Differential Handling of Urea and Its Analogues Suggests Carrier-Mediated Urea Excretion in Freshwater Rainbow Trout

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ABSTRACT

The possible presence of urea transport mechanisms in the gill and kidney of the freshwater rainbow trout (*Oncorhynchus mykiss*) was investigated in vivo by comparing the branchial and renal handling of analogues acetamide and thiourea with the handling of urea. Trout were fitted with indwelling dorsal aortic catheters and urinary catheters and injected with an isosmotic dose of [^{14}C]-labeled urea analogue (acetamide or thiourea) calculated to bring plasma analogue concentrations close to plasma urea concentrations. Urea and analogue concentrations were significantly greater in the urine than in the plasma. Branchial clearance rate of acetamide was only 48% of urea clearance, whereas the clearance of thiourea was only 22%, a pattern that was also observed in branchial uptake of these substances and was similar to our previous observations in toadfish and midshipmen. The renal secretion clearance rates of urea and acetamide were similar, and on average, both substances were secreted on a net basis, although reabsorption did occur in some cases. In contrast, thiourea was neither reabsorbed nor secreted by the kidney tubule. The secretion clearance rates of both acetamide and urea were well correlated with the secretion clearance rates of Na^+, Cl^-, and water, whereas there was no relationship between thiourea and these substances. The pattern of acetamide, thiourea, and urea handling by the gill of the trout is similar to that found in the gills of the midshipman and the gulf toadfish and strongly suggests the presence of a UT-type facilitated diffusion urea transport mechanism. The pattern of differential handling in the kidney is unlike that in the gill and also unlike that in the kidney of the midshipman and the gulf toadfish, suggesting a different mechanism. In addition, renal urea secretion occurs against a concentration gradient, suggesting the involvement of an active transport mechanism.

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Introduction

Recently, urea transport mechanisms have been cloned and physiologically characterized in the gulf toadfish (*Opsanus beta*; tUT; Walsh et al. 2000) and the Lake Magadi tilapia (*Alcolapia grahami*; mtUT; Walsh et al. 2001*a*). However, these two teleosts are unlike most other teleosts in that they can excrete urea as their primary nitrogenous waste (Randall et al. 1989; Wood et al. 1989; Walsh et al. 1990; Walsh and Milligan 1995). Teleost fish usually excrete the majority of their nitrogenous wastes as ammonia. However, many teleosts do retain circulating concentrations of urea many times higher than those of ammonia (reviewed by Wood 1993), suggesting a role for urea transport mechanisms even in ammoniotelic species. Reflecting this, a urea transporter has recently been cloned in the ammoniotelic eel *Anguilla japonica* (eUT; Mistry et al. 2001). In the ammoniotelic plainfin midshipman (*Porichthys notatus*), a close relative of the facultatively ureotelic gulf toadfish, there is evidence for urea transporters in both the gill and the kidney (Walsh et al. 2001*b*; McDonald et al. 2002). In addition, there is circumstantial evidence suggesting that adult rainbow trout (*Oncorhynchus mykiss*) may regulate endogenous urea concentrations by urea transport mechanisms in the gill and kidney (McDonald and Wood 1998).

The retention of urea synthesis and transport mechanisms in ammoniotelic teleosts might have stemmed from a need in early life-stage development. Unlike adults, larval trout express a full complement of ornithine urea cycle (OUC) enzymes, and urea synthesis during these early life stages is believed to be a strategy to avoid ammonia toxicity and a mechanism for ammonia detoxification (Wright et al. 1995; Pilley and Wright 2000; Steele et al. 2001). In trout embryos, a bidirectional facilitated diffusion urea transport mechanism has been described that is characteristically similar to the toadfish UT (Pilley and Wright 2000; Walsh et al. 2000). It is possible that the ability to upregulate urea synthesis during periods of elevated ammonia has been extended from early life stages into adults and can be called upon when needed. And while urea transport mechanisms might have followed the same evolutionary path as urea synthesis pathways, expression of urea transport mech-

anisms continued in adult teleosts long after they were no longer necessary.

Urea analogues (i.e., thiourea, acetamide, N-methylurea) are often used to identify urea transport mechanisms. Their structural similarity to urea enables them to interact competitively at urea transport sites, thereby inhibiting urea transport (Chou and Knepper 1989; Gillan and Sands 1993). Although they are often found to interfere with the movement of urea, analogues are not always transported as effectively as urea. Studies characterizing urea transport mechanisms in other organisms have shown that urea transporters customarily move analogues less effectively than urea and can often distinguish between analogues. In mammalian inner medullary collecting ducts (via UT-A1) and erythrocytes (via UT-B transporters), the permeability of thiourea was much lower than the permeability of urea (Naccache and Sha'afi 1973; Chou et al. 1990). In the kidney of the frog *Rana catesbeiana*, there appeared to be active secretion of thiourea but a lack of acetamide transport (Schmidt-Nielsen and Shrauger 1963). The opposite pattern was observed in the kidney of the spiny dogfish, *Squalus acanthias* (potentially via shUT), in which acetamide and methylurea were reabsorbed nearly as well as urea but thiourea was not (Schmidt-Nielsen and Rabinowitz 1964).

With respect to teleost fish, the permeability of urea through the facilitated diffusion urea transporter in the gill of the gulf toadfish (tUT) is twofold greater than that of acetamide and sixfold greater than the permeability of thiourea (Wood et al. 1998; McDonald et al. 2000). Furthermore, in hepatocytes of the gulf toadfish, phloretin-sensitive urea transport demonstrated a high specificity for urea compared with acetamide, thiourea, and N-methylurea (Walsh et al. 1994). In the Lake Magadi tilapia, the mtUT transporter exhibits a high specificity for urea over thiourea (Walsh et al. 2001*a*).

One of the two main objectives of this study was to investigate the possibility of urea transport mechanisms in the gill and kidney of the adult rainbow trout by quantitatively comparing the handling of urea and two urea analogues, acetamide and thiourea. To address the second objective, the renal handling of urea and analogues was compared with that of other substances such as Na^+, Cl^-, and water. Our results indicate that rainbow trout have distinct urea excretion transport mechanisms that are found in the gill and the kidney, each of which differentially handles urea, acetamide, and thiourea.

Material and Methods

Experimental Animals

Rainbow trout (*Oncorhynchus mykiss*) were obtained from Humber Springs Trout Farm in Mono Mills, Ontario. The fish were acclimated to seasonal water temperatures (11°–14°C) and were fed with commercial trout pellets every second day until the time of surgery. Acclimation was carried out in dechlorinated Hamilton tap water (in mmol L^{-1}: $Ca^{++} = 1.8$; $Cl^- = 0.8$; $Na^+ = 0.6$; $Mg^{++} = 0.5$; $K^+ = 0.04$; titration alkalinity [to pH 4.0] = 1.9; total hardness = 140 mg L^{-1} as $CaCO_3$; pH = 8.0).

