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Urea and Water Permeability in Dogfish (*Squalus acanthias*) Gills

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ABSTRACT. We used a perfused gill preparation from dogfish to investigate the origin of low branchial permeability to urea. Urea permeability (^{14}C-urea) was measured simultaneously with diffusional water permeability (^3H_2O). Permeability coefficients for urea and ammonia in the perfused preparation were almost identical to *in vivo* values. The permeability coefficient of urea was 0.032×10^{-6} cm/sec and of ^3H_2O 6.55×10^{-6} cm/sec. Adrenalin (1×10^{-6} M) increased water and ammonia effluxes by a factor of 1.5 and urea efflux by a factor of 3.1. Urea efflux was almost independent of the urea concentration in the perfusion medium. The urea analogue thiourea in the perfusate had no effect on urea efflux, whereas the non-competitive inhibitor of urea transport, phloretin, increased efflux markedly. The basolateral membrane is approximately 14 times more permeable to urea than the apical membrane. We conclude that the dogfish apical membrane is extremely tight to urea, but the low apparent branchial permeability may also relate to the presence of an active urea transporter on the basolateral membrane that returns urea to the blood and hence reduces the apical urea gradient. COMP BIOCHEM PHYSIOL 119A;1:117–123, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. *Squalus acanthias*, gill, urea, ^3H_2O, permeability, gill perfusion, adrenalin, thiourea, phloretin

INTRODUCTION

Elasmobranchs retain relatively large amounts of urea in their body fluids for osmoregulatory purposes. One unsolved problem is how they are able to maintain these urea levels without continuous loss of urea to the environment. The gills in particular, with their large surface area and short diffusion distances, would be expected to be a site for diffusional losses. The urea gradient across the gill epithelium is approximately 350 mmol/l. If the permeability of the epithelium to urea was similar to that of teleosts (2.6×10^{-6} cm/sec) (10; Pärt, unpublished data), this would result in a urea efflux of close to 10,000 μmol urea/kg/hr, which is 40 times higher than the actual urea efflux in dogfish *in vivo* (270 μmol urea/kg/hr) (26). It is clear from this example that the dogfish gill epithelium is exceptionally tight to urea compared with other gill breathing organisms—a conclusion also reached by Boylan (3) in his pioneering work on dogfish gill urea permeability.

The mechanisms for this apparent impermeability are unknown. Based on information on urea metabolism and compartmentalization in elasmobranchs, on urea transport in vertebrate cells and on urea permeability in artificial bilayer lipid membranes, we formulated two hypotheses. The first assumes that the gill epithelium and particularly the apical epithelial cell membrane is impermeable to urea. This hypothesis is supported by recent work showing that it is indeed possible to get urea impermeable biomembranes by modifying the composition of membrane lipids (11). By this scenario, the urea efflux would be a process of free diffusion with the blood-water urea gradient being the driving force. Modifications of the gradient (changes in blood urea concentration) should lead to a predictable change in efflux according to Fick's law of diffusion. The efflux should not be affected by urea analogues like thiourea or acetamide, which are potential competitive inhibitors of urea transport (13), and by non-competitive urea transport inhibitors like phloretin (4).

The second hypothesis assumes that the gill epithelium forms an intermediary compartment between blood and water. The apical diffusion gradient is reduced by the gill intracellular urea concentration being lower than in the body fluids. This lower concentration would be achieved by a basolateral urea transporter at the epithelial membrane facing the blood. The intracellular urea concentration would then be determined by passive influx from the blood, possible intracellular urea synthesis and active urea efflux to the blood. Modifications of each of these three components will affect the urea efflux to the water. Active urea transport has been implicated in some tissues in the dogfish. Best estab-

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lished is the reabsorption of urea in the dogfish kidney, where urea is absorbed from the urine against a concentration gradient (8,20). According to this hypothesis, urea excretion would not be expected to directly follow blood urea concentrations. Inhibitors, competitive or non-competitive, would lead to increased urea efflux because of increasing intracellular urea concentrations when the back transport is inhibited.

