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THE MECHANISMS OF UREA TRANSPORT BY EARLY LIFE STAGES OF RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

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Summary

We tested the hypothesis that urea transport in rainbow trout (*Oncorhynchus mykiss*) embryos is dependent, in part, on a bidirectional urea-transport protein. Acute exposure to phloretin and urea analogs [acetamide, thiourea, 1,(4-nitrophenyl)-2-thiourea] reversibly inhibited urea excretion from the embryos to the external water. Unidirectional urea influx was inhibited by acetamide and thiourea, with IC_{50} values of 0.04 and 0.05 mmol l^{-1}, respectively. Influx of urea from the external water to the embryo tended to saturate at elevated external urea concentrations (V_{max}=10.50 nmol g^{-1} h^{-1}; K_m=2 mmol l^{-1}). At very high urea concentrations (20 mmol l^{-1}), however, a second, non-saturable component was apparent. These results indicate that urea excretion in trout embryos is dependent, in part, on a phloretin-sensitive facilitated urea transporter similar to that reported in mammalian inner medullary collecting ducts and elasmobranch kidney.

Key words: urea excretion, phloretin, thiourea, acetamide, ammonia, embryo, nitrogen excretion, rainbow trout, *Oncorhynchus mykiss*.

Introduction

Yolk proteins are the major fuel utilised by developing teleost embryos (Kamler, 1992), and the removal of nitrogenous waste products is vital to avoid accumulation to toxic levels. Studies of nitrogen excretion in rainbow trout embryos have clearly demonstrated ammonia excretion soon after fertilization (Smith, 1947; Rice and Stokes, 1974; Wright et al., 1995a; Rahaman-Noronha et al., 1996). In earlier studies, we demonstrated that ammonia diffusion from the embryo is dependent primarily on the partial pressure gradient of ammonia (NH_3), facilitated by an acidic unstirred layer of water on the external water side of the chorion (Rahaman-Noronha et al., 1996). Although early stages are ammoniotelic, a significant proportion (up to 26 %) of nitrogenous wastes are excreted as urea in rainbow trout (Wright and Land, 1998). In adult trout, urea accounts for approximately 10 % of total nitrogen excreted (Wood, 1993).

Traditionally, urea was thought to permeate cell membranes solely by simple diffusion through unspecialized routes. However, urea solubility is quite low in lipid bilayers (Gallucci et al., 1971) and, in conditions where a high rate of urea transport is necessary, such as in the mammalian kidney, urea permeability occurs at much higher rates than can be explained by simple diffusion (Gillin and Sands, 1993). In recent years, several different mechanisms of urea transport have been reported in tissues of various animals, and it is now clear that the transport of this molecule is far from simple. For example, Na^+-independent (primary) active urea transporters have been reported in the skin of frogs *Rana esculenta* (Lacoste et al., 1991) and toads *Bufo marinus* (Dytko et al., 1993). Active, Na^+-dependent (secondary) urea transport occurs in the initial inner medullary collecting ducts (IMCDs) of rats fed a low-protein diet (Isozaki et al., 1994; Sands et al., 1996). Simple, or non-mediated, diffusion of urea has been identified across the gills of a teleost fish (*Oligocottus maculosus*) (Wright et al., 1995b) and in various other species and tissues (Kaplan et al., 1974; Wood et al., 1989; Walsh et al., 1994; Carlson and Goldstein, 1997).

Finally, there is evidence for facilitated urea transport in mammalian tissues such as the rat kidney (Chou and Knepper, 1989; Chou et al., 1990), human red blood cells (Brahm, 1983; Mayrand and Levitt, 1983) and the rat liver (Effros et al., 1993). In addition, facilitated urea transport has been reported in the urinary bladder of toads and frogs (Levine et al., 1973a,b; Shpun and Katz, 1990; Martial et al., 1991) and in the kidney of the dogfish *Squalus acanthias* (Hays et al., 1976; Smith and Wright, 1999). In teleosts, a facilitated urea transporter has been identified in the red blood cells of redfish *Scianops ocellatus* and in the hepatocytes of two species of toadfish, *Opsanus beta* and *O. tau* (Walsh et al., 1994; Walsh and Wood, 1996). Wood et al. (1998) proposed that pulsatile urea excretion across the gills of the gulf toadfish *Opsanus beta* was linked to the activation of facilitated branchial urea transporters. Further, bidirectional diffusion appears to be a key property of facilitated urea transporters (Chou et al., 1990), including the gulf toadfish gill transporter (Wood et al., 1998). The cDNAs for several facilitated urea-transport proteins have been isolated and

characterized (for a review, see Sands et al., 1997). More recently, a facilitated transporter in the dogfish kidney (*Squalus acanthias*; Smith and Wright, 1999) and in the gulf toadfish gill (*Opsanus beta*; Walsh, 1999) have been cloned.

