Active urea transport and an unusual basolateral membrane composition in the gills of a marine elasmobranch

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Fines, Glenn A., James S. Ballantyne, and Patricia A. Wright. Active urea transport and an unusual basolateral membrane composition in the gills of a marine elasmobranch. Am J Physiol Regulatory Integrative Comp Physiol 280: R16–R24, 2001.—In elasmobranch fishes, urea occurs at high concentrations (350–600 mM) in the body fluids and tissues, where it plays an important role in osmoregulation. Retention of urea by the gill against this huge blood-to-water diffusion gradient requires specialized adaptations to the epithelial cell membranes. Experiments were performed to determine the mechanisms and structural features that facilitate urea retention by the gill of the spiny dogfish *Squalus acanthias*. Analysis of urea uptake by gill basolateral membrane vesicles revealed the presence of a phloretin-sensitive (half inhibition 0.09 mM), sodium-coupled, secondary active urea transporter (Michaelis constant = 10.1 mM, maximal velocity = 0.34 μmol·h⁻¹·mg protein⁻¹). We propose that this system actively transports urea out of the gill epithelial cells back into the blood against the urea concentration gradient. Lipid analyses of the basolateral membrane revealed high levels of cholesterol contributing to the highest reported cholesterol-to-phospholipid molar ratio (3.68). This unique combination of active urea transport and modification of the phospholipid bilayer membrane is responsible for decreasing the gill permeability to urea and facilitating urea retention by the gill of *Squalus acanthias*.

Basolateral plasma membrane; cholesterol; phloretin; urea permeability

**UREA IS A MOLECULE** found in many organisms with a range of functions, from acting as a nitrogen source in prokaryotes to a prime waste product in many vertebrates. In 1858, Stadeler and Frerichs (35) first discovered the presence of “colossal quantities” of urea in muscle tissue of marine elasmobranch fish (sharks, skates, and rays), sparking research that has continued for over 140 years. At levels from 350 to 600 mM (28), urea functions as an osmolyte in the elasmobranchs, balancing the osmotic pressure of seawater (34). An important unsolved question concerns how elasmobranchs maintain these elevated levels of urea in their body fluids and tissues against an essentially infinite gradient with seawater. Homer W. Smith (34) first provided indirect evidence that the elasmobranch kidney efficiently reabsorbs urea, preventing loss via the urine. The gill, with its huge surface area, is consequently the most important site of urea loss to the environment (41). The elasmobranch gill is relatively impermeable to urea compared with the gills of most teleost fishes (6, 34), but the mechanism(s) responsible for this impermeability are still unknown.

One possible mechanism that may confer low urea permeability to the elasmobranch gill is the incorporation of a carrier-mediated urea transport system into the basolateral membrane. In this case, a urea transport protein would have to be oriented to return urea to the blood from the gill epithelium against the gradient. A variety of urea transporters has been described from tissues of different species, including mammalian kidney (30), amphibian skin (15), toadfish gill (40), and dogfish kidney (32), where they perform various functions. An experiment using the isolated perfused dogfish head preparation revealed that phloretin increases urea efflux across the gill (25), providing physiological evidence for the presence of a gill urea transporter. In addition, low stringency Northern analysis of dogfish gill tissue revealed a possible homologue to the dogfish kidney phloretin-sensitive urea transporter (32). Taken together, these previous studies provide circumstantial evidence for a specialized gill urea transport protein.

A second possible mechanism that may confer low urea permeability to the elasmobranch gill is the incorporation of cholesterol into phospholipid bilayer membranes of the gill epithelial cells. Cholesterol is directly correlated with the permeability of biological membranes to urea (27). The additional low permeability of elasmobranch gills to sodium and water, relative to teleost gills (6), suggests that the low permeability to urea is not due to a single mechanism and that a general impermeability may exist.

We isolated the basolateral membrane of the gill epithelial cells from *Squalus acanthias* using differential centrifugation. Characterization of urea transport was performed using vesicles prepared from enriched basolateral membrane vesicles (BLMV) by measuring [¹⁴C]urea uptake in the presence of various urea concentrations, known urea transport inhibitors, and energy sources (ATP or ion gradients), using a rapid filtration technique. The lipid composition and cholesterol...
terol content of the BLMV were determined using chromatographic and spectrophotometric methods.

