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OXYGEN TRANSPORT IN FISH

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I. INTRODUCTION

The solubility of oxygen in water is only ca. 1/30, and the rate of oxygen diffusion only 1/10,000, of that in air. Because of these properties of water,

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the oxidation of organic materials and the respiration of organisms in the water and in the sediments can result in a marked depletion of oxygen from freshwater bodies. In eutrophic waters with dense green vegetation, not only hypoxia is common, but active photosynthesis by green plants can also cause oxygen supersaturation. Life in aquatic environments thus requires efficient respiratory and metabolic adjustments to changes in oxygen availability. In addition, the oxygen demand by animals varies markedly. The oxygen consumption of fishes increases with increasing temperature, often more than doubling for every 10° increase in temperature (e.g., Brett and Glass, 1973). The oxygen consumption of fish also increases markedly with exercise: at optimum temperature, the maximal oxygen consumption can be more than ten times the standard oxygen consumption (Brett and Glass, 1973).

The flux of oxygen from the environment to the sites of consumption in the tissues consists of the following steps: (1) Breathing movements continuously bring new oxygen molecules in contact with the respiratory surface. (2) Oxygen diffuses down its partial pressure gradient from the ambient water into the capillaries of respiratory organs. (3) Oxygen binds to hemoglobin in the erythrocytes. The amount of oxygen bound per unit volume of blood depends on the number of erythrocytes, the concentration of hemoglobin within the erythrocyte, the prevailing oxygen partial pressures, and the oxygen-binding properties of the hemoglobin molecule. (4) Oxygen is transported in the bloodstream from the gills to the sites of consumption. (5) In tissue capillaries, the partial pressure of oxygen decreases and, consequently, oxygen dissociates from hemoglobin. (6) Oxygen diffuses from the capillaries to the oxygen-requiring sites, mainly mitochondria, within the cells. Since the mitochondrial oxygen tension is very close to zero, the rate of oxygen diffusion per unit area in a given tissue (with a unique diffusion coefficient for oxygen) is the function of the diffusion distance between the capillaries and the mitochondria and the oxygen tension of capillary blood.

Hemoglobin plays a decisive role in the oxygen transport cascade. In the blood spaces of gill epithelium, hemoglobin has to bind oxygen effectively, and in the tissues, the release of oxygen should take place at a high partial pressure to produce a large oxygen partial pressure gradient from capillaries to mitochondria. Furthermore, both oxygen loading in gills and oxygen unloading in tissues should respond in an appropriate manner to changes in oxygen availability and oxygen demand.

The erythrocyte provides the environment in which hemoglobin functions. Thus, hemoglobin function within the animal can be modified by changing the properties of the erythrocyte. Indeed, one major advantage of having an intracellular respiratory pigment (as in vertebrates) as opposed

to an extracellular one (as in many invertebrates) is that the oxygen-binding properties of the pigment can be adjusted at the cellular level without the need to affect the properties of the whole blood volume. As a consequence, the oxygen-binding properties of blood can be adjusted very rapidly, as seen in exercised or hypoxic teleost fish (Nikinmaa *et al.*, 1984; Tetens and Christensen, 1987). The presence of an intracellular oxygen-carrying pigment also provides other advantages over the pigments that are free in solution. First, the number of erythrocytes (and oxygen-carrying capacity) can be changed by sequestration in or liberation from storage organs such as the spleen (Stevens, 1968; Nilsson and Grove, 1974; Yamamoto *et al.*, 1980). Second, the flow of erythrocytes (and oxygen) in different capillary beds and, in the case of teleost fish, in the secondary circulation, can be modulated by selective opening and closing of precapillary sphincters and/or arterio–venous anastomoses (Olson, 1984; Steffensen *et al.*, 1986).

In view of the critical role of the intraerythrocytic environment in controlling hemoglobin function, this chapter discusses how the oxygen affinity of hemoglobin is controlled by the properties of the erythrocyte. Throughout the chapter, similarities and differences in the responses of different groups of fish, from hagfish to teleosts, are presented.

II. HEMOGLOBIN FUNCTION: BASIC PRINCIPLES

The oxygen-binding properties of hemoglobin can be described using the oxygen equilibrium curve (Fig. 1), which relates the prevailing oxygen tension to the oxygen saturation of hemoglobin. Oxygen transport to tissues can be affected both by changes in the arterial oxygen tension and by changes in the oxygen affinity of hemoglobin.

There are several examples of situations in which the *oxygen tension* of postbranchial blood is regulated by fish. For example, tench (*Tinca tinca*) and carp (*Cyprinus carpio*) breathe intermittently under normoxic, resting conditions (e.g., Hughes 1981), and the arterial oxygen tension of blood is low (20–40 torr; Jensen *et al.*, 1983, 1987; Jensen, 1987). In exercised animals, the oxygen tension increases markedly (up to 100 torr) owing to hyperventilation (Jensen *et al.*, 1983; Jensen, 1987). Thus, oxygen saturation of hemoglobin can be maintained even if the oxygen equilibrium curve is shifted to the right (Fig. 1). Another mechanism of elevating the oxygen tension of postbranchial blood utilizes the fact that arterial blood is in a closed system. Any acidification of blood that decreases the hemoglobin–oxygen affinity will liberate hemoglobin-bound oxygen in solution and increase the arterial oxygen tension. Pronounced acidification-induced increases in the

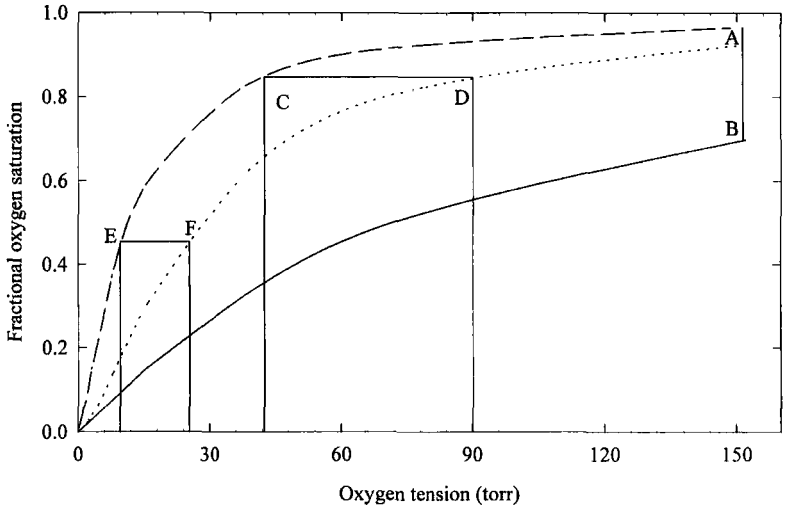

Fig. 1. Oxygen equilibrium curves for fish hemoglobin with the fractional oxygen saturation on the *y* axis and blood oxygen tension on the *x* axis. A decrease in pH or an increase in cellular NTP concentration shifts the equilibrium curve to the right, thus reducing oxygen affinity. In the case of teleost fish and lampreys, the oxygen saturation at low pH values is reduced even at atmospheric oxygen tension (A–B). A shift of the oxygen equilibrium curve to the right can be compensated for by fish that have a low resting blood oxygen tension: by ventilatory adjustments the arterial oxygen tension can be increased and arterial saturation maintained (C–D). As long as the arterial saturation can be maintained, a rightward shift of the oxygen equilibrium curve is beneficial to the fish because the same amount of oxygen is given up at a higher oxygen tension (E–F).

blood oxygen tension are observed in the blood entering the retina and swim bladder of teleost fish (Fairbanks *et al.*, 1969; Pelster and Scheid, 1992).

The oxygen affinity of hemoglobin within the erythrocyte is a function of the intrinsic oxygen affinity of hemoglobin, the sensitivity of hemoglobin–oxygen affinity to heterotrophic ligands (molecules that bind to hemoglobin at a site different from the oxygen-binding site), the concentration of hemoglobin within the cell, and the concentration of heterotrophic ligands within the erythrocyte. The heterotrophic ligands that are most important in physiological regulation of hemoglobin–oxygen affinity in fishes are protons and organic phosphates [mainly adenosine triphosphate (ATP) and guanosine triphosphate (GTP); e.g., Jensen *et al.*, 1998]. The intracellular pH and the concentrations of organic phosphates within the cell are regulated in response to environmental changes, and therefore, their effects on hemoglobin function are discussed later in this chapter. In addition, several other effectors such as lactate (Guesnon *et al.*, 1979), chloride (Fronticelli *et al.*,

1984), carbon dioxide (Jensen and Weber, 1982), and urea (Aschauer *et al.*, 1985) have been shown to affect the oxygen affinity of various vertebrate hemoglobins.

Both protons and organic phosphates bind preferentially to the deoxygenated, low-affinity conformation of hemoglobin and stabilize it (see Weber and Jensen, 1988; Jensen *et al.*, 1998). Consequently, an increase in organic phosphate concentration or a decrease in pH will decrease the hemoglobin–oxygen affinity (shift the oxygen equilibrium curve to the right). In many teleosts and in lampreys, the stabilization of the low-affinity conformation by protons is so strong that the maximal hemoglobin–oxygen saturation can be drastically reduced at atmospheric oxygen tension (Root effect; see Fig. 1 and, e.g., Jensen *et al.*, 1998). Since the reaction between hemoglobin and oxygen is exothermic under physiological conditions, the oxygen equilibrium curve is shifted to the right as temperature increases. However, there are pronounced differences between species in the temperature sensitivity of the reaction between hemoglobin and oxygen (e.g., Carey and Gibson, 1977; Weber *et al.*, 1976b; Jensen and Weber, 1982). The temperature dependence of the reaction between hemoglobin and oxygen is modulated by heterotrophic ligands. Both a decrease in pH and an increase in organic phosphate concentration diminish the effect of temperature on the oxygen affinity of hemoglobin because the liberation of these allosteric co-factors from hemoglobin, which takes place during oxygenation, is an endothermic reaction and thus reduces the overall heat of oxygenation (e.g., Jensen and Weber, 1982).

III. REGULATION OF HEMOGLOBIN FUNCTION BY CHANGES IN ERYTHROCYTIC ORGANIC PHOSPHATE CONCENTRATIONS

Organic phosphates (mainly ATP and GTP) affect the hemoglobin function of fishes (1) by direct binding preferentially to the deoxygenated form of hemoglobin and (2) by an indirect effect on intracellular pH (Wood and Johansen, 1973). Organic phosphates, however, do not bind to agnathan hemoglobins, which do not form stable tetramers (Bauer *et al.*, 1975; Nikinmaa and Weber, 1993). The binding of organic phosphates to hemoglobin is affected by complex formation with other intracellular components. ATP readily complexes with magnesium ions, and consequently, the effect of ATP on the oxygen equilibrium curve is reduced (Bunn *et al.*, 1971). In contrast, a considerable effect of GTP on the oxygen equilibrium curve of carp remains after GTP interacts with magnesium ions (Weber, 1978). The

erythrocytic ATP and GTP concentrations vary markedly between species. Erythrocyte GTP concentration varies from nearly undetectable levels as in rainbow trout (e.g., Tetens, 1987) to values clearly exceeding those of ATP as in eel (e.g., Geoghegan and Poluhowich, 1974). ATP in fish erythrocytes is mainly produced by the aerobic metabolism of the erythrocytes (Ferguson and Boutilier, 1988), using glucose, monocarboxylic acids, or glutamine as substrates (Walsh *et al.*, 1990; Sephton *et al.*, 1991; Tiihonen and Nikinmaa, 1991a). Both monocarboxylic acids and glutamine appear to be effectively transported across the erythrocyte membrane via specific carriers in all fishes from hagfish to teleosts (Tiihonen, 1995), whereas glucose transport appears to be exceedingly slow in most teleost erythrocytes (Ingermann *et al.*, 1985; Tse and Young, 1990; Tiihonen and Nikinmaa, 1991b). Despite this, the high plasma glucose concentration generates an adequate flux of glucose that supports a significant utilization of glucose as a metabolic fuel.

