Ann. Zool. Fennici 23:223–235. 1986

Control of red cell pH in teleost fishes

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Nikinmaa, M. 1986: Control of red cell pH in teleost fishes. – Ann. Zool. Fennici 23:223–235.

Although the chloride/bicarbonate exchange is present in fish red cells whereby protons are, in most instances, distributed passively across the red cell membrane, changes in organic phosphate metabolism, sodium/proton exchange, and cell volume provide the animals with a means to control red cell pH. In many instances the red cell pH is maintained in exercise despite marked extracellular acidosis, whereas it is increased in hypoxia, in order to maintain or enhance oxygen loading in gills. Often, the control of red cell pH is achieved by hormonal, notably adrenergic, stimulation. Catecholamines affect both the organic phosphate concentrations and cell volume. An important effect of adrenaline is the stimulation of sodium proton exchange, which in suitable conditions is capable of transporting protons against their electrochemical gradient, and appears to initiate adrenergic cell swelling.

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1. Effects of protons on cellular functions

Many cellular functions are affected by intracellular pH (see Table 1 for examples). Most of these effects are due to pH-dependent changes in the function of proteins. Proteins are amphoteric molecules, thus being able to accept and lose protons. The acid-base reactions determine the ionization state of the molecule and thus determine the activity of its functional groups.

Within the physiological pH-range (6–8) the most important ionizable groups in proteins are the alpha-amino groups (pK 7.4–7.9) and histidine imidazole groups (pK 6.4–7.0; see Reeves & Rahn 1979). Histidine imidazole plays three distinct roles in enzyme systems (Somero 1981). Firstly, imidazole groups may be essential for ligand binding, as in lactate dehydrogenase. Secondly, imidazole groups frequently function as proton donors and acceptors in the catalytic reaction sequence. Thirdly, the charge states of imidazole groups may affect the conformations and structural stabilities of proteins.

Control of intracellular pH aims at optimizing the protein function in any given situation. Often this means that the imidazole charge is conserved. At constant temperature the pH is maintained within narrow limits, and with changing temperature the pH of cells, tissue fluids and blood plasma changes in the same manner as the pK value of the imidazole group (Reeves 1972; Rahn & Baumgardner 1972; Reeves & Rahn 1979). Often, however, changes in extracellular and intracellular pH during adaptation to the changing external or internal environment of the animal do not follow the concept of constant imidazole charge. Heisler (1984) has reviewed the data on temperature-induced pH changes in fish, and found that with few exceptions changes in both the extracellular and intracellular pH are different from what would be expected if the imidazole charge were kept constant. In proliferating mouse lymphocytes the intracellular pH is higher than in resting lymphocytes (Gerson 1982a, b; Gerson & Kiefer 1983), and the intracellular pH of sea urchin eggs rapidly increases after fertilization (Johnson et al. 1976). In fish, the red cell pH increases when the animals are subjected to hypoxia (Wood & Johansen 1973; Nikinmaa & Soivio 1982).

2. Red cell pH and oxygen transport

The major function of vertebrate red cells is to transport oxygen from the respiratory surfaces (gills, skin, lungs) to the oxygen requiring tissues. Oxygen binds to haemoglobin

Table 1. Examples of cellular functions affected by changes in intracellular pH. For details, see Roos & Boron (1981), Gerson (1982b), Bauer (1974)

Membrane permeability and conductance
Cell-to-cell coupling (cardiac Purkinje fibres are uncoupled by intracellular injections of protons)
Epithelial secretion of acid
Contraction of muscle (low intracellular pH has negative inotropic effect)
Glycolysis (e.g. phosphorylase a and phosphofructokinase activities are decreased by low intracellular pH)
Fertilization (fertilization is associated with marked increase in intracellular pH)
Proliferation of lymphocytes
Haemoglobin function (oxygen affinity is generally decreased at low pH values; alkaline Bohr effect)

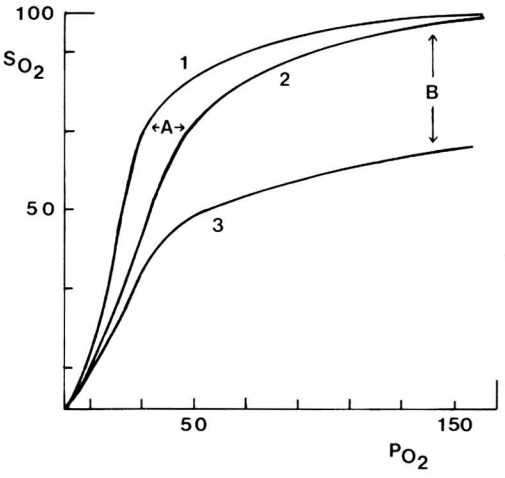

Fig. 1. Examples of oxygen dissociation curves of fish haemoglobin. The percentage oxygen saturation of haemoglobin (S_{O2}) is given as the function of blood oxygen tension (in mmHg; P_{O2}). Curves (1–3) are drawn in order of decreasing pH. A indicates Bohr effect, B shows Root effect.