**METHODS AND MATERIALS**

**Experimental Animals**

Dogfish (Squalus acanthias) were obtained by otter trawl in Passamaquoddy Bay, New Brunswick, between mid-July and the end of August 1999 and maintained at the Huntsman Marine Science Centre in 1,000-liter outdoor tanks under natural photoperiod and supplied with filtered seawater. Dogfish do not feed in captivity and were thus held for no more than 10 days before use.

**Gill Basolateral Plasma Membrane Vesicles**

BLMV were prepared using the method of Perry and Flik (26), with the following modifications. All steps were performed at 0–4°C. Adult dogfish were killed by a blow to the head, and the gill arches were removed. The soft tissue of the gill arches was scraped from the cartilaginous tissue and homogenized with a Dounce homogenizer first with a loosely and then with a tightly fitting pestle (30 strokes each) in 15 ml of hypotonic homogenization buffer containing (in mM) 25 NaCl, 1 dithiothreitol, 0.5 EDTA, 1 HEPES, 1 Tris-HCl (pH 8.0), plus 100 U/ml aprotinin. After homogenization, the volume was adjusted to a final volume of 50 ml with the same buffer. A sample (0.5 ml) was saved for later enzyme analysis. This homogenate was divided into two centrifuge tubes and centrifuged at 550 g for 10 min, producing a pellet containing the remaining contaminating membranes. The supernatant from the first spin was decanted into a clean centrifuge tube and then centrifuged at 50,000 g for 1 h, producing a pellet with a light portion (plasma membranes) and a dark portion (mitochondria). The light portion of the pellet was shaken loose with 15 ml of sucrose buffer containing (in mM) 250 sucrose, 5 MgCl₂, 5 HEPES, and 5 Tris (pH 7.4) and homogenized (Dounce homogenizer, tight pestle, 100 strokes). This second homogenate was centrifuged at 1,000 g for 10 min and then at 10,000 g for 10 min, producing a pellet containing the remaining contaminating membranes. The supernatant was decanted into a clean centrifuge tube and centrifuged at 30,000 g for 45 min to produce a final pellet of enriched basolateral membranes. This final pellet was resuspended in 0.5 ml of suspension medium containing (in mM) 5 MgCl₂, 150 NaCl, 20 HEPES, and 20 Tris-HCl (pH 7.4) and was used immediately for enzyme analysis, vesicle volume determinations, urea transport assays, protein concentration determinations, cholesterol assays, and phospholipid analysis.

**Marker Enzymes**

Enzyme assays (n = 6) were performed on the initial homogenate and the final pellet of enriched BLMV to determine the relative purity of the final preparation and the relative contamination of the final preparation by other cellular membranes. In accordance with published methods, Na⁺-K⁺-ATPase (22), glucose-6-phosphatase (36), cytochrome-c oxidase (3), and nicotinamide mononucleotide (NMN)-adenyllyltransferase (29) were used as marker enzymes for the basolateral membrane, endoplasmic reticulum (ER), inner mitochondrial membrane (IMM), and nuclear membrane, respectively. All measurements were made in duplicate at 25°C in a temperature-controlled Perkin Elmer Lambda 2 spectrophotometer (Perkin-Elmer, Norwalk, CT).

**Vesicle Resealing and Orientation**

The extent of resealing and the orientation of the basolateral membranes were determined using a previously described method (38). The vesicles were assayed for Na⁺-K⁺-ATPase activity in the presence and absence of the detergent digitonin (0.04%) to determine the percentage of resealed vesicles. To unmask inside-out (IO)-oriented resealed vesicles, Na⁺-K⁺-ATPase activity was determined in the presence of 0.5 mM ouabain, and the difference in ATP hydrolysis in the presence and absence of 10 mM KCl was determined. Vesicles assayed in KCl-containing medium were preincubated with 10 mM KCl for 10 min on ice. The percentage of right side-out (RO) vesicles can then be calculated as the difference between resealed and IO vesicles. Vesicle resealing was also determined in BLMV preparations that were frozen at −80°C, thawed, and passed through a 23-gauge needle and 1-ml syringe 10 times to determine the viability of frozen BLMV preparations.