Very little is known about the metabolic reasons for the high GTP concentration in fish erythrocytes. The activities of key synthetic or catabolic enzymes in the guanosine phosphate pathway are not known for fish. Based on information from rabbit erythrocytes (Hershko *et al.*, 1967; Fig. 2), the

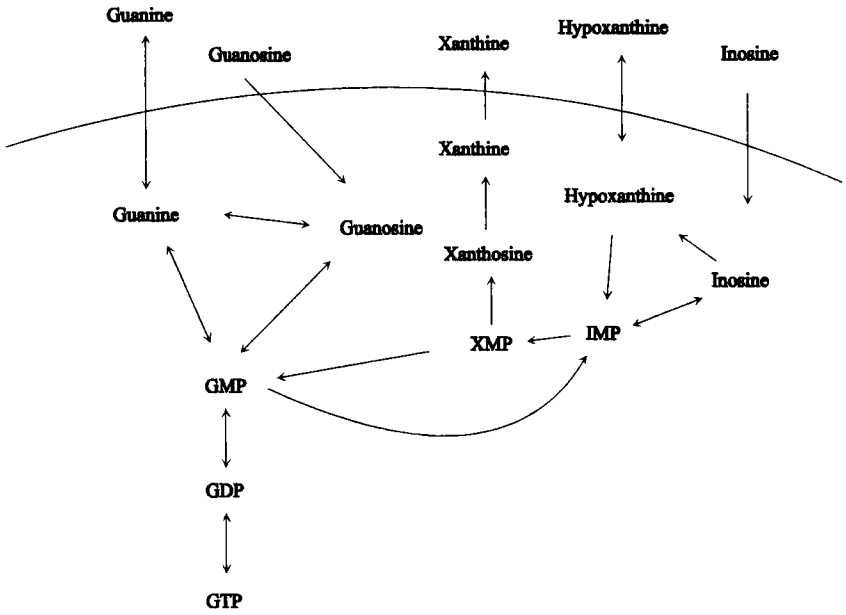

Fig. 2. Possible metabolic pathways involved in the production and breakdown of guanosine phosphates within the erythrocytes.

initial formation of guanine nucleotides may be limited by the availability of intracellular phosphoribose pyrophosphate concentration, the activity of guanine phosphoribosyl transferase (which adds the phosphoribosyl group to guanine), or the activities of inosinate dehydrogenase and guanylate synthetase (which catalyze the conversion of inosine monophosphate to guanosine monophosphate). A high concentration of intracellular guanosine nucleotides could also be caused by a low activity of the catabolic pathway, mainly the GMP reductase, which converts guanosine monophosphate to inosine monophoshate and initiates the further catabolism of purine nucleotides (Hershko *et al.*, 1967; see Nikinmaa, 1990, for discussion). Once guanosine monophosphate is formed, it appears that in species with high GTP concentration, the guanosine monophosphate kinase/adenosine monophosphate kinase activity ratio is greater than that in species with low GTP concentration. At least in the eel, *Anguilla rostrata,* which has a high GTP concentration, the GMPK/AMPK ratio is much higher than that in hagfish and dogfish with low or negligible GTP concentration (Parks *et al.*, 1973). Guanosine diphosphate is subsequently converted to GTP in a reaction catalyzed by nucleoside diphosphokinase, which has a much higher activity than GMPK (Parks *et al.*, 1973), suggesting that the step from monophosphate to diphosphate is rate limiting in the synthesis of GTP.

Despite the importance of organic phosphates in regulating hemoglobin function, the mechanisms by which the ATP and GTP levels are adjusted to respond to respiratory requirements are not known. Initially, it was suggested (Greaney and Powers, 1978) that the hypoxia-induced decrease in red cell ATP concentration would be a direct consequence of decreased oxidative phosphorylation because of inadequate oxygen supply. However, this is unlikely since the erythrocyte ATP levels *in vivo* decrease at relatively high oxygen tensions (e.g., 35–40 torr in rainbow trout, *Oncorhynchus mykiss;* Soivio *et al.*, 1980) at which more than 50% of the total hemoglobin-bound oxygen stores are still available for erythrocyte metabolism. In other cell types without such oxygen stores, e.g., liver cells, oxidative phosphorylation is not affected until oxygen tension is reduced below 5 torr (DeGroot and Noll, 1987). Furthermore, the red cell ATP concentration *in vitro* decreases only in nearly complete anoxia (Greaney and Powers, 1978; Tetens and Lykkeboe, 1981).

Tetens and Lykkeboe (1981) suggested that a humoral factor would induce the decrease in the erythrocyte ATP concentration in hypoxia. Catecholamines were prime candidates for such hormones since their concentrations increase in hypoxia (Tetens and Christensen, 1987) and since adrenaline decreases erythrocytic ATP concentration (Nikinmaa, 1983). The decrease is probably due to the activation of sodium/proton exchange

and a subsequent increase in the activity of the sodium pump, which increases ATP consumption (Tufts and Boutilier, 1991). However, a significant role for catecholamines in the regulation of erythrocytic NTP concentration is unlikely for two reasons. First, the catecholamine-induced reduction of cellular ATP concentration is entirely due to the conversion of ATP to ADP; i.e. the total cellular pool of adenylates remains constant, but in hypoxia the total pool of nucleotide phosphates is reduced (Tetens, 1987; Fig. 3). Second, catecholamines do not affect the GTP concentration in carp (Salama and Nikinmaa, 1988), although GTP concentration is preferentially decreased in hypoxia *in vivo* (Weber and Lykkeboe, 1978; Lykkeboe and Weber, 1978) and *in vitro* (Jensen and Weber, 1985). The decrease of NTP concentration can also be due to an elevated level of cortisol in the blood: an injection of cortisol caused a reduction of erythrocyte ATP concentration in red snapper, *Pagrus auratus* (Bollard *et al.*, 1993). However, it is not known whether the GTP concentration is affected, and the relationship between cortisol and ATP levels under physiological conditions (hypoxia, etc.) has not been, to our knowledge, examined. Thus, the nature of the humoral factor controlling the cellular organic phosphate levels is still unknown.

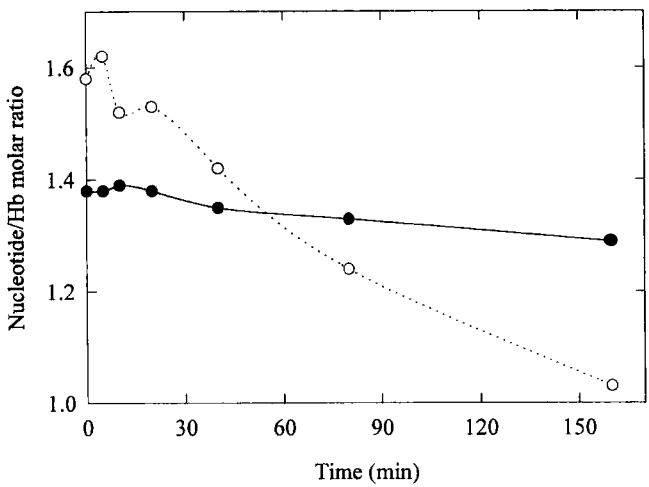

Fig. 3. The nucleotide phosphate/hemoglobin molar ratio as a function of time in rainbow trout erythrocytes subjected to hypoxia *in vivo* (oxygen tension 30 torr; empty circles) or stimulated with 10^{-5} M noradrenaline *in vitro* (filled circles). Data from Tetens (1987).

IV. EFFECTS OF CELLULAR HEMOGLOBIN CONCENTRATION AND RED CELL VOLUME ON OXYGEN TRANSPORT

The erythrocytic concentration of agnathan hemoglobins, which do not form stable tetramers, affects their affinity for oxygen. In the lamprey, *Lampetra fluviatilis,* intracellular hemoglobin concentrations higher than normal decrease the hemoglobin–oxygen affinity (Airaksinen and Nikinmaa, 1995). An increase in hemoglobin concentration within intact erythrocytes shifts the monomer–oligomer equilibrium toward oligomers. Since the oligomeric forms of hemoglobin have a much lower oxygen affinity than the monomers, the overall oxygen affinity will be decreased (for detailed mechanisms of oxygen binding in agnathan erythrocytes, see Perutz, 1990; Nikinmaa *et al.,* 1995). Such direct effects of hemoglobin concentration on oxygen affinity appear to be uncommon in other fishes, although dissociation–association reactions have been described for elasmobranch hemoglobins (Fyhn and Sullivan, 1975). The observed concentration effects—an increase in hemoglobin–oxygen affinity with a decrease in hemoglobin concentration within the cell (Soivio and Nikinmaa, 1981) and a decrease in hemoglobin–oxygen affinity with an increase in hemoglobin concentration (Jensen, 1990)—may be caused either via the concomitant changes in the intracellular pH or via alterations in the interaction between organic phosphates and hemoglobin. A reduction in the concentration of both hemoglobin and ATP/GTP will reduce the likelihood of complex formation between the two, and thus the oxygen affinity will increase (Lykkeboe and Weber, 1978).

The red cell volume may also affect oxygen transport by influencing the deformability of erythrocytes, and thus their behavior in circulation (e.g., entrance and movement in capillaries). The deformability of erythrocytes is influenced by the surface-to-volume ratio and the internal viscosity (which is a function of internal hemoglobin concentration; see Nikinmaa, 1990). If the red cell volume is reduced, there is an increase in the surface-to-volume ratio of the cells that tends to increase deformability. However, the simultaneously occurring increase in internal viscosity tends to decrease deformability. The net effect depends on which of the two factors predominates. This depends on the size of the pore through which the cell must traverse. Reinhart and Chien (1985) have shown that when human erythrocytes enter pores with a diameter close to the critical size for red cell passage, a reduced volume is beneficial (indicating a major role for surface-to-volume ratio), but when the cells traverse larger pores, swollen cells

have smaller resistance than normal-sized cells (indicating that internal viscosity dominates in determining the deformability of the cells). Thus, the observation (Hughes and Kikuchi, 1984) that swollen, hypoxic erythrocytes of rainbow trout traversed through 8-μm Nuclepore filters faster than the normal-sized erythrocytes of control fish suggests that the behavior of fish erythrocytes in this instance is mainly determined by changes in internal viscosity.

