

AMMONIA STORES AND EXCRETION IN FISH:
RELATIONSHIP TO pH

by

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ABSTRACT

The distribution and transfer of ammonia between intracellular and extracellular compartments of fish and the external water environment was investigated. In vivo and in vitro experiments were performed on the freshwater rainbow trout (Salmo gairdneri) and the intact, seawater lemon sole (Parophrys vetulus).

The distribution of ammonia and H⁺ ions were compared between red cells and plasma (in vivo and in vitro) taken from rainbow trout at rest and during hypercapnia. At rest (in vivo and in vitro), measured intracellular ammonia levels were equal to those predicted by the plasma to red cell pH gradient. The same was not true during hypercapnia, where measured red cell ammonia levels were greater than predicted levels. The addition of the Na⁺/K⁺ ATPase inhibitor, ouabain, had no effect on ammonia accumulation during hypercapnia. It was concluded that ammonia is passively distributed according to plasma-to-red cell H⁺ ion distribution in blood at resting pH values, but under hypercapnic conditions, ammonia accumulation must be due to some other active uptake mechanism.

The distribution of ammonia and ¹⁴C-DMO were compared in white muscle, heart, brain, red cells, and plasma of lemon sole (in vivo) at rest, during hypercapnia, and following exercise. The red cell ammonia distribution at rest and during an extracellular acidosis (hypercapnia and exercise) was similar to

that found in rainbow trout. Red cells are unusual in that H^+ ions are passively distributed according to membrane potential (E_m), whereas in other tissues, this is not the case. In white muscle, heart, and brain under all experimental conditions, intracellular ammonia levels far exceeded those predicted by transmembrane pH gradients. Calculated $E_{NH_4^+}$ values in these tissues were very close to published resting values of E_m . It was concluded that NH_4^+ is permeable across cell membranes and that intracellular ammonia stores are not determined by transmembrane pH gradients in lemon sole.

The pH of interlamellar water was investigated in rainbow trout by following changes in the downstream pH of expired water using a stopped-flow method. As water flowed over the gills of control fish, there was a significant decrease in water pH. Acetazolamide (carbonic anhydrase (CA) inhibitor) added to the water increased the CO_2 disequilibrium, while CA eliminated the CO_2 disequilibrium relative to control water. Mucus excreted by the fish was found to contain CA activity by the pH-stat technique. It was concluded that water acidification is due to the conversion of excreted CO_2 to HCO_3^- and H^+ at the gill surface.

A possible function of CA at the external gill surface is to facilitate carbon dioxide and ammonia excretion. Acetazolamide or CA added to the water did not alter carbon dioxide (\dot{M}_{CO_2}) or ammonia (\dot{M}_{Amm}) excretion in intact rainbow trout. Methazolamide (CA inhibitor) or methazolamide + amiloride (Na^+

uptake inhibitor) added to the water had no effect on plasma NH_3 tensions (P_{NH_3}), but increased \dot{M}_{Amm} slightly compared to control fish. In general, methazolamide resulted in an increase in the diffusing capacity of ammonia. The interpretation of these results was complicated by the fact that rapid serial blood sampling resulted in a universal blood alkalosis. The intact resting fish is unsuitable for studying the interaction between water boundary layer chemistry and excretion across the gill.

With the blood-perfused trout head preparation it was demonstrated that \dot{M}_{CO_2} and \dot{M}_{Amm} are linked through chemical reactions in the external water boundary layer adjacent to the gill. Pre-incubation of blood with acetazolamide reduced \dot{M}_{CO_2} and \dot{M}_{Amm} in the blood-perfused head. Increasing the buffering capacity of inspired water, significantly reduced \dot{M}_{Amm} , but \dot{M}_{CO_2} was unaffected. Each of these experimental treatments significantly reduced the acidification of ventilatory water flowing over the gills. It is proposed that the catalysed conversion of excreted CO_2 to form HCO_3^- and H^+ ions in the gill boundary layer provides a continual supply of H^+ ions needed for the removal of NH_3 to NH_4^+ , which reduces water NH_3 levels and facilitates ammonia excretion.

Gas transfer variables in the blood-perfused head preparation were compared to intact cannulated fish with and without oral masks. Oxygen uptake (\dot{M}_{O_2}) and \dot{M}_{CO_2} were lower, and \dot{M}_{Amm} , higher in the blood-perfused head compared to in vivo values.

these discrepancies were due to differences in venous O_2 , CO_2 , and ammonia levels, which determine mean gradients across the gills. It was concluded that the blood-perfused head is a suitable preparation for studying the interaction between \dot{M}_{CO_2} and \dot{M}_{Amm} because the overall efficiency of transfer of $NH_3:CO_2$ was very similar between in vitro and in vivo preparations.

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GENERAL INTRODUCTION

Carbon dioxide, oxygen, and ammonia¹ are the three respiratory gases transferred across fish gills. Oxygen is extracted from water flowing over the gills and is carried in the blood to the tissues. Carbon dioxide and ammonia are metabolic endproducts which are carried away from the tissues in the blood and are eliminated across the gills. Gas transfer across the gills is modified by varying blood and water flow, the characteristics of the gill epithelium, the distribution of blood within the gills, and the properties and numbers of red blood cells (Randall & Daxboeck, 1984).

The different physical and chemical properties of oxygen, carbon dioxide, and ammonia have numerous physiological consequences. The rate of molecular diffusion through a given medium is related to the molecular weight of the molecules involved. Carbon dioxide is slightly larger than oxygen, so that in the gas phase, the diffusion coefficient for CO₂ is somewhat lower. In an aqueous medium, however, transfer of a molecule is also related to its solubility. The Krogh's permeation coefficient ($K = \text{diffusion coefficient} \times \text{solubility}$) for CO₂ is much greater than that for O₂ because CO₂ is about 30 times

1. The term ammonia or Tamm will be used to indicate the total ammonia concentration, while NH₄⁺ and NH₃ will refer to ammonium ion and nonionic ammonia, respectively.

more soluble. Ammonia's molecular weight is lower than that of either CO_2 and O_2 and in water, is about 900 times more soluble than CO_2 and 24,000 times more soluble than O_2 . The Krogh's permeation coefficient for NH_3 , therefore, will be several orders of magnitude larger than that for either CO_2 or O_2 . The diffusion of gases through an aqueous medium may be influenced by factors other than diffusion coefficients and solubility factors. The facilitated diffusion of O_2 and CO_2 modifies the effective value of the Krogh's constant. The diffusion of O_2 through water is enhanced by the presence of haemoglobin or myoglobin in the water solution (Scholander, 1960). The enzyme carbonic anhydrase, which catalyses the interconversion reaction of $\text{CO}_2:\text{HCO}_3^-$, facilitates CO_2 diffusion through water (Longmuir *et al.*, 1966).

The chemical properties of oxygen, carbon dioxide, and ammonia in solution are quite dissimilar. Oxygen is a nonpolar gas, while carbon dioxide and ammonia are polar molecules which undergo reversible hydration reactions in water. The apparent pK' of the $\text{CO}_2:\text{HCO}_3^-$ reaction at fish temperatures ($5-15^\circ\text{C}$) is about 6 (Boutilier *et al.*, 1985), whereas the pK for the $\text{NH}_3:\text{NH}_4^+$ reaction is about 10 (Cameron & Heisler, 1983). The pH of fish plasma is midway between these two values such that the $\text{CO}_2/\text{HCO}_3^-$ and $\text{NH}_3/\text{NH}_4^+$ ratios are very small, as described by the Henderson-Hasselbalch equation:

$$\text{pH} = \text{pK} + \log [\text{NH}_3]/[\text{NH}_4^+] \quad (1)$$

$$\text{pH} = \text{pK} + \log [\text{HCO}_3^-]/[\text{CO}_2] \quad (2)$$

Cell membranes are permeable to the un-ionized CO_2 and NH_3 , but relatively impermeable to the ionized forms of these compounds, NH_4^+ and HCO_3^- . The fact that body pH is intermediate between the pK of the $\text{CO}_2:\text{HCO}_3^-$ and $\text{NH}_3:\text{NH}_4^+$ reactions ensures adequate transfer of the un-ionized forms and minimizes the stores of both metabolic endproducts (Wright & Randall, 1987). Accumulation of ammonia or H^+ (OH^-) ions will have deleterious effects to the animal, while HCO_3^- , CO_2 , and O_2 are not particularly toxic. Increases in body ammonia levels interfere with neural function and may cause convulsions and death in vertebrates (Visek, 1968). Large fluctuations in body pH will alter the ionization state of proteins and other weak electrolytes, which may inhibit enzyme activity, alter cell membrane function, and disrupt protein subunit aggregations.

The theory of nonionic diffusion (Jacobs & Stewart, 1936; Milne et al., 1958; Pitts, 1973) describes the distribution of weak electrolytes, such as ammonia and carbon dioxide, between body compartments. In terms of ammonia, the theory states that cell membranes are relatively impermeable to NH_4^+ ions, but highly permeable to the non-ionic form of ammonia, NH_3 . The transfer of ammonia between body compartments, therefore, will depend on NH_3 diffusion gradients. NH_3 levels will be larger in a high pH compartment compared to a low pH compartment, and hence, ammonia levels increase in the low pH compartment as NH_3

enters, combines with a H^+ ion and is trapped in the impermeant NH_4^+ form. Thus, the distribution of ammonia between tissue compartments will be determined by intracellular-to-extracellular gradients, described by the equation:

(3)

$$\frac{[\text{intracellular ammonia}]}{[\text{extracellular ammonia}]} = \frac{1 + 10^{(pK - pH \text{ intracellular})}}{1 + 10^{(pK - pH \text{ extracellular})}}$$

As the pH of most intracellular compartments is lower than extracellular fluid, tissue stores of ammonia will be large. The pH dependence of the CO_2 distribution is the reverse of that of ammonia, that is, acid conditions trap NH_4^+ , while alkaline conditions trap HCO_3^- .

The above theory of non-ionic diffusion describes transfer of carbon dioxide and ammonia across membranes within the fish. Due to oxygen's nonpolar nature, O_2 transfer between body compartments and the environment is dependent on O_2 partial pressure gradients. Ammonia and carbon dioxide transfer between the body and the environment are also dependent on partial pressure gradients, as well as, NH_4^+ and HCO_3^- electrochemical gradients. Ion exchange processes at the gills (eg., Na^+/NH_4^+ , Cl^-/HCO_3^-) are essential for acid-base balance and ion regulation in fish. The relative branchial excretion rates of NH_3 versus NH_4^+ and CO_2 versus HCO_3^- varies under different conditions. In freshwater fish, NH_3

excretion may be between 100% (Cameron & Heisler, 1983) and less than 50% (Wright & Wood, 1985), with the remainder of ammonia eliminated as NH_4^+ . Carbon dioxide is primarily excreted as CO_2 (Perry et al., 1982), with less than 10% as HCO_3^- (Cameron, 1976; Perry, 1986).

The ratio of carbon dioxide production to oxygen consumption in resting fish is near unity (0.7-1.0). In contrast, ammonia production is considerably lower than oxygen consumption, with a ratio of about 0.1 in starved fish (Brett & Zala, 1975). Blood levels of oxygen, carbon dioxide, and ammonia are not only determined by uptake or production rates, but also by the efficiency of transfer between the body and water environment. In fish, a large volume of water must come in contact with the gills to maintain adequate O_2 uptake because of the low solubility of O_2 in aqueous media. Consequently, blood carbon dioxide and ammonia concentrations will be comparatively low because of their greater solubility coefficients.

The chemical composition and rate of flow of environmental water also strongly influences gas transfer across the gill. Water quality may vary greatly in freshwater systems, both spatially and temporally, in terms of water pH, gas tensions (O_2 , CO_2 , ammonia), and temperature. In general, an increase in the convection of water over the gills will enhance diffusion gradients (O_2 , CO_2 , NH_3) across the gill by renewing water at the branchial surface. Next to the branchial surface there is thin layer of mucus and an associated layer of unstirred water or

boundary layer (Scheid & Piiper, 1971). Increases in ventilatory flow which diminish the thickness of the boundary layer, will enhance gas transfer across the gill (Piiper et al., 1986).

The objectives of the present study were to investigate the transfer of ammonia in fish, in light of its unique chemical and physical properties. The following experiments can be divided into two parts; those designed to investigate the transfer of ammonia between tissue compartments in the body and those designed to explore ammonia transfer between the body and the environment. In the first section, I propose that the theory of non-ionic diffusion describes the distribution of ammonia between various tissue compartments in fish. More specifically, the hypothesis tested was that intracellular ammonia stores will be determined by transmembrane pH gradients. In the second section on ammonia transfer across the gills of fish, the experiments were concerned with two problems. First, it was known that ammonia excretion in fish was influenced by environmental pH (Wright & Wood, 1985), but it was not known if water in contact with the gill surface was representative of bulk water. The proposed hypothesis was that water leaving the gill becomes progressively more acidic as excreted CO_2 is hydrated to form HCO_3^- and H^+ ions in the expired water. The second problem concerning ammonia transfer across the gills developed from the discovery that carbonic anhydrase was present on the external gill surface. The final hypothesis tested was that the catalysed conversion of CO_2 to form H^+ and HCO_3^- ions in the gill water

boundary layer facilitates ammonia excretion by chemically removing NH_3 from the water next to the gill surface.

GENERAL MATERIALS AND METHODS

Surgical Procedures

Trout dorsal aortic cannulation

Trout were anaesthetized in a buffered (NaHCO_3) tricanemethanesulphonate (MS222) solution at a concentration of 1:10,000 and then transferred to the operating table where they were maintained at a MS222 concentration of 1:20,000. Dorsal aortic (DA) cannulae were implanted using the technique of Sovio et al. (1972). In this method a sharpened guitar wire was fitted inside a piece (~ 20cm) of PE50 tubing and used to pierce the DA superficially, at the level of the first or second dorsal gill arch. The wire was then removed and the DA cannula was advanced before being secured to the roof of the mouth with two stitches. The cannula was led out of the buccal cavity through a heat-flared sleeve (PE 160).

Trout Ventral Aortic cannulation

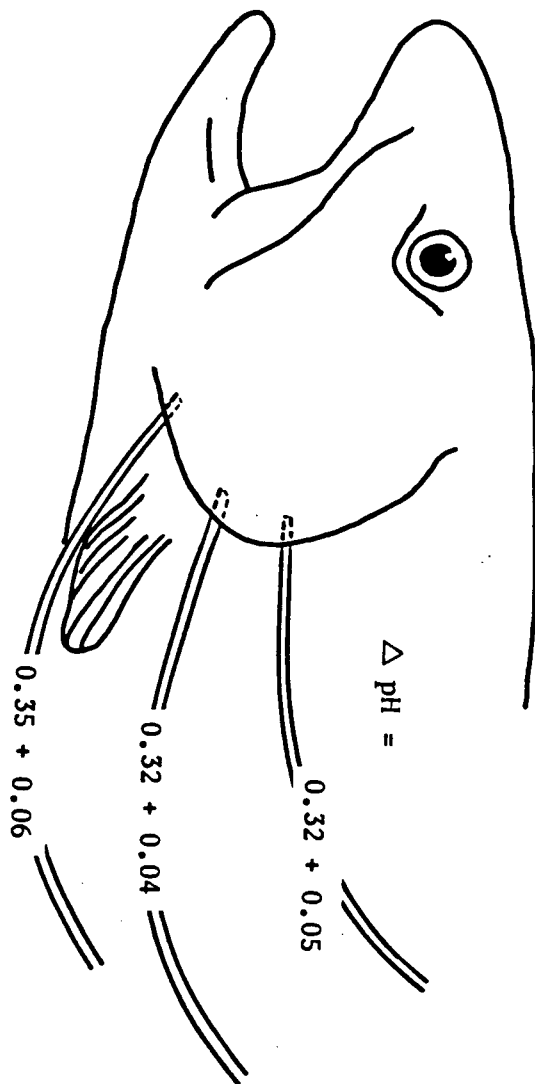
Fish were anaesthetized, as described above and the ventral aorta (VA) was cannulated by one of the following two techniques. A trocar (needle gage 20) was used to puncture the VA at an angle through the soft tissue at the side of the tongue at the level of the second ventral gill arch. Upon removing the trocar needle, a stretched piece of PE 50 tubing was inserted into the VA through the trocar catheter (gage 18). The trocar

catheter was then carefully removed and the cannula was secured to the side of the tongue with two stitches. Alternatively, the VA was cannulated with a sharpened guitar wire inside a stretched piece of PE50 tubing. The VA was punctured from the dorsal surface of the tongue at the level of the first gill arch. As before, the VA was secured in place with two stitches and was led out of the buccal cavity via a heat-flared sleeve (PE160). The overall success rate of these two techniques was approximately equal: the surgery was more difficult in the trocar technique, while there was a lower rate of survival for the guitar wire technique.

Trout opercular cannulae

In order to measure pH of expired water just leaving the opercular cavity, a polyethylene cannula (PE 90) was stitched in position under and midway along the opercular opening. The presence of the cannula just inside the opercular cavity did not appear to prevent proper closure of the opercular valve and to our knowledge there was no interference with the normal pumping of water over the gills. It has been suggested that the opercular catheterization method is a poor method for sampling mean expired water in trout (Davis & Watters, 1970; Davis & Cameron, 1971). It was important, therefore, to determine if water pH varied with the position of the opercular cannulae. Six fish were surgically fitted with three opercular cannula which were positioned in different locations along the opercular valve

Figure 1. Trout were surgically fitted with 3 cannulae placed just under the opercular valve (dotted line). The difference between inspired water pH and expired water pH at equilibrium (equilibrium= water pH 8 min after sample was taken from the opercular chamber, see chapter 2), (Δ pH), were measured on six fish. Numbers represent means \pm standard errors.



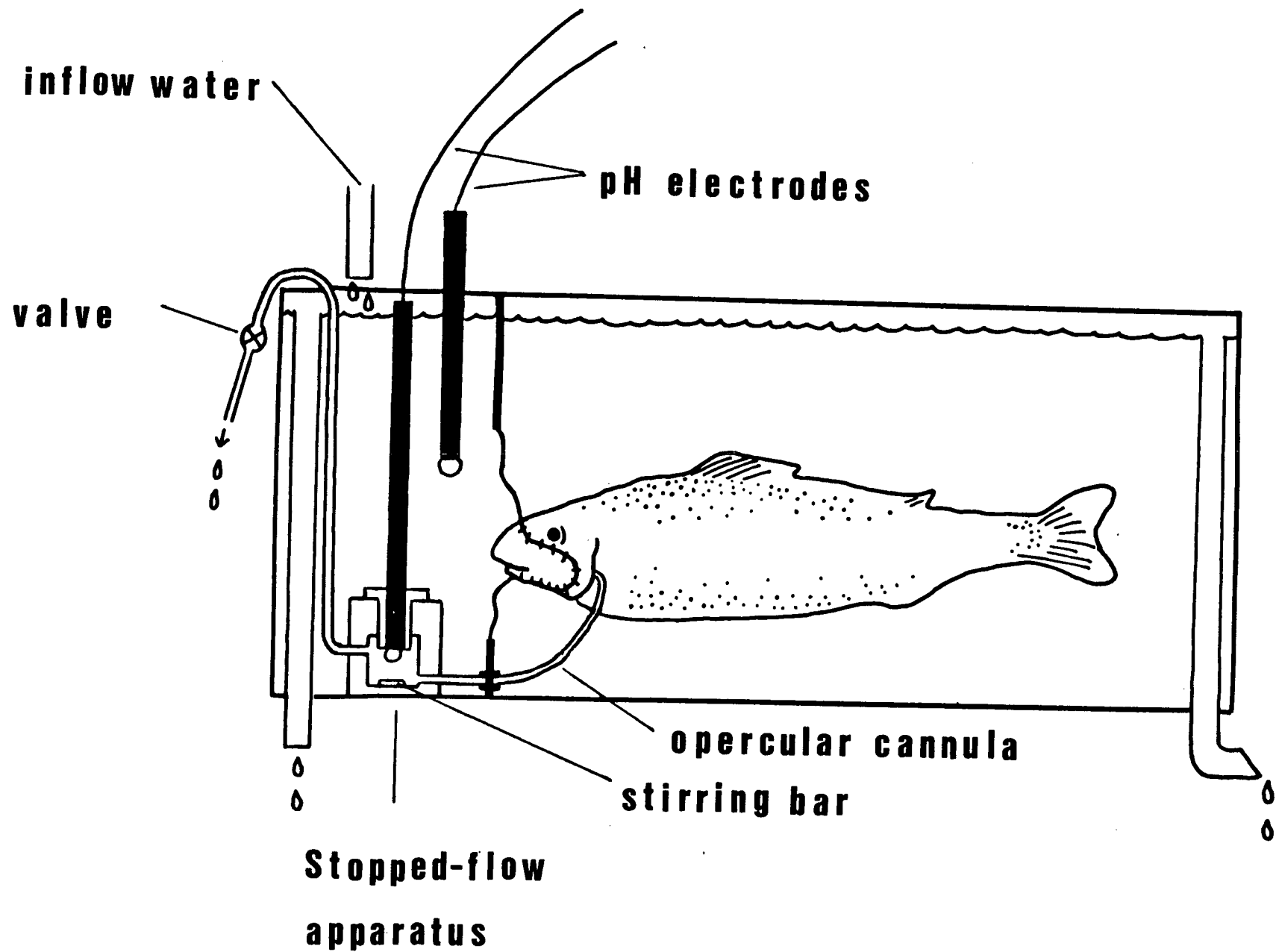
(Fig. 1). The difference between inspired water pH and expired water pH at equilibrium (equilibrium= water pH 8 min after collection) were measured from individual cannulae. The unpaired t-test for two means was employed to compare the three possible pairs of mean values, and pH did not vary with the position of the cannula.

In Chapter 2 and 3, fish were fitted with one opercular cannula (right gill), while in Chapter 4, right and left opercular cannulae were used in the blood-perfused trout head preparation and in intact fish. The rate of water flow over the gills and the rate of gas exchange between gill tissues and the environment are approximately matched between the right and left gills in teleost fish. Thus, there is probably little difference between water samples collected from single or double opercular cannulated fish.

Trout rubber dam

Fish were fitted with rubber dams prepared from latex surgeon gloves using the technique of Cameron and Davis (1970). This technique involves cutting the thumb of the glove to form a snug mask that is sewn posterior to the mouth and anterior to the gills of the fish. Following surgery, fish were inserted into a narrow black perspex box in the posterior end of a two chambered box (Fig. 2). The rubber dam was secured to a dividing perspex O-ring so as to separate the front compartment (inspired water) from the back compartment (expired water), in this continuous

Figure 2. Fish were placed in the two-chambered plexiglass box. A latex dam was stitched around the mouth of the fish to separate inspiratory water from mixed expiratory water. The opercular cannula was stitched against the body of the fish and positioned just inside the opercular cavity. The small, glass stopped-flow chamber contained a pH electrode and magnetic stirring bar. Expired water flowed through the opercular cannula, past the pH electrode and out through the outlet valve by gravitational forces.



flow-through apparatus. Fish were left to recover in the experimental apparatus for 48 hrs following surgery.

Sole caudal artery cannulation

Sole were fitted with caudal artery cannulae (PE50) after anaesthetization in MS222 (1:15,000), as described by Watters and Smith (1973). Briefly, a 2-3 cm incision was made just above the caudal lateral line. The artery lying beneath the vertebrae was pricked with a 26 gage needle and a stretched piece of PE50 tubing (~30 cm) was fed into the artery in the cephalic direction. A small amount of oxytetracycline HCl, a general fish antibiotic, was placed in each wound before the wound was closed with surgical silk. After surgery, the sole were placed in small plastic chambers (approx. 8 L) covered with sand, and allowed to recover in flowing seawater for 72 hours prior to experimentation. To re-establish resting conditions, flatfish require long recovery periods and available sand in which to bury themselves (Wood et al., 1979).

Analytical techniques

Gas chromatography

Plasma and water samples were analysed for total carbon dioxide content (C_{CO_2}) with a Carle gas chromatograph (Model III) containing a CO_2 discriminating column (porapak Q) coupled to a data acquisition unit (Olivetti PC). The principle of the

technique is given by Lenfant and Aucutt (1966) and Boutilier et al. (1985). Plasma samples were prepared by injecting 50 μ l of plasma (or standard) into a gas tight Hamilton syringe containing 7 ml of N_2 and 1ml of N_2 -equilibrated 0.1 N HCl. Alternatively, 1 ml water samples (or standard) were mixed with 7 ml of N_2 and 50 μ l of 0.1 N HCl. The samples were automatically shaken for 3 min in order to liberate the CO_2 into the gas phase and ensure equilibration of the gas and liquid phase. The gas phase (6 ml) was then introduced into a drying filter and flushed through a 1ml sample loop, ensuring complete washout of the previous sample. The area under each peak was integrated and compared to standard peak areas.

Plasma CO_2 tensions (P_{CO_2}) and HCO_3^- concentrations were calculated by manipulating the Henderson-Hasselbalch equation as follows:

$$\text{plasma } P_{CO_2} = \frac{\text{plasma } C_{CO_2}}{(\alpha_{CO_2}) \cdot [1 + \text{antilog} (pH - pK)]} \quad (4)$$

(5)

$$\text{plasma } HCO_3^- = C_{CO_2} - (\alpha_{CO_2} \cdot P_{CO_2})$$

where pK is the apparent pK of plasma and α_{CO_2} is the solubility of CO_2 in plasma taken from Boutilier et al. (1984).

Statistics

Data are presented as means \pm 1 S.E.M. (N). The Student's paired and unpaired t-test was employed, where appropriate, to compare the difference between mean values ($P < 0.05$).

CHAPTER 1

The distribution of ammonia and H^+ ions between intracellular and extracellular compartments in Salmo gairdneri and Parophrys vetulus

INTRODUCTION

Ammonia is the endproduct of protein, amino acid, and adenylate catabolism in fish. Ammonia formed in the tissues is either re-utilized in situ or enters the blood where it remains until excreted across the gills. There is a substantial body of knowledge on the mechanisms of ammonia excretion across the gills of various fish (eg. Maetz & Garcia Romeu, 1964; Maetz, 1973, Evans, 1977; Payan, 1978; Cameron & Heisler, 1983; Wright & Wood, 1985). However, little is known about ammonia stores in body tissues of fish. Ammonia is a weak base that exists in solution as ammonia (NH_3) and ammonium ions (NH_4^+). With a pK of 9.7 at 10°C , the majority of ammonia in body fluids ($\text{pH} = 6.5\text{--}8.0$) will be in the ionized form, as NH_4^+ . Movement of ammonia between tissue compartments is thought to be primarily dependent on NH_3 partial pressure gradients (P_{NH_3}), because biological membranes are highly permeable to NH_3 (Castell & Moore, 1971; Klocke et al., 1972; Boron, 1980; Lockwood et al., 1980; see also Good & Knepper, 1985). Despite NH_3 's greater permeability, there is evidence that NH_4^+ electrochemical gradients also play a role in ammonia transfer across cell membranes (Thomas, 1974; Boron & DeWeer, 1976; Aickin & Thomas, 1977). Transfer of ammonium ions will depend on the diffusive permeability of the membrane for NH_4^+ , the availability of ion carriers in the membrane, and their affinity for NH_4^+ ions. A significant diffusive permeability to NH_4^+ is now recognized or

hypothesized in many biological membranes (Schwartz & Tripolone, 1983; Arruda et al., 1984; Evans & Cameron, 1986). Ammonium ions can substitute for K^+ in the $Na^+/K^+/Cl^-$ cotransporter (Kinne et al., 1986), the Na^+/K^+ ATPase pump (eg. Post & Jolly, 1957; Skou, 1960; Robinson, 1970; Aickin & Thomas, 1977; Sorensen, 1981; Kurtz & Balaban, 1986), and for H^+ ions in the Na^+/H^+ ion exchange mechanism (eg. Kinsella & Aronson, 1981; Aronson, 1983). As well, NH_4^+ can replace K^+ in nerve K^+ channels (Binstock & Lecar, 1969; Hille, 1973). Thus, it appears that transfer of ammonia between tissue compartments may involve both NH_3 and NH_4^+ movements.

In a closed system at equilibrium, the distribution of ammonia across biological membranes will be determined by the pH gradient across the membrane, as long as the effective permeability to NH_3 is much greater than that to NH_4^+ , and there is no chemical binding of the species. However, the greater the effective NH_4^+ permeability, the greater will be the influence of the electrical gradient on the equilibrium distribution. Living animals are certainly not closed systems at equilibrium. Nevertheless, the assumption has often been made that the distribution of ammonia between intracellular and extracellular tissue compartments is largely a function of the intracellular to extracellular pH gradient, in both mammals (eg. Visek, 1968; Pitts, 1973; Meyer et al., 1980; Mutch & Bannister, 1983; Remesy et al., 1986) and fish (Randall & Wright, 1987; Dobson & Hochachka, 1987).

The purpose of this study was to determine whether ammonia was distributed between intracellular and extracellular compartments according to transmembrane pH gradients. The first set of experiments were performed on blood of rainbow trout (Salmo gairdneri, in vivo and in vitro), to investigate whether the distribution of ammonia followed the pH gradient between red cells and plasma at rest, to establish the situation under conditions as close to steady state as possible, and during a perturbed situation, extracellular acidosis. Red cells are unusual compared with many other tissues in that H^+ ion distribution is passive and follows the membrane potential over a range of pH values (Heming et al., 1986; Nikinmaa et al., 1987; Lassen, 1977). Thus, if the ammonia distribution does follow the red cell-to-plasma pH gradient, this will then indicate that the distribution is dependent on transmembrane H^+ ion gradients or electrical gradients, or both. To distinguish between the two it is necessary to look at tissues other than red cells, where H^+ ions are not passively distributed (see Roos & Boron, 1981). If ammonia movements across cell membranes are solely dependent on P_{NH_3} gradients, that is, NH_4^+ movements are negligible, then one would expect the distribution of ammonia between intra- and extracellular compartments to follow transmembrane pH gradients. On the other hand, if NH_4^+ is permeant, then one would expect the ammonia distribution to follow the electrochemical NH_4^+ gradient between intra- and extracellular compartments. A second set of experiments was performed on lemon sole (Parophrys

vetulus) to investigate the distribution of ammonia and H^+ ions across white muscle, heart, and brain, as well as red blood cells. Measurements were made at rest, and under two treatments designed to perturb the normal acid-base status by very different mechanisms- respiratory acidosis (hypercapnia) and metabolic acidosis (exhaustive exercise). Lemon sole were chosen for these experiments as flatfish have a discrete white muscle mass uncontaminated by pink or red fibres, in contrast to salmonids (Mosse, 1979), and because their blood pH is somewhat lower (approx. 0.3 units) than in trout. Instructive comparisons could therefore be made with the first set of experiments on ammonia distribution in trout blood, as well as with a previous theoretical analysis of ammonia distribution in trout muscle (Randall & Wright, 1987).

MATERIALS & METHODS

Experimental animals

Rainbow trout (Salmo gairdneri) weighing approximately 300g were obtained from Sun Valley Trout Hatchery (Mission, B.C.) and transported to the University of British Columbia. Fish were held in outdoor, fiberglass tanks supplied with dechlorinated Vancouver tapwater (pH approx. 7, $[Na^+] = 40 \text{ uequiv.l}^{-1}$, $[Cl^-] = 20 \text{ uequiv.l}^{-1}$, hardness = 12 ppm $CaCO_3$, temperature = $7-9^\circ C$), and fed a diet of commercial trout pellets. Lemon sole (Paraphrys vetulus) weighing 292 ± 14 grams were collected by two fifteen minute otter trawls in Barclay Sound, British Columbia (August). Fish were held at the Bamfield Marine Station in a sandy-bottomed, outdoor, fiberglass tank supplied with flow through seawater (pH approx. 7.8, salinity approx. 31 o/oo, temperature = $11^\circ C$) for at least 7 days before experimentation. Trout were starved a minimum of 72 hours prior to experimentation and sole were not fed during captivity to eliminate variations in body ammonia levels due to feeding history (Fromm, 1963; Brett & Zala, 1975).

The experiments in this chapter can be divided into two sections. Section A describes the ammonia distribution across rainbow trout red cells, and section B covers the distribution of ammonia between muscle, heart, brain, red cells, and plasma of lemon sole.

SECTION A: Trout red cells

Experimental Protocol

In the in vitro experiment blood was collected from several cannulated donor fish (see General Materials and Methods), pooled, and then divided between tonometers (4 ml/tonometer). Each tonometer received either a 0.2% CO₂ (control) or a 1.0% CO₂ (hypercapnia) humidified gas mixture in air and was shaken for 90 min in a 9° C water bath before the measurements were taken. In one experiment, blood was equilibrated with 1.0% CO₂ and the Na⁺/K⁺ ATPase inhibitor, ouabain (10⁻⁴ M) for 90 min before the measurements were taken. Blood was analysed for whole blood pH (pHe), red cell pH (pHi), whole blood and plasma ammonia concentrations (Tamm), plasma and red cell water content, and haematocrit (hct). In the in vivo trout experiment, cannulated fish were placed in individual, low volume (2 l) flow-through chambers to recover for 48 h. In the 30 min prior to sampling, inflow water was turned off and fish chambers were aerated with either 100% air (water pH= 7.0, control) or switched to 1% CO₂ in air (water pH= 5.6, hypercapnia). A 2 ml blood sample was withdrawn from each fish at the end of 30 min and analysed for pHe, pHi, whole blood and plasma Tamm, plasma and red cell water content, and hct.

Analytic techniques and calculations

Arterial pHe was measured immediately upon collection using a

Radiometer microelectrode (type E5021) and acid-base analyzer (PHM 72), maintained at 90° C. Red cell pH_i was directly measured by the freeze-thaw technique (Zeidler & Kim, 1977) where a 400 ul whole blood sample was centrifuged in a bullet tube and then immediately frozen in either liquid N₂ or dry ice chips in ethanol. The frozen packed red cells were then cut away from the plasma, thawed, and the pH of the red cell slurry was measured using the Radiometer pH microelectrode assembly. Haematocrit was determined after centrifugation (5000 G). Plasma and red cell water content were calculated by drying to constant weight in an oven (100° C). Whole blood and plasma samples (250 ul) were assayed for Tamm after deproteinization in 500 uL of iced 8% HClO₃. Samples were then centrifuged and the supernatant was neutralized with 2 M KHCO₃. Tamm of this neutralized supernatant was measured by the glutamate dehydrogenase enzymatic assay (Kun & Kearney, 1971). Internal ammonia standards were routinely made in whole blood because it was found that distilled water standards did not agree with the internal standards. Whole blood internal standards were found to give the same results as plasma internal standards, and therefore, whole blood was used as the reference medium. Plasma Tamm was corrected for water content, and then red cell ammonia levels were calculated with the following formula:

$$\text{Red cell Tamm} = \frac{\text{whole blood Tamm} - [(1 - \text{hct}/100) \times \text{plasma Tamm}]}{\text{hct}/100} \quad (6)$$

Red cell Tamm calculated above was then corrected for red cell water content, and the final concentration ('measured') was expressed as $\mu\text{mol.l}^{-1}$ cell water.

Plasma NH_3 concentration ($[\text{NH}_3]$) was calculated by the following manipulation of the Henderson-Hasselbalch equation, using pK values given by Cameron and Heisler (1983):

$$\text{Plasma } [\text{NH}_3] = \frac{\text{plasma Tamm} \cdot (\text{antilog } (\text{pHe} - \text{pK}))}{1 + (\text{antilog } (\text{pHe} - \text{pK}))} \quad (7)$$

Intracellular $[\text{NH}_3]$ could also be calculated from red cell Tamm and pHi by a similar equation:

$$\text{Intracellular } [\text{NH}_3] = \frac{\text{intracellular Tamm} \cdot (\text{antilog } (\text{pHi} - \text{pK}))}{1 + (\text{antilog } (\text{pHi} - \text{pK}))} \quad (8)$$

Ammonium ion concentration ($[\text{NH}_4^+]$) in either compartment could be calculated as:

$$[\text{NH}_4^+] = \text{Tamm} - [\text{NH}_3] \quad (9)$$

Henry's law was applied to calculate the partial pressure of NH_3 in plasma and red cells (P_{NH_3} in uTorr: 1 Torr = 133.32 Pa), using the appropriate solubility coefficient (α_{NH_3} ; Cameron and Heisler, 1983).

$$P_{\text{NH}_3} = \frac{[\text{NH}_3]}{\alpha_{\text{NH}_3}} \quad (10)$$

If ammonia is distributed between plasma and red cells entirely according to the pH gradient, then plasma $[\text{NH}_3]$ will equal red cell $[\text{NH}_3]$ and red cell pH_i can be predicted from measured plasma and red cell Tamm using the Henderson-Hasselbalch equation:

$$\text{predicted intracellular } \text{pH}_i = \text{pK} + \log \frac{\text{intracellular } [\text{NH}_3]}{\text{intracellular } [\text{NH}_4^+]} \quad (11)$$

Predicted red cell Tamm could also be calculated by a further manipulation of the Henderson-Hasselbalch equation, employing the measured intracellular pH and again assuming that plasma $[\text{NH}_3]$ was equal to red cell $[\text{NH}_3]$.

$$\text{predicted red cell Tamm} = [\text{NH}_3] + \frac{[\text{NH}_3]}{\text{antilog}(\text{measured } \text{pH}_i - \text{pK})} \quad (12)$$

This 'predicted' red cell Tamm was then compared to 'measured' red cell Tamm determined from the direct measurement of plasma and whole blood ammonia (equation 6).

Data are expressed as mean \pm 1 S.E.M. (N), where N equals the number of animals sampled (in vivo) or the number of tonometers containing blood (in vitro).

The Student's two-tailed paired and unpaired t-test was employed to evaluate the significance of differences between mean values, where appropriate ($P < 0.05$).

SECTION B: Sole red cells, muscle, ventricle, and brain tissue

Experimental Protocol

Lemon sole with caudal artery cannulae (see General Materials and Methods) were placed in individual, flow-through, sandy-bottomed chambers (~ 8 l).