**BLMV Volume Measurement**

BLMV volumes were determined using a previously described method (8). In brief, 40 µl of BLMV (~0.1 mg of protein) were incubated in 1 ml of medium containing 10 µl (1 µCi) of ³H₂O and 10 µl (0.1 µCi) of [¹⁴C]polyethylene glycol-4000 (PEG-4000) for 2 min at 37°C. The BLMV were sedimented by centrifugation at 12,000 g for 4 min. The supernatant (500 µl) was then transferred to a scintillation vial, and 15 ml of Scintisafe Econo F scintillation cocktail (Fisher Scientific, Fair Lawn, NJ) was added. The remainder of the supernatant was discarded, the pellet was resuspended in 40 µl of 20% Triton X-100 (vol/vol) and mixed by vigorous vortex mixing. The base of the centrifuge tube was then cut off and placed in a scintillation vial with 15 ml of scintillation cocktail, and the radioactivity was counted with a liquid scintillation spectrometer (model 1211 Rack Beta, LKB-Wallac). Vesicle volume was calculated in microliters per milligram protein as ³H₂O space minus [¹⁴C]PEG-4000 space per milligram protein added.

**Urea Transport Assays**

Transport of [¹⁴C]urea was performed in duplicate at 10°C by a rapid filtration technique as previously described (26). Freshly prepared BLMV pellets were resuspended at a protein concentration of 0.5 mg/ml in resuspension buffer containing (in mM) 300 NaCl, 5.2 KCl, 2.7 MgSO₄, 5 CaCl₂, 370 D-mannitol, and 15 Tris-HCl (pH 7.4) and allowed to equilibrate for 1 h on ice. The BLMV were then collected by centrifugation at 30,000 g for 45 min and again resuspended at a protein concentration of ~6 mg/ml. Thorough mixing was achieved by passage through a 23-gauge needle (10 times). Transport experiments were initiated by mixing 10-µl aliquots of BLMV with 40-µl aliquots of the radioactive elasmobranch incubation medium (EIM) (containing 50 µCi/ml [¹⁴C]urea), vortexing, and incubating for 15 s at 10°C. EIM contained (in mM) 300 NaCl, 5.2 KCl, 2.7 MgSO₄, 5 CaCl₂, 370 urea, and 15 Tris-HCl (pH 7.4). With D-mannitol as an osmotic substitute, EIM solutions containing urea concentrations of 1–370 mM were prepared. The detailed composition of the EIM used for each incubation is described in the corresponding figure for each experiment. Incubations were terminated by the addition of 1 ml of ice-cold stop solution (EIM containing 370 mM urea). The diluted mixtures were immediately filtered through prewetted Millipore Isopore filters (Millipore, 0.4 µm HTTP type). Filters were washed with two 3-ml aliquots of ice-cold stop solution and...
placed in a vial with 15 ml of Scintisafe Econo F scintillation fluid. Vials were counted in a liquid scintillation spectrometer (model 1211 Rack Beta, LKB-Wallac, or Beckman 6400).

Concentration dependence. Urea uptake was measured over a range of urea concentrations (1–370 mM). The EIM solutions containing concentrations of urea <370 mM contained appropriate concentrations of d-mannitol, which functions as an osmotic replacement, thereby eliminating the effects of osmotic differences. Urea uptake was measured as described above using each individual EIM solution.

Inhibition assays. Competitive and noncompetitive inhibition of urea transport was examined to further define the properties of the transporter. Four urea analogs, acetamide, N-methylurea, thiourea, and nitrophenylthiourea (NPTU), were tested. BLMV were prepared in the same manner as above, but individual EIM solutions were made by substituting each of these compounds at a concentration of 370 mM in place of urea and used in place of the mannitol-containing resuspension buffer. The vesicles containing the respective analog were then incubated with a radioactive mixture containing ATP (10 mM) and ouabain (1 mM), plus a control. Urea uptake was measured as described above.

ATP dependence. ATP dependence of urea uptake was determined by measuring urea uptake in 15 mM urea EIM containing ATP (10 mM), ATP (10 mM) and ouabain (1 mM), ATP (10 mM) and NEM (1 mM), plus a control. Urea uptake was measured as described above.