Experimental Protocol

Dorsal aortic and urinary catheterizations were performed simultaneously on fish anaesthetized with MS-222 (0.07 g L^{-1}; Sigma-Aldrich Canada) and artificially ventilated on an operating table. Dorsal aortic catheters (Clay Adams PE-50 tubing) were implanted as described by Soivio et al. (1972) and were filled with Cortland saline (Wolf 1963) containing 50 IU/mL lithium heparin (Sigma-Aldrich Canada). Internal urinary catheters were inserted using a technique described by Curtis and Wood (1991) and Wood and Patrick (1994), in which the catheter (heat-molded Clay Adams PE-60 tubing) is placed in the urinary bladder to drain urine continuously as it is produced. Any reabsorptive/secretory role of the bladder is negated so as to examine the function of the kidney alone. During recovery and experimentation, urine was collected continuously, with the catheter emptying into a vial approximately 3 cm below the water level of the box. Following surgery, fish were kept in darkened individual containers (minimum volume = 4 L) that were continually aerated and supplied with flowing freshwater (200 mL min^{-1}).

Following the procedure outlined by McDonald and Wood (1998), [^{3}H]–polyethylene glycol (PEG; Mr 4,000) was used as a marker for glomerular filtration rate (GFR). PEG 4000 was chosen because it is considered a more accurate and conservative indicator of GFR in fish than other commonly used markers (e.g., inulin derivatives) as it undergoes minimal radioautolysis, metabolic breakdown, or postfiltration reabsorption across tubules and bladder (Beyenbach and Kirschner 1976; Erickson and Gingerich 1986; Curtis and Wood 1991). Before 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 5 μCi 100 g body weight^{-1} of [^{3}H]-PEG 4000 (New England Nuclear) in 0.25 mL 100 g body weight^{-1} was injected via the dorsal aortic catheter followed by an additional 0.3 mL of saline in fish where both catheters were deemed successful. The [^{3}H]-PEG 4000 was then allowed to equilibrate throughout the extracellular space for 24 h before sampling commenced.

Series i: Urea Handling Compared with Analogues

In this series, the patterns of branchial and renal urea excretion were compared with the handling of acetamide and thiourea, which are similar in size and structure to urea. Two trials were performed: six fish treated with acetamide and five fish treated with thiourea. Mean weights of fish in the acetamide trial were 0.259 ± 0.017 kg (number of fish [*N*] = 6), ranging from 0.187 to 0.296 kg. Mean weights of fish in the thiourea trial were

0.268 ± 0.004 kg ($N = 5$), ranging from 0.256 to 0.276 kg. Following the [^{3}H]-PEG 4000 injection and a 24-h equilibration period, a blood sample was taken (200 μL with saline plus red blood cell replacement), a fresh urine collection was started, water flow to the fish box was stopped, and the water level was set to an exact volume mark of 4 L. An initial water sample was taken for measurement of urea concentration. Thereafter, water, urine, and blood samples were taken every 12 h, and the fish box was flushed thoroughly over a 15-min period at 12-h intervals. Vigorous aeration maintained Po_2 close to air saturation during times when water flow to the box was stopped. Twenty-four hours after sampling commenced, the fish were injected with a dose of 5 μCi 100 g body weight^{-1} of [^{14}C]-labeled thiourea or acetamide in 160 μmol 100 g body weight^{-1} of isosmotic cold urea analogue (concentration = 300 mmol L^{-1}) to render internal analogue concentrations approximately equal to internal urea concentrations (1.8 mmol L^{-1}). Sampling continued for the remainder of the experiment (72 h) to make a total of six water samples, six blood samples, and six urine samples. Two of the six samples were taken before the analogue injection and provided control values; the four that were taken after the analogue injection provided experimental values. 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 and [^{14}C]-analogue only.

Series ii: Total Uptake of Urea from the Surrounding Water

Two different trials were performed using a total of 30 fish. Similar to the protocol of McDonald et al. (2002), small rainbow trout (average weight = 0.025 ± 0.004 kg; $N = 30$) in groups of six were placed into aerated flux chambers with flow-through freshwater and were left to acclimate for 12 h. After the acclimation period, water flow was stopped, the volume of the chamber was set to 2 L, and the fish became a part of one of the following trials.

In trial A, a direct comparison was made between the uptake rates of urea and two urea analogues, acetamide and thiourea, using concentrations that were comparable to urea concentrations found in blood plasma in vivo. In this trial, one group of fish was exposed to 2 mmol L^{-1} urea + 25 μCi L^{-1} [^{14}C]-urea in the surrounding water; a second group was exposed to 2 mmol L^{-1} acetamide + 25 μCi L^{-1} [^{14}C]-acetamide; and a third group was exposed to 2 mmol L^{-1} thiourea + 25 μCi L^{-1} [^{14}C]-thiourea in the surrounding water. Trial B investigated analogue competition with urea using urea concentrations slightly greater than internal concentrations and analogue concentrations five times greater than urea concentrations. The first group of fish was exposed to 2 mmol L^{-1} urea + 25 μCi L^{-1} [^{14}C]-urea; the second set was exposed to 2 mmol L^{-1} urea + 25 μCi L^{-1} [^{14}C]-urea + 10 mmol L^{-1} acetamide; and the third set was exposed to 2 mmol L^{-1} urea + 25 μCi L^{-1} [^{14}C]-urea + 10 mmol L^{-1} thiourea in the external water. An examination of the effect of phloretin on urea uptake was attempted; however, phloretin at 0.250 mM proved toxic to the fish.

In both trials, after a 12-h flux period, the fish were killed by a blow to the head, weighed, placed in 50 mmol L^{-1} of cold urea or analogue solution (to displace any surface binding of [^{14}C]-label), blotted dry, and then homogenized in 8% perchloric acid (2 parts acid to 1 part fish) using a Proctor-Silex Blend Master blender. A sample of this solution was centrifuged, and the supernatant was analyzed for [^{14}C] counts.

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 done by adding the calculated concentration of analogue present in the urine, plasma, or water (Eqq. 1 and 2, below) in the same volume as the sample to each point of the standard curve of the urea assay. Any interference to the colorimetric assay by thiourea or acetamide would thus be automatically accounted for when using the respective standard curve, a point that was validated by urea addition/recovery tests. 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 + 5 mL of freshwater) or water samples (5 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. Samples of [^{14}C]-urea or [^{14}C]-analogue in supernatant (5 mL) were added to 10 mL ACS fluor (Amersham) and analyzed by scintillation counting on an LKB Rackbeta 1217 Counter. Quench curves using various amounts of supernatant plus 1 μCi [^{14}C]-urea or [^{14}C]-analogue were made to account for the quench associated with supernatant color.