V. REGULATION OF ERYTHROCYTE VOLUME

The intracellular impermeable polyions (mainly hemoglobin and organic phosphates that are negatively charged at the physiological pH range of 6.5 to 8) and their counterions generate an osmotic pressure difference across the erythrocyte membrane. Unopposed, this would lead to continuous influx of water (and permeable solutes) into the cell, until the cell would burst. The effect of impermeable polyions is counterbalanced by the sodium pump, which actively extrudes sodium ions from the erythrocyte at the same rate as they enter the cells. Thus, sodium can be treated as a "functionally impermeable" solute and provides the osmotic force outside the cell that is required for maintenance of steady-state volume (Tosteson and Hoffman, 1960).

The volume of erythrocytes is influenced by the charge of organic phosphates and hemoglobin. If the negative charge of hemoglobin and organic phosphates decreases, either permeable cations must leave the cell or permeable anions enter the cell to maintain electroneutrality. Similarly, if the negative charge of hemoglobin increases, permeable cations must enter or permeable anions leave the cell. In teleost and elasmobranch fish, the permeability of erythrocyte membrane to small anions, chloride and bicarbonate, is much greater than the permeability to potassium or sodium (see, e.g., Nikinmaa, 1992a). Thus, changes in the charge of hemoglobin and organic phosphates are mainly compensated for by a net influx or efflux of chloride. In lampreys, on the other hand, it appears that potassium permeability may predominate (Virkki and Nikinmaa, 1995). Furthermore, in lamprey erythrocytes, association–dissociation reactions of hemoglobin occur under physiological conditions (Nikinmaa *et al.*, 1995). The volume of lamprey erythrocytes, therefore, responds to changes in the charge of hemoglobin or organic phosphates in a fashion different from that of other fish erythrocytes.

Physiologically, the major changes that affect the charge of impermeable polyions are variations of pH and oxygen tension. A decrease in extracellu-

lar pH will cause a decrease in erythrocyte pH, which will reduce the negative charge of hemoglobin (mainly the charge of histidine imidazole) and organic phosphates. Consequently, chloride will enter the cell, and the cell swells (Hladky and Rink, 1977). A reduction of oxygen tension increases erythrocyte volume since hemoglobin takes up protons upon deoxygenation, its negative charge decreases, and chloride enters the cells, (Hladky and Rink, 1977). In contrast to other fishes, the volume of lamprey erythrocytes is hardly affected by a decrease in extracellular pH or oxygen tension (e.g., Tufts and Boutilier, 1989; Nikinmaa and Mattsoff, 1992).

A. Volume Regulation after Osmotic Disturbances

A reduction in the osmolality of the medium will immediately result in cell swelling and an increase in osmolality will result in cell shrinking because the rapid transport of water tends to abolish differences in the osmotic pressure between the cell and plasma. These volume changes are sensed by the cells because of either the stretch of the cell membrane (Sackin, 1989) or the dilution/concentration of intracellular macromolecules (Colclasure and Parker, 1991, 1992). In most fish erythrocytes, transport pathways are then activated and tend to restore the original cell volume. A notable exception are the erythrocytes of myxinoids, which do not seem to respond to osmotically induced volume changes (Nikinmaa *et al.,* 1993). The recovery of erythrocyte volume after the cells are initially swollen in hypoosmotic medium is called regulatory volume decrease (RVD), and the recovery of volume after the cells are shrunk in hyperosmotic medium is called regulatory volume increase (RVI). The mechanisms of RVD and RVI are depicted in Fig. 4. The activation pathways for RVI and RVD are reciprocal and involve a complex cascade of phosphorylation–dephosphorylation reactions (Cossins and Gibson, 1997).

RVD is achieved either by the loss of organic osmolytes, mainly taurine, or by the loss of potassium and chloride from the erythrocytes, followed by osmotically obliged water. In the case of lampreys, RVD is solely due to the loss of potassium and chloride (Nikinmaa *et al.,* 1993) via electrically coupled conductive pathways (Virkki and Nikinmaa, 1995). In elasmobranch and teleost fish, the loss of taurine is an important component of RVD (Boyd *et al.,* 1977; Fugelli and Zachariassen, 1976; Fugelli and Rohrs, 1980). Erythrocytes accumulate taurine via a sodium-dependent pathway (Fugelli and Thoroed, 1986; Fincham *et al.,* 1987). When the cell volume is increased, a channel-like transport pathway is opened, and there is a pronounced efflux of taurine down its concentration gradient (Fugelli and Thoroed, 1986; Fincham *et al.,* 1987; Kirk *et al.,* 1992; Goldstein and Musch, 1994). The loss of potassium during RVD in teleost fish appears to take place

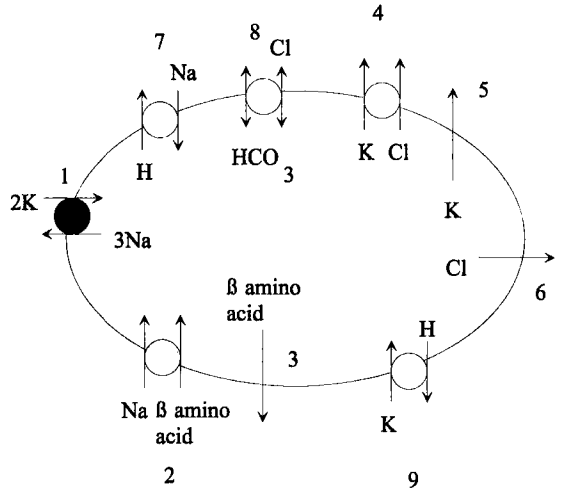

Fig. 4. Transport pathways involved in the control of erythrocyte volume in fish. (1) The sodium pump is involved in the maintenance of steady-state volume and generates the sodium and potassium gradients utilized in volume regulation. (2). A sodium-dependent transport system generates the high intraerythrocytic taurine concentration. In regulatory volume decrease (RVD), taurine is transported via a channel-like pathway (3) and potassium and chloride either via a K–Cl co-transport pathway (4) or via separate channels (5, 6). Regulatory volume increase (RVI) occurs mainly by activation of sodium/proton exchange (7) and consecutive net transport of chloride into the cell via the anion exchanger (8). A potassium/proton exchange pathway (9) has also been described for fish erythrocytes. However, its role in volume regulation is not clear. In every case, the net movement of osmotically active particles (taurine, potassium, chloride and sodium) is followed by osmotically obliged water, and hence cell volume is changed.

via two separate pathways. One of these pathways is chloride-independent (Garcia-Romeu *et al.*, 1991; Cossins *et al.*, 1994; Bursell and Kirk, 1996), and could be the channel involved in taurine transport (Bursell and Kirk, 1996). The other pathway for chloride efflux is a swelling-activated potassium/chloride co-transporter (Lauf, 1982; Garcia-Romeu *et al.*, 1991; Guizouarn *et al.*, 1993; Cossins *et al.*, 1994).

RVI involves the osmotic activation of sodium/proton exchange. The sodium/proton exchange is usually coupled to chloride/bicarbonate exchange resulting in the uptake of sodium and chloride (and osmotically obliged water). Net transport of chloride into the cell via the anion exchanger is not due to the increase in the turnover rate of the exchanger (which far exceeds that of the osmotically activated sodium/proton exchanger under all conditions). Rather, it is due to the following sequence of events. First, there is a net flux of protons from the cell via the sodium/proton exchange. The proton efflux shifts the reaction catalyzed by carbonic

anhydrase toward bicarbonate and protons. Thus, there is a transient increase in bicarbonate concentration within the cells. An increase in intracellular bicarbonate concentration reduces the concentration gradient for bicarbonate, and consequently, the anion exhanger is temporarily out of equilibrium. The equilibrium is regained by a net transport of chloride into the cell and bicarbonate out of the cell. This net transport continues as long as the proton extrusion via the sodium/proton exchange persists.

The degree to which volume recovery after osmotic shrinkage occurs differs markedly between species. In lamprey erythrocytes, although sodium/proton exchange is activated by osmotic shrinking, there is no net transport of sodium into the cell because of the lack of driving force for the exchanger (Virkki and Nikinmaa, 1994). Among teleost fish, at least the erythrocytes of the winter flounder (*Pseudopleuronectes platessa;* Cala, 1977), eel (*Anguilla anguilla;* Gallardo Romero *et al.,* 1996), and carp (*Cyprinus carpio;* Orlov and Skryabin, 1993) effectively regulate red cell volume after osmotic shrinkage. In the salmonids, brown trout (*Salmo trutta*), and rainbow trout (*Oncorhynchus mykiss*), volume activation of the sodium/proton exchange is weaker (Orlov *et al.,* 1994; Gallardo Romero *et al.,* 1996), and volume recovery is not complete.

B. Adrenergic Volume Changes

Adrenergic stimulation of rainbow trout erythrocytes leads to cell swelling (Nikinmaa, 1982). Cell swelling is caused by the activation of sodium/proton exchange (Nikinmaa and Huestis, 1984; Cossins and Richardson, 1985). The adrenergically activated sodium/proton exchange functions in parallel with anion exchange (Nikinmaa and Huestis, 1984; Borgese *et al.,* 1986), resulting in net sodium and chloride influx. Catecholamine stimulation of sodium/proton exchange has since been observed in many teleost fish (Salama and Nikinmaa, 1989; Cossins and Kilbey, 1991) with notable exceptions such as the eel (e.g., Hyde and Perry, 1990). Apart from teleost fish, it appears that there is a slight activation of the sodium/proton exchanger by catecholamines in lamprey (Gusev *et al.,* 1992; Virkki and Nikinmaa, 1994). However, the activation is too weak to cause significant changes in cell volume under physiological conditions (Tufts, 1991; Virkki and Nikinmaa, 1994). Up to the present, data for elasmobranch fish do not indicate that there are catecholamine-stimulated transport pathways affecting erythrocyte volume (see Tufts and Randall, 1989).

C. Effects of Oxygenation-Sensitive Ion Transport Pathways

There is a pronounced efflux of potassium, together with chloride, from fish erythrocytes at high oxygen tensions even in isoosmotic medium (Jen-

sen, 1990). The efflux of potassium and chloride causes a decrease in erythrocyte volume far below that expected on the basis of the oxygenation-dependent change in the charge of hemoglobin (Borgese *et al.*, 1991; Fig. 5). The transport pathway is potassium/chloride co-transport, as indicated by the nearly absolute requirement of oxygenation-induced potassium efflux on chloride (Borgese *et al.*, 1991; Nielsen *et al.*, 1992). As discussed earlier, the potassium/chloride co-transporter is also involved in RVD. Because this pathway is oxygenation-sensitive, RVD is much more effective at high than at low oxygen tension (Nielsen *et al.*, 1992; Jensen, 1995). In addition to oxygenation, treatment of cells with CO or nitrite (which oxidizes heme groups) activates the transporter (Jensen, 1990; Nielsen and Lykkeboe, 1992). These findings indicate that a heme group is involved in the activation step. The actual activation sequence is not known, although interaction between hemoglobin and band 3 protein could be involved (Jensen, 1990; Nielsen *et al.*, 1992; Jensen, 1995).

