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UREA TRANSPORT BY HEPATOCYTES AND RED BLOOD CELLS OF SELECTED ELASMOBRANCH AND TELEOST FISHES

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Summary

Although urea transport is receiving increased attention in mammalian systems, very little is known about urea transport in fish tissues. This study examined mechanisms of urea transport in red blood cells and hepatocytes from the lesser spotted dogfish (*Scyliorhinus canicula*), Atlantic stingray (*Dasyatis sabina*), turbot (*Scopthalmus maximus*), redfish (*Scianops ocellatus*), gulf toadfish (*Opsanus beta*) and oyster toadfish (*Opsanus tau*). Urea appeared to be passively distributed in both tissues (i.e. there was no difference between plasma and tissue urea concentrations). Additionally, a number of *in vitro* experiments examining [^{14}C]urea flux were performed. In red blood cells from all species except redfish, urea transport occurred *via* simple passive diffusion, but redfish red blood cells showed a small (25 %) phloretin-sensitive uptake component. In hepatocytes of the two elasmobranch species (dogfish and stingray), urea efflux was also by simple passive diffusion. However, urea efflux in toadfish (both *O. beta* and *O. tau*) hepatocytes exhibited a marked phloretin-sensitivity, and *O. beta* hepatocytes were used in further experiments with other inhibitors and treatments. Urea transport in *O. beta* had a relatively high specificity for urea compared with the urea analogues acetamide, thiourea and *N*-methylurea, was unaffected by phloridzin and extracellular Na^+ removal, and was not inhibited by physiological levels of glucose (0.5–10 mmol l^{-1}). A phloretin-sensitive glucose transport, that was not inhibited by physiological levels of urea, was discovered in *O. beta* hepatocytes. The results are discussed in terms of patterns of species distribution and similarities between urea and glucose transport.

Key words: urea transport, red blood cells, hepatocytes, *Opsanus beta*, *Opsanus tau*, *Dasyatis sabina*, *Scyliorhinus canicula*, *Scopthalmus maximus*, *Scianops ocellatus*, phloretin-sensitive transport, glucose transport.

Introduction

Until recently, the generally held views were that biological membranes are highly permeable to the urea molecule and that urea is passively distributed between tissue and extracellular compartments; these views are, in fact, pervasive in physiology textbooks. However, the urea permeability of artificial bilayer membranes is relatively low (4×10^{-6} cm s^{-1}) (Galluci *et al.* 1971), and it has been known for some time that urea transport in human red blood cells occurs largely by facilitated diffusion (see references in Stein, 1986). Recently, a number of physiological observations in the mammalian kidney have rekindled interest in urea transport and have led to the view that urea is transported by facilitated diffusion and perhaps also by secondary active cotransport with Na^+ (see review by Gillin and Sands, 1993). Urea transport by kidney tubules is inhibited by phloretin and a variety of competitive urea analogues (Chou and Knepper, 1989; Zhang and Verkman, 1990). An apparently active reabsorption of urea by the kidney of elasmobranchs has been recognized for many years (Smith, 1936; Kempton, 1953). However, opinions still vary as to whether this is a true active reabsorption (e.g. Schmidt-Nielsen and Rabinowitz, 1964; Schmidt-Nielsen *et al.* 1972), a facilitated diffusion (e.g. Hays *et al.* 1976) or a simple passive diffusion (e.g. Boylan, 1972), any of which could be facilitated by the complex countercurrent anatomy of the elasmobranch nephron (Lacy *et al.* 1985; Lacy and Reale, 1991). Nevertheless, it is clear that the elasmobranch kidney transport system can discriminate amongst various competitive urea analogues (Schmidt-Nielsen and Rabinowitz, 1964) and is inhibited by phloretin (Hays *et al.* 1976), which strongly suggests that a urea carrier is involved.

Recent evidence suggests that urea carriers also occur in mammalian liver; phloretin-sensitivity of urea efflux has been documented in mammalian hepatocytes (Effros *et al.* 1993). However, urea transport in the liver of elasmobranch or teleost fishes has not been examined. Urea efflux has been assumed to be passive, since urea production occurs in the liver and this supply could provide an outwardly directed gradient for simple passive diffusion. This strategy may be useful for elasmobranchs, in which total urea concentrations are already high, and other biochemical strategies for coping with the effects of high intracellular concentrations on metabolism have evolved (i.e. the co-accumulation of trimethylamine oxide, Yancey *et al.* 1982). However, for teleosts, especially ureogenic species, it may be advantageous to have specific transport systems to remove urea from the liver and thus to avoid deleterious effects on enzyme systems.

Fish might benefit from urea transport mechanisms other than passive diffusion in a second tissue, red blood cells. For example, as fish encounter more dilute environments in estuarine habitats, the red blood cells may need to lose urea rapidly for volume regulation. Additionally, current models of the countercurrent arrangement of tubule and capillary segments in the elasmobranch nephron (Lacy *et al.* 1985; Lacy and Reale, 1989) strongly suggest that red blood cells must encounter marked variations in extracellular urea concentrations as they traverse the peritubular capillary bed. The cells may need a mechanism to gain or lose urea rapidly in order to maintain their osmotic balance. Urea transport has been studied several times in the red blood cells of the spiny dogfish *Squalus*

acanthias. The results appear to be conflicting, suggesting both carrier-mediated (Murdaugh *et al.* 1964) and passive (Rabinowitz and Gunther, 1973; Kaplan *et al.* 1974) diffusion. Results with phloretin were particularly unusual, inasmuch as this normally non-competitive blocker appeared to accelerate urea transport at high concentrations and to have no effect at low concentrations (Kaplan *et al.* 1974).