contained within the cell at the respiratory surface, and is given up in the tissues. The binding of oxygen to haemoglobin and its dissociation can be described in terms of the oxygen equilibrium curve (Fig 1) which gives the haemoglobin oxygen saturation as a function of the oxygen tension in blood. Since the oxygen availability in the environment and oxygen demand in the tissues vary greatly, animals must be able to adjust the transport of oxygen to meet the changing requirements. In most vertebrates more than 90% of oxygen is transported to the tissues bound to haemoglobin. Therefore, regulation of oxygen transport at red cell level is an extremely important mechanism in the adaptation to changes in oxygen availability and oxygen demand.

Generally, the most important factors regulating the haemoglobin-oxygen affinity within the red cells are pH and organic phosphate concentrations (for review see e.g. Bauer 1974). With few exceptions (notably the electrophoretically-cathodic haemoglobin components of eel and trout; see Gillen & Riggs 1973; Weber et al. 1976a; 1976b) decreasing pH reduces the affinity of haemoglobin for oxygen within the physiological pH range. This so called Bohr effect varies markedly from species to species (Table 2), In addition, increasing proton concentration decreases the maximal oxygen saturation at atmospheric or even higher oxygen partial pressure in several fish species (see e.g. Root 1931; Baines 1975; Nikinmaa & Soivio 1980; Jensen & Weber 1982). In view of the pronounced effects of protons on haemoglobin function, the factors controlling intracellular pH are of prime importance in the control of red cell function.

In addition to intracellular pH, organic phosphates exert a major influence on the oxygen affinity of haemoglobin (Benesch & Benesch 1967; Chanutin & Curnish 1967). In fishes the most important organic phosphates regulating haemoglobin function are ATP (adenosine triphosphate) and GTP (guanosine triphosphate), although, additionally, 2,3-DPG (2,3-diphosphoglycerate), UTP (uridosine triphosphate), IDP (inositol diphosphate) and IPP (inositol pentaphosphate) are present in some species (for review see Weber 1982). These organic phosphates bind preferentially to deoxyhaemoglobin (for review see Bauer 1974), stabilizing it and thus decreasing the affinity of haemoglobin for oxygen. In addition to the effect of organic phosphates on the oxygen affinity per se, their concentration within the cell influences the intracellular pH via the Donnan effect (e.g. Duhm 1972 and below) and markedly affects the Bohr effect (see Table 2 and Gillen & Riggs 1977). Thus, the effects of the most important effectors of red cell oxygen transport are interactive.

The adaptive significance of regulating haemoglobin oxygen affinity by controlling

Table 2. Bohr effects (B.E. = dlogP50/dpH) of blood and haemoglobin solutions of several fish species in different conditions.

Species	B.E.	pHe	T (°C)	Source	
Dissostichus mawsoni	−0.39	7.6−7.8	− 2	Blood	1
	−0.5	8.1	− 2	Hb	1
	−1.1	7.7	− 2	Hb	1
Cephaloscyllium isabella	−0.49	7.5−7.9	+ 5	Blood	2
	−0.32	7.5−7.9	+15	Blood	2
Salmo gairdneri	−0.52	7.3−7.9	+15	Blood	3
	+0.19	7.8	+15	Hb I	3
	+0.10	7.8	+15	Hb III	3
	−0.73	7.8	+15	Hb IV	3
	−0.48	7.8	+15	Hb V	3
Anguilla anguilla	+0.34	7.4	+15	Hb I	4
	−0.40	7.4	+15	Hb II	4
	−0.51	7.4	+15	Hb IV/V	4
Serrasalmus ahombius	0	7.3	+30	Hb	5
	−1.0	<7.0	+30	Hb	5
	−0.38	6.8−7.7	+30	Blood	5
Tinca tinca	−0.72	7.3−8.5	+15	Blood	6
	−0.48	7.35	+15	Hb	6
	−1.22	7.35	+15	ATP/Hb	6
	−1.27	7.35	+15	GTP/Hb	6
Cyprinus carpio	−0.93		+20	Blood	7
	−1.17		+10	Blood	7
Clupea harengus juv.	−0.48	7.4−7.8	+15	Blood	8
Clupea harengus ad.	−0.29	7.4−7.8	+15	Blood	8

1. Qvist et al. 1977. − 2. Tetens & Wells 1984. − 3. Weber et al. 1976a. − 4. Weber et al. 1976b. − 5. Wood et al. 1979. − 6. Jensen & Weber 1982. − 7. Albers et al. 1983a. − 8. Everaarts 1978.

