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**INTRACELLULAR pH REGULATION IN HEPATOCYTES
OF TELEOST FISH**

By

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School of Graduate Studies and Research
University of Ottawa
in partial fulfillment of the requirements for the
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Doctor of Philosophy (2000), University of Ottawa (Biology)

Title: Intracellular pH Regulation in Hepatocytes of Teleost Fish

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ABSTRACT

Teleost fish are very sensitive to changes that occur in their aquatic environment, including those that influence blood acid-base status. The intracellular pH (pH_i) of the liver is strongly influenced by the pH of circulating blood and must be able to regulate any changes that occur. The main goal of this thesis was to examine the mechanisms of pH_i regulation in hepatocytes (liver cells) isolated from different fish species.

Isolated hepatocytes were validated as an appropriate cell model system by examining intracellular and plasma ion levels in three teleost species, rainbow trout, *Oncorhynchus mykiss* (Walbaum), black bullhead, *Ameiurus melas* (Rafinesque) and American eel, *Anguilla rostrata* (Lesueur). Although the electrochemical gradients for Na^+ and K^+ were different in isolated cells as compared to the intact tissue, these gradients were directionally correct.

The mechanism of intracellular pH (pH_i) regulation was studied in trout, bullhead and eel hepatocytes using the pH-sensitive fluorescent dye BCECF. In the trout and bullhead, recovery to an acid load occurred principally by way of $\text{Na}^+\text{-H}^+$ exchange. In trout hepatocytes, an electrogenic $\text{Na}^+\text{-HCO}_3^-$ cotransporter also plays a significant role in the maintenance of steady-state pH_i and recovery from intracellular acidosis. In eel, recovery to an acid load occurred by a Cl^- -dependent mechanism. Recovery to a base load in the trout was diminished by blocking the $\text{Na}^+\text{-H}^+$ exchanger.

Partial sequences of the $\text{Na}^+\text{-HCO}_3^-$ (NBC) gene were obtained independently from trout liver and gill/kidney cDNA libraries. The fish NBC sequences were identical and showed high homology with mammalian and amphibian sequences. The most important

differences between the fish and these other sequences occurred in the large extracellular loop as well as in the DIDS binding motif.

Hypercapnia exposure resulting in decreases in blood pH significantly decreases hepatocyte pH_i . Chronic *in vivo* or *in vitro* hypercapnia did not enhance the ability of hepatocytes to recover from subsequent intracellular acidoses.

In summary, hepatocytes of all three teleost species studied appear to have the ability to regulate pH_i , though they do not exploit identical mechanisms. In addition to the role of a $\text{Na}^+\text{-H}^+$ exchanger and $\text{Cl}^-\text{-HCO}_3^-$ exchanger, a $\text{Na}^+\text{-HCO}_3^-$ cotransporter may contribute significantly to hepatocyte pH_i regulation. The primary molecular data obtained in this thesis may allow for a more complete and in depth determination of a role of the NBC in regulatory mechanisms in teleost fish.

RÉSUMÉ

Les poissons téléostéens sont très sensibles aux changements dans leurs environnements aquatiques, incluant ceux qui influencent le niveau acido-basique du sang. Le foie est fortement influencé par le pH du sang et doit avoir la capacité de régulariser son milieu intracellulaire. Le but principal de cette thèse était d'examiner le mécanisme de régulation du pH_i dans les hépatocytes de différentes espèces de poissons.

Les hépatocytes isolés ont été validés comme système expérimental approprié dans nos études en examinant les niveaux d'ions intracellulaire et les niveaux d'ions du plasma chez trois espèces de poissons téléostéens; la truite arc-en-ciel (*Oncorhynchus mykiss*), la barbotte noire (*Ictalurus melas*) et l'anguille d'Amérique (*Anguilla rostrata*). La comparaison des cellules isolées avec le foie intacte a démontré que les gradients électrochimiques du Na^+ et du K^+ sont en fait différents mais les gradients directionnels sont demeurés constants.

Le mécanisme de régulation du pH intracellulaire fut étudié chez la truite, la barbotte et l'anguille en utilisant un fluorophore sensible au pH (BCECF). Chez la truite et la barbotte, l'échangeur $\text{Na}^+ - \text{H}^+$ joue un rôle important dans la récupération du pH face à une charge acide. De plus, chez la truite, un cotransporteur $\text{Na}^+ - \text{HCO}_3^-$ électrogénique joue un rôle significatif dans la régulation du pH_i . Chez l'anguille, la récupération suite à une charge acide fut régulée par un mécanisme dépendant du Cl^- . La récupération du pH_i suite à une charge basique implique l'échangeur $\text{Na}^+ - \text{H}^+$.

Les ordres partiels du gène pour le cotransporteur $\text{Na}^+ - \text{HCO}_3^-$ (NBC) obtenu de l'ADN complémentaire du foie et d'un mélange branchie/rein de la truite était très similaire aux mêmes ordres NBC des mammifères et des amphibiens. Les différences les plus

importantes entre les poissons et les autres séquences surviennent dans les grandes loupes extracellulaires ainsi que dans les motifs pour l'attachement du DIDS. Les portions qui se chevauchent entre les séquences du foie et du mélange branchie/rein étaient identiques.

Durant l'exposition à l'hypercapnie, la baisse du pH du sang a eu un effet significatif en baissant le pH_i des hépatocytes. L'hypercapnie chronique *in vivo* ou *in vitro* n'a pas augmentée l'habileté des hépatocytes de récupérer d'une acidose intracellulaire subséquente.

En somme, les résultats montrent que les hépatocytes des espèces de téléostéens de cette étude régularisent le pH_i , par contre, les espèces utilisent des mécanismes non-identiques. En addition aux rôles connus des échangeurs $\text{Na}^+ - \text{H}^+$ et $\text{Cl}^- - \text{HCO}_3^-$ dans le mécanisme de récupération du pH_i chez les hépatocytes de la truite, un symporteur $\text{Na}^+ \text{HCO}_3^-$ semble avoir un rôle significatif. Les résultats primaires obtenus dans cette thèse peuvent aider à déterminer le rôle du NBC dans les mécanismes régulatifs chez les poissons téléostéens.

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LIST OF ABBREVIATIONS

ACZ, acetazolamide
AE, anion exchanger
AM, acetoxy methyl ester
ANOVA, analysis of variance
 β , Greek letter beta
BCECF, 2', 7'-bis-(2-carboxyethyl)-5-(and 6)-carboxyfluorescein
BSA, bovine serum albumin
CA, carbonic anhydrase
CPM, counts per minute
DIDS, 4, 4' -diisothiocyanostilbene-2, 2' - disulfonic acid
DMO, 5,5-dimethyl-2,4-oxazolidine dione
EGTA, ethylene glycol-bis (β -aminoethyl ether) N,N,N',N'-tetraacetic acid
h, hours
HCT, haematocrit
Min, minutes
n, number of animals or preparations
NBC, Na^+ - HCO_3^- cotransporter
NHE, Na^+ - H^+ exchanger
 PaCO_2 , partial pressure of arterial carbon dioxide
 PCO_2 , partial pressure of carbon dioxide
 PaO_2 , partial pressure of arterial oxygen
PE, polyethylene tubing
Sec, seconds
SEM, standard error of the mean
SITS, 4-acetamido-4' -isothiocyanatostilbene-2,2' -disulfonic acid
U, units of enzyme activity
w/v, weight per volume

CHAPTER 1

GENERAL INTRODUCTION

OVERALL RATIONALE

The physiology of the liver has been extensively studied in many vertebrate species. It is not surprising that this organ would be the focus of intensive research as it is an important metabolic tissue involved in numerous necessary functions. The ability of the liver to function to its full capacity is dependent on the proper functioning of enzyme pathways that are in turn dependent on intracellular conditions including pH. The maintenance of intracellular pH (pH_i) is key to the integrity of enzyme pathways and other systems, thus the mechanisms of liver pH_i regulation are of much interest. Unlike mammals, the blood acid-base status of teleost fish is very sensitive to their immediate environment as a result of the use of water as the respiratory medium and the high surface area and permeability of the gills. Given close contact between the liver and the circulating blood, the intracellular conditions are strongly influenced by any changes that occur in blood acid-base status. Evidence clearly shows in fish that acid-base stresses compromise the functions of crucial metabolic enzymes. Though the mechanism of pH_i regulation in mammalian liver cells has been extensively studied, information regarding similar mechanisms in lower vertebrates is limited. Prior to the publications arising from this thesis, the mechanism of pH_i regulation had been examined in liver cells (hepatocytes) of only two different teleost species, the rainbow trout *Oncorhynchus mykiss* (Walsh, 1986) and the Gulf toadfish *Opsanus beta* (Walsh, 1989), and these two systems demonstrated significant differences. The results of this thesis will hopefully provide a better understanding of the mechanisms of pH_i regulation in teleost fish hepatocytes and especially the membrane transporting components associated with this regulation.

BACKGROUND KNOWLEDGE

1. Acid-base disturbances in fish

Teleost fish exploit many different habitats that vary in salinity, pH, altitude (hydrostatic pressure) and temperature, requiring specific adaptations to suit their relative needs. Some fish are also able to move from one environment to another that vary in salinity (euryhaline fish e.g., salmon and eel). Fish have, therefore, developed mechanisms by which they can survive in their respective aquatic systems and defend against (within certain limits) physical or environmental stressors that may be encountered. Though some stressors result from human activities (e.g. toxicants, pulp effluents, acid rain), others are encountered naturally as a regular part of their lifestyles (migration, predation).

Specialised gill structures allow for efficient gas and ion exchange between the blood and the aquatic environment and are a key regulatory site for responses to different stressors (Perry and Wood, 1989; Perry and McDonald, 1993; Perry and Laurent, 1993; Laurent and Perry, 1995). Unlike mammals where the kidneys play a primary role in both osmo- and acid-base regulation, this organ plays only a secondary role in acid-base regulation in fish (Perry *et al.*, 1987). The kidneys are, however, the principal site for osmoregulation in fish. As a result of these regulatory mechanisms, the osmolarity of teleost fish plasma is kept constant (~300 mOsm) in comparison to their external environment that can vary from a few mOsm (freshwater) to 1,000 mOsm (sea water). Due to the low capacitance of water for oxygen, fish require a high ventilation rate in order to meet their oxygen demand. The countercurrent exchange at the gills results in a high PaO_2 (~140 mm Hg) and a PaCO_2 that is very low (~1-4 mm Hg) compared with that in terrestrial animals (30-45 mm Hg).

Any significant changes that occur in the pH, temperature and/or dissolved gas composition of the water can lead to changes in blood acid-base status in fish (Heisler, 1993). When a change in blood acid-base status is detected, several regulatory responses are elicited that work together during an acute or chronic disturbance with the common goal of returning the blood to a normal steady-state. The nature of the cellular responses depends on the nature

of the stress, but includes hormonal, ventilatory and molecular mechanisms. In response to an acid-base disturbance, the morphology of the gill cells (mitochondria-rich chloride cells and pavement cells) is modulated in order to enhance or diminish the movement of acid or base equivalents between the blood and the water (Perry and Goss, 1992; Goss and Perry, 1993).

The degree of blood acid-base disturbance can vary from the tonic release of low amounts of metabolic acids under resting conditions, to drastic changes in blood pH due to more severe stress. In general, these blood acid-base disturbances involve changes in the acid-base variables, pH, PCO_2 and $[\text{HCO}_3^-]$. Small changes in pH can be easily corrected by a combination of plasma buffers and ventilatory mechanisms. More severe acute or chronic stress, however, requires a greater regulatory effort. Hypercapnia (respiratory acidosis) is experienced in aquatic environments where a lack of surface gas exchange exists due to dense surface vegetation or where there is increased CO_2 production by anaerobic microbes. Since the P_aCO_2 is so low in fish, even a relatively small increase in water PCO_2 during hypercapnia may cause a significant increase in P_aCO_2 and a decrease in blood pH (Goss and Perry, 1993). The compensatory hyperventilatory regulation that occurs during hypoxia (decreased O_2) leads to an associated respiratory alkalosis (Glass *et al.*, 1990). Chronic hypoxia, however, can lead to anaerobic energy production resulting in a metabolic acidosis (Thomas and Motais, 1990). Metabolic acidosis can occur as a consequence of severe physical activity, including chasing by predators or migration against strong currents, where energy expenditure is high (Milligan and Wood, 1986). The increase in lactic acid production and its release into the blood may significantly decrease blood pH, though having no effect on PaCO_2 .

2. Liver pH_i regulation

The physiology of the liver has been intensely studied in both fish and mammalian systems as a result of its importance as a metabolic tissue. Approximately 80% of the fish liver consists of hepatocytes that are the main site of glucose storage (as glycogen), metabolic pathways (glycolysis, gluconeogenesis, ureogenesis, lipogenesis), as well as protein, bile and hormone production and detoxification processes. Unlike the lobular morphology of mammalian livers, fish livers generally have a tubular morphology consisting of cords of hepatocytes surrounding the bile canaliculus (Hampton *et al.*, 1985). The hepatocyte is organised as a classic epithelium in that it is a polarised cell exposed to blood on the basolateral side and to the bile canaliculus on their apical side (see Fig. 1-1). Mammalian and fish hepatocytes are further subdivided into two types, termed 'upstream' (close to portal vein) or 'downstream' (closer to hepatovenous circulation). This distinction is based upon their location within the liver, their pattern of enzyme activities and subcellular structures (Jungemann and Katz, 1989; Mommsen *et al.*, 1991; Ottolenghi *et al.*, 1991; Benedetti *et al.*, 1993, 1995). As a result of their specialised roles and ease of isolation, hepatocytes have been extensively utilised as model cell systems (Moon *et al.*, 1985; Mommsen *et al.*, 1994).

Like the role played by gills and kidneys in maintaining the steady-state blood osmotic, ionic and acid-base status different from that of the water, regulatory mechanisms at the cellular (hepatocyte) level are necessary to maintain homeostasis of cytosolic environments that are in turn very different from the blood (see Fig. 1-1). Different regulatory mechanisms exist in hepatocytes to regulate cell volume and pH_i (Boron, 1986; Boyer *et al.*, 1992). The intra- and extracellular (plasma) environments are osmotically balanced, though the ion compositions are different. In general, K^+ levels are high and Na^+ levels low in the cytosol, compared with their opposite concentrations in plasma. Also, the cytosol contains less Cl^- than the plasma, with a significant negative charge in the cytoplasm

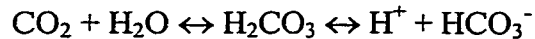
being attributed to proteins. These ion gradients are key to the function of the different membrane transporting mechanisms (Boyer *et al.*, 1992).

Since hepatocytes are in direct contact with the circulation, they are strongly influenced by the acid-base status of the blood. The ability of the liver to regulate pH_i is important as cell systems are pH sensitive. Significant changes in pH_i can have a negative impact on pH-sensitive processes (e.g. metabolic enzymes), hence affecting normal cell function. The pH_i in trout tissues exposed to extracellular acidosis is known to decrease significantly (Milligan and Wood, 1985; 1986). The return of fish liver pH_i to normal involves membrane transport proteins, intracellular buffers and metabolic acid-base control (Walsh and Milligan, 1989; Walsh and Mommsen, 1992). *In vivo* hypercapnic exposure decreased the activity of glycogen phosphorylase (GPase), while increasing the activity of the enzyme pyruvate kinase (PyK), though these effects were compensated for by catecholamines (Perry *et al.*, 1988). *In vitro* hypercapnia decreased CO_2 production and gluconeogenesis from lactate in isolated trout hepatocytes (Walsh *et al.*, 1988). In that same study, compensated hypercapnia (i.e., increased $[\text{HCO}_3^-]$) did not return metabolic activity to normal.

The mechanism of pH_i regulation in hepatocytes is now fairly well understood in fish and mammals (see reviews by Walsh and Mommsen, 1992; Strazzabasco and Boyer, 1996). Much focus has concentrated on the membrane transport proteins participating in acid and base movement across hepatocyte plasma membranes. The $\text{Na}^+\text{-H}^+$ exchanger and the $\text{Na}^+\text{-HCO}_3^-$ cotransporter work as acid extruders and the $\text{Cl}^-\text{-HCO}_3^-$ exchanger as a base extruder (the physiology and molecular biology of these transporters is described in more detail below). In mammals, a role for all three of these transporters is well documented in hepatocyte pH_i regulation (Gleeson *et al.*, 1989; Strazzabasco and Boyer, 1996). The trout hepatocyte utilises both $\text{Na}^+\text{-H}^+$ and $\text{Cl}^-\text{-HCO}_3^-$ exchange in pH_i regulation (Walsh, 1986), while the toadfish only utilises $\text{Na}^+\text{-H}^+$ exchange (Walsh, 1989). It was postulated that the

ability of toadfish to produce and excrete urea is enhanced by the retention of HCO_3^- that is a substrate of urea synthesis.

In addition to membrane transport proteins, buffers are also involved in pH_i regulation. Like all vertebrate tissues, the $\text{CO}_2\text{-HCO}_3^-$ buffer system is very important as both an intracellular and extracellular buffer in fish hepatocytes. The reversible reaction



is well known and critical in both the cytoplasm and the plasma (Henry, 1996). The $\text{CO}_2\text{-HCO}_3^-$ buffer system is key to many processes, including CO_2 excretion, HCO_3^- transport and intracellular buffering. The enzyme carbonic anhydrase (CA) catalyses the above reaction that would otherwise occur very slowly at its non-catalysed rate (Henry and Heming, 1998). In fish gill epithelial cells and in erythrocytes, CA is key to CO_2 excretion, pH regulation and ion regulation (Perry and Laurent, 1990; Henry and Heming, 1998; Tufts and Perry, 1998). In liver, intracellular CA supplies intracellular HCO_3^- as a buffer, and as a substrate for ureogenesis in the gulf toadfish (Walsh *et al.*, 1989). Non-bicarbonate buffers such as proteins and phosphates are mainly located inside the cytoplasm, and are not involved in extracellular pH regulation (Burton, 1978). The role of non-bicarbonate buffers has not specifically been examined in fish hepatocytes.

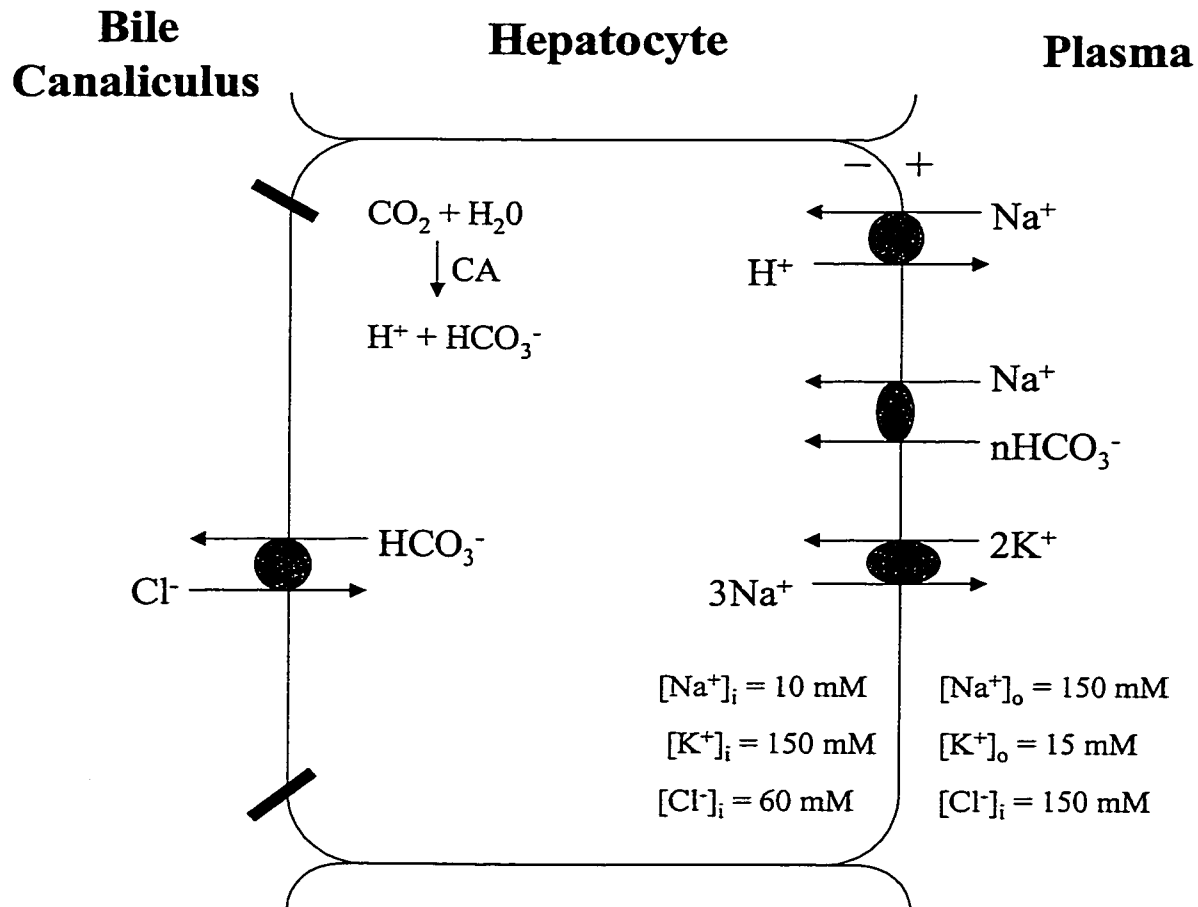


Figure 1-1. Model of the polarised hepatocyte demonstrating the mechanisms involved in pH_i regulation. Note that the Na⁺-HCO₃⁻ cotransporter is indicated, though previous experiments have only shown its presence in the mammalian cell. The relative ion concentrations in the cytosol and plasma are generalised to demonstrate the existing ion gradients across the cell membrane. This figure is based on similar diagrams by Walsh and Milligan (1989), and Walsh and Mommsen (1992).

3. Physiology and Molecular Biology of Membrane Transport Proteins

Ions and organic solutes are able to move across otherwise impermeable cell membranes by way of membrane transport proteins. These proteins are classified as either channels, pumps or carriers (uniporters, antiporters or cotransporters), and are generally specific to the substrates that they transport (Harvey and Slayman, 1994; Hediger, 1994; Nelson, 1994; Wolfersberger, 1994). The movement of molecules down their respective electrochemical gradients requires no input of energy, and is referred to as passive transport. When ions are being moved across the cell membrane against their respective electrochemical gradients, however, it is referred to as primary active transport, since a direct input of energy is required in the form of ATP hydrolysis. Secondary active transport involves the uphill transport of a solute at the expense of the electrochemical potential of another solute. Membrane transporters can consist of either protein subunits (in the case of some primary active transport) or peptide chains that form membrane-spanning domains. In either case, the tertiary protein structures form pores of specific size and charge based on the amino acid properties at the transport sites, conferring selectivity for certain solute species. The pharmacological study of membrane transport is possible by the wide array of compounds that are capable of inhibiting the movement of ions through these specific transport sites.

The relative permeability of ion channels, together with the activity of the $\text{Na}^+\text{-K}^+$ ATPase is the driving force behind the maintenance of the electrochemical gradient across the cell membrane. Under steady state conditions, the electrochemical gradient across animal cells is characterised by a large gradient for Na^+ and Cl^- entry into the cell, a large gradient for K^+ to exit from the cell interior and a negative membrane potential (E_m) across the cell membrane (inside negative). Since the majority of transport across cell membranes is driven by the electrochemical gradient, the integrity of the cell membrane is crucial. The movement of many physiologically important molecules (e.g., glucose, amino acids) or ions into or out

of the cell is dependent on cotransport or exchange with extracellular Na^+ that moves down its chemical gradient from the plasma to the cytosol.

Na^+ - H^+ exchanger (NHE gene family)

The Na^+ - H^+ exchanger (or antiporter) catalyses the passive electroneutral 1:1 exchange of Na^+ and H^+ . It is found in almost every eukaryotic cell type and is an important regulator of intracellular pH (pH_i), cell volume and transepithelial Na^+ transport (see reviews by Bianchini and Pouyssegur, 1994; Demaurex and Grinstein, 1994; Noël and Pouyssegur, 1995; Yun *et al.*, 1995; Wakabayashi *et al.*, 1997). The Na^+ - H^+ exchanger (NHE) gene family includes several isoforms that are similar in their basic structural and functional properties, but vary according to their sensitivity to the diuretic amiloride and its derivatives, their tissue distribution and their kinetic properties.

Although the physiological properties of the different Na^+ - H^+ exchangers have been known for some time, the cloning of the human Na^+ - H^+ exchanger by Sardet *et al.* (1989) allowed for the elucidation of molecular differences between known NHE isoforms and also the discovery of new isoforms. The NHE has two functional domains, a transmembrane domain (~500 amino acids), including the 12 membrane-spanning segments involved in amiloride-sensitive Na^+ - H^+ exchange, and a carboxy terminal cytoplasmic domain (~300 amino acids) involved in hormonal regulation (see Fig. 1-2). Amongst all NHE isoforms, the highest degree of homology occurs at the membrane-spanning domain. Site-directed mutagenesis of the membrane-spanning domain has shown that Na^+ and H^+ binding occurs at a specific site and that amiloride competes with extracellular Na^+ at a specific site on the exterior surface of the cell membrane.

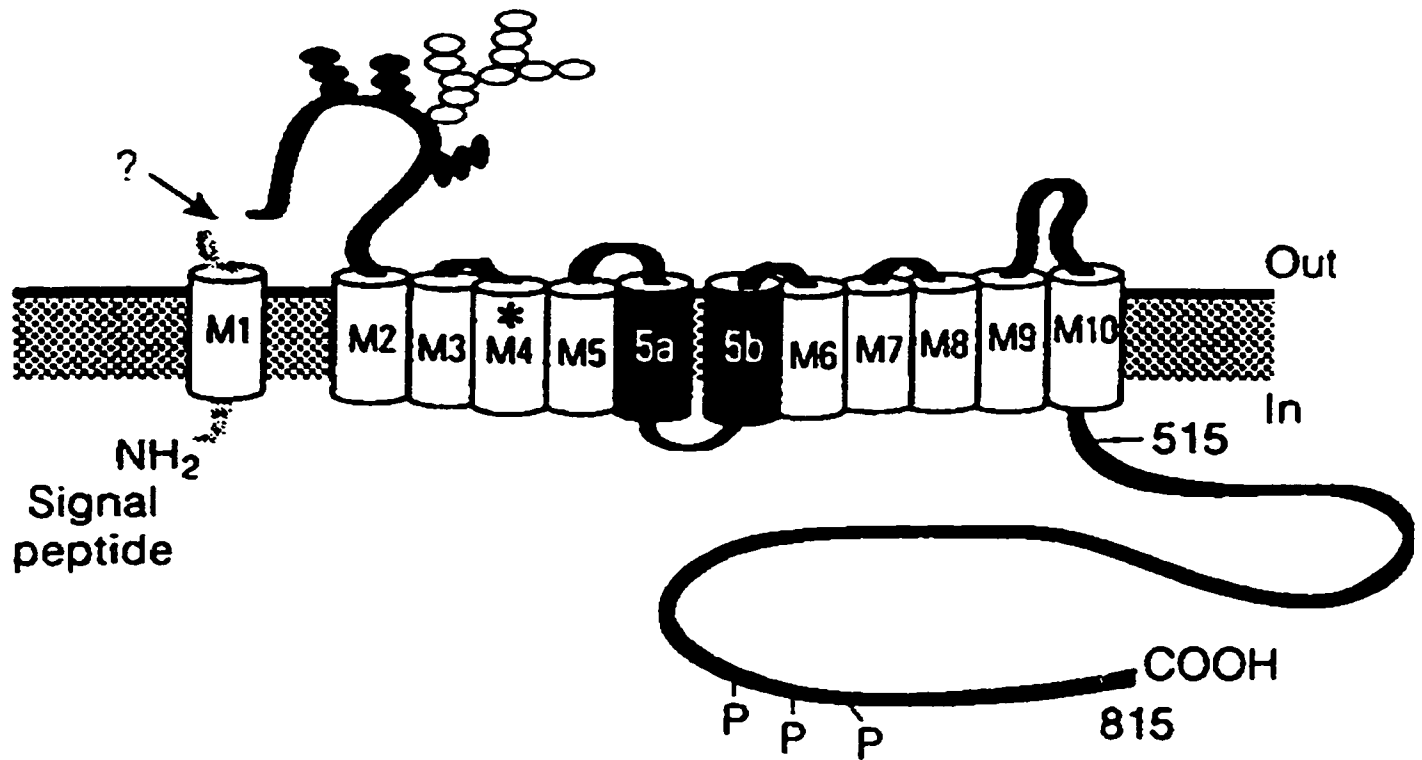


Figure 1-2. The structure of the Na⁺-H⁺ exchanger isoform NHE1. The protein is 815 amino acids long, consisting of putative membrane-spanning domains and the cytoplasmic tail. This diagram is from the paper by Noël and Pouyssegur (1995).

The $\text{Na}^+\text{-H}^+$ exchanger known as isoform 1 (NHE1) is ubiquitously distributed and is involved in pH_i and cell volume regulation. The other NHE isoforms (NHE2-5 and βNHE) differ in their structure and regulation, and are more restricted in their tissue distribution (Klanke *et al.*, 1995; Wakabayashi *et al.*, 1997). NHE-1 and NHE-2 are mainly located on the basolateral surface of polarised epithelial cells and are highly sensitive to amiloride. NHE-3, on the other hand, is distinct in that it is located mainly on the apical surface of epithelial cells in kidney, intestine and stomach and is highly resistant to amiloride or its analogues. βNHE is unique in that it exists only on the red blood cells of some species of teleost fish and is activated by cAMP following the stimulation of membrane surface β -adrenoreceptors (Borgese *et al.*, 1992; Borgese *et al.*, 1994; Malapert *et al.*, 1997).

The main regulator of NHE activity is pH_i . The exchanger has a H^+ modifier site that is sensitive to the pH of the cytosol (Wakabayashi *et al.*, 1994a). The activity of the exchanger is very low under normal cell conditions when pH_i is at its normal steady state, but is activated by a decrease in pH_i . An auto-inhibitory mechanism exists for NHE that is abolished when pH_i decreases (Wakabayashi *et al.*, 1994b). The cytosolic region of the exchanger reacts with the modifier site located on the membrane-spanning domain and leads to an increase in activity that ultimately extrudes protons until the resting pH_i values are re-established. The pH sensitivity of the exchanger is also regulated so as to turn off at higher pH_i values, thus not exceeding baseline pH_i values.

HCO_3^- Transporter Superfamily

As more membrane transporters are being discovered, strong evidence supports the existence of a bicarbonate transporter superfamily (BTS) (Romero and Boron, 1999). Several different HCO_3^- transporters exist, namely the $\text{Cl}^-\text{-HCO}_3^-$ exchanger (including Band 3 protein), the Na^+ -dependent $\text{Cl}^-\text{-HCO}_3^-$ exchanger, $\text{K}^+\text{-HCO}_3^-$ cotransporter and $\text{Na}^+\text{-HCO}_3^-$ cotransporter. Although the main distinguishing features of these transporters is their ability to transport HCO_3^- , the elucidation of their primary amino acid sequences has yielded

evidence demonstrating similarities related to their function. The first two gene families identified as branches of this superfamily are the anion exchanger (AE) gene family, including the Cl^- - HCO_3^- exchanger, and the Na^+ - HCO_3^- cotransporter (NBC) gene family. These two gene families share 30-35% homology at the protein level and are discussed in more detail below. Recently, a Na^+ driven anion exchanger (NDAE1) was cloned and characterized in *Drosophila* (Romero *et al.*, 2000).

Anion Exchanger (AE gene family)

Anion exchange occurs in all cells. Transporters belonging to the anion exchanger (AE) gene family demonstrate characteristic electroneutrality of transport, sensitivity to stilbene disulfonate antagonists, and provide linkage between the plasma membrane and the cytoskeleton. The dominant form of anion exchanger is the Cl^- - HCO_3^- exchanger that catalyzes the 1:1 electroneutral exchange of Cl^- and HCO_3^- (see reviews by Alper, 1991; Hamasaki and Okubo, 1996). In most cell types, the electrochemical gradient moves Cl^- into the cell in exchange for intracellular HCO_3^- . The band 3 protein, or AE1 is the most abundant membrane protein in the vertebrate red blood cell (erythrocyte) where it is important in increasing the CO_2 carrying capacity of the blood. The non-erythroid Cl^- - HCO_3^- exchangers work with other membrane transport systems in the regulation of pH_i , cell volume, and intracellular $[\text{Cl}^-]$. Cl^- - HCO_3^- exchangers are activated at alkaline pH_i and are inhibited at lower pH. Their location can also vary as in the renal collecting duct cells, where it is located on the basolateral membrane of the A-type acid extruding cells, and on the apical membrane of the B-type base extruding cells (Madsen and Tisher, 1986; Schuster *et al.*, 1986).

The secondary structure of the AE proteins is similar, and consists of two domains. The C-terminal domain, containing up to 14 membrane-spanning segments, catalyses the stilbene disulfonate-sensitive anion exchange (Alper, 1991). The N-terminal domain participates in the binding of the protein to intracellular and cytoskeletal proteins. The homology between the different AE isoforms (AE 1-3) is high in the membrane-spanning

domain (64-69%), with greater differences found in the cytosolic domain. Anion exchange by the AE-2 and AE-3 isoforms is activated at alkaline pH, and participates as an acid loader in pH_i regulation by extruding intracellular HCO₃⁻ in exchange for extracellular Cl⁻.

The Na⁺-HCO₃⁻ cotransporter (NBC gene family)

The Na⁺-HCO₃⁻ cotransporter (or symporter) mediates the unidirectional movement of Na⁺ and HCO₃⁻ across the cell membrane in a fashion that can be either electroneutral or electrogenic (see reviews by Boron *et al.*, 1997; Romero and Boron, 1999; Soleimani and Burnham, 2000). The protein is also distinguished by the fact that it is dependent on Na⁺ and HCO₃⁻, independent of Cl⁻ and blocked by stilbene disulfonate derivatives such as SITS and DIDS. The Na⁺:HCO₃⁻ stoichiometry is generally 1:3 or less, depending on the direction of HCO₃⁻ movement and the tissue type. The electrogenic stoichiometry that results in the movement of net negative charge out of a cell, such as in the case of the proximal tubule, is generally 1Na⁺:3HCO₃⁻ (Yoshitomi *et al.*, 1985; Soleimani *et al.*, 1987). In tissues where HCO₃⁻ is transported into the cytoplasm, such as in the liver (Fitz *et al.*, 1989, 1992; Gleeson *et al.*, 1989) or pancreas (Shumaker *et al.*, 1999), the stoichiometry is estimated to be 1:2. Clear evidence for electroneutral activity has only been found in the heart (Dart and Vaughan-Jones, 1992; Lagadic-Gossmann *et al.*, 1992). Unlike the anion exchangers, Na⁺-HCO₃⁻ cotransporters do not move anions other than HCO₃⁻ (Gritchenko *et al.*, 2000).

The Na⁺-HCO₃⁻ cotransporter was first found to play a crucial role in Na⁺ and HCO₃⁻ reabsorption from the lumen across the basolateral membrane of proximal renal tubule cells (Boron and Boulpaep, 1983; Alpern, 1985). In the pancreas, NBC plays a role in HCO₃⁻ transport from blood to the pancreatic duct (Shumaker *et al.*, 1999). It is also important as an alkaline loader in pH_i regulation of several tissues, namely brain (Bevensee *et al.*, 1997a,b), liver (Fitz *et al.*, 1989; Gleeson *et al.*, 1989) and heart (Dart and Vaughan-Jones, 1992; Lagadic-Gossmann *et al.*, 1992).

The cloning of the $\text{Na}^+\text{-HCO}_3^-$ cotransporter gene (NBC) has lead to novel findings as to the structure and function of this transporter and its relation to other HCO_3^- transporters (Romero and Boron, 1999). The protein generally consists of 1035 amino acids and has a hydropathy profile that suggests at least 10 membrane-spanning domains. Areas of this protein are well conserved amongst all the known NBC and AE isoforms, suggesting DIDS binding motifs and amino acids important for HCO_3^- transport.

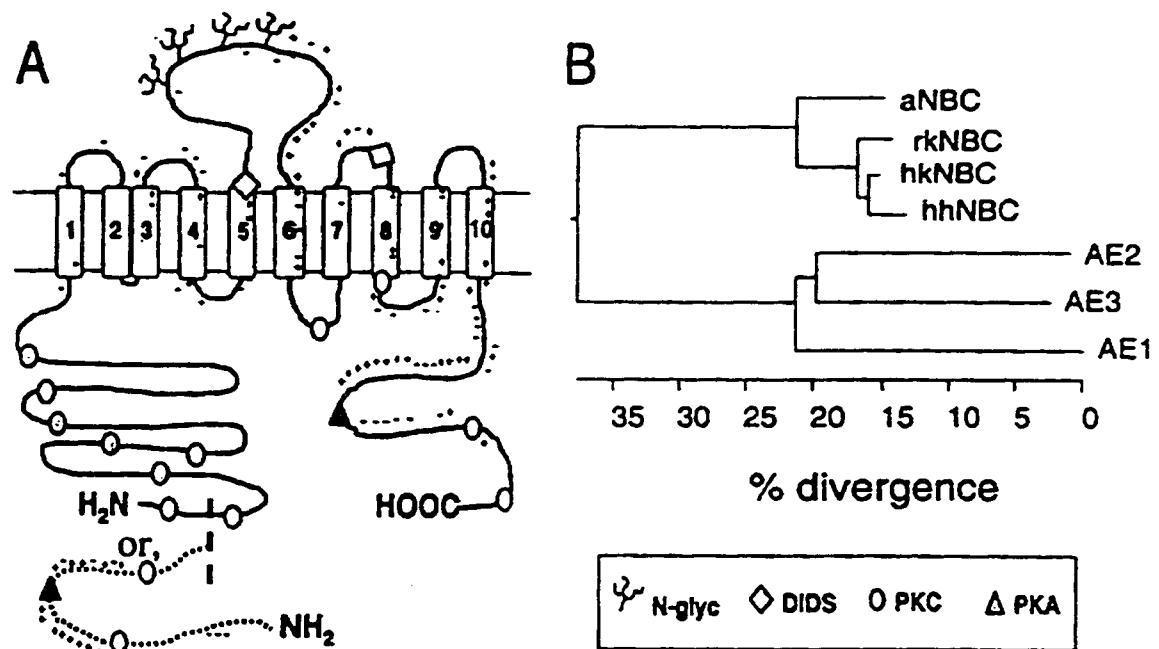


Figure 1.3. Diagram showing (A) the structure of the $\text{Na}^+\text{-HCO}_3^-$ cotransporter and (B) the evolutionary relationship between known NBC and AE isoforms. This diagram is from the review by Romero and Boron (1999).

Regulation of Membrane Transport Proteins

Acid-base disturbances have direct effects on the expression of membrane transport proteins involved in acid-base and ionic regulation. In general, strategies are employed to either enhance (up-regulate) or diminish (down-regulate) activity of certain proteins, depending on the physiological situation. In mammalian kidneys, respiratory or metabolic acidosis enhance the activity of basolateral NBC and apical NHE (increase V_{max}), in order to promote acid extrusion and base uptake (Akiba *et al.*, 1987; Presig and Alpern, 1988; Krapf *et al.*, 1991). Metabolic alkalosis, on the other hand, decreases the activity of both transporters (Akiba *et al.*, 1987). In fish, respiratory acidosis enhances the expression of gill H^+ -ATPase that is involved in acid-secretion (Sullivan *et al.*, 1995, 1996; Perry *et al.*, 2000). The expression of these transporters is determined either by changes in their activity, or by changes in mRNA expression.

HYPOTHESESE FOR THE THESIS

GENERAL HYPOTHESIS: Teleost hepatocytes regulate intracellular pH through the action of membrane transport mechanisms.

CHAPTER 2: The hepatocyte isolation procedure, by collagenase digestion, causes an intracellular ion disturbance. The chemical gradient across the plasma membrane of isolated hepatocytes will be different from those in the intact liver.

CHAPTER 3: The mechanisms of hepatocyte pH_i regulation will differ between teleost species that live in differing environments.

CHAPTER 4: In addition to the $\text{Na}^+\text{-H}^+$ exchanger, another Na^+ -dependent mechanism, likely a $\text{Na}^+\text{-HCO}_3^-$ cotransporter, plays an active role in pH_i regulation of the trout hepatocyte.

CHAPTER 5: Exposure of trout hepatocytes to hypercapnia (respiratory acidosis) will enhance the ability of the cell to regulate pH_i following subsequent acid loads. The increased ability to regulate pH_i is due to an increase in the expression of pH_i regulatory proteins.

CHAPTER 6: The $\text{Na}^+\text{-HCO}_3^-$ cotransporter gene (NBC) will be expressed in the trout liver.

CHAPTER 2
INTRACELLULAR ION LEVELS IN ERYTHROCYTES AND HEPATOCYTES
ISOLATED FROM THREE TELEOST SPECIES

Taken from:

Furimsky, M., Perry, S.F. and Moon, T.W. (1999). Intracellular ion levels in erythrocytes and hepatocytes isolated from three teleost species. *J. Fish Biol.* **55**: 1064-74.

ABSTRACT

Plasma and intracellular ion levels in liver tissue, isolated hepatocytes and erythrocytes were determined in three teleost species, rainbow trout, *Oncorhynchus mykiss* (Walbaum), black bullhead, *Ameiurus melas* (Rafinesque) and American eel, *Anguilla rostrata* (Lesueur). The purpose of this study was to determine whether the isolation from their respective tissues affects transmembrane gradients in cells in suspension owing to disruption of intracellular ion levels. Intracellular concentrations of Na^+ and K^+ were similar ($\sim 75 \text{ mmol l}^{-1}$) in rainbow trout hepatocytes directly following isolation by collagenase digestion, but partial recovery occurred over 6 hours with K^+ levels increasing to 110 mmol l^{-1} and Na^+ levels decreasing to 42 mmol l^{-1} . Black bullhead hepatocytes exhibited higher intracellular concentrations of K^+ (90 mmol l^{-1}) than Na^+ (55 mmol l^{-1}) with no recovery occurring over six hours following cell isolation. Concentrations of Na^+ , K^+ and Cl^- in eel hepatocytes were similar ($\sim 55 \text{ mmol l}^{-1}$) following isolation, with no recovery occurring over time. Erythrocytes from all species apparently did not experience an intracellular ion imbalance following isolation as indicated by high K^+ levels ($\sim 140 \text{ mmol l}^{-1}$) and low Na^+ levels ($<40 \text{ mmol l}^{-1}$) during the entire 24 h monitoring period. Although hepatocytes from all species exhibited an ion imbalance post-isolation, comparison of their *in vitro* intracellular Na^+ and K^+ concentrations to those in plasma demonstrated that directionally correct ion gradients still exist across the cell membrane, albeit different than those that would be found in the tissue *in vivo*.

INTRODUCTION

The maintenance of an appropriate electrochemical gradient across the plasma membrane is essential in maintaining proper cell function. The intracellular environment is kept at a steady-state by the actions of different membrane proteins (channels, carriers and pumps) that act to move ions across the otherwise impermeable lipid bilayer. This includes the maintenance of intracellular pH that is important to cytosolic enzyme function. Though some membrane proteins (e.g. $\text{Na}^+\text{-K}^+$ ATPase) participate in maintaining the ion gradient, others (e.g. $\text{Na}^+\text{-H}^+$ and $\text{Cl}^-\text{-HCO}_3^-$ exchangers) rely on the ion gradient for their function.

In freshwater fishes, transmembrane ion gradients are key to several physiological processes. Gill cells are exposed to different ion gradients across their apical and basolateral surfaces as a result of their exposure to water and blood, respectively, requiring different membrane transport mechanisms for intracellular and blood ionic balance (Perry, 1997). With Na^+ concentrations generally high and K^+ concentrations low in fish plasma compared with those in the cytosol of different tissues, these gradients serve important functions in the maintenance and adjustment of osmoregulatory status of cells (Bianchini *et al.*, 1991; Cossins and Gibson, 1997). Though ionic gradients are generally maintained across basolateral membranes, they can also be influenced by different environmental and chemical factors (Albers and Goetz, 1985; Fuchs and Albers, 1988; Morgan and Potts, 1995).

In vitro model cell systems, including erythrocyte and hepatocyte preparations, have been intensively used in fish physiology. Studies using isolated erythrocytes and hepatocytes have addressed many physiological processes. These include membrane transport (erythrocytes: Garcia-Romeu *et al.*, 1991; Cossins and Gibson, 1997; hepatocytes: Bianchini *et al.*, 1991; Fossat *et al.*, 1997), metabolism (erythrocytes: Soengas and Moon, 1995;

hepatocytes: Peterson *et al.*, 1987; Walsh *et al.*, 1988; Pereira *et al.*, 1995) and cell signalling (erythrocytes: Perry *et al.*, 1996; hepatocytes: Fabbri *et al.*, 1995).

If isolated cells are in an “unhealthy” state or have experienced cellular injury, alterations in intracellular ion levels may occur. In hepatocytes exposed to anoxia *in vitro*, intracellular levels of strong ions such as Ca^{2+} , Mg^{2+} and Na^{+} increase, whereas K^{+} decreases (Berger *et al.*, 1989; Gasbarrini *et al.*, 1990). The increase in intracellular Na^{+} is an important characteristic of cellular injury following ATP depletion, demonstrating that the mechanism for active Na^{+} transport (the Na^{+} - K^{+} ATPase) is no longer able to remove intracellular Na^{+} (Carini *et al.*, 1995). The mechanical stress experienced by hepatocytes during cell isolation also can affect cytosolic content. In hepatocytes, K^{+} permeability (leakage to the medium) is an issue that has been addressed along with other indicators of cell health (Mommensen *et al.*, 1994). Intracellular amino acids are also depleted following isolation (Seglen, 1976), indicating some stress is experienced by hepatocytes leading to membrane instability and leakiness.

