ION EXCHANGE MECHANISMS FOR THE CONTROL OF VOLUME AND PH IN FISH AND AMPHIBIAN ERYTHROCYTES

By

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ABSTRACT

The characteristics of the ion exchange mechanisms which regulate volume and pH in fish and amphibian erythrocytes were investigated and compared. Experiments were carried out under steady state conditions and also following adrenergic stimulation both *in vivo* and *in vitro*.

Under steady state conditions, a decrease in extracellular pH caused an increase in the volume of rainbow trout erythrocytes, and a decrease in the intracellular pH. These pH-induced volume changes were mainly associated with movements of chloride across the chloride/bicarbonate exchange pathway. The sodium/proton exchange mechanism is quiescent at all pH's studied under steady state conditions.

Beta adrenergic stimulation of rainbow trout erythrocytes promoted cell swelling and proton extrusion from the erythrocytes. Amiloride inhibited both the volume and pH changes associated with adrenergic stimulation indicating that this response is associated with an increase in the activity of the sodium/proton

exchange mechanism on the erythrocyte membrane. The adrenergic swelling and pH responses are enhanced by a decrease in extracellular pH. An increase in bicarbonate concentration reduces the adrenergic pH response, but it is still significant even at 10 mM bicarbonate. DIDS markedly enhanced the beta adrenergic effect on the erythrocyte pH, but abolished the increase in erythrocyte volume. The adrenergic response was independent of temperature between 10 and 18^{o}C. These results support a loosely coupled sodium/proton and chloride/bicarbonate exchange model for the adrenergic response in rainbow trout erythrocytes.

The increases in erythrocyte pH and volume following adrenergic stimulation are associated with increases in the haemoglobin:oxygen affinity. The oxygen carrying capacity of the blood is, therefore, increased following adrenergic stimulation in rainbow trout. Carbon dioxide excretion, however, was not significantly affected by adrenergic stimulation. The functional significance of the adrenergic response of fish erythrocytes may be to offset the effects of the Root shift on the oxygen carrying capacity of the blood during acute changes in extracellular pH.

In contrast to fish erythrocytes, the

sodium/proton exchange mechanism in amphibian erythrocytes is active under steady state conditions. In the presence of bicarbonate movements, this exchange significantly affects the erythrocyte volume, but not the erythrocyte pH. Similar to fish erythrocytes, protons are passively distributed in amphibian erythrocytes under steady state conditions and in Donnan equilibrium with chloride ions. The erythrocyte volume also increases with decreases in extracellular pH as in fish erythrocytes, due to changes in the chloride distribution across the erythrocyte membrane.

Adrenergic stimulation does not affect the volume or pH of amphibian erythrocytes either *in vivo* or *in vitro*. These animals, therefore, do not appear to regulate erythrocyte pH adrenergically. Amphibians are able to efficiently utilize oxygen stores via both central and peripheral shunting. In addition, the blood of these animals does not have a Root shift. Adrenergic regulation of erythrocyte pH in order to enhance oxygen transport during fluctuations in ambient and internal gas tensions, therefore, is probably less important than it would be in fish.

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LIST OF ABBREVIATIONS

DIDS	4,4-Diisothiocyanostilbene-2,2-Disulfonic acid.
2,4-D.N.P.	2,4-Dinitrophenol.
DMO	Dimethyl oxazolidine-2,4-dione.
pH_e	Extracellular pH.
pH_i	Erythrocyte pH.
RE	Respiratory exchange ratio.
SITS	4-acetamido-4-iso-thiocyanato-stilbene-2, 2-disulphonic acid.
VCO_2	Carbon dioxide excretion.
VO_2	Oxygen consumption.

GENERAL INTRODUCTION

In most vertebrate species, more than 90% of the total oxygen content of the blood is bound to haemoglobin and changes in haemoglobin oxygen affinity have a large effect on the amount of oxygen delivered to the tissues at any given blood flow. It is not surprising, therefore, that vertebrate haemoglobin systems possess adaptations to regulate haemoglobin oxygen affinity and ensure adequate delivery of oxygen to aerobically respiring tissues. The adaptations which regulate haemoglobin oxygen affinity in the nonnucleated erythrocytes of mammals are well known (Bauer, 1974). The majority of vertebrate species, however, possess nucleated erythrocytes. Relative to their nonnucleated counterparts, the adaptations regulating haemoglobin oxygen affinity in nucleated erythrocytes are poorly described and in particular, very little is known about the functional diversity in these systems.

Haemoglobin:

The respiratory pigment found in the circulatory system of vertebrates is haemoglobin. Its basic function is similar to that of other circulating respiratory pigments which is to combine with oxygen at the respiratory surface and unload it at the tissues. The

capacity of a specific haemoglobin to perform this function will depend on its affinity for oxygen. This affinity for oxygen must be high enough to allow efficient loading at the respiratory surface but not so high that it impairs efficient release of oxygen at the tissues. It has been demonstrated that the intrinsic affinity of haemoglobin for oxygen may vary depending on either the species examined or the particular haemoglobin component in multiple haemoglobin systems (see reviews by Johansen and Weber, 1976; Riggs, 1979; Powers, 1980; Hochachka and Somero, 1984).

It has often been suggested that haemoglobin oxygen affinity decreases with increasing dependence on aerial gas exchange (McCutcheon and Hall, 1937; Lenfant and Johansen, 1967; Johansen et al., 1979a,b). Powers (1980) points out that, among fish, this conclusion may only be valid for closely related species. Additional trends in haemoglobin oxygen affinity are also apparent. Fish which live in a low oxygen environment (excluding air breathers) generally have haemoglobins with high oxygen affinities while pelagic fish living in an environment with a constant high oxygen level have haemoglobins with lower oxygen affinities (Riggs, 1970;

Powers, 1980). There is also a correlation between an organisms metabolism and the affinity of its haemoglobin for oxygen. Active fish such as trout and mackerel tend to have lower haemoglobin oxygen affinities than less active fish (Riggs, 1970). This is thought to be an adaptation which increases the unloading of oxygen at the tissues. Haemoglobins with a very low temperature sensitivity have also been found in a variety of vertebrates. Barcroft and King (1909) first demonstrated that an increase in temperature will decrease the affinity of mammalian haemoglobin for oxygen. This temperature effect on haemoglobin oxygen affinity has since been found in several species of poikilothermic vertebrates (Gillen and Riggs, 1971, 1972: Greenwald, 1974; Mied and Powers, 1977). In these animals, the resulting problems of oxygen uptake are compounded by the fact that the rising temperature also increases both metabolism and the resulting tissue oxygen requirements. Furthermore, water breathers face an additional problem because of the reduction in the solubility of oxygen in water at higher temperatures. It has been shown, however, that individual haemoglobins from some species such as the trout (Brunori, 1975), chum salmon (Hashimoto et al., 1960) and tuna (Rossi-Fanelli and Antonini, 1960)

have a reduced temperature sensitivity. The accepted view is that these temperature insensitive haemoglobins are adaptations found in eurythermal species experiencing large fluctuations in body temperature (Johansen and Weber, 1976).

Most vertebrates also possess more than one type of haemoglobin (Hochachka and Somero, 1973). This tendency is more pronounced in fish, reptiles and amphibians than in mammals and birds (Gratzer and Allison, 1960). These different haemoglobins can have radically different oxygen binding properties allowing organisms to maintain oxygen transport in the face of changing oxygen requirements or availability. The relative proportions of different haemoglobin components may also change during the life cycle of an organism (Herner and Frieden, 1961; Wilkens, 1968). For example, Giles and Randall (1980) found that in coho salmon, O. kisutch, haemoglobin from the fry had a much higher oxygen affinity than the adult haemoglobin.

Differences in the oxygen requirements of organisms and in the availablity of environmental oxygen have resulted in the evolution of both interspecific and intraspecific variation in the intrinsic affinity of

haemoglobin for oxygen. Generally, the intrinsic affinity of specific haemoglobins for oxygen are suited to efficiently extract oxygen from the environment and deliver it to the respiring tissues. The oxygen affinity of most haemoglobins is also sensitive to changes in the physical and chemical properties of the haemoglobin environment, however, representing another level of adaptation and therefore variation in haemoglobin systems.

Modulation of Haemoglobin Oxygen Affinity:

In response to changing demands on the oxygen transport system, haemoglobin oxygen affinity may be adjusted at the cellular level by altering the concentration of certain organic phosphate compounds within the erythrocyte. These are known as negative modulators of haemoglobin oxygen affinity (Hochachka and Somero, 1973) since decreases in the cellular concentrations of these compounds decreases the affinity of haemoglobin for oxygen. In mammalian erythrocytes, the organic phosphate compound which modulates haemoglobin oxygen affinity is 2,3-diphosphoglycerate (2,3-DPG; Benesch and Benesch, 1967; Bunn, 1980). The distribution and concentrations of the organic phosphate constituents of other vertebrate erythrocytes are

summarized in a review by Bartlett (1980). Inositol pentaphosphate (IP_5) and adenosine triphosphate (ATP) are the main inorganic phosphates in both bird and reptile erythrocytes. In amphibians, the most common erythrocytic organic phosphates are ATP and 2,3-DPG, and in fish, ATP and guanosine triphosphate (GTP) are believed to be important in modulating haemoglobin oxygen affinity.

An increase in either the proton or the carbon dioxide partial pressure of the haemoglobin environment will also cause a reduction in the haemoglobin oxygen affinity. The reduction in affinity caused by protons is known as the Bohr effect or Bohr shift (Dejours, 1981). Carbon dioxide causes a reduction in affinity both indirectly by its effect on pH and directly by reacting with haemoglobin to form carbamino compounds (see review by Roughton, 1970). At the respiring tissues, the combined effects of protons and carbon dioxide, end products of respiration, on haemoglobin oxygen affinity facilitates the unloading of oxygen. Similarly, at the gas exchange organ, removal of carbon dioxide from the blood facilitates the uptake of oxygen.

Root and Irving (1943) demonstrated that a

reduction in the pH of the haemoglobin environment *in vitro* also lowered the oxygen capacity of the blood in teleost fish. This 'Root' effect is important in the delivery of oxygen to the swim bladder (Berg and Steen, 1968) and possibly also to the retinal tissue in some fish (Wittenberg and Wittenberg, 1974). Such an effect, however, may also limit delivery of oxygen to the respiring tissues under conditions of low blood pH and, therefore, limit aerobic performance. Jones (1971), for example, showed that a 30-40% reduction in blood oxygen content caused by hypoxia led to a 30% reduction in maximum sustainable swimming speed in the rainbow trout, *Salmo gairdneri*. It has been hypothesized that the Root effect may even be a factor contributing to the death of hyperactive fish (Black, 1958).

Some species of fish having multiple haemoglobin components have particular components which are insensitive to pH (Hashimoto et al., 1960; Powers, 1972; Brunori, 1975). This intrinsic adaptation may act as a safeguard to maintain a minimum level of oxygen transport immediately following hyperactivity in these species (Hochachka and Somero, 1984). The reduction in the affinity of the pH sensitive components, however, would still result in a reduction in total aerobic capacity.

In summary, the effects of organic phosphates, hydrogen ions and carbon dioxide on haemoglobin oxygen affinity represent an important level for adaptation of vertebrate oxygen transport systems. At this level, the intrinsic affinity of haemoglobin for oxygen may be re-adjusted according to environmental or organismic challenge to enhance its ability to transport oxygen. Bohr and Root shifts also have the potential to negatively influence oxygen transport in some species by reducing the total oxygen carrying capacity of the blood. Some animals, however, appear to have evolved erythrocytic adaptations to ameliorate this potential problem.

Regulation of Erythrocyte pH:

Burst swimming in the rainbow trout (Primmett et al., 1986) and the striped bass (Nikinmaa et al., 1984) causes a decrease in the erythrocytic transmembrane pH gradient (as the extracellular pH falls, the intracellular pH is maintained or slightly increased). A similar response occurs in vivo following acid infusion in rainbow trout (Boutilier et al., 1986b) or in vitro, upon addition of catecholamines to erythrocytes (Nikinmaa and Huestis, 1984; Heming et al., 1987). It has been

concluded that beta-adrenergic stimulation of these erythrocytes results in a net loss of protons from the erythrocyte. This response is thought to help maintain blood oxygen carrying capacity in the presence of a plasma acidosis (Nikinmaa et al., 1984; Primmett et al., 1986).

The addition of catecholamines to rainbow trout erythrocytes in vitro not only causes a net loss of protons from the erythrocytes, but also a pronounced cell swelling (Baroin et al., 1984a,b; Nikinmaa and Huestis, 1984; Heming et al., 1987). The cell swelling is apparently due to an increase in erythrocyte sodium and chloride levels with water following passively (Baroin et al., 1984a; Heming et al., 1987). The exact mechanism by which adrenergic stimulation elevates the erythrocyte pH and water content, however, have not been determined.

The pH of lamprey erythrocytes is also regulated, but the characteristics of pH regulation in these erythrocytes are quite different than in teleost fish. In the lamprey, protons and chloride ions are not passively distributed as in most other vertebrates (Nikinmaa, 1986). Instead, there is a gradient for protons which is actively maintained such that the erythrocyte pH is higher than the plasma pH in most instances (Nikinmaa and

Weber, 1984; Nikinmaa, 1986). Evidently, this gradient is maintained by having a continuously and highly active sodium/proton exchange mechanism (Nikinmaa et al., 1986) coupled to a chloride/bicarbonate exchanger with extremely low levels of activity (Nikinmaa, pers. comm.). Indeed, Nikinmaa (1986) has suggested that the functional significance of pH regulation in lamprey erythrocytes is to offset the large Bohr effect of lamprey haemoglobin during changes in extracellular pH.

Avian erythrocytes also exhibit several unique characteristics. These cells do not have a sodium/proton exchange mechanism capable of influencing the volume or pH of the cell (Nikinmaa and Huestis, 1984). However, there is a sodium/potassium/chloride cotransport system on the membrane of avian erythrocytes which regulates the cell volume (Palfrey and Greengard, 1981). While this mechanism is sensitive to beta-adrenergic stimulation, it will only cause the erythrocytes to swell in the presence of elevated extracellular potassium and it does not influence the pH of the cell (Riddick et al., 1971; Palfrey and Greengard, 1981; Nikinmaa and Huestis, 1984).

The ionic characteristics of volume regulation have also been described in amphibian erythrocytes.

Although these erythrocytes possess a sodium/proton exchange mechanism which is capable of influencing the cell volume (Cala, 1980, 1985; Rudolph and Greengard, 1980; Palfrey and Greengard, 1981), the ability of this exchanger to influence the erythrocyte pH has not been investigated. It is possible, therefore, that this exchanger also regulates erythrocyte pH as in fish and lamprey erythrocytes.

Summary:

The preceding discussion has attempted to outline the documented inter- and intraspecific variation in the oxygen transport characteristics of nucleated erythrocytes. It has recently been demonstrated that the interspecific variation must also include differences in ion exchange mechanisms on the erythrocyte membrane. These mechanisms have the potential to influence the oxygen transport charcteristics of these cells by altering the chemical state of the haemoglobin environment. The hypothesis of this study is that the ion exchange mechansims on the erythrocyte membrane of fish and amphibian erythrocytes will each exhibit unique adaptive features whose functional significance will be attributable to both environmental and evolutionary pressures.

GENERAL MATERIALS AND METHODS

Animals and Surgical Procedures:

Rainbow trout (Salmo gairdneri), weighing between 285 and 700g were obtained from the Sun Valley Trout Farm (Mission, B.C.) and maintained in large outdoor tanks. Water temperature varied from 10°C to 15°C. The fish were regularly fed and in good health.

In experiments which required cannulated trout, the animals were anaesthetized in an aerated and pH balanced solution of tricaine methane sulphonate (66.7 mg L^{-1} MS-222 and 133.3 mg L^{-1} $NaHCO_3$). The cannulation was then performed on a surgical table where a lighter dose of the aerated anaesthetic solution (50 mg L^{-1} MS-222 and 100 mg L^{-1} $NaHCO_3$) was recirculated over the gills. A catheter (Sovereign indwelling canine catheter, 2 inch, 18 gauge) was used to make a blind puncture into the dorsal aorta through the mouth of the fish. The puncture was made on a 45 degree angle (in the caudal direction) at the midline of the branchial basket between the first and second gill arches. A cannula of polyethylene tubing (PE-50) was fed into the aorta through the implanted catheter. The cannula was then led out of the roof of the mouth via a flanged section of tubing (PE-200).

Following the cannulation, the fish was recovered in a light proof Perspex box for at least 48h prior to the experiment. The cannula was filled with heparinized (20 U.S.P. units mL^{-1}) Cortland saline (Wolf, 1963) and flushed daily in order to maintain patency.

In certain instances, fish blood was also obtained via cardiac or caudal puncture. In these instances, the fish were lightly anaesthetized in the MS-222 solution or stunned by a blow to the head. Blood was then slowly withdrawn into heparinized syringes inserted ventrally into the caudal vessel or the heart.

Adult toads, Bufo marinus, of both sexes (250-500g) were obtained from Charles D. Sullivan Co. Inc. (Nashville, TN, U.S.A.). The animals were kept in large fiberglass aquaria with 2-4 cm of dechlorinated tap water at the experimental temperature ($22^{o}C$) for at least a week prior to the experiments. Toads were anaesthetized by immersion in an MS-222 solution (1.5 g L^{-1} MS-222 and 3 g L^{-1} $NaHCO_3$) before surgery. The femoral cannulation involved making a 2-3 cm incision in the dorsal skin of the right leg and exposing the femoral artery between the triceps femoris and semimembranous muscles. A section of the vessel was clamped off and a small hole was made downstream from the clamp. Polyethylene tubing (PE-60)

was inserted via this hole upstream approximately 1-2 cm. This cannula was filled at all times with heparinized (125 U.S.P. units mL^{-1}) MacKenzie's amphibian saline (de la Lande et al., 1962) to prevent clotting. Once in place, the cannula was secured to the dorsal skin and surrounding musculature with surgical suture. Following surgery, the animals were transferred to light-proof Perspex boxes with 500 mL of dechlorinated tap water and allowed to recover for 24h.

Specimens of Amphiuma tridactylum (450-1600g) were also obtained from Charles D. Sullivan Co. Inc. (Nashville, TN, U.S.A.). The salamanders were kept in large fiberglass aquaria at 25°C for at least a week prior to the experiments. Animals were anaesthetized by immersion in an MS-222 solution (5 g L^{-1} MS-222 and 10 g L^{-1} $NaHCO_3$). A mid-ventral incision was made and a cannula of polyethylene tubing (PE-50) was inserted into one of the celiac arteries. The cannula was pushed forward into the dorsal aorta approximately 10 - 15 cm, tied occlusively into the celiac artery and secured to the surrounding musculature with surgical suture. The incision was then closed with suture and the animal was recovered in a light-proof glass aquarium in 10 L of

water for at least 24h.

Calculations:

The cell water content was determined by weighing the wet cell pellet, drying it to a constant weight at 90°C and reweighing it. The water content was then calculated using the following formula:

$$\%H_2O=100-(100 \times \text{dry weight/wet weight}).$$

The intracellular pH may be determined from the distribution of the weak acid 5,5-dimethyl-2,4-oxazolidinedione (DMO) (Waddell and Butler, 1959). In the present experiments, the erythrocyte pH (pH_i) was calculated from the distribution of DMO across the erythrocyte membrane. Initially, 10 uL of 1 uCi mL^{-1} ^{14}C-DMO (New England Nuclear, specific activity 50 mCi $mmol^{-1}$) was added to the suspension (erythrocytes in plasma or saline). Following the incubation period, 0.4 mL samples of blood were taken and centrifuged. A 100 uL sample of extracellular fluid (plasma or saline) was taken for the determination of the extracellular DMO concentration and the remaining extracellular fluid discarded. The erythrocyte pellet was saved for the determination of the intracellular DMO concentration. Both the extracellular and intracellular DMO samples were

deproteinized with 0.2 mL of 0.6 M perchloric acid to reduce quenching and left for 24h. The resulting suspensions were centrifuged and 0.1 mL of supernatant was added to scintillation vials containing 7 mLs of aqueous counting scintillant (Amersham corp, Ill., U.S.A.). The samples were then analysed for ^{14}C by a Beckman LS 9000 Liquid Scintillation System (Beckman Instruments, Inc., CA, U.S.A.). It should be noted that the deproteinized samples were also used for the determination of the chloride concentration of the extracellular fluid and the erythrocytes. Chloride concentrations were determined with a Radiometer CMT 10 chloride titrator. The pH_i was calculated from the distribution of DMO using the following formula:

$$pH_i = pK_{DMO} + \log([DMO]_i/[DMO]_e(1+10\exp(pH_e - pK_{DMO})) - 1)$$

(Heisler, 1975; Albers and Goetz, 1985) where pH_i and pH_e are the intracellular and extracellular pH respectively and DMO_i and DMO_e are the intracellular and extracellular DMO concentrations respectively. The pK_{DMO} was taken as 6.377 for Salmo gairdneri at 10°C, 6.307 for Salmo gairdneri at 18°C, 6.272 for Bufo marinus and 6.245 for Amphiuma tridactylum according to Albers et al., (1971).

The intracellular and extracellular concentration of DMO and chloride were calculated as:

$$C_i = [((fwc \times wet\ wt) + PCA)/(fwc \times wet\ wt)] \times C_m$$

$$C_e = [((fwc \times S_{vol}) + PCA)/(fwc \times S_{vol})] \times C_m$$

where C_i, C_e and C_m are the intracellular, extracellular and measured sample concentrations respectively, fwc is the fractional water content ($\%H_2O/100$) of the sample, wet wt is the wet weight (mg) of the erythrocytes, PCA is the volume (uL) of perchloric acid added to the sample and S_{vol} is the sample volume (uL) of extracellular fluid.

It should be noted that trapped extracellular fluid was not taken into account in the intracellular concentration calculation. This value has been found to be 2-3% in fish erythrocytes (Houston, 1985) and 2-3% in amphibian erythrocytes (Emilio and Shelton, 1980).

It is well established that chloride ions are passively distributed across the membrane of vertebrate erythrocytes (Lassen, 1977) and the membrane potential in these cells is equal to the chloride equilibrium potential (Fortes, 1977). The erythrocyte pH (pH_i) was, therefore, calculated from the extracellular pH (pH_e),

and the intracellular [Cl^-_i] and extracellular [Cl^-_e] chloride concentrations assuming a Donnan equilibrium distribution for protons and chloride ions using the following equation:

$$pH_i = pH_e + \log[Cl^-_i] - \log [Cl^-_e]$$

(Albers and Goetz, 1985).

SECTION I: FISH ERYTHROCYTES

OVERVIEW

Under normal steady state conditions, the pH of the body compartments are kept constant in vertebrates. This does not include transient pH changes normally occurring in the blood at the gas exchange organ and at the tissues which enhance oxygen loading and unloading respectively. Several types of stress, however, may result in deviations in pH away from optimal steady state levels both in the blood and other body compartments. In fish, environmental factors commonly causing acid-base disturbances include temperature changes, hypercapnia and acid waters. In addition, burst activity (supported by anaerobic glycolysis in contracting white muscle fibers) is a frequent cause of acidosis due to the increased production of lactate and associated protons (Hochachka and Somero, 1984). Fish have a large proportion of poorly perfused white muscle (Heisler, 1984), and therefore, burst activity often results in a large number of protons added to the circulation (Holeton et al., 1983; Nikinmaa et al., 1984; Primmett et al., 1986). The acid-base stress imposed on an animal by excess H^+ or OH^- ions may be reduced by the body buffer systems until excreted. Biological buffer systems consist of

nonbicarbonate and CO_2/bicarbonate buffers. There is considerably less bicarbonate in the extracellular space of water breathing fish than other vertebrates (Rahn and Garey, 1973; Heisler, 1980). In addition, the nonbicarbonate buffer value is relatively low in fish (Albers, 1970). The intracellular compartments in fish are better buffered than the extracellular space (Heisler, 1984). However, these are still lower than in similar mammalian tissues (Heisler and Piiper, 1971). Indeed, the total buffering capacity in fish is low compared to other vertebrates. Thus, relatively large acidoses are probably common in fish.

It has been demonstrated that during acute extracellular acidoses in fish, the pH of the erythrocyte is regulated adrenergically and blood oxygen carrying capacity is, therefore, maintained (Nikinmaa et al., 1984; Primmett et al., 1986; Boutilier et al., 1986a). Indeed, Randall et al., (1987) found that chinook salmon were able to perform aerobic exercise as well after as before a burst swim. However, the mechanism by which adrenergic stimulation elevates the water content and pH in teleost fish erythrocytes is not clear. It has been demonstrated that adrenergic stimulation effects ion movements across both

sodium/proton and chloride/bicarbonate mechanisms on the erythrocyte membrane (Nikinmaa and Huestis, 1984; Baroin et al., 1984a,b; Wood and Perry, 1985; Heming et al., 1987). In fish, the rate of carbon dioxide excretion is dependent in large part on the catalysed dehydration of plasma bicarbonate by erythrocytic carbonic anhydrase (Randall and Daxboeck, 1984). As in mammalian erythrocytes, plasma bicarbonate enters fish erythrocytes via a chloride/bicarbonate exchange mechanism on the erythrocyte membrane (Cameron, 1978; Obaid et al., 1979; Heming et al., 1986). Adrenergic effects on erythrocytic chloride/bicarbonate exchange would, therefore, be expected to alter the rate of carbon dioxide excretion in the intact animal. There are no documented studies, however, which investigate the effects of catecholamines on carbon dioxide excretion in fish in vivo.

The purpose of this section is to describe the characteristics of the ion exchange mechanisms which are involved in the adrenergic response in fish erythrocytes. In addition, this section will examine the effect of catecholamines on carbon dioxide transport and excretion in fish both in vivo and in vitro.

CHAPTER.1: CONTROL OF ERYTHROCYTE VOLUME AND pH IN THE RAINBOW TROUT, SALMO GAIRDNERI

Introduction:

Nikinmaa and Huestis (1984) described the mechanism of the adrenergic response in rainbow trout erythrocytes as a loosely coupled sodium/proton and chloride/bicarbonate exchange. In contrast, Heming et al., (1987) proposed that the adrenergic swelling and pH changes were caused by stimulation of tightly coupled sodium/proton and chloride/bicarbonate exchange mechanisms. Finally, according to Baroin et al., (1984a), the adrenergic response in rainbow trout erythrocytes involved stimulation of a sodium/proton exchange mechanism and a sodium:chloride co-transport mechanism. The experimental conditions were very different in these studies. The experiments of Heming et al., (1987) were carried out at 10^{o}C on erythrocytes suspended in plasma and the pH adjusted with carbon dioxide. The extracellular pH in their study was never lower than 7.5. Nikinmaa and Huestis (1984) on the other hand, performed their experiments at 22^{o}C and at low extracellular pH (7.2 - 7.3) in HEPES buffered media. Finally, in the experiments of Baroin et al., (1984a),

the temperature was 15°C and the pH was approximately 7.8 (adjusted with HEPES buffer). Clearly, the variable conclusions of these studies may have occurred as a result of different effects of temperature, extracellular pH or extracellular bicarbonate ion concentration on the adrenergic response. The purpose of this study was, therefore, to examine the effects of these variables on the characteristics of the adrenergic response in rainbow trout erythrocytes in an attempt to resolve these apparent discrepancies.