Because of these observations and the generally small amount of information available on urea transport in fish tissues, we undertook this study of urea transport in red blood cells and hepatocytes from several teleost and elasmobranch fishes. The species chosen were representative elasmobranchs (the Atlantic stingray *Dasyatis sabina* and the lesser spotted dogfish *Scyliorhinus canicula*), with one (the dogfish) known to make at least some estuarine forays, representative ammoniotelic teleosts (the turbot *Scopthalmus maximus*, the redfish *Scianops ocellatus* and the oyster toadfish, *Opsanus tau*) and a representative ureogenic teleost (the gulf toadfish *Opsanus beta*).

Materials and methods

Experimental organisms

Lesser spotted dogfish *Scyliorhinus canicula* (350–1100 g) and turbot *Scopthalmus maximus* (250–450 g) were obtained from the Brittany coast of France between May and June, 1993. Fish were briefly held at IFREMER in running sea water (at 15 °C) and fed *ad libitum* on chopped squid. Fish were then transported to Brest, where they were held in a recirculating seawater system at 15±2.0 °C and ambient photoperiod without feeding for up to 1 week prior to experimentation. Gulf toadfish *Opsanus beta* were captured by roller trawl by commercial shrimpers in Biscayne Bay, Florida, USA, between July and August, 1993. Toadfish (75–200 g) were held in an outdoor tank with running sea water at ambient seasonal conditions at the shrimpers' holding facility for up to 4 days following capture, then transferred to the laboratory, where they were held in 80 l glass aquaria with running sea water at 24±2 °C without food at ambient photoperiod for up to 1 week prior to experiments. Redfish (*Scianops ocellatus*) (100–150 g) were raised by the University of Miami Experimental Fish Hatchery and fed a commercial chow. Atlantic stingrays (*Dasyatis sabina*) (600–700 g) were supplied by Gulf Specimen Marine Laboratories, Inc. (Panacea, FL), and oyster toadfish (*Opsanus tau*) (500–700 g) were supplied by Marine Biological Laboratories (Woods Hole, MA) and were not fed after arrival in Miami.

Harvesting of red blood cells and hepatocytes

Red blood cells were obtained through caudal artery catheters (Watters and Smith, 1973) in the case of turbot, by anterior mesenteric artery catheters (Graham *et al.* 1990) in the case of dogfish, and by caudal puncture of anaesthetized redfish and toadfish. Red blood cells were typically washed (centrifuged at up to 500 ***g*** and resuspended in saline) copiously to remove native urea. In Brest, these washing centrifugations were performed at 10 °C in a Sigma 3K30 centrifuge; in Miami, they were performed at 22 °C in an IEC Clinical Centrifuge. Teleost washing saline contained: 138 mmol l^{-1} NaCl, 5.4 mmol l^{-1} KCl, 0.8 mmol l^{-1} $MgSO_4$, 0.42 mmol l^{-1} Na_2HPO_4, 0.44 mmol l^{-1} KH_2PO_4, 8 mmol l^{-1} $NaHCO_3$, 1 mmol l^{-1} $CaCl_2$, 3 mmol l^{-1} glucose, 50 i.u. ml^{-1}

lithium heparin, and 5 mmol l^{-1} [*N*-(2-hydroxyethyl)piperazine-*N*′-(3-propanesulfonic acid)] (EPPS) (Brest) or Hepes (Miami), pH 7.8 at 20 °C. Elasmobranch washing saline contained: 456 mmol l^{-1} NaCl, 80 mmol l^{-1} trimethylamine oxide (TMAO), 6 mmol l^{-1} KCl, 3 mmol l^{-1} $MgSO_4$, 1 mmol l^{-1} Na_2HPO_4, 8 mmol l^{-1} $NaHCO_3$, 1 mmol l^{-1} $CaCl_2$, 3 mmol l^{-1} glucose, 5 mmol l^{-1} EPPS, pH 7.8 at 20 °C, and 50 i.u. ml^{-1} lithium heparin. In order to remove the bulk of urea from elasmobranch red blood cells, it was necessary, after several washings, to store them in washing saline for up to 24 h at 5–10 °C. This storage period was only about 30 min for teleosts.

Toadfish hepatocytes were isolated as described by Walsh (1987), except that glucose was omitted from the medium and substrates were 0.25 mmol l^{-1} aspartate, 1 mmol l^{-1} alanine and 1 mmol l^{-1} ornithine, with no glutamine added. Elasmobranch hepatocytes were isolated by similar methods, as modified by Mommsen and Moon (1987) and Nener (1984). Perfusion solution was similar to the elasmobranch washing saline above, but $CaCl_2$ and TMAO were omitted and 350 mmol l^{-1} urea was added with [NaCl] reduced to 281 mmol l^{-1} (and Hepes substituted for EPPS in Miami). The resuspension solution for elasmobranch hepatocytes was similar to the perfusion solution, but the following were added: 1 mmol l^{-1} $CaCl_2$, 2 % bovine serum albumin, 0.13 mmol l^{-1} taurine, 0.06 mmol l^{-1} asparagine, 0.03 mmol l^{-1} β-alanine, 0.1 mmol l^{-1} aspartate, 0.8 mmol l^{-1} ornithine and 0.1 mmol l^{-1} glutamine. Specific modifications to these solutions are noted for each experiment in the Results.