The concentration of analogue [A] in the plasma, urine, water, or whole body was determined from the original specific activity (S; cpm μmol^{-1} [cpm = counts per minute]) of the injected solution by converting the radioactivity found in the samples into concentration (μmol mL^{-1}):

$$S = \frac{\text{cpm}_{\text{in}}}{[A]_{\text{in}}}, \tag{1}$$

$$[A]_{\text{s}} = \frac{\text{cpm}_{\text{s}}}{S}, \tag{2}$$

where cpm_{in} (cpm mL^{-1}) indicates the radioactivity of the injected solution, $[A]_{in}$ indicates the total analogue concentration of the injected solution, cpm_s is the radioactivity in the sample and $[A]_s$ is the analogue concentration in the sample. These analogues are not known to occur endogenously in fish.

The branchial clearance rate (C_B; mL kg^{-1} h^{-1}) of any substance (X) was calculated by dividing the concentration of the substance appearing in the water $[X]_w$ by fish body weight (wt), plasma concentration $[X]_p$, and time (t):

$$C_{B_X} = \frac{[X]_w \times V_f}{wt \times [X]_p \times t}, \quad (3)$$

where V_f is the volume of water surrounding the fish. The amount of any substance X taken up from the water was determined from the specific activity of the surrounding water (S_w; cpm μmol^{-1}) by converting the radioactivity measured in fish (cpm_f) into concentration (μmol kg^{-1} body weight). The relative uptake (R_U; mL kg^{-1} h^{-1}) of a substance X was then determined by dividing the amount of X taken up from the water by $[X]_w$ and by time (t):

$$X = \frac{cpm_f}{S_w}, \quad (4)$$

$$R_{U_X} = \frac{X}{[X]_w \times t}. \quad (5)$$

All the following renal rates were related to fish body weight by expressing urinary flow rate (UFR) in milliliters per kilogram per hour. Urinary excretion rates (U) of any substance (X) were calculated as

$$U_X = [X]_u \times UFR, \quad (6)$$

using measured values of UFR and urine concentrations $[X]_u$.

Glomerular filtration rates (GFR) were calculated as the clearance of [^{3}H]-PEG 4000, that is, the excretion of radioactivity in the urine (cpm_u) relative to its concentration in the blood plasma (cpm_p):

$$GFR = \frac{cpm_u \times UFR}{cpm_p}. \quad (7)$$

The filtration rate (FR; μmol kg^{-1} h^{-1}) of a substance X at the glomeruli was calculated as

$$FR_X = [X]_p \times GFR, \quad (8)$$

and consequently the tubular secretion rate (TS; μmol kg^{-1} h^{-1}) of X was calculated as

$$TS_X = U_X - FR_X. \quad (9)$$

A $FR_X > U_X$ results in a negative TS_X, indicating net tubular reabsorption. As a way to compare the handling of a variety substances (urea, analogues, and ions) at different circulating concentrations on a common basis, the renal clearance rate by tubular secretion (SCR; mL kg^{-1} h^{-1}) of X was calculated as

$$SCR_X = \frac{TS_X}{[X]_p}. \quad (10)$$

A negative SCR value signifies a negative renal "clearance rate" by tubular reabsorption.

The concept of clearance ratio (CR_X; see Wood 1995) relates the clearance of a substance X to the GFR (that is, to the clearance of [^{3}H]-PEG 4000):

$$CR_X = \frac{[X]_u \times UFR}{[X]_p \times GFR} = \frac{U_X}{FR_X}. \quad (11)$$

Assuming that a substance X is filtered at the glomeruli with the same efficiency as [^{3}H]-PEG 4000, a relatively safe assumption with the small neutral molecules (urea, acetamide, thiourea, water) and monovalent ions (Na^+, Cl^-) of this study, then the clearance ratio provides quantitative information on the tubular handling of X. If the clearance ratio is greater than 1, then there is a net secretion of X; if less than 1, then net reabsorption has occurred. For example, $CR_X = 0.05$ would indicate 95% net reabsorption of the filtered load of X.

Statistics

Data have been reported as means ± 1 SEM (N = number of fish). Regression lines were fitted by the method of least squares, and the significance ($P < 0.05$) of the slope assessed. The significance of differences between means was evaluated using Student's paired or unpaired, two-tailed or one-tailed t-test ($P < 0.05$) as appropriate (Nemenyi et al. 1977). ANOVA with time as the main factor was followed by a comparison of individual means using the Bonferroni correction for multiple sample comparisons.

Results

The theory behind this study is that if branchial and renal handling of urea were carrier mediated, then the transporter involved would differentially transport urea versus analogues. However, if urea moves through the gill and kidney by passive diffusion, urea would not be differentially transported. In fish treated with acetamide, the branchial clearance rate of urea

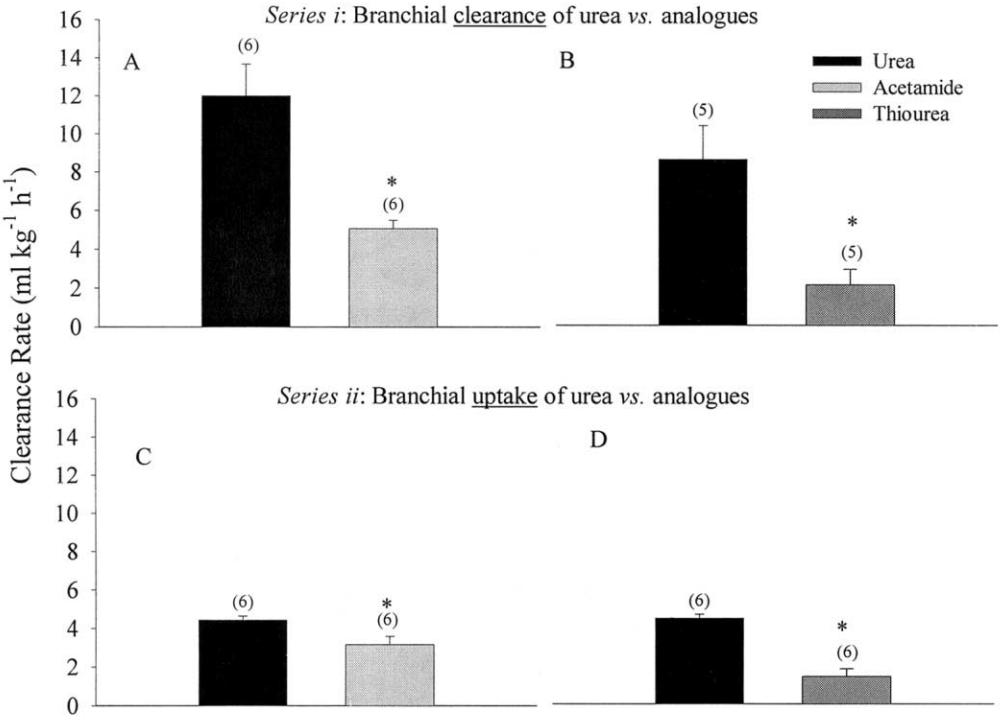

Figure 1. The branchial clearance rates (mL kg^{-1} h^{-1}) of fish in the (*A*) acetamide series and (*B*) thiourea series showing a significantly lower branchial clearance of acetamide and thiourea compared to urea. A similar trend is illustrated by measured branchial uptake clearance rates of (*C*) urea versus acetamide and (*D*) urea versus thiourea. Values are means ± 1 SEM (N = number of fish); asterisk denotes $P < 0.05$ significantly different from urea.