Materials and Methods

Rainbow trout, Salmo gairdneri, of both sexes (200-300g) were obtained from Sun Valley Hatchery, Mission, B.C., and were held outdoors at 13-15°C. Blood was taken into heparinized syringes from stunned fish via caudal or cardiac puncture, and the red cells washed twice in Ringer's solution. The Ringer's solution used had the following composition: 145 mM NaCl, 4 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgCl_2$, 5 mM glucose and 5 or 10 mM $NaHCO_3$. The pH of the extracellular medium was adjusted by varying the carbon dioxide tension, which ranged from atmospheric (pH 8.2; in this case 10 mM HEPES was added to the buffer to stabilize the pH) to 4% CO_2 to obtain the desired pH of 7.1 in 10 mM HCO_3^-.

Following the washes, the cells were resuspended to a haematocrit of 15% and the sample was divided into four portions. Two of the subsamples were incubated for 30 minutes in a shaking tonometer in the Ringer's alone, in another sample 0.1 mM DIDS (4,4-Diisothiocyanostilbene-2,2-Disulfonic acid) was added, and in the fourth sample 1 mM amiloride was added. The cells were incubated at different extracellular pH values (7.1-8.2),

bicarbonate concentrations (5 and 10 mM), and temperatures (10 and 18^{o}C). After the 30 min incubation, 0.4 ml samples were taken for extra- and intracellular pH determinations, and determinations of cellular water content. Immediately after sampling, 10^{-5} M (final concentration) isoproterenol, freshly made for each experiment, was added to the DIDS and amiloride incubations and one of the incubations with Ringer's solution alone. The cells were then incubated for an additional 30 min. At this point, another set of samples was taken for the pH and cell water determinations. Similar experiments were carried out in which either choline was substituted for sodium, or in which 0.1 mM 2,4-dinitrophenol (a protonophore, see McLaughlin & Dilger, 1980) was added to the Ringer's solution. The procedure for the analysis of the samples is described in the general materials and methods section.

Results

Below the extracellular pH of 7.6, the cell volume (% water content) increased linearly with decreasing extracellular pH by 1%/0.1 pH unit (from 67-68 % at pH 7.6 to 72-73 % at pH 7.1; Fig.1). The volume of amiloride-treated cells changed as that of control cells, whereas DIDS effectively abolished the pH dependent changes in cell volume. At each pH studied, isoproterenol caused an increase in the cell volume. This effect was also pH dependent and increased at low pH (Fig.2). The isoproterenol-induced increase in cell volume was similar in both 5 and 10 mM bicarbonate. The cell water content at extracellular pH 7.10-7.15 was 72.4$\pm$0.4% in 5 mM bicarbonate and 72.3$\pm$0.5% in 10 mM bicarbonate before the addition of isoproterenol, and increased to 78.2$\pm$0.3% in 5 mM bicarbonate and to 78.4$\pm$0.3 % in 10 mM bicarbonate after the addition of isoproterenol (N = 8 in each case). Together, the beta-adrenergic and pH-induced changes represent a 15 % increase in cellular water content between pH 8.2 and 7.1. The beta-adrenergic cell swelling was abolished by addition of DIDS or amiloride (Fig.2).

Figure 1. Effect of external pH on the cellular water content (%) of rainbow trout erythrocytes. open circles = control cells, triangles = amiloride (1 mM)-treated cells, closed circles = DIDS (0.1 mM)-treated cells. Lines are fitted by eye. Each point represents a mean of 8 determinations. The cell water content of control and amiloride treated cells increased significantly (unpaired t-test; $p<0.05$) below pH 7.6. No significant changes were observed in DIDS-treated cells.

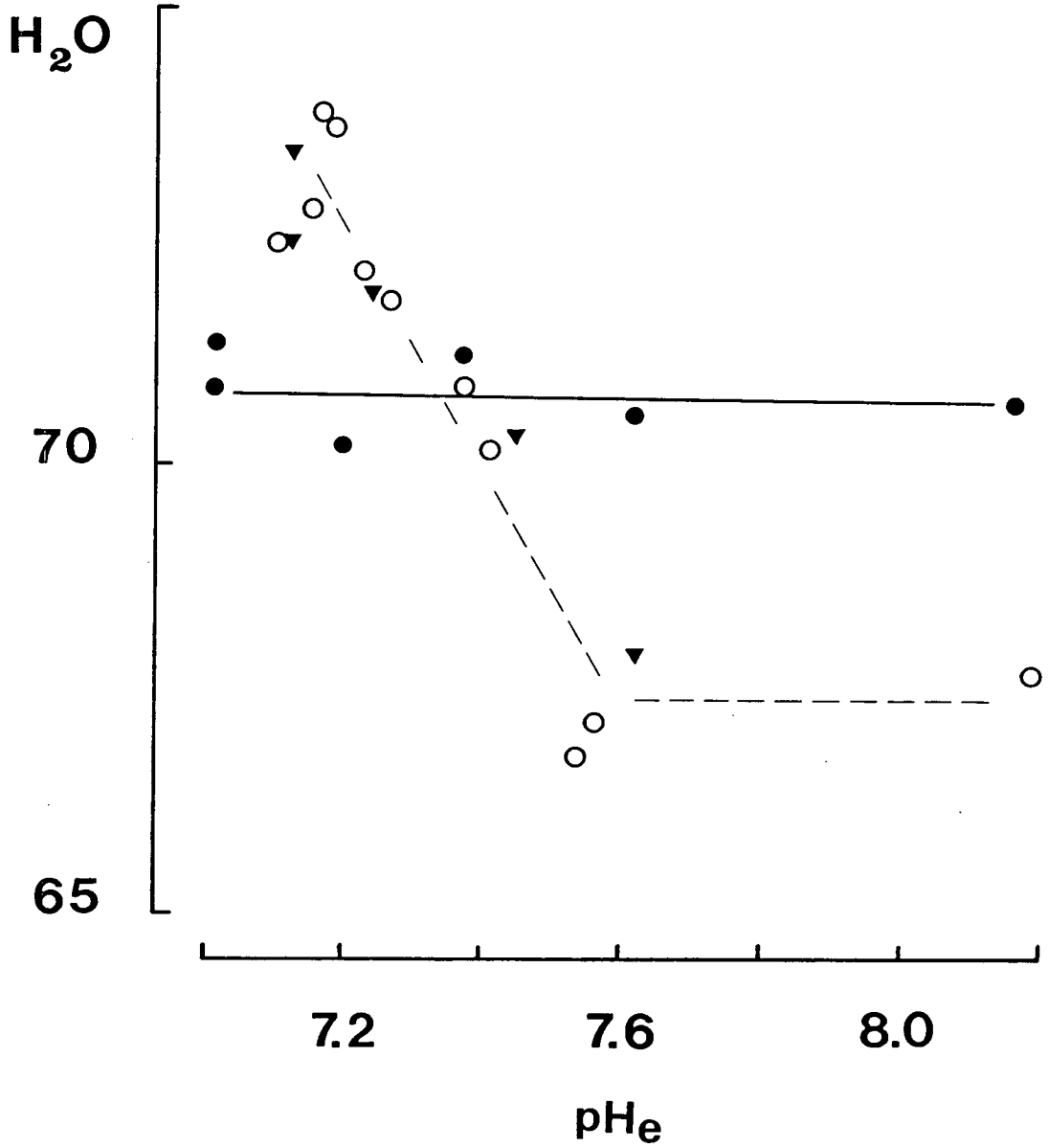

H2O
70
65
7.2
7.6
8.0
pHe

Figure 2. Isoproterenol-induced increase in cellular water content of rainbow trout erythrocytes. Erythrocytes were initially incubated for 30 min and their water content ($\%H_2O$) determined. Thereafter, 10^{-5} M isoproterenol was added to the medium and the water content determined following a further 30 min of incubation ($\%H_2O_{isoprot.}$). The change in water content was calculated as:

$$dH_2O\ (\%) = (\%H_2O_{isoprot.} - \%\ H_2O)/\%H_2O$$

Line is fitted by eye. Each point represents 8 determinations. open circles = control cells (10^{-5} M isoproterenol only), triangles = amiloride cells (1 mM amiloride + 10^{-5} M isoproterenol), closed circles = DIDS-treated cells (0.1 mM DIDS + 10^{-5} M isoproterenol).

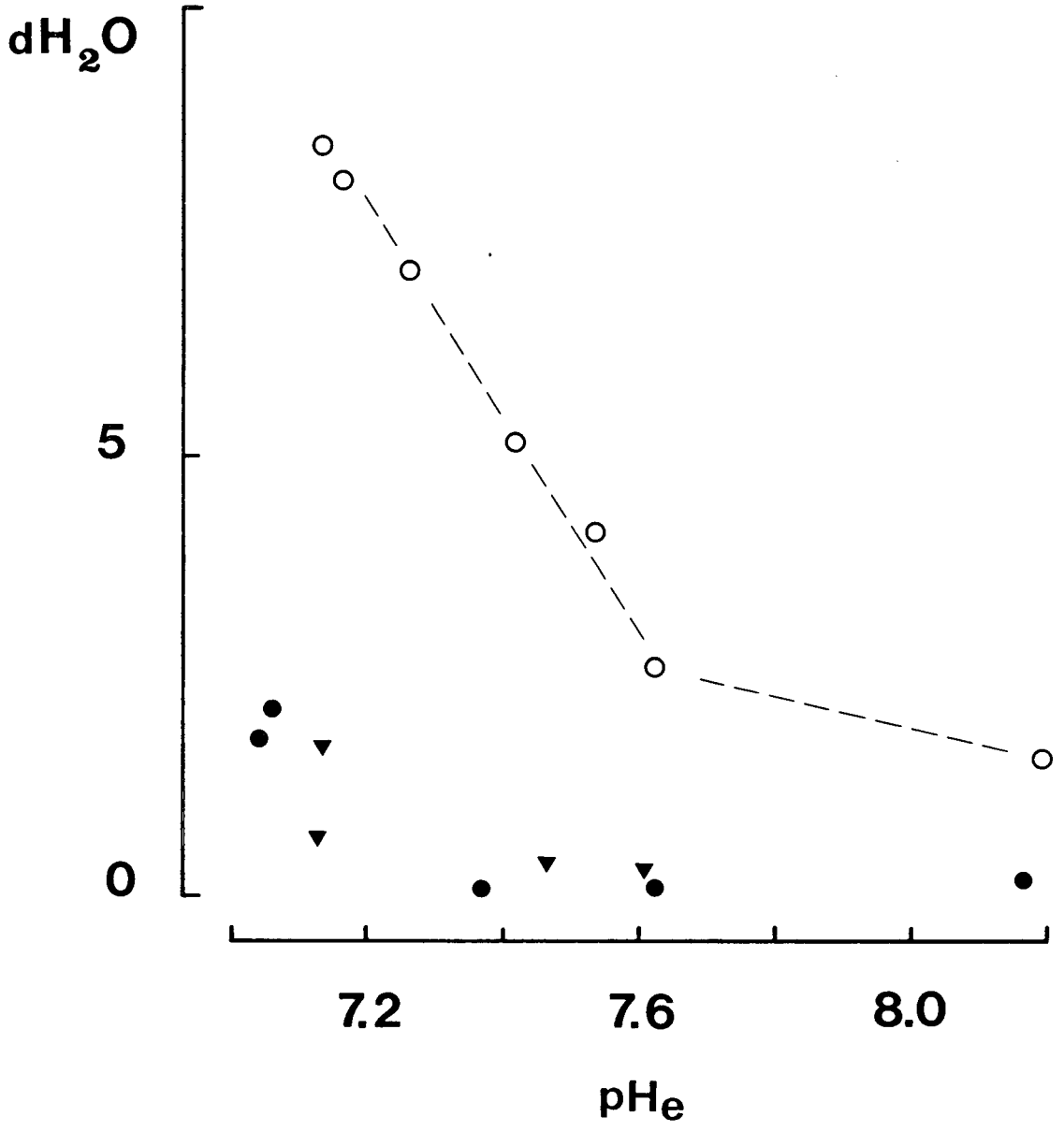

dH2O
5
0
7.2
7.6
8.0
pHe

Figure 3. Intracellular pH (pH_i) vs extracellular pH (pH_e) of rainbow trout erythrocyte suspensions. Each point represents a mean of 3 to 8 determinations. open circles = values measured using the DMO method; triangles = values calculated from the pH_e and the chloride distribution across the erythrocyte membrane. The dependence of intracellular pH on extracellular pH, calculated for individual data points, is given by the following regression lines: DMO method; $pH_i = 0.725 \times pH_e + 1.80$, $r = 0.93$ (n=116). Chloride distribution method; $pH_i = 0.821 \times pH_e + 1.05$, $r = 0.95$, (n=77).

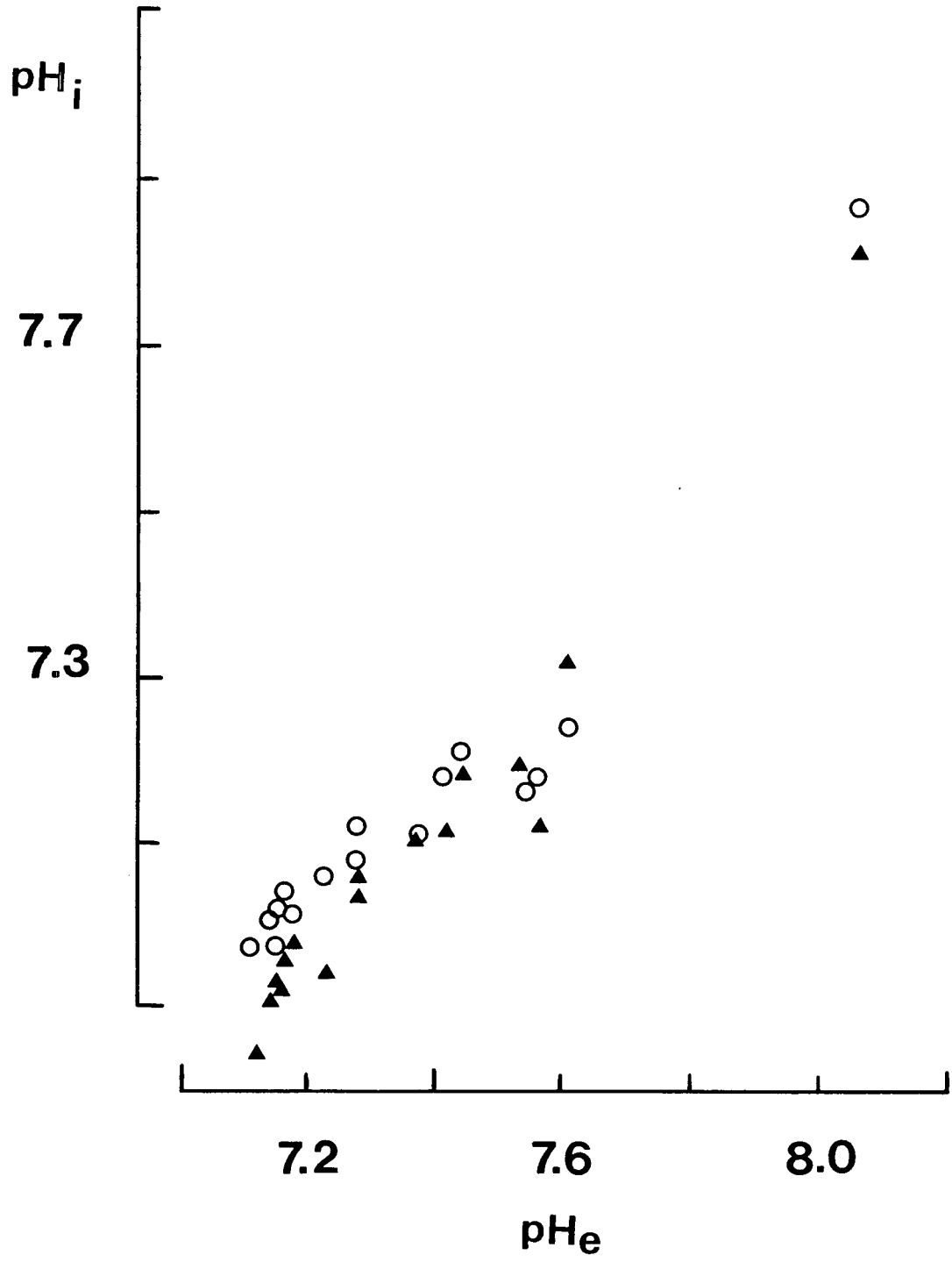

pH$_i$
7.7
7.3
7.2
7.6
8.0
pH$_e$

Figure 4. Effects of isoproterenol on the intracellular vs. extracellular pH of rainbow trout erythrocytes. Erythrocytes were first incubated for 30 min and their pH measured (open circles = control). Thereafter, 10^{-5}M isoproterenol was added and determinations were repeated after another 30 min (closed circles = control + isoproterenol). Lines were fitted by eye. Each point represents a mean of 8 determinations. Apart from pH 8.2, isoproterenol caused a significant (paired t-test; $p<0.05$) decrease in the pH gradient across the erythrocyte membrane at every pH studied.

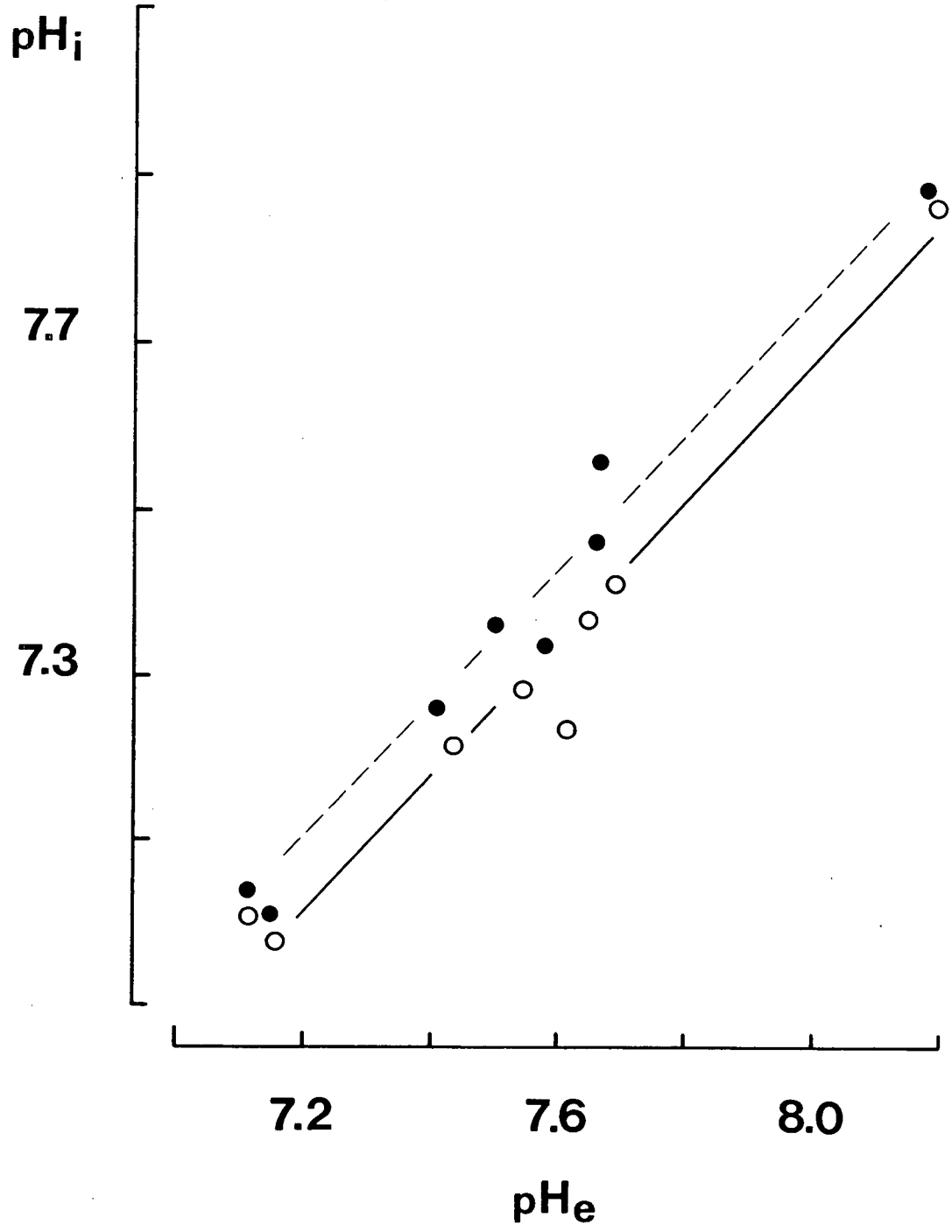
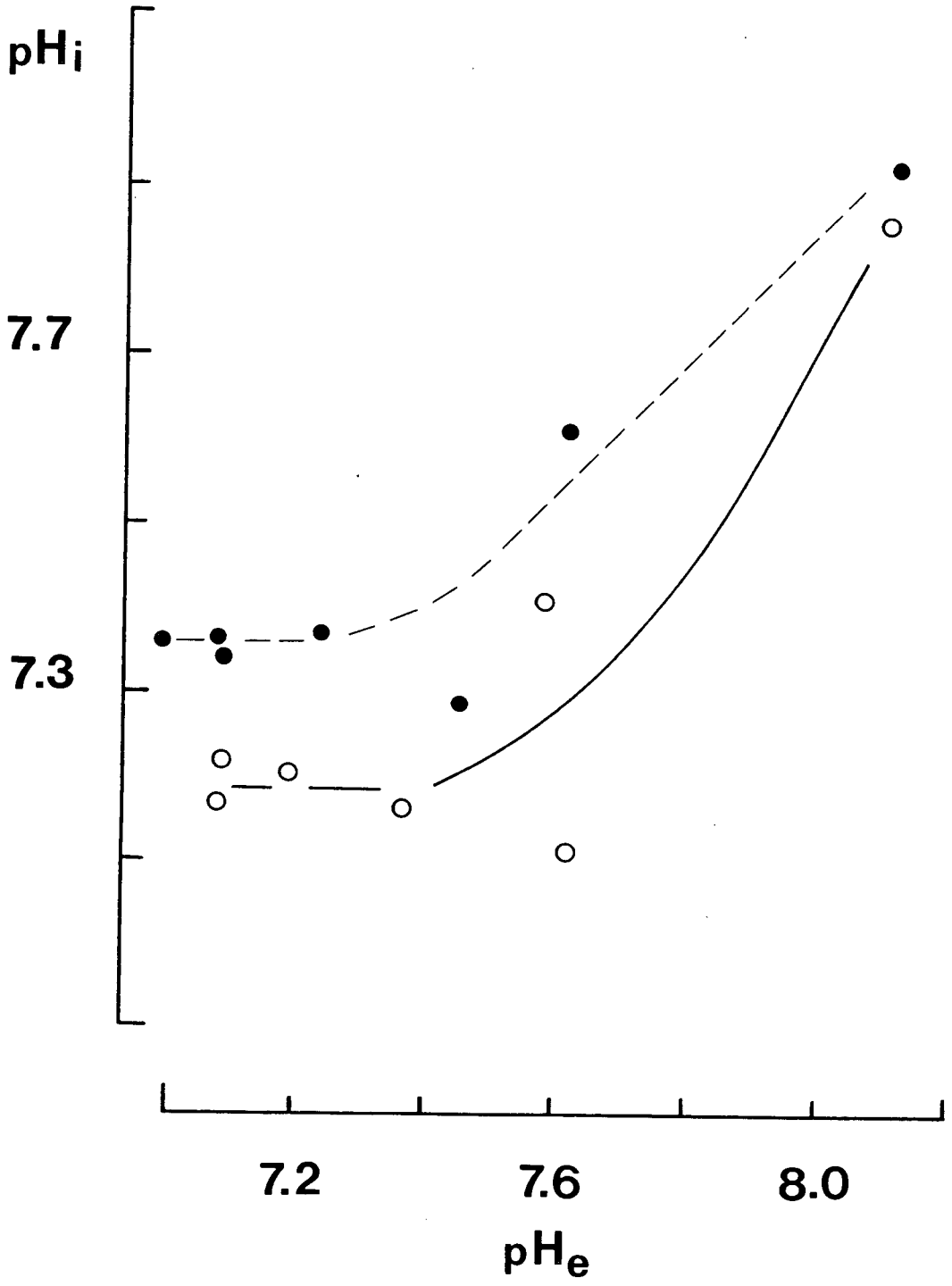

pH$_i$
7.7
7.3
7.2
7.6
8.0
pH$_e$

Figure 5. Extracellular pH (pH_e) vs. intracellular pH (pH_i) in DIDS-treated rainbow trout erythrocytes before (open circles) and after (closed circles) the addition of 10^{-5} M isoproterenol. Lines fitted by eye. Each point represents the mean of 8 determinations.


pHi
7.7
7.3
7.2
7.6
8.0
pHe

The intracellular pH of the untreated cells, measured by the DMO method and the intracellular pH calculated from the distribution of chloride (assuming a Donnan equilibrium) were not significantly different at pH values above 7.3 (Fig.3). At lower extracellular pH values, however, the DMO method gave consistently higher values for the intracellular pH. The intracellular pH of non-treated cells at low extracellular pH was not affected by amiloride or by removing sodium from the incubation medium (Table 1) suggesting that sodium/proton exchange does not occur under these conditions.

Isoproterenol caused a significant reduction in the pH gradient across the erythrocyte membrane at every pH studied except pH 8.2 (Fig.4). This reduction was inhibited by amiloride (Table 2). In contrast, this adrenergic response was enhanced by DIDS (Table 2, Fig. 5). The magnitude of the combined effect of DIDS and isoproterenol was dependent on the bicarbonate concentration. In 10 mM bicarbonate, the cellular alkalinization was only half of that at 5 mM bicarbonate (Table 2). The adrenergic response was independent of temperature over the range tested. The volume change observed at $10^{o}C$ was not significantly different than that observed at $18^{o}C$, nor was the pH change (Table 3).

Table 1. Extracellular and intracellular pH and the pH gradient across the erythrocyte membrane in control cells, in amiloride (1 mM)-treated cells, and in cells incubated in the absence of sodium.

treatment	pH_e	pH_i	dpH
Control (N=4)	7.231±0.018	7.051±0.030	0.180
Amiloride (N=4)	7.262±0.008	7.070±0.012	0.192
No Sodium	7.207±0.009	7.037±0.012	0.170

Values are means ± one S.E.M.

Table 2. Changes in the pH gradient across the erythrocyte membrane induced by isoproterenol.