intracellular pH and organic phosphate concentration can be seen when fishes are subjected to low oxygen concentrations. In a hypoxic environment the oxygen affinity of fish haemoglobins is raised because of increases in intracellular pH and decreases in organic phosphate concentrations (for review see Nikinmaa 1981; Weber 1982). When present, GTP is a more potent modifier of haemoglobin oxygen affinity than ATP (e.g. Weber 1982). As a result, the oxygen transport to tissues is little affected by hypoxia, although the environmental oxygen availability may be markedly reduced (see e.g. Nikinmaa & Soivio 1982).

3. Control of red cell pH in fishes

3.1. Acid-base equilibria in resting animals

In mammalian red cells, the factors influencing intracellular pH at any given moment are the properties of intracellular buffers (especially haemoglobin), the presence of carbonic anhydrase in the cell, and three types of passive transport across the red cell membrane: (1) diffusion of carbon dioxide, (2) movement of water molecules and (3) stoichiometric electrically neutral exchange of chloride for bicarbonate (see Hladky & Rink 1977). The passive movement of bicarbonate is the major pathway for transferring acid/base equivalents across the red cell membrane, because:

1. The movements of chloride and bicarbonate are a million times faster than those of cations (Na^+ and K^+) with similar hydrated radii (e.g. Fortes 1977). Thus, Cl^- and HCO_3^- rapidly reach equilibrium, so that

$$(HCO_3^-)_i/(HCO_3^-)_o = (Cl^-)_i/(Cl^-)_o.$$

2. The hydroxyl ion concentration is tied to the bicarbonate ion concentration ratio by the chemical reaction between CO_2 and OH^- so that

$$(OH^-)_i/(OH^-)_o = (HCO_3^-)_i/(HCO_3^-)_o.$$

3. Because the ionic product of water K_w is a

constant and $K_w = (H^+)(OH^-)$, the proton distribution ratio

$$(H^+)_o/(H^+)_i = (OH^-)_i/(OH^-)_o.$$

Thus, the intracellular pH can be obtained from the relation

$$pH_i = pH_o + \log(Cl^-)_i - \log(Cl^-)_o.$$

The chloride distribution ratio is dependent on the number of cations in the cell, the amount of impermeant protein and organic phosphate, and the net charge on these impermeant ions (for a more detailed account see e.g. Hladky & Rink 1977). Experimental measurements of intracellular pH, and calculations from chloride distribution ratio have confirmed the above scheme: at an extracellular pH of 7.4 both the measured intracellular pH and the intracellular pH calculated from the chloride distribution ratio were 7.27 in rabbit red cells (Calvey 1970).

In mammalian red cells, the presence of carbonic anhydrase within the cell and the rapid chloride/bicarbonate exchange across the red cell membrane are required for effective carbon dioxide excretion. The carbon dioxide produced in the tissues diffuses through the red cell membrane, and is rapidly hydrated to form bicarbonate and protons in a reaction catalysed by carbonic anhydrase. Protons are effectively taken up by the most important cellular buffer, haemoglobin, and bicarbonate enters the plasma in exchange for chloride entering the cell. In the lungs, the Jacobs-Stewart cycle functions in the opposite direction: plasma bicarbonate enters the cell in exchange for chloride, and is dehydrated to carbon dioxide which diffuses out of the animal (For review of chloride/bicarbonate exchange see e.g. Wieth et al. 1980; Lowe & Lambert 1983).

The chloride/bicarbonate exchange and function of cellular carbonic anhydrase are also involved in the carbon dioxide excretion of fishes (see e.g. Randall 1982; Randall et al. 1982; Randall & Daxboeck 1984; Heming 1984), the mechanism being similar to that observed in mammals. Data on rainbow trout, cannulated both pre- and postbranchially (Nikinmaa & Jensen 1986) show that during the passage of blood through the gills, the red cell chloride concentration decreases by 25%, and total plasma carbon dioxide concentration by 24%. This illustrates the implication of the Jacobs-Stewart cycle in carbon dioxide excretion in rainbow trout. In rainbow trout the anion movements are mediated by the DIDS-sensitive anion exchange pathway, which appears to be very similar to the band 3 protein of human red cells (Romano & Passow 1984). In the physiological temperatures for trout (10−15°C) the half-time for chloride equilibration is 0.8−1.3 s (Romano & Passow 1984), as compared to the 1−3 s passage time of blood through the gills (Hughes et al. 1981). This indicates that despite its relative rapidity, chloride/bicarbonate exchange may be a limiting factor in carbon dioxide excretion in rainbow trout. Rapid chloride movements across the red cell membrane also occur in other fish like the red snapper (Cameron 1978) and tilapia (Haswell et al. 1979), indicating that the anion exchange pathway is generally present in fish red cells, although contrasting data has also been presented (Ohnishi & Asai 1985).

