PROTON-ATPASE IN FISH GILLS

BY

HONG LIN

B.A.Sc., Zhejiang University, 1986
M.Sc., The University of British Columbia, 1989

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
in
THE FACULTY OF GRADUATE STUDIES
DEPARTMENT OF ZOOLOGY

We accept this thesis as conforming
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA
MARCH 1993
© Hong Lin, 1993
In presenting this thesis in partial fulfilment of the requirements for an advanced
degree at the University of British Columbia, I agree that the Library shall make it
freely available for reference and study. I further agree that permission for extensive
copying of this thesis for scholarly purposes may be granted by the head of my
department or by his or her representatives. It is understood that copying or
publication of this thesis for financial gain shall not be allowed without my written
permission.

(Signature)

Department of Zoology
The University of British Columbia
Vancouver, Canada

Date April 29, 1993
ABSTRACT

The cellular mechanisms responsible for branchial proton excretion and sodium absorption in freshwater rainbow trout, *Oncorhynchus mykiss* (Walbaum), were investigated by monitoring the proton excretion rate and determining the proton ATPase activity in gill tissue under different inhibitor treatments and environmental conditions. Evidence for the existence of an electrogentic proton pump in fish gills was accumulated.

Branchial proton excretion rate was estimated by measuring the total CO\(_2\), total ammonia, pH and buffer capacity of the inspired and expired water of the fish. The net proton excretion across fish gills was resistant to 0.1 mM amiloride which could completely abolish the branchial sodium uptake, indicating that a Na\(^+\)/H\(^+\) exchanger was not responsible for this proton transport. Branchial proton excretion, however, was sensitive to vanadate and acetazolamide, suppressed by low external water pH and sodium levels, and stimulated by elevated ambient Pco\(_2\). All these characteristics are typical for proton transport mediated by an electrogentic proton pump, as demonstrated in frog skin, turtle bladder and mammalian kidney.

N-ethymaleimide-sensitive ATPase activity was measured in crude homogenates of gill tissue from rainbow trout using a coupled-enzyme ATPase assay in the presence of EGTA, ouabain and azide. This NEM-sensitive ATPase activity, determined to be about 1.5 umol/mg.pr./h. at 15°C for freshwater trout, is also
inhibited by other proton-ATPase blockers such as DCCD, DES, PCMBS and Bafilomycins. It was concluded, therefore, that the NEM-sensitive ATPase activity was generated by a proton-translocating ATPase. Since this NEM-sensitive ATPase was also sensitive to the plasma membrane ATPase inhibitor vanadate, the H⁺-ATPase in fish gill was speculated to be a plasma membrane type. Sodium concentration in the external media was the primary regulator of the H⁺-ATPase in fish gills, with low water sodium levels associated with high H⁺-ATPase activity. High external calcium concentration and plasma cortisol levels had a marked stimulating effect on H⁺-ATPase activity in fish gills only when the water sodium level was low. Thus the major role of the H⁺-ATPase in the gill epithelium is to facilitate Na⁺ uptake from fresh water. The H⁺-ATPase in the gills also plays a role in acid-base regulation.

It was concluded that an electrogenic proton pump (H⁺-ATPase) indirectly coupled to a sodium conductive channel in the gill epithelium is the ion transport pathway which mediates proton excretion and energies sodium absorption in freshwater fish.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Abstract</th>
<th>ii</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table of Contents</td>
<td>iv</td>
</tr>
<tr>
<td>List of Tables</td>
<td>v</td>
</tr>
<tr>
<td>List of Figures</td>
<td>vi</td>
</tr>
<tr>
<td>List of Abbreviations</td>
<td>vii</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>ix</td>
</tr>
<tr>
<td>General Introduction</td>
<td>1</td>
</tr>
<tr>
<td>Section I: Branchial proton excretion in rainbow trout mediated by an electrogenic proton pump.</td>
<td>26</td>
</tr>
<tr>
<td>Chapter 1. Inhibitor sensitivity of branchial proton excretion and \textit{in vivo} evidence for the existence of a proton pump.</td>
<td>26</td>
</tr>
<tr>
<td>Summary</td>
<td>27</td>
</tr>
<tr>
<td>Introduction</td>
<td>28</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>30</td>
</tr>
<tr>
<td>Results</td>
<td>38</td>
</tr>
<tr>
<td>Discussion</td>
<td>47</td>
</tr>
<tr>
<td>Chapter 2. Effects of external water pH, Pco$_2$ and ion levels on branchial proton excretion mediated by proton pump.</td>
<td>57</td>
</tr>
<tr>
<td>Summary</td>
<td>58</td>
</tr>
<tr>
<td>Introduction</td>
<td>59</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>61</td>
</tr>
<tr>
<td>Results</td>
<td>64</td>
</tr>
<tr>
<td>Discussion</td>
<td>76</td>
</tr>
<tr>
<td>Section II: Proton-ATPase activity in gill tissue of rainbow trout.</td>
<td>80</td>
</tr>
<tr>
<td>Chapter 3. Inhibitor sensitivity and classification of the proton-ATPase in gill tissue.</td>
<td>80</td>
</tr>
<tr>
<td>Summary</td>
<td>81</td>
</tr>
<tr>
<td>Introduction</td>
<td>82</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>84</td>
</tr>
<tr>
<td>Results</td>
<td>90</td>
</tr>
<tr>
<td>Discussion</td>
<td>97</td>
</tr>
<tr>
<td>Chapter 4. Environmental and hormonal regulation of the proton-ATPase in fish gills.</td>
<td>101</td>
</tr>
<tr>
<td>Summary</td>
<td>102</td>
</tr>
<tr>
<td>Introduction</td>
<td>103</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>105</td>
</tr>
<tr>
<td>Results</td>
<td>108</td>
</tr>
<tr>
<td>Discussion</td>
<td>125</td>
</tr>
<tr>
<td>General Discussion</td>
<td>130</td>
</tr>
<tr>
<td>References</td>
<td>145</td>
</tr>
</tbody>
</table>
LIST OF TABLES

Table 1. Total CO$_2$ excretion by fish.  

Table 2. Comparison between the reduction in the rate of ammonia excretion and the reduction in the rate of net proton excretion under amiloride treatment.

Table 3. Test of inhibitor interference with PK or LDH in the NADH oxidation reaction.

Table 4. Effects of inhibitors on ATPase activity in crude homogenate of trout gill tissue.

Table 5. Plasma pH and NEM-sensitive ATPase activity in gill tissue of NH$_4$Cl-injected rainbow trout.
LIST OF FIGURES

Figure 1. Simplified cross section of gill epithelium of freshwater fish with the gas exchange and ion transport pathways. 2

Figure 2. A schematic view of the outermost living cell layer of frog skin, with two distinct cellular pathways for Na⁺ absorption. 23

Figure 3. The recirculating system with a black chamber. 32

Figure 4. Bicarbonate concentration differences between inspired and expired water of rainbow trout under control, amiloride, vanadate and acetazolamide treatments. 39

Figure 5. Net proton excretion across the gill epithelium of rainbow trout under control, amiloride, vanadate and acetazolamide treatments. 41

Figure 6. Branchial ammonia excretion rates of rainbow trout under control, amiloride, vanadate and acetazolamide treatments. 45

Figure 7. Schematic representation of gas and ion transport across the gill epithelium of rainbow trout. 50

Figure 8. The relationship between the branchial net proton excretion and the inspired water pH. 65

Figure 9. The relationship between branchial net proton excretion and expired water carbon dioxide levels. 67

Figure 10. Branchial proton excretion rate of rainbow trout in control and sodium-free water. 69

Figure 11. Branchial proton excretion rate of rainbow trout in control, sodium-free water and sodium-free water plus 0.1 mM 9-anthroic acid. 72

Figure 12. Branchial proton excretion rate of rainbow trout in control and high calcium (10 mM) water. 74

Figure 13. NEM-sensitive ATPase activity in the crude homogenates of trout gill tissue in response to various concentrations of NEM. 91
Figure 14. DCCD-sensitive ATPase activity in the crude homogenates of trout gill tissue in response to various concentrations of DCCD.

Figure 15. NEM-sensitive ATPase activity in the gill tissue of rainbow trout acclimated to various Na$^+$ and Ca$^{2+}$ levels in the external media for 10-14 days.

Figure 16. Ouabain-sensitive ATPase activity in the gill tissue of rainbow trout acclimated to various Na$^+$ and Ca$^{2+}$ levels in the external media for 10-14 days.

Figure 17. Plasma cortisol concentration in freshwater and seawater rainbow trout after 7 days of chronic cortisol treatment.

Figure 18. NEM-sensitive ATPase activity in the gill tissue of freshwater and seawater rainbow trout after 7 days of chronic cortisol treatment.

Figure 19. Ouabain-sensitive ATPase activity in the gill tissue of freshwater and seawater rainbow trout after 7 days of chronic cortisol treatment.

Figure 20. NEM-sensitive ATPase activity in the gill tissue of freshwater rainbow trout during 48 hours of hypercapnia treatment and 24 hours recovery.

Figure 21. NEM-sensitive ATPase activity in the gill tissue of freshwater rainbow trout during control and 16 days exposure to soft and hard alkaline water.

Figure 22. Hypothetical model of the gas and ion transport pathways in gill epithelium of freshwater rainbow trout.

Figure 23. The relationship between the NEM-sensitive ATPase activity and the ouabain-sensitive ATPase activity in freshwater rainbow trout under different treatments.

Figure 24. The relationship between the NEM-sensitive ATPase activity and the ouabain-sensitive ATPase activity in seawater-adapted rainbow trout under control and cortisol treatments.
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>9-AA</td>
<td>9-anthroic acid</td>
</tr>
<tr>
<td>CA</td>
<td>carbonic anhydrase</td>
</tr>
<tr>
<td>CHAPS</td>
<td>3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate</td>
</tr>
<tr>
<td>DCCD</td>
<td>N,N'-dicyclohexylcarbodiimide</td>
</tr>
<tr>
<td>DES</td>
<td>diethylstilbestrol</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethyleneglycol-bis-(B-aminoethyl)ether)N,N,N',N'-tetraacetic acid</td>
</tr>
<tr>
<td>LDH</td>
<td>lactic dehydrogenase</td>
</tr>
<tr>
<td>NBD-Cl</td>
<td>7-chloro-4-nitrobenz-2-oxa-1,3-diazone</td>
</tr>
<tr>
<td>PCMBS</td>
<td>p-chloromercuri-benzenesulfonate</td>
</tr>
<tr>
<td>PK</td>
<td>pyruvate kinase</td>
</tr>
<tr>
<td>SITS</td>
<td>4-acetamido-4'-isothiocyanatostilbene-2-2'-disulfonic acid</td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS

I would like to thank Dr. David Randall, my supervisor, for his inspiration and support during these studies. It has truly been an enjoyable experience. I am grateful to my supervisory committees Dr. J.E. Phillips, Dr. G.K. Iwama, Dr. A.M. Perks and Dr. J.D. McPhail for valuable comments and discussion on the manuscript. My appreciation extends to all the members in Dr. Randall's lab, specially Mark Shrimpton, Colin Brauner, Sumi Aota and Nick Bernier, for their pleasant company and help. I also like to thank Dr. K. Iwata and Mr. J. Wilson who collaborated on the alkaline study in Chapter 4. Appreciation is also expressed to Dr. Hochachka's Lab for generous use of their spectrophotometer.

I thank my husband Lee, and my parents for always being there for me.
Gas exchange and ion transport in fish gill epithelia

The gills of the fish are the primary site of gas exchange, acid-base regulation and osmoregulation (Maetz and Garcia-Romeu, 1964; McDonald et al, 1989; Randall, 1990). The gill lamellar epithelium is permeable to \( \text{O}_2 \), \( \text{CO}_2 \) and \( \text{NH}_3 \) (Figure 1), and 100% of oxygen uptake, 90% of carbon dioxide and at least 60% of ammonia excretion relies on passive diffusion of the gases (Randall, 1990). Ion transfer across the gill epithelium, on the other hand, is usually mediated by active or passive ion transport processes (Figure 1). In freshwater teleosts, branchial absorption of \( \text{Na}^+ \) and \( \text{Cl}^- \) from the hypo-osmotic environment compensates for the constant loss of ions from the body by diffusion. The mechanisms for \( \text{Na}^+ \) and \( \text{Cl}^- \) uptake are independent (Maetz and Garcia-Romeu, 1964) and the counterions for \( \text{Na}^+ \) and \( \text{Cl}^- \), presumably \( \text{H}^+(\text{NH}_4^+) \) and \( \text{HCO}_3^- \) respectively, are extruded to the water simultaneously (Perry and Randall, 1981; McDonald et al, 1989). These electroneutral ion exchange pathways have been suggested to be involved in acid-base regulation and a portion of carbon dioxide and ammonia excretions.

Carbon dioxide (\( \text{CO}_2 \)) excretion acidifies water as it passes over the gills, due to the hydration of \( \text{CO}_2 \) forming \( \text{HCO}_3^- \) and \( \text{H}^+ \). (Wright et al, 1986; Lin & Randall, 1990). Ammonia (\( \text{NH}_3 \)) excretion on the other hand, can alkalize the expired water of fish due to the conversion of \( \text{NH}_3 \) to \( \text{NH}_4^+ \), consuming protons. The
Figure 1. Simplified cross section of gill epithelium of freshwater fish with the gas exchange and ion transport pathways. ATP-driven pumps are denoted by half-filled circles, ion exchanger by opened circles and passive diffusion by arrows. (Adapted from McDonald et al, 1989).
WATER

BRANCHIAL EPITHELIUM

PLASMA

Na^+

NH_4^+, H^+

NH_3

Cl^-

HCO_3^-

O_2

HCO_3^-

NH_4^+, HCO_3^-, Na^+, Cl^-

K^+ (H^+, NH_4^+)

ATP

H^+

CO_2

CA

H^+

NH_3

CO_2
degree of acidification or alkalinization in expired water depends on the pH and buffer capacity of the external water, the CO$_2$ and NH$_3$ excretion rate and the amount of acid that is excreted into the water by the fish.

The gill lamellar epithelium separating the blood from the external water consists mainly of three cell types - mucous (also called goblet), epithelial (also called pavement) and chloride cells (Laurent and Dunel, 1980). Mucous cells secrete mucous that forms a thin layer on the gill surface. Epithelial cells are permeable to respiratory gases such as O$_2$, CO$_2$ and NH$_3$ and play a prominent role in gas exchange. Chloride cells, also known as mitochondria-rich cells, house most of the energy consuming ion transport pathways such as Na$^+$-K$^+$-ATPase and are involved in osmoregulation by euryhaline teleost (Pisam, et al, 1987). In freshwater fish the epithelium and chloride cells are joined by tight junctions, which act as a minimal barrier for diffusion of gases, but has a high resistance to the transfer of ions and water. Thus gill epithelia in freshwater fish are considered to be "tight" epithelia (Sardet, 1980).

This tight epithelium consists of two cell membranes. Studies of turtle bladder epithelia showed that the apical membrane has a very low permeability to H$^+$, OH$^-$ and is capable of generating an electrochemical potential gradient for protons either by a proton pump or by an exchange that is driven by a concentration gradient for another ion. The second cell membrane is the basolateral cell membrane which is permeable to HCO$_3^-$, OH$^-$. 
and H' and permits the passive movement of these ions due to the existence of transport proteins specialized in the transfer of anions (Steinmetz, 1985).

Chloride cells in the gill epithelia manage the role of acid-base regulation and osmoregulation because they have high mitochondrial activity and contain primary and secondary active ion transport pathways. These pathways (see Figure 1) have been postulated to include:

1) Na⁺-K⁺-ATPase in the basolateral membrane. This primary active transport pathway maintains a low intracellular Na⁺ concentration by continuously pumping Na⁺ out to serosal side in exchange for K⁺. For each ATP consumed, 3 Na⁺ is pumped out and 2 K⁺ is pumped in. K⁺ can be replaced by H⁺ or NH₄⁺ (Evans et al, 1989) and enter the cell through this pathway. Ouabain is the specific inhibitor for Na⁺-K⁺-ATPase (Pedersen and Carafoli, 1987). Ouabain applied to the extracellular surface of the membrane blocks potassium dependent Na⁺-K⁺-ATPase by competing for the K⁺ binding site. Ammonia excretion in Opsanus beta perfused head was reduced by 22% when 0.1 mM ouabain was added to the perfusate, possibly due to the blockage of Na⁺-K⁺(NH₄⁺)-ATPase by ouabain (Evans et al, 1989).

2) Cl⁻/HCO₃⁻ exchange is located in the apical membrane, through which chloride is absorbed and bicarbonate excreted. Since the gill epithelium is impermeable to HCO₃⁻ (Perry et al. 1982) and Cl⁻, it has been suggested that a portion of the carbon dioxide entering the gill epithelium is hydrated, forming
bicarbonate, which is then exchanged for chloride across the apical membrane. The addition of 4-acetamido-4'-isothiocyanato stilbene-2-2'-disulfonic acid (SITS), (known to block Cl⁻/HCO₃⁻ exchange in red blood cells), to the water results in a rise in blood pH in trout (Perry et al. 1981) and a 71% reduction in Cl⁻ uptake (Perry & Randall, 1981). Although no more than 10% of the total carbon dioxide is transferred to water by this means, it is a primary pathway for chloride uptake in freshwater fish. SITS inhibits this anion transport by binding to a specific membrane protein.