Our recent findings (26) favor this second hypothesis to some extent. Infusion of urea into dogfish *in vivo* did not increase urea efflux, although plasma levels of urea were increased by 15%. Infusion of the thiourea and acetamide (competitive urea transport blockers) resulted in a slight increase in efflux. Furthermore, infusion with ammonia (NH_4Cl) markedly increased urea efflux despite no change in plasma urea concentration. These findings show no direct connection between plasma urea concentrations and urea efflux across the gills and suggests that processes within the gill epithelium regulate branchial efflux.

Here, we used a perfused dogfish gill preparation to evaluate these two hypotheses to explain the mechanism of urea retention in dogfish gills. This isolated gill preparation allows experimental manipulation of both the blood and water side of the epithelium in a more controlled way than in the *in vivo* models. Furthermore, diffusional water permeability (^3H_2O) was measured simultaneously with urea permeability as a reference for functional gill area.

MATERIALS AND METHODS

Experimental Animals

Spiny dogfish (*Squalus acanthias*, L.), weighing 0.6–2.1 kg, were obtained by trawling in Berkeley Sound (British Columbia, Canada) in July and August 1992 and 1993. At Bamfield Marine Station, the fish were held for 1–3 weeks in large outdoor tanks supplied with running sea water at the experimental temperature of 12 ± 1 EC, salinity (30 ± 3 ppt) and pH (7.90 ± 0.15). The fish were not fed.

Method

For the perfused gill preparation we basically adopted the perfused head methodology developed for rainbow trout (18). Before surgery, the fish were injected intracardially with 5000 IU Na–heparin/kg and left for 15–20 min. The fish were decapitated behind the pectoral fins, pithed and the ventral and dorsal aortas cannulated with PE-90 and PE-280 (Intramedic) cannulae. The ventral aortic cannula was connected to a pulsatile perfusion pump (Harvard model 1100) in the working mode resembling the fish heart (1/3 systole, 2/3 diastole). A pressure transducer (Statham P23 Db) monitored the afferent perfusion pressure. The perfusion medium returning from the gills was collected from the cannula in the dorsal aorta. No back pressure was applied on the dorsal side.

TABLE 1. Perfusion medium for dogfish gills

	Concentrations
NaCl	257 mmol/l
$NaSO_4$	7 mmol/l
$NaHCO_3$	6 mmol/l
Na_2HPO_4	0.1 mmol/l
KCl	4 mmol/l
$MgSO_4 \times 7H_2O$	3 mmol/l
$CaCl_2 \times 2H_2O$	2 mmol/l
Urea	350 mmol/l
TMAO[c]	15 mmol/l
Glucose	5 mmol/l
PVP-40[a]	20 g/l
Albumin[b]	10 g/l

[a]Polyvinylpyrrolidone, MW = 40,000 Da.
[b]Albumin, fraction V.
[c]Trimethylamine oxide.

The gills were immersed in sea water (volume 0.8 l) during perfusion, and the water was recirculated (2.5 l/min) over the gills through silastic tubing inserted in the spiracles. A piece of a thin latex sheet around the body prevented contamination of the water by perfusion medium. Care was taken to check the tightness of this arrangement because even minute leaks of perfusion medium to the water would impair efflux measurements.

The perfusion medium was dogfish saline (Table 1). The perfusion flow rate was adjusted to 25 ml/min/kg, which corresponds to measured *in vivo* values (14). The saline was equilibrated with a gas mixture (0.4% CO_2 in air) that resulted in a pH of 7.80 ± 0.1.

Chemicals

All chemicals were of analytical grade and from Sigma Chemical Company (St Louis, MO, U.S.A.). The adrenaline was made as fresh 1 mmol/l stock daily from l-epinephrine-bitartrate salt. Heparin was Na–heparin and was dissolved in dogfish saline. Phloretin was made up as a 250 mmol/l stock solution in dimethyl sulphoxide (DMSO). ^3H_2O and ^{14}C-urea were purchased from NEN-DuPont (U.S.A.). Scintillation fluor ACS was purchased from Amersham (U.K.).

Experimental Procedures

In influx experiments, ^3H_2O and ^{14}C-urea were added to the water, irrigating the gills to final activities of 0.1 and 0.025 μCi/ml, respectively. Urea was added to the water to a final concentration of 100 μmol/l. The perfusion lasted for 30 min. The first 10 min was a stabilization period during which the blood was washed out of the gills. The experiment started with the addition of the radionuclides to the water. Perfusion medium was sampled (5 ml) every second minute during the 20-min experimental period. The experimental period was divided into two parts. During the first

10 min, the perfusion medium contained no adrenaline, whereas the medium contained 1 μmol/l adrenaline (final concentration) during the second 10-min period. Water samples (1 ml) were taken at 0, 10 and 20 min.

