Metabolism and Ion Exchange in Nucleated Erythrocytes of the Hypoxia-tolerant Goldfish, *Carassius auratus*, and Hypoxia-sensitive Rainbow Trout, *Oncorhynchus mykiss*.

by

Nancy L. McAllister-Irwin

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Dedication

To my children, Jason and Donnie, for their patience and pride in their Mom; to my family for their support and understanding; to David, for letting me be who I am.

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Abstract

A consequence of variable oxygen partial pressures in aquatic environments, is that all fish share some degree of hypoxia tolerance. The hierarchical recruitment of physiological and biochemical defence mechanisms and the degree of their success in protecting the animal against losses of oxygen to vital tissues, appears to be species dependent. Animals, like the goldfish (*Carassius auratus*) that thrive in low oxygen environments, employ a whole host of mechanisms to ensure continued viability. The two major strategies are (1) metabolic depression and (2) maintenance of intracellular ion homeostasis *via* channel regulation.

Goldfish and trout red blood cells display similar metabolic and ionic responses to an acute hypoxic exposure. However, goldfish RBC's incubated in nitrogen for 60 minutes prior to sampling do not display changes of energy concentrations (ATP) or intracellular sodium and potassium ions typical of the less hypoxia-tolerant trout. Even when adrenergically challenged, these red cells maintained their metabolic-membrane coupling. This suggests a reduced metabolism as energy consumption and energy production are matched. The absence of an adrenergic response is also typical of animals that are 'good animal anaerobes'.

Red cells incubated with ouabain (a sodium-potassium ATPase blocker) do not show the changes in intracellular ion concentrations seen in the rainbow trout. This supports the notion that channel arrest is integral to survival in low ambient oxygen concentrations for the goldfish.

Another evolutionary adaptation for a lifestyle which requires an ability to survive extended hypoxia is an organism's ability to deal with acid-base imbalances. This is reflected in their buffering capacity and Haldane effect.

List of Abbreviations and Symbols

ATP ..... adenosine 5'-triphosphate

cAMP ..... (cyclic) adenosine 3',5'-monophosphate

DPG ..... diphosphoglyceric acid

EDTA ..... ethylenediamine-tetraacetic acid

GTP ..... guanosine 5'-diphosphate

h ..... hours

Hb ..... haemoglobin

Hct ..... haematocrit

$[K^+]_i$ ..... intracellular potassium concentration

M ..... molar

MCHC ..... mean cellular haemoglobin content

min ..... minute

MS-222 ..... 3-aminobenzoic acid ethyl ester

$[Na^+]_i$ ..... intracellular sodium concentration

NTP ..... nucleotide 5'-triphosphate

O_2 ..... oxygen

pH_e ..... plasma (i.e. extracellular) pH

pH_i ..... intra-erythrocytic pH

RBC ..... erythrocyte/red blood cell/red cell

sec ..... seconds

S.E. ..... standard error of the mean

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GENERAL INTRODUCTION

I. Preface

All fish share some degree of hypoxia tolerance due to the variability of oxygen partial pressures inherent in aquatic environments. Hypoxia-tolerant animals exhibit a variety of physiological and biochemical strategies which ensure that adequate supplies of oxygen reach critical tissues when ambient levels become limiting. Should inadequate oxygen be available, alternative measures (e.g. reduced ion permeabilities and metabolic depression) can be elicited. Exposure to hypoxia evokes a hierarchical recruitment of defense mechanisms. The degree to which these mechanisms are recruited, and the success of the particular strategy, is dependent on the severity and the duration of the oxygen deprivation. Hypoxia tolerance also appears to be species dependent, since some animals are far more capable of surviving severe, extended periods of oxygen lack.

Defense strategies against hypoxia fall into two categories; those operating at the systemic level and those operating at the cellular level. Regulation of oxygen delivery to the tissues can be maintained by the animal through systemic mechanisms until environmental oxygen concentrations reach a critical partial pressure. Beyond this point, oxygen consumption decreases in proportion to PO_2. Survival then depends on the initiation of cellular defenses, such as lowering the aerobic metabolic rates of certain

tissues. It is often at this point that anaerobiosis is recruited into the overall energy budget of the animal.

Systemic mechanisms of hypoxia tolerance include changes in ventilation and perfusion of the gill (Randall, 1982), changes in total haemoglobin content (Murad *et al.*, 1990), haemoglobin isomorphs (Houston and Murad, 1992) and Hb-O_2 affinity (Weber and Jensen, 1988), in addition to rate-limiting changes in enzymes of intermediary metabolism (Hochachka and Somero, 1984). Systemic mechanisms are adequate for short-term bouts of moderate hypoxia, but will not provide protection against severe exposure. Boutilier *et al.* (1988) examined the metabolic and respiratory adaptations of rainbow trout (*Oncorhynchus mykiss*) exposed to acute graded levels of hypoxia. Although generally considered to be a highly aerobic and 'hypoxia-sensitive' animal, the trout were able to successfully recruit anaerobic metabolism when exposed to 24 h of acute graded hypoxia. Oxygen consumption (M_{O2}) remained unchanged over a broad range of ambient PO_2, however, a significant decrease in M_{O2} was detected when environmental O_2 was lowered to severe levels of approximately 30 Torr. As well, the animals were able to offset an initial plasma lactacidosis and to maintain red blood cell pH at a constant level. At the most severe levels of hypoxia, anaerobic metabolism was well advanced, as muscle lactate increased and cellular adenosine triphosphate (ATP) levels began to fall. This study

indicated that the initiation and hierarchical order of responses to hypoxia were dependent upon the duration of exposure and the severity of the oxygen deprivation.

Although anaerobic glycolysis can generate ATP, it cannot provide sufficient ATP to maintain cellular function at pre-hypoxic rates over long periods of time. Falling ATP concentrations result in the failure of active transport processes involved in the maintenance of ionic homeostasis. Such failure leads to a dissipation of ion gradients across most cell membranes, eventually resulting in cell death. Anaerobiosis also leads to a marked lactacidosis, and the decreased pH can compromise the action of certain pH-sensitive enzymes (Hochachka and Somero, 1984). Because anaerobic metabolism is much less energetically efficient than aerobic metabolism, animals that attempt to defend pre-hypoxic rates of ATP production must use vast quantities of glycogen and/or glucose, thus threatening the long-term viability of the organism (Hochachka, 1986; Hansen, 1987).

Animals that thrive in environments of low oxygen are said to be hypoxia-tolerant and have been referred to as 'good animal anaerobes' (Hochachka and Somero, 1984). They employ a whole host of mechanisms to ensure continued viability: (1) the conservation of limited glycolytic stores through metabolic suppression (Sick *et al.*, 1982), (2) the maintenance of intracellular

ionic homeostasis by down-regulation of ion channels (Ching-Ping *et al.*, 1989), (3) changes in the levels and activity of 'second messengers' (Nilsson *et al.*, 1991) which mediate the lowering of brain activity and cellular energy consumption, and (4) stabilization of acid-base balance through increased buffering capacity, H^+ consuming metabolism and/or the production of novel metabolic end products such as ethanol (Shoubridge & Hochachka, 1980). One or more of these mechanisms are employed by all hypoxia-tolerant species. This thesis will examine the processes involved in extending the hypoxia-tolerance of ectotherms, with particular emphasis on the cyprinids, the so-called champions of hypoxia-tolerance in water breathing fish.

II. The 'Metabolic Arrest' Hypothesis

Normal rates of aerobic metabolism are impossible to maintain during periods of low oxygen availability. ATP synthesis can no longer be derived from energetically efficient oxidative phosphorylation, but must rely instead on less efficient anaerobic glycolysis (Pasteur Effect). If energy consumption remains the same, anaerobiosis will lead to a rapid depletion of the limited cellular glycogen stores (Hochachka, 1986; Hansen, 1987). Potentially large and life-threatening carbohydrate depletion may be minimized in

hypoxia-tolerant species in a number of ways: 1) by storing larger quantities of glycogen, 2) by utilizing more efficient fermentation pathways, or 3) by reversing the Pasteur Effect.

The first two mechanisms in principle could not extend hypoxia tolerance by more than a factor of 3- to 4-fold (Hochachka, 1986). It can be argued that a greater anaerobic scope, and thus a larger anaerobic capacity, can be supported with high concentrations of substrate for conversion into metabolic energy, and indeed many studies have shown that anoxia tolerant species have substantially higher levels of glycogen than their hypoxia-sensitive counterparts. Goldfish and turtles have liver glycogen stores four to six times higher than those observed in various anoxia-sensitive animals (Hochachka and Somero, 1984). Glycogen levels in the brain of the bullhead catfish (*Ictalurus nebulosus*), another anoxia-tolerant species, are five times higher than those of the rainbow trout (Heath, 1988). Hansen (1985) suggested there was a potential for high energy production in the anoxia-tolerant turtle brain because of unusually large glycogen stores (eight times that of the rat).

Even though many hypoxia-tolerant animals possess high levels of glycogen, this strategy alone cannot provide the energy required for prolonged periods of O_2 lack. Thus, although catfish survive five times longer than the trout under anoxia, lactate accumulation and glycogen depletion in the bullhead occurs much

more gradually than in the more hypoxia-sensitive animal. ATP generation in anoxic catfish was also much lower (20%) than in trout suggesting a slowing down of glycolysis (Heath, 1988). Similarly, Sick *et al.* (1982) found no dependence on cellular stores of high energy compounds (e.g. glycogen) for extended anoxia tolerance in turtle brain tissue. Glycogen utilization in the anoxic goldfish also does not occur to the extent of that observed in less tolerant species (Van Waversveld *et al.*, 1989). The decrease in ATP production and gradual accumulation of anaerobic end-products in these hypoxia-tolerant organisms suggests other factors may be involved.

Alternative fermentation pathways exist within certain groups of good invertebrate anaerobes such as the bivalve molluscs (De Zwaan, 1977). The only vertebrate known to employ such novel fermentative pathways is the goldfish (*Carassius auratus*) (Van den Thillart and Van Waarde, 1985) which utilize one or more of these pathways at different times during anoxia. This enables the goldfish to increase ATP yields two or four times above that of the classic glucose - lactate fermentation. Nevertheless, there is still an order of magnitude difference in energy production between anaerobic glycolysis and oxidative glucose metabolism.

In contrast, reversing the Pasteur Effect (i.e. suppressing metabolic rate) so as to allow ATP turnover rates to drop appears to be the most effective strategy for solving the problem of substrate

conservation. This not only reduces the rates of substrate depletion, but also automatically reduces the rates of formation of potentially deleterious anaerobic end products. Comparisons of energy production/utilization between hypoxia-sensitive and hypoxia-tolerant animals indicates a reduced dependence upon high ATP turnover rates to sustain cellular metabolism (Hochachka, 1986; Hochachka and Guppy, 1986; Sick *et al.*, 1982). For instance, metabolic arrest in diving turtles increases anoxia tolerance by 60-fold, as compared to the hypoxia-sensitive rat (Sick *et al.*, 1982) and Maginnis and Hitzig (1987) have shown that, when compared with control values, submerged anoxic turtles undergo a 77% reduction in ATP production. In addition, Robin *et al.* (1979) also reported no appreciable increase in the rate of anaerobic energy production during anoxia in turtles, concluding that energetic balance must result from an overall decrease in energy consumption. While the results of many such studies support the general notion of the metabolic arrest concept, stabilized membrane function must also occur during O_2 deprivation in order to prevent ions from drifting to their electro-chemical equilibrium. Energy production cannot be reduced unless energetically expensive ion pumping (Na^+/K^+-ATPase) also declines.

III. The 'Channel Arrest' Hypothesis

Studies of ischemic mammalian brain (Hansen, 1985), reveal a massive efflux of K^+ from the neurons and a corresponding influx of Na^+ into the intracellular space. Similar K^+ efflux from the brain of the hypoxia-sensitive rainbow trout has also been reported (Girard, 1989) when animals were deprived of ambient O_2. Comparable studies, using hypoxia-tolerant ectotherms, have shown that the massive K^+ efflux typical of hypoxia-sensitive animals either does not occur at all (Surlykke, 1983) or develops much more slowly (Sick *et al.*, 1982). For example, Ching-Ping *et al.* (1989) measured passive ion leakage rates in turtle brains treated with ouabain (a Na^+/K^+-ATPase inhibitor). They found that the rates of K^+ leakage were 50% lower in brains subjected to 2 h of anoxia than in their normoxic counterparts. Suppression of EEG activity was also noted, indicating reduced ionic traffic across the neuronal membranes.

There is evidence that certain species of hibernating mammals may also display channel arrest. Hall and Willis (1984), for example, characterized the effect of temperature on the ouabain-insensitive fluxes of K^+ ions in red blood cells in the hibernating ground squirrel and the non-hibernating guinea pig. Cold-adapted erythrocytes from

the ground squirrel retain K^+ ions better than cells from the guinea pig. Potassium flux was resolved into three components; basic leak, co-transport and Gardos channels (Ca^{2+} sensitive K^+ channels). They conclude that ion stabilization could not all be accounted for by the first two components, but that the more efficient regulation of cytoplasmic Ca^{2+} in the hibernator was largely responsible for reduced channel movement of K^+. Lowered Na^+/K^+-ATPase activity also appears to be a contributing factor to reduced K^+ loss (Willis *et al.*, 1980).

Ion homeostasis and cellular energy metabolism has also been investigated in the red cells of common carp (*Cyprinus carpio*) subjected to acute hypoxia. Nikinmaa *et al.*, (1987) observed slow changes of intracellular ion concentrations (increased Na^+ and decreased K^+) over the six hour time course of their experiment, in blood samples withdrawn from chronically catheterized animals. Exposure of the fish to decreasing environmental oxygen saturations (from 100% to 15-20%) had no effect on erythrocyte ATP concentrations. Clearly, the coupling of metabolic arrest with channel arrest allows good animal anaerobes to maintain cellular viability in potentially hypoxic environments.

IV. Chemical Messengers

Hormones, neurotransmitters and cellular 'second messengers' are essential for the regulation and integration of cellular, tissue and system activity. For example, the nervous system provides the body with a rapid means of internal communication that is critically important in regulating and coordinating the activities of the cells. As a result, neural activity is essential to an organism's ability to maintain homeostasis. Chemical transmission of information across synapses from one neuron to another is accomplished with neurotransmitters. The action of these substances may be altered by neuromodulators. Anoxia-tolerant vertebrates, like the loggerhead sea turtle (*Caretta caretta*), the freshwater turtle (*Pseudemys scriptaelegans*) and the crucian carp (*Carassius carassius*), display changes in brain neurotransmitter levels in response to reduced concentrations of environmental oxygen (Nilsson *et al.*, 1991). When exposed to 4 h of nitrogen, levels of the inhibitory amino acids gamma-aminobutyric acid (GABA), taurine and glycine increased, while the level of the excitatory amino acid glutamate decreased. It is suggested that the combined effects of an increase in inhibitory amino acids and a corresponding decrease in the excitatory neurotransmitter, may facilitate a lowering of brain activity and therefore energy consumption. In anoxia-intolerant species (the

anole lizard, *Anolis sagrei*, and the dog) GABA levels increase to only a moderate extent while glutamate levels either remain unchanged or increase slightly. Anoxia-induced increases of the inhibitor GABA may act to protect the brain from immediate ischemic damage. However, the corresponding increase of the excitatory amino acid, glutamate, predominates and prohibits long-term protection for these more sensitive organisms.

Other neuronal inhibitors such as norepinephrine and serotonin (Nilsson, 1989, 1990) may play a similar role in protecting the brain against anoxia damage. Serotonin synthesis and degradation requires the simultaneous reduction of molecular oxygen and should therefore be strongly affected by anoxia. Species which exhibit extreme anoxia tolerance (e.g. cyprinid fish and turtles) display little or no decrease in serotonin, which suggests they have developed effective mechnisms for the uptake or storage of monoamines. For example, serotonin levels were maintained for up to 13 h of anoxia in both *Pseudemys* and *Caretta* , but fell by 40% in the anole lizard after only 40 min of similar treatment (Nilsson *et al.*, 1991). In an earlier study, Nilsson (1989) measured serotonin levels in the brain of the crucian carp subjected to anoxia (oxygen levels less than 0.1 mg L^{-1}). Serotonin decreased only 15%, while the two main metabolites of serotonin, 5-HIAA and 5-HTOH, decreased 80-90%. Crucian carp are

therefore capable of conserving this inhibitory neurotransmitter responsible for lowering activity levels in the brain.

Phosphorylation is a major mechanism of ion channel regulation (Rossie *et al.*, 1987). Cyclic AMP-mediated phosphorylation is known to be involved in modulating the behaviour of calcium, potassium and sodium ion channels in nerve, muscle and heart tissues (Costa and Catterall, 1984).

Adenosine is known to initiate transmembrane signals which influence the activity of adenylate cyclase, phospholipase C or K^+ ion channels (Stiles, 1991). Recently, it has been implicated in Na^+ channel regulation (M. Rosenthal, pers. comm.). Their results indicate that exposure of isolated turtle cerebellum to anoxia leads to a decrease in the density of voltage-gated sodium channels. Ion homeostasis, under this condition, is only lost when adenosine receptors are blocked. It should be noted, however, that it is still unclear if reduced ion movement is a function of channels being removed from the plasma membrane by endocytosis or that channel activity rate is affected.

Aldosterone is a well known regulator of passive sodium movements and affects recruitment of pre-existing Na^+ channels in the toad bladder through a mechanism involving aerobic metabolism (Palmer *et al.*, 1982). Under anaerobic conditions, permeability is

impaired through inactivation of these channels.