Considerable evidence has been gathered to support the existence of Cl⁻/HCO₃⁻ exchangers in the basolateral cell membranes of tight epithelia such as in turtle bladder and frog gastric mucosa (Steinmetz, 1985). In turtle bladder, this Cl⁻/HCO₃⁻ exchanger is paralleled by a Cl⁻ conductive channel which permits the Cl⁻ to recycle across the basolateral membrane so that the exit of HCO₃⁻ is associated with the transfer of an electron.

(3) Na⁺/H⁺(NH₄⁺) exchange in the apical membrane was concluded to be the major pathway for sodium uptake and proton excretion and an optional excretory pathway for ammonia (Wright and Wood, 1985). Amiloride is a very potent and specific inhibitor of sodium transport, including both the sodium conductive channels and Na⁺/H⁺(NH₄⁺) exchange, in a wide variety of cellular and epithelial transport systems, by competing for the Na⁺ transport site (Benos, 1982). Addition of amiloride to
water reduced sodium uptake in the gill epithelium of trout by 84% (Perry and Randall, 1981) and 94% (Wright and Wood, 1985).

The trout gill NH$_3$ permeability coefficient of 6X10$^{-3}$ cms$^{-1}$ (Avella & Bornancin, 1989) is intermediate between values reported for the toad bladder and mammalian kidney tubule. It has been suggested that ammonia excretion, although dominated by NH$_3$ diffusion (Hillaby & Randall, 1979; Cameron & Heisler, 1983), is also mediated by Na$^\dagger$/NH$_4^\dagger$ exchange on the apical surface (Payan, 1978; Wright & Wood, 1985). The stimulating effect of NH$_4^\dagger$ on sodium flux, however, could be explained in terms of a pH effect of the ammonia addition (Cameron & Kormanik, 1982), and Avella and Bornancin (1989) and Heisler (1990) concluded that the balance of evidence was against the presence of Na$^\dagger$/NH$_4^\dagger$ exchange across the apical surface of trout gills. Avella and Bornancin (1989) considered the trout gill to be similar to other tight epithelia, such as frog skin and toad bladder, in that passive sodium uptake from water is indirectly coupled to an active electrogenic proton transport system.

Possibility of the existence of H$^\dagger$-ATPase in fish gills

The most recent evidence for the existence of Na$^\dagger$/H$^\dagger$(NH$_4^\dagger$) exchange in the fish gills comes from the in vivo studies of Wright and Wood (1985), who demonstrated that sodium influx and ammonia efflux is coupled with approximately 1:1 stoichiometry in the external water pH range of 4-9 and concluded that a flexible combination of NH$_3$ diffusion and Na$^\dagger$/NH$_4^\dagger$ exchange was the major
mechanism of ammonia excretion. 94% of the Na\(^+\) uptake by the gills of freshwater trout was inhibited by 0.1 mM amiloride in the external media. However, without altering $\Delta \text{PNH}_3$ or $\Delta [\text{NH}_4^+]$ across the epithelium, which means no ammonia transfer should have been shifted from $\text{Na}^+ / \text{NH}_4^+$ exchange to $\text{NH}_3$ diffusion, amiloride exposure caused only 23% reduction in ammonia excretion. These authors provided no clear explanation for the contrary result. The degree of coupling between sodium absorption and ammonia excretion was also found to be rather loose in goldfish, carp and trout in vivo (Payan, 1978). Sodium uptake from a diluted medium such as freshwater, will usually require an active transport process. Although intracellular sodium concentration in the gill epithelium cell may be lowered by the $\text{Na}^+-\text{K}^+-\text{ATPase}$ in the basolateral membrane to about 10 mM (intracellular sodium ion activity in frog skin epithelium was measured to be 6.2 mmol/l using a double-barrelled ion sensitive microelectrodes, by Harvey and Ehrenfeld, 1986), it is still higher than that in fresh water (usually < 1 mM) and the sodium electrochemical gradient across the apical membrane could not possibly drive the $\text{Na}^+/\text{H}^+$ exchange (Avella and Bornancin, 1989). Therefore, the argument for a $\text{Na}^+/\text{H}^+(\text{NH}_4^+)$ exchange is not strong. The $\text{NH}_4^+$ concentration gradient might provide the driving for $\text{Na}^+/\text{H}^+(\text{NH}_4^+)$ exchange. However, Avella and Bornancin (1989) re-examined the mechanism of ammonia excretion and sodium absorption using an isolated-perfused head preparation. They found that ammonia excretion was basically dependent on passive $\text{NH}_3$
diffusion, sodium absorption was indirectly modified by intracellular pH and sodium uptake and ammonia excretion were uncoupled. An indirect coupling of an electrogenic proton pump and a passive sodium entry, instead of a Na"H\textsuperscript{+}(NH\textsubscript{4}\textsuperscript{+}) exchange, was proposed by these authors as the mechanism of sodium and proton transport across the fish gill epithelium.

Na"H\textsuperscript{+} exchange is only one of the two fundamentally different mechanisms that has been proposed to account for Na" and H" transport in opposite directions in ion-transporting epithelia. Na"H" countertransport, a passive and electroneutral process, exists in certain isolated cells such as sea urchin eggs and red blood cells, and in "leaky" epithelia such as rabbit gall-bladder, small intestine and renal proximal tubule. The other mechanism is that of an active proton transport, which has been described in "tight" epithelia such as the turtle and toad urinary bladder (Steinmetz, 1986; Al-Awqati, 1978), frog skin (Ehrenfeld et al, 1985) and mammalian renal collecting tubule (Ait-Mohamed et al, 1986). This proton transport is initiated by a electrogenic proton-translocating-ATPase which pumps hydrogen ion to one side of the membrane and generates a negative potential in the other side of the membrane that, in some cases, drives sodium flux via a sodium conductive channel (Ehrenfeld et al, 1985).

The gill epithelium of freshwater fish is considered to be a tight epithelium (Sardet, 1980; Avella and Bornancin, 1989). It resembles freshwater frog skin and turtle urinary bladder
epithelia in many features. Functionally they are all capable of acid-base regulation and electrolyte transport. Both freshwater frogs and freshwater fish are hyperosmotic to their aqueous surroundings and face the problem of continual loss of body salt to the environment. The lost salts have to be replaced through an active transport system across the epithelia in the skin of frogs or the gills of fish. Morphologically they contain analogous cell types. The epithelium of turtle urinary bladder consists of basal cells, granular cells and carbonic anhydrase (CA)-rich cells. CA-rich cells, which contain numerous mitochondria and tubulovesicular membrane structures, are responsible for H⁺ secretion in turtle bladder (Madsen and Tisher, 1985). It could be equivalent to the chloride cell in the gill epithelium.

The apical membrane of frog skin comprises stratum granulosum firmly interconnected by tight junctions that form a barrier separating the apical bathing solution from the basolateral solution (Nielsen, 1982), similar to those in gill lamellae. The outermost living cell layer of the frog skin epithelium is composed of cuboidal granular (GR) cells and mitochondria-rich (MR) cells (Ehrenfeld et al, 1989). The latter cells can be identified by their long flask-like shape and narrow apical pole beneath the stratum corneum, the exclusive localization of carbonic anhydrase and the rod-shaped intraplasmic membrane particles. The intercalated cells in mammalian renal collecting tubule also share similar characteristics described above for the MR cell of frog epithelium and
responsible for proton transport across kidney tubular epithelium (Brown et al, 1988). Bartels (1989) suggested that, in lamprey, the morphological characteristics of the epithelial cell were identical to those of the frog skin GR cell. Thus, it is logical to suggest that the same ion transport mechanism, namely H⁺-ATPase indirectly coupled with a sodium channel, will exist in gill epithelium to account for the proton and sodium transport.

Differences do exist between the gill epithelium and other tight epithelia. Carbonic anhydrase for example, is generally distributed in the chloride cells, epithelial cells and mucous cells in fish gills (Rahim et al, 1988), but restricted to MR cells of frog skin (Rosen and Friedley, 1973) and CA-rich cells of turtle bladder (Madsen and Tisher, 1985). The percentage of chloride cells in gill epithelium of freshwater fish is only 1% (Perry and Walsh, 1989), whereas the ratio of MR to GR cells in frog skin is much higher and MR cells can represent between 13 to 60% of the exposed apical area depending on salt adaptation (Ehrenfeld et al, 1989), since frog skin plays a minor role in gas transfer. The location and distribution of ion transport pathways in different cell types in fish gill epithelium, therefore, might not be exactly the same as that in frog skin epithelium.

**Background information about H⁺-ATPase**

Proton-Translocating-ATPases are integral membrane proteins that vectorially translocate H⁺ from one surface to the other
(Pedersen and Carafoli, 1987). They can be classified into three categories: Mitochondria H⁺-ATPase (F-type), vacuolar H⁺-ATPase (V-type) and plasma membrane H⁺-ATPase (P-type). Mitochondria H⁺-ATPase utilizes the proton gradient generated by the cytochrome chain in the inner mitochondrial membrane for ATP synthesis and provides the energy source for other ATPases in the cell.

Vacuolar H⁺-ATPase and mitochondria H⁺-ATPase share a number of important structural properties, including complexity of subunit composition and probably are derived from a common evolutionary ancestor (Forgac, 1989). Vacuolar H⁺-ATPase consumes ATP and actively pumps protons against an electrochemical gradient into the vacuoles. The function of plasma membrane H⁺-ATPase is similar to that of vacuolar H⁺-ATPase but it has a lower molecular weight and a simpler subunit structure, with only 2 subunits instead of 18 as in mitochondria H⁺-ATPase and 16 as in vacuolar H⁺-ATPase (Forgac, 1989).

The H⁺-ATPase in the plasma membrane of eukaryotic cells are classified as phosphorylated ion motive enzymes because they form a covalent phosphorylated intermediate as part of the reaction cycle (Pedersen and Carafoli, 1987). Na⁺-K⁺-ATPase and Ca⁺⁺-ATPase are also phosphorylated ion motive ATPases. Vanadate, a transition state analog of phosphate, inhibits P-type ATPase by blocking the formation of phosphorylated intermediates in the ATPase.

Mitochondria H⁺-ATPase functions obligatorily in the direction of ATP synthesis and supplies ATP to the plasma membrane H⁺-ATPase and vacuolar H⁺-ATPase which function obligatorily in the direction of ATP hydrolysis to transport protons actively (Pedersen and Carafoli, 1987).

Many epithelial membranes have the capacity to transport hydrogen ions. In the "tight" epithelial membranes, such as in mammalian renal collecting tubules (Gluck and Al-Awqati, 1984; Ait-Mohamed et al., 1986), turtle urinary bladder (Steinmetz and Andersen, 1982; Steinmetz, 1985) and frog skin (Ehrenfeld et al., 1990), the P-type or V-type proton-translocating-ATPase, also termed electrogenic proton pump, is responsible for the transport of protons. The proton pump is characterized by tight coupling to the energy of ATP hydrolysis but not directly dependent on movement of other ions. It plays a vital role in acid-base and osmotic homeostasis in animals.

Proton pumps located in the luminal membrane of freshwater turtle urinary bladder and apical membrane of freshwater frog skin have been extensively studied because the turtle bladder and frog skin permit some major simplifications, eg. they can be stretched as a flat epithelium sheet between two bulk solutions, and the passive H⁺ permeability in these epithelia is so low that net H⁺ transport rates can be used directly to study the behaviour of the proton pump (Steinmetz and Andersen, 1982).

The proton transport mediated by this active pump has several important properties:
(1) The proton pump is electrogenic. In the short-circuited turtle bladder, proton secretion takes place against a steep pH gradient and generates 40 mV negative intracellular potential (Steinmetz and Andersen, 1982). Proton excretion in frog skin generates a negative potential (about -50 mV) which is a driving force for passive sodium uptake via a conductive Na⁺ entry channel from dilute mucosal solutions. 0.05 mM amiloride added to the mucosal solution inhibited sodium influx in frog skin under voltage clamp conditions but had no effect on proton excretion (Ehrenfeld et al, 1985). However, addition of 0.01 mM amiloride or substitution of external Na⁺ by Mg²⁺ or K⁺ caused a hyperpolarization of apical membrane potential and inhibited proton excretion under open circuit conditions in frog skin (Harvey and Ehrenfeld, 1986). Proton excretion in frog skin does not require sodium transport but they can be electrically coupled with a 1:1 stoichiometry.

(2) Proton pump operation is regulated primarily by the proton electrochemical gradient across the membrane. Proton secretion in turtle bladder as well as the proton excretion in frog skin increases with increasing mucosa-negative voltage and increasing luminal pH (Steinmetz and Andersen, 1982; Ehrenfeld et al, 1985). ΔpH (serosal pH - mucosal pH) and Δψ (the electric potential difference between the serosal side and the mucosal side of the epithelium) have equivalent inhibitory or stimulating effects on proton flux. Frog skin proton transport is annulled at ΔpH of 1.59 unit or Δψ of -80 mV, but proton secretion in turtle
bladder continues until a ΔpH of up to 2.7 pH units or ΔΨ down to -180 mV. Studies of turtle urinary bladder showed that the instantaneous changes in the proton transport rate induced by varying pH involved changes of intrinsic properties of the proton pump but not the number of pumps (Steinmetz, 1986).

(3) The cellular acid-base conditions are the second major determinant of the rate of proton transport. Cell [H+] is the final common pathway by which changes in serosal or mucosal Pco2 and HCO$_3^-$ can exert their effects on the pump (Cannon et al, 1985). Proton transport rate could be related to cell [H+] as a Michaelis-Menten function in which hydrogen ions serve as the substrate for the proton pump. The proton pump in turtle bladder is far more sensitive to pH changes on its cytoplasmic side than on the luminal side. The regulatory pH range on the cell side is about 1 pH unit, whereas it is at least 3 pH units on the luminal side (Steinmetz, 1985).

(4) Protons transported by the proton pump have an intracellular origin. Protons are generated in the cellular compartment from CO$_2$ hydration under the catalysis of carbonic anhydrase (CA). CA-rich cells, which constitute 10-15 percent of luminal surface area, are responsible for proton secretion in turtle bladder (Steinmetz and Andersen, 1982). Proton transport via the proton pump is stimulated by an increase in the ambient CO$_2$ in both turtle bladder and frog skin (Cannon et al, 1985; Ehrenfeld and Garcia-Romeu, 1977). Addition of 1 mM acetazolamide (CA inhibitor) to the serosal side of frog skin caused
considerable inhibition of the Na\(^+\) and H\(^+\) fluxes without changing the relationship between the two (Ehrenfeld and Garcia-Romeu, 1977). Administration of 0.1 mM ethoxyolamide, a lipophilic CA inhibitor, to either the serosal or the mucosal side of frog skin, induced a 70% reduction in proton excretion which in turn reduce sodium influx (Ehrenfeld et al., 1985). It is suggested that CA inhibition affects proton transport indirectly by inhibiting the intracellular CO\(_2\) hydration reaction which supplies hydrogen ions to the proton pump. The by-product, bicarbonate ion, passes into the serosal medium by Cl\(^-\)/HCO\(_3\)^- exchange in the basolateral membrane. SITS applied to the serosal site of turtle bladder caused secondary inhibition on H\(^+\) transport which was associated with increased alkalinity of the cell. Cl\(^-\) was required in the serosal compartment for H\(^+\) transport in the urinary bladder epithelium (Steinmetz and Andersen, 1982).

(5) H\(^+\)-ATPase has been noted to be either bound to membranes or packaged in cytoplasmic vesicles of CA-rich cells in turtle bladder (Arruda et al., 1990) and intercalated cells in rat kidney (Brown et al., 1988). Proton pumps can be inserted in the membrane by exocytosis of vesicles containing the ion motive enzyme or removed from the membrane by endocytosis of segments of the membrane in which H\(^+\)-ATPases are concentrated, and the process can be induced by environmental stimuli (Brown, 1989; Schwartz and Al-Awqati, 1985; Stetson, 1989). Increased ambient CO\(_2\) for example, causes cytoplasmic acidification in both turtle bladder
and mammalian renal tubules which in turn raises cell calcium. Calcium causes rapid fusion of the vesicles to the luminal membrane and the proton pump is inserted exocytically. The pumps then turnover in the luminal membrane and pump protons out of the cell, recovering the cell pH towards its original level (Cannon et al, 1985; Arruda et al, 1990; Schwartz and Al-Awqati, 1985).

(6) Proton pump activity relies on the energy of ATP hydrolysis. The stoichiometry of the pump is estimated to be $3 \text{H}^+ / \text{ATP}$ (Al-Awqati and Dixon, 1982). The CA-rich cells, MR cells and intercalated cells in which $\text{H}^+-\text{ATPase}$ is located, have abundant mitochondria (Madsen and Tisher, 1985). Turtle urinary bladder is capable of using both aerobic and anaerobic metabolism as energy sources to drive active proton transport. Oligomycin, a classical inhibitor of mitochondrial ATPase, has no effect on turtle bladder $\text{H}^+-\text{ATPase}$ (Steinmetz and Andersen, 1982). However, the proton pump in frog skin depends completely on oxidative metabolism, since anaerobic conditions totally block proton excretion, and 83% of the proton pump activity in frog skin was inhibited by 14 ug/ml oligomycin in either mucosal or serosal solutions, presumably due to an indirect effect on mitochondrial respiration (Ehrenfeld et al, 1985).