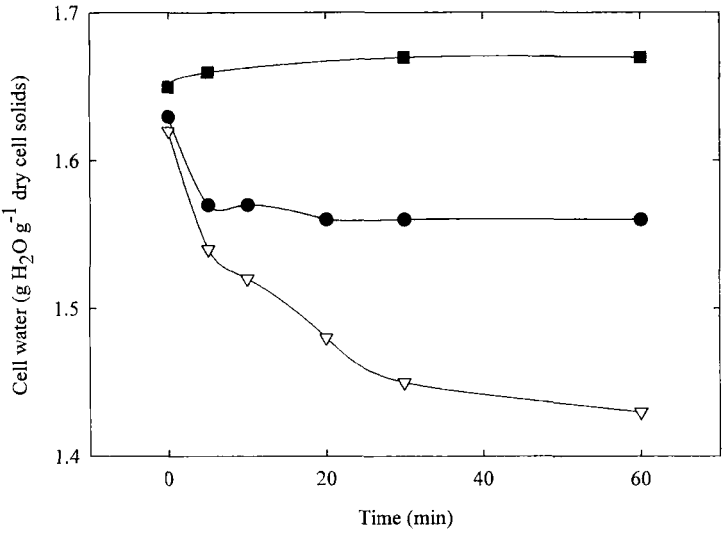

Fig. 5. Changes in erythrocyte water content of rainbow trout brought about by oxygenation as a function of time (data from Borgese *et al.*, 1991). Squares: erythrocytic water content in deoxygenated erythrocytes. Triangles: erythrocytic water content in cells oxygenated at time 0. Circles: erythrocyte water content in cells maintained in a medium in which nitrate has been substituted for chloride precluding the activation of potassium/chloride co-transport pathway and oxygenated at time 0. The difference in water content between squares and circles indicates the effect of the oxygenation-induced change in the charge of hemoglobin on cellular water content. The further decrease shown by the curve with triangles shows the effect of the oxygenation-activated potassium/chloride co-transport pathway on the cellular water content.

Since the activation of the potassium/chloride co-transporter by oxygenation occurs under isotonic conditions, it is probable that the potassium/chloride co-transport has other physiological functions in addition to osmotic volume regulation. Effects on oxygen transport are possible: a reduction of volume at high blood oxygen tensions, caused, e.g., by diurnal hyperoxia, would decrease the hemoglobin–oxygen affinity by increasing complex formation between hemoglobin and organic phosphates, thus facilitating the initial phases of oxygen delivery to tissues. Alternatively, a reduction of volume at high oxygen tensions could facilitate the entrance of erythrocytes to capillaries if the surface-to-volume ratio of the erythrocyte is the major determinant of the overall deformability of the erythrocyte.

VI. EFFECTS OF PROTONS ON HEMOGLOBIN FUNCTION

Protons affect the hemoglobin–oxygen affinity of practically all fishes. One important point to remember is that hemoglobin function must be related to intraerythrocytic pH. This point is demonstrated by data on lampreys—early reports (Bird *et al.*, 1976; Nikinmaa and Weber, 1984) gave low Bohr factors (-0.1 to -0.3). However, these values were measured as a function of extracellular pH. Later studies (Nikinmaa, 1986) showed that the erythrocyte pH of lamprey decreased only slightly with a decrease in extracellular pH (Fig. 6). Thus, when intracellular pH was used in determining the pH dependence of hemoglobin–oxygen affinity of lamprey, it was found that the hemoglobins were highly sensitive to protons, with Bohr factors above -0.6 (Ferguson *et al.*, 1992; Nikinmaa, 1993). In lampreys, the effect of protons on hemoglobin–oxygen affinity is due to the stabilization of low-affinity oligomers that dissociate to high-affinity monomers upon oxygenation (see Perutz, 1990; Nikinmaa *et al.*, 1995). In contrast to lampreys, the effect of protons on oxygen affinity of hemoglobins within the erythrocytes of myxinoids is marginal (as are the effects caused by changes in the hemoglobin concentration; see Hardisty, 1979), suggesting that, in these animals, the aggregation state of hemoglobin is hardly affected by changes in the red cell pH or volume within the physiological range.

In elasmobranch and teleost fish, protons stabilize the low-affinity conformation of the tetrameric hemoglobins. Thus, a decrease in pH moves the oxygen equilibrium curve to the right. The effect of protons (Bohr effect) varies markedly between species, and even within species, as exemplified by the fact that in rainbow trout and eel there are hemoglobin components both with very large Bohr factors and with reversed Bohr factors (oxygen affinity increases with decreasing pH) within the pH range

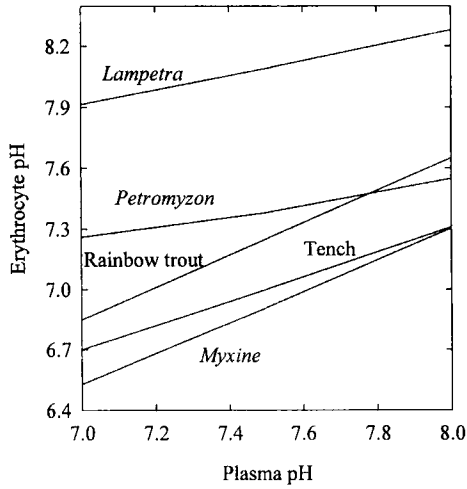

Fig. 6. Relationship between erythrocyte pH and plasma pH in a myxinoid (*Myxine glutinosa*), two lampreys (*Petromyzon marinus* and *Lampetra fluviatilis*), and two teleosts (tench, *Tinca tinca*, and rainbow trout, *Oncorhynchus mykiss*). Figure modified from Nikinmaa (1997).

7–8 in the absence of organic phosphates (Gillen and Riggs, 1973; Weber *et al.*, 1976a,b). As a broad generalization, however, it can be stated that the effect of protons on hemoglobin function is greater in teleost than in elasmobranch fish (cf. Nikinmaa, 1997).

The effect of protons on hemoglobin–oxygen affinity provides the animals with a means to rapidly respond to the conflicting demands for an oxygen equilibrium curve at the respiratory surface and within the tissues—at the gills a high oxygen affinity is required for effective oxygen loading, whereas within the tissues a low oxygen affinity ensures a high unloading partial pressure for oxygen. Proton excretion across the gills (either via ion exchanges or as carbon dioxide excretion) will result in an increase in erythrocyte pH at a constant oxygen saturation, thus increasing the hemoglobin–oxygen affinity. In the tissues, production of metabolic acids and carbon dioxide will decrease the erythrocyte pH at a constant oxygen saturation. This shifts the oxygen equilibrium curve to the right, which will increase the amount of oxygen dissociating from hemoglobin at a given oxygen tension. The greater the Bohr effect, and the decrease of pH in the capillary blood at a constant oxygen saturation, the more oxygen will be delivered. Thus, the physiological oxygen equilibrium curve will be much steeper than the oxygen equilibrium curve in the absence of proton loads from the tissues and proton excretion across the gills.

It is the blood pH at constant oxygen saturation that is important in terms of the physiological oxygen equilibrium curve. Changes in the oxygen saturation of hemoglobin also have a marked effect on blood pH, especially in teleost fish and lampreys, the hemoglobins of which often have large Haldane effects (i.e., oxygenation-dependent proton uptake and release). The deoxygenation-dependent proton uptake can be so large that, despite the carbon dioxide/metabolic acid load from the tissues, venous blood has a higher pH than arterial blood (see Milligan and Wood, 1986; Nikinmaa *et al.*, 1990; Tufts *et al.*, 1992). However, the proton uptake upon deoxygenation cannot affect hemoglobin–oxygen affinity since it is a part of the hemoglobin mechanism and the protons taken up will be released as soon as hemoglobin is again oxygenated.

VII. CONTROL OF ERYTHROCYTE pH

In the simplest case, the erythrocyte pH is determined by (1) the concentration and charge of the impermeable polyions, (2) the electrically silent one-to-one exchange of chloride for bicarbonate, and (3) the diffusion of carbon dioxide across the erythrocyte membrane and its hydration–dehydration reactions in the erythrocyte and blood plasma (see, e.g., Nikinmaa, 1990). Any extracellular proton load (decrease in pH) is transferred into the intracellular compartment via the Jacobs–Stewart cycle. Extracellular protons react with bicarbonate, forming carbon dioxide. Carbon dioxide diffuses into the erythrocyte and is hydrated to bicarbonate and protons. The protons are buffered by hemoglobin, the charge of which decreases. The bicarbonate ions formed leave the cell in exchange for chloride. Because the charge of hemoglobin decreases, the distribution ratio ($[A^-]_i/[A^-]_e$) for the permeable anions, chloride and bicarbonate, must increase in order to maintain electroneutrality. Since the proton distribution ratio is the inverse of the anion distribution ratio, the pH gradient across the erythrocyte membrane decreases.

The effect of extracellular acid loads on both the plasma and erythrocyte pH is determined largely by the buffering capacity of hemoglobin. The major determinant of the buffering capacity of hemoglobins at physiological pH values is the number of histidine residues per hemoglobin chain. The number of histidine residues per hemoglobin chain varies greatly in fishes, being small (2–6) in agnathans and teleosts and large (around 10) in elasmobranchs (see Jensen, 1989; Nikinmaa, 1990). Correspondingly, the buffering capacity of hemoglobin molecules, as measured by direct titration, is much lower in lampreys and teleosts than in elasmobranchs (Jensen, 1989; F. B. Jensen, unpublished data).

Oxygenation of hemoglobin also affects the erythrocyte pH. Hemoglobin binds protons upon deoxygenation, its charge decreases, and the anion ratio and intracellular pH increase. Conversely, protons are released upon oxygenation, whereby the anion ratio and intracellular pH decrease. The Haldane effect is very large in lamprey and many teleost fish—the intraerythrocytic pH at a constant extracellular pH increases up to 0.3–0.4 unit in tench and in the lampreys *Petromyzon marinus* and *Lampetra fluviatilis* when hemoglobin is deoxygenated (Jensen, 1986, 1989; Nikinmaa and Mattsoff, 1992; Ferguson *et al.*, 1992). In contrast, the Haldane effect of elasmobranch hemoglobins is very small—when determined by direct titration, the maximal proton uptake of carp hemoglobin upon deoxygenation is 0.95 proton per hemoglobin chain, but that of dogfish hemoglobin only 0.19 proton per chain (Jensen, 1989).

A. Agnathans

The control of pH in hagfish erythrocytes is influenced by the fact that the traditional anion exchange pathway is absent (Ellory *et al.*, 1987). Thus, the buffering of extracellular fixed acid loads by hemoglobin is slowed down. However, although the cells lack DIDS-sensitive chloride and bicarbonate transport, some equilibration of bicarbonate between plasma and erythrocytes occurs (Tufts and Boutilier, 1990a). One possible route for bicarbonate is the sodium-dependent carboxylic acid transporter of hagfish erythrocytes (Tiihonen, 1995).