(11.39 ± 1.50 [$N = 6$] mL kg^{-1} h^{-1}) was twofold greater than the clearance of acetamide (4.97 ± 0.60 [$N = 6$] mL kg^{-1} h^{-1}; Fig. 1*A*). In thiourea-treated fish, the branchial clearance rate of urea (8.59 ± 1.77 [$N = 5$] mL kg^{-1} h^{-1}) was approximately 4× greater than the clearance rate of thiourea (2.10 ± 0.81 [$N = 5$] mL kg^{-1} h^{-1}; Fig. 1*B*). The branchial clearance rates of urea in both trials were not significantly different and the branchial clearance in the presence of analogue was not significantly different from that measured during control periods (control in acetamide trial: 10.89 ± 3.41 [$N = 6$] mL kg^{-1} h^{-1}; control in thiourea trial: 6.84 ± 1.25 [$N = 5$] mL kg^{-1} h^{-1}). When expressed as a clearance ratio (analogue : urea) for all collection periods where simultaneous measurements were made, the ratios were 0.48 ± 0.08 ($N = 6$) for acetamide and 0.22 ± 0.05 ($N = 5$) for thiourea, both showing a significant difference from unity.

Although a similar pattern to clearance was observed when measuring the total uptake rate of these substances, the relative uptake ratio was substantially greater than the branchial clearance ratio in the case of acetamide : urea (acetamide : urea = 0.71; thiourea : urea = 0.32). The total uptake rates of urea (4.44 ± 0.22 [$N = 6$] mL kg^{-1} h^{-1}) and acetamide (3.16 ± 0.44 [$N = 6$] mL kg^{-1} h^{-1}; Fig. 1*C*) were significantly lower than their clearance rates (10.12 ± 1.17 [$N = 11$] mL kg^{-1} h^{-1} and 4.97 ± 0.60 [$N = 6$] mL kg^{-1} h^{-1}, respectively), whereas the total uptake rate of thiourea (1.41 ± 0.41 [$N = 6$] mL kg^{-1} h^{-1}; Fig. 1*D*) was not significantly different from its clearance rate (2.10 ± 0.81 [$N = 5$] mL kg^{-1} h^{-1}).

The uptake protocol was also used to investigate the possibility of competitive interactions between urea and urea analogues. Urea movement was found to be unaffected by the presence of analogues, even at analogue concentrations in the external water that were fivefold greater than urea concentrations (data not shown). Thus there appears to be no competitive effect, in accord with the clearance data. No conclusions could be drawn about the effect of phloretin, a urea transport blocker, on the rate of urea uptake because of the apparent toxicity of this drug in vivo.

Over the entire experimental period of both analogue trials, the mean plasma urea concentration (1.20 ± 0.13 [$N = 11$] mmol L^{-1}) was only about 48% of that in the urine (2.51 ± 0.30 [$N = 11$] mmol L^{-1}; Table 1). Specifically in the acetamide series, urea levels remained relatively stable in the urine (2.01 ± 0.33 [$N = 6$] mmol L^{-1}) and in the plasma (1.10 ± 0.17 [$N = 6$] mmol L^{-1}); urine levels were significantly greater than plasma levels throughout most of the experiment (Fig.

Table 1: Average values for plasma and urinary ion composition, urine flow rate, and glomerular filtration rate

CR (mL kg^{-1} h^{-1})	GFR (mL kg^{-1} h^{-1})	$[Urea]_p$ (mmol L^{-1})	$[Urea]_u$ (mmol L^{-1})	$[Na^+]_p$ (mmol L^{-1})	$[Na^+]_u$ (mmol L^{-1})	$[Cl^-]_p$ (mmol L^{-1})	$[Cl^-]_u$ (mmol L^{-1})
2.1 ± .1	3.7 ± .5	1.20 ± .13	2.51 ± .30	140.8 ± 2.8	8.3 ± 1.7	133.0 ± 2.8	7.2 ± .3

Note. Data are shown as mean ± 1 SEM ($N = 11$); UFR = urine flow rate; GFR = glomerular filtration rate; p = plasma; u = urine.

2*A*). Acetamide showed a similar trend, being significantly more concentrated in the urine (1.79 ± 0.06 [$N = 6$] mmol L^{-1}) than in the plasma (0.98 ± 0.05 [$N = 6$] mmol L^{-1}; Fig. 2*B*). However, the concentration of acetamide in the plasma and the urine steadily declined as it was cleared by the kidney and gills ($P < 0.005$).

As in the acetamide series, fish treated with thiourea had relatively stable urine (3.08 ± 0.28 [$N = 5$] mmol L^{-1}) and plasma (1.32 ± 0.15 [$N = 5$] mmol L^{-1}) urea concentrations, with urine levels being significantly greater than plasma levels at all times (Fig. 2*C*). Thiourea was also found in greater concentrations in the urine (1.55 ± 0.17 [$N = 5$] mmol L^{-1}) than in the plasma (1.00 ± 0.07 [$N = 5$] mmol L^{-1}), although early in the experiment, urine and plasma thiourea concentrations were the same (Fig. 2*D*). Unlike acetamide, thiourea concentrations did not fall with time.

Urine flow rate was about two-thirds of the measured GFR, and the concentrations of $Na^+$and Cl^- in the ureteral urine were <10% of plasma levels (Table 1) and were constant in both injection trials throughout the experiment (data not shown). During analogue treatment, both Na^+ and Cl^- on average had very low clearance ratios (CR), indicating close to 95% reabsorption from the kidney tubules (Table 2). In contrast, CR_{water} during analogue treatment was 0.71, indicating only 30% reabsorption by the kidney tubules. In the presence of acetamide, CR_{urea} was 1.36, whereas $CR_{acetamide}$ was 1.33, indicating 36% and 33% net secretion by the kidney tubules, respectively. Similarly, in the presence of thiourea, CR_{urea} was