	5 mM HCO_3^-			10 mM HCO_3^-		
	dpHO	dpHISO	Change	dpHO	dpHISO	Change
pH_e 7.1-7.2						
ISO only	0.172	0.102	-0.070	0.131	0.088	-0.043
Amil	0.155	0.126	-0.029	0.121	0.112	-0.009
DIDS	-0.111	-0.393	-0.282	-0.149	-0.307	-0.158
pH_e 7.3-7.4						
ISO only	0.220	0.150	-0.070			
Amil	0.247	0.243	-0.006			
DIDS	0.203	-0.100	-0.303			
pH_e 7.5-7.6						
ISO only	0.377	0.267	-0.110			
Amil	0.299	0.228	-0.071			
DIDS	0.505	0.174	-0.159	0.159	0.013	-0.146

Values are means ± one S.E.M. The cells were initially incubated for 30 min in the Ringer's solution or in the presence of the transport inhibitors (O). Thereafter, 10^{-5} M isoproterenol was added to the incubations and the cells incubated for an additional 30 min (ISO). At both times (O and ISO), the pH_e and pH_i were determined, and the pH gradient ($dpH=pH_e-pH_i$) calculated. The change in pH gradient (Change=dpHISO-dpHO) shows the effect of isoproterenol in the different treatments.

Table 3. Effects of temperature on the adrenergic response of rainbow trout erythrocytes.

treatment	pH_e	pH_i	dpH	H_2O
10°C Cont	7.539±0.019	7.286±0.027	0.216	70.4±0.3
Isoprot	7.491±0.025	7.364±0.019*	0.127	73.3±0.2*
18°C Cont	7.428±0.005	7.208±0.013	0.220	70.0±0.3
Isoprot	7.402±0.011	7.253±0.041*	0.150	73.6±0.23*

Values are means ± one S.E.M. Asterisk denotes significant (paired t-test; $p<0.05$) differences between isoproterenol and control values.

Discussion

The erythrocyte volume of rainbow trout increases as a result of extracellular acidification below a pH of 7.6 (Fig.1). During extracellular acidification, the intracellular pH also decreases, decreasing the negative charge on the haemoglobin. To maintain electroneutrality, the intracellular concentration of chloride increases and water follows osmotically (For review, see e.g. Hladky and Rink, 1977). The volume changes associated with changes in pH could be prevented by blocking the anion exchange pathway with DIDS. When anion movements are inhibited in this manner, the net influxes (at low pH) or effluxes (at high pH) of Cl- and water do not occur. Thus, at low pH, the volume of DIDS-treated cells is smaller and at high pH greater than that of control cells. Cala (1983, 1985) has pointed out that the net movements of chloride are important in the cell volume changes.

The observed decrease in intracellular pH did not activate the amiloride-sensitive sodium/proton exchange. Thus, the sodium/proton exchanger of trout erythrocytes

differs from that of lymphocytes (Grinstein et al., 1986) and lamprey red cells (Nikinmaa et al., 1986) in which acidification of the intracellular compartment activates this exchanger. Adrenergic agonists activate sodium/proton exchange in trout erythrocytes in vitro (Nikinmaa and Huestis, 1984; Baroin et al., 1984b). In nominally bicarbonate free media, this activation increases the intracellular pH by 0.1-0.2 units (Nikinmaa and Huestis, 1984). Heming et al., (1987) showed that an adrenergically-induced increase in intracellular pH also occurs in erythrocytes incubated in plasma. Our study confirms that the intracellular pH may increase when a bicarbonate/carbon dioxide system is used as a buffer. Furthermore, this increase is significant even at a bicarbonate concentration of 10 mM. The magnitude of this response, however, appears to decrease with increasing bicarbonate concentration. Amiloride inhibits this intracellular alkalinization. Together, these data strongly suggest that adrenaline-activated sodium/proton exchange plays a significant role in the control of cytoplasmic pH in trout erythrocytes.

The present results show that DIDS inhibits the cell volume increase in isoproterenol-treated cells, but

causes a marked intracellular alkalinization. Thus, the activity of the sodium/proton exchange does not appear to be altered by the inhibition of the chloride/bicarbonate exchanger. Indeed, the adrenergically stimulated sodium/proton exchange causes a larger intracellular pH change in the absence of bicarbonate movements. This finding supports the results of Nikinmaa and Huestis (1984) who describe these ion exchange mechanisms as loosely coupled. The adrenergic responses of trout erythrocytes are more pronounced at low extracellular pH indicating that the adrenergically activated sodium/proton transporter is sensitive to protons. These findings are consistent with the results of Nikinmaa (1983) and Heming et al., (1986) for cells incubated in TRIS-HCl buffered saline or plasma, respectively. Stress is invariably associated with a decrease in plasma pH in fish (Holeton & Randall, 1967; Soivio and Nikinmaa, 1981; Holeton et al., 1983; Jensen et al., 1983; Nikinmaa et al., 1984; Primmett et al., 1986). The present results, therefore, indicate that the adrenergic response partially offsets the detrimental effects which severe exercise and low pH would otherwise have on erythrocytic oxygen transport.

The adrenergic response was not affected by

temperature in the range 10 to 18°C. This finding shows that the in vivo differences in the erythrocyte responses to adrenaline or strenuous exercise between different temperatures (Nikinmaa,1982b) or different seasons are not caused by a simple temperature dependence of the adrenergic response.

Cossins and Richardson (1985) have documented that the cellular alkalinization in trout erythrocytes following adrenergic activation is caused by stimulation of sodium/proton exchange with a stoichiometry of 1. In addition, Borgese et al., (1986) have reported that nitrate inhibits sodium/proton exchange. The co-transport mechanism for adrenergic swelling in trout erythrocytes proposed by Baroin et al., (1984a) can, therefore, be rejected. Baroin et al., (1984a) based their argument on the assumption that nitrate does not inhibit sodium/proton exchange. Together, these more recent studies have provided further support for the double exchanger mechanism for the adrenergic response which is proposed in this study.

Summary

1. A decrease in extracellular pH caused an increase in the volume of rainbow trout erythrocytes, and a decrease in the intracellular pH.
2. These pH-induced volume changes are mainly associated with movements of chloride across the chloride/bicarbonate exchange pathway.
3. The sodium/proton exchanger is quiescent at all pH's studied.
4. The adrenergic drug, isoproterenol, promoted cell swelling and proton extrusion even in the presence of 10 mM bicarbonate, although the magnitude of the adrenergic pH response decreases with increasing bicarbonate concentration.
5. The adrenergic response was enhanced by a decrease in extracellular pH.
6. DIDS markedly enhanced the effect of isoproterenol on the intracellular pH, but abolished the increase in erythrocyte volume.
7. Amiloride inhibited both the volume and the pH changes associated with adrenergic stimulation.
8. The adrenergic response was independent of temperature between 10 and 18^{o}C.

9. The results support a loosely coupled sodium/proton and chloride/bicarbonate exchange model for the adrenergic response in rainbow trout erythrocytes.

CHAPTER.2: EFFECT OF BURST SWIMMING AND ADRENALINE INFUSION ON OXYGEN CONSUMPTION AND CARBON DIOXIDE EXCRETION IN THE RAINBOW TROUT, SALMO GAIRDNERI.

Introduction:

Wood and Perry (1985) have reported that adrenaline inhibits bicarbonate entry into rainbow trout erythrocytes *in vitro*. In the intact animal, this inhibition would result in the retention of plasma bicarbonate during branchial blood transit, and therefore a reduction in carbon dioxide excretion. At present, however, there is little evidence that catecholamines modulate carbon dioxide excretion in the intact animal. Van den Thillart *et al.*, (1983) have reported low respiratory exchange ratios (carbon dioxide retention) in exercising coho salmon (*Oncorhynchus kisutch*) which would indicate a reduction in erythrocytic chloride/bicarbonate exchange. The effect of their experimental protocol on circulating catecholamine levels in these fish, however, is unknown.

The purpose of these experiments was to determine if catecholamines modulate carbon dioxide excretion and therefore the respiratory exchange ratio in the rainbow trout *in vivo*. Burst swimming is known to cause a large

increase in circulating catecholamines and an acid-base disturbance in the rainbow trout Salmo gairdneri (Primmett et al., 1986). I have, therefore, examined the effect of 1) burst swimming and 2) adrenaline infusion on carbon dioxide excretion and the respiratory exchange ratio in these animals.

Materials and Methods

The first series of experiments were performed at 15°C in a Brett-type swimming respirometer (Brett, 1964) with a total water volume of 37.5 liters. Oxygen consumption was determined at 20 min intervals by measuring the decline in oxygen tension of the recirculating water in the closed respirometer, and the amount of pure oxygen injected into the system, as described by van den Thillart et al., (1983). Oxygen tension of the water was measured continuously by recirculating a small fraction of the water through an oxygen electrode mounted in a cuvette (Radiometer, E-5046 and D-616), as described by Steffensen et al. (1984). The oxygen electrode was connected to a Radiometer PHM 71 acid-base analyzer and a chart recorder.

Carbon dioxide excretion of the fish was calculated from the difference in total carbon dioxide content of the water, determined every 20 min by analyzing water samples using a Carle Series 111 Analytical Gas Chromatograph with a Poropak Q column. Total carbon dioxide content was determined as described by Boutilier et al., (1985) with the following modifications. A water sample of 2.0 ml was injected

into a glass syringe (10 ml) containing pure nitrogen, and acidified with 50 ul of 1.0 N HCl. After 2 min of shaking, at least 6 ml of the gas in the syringe was injected into the gas chromatograph loop (volume = 1.0 ml) to assure complete washout of the loop. The carbon dioxide concentration was calculated by integrating the signal from the gas chromatograph with a HP 3497 Data Acquisition/Control Unit and a HP 9135A computer.

The water pH in the swimming respirometer was kept constant at 6.80. The water pH was continuously monitored with a pH electrode (Canlab, GK2401C) permanently mounted in the system, connected to a Radiometer PHM 71 acid-base analyzer and a comparator. The recorder output was connected to a circuit controlling a Harvard Linear displacement pump injecting 0.25 M NaOH, as described by van den Thillart et al. (1983). When the pH of the water decreased below 6.80, the pump was activated and NaOH injected until a pH of 6.80 was re-established.

The fish were acclimated in the respirometer for 24 h before the experiment. During this period, the respirometer was continuously flushed with thermostatted aerated water having a pH of 6.80. The experiment was

started by closing the respirometer. Control oxygen consumption and carbon dioxide excretion were measured over 20 min periods for 80 min at the acclimation swimming speed (40 cm/sec). The swimming speed was then increased to 80 - 85 cm/sec for 10 min causing the fish to burst swim to exhaustion. Burst swimming causes an increase in plasma catecholamines in trout (Primmett et al., 1986). After 10 min, the speed was returned to 40 cm/sec and the same parameters were measured during the following 80 min, at which point the experiment was terminated.

In the second series of experiments, fish were anaesthetized with MS-222 and the dorsal aorta cannulated with PE-50 polyethylene tubing as described by Soivio and Oikari (1976). The cannula was used to sample blood for the determination of erythrocyte pH (pH_i) via the freeze-thaw method of Zeidler and Kim (1977) with a Radiometer PHM 71 acid-base analyzer and a micro-pH unit. Oxygen consumption, carbon dioxide excretion and erythrocyte pH (pH_i) were measured in resting fish before and after infusion of adrenaline (0.25 ml of 10^{-4}M adrenaline solution). The fish were housed in a flow through respirometer with a volume of 3.0 liters. The respirometer was constructed with a recirculating circuit

to assure adequate mixing. Water temperature was 10°C.

The "mega" dose of adrenaline was used to make sure that the beta-adrenergic receptors were stimulated and saturated. The increased pH_i indicated that the bolus acted on the beta-adrenergic receptors of the erythrocyte. The adrenaline solution was prepared less than 10 min before the infusion. After each fish was acclimated for at least 24 h in the respirometer, control measurements were taken at 10 min intervals for 70 min. Adrenaline was then injected and the measurements continued for the following 60 min.

Oxygen tension, PO_2, and total CO_2 were measured every 10 min on samples of water entering and leaving the respirometer. Oxygen consumption was calculated from the measured incurrent and excurrent PO_2 and water flow (V) according to the following equation:

$$VO_2 = B \times ((P_{IO2}-P_{EO2t=1/2}) \times V + (P_{EO2}/t) \times V)\ bw^{-1}$$

(Ultsch et al., 1980) where B = solubility of oxygen in water; P_{IO2} = partial pressure of oxygen in the incurrent water, P_{EO2} = partial pressure in the excurrent water; $P_{EO2t=1/2}$ = partial pressure of oxygen in the excurrent water after the elapse time of one half the time interval

t. V = volume of respirometer. bw = body weight. Carbon dioxide excretion was calculated in a similar manner.

In both series of experiments, the ambient oxygen tension was always kept above 110 mmHg.

Results

Measurements of oxygen consumption and CO_2 excretion for 6 fish swimming at 40 cm/sec before and after burst swimming are illustrated in Figure 6. Control oxygen consumption at a swimming speed of 40 cm/sec ranged from 118.3 to 135.6 um kg^{-1} min^{-1}. CO_2 excretion varied from 86.7 to 102.1 um kg^{-1} min^{-1}. The calculated respiratory exchange ratio (RE; VCO_2/VO_2) varied from 0.68 to 0.80 (Fig. 7).

After burst swimming for 10 min, oxygen consumption increased significantly (71%) to 217.3 $\pm$ 32.1 um kg^{-1} min^{-1} during the first 20 min of recovery. CO_2 excretion increased 104% to 192.1 $\pm$ 49.9 um kg^{-1} min^{-1}, thus RE increased significantly (17%) to 0.87 $\pm$ 0.11.

20 - 40 min after burst exercise O_2 consumption and CO_2 excretion were still significantly elevated as compared to the control values, 32% (167 $\pm$ 26.9 um kg^{-1} min^{-1}) and 50% (141.6 $\pm$ 23.8 um kg^{-1} min^{-1}), respectively. The respiratory exchange ratio was not significantly different from the control.

During the following 20 min, O_2 consumption was 152.8 $\pm$ 25.7 um kg^{-1} min^{-1} and CO_2 excretion was 118.4 $\pm$ 34.5 um kg^{-1} min^{-1}, or 21% and 26% higher than control,

Figure 6. Oxygen consumption and carbon dioxide excretion of rainbow trout swimming 40 cm sec^{-1} before and after burst swimmming. Values are means + standard error (N=6). Paired t-test was used to compare values following the burst swim to the mean of the control values. Differences were accepted as significant at the $p<0.05$ level and are indicated by an asterisk.

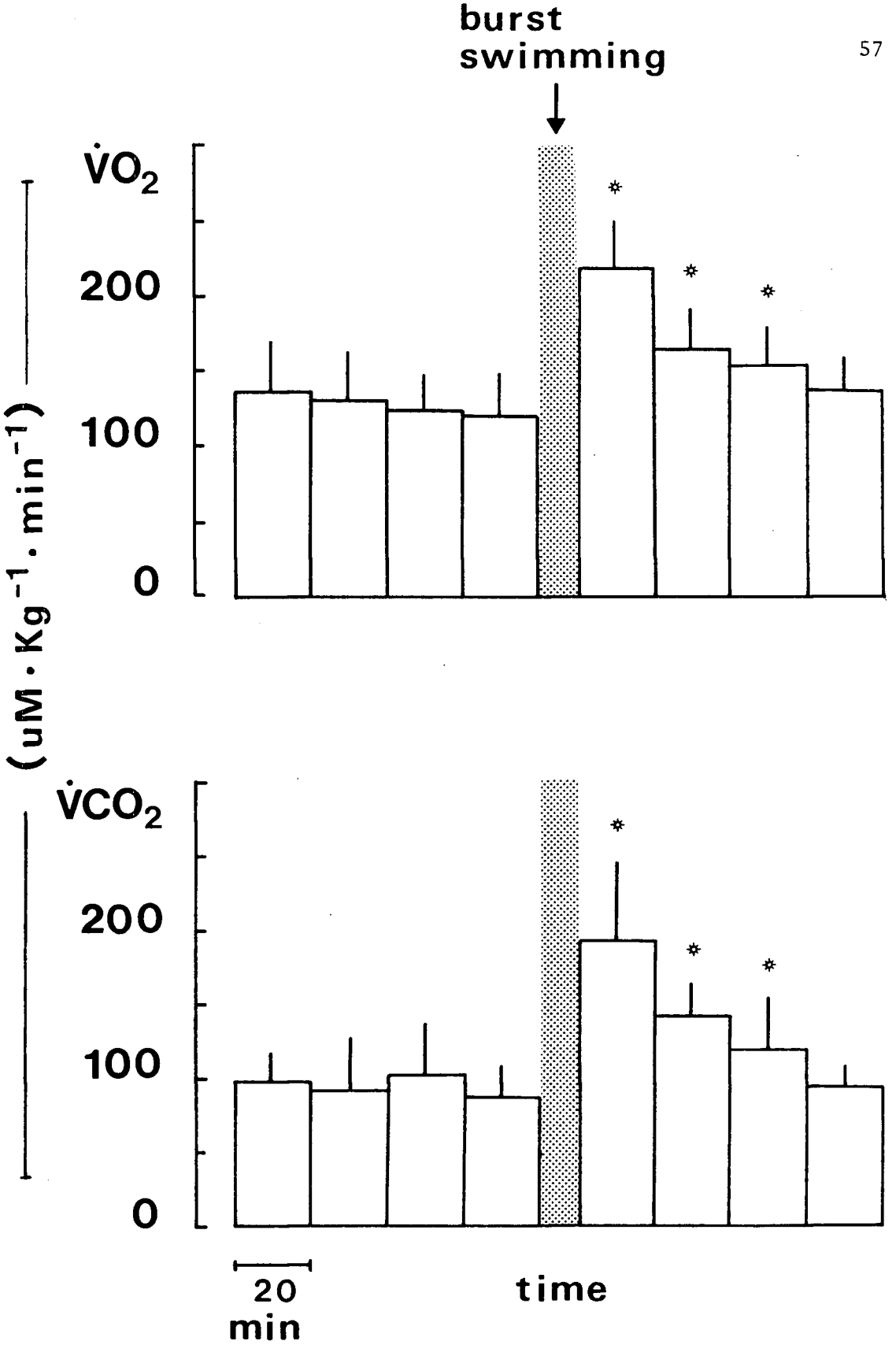

burst
swimming
$\dot{V}O_2$
200
100
0
$(uM \cdot Kg^{-1} \cdot min^{-1})$
$\dot{V}CO_2$
200
100
0
20
min
time

Figure 7. Respiratory exchange ratio of rainbow trout swimming 40 cm sec^{-1} before and after burst swimming. Values are means + standard error (N=6). Paired t-test was used to compare values following a burst swim to the average of the control values. Differences were accepted as significant at the $p<0.05$ level and are indicated by an asterisk.

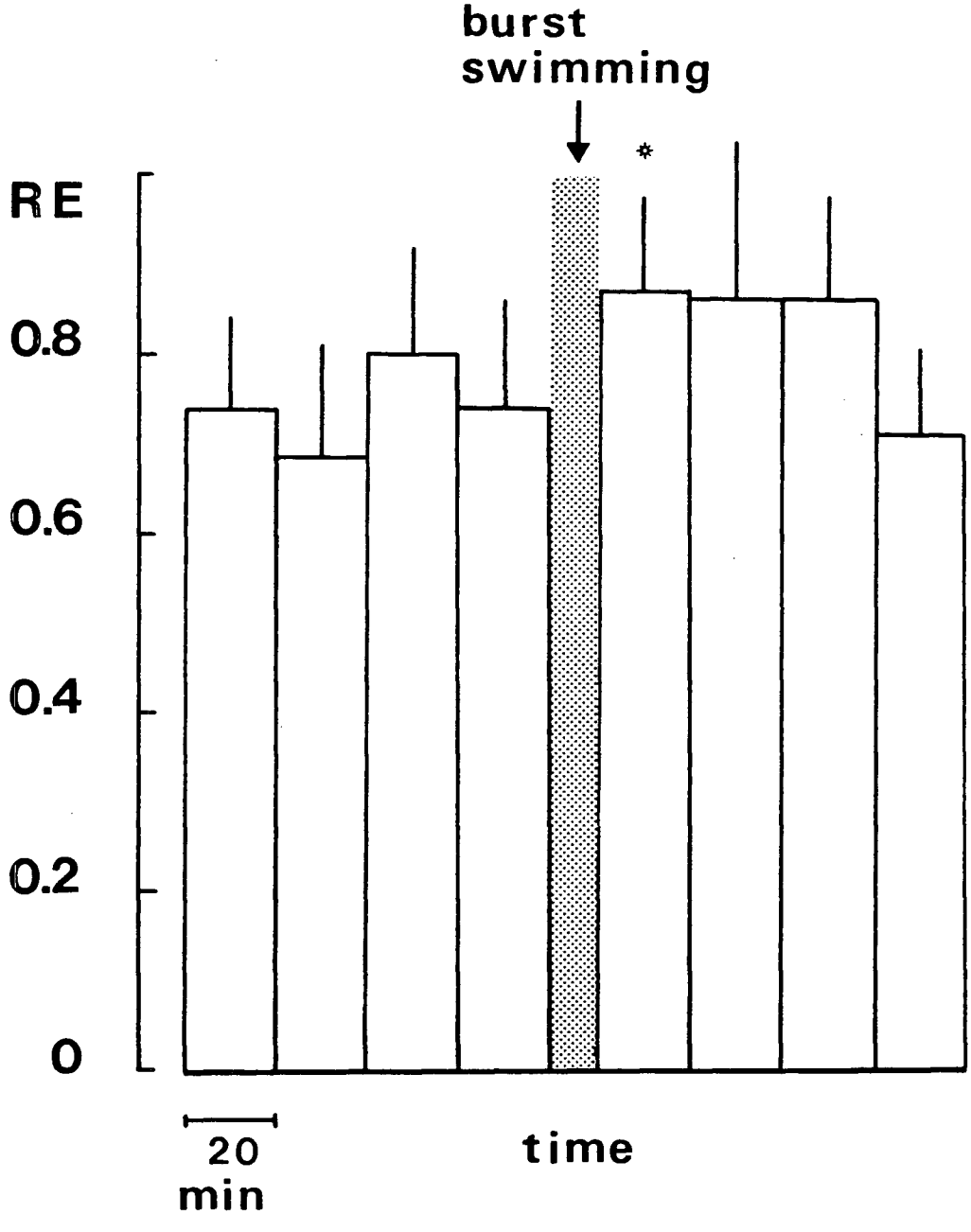

burst
swimming
RE
0.8
0.6
0.4
0.2
0
20
min
time

Figure 8. Oxygen consumption and carbon dioxide excretion of rainbow trout before and after infusion of adrenaline. Values are means + standard error (N=9). Paired t-test was used to compare post-infusion values to the average of all pre-infusion values. Differences were accepted as significant at the $p<0.05$ level and are indicated by an asterisk.

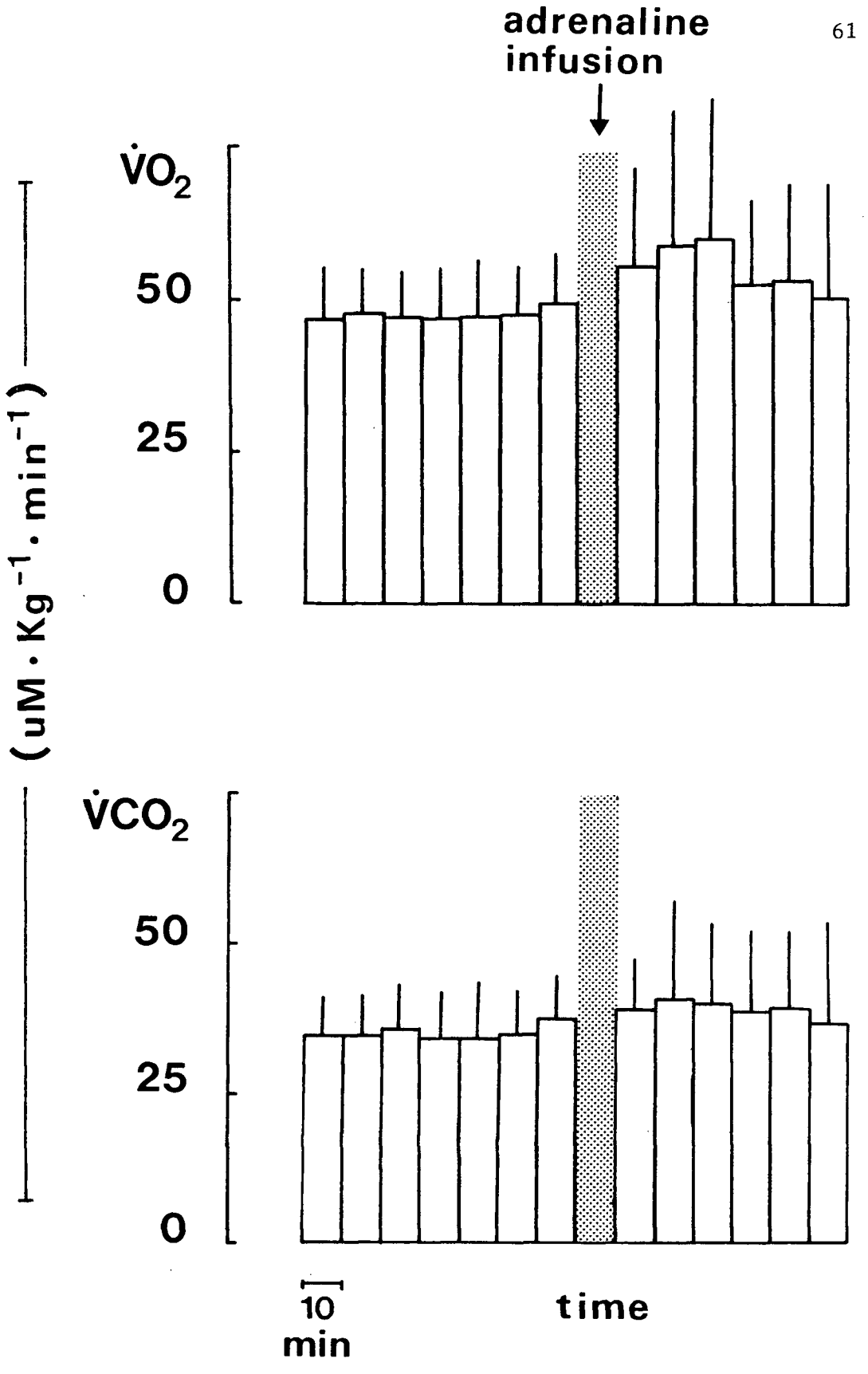
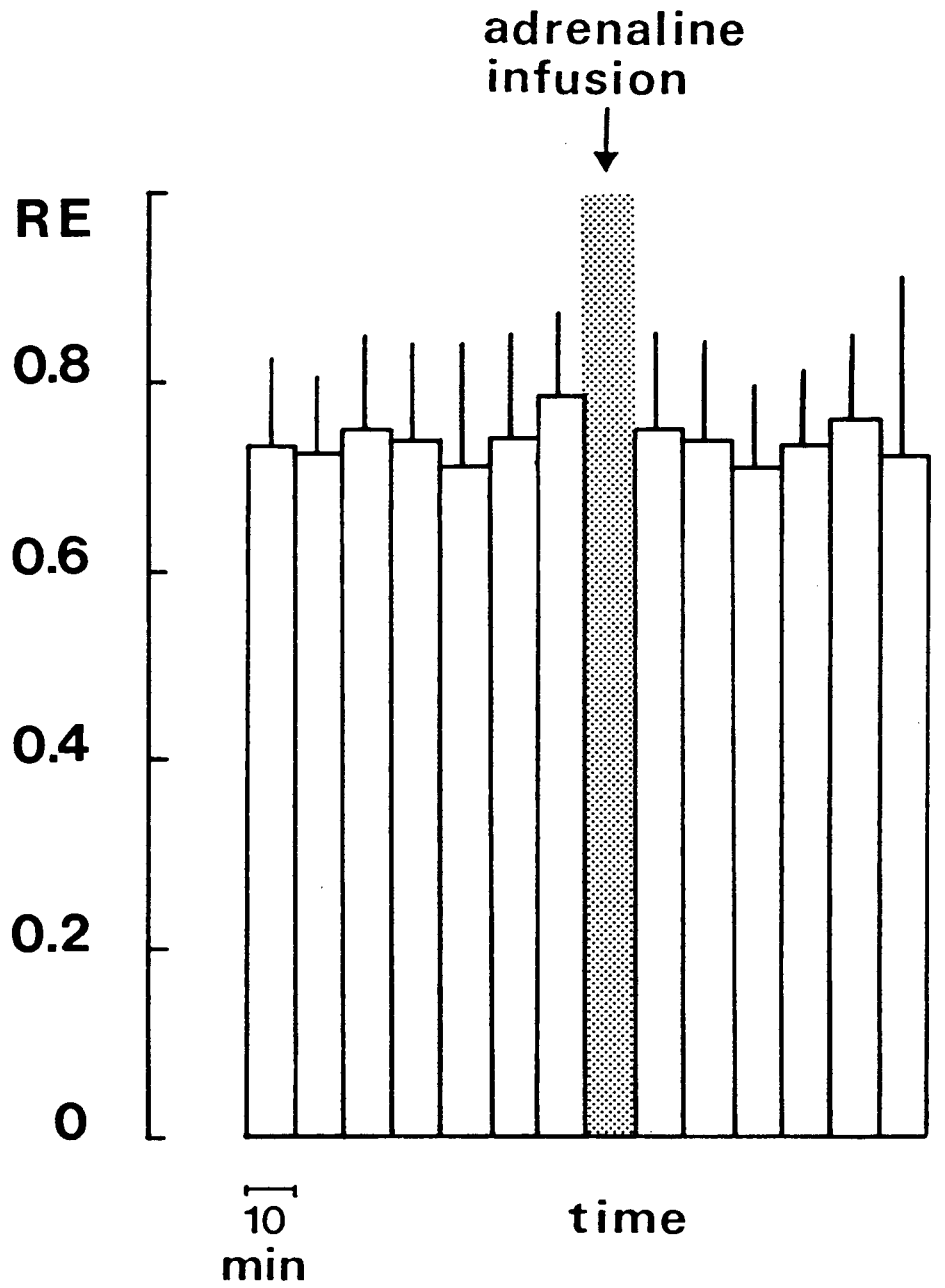

adrenaline
infusion
$\dot{V}O_2$
50
25
0
$\dot{V}CO_2$
50
25
0
(uM · Kg^{-1} · min^{-1})
10
min
time

Figure 9. Respiratory exchange ratio of rainbow trout before and after infusion of adrenaline. Values are means + standard error (N=9). Paired t-test was used to compare post-infusion values to the mean of all pre-infusion values. Differences were accepted as significantly different at the $p<0.05$ level and are indicated by an asterisk.


adrenaline
infusion
RE
0.8
0.6
0.4
0.2
0
10
min
time

respectively. RE was not significantly different from the control.