As a consequence of the rapid chloride/bicarbonate exchange in fish red cells, the red cell pH of fish is, in most situations, determined by anion movements, as stated by Randall (1982). The in vivo data of Nikinmaa &

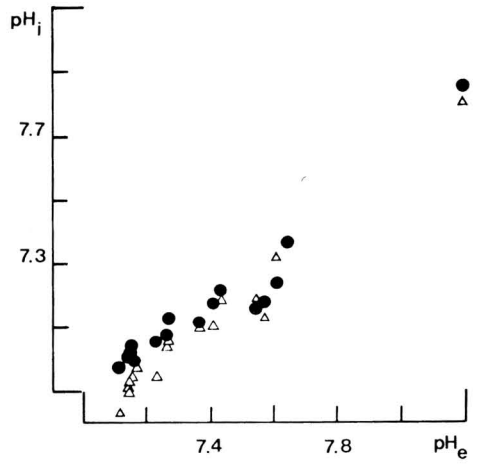

Fig. 2.Intracellular (pHi) vs. extracellular pH (pHe) of rainbow trout red cell suspensions. Data from M. Nikinmaa, J. F. Steffensen, B. L. Tufts and D. J. Randall (unpubl.). Each point represents a mean of 3−8 determinations, ● = values measured using DMO method, △ = values calculated from the chloride distribution across red cell membrane. The dependence of intracellular pH on extracellular pH, calculated for individual data points, is given by the regression lines

A. (for DMO method) pHi=0.725 × pHe + 1.80, r=0.93, n=116

B. (for Cl distr.) pHi=0.821 × pHe + 1.05, r=0.95, n=77

The slope of regression line B is significantly ($P<0.001$) steeper than the slope of regression line A. For further details, see text.

Jensen (1986) show that the intracellular pH measured by the DMO method is similar to that determined from the chloride distribution ratio. Nikinmaa et al. (Fig. 2, unpubl.) have determined the pH values of rainbow trout red cells in vitro by the DMO method, and from the chloride distribution ratio over the extracellular pH range 7.1 to 8.2 (manipulated by changing the carbon dioxide tension of the incubation) noting a close similarity between the two at extracellular pH values above 7.3. At lower extracellular pH values, the intracellular pH calculated from chloride distribution is consistently lower than that measured with the DMO method (see Figure 2). This suggests that intracellular pH may be actively maintained at low pH values.

3.2. Mechanisms of adjusting red cell pH

3.2.1. Organic phosphate metabolism

The passive distribution of chloride and bicarbonate (and thereby protons) is dependent on the concentration of impermeable polyions within the cell, of which haemoglobin and organic phosphates are the most important (see Hladky & Rink 1977; and above). This dependence provides the animals with a means to adjust cellular pH during adaptation to environmental changes without the need to have an active membrane-associated acid extrusion mechanism. Duhm (1972) showed that in mammalian red cells the intracellular pH could be decreased by incubation of cells in a medium that caused an elevation of cellular organic phosphate concentrations. Wood & Johansen (1973) showed that the ATP concentration of eel blood decreases when the animals are subjected to hypoxia. Simultaneously the intracellular pH increased. Later, Weber et al. (1976b) showed that in the eel the GTP concentrations changed more than the ATP concentration. Wood & Johansen (1973) further showed that in eel blood the indirect effect of the organic phosphates on the intracellular pH was a more important modifier of haemoglobin oxygen affinity than the direct binding of the organic phosphates to haemoglobin. The decrease in cellular ATP and/or GTP concentration, and increase in intracellular pH have been later shown to be major adaptations to hypoxia in several other fish species, including carp (Weber & Lykkeboe 1978; Lykkeboe & Weber 1978), killifish (Greaney & Powers 1978), rainbow trout (Nikinmaa & Soivio 1982) and tench (Jensen & Weber 1982; 1985a; 1985b).

The mechanism by which cellular ATP and GTP concentrations are decreased is not, as yet, fully clarified. Powers (1980) suggested that decreased cellular ATP concentration was a result of decreased oxidative phosphorylation within the red cells. However, as pointed out by Tetens & Lykkeboe (1981), the ATP concentration in rainbow trout red cells in vitro decreases only in complete anoxia, whereas in vivo the hypoxia-induced drop in ATP concentration occurs at arterial oxygen tensions of more than 30 mmHg (Soivio et al. 1980). Also, Lykkeboe & Weber (1978) showed that the GTP concentration of carp blood decreases as much during a week's 12 h/12 h cycling of normoxia and hypoxia as in a week's continuous hypoxia. Thus, it is likely, as suggested by Tetens & Lykkeboe (1981) that some other factors, e.g. hormones, influence the organic phosphate concentrations.