In efflux experiments ^3H_2O and ^{14}C-urea were added to the perfusion medium to final concentrations of 0.14 and 0.05 μCi/ml, respectively. Furthermore, NH_4Cl was added to obtain a measurable ammonia efflux. The analyzed total ammonia concentration (T_{Amm}) in the medium was 730 ± 21 μmol/l ($n = 7$). The experiment started with a switch from non-radioactive to the radioactive perfusion medium. Samples (5 ml) of the water flushing the gills were taken every second minute for measurements of radioactivity. A 0.1-ml sample of the perfusion medium was taken to measure the exact specific activities for the radionucleids. At 0, 10 and 20 min, a 5-ml water sample was taken for ammonia efflux. As in the influx experiments, the experimental period was divided into two 10-min periods interrupted by a 2-min flush period when the water was replaced. The first period was without adrenaline, and the second period used 1 μmol/l adrenaline in the perfusion medium.

Efflux experiments with a reduced perfusion medium urea concentration, phloretin and thiourea followed the general efflux protocol except that the 10-min adrenaline period was replaced by a 10-min period with either 0.25 mmol/l phloretin in a final concentration of 0.1% DMSO or 175 mmol/l thiourea in the perfusion medium. The perfusion medium during the control period in the phloretin experiments contained 0.1% DMSO to account for DMSO artifacts. The urea concentration in the perfusion medium was reduced to 175 mmol/l in the thiourea experiments so that urea + thiourea together made up to 350 mmol/l. In the experiments where only urea was decreased to 175 mM, the medium was osmotically compensated with NaCl.

The washout of urea from gill tissue was measured in one series of experiments. The aim was to simultaneously measure the urea fluxes across the apical and basolateral epithelial membranes and thus to get an idea of the relative permeability to urea of both these membranes. The gills were perfused with a perfusion medium without urea but osmotically compensated with NaCl. The experiment lasted for 40 min. During the last 10 min of this period, effluxes of non-radioactive urea to the water and to the perfusion medium were measured simultaneously by sampling the water and the perfusion medium.

Analysis

For measurements of radioactivity, the samples were made up to 5 ml with sea water or perfusion medium when necessary. This was done to ensure identical quenching in all samples. Scintillation fluor (ACS 15 ml) was added, and the samples were counted for ^3H and ^{14}C in a Nuclear Chicago Mark I liquid scintillation counter using a double-label program. Urea in the water and in the perfusion medium was analyzed with the diacetyl monoxime method (17). T_{Amm} in water was measured with the salicylate-hypochlorite method (24) and in perfusion medium enzymatically with the glutamate dehydrogenase (15) method using a Sigma kit.

Calculations

The influx and efflux values of urea are presented as μmol urea/kg/hr and were calculated from the appearance rate (cpm/min) of ^{14}C-urea in the perfusion medium or water, the measured specific activity and the weight of the fish. Water fluxes are presented as ml H_2O/kg/hr and were calculated as for urea. For calculations of permeability coefficients, a gill area of 3000 cm^2/kg was used (9). Values are presented as means ± SEM with number of experiments in brackets (n). Statistical analysis was based on t-test (two-tailed) for independent observations or for paired observations when appropriate.

RESULTS

The dogfish 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-min perfusion period independent of the presence of adrenaline. Furthermore, vascular resistance was unaffected by the experimental treatments (lowering of perfusion medium urea or the addition of thiourea or phloretin) invoked in the present study. By comparison, rainbow trout gills perfused under the same condition would show a pronounced vasoconstriction after 10–15 min perfusion if adrenaline is not included in the perfusion medium (18,19). Urea and ^3H_2O fluxes equilibrated rapidly in the preparation. Both influx and efflux reached a constant rate within 2–4 min after the addition of radioactivity. This observation shows that the radioactivity equilibrates rapidly within in the tissue. Moreover, it is also shows 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.