Because the equilibration of anions across the erythrocyte membrane of hagfish is slow, even relatively slow secondarily active proton transport could influence erythrocyte pH. However, there is no indication that either pH or volume disturbances would activate ion transport pathways in the red blood cell membrane (Nikinmaa *et al.*, 1993). Thus, the intraerythrocytic pH of hagfish at the physiological pH range is much lower than the extracellular pH (Tufts and Boutilier, 1990a; Fig. 6).

Lamprey erythrocytes also lack a functional anion exchange, although a protein immunologically related to the anion exchanger has been described (Kay *et al.*, 1995; Cameron *et al.*, 1996). The erythrocyte membrane of lampreys thus has a very low permeability to bicarbonate and acid equivalents (Nikinmaa and Railo, 1987; Tufts and Boutilier, 1989). Lamprey erythrocytes are able to maintain a high intracellular pH (Nikinmaa, 1986; Fig. 6) by secondarily active sodium/proton exchange. The sodium/proton exchange is markedly activated by acidification (Virkki and Nikinmaa, 1994).

Since the intrinsic hemoglobin–oxygen affinity of lampreys is low and the Bohr effect large, the high erythrocyte pH is required to achieve effective oxygen loading in gills (Nikinmaa *et al.*, 1995). If the intracellular pH

values were similar to those of rainbow trout, hemoglobin would reach only 60% oxygen saturation in the gills of the lamprey, *Lampetra fluviatilis,* in normoxia. With the high intracellular pH, the oxygen affinity of hemoglobin within lamprey erythrocytes is in the range expected for active teleost fish such as the salmonids (Nikinmaa *et al.,* 1995). The high intracellular pH is also required to ensure that the dissociation–association reactions of hemoglobin occur in intact erythrocytes since these reactions are the basis of any cooperative phenomena and of Bohr and Haldane effects of lamprey hemoglobins (see Perutz, 1990; Nikinmaa *et al.,* 1995).

B. Elasmobranchs

Elasmobranch erythrocytes conform to the basic pattern of erythrocytic pH control. The erythrocytes are characterized by a very rapid anion exchange (Obaid *et al.,* 1979) and high buffering capacity of hemoglobin (Jensen, 1989, 1991). Both of these factors facilitate the buffering of extracellular acid loads, but reduce the possibility of regulating hemoglobin function by secondarily active ion transport pathways such as sodium/proton exchange (see Nikinmaa, 1997). If changes in the intracellular pH play a role in the physiological adjustments of hemoglobin function, they are probably caused by changes in the organic phosphate concentrations within the erythrocyte and consecutive changes in the anion and proton distribution ratio since no published data are available indicating a role for secondarily active transport in the control of intracellular pH.

C. Teleosts

Similar to elasmobranch erythrocytes, the pH of unstimulated teleost erythrocytes conforms to the basic pattern of pH control. The major difference between teleost and elasmobranch fish is that the buffering capacity of teleost hemoglobins is generally much smaller, and the Haldane effects much larger, than those of elasmobranch fish (see, e.g., Jensen, 1991). As a consequence, extracellular acid loads should cause larger changes in the erythrocyte and plasma pH of teleosts than of elasmobranchs. Furthermore, the effect of oxygenation on the intracellular pH should be larger in teleost than in elasmobranch fish.

In many instances, the catecholamine-activated sodium/proton exchange influences the erythrocyte pH of teleost fish. The effects of catecholamine-sensitive sodium/proton exchange on the intraerythrocytic pH are known in great detail (for reviews, see, e.g., Motais *et al.,* 1992; Nikinmaa, 1992a; Thomas and Perry, 1992; Fig. 7). A reduction in the oxygen availability (normally a reduction in the arterial oxygen content; see Perry *et al.,*

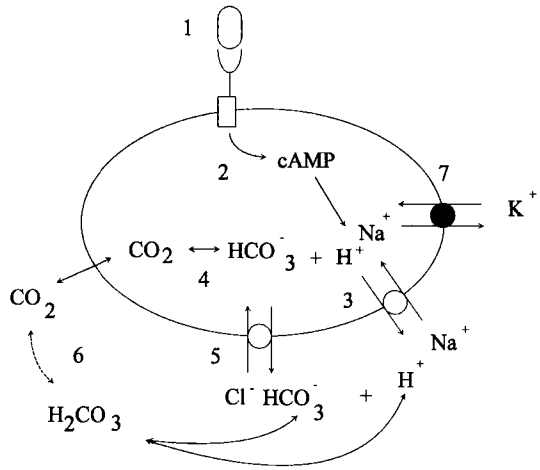

Fig. 7. Adrenergic effects on teleost erythrocytes. (1) Binding of catecholamines to β-adrenergic receptors on the erythrocyte membrane activates adenylate cyclase, and (2) the cAMP concentration increases. This leads to the activation of sodium/proton exchange (3) with the consequence that intraerythrocytic pH increases. As a result (4), more bicarbonate is formed from carbon dioxide, causing a disequilibrium for the anion exchanger. Thus, (5) chloride enters the cell in exchange for bicarbonate. The increase in intracellular pH and the decrease in extracellular pH, observed after adrenergic stimulation, occur because the extracellular buffering of protons is slow owing to the slow rate of dehydration of extracellular carbonic acid to carbon dioxide (6). The increase in intracellular sodium concentration increases the activity of the sodium pump (7), leading to a reduction in the cellular ATP concentration.

1989) /oxygen demand ratio at the level of chromaffin tissue causes liberation of catecholamines to the bloodstream (Perry *et al.*, 1991). Catecholamines bind to the β-adrenergic receptors on the red blood cell membrane. Binding of catecholamines to the receptors causes an accumulation of cyclic AMP (Mahé *et al.*, 1985) and activation of the sodium/proton exchange. Early studies (mainly on rainbow trout and carp) suggested that noradrenaline would always be a more potent activator of the system than adrenaline (e.g., Tetens *et al.*, 1988; Salama and Nikinmaa, 1990; Nikinmaa, 1992a). However, recent data (Berenbrink and Bridges, 1994) suggest that in some species such as the cod, *Gadus morhua,* adrenaline can be more potent than noradrenaline.

The sodium/proton exchange carries out proton extrusion and displaces protons from electrochemical equilibrium during the initial minutes of activation. This is possible even though the initial efflux of protons and influx of sodium via the sodium/proton exchange is only ca. 1/200 of the chloride and bicarbonate exchange fluxes via anion exchange (see Nikinmaa

and Boutilier, 1995), as long as the turnover rate of the sodium/proton exchange approaches the extracellular uncatalyzed rate of dehydration of bicarbonate and protons to carbon dioxide, which is the speed by which protons are buffered extracellularly (Motais *et al.*, 1989; Nikinmaa *et al.*, 1990b; Nikinmaa, 1992a; Nikinmaa and Boutilier, 1995). Thus, a large number of protons can be extruded via the sodium/proton exchanger, and can accumulate in the incubation medium, before the carbonic acid formed from the accumulated protons and bicarbonate is dehydrated to carbon dioxide to a significant extent. After this, the proton and bicarbonate movements are coupled, and no further changes in the erythrocyte pH occur until the sodium/proton exchange is inactivated. However, during this time, there is a continuous net influx of sodium and chloride into the cell (for further details on the mechanism of adrenergic pH changes, see Nikinmaa, 1992a; Nikinmaa and Boutilier, 1995). Notably, the low buffering capacity of teleost hemoglobins contributes to the observed adrenergic pH changes: in elasmobranch fish, much more pronounced proton fluxes would be required for a similar change in intracellular pH because of the large buffering capacity.

There are pronounced species differences in the activity of the sodium/proton exchanger at any given temperature, and in the pH dependence of the activity. In salmonids, the adrenergic net sodium influx is maximal at around extracellular pH 7.3, whereas in carp and in the percid, *Stizostedion lucioperca,* the activity continues to increase at least down to pH 7 (Salama and Nikinmaa, 1989; Cossins and Kilbey, 1991). At physiological extracellular pH values under normoxic conditions, the activity of the sodium/proton exchange is much smaller in the cyprinids and percids than in the salmonids, and practically nonexistent in the eel (Hyde and Perry, 1990). Whenever the activity of the exchanger is small, there is almost exactly one-to-one coupling of the net sodium and chloride influxes after adrenergic stimulation, but when the activity of the sodium/proton exchange is large, the ratio between net sodium influx and net chloride influx clearly exceeds one, and can be more than two (Fig. 8). Thus the ratio of net sodium influx/net chloride influx can be used to obtain an idea of the activity of adrenergic sodium/proton exchanger.

In the case of the adrenergic sodium/proton exchange, the loose coupling of this transport pathway to the anion exchanger diminishes the intra- and extracellular pH changes. However, in the case of the coupling of the potassium/chloride co-transporter and the anion exchanger, pH changes will be generated. The potassium/chloride co-transporter reduces the intracellular and increases the extracellular chloride concentration. This causes a disequilibrium for the anion exchanger, and chloride reenters the cell in exchange for bicarbonate. A consequence of the net bicarbonate efflux is

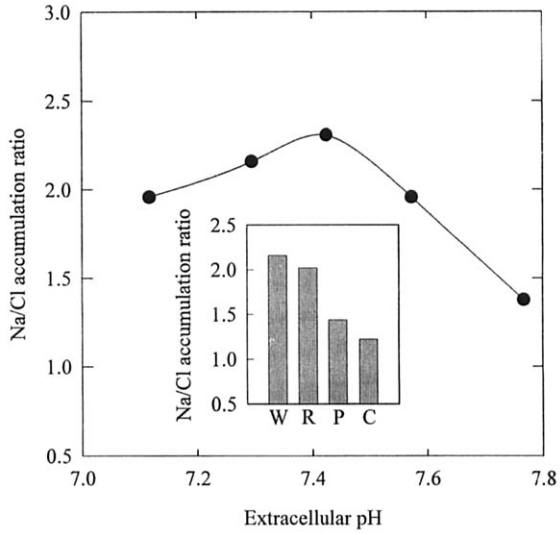

Fig. 8. The molar accumulation ratio of sodium and chloride during a 30-min incubation with 10^{-5} M isoproterenol as a function of pH in whitefish. An increase in the ratio indicates an increase in the activity of the sodium/proton exchanger and, consequently, larger intra- and extracellular pH changes. The inset shows the molar accumulation ratio of sodium and chloride during a 30-min incubation with 10^{-5} M isoproterenol at extracellular pH of 7.1 in whitefish (W), rainbow trout (R), pikeperch (P), and carp (C), indicating that in normoxic carp only slight pH effects are produced because of the modest activation of the sodium/proton exchange. Data from Salama and Nikinmaa (1989).

a reduction of intracellular pH. To date, changes in the intracellular pH as a consequence of the potassium and chloride efflux have not been measured. However, alkalinization of the extracellular compartment has been observed in nitrite-treated and hypotonically treated carp erythrocytes (Jensen, 1990, 1995), and in both cases, net potassium and chloride effluxes are seen. In view of this, one possible reason for the oxygenation-sensitive potassium/chloride efflux could be to reduce the intraerythrocytic pH at high oxygen tensions. This could, by reducing the oxygen affinity of hemoglobin, facilitate oxygen unloading in the arterial end of the capillary bed whenever fish are exposed to diurnally hyperoxic conditions as in many eutrophic lakes.