Cation specificity. Cationic specificity of urea transport was also examined in the BLMV, using modified resuspension buffer and radioactive mixture containing only one of the following salts: NaCl or KCl. BLMV were prepared as described above. The final pellet was resuspended in medium containing 15 mM NaCl and 2.5 mM urea and 3 μCi of [14C]urea and treated as described above. Stock solutions of the noncompetitive inhibitors phloretin (0.03–0.16 mM in ethanol), amiloride (0.1 mM), bumetanide (0.1 mM in ethanol), N-ethylmaleimide (NEM) (0.1 mM in ethanol), p-chloromercuriphenylsulfonic acid (pCMPS) (0.1 mM), and phenylmethylsulfonyl fluoride (PMSF) (0.1 mM in ethanol) were prepared in ethanol or water at a concentration of 250 mM. These stock solutions were diluted to give the final concentrations in parentheses above (final ethanol concentrations were <0.1%). Before the addition of the radioactive mixture, 10 μl of each inhibitor was added to the BLMV and vortexed. Then urea uptake was measured as described above.

RESULTS

Marker Enzymes

The measurement of the four marker enzymes demonstrated that the basolateral membrane preparation was highly purified with very little contamination by other membranes (Tables 1 and 2). The marker enzyme for basolateral membrane, Na^+/K^+ -ATPase, was enriched 4.2-fold. The specific activity for the marker enzymes glucose-6-phosphatase and cytochrome-c oxidase corresponds to a reduction in ER and IMM of 9.5- and 34.3-fold, respectively (Table 1). The nuclear membrane was slightly enriched in the BLMV preparation with an increase in specific activity of 1.2-fold (Table 1). However, the total activity of NNM-adenyllytransferase was only 0.55 μmol substrate/h, which corresponds to 1.15% recovery of the initial amount of enzyme and 2.96% contamination [percentage of the total Na^+/K^+ -ATPase activity (Table 2)]. The recoveries of the ER and IMM were 0.15 and 0.05%, respectively. Contamination of the final pellet by ER and IMM was <6% (Table 2). Final recovery of basolateral membrane was 6.15% (Table 2), consistent with previous studies.

Vesicle Resealing, Orientation, and Volume

Of the total membrane population, 35.00 ± 7.49% formed resealed vesicles. The resealed vesicles were

Table 1. Marker enzyme specific activities and magnitude of purification of basolateral membrane

<table>
<thead>
<tr>
<th></th>
<th>Na^+/K^+ -ATPase</th>
<th>Glucose-6-Phosphatase</th>
<th>Cytochrome C Oxidase</th>
<th>NNM-Adenyllytransferase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>1.02 ± 0.25</td>
<td>0.67 ± 0.25</td>
<td>5.72 ± 1.98</td>
<td>0.11 ± 0.10</td>
</tr>
<tr>
<td>BLMV</td>
<td>4.20 ± 1.71</td>
<td>0.07 ± 0.07</td>
<td>0.17 ± 0.06</td>
<td>0.13 ± 0.10</td>
</tr>
<tr>
<td>Magnitude of purification</td>
<td>4.12-fold</td>
<td>0.11-fold</td>
<td>0.05-fold</td>
<td>1.18-fold</td>
</tr>
</tbody>
</table>

Values are means ± SE (n = 6) in μmol substrate·h⁻¹·mg protein⁻¹. BLMV, basolateral membrane vesicle.
composed of 27% IO and 73% RO orientation (Table 3). Freezing and thawing of the final BLMV preparation resulted in a decrease in the percentage of resealed vesicles of both orientations (Table 3). The volume of the BLMV was relatively large, 8.6 ± 1.8 μl/mg protein, providing additional confirmation of vesicle resealing.

### Concentration Dependence of Urea Uptake by BLMV

Urea uptake by BLMV was measured over a range of urea concentrations in the incubation medium, revealing two components of uptake (Fig. 1A). At high concentrations (25–370 mM urea) urea uptake was linearly dependent on the urea concentration (Fig. 1A). However, at low urea concentrations (1–15 mM) urea uptake exhibited saturation (Fig. 1B). The 5 mM value is somewhat lower than expected from the best fit curve, but the high $r^2$ value (0.9064) justifies the inclusion of this point.