(7) Inhibitor sensitivity:

Orthovanadate is a specific inhibitor of plasma membrane ATPase. Micromolar concentrations of vanadate can completely abolish the plasma membrane ATPase of Neurospora, but even millimolar concentrations had no effect on mitochondrial ATPase
from the same organism (Goffeau and Slayman, 1981). Vanadate, however, inhibits not only H⁺-ATPase in plasma membrane, but many other transport ATPases, including Na⁺-K⁺-ATPase and Ca²⁺-ATPase. Orthovanadate ion, VO₄³⁻, acting as a phosphate transition analogue, blocks the formation of phosphorylated intermediates in these ATPase (Pedersen and Carafoli, 1987). Vanadate in the serosal solution of toad bladder (Beauwens et al., 1981) and turtle bladder decreases proton secretion markedly under anaerobic and aerobic conditions (Arruda et al., 1981). Proton excretion through frog skin is completely abolished when 1 mM vanadate is applied to the serosal site (Ehrenfeld et al., 1985). However, vanadate exerts no inhibitory effect on either the proton-ATPase activity or the proton transport in mammalian renal tubules (Gluck and Caldwell, 1987; Turrini et al., 1989) because the H⁺-ATPase in mammalian kidney belongs to the vacuolar type.

N-ethylmaleimide (NEM) is another metabolic inhibitor that effects V-type and P-type H⁺-ATPase, with much more potent inhibition on vacuolar H⁺-ATPase (1-2 uM) than plasma membrane H⁺-ATPase (0.1-1 mM). Mitochondrial ATPase is virtually resistant to NEM (Forgac, 1989). NEM is an alkylating agent that is relatively selective for sulfhydryl groups (SH-) and inhibits H⁺-ATPase in an ATP-protetable manner. So-called NEM-sensitive ATPase is found in all segments of mammalian kidney (Ait-Mohamed et al., 1986; Gluck and Al-Awqati, 1984; Gluck and Caldwell, 1987). Proton excretion across frog skin is also inhibited by 1 mM NEM (Ehrenfeld et al., 1990). PCMBS is also a SH-group reagent
and affects proton-ATPase in rat and bovine kidney (Turrini et al., 1989; Gluck and Al-Awqati, 1984).

Dicyclohexylcarbodiimide (DCCD) can bind to a subunit (the DCCD binding protein) of the hydrophobic channel portion and inhibit H⁺-ATPase in mitochondria, vacuolar and plasma membrane (Pedersen and Carafoli, 1987). The sensitivity was highest in F-type, followed by V-type and then P-type (Forgac, 1989). H⁺-ATPase in mammalian kidney (Ait-Mohamed et al., 1886), turtle bladder (Steinmetz and Andersen, 1982) and frog skin (Ehrenfeld et al., 1985) is sensitive to DCCD. Diethylstibestrol (DES) has a very similar effect on H⁺-ATPase as DCCD (Pedersen and Carafoli, 1987) and 0.1 mM DES was found to inhibit proton excretion in frog skin (Ehrenfeld et al., 1990).

7-chloro-4-nitrobenz-2-oxa-1,3-diazole (NBD-Cl) is also an alkylating agent that potently inhibits H⁺-ATPases of all types (Forgac, 1989), by affecting the NH₂-group in an ATP-protetable manner. Proton-ATPase in mammalian kidney was reported to be sensitive to 10-20 uM NBD-Cl (Gluck and Caldwell, 1987; Turrini et al., 1989).

Bafilomycins is a macrolide antibiotic that appears to be a very specific and potent inhibitor to vacuolar H⁺-ATPase (Bowman et al., 1988). Mitochondrial H⁺-ATPase is resistant to it and plasma membrane H⁺-ATPase is moderately sensitive to it. It can be used as a valuable tool for distinguishing among the three different types of H⁺-ATPases. Another reagent demonstrating unique specificity to V-type H⁺-ATPase is potassium nitrate
Vacuolar H⁺-ATPase from Neurospora was inhibited by KNO₃ with a half-maximal inhibition seen at 50 mM, whereas plasma membrane and mitochondria H⁺-ATPase from the same organism was completely resistant to KNO₃ up to 100 mM (Bowman, 1983).

(8) Hormonal regulation:

Among the hormonal factors regulating proton secretion, the mineralocorticoid hormones play a central role. The rate of acidification by the urinary bladder is stimulated by aldosterone in toad (Ludens et al., 1974) and turtle (Al-Awqati et al., 1976) and the effects were independent of the stimulation of sodium transport. The stimulation of proton secretion is not associated with a change in the apparent proton-motive force (the intrinsic property of the pump; Al-Awqati et al., 1976). Instead, aldosterone has been shown to be preferentially bound to MR cells of urinary bladder (Sapirstein and Scott, 1975) and frog skin (Harvey, 1992) and produce rapid exocytotic insertion of the proton pump (Harvey, 1992). Aldosterone was also reported to have both a long-term and short-term stimulating effect on proton-ATPase in renal collecting tubules of mammals (Garg and Narang, 1988; Khadouri et al., 1989; Mujais, 1987).

Deoxycorticosterone acetate (DOCA), a potent mineralocorticoid, elicits a stimulation of proton transport in amphibian skin (Ehrenfeld et al., 1989), which also induces morphological changes in the MR cells of frog skin epithelia (Voute et al., 1972; Voute et al., 1975). Serosal 10⁻⁶ M DOCA
addition induced a 31% proton secretion and a 28% sodium absorption. The action of DOCA on H'-ATPase appears to be independent of the sodium effect since stimulation of proton efflux was also observed in the absence of sodium transport (Ehrenfeld et al, 1989).

Neurohypophyseal hormones, including arginine vasotocin (AVT) and oxytocin, enhance transepithelial sodium transport, osmotic response and permeability to a variety of solutes in frog or toad urinary bladder and frog skin (De Sousa and Grosso, 1981). Sodium transport in frog skin was only enhanced by AVT (10^-6 M) or oxytocin (100 mU/ml) when the skin was bathed on its apical side with high sodium containing solution (115 mmol/l), while sodium influx is independent of proton secretion since chloride ions can provide the permeant counterion for sodium absorption (Ehrenfeld et al, 1989). Conversely, in dilute NaCl solutions, where sodium absorption is tightly coupled to proton secretion, neither oxytocin nor AVT stimulated sodium transport or proton secretion (Ehrenfeld et al, 1989). This indicates that neurohypophyseal hormones increase sodium permeability in frog skin, but exert no effect on the H'-ATPase in MR cells. Studies on three amphibian species by Brown et al (1981) showed that there was no relationship between MR cell morphology and oxytocin treatments, and suggested that there was a lack of neurohypophyseal receptors on the MR cell membranes.

(9) Cellular model for Na^+ absorption and H^+ excretion in frog skin:
Two types of sodium absorption kinetics have been described in frog skin under open-circuit conditions (Ehrenfeld et al., 1989; Ehrenfeld et al., 1990). The first one of high capacity and low affinity, is predominant in high NaCl-containing water, and is mainly mediated by the granular (GR) cells (Figure 2). Cl\textsuperscript{-} is the accompanying anion and proceeds through voltage-gated Cl\textsuperscript{-} channels and Cl\textsuperscript{-}/HCO\textsubscript{3}\textsuperscript{-} exchangers (not represented in the figure) located on the apical membranes of the mitochondria-rich (MR) cells and/or through a paracellular pathway. An acid load blocks the sodium transport by the gating effects of protons on the Na\textsuperscript{+} and K\textsuperscript{+} conductance of the GR cells. Basolateral exchangers (Na\textsuperscript{+}/H\textsuperscript{+} and Cl\textsuperscript{-}/HCO\textsubscript{3}\textsuperscript{-}) regulate cell pH and thereby participate in the control of Na\textsuperscript{+} transport. The second sodium absorption mechanism, of high affinity and low capacity, is predominant in dilute apical NaCl solutions, which is the physiological condition for freshwater frogs. Sodium absorption here is energized by a favourable apical electrical potential generated by the electrogenic proton pump located in the MR cells, and this electrical effect should occur in both cell types. After an acid load, Na\textsuperscript{+} transport and H\textsuperscript{+} secretion are increased and Na\textsuperscript{+} transport via MR cells may be favoured, since the Na\textsuperscript{+} and K\textsuperscript{+} conductances of the GR cells are inhibited by intracellular protons. The two cell types are targets for aldosterone whereas the neurohypophyseal hormone, AVT and oxytocin affect only the GR cells.
Figure 2. A schematic view of the outermost living cell layer of frog skin, with two distinct cellular pathways for Na⁺ absorption. (Adapted from Ehrenfeld et al, 1990).
The key question I tried to answer in this study was: Does H+-ATPase exist in fish gills as suggested by Avella and Bornancin (1989)? Most investigators have supported Maetz's model (1964) of Na'/H+ and Cl'/HCO3−, but the H+-ATPase model has not been investigated. Gills of freshwater fish behave like frog skin in many ways: branchial sodium influx is sensitive to amiloride and low pH (Wright and Wood, 1985); proton efflux is inhibited by low pH (McDonald and Wood, 1981); transepithelium potential changes with external water pH (McWilliams and Potts, 1978; Ye et al, 1991). These are all consistent with the presence of a proton pump in fish gills. I have attempted to gather evidence for the existence of a proton pump in fish gills, and to study its inhibitor sensitivities, function, regulation and relationship with other ion transport pathways in gill epithelium.

Due to the structural complexity of fish gills, gill epithelium can not be stretched out as a flat sheet; an isolated gill preparation does not behave in the same way as in vivo (Perry et al, 1984); many conventional methods for studying ion transport pathways such as the Ussing chamber, cannot be applied to gills. My approach to the problem therefore, was limited to examining the branchial proton excretion of intact fish, and assaying H+-ATPase activity in homogenates of gill tissue.
SECTION I: BRANCHIAL PROTON EXCRETION IN RAINBOW TROUT MEDIATED
BY AN ELECTROGENIC PROTON PUMP

Chapter 1: Inhibitor sensitivity of branchial proton excretion
and *in vivo* evidence for the existence of a proton pump
SUMMARY

Total CO₂, total ammonia, pH and buffering capacity of the inspired and expired water in rainbow trout were measured and branchial proton excretion rate was calculated from these data. Ion transport inhibitors such as amiloride, vanadate and acetazolamide were added to the water to determine their effects on branchial proton excretion. There was a substantial proton excretion across the gill epithelium which caused dehydration of bicarbonate in expired water and was responsible for the expired water acidification. Proton excretion across the gills is insensitive to 0.1 mM amiloride but sensitive to vanadate, acetazolamide and higher concentration of amiloride; thus, we conclude that proton excretion is probably mediated by an active proton pump, instead of a Na⁺/H⁺ exchanger, on the apical membrane of gill epithelium similar to that reported for the frog skin and turtle bladder.
INTRODUCTION

Water is acidified as it passes over the gills of fish (Wright et al., 1986; Lin and Randall, 1990). The acidification of expired water is inhibited by acetazolamide, a carbonic anhydrase inhibitor in external water. It was concluded therefore, that the acidification of water was caused by the hydration of excreted CO$_2$, resulting in increased concentration of bicarbonate and protons (Wright et al., 1986). Carbonic anhydrase in the gill mucus (Wright et al., 1986) and on the apical surface of the gill epithelium (Rahim et al., 1988) will catalyse the CO$_2$ hydration reaction.

Acidification of expired water could also be caused by excretion of acid equivalents across the gills. Excretion of protons, NH$_4^+$ or absorption of HCO$_3^-$ or any combination of the three would also result in a decrease of expired water pH. HCO$_3^-$ absorption is unlikely to be the cause of expired water acidification because this acidification was not affected by SITS, a Cl$^-$/HCO$_3^-$ exchange inhibitor (Lin and Randall, 1991). Proton transport in some epithelia is inhibited by acetazolamide (Steinmetz and Anderson, 1982; Ehrenfeld and Garcia-Romeu, 1977), and this could be an alternate interpretation of the acidification of expired water.

Na$^+$/H$^+$(NH$_4^+$) exchange in the apical membrane of gill epithelia has been concluded to be the major pathway for sodium uptake and proton excretion and an optional excretory pathway for
ammonia (Maetz, 1964; Wright and Wood, 1985). This conclusion was challenged by Avella and Bornancin (1989) who demonstrated that sodium uptake and ammonia excretion were uncoupled. An indirect coupling of an electrogenic proton pump and a passive sodium entry was proposed by these authors as the mechanism of sodium and proton transport across the fish gill epithelium. In fact, proton transport in frog skin and turtle bladder is mediated by a proton pump and it is sensitive to acetazolamide and vanadate. The objective of this study is first, to clarify whether the acidification of expired water in fish can be attributed to excreted CO$_2$ hydration, by analyzing the bicarbonate level in both inspired and expired water; and secondly, to identify the pathway through which protons are excreted by applying ion transport inhibitors such as amiloride, vanadate and acetazolamide to external water.
Animals and Preparation

Rainbow trout *Oncorhynchus mykiss* (Walbaum), weighing 202-592g, were maintained in outdoor fibreglass tanks supplied with flowing dechlorinated Vancouver tap water (pH 6-7; [Ca$^{2+}$], 0.03 mM; [Na$^+$], 0.89 mM; [Cl$^-$], 0.92 mM; [K$^+$], 0.03 mM, [Mg$^{2+}$], 0.01 mM). Fish were fed daily with commercial trout pellets and feeding was suspended for at least 48 h prior to experimentation.

Surgery was performed on each fish under general anaesthesia (1:10,000 MS222 solution, pH adjusted to 7.5 with NaHCO$_3$) to fix an opercular cannula for sampling expired water. Fish were then confined, but not physically restrained, in a black chamber to recover for at least 24 h. This black chamber was supplied with aerated dechlorinated tap water during the recovery period. Three hours prior to the experiment, the water supply was switched to the aerated test solution (40 mM NaCl and 0.5 mM CaCl$_2$ in dechlorinated tap water, from Wright *et al.* 1986) with a buffering capacity (B) of 81 unequiv.L$^{-1}$.pH unit$^{-1}$. The test solution had the same ionic strength as the buffer solution used to calibrate the pH electrodes. By using this test solution, we reduced the response time of the pH electrode, increased its stability and thus obtained more precise water pH measurements. Temperature was regulated to that of tap water with a cooling coil.

Experimental Protocols
Experiments were carried out using a recirculating system connected to a black chamber (Figure 2). The volume of the recirculating system was 6 litres and it was aerated and controlled to ambient temperature. A magnetic stirring bar was used in the reservoir to ensure complete mixing. Ion transport inhibitors such as amiloride, vanadate and acetazolamide were added to the recirculating system and their effects on CO$_2$, ammonia and net proton excretion determined. Inspired and expired water samples (approximately 5 ml each) were withdrawn from the outlets of the glass electrode chambers for total CO$_2$, ammonia and buffer capacity analysis.

(1) Amiloride treatment

Three concentrations of amiloride were utilized. Each experiment started with a one hour control period, with the fish rested in the recirculating system containing the test solution alone and inspired and expired water were sampled at 30 minutes and 60 minutes. The system was flushed with fresh test solution and amiloride was added to the system to give a final concentration of 0.1, 0.5 or 1 mM. Recirculation was restored and the amiloride treatment lasted for another hour. Water samples were taken at 30 minutes and 60 minutes. The results of the 30 minutes and 60 minutes sampling were pooled for data analysis since there was no significant difference between them.

(2) Vanadate treatment

Experiment started with a one hour control period followed by a one hour treatment period. Freshly made 3 mM sodium
Figure 3. The recirculating system with a black chamber. Fish were prepared with an opercular cannula. Inspired and expired water samples were withdrawn from the outlets of the glass electrode chambers.
orthovanadate (Na$_3$VO$_4$) solution was boiled and the cooled solution was neutralized with 0.1 mol l$^{-1}$ HCl. After the control period, 200 ml of the 3 mM vanadate solution was added to the 6 litre recirculating system to obtain a final concentration of 0.1 mM. Since the ammonia accumulation in the system was very low (less than 100 uM after 2 hours), flushing the system with fresh test solution at the beginning of the treatment period was considered unnecessary. External water pH was adjusted to the control value during the two 30 minutes treatment periods. Inspired and expired water samples were taken at 30 minutes intervals and the 30-min and 60-min samples were pooled for data analysis since no significant differences were observed.

(3) Acetazolamide treatment

For the acetazolamide treatment, acetazolamide was added to the system to give a final concentration of 0.1 mM after a 30-min control period. Inspired and expired water were sampled at the end of control period and 30 minutes and 60 minutes after acetazolamide treatment. Again the 30-min and 60-min treatment samples were pooled for analysis.

Analytical techniques and calculations

Inspired and expired water pH were monitored during the whole experimental period with combination glass pH electrodes housed in two water-jacketed glass chambers (Wright et al. 1986). Inspired pH (pH$_{in}$) and expired pH (pH$_{ex}$) values were recorded at each sampling.

Total carbon dioxide contents of inspired water [$CO_2$]$_{in}$ and
expired water \([\text{CO}_2]_{\text{ex}}\) were measured immediately with a Carle gas chromatograph (model III) containing a \(\text{CO}_2\) discriminating column (porapak Q) (Boutilier et al. 1985; Lenfant & Aucutt, 1966).