VIII. HEMOGLOBIN OXIDATION

Hemoglobin subunits that have been oxidized (the ferrous ion of heme is oxidized to ferric ion) are not capable of binding oxygen. Thus, methemo-

globin formation either by autoxidation or during exposure to nitrite, reactive oxygen species, or thiols causes a reduction in the oxygen-carrying capacity of blood. In addition, partial oxidation of hemoglobin in fishes appears to decrease the oxygen affinity of the remaining functional hemoglobin molecules (Jensen *et al.,* 1987; Jensen, 1990). A major reason for this decrease appears to be the concomitant reduction of red cell volume, and, possibly, decrease in intraerythrocytic pH (Jensen, 1990; Jensen *et al.,* 1993). At the cellular level, methemoglobin reduction back to functional hemoglobin involves two systems (see Nikinmaa, 1990): the NADPH methemoglobin reductase system and NADH methemoglobin reductase system (Fig. 9). At present, the regulation of these reductive pathways is not known.

IX. RESPONSES OF HEMOGLOBIN FUNCTION TO CHANGES IN THE EXTERNAL AND THE INTERNAL ENVIRONMENT OF FISH

A. Temperature

An increase in temperature decreases the hemoglobin–oxygen affinity. This affinity decrease is beneficial as long as oxygen loading can be secured in the gills because a reduction in the hemoglobin–oxygen affinity increases the amount of oxygen given up at any capillary oxygen tension (or increases the oxygen partial pressure in the capillaries for a given amount of oxygen delivered). It appears that the oxygen affinity of hemoglobin is further decreased with an increasing temperature by an increase in the erythrocytic organic phosphate concentration, as long as arterial oxygen saturation can be secured (Nikinmaa *et al.,* 1980; Laursen *et al.,* 1985). However, at high temperatures, where the temperature-induced decrease in hemoglobin–oxygen affinity would significantly reduce oxygen loading in gills, the cellular NTP concentration is reduced (Nikinmaa *et al.,* 1980; Albers *et al.,* 1983; Laursen *et al.,* 1985). Furthermore, the red cell magnesium concentration is increased (Houston and Koss, 1984), and since magnesium complexes with NTP, the binding of NTP to hemoglobin is reduced. Both responses increase hemoglobin–oxygen affinity. Indeed, Grigg (1969) reported that the hemoglobin–oxygen affinity of fish acclimated to high temperatures was higher than that of fish acclimated to low temperatures, when measurements were made at the same temperature.

An increase in temperature is usually associated with a decrease in blood (and erythrocyte) pH (Heisler, 1984). As long as the decrease in pH is such that the protein charge of hemoglobin is not affected, it does not

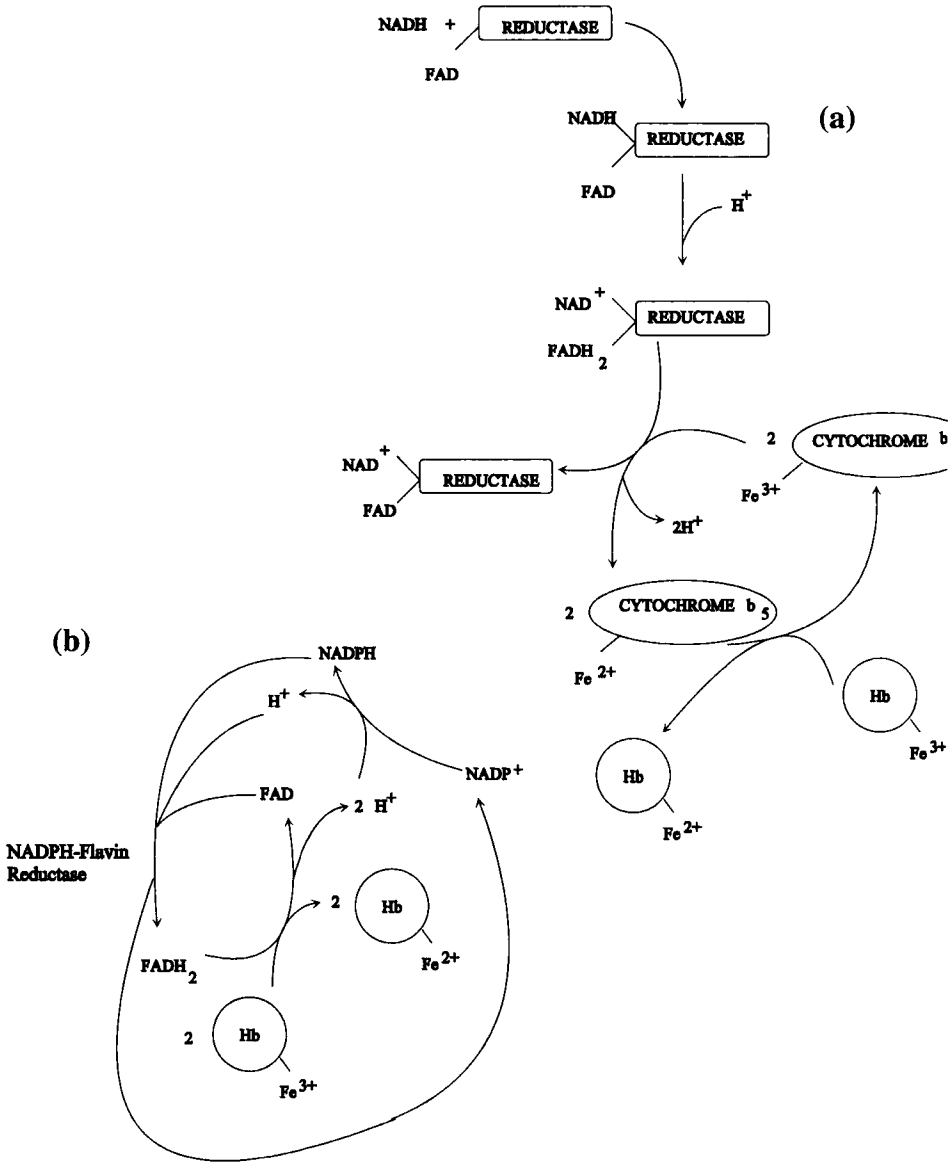

Fig. 9. Reduction of methemoglobin within the erythrocytes. (a) Cytochrome b_5 reductase pathway. (b) NADPH-flavin reductase pathway. Modified from Nikinmaa (1990).

cause an additional decrease in hemoglobin–oxygen affinity on top of the intrinsic temperature sensitivity of hemoglobin (see Nikinmaa, 1990). Only if the erythrocyte pH decreases more with a temperature increase than the pK value of histidine imidazole will the Bohr effect contribute to the observed decrease in hemoglobin–oxygen affinity.

In addition to the effects of temperature on hemoglobin function as such, seasonal phenomena may modify gas transport. It appears that erythrocyte methemoglobin concentrations are higher in temperate fish in winter than in summer (Graham and Fletcher, 1986), and that the β-adrenergic responsiveness of rainbow trout erythrocytes is reduced in winter (Nikinmaa and Jensen, 1986; Cossins and Kilbey, 1989). These responses reduce the effectiveness of oxygen transport by blood in cold environments and reduce the scope of activity in winter when food availability may be limited. In spring, it is probable that production of new erythrocytes increases, cellular methemoglobin levels fall, and the β-adrenergic activity of the sodium/proton exchange increases with increasing temperature. Thus, blood oxygen transport can respond appropriately to the large increases in activity brought about by the increase in temperature.

B. Hypoxia

The occurrence of environmental hypoxia is common especially in freshwater environments. Hypoxic conditions may be intermittent, occurring in eutrophied waters during the night, in which case the water may be hyperoxic during the day. In most cases, hypoxic conditions occur together with hypercapnia since oxygen depletion is normally due to the oxidation of organic matter and to the respiration of organisms with carbon dioxide as an end product. In addition, internal hypoxia that results from an increased diffusion distance between water and blood across the gills, as occurs during exposure to many pollutants, may develop in fishes (see Hughes, 1981). This section deals with responses to pure hypoxia; other forms of hypoxia are treated in a later sections.

When fish are subjected to a hypoxic environment, the hemoglobin oxygen saturation decreases, and the erythrocyte pH increases owing to the Haldane effect. As discussed earlier, this increase of erythrocyte pH will not affect the oxygen-binding properties of hemoglobin, since it is part of the hemoglobin mechanism. Fish also respond to hypoxia with immediate hyperventilation (e.g., Holeton and Randall, 1967). Thus, hypoxia will result in an increase in plasma pH as long as the repiratory alkalosis is greater than the metabolic acidosis that results from the inadequate oxygen supply to the tissues and consequent lactacidosis. The increase in plasma pH will,

to some degree, be transmitted to erythrocytes, and the hemoglobin–oxygen affinity will increase.

In many teleost fish, the general alkalinization of plasma in acute hypoxia is interrupted 1–2 min after the onset of hypoxia by a marked acidification (Thomas and Hughes, 1982)—up to 0.5 pH unit. This acidification represents the activation of adrenergic sodium/proton exchange and is associated with a rapid increase in erythrocyte pH (Tetens and Christensen, 1987). The adrenergic increase in erythrocytic pH can be differentiated from the increase caused by deoxygenation of hemoglobin: in the adrenergic increase, there is an amiloride- and propranolol-inhibitable increase in cellular sodium concentration and an increase in chloride concentration that is partially inhibited by the aforementioned inhibitors; in the deoxygenation-induced increase only the chloride concentration increases, and the increase is not blocked by amiloride or propranolol (Nikinmaa *et al.*, 1987).

The adrenergic sodium/proton exchange is uniquely suited to modulate erythrocyte pH under hypoxic conditions since most of the components of the receptor–effector cascade (Fig. 7) are hypoxia-sensitive. Catecholamines are released to the bloodstream in response to hypoxia (Tetens and Christensen, 1987). The number of β-adrenergic receptors on the erythrocyte membrane increases under hypoxic conditions (Marttila and Nikinmaa, 1988; Reid and Perry, 1991). The concentration of cAMP formed under hypoxic conditions appears to be higher than the formation under normoxic conditions in some species (Salama and Nikinmaa, 1990; Salama, 1993). The activity of sodium/proton exchange as such is increased in hypoxia (Motais *et al.*, 1987; Salama and Nikinmaa, 1988), and the number of exchangers on the cell surface may also increase (Reid *et al.*, 1993; Guizouarn *et al.*, 1995). Notably, in normoxic tench and carp erythrocytes, catecholamines do not cause an increase in intracellular pH either *in vivo* or *in vitro* at normal plasma pH values (Jensen, 1987; Nikinmaa *et al.*, 1987; Salama and Nikinmaa, 1988). However, when the arterial oxygen tension of carp is reduced, a pronounced increase in erythrocyte pH that is associated with a propranolol-inhibitable increase in intracellular sodium concentration and water content is seen (Nikinmaa *et al.*, 1987). The increase in erythrocyte pH is the major reason for the rapid increase in hemoglobin–oxygen affinity of teleost fish in acute hypoxia (Nikinmaa, 1983; Tetens and Lykkeboe, 1985), although the dilution of hemoglobin and organic phosphates may also play a role (Soivio and Nikinmaa, 1981). In chronic hypoxia, other mechanisms must take over since a prolonged elevation of blood catecholamine levels diminishes the red cell adrenergic response (Thomas *et al.*, 1991).