Three experimental conditions were studied:

(i) A control in which fish were subjected to unaltered, aerated seawater (pH= 7.8) prior to sampling.

ii) A second group of fish were placed in a seawater bath equilibrated to approximately 1% CO₂ (pH= 6.7) for 30 minutes before sampling in order to rapidly alter extracellular and intracellular pH via high P_{CO2} levels (respiratory acidosis).

(iii) The third group of fish were transferred to a large plastic tank and chased for 20 minutes. The aim in subjecting fish to exhaustive exercise was to induce a metabolic acidosis in both extracellular and intracellular compartments. These fish were allowed to recover for 30 min prior to sampling.

In order to determine intracellular pH (p_{Hi}) by the DMO (5,5 dimethyl-2,4-oxazolidinedione) distribution technique (Waddell & Butler, 1959), fish were injected with 1 ml.kg^{-1} of 5 uCi.ml^{-1} [^{14}C] DMO (New England Nuclear, specific activity 50 mCi.mmol^{-1}) and 20 uCi.ml^{-1} of the extracellular marker, [^3H] mannitol (New England Nuclear, specific activity $27.4 \text{ mCi.mmol}^{-1}$) in Cortland saline (adjusted to 160 mmol.l^{-1} NaCl) approximately 12 h prior to sampling (Milligan & Wood, 1986a,b).

At sampling, 2 ml of blood were withdrawn from each fish and replaced with an equal volume of saline. Blood was immediately analysed for p_{He} , p_{Hi} , plasma and whole blood total carbon dioxide content (C_{CO_2}) and hct. Samples were appropriately fixed and stored for later determination of whole blood and plasma Tamm, whole blood lactate ([La-]) and hemoglobin ([Hb]) concentrations and plasma and red cell water content. In less than 5 minutes after blood withdrawal, fish were removed from the water and the spinal cord quickly severed with a scalpel. Epaxial white muscle samples were excised and a thin slice of tissue immediately frozen with freeze-clamp tongs, cooled by immersion into dry ice chips. The frozen samples were stored on dry ice for later determination of intracellular Tamm and [La-], while the unfrozen tissue samples were later analysed for [^{14}C] DMO and [^3H] mannitol. The heart ventricle was then quickly removed. Part of the ventricle was freeze-clamped for subsequent analysis of intracellular Tamm, while the remaining tissue was

analysed for [^{14}C] DMO, [^3H] mannitol, and water content. Brain tissue was then collected after cutting through the cranium and, as before, part of the tissue was immediately frozen and later assayed for intracellular Tamm, while the rest of the tissue was reserved for [^{14}C] DMO, [^3H] mannitol, and water content determination. The entire tissue excision and freeze-clamping procedure took approximately 1-2 minutes. At the end of this procedure, large samples of white muscle tissue were taken for water content analysis.

Analytical Techniques and Calculations

Arterial pHe was measured immediately upon collection using a Radiometer microelectrode (type E5021) maintained at 11°C and linked to a Radiometer PHM71 acid-base analyzer. Plasma and whole blood CCO_2 were measured on 50 μl samples by the technique of Cameron (1971), using a Radiometer E5036 CO_2 electrode and the same acid-base analyser. Plasma PCO_2 and HCO_3^- were calculated by standard manipulations of the Henderson-Hasselbalsch equation (see General Materials & Methods). Red cell pHi was directly measured by the freeze-thaw technique of Zeidler & Kim (1977) using the same microelectrode assembly. The freeze-thaw method and not the DMO method was used to measure red cell pHi because the former technique is a more direct method for pHi determination of isolated cells, it is a simpler method, and there are no significant differences between red cell pHi values from the two techniques in fish blood

(Milligan & Wood, 1985). Plasma and red cell water content were calculated by drying to constant weight in an oven at 100° C.

Lactate levels ([La-]) were assayed enzymatically (L-lactic dehydrogenase/ NADH method, Sigma reagents) after deproteinizing 100 ul whole blood in 200 ul 8 % HClO₃, or homogenizing 100 mg white muscle in 1 ml 8 % HClO₃ with a glass homogenizer (cf. Turner et al., 1983). Muscle intracellular [La-] were expressed as mmol.l⁻¹ of ICF as outlined for muscle intracellular Tamm below (equation 9). hct was determined by centrifugation (5000 G for 5 min) and Hb by the cyanmethaemoglobin method of Blaxhall and Daisley (1973) using Sigma reagents.

Whole blood and plasma samples (250 ul) were assayed for Tamm as described above for trout blood. Red cell Tamm, plasma [NH₃], red cell [NH₃], P_{NH3}, predicted red cell pHi, and red cell Tamm were calculated using equations 6-12 (see above).

Intracellular muscle, heart, and brain Tamm were determined in the following manner. Frozen tissue samples were weighed, deproteinized in iced 8% HClO₃ (1:20 dilution), and then homogenized (Tekmar Tissumizer with microprobe head). Samples were centrifuged, and the supernatant was extracted and neutralized with saturated Tris buffer. The enzymatic assay technique was identical to that described above for blood, except that muscle tissue extract provided the reference medium for internal standards. (There were no differences between internal standards made up in muscle, heart, and brain tissue extracts.)

Muscle, heart, and brain levels of [^3H] and [^{14}C] radioactivity were measured by digesting 50-150 mg of tissue in 2 ml NCS tissue solubilizer (Amersham) for 1-2 days until the solution was clear. Extracellular fluid [^3H] mannitol and [^{14}C] DMO activity were determined in the same manner, except 100 μL of plasma were added to 2 ml of tissue solubilizer. The tissue digests were neutralized with 60 μl glacial acetic acid and 10 ml of fluor (OCS; Amersham) was added. To decrease chemiluminescence, all samples were kept in the dark overnight before being counted on a liquid scintillation counter. Dual-label quench correction was performed using quench standards prepared from each type of tissue and the external standard ratio method (Kobayashi & Maudsley, 1974).

Tissue extracellular fluid volume (ECFV, ml.g^{-1}) was calculated according to the equation:

(13)

$$\text{ECFV} = \frac{\text{Tissue } [^3\text{H}] \text{ mannitol (d.p.m.g}^{-1}\text{)}}{\text{Plasma } [^3\text{H}] \text{ mannitol (d.p.m.g}^{-1}\text{)} / \text{plasma H}_2\text{O (ml.g}^{-1}\text{)}}$$

Total tissue water was determined as previously described for plasma and whole blood samples. Intracellular fluid volume (ICFV, ml.g^{-1}) was calculated as the difference between total tissue water and ECFV.

Intracellular Tamm was determined by first accounting for ammonia trapped in the extracellular fluid and then correcting for intracellular water, as follows:

$$\text{Intracellular Tamm} = \frac{\text{Tissue Tamm} - (\text{plasma Tamm} \cdot \text{ECFV})}{\text{ICFV}} \quad (14)$$

Tissue pHi was calculated according to the equation:

$$\text{pHi} = \frac{\text{pK}_{\text{DMO}} + \log \{ [\text{DMO}]_i \cdot (10^{(\text{pHe} - \text{pK}_{\text{DMO}})} + 1) - 1 \}}{[\text{DMO}]_e} \quad (15)$$

where pK_{DMO} was taken from Malan et al. (1976) and $[\text{DMO}]_e$ and $[\text{DMO}]_i$ represent extracellular and intracellular $[\text{DMO}]$, respectively. These two values were calculated as:

$$[\text{DMO}]_e \text{ (d.p.m.ml)} = \frac{\text{plasma } [^{14}\text{C}] \text{ DMO (d.p.m.ml}^{-1}\text{)}}{\text{plasma H}_2\text{O (ml.g}^{-1}\text{)}} \quad (16)$$

and

$$[\text{DMO}]_i = \frac{\text{tissue } [^{14}\text{C}] \text{ DMO (d.p.m.ml)} - (\text{ECFV} \cdot [\text{DMO}]_e)}{\text{ICFV}} \quad (17)$$

Tissue $[\text{NH}_3]$, $[\text{NH}_4^+]$, and P_{NH_3} levels were calculated as described previously using equations (8), (9), and (10), respectively.

To determine whether the distribution of ammonia between extracellular and intracellular compartments in various tissues was according to the pH gradient, tissue pHi's were predicted

from the measured ammonia distribution by equation (11), assuming intracellular $[\text{NH}_3] = \text{plasma } [\text{NH}_3]$. These predicted pH_i 's were compared to the measured pH_i 's determined from the DMO distributions. A predicted intracellular Tamm could also be calculated by equation (12), again assuming intracellular $[\text{NH}_3] = \text{plasma } [\text{NH}_3]$.

To determine whether the distribution of ammonia between extracellular and intracellular compartments in various tissues could alternatively be a function of the membrane potential, $E_{\text{NH}_4^+}$ was calculated from the Nernst equation:

$$E_{\text{NH}_4^+} = \frac{RT}{ZF} \ln \frac{[\text{NH}_4^+]_e}{[\text{NH}_4^+]_i} \quad (18)$$

where R, T, Z , and F have their usual values, and $[\text{NH}_4^+]_e$ and $[\text{NH}_4^+]_i$ represent plasma and intracellular levels based on measured extracellular and intracellular pH and Tamm values (equation 8,9,14, and 15).

Data are presented as means ± 1 S.E.M.(N). The Student's two-tailed unpaired t-test was used to determine the significance of differences between mean values ($P < 0.05$).

RESULTS

A. Rainbow trout

Slight quantitative differences between rainbow trout in vitro and in vivo data are shown in Table 1, but the overall results and conclusions are the same whether blood was held in tonometers (in vitro) or in live animals (in vivo) prior to analysis. Red cell ammonia levels are consistently higher than plasma levels, resulting in ammonia concentration ratios of between 0.29 and 0.40 (plasma-to-red cell, Table 1). Red cell pH_i predicted from the plasma-to-red cell ammonia distribution was not significantly different from measured pH_i in the control experiment, but during hypercapnia, predicted pH_i was significantly less than measured pH_i (Table 1). NH_3 was in equilibrium between plasma and red cell at rest, but not during hypercapnia (Fig. 3). The P_{NH_3} gradient from red cell-to-plasma during hypercapnia was 17 uTorr, in vitro, and 20 uTorr, in vivo (Fig. 3). Our calculations of P_{NH_3} levels assume an equilibrium between NH_3 and NH_4^+ in the red cell as well as the plasma, because the NH_3 NH_4^+ reaction is considered instantaneous (<50 msec., Stumm & Morgan, 1981). If there is an NH_3 gradient from red cell to plasma and if H^+ ions are passively distributed across red cell membranes as commonly believed (Lassen, 1977; Heming et al., 1986; Nikinmaa et al., 1987), then there must also be an electrochemical gradient for NH_4^+ . As red cell NH_3 levels are elevated, NH_4^+

TABLE 1. Measured pHe, red cell pH_i, plasma and red cell Tamm, and the ratio of plasma:red cell Tamm, in vitro and in vivo, under control and hypercapnic conditions. Predicted red cell pH_i calculated from the ratio of NH₂:NH₄⁺ in the red cell using equation 11 is compared to measured pH_i (freeze-thaw method). Predicted red cell Tamm (equation 12) is compared to measured red cell Tamm. E_{NH₄⁺} and E_{H⁺} were calculated from equation 18. Means \pm S.E.M.

TREATMENT	MEASURED				AMMONIA CONCENTRATION RATIO	PREDICTED		CALCULATED	
	pHe	pHi (freeze- thaw method)	PLASMA Tamm (umoll ⁻¹)	RED CELL Tamm (umoll ⁻¹)		pHi	RED CELL Tamm (umoll ⁻¹)	E _{NH4+} (mV)	E _{H+} (mV)
.....									
CONTROL									
<u>in vitro</u> (N=6)	8.03 ±.05	7.48 ±.02	304 ± 8	1048 ± 81	.29	7.46 ±.05	1018 ±124	-30.0 ±1.7	-31.2 ±2.8
<u>in vivo</u> (N=7)	8.02 ±.03	7.50 ±.01	311 ±19	782 ±55	.40	7.51 ±.04	943 ±86	-24.9 ±2.2	-29.3 ±1.6
.....									
HYPERCAPNIA									
<u>in vitro</u> (N=7)	7.63 [*] ±.02	7.25 [*] ±.01	318 ± 9	969 ±52	.33	7.11 [^] ±.04	704 [#] ±36	-27.8 ±1.4	-21.3 [*] ±1.1
<u>in vivo</u> (N=7)	7.55 ^{**} ±.02	7.28 ^{**} ±.01	323 ± 3	872 ±30	.37	7.09 [^] ±.01	560 [#] ±34	-24.2 ±0.9	-15.3 ^{**} ±1.6

* significantly different from in vitro control, paired t-test

** significantly different from in vivo control, unpaired t-test

[^] significantly different from measured pH_i, unpaired t-test

[#] significantly different from "measured" red cell Tamm, unpaired t-test, (P < 0.05)

Figure 3. Plasma P_{NH_3} (stipled bars) is compared to red cell P_{NH_3} (open bars) in control and hypercapnic treatments of both in vitro and in vivo experiments. ■ denotes significant difference from plasma P_{NH_3} . ▲ denotes significant difference from control P_{NH_3} . means \pm 1 S.E.M.

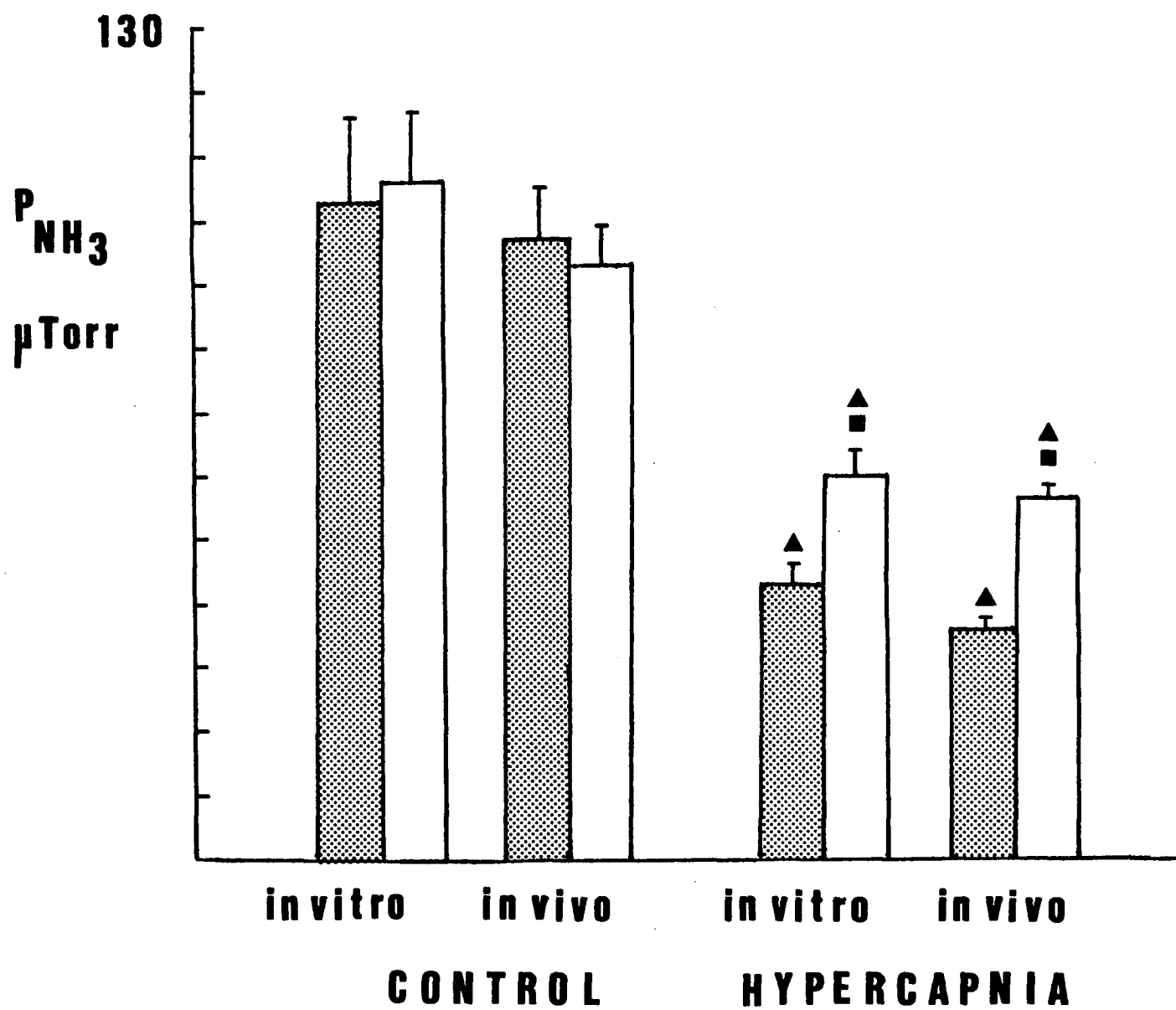


TABLE 2. Measured pHe, red cell pH_i, plasma and red cell Tamm, and the ratio of plasma:red cell Tamm, in vitro, under hypercapnic conditions. Values for control (no ouabain) and ouabain (10⁻⁴ M) are shown. See Table 1 for details.

TREATMENT	MEASURED					PREDICTED		CALCULATED	
	pHe	pH _i (freeze-thaw method)	PLASMA Tamm (umol l ⁻¹)	RED CELL Tamm (umol l ⁻¹)	AMMONIA CONCENTRATION RATIO	pH _i	RED CELL Tamm (umol l ⁻¹)	E _{Na+} (mV)	E _{K+} (mV)
control (N=7)	7.64 ±.03	7.25 ±.03	346 ±17	1016 ± 61	.34	7.13 [^] ±.03	777 [#] ±45	-26.3 ±1.1	-22.1 ±1.4
ouabain (N=7)	7.64 ±.01	7.24 ±.01	354 ±16	1000 ± 82	.35	7.16 [^] ±.03	808 [#] ±40	-25.1 ±1.7	-22.6 ±0.4

[^] significantly different from measured pH_i, unpaired t-test

[#] significantly different from "measured" red cell Tamm, unpaired t-test, (P < 0.05)

ions will also be elevated as NH_3 molecules combine with H^+ to form NH_4^+ . In hypercapnia, therefore, there is a net diffusion gradient for both NH_3 and NH_4^+ out of the red cell. This is illustrated by calculations of $E_{\text{NH}_4^+}$ and E_{H^+} from the Nernst equation (equation 18) in Table 1. $E_{\text{NH}_4^+}$ and E_{H^+} agreed under control conditions, however, E_{H^+} becomes less negative in the expected manner (eg. Lassen, 1977) during hypercapnia, while $E_{\text{NH}_4^+}$ did not change.

Incubation of the blood with the Na^+/K^+ ATPase inhibitor, ouabain, did not alter the ammonia distribution during hypercapnia (Table 2).

Lemon Sole

Acid-Base and Fluid Volume changes

The extracellular acid-base status of the three experimental groups is given in Table 3A. The lower arterial pHe associated with hypercapnia was due to a respiratory acidosis. Elevated plasma PCO_2 was associated with a significantly greater plasma $[\text{HCO}_3^-]$ relative to control fish. This elevated $[\text{HCO}_3^-]$ was equal to the product of the difference in pH and the buffer value (β , taken from in vitro flathead sole data, Turner et al., 1983b), which indicates that the higher plasma $[\text{HCO}_3^-]$ levels were probably due to the elevated PCO_2 alone and not to other factors. The lower pHe in exercised fish was the result of a

TABLE 3. Acid-base status in sole during control, hypercapnia and exercise regimes. Means \pm S.E.M.

A

TREATMENT	pHe	PLASMA P _{CO2} torr	PLASMA [HCO ₃ ⁻] mM/L	WHOLE BLOOD [La ⁻] mM/L	MUSCLE [La ⁻] mM/L
CONTROL (N=10)	7.73 \pm .01	1.94 \pm .08	4.64 \pm .19	0.11 \pm .03 (N=6)	3.22 \pm .09 (N=6)
HYPERCAP. (N=9)	* 7.32 \pm .04	* 7.44 \pm .74	* 5.92 \pm .14	0.19 \pm .05 (N=7)	6.00 \pm 2.16 (N=7)
EXERCISE (N=9)	* 7.48 \pm .04	1.99 \pm .35	* 2.40 \pm .23	* 0.98 \pm .16 (N=10)	* 19.57 \pm 1.55 (N=10)

B

TREATMENT	RED CELL pHi	WHITE MUSCLE pHi	HEART pHi	BRAIN pHi
CONTROL (N=10)	7.25 \pm .02	7.29 \pm .03	7.32 \pm .06	7.75 \pm .03
HYPERCAP. (N=9)	* 7.01 \pm .02	* 6.95 \pm .03	* 7.10 \pm .03	* 7.44 \pm .02
EXERCISE (N=9)	7.22 \pm .02	* 7.12 \pm .03	7.27 \pm .07	7.71 \pm .05

* significantly different from control value, t-test $p < 0.05$

metabolic acidosis, because plasma $[\text{HCO}_3^-]$ was significantly lower, but P_{CO_2} remained unchanged from control values. There was a significant elevation of blood $[\text{La}^-]$ in the exercise group over resting levels ($+ 0.9 \text{ mmol.l}^{-1}$), but the absolute difference was small compared to the differences in muscle $[\text{La}^-]$ levels ($+ 16.3 \text{ mmol.l}^{-1}$, Table 3A). The elevation of blood $[\text{La}^-]$ ($+0.9 \text{ mmol.l}^{-1}$) was also small relative to the metabolic acid load ($\text{H}^+_{\text{m}} = 3.0 \text{ mmol.l}^{-1}$) which can be calculated from the differences in pH_e and $[\text{HCO}_3^-]$ (cf. Turner et al., 1983a).

Of the various tissues examined, pH_i was by far the highest in brain, with a value approximately equal to pH_e (Table 3B). Red cells, white muscle, and heart ventricle all maintained a pH_e - pH_i gradient of 0.3-0.4 pH units. Hypercapnia resulted in lower pH_i in all tissues relative to control fish, with greater decreases in brain and white muscle (0.3 units) than in red cells or heart (0.2 units). The acidifying effect of exhaustive exercise was much more selective, with pH_i significantly lower only in the working muscle. The acid-base status of the other tissues was unaffected.

The changes in haematological variables after hypercapnia were very similar to those after exercise: $[\text{Hb}]$ remained unchanged, while hct was significantly greater, relative to control values (Table 4). Mean cellular haemoglobin concentration ($\text{MCHC} = [\text{Hb}]/\text{hct}$) was significantly less in the hypercapnia and exercise groups, which suggests that the red

TABLE 4. Blood haemoglobin, haematocrit, mean cellular haemoglobin concentration (MCHC) and red cell and plasma water content in three groups of lemon sole; control fish, hypercapnic fish, and fish swum to exhaustion. Means \pm S.E.M.

TREATMENT	Hb (g/100 mL blood)	Hct (%)	MCHC (g/mL cells)	Red cell water (%)	Plasma water (%)
CONTROL (N=10)	3.0 \pm 0.2	11.0 \pm 1.0	0.28 \pm 0.01	76.3 \pm 1.4	97.4 \pm 0.3
HYPERCAPNIA (N=9)	3.7 \pm 0.2	15.9 \pm 1.1	0.24 \pm 0.01	73.6 \pm 0.5	96.6 \pm 0.0
EXERCISE (N=9)	3.7 \pm 0.6	17.2 \pm 2.2	0.24 \pm 0.00	73.9 \pm 1.4	95.9 \pm 0.2

* significantly different from control, unpaired t-test, $P < 0.05$

cells swelled. However, this was not confirmed by the red cell water content measurements, which did not differ significantly amongst groups (Table 4). Plasma % water was significantly less in both exercised and hypercapnic fish. The higher hct values in the hypercapnic and exercised fish relative to control fish could be explained by either inherent differences between the groups or the effects of the experimental treatments.

ECFV was much greater in the heart ventricle tissue than in brain or white muscle, while ICFV demonstrated the opposite trend (Table 5). Hypercapnia had no effect on ECFV AND ICFV. Following exhaustive exercise, however, ECFV was significantly lower and ICFV, higher in white muscle, suggesting a net water flux into the cells, probably in response to the osmotic effect of the increased intracellular $[La^-]$ (Table 3A). Total water contents (ECFV + ICFV) did not vary significantly amongst the three tissues, nor as a result of hypercapnia and exercise.

Ammonia distribution

The distribution of ammonia between plasma and tissues under control, hypercapnia, and exercise conditions is presented for red cells in Table 6, white muscle in Table 7, heart ventricle in Table 8, and brain in Table 9. In hypercapnic fish, plasma ammonia levels were significantly lower than in the control group by about 25%, while tissues showed generally smaller, non-significant reductions. In exercised fish, intracellular

TABLE 5. Fluid volume distribution (g H₂O/g tissue) in various tissues of lemon sole during control, hypercapnia, and exercise regimes. Total H₂O refers to the sum of ECFV and IVFV. Means \pm S.E.M.

TREATMENTS	MUSCLE			HEART			BRAIN		
	ECFV	ICFV	TOTAL H ₂ O	ECFV	ICFV	TOTAL H ₂ O	ECFV	ICFV	TOTAL H ₂ O
CONTROL (N=10)	0.0997 \pm .0113	0.7174 \pm .0129	0.8171 \pm .0029	0.2594 \pm .0243	0.5529 \pm .0241	0.8123 \pm .0042	0.1206 \pm .0149	0.6901 \pm .0171	0.8107 \pm .0045
HYPERCAPNIA (N=9)	0.0875 \pm .0192	0.7253 \pm .0197	0.8129 \pm .0012	0.2765 \pm .0103	0.5348 \pm .0141	0.8110 \pm .0041	0.1077 \pm .0051	0.6832 \pm .0118	0.7909 \pm .0090
EXERCISE (N=9)	0.0599 \pm .0090	0.7552 \pm .0097	0.8152 \pm .0027	0.2837 \pm .0278	0.5237 \pm .0261	0.8074 \pm .0043	0.1235 \pm .0234	0.6716 \pm .0277	0.7951 \pm .0080

* significantly different from control, unpaired t-test $P < 0.05$.

TABLE 6. Measured pHe, red cell pH_i, plasma and red cell Tamm, and the ratio of plasma:red cell Tamm at rest, during hypercapnia, and following exercise in lemon sole. Predicted red cell pH_i calculated from the ratio of NH₃:NH₄⁺ in the red cell using equation 11 is compared to measured pH_i (freeze-thaw method). Predicted red cell Tamm (equation 12) is compared to measured red cell Tamm. E_{NH₄⁺} and E_{H⁺} were calculated from equation 18. Means ± S.E.M.

TREATMENT	MEASURED					PREDICTED		CALCULATED	
	pHe	pH _i (freeze-thaw method)	PLASMA Tamm uM/L	RED CELL Tamm uM/L	AMMONIA CONCENTRATION RATIO	pH _i	RED CELL Tamm (umol/L)	E _{NH₄⁺} (mV)	E _{H⁺} (mV)
CONTROL (N=10)	7.73 ±.01	7.25 ±.02	192 ±13	657 ±93	.29	7.23 ±.07	588 ±52	-29.1 ±3.8	-27.9 ±1.1
HYPERCAPNIA (N=9)	* 7.32 ±.04	* 7.01 ±.02	* 142 ±11			^ 6.79 ±.08	** 302 ±24		* -31.1 ±5.1
EXERCISE (N=9)	* 7.48 ±.04			* 1029 ±213	.22	^ 6.89 ±.05	** 453 ±90		* -15.7 ±1.3

* significantly different from control, P<0.05

^ significantly different from measured pH_i, P<0.05

** significantly different from "measured" red cell Tamm, P<0.05.

TABLE 7. Measured pHe, muscle pH_i, plasma and muscle Tamm, and the ratio of plasma:muscle Tamm at rest, during hypercapnia, and following exercise in lemon sole. Predicted muscle pH_i calculated from the ratio of NH₃:NH₄⁺ in muscle using equation 11 is compared to measured pH_i (DMO method). Predicted muscle Tamm (equation 12) is compared to measured muscle Tamm. E_{NH₄⁺} was calculated from equation 18.

TREATMENT	MEASURED				AMMONIA CONCENTRATION RATIO	PREDICTED		CALCULATED E _{NH₄⁺} (mV)
	pHe	pH _i (DMO)	PLASMA Tamm uM/L	MUSCLE Tamm uM/L		pH _i	MUSCLE Tamm (uM/L)	
CONTROL (N=9)	7.73 ±.01	7.29 ±.03	192 ±13	6005 ±577	.03	6.24 ±.05	550 ±35	-83 ± 3
HYPERCAPNIA (N=9)	* 7.32 ±.04	* 6.95 ±.03	* 142 ±11		.03	^ 5.83 ±.06	** 343 ±26	-83 ± 3
EXERCISE (N=9)	* 7.48 ±.04	* 7.12 ±.03		* 9344 ±1256	.02	^ 5.84 ±.12	** 560 ±112	-88 ± 4

*, ^, ** see TABLE 4

TABLE 8. Measured pHe, heart pH_i, plasma and heart Tamm, and the ratio of plasma:heart Tamm at rest, during hypercapnia, and following exercise in lemon sole. Predicted heart pH_i calculated from the ratio of NH₃:NH₄⁺ in heart using equation 11 is compared to measured pH_i (DMO method). Predicted heart Tamm (equation 12) is compared to measured heart Tamm. E_{NH₄⁺} was calculated from equation 18.

TREATMENT	MEASURED				AMMONIA CONCENTRATION RATIO	PREDICTED		CALCULATED E _{NH₄⁺} (mV)
	pHe	pH _i (DMO)	PLASMA Tamm (uM/L)	HEART Tamm (uM/L)		pH _i	HEART Tamm uM/L	
CONTROL (N=9)	7.73 ±.01	7.32 ±.06	192 ±13	7891 ±598	0.02	6.11 ±.05	543 ±78	-90 ± 3
HYPERCAPNIA (N=9)	7.32 ±.04	7.10 ±.03	142 ±11	6941 ±998	0.02	5.67 ±.08	254 ±33	-94 ± 4
EXERCISE (N=9)	7.48 ±.04	7.27 ±.07	228 ±31	12,355 ±1071	0.02	5.70 ±.10	403 ±81	-96 ± 2

*, ^, ** see TABLE 4

TABLE 9. Measured pHe, brain pH_i, plasma and brain Tamm, and the ratio of plasma:brain Tamm at rest, during hypercapnia, and following exercise in lemon sole. Predicted brain pH_i calculated from the ratio of NH₃:NH₄⁺ in brain using equation 11 is compared to measured pH_i (DMO method). Predicted brain Tamm (equation 12) is compared to measured brain Tamm. ENH₄⁺ was calculated from equation 18.

TREATMENT	MEASURED				AMMONIA CONCENTRATION RATIO	PREDICTED		CALCULATED ENH ₄ ⁺ (mV)
	pHe	pH _i (DMO)	PLASMA Tamm uM/L	BRAIN Tamm uM/L		pH _i	BRAIN Tamm (uM/L)	
CONTROL (N=9)	7.73 ±.01	7.75 ±.03	192 ±13	3419 ±434	0.06	6.48 ±.06	195 ±21	-69 ±3
HYPERCAPNIA (N=9)	7.32 ±.04	7.44 ±.02	142 ±11	2787 ±948	0.05	6.19 ±.11	115 ±11	-65 ±6
EXERCISE (N=9)	7.48 ±.04	7.71 ±.05	228 ±31	4970 ±864	0.05	6.12 ±.12	144 ±2	-72 ±4

*, ^, ** see TABLE 4

ammonia levels were elevated by about 55% over control levels, an effect which was significant in all tissues except brain. Plasma ammonia levels were not significantly altered.

As in trout red cells (Table 1), ammonia was distributed between red cells and plasma in sole according to pH gradients, at rest, but not during an extracellular acidosis (hypercapnia and exercise, Table 6). Absolute Tamm levels in the red cell and plasma were lower in sole compared to trout by about 16% and 40%, respectively. Despite these differences, predicted red cell pH_i and Tamm were not significantly different from measured values in the control experiment, but were significantly less during an extracellular acidosis (hypercapnia and exercise, Table 6), as in trout. As well, NH_3 was in equilibrium between plasma and red cells at rest, but not during either hypercapnia and exercise (Fig. 4). The P_{NH_3} gradient from red cell-to-plasma during hypercapnia was 10 uTorr and following exercise about 30 uTorr. $E_{NH_4^+}$ and E_{H^+} agreed under control conditions, however, E_{H^+} was less negative during both acidotic treatments, while $E_{NH_4^+}$ was more negative.

Ammonia distribution ratios between muscle and plasma (control ratio= 0.03, Table 7), heart and plasma (control ratio= 0.02, Table 8), and brain and plasma (control ratio= 0.06, Table 9) were considerably lower than those between red cells and plasma (ratios > 0.20, Table 6). Ammonia was clearly not distributed across tissue compartments according to the H^+ distribution. In all tissues and treatments, intracellular pH_i

Figure 4. Plasma P_{NH_3} (stipled bars) is compared to red cell P_{NH_3} (open bars) in control fish (C), fish exposed to hypercapnia (H), and exercised fish (E). ■ denotes significant difference from plasma P_{NH_3} . ▲ denotes significant difference from control P_{NH_3} . means \pm 1 S.E.M.

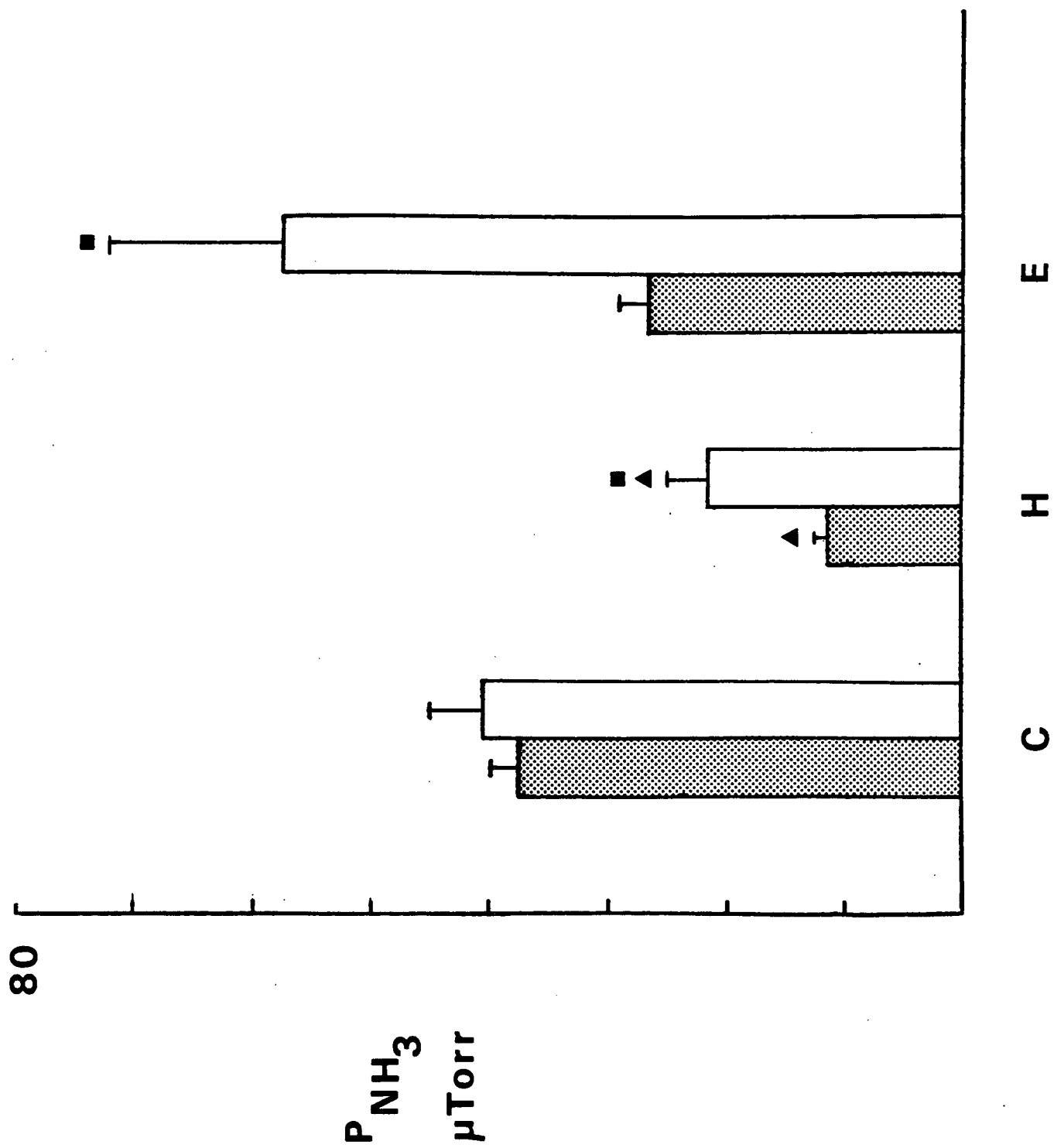
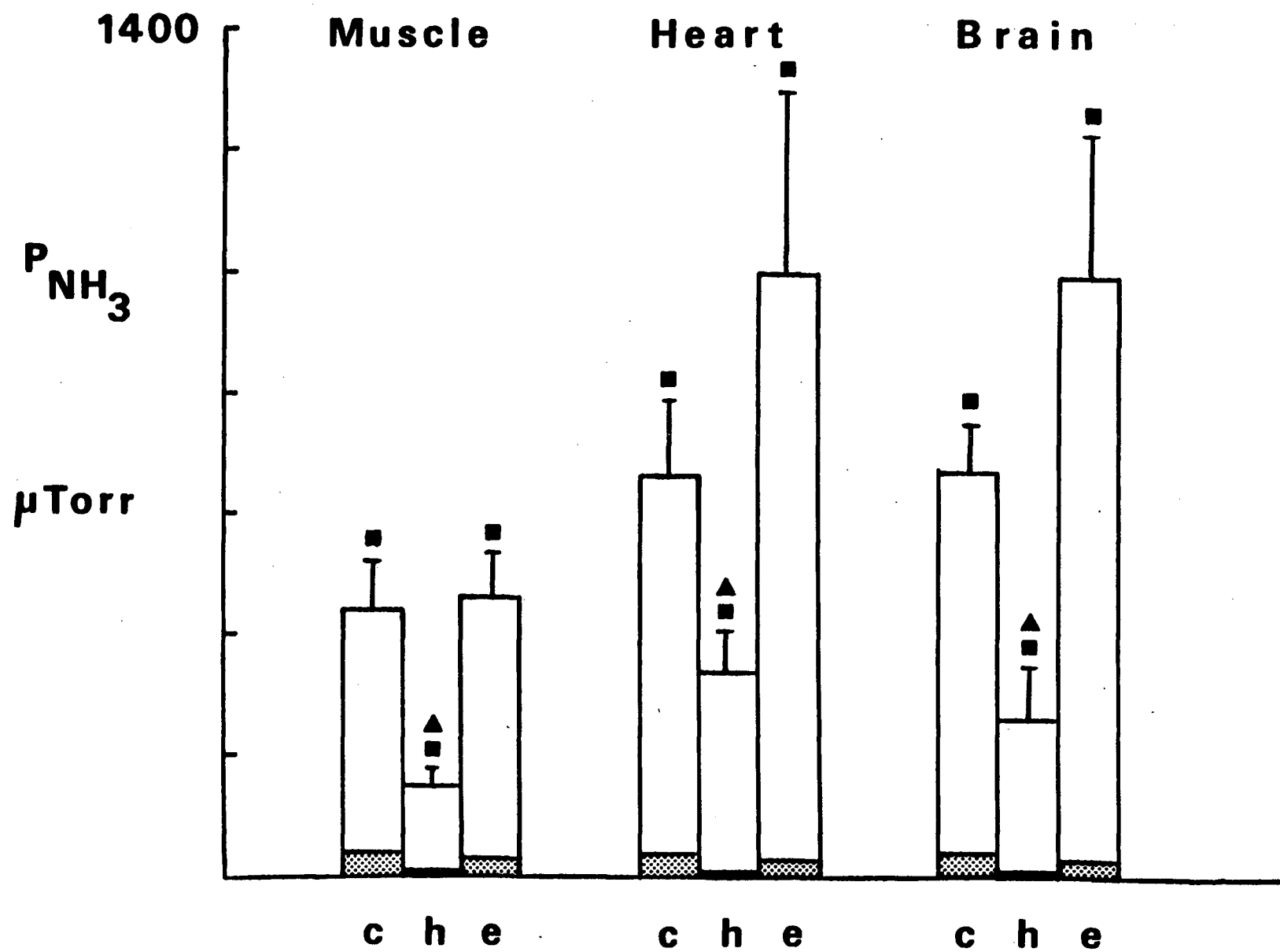


Figure 5.. White muscle, ventricular heart muscle, and brain P_{NH_3} levels (open bars) are compared to plasma P_{NH_3} levels (stippled bars) in control fish (C), fish exposed to hypercapnia (H), and exercised fish (E). ■ denotes significant difference from plasma P_{NH_3} . ▲ denotes significant difference from control P_{NH_3} . means ± 1 S.E.M.



calculated from the plasma-to-tissue ammonia distribution was significantly less (by 1.0-2.0 pH units) than pH_i calculated from the [^{14}C] DMO distribution (Table 7, 8, 9). As well, predicted tissue Tamm was significantly less than measured tissue Tamm in each treatment (Table 7, 8, 9). The tissue intracellular-to-extracellular P_{NH_3} gradients (Fig. 5) were an order of magnitude greater than red cell-to-plasma gradients (Fig. 4). As stated above for red cells, our calculations of P_{NH_3} levels assume an equilibrium between NH_3 and NH_4^+ in each tissue compartment. The difference between red cells and other intracellular compartments, however, is that H^+ ion distribution is not passive (Roos & Boron, 1981). Hence, NH_3 gradients can exist in the absence of NH_4^+ electrochemical gradients, in a situation where NH_4^+ is passively distributed across the membrane, but H^+ ions are not (see Discussion). Control P_{NH_3} gradients were smaller between muscle and plasma (410 uTorr), than heart (630 uTorr) or brain (640 uTorr) and plasma. In all tissues, P_{NH_3} was significantly reduced during hypercapnia, largely because of intracellular acidosis, although intracellular Tamm did decrease slightly in all tissues. There were no significant changes in P_{NH_3} gradients after exercise (Fig. 5), although Tamm gradients from tissue to plasma were greatly increased in all tissues (Table 7, 8, 9).