During the last period (60 - 80 min), none of the measured variables were significantly different from the controls.

The effect of infusing adrenaline into 9 resting rainbow trout is shown in Figures 8 and 9. Oxygen consumption during the 70 min control period was 47.9 $\pm$ 8.0 um kg^{-1} min^{-1}, CO_2 excretion was 35.2 $\pm$ 6.7 um kg^{-1} min^{-1}, and RE consequently 0.74 $\pm$ 0.09. Erythrocyte pH measured prior to adrenaline infusion was 7.391 $\pm$ 0.017. Adrenaline had no significant effect on either O_2 consumption, CO_2 excretion or RE during the following 60 min. Ten min after injection of adrenaline, pH_i had significantly ($P<0.05$) increased to 7.447$\pm$0.034 and remained elevated (7.432$\pm$0.051) for 60 min after adrenaline infusion.

Discussion

Several types of stress increase the levels of circulating catecholamines in fish (Nakano and Tomlinson, 1968; Mazeaud and Mazeaud, 1981; Boutilier et al., 1986a; Perry, 1986). Recently, Primmett et al., (1986) have documented 25 to 35 fold increases in circulating adrenaline and noradrenaline levels in the rainbow trout following a burst swim. It was assumed, therefore, that the burst swim in the present experiments would cause a similar increase in the levels of circulating catecholamines.

It has been suggested that catecholamines modulate CO_2 transport by inhibition of bicarbonate flux through the erythrocyte (Wood and Perry, 1985). This inhibition of erythrocytic chloride/bicarbonate exchange could explain the low RE values reported by van den Thillart et al., (1983) in the coho salmon (Oncorhyncus kisutch). In coho salmon exercised in seawater at pH 7.0, the RE was found to be 0.21. van den Thillart et al., (1983) also measured VO_2 and VCO_2 after burst swimming in normal seawater, but only as mean rates for a 6h period "since most rates did not change very much

during each run". They determined RE to be 0.64 (N = 4). It appears, however, that in their representative fish (Fig. 3), the bicarbonate excretion during the first hour was only 1/15 of the following 3 hours. Accordingly, RE must have been approximately 0.1 during the first hour. The results of the present experiments, however, provide no evidence that increased circulating catecholamines lead to a reduction in carbon dioxide excretion in the whole animal.

Infusion of adrenaline into resting rainbow trout *in vivo* caused no significant changes in either CO_2 excretion or O_2 consumption and consequently the RE value did not change significantly from its control value of 0.74. In addition, burst swimming, which is associated with an increase in blood catecholamines (Primmett *et al.*, 1986) caused an increase in both O_2 consumption (71%) and carbon dioxide excretion (104%) during the recovery period. Thus, in contrast to the "CO_2 retention" proposal of Wood and Perry (1985), there was, in fact, a significant increase in the respiratory exchange ratio to 0.87. The increased RE, following the burst swim is likely attributable as in other animals to the titration of the blood bicarbonate pool by protons entering the blood from the exercising tissues.

It is possible that the absence of an effect of catecholamines on CO_2 excretion in this study could have been due to seasonal variation in inhibition of erythrocytic chloride/bicarbonate exchange due to seasonal variation in B-receptor activity. For example, it has been documented that the gills and heart of eels may lose B-adrenergic sensitivity during the winter (Peyraud-Waitzenegger et al., 1980). Similarly, Nikinmaa and Jensen (1986) have suggested that this may also be the case for the erythrocytes of the rainbow trout. The fact, however, that an elevation in catecholamine levels caused a significant increase in the erythrocyte pH, as shown in other studies in vitro (Nikinmaa, 1983; Nikinmaa and Huestis, 1984; Heming et al., 1987) and in in vivo, indicates that the B-adrenergic receptors were still functional in the trout used in this study. Thus, the absence of an effect of catecholamines on CO_2 excretion in the present study cannot be attributed to reduced activity of erythrocytic B-adrenergic receptors.

There is also a discrepancy between the present experiments and those of van den Thillart et al., (1983) concerning oxygen uptake following burst swimming. The

latter reported no change in oxygen consumption during the approximately 4h recovery period, whereas in this study, O_2 consumption initially increased and then decreased to control levels in the following 80 min. Brett (1964) reported an O_2 debt replacement up to 5h after fatigue in yearling sockeye salmon (Oncorhynchus nerka). Likewise Stevens and Randall (1967) and Steffensen et al., (1984) found that the O_2 consumption of rainbow trout initially increased after strenuous exercise and then decreased to control values within 0.5 to 4 hours. Why van den Thillart et al., (1983) found no such increase in O_2 consumption after burst swimming (i.e repayment of an oxygen debt) is not clear. An oxygen debt after anaerobic exercise can be expected, since the end product lactate must be removed metabolically. Holeton et al., (1983) found strenuous exercise resulted in a severe lactacidosis, which was corrected within 4h by a transient net transfer of H^+ ions to the environmental water. The lactate was removed metabolically within 6-8h. The curious lack of an O_2 debt after burst swimming in the study of van den Thillart et al., (1983) indicates that their suprisingly low RE values may in fact be due to technical limitations.

In conclusion, the present experiments provide no evidence to support the view that increased catecholamine levels in fish will cause a reduction in carbon dioxide excretion (Wood and Perry, 1985; Perry, 1986). Further, the present experiments also provide no evidence that erythrocytic chloride/bicarbonate exchange is inhibited by elevated catecholamines in the rainbow trout *in vivo*, in contrast to what has been reported *in vitro* (Wood and Perry, 1985).

Summary

1. Immediately following burst swimming, the oxygen consumption of rainbow trout increased 71%, carbon dioxide excretion increased 104% and the respiratory exchange ratio increased 17%.
2. Infusion of adrenaline into resting fish had no significant effect on oxygen consumption or carbon dioxide excretion and therefore, there was no significant change in the respiratory exchange ratio.
3. This infusion of adrenaline did cause a significant elevation in the erythrocyte pH which was still present 80 min later.
4. The present results are in contrast to those of van den Thillart et al., (1983) who found carbon dioxide retention in coho salmon following a burst swim.

CHAPTER. 3: THE EFFECT OF CATECHOLAMINES ON CHLORIDE/BICARBONATE EXCHANGE IN RAINBOW TROUT ERYTHROCYTES

Introduction:

In the previous chapter, it was demonstrated that catecholamines do not cause a reduction in carbon dioxide excretion in the rainbow trout *in vivo*. Wood and Perry (1985), on the other hand, have reported that erythrocytic chloride/bicarbonate exchange is inhibited by catecholamines in rainbow trout blood *in vitro*. Why this adrenergic inhibition would be functional *in vitro* and yet absent *in vivo* is unclear.

The experiments reported by Wood and Perry (1985) were conducted using the modified boat assay which measures the rate of bicarbonate flux through intact erythrocytes as described by Heming and Randall (1982) and Haswell and Randall (1976). This assay was originally described and criticized by Booth (1938) who concluded that the carbonic anhydrase activity of intact erythrocytes cannot be measured manometrically due to enzyme substrate accessibility factors. Although recent investigators since Booth (1938) have produced modified versions of the assay which give repeatable results,

there has been no conclusive evidence in these studies that the assay is an actual measure of the bicarbonate flux through intact erythrocytes. It is possible, therefore, that the apparent conflict between the *in vitro* (Wood and Perry, 1985) and the *in vivo* (chapter.2) experiments may be a result of the technical limitations in the *in vitro* experiments. It is also possible that the effect occurs *in vitro* and *in vivo*, but is counteracted by some other adjustment *in vivo*. In the present study, a further modified boat assay which measures bicarbonate flux through intact erythrocytes is demonstrated and the effect of catecholamines on chloride/bicarbonate exchange in rainbow trout erythrocytes *in vitro* is re-examined.

Materials and Methods

Experimental Protocol:

The blood used in these experiments was obtained from lightly anaesthetized rainbow trout (150 - 250g) via caudal vessel puncture with the exception of the experiments which examined the effects of adrenergic stimulation. In this case, blood was obtained from resting fish via a dorsal aortic cannula which had been surgically implanted 48 h prior to the experiment (see the General Materials and Methods section for a detailed description of these procedures). In all experiments, the collected blood was pooled and equilibrated in an intermittently rotating glass tonometer at 10°C with humidified 0.2% CO_2 in air or 1% CO_2 in air (delivered by Wosthoff gas mixing pumps).

The first series of experiments examined the effect of haematocrit on carbon dioxide evolution. In these experiments, the blood pool was equilibrated with 0.2% CO_2 in air for at least 1 h at which time a blood sample to be assayed was removed. The haematocrit of the sample was adjusted with an appropriate volume of plasma from the same blood pool and the final volume of the whole blood sample to be assayed was always 0.4 ml.

For each whole blood assay, an additional assay was also performed which measured the rate of carbon dioxide evolution of a plasma sample from the blood pool. The volume of plasma assayed was equivalent to the plasma volume in the previous whole blood sample. These experiments were performed at haematocrits of 10, 15, 20, 25 and 30 %.

A second series of experiments examined the effect of the anion exchange inhibitors, DIDS and SITS (4-Acetamido-4-isothiocyanatostilbene-2,2-disulfonic acid), and the carbonic anhydrase inhibitor, acetazolamide on the rate of carbon dioxide evolution from intact erythrocytes. In these experiments, a 2 ml aliquot of whole blood (20% haematocrit) was removed from the blood pool and equilibrated in a separate tonometer in the presence of 0.1 mM DIDS, SITS or acetazolamide for 30 min prior to the boat assay. A control set of experiments was also performed in which only the saline vehicle (100 ul of Cortland's saline) for the above solutions was added to the tonometer.

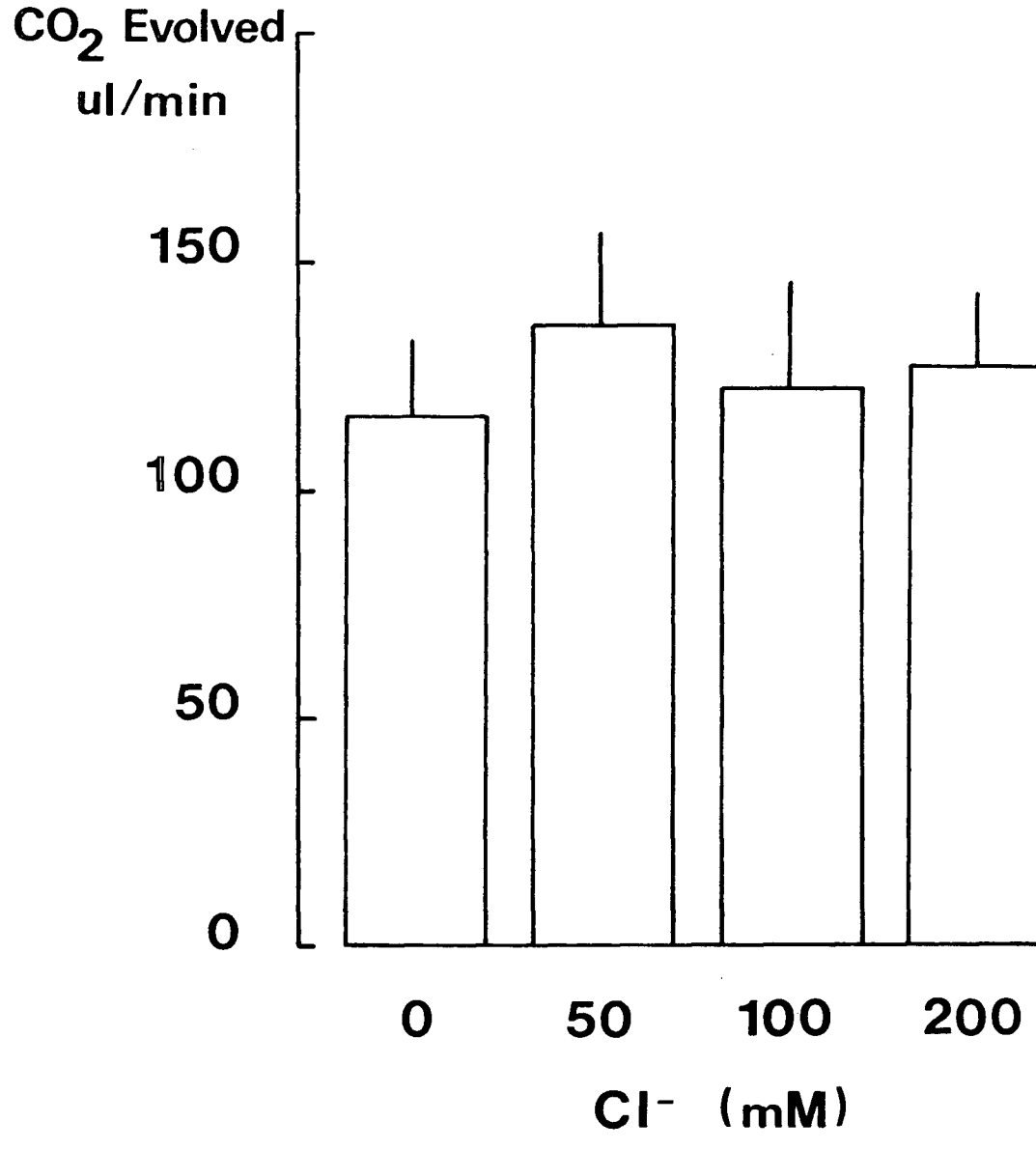
In the final series of experiments, the effects of the adrenergic agonists, isoproterenol (0.1mM), adrenaline (0.1mM) and noradrenaline (0.1mM) on carbon

dioxide evolution from intact erythrocytes were investigated. This series of experiments was similar to the second series with the following exceptions: 1) The equilibration gas mixture was 1% CO_2 in air since the effects of adrenergic agents on trout erythrocytes are enhanced at lower pH (see Chapter 1). 2) 10^{-4} M isoproterenol, adrenaline or noradrenaline were added to the tonometers. 3) The vehicle for these agonists was 100 mM perchloric acid, diluted to 4 vol% with Cortland saline; final volume 100 ul. 4) The extracellular and erythrocyte pH were also determined following the 30 min equilibration period.

Modified Boat Assay:

The assays were performed in a 50 ml ehrlenmeyer flask which had been partitioned along the bottom by a raised glass ridge. Two ml of bicarbonate solution (200 mM $NaHCO_3$ in 20 mM NaOH) were placed on one side of the ridge and 2 ml of phosphate buffer solution (61% 200 mM Na_2HPO_4 and 39% 200 mM KH_2PO_4) were placed on the other side. It is noteworthy that in a recent study, Nikinmaa et al., (1986) included 20 mM NaCl in the bicarbonate solution. The effect of sodium chloride addition was, therefore, examined in the present study (Fig.10), but was found to have no significant effect on the assay

Figure 10. The effect of extracellular chloride concentration on the carbon dioxide evolution by rainbow trout erythrocytes. Values are means ± standard error (N=6).


CO_2 Evolved
ul/min
150
100
50
0
0
50
100
200
Cl^- (mM)

between 0 and 200 mM NaCl. Thus, no NaCl was added in the present experiments. The material to be assayed was added to the side containing the buffer solution.

The top of the boat was fitted with a ground glass joint connected to a differential pressure transducing system (Validyne DP 103 with CD 16 carrier demodulator) by latex tubing. The output from the pressure transducing system was displayed on a chart recorder (Gould TA 600). The boat was also connected to a shaker. Partial immersion of the boat in a constant temperature bath as in previous studies (Haswell and Randall, 1976; Heming and Randall, 1982) was found to cause a temperature related artifact in the system. The entire system was, therefore, kept at 10^{o}C in a constant temperature room.

Upon addition of a sample to the buffer solution, the boat was sealed and left 5 - 10 min until the output from the recorder stabilized (manual sealing of the vessel caused a temporary temperature related pressure change). The shaker was then turned on and the pressure in the system was followed as the dehydration reaction proceeded. The system was calibrated with known volumes of air from 50 to 400 ul at the experimental temperature.

The increase in pressure was linear over this range and there was no detectable leakage in the system.

Reaction Rate:

Reaction rates (ml CO_2 evolved min^{-1}) were calculated from the time required for the sample mixture to evolve 200 uL of gas. The erythrocyte rate was obtained by subtraction of each sample's true plasma rate from the whole blood rate. This eliminated any error that could be attributed to haemolysis in the system.

Results

The rate of CO_2 evolution was proportional to the number of erythrocytes in the boat (Fig.11). At a haematocrit of 10%, the rate of carbon dioxide evolution was 30.9 $\pm$ 13.8 uL min^{-1}. As the haematocitrit increased, the rate of CO_2 evolution also increased and at a haematocrit of 30%, the CO_2 evolution had reached 259 $\pm$ 48.2 ul min^{-1}.

The effects of the anion exchange inhibitors, DIDS and SITS, and the carbonic anhydrase inhibitor, acetazolamide, on the rate of CO_2 evolution are shown in Figure 12. The anion exchange inhibitors, DIDS and SITS, caused the CO_2 evolution to decrease from the control value of 214.7 $\pm$ 13.4 ul min^{-1} to 93.7 $\pm$ 17.0 ul min^{-1} and 126 $\pm$ 18.9 ul min^{-1} respectively. DIDS, therefore, caused a 54% reduction in CO_2 evolution and SITS inhibited the system by 41%. This inhibition was due to a change in the characteristics of the erythrocytes since the plasma rate of CO_2 evolution was not significantly different after addition of these blockers. The carbonic anhydrase inhibitor, acetazolamide, decreased the rate of CO_2 evolution to 14.7% (95% inhibition).

Niether adrenaline, isoproterenol or

Figure 11. Effect of changing haematocrit on carbon dioxide evolution by rainbow trout erythrocytes. Values are means ± standard error (N=6 in all cases except haematocrit 10 where N=5).

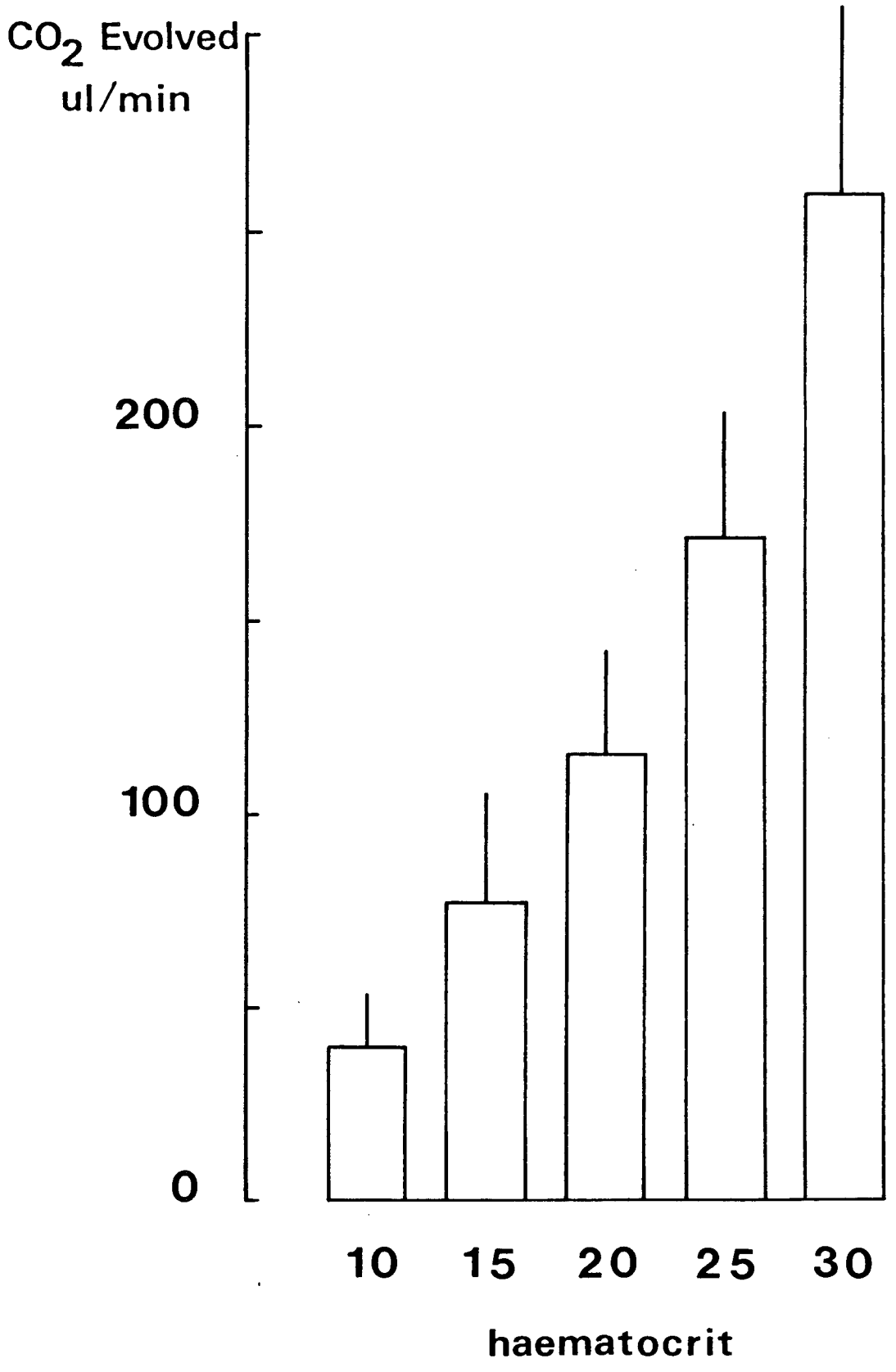

CO_2 Evolved
ul/min
200
100
0
10
15
20
25
30
haematocrit

Figure 12. The effect of SITS (0.1 mM), DIDS (0.1 mM) or acetazolamide (0.1mM) on the carbon dioxide evolution of rainbow trout erythrocytes (haematocrit 25%). Values are means + standard error (N=6). Unpaired t-tests were used to compare blocker values to control values. Differences were accepted as significant at the $p<0.05$ level and are indicated by an asterisk.