Urea efflux in the perfused dogfish gill preparation was 182 ± 25 ($n = 5$) μmol urea/kg/hr, similar to the gill urea efflux of 250 μmol urea/kg/hr reported by us for dogfish *in vivo* (26). Similarity, the ammonia permeability in the perfused preparation, $3.92 \pm 0.34 \times 10^{-5}$ cm/sec ($n = 5$), was very close to the permeability reported by us *in vivo*, 2.65×10^{-5} cm/sec. We therefore conclude that the perfused dogfish gill preparation can be used to investigate mechanisms of urea retention *in vivo*.

Adrenaline had a pronounced effect on ^3H_2O, urea and ammonia effluxes (Table 2). The response to adrenaline was clearly different for the different substances. In the presence of adrenaline, the efflux of both ^3H_2O and ammonia increased to the same extent, the AD/control ratio being

TABLE 2. Efflux of ^3H_2O (ml/kg/hr) and of urea and total ammonia (T_{Amm} μmol/kg/hr) across perfused gills from dogfish

	Control	Adrenalin (1×10^{-6})	Ratio Adrenalin/Control
^3H_2O	71.2 ± 8.43	109 ± 19*	1.53 ± 0.05
^{14}C-Urea	182 ± 25	572 ± 97*	3.14 ± 0.51
T_{Amm}	315 ± 33	449 ± 16*	1.48 ± 0.13

Values are means ± SEM ($n = 5$).

*$P < 0.05$ (t-test for paired observations) for comparisons within the experiment. The adrenalin/control ratio for ^{14}C-urea is significantly ($P < 0.01$) different from the ratios of ^3H_2O and T_{Amm} (t-test for independent observations).

about 1.5 for both. Urea efflux, however, increased markedly with a ratio of 3.14. This observation indicated that adrenaline had a selective effect on urea efflux. This adrenaline effect becomes even more apparent when comparing the influx and efflux permeabilities (Table 3). First, in the absence of adrenaline, no difference was seen between influx and efflux permeabilities either for ^3H_2O or for urea. Both diffusional water and urea permeability increased in the presence of adrenaline independent of whether permeability was measured in the influx or efflux direction. However, for urea, adrenaline caused a markedly larger increase in efflux permeability than in influx permeability, which underlines the previous observation that adrenaline selectively increases urea efflux through the gills.

Urea efflux was not strongly dependent on the urea concentration of the perfusion medium. A reduction of the urea concentration from 350 to 175 mmol/l resulted in no significant reduction in efflux (Fig. 1). A significant reduction was observed when the medium urea was further reduced to 0 mmol/l. Replacement of half of the perfusion medium urea with thiourea, a potential competitive inhibitor of urea transport, had no effect on urea efflux (Fig. 2). However, urea efflux increased markedly (2-fold) in the presence of 0.25 mmol/l phloretin, a non-competitive inhibitor of urea transport (Fig. 3). The effect of phloretin was selective for urea. ^3H_2O efflux was not affected, which shows that phloretin effects on functional gill area could be excluded as the cause for the increased urea efflux.

TABLE 3. Effect of adrenaline (1×10^{-6} mol/l) on permeability coefficients for ^3H_2O and urea in perfused gills from dogfish

	Permeability coefficient × 10^{-6} cm/sec		
	Control	Adrenalin (1×10^{-6} M)	Ratio Adrenalin/Control
		Influx	
^3H_2O ($n = 7$)	6.55 ± 1.24	11.41 ± 1.74	1.74 ± 0.12
Urea ($n = 7$)	0.032 ± 0.004	0.059 ± 0.003	1.84 ± 0.09
		Efflux	
^3H_2O ($n = 5$)	6.59 ± 0.78**	10.20 ± 1.76**	1.55 ± 0.08
Urea ($n = 5$)	0.048 ± 0.006**	0.151 ± 0.02*	3.15 ± 0.18*

Influx is from water to perfusate, efflux from perfusate to water. Values are means ± SEM, n = number of experiments.

*$P < 0.05$, for comparisons between influx and efflux groups (t-test for independent observations).

