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Urea and Water Permeability in the Ureotelic Gulf Toadfish (*Opsanus beta*)

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ABSTRACT The permeability of toadfish gills and skin to urea and water has been measured in order to investigate the mechanisms behind the pulsatile excretion of urea previously described in this species. A perfused gill preparation was used in the gill studies and isolated pieces of skin mounted in an Ussing chamber in the skin studies. Simultaneously, urea and water permeability was measured in vivo in free swimming fish. In vivo the nonpulsing urea permeability was exceptionally low compared to other teleosts, while the tritiated water permeability was similar to that of other teleosts. The urea permeability increased 30-fold during a pulse while water permeability stayed unaffected. Compared to in vivo, tritiated water permeability was approximately 50% lower in the gills and the skin when measured directly in the isolated preparations. The urea permeability was almost identical between the three preparations. Four out of 20 perfused gill preparation showed a spontaneous urea pulse during perfusion. Several treatments were tested to elicit the pulse artificially but without success. Hormones and drugs tested were: arginine-vasotocin (AVT), 10^{-10} M; adrenaline, 10^{-7} M; isoprenaline, 10^{-5} M; acetylcholine, 10^{-7} and 10^{-6} M; serotonin, 10^{-7} and 10^{-6} M; adenosine, 10^{-6} M; cortisol, 10^{-7} M; and combinations of AVT, adrenaline, and cortisol. Adrenaline and isoprenaline increased tritiated water permeability without affecting urea permeability. Gradually increasing the ammonia levels in the perfusate from 0.1 mM to 1.6 mM caused a slight increase in water permeability but a marked and progressive increase in urea permeability. No indications of an ammonia trapping mechanism in the gills were found. There was no effect of AVT (10^{-10} mol l^{-1}) in the urea permeability of the skin preparation while cortisol (10^{-7} M) led to a modest increase in urea permeability. Based on a comparison between the in vivo and in vitro preparations used here, we conclude that the urea pulse in a urea-pulsing toadfish occurs through the gills and not the skin. We still do not know which internal mechanism or signal triggers the urea pulse in the toadfish. *J. Exp. Zool. 283:1–12, 1999.*

Although the majority of teleost fishes excrete ammonia as their principal nitrogenous waste product, the list of teleosts excreting a substantial portion of waste as urea is expanding (see reviews by Mommsen and Walsh, '92; Wright, '95). The gulf toadfish, *Opsanus beta,* is one such teleost, excreting over 90% of its waste nitrogen as urea under certain situations in the laboratory (e.g., confinement stress; see Walsh, '97 for review). Additionally, at least one population of the species (Biscayne Bay, FL) is believed to be at least 50% ureotelic in

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nature (Hopkins et al., '97), although the evolutionary/ecological significance of this phenomenon remains speculative (Hopkins et al., '97; Walsh, '97). Considerably more is known about the biochemical pathways leading to urea production (i.e., a complete hepatic ornithine-urea cycle, Mommsen and Walsh, '89; Anderson and Walsh, '95; see review by Anderson, '95) and the mechanisms of urea excretion (Walsh, '97). The pituitary-hypothalamus-interrenal axis (or stress response) is involved in regulating at least the biochemical transition to ureogenesis (Hopkins et al., '95), and possibly mechanisms of urea excretion (see below).

Urea excretion in the gulf toadfish occurs in discrete pulses (Wood et al., '95a), with fish eliminating virtually all of their daily nitrogen waste in one to three pulses per day, each pulse lasting 0.5–3 hr (Wood et al., '95a, '97; Gilmour et al., '98). In the long periods between pulses, there is virtually no urea excretion, despite the persistence of high urea levels in the blood. The pulsatile excretion phenomenon is known to be due to activation of a urea transfer mechanism from the plasma to the water, with urea synthesis and accumulation in the plasma being continuous, rather than pulsatile (Wood et al., '97). Furthermore, by divided chamber experiments, the site of urea excretion has been localized to the anterior end of the toadfish (Wood et al., '95a), rather than the earlier hypothesized periodic micturation of the urinary bladder (Walsh et al., '90; Griffith, '91). Most recently, experiments directly measuring expired water from the opercular chamber (Gilmour et al., '98) have implicated the gills as the site of urea excretion.

The control of pulsatile urea excretion in the toadfish appears to be at least partly under hormonal influence, but direct causal links in vivo have yet to be established. For example, urea pulses occur following a marked drop in plasma cortisol level, followed then by an increase to prepulse levels (Wood et al., '97). Additionally, administration of the hormone arginine vasotocin (AVT), a piscine analogue of mammalian vasopressin, elicits urea pulses at physiological doses (Perry et al., '98). Furthermore, the studies of Wood et al. ('98), Perry et al. ('98) and Gilmour et al. ('98) all appear to rule out a generalized increase in gill permeability (e.g., by catecholaminergic vasodilation) as the mechanism for pulsatile urea excretion. Instead, all three studies point to the activation of a highly specific, facilitated diffusion type transport system for urea in the gills at the time of pulse events.

Despite this extensive information, permeability values for urea in the gulf toadfish have not yet been reported, and the hypothesis that the gills are the organ of urea excretion (Wood et al., '95a; Gilmour et al., '98) requires additional experimental evaluation, particularly under in vitro circumstances where additional variables can be controlled. To further examine the mechanisms of urea excretion in the gulf toadfish, the present study employed radioisotopic methods to measure the permeability to urea ([^{14}C]) and tritiated H_2O ([^{3}H]; as a reference) in vivo and in two in vitro preparations, namely the isolated perfused head preparation (IPHP; Odulye and Evans, '82; Odulye et al., '82), and an Ussing-type chamber preparation of skin from the head region (Zadunaisky, '84). The effects of key hormones examined previously in vivo (catecholamines, AVT) were also examined in the two in vitro preparations.