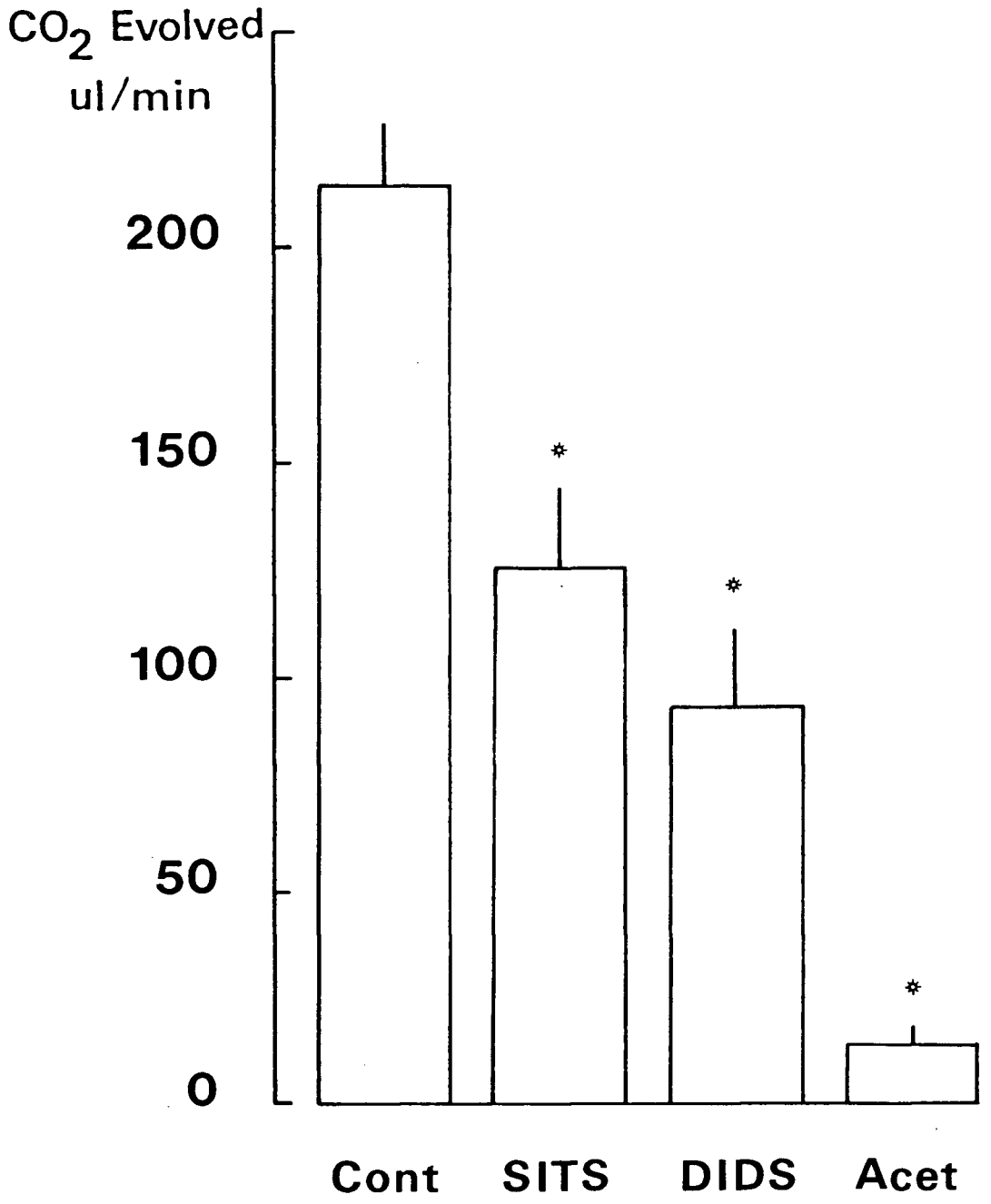
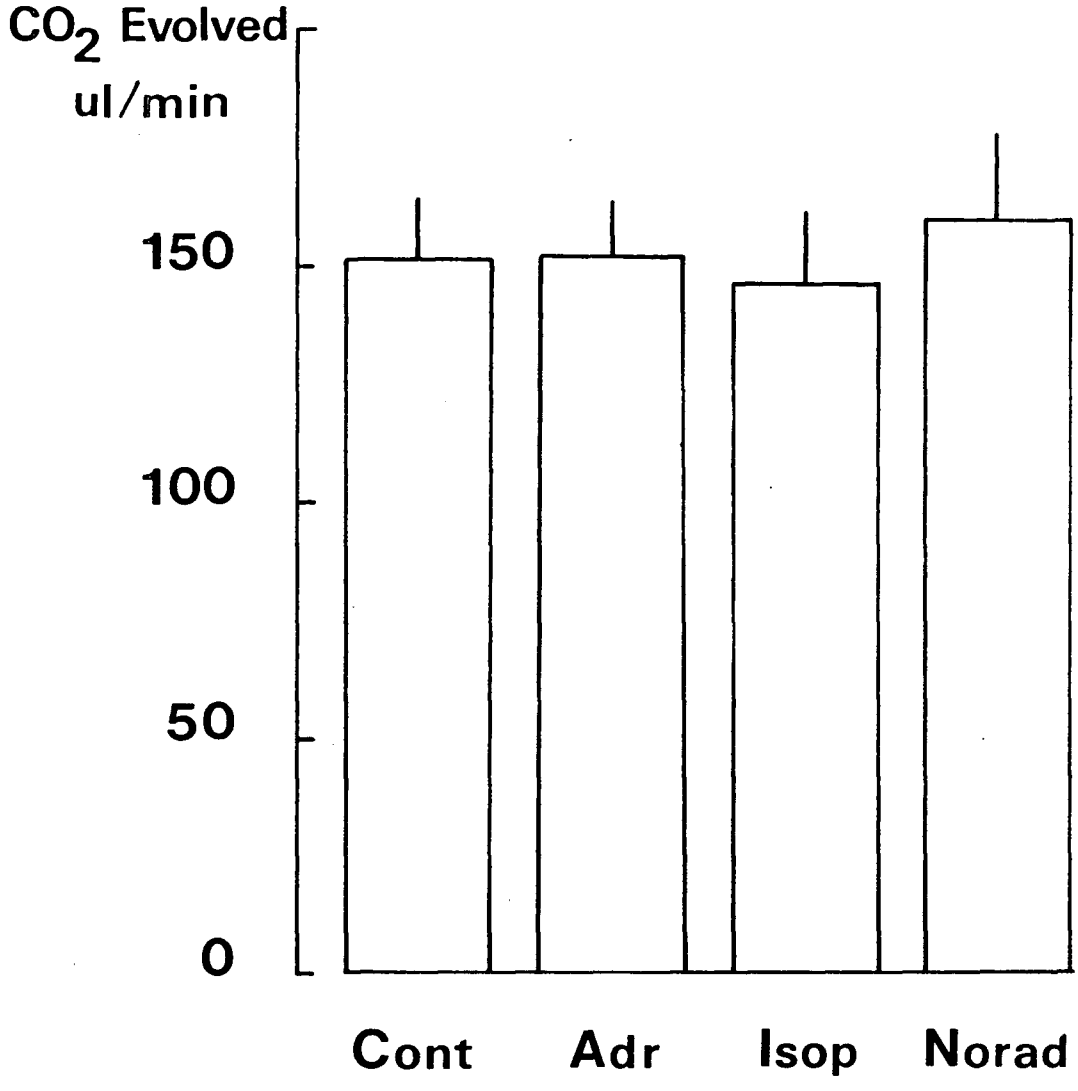

CO_2 Evolved
ul/min
200
150
100
50
0
Cont
SITS
DIDS
Acet

Figure 13. The effect of adrenaline (0.1 mM), isoproterenol (0.1 mM) and noradrenaline (0.1 mM) on the carbon dioxide evolution of rainbow trout erythrocytes (haematocrit 25%). Values are means + standard error (N=8). Unpaired t-tests were used to compare agonist values to control values. Differences were accepted as significant at the $p<0.05$ level and are indicated by an asterisk.


CO_2 Evolved
ul/min
150
100
50
0
Cont
Adr
Isop
Norad

noradrenaline had any significant effect on the rate of CO_2 evolution from the erythrocytes (Figure 13). The control rate was 150.9 $\pm$ 13.2 ul min^{-1} while the rates for the agonists were 151.5 $\pm$ 13.8 ul min^{-1}, 145.5 $\pm$ 15.8 ul min^{-1} and 160.5 $\pm$ 18.1 ul min^{-1} respectively. These agonists did, however, have a large effect on the pH gradients across the erythrocyte membrane (Table 4). In each case, the erythrocyte pH increased while the extracellular pH decreased. In the control situation, the extracellular pH was 7.601 $\pm$ 0.049, the intracellular pH was 7.273 $\pm$ 0.082 and the pH gradient was 0.328. In the presence of adrenaline, the extracellular pH was 7.405 $\pm$ 0.028, the intracellular pH was 7.300 $\pm$ 0.016 and thus, the pH gradient was 0.105. Isoproterenol caused the extracellular pH to fall to 7.371 $\pm$ 0.029 and an increase in the intracellular pH to 7.323 $\pm$ 0.011, and therefore, the pH gradient was only 0.048. Finally, in the case of the cells stimulated with noradrenaline, the extracellular pH was 7.446 $\pm$ 0.029 and the intracellular pH and the pH gradient were 7.323 $\pm$ 0.020 and 0.123 respectively.

Table 4. Effect of adrenaline, isoproterenol and noradrenaline on the plasma pH (pH_e), erythrocyte pH (pH_i) and on the pH gradient (dpH) of rainbow trout blood.

treatment	pH_e	pH_i	dpH
control	7.601±0.049	7.273±0.082	0.328
adrenaline	7.405±0.080*	7.300±0.046	0.105
isoproterenol	7.371±0.082*	7.323±0.030	0.048
noradrenaline	7.446±0.083*	7.323±0.056	0.123

Values are means ± one S.E.M. Asterisk denotes significant (unpaired t-test; $p<0.05$) difference from control.

Discussion

According to Booth (1938), carbonic anhydrase activity of intact erythrocytes cannot be measured manometrically due to enzyme substrate accessibility factors. Haswell and Randall (1976), however, were able to demonstrate enzyme activity in rat whole blood using a modified boat assay similar to that described by Booth (1938). Haswell and Randall (1976) were also able to measure enzyme activity from trout erythrocytes suspended in saline, but found only very occasional activity in trout whole blood. These authors concluded that the absence of enzyme activity in trout whole blood was due to a plasma inhibitor which caused erythrocytes to be bicarbonate impermeable *in vivo*. Later, Heming and Randall (1982) demonstrated that the absence of activity in the experiments of Haswell and Randall (1976) was, in fact, caused by foaming in the assay and trout whole blood could catalyze the dehydration of plasma bicarbonate in the typical mammalian fashion in the presence of defoaming agents. The modified boat technique described by Heming and Randall (1982) was also used in the experiments cited by Wood and Perry (1985)

which demonstrated that catecholamines inhibit the dehydration of plasma bicarbonate by intact trout erythrocytes.

The present study was done using a boat technique similar to that described by Heming and Randall (1982) with further modifications (see Methods). The difficulties encountered in the previously described studies made it imperative, however, to thoroughly examine the system prior investigating the effects of catecholamines on trout erythrocytes.

The evolution of carbon dioxide in these experiments was clearly dependent on haematocrit. This is further confirmation that fish erythrocytes are bicarbonate permeable (Cameron, 1978; Obaid et al., 1979; Heming and Randall, 1982). In addition, this result demonstrates that the system was linear in the chosen range of volume and haematocrit for the subsequent experiments. Inhibition of the CO_2 evolution from whole blood in the present system by acetazolamide indicates that the assay was, in fact, measuring erythrocytic carbonic anhydrase activity. Furthermore, the large degree of inhibition obtained when the anion exchange inhibitors, DIDS and SITS, were added to the blood provided evidence that the rate of carbon dioxide

evolution in this assay was dependent on erythrocytic chloride/bicarbonate exchange. Together, these results indicated that the present system could be employed to examine the effects of catecholamines on chloride/bicarbonate exchange in trout erythrocytes.

In the present study, catecholamines had no effect on bicarbonate flux through trout erythrocytes. This result is in sharp contrast to the results cited by Wood and Perry (1985). According to these authors, the dosages of the adrenergic agonists used in this study should have caused a large reduction in the CO_2 evolution from the erythrocytes. These agonists did, however, have an effect on the pH gradient across the erythrocyte membrane as in previous studies (Nikinmaa and Huestis, 1984; Heming et al., 1986; Primmett et al., 1986). The effect of catecholamines on the pH gradient demonstrated, therefore, that the erythrocytes were adrenergically stimulated.

The reason for the disparity between the present results and those of Wood and Perry (1985) is unclear. Haemolysis increases the carbonic anhydrase activity of blood several fold (Meldrum and Roughton, 1933; Haswell and Randall, 1976). Any haemolysis in the boat assay

would, therefore, be an important source of error. This problem was avoided in the present experiments by subtracting the rate of the true plasma from that of whole blood to obtain the erythrocyte rate. This also ensured that the effects of pharmacological agents seen in the assay were a result of changes in the characteristics of the intact erythrocytes and not merely an effect of these agents on the free enzyme in the plasma. In previous studies, only the whole blood rate was determined and this was compared to a saline control rather than to the blood's true plasma. Heming and Randall (1982) monitored haemolysis by haemoglobin assay. In preliminary experiments in this study, however, it was found that haemolysis that was undetectable by haemoglobin analysis was apparent if the plasma was assayed in the boat. Furthermore, in the present study, it was never possible to wash erythrocytes without some detectable haemolysis. It is therefore possible that the results cited by Wood and Perry (1985) may have been a result of haemolysis in the assay. The study reported by Wood and Perry (1985) also failed to monitor the erythrocyte pH during the experiment. As explained by Booth (1938), this variable has a large effect on the bicarbonate flux through erythrocytes. The documented

adrenergic inhibition of bicarbonate flux could, therefore, be representative of erythrocyte pH changes during their experiment. Unfortunately, without these measurements, the source of error in the experiments described by Wood and Perry (1985) is impossible to determine. Regardless, the present results also would explain why catecholamines had no effect on the respiratory exchange ratio of trout in vivo (Chapter.2).

Summary

1. A further modified boat assay is described. This assay is demonstrated to measure bicarbonate flux through intact trout erythrocytes.

2. The results confirm that erythrocytes in fish are bicarbonate permeable.

3. In contrast to results cited by Wood and Perry (1985), this study demonstrated that catecholamines do not inhibit bicarbonate flux through intact trout erythrocytes.

4. Catecholamines did, however, cause a reduction in the pH gradient across the erythrocyte membrane as in previous studies.

SECTION II: AMPHIBIAN ERYTHROCYTES

OVERVIEW

Adrenergic regulation of erythrocyte pH and volume has been documented in several species of fish (Nikinmaa and Huestis, 1984; Nikinmaa et al., 1984; Jensen and Weber, 1985a,b; Primmett et al., 1986; Heming et al., 1987). This response is absent, however, in domestic goose erythrocytes and the ion exchange mechanisms on the membrane of these nucleated erythrocytes are quite different from those of fish erythrocytes (Nikinmaa and Huestis, 1984). In the lamprey, the pH of the erythrocyte is actively regulated in the absence of adrenergic stimulation (Nikinmaa, 1986; Nikinmaa et al., 1986). At the present time, however, it is not known whether any other species of vertebrates regulate their erythrocyte pH.

The amphibians are a diverse group of animals which have adapted to a wide range of habitats. The anuran species are able to endure periods of environmental adversity by remaining dormant in burrows (Lee and Mercer, 1967; Seymour, 1973; Boutilier et al., 1979b). The reduced capacity for the convection of gases under these conditions may impose both hypoxic and hypercapnic stress upon the animal. Others, like the

semi-aquatic urodeles may daily encounter widely fluctuating oxygen and carbon dioxide levels in their environment (Ultsch, 1976; Heisler et al., 1982). Although routine dives are mainly aerobic in the semi-aquatic amphibians (Toews et al ., 1971; Boutilier and Shelton, 1986), extended dives to avoid predation may also result in hypoxic stress. Studies on acid-base regulation in amphibians have revealed that these animals generally tolerate wide oscillations in extracellular pH in favour of regulating the intracellular pH of certain body compartments (Toews and Heisler, 1982; Heisler et al., 1982). There are no studies, however, which follow changes in the erythrocyte pH during acid-base disturbances in these animals. The ability to control haemeglobin:oxygen affinity via the regulation of erythrocyte pH may be an adaptation which enables amphibians to more efficiently transport oxygen throughout this broad range of ambient and internal gas tensions.

The ion exchange mechanisms involved in volume regulation in amphibian erythrocytes have been described in detail (Cala, 1980, 1985; Rudolph and Greengard, 1980; Palfrey and Greengard, 1981). These mechanisms resemble

those involved in volume regulation following adrenergic stimulation in teleost erythrocytes (Baroin et al., 1984a,b; Nikinmaa and Huestis, 1984; Heming et al., 1987). Frog erythrocytes also exhibit some degree of adrenergic sensitivity. Cell swelling has been documented in frog erythrocytes after addition of isoproterenol, but this was only significant in the presence of a phosphodiesterase inhibitor (Rudolph and Greengard, 1980). Adrenergic stimulation in fish erythrocytes results in both a swelling and a pH response (Nikinmaa and Huestis, 1984; Heming et al., 1987). There are no studies, however, which have examined the effects of adrenergic stimulation on the pH in amphibian erythrocytes.

The purpose of this section is to determine whether amphibians are also capable of regulating their erythrocyte pH as in the lamprey and in fish. The animals chosen for these studies are the semi-terrestrial anuran, Bufo marinus and the semi-aquatic urodele, Amphiuma tridactylum.

CHAPTER.4: THE EFFECTS OF FORCED ACTIVITY ON CIRCULATING CATECHOLAMINES AND pH AND WATER CONTENT OF ERYTHROCYTES IN THE TOAD, BUFO MARINUS.

Introduction:

Exhaustive exercise in fish results in an extracellular acidosis (Holeton et al., 1983; Nikinmaa et al., 1984; Primmett et al., 1986). In addition, this type of activity is associated with an increase in circulating catecholamines which preserves the oxygen carrying capacity of the blood via beta-adrenergic effects on the erythrocyte (Nikinmaa et al., 1984; Primmett et al., 1986). Forced activity in the semi-terrestrial toad, Bufo marinus also results in an extracellular acidosis (McDonald et al., 1980). During an extracellular acidosis induced by exposure to hypercapnia, Toews and Heisler (1982) demonstrated that Bufo marinus regulated the pH of the analysed intracellular compartments more than the extracellular compartment. There are no studies, however, which follow the changes in the erythrocyte pH or the levels of circulating catecholamines during an acidosis in amphibians.

Bufo marinus has a substantial Bohr effect (-

0.230; Boutilier and Toews, 1981). Changes in erythrocytic pH will, therefore, have a marked effect on haemoglobin:oxygen affinity. While facilitating the unloading of oxygen to the tissues under normal circumstances, a fall in erythrocyte pH could in fact compromise oxygen uptake in conditions where an extracellular acidosis is associated with hypoxia. Boutilier et al., (1979b) demonstrated that burrowing in these animals results in both a decrease in the extracellular pH and a reduction in the arterial PO_2. Thus, during an extracellular acidosis, these animals may derive some benefit from the ability to regulate erythrocyte pH.

The purpose of these experiments was to produce an extracellular acidosis in the toad, Bufo marinus and to determine if erythrocyte pH and, therefore, the characteristics of haemoglobin:oxygen binding, are modulated during an extracellular acidosis in amphibians.

Methods

In Vivo Experiments:

Adult Bufo marinus were cannulated in the femoral artery (see General Materials and Methods). Following overnight recovery, a 1 ml control blood sample was removed from the femoral cannula and immediately analysed for pH and haematocrit. The sample was then centrifuged and the plasma was removed for catecholamine analysis. The packed erythrocytes were immediately frozen in liquid nitrogen for later analysis of intracellular pH. The animal was then subjected to 30 min of vigorous exercise. This involved the manual manipulation of the animal by the experimenter so that continuous righting movements were elicited. Upon completion of this exercise period, the animal was returned to the chamber and another blood sample was immediately taken. Blood samples were also taken 0.5, 1 and 4h after exercise and analysed as described above for the control sample. An additional set of experiments was conducted to determine any relationship between changes in haematocrit and changes in the erythrocyte water content. These two variables were measured in five animals subjected to the previously

described protocol with the exception that only two samples (control and 0h post-exercise) were taken.

In Vitro Experiments:

Following the recovery period from surgery, blood was collected from several cannulated toads and pooled. Samples (2.5 ml) of blood were then transferred to glass tonometers and equilibrated with a humidified 5% CO_2/95% air mixture delivered by Wosthoff gas mixing pumps. This gas mixture resulted in blood pH values close to those of the animals immediately following exhaustive exercise (see Fig.16). Following a 90 min equilibration period, a 1 ml blood sample was taken from each tonometer and centrifuged; 0.7 ml in one tube for the determination of plasma and erythrocyte pH and the remaining 0.3 ml in another tube for the determination of cell water content. The pH of the plasma (pH_e) was measured immediately and the remaining plasma from both sample tubes was discarded. The packed erythrocytes from the first sample tube were then frozen in liquid nitrogen for later determination of erythrocyte pH while those from the second sample tube were saved for the determination of cell water content. Baroin et al., (1984a) have demonstrated that rainbow trout erythrocytes exhibit

significant beta-adrenergic sensitivity following 3h of equilibration *in vitro* at 15°C and therefore it was assumed that the equilibration time (90 min) would not alter the beta-adrenergic sensitivity of the cells. At this point, each tonometer received either 10^{-5} mol l^{-1} isoproterenol (final concentration) or an equivalent volume (50 ul) of Mackenzie's amphibian saline. Isoproterenol was chosen since the regulation of pH in teleost erythrocytes appears to be a beta-adrenergic effect (Nikinmaa *et al.*, 1984; Primmett *et al.*, 1986) and beta-adrenergic effects are most potently stimulated by isoproterenol (Lefkowitz, 1976). The concentration of 10^{-5} mol l^{-1} was used in order to fully saturate the beta-adrenergic receptors. The sampling procedure was then repeated following another 30 min of equilibration. It should be noted that in representative cases (N=3), plasma catecholamine measurements were made at this point in the experiment. In these cases, plasma adrenaline and noradrenaline levels from the control tonometers were very similar to those of resting animals *in vivo*.

Analytical Procedures:

Measurements of pH were made using a Radiometer PHM 72 acid-base analyzer and associated micro-pH unit (Radiometer, Copenhagen, Denmark). Erythrocytic pH

measurements were made using the freeze-thaw method of Zeidler and Kim (1977). Plasma adrenaline and noradrenaline levels were determined by high pressure liquid chromatography (Spectra Physics, model SP8700) with electrochemical (Bioanalytical Systems) detection (Woodward, 1982; Primmett et al., 1986). The determination of the erythrocyte water content has been described in the General Materials and Methods.

Results

Exhaustive exercise in the toad was associated with large increases in circulating levels of adrenaline and noradrenaline (Table 5). Following the exercise period, the circulating adrenaline level had significantly increased from the control value of 2.2 ± 0.4 nmol l^{-1} to 36.5 ± 3.2 nmol l^{-1}. Similarly, noradrenaline levels significantly increased from 0.3 ± 0.1 nmol l^{-1} to 2.7 ± 0.5 nmol l^{-1} immediately after exercise. Both of these parameters had returned to levels which were not significantly different from their respective control levels after 30 min of recovery.

The exercise period also caused an increase in the haematocrit (Fig. 14). The control haematocrit was 22.3 ± 1.4. Immediately following exercise, this increased significantly to 33.5 ± 1.4 and after 30 min of recovery, it was still significantly elevated (26.2 ± 1.8). After 1h of recovery, the haematocrit had returned to a value (22.1 ± 1.4) which was not significantly different from the control. This increase in haematocrit was not associated with an increase in the cell water

Table 5. Effect of forced activity on the circulating adrenaline and noradrenaline levels (nanomoles/L) in the toad.

	Time (h)				
	C	0	0.5	1	4
adr (N=11)	2.2±0.4	36.5±3.2*	1.7±0.2	1.7±0.2	1.4±0.3
nor (N=6)	0.3±0.1	2.7±0.5*	0.2±0.1	0.1±0.1	0.1±0.1

C = control; 0, 0.5, 1 and 4 h = hours following 30 minutes of forced activity. Values are means ± one S.E.M. Asterisk denotes significant (paired t-test; $p<0.05$) difference from control.

Figure 14. Haematocrit (%) values in eleven toads before (C) forced activity and at 0, 0.5, 1 and 4 h following forced activity. Values are means + standard error. Paired t-test was used to compare post activity values to control value. Differences were accepted as significantly different at the $p<0.05$ level and are indicated by an asterisk.

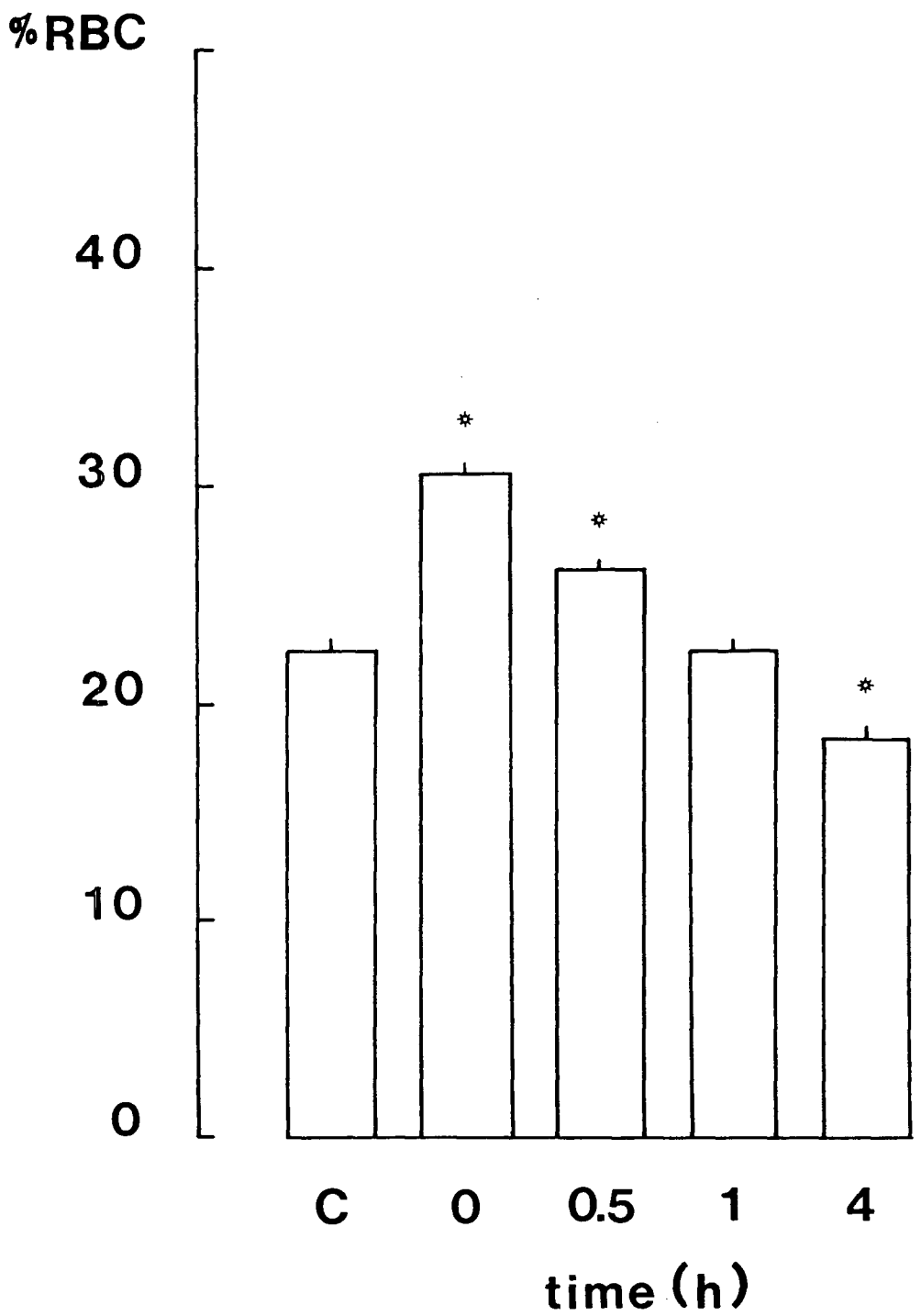

%RBC
40
30
20
10
0
C
0
0.5
1
4
time (h)

Figure 15. Haematocrit (hatched) and cell water content (solid) in five toads before (C) and immediately following (E) forced activity. Values are means + standard error. Paired t-test was used to compare the post activity value to the control value. Differences were accepted as significantly different at the $p<0.05$ level and are indicated by an asterisk.