The equilibrium potentials for NH_4^+ ($E_{NH_4^+}$) across muscle, heart, and brain cell membranes were calculated from the Nernst equation and are presented in Tables 7, 8, and 9,

respectively. These values were 2-3 fold greater than those in calculated red blood cells (cf. Table 6). There were no significant differences in $E_{NH_4^+}$ between treatments.

DISCUSSION

Evaluation of methods

The accuracy of red cell Tamm calculations depends largely on hct, increasing with increasing hct (equation 6). In the trout experiments hct values were greater than 23%, and therefore the calculation error was negligible. However, in the control sole experiment, the error in red cell Tamm may be as great as 7% if hct is off by 1%, at the mean hct value of 11%. A 7% error in 'measured' red cell Tamm would not alter the conclusions that ammonia is distributed according to the H⁺ ion distribution across red cell membranes at rest. The significance of this problem diminishes in the sole hypercapnia (hct= 16%) and exercise (hct= 17%) treatments, where the error in red cell Tamm is at most 4% for a 1% digression in hct. The potential for error in 'measured' red cell Tamm calculations in the hypercapnia and exercise experiments is small relative to the large discrepancy between 'measured' and 'predicted' red cell Tamm. The potential for error in 'measured' intracellular Tamm for muscle, heart, and brain tissue (equation 18) is even smaller; for instance, for a 1% digression in ICFV, the error in tissue Tamm is less than 2%. Thus, ICFV is not a critical variable in the calculation of intracellular Tamm.

The accuracy and limitations of the ¹⁴C-DMO/³H-mannitol technique for intracellular pH measurements in fish have been assessed in detail previously (Milligan & Wood, 1985, 1986a,b),

and will not be discussed in the present paper.

Acid-base changes in the sole: comparisons to other studies

Resting blood acid-base parameters in lemon sole were very similar to those measured by McDonald et al. (1982) on the same species. Hypercapnia resulted in a plasma acidosis that was typical of other saltwater species (Conger conger, Toews et al., 1983; Scyliorhinus stellaris, Heisler et al., 1976) when compared over the same period of time. The metabolic acidosis incurred after exhaustive exercise in lemon sole was typical of other benthic, sluggish-type fish swum to exhaustion, with very low blood [La-] despite high muscle [La-] and a considerable blood metabolic acid load (Wood & Perry, 1985). In other fish, however, a respiratory component (P_{CO_2} elevation) often persists for 1-2 h following exercise, which was not seen here. Hence, if lemon sole did suffer a respiratory acidosis in addition to the metabolic acidosis following exercise, full recovery must have occurred within 30 min.

White muscle, brain, and red cell pH_i in resting lemon sole were identical to those reported for another flatfish, the starry flounder (Platichthys stellatus), using the same DMO and freeze-thaw techniques (Wood & Milligan, 1987), while heart pH_i was approximately 0.2 pH units lower. Exhaustive exercise in lemon sole resulted in a less marked intracellular acidosis in white muscle, and no acidosis in heart or brain tissue, in contrast to the starry flounder. This difference undoubtedly

reflects the lack of a respiratory component in sole relative to the large P_{CO_2} elevation in flounder at a comparable time after exercise. Thus, in the sole, the intracellular acidosis was limited to the working muscle itself and was completely metabolic in nature, as signalled by the large $[La^-]$ elevation in white muscle. I am aware of no comparable studies on the intracellular responses to hypercapnia in fish over this time period.

Red Cell Ammonia Distribution: trout and sole

The results show that ammonia is distributed according to the pH gradient across red cells under resting conditions in trout and sole, which agrees with a study on human red cells (Bone et al., 1976). If NH_4^+ transfer across red cell membranes is negligible, and ammonia distribution is solely dependent on P_{NH_3} gradients, then one would expect the red cell-to-plasma ammonia distribution to follow the H^+ distribution, as for other weak acids and bases with impermeant ion forms. Red cells, however, are unusual in that H^+ ions are passively distributed across the membrane according to the membrane potential (Lassen, 1977; Heming et al., 1986; Nikinmaa et al., 1987), unlike other tissues (see Roos & Boron, 1981). Therefore, exactly the same, distribution would occur if the membrane was permeable only to NH_4^+ , and the ammonia distribution was set entirely by the membrane potential. We conclude that ammonia is passively distributed between red cells and plasma under control conditions, however, we cannot determine if ammonia flux across

red cell membranes depends upon P_{NH_3} gradients solely, or if NH_4^+ electrochemical gradients are also important.

Ammonia is not distributed across red cell membranes according to the pH gradient during an extracellular acidosis (hypercapnia in trout (in vitro and in vivo), hypercapnia and exercise in sole), which agrees with data on avian red cells in vitro over the pH range 7-8 (Bone et al., 1976). Ammonia accumulation in the red cell, could be maintained by the active uptake of NH_4^+ in the face of NH_3 diffusion out of the red cell down the P_{NH_3} gradient and NH_4^+ electrochemical gradients. The ability of NH_4^+ to replace K^+ in Na^+/K^+ ATPase is well established in many tissues, including red cell membranes (Post & Jolly, 1957; Sorensen, 1981). We tested the possibility that NH_4^+ was replacing K^+ in the Na^+/K^+ ATPase by adding the specific Na^+/K^+ ATPase inhibitor, ouabain, to hypercapnic trout blood, in vitro (Table 2). The addition of ouabain did not alter the distribution of ammonia between red cells and plasma and red cell-to-plasma P_{NH_3} gradients were not abolished. This implies that even if Na^+/K^+ ATPase plays a role in ammonia accumulation within the red cell during hypercapnia, it cannot be a major one. Furthermore, trout red cell membrane Na^+/H^+ exchange mechanism is known to be active during an acidosis (Nikinmaa et al., 1987). If NH_4^+ ions can replace H^+ in exchange for Na^+ , as in other cells (eg. Maetz & Garcia Romeu, 1964; Kinsella & Aronson, 1981; Wright & Wood, 1985), then red cell ammonia stores would be depleted during hypercapnia. Instead, we observed an

accumulation of ammonia during hypercapnia and therefore, NH_4^+ substitution for H^+ in Na^+/H^+ exchange cannot be involved.

Ammonia gradients during hypercapnia may develop between intra- and extracellular compartments because of high rates of ammonia production. We tested this possibility in trout blood in vitro, by following whole blood Tamm levels over time during hypercapnic exposure and found that ammonia levels did not change. Thus, intracellular ammoniogenesis is not a factor in the development of P_{NH_3} gradients during hypercapnia in trout, and because of the similarity of the trout and sole data, it is probably not a factor in sole, as well.

It is possible that changes in pH and water content, which will lead to changes in ammonia distribution, may have caused the development of ammonia gradients between red cell and plasma during blood acidosis. In trout, whole blood pH remained stable after 30 min. of hypercapnia in vitro. Red cell water content increased significantly between control (in vitro, $65.1\% \pm .4$, in vivo, $65.7\% \pm .3$) and hypercapnia (in vitro, $68.4\% \pm .3$, in vivo, $68.9\% \pm .3$) experiments. It seems likely that water content of red cells was stable following 90 min of exposure to hypercapnia in vitro. Thus it appears that nonsteady states for pH and water content cannot account red cell-to-plasma P_{NH_3} gradients in vitro during hypercapnia. It also seems to be an unlikely explanation of the trout and sole in vivo results because of the similarity of the in vitro and in vivo data.

Another possible explanation for red cell-to-plasma ammonia

gradients under acid conditions is binding of ammonia to proteins (Barker, 1968) in the red cell. It seems unlikely, however, that differential binding would occur, that is, binding at low cell pH_i values (hypercapnia and exercise) but not at control pH values. Furthermore there is evidence that ammonia binds in some mammalian intracellular compartments (see below), but we are unaware of any reports of ammonia binding in red cells.

Finally, Bone et al. (1976) proposed that ammonia and DMO would distribute differently between the cytosol and the acidic nucleus, resulting in a discrepancy between pH_i calculated from the DMO distribution versus the ammonia distribution. Roos and Boron (1981), however, reanalysed their data and found that intracellular compartmentalization could not fully account for the discrepancy between the two calculated pH_i values.

In conclusion, ammonia is passively distributed according to the plasma-to-red cell H^+ distribution in blood at resting pH values, but not during an extracellular acidosis. Various possibilities for ammonia accumulation during blood acidosis have been discussed. I propose that some other active NH_4^+ uptake process must be operating to maintain red cell Tamm levels above those expected from pH considerations.

Muscle, Heart, and Brain Ammonia Distribution

Ammonia was not distributed between plasma and muscle, heart, and brain tissue according to the H^+ distribution at rest, during

hypercapnia, and following exercise in lemon sole. These findings are consistent with studies on mammalian hepatocytes where at physiological pH and ammonia concentrations, intracellular ammonia levels are much higher than predicted from the H^+ distribution (Sainsbury, 1980; Remesy et al., 1986). Ammonia also accumulates in mammalian brain tissue (Hindfelt, 1975; Benjamin, 1982) and colon (Bown et al., 1975) in concentrations above those expected from extracellular ammonia and pH considerations. These studies and the present results indicate that ammonia distribution across tissue membranes cannot be described by the classic diffusion trapping model (Visek, 1968; Pitts, 1973), which predicts intra- to extracellular ammonia distribution according to pH gradients, assuming only NH_3 is transferred across cell membranes and NH_4^+ transfer is negligible. If we assume that NH_4^+ permeability is significant, then the distribution of ammonia at equilibrium will be influenced by the membrane potential (E_m) as well as the pH gradient. The greater the permeability to NH_4^+ , the greater the influence of E_m .

Boron and Roos (1976) and Roos and Boron (1981) derived a general equation describing the equilibrium distribution of any weak base for which there is significant permeability to the charged as well as the uncharged form. Adopting this equation for ammonia, and correcting typographical errors in both original publications, this equation becomes:

(19)

$$\frac{[Tamm]_e}{[Tamm]_i} = \frac{([H+]_e + k)}{([H+]_i + k)} \times$$

$$\frac{(pNH_3/pNH_4+) - (F \cdot Em \cdot \gamma / R \cdot T(1-\gamma)) \cdot ([H+]_i/k)}{(pNH_3/pNH_4+) - (F \cdot Em / R \cdot T(1-\gamma)) \cdot ([H+]_e/k)}$$

where k is the NH_3/NH_4+ dissociation constant, pNH_3 is the permeability to NH_3 , pNH_4+ is the permeability to NH_4+ and:

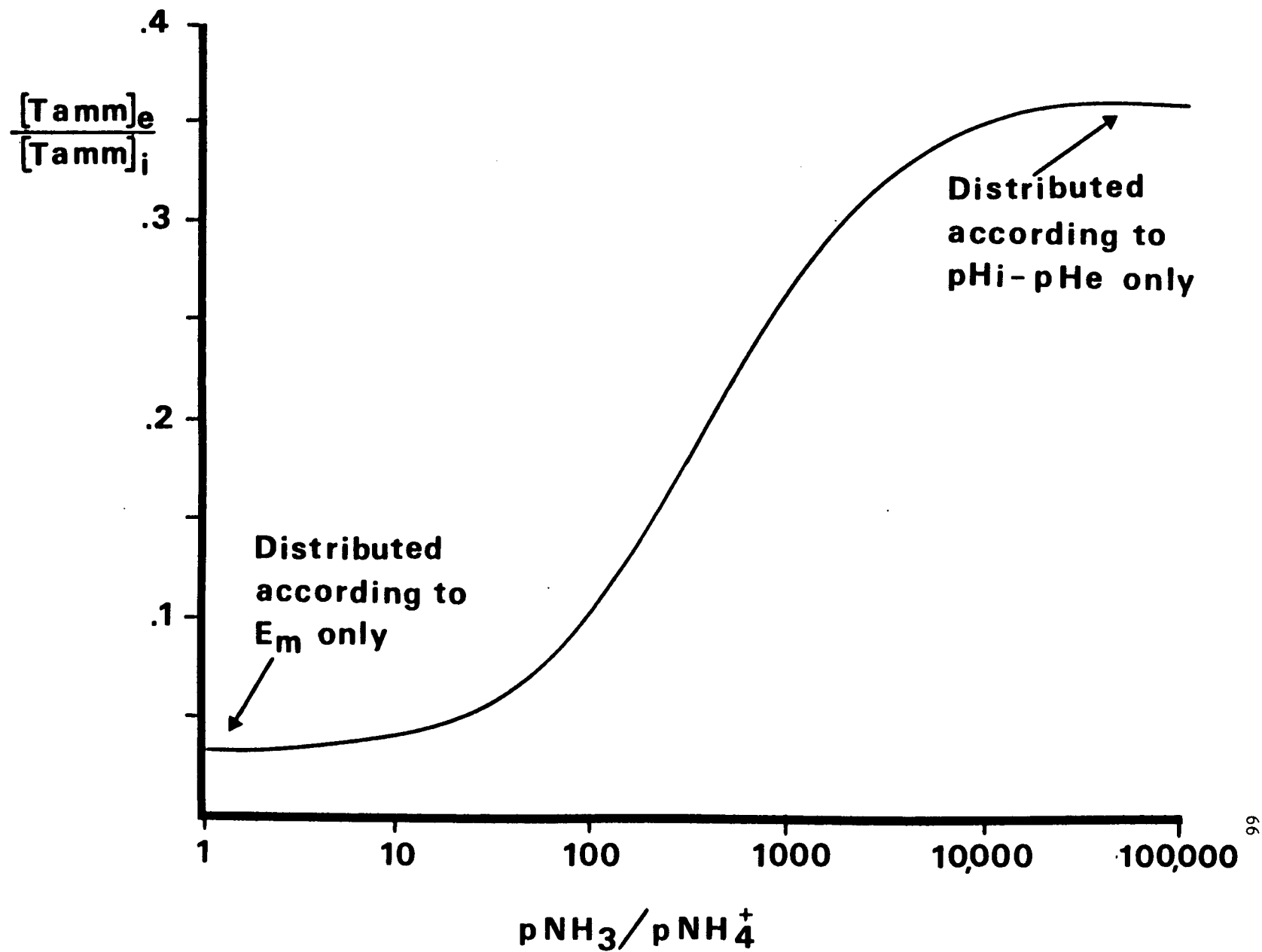
(20)

$$\gamma = \exp \cdot \{ (Em \cdot F) / (R \cdot T) \}$$

If Em and the pH_i - pH_e gradient are known, then

$[Tamm]_e/[Tamm]_i$ can be calculated as a function of the pNH_3/pNH_4+ ratio using these equations. Em in fish white muscle, as in most vertebrate muscle, is uniformly in the range of -80 to -85 mV (Hagiwara & Takahashi, 1967; Hidaka & Toida, 1969; Yamamoto, 1972). In Fig 6, $Em = -83$ mV has been employed along with the measured pH_e and pH_i values for resting white muscle (Table 7) to calculate $[Tamm]_e/[Tamm]_i$ as a function of muscle cell membrane permeability ratio (pNH_3/pNH_4+). The analysis shows that at $pNH_3/pNH_4+ > 10,000$, the distribution asymptotically approaches the theoretical maximum of ~ 0.36 , where it is entirely a function of the pH gradient, while at $pNH_3/pNH_4+ < 10$, the distribution asymptotically

Figure 6. The relationship between the distribution of ammonia at equilibrium between extracellular and intracellular compartments of resting sole white muscle ($[Tamm]_o/[Tamm]_i$) and the relative permeability (pNH_3/pNH_4^+) of the cell membrane to NH_3 and NH_4^+ . A membrane potential (E_m) of -83 mV and the measured pH_i and pH_e values of Table 7 have been assumed. See text for additional details.



approaches the theoretical minimum of ~ 0.03 , where it is entirely a function of the membrane potential (E_m). In between these limits there is an approximately a log/linear relationship between pNH_3/pNH_4^+ and equilibrium distribution ratio. The measured distribution ratio was 0.03 (Table 7), strongly suggesting that it was entirely a function of E_m , and therefore pNH_3/pNH_4^+ was low, i.e., permeability to NH_4^+ was relatively high. It is probably safe to assume that pNH_3/pNH_4^+ was at least below 25, where the ratio would be < 0.06 . Reversing the analysis, and using the measured distribution to predict E_m by the Nernst equation produced the answer $E_{NH_4^+} = -83 \pm 3$ mV (Table 7) in agreement with literature values for fish white muscle E_m .

Similar analyses have been performed with measured and predicted ammonia distributions in heart ventricle and brain, with the same conclusions, i.e., that pNH_3/pNH_4^+ is low, and the distribution is almost entirely a function of E_m . Calculated $E_{NH_4^+}$ for resting heart ventricle was -90 ± 3 mV (Table 8) in agreement with Jaeger's (1962) measured $E_m = -84$ mV in Leucisus rutilus ventricle, while calculated $E_{NH_4^+}$ for resting brain tissue was -69 ± 3 mV, in close accord with the generally accepted value of -70 mV for nervous tissue (Hille, 1984). The similarity between calculated $E_{NH_4^+}$ values for muscle, heart, and brain tissue and published E_m values imply that ammonia is passively distributed according to E_m in these tissues at rest, and that the permeability of tissue membranes to

NH₄⁺ ions is significant.

It is possible that NH₄⁺ permeability is not as great as is suggested by this analysis and that intracellular binding of ammonia accounts for the high intracellular Tamm concentrations. Wanders *et al.* (1980) presented evidence that a large fraction of intracellular ammonia was bound to mitochondrial protein in rat heart tissue, while smaller fractions of ammonia were associated with protein in skeletal muscle and brain. Thus, deproteinization of tissues during the extraction method would liberate this protein-bound ammonia, and would result in an overestimation of free ammonia in the cytosol. Mean cellular Tamm levels in sole heart, muscle, and brain have been calculated assuming that 1) the values given by Wanders *et al.* (1980) for bound ammonia in mitochondria of rat are valid for fish, 2) that mitochondrial volume is between 9 and 13% (Hoek *et al.*, 1980), 3) that the cytosol constitutes the major portion of the remaining volume, and 4) that Tamm levels in the cytosol are determined by extra- to intracellular pH gradients. Tamm levels calculated with these assumptions constitute only 8-13% of our measured values, which falls within ± 1 S.E.M.. Hence, even if some fraction of cellular ammonia binds to mitochondrial proteins in fish, it cannot account for the high Tamm levels measured in the present study.

Resting levels of ammonia in sole white muscle measured in this study, are between published values for cod, *Gadus morrhua*, (Fraser *et al.*, 1966) and carp, *Cyprinus carpio*, (Dreidzic &

Hochachka, 1976), but are about 4-8 times higher than resting levels in rainbow trout (Dobson & Hochachka, 1987; Mommsen & Hochachka, unpublished data). In a recent review, Randall & Wright (1987) analysed the distribution of ammonia between trout white muscle and plasma, at rest and following exhaustive exercise (data taken from Mommsen & Hochachka, unpub. and Milligan & Wood, 1986b). The analysis was based on the principle that ammonia was distributed across muscle cells relative to the H^+ distribution, assuming that muscle cells were essentially impermeable to NH_4^+ . From their calculations, ammonia was distributed according to the H^+ distribution in trout, at rest, but not following exercise, where NH_3 gradients existed between muscle and blood. It seems odd that the ammonia distribution in resting trout white muscle apparently follows the H^+ distribution, while in sole it does not. The problem with this analysis is that the trout muscle Tamm levels (Mommsen & Hochachka, unpub.) and pH_i values (Milligan & Wood, 1986b) were obtained from two separate studies (see Randall & Wright, 1987). When these measurements are performed on a single set of fish (C.M. Wood, personal communication) the trout muscle ammonia distribution parallels the findings of the present study on sole muscle tissue. At rest and following exercise in trout, Wood finds that ammonia is not distributed according to H^+ ion gradients and calculated $E_{NH_4^+}$ values (rest -81 ± 9 (N=8), exercise -86 ± 4 (N=7)) are close to those calculated for sole and similar to published muscle E_m values.

Following exercise, muscle ammonia levels increased by ~ 55 % compared to control. An increase in white muscle ammonia levels with exercise is consistent with other studies on fish (Suyama et al., 1960; Fraser et al., 1966; Driedzic, 1975, Driedzic & Hochachka, 1976; Dobson & Hochachka, 1987) and is due to deamination of the adenylate pool (Driedzic & Hochachka, 1976; Dobson & Hochachka, 1987). In mammalian muscle, it is recognized that ammonia production and glycolysis may be functionally coupled in several ways. Ammonia may enhance glycolysis by activating phosphofructokinase, H^+ production by glycolysis may in turn enhance ammonia production by activating AMP deaminase, and ammonia production (as NH_3) may be important in buffering the H^+ ions produced by glycolysis (Lowenstein, 1972; Mutch & Bannister, 1983; Dudley & Terjung, 1985). The quantitative importance of the latter has recently been discounted by the detailed study of Katz et al. (1986) on working human leg muscle, because ammonia production was less than 4% of lactate production. In the lemon sole, however, 30 min after exhaustive exercise, ammonia accumulation in white muscle (~ 3.3 mmol.l⁻¹, Table 7) was 20% of lactate accumulation (~ 16.3 mmol.l⁻¹, Table 3). Identical stoichiometry was reported by Dobson and Hochachka (1987) in trout white muscle after exhaustive exercise. Thus in ammoniotelic teleost fish, deamination/glycolysis coupling may be more important than in mammals, especially in limiting intracellular acidosis.

Ammonia levels also increased in heart (~55%) and brain

(~45%) tissue following strenuous exercise, although it is unlikely that ammonia production can account for this. Heart is predominantly an aerobic tissue, and therefore, unlike white muscle, the deamination of adenylates during exercise would be negligible. In nervous tissue, ammonia is known to be extremely toxic (see Randall & Wright, 1987) and therefore, high ammonia production rates are improbable. As well, it is doubtful that increased body ammonia levels after exercise are related to reduced ammonia excretion because in other teleosts, ammonia excretion either increases or remains unchanged after strenuous exercise (Holeton et al., 1983; Heisler, 1984; Milligan & Wood, 1986a; Milligan & Wood, 1987). I propose that elevated ammonia levels in heart and brain tissue after exercise are simply due to the passive uptake of ammonia from the increased plasma ammonia pool.

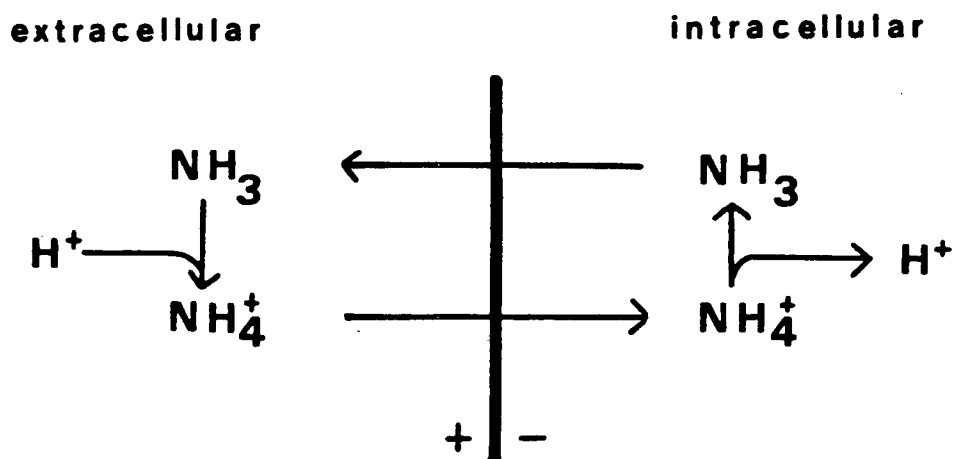
With hypercapnia there were no significant changes in muscle, heart, and brain ammonia stores, although mean levels decreased in all tissues, as well as in the plasma. A reduction in total body ammonia stores may have been due to a decrease in ammonia production and/or an increase in ammonia excretion rates. The decrease in water pH (7.8 to 6.7) caused by hypercapnia would certainly favour increased diffusive loss of NH_3 across the gills. Ammonia excretion rates have been reported to increase with hypercapnia in some teleosts (trout, Lloyd & Swift, 1976; Perry et al., 1987; carp, Claiborne & Heisler, 1984), and decrease in others (Conger conger, Toews et al., 1983; Opsanus

Figure 7. A model of ammonia (NH_3 and NH_4^+) movements

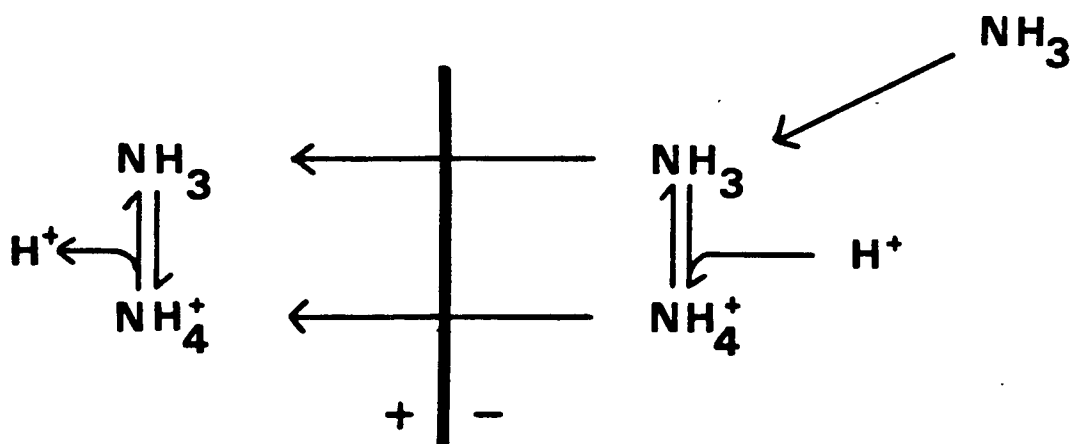
between the extracellular and intracellular compartments of a hypothetical tissue where NH_4^+ ions are passively distributed across the membrane according to the membrane potential. A. In the intracellular compartment where there is no ammonia production, the direction of the $\text{NH}_4^+ \rightleftharpoons \text{NH}_3$ reaction will always be towards the formation of NH_3 and H^+ because intracellular H^+ ions levels are lower than expected for a passive distribution. The net result being an increase in intracellular H^+ ion levels and PNH_3 gradients directed from cell to plasma. B. In the active state, there may be both P_{NH_3} and NH_4^+ gradients from cell to plasma. Intracellular NH_3 production will trap H^+ ions and ammonia leaving as NH_4^+ will act to export H^+ ions from the cell, while NH_3 diffusion out of the cell will not affect H^+ ion levels. See text for additional details.

A. Resting state

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B. Active state



beta, Evans, 1982). Based on these studies it is difficult to predict how ammonia excretion would be affected by hypercapnia in lemon sole. Metabolism may be suppressed by high P_{CO_2} levels in cell culture (Folbergrova et al., 1972) and in whole animals (Bickler, 1986). It is likely, therefore, that a component of decreased Tamm levels in tissues is due to suppressed metabolism during hypercapnia.

The analysis of the distribution of ammonia between extracellular and intracellular compartments strongly suggests that muscle, heart, and brain cell membranes are permeable to NH_4^+ . It is assumed that NH_3 permeability is also significant based on the fact that $NH_3:NH_4^+$ permeability ratios for other tissues are at least 5:1 (Castell & Moore, 1971; Bown et al., 1975; Kleiner, 1981; Schwatz & Tripolone, 1983; Arruda et al., 1984; Cameron & Evans, 1986). Despite the relatively high permeability of NH_3 , P_{NH_3} gradients (0.0002–0.001 Torr) do exist between intracellular and extracellular compartments (Fig. 5). Figure 7 describes a model of NH_3 and NH_4^+ movements across a hypothetical tissue where NH_4^+ ions are passively distributed according to E_m , intracellular NH_3 and NH_4^+ are in equilibrium (interconversion reaction rate for $NH_3:NH_4^+$ < 50 msec, Stumm & Morgan, 1981), and ammonia production is insignificant (Fig. 7A). NH_3 gradients will form in the intracellular compartment as NH_4^+ ions enter the tissue and dissociate to form NH_3 and H^+ ions. The direction of this reaction will always be towards the formation of NH_3 and H^+

because intracellular H^+ ions levels are lower (i.e., pH_i higher) than expected for a passive distribution (Roos & Boron, 1981). The rate of NH_3 'back diffusion' out of the tissue will depend on the magnitude of the P_{NH_3} gradient and the NH_3 permeability (p_{NH_3}). As NH_3 diffuses into the extracellular compartment, NH_4^+ flux into the intracellular compartment will be driven by the transmembrane voltage. The result of this cycle will be a net transfer of H^+ ions to the intracellular compartment. The net effect would be an acidification of the intracellular compartment and alkalization of the extracellular compartment, were there not active H^+ extrusion mechanisms which keep $[H^+]_i$ low and well out of electrochemical equilibrium under steady state conditions.

With ammonia production by muscle during exercise, it seems likely that P_{NH_3} and NH_4^+ electrochemical gradients would form between muscle and plasma (Fig. 7B). If ammonia is produced as NH_3 , then intracellular ammonia will raise pH_i by trapping H^+ ions (from glycolysis) with an almost 1:1 stoichiometry. Ammonia may diffuse out of the cell as either NH_3 or NH_4^+ . Ammonia leaving as NH_3 will not affect the H^+ ion budget of the intracellular compartment. Alternatively, ammonia leaving as NH_4^+ would act as a mechanism to export H^+ ions, which will also help to regulate intracellular pH.

In summary, the results of this study show that the distribution of ammonia between plasma and muscle, heart, and brain tissue in lemon sole is not dependent on the H^+

distribution, which indicates that the cell membranes of these tissues have a significant permeability to NH_4^+ ions. The ammonia distribution appears to follow the membrane potential at rest; calculated $E_{\text{NH}_4^+}$ values generally agree with published values for E_m in these tissues. During hypercapnia and following exercise NH_4^+ as well as NH_3 gradients may exist between tissue and plasma, particularly in tissues where ammonia production is stimulated, such as, white muscle after exhaustive exercise.

CHAPTER 2

Downstream pH changes in water flowing over the gills of.

Salmo gairdneri

INTRODUCTION

The apparent mean pH gradient across the gills of fish is usually determined as the difference between bulk water pH and blood pH. The precise gradient, however, is dependent upon possible pH gradients within the interlamellar space. Water flow is laminar through the mouth and over the gills (Randall & Daxboeck, 1984). Boundary layers are undoubtedly present next to the mucous layer covering the gill surface since water flow through the gill channels in relation to their dimensions results in Reynolds numbers that have been reported by Hughes (1984) to be very small (<10). It has been suggested (Lloyd & Herbert, 1960; Szumski et al., 1982) that the pH of water within the gill chamber is significantly more acidic than that of bulk water due to CO_2 excretion and subsequent hydration to HCO_3^- and H^+ . If this theory is correct it would have important implications to ammonia excretion, which is pH-dependent (Wright and Wood, 1985). For hydration of excreted CO_2 to have such an appreciable effect on interlamellar pH, CO_2 hydration must occur rapidly enough to alter the proton activity within the interlamellar transit time of gill water (100 to 400 msec, Randall, 1982a,b). This is much faster than the uncatalysed rate of CO_2 hydration, which is on the order of minutes (Kern, 1960) at typical fish water pH and temperatures. Carbonic anhydrase, the enzyme responsible for catalyzing CO_2 hydration and dehydration reactions, has been located within gill epithelial

cells of fish (Lacy, 1983; Dimberg et al, 1981). Thus, it seems possible that the apical surface of epithelial cells and/or branchial mucus could contain carbonic anhydrase.

The aim of this study was to investigate interlamellar water pH of trout (Salmo gairdneri) by following changes in the pH of expired water (pH_E) in order to determine the contribution of carbonic anhydrase activity to the hydration of CO_2 eliminated at the branchial epithelium. Downstream changes in pH_E were followed using a stopped-flow apparatus. Effects of carbonic anhydrase and acetazolamide, a specific inhibitor of carbonic anhydrase, on downstream pH_E changes were also studied. In addition, mucus excreted by fish was assayed for carbonic anhydrase activity by the pH-stat technique (Henry & Cameron, 1982).

MATERIALS AND METHODS

Experimental animals

The experiments were divided into two series. In the first experiment (Series I), fish were allowed at least 5 days to acclimate to a test solution of $40 \text{ mmol.l}^{-1} \text{ NaCl}$ and $0.5 \text{ mmol.l}^{-1} \text{ CaCl}_2$ in dechlorinated tapwater ($11.7^\circ \text{C} \pm 0.2$) with a B value of $81 \text{ uequiv.l}^{-1}.\text{pH}^{-1}$ unit, prior to surgery and experimentation. Fish were starved during this acclimation period and throughout the experiment. This period of time is adequate to allow rainbow trout to re-establish ionic, osmotic and respiratory steady-state conditions after transfer from freshwater to a balanced salt solution (Perry & Heming, 1981). The second type of experiment (Series II) was an in vitro experiment where mucus samples were collected from fish held in dechlorinated tapwater.

Measurements

In order to measure pH of expired water just leaving the opercular cavity of fish in Series I, an opercular cannula (PE 90) was stitched in position under and midway along the opercular opening (see General Materials and Methods). Fish were then fitted with rubber dams (see General Materials and Methods) and placed in a two-chambered Perspex box (Fig. 2), where they were left to recover for 48 hrs following surgery.