Recent studies in our laboratory suggested that a specialized urea-transport process might be present in embryos of rainbow trout *Oncorhynchus mykiss*. Urea concentrations in the yolk of rainbow trout embryos were five times higher than in the perivitelline fluid, indicating that the gradient for urea was substantial (Rahaman-Noronha, 1995). When embryos were exposed to elevated levels of external urea (2 mmol l^{-1}), the level of urea in the perivitelline fluid increased fourfold to match external levels, but no changes were observed in the yolk urea levels. The fact that yolk urea concentrations were unaltered by a large increase in the urea gradient from the perivitelline fluid to the yolk suggested that the yolk-sac membrane was relatively impermeable to urea. Rahaman-Noronha (1995) suggested that a specialized urea transporter could be involved in the regulation of urea transport across this membrane. Furthermore, when trout embryos were exposed to phloretin (0.2 mmol l^{-1}), an inhibitor of the facilitated urea-transport protein in other tissues (Sands et al., 1997), a complete inhibition of urea excretion was observed (K. K. Moore and P. A. Wright, unpublished observations).

The objective of this study was to determine the mechanisms of urea transport in embryonic rainbow trout. We tested the null hypothesis that urea transport occurs solely by simple diffusion. The alternative hypothesis was that urea transport is dependent, in part, on a urea-transport protein. A series of net excretion experiments was performed by exposing embryos to an inhibitor (phloretin) and to analogs of urea [acetamide, thiourea or 1,(4-nitrophenyl)-2-thiourea (NPTU)], added to the external water. A series of unidirectional influx experiments was also performed using radiolabelled urea. If the putative urea transporter in the embryos is bidirectional, then the flux of urea into the embryos can provide information regarding the mechanism of transport. Urea transporters in rat kidney (Chou et al., 1990) and the gills (Wood et al., 1998) of the gulf toadfish are bidirectional, and urea is transported efficiently in both directions, depending on the urea concentration. The rate of urea influx into the embryos was measured under control conditions and during exposure to varying concentrations of external acetamide or thiourea. To determine whether urea influx was saturable, the rate of urea influx was measured in the presence of different concentrations of external urea. We also compared the rate of thiourea and urea influx to determine whether thiourea influx saturates at high external thiourea concentrations.

Materials and methods

Animals

Rainbow trout [*Oncorhynchus mykiss* (Walbaum)] embryos at the eyed stage of development (16–32 days post-fertilization) were purchased from Rainbow Springs Trout Farm (Thamesford, Ontario, Canada) or donated from Alma Research Station (Alma, Ontario, Canada) or Blue Springs Trout Hatchery (Hanover, Ontario, Canada). The embryos were maintained in incubation trays with a continuous flow of well water. Water temperature and photoperiod varied with season (8.5–15.5 °C, pH 7.9).

Experimental protocol

Net excretion experiments

Two series of excretion experiments were performed to determine the ammonia and urea excretion rates of the embryos under control conditions (series A; 28 days post-fertilization, 13.0 °C) and during exposure to various treatments (series B). Urea and/or ammonia excretion to the water was measured over three consecutive periods of 30 min each; identified as initial, treatment and recovery flux periods. To determine whether excretion rates varied over these three flux periods, a control experiment (series A) was performed by exposing the embryos to fresh water for each of the three flux periods. For experiments involving urea analogs or inhibitors (series B), the initial control period (fresh water) was followed by exposure to the 'treatment' water, followed by a final recovery control period (fresh water). The experiments described below were all performed at different times of the year, and the experimental temperature was therefore adjusted to match the temperature at which the embryos were maintained at the fish farm. This resulted in different experimental temperatures for each treatment. Thus, the initial and recovery flux periods for each experiment represented intrinsic controls that could be compared directly with the treatment period. The small differences in water temperature and developmental stage in each experiment may result in some variability when comparing excretion rates between the experiments. The embryos were exposed to the following treatments: (1) phloretin, 0.05 mmol l^{-1} in a final concentration of 0.02 % ethanol (36 days post-fertilization, 9.0 °C); (2) ethanol (0.02 %) control (28 days post-fertilization, 13.0 °C); (3) 1,(4-nitrophenyl)-2-thiourea (NPTU), 0.050 or 0.075 mmol l^{-1} (25 days post-fertilization, 11.0 °C); (4) acetamide, 1.80 mmol l^{-1} (35 days post-fertilization, 9.0 °C); and (5) thiourea, 1.80 mmol l^{-1} (33 days post-fertilization, 8.5 °C).

On the day prior to the experiments, embryos (2 g, approximately 30–34 embryos) were placed in perforated polystyrene beakers supplied with flowing water and allowed to acclimate for 18–24 h. At the onset of the experiment, water inflow was stopped, and individual beaker volume was fixed at 8 ml (by placing the perforated beaker into an intact beaker). The water was aerated gently for the duration of the experiment.

Preliminary experiments demonstrated that the slight change in pH (approximately 0.35 units over 30 min) that occurred over the experimental period had no effect on ammonia or urea excretion rates; thus, the pH of the experimental water was not maintained. The relatively short flux period of 30 min was chosen to minimize the exposure to analogs and inhibitors that could potentially influence nitrogen metabolism, while ensuring a sufficient period in which to measure net excretion

rates to the water. The recovery period was necessary, therefore, to determine whether the effects of the inhibitors or analogs were reversible and whether they had any marked metabolic effects on the embryos. In addition, the influence of urea analogs on ammonia excretion rates were also determined using the above protocol. If exposure to these analogs had any influence on the overall metabolism of the embryos, then it would be likely also to influence ammonia excretion.