A related interesting phenomenon in the physiology of nitrogen excretion of the toadfish occurs in conjunction with the switchover to ureotely, namely the virtual cessation of ammonia excretion. This takes place despite an unchanged plasma ammonia concentration (Walsh and Milligan, '95; Wood et al., '95a), and presumably the persistence of an outwardly directed gradient for ammonia, which is normally considered to be a highly diffusible molecule. This suggests that either there is a capacity for greatly reducing gill ammonia permeability (as proposed for mammalian kidney—c.f. Kikeri et al., '89), or there is a biochemical mechanism in the gills which can "trap" ammonia (as proposed for elasmobranch gill—cf. Wood et al., '95b). Therefore a second aim of the present study was to compare urea, ammonia, and tritiated water permeability values of the toadfish IPHP to those of the dogfish shark IPHP (Pärt et al., '98) and to examine ammonia permeability at progressively higher ammonia concentrations in the internal perfusate. We predicted that if a biochemical trapping mechanism for ammonia were present in the gills, it would become less efficient at higher input concentrations, leading to higher apparent ammonia permeability.

Our principal findings are that urea permeability is exceptionally low in ureotelic toadfish, but increases markedly during pulse events, whereas tritiated water permeability is normal and does not show such increases. Furthermore, high urea permeabilities are observed occasionally in specific IPHPs at the approximate spontaneous fre-

quency of occurrence predicted from in vivo monitoring. This does not occur in the skin preparation, lending further support to the hypothesis that the gill is the site of urea excretion.

MATERIALS AND METHODS

Animals and holding conditions

Local trawl fisherman captured sexually mature gulf toadfish (*Opsanus beta*) in Biscayne Bay, Florida, in May 1996. At the University of Miami, fish were maintained initially in glass aquaria supplied with flowing Biscayne Bay sea water (29–34 ppt, temperature = 25 ± 1°C). Each aquarium contained a sand/gravel substrate (2–5 cm depth) and several polyvinyl chloride tubes that acted as individual shelters. Typically, 3–4 fish inhabited a single 45-l aquarium corresponding to a density of approximately 7–10 g fish l^{-1}. On days 1 and 3 after arrival, the fish were bathed in a mixture of Malachite Green and formalin (Wood et al., '95a) as a prophylactic treatment against the ciliate *Cryptocaryon irritans*—"uncrowded" treatment. Fish were maintained under these conditions for at least 1 week and were fed ad libitum with live shrimp on alternate days. Food was witheld 48 hr prior to experimentation.

To induce ureotelism, fish, weighing between 42 and 88 g (mean mass = 63 ± 2.0 g; n = 33), were subjected to a standardized "crowding" protocol (Walsh et al., '94; Hopkins et al., '95; Wood et al., '95a) 48–72 hr prior to experimentation. Briefly, this procedure involved placing fish and their tube shelters in small plastic tubs (volume approximately 6 l) to achieve densities exceeding 80 g fish l^{-1}. The tubs were aerated continually and supplied with flowing sea water. The fish were not fed during the period of crowding.

In vivo studies

Crowded toadfish were fitted with indwelling caudal artery catheters (PE-50) as described in Wood et al. ('97). After at least 24 hr of recovery from surgery, excretion of urea and ammonia to the water was monitored on an hourly basis by pump and fraction collection of water as described in Wood et al. ('97) to insure that the fish were still ureotelic and pulsatile. Once pulsatility was confirmed, at time zero, fish were injected with 800 μCi kg^{-1} of $^3H_20$ (1 μCi of 1.0 mCi g^{-1}, New England Nuclear, Boston, MA) and 400 μCi $kg^{-1}$of ^{14}C-urea (2 μCi of 40 mCi $mmol^{-1}$, New England Nuclear). Plasma was sampled every 2 hr, and hourly water sampling as above was continued. Plasma was analyzed for radioactivity and total urea concentration, and water samples were subjected to liquid scintillation counting as detailed below.

IPHP preparation

Isolated saline-perfused heads of toadfish were prepared using the basic method of Payan and Matty ('75) originally developed for rainbow trout. Fish were anesthetized with MS-222 (1.0 g l^{-1}) adjusted to pH 7.0 with $NaHCO_3$. The fish was then decapitated just posterior to the pectoral fins. Cannulae (PE 50) were inserted into the bulbus arteriosus and dorsal aorta to permit perfusion of the gills and collection of post-branchial perfusate, respectively. The head was placed into a stainless steel container and sealed in place with a latex condom. The gills were irrigated continuously (flow rate = 170 ml min^{-1} 100 g^{-1}) with temperature-controlled seawater (T = 26°C) and during flux experiments, irrigation water was recirculated using a pump. The gills were perfused under constant pulsatile flow conditions using a peristaltic pump (flow rate = 1.1 ml min^{-1} 100 g^{-1}). Afferent perfusion (input) pressure was monitored continuously via a T-connection on the input cannula that was connected to a pressure transducer. The pressure transducer output was displayed on a chart recorder and calibrated daily against a static column of water. To reduce afferent pulse pressure to physiological values (approximately 10 cm H_2O), a pulse-dampening windkessel was connected to the input cannula (see Perry et al., '84).