**No significant difference.

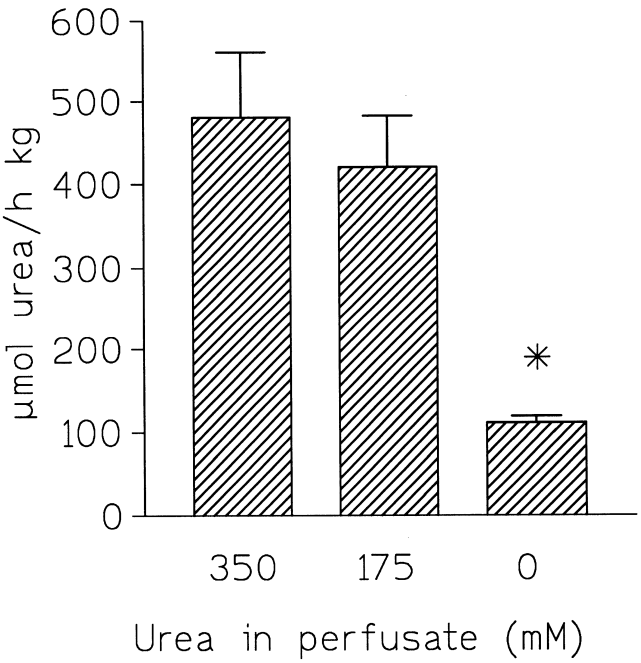


FIG. 1. Urea efflux (μmol/kg/hr) across perfused dogfish gills at different urea concentrations in the perfusion medium. Values are means ± SEM. ($n = 5$). *$P < 0.05$, t-test for independent observations.

A washout experiment was performed to estimate the relative permeabilities of the apical and basolateral epithelial cell membranes to urea. The gills were perfused with a medium without urea and osmotically compensated with NaCl. After a 30-min washout and stabilization period, the fluxes of urea to the perfusion medium and to the water were measured during 10 min. The apical efflux to the water was 111 ± 7.6 μmol urea/kg/hr ($n = 5$), whereas the basolateral flux to the perfusion medium was 1525 ± 161 μmol urea/kg/hr. The basolateral/apical flux ratio is close to 14, showing that the effective permeability of the basolateral epithelial membrane to urea is approximately 14-fold greater than that of the apical membrane.

DISCUSSION

The apparent urea permeability of the dogfish gill epithelium is indeed very low compared with gill epithelia from

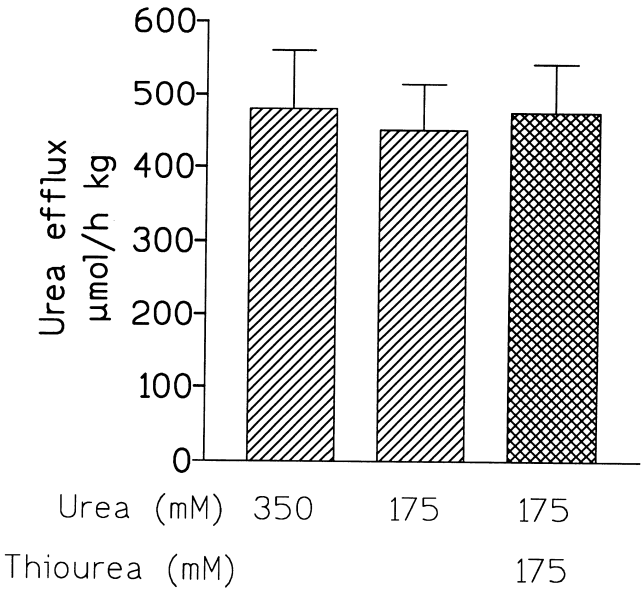
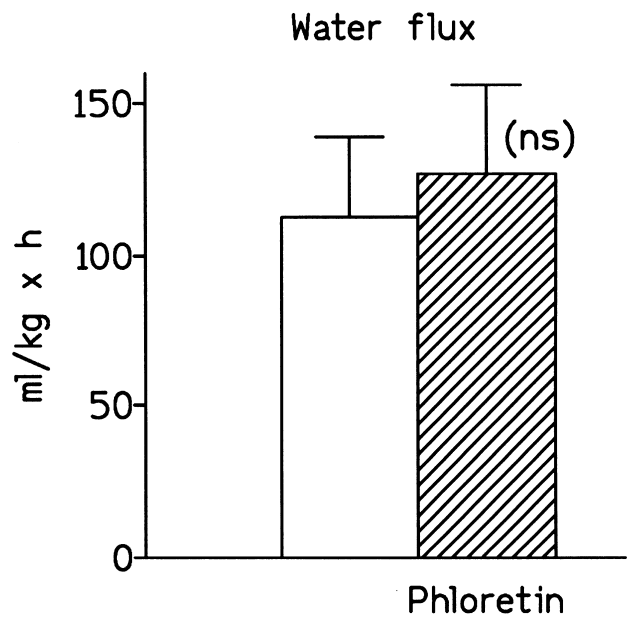