To eliminate any possible carry-over of water from one flux period to the next, the embryos were carefully rinsed three times with fresh water, or the treatment water, between each flux period. Agitation of the embryos was kept to a minimum, and the rinsing procedure was performed in less than 1 min. An initial (0 h) water sample (8 ml) was collected from the bulk water container, and a final sample (8 ml) was collected at the end of the flux period (0.5 h) for determination of urea and ammonia concentrations.

Preliminary experiments were conducted with phloretin concentrations (0.5 mmol l^{-1}: Brahm, 1983; Chou and Knepper, 1989; Walsh et al., 1994) and NPTU concentrations (0.1 mmol l^{-1}: Mayrand and Levitt, 1983; Martial et al., 1991) commonly used in experiments on tissues from other animals. Although a significant inhibition of urea excretion was observed at these elevated inhibitor and analog concentrations, there was no recovery upon return to fresh water. As a result, lower concentrations of phloretin and NPTU were used in the present study. Ethanol was chosen as a vehicle for dissolving phloretin because preliminary experiments using the organic solvent dimethyl sulphoxide (DMSO, 0.2 %; Sigma Chemical Co., Oakville, Ontario, Canada) resulted in highly inconsistent results. The minimal ethanol concentration (0.02 %) necessary to dissolve the phloretin was determined.

In the present study, the concentrations of the urea analogs (thiourea and acetamide, 1.80 mmol l^{-1}) were chosen to be approximately twice the tissue concentration of urea in just-hatched free embryos (0.90±0.001 mmol l^{-1}; mean ± S.E.M., N=3). Other studies have used analog concentrations that were three times the tissue urea levels or that matched tissue urea levels (Wood et al., 1998; Wright et al., 1995b). NPTU is one of the most potent urea analogs (Mayrand and Levitt, 1983), and much lower NPTU concentrations were chosen, as described above. All inhibitor and analogs were purchased from Sigma Chemical Co. (Oakville, Ontario, Canada).

Unidirectional flux experiments

Five series of experiments (series C–F) involving the use of ^{14}C radioisotopes were performed to characterize the saturation kinetics of urea and thiourea transport and to examine the effects of urea analogs on the unidirectional flux of urea. Just-hatched or free embryos (approximately 29–40 days post-fertilization) within 3 days of hatching were used for all the radioisotope experiments. Post-hatch embryos were chosen for these experiments to focus on the putative transport processes on the external surface (i.e. cutaneous and yolk-sac membranes), without the potentially complicating influence of uptake across the chorion and into the perivitelline fluid.

Series C. To determine whether urea influx is saturable, unidirectional urea influx (J^{in}_{urea}) was measured over a range of urea concentrations in the external water. Free embryos (N=7; 35 days post-fertilization, 13.5 °C) were exposed to 0.05, 0.5, 2.5, 5, 10 or 20 mmol l^{-1} urea for 8 h. The concentrations were chosen to ensure that the urea concentrations in the external water were considerably higher than internal levels. An external urea concentration of 20 mmol l^{-1} is more than 20 times higher than tissue urea levels. In the rat terminal IMCD, urea concentrations were used that were 2–3 times higher than the concentration of urea in that tissue (Chou et al., 1990). Previous studies in other tissues have demonstrated that the facilitated urea transporter saturates only in the presence of relatively large urea gradients (Mayrand and Levitt, 1983; Chou et al., 1990; Couriaud et al., 1996).

Series D. The effects of urea analogs on unidirectional urea influx (J^{in}_{urea}) were investigated. Free embryos (N=7) were exposed (8 h) to either external acetamide (0, 0.05, 0.5, 5 or 20 mmol l^{-1}; 40 days post-fertilization, 11.0 °C) or thiourea (0, 0.05, 0.5, 5 or 20 mmol l^{-1}; 38 days post-fertilization, 12.5 °C) in the presence of 20 mmol l^{-1} urea, and J^{in}_{urea} was measured. The IC_{50} values were obtained by interpolation, as described by Pallone (1994). The IC_{50} value represents the concentration of the analog at which urea flux is inhibited by 50 %.

Series E. To compare the concentration-dependence of thiourea flux with that of urea, similar unidirectional flux experiments were conducted as described in series C. Chou et al. (1990) reported that thiourea transport in the rat IMCD saturated at high thiourea concentrations. Varying concentrations of thiourea (0.50, 2.5, 5, 10 or 20 mmol l^{-1}) were added to the external water for 8 h, and $J^{in}_{thiourea}$ was measured (N=7; 40 days post-fertilization, 11.0 °C).

Series F. Unidirectional influx (J^{in}) rates of thiourea and urea were compared. In previous studies in different tissues, the urea permeability coefficient of thiourea was found to be considerably lower (less than 25 % of urea permeability) than that of urea (Chou et al., 1990; Verbavatz et al., 1996; Wood et al., 1998), suggesting that thiourea was probably transported *via* the urea transporter, but at a lower rate. Thiourea (20 mmol l^{-1}) or urea (20 mmol l^{-1}) was added to the external water for 8 h, and J^{in} was measured (N=7; 38 days post-fertilization, 9.5 °C; 29 days post-fertilization, 15.5 °C).