It is clear from these examples that a myriad of hormones, neurotransmitters and neuromodulators may effect ion channel densities and activity, thereby enhancing the tolerance capabilities in some species. The complexity of the interactions makes it virtually impossible to generalize direct cause and effect mechanisms, but their importance in anoxia tolerance is apparent.

V. Novel Metabolic End Products

In 1980, Shoubridge and Hochachka suggested that lactate produced during anaerobiosis in the glycolytic tissues of the goldfish could be metabolized further to ethanol. They reasoned that this could account for the discrepancy they saw between the rate of glycogen utilization and the low accumulation of lactate, as well as the presence of the enzyme alcohol dehydrogenase in red and white muscle tissues. Johnston and Bernard (1983) also found that lactate accumulation could only account for 18.5% of the glycogen stores utilized, with the major end-product of anaerobic metabolism being ethanol. Ethanol is freely diffusible, is easily removed across the gills, and therefore never reaches toxic levels in the fish. However, this strategy is quite wasteful of carbon and is probably called upon only in life-threatening conditions.

Further studies by Van den Thillart and colleagues (1983),

found a significant 'gap' between the amount of glycogen utilized as substrate and the amount of ethanol produced after lactate concentrations were taken into account. This would suggest still another factor involved in substrate depletion. Van den Thillart *et al.* (1983) observed anaerobic carbon dioxide production in goldfish. Under hypoxic conditions, carbon dioxide and ethanol were presumed to be produced in a 1:1 ratio according to the combined action of pyruvate dehydrogenase and ethanol dehydrogenase (Van den Thillart, 1982). The excretion rates would be slightly different because bicarbonate and carbonic anhydrase facilitate CO_2 diffusion, while ethanol equilibration is a function of blood perfusion and diffusion distances across the gill. Assuming a time constant for ethanol equilibration of approximately 15 minutes as opposed to 2 minutes for CO_2, Van den Thillart (1982) reasoned that steady states should still be reached within 1 hour after anoxic exposure. However, CO_2 excretion stayed well above that of ethanol, suggesting the existence of other CO_2 producing pathways. One explanation is anaerobic tricarboxylic acid (TCA) cycle activity, as long as there was a mitochondrial sink available for reducing equivalents. The discrepancy between CO_2 and ethanol excretion could also simply be explained by the generation of CO_2 from dehydration of plasma bicarbonate stores by protons of anaerobic origin.

The mitochondria of anoxic goldfish are also known to be the

site of decarboxylation of pyruvate to form acetaldehyde instead of acetyl coenzyme A (Mourik *et al.*, 1982). Alcohol dehydrogenase would further reduce acetaldehyde to ethanol. If total ethanol excretion cannot be explained by glycogen depletion alone, the balance may be comprised from protein as a substrate (Van den Thillart, 1983). Protein is normally catabolized under aerobic conditions resulting in ammonia production; however, anaerobic ammonia excretion has been described in goldfish by Van den Thillart and Kesbeke (1978), and in crucian carp by Johnston and Bernard (1983).

Crucian carp also excrete small amounts of acetic acid (Holopainen *et al.*, 1986). The origin of this material is thought to be through an intermediate pathway involving acetate. Acetate is not easily converted to acetaldehyde and so is excreted, which may aleviate the problem of acid accumulation during long-term anoxia. The acetate pathway also has the energetic advantage of forming one additional ATP (Van den Thillart, 1982).

VI. Goldfish Strategies

As already mentioned, the goldfish is remarkably resistant to anoxia, surviving from 22 h at 20°C (Van den Thillart *et al.*, 1983) to several days at 2-3°C (Shoubridge & Hochachka, 1980). Goldfish

exhibit a novel metabolic solution to oxygen lack via production of ethanol and carbon dioxide as anaerobic end products, which effectively retard metabolic acidosis, but do not eliminate it. Decreases in tissue pH are minor and stabilize around 7.4 after 1.5 h of anoxia at 15°C. Lactate accumulation plateaus at 7.5 mMol L^{-1}, and a rapid alcohol production is established (Van den Thillart and Van Waarde, 1990).

Indirect calorimetry experiments on small, starving goldfish have shown the animals are capable of producing fat during anoxic episodes (Van Waversveld *et al.*, 1989). Fat accumulation has also been reported in the crucian carp (Blazka, 1958). Lipid metabolism may act as a sink for anaerobically produced CO_2 through a reversal of the β-oxidation process (Van Waversveld *et al.*, 1989). Instead of a series of reactions where two-carbon fragments are removed from fatty acids, carbon would be used for chain elongation.

Anderson (1975, as cited in Van den Thillart and Van Waarde, 1990) first noted a reduction in the rate of internal heat production in goldfish subjected to anoxia. Direct calorimetry and metabolite determinations have shown that, during anoxia, goldfish reach a lower thermodynamic steady state (Van Waversveld *et al.*, 1989) indicating a sustained, stable suppression of metabolism. This hypometabolism is important in extending anoxia tolerance by

reducing the amount of ATP required for routine activity.

Other muscle metabolites, such as creatine phosphate and inorganic phosphate, have been investigated using ^{31}P-NMR (Van den Thillart *et al.*, 1989) and suggest that a 'metabolic switch' is activated after 20 min of anoxia. At this point, hydrolysis of creatine phosphate and production of inorganic phosphate stabilize at 50% of control levels indicative of a slowing of energy consuming processes.

Walker and Johansen (1977) observed an increase in liver glycogen stores of cold acclimated goldfish. These animals exhibited elevated liver glucose-6-phosphatase activity, suggesting an increase in glycogenolysis. This ability to produce and store potential sources of energy may be an adaptive strategy to cold, low-oxygen environments.

VII. Hypothesis

Among the cyprinids, the relationship between energy metabolism (ATP production) and membrane transport function (ion channel arrest) is the least studied and the most poorly understood of the processes involved in hypoxia tolerance. The suppression of metabolic rate and ion homeostasis has been investigated extensively in various other hypoxia-tolerant species; however, much of that work has utilized tissue slice or perfused head preparations. These

methods limit ion transport and metabolic energy studies for a number of reasons, not the least of which is damage to membrane integrity during cell culture or tissue slice preparation (e.g. Else and Hulbert, 1987).

Nucleated erythrocytes, like those found in fish, are a naturally occurring tissue cult' re which contain many organelles found in somatic cells (Boutilier and Ferguson, 1988). Because they are free of any connective tissue and are of a small size, ion movement into and out of the cells can readily be assessed. They are often considered to resemble somatic cells in that they contain a nucleus and mitochondria, making them a model cell culture system which may reflect general properties of the whole animal. However, this notion deserves some critical comment. Firstly, much of the organellar complement is expelled or broken down *in situ* (Sekhon and Beams, 1969). It is true that the mature erythrocytes of both trout and goldfish do retain mitochondria. These are, however, considerably smaller and much less numerous than those of juvenile and developing cells. Consequently, and consistent with their mode of maturation, circulating mature erythrocytes are, to some extent, metabolically impaired (Houston, pers. comm.). They do not, for example, take up ^{55}Fe, and apparently cannot synthesize haemoglobin (Murad *et al.*, 1993). Secondly, both goldfish and trout

respond to stress through the rapid release of stored erythrocytes. This response alters circulating erythrocyte composition in favour of more metabolically competent juvenile cells (Pearson and Stevens, 1991) and could be a problem of significant proportions when there is a need to pool blood by the successive sampling of several specimens. It is quite common in such instances to find increases in Hb, RBC and Hct as sequential stress responses accumulate in the last-sampled animal. Thus, there is a likelihood that cell samples may differ in ontogenic composition. Nevertheless, precedence for such similarities between erythrocyte and somatic cell responses has been established in studies on rainbow trout (Boutilier and Ferguson, 1988), turtles (Maginnis and Hitzig, 1987) and rodents (Hall and Willis 1984; Zou and Willis, 1989).

The aim of this study is:

1) to examine and compare ion transport and cellular metabolism in goldfish and trout erythrocytes in response to an cute hypoxia exposure.

2) to examine acid-base regulation in both species under circumstances of normoxia and hypoxia with adrenergic stimulation.

3) to demonstrate if metabolic-membrane events in adrenergically stimulated hypoxic goldfish erythrocytes experience an 'uncoupling' similar to that observed in the trout.

4) to examine the link between buffering capacity, Haldane effect,

adrenergic response and anoxia tolerance.

5) to determine whether goldfish erythrocytes alter ion permeabilities during exposure to anoxic conditions as compared to those of the trout (direct test of the channel arrest hypothesis).

GENERAL MATERIALS AND METHODS

I. Animals

I.A. Acquisition and maintenance of experimental animals.

Freshwater rainbow trout (*Oncorhynchus mykiss*) ranging in weight from 540-1,300 g were purchased from Sugarloaf Farms, Wentworth, Nova Scotia. Mt. Parnell Fisheries, Mercerburg, Pennsylvania, supplied the goldfish (*Carassius auratus*). Goldfish weights ranged from 40-260 g. Trout were transported to the Department of Biology at Dalhousie by the supplier and the goldfish arrived by air freight. Following arrival, the fish were allowed to acclimate for at least one month prior to experimentation.

The trout were held in 1.5 x 1.5 x 1.0 m fiberglass tanks supplied with dechlorinated tap water from the Dalhousie University Aquatron facility. The goldfish were supplied with the same water in Living Stream aquaria (Frigid Units Inc., Toledo, Ohio). Preliminary studies for this thesis were conducted during two seasons: spring/summer and fall/winter. Water temperatures were held at 15 $\pm 1^{o}C$ using either submersible chillers or heaters. Although temperatures were maintained within a few degrees, seasonal variability in several blood parameters (e.g. Hct, Hb, [ATP]) were noted (Table 1). Acclimation temperature and season are known to influence the activity of Na^{+}/K^{+}-ATPase and carbonic anhydrase in the goldfish (Houston and Mearow, 1981). As well,

erythropoiesis is temperature-dependent; virtually ceasing at temperatures < 5°- $10^{o}C$ (Houston, pers. comm.). In winter animals, the circulating erythrocytes are predominantly composed of mature red cells. During maturation, juvenile ovate cells elongate with a reduction in volume and accumulation of haemoglobin. This is confirmed in the observed low Hct/high Hb in the fall/winter goldfish red cells. In contrast, juvenile and developing cells are more prevalent in the blood of summer fish resulting in high Hct/low Hb. The reduced NTP:Hb concentrations also observed in the fall/winter fish may reflect increased Na^{+}/K^{+}-ATPase activity similar to that reported in gill tissues of cold-acclimated roach (Schwarzbaum *et al.*, 1991) and kidney of goldfish (Houston and Mearow, 1982). As a result of these findings, all experimental data for this thesis was collected during the spring/summer to minimize the influence of seasonal variability.

Commercially prepared fish pellets and food flakes were obtained from local sources (Corey Feeds and The Animal House pet store) and were fed to the fish on a daily basis. Feeding was suspended for 24 h prior to blood sampling. Occasionally, the goldfish were treated with a 10% Methylene Blue solution combined wiht 10% Formalin to control for external parasites and fungus. Fish were allowed to recover from treatment for at least one week prior

Table 1

Seasonal variation in several blood parameters of the goldfish. Values are mean ± standard error of the mean. N = 5 independent blood pools. All measurements were done in duplicate for a total of n = 10 replicates.

Table 1

	Spring/summer	Fall/winter
[Hb], g 100 mL^{-1} blood	5.0808 ±.2457	6.8701 ±.3088
Hct, (%)	23.6 ±1.4	17.4 ±1.6
NTP:HB, mmol g^{-1} Hb	21.2 ±1.4	16.7 ±2.6

to resumption of experimentation.

I.B. Blood sampling

Animals were anaesthetized in neutralized MS-222 (Sigma A-5040) at a concentration of 1:10,000 for the trout and 1:8,000 for the goldfish. Buffering was accomplished by adding $NaHCO_3$ to the solution to achieve a pH of approximately 7.0. Following anaesthesia the fish were placed on a surgical table, ventral side up. Blood samples were taken into heparinized syringes by caudal puncture and pooled in chilled, round-bottomed flasks. Sample volumes ranged from 0.5-1.5 mL from each goldfish and 1.5-2.0 mL from each trout. Typically, blood from 4-5 goldfish or 3-4 trout was combined for each of 5 independent blood pools used in individual experiments. Sub-sampling for analytical purposes was made from each of these 5 independent pools. (The number of replicates of each measurement will be discussed in the following sections of the General Materials and Methods). Once blood was removed, the fish were then returned to a recovery tank. Fish were re-used at 2-3 month intervals to negate the effects of erythropoiesis caused by sampling.

The blood was centrifuged and the buffy coat and plasma were discarded. The red cells were washed three times in physiological

saline (prepared according to Hoar and Hickman, 1983), resuspended to a haematocrit of 20-25% and left overnight in saline at 5°C. Storage of cells overnight at low temperatures does not alter the levels of high energy phosphates (Boutilier *et al.*, 1993) and allows the washed cells to come into complete equilibrium with the saline solution. The following day the haematocrit was rechecked before the experimental procedure began. The collected blood was then distributed to humidified intermittently rotating glass tonometers supplied with an appropriate gas mixture (Wosthoff gas-mixing pumps, Bochum, Germany).

II. Analytical Measurements

II.A. Measurement of intracellular (i) and extracellular (e) pH.

pH measurements were made with a Radiometer G279/G2 glass capillary electrode (15°C) coupled to a Radiometer PHM 84 pH meter. The electrode was calibrated with Radiometer precision buffers (S1500 and S1510) prior to each sampling series. pH_e was determined on 50 µL samples of red cell suspension whereas pH_i was measured on haemolysates obtained from 500 µL of centrifuged red cell suspension according to the freeze-thaw method of Zeidler & Kim (1977).

II.B. Haematocrit and haemoglobin content.

Haematocrit (Hct) determinations were made in duplicate by centrifuging 50 μL samples of suspension in microcapillary tubes in an Autocrit centrifuge (3 min, Clay-Adams). Duplicate haemoglobin (Hb) measurements were made using the cyanomethaemoglobin method (per Sigma Bulletin no. 525) by adding 25 μL of re-suspended red cells to 3.0 mL of Drabkin's reagent (Sigma 525) and measuring the absorbance at 540 nm.

Mean cellular haemoglobin content (MCHC) was estimated according to the following formula:

$$\text{MCHC (g Hb.100 mL}^{-1}\text{ RBC)} =$$

$$[\text{Hb (g.100 mL}^{-1}\text{ suspension) . Hct (\%)}^{-1}] \text{ . } 100$$

These values are subsequently used in calculating the ratio of nucleotide triphosphates to haemoglobin (NTP:Hb). MCHC can be used to detect changes in red cell volume since absolute amounts of haemoglobin within the cells will remain constant over the course of several hours.

II.C. Red cell ion (Na^+ and K^+) and water content.

Cellular water content was determined by centrifuging 250 μL

samples of the suspension for three minutes (Fisher Microfuge) in a previously dried and weighed Eppendorf tube. After discarding the supernatant and weighing the wet cell pellet, the erythrocyte fraction was then dried to a constant mass at 80°C (48-72 h) and reweighed. The water content was then calculated using the formula:

$$\%H20=100-(100xdry\ \ weight/wet\ \ weight)$$

Intracellular ion concentrations were determined in quadruplicate by centrifuging 300 µL samples of red cell suspension for three minutes, discarding the supernatant and digesting the packed red cells in 300 µL perchloric acid (8%) prior to analysis by flame photometry (Corning 410). No correction for trapped extracellular fluid was made. In previous studies, trapped extracellular fluid has been reported as a constant (e.g. Borgese *et al.*, 1986) amounting to 2-3% of the packed red cell volume. The large relative changes observed in my studies would not be affected by omitting this.

II.D. Enzymatic analysis of nucleotide triphosphates.

Coupled NAD/NADH enzymatic assays were used to measure total nucleotide triphosphate (NTP). The absorbance was measured

on a Gilford Response narrow-beam UV-VIS spectrophotometer at 340 nm (Sigma). Samples were prepared by adding 200 µL of red cell suspension to the same volume of ice-cold perchlorioc acid (6% w/v) with 1 mM EDTA. EDTA will remove Ca^{2+} which can act to lower ATP content. The 'slurry' was immediately vortexed and centrifuged for three minutes. The supernatant was removed and added to 80 µL of a neutralizing solution containing 2 M KOH, 400 mM KCl and 400 mM Imidazole. The sample was vortexed and centrifuged once more. The resulting supernatant was transferred to a third 0.5 ml Eppendorf tube and immersed in liquid nitrogen. These samples were stored in an ultra-cold freezer (-80°C) until an analysis could be performed. Nucleotide triphosphate levels were determined in duplicate at room temperature.

III. Blood-Gas Equilibration

5 mL samples of red cell suspension were equilibrated in thermostated heparinized tonometers. These vessels rotated intermittently causing the suspension to form a thin film on the glass walls thus facilitating equilibration with humidified gases of known composition (i.e. CO_2, N_2, or air).

IV. Data Presentation and Statistical Analysis

All data are presented as the mean ± 1 standard error of the mean. The number of replicates is designated 'n' whereas the total number of independent trials is designated 'N'. A multivariate repeated measures test was used to analyze the data unless otherwise specified (Sokal and Rohlf, 1981). A fiducial limit of significance of 5% was applied to determine differences between mean values.