Elasmobranchs and agnathans do not respond to adrenergic stimulation via a significant activation of sodium/proton exchanger. However, in lam-

prey it is possible that hypoxia affects erythrocyte pH via activating sodium/proton exchange by some other mechanism—at least hypoxic conditions lead to an elevation of cellular sodium concentration, water content, and intracellular pH (Nikinmaa and Weber, 1984).

In chronic hypoxia, the most important mechanism for regulating hemoglobin function of fish (apart from agnathans) is the reduction of erythrocyte NTP concentrations (Wood and Johansen, 1972; Weber and Lykkeboe, 1978; Soivio *et al.*, 1980). A reduction in cellular ATP and GTP concentrations increases the blood oxygen affinity both by reducing allosteric interaction of phosphates with hemoglobin and by increasing intraerythrocytic pH. The erythrocytic pH increases only if the total pool of nucleotide phosphates decreases since the negative charge of the hydrolysis products of NTPs is similar to that of NTPs themselves (see Nikinmaa, 1990). A reduction in the total pool of nucleotide phosphates does, indeed, take place under hypoxic conditions (Tetens, 1987; Fig. 3). A considerable decrease in NTP concentrations occurs within a few hours from the onset of hypoxia (Tetens and Lykkeboe, 1985; Jensen and Weber, 1985). Whenever both ATP and GTP are present in fish erythrocytes, the GTP concentrations decrease more rapidly and to a greater extent than the ATP concentrations (Weber and Lykkeboe, 1978). It appears that high red cell concentrations of GTP have been selected for in species, such as carp, tench, and eel, that encounter large fluctuations in oxygen tension in their natural habitats (Weber and Jensen, 1988).

C. Hypercapnia and Hypercapnic Hypoxia

An elevation of environmental carbon dioxide tension causes a respiratory acidosis (Heisler *et al.*, 1976; Toews *et al.*, 1983). Respiratory acidosis causes liberation of catecholamines to the bloodstream of rainbow trout under both hypoxic and normoxic conditions, but not under hyperoxic conditions (Perry and Kinkead, 1989; Perry *et al.*, 1989). However, if hyperoxic hypercapnia is induced to fish made anemic before the exposure, a liberation of catecholamines to the bloodstream also occurs, indicating that a reduction in arterial oxygen content is the primary reason for catecholamine release (Perry *et al.*, 1989). The liberation of catecholamines to the bloodstream causes an activation of the adrenergic sodium/proton exchanger, as indicated by the propranolol-inhibitable elevation of erythrocyte pH in normoxic hypercapnic fish compared to hyperoxic hypercapnic fish in which plasma catecholamine levels did not increase (Perry and Kinkead, 1989). As a consequence, the oxygen affinity of hemoglobin can be maintained. The situation in carp is somewhat different since the animals can significantly increase the arterial oxygen tension in response to hyper-

capnic conditions (Takeda, 1991). In hypercapnic carp, the change in oxygen tension is the major mechanism for maintaining constant oxygen saturation of blood (Takeda, 1991).

Hypercapnic hypoxia reduces arterial oxygen saturation, and despite the hyperventilation, a respiratory acidosis is induced owing to the elevated carbon dioxide tension of water (Jensen and Weber, 1982). Thus, opposite to "pure" hypoxia there is a tendency for a reduction of erythrocyte pH and for a consecutive decrease of hemoglobin–oxygen affinity (e.g., Jensen *et al.*, 1993). Apart from this difference, the responses to hypoxia in hypercapnic conditions are the same as in normocapnic hypoxia, i.e., initial activation of the sodium/proton exchange, which elevates intraerythrocytic pH (Thomas and Perry, 1994), and a slower reduction of erythrocytic NTP concentration, which, in the case of the tench *Tinca tinca,* is caused by the reduced GTP content (Jensen and Weber, 1985). Thus, there is a leftward shift of the oxygen equilibrium curve in hypercapnic, hypoxic animals compared to normoxic, normocapnic animals.

D. Hyperoxia

When fish experience environmental hyperoxia, their ventilatory drive is reduced since the regulation of ventilation is mainly governed by the oxygen demand of fish (Dejours, 1973). As a consequence of the reduced ventilatory volume, there is an accumulation of carbon dioxide in the blood and a decrease in plasma pH (Randall and Jones, 1973; Wood and Lemoigne, 1991). However, blood oxygenation can easily be maintained despite the acidification since blood oxygen tension increases (Fig. 10). The fact that oxygen saturation in the arterial blood does not increase despite the pronounced increase in arterial oxygen tension in rainbow trout suggests that the oxygen affinity of hemoglobin within the erythrocytes decreases in hyperoxia. It is possible that the oxygenation-dependent potassium/chloride co-transport and consecutive efflux of bicarbonate from the erythrocytes in exchange for chloride contribute to this, as there is an increase in plasma potassium concentration (Wheatly *et al.*, 1984), and the erythrocyte pH decreases during initial stages of hyperoxia (Wood and Lemoigne, 1991).

E. Environmental Acidification

In the absence of other disturbances, a decrease of water pH to values below 5 causes only small changes in the blood pH or oxygen tension of teleost fish, although there are reports of gill damage in acid-exposed animals (Daye and Garside, 1976). Malte (1986) observed a 0.15-unit decrease

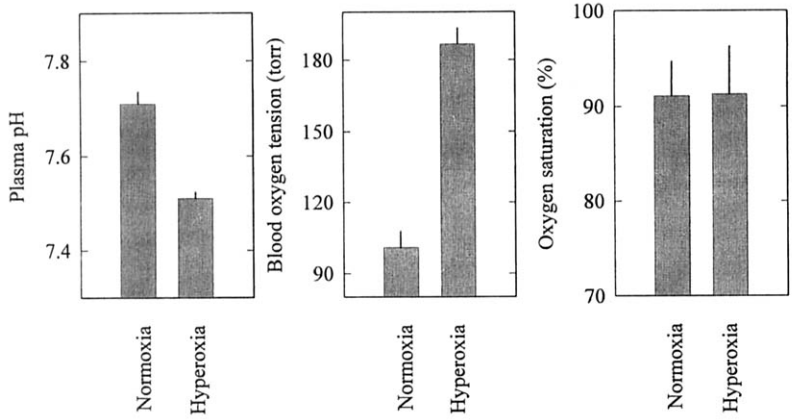

Fig. 10. Plasma pH, blood oxygen tension and blood oxygen saturation of six rainbow trout held in normoxic water (oxygen tension 140–155 torr) or subjected to 3-h hyperoxia (oxygen tension 240 torr) at 15°C. Hyperoxia induced a significant ($P < 0.05$; t test was used for comparisons) decrease in plasma pH and an increase in blood oxygen tension, but did not affect arterial oxygen saturation. M. Nikinmaa, unpublished data.

in the pH of rainbow trout during a 102-h exposure to pH 5; Wood *et al.* (1988a) observed a 0.1-unit decrease in the plasma pH of brook trout (*Salvelinus fontinalis*) during the first 40 h of a 10-day exposure to pH 4.8; Nikinmaa *et al.* (1990a), working on rainbow trout, and Van Dijk *et al.* (1993a), working on carp, did not see any effect of acidification on the pH of arterial blood during short-term exposures to pH 5 and 4, respectively. In contrast, similar treatment of the lamprey, *Lampetra fluviatilis,* caused a marked reduction in plasma pH, by 0.6 unit when the water pH was decreased from 7.8 to 5 for 24 h, and by 0.8 unit when the pH was decreased to 4 (Mattsoff and Nikinmaa, 1988). This difference between lamprey and teleost fish is largely because, in contrast to teleost fish in which extracellular acid loads can be buffered by hemoglobin, lamprey hemoglobin does not participate in rapid buffering of extracellular acid loads owing to the low permeability of erythrocyte membrane to acid equivalents (for a review, see Nikinmaa *et al.,* 1995).

If additional stresses occur simultaneously with acidification, the respiratory physiology of teleost fish is markedly affected. For example, the aluminum concentration of water commonly increases with acidification. In this case, the diffusion distance between water and blood increases markedly both because of gill damage and because of precipitation of aluminum hydroxide on the gill surface (see McDonald and Wood, 1993). Conse-

quently, there is a marked reduction in arterial oxygen tension, oxygen saturation, and pH (Malte, 1986; Jensen and Weber, 1987). Similar gill damage occurs if the iron concentration of water is high, as is the case in many water bodies in Finland (Peuranen *et al.*, 1994).

Fish respond to this internal hypoxia as to an externally induced hypercapnic hypoxia: there is a liberation of catecholamines to the bloodstream (Witters *et al.*, 1991) and the erythrocyte volume increases (Milligan and Wood, 1982). The erythrocyte pH increases or is maintained in the face of marked extracellular acidification. However, it is likely that the erythrocyte pH at a constant oxygen saturation would be decreased, since, owing to the decrease in hemoglobin oxygen saturation, the change in erythrocyte pH includes the contribution of the marked deoxygenation-induced proton uptake by hemoglobin. The NTP concentration of the erythrocytes decreases, which in the case of tench is mainly due to a selective decrease on GTP concentration (Jensen and Weber, 1987). Thus, the oxygen affinity of hemoglobin at a given pH is increased. However, the increase cannot fully offset the effects of a marked decrease in oxygen tension and a reduction of erythrocyte pH at a given oxygen saturation that occur in acid-exposed fish since the blood oxygen saturation of acid-exposed fish remains much lower than that of nonexposed fish (Malte, 1986; Jensen and Weber, 1987; Malte and Weber, 1988). To some degree, preexposure to the metal results in increased tolerance (see MacDonald and Wood, 1993): there was no reduction in arterial oxygen tension in fish preacclimated to aluminum-containing (150 μg L^{-1}) water at pH 5.2, when they were further exposed to pH 4.8 and 333 μg L^{-1} aluminum (Wood *et al.*, 1988b).