Adrenaline is now known to affect the ATP concentrations. The ATP/Hb ratio of adrenaline-treated cells decreased during 1-h in vitro incubations at 22°C (Nikinmaa 1983). Later, the same trend has been observed at 10°C (Salama 1986). This decrease may be caused either by increased breakdown or by decreased production of ATP. No conclusive evidence as to which effect is more important has yet been obtained. However, adrenaline causes an increase in intracellular pH (Nikinmaa 1982; 1983; and below), and increased pH inhibits red cell pyruvate kinase which is required for net glycolytic ATP production (Salama 1986). Adrenergic control has also been implicated in the regulation of aerobic energy production: the activity of the pyruvate dehydrogenase complex in heart cells can be affected by beta-adrenergic stimulation (Hiraoka et al. 1980). Nucleated fish red cells can produce ATP aerobically (see e.g. Powers 1980; Zapata & Carrato 1981; Salama 1986). However, no clear effect of adrenaline on aerobic metabolism has, as yet, been demonstrated (Salama 1986). On the other hand, the adrenaline-induced activation of sodium/proton exchange in red cells (see below; Palfrey & Greengard 1981; Nikinmaa & Huestis 1984) leads to a secondary increase in the activity of sodium/potassium ATPase (Palfrey & Greengard 1981) which may affect ATP concentration.

No clear hormonal involvement in the regu-

lation of GTP concentration in fish red cells has yet been implicated. However, the function of the beta-adrenergic receptor, present in fish red cells (e.g. Nikinmaa 1982), is associated with considerable GTPase activity (for review see e.g. Lefkowitz et al. 1982). Thus, adrenergic stimulation could directly affect cellular GTP concentration. On the other hand, the regulation of GTP concentration may be achieved by enzymatic means. The formation of GTP from GMP is catalysed by two phosphokinases, guanosine monophosphokinase and nucleoside diphosphokinase (see Weber 1982). These enzymes will mediate the conversion of ATP to GTP which is a more potent modulator of haemoglobin oxygen affinity than ATP (Weber 1982).

3.2.2. Cell volume changes

Weber et al. (1976a) first suggested that cell volume changes could affect the red cell oxygen affinity of hypoxic fish. The importance of red cell volume in modulating oxygen affinity in fish has also been demonstrated in stress (Soivio & Nikinmaa 1981) and in maternal-fetal oxygen transfer of a viviparous fish (Ingermann & Terwilliger 1982). Earlier, Bellingham et al. (1971) showed that the oxygen affinity of human red cells was dependent on the mean cellular haemoglobin concentration. Lichtman et al. (1974) showed that propranolol at high concentration decreased the volume of human red cells, causing a decrease in red cell pH and oxygen affinity. On the basis of the above data, the effect of red cell volume on the cellular oxygen affinity depends on two factors: Firstly, the increase in cell volume dilutes both the haemoglobin and organic phosphates within the cell, thus decreasing the direct binding of ATP and GTP to haemoglobin (see e.g. Weber 1982). Secondly, the dilution of impermeable polyions within the cells will affect the distribution of permeable ions in the cells (see Hladky & Rink 1977). The chloride distribution ratio across the red cell membrane will increase, and thus, at constant extracellular pH, the intracellular pH will also increase (see above). Thereby any disturbance affecting the cell volume will affect acid-base equilibrium, and intracellular pH.