### Table 2. Total activity of marker enzymes, percent recovery, and percent contamination in the final basolateral membrane vesicle preparation

<table>
<thead>
<tr>
<th>Protein Activity</th>
<th>Homogenate</th>
<th>BLMV</th>
<th>Percent recovery</th>
<th>Percent contamination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na⁺-K⁺-ATPase</td>
<td>302.7 ± 35.9</td>
<td>18.6 ± 8.2</td>
<td>6.15</td>
<td>1.56</td>
</tr>
<tr>
<td>Glucose-6-Phosphatase</td>
<td>197.4 ± 51.5</td>
<td>0.29 ± 0.23</td>
<td>0.15</td>
<td>4.25</td>
</tr>
<tr>
<td>Cytochrome C Oxidase</td>
<td>1,701 ± 482</td>
<td>0.79 ± 0.47</td>
<td>0.55</td>
<td>1.16</td>
</tr>
<tr>
<td>NAD-Adenylyltransferase</td>
<td>47.6 ± 43.6</td>
<td>0.55 ± 0.46</td>
<td>1.56</td>
<td>2.96</td>
</tr>
</tbody>
</table>

Values are means ± SE ($n = 6$) in μmol substrate/h.

### Table 3. Percentage of the basolateral membrane fraction as resealed vesicles and vesicle orientation

<table>
<thead>
<tr>
<th>Orientation</th>
<th>Fresh</th>
<th>Freeze/thaw</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaky</td>
<td>65.0 ± 7.5</td>
<td>87.9 ± 3.9</td>
</tr>
<tr>
<td>IO</td>
<td>9.4 ± 2.8</td>
<td>7.0 ± 3.0</td>
</tr>
<tr>
<td>RO</td>
<td>25.6 ± 9.8</td>
<td>5.1 ± 0.9</td>
</tr>
</tbody>
</table>

Values are means ± SE ($n = 6$). IO, inside out; RO, right side out.

---

**Fig. 1.** A: rates of urea uptake at variable urea concentrations in basolateral membrane vesicles (BLMV) from the gill of the dogfish, *Squalus acanthias*. Means ± SE, $n = 8$. B: expansion of the low end of the urea concentration ([urea]) range from A. The regression is $y = 0.0774\ln(x) + 0.0116$, $r^2 = 0.9064$. Means ± SE, $n = 7$. C: Lineweaver-Burk transformation of the relationship between urea concentration and urea uptake by BLMV. The regression is $y = 2.9612 + 29.81x$, $r^2 = 0.9778$. Means ± SE, $n = 7$. 

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sion of all values up to 15 mM. When data from Fig. 1B were transformed using a Lineweaver-Burk plot, the Michaelis constant \((K_m)\) was 10.1 mM and the maximal velocity \((V_{\text{max}})\) was 0.34 \(\mu\text{mol} \cdot \text{h}^{-1} \cdot \text{mg protein}^{-1}\) (Fig. 1C).

**Inhibition of Urea Uptake by BLMV**

Urea uptake by BLMV demonstrated sensitivity to the noncompetitive inhibitor phloretin (Fig. 2). Phloretin produced a dose-dependent inhibition of urea uptake by BLMV, with 50% inhibition occurring at a concentration of 0.09 mM. The use of urea analogs demonstrated competitive inhibition of urea uptake by BLMV. \(N\)-methylurea and NPTU significantly reduced the rate of urea uptake (Fig. 3). Thiourea and acetamide also tended to reduce the rate of urea uptake, although the change was not significantly different from the control. Amiloride, bumetanide, and thiol-reagents \(\text{pCMBS}, \text{PMSF}, \text{and NEM}\) did not inhibit urea uptake by BLMV (data not shown).

**ATP Dependence and Inhibition of Urea Uptake by BLMV**

Urea uptake was significantly stimulated by the addition of ATP to the incubation medium (Fig. 4A). On the addition of ouabain, the rate of urea uptake by BLMV decreased to control levels (Fig. 4A). The addition of NEM had no effect on ATP-stimulated urea uptake (Fig. 4A).