Transport studies

Transport was measured in red blood cells and hepatocytes using [^{14}C]urea (2.04 GBq $mmol^{-1}$, Amersham, UK) and, in a limited number of experiments, 2-deoxy-D-[U-^{14}C]glucose (10.7 GBq $mmol^{-1}$, Amersham), using methods previously applied to fish hepatocytes (Walsh, 1987, 1990) and red blood cells (Moon *et al.* 1987), reviewed recently by Moon and Walsh (1993). Briefly, for uptake studies, 50–100 μl of cell suspension was injected into the topmost of three layers in a 1.8 ml microcentrifuge tube. This top layer contained 0.9 ml of saline plus radioactive (0.0185 MBq ml^{-1}) and cold transport molecules and had been layered onto a middle layer of 0.25–0.4 ml of a phthalate ester or bromododecane oil with an appropriate density for the tissue and species in question. The bottom layer was 50–75 μl of 80 % perchloric acid. After various timed intervals, the tube was centrifuged at 11 000–13 000 ***g*** to move the cells to the acid layer, the cells being accompanied by minimal extracellular water (typically <10 %; Walsh, 1987, 1990). Radioactivity was then counted in both the supernatant and pellet. The two layers were separated by aspiration and the bottom 1 cm of the tube with the pellet was clipped with a dog pedicure tool. The bottom of the tube or a sample of supernatant was placed in ACS (Amersham) and counted on a Packard 1600TR Tri Carb LSC with an onboard quench correction program (Brest). For experiments in Miami, Ecolume (ICN) was used as the fluor, and counts were made on a Beckman LS1801 LSC with an onboard quench correction program. Efflux experiments were performed by preloading concentrated suspensions of cells in high concentrations of radioactivity (typically 200 μl of packed cells plus 0.37 MBq of labelled substrate) for 30 min, then injecting small volumes of prelabelled cells into a 100-fold larger volume of non-

radioactive medium. Typically 12 μl (containing up to 9 mg wet mass of cells) was injected into 1.2 ml of saline without radioactive substrate and layered and separated as described above for uptake experiments. Inhibitors were added in the uptake or efflux medium only, or by preincubation with cells for 10 min prior to the flux measurements; in efflux experiments, this followed the 30 min loading period. No differences were observed in these two treatments. Several other variations on this basic protocol, e.g. changes in oil composition and changes in non-radioactive substrate concentration, were necessary to accommodate the different tissues, species and experimental aims. These details are contained in the Results section for the relevant experiments. Experiments were conducted at 20 °C (Brest) or 22 °C (Miami).

Analytical methods

Urea concentrations in plasma and tissues were measured using the diacetyl monoxime method (Price and Harrison, 1987). ATP measurements were made with standard kits (no. 366-UV, Sigma Chemical Co., St Louis, MO). All chemicals were reagent grade, and biochemicals were purchased from Sigma Chemical Co. (St Louis, MO). Statistical differences between treatments were determined using an unpaired Student's *t*-test (Zar, 1974).

Results

Red blood cells were judged to be viable since ATP contents were close to *in vivo* values, even after 24 h incubations, undoubtedly because glucose was available as a metabolic fuel. Hepatocytes from dogfish, stingrays and toadfish were judged to be viable as they exhibited Trypan Blue staining of less than 2 %. Interestingly, however, our isolation methods for dogfish may have subsampled a particular population of cells from the liver. Compared with benthic stingrays (this study) and skates (Mommsen and Moon, 1987; Nener, 1984), dogfish livers appear to have a much higher fat content, as many of the tan-coloured hepatocytes from dogfish liver floated to the top of the buffer during centrifugation. However, we consistently obtained a fraction of cells which pelleted as a mixture of some tan cells and some darkly pigmented cells. These cells were ureogenic (producing urea at a rate of more than 0.3 μmol g^{-1} h^{-1}), so we judged them to be suitable for urea transport studies. Atlantic stingrays yielded uniform populations of darkly pigmented cells from livers which were uniformly dark prior to perfusion, with virtually no floating cells.

Urea concentrations in plasma, red blood cells and hepatocytes of several species used in this study are given in Table 1. As expected, concentrations in elasmobranchs were high and in teleosts were low, but there were no statistically significant differences between the tissue and plasma compartments (Table 1).

Urea uptake by turbot red blood cells followed a simple hyperbolic pattern during a 1 min period, but there was no effect of the competitive inhibitor acetamide, nor of a non-competitive inhibitor, phloretin (Fig. 1). Additionally, when transport over the first 15 s was compared for a range of urea concentrations, the *y*-intercept of a Lineweaver–Burke transformation of the data was not significantly different from zero (Fig. 2).