3.2.3. Adrenergic stimulation

Adrenergic stimulation causes an increase in cell volume in nucleated red cells. Riddick et al. (1971) reported that adrenergic drugs affect the cell volume of duck. Later, similar findings have been made with other species of birds (e.g. Palfrey et al. 1980), in amphibians (Rudolph & Greengard 1981) and in fish (Nikinmaa 1982; Nikinmaa & Huestis 1984; Baroin et al. 1984). The mechanisms of cellular swelling in birds on the one hand, and in amphibians and fish on the other have proved to be different. In birds, the volume increase involves a sodium/potassium/chloride co-trans-port, which can be inhibited by bumetanide or furosemide, two 'loop' diuretics, but is not affected by DIDS, an inhibitor of anion exchange, or by amiloride, an inhibitor of sodium/proton exchage (for review see Palfrey & Greengard 1981). Generally, an elevated plasma concentration of potassium is required for adrenergic volume changes in birds (e.g. Nikinmaa & Huestis 1984). In amphibians, extracellular potassium is not required for cellular swelling. The mechanism is an amiloride-sensitive sodium/proton exchange (Palfrey & Greengard 1981). However, at least in the adult frog, *Rana catesbiana*, inhibition of cellular phosphodiesterase activity is required for the adrenergic cell volume changes (Rudolph & Greengard 1980). In fish, the cell volume changes are associated with accumulation of sodium and chloride in the cell (Baroin et al. 1984), can be inhibited by amiloride, furosemide and DIDS, or by removing sodium from the incubation medium, and are drastically reduced by substituting nitrate for chloride (Baroin et al. 1984; Nikinmaa & Huestis 1984). On the basis of the above, Baroin et al. (1984) suggested that a major proportion of the cell volume changes would be due to a sodium/chloride cotransport with specific pharmacological properties. They also suggested that both the sodium and chloride movements might occur through the band three anion exchange pathway. Nikinmaa & Huestis (1984) additionally measured the intracellular pH values of adrenaline treated cells in the presence and absence of different transport inhibitors and using ion substitutions (Table 3). From these data and more recent data by M. Nikinmaa, J. F. Steffensen, B. L. Tuffs and D. J. Randall (unpubl.) it is clear that although treatment with DIDS or furosemide inhibits adrenergic swelling, pronounced intracellular alkalinization occurs in both cases during adrenaline treatment. This alkalinization is inhibited either by amiloride treatment or by

Table 3. Effects of 10 micromolar isoproterenol on the extra- (pHe) and intracellular pH (pHi) of rainbow trout red cells. Data from Nikinmaa & Huestis (1984) and M. Nikinmaa, J. F. Steffensen, B. L. Tufts & D. J. Randall, unpubl. Wilcoxon's two-sample rank sum test was used for statistical comparisons between groups treated with and without isoproterenol.

Incubation		No isoprot.	Isoprot.	$P<$
Control	pHe	7.282±0.009	7.222±0.011	0.01
	pHi	7.089±0.012	7.128±0.013	0.01
0 mM Na^+	pHe	7.250±0.002	7.231±0.024	0.05
	pHi	7.007±0.017	7.024±0.008	NS
0.1 mM	pHe	7.277±0.011	7.143±0.020	0.01
DIDS	pHi	7.118±0.027	7.276±0.023	0.01
0.1 mM	pHe	7.281±0.011	7.223±0.012	0.01
Furosemide	pHi	7.095±0.035	7.195±0.031	0.01
0 mM Na^++	pHe	7.242±0.004	7.212±0.004	0.01
0.1 mM DIDS	pHi	7.028±0.035	7.049±0.020	NS

removing sodium from the incubation movement. The pH-stat experiments of Cossins & Richardson (1984) have also shown that adrenaline induces sodium-dependent acid extrusion in SITS-treated cells. From these data it appears that adrenergic stimulation of fish red cells initially activates the sodium/proton exchange. Consequently, chloride is accumulated in the cell either because of sodium/chloride cotransport as suggested by Baroin et al. (1984) or in exchange for HCO_3^-, as suggested by Nikinmaa & Huestis (1984). The accumulation of sodium and, especially chloride leads to an increase in cell volume. The adrenergic changes are pH dependent; the water content of rainbow trout red cells increased more at low than at high extracellular pH values (M. Nikinmaa, J. F. Steffensen, B. L. Tufts & D. J. Randall, unpubl.). Also, at a high pH (8.2), 10^{-5} M isoproterenol (a beta-adrenergic agonist) did not affect the intracellular pH.

The mechanism by which adrenergic drugs affect the sodium/proton exchange in fish red cells is not known. However, generally sodium/proton exchange is activated by a decrease in intracellular pH (e.g. Aronson et al. 1982; Grinstein et al. 1984; Moolenaar et al. 1984; Vigne et al. 1984). It is conceivable that this general dependence of sodium/proton exchange on internal pH may be important also in fish red cells, making it possible that sodium/proton exchange be activated without adrenergic stimulation as a response to acid load.

Cytoplasmic alkalinization is affected by the epidermal growth factor in A431 (an epidermoid carcinoma) cells (Rothenberg et al. 1983), by alpha-thrombin and insulin in human fibroblasts (L'Allemain et al. 1984) and phorbol esters in lymphocytes (Grinstein et al. 1985). Thus, in all of these cells external factors cause a similar response to adrenergic stimulation of fish red cells. Grinstein et al. (1985) have proposed the following mechanism for internal alkalinization and consecutive volume increase in lymphocytes after phorbol ester treatment: (1) phorbol ester stimulates cellular protein kinase C, which leads to (2) activation of sodium/proton exchange and (3) cytoplasmic alkalinization. As a result of the cytoplasmic alkalinization (4) bicarbonate and chloride are accumulated in the cell followed by (5) osmotically obliged water which causes (6) increase in cell volume. These responses are very similar to the changes observed in fish red cells. The similarity becomes even more pronounced, as beta-adrenergic stimulation commonly activates cellular protein kinases (e.g. Lehninger 1975).