In Series I, water pH was measured at two locations; inspired

water pH was measured continuously with a combination glass pH electrode (Radiometer GK2401C) placed in the front chamber of the fish box, and expired water was drawn from the opercular cavity through the opercular cannula and into a glass 'stopped-flow' chamber (volume, 1 ml), housing a pH electrode (Canlab pH semi-microelectrode, H5503-21) and micro teflon stirring bar. The stopped-flow chamber was designed to measure pH changes in the expired water. The opercular cannula fed into the inlet port (inside diameter 1.27 mm) of the stopped-flow chamber and another cannula (PE 160) inserted into the outlet port (inside diameter 1.70 mm) carried the water out of the chamber. The pH electrode fit tightly into a teflon sleeve which then fit into the glass chamber so as to form a seal to ensure the chamber was gas tight.

The pH electrodes were calibrated with standard Radiometer buffer solutions at pH 7 and 4. Vancouver tapwater has a low ionic strength ($I \sim 0.002 \text{ M}$), relative to other freshwaters, and therefore the addition of dissolved salts to the test water greatly increased the stability and decreased the lag time of the pH electrode response. The error associated with ionic strength differences between the commercial buffers and the test solution was not corrected for and is estimated to be approximately 0.05 to 0.25 pH units depending on pH. Although this introduces an error to the absolute pH values, the relative changes in pH were of greater importance to this study. The lag time of the Canlab microelectrode was determined for a step change in water pH at the same ionic strength and temperature as the experimental

solution.

Fish were exposed to three different water regimes: test solution (control water), acetazolamide (1.6 mmol.l^{-1}) in test solution, and carbonic anhydrase (6.8 mg.l^{-1} or 20,400 Wilbur-Anderson units. l^{-1} bovine carbonic anhydrase) in test solution. The buffer values of these solutions were 81, 179, 78 $\mu\text{equiv.l}^{-1}.\text{pH}^{-1}$ unit, respectively. Two complete sets of measurements were obtained while fish were in control water. The inflow was then changed over to either the acetazolamide or carbonic anhydrase test solution (fish were exposed to one or the other, but not both) and the same duplicate measurements were repeated after a 30 minute period.

Inspired and expired water pH were recorded for an hour preceding the experiment to ensure that the water pH was stable over time. Inspired water pH (pH_I) and expired continuous flow pH (pH_E) were measured simultaneously and recorded immediately before the flow of water through the stopped-flow chamber was stopped. The rate of water flow through the chamber ($8\text{--}10 \text{ ml.min}^{-1}$) was such that water transit time through the opercular cannula to the pH electrode was less than two seconds. Once the flow of water was stopped, expired water pH was recorded over an 8 minute period with a Hewlett Packard data acquisition unit and a strip chart recorder. If a chemical disequilibrium existed in the water, then a change in pH with time (dpH/dt) was recorded. The final equilibrium pH after 8 minutes was referred to as the expired stopped-flow pH (pH_{St}).

After the response time of the electrode was accounted for, the half time ($t_{1/2}$) of the equilibrium reaction was calculated.

To ensure that inspired water was in complete pH equilibrium, water was vigorously bubbled with compressed air in a 100 l tank before it flowed into the experimental apparatus. The pH equilibrium of inspired water was periodically checked in the stopped flow chamber as described above.

Immediately before the flow of water was stopped in the chamber, ventilation (\dot{V}_w) and carbon dioxide content of the inspired (C_{ICO_2}) and expired (C_{ECO_2}) water were measured. \dot{V}_w was determined by collecting the outflow water from the standpipe in the back chamber of the experimental chamber. Collections were made over 1 minute periods and volumes determined by weight. Measurements of water C_{ICO_2} and C_{ECO_2} were made by gas chromatography (see General Materials and Methods). The rate of carbon dioxide excretion (\dot{M}_{CO_2}) was calculated by application of the Fick equation.

In Series II, mucus samples taken from the body of trout were assayed for carbonic anhydrase (CA). Initially we tried to obtain mucus samples from the gill surface; it was impossible, however, to collect samples that were large enough for the assay technique. The pH stat technique was chosen to measure CA activity over other methods (modified boat and esterase assay) because previous work had shown that the pH stat method exhibited the lowest limit of detection and the highest degree of sensitivity (Heming, 1984; see also Henry & Cameron, 1982). A

mixture of phosphate buffer and a bicarbonate solution initiates the conversion of HCO_3^- and H^+ ions to CO_2 gas. As CO_2 is liberated from the reaction solution, the pH will increase and the volume of acid (HCl) that is required to maintain the reaction mixture at a given pH will be proportional to the production of CO_2 , since the stoichiometry of $\text{H}^+:\text{CO}_2$ is 1:1. The reaction rate, moles CO_2/min , will be greater in the presence of the catalyst, CA, relative to the uncatalysed reaction.

Mucus samples (~0.5 ml) were collected from the body of 6 fish by lightly stroking the fish with a metal spatula, and these samples were then assayed for CA. The addition of mucus to the reaction vessel caused foaming, a problem which was eliminated with a defoaming agent (50 μl octan-2-ol). The dehydration reaction rate was not affected by the addition of octan-2-ol. The catalysed (mucus) reaction rate was compared to the uncatalysed rate, with or without acetazolamide. Anhydrous acetazolamide (5 mmol.l^{-1}) was added directly to the phosphate buffer solution. To test for the presence of cellular material, mucus samples were stained with eosin and crystal violet and examined under a microscope.

Determination of half-time values

Half-time values ($t_{1/2}$) were calculated by taking several points along individual kinetic curves (dpH/dt) and converting

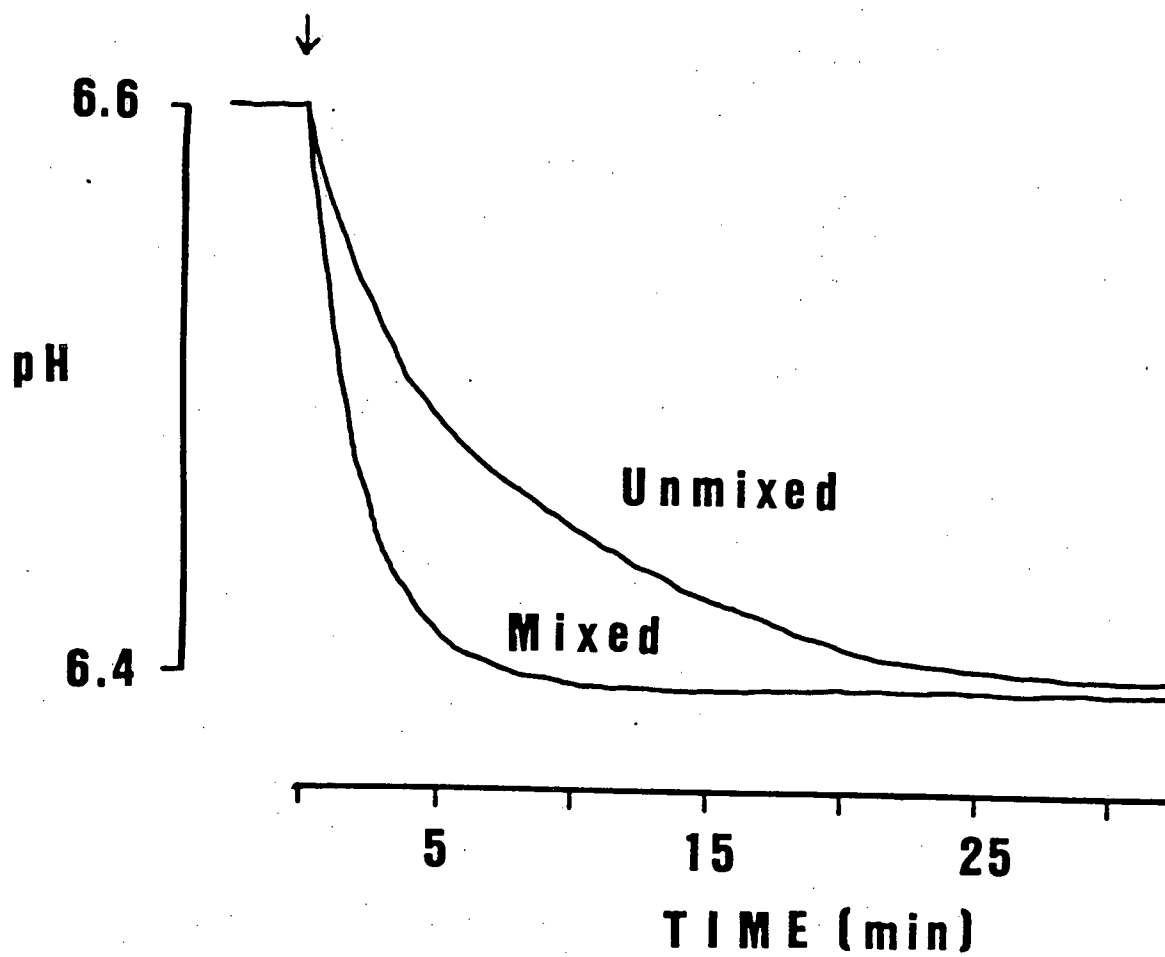
these values to percentage change in pH (assuming that the pH_{st} value at 8 minutes was the equilibrium pH or very close to it). These points were then plotted on semilog paper as log % change in pH versus time giving straight lines over the first 60% of the reaction. The $t_{1/2}$ values were determined graphically.

Data are presented as means ± 1 S.E.M. (N). The Student's two-tailed paired t-test was used to evaluate the significance of differences between mean values ($P < 0.05$).

RESULTS

An important factor in the lag time of the pH electrode was the degree of mixing in the stopped-flow chamber. In order to test the effects of mixing under similar conditions to those in the experiment, a small volume of pure CO_2 gas was introduced into the test solution before it flowed into the stopped-flow chamber. The flow of water past the electrode was then stopped, and the CO_2 hydration reaction was then followed over time, with and without a stirring bar in the chamber. Fig 8 depicts the change in pH with time for the mixed ($t_{1/2} = 76$ secs) and the unmixed ($t_{1/2} = 300$ secs) solutions. The difference of 224 seconds was presumably due to slow diffusion of H^+ ions through the unmixed solution. To reduce the effects of mixing on the lag time of the pH electrode, a stirring bar was placed in the stopped-flow chamber and a magnetic stirrer maintained a constant rotation of the stirring bar. This test also demonstrated that

Figure 8. Water flow in the stopped-flow chamber was stopped (arrow) and the kinetics of the CO_2 hydration reaction was recorded. In one case, a teflon-coated, magnetic stirring bar continuously mixed the solution (mixed), while in the other solution, no stirring bar was present (unmixed).

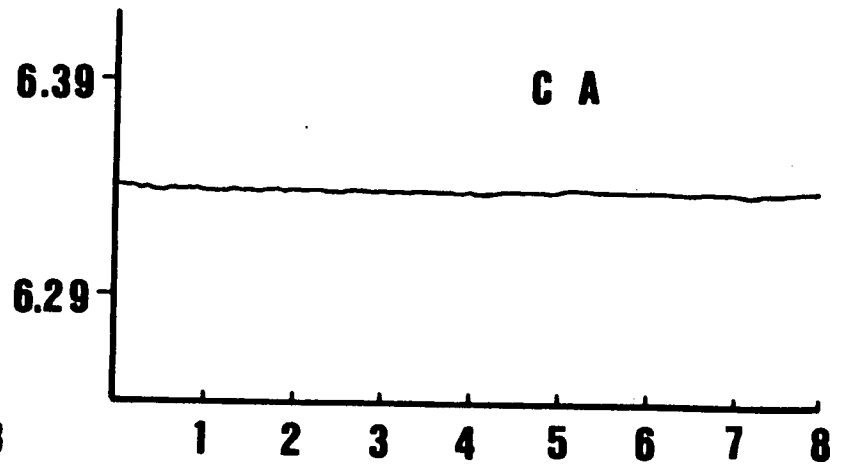
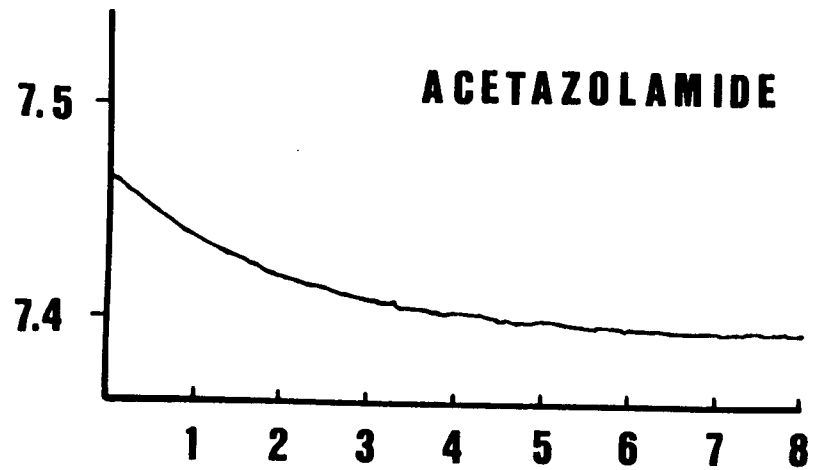
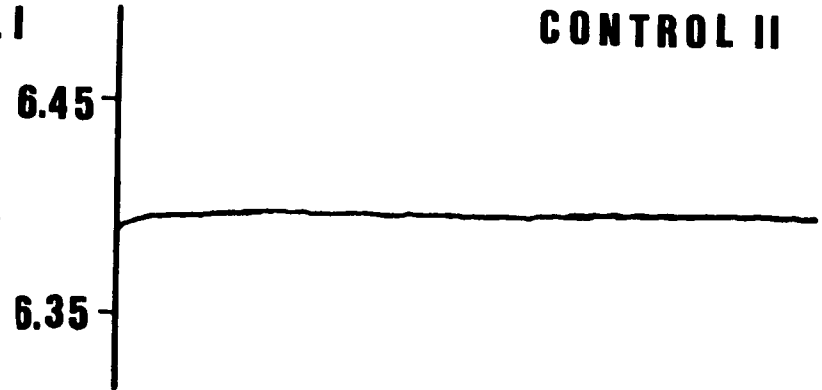
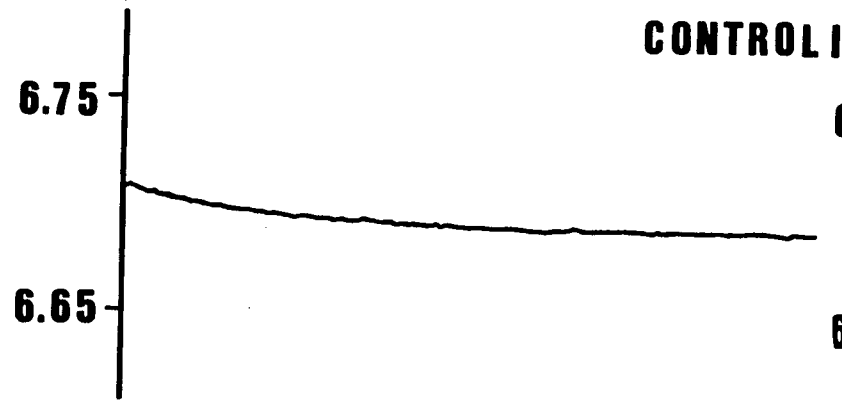


the system detects pH differences due to CO₂ hydration, and the $t_{1/2}$ value for the mixed reaction is in approximate agreement with values given by Kern (1960) at the test pH and temperature.

The results from Series I are presented in Table 10 and 11. Figure 9 shows representative stopped-flow traces from two fish. As water flowed over the gills of control fish, there was a significant decrease from inspired water (pH_E) to expired water at equilibrium (pH_{St}) (Table 10, 11). Expired control water was almost completely equilibrated by the time it reached the pH electrode, because pH_E was not significantly different from pH_{St} in both the first and second control sets. Even though the overall expired water pH disequilibrium in control water was small, in 11 out of 30 stopped-flow pH traces there was a decrease in pH once water flow was stopped in the chamber, in a few cases pH increased after the flow had been stopped in the chamber (2/30 stopped-flow pH traces), and in the remainder of traces there was no measureable change in water pH (16/30 traces). The addition of acetazolamide significantly increased the CO₂ disequilibrium relative to control water (Fig 9, Table 10). In contrast, carbonic anhydrase eliminated the CO₂ disequilibrium except in one stopped-flow pH trace where a small pH change ($pH_E - pH_{St} = 0.01$) was observed (Fig. 9, Table 11).

There was no significant change in ventilation and carbon dioxide excretion rates between control I and acetazolamide measurements and the control II and carbonic anhydrase values

Figure 9. Representative changes in expired water pH of two fish (control I and acetazolamide; control II and carbonic anhydrase) after water flow past the pH electrode was stopped at time 0.



TIME (min)

TABLE 10. Water pH values (inspired water, pH_i ; expired water immediately leaving the opercular valves pH_e ; equilibrated expired water, pH_{eT} , values given as mean \pm SEM) and half-time values for $\text{CO}_2\text{:HCO}_3^-$ interconversions measured on rainbow trout in CONTROL water and after ACETAZOLAMIDE (1.6 mmol.l^{-1}) was added to the water.

CONTROL				ACETAZOLAMIDE
.....				
TEMPERATURE (°C)	10.4 ± 0.1			10.7 ± 0.2
DIRECTION OF	↓ (7)	↑ (2)	no Δ (5)	(12)
pH CHANGE ¹ (N)				
pH _i	7.41±.01	7.39±.00	7.41±.02	7.42±.02
pH _e	6.84±.06	6.70±.05	6.60±.01	7.38±.02*
pH _{BT}	6.82±.06	6.72±.05	6.60±.01	7.31±.02*
pH _e -pH _{BT}	0.03±.00	0.02±.01	0.00±.00	0.07±.01*
t _{1/2} (sec)	90 + 6	66 + 4	-	88 + 3
.....				

1. 'DIRECTION OF THE pH CHANGE' refers to whether pH increased or decreased once the flow of water was stopped in the stopped-flow chamber.

* significantly different from pH CONTROL, $p \leq 0.05$

TABLE 11. Water pH values (inspired water, pH_i ; expired water immediately leaving the opercular valve, pH_e ; equilibrated expired water, pH_{eT} , values given as mean \pm SEM) and half-time values for CO_2 : HCO_3^- interconversions, measured on trout in CONTROL water and after CARBONIC ANHYDRASE (6.8 mg l^{-1}) was added to the water.

	CONTROL		CARBONIC ANHYDRASE
.....			
TEMPERATURE ($^{\circ}\text{C}$)	12.7 ± 0.1		13.1 ± 0.1
DIRECTION OF pH CHANGE ¹ (N)	\downarrow (4)	no Δ (11)	no (12)
pH_i	$7.35 \pm .10$	$7.13 \pm .03$	$7.19 \pm .06$
pH_e	$6.44 \pm .05$	$6.35 \pm .03$	$6.39 \pm .03$
pH_{eT}	$6.43 \pm .04$	$6.35 \pm .03$	$6.39 \pm .03$
$\text{pH}_e - \text{pH}_{eT}$	$0.02 \pm .01$	$0.00 \pm .00$	$0.00 \pm .00$
$t_{1/2}$ (sec)	48 ± 13	-	-
.....			

1. see TABLE 1

Experimental values were not statistically significant from control values using the Student's paired t-test.

TABLE 12. Ventilation (\dot{V}_w) and carbon dioxide excretion (\dot{M}_{CO_2}) rates from experiment one (CONTROL I and ACETAZOLAMIDE) and experiment two (CONTROL II and CARBONIC ANHYDRASE)

	TEMP. (°C)	\dot{V}_w (l.h ⁻¹)	\dot{M}_{CO_2} ($\mu\text{mol} \cdot 100\text{g}^{-1} \cdot \text{h}^{-1}$)
.....			
CONTROL I	10.4 \pm 0.1	6.45 \pm 0.37	239.39 \pm 27.28
ACETAZOLAMIDE	10.7 \pm 0.2	6.18 \pm 0.18	261.56 \pm 28.26
.....			
CONTROL II	12.7 \pm 0.1	9.95 \pm 0.97	380.83 \pm 38.96
CARBONIC ANHYDRASE	13.1 \pm 0.1	10.21 \pm 0.77	393.11 \pm 42.02
.....			

Experimental values (acetazolamide, carbonic anhydrase) were not statistically significant from the respective control values using the Student's paired t-test.

TABLE 13. CO₂ Dehydration reaction rate in cataylsed (mucus samples) and uncatalysed solutions, with or without caetazolamide at 22° C. Mucus samples taken from the body of six fish. Means \pm S.E.M. (N= 6)

	CONTROL (CO ₂ umol.min ⁻¹)	ACETAZOLAMIDE (5mM) (CO ₂ umol.min ⁻¹)
.....		
	*	#
CATALYSED (MUCUS)	48.0 \pm 7.7	20.7 \pm 0.8
		#
UNCATALYSED	26.0 \pm 1.6	21.4 \pm 0.7
.....		

* significantly different from control

significantly different from uncatalysed control

(Table 12). It is interesting to note, however, that fish in the first experiment (April-May, 10.5° C) had considerably lower \dot{V}_w and \dot{M}_{CO_2} rates compared to fish in the second experiment (Sept.-Oct., 13.0° C), and these differences may be related to seasonal variations.

Mucus excreted by the fish contained carbonic anhydrase activity. The addition of mucus doubled the CO₂ dehydration reaction rate relative to the uncatalysed rate and acetazolamide reduced the reaction rate to the uncatalysed rate (Table 13). There was a small difference in the reaction rate between control and acetazolamide uncatalysed reactions. In addition mucus samples were found to contain cellular material.

DISCUSSION

These experiments present the first documented measurement of the pH change in water flowing over the gills of freshwater fish. Holeton and Randall (1967) reported a rough estimate of the inspired to expired water pH difference of about 0.2 to 0.5 pH units, in similar Vancouver tapwater. The actual difference measured in this study, however, was 0.7-0.9 pH units. In both cases, these differences were measured at point sources from the opercular outflow, but preliminary tests in this study, to determine if water pH varied with the position of the cannula, showed that there was no significant differences between the pH measured in three locations along the opercular valve. Changes in

water sampled from a single point, therefore, are representative of changes in expired water in general. Differences in the magnitude of the pH change reported in our study and that of Holeyton and Randall (1967) are probably related to several factors, including differences in temperature, the presence or absence of latex masks, fluctuations in calcium carbonate and buffering capacity of the water, and differences in the amount of carbon dioxide eliminated by the fish.

Evidence for the presence of carbonic anhydrase in the mucus is supported by the fact that expired water was almost completely equilibrated as it left the opercular cavity. Water is only in contact with the gill for about 100 to 400 msec (Randall, 1982a, 1982b) and the time required for complete CO₂ hydration is on the order of minutes (Kern, 1960), therefore, the CO₂ reaction must be catalysed at the gill surface. Acetazolamide inhibits CA and increased the expired water CO₂ disequilibrium, whereas, the addition of CA to the water completely eliminated the small disequilibrium that was observed in the expired control water of some fish. The cause of this small disequilibrium downstream from the gills in control water will be discussed later, but is probably not related to any nonequilibrium state at the gill surface.

Carbonic anhydrase activity is present in mucus covering the epidermis of rainbow trout. Although it was not feasible to measure CA activity in mucus covering the gill surface, it is

probable that CA activity is present in the branchial mucus excreted by gill epithelial cells. Fletcher et al (1976) compared epidermal (body) and gill filament mucus-secreting goblet cells of rainbow trout and found that cell density and glycoproteins composition (predominantly acid glycoproteins) were very similar. Branchial epithelium contains high levels of carbonic anhydrase (Haswell et al, 1980), which appears to be concentrated in the apical regions of the epithelial cells (Dimberg et al., 1981). Also, CA has been located in the cytoplasm of chloride cells, vesicular cells, supportive cells, in the cytoplasm and mucous granules of goblet cells, and in the extracellular spaces between vesicular and supportive cells of the opercular membrane of the teleost, Fundulus heteroclitus (Lacy, 1983). It is reasonable to assume, therefore, that carbonic anhydrase activity is also present in the mucus covering the gill filaments of rainbow trout. Mucus samples were found to contain cellular material, which is not surprising considering that the epidermal cells are regularly sloughed off to allow for new growth. The presence of cells does not invalidate the experimental findings. Enzyme activity in the mucus may originate from CA expelled from mucous cells into the mucus covering the gills and body of the fish, or from epithelial cell fragments which have been sloughed off into the mucous layer.

The elimination rate of CO₂ far exceeds that of other branchially excreted molecules, and it is well established that CO₂ diffusion is facilitated in the presence of CA (Longmuir et

al., 1966; Enns, 1967; Zborowska-Sluis et al., 1974; Gutknecht et al., 1977; Burnett, 1984). Carbon dioxide is primarily excreted in the gaseous form (Perry et al., 1982), with approximately 10% of total excretion as HCO_3^- exchanging for Cl^- (Cameron, 1976). Gutknecht et al (1977) used artificial lipid bilayer membranes to study properties of CO_2 flux and found that at pH 7-8, CA (1-1.6 mg/ml) caused a 10- to 80-fold stimulation of CO_2 flux. They suggested that the presence of CA in the unstirred layer renders the CO_2 hydration-dehydration reaction so fast that chemical equilibrium between CO_2 and HCO_3^- exists throughout the unstirred layer. Furthermore, the rate-limiting step in CO_2 transport in a situation where HCO_3^- cannot readily cross the membrane and the solutions are poorly buffered is the diffusion of HCO_3^- and H^+ through the unstirred layer.

It is interesting that a small disequilibrium was observed in expired control water, even though we postulate complete equilibration of the $\text{CO}_2/\text{HCO}_3^-$ reaction at the gill surface. The fact that CA eliminated the pH disequilibrium observed downstream of the gills indicates that it was, in fact, due to a $\text{CO}_2/\text{HCO}_3^-$ disequilibrium in the water. CO_2 may be in complete equilibrium with HCO_3^- and H^+ in the mucus and associated unstirred layer next to the gill, but the differential diffusion rates of these molecules across the unstirred layer may create a small disequilibrium in the mainstream of water flowing over the gills. In addition, mixing of waters of different carbon dioxide content may result in

$\text{CO}_2/\text{HCO}_3^-$ disequilibrium downstream of the gills, indeed this may account for the two traces collected from one fish which showed a reverse reaction, that is, the expired water pH increased slightly when the flow of water was stopped in the chamber. It may be that respiratory water at equilibrium was mixed with nonrespiratory 'shunt' water just after it entered the opercular cavity. This would result in a small increase in pH as the shunt water would contain a lower H^+ activity compared to the respiratory water. In one trace, slow pH changes were observed in the presence of CA, presumably due to the uncatalysed CO_2 hydration reaction. The M_{CO_2} rate was extremely high in this fish and therefore the level of CA in relation to the concentration of CO_2 was probably inadequate to completely catalyse the reaction.

Variations in $t_{1/2}$ values for a given reaction are the result of differences in temperature, ionic strength, and buffering capacity. Ionic strength influences the ionic mobility of the solution, as well as effecting the stability of the pH electrode. The $t_{1/2}$ values for the control experiments ranged from 48 to 90 secs. These variations in $t_{1/2}$ may be due to ionic strength differences related to small changes in the ionic compositions of the Vancouver tapwater during different times of the year. These seasonal variations in water composition are also reflected in the slightly different pH_{in} values between the two control experiments. Increases in the buffering capacity of a solution results in longer $t_{1/2}$ values for the uncatalysed

CO₂ hydration reaction (Gray, 1971). Even though acetazolamide increased the B value in this study, the mean $t_{1/2}$ value was not altered, which may be a reflection of the complexity of pH changes in water downstream of the gills.

The final equilibrium pH for acetazolamide-treated fish was significantly higher than the final control pH. The addition of acetazolamide altered the ionic strength of the test solution slightly. The error in the absolute pH value associated with ionic strength differences was estimated to be no more than 0.01 pH units, and this can not account for the relatively high expired water pH value. The three parameters that would effect the expired water pH value are \dot{M}_{CO_2} , \dot{V}_w , and the β value. There were no changes in either \dot{M}_{CO_2} or \dot{V}_w and the results can be explained only in terms of changes in the β value with the addition of acetazolamide, a weak acid. Acetazolamide increased the β value two-fold compared to that of control water.

Heming (1985) performed similar experiments to this study on rainbow trout acclimated to a saline test solution (in mmol.l⁻¹: NaCl, 40; KCl, 1.6; CaCl₂, 0.47; MgSO₄, 0.62; NaHCO₃, 5.5; NaH₂PO₄, 0.95)with different results and conclusions. He found expired pH was almost identical to inspired pH and water downstream from the gill became progressively more acidic at the uncatalysed rate of CO₂ hydration, and concluded that CA was not present on the gill surface. An explanation for the discrepancy between the two studies may be related to the fact that the fluid in Heming's stopped-flow chamber was static

after water flow stopped, whereas in the present study the fluid was continually mixed via a stir bar. We have found that the dpH/dt in static fluids has much larger $t_{1/2}$ than when mixed and this accounts for the slow responses observed.

Carbon dioxide is not the only molecule eliminated at the gill which will influence water pH. Fish excrete NH_3 , NH_4^+ , H^+ , and HCO_3^- in addition to CO_2 across their gills. All of these molecules will affect the water pH and the magnitude of the effect will depend on the relative rates of excretion, the rates of chemical equilibrium, the water-buffering capacity, and the rate of water flow past the gills. Protonation of NH_3 is extremely rapid, so that NH_3 excretion will result in the immediate elevation of water pH at the gill surface. Alternatively, NH_4^+ excretion will have a negligible effect on water pH. The efflux of H^+ (or OH^- influx) will alter the pH of water instantaneously, but the gradient for H^+ ions is generally very small (H^+ gradient from blood (pH 8) to expired water (pH 6) = 9.9×10^{-7} equiv. $\cdot\text{l}^{-1}$) thus, net excretion of H^+ , in exchange for Na^+ or NH_4^+ , will be low under most conditions, even if the gills are very permeable to protons (McWilliams & Potts, 1978).

It is clear that interlamellar water pH is significantly more acidic than that of bulk water in the gills of rainbow trout. Water acidification is due to the catalysed conversion of excreted CO_2 to HCO_3^- and H^+ at the gill surface. Several authors (Lloyd & Herbert, 1960; Szumski *et al.*, 1982) have

modelled the observed effects of bulk water pH on the aquatic toxicity of ammonia in terms of a large CO₂-induced acidification of interlamellar water. Such models are valid under the water conditions tested in this study, however, water acidification at the gill may not occur in all situations. For example, in very well buffered lakes and rivers CO₂ excretion may have very little effect on interlamellar water pH. Furthermore, the degree of acidification at the gill surface may vary greatly in fish depending on the environmental water pH, temperature, the relative branchial molecular excretion rates and the rate of water flow past the gills. All these factors must be accounted for before one can assess the effect of CO₂ excretion by the fish on interlamellar water pH in a given body of water.

CHAPTER 3

Effects of inhibition of external gill carbonic anhydrase
on carbon dioxide and ammonia excretion in intact

Salmo gairdneri

INTRODUCTION

The ubiquitous enzyme carbonic anhydrase (CA) catalyses the interconversion of bicarbonate and carbon dioxide in animal tissues (Maren, 1967; Lindskog et al., 1971; Carter, 1972; Bauer et al., 1980). Intracellular localization of CA in fish gills is well known (Haswell et al., 1980; Dimberg et al., 1981; Lacy, 1983; Swenson & Maren, 1987), but recent advances suggest that CA is also present on the external surface of the gill. Wright et al. (1986) concluded that mucus adjacent to the gill epithelial surface contained CA activity because 1) water downstream from the gill was more acidic than inspired water but was in pH equilibrium and 2) epidermal mucus, which is chemically similar to gill mucus, contained CA activity (see Chapter 2, Discussion). Rohim, Delaunoy, & Laurent (1987) have demonstrated by immunoelectronmicroscopic techniques that CA molecules are bound to the apical epithelial membrane of fish gills.

There are several possible physiological roles for external gill CA in the mucous layer or bound to the apical epithelial membrane. In all likelihood, external CA facilitates CO₂ excretion from blood to the environment. It is well documented that CA facilitates CO₂ diffusion across a variety of tissues, including muscle tissue (Zborowska-Sluis et al., 1974; Kawashiro & Scheid, 1976), lung tissue (Klocke, 1980; Effros et al., 1980; Enns & Hill, 1983), elasmobranch rectal gland (Swenson & Maren,

1984), and crab gill epithelium (McMahon et al., 1984; Burnett & McMahon, 1985; Henry, 1987).

Another possible role of external CA is to maintain an acid boundary layer next to the gill surface to facilitate ammonia excretion. In freshwater fish, ammonia is primarily excreted across the gills by simple diffusion of non-ionic NH_3 and via the $\text{Na}^+/\text{NH}_4^+$ ion exchange mechanism (Maetz & Garcia Romeu, 1964; Maetz, 1973; Kirschner et al., 1973; Evans, 1977; Payan, 1978; Cameron & Heisler, 1983; Wright & Wood, 1985). Sustained NH_3 diffusion is dependent upon NH_3 removal from the boundary layer because the accumulation of excreted NH_3 in the boundary layer will reduce the blood-to-water NH_3 partial pressure (P_{NH_3}) diffusion gradient. NH_3 can be removed from the boundary layer physically by diffusion into the water flowing past the gills and/or removed chemically by combining with a H^+ ion to form NH_4^+ in the boundary layer. The maintenance of P_{NH_3} gradients across the gill therefore, will depend, in part, upon the availability of H^+ ions in the boundary layer to facilitate interconversion of NH_3 to NH_4^+ . The role of external gill CA, therefore, is to catalyse the conversion of excreted CO_2 to HCO_3^- and H^+ ions in the boundary layer to supply H^+ ions for the $\text{NH}_3 \rightleftharpoons \text{NH}_4^+$ reaction.

For external gill CA to affect branchial carbon dioxide and ammonia excretion the transfer of carbon dioxide and ammonia must be diffusion limited. In other words, no matter how quickly carbon dioxide or ammonia are delivered to the gills, transfer is

restricted at one or more points along the diffusion pathway, that is, from blood-to-gill epithelium-to-water. On the other hand, if CO_2 and ammonia are perfusion limited, diffusion is unrestricted and blood flow rates will be inversely proportional to the rate of transfer. In the literature there are conflicting reports as to whether gas transfer is diffusion or perfusion limited (Daxboeck et al., 1982; Piiper & Scheid, 1982; Part et al., 1984; Perry et al., 1985c). It is probable that gas transfer across the gill is limited by both perfusion and diffusion components, depending on the physiological conditions.

If one is considering gas diffusion in a simple system, diffusion will be more limiting in oxygen transfer processes than carbon dioxide, due to CO_2 's higher Krogh's permeation coefficient (Piiper & Scheid, 1982). It follows that ammonia transfer will be least affected by diffusion limitations because the Krogh's permeation coefficient for ammonia is considerably greater than that for O_2 or CO_2 (see General Introduction). Diffusion limitations, however, not only include physical differences between gases, but also involve reaction velocities (eg., $\text{CO}_2:\text{HCO}_3^-$ interconversions) and ion transfer processes (eg., $\text{Cl}^-/\text{HCO}_3^-$ and $\text{Na}^+/\text{NH}_4^+$). Hence, if gas exchange at the gills is significantly effected by diffusion limitations, then enzymatic reactions and electrolyte permeabilities will be important components of gas exchange efficiency (Piiper & Scheid, 1982).

The experiments described in this chapter were designed to

test the theory that carbonic anhydrase at the external gill surface maintains an acid boundary layer which facilitates ammonia excretion. The first set of experiments were designed to investigate the effects of inhibition of CA at the external surface of the gill on branchial carbon dioxide and ammonia excretion (30 min exposure to acetazolamide or carbonic anhydrase). A second set of experiments were designed to determine the acute effects (0-60 min) of inhibition of external gill CA on blood ammonia levels and branchial ammonia excretion. Methazolamide was used to replace the more common CA inhibitor, acetazolamide, in the second set of experiments because methazolamide is three times more water soluble and a more potent inhibitor of CA (Maren, 1967). If ammonia excretion is influenced by the pH of boundary layer water, then an inhibition of NH_3 diffusion may result in a compensatory increase in NH_4^+ efflux to maintain net ammonia excretion (Wright & Wood, 1985). To eliminate this possibility, the Na^+ uptake blocker, amiloride, was also added to the water along with methazolamide to eliminate NH_4^+ excretion via the $\text{Na}^+/\text{NH}_4^+$ ion exchange mechanism. Passive NH_4^+ efflux is of minor quantitative importance in freshwater fish (Kormanik & Cameron, 1981a; Wright & Wood, 1985), and therefore, was ignored in this study.

MATERIALS AND METHODS

Experiments in this chapter can be divided into two sections, A and B:

SECTION A: Effects of acetazolamide and carbonic anhydrase on \dot{M}_{Amm} and \dot{M}_{CO_2} .

Experimental animals

Rainbow trout (Salmo gairdneri) were removed from holding tanks (Vancouver tapwater) and held for at least 5 days in dechlorinated tapwater containing 40 mmol.l⁻¹ NaCl and 0.5 mmol.l⁻¹ CaCl₂ (11.7° C ± 0.2, see Chapter 2, Experimental animals). After anaesthetization, an opercular cannula and oral mask were stitched in place (see General Materials and Methods). Following surgery, fish were left to recover in a two-chambered, flow-through experimental apparatus (Fig. 2) for 48 h.

Experimental Protocol and Measurements

Fish (mean weight = 257 ± 9 g (16)) were exposed to three different water regimes: control water, acetazolamide (1.6 mmol.l⁻¹) added to the water, and carbonic anhydrase (6.8 mg.l⁻¹ or 20,400 Wilbur-Anderson units.l⁻¹ bovine carbonic anhydrase) added to the water. Two complete sets of measurements were obtained while fish were in control water. The inflow water was then changed over to either the acetazolamide or the carbonic

anhydrase solution (fish were exposed to one or the other, but not to both) and the same duplicate measurements were repeated after 30 min.