Despite these findings, it is often taken for granted on the basis of established protocols that the cells commonly used in experimentation are in a healthy state. The purpose of this experiment was to examine the effects of cell isolation on ion levels in erythrocytes and hepatocytes of rainbow trout, black bullhead and American eel, and to determine whether isolated cells *in vitro* are able to regulate ions to the levels found in the animal *in vivo*. These two different cell types served the purpose of an intercellular comparison and the examination within each species of the effects of direct removal of cells (erythrocytes) and cell isolation by collagenase digestion of a tissue (hepatocytes). In addition, plasma ion levels

of the three species were measured, as were intracellular ion levels in hepatocytes as compared to liver tissue.

MATERIALS AND METHODS

Experimental Animals

Rainbow trout, *Oncorhynchus mykiss* (Walbaum), weighing 200 to 300 g were obtained from Linwood Acres Trout Farm (Campbellcroft, Ontario, Canada). Black bullheads, *Ameiurus melas* (Rafinesque), weighing 200 to 350 g were obtained from the State University of New York at Brockport (Brockport, NY, USA). American eels, *Anguilla rostrata* (Lesueur), weighing 300 to 450 g were obtained from the St. Lawrence River by a commercial fisherman (Lancaster, Ontario, Canada). Rainbow trout and black bullhead were transported to the University of Ottawa in oxygenated water and eels were transported on ice. Fish were maintained on a 12L:12D photoperiod in fiberglass aquaria (water volume 500 l) supplied with flowing, aerated and dechloraminated City of Ottawa tap water ($[Na^+] = 0.10 \text{ mmol l}^{-1}$, $[Cl^-] = 0.15 \text{ mmol l}^{-1}$, $[Ca^{2+}] = 0.35 - 0.40 \text{ mmol l}^{-1}$, $[K^+] = 0.03 \text{ mmol l}^{-1}$, pH 7.7 - 8.0). Trout and eels were kept at a water temperature of 15 °C, whereas black bullheads were kept at 20 °C. Trout and bullheads were fed a commercial fish chow diet daily and eels were not fed, but were used within 3 months of their arrival. Fish were held under these conditions for at least 4 weeks prior to experimentation. All experiments were performed in late summer and early fall.

Blood Sampling

Trout and bullhead blood was removed by caudal puncture using needles primed with saline containing 6.25 U/ml of the sodium salt of heparin (Sigma). The saline used for all blood washes and incubations was Hanks' medium (in mmol l⁻¹: NaCl, 136.9; KCl, 5.4; MgSO₄, 0.8; NaHCO₃, 5; Na₂HPO₄, 0.33; KH₂PO₄, 0.44; Hepes, 5; Na-Hepes, 5) containing 1.5 mmol.l⁻¹ CaCl₂, 3 mmol l⁻¹ glucose, 2 mmol l⁻¹ alanine and 2 mmol l⁻¹ lactate. The pH of all experimental media was adjusted to 7.9 at 20 °C. Media were not gassed prior to or during experimentation, although PO₂ and PCO₂ were consistently found to be 144 and 0.75 Torr, respectively. Eel blood was collected post-decapitation from the trunk into a beaker containing the same heparinised saline as above. Blood was transferred to 15 ml capacity tubes and centrifuged (2 min, 1500 rpm; Sorvall RC centrifuge with SS-34 rotor) at 4 °C. Following the removal of plasma, erythrocytes were rinsed twice by centrifugation with non-heparinised saline removing the buffy coat layer in the process. Erythrocyte cell suspensions were kept refrigerated at 4 °C until used during the 24 h experimental period.

Plasma Ion Contents

Plasma osmolarity was determined using a vapour pressure osmometer (Wescor, Inc.) immediately following collection. Na⁺, K⁺ and total calcium (Ca_{total}) concentrations were determined using an atomic absorption flame spectrometer (Varian, SpectrAA-250 Plus) and Cl⁻ concentrations were determined spectrophotometrically (Milton Roy Spectronic 1001 Plus) (Zall *et al.*, 1956). Strong ion difference (SID) was calculated by determining the difference between strong cations (Na⁺, K⁺ and Ca²⁺) and strong anions (Cl⁻). The

concentration of ionic calcium (Ca^{2+}) was calculated as being 50% of Ca_{total} , which is an approximation based on values in rainbow trout blood (Andreasen, 1985).

Hepatocyte Isolation

Rainbow trout and black bullheads were killed by a blow to the head, while eels were decapitated. Following blood sampling, hepatocytes were isolated according to the method of Mommsen *et al.* (1994). All solutions are modifications of complete Hanks' medium (in mmol l^{-1} : NaCl, 136.9; KCl, 5.4; MgSO_4 , 0.8; NaHCO_3 , 5; Na_2HPO_4 , 0.33; KH_2PO_4 , 0.44; Hepes, 5; Na-Hepes, 5). The pH of all experimental media was adjusted to 7.9 at room temperature (20 °C). A cannula was inserted into the hepatic portal vein and the connection between the liver and the heart was cut to relieve vessel pressure. Blood was then cleared from the liver by perfusion with a calcium free rinsing medium containing 1 mmol l^{-1} EGTA in Hanks' medium for 5 min. Liver digestion occurred enzymatically using collagenase (Sigma type IV, 7.5 mg per 50 ml^{-1} Hanks' for trout and bullhead and 13 mg per 50 ml^{-1} for eel). Collagenase perfusion of the liver occurred *in situ* in trout and bullhead. For eel, the liver was removed post-cannulation and collagenase perfusion was performed on a watch glass. Liver digestion was indicated by a softening of the liver and dispersal of the tissue, at which time perfusion with collagenase was terminated. Livers were then minced using a razor blade and filtered through a 253 and a 73 μm nylon mesh, respectively. The initial cell suspension was added to a 30 ml capacity centrifuge tube and washed by centrifugation (3 min, 1500 rpm in a Sorvall RC centrifuge with SS-34 rotor) at 4 °C. Cells were finally suspended and incubated in Hanks' buffer containing 1.5 mmol l^{-1} CaCl_2 , 3 mmol l^{-1} glucose, 2 mmol l^{-1} alanine, 2 mmol l^{-1} lactate and 1% bovine serum albumin (ICN, clinical reagent grade, 98% fat free). Cells were counted using a Bright-Line haemocytometer (Spencer) and

viabilities were determined using the trypan blue exclusion method (Mommensen *et al.*, 1994). Cell populations in which viabilities were less than 70% post-isolation were discarded.

Cell ion Contents

Cell ion contents were measured according to Garcia-Romeu *et al.* (1991) with slight modifications. Cell samples were collected from the suspension at various times following isolation (for hepatocytes) or following removal from the fish (for erythrocytes). The hematocrit (cytocrit for hepatocytes) was determined by centrifuging cells in micro-hematocrit capillary tubes (VWR). Cell concentrations were modified so that a hematocrit or cytocrit of approximately 25% was used to determine ion concentrations. Hepatocytes or erythrocytes were removed from the suspension using a 1 ml syringe fitted with an 18 gauge needle, then emptied into thin teflon tubing with a capacity of 0.1 ml and then centrifuged at 10,000 rpm for 10 min in a Sorvall RC refrigerated centrifuge. All experiments were performed in triplicate. The densely-packed cell pellet was cut from the tube using a razor blade and the wet solids were pushed into a pre-weighed aluminium weigh boat. Cell wet weights were determined, and the cells were allowed to dry overnight in an oven at 50 °C. Dry weights were determined and weigh boats containing cell solids were placed into 5 ml of distilled water and allowed to sit for 24 h. Perchloric acid (final concentration 2%) was added to the resultant fluid to precipitate protein. The fluid was centrifuged, the supernatant was collected and diluted for the determination of ion contents. Na^+ and K^+ contents were determined using flame spectrometry and Cl^- was determined using colourimetry, as noted above.

Extracellular Water and Ion Determinations

Contamination of cell pellets by the extracellular medium was measured using radio-labelled polyethylene glycol (^{14}C -PEG) (Dupont, Boston, MA.). The percentage of extracellular water was determined by comparing the radioactivity in the cell pellet to that in the supernatant following a 10 min incubation and centrifugation at 10,000 rpm. Because each tube contained a volume of 0.1 ml, an approximation of the volume of cells was determined based on the cytocrit or hematocrit, with each percent representing 1 μl of fluid volume. The extracellular water contamination in hepatocyte pellets varied between species (trout, 20%; bullhead, 15%; eel, 25%) due to differences in cell morphology and packing properties. As a result of the packing properties of the oval-shaped erythrocytes, the extracellular water contamination of pellets for all species was below 1%, so no correction factor was applied. The ion content of the determined volume of medium was obtained from a standard curve constructed with different volumes of medium placed on weigh boats, dried and processed as above for the cell samples. Corrections were made for the Na^+ and K^+ contribution of the extracellular fluid from values determined from the standard curve and Cl^- was corrected by subtracting the same value determined for Na^+ , since concentrations of both ions are almost identical in the incubation medium. Ion concentrations were determined as per L of cell water since cell volumes were known.

Tissue Ion Contents

Livers were perfused with an approximately isosmolar (300 mOsm l⁻¹) solution of sucrose until they were clear of blood (~ 30 seconds maximum). Three small pieces of tissue (~150 mg) were immediately cut from random areas of the liver. Samples were placed in pre-weighed aluminium weigh boats and allowed to dry overnight at 50 °C. The mass of dry tissue solids was determined and the same procedure was followed as above for the determination of cell ions. Ion concentrations were determined as per g of dry tissue since the actual volume could not be accurately estimated as a result of tissue redistribution by sucrose perfusion.

Statistics

All data are presented as the mean $\pm$ one standard error of the mean (SEM). Statistical differences were determined by performing either a Student's t-test, or a one way ANOVA followed by the Student-Newman-Keuls test for the pairwise comparison of means ($p < 0.05$).

RESULTS

Ion levels in plasma of the three species are presented on Table 2-1. Plasma of all species displayed characteristic high Na^+ and low K^+ levels, although there were some inter-specific differences; notably the lower Na^+ concentration and osmolarity of bullhead plasma and the higher Cl^- levels in trout blood. Also of note is the larger strong ion difference (SID) (Farrell and Lutz, 1975) in eel plasma.

Erythrocytes were analysed at 2, 6 and 24 hours post-removal in order to determine if any changes occurred during the normal rest period often used before experimentation with erythrocytes. Erythrocytes removed from all 3 species demonstrated high K^+ and low Na^+ concentrations and appropriate Cl^- concentrations that did not change over a 24 h period (Fig. 2-1) with the exception of the eel which displayed a slight change in intracellular $[\text{K}^+]$ after 24 h. The only significant species difference was a lower intracellular Na^+ concentration in trout erythrocytes compared with the other two species (Fig. 2-1).

Following the isolation of hepatocytes from the different species, ion levels were monitored over the course of 6 h, the period within which experimentation with freshly isolated hepatocytes is routinely undertaken. In trout hepatocytes, Na^+ levels were initially slightly lower than K^+ , but decreased over time as K^+ increased (Fig. 2-2). The Na^+ and K^+ levels were significantly changed after 4 and 6 h from the first measurement taken 30 min post isolation. In bullhead hepatocytes, intracellular K^+ was higher than Na^+ , and no change occurred over the 6 h experimental period (Fig. 2-2). In eel hepatocytes, Na^+ and K^+ concentrations were similar and did not change over time (Fig. 2-2). The levels of Cl^- did not change over time in any species.

Table 2-1: Ion levels, strong ion difference (SID) and osmolarity in plasma of three species of teleost fish.

	Rainbow trout (n = 11)	Black bullhead (n = 7)	American eel (n = 6)
Na ⁺ (mmol l ⁻¹)	135 ± 3	119 ± 4 *	146 ± 5
K ⁺ (mmol l ⁻¹)	5.68 ± 0.64 #	4.29 ± 0.51	3.75 ± 0.28 #
Cl ⁻ (mmol l ⁻¹)	133 ± 3 *	112 ± 4	116 ± 3
Ca _{total} (mmol l ⁻¹)	1.53 ± 0.04 #	1.76 ± 0.15	1.97 ± 0.10 #
SID (mmol l ⁻¹)	7.6 ± 2.2	12.5 ± 3.6	32.5 ± 5.4 *
Osmolarity (mOsm)	312 ± 3 *	274 ± 4 *	330 ± 6 *

Data are presented as the mean ± SEM.

A one way ANOVA followed by the Student-Newman-Keuls test for a pairwise comparison of means was performed.

* indicates a significant difference from the two other species. # indicates a significant difference between the two marked species.

Figure 2-1. Intracellular ion concentrations (in mmol l⁻¹) in erythrocytes following isolation from the different teleost species. Samples were collected at 2, 6 and 24 h post-isolation. Data are presented as the mean $\pm$ SEM ($n = 6$). * indicates a significant difference from ion levels at 2 hours post-removal.

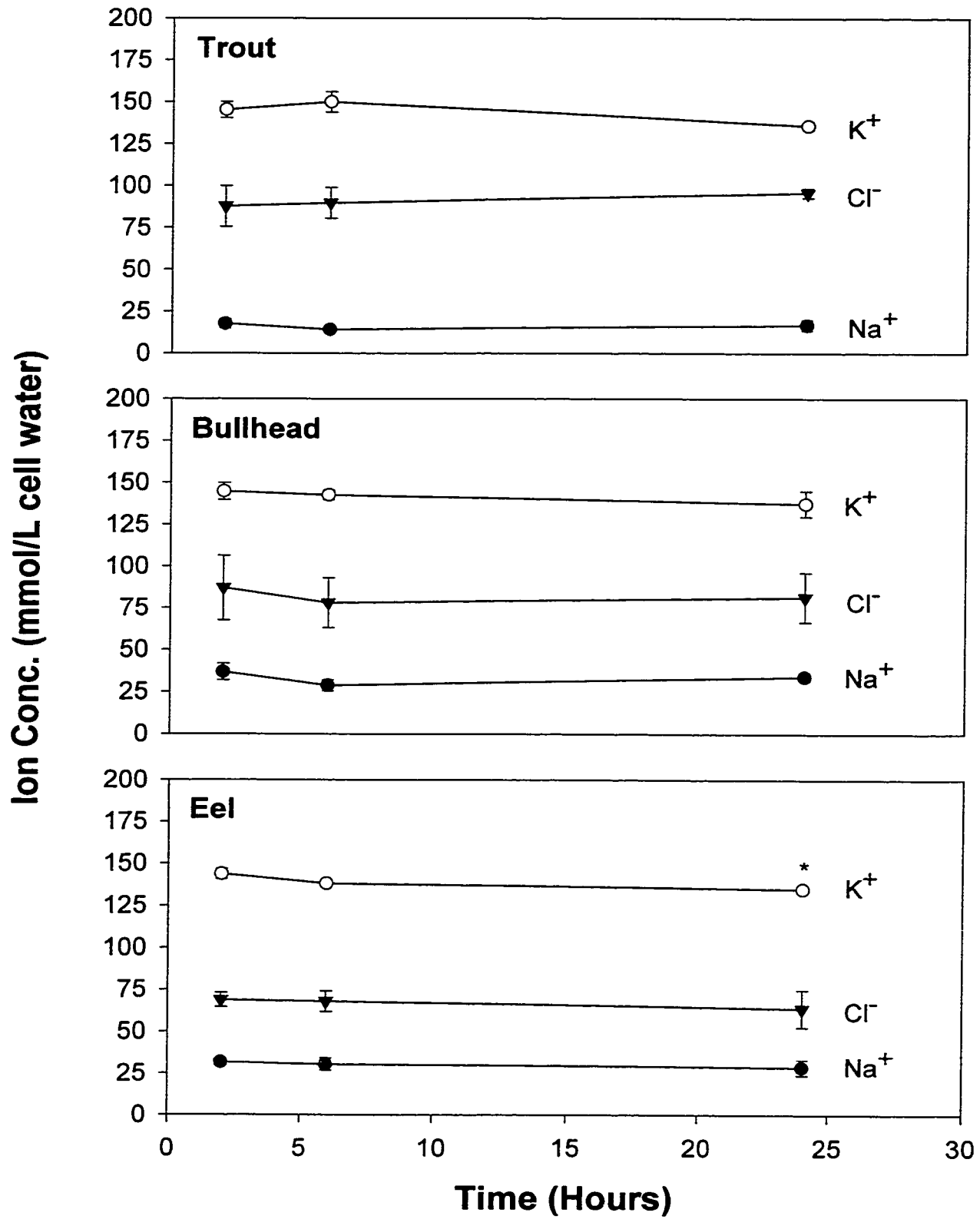
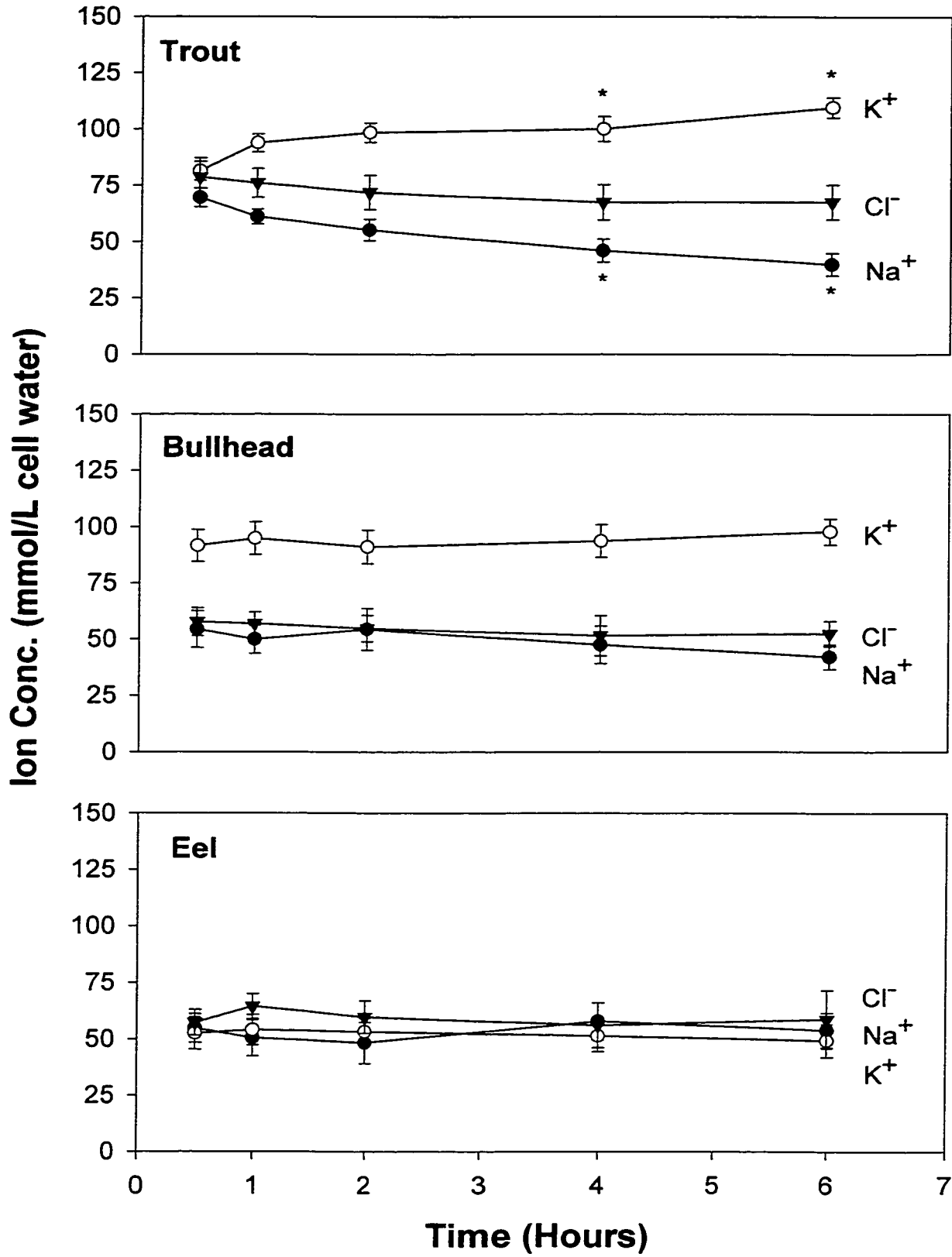


Figure 2-2. Intracellular ion concentrations in hepatocytes following isolation from the livers of three teleost species. Samples were collected at 30 min, then at 1, 2, 4 and 6 h post-isolation. Data are presented as the mean $\pm$ SEM ($n = 6$). * indicates a significant difference from ion levels at 30 min post-isolation.



As a routine procedure, hepatocyte viabilities were determined post-isolation using the trypan blue exclusion method (see Mommsen *et al.*, 1994). Following a 2 hr rest period, hepatocytes isolated from bullhead demonstrated high viabilities (~95%), followed by eels (~85%) and trout (75-80%).

The ion levels in liver tissue from the 3 species were determined in order to compare with isolated cells (Fig. 2-3). Since different experimental procedures were used for liver tissue and hepatocytes and different units of concentration were obtained, this necessitated the comparison of $K^+ : Na^+$ and $K^+ : Cl^-$ ratios (Table 2-2). The ratios observed in the intact organ demonstrate that the intracellular levels of K^+ are higher than Na^+ in all species, though this difference is decreased in trout and eel *in vitro*.

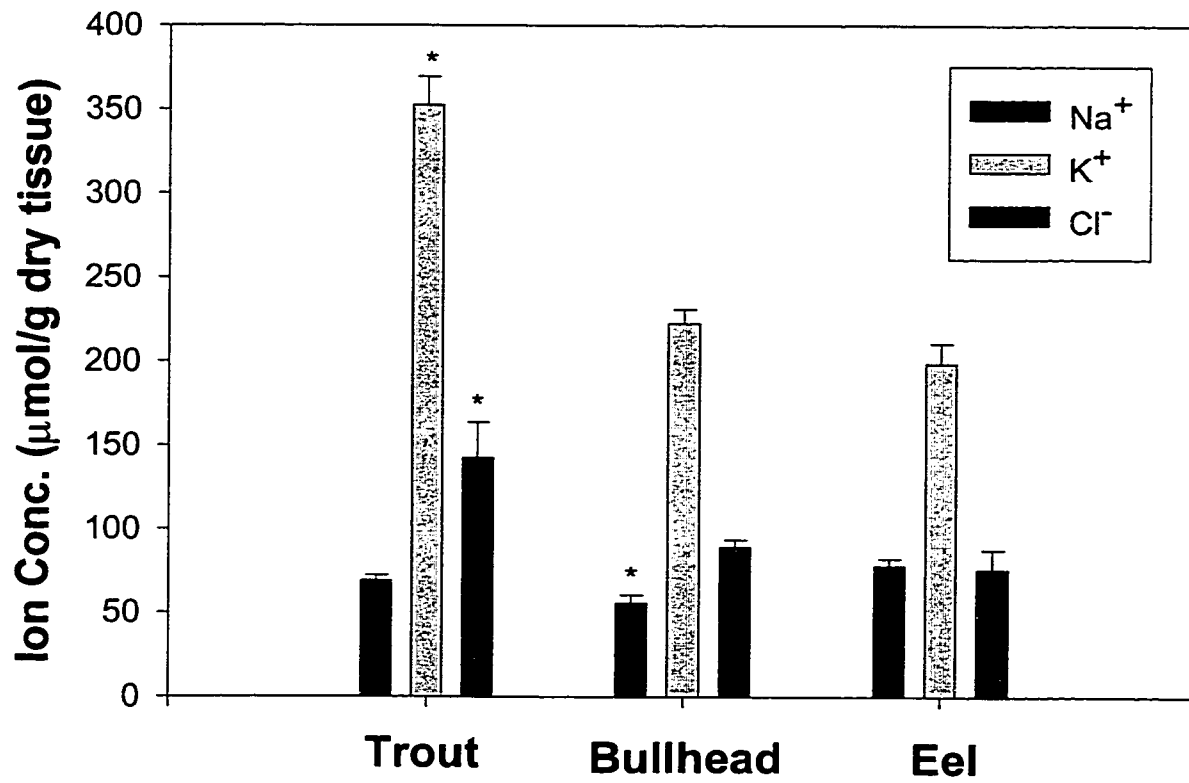


Figure 2-3. Ion levels in liver tissue of the three species of fish. Data are presented as the means $\pm$ SEM ($n = 6$). * indicates a difference from the two other species.

Table 2-2: A comparison of the ratios of strong ions in liver tissue and hepatocytes (6 h post isolation).

	$K^+:Na^+$			$K^+:Cl^-$		
	<u>Tissue</u>	<u>Cells (0.5 h)</u>	<u>Cells (6 h)</u>	<u>Tissue</u>	<u>Cells (0.5 h)</u>	<u>Cells (6 h)</u>
Trout	5.12 ± 0.14	$1.22 \pm 0.08 *$	$3.14 \pm 0.23 * \#$	2.85 ± 0.59	$1.07 \pm 0.10 *$	$1.70 \pm 0.12 * \#$
Bullhead	4.16 ± 0.39	$1.88 \pm 0.30 *$	2.64 ± 0.49	2.54 ± 0.16	$1.56 \pm 0.11 *$	1.90 ± 0.29
Eel	2.58 ± 0.15	$1.31 \pm 0.32 *$	$1.32 \pm 0.23 *$	3.06 ± 0.64	$1.11 \pm 0.29 *$	$1.07 \pm 0.25 *$

Data are presented as the mean $\pm$ SEM ($n = 6$).

* indicates a significant difference between cell and tissue ion ratios as determined by a Student's t-test ($p < 0.05$). # indicates a difference in ratios between cells at 30 min post-isolation and 6 h post-isolation.

DISCUSSION

This study demonstrates the behaviour of intracellular ions in erythrocytes and hepatocytes of three different teleost species following commonly used isolation procedures. The typical incubation and experimental protocols involving the two cell types were closely followed in order to represent the ionic state of the cells during actual experimentation. The results demonstrate that the effects of the isolation procedure on intracellular ion levels is tissue (i.e., liver versus blood) and species dependent. When cells are isolated from their respective tissues, transmembrane gradients can be disrupted.

The straightforward procedure of isolating erythrocytes from blood by centrifugation and washing has little effect on their intracellular ion levels in any of the species used in our study. The intracellular levels of K^+ are high and those of Na^+ are low as would be expected (Garcia-Romeu *et al.*, 1991) and change very little over the 24 h test period. When hepatocytes are isolated from liver tissue, the cells must undergo a more stressful mechanical isolation procedure, but a time period termed “metabolic rest” does allow for some recovery from the stresses incurred. In particular, cells can replenish ATP levels when incubated in a medium containing glucose, amino acids and BSA (Mommensen *et al.*, 1994) as occurred in this study. The enzyme digestion using collagenase attacks collagen that holds the hepatocytes together and this disruption could affect the properties of the cell membrane that in turn may influence the intracellular environment. During the isolation period, cells are also deprived of extracellular Ca^{2+} as a result of perfusion with solutions that are free of Ca^{2+} and that contain the Ca^{2+} chelator EGTA. Since Ca^{2+} is necessary for mechanisms regulating membrane transport, its absence may be a factor influencing the observed ion imbalances. If cell iono-regulatory mechanisms are disrupted and the plasma membrane becomes “leaky”,

the plasma or buffer ions may have an influence by increasing intracellular Na^+ and decreasing K^+ levels. This is supported by a previous study in which the disaggregation of trout liver tissue caused the depolarisation of cells with a loss of intracellular K^+ and normal K^+ uptake did not occur despite a functional Na^+-K^+ ATPase (Eddy *et al.*, 1995). Despite any negative effects that collagenase digestion might have on isolated cells, the advantages of its employment may outweigh its disadvantages. An alternative collagenase-free method of cell isolation used by Foster and Moon (1993) yielded sea raven hepatocytes with lower metabolic activity, and lower sensitivity and responsiveness to hormones than those isolated with collagenase. Ion levels, unfortunately were not investigated by these authors. The incubation buffer used for hepatocytes was identical for all species, so even though extracellular ions could influence those in the cytosol it would not explain the species differences observed in Figure 2-2.

The intracellular ion levels observed in trout hepatocytes 30 min post-isolation showed that Na^+ levels were higher and K^+ levels were lower than would be expected from the intact liver values (Fig. 2-2). If hepatocytes are more permeable following isolation as suggested by Seglen (1976) and Mommsen *et al.* (1994), extracellular ions may indeed influence cytosolic levels. Recovery did occur over the course of the 6 h period following isolation, with Na^+ levels decreasing and K^+ levels increasing. Only in trout hepatocytes was it obvious that intracellular ion levels were being partially re-established, with an important role likely being played by the Na^+-K^+ ATPase that maintains the electrochemical gradient under normal conditions. In bullhead hepatocytes, K^+ levels remained higher relative to those of Na^+ during the 6 h monitoring period without any change occurring (Fig. 2-2), whereas eel ion levels remained the same (Fig. 2-2). The reasons for the lack in intracellular ion recovery

in eel and bullhead hepatocytes are not clear, but several explanations may be proposed. It is possible that eel and bullhead are unable to regulate ion levels back to “normal” as is occurring in the trout, or else this regulation may be much slower in eel and bullhead. It is also possible that eel and bullhead hepatocytes do not experience the same mechanical stress during the isolation process as trout, as indicated by their respective membrane integrities determined by trypan blue exclusion (see Results). The ion levels observed in bullhead and eel hepatocytes are likely those that are established in the isolated cells. The relative amounts of intracellular Na^+ , K^+ and Cl^- ions differ between isolated hepatocytes (Fig. 2-2) and intact liver tissue (Fig. 2-3) of all species, meaning that an ionic imbalance may be a common occurrence following the cell isolation procedure. The liver tissue ion levels were determined immediately following the clearance of blood by perfusion with an isosmolar sucrose solution, so redistribution of tissue ions should not have been significant. The comparison of tissue to cell ion levels should have been representative of those in the intact organism versus those in the individual cells in suspension (Table 2-2).

The ion gradients that exist in a tissue *in vitro* can differ from those in the intact tissue (Table 2-2), but can still be appropriate for the proper function of particular membrane transport systems. The Na^+/H^+ exchanger and the $\text{Cl}^-/\text{HCO}_3^-$ exchanger, which are known to actively participate in pH_i regulation of teleost hepatocytes (Walsh, 1986; Walsh, 1989; Walsh and Mommsen, 1992), can only function if appropriate Na^+ and Cl^- gradients exist. Extracellular Na^+ and Cl^- concentrations are significantly increased compared with those of the cytosol. In a recent study performed in our lab, hepatocytes of all three species examined in this study were successfully employed in the study of pH_i regulation showing functional Na^+/H^+ and $\text{Cl}^-/\text{HCO}_3^-$ exchanger activity (Chapter 3; Furimsky *et al.*, 1999a). In the same

study, baseline pH_i values were similar to those that would be found in the liver *in vivo*. The $\text{K}^+:\text{Na}^+$ ratio in trout hepatocytes, though different from that in the tissue, is almost identical to that in a study by Bianchini *et al.* (1988), suggesting that the ion imbalance following cell isolation is typical of trout hepatocytes. Though an imbalance existed immediately following isolation in bullhead, $\text{K}^+:\text{Na}^+$ and $\text{K}^+:\text{Cl}^-$ ratios in tissue and hepatocytes at 6 hr post-isolation did not differ. Regardless of the ion imbalances that may exist, eel hepatocytes following isolation have the ability to function metabolically (Foster and Moon, 1986). When comparing the ionic composition of the plasma of the three species examined (Table 2-1) to the hepatocyte intracellular ion levels determined (Fig. 2-2), an appropriate chemical gradient exists for Na^+ , K^+ and Cl^- , though it is different to that existing in the intact organism.

Though isolated cells in suspension are routinely used in labs following well-established protocols, it is also important to be confident in the integrity of the respective experimental model. The trypan blue exclusion method provides a good indication of membrane integrity, measurement of ATP levels in cells indicates the metabolic state of the cells and determination of lactate dehydrogenase (LDH) leakage shows the leakiness of the cell membranes to intracellular enzymes. It is a challenge to duplicate exact *in vivo* physiological conditions *in vitro* when using isolated cells, but hepatocyte cell suspensions have proven in past studies to be a reliable cell model. It is, however, important to be aware of the ionic state of cells especially when transmembrane gradients are studied, or when studies are being performed in which electrochemical ion gradients play an important role.

CHAPTER 3
INTRACELLULAR pH REGULATION IN HEPATOCYTES ISOLATED FROM THREE
TELEOST SPECIES

Taken from:

Furimsky, M., Moon, T.W. and Perry, S.F. (1999). Intracellular pH regulation in hepatocytes isolated from three teleost species. *J. Exp. Zool.* **28**: 361-367.

ABSTRACT

The mechanisms of intracellular pH (pH_i) regulation were studied in hepatocytes isolated from three species of teleost, rainbow trout (*Oncorhynchus mykiss*), black bullhead (*Ameiurus melas*) and American eel (*Anguilla rostrata*). Intracellular pH was monitored over time using the pH-sensitive fluorescent dye BCECF. Responses to acid or base loading were examined under control conditions and in different experimental media containing either low Na^+ or Cl^- concentrations, the Na^+ - H^+ exchanger blocker amiloride, the anion exchanger blocker SITS, or the blocker of the V-type H^+ -ATPase, bafilomycin A_1 . In trout and bullhead hepatocytes, recovery to an intracellular acid load occurred principally by way of a Na^+ -dependent amiloride-sensitive Na^+ - H^+ exchanger. In eel hepatocytes, the Na^+ - H^+ exchanger did not contribute to recovery to an acid load though evidence suggests that it is present on the cell membrane and participates in the maintenance of steady-state pH_i . The V-type H^+ -ATPase did not participate in recovery to an acid load in any species. A Cl^- - HCO_3^- exchanger may play a role in recovery to an acid load in eel hepatocytes by switching off and retaining base that would normally be tonically extruded. The recovery to a base load in trout hepatocytes includes an amiloride-sensitive component that was not evident in the other two species. Baseline hepatocyte pH_i of all species was strongly influenced by pH_e and recovery to acid or base loads was affected at extreme pH_e values. Thus, it is clear that hepatocytes isolated from the three species are capable of regulating pH_i , principally by way of a Na^+ - H^+ exchanger and a Cl^- - HCO_3^- exchanger, but do not exploit identical mechanisms for pH_i recovery.

INTRODUCTION

The ability to regulate blood acid-base balance allows fish to move between environments that vary in temperature, salinity and acidity and permits recovery from acid-base disturbances associated with exhaustive exercise (Milligan and Wood, 1986). Extracellular pH (pH_e) is regulated principally at the gills, which are the main site of gas and ion exchanges (Goss *et al.*, 1992; Laurent and Perry, 1995). Although pH_e is regulated principally by branchial mechanisms, tissues are also capable of regulating their own acid-base status.

The liver is an important metabolic tissue, and it is essential that intracellular pH (pH_i) be regulated as acid-base disturbances can impact negatively on metabolic pathways (Mommensen *et al.*, 1988; Walsh *et al.*, 1988). In fish hepatocytes, the mechanisms of pH_i regulation, although species dependent, are known to involve different membrane exchange proteins that act in concert to keep pH_i constant (see review by Walsh and Mommensen, 1992). The principal exchange proteins participating in the regulation of pH_i in teleost hepatocytes are the $\text{Na}^+ \text{-H}^+$ exchanger that extrudes acid and the $\text{Cl}^- \text{-HCO}_3^-$ exchanger that extrudes base. In rat hepatocytes, a $\text{Na}^+ \text{-HCO}_3^-$ symporter additionally contributes to pH_i regulation by buffering intracellular protons (Gleeson *et al.*, 1989). Regulation of hepatocyte pH_i involves both $\text{Na}^+ \text{-H}^+$ and $\text{Cl}^- \text{-HCO}_3^-$ exchangers in trout (Walsh, 1986) but only the $\text{Na}^+ \text{-H}^+$ exchanger in a marine species, the Gulf toadfish *Opsanus beta* (Walsh, 1989).

Our understanding of pH_i regulation in teleost hepatocytes, therefore, is based on experiments performed on only trout and toadfish, and significant differences in the roles of the different exchangers apparently exist. The purpose of this study was to examine the mechanisms of pH_i regulation in hepatocytes of three species of freshwater teleost fish, rainbow trout, black bullhead and American eel. These three species are evolutionarily distinct as well as differing in lifestyles and in the habitats where they are found. Using a comparative approach, the specific

role of the $\text{Na}^+\text{-H}^+$ exchanger, $\text{Cl}^-\text{-HCO}_3^-$ exchanger and V-type $\text{H}^+\text{-ATPase}$ in hepatic pH_i regulation was established.

MATERIALS AND METHODS

Experimental Animals

Rainbow trout, *Oncorhynchus mykiss*, weighing 200 to 300 g were obtained from Linwood Acres Trout Farm (Campbellcroft, Ontario, Canada). Black bullhead, *Ameiurus melas*, weighing 200 to 350 g were obtained from the State University of New York at Brockport (Brockport, NY, USA). American eels, *Anguilla rostrata*, weighing 350 to 500 g were obtained from the St. Lawrence River by a commercial fisherman (Lancaster, Ontario, Canada). Fish were transported and kept in conditions as described in Chapter 2. In the temperature acclimation experiment, bullheads were acclimated to either 15 °C or 20 °C. All experiments were performed in the fall and winter.

Cell Isolation

Hepatocytes were isolated according to the method of Mommsen *et al.* (1994). See Chapter 2 for the full description of solutions and procedures. Following the isolation procedure, cells were allowed to “rest” for two hours on ice before proceeding with the experiment. Based on a previous study by Furimsky *et al.* (1999b; Chapter 2), hepatocytes isolated using this procedure maintained appropriate directionally correct Na^+ and Cl^- gradients.

Measurement of pH_i

Cell suspensions at a concentration of 10^6 cells ml^{-1} were used in experiments. Cells were washed and resuspended in Hanks' medium (pH 7.9) containing 1.5 mmol l^{-1} CaCl_2 and then

incubated in the same solution containing $1 \mu\text{mol l}^{-1}$ ($3 \mu\text{l}$ of 1 mmol l^{-1} stock dissolved in dimethylsulfoxide) of the ester form of the pH-sensitive dye 2', 7 - bis- (2-carboxyethyl)-5-(and 6)-carboxyfluorescein (BCECF-AM) for 20 min. Cells were then rinsed and resuspended in the experimental medium. All experimental media were modifications of the resuspension medium described above, containing (or a combination of) the following: the $\text{Na}^+\text{-H}^+$ exchanger blocker, amiloride hydrochloride (Sigma); the anion exchanger blocker SITS (Sigma); or, the blocker of the V-type $\text{H}^+\text{-ATPase}$, bafilomycin A_1 (Sigma). Amiloride (1 mmol l^{-1}) and SITS (0.5 mmol l^{-1}) did not dissolve freely at the concentration used, so the medium was sonicated until all solids were in solution. Bafilomycin A_1 was dissolved in DMSO; the DMSO was found to have no effect on the pH of the medium or pH_i recovery at the low volumes added. In the Na^+ -free medium, NaCl , Na_2HPO_4 , Na-HEPES and NaHCO_3 were replaced by equal concentrations of choline chloride, K_2HPO_4 , HEPES and KHCO_3 , respectively (see Gleeson *et al.*, 1989). In the Cl^- -free medium, NaCl , KCl and CaCl_2 were replaced by equal concentrations of Na-gluconate , K-gluconate and Ca-gluconate , respectively. The nominal concentrations of Na^+ and Cl^- were not measured, though they can be estimated to be lower than 3 mmol l^{-1} and 1 mmol l^{-1} , respectively as a result of trace amounts in BSA (Walsh, 1986). These media were all pH adjusted to 7.9 at room temperature (20°C). All experiments were performed at room temperature using a fluorescence spectrophotometer (Photon Technology International, South Brunswick, New Jersey). Cell suspensions were stirred continuously in a quartz cuvette during data collection. Data were obtained as the ratio of the pH-sensitive excitation wavelength (490 nm) to the pH-insensitive excitation wavelength (440 nm), with the emission wavelength set at 535 nm. An acid load was induced by the addition of Na-propionate ($150 \mu\text{l}$ of 1 mol l^{-1} stock; 50 mmol l^{-1} final concentration) directly into the cuvette containing the cell suspension. A base load was induced

by the addition of NH_4Cl (60 μl of 1 mol l^{-1} stock; 20 mmol l^{-1} final concentration). An acid load was also induced by the NH_4Cl washout method (Walsh, 1986; 1989), where the cells were initially alkaline-loaded by the addition of NH_4Cl (20 mmol l^{-1} final concentration) to the cell suspension, then acidified by replacing this medium with the normal (pH 7.9) extracellular medium. Recovery rates were followed over the initial 200 seconds (the linear portion of the curve) after the lowest measured pH_i following the acid load or the highest measurement of pH_i following base load.

Standard curves were constructed using an *in situ* calibration method. Cells were first loaded with BCECF-AM, washed, and then re-suspended in calibration media of varying pH's. The calibration medium was the same as the resuspension medium, except that the concentrations of NaCl and KCl were reversed (NaCl, 5.4 mmol l^{-1} ; KCl, 136.9 mmol l^{-1}) to represent intracellular ion concentrations, there was no BSA in the medium and the medium contained 14 $\mu\text{mol l}^{-1}$ of the K^+/H^+ ionophore nigericin (Molecular Probes). The nigericin stock was dissolved in 95% ethanol. Ratios obtained were representative of the different intracellular pH values and a standard curve was constructed using the appropriate software. Separate standard curves were constructed using hepatocytes of the three different species.

Statistical Analysis

Data are presented as the mean $\pm$ the standard error of the mean (SEM). Statistical analysis was conducted using a one way ANOVA followed by a Student-Newman-Keuls test for the pairwise analysis of means (Sigma Stat 2.0).

RESULTS

Resting pH_i

The resting baseline pH_i of eel hepatocytes was significantly lower than that of trout and bullhead (Table 3-1). The effect of exchanger blockers and different media on resting pH_i varied between species. Amiloride caused a decrease in resting pH_i in the rainbow trout and the eel, but not the bullhead (Table 3-1). Bafilomycin A_1 had no individual effect on baseline pH_i in any species. In bullhead, the baseline decreased only when amiloride and bafilomycin were in the media together. Exposure of hepatocytes to a Na^+ -free medium lead to a decrease in resting pH_i in the trout and bullhead, but not the eel (Table 3-1). Exposure of the cells to a Cl^- -free medium lead to an increase in resting pH_i in all species (Table 3-1).

Recovery to an acid load

Recovery rates were determined in isolated hepatocytes in response to an acid load induced by either 50 mmol l^{-1} Na-propionate (Fig. 3-1A) or the NH_4Cl washout method (Fig. 3-5). The Na^+-H^+ exchanger blocker, amiloride (1.0 mmol l^{-1}) significantly reduced the rate of recovery in rainbow trout and bullhead hepatocytes, but not those isolated from eel (Fig. 3-2). The V-type H^+ ATPase blocker, bafilomycin A_1 (10 nmol l^{-1}) and the anion exchange blocker SITS (0.5 mmol l^{-1}), had no individual effect on recovery in any species. The recovery rate when both amiloride and bafilomycin A_1 were in the medium was not different from recovery in the presence of amiloride alone for any species (Fig. 3-2). Since bullhead were kept in holding conditions different to those of the other two species, any possible effect of temperature acclimation had to be determined. The response to acid or base loading of hepatocytes isolated from bullhead acclimated to a water temperature of 20 °C did not differ from that of the same

species acclimated to 15 °C (Fig. 3-3). Increasing the concentration of bafilomycin A₁ had no effect on recovery to an acid load in bullhead hepatocytes (Fig. 3-4). Recovery to an acid load induced by an NH₄Cl prepulse was impaired in trout and bullhead hepatocytes when in a Na⁺-free medium, whereas the lack of extracellular Na⁺ had no effect on recovery in eel hepatocytes (Fig. 3-6). When cells were incubated in a Cl⁻-free medium, baseline pH_i increased in all species (Table 3-1), but a significant decrease in the rate of recovery following an acid load was observed only in the eel (Fig. 3-7).

Recovery to base load

Recovery to base loading was monitored in cells following the addition of 20 mmol l⁻¹ NH₄Cl (Fig. 3-1B). The addition of the anion exchanger blocker SITS had no effect on the recovery rates in any species (Fig. 3-8). The addition of amiloride decreased the rate of recovery in trout hepatocytes, suggesting a role for Na⁺-H⁺ exchange.

Effects of varying extracellular pH

Varying the extracellular pH had a direct effect on pH_i in the hepatocytes of all species (Tables 3-2, 3-3). Intracellular pH was always maintained at a lower value (i.e. more acidic) than extracellular pH, except at lower extreme pH values where pH_i was higher. The recovery to acid and base loading was also affected by external pH, though this effect could have been partially attributed to the degree of acidification experienced.

Table 3-1: Baseline pH_i values under different experimental conditions.