In rainbow trout red cells, the adrenergic activation of sodium/proton exchange and consecutive events may function in the control of intracellular pH in the following fashion (see Fig. 3):

1. Adrenaline activates the sodium/proton exchange. The actively maintained sodium gradient tends to extrude protons against their electrochemical gradient.

2. As a result of the initiation of proton extrusion, the equilibrium of the reaction

$$CO_2 + H_2O \gtrless H^+ + HCO_3^-,$$

catalysed by carbonic anhydrase, is shifted to the right, and more bicarbonate and protons are formed.

3. Accumulation of bicarbonate and con-

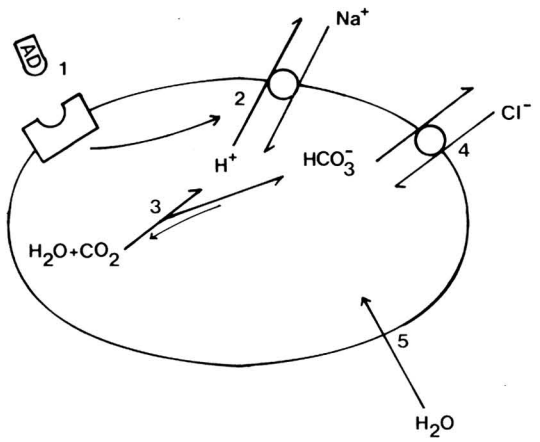

Fig. 3. A model for the adrenergic response of rainbow trout red cells. – Binding of adrenaline to membrane receptor (1) activates the sodium/proton exchange (2) which extrudes protons and shifts (3) the reaction $H_2O + CO_2 = H^+ + HCO_3^-$ to the right by removing one of the end products. Accumulation of bicarbonate (4) leads to increased efflux of HCO_3^- and influx of chloride. The accumulation of sodium and chloride draws (5) osmotically obliged water into the cell, and cell volume increases.

tinuing proton extrusion increase the intracellular pH markedly in the cells treated with anion exchange inhibitor DIDS, as observed by Nikinmaa & Huestis (1984).

4. When the anion exchange pathway is functional, as in saline-incubated rainbow trout red cells, the bicarbonate formed is rapidly exchanged with chloride, causing chloride accumulation. Chloride (and sodium) may also be accumulated via the postulated sodium/chloride cotransport pathway (Baroin et al. 1984). The accumulation of sodium, bicarbonate and chloride, leads to cell swelling. The role of anion, especially chloride, movements appears to be of prime importance, as DIDS treatment abolishes adrenergic cell volume changes even though the sodium/proton exchange is unaffected.

5. The chloride/bicarbonate exchange brings protons towards electrochemical equilibrium (as shown by the fact that the intracellular pH measurements with the DMO method, and intracellular pH calculated from the chloride distribution ratio give similar values in adrenaline-treated cells; M. Nikinmaa, J. F. Steffensen, B. L. Tufts and D. J. Randall (unpubl.). However, even if reached this new equilibrium is dependent on the function of the adrenaline-stimulated, secondarily active sodium/proton exchange, consecutive rightward shift in the equilibrium of the carbonic anhydrase reaction, accumulation of sodium, bicarbonate and chloride in the cell, and increase in cell volume. Thus, the intracellular pH increases because of adrenergic stimulation.

The mechanism suggested above can explain Heming's (1984) finding that adrenaline decreases the carbonic anhydrase activity in intact red cells. Heming measured the carbonic anhydrase activity using the modified boat assay. In this method, the production of carbon dioxide from bicarbonate is followed: if the equilibrium of the dehydration reaction is shifted towards bicarbonate, this will be seen as a decrease in apparent carbonic anhydrase activity. Also, the efflux of bicarbonate from the cells will be increased, leading to a decrease in the net influx of bicarbonate. However, this does not rule out the possibility that adrenaline would directly affect bicarbonate movements in and out of the cell.

Regardless of the mechanism by which adrenaline affects the bicarbonate and carbon dioxide equilibria, the adrenaline-induced changes in red cell metabolism may both improve the oxygen transport (by increasing intracellular pH, as suggested by Nikinmaa 1982; 1983), and limit the loss of buffer base (by decreasing the bicarbonate dehydration and consecutive diffusion of CO_2 out of the animal, as suggested by Heming 1984).