Total ammonia contents of inspired water \([\text{Amm}]_{\text{i}}\) and expired water \([\text{Amm}]_{\text{ex}}\) were measured by a micro-modification of the salicylate-hypochlorite assay with frozen water samples (Verdouw et al. 1978). To ensure that there was no ammonia loss from water in the recirculating system, two experiments were carried out in which known amounts of \(\text{NH}_4\text{Cl}\) were added to the system without fish. No loss from the system occurred.

The ammonia excretion rate of the fish was calculated as:

\[
\text{Ammonia excretion rate} = ([\text{Amm}]_{\text{i}} - [\text{Amm}]_{\text{f}}) \times \frac{V}{t} \times \frac{W}{t},
\]

where \(i\) and \(f\) refer to the initial and final ammonia concentration in inspired water in \(\text{umol}^{-1}\), \(V\) is the volume of the system (6 l in this study), \(t\) is the elapsed time in hours and \(W\) is the mass of the fish in kilograms.

Bicarbonate concentrations in inspired water \([\text{HCO}_3^-]_{\text{i}}\) and expired water \([\text{HCO}_3^-]_{\text{ex}}\) were calculated from \([\text{CO}_2]_{\text{i}}\), \(\text{pH}_{\text{i}}\) and \([\text{CO}_2]_{\text{ex}}, \text{pH}_{\text{ex}}\), respectively, by the Henderson-Hasselbalch equation, using the \(pK_{\text{co}_2}\) and \(\alpha_{\text{co}_2}\) values from Boutilier et al. (1985). Carbonate formation is negligible over this pH range. Ammonium ion concentrations in inspired water \([\text{NH}_4^+]_{\text{i}}\) and expired water \([\text{NH}_4^+]_{\text{ex}}\) were calculated from \([\text{Amm}]_{\text{i}}, \text{pH}_{\text{i}}\) and \([\text{Amm}]_{\text{ex}}, \text{pH}_{\text{ex}}\), respectively, by the Henderson-Hasselbalch equation, using the \(pK_{\text{Amm}}\) value from Cameron & Heisler (1983).

If we assume all the carbon dioxide is excreted as \(\text{CO}_2\) and
all the ammonia is excreted as NH₃, the pH change occurring in
the water passing over the gills of fish could be due to CO₂
hydration, ammonium ion formation or acid excretion.

\[ \text{CO}_2 + \text{H}_2\text{O} \rightarrow \text{HCO}_3^- + \text{H}^+ \]
\[ \text{NH}_3 + \text{H}^+ \rightarrow \text{NH}_4^+ \]

The proton added by CO₂ hydration can be estimated from the
difference of bicarbonate levels in inspired and expired water:
\([\text{HCO}_3^-]_{ex} - [\text{HCO}_3^-]_{in}\). The proton consumed by ammonium ion formation
can be estimated from the difference of ammonium ion levels in
inspired and expired water: \([\text{NH}_4^+]_{ex} - [\text{NH}_4^+]_{in}\).

The total acid equivalents added to the water, \([\text{H}^+]_{total}\), can
be calculated from the appropriate buffer curve and \(\text{pH}_{in}\) and \(\text{pH}_{ex}\).
The buffer capacities of inspired and expired water were measured
by titrating the stored water samples with 0.1 mol·l⁻¹ HCl. 20 ml
of water sample was held in a glass beaker with a water jacket
for temperature control and its pH was increased to approximately
8.0 by addition of 0.1 mol·l⁻¹ NaOH as the sample was aerated. HCl
then was manually added to the aerated sample with a syringe and
pH was recorded 2-3 minutes after each addition to ensure
complete equilibrium. Buffer curves were constructed from the pH
and the amount of acid added for each individual water sample.
The buffer curves of inspired and expired water were very similar
and only inspired water buffer curves were used for subsequent
calculations.

Since \([\text{H}^+]_{total} = ([\text{HCO}_3^-]_{ex} - [\text{HCO}_3^-]_{in}) - ([\text{NH}_4^+]_{ex} - [\text{NH}_4^+]_{in}) + \text{net}
proton excretion, the net branchial proton excretion rate,
therefore, is equal to: $[H']_{\text{total}} - ([HCO_3^-]_{\text{ex}} - [HCO_3^-]_{\text{in}}) + ([NH_4^+]_{\text{ex}} - [NH_4^+]_{\text{in}})$. All the measurements and calculations were conducted with each individual inspired and expired water samples.

Data are presented as means ± standard error. Student's two-tailed $t$-test and analysis of variance (ANOVA) was used to test for significant differences between means. Tests of significance were conducted at the 5% level of rejection.
RESULTS

When water pH was approximately neutral, \([\text{HCO}_3^-]_{\text{in}}\) was always greater than \([\text{HCO}_3^-]_{\text{ex}}\) (Figure 4, \([\text{HCO}_3^-]_{\text{in}} - [\text{HCO}_3^-]_{\text{ex}}\) gives positive values), indicating that there was no CO\(_2\) converted to \(\text{HCO}_3^-\) as water passed over the gills. Instead, bicarbonate level was reduced due to \(\text{HCO}_3^-\) dehydration induced by proton excretion or bicarbonate absorption. When proton excretion was inhibited by 0.1 mM vanadate, 0.1 mM acetazolamide or 0.5 or 1 mM amiloride (see Figure 5), the extent of bicarbonate dehydration decreased and \([\text{HCO}_3^-]_{\text{ex}}\) increased. Therefore, \([\text{HCO}_3^-]_{\text{in}} - [\text{HCO}_3^-]_{\text{ex}}\) was significantly lower than the control value. This suggests that branchial proton excretion not only caused expired water acidification but also dehydrates \(\text{HCO}_3^-\) as water passed over the gills. The control \([\text{HCO}_3^-]_{\text{in}} - [\text{HCO}_3^-]_{\text{ex}}\) value at pH 7.6 was greater than that at pH 6.6 because the excretion of acid equivalents was higher at pH 7.6 (Figure 5).

There is a substantial net proton excretion across the gill of freshwater rainbow trout when external water pH was about neutral. This proton excretion was not affected by 0.1 mmoll\(^{-1}\) amiloride (Figure 5). Increasing amiloride concentration in the external medium induced a reduction in proton excretion, but more than 50% of the net proton excretion was still sustained even in 1 mM amiloride (Figure 5).

0.1 mM vanadate treatment resulted in reductions of net proton excretion by 58% (Figure 5). Acetazolamide treatment also
Figure 4. Bicarbonate concentration differences between inspired and expired water of rainbow trout under control, amiloride, vanadate and acetazolamide treatments. * indicates a significant difference between the control and treatment values (P<0.05); Bars show standard errors; N=6.
The graph shows the change in [HCO₃⁻] in - [HCO₃⁻]ex (µmol/L kg) for different concentrations of Amiloride, Acetazolamide, and Vanadate at pH 6.6 and pH 7.0. The bars represent the control and treatment conditions. Asterisks indicate significant differences between the control and treatment conditions.
Figure 5. Net proton excretion across the gill epithelium of rainbow trout under control, amiloride, vanadate and acetazolamide treatments. * indicates a significant difference between the control and treatment values (P<0.05); Bars show standard errors; N=6.
caused a 48% reduction in net proton excretion (Figure 5). The differences between control proton excretion values were due to different pH in external water.

The differences in total CO₂ content between inspired water and expired water with different treatments are presented in Table 1. \([\text{CO}_2]_{ex} - [\text{CO}_2]_{in}\) represents the CO₂ excretion rate if we assume that ventilation rate is constant. There was no significant difference in \([\text{CO}_2]_{ex} - [\text{CO}_2]_{in}\) between control and drug-treated animals except in the case of acetazolamide. CO₂ excretion increased when fish were exposed to 0.1 mM acetazolamide in water.

Ammonia excretion of fish was not significantly inhibited by 0.1 mM amiloride (Figure 6). However, higher concentrations of amiloride induced a reduction in ammonia excretion, by 58% with 0.5 mM amiloride and by 87% with 1 mM amiloride (Figure 6). Vanadate and acetazolamide had no significant effect on ammonia excretion.
Table 1. Total CO$_2$ Excretion By Fish (umol/l.kg)

\[
[\text{CO}_2]_{\text{ex}} - [\text{CO}_2]_{\text{in}}
\]

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 mM Amiloride</td>
<td>39.73 ± 6.57</td>
<td>32.75 ± 5.30</td>
</tr>
<tr>
<td>(n=6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 mM Amiloride</td>
<td>71.00 ± 8.04</td>
<td>71.48 ± 10.43</td>
</tr>
<tr>
<td>(n=6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 mM Amiloride</td>
<td>62.46 ± 8.11</td>
<td>57.77 ± 7.49</td>
</tr>
<tr>
<td>(n=6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1 mM Vanadate</td>
<td>76.94 ± 4.41</td>
<td>63.68 ± 5.60</td>
</tr>
<tr>
<td>(n=10)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1 mM Acetazolamide</td>
<td>55.11 ± 9.91</td>
<td>82.90 ± 10.32*</td>
</tr>
<tr>
<td>(n=4)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* indicates a significant difference from the control value.
Figure 6. Branchial ammonia excretion rates of rainbow trout under control, amiloride, vanadate and acetazolamide treatments. * indicates a significant difference between control and treatment values (P<0.05); Bars show standard errors; N=6.
DISCUSSION

The fact that the bicarbonate level in inspired water of fish was always higher than that in expired water indicates either bicarbonate was converted to CO$_2$ as water passed over the gills, or bicarbonate was absorbed from the water, probably via Cl$^-$/HCO$_3^-$ exchange. The later possibility was eliminated by the observation that SITS, an inhibitor of Cl$^-$/HCO$_3^-$ exchange, had no effect on acid excretion across fish gills (Lin and Randall, 1991). Bicarbonate could be converted to CO$_2$ when CO$_2$ was excreted to the water by the fish if a substantial amount of acid was also excreted simultaneously into the water. Therefore, the expired water acidification reported by Wright et al (1986) and Lin and Randall (1990) was not due to the hydration of excreted CO$_2$ but due to acid excretion across the gills. The excreted acid was partially consumed by HCO$_3^-$ dehydration and ammonia protonation, and the rest contributed to expired water acidification.

It has long been hypothesized that Na$^+$/H'(NH$_4^+$) electroneutral exchange is the principal mechanism of sodium uptake and proton excretion in the gill epithelium of fish (Wright & Wood, 1985). This antiport exchange process is blocked by 0.1 mM amiloride, a very potent and relatively specific inhibitor of sodium transport in a wide variety of cellular and epithelial transport systems (Benos, 1982). 84% and 94% reduction of the Na$^+$ uptake by the gills of intact freshwater rainbow trout
exposed to 0.1 mM amiloride in the external media were reported by Perry & Randall (1981) and Wright & Wood (1985), respectively. In our studies, this concentration of amiloride had no effect on either net proton excretion or ammonia excretion when compared with control values from the same animals (Figure 5 and 6). Our experimental conditions are similar to those of Perry & Randall and Wright & Wood. This indicates that sodium influx and proton ion efflux are not directly coupled (see also Avella and Bornancin, 1989).

It is well documented that proton transport in mammalian kidney (Steinmetz, 1985), amphibian urinary bladder (Al-Awqati, 1978; Steinmetz, 1986) and frog skin (Ehrenfeld et al. 1985) is mediated by an electrogenic proton pump. The gill epithelium in freshwater fish is considered to be "tight" (Sardet, 1980) and resembles frog skin and turtle bladder epithelia functionally and morphologically. My studies demonstrated that proton excretion in trout was unaffected by low concentrations of amiloride but was inhibited by vanadate and acetazolamide. The same have been reported for the proton transport in frog skin (Ehrenfeld et al. 1985; Ehrenfeld & Garcia-Romeu, 1977) and turtle bladder (Al-Awqati, 1978; Steinmetz, 1986). I therefore agree with Avella and Bornancin (1989) and similarly postulate that the fish gill has an electrogenic proton pump in the mucosal membrane, similar to that reported for frog skin and toad bladder, rather than a Na⁺/H⁺ exchange mechanism.

The electrogenic proton pump, or H⁺-translocating ATPase,
on the apical membrane removes protons from the cell and generates a negative potential in the inner side of the apical membrane (Figure 7). Sodium influx, driven by the negative potential, occurs via a sodium channel that is highly sensitive to amiloride. Na⁺/K⁺(NH₄⁺)-ATPase in the basolateral membrane pumps sodium out of the cell into the blood. Thus, proton excretion and sodium uptake are intimately, but indirectly, linked. Since Avella et al. (1987) showed that branchial sodium uptake was proportional to the number of chloride cells in the gills and, since proton pumps consume energy and chloride cells are rich in mitochondrial and can supply the energy demand, I speculate that the electrogenic proton pump is located in the chloride cell.

Ammonium ions can replace potassium on the Na⁺/K⁺-ATPase and thus enter the cell and form NH₃ and protons (Evans et al. 1989). The deprotonation of NH₄⁺ could supply the proton pump and NH₃ could diffuse passively across the apical membrane into the water. Although much less sensitive than the Na⁺ channel, Na⁺/K⁺-ATPase in the basolateral membrane can be inhibited by amiloride that has entered the cell when applied in high concentrations to the mucosal side (Knauf et al. 1976; Kleyman & Cragoe, 1988). Thus, the reduced proton and ammonia excretion in 0.5 and 1 mM amiloride treatments could be accounted for by the inhibitory effect of amiloride on Na⁺/K⁺(NH₄⁺)-ATPase in the basolateral membrane. In support of this contention, Evans et al. (1989) showed that amiloride did not affect ammonia excretion if
Figure 7. Schematic representation of gas and ion transport across the gill epithelium of rainbow trout. ATP-driven pumps are denoted by filled circles, ion exchanger by opened circles and passive diffusion by arrows. See text for details.
the perfused head of the toadfish was pretreated with ouabain, which blocks Na\textsuperscript{+}/K\textsuperscript{+}(NH\textsubscript{4}\textsuperscript{+})-ATPase. Proton excretion in frog skin was inhibited by 0.5 mM amiloride by 35% but was not affected by 0.05 mM amiloride, whereas sodium uptake was completely abolished. If we assume that the ventilation rate of the fish was 100 ml/min (Lin and Randall, 1990), we can compare the ammonia excretion rate with the net proton excretion rate under amiloride treatments (Table 2). The reduction in ammonia excretion was equivalent to that in proton excretion, indicating the possibility that NH\textsubscript{3} and protons were both originating from NH\textsubscript{4}\textsuperscript{+} transported into the epithelium via Na\textsuperscript{+}/K\textsuperscript{+}(NH\textsubscript{4}\textsuperscript{+})-ATPase in the basolateral membrane (Figure 7).

Ammonia elimination was not affected by vanadate or acetazolamide, indicating that proton and ammonia efflux from the gill epithelium are through different pathways. Thus, ammonium entry into the gill epithelium may affect proton excretion (Table 2), but variations in proton excretion do not appear to affect ammonia excretion. Ammonium cannot be the sole source of protons however, because proton excretion can be more than twice ammonia excretion in some instances. Acetazolamide, a traditional carbonic anhydrase inhibitor, inhibits proton excretion in fish gills (Figure 5), as demonstrated in frog skin and turtle bladder (Ehrenfeld & Garcia-Romeu, 1977; Steinmetz, 1986). This suggests the possible contribution of intracellular CO\textsubscript{2} hydration to the proton supply. The apical addition of 0.01 mM ethoxzolamide, a lipid soluble CA inhibitor, blocked net H\textsuperscript{+} excretion as well as
Table 2. Comparison between the reduction in the rate of ammonia excretion and the reduction in the rate of net proton excretion under amiloride treatment.

<table>
<thead>
<tr>
<th>Amiloride concentration (mM)</th>
<th>Reduction in the rate of ammonia excretion (umol/kg.h)</th>
<th>Reduction in the rate of net proton excretion (umol/kg.h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>47.968 ± 20.568*</td>
<td>60.806 ± 36.178*</td>
</tr>
<tr>
<td>0.5</td>
<td>130.542 ± 9.346</td>
<td>143.169 ± 61.059</td>
</tr>
<tr>
<td>1</td>
<td>280.560 ± 35.561</td>
<td>298.105 ± 86.911</td>
</tr>
</tbody>
</table>

Net proton excretion was converted to umol/kg.h by assuming ventilation rate = 100 ml/min (Lin & Randall, 1990). * indicates a value not significantly different from zero.
Na⁺ absorption in frog skin (Harvey and Ehrenfeld, 1986). While inhibiting proton excretion, acetazolamide also elevates carbon dioxide excretion across fish gills (Table 1). Because CO₂ excretion is not diffusion limited (Perry et al., 1982), elevated CO₂ excretion usually involves an increase in CO₂ production or a decrease in CO₂ storage in the fish. There is not enough information from this study to explain it.