There are several critical assumptions which needed to be satisfied prior to statistical analysis of the data. These assumptions are:

1. the fundamental assumption of random sampling.
2. independence of errors
3. homogeneity of variance

CHAPTER ONE

A COMPARISON OF ENERGY METABOLISM AND pH REGULATION BETWEEN GOLDFISH (*CARASSIUS AURATUS*) AND RAINBOW TROUT (*ONCORHYNCHUS MYKISS*): THE EFFECTS OF ACUTE ANOXIA EXPOSURE

Introduction

Previous experiments have shown that hypoxia induces changes in cellular energy metabolism in the erythrocytes of salmonids (Ferguson and Boutilier, 1989; Tufts and Boutilier, 1991), chelonids (Sick *et al.*, 1982), and bullhead catfish (Heath, 1988). Exposure to decreases in ambient PO_2 initially caused a decline of cellular NTP concentrations since red cells incubated under anaerobic conditions are limited to glycolytic energy production. Thereafter, NTP was maintained at a new, lower, steady-state level. Although salmonids are characterized as hypoxia-sensitive, Ferguson *et al.* (1989) observed only a 20% decline in salmonid red cell NTP:Hb ratios after 90 minutes of anoxia. NTP depletion did not proceed at the expected rate; NTP would have declined to approximately 50% of normoxic values had cellular energy demands remained at aerobic levels. Ferguson *et al.* (1989) suggest that salmonid red cells may be adapted for some degree of hypoxia and/or anoxia tolerance and that metabolic arrest strategies may be functioning. Tetens and Lykkeboe (1981) also report rainbow trout red cell ATP concentrations were maintained in air with 7% and 2% oxygen. The degree to which NTP levels are reduced appears to be species-specific with hypoxia-tolerant organisms experiencing the least decline. For example, ATP concentrations in red cells of common

carp were unaffected after six hours of exposure to air with 15-20% oxygen (Nikinmaa *et al.*, 1987).

In teleost fish, deoxygenation is accompanied by an increase in red cell pH as protons are taken up by deoxyhaemoglobin. As a result, the charge on the haemoglobin molecule decreases while the anion ratio, red cell volume and intracellular pH increase (Wood and Johansen, 1972; Nikinmaa and Boutilier, 1992). In the anoxia-resistant tench, deoxygenation-induced increases of pH_i at constant pH_e (Haldane effect) are more pronounced (Jensen, 1986).

Although the remarkable hypoxia-tolerance of goldfish, *Carassius auratus*, has been well documented (Shoubridge and Hochachka, 1980, Van den Thillart *et al.*, 1983), little is known concerning their pH regulation, changes in cellular energy metabolism or intracellular ion regulation. The following experiments were undertaken to determine if hypoxia will induce changes in red cell volume, in intracellular pH and in cellular energy levels similar to those observed in the less hypoxia-tolerant trout and common carp.

Materials and Methods

Animals Rainbow trout (*Oncorhynchus mykiss*) and goldfish (*Carassius auratus*) were maintained and blood samples prepared as

described in the General Materials and Methods.

Experimental protocol In the first series of experiments, washed red cells from either the trout or the goldfish were distributed in 5 mL aliquots to each of two intermittently rotating glass tonometers. Both tonometers were supplied with a humidified mixture of 1% CO_2 in air delivered by gas mixing pumps (Wosthoff, Bochum, FRG). the suspension was equilibrated for a period of 60 minutes, after which samples were removed with gas-tight Hamilton syringes and distributed for the determination of NTP, Hb concentration, haematocrit, red cell and plasma pH, as well as cell water and erythrocyte ion concentrations. After a further 5 min, one of the tonometers was switched to a 1% CO_2/N_2 gas mixture. Washed blood cell samples were subsequently removed at 10, 30 and 60 minutes following the change to anaerobic conditions. N = 5 for both control and experimental pools.

The procedures for the analytical measurements employed in this study are described in the General Materials and Methods.

Results

Deoxygenation led to an increased erythrocyte water content in both the goldfish and trout (Fig. 1). Significant changes from control

values ($p < 0.05$) were observed in both species at 30 min and 60 min. The mean percent increase in trout RBC water content at 30 min was 3.8% and remained at this value to the end of the experiment. Goldfish mean percent increase was 4.3% at 30 minutes and 5.8% at 60 minutes, significantly greater than the trout 60 minute value.

The effect of decreased environmental PO_2 on red cell nucleotide triphosphate content of rainbow trout and goldfish erythrocytes is shown in Fig. 2. It is clear that after 60 min of deoxygenation, erythrocyte energy concentrations were unchanged in either species.

The changes in concentration of intracellular Na^+ ($[Na^+]_i$) are presented in Fig. 3A. Although $[Na^+]_i$ increased in both species, significant differences from control values were never observed ($p > 0.05$). This indicates that deoxygenation itself had no appreciable influence on intracellular sodium concentration in either trout or goldfish erythrocytes. Similarly, intracellular K^+ concentrations ($[K^+]_i$) remained constant over the course of the experiment in both species (Fig. 3B).

Deoxygenation induced a significant extracellular acidification in the trout whole blood at 10 min which remained constant to 60 min ($p > 0.05$) (Fig. 4A). The pH_e of the goldfish red cell suspension

did not change significantly. The pH_i of the deoxygenated trout erythrocytes showed a progressive alkalinization which was significant at 30 min. The increase of goldfish pH_i was only significant after 60 min of continuous hypoxic exposure (Fig. 4B).

Fig. 5 shows the relationship between pH_e and pH_i in resuspended red blood cells from trout and goldfish subjected to acute deoxygenation. pH_e decreased as pH_i increased in both species. Linear regression analysis shows there is no significant difference between the slopes of the two lines ($p > 0.05$). Thus, there is a strong correlation between pH_i and pH_e and intracellular alkalinization in response to an acute hypoxia exposure is typical of both the hypoxia-sensitive trout and hypoxia-tolerant goldfish. However, the range of the response is larger in trout RBC's - 0.1 pH_e units over 0.21 pH_i units, as opposed to 0.05 pH_e units over 0.18 pH_i units in the goldfish.

Figure 1

Changes in red cell water content of erythrocytes in aerobic (trout (o); goldfish (▽)) and anaerobic (trout (●); goldfish (▼)) environments. The cells were preincubated for 60 min in a 1% CO_2 in air gas mix at 15°C. Samples for analysis of both control and experimental pools were taken at the end of the incubation period (time '0'). After a further 5 min, one tonometer was switched to 1% CO_2 in N_2 (indicated by the arrow) and samples were taken at 10, 30 and 60 minutes following the switch to hypoxia. Anaerobiosis caused significant cell swelling in both species by 30 min (multivariate repeated measures test, $p < 0.05$). See text for further details ($N = 5$).

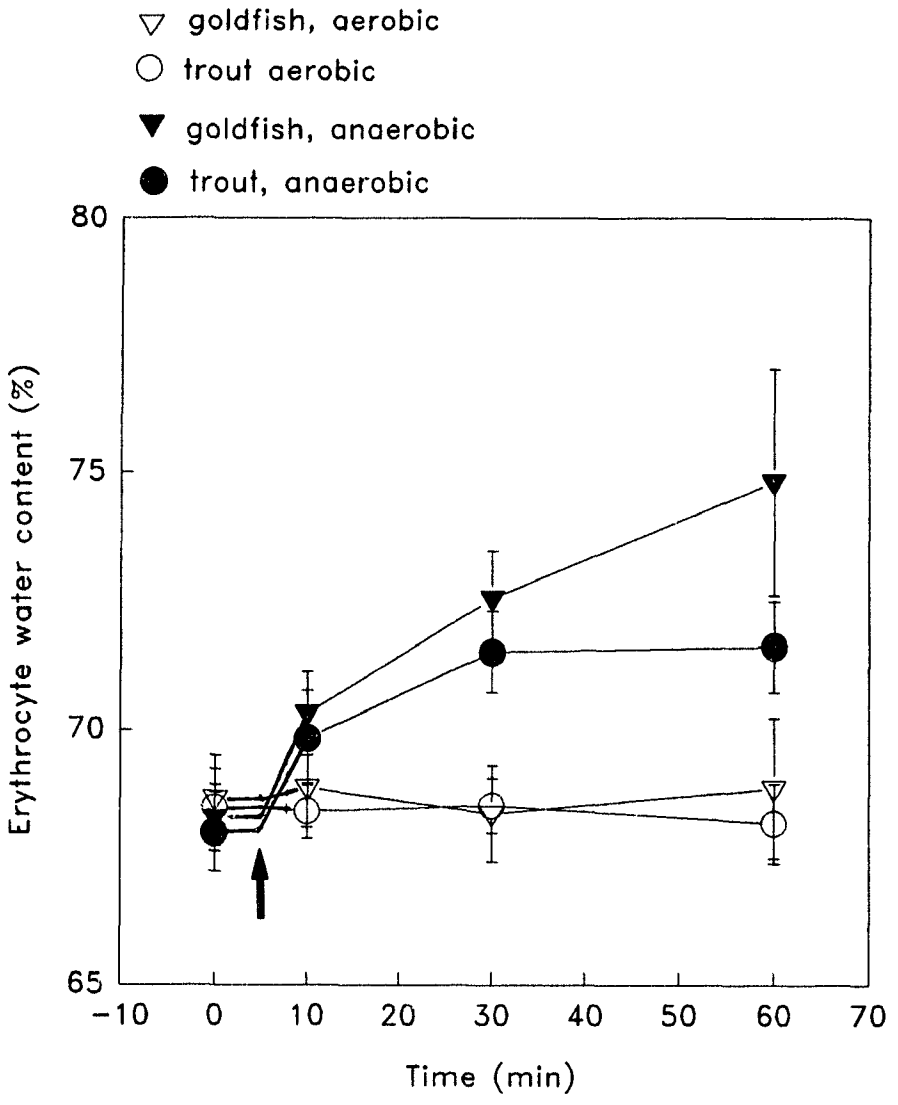
goldfish, aerobic
trout aerobic
goldfish, anaerobic
trout, anaerobic
Erythrocyte water content (%)
80
75
70
65
−10
0
10
20
30
40
50
60
70
Time (min)

FIGURE 1

Figure 2

Cellular nucleotide triphosphate (NTP) concentrations of goldfish (aerobic (▽); anaerobic (▼)) and trout (aerobic (○); anaerobic (●)) subjected to acute hypoxia. No significant changes from control values were observed in either species over the time course of the experiment (multivariate repeated measures test, $p > 0.05$). See text for further details.

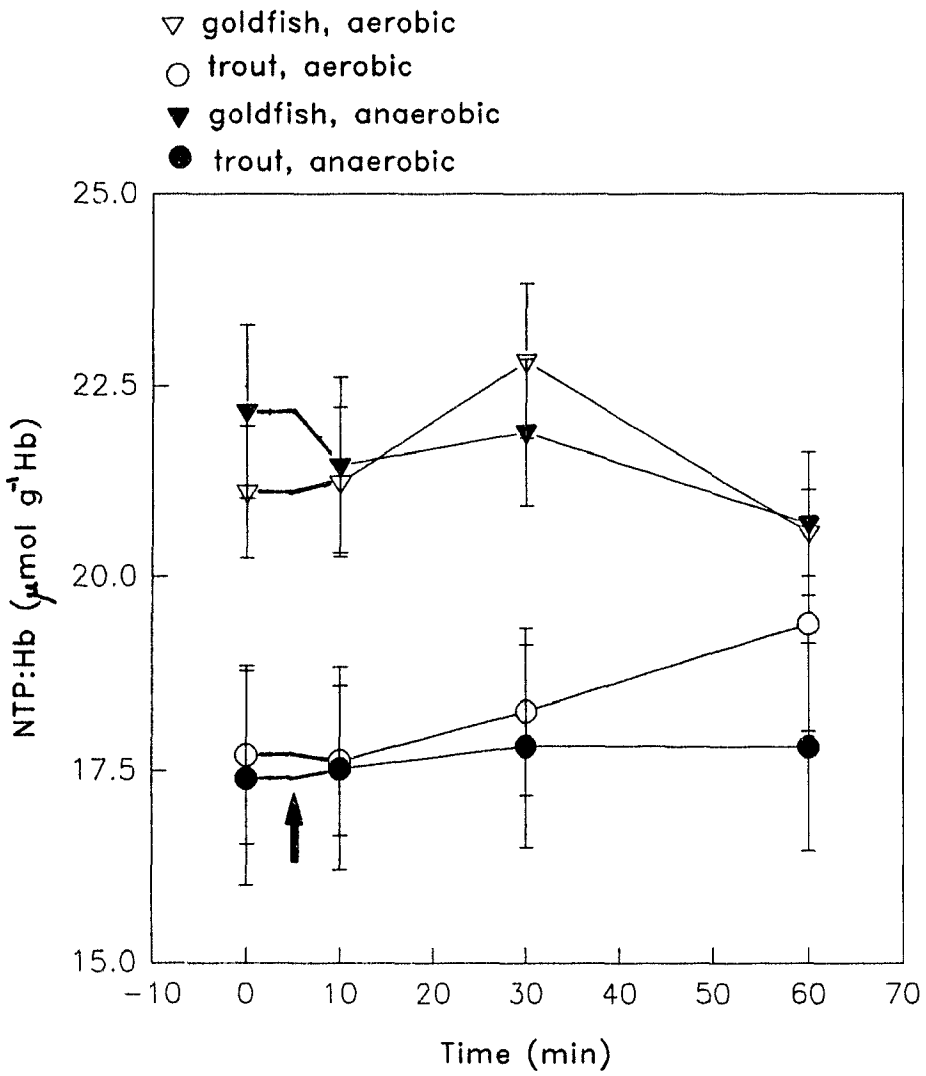
goldfish, aerobic
trout, aerobic
goldfish, anaerobic
trout, anaerobic
25.0
22.5
20.0
17.5
15.0
NTP:Hb (µmol g⁻¹Hb)
−10
0
10
20
30
40
50
60
70
Time (min)

FIGURE 2

Figure 3

Responses of intracellular sodium (A) and potassium (B) concentrations in erythrocytes of the goldfish and the trout. The red cells were incubated under aerobic conditions (goldfish (▽); trout (○)) prior to hypoxia exposure (goldfish (▼); trout (●)). See text for futher details.

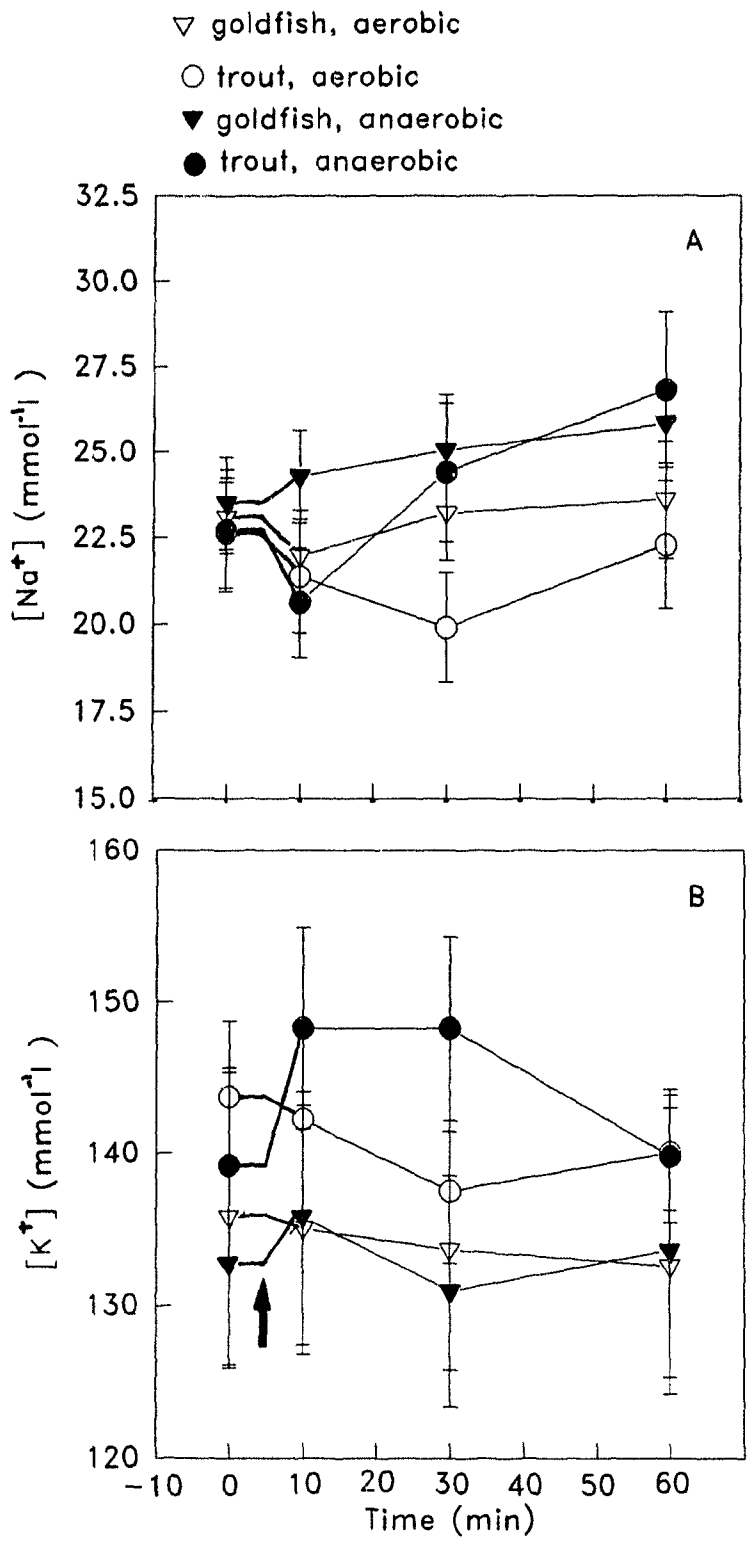
goldfish, aerobic
trout, aerobic
goldfish, anaerobic
trout, anaerobic
A
B
[Na+] (mmol-1l)
[K+] (mmol-1l)
32.5
30.0
27.5
25.0
22.5
20.0
17.5
15.0
160
150
140
130
120
-10
0
10
20
30
40
50
60
Time (min)

FIGURE 3

Figure 4

pH_e (A) and pH_i (B) in aerobic (goldfish (▽); trout (○)) and anaerobic (goldfish (▼); trout (●)) treatments. Significant differences from control values were observed in trout pH_i (multivariate measures test, $p < 0.05$) by 30 min. Goldfish pH_i was not significant until 60 min post exposure. Trout pH_e was also significantly different from the control by 30 min post exposure, however goldfish pH_e never reached statistically different levels. See text for further details.