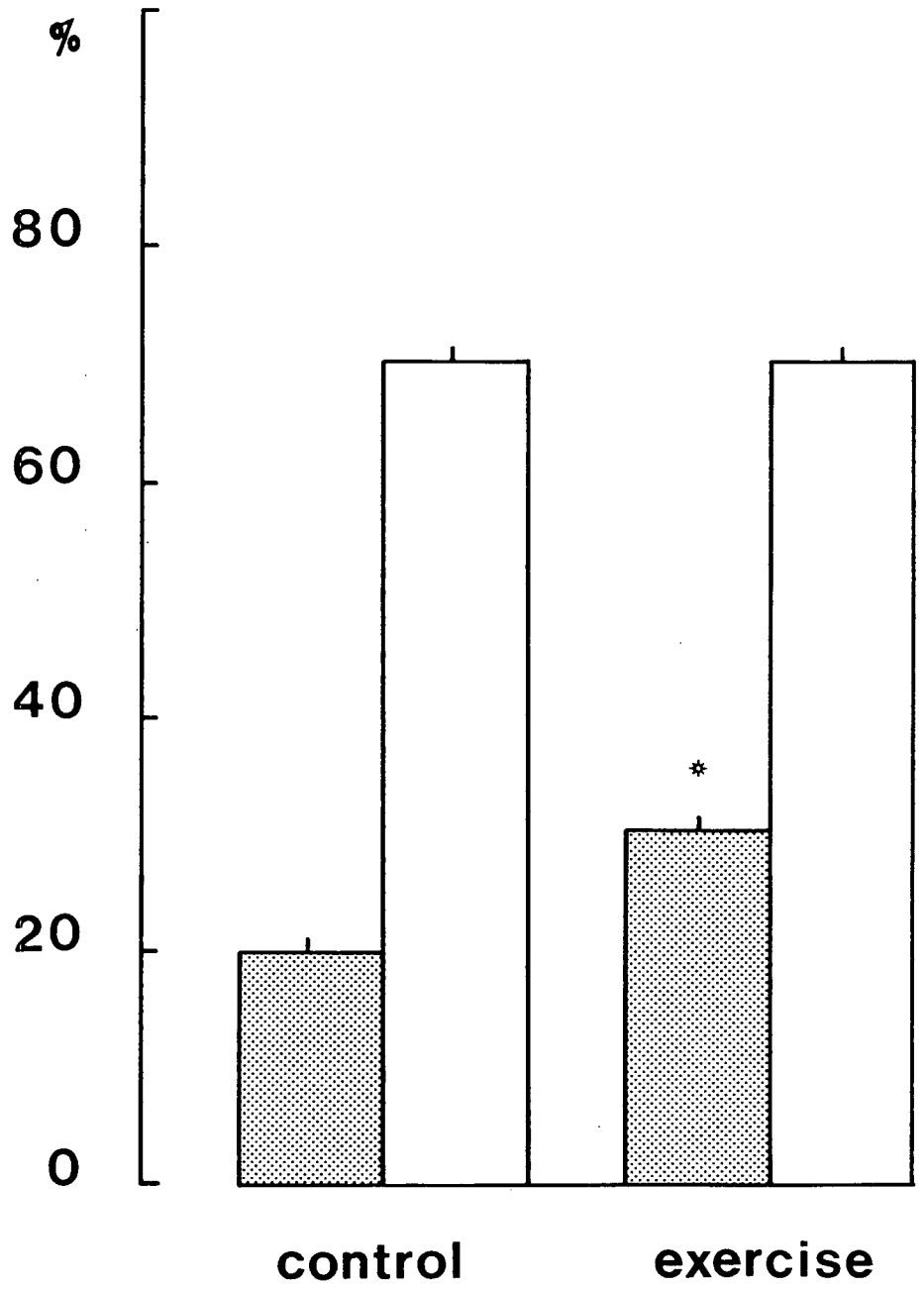

%
80
60
40
20
0
*
control
exercise

Figure 16. Whole blood (circles) and erythrocyte (triangles) pH in 11 toads before (C) forced activity and at 0, 0.5, 1 and 4h following forced activity. Values are means $\pm$ standard error. Paired t-test was used to compare the post activity values to the control value. Differences were accepted as significantly different at the $p<0.05$ level and are indicated by an asterisk.

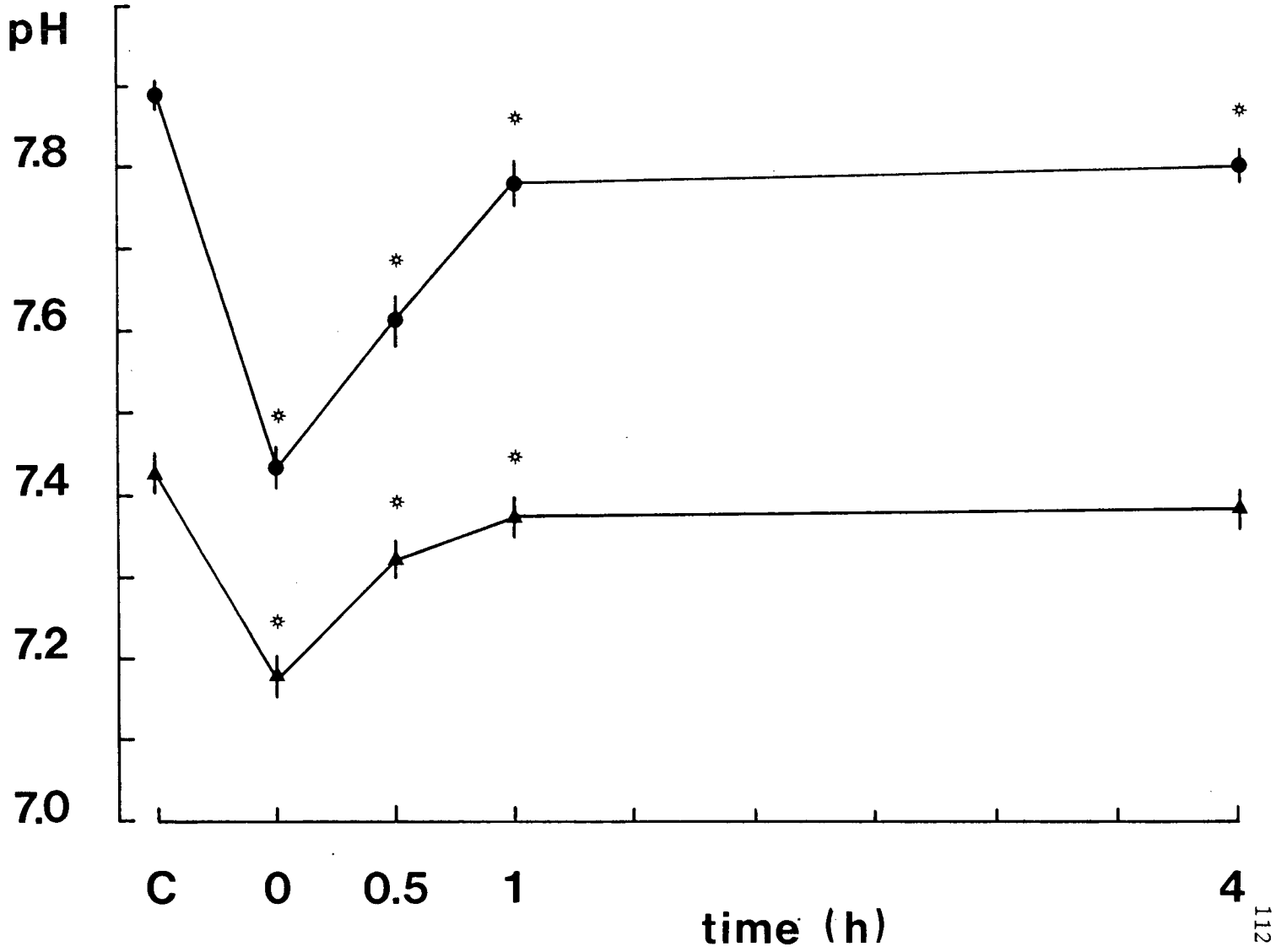

pH
7.8
7.6
7.4
7.2
7.0
C
0
0.5
1
4
time (h)

Table 6. Effect of isoproterenol on the water content (%), extracellular pH (pH_e), intracellular pH (pH_i) and on the pH gradient (dpH) of toad blood equilibrated with 5% CO_2/95% air.

	90 min	treatment	120 min
control (N=9)			
cell water	69.1±0.4	saline	67.9±1.4*
pH_e	7.516±0.017		7.511±0.016
pH_i	7.124±0.010		7.134±0.017
dpH	+0.392		+0.377
experimental (N=9)			
cell water	68.5±0.52	isoprot.	67.9±0.4*
pH_e	7.514±0.016		7.511±0.015
pH_i	7.128±0.011	(10^{-5})	7.140±0.018
dpH	+0.386		+0.371

Values are means ± one S.E.M. Asterisk denotes significant (paired t-test; $p<0.05$) difference from 90 min value.

content (Fig. 15). Following the exercise period, the cell water content was identical to the value prior to exercise (68.4 $\pm$ 1.0).

The effect of forced activity on the whole blood and erythrocyte pH is illustrated in Figure 16. Both of these parameters significantly decreased following the activity period. The magnitude of the drop in erythrocyte pH (0.270 pH units) was less than the drop in extracellular pH (0.429 pH units). Thus, the pH difference across the erythrocyte membrane was 0.443 pH units at rest and was reduced to 0.284 pH units after exercise. By the 4h recovery sample, the extracellular pH was still significantly reduced, but the erythrocyte pH had returned to a value which was not significantly different from the control value.

Isoproterenol had no effect on the pH or water content of erythrocytes equilibrated *in vitro* (Table 6). There was a small, but significant decrease in cell water content *in vitro* at 120 min both in erythrocytes treated with saline and in those treated with isoproterenol. The cause of this effect is not clear.

Discussion

Control (resting) levels of adrenaline and noradrenaline for Bufo marinus were slightly lower than resting levels recorded for Bufo arenarum (Donoso and Segura, 1965). While there are no values available from the literature for catecholamine levels following activity in amphibians, the magnitudes of the increases in the present experiments was similar to that found in the rainbow trout following exhaustive exercise (Primmett et al., 1986). In the present experiments these levels had returned to the control levels within 30 min. In contrast, 4h of recovery were required in the rainbow trout before catecholamine levels had returned to values near the control levels. Boutilier et al., (1986a) have demonstrated that increases in circulating catecholamine levels in the rainbow trout are proportional to acute decreases in blood pH caused by acid infusion. The results of the present experiments indicate, however, that in the toad, increases in catecholamine levels are not proportional to the degree of metabolic acidosis since whole blood pH was still significantly reduced after 4h of recovery and circulating catecholamine levels

had returned to normal within 30 min.

The increase in haematocrit in the present study was similar to that documented by McDonald et al., (1980) for Bufo marinus following forced activity. In Rana catesbeiana, it has also been shown that infusion of adrenaline or noradrenaline (Mbangkollo and deRoos, 1983; Herman, 1977) or a period of handling (Mbangkollo and deRoos, 1983) will cause an increase in the haematocrit. There are several factors which may have contributed to this increase in haematocrit. In teleosts, increased haematocrit levels following exhaustive exercise are due to erythrocyte swelling as well as an increase in the circulating number of erythrocytes relative to the plasma volume (Nikinmaa et al., 1984; Primmett et al., 1986). In Bufo, the absence of erythrocyte swelling in the presence of increased catecholamine levels in vivo (Table 6) is interesting in view of the study of Rudolph and Greengard (1980) in which catecholamines were found to stimulate swelling in frog erythrocytes in vitro. The ion exchanges involved in the adrenergic swelling response in frog erythrocytes (Palfrey and Greengard, 1981) resemble those in teleosts (Baroin et al., 1984a,b; Nikinmaa and Huestis, 1984; Heming et al., 1987).

Swelling has only been documented in frog erythrocytes in vitro, however, if the cells are adrenergically stimulated in the presence of a phosphodiesterase inhibitor. The present experiments indicate that these ion exchange processes are not capable of raising the erythrocyte water content in the toad during beta-adrenergic stimulation in vivo. This may be due to other factors that are capable in vivo of influencing erythrocyte water content, such as plasma osmotic pressure. However, the beta-adrenergic agonist isoproterenol caused no increase in the cell water content in vitro. Together, these results seem to indicate that beta-adrenergic stimulation of toad erythrocytes does not result in changes in water content under physiological conditions. The increase in haematocrit in the present experiments is, therefore, probably due to an increase in the circulating number of erythrocytes relative to the plasma volume. According to Boutilier et al., (1986b), uptake of plasma water by osmotically enriched muscle cells may account for a portion of the haematocrit increase. Erythrocyte recruitment may also have been a contributing factor, since Nilsson and Grove (1974) have demonstrated that constriction of the spleen may be induced in teleosts by

perfusion of the splenic artery with adrenaline or noradrenaline.

The acidosis in whole blood following forced activity in Bufo marinus is similar to that found by McDonald et al., (1980). In addition, the results of the present study indicate that the erythrocyte pH also falls during this period. Hladky and Rink (1977) explain that a reduction in pH reduces the net charge on the haemoglobin inside the erythrocyte. This would result in a reduction in the erythrocyte proton concentration (relative to the extracellular concentration) and, therefore, in the erythrocyte transmembrane pH gradient. The erythrocyte transmembrane pH gradient also decreases with decreasing pH in rainbow trout erythrocytes equilibrated in vitro in the absence of catecholamines (Heming et al., 1987).

It has been demonstrated that while plasma pH falls after exhaustive exercise in teleosts, an increase in circulating catecholamine levels causes the erythrocyte pH to be maintained (Nikinmaa et al., 1984) or increased (Primmett et al., 1986). Beta-adrenergic agonists also cause an increase in the pH of teleost erythrocytes in vitro (Nikinmaa and Huestis, 1984;

Cossins and Richardson, 1985; Heming et al., 1987; Chapter 1). Nevertheless, the erythrocyte pH of toads fell significantly in the present experiments, even though circulating catecholamine levels increased. In addition, isoproterenol had no effect on the pH of toad erythrocytes equilibrated in vitro (Table 6). The effect of catecholamines on the pH of toad erythrocytes is, therefore, quite different from the effects which have been documented in teleosts.

It is possible that the regulation of erythrocyte pH via adrenergic mechanisms is an adaptation which may be specific to water-breathing vertebrates. The difference in the adrenergic effects on the erythrocyte between the toad and teleost fish may also be explained if the subsequent effects on oxygen carrying capacity in the blood of each of these animals are considered. In teleost fish, a fall in the erythrocyte pH lowers the oxygen-carrying capacity of the blood because of the Root shift (Cameron, 1971; Nikinmaa et al., 1984; Boutilier et al., 1986a). Amphibians, however, do not possess a Root shift (Bridges et al., 1985) and a fall in the erythrocyte pH would not lower the oxygen carrying capacity of the blood of Bufo marinus as it does in teleosts. The functional significance of the adrenergic

response in teleosts may, therefore, be to offset the Root shift.

Summary

1. Circulating catecholamine levels increase in the toad following forced activity.

2. The activity period also caused an increase in haematocrit and a plasma acidosis.

3. The increase in circulating catecholamine levels was not proportional to the drop in extracellular pH as documented in fish.

4. The increase in haematocrit was not associated with any increase in the erythrocyte water content.

5. There was an absence of any beta-adrenergic regulation of pH or water content in the erythrocytes of the toad. This is in sharp contrast to the response of teleost fish erythrocytes to beta-adrenergic stimulation and may be due to the fact that amphibians do not have a Root shift.

CHAPTER.5: THE DISTRIBUTION OF PROTONS AND CHLORIDE IONS IN AMPHIBIAN ERYTHROCYTES

Introduction:

The distribution of protons is in Donnan equilibrium with the chloride ion distribution in nonnucleated mammalian erythrocytes (Hladky and Rink, 1977). Similarly, in nucleated erythrocytes, it has been demonstrated that protons and chloride ions may be in Donnan equilibrium (Albers and Goetz, 1985; Heming et al., 1986). However, in the nucleated erythrocytes of the lamprey (Nikinmaa, 1986) and the eel (Steen and Turitzin, 1968), protons and chloride ions are not in Donnan equilibrium. Indeed, Nikinmaa et al., (1986) has further demonstrated that the erythrocyte pH in the lamprey is actively regulated by a sodium/proton exchange mechanism. Albers and Goetz (1985) have shown that these types of studies may be complicated by differences encountered between the DMO method and the freeze-thaw method (Zeidler and Kim, 1977), both frequently used for the measurement of erythrocyte pH.

McDonald et al., (1980) have determined that chloride ions and bicarbonate ions are in Donnan equilibrium in the erythrocytes of the amphibian, Bufo

marinus. There have been no studies, however, which simultaneously determine the proton and chloride distribution ratios over the physiological pH range in amphibian erythrocytes. It was, therefore, the purpose of this study to determine if protons and chloride ions are distributed according to a Donnan equilibrium across the erythrocyte membrane in the amphibian, Bufo marinus. The relationship between the DMO method and the freeze-thaw method for measuring the erythrocyte pH in these cells was also investigated.

Methods

Arterial blood was collected from several cannulated toads and pooled. Aliquots (2 mL) of blood were then transferred to glass tonometers and equilibrated for one hour with a humidified 1% CO_2/99% air mixture (delivered by Wosthoff gas mixing pumps) and 0, 50, 100, 150 or 200 uL of 0.2N HCl. In addition, 10 uL of 1 uCi ml^{-1} ^{14}C-DMO (New England Nuclear, specific activity 50 mCi $mmol^{-1}$) was added for later determination of intracellular pH. Following the equilibration period (2h), the blood was removed from the tonometer and distributed among four eppendorf sample tubes. The first tube received 0.8 mL of blood while each of the remaining three tubes received 0.4 mL. The tubes were then centrifuged and the plasma pH was measured from the first sample tube immediately using a Radiometer PHM 72 acid-base analyser and associated micro-pH unit (Radiometer, Copenhagen, Denmark). The remaining plasma from this sample tube was discarded and the packed erythrocytes were frozen in liquid nitrogen for the later determination of erythrocyte pH. The pH of this sample was determined via the freeze-thaw method of Ziedler and Kim (1977) with the exception that, in one series of

experiments, this measurement was also made on a separate sample with a bridge of physiological saline between the sample and the saturated KCl solution of the pH electrode (Sigaard-Andersen, 1961; 1974; Boutilier et al., 1985). This saline bridge eliminates the junction potential which may develop between the sample and the KCl solution of the reference electrode during the measurement of pH in slurries (1974). A 100 uL sample of plasma and the packed erythrocytes were saved from each of the other three sample tubes for the determination of water content and chloride and DMO concentrations respectively. The procedure for the determination of both the erythrocyte pH via the DMO method and the erythrocyte water content have been described in the General Materials and Methods. Chloride analyses were performed using a Radiometer CMT 10 chloride titrator (Radiometer, Copenhagen, Denmark).

Results

The water content of toad erythrocytes increased linearly with decreasing pH (Figure 17). The minimum water content was 68% at a pH_e of 7.948 and this increased to 75.5% at a pH_e of 6.913. This represented an 11% change in the water content over the pH range studied or 1.1%/0.1 pH unit.

The regression lines generated for the distribution ratios of chloride ions (rCl^-) and protons with the erythrocyte pH determined via both the DMO method (rH^+_{DMO}) and the freeze-thaw method (rH^+_{FT}) are displayed in Figure 18. Both the distribution ratios for protons vary inversely to the chloride distribution ratio with changes in extracellular pH. The slope of each line, however, is significantly different ($p<0.05$) from that of the other 2 lines. The y-intercepts of rH^+_{DMO} vs rH^+_{FT} and rH^+_{FT} vs rCl^- are also significantly different ($p<0.05$). The relationship between the overall distribution ratios (neglecting the effects of extracellular pH) are shown in Table 7. The overall distribution ratio for rCl^- is 0.607 $\pm$ 0.025 while the values for rH^+_{FT} and rH^+_{DMO} are 0.470 $\pm$ 0.023 and 0.635 $\pm$

Figure 17. Erythrocyte water content versus extracellular pH (pH_e) of toad blood. The dependence of erythrocyte water content on pH_e is given by the following regression line: $\%H_2O = -5.706 \times pH_e + 114.0$, $r = 0.89$ (n=32).

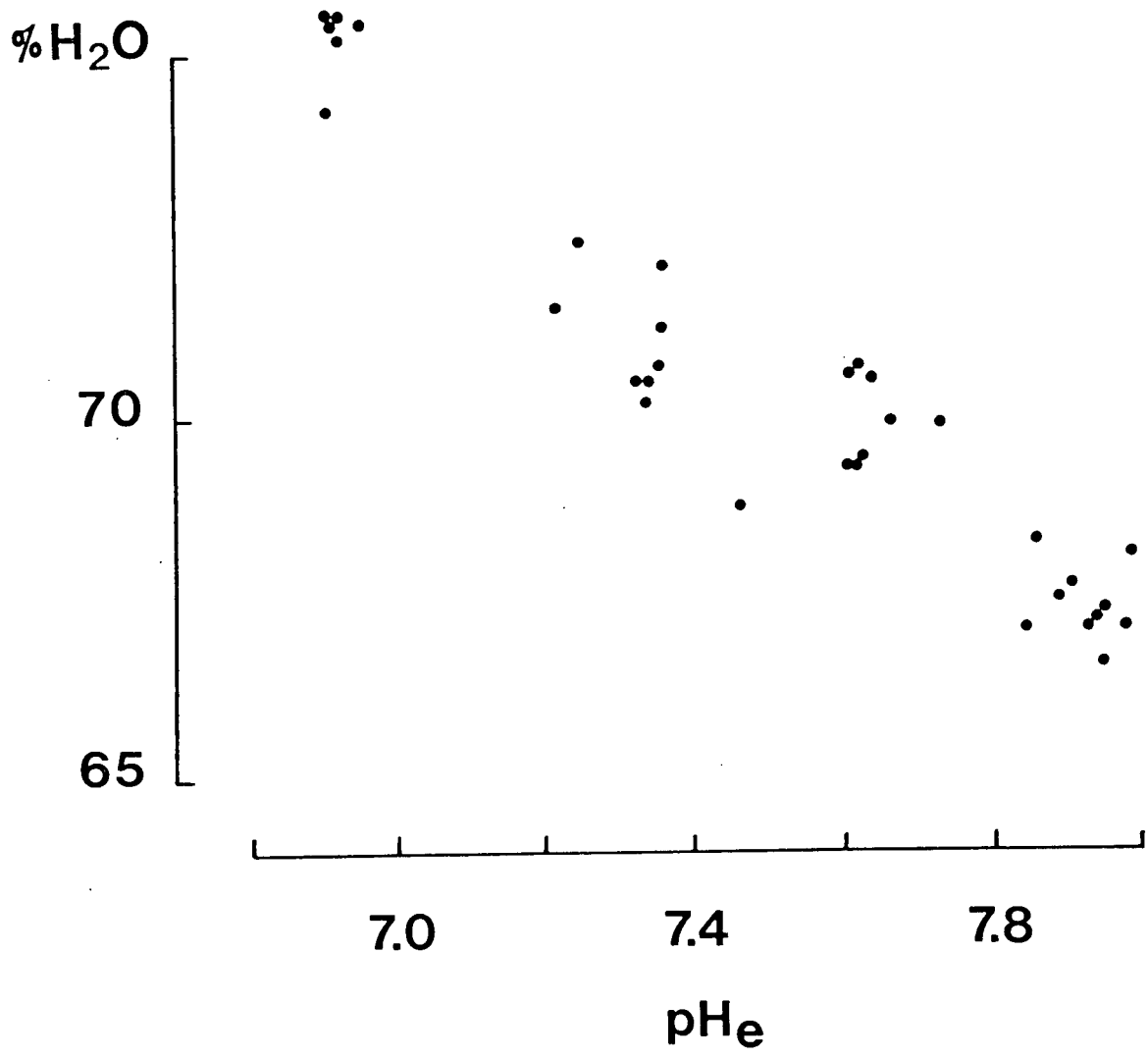

%H2O
70
65
7.0
7.4
7.8
pHe

Figure 18. Proton and Chloride distribution ratios (r) vs extracellular pH (pH_e) of toad blood. triangles = proton distribution ratio using the DMO method for pH_i; asterices = proton distribution ratio using the freeze-thaw method for pH_i; circles = chloride distribution ratio. The dependence of the distribution ratios on extracellular pH is given by the following regression lines: DMO method for pH_i; $rH^+ = -0.432 \times pH_e + 3.878$, $r = 0.97$ $(n=32)$. Freeze-thaw method for pH_i; $rH^+ = -0.350 \times pH_e + 3.101$, $r = 0.97$ $(n=32)$. Chloride distribution; $rCl^- = -0.390 \times pH_e + 3.535$, $r = 0.99$ $(n=32)$. The slope of each line is significantly $(p<0.05)$ different from the that of the other 2 lines. The y intercept of rH^+_{DMO} vs rH^+_{FT} and rH^+_{FT} vs rCl^- are also significantly $(p<0.05)$ different.