The amount of proton excretion in this study is of the same magnitude as that reported by Avella and Bornancin (1989), but in excess of that measured by McDonald and Wood (1981). Comparison was made assuming the ventilation rate of the fish is 100 ml/min (see Lin and Randall, 1990). The method I used in this study to estimate branchial proton excretion rate is different from that used by the above-mentioned workers, which deals with whole animals in a 30-60 min time period. In my method I focus on the instantaneous proton excretion across fish gill epithelium. The detailed analysis of inspired and expired water chemistry was designed to reveal changes occurring in the gill surface that might not be picked up in whole animals studies. I accounted for the proton addition / consumption from the CO₂ hydration / dehydration by measuring the bicarbonate levels in inspired and expired water. However, during the titration process a portion of the CO₂ component was also involved, and the proton excretion rate might have been over-estimated. In the method used by the other authors, the hydrogen ions consumed by HCO₃⁻ dehydration was not accounted for and proton excretion rate might had been
under-estimated. The other reason that the proton excretion reported here is high is that, proton excretion was probably stimulated under the high salt experimental condition. 40 mM NaCl was added to the external water in order to obtain precise pH measurements. The operation of the putative proton pump was probably stimulated by the sudden increase of Na\(^+\) levels in the water and 3 hours was not long enough for endocytotic retreat of proton pump. When blood pH was measured under similar condition (Lin and Randall, 1990), fish were suffering from a slight alkalosis (blood pH was 7.95-8.00 compared to the normal value of 7.80).

Vanadate has a nonspecific inhibitory effect on ATPases and could be acting on Na\(^+-K^+\)-ATPase on the basolateral border of the fish gill. In my studies, more than 50% of the net proton excretion across the gill epithelium was inhibited by 0.1 mM vanadate applied to the mucosal membrane. De Sousa & Grosso (1979) showed that applying 1 mM vanadate to the outer surface did not affect the Na\(^+-K^+\)-ATPase in the basolateral membrane of frog skin. Arruda et al. (1981) showed that vanadate had no effect on the backleak of proton or bicarbonate secretion but had a direct effect on H\(^+\)-translocating ATPase in turtle bladder. Thus, I conclude that the reduction in proton excretion observed in my studies was induced by the inhibitory effect of vanadate on H\(^+\)-translocating-ATPase in the apical membrane. The reason that the proton excretion was not completely abolished was, presumably, because of the difficulty of vanadate reaching the
action site from the mucosal side (Arruda et al. 1981).

0.1 mM amiloride had no effect on the putative fish gill proton pump in open-circuit conditions. In frog skin epithelium under open-circuit conditions however, application of 0.01 mM amiloride caused a hyper-polarization of membrane potential, a fall in intracellular sodium ion activity, an inhibition of H⁺ excretion and a decrease in pHi (Harvey and Ehrenfeld, 1986). Inhibition of sodium influx should have increased membrane potential and reduced proton excretion in fish gill epithelium. This did not happen; therefore, if the proton pump does exist, there must be some other counter-ion that can replace sodium. Perry and Randall (1981) found that 0.1 mM amiloride inhibited chloride influx in the fish gill. Inhibition of both chloride and sodium influx, when fish are exposed to 0.1 mM amiloride, would tend to ameliorate any rise in potential across the apical membrane and, therefore, permit continued functioning of the proton pump.

In conclusion, my results provide preliminary evidence that the acidification of expired water in rainbow trout in neutral water is mainly caused by a net proton excretion, probably mediated by an active proton pump on the apical membrane of gill lamellae. This proton pump is sensitive to vanadate and acetazolamide, and resembles the electrogenic proton pump in frog skin and turtle bladder.
Chapter 2: Effects of external water pH, Pco₂ and ion levels on branchial proton excretion mediated by proton pump
SUMMARY

Branchial proton excretion was sensitive to external water pH. When water pH is below 5.5 (about 2.5 units lower than blood pH), proton excretion was inhibited. In water pH range of 5.5-8.0, proton excretion increased linearly with water pH. In pH above 8.0, proton excretion appeared saturated. Elevated water \( \text{PCO}_2 \) levels stimulated branchial proton excretion, indicating intracellular \( \text{CO}_2 \) hydration provides hydrogen ion to the putative proton pump. Sodium-free water suppressed proton excretion across the gills but addition of the chloride channel inhibitor 9-anthroic acid transiently lessened the suppression, which indicates that both sodium and chloride influx across the gill epithelium probably play a role in justifying the electrical potential generated by active proton excretion.
INTRODUCTION

It was concluded from the studies in Chapter 1 that the acidification of expired water across fish gills was induced by a net branchial proton excretion. Inhibitor sensitivity studies ruled out the possibility that a Na'/H' exchanger was responsible for the proton flux, and indicated the existent of a primary proton pump in the gill epithelium that mediated the measured proton excretion, but many questions remain unanswered. For example: Proton transport mediated by a proton pump in turtle bladder (Steinmetz, 1986) and frog skin (Ehrenfeld et al., 1985) was regulated by the transepithelial pH gradient. The fact that expired water acidification was sensitive to external water pH (Lin and Randall, 1990) suggests that branchial proton excretion may also be pH-sensitive, but it remains to be tested. Ammonium ion deprotonation in the intracellular compartment was considered to be the supply of hydrogen ions for the proton pump but could only account for 50% or less of the proton excretion. What is the other source of protons? Is it from carbon dioxide hydration as observed in frog skin (Ehrenfeld and Garcia-Romeu, 1977) and toad bladder (Al-Awqati, 1978)? What is the counterion for the electrogenic proton pump? The sodium channel inhibitor, amiloride, appeared to have no effect on branchial proton excretion but the application of this drug (and also SITS, a Cl-/HCO₃⁻ exchanger inhibitor) was complicated by its double effect on both Na' and Cl' flux across fish gills (Perry and Randall,
1981). Other approaches, such as removing sodium from external water and / or employing a chloride channel inhibitor, have to be used to clarify these points.

Experiments reported in this chapter were designed to examine these problems. Attempts were also made to investigate the effect of high external calcium levels on the proton excretion across fish gills. Fish appear to be able to maintain acid-base and osmotic homeostasis better in hard water (McDonald et al, 1980; McDonald 1983; McDonald et al, 1983), it is interesting to know whether the proton pump was playing a role in this process.
MATERIALS AND METHODS

Experiments were carried out on rainbow trout of both sexes, weighing between 200 and 500 g. Fish were maintained in large outdoor tanks and regularly fed. Feeding was suspended two days before surgery. To sample expired water from fish gills, an opercular cannula was fixed right next to the opercular opening in the fish using the method described in chapter 1. The fish was allowed to recover from anaesthesia for 24 hours in a black chamber supplied with aerated dechlorinated tap water. Two hours prior to the experiment, this black chamber was hooked up to a recirculating system and the water supply was switched to the aerated test solution (40 mmol·l⁻¹ NaCl and 0.5 mmol·l⁻¹ CaCl₂, pH 6.7, see Chapter 1). Inspired and expired water samples were taken for pH, total CO₂, total ammonia and buffer capacity analysis and net branchial proton excretion rate can be calculated with these data. Detailed analytical techniques and calculations are described in Chapter 1. Control water samples were obtained two hours after the fish was acclimated to the test solution and experimental water samples were obtained after one of the following treatments.

(1) Varying external water pH

The pH of the test solution was adjusted to the 4, 5, 8 or 9 by adding 0.1 mmol·l⁻¹ NaOH or 0.1 mmol·l⁻¹ HCl at the beginning of each different environmental pH exposure. A magnetic stirring bar was used in the reservoir to ensure quick and complete
mixing. The water pH of the recirculating system changed slightly (<0.3 unit) over the 30 minutes experimental period, and no attempt was made to stabilize pH during this period. Inspired and expired water samples were taken 30 minutes after the fish were exposed to the desired external pH.

(2) Sodium-free external water

Sodium-free test solution was prepared by replacing 40 mmoll$^{-1}$ NaCl with 40 mmoll$^{-1}$ choline chloride. The recirculating system was flushed with sodium-free test solution after control sampling and fish were maintained in the recirculating sodium-free test solution for one hour. Water samples were taken at 30 minutes and 60 minutes. The recirculating system was then flushed with regular test solution and inspired and expired water were sampled 30 minutes later.

(3) 9-anthroic acid treatment

Fish were maintained in sodium-free test solution for 30 minutes after the control period. 9-anthroic acid (9-AA) was then added to the recirculating system to a final concentration of 0.1 mM. Water samples were taken 30 minutes and 60 minutes after the fish were exposed to 9-AA in sodium-free external water.

(4) High external calcium levels

Calcium chloride was added to the recirculation system to a final concentration of 10 mM after control sampling. Inspired and expired water samples were obtained 30 minutes and 60 minutes during high calcium treatment.

All branchial net proton excretion rate are expressed as
umol/L.kg and are reported as mean values ± standard error. Statistical comparisons were made with Student's t-test for paired samples. Regression analyses were used to describe relationships between variables.
RESULTS

The relationship between net proton excretion across fish gills and the inspired water pH is illustrated in Figure 8. When water pH is below 5.5, there is no net proton excretion; that is, any change in water pH can be accounted for by CO₂ hydration / HCO₃⁻ dehydration and / or NH₃ protonation. Net proton excretion was completely inhibited at low external water pH. When water pH is between 5.5 and 8.0, net proton excretion increased linearly with inspired water pH. Branchial net proton excretion was maximal when inspired water pH was above 8.0, which is higher than blood pH in the fish.

Figure 9 shows the relationship between net proton excretion and expired water carbon dioxide levels in a neutral environment. The figure contains all the control net proton excretion rates and the associated Pco₂ values in expired water. There is a general tendency for increased Pco₂ in expired water to be associated with an increase in net proton excretion.

The removal of sodium from the external water caused a marked decrease in branchial proton excretion (Figure 10). This reduction persisted during the total 60 minutes of the choline chloride treatment period and proton excretion was completely recovered 30 minutes after sodium was returned to the external water. Addition of 9-AA to sodium-free external water caused a transient recovery in net proton excretion across fish gills, but 60 minutes after the onset of the 9-AA treatment.
Figure 8. The relationship between the branchial net proton excretion and the inspired water pH, expressed by a regression curve ($R^2 = 0.853$).
$R^2 = 0.853$
Figure 9. The relationship between branchial net proton excretion and expired water carbon dioxide levels. The linear regression line has a $R^2$ value of 0.77. The slope of the regression line is significantly different from zero ($P<0.05$).
Net Proton Excretion (umol/L/kg)

Expired Water $P_{CO_2}$ (kPa)

$Y = 505.86X - 84.93$

$R^2 = 0.77$
Figure 10. Branchial proton excretion rate of rainbow trout in control and sodium-free water. * indicates a significant difference between control and treatment values (P<0.05); Bars show standard errors; N=6.
Net Proton Excretion (umol/L.kg)

Control 0min | Choline 30min | Choline 60min | Recovery 90min

* indicates significant difference from control.
proton excretion was again significantly lower than control levels (Figure 11).

Branchial proton excretion rate was slightly elevated during the one hour exposure of fish to high calcium water (Figure 12). The differences, however, are not statistically significant.
Figure 11. Branchial proton excretion rate of rainbow trout in control, sodium-free water and sodium-free water plus 0.1 mM 9-anthroic acid. * indicates a significant difference between control and treatment values (P<0.05); Bars show standard errors; N=6.
Control 0 min  Choline 30 min  9-AA 60 min  9-AA 90 min

Net Proton Excretion (umol/L/kg)
Figure 12. Branchial proton excretion rate of rainbow trout in control and high calcium (10 mM) water. Bars show standard errors; N=6.
DISCUSSION

The relationship between net proton excretion across fish gills and inspired water pH, shown in Figure 8, is very typical of proton transport mediated by an electrogenic proton pump. At constant serosal pH, net proton secretion in turtle bladder increased linearly with luminal pH over the physiological range of urine pH (4.4 - 7.4). Proton secretion was maximal at higher pH (Steinmetz, 1986). A linear relationship between proton excretion and mucosal pH over a limited range was also reported in frog skin by Ehrenfeld et al (1985). This indicates that the electrochemical gradient for protons across the membrane is a fundamental regulator of proton transport.

When external water pH was lower than 5.5, the ΔpH across the epithelial membrane is > 2.4 pH unit (assuming blood pH of fish was 7.9, see Lin and Randall, 1990). The electrochemical gradient for protons was too great for the proton pump to work against, therefore no hydrogen ions excluded across the gill epithelium. The reversal of gill transepithelial potential associated with low water pH (McWilliams and Potts, 1978; Ye et al, 1991) probably resulted from the shut-down of the proton pump. Within the pH range of 5.5 to 8.0, more protons were transported by the active pump as the proton electrochemical gradient apposing on the pump decreased. Above inspired water pH of 8.0, the proton electrochemical gradient was favourable to proton excretion, however, branchial proton excretion plateaued,
probably because the electrogenic proton pump was now operating at its maximal capacity.

Elevated carbon dioxide levels in expired water, which is an indicator of the CO₂ level in venous blood, appear to enhance proton excretion (Figure 9). This was also observed in toad bladder (Al-awqati, 1978), turtle bladder (Arruda et al, 1990) and frog skin (Ehrenfeld and Garcia-Romeu, 1977). In all these epithelia carbon dioxide provides a source of hydrogen ions for the proton pump when it is hydrated, the reaction catalysed by carbonic anhydrase in the intercellular compartment. The same theory probably applies to the fish gill epithelium because acetazolamide, a carbonic anhydrase inhibitor, also inhibits proton excretion in fish gills (Chapter 1, Figure 5).

Removal of sodium from the external water resulted in a reduction of branchial proton excretion (Figure 10), probably due to the lack of a counterion to diminish the negative potential generated by the proton pump on the inner side of the apical membrane. This then increases the electrochemical gradient against which the proton pump must operate and accordingly proton excretion is reduced. Substitution of external Na⁺ by Mg²⁺ or K⁺ was observed to cause a hyper-polarization of the apical membrane and inhibited proton excretion in frog skin under open circuit conditions (Harvey and Ehrenfeld, 1986).

Accumulation of negative potential in the apical membrane of fish gills was also expected when 0.1 mM amiloride was added to the external water and blocked the sodium conductive channels.
However 0.1 mM amiloride also inhibited chloride influx across fish gills (Perry and Randall, 1981), which might alleviate the build-up of the negative potential against the proton pump. This explanation was supported by the fact that addition of 9-anthroic acid (9-AA) caused a transient recovery of branchial proton excretion under sodium-free conditions (Figure 11). 9-AA was shown to inhibit a Cl⁻ conductance in the apical membrane of canine tracheal epithelium (Welsh, 1984) and frog kidney peritubular cell (Oberleithner et al, 1983). Luminal addition of 0.1 mM 9-AA inhibited the electrogenic \( HCO_3^- \) secretion initiated by the proton pump in turtle bladder by blocking the recycling of Cl⁻ via a chloride channel in the luminal membrane (Stetson et al, 1985). Application of 9-AA to the mucosal side of fish gills might inhibit chloride influx and therefore temporary reduce the electrical gradient against which the proton pump must operate.

Elevated calcium levels in external water had no significant stimulating effect on proton excretion across fish gills during the one hour experimental period (Figure 12). It was demonstrated by Avella et al (1987) that high calcium levels in freshwater (10 mM) stimulated the proliferation of chloride cells and the sodium influx by 2.5 times. But this stimulating effect only appeared after a long-term acclimation (7-15 days). Insertion of a proton pump into the apical membrane of turtle bladder epithelium was mediated by cell calcium (Adelsberg and Al-Awqati, 1986). The time course of the study reported here was probably too short to cause any change in chloride cell numbers or proton pump activity.

78
in fish gills.
SECTION II: PROTON-ATPASE ACTIVITY IN GILL TISSUE OF RAINBOW TROUT

Chapter 3: Inhibitor sensitivity and classification of the proton-ATPase in gill tissue
SUMMARY

N-ethymaleimide-sensitive ATPase activity was measured in crude homogenates of gill tissue from rainbow trout using a coupled-enzyme ATPase assay in the presence of EGTA, ouabain and azide. This NEM-sensitive ATPase activity, determined to be about 1.5 umol/mg.pr./h. at 15°C for freshwater trout, was also inhibited by other proton-ATPase blockers such as DCCD, DES, PCMBS and Bafilomycins. It is concluded, therefore, that the NEM-sensitive ATPase activity was generated by a proton-translocating ATPase. Since this NEM-sensitive ATPase was also sensitive to the plasma membrane ATPase inhibitor vanadate, I speculate the H⁺-ATPase in fish gill is a plasma membrane type.
INTRODUCTION

The Na⁺/H⁺ exchanger in fish gill epithelium was postulated to be the major pathway for Na⁺ uptake and acid excretion (Wright and Wood, 1985). The sodium concentration in fresh water, however, is usually lower than 1 mM, and the intracellular sodium concentration in the gill epithelial cell, although lowered by the Na⁺-K⁺-ATPase in the basolateral membrane, is much higher than 1 mM. Intracellular sodium ion activity in frog skin epithelium was reported to be 6.2 mmol/l (Harvey and Ehrenfeld, 1986). The sodium electrochemical gradient across the apical membrane could not drive Na⁺/H⁺ exchange. An alternative mechanism which will account for Na⁺ and H⁺ transport in opposite directions is an electrogenic H⁺-translocating-ATPase coupled with a sodium conductive channel (Chapter 1), as demonstrated in freshwater frog skin (Ehrenfeld et al. 1985). This so called proton pump will consume ATP, actively exclude hydrogen ions across the membrane and generate a negative potential inside the apical membrane, which will then drive sodium influx via the sodium channel.