**DISCUSSION**

**Methodology**

The method (26) we used to prepare basolateral plasma membranes from the gill epithelium of the spiny dogfish \(Squalus acanthias\) yielded a specific enrichment of \(Na^+-K^+\)-ATPase, indicating selective isolation of basolateral plasma membranes. Although there was only minor contamination \(<5\%\) of membranes from the ER, mitochondria, and nucleus, this may have led to a slight underestimation of urea uptake by the gill BLMV. The final recovery of \(Na^+-K^+\)-ATPase activity \((6.2\%)\) is consistent with previous studies (26), whereas the vesicle orientation \((\text{IO} = \ldots\)
9.4%; RO = 25.6%) and resealing efficiency (35%) were somewhat lower than reported values for eel (IO = 33%; RO = 23%; resealing efficiency 56%) (16). The volume measurements confirmed that the vesicles successfully resealed. The decrease in resealing efficiency after freezing and thawing of the final BLMV preparation suggested decreased viability of the membranes, and therefore only freshly prepared BLMV were used for transport experiments. Significant time was saved by omitting the gill perfusion step used to clear the gills of red blood cells (26), because there are no urea transporters present in elasmobranch erythrocyte plasma membranes (10).

**Urea Transport**

The measurement of urea uptake by enriched BLMV revealed saturation kinetics at low urea concentrations ($K_m = 10.1 \text{ mM}$, $V_{max} = 0.34 \mu\text{mol} \cdot \text{h}^{-1} \cdot \text{mg protein}^{-1}$), suggesting the presence of carrier-mediated urea transport. The low $K_m$, relative to the urea concentration in the blood, indicates that the transporter has a relatively high affinity for urea. This implies that the putative urea transporter acts to “scavenge” intracellular urea, actively returning it to the blood and thereby maintaining a low urea concentration within the gill epithelial cells. To further characterize the saturable component of urea uptake by the BLMV, the effects of several known inhibitors of urea transport were examined. Inhibition by the noncompetitive inhibitor phloretin is diagnostic of both facilitated and secondary active, urea transport systems (19, 21, 32, 39). The dose-dependent inhibition of urea uptake in shark gill BLMV by phloretin in this study is consistent with a previous study on the isolated perfused dogfish head preparation (25), which demonstrated that phloretin infusion significantly increased urea efflux across the gill. The urea analogs N-methylurea and NPTU also significantly inhibited urea uptake in shark gill BLMV. Acetamide and thiourea had a slight, but nonsignificant, effect on urea uptake rates. However, these results are inconclusive until the analog concentrations are optimized, because studies have shown that under different conditions urea analogs may or may not have statistically significant effects on the branchial urea efflux in the spiny dogfish (25, 40). In mammalian inner medullary collecting duct, methylurea and thiourea significantly reduced urea permeability, whereas acetamide did not (11). These results demonstrate that urea transporters in different tissues and animals exhibit different sensitivities to urea analogs. The inhibition of urea uptake in shark gill BLMV by phloretin and urea analogs supports previous hypotheses (32, 40) for a carrier-mediated urea transport system in the dogfish shark gill.

**Urea uptake is energy dependent in shark gill BLMV.** The addition of ATP to the incubation medium

**Table 4. Percentage of phospholipid types and total phospholipid and cholesterol in the basolateral membrane of gill epithelium from the Squalus acanthias**

<table>
<thead>
<tr>
<th>Phospholipid Type</th>
<th>Percentage (%) ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cardiolipin</td>
<td>2.6 ± 0.5</td>
</tr>
<tr>
<td>Phosphatidylcholine</td>
<td>45.6 ± 1.1</td>
</tr>
<tr>
<td>Phosphatidylethanolamine</td>
<td>26.5 ± 1.3</td>
</tr>
<tr>
<td>Phosphatidylinositol</td>
<td>8.6 ± 0.8</td>
</tr>
<tr>
<td>Phosphatidylserine</td>
<td>12.3 ± 0.6</td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td>4.4 ± 0.6</td>
</tr>
<tr>
<td>PC/PE</td>
<td>1.7 ± 0.1</td>
</tr>
<tr>
<td>Total (nmol/mg protein)</td>
<td>1,248 ± 96</td>
</tr>
<tr>
<td>Cholesterol (nmol/mg protein)</td>
<td>4,768 ± 310</td>
</tr>
</tbody>
</table>