	<i>Rainbow Trout</i>	<i>Black Bullhead</i>	<i>American Eel</i>
Normal medium	7.71 ± 0.013	7.64 ± 0.026	$7.33 \pm 0.031 \ddagger$
Amiloride (1 mM)	7.54 ± 0.020 *	7.54 ± 0.027	7.24 ± 0.026 *
SITS (0.5 mM)	7.73 ± 0.028	7.56 ± 0.055	7.33 ± 0.016
Bafil. (10 nM)	7.73 ± 0.024	7.62 ± 0.029	7.34 ± 0.025
Amil. + Bafil.	7.55 ± 0.027 *	7.51 ± 0.030 *	7.25 ± 0.015 *
Normal medium	7.70 ± 0.042	7.67 ± 0.027	7.21 ± 0.053
Na^+ -free medium	7.47 ± 0.070 *	7.56 ± 0.016 *	7.24 ± 0.050
Normal medium	7.68 ± 0.050	7.59 ± 0.042	7.32 ± 0.022
Cl^- -free medium	8.01 ± 0.080 *	7.92 ± 0.053 *	7.54 ± 0.055 *

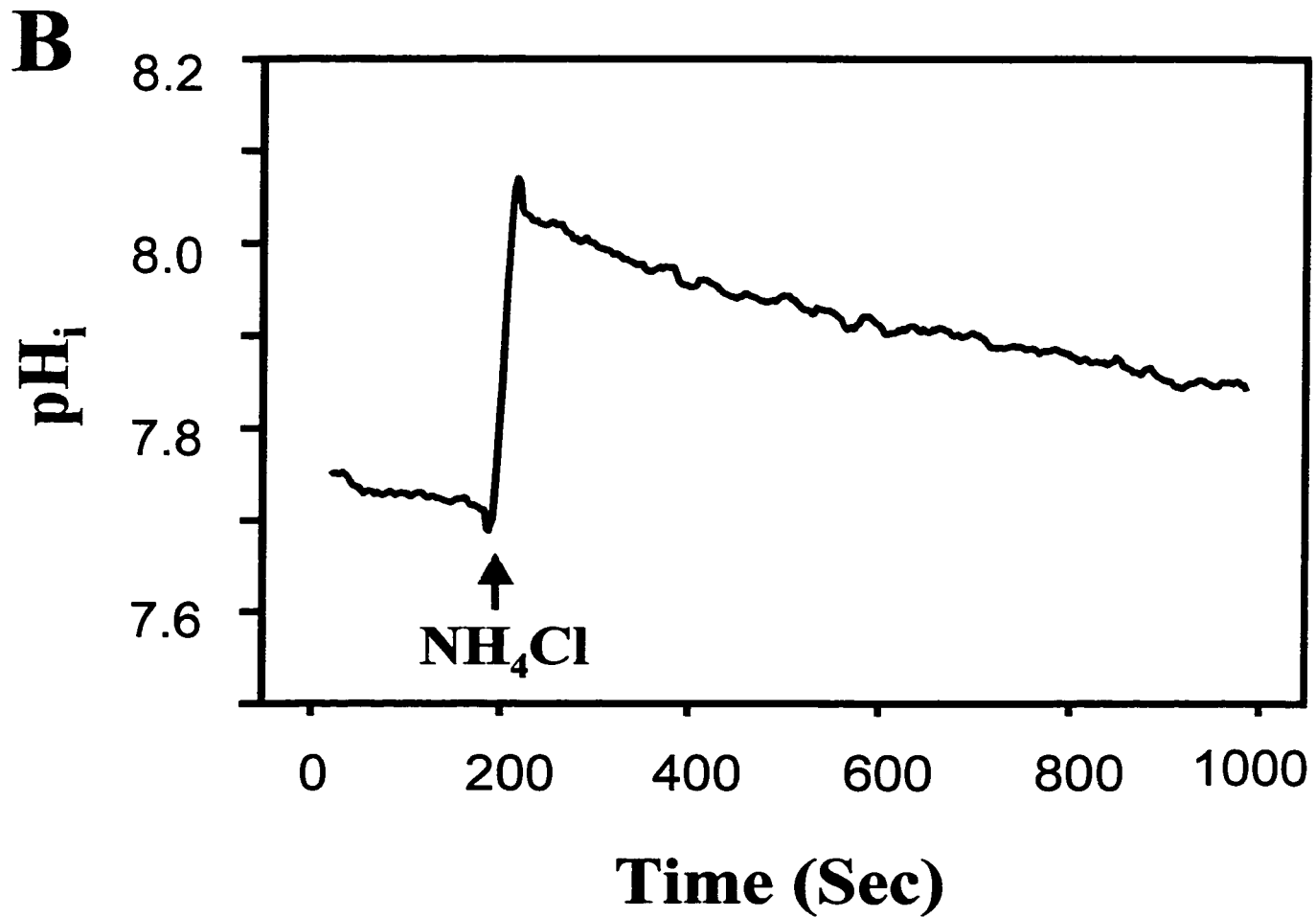
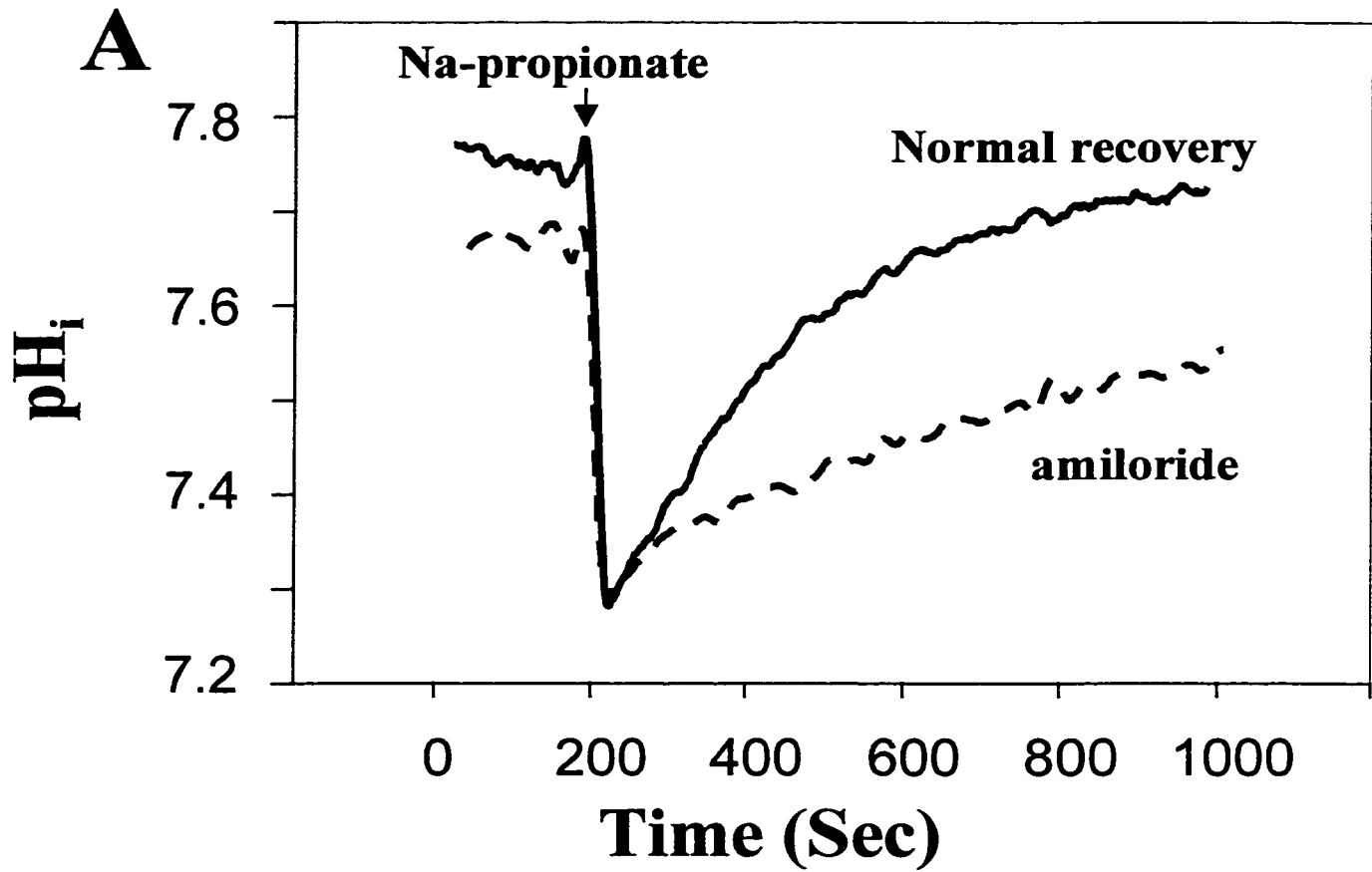
Values are means $\pm$ S.E.M. (n=6)

Three separate studies were performed using 6 different fish in each study.

* denotes a difference from pH_i values in normal medium.

$\ddagger$ denotes a difference in resting pH_i from other two species.

Figure 3-1. Representative traces showing (A) intracellular acidification of trout hepatocytes by Na-propionate (50 mmol l⁻¹) and subsequent recovery by cells in normal medium and medium containing the Na⁺-H⁺ exchanger blocker amiloride (1 mmol l⁻¹), as well as intracellular alkalization following addition of NH₄Cl (20 mmol l⁻¹) and subsequent recovery (B).



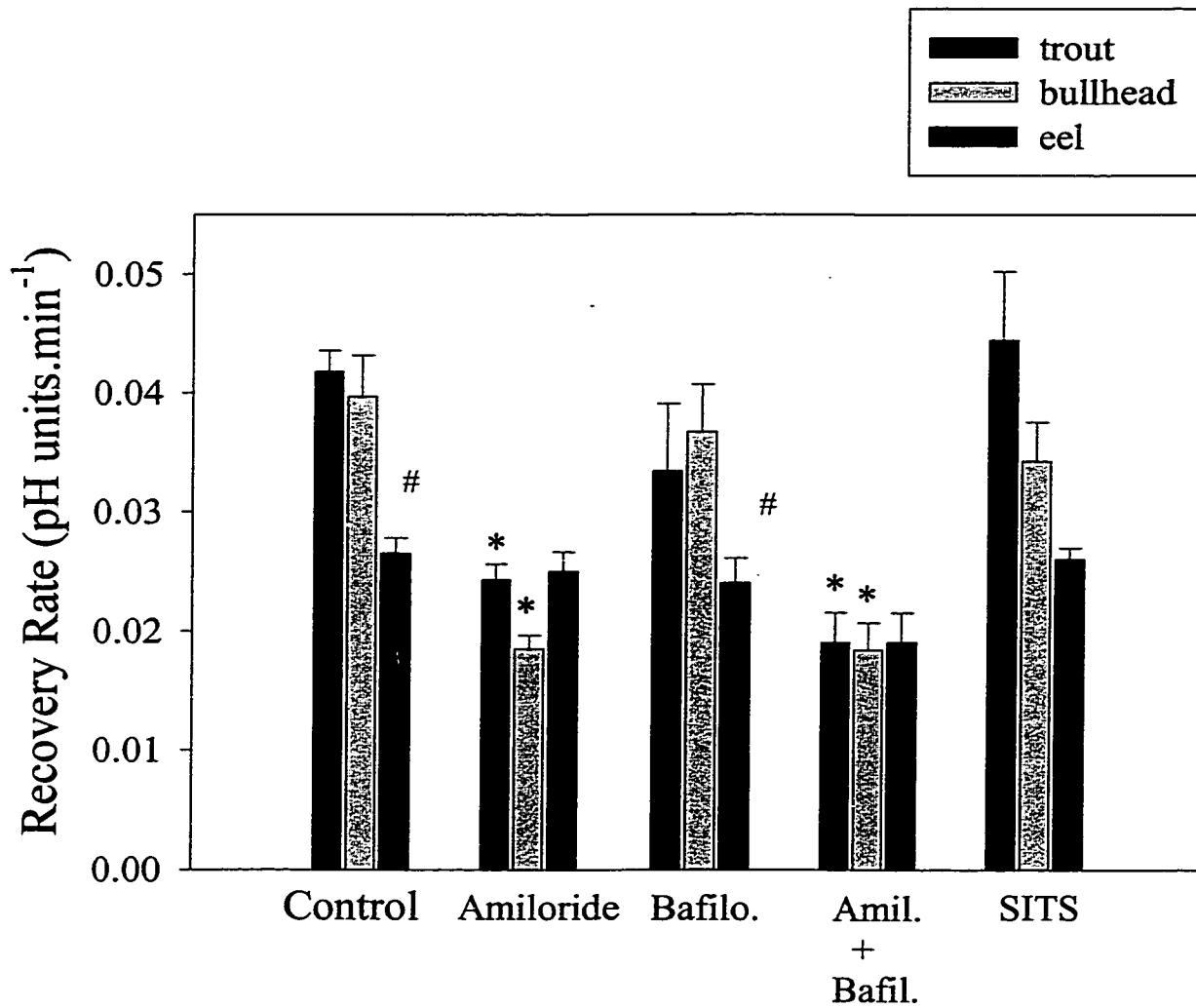
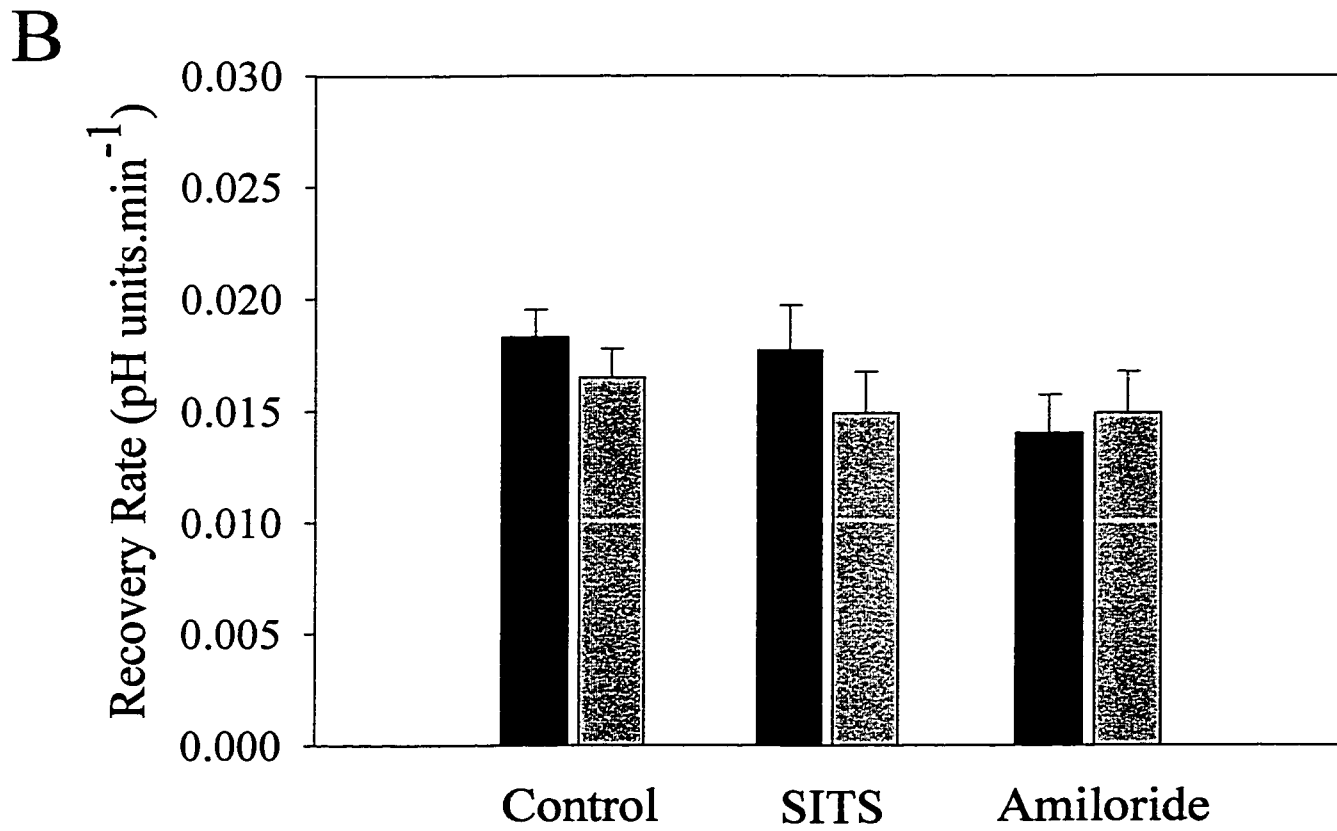
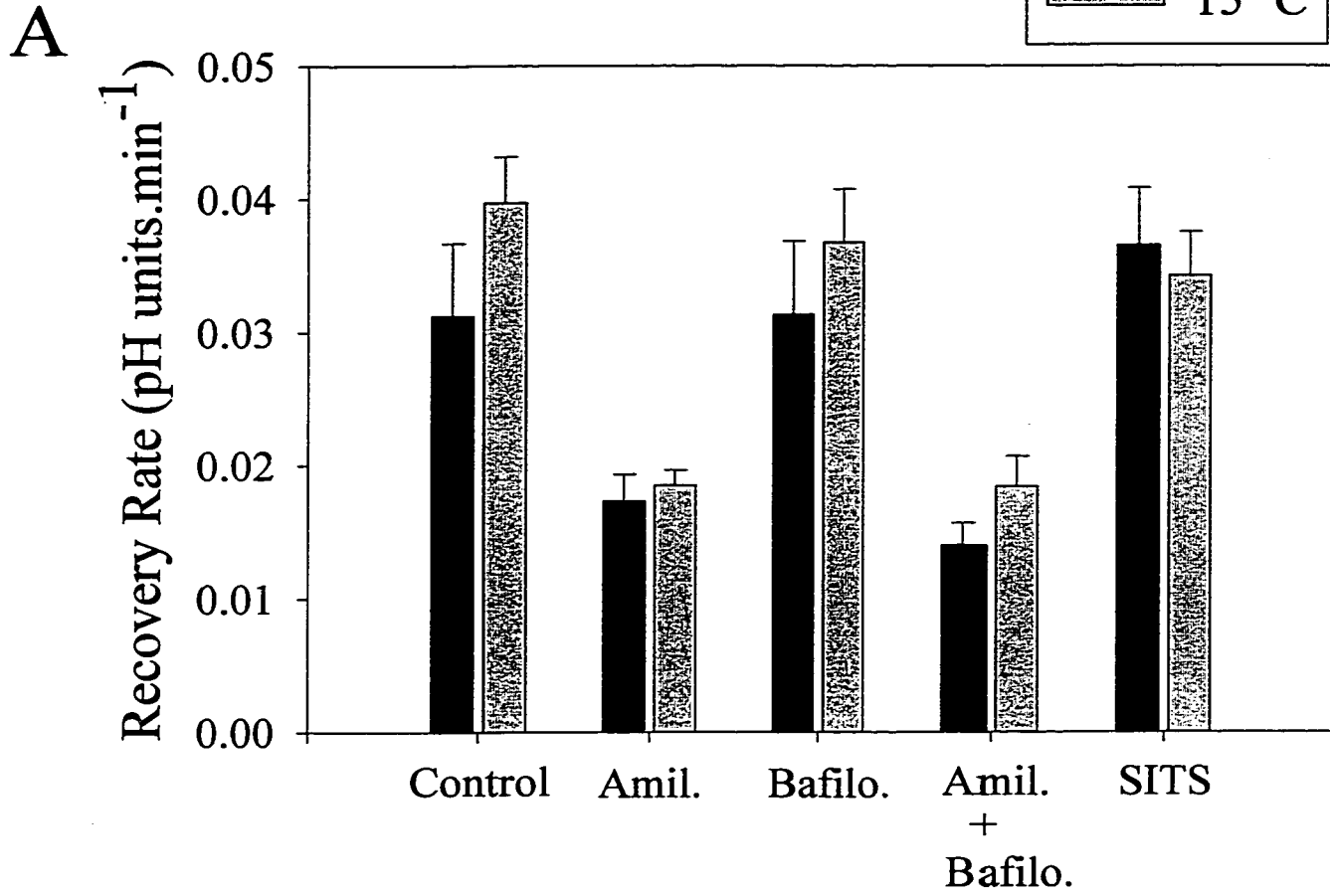


Figure 3-2. Rate of pH_i recovery in hepatocytes from three species of teleost fish in response to acidification by Na-propionate (50 mmol l^{-1}). * denotes a significant difference from normal recovery. # denotes a species difference. Values are means $\pm$ SEM ($n=6$). Bafilomycin A_1 is represented as Bafil or Bafil.

Figure 3-3. Effects of temperature acclimation on pH_i regulation in bullhead hepatocytes following (A) intracellular acidification or (B) intracellular alkalinisation. Values are presented as the means $\pm$ SEM (n=6). No significant differences were detected between similar treatments performed on hepatocytes from fish acclimated to 15 °C vs 20 °C).



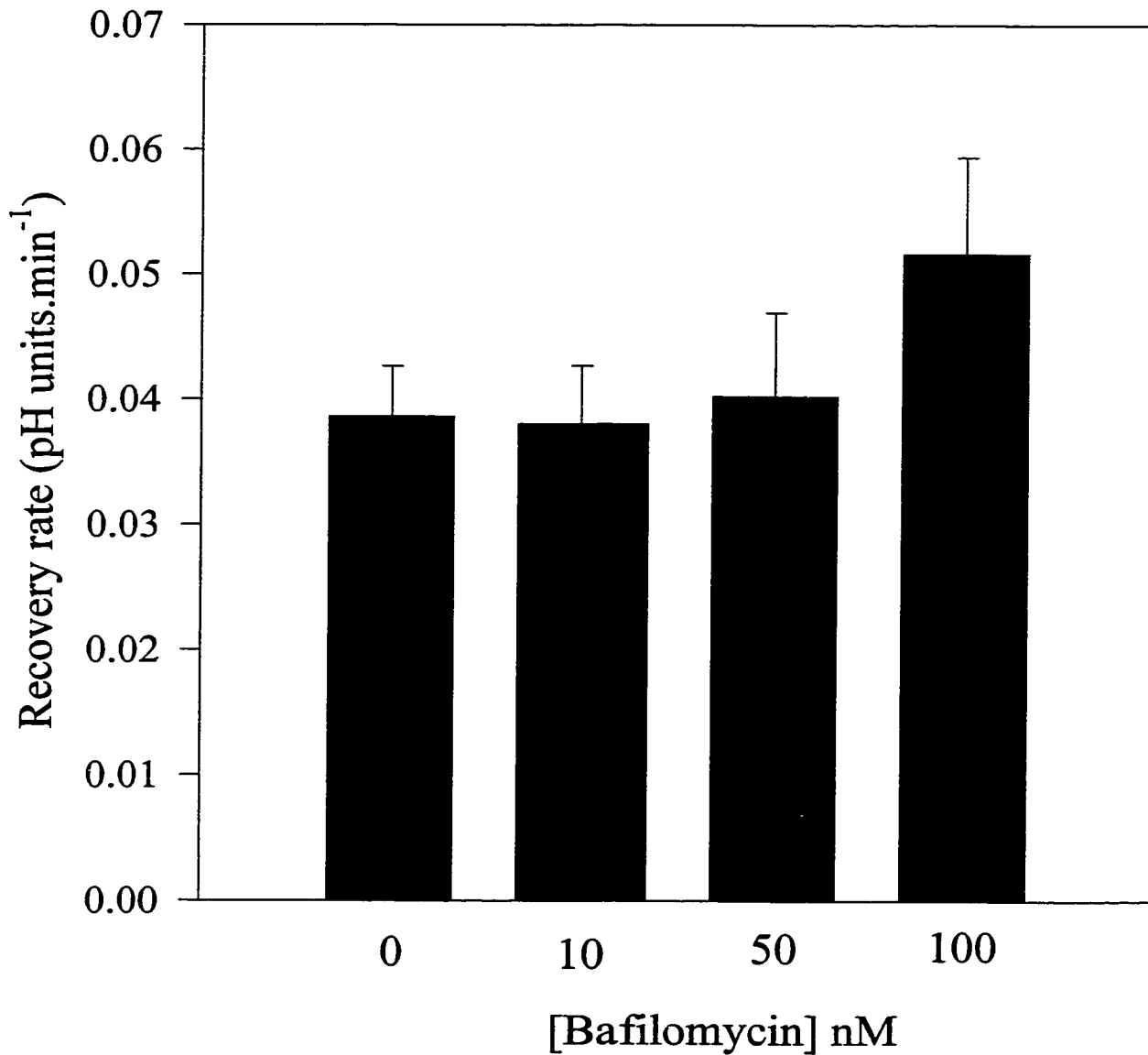


Figure 3-4. Effects of bafilomycin A₁ concentration on pH_i recovery following acid loading in bullhead hepatocytes. Values are presented as means $\pm$ SEM (n=6). No significant differences were detected.

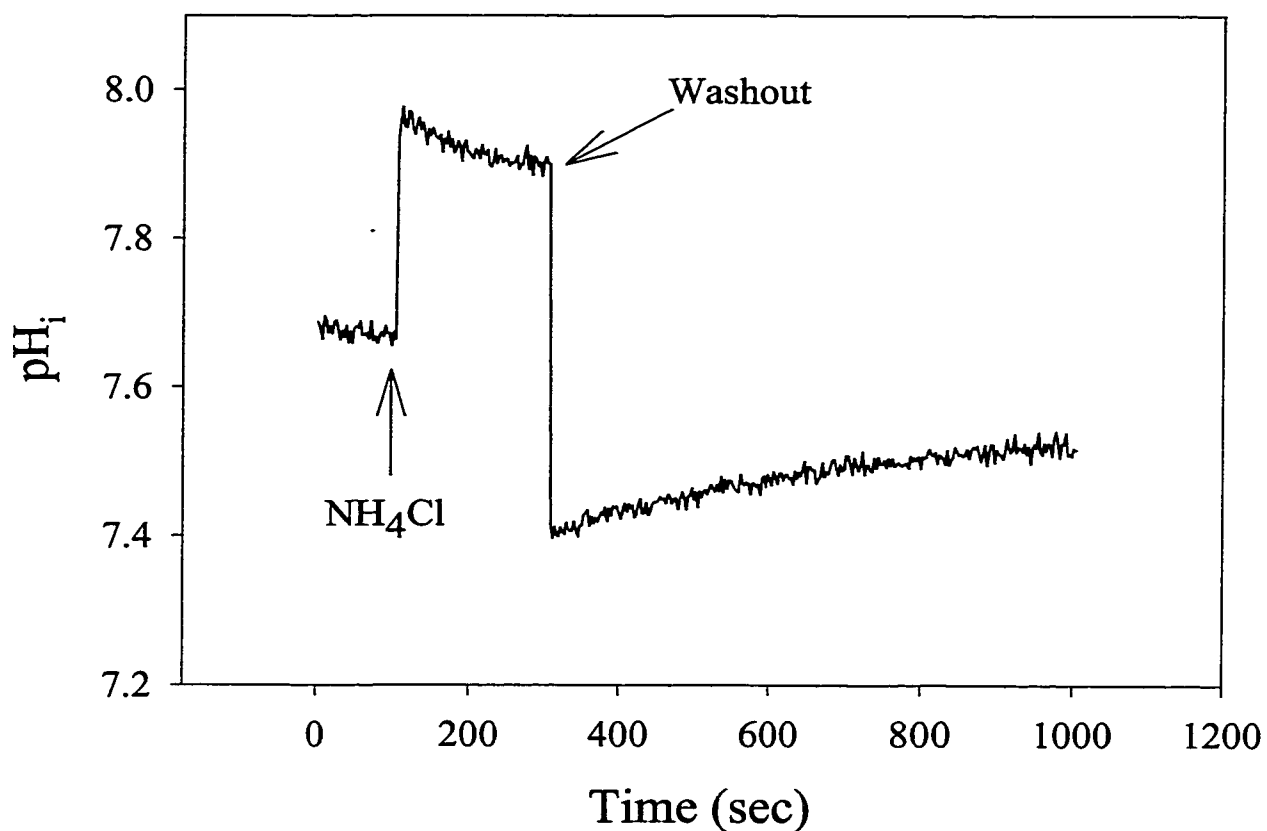


Figure 3-5. Representative trace of pH_i recovery in trout hepatocytes following intracellular acidification by the NH_4Cl washout method. The addition of NH_4Cl (20 mmol l^{-1}) alkalises the cytoplasm by the buffering of intracellular H^+ with NH_3 . Washing the cells in fresh medium, causes the exit of intracellular NH_3 and release of H^+ , hence acidifying the cytoplasm.

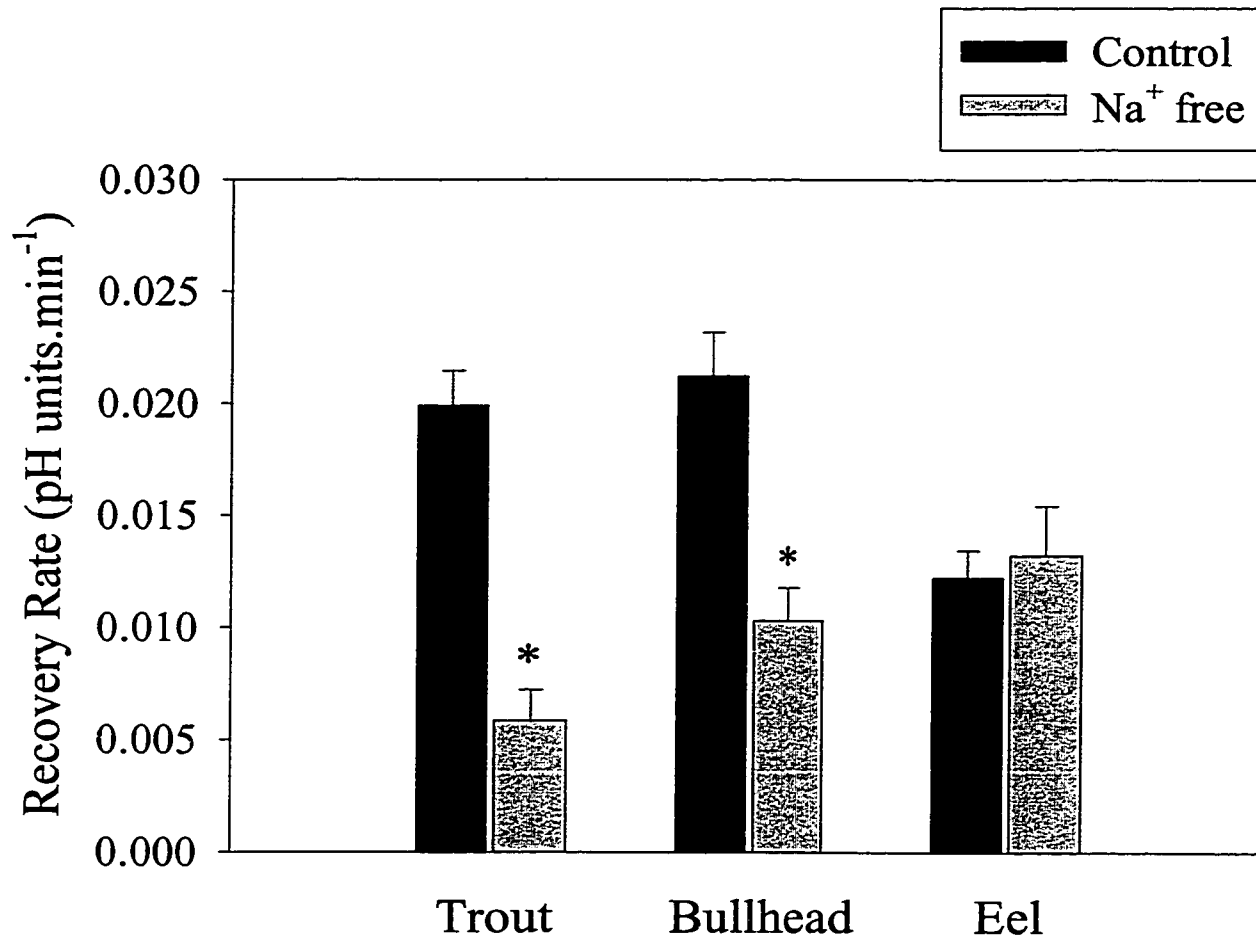


Figure 3-6. Effects of extracellular Na⁺ on pH_i recovery in hepatocytes from three teleost species in response to acidification by NH₄Cl prepulse. * denotes a significant difference from recovery in normal Na⁺-containing medium. Values are means ± SEM (n= 6).

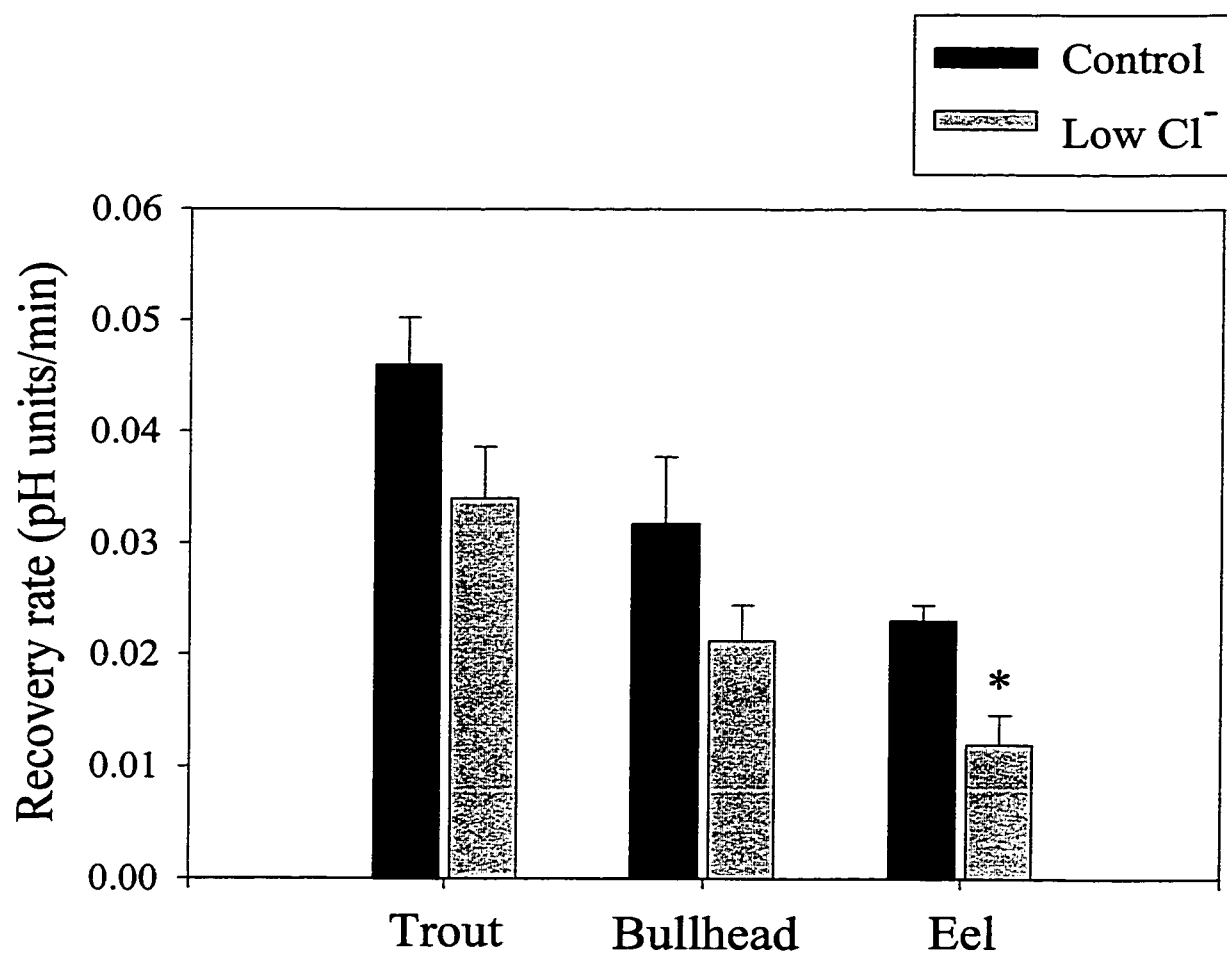


Figure 3-7. Effects of extracellular Cl^- on the rate of pH_i recovery in response to acidification by Na-propionate (50 mmol l^{-1}). * denotes a significant difference from recovery in normal medium. Values are means $\pm$ SEM ($n=6$).

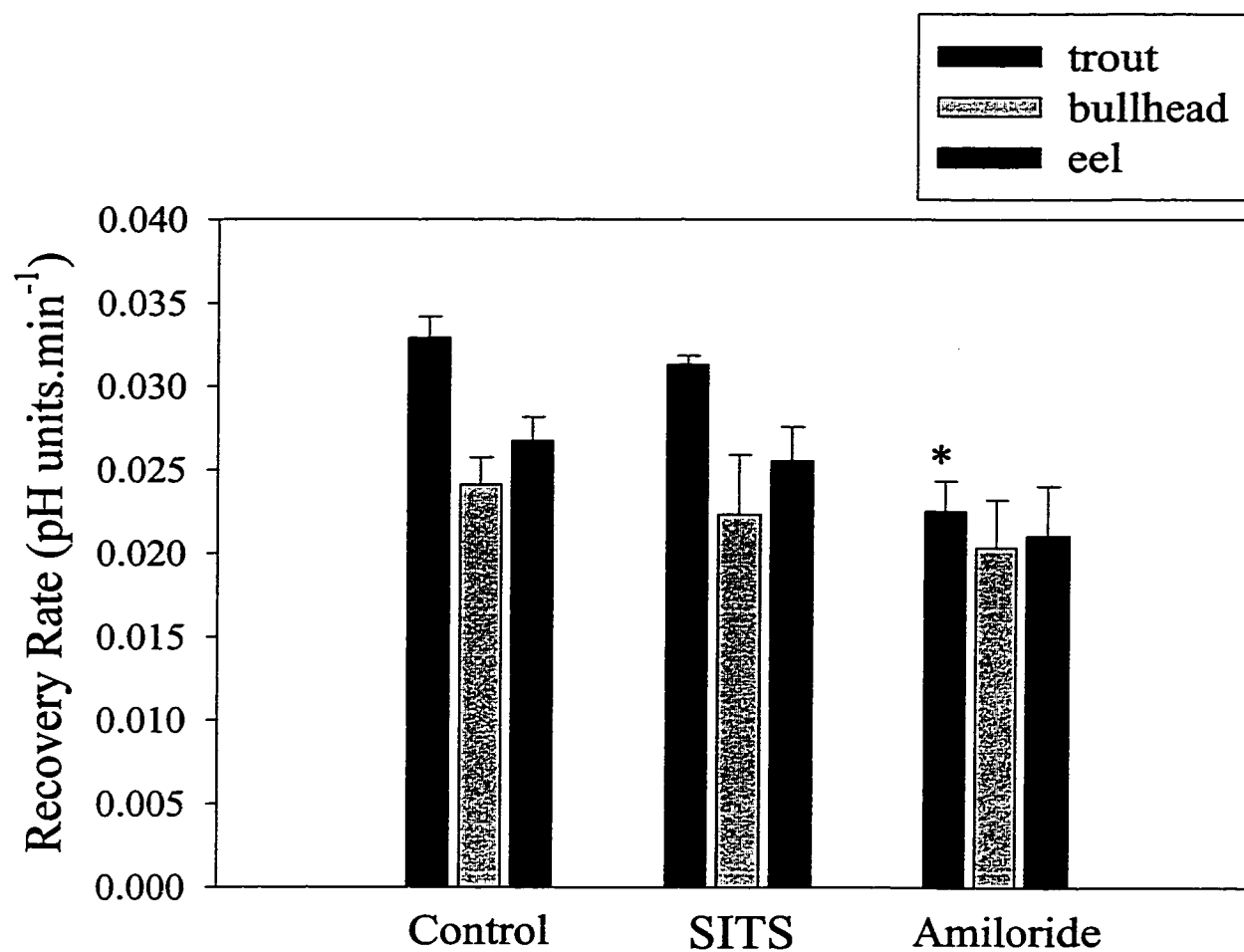


Figure 3-8. Intracellular pH recovery following base load in hepatocytes due to addition of 20 mmol l⁻¹ NH₄Cl. * denotes a significant difference from normal recovery. Values are means $\pm$ SEM (n=6).

Table 3-2. The effects of extracellular pH (pHe) on recovery of hepatocytes from three species of teleost fish to an acid load (50 mmol l⁻¹ Na-propionate). Acid pH_i is the lowest pH_i following acid loading.

a) Rainbow trout (n=6)

<u>pHe</u>	<u>pHi</u>	<u>acid pHi</u>	<u>Recovery rate</u> (pH units min ⁻¹)
6.9	7.28 ± 0.02	6.70 ± 0.03	0.027 ± 0.003
7.4	7.49 ± 0.02	7.01 ± 0.02	0.038 ± 0.006
7.9	7.73 ± 0.04	7.34 ± 0.03	0.039 ± 0.004
8.4	8.02 ± 0.04	7.80 ± 0.02	0.034 ± 0.003

b) Black bullhead (n=6)

<u>pHe</u>	<u>pHi</u>	<u>acid pHi</u>	<u>Recovery rate</u> (pH units min ⁻¹)
6.9	7.22 ± 0.05	6.58 ± 0.04	0.021 ± 0.003
7.4	7.50 ± 0.04	6.92 ± 0.02	0.030 ± 0.004
7.9	7.72 ± 0.04	7.30 ± 0.01	0.032 ± 0.004
8.4	7.86 ± 0.03	7.64 ± 0.03	0.017 ± 0.003 *

c) American eel (n=6)

<u>pHe</u>	<u>pHi</u>	<u>acid pHi</u>	<u>Recovery rate</u> (pH units min ⁻¹)
6.9	6.87 ± 0.02	6.50 ± 0.02	0.021 ± 0.001
7.4	7.05 ± 0.04	6.76 ± 0.01	0.024 ± 0.002
7.9	7.30 ± 0.04	7.08 ± 0.01	0.024 ± 0.001
8.4	7.39 ± 0.04	7.39 ± 0.01	0.018 ± 0.001 *

Data are presented as the mean ± SEM.

* denotes a significant difference from the control (pHe 7.9) using one way ANOVA followed by Bonferonnis t-test for the multiple comparison of means.

Table 3-3. The effects of extracellular pH (pH_e) on recovery of hepatocytes from three species of teleost fish to an alkaline load ($20 \text{ mmol l}^{-1} \text{ NH}_4\text{Cl}$). Alkaline pH_i is the highest value of pH_i following base loading.

a) Rainbow trout (n=6)

<u>pH_e</u>	<u>pH_i</u>	<u>alkaline pH_i</u>	<u>Recovery rate</u> ($pH \text{ units min}^{-1}$)
6.9	7.28 ± 0.02	7.67 ± 0.02	0.024 ± 0.001 *
7.4	7.49 ± 0.02	7.92 ± 0.02	0.040 ± 0.002
7.9	7.72 ± 0.04	8.19 ± 0.03	0.041 ± 0.003
8.4	8.08 ± 0.06	8.42 ± 0.02	0.040 ± 0.002

b) Black bullhead (n=6)

<u>pH_e</u>	<u>pH_i</u>	<u>alkaline pH_i</u>	<u>Recovery rate</u> ($pH \text{ units min}^{-1}$)
6.9	7.27 ± 0.05	7.70 ± 0.03	0.020 ± 0.001 *
7.4	7.51 ± 0.03	7.92 ± 0.03	0.030 ± 0.001
7.9	7.75 ± 0.03	8.16 ± 0.02	0.036 ± 0.002
8.4	7.86 ± 0.04	8.13 ± 0.03	0.027 ± 0.003 *

c) American eel (n= 6)

<u>pH_e</u>	<u>pH_i</u>	<u>alkaline pH_i</u>	<u>Recovery rate</u> ($pH \text{ units min}^{-1}$)
6.9	6.85 ± 0.04	7.24 ± 0.04	0.029 ± 0.001
7.4	7.07 ± 0.05	7.53 ± 0.01	0.037 ± 0.002 *
7.9	7.30 ± 0.04	7.96 ± 0.02	0.027 ± 0.002
8.4	7.50 ± 0.06	7.84 ± 0.02	0.018 ± 0.002 *

Data are presented as the mean $\pm$ SEM.

* denotes a significant difference from control (pH_e 7.9) using one way ANOVA followed by Bonferonnis t-test for the multiple comparison of means.

DISCUSSION

The present study has confirmed the importance of the $\text{Na}^+\text{-H}^+$ exchanger in the mechanism of pH_i regulation in hepatocytes of the trout (Walsh, 1986; Fossat *et al.*, 1997). Similarly, this exchanger is important in pH_i recovery in the black bullhead, but not in the eel. The pH_i recovery rates of eel hepatocytes to an acid load were significantly slower than those found in the other two species under control conditions but similar to those of the other two species when amiloride was in the medium or the cells were in a Na^+ -free medium. Two different experiments lead to the same conclusion that recovery of trout and bullhead hepatocytes to an acid load occurred principally by a Na^+ -dependent, amiloride-sensitive mechanism and that this mechanism was not active in the eel hepatocyte. The $\text{Na}^+\text{-H}^+$ exchanger does appear to be present in eel hepatocytes as amiloride decreased baseline pH_i , however, the same result was not obtained by removing Na^+ from the extracellular medium. A separate study showed a decrease in the rate of tonic Na^+ uptake in hepatocytes of rainbow trout and the European eel (*Anguilla anguilla*) when exposed to amiloride (M. Furimsky and A. R. Cossins, unpublished data). Unless different mechanisms exist between the American and the European eels, these findings suggest that the $\text{Na}^+\text{-H}^+$ exchanger in eel hepatocytes may not be activated by a decrease in cytoplasmic pH and thus does not participate in recovery to an intracellular acid load. The cytoplasmic domain of the $\text{Na}^+\text{-H}^+$ exchanger (NHE1 human isoform) is known to have a " H^+ -sensing" ability (Wakabayashi *et al.*, 1992), but the amino acid sequence responsible for " H^+ -sensing" may not be present in the eel exchanger. It is also possible that the eel, the most primitive of the three species studied (Moyle and Cech, 1996), is an important link in the evolution of the $\text{Na}^+\text{-H}^+$ exchanger on the teleost hepatocyte. This observed species difference is not surprising considering that the regulatory properties of the $\text{Na}^+\text{-H}^+$ exchanger in erythrocytes of eel and trout also differ (Romero *et al.*, 1996).