Analysis and calculations

Colorimetric assays were used to determine levels of ammonia and urea in the water samples. The salicylate–hypochlorite assay described by Verdoux et al. (1978) and the diacetyl monoxime method described by Rahmatullah and Boyde (1980) were used to measure ammonia and urea concentrations, respectively. Net excretion rates (J^{net}; μmol g^{-1} h^{-1}) for ammonia and urea were calculated as the difference in concentration (μmol-N l^{-1}) multiplied by the volume of the chamber (l) divided by the mass of the embryos (g) and the flux period (h; 1 μmol l^{-1} of urea=2 μmol-N l^{-1} urea, 1 μmol l^{-1} ammonia=1 μmol-N l^{-1} ammonia;, N is nitrogen). The percentage of total nitrogen (ammonia+urea) excreted as

urea was expressed as the urea concentration (μmol-N l^{-1}) divided by the total ammonia-N plus urea-N concentration (μmol-N l^{-1}), multiplied by 100.

In preliminary experiments, the urea excretion rate and, consequently, the final water concentration was very low, approaching the limit of detection of the urea assay (<0.5 μmol l^{-1}). Water samples (5 ml) and standards (5 ml) were freeze-dried and reconstituted in a smaller volume of distilled water (2 ml) to concentrate the urea. To eliminate any possible effect of the inhibitors or analogs on the colorimetric assay, inhibitors or analogs were added to the urea standards at the same concentration as in the original experiment. Urea influx (J^{in}_{urea}) was measured by adding 0.48 GBq of [^{14}C]urea (Mandel Scientific Ltd, Guelph, Ontario, Canada) or [^{14}C]thiourea (Sigma Chemical Co., Oakville, Ontario, Canada) per μmol l^{-1} of unlabelled urea (or thiourea) to gently aerated beakers of water (12.2 ml). Five free embryos (approximately 0.3 g) were placed in each beaker and left for 10 min to ensure adequate mixing of the water. Water samples (1.4 ml) were removed at 0 h and 8 h. To displace any radiolabelled urea that might be bound to the body surface, the free embryos were placed in a solution containing a very high urea concentration (50 mmol l^{-1}) for 1 min. The free embryos were then blotted and weighed. Standard procedures for tissue digestion and scintillation counting were followed, as described by Wright et al. (1995b). Urea influx, J^{in}_{urea} (μmol g^{-1} h^{-1}), was calculated as:

$$J^{in}_{urea} = \frac{^{14}\text{C radioactivity in free embryo}}{Wt} \times \frac{1}{SA_{ext}} ,$$

where W is the mass of the free embryos (g), t is the duration of the flux period (h) and SA_{ext} is the mean specific activity of [^{14}C]urea (cts min^{-1} ml^{-1} water per μmol $^{-1}$ urea ml^{-1} water). The radioactivity of the free embryo was measured in cts min^{-1}.

Statistical analyses

All data are expressed as means ± the standard error of the mean (S.E.M.). A one-way analysis of variance (ANOVA) was used to compare differences in mean excretion rates among the three flux periods (excretion experiments) and to compare urea influx (J^{in}_{urea}) of free embryos exposed to various concentrations of external acetamide or thiourea. If significant differences were observed, a multiple-comparison test was performed using Tukey's HSD method (Kuehl, 1994). A Student's unpaired t-test was used to compare influx (J^{in}) between urea and thiourea (P<0.05). For saturation experiments, linear regression analysis was performed, using the method of least squares, and the significance of the Pearson's correlation coefficient, r, was evaluated.

Results

Net excretion experiments

Excretion rates for both ammonia and urea were unchanged throughout the three control flux periods (Table 1). Under these control conditions, urea excretion accounted for 6–8 % of the total nitrogenous waste excretion for the initial, control and recovery flux periods.

Table 1. *Ammonia and urea excretion rates of the initial, control and recovery flux periods (30 min) of eyed rainbow trout embryos under control conditions*

	Ammonia excretion rate (nmol g^{-1} h^{-1})	Urea excretion rate (nmol g^{-1} h^{-1})
Initial	143.39±9.13	4.81±0.69
Control	139.47±8.52	4.61±0.31
Recovery	131.42±6.62	5.41±0.95

Values are means ± 1 S.E.M. (N=8).

Exposure of the embryos to phloretin (0.05 mmol l^{-1}) resulted in 100 % inhibition of urea excretion, followed by a recovery to control excretion rates upon return to fresh water (Fig. 1A). Net urea excretion was negative in the presence of phloretin, indicating that the concentration of urea in the experimental chamber at the end of the 30 min flux period was lower than the initial concentration at time 0 h. This phenomenon was reproducible (six separate experiments, N=6–8 each time) and was also observed in the analog experiments described below. Ethanol (0.02 %), which was used as a vehicle to dissolve the phloretin, had no effect on the urea excretion rates of the embryos (data not shown).

Both acetamide (1.80 mmol l^{-1}) and thiourea (1.80 mmol l^{-1}) completely inhibited urea excretion with full recovery (Fig. 1B,C). As with the inhibitor phloretin, excretion rates were negative during the experimental flux period, a phenomenon that was reproducible. Acetamide (1.80 mmol l^{-1}) and thiourea (1.80 mmol l^{-1}) had no effect on ammonia excretion rates (data not shown).

Exposure to 0.05 mmol l^{-1} NPTU did not significantly lower the excretion rates from values observed in the initial and recovery flux periods. However, at a slightly higher NPTU concentration (0.075 mmol l^{-1}), exposure to this analog resulted in a 58 % inhibition of urea excretion (Fig. 1D).