The existence of an H⁺-ATPase is well documented not only in freshwater frog skin, which has the same Na⁺ uptake function as FW fish gills, but also in other tight epithelia such as turtle urinary bladder (Steinmetz and Andersen, 1982) and mammalian renal collecting tubule (Gluck and Al-Awqati, 1984; Ait-Mohamed et al. 1986). N-ethymaleimide, a covalent SH-reactive reagent, is
a H⁺-ATPase inhibitor commonly used to identify H⁺-ATPase in different organisms (Pedersen and Carafoli, 1987).

My previous in vivo studies (Chapter 1 and 2) showed that proton excretion across the gill epithelium of freshwater trout was sensitive to external pH, Pco2, vanadate (a plasma membrane ATPase inhibitor) and acetazolamide (a carbonic anhydrase inhibitor), but was not sensitive to 0.1 mM of amiloride which will block the Na⁺ influx across the gills of rainbow trout completely (Wright and Wood, 1985). All these characteristics are typical for H⁺ transport mediated by H⁺-ATPase in other tight epithelia and thus indicated the presence of a proton pump in the gill epithelium. The objective of these studies was to measure H⁺-ATPase activity directly in crude homogenates of gill tissue and examine its sensitivity to proton-ATPase inhibitors including NEM, DCCD, PBMBs and bafilomycins. These drugs are usually too toxic for in vivo application.
MATERIALS AND METHODS

Experimental animals

Rainbow trout, *Oncorhynchus mykiss* (Walbaum), weighing 300-500g were kept in aerated, dechlorinated Vancouver tap water ([Na⁺], 0.89 mM; [Ca⁺⁺], 0.03 mM; [Cl⁻], 0.92 mM) at 10-15°C ambient temperature. Animals were fed commercial trout pellets twice a week and terminated at least one day before usage.

Preparation of gill tissue homogenates

A crude homogenate of rainbow trout gill tissue was prepared using a method modified from Zaugg (1981). Fish were killed with a blow to the head. The gills were perfused through the heart with heparinized saline in order to clear red blood cells. Gill filaments (approximately 1 g wet weight) were trimmed from supporting arches and immersed in 2 ml of a cool homogenate medium I containing 300 mM sucrose, 2 mM EGTA, 1 mM dithiothreitol and 100 mM tris-HCl at pH 7.3. Tissue was then homogenized with a Kontes micro ultrasonic cell disrupter for 20 strokes, 2 ml distilled water was added to the homogenates and another 20 strokes were employed to ensure all filaments were disintegrated. The diluted homogenates were centrifuged for 7 min in a Janetzli laboratory table centrifuge (model T32c) at about 4000 rpm (2000 RCF). Supernatant solutions were discarded, and pellets suspended in 1 ml of homogenate medium II (homogenate medium I containing 6% CHAPS, a zwitterionic detergent) were homogenized twice for 20 strokes each. The resulting homogenates
were centrifuged as before and supernatant solutions were removed for ATPase assay usage. We found the ATPase activity in the supernatant was stable for at least one month when stored in a -80°C freezer. Protein concentration of the supernatant was around 6-8 mg/ml.

**Determination of NEM-sensitive ATPase activity**

NEM-sensitive ATPase activity was measured by a modified coupled-enzyme ATPase assay used for determination of Na⁺-K⁺-ATPase activity (Scharschmidt et al. 1979). The formation of ADP catalyzed by ATPase is coupled with NADH oxidation in the presence of excess pyruvate kinase (PK), lactate dehydrogenase (LDH) and phosphoenolpyruvate.

\[
\text{ATPase} \\
\text{ATP} \rightarrow \text{ADP} + \text{Pi} \quad (A) \\
\text{PK} \\
\text{ADP} + \text{phosphoenolpyruvate} \rightarrow \text{pyruvate} + \text{ATP} \quad (B) \\
\text{LDH} \\
\text{Pyruvate} + \text{NADH} + \text{H}^+ \rightarrow \text{lactate} + \text{NAD}^+ \quad (C)
\]

Stock reaction buffer containing 130.9 mM Tris (pH 7.4 at 15°C), 1.05 mM EGTA and 13.09 mM KCl was prepared in advance. On the day of assay NADH, phosphoenolpyruvate, ouabain and sodium azide were added to the reaction buffer in an amount necessary to bring their concentrations to 0.52 mM, 2.62 mM, 2.12 mM and 5.24 mM, respectively. NEM was added to half of the reaction buffer to a concentration of 1.06 mM. Tris ATP was dissolved in 200 mM MgCl₂ solution to yield a 200 mM concentration. LDH-PK enzyme mixture (1000 units each per ml) was purchased from Sigma. All
reagents were kept on ice. To perform the assay, 0.945 ml reaction buffer containing NADH and phosphoenolpyruvate, 0.025 ml ATP-MgCl solution and 0.01 ml LDH-PK mixture were added to a 1.5 ml cuvette. The reaction was begun by adding 0.02 ml crude homogenate and mixing the contents of the cuvette by inversion. The final 1 ml reaction mixture thus contained 125 mM Tris buffer, 1 mM EGTA, 12.5 mM KCl, 5 mM NaN₃, 2 mM ouabain, 5 mM MgCl₂, 5 mM ATP, 2.5 mM phosphoenolpyruvate, 0.5 mM NADH and 10 units each of LDH and PK (with or without 1 mM NEM). The oxidation of NADH was continuously monitored at 340 nm at 15°C in the temperature-controlled cuvette compartment of a continuously recording spectrophotometer (Perlin-Elmer Lambda 2). ATPase activity was calculated from the slope of the linear portion of the tracing, the NADH mM extinction coefficient, the volume of the reaction mixture, and the milligrams of crude homogenate protein added:

ATPase activity (umol pi/mg.pr.h.)

\[
\text{slope (OD units/h.)} \times \frac{1 \text{ ml}}{\text{(OD units.ml)}} \times \frac{\text{protein (mg)}}{6.22 \text{ umol}}
\]

Protein concentrations in the crude homogenates were determined by the method of Bradford (1976). The differences between the ATPase activity with and without NEM represent the NEM-sensitive ATPase activity.

Preliminary tests were conducted to determine the appropriated amount of enzyme or homogenate for the assay and the
relationship between activity and temperature in the range of 10-15°C was tested to be linear. pH optimum was not tested but according to the literatures, H⁻-ATPase has a broad pH optimal range 7.0-7.5. My reaction mixture was well buffered to 7.3.

Application of other inhibitors

In experiments with the inhibitors DCCD, DES and PCMBS, stock solutions were made by dissolving the drugs in 100% ethanol and then adding them to the reaction mixture to the required concentration. Stock solution of bafilomycins (purchased from Dr. Altendorf, Fachbereich Biologie/Chemie, U. Osnabruck, FRG) was prepared in dimethylsulfoxide (Bowman et al. 1988). Control samples containing the proper amount of solvent were assayed simultaneously. KNO₃, acetazolamide and sodium vanadate are water soluble and assays were performed in the same way as for NEM.

Since the effect of all these inhibitors on PK and LDH was unknown, an experiment was designed to test their interference with the NADH oxidation reaction. To omit the reaction (A), I used ADP to replace ATP in the original assay. The initial O.D. of the 1 ml reaction mixture containing 125 mM Tris buffer, 1 mM EGTA, 12.5 mM KCl, 5 mM NaN₃, 2 mM ouabain, 5 mM MgCl₂, 2.5 mM phosphoenolpyruvate, 0.5 mM NADH and 10 units each of LDH and PK and appropriate amount of inhibitor was recorded. Then 25 ul 200 mM ADP was added to the reaction mixture and final O.D. was recorded 1 minute later. A significant decrease of O.D. indicates a complete reaction without interference. If O.D appeared to be unchanged, the drug being tested was effecting the NADH oxidation.
Table 3. Test of inhibitor interference with PK or LDH in the NADH oxidation reaction. - indicates no interference; + indicates minus interference; ++ indicates serious interference.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Interference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 mM NEM</td>
<td>-</td>
</tr>
<tr>
<td>1.2 mM NEM</td>
<td>-</td>
</tr>
<tr>
<td>0.1 mM Vanadate</td>
<td>-</td>
</tr>
<tr>
<td>0.5 mM Vanadate</td>
<td>+</td>
</tr>
<tr>
<td>5 mM Vanadate</td>
<td>++</td>
</tr>
<tr>
<td>1 mM DCCD</td>
<td>-</td>
</tr>
<tr>
<td>0.025 mM DES</td>
<td>-</td>
</tr>
<tr>
<td>0.01 mM PBMBS</td>
<td>-</td>
</tr>
<tr>
<td>0.025 Bafilomycins</td>
<td>-</td>
</tr>
<tr>
<td>100 mM KNO₃</td>
<td>-</td>
</tr>
<tr>
<td>0.1 mM Acetazolamide</td>
<td>-</td>
</tr>
<tr>
<td>0.25 mM NDB-Cl</td>
<td>+</td>
</tr>
<tr>
<td>0.5 mM NDB-Cl</td>
<td>++</td>
</tr>
<tr>
<td>0.1 mM Amiloride</td>
<td>+</td>
</tr>
<tr>
<td>0.5 mM Amiloride</td>
<td>++</td>
</tr>
<tr>
<td>1 mM Amiloride</td>
<td>++</td>
</tr>
</tbody>
</table>
reaction itself and can not be used in the ATPase assay. Table 3 summarized the result of this drug test. The effect of NDB-Cl, a specific inhibitor for H⁺-ATPase, and amiloride, which appeared to inhibit branchial proton excretion (Chapter 1), on gill tissue H⁺-ATPase activity cannot be examined. But all other inhibitors can be trusted to appose their effect on ATPase only.

**Statistical analysis**

All ATPase assays were performed in triplicate. In Figures and Tables, data are presented as mean ± standard error. Results have been statistically analyzed using unpaired Student's t-test between appropriate sample means. 5% was taken as the fiducial limit of confidence.
RESULTS

A substantial amount of the ATPase activity in the crude homogenates of gill tissue, in the presence of azide, ouabain and EGTA, was sensitive to NEM. Figure 13 shows the ATPase activity of gill tissue in response to various concentration of NEM. NEM causes a dose-dependent inhibition of ATPase activity. Maximal inhibition is observed in 1 mM NEM, which accounted for more than 70% of the total ATPase activity (Table 4). The difference of ATPase activity with and without 1 mM NEM is referred to as the NEM-sensitive ATPase activity and, at 15°C, it was determined to be about 1.5 ± 0.09 umol/mg.pr.h. for freshwater adapted trout.

Vanadate (0.1 mM) suppressed 60% of the ATPase activity in gill tissue (Table 4). When 1 mM NEM and 0.1 mM vanadate were applied together, the percentage of ATPase affected increased slightly only. The combination of 1 mM of NEM and 0.1 mM vanadate suppressed 80% of the ATPase in crude homogenates, which indicates that of the 60% ATPase that was sensitive to vanadate, 50% is from the NEM-sensitive ATPase.

The effect of DCCD was also examined, a maximum of 52% of the total ATPase activity was suppressed by 1 mM of DCCD (Table 4). A similar dose response curve to that produced by NEM was observed with DCCD (Figure 14). The sensitivity profiles of fish gill ATPase towards both NEM and DCCD are similar to those found for the inhibition of proton-ATPase in rat kidney (Ait-Mohamed et al, 1986).
Figure 13. NEM-sensitive ATPase activity in the crude homogenates of trout gill tissue in response to various concentrations of NEM. Activity is expressed in % assuming the ATPase activity is 0 with 1 mM of NEM and 100% without NEM. Each point is the mean ± S.E. of four fish.
Figure 14. DCCD-sensitive ATPase activity in the crude homogenates of trout gill tissue in response to various concentrations of DCCD. Activity is expressed in % assuming the ATPase activity is 0 with 1 mM of DCCD and 100% without DCCD. Each point is the mean ± S.E. of four fish.
Table 4. Effects of inhibitors on ATPase activity in crude homogenate of trout gill tissue.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration mM</th>
<th>Relative activity %</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>NEM</td>
<td>1</td>
<td>27.08 ± 2.36</td>
<td>6</td>
</tr>
<tr>
<td>Vanadate</td>
<td>0.1</td>
<td>38.18 ± 3.01</td>
<td>6</td>
</tr>
<tr>
<td>NEM + Vanadate</td>
<td>1 + 0.1</td>
<td>20.87 ± 2.37</td>
<td>6</td>
</tr>
<tr>
<td>DCCD</td>
<td>1</td>
<td>47.43 ± 0.65</td>
<td>4</td>
</tr>
<tr>
<td>DES</td>
<td>0.025</td>
<td>36.88 ± 0.82</td>
<td>3</td>
</tr>
<tr>
<td>PCMBS</td>
<td>0.010</td>
<td>55.35 ± 4.36</td>
<td>3</td>
</tr>
<tr>
<td>Bafilomycins</td>
<td>0.025</td>
<td>58.02 ± 2.25</td>
<td>3</td>
</tr>
<tr>
<td>KNO₃</td>
<td>100</td>
<td>72.25 ± 2.61</td>
<td>3</td>
</tr>
<tr>
<td>Acetazolamide</td>
<td>0.1</td>
<td>97.19 ± 5.66</td>
<td>3</td>
</tr>
</tbody>
</table>
DES and PCMBS had maximal inhibitory effects at much lower concentrations and also accounted for inhibition of 63% and 45% of the total ATPase activity, respectively (Table 4). Bafilomycins, a very specific and potent inhibitor of vacuolar H⁺-ATPase (Bowman et al., 1988), significantly inhibited ATPase activity of fish gills only at concentrations above 25 µM (Table 4), whereas vacuolar H⁺-ATPase was completely blocked at concentrations as low as 0.1 µM (Bowman, et al., 1988). Potassium nitrate, another inhibitor used to distinguish vacuolar H⁺-ATPase from plasma membrane H⁺-ATPase (Bowman, 1983), caused less than a 30% reduction in fish gill ATPase activity at a concentration of 100 mM (Table 4), a dosage that is sufficient to inhibit 80% of the vacuolar H⁺-ATPase. 0.1 mM of acetazolamide however, had no effect on the ATPase activity of fish gills.
DISCUSSION

These studies demonstrate for the first time the existence of a NEM-sensitive ATPase in crude homogenates of fish gill tissue. H⁺-ATPase has been noted to be either bound to membranes or packaged in cytoplasmic vesicles (Arruda et al. 1990). The crude homogenates prepared with the current method contained mainly the membrane faction of gill cells, and the NEM-sensitive ATPase that I detected in this study was released by a protein solubilizer from the membrane faction. Soluble cell material, mitochondria, cytoplasmic vesicles and other organelles would have been discarded in the supernatant of the first centrifugation (2000g) because much higher relative centrifugation forces are required to spin down this material. Little ATPase activity was found in the discarded supernatant but protein solubilizer was never applied. If there was H⁺-ATPase packaged in cytoplasmic vesicles, perhaps protein solubilizer was required to release them for subsequent detection.

NEM is an alkylating agent that is relatively selective for sulphydryl groups (SH⁻). It inhibits vacuolar H⁺-ATPase in an ATP-protetable manner in concentrations under 10 µM (Forgac, 1989; Pedersen and Carafoli, 1987). Phosphorylated ATPases (including Na⁺-K⁺-ATPase, Ca⁺⁺-ATPase and plasma membrane H⁺-ATPase) are sensitive to higher concentrations (100 µM - 1 mM) of NEM (Forgac, 1989). Since the assay was carried on in the presence of EGTA, a Ca⁺⁺ chelator which should abolish Ca⁺⁺-ATPase
activity; azide, a mitochondrial H⁺-ATPase inhibitor; and ouabain, a Na⁺-K⁺-ATPase inhibitor; the contribution of unrelated ATPase activity was minimal. Thus, the ATPase activity in the crude homogenate of gill tissue that was sensitive to 1 mM of NEM probably originated from plasma membrane H⁺-ATPase.

30% of the ATPase activity was NEM-insensitive and is of unknown origin. Unidentified NEM-insensitive ATPase was also detected in mammalian kidney (Ait-Mohamed et al. 1986; Garg and Narang, 1988). Bornancin et al. (1980) presented evidence of Cl⁻-HCO₃⁻-ATPase in microsomes from the gill plasma membrane of rainbow trout. This might account for the NEM-insensitive ATPase activity in the gill tissue crude homogenates.

The gill ATPase sensitive to NEM was also sensitive to vanadate. Orthovanadate ion, VO₄³⁻, acting as a phosphate transition analogue, blocks the formation of phosphorylated intermediates in all P-type ATPases. 0.1 mM vanadate was reported to inhibit branchial proton excretion in freshwater trout (Chapter 1). Urinary acidification by turtle bladder (Arruda, et al. 1981) and proton transport across freshwater frog skin (Ehrenfeld, et al. 1985), both mediated by H⁺-ATPase, are also vanadate-sensitive. However, vanadate fails to inhibit NEM-sensitive ATPase activity and proton transport in mammalian kidney (Gluck and Al-Awqati, 1984; Ait-Mohamed et al. 1986) which was believed to be a vacuolar H⁺-ATPase (Forgac, 1989).