Although the $\text{Na}^+\text{-H}^+$ exchanger is clearly the most important mechanism by which protons are extruded from the cytosol, some recovery persists despite blockade with amiloride or the removal of Na^+ from the extracellular medium. Protons may be diffusing from the cells or leaving by way of other transport mechanisms. The $\text{Na}^+\text{-HCO}_3^-$ symporter plays a role in pH_i regulation in rat hepatocytes (Gleeson *et al.*, 1989), but it was previously shown not to be active on trout hepatocytes (Walsh, 1986). It was thought unlikely that this symporter played a major role in teleost hepatocytes as a result of the low extracellular HCO_3^- concentration ($\sim 5 \text{ mmol l}^{-1}$) in fish plasma, but recent findings have shown otherwise (see Chapter 4; Furimsky *et al.*, 2000). A further mechanism of proton extrusion is by way of a V-type $\text{H}^+\text{-ATPase}$, which is specifically blocked by the antibiotic bafilomycin A_1 . This pump could be a complementary Na^+ -independent method of proton extrusion, as shown in mammalian outer medullary collecting duct cells (Manger and Koeppen, 1992). Bafilomycin A_1 , added to the cell suspensions at a final concentration of 10 nmol l^{-1} , had no impact on the pH_i ~~recovery~~ recovery rates to an acid load in any species. The same concentration of blocker, when used by Manger and Koeppen (1992) significantly decreased recovery to an acid load. In addition, bafilomycin A_1 did not affect pH_i recovery when added to the medium containing amiloride, so the additional recovery of pH_i is by way of another mechanism. Higher concentrations of bafilomycin A_1 ($10 - 200 \text{ nmol l}^{-1}$) did not affect recovery rates in bullhead hepatocytes (Fig. 3-4).

Although membrane exchangers are often activated in response to specific signals, including hormone stimulation or changes in pH_i , these exchangers generally are tonically active under steady-state conditions. One factor influencing the pH set-point of the membrane exchangers in teleost hepatocytes is pH_e , as demonstrated by the linear relationship between pH_i and pH_e (Tables 3-2, 3-3), with pH_i always being lower over a range of physiological pH (Walsh and Moon, 1983; Walsh 1986). A similar relationship holds true for liver pH_i *in vivo*, as

determined in trout (Milligan and Wood, 1986) and eel (Walsh and Moon, 1982). Though the pH_i values determined *in vivo* differ from those *in vitro*, they are very similar and any difference may be due to the different methods used in the separate studies. The eel demonstrated a greater difference between pH_e and pH_i *in vitro* and *in vivo* compared to the trout in previous studies, and the values obtained in this study are in agreement. Hepatocytes are capable of maintaining a constant difference between pH_e and pH_i , and when this difference increases, such as in response to an acid load, the exchanger is activated to return this difference to its initial value. Blocking the $\text{Na}^+\text{-H}^+$ exchanger significantly reduced baseline pH_i in trout and eel, but not in bullhead, although any difference may be masked by the variation in the data. A reduction of extracellular Na^+ , which should have had an effect similar to that observed with amiloride, caused a decrease in pH_i in trout and bullhead, but not the eel. Results for the trout are consistent with a study by Fossat *et al.* (1997) in which amiloride caused a decrease in Na^+ uptake in trout hepatocytes under steady-state conditions. The $\text{Na}^+\text{-H}^+$ exchanger also participates in maintaining steady-state pH_i in trout gill epithelial cells (Pärt and Wood, 1996), so this regulatory role may be typical in most cell types. The data for eel baseline pH_i differed among the three different experimental groups, with that in the Na^+ -free experiment demonstrating lower mean pH_i under normal conditions. No difference was observed in eel baseline pH_i between normal and Na^+ -free media, so the result in which baseline pH_i decreased with amiloride exposure was not adequately supported.

Also of note is the difference in resting pH_i between the eel and the trout and bullhead. The $\text{pH}_i - \text{pH}_e$ difference is much greater in eel than the other two species, meaning that proton retention or a lack of proton extrusion occurs in eel hepatocytes. The pH_i to pH_e relationship in this study was very similar to that previously obtained for trout (Walsh, 1986) but different than that of eel (Walsh and Moon, 1983) at similar temperatures, but using the DMO (5,5-dimethyl-

2,4-oxazolidine dione) method. At a pH_e of 7.90, the resting pH_i of eel hepatocytes in our study was 7.33 using BCECF, whereas Walsh and Moon (1983) observed a value of approximately 7.50 with the DMO method. The ionic composition of the media in our experiments was more similar to trout and bullhead plasma than to that of the freshwater adapted eel, which exhibits an “anion gap”, where Cl^- levels are much lower compared to Na^+ (Farrell and Lutz, 1975; see Chapter 2, Table 2-1). Whether or not the media used in this study influenced resting pH_i levels in the eel hepatocyte is unclear, but it is possible since Walsh and Moon (1983) used a more appropriate medium for their study. The ionic composition of the experimental media was consistent for all species, though media representing the species-specific differences in plasma may have provided differing results. We can speculate that the lower Cl^- concentration in eel plasma may serve to promote base retention as a result of a decrease in tonic $Cl^-HCO_3^-$ exchanger activity. However, this is unlikely since the K_m of this exchanger is much lower than the Cl^- levels in freshwater adapted eel plasma that are 30-40 $mmol\ l^{-1}$ lower than those of Na^+ . Walsh (1989) determined that the membrane potential of toadfish hepatocytes is approximately -30 mV and deduced that pH_i would be 0.6 units below pH_e if protons were not actively extruded. If the membrane potential of eel hepatocytes is similar to those of toadfish, skate (Wondergem *et al.*, 1985) and rat (Hoek *et al.*, 1980), protons should be very close to electrochemical equilibrium. The advantage, if any, that the lower hepatocyte pH_i may have to the eel is unclear. The use of eel hepatocytes in cell metabolism experiments (e.g., Foster and Moon, 1986) indicates that eel liver enzymes are active even at this lower pH_i .

When Cl^- was absent from the extracellular medium, baseline pH_i increased in all species. When Cl^- is not available, $Cl^-HCO_3^-$ exchanger activity is blocked and intracellular HCO_3^- is retained, hence buffering intracellular protons. The significant increase in baseline pH_i in all species suggests that a Cl^- dependent mechanism, likely the $Cl^-HCO_3^-$ exchanger, is participating

in the maintenance of steady-state pH_i . Direct evidence exists for the role of a Cl^- - HCO_3^- exchanger in trout hepatocytes (Walsh, 1986), where the specific blocker of the Cl^- - HCO_3^- exchanger SITS decreased tonic Cl^- uptake and caused an overshoot of baseline pH_i following recovery to an acid load. The removal of Cl^- from the extracellular medium had a similar impact on pH_i recovery in the study by Walsh (1986). In eel hepatocytes, the decrease in the rate of recovery to an acid load in low Cl^- media suggests that the cessation of Cl^- - HCO_3^- exchanger activity as a result of the decreased pH_i is important in the recovery by retaining base and buffering intracellular protons. The same mechanism is not as significant in recovery in the other two species where the Na^+ - H^+ exchanger is the main method of proton extrusion.

Since the anion exchanger blocker SITS had no effect on recovery rate following a base load in any species, this study does not support a role for Cl^- - HCO_3^- exchange in this process. Following a base load, the decrease in pH_i recovery rate observed with amiloride in trout hepatocytes suggests that under control conditions, the protons being retained when the Na^+ - H^+ exchanger turns off contribute to buffering intracellular base. The fact that membrane transporters are tonically active means that they can contribute to pH_i regulation not only by increasing their activity, but also by decreasing their activity under certain conditions.

CHAPTER 4
EVIDENCE FOR A ROLE OF THE $\text{Na}^+\text{-HCO}_3^-$ COTRANSPORTER IN TROUT
HEPATOCYTE pH_i REGULATION

Taken from:

Furimsky, M., Moon, T.W. and Perry, S.F. (2000). Evidence for the role of a $\text{Na}^+\text{-HCO}_3^-$ cotransporter in trout hepatocyte pH_i regulation. *J. exp. Biol.* 203 : 2201-2208.

ABSTRACT

The mechanisms of intracellular pH (pH_i) regulation were examined in hepatocytes of the rainbow trout, *Oncorhynchus mykiss*. Intracellular pH was monitored using the pH-sensitive fluorescent dye BCECF and the effects of varying media and pharmacological agents were examined for their influence on baseline pH_i and recovery rates to acid- and base-loading. Rates of Na^+ uptake were measured using ^{22}Na and changes in membrane potential were examined using the potentiometric fluorescent dye Oxonol VI. The rate of proton extrusion following acid loading was diminished by the blockade of either $\text{Na}^+\text{-H}^+$ exchange (using amiloride) or anion transport (using DIDS). The removal of external HCO_3^- and the abolishment of outward K^+ diffusion by the channel blocker Ba^{2+} also decreased the rate of proton extrusion following acid loading. Depolarisation of the cell membrane with $50 \text{ mmol l}^{-1} \text{ K}^+$, however, did not affect pH_i . The rate of recovery to base loading was significantly diminished by the blockade of anion transport, removal of external HCO_3^- and to a lesser extent by blocking $\text{Na}^+\text{-H}^+$ exchange. The blockade of K^+ conductance had no effect. The decrease of Na^+ uptake rate observed with the anion transport blocker DIDS and the DIDS-sensitive hyperpolarisation of membrane potential during recovery to acid loading suggests that a Na^+ -dependent electrogenic transport system is involved in the restoration of pH_i after intracellular acidification. The effects on baseline pH_i indicate that the different membrane exchangers are tonically active in the maintenance of steady-state pH_i . This study confirms the roles of a $\text{Na}^+\text{-H}^+$ exchanger and a $\text{Cl}^-\text{-HCO}_3^-$ exchanger in trout hepatocyte pH_i regulation and provides new evidence that a $\text{Na}^+\text{-HCO}_3^-$ cotransporter contributes to pH_i regulation.

INTRODUCTION

The regulation of intracellular pH (pH_i) in eukaryotic cells is a concerted effort involving cytosolic buffers and different membrane transport proteins. The participation of the specific proteins in pH_i regulation varies according to tissue and species. The Na^+ - H^+ exchanger (NHE gene family) is presumed to be present in all eukaryotic cells and participates in acid extrusion by way of 1:1 electroneutral exchange of intracellular H^+ with extracellular Na^+ (Noël and Pouyssegur, 1995; Yun *et al.*, 1995; Wakabayashi *et al.*, 1997; see Chapter 1, Fig. 1-1). Several HCO_3^- -dependent mechanisms are known to participate in pH_i regulation, namely Na^+ -dependent and Na^+ -independent Cl^- - HCO_3^- exchange and Na^+ - HCO_3^- cotransport (Madshus, 1988). The Cl^- - HCO_3^- exchanger (AE or anion exchanger gene family) plays a role in recovery to intracellular alkalinisation by removing HCO_3^- from the cytoplasm in exchange for extracellular Cl^- in a 1:1 electroneutral fashion (Tonnessen *et al.*, 1990; Alper, 1994). The role of the electrogenic Na^+ - HCO_3^- cotransporter (NBC gene family) has been well documented in the mammalian kidney where it participates in Na^+ and HCO_3^- reabsorption (Boron *et al.*, 1997). The Na^+ - HCO_3^- cotransporter can also indirectly behave as an acid extruder in some tissues (Gleeson *et al.*, 1989; Dart and Vaughan-Jones, 1992; de la Rosa *et al.*, 1999) as a result of the buffering of intracellular H^+ by the HCO_3^- entering the cell. The recent cloning of the Na^+ - HCO_3^- cotransporter (Burnham *et al.*, 1997, 1998; Romero *et al.*, 1997, 1998) facilitates studies of the expression and regulation of this protein in kidney and other tissues.

Previous studies have examined pH_i regulation in teleost hepatocytes with varying results. In trout (*Oncorhynchus mykiss*) hepatocytes, a Na^+ - H^+ exchanger and a Cl^- - HCO_3^- exchanger are important in regulation of pH_i (Walsh, 1986; Fossat *et al.*, 1997), whereas in

the gulf toadfish (*Opsanus beta*), pH_i regulation is achieved exclusively by modulation of $\text{Na}^+\text{-H}^+$ exchange (Walsh, 1989). A more recent comparative study (Chap. 3; Furimsky *et al.*, 1999a) has demonstrated that in addition to trout hepatocytes, those of black bullhead (*Ameiurus melas*) and American eel (*Anguilla rostrata*) also rely on the $\text{Na}^+\text{-H}^+$ exchanger and $\text{Cl}^-\text{-HCO}_3^-$ exchanger for pH_i regulation. In that same study, the $\text{Na}^+\text{-H}^+$ exchanger, though present in the eel, was not activated by a decrease in pH_i , as it was in the other species. In rat hepatocytes, an electroneutral $\text{Na}^+\text{-HCO}_3^-$ cotransporter participates significantly along with the $\text{Na}^+\text{-H}^+$ exchanger in net acid extrusion following a decrease in cytosolic pH (Gleeson *et al.*, 1989). The activity of the $\text{Na}^+\text{-HCO}_3^-$ cotransporter in rat hepatocytes has been linked to that of a K^+ channel, where a decrease in pH_i leads to a decrease in K^+ conductance, with the resulting membrane depolarization activating $\text{Na}^+\text{-HCO}_3^-$ cotransport (Fitz *et al.*, 1992).

In the few studies that have investigated pH_i regulation in teleost hepatocytes, no evidence has been provided for a possible role of a $\text{Na}^+\text{-HCO}_3^-$ cotransporter. A recent study by Furimsky *et al.* (1999a; Chapter 3), however, suggested that an additional Na^+ -dependent ion transport mechanism may be participating in proton extrusion following an acid load in trout. This hypothesis was proposed because removal of extracellular Na^+ had a more pronounced effect on pH_i recovery to an acid load than did the $\text{Na}^+\text{-H}^+$ exchanger blocker amiloride given alone.

The goal of this chapter was to dissect the mechanisms of pH_i regulation in trout hepatocytes and specifically to provide evidence that a $\text{Na}^+\text{-HCO}_3^-$ cotransporter is an integral component of this regulatory mechanism.

MATERIALS AND METHODS

Experimental animals

Rainbow trout, *Oncorhynchus mykiss* (Walbaum), weighing 200 to 300 g were obtained from Linwood Acres Trout Farm (Campbellcroft, Ontario, Canada). Fish were transported and kept as described in Chapter 2. Experiments were performed between July and September, following at least two months of acclimation to the holding facility.

Cell isolation

Hepatocytes were isolated according to the method of Mommsen *et al.* (1994) as described in detail in Chapter 2.

Measurement of pH_i

Intracellular pH was measured using the pH-sensitive fluorescent dye BCECF as described in detail in Chapter 3. Experimental media contained either (or a combination of) the Na^+ - H^+ exchanger blocker amiloride (1 mmol l^{-1}), the anion transporter blocker 4, 4'-diisothiocyanostilbene-2, 2'-disulfonic acid (DIDS) (0.5 mmol l^{-1}), the K^+ channel blocker Ba^{2+} ($1 \text{ mmol l}^{-1} \text{ BaCl}_2$) or a lack of $NaHCO_3$. All blockers were dissolved in the experimental media at the experimental concentrations, with sonication being necessary for amiloride and DIDS. Membrane depolarization was induced by washing cells in a medium in which $44 \text{ mmol l}^{-1} \text{ NaCl}$ was replaced with KCl to give a final $[K^+]$ of 50 mmol l^{-1} . Baseline pH_i values were established prior to the acid or base challenge (see Fig. 4-1). Acid loading was induced by the addition of Na-propionate ($150 \text{ }\mu\text{l}$ of 1 mol l^{-1} stock; 50 mmol l^{-1} final concentration) directly to the cuvette containing the cell suspension. Base loading was

induced by the addition of NH_4Cl (60 μl of 1 mol l^{-1} stock; 20 mmol l^{-1} final concentration) to the cell suspension (see Fig. 4-1).

Intracellular Buffer Capacity

The total apparent intracellular buffer capacity (β), reported as $\text{mmol H}^+ \text{pH unit}^{-1} \text{l}^{-1}$ was measured in hepatocytes incubated in either a HCO_3^- -containing medium (5 mmol.l^{-1} NaHCO_3) or a HCO_3^- -free medium. Buffer capacities were determined using the following equation

$$\beta = ([\text{Propionate}]10(\text{pH}_{\min} - \text{pK}_a)) / (1 + 10 (\text{pH}_{\text{input}} - \text{pK})) \times (\text{pH}_{\text{cyto}} - \text{pH}_{\min})$$

where $[\text{Propionate}]$ is 50 mmol l^{-1} , pK_a of propionate at room temperature is 4.87, $\text{pH}_{\min}$ is the minimal pH_{cyto} following acid loading, pH_{input} is the pH of the medium (7.9) and pH_{cyto} is baseline pH_i prior to acid load.

The rate of influx or extrusion of H^+ equivalents were determined as

$$J = k.\beta$$

where k is the initial rate of recovery (pH units min^{-1}) to an acid or a base load and β is the apparent intracellular buffer capacity ($\text{mmol H}^+ \text{pH unit}^{-1} \text{l}^{-1}$).

Carbonic Anhydrase (CA) Assay

The delta pH method was followed to measure CA activity, as described by Henry (1991). CA activities were measured in lysates produced from erythrocytes, isolated hepatocytes and liver tissue. The volume or weight of the tissue was determined and diluted in appropriate volumes of ddH₂O in order to lyse the cells and release intracellular CA into the supernatant.

The reaction occurred in a chamber in which all solutions were constantly mixed and kept at 4°C using a temperature controlled water bath. The standard reaction consisted of 10 ml of reaction medium (10 mmol l⁻¹ Tris base, 225 mmol l⁻¹ mannitol, 75 mmol l⁻¹ sucrose; pH 7.4), 50 µl of the enzyme source (CA from lysates), and 400 µl of the substrate (CO₂-saturated H₂O) that gave a concentration of approximately 2.7 mM CO₂ in the chamber. The pH of the reaction was measured using a pH glass electrode (Metrohm). The buffer capacity of the solution was determined by measuring the change in pH following the addition of a known concentration of acid (50 µl of 0.1 mol l⁻¹ HCl) to the reaction medium. The non-catalysed rates of CA activity were calculated by the change in pH following the addition of CO₂ saturated water (400 µl of ~ 2.7 mmol l⁻¹ CO₂) to reaction medium without CA. The rates of CA activity were determined from the slope of the pH change over time. These values were continuously verified throughout the experiment and subtracted from the values for the catalysed rates.

Na⁺ flux studies

The rate of Na⁺ uptake was measured at room temperature (20°C) using sodium-22 (²²Na⁺) as tracer. Experiments were performed on 4 ml of cell suspension at a cytocrit of ~3%, representing approximately 15 million cells ml⁻¹. Cells were incubated in their respective media for 20 min and then exposed to ouabain (4 µl of 1 mol l⁻¹ stock in DMSO; final concentration of 1 mmol l⁻¹) for 20 min to block Na⁺-K⁺ ATPase activity. Uptake measurements were initiated with the addition of ²²Na⁺ directly to the cell suspension. At different time intervals, 300 µl aliquots were removed in triplicate and added to microcentrifuge tubes (1.5 ml capacity) containing ice-cold stopping buffer (110 mmol l⁻¹ MgCl₂, 10 mmol l⁻¹ imidazole, pH 7.90) and washed twice in this same medium. Cell pellets were lysed by the addition of 0.5 ml 0.005% Triton-X and deproteinised with 0.5 ml 1% trichloroacetic acid (TCA). Following centrifugation, the supernatant was collected into 25 ml capacity plastic scintillation vials. Radioactivity was measured in a liquid scintillant (Safety Solve High Flash Point Scintillation Cocktail, RPI, Mount Prospect, IL) using a 2500 TR Liquid Scintillation Analyzer (Packard). Total ²²Na⁺ uptake was measured at 5 min intervals for 20 min following addition of the isotope.

Membrane Potential

Changes in membrane potential were determined using the fluorescent potentiometric slow-response dye Oxonol VI (Molecular Probes, Eugene, OR). Unlike BCECF, this dye is not contained exclusively within cells during an experiment, but is free to move between the medium and the cytosol following changes in membrane potential. Oxonol is a lipophilic anion molecule that fluoresces only when in contact with the cell membrane. When the

membrane becomes more positive, Oxonol enters the cell and accumulates on the inside of the membrane resulting in an increase in fluorescence. A 3 μl volume of a concentrated stock solution of the dye ($500 \mu\text{mol l}^{-1}$ in 95% ethanol) was added to the cell suspension (3 ml at $10^6 \text{ cells ml}^{-1}$) to give a final concentration of approximately 500 nmol l^{-1} . The dye was allowed to equilibrate with the cells for 15 min prior to the beginning of the experiment (see Fig. 4-9). The excitation and emission wavelengths were 614 nm and 646 nm, respectively; fluorescence was measured using a fluorescence spectrophotometer as noted for BCECF in Chapter 3. The fluorescence intensities varied slightly from experiment to experiment, so data are presented as relative fluorescence with all values being divided by the baseline fluorescence values.

Statistical Analysis

Data are presented as the mean $\pm$ one standard error of the mean (SEM). Each trial represents an experiment performed on a single cell preparation from one individual fish. Statistical analysis was conducted using either a one way ANOVA followed by a Student-Newman-Keuls test for the pair-wise analysis of means, or a two way ANOVA with repeated measures using the Student-Newman-Keuls test for the pair-wise analysis of means. Significant differences were accepted as $p < 0.05$.

RESULTS

Steady-state pH_i

The different experimental treatments had varying effects on baseline pH_i suggesting that different membrane transporters are tonically active under resting conditions (Table 4-1). All experimental media, though chemically different, were adjusted to the same pH of 7.9. Amiloride and DIDS decreased baseline pH_i, an effect that was exaggerated when both were in the medium together. The significant decrease in baseline pH_i observed when extracellular HCO₃⁻ was removed from the experimental medium was enhanced by the addition of amiloride to this same medium. In all cases, the addition of Ba²⁺ to the medium decreased baseline pH_i.

Intracellular buffer capacity

The total apparent intracellular buffer capacity (β) was determined in hepatocytes under control (medium containing 5 mmol l⁻¹ NaHCO₃) and HCO₃⁻-free conditions. The buffer capacity in HCO₃⁻-containing medium was 45.3 ± 3.2 mmol H⁺ pH unit⁻¹ l⁻¹ (n=6), while that in HCO₃⁻-free medium was 32.7 ± 1.5 mmol H⁺ pH unit⁻¹ l⁻¹. These values were taken into account when determining the influx or extrusion of H⁺ equivalents in the different experimental media.

Table 4-1: Resting pH_i values, maximal pH_i changes (ΔpH_i) and relative recoveries in hepatocyte recovery experiments.

	pH_i ($n=12$)	ΔpH_i (acid) ($n=6$)	% Recovery (acid)	ΔpH_i (alkaline) ($n=6$)	% Recovery (alkaline)
Control	7.71 ± 0.01	0.29 ± 0.02	97.8 ± 5.1	0.40 ± 0.02	51.0 ± 2.2
Amiloride (1 mmol.l^{-1})	$7.62 \pm 0.01^*$	0.29 ± 0.01	$63.2 \pm 7.6^*$	0.39 ± 0.02	$38.8 \pm 3.5^*$
DIDS (0.5 mmol.l^{-1})	$7.61 \pm 0.02^*$	0.25 ± 0.02	$66.5 \pm 3.0^*$	0.43 ± 0.02	$23.7 \pm 1.4^*$
Amiloride (1 mmol.l^{-1}) + DIDS (0.5 mmol.l^{-1})	$7.43 \pm 0.02^*$	$0.22 \pm 0.02^*$	$43.2 \pm 8.4^*$	0.36 ± 0.03	$24.5 \pm 2.7^*$
Control	7.72 ± 0.01	0.29 ± 0.01	95.0 ± 5.3	0.38 ± 0.03	59.7 ± 5.9
HCO_3^- free	$7.67 \pm 0.01^*$	$0.37 \pm 0.02^*$	$77.5 \pm 4.0^*$	0.37 ± 0.02	$32.7 \pm 0.8^*$
HCO_3^- free + Amiloride (1 mmol.l^{-1})	$7.63 \pm 0.01^*$	$0.35 \pm 0.02^*$	$50.8 \pm 5.1^*$	0.35 ± 0.02	$25.2 \pm 1.9^*$
Ba^{2+} (1 mmol.l^{-1})	$7.62 \pm 0.02^*$	0.28 ± 0.01	88.5 ± 5.7	0.32 ± 0.02	56.0 ± 5.0
HCO_3^- free + Ba^{2+} (1 mmol.l^{-1})	$7.64 \pm 0.01^*$	$0.37 \pm 0.03^*$	$67.5 \pm 6.1^*$	0.34 ± 0.02	$37.0 \pm 3.0^*$

Values are means $\pm$ SEM.

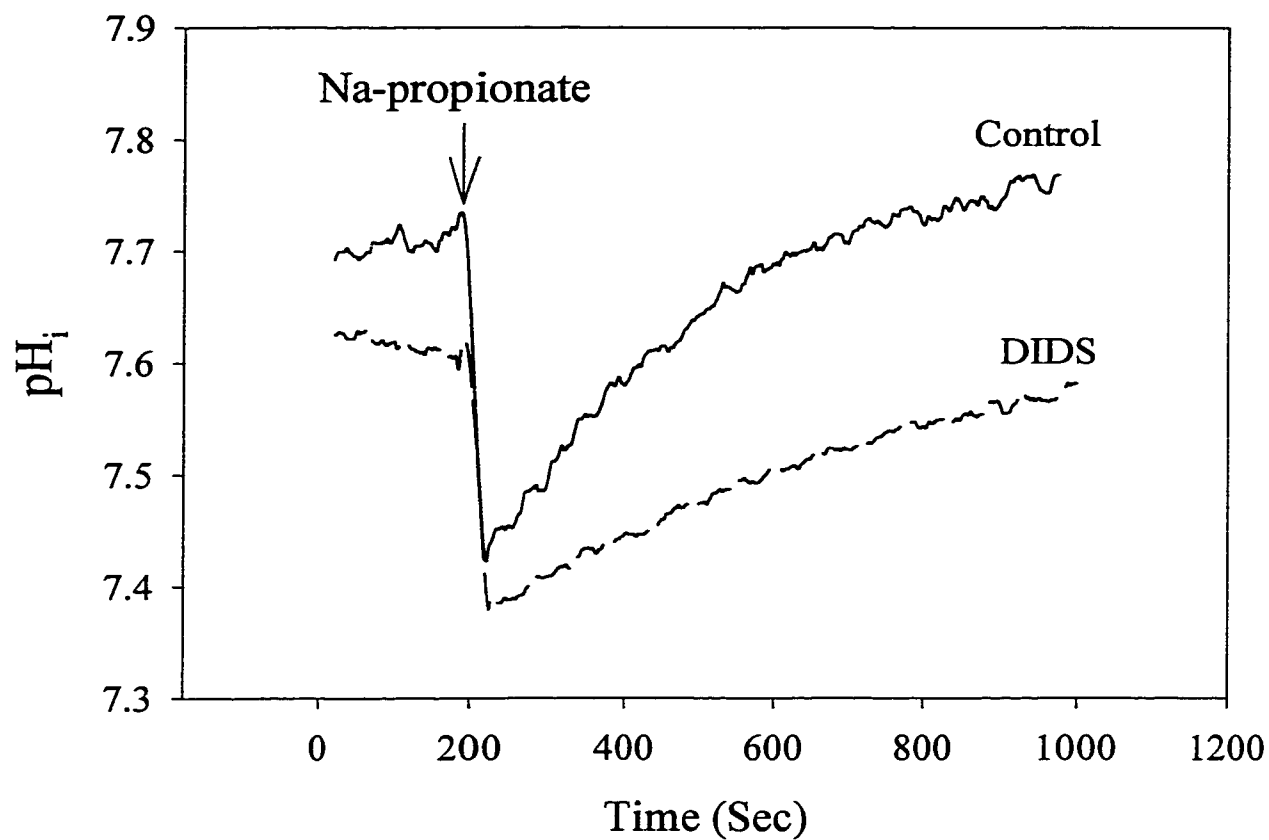
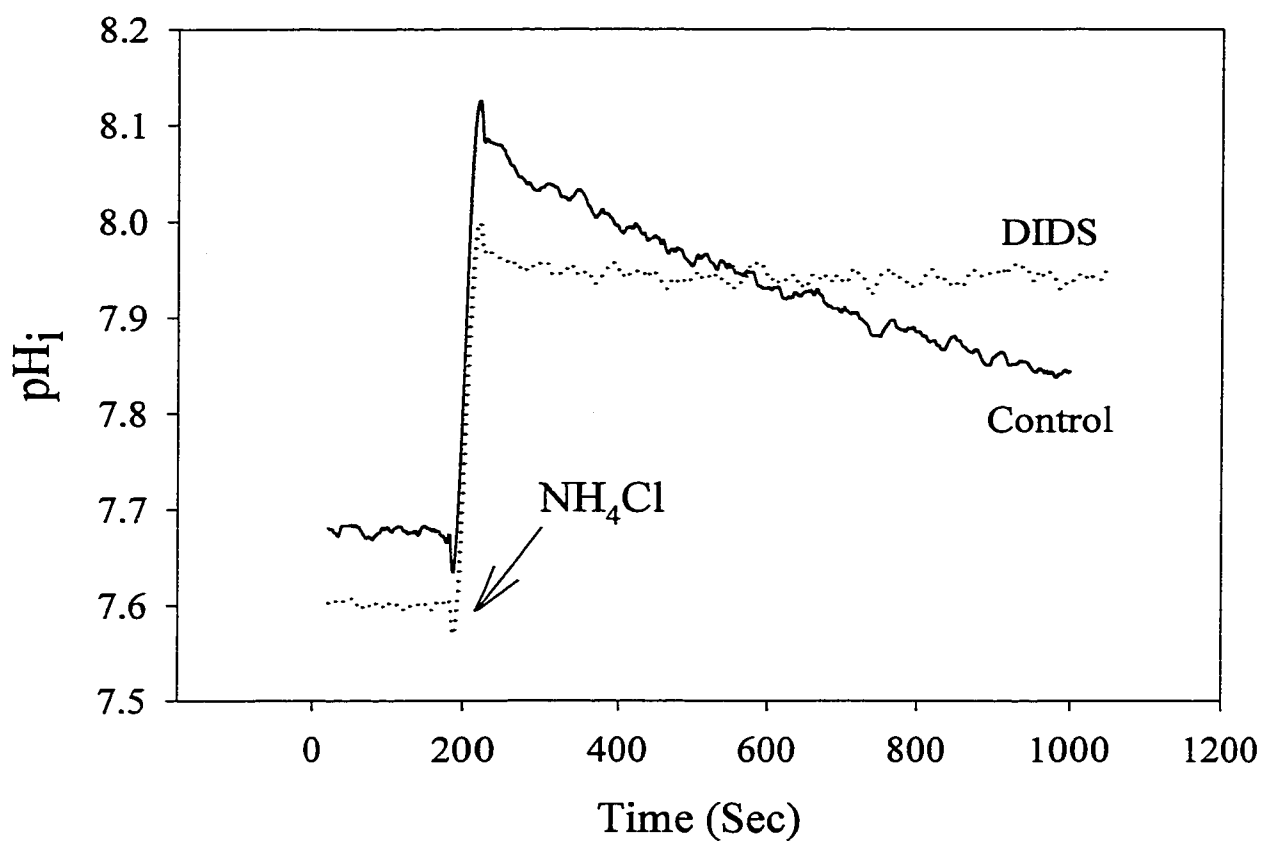
The relative recoveries (% recovery back to baseline pH_i) were determined 8 min following intracellular acidification or alkalization.

* indicates a significant difference from respective control values.

Recovery to acid loading

The mechanism of pH_i recovery to an acid load involves different membrane transporters whose roles can be dissected pharmacologically (see Fig. 4-1A). The rate of proton extrusion following acid loading was decreased by the Na^+ - H^+ exchanger blocker amiloride (1 mmol l^{-1}), as well as by DIDS (0.5 mmol l^{-1}), a compound that inhibits activity of both the Cl^- - HCO_3^- exchanger and Na^+ - HCO_3^- cotransporter (Fig. 4-2). When amiloride and DIDS were combined in the experimental medium, the rate of proton extrusion following acid loading was further decreased. Removal of HCO_3^- from the extracellular medium diminished proton extrusion following acid loading (Fig. 4-3). The K^+ channel blocker Ba^{2+} also decreased proton extrusion, but to a lesser extent. When Ba^{2+} was added to the HCO_3^- -free medium, the rate of proton extrusion was not different from that in the HCO_3^- -free medium alone. However, the addition of amiloride to the HCO_3^- -free medium decreased the rate of recovery significantly compared to those same treatments individually (Fig. 4-3). The relative recoveries to baseline pH_i at 8 min (the time where full recovery usually occurred following an acid load under control conditions) showed that, with the exception of Ba^{2+} (1 mmol l^{-1}), the inhibitory effects of the different treatments were consistent over time (Table 4-1). The addition of the carbonic anhydrase (CA) inhibitor acetazolamide (0.1 mmol l^{-1}) had no significant effects on recovery to an acid load, even in combination with amiloride in a HCO_3^- free medium (Fig. 4-4). Despite not having a direct effect on pH_i regulation, significant CA activity was measured in hepatocytes (though activities were much lower than those found in erythrocytes) that was almost completely blocked by acetazolamide (Fig. 4-5).

Figure 4-1. Representative time course traces of intracellular pH in trout hepatocytes under control conditions (solid lines) and DIDS inhibition (dotted lines) following (A) acid loading with 50 mmol l⁻¹ Na propionate or (B) base loading with 20 mmol l⁻¹ NH₄Cl.

A**B**

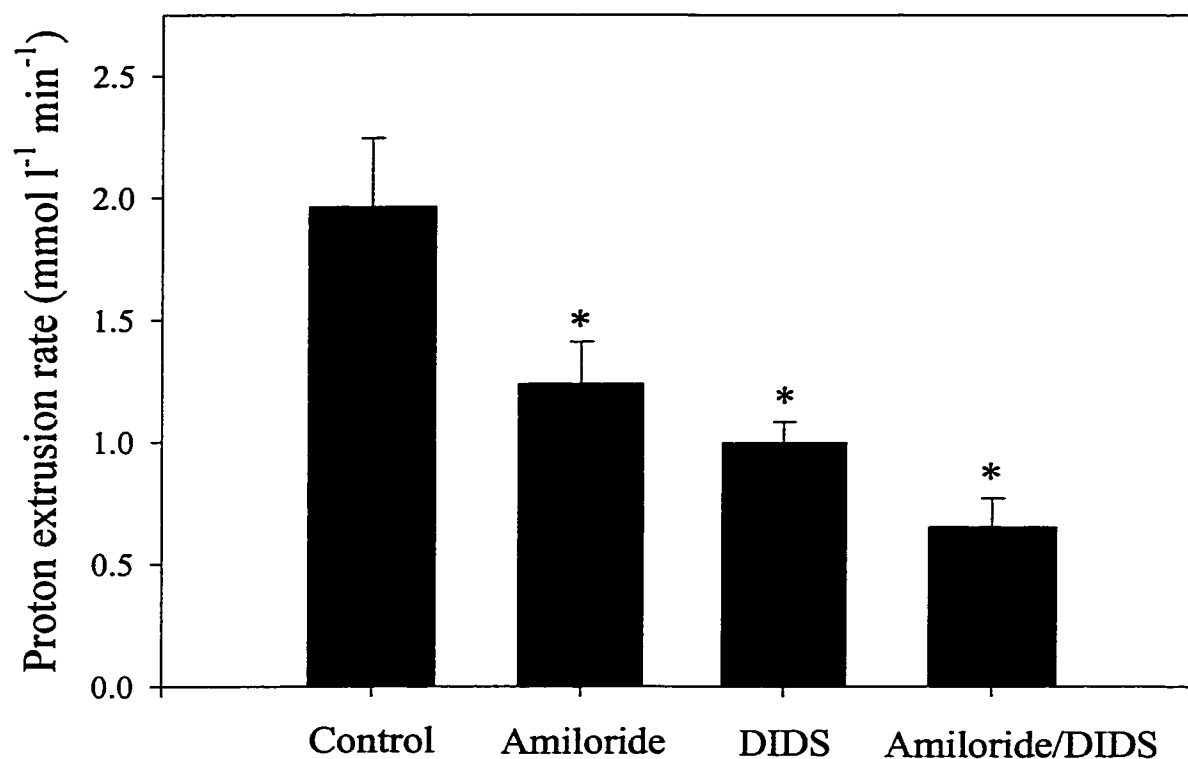


Figure 4-2. Rates of extrusion of proton equivalents following intracellular acid loading as influenced by amiloride (1 mmol l⁻¹) and DIDS (0.5 mmol l⁻¹). Data are presented as the means $\pm$ SEM of 6 trials. * indicates a significant difference from control values.

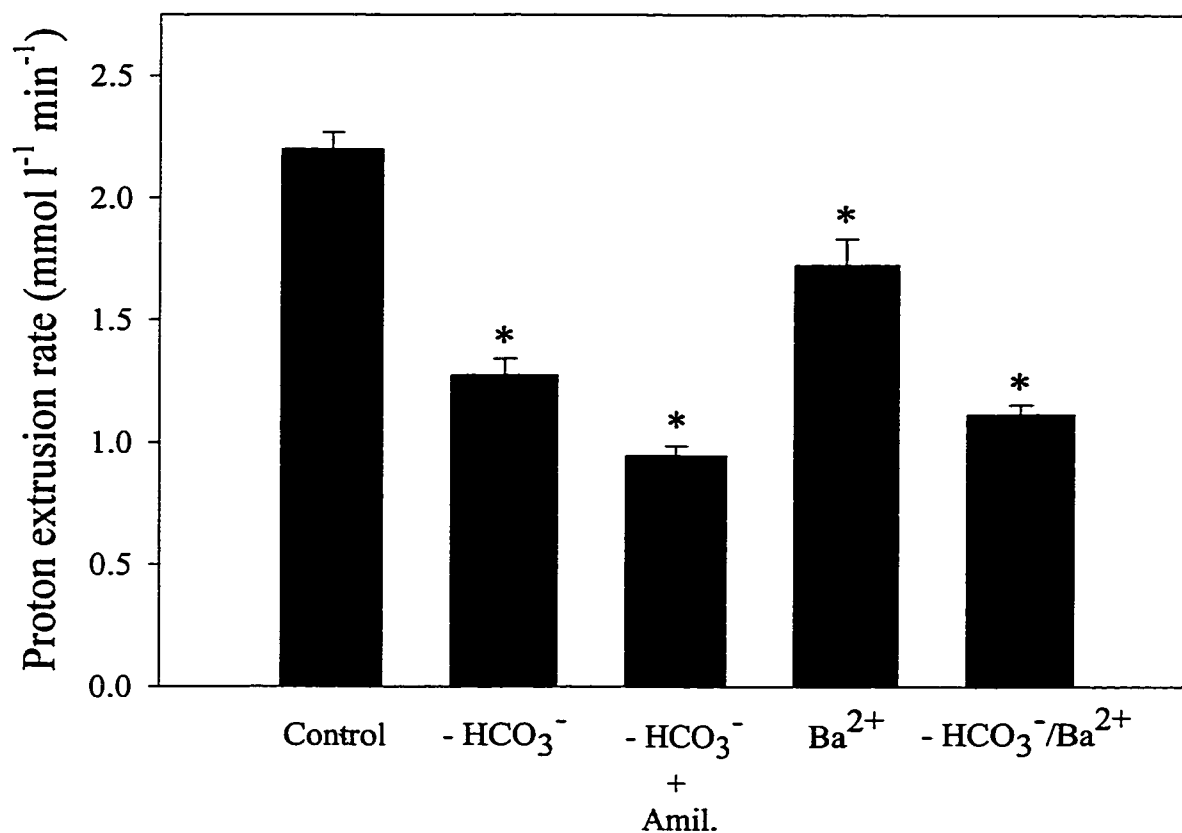


Figure 4-3. Rates of extrusion of proton equivalents following intracellular acid loading as influenced by extracellular HCO₃⁻, amiloride (Amil., 1 mmol l⁻¹) and Ba²⁺ (1 mmol l⁻¹). Data are presented as the means ± SEM of 6 trials. * indicates a significant difference from control values.

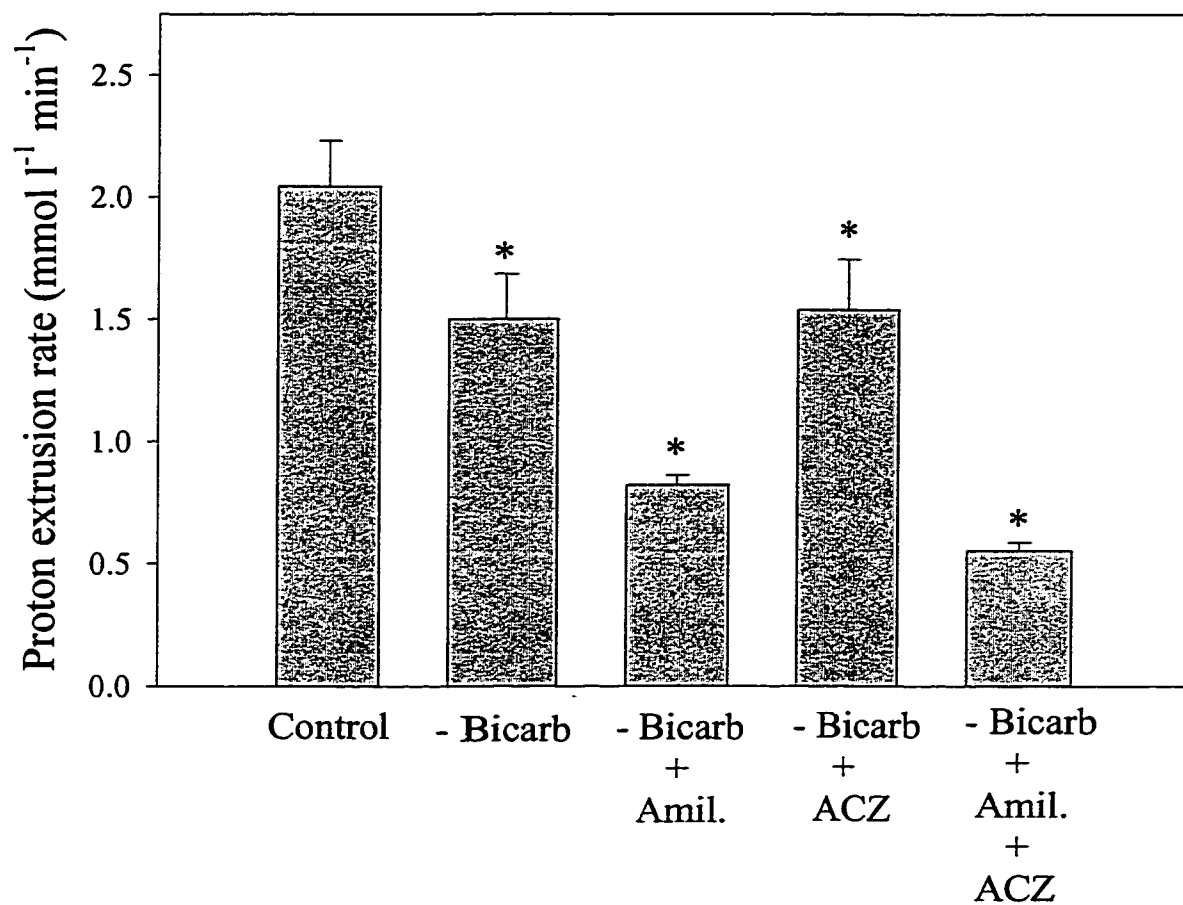


Figure 4-4. Rates of proton extrusion in hepatocytes following an acid load as influenced by extracellular HCO_3^- , amiloride (Amil.) and acetazolamide (ACZ, 0.1 mmol l⁻¹). Values are presented as the means $\pm$ SEM (n=6). * represents a significant difference from control values.