Unidirectional flux experiments

When free embryos were exposed to varying concentrations of thiourea in the presence of 20 mmol l^{-1} urea, the rate of urea influx decreased significantly at relatively low external thiourea concentrations (Fig. 2). The greatest inhibition of urea influx (76 %) occurred at 0.05 mmol l^{-1} thiourea. The IC_{50} for thiourea was approximately 0.5 mmol l^{-1}. A similar trend was observed for free embryos exposed to different concentrations of acetamide, with the greatest inhibition (72 %) also occurring at 0.5 mmol l^{-1} acetamide (Fig. 3). The IC_{50} for acetamide was approximately 0.04 mmol l^{-1}. Two overlapping trends were observed in both the urea and thiourea influx experiments. Urea influx (J^{in}_{urea}) appeared to saturate, with a

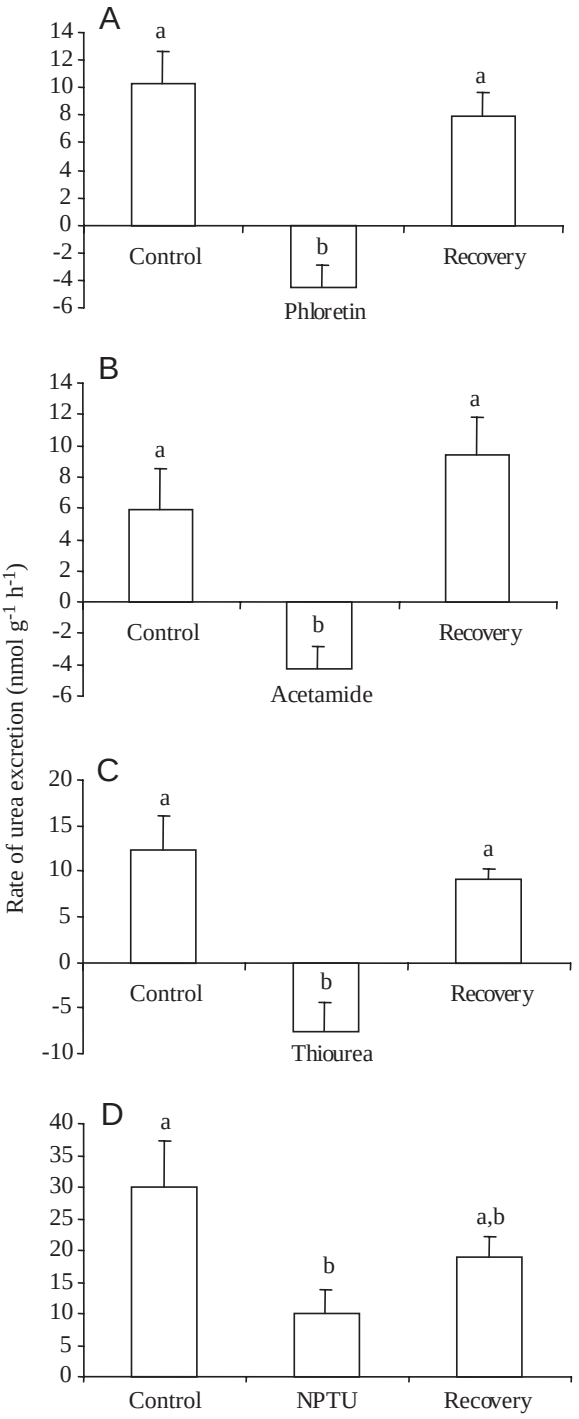

Fig. 1. Urea excretion rates (means + S.E.M.) of eyed rainbow trout embryos exposed for 30 min to (A) the inhibitor of facilitated urea transport phloretin (0.05 mmol l^{-1}; 36 days post-fertilization, 9.0 °C), (B) the urea analog acetamide (1.80 mmol l^{-1}; 35 days post-fertilization, 9.0 °C), (C) the urea analog thiourea (1.80 mmol l^{-1}; 33 days post-fertilization, 8.5 °C) or (D) the urea analog 1,(4-nitrophenyl)-2-thiourea (NPTU; 0.075 mmol l^{-1}; 25 days post-fertilization, 11.0 °C). Phloretin was dissolved in ethanol to a final concentration of 0.02 %. Urea excretion in the control and recovery flux periods was measured during exposure (for 30 min) to fresh water. Identical letters indicate values that are not significantly different (P<0.05; N=8).

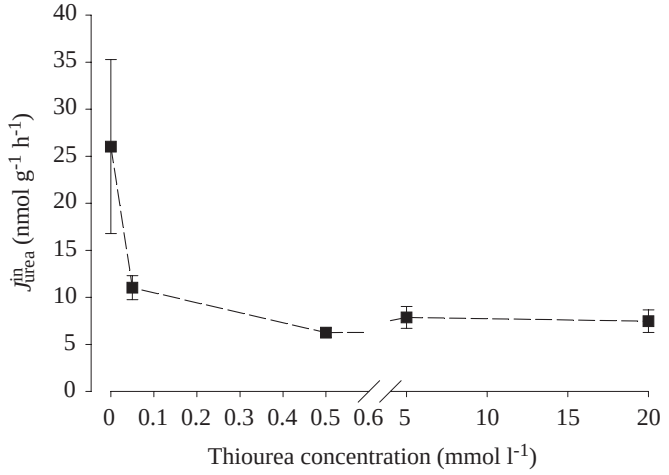

Fig. 2. The rate (mean ± S.E.M.) of urea influx (J^{in}_{urea}) of just-hatched rainbow trout embryos exposed (for 8 h) to varying concentrations of external thiourea (0, 0.05, 0.5, 5 or 20 mmol l^{-1}) in the presence of 20 mmol l^{-1} urea (N=7).