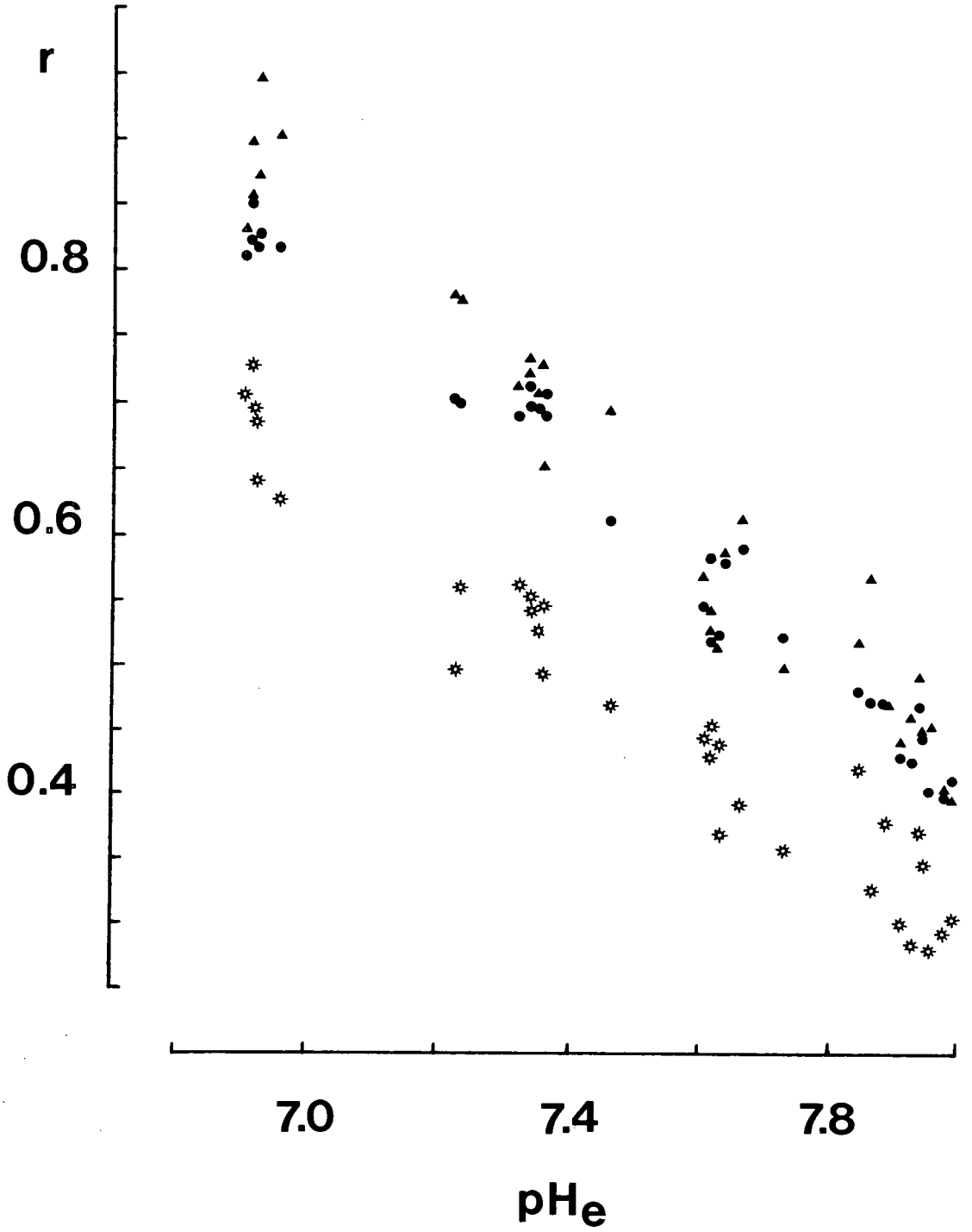
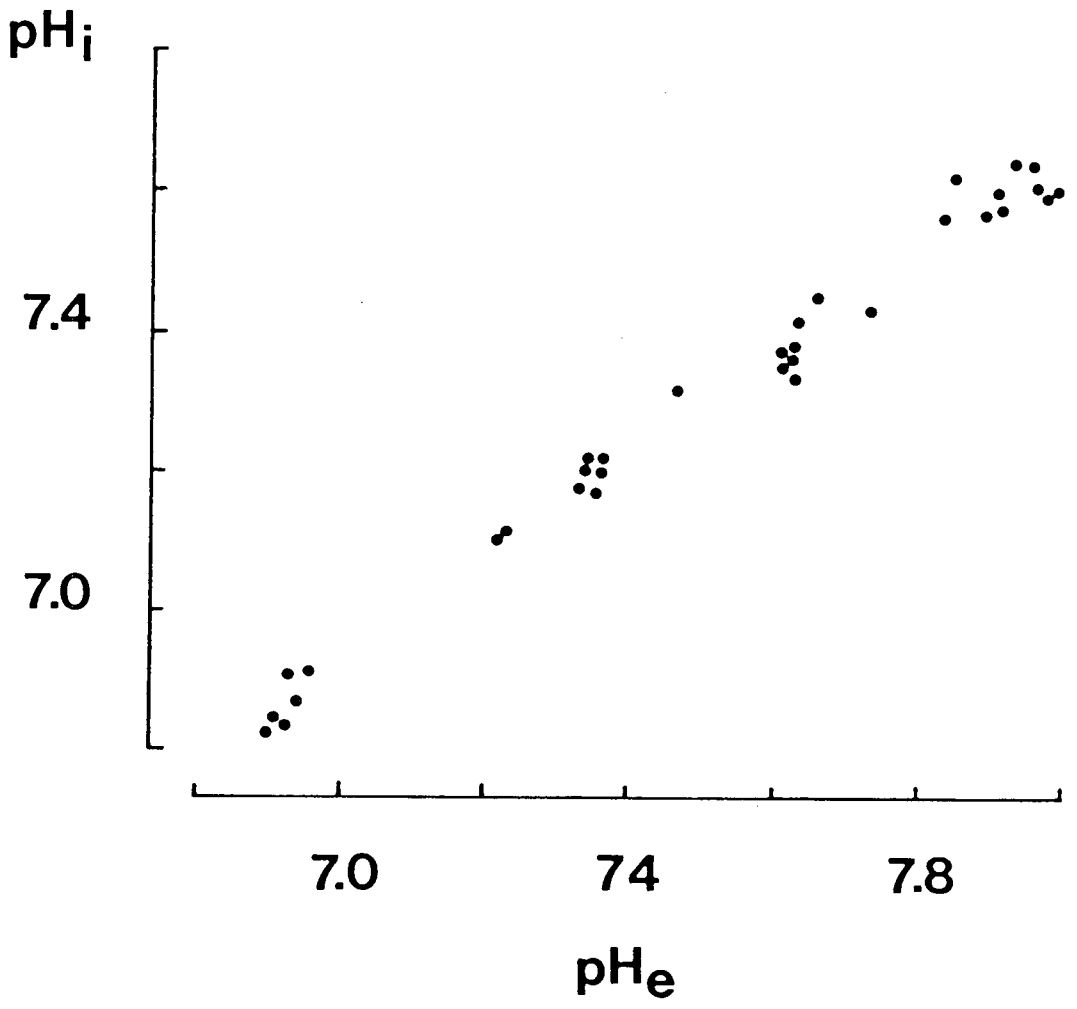

r
0.8
0.6
0.4
7.0
7.4
7.8
pHe

Figure 19. Erythrocyte pH (pH_i) versus extracellular pH (pH_e) of toad blood. Erythrocyte pH was determined via the DMO method. The dependence of erythrocyte pH on the extracellular pH is given by the following regression line:

$$pH_i = 0.706 \times pH_e + 1.998,\ r = 0.99\ (n=32).$$


pH$_i$
7.4
7.0
7.0
7.4
7.8
pH$_e$

0.029 respectively. The difference between the rCl^- and the rH^+_{DMO} is, therefore, only 20% of the difference between rCl^- and rH^+_{DMO}.

The discrepancy between the distribution ratios for protons results from a consistent difference obtained for the erythrocyte pH between the DMO method and the freeze-thaw method (Table 8). The difference in these two values was 0.13 pH units at an extracellular pH of 7.732. This table also shows the effect of a saline bridge during pH_i measurements via the freeze-thaw method on this difference. The addition of the saline bridge clearly eliminated this difference and the pH_i values were no longer significantly different.

The relationship between pH_e and erythrocyte pH (pH_i) is plotted in Figure 19. The erythrocyte pH increases linearly with increasing extracellular pH.

Table 7. Overall means of distribution ratios for chloride (rCl^-) and for protons with erythrocyte pH determined via the freeze-thaw method (rH^+_{FT}) and by the DMO method (rH^+_{DMO}).

	Mean	S.E.M.
rCl^-	0.607	0.025
rH^+_{FT}	0.470	0.023
rH^+_{DMO}	0.635	0.029

Table 8. Erythrocyte pH (pH_i) determined by the freeze-thaw method (FT), freeze-thaw method with saline bridge (FT+sal), DMO distribution method (DMO) and predicted from the extracellular pH and the chloride distribution (Cl^-).

pH_e	$pH_{i(FT)}$	$pH_{i(DMO)}$	$pH_{i(Cl-)}$
7.732±0.012	7.731±0.018	7.449±0.012*	7.443±0.010*
pH_e	$pH_{i(FT+sal)}$	$pH_{i(DMO)}$	$pH_{i(Cl-)}$
7.738±0.010	7.469±0.023	7.447±0.011	7.455±0.015

Values (n=9) are means ± one S.E.M. Asterisk indicates significant (unpaired t-test; $p<0.05$) difference from the freeze-thaw or freeze-thaw + saline value.

Discussion

The water content of Bufo marinus erythrocytes increases with decreasing pH (Fig. 17) as it does in the rainbow trout, Salmo gairdneri (Heming et al., 1986). In the trout, the increase in water content is associated with an increase in the chloride ratio (Cl^-_{in}/Cl^-_{out}) across the erythrocyte membrane (Heming et al., 1986). The present study demonstrates that the chloride ratio across the erythrocyte membrane in Bufo marinus also increases with decreasing pH. Similar to mammalian nonnucleated erythrocytes, the nucleated erythrocytes of amphibians possess a chloride/bicarbonate exchange mechanism (Cala, 1980; Boutilier and Toews, 1981), and chloride and bicarbonate are passively distributed across the erythrocyte membrane (McDonald et al., 1980). According to Hladky and Rink (1977), decreasing pH in nucleated erythrocytes causes chloride to enter the cell in response to charge changes on the haemoglobin and water is drawn into the cell osmotically. The results of this study indicate that the water content of amphibian erythrocytes is influenced by similar mechanisms.

The distribution ratio for protons (rH^+) varies

inversely to the chloride distribution ratio (rCl^-) with changes in extracellular pH. The regression lines generated for rH^+_{DMO} versus pH_e and rCl^- versus pH_e are significantly different over the entire pH range studied. In the physiological pH range (above pH 7.0), however, these lines are identical. These results indicate that protons and chloride ions are in a Donnan equilibrium in the physiological pH range in Bufo marinus erythrocytes and protons are also passively distributed. In contrast, the regression line generated for rH^+_{FT} versus pH_e is significantly different than the regression lines for rCl^- and rH^+_{DMO} versus pH_e. This result indicates that protons may not be passively distributed in amphibian erythrocytes.

The significant difference between the lines generated for the distribution ratios for protons is a result of the consistent difference obtained for the erythrocyte pH between the DMO method and the freeze-thaw method. While these two methods give similar values for the erythrocyte pH in nonnucleated erythrocytes (Waddel and Bates, 1969; Roos and Boron, 1981) and in nucleated trout erythrocytes (Milligan and Wood, 1984), a similar discrepancy occurs in the erythrocytes of the carp (Albers and Goetz, 1985). In the latter, erythrocyte pH

determined by the freeze-thaw method was found to be 0.15 pH units lower than that obtained from the DMO method. At an extracellular pH of 7.732, the difference between these two values in this study was 0.13 pH units. Trapped extracellular fluid in the packed erythrocytes was not responsible for this difference. Indeed, calculations which correct for trapped extracellular fluid predict a greater pH difference. Albers and Goetz (1985) hypothesized that this discrepancy could be due to a 'suspension effect' at the liquid junction potential between the sample and the KCl solution of the reference electrode. This potential is caused by the suspended negatively charged particles in the haemolysate and is similar to the 'haemolysis effect' described in the measurement of whole blood pH (Siggaard-Andersen, 1961; 1974; Maas, 1970; Salling and Siggaard-Andersen, 1971; Boutilier et al., 1985). In whole blood, Siggaard-Andersen (1961) demonstrated that this effect disappeared when physiological saline was placed between the saturated KCl solution and the sample in the pH electrode. Presumably, the saline bridge eliminates the charge difference and, therefore, the potential between the sample and the KCl solution. When this technique was

employed in the present study to measure the pH of the erythrocyte haemolysate, the significant difference between the two methods of pH_i measurement was eliminated. Thus, the difference is apparently due to a 'suspension effect' at the liquid junction potential of the pH electrode. Thus, protons are in Donnan equilibrium with chloride ions in amphibian erythrocytes and are passively distributed.

The slope of the erythrocyte pH versus pH_e regression line for Bufo marinus (0.706) blood is similar to that reported by Nikinmaa et al., (1987) for rainbow trout blood (0.721). Both of these values, however, are quite different from the slope (0.471) for this relationship reported by Heming et al., (1986). Erythrocytic NTP levels influence the net charge within the erythrocyte and, therefore, the distribution of protons across the erythrocyte membrane if protons are passively distributed. Indeed, Wood et al., (1975) has demonstrated that the blood ATP concentration affects the pH_i versus pH_e relationship. It is therefore possible that differences in erythrocytic NTP levels resulting from different experimental protocols caused the differences in the slopes of erythrocyte pH versus pH_e in these studies.

Summary

1. The water content of Bufo marinus erythrocytes increases with decreasing extracellular pH.

2. Protons and chloride ions are in Donnan equilibrium and are both passively distributed across the erythrocyte membrane.

3. There is a consistent difference in the measurement of the erythrocyte pH in this species between the DMO method and the freeze-thaw method.

4. This difference is apparently due to a 'suspension effect' at the liquid junction potential of the pH electrode encountered when measuring the haemolysate pH.

CHAPTER.6: ION EXCHANGE MECHANISMS ON THE ERYTHROCYTE MEMBRANE OF THE AQUATIC SALAMANDER, AMPHIUMA TRIDACTYLUM

Introduction:

The aquatic salamander, Amphiuma lives in an environment which is part open water and part vegetation covered water in which water PCO_2's of 50-60 mm Hg may be associated with hypoxia (Ultsch, 1976; Heisler et al., 1982). According to Toews et al., (1971), most voluntary dives in these air breathing animals are probably aerobic. Avoidance of predators in this type of environment, however, undoubtedly involves dives of longer duration during which time oxygen stores may be depleted. These animals also have a substantial Bohr effect (-0.205; Lenfant and Johansen, 1967) which may exacerbate the problems of oxygen transport under these conditions. As in Bufo marinus, these animals preferentially defend the pH of the intracellular compartment during acid-base disturbances (Heisler et al., 1982). It seems that Amphiuma might also benefit from a mechanism which regulated the erythrocyte pH as in salmonid fish and the lamprey. Again, however, the pH of the erythrocyte has not been monitored during an acidosis in Amphiuma.

Cala (1980, 1985) has described the ion exchange mechanisms involved in volume regulation in Amphiuma erythrocytes. Volume regulatory decrease after osmotic swelling is associated with a net loss of erythrocyte potassium and chloride whereas volume regulatory increase after osmotic shrinkage involves loosely coupled sodium/proton and chloride/bicarbonate exchange mechanisms. During such volume regulatory increases, the extracellular medium is acidified. Thus, the observed volume increase may be associated with changes in erythrocyte pH as in fish erythrocytes. The effects of these processes on the pH in these erythrocytes, however, has not been documented.

This study examined the characteristics of the ion exchange mechanisms in Amphiuma erythrocytes by using different transport and metabolic inhibitors, and ion substitutions. The sensitivity of these ion exchange mechanisms to beta-adrenergic stimulation was also investigated. The purpose of these experiments was to determine if Amphiuma is capable of regulating erythrocyte pH as documented in fish and lampreys.

Methods

Samples of arterial blood were collected from cannulated salamanders and centrifuged in heparinized 1.5 mL eppendorf tubes. The plasma was discarded following centrifugation. The cells were then washed in Mackenzie's amphibian saline adjusted to 30 mM bicarbonate with the exception that in one set of experiments in which the saline contained the impermeant cation choline instead of sodium. The protocol for the washing of the cells was variable depending on the experiment. In the experiments in which choline replaced sodium, the cells were left suspended for 10 minutes during each wash to improve the removal of sodium. If ouabain (10^{-4}M) or 2,4-dinitrophenol (2,4-D.N.P.; 10^{-4}M) were to be used in the experiment, the drug would be present in all washings. If amiloride (10^{-3}M) or DIDS (10^{-4}) were to be used, they were only added to the final suspension. In all cases, the erythrocytes were resuspended to approximately 15% haematocrit following the second wash in the appropriate incubation media and 10 uL of 1 uCi/mL ^{14}C-DMO (New England Nuclear, specific activity 50 mCi/mmol) was added to the suspension for

later determination of intracellular pH. The suspensions were then incubated in 50 mL tonometers at 25^{o}C and gassed with humidified mixtures of either 4% carbon dioxide/96% air or 8% carbon dioxide/92% air. The carbon dioxide mixtures were delivered by Wosthoff gas mixing pumps (Wosthoff Digamix, Type M-300a and M-30f, Bochum, FRG). Following a 30 minute equilibration period, two 0.4 mL samples were taken from the tonometer and centrifuged. The pH of the extracellular fluid was then measured immediately from the first sample tube using a Radiometer PHM 72 and associated micro-pH unit (Radiometer, Copenhagen, Denmark). A 100 uL sample of extracellular fluid was taken from the second sample tube for the determination of DMO levels for subsequent calculation of pHi. The erythrocytes from the first sample tube were saved for the pHi determination and those from the second sample tube were saved for the determination of cellular water content.

At this point, the experiment was either terminated, or in those experiments in which the effects of adrenaline were to be assessed, $5x10^{-5}$M adrenaline was added to all tonometers except the control. In the adrenaline experiments, the sampling procedure was then repeated following another 30 minutes of equilibration.

Results

The pH and volume of Amphiuma tridactylum erythrocytes were not influenced by the incubation times in the range of 30 to 60 min. The presence of the anion exchange inhibitor DIDS, however, caused a marked intracellular alkalinization and decrease in the erythrocyte water content at both carbon dioxide tensions studied (Table 9, 10). The intracellular alkalinization could be prevented or reduced by (1) removing sodium from the incubation medium (2) treating the cells with amiloride (3) blocking the sodium/potassium pump with ouabain (Table 11). These results indicate that there is a sodium/proton exchange mechanism on the erythrocyte membrane which is functional under steady-state conditions. This exchange is driven by the sodium gradient produced by the sodium/potassium pump. In the absence of bicarbonate movements as occurs in the DIDS experiments (Table 9, 10), the intracellular pH is controlled by the sodium/proton exchange.

The presence of amiloride alone in the incubation medium caused only slight changes in the pH gradient across the erythrocyte membrane, but did cause a

Table 9. Effects of adrenaline, DIDS and amiloride on the water content, extracellular pH (pH_e), erythrocyte pH (pH_i) and on the pH gradient (dpH) of Amphiuma tridactylum erythrocytes equilibrated with 4% CO_2/96% air.

incubation medium	30 min	treatment	60 min
saline (cont.)			
H_2O	74.0±0.6 (8)	A	74.0±0.7 (8)
pH_e	7.62±0.01 (10)		7.63±0.02 (10)
pH_i	7.39±0.02 (10)		7.37±0.02 (10)
dpH	+0.23 (10)		+0.26 (10)
saline			
H_2O	73.2±0.6 (7)	B	75.3±0.09 (7)
pH_e	7.64±0.01 (9)		7.66±0.01 (9)
pH_i	7.40±0.02 (9)		7.42±0.01 (9)+
dpH	+0.24 (9)		+0.24 (9)
saline + DIDS			
H_2O	70.8±0.4 (8)+	B	72.0±0.8 (8)*
pH_e	7.64±0.02 (9)		7.64±0.02 (9)
pH_i	7.64±0.03 (9)+		7.62±0.03 (9)+
dpH	+0.01 (9)		+0.02 (9)
saline + amil.			
H_2O	68.5±0.8 (8)+	B	70.5±0.9 (8)+
pH_e	7.68±0.02 (10)+		7.66±0.01 (10)
pH_i	7.39±0.03 (10)		7.42±0.03 (10)
dpH	+0.29 (10)		+0.24 (10)

Values are means ± one S.E.M. (n). Asterisk denotes significant (paired t-test; $p<0.05$) difference from 30 min value. + denotes significant (unpaired t-test; $p<0.05$) difference from treatment A. Treatment A = control, treatment B = 5×10^{-5} M adrenaline.

Table 10. Effects of adrenaline, DIDS and amiloride on the water content, extracellular pH (pH_e), erythrocyte pH (pH_i) and on the pH gradient (dpH) of Amphiuma tridactylum erythrocytes equilibrated with 8% CO_2/92% air.

incubation medium	30 min	treatment	60 min
saline (cont.)			
H_2O	72.6±0.4 (7)	A	73.8±0.2 (7)
pH_e	7.43±0.02 (9)		7.44±0.02 (9)
pH_i	7.26±0.02 (9)		7.32±).04 (9)
dpH	+0.17 (9)		+0.11 (9)
saline			
H_2O	73.0±0.6 (8)	B	74.1±0.3 (8)
pH_e	7.45±0.02 (10)		7.43±0.02 (10)
pH_i	7.31±0.04 (10)		7.27±0.02 (10)
dpH	+0.14 (10)		+0.16 (10)
saline + DIDS			
H_2O	72.0±0.02 (8)	B	72.4±0.4 (8)+
pH_e	7.43±0.03 (10)		7.41±0.02 (10)
pH_i	7.48±0.02 (10)+		7.45±0.02 (10)+
dpH	-0.05 (10)		-0.04 (10)
saline + amil.			
H_2O	71.7±0.6 (8)	B	71.8±0.8 (8)+
pH_e	7.48±0.02 (10)		7.46±0.02 (10)*
pH_i	7.29±0.04 (10)		7.31±0.03 (10)
dpH	+0.19 (10)		+0.14 (10)

Values are means ± one S.E.M. (n). Asterisk denotes significant (paired t-test; $p<0.05$) difference from treatment A. Treatment A = control, treatment B = 5 x 10^{-5} M adrenaline.

Table 11. Effects of DIDS, amiloride, ouabain and absence of extracellular sodium on the water content, extracellular pH (pH_e), intracellular pH (pH_i) and on the pH gradient (dpH) of Amphiuma tridactylum erythrocytes equilibrated with 4% CO_2/96% air.

	saline control	saline + DIDS	saline, DIDS + amiloride
H_2O	73.8±0.6 (4)	70.9±0.6 (4)*	67.0±1.0 (8)*
pH_e	7.54±0.01 (4)	7.51±0.04 (4)	7.54±0.01 (8)
pH_i	7.36±0.02 (4)	7.53±0.05 (4)*	7.35±0.03 (8)
dpH	+0.17 (4)	-0.02 (4)	+0.20 (8)

	saline control	saline + ouabain	saline, ouabain + DIDS
H_2O	75.5±1.1 (6)	73.9±0.6 (6)	72.2±0.7 (6)*
pH_e	7.52±0.01 (6)	7.54±0.01 (6)	7.46±0.01 (6)*
pH_i	7.32±0.04 (6)	7.24±0.03 (6)	7.33±0.02 (6)
dpH	+0.20 (6)	+0.29 (6)	+0.13 (6)

	saline (no Na^+) control	saline (no Na^+) + DIDS
H_2O	67.7±0.4 (20)	67.9±0.4 (24)
pH_e	7.63±0.03 (4)	7.66±0.01 (8)
pH_i	7.32±0.06 (4)	7.32±0.03 (8)
dpH	+0.32 (4)	+0.34 (8)

Values are means ± one S.E.M. (n). Asterisk denotes significant (unpaired t-test; $p<0.05$) difference from saline control.

Table 12. Comparison of the erythrocyte pH determined by the DMO distribution ($pH_{i(DMO)}$) and the erythrocyte pH predicted from the extracellular pH and the chloride distribution ($pH_{i(Cl-)}$) in Amphiuma tridactylum erythrocytes.

incubation medium	$pH_{i(DMO)}$	$pH_{i(Cl-)}$
4% CO_2/96% air		
saline	7.37±0.02 (10)	7.32±0.03 (10)*
saline + DIDS	7.60±0.04 (6)	7.08±0.05 (6)*
saline + amiloride	7.38±0.04 (6)	7.32±0.05 (6)*
saline + 2,4-D.N.P.	7.26±0.02 (7)	7.28±0.02 (7)
8% CO_2/92% air		
saline	7.23±0.02 (10)	7.16±0.03 (10)*
saline + DIDS	7.43±0.01 (6)	6.93±0.08 (6)*
saline + amiloride	7.22±0.03 (6)	7.14±0.06 (6)

Values are means ± one S.E.M. (n). Asterisk denotes significant (unpaired t-test; $p<0.05$) difference between $pH_{i(DMO)}$ and $pH_{i(Cl-)}$.

reduction in the erythrocyte water content (Table 9, 10). These results indicate that the chloride/bicarbonate exchange is capable of equilibrating acid equivalents under normal circumstances.

The sodium/proton exchange mechanism on the erythrocyte membrane did not appear to be sensitive to beta-adrenergic stimulation. Adrenaline alone had no significant effects on any of the measured variables. In the presence of DIDS or amiloride, significant differences were seen between control and adrenaline samples. These differences, however, were not consistent.

Table 12 compares the erythrocyte pH determined by the DMO method in several experiments with the erythrocyte pH predicted by the distribution of chloride across the erythrocyte membrane using the Donnan equation. These results showed that there was a significant difference (0.05-0.07 pH units) between the two determinations, even in the controls which was not affected by amiloride. This difference was exacerbated (0.50-0.52 pH units) in the presence of DIDS, and completely abolished when the ionophore 2,4-DNP was added to the medium.

Discussion

Cala (1980, 1985) has demonstrated that Amphiuma erythrocytes use loosely coupled, electrically silent sodium/proton and chloride/bicarbonate exchange mechanisms to re-establish cell volume after osmotic shrinkage. During this process, sodium and chloride enter the cell in exchange for protons and bicarbonate ions respectively and cell volume is restored passively due to osmotically obligated water flow. From the present experiments, it also appears that sodium/proton and chloride/bicarbonate exchangers are involved in steady-state cell volume regulation. Inhibition of either of these exchanger mechanisms causes significant reductions in cell volume which are most pronounced (9.2%) when both ion exchange pathways are blocked. The large decrease in the transmembrane pH gradient in the presence of DIDS (Table 10, 11) also indicates that the sodium/proton antiporter is constantly functioning and removing protons from the cell interior in exchange for sodium. DIDS blockade of chloride/bicarbonate exchange enables this proton extrusion mechanism to alkalinize the intracellular compartment relative to the extracellular

medium since bicarbonate can no longer equilibrate across the erythrocyte membrane. Replacement of sodium with the impermeant cation choline or the presence of amiloride in the incubation medium inhibits this intracellular alkalinization. This supports the hypothesis that the intracellular alkalinization occurs due to sodium dependent proton extrusion as is the case in teleost erythrocytes following beta-adrenergic stimulation (Nikinmaa and Huestis, 1984).