The respiratory function of blood is also markedly affected if acid-exposed fish are exercised or exposed to hypoxia. Nikinmaa *et al.* (1990a) exposed rainbow trout to either pH 5 for 24 h or to hypoxia (environmental oxygen tension 55–60 torr) or their combination. Neither treatment alone caused any changes in the respiratory function of the erythrocytes. However, hypoxia in acid water caused a pronounced release of catecholamines to blood, a marked reduction in blood NTP concentration, and an increase in erythrocytic sodium concentration. Furthermore, there was a pronounced loss of sodium and chloride from plasma. Similarly, Van Dijk *et al.* (1993a,b) exposed carp to a gradual decrease of pH from 7 to 4 in 4 h. In resting animals, this did not influence plasma pH or sodium and chloride concentrations. However, when animals were exercised, there was a decrease in plasma pH and sodium and chloride concentrations. These results indicate that hyperventilation, when occurring in acid-exposed fish, plays an important role in proton accumulation into the animal and ion loss to the environment and that, in addition to aluminum, many other physiological stresses influence "acid toxicity."

F. Salinity Changes

A transfer of fish from freshwater to seawater (rainbow trout) or to brackish water (2 or 2.5% salinity; whitefish) causes both a transient reduction of arterial oxygen content and a respiratory acidosis, probably because of a reduced diffusion conductance in gills (Maxime *et al.,* 1991; Larsen and Jensen, 1993; Madsen *et al.,* 1996). In the whitefish (*Coregonus lavaretus*), the acidosis persists for at least 48 h, but shifts from being predominantly respiratory to predominantly metabolic (Madsen *et al.,* 1996). It is, as yet, uncertain whether the respiratory acidosis observed in seawater elicits the normal rapid response to hypoxia, i.e., the compensatory increase in erythrocyte pH via catecholamine stimulation of the sodium/proton exchange. In the whitefish, some protection of the erythrocyte pH may have occurred since the slope of the relationship between erythrocyte pH and plasma pH ($\Delta pH_i/\Delta pH_e$) was only 0.26 (Madsen *et al.,* 1996), i.e., much shallower than in teleost fish in general (0.6–0.9). However, no data are available on catecholamine concentrations nor on the effects of β-adrenergic blockade on the responses to seawater-induced changes in the oxygen transport parameters.

G. Exercise

The responses of hemoglobin function to exercise depend on the severity of exercise. As long as the increased oxygen demand of muscle work can be fulfilled by an increase in the ventilatory volume and in the cardiac output, a rightward shift of the oxygen equilibrium curve in the capillary bed is beneficial since more oxygen can be unloaded from hemoglobin at a given oxygen tension. A facilitation of oxygen delivery requires that the pH of capillary blood at a given oxygen saturation of hemoglobin decreases. Such a decrease is caused by the flux of carbon dioxide and metabolic acid from the working muscles into the capillaries.

During exhaustive exercise, a mixed respiratory and metabolic acidosis develops (e.g., Jensen *et al.,* 1983). In the absence of compensatory responses in the oxygen transport system, this would seriously affect the oxygen loading in gills. Teleost fish have two types of responses enhancing oxygen transport. In species with high resting blood oxygen tensions, such as the salmonids and the striped bass, *Morone saxatilis,* the plasma catecholamine concentration is increased (Ristori and Laurent, 1985; Butler *et al.,* 1986), activating the adrenergic sodium/proton exchange. This prevents or reduces the decrease in the erythrocyte pH that would otherwise take place (Nikinmaa *et al.,* 1984; Primmett *et al.,* 1986; Milligan and Wood, 1987). Plasma catecholamine concentration increases much more when there is a

"psychological" component (tail grabbing or chasing with a stick) in the stress than in burst swimming alone (cf. Ristori and Laurent, 1985; Butler *et al.*, 1986; Milligan *et al.*, 1989). Repeated stresses (e.g., chasing until exhaustion daily for a week) cause an increase in the responsiveness of erythrocytes to catecholamines (Perry *et al.*, 1996). It appears that this effect is due to an increase in the sensitivity of the sodium/proton exchanger to cAMP, since the accumulation of cAMP was not affected, but the dose–response curve for the cAMP-sensitive sodium accumulation was shifted to lower cAMP concentrations (Perry *et al.*, 1996).

In species with a low resting oxygen tension of blood, such as tench and carp, the major blood response to normoxic exercise is a marked elevation of arterial oxygen tension, from 20–40 up to 100 torr (e.g., Jensen *et al.*, 1983). Thus, although the oxygen equilibrium curve is shifted to the right owing to the exercise-induced acidification, the increase in arterial oxygen tension limits the reduction in hemoglobin–oxygen saturation. In these species, catecholamines are not capable of activating the sodium/proton exchange at normal blood pH values under normoxic conditions (Jensen, 1987; Salama and Nikinmaa, 1988).

In lampreys, the permeability of the red blood cell membrane to acid equivalents is very low (e.g., Nikinmaa and Railo, 1987). Thus, the metabolic proton production that takes place during exhaustive exercise will only acidify the plasma compartment. Indeed, exhaustive exercise causes a pronounced reduction in both arterial and venous plasma pH in *Petromyzon marinus* by 0.36 and 0.46 pH unit, respectively, but does not affect erythrocyte pH (Tufts *et al.*, 1992). However, exhaustive exercise also causes a pronounced carbon dioxide load, as evident by the increase of carbon dioxide tension by 0.39 kPa in venous and by 0.17 kPa in arterial blood (Tufts *et al.*, 1992), which should be transmitted to the erythrocyte in lamprey and cause a reduction in erythrocyte pH. This is the case if the erythrocyte pH is extrapolated to a constant oxygen saturation. The data of Tufts *et al.* (1992) show that the arterial oxygen saturation decreased from ca. 95 to ca. 75%, and the venous saturation from ca. 75 to ca. 18% following exhaustive exercise. In the absence of a carbon dioxide load, these decreases in oxygen saturation would have caused approximately 0.1- and 0.25-unit increases in the pH of arterial and venous erythrocytes, respectively, owing to the pronounced Haldane effect of lamprey hemoglobin (calculations based on the data of Ferguson *et al.*, 1992). Thus, the carbon dioxide load causes a 0.1- to 0.25-pH-unit decrease in the erythrocyte pH, relative to that in resting lampreys. This decrease causes a reduction in arterial oxygen saturation (Tufts *et al.*, 1992). Thus, it appears that the sodium/proton exchange of lamprey erythrocytes, which is activated by

acidification (Virkki and Nikinmaa, 1994), is not able to correct the decrease in erythrocyte pH, at least during short-term exhaustive exercise.

H. Anemia

When fish become anemic, they hyperventilate, the carbon dioxide tension of dorsal aortic blood decreases, and pH increases (Fig. 11). These changes tend to increase the oxygen affinity of blood. However, in anemic fish such a response would be maladaptive since a reduction of blood oxygen capacity as such will, in addition to reducing the amount of oxygen carried by unit volume of blood, reduce the partial pressure of oxygen at which a given amount of oxygen is given up in the tissues. Thus, if a further increase in oxygen affinity took place, the capillary oxygen tension would be further reduced, as would the diffusion of oxygen from capillary blood to tissues. In view of this, a decrease of oxygen affinity would be beneficial, and this is, indeed, what is observed in anemic fish. Anemia is associated with an increase in erythrocytic NTP concentration (Lane *et al.*, 1981; Vorger and Ristori, 1985; Jensen *et al.*, 1990), and consequently, a significant increase in the P_{50} value of blood (Vorger and Ristori, 1985).

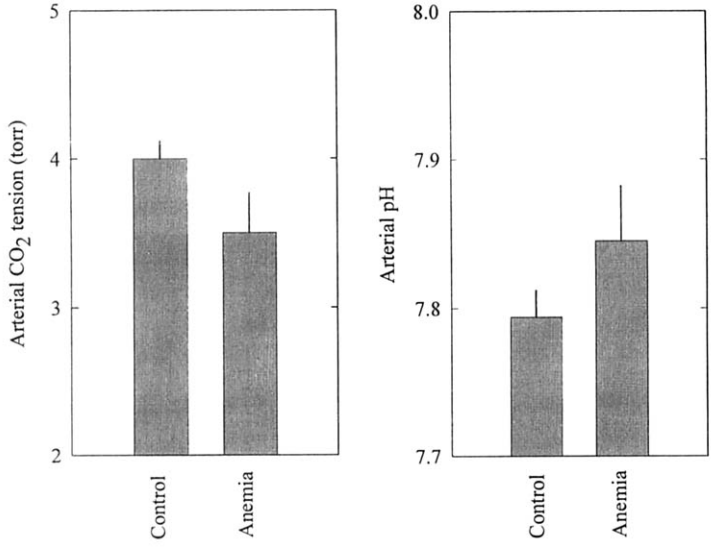

Fig. 11. The arterial carbon dioxide tension and pH of normal (Hct value 28%; $N = 14$) and anemic (Hct value 15%; $N = 8$) rainbow trout at 10°C. Anemia induced a significant ($P < 0.05$; t test was used for comparisons) decrease in arterial carbon dioxide tension and an increase in pH. A. Soivio, K. Nyholm, K. Westman, and M. Nikinmaa, unpublished data.

I. Environmental Pollutants

Various toxicants affect blood oxygen transport by causing gill damage and consecutive internal hypoxia (e.g., Mallatt, 1985; McDonald and Wood, 1993). As illustrated by the aluminum and acidification-induced internal hypoxia, the responses are similar to the ones caused by environmental hypoxia (see also Hughes, 1981). In other cases, effects on blood oxygen transport occur independently from effects on other tissues. The effects of pollutants on erythrocyte function have been discussed by Nikinmaa (1992b). *Nitrite* and other oxidizing agents convert functional hemoglobin to methemoglobin, which does not bind oxygen (e.g., Jensen *et al.*, 1987; Jensen, 1990). This decreases the oxygen capacity of blood markedly. Furthermore, there is a significant reduction in the oxygen affinity of the remaining functional heme groups (Jensen *et al.*, 1987; Jensen, 1990). Both effects reduce oxygen transport to the tissues. Nitrite also inhibits the adrenergically activated sodium/proton exchange of erythrocyte membranes (Nikinmaa and Jensen, 1992), thus reducing the possibility of utilizing the β-adrenergic increase in erythrocyte pH in acute hypoxia or exercise. *Tributyltin chloride* similarly inhibits sodium/proton exchange across the erythrocyte membrane (Virkki and Nikinmaa, 1993). Alkyltin compounds also function as chloride/hydroxyl ion exchangers, causing a marked reduction of the erythrocyte pH in lamprey (Tufts and Boutilier, 1990b; Nikinmaa *et al.*, 1995) and consecutive reduction in hemoglobin–oxygen affinity. *Resin acids,* important constituents of paper and pulp mill effluents, decrease erythrocyte pH in lampreys (A. Bogdanova and M. Nikinmaa, unpublished data) since they behave as protonophores. Resin acids also reduce the energy production of fish erythrocytes (Bushnell *et al.*, 1985), which leads to a reduction in cellular ATP levels, and in the long term to altered red cell shape and red cell breakdown (see Nikinmaa, 1992b). These examples indicate that many toxicants affect red cell function and, consequently, oxygen transport. These changes, when occurring together with natural variations in environmental temperature and oxygen tension, may be a decisive factor behind fish deaths and reduction in fish growth and reproduction in contaminated environments.

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