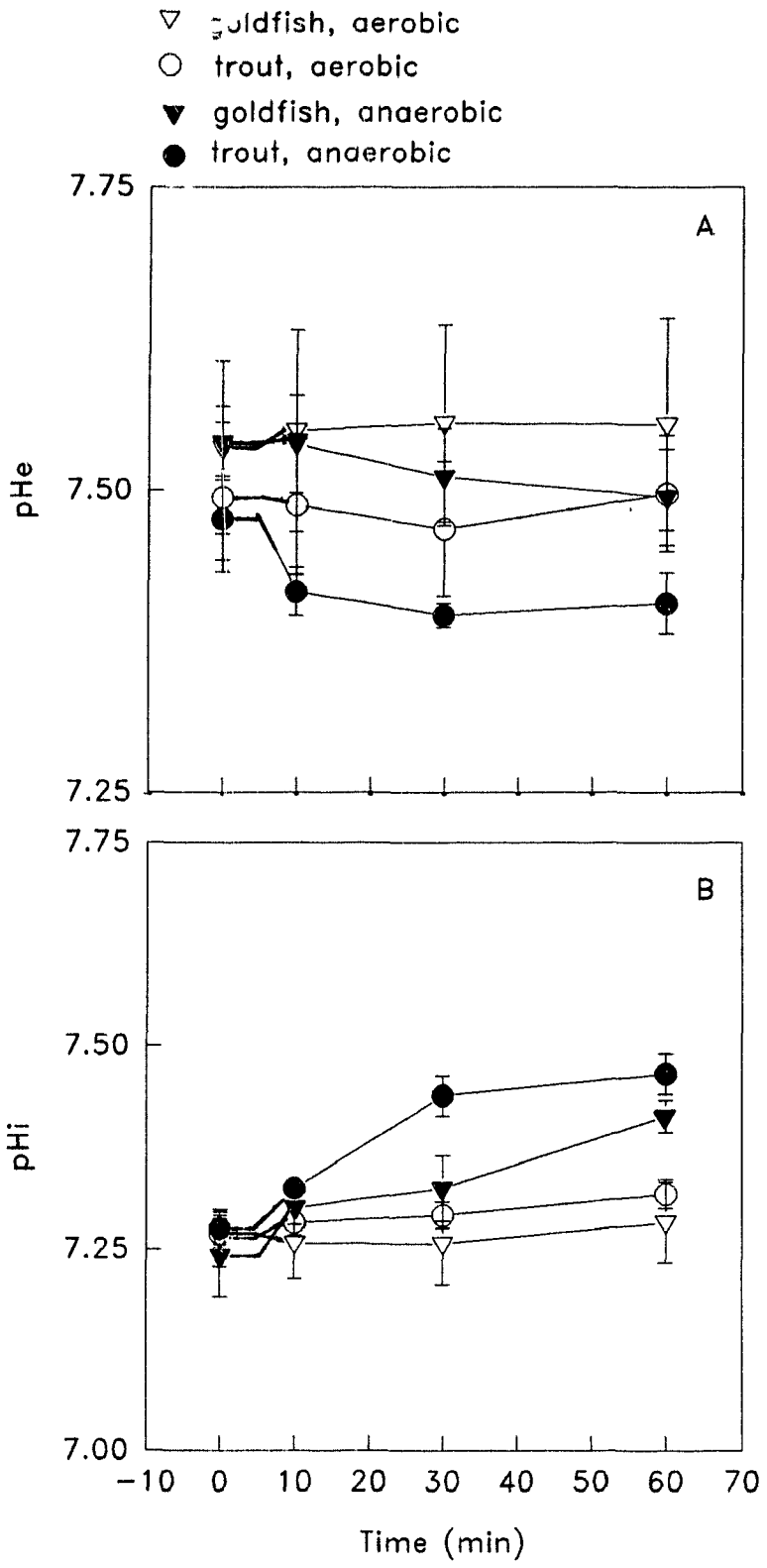
goldfish, aerobic
trout, aerobic
goldfish, anaerobic
trout, anaerobic
7.75
7.50
7.25
pHe
A
7.75
7.50
7.25
7.00
pHi
B
−10
0
10
20
30
40
50
60
70
Time (min)

FIGURE 4

Figure 5

The relationship between pH_e and pH_i in goldfish (▼) and trout (●) erythrocytes exposed to an hypoxic environment. Each data point is the mean pHe at the mean pHi for that sampling time in N = 5 independent experiments. Linear regression analysis established that there was no significant difference ($p > 0.05$) between the slopes of the two lines. See text for details.

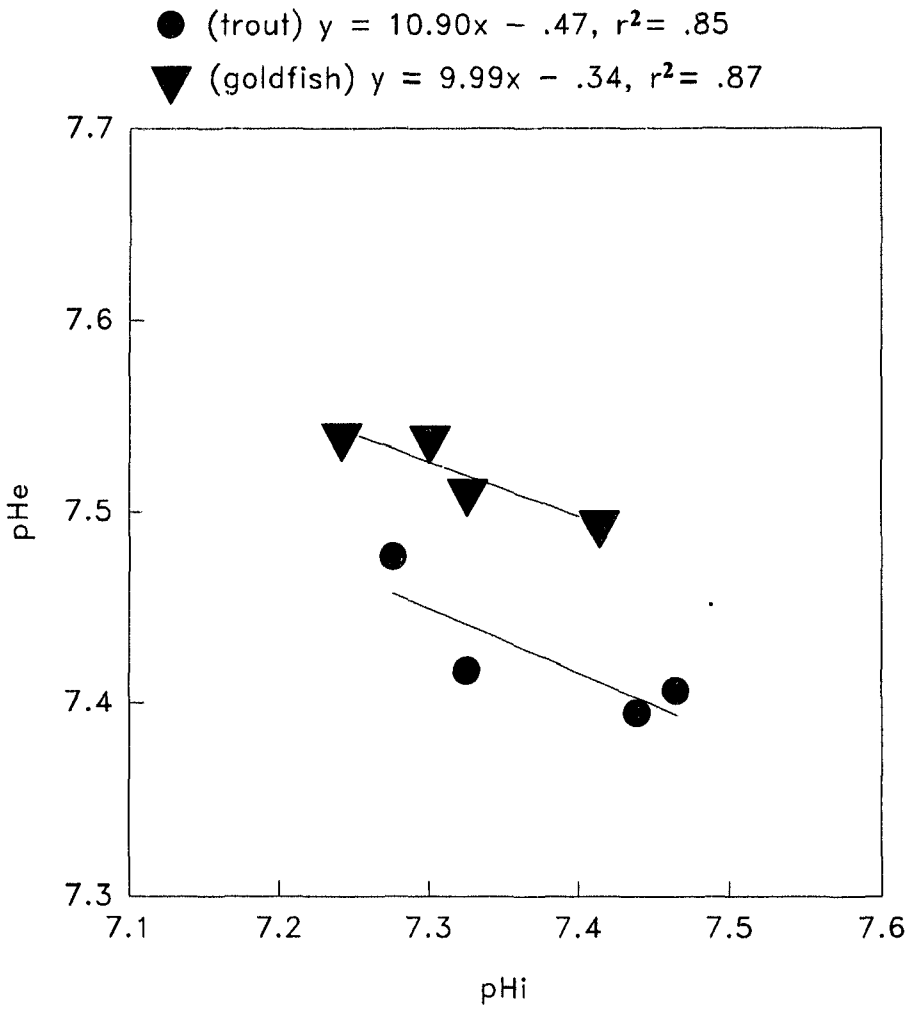
(trout) y = 10.90x − .47, r²= .85
(goldfish) y = 9.99x − .34, r²= .87
7.7
7.6
7.5
7.4
7.3
pHe
7.1
7.2
7.3
7.4
7.5
7.6
pHi

FIGURE 5

Discussion

The nucleated erythrocytes of teleosts possess the capacity for both aerobic and anaerobic energy production (Ferguson and Boutilier, 1988; Heath, 1988; Ferguson *et al.*, 1989). Ferguson and co-workers (1989) have shown that salmonid erythrocytes derive over 99% of their total energy requirements (ATP) from aerobic respiration. They estimated the NTP demand in aerobic cells to be approximately 430 nmol g^{-1} Hb min^{-1}. Since red cells incubated under anaerobic conditions are limited to NTP production *via* anaerobic glycolysis, one would expect a rapid decline of [NTP]. However, they observed NTP:Hb values in cells incubated for 90 min under anaerobic conditions which were 80% of those observed in normoxia. In this experiment, NTP:Hb levels remained stable in both trout and goldfish throughout the 60 minute time course of the experiment (Fig. 2). The discrepancy between trout response in the present study and the work of Ferguson *et al.* (1989) may lie in the time frame over which samples were taken (90 min as opposed to 60 min in this experiment). The absence of NTP depletion in the goldfish red cells may also be due to the time factor. However, this response concurs with results in other hypoxia resistant animals (Nikimaa *et al.*, 1987; Ching-Ping *et al.*, 1989; Buck and Hochachka, 1993a).

These data may suggest that both trout and goldfish have some intrinsic ability to withstand hypoxia exposure. Further support can be found in the lack of accelerated lactate production in trout RBC's (Ferguson *et al.*, 1989). Although comparable in vitro studies of substrate utilization are not available for goldfish, Storey (1987b) reports tissue-specific decreases in glycogen phosphorylase activity indicative of an overall metabolic rate depression.

Adaptations for hypoxia and/or anoxia tolerance include an ability to undergo a coordinated depression of metabolic rate and the stabilization of membrane function (Hochachka, 1986). The 'coupling' of these two processes minimizes energy expenditures for maintenance of intracellular ion homeostasis and hypoxia tolerance is achieved. In this experiment, intracellular sodium and potassium concentrations in both anaerobic trout and goldfish RBC's were never significantly different from their aerobic counterparts. This stabilization of intracellular ions reduces the dependence on the energetically expensive Na^+/K^+-ATPase for maintenance of ion concentrations.

It is generally accepted that upon deoxygenation, haemoglobin becomes a buffer and tends to accept H^+ ions. Thus, at a constant PCO_2 level, the release of oxygen from haemoglobin leads to an increase in pH_i (Fig. 4B) as more protons can be bound by the deoxy-

form and HCO_3^- concentrations rise. However, the associated decrease in extracellular pH, upon deoxygenation, is more difficult to explain. One explanation may be that the extracellular compartment has very little buffering capacity. Any anaerobically produced protons which may be removed from the cytosol will cause an extracellular acidification. Another explanation would also account for the changes in cell volume observed in red cells incubated in nitrogen. Motais *et al.* (1987) argue that molecular oxygen controls Na^+/H^+ antiporter activity. It is well known that haemoglobin binds to the cytoplasmic domain of the anion exchanger (i.e. the $Cl^-/HCO3^-$ exchanger) and deoxyhaemoglobin and oxyhaemoglobin have different affinities for these specific sites (Motais *et al.*, 1990). Though their results are not entirely conclusive, it appears that changes in the degree of oxygenation of haemoglobin influences the affinity of Hb interaction with membrane proteins, leading to an acceleration of Na^+/H^+ activity when O_2 levels become limiting. This would serve to explain the slightly elevated Na^+ levels in anoxic cells and the corresponding changes in cell volume, but would also clarify why deoxygenation of the cells leads to a decrease in extracellular pH. The type and magnitude of ionic exchange mechanisms that are set in motion by deoxygenation of erythrocytes have not been studied in any detail and there are still many unanswered questions

as to the molecular interactions that may occur between haemoglobin and transporter segments in the cytosolic domain. Nevertheless, the present results suggest that the Na^+/H^+ exchanger may be activated by the deoxy-form of haemoglobin, consistent with the findings of Motais *et al.* (1987).

Summary

1. Erythrocytes from hypoxia-sensitive and hypoxia-tolerant teleost fish respond similarly to acute hypoxia exposure.
2. Increases in cell water content reflect changes of, and sensitivity to, intracellular ions (i.e. Na^+) and pH_i. Percent increase in water content at 60 min post-exposure was 2% higher in the goldfish than in the trout.
3. Deoxygenation itself had no significant affect on intracellular $[Na^+]$ or $[K^+]$ in either the trout or the goldfish.
4. NTP:Hb concentrations decreased, but were not significant in either species within the 60 min time course of this experiment.
5. Hypoxia induced an extracellular acidification in trout erythrocytes and a marked intracellular alkalinization. Goldfish pH_i was only significantly different by 60 min post-exposure.

CHAPTER TWO - PART A

ENERGY METABOLISM, ION TRANSPORT AND pH REGULATION OF RED BLOOD CELLS FOLLOWING ADRENOCEPTOR STIMULATION UNDER NORMOXIC AND HYPOXIC CONDITIONS.

Introduction

β-adrenergic stimulation of trout erythrocytes under aerobic conditions induces changes in cellular energy metabolism, causes cell swelling and reduces the proton gradient across the cell membrane through activation of the sodium/proton exchanger (Tetens *et al.*, 1988; Ferguson and Boutilier, 1989). This adrenergic response has the advantage of enhancing haemoglobin-oxygen affinity for increased oxygen delivery to tissues (Cossins and Richardson, 1985). Catecholamine release occurs as a result of stress such as burst swimming for predator avoidance. The disadvantage of adrenoceptor stimulation is the increase of intracellular sodium due to accelerated Na^+-H^+ antiporter exchange rates. The increased $[Na^+]_i$ activates the energy consuming Na^+/K^+-ATPase and cellular NTP demands by this ion transport system also increase. However, the decline in the NTP:Hb ratio is only observed within the first five minutes after stimulation (Ferguson *et al.*, 1989). Thereafter, the ratio is maintained at a lower steady-state level and ionic homeostasis is re-established. Stimulation under aerobic conditions, therefore, does not affect the balance between energy-consumption and energy-production, nor is coordination between metabolic and membrane events lost.

Exposing stimulated red cells to anoxia, however, leads to a functional uncoupling of membrane ion transport and energy metabolism. Boutilier and Ferguson (1989) and Nikinmaa *et al.* (1987) investigated ion homeostasis and cellular energy metabolism in adrenergically stimulated anoxic red cells of the rainbow trout *(Oncorhynchus mykiss)* and the common carp *(Cyprinus carpio)* , respectively. Although the trout is hypoxia-sensitive and the carp hypoxia-tolerant, both studies indicate that ion homeostasis is not maintained under these conditions. However, the changes in carp red cell ions appear to be quantitatively much smaller than in trout. As well, ATP concentrations drop drastically in the trout while carp erythrocyte ATP was unchanged after six hours of anoxia exposure.

The adrenergic response appears to be absent in exercised tench, *Tinca tinca* (Jensen, 1987), the American eel, *Anguilla rostrata*(Hyde *et al.*, 1987) and the starry flounder, *Platichthys stellatus* (Milliga ·nd Wood, 1987) in normoxia. All three species are hypoxia toler It was suggested that the differences in adrenergic response may be due to either the differences in oxygen requirements or the activity pattern of the fish.

The purpose of this experiment was threefold. First, it must be determined whether an adrenergic response can be elicited from goldfish RBC's in either an aerobic or anaerobic environment. We know from previous experiments that hypoxic carp do respond to

adrenoceptor stimulation while other tolerant organisms do not. Investigating an organism so closely related to the carp may further reinforce the idea of species specific differences in hypoxia tolerance. The goldfish response may indeed be similar to that of the carp because of their phylogenetic relationship. However, their extreme tolerance to low environmental oxygen concentrations may place their response pattern more similar to that of the tench or flounder. Secondly, if the response is present, to compare changes in pH, NTP, and intracellular ions with those of the trout. Thirdly, again if the response is present, to determine if metabolism and membrane transport can be uncoupled under anaerobic conditions in a species which is highly tolerant of very low levels of ambient oxygen.

Materials and Methods

Animals Animal maintenance and blood sample preparation are described in the General Materials and Methods. Rainbow trout (540 - 1300 g) and common goldfish (40 - 260 g) were used in this series of experiments.

Experimental protocol Washed red blood cells from either trout or goldfish were distributed in 5 mL aliquots to each of four tonometers. Two of these tonometers were supplied with 1% CO_2 in

air and two were supplied with 1% CO_2 in N_2. Following an equilibration period, samples were removed for analysis as described in Chapter One. At this point, 100 µL of saline (sham) was introduced into one tonometer from each set while the other received 100 µL of isoproteronol (final concentration $1x10^{-5}$ mol.L^{-1}). Samples were subsequently removed at 10, 30 and 60 minutes after the additions for further analysis. N = 5 independent blood pools for both control and experimental conditions.

Results

Erythrocyte water content

Isoproterenol treatment of aerobic trout red cells induced a marked increase of intracellular water content compared with control values within the first 10 minutes, following which, no further change was apparent (Fig. 6A). Treatment of anaerobic trout erythrocytes with isoproterenol also resulted in significant increases within the first 10 minutes ($p < 0.05$). However, water contents were approximately 1% higher when compared with the aerobic cells at the same sampling time and increased over the time course of the experiment (Fig. 6B). No significant changes in red cell water content were observed as a result of sham treatment in either the

oxygenated or deoxygenated preparations ($p > 0.05$).

In contrast with the trout cells, no significant changes could be detected in goldfish erythrocyte water content following isoproterenol treatment under either the aerobic or anaerobic condition.