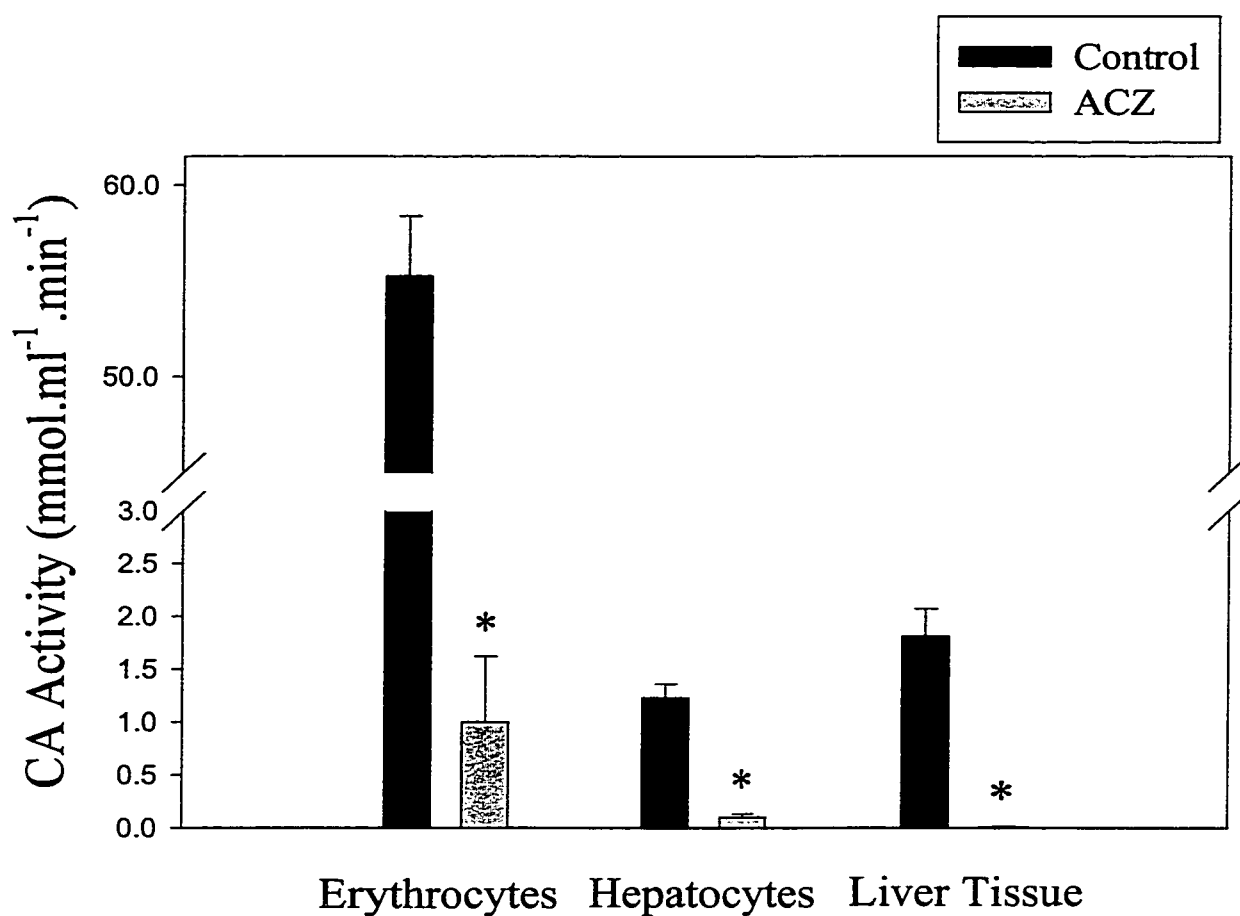


Figure 4-5. Carbonic anhydrase activity measured in isolated erythrocytes, hepatocytes, and liver tissue. The effect of the CA inhibitor, acetazolamide (0.1 mmol l^{-1}) was examined in all preparations. * represents a significant decrease in CA activity by acetazolamide (ACZ). Values are presented as the means $\pm$ SEM (n=6).

Recovery to base loading

The rate of influx of proton equivalents following base loading was decreased by the $\text{Na}^+\text{-H}^+$ exchange blocker amiloride and to a greater extent by the anion transport blocker DIDS (Fig. 4-6). Recovery to base loading was also dependent on extracellular HCO_3^- , as shown by the decrease in recovery in its absence (Fig. 4-7). The rate of proton influx in HCO_3^- -free medium that contained amiloride or Ba^{2+} was not different from that in HCO_3^- -free medium alone. The K^+ channel blocker Ba^{2+} had no effect on recovery rate in normal medium. The relative recoveries 8 min following the base load were consistent with the inhibitory effects observed initially (Table 4-1). The degree of intracellular alkalization was similar in all treatments (Table 4-1).

Na^+ fluxes

The rate of unidirectional Na^+ uptake into hepatocytes was calculated from the regression of total Na^+ uptake at each time point from 5 to 20 min. The Na^+ uptake rate was unstable during the initial 5 min assessment period but remained constant in the subsequent 15 min period. The activity was not assumed to be zero at time zero because trapped extracellular fluids in the cell pellets or non-specific binding could increase baseline levels of $^{22}\text{Na}^+$. Indeed, the regression analyses demonstrated a positive y-intercept. Thus, the rates of Na^+ uptake were significantly decreased in hepatocytes in the presence of amiloride or DIDS (Fig. 4-8).

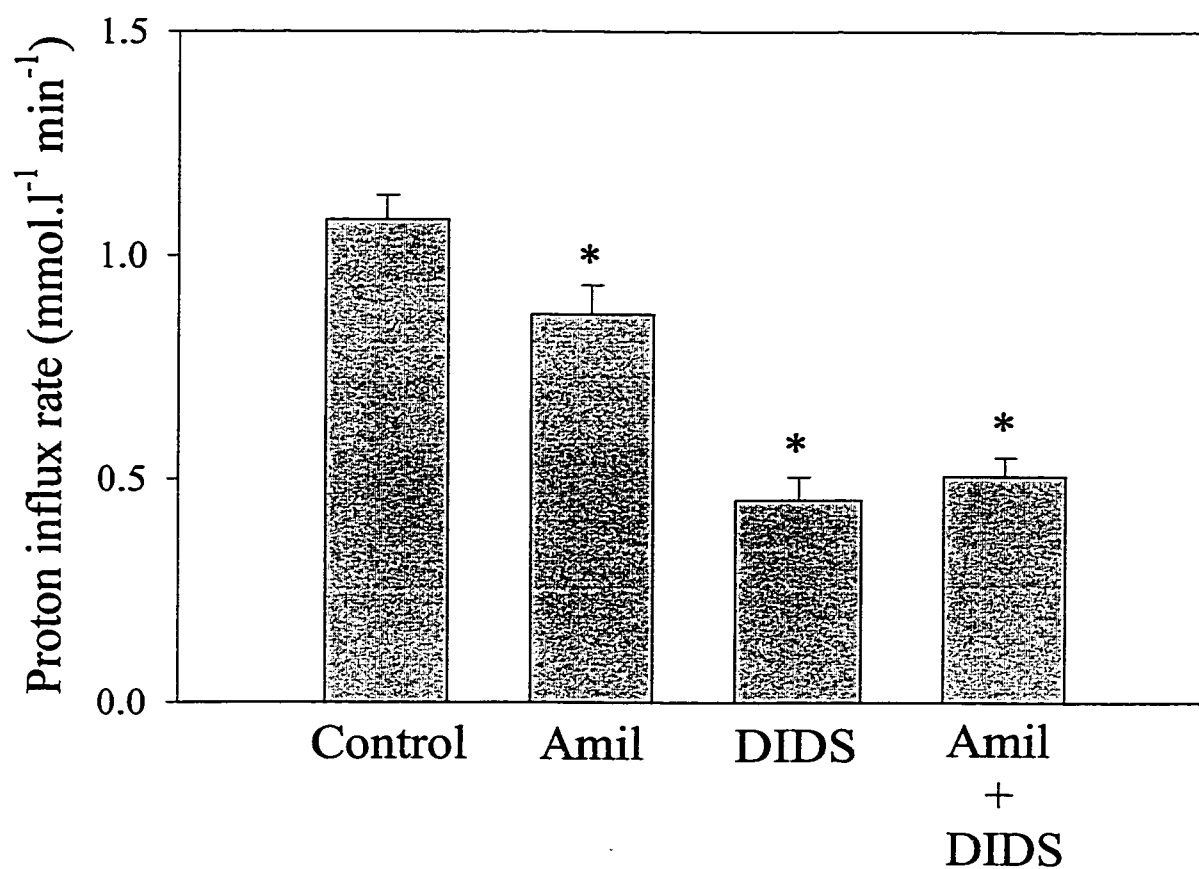


Figure 4-6. Rates of proton equivalent influx following intracellular base loading as influenced by amiloride (Amil, 1 mmol l⁻¹) and DIDS (0.5 mmol l⁻¹). Data are presented as the mean $\pm$ SEM of 6 trials. * indicates a significant difference from control values.

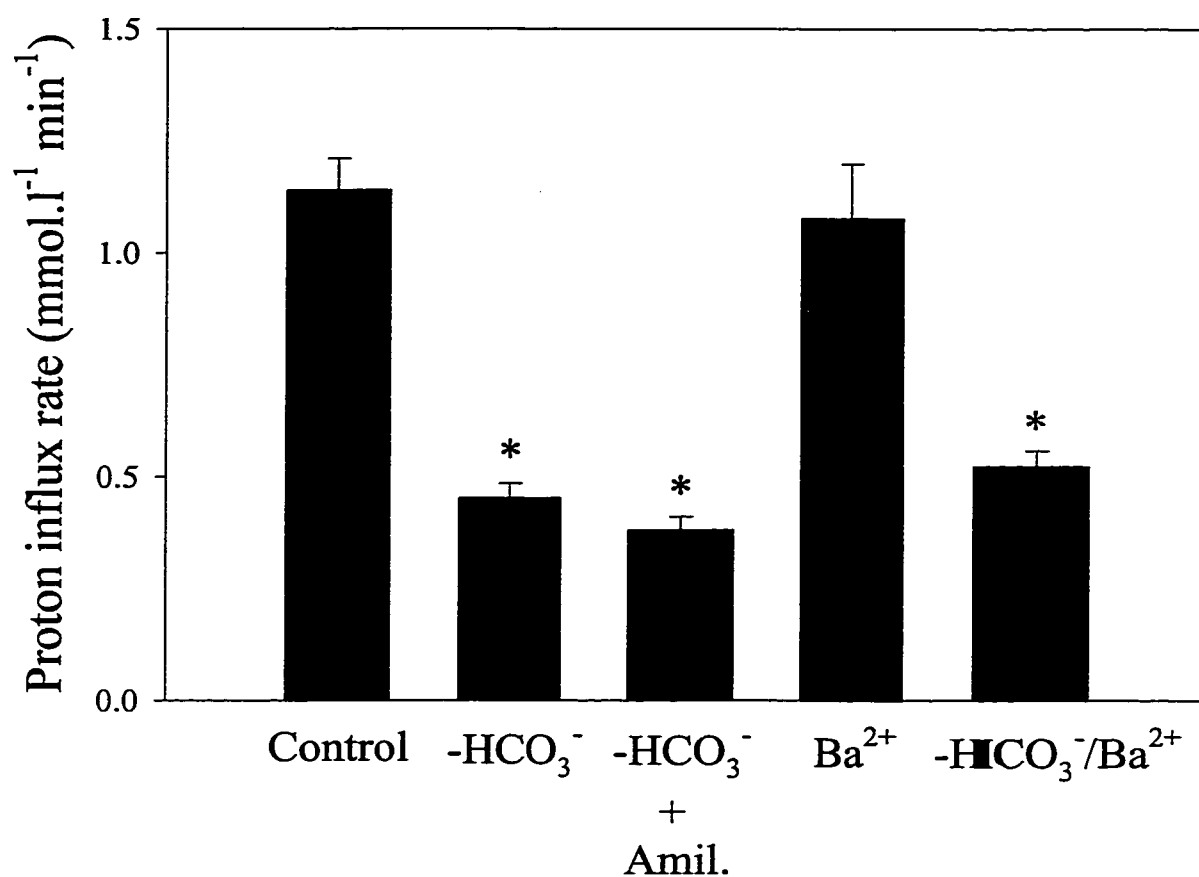


Figure 4-7. Rates of influx of proton equivalents following intracellular base loading as influenced by extracellular HCO₃⁻, amiloride (Amil., 1 mmol l⁻¹) and Ba²⁺ (1 mmol l⁻¹). Data are presented as the mean ± SEM for 6 trials. * indicates a significant difference from control values.

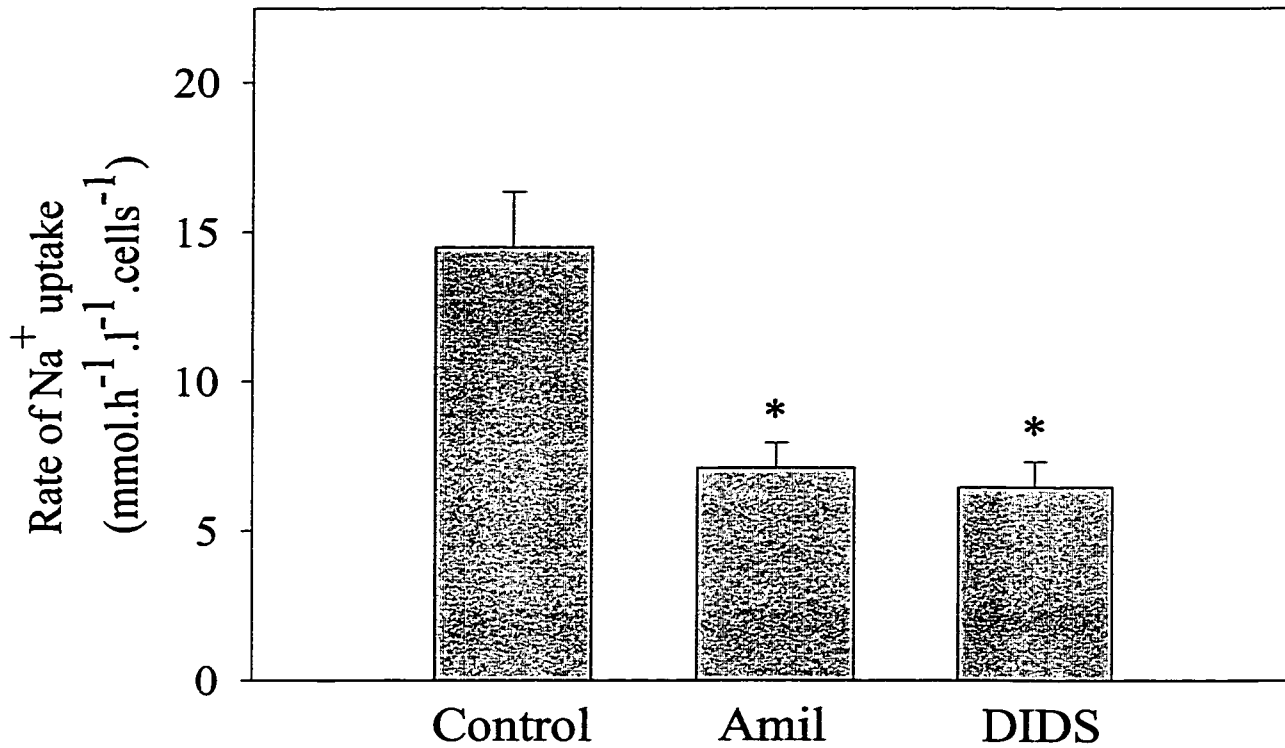


Figure 4-8. Rates of Na⁺ uptake in hepatocytes, as influenced by amiloride (Amil) and DIDS. Rates were determined over a 20 min experimental period. Data are presented as the mean $\pm$ SEM for 5 trials. * indicates a significant difference from control values.

Membrane depolarisation

The relationship between a HCO_3^- -dependent transport protein and a K^+ channel was investigated by observing the direct effects of cell depolarization on pH_i . When cells were depolarized by the addition of $50 \text{ mmol l}^{-1} \text{ K}^+$ to the extracellular medium, no significant effect on pH_i was observed in either normal or HCO_3^- -free media. The mean change in baseline pH_i (ΔpH_i) following cell depolarization in normal medium was -0.015 ± 0.02 , whereas that in a HCO_3^- -free medium was 0.001 ± 0.01 ($n=5$).

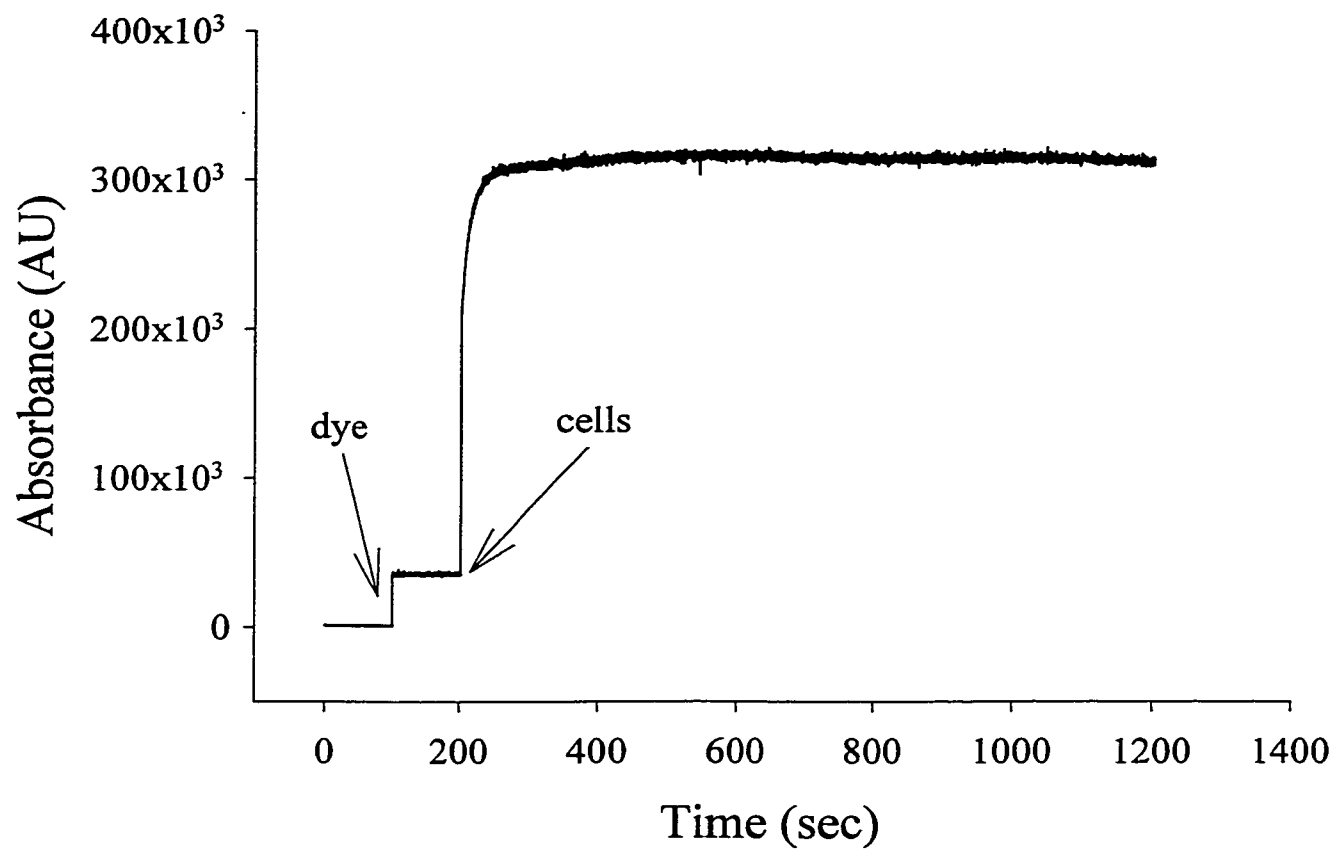
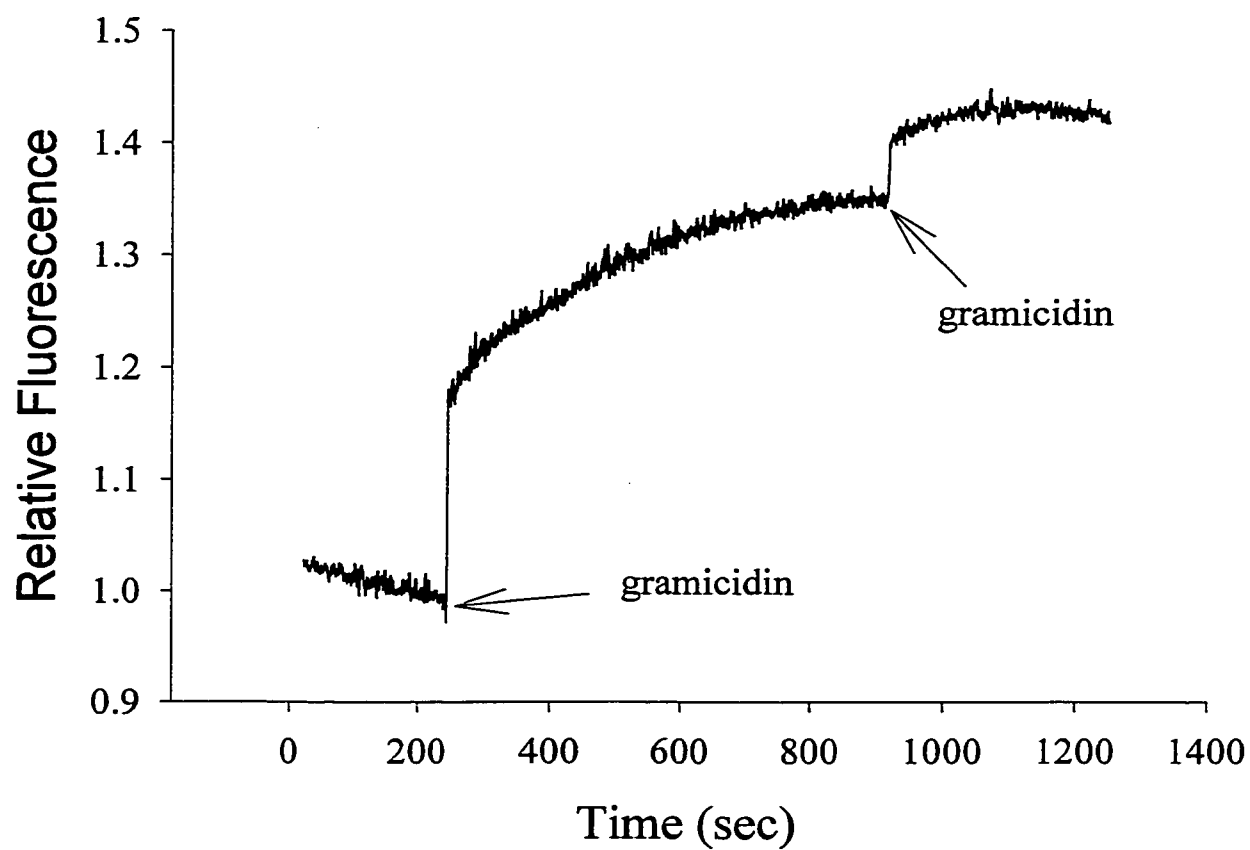
Membrane potential

The potentiometric fluorescent dye Oxonol VI was employed to measure changes in membrane potential (E_m). This dye does not fluoresce unless in direct contact with the cell membrane (Fig. 4-9A). An increase in fluorescence indicates a more positive membrane potential (depolarisation) and vice versa. The membrane potentials were not directly measured, but baseline values were at a negative potential, indicated by the addition of $1 \text{ } \mu\text{mol l}^{-1}$ gramicidin (Fig. 4-9B). This antibiotic forms non-selective cation pores in the cell membrane, exchanging Na^+ and K^+ , causing total depolarisation indicated by the increased fluorescence to a value representing a E_m of 0 (Fig. 4-9B).

Directly following the addition of Na-propionate, a slight decrease in fluorescence was observed (Fig. 4-10). This immediate decrease can be explained by dilution of dye signal and does not reflect a change in membrane potential. The addition of an equal volume of saline ($150 \text{ } \mu\text{l}$) to the medium had no effect other than decreasing the baseline fluorescence ($\sim 5\%$). During the recovery phase to acid loading, the hepatocyte membrane experienced an

initial slight depolarization and then a hyperpolarisation (potential became more negative) that was diminished when DIDS was in the medium (Fig. 4-10).

Figure 4-9. Representative traces showing (A) the addition of trout hepatocytes to Oxonol VI and (B) the effects of the addition of gramicidin ($1 \mu\text{mol l}^{-1}$) to medium containing cells and Oxonol VI. Gramicidin forms non-specific cation pores and depolarises the cell membrane.

A**B**

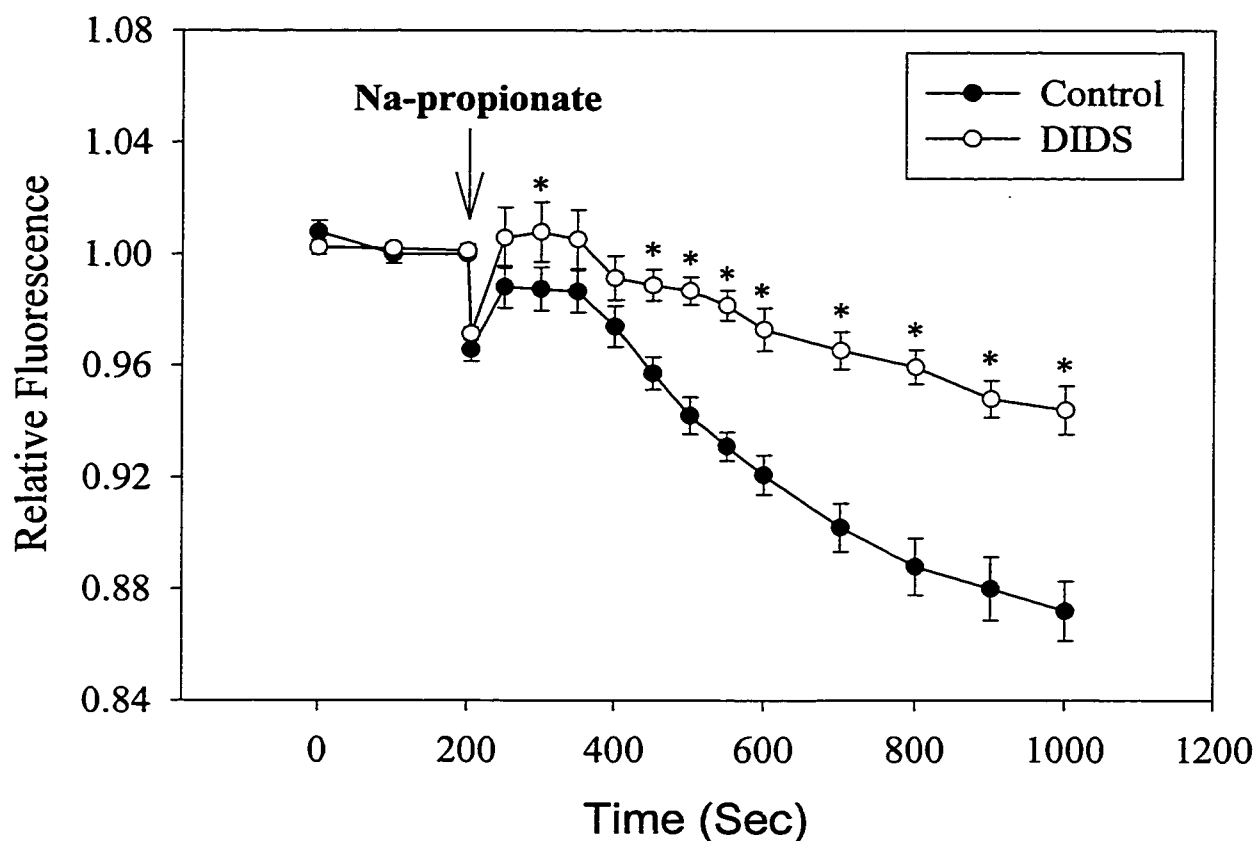


Figure 4-10. Changes in membrane potential of hepatocytes. The effects of cell acidification by Na-propionate were monitored using the fluorescent potentiometric dye Oxonol VI (500 nmol l⁻¹). Changes in membrane potential are compared in hepatocytes in normal experimental medium or in a medium containing the anion transporter blocker DIDS (0.5 mmol l⁻¹). Data are presented as the mean relative fluorescence $\pm$ SEM of 5 trials. * indicates a significant difference between control and treatment (DIDS) values.

DISCUSSION

The participation of $\text{Na}^+\text{-H}^+$ exchange and $\text{Na}^+\text{-HCO}_3^-$ cotransport in pH_i regulation has been reported in several mammalian cell types including hepatocytes (Gleeson *et al.*, 1989; Kikeri *et al.*, 1990; De la Rosa *et al.*, 1999). Prior to this study, the role of a $\text{Na}^+\text{-HCO}_3^-$ cotransporter in pH_i regulation in fish hepatocytes had not been examined specifically. Thus, the generally accepted model of pH_i regulation in fish hepatocytes (Walsh and Mommsen, 1992) incorporates only $\text{Na}^+\text{-H}^+$ and $\text{Cl}^-\text{-HCO}_3^-$ exchangers. Evidence from this study, however, suggests that at least in trout, the $\text{Na}^+\text{-HCO}_3^-$ cotransporter may play a significant role in H^+ extrusion that is complementary to that of the $\text{Na}^+\text{-H}^+$ exchanger. Plasma HCO_3^- levels are low in teleosts ($\sim 5\text{-}10 \text{ mmol l}^{-1}$) compared with those in mammals ($\sim 25 \text{ mmol l}^{-1}$). Regardless, extracellular HCO_3^- , albeit at low concentrations, still appears to play a significant role in the ability of trout hepatocytes to regulate pH_i . Despite the low levels of HCO_3^- in fish plasma, HCO_3^- -dependent acid extrusion is not unheard of in fish tissues. Evidence does exist for the role of a Na^+ -dependent $\text{Cl}^-\text{-HCO}_3^-$ exchanger in Atlantic cod (*Gadus morhua*) red blood cells (Berenbrink and Bridges, 1994) as well as in trout and carp thrombocytes (Nikinmaa *et al.*, 1999).

The specific roles of ion transporters such as the $\text{Na}^+\text{-HCO}_3^-$ cotransporter and the $\text{Cl}^-\text{-HCO}_3^-$ exchanger are often examined pharmacologically using stilbene derivatives such as SITS and DIDS. These compounds are similar in structure and both are potent inhibitors of anion transport. The binding of stilbene derivatives with the $\text{Na}^+\text{-HCO}_3^-$ cotransporter and $\text{Cl}^-\text{-HCO}_3^-$ exchanger are believed to involve interaction with consensus sites on the transporters (Bartel *et al.*, 1989; Okubo *et al.*, 1994; Romero *et al.*, 1998). In the study by Walsh (1986), addition of the anion transport blocker SITS increased baseline pH_i in trout hepatocytes, but

did not influence the rate of recovery from acid or base loading. Similarly, SITS did not significantly affect acid extrusion in toadfish (*Opsanus beta*) hepatocytes after acid loading (Walsh, 1989). In the study by Furimsky *et al.* (1999a; Chapter 3), SITS did not affect baseline pH_i or recovery rates from acid or base loading in 3 teleost species (trout, eel, bullhead). In the present study, DIDS significantly decreased baseline pH_i , significantly decreased pH_i recovery rates following acid and base loading (Table 4-1; Figs 4-2, 4-5), and decreased Na^+ uptake rates (Fig. 4-8). The different results obtained using SITS versus DIDS are unexplained but may reflect their different chemical properties influencing binding interactions with the transporters on the teleost hepatocyte. Though it had no effect in the fish model, SITS did significantly inhibit recovery to an acid load induced by gassing rat hepatocytes with 10% CO_2 (Fitz *et al.*, 1992).

The pH_i recovery rate to acid loading in trout hepatocytes is amiloride-sensitive indicating that the Na^+-H^+ exchanger is active in acid extrusion as it is in most cells types. Similar results were observed in hepatocytes of the gulf toadfish (Walsh, 1989) and black bullhead (Furimsky *et al.*, 1999a; Chapter 3). However, the fact that pH_i recovery was not totally abolished during blockade of the Na^+-H^+ exchanger suggested that another transporter might be involved in H^+ extrusion. The study by Furimsky *et al.* (1999a; Chapter 3) showed that a V-type H^+-ATPase was not involved in recovery to acid loading. However the study did suggest the contribution of another Na^+ -dependent mechanism because Na^+ removal caused a 75% decrease in recovery rate compared to a 50% decrease in the presence of amiloride. Walsh (1986) did not observe such a difference in the rate of pH_i recovery between Na^+ -free medium and amiloride. The discrepancies between the two studies may be related to the different methods that were used to measure pH_i , namely the BCECF method

(this study) that measures exclusively cytoplasmic pH versus the DMO method (Walsh, 1986) that measures pH of all intracellular compartments.

During blockade of anion transport using DIDS, an increase in baseline pH_i would be expected if it was acting exclusively upon a Cl^- - HCO_3^- exchanger owing to accumulation of intracellular HCO_3^- . Indeed, such an increase in baseline pH_i was observed previously after the removal of extracellular Cl^- (Furimsky *et al.*, 1999a; Chapter 3). The decrease in baseline pH_i and the inhibitory effects on proton extrusion rate that were observed in this study indicate that a Na^+ - HCO_3^- cotransporter is present and contributing to pH_i regulation during steady-state conditions and in response to acid loading (Table 4-1; Fig. 4-3). This observation also agrees with the fact that the Na^+ - HCO_3^- cotransporter plays a major role in the maintenance of pH_i near baseline values, as opposed to Na^+ - H^+ and Cl^- - HCO_3^- exchange that are most active at low and high pH_i values, respectively (Alper, 1990). The importance of HCO_3^- in hepatocyte pH_i regulation is not restricted to its role as a significant intracellular buffer, but also as an anion needed to drive Na^+ - HCO_3^- cotransport. The reduction in proton extrusion observed following acid loading in HCO_3^- -free media is similar to that observed with DIDS, providing further evidence for the involvement of a Na^+ - HCO_3^- cotransporter (Fig. 4-3). The intracellular HCO_3^- being moved to the medium as a result of cell metabolism may be sufficient to allow for some transport activity to occur. In mammals, where the extracellular HCO_3^- concentration is much higher than in fish, the effect of removal from the medium has a more pronounced effect by significantly decreasing baseline pH_i and recovery to acid loading (Gleeson *et al.*, 1989). Studies on perfused rat liver, however, have provided evidence that metabolic HCO_3^- is participating in pH_i regulation to a certain extent, since H^+ efflux in a HCO_3^- free medium is decreased even further by the inhibition of carbonic

anhydrase (CA) by acetazolamide (ACZ) (Vidal *et al.*, 1998). Even though CA activity in trout hepatocytes is almost completely blocked by ACZ (see Fig. 4-5), exposure to this drug does not significantly affect the ability of these cells to recover from an acid load (Fig. 4-4).

In the model of pH_i regulation in rat hepatocytes, the activity of the $\text{Na}^+\text{-HCO}_3^-$ cotransporter is linked to a pH_i -sensitive K^+ channel (Fitz *et al.*, 1992). In this study, the blockade of K^+ channels on trout hepatocytes had a similar effect on reducing the rate of proton extrusion following an acid load as did the removal of extracellular HCO_3^- , whereas Ba^{2+} addition and HCO_3^- removal had no cumulative effects on recovery. These data provide evidence that the presumptive $\text{Na}^+\text{-HCO}_3^-$ cotransporter on trout hepatocytes, as in rat, is linked to K^+ conductance. The model of $\text{Na}^+\text{-HCO}_3^-$ cotransporter activation proposed by Fitz *et al.* (1992) developed from the demonstration that blockade of K^+ channels with Ba^{2+} decreased recovery to acid loading and cell depolarization caused an increase in pH_i that did not occur when extracellular HCO_3^- was removed. Such a direct link could not be established in our study, meaning that the effects of K^+ channel blockade on baseline pH_i and recovery to an acid load may occur by way of a different mechanism.

Of the different HCO_3^- transporters, Na^+ -dependent and Na^+ -independent $\text{Cl}^-\text{-HCO}_3^-$ exchangers are electroneutral whereas the $\text{Na}^+\text{-HCO}_3^-$ cotransporter can have a $\text{Na}^+:\text{HCO}_3^-$ stoichiometry of either, 1:3, 1:2 or 1:1 depending on the tissue (Boron, 1997). In the proximal tubule of the mammalian kidney, a $1\text{Na}^+:3\text{HCO}_3^-$ cotransporter is crucial in HCO_3^- reabsorption from the lumen. The stoichiometry of $\text{Na}^+\text{-HCO}_3^-$ cotransport is not clear when participating in acid extrusion, but it is suggested to be either electroneutral or 1:2 (Boron, 1997). Though we were not specifically examining the stoichiometry of the putative cotransporter, it was of interest to determine if recovery to acid loading was an electrogenic

process. If recovery to acid loading occurred uniquely by way of a $\text{Na}^+\text{-H}^+$ exchanger, no change in membrane potential would be expected. However, hyperpolarisation (more negative membrane potential) should occur during the activation of an electrogenic $\text{Na}^+\text{-HCO}_3^-$ cotransporter. The results of the present study using the potentiometric dye Oxonol VI indicated that during the time of recovery following acid loading, the membrane potential did indeed decrease and this decrease was blocked by DIDS (Fig. 4-10).

In response to intracellular base loading induced by NH_4Cl , normal pH_i recovery principally involves the $\text{Cl}^-\text{-HCO}_3^-$ exchanger, but may also involve, to a lesser extent, the $\text{Na}^+\text{-H}^+$ exchanger and $\text{Na}^+\text{-HCO}_3^-$ cotransporter (Fig. 4-6). The decrease in pH_i recovery following base load observed with amiloride was also observed in an independent experiment in Chapter 3 (see Fig. 3-3; Furimsky *et al.*, 1999a). When both DIDS and amiloride were in the medium, recovery rates were not different than when DIDS was present alone, suggesting that the $\text{Cl}^-\text{-HCO}_3^-$ exchanger is dominant over the $\text{Na}^+\text{-H}^+$ exchanger in its role in base extrusion. Extracellular HCO_3^- , as in recovery to an acid load, is also important in recovery to a base load (Fig. 4-7). When intracellular HCO_3^- levels are diminished as a result of washing cells into HCO_3^- -free medium, this has two consequences on the ability of the cell to regulate pH_i . Not only does the apparent intracellular buffer capacity decrease, but recovery to base loading by the $\text{Cl}^-\text{-HCO}_3^-$ exchanger also appears to be inhibited by the very low concentrations of intracellular HCO_3^- needed to drive the transporter. Blocking K^+ channels with Ba^{2+} had no effect on recovery to a base load.

The interplay and co-operation that exist between different membrane ion transporters (pumps, channels) have been well documented in different experimental models. Thus, the fact that a $\text{Na}^+\text{-HCO}_3^-$ cotransporter appears to participate in pH_i regulation in the trout

hepatocyte along with a $\text{Na}^+\text{-H}^+$ exchanger and $\text{Cl}^-\text{-HCO}_3^-$ exchanger is not surprising. However, while it appears that the model of pH_i regulation in the trout hepatocyte may be more similar to the mammalian model than previously believed, significant differences still remain. These include the degree of reliance on extracellular HCO_3^- and the relationship that exists between $\text{Na}^+\text{-HCO}_3^-$ cotransport and the activity of a K^+ channel. As different organisms have evolved to live in different environments, the mechanisms by which different tissues deal with environmental changes have evolved accordingly.

CHAPTER 5**THE EFFECT OF RESPIRATORY ACIDOSIS ON pH_i AND pH_i REGULATION IN
TROUT HEPATOCYTES**

ABSTRACT

The effect of respiratory acidosis was examined on isolated trout hepatocytes. The decrease in blood pH that occurs *in vivo* is immediate following the onset of hypercapnia exposure (1% CO₂). The acidosis is, however regulated following 48 h with an accompanying increase in plasma [HCO₃⁻]. The decrease in blood pH directly affects pH_i in hepatocytes. Following the onset of *in vitro* hypercapnia, the decrease in pH_i is greater when hepatocytes are incubated with amiloride. Hepatocytes do not regulate pH_i to baseline levels during acute hypercapnia exposure, though regulatory mechanisms (e.g. Na⁺-H⁺ exchanger) appear to be activated to alleviate the degree of intracellular acidification. Chronic exposure of hepatocytes to hypercapnia, *in vivo* or *in vitro* had no effect on the ability of the cells to recover from a subsequent acid load. These results suggest that liver pH_i ultimately depends on the regulation of blood acid-base status by the gills and kidneys.

INTRODUCTION

Environmental stressors such as hypercapnia (increased PCO_2 of water) are well known to significantly change blood acid-base status of fish. The coincident increase in PaCO_2 during hypercapnia causes a decrease in blood pH (respiratory acidosis) that is regulated over time principally at the gills (Goss and Perry, 1993; Goss *et al.*, 1995; Laurent and Perry, 1995). Morphological changes in the gills involving chloride and pavement cells allow for the more efficient excretion of acids during blood acidosis. In trout and bullhead catfish, the chloride cell surface area is reduced during hypercapnia by covering pavement cells, a morphological change that uncouples base excretion by Cl^- - HCO_3^- exchange and promotes acid extrusion by H^+ ATPase (Goss *et al.*, 1992; Goss and Perry, 1993).

When significant changes in blood pH occur, the intracellular pH of various tissues can also be directly affected (Milligan and Wood, 1985). The liver cells (hepatocytes) are in very close contact with the circulation and may experience very significant pH changes during an acid-base disturbance. Since most enzymes function at an optimal pH, it is not surprising that an acid-base disturbance such as hypercapnia, that causes an intracellular acid load, could have a negative impact on hepatic enzyme activity and metabolism (Mommsen *et al.*, 1988; Perry *et al.*, 1988).

The mechanisms by which teleost hepatocytes regulate pH_i in response to an acid load are now well understood and involve the removal of acid equivalents by intracellular buffers and cell surface membrane exchangers (Walsh, 1986, 1989; Walsh and Mommsen, 1992; Furimsky *et al.*, 1999b, 2000; Chapters 3 and 4). The Na^+ - H^+ exchanger (NHE) and Na^+ - HCO_3^- cotransporter (NBC), in particular, play key roles in the removal of intracellular protons. Although regulatory mechanisms are in place in hepatocytes, pH_i is strongly

dependent on extracellular pH. In erythrocytes of several teleost species, on the other hand, activation of the β NHE by catecholamines can maintain high pH_i even during a significant blood acidosis (Thomas and Motais, 1990; Thomas and Perry, 1992). As a result, the ability of the blood to transport O_2 is not compromised.

In tissues that actively regulate blood pH, chronic acidosis generally increases their ability to remove acids by up-regulation of the various transport mechanisms. For example, in the mammalian kidney the expression of $\text{Na}^+\text{-H}^+$ exchangers and $\text{Na}^+\text{-HCO}_3^-$ cotransporters increases in order to extrude protons and reabsorb HCO_3^- (Akiba *et al.*, 1987; Preisig and Alpern, 1988; Krapf *et al.*, 1991). In fish, an increase in the branchial and renal expression of $\text{H}^+\text{-ATPase}$ has been reported during hypercapnia exposure (Sullivan *et al.*, 1996; Perry *et al.*, 2000).

Although it is well known that the tissues most directly involved in acid-base regulation (e.g., gills, kidney) can modify the expression of their membrane transport mechanisms, it is not known if other tissues can enhance their ability to regulate pH_i during a chronic acid stress. In this study, the ability of isolated trout hepatocytes to regulate pH_i was examined during acute hypercapnia exposure and following chronic *in vivo* and *in vitro* exposures. The purpose of this study was to determine whether the liver is able to enhance mechanisms of pH_i regulation in response to acid exposure.

METHODS AND MATERIALS

Experimental animals

Rainbow trout, *Oncorhynchus mykiss*, weighing between 200 and 600 g were obtained from Linwood Acres Trout Farm (Campbellcroft, Ontario, Canada). Rainbow trout were transported to the University of Ottawa in oxygenated water. Fish were maintained on a 12h:12h L:D photoperiod in fiberglass aquaria (water volume = 500 l) supplied with flowing, aerated and dechloraminated City of Ottawa tap water. Water temperature was kept at 15 °C. Fish were fed a commercial trout diet daily. Experiments were performed during the late spring and summer months.

Blood acid-base analysis

Extracorporeal Blood Loop

Surgical procedures

Large rainbow trout (~600 g) were anaesthetized in oxygenated and buffered water containing 1:1000 benzocaine until the cessation of breathing movement. Fish were then placed on the operating table where the gills were continuously irrigated with the anaesthetic solution. An incision was made in the caudal region of the fish, anterior to the tail and slightly above the lateral line and the caudal vein and caudal artery were cannulated with polyethylene tubing (PE50; Clay Adams). The dorsal aorta was also cannulated with polyethylene tubing (PE50; Clay Adams). Fish were allowed to recover from anaesthesia and were then placed in black perspex boxes (6 L capacity) with inserts to reduce movement. Fish were allowed to recover in these boxes for 24 h prior to experimentation.

Hypercapnia exposure and blood analysis

Blood acid-base variables were examined using the extracorporeal circulation loop as described by Thomas (1994). Blood was continuously drawn from the caudal artery by a peristaltic pump and passed over temperature controlled O₂ (Radiometer E5046), CO₂ (Radiometer E5036) and pH (Metrohm) electrodes. The blood flow rate was 0.6 ml min⁻¹ and the total blood volume in the extracorporeal loop was 1 ml. Inflow H₂O was rendered hypercapnic by gassing with CO₂ until a P_wCO₂ of 7.6 Torr was obtained. All data were collected using the Acqknowledge™ data acquisition system that was calibrated prior to each experiment.

Chronic hypercapnia exposure

Surgical Procedures

Rainbow trout weighing 200 to 300 g were anaesthetized as above. For these experiments, only the dorsal aorta was cannulated. Fish were allowed to recover by irrigating the gills with freshwater and then placed in black perspex boxes (4 L capacity) to recover for 24 h prior to experimentation.

Hypercapnia exposure and blood analysis

Experimental fish were exposed to hypercapnia by gassing the inflow water to their boxes with 1% CO₂, giving a final P_wCO₂ of 7.6 mm Hg. Blood was collected via the dorsal aorta cannula before exposure (time 0) to hypercapnia and then every 24 h during the 72 h exposure. Blood pH was measured using a pH meter (Radiometer) and plasma total CO₂ was measured using a CO₂ analyser (Capni-Con).

Cell isolation

Hepatocytes were isolated according to the method of Mommsen *et al.* (1994) and described in detail in Chapter 2. Cells were isolated either previously to *in vitro* hypercapnic exposure or immediately following *in vivo* exposure.

pH_i measurement

Hepatocyte pH_i was measured using BCECF as described in detail in Chapter 3. The experimental media contained either 5 mmol l⁻¹ of NaHCO₃ under control conditions or 15 mmol l⁻¹ NaHCO₃ to represent compensated hypercapnia.