Table 1. *Urea concentrations in plasma, red blood cells and liver of selected fish species*

Species	Common name	Urea concentration		
		Plasma (mmol l^{-1})	Red blood cells (μmol g^{-1} tissue water)	Liver (μmol g^{-1} tissue water)
Scyliorhinus canicula	Lesser spotted dogfish	449.0±13.6 (18)	435.0±32.3 (9)	470.3±37.4 (7)
Scopthalmus maximus	Turbot	7.6±0.6 (3)	–	–
Opsanus beta	Gulf toadfish	7.7±0.7 (5)	7.8±0.9 (5)	9.7±0.7 (5)
Opsanus tau	Oyster toadfish	8.2±1.5 (5)	10.9±3.5 (5)	9.4±2.7 (4)

Values are means ±1 S.E.M. (*N*).

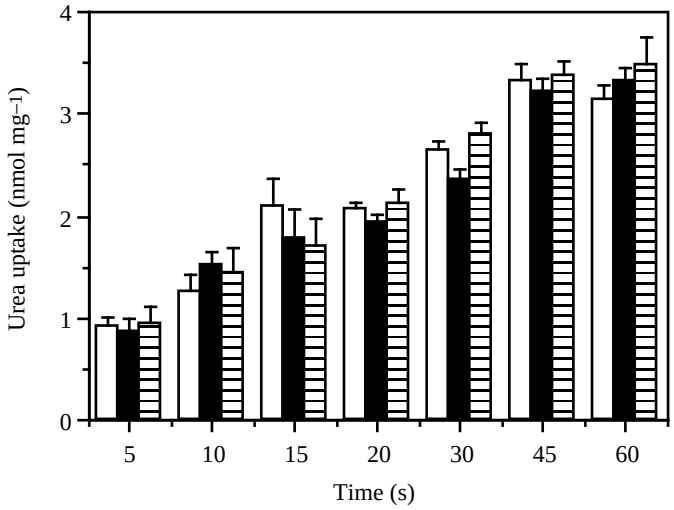

Fig. 1. Uptake of urea by turbot red blood cells *versus* time for control (open bars), phloretin (filled bars) and acetamide (hatched bars) treatments. Values are means + 1 S.E.M. (*N*=3). Red blood cells had no detectable urea prior to injection into uptake medium, which was teleost saline plus 10 mmol l^{-1} urea and 0.0185 MBq ml^{-1} of [^{14}C]urea. [Acetamide] was 10 mmol l^{-1}, [phloretin] was 250 μmol l^{-1}. Phloretin was added as small volumes (1 μl ml^{-1}) of concentrated stock in dimethylsulphoxide (DMSO). Control and acetamide treatments contained equal amounts of DMSO. The oil layer was a 2:1 mixture of dibutylphthalate:dinonylphthalate. Uptake rates were calculated using the measured specific activity in the uptake medium, and the wet mass of cells determined in parallel tubes.

Urea uptake by dogfish red blood cells also followed a hyperbolic pattern *versus* time; however, the time course was longer and the rate was higher, presumably because of the higher concentration used (Fig. 3). There was also no effect of phloretin on this uptake rate (Fig. 3), nor was there an effect of acetamide at equivalent concentrations to urea (Fig. 4). Note that the control rate in Fig. 4 is approximately 50 % of that in Fig. 3, reflecting the lower concentration of urea used (200 *versus* 400 mmol l^{-1}). As in turbot

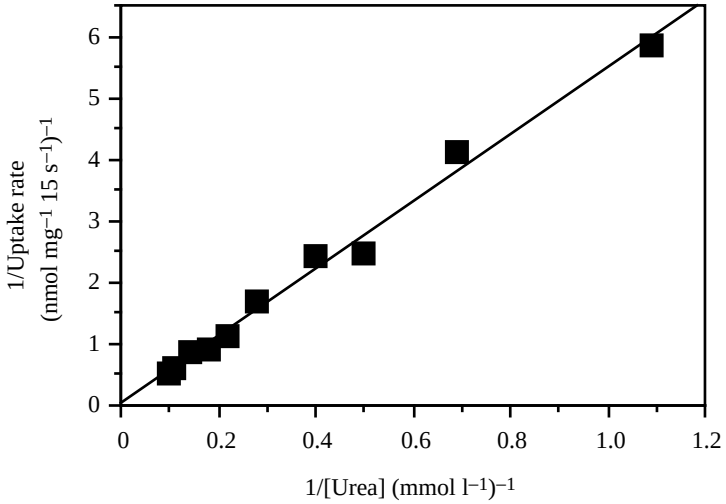

Fig. 2. Lineweaver–Burke transformation of the effect of variable [urea] on uptake rate by turbot red blood cells. The regression is y=0.037+5.46x, r^2=0.989; the y-intercept is not significantly different from zero. Conditions as in Fig. 1 control, except [urea] was variable. Points are means of duplicates for each substrate concentration.