DCCD inhibits mitochondrial, vacuolar and plasma membrane H⁺-ATPase by binding to the c-subunit of the hydrophobic channel.
portion (Pedersen and Carafoli, 1987). Mitochondrial H⁺-ATPase has the highest sensitivity to DCCD (0.1-0.5 uM), followed by vacuolar H⁺-ATPase (1-10 uM) and then plasma membrane H⁺-ATPase (10-100 uM). The dose response curves of the ATPase activity in gill tissue towards NEM and DCCD are very similar to those reported for rat kidney H⁺-ATPase (Ait-Mohamed, et al., 1986). 0.05 mM of DCCD inhibited proton excretion and decreased pHi in frog skin (Harvey and Ehrenfeld, 1986). DES was also reported to inactivate H⁺-ATPase in the Fₖ moiety level (Pedersen and Carafoli, 1987). 0.1 mM DES markedly reduced the proton excretion across frog skin. Proton transport mediated by H⁺-ATPase in bovine kidney medulla was completely blocked by 10 uM PCMBS, a SH-group reagent, (Gluck and Al-Awqati, 1984), which partially inhibited H⁺-ATPase activity in fish gills.

Bafilomycins is a macrolide antibiotic that has a specific and potent inhibitory effect on vacuolar H⁺-ATPase (Bowman et al. 1988). Mitochondrial H⁺-ATPase is resistant to up to 1 mM of bafilomycins whereas vacuolar H⁺-ATPase is completely inhibited by <0.1 uM of the antibiotic. Phosphorylated ATPase exhibits intermediate sensitivities with I₅₀ values between 10-100 uM. The sensitivity level of the ATPase in gill tissue to bafilomycins is within this intermediate range.

Vacuolar H⁺-ATPase also demonstrates a unique sensitivity to KNO₃ with a I₅₀ value of about 50 mM (Bowman, 1983). The resistance of fish gill ATPase to nitrate indicates that the H⁺-ATPase we measured is not a vacuolar type. The balance of all
these pharmacological properties indicates that H⁺-ATPase in fish gills is a plasma membrane type and not a vacuolar type.

Acetazolamide was demonstrated to inhibit luminal acidification in turtle bladder by stimulating the endocytosis of apical membrane (Dixon et al. 1988; Graber et al. 1989). The inhibition appeared to be independent of cell pH, which ruled out the possibility of a secondary affect due to carbonic anhydrase inhibition. The inhibitory effect of acetazolamide on in vivo proton excretion (Lin and Randall, 1991) can not be reproduced in the in vitro ATPase assay, indicating that acetazolamide has no direct effect on H⁺-ATPase itself.
Chapter 4: Environmental and hormonal regulation of the proton-ATPase in fish gills
The effects of sodium, calcium and CO$_2$ levels in environmental water on the proton-ATPase activity in fish gills were investigated. Sodium concentration in the external media was the primary regulator of the H$^+$-ATPase in fish gills, with low sodium levels associated with high H$^+$-ATPase activity. It was concluded therefore, that the major role of the H$^+$-ATPase in the gill epithelium was to facilitate Na$^+$ uptake from fresh water. High external calcium concentration had a marked stimulating effect on H$^+$-ATPase activity in fish gills when the sodium level was low. Environmental hypercapnia induced a 70% increase in the H$^+$-ATPase activity in fish gills. The effect of the steroid hormone cortisol was also investigated. H$^+$-ATPase activity was elevated in freshwater fish after chronic cortisol infusion, but not in seawater fish. The H$^+$-ATPase in fish gills also plays a role in acid-base regulation.
INTRODUCTION

The results from Chapter 3 confirm the existence of a proton pump, or proton-ATPase in the gill epithelium of freshwater rainbow trout. The first apparent function of the proton-ATPase in fish gill is to generate an electrical gradient favourable for sodium uptake from freshwater, and the effective operation of this active pump will be critical for the maintenance of osmotic homeostasis in fish living in a very dilute media. For seawater fish, however, the negative potential generated by H⁺-ATPase will retard the animal's ability to excrete sodium in seawater. Euryhaline fish such as rainbow trout, therefore, should be able to regulate the proton-ATPase activity in the gill epithelium according to environmental salinity.

Influx of sodium in freshwater fish is effected by different environmental factors such as hypercapnia (Goss et al. 1992) and water Ca²⁺ levels (Avella et al. 1987). If the proton pump does provide the driving force for the sodium uptake in freshwater fish, these factors could act on H⁺-ATPase and variation of Na⁺ influx would be the secondary outcome. The objective of these studies, therefore, was to examine the effect of environmental variations on H⁺-ATPase activity in fish gills.

It has also been suggested that the molecular target of the mineralocorticoid hormone aldosterone on urine acidification is H⁺-ATPase in the kidney collecting tubule of mammals (Mujais, 1987; Garg and Narang, 1988; Khadouri et al. 1989). Cortisol, the
equivalent steroid hormone in fish, increased in rainbow trout when they were transferred from freshwater to seawater (Foskett et al., 1983) and when they were acclimated to deionized water (Perry and Laurent, 1989). The effect of cortisol on gill H'-ATPase activity has yet to be investigated.

H'-ATPase in fish gills might also play a role in the acid-base balance of the whole animal and in the regulation of intracellular pH, as reported in frog skin (Ehrenfeld et al., 1990), turtle bladder (Cohen and Steinmetz, 1980) and mammalian kidney (Sabatini et al., 1990). An acidosis, caused by environmental hypercapnia or NH₄Cl injection and an alkalosis, caused by alkaline water exposure, was induced in trout and effects on H'-ATPase activity was determined to illuminate the significance of gill proton-ATPase in acid-base regulation.
MATERIALS AND METHODS

Freshwater rainbow trout weighing between 150-500 g were maintained in large fibreglass tanks supplied with flowing dechlorinated Vancouver tap water. Seawater adapted rainbow trout (400-600 g) were obtained by acclimating freshwater rainbow trout to seawater (34-38%\textsubscript{o}) for 8 to 10 weeks in the large fibreglass tanks at DFO West Vancouver laboratory. After the fish were subjected to one of the treatments described below, they were sacrificed and crude homogenates of gill tissue containing mainly the membrane faction, were obtained by the method described in Chapter 3.

The $\text{H}^+\text{-ATPase}$ activity in gill tissue of fish under different treatments was determined by the modified couple-enzyme assay in a temperature-controlled spectrophotometer using N-ethymaleimide as a specific inhibitor (see Chapter 3 for details). NEM-sensitive ATPase is equivalent to $\text{H}^+\text{-ATPase}$ activity (Chapter 3). Ouabain-sensitive ATPase, which is equivalent to $\text{Na}^+\text{-K}^+\text{-ATPase}$, was measured in gill tissue using the method from Scharschmidt et al (1979). All ATPase assays were performed in triplicate and data are presented as mean ± standard error. Student's two-tailed t-test and one-way ANOVAs (analysis of variance) were used to test for significant differences at the 5% level rejection between means.

Acclimation to various external sodium and calcium concentrations

Four kinds of external media were prepared by dissolving
NaCl and/or CaCl$_2$ into dechlorinated Vancouver tap water: 100 mM NaCl; 100 mM NaCl plus 1 mM CaCl$_2$; 1 mM CaCl$_2$ and 10 mM CaCl$_2$.

Fish were placed into a 100 litre opaque fibreglass tanks (density < 25g/L) filled with different external media for acclimation period of 10-14 days. The tanks were well-aerated and temperature was maintained at ambient levels with cooling coils. The external media were changed daily to prevent ammonia accumulation.

**Chronic cortisol treatment**

Plasma cortisol levels were elevated in both freshwater and seawater adapted trout by implantation of Alzet mini osmotic pumps containing cortisol (Reid and Perry, 1991). Mini osmotic pumps were loaded with cortisol (hydrocortisone 21-hemisuccinate, Sigma) or saline (in sham treatments) and surgically implanted into the peritoneal cavity of anaesthetized fish. The nominal calculated plasma cortisol concentration was 200 ngml$^{-1}$ and fish were sampled for gill tissue and blood (by caudal puncture) 7 days after the implantation. Plasma cortisol level was measured using a Gammacoat $^{125}$I cortisol radioimmunoassay kit (Incstar Corp.).

**Hypercapnia treatment**

Rainbow trout were placed into a 100 litre opaque fibreglass tank supplied with flowing aerated dechlorinated Vancouver tap water (water pH 5.8-6.2). Fish were allowed to acclimate for 24h. Control fish were sampled right before the 48h hypercapnia treatment (2% CO$_2$ in air, mixed using a Wosthoff gas mixing pump,
water pH 5.0-5.5) and fish were sampled at 6, 24 and 48h following hypercapnia. Fish were then allowed to recover for 24h and samples were taken at 6 and 24h of recovery.

**NH₄Cl injection**

Rainbow trout were fitted with dorsal aortic catheters under MS-222 anaesthesia (1:10000 in NaHCO₃-buffered freshwater). Fish were then allowed to recover for 24h in a sectioned plexiglas box. 2 mlkg⁻¹ body mass of saline (control group) or 1 mol·l⁻¹ NH₄Cl in saline was injected daily for 2 days into the dorsal aorta of the fish over a period of approximately 5 min. 48h after the first injection, blood samples were taken for pH measurement using a microcapillary pH electrode (Radiometer G279/G2) coupled to a PHM84 pH meter, and fish were sacrificed for gill tissue sampling.

**Exposure to alkaline water**

Freshwater trout were held in a 200 litre fibreglass tank supplied with flowing aerated dechlorinated tap water (pH 6-7). After 24 hours of acclimation, control fish were sampled. Then water pH was increased to 10 by metering a concentrated NaOH solution into the tank using a peristaltic pump. In another set of experiments, water hardness was also increased to 50 ppm ([Ca⁺²] = 1.25 mM) by metering a concentrated CaCl₂ solution into the tank with a separate peristaltic pump. Fish were sampled on the first, second, third, fourth and sixteenth day of the alkaline soft water or alkaline hard water exposure.
RESULTS

In rainbow trout acclimated to different external levels of Na⁺ and / or Ca²⁺, gill NEM-sensitive ATPase activity decreased as Na⁺ acclimation level increased (Figure 15). NEM-sensitive ATPase activity was significantly lower in fish acclimated to 100 mM of Na⁺ (with or without Ca²⁺). Seawater adapted rainbow trout had only 1/3 of the NEM-sensitive ATPase activity of their freshwater counterparts. The addition of Ca²⁺ to high Na⁺ water made no difference to NEM-sensitive ATPase activity. Increased Ca²⁺ level in low Na⁺ media, however, had a marked stimulating effect and resulted in a two fold increase on NEM-sensitive ATPase activity in fish gills (Figure 15).

Ouabain-sensitive ATPase activity, which is equivalent to Na⁺-K⁺-ATPase activity, decreased when trout were acclimated to freshwater containing high sodium and high calcium levels, but increased when they were acclimated to low sodium but high calcium water (Figure 16).

Chronic cortisol infusion in freshwater rainbow trout caused a 170% increase in plasma cortisol level (Figure 17) and a 30% increase in NEM-sensitive ATPase activity in gill tissue (Figure 18). Seawater adapted animals on the other hand, showed no response of NEM-sensitive ATPase activity (Figure 18) to a similar cortisol treatment although their plasma cortisol levels increased four-fold (Figure 17). There was no significant difference in gill tissue Na⁺-K⁺-ATPase activity between sham and
Figure 15. NEM-sensitive ATPase activity in the gill tissue of rainbow trout acclimated to various Na' and Ca'' levels in the external media for 10-14 days. Mean ± S.E. * indicates a significant difference from the control value (P < 0.05). Number in brackets indicates the sample size.
Figure 16. Ouabain-sensitive ATPase activity in the gill tissue of rainbow trout acclimated to various Na\(^+\) and Ca\(^{++}\) levels in the external media for 10-14 days. Mean ± S.E. * indicates a significant difference from the control value (P < 0.05). Number in bracket indicates the sample size.
Figure 17. Plasma cortisol concentration in freshwater and seawater rainbow trout after 7 days of chronic cortisol treatment. Mean ± S.E. * indicates a significant difference from the sham treatment value (P < 0.05). Number in bracket indicates the sample size.
Sham Cortisol
Freshwater
Sham Cortisol
Seawater

Plasma Cortisol Concentration (ng/ml)

(6)

(3)

(6)

(3)

Sham Freshwater
Cortisol
Sham Seawater
Cortisol

*
Figure 18. NEM-sensitive ATPase activity in the gill tissue of freshwater and seawater rainbow trout after 7 days of chronic cortisol treatment. Mean ± S.E. * indicates a significant difference from the sham treatment value (P < 0.05). Number in bracket indicates the sample size.
cortisol treatments in either freshwater or seawater animals (Figure 19).

Hypercapnia treatment in freshwater fish induced a rapid increase in the NEM-sensitive ATPase activity which stabilized at a level twice that of normocapnia (Figure 20). The elevated NEM-sensitive ATPase activity returned to control levels after 24h recovery.

A blood acidosis was associated with NH$_4$Cl injection (Table 5). NEM-sensitive ATPase activity, however, was not altered by NH$_4$Cl injection. Daily injection of NH$_4$Cl was performed on two fish for five days and no change of NEM-sensitive ATPase activity was observed (data not shown).

Short term (4 days) exposure to alkaline soft water induced no change in NEM-sensitive ATPase activity in fish gill tissue (Figure 21). But long term (16 days) exposure resulted in a significant decrease of H$^+$-ATPase activity. Alkaline hard water, on the other hand, stabilized the H$^+$-ATPase activity in the control level for the whole period except for a transient decrease in activity at the beginning of the exposure (Figure 21). The fish subjected to soft water and hard water treatments were from different fish stocks, which may account for the different control levels of H$^+$-ATPase activity observed.
Figure 19. Ouabain-sensitive ATPase activity in the gill tissue of freshwater and seawater rainbow trout after 7 days of chronic cortisol treatment. Mean ± S.E. Number in bracket indicates the sample size.
Figure 20. NEM-sensitive ATPase activity in the gill tissue of freshwater rainbow trout during 48 hours of hypercapnia treatment and 24 hours recovery. Mean ± S.E. (N=6) * indicates a significant difference from the control value (P < 0.05).
Figure 21. NEM-sensitive ATPase activity in the gill tissue of freshwater rainbow trout during control and 16 days exposure to soft (N=6) and hard (N=4) alkaline water (pH=10). Mean ± S.E. * indicates a significant difference from the control value (P < 0.05).
Table 5. Plasma pH and NEM-sensitive ATPase activity in gill tissue of NH₄Cl-injected rainbow trout (n=6).

<table>
<thead>
<tr>
<th></th>
<th>Plasma pH</th>
<th>NEM-sensitive ATPase Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>umol/mg.pr.h</td>
</tr>
<tr>
<td>Saline-Injected</td>
<td>7.70 ± 0.12</td>
<td>0.985 ± 0.123</td>
</tr>
<tr>
<td>NH₄Cl-Injected</td>
<td>7.37 ± 0.22*</td>
<td>1.020 ± 0.224</td>
</tr>
</tbody>
</table>

*Indicates a significant difference between saline and NH₄Cl injection values.
DISCUSSION

Ehrenfeld et al. (1985) demonstrated that the sodium absorption across freshwater frog skin was mediated by an active proton pump indirectly coupled with a sodium channel, instead of a Na⁺/H⁺ exchange. The proton pump in frog skin was inhibited by DCCD and vanadate. Since the gill epithelium in freshwater fish is a tight epithelium similar to that of freshwater frog skin, it is reasonable that they have the same mechanism to solve the same osmoregulatory problem. The fact that fish acclimated to water with low Na⁺ level have higher H⁺-ATPase activity suggests that the functional significance of the H⁺-ATPase in fish gills is to generate a electrochemical gradient for Na⁺ uptake from a dilute media. When external Na⁺ level is high, H⁺-ATPase is down-regulated, possibly by endocytosis of membrane protein into intracellular vesicles (Schwartz and Al-Awqati, 1986). The residual H⁺-ATPase in fish gills may play a role in acid-base regulation, although it is not required for sodium absorption or sodium excretion in fish living in a high sodium environment.

The stimulating effect of Ca²⁺ on H⁺-ATPase could be explained using the cellular model of Wendelaar Bonga et al. (1992), who proposed the existence of an apical Ca²⁺ channel. When the external medium has a high Ca²⁺ but low Na⁺ level, Ca²⁺ could enter the cell via the Ca²⁺ channel and reduce the potential gradient generated by the H⁺-ATPase and therefore Na⁺ influx. The resulting high Ca²⁺ concentration in the cell might
stimulate H+-pump insertion from intracellular vesicles into the apical membrane in order to maintain Na+ influx. Exocytosis of proton pump containing vesicles into the cell membrane is Ca++ dependent in turtle bladder epithelium (Adelsberg and Al-awgati, 1986).

Variations in Ca++ level in freshwater environments were reported to affect the gill morphology and sodium influx in rainbow trout (Avella et al. 1987). Na+ influx increased 2.5 times in fish acclimated to FW + 10 mM CaCl₂ for 15 days and new globular chloride cells appeared and proliferated in the secondary lamellae. Fish acclimated to FW + 5 mM CaCl₂ for 5 days showed no change in gill morphology or sodium flux, perhaps because the duration was too short for morphological modification. In earlier studies (Chapter 2) I found no effect of calcium exposure on branchial proton excretion after 2 hours.