The rate of ventilatory water flow (\dot{V}_w), carbon dioxide content of the inspired (C_{ICO_2}) and expired (C_{ECO_2}) water, and the total ammonia concentration of inspired ($Tamm_I$) and expired ($Tamm_E$) water were measured. \dot{V}_w was determined by collecting outflow water from the back chamber standpipe over 1 min intervals and determining the volume by weight. Measurements of C_{ICO_2} and C_{ECO_2} were made by gas chromatography (see General Materials and Methods). Tamm levels in the water were measured by a modification of the salicylate-hypochlorite assay (Verdouw et al., 1978). The rate of carbon dioxide (\dot{M}_{CO_2}) and ammonia (\dot{M}_{Amm}) excretion were calculated by application of the Fick principle.

Data are presented as means \pm 1 S.E.M. Student's two-tailed, paired t-test was used to compare relationships in the data ($P < 0.05$).

SECTION B: Effects of methazolamide and amiloride on \dot{M}_{Amm}

Experimental animals

Rainbow trout (Salmo gairdneri) of both sexes were obtained from Spring Valley Trout Farm (Mission, B.C.) and held in outdoor, circular fiberglass tanks. The tanks were supplied with flowing, aerated and dechlorinated tapwater (pH approx. 7.0,

[Na⁺]= 40 uequiv.l⁻¹, [Cl⁻]= 20 uequiv.l⁻¹, hardness= 12 p.p.m. CaCO₃, temperature 9-10° C). Fish were fed a diet of commercial trout pellets while in the outdoor holding tanks. Prior to experimentation (4-5 days) fish were transferred to the laboratory holding facility (supplied with the same water) and were not fed during this period to minimize the influence of diet on ammonia excretion (Fromm, 1963).

Trout (mean weight= 300 ± 9 g (N=28)) were fitted with dorsal aortic cannulae (see General Materials and Methods) and were placed in individual, low volume (2-2.5 l), flow-through chambers to recover for at least 48 h.

Experimental Protocol and Measurements

Four experimental conditions were studied:

Series I. A control experiment was performed in which fish were subjected to unaltered freshwater (pH= 7.2). Series I fish served as control animals for Series II experiments.

Series II. In a second group of fish, the CA inhibitor, methazolamide (Neptazane, Lederle Laboratories, 0.5 mmol.l⁻¹) was added to the water during the experimental period to determine if CA inhibition at the gill external surface affected ammonia movements across the gill.

Series III. In the third group of fish, amiloride control, the

Na⁺ uptake blocker, amiloride (0.1 mmol.l⁻¹) was added to the water during the experimental period to inhibit NH₄⁺ excretion via the branchial Na⁺/NH₄⁺ ion exchange mechanism. In previous experiments (Wright & Wood, 1985), the same concentration of amiloride was found to completely abolish Na⁺ uptake across gill epithelium of rainbow trout. Fish in Series III served as control animals for Series IV experiments.

Series IV. In the final group of fish, methazolamide (0.5 mmol.l⁻¹), was added to the water during the experimental period along with amiloride (0.1 mmol.l⁻¹). The aim of this experiment was to investigate the effects of the inhibition of external gill CA on ammonia excretion, in the absence of NH₄⁺ excretion via the Na⁺/NH₄⁺ ion exchange.

The experimental protocol was identical in Series I-IV. Fish were first subjected to a 30 min control period (unaltered freshwater); water flow was switched off so that ammonia excretion could be measured by appearance of ammonia in the water over time. Water samples (5 ml) for ammonia determination were collected at 0, 15, and 30 min. The water was vigorously aerated throughout the flow-through and closed-circuit period. Preliminary tests in the absence of fish showed that ammonia was not lost from the chamber due to aeration alone. A 0.45 ml arterial blood sample was collected at 15 min and replaced with an equal volume of heparinized Cortland saline (Wolf, 1963, pH=

7.0). Following the initial control period, water flow through the box was resumed for 1 h to flush through accumulated waste products. Fish were then subjected to a 1 h experimental period. Water flow was switched off and in 3 out of 4 of the experiments amiloride and/or methazolamide were added to the water. Ammonia excretion was followed over a 1 h period by collecting water samples every 15 min. Arterial blood samples (0.45 ml) were taken at 1, 4, 10, 30, and 60 min and were replaced with equal volumes of heparinized saline. The volume of blood removed from each fish (2.25 ml) represented about 15% of total blood volume.

Whole blood samples were immediately analysed for extracellular pH (pHe) and haematocrit (hct). pHe was determined with a Radiometer microelectrode (type E5021) maintained at 10° C and linked to a Radiometer PHM72 acid-base analyser. Total carbon dioxide content (C_{CO_2}) was measured on plasma samples by gas chromatography (see General Materials and Methods). Plasma CO_2 tensions (P_{CO_2}) and HCO_3^- concentrations were calculated by manipulations of the Henderson-Hasselbalch equation (see General Materials and Methods). Plasma samples (175 μ l) were stored for less than 24 h in 10% trichloroacetic acid (350 μ l) at -4° C and then analysed for ammonia content (Tamm), as follows. Samples were thawed, centrifuged, and the supernatant was neutralized with saturated Tris buffer. Tamm of this neutralized solution was measured by the glutamate dehydrogenase enzymatic assay (Kun and Kearney, 1971). Plasma NH_3 and P_{NH_3}

levels were then calculated (see Chapter 1, Materials and Methods).

Water Tamm was determined as described in Section A. Ammonia excretion rates (\dot{M}_{Amm}) were calculated as:

(21)

$$M_{Amm} = \frac{(Tamm_i - Tamm_f) \times V}{t \times W}$$

where i and f refer to initial and final concentrations in water in $\mu\text{mol.ml}^{-1}$, V is the volume of the system in ml (corrected for sampling deficits), t is the elapsed time in h, and W is the fish weight in kg. Water samples for Tamm determination were collected at 15 min intervals during the experimental period, however, values were averaged over 30 min periods (0-30 min and 30-60 min).

Data are presented as means \pm 1 S.E.M. (N). The Student's two-tailed, paired and unpaired t-test was employed to compare the difference between mean values ($P < 0.05$). A paired t-test was used to compare initial control values with subsequent experimental values within a Series. The unpaired t-test was used to compare values between different Series.

RESULTS

SECTION A: Effects of acetazolamide and carbonic anhydrase.

Data from the acetazolamide and carbonic anhydrase experiments are presented in Figures 10 and 11, respectively. There were no significant changes in ventilation and carbon dioxide and ammonia excretion rates between control I and acetazolamide and between control II and carbonic anhydrase values.

SECTION B: Effects of methazolamide and amiloride.

The results of Series I-IV are presented in Figures 12-14 and Tables 14 and 15. The initial control values of Series I-IV were not statistically different from each other. In all series, pHe of the 4 min blood sample (experimental period) was significantly higher compared to initial control values (+0.13 to +0.18 pH units, Fig. 12 and 14). This blood alkalosis was a result of a respiratory alkalosis, because plasma P_{CO_2} decreased while plasma HCO_3^- levels remained constant.

In Series I where the chemical composition of the water was not altered, \dot{M}_{amm} decreased during the 30-60 min experimental period by 32% relative to the initial control rate (Fig 14). Wright and Wood (1985) attribute the step-wise decrease in ammonia excretion in a closed system to the progressive build up

Figure 10. The rate of carbon dioxide excretion (\dot{M}_{CO_2}) in $\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$, ammonia excretion (\dot{M}_{Amm}) in $\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$, and the rate of ventilatory water flow (\dot{V}_w) in $\text{ml} \cdot \text{min}^{-1} \cdot \text{fish}$ under control (CI) and acetazolamide (A) treatments. Means \pm S.E.M. (N=8)

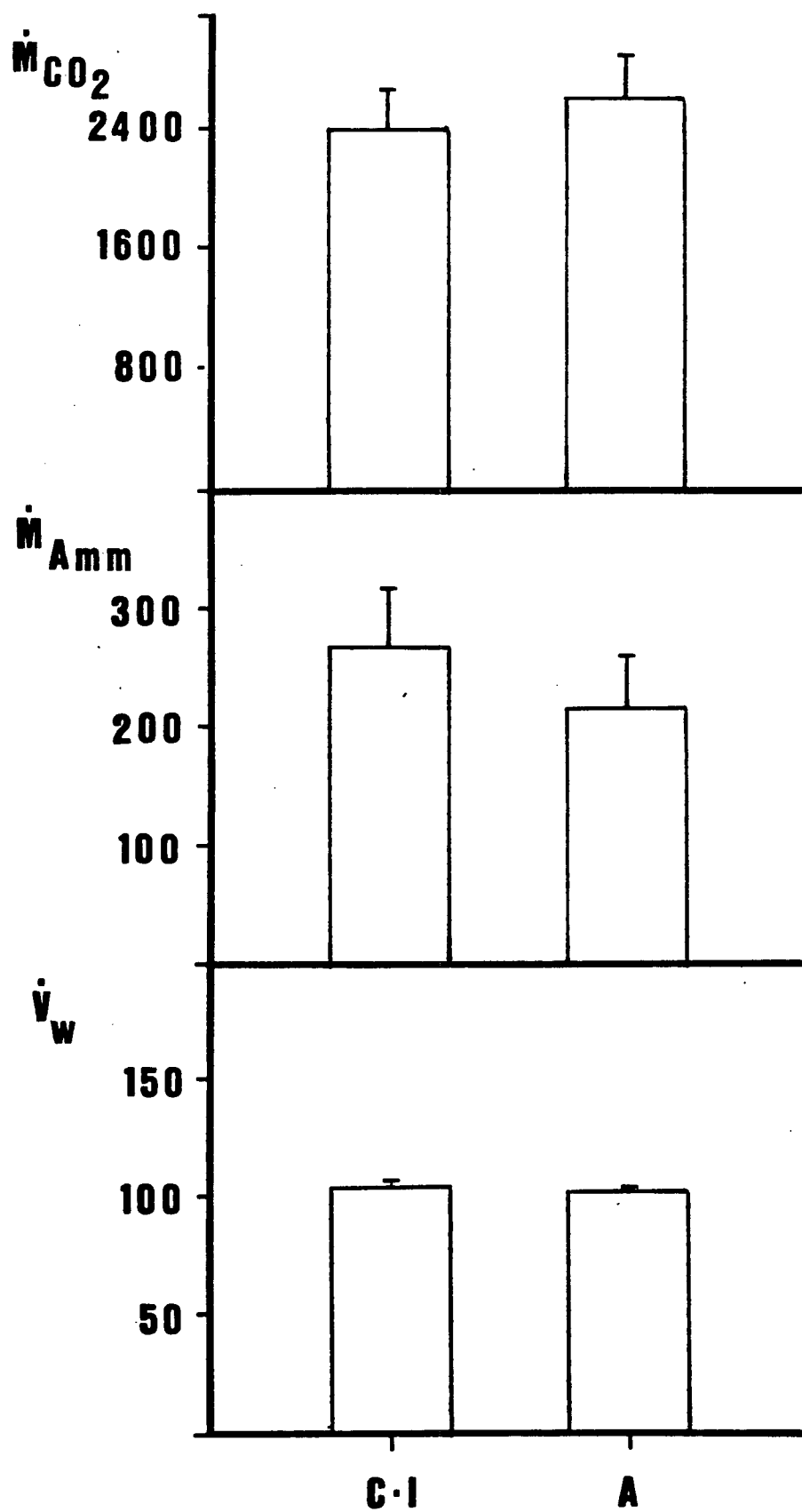


Figure 11. The rate of carbon dioxide excretion (\dot{M}_{CO_2}) in $\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$, ammonia excretion (\dot{M}_{Amm}) in $\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$, and the rate of ventilatory water flow (\dot{V}_w) in $\text{ml} \cdot \text{min}^{-1} \cdot \text{fish}$ measurements under control (CII) and carbonic anhydrase (CA) treatments. Means \pm S.E.M. (N=8)

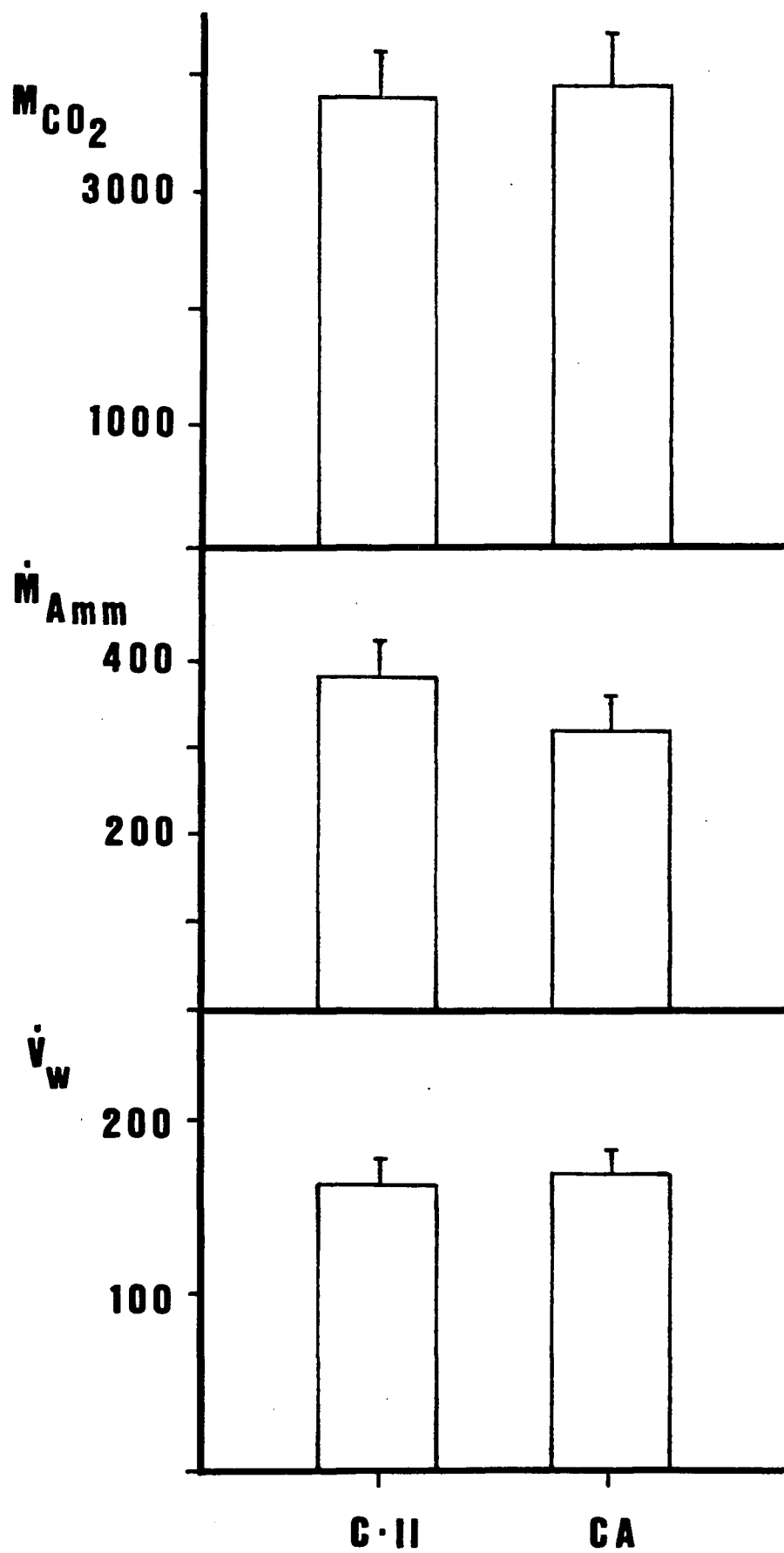


Figure 12. Blood pH (pHe), total ammonia concentration (Tamm) in $\mu\text{mol.l}^{-1}$, and NH_3 tensions (P_{NH_3}) in uTorr are shown for fish under control conditions (\bullet , $N=7$) and fish exposed to methazolamide (\blacktriangle , $N=7$). Measurements were made under control conditions (C) and throughout the experimental period (0-60 min). * denotes statistical difference from the initial control value (C). Means \pm S.E.M.

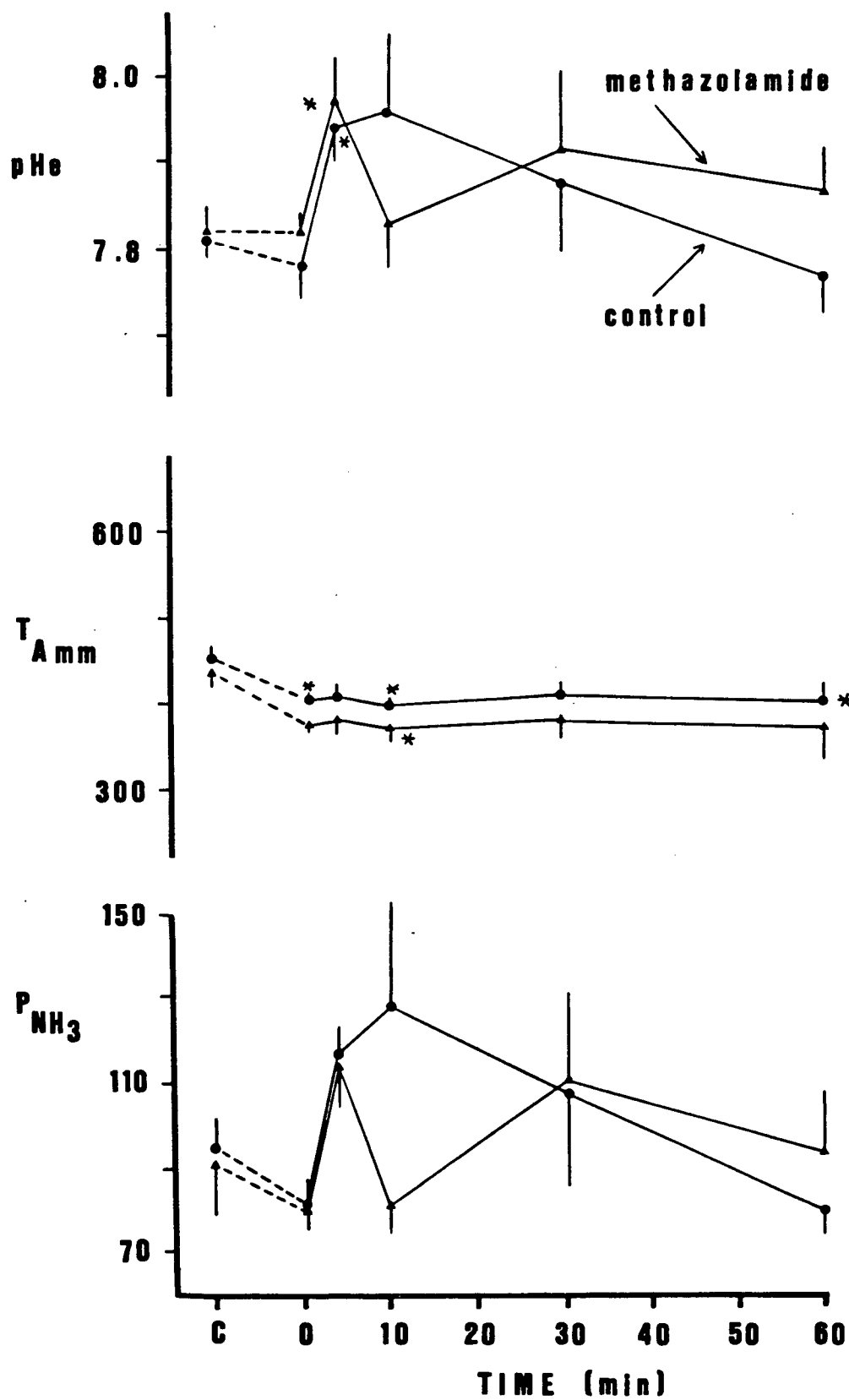


Figure 13. Blood pH (pHe), total ammonia concentration (Tamm) in $\mu\text{mol.l}^{-1}$, and NH_3 tensions (P_{NH_3}) in uTorr are shown for fish exposed to amiloride (●, N=8) and to amiloride and methazolamide together (▲, N=7). Measurements were made under control conditions (C) and throughout the experimental period (0-60 min). * denotes statistical difference from the initial control value (C). Means \pm S.E.M.

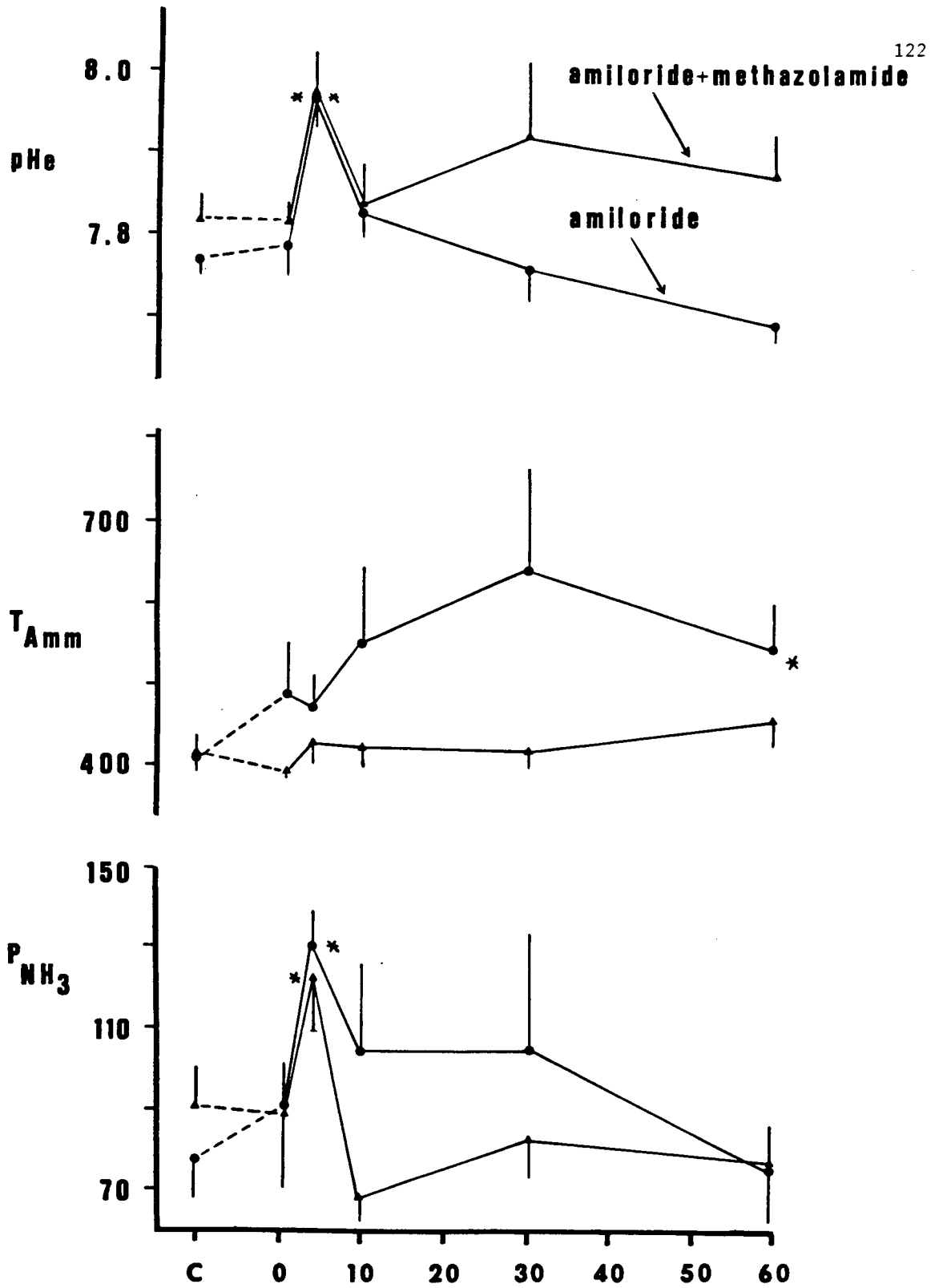


Figure 14. Ammonia excretion rates (\dot{M}_{amm}) in $\mu\text{mol.kg}^{-1}.\text{h}^{-1}$

are shown for, control (Series I (N=7)), methazolamide (Series II (N=7)), amiloride (Series III (N=8)), and amiloride + methazolamide (Series IV (N=7)) experiments.

Measurements were made under control conditions (C) and during the experimental period (0-30 min, 30-60 min).

* denotes statistical difference from initial control values, \square denotes statistical difference from Series I control experiment 0-30 min. \blacksquare denotes statistical significance from amiloride Series III experiment 0-30 min. Means \pm S.E.M.

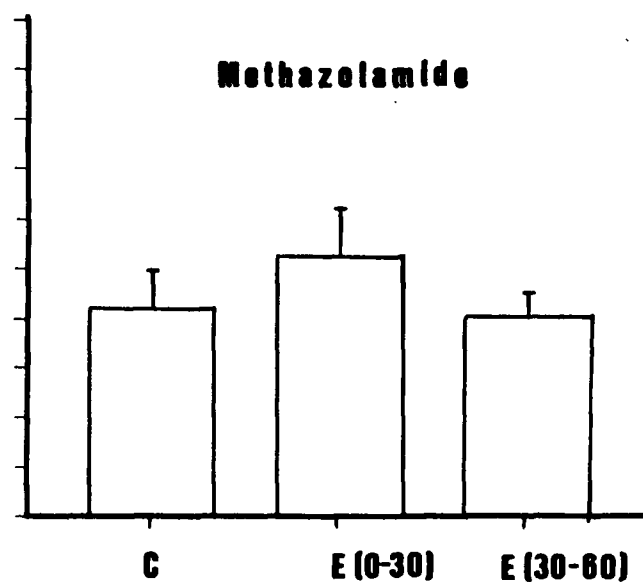
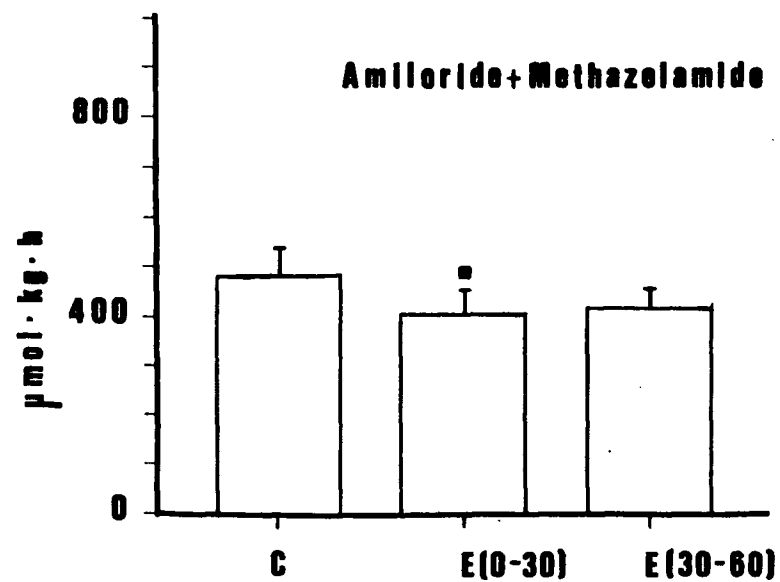
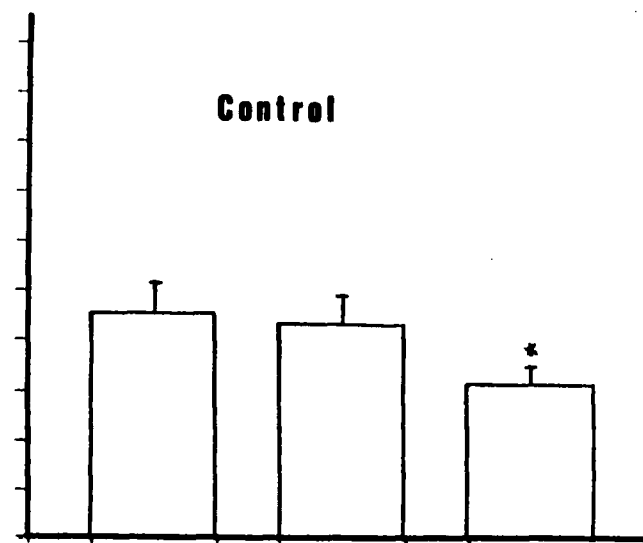
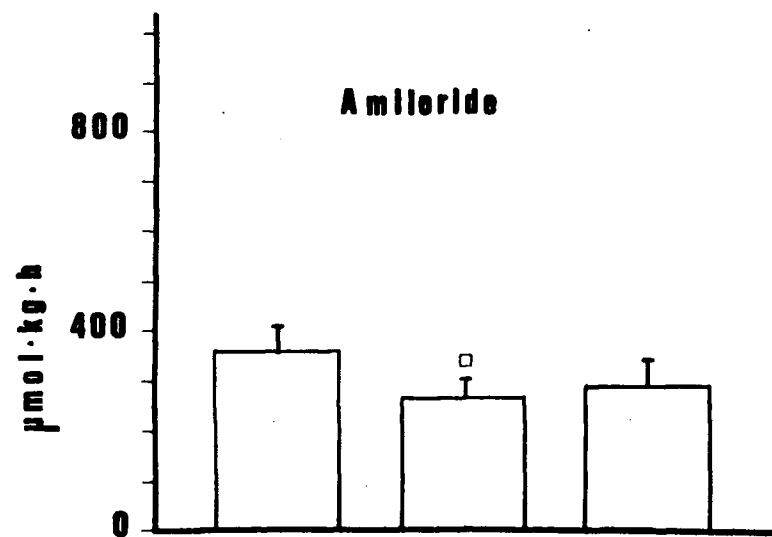


TABLE 14. Carbon dioxide content (C_{CO_2}), CO_2 tensions (P_{CO_2}), and bicarbonate levels (HCO_3^-) in plasma of Series I (control, N=7) and Series II (methazolamide, N=7) fish under control and experimental conditions (0-60 min). Means ± 1 S.E.M.

TIME	SERIES I			SERIES II		
	C_{CO_2} mmol/l	P_{CO_2} Torr	HCO_3^- mmol/l	C_{CO_2} mmol/l	P_{CO_2} Torr	HCO_3^- mmol/l
CONTROL	7.8 $\pm .3$	2.6 $\pm .2$	7.7 $\pm .3$	8.3 $\pm .4$	2.8 $\pm .2$	8.1 $\pm .4$
EXPERIMENTAL						
1 min	7.7 $\pm .4$	2.8 $\pm .2$	7.5 $\pm .4$	8.2 $\pm .5$	2.7 $\pm .2$	8.0 $\pm .5$
4 min	7.3 $\pm .1$	1.9 $\pm .2$	7.2 $\pm .1$	8.1 $\pm .3$	1.7 $\pm .2$	8.0 $\pm .3$
10 min	7.8 $\pm .6$	1.6 $\pm .3$	7.7 $\pm .6$	9.2 $\pm .5$	2.8 $\pm .3$	9.0 $\pm .5$
30 min	7.6 $\pm .5$	2.0 $\pm .3$	7.4 $\pm .5$	9.3 $\pm .5$	2.3 $\pm .4$	9.2 $\pm .5$
60 min	7.6 $\pm .3$	2.4 $\pm .2$	7.4 $\pm .3$	9.3 $\pm .5$	2.6 $\pm .3$	9.0 $\pm .5$

* significantly different from initial control value, paired t-test ($P < 0.05$).

^ significantly different from Series III values at the same time, unpaired t-test ($P < 0.05$)

TABLE 15. Carbon dioxide content (C_{CO_2}), CO_2 tensions (P_{CO_2}), and bicarbonate levels (HCO_3^-) in plasma of Series III (amiloride control, N=8) and Series IV (amiloride + methazolamide, N=7) fish under control and experimental conditions (0-60 min). Means \pm 1 S.E.M.

TIME	SERIES III			SERIES IV		
	C_{CO_2} mmol/l	P_{CO_2} Torr	HCO_3^- mmol/l	C_{CO_2} mmol/l	P_{CO_2} Torr	HCO_3^- mmol/l
CONTROL	7.9 \pm .5	3.0 \pm .2	7.7 \pm .5	7.5 \pm .3	2.5 \pm .3	7.4 \pm .3
EXPERIMENTAL						
1 min	8.0 \pm .6	2.9 \pm .2	8.1 \pm .6	7.4 \pm .6	2.2 \pm .4	7.3 \pm .6
4 min	7.9 \pm .5	* 2.1 \pm .2	8.2 \pm .6	7.7 \pm .2	1.8 \pm .2	7.6 \pm .2
10 min	7.8 \pm .5	2.6 \pm .3	7.6 \pm .5	7.2 \pm .4	2.6 \pm .2	7.0 \pm .4
30 min	7.5 \pm .4	2.9 \pm .3	7.3 \pm .4	7.1 \pm .3	2.3 \pm .4	6.9 \pm .3
60 min	6.7 \pm .9	3.0 \pm .6	6.4 \pm .9	7.1 \pm .3	2.8 \pm .4	7.0 \pm .3

* significantly different from initial control value, paired t-test ($P < 0.05$).

of ammonia levels in the water, which results in reduced blood-to-water ammonia gradients. Plasma Tamm levels were depressed by 9-12% during the experimental period compared to the initial control value, while plasma P_{NH_3} levels did not change (Fig 12). There were no significant changes in CCO_2 , HCO_3^- , and PCO_2 between control and experimental values (Table 14).

With methazolamide in the water in Series II, plasma Tamm levels were reduced by 10-12% and there were no changes in P_{NH_3} levels (Fig. 12) compared to initial control values. Ammonia excretion increased slightly with methazolamide, however, this change was not significant (Fig. 14). Plasma PCO_2 tensions were reduced by 40% at the 4 min sample, but returned to initial control levels after 10 min. This PCO_2 level at 10 min was significantly greater than the Series I values at the same time. Plasma CCO_2 and HCO_3^- values were significantly greater than both initial control (Series II) and Series I values, after 30 min of the methazolamide exposure.

In the amiloride control experiment, Series III, amiloride depressed ammonia excretion (Fig. 14), although the decrease was not statistically significant from the initial control period. (The decrease was statistically different relative to Series I (0-30 min)). Concomittant with the decrease in \dot{M}_{Amm} , plasma Tamm levels progressively increased with amiloride exposure and at 60 min were 32% greater than the initial control level (Fig. 13). Plasma P_{NH_3} levels significantly increased (+69%) at the 4 min sample, due to a combination of blood alkalosis and

elevated plasma Tamm. P_{CO_2} levels were significantly reduced at the 4 min sample relative to initial control values. There were no changes in CCO_2 or HCO_3^- levels (Table 15).

In Series IV, the addition of methazolamide and amiloride to the water resulted in very similar trends to those observed in Series III, however, the changes were less marked. The only statistically significant difference between Series III and IV data was that in Series IV, \dot{M}_{amm} was 50% greater in the first 30 min of the experimental period (Fig. 14). CCO_2 , HCO_3^- , and P_{CO_2} were unaltered with the amiloride and methazolamide treatment (Table 15).

DISCUSSION

Penetration of CA inhibitors

Carbonic anhydrase inhibitors, such as acetazolamide and methazolamide, are permeable to cell membranes. One of the difficulties in adding CA inhibitors to the external water is that these drugs may enter the fish and CA inhibition within the fish may confound the interpretation of the results. Henry and Cameron (1983) have measured the time for 95% inhibition of branchial epithelium CA in crabs after an injection of acetazolamide and found that there was a lag of about 4 h. They speculated that the lag time was equal to the time needed for the drug to cross the basal membrane of the gill and affect inhibition. Acetazolamide added to the external water achieves equilibrium between water and haemolymph of crab in about 1 h (Burnett *et al.*, 1981). In Section A experiments, acetazolamide (1.6 mmol.l^{-1}) was added to the water reservoir supplying the flow-through experimental chamber, for 30 min prior to sampling. The permeability of gills is significantly less in freshwater than in seawater (see Girard & Payan, 1980) and therefore, one would expect acetazolamide to diffuse slower across freshwater trout gills compared to seawater crab gills. If some acetazolamide did diffuse into gill tissue or the blood, there was no apparent effect on CO_2 excretion across the gill. Complete inhibition of gill epithelium CA does not affect CO_2

excretion, while complete inhibition of red cell CA causes an increase in plasma P_{CO_2} , sufficient to maintain steady-state CO_2 excretion (Swenson & Maren, 1987). There was no elevation of plasma P_{CO_2} during 60 min exposure to methazolamide (Section B). Methazolamide diffuses more quickly into tissues than acetazolamide (Maren, 1967), and therefore, it can be assumed that acetazolamide did not inhibit red cell carbonic anhydrase in the Section A experiments.

Effects of Acetazolamide and Carbonic Anhydrase on \dot{M}_{CO_2} and \dot{M}_{Amm}

Acetazolamide or carbonic anhydrase exposure in trout did not alter branchial carbon dioxide or ammonia excretion. The theory proposed was that carbonic anhydrase in the external water boundary layer facilitates the diffusion of CO_2 and NH_3 from the blood compartment to the external water environment. The rapid conversion of excreted CO_2 to HCO_3^- and H^+ ions in the gill water boundary layer would lower water CO_2 levels and serve to enhance blood-to-water P_{CO_2} diffusion gradients. In addition, catalysed CO_2 hydration in the boundary layer would facilitate NH_3 diffusion by providing H^+ ions to combine with NH_3 to form NH_4^+ , thereby chemically removing NH_3 from the boundary water layer, and enhancing blood-to-water P_{NH_3} diffusion gradients.

There are three possible explanations for the observed

results. First, acetazolamide or carbonic anhydrase did not contact CA at the external surface of the gill epithelium. Second, carbon dioxide and ammonia excretion are not diffusion limited under the conditions tested in this study. The third possibility is that carbon dioxide and ammonia excretion are diffusion limited, but compensatory mechanisms were quickly mobilized which masked any effects of acetazolamide and carbonic anhydrase at the time of sampling, 30 min later.