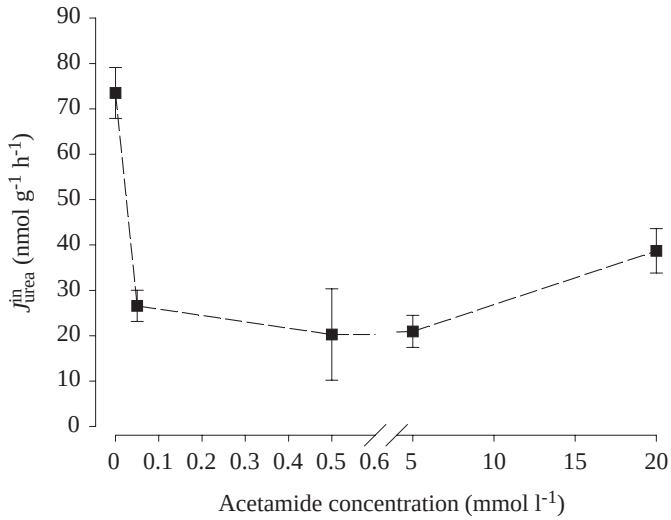

Fig. 3. The rate (mean ± S.E.M.) of urea influx (J^{in}_{urea}) of just-hatched rainbow trout embryos exposed (for 8 h) to varying concentrations of external acetamide (0, 0.05, 0.5, 5 or 20 mmol l^{-1}) in the presence of 20 mmol l^{-1} urea (N=7).

V_{max} of 10.50 nmol g^{-1} h^{-1}. The K_m, determined by interpolation, was 2 mmol l^{-1}. At the highest external urea concentration (20 mmol l^{-1}), a second transport process was apparent because urea influx did not remain saturated (Fig. 4). Thiourea influx appeared to saturate with a V_{max} of 7.80 nmol g^{-1} h^{-1} and a K_m of 0.4 mmol l^{-1} (Fig. 5). A second non-saturable transport process for thiourea influx was observed at relatively high external thiourea concentrations (10–20 mmol l^{-1}; Fig. 5), as in the urea influx experiment.

Urea and thiourea influx were compared, but there were no significant differences (P>0.05): J^{in}_{urea}=28.32±4.37 nmol g^{-1} h^{-1} and $J^{in}_{thiourea}$=18.54±4.05 nmol g^{-1} h^{-1}; N=7.

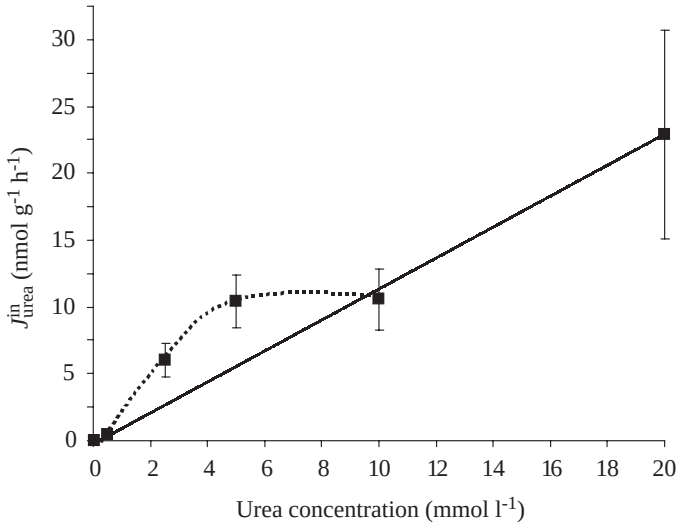

Fig. 4. The rate (mean ± S.E.M.) of urea influx (J^{in}_{urea}) of just-hatched rainbow trout embryos exposed to varying concentrations of external urea (0.05, 0.5, 2.5, 5, 10 and 20 mmol l^{-1}) for 8 h (N=7). The dotted line represents the saturable component of urea influx, and the solid line represents the simple diffusion component (the origin is equal to zero).

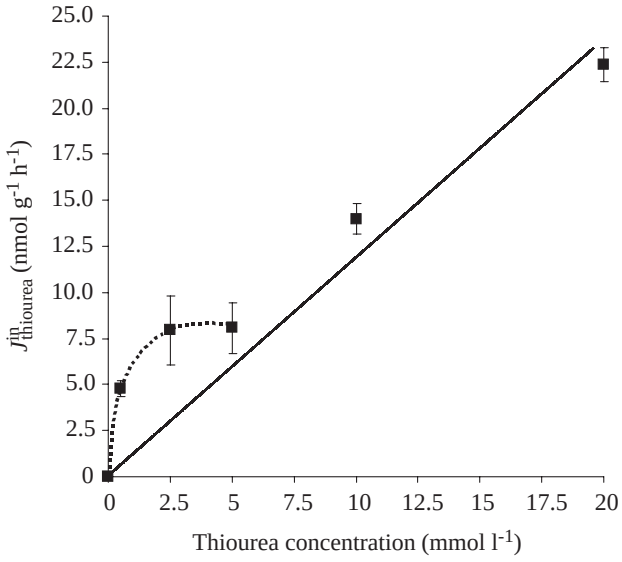

Fig. 5. The rate of thiourea influx ($J^{in}_{thiourea}$) of just-hatched rainbow trout embryos exposed to varying concentrations of external thiourea (0.5, 2.5, 5, 10 and 20 mmol l^{-1}) for 8 h (N=7). The dotted line represents the saturable component of thiourea influx, and the solid line represents the simple diffusion component (the origin is equal to zero).