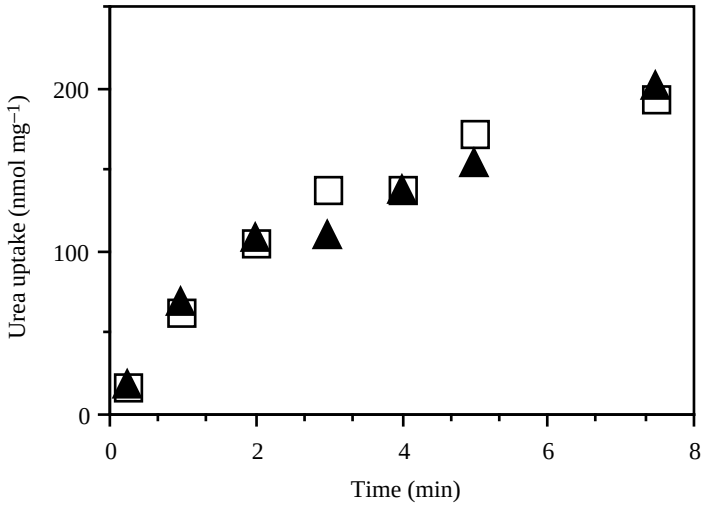

Fig. 3. Uptake of urea by dogfish red blood cells *versus* time for control (open squares) and phloretin (filled triangles) treatments. Values are means of duplicate experiments. In elasmobranch red blood cells, urea was not completely removed by washing, but was greatly reduced (to 6.21±1.31 μmol g^{-1}, N=5). Uptake medium was elasmobranch saline with 400 mmol l^{-1} urea, 200 mmol l^{-1} TMAO and 175 mmol l^{-1} NaCl. Other conditions and calculations as in Fig. 1.

red blood cells, a Lineweaver–Burke transformation of the effect of variable urea concentration showed a y-intercept not significantly different from zero (Fig. 5).

Red blood cells from other teleosts were examined in less extensive experiments. Uptake rates of urea by gulf toadfish, *Opsanus beta*, red blood cells

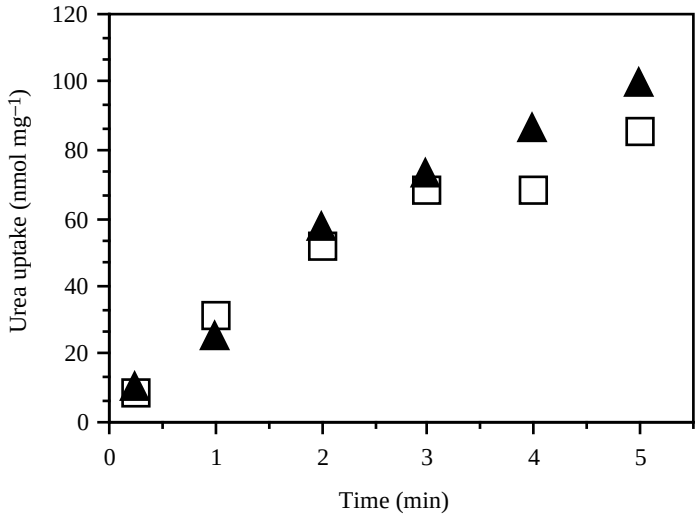

Fig. 4. Uptake of urea by dogfish red blood cells *versus* time for control (open squares) and acetamide (filled triangles) treatments. Values are means of duplicate experiments. Uptake medium was elasmobranch saline containing: control, 200 mmol l^{-1} urea, 200 mmol l^{-1} TMAO, 275 mmol l^{-1} NaCl; acetamide treatment, as in control but with 200 mmol l^{-1} acetamide added and [NaCl] reduced to 175 mmol l^{-1}. Other conditions and calculations as in Fig. 1.

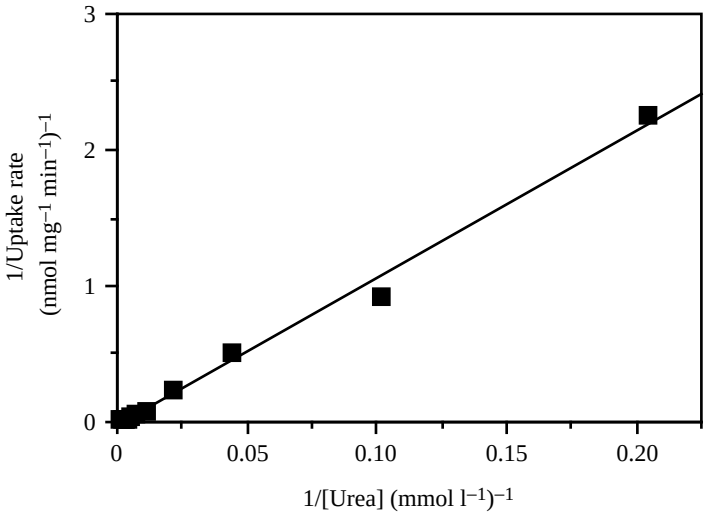

Fig. 5. Lineweaver–Burke transformation of the effect of variable [urea] on uptake rate by dogfish red blood cells. The regression is $y=-0.019+10.80x$, $r^2=0.992$; the y-intercept is not significantly different from zero. Conditions as in Fig. 4 control, except [urea] was variable and [NaCl] was varied in a 0.5:1 ratio with urea to maintain constant total osmolality. Points

(control=3.56±0.19 nmol mg^{-1} min^{-1}, N=3) were unaffected by phloretin (3.38±0.53 nmol mg^{-1} min^{-1}, N=3) and acetamide (3.65±0.35 nmol mg^{-1} min^{-1}, N=3) treatment (conditions as in Fig. 1). However, uptake rates of urea by redfish, *Scianops*