In vitro hypercapnia exposure

Hepatocytes were exposed to hypercapnia *in vitro* by gassing (bubbling) BCECF-loaded cell suspensions in the cuvette directly with humidified 1% CO₂ in air. The experimental media were as above, except that Hepes was omitted. Chronic *in vitro* hypercapnia occurred by gassing cells in suspension for 18 h while in a shaker bath kept at 15 °C.

NHE Expression

Total RNA was isolated from a selection of trout tissues, and specifically from liver of hypercapnia exposed trout (see full protocol for RNA isolation and Northern Blots in Chapter 6; Methods and Materials). Northern blots were probed with either a 1.3 Kb KpnI fragment from the N-terminal end of the rat NHE-1 gene (clone kindly provided by Dr. John Orlowski, McGill University) or a 258 bp NHE probe cloned from a trout gill/kidney library

(see Chapter 6 for details). Slot blot analysis was also performed using 20 µg total RNA per slot probed with the rat NHE-1 probe.

Statistical Analysis

Data are presented as the mean $\pm$ one standard error of the mean (SEM). Each trial represents an experiment performed on a single cell preparation from one individual fish. Statistical analysis was conducted using either a one way ANOVA followed by a Student-Newman-Keuls test for the pair-wise analysis of means, or a two way ANOVA with repeated measures using the Student-Newman-Keuls test for the pair-wise analysis of means. The level of significance was accepted as $p < 0.05$.

RESULTS

Hypercapnia is distinguished by an increase in the $P_w\text{CO}_2$ and a decrease in pH_w , with an accompanying increase in blood PaCO_2 and decrease in pH_a (Fig. 5-1). During chronic hypercapnia exposure, the regulation of the blood acid-base status by increasing blood total CO_2 (principally HCO_3^-) is evident after 24 h, increasing during the 72 h experimental period until blood pH returned to baseline levels (Fig. 5-2).

Gassing the extracellular medium with 1% CO_2 *in vitro* caused a significant decrease in hepatocyte pH (Fig. 5-3). This decrease was mainly due to the movement of CO_2 across the cell membrane and subsequent production of H^+ by the hydration reaction that also forms HCO_3^- (De Weer, 1978). The degree of pH_i decrease was accentuated by the addition of the Na^+/H^+ exchanger blocker amiloride.

Hepatocytes isolated from trout exposed to chronic (48 h) *in vivo* hypercapnia did not demonstrate an enhanced ability to recover from a subsequent acid loading. The medium that represents partially compensated hypercapnia (i.e. $15 \text{ mmol l}^{-1} \text{ HCO}_3^-$) did not affect the ability of cells to recover from an acid load (Fig. 5-4). *In vitro* hypercapnia exposure (18 h) also had no effect on baseline pH_i (Table 5-1) or pH_i recovery (Fig. 5-5).

Studies of the expression of NHE were unsuccessful since a specific signal could not be detected in trout liver using the available NHE probes. The tissue distribution of trout RNA showed no signal when probed with a 258 bp fragment of NHE that was cloned from a trout mixed gill/kidney cDNA library (Fig. 5-6). Slot blot analysis of liver total RNA demonstrated a signal with the rat NHE-1 probe, but lack of signal from the same RNA by Northern blot analysis demonstrated that hybridisation in slot blots was most likely non-specific.

Figure 5-1. Representative data showing the effects of a 3 h hypercapnia exposure on trout blood acid-base status. Trout were cannulated to allow for an extracorporeal blood loop as noted in the Materials and Methods. Hypercapnia exposure (gassing water with CO₂) was initiated at 10 min. The different variables shown are (A) P_wCO₂, (B) pH_w, (C) P_aCO₂ and (D) pH_a. All data were obtained from one fish during the same time period.

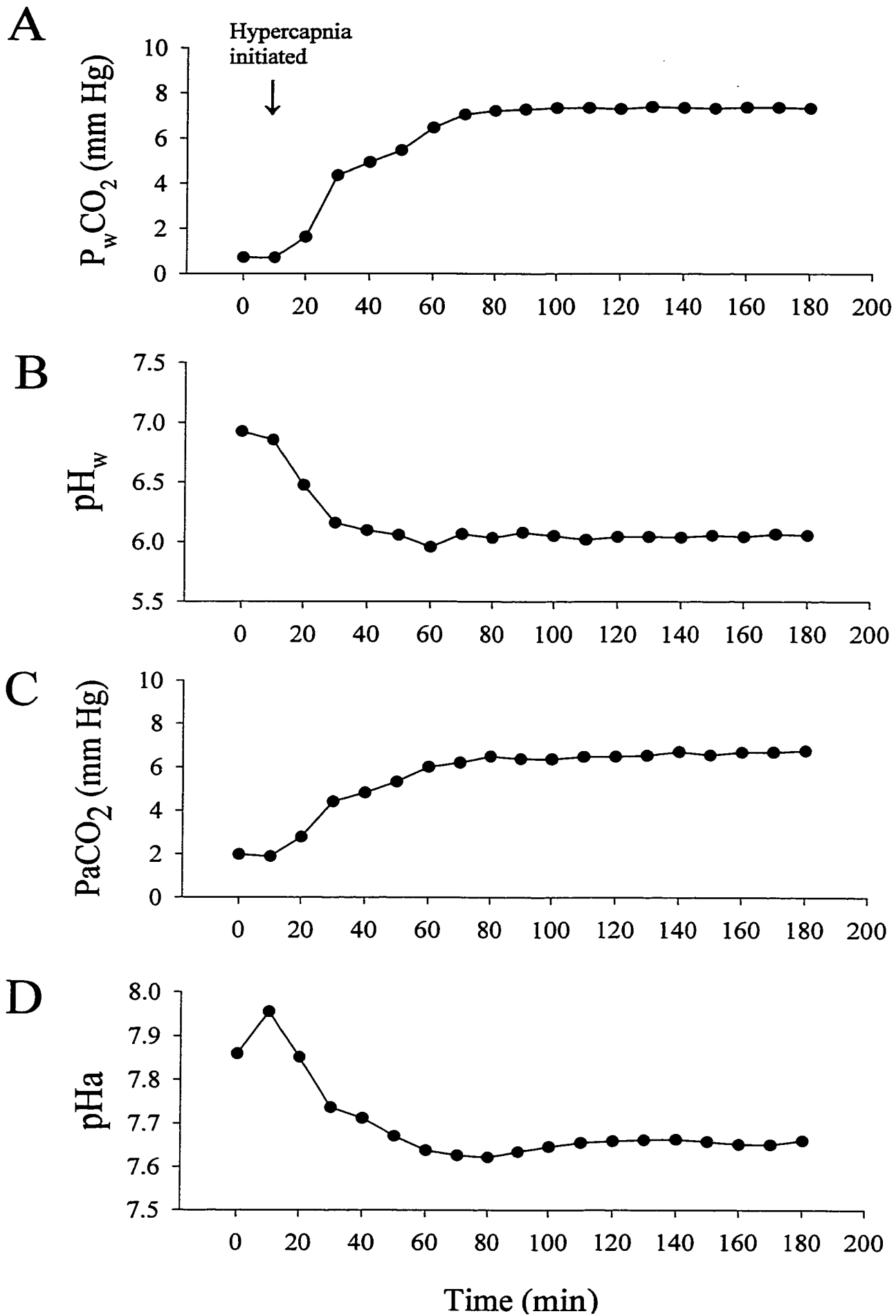
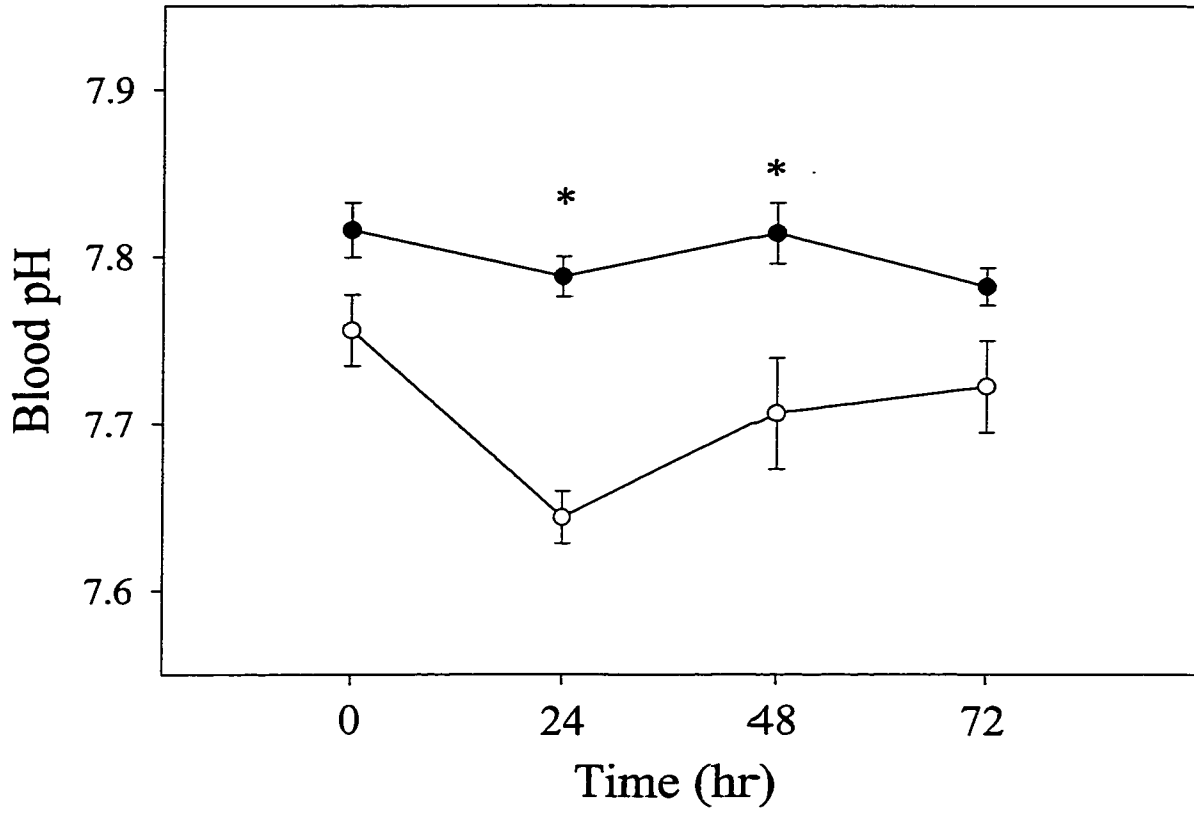
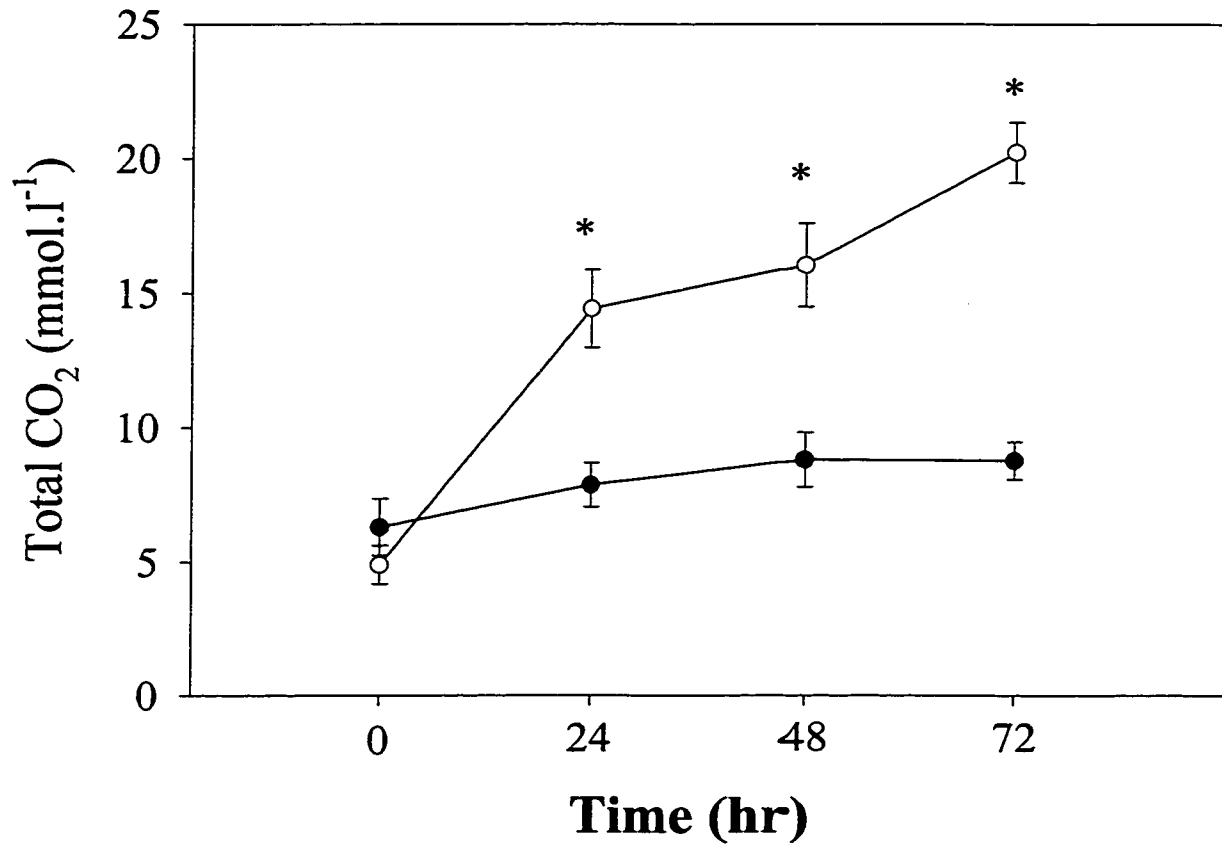


Figure 5-2: The effects of chronic hypercapnia exposure on (A) blood pH and (B) plasma total CO₂ in the rainbow trout. Closed symbols (●) represent control values and open symbols (○) represent hypercapnic exposure values. Following the collection of pre-samples (time 0), blood was collected from fish every 24 h for 72 h. Values are presented as the means $\pm$ SEM (n=6). * represents a significant difference between control and experimental values.

A**B**

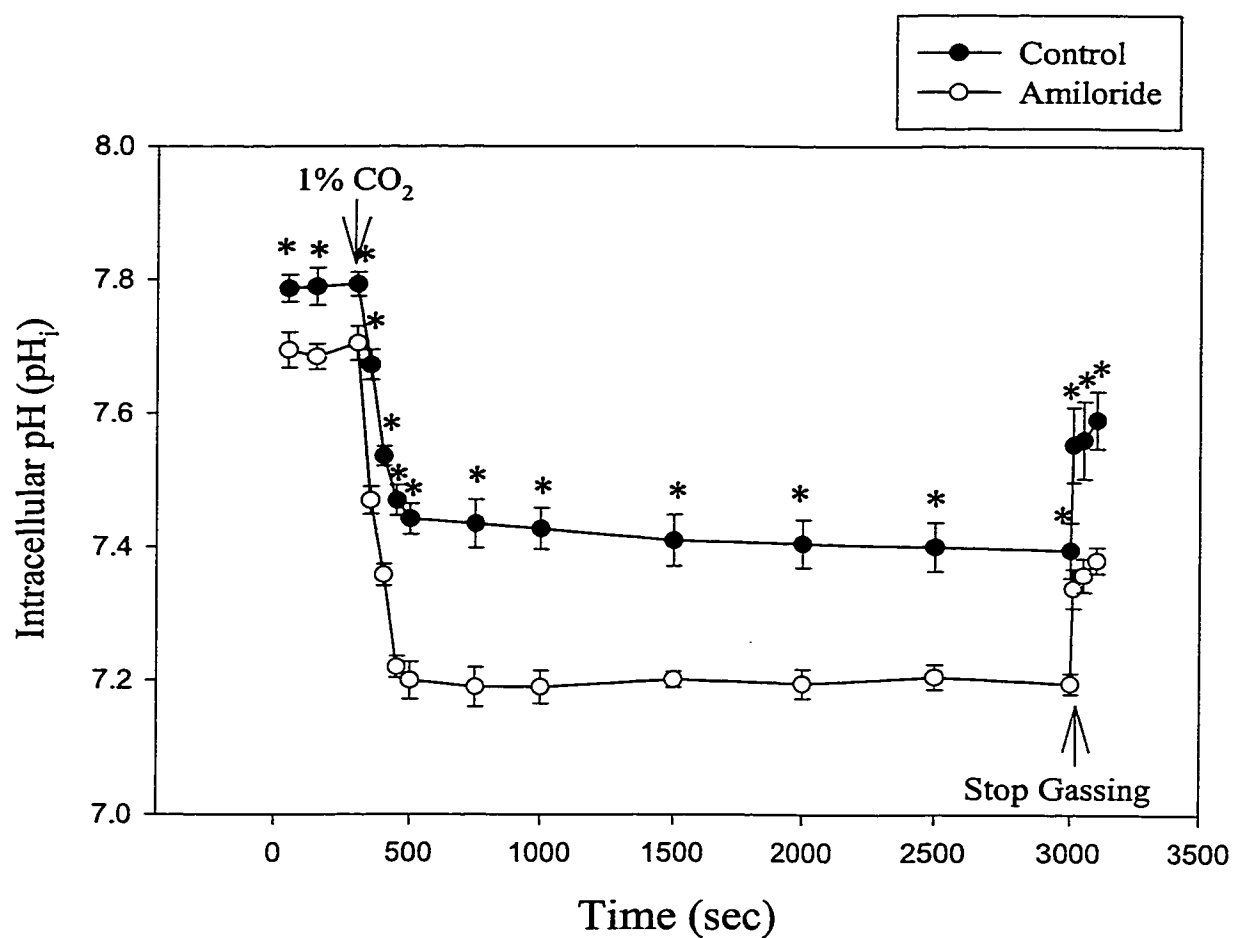


Figure 5-3: The effect of *in vitro* hypercapnia exposure (gassing by 1% CO₂) on hepatocyte pH_i. Values are presented as the means $\pm$ SEM (n=4). * represents a significant difference between control and amiloride treatments.

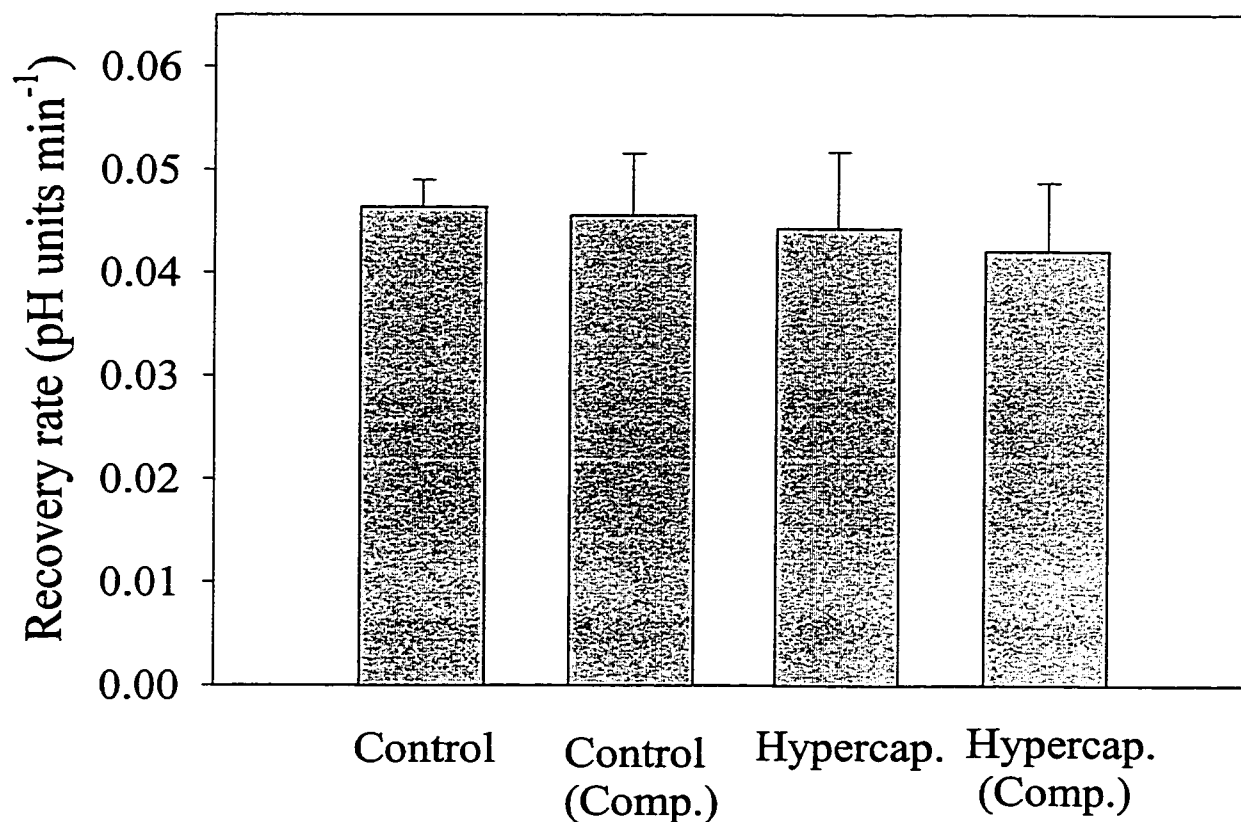


Figure 5-4: Hepatocyte pH_i recovery rates following acid loading with Na-propionate (50 mmol l^{-1}). Hepatocytes were isolated from control trout or trout exposed to hypercapnia ($1\% \text{ CO}_2$) for 48 h. Control medium contained $5 \text{ mmol l}^{-1} \text{ NaHCO}_3$ and compensated medium contained $15 \text{ mmol l}^{-1} \text{ NaHCO}_3$. Values are presented as the means $\pm$ SEM ($n=6$). No significant differences between control and experimental values were detected.

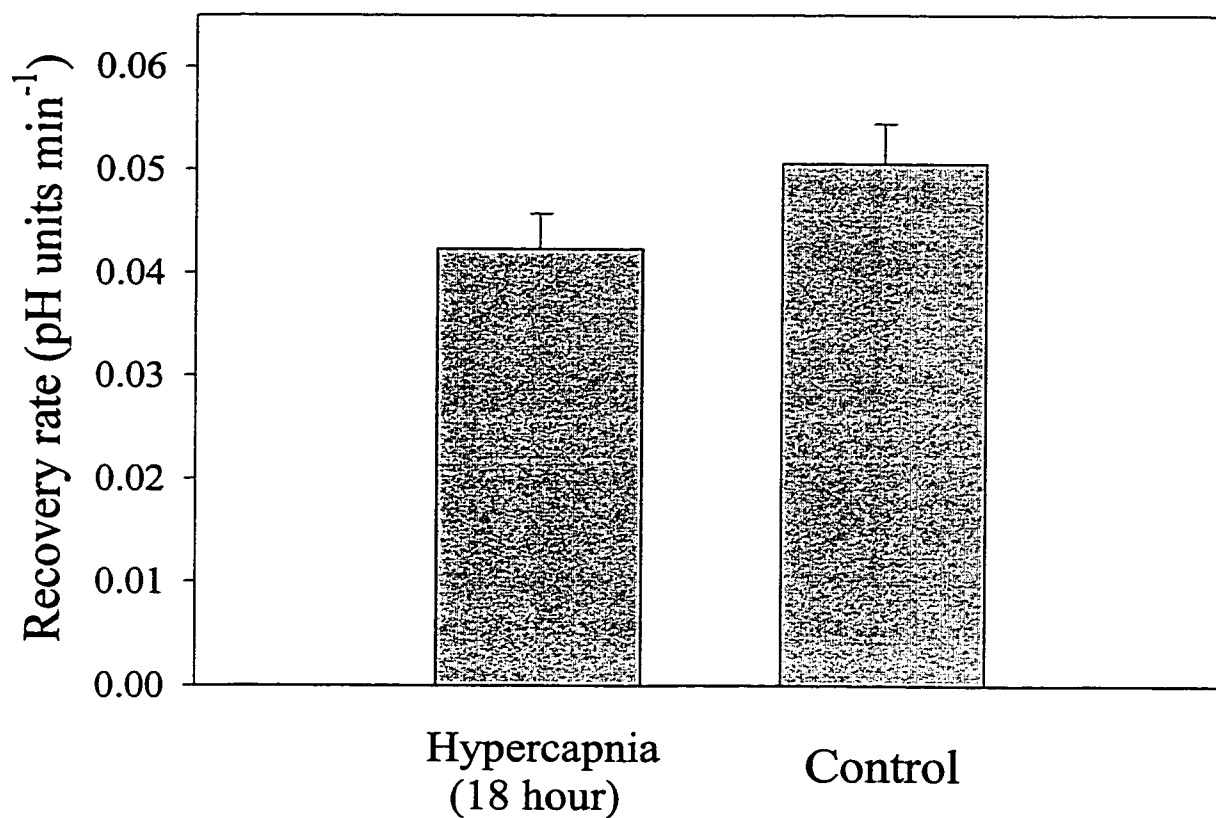


Figure 5-5: Recovery rates in hypercapnia-exposed trout hepatocytes following acid load by Na-propionate (50 mmol l⁻¹). Hypercapnia-exposed cells were previously subjected to *in vitro* hypercapnia (1% CO₂) for 18 h. Values are presented as the means ± SEM (n=6). No significant difference between the control and experimental values was detected.

Table 5-1. Baseline pH_i values in hepatocytes following an 18 hr *in vitro* hypercapnia exposure. Under control conditions, cells were bubbled with air instead of with 1% CO_2 in air.

	<i>Control</i>	<i>Hypercapnia Exposed</i>
pH_i	7.59 ± 0.049	7.55 ± 0.076
ΔpH_i (acid)	0.33 ± 0.014	0.29 ± 0.027

Values are means $\pm$ S.E.M. (N=6)

No significant difference from pH_i values in normal medium was detected in hypercapnia exposed hepatocytes.

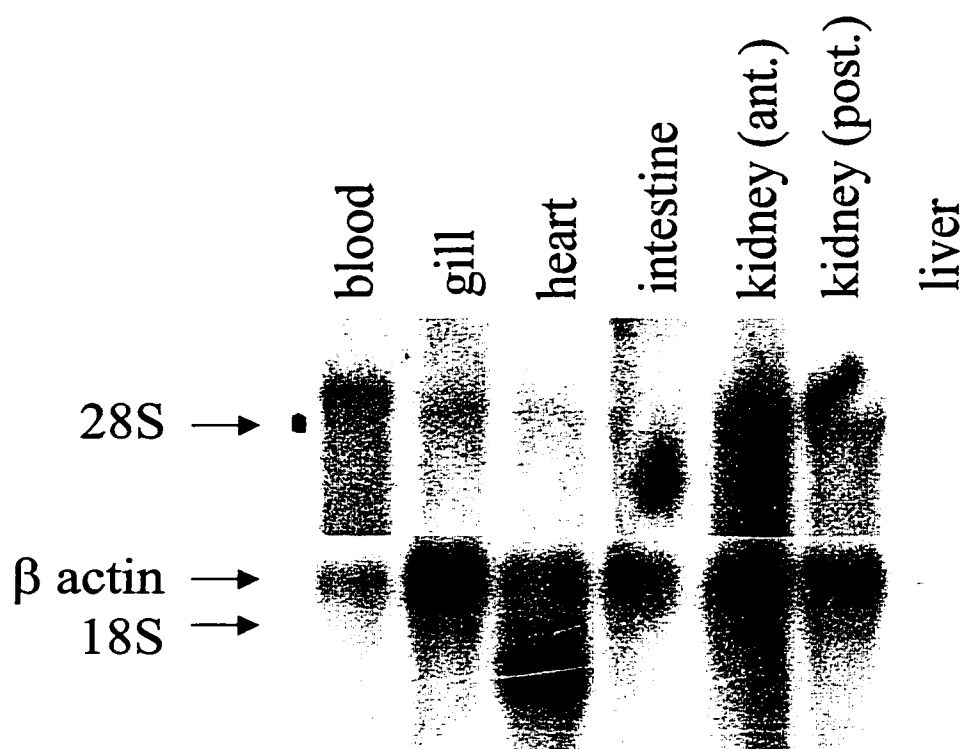


Figure 5-6. Tissue distribution of NHE expression in the rainbow trout. The blot was probed with the 258 bp fragment of NHE isolated from the gill/kidney cDNA library. To confirm equal loading between samples, membranes were re-probed with an β actin probe from the rainbow trout.

DISCUSSION

Hypercapnia (respiratory acidosis) has been extensively studied as an environmental stressor that causes a blood acid-base disturbance in fish. The effects of hypercapnia on blood acid-base status are well understood, as are the mechanisms that are involved in the regulation of this stress at the level of the gills (Goss and Perry, 1993; Goss *et al.*, 1995; Laurent and Perry, 1995). These regulatory mechanisms involve morphological changes at the gills that promote acid excretion by the pavement cells, and that physically cover the HCO_3^- secreting chloride cells, decreasing their surface area and enabling the retention of plasma HCO_3^- . Regulation during acidosis also occurs at the molecular level, as shown by the increase in expression of the acid-secreting H^+ -ATPase in gill cells as a result of hypercapnia (Sullivan *et al.*, 1996; Perry *et al.*, 2000). Similarly in mammals, the expression of apical NHE and basolateral NBC is increased in kidney cells to promote Na^+ and HCO_3^- uptake and H^+ excretion during metabolic and respiratory acidosis (Akiba *et al.*, 1987; Preisig and Alpern, 1988; Krapf *et al.*, 1991).

Although as noted in this study blood pH is eventually regulated back to baseline values during chronic hypercapnia exposure (Fig. 5-2), the initial decrease in blood pH would cause a significant intracellular acidosis in visceral tissues (Milligan and Wood, 1985). As a result, pH-sensitive cellular processes may be compromised, impacting upon the function of the entire organ. It is clear that pH regulatory organs (e.g., gills and kidney) are able to efficiently counteract acid-base stress more or less rapidly regulating blood pH back to baseline values, but it is not clear if a tissue such as liver also activates similar mechanisms to regulate intracellular pH during the face of an extracellular pH challenge.

When trout hepatocytes are exposed *in vitro* to hypercapnia (gassing with 1% CO₂), the decrease in pH_i is regulated to a degree by the Na⁺-H⁺ exchanger as evidenced by the use of amiloride (Fig. 5-3). When the NHE was blocked by amiloride, pH_i was maintained at a lower level compared with that in the extracellular medium. The extent of the pH_i decrease is accentuated by the addition of amiloride, suggesting that this exchanger is indeed activated during the initial onset of acidosis and participates in regulating pH_i under these circumstances. NHE may not only be activated by a decrease in pH_i as would occur during an acid load, but is also active in maintaining the pH difference between the blood and the cytosol. The pH gap between the blood and cytosol during hypercapnia is likely decreased by the activity of NHE. It is clear that this exchanger regulates pH_i during hypercapnia, but not to the same extent as observed for the erythrocyte βNHE (Thomas and Motais, 1990; Thomas and Perry, 1992). It does appear to play at least a limited role in reducing the difference between intra- and extracellular pH.

The pH_i recovery observed by Walsh (1986) during persistent hypercapnia exposure (gassing with CO₂) was not observed in this study (Fig. 5-3). The reason for this difference likely lies in the methods that were employed, namely the BCECF method (this study) vs. the DMO method (Walsh, 1986). Since pH_i values measured by the DMO method include the pH of all cytoplasmic components, including organelles, the pH_i recovery observed using that method can be attributed to alkalization of an organelle such as mitochondria that are a large component of hepatocytes. No changes in cytosolic pH were measured with BCECF.

The hypothesis that hepatocytes up-regulate their acid extrusion mechanism (principally Na⁺-H⁺ exchange) during chronic intracellular acidosis was not supported by this study. In hepatocytes isolated from hypercapnia-exposed fish, or hepatocytes exposed to *in*

vitro hypercapnia, no difference was observed in comparison to those from normocapnic fish in the ability to extrude intracellular acid following a subsequent acid load. Although, the physiological evidence showed no effect of hypercapnia exposure, it is possible that the cells are up-regulating their pH_i regulatory mechanisms as would be indicated by an increase in mRNA or protein expression. We were unable to obtain any positive hybridisation signals with our liver RNA so an analysis of NHE expression was not possible. The probes used contained very highly conserved NHE sequences and low-stringency wash procedures were followed. In our opinion, low sequence identity of our probes with the RNA of interest should not have been an issue. It is possible that since liver provides a very high yield of RNA per gram of tissue, the high concentration of RNA may only contain a low copy number of the mRNA of interest.

This study was performed under the assumption that only NHE would participate in regulating intracellular acidosis, although it is now known that the $\text{Na}^+\text{-HCO}_3^-$ cotransporter also contributes to this regulatory mechanism (Furimsky *et al.*, 2000; Chapters 4 and 6). It is very likely that NBC also plays an active role under these circumstances since it is known to regulate pH closer to baseline levels.

In summary, though it is clear that trout hepatocytes regulate pH_i following acid and base loads, it is not clear whether they up-regulate pH_i regulatory mechanisms during a chronic acidosis. Physiological evidence from this study did not demonstrate an enhanced ability of cells to recover from a subsequent acidosis. It is possible that the pH of different tissues is inherently dependent on the regulation of pH_e that is regulated over time. The molecular examination of mRNA expression of NHE and NBC during hypercapnia exposure could indicate whether different regulatory systems are enhanced.

CHAPTER 6

CLONING AND EXPRESSION OF THE Na^+ - HCO_3^- COTRANSPORTER IN

RAINBOW TROUT (*Oncorhynchus mykiss*)

ABSTRACT

The $\text{Na}^+\text{-HCO}_3^-$ cotransporter gene (NBC) was cloned from both a trout gill/kidney cDNA library as well as a trout liver cDNA library using homology cloning. The 731 bp sequence of gill/kidney cDNA showed identical sequence homologies to the 437 bp sequence sequenced from the liver cDNA library. These fish NBC sequences showed high homology to known NBC sequences from other vertebrate species at both the nucleotide (78-79%) and amino acid (44-71%) levels. The highest homology was in the membrane-spanning domain, whereas the lowest homology among all six protein sequences aligned, occurred at the putative extracellular loop. The DIDS binding motif that is highly conserved in mammalian NBC (KMIK), is different in the fish sequence with isoleucine (I) substituted by leucine (L). Northern Blot analysis showed high NBC expression in trout gill and kidney, with lower expression in liver. These results support functional evidence for the expression of NBC in the trout liver while providing evidence that this transporter is present in gill and kidney.

INTRODUCTION

The $\text{Na}^+\text{-HCO}_3^-$ cotransporter (NBC) belongs to the superfamily of HCO_3^- cotransporters that also includes the Na^+ -dependent and Na^+ -independent $\text{Cl}^-\text{-HCO}_3^-$ exchangers. The $\text{Na}^+\text{-HCO}_3^-$ cotransporter is expressed in several different tissues where it mediates Na^+ and HCO_3^- movements across cell membranes for the purpose of Na^+ and HCO_3^- reabsorption or pH_i regulation (see reviews by Boron *et al.*, 1997; Romero and Boron, 1999; Soleimani and Burnham, 2000). The cloning of NBC from the kidney of the tiger salamander *Amphystoma tigrinum* (Romero *et al.*, 1997) was followed by the cloning of this same gene in several mammalian tissues. These included human kidney (Burnham *et al.*, 1997), human heart (Choi *et al.*, 1999), human pancreas (Abuladze *et al.*, 1998), rat kidney (Romero *et al.*, 1998), rat pancreas (Thevenod *et al.*, 1999), mouse pancreas (Abuladze *et al.*, 1998) and rabbit duodenum (Rossmann *et al.*, 1999). A similar sequence has been reported from the nematode *Caenorhabditis elegans* (Romero and Boron, Genbank accession no. AF004926), although homology with the vertebrate NBC gene is relatively low.

The activity of NBC is prominent on the basolateral membranes of renal proximal tubules, participating in the reabsorption of the majority of filtered HCO_3^- and a significant amount of Na^+ back into the circulation (Boron and Boulpaep, 1983; Alpern, 1985). In the pancreas, NBC mediates HCO_3^- transport from blood to the pancreatic duct (Shumaker *et al.*, 1999). This protein is also important as an alkaline loader in pH_i regulation of several tissues, namely brain (Bevensee *et al.*, 1994a,b; Boussouf *et al.*, 1997), liver (Fitz *et al.*, 1989; Gleeson *et al.*, 1989) and heart (Dart and Vaughan-Jones, 1992; Lagadic-Gossmann *et al.*, 1992).

In fish, HCO_3^- transporters have been identified in several tissues. In the gill, which has a primary role in ionic and acid-base regulation, strong evidence exists for the roles of $\text{Na}^+\text{-H}^+$ exchangers, $\text{Cl}^-\text{-HCO}_3^-$ exchangers, $\text{H}^+\text{-ATPase}$ and Na^+ channel (Avella and Bornancin, 1989; Goss *et al.*, 1992; Heisler, 1993; Claiborne, 1998). The uptake of Na^+ is linked to the excretion of H^+ , and the uptake of Cl^- is linked to the excretion of HCO_3^- by a

Cl^- - HCO_3^- exchanger (see Chapter 1, Fig. 1-1). The kidneys, though having a less important role in acid-base regulation in fish than in their mammalian counterparts, are also important sites for Na^+ and HCO_3^- movement. The Band 3 protein (AE1) is also one of the major proteins in fish erythrocytes, being responsible for both Cl^- - HCO_3^- exchange and anchorage to the cytoskeletal network. The primary structure of the trout Band 3 protein has been deduced (Hübner *et al.*, 1992). Physiological evidence also exists for the role of Na^+ -dependent Cl^- - HCO_3^- exchange in trout erythrocytes (Berenbrink and Bridges, 1994) and thrombocytes (Nikinmaa *et al.*, 1999). The role of Na^+ - HCO_3^- cotransporter had not been specifically determined in the tissue of any fish species until recently, when Furimsky *et al.* (2000) provided evidence for its role in liver pH_i regulation, and Wood and Pärt (2000) provided evidence for its role in gill (pavement cell) pH_i regulation.

The cloning of this integral membrane transport protein has led to the determination of its expression patterns in the tissues of several vertebrate species. Although much attention has focused on the role of NBC in mammalian systems, little is known regarding its expression in lower vertebrates such as fish. The purpose of the present study was to clone the trout Na^+ - HCO_3^- cotransporter and to use a homologous probe to determine its distribution in selected trout tissues.

MATERIALS AND METHODS

Experimental Animals

Rainbow trout, *Oncorhynchus mykiss* (Walbaum), weighing 200 to 300 g were obtained from Linwood Acres Trout Farm (Campbellcroft, Ontario, Canada). Rainbow trout were transported to the University of Ottawa in oxygenated water. Fish were maintained on a 12L:12D photoperiod in fiberglass aquaria (water volume 500 l) supplied with flowing, aerated and dechloraminated City of Ottawa tap water ($[\text{Na}^+] = 0.10 \text{ mmol l}^{-1}$, $[\text{Cl}^-] = 0.15 \text{ mmol l}^{-1}$, $[\text{Ca}^{2+}] = 0.35 - 0.40 \text{ mmol l}^{-1}$, $[\text{K}^+] = 0.03 \text{ mmol l}^{-1}$, pH 7.7 - 8.0). Trout were kept at a water temperature of 15 °C and were fed a commercial trout chow diet daily.

Polymerase Chain Reaction using Library cDNA as Template

Degenerate primers were designed for PCR based on conserved gene sequences determined from an alignment of the NBC gene from different vertebrate species (Romero *et al.*, 1997, 1998; Burnham *et al.*, 1997; Rae, unpublished sequence) obtained from GenBank. Primer sites were designed to give a maximum fragment size of 1 Kb. The outer primers were the forward 5' primer (NBCMF-F1) that was the 21mer 5'-CATGCAGGGCGTGTGGAGAG-3' and the 3' primer (NBCMF-R1) that was the 21mer 5'-GTCTCTGTCTCCATCTTCAAR-3'. These primers correspond to the nucleotides at positions (1492 - 1512) and (2566 - 2586), respectively, of the *Amphystoma* kidney Na^+ - HCO_3^- cotransporter (Romero *et al.*, 1997). The inner primers were the forward 5' primer (NBCMF-F2) that was the 21mer 5'-TAYGATGCTTTCAAGAAGATG-3' and the 3' primer (NBCMF-R2) that was the 21mer 5'-GATAATGGCAAARTCACTGAT-3'. These primers correspond to the nucleotides at positions (1793-1813) and (2183- 2203) of the *Amphystoma* kidney Na^+ - HCO_3^- cotransporter, respectively. The position of these primers is indicated on Figure 6-1. Different primer combinations were used rather than using a nested PCR approach.

PCR was performed in 25 μ l reaction mixtures containing 2.5 mmol l⁻¹ MgCl₂, 200 μ mol l⁻¹ of each dNTP, 200 nmol l⁻¹ of each primer (in different combinations) and 1 unit of Taq polymerase (Life Technologies) in PCR buffer supplied with the enzyme. The templates for the reactions were 1 μ l of either a mixed gill/kidney cDNA library from the rainbow trout (Perry *et al.*, 2000) or a rainbow trout liver cDNA library (generously provided by JF Leatherland, University of Guelph). The PCR protocol was as follows: an initial 5 min denaturation step at 94°C followed by 40 cycles of 94°C for 0.5 min (denaturing), 55.9°C for 0.5 min (annealing), and 72°C for 1 min (extension), with a final extension for 5 min at 72°C in a PTC-100 (M.J. Research) thermal cycler. The primer combinations NBCMF-F1/NBCMF-R2 and NBCMF-F2/NBCMF-R2 yielded products of the expected size.

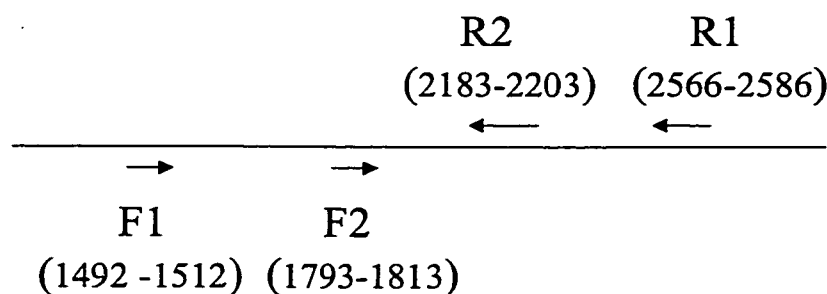


Figure 6-1. The primer strategy utilised to clone the Na⁺-HCO₃⁻ cotransporter from trout (*Oncorhynchus mykiss*) tissues. The nucleotide sequences are based on those in the full length salamander kidney NBC sequence (*Ambystoma tigrinum*, Genbank accession no. AF001958).

Clones were produced using the pGEM®-T Easy cloning vector system (Promega). The pGEM® vector has a size of 3 Kb and DNA is inserted into the EcoR1 site of the polylinker. The PCR products were ligated into the cloning vector [5 µl 2X Rapid Ligation Buffer, 1 µl of pGEM®-T Easy Vector (50 ng), 3 µl of PCR product and 1 µl T4 DNA Ligase (3 Weiss units/µl)] and incubated overnight at 4°C. Plasmid DNA containing the inserts was transformed into competent DH5αF' cells according to the procedure of Hanahan (1985). Briefly, competent cells were permeabilised and cells were “heat-shocked” to allow for plasmid uptake. Cell growth was encouraged by incubating in SOC Broth for 1 h. Cells were then grown on agar plates (+ ampicillin) overnight. Positive (white) colonies were chosen and patched onto another plate with a grid. Following overnight culture, colony PCR was performed to determine the size of the relative inserts in selected cell colonies. Plasmids containing inserts (from white colonies) were used as templates, the primers were the M13 forward and reverse, and the reaction was catalysed by Vent polymerase (New England Biolabs). Colonies containing inserts of the correct size were chosen and grown overnight in 2X YT Broth (+ ampicillin). Plasmids were isolated from the cells using the Wizard Plasmid prep kit (Promega). Restriction analysis was performed to determine the size of the PCR product inserted into the EcoR1 site of the polylinker of the pGEM®-T-Easy vector.

DNA Sequencing

Sequencing of the cDNA was performed using the dideoxy chain termination technique. DNA sequences were analysed using the BLAST program at the National Center for Biotechnology Information (NCBI).

Southern Blot

The presence of NBC in trout liver was initially determined using Southern Blot analysis. The PCR products from the liver cDNA library or the restriction fragments were run on a gel and transferred to a nylon membrane (Hybond) by capillary action overnight following denaturation and neutralisation steps. Blots were hybridised with the 730 bp NBC fragment isolated from the trout gill/kidney cDNA library.

Northern Blot Analysis

Preparation of RNA

Following the different treatments, fish were terminally anaesthetised in 1:12 000 (mass:volume) solution of benzocaine (ethyl-*p*-aminobenzoate). Blood samples were collected from each fish by caudal puncture with a heparinised syringe. The remainder of the blood was removed from the entire fish by perfusion via the heart with 0.9% NaCl solution cooled to 10°C. The perfusion procedure was very effective at clearing blood from gill and liver, though some residual blood contamination was present in the kidney. Tissues were then removed from the fish, placed in labelled aluminum weigh dishes and frozen immediately in liquid N₂. All tissues, blood and plasma samples were stored at -80°C until needed.

RNA was isolated by the acid guanidium thiocyanate-phenol-chloroform extraction. All solutions used during the RNA isolation procedure were made in DEPC (diethylpyrocarbonate)-treated water to ensure that they were RNase free. The solutions used were: phenol (saturated with DEPC water); Solution D [73 ml DEPC water, 62.5 g of solid GITC (Guanidine Isothiocyanate); 4.4 ml 0.75 sodium citrate; 6.6 ml of 10% Sarkosyl, 0.3 ml of 2 -β-mercaptoethanol]; sodium acetate (2.0 mol l⁻¹, pH 4.0); chloroform; high salt

buffer (1.2 mol l⁻¹ NaCl, 0.8 mol l⁻¹ Na-citrate); iso-propanol; 80% ethanol; and, 3.0 mol l⁻¹ sodium acetate (pH 5.5).