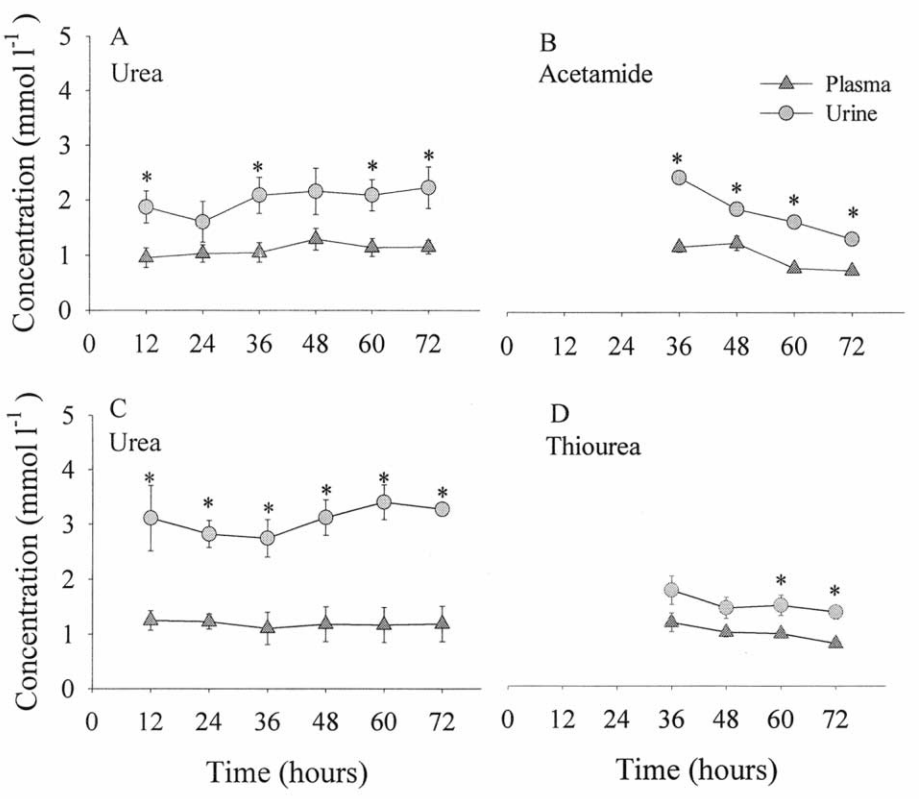

Figure 2. Urine and plasma concentrations of (*A*) urea and (*B*) acetamide in the same fish and (*C*) urea and (*D*) thiourea in the same fish demonstrating that all three substances are more concentrated in the urine than in the plasma. Values are means ± 1 SEM ($N = 6$ for acetamide series; $N = 5$ for thiourea series); asterisk denotes $P < 0.05$ significantly different from plasma concentrations.

Table 2: The calculated clearance ratios for water, Na^+, Cl^-, urea, acetamide, and thiourea for fish in Series i

			Acetamide Series		Thiourea Series	
CR_{water}	CR_{Na+}	CR_{Cl-}	CR_{urea}	$CR_{acetamide}$	CR_{urea}	$CR_{thiourea}$
.71 ± .07* (11)	.05 ± .01* (11)	.04 ± .01* (11)	1.36 ± .16* (6)	1.33 ± .12* (6)	1.57 ± .13* (5)	1.25 ± .29 (5)

Note. Data are shown as mean ± 1 SEM (N = number of fish).

* $P < 0.05$ significantly different from 1.

1.57, indicating 57% net secretion by the kidney tubules. However, $CR_{thiourea}$ was not significantly different from 1.0, indicating that it was neither net secreted nor net reabsorbed by the kidney tubule (Table 2). CR_{urea} was unaffected by the injection of each respective analogue (data not shown).

The handling of urea and acetamide by the kidney was complicated by the fact that the two substances were both reabsorbed and secreted. When comparing the relative renal secretion clearance rates (SCR_x, Eq. [10]), the $SCR_{acetamide}$ exhibited a strong linear, proportional relationship ($r = 0.98$) to the SCR_{urea} with slope = 1.08 ($P < 0.001$), indicating that the secretion/reabsorption clearance rate of acetamide was approximately 100% of the rate for urea (Fig. 3). In contrast, there was no relationship between the $SCR_{thiourea}$ and SCR_{urea} ($r = 0.17$; slope not significantly different from 0).

Plasma Na^+ and Cl^- concentrations in both the acetamide and thiourea series were greater than urine concentrations and on average had very similar SCR values (Na^+: -3.58 ± 0.48 [$N = 11$] mL kg^{-1} h^{-1}; Cl^-: -3.57 ± 0.48 [$N = 11$] mL kg^{-1} h^{-1}), the negative values indicating net reabsorption and not secretion. Using SCR values measured during analogue treatment, both SCR_{Na+} and SCR_{Cl-} exhibited linear proportional relationships ($r = 0.93$ for both) to negative SCR_{urea} values (urea reabsorption) with slopes of 0.624 ($P < 0.005$; Fig. 4*A*) and 0.616 ($P < 0.005$; Fig. 4*B*), respectively. However, SCR_{Na+} and SCR_{Cl-} exhibited no relationship to positive SCR_{urea} values (urea secretion; Fig. 4*A*, 4*B*). In contrast, SCR_{water}(-1.74 ± 0.32 [$N = 11$] mL kg^{-1} h^{-1}) showed a strong, linear correlation to negative SCR_{urea} values ($r = 0.97$) with slope = 0.849 ($P < 0.005$; Fig. 4*C*). However, positive SCR_{urea} values showed no relationship to the movement of water, when both positive and negative SCR_{water} values were taken into account separately (Fig. 4*C*). Thus on a relative basis, the reabsorption of urea was approximately 62% of SCR values of Na^+ and Cl^- and 85% that of water. However, the secretion of urea was independent of water, Na^+, and Cl^- movement.

In comparison, negative $SCR_{acetamide}$ values (reabsorption) exhibited a linear relationship with both Na^+ ($r = 0.93$; slope = 0.754; $P < 0.0001$) and Cl^- ($r = 0.93$; slope = 0.752; $P < 0.0001$; Fig. 5*A*, 5*B*). Negative $SCR_{acetamide}$ values exhibited a linear proportional relationship ($r = 0.93$) to SCR_{water} with slope = 0.880 ($P < 0.0001$; Fig. 5*C*). However, positive $SCR_{acetamide}$ values showed no relationship to water movement, when both positive and negative SCR_{water} values were taken into account separately (Fig. 5*C*). Thus, the reabsorption of acetamide was approximately 75% of the SCR values of Na^+ and Cl^- and 88% the movement of water. However, the secretion of acetamide was independent of water, Na^+, and Cl^- movement. In contrast, there was no relationship between the negligible $SCR_{thiourea}$ and the SCR values of Na^+, Cl^-, or water (data not shown).