Erythrocyte NTP content

Trout red cell NTP:Hb ratios remained constant following sham treatment in both oxygenated and deoxygenated conditions, whereas a steady decline was observed for cells treated with isoproterenol (Fig. 7A and 7B). NTP concentrations were not significantly different in the isoproterenol-treated oxygenated cells until 60 minutes post-treatment, whereas concentrations in the deoxygenated cells were significantly different by 10 minutes ($P < 0.05$) and continued to fall throughout the experiment.

Goldfish red cell NTP:Hb ratios were affected by neither sham nor isoproterenol addition in either of the aerobic and anaerobic preparations.

Erythrocyte ion concentrations

Stimulation of goldfish erythrocytes under oxygenated and

deoxygenated conditions did not significantly change intracellular sodium concentrations ($p > 0.05$) (Fig. 8A and 8C). In contrast, aerobic trout RBC's displayed significant increases of $[Na^+]_i$ in the isoproterenol-treated pool. Deoxygenated trout red cells showed a much greater increase of $[Na^+]_i$ (approximately double the isoproterenol-treated oxygenated RBC's).

Intracellular potassium concentrations ($[K^+]_i$) remained constant in sham- and isoproterenol-treated goldfish red cells under both aerobic and anaerobic conditions (Fig. 8B and 8D). Trout red cell $[K^+]_i$ also remained at nearly control values except when treated with isoproterenol in the anaerobic preparation.

Erythrocyte and plasma pH

Isoproterenol treatment of oxygenated trout and goldfish resuspended red cell preparations caused no significant differences in extracellular pH in either species (Fig. 9B). Under deoxygenated conditions, trout red cell suspension displayed a marked extracellular acidosis by the 10 minute sampling time, whereas pH in the goldfish red cell suspension again remained stable throughout (Fig. 9D).

Intracellular pH in the goldfish was also unaffected by adrenergic stimulation under either aerobic or anaerobic conditions.

pH_i of both sham- and isoproterenol-treated cells remained close to pre-treatment values. The pH of the oxygenated and deoxygenated trout RBC's (Fig. 9A and 9C, respectively) showed a small, but progressive, alkalinization that was not significantly different ($p > 0.05$) following the addition of isoproterenol.

The transmembrane pH gradient (ΔpH) of both oxygenated and deoxygenated trout cells at 60 minutes was significantly reduced upon isoproterenol administration, while goldfish ΔpH was unaffected by isoproterenol addition under either condition (Fig. 10).

Figure 6

Changes in water content (%) of sham- (trout (○); goldfish (▽)) and isoproterenol-treated (trout (●); goldfish (▼)) erythrocytes under (A) aerobic and (B) anaerobic conditions. The arrow denotes time of addition of isoproterenol. The values are mean ± 1 standard error.

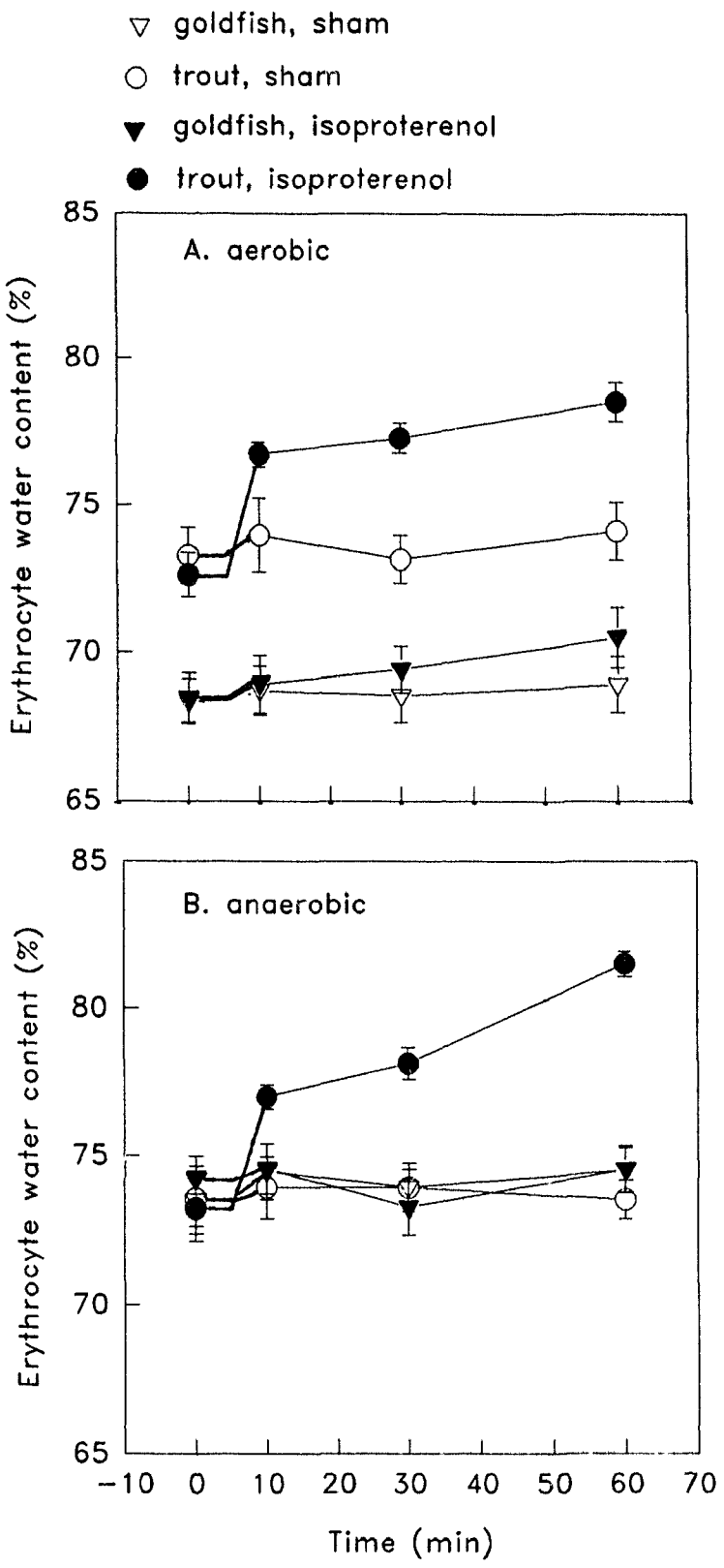
goldfish, sham
trout, sham
goldfish, isoproterenol
trout, isoproterenol
A. aerobic
B. anaerobic
Erythrocyte water content (%)
85
80
75
70
65
−10
0
10
20
30
40
50
60
70
Time (min)

FIGURE 6

Figure 7

The response of red blood cell NTP (μmol g^{-1} Hb) in the trout (sham (○) ; isoproterenol (●)) and the goldfish (sham (▽); isoproterenol (▼)) following adrenoceptor stimulation. Cells were incubated for 60 minutes under (A) aerobic and (B) anaerobic conditions prior to stimulation. See text for further details.

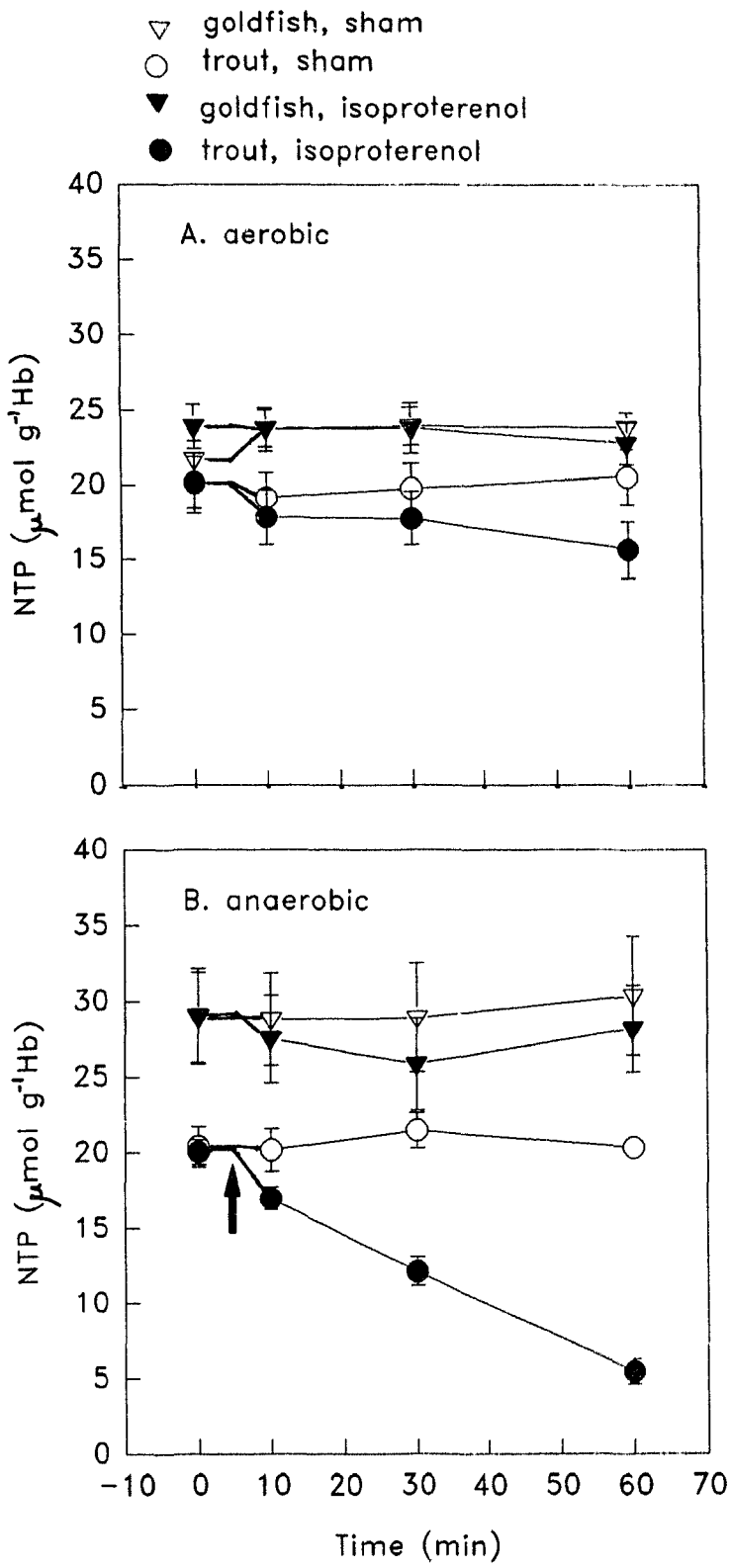
goldfish, sham
trout, sham
goldfish, isoproterenol
trout, isoproterenol
A. aerobic
B. anaerobic
NTP (μmol g⁻¹Hb)
Time (min)
40
35
30
25
20
15
10
5
0
−10
0
10
20
30
40
50
60
70

FIGURE 7

Figure 8

The effect of adrenoceptor stimulation on intracellular sodium and potassium. Red cells from rainbow trout (sham (○); isoproterenol (●)) and goldfish (sham (▽); isoproterenol (▼)) were equilibrated with either 1% CO_2/air (A and B) or 1%CO_2/ N_2 (C and D).

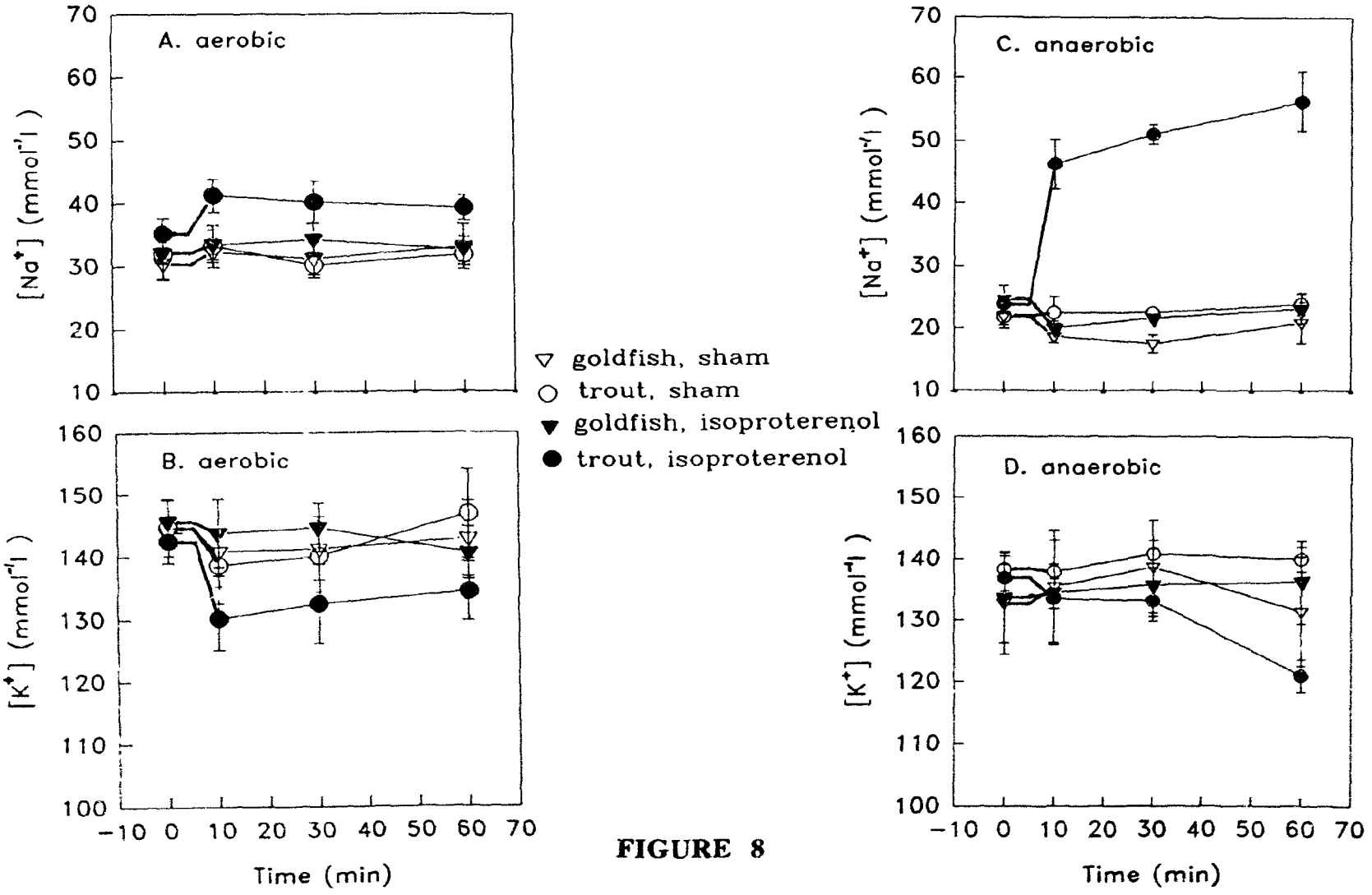
A. aerobic
B. aerobic
C. anaerobic
D. anaerobic
[Na+] (mmol·l-1)
[K+] (mmol·l-1)
Time (min)
goldfish, sham
trout, sham
goldfish, isoproterenol
trout, isoproterenol

FIGURE 8

Figure 9

pH_i (A and C) and pH_e (B and D) responses of sham (trout (○); goldfish (▽)) and isoproterenol-treated (trout (●); goldfish (▼)) red cells following incubation in either an aerobic (A and B) or anaerobic (C and D) environment. See text for further details.