Frozen tissues were weighed, placed in a metal homogenising bucket containing solution D (5 ml g⁻¹ of tissue) and homogenised. The successive addition of 2 mol l⁻¹ Na-acetate (0.5 ml g⁻¹) and phenol (2.5 ml g⁻¹), and then chloroform (1 ml g⁻¹) were always followed by intermittent homogenisation. Homogenates were emptied into labelled 17 x 100 round bottom conical tubes and the pH verified to be approximately 4.0. Following a 20 min centrifugation at 8,000 rpm, the supernatant was transferred to a new conical tube and the volume measured. RNA was precipitated from this supernatant by the addition of high salt buffer (0.2 vol) and iso-propanol (0.2 vol) that were then mixed and incubated for 10 min at room temperature. The solutions were then centrifuged at 8,000 rpm for 15 min. The pellets were repeatedly washed with 10 ml 80 % ethanol, shaken for 10 min and finally centrifuged for 10 min at 8,000 rpm. Pellets were dried in a vacuum dessicator for 5 min. Dry RNA pellets were resuspended in minimal volumes of DEPC water. The RNA was aliquoted for storage in DEPC water, as well as being precipitated with 1/10 vol of 3 mol l⁻¹ Na-acetate and 2.5 vol of 95% ethanol.

Poly A⁺ RNA was isolated from liver, gill and kidney using the straight A's mRNA isolation kit (Novagen) according to the manufacturers instructions.

In order to determine purity and amount of total or poly A⁺ RNA, optical density (OD) readings were made using a GeneQuant RNA/DNA Calculator (Pharmacia). Samples were diluted so as to give reading between 0.1 and 1.0 absorbance units (AU). A 260/280 ratio of 2 indicated high purity of RNA. RNA was also run on denaturing gels (see below for

specific details) in order to test for purity (determined by the presence of distinct 18S and 28S ribosomal RNA bands).

RNA samples were incubated in 2.25 vol of RNA running buffer (Bromophenol Blue, Ethidium Bromide) for 20 min at 65°C. Samples were then loaded on to 100 ml volume denaturing gels (1% Agarose, 1X MOPS running buffer) and run so as to give a good distribution of RNA. Total RNA (20 µg) from the following tissues was run on these gels: blood, blood (cortisol-treated), gill, gill (cortisol-treated), heart, intestine, kidney (anterior), kidney (posterior), liver, liver (cortisol-treated), spleen, and white muscle. Poly A⁺ RNA (10 µg) from liver, kidney and gill was also run on Agarose gels. RNA was transferred by capillary action onto nylon membranes (GeneScreen) for 48 h. The membrane was exposed to UV for 5 min to cross-link the RNA to the membrane.

PCR products containing the fragment of the NBC gene of interest were run on a gel and then isolated using the QIAquick PCR band isolation kit (QIAGEN) according to the manufacturer's instructions. The isolated DNA was used to probe the tissues of interest. DNA was radio-labelled with ³²P-dCTP (Amersham) using the random prime (Klenow) method. Initially, 7 µl DNA was added to 5 µl H₂O (final volume 12 µl), boiled for 5 min and then chilled on ice for 3 min. Probe was labelled by the Klenow reaction consisting of 6 µl 5X buffer, 5X nucleotides, 5 µl (50 µCi) ³²P-dCTP and 1 µl large fragment DNA polymerase (GibcoBRL). The reaction progressed for 30 min and then was stopped by the addition of 70 µl STE buffer (50 mmol l⁻¹ Tris, 10 mmol l⁻¹ EDTA, 150 mmol l⁻¹ NaCl, 0.1% SDS). This mixture was added to a Sephadex spin column (made using a 1.0 ml syringe) and centrifuged at maximum speed for 3 min to remove unincorporated isotope. The reaction mixture containing the labelled probe was boiled for 10 min to denature the DNA strands and

chilled on ice. Probe was then added directly to the tube containing the blots and hybridisation solution.

Blots were pre-washed in 50 ml of 0.1 X SSC, 0.1% SDS at 65°C for 20 min. The hybridisation solution (made up in ddH₂O containing 10% dextran sulfate, 1% SDS, 6X SSC, 100 µg ml⁻¹ ssDNA and 5X Denhardt's), was added to the membranes at 65°C (10 ml hybridisation solution per 100 cm²). After 4 h, the denatured probe was added to the pre-hybridisation solution and incubated at 65°C for 20 h. The blots were washed at 65°C sequentially with 2 X SSC (2 washes of 50 ml each for 15 min each wash) followed by 2 X SSC, 0.1% SDS (2 washes of 50 ml each for 30 min each wash).

RESULTS

A homology cloning approach was used to clone a portion of the cDNA encoding the Na⁺-HCO₃⁻ cotransporter in the rainbow trout (*Oncorhynchus mykiss*). Based on conserved nucleotide sequences of other vertebrate species, primers were designed for PCR experiments involving trout gill/kidney and liver cDNA libraries (Fig. 6-1). PCR products of the expected size (731 bp and 420 bp) were obtained from the gill/kidney cDNA library, demonstrating high homology with the known NBC sequences from other species (Figs. 6-2 and 6-3). The 731 bp cDNA fragment (that included identical sequences from the smaller fragment) was used as a probe for the hybridisation experiments. Hybridisation of a Southern Blot containing PCR products obtained using the same primers but the liver cDNA library as a template yielded positive signals of the expected sizes (Fig. 6-4A), as did the cloned liver DNA restricted from the EcoRI site of the pGEM vector (Fig. 6-4B). The 420 bp fragment of liver DNA cloned from the liver cDNA (Fig. 6-5), demonstrated 100% homology with the same portion of NBC sequence from the gill/kidney (Fig. 6-6).

A Blastn search of the Genbank database yielded 17 matches corresponding to vertebrate NBC sequences, all having E values less than 10^{-16} . Nucleotide identity with the trout gill/kidney NBC sequence was 78-79%. The highest score occurred with the rabbit duodenal $\text{Na}^+\text{-HCO}_3^-$ cotransporter sequence. A Blastp search of the translated nucleotide sequence yielded 25 matches with E values less than 10^{-50} . Amino acid identities were between 44-71%. The highest identity was with a fragment of the *Ambystoma* NBC protein. The 420 bp trout liver sequence was ~ 100% identical to a portion of the trout gill/kidney sequence.

The tissue distribution Northern blot using 20 μg of total RNA isolated from different fish tissues showed some non-specific signals with gill, intestine and kidney, but not in liver (Fig. 6-7). A Northern blot of 15 μg of mRNA isolated from liver, gill and kidney showed distinct bands above that of the 28S ribosomal RNA (~4 Kb) that were estimated to be approximately 6-8 Kb in size. The expression appears high in gill and kidney, with lower expression in the liver (Fig. 6-8). Non-specific binding is visible to the 18S and 28S ribosomal bands and the size of the NBC could only be derived from their known sizes.

Figure 6-2. Nucleotide sequence and translated amino acids of the trout gill/kidney NBC partial sequence (731 bp). The primer sites are indicated (bold). The sequence was prepared using DNAMAN (Lynnon Biosoft).

1 GCCTCCCATGGCGGCCGCGGGAATTCGATT**CATGCAGGGCGTGTGAGAG**
 1 P P M A A A G I R F M Q G V L E S
 52 TTTCCTGGGCACGGCACTGACGGGGGCCATATTCTGTCTGCTGGGTGGCCA
 18 F L G T A L T G A I F C L L G G Q
 103 GCCTCTCACCATTCTCAGCAGCACAGGTCCTGTACTGGTGTTTGAAAGGCT
 35 P L T I L S S T G P V L V F E R L
 154 CCTCTTCAACTTCAGCAAAGACCATGACTTTGACTATCTGGAGTTCAGGCT
 52 L F N F S K D H D F D Y L E F R L
 205 GTGGATCGGCCTTTGGTCAGCCTTCTTCTGCCTGGTGCTGGTTGCCACAGA
 69 W I G L W S A F F C L V L V A T D
 256 TGCCAGCACCTGGTCAAGTACTTCACACGCTTCACAGAGGAGGGCTTTTC
 86 A S T L V K Y F T R F T E E G F S
 307 TTCCCTCATCTCCTTCATCTTCATCTACGACGCCTTCAAGAAGATGCTGAA
 103 S L I S F I F I Y D A F K K M L K
 358 GCTGGCCCACTACAACCCCATCAACGCTGACTATGACCCCAACTACGTCAC
 120 L A H Y N P I N A D Y D P N Y V T
 409 AATGTACGACTGCCGCTGCATGCCCCACCGGACGACGGCAATATGACGGG
 137 M Y D C R C M P P P D D G N M T G
 460 TCTCTATATCGATGCCTCTGCCTGGATAAACAATGCTGATCTGTCGGTAAA
 154 L Y I D A S A W I N N A D L S V N
 511 TGCCACATGGGCGTCTCTCGACAAGAAGCAGTGTGTGGTGTACGGCGGCGA
 171 A T W A S L D K K Q C V V Y G G E
 562 GCTGATTGGTCCAGCCTGTGGCTTCGTCCCTGACGTCACTCTGATGTCATT
 188 L I G P A C G F V P D V T L M S F
 613 CATCCTTTTCCTGGGGACCTACACCTGCTCCATGTCCCTCAAGAAGTTTAA
 205 I L F L G T Y T C S M S L K K F K
 664 GTTCAGCCGCTTCTTCCCCACCACTGTGAGGAAGCTG**ATCAGTGACTTTGC**
 222 F S R F F P T T V R K L I S D F A
 715 **CATTATCAATCACTAGT**
 239 I I N H *

Figure 6-3. Protein alignment of the trout gill/kidney NBC amino acid sequence with different vertebrate NBC sequences, including human kidney (*Homo sapiens*, Genbank accession no. AF007216), rat kidney (*Rattus norvegicus*, Genbank accession no. AF004017), mouse pancreas (*Mus musculus*, Genbank accession no. AF020195), rabbit cornea (*Oryctolagus cuniculus*, Genbank accession no. AF119816) and salamander kidney (*Ambystoma tigrinum*, Genbank accession no. AF001958). The alignment was prepared using DNAMAN (Lynnon Biosoft) and the alignment was shaded using GeneDoc® (available free at www.psc.edu/biomed/genedoc). The consensus of the six sequences is indicated below the alignments. Capital letter represents identity among all six sequences; small letter represents identity among five of six; numbers indicate that all six have an amino acid of the same functional group. Functional amino acids are defined in six groups: 1 (DN), 2 (EQ), 3 (ST), 4 (KR), 5 (FYW), and 6 (LIVM). Black shading represents identity among all six sequences, grey shading represents identity among five of six sequences, and light shading represents identity among 4 of 6 sequences.

```

      *      20      *      40      *      60      *      80
human : -----MSTENVEGKPSNLGRGRARSTFTIRVQPMFN-HSTSA : 41
rat : -----MSTENVEGKPNNLGRGRARSTFTIRVQPMFN-HSTSA : 41
mouse : -----MSTENVEGKPNNLGRGRARSTFTIRVQPMFN-HSTSA : 41
rabbit : MEDEAVLDRGASFLKHVCDEEEVEGHHTIYIGVHVPKSYRRRRRHKKRTGHEKREKRISENYSKSDENADESSSLKPL : 85
salaman : -----MSSEKCEINMLNGYASQVLRISITVNAVNR--EST : 42
trout : -----e si-----

      *      100      *      120      *      140      *      160      *
human : ----- : 126
rat : ----- : 126
mouse : ----- : 126
rabbit : ----- : 170
salaman : ----- : 127
trout : -----
      spaaerirfilgeedspappqlfteldellavdgqemewketarwikfeekveqggrwskphvatlsihlsifelrtc ekq i

      180      *      200      *      220      *      240      *
human : ----- : 211
rat : ----- : 211
mouse : ----- : 211
rabbit : ----- : 255
salaman : ----- : 212
trout : -----
      ld ea slpq ve qie gllk k vt tllrkhrrhqtksnlrsladigktvssasr f pdngsp mthrnltS

      260      *      280      *      300      *      320      *      340
human : ----- : 296
rat : ----- : 296
mouse : ----- : 296
rabbit : ----- : 340
salaman : ----- : 297
trout : -----
      slnd sdkp k qlknkfmkklprdaeeasnlvgevdfI pfiafvrlqqavmlg ltevpvptrflfillgpkqkaksyhei

      *      360      *      380      *      400      *      420
human : ----- : 381
rat : ----- : 381
mouse : ----- : 381
rabbit : ----- : 425
salaman : ----- : 380
trout : -----
      gr iatlmSdevfhdiaykak r dliagidefldevilp gewdp irieppkslpssdkrknmysgg n qmngd phd

      *      440      *      460      *      480      *      500      *
human : ----- : 466
rat : ----- : 466
mouse : ----- : 466
rabbit : ----- : 510
salaman : ----- : 465
trout : -----
      ggggh d eelqtrgrfcgglikd rkapffasdfydal iq lsailfiyl tvtnaitfgg6lgdat nMQGVLESFLGTA6

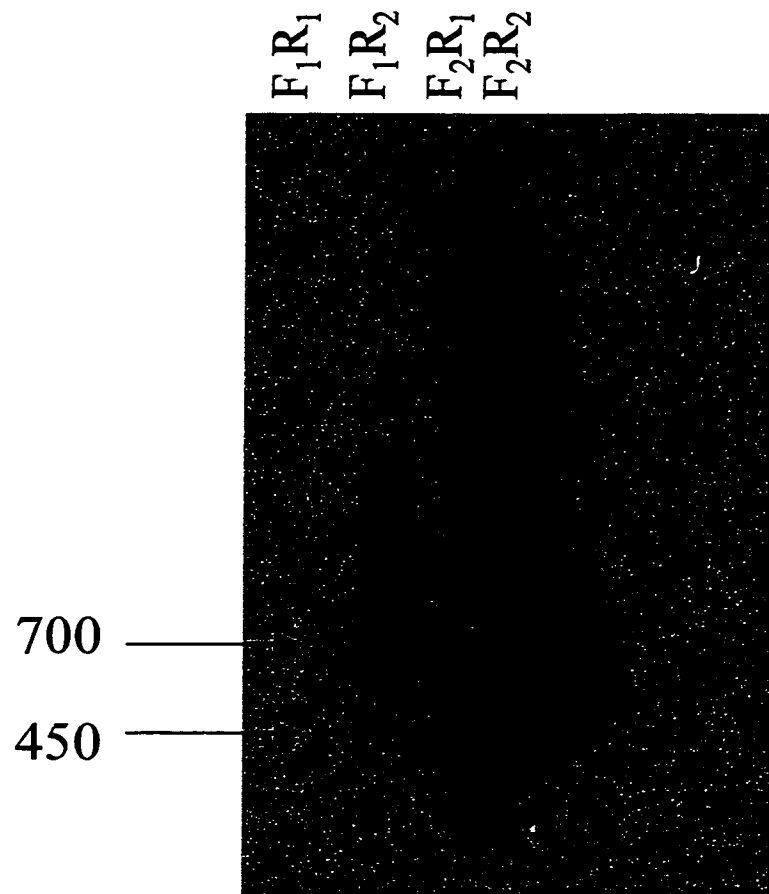
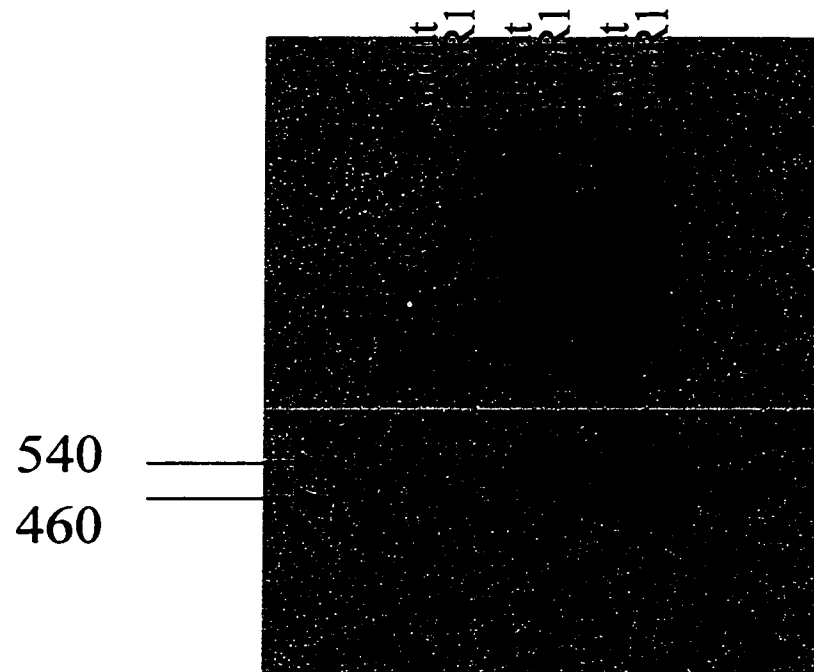
      520      *      540      *      560      *      580      *
human : ----- : 551
rat : ----- : 551
mouse : ----- : 551
rabbit : ----- : 595
salaman : ----- : 550
trout : -----
      3GA6ECLf GQPLTILSSTGPFVLVFERLLENFSKD FDYLEFRLWIGLWSAF CL6LVATDASfLV YFTRFTEEGFSSLISFI

      600      *      620      *      640      *      660      *      680
human : ----- : 635
rat : ----- : 635
mouse : ----- : 635
rabbit : ----- : 679
salaman : ----- : 635
trout : -----
      FIYDAFKKM6KLAdYyPINS 5 v Y T 5sC C PP P l s N Tt a std6 N T dwa LsKK2C 5GG

      *      700      *      720      *      740      *      760
human : ----- : 720
rat : ----- : 720
mouse : ----- : 720
rabbit : ----- : 764
salaman : ----- : 720
trout : -----
      L6G nC 5VPD6TLMSFILFLGTYT SMa6KKFKtSr5FPTTaRKLISDFAIILsilifc dal gvdtpkiliVpsefkptsp

```


Figure 6-4. Southern blot analysis of PCR products from the trout liver cDNA library. Blots were probed with the 731 bp NBC cDNA cloned from the trout gill/kidney cDNA library. Positive signals were obtained with (A) initial PCR products using the F₁R₂ and F₂R₂ primer combinations, as well as (B) EcoR1 restriction fragments from pGEM vector containing cloned liver cDNA. Approximate size of cDNA is indicated based on size ladder PhiXHaeIII.

A**B**

```

1      GATTTATGATGCTTTCAAGAAGATGCTGAAGCTGGCCCCACTACAACCCCAT
      I Y D A F K K M L K L A H Y N P I

52     CAACGCTGACTATGACCCCAACTACGTCACAATGTACGACTGCCGCTGCAT
18     N A D Y D P N Y V T M Y D C R C M

103    GCCCCACCGGACGACGGCAATATGACGGGTCTCTATATCGATGCCTCTGC
35     P P P D D G N M T G L Y I D A S A

154    CTGGATAACAATGCTGATCTGTTCGGTAAATGCCACATGGGCGTCTCTCGA
52     W I N N A D L S V N A T W A S L D

205    CAAGAAGCAGTGTGTGGTGTACGGCGGCGAGCTGATTGGTCCAGCCTGTGG
69     K K Q C V V Y G G E L I G P A C G

256    CTTTCGTCCTGACGTCACCTCTGATGTCATTCATCCTTTTCCTGGGGACCTA
86     F V P D V T L M S F I L F L G T Y

307    CACCTGCTCCATGTCCTCAAGAAGTTTAAGTTCAGCCGCTTCTTCCCCAC
103    T C S M S L K K F K F S R F F P T

358    CACTGTGAGGAAGCTGATCAGTGACTTTGCCATTATCAGTGATTTTGCCAT
120    T V R K L I S D F A I I S D F A I

409    TATCAATCACTAGT
137    I N H *

```

Figure 6-5. Nucleotide sequence and translated amino acids of the trout liver NBC partial sequence (422 bp). The primer sites are indicated (bold). The sequence was prepared using DNAMAN (Lynnon Biosoft).

Figure 6-6. Amino acid sequence alignment of the partial $\text{Na}^+\text{-HCO}_3^-$ cotransporters from trout gill/kidney and liver. The alignment was prepared using DNAMAN (Lynnon Biosoft) and the alignment was shaded using GeneDoc© (available free at www.psc.edu/biomed/genedoc). The consensus of both sequences is indicated below the alignment. Capital letter and black shading represent identity between the two sequences.

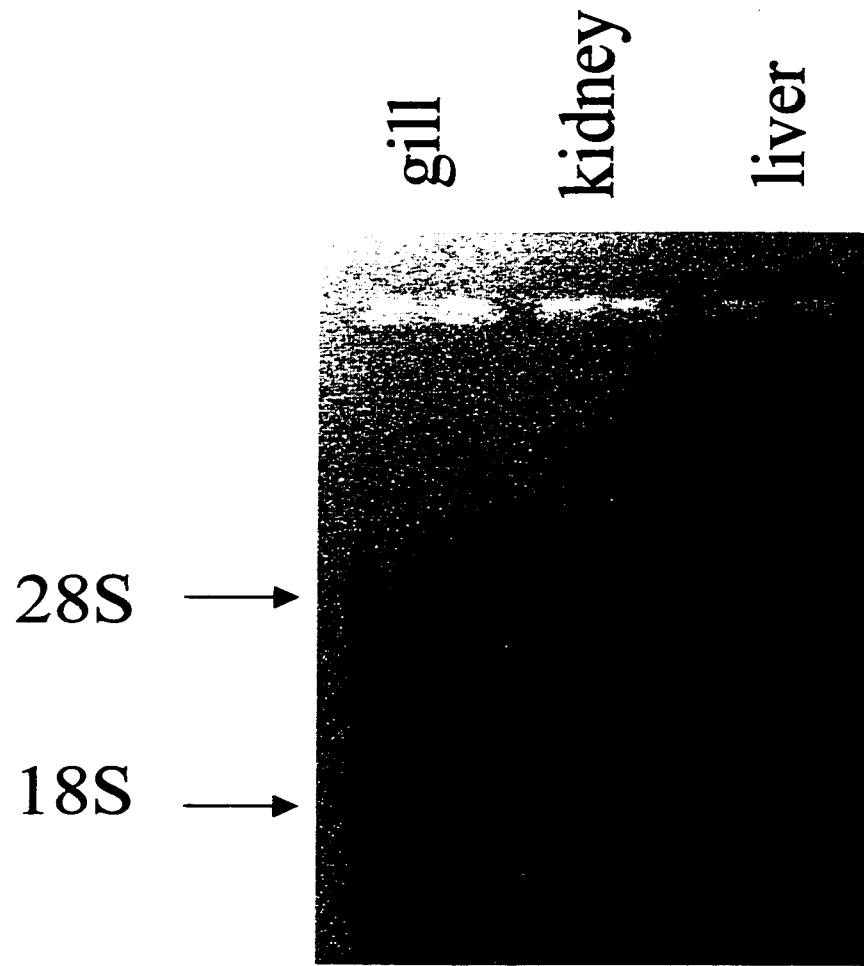
Figure 6-7. Northern blot analysis of total RNA (20 µg per lane) isolated from different trout (*Oncorhynchus mykiss*) tissues. Blot was hybridised with the 731 bp NBC cDNA from trout gill/kidney under low stringency conditions.



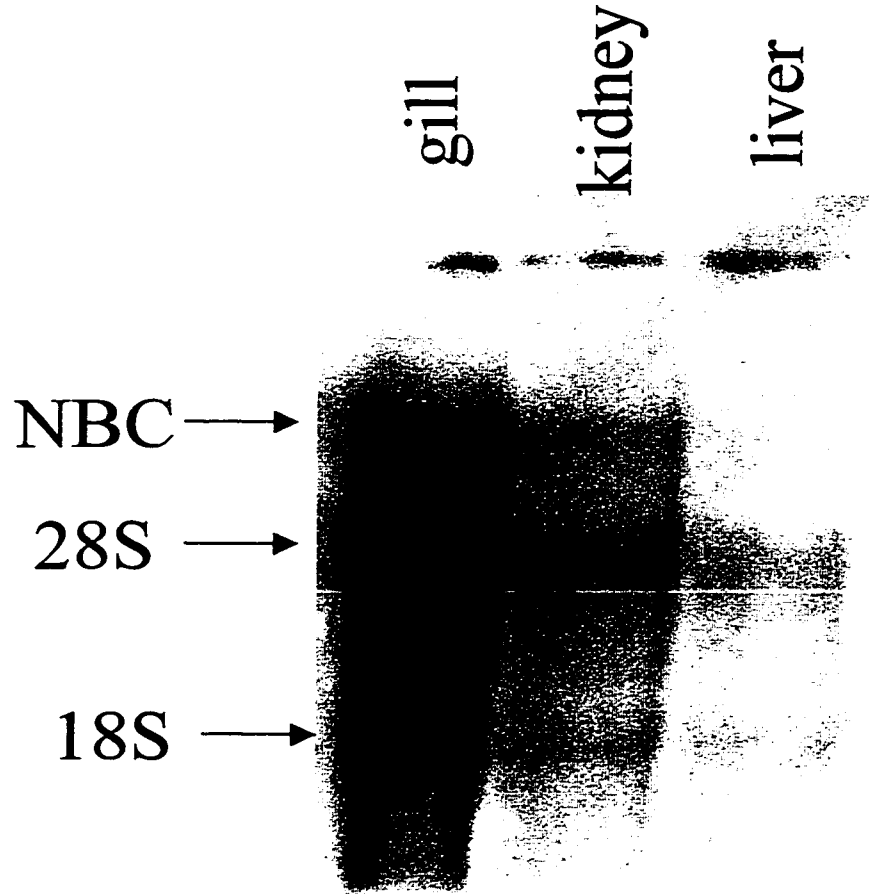
blood
blood (cort.)
gill
gill (cort.)
heart
intestine
kidney (ant.)
kidney (post.)
liver
liver (cort.)
muscle (white)
spleen

Figure 6-8. Northern blot analysis of mRNA (15 µg per lane) isolated from trout (*Oncorhynchus mykiss*) gill, kidney and liver. Blot was hybridised with the 731 bp NBC cDNA from the gill/kidney under high stringency conditions.

A



B



DISCUSSION

The molecular biology of the $\text{Na}^+\text{-HCO}_3^-$ cotransporter has only very recently come to the forefront after years of pharmacological studies. Since the gene was first cloned from *Ambystoma* kidney (Boron *et al.*, 1997), its expression has been observed in several species and tissues, and different isoforms have been elucidated (see reviews by Boron *et al.*, 1997; Romero and Boron, 1999; Soleimani and Burnham, 2000). Since the initial discovery of the salamander NBC, all further focus has been on the cloning and expression of this protein in mammalian systems. A gene coding for a similar transporter has been found in an invertebrate, the nematode *C. elegans* (Romero and Boron, Genbank accession no. AF004926).

The $\text{Na}^+\text{-HCO}_3^-$ cotransporter is known to play an important role in the reabsorption of the majority of HCO_3^- from the lumen of the kidney (Boron and Boulpaep, 1983; Alpern, 1985). The stoichiometry of NBC in the kidney is $1\text{Na}^+:3\text{HCO}_3^-$ that favours the movement of HCO_3^- out of kidney cells and into the circulation. When participating in the regulation of pH_i , transport occurs by the more favourable $1\text{Na}^+:2\text{HCO}_3^-$ stoichiometry that allows for the entrance of HCO_3^- into the cytoplasm along with the Na^+ gradient. The same NBC isoform is capable of working in both directions. In its role in pH_i regulation, it is involved in maintaining pH_i at baseline levels rather than being mainly activated by decreases in pH_i as is the $\text{Na}^+\text{-H}^+$ exchanger.

The $\text{Na}^+\text{-HCO}_3^-$ cotransporter is a single protein that displays a hydropathy profile of 10 transmembrane domains (Romero and Boron, 1999). The membrane-spanning domain contains the sites for Na^+ and HCO_3^- transport, and incurs DIDS sensitivity. The 230 amino acid fragment of trout gill/kidney NBC represents the sequence spanning from the third to the seventh transmembrane domain. This sequence includes the large putative extracellular loop as well as a DIDS binding motif. The greatest heterology that exists between all the sequences studied is at the large extracellular loop located between the putative fifth and sixth transmembrane domains. The DIDS motif containing four amino acids (KMIK;

K=lysine, M=methionine, I=isoleucine) is conserved in all mammalian NBC sequences. In the trout sequences (liver and gill/kidney) this motif, located prior to the extracellular loop, is KMLK (L=leucine). Anion exchangers (AE) have a different DIDS motif KL(X)K (X=I,V,Y) (Bartel *et al.*, 1989; Okubo *et al.*, 1994). The size of the NBC gene in gill, kidney and liver, approximated by comparison to the size of fish rRNA (Hervio *et al.*, 1997), is comparable to the known complete sequences.

Since a complete NBC sequence has yet to be obtained from any fish tissue, it is not possible to make a proper phylogenetic comparison with other vertebrate species. The differences observed can only suggest an evolutionary difference. Not only would the translated sequences of the gene be necessary for a complete analysis, but also the 5' and 3' untranslated sequences. The cloned liver and gill/kidney cDNA sequences represent amino acids located in the middle of the protein, so a good strategy to employ from this point forward would be to use RACE (Rapid Amplification of cDNA Ends) PCR to clone the 5' and 3' segments.

The identification of the role of $\text{Na}^+\text{-HCO}_3^-$ cotransport as a principal mechanism of pH_i regulation in trout hepatocytes (Furimsky *et al.*, 2000), provided some initial evidence that this protein was active in fish tissues. Though a complete liver NBC sequence has yet to be obtained from any vertebrate species, the fragment cloned in this study provides further evidence for its presence in the trout liver (Fig. 6-2, 6-3). This transporter has also recently been identified to play a role in the pavement cells of trout gills (Wood and Pärt, 2000), though it is not yet clear how NBC is directly involved in acid-base or ionoregulatory mechanisms. The accepted model for Na^+ uptake and H^+ excretion in the pavement cells of freshwater fish involves a linked $\text{H}^+\text{-ATPase}$ and Na^+ channel (Avella and Bornancin, 1989). It is likely that NBC is located on the basolateral membrane of chloride and pavement cells and is involved in HCO_3^- excretion and/or pH_i regulation of gill cells as a result of the appropriate gradients that exist for Na^+ and HCO_3^- between blood and the cytoplasm. It is possible that similar to the mammalian and amphibian kidney, this protein is also involved in

osmoregulation by reabsorbing HCO_3^- from the nephron. During hypercapnia, the kidney excretes acids and takes up more HCO_3^- to avoid its loss in the urine. The reabsorption of tubular HCO_3^- may be mediated by NBC as this activity is up-regulated during an acid stress. A good strategy to detect any subtle changes in NBC mRNA expression, as may be occurring during an acid-base disturbance, would be to employ ribonuclease protection assay.

In summary, the trout gill, kidney and liver express the $\text{Na}^+\text{-HCO}_3^-$ cotransporter. Although only a partial sequence has been obtained, it is clear that very high homology exists between trout and the other vertebrate species sequences examined to date. The transmembrane domains are well conserved, though one amino acid substitution occurs in the DIDS motif of the fish sequence. Though the molecular evidence obtained in this study supports physiological evidence obtained from trout liver (Furimsky *et al.*, 2000), it also suggests a novel mechanism involving this protein in Na^+ and HCO_3^- transport in the gills and kidneys of teleost fish.

CHAPTER 7

GENERAL DISCUSSION

SUMMARY OF PRINCIPLE FINDINGS

CHAPTER 2: Isolated hepatocytes experience an intracellular ion imbalance following isolation. Ion gradients for Na^+ , K^+ and Cl^- are directionally correct, though different from those found in the intact tissue.

CHAPTER 3: The hepatocytes of *O. mykiss*, *A. melas* and *A. rostrata* are able to regulate pH_i , though they exploit different mechanisms. Regulation of pH_i in *O. mykiss* and *A. melas* occur principally by Na^+/H^+ exchange, whereas *A. rostrata* appears to exploit a Cl^- -dependent mechanism.

CHAPTER 4: A $\text{Na}^+/\text{HCO}_3^-$ cotransporter is a principal component in pH_i regulation in the trout hepatocyte. This transporter regulates baseline pH_i and is complementary to the Na^+/H^+ exchanger in recovery to an acid load.

CHAPTER 5: The ability of trout hepatocytes to recover from an acid load is not influenced by respiratory acidosis. Though a decrease in blood pH results in a decrease in pH_i , no physiological evidence was obtained supporting the hypothesis that chronic exposure to acidosis up-regulates the mechanisms involved in recovery to acid loading.

CHAPTER 6: The trout liver and gill/kidney express the gene that codes for the $\text{Na}^+/\text{HCO}_3^-$ cotransporter. A partial clone was obtained from both a liver cDNA library as well as a mixed gill/kidney library. A Northern blot of a liver, gill and kidney mRNA probed with a homologous 734 bp NBC fragment gave a positive signal.

Intracellular pH regulation in teleost hepatocytes

Fish hepatocyte as a cell model

The use of hepatocytes as a cell model has yielded countless data regarding many aspects of mammalian and comparative physiology. It is, however, necessary to appreciate that these cells are not in their natural physiological state and that results may not be truly representative of what occurs in the intact organism. The morphology of the liver *in vivo* consists of hepatocytes in a polarised arrangement with the basolateral membrane facing the circulating blood and the apical membrane facing the bile canaliculus. Hepatocytes in suspension would clearly display a non-polarised distribution of membrane transporters and loss of the physiological steady-state dependent on an association with other cells in a tissue. The cell membrane targeting (polarity) of the membrane transporters was appreciated, though not crucial to this thesis. The main interest of the pharmacological studies was the activity of the mechanism of pH_i regulation as a whole, though experimental systems in which the polarity of fish hepatocytes could be established would be of great benefit. Cell viability was always high, though the gradients that existed across the cell membrane differed from those found in the intact tissue (Chapter 2). The measurements of plasma ion levels, as well as intracellular erythrocyte and hepatocyte ion levels demonstrated that differences do exist between species in their blood chemistry and its regulation. The basis for different plasma ion levels in fish are not understood, but are interesting species differences nonetheless. The ionic gradients that existed between the plasma and the hepatocyte intracellular environments did, in all cases, display favourable Na^+ and K^+ gradients.

Interspecific Comparisons

Several different regulatory mechanisms participate in concert to regulate hepatocyte pH_i . The different regulatory mechanisms, be they membrane transport proteins or intracellular buffers, are in turn regulated independently. The $\text{Na}^+\text{-H}^+$ exchanger is activated by low pH_i , the $\text{Cl}^-\text{-HCO}_3^-$ exchanger is activated by high pH_i and the $\text{Na}^+\text{-HCO}_3^-$ cotransporter is most highly active at baseline pH_i . Though all mechanisms work towards the main goal of regulating pH_i , their roles are distinct and may vary between different cell types.

The membrane transport proteins that contribute to hepatocyte pH_i regulation were examined in the rainbow trout, black bullhead and American eel (Chapter 3). Previous studies involving the rainbow trout and the marine Gulf toadfish showed significant differences in the mechanisms employed, namely the absence of $\text{Cl}^-\text{-HCO}_3^-$ exchange in the toadfish (Walsh, 1986; 1989). Pharmacological studies showed that trout and bullhead employed similar mechanisms to regulate hepatocyte pH_i , but the eel differed significantly. A decrease in pH_i activated the $\text{Na}^+\text{-H}^+$ exchanger (NHE) in trout and bullhead. In these two species, recovery was dependent on extracellular Na^+ , though trout recovery was even more strongly dependent, suggesting another mechanism that upon further study (Chapter 4) was determined to be a $\text{Na}^+\text{-HCO}_3^-$ cotransporter (NBC). The eel did not demonstrate any Na^+ -dependent or amiloride-sensitive recovery to an acid load. Though amiloride did decrease baseline pH_i in eel hepatocytes, NHE appears to be functionally absent. Recovery to an acid load was Cl^- -dependent in eel, likely involving the decrease in activity of a Na^+ -independent $\text{Cl}^-\text{-HCO}_3^-$ exchanger. Recovery to a base load in trout hepatocytes involved the turning off of NHE and the activity of $\text{Cl}^-\text{-HCO}_3^-$ exchange. Significant morphological, physiological, and pharmacological differences are known to exist between eel and other teleost species

(gill acid-base and ionic regulation: Laurent and Perry, 1995; chromaffin cell pharmacology: Reid and Perry, 1994; erythrocyte NHE: Romero *et al.*, 1996). The eel is considered a more primitive teleost species, and is well known to be resistant to stress and able to cope with severe environmental changes. It is not yet clear if the differences observed between the different species are true evolutionary differences or whether they represent different strategies that best suit the lifestyles of the particular fish. It is possible that the eel has retained some basic ionoregulatory characteristics, whereas more advanced species have become more specialised to exploit different environments and lifestyles, requiring new methods of cell and blood regulation.

The three teleost species compared in chapters 2 and 3 of this thesis (trout, bullhead and eel) do not represent the entire spectrum of fish evolution, but rather only a relatively short period of the early evolution of the teleost lineage. A more representative comparative study would include agnathans (the predecessors of fish), elasmobranch species such as sharks and rays, and a wider range of teleost fish.

Role of the $\text{Na}^+\text{-HCO}_3^-$ cotransporter

Though the $\text{Na}^+\text{-HCO}_3^-$ cotransporter (NBC) is well known in mammalian tissues for its role in HCO_3^- reabsorption and pH_i regulation, this transporter had never before been identified in any fish tissue. Though the role of this transporter was suggested from data in trout (Chapter 3), only further investigation lead to the conclusion that it was actually present in the trout hepatocyte and integral to pH_i regulation (Chapter 4). Previous studies provided no evidence for its expression and activity in fish, and it was suggested, quite reasonably that the low $[\text{HCO}_3^-]$ would not favour the activity of this HCO_3^- -dependent transporter. The

transport of HCO_3^- had been attributed only to Na^+ -dependent and -independent Cl^- - HCO_3^- exchange. Evidence for the role of a Na^+ - HCO_3^- cotransporter was obtained using both physiological and molecular techniques (Chapters 4 and 6, respectively). In trout hepatocytes, all the factors that distinguish NBC from other membrane transport proteins were observed, namely DIDS-sensitivity, Na^+ -and HCO_3^- -dependence, and electrogenicity. The recovery to acid loading did not appear to have an additional Na^+ -dependent mechanism in bullhead or eel hepatocytes, though further investigation would be necessary to draw any positive conclusions.

The analysis of putative NBC sequences cloned from both liver and gill/kidney cDNA libraries showed highest homology to known NBC sequences in mammalian tissues. Since the NBC gene family shares high sequence homology to the anion exchanger (AE) gene family, primers were designed from NBC-specific sequences in order to avoid cloning a Cl^- - HCO_3^- exchanger that is known to be active in most fish tissues including liver. The homology between AE and NBC is clear, necessitating the formation of a Bicarbonate Transporter Superfamily (BTS) (Romero and Boron, 1999). The initial primer strategy did indeed provide correct inserts, though the full-length sequence is still to be determined. The NBC clones obtained were similar to the mammalian sequences, though the differences observed in the DIDS-binding motif and the external loop could provide evidence as to the evolution of this gene in vertebrates.

The experiments examining Na^+ - HCO_3^- cotransporter function and expression focused only on one species, the rainbow trout. It is realistic to believe that NBC is also expressed in other fish species. It is surprising that no concrete evidence has been presented for this transporter in any fish species until now, but these discoveries can provoke a re-

evaluation of some well known ionoregulatory models in fish tissues. Though it was beyond the scope of this thesis, the putative role of NBC in the gill, in particular could add another dimension to the wealth of knowledge concerning Na^+ and HCO_3^- excretion in fish. The primary genetic information that exists for the trout not only provides a greater understanding of this membrane transporting mechanism in this salmonid, but also in all other fish species. Though high homology exists between the trout and salamander NBC, it is possible that the differences that occurred over evolutionary time can be traced to more advanced species within the fish lineage.

Expression of membrane transport proteins

The expression of regulatory proteins in fish and mammals is known to be strongly influenced by external (environmental) factors or stressors that result in blood acid-base disturbances. The membrane transport mechanisms involved in counteracting these stresses are expressed in such a way as to enhance the ability of the organism to regulate these disturbances and return conditions back to their normal steady-state. One goal of this thesis was to determine if the hepatocyte pH_i regulatory mechanisms are regulated by blood acid-base status, but no physiological differences were observed following exposure to chronic hypercapnia. The expression of genes for membrane transport proteins, namely NHE, could not be determined as a result of a lack of signal in Northern Blots where liver RNA was probed with homologous and NHE heterologous probes. The ribonuclease protection assay may be a valuable tool in assessing the more subtle changes in mRNA expression. The physiological data do not demonstrate that regulation occurs at the liver during an acid-base

disturbance, but the data do suggest that this organ would depend on the actions of the gills and kidney to regulate blood status

CONCLUSIONS

The focus of this thesis was an investigation of the mechanisms involved in regulating hepatocyte pH_i in teleost fish. Experimental evidence suggested that several membrane transport proteins are involved in the regulation of pH_i and that their relative roles differ in evolutionarily distinct fish species. Compared to the trout and bullhead, where the Na^+/H^+ exchanger played a significant role in recovery to an acid load, the eel hepatocyte exploited a Cl^- -dependent mechanism. The significant role of a $\text{Na}^+/\text{HCO}_3^-$ cotransporter in the maintenance of baseline pH_i and the recovery from an acid load in trout hepatocytes is a novel finding in a fish tissue. The partial cloning of the $\text{Na}^+/\text{HCO}_3^-$ cotransporter (NBC) gene from fish tissues demonstrated that this membrane transport protein is highly conserved amongst the vertebrate species so far examined. Therefore, while several different membrane transport mechanisms are involved in regulating pH_i of fish hepatocytes, it is still not known if these mechanisms are influenced by environmental factors as is the H^+ -ATPase that is a principal iono-regulatory membrane transport mechanism of the gill and kidney (Perry *et al.*, 2000). The significant differences observed among teleost species in this study and the molecular evidence for the presence of a $\text{Na}^+/\text{HCO}_3^-$ cotransporter in fish provide a significant starting point for future research into the evolution of the regulatory mechanisms in lower vertebrates. Comparative physiological studies involving complementary experiments at the whole animal, cell and molecular levels should be the focus of future work in examining the mechanisms of pH_i regulation in evolutionarily distinct species of fish.

REFERENCES

- Abuladze, N., Lee, I., Newman, D., Hwang, J., Boorer, K., Pushkin, A. and Kurtz, I.(1998). Molecular cloning, chromosomal localisation, tissue distribution, and functional expression of the human pancreatic sodium bicarbonate cotransporter. *J. Biol. Chem.* **273**: 17689-17695.
- Aiello, E.A., Petroff, M.G., Mattiazzi, A.R. and Cingolani, H.E. (1998). Evidence for an electrogenic Na^+ - HCO_3^- symport in rat cardiac myocytes. *J. Physiol. (Lond)* **512**: 137-148.
- Akiba, T., Rocco, V.K. and Warnock, D.G. (1987). Parallel adaptation of the rabbit renal cortical sodium/proton antiporter and sodium/bicarbonate cotransporter in metabolic acidosis and alkalosis. *J. Clin. Invest.* **80** : 308-315.
- Albers, C. and Goetz, K.-H. (1985). H^+ and Cl^- ion equilibrium across the red cell membrane in the carp. *Resp. Physiol.* **61**: 209-219.
- Alper, S.L. (1991). The band 3-related anion exchanger (AE) gene family. *Annu. Rev. Physiol.* **53**: 549-564.
- Alper, S.L. (1994). The band 3-related AE anion exchanger gene family. *Cell Physiol. Biochem.* **4**: 265-281.
- Alpern, R.J. (1985). Mechanism of basolateral membrane $\text{H}^+/\text{OH}^-/\text{HCO}_3^-$ transport in the rat proximal convoluted tubule. A sodium-coupled electrogenic process. *J. Gen. Physiol.* **86**: 613-636.

- Alvaro, D., Della Guardia, P., Bini, A., Gigliozzi, A., Furfaro, S., La Rosa, T., Piat, C. and Capocaccia, L. (1995). Effect of glucagon on intracellular pH regulation in isolated rat hepatocytes couplets. *J. Clin. Invest.* **96**: 665-675.
- Ambuhl, P.M., Yang, X., Peng, Y., Preisig, P.A., Moe, O.W. and Alpern, R.J. (1999). Glucocorticoids enhance acid activation of the Na^+/H^+ exchanger 3 (NHE3). *J. Clin. Invest.* **103**: 429-435.
- Amemiya, M., Yamaji, Y., Cano, A., Moe, O.W. and Alpern, R.J. (1995). Acid incubation increases NHE -3 mRNA abundance in OKP cells. *Am. J. Physiol.* **269**: C126-C133.
- Amlal, H., Wang, Z., Burnham, C. and Soleimani, M. (1998). Functional characterization of a cloned human kidney $\text{Na}^+:\text{HCO}_3^-$ cotransporter. *J. Biol. Chem.* **273**: 16810-16815.
- Amlal, H., Burnham, C.E. and Soleimani, M. (1999). Characterization of $\text{Na}^+:\text{HCO}_3^-$ cotransporter isoform NBC-3. *Am. J. Physiol.* **276**: F903-F913.
- Andreasen, P. (1985). Free and total calcium concentrations in the blood of rainbow trout, *Salmo gairdneri*, during "stress" conditions. *J. exp. Biol.* **118**: 111-120.
- Attaphitaya, S., Park, K. and Melvin, J.E. (1999). Molecular cloning and functional expression of a rat Na^+/H^+ exchanger (NHE5) highly expressed in brain. *J. Biol. Chem.* **274**: 4383-4388.
- Avella, M. and Bornancin, M. (1989). A new analysis of ammonia and sodium transport through the gills of the freshwater rainbow trout (*Salmo gairdneri*). *J. exp. Biol.* **142** : 155-175.