Values are means ± SE ($n = 8$). PC, phosphatidylcholine; PE, phosphatidylethanolamine.
directed concentration gradient of Na⁺ to function in a symport fashion. By using the inwardly 
scribed from the dogfish kidney (31), which is thought
from the putative sodium-linked urea transporter de-
in the rat inner medullary collecting duct, but differs
antiport fashion, similar to the transporter described
lian kidney (19).

transporter, similar to the type found in the mamma-
1
transporting Na⁺ and urea are reabsorbed at a fixed ratio of 1.6 mol
cule returned to the blood. In the dogfish kidney, so-
aming 1 urea molecule via the ornithine-urea cycle
up to 5 ATP equivalents (the metabolic cost of synthe-
ctions (37). In most temperate species, the ratio of PC to
in the gill is low, but the metabolic savings are signif-
that the metabolic cost associated with urea transport
in the gill is low, but the metabolic savings are signif-
ificant.

Gill Basolateral Membrane Composition

In the basolateral membrane of the gill from the
spiny dogfish, *Squalus acanthias*, PC and PE were the
main phospholipids, typical of most eukaryotic mem-
trated by the organism. For example, cold-acclimated
organisms (Arctic char, molluscs) increase the propor-
tion of PE in their membranes to maintain membrane
fluiddity at low temperature (17). This results in a
PC-to-PE ratio of <1 (0.3–0.5). However, in elasmo-
branchs, the cellular membranes face the opposite
problem, increased fluidity due to urea (2). One adap-
tation elasmobranchs have evolved to deal with this
effect of urea is the presence of trimethylamine oxide
(TMAO). TMAO counteracts the negative effects of
urea on both proteins (43) and phospholipid mem-
branes (2). Another strategy, which may work in con-
junction with TMAO, is increasing the ratio of PC to
PE, which would provide additional stability to the
membrane.

The very high cholesterol-to-phospholipid molar ra-
tio (3.68) reported here is the highest for native mem-
branes (Table 5). Cholesterol decreases the permeabil-
ity of biological membranes to urea (27) by inducing an
increased order of the phospholipid molecules that
compose the bilayer membrane, allowing them to pack
closer together forming a tighter barrier (24). Choles-

<table>
<thead>
<tr>
<th>Species</th>
<th>Tissue</th>
<th>Subcellular Fraction</th>
<th>C:P, mol/mol</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spiny Dogfish (<em>Squalus acanthias</em>)</td>
<td>Gill epithelium</td>
<td>Basolateral membrane</td>
<td>3.68 ± 0.26</td>
<td>Present study</td>
</tr>
<tr>
<td>Human</td>
<td>Lens</td>
<td>Plasma membrane</td>
<td>3.50</td>
<td>5</td>
</tr>
<tr>
<td>Rat (<em>Rattus norvegicus</em>)</td>
<td>Intestine</td>
<td>Brush-border membrane</td>
<td>0.95 ± 0.03</td>
<td>23</td>
</tr>
<tr>
<td>Little Skate (<em>Raja erinacea</em>)</td>
<td>Liver</td>
<td>Basolateral membrane</td>
<td>0.46 ± 0.01</td>
<td>23</td>
</tr>
<tr>
<td>Rainbow Trout (<em>Oncorhynchus mykiss</em>)</td>
<td>Intestine</td>
<td>Brush-border membrane</td>
<td>0.41 ± 0.12</td>
<td>34</td>
</tr>
<tr>
<td>Squid (<em>Loligo pealei</em>)</td>
<td>Cerebral and optic lobes</td>
<td>Plasma membrane</td>
<td>0.34 ± 0.04</td>
<td>12</td>
</tr>
<tr>
<td>Crab (<em>Cancer pagurus</em>)</td>
<td>Leg muscle</td>
<td>Plasma membrane</td>
<td>0.24 ± 0.02</td>
<td>12</td>
</tr>
<tr>
<td>Crab (<em>Carcinus maenas</em>)</td>
<td>Leg muscle</td>
<td>Plasma membrane</td>
<td>0.34 ± 0.04</td>
<td>12</td>
</tr>
<tr>
<td>Cucumber (<em>Cucurbita ficifolia</em>)</td>
<td>Root</td>
<td>Plasma membrane</td>
<td>0.24 ± 0.02</td>
<td>12</td>
</tr>
<tr>
<td>Fungus (<em>Botrytis cinerea</em>)</td>
<td>Hypha</td>
<td>Plasma membrane</td>
<td>0.60</td>
<td>20</td>
</tr>
<tr>
<td>Bacteria (<em>Methylococcus capsulatus</em>)</td>
<td>Plasma membrane</td>
<td>0.08</td>
<td>18</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ±SE as reported in the literature cited, or means if SE were not reported. C:P, cholesterol-to-phospholipid ratio.
terol, when inserted in the appropriate place in the membrane, also increases membrane permeability to oxygen (14); therefore, the elevated cholesterol levels in shark gill basolateral membranes would not impair gas exchange. We propose that the high cholesterol content of shark gill basolateral membrane provides a physical barrier that retards passive loss of urea at the gill without affecting oxygen permeability. This high cholesterol-to-phospholipid molar ratio may also explain the low permeability of the shark gill to water (6, 25) and sodium (6) relative to teleost fishes.