Discussion

The objectives of this study were to characterize the branchial and renal handling of urea in the rainbow trout to determine whether the excretion of urea through the gills or kidney is in fact carrier mediated. Our results suggest that there is evidence for a distinct transport mechanism involved in the excretion of urea through the gill that has characteristics similar to those of the UT family of facilitated diffusion urea transporters and therefore similar to those found in the gills of the gulf toadfish (McDonald et al. 2000) and the plainfin midshipman (McDonald et al. 2002). There is also evidence for carrier-mediated

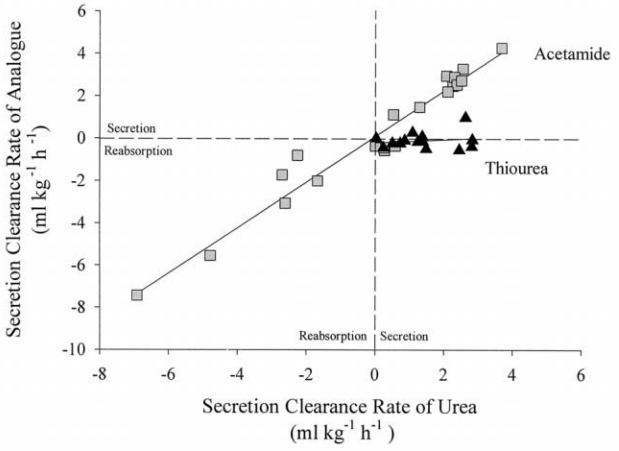

Figure 3. Linear regressions of the secretion clearance rates (SCR_x; Eq. [10]) of analogue (*Y*-axis) and urea (*X*-axis) demonstrating a $SCR_{acetamide}$ that is equal to that of urea. The equation of the acetamide line and the significance of the correlation is $y = 1.08x + 0.088$, $r = 0.98$, $P < 0.001$ ($N = 20$ simultaneous measurements from six fish). In contrast, thiourea does not appear to be transported by the kidney tubule. The equation of the thiourea line is $y = 0.070x - 0.200$, $r = 0.17$, slope = NS ($N = 13$ simultaneous measurements from five fish).

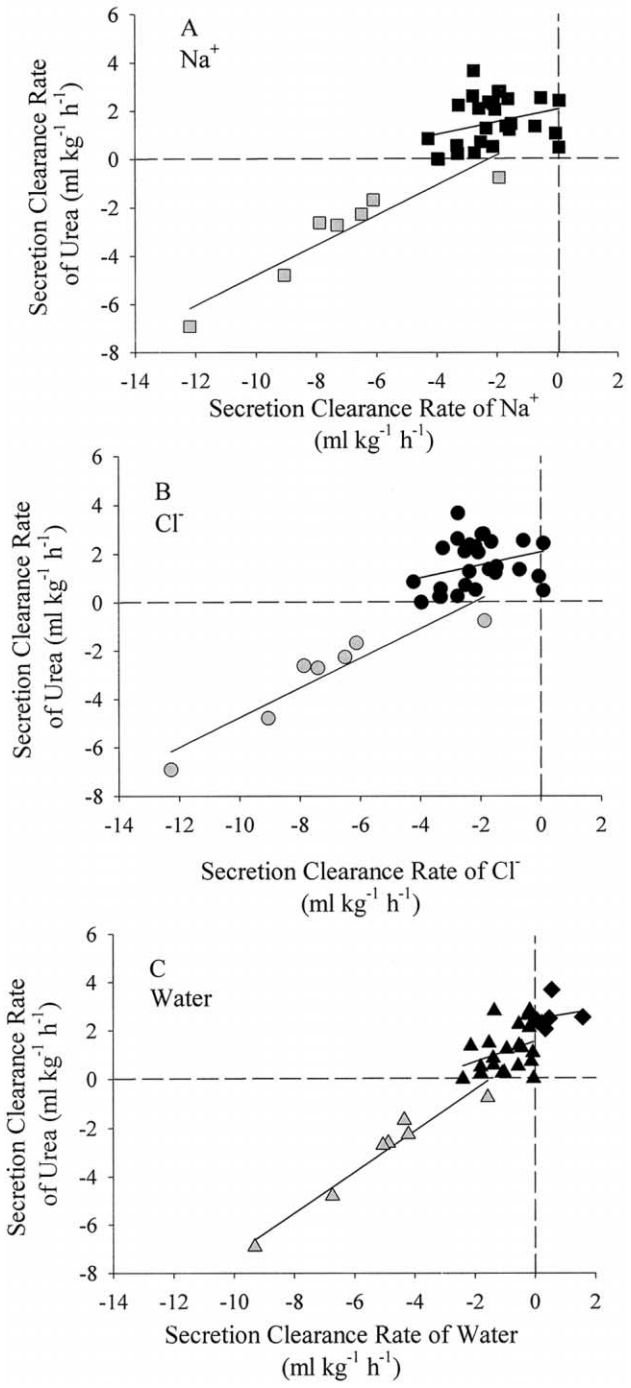

Figure 4. Linear regressions of the secretion clearance rate (SCR_x; Eq. [10]) of urea (*Y*-axis) versus (*A*) Na^+, (*B*) Cl^-, and (*C*) water (*X*-axis) during analogue treatment. Urea and water are both reabsorbed and secreted by the kidney, whereas Na^+ and Cl^- are only reabsorbed. Negative SCR_{urea} values (representing reabsorption, *gray symbols*) are highly correlated with the SCR values of all three substances. Positive SCR_{urea} values (representing secretion, *black symbols*) are not correlated with any of the three substances (black diamonds represent the relationship with water secretion). The equation of the urea reabsorption versus Na^+ line and the significance of the correlation is $y = 0.624x + 1.44$, $r = 0.93$, $P < 0.005$. The equation of the urea reabsorption versus Cl^- line and the significance of the correlation is $y = 0.616x + 1.39$, $r = 0.93$, $P < 0.005$. The equation of the urea reabsorption versus water line is $y = 0.849x + 1.28$, $r = 0.97$, $P < 0.005$ ($N = 7$ simultaneous measurements from three fish for reabsorption values, and $N = 28$ simultaneous measurements from 10 fish for secretion values).

renal secretion of urea via a transport mechanism that is distinct from that in the gill.

The trout gill exhibited the same pattern of differential handling as observed in the facultatively ureotelic gulf toadfish and the ammoniotelic plainfin midshipman, where the branchial clearance rate of urea > acetamide > thiourea. Through the trout gill, the ratio of analogue : urea clearance was 48% for acetamide and only 22% for thiourea. In the influx direction, these ratios were 71% and 32%, respectively. In the gulf toadfish (McDonald et al. 2000), a similar pattern is observed through tUT, a hormonally controlled, pulsatile, facilitated diffusion mechanism in the gill that shows 62% homology at the amino acid level to mammalian UT urea transporters (Smith et al. 1998; Walsh et al. 2000). In the toadfish, the branchial clearance of acetamide when tUT is activated is 35%–50% that of urea clearance, and the relative thiourea clearance is at most only 16% of urea clearance during pulsing events. However, all three substances exhibit similar permeabilities during nonpulsing periods, suggesting that the gill handles urea, acetamide, and thiourea identically when tUT is not activated, despite small differences in calculated oil/water partition coefficients and lipid permeabilities of the three substances (Goldstein and Solomon 1960; Lippe 1969; Galluci et al. 1971).