I have also investigated the effects of chronic infusion of cortisol on H+-ATPase activity in gill tissue in FW and SW rainbow trout. Aldosterone treatments, either long term (7 days) or short term, were reported to stimulate H+ secretion mediated by H+-ATPase in the collecting duct of the mammalian kidney (Garg and Narang, 1988; Mujais, 1987). The functionally parallel steroid hormone in fish is cortisol. Whether aldosterone is present in teleosts is uncertain, and little information is available about its function in fish (Butler, 1973). Perry and Laurent (1989) have shown that plasma cortisol levels rose transiently in fish exposed to deionized water. Daily
intramuscular injections of cortisol for 10 days caused an increase in Na' uptake (Perry and Laurent, 1989). The 30% increase of H⁺-ATPase activity observed in freshwater trout following chronic cortisol treatment is probably responsible for this increased Na' uptake. Similar cortisol treatment had no effect on SW acclimated rainbow trout, indicating that Na' concentration is the predominant regulator of the H⁺-ATPase in fish gills.

Another possible function of the H⁺-ATPase in the fish gill epithelium is acid-base regulation. I induced respiratory acidosis through hypercapnia treatment and observed an increased H⁺-ATPase activity in fish gills under this treatment. Similar hypercapnia treatment in FW catfish was reported to cause a marked increase in Na' influx, which might be correlated with the increased H⁺-ATPase activity. The elevated H⁺-ATPase activity could be induced by CO₂ mediated proton pump insertion via exocytosis, as demonstrated in turtle bladder epithelium (Cannon et al. 1985; Arruda et al. 1990). High CO₂ levels reduced the intracellular pH of the proton secreting cell, which increased the intracellular Ca⁡²⁺ concentration and in turn stimulated the fusion of cytoplasmic vesicles containing proton pump into the apical membrane. This would then correct the intracellular acidosis.

Chronic metabolic acidosis was induced in the fish by NH₄Cl injection and resulted in no significant change in H⁺-ATPase activity. This indicates that high plasma hydrogen ion levels
alone can not stimulate H'-ATPase activity in gill tissue or proton pump insertion to the epithelium. I concluded that elevated CO₂ levels increased H'-ATPase activity via a depression of epithelial pH. A metabolic acidosis will only activate H'-ATPase activity if the acidosis is transferred into the gill intracellular compartment. If I follow this argument, then presumably NH₄Cl infusion has little or no effect on epithelial pH because H'-ATPase activity was unchanged. Unfortunately, I was not able to measure the effects of NH₄Cl injection on gill epithelial pH. The operation of the existing proton pumps was probably sufficient to correct the acidosis.

The alkalosis, reported to occur immediately in rainbow trout exposed to pH 10 alkaline water (Yesaki and Iwama, 1992), is more severe in soft water than in hard water. Fish in hard water were able to maintain acid-base balance because external calcium helps to stabilize biological membranes, maintain the integrity of cell to cell junctions and control ion and water permeability across epithelial tissue (McDonald et al, 1983). It is possible that high gill intracellular calcium levels (due to the high calcium level in the external water) prevented the withdrawal of proton pumps from the apical membrane in the face of a mild gill epithelial alkalosis. Proton-ATPase activity decreased in fish maintained in soft alkaline water, probably due to the intracellular alkalosis, but the process occurred very slowly. This adjustment of H'-ATPase activity in the gill epithelium might lessen the acid-base disturbance in the fish by
reducing proton excretion and contribute to their long term survival. Apparently proton pump withdrawal was unnecessary for fish in alkaline hard water when Ca\(^{2+}\) reduced the permeability of gill epithelium to base equivalents. But reduction of H\(^+-ATPase\) activity in gill apical membrane might be a critical adaptational response to fish in alkaline soft water in order to retain hydrogen ions. Yesaki and Iwama (1992) reported a 100% mortality 4 days after fish were exposed to soft alkaline water. No loss of fish occurred in my studies, possibly because no surgery was preformed on these animals.
GENERAL DISCUSSION

The studies reported in this thesis provide us, for the first time, with direct \textit{in vivo} and \textit{in vitro} evidence of the existence of a active electrogenic proton pump, or $\text{H}^+\text{-translocating-ATPase}$, in fish gills. This proton pump, indirectly coupled with a sodium conductive channel, instead of a $\text{Na}^+\text{H}^+(\text{NH}_4^+)$ exchanger, is responsible for the sodium uptake and proton excretion in freshwater fish. As discussed in the General Introduction, the operation of a $\text{Na}^+\text{H}^+(\text{NH}_4^+)$ exchanger in the apical membrane of the gill epithelia of freshwater fish is energetically improbable, because the $\text{Na}^+$ electrochemical gradient is unfavourable for the exchanger. Studies on ammonia excretion in fish found ammonia excretion was not always coupled with sodium absorption (Payan, 1978; Avella and Bornancin, 1989) and when it was, the coupling could be explained in terms of an intracellular pH effect (Cameron and Kormanik, 1982; Avella and Bornancin, 1989). My studies confirm the absence of the $\text{Na}^+\text{H}^+(\text{NH}_4^+)$ exchange in the gills of freshwater fish and establish a new model for sodium and proton transport across fish gill epithelia (Figure 22).

The relationship of the proton pump and the other ion transport pathways in the gill epithelium of freshwater fish is summarized in Figure 22. The primary electrogenic proton pump in the apical membrane consumes ATP and excludes hydrogen ion from the intracellular compartment to the external water, and
Figure 22. Hypothetical model of the gas and ion transport pathways in gill epithelium of freshwater rainbow trout. ATP-driven pumps are denoted by filled circles, ion exchanger by opened circles and passive diffusion by arrows. See text for details.
generates a negative potential in the inner side of the apical membrane. This negative potential will serve as a driving force for sodium uptake from freshwater via a sodium conductive channel. CO₂ hydration in the intracellular compartment provides one of the sources of protons to the proton pump. HCO₃⁻ generated by CO₂ hydration can either be excreted to the external water by a Cl⁻/HCO₃⁻ exchanger or recycled to the serosal side via an anion channel. The deprotonation of NH₄⁺ which entered the cell from the serosal side through the Na⁺-K⁺(NH₄⁺)-ATPase pathway in the basolateral membrane provides another source of protons to the pump. The resulting NH₃ produced will diffuse across the apical membrane and be excreted to the water. Na⁺ and sometimes Ca²⁺ enter the cell from the mucosal side via the conductive channels and act as the counterion for the proton pump. Cl⁻ transferred across the apical membrane into the cell can also affect the electric potential generated by the pump and therefore affect the proton pump's operation.

The proton pump model solves the energetic problem for sodium uptake from a very dilute medium in fish, but still explains the 1:1 stoichiometry for Na⁺ uptake and proton/ammonia excretion as demonstrated in freshwater fish by Wright and Wood (1985). Proton excretion via the proton pump can facilitate ammonia excretion either by converting NH₃ to NH₄⁺ in the unstirred water layer right next to the gill epithelium or by lowering the intracellular pH and enhancing NH₄⁺ deprotonation in the epithelial compartment. The operation of the proton pump
however, is unfavourable to CO₂ excretion because it facilitates HCO₃⁻ dehydration in the external water and CO₂ hydration in the intracellular compartment. Since the gill epithelial membrane is very permeable to CO₂ and CO₂ is very soluble in water, fish can maintain a CO₂ excretion despite this obstruction.

The studies of the inhibitor sensitivity, environmental and hormonal regulation of the H⁺-ATPase in fish gills show that the H⁺-ATPase in gill tissue is very similar to the H⁺-ATPase in turtle bladder and mammalian kidney and that branchial proton excretion is very similar to the proton transport mediated by the proton pump in frog skin and turtle bladder. H⁺-ATPase in fish gills is sensitive to NEM, DCCD, DES, PCMB and bafilomycins, to an extent that is characteristic for plasma membrane H⁺-ATPase (Chapter 3). Both the H⁺-ATPase in gill tissue and the branchial proton excretion are inhibited by vanadate (Chapter 1 and 3), indicating that the H⁺-ATPase in fish gills is probably a P-type as in frog skin and not a V-type as in mammalian kidney. Branchial proton excretion is sensitive to external water pH (Chapter 2), or the electrochemical gradient of hydrogen ion (the pH change in fish blood is negligible), which is a standard feature of proton transport via proton pumps (Steinmetz, 1985). The effect of ambient CO₂ levels and acetazolamide (Chapter 1 and 3) on the branchial proton excretion is also typical for proton transport mediated by proton pumps. The effect of acetazolamide can be explained either by its direct inhibition on carbonic anhydrase in the gill epithelium or by stimulating the
endocytosis of apical membrane (Dixon et al., 1988; Graber et al., 1989), but not by its direct effect on H⁺-ATPase (Chapter 3). The effects of amiloride, 9-anthroic acid and sodium-free water on branchial proton excretion reveal the electrical linkage between \( \text{Na}^+ \), \( \text{Cl}^- \) and \( \text{H}^+ \) in the gill epithelial compartment (Chapter 1 and 2). Under open circuit conditions the negative potential generated by the proton pump will slow down its own operation, unless counterions are available to balance the potential. \( \text{Na}^+ \) is usually playing this role whereas \( \text{Cl}^- \) rewards the problem, and the balance of the two effects determines the electric potential imposed on the proton pump.

The major role of the H⁺-ATPase in the gill epithelium is to facilitate \( \text{Na}^+ \) uptake from freshwater. Thus the external \( \text{Na}^+ \) level is the primary regulator of H⁺-ATPase activity in gill tissue (Chapter 4). The higher the external \( \text{Na}^+ \) level, the lower the H⁺-ATPase activity in gill tissue. The proton pump appeared to be removed from the membrane as external sodium levels increased, through an unknown mechanism. This phenomenon has not been reported in other H⁺-ATPase transport systems, not even in freshwater frog skin which shares the same sodium uptake function as freshwater fish gills. \( \text{Ca}^{++} \) and cortisol stimulate the recruitment of H⁺-ATPase to the apical membrane when external \( \text{Na}^+ \) levels are low (Chapter 4), and this is probably associated with the gill morphological changes induced by \( \text{Ca}^{++} \) (Avella et al., 1987) and cortisol (Perry and Laurent, 1989) under similar experimental conditions. Avella et al. (1987) discovered that
after 5 days of acclimation in distilled water or 5 to 15 days of acclimation in freshwater plus 10 mM of CaCl₂, there was a significant proliferation of new protruding chloride cells in the secondary lamellae of rainbow trout. The maximal rate of branchial sodium uptake (V_max) was also increased in both cases, suggesting an increase of the number of carriers that might locate in chloride cells. Perry and Laurent (1989) demonstrated that the increased sodium and chloride absorption capacity across trout gills after long term cortisol treatment and deionized water exposure was also correlated to the proliferation of chloride cells. It is possible that the proton pump is located in the branchial chloride cell, and the recruitment of H⁺-ATPase induced by Ca²⁺ or cortisol stimulates the branchial sodium absorption.

The H⁺-ATPase in fish gills is also involved in acid-base regulation. It appears that the acid-base disturbance of the animal has to be transferred to the intracellular compartment in the gill epithelium to cause any adjustment of H⁺-ATPase activity in gill tissue (Chapter 4). The acidosis induced by NH₄Cl injection for example, did not result in an increase of H⁺-ATPase activity in gill tissue because gill epithelium pH was probably unchanged (Chapter 4). Distinction must be made between the branchial proton excretion rate, and the H⁺-ATPase activity in gill tissue, which refers to the amount of enzyme incorporated in the apical membrane of gill epithelium. When fish were exposed to high pH water, an alkalosis was induced in the epithelial cells.
H⁺-ATPase activity in gill tissue was lower due to the removal of proton pump from the apical membrane in order to retain hydrogen ions (Chapter 4). The branchial proton excretion under this condition, on the other hand, was maximal because the proton electrochemical gradient imposed on the pump favoured the operation of the remaining proton pumps (Chapter 1). I was not able to examine branchial proton excretion and H⁺-ATPase activity at the same time, since fish subjected to surgery can not survive pH 10 exposure over 4 days (Yesaki and Iwama, 1992), but proton pump withdrawal by membrane endocytosis did not occur in the first 4 days of exposure (Chapter 4). Nevertheless the problem can be illuminated by the current approaches. The reverse is expected in fish exposed to acid water. Branchial proton excretion was completely suppressed by low environmental pH (Chapter 2), but even intact fish had difficulty surviving a chronic exposure of pH 4 water and the H⁺-ATPase activity in gill tissue under this condition can not be examined.

The localization of the proton pump in fish gills is an interesting but unanswered question. It is not difficult to speculate that the pump is in the apical membrane, because that is where the H⁺-ATPase will function as in frog skin, turtle bladder and mammalian kidney. Experimentally drugs and treatments applied to the apical side of fish gills affect the operation of the pump (Chapter 1 and 2). External water pH, for instant, exerts an immediate effect on the operation of the proton pump and the apical membrane is known to be impermeable to hydrogen
Whether the proton pump is housed in the chloride or epithelium cell is difficult to predict. Attempts have been made to separate chloride cells from epithelial cells using a FACStar cell sorter, but the size and density differences of these two cell lines in freshwater fish gills are too small to permit separation. Results of morphological studies by other workers are controversial. Freshwater fish exposed to deionized water, high calcium water or treated cortisol shown proliferation and enlargement of branchial chloride cells and enhanced NaCl transporting capacity (Avella et al, 1987; Laurent and Perry, 1990; Perry and Laurent, 1989). Environmental hypercapnia, however, caused density increases of epithelium cells in freshwater fish gills, which was apparently associated with elevated Na⁺ uptake rate (Goss et al, 1992). All the above mentioned treatments were tested for H⁺-ATPase activity in freshwater trout in my studies and all resulted in an increase of H⁺-ATPase activity (Chapter 4). Membrane protein with a surface structure similar to the proton pump in turtle bladder and mammalian kidney as described by Brown et al (1987) was observed in epithelium cells but not in chloride cells of freshwater fish gills under electron microscope (Perry, personal communications). It is possible that H⁺-ATPase and sodium channels are housed in different cell types, as long as gap junctions exist laterally so all the cells are electrically coupled, as in frog skin. It is also possible that sodium channels are present in both chloride cells and epithelium cells, and under certain conditions (eg.
respiratory acidosis) those in one particular cell type (eg. epithelium cell) become predominant, as demonstrated in frog skin (Ehrenfeld et al, 1990). More powerful tools such as immunocytochemistry have to be employed before any conclusion can be drawn in this matter.

Na\(^+\)-K\(^-\)-ATPase plays an important role in osmoregulation of seawater fish and the cellular localization of this primary ion transport pathway was concluded to be in the basolateral membrane of chloride cells in fish gills (Karnaky et al, 1976). There is a weak correlation between the H\(^+\)-ATPase activity and the Na\(^+\)-K\(^-\)-ATPase activity in freshwater rainbow trout (Figure 23) but this correlation disappeared in seawater animals (Figure 24). The major morphological change in gill epithelium when fish are transferred from freshwater to seawater is an increase in the size and number of chloride cell (from 1% to 13%, Perry and Walsh, 1989). The lack of correlation between H\(^+\)-ATPase and Na\(^+\)-K\(^-\)-ATPase in seawater fish argues against the notation that H\(^+\)-ATPase is housed in the chloride cell, as suggested by the weak correlation between the two in freshwater fish. Ca\(^++\)-ATPase in fish gills was also located in the basolateral membrane of chloride cells and provides the major driving force for calcium uptake (Perry and Wood, 1985; Wendellaar Bonga et al, 1992). As long as gap junctions exist between chloride cells and epithelium cells, all the primary and secondary ion transport pathways can be coupled and the gill epithelium can be viewed as a general compartment (Figure 22). When external sodium and calcium levels
Figure 23. The relationship between the NEM-sensitive ATPase activity and the ouabain-sensitive ATPase activity in freshwater rainbow trout under different treatments. The linear regression line has a $R^2$ value of 0.626. The slope of the regression line is significantly different from zero ($P<0.05$).
$Y = 1.012X + 0.426$

$R^2 = 0.626$
Figure 24. The relationship between the NEM-sensitive ATPase activity and the ouabain-sensitive ATPase activity in seawater-adapted rainbow trout under control and cortisol treatments.
\[ Y = 1.950X - 0.171 \]

\[ R^2 = 0.054 \]
are not too low, \( \text{Na}^+-\text{K}^-\text{ATPase} \) and \( \text{Ca}^{2+}\text{ATPase} \) in the basolateral membrane may be sufficient to energize the uptake of sodium and calcium from external water. However, when external sodium and calcium levels are very low, \( \text{H}^+\text{ATPase} \) is needed in addition to generate an immediate driving force from apical membrane and the deterioration of the intracellular ion concentration on the driving capacity can be avoid.

In summary, substantial evidence was accumulated in these studies for the existence of an electrogenic proton pump in fish gill epithelium. Branchial proton excretion exhibits characteristics typical of proton transport mediated by proton pump in frog skin, turtle bladder and mammalian kidney. The balance of pharmacological studies indicates that the \( \text{H}^+\text{ATPase} \) in fish gills is probably a plasma membrane type. The major role of the proton pump in fish gills is to facilitate sodium uptake from fresh water. Thus the external sodium level is the primary regulator of its activity and it could also be affected by external calcium or cortisol level of the fish. The proton pump in fish gills is also involved in acid-base regulation. The old model of \( \text{Na}^+\text{H}^+(\text{NH}_4^+) \) exchanger is rejected because it is energetically improbable and branchial proton excretion is resistant to 0.1 mM amiloride.
REFERENCES