In conclusion, on the basis of the results of this study we propose that a unique combination of physiological and structural mechanisms is at least in part responsible for the low urea permeability of the dogfish gill. The marine elasmobranch gill is ~80 times less permeable to urea than the rainbow trout gill, resulting in a urea efflux of 270 μmol·kg⁻¹·h⁻¹ (25). If the elasmobranch gill were as permeable to urea as the rainbow trout gill, the resulting urea efflux would be immense (10,000 μmol·kg⁻¹·h⁻¹) (25), due to the enormous blood-to-water gradient. With data from the present study, it is possible to calculate the relative contribution of active urea transport to the difference between observed and predicted (based on teleost gill urea permeability) urea efflux rates. The $V_{\text{max}}$ of urea uptake (0.34 μmol·h⁻¹·mg protein⁻¹) was corrected for vesicle resealing (35%), vesicle orientation (27% of resealed vesicles), membrane recovery (6.15%), protein level (9 mg/animal), and animal mass (1.3 kg). This results in a total rate of active transport of urea back into the blood from within the gill epithelium of 535 μmol·kg⁻¹·h⁻¹. This value is ~6% of the difference in urea permeability between the teleost and elasmobranch gill (i.e., 10,000–270 μmol·kg⁻¹·h⁻¹). The remaining 94% may be due in part, or in whole, to the elevated cholesterol-to-phospholipid molar ratio in the basolateral membrane. Thus we envision that the primary role of the basolateral membrane is to substantially reduce the influx of urea into the gill epithelial cells, thereby maintaining low intracellular urea concentrations at which the urea transport system functions. The urea transport system actively transports urea out of the epithelial cells back into the blood, maintaining low intracellular urea concentrations. The low intracellular urea concentrations achieved by these complementary mechanisms lead to a reduced diffusion gradient for urea across the apical membrane and thus a lower effective permeability of the gill to urea. One could argue, therefore, that high cholesterol levels and active urea transport in the gill basolateral membranes probably coevolved in elasmobranchs, enabling the retention of urea and its use as a key component of their osmoregulatory strategy, while minimizing the energetic cost. However, the high nonspecific urea uptake by the BLMV suggests that there are other mechanisms and/or structures, particularly the composition of the apical membrane, which may contribute significantly to the overall low urea permeability of the elasmobranch gill. Further studies of the elasmobranch gill are thus required to completely resolve this issue.

**Perspectives**

Previous attempts to identify the mechanisms responsible for the low urea permeability of the elasmobranch gill have focused primarily on the involvement of urea transport, despite a previous suggestion for the importance of structural modifications to the membranes (6). In this study we used a combination of techniques to reveal the involvement of two complementary mechanisms (urea transport and membrane composition) in conferring low urea permeability to the elasmobranch gill, a phenomenon first described by Homer Smith (34) over 60 years ago. These results demonstrate the importance of using different techniques or approaches to address the same question and that physiological phenomenon cannot always be simply explained by one mechanism. The approach used in this study may be useful in investigating the selective permeability of other epithelial tissues (e.g., kidney, bladder, intestine, and gall bladder) to various electrolytes and solutes. A wide range of studies has focused on the presence of protein transporters in these tissues, but analysis of the phospholipid and fatty acid composition of the membranes from these tissues may provide additional information essential to the full understanding of their selective permeability.

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