The gills were perfused with physiological saline containing148 mM NaCl, 2.7 mM Na_2HPO_4, 5 mM $NaHCO_3$, 2.6 mM KCl, 1.24 mM $MgSO_4$, 1.26 mM $CaCl_2$, 0.1 mM NH_4Cl, 3 mM Glucose, 10 mM Urea, and 20 g l^{-1} Fraction V bovine serum albumin, pH 7.8 (at 25°C), equilibrated with 0.3% CO_2 in air. The gills were perfused for 15 min prior to the beginning of the experiment. The experiment started with the addition of 0.20 μCi ml^{-1} ^3H_2O and 0.05 μCi ml^{-1} ^{14}C-urea to the perfusion medium. The water irrigating the gills was sampled every second minute for measurement of radioactivity and the efflux of ^3H_2O and ^{14}C-urea (cpm min^{-1} 100 g^{-1}) was calculated from the rate of appearance and the total volume of the irrigation water. The experiment was divided into two 20-min periods. The first period was a control period to establish baseline efflux values, the second was a treatment period when effluxes were measured in the presence of hormones or drugs added to the perfusion medium.

One series of experiments was devoted to measure the efflux of ammonia, simultaneously with ^3H_2O and ^{14}C-urea, at different total ammonia concentrations in the perfusate. The experiment consisted of five consecutive 20-min periods. The total ammonia concentration in the perfusion medium was increased from 100 μmol l^{-1} (during the first period) progressively to 200, 400, 800, and 1,600 μmol l^{-1}. The preparation was allowed to stabilize for 10 min after each change of the ammonia concentration and the effluxes were measured during the last 10 min.

Skin preparation

Toadfish (from 70 to 90 g) were anesthetized in 0.67 g l^{-1} of tricaine methanesulfonate buffered with $NaHCO_3$, for 3–5 min. A shallow incision with a scalpel was made around a roughly circular section of skin on the dorsal surface of the head, ranging laterally between the eyes, and anteriorly/posteriorly for 1.5 cm ahead of and behind the eyes. The skin was then gently peeled away from the underlying muscle with a gripping forceps, freeing the skin from the underlying tissue with a gentle touch of the scalpel as needed. The skin was then weighed and placed in physiological saline (identical to that for the IPHP, except for the omission of bovine serum albumin). The skin was then mounted in an Ussing-type chamber constructed of plexiglass, with an aperture of 2.986 cm^2 of skin exposed to the fluids on each side, and a chamber volume of 3.0 ml on each side. The serosal side of the skin contained the saline, and the mucosal side contained sterile filtered seawater. The fluids were gently circulated and aerated by air-lift.

Prior to the start of actual measurements, ^3H_2O and ^{14}C-urea were added to the serosal saline, and allowed to permeate the skin for 1 hr. This preequilibration period was necessary to avoid artificially low permeabilities in the first flux period due to the fact that the skin absorbs a non-trivial amount of label prior to achieving steady state flux rates of label. Flux was then measured in a 1-hr control period by taking a 100 μl aliquot of fluid from each side of the Ussing-type chamber every 15 min, and counting for radioactivity. In a second 1-hr flux period, AVT or cortisol was added as small volumes of concentrated stock solutions to the serosal side of the preparation, and samples were taken as above.

Samples for radioactivity were dissolved in 10 ml Ecolume fluor and counted in a Beckman LS1801 liquid scintillation counter for simultaneous determination of ^{14}C and ^3H activities employing an onboard program for count separation and quench correction. Urea and ammonia were measured as previously (Wood et al., '97).

Chemicals, calculations, data presentation, and statistical analyses

All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). For skin preparations, permeability (cm sec^{-1}) was calculated as follows:

$$\text{Flux } (\mu\text{mols cm}^{-2}\text{ sec}^{-1}) = \frac{(\Delta\text{dpms in 3 ml chamber volume})}{\text{time (sec)}\cdot 2.986\ \text{cm}^2\cdot\text{specific activity (dpms }\mu\text{mol}^{-1})}$$

$$\text{Permeability (cm sec}^{-1}) = \text{Flux/Concentration gradient } (\mu\text{mols/cm}^3).$$

Calculations for IPHP's were similar except that an area of 1.92 cm^2 g^{-1} body weight was used based on the results of Hughes and Gray ('72). Calculations for the in vivo preparations were similar, except that instead of nominal values for urea in saline, actual plasma values were used to calculate the concentration gradient. Values are presented as means ± 1 S.E.M. and significance was tested by Student's paired or unpaired *t*-test, as appropriate, at the $P = 0.05$ level.

RESULTS

Urea and tritiated water permeability in vivo

Urea and tritiated water permeability in toadfish are presented in Table 1. The nonpulsing values for urea are exceptionally low relative to other teleosts, while the nonpulsing values for tritiated water are similar to other fish, which are typically in the range of 2×10^{-5} cm/sec (see Discussion). While there was no change in tritiated water permeability during pulse periods, there was a 30-fold increase in urea permeability at these times,

TABLE 1. Permeability values for urea and water in the gulf toadfish, Opsanus beta*, in pulsing and nonpulsing periods (3 hr each)*[1]

	Permeability (cm sec^{-1})		
	Pre-pulse	Pulse	Post-pulse
$P_{urea} \times 10^{-7}$	2.85 ± 0.17	87.1 ± 13.2*	4.87 ± 1.29
$P_{H_2O} \times 10^{-5}$	1.42 ± 0.15	1.45 ± 0.22	1.54 ± 0.32

[1]Values are means ± 1 S.E.M. (n = 6 pulse events for 4 fish). Mean pulse size was 2431 ± 458 μmol urea-N kg^{-1}.
*Significantly different from pre- and post-pulse at $P < 0.05$ level.

such that urea permeabilities approached basal water permeabilities.