FIG. 2. Effect of thiourea on urea efflux (µmol/kg/hr) across perfused dogfish gills. The thiourea was added to the perfusion medium. Values are means ± SEM. ($n = 5$). No significant difference between the groups (*t*-test for independent samples).



other water-breathing organisms. In contrast, the diffusional water permeability is in the range found in teleosts. Table 4 is a compilation of water and urea permeabilities in teleost gills and in other types of vertebrate epithelia and cell membranes. We also calculated the P_{water}/P_{urea} ratio. The ratio in dogfish based on influx permeabilities is 205, whereas the ratios in many other cell—or epithelial preparations—are in the range of 5–15. Some toad urinary bladder preparations and gastric vesicle preparations show P_{water}/P_{urea} ratios even higher than the dogfish gill, but these high ratios are the result of an exceptionally high water permeability, probably because the presence of water channels (aquaporins). The dogfish branchial water permeability is low by comparison, but the exceptionally low urea permability is the primary cause of the high permeability ratio.

Several of the present results support our hypothesis that the gill epithelium is an intermediary compartment that controls urea efflux. A 50% reduction in perfusion medium urea concentration had no effect on urea efflux. A significant reduction in efflux was observed only when the perfusion medium did not contain urea. Together with our previous *in vivo* observation (26) that urea infusion did not increase urea efflux despite an increased plasma concentration, these observations show no direct relationship between plasma urea levels and urea efflux across the gills. A similar conclusion was reached by Boylan (3) from urea infusion experiments, where he found a nonlinear relationship between urea efflux and plasma concentration. An increased efflux was observed *in vivo* with the competitive urea transport inhibitors acetamide and thiourea (26) but could

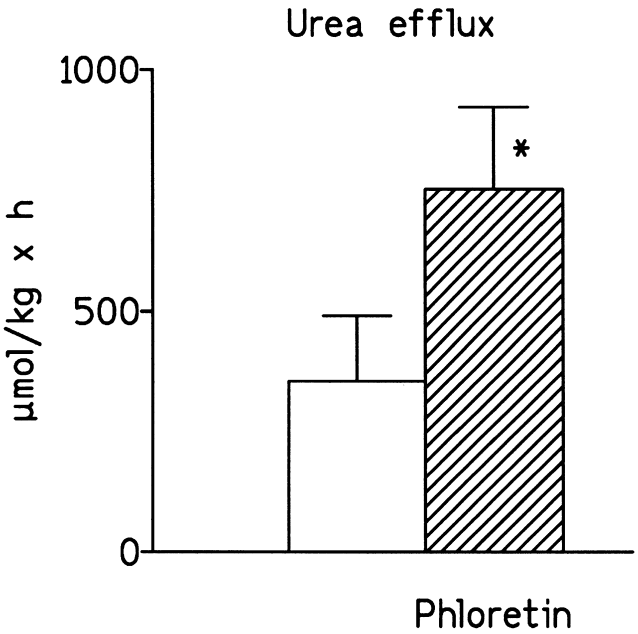


FIG. 3. Effect of 0.25 mmol/l phloretin in the perfusion medium on ^3H_2O (ml/kg/hr) and urea (µmol/kg/hr) efflux across perfused dogfish gills. *$P < 0.05$, ns, not significant, *t*-test for paired observations. Values are means ± SEM. ($n = 7$).