Fish were exposed to acetazolamide or carbonic anhydrase for 30 min, which is probably sufficient time for diffusion of these drugs into the gill water boundary layer given the rate of ventilatory flow ($\sim 100\text{--}170\text{ ml}\cdot\text{min}^{-1}$) and the relatively high drug concentrations in the water ($1.6\text{ mmol}\cdot\text{l}^{-1}$, acetazolamide and $6.8\text{ mg}\cdot\text{l}^{-1}$, carbonic anhydrase). Moreover, acetazolamide treatment resulted in an increase in the pH disequilibrium in downstream water (see Chapter 2), implying that CA located on the external gill surface was inhibited.

It is possible that carbon dioxide and ammonia excretion are not diffusion limited under the experimental conditions of the present study. Carbon dioxide transfer is limited by the rate of $\text{Cl}^-/\text{HCO}_3^-$ exchange at the red cell membrane, and therefore, there is an inverse relationship between hct and \dot{M}_{CO_2} (Perry et al., 1982). Increases in blood HCO_3^- , however, result in increases in \dot{M}_{CO_2} (hct = 11%, Perry et al., 1982), indicating that branchial CO_2 transfer is primarily perfusion limited at higher hct values. It was suggested by Zborowska-Sluis et al.

(1974) that the function of extravascular CA was to facilitate CO_2 transport from muscle cells during periods of high muscular activity when CO_2 production rates are maximal. During resting conditions, as in the present study, theoretical considerations suggest that CO_2 (Piiper & Scheid, 1982) and ammonia transfer are perfusion limited. It may be, however, that under extreme conditions, such as, high carbon dioxide and ammonia production, elevated blood catecholamine levels, and water conditions that reduce blood-to-water diffusion gradients, diffusion limitations may become significant and facilitation of CO_2 and NH_3 transfer by external gill CA may play an important role.

Whole animal physiology is often difficult because of the complexity of physiological problems due to the multitudinous interactions between body systems. For instance, if ammonia excretion is diffusion limited, then the proposed theory suggests that inhibition of external gill CA would reduce the availability of H^+ ions in the boundary layer, reduce the rate of chemical removal of NH_3 from the boundary layer, raise water NH_3 levels, and decrease the blood-to-water P_{NH_3} gradient. The net result would be a decrease in total ammonia excretion. In a steady state system, however, blood-to-water P_{NH_3} gradients are probably quickly re-established at higher blood P_{NH_3} levels, which would maintain ammonia excretion across the gills. There are other mechanisms which may act to compensate for diffusion limitations. For instance, increases in gill permeability to NH_3 , CO_2 , H^+ , or NH_4^+ would all play a role in maintaining

ammonia excretion in the face of an inhibition of external gill carbonic anhydrase. Hormones, such as adrenaline, selectively increase gill cell membrane permeability to lipophilic substances (Isaia, 1984), such as, NH_3 and CO_2 . An increase in CO_2 or H^+ ion excretion would sustain H^+ ion concentrations in the boundary layer in a situation where the CO_2 hydration reaction was uncatalysed, thereby maintaining P_{NH_3} gradients across the gills. CO_2 excretion was unchanged in the acetazolamide experiment, however, which rules out this possibility. Finally, an increase in NH_4^+ excretion (via the $\text{Na}^+/\text{NH}_4^+$ exchange) may occur in situations where NH_3 diffusion gradients are less favourable (Wright & Wood, 1985). Similar arguments can be made to explain the apparent lack of effect of acetazolamide and CA treatments on carbon dioxide excretion.

Effects of Methazolamide on plasma ammonia levels and \dot{M}_{Amm}

Experiments in this section focused specifically on whether external gill CA acts to facilitate ammonia excretion. These experiments were designed to avoid some of the complicating factors inherent in the design of Section A experiments, such as, the 30 min delay following exposure to drug, prior to sampling, and NH_4^+ excretion as an alternate pathway for ammonia excretion. Despite these modifications in experimental design, inhibition of external gill CA did not alter total ammonia excretion, as expected. Methazolamide added to the water

increased the diffusivity of the gills to NH_3 ($D_{\text{NH}_3} = \dot{M}_{\text{Amm}}/P_{\text{NH}_3}$ gradient blood-to-water) by about 20% in the first 30 min of exposure (4.6 vs 5.4 $\text{umol kg}^{-1} \text{h}^{-1} \text{uTorr}^{-1}$, Series II). This increase in D_{NH_3} is unexpected because an inhibition of external gill CA should elevate water boundary layer pH, elevate NH_3 levels in the water, and in the short term, reduce NH_3 diffusion across the gill. One possible explanation for these results is that a stimulation of the $\text{Na}^+/\text{NH}_4^+$ exchange maintained total ammonia excretion in the face of less favourable blood-to-water P_{NH_3} gradients. Alkaline waters increase the rate of $\text{Na}^+/\text{NH}_4^+$ exchange relative to NH_3 diffusion (Wright & Wood, 1985), which is consistent with the proposed increase in boundary water pH with an inhibition of external gill CA. It should be mentioned, however, that some studies report an inhibition of Na^+ uptake with the CA inhibitor, acetazolamide (Maetz & Garcia Romeu, 1964; Henry & Cameron, 1983), which would argue against an increase in $\text{Na}^+/\text{NH}_4^+$ exchange if methazolamide acts in a similar fashion. To clarify the role of $\text{Na}^+/\text{NH}_4^+$ in ammonia excretion during methazolamide treatment it is of value to look at the experiments where amiloride was also added to the water.

Amiloride decreased \dot{M}_{Amm} by 22% (amiloride control, mean value over 60 min.), a figure consistent with 23% reductions in intact rainbow trout (Wright & Wood, 1985) and 30% reductions reported for perfused in vitro rainbow trout preparations (Kirschner et al., 1973; Payan, 1978). In the present study,

amiloride rapidly and progressively increased plasma Tamm and P_{NH_3} levels, resulting in a 50% reduction in D_{NH_3} over the first 30 min. If it is true that the increase in D_{NH_3} with methazolamide treatment (Series II) was due to a stimulation of Na^+/NH_4^+ exchange, then by adding amiloride together with methazolamide D_{NH_3} should equal or be less than the value with amiloride alone. This was not the case, D_{NH_3} with both drugs in the water only decreased by 20% (5.3 vs 4.3 $\mu\text{mol.kg}^{-1}.\text{h}^{-1}.\text{uTorr}^{-1}$), compared to the 50% reduction with amiloride alone. Hence, the increase in D_{NH_3} with methazolamide cannot be explained by a stimulation of Na^+/NH_4^+ exchange, and is due to unknown factors.

The blood alkalosis which was observed in all series of experiments (4 min, experimental sample) dominated any changes in blood parameters and complicated interpretation of the results. The respiratory alkalosis (decrease in P_{CO_2} , no change in HCO_3^-) was likely due to hyperventilation as a result of rapid responses (<3 min) to blood volume changes. Hyperventilation immediately follows the removal of ~0.5 ml of blood from the dorsal aorta of cannulated trout under similar conditions (S.F. Perry, personal communication). The presence and location of baroreceptors in fish are unknown, but haemorrhage results in an increase in renin activity in fish (Bailey & Randall, 1981), as in other vertebrates. The renin-angiotensin system effects many changes in the body, and in the present study these changes may be related to the increase in ventilation.

To summarize, the addition of acetazolamide and carbonic anhydrase to the water did not alter CO_2 and ammonia excretion in vivo. The interpretation of these results is complicated by the fact that the interval of time prior to sampling may have been too long to observe any short term effects of these drugs. Methazolamide resulted in an increase in the diffusing capacity of ammonia, unrelated to NH_4^+ efflux via $\text{Na}^+/\text{NH}_4^+$ exchange. Rapid serial blood sampling resulted in a universal blood alkalosis, probably as a consequence of hyperventilation. Because blood pH was not constant during the experiment it was impossible to determine the validity of the proposed hypothesis. It is concluded that the intact resting animal is unsuitable for studying the interaction between water boundary layer chemistry and excretion across the gill.

CHAPTER 4

The linkage between carbon dioxide and ammonia excretion in the isolated, blood-perfused trout head preparation.

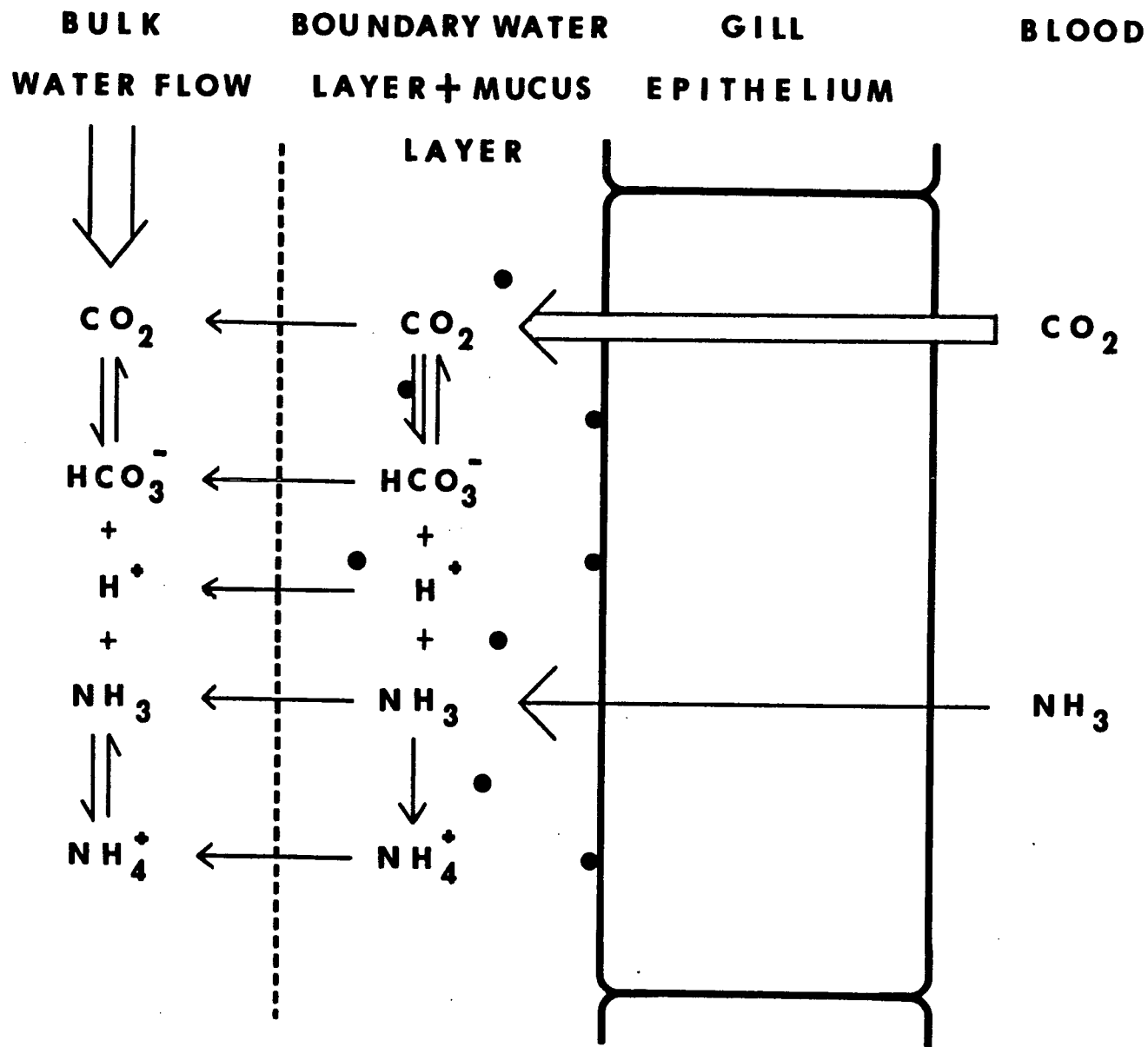
INTRODUCTION

Transport across epithelial surfaces is influenced by the presence of boundary layers associated with the epithelium (Knepper et al., 1984, 1985; Arruda & Dytko, 1985). Boundary layers are considered "unstirred layers", and therefore the chemical composition of the boundary layer is distinct from the bulk phase because materials that are excreted into the boundary layer only slowly diffuse into the bulk phase. External boundary layers next to the fish gill epithelium exist because water flow between gill lamellae is slow and the pattern of water flow through the mouth and over the gills is laminar, rather than turbulent (Scheid & Piiper, 1971; Piiper et al., 1986).

A significant component of ammonia is eliminated at the gill of freshwater fish as NH_3 , by diffusion down the blood-to-water P_{NH_3} gradient (deVooy, 1968; Cameron & Heisler, 1983; Wright & Wood, 1985; Cameron, 1986). NH_3 levels may build up in the gill water boundary layer unless diffusion out of the boundary layer into the bulk water is rapid or NH_3 is removed by other means. The excretion of carbon dioxide and subsequent catalysed hydration to HCO_3^- and H^+ may ensure continual availability of H^+ ions in the boundary layer to combine with NH_3 to form NH_4^+ . This chemical removal of NH_3 would enhance NH_3 transfer and functionally link carbon dioxide and ammonia excretion across the gill (Fig. 15).

To investigate the possible linkage of CO_2 and ammonia

Figure 15. A simplified cross-section through the gill epithelium showing the bulk water flow, the boundary water layer and associated mucus layer containing carbonic anhydrase molecules represented by ●. The catalysed hydration of CO_2 in the boundary layer to $\text{HCO}_3^- + \text{H}^+$ facilitates ammonia excretion by promoting the interconversion of NH_3 to NH_4^+ thereby preventing reductions in the trans-epithelial NH_3 diffusion gradient due to accumulation of NH_3 . The thickness of the arrows denotes the magnitude of the particular process illustrated (not precisely drawn to scale).



excretion at the gill it was necessary to employ an in vitro preparation in which the independent variables could be precisely controlled. In chapter 3, it was impossible to determine whether or not carbonic anhydrase present on the external surface of the gill facilitated carbon dioxide and/or ammonia excretion because of the difficulties in studying boundary layer phenomena in intact animals. With the blood-perfused trout head preparation the convective components of gas exchange (blood flow rate through the gills and water flow rate over the gills), as well as the chemical composition of the blood perfusate and ventilatory water, could be controlled.

Perfused preparations are valuable in elucidating mechanisms which cannot be effectively examined in the intact animal. The primary criterion by which to judge a preparation is its ability to match the physiological response as it occurs in vivo. To evaluate the physiological performance of the blood-perfused trout head preparation, blood and water variables and gas exchange rates were compared between in vivo and in vitro preparations.

MATERIALS & METHODS

Experiments were divided into two sections. Section A describes the blood-perfused trout head preparation used to investigate the linkage between CO_2 and NH_3 in the gill water boundary layer. Section B in vivo experiments were designed to emulate the conditions of the blood-perfused head experiment, so that comparisons between in vivo and in vitro parameters could be made.

Experimental animals

Rainbow trout (Salmo gairdneri) of both sexes were obtained from Thistle Springs Trout Farm (Ashton, Ontario) and transported to the University of Ottawa. Fish were held in indoor rectangular fiberglass tanks supplied with flowing, aerated and dechlorinated tapwater (pH= 7.0-8.0, $[\text{Na}^+]= 0.10$; $[\text{Cl}^-]= 0.10$; $[\text{Ca}^{++}]= 0.35$; $[\text{K}^+]= 0.03 \text{ mmol.l}^{-1}$, temperature= 6-9° C). Fish were fed a daily diet of commercial trout pellets.

Section A: Blood-perfused trout head preparation

Blood collection and preparation

Isolated trout heads were perfused with whole blood, collected from fish with dorsal aortic cannulae implanted 24 h

earlier (see General Materials and Methods). Blood was collected immediately prior to each experiment. In general, 6-7 cannulated donor fish provided enough blood for one perfused head experiment. To increase the volume of blood removed from each donor fish, 2-3 ml of heparinized saline (ammonium heparin, Sigma 10 units.ml⁻¹) was initially injected into each fish via the dorsal aortic cannulae. After a short time to allow for mixing (approx. 1-2 min), blood was withdrawn and pooled. In most cases, 5 to 10 ml of blood could be collected from each fish by this method. In some cases, it was necessary to augment the total blood volume for a single experiment (50-60 ml) by adding small volumes (3-5 ml) of blood removed by external puncture from the caudal vein-artery of a few uncannulated fish. The haematocrit (hct) of the pooled blood was measured (mean hct = 17.1% \pm 0.5) and adjusted to 13-15% (mean = 14.4% \pm 0.3) with Cortland saline (Wolf, 1963) containing 2.2% Bovine Serum Albumin (BSA, Sigma). In trial experiments the hct of the pooled blood was adjusted to 10%; however, we found that gas exchange across the gills was considerably less than at a hct of 13-15 %, and the higher hct was adopted. The extent of dilution depended on the hct of the pooled donor blood, but plasma osmotic pressure was likely constant in all experiments since 2.2% Bovine Serum Albumin (BSA, Sigma) was present in the saline. In preliminary experiments, we also found that the addition of the colloid osmotic filler, polyvinylpyrrolidone (see Perry et al., 1984a), to the saline in place of BSA resulted in extremely high blood pressure in the

isolated head preparation. Thus, saline which was used to dilute the blood as well as perfuse the head on the operating table (see below), only contained BSA. Diluted blood was divided into two reservoirs and gently stirred for 90 min in an ice bath. The blood was equilibrated with 0.4% CO₂ and 4% O₂, balanced with N₂ (PCO₂ 3 Torr, PO₂ 30 Torr), for 90 minutes. Blood was gassed for 90 min with identical gas mixtures to simulate venous blood gas tensions.

Surgical procedure for preparing isolated perfused head

Trout (216-353g, mean weight 269g \pm 6, N= 31) were removed from holding tanks and immediately injected with 1 ml of saline containing 2500 units.ml⁻¹ ammonium heparin. Fish were left for approximately 10 minutes to ensure complete mixing of the heparin in the body, and then removed from the water and quickly decapitated by cutting 2cm posterior to the pectoral fins. The head (mean weight 74 g \pm 1) was immediately transferred to the operating table where a tube containing flowing tapwater was placed in the mouth in order to irrigate the gills. The pericardium was quickly torn and an incision was made in the ventricle muscle so as to prevent air being pumped into the gill vasculature. The viscera were removed from the body cavity and a saline-filled 4 cm piece of PE50 tubing was introduced into the bulbous arteriosus by way of the ventricular incision and secured in place with surgical silk. Next a 3 cm piece of PE 160 tubing

was placed in the dorsal aorta (DA), and blood was cleared from the gills by perfusing in the retrograde direction for approximately 5 sec with filtered (Millipore 0.45 μ m) saline containing 2.2% BSA and 10^{-7} M epinephrine (Sigma) at a constant pressure of 50 cm H₂O (see Appendix I for saline pH, Tamm, CCO₂, and protein levels). Perfusing in the retrograde direction cleared any air bubbles trapped in the ventral aorta (VA). The direction of perfusion was then switched to orthograde by connecting the saline reservoir with the VA catheter for the remainder of the surgical procedure. The gills were ischemic for no longer than 2 minutes. Next the esophagus was firmly ligated to prevent water contamination in the body cavity. The body wall was then supported by stitching a plastic semicircular collar (made from a 60 ml disposable syringe) to the inside of the ventral body wall. The DA catheter was secured by cutting a half circle in the surrounding muscle and slipping a piece of silk in the incision and tying the silk firmly around the tissue and catheter. This completed the cardiovascular surgery in approximately 8 min.

Opercular cannulae were affixed to the isolated head in order to sample mixed expired water downstream from the gill (see General Materials and Methods). A small tube (Tygon tubing, 0.5 cm diameter) was then stitched to the floor of the buccal cavity and the tongue just at the opening of the mouth to ensure proper water flow over the gills during the subsequent experiment. The mouth was then sealed shut around this tube by two large stitches

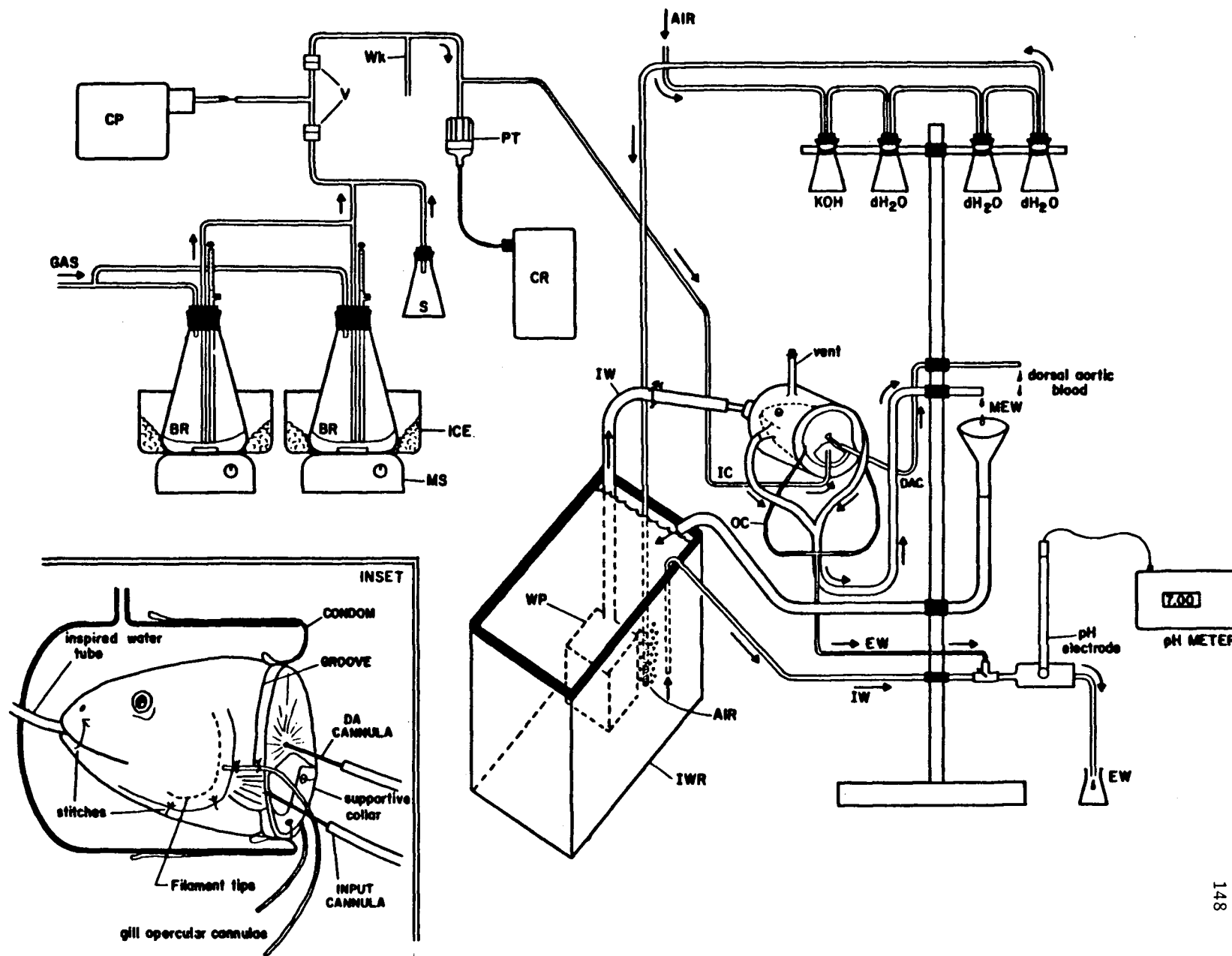
between the upper and lower jaw on either side. The opercular openings were loosely closed by two stitches along the ventral openings to prevent the opercular valve from flaring open and resulting in poor ventilatory flow through the gill arches. Different methods of gill irrigation were initially tried, for example, an inflow tube was simply placed in the fish's mouth without securing the mouth or the opercular valves closed. This method has been widely used in other isolated head studies (eg., Perry & Wood, 1985; Perry et al., 1984c; Bornancin et al., 1985), however, we found that water tended to flow back out of the mouth and what water did pass through the opercular cavity did not flow in a uniform pattern over the gills.

Finally, a rubber membrane (condom) was fitted around the head by first making a groove on the external surface with a heavy piece of silk tied snugly around the fish, posterior to the pectoral fins. This rubber seal prevented mixing of the blood perfusate with the ventilatory water once the fish was placed in the experimental apparatus. Surgery was then complete in a total time of approximately 15 minutes. The gills were visually examined to assess the degree of blood clearance and if less than 75%, the preparation was discarded.

Experimental apparatus

The isolated head was immediately transferred to the small plastic chamber shown in the inset of Figure 16 and the condom

Figure 16. The experimental apparatus for the isolated head preparation is shown. The inset illustrates a close-up of the head which was placed in the plastic experimental chamber after surgery and sealed with a condom. The placement of the dorsal aortic (DA), venous (input), and opercular cannulae is shown. BR= blood reservoir, MS= magnetic stirrers, CP= cardiac pump, S= saline, V= one-way valves, Wk= Windkessel, PT= pressure transducer, CR= chart recorder, IC= input cannulae, DAC= dorsal aortic cannula, OC= opercular cannulae, EW or MEW= mixed expired water, IW= inspired water, WP= water pump.



was fitted around the outside of the chamber to make a tight seal. Water flow (\dot{V}_w) over the gills was set to approx. 840 ml.min⁻¹.kg⁻¹ (water temp.= 8° C). Tapwater was air equilibrated to ensure that water carbon dioxide levels were in equilibrium between water and air. The gills were perfused at constant pulsatile flow (blood flow rate, \dot{V}_b = 11 ml.min⁻¹.kg⁻¹) with gas equilibrated fish whole blood from one of the two blood reservoirs. For details of the modified cardiac pump refer to Davie and Daxboeck (1983). Input (afferent) pressure (P_i) was monitored via a T-junction in the input catheter connected to a pressure transducer (Bell and Howell) and displayed on a Harvard chart recorder. Pulse pressure was kept constant at 10 cm H₂O by adjusting the size of a gas space at the top of a wide-bore side-arm (Windkessel) in the perfusion line. The pressure drop across the input catheter was measured with ligatures still in place after each experiment. This catheter resistance (R_s = 13 \pm 1 (N=18)) was subtracted from measured total input pressure (P_t = 81 \pm 3 (N=18)) for each preparation to determine corrected input pressure (P_i = 69 \pm 4 cm H₂O (N=18)). Dorsal aortic pressure (P_{da}) was maintained between 10 and 15 cm H₂O (Perry et al., 1985a).

To test whether water flow was evenly distributed between both gills, a non-toxic dye (food colour) was added to the inflow water, and the pattern of flow from each opercular opening was visually assessed at the onset and termination of each experiment. Preparations were discarded if the water flow

pattern was not approximately matched on both sides. The head was removed from the chamber at the end of the experiment, and the gills were examined once again to ensure that the perfusion reached at least 75 % of the tissue. The placement of the mouth tube was also checked, along with the position of the opercular catheters.

Experimental Protocol and measurements

Each experiment involved two sets of paired blood (arterial and venous) and water (inspired and mixed expired) samples. The first samples (control) were collected 7 minutes after blood flow was initiated in the head preparation. At 8 min (elapsed time) the blood flow was derived from the second blood reservoir and depending on the experiment, the chemical composition of the inflow water was altered. The second samples (experimental), were collected 7 min later or after 15 min of total elapsed time. The measurement times were chosen to allow adequate time for stabilization of the head preparation (based on perfusion pressure measurements) after the initiation of blood flow and after a change in the composition of blood or water.

Four experimental treatments were performed:

1. In the first experimental treatment the chemical composition of the blood and water was not altered (control) to provide baseline excretion rates, as well as determining the stability of

the head preparation over the duration of the experiment.

2. In subsequent experiments it was important to isolate NH_3 from NH_4^+ excretion in order to determine if NH_3 diffusion across the gill was linked to CO_2 excretion. In the second experiment, referred to as amiloride control, the Na^+ uptake blocker, amiloride (10^{-4} M) was added to the water in both the control and experimental periods to inhibit the excretion of the NH_4^+ , via the $\text{Na}^+/\text{NH}_4^+$ (H^+) ion exchange mechanism (e.g. Kirschner et al., 1973; Wright & Wood, 1985). In freshwater fish, passive NH_4^+ flux across the gill is of minor quantitative importance (Kormanik & Cameron, 1981a; Wright & Wood, 1985). The chemical composition of the blood was not altered.

3. To determine a possible link between CO_2 excretion and NH_3 diffusion in the third experiment one of the two blood reservoirs was incubated (90 min) with the carbonic anhydrase inhibitor, acetazolamide (10^{-4} M) (Maren, 1977), in order to inhibit carbon dioxide excretion across the gills.

4. To test whether the possible link between CO_2 and ammonia excretion was due, in fact, to chemical reactions in the boundary layer, a final experiment was performed in which the buffering capacity of expired water was raised by adding Tris buffer to the water during the second half of the experiment. A combination of

Trizma base ($C_{14}H_{11}NO_3$, Sigma) and Trizma hydrochloride ($C_4H_{12}ClNO_3$, Sigma) was used to make a $4 \times 10^{-3}M$ solution at pH= 8.0.

Blood pH, oxygen tension (P_{O_2}) and total O_2 content (C_{O_2}), carbon dioxide content (C_{CO_2}), plasma Tamm, and hct were measured on both arterial and venous blood samples, along with \dot{V}_b and perfusion pressure. Plasma epinephrine levels were measured on venous samples ($1.8 \times 10^{-7} M \pm 0.2 \times 10^{-7}$). Blood pH and P_{O_2} were measured with a Radiometer Blood Microsystem (BMS3 Mk2) and associated acid-base analyser (PHM 71), maintained at the experimental temperature. C_{O_2} was determined by the Tucker method (Tucker, 1967). C_{CO_2} was measured using a total CO_2 analyser (Corning). Plasma Tamm levels were determined enzymatically as described by Kun & Kearney (1971). Plasma samples were frozen in liquid N_2 and stored at $-80^\circ C$ for later determination of epinephrine levels with a high pressure liquid chromatograph (Woodward, 1982). \dot{V}_b was measured at the beginning and the end of each experiment and these values were averaged, as there were only very small differences between them ($< 5\%$). As well, the specific gravity (or refractive index) of plasma was determined with a refractometer (Scientific Instruments) to assess any dilution of the blood by water contamination ($2.3\% \pm 0.0$ (N=23)). The rates of oxygen uptake (\dot{M}_{O_2}) and carbon dioxide (\dot{M}_{CO_2}) and ammonia (\dot{M}_{Amm}) excretion were determined by the Fick principle, where

arterial-venous differences were multiplied by V_b .

At the same time that blood samples were collected, inspired water pH (pH_I) and mixed expired pH (pH_E) were measured with a Fischer Accumet pH meter (829MP). Ventilatory water flow (\dot{V}_w) was determined by collecting mixed expired water leaving the head chamber in a given period of time and accounting for the small flow ($\sim 30 \text{ ml} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$) through the opercular cannula. Inspired and mixed expired water samples were also collected for total carbon dioxide and ammonia levels, as well as oxygen tensions. These values, along with \dot{V}_w were used to calculate \dot{M}_{CO_2} , \dot{M}_{Amm} , and \dot{M}_{O_2} by the Fick principle. Although the trends in the \dot{M}_{CO_2} , \dot{M}_{Amm} , and \dot{M}_{O_2} values calculated from water parameters were similar to those calculated from blood parameters, there was more scatter in the data, which will be discussed as a part of Section B.

Data are presented as means ± 1 S.E.M. The Student's paired t-test was used to determine significance between control and experimental values.

Section B: Trout in vivo experiments

Surgical procedures

Two groups of fish were prepared, those with oral masks, opercular cannulae, and ventral aortic (VA) and dorsal aortic (DA) cannulae, and those VA and DA cannulae only. To avoid undue

stress, surgery on the first group of fish was performed in two sessions. Fish were anaesthetized, the VA was cannulated (see General Materials and Methods), and fish were left to recover for 24 h in opaque plexiglass chambers (~10 l). If the VA catheter was patent, fish were re-anaesthetized, and a DA catheter, oral mask and opercular cannulae were stitched in place (see General Materials and Methods). Fish were left to recover in the two-chambered, flow-through experimental apparatus for 48 h following surgery.

In the second group of fish, VA and DA catheters were implanted together and fish recovered for 24 h in single-chamber, opaque plexiglass chambers.

Protocol and Measurements

Fish with oral masks:

The aim of this experiment was to investigate arterial-venous blood and inspired-mixed expired water differences in vivo, so as to compare these values with those in the isolated blood-perfused trout head preparation. Ventilatory water flow (\dot{V}_w) could be manipulated in the two-chambered box by adjusting the height of the front compartment standpipe relative to the back compartment standpipe (Fig 2). A pressure head across the gills was established in the anterior compartment to raise \dot{V}_w to levels comparable to the blood-perfused head (850-900 ml.kg⁻¹.min⁻¹). After 2 h, arterial and venous blood samples

(0.6 ml) and inspired and mixed expired water samples (10 ml) were collected.

Arterial and venous blood was analysed for pH, P_{O_2} , C_{O_2} , CCO_2 , Tamm, and hct, by methods outlined in Section A. Inspired and expired water samples were analysed for pH (see Section A), Tamm (see Chapter 3, Section A), CCO_2 (see General Material and Methods), and P_{O_2} . Water P_{O_2} was measured with microelectrodes as described for blood in Section A of this chapter. \dot{V}_w was measured by collecting outflow water from the back chamber standpipe over 1 min intervals.

Fish without oral masks:

The purpose of this experiment was to determine resting arterial -venous differences in fish unencumbered by oral masks and opercular cannulae. Arterial and venous blood samples were withdrawn and analysed in the same manner as outlined above.

Calculations

The efficiency of oxygen extraction is expressed by the following equation:

(22)

$$O_2 \text{ extraction efficiency (\%)} = \frac{Pa_{O_2} - Pv_{O_2}}{P_{IO_2} - Pv_{O_2}}$$

where Pa_{O_2} and Pv_{O_2} represent arterial and venous O_2 tensions, respectively, and P_{IO_2} denotes inspired water O_2

tension.

The utilization of oxygen in the water passing over the gill was calculated as follows:

(23)

$$O_2 \text{ utilization (\%)} = 100 \cdot \left[\frac{P_{IO_2} - P_{EO_2}}{P_{IO_2}} \right]$$

where P_{IO_2} and P_{EO_2} represent inspired and mixed expired water O_2 tensions, respectively.

The transfer factor (T) is a measure of the relative ability of the gill respiratory surface to exchange gases and is defined as:

(24)

$$T_{O_2} = \frac{M_{O_2}}{1/2(P_{IO_2} + P_{EO_2}) - 1/2(P_{aO_2} + P_{vO_2})}$$

$$T_g = \frac{M_g}{1/2(P_{a_g} + P_{v_g}) - 1/2(P_{IO_2g} + P_{EO_2g})} \quad (25)$$

where g represents CO_2 or NH_3 . Excretion or uptake rates were calculated from arterial-venous differences and \dot{V}_b . A large T value for a given gas indicates a more effective exchange across the gill. The ratio of transfer factors for two gas species was compared to the ratio of Krogh's diffusion coefficients, where,

$$\frac{K_{g1}}{K_{g2}} = \frac{d_{g1} \cdot \alpha_{g1}}{d_{g2} \cdot \alpha_{g2}}$$

$$K_{g2} \quad d_{g2} \cdot \alpha_{g2}$$

and g_1 and g_2 are equal to two different gases (CO_2 , O_2 , or NH_3), d is the physical diffusion coefficient in water, and α is the solubility coefficient in water. Diffusion coefficients were calculated using Graham's law which states that the ratio of the diffusion of two gases x and y , dx/y , is inversely related to the square root of their molar masses.

Data are presented as means \pm 1 S.E.M.

RESULTS

Section A: Linkage between \dot{M}_{CO_2} and \dot{M}_{Amm}

The results of the control experiment, where the chemical composition of the blood and water was not altered, are displayed in the first column of Figs. 17 and 18. The head preparation was stable throughout the experiment because there was no significant difference between the values in the first (control) and second (experimental) measurement periods. The mean control gas exchange values over the two measurement periods were as follows: CO_2 excretion (\dot{M}_{CO_2}) = $799 \pm 50 \text{ umol.kg}^{-1}.\text{h}^{-1}$, oxygen uptake (\dot{M}_{O_2}) = $324 \pm 31 \text{ umol.kg}^{-1}.\text{h}^{-1}$, and ammonia excretion (\dot{M}_{Amm}) = $263 \pm 26 \text{ umol.kg}^{-1}.\text{h}^{-1}$. The RE value ($\dot{M}_{CO_2}/\dot{M}_{O_2}$) was $2.47 \pm .50$. The mean arterial to venous pH difference ($pH_a - pH_v$) was 0.07 ± 0.01 and the inspired to expired water pH difference ($pH_I - pH_E$) was 0.28 ± 0.03 . Ventilation was approximately the same in all experiments ($\sim 210 \text{ ml.min}^{-1}$, Fig. 18).

In the second treatment where amiloride was present in the water throughout the experiment and blood chemistry was not altered (amiloride control), there were no changes in \dot{M}_{CO_2} , \dot{M}_{Amm} , \dot{M}_{O_2} , (Fig. 17) and $pH_I - pH_E$ (Fig. 18) between the control and experimental measurement periods. There was a small, but significant, decrease in the $pH_a - pH_v$ difference between the control and the experimental period (Fig. 17). In general,

Figure 17. The first (control) and second (experimental) measurements of carbon dioxide (\dot{M}_{CO_2}) and ammonia excretion (\dot{M}_{amm}) and oxygen uptake (\dot{M}_{O_2}) in $\mu\text{mol.kg}^{-1}.\text{h}^{-1}$, are shown along with $\text{pH}_a\text{-pH}_v$ differences, in the control (N=10), amiloride control (N=8), amiloride + Diamox (N=6), and amiloride + Tris (N=4) experiments. * denotes statistical significance ($P \leq 0.05$) between the first and second measurement. Means \pm S.E.M.