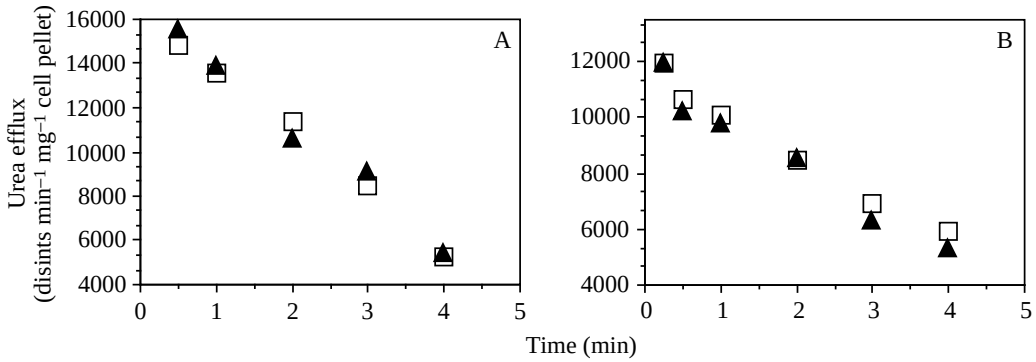

Fig. 6. Urea efflux from dogfish hepatocytes *versus* time. Hepatocytes were concentrated in suspension medium (with 350 mmol l^{-1} urea) and were loaded with [^{14}C]urea for 30 min. Urea content of the cells was stable by 15 min and for up to 1 h. Control (open squares); treatment (filled triangles). (A) Effect of phloretin. Efflux medium was suspension medium without radioactive urea but with 350 mmol l^{-1} urea. Phloretin was added to efflux medium as stated in Fig. 1, and DMSO was added to controls. (B) Effect of acetamide. Efflux medium was suspension medium without radioactive urea and with 200 mmol l^{-1} urea and 316 mmol l^{-1} NaCl for controls, and with added 200 mmol l^{-1} acetamide and reduced [NaCl] to 216 mmol l^{-1} for the acetamide treatment.

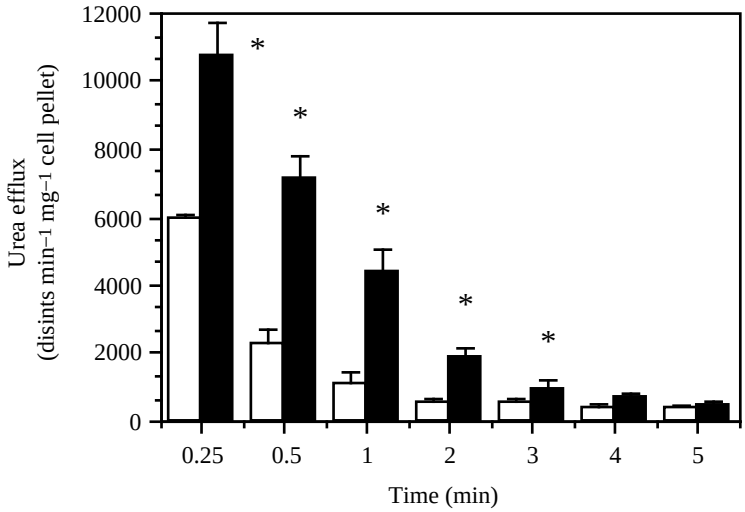

Fig. 7. Efflux of urea *versus* time from gulf toadfish hepatocytes for control (open bars) and phloretin (filled bars) treatments. Hepatocytes were concentrated in suspension medium with 10 mmol l^{-1} urea and loaded for 30 min, then efflux took place in the same medium without radioactivity. Phloretin and DMSO were added as in Fig. 1; the oil layer was 1-bromododecane. Values are means + 1 S.E.M. (N=3) and an asterisk denotes a significant difference from the corresponding control, P<0.05.

ocellatus, red blood cells (control=3.50±0.08 nmol mg^{-1} min^{-1}, N=3) were slightly decreased by phloretin treatment (2.64±0.14 nmol mg^{-1} min^{-1}, N=3) (P<0.05; conditions as in Fig. 1).

Table 2. *Effect of various treatments on urea efflux from gulf toadfish (*Opsanus beta, *except where noted) hepatocytes*

	Urea efflux (disints $min^{-1}mg^{-1}$ remaining after 1 min)	
	Control	Treatment
Phloretin[a]	1115±309	4368±675*
Phloridzin[a]	2763±371	2108±609
Acetamide[b]	1091±258	1584±433
N-Methylurea[b]	2810±323	2957±465
Thiourea[b]	2810±323	2604±323
Sodium removal[c]	841±221	906±206
Opsanus tau		
Phloretin[a]	2160±123	5013±284*

Conditions as in Fig. 7 legend.
Values are means ±1 S.E.M. (*N*=3).
* indicates a value significantly different from the respective control, $P<0.05$.
[a]250 μmol l^{-1}; [b]10 mmol l^{-1}; [c]NaCl in efflux medium replaced with *N*-methyl-D-glucamine, other Na^+ salts replaced with K^+ salts, leaving a nominal [Na^+] in the cell suspension of <1.4 mmol l^{-1}.