Urea and tritiated water permeability in the IPHP

Toadfish gills proved to be easy to perfuse and to keep viable during perfusion. Small or no changes in vascular resistance were observed during the 40- or 100-min (ammonia experiments) perfusion period. By comparison, rainbow trout gills perfused under the same condition would have shown a pronounced vasoconstriction after 10–15 min perfusion if adrenaline is not included in the perfusion medium (Pärt, '84). In the present experiments adrenaline was only used as an experimental treatment and it was not necessary to include adrenaline to keep the preparation viable. Urea and ^3H_2O fluxes rapidly stabilized in the preparation. Efflux reached a constant rate within 2 to 4 min after the addition of radioactivity and the rate stayed constant during the experimental period (8 control experiments without treatment). The short time needed to obtain a constant rate shows that the radioactivity equilibrates rapidly within the tissue and that there is little or no inhomogenity in the perfusion of the vascular bed, i.e., no vascular shunts. Such an inhomogenity could otherwise lead to delayed responses in the measured fluxes when changing experimental conditions. That efflux rates does not change over time shows that the diffusive properties of the tissue remain intact (no edema).

The standard crowding/confinement protocol used in this and other studies resulted in 2- to 2.5-fold increases in the permeability of the toadfish IPHP to both urea and tritiated water (Table 2). Interestingly, while urea permeabilities were very comparable in the IPHP relative to intact fish, tritiated water permeabilities were significantly lower by about 50% in the IPHP (c.f. Table 2 vs. Table 1). Of even greater interest, amongst all the IPHPs studied, there were four (all from crowded fish) which were apparently prepared in the midst of a spontaneous pulse event. A typical plot of urea and tritiated water appearance in the bath from one of these spontaneously pulsing fish is shown in Figure 1 relative to a typical experiment from a nonpulsing fish. In these spontaneously pulsing preparations, tritiated water permeability was unchanged, but urea permeability was elevated over 16-fold (Table 2). This elevation and the absolute values are very comparable to those seen in vivo during spontaneous pulse events (Table 1).

TABLE 2. Urea and water permeabilities and perfusion pressure in IPHPs in gulf toadfish under different conditions[1]

		Crowded	
	Noncrowded (n = 13)	Nonpulsing (n = 16)	Pulsing (n = 4)
Pressure (cm H_2O)	25.2 ± 2.46	28.7 ± 3.29	22.8 ± 3.1
$P_{H_2O} \times 10^{-5}$ (cm sec^{-1})	0.32 ± 0.04	0.80 ± 0.08*	0.60 ± 0.10*
$P_{urea} \times 10^{-7}$ (cm sec^{-1})	2.03 ± 0.41	4.16 ± 0.40*	68.22 ± 26.56**
$R_{H_2O/urea}$	26.0 ± 5.6	19.5 ± 5.8	1.77 ± 0.76**

[1]Values are means ± 1. S.E.M.
*Significantly different from noncrowded, $P < 0.05$.
**Significantly different from noncrowded and crowded, nonpulsing, $P < 0.05$.

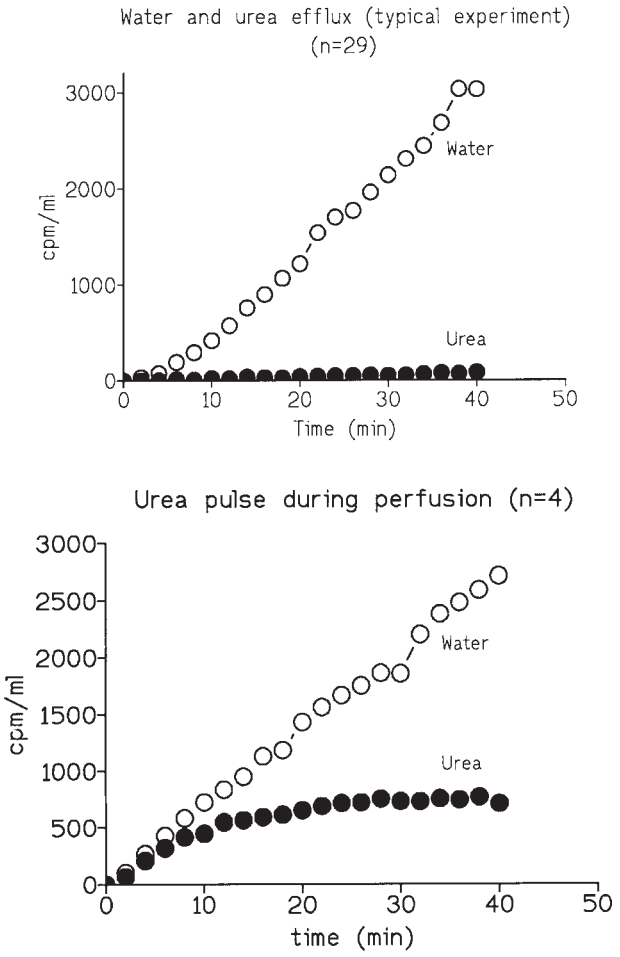

Fig. 1. Appearance of urea and water radioactivity in the bath water as a function of time after injection of label into the perfusate in gulf toadfish (*Opsanus beta*) IPHPs which were prepared while not pulsing (upper panel) or pulsing (lower panel) urea excretion.