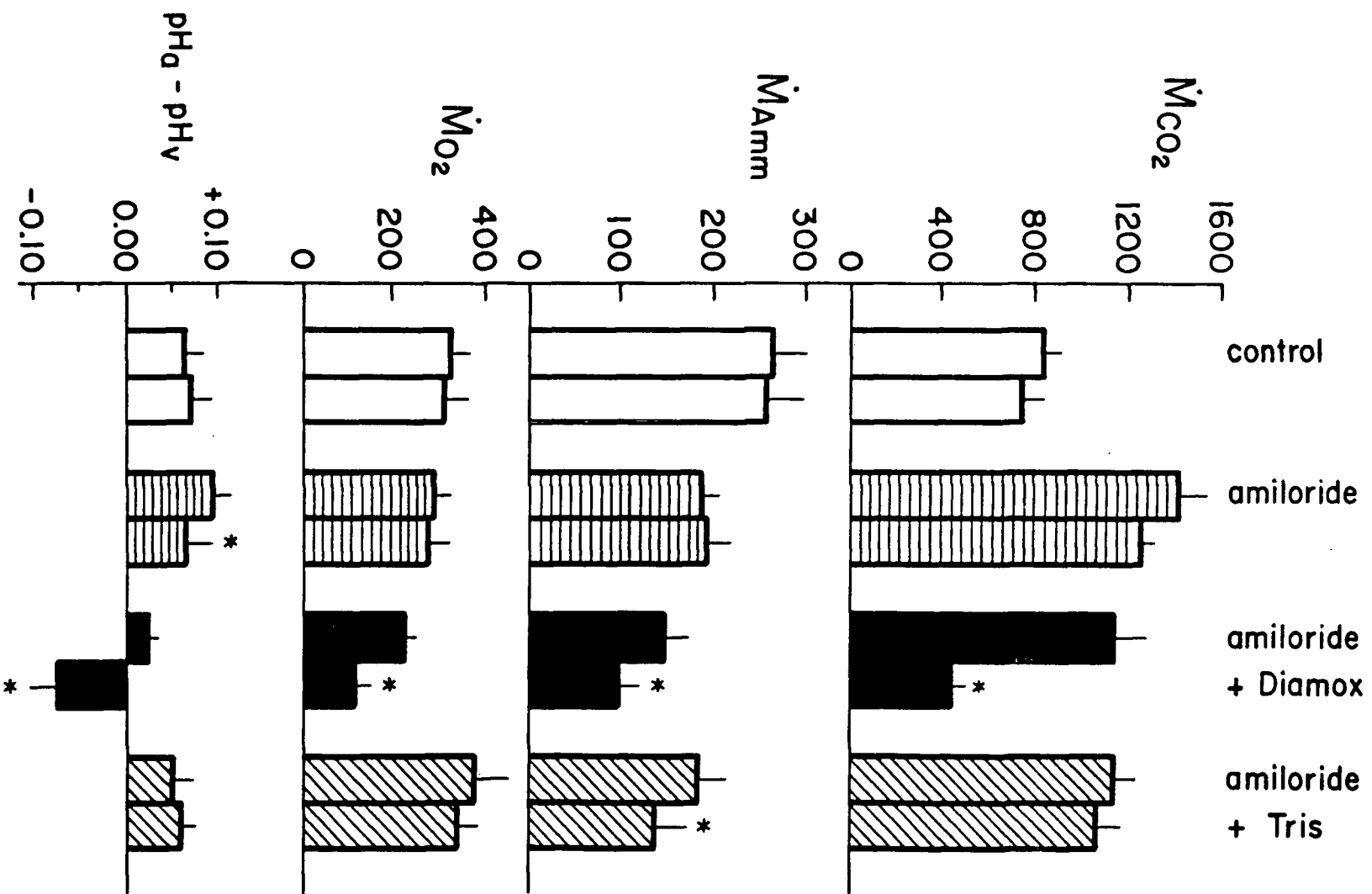
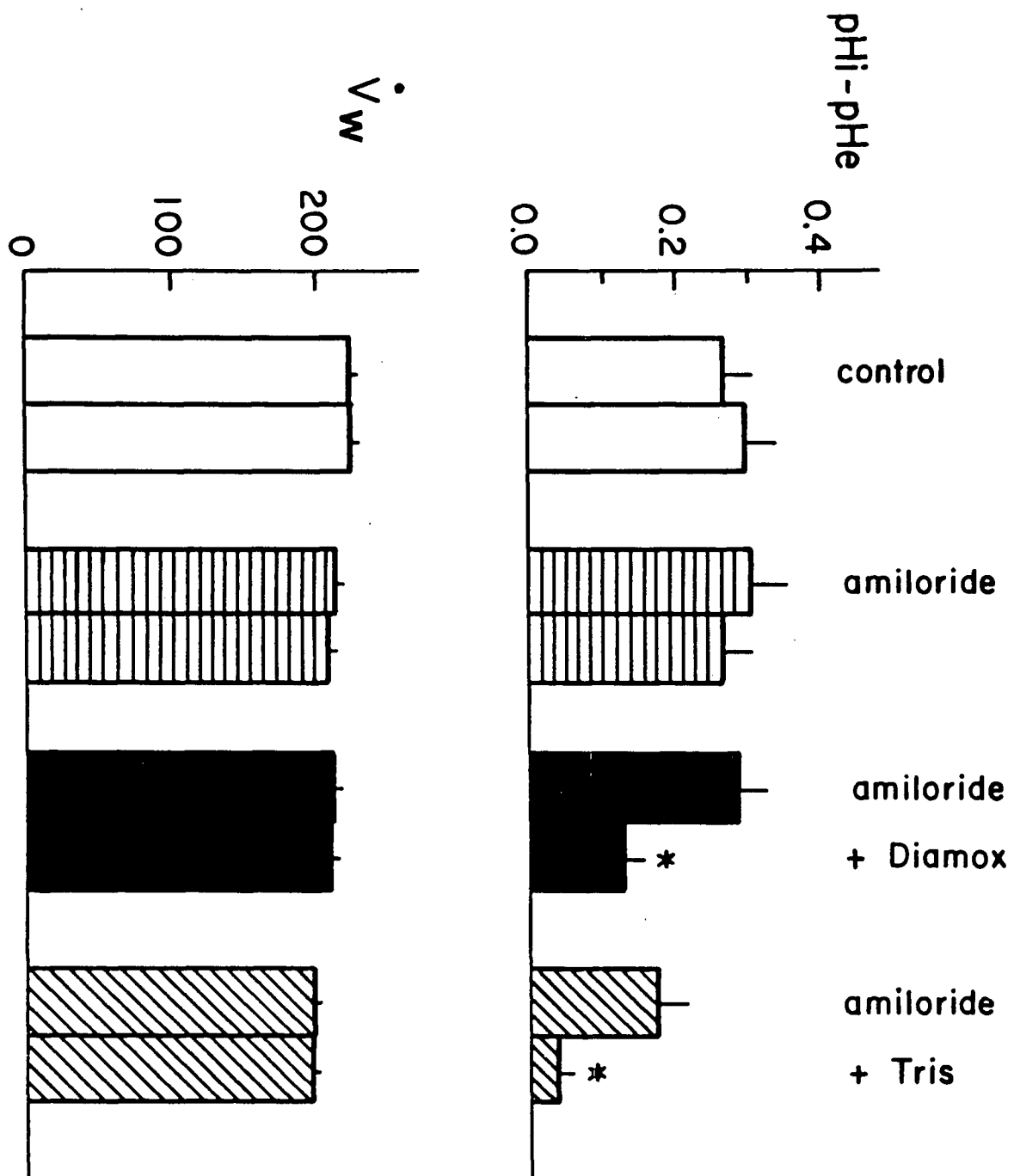


Figure 18. Water pH_I - pH_E difference and ventilation (\dot{V}_W) in $\text{ml} \cdot \text{min}^{-1} \cdot \text{fish}$. See Fig. 16 legend for details.



amiloride in the water reduced \dot{M}_{Amm} by about 30% relative to the initial control experiment, and resulted in a stimulation of \dot{M}_{CO_2} (~+70%).

With amiloride in the water throughout and acetazolamide in the blood in the experimental period (Diamox experiment), \dot{M}_{CO_2} and \dot{M}_{Amm} were reduced by 60% and 55%, respectively, relative to the control period (Fig. 17). As well, there was a significant decrease in \dot{M}_{O_2} (50%, Fig. 17). The pH_a-pH_v difference was reversed with the acetazolamide treatment (Fig. 17), while the pH_I-pH_E difference was cut in half (Fig. 18).

In the fourth experiment, Tris was added to the water in the experimental period (amiloride was present throughout the experiment). This resulted in a significant reduction in \dot{M}_{Amm} (~30%), in the absence of any change in \dot{M}_{CO_2} (Fig. 17). There were no changes in \dot{M}_{O_2} nor the pH_a-pH_v difference (Fig. 17). The pH_I-pH_E difference was almost eliminated with Tris buffer in the water (Fig. 18).

Section B: Comparisons between the blood-perfused trout head and in vivo gas exchange values

Table 16 compares gas transfer variables in the blood-perfused head preparation (in vitro) with those in intact fish (in vivo) with oral masks, opercular cannulae, arterial and venous cannulae, or in fish with only arterial and venous cannulae. \dot{M}_{O_2} and CaO_2-CvO_2 in the blood-perfused head.

TABLE 16. Comparison of gas transfer variables in vivo and blood perfused trout head based on arterial-venous differences. Data are expressed as means \pm S.E.M (N)

PARAMETER	BLOOD-PERFUSED HEAD	* <u>IN VIVO</u>	* <u>IN VIVO</u>
\dot{V}_b (ml.min ⁻¹ kg ⁻¹)	11.10 \pm 0.39 (10)	~17.6	~17.6
CaO ₂ (mmol.l ⁻¹)	1.73 \pm 0.06 (10)	1.84 \pm 0.42 (8)	2.63 \pm 0.23 (5)
CvO ₂ (mmol.l ⁻¹)	1.26 \pm 0.05 (10)	0.31 \pm 0.08 (8)	0.53 \pm 0.12 (5)
CaO ₂ -CvO ₂ (mmol.l ⁻¹)	0.48 \pm 0.06 (10)	1.53 \pm 0.35 (8)	2.10 \pm 0.25 (5)
\dot{M}_{O_2} (mmol.kg ⁻¹ h ⁻¹)	0.33 \pm 0.04 (10)	¹ 1.81 (8)	¹ 2.48 (5)
PaO ₂ (Torr)	86.5 \pm 7.0 (10)	52.1 \pm 12.1 (8)	85.3 \pm 12.6 (6)
PvO ₂ (Torr)	37.2 \pm 1.6 (10)	12.0 \pm 1.1 (8)	13.8 \pm 1.5 (6)
PaO ₂ -PvO ₂ (Torr)	49.0 \pm 3.7 (10)	37.8 \pm 12.3 (8)	73.2 \pm 14.4 (6)
O ₂ Extraction efficiency (%)	41.6 (10)	28.5 (8)	51.8 (6)
CaCO ₂ (mmol.l ⁻¹)	9.68 \pm 0.32 (10)	7.53 \pm 0.72 (8)	6.35 \pm 0.56 (6)
CvCO ₂ (mmol.l ⁻¹)	10.88 \pm 0.30 (10)	8.81 \pm 0.78 (8)	8.63 \pm 0.50 (6)
CaCO ₂ -CvCO ₂ (mmol.l ⁻¹)	1.25 \pm 0.08 (8)	1.29 \pm 0.20 (8)	2.06 \pm 0.45 (5)

TABLE 16. (continued)

PARAMETER	BLOOD-PERFUSED HEAD	* <u>IN VIVO</u>	* <u>IN VIVO</u>
\dot{M}_{CO_2} (mmol.kg ⁻¹ h ⁻¹)	0.80 ± 0.07 (10)	¹ 1.52 (8)	² 2.43 (5)
RE	2.91 ± 0.5 (10)	0.84 (8)	0.98 (5)
[Tamm]a (mmol.l ⁻¹)	0.74 ± 0.07 (10)	0.40 ± 0.06 (7)	0.35 ± 0.03 (6)
[Tamm]v (mmol.l ⁻¹)	1.13 ± 0.07 (10)	0.53 ± 0.06 (7)	0.51 ± 0.05 (6)
[Tamm]a-[Tamm]v (mmol.l ⁻¹)	0.40 ± 0.05 (10)	0.13 ± 0.02 (7)	0.16 ± 0.03 (6)
\dot{M}_{amm} (mmol.kg ⁻¹ h ⁻¹)	0.26 ± 0.04 (10)	¹ 0.15 (7)	² 0.18 (6)
pHa	8.08 ± 0.02 (10)	7.87 ± 0.06 (8)	8.01 ± 0.03 (6)
pHv	8.01 ± 0.02 (10)	7.79 ± 0.06 (8)	7.85 ± 0.06 (6)
pHa-pHv	0.07 ± 0.02 (10)	0.09 ± 0.03 (8)	0.15 ± 0.05 (6)
hct (%)	14.3 ± 0.3 (10)	~20	23.3 ± 1.4 (6)
water temp (°C)	9.5	12	12

* Fish were fitted with oral masks, opercular cannulae, and dorsal aortic and ventral aortic cannulae.

^ Kiceniuk & Jones (1977)

1 estimated values based on \dot{V}_b from Kiceniuk & Jones (1977).

2 Fish fitted with dorsal and ventral aortic cannulae only.

were 70-80% lower and Cv_{O_2} , 2-4 times higher, than in vivo values. Oxygen tensions (Pa_{O_2} , Pv_{O_2}) and O_2 extraction efficiencies in vitro and in vivo were approximately equal. Carbon dioxide contents (Ca_{CO_2} , Cv_{CO_2}) in the blood were slightly higher in vitro compared to in vivo, and \dot{M}_{CO_2} was 50-70% lower in the blood-perfused head. The respiratory exchange ratio ($RE = \dot{M}_{CO_2} / \dot{M}_{O_2}$) in vitro was 2.91, a value that was three times greater compared to the in vivo value. Arterial Tamm, venous Tamm, and the arterial-venous Tamm difference were 2-3 times greater in vitro relative to in vivo. \dot{M}_{Amm} in vitro was ~60% greater than in vivo values. The $pHa-pHv$ difference was slightly lower in vitro (-0.02 to -0.08 pH units), as a result of the higher pHv value (+0.2 pH units). The hct value was 10% lower in vitro relative to in vivo.

The water gas transfer variables (in vitro and in vivo) are given in Table 17. The in vitro ventilation rate (\dot{V}_w) was relatively high (~900 ml.kg⁻¹.min⁻¹), but \dot{V}_w in vivo was set to a similar level (~1200 ml.kg⁻¹.min⁻¹), whereas resting in vivo values were considerably lower (~400 ml.kg⁻¹.min⁻¹, Wright et al. (1986)). \dot{M}_{O_2} in vivo was 2-3 fold and \dot{M}_{CO_2} about 1.4 fold that measured in the blood-perfused preparation. The RE value in vitro was 1.93, while in vivo values were approximately 1. \dot{M}_{Amm} was approximately 0.6 mmol.kg⁻¹.h⁻¹ in both in vitro and in vivo at similar \dot{V}_w rates, but was 50% less at lower \dot{V}_w rates in vivo (Wright et al., 1986). O_2 utilization was 6% in vitro, 14% in

TABLE 17. Comparison of gas transfer variables in vivo and perfused preparations based on inspired-mixed expired water differences. Means \pm S.E.M. (N)

PARAMETER	BLOOD-PERFUSED HEAD	* <u>IN VIVO</u>	^{1,2} <u>IN VIVO</u>
\dot{V}_w (ml kg ⁻¹ min ⁻¹)	869.0 \pm 35.3 (7)	1167.0 \pm 52.7 (10)	418.3 \pm 24.0 (8)
\dot{M}_{O_2} (mmol kg ⁻¹ h ⁻¹)	1.00 \pm 0.19 (6)	3.14 \pm 0.32 (10)	1.73 \pm 0.06 (18)
\dot{M}_{CO_2} (mmol kg ⁻¹ h ⁻¹)	1.93 \pm 0.22 (6)	2.90 \pm 0.50 (10)	2.39 \pm 0.27 (8)
RE = $\frac{\dot{M}_{CO_2}}{\dot{M}_{O_2}}$	1.93	0.94	1.38
\dot{M}_{amm} (mmol · kg ⁻¹ · h ⁻¹)	0.57 \pm 0.08 (7)	0.64 \pm 0.13 (10)	0.27 \pm 0.05 (8)
P _i O ₂ (Torr)	155.9 \pm 2.4 (7)	144.4 \pm 2.0 (10)	160.3 \pm 0.5 (18)
P _e O ₂ (Torr)	147.1 \pm 3.0 (7)	124.0 \pm 3.4 (10)	86.1 \pm 2.3 (18)
O ₂ Utilization (%)	5.7 \pm 0.9 (7)	14.0 \pm 1.4 (10)	46.0 \pm 1.5 (18)
pH _i -pH _e	0.28 \pm 0.04 (7)	0.74 \pm 0.11 (10)	0.69 \pm 0.04 (8)

* Fish were fitted with oral masks, opercular catheters, and dorsal aortic and ventral aortic cannulae. A pressure head was established in the anterior compartment of the two-chambered box to raise \dot{V}_w to levels comparable to the blood-perfused head.

1. Data from Wright et al. (1986) also see Chapter 2 and 3A. Trout were fitted with oral masks and opercular catheters.

2. Data from Cameron and Davis (1970). Trout were fitted with oral masks and dorsal aortic cannulae.

TABLE 18. Comparison between gas transfer variables calculated from arterial-venous blood differences with those calculated from inspired-expired water differences in the isolated blood-perfused head preparation (in vitro) and in intact fish (in vivo). Means \pm S.E.M. (N)

<u>IN VITRO</u>	<u>WATER</u>	<u>BLOOD</u>
\dot{V}_w, \dot{V}_b (ml.kg ⁻¹ min ⁻¹)	869.5 \pm 35.3 (7)	11.61 \pm 0.36 (7)
\dot{M}_{O_2} (mmol.kg ⁻¹ h ⁻¹)	1.00 \pm 0.19 (6)	0.32 \pm 0.04 (10)
\dot{M}_{CO_2} (mmol.kg ⁻¹ h ⁻¹)	1.93 \pm 0.22 (6)	0.80 \pm 0.07 (10)
RE	1.93	2.42
\dot{M}_{amm} (mmol.kg ⁻¹ h ⁻¹)	0.57 \pm 0.08 (7)	0.26 \pm 0.04 (10)
<u>IN VIVO</u>		
\dot{V}_w, \dot{V}_b (ml.kg ⁻¹ min ⁻¹)	1167.0 \pm 52.7 (10)	17.6 ¹
\dot{M}_{O_2} (mmol.kg ⁻¹ h ⁻¹)	3.14 \pm 0.32 (10)	1.81 ¹ (8)
\dot{M}_{CO_2} (mmol.kg ⁻¹ h ⁻¹)	2.90 \pm 0.50 (10)	1.52 ¹ (8)
RE	0.94	2.42 ¹
\dot{M}_{amm} (mmol.kg ⁻¹ h ⁻¹)	0.64 \pm 0.13 (10)	0.15 ¹ (7)

1. estimated values based on \dot{V}_b from Kiceniuk & Jones (1977)

vivo at high \dot{V}_w , and 46% at lower \dot{V}_w rates. The pH_I - pH_E difference was about 0.7 pH units in vivo, but only 0.3 pH units in vitro.

A comparison between gas transfer variables calculated from arterial-venous blood differences and inspired-mixed expired water differences is given in Table 18 for the blood-perfused head preparation and intact trout. \dot{M}_{O_2} , \dot{M}_{CO_2} , and \dot{M}_{Amm} calculated from water variables were 2-4 times greater than those values calculated from blood parameters. Respiratory exchange ratios (RE) calculated from blood or water considerations were similar (RE= in vitro 1.93 vs 2.42, in vivo 0.94 vs 0.84).

In Table 19, transfer factors were calculated for CO_2 , O_2 , and NH_3 in the blood-perfused head preparation and in intact fish. T_{O_2} was 5 times greater in intact fish (~0.007) compared to the blood-perfused head (0.0014). T_{CO_2} in vitro was 0.15 and in vivo was 0.22. T_{NH_3} values were several orders of magnitude greater than transfer factors for O_2 and CO_2 . The in vitro value was 288 versus 455, in vivo. The in vitro ratio for T_{CO_2}/T_{O_2} and T_{NH_3}/T_{O_2} were 3 times those in vivo, while T_{NH_3}/T_{CO_2} were approximately equal between the perfused head and intact animal.

TABLE 19. Transfer factor, T, for O₂, CO₂, and ammonia across the gill, in ml.min⁻¹.kg⁻¹.h⁻¹.Torr⁻¹.
 T = (rate of uptake or excretion)/(mean gradient across the gills). Theoretical ratios were calculated from the ratio of Krogh's diffusion coefficients for respective gases in water (K= diffusion coefficient x solubility coefficients). See text for details.

PARAMETER	BLOOD PERFUSED TROUT HEAD	<u>IN VIVO</u>	<u>IN VIVO</u> ^{1,2,3}	
T _{O2}	0.0014	0.0066	0.006 ¹ 0.02-0.03 ² 0.0092 ³	
T _{CO2}	0.1522	0.2247	0.42 ³	
T _{NH3}	288	455	-	
				<u>THEORETICAL</u>
$\frac{T_{CO2}}{T_{O2}}$	109	34	46-58 ³	23
$\frac{T_{NH3}}{T_{O2}}$	206,000	69,000	-	32,600
$\frac{T_{NH3}}{T_{CO2}}$	1890	2020	-	1400

1. Stevens & Randall (1967), rainbow trout (Salmo gairdneri)
2. Holeyton & Randall (1967); Randall, Holeyton, & Stevens (1967), rainbow trout (Salmo gairdneri)
3. Piiper & Baumgarten-Schumann (1968), dogfish (Scyliorhinus stellaris)

DISCUSSION

Evaluation of the blood-perfused trout head preparation

The present study demonstrates that the blood-perfused trout head preparation is a viable technique for studying the relationship between carbon dioxide and ammonia excretion in fish. Several modifications have been made to the preparation which enhance the preparation's ability to simulate in vivo conditions. In preliminary experiments, the head was initially perfused with saline containing the colloid osmotic filler, PVP (see Materials & Methods), also used by Wood (1974), Davie (1981), Perry et al. (1984b), and Bornancin et al. (1985). Many problems arose with PVP in the saline including blood leaks at the gills, incomplete perfusion of gill arches, and very high blood pressure values. In later experiments, these problems were eradicated by replacing PVP with the natural protein, bovine serum albumin (BSA).

In terms of ventilation, inspired water was delivered through a mouth tube tightly secured inside the buccal chamber, in a central position, and at a rate comparable to swimming fish (Kiceniuk & Jones, 1977). The pattern of water flow was assessed visually before and after measurements by adding a nontoxic dye to the inspired water to ensure that both opercular chambers were evenly irrigated. In other perfused-head preparations (see Perry et al., 1984a for review), a mouth tube is simply placed in the

opening of the mouth. In preliminary tests (see Materials & Methods), this technique was shown to be far less effective at delivering an even water flow through both opercular chambers. Also, previous investigators have typically set \dot{V}_w at 500-1000 ml.min⁻¹ (Payan & Matty, 1975; Payan, 1978; Perry et al., 1984b; Bornancin et al., 1985; Perry et al., 1985a,b), rates 2.5 times \dot{V}_w in the present study and far in excess of the normal range (Kiceniuk & Jones, 1977; Wright et al., 1986; Iwama et al., 1987). The perfused head in the present study was irrigated with a large recirculating water reservoir (10 l) or flow-through water (control), in contrast to other studies which report a recirculating volume of 100-200 ml (Payan & Matty, 1975; Payan, 1978; Perry et al., 1984b; Bornancin et al., 1985; Perry et al., 1985a,b). These low volumes are too small to maintain optimal O₂, CO₂, and NH₃ gradients across the gills over the experimental period.

The blood-perfused trout head has been shown to be far superior to the saline-perfused trout head preparation in terms of gas transfer and acid-base status (Perry et al., 1985a). It was the purpose of this study, however, to investigate the ability of the blood-perfused head preparation to exchange gases compared to intact fish. The rate of O₂ uptake in the blood-perfused preparation was considerably lower than in vivo, but equal to that reported in other blood-perfused preparations (Perry et al., 1982; Perry et al., 1985a). The arterial-venous P_{O2} difference and O₂ extraction efficiency were analogous to

in vivo values, however, the arterial-venous O_2 content difference was lower. The lower \dot{M}_{O_2} in the blood-perfused head is probably not related to impaired diffusion because of the normal gill extraction efficiency, but may be due to the low O_2 carrying capacity of the blood. Cameron and Davis (1970) reported that \dot{M}_{O_2} in anaemic fish (hct= 8%) was not different from normal fish (hct= 27%), however, cardiac output was 8 times higher, and 5 times greater compared to \dot{V}_b in the present study. Hence, the reduced \dot{M}_{O_2} in the perfused head is probably due to low blood hct values which resulted in perfusion limitations under resting blood flow (\dot{V}_b) conditions.

Carbon dioxide excretion was also less in the blood-perfused head compared to in vivo, although similar to published values for perfused preparations with blood of the same hct (Perry et al., 1982). There is a strong positive correlation between \dot{M}_{CO_2} and hct (Perry et al., 1982), which probably explains the lower in vitro \dot{M}_{CO_2} values in the present study. The hct value used in this study (14%) was higher than in other blood-perfused trout preparations (9-11%, Perry et al. (1984a); Perry et al. (1985a,b)); however, it is obvious that to achieve in vivo gas exchange rates hct must be closer to in vivo values (20-30%).

RE values in perfused preparations are consistently greater than 1.0 (this study, 2.9; Perry et al., 1982, 1.7; Perry et al., 1985a, 2.5), while in vivo values range between 0.8 and 1.0. RE values in vivo reflect the rate of CO_2 production relative to O_2 consumption, while those in vitro are generally related to

O₂ and CO₂ venous input levels which will determine \dot{M}_{O_2} and \dot{M}_{CO_2} .

In contrast to \dot{M}_{O_2} and \dot{M}_{CO_2} , ammonia excretion was 60% higher in the blood-perfused preparation compared to in vivo, which is related to the greater venous Tamm levels. Blood Tamm levels vary greatly in fish depending on feeding behaviour, ranging from 0.13 mmol.l⁻¹ in starved fish (Wright & Wood, 1985) to 0.85 mmol.l⁻¹ in fed fish (Perry & Vermette, 1987). Venous Tamm levels were set to a relatively high level in the perfused head to ensure \dot{M}_{Amm} was in the normal range (200-350 μ mol.kg⁻¹.h⁻¹, McDonald & Wood, 1981; Cameron & Heisler, 1983; Wright & Wood, 1985; Vermette & Perry, 1987).

In general, the rates of gas transfer calculated from inspired-mixed expired water differences were lower in vitro versus in vivo. As before, the differences were not proportional, that is, \dot{M}_{O_2} was most affected, followed by \dot{M}_{CO_2} , and then \dot{M}_{Amm} . The % utilization of O₂ from water flowing over the gills is a reflection of the amount of water involved in gas exchange. Oxygen utilization was low in vitro (6%), but was closer to the high \dot{V}_w in vivo value (14%), than the resting \dot{V}_w value (46%). Percent utilization is inversely related to \dot{V}_w in trout restrained in VanDam-type chambers (similar to Fig. 2) (Cameron & Davis, 1970).

Comparisons between the inspired-mixed expired water pH difference between in vivo and in vitro values are difficult. The magnitude of water acidification will depend on \dot{V}_w , \dot{M}_{CO_2} ,

and \dot{M}_{Amm} , among other things, and these values were not the same between in vitro and in vivo experiments.

A comparison between gas transfer rates calculated from water versus blood differences in the blood-perfused preparation and intact fish shows that \dot{M}_{O_2} , \dot{M}_{CO_2} , and \dot{M}_{Amm} are greater when calculated from water considerations (Table 18). Payan & Matty (1975), in the saline-perfused trout head, and Cameron & Heisler (1983) in intact trout, found that \dot{M}_{Amm} was 5-20% greater when calculated from inspired and mixed expired water differences compared to arterial-venous differences. They proposed that the discrepancy between calculated \dot{M}_{Amm} values was due to ammonia production in gill epithelium. The reason for the large discrepancies in the present study cannot be solely related to endogenous metabolism in gill epithelial tissue and may be related to the sampling technique. Blood samples are assumed to be representative because they are collected from a small, homogenous pool. On the other hand, mixed expired water samples are collected from a larger pool, which may or may not be homogenous. Although the opercular cannulation technique has been validated for $\text{pH}_I\text{-pH}_E$ differences at resting V_w rates in intact fish (see General Materials & Methods and chapter 2), in the absence of rhythmical ventilatory movements and at higher ventilation rates, a greater proportion of ventilatory water is shunted past respiratory surfaces and water transit time in the opercular chamber is reduced. Thus, samples collected from the opercular cannula may not always be representative of mixed

expired water. It is assumed, therefore, that calculated \dot{M}_{CO_2} , \dot{M}_{O_2} , and \dot{M}_{Amm} values are more valid when determined from arterial-venous differences than inspired-mixed expired differences in the present study.

The calculation of transfer factors (T) allows comparisons between the ability of a respiratory surface to exchange gases. Carbon dioxide and ammonia were transferred far more efficiently than oxygen. Oxygen is much less soluble in water relative to the two other respiratory gases, CO_2 ($\alpha_{O_2}/\alpha_{CO_2} = 1/30$) and NH_3 ($\alpha_{O_2}/\alpha_{NH_3} = 1/24,000$). Transfer is also dependent on the magnitude of the gradient across the gills and the rate of reactions, such as, the O_2 -haemoglobin and $HCO_3^-:CO_2$ interconversion reactions. In general, for a given gradient between blood and water, less O_2 will be transferred compared to CO_2 or NH_3 . This also implies that if gas transfer across the gills is diffusion limited, then O_2 uptake will be most effected. It is also interesting to note the tremendous difference between T_{NH_3} and either T_{CO_2} or T_{O_2} . Part of this difference may be due to the fact that ammonia is also eliminated at the gills as NH_4^+ , which would overestimate the T_{NH_3} value. However, even if 50% of ammonia was excreted as NH_4^+ and this was accounted for, T_{NH_3} would only be reduced by 1/2 and would still be several orders of magnitude greater than T_{CO_2} or T_{O_2} . Thus, the large T_{NH_3} value is primarily due to ammonia's greater solubility in water compared to O_2 or CO_2 .

Theoretical ratios of transfer factors were calculated in Table 19, from the ratios of Krogh's permeation coefficients ($K = \text{diffusion coefficient} \times \text{solubility coefficient}$). This analysis compares transfer of gas in water and does not account for diffusion through various tissue membranes, transfer of ion species, as well as chemical reactions implicit in O_2 , CO_2 , and NH_3 transport. As well, this analysis does not account for perfusion limitations to gas exchange. Gas exchange may be either perfusion or diffusion limited, or both, depending on the conditions for transfer (see Chapter 3). Any combination of these factors may lead to inequalities between T factors. Despite the simplicity of the analysis, it is amazing that T_{CO_2}/T_{O_2} and T_{NH_3}/T_{CO_2} ratios in trout only vary by a factor of ~ 1.4 from the theoretical ratios expected for simple diffusion in an "ideal" system. Piiper and Baumgarten-Schumann (1967) found that in dogfish the T_{CO_2}/T_{O_2} ratio was 2 times that expected from a similar theoretical analysis. The similarity between theoretical and in vivo or in vitro values implies that physical properties of gases play a major role in the efficiency of transfer.

To summarize, oxygen transfer is limited in the isolated blood-perfused head probably because of the low carrying-capacity of the blood. Carbon dioxide and ammonia transfer were quantitatively less in the perfused head than in intact animals, but the ratio of T_{NH_3}/T_{CO_2} was equal to that found in vivo. It can be concluded, therefore, that the blood-perfused trout

head is a suitable preparation for the study of the relationship between carbon dioxide and ammonia excretion.

The linkage between carbon dioxide and ammonia excretion

In the perfused head experiments, it was important to isolate NH_3 from NH_4^+ excretion in order to determine if NH_3 diffusion across the gill was linked to CO_2 excretion. In the amiloride control experiment, mean ammonia excretion decreased by 30% relative to the control experiment, which equals that reported by Kirschner et al. (1973) for the saline perfused trout head preparation and compares well with the 23% reduction reported in vivo (Wright & Wood, 1985; also see Chapter 3). The stimulation of carbon dioxide excretion with amiloride may be related to removal of CO_2 from the gill epithelium due to acidification as a result of inhibition of H^+ excretion. (H^+ ions may be excreted directly by Na^+/H^+ exchange, or may be excreted as NH_4^+ , also linked to Na^+ uptake.) The significant reduction in the $\text{pH}_a\text{-pH}_v$ difference indicates that the effects of the inhibition of H^+ ion excretion on the blood compartment were delayed and only apparent in the second amiloride measurement.

Acetazolamide dramatically reduced \dot{M}_{CO_2} and \dot{M}_{Amm} (Fig. 17) which demonstrates chemical coupling between ammonia and carbon dioxide excretion. The concomittant decrease in oxygen uptake was probably a direct result of the decrease in \dot{M}_{CO_2} , because O_2 and CO_2 gas exchange are related through chemical

reactions involving haemoglobin in the red cell (Maren & Swenson, 1980). The reversal of the pH_a - pH_v difference with acetazolamide is attributable to higher P_{CO_2} levels in arterial blood due to the inhibition of \dot{M}_{CO_2} . It should be noted that the decrease in arterial pH did not effect NH_3 diffusion gradients because the calculated arithmetic arterial-venous mean P_{NH_3} values did not change significantly between the control (214 ± 23 uTorr) and acetazolamide (190 ± 20 uTorr) measurement. The pH_I - pH_E difference (Fig. 18) was reduced as a direct result of the depression in CO_2 excretion and subsequent reduction in CO_2 hydration in the expired water.

It was possible that the link between CO_2 and ammonia excretion occurred internally, similar to the coupling between CO_2 and O_2 in the red cell, rather than in the boundary layer on the external surface of the gill. Tris buffer was added to the water in the final experiment to test whether the link between CO_2 and ammonia was due to chemical reactions in the boundary layer. (It was assumed that given the flow rate of water over the gills and the relatively small volume of the buccal and opercular chambers, that Tris in the bulk phase water reached the boundary layer water in a relatively short time.) Tris caused a significant reduction in ammonia excretion in the absence of any change in CO_2 excretion. Since CO_2 excretion and O_2 uptake did not change with the Tris treatment, it is unlikely that the change in \dot{M}_{Amm} was due to direct effects of Tris on gill cell membrane permeability. Rather the decrease in

M_{Amm} was probably because H^+ ions normally available in the boundary layer from the CO_2 hydration reaction were buffered by Tris. This is also demonstrated by the fact that the pH_I - pH_E difference was virtually eliminated with Tris in the water (Fig. 18). These experiments establish that NH_3 diffusion is linked to CO_2 excretion in the gill boundary layer, and that H^+ ions produced from the catalyzed CO_2 hydration reaction are used to protonate NH_3 , which in turn facilitates NH_3 excretion (Fig. 15). These results do not preclude the possibility of an internal link between CO_2 and ammonia excretion, however they demonstrate that the external boundary layer is a site of linkage between carbon dioxide and ammonia excretion.

The CO_2 hydration reaction in the external boundary layer enhances the blood-to-water NH_3 diffusion gradient and facilitates ammonia excretion across the gill. This facilitation of ammonia excretion indicates that under the conditions of the present study, ammonia transfer was diffusion limited. This does not necessarily mean that ammonia excretion is not also perfusion limited (see Chapter 3). Based on theoretical considerations, one would expect that transfer of ammonia would be largely perfusion limited due to ammonia's relatively high Krogh's permeation coefficient. This study demonstrates, however, that a significant component of ammonia transfer is diffusion limited. One would predict that the effect of changes in CO_2 excretion on NH_3 diffusion will be more pronounced at lower \dot{V}_w rates because boundary layer thickness will be increased. Piiper et

al. (1986) have estimated the thickness of the boundary layer in dogfish (Scyliorhinus stellaris) at rest ($\dot{V}_w = \sim 200$ mlmin⁻¹kg⁻¹) and during swimming, where \dot{V}_w rates were similar to the present study ($\dot{V}_w = \sim 900$ mlmin⁻¹kg⁻¹). They found that at rest the thickness of the boundary layer was about 20% greater than that in swimming fish. A 20% increase in the boundary layer would increase the diffusion pathway for NH₃ and accentuate the importance of chemical removal of NH₃ through interactions with the CO₂ hydration reaction in the boundary layer.

For ammonia excretion to be facilitated by the catalysed CO₂ hydration reaction in the gill water boundary layer, the apical epithelial membrane must be relatively impermeable to ion species. If the apical membrane was highly permeable to NH₄⁺, HCO₃⁻, or H⁺ ions, then NH₃ levels in the water boundary layer would always be low, and there would be no link between carbon dioxide and ammonia excretion. The fact that this linkage has been demonstrated indicates that the gill water boundary layer is a distinct "microclimate" and that the apical membrane is relatively impermeable to ions. This fits in with accepted theory of freshwater gill epithelium ion permeabilities (see Girard & Payan, 1980 and Potts, 1984 for reviews).

The significance of the link between carbon dioxide and ammonia will be greatest for fish in alkaline waters. For instance, several species of Tilapia inhabit the alkaline lakes of the Great Rift Valley in Africa where water pH may range

between 9.6 and 10.5 (Johansen et al., 1975). In these waters, the pK of ammonia will be approximately 9, the $\text{NH}_3:\text{NH}_4^+$ ratio will be 50% or greater, and NH_3 levels in water near the gill surface may be extremely high (see General Discussion). Hence, the rate of CO_2 excretion may become extremely important in order to maintain an acid boundary layer next to the gill which in turn will facilitate ammonia removal from the blood. The interaction between carbon dioxide and ammonia excretion would enable these fish to survive in such extreme environmental conditions.