In the ammoniotelic midshipman, a cDNA probe based on tUT produced a strong signal when tested against gill mRNA, suggesting that there is a message for a UT-type transporter in the gills (Walsh et al. 2001*b*). The clearance of acetamide through midshipman gill was 74% of urea clearance whereas thiourea clearance was only 55%, with similar patterns in the influx direction (McDonald et al. 2002). In the obligately ureotelic Lake Magadi tilapia, mtUT (with 75% identity to tUT) demonstrated a permeability to thiourea that was 19% that of urea permeability; acetamide was not tested (Walsh et al. 2001*a*). Thus, this pattern of analogue versus urea handling appears to be characteristic of branchial UT-type transport mechanisms in both ureotelic and ammoniotelic organisms. That this pattern is also evident in rainbow trout strongly suggests the presence of a UT-type facilitated diffusion transporter in the gill and supports preliminary Northern blot and sequence analysis that revealed an mRNA from trout gill with approximately 1,200 bases homologous to the mRNA of tUT (C. M. Pilley, P. W. Wright, and P. J. Walsh, unpublished data).

Recent molecular evidence (mRNA hybridization with a

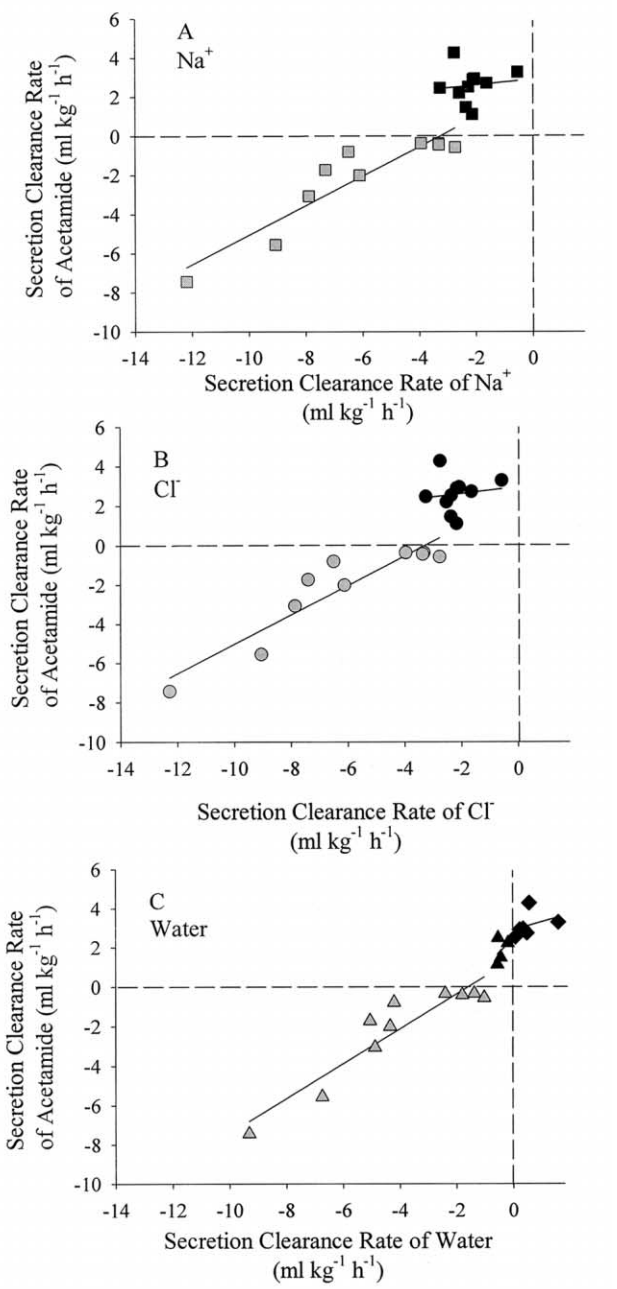

Figure 5. Linear regressions of the secretion clearance rate (SCR_x; Eq. [10]) of acetamide (*Y*-axis) versus (A) Na^+, (B) Cl^-, and (C) water (*X*-axis). Acetamide and water, like urea, are both reabsorbed and secreted by the kidney, whereas Na^+ and Cl^- are only reabsorbed. Negative $SCR_{acetamide}$ values (representing reabsorption, *gray symbols*) are highly correlated to the SCR values of all three substances. Positive SCR_{urea} values (representing secretion, *black symbols*) are not correlated with any of the three substances (black diamonds represent the relationship with water secretion). The equation of the acetamide reabsorption versus Na^+ line and the significance of the correlation is $y = 0.754x + 2.48$, $r = 0.93$, $P < 0.0001$. The equation of the acetamide reabsorption versus Cl^- line and the significance of the correlation is $y = 0.752x + 2.48$, $r = 0.93$, $P < 0.0001$ ($N = 20$ simultaneous measurements from six fish). The equation of the acetamide reabsorption versus water line is $y = 0.880x + 1.40$, $r = 0.93$, $P < 0.0001$ ($N = 10$ simultaneous measurements from five fish for reabsorption values, and $N = 10$ simultaneous measurements from six fish for secretion values).

cDNA based on tUT) indicates that facilitated diffusion transporters may be present in the gills of many other ammoniotelic teleosts (Walsh et al. 2001*b*). In addition, trout embryos appear to have a UT-type transport mechanism, facilitating the diffusion of urea through the chorion, because a significant proportion (26%) of their nitrogenous wastes is excreted as urea (Wright and Land 1998; Pilley and Wright 2000). Trout embryos also appear to have relatively high levels of at least four OUC enzymes at the time of hatching, which suggests the possibility that ammoniotelic teleosts may have retained the genes for OUC enzymes and urea transporters because they are important during embryogenesis (Wright et al. 1995).

A common tool for characterizing UT-type transport mechanisms is to determine whether analogues will competitively inhibit urea movement. Competition between thiourea and urea has been observed through tUT (Wood et al. 1998; Walsh et al. 2000). In addition, urea transport through the Lake Magadi tilapia mtUT was inhibited by the presence of acetamide, N-methylurea, and thiourea (Walsh et al. 2001*a*). However, there was no competitive inhibition of analogues on urea transport in the trout either when analogues were present internally (injected) or when present externally (uptake of [^{14}C]-urea in the presence of an excess of cold thiourea or acetamide). These results are similar to those determined in vivo for the plainfin midshipman (McDonald et al. 2002).

Interestingly, these in vivo patterns with analogue (differential handling but no competitive interactions) differ from those seen in vitro in a study of transport by gill basolateral membrane vesicles of trout (McDonald 2002). In that study, thiourea was transported at the same rate as urea, but urea transport was inhibited by a fivefold excess of thiourea. In contrast, acetamide was transported at a lower rate than urea, but urea transport was not inhibited by a fivefold excess of acetamide. Perhaps the analogue concentrations in this study were simply not high enough to cause a substantial effect on urea transport in vivo, especially when dealing with both apical and basolateral membranes. In both the toadfish and the tilapia competition uptake studies, the urea and analogue concentrations in the external water were much higher than those used in this study (Wood et al. 1998; Walsh et al. 2001*a*). In the toadfish study, 30 mmol L^{-1} urea was placed in the external water with double the amount of thiourea, and in the tilapia study, 20 mmol L^{-1} urea was placed with triple the amount of analogue (Wood et al. 1998; Walsh et al. 2001*a*). Alternatively,