Discussion

The results of this study indicate that urea excretion in embryos of rainbow trout (*Oncorhynchus mykiss*) does not occur solely by passive diffusion, and we therefore reject the null hypothesis. The data provide strong evidence for our alternative hypothesis that the diffusion of urea is dependent, in part, on a specialized urea transporter with characteristics similar to those of urea transporter proteins found in mammalian kidney and red blood cells. Urea excretion by embryonic trout was completely (100 %) inhibited by phloretin (0.05 mmol l^{-1}), and this phenomenon was reversible. In other animals and tissues, reversible inhibition of urea flux by phloretin has been accepted as one of the hallmarks of facilitated urea transport. For example, in human red blood cells, phloretin inhibited urea permeability by more than 97 % (Brahm, 1983). Similarly, phloretin significantly inhibited urea permeability in the rat terminal IMCD and in the toad urinary bladder (Chou and Knepper, 1989; Levine et al., 1973a).

Another well-described characteristic of facilitated urea transporters is their sensitivity to various urea analogs. Urea excretion was completely and reversibly inhibited by exposure to external thiourea (1.80 mmol l^{-1}) and acetamide (1.80 mmol l^{-1}). In addition, exposure to NPTU (0.075 mmol l^{-1}) inhibited urea excretion by 58 %. In the rat kidney and brain, thiourea significantly inhibited urea transport (Chou and Knepper, 1989; Couriaud et al., 1996). Similar levels of inhibition of urea transport by these urea analogs has been reported in human red blood cells (Olives et al., 1994), frog urinary bladder (Levine et al., 1973a,b; Martial et al., 1991) and toadfish gills (Wood et al., 1998). Exposure of embryos to acetamide (1.80 mmol l^{-1}) and thiourea (1.80 mmol l^{-1}) had no effect on ammonia excretion rates (results not shown), suggesting that nitrogen metabolism was not markedly affected by exposure to urea analogs.

In the net excretion experiments, the inhibitors were added to the external water (*trans*), whereas urea, the substrate, was naturally present within the embryo. According to kinetic theory, the observed inhibition would be non-competitive even though these urea analogs are considered to be competitive inhibitors (Krupka and Devés, 1983). The analog is not directly competing with urea for the binding site on the transport protein. Hence, because a non-competitive inhibitor essentially removes the functional carrier from the system, a greater inhibition of urea flux was observed than would be expected with a competitive inhibitor at the same concentration (Stein, 1990).

The concentration-dependent inhibition of urea flux by acetamide and thiourea was also examined. Urea influx was maximally inhibited by acetamide (72 % inhibition) and thiourea (76 % inhibition), with IC_{50} values of 0.04 and 0.05 mmol l^{-1}, respectively. K_i (half-inhibition constant; same as IC_{50}) values for thiourea inhibition have been determined in rat IMCDs (27 mmol l^{-1}, Chou and Knepper, 1989) and human red blood cells (12 mmol l^{-1}, Mayrand and Levitt, 1983; 13 mmol l^{-1}, Brahm, 1983). In human red blood cells, the K_i value for acetamide inhibition was 100 mmol l^{-1} (Mayrand and Levitt, 1983). These values are all notably higher than the IC_{50} values determined in the present study. This is not surprising considering the much lower tissue urea concentrations in trout embryos (approximately 0.9 mmol l^{-1}) compared with mammalian tissues such as the renal inner medulla (400–1500 mmol l^{-1}; Marsh and Knepper, 1992).

If a transport protein were involved in urea transport in rainbow trout embryos, it would be expected that urea influx would saturate at elevated concentrations of urea in the external water. In the present study, it appears that there may be two overlapping mechanisms involved in urea transport in these embryos. First, there was evidence for saturation of urea influx with a K_m of approximately 2 mmol l^{-1}. Typically, K_m values are very similar to the *in vivo* substrate concentration (Hochachka and Somero, 1984). The K_m value calculated in the present study was approximately twice the tissue level of urea found in whole rainbow trout embryos (0.9 mmol l^{-1}).

At the highest external urea concentration (20 mmol l^{-1}), a second non-saturable process appears to be involved that resembles simple urea diffusion. The external urea concentration was 20 times greater than the tissue urea level in the whole embryos (0.9 mmol l^{-1}) and, therefore, quite unphysiological. The large gradient from the external water to the embryo would undoubtedly result in a massive influx of urea through unspecialized pathways (i.e. aqueous pores). Saturation of urea flux has been reported in the frog urinary bladder (K_m=88 mmol l^{-1}; Shpun and Katz, 1990), the rat IMCD (K_m=150 mmol l^{-1}; Chou et al., 1990) and human erythrocytes (K_m=218 mmol l^{-1}; Mayrand and Levitt, 1983). Saturation of urea transport has not been demonstrated in mammalian red blood cells (Brahm, 1983) and dogfish kidney tubules (Kempton, 1953).