The mechanism of proton extrusion in Amphiuma erythrocytes is also ouabain sensitive. The sodium/proton exchange mechanism therefore seems to be dependent on the sodium/potassium pump. The association between these two mechanisms has also been documented in the frog (Palfrey and Greengard, 1981). It has been suggested (Palfrey and Greengard, 1981) that beta-adrenergic stimulation of sodium/proton exchange in the frog erythrocyte results in subsequent stimulation of the sodium/potassium pump due to sodium entry into the cell. Our experiments indicate that the intracellular alkalinization occurring in Amphiuma erythrocytes in the presence of DIDS is also dependent on the continuous cycling of these two mechanisms.

There is a significant difference at both carbon dioxide tensions between the measured erythrocyte pH and that calculated from the distribution of chloride (Table 12). This difference may be removed by treatment with the protonophore 2,4 DNP, but not by treatment with amiloride indicating that it is probably not due to the sodium/proton exchange mechanism. The small magnitude of this difference indicates that it is probably not caused by a disequilibrium of protons between the cytoplasm and the extracellular space. It is possibly due to the fact that protons are not passively distributed according to a Donnan equilibrium in all the intracellular compartments.

Upon beta-adrenergic stimulation, teleost erythrocytes show a marked increase in both cell water content and pH (Nikinmaa, 1982; Baroin et al.,1984a,b; Nikinmaa and Huestis, 1984; Heming et al., 1987). These adrenergic effects contribute to an increase in haemoglobin oxygen-affinity during stress (Nikinmaa et al., 1984; Primmett et al., 1986). There have been very few studies, however, documenting the effects of beta-adrenergic stimulation of amphibian erythrocytes. Rudolf and Greengard (1980) have found that Rana pipiens erythrocytes will swell if exposed to isoproterenol in the presence of a phosphodiesterase inhibitor. The

results of the present investigation, however, indicate that beta-adrenergic stimulation does not affect either the cell water content or the pH of erythrocytes from the aquatic air-breathing urodele, Amphiuma tridactylum. These results are similar to those found in toads (Chapter 5). The functional significance of the difference in beta-adrenergic sensitivity between amphibian and teleost erythrocytes is not clear. Both Amphiuma and Bufo are air breathers. It is possible, therefore, that the beta-adrenergic response may be an adaptation found only among exclusive water breathers. The difference between amphibian and teleost erythrocytes may also be related to differences in the oxygen transport characteristics of the blood. In teleosts, a reduction in the erythrocyte pH lowers the oxygen carrying capacity of the blood, known as the Root shift (Root and Irving, 1943). Therefore, it would be beneficial for teleosts to regulate erythrocyte pH via adrenergic mechanisms during stress in order to maintain the oxygen carrying capacity of the blood. Amphibian blood does not appear to have a Root shift (Bridges et al., 1985) and a reduction in erythrocyte pH does not affect the total oxygen carrying capacity of the

erythrocyte.

Summary

1. This study suports the view that the membrane of the Amphiuma erythrocyte contains sodium/proton and chloride/bicarbonate exchange mechanisms.

2. These ion exchange mechanisms are involved in steady-state volume regulation.

3. The secondarily active sodium/proton exchanger may cause a marked intracellular alkalinization in the absence of bicarbonate movements.

4. In contrast to the sodium/proton exchanger on the teleost erythrocyte membrane, the sodium/proton exchange mechanism on the Amphiuma erythrocyte membrane does not exhibit beta-adrenergic sensitivity, but is active under steady-state conditions.

GENERAL DISCUSSION

Protons are passively distributed in both fish (Albers and Goetz, 1985; Heming et al., 1986; Chapter 1) and amphibian erythrocytes (Chapter 5, 6) as they are in mammalian erythrocytes (Fitzsimons and Sendroy, 1961; Hladky and Rink, 1977). The erythrocyte pH will, therefore, be influenced by the extracellular pH and the net charge on the impermeable ions within the erythrocyte (Steen and Turitzin, 1968). Haemoglobin and organic phosphates are the predominant impermeable erythrocytic ions. In nucleated erythrocytes, it has recently become apparent that the erythrocyte pH may also be determined by the activity of the sodium/proton exchange mechanism on the erythrocyte membrane (Nikinmaa and Huestis, 1984; Nikinmaa, 1986; Heming et al., 1987 Chapter 1).

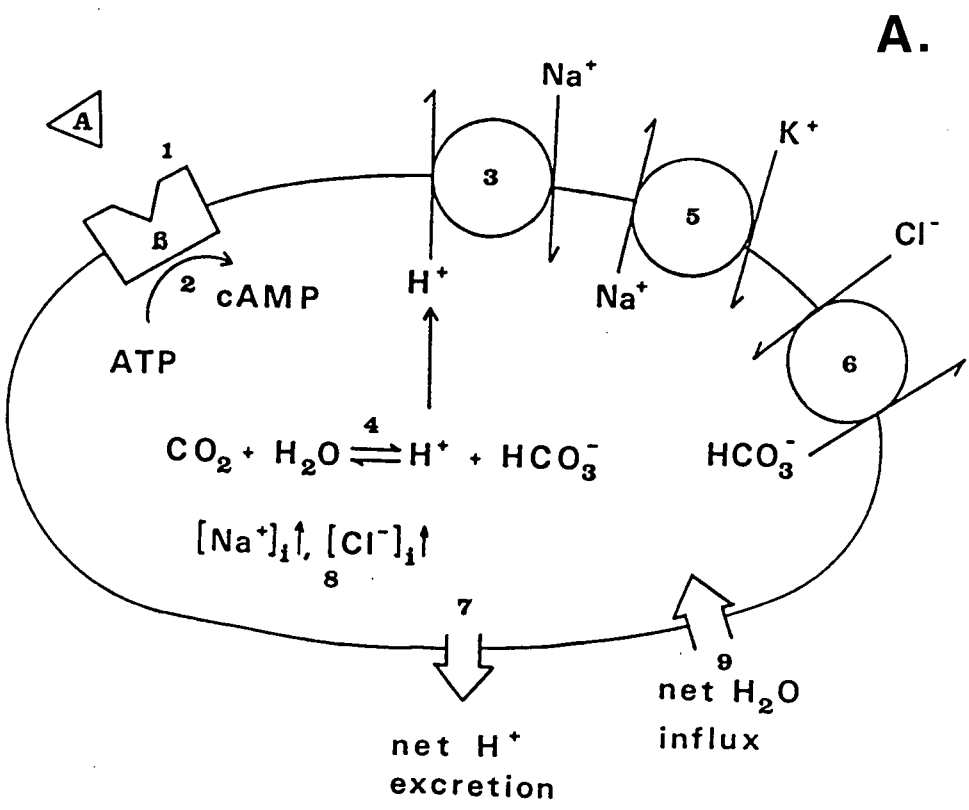
Na^+/H^+ Exchange in Fish Erythrocytes:

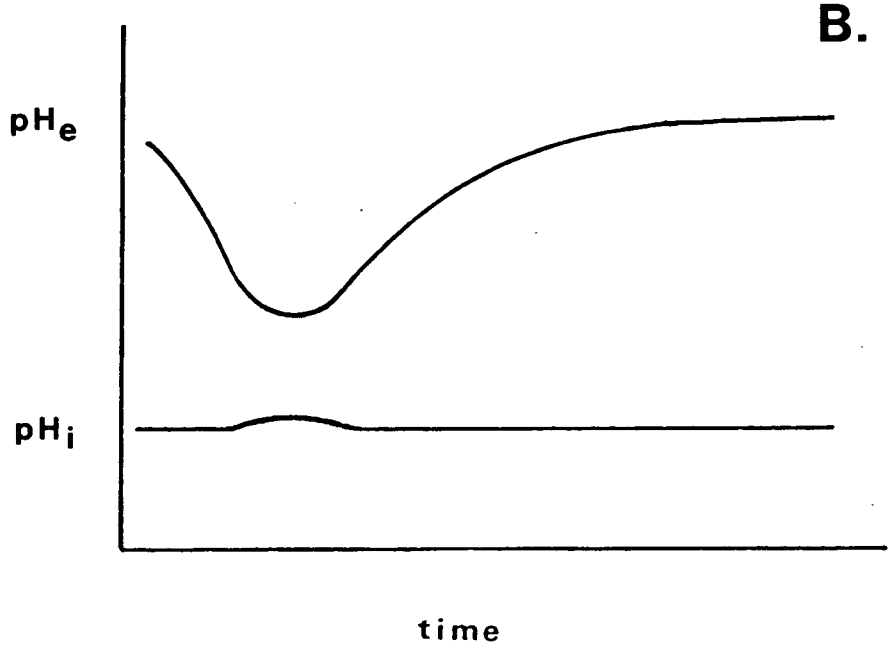
Under steady-state conditions, the sodium/proton exchanger in fish erythrocytes is quiescent and it does not affect the volume or pH of the erythrocytes (Nikinmaa and Huestis, 1984; Chapter 1). Beta-adrenergic stimulation, however, causes an increase in the activity of this exchanger and sodium enters the cell in exchange for protons (Nikinmaa and Huestis, 1984; Cossins and

Richardson, 1985; Chapter 1; Fig. 20). This exchanger is secondarily active and is dependent on the sodium gradient established by the sodium/potassium ATPase. Indeed, Bourne and Cossins (1982) have demonstrated that beta-adrenergic stimulation of rainbow trout erythrocytes results in a 100-250% increase in the activity of the sodium/potassium ATPase. The response also involves movement of chloride ions into the cell via the chloride/bicarbonate exchange mechanism. The increased concentrations of both sodium and chloride ions causes water to be drawn into the cell osmotically and the erythrocyte volume increases. There is also a significant increase in the erythrocyte pH. This increase in erythrocyte pH is clearly dependent on the activation of the sodium/proton exchanger (Nikinmaa and Huestis, 1984; Cossins and Richardson, 1985; Chapter 1). The observed intracellular alkalinization may be both a direct and an indirect effect of the activation of this exchanger. In addition, it has been demonstrated that adrenergic stimulation of fish erythrocytes results in a decrease in cellular NTP levels (Ferguson and Boutilier, 1987) and ATP (Nikinmaa, 1983). Ferguson and Boutilier (1987) propose that this decrease in NTP concentration is due to the increased demand for NTP equivalents by

Figure 20. A: Model of adrenergically stimulated fish erythrocyte. 1. Adrenaline binds to beta receptor. 2. Beta adrenergic stimulation causes an increase in cAMP. 3. Na^+/H^+ exchange is activated. 4. The CO_2 hydration reaction is shifted towards formation of protons and bicarbonate. 5. The activity of the Na^+/K^+ ATPase is increased. 6. Chloride moves into the cell in exchange for bicarbonate. 7. There is a net proton excretion. 8. There is a net increase in sodium and chloride in the cell. 9. There is a net influx of water.

B: Schematic of beta-adrenergic regulation of erythrocyte pH during an extracellular acidosis in fish erythrocytes.


A.
A
1
B
2 cAMP
ATP
3
Na+
H+
4
CO2 + H2O ⇌ H+ + HCO3-
5
Na+
K+
Cl-
6
HCO3-
[Na+]i↑, [Cl-]i↑
8
7
net H+ excretion
9
net H2O influx


B.
pHe
pHi
time

adrenergically-enhanced membrane ion exchanger activity. This decrease in NTP would reduce the concentration of impermeable anions within the cell and thereby elevate the pH. The ability of the sodium/proton exchanger to influence the pH of the erythrocyte directly via the extrusion of cytoplasmic protons is difficult to determine. According to Baroin et al., (1984b), the sodium/proton exchange mechanism would be unable to influence the erythrocyte pH directly because of the presence of the high-capacity anion exchanger (Romano and Passow, 1984; Baroin et al., 1984a; Cossins and Richardson, 1985; Heming et al., 1986). Protons are passively distributed in rainbow trout erythrocytes 30 minutes subsequent to adrenergic stimulation (Heming et al., 1987). Cossins and Richardson have demonstrated, however, that the activity of the adrenergically stimulated sodium/proton exchanger does approach that of the chloride/bicarbonate exchanger. Furthermore, Borgese et al., (1986) have found that chloride/bicarbonate exchange lags behind sodium/proton exchange in trout erythrocytes at the onset of adrenergic stimulation. It is therefore possible that this exchanger may directly influence the pH of the cell even in the presence of the

chloride/bicarbonate exchange mechanism via the extrusion of protons and also by a shift in the carbon dioxide hydration reaction towards the formation of bicarbonate. The exact contribution of each of these processes to the increase in erythrocyte pH is not known. The combined effect of these processes is, however, to increase the pH of the cell even in the presence of 10 mM bicarbonate (Chapter 1) Thus, this elevation in erythrocyte pH contributes to the increase in haemoglobin oxygen affinity (Nikinmaa et al., 1984; Cossins and Richardson, 1985; Primmett et al., 1986; Boutilier et al., 1986a) in the intact animal after an increase in circulating catecholamines. In addition, this response is enhanced at lower pH values (Chapter 1) which indicates that it provides the highest benefit to animals which have been stressed and in which blood pH is reduced.

The cellular mechanisms involved in the beta-adrenergic stimulation of the sodium/proton exchange mechanism in fish erythrocytes have not been clearly defined. Mahe et al ., (1985) have demonstrated that beta-adrenergic stimulation of rainbow trout erythrocytes results in an increase in the beta-adrenergic second messenger 3', 5'-cyclic AMP (cAMP). Activation of specific protein kinases is mediated by levels cAMP in

other tissues (Cohen, 1982) and, therefore, this system may also be operative in fish erythrocytes. Grinstein et al., (1985) proposes that the increase in volume and intracellular alkalinization occurring in lymphocytes following phorbol ester treatment is a result of stimulation of a cellular protein kinase which regulates the activity of the sodium/proton exchanger. It is possible, therefore, that cAMP levels also regulate the activity of the sodium/proton exchanger in fish erythrocytes via specific protein kinases. This system, however, requires further study.

Cl^-/HCO_3^- Exchange in Fish Erythrocytes:

Generally, air breathing vertebrates regulate body pH by an initial ventilatory adjustment of body CO_2 levels followed by a long term renal adjustment of body bicarbonate levels (Davenport, 1974; Woodbury, 1974). Changes in ventilation in water breathing fish, however, have less of an effect on blood PCO_2 or pH (Janssen and Randall, 1975; Iwama et al., 1987). Instead, water breathing fish regulate blood pH during acid-base disturbances by adjustments in body bicarbonate levels (Cameron and Randall, 1972; Randall and Cameron, 1973; Eddy et al., 1977; Cameron, 1978). Wood and Perry (1985)

have reported that adrenaline inhibits bicarbonate entry into rainbow trout erythrocytes in vitro. Based on this data, Perry (1986) has proposed that adrenergic control of erythrocytic chloride/bicarbonate exchange may play a role in this increase in internal bicarbonate stores in fish and, therefore, in acid-base regulation. An adrenergically mediated reduction in the activity of the chloride/bicarbonate exchange pathway would also be expected to result in a decrease in carbon dioxide excretion by the intact animal. In the present experiments, however, there was no reduction in carbon dioxide excretion or in the respiratory exchange ratio after burst swimming (known to cause an increase in circulating catecholamines; Primmett et al., 1986) or adrenaline infusion. Furthermore, in the present study, neither beta nor alpha agonists could reduce the rate of chloride/bicarbonate exchange in rainbow trout erythrocytes in vitro whereas this exchange could be largely inhibited by acetazolamide and the anion exchange blockers DIDS and SITS. Based on these results, it can be concluded that catecholamines do not inhibit erythrocytic chloride/bicarbonate exchange in fish. Hence, increases in plasma bicarbonate in response to acidoses in these animals are not achieved by adrenergic

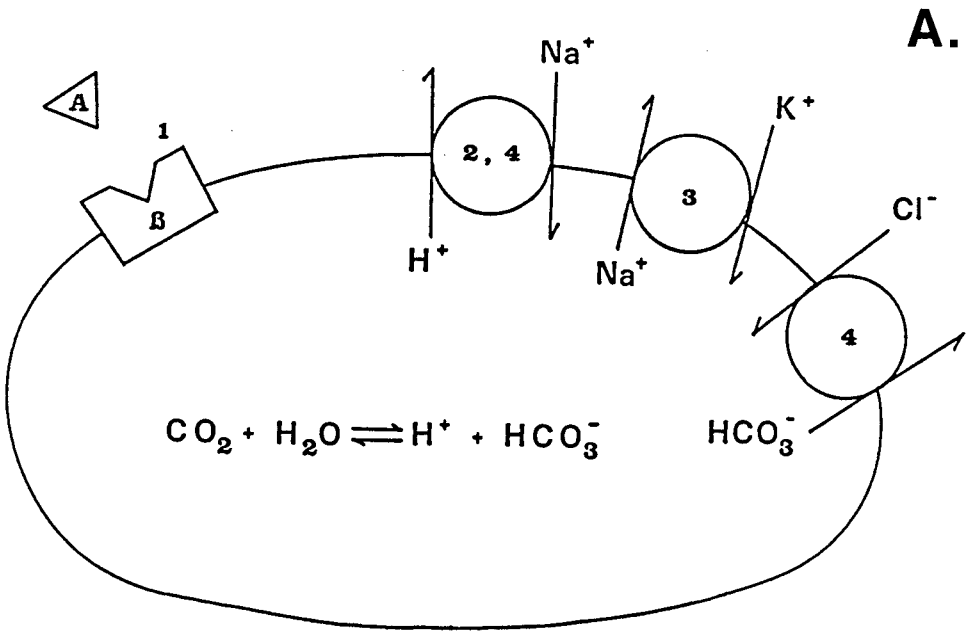
regulation at the level of the erythrocyte.

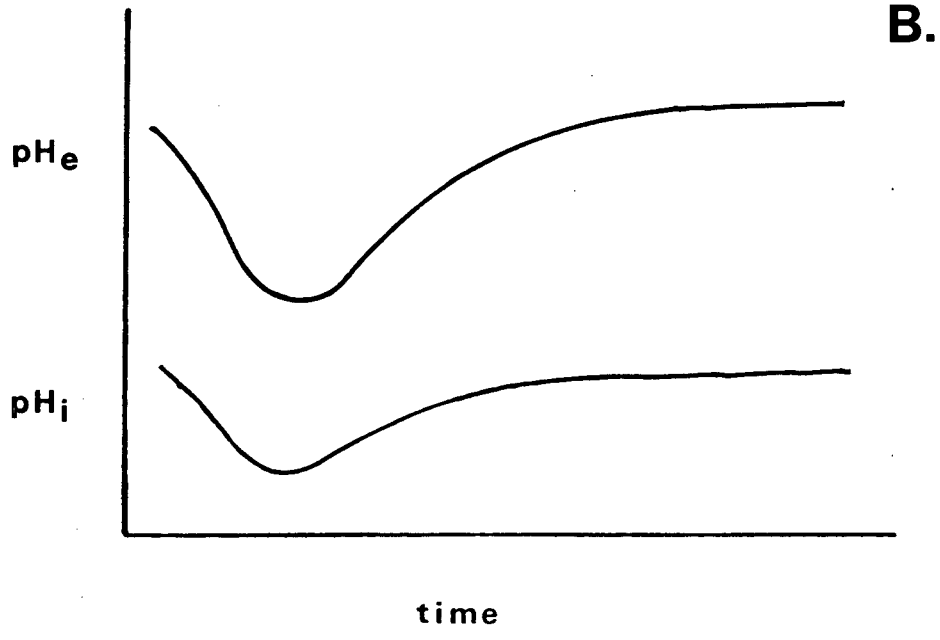
Na^+/H^+ and Cl^-/HCO_3^- Exchange in Amphibian Erythrocytes:

In contrast to the exchanger in fish erythrocytes, the sodium/proton exchange mechanism in amphibian erythrocytes is active under steady state conditions (Chapter 6; Fig. 21). Sodium ions continuously enter the cell in exchange for protons. In the presence of bicarbonate movements, this exchange significantly affects the erythrocyte volume, but not the cytoplasmic pH. According to Hladky and Rink (1977), the chloride distribution across the erythrocyte membrane is an indicator of the net charge on the impermeable ions within the erythrocyte since chloride ions are passively distributed across the erythrocyte membrane. Changes in the activity of the sodium/proton exchange mechanism in amphibian erythrocytes are not associated with changes in the chloride distribution (Chapter 6), and, therefore, are not associated with changes in the net charge in amphibian erythrocytes. In contrast, changes in the activity of the sodium/proton exchanger in fish erythrocytes are associated with changes in the chloride distribution across the erythrocyte membrane (Heming et al., 1987) and changes in the concentrations of

Figure 21. A: Model of adrenergically stimulated amphibian erythrocyte. 1. Adrenaline binds to beta receptor. 2. The Na^+/H^+ exchange mechanism is continuously active, but this activity is not altered by adrenergic stimulation. 3. The Na^+/K^+ ATPase establishes the sodium gradient which drives the continuously active Na^+/H^+ exchange. 4. The steady-state cell volume is regulated by the Na^+/H^+ and Cl^-/HCO_3^- exchange mechanisms, but the cell volume is not affected by adrenergic stimulation.

B: Schematic of changes in erythrocyte pH during an extracellular acidosis in beta-adrenergically stimulated amphibian erythrocytes.


A.
A
1
ß
2, 4
3
4
Na+
K+
Cl-
H+
Na+
CO2 + H2O ⇌ H+ + HCO3-
HCO3-


B.
pHe
pHi
time

impermeable anions within the erythrocyte (Nikinmaa, 1983; Ferguson and Boutilier, 1987). These results suggest that the influence of the sodium/proton exchange mechanism in determining the erythrocyte pH may be related to changes in impermeable ion concentration within the cell. Thus, the inability of the sodium/proton exchanger to influence the pH in amphibian cells may be due to the lack of an association between the activity of this exchanger and the impermeable ion concentrations within these cells. Possibly, the number or the activity of sodium/proton exchangers in amphibian cells may be reduced relative to fish cells such that the activity of these exchangers does not significantly effect cellular NTP levels as in fish erythrocytes. These results further suggest that much of the pH change in fish erythrocytes following adrenergic stimulation may be due to changes in cellular NTP levels and a redistribution of protons rather than a direct effect of the sodium/proton exchange on the intracellular proton concentration.

Adrenergic stimulation did not affect the pH or volume of erythrocytes from either Amphiuma or Bufo (Chapter 4, 6). These animals, therefore, do not appear to regulate erythrocyte pH adrenergically. In contrast

to the undivided circulatory system in fish, amphibians possess a partially divided double circulation. This system allows selective distribution of blood between pulmonary and cutaneous circuits via cardiac shunting (Shelton, 1985). In addition, Boutilier et al., (1986c) have demonstrated that the relative distribution of the pulmocutaneous heart output between lungs and skin in Rana catesbeiana may be adjusted depending on the pattern of environmental oxygen partial pressure. Thus, amphibians are able to efficiently utilize oxygen stores via both central and peripheral shunting. Adrenergic regulation of erythrocyte pH in order to enhance oxygen transport during fluctuations in ambient and internal gas tensions, therefore, is probably less important than it would be in fish.

Functional Significance of Adrenergic pH Regulation:

What is the functional significance of the adrenergic regulation of pH and volume in nucleated erythrocytes? Root and Irving (1943) demonstrated that a reduction in the pH of the haemoglobin environment in vitro also lowers the oxygen capacity of the blood in certain species of fish. This effect is important in the delivery of oxygen to the swimbladder (Berg and Steen,

1968) which controls buoyancy in some species of fish. Glycolysis occurs in the secretory epithelium of the swimbladder and acidifies the blood as it passes through the rete structure in its wall. Consequently, an increase in blood P_{O2} results as oxygen is released from haemoglobin due to the Root effect. Thus, a P_{O2} gradient is established between the blood and the swim bladder and oxygen diffuses into the swimbladder. The Root effect has also been implicated in the delivery of oxygen to the retinal tissue in fish (Wittenberg and Wittenberg, 1974). This effect, however, if general throughout the body may also limit the blood oxygen carrying capacity under conditions of low pH and thereby limit aerobic performance. It has been suggested (Chapter 4, 6) that the functional significance of the adrenergic response in fish erythrocytes may be to offset the detrimental effects that the Root effect would have on the oxygen carrying capacity of fish blood. In the face of an extracellular acidosis, this response would maintain the erythrocyte pH and, therefore, the oxygen carrying capacity of the blood. In addition, oxygen delivery to the swim bladder could continue at the same rate as in unstressed fish. It can be seen from Table 13 that the adrenergic response has not been documented in any

Table 13. The presence or absence of the adrenergic response in erythrocytes of fish versus the presence or absence of a Root shift.

Species	Adrenergic Response Yes/No	Root Shift Yes/No
Salmo gairdneri	Yes, Nikinmaa, 1982a; Nikinmaa and Huestis, 1984; Baroin *et al.*, 1984a,b; Heming *et al.*, 1987; Chapter 1. No, Nikinmaa and Jensen, 1986	Yes, Cameron 1971
Salmo salar	Yes, Ferguson and Boutilier, 1987	Yes Borjeson and Haglund, 1976
Cyprinus carpio	Yes, Nikinmaa *et al.*, 1987	Yes, Tan *et al.*, 1972
Squalus suckleyi	No, Tufts and Randall, unpubl.	No, Lenfant and Johansen 1966
Raja sp.	No, Tufts and Randall, unpubl.	No, Lenfant and Johansen 1966
Tinca tinca	Yes, Tufts and Randall, unpubl.	Yes, Jensen Weber, 1982
Oncorhynchus tshawytscha	No, Tufts and Randall, unpubl.	Yes, Randall *et al.*, 1987
Platichthys flessus	Yes, Fugelli and Reiersen, 1978	Yes, Weber and Wilde, 1975

species of fish which does not have a Root effect. Equally, the response is not found in all species which do have a Root effect. This absence of an adrenergic response in some species may be due, however, to the labile nature of the beta adrenergic receptors. Beta-adrenergic receptor systems may down-regulate and become desensitized (Harden, 1983; Sibley and Lefkowitz, 1985). It has been documented that eel gills (Peyraud-Waitzenegger et al., 1980) and the eel heart (Pennec and Peyraud, 1983) lose beta adrenergic sensitivity during the winter. Indeed, Nikinmaa and Jensen (1986) found that the erythrocytes from the rainbow trout, Salmo gairdneri may be insensitive to adrenergic stimulation under certain conditions. It is possible, therefore, that the absence or presence of the adrenergic response may be determined by other variables such as season or temperature. Certainly, it would be conceivable that this energy-requiring process may only be functional during periods when it would benefit the animal, such as during the spawning migration in Salmonid species. Thus, the evidence to date is consistent with the view that the functional significance of the adrenergic response may be to offset the Root effect, but it is also apparent that

other factors may influence this response.

In summary, interspecific variation in the ionic exchange mechanisms on the membrane of nucleated erythrocytes is more widespread than previously thought. This variation is apparently yet another level of adaptation for modulating haemoglobin oxygen affinity and, therefore, blood oxygen transport. It would seem that this level of adaptation may have arisen due to different selective pressures on the erythrocyte ion exchange processes caused by haemoglobin heterogeniety as well as different strategies among animals for the regulation of acid-base balance and blood oxygen transport.

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