AVT (10^{-10} M) elicited the anticipated vasopressor response (i.e., constriction of the preparation resulting in a rise in perfusion pressure) in IPHPs from crowded toadfish (Table 3). However, AVT had no effects on urea or tritiated water permeabilities. L-adrenaline (10^{-7} M) significantly increased tritiated water permeability by about 80% in IPHPs from noncrowded toadfish, but did not significantly alter urea permeability or perfusion pressure (Table 4). In crowded toadfish, the synthetic B-adrenergic agonist l-isoprenaline (10^{-5} M) significantly decreased perfusion pressure (i.e., caused vasodilation of the preparation) and significantly increased tritiated water permeability (Table 5), the latter effect comparable to that of L-adrenaline in noncrowded animals (Table 4). However, l-isoprenaline had no effect on urea permeability (Table 5). When AVT (10^{-10} M) and l-adrenaline (10^{-7} M) were tested in combination, there was a modest (2-fold), statistically significant increase in urea permeability relative to either l-adrenaline alone or the control condition in these same IPHPs (Table 6). However, this stimulation fell far short of the 16- to 30-fold increase seen in spontaneous pulses (Tables 1 and 2). Under these conditions, AVT did not further increase tritiated water permeability above the level caused by l-adrenaline, and there was no effect on perfusion pressure (Table 6).

TABLE 3. Effect of 10^{-10} M arginine vasotocin (AVT) in the perfusion medium on perfusion pressure, and urea and water permeabilities in IPHPs of gulf toadfish from noncrowded and crowded conditions[1]

	Noncrowded (n = 3)	
	Control	AVT
Pressure (cm H_2O)	20.6 ± 4.7	27.3 ± 11.4
$P_{H2O} \times 10^{-5}$ (cm sec^{-1})	0.34 ± 0.05	0.38 ± 0.06
$P_{urea} \times 10^{-7}$ (cm sec^{-1})	1.88 ± 0.36	2.18 ± 1.04
$R_{H2O/urea}$	18.3 ± 2.0	22.7 ± 6.0
	Crowded (n = 10)	
Pressure (cm H_2O)	25.7 ± 7.0	31.9 ± 4.0*
$P_{H2O} \times 10^{-5}$ (cm sec^{-1})	0.82 ± 0.09	0.77 ± 0.08
$P_{urea} \times 10^{-7}$ (cm sec^{-1})	4.58 ± 0.42	4.47 ± 0.47
R_{H2O}/urea	18.6 ± 1.9	17.8 ± 1.3

[1]Values are means ± 1 S.E.M. Comparisons are paired samples, 20 min control perfusion followed by 20 min AVT perfusion.
*Significantly different from control, $P < 0.05$.

TABLE 4. Effect of 10^{-7} M adrenaline in the perfusion medium on perfusion pressure, and urea and water permeabilities in IPHPs of gulf toadfish from noncrowded conditions[1]

	Control	Adrenaline
Pressure (cm H_2O)	28.0 ± 5.6	24.0 ± 3.1
$P_{H2O} \times 10^{-5}$ (cm sec^{-1})	0.28 ± 0.06	0.49 ± 0.10*
$P_{urea} \times 10^{-7}$ (cm sec^{-1})	1.41 ± 0.41	2.08 ± 0.52
$R_{H2O/urea}$	29.5 ± 9.2	27.2 ± 7.2

[1]Values are means ± 1 S.E.M. (n = 5). Comparisons are for paired samples, 20 min control perfusion followed by 20 min adrenaline perfusion.
*Significantly different from control, $P < 0.05$.

Several other potential effectors (acetylcholine: 10^{-7} and 10^{-6} M; serotonin: 10^{-7} and 10^{-6} M; adenosine: 10^{-6} M, AVT 10^{-12} M, and serotonin 10^{-7} M; cortisol: 10^{-7} M; cortisol 10^{-7} M + AVT 10^{-12} M) were screened (n = 1–2 for each) for effects on urea permeability, with no effects observed (results not shown).

Ammonia permeability in the IPHP from crowded fish was approximately 5×10^{-5} M, rather lower than in most teleost fish (see Discussion). Progressively raising the perfusate ammonia concentration from 100 to 1,600 μM did not alter the ammonia permeability (Fig. 2B), arguing against the presence of a biochemical trapping mechanism as the source of this low permeability (see Introduction). Higher levels of ammonia did slightly increase tritiated water permeability (less than 2-fold), but most surprisingly, caused a marked and progressive elevation in urea permeability (Fig. 2A). This stimulation reached more than 30-fold at 1600 μM ammonia,

TABLE 5. Effect of 10^{-5} M isoprenaline in the perfusion medium on perfusion pressure, and urea and water permeabilities in IPHPs of gulf toadfish from crowded conditions[1]

	Control	Isoprenaline
Pressure (cm H_2O)	25.6 ± 0.6	20.1 ± 1.7*
$P_{H2O} \times 10^{-5}$ (cm sec^{-1})	0.73 ± 0.08	1.09 ± 0.10*
$P_{urea} \times 10^{-7}$ (cm sec^{-1})	4.17 ± 0.41	4.22 ± 0.57
$R_{H2O/urea}$	18.0 ± 5.7	25.4 ± 5.6

[1]Values are means ± 1 S.E.M. (n = 3). Comparisons are for paired samples, 20 min control perfusion followed by 20 min isoprenaline perfusion.
*Significantly different from control, $P < 0.05$.

TABLE 6. Effect of 10^{-7} M adrenaline and 10^{-10} M AVT in the perfusion medium of perfusion pressure, and urea and water permeabilities in IPHPs from crowded gulf toadfish under different conditions[1]

	Control	Adrenaline	Adrenaline + AVT
Pressure (cm H_2O)	31.3 ± 6.1	26.3 ± 3.0	26.5 ± 2.7
$P_{H_2O} \times 10^{-5}$ (cm sec^{-1})	0.23 ± 0.06	0.44 ± 0.11*	0.56 ± 0.12*
$P_{urea} \times 10^{-7}$ (cm sec^{-1})	1.46 ± 0.52	1.82 ± 0.57	3.64 ± 0.52**
$R_{H_2O/urea}$	28.1 ± 11.0	28.8 ± 9.1	1.53 ± 3.0**

[1]Values are means ± 1. S.E.M. (n = 4). Comparison are for paired samples, 20 min control perfusion followed by 20 min adrenaline perfusion, followed by 20 min adrenaline + AVT perfusion.
*Significantly different from control, $P < 0.05$.
**Significantly different from adrenaline, nonpulsing, $P < 0.05$.

comparable to that seen during spontaneous pulse events (Tables 1 and 2).