Dogfish hepatocytes exhibited a rapid efflux of labelled urea (Fig. 6); however, efflux rate was unaffected by phloretin (Fig. 6A) or acetamide (Fig. 6B). Stingray hepatocytes were similarly unaffected (results not shown). Note that, in all efflux experiments, absolute rates were not calculated since the internal concentration of urea, and hence a specific activity, was not known.

Gulf toadfish (*O. beta*) hepatocytes also showed similar rapid urea efflux, but the efflux was sensitive to phloretin (Fig. 7). The phloretin-sensitivity of urea efflux was also apparent in oyster toadfish (*O. tau*) hepatocytes (Table 2). The effects of several other treatments on urea efflux rate from *Opsanus beta* hepatocytes were examined, including the effects of phloridzin, three urea analogues (acetamide, *N*-methylurea and thiourea) and replacement of extracellular sodium with *N*-methyl-D-glucamine; these were all without effect (Table 2). There was also an effect of phloretin on urea uptake by *O. beta* hepatocytes and a similar lack of effect of the other treatments (Table 3).

Since phloretin-sensitive transporters are known to transport glucose in other systems (Stein, 1986), we examined the interactions of glucose and urea transport in gulf toadfish hepatocytes. High concentrations of glucose caused a slight decrease in urea efflux rates from toadfish hepatocytes (Table 4), but this effect was not seen in the physiological range of 0.5–10 mmol l^{-1} glucose (results not shown), nor was there any effect of the non-metabolized glucose analogue 2-deoxy-D-glucose at 10 mmol l^{-1} (Table 4). The efflux of 2-deoxy-D-glucose from toadfish hepatocytes was phloretin-sensitive, but was not inhibited by physiological concentrations (10 mmol l^{-1}) of urea (Table 4).

Discussion

Although the study of the transport of urea in higher vertebrates is still at a relatively early stage, there is enough of a mechanistic framework within which to interpret the data

Table 3. *Effect of various treatments on urea uptake by gulf toadfish (*Opsanus beta*) hepatocytes*

	Urea uptake (nmol mg^{-1} min^{-1})	
	Control	Treatment
Phloretin[a]	1.76±0.10	1.43±0.05*
Phloridzin[a]	1.72±0.15	1.73±0.08
N-Methylurea[b]	1.57±0.13	1.57±0.15
Thiourea[b]	1.57±0.13	1.64±0.18
Sodium removal[c]	1.71±0.14	1.89±0.30

Values are means ±1 S.E.M. (*N*=3).

* indicates a value significantly different from the respective control, $P<0.05$.

[a]250 μmol l^{-1}; [b]10 mmol l^{-1}; [c]NaCl in efflux medium replaced with *N*-methyl-D-glucamine, other Na^+ salts replaced with K^+ salts, leaving a nominal [Na^+] in the cell suspension of <1.4 mmol l^{-1}.

Table 4. *Interactions of urea and glucose efflux in* Opsanus beta *hepatocytes*

Urea efflux (disints min^{-1} mg^{-1} remaining after 15 s)	
Control (plus 25 mmol l^{-1} NaCl)	Glucose (50 mmol l^{-1})
4551±508	6178±525*
Control	2-Deoxy-D-glucose (10 mmol l^{-1})
5196±466	5888±484
2-Deoxy-D-glucose efflux (disints min^{-1} mg^{-1} remaining after 15 s)	
Control	6298±403
Phloretin (250 μmol l^{-1})	11627±967*
Urea (10 mmol l^{-1})	7478±608

Values are means ±1 S.E.M. (*N*=3).

* indicates a significant difference from the respective control value ($P<0.05$).

of the current study. In human erythrocytes, urea transport occurs by two pathways. There is a phloretin-sensitive (carrier-mediated) facilitated diffusion pathway, which accounts for up to 97 % of transport, and a residual transport by dissolution and diffusion through the lipid bilayer, i.e. passive diffusion (Brahm, 1983; see references in Stein, 1986).

Although models of urea transport in epithelial tissues such as liver and kidney are not fully developed, the analogy with glucose transport in the intestine is useful for comparative purposes. In chicken intestine, glucose is accumulated from the lumen into the intestinal cells by phloridzin-sensitive secondary active cotransport with Na^+ at the apical membrane, and then ejected down a concentration gradient to the blood *via* phloretin-sensitive (carrier-mediated) facilitated diffusion at the basolateral membrane (summarised in Kimmich, 1981). At least one aspect of this system, the phloretin-sensitive efflux of urea, has been found in mammalian liver (Effros *et al.* 1993).

In the present study, the initial survey of urea concentrations in plasma, red blood cells

and hepatocytes (Table 1) indicates that there does not appear to be any active, i.e. against a gradient, accumulation or ejection of urea from red blood cells and hepatocytes in the fish species examined. This conclusion is supported by additional observations on toadfish hepatocytes in which addition of phloridzin or removal of Na^+ had no effect on transport (Tables 2 and 3). It would be interesting to determine whether a secondary active cotransport system (phloridzin-sensitive) is expressed in other epithelial tissues (e.g. intestine, kidney, etc.) of fish. In this respect, it is relevant that several studies have reported a close coupling between Na^+ and urea reabsorption in the elasmobranch kidney (Schmidt-Nielsen *et al.* 1972; Hays *et al.* 1976).