TABLE 4. Diffusional permeabilities of different cells and epithelia to ^3H_2O and urea

	^3H_2O	Urea	Ratio ^3H_2O/urea	Reference
		$\times 10^{-6}$ cm/sec		
Dogfish gill	6.55	0.032	205	Present study
Rainbow trout gill	15.6	2.62	6	Pärt, unpublished results
Eel gill	14.6	2.2	6	23
Lake Magadi Tilapia gill		14.4		25[a]
Inner medullary collecting duct cells, rat		6		21
Inner medullary collecting duct cells, rat + ADH		30		
Hepatocytes, rat	986	182	5	1
Gastric apical vesicles, pig	280	0.54	519	12
Apical membrane urinary bladder, toad	390	0.21	1857	7
Urinary bladder, toad	105	0.61	171	2
Urinary bladder, toad	100	1.4	71	16
Red blood cell, human	5300	360	15	22

[a]Calculated from data.

not be reproduced with thiourea in the perfused preparation. However, phloretin clearly increased urea efflux. The effect was specific for urea because water flux was not affected. Caution is warranted, however, because phloretin can change passive membrane permeability for solutes (5), so the phloretin effect does not necessarily imply the presence of a mediated urea transport.

Our observation of the basolateral epithelial membrane being 14 times more permeable to urea on a net basis than the apical could be because of the presence of a basolateral urea transporter, but the observation is not conclusive. The larger basolateral permeability could also be the result of a larger basolateral surface area. The fact that adrenaline had a stronger effect on urea efflux than on water or ammonia efflux (Table 2) could be taken as support for a basolateral transporter. The adrenaline effect on water and ammonia fluxes are likely an effect of an increased functional surface area, whereas the effect on urea efflux implies the involvement of an additional component. Forster *et al.* (6) reported that adrenaline in dogfish increased urinary urea efflux. The effect was not related to an increased glomerular filtration rate. Because urea is actively reabsorbed in the dogfish kidney (20), this observation may indicate that adrenaline inhibits the active reabsorption mechanisms. The adrenaline effect on urea efflux across gills could therefore be because adrenaline is similarily inhibiting a putative back-transport across the basolateral membrane.

In favor of this assumption is also that adrenaline increased the permeability measured in the efflux direction more than the permeability measured in the influx direction. This result is to be expected if the intracellular urea concentration is the determining factor for urea efflux and if adrenaline is controlling intracellular urea. Finally, our result from the *in vivo* study (26) that NH_4^+ infusion increased urea efflux without changing plasma urea further identifies the gill epithelial cells as the primary source for urea efflux. Combined, our *in vivo* and *in vitro* results could be taken as evidence for the presence of some type of mediated urea transport in the gills involving a back transport mechanism at the basolateral membrane, but there are also observations contradicting this conclusion. The issue could be finally settled by using molecular probes, for example, the cDNA for the dogfish kidney urea transporter (C. P. Smith, M. A. Hediger, and P. A. Wright, unpublished data), which may shed new light on the presence or absence of a specialized urea transport protein in the gill.

Our washout experiment showed that the apical epithelial membrane is likely less permeable to urea than the basolateral membrane. In this respect, the dogfish gill epithelium is no exception from other barrier epithelia (12). Looking at urea transflux across the epithelium, it appears that the apical membrane will be the rate-limiting barrier. We can therefore assume that the permeability measured in our influx measurements (when we know the exact specific activity of urea) represents the apical membrane permeability. The permeability coefficient is then 3.2×10^{-8} cm/sec, which is among the lowest urea permeabilities ever recorded. Surprisingly, this membrane still has a diffusional water permeability that is in the same range as most other gill-breathing organisms. Apparently, the dogfish gill apical membrane is selectively impermeable to urea. In theory, this situation can be obtained by modifying the lipid composition of the membrane. In a recent article, Lande *et al.* (11) showed that by increasing the sphingomyelin content in artificial bilayer membranes, it is possible to construct a lipid membrane that is selectively impermeable to urea over water. This could be the strategy adopted by the dogfish, but as long as the chemical composition of the apical membrane is undefined, this remains an unanswered question.

In conclusion, we propose that the dogfish apical gill membrane is very tight to urea and will therefore limit efflux. We further speculate that efflux is reduced by lowering

the diffusion gradient over the membrane by maintaining intracellular urea concentration in the gill epithelium below that of the plasma through a putative back-transport system in the basolateral membrane. Obviously, these ideas can be evaluated experimentally in several ways—for example, by measuring the urea permeability of apical and basolateral membrane vesicles, by looking for carrier-mediated transport in such vesicles, by measuring intracellular urea concentrations in the cells and/or by molecular biology strategies.

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