Urea and water permeability in toadfish skin

Urea permeability in skin of crowded toadfish was approximately two orders of magnitude lower than tritiated water permeability (Table 7), a similar sort of difference to that seen in vivo (Table 1) or in the IPHP for nonpulsing fish (Table 2). Notably, skin urea permeabilities were very comparable to the absolute values seen both in vivo (Table 1) and in the IPHP (Table 2) for nonpulsing fish, whereas tritiated water permeabilities were comparable to those in the IPHP but lower than those in vivo. Unlike the IPHP, none of the skin preparations from crowded fish exhibited high spontaneous urea permeability. There were no significant effects of AVT (10^{-12} M) on either urea or tritiated water permeability, but cortisol treatment (10^{-7} M) led to a modest increase in urea permeability of the skin (Table 7).

DISCUSSION

In order to control the convective components (ventilation and perfusion) of branchial solute transport, an isolated saline-perfused head preparation (IPHP) was utilized (Payan and Matty, '75). In general, perfused preparations permit the individual components of complex physiological systems to be isolated. In the IPHP, gill external water flow and internal perfusion rate are held constant and are thereby eliminated as potential factors contributing to any measured changes in solute transfer. The goal of the present study was to specifically evaluate

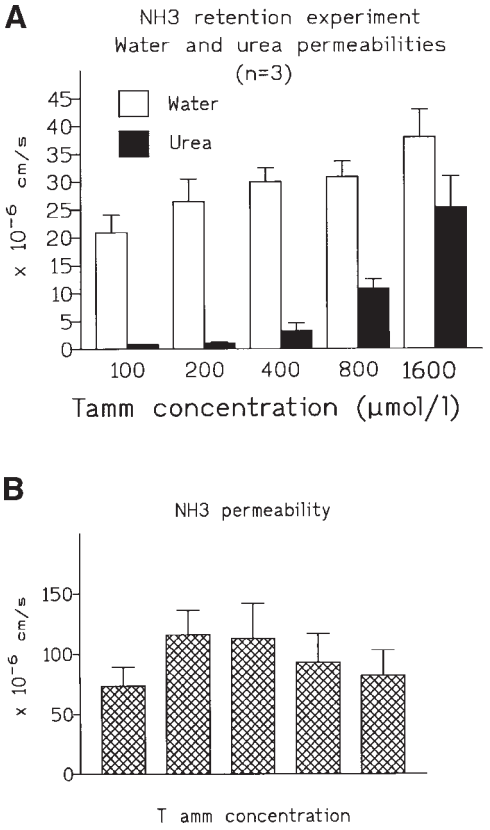

Fig. 2. Plots of permeability for water and urea (**A**) and ammonia (**B**) in IPHPs of gulf toadfish (*Opsanus beta*) as a function of perfusate ammonia concentration.

hormone-dependent and -independent effects on branchial solute permeabilities and thus it was necessary to use a perfused preparation. The merits and disadvantages of using such preparations to assess physiological functions have been addressed in numerous publications (Evans et al., '82; Perry et al., '84; Bornancin et al., '85; Perry and Farrell, '88; Wright and Perry, '89). The principal disadvantage associated with perfused preparations is the inability to exactly match the conditions of gill ventilation and perfusion as they occur in vivo. In addition, certain

*TABLE 7. Effects of arginine vasotocin (AVT) and cortisol (Cort) on skin permeability values (cm sec^{-1}) from Ussing chamber fluxes in gulf toadfish (*Opsanus beta*)*[1]

	Treatment		
	Control	AVT (10^{-12} M)	Cort (10^{-7} M)
$P_{urea} \times 10^{-7}$	5.07 ± 0.56	6.53 ± 0.82	7.07 ± 0.40*
$P_{H_2O} \times 10^{-5}$	0.72 ± 0.15	1.03 ± 0.31	0.60 ± 0.10

[1]Values are means ± S.E.M. (n = 8).
*Significantly different from control at $P < 0.05$ level.

perfused gill preparations tend to deteriorate with time making it difficult to evaluate time-dependent events. Unlike other preparations, however, the IPHP of toadfish is stable over time and has been used with considerable success to elucidate patterns of blood flow in the gill (Oduleye et al., '82) and to demonstrate adrenergic control of gill permeability (Oduleye and Evans, '82). Although it is probable that the absolute values of flux rates and permeabilities arising from the IPHP do not exactly match those in vivo, the technique is well suited to discern relative changes as in the present study.

Permeability coefficients for tritiated water and urea have been summarized for a variety of fish by Isaia ('84) and Pärt et al. ('98). In teleost gills, most values are typically about 1×10^{-5} cm sec^{-1} for water and 2×10^{-6} cm sec^{-1} for urea. Thus, in the present study, values measured both in vivo (Table 1) and in the IPHP for nonpulsing crowded toadfish (Table 2) were unusually low (by about one order of magnitude) for urea permeability but within the normal range for tritiated water permeability. Interestingly, the gills of the dogfish shark, which are extremely impermeable to urea ($3–8 \times 10^{-8}$ cm sec^{-1}; Boylan, '67; Wood et al., '95b; Pärt et al., '98) also exhibit a diffusive water permeability within the normal range (Pärt et al., '97).