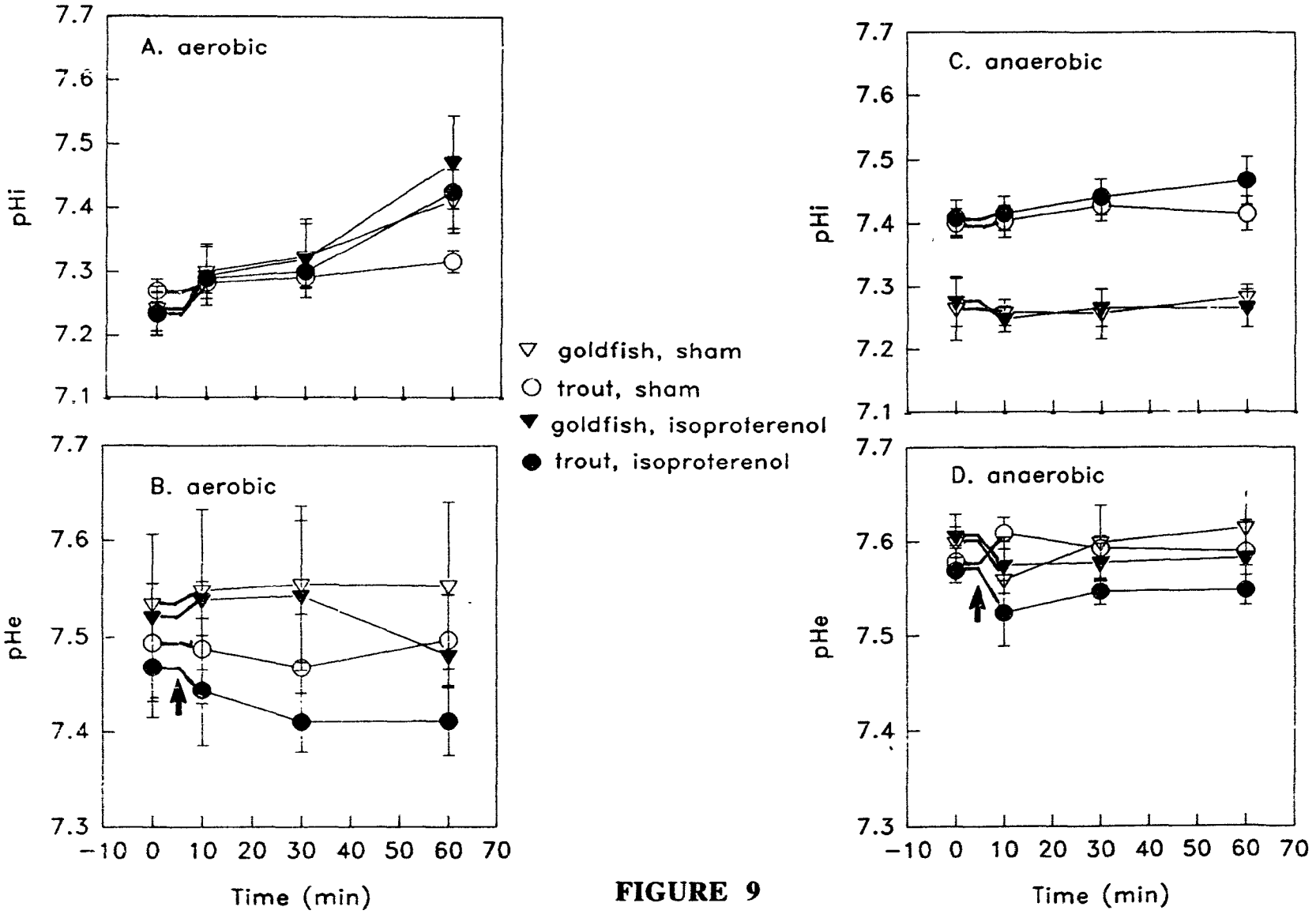
A. aerobic
B. aerobic
C. anaerobic
D. anaerobic
pHi
pHe
7.7
7.6
7.5
7.4
7.3
7.2
7.1
−10 0 10 20 30 40 50 60 70
Time (min)
goldfish, sham
trout, sham
goldfish, isoproterenol
trout, isoproterenol

FIGURE 9

Figure 10

Transmembrane pH gradients in aerobic and anaerobic trout and goldfish red blood cells at the 60 min sample time following the 100 μL addition of either a sham or isoproterenol (final concentration 1×10^{-5} mol L^{-1}).

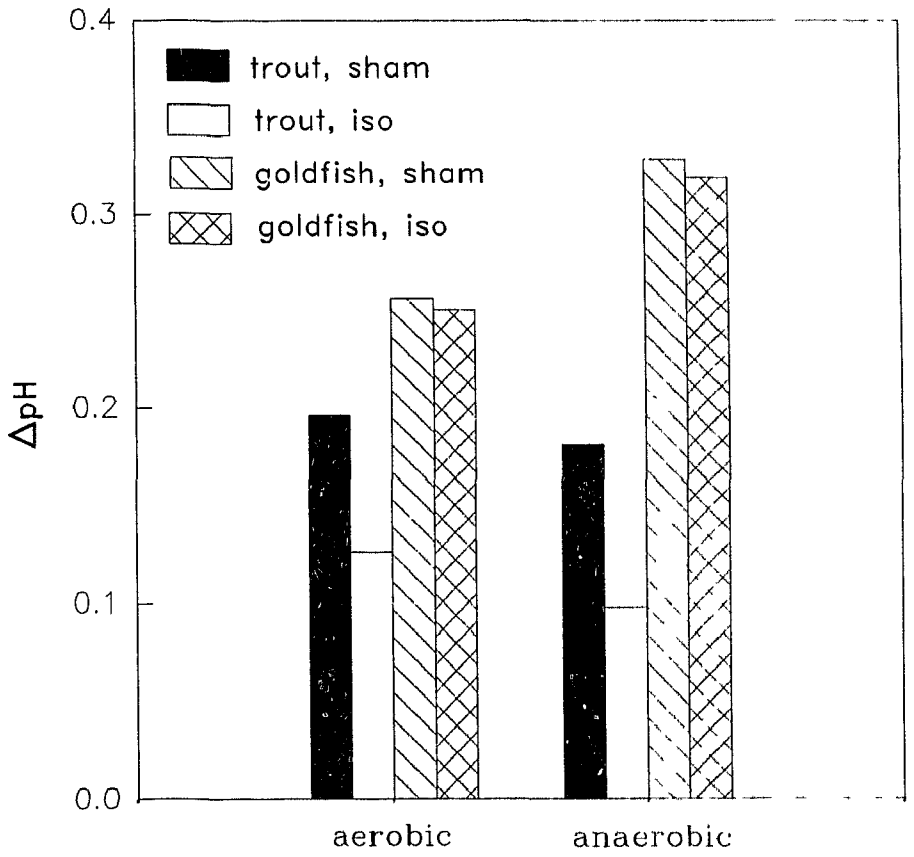
0.4
0.3
0.2
0.1
0.0
ΔpH
trout, sham
trout, iso
goldfish, sham
goldfish, iso
aerobic
anaerobic

FIGURE 10

Discussion

Fish subjected to severe hypoxia show a marked lactacidosis and an increase in circulating catecholamines (Boutilier *et al.*, 1988). The latter act as agonists towards various target tissues that serve to promote oxygen uptake. For example, stimulation of β-receptors in the cardiorespiratory system effect increased perfusion and capillary recruitment of the gill vasculature leading to an overall increase in the diffusing capacity of the branchial gas exchanger (Randall and Daxboek, 1984). The erythrocytes of certain teleosts have membrane bound β-receptors which initiate an adenylate-cyclase cAMP cascade (Ferguson and Boutilier, 1989). The increased cAMP levels activate a Na^+/H^+ antiporter which excretes protons from the cytosol, thereby alkalinizing the intracellular compartment and leading to a Bohr-induced increase in haemoglobin-oxygen affinity (Nikinmaa, 1983) and offsetting any potential complications that a Root effect might have on O_2 carrying capacity (Boutilier *et al.*, 1988). The Na^+/H^+ exchange mechanism is considered to be of vital importance to the preservation of oxygen transport and delivery in a number of hypoxia-sensitive teleosts. However, this so-called 'adrenergic pH regulation' of erythrocytes is not a universal response in fish, and there are several examples of hypoxia-tolerant species which appear

to lack such a mechanism. Indeed, one might postulate that certain fish have selected against such hypoxia-induced activation of erythrocyte ion exchange in order to 'avoid' excess ATP consumption by the Na^+/K^+ pump at a time when the energy currency is limiting.

While 'adrenergic pH regulation' may prove beneficial at times when O_2 is not limiting (e.g. burst exercise), there is no obvious long-term benefit to be gained when animals experience ionic exchange, and energy consumption by the Na^+/K^+ pump would serve only to lower cell ATP levels. In the present study, adrenergic stimulation of goldfish erythrocytes failed to elicit changes in cell water, intracellular ions, NTP:Hb ratios pH_i or pH_e in either the oxygenated or deoxygenated preparations. The absence of an adrenergic response has been demonstrated in a number of organisms, all of which are extremely hypoxia-tolerant (Hyde *et al.*, 1987; Jensen, 1987). A delayed and much reduced response has also been demonstrated with red blood cells from common carp, *Cyprinus carpio* (Salama and Nikinmaa, 1989). Salama and Nikinmaa (1988, 1989) suggest that the rapidity and magnitude of the adrenergic response is closely linked to the Root effect. To test this hypothesis, they varied the extracellular pH of the incubation medium by manipulating the PCO_2 and observed that a lower extracellular pH was required before changes in cellular water content, sodium content and pH_i could be elicited in several hypoxia-tolerant species

compared to trout and whitefish (pH 7.3 versus 7.6 at 20°C, respectively). Carp red cells did not display an adrenergic response until PCO_2 levels rose above 7.6 mmHg. Similar results were found by Orlov and Skryabin (1993). Noradrenaline was applied to carp erythrocytes to study the effect of catecholamines on volume changes and ion fluxes. Adrenergic stimulation of the Na^+/H^+ antiporter and cell swelling was initiated only when the pH of the medium was decreased far below normal physiological values.

The PCO_2 in the present experiment was held constant at approximately 7.6 mmHg which may account for the absence of any detectable adrenergic reaction in the goldfish RBC's. Since pH_e values of maximally exercised hypoxia-tolerant animals do not appear to drop much below 7.5 (Jensen *et al.*, 1983; Milligan and Wood, 1987), one can surmise that enhancement of oxygen transport *via* the adrenergic response is not a priority with tolerant species. In contrast, adrenoceptor stimulation of normoxic rainbow trout red blood cells promoted a slow but steady increase of intracellular pH which was significantly different from control values by 60 min following isoproterenol addition, presumably *via* the acceleration of the sodium-hydrogen antiporter. The resultant increase of intracellular sodium in turn contributed to increased activity of the Na^+/K^+-ATPase. Evidence that total disruption of metabolic and

membrane coupling did not occur can be observed as intracellular ions came to a new steady state level. Integration between metabolism and ion homeostasis was lost only when the trout cells were stimulated in an anoxic environment. This concurs with the present body of knowledge of the adrenergic response typical of salmonids (Cossins and Richardson, 1985; Nikinmaa and Heustis, 1984; Ferguson and Boutilier, 1989). No such "uncoupling" could be induced in the anoxic goldfish RBC's.

Niina and co-workers (1988) quantified the number of β-adrenergic binding sites on the red cell membrane of trout and common carp. Normoxic carp erythrocytes had significantly fewer β-receptors than any other cell which was studied. The number of binding sites in hypoxic carp red cells increased, yet remained lower in number than those observed in either normoxic or hypoxic trout. The rapid rate at which the β-adrenergic sites of hypoxic carp red cells increased indicated that the sites were cytosolic in origin and were recycled to the membrane during hypoxia. A structural rearrangement of the membrane would have to occur as this recycling proceeded. The triggers for this recycling are unknown.

During chronic anoxia exposure, goldfish are known to switch from lactic acid production as an anaerobic end-product to ethanol and CO_2 (Van den Thillart *et al.*, 1983). With decreased proton

production, there would be a reduced dependence on the Na^+/H^+ exchanger to regulate intracellular acid-base disturbances in RBC's. One can speculate this may also influence the number of adrenergic binding sites.

Activation of the Na^+/H^+ exchanger in both trout and carp is influenced by the activity and/or concentrations of adenylate cyclase, cAMP and protein kinase A (Boutilier and Ferguson, 1988; Salama, 1993; Orlov and Skryabin, 1993). To date, no data are available on any of these factors in goldfish. With fewer adrenergic receptors, it would be reasonable to assume there would be less demand for the second messenger, cAMP, resulting in a reduced or absent adrenergic response. Buffering capacity and Haldane effect may also be factors. These will be dealt with in the following chapter.

CHAPTER TWO - PART B

BUFFERING CAPACITY, THE ADRENERGIC RESPONSE AND THE HALDANE EFFECT: ADAPTATIONS FOR LIFE IN HYPOXIC ENVIRONMENTS

Carbon dioxide carriage and bicarbonate buffering

Carbon dioxide is transported by the blood in several different ways. Approximately 8% is transported in physical solution dissolved in the plasma, 20% is in reversible association with various blood proteins (e.g. haemoglobin), and 72% is transported as bicarbonate ions (Mason, 1983). Within the red blood cells, the enzyme carbonic anhydrase facilitates the combination of carbon dioxide and water to form carbonic acid. The carbonic acid can dissociate into hydrogen ions and bicarbonate ions, and most of the hydrogen ions associate with Hb molecules.

$$CO_2 + H_2O \underset{}{\overset{\text{carbonic anhydrase}}{\rightleftharpoons}} H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$$

The overall direction of the reaction in the above equation depends on the partial pressure of carbon dioxide, the pH and the bicarbonate ion concentration. The extent of bicarbonate formation at any given $[CO_2]$ depends inversely on the H^+ concentration. If $[H^+]$ is kept relatively low, a lot of HCO_3^- will be formed, but if it is allowed to rise to high levels, little HCO_3^- will be formed. The buffering capacity of the blood, therefore, is also a major determinant of the extent of bicarbonate formation. Buffering augments formation of HCO_3^-, and

consequently it enhances the total amount of CO_2 taken on by a solution.

The bicarbonate ions resulting from the dissociation of carbonic acid molecules diffuse out of the erythrocyte into the plasma, and in response to this movement of ions, chloride ions from the plasma enter the erythrocyte (the 'chloride shift').

Haemoglobin as a buffer

The role of vertebrate haemoglobin in the transport of oxygen and carbon dioxide has been extensively investigated (for review see Weber and Jensen, 1988; Jensen, 1991). In addition, buffering is achieved through reversible hydrogen ion exchange between the haemoglobin protein and the cytoplasm (the Haldane effect) (Weber and Jensen, 1988; Jensen. 1989). Ectothermic vertebrate Hb's exhibit a molecular and functional multiplicity which, in turn, reflects their adaptations to environmental conditions and metabolic requirements.

The modulation of Hb function is elicited *via* changes within the erythrocyte which involve acid-base balance, PCO_2 and PO_2. In ectothermic vertebrates, the major modulators of Hb are the concentrations of ATP, GTP, DPG and intracellular phosphates. Whereas ATP is the major NTP effector in the trout and dogfish shark, GTP is the major modulator in eel, carp, tench and goldfish (Weber and Jensen, 1988). These species also have a lower buffer

capacity due to a smaller number of titratable groups in both the oxy- and deoxy- conformations at physiological pH. The reduced H^+ binding at fixed protein conformation is compensated for by having a larger Haldane effect (i.e. a greater proton uptake upon deoxygenation). The largest Haldane effect and smallest buffer values are seen in carp (Weber and Jensen, 1988; Jensen, 1989). The lower buffer values are also correlated with lower content of histidine residues and α-amino groups. The free histidine at physiological pH is largely in the non-protonated state and can accept protons that are generated by anaerobically working muscles. This histidine proton buffering has been reported in the white muscle of goldfish (Van der Boon *et al.*, 1989).

The adrenergic response and the Haldane effect

Regulation of pH_i is achieved through a combination of buffering, transport of acids across the plasma membrane, and production and consumption of acids and bases by metabolism. In many teleosts, compensation of acid-base disturbances is linked to catecholamines (Boutilier and Ferguson, 1988). In general, fish exhibiting the 'adrenergic response' are those which are active and fast swimming. The response may have arisen evolutionarily as a mechanism to combat against a sudden loss in oxygen transport capacity during burst or escape activity. However, this response is

also turned on by hypoxia. In fact, it might be considered maladaptive and therefore restricts the hypoxia tolerance of the animal - a trade-off of sustained O_2 during emergencies against low hypoxia tolerance. On the other hand, hypoxia tolerant species like the goldfish, have a reduced or absent adrenergic response (see Chapter 2 Part A). These animals have a more sedentary lifestyle and inhabit waters where oxygen concentrations may be chronically low.

Table 2 summarizes data on Haldane effect, adrenergic response and buffering capacity for a number of species. Multiplicity in poikilothermic vertebrate haemoglobin is correlated with adaptations to environmental conditions and metabolic requirements. For example, intraspecific differences in non-bicarbonate buffering and Haldane effect have been recently investigated (Jensen, 1989). Jensen observed that the maximum number of H^+ taken up by carp deoxyhemoglobin at constant pH was 3.8 per tetramer, whereas it was 2.7 in rainbow trout. He suggests one reason for high Haldane effect and low buffer capacity would be to protect H^+ uptake necessary for carbon dioxide transport. It allows a large proton uptake without disturbing pH balance, thereby decreasing the formation of HCO_3^- when CO_2 is added to the tissues to compensate for slow HCO_3^-/Cl^- exchange across the plasma membrane. The eel

and tench also have lower non-bicarbonate buffer values due to the smaller number of titratable groups and exhibit a higher Haldane effect. The non-bicarbonate buffering contributed by the haemoglobin has not been determined for goldfish, and is worthy of future investigation. In goldfish, there are two major haemoglobin isomorphs at temperatures less than 10°C and three isomorphs at higher temperatures (Weber and Jensen, 1988).

The rapid swelling of hypoxia-exposed fish erythrocytes *in vitro* has been described by a number of researchers (Weber and Lykkeboe, 1978; Soivio and Nakinmaa, 1981). This response has been shown to decrease blood P_{50} indicating an increased oxygen affinity (Claireaux *et al.*, 1988). Swelling occurs as a result of increased intracellular sodium concentrations and changes in pH_i. Briefly, Na^+ uptake occurs *via* a cyclic AMP-dependent Na^+-H^+ antiport. This antiporter serves to permit the outward transport of H^+ ion utilizing the energy derived from the inward movement of sodium down the chemical gradient created by the active extrusion of Na^+ by Na^+/K^+-ATPase. Anaerobic metabolism cannot maintain energy production with energy consumption demands, therefore intracellular ATP concentrations are reduced. Intracellular sodium levels will increase as the Na^+/K^+-ATPase is no longer capable of maximum efficiency. This net uptake of Na^+ ions is accompanied by

osmotically obliged water molecules. The sodium-hydrogen antiporter is also subject to regulation by intracellular hydrogen ion concentrations (Lowenstein, 1993). A regulatory site on the cytoplasmic portion of the antiporter is highly sensitive to the $[H^+]$ of the cytoplasm. An increase in the cytosolic H^+ ions accelerates antiporter activity. This will increase the turnover rates of the Na^+-H^+ exchanger and pH_i increases.

The magnitude of this response, however, appears to be species-specific. For example, tench (Jensen, 1987) and goldfish (Chapter Two, Part A) show no response to adrenergic stimulation. Salama and Nikinmaa (1988) have also shown the β-adrenergic response in trout and carp depends on season and low oxygen tensions.