- Baird, N.R., Orlowski, J., Szabo, E.Z., Zaun, H.C., Schultheis, P.J., Menon, A.G. and Schull, G.E. (1999). Molecular cloning, genomic organization, and functional expression of Na^+/H^+ exchanger isoform 5 (NHE5) from human brain. *J. Biol. Chem.* **274**: 4377-4382.
- Bartel, D., Hans, H. and Passow, H. (1989). Identification by site directed mutagenesis of Lys-558 as the covalent attachment site of dihydro DIDS in the mouse erythroid band 3 protein. *Biochim. Biophys. Acta* **985** : 355-358.
- Baum, M., Cano, A. and Alpern, R.J. (1993): Glucocorticoids stimulate Na^+/H^+ antiporter in OKP cells. *Am. J. Physiol.* **264**: F1027-F1031.
- Baum, M., Moe, O.W., Gentry, D.L. and Alpern, J. (1994). Effect of glucocorticoids on renal cortical NHE-3 and NHE-1 mRNA. *Am. J. Physiol.* **267**: F437-F442.
- Benedetti, A., Baroni, G.S., Marucci, L., Mancini, R., Jezequel, A.M. and Orlandi, F. (1993). Regulation of intracellular pH in isolated periportal and perivenular rat hepatocytes. *Gastroenterology* **105**: 1797-1805.
- Benedetti, A., Baroni, G.S., Marucci, L., Mancini, R., Bassotti, C. and Macarri, G. (1995). Regulation of intracellular pH in periportal and perivenular hepatocytes isolated from ethanol-treated rats. *Alcohol. Clin. Exp. Res.* **19**: 216-225.
- Berenbrink, M. and Bridges, C.R. (1994). Active Na^+ -, Cl^- - and HCO_3^- -dependent acid extrusion in Atlantic cod red blood cells in winter activated by hypercapnia. *J. exp. Biol.* **192**: 239-252.
- Berger, M.L., Reynolds, R.C., Hagler, H.K., Bellotto, D., Parsons, D., Mulligan, K.J. and Buja, M. (1989). Anoxic hepatocellular injury: role of reversible changes in elemental content and distribution. *Hepatology*. **9**: 219-228.

- Berk, B.C., Vallega, G., Griendling, K.K., Gordon, J.B., Cragoe, Jr., E.J., Canessa, M. and Alexander, R.W. (1988). Effects of glucocorticoids on Na^+/H^+ exchange and growth in cultured vascular smooth muscle cells. *J. Cell. Physiol.* **137**: 391-401.
- Bernardo, A.A., Kear, F.T., Santos, A.V., Ma, J., Steplock, D., Robey, R.B. and Weinman, E.J. (1999). Basolateral $\text{Na}^+/\text{HCO}_3^-$ cotransport activity is regulated by the dissociable Na^+/H^+ exchanger regulatory factor. *J. Clin. Invest.* **104**: 195-201.
- Bevensee, M.O., Weed, R.A. and Boron, W.F. (1997a). Intracellular pH regulation in cultured astrocytes from rat hippocampus. I. Role of HCO_3^- . *J. Gen. Physiol.* **110**: 453-465.
- Bevensee, M.O., Apkon, M. and Boron, W.F. (1997b). Intracellular pH regulation in cultured astrocytes from rat hippocampus. II. Electrogenic Na/HCO_3 cotransport. *J. Gen. Physiol.* **110**: 467-83.
- Bianchini, L., Fossat, B., Porthé-Nibelle, J., Ellory, J.C. and Lahlou, B. (1988). Effects of hyposmotic shock on ion fluxes in isolated trout hepatocytes. *J. exp. Biol.* **137**: 303-318.
- Bianchini, L., Fossat, B., Porthé-Nibelle, J. and Lahlou, B. (1991). Activation by N-ethylmaleimide of a Cl^- -dependent K^+ flux in isolated trout hepatocytes. *J. exp. Biol.* **157**: 335-348.
- Bianchini, L. and Pouysségur, J. (1994). Molecular structure and regulation of vertebrate Na^+/H^+ exchangers. *J. exp. Biol.* **196**: 337-345.
- Bidani, A., Brown, S.E.S. and Heming, T.A. (1994). pH_i regulation in alveolar macrophages: relative roles of Na^+-H^+ antiport and H^+ -ATPase. *Am. J. Physiol.* **266**: L681-L688.

- Blair, J.B., Miller, M.R., Pack, D., Barnes, R., The, S.J. and Hinton, D.E. (1990). Isolated trout liver cells: Establishing short-term primary cultures exhibiting cell-to-cell interactions. *In Vitro Cell Dev. Biol.* **26**: 237-249.
- Borgese, F., Sardet, C., Cappadoro, M., Pouyssegur, J. and Motaïs, R. (1992). Cloning and expression of a cAMP-activated Na^+/H^+ exchanger: Evidence that the cytoplasmic domain mediates hormonal regulation. *Proc. Natl. Acad. Sci. USA.* **89**: 6765-6769.
- Borgese, F., Malapert, M., Fievet, B., Pouyssegur, J. and Motaïs, R. (1994). The cytoplasmic domain of the Na^+/H^+ exchangers (NHEs) dictates the nature of the hormonal response: Behaviour of a chimeric human NHE1/trout βNHE antiporter. *Proc. Natl. Acad. Sci. USA.* **91**: 5431-5435.
- Boron, W.F. (1986). Intracellular pH regulation in epithelial cells. *Annu. Rev. Physiol.* **48** : 377-388.
- Boron, W.F. and Boulpaep, E.L. (1983). Intracellular pH regulation in the renal proximal tubule of the salamander: basolateral HCO_3^- transport. *J. Gen. Physiol.* **81**: 53-94.
- Boron, W.F., Hediger, M.A., Boulpaep, E.L. and Romero, M.F. (1997). The renal electrogenic $\text{Na}^+:\text{HCO}_3^-$ cotransporter. *J. exp. Biol.* **200**: 263-268.
- Boussouf, A., Lambert, R.C. and Gaillard, S. (1997). Voltage-dependent $\text{Na}^+/\text{HCO}_3^-$ cotransporter and Na^+/H^+ exchanger are involved in intracellular pH regulation of cultured mature rat cerebellar oligodendrocytes. *Glia* **19**: 74-84.
- Boyer, J.L., Graf, J. and Meier, P.J. (1992). Hepatic transport systems regulating pH_i , cell volume, and bile secretion. *Annu. Rev. Physiol.* **54** : 415-438.

- Burnham, C. E., Amlal, H., Wang, Z., Shull, G. E. and Soleimani, M. (1997). Cloning and functional expression of a human kidney $\text{Na}^+:\text{HCO}_3^-$ cotransporter. *J. Biol. Chem.* **272**: 19111-19114.
- Burnham, C.E., Flagella, M., Wang, Z., Amlal, H. Shull, G.E. and Soleimani, M. (1998). Cloning, renal distribution, and regulation of the rat $\text{Na}^+:\text{HCO}_3^-$ cotransporter. *Am. J. Physiol.* **274**: F1119-F1126.
- Burton, R.F. (1978). Intracellular buffering. *Resp. Physiol.* **33**: 51-58.
- Carini, R., Bellomo, G., Benedetti, A., Fulceri, R., Gamberucci, A., Parola, M., Dianzani, M.U. and Albano, E. (1995). Alteration of Na^+ homeostasis as a critical step in the development of irreversible hepatocyte injury after adenosine triphosphate depletion. *Hepatology* **21**: 1089-1098.
- Choi, I., Romero, M.F., Khandoudi, N., Bril, A. and Boron, W.F. (1999). Cloning and characterization of a human electrogenic $\text{Na}^+:\text{HCO}_3^-$ cotransporter isoform (hhNBC). *Am. J. Physiol.* **276**: C576-84.
- Claiborne, J.B. (1998). Acid-base regulation. In *The Physiology of Fishes*, second edition (ed. D.H. Evans). pp. 171-198. Boca Raton. FL : CRC Press.
- Claiborne, J.B., Blackston, C.R., Choe, K.P., Dawson, D.C., Harris, S.P., Mackenzie, L.A. and Morrison-Shetlar, A.I. (1999). A mechanism for branchial acid excretion in marine fish: identification of multiple Na^+/H^+ antiporter (NHE) isoforms in gills of two seawater teleosts. *J. exp. Biol.* **202**: 315-324.

- Claiborne, J.B., Walton, J.S. and Compton-McCullough, D. (1994). Acid-base regulation, branchial transfers and renal output in a marine teleost fish (the long-horned sculpin *myoxocephalus octodecimspinosus*) during exposure to low salinities. *J. exp. Biol.* **193**: 79-95.
- Cho, J.H., Musch, M.W., DePaoli, A.M., Bookstein, C.M., Xie, Y., Burant, C.F., Rao, M.C. and Chang, E.B. (1994). Glucocorticoids regulate Na^+/H^+ exchange expression and activity in region- and tissue-specific manner. *Am. J. Physiol.* **267**: C796-C803.
- Collins, J.F., Honda, T., Knobel, S., Bulus, N.M., Conary, J., Dubois, R. and Ghishan, F.K. (1993). Molecular cloning, sequencing, tissue distribution, and functional expression of a Na^+/H^+ exchanger (NHE-2). *Proc. Natl. Sci. USA.* **90**: 3938-3942.
- Cossins, A.R. and Kilbey, R.V. (1990). The temperature dependance of the adrenergic Na^+/H^+ exchanger of trout erythrocytes. *J. exp. Biol.* **148**: 303-312.
- Cossins, A.R. and Gibson, J.S. (1997). Volume-sensitive transport systems and volume homeostasis in vertebrate red blood cells. *J. exp. Biol.* **200**: 343-352.
- Dart, C. and Vaughan-Jones, R.D. (1992). $\text{Na}^+/\text{HCO}_3^-$ symport in the sheep cardiac Purkinje fibre. *J. Physiol. (Lond)*. **451**:365-385.
- De la Rosa, L. A., Cabado, A. G., Botana, M. A., Vieytes, M. R., Vidal, J. I. and Botana, L. M. (1999). Evidence for an electrogenic, negatively protein-kinase-A-modulated, Na^+ -dependent HCO_3^- transporter in human lymphocytes. *Pflügers Arch. - Eur. J. Physiol.* **437** : 935-943.
- Demaurex, N. and Grinstein, S. Na^+/H^+ antiport : modulation by ATP and role in cell volume regulation. *J. exp. Biol.* **196** : 389-404.

- De Weer, P. (1978). Intracellular pH transients induced by CO₂ or NH₃. *Respir. Physiol.* **33**: 41-50.
- Dibrov, P. and Fliegel, L. (1998). Comparative molecular analysis of Na⁺/H⁺ exchangers: a unified model for Na⁺/H⁺ antiport? *FEBS Lett.* **424**: 1-5.
- Eddy, F.B., Penrice, W.S., Fernandes, M.N. and Hill, G.F. (1995). K⁺ balance in rainbow trout gill and liver tissue, cell suspensions and cultured cells. *Braz. J. Med. Biol. Res.* **28**: 1319-1325.
- Farrell, A.P. and Lutz, P.L. (1975). Apparent anion imbalance in the freshwater adapted eel. *J. Comp. Physiol.* **102**: 159-166.
- Fitz, J.G., Persico, M., and Scharschmidt, B.F. (1989). Electrophysiological evidence for Na-coupled bicarbonate transport in cultured rat hepatocyte. *Am. J. Physiol.* **256**: G491-G500.
- Fitz, J.G., Lidovsky, S.D., Xie, M.-H. and Scharshmidt, B.F. (1992). Transmembrane electrical potential differences regulates Na⁺/HCO₃⁻ cotransport and intracellular pH in hepatocytes. *Proc. Natl. Acad. Sci. USA* **89**: 4197-4201.
- Fossat, B., Porthé-Nibelle, J., Pederson, S. and Lahlou, B. (1997) Na⁺/H⁺ exchange and osmotic shrinkage in isolated trout hepatocytes. *J. exp. Biol.* **200**: 2369-2376.
- Foster, G.D. and Moon, T.W. (1986) Cortisol and liver metabolism of immature American eels, *Anguilla rostrata* (LeSueur). *Fish Physiol. Biochem.* **1**: 113-124.
- Foster, G.D. and Moon, T.W. (1993). Hormonal sensitivity and responsiveness in sea raven hepatocytes: changes with fasting and collagenase exposure. *Can. J. Zool.* **71**: 1755-1762.

- Fuchs, D.A. and Albers, C. (1988). Effect of adrenaline and blood gas conditions on red cell volume and intraerythrocytic electrolytes in the carp, *Cyprinus carpio*. *J. exp. Biol.* **137**: 457-475.
- Furimsky, M., Moon, T.W. and Perry, S.F. (1999a). Intracellular pH regulation in hepatocytes isolated from three teleost species. *J. Exp. Zool.* **28**: 361-367.
- Furimsky, M., Moon, T.W. and Perry, S.F. (2000). Evidence for the role of a $\text{Na}^+\text{-HCO}_3^-$ cotransporter in trout hepatocyte pH_i regulation. *J. exp. Biol.* **203** : 2201-2208.
- Furimsky, M., Perry, S.F. and Moon, T.W. (1999b). Intracellular ion levels in erythrocytes and hepatocytes isolated from three teleost species. *J. Fish Biol.* **55**: 1064-74.
- Garcia-Romeu, F., Cossins, A.R. and Motais, R. (1991). Cell volume regulation by trout erythrocytes: characteristics of the transport systems activated by hypotonic swelling. *J. Physiol* **440**: 547-567.
- Gasbarrini, A., Borle, A.B., Farghali, H., Francavilla, A. and Van Thiel, D. (1990). Effect of anoxia on intracellular ATP, Na^+ , Ca^{2+} , Mg^{2+} , and cytotoxicity in rat hepatocytes. *J. Biol. Chem.* **267**: 6654-6663.
- Glass, M.L., Andersen, N.A., Kruhoffer, M., Williams, E.M. and Heisler, N. (1990). Combined effects of environmental PO_2 and temperature on ventilation and blood gases in the carp *Cyprinus carpio* L. *J. exp. Biol.* **148** : 1- 11.
- Gleeson, D., Smith, N.D. and Boyer, J.L. (1989). Bicarbonate-dependent and -independent intracellular pH regulatory mechanisms in rat hepatocytes. Evidence for $\text{Na}^+\text{-HCO}_3^-$ cotransport. *J. Clin. Invest.* **84**: 312-321.
- Goss, G.G., Perry, S.F., Wood, C.M. and Laurent, P. (1992). Mechanisms of ion and acid-base regulation at the gills of freshwater fish. *J. Exp. Zool.* **263** : 143-159.

- Goss, G.G. and Perry, S.F. (1993). Physiological and morphological regulation of acid-base status during hypercapnia in rainbow trout (*Oncorhynchus mykiss*). *Can. J. Zool.* **71**: 1673-1680.
- Goss, G.G., Wood, C.M., Laurent, P. and Perry, S.F. (1994). Morphological responses to the rainbow trout (*Oncorhynchus mykiss*) gill to hyperoxia, base (NaHCO_3) and acid (HCl) infusions. *Fish Physiol. Biochem.* **12**: 465-477.
- Goss, G.G., Perry, S.F. and Laurent, P. (1995). Gill morphology and acid-base regulation. In *Fish Physiology*, (eds. C.M. Wood and T.J. Shuttleworth) **14** : 257-284. New York : Academic Press.
- Gritchenko, I.I., Romero, M.F. and Boron, W.F. (2000). Extracellular HCO_3^- dependence of electrogenic Na/HCO_3 cotransporters cloned from salamander and rat kidney. *J. Gen. Physiol.* **115**: 533-545.
- Gross, E. and Hopfer, U. (1998). Voltage and cosubstrate dependence of the $\text{Na}-\text{HCO}_3$ cotransporter kinetics in renal proximal tubule cells. *Biophys. J.* **75**: 810-24.
- Hamasaki, N. and Okubo, K. (1996). Band 3 protein: physiology, function and structure. *Cell. Mol. Biol.* **42**: 1025-1039.
- Hampton, J.A., McCuskey, P.A., McCuskey, R.S. and Hinton, D.E. (1985). Functional units in rainbow trout (*Salmo gairdneri*) liver : I. Arrangement and histochemical properties of hepatocytes. *Anat. Rec.* **213**: 166-175.
- Hanahan, D. (1985). Techniques for transformation of *E. Coli*. In *DNA Cloning*, vol. 1, *A Practical Approach* (Ed. D.M. Glover), pp. 109-135. Oxford, Washington : IRL Press.

- Harris, C. and Fliegel, L. (1999). Amiloride and the Na^+/H^+ exchanger protein: Mechanism and significance of inhibition of the Na^+/H^+ exchanger (Review). *Int. J. Mol. Med.* **3**: 315-321.
- Harvey, W.R. and Slayman, C.L. (1994). Coupling as a way of life. *J. exp. Biol.* **196**: 1-5.
- Hediger, M.A. (1994). Structure, function and evolution of solute transporters in prokaryotes and eukaryotes. *J. exp. Biol.* **196**: 15-49.
- Heisler, N. (1993). Acid-base regulation. In *The Physiology of Fishes* (ed. D.H. Evans). pp. 343-378. Boca Raton, FL: CRC Press.
- Henry, R.P. (1991). Techniques for measuring carbonic anhydrase activity in vitro. In *The Carbonic Anhydrases* (ed. S.J. Dodgson, R.E. Tashian, G. Gros, and N.D. Carter), pp. 119-131. New York : Plenum Press.
- Henry, R.P. (1996). Multiple roles of carbonic anhydrase in cellular transport and metabolism. *Ann. Rev. Physiol.* **58**: 523-538.
- Henry, R.P. and Heming, T.A. (1998). Carbonic anhydrase and respiratory gas exchange. In *Fish Respiration*. (eds. S.F. Perry and B.L. Tufts), Fish Physiology Volume 17 (eds. W.S. Hoar, D.J. Randall and A.P. Farrell), Chapter 3, p. 75-111. New York : Academic Press.
- Hervio, D.M.L., Kent, M.L., Khattra, J., Sakanari, J., Yokoyama, H. and Devlin, R.H. (1997). Taxonomy of *Kudoa* species (*Myxosporea*), using a small-subunit ribosomal DNA sequence. *Can. J. Zool.* **75** : 2112-2119.
- Hoek, J.B., Nicholls, D.B. and Williams, J.R. (1980). Determination of mitochondrial protonmotive force in isolated hepatocytes. *J. Biol. Chem.* **255** : 1458-1464.

- Hübner, S., Michel, F., Rudloff, V. and Appelhans, H. (1992). Amino acid sequence of band-3 protein from rainbow trout erythrocytes derived from cDNA. *Biochem. J.* **285**: 17-23.
- Igarashi, I., Freed, M.I., Ganz, M.B. and Reilly, R.F. (1992). Effects of chronic metabolic acidosis on Na^+ - H^+ exchangers in LLC-PK1 renal epithelial cells. *Am. J. Physiol.* **263**: F83-F88.
- Jungermann, K. and Katz, N. (1989). Functional specialization of different hepatocyte populations. *Physiol. Rev.* **69**: 708-764.
- Kikeri, D., Azar, S., Sun, A., Zeidel, M.L. and Hebert, S.C. (1990). Na^+ - H^+ antiporter and Na^+ -(HCO_3^-)n symporter regulate intracellular pH in mouse medullary thick limbs of Henle. *Am. J. Physiol.* **258**: F445-F456.
- Kinkead, R. and Perry, S.F. (1991). The effects of catecholamines on ventilation in rainbow trout during hypoxia or hypercapnia. *Resp. Physiol.* **84**: 77-92.
- Kinsella, J., Cujdik, T. and Sacktor, B. (1984). Na^+ - H^+ exchange activity in renal brush border membrane vesicles in response to metabolic acidosis: The role of glucocorticoids. *Proc. Natl. Acad. Sci. USA.* **81**: 630-634.
- Kinsella, J.L., Freiberg, J.M. and Sacktor, B. (1985). Glucocorticoid activation of Na^+ / H^+ exchange in renal brush border vesicles: kinetic effects. *Am. J. Physiol.* **248**: F233-F239.
- Kinsella, J.L., Heller, P. and Froehlich, J.P. (1998). Na^+ / H^+ exchanger: proton modifier site regulation of activity. *Biochem. Cell. Biol.* **76**: 743-749.

- Klanke, C.A., Su, Y.R., Callen, D.F., Wang, Z., Meneton, P., Baird, N., Kandasamy, R.A., Orlowski, J., Otterud, B.E., Leppert, M., Shull, G.E. and Menon, A.G. (1995). Molecular cloning and physical and genetic mapping of a novel human Na^+/H^+ exchanger (NHE5/SLC9A5) to chromosome 16q22.1. *Genomics* **25**: 615-622.
- Krapf, R., Pearce, D., Lynch, C., Xi, X.-P., Reudelhuber, T.L., Pouysségur, J. and Rector, Jr., F.C. (1991). Expression of rat renal Na/H antiporter mRNA levels in response to respiratory and metabolic acidosis. *J. Clin. Invest.* **87**: 747-751.
- Krumschnabel, G., Biasi, C., Schwarzbaum, P.J. and Wieser, W. (1997). Acute and chronic effects of temperature, and of nutritional state, on ion homeostasis and energy metabolism in teleost hepatocytes. *J. Comp. Physiol. B* **167**: 280-286.
- Lagadic-Gossmann, D., Buckler, K.J. and Vaughan-Jones, R.D. (1992). Role of HCO_3^- in pH recovery from intracellular acidosis in the guinea-pig ventricular myocyte. *J. Physiol. (Lond)*. **458**: 361-384.
- Laurent, P. and Perry, S.F. (1995). Morphological basis of acid-base and ionic regulation in fish. In *Advances in Comparative and Environmental Physiology. Mechanisms of Systemic Regulation : Acid-Base Regulation, Ion Transfer and Metabolism* (ed. N. Heisler), pp. 91-118. Heidelberg : Springer-Verlag.
- L' Allemain, G., Paris, S. and Pouysségur, J. (1985). Role of Na^+ -dependent/ HCO_3^- exchange in regulation of intracellular pH in fibroblasts. *J. Biol. Chem.* **260**: 4877-4883.
- Madsen, K.M. and Tisher, C.C. (1986). Structural-functional relationships along the distal nephron. *Am. J. Physiol.* **250**: F1-F15.
- Madhus, I.H. (1988). Regulation of intracellular pH in eukaryotic cells. *Biochem. J.* **250**: 1-8.

- Malapert, M., Guizouarn, H., Fievet, B., Jahns, R., Garcia-Romeu, F., Motais, R. and Borgese, F. (1997). Regulation of Na^+/H^+ antiporter in trout red blood cells. *J. exp. Biol.* **200**: 353-360.
- Malakooti, J., Dahdal, R.Y., Schmidt, L., Layden, T.J., Dudeja, P.K. and Ramaswamy, K. (1999). Molecular cloning, tissue distribution , and functional expression of the human Na^+/H^+ exchanger NHE2. *Am. J. Physiol.* **277**: G383-G390.
- Manger, T.M. and Koeppen, B.M. (1992). Characterization of acid-base transporters in cultured outer medullary collecting duct cells. *Am. J. Physiol.* **263**: F996-F1003.
- Martin-Requero, A., Daza, F.J., Hermida, O.G., Butta, N. and Parrilla, R. (1997). Role of Ca^{2+} and protein kinase C in the receptor-mediated activation of Na^+/H^+ exchange in isolated liver cells. *Biochem. J.* **325**: 631-636.
- McLean, L.A., Zia, S., Gorin, F.A. and Cala, P.M. (1999). Cloning and expression of the Na^+/H^+ exchanger from *Amphiuma* RBCs: resemblance to mammalian NHE1. *Am. J. Physiol.* **276**: C1025-C1037.
- Milligan, C.L. and Wood, C.M. (1985). Intracellular pH transients in rainbow trout tissues measured by dimethadione distribution. *Am. J. Physiol.* **248**: R668-R673.
- Milligan, C.L. and Wood, C.M. (1986). Intracellular and extracellular acid-base status and H^+ exchange with the environment after exhaustive exercise in the rainbow trout. *J. exp. Biol.* **123**: 93-121.
- Mommsen, T.P., Danulat, E., Gavioli, M.E., Foster, G.D. and Moon, T.W. (1991). Separation of enzymatically distinct populations of trout hepatocytes. *Can. J. Zool.* **69**: 420-426.

- Mommsen, T.P., Moon, T.W. and Walsh, P.J. (1994). Hepatocytes: isolation, maintenance and utilization. In: *Biochemistry and molecular biology of fishes, vol.3.* (ed. P.W. Hochachka and T.P. Mommsen), pp. 355-373. Amsterdam: Elsevier.
- Mommsen, T.P., Walsh, P.J., Perry, S.F. and Moon, T.W. (1988). Interactive effects of catecholamines and hypercapnia on glucose production in isolated trout hepatocytes. *Gen. Comp. Endocrinol.* **70**: 63-73.
- Moon, T.W., Walsh, P.J. and Mommsen, T.P. (1985). Fish hepatocytes: A model metabolic system. *Can. J. Fish. Aquatic. Sci.* **42**: 1772- 1782.
- Morgan, I.J. and Potts, W.T.W. (1995). The effects of adrenoreceptor agonists phenylephrine and isoproterenol on the intracellular ion concentration of branchial epithelial cells of brown trout (*Salmo trutta* L.). *J. Comp. Physiol. B* **165**: 458-463.
- Moule, S.K. and McGivan, J.D. (1990). Epidermal growth factor and cyclic AMP stimulate Na^+/H^+ exchange in isolated rat hepatocytes. *Eur. J. Biochem.* **187**: 677-682.
- Nelson, N. (1994). Energizing porters by proton-motive force. *J. exp. Biol.* **196** : 7-13.
- Nikinmaa, N., Bogdanova, A. and Virkii, L.V. (1999). Intracellular pH regulation of rainbow trout and carp thrombocytes. *Fish Physiol. Biochem.* **21**: 269-275.
- Noël, J. and Pouysségur, J. (1995). Hormonal regulation, pharmacology, and membrane sorting of vertebrate Na^+/H^+ exchanger isoforms. *Am. J. Physiol.* **268**: C283-C296.
- Okubo, K., Kang, N., Hamasaki, N. and Jennings, M. (1994). Red blood cell band 3: lysine-539 and lysine-851 react with the same 4,4'-diisosthiocyanodihydrostilbene-2,2'-disulfonate molecule. *J. Biol. Chem.* **269**: 1918-1926.
- Orlowski, J., Kandasamy, R.A. and Shull, G.E. (1992). Molecular cloning of putative members of of the Na^+/H^+ exchanger gene family. *J. Biol. Chem.* **267**: 9331-9339.

- Otto, D.M.E. and Moon, T.W. (1995). 3,3',4,4'-Tetrachlorobiphenyl effects on antioxidant enzymes and glutathione status in different tissues of rainbow trout. *Pharmacol. Toxicol.* **77**: 281-287.
- Ottolenghi, C., Ricci, D., Gavioli, M.E., Puviani, A.C., Fabbri, E., Capuzzo, A., Brighenti, L. and Plisetskaya, E.M. (1991). Separation of two populations of fish hepatocytes by digitonin infusion : some metabolic patterns and hormonal responsiveness. *Can. J. Zool.* **69**: 427-435.
- Pärt, P. and Wood, C.M. (1996). Na/H exchange in cultured epithelial cells from fish gills. *J. Comp. Physiol. B* **166** : 37-45.
- Pereira, C., Vijayan, M.M. and Moon, T.W. (1995). In vitro hepatocyte metabolism of alanine and glucose and the response to insulin in fed and fasted rainbow trout. *J. exp. Zool.* **271**: 425-431.
- Perry, S.F. (1997). The chloride cell: structure and function in the gills of freshwater fishes. *Ann. Rev. Physiol.* **59**: 325-347.
- Perry, S.F., Goss, G.G. and Laurent, P. (1992). The interrelationships between gill chloride cell morphology and ionic uptake in four freshwater teleosts. *Can. J. Zool.* **70**: 1775-1786.
- Perry, S.F. and Laurent, P. (1990). The role of carbonic anhydrase in carbon dioxide excretion, acid-base balance and ionic regulation in aquatic gill breathers. (ed. J.-P. Truchot and B. Lahlou) In *Animal Nutrition and Transport Processes. 2. Transport, Respiration and Excretion : comparative and Environmental Aspects. Comp. Physiol.* vol. 6, pp. 39-57 Basel : Karger.

- Perry, S.F. and Laurent, P. (1993). Environmental effects on fish gill structure and function. In *Fish Ecophysiology* (ed. J.C. Rankin and F.B. Jensen), pp. 231-264. London : Chapman & Hall.
- Perry, S.F., Malone, S. and Ewing, D. (1987). Hypercapnic acidosis in the rainbow trout (*Salmo gairdneri*). I. Branchial ionic fluxes and blood acid-base status. *Can. J. Zool.* **65**: 888-895.
- Perry, S.F. and McDonald, G. (1993). Gas Exchange. In *The Physiology of Fishes* (ed. D.H. Evans), pp. 251-278. Boca Raton, FL : CRC Press Inc.
- Perry, S.F., Malone, S. and Ewing, D. (1987). Hypercapnic acidosis in the rainbow trout (*Salmo gairdneri*). II. Renal ionic fluxes. *Can. J. Zool.* **65**: 896-902.
- Perry, S.F., Walsh, P.J., Mommsen, T.P. and Moon, T.W. (1988). Metabolic consequences of hypercapnia in the rainbow trout, *Salmo gairdneri*: beta adrenergic effects. *Gen. Comp. Endocrinol.* **69**: 439-447.
- Perry, S.F. and Wood, C.M. (1989). Control and coordination of gas transfer in fishes. *Can. J. Zool.* **67**: 2961-2970.
- Perry, S.F. and Reid, S.D. (1993). β -adrenergic signal transduction in fish: interactive effects of catecholamines and cortisol. *Fish Physiol. Biochem.* **11**: 195-203.
- Perry, S.F., Reid, S.G. and Salama, A. (1996). The effects of repeated physical stress on the β -adrenergic response of the rainbow trout red blood cell. *J. exp. Biol.* **199**: 549-562.
- Perry, S.F., Beyers, M.L. and Johnson, D.A. (2000). Cloning and molecular characterisation of the trout (*Oncorhynchus mykiss*) vacuolar H^+ -ATPase B subunit. *J. exp. Biol.* **203**: 459-470.

- Peterson, T.D.P., Hochachka, P.W. and Suarez, R.K. (1987). Hormonal control of gluconeogenesis in rainbow trout hepatocytes; regulatory role of pyruvate kinase. *J. Exp. Zool.* **243**: 173-180.
- Preisig, P.A. and Alpern, R.J. (1988). Chronic metabolic acidosis causes an adaptation in the apical membrane Na/H antiporter and basolateral membrane Na(HCO₃)₃ symporter in the rat proximal convoluted tubule. *J. Clin. Invest.* **82**: 1445-1453.
- Pushkin, A. Abuladze, Lee, I., Newman, D., Hwang, J. and Kurtz, I. (1999). Cloning, tissue distribution, genomic organization, and functional characterization of NBC3, a new member of the sodium bicarbonate cotransporter family. *J. Biol. Chem.* **274**: 16569-16575.
- Quedneau, B., Rosskopf, D., Reusch, H.P., Luft, F.C. and Siffert, W. (1994). Enhanced Na⁺-H⁺ exchanger activity and NHE-1 mRNA levels in human lymphocytes during metabolic acidosis. *Am. J. Physiol.* **266**: C480-C488.
- Reader, S., Steen, H.B. and Denizeau, F. (1994). Intracellular calcium and pH induced by tri-n-butyltin chloride in isolated rainbow trout hepatocytes: A flow cytometric analysis. *Arch. Biochem. Biophys.* **312**: 407-413.
- Reid, S.D. and Perry, S.F. (1991). The effects and physiological consequences of raised levels of cortisol on rainbow trout (*Oncorhynchus mykiss*) erythrocyte β -adrenoreceptors. *J. exp. Biol.* **158**: 217-240.
- Reid, S.G., Fritsche, R. and Jönsson, A.-C. (1995). Immunohistochemical localisation of bioactive peptides and amines associated with the chromaffin tissue of five species of fish. *Cell Tiss. Res.* **280**: 499-512.

- Romero, M.F. and Boron, W.F. (unpublished). Identification of a *C. elegans* protein similar to the electrogenic Na/HCO₃ cotransporters.
- Romero, M.F. and Boron, W.F. (1999). Electrogenic Na⁺/HCO₃⁻ cotransporters: cloning and physiology. *Annu. Rev. Physiol.* **61**: 699-723.
- Romero, M.F., Fong, P., Berger, U.V., Hediger, M.A. and Boron, W.F. (1998). Cloning and functional expression of rNBC, an electrogenic Na⁺-HCO₃⁻ cotransporter from rat kidney. *Am. J. Physiol.* **274**: F425-F432.
- Romero, M.F., Hediger, M.A., Boulpaep, E.L. and Boron, W.F. (1997). Expression cloning and characterization of a renal electrogenic Na⁺-HCO₃⁻ cotransporter. *Nature*. **387**: 409-413.
- Romero, M.F., Henry, D., Nelson, S., Harte, P.J., Dillon, A.K. and Sciortino, C.M. (2000). Cloning and characterization of a Na⁺ driven anion exchanger (NDAE1): a new bicarbonate transporter. *J. Biol. Chem.* **275** : 24552-24559.
- Romero, M.G., Guizouarn, H., Pellissier, B., Garcia-Romeu, F. and Motaïs, R. (1996). The erythrocyte Na⁺-H⁺ exchangers of eel (*Anguilla anguilla*) and rainbow trout (*Oncorhynchus mykiss*): a comparative study. *J. exp. Biol.* **199** : 415-426.
- Roskopf, D., Siffert, G., Osswald, U., Witte, K., Düsing, R., Akkerman, J.W.N. and Siffert, W. (1992). Platelet Na⁺-H⁺ exchanger activity in normotensive and hypertensive subjects: effect of enalapril therapy upon antiport activity. *J. Hypertension*. **10**: 839-847.
- Rossmann, H., Bachmann, O., Vieillard-Baron, D., Gregor, M. and Seidler, U. (1999). Na⁺/HCO₃ cotransport and expression of NBC1 and NBC2 in rabbit gastric parietal and mucous cells. *Gastroenterology* **116**: 1389-1398.

- Ruiz, O.S., Qiu, Y.Y., Wang, L.J., Cardoso, L.R. and Arruda, J.A.L. (1998). Regulation of renal Na-HCO₃ cotransporter: VIII. Mechanism of stimulatory effect of respiratory acidosis. *J. Membr. Biol.* **162**: 201-208.
- Ruiz, O. S., Robey, R.B., Qui, Y.Y., Wang, L.J., Li, C.J., Ma, J. and Arruda, J.A. (1999). Regulation of the renal Na-HCO₃ cotransporter. XI. Signal transduction underlying CO₂ stimulation. *Am. J. Physiol.* **277**: F580-6.
- Salama, A., Morgan, I.J. and Wood, C.M. (1999). The linkage between Na⁺ uptake and ammonia excretion in rainbow trout: kinetic analysis, the effects of (NH₄)SO₄ and NH₄HCO₃ infusion and the influence of gill boundary layer pH. *J. exp. Biol.* **202**: 697-709.
- Schmitt, B.M., Biemsderfer, D., Romero, M.F., Boulpaep, E.L. and Boron, W.F. (1999). Immunolocalization of the electrogenic Na⁺-HCO₃⁻ cotransporter in mammalian and amphibian kidney. *Am. J. Physiol.* **276**: F27-F38.
- Schuster, V.L., Bonsib, S.M. and Jennings, M.L. (1986). Two types of collecting duct mitochondria-rich (intercalated) cells : lectin and band 3 cytochemistry. *Am. J. Physiol.* **251**: C347-C355.
- Sciortino, C.M. and Boron, M.F. (1999). Cation and voltage dependence of rat kidney electrogenic Na⁺-HCO₃⁻ cotransporter, rkNBC, expressed in oocytes. *Am. J. Physiol.* **277**: F611-F623.
- Seglen, P.O. (1976). Preparation of isolated rat liver cells. *Meth. Cell Biol.* **13**: 29-83.
- Seifter, J.L. and Aronson, P.S. (1986). Properties and physiologic roles of the plasma membrane sodium-hydrogen exchanger. *J. Clin. Invest.* **78**: 859-864.

- Shumaker, H., Amlal, H., Frizzell, R., Ulrich, C.D. II, and Soleimani, M. (1999). CFTR drives $\text{Na}^+/\text{HCO}_3^-$ cotransport in pancreatic duct cells: A basis for defective HCO_3^- secretion in CF. *Am. J. Physiol.* **276**: C16-C25.
- Singh, G., McAteer, J.A. and Soleimani, M. (1995). The effect of lethal acid stress on Na^+/H^+ exchanger isoforms in cultured inner medullary collecting duct cells: deletion of NHE-2 and over expression of NHE-1. *Biochim. Biophys. Acta* **1239**: 74-80.
- Soengas, J.L. and Moon, T.W. (1995). Uptake and metabolism of glucose, alanine and lactate by red blood cells of the American eel *Anguilla rostrata*. *J. exp. Biol.* **198**: 877-888.
- Soleimani, M. and Burnham, C.E. (2000). Physiologic and molecular aspects of the $\text{Na}^+:\text{HCO}_3^-$ cotransporter in health and disease processes. *Kidney Int.* **57**: 371-384.
- Soleimani, M., Grassl, S.M., and Aronson, P.S. (1987). Stoichiometry of the $\text{Na}^+/\text{HCO}_3^-$ co-transporter in basolateral membrane vesicles isolated from rabbit renal cortex. *J. Clin. Invest.* **79**: 1276-1280.
- Strazzabosco & Boyer (1996). Regulation of intracellular pH in the hepatocyte : Mechanisms and physiological implications. *J. Hepatol.* **24**: 631-644.
- Sullivan, G.V., Fryer, J.N. and Perry, S.F. (1995). Immunolocalization of proton pumps (H^+ -ATPase) in pavement cells of rainbow trout gill. *J. Exp. Biol.* **198**: 2619-2629.
- Sullivan, G. V., Fryer, J.N. and Perry, S.F. (1996). Localization of mRNA for proton pump (H^+ ATPase) and $\text{Cl}^-/\text{HCO}_3^-$ exchanger in rainbow trout gill. *Can. J. Zool.* **74** : 2095-2103.
- Thevenod, F., Roussa, E., Schmitt, B.M. and Romero, M.F. (1999). Cloning and immunolocalization of a rat pancreatic Na^+ bicarbonate cotransporter. *Biochem. Biophys. Res. Commun.* **264**: 291-298.

- Thomas, S. 1994. Extracorporeal circulation. In *Biochemistry and Molecular Biology of Fishes*, Vol. 3 Analytical Techniques (Ed by Hochachka, P.W. and Mommsen, T.P.), pp. 161-167. Elsevier Science B.V., Amsterdam.
- Thomas, S. and Motaïs, R. (1990). Acid-base balance and oxygen transport during acute hypoxia in fish. *Comp. Physiol.*, **6**: 76- 86.
- Thomas, S. and Perry, S.F. (1992). Control and consequences of adrenergic activation of red blood cell Na^+/H^+ exchange on blood oxygen and carbon dioxide transport in fish. *J. Exp. Zool.* **263**: 160-175.
- Thomas, S., Fritsche, R. and Perry, S.F. (1994). Pre- and post-branchial respiratory status during acute hypercapnia or hypoxia in rainbow trout, *Oncorhynchus mykiss*. *J. Comp. Physiol. B.* **164**: 451-458.
- Tonnessen, T.I., Sandvig, K. and Olsnes, S. (1990). Role of Na^+/H^+ and $\text{Cl}^-/\text{HCO}_3^-$ antiports in the regulation of cytosolic pH near neutrality. *Am. J. Physiol.* **27**: C1117-C1126.
- Tufts, B.L. and Perry, S.F. (1998). Blood carbon dioxide transport. In 'Fish Physiology, Haemoglobin and Respiration' (B.L. Tufts and S.F. Perry, eds.), vol. 17, Academic Press, New York.
- Vidal, G., Durand, T., Canioni, P. and Gallis, J.L. (1998). Cytosolic pH regulation in perfused rat liver: role of intracellular bicarbonate production. *Biochim. Biophys. Acta* **1425**: 224-234.
- Vijayan, M.M., Reddy, P.K., Leatherland, J.F. and Moon, T.W. (1994). The effects of cortisol on hepatocyte metabolism in rainbow trout: A study using the steroid analogue RU486. *Gen. Comp. Endocrinol.* **96**: 75-84.

- Virkki, L.V. and Nikinmaa, M. (1994). Activation and physiological role of Na^+/H^+ exchange in lamprey (*lampetra fluviatilis*) erythrocytes. *J. exp. Biol.* **191**: 89-105.
- Wakabayashi, S., Fafournoux, P., Sardet, C. and Pouyssegur, J. (1992). The Na^+/H^+ antiporter cytoplasmic domain mediates growth factor signals and controls « H^+ -sensing ». *Proc. Natl. Acad. Sci. USA* **89** : 2424-2428.
- Wakabayashi, S., Bertrand, B., Shigekawa, M., Fafournoux, P. and Pouyssegur, J. (1994a). Growth factor activation and ' H^+ -sensing' of the Na^+/H^+ exchanger isoform 1 (NHE1). Evidence for an additional mechanism not requiring direct phosphorylation. *J. Biol. Chem.* **269** : 5583-5588.
- Wakabayashi, S., Bertrand, B., Ikeda, T., Pouyssegur, J. and Shigekawa, M. (1994b). Mutation of calmodulin-binding site renders the Na^+/H^+ exchanger (NHE1) highly H^+ -sensitive and Ca^{2+} regulation-defective. *J. Biol. Chem.* **269**: 13710-13715.
- Wakabayashi, S., Shigekawa, M. and Pouyssegur, J. (1997). Molecular physiology of vertebrate Na^+/H^+ exchangers. *Physiol. Rev.* **77**: 51-74.
- Walsh, P.J. (1986). Ionic requirements for intracellular pH regulation in rainbow trout hepatocytes. *Am. J. Physiol.* **250**: R24-R29.
- Walsh, P.J. (1989). Regulation of intracellular pH by toadfish (*Opsanus beta*) hepatocytes. *J. exp. Biol.* **147**: 407-419.
- Walsh, P.J. (1994). Measurement of intracellular pH in fish hepatocytes. In: *Biochemistry and molecular biology of fishes*, vol. 3. (eds. P.W. Hochachka and T.P. Mommsen) pp. 399-406. Amsterdam : Elsevier.
- Walsh, P.J. and Milligan, C.L. (1989). Coordination of metabolism and intracellular acid-base status: ionic regulation and metabolic consequences. *Can. J. Zool.* **67**: 2994-3004.

- Walsh, P.J. , Mommsen, T.P., Moon, T.W. and Perry, S.F. (1988). Effects of acid-base variables on in vitro hepatic metabolism in the rainbow trout, *Salmo gairdneri*. *J. exp. Biol.* **135**: 231-241.
- Walsh, P.J. and Mommsen, T.P. (1992). Intracellular pH regulation and metabolic interactions in hepatic tissues. *J. exp. Biol.* **263**: 176-185.
- Walsh, P.J., Moon, T.W. and Mommsen, T.P. (1985). Interactive effects of acute changes in temperature and pH on metabolism in hepatocytes from the sea raven *Hemitripterus americanus*. *Physiol. Zool.* **58** (6): 727-735.
- Walsh, P.J., Parent, J.J. and Henry, R.P. (1989). Carbonic anhydrase supplies bicarbonate for urea synthesis in toadfish (*Opsanus beta*) hepatocytes. *Physiol. Zool.* **62** : 1257-1272.
- Weaver, Y.R., Kiessling, K. and Cossins, A.R. (1999). Responses of the Na^+/H^+ exchanger of european flounder red blood cells to hypertonic, β -adrenergic and acidotic stimuli. *J. exp. Biol.* **202**: 21-32.
- Wolfersberger, M.G. (1994). Uniporters, symporters and antiporters. *J. exp. Biol.* **196** : 5-6.
- Wundergem, R., Smith, D.J. and Boyer, J.L. (1985). Transmembrane potential of hepatocytes isolated from the livers of little skates (*Raja erinacea*). *Bull. Mt. Des. Island Lab.* **25** : 2-43.
- Wood, C.M. and Pärt, P. (2000). Intracellular pH regulation and buffer capacity in $\text{CO}_2/\text{HCO}_3^-$ buffered media in cultured epithelial cells from rainbow trout gills. *J. Comp. Physiol. B* **170** : 175-184.
- Wright, P.A., Perry, S.F. and Moon, T.W. (1989). Regulation of hepatic gluconeogenesis and glycogenolysis by catecholamines in rainbow trout during environmental hypoxia. *J. exp. Biol.* **147**: 169-188.

- Yoshitomi, K., Burckhardt, B.C. and Frömter, E. (1985). Rheogenic sodium-bicarbonate cotransport in the peritubular cell membrane of rate renal proximal tubule. *Pflügers Arch.* **405**: 360-366.
- Yun, C.H.C., Tse, C.M., Nath, S.K., Levine, S.A., Brant, S.R. and Donowitz, M. (1995). Mammalian Na^+/H^+ exchanger gene family: structure and function studies. *Am. J. Physiol.* **269**: G1-G11.
- Zall, D.M., Fisher, M.D. and Gerner, Q.M. (1956). Photometric determination of chloride in water. *Analyt. Chem.* **28**: 1665-1678.