In the present study, thiourea influx appeared to saturate (K_m=0.4 mmol l^{-1}) as the external thiourea concentration was increased, suggesting that thiourea is being transported by the putative urea transporter. As in the urea influx experiment, there was also a second, non-saturable component of thiourea transport. Saturation of thiourea flux has been demonstrated in the rat kidney (Chou et al., 1990) and brain (Couriaud et al., 1996) and in mammalian red blood cells (Mayrand and Levitt, 1983; Martial et al., 1996). The K_m for thiourea saturation was somewhat lower than the K_m for urea, suggesting that the urea transporter has a higher affinity for thiourea. Mayrand and Levitt (1983) suggest that the more efficient transport of urea, compared with thiourea, could be due to a conformational change that occurs in the carrier protein after binding to urea resulting in an increase in the rate of transport across the membrane. Urea would then have a low affinity and high transport rate, whereas thiourea would have a higher binding affinity, but a lower rate of transport. Studies on the rat IMCD, mammalian red blood cell and toadfish gill have shown that permeability to thiourea was lower than permeability to urea (Mayrand and Levitt, 1983; Chou et al., 1990; Wood et al., 1998). In our study, the rates of urea and thiourea transport were the same, although thiourea tended to have a lower K_m (a higher affinity) and thiourea influx saturated at high external thiourea concentrations, suggesting carrier-mediated transport of thiourea.

In the case of the unidirectional flux experiments, the possibility of urea entering the free embryos *via* pathways other than simple or facilitated diffusion must not be dismissed. For instance, if the fish were drinking at this early stage, then some additional label may have been imbibed. Many fish species hatch without a mouth, but it develops very quickly after hatching (Blaxter, 1988). Tytler et al. (1990) provided evidence for drinking by free embryos of rainbow trout at 4 days after hatching (0.22±0.01 μl g^{-1} h^{-1}), and the drinking rate increased in proportion to the increase in age. All influx experiments in the present study were performed within 1–3 days after hatching. By using the drinking rate reported by Tytler et al. (1990), the intake of [^{14}C]urea due to drinking may account for 2–20 % of the total [^{14}C]urea found in the whole embryos after the 8 h experiment. If embryos were, in fact, drinking immediately after hatching, then it is likely that the drinking rate would be somewhat lower than that reported by Tytler et al. (1990). Thus, in the present study, drinking probably represents an even smaller estimated 'error' than 2–20 %. However, if drinking did represent rates as high as those reported by Tytler et al. (1990), then the inhibitory effects of thiourea and acetamide on urea influx would not be as marked. Overall, we presume that, if urea was imbibed, it constituted a relatively small component of the total uptake in these experiments.

One interesting finding was that the net flux of urea was reversed in the presence of the inhibitor phloretin and the two urea analogs acetamide and thiourea (Fig. 1A–C), but not in response to exposure to NPTU (Fig. 1D). These (repeatable) results are unlikely to be due to an artefact because of the precautions taken with the urea assay (see Material and methods). Also, we ruled out the possibility that the urea analogs were contaminated with urea. Regardless, the data support the conclusion that, when inhibitors or analogs bind to or modify the putative urea transporter, it is no longer available for urea transport.

It should be noted that the urea excretion rates for the control and recovery periods were higher for the NPTU experiment (Fig. 1D) than for other experiments (Table 1, Fig. 1A–C). This variability could result from genetic variation or from differences in developmental stage or metabolic rates due to temperature. The experimental temperature was 2.0–2.5 °C higher for the NPTU experiment than for the other net excretion experiments and this cannot, therefore, be the sole factor involved. This highlights the importance of having intrinsic controls for each experiment that can be directly compared with the treatment period.

Where might a facilitated urea transport protein be located? The cutaneous surface is thought to be the primary site of respiratory gas exchange in trout larvae (Rombough, 1998), although the sites of ammonia and urea nitrogen excretion have not been determined. Nitrogen excretion occurs primarily at the gills in adult fish, and the site of nitrogen excretion therefore probably varies during development. Preliminary northern analysis of mRNA isolated from the gills of adult trout revealed an mRNA of approximately 2000 bases homologous to the mRNA of the gulf toadfish (*Opsanus beta*) gill urea transporter (tUT) (C. Pilley, P. Wright and P. Walsh, unpublished data). Further investigations of the molecular nature of urea transporters in early developmental stages using

techniques such as *in situ* hybridization should provide answers to these interesting questions.

In conclusion, we tested the null hypothesis that urea excretion in embryos of rainbow trout occurs by simple diffusion. Urea excretion was significantly inhibited after acute exposure to the facilitated urea transport inhibitor phloretin and after exposure to the urea analogs NPTU, acetamide and thiourea. Further, unidirectional urea influx appeared to saturate as external urea concentration increased. At the highest urea concentration (20 mmol l^{-1}), however, a second, non-saturable component involving non-specific pathways was evident. These results provide evidence for our alternative hypothesis that urea transport in rainbow trout (*Oncorhynchus mykiss*) embryos is dependent, in part, on a facilitated urea transporter protein.

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