It is clear that there are an immeasureable number of adaptations for coping with hypoxia which involve a variety of different strategies for buffering capacities, adrenergic response and Haldane effect. The plasticity of these features allows animals an adaptive advantage for survival in extreme environments.

Table 2

Summary of buffering capacity, Haldane effect and adrenergic response in several teleosts. The asterisks (*) denote the degree to which each feature is expressed with the least being one (e.g. *) and increasing as the response is increased in expression (e.g. *******).

TABLE 2

Species	Buffering capacity	Haldane effect	Adrenergic response
Cyprinus carpio	**	**	*
Ictalurus punctatus	*	**	**
Tinca tinca	*	***	ABS[1]
Oncorhynchus mykiss	**	*	***
Carassius auratus	*	***	ABS[1]

[1] Response is absent

Summary

1. No adrenergic response could be elicited from either aerobic or anaerobic goldfish erythrocytes over the 60 minute time course of these experiments. There were no changes of cell water content, NTP:Hb ratios, intracellular ion concentrations or pH. In contrast, trout erythrocytes displayed an immediate response under both conditions.

2. The adrenergic response in trout RBC's appears to be linked to pH regulation and intracellular ion homeostasis. The absence of any response in goldfish RBC's may benefit the animal in that excess ATP consumption is avoided. No functional uncoupling of metabolism and membrane transport was observed under either experimental condition. Trout RBC's displayed a characteristic loss of intracellular ion homeostasis and rapidly declining NTP:Hb ratios under the anaerobic condition.

3. The Haldane effect, adrenergic response and buffering capacity appear to be linked in that hypoxia–sensitive teleosts have a smaller Haldane effect, display a rapid adrenergic response and high buffering capacity. Hypoxia–tolerant teleosts have a much larger Haldane effect, are much slower to respond to adrenergic stimulation if at all and have a much lower buffering capacity. These characteristics may be necessary for adaptations to their particular life styles and environments.

CHAPTER THREE

REDUCED ION LEAKAGE IN GOLDFISH ERYTHROCYTES: TESTING THE CHANNEL ARREST HYPOTHESIS

Introduction

The relationship between ion exchange and energy metabolism in the erythrocytes of salmonids (Tufts and Boutilier, 1991) and cyprinids (Nikinmaa *et al.*, 1987; Chapter 2, Part A) has been clearly established. Under oxygenated conditions, the red cells of rainbow trout exhibit a tight coupling between metabolic and membrane events (Ferguson and Boutilier, 1989). Disruption of this coupling, characterized by depletion of cellular energy stores (ATP) and loss of intracellular ion homeostasis, can be induced by subjecting RBC's to anoxia and administering a β-adrenergic agonist (e.g. isoproterenol). Carp red cells are subject to a similar ion disruption, however, the response is not as immediate and ATP concentrations remain close to control values. Goldfish do not appear to experience the same 'uncoupling' as energy levels and ion homeostasis are maintained (see Chapter 2, Part A).

Stabilized ion gradients in the goldfish cannot be a result of accelerated ion pumping as ATP turnover rates reflect a metabolically arrested state (Hochachka, 1986). Membrane-based differences between the goldfish and the trout may result from basic dissimilarities in the permeability of the two membranes. For example, downregulation of membrane protein channel densities as an energy conserving mechanism has been established in anoxic

turtle hepatocytes (Buck and Hochachka, 1993b) and turtle brain (Ching-Ping *et al.*, 1989).

In order to test this hypothesis, ouabain, a Na^+/K^+-ATPase inhibitor, was applied to goldfish erythrocytes. By shutting down this active transport site and measuring the change of intracellular ion concentrations, we can determine if these cells are indeed 'less leaky' under anoxic conditions, thereby contributing to overall stability and lending support to the channel arrest concept. If this hypothesis is correct, we should observe no change of intracellular Na^+ or K^+ ion concentrations. Adrenergically stimulating these same cells will further determine to what extent, if any, catecholamines contribute to the metabolic-membrane coupling in goldfish erythrocytes.

Materials and Methods

Animals. The animals used in this study were goldfish (*Carassius auratus*) obtained from a commercial supplier. The maintenance of the fish and blood removal and preparation were as described in the General Methods and Materials.

Experimental protocol. Prepared red blood cells from the goldfish were distributed to each of four tonometers in 2.5 mL

aliquots. Two tonometers were supplied with humidified 1% CO_2 in air and two with 1% CO_2 in N_2 to simulate oxygenated and deoxygenated conditions, respecitvely. All four tonometers were equilibrated for 60 minutes at 15°C with ouabain. Samples were then removed from each tonometer for measurement of haematocrit, NTP:Hb, and intracellular [Na^+] and [K^+]. At this time, 100 µL of either a saline sham or isoproterenol (final concentration 1×10^{-5} mol L^{-1}) was added. Samples were again removed for analysis 30 and 60 minutes later. N = 5 independent blood pools.

Data for control and isoproterenol groups was used from previous experiments (Chapter One and Chapter Two, Part A)

Results

The effects of ouabain and/or isoproterenol on goldfish erythrocyte water content is illustrated in Fig. 11A and 11B. By itself, ouabain incubation under oxygenated conditions caused a significant increase in cell water as compared to the control and isoproterenol groups. In the presence of ouabain plus isoproterenol, no further increase was observed. In fact, a slight decline in percent water was seen. In contrast to this, no significant increases could be detected in the isoproterenol, the ouabain or the ouabain plus

isoproterenol preparations under deoxygenated conditions.

Blocking the sodium/potassium pump with ouabain results in no significant changes of NTP:Hb ratio in either aerobic or anaerobic red cells (Fig. 14A and 14B) when compared to control and isoproterenol preparations. Likewise, the β-agonist produced no significant changes in cells previously incubated in ouabain.

Inhibition of the pump under both oxygenated and deoxygenated conditions failed to cause a decrease in the erythrocyte potassium concentration (Fig. 13A and 12A) or an increase in intracellular sodium (Fig. 13B and 12B). Addition of isoproterenol and ouabain plus isoproterenol also had no effect on ion homeostasis.

Figure 11

Water content in goldfish erythrocytes prior to (time 0) and 30 and 60 min following treatment with either isoproterenol (10^{-5} mol L^{-1}) or saline (control). C, saline control; I, isoproterenol; O, ouabain (10^{-4} mol L^{-1}); O+I, ouabain plus isoproterenol. Treatments were applied under aerobic (A) and anaerobic (B) conditions.

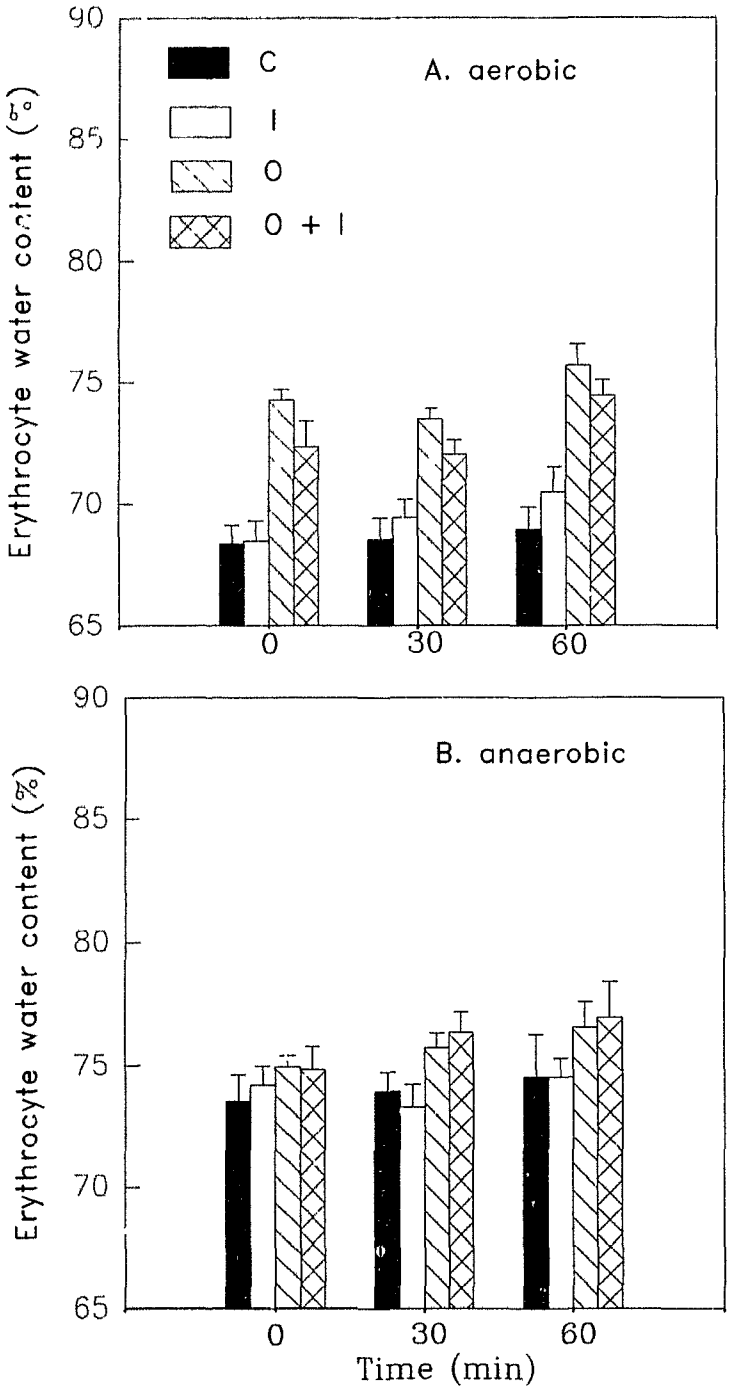
A. aerobic
B. anaerobic
C
I
O
O + I
Erythrocyte water content (%)
90
85
80
75
70
65
0
30
60
Time (min)

FIGURE 11

Figure 12

Intracellular sodium concentrations (mmol L^{-1}) of goldfish red cells equilibrated to oxygenated (A) or deoxygenated (B) conditions. Cells were treated with either a saline sham or isoproterenol (final concentration $1x10^{-5}$ mol L^{-1}). C, saline control; I, isoproterenol; O, ouabain (final concentration $1x10^{-4}$ mol L^{-1}); O+I, ouabain plus isoproterenol. All values are mean $\pm$ S.E.

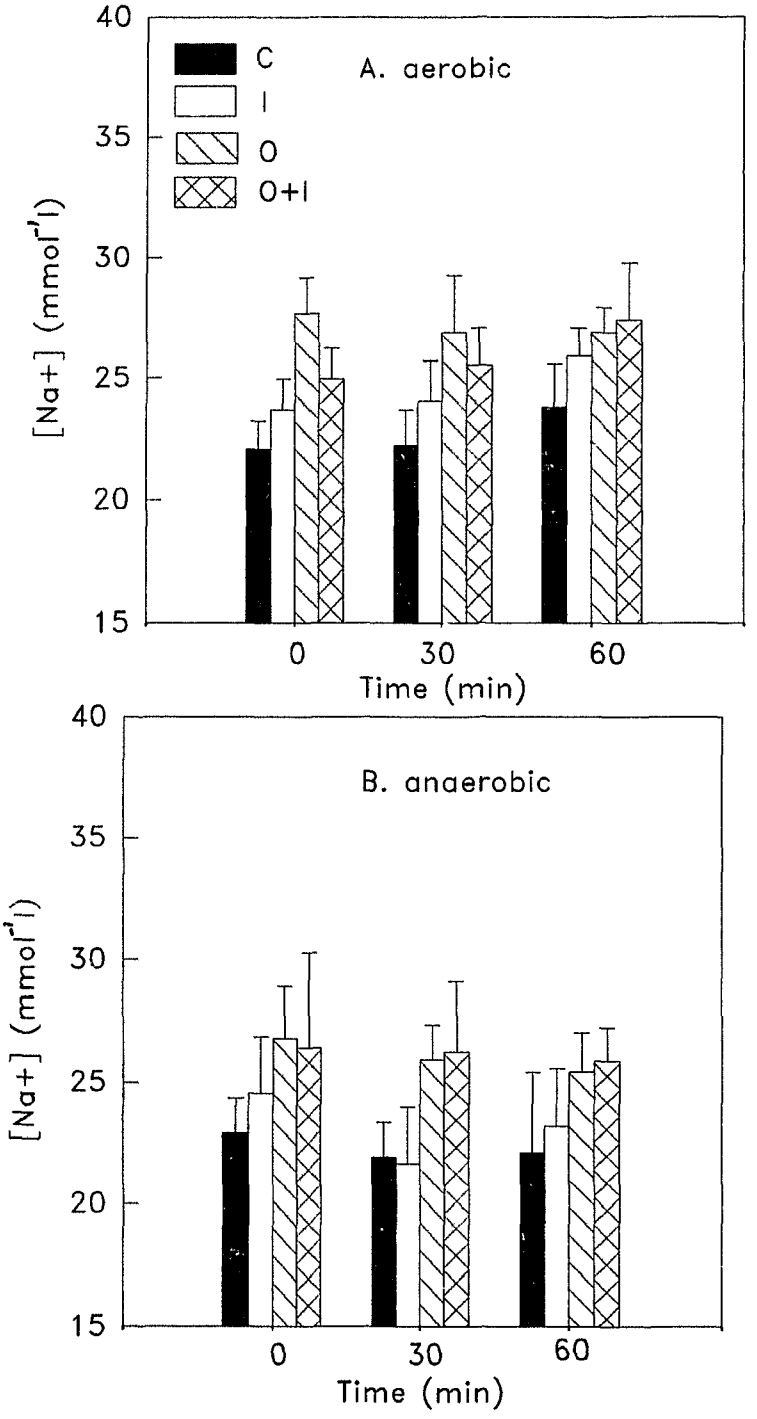
A. aerobic
C
I
O
O+I
[Na+] (mmol⁻¹l)
40
35
30
25
20
15
0
30
60
Time (min)
B. anaerobic
[Na+] (mmol⁻¹l)
40
35
30
25
20
15
0
30
60
Time (min)

FIGURE 12

Figure 13

Intracellular potassium ($[K^+_i]$) in mmol L^{-1}. Goldfish erythrocytes were equilibrated with either 1% CO_2 in air (A) or 1% CO_2 in N_2 (B). Cells were treated with C, control saline; I, isoproterenol (10^{-5} mol L^{-1}); O, ouabain (10^{-4} mol L^{-1}); O+I, ouabain plus isoproterenol.

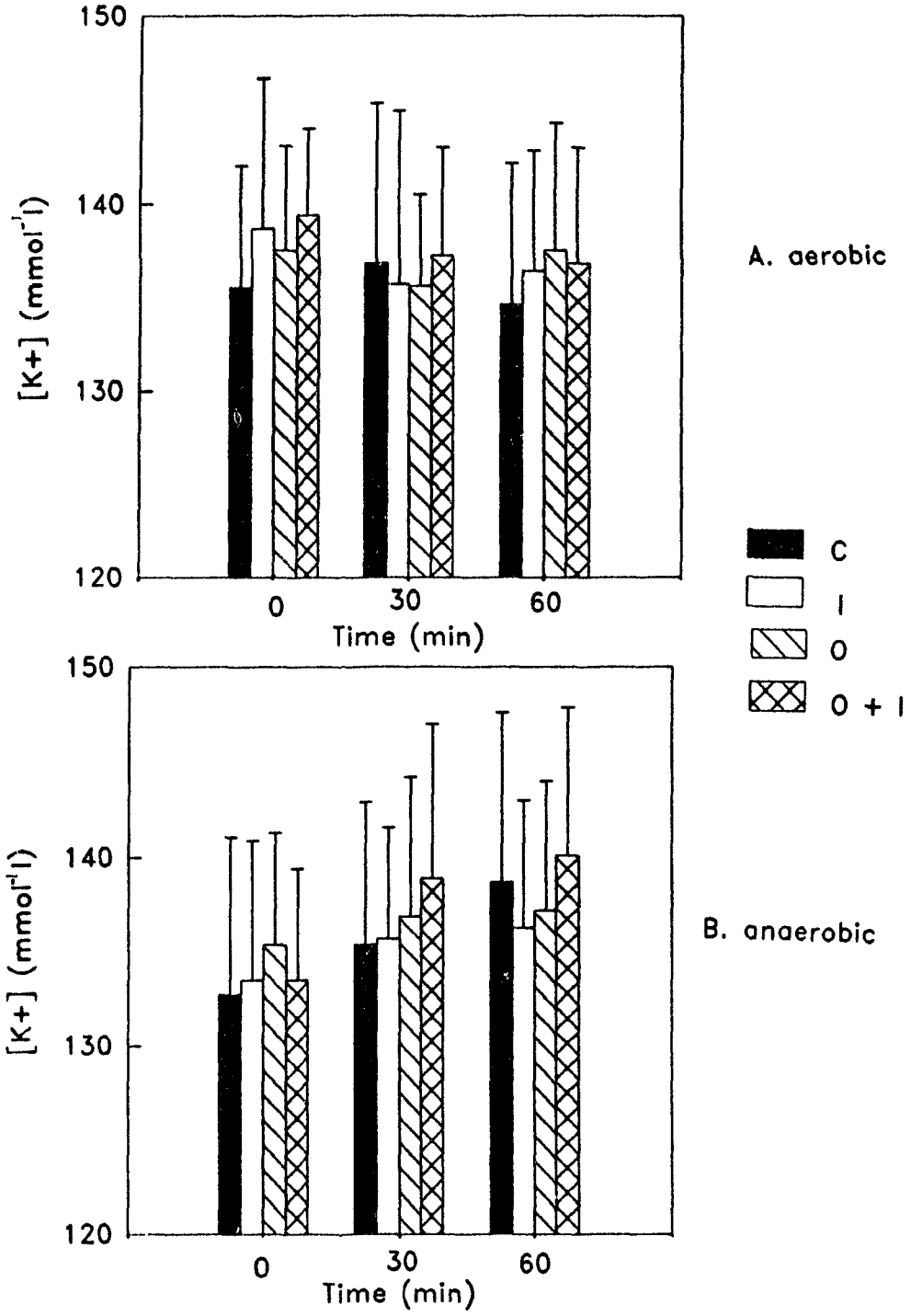
150
140
130
120
[K+] (mmol-1l)
0
30
60
Time (min)
A. aerobic
C
I
O
O + I
150
140
130
120
[K+] (mmol-1l)
0
30
60
Time (min)
B. anaerobic

FIGURE 13

Figure 14

The NTP/Hb concentrations in goldfish red blood cells prior to treatment (time 0), and 30 and 60 min following treatment with a saline sham or isoproterenol (final concentration 1×10^{-5} mol L^{-1}). Cells were subjected to either an aerobic (A) or anaerobic (B) environment. All values are mean $\pm$ S.E. (N=5 individual experiments).