Surprisingly, when IPHPs were made from crowded, presumably ureotelic toadfish, both water and urea permeabilities were elevated 2- to 2.5-fold relative to noncrowded, presumably ammoniotelic fish, rather than reduced as might otherwise have been predicted (Table 2). The explanation may relate to generally higher levels of "stress" in crowded toadfish, with associated mobilization of hormones such as cortisol (Hopkins et al., '95; Wood et al., '95a) and catecholamines which could increase permeability (see below). In any event, the key point is that an intrinsically low permeability to urea seems to be a basic property of toadfish gills, and is present prior to crowding and the transition to ureotelism.

How are such low permeabilities to urea achieved in the gills of the nonpulsing toadfish and the elasmobranch? As discussed by Walsh ('97), it is a common misconception that biological membranes are highly permeable to urea. Permeability in artificial lipid bi-layers is about 4×10^{-6} cm sec^{-1}, similar to the values in most teleost gills. Nevertheless, given its low oil/water partition coefficient (10^{-4}), urea should diffuse preferentially through aqueous pathways (Wood, '93), the presence of which would be expected to raise rather than lower the overall permeability of the gills. Therefore, it is difficult to see how gill diffusive urea permeability can be reduced, while diffusive water permeability remains within the normal range.

We suggest several possible explanations, which relate to the increasingly common observation that urea may not always move across biological membranes by simple diffusion (Marsh and Knepper, '92; Knepper and Chou, '95). One possibility, proposed for toadfish by Wood et al. ('97), is the presence of an active reabsorptive "back-transport" mechanism in the gills, which reabsorbs urea as fast as it leaks out across the basolateral membrane. While indirect evidence for such a mechanism has been found in shark gills (Wood et al., '95b; Pärt et al., '98), recent experiments designed to reveal such a system in the toadfish yielded negative results (Wood et al., '98). Another possibility is that the *true* diffusive permeability of all gills is in the elasmobranch-toadfish range (i.e., $10^{-8}–10^{-7}$ cm sec^{-1}, lower than current theory would predict), and that the higher levels actually seen in most fish (2×10^{-6} cm sec^{-1}) reflect the presence of at least a modest number of facilitated diffusion transporters in the epithelium. This theory is attractive inasmuch as the high urea permeability seen in toadfish during pulse events seems to reflect the expression of a large number of such transporters (see below). However, the only detailed search for such transporters in an ammoniotelic teleost, the tidepool sculpin, proved negative (Wright et al., '95). Clearly, more work is needed.

An interesting aspect of the IPHP (Table 2) vs. intact toadfish comparison (Table 1) was the fact that while urea permeabilities agreed quantitatively, tritiated water permeabilities in the IPHP were only about half of those in vivo. A similar discrepancy was noted by Odulye and Evans ('82). One possible explanation relates to the fact that permeabilities were calculated assuming that only the gill area (1.92 cm^2 g^{-1}) was responsible for the observed fluxes, which is a safe assumption for the IPHP but perhaps not for the intact toadfish which lacks scales and has a well-vascularized skin. Total skin area is likely comparable to total gill area, so if this is also a route of tritiated water flux but not urea flux in vivo, it would explain the discrepancy. On the other hand, direct Ussing chamber measurements (Table 7) indicated that baseline urea and water permeabilities of the skin, when removed from the body, were both simi-

lar to those seen in the IPHP, so the situation remains unresolved. One point of interest with respect to skin permeability was the stimulatory effect of cortisol (Table 7). This observation is consistent with the general "leakiness," to both ammonia and urea, of the whole body surface of the intact toadfish which develops during the presence of high cortisol levels under extremely stressful (i.e., unnatural) laboratory conditions such as physical restraint, extreme surgical intervention, disease, etc. (Hopkins et al., '95; Wood et al., '95a).

Perhaps the most important observation of the present study was the finding that in four IPHPs, all from the crowded, presumably ureotelic group, there was a very high permeability to urea, suggesting that at the time of preparation, these fish were undergoing spontaneous pulse events (Fig. 1, Table 2). These striking results compare well with in vivo studies in two important respects. First, the elevation in urea permeability was specific (no accompanying elevation of tritiated water permeability) and similar in magnitude to that seen during pulses from intact animals (Table 1; Gilmour et al., '98; Wood et al., '98). Second, the frequency of the pulses (4 of 20 preparations) was roughly that expected based on pulses lasting about 0.5–3 hr, and occurring 1 to 3 times per day. These were never seen in IPHPs of noncrowded fish (23 preparations), and were never seen in isolated skin from crowded fish (8 preparations).

These observations provide the first concrete evidence that the gills rather than the head skin are the actual sites of pulsatile urea excretion, thereby confirming previous studies which previously localized the phenomenon to first the anterior end of the fish (Wood et al., '95a) and then to the gill region (Gilmour et al., '98). If the skin also contributed to pulsatile urea excretion, one would have expected to see at least one skin preparation with a much higher urea permeability than the mean observed, based on the proportion of toadfish exhibiting urea pulses in vivo and in the IPHP.