Urea transport in the tissues examined in the present study appears to occur *via* either passive diffusion or a combination of passive diffusion and facilitated diffusion. In red blood cells of nearly all the species examined in the present study, urea transport appears to occur *via* passive diffusion. In dogfish and turbot, there was no effect of phloretin (a potential non-competitive inhibitor), nor of the urea analogue acetamide (a potential competitive inhibitor) (Figs 1, 3 and 4). Additionally, Lineweaver–Burke plots for both species passed through the origin (Figs 2 and 5), yielding no measurable V_{max} or K_m, another diagnostic indicator of passive diffusion. These results are similar to those found for lactate transport in fish red blood cells (Moon *et al.* 1987) and hepatocytes (Walsh, 1987). These kinetic results are also in basic agreement with those obtained using other approaches by both Rabinowitz and Gunter (1973) and Kaplan *et al.* (1974). These authors could find no evidence for a urea transporter in elasmobranch red blood cells. Kaplan *et al.* (1974) did note an unexplained stimulation of transport with phloretin concentrations 2–3 times higher than that used in the present study (250 μmol l^{-1}), but no effect at a concentration approximately half the present level. Kaplan *et al.* (1974) also found no effects of acetamide or phloretin on urea transport in the red blood cells of several teleosts. In the present study, evidence for a urea transporter was found in only one of the four teleosts surveyed, the redfish *Scianops ocellatus*, where there was a small but measurable phloretin-sensitive component. Interestingly, a glucose transporter has been found in teleost red blood cells, but only in a limited number of the species so far examined, occurring in sea perch *Embiotoca lateralis* and Japanese eel *Anguilla japonica*, but not in rainbow trout *Oncorhynchus mykiss*, brown trout *Salmo trutta* and paddyfield eels *Monopterus albus* (Tse and Young, 1990; Bolis *et al.* 1971; Ingermann *et al.* 1985). It would be interesting to examine red blood cells in other teleosts to determine the distribution and coincidence of glucose and urea transport and the mechanistic and functional significance of the presence or absence of the transporters.

Urea efflux from elasmobranch hepatocytes was not sensitive to inhibition by phloretin or acetamide (Fig. 6). As in red blood cells, urea efflux from the liver appears to be a simple passive phenomenon. This observation fits well with current paradigms of osmoregulation in this group. Urea is accumulated in elasmobranch tissues, along with other solutes, in a coordinated strategy that maintains the biochemical integrity of the cell. Thus, the metabolic machinery of the elasmobranch liver is adapted to high intracellular urea levels, and it is not necessary for the liver to rid itself rapidly of urea. Apparently, the rate of efflux *via* passive means is sufficient to keep pace with metabolic production in the liver, as urea does not accumulate there (Table 1). The apparent absence of a

transporter for urea in elasmobranch liver may also be related to glucose metabolism. Unlike many other vertebrates, elasmobranch tissues may preferentially use other substrates (ketones, amino acids, etc.) instead of glucose, and the liver may be less of a 'glucostat' organ in this group (deRoos *et al.* 1985; Moon and Mommsen, 1987; Moyes *et al.* 1990).

Unlike the other tissues and species studied, urea transport in toadfish hepatocytes exhibited marked phloretin-sensitivity (Fig. 7; Tables 2 and 3). Thus, the toadfish liver appears to be similar to mammalian liver (Murphy *et al.* 1993) in this regard. There was no evidence for a phloridzin-sensitive, Na^+-dependent transporter (Tables 2 and 3). Interestingly, although *Opsanus tau* is only minimally ureogenic (Mommsen and Walsh, 1989; P. M. Anderson and P. J. Walsh, unpublished data) compared with *Opsanus beta*, it too showed phloretin-sensitive urea efflux (Table 2). This observation and the above discussion suggest that this transport is not necessarily specific for urea alone, but may be able to transport other small neutral molecules such as glucose. Further preliminary experiments with *Opsanus beta* hepatocytes were designed to examine this suggestion. First, uptake and efflux of urea were both unaffected by urea analogues (acetamide, *N*-methylurea and thiourea, Tables 2 and 3), suggesting that transport has a relatively high specificity for urea, i.e. the differences between the analogues and the urea molecule were enough to make them unrecognisable to the putative transporter. Toadfish hepatocytes also appear to have a glucose transporter that is phloretin-sensitive, but transport is unaffected by physiological concentrations of urea (Table 4). Furthermore, glucose and 2-deoxyglucose appeared not to inhibit urea transport but, at supraphysiological concentrations, glucose modestly enhanced urea transport (Table 4). The simplest conclusion to draw from these data is that glucose and urea are transported separately, each with a phloretin-sensitive component. However, this suggestion clearly needs to be fully tested under more defined conditions (e.g. using membrane vesicles preparations).

In conclusion, although urea appears to be passively distributed across the membranes of hepatocytes and red blood cells in a variety of fish species, there is evidence to support the presence of systems for the facilitated diffusion of urea molecules in selected tissues and species. These cells offer excellent opportunities for further study of the mechanisms and evolutionary patterns of urea (and glucose) transport.

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