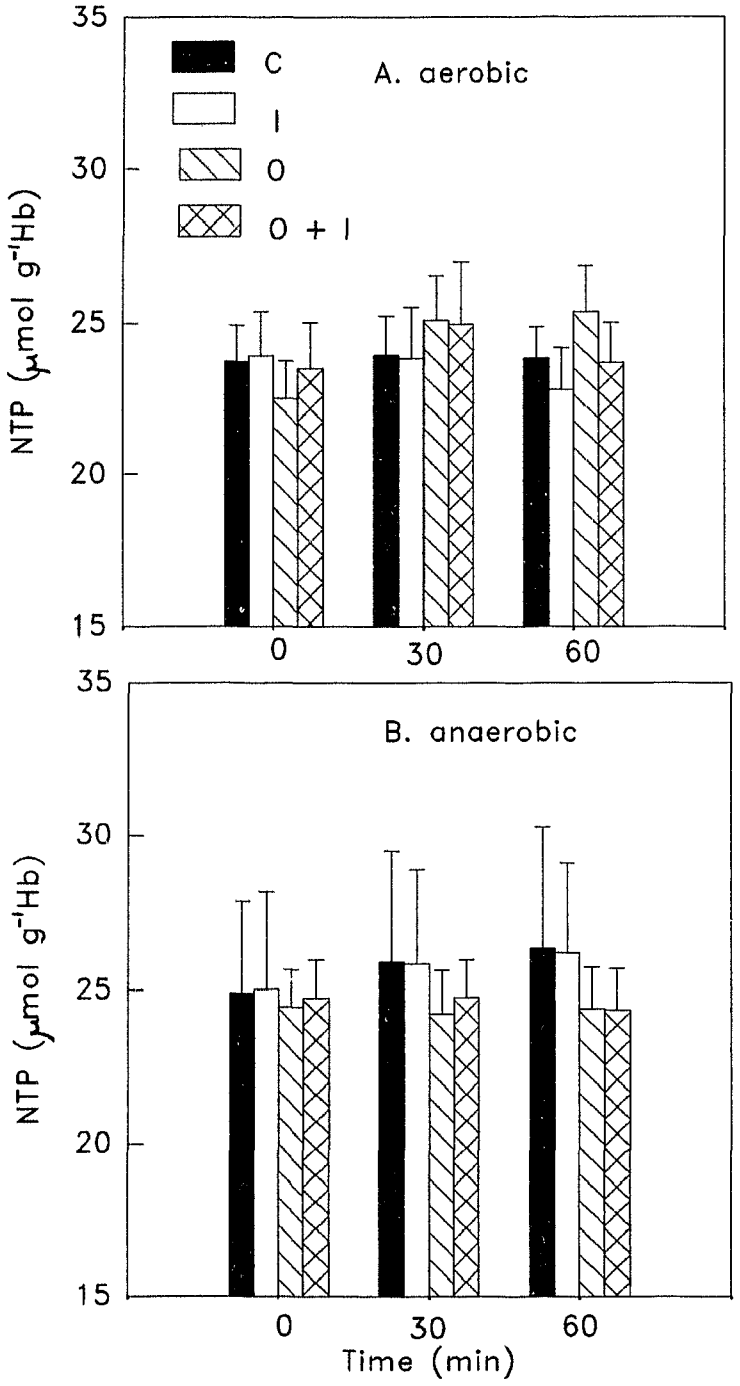
A. aerobic
C
I
O
O + I
NTP (μmol g⁻¹Hb)
35
30
25
20
15
0
30
60
B. anaerobic
NTP (μmol g⁻¹Hb)
35
30
25
20
15
0
30
60
Time (min)

FIGURE 14

Discussion

Ionic gradients across the erythrocyte membrane are sustained by continuous input of energy to the Na^+/K^+-ATPase. Intracellular sodium must be maintained at a level one-fifth that of the surrounding fluid, while intracellular potassium concentrations are almost 10 times higher than extracellular values. Following adrenoceptor stimulation, the energy demands of oxygenated salmonid red cells can be met through increased aerobic metabolism, thereby maintaining intracellular ion concentrations close to pre-stimulation levels (Ferguson and Boutilier, 1989). Adrenergically stimulating trout red blood cells under this condition results in the loss of metabolic-membrane 'coupling' as energy production and consumption cannot be balanced and intracellular sodium levels soar. In a recent study by Tufts and Boutilier (1991), ouabain inhibition of the Na^+/K^+-ATPase of oxygenated rainbow trout red cells resulted in increased concentrations of intracellular sodium and decreased concentrations of intracellular potassium (Fig. 15). Their experiment indicated that no changes were detected in NTP:Hb ratios or erythrocyte water content. In addition, isoproterenol and ouabain increased cell water content and $[Na^+]_i$ more than those seen in samples which just had isoproterenol added. Similarly, $[K^+]_i$ fell to

much lower levels in those aliquots where both ouabain and isoproterenol were added. Leakage of ions across the cell membrane is evident from these results. Although the time course for their experiment differs from this investigation, it serves to illustrate the pronounced differences in ion leakage between hypoxia-sensitive and hypoxia-tolerant organisms.

In contrast to salmonids, carp red cells have a much more modest response to adrenoceptor stimulation (Salama and Nikinmaa, 1988). Goldfish, although closely related to the common carp, display no adrenergic response, and no uncoupling of metabolic-membrane events can be detected as energy levels and intracellular ion homeostasis are maintained (see Chapter Two, Part A). Impairment of the Na^+/K^+-ATPase with ouabain, and the subsequent lack of change of intracellular ions, supports channel shut-down as a means of hypoxia protection. Concomitant with this is the reduction in energetic costs of maintaining ion gradients as observed in the maintenance of NTP:Hb which was evident in hypoxic cells (Chapter 2, Part A). Similar results have been reported by Buck and Hochachka (1993) in isolated turtle hepatocytes. Downregulation of sodium channels in isolated turtle cerebellum have also been reported (Perez-Pinzon *et al.*, 1992).

Figure 15

(A) Water content, (B) NTP/Hb concentrations, and intracellular ion concentrations (mequiv L^{-1} cell water) of (C) sodium and (D) potassium in oxygenated rainbow trout red blood cells. Erythrocytes were treated with either a saline sham or isoproterenol ($1x10^{-5}$ mol L^{-1}). C, saline control; O, ouabain ($1x10^{-4}$ mol L^{-1}); I, isoproterenol; I+O, isoproterenol plus ouabain. Data adapted from Tufts and Boutilier, 1991.

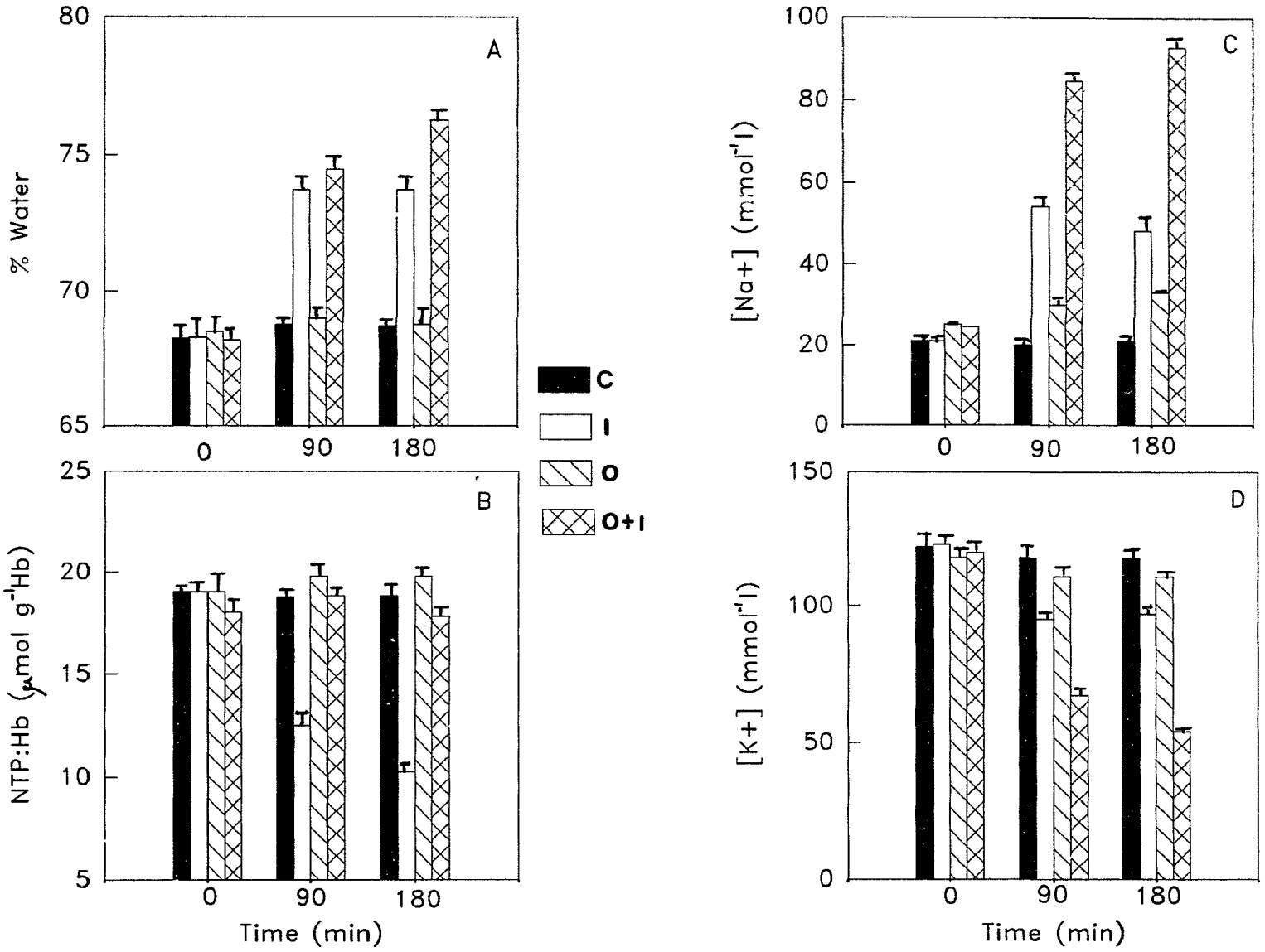
A
% Water
80
75
70
65
0
90
180
B
NTP:Hb (μmol g⁻¹Hb)
25
20
15
10
5
Time (min)
C
I
O
O+I
C
[Na+] (mmol⁻¹l)
100
80
60
40
20
0
D
[K+] (mmol⁻¹l)
150
100
50
0
Time (min)

FIGURE 15

A significant increase in erythrocyte cell water can be seen in normoxic goldfish RBC's incubated in ouabain, while no significant increase was observed in those cells equilibrated to 1% CO_2 in N_2. This lends strength to my argument for a reduction in ion channels.

Summary

1. Sodium/potassium pump blockade resulted in no significant changes in goldfish erythrocytes under either oxygenated or deoxygenated conditions. There was no response to isoproterenol under either of these conditions.
2. Intracellular sodium and potassium concentrations showed no significant change in aerobic or anaerobic goldfish RBC's incubated in ouabain. The addition of the β-agonist, isoproterenol, also resulted in no significant change over the 60 minute time course of the experiment.
3. Ouabain incubation caused a significant increase from control values in aerobic goldfish erythrocyte water content at 30 and 60 minutes. Isoproterenol treatment did ot exacerbate the disturbance, in fact, water content decreased slightly. Red blood cells equilibrated to 1% CO_2/N_2 did not display the same swelling, even when isoproterenol was added to the tonometer.
4. These results indicate that membrane permeability is low in goldfish red blood cells, supporting the notion of channel arrest in this teleost species.

GENERAL DISCUSSION

Preface

Down regulation of cellular metabolism and ion homeostasis as a hypoxia survival strategy may be present in some degree in most teleosts. Metabolic depression concomitant with reduced membrane permeability maximize survival time of an individual exposed to low oxygen concentrations. A new balanced steady state is achieved provided oxygen deprivation is neither extended nor severe. Ultimately, prolonged hypoxia is incompatible with the survival of most tissues. The remarkable hypoxia tolerance of some organisms has been documented and attributed to their enhanced ability to slow down energy turnover by maintaining membranes of low permeability (Sick *et al.*, 1982; Maginniss and Hitzig, 1987; Else and Hulbert, 1987). In effect, they make themselves 'less leaky'. The common goldfish has achieved an extremely high degree of hypoxia tolerance by fine tuning these two mechanisms.

Metabolic depression

The ability to reduce metabolic rates as a mechanism for surviving harsh environmental conditions occurs widely in nature. It has been shown to be a major factor during periods of hibernation and estivation and is characteristic of certain life stages in some organisms (Hochachka and Guppy, 1987). It allows the conservation

of substrates by lowering energy utilization demands. Decreased demand for ATP is especially crucial during extended periods of oxygen deprivation because glycolytic ATP generation would fall short of cellular requirements. An arrested metabolic state has been described in the diving turtle (Lutz *et al.*, 1985; Buck *et al.*, 1993) and the bullhead catfish (Heath, 1988). Metabolic depression in the goldfish is also evident as NTP:Hb ratios remained stable following exposure to hypoxia (Fig. 2). NTP:Hb in erythrocytes exposed to two hours of hypoxia (one hour equilibration and one hour with an added saline sham) likewise did not change, nor was there a decline even with the addition of isoproterenol (Fig. 7B).

Signalling mechanisms

How hypoxia tolerant organisms coordinate the response to low oxygen concentrations has been a question of interest. Coordinated control of metabolism via phosphorylation of proteins, by protein kinases, and changes in nucleotide concentrations is frequently regulated by hormones or other extracellular agents (Storey and Storey, 1990). Although mammalian cAMP-dependent protein kinases have been shown to mimic the effects of anoxia on the kinetic properties of phosphofructokinase and pyruvate kinase in goldfish liver *in vitro*, this enzyme has not been shown to mediate

the signal *in vivo*. Normoxic and hypoxic red blood cells of rainbow trout showed no difference in accumulation of cAMP following adrenergic stimulation with physiological concentrations of isoproterenol, noradrenaline and adrenaline (Salama, 1992). Consideration has also been given to the role of the Na^+/H^+ exchanger and pH in regulating the transition from aerobic to anaerobic states. The fact that some facultative anaerobes have developed alternate glycolytic end products to guard against acidosis (Hochachka and Somero, 1984) and that pH change during anoxia is gradual (Fig. 4), may indicate pH is not a control mechanism. The low pH that is developed as anoxia progresses may suppress other enzyme activities which, in turn, signal the reduction of metabolism (Storey and Storey, 1990).

Channel arrest

Long-term survival in a hypometabolic state requires a reduction of membrane permeability to avoid the loss of intracellular ion homeostasis. Evidence points to the regulation of membrane channel densities and activity (Hochachka, 1986; Perez-Pinzon *et al.*, 1992). This serves to both conserve energy and maintain intracellular ion concentrations. Stabilized ion gradients cannot be a result of increased ATP turnover in the goldfish erythrocyte as

NTP:Hb ratios are maintained. Goldfish erythrocytes were incubated with ouabain in order to test whether ion permeabilities could be altered during exposure to anoxic conditions (Chapter 3). Concentrations of intracellular sodium and potassium never reached significantly different levels from control values (Fig. 13A and B) whereas there were significant changes in normoxia. Whether this response is a result of changes in ion channel densities or activity has yet to be determined. In either event, these results support the channel arrest concept.

Metabolic-membrane coupling in nucleated erythrocytes

Active maintenance of transmembrane ion gradients is established by direct investments of metabolic energy. In turn, these gradients serve as an energy source for secondarily active membrane channels and exchangers. Coordination, or 'coupling', of metabolic energy production and membrane function is essential for the preservation of cell function. Adrenoceptor stimulation of salmonid red cells under normoxic conditions fails to uncouple metabolic-membrane events as ATP production/consumption and intracellular ion concentrations are maintained (Ferguson and Boutilier, 1989). Stimulating these cells under hypoxic conditions, however, leads to reduced ATP concentrations and loss of ion

homeostasis This adrenergic reaction appears to be absent in the red cells of goldfish as stimulation of the β-receptor has no effect on either NTP:Hb (Fig. 7A, 7B) or concentrations of intracellular sodium and potassium (Fig. 8A, 8B). The absence of an adrenergic reaction or a much reduced response has also been reported for a number of other teleosts (Hyde *et al.*, 1987; Jensen, 1987; Nikinmaa *et al.*, 1987). This correlates with the inverse relationship observed between buffer values and Haldane effects. Animals with a large Haldane effect and low buffer values have a reduced or absent adrenergic response - all characteristics of hypoxia-tolerant organisms.

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