This confirmation of the gills as the site of pulsatile urea excretion must be considered against a background of other recently acquired information. Gilmour et al. ('98) and Perry et al. ('98) have shown that the event is highly specific to urea, and does not involve a generalized increase in gill permeability. Wood et al. ('98) have shown that the event represents the activation of a facilitated diffusion transport system for urea which shows many similarities (bidirectionality of transport, transport of thiourea, inhibition by high levels of thiourea) to the facilitated diffusion transporter (UT-2) in the mammalian kidney (Marsh and Knepper, '92; Knepper and Chou, '95). Walsh ('97) has reported preliminary molecular evidence showing that the gill expresses 2.0 and 4.0 Kb mRNAs with sequence similarity to the mammalian UT-2 transporter (You et al., '93; Smith et al., '95). Laurent et al. (unpublished observations) have reported preliminary ultrastructural evidence in gill pavement cells that during natural pulse events, vesicles emanating from the Golgi appear to fuse with the apical membranes. This latter observation helps explain the finding that treament of toadfish with colchicine, a blocker of microtubule assembly and vesicle traffic, abolishes pulsatile urea excretion (Gilmour et al., '98), and is in accord with recent findings on the mammalian kidney that the UT-2 protein is inserted via vesicles into the apical membranes of renal tubule cells under hormonal stimulation (Nielsen et al., '96).

In mammals, the hormone which controls UT-2 activation is arginine vasopressin (ADH; Marsh and Knepper, '92; Chou and Knepper, '95), and administration of physiological doses of the teleost analogue arginine vasotocin (AVT; plasma concentrations of 10^{-11} M to 10^{-9} M) induces pulsatile urea excretion events in ureotelic toadfish (Perry et al., '98). In the present study, AVT by itself (10^{-10} M) elicited the expected vasoconstriction (reviewed by Perry et al., '98) of the gills in the IPHP, but had no effect on urea excretion in the IPHP (Table 3) or isolated skin (Table 7). However, in combination with a high physiological level of adrenaline (10^{-7} M), this same level of AVT induced a modest increase in urea permeability of the IPHP (Table 6). These results suggest that key parts of the AVT response are missing in this in vitro preparation, such as other neurohormonal factors acting in concert, either by the circulation or via direct innervation of gill tissue. Indeed, in vivo, AVT may not exert direct control, but may be synergistic with other neuroendocrine influences such as catecholamine mobilization and decreasing plasma cortisol levels (Wood et al., '97). Catecholamines by themselves exerted the anticipated effects on both vascular resistance and diffusive water permeability of the IPHP (Tables 4 and 5), confirming the results of Odulye and Evans ('82) and Odulye et al. ('82). Catecholamines did not affect urea permeability, in general accord with recent in vivo studies on ureotelic toadfish, which have shown either no effect (Wood et al., '98) or a very small stimulatory action of isoprenaline only (Perry et al., '98) on urea permeability.

The permeability of the toadfish gill to ammonia has been studied extensively both in vivo and in the IPHP (Evans, '78; Claiborne et al., '82; Goldstein et al., '82; Claiborne and Evans, '84; Evans et al., '89), though no distinction was made between ammoniotelic and ureotelic fish. In our experience, based on the holding conditions described, the toadfish would have been predominantly ureotelic in most of those studies, and therefore comparable to the crowded fish of the present investigation (Fig. 2). In total, the previous studies have presented evidence for multiple routes of ammonia permeation (basolateral Na^+/NH_4^+ exchange, NH_4^+ diffusion, NH_3 diffusion, with the latter predominant under most circumstances). The present study was not designed to separate these various pathways, and therefore we have calculated "lumped" permeability coefficients to ammonia averaging about 5×10^{-5} cm sec^{-1} (Fig. 2B), similar to those derivable from the data of Goldstein et al. ('82) and Evans et al. ('89). This value is about an order of magnitude lower than reported in most standard teleosts (e.g., Cameron and Heisler, '83; cf. calculations in Wood et al., '95b). However it is almost identical to recent determinations in the dogfish shark gill, both in vivo (Wood et al., '95b) and in the IPHP (Pärt et al., '98). Thus gill ammonia permeability in the ureotelic toadfish really is unusually low, as in the elasmobranch. Clearly, there is a need for comparable direct determinations on IPHPs from ammoniotelic toadfish; indirect calculations based on in vivo data from such fish (Walsh and Milligan, '95; Wood et al., '95a) indicate that gill ammonia permeability really is considerably higher than in ureotelic toadfish.

The key finding of the present study was that ammonia permeability of the toadfish gill was independent of input total ammonia levels (Fig. 2B). In the dogfish shark, ammonia loading tends to increase gill ammonia permeability, suggesting that a biochemical trapping mechanism becomes overwhelmed (Wood et al., '95b). Walsh ('97) has speculated that a similar trapping or "scavenging" mechanism is present in the gills of ureotelic toadfish, based on measured glutamine synthetase and glutamate dehydrogenase activities in branchial tissue. If this is the case, the present experiments suggest that it must have a much higher trapping potential than estimated by Walsh ('97), or else that another mechanism must be present for greatly reducing gill permeability, as in certain parts of the mammalian kidney (Kikeri et al., '89).

A completely unexpected finding of the present study was the progressive and dramatic stimulation of urea permeability in the IPHP caused by increasing levels of ammonia in the perfusate, accompanied by only very modest changes in tritiated water permeability (Fig. 2A). At present, we have no clear explanation for the response, which at least starts to occur within the physiological range of plasma ammonia concentrations. In general, ammonium ions tend to stabilize protein structure (Somero, '86), though it is well recognized that general and nonspecific toxic responses will develop at very high levels. There has been some suggestion that ammonium loading may stimulate branchial paracellular permeability in teleost fish (McDonald and Prior, '88) and ammonium loading is reported to increase branchial Na^+ exchange in the intact toadfish (Evans, '78). However, if paracellular permeability is opened up by ammonia, it is unclear why tritiated water permeability should not exhibit a comparable response. Another more exciting possibility is that high ammonia may activate the facilitated diffusion transport system for urea in the gills. A recent in vivo test of this hypothesis yielded negative results (Wood et al., '98), but there is clearly much we yet need to learn about ammonia vs. urea interactions in this most interesting system.

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