In acidic environments, there will be little HCO_3^- formed as CO_2 is excreted into the gill water boundary layer. Under these conditions NH_3 excretion may alkalize the boundary layer relative to the bulk water. The transition from an acid to a more alkaline water boundary layer will depend on the relative excretion rates of CO_2 and NH_3 and the pK of these molecules.

Is the reverse situation also true, that is, that NH_3 diffusion into the boundary layer facilitates CO_2 excretion by mopping up H^+ ions and enhancing the blood-to-water P_{CO_2} gradients? The fact that \dot{M}_{CO_2} did not change with an increase in inspired water buffering capacity by ~ 3 times (Tris experiment), implies that under these conditions, increases in \dot{M}_{Amm} would probably not affect the rate of CO_2 excretion. It may be, however, that NH_3 diffusion acts as an important buffer in the boundary layer under other conditions, such as, in poorly buffered waters, at maximal CO_2 excretion rates, at low \dot{V}_w

rates, and when boundary layer thickness is increased.

GENERAL DISCUSSION

This thesis has examined ammonia stores and excretion in fish, with respect to pH gradients within the body and between the body and the environment. The results demonstrate that the distribution of ammonia between red cells and plasma in fish is dependent on transmembrane pH gradients under resting conditions. Red cells are the exception, however, and in other tissue compartments ammonia stores are not determined by pH gradients, indicating that the general theory of non-ionic diffusion for weak electrolytes is not valid in these tissues. Although shifts in intracellular-to- extracellular pH gradients will not directly alter intracellular ammonia levels in these tissues, changes in extracellular pH will indirectly alter tissue ammonia stores if they are accompanied by changes in plasma ammonia concentrations. For example, when fish are exposed to alkaline waters, blood pH and plasma ammonia content increase, while the reverse is true for fish in acid waters (Wright & Wood, 1985). Because ammonia is passively distributed between plasma and intracellular compartments according to the membrane potential, alkaline conditions will increase, and acid conditions will reduce intracellular ammonia levels.

It is commonly stated in the literature that ammonia, as well as carbon dioxide, are distributed in the body according to pH gradients between tissue compartments (eg. Jacobs & Stewart, 1936; Milne et al., 1958; Pitts, 1973; Cameron & Heisler, 1983;

Milligan & Wood, 1986b; Remesey et al., 1986; Randall & Wright, 1987; Wright & Randall, 1987). Measured muscle, heart, and brain ammonia levels in sole were 10-20 times higher than those predicted from pH gradients. Bicarbonate tissue stores were also calculated from pH gradients assuming that the non-ionic diffusion theory describes the distribution of carbon dioxide between body tissues. Muscle and heart levels were about 4 times greater than calculated bicarbonate levels in these tissues ($1.5-1.9 \text{ mmol.l}^{-1}$), and measured brain ammonia levels were slightly less than calculated brain bicarbonate concentrations (4.7 mmol.l^{-1}). Bicarbonate ions are transferred across tissue membranes by ion exchange processes (eg. $\text{Cl}^-/\text{HCO}_3^-$), but the rate of transfer across membranes is thought to be extremely slow, with the exception of red cell membranes (Heming, 1984). If HCO_3^- ions were more permeable across muscle, heart, and brain tissue membranes than generally accepted, the membrane potential would influence intracellular HCO_3^- levels. If this is the case, the measured total carbon dioxide tissue levels will be lower than predicted from pH gradients. Intuitively this makes sense because cells are negatively charged with respect to extracellular fluid ($-E_m$), and therefore cells will sequester NH_4^+ ions and repel HCO_3^- ions. Hence, if HCO_3^- permeability approaches NH_4^+ permeability in fish tissues, then the $[\text{NH}_4^+]/[\text{HCO}_3^-]$ ratio will be as high as 50 for heart tissue, and as low as 10 for brain tissue, with the ratio for muscle tissue between these values. The actual ratios in fish

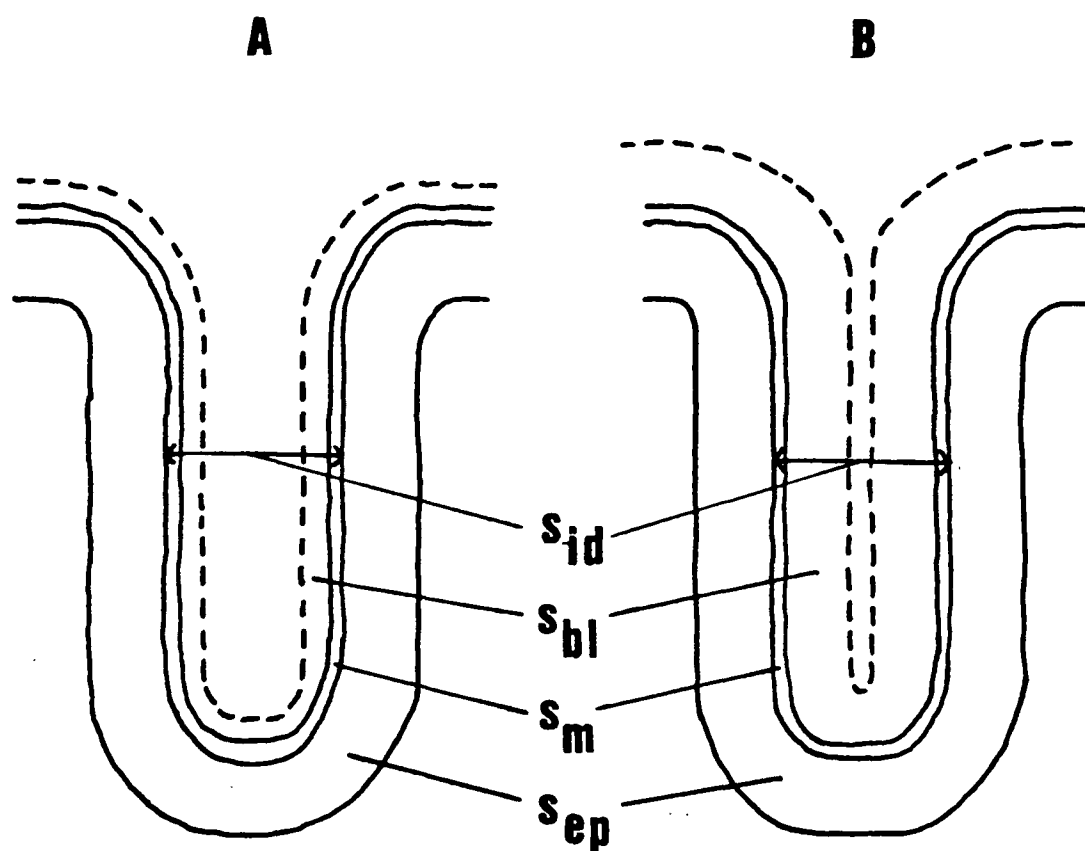
are not known, for HCO_3^- tissue levels are typically derived from the non-ionic diffusion theory (Milligan & Wood, 1986b) or calculated from pH_i and the intracellular nonbicarbonate buffer values (Heisler, 1978). Thus, depending on the HCO_3^- permeability value, the $[\text{NH}_4^+]/[\text{HCO}_3^-]$ will range between 4 and 50 for muscle and ventricle, and between 0.7 and 10 in brain tissue.

The results also show that fish are able to maintain extremely high resting brain ammonia levels with no toxic effect. Mammalian plasma ammonia concentrations are extremely low ($\sim 10 \text{ } \mu\text{mol.l}^{-1}$, Mutch & Bannister, 1983) and only during disease states does ammonia increase in the body with extremely deleterious effects (Lawrence et al., 1957; Stauffer & Scribner, 1957; Sherlock, 1960). The toxic effect of ammonia in vertebrates is linked to an interruption of neural function through the direct action of NH_4^+ ions on electrical activities of neurons (Binstock & Lecar, 1969), diminution of neurotransmitter synthesis (Mutch & Bannister, 1983), suppression of energy-generating processes and depletion of brain ATP levels (Arillo et al., 1981; Benjamin, 1982). While profound toxicity in mammals (convulsions) occurs at brain ammonia levels of $\sim 1 \text{ mmol.l}^{-1}$ (Bannister & Singh, 1980), lemon sole were able to accommodate brain ammonia concentrations of between 2.8 and 5.0 mmol.l^{-1} in the present study. What accounts for this tolerance difference between fish and mammals? The answer to this is unknown, however, ammonia is more toxic to some brain

cells than others (McGilvery, 1983), and there are vast differences between relative quantity and type of neural tissue in mammal and fish. As well, fish may be able to withstand larger fluctuations in body ammonia levels because of efficient detoxifications mechanisms, such as, glutamine and/or urea synthesis (Arillo et al., 1981).

The distribution of ammonia between body compartments in fish is dependent on electrical potential differences across tissue membranes and transfer of ammonia in the body is dependent on both NH_3 and NH_4^+ gradients. Studies have shown that transfer of ammonia across gill epithelium of freshwater fish is also in the NH_3 form (deVooy, 1968; Cameron & Heisler, 1983; Wright & Wood, 1985) and NH_4^+ form (Maetz & Garcia Romeu, 1964; Maetz, 1973; Evans, 1977; Payan, 1978; Wright & Wood, 1985). The results of this thesis demonstrate that ammonia and carbon dioxide excretion are linked through chemical reactions in the gill water boundary layer. This interaction between NH_3 diffusion and the catalyzed CO_2 hydration reaction will be influenced by the depth of the boundary layer at the gill surface. The thickness of the boundary layer was calculated by a method outlined in Appendix II, first described by Piiper et al. (1986). Briefly, the thickness of the unstirred layer next to the gill can be determined by the Fick diffusion equation, that is, the product of the O_2 diffusion and solubility coefficients and the ratio of the secondary lamellar surface area to the diffusing capacity of oxygen in the unstirred water layer. Fig.

Figure 19. A schematic representation of a cross-section through the channel between two secondary lamellae under two different flow conditions; the high flow value is similar to ventilatory flow (\dot{V}_w) during exhaustive exercise, and the low flow is representative of resting conditions. The thickness of the interlamellar distance (s_{ld}), boundary layer (s_{bl}), mucus layer (s_m), and epithelium (s_{ep}) are shown, drawn to scale.



\dot{V}_w ($\text{ml min}^{-1} \text{kg}^{-1}$)	1750	250
initial s_{bl} (μm)	5.7	20.0
adjusted s_{bl} (μm)	4.6	9.6
$1/2 s_{id}$ (μm)	11.5	11.5
ratio $s_{bl}/1/2 s_{id}$	0.43	0.87

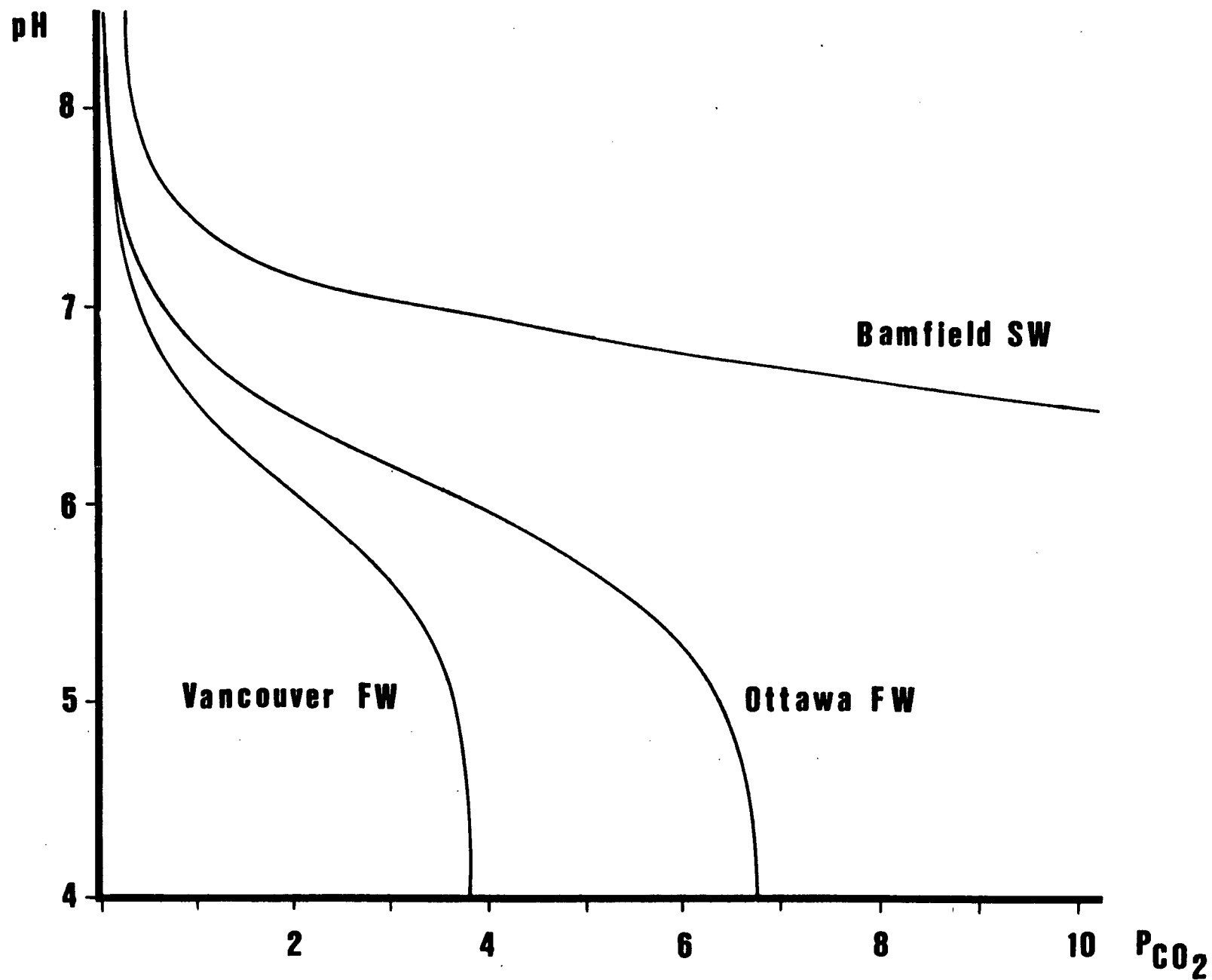
19 illustrates the ratio of the calculated gill water boundary layer to the interlamellar distance under high and low water flow conditions. The gill epithelium is approximately 10 μm thick (Piiper et al., 1986), and the associated external mucus layer is estimated to be about 2 μm in depth. Mucus production varies under different conditions in fish (Ultsch & Gros, 1979) and probably at different gill locations, although exact measurements have not been reported. It is likely that under normal conditions, the mucus layer thickness is in the range of 1-3 μm , because in freeze-fracture methods the mucus layer separates from the epithelium and appears to just cover the morphological heterogeneity of the lamellar surface (Randall, personal communication). The interlamellar distance depends largely on the species of fish; in trout it is about 23 μm (Hughes & Morgan, 1973; Randall, 1982a). The boundary layer thickness calculated at an exercise and a resting ventilation rate was 5.7 and 20.0 μm , respectively. These values are likely to be overestimates of the true boundary layer thickness because the analysis does not account for the fact that 20% of the total surface area of secondary lamellae is made up of nonrespiratory pillar cells (Farrell et al., 1980). As well, only about 60% of the secondary lamellae are perfused with blood in resting trout (Booth, 1978). Hence, the initial calculations were adjusted for these surface area considerations, and the adjusted boundary layer thickness ranged from 4.6 to 9.6 μm , between rest and exercise water flow conditions. At rest, the channel for free flowing water between

two lamellae is narrow (~ 4 μm , Fig. 19). Piiper et al. (1986) suggested that diffusion across interlamellar water is more important than diffusion across the blood-water barrier in limiting the transfer of O_2 between water and blood. It is not surprising that at high \dot{V}_w rates typical of exercise conditions there is a decrease in the ratio of the boundary layer thickness to the interlamellar distance, which undoubtedly enhances gas transfer during increased metabolic demands.

Piiper et al. (1986) report much larger values for estimated boundary layer thickness (32 μm at rest, $\dot{V}_w = 195$ $\text{ml} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$ and 26 μm during swimming, $\dot{V}_w = 917$ $\text{ml} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$) than those calculated above. One difference between these studies is that Piiper and coworkers used large Scyliorhinus stellaris (>2 kg), where interlamellar distance was 4 times greater than in trout. To my knowledge these authors did not account for the difference between functional respiratory surface area and total surface area, which would overestimate their calculated values. Other techniques have been used to estimate boundary layer thickness in rat jejunum (Lucas et al., 1975) and ileum (Jackson et al., 1978), and these values range between 60 and 155 μm . One would expect much larger boundary layers associated with epithelium lining intestine, stomach, or kidney tubules because the rate of fluid flow is considerably lower relative to fish gills.

The pH of boundary water at the gill surface will be more acidic than expired water. Gill water boundary layer pH can be

Figure 20. The relationship between water carbon dioxide tensions (P_{CO_2}) and pH are given for Vancouver tapwater (10°C , $C_{CO_2} = 0.224\text{ mmol.l}^{-1}$), Ottawa tapwater (12°C , $C_{CO_2} = 0.455\text{ mmol.l}^{-1}$), and Bamfield seawater (11°C , $C_{CO_2} = 2.0\text{ mmol.l}^{-1}$).



estimated from the following assumptions: 1) the CO_2 diffusion coefficient in intracellular fluid and interlamellar water is approximately equal, since both media contain carbonic anhydrase, which facilitates CO_2 diffusion (see Chapter 3). 2) that P_{CO_2} levels decrease from blood-to-water in proportion to the distance. This last assumption means that because CO_2 must diffuse through the gill epithelium and the boundary water layer before reaching the bulk medium, boundary water P_{CO_2} levels will be equal to about 1/3 blood-to-bulk water mean P_{CO_2} gradient. Figure 20 illustrates the relationship between water P_{CO_2} levels and pH for three different waters; Vancouver tapwater ($C_{\text{CO}_2} = 0.224 \text{ mmol.l}^{-1}$), Ottawa tapwater ($C_{\text{CO}_2} = 0.455 \text{ mmol.l}^{-1}$), and Bamfield seawater ($C_{\text{CO}_2} = 2.0 \text{ mmol.l}^{-1}$). Mean boundary layer pH (pH_{bl}) was estimated from predicted mean boundary layer P_{CO_2} using Fig. 20; in resting trout pH_{bl} is 6.75 in Ottawa tapwater and 6.32 in Vancouver water. The small differences between these pH values relate to variations in P_{CO_2} gradients and water buffering capacity. In seawater, where the water buffering capacity is relatively large, mean boundary water pH will be higher; in lemon sole the value is estimated to be 7.60 (Table 20). Blood P_{CO_2} levels are known to increase dramatically immediately following exhaustive exercise (Milligan & Wood, 1986a; Wood & Milligan, 1987), and mean water boundary layer pH may be as low as 5.62 (Vancouver) and 6.20 (Ottawa) in freshwater, and 7.22 in seawater (Table 20). The consequence of these differences in boundary water pH

TABLE 20. Estimates of gill water boundary layer pH (pH_{bl}) from P_{CO_2} gradients across the gill at rest and exercise in freshwater (FW) trout in two different waters (Ottawa, $\text{C}_{\text{CO}_2} = 0.455 \text{ mmol.l}^{-1}$, Vancouver, $\text{C}_{\text{CO}_2} = 0.224 \text{ mmol.l}^{-1}$) and in seawater (SW) sole. The pH of inspired water (pH_i) is also shown.

CONDITIONS	P_{CO_2} gradients blood-to-water	Boundary layer P_{CO_2}	pH_i	pH_{bl}
<u>Trout</u>				
Ottawa FW	^			
Rest	2.53 (Chapter 4)	1.05	8.04	6.75
	*			
Exercise	9.00 (Milligan & Wood, 1986a; trout)	3.00	8.04	6.2
Vancouver FW	^			
Rest	2.57 (Chapter 3)	1.34	7.41	6.32
	*			
Exercise	9.00 (Milligan & Wood, 1986a; trout)	3.00	7.41	5.62
<u>Sole</u>				
Bamfield SW	*			
Rest	1.94 (Chapter 1)	0.65	7.80	7.60
	*#			
Exercise	5.00 (Wood & Milligan, 1987; SW flounder)	1.67	7.80	7.22

^ P_{CO_2} gradient = $1/2 (\text{Pa}_{\text{CO}_2} + \text{Pv}_{\text{CO}_2}) - 1/2 (\text{Pi}_{\text{CO}_2} + \text{Pe}_{\text{CO}_2})$

* the P_{CO_2} gradient is estimated in these fish assuming water $\text{P}_{\text{CO}_2} = 0$ and Pa_{CO_2} is approximately equal to mean blood P_{CO_2} .

exercise data from Wood & Milligan (1987) was used instead of sole exercise data (Chapter 1) because increases in P_{CO_2} are observed immediately following exhaustive exercise, while the sole experiments samples were taken 30 min following exercise.

between freshwater and seawater is that for fish in seawater the link between NH_3 diffusion and CO_2 excretion across the gills is less important. In other words, the facilitation of NH_3 diffusion by the CO_2 hydration reaction in the boundary layer will be minimal in seawater where H^+ ions will be quickly absorbed by other buffers. It is not surprising, therefore, that in Opsanus beta and Myoxocephalus octodecimspinosus there is no evidence for NH_3 diffusion across the gill (Goldstein et al., 1982; Claiborne et al., 1982). Furthermore, changes in blood-to-water pH gradients, which would be expected to alter P_{NH_3} gradients, have no affect on ammonia excretion in the seawater S. stellaris, S. canicula, and Conger conger (Payan & Maetz, 1973; Heisler, 1984).

An analysis of ammonia levels in gill tissue and boundary water layer is possible using the estimated mean water boundary layer pH values (Figure 21). If one assumes that, like CO_2 , the NH_3 diffusion coefficients in water and intracellular fluid are similar and P_{NH_3} levels from blood to bulk phase water decrease with distance from the blood, then for a blood-to-water P_{NH_3} gradient of 124 uTorr, P_{NH_3} in the gill epithelium will be about 83 uTorr and in the boundary layer, 42 uTorr. Gill intracellular ammonia levels are probably determined by the membrane potential (E_m) across the basal membrane, as they are in other fish tissues (Chapter 1). This is because the activity of Na^+/K^+ ATPase in gill cells is relatively high (Karnaky, 1980) and the Na^+/K^+ ATPase inhibitor, ouabain, decreases ammonia

Figure 21. A schematic representation of a cross-section through the gill epithelium showing measured mean arterial-venous blood and inspired-expired water parameters (taken from Chapter 4, in vivo trout experiments), calculations of boundary water and gill cell pH, and calculations of ammonia levels in the gill cell and water boundary layer. TEP represents the transepithelial potential.

mean inspired- expired water		water boundary layer	gill epithelium	mean arterial- venous blood
pH	7.66	6.75	6.94	7.83
P _{CO₂} (Torr)	0.2	1.1	1.9	2.7
T _{Amm} (μM)	4	1446	2360	461
P _{NH₃} (μTorr)	1	42	83	125
Em TEP (mV)		-60	-40 -20	

excretion across fish gills (Payan, 1978; Claiborne et al., 1982; Cameron, 1986), indicating the replacement of NH_4^+ for K^+ ions in this active exchange mechanism at the basal membrane. To calculate gill intracellular ammonia levels from the Nernst equation (13), an E_m value of -40 mV was assumed which is the difference between the measured transepithelial potential (blood-to-water = -20 mV; Iwama, 1986) and the measured water-to-gill cell potential (-60 mV; Randall, unpublished data). With these values gill intracellular ammonia concentration was calculated to be $2360 \text{ } \mu\text{mol.l}^{-1}$. Gill cell pH_i was then calculated from the ratio of $\text{NH}_3:\text{NH}_4^+$ with the Henderson-Hasselbalch equation (3). There are no published values of gill cell pH_i to compare with this calculated value of 6.94, however, because gill cells have a relatively high permeability to H^+ ions (McWilliams & Potts, 1978) it is expected that gill epithelium pH will be lower than other intracellular compartments (~ 7.2 – 7.4). Furthermore, if the estimated E_m value of -40 mV is a good approximation of gill cell membrane potential, then this value is closer to measured red cell E_m (-30 mV, Lassen, 1977), where H^+ ions are known to be passively distributed (Lassen, 1977; Heming et al., 1986; Nikinmaa et al., 1987; Heming et al., 1987), than to other tissues (E_m -70 to -90 mV) where H^+ ions are relatively impermeable (Roos & Boron, 1981).

Boundary layer total ammonia levels were calculated assuming that the apical gill membrane in freshwater fish is relatively

impermeable to NH_4^+ ions (Kormanik & Cameron, 1981a; Wright & Wood, 1985), as it is to other ion species (Girard & Payan, 1980). With the estimated mean boundary water pH ($\text{pH} = 6.75$) and mean P_{NH_3} level (42 uTorr) the boundary layer ammonia concentration was calculated with the Henderson-Hasselbalch equation (3) to be $1446 \text{ } \mu\text{mol.l}^{-1}$. On the other hand, if the apical membrane was highly permeable to NH_4^+ ions, then boundary water ammonia levels calculated from the Nernst equation would be $964 \text{ } \mu\text{mol.l}^{-1}$. As stated above, however, it is unlikely that apical membrane NH_4^+ permeability is significant in freshwater fish. Moreover, for the boundary layer to be maintained as a distinct "microclimate", the apical membrane must be relatively impermeable to ion species (see Chapter 4).

The above analysis of ammonia levels fits in with accepted theory of ammonia transfer in freshwater fish. Ammonia may diffuse as NH_3 across the gills down the blood-to-water P_{NH_3} gradient into the interlamellar water where an acidic boundary layer maintains low $\text{NH}_3:\text{NH}_4^+$ ratios. Alternatively, NH_4^+ ions may cross the gills in exchange for Na^+ ions down the NH_4^+ gill cell-to-water gradient. Ammonia concentrations in the gill epithelium would be maintained by a shuttling of NH_4^+ ions across the basolateral membrane, as ammonia was lost to the environment across the apical membrane. Unlike the membrane potential of most body cells, transepithelial potentials across fish gills vary widely with changing water conditions (Iwama, 1986; Ye, 1986; McWilliams & Potts, 1978; Kerstetter et al.,

1970). Changes in transepithelial potential may directly affect ammonia transfer by altering NH_4^+ electrochemical gradients, or indirectly affect ammonia flux by altering gill intracellular ammonia concentrations.

The demonstrated linkage between ammonia and carbon dioxide excretion in this study makes it necessary to re-evaluate the experimental interpretation of earlier studies concerning ammonia excretion where experimental treatments may have induced changes in CO_2 excretion, and therefore chemical reactions in the boundary layer. For instance, Payan and Maetz (1973) injected ammonium salts into dogfish (*S. canicula*) and observed a decrease in branchial ammonia efflux after an injection of the carbonic anhydrase inhibitor, acetazolamide. Payan and Matty (1975) removed carbon dioxide from the saline perfusing isolated trout heads and found that ammonia efflux was significantly reduced. In both these studies, the reduction in ammonia excretion may be partly explained by the inhibition of CO_2 excretion across the gills, which would reduce the amount of H^+ ions available in the gill water boundary layer and elevate water P_{NH_3} tensions. It is also feasible that the observed decrease in ammonia excretion in these studies was also partly due to a reduction in $\text{Na}^+/\text{NH}_4^+$ exchange. Payan and Matty (1975) point out that reduced CO_2 flux across the gills would reduce the availability of H^+ ions in the gill epithelium, which combine with NH_3 to supply NH_4^+ as a counterion for the Na^+ uptake mechanism. Although this latter explanation is also a viable one, the significance of the

catalysed CO_2 hydration reaction in the boundary layer to ammonia excretion was not previously recognized.

The physiological significance of a distinct water pH microclimate at the gill surface to ammonia excretion will vary depending on the physiological state of the animal and water conditions. For instance, if water pH at the gill surface was suddenly increased to inspired water values ($\text{pH}_I = 8.0$), which might occur if external gill carbonic anhydrase was removed and the CO_2 hydration reaction was completely uncatalysed, then P_{NH_3} levels in the gill boundary layer would be 760 uTorr, instead of 42 uTorr, as predicted in Fig. 21. Such a high P_{NH_3} level in the water would make ammonia excretion very difficult. In the short term, a reversal of the blood-to-water P_{NH_3} gradient would result in an uptake of ammonia. To maintain steady state excretion, however, blood ammonia levels would have to exceed water ammonia levels. Blood P_{NH_3} levels of 800 uTorr would be toxic to the fish (see Randall & Wright, 1987), and therefore, carbonic anhydrase at the gill surface may be of considerable importance to all aquatic animals that excrete significant amounts of ammonia. In more alkaline waters ($\text{pH} = 9.5$, (see chapter 4), P_{NH_3} levels in the boundary layer could be as high as 12,000 uTorr (given boundary water Tamm levels in Fig. 21). One would predict that fish living in alkaline waters maintain high rates of carbon dioxide excretion and carbonic anhydrase activity at the gill surface to ensure adequate removal

of ammonia from the body.

Another interesting situation is the effects of CO_2 excretion on ammonia efflux in starving fish. Hillaby and Randall (1979) report that blood ammonia levels progressively increase from day 2 to day 12 of starvation, a curious phenomena in unfed fish. Brett and Zala (1975) found that oxygen consumption decreased in starved fish, and this implies that there was also a concurrent reduction in carbon dioxide excretion. It may be that the increase in blood ammonia levels was a compensatory mechanism to increase blood-to-water P_{NH_3} gradients in the face of reduced CO_2 excretion.

In conclusion, ammonia transfer in fish is dependent on both NH_4^+ electrochemical and NH_3 partial pressure gradients. Changes in tissue membrane potentials or gill transepithelial potential will alter intracellular ammonia stores and may result in alterations of ammonia efflux across the gill. Changes in red cell membrane potential or transmembrane pH gradients will alter the intracellular ammonia content, since H^+ ions are passively distributed between plasma and red cells. Ammonia may be eliminated across the gills of freshwater fish as both NH_3 and NH_4^+ ; the rate of NH_3 diffusion is influenced by the availability of H^+ ions in the gill water boundary layer. The availability of H^+ ions is dependent on the rate of CO_2/NH_3 excretion across the gill, the rate of the CO_2 hydration reaction in the boundary layer, and the buffering capacity of the bulk water, among other things. The identification of the

interaction between CO_2 hydration and NH_3 diffusion in the water boundary layer may have many physiological consequences and opens new areas of investigation of gas transfer in fish.

Furthermore, the gill water boundary is a common component of many transfer processes and there are many interesting problems to address in future research concerning the ability of aquatic animals to maintain a gill microenvironment distinct from the general environment.

APPENDIX I

Characteristics of the initial saline used to perfuse trout heads while on the operating table.

$$\text{pH} = 7.86 \pm 0.02 \text{ (N=26)}$$

$$\text{C}_{\text{CO}_2} = 5.00 \pm 0.02 \text{ (N=28) mmol.l}^{-1}$$

$$\text{Tamm} = 1028 \pm 44 \text{ (N=32) } \mu\text{mol.l}^{-1}$$

$$\text{Protein} = 2.2 \pm 0.0 \text{ (N=28) \%}$$

APPENDIX II

The thickness of the gill water boundary layer, s_{bl} , can be calculated as outlined by Piiper et al. (1986) by the following formula:

(27)

$$s_{bl} = d \times \alpha \times A / D_w$$

where d is the O_2 diffusion coefficient at $10^\circ C$ in water, α is the O_2 solubility coefficient, A is the secondary lammellar surface area (see Table 21 for constants). D_w is the diffusing capacity of interlamellar water, where

(28)

$$D_w = \dot{V}_w \times \alpha \times \ln (1 / \epsilon)$$

and \dot{V}_w is the ventilatory water flow, and ϵ is the equilibration inefficiency. ϵ defines the fraction of respiratory water that is shunted past the respiratory surface, while the remainder of water equilibrates with the secondary lamellae.

(29)

$$\epsilon = \frac{P_{EO_2} - P_o}{P_{IO_2} - P_o}$$

P_{IO_2} and P_{EO_2} are the oxygen tensions of inspired and mixed expired water and P_o is P_{O_2} at the secondary lamellar surface which is constant. Scheid and Piiper (1971) have related ϵ to

TABLE 21. Values used to calculate the boundary layer thickness. See Appendix II for details.

PARAMETER	VALUES	REFERENCE
d	0.0011 cm ² .min ⁻¹	Piiper <u>et al.</u> , 1986 (extrapolated 10° C)
α	0.00224 mmol.l ⁻¹ .torr ⁻¹	Boutilier <u>et al.</u> , 1986
A	837.8 cm ² for 400g fish	Hughes, 1980
b	0.0115 mm	Hughes, 1966
l	0.7 mm	"
h	0.07 mm	"
N	380,487	calculated from data taken from Hughes & Morgan, 1973 and Hughes, 1980

the dimensionless equilibration resistance index, ϕ . They have shown that the value of ϕ for a certain geometry of the secondary lamellae and water velocity profile through the interlamellar channels can be described as a function of :

(30)

$$\phi = \frac{b^2 \times \bar{v}}{l \times d}$$

where b is one half the interlamellar distance, \bar{v} is the mean water velocity, l is the length of the secondary lamellae at the base. If water conditions for O_2 equilibration across the gill are poor, then ϕ will be large. Fig. 22 shows this relationship, redrawn from Piiper et al. (1986).

The mean velocity was calculated:

(31)

$$\bar{v} = \dot{V}_w / F$$

F denotes the total cross-sectional area of the interlamellar spaces:

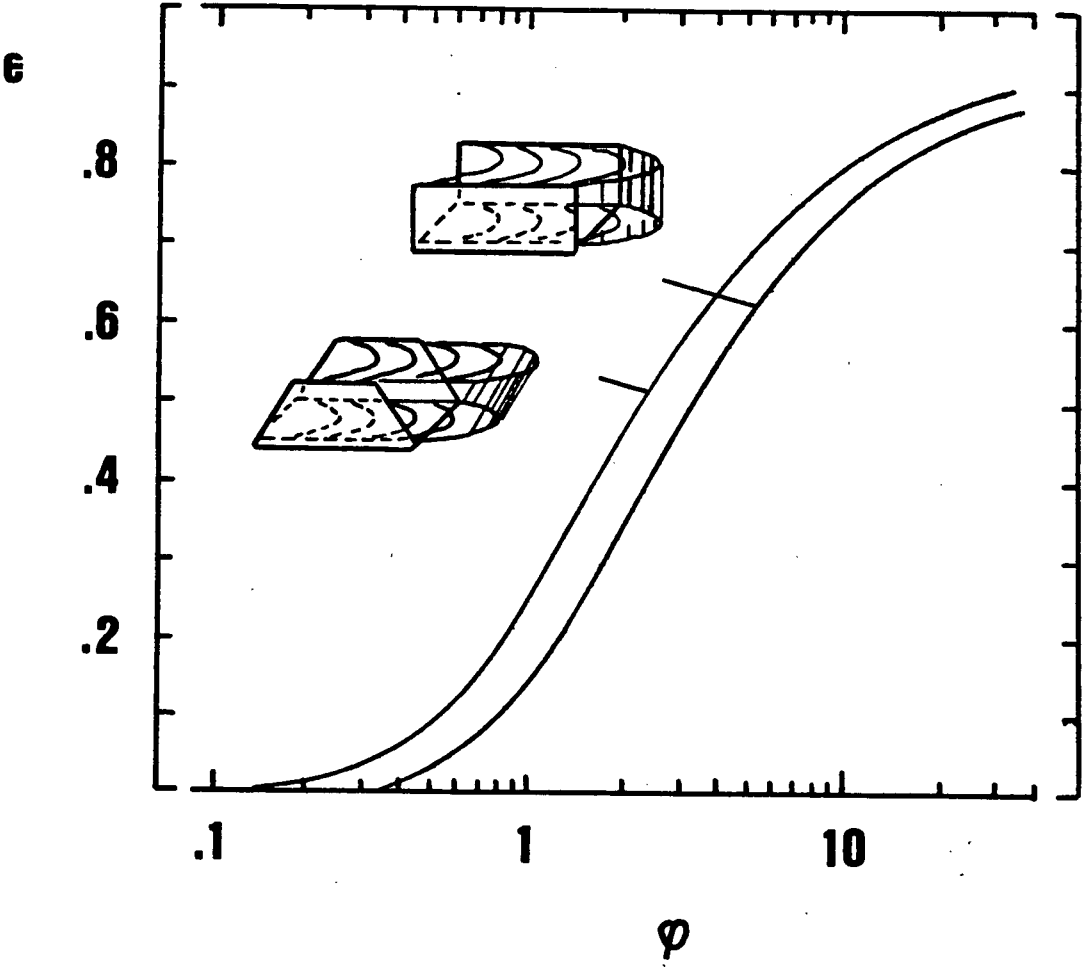
(32)

$$F = 2b \times h \times N$$

where h is the mean height of the secondary lamella and N is the total number of secondary lamellae.

The ϕ value was calculated and an approximate ξ value was read from Fig. 22 between the curves for the two different

Figure 22. Plot of 'equilibration inefficiency', \mathcal{E} (equation 29), against 'equilibration resistance index', φ (equation 30). Abscissa (φ), logarithmic; ordinate (\mathcal{E}), linear. The two curves are for a rectangular lamella (top diagram) and for a trapezoidal lamella, of same base length, but tapering to one-half the base length at the top edge (bottom diagram). Estimates of \mathcal{E} were taken midway between the two curves (see Piiper et al., 1986) for calculated values in resting and active fish. Figure redrawn from Piiper et al., 1986.



geometric gill models proposed by Piiper et al. (1986). In dogfish, the measured ε versus ϕ values were situated halfway between the two theoretical geometric models and therefore, it is assumed that similar situation occurs in trout because gas transfer rates between the two species are not unlike. D_w and s_{b1} were then calculated.

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