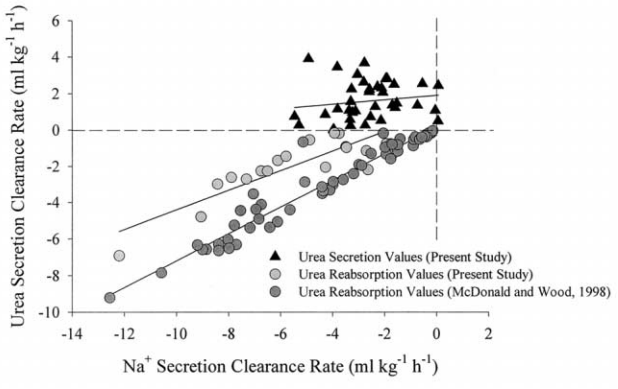

Figure 6. Linear regressions of the positive (secretion) and negative (reabsorption) secretion clearance rates (SCR_x; Eq. [10]) of Na^+ (*X*-axis) and urea (*Y*-axis) from this study compared with the relationship found by McDonald and Wood (1998). Similar to the findings of McDonald and Wood (1998), there is a strong correlation between the reabsorption of urea and Na^+ in this study. However, there is not a significant correlation between urea secretion and Na^+ reabsorption. The equation of the secretion line from this study and the significance of the correlation is $y = 0.122x + 1.91$, $r = 0.14$, NS ($N = 48$ simultaneous measurements from 11 fish). The equation of the reabsorption line from this study is $y = 0.541x + 1.01$, $r = 0.87$, $P < 0.0001$ ($N = 18$ simultaneous measurements from eight fish). The equation of the line from McDonald and Wood (1998) is $y = 0.734x + 0.155$, $r = 0.97$, $P < 0.0001$ ($N = 59$ simultaneous measurements from 15 fish).

the lack of competitive interaction between analogues and urea could be a reflection of urea moving through a specific transport mechanism while the analogues are moving via simple diffusion or through a separate mechanism (a second urea transporter isoform) and thus not interfering with urea movement. Evidence suggests that this might be the case for acetamide across the isolated basolateral membrane of rainbow trout gill (McDonald 2002).

The trout kidney appeared to handle urea and acetamide similarly. On average, both were added to the kidney ultrafiltrate by net secretion by the kidney tubule, although reabsorption occurred in some circumstances. In contrast, there was no net transport of thiourea by the kidney tubule, and thiourea entered the urine entirely by filtration. This pattern of urea and analogue handling is similar to that of the elasmobranch kidney in which acetamide and urea were both reabsorbed while thiourea was not (Schmidt-Nielsen and Rabinowitz 1964; Schmidt-Nielsen et al. 1972). Although the pattern similarity in the trout and shark kidney could be characteristic of a similar mechanism, this finding is complicated by the fact that the elasmobranch kidney has been shown to have a facilitated diffusion urea transporter (ShUT; Smith and Wright 1999) and is believed to reabsorb urea via a Na^+-coupled secondary active mechanism (Schmidt-Nielsen et al. 1972). In comparison, in trout of this study, urea was moving in the secretory direction on a net basis, independent of Na^+ and against a concentration gradient. It is unlikely that the urea transport mechanisms in the gill and kidney are the same, based on the different pattern observed in the kidney plus the fact that urea is moving against a concentration gradient. In addition, the pattern of urea and analogue handling by the trout kidney is different from that observed in the toadfish and midshipman where $SCR_{thiourea} > SCR_{urea} > SCR_{acetamide}$ (McDonald et al. 2000, 2002). This latter difference is most likely a reflection of different urea transport mechanisms and morphology; the handling of urea in batrachoidides is not correlated with Na^+ as observed in trout, and the kidney of toadfish and midshipmen is aglomerular, whereas the kidney of the trout is glomerular.

Interestingly, previous evidence indicated that rainbow trout reabsorbed urea against a concentration gradient, and urea concentrations in the plasma were almost double the concentrations in the urine (reviewed by Wood 1993; McDonald and Wood 1998). In McDonald and Wood 1998, the mean clearance ratio of urea (0.27) was significantly less than 1.0, indicating that 73% of the urea filtered at the glomerulus was then reabsorbed by the kidney tubule. In contrast, in this study, plasma urea concentrations are significantly less than urine urea concentrations, and the mean clearance ratio for urea is 1.46. This indicates that in addition to the filtered urea load, 46% more urea is secreted into the urine against a concentration gradient. Consistent in both studies is the strong correlation between the reabsorption of urea and Na^+ (Figs. 4, 6). The linear relationships of Na^+ reabsorption and urea reabsorption are statistically similar in both studies (slope = 0.73; $P < 0.001$; *y*-intercept not significantly different from 0; previously unreported data from McDonald and Wood 1998; Fig. 6), suggesting the same reabsorptive mechanism for urea is acting in both cases. However, it is clear that no relationship exists between urea secretion and Na^+ or Cl^- reabsorption, perhaps suggesting a second, unique mechanism mediating urea secretion.

It is interesting that under conditions of high Na^+, Cl^-, and water reabsorption there was tubular reabsorption of urea (negative SCR_{urea}), whereas tubular secretion of urea (positive SCR_{urea}) appeared only to occur when Na^+, Cl^-, or water reabsorption was reduced (low negative values of SCR). Dichotomies in urea handling have also been observed in mammals, in which different urea transport mechanisms are induced when urine-concentrating ability is altered. For instance a Na^+-dependent, secondary active, urea-reabsorptive mechanism is present in initial inner medullary collecting ducts (IMCD) from rats fed a low-protein diet for at least 3 wk (Isozaki et al. 1994*a*, 1994*b*). However, this mechanism is not expressed in rats fed a normal (18%) protein diet. In addition, urea can move in the secretory direction via a Na^+-urea counter transport mechanism found in the terminal IMCD of rats given free access to food and water (Kato and Sands 1998). Whereas trout of the recent study were fed up until the time of surgery, trout used

in McDonald and Wood 1998 were starved for 1 wk before surgery. It would be interesting to investigate the long-term effect of a low-protein diet on trout and its implications for urea metabolism and excretion. Another possible factor, stress, through the mobilization of cortisol, may effect urea transport and metabolism (Naruse et al. 1997; Mommsen et al. 1999; Peng et al. 2002).

In summary, it appears that the freshwater rainbow trout has urea transporters in both the gill and the kidney. The patterns of analogue versus urea handling by the gill are similar to observations in three other teleost species, suggesting not only the involvement of a UT-type transporter in the gill of the trout but a characteristic pattern of analogue handling by this type of branchial urea transporter, across a broad range of teleost species. The gill transporter appears to be of the facilitated diffusion type. Urea and analogue handling by the trout kidney exhibits a different pattern from the gill, suggesting different transport mechanisms in the two organs. Indeed, at least one kidney transporter of the trout appears to be of the active type and a second may be Na^+-linked. Further investigation is required to fully understand the implications for urea handling at the level of the kidney with respect to the physiological state of the whole animal.

Acknowledgments

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