Interestingly, the intracellular pH increases more when isoproterenol (final concentration 10^{-5}) is added to the in vitro incubations diluted in plasma than when it is added diluted in saline (M. Nikinmaa, J. F. Steffensen, B. L. Tufts & D. J. Randall, unpubl.), although the red cell volume increases similarly in both treatments. The residual catecholamine concentration remaining in the added plasma is likely to be less than 1 % of the total amount of catecholamines added to the incubation, and cannot, therefore, be the cause of the observed difference. This indicates a role for an unknown plasma component in the adrenergic responses of red cells, and may partly explain why the effects of adrenaline in vitro (e.g. Nikinmaa 1982) seem to require higher adrenaline concentrations than adrenergic responses in vivo (e.g. Primmett et al. 1985). It is possible that other hormones, like thyroid hormone or corticosteroids, or prostaglandins will enhance the effects of catecholamines on the red cells.

3.3. Adjustments of red cell pH to changes in the external and internal environment of the fish

3.3.1. Temperature changes

The effect of temperature on the red cell pH of fish has received little attention. Albers et al. (1983) have shown that, measured at a constant temperature, the intracellular pH of carp is not affected by acclimation temperature. Also, the relation between intra- and extracellular pH is independent of the acclimation temperature (10 or 20°C), being

$$pHi = 6.1+(0.853-0.159\times S)\times(pHe-6.21)$$

where S is the fractional oxygen saturation of haemoglobin. This would indicate that the red cell pH changes passively with changes in temperature.

In many cases changes in acclimation temperature are associated with changes in the total organic phosphate concentration of blood (see e.g. Nikinmaa et al. 1980; Laursen et al. 1985). Also, the cell volume appears to be different in fish acclimated to high and to low temperatures (for review see Houston 1981). The effects of these changes on the intracellular pH have not, however, been investigated. Nor is it known how acute temperature changes affect red cell pH.

3.3.2. Effects of environmental acidification (external acid loads)

As a result of external acidification, the plasma pH tends to drop (for review see Heisler 1984). At low environmental pH values the red cells swell markedly (see e.g. Neville 1979; McWilliams 1980; Hobe et al. 1984; Milligan & Wood 1982). A decrease in plasma osmolarity and consequent influx of water into the red cells may partly account for this (Milligan & Wood 1982). Also, the plasma acidosis causes an increase in the plasma catecholamine concentration (Boutilier et al. 1985), which will affect cellular volume. Furthermore, it is possible that the plasma acidification per se could activate the sodium/proton exchange (see above) and lead to an increase in cell volume. Whatever the mechanism, the cellular swelling reduces the pH changes within the red cells, thus improving oxygen loading in gills in an acid medium.

3.3.3. Effects of hypoxia and hypercapnic hypoxia

Hypoxia commonly leads to hyperventilation (e.g. Hughes 1973) which may cause a respiratory alkalosis, as in carp (Dejours 1973), or may counteract the effects of lactate accumulation so that plasma pH remains unchanged, as in rainbow trout (Nikinmaa & Soivio 1982). In hypercapnic hypoxia the plasma pH decreases (Jensen & Weber 1982; 1985b). In most cases, acclimation to hypoxia causes an increase in red cell pH (Wood & Johansen 1973; Nikinmaa & Soivio 1982). As discussed above, the mechanism involves changes in the organic phosphate metabolism (for review see also Nikinmaa 1981; Weber 1982) as well as cell volume changes (Holeton & Randall 1967; Soivio & Nikinmaa 1981; Nikinmaa & Soivio 1982; Jensen & Weber 1982; 1985a; 1985b). Nikinmaa, Cech & Ryhänen (to be published) have recently shown that the hypoxia-induced increase in cellular water content of carp can be almost completely abolished by injecting the beta-blocker, propranolol, into the circulation before the stress, suggesting a role for beta-adrenergic responses in hypoxia. Jensen & Weber (1985a; 1985b) have similarly suggested that beta-adrenergic responses may play a role in the acclimation of tench to hypoxia. Notably, the adrenaline concentration of at least one elasmobranch fish increases in hypoxia (Butler et al. 1978).

The studies of Jensen & Weber (1985a; 1985b) on tench subjected to hypercapnic hypoxia showed that despite the marked decrease of plasma pH, the red cell pH increased, as did the cell volume. The intracellular pH was 0.3 units higher than that expected from the in vitro behaviour of tench blood. It appears that the changes in cell volume (and consequent changes in Donnan distribution of protons) are not adequate to completely account for the increase in intracellular pH. These data suggest that the effects of active acid extrusion in hypoxic-hypercapnic tench may exceed those of the chloride/bicarbonate exchange. However, more conclusive data on this point are required.

3.3.4. Effects of exercise (internal acid loads)

The catecholamine-mediated control of intracellular pH has been clearly demonstrated

in response to exhausting swimming exercise in trout (Primmett et al. 1985) and to escape activity in striped bass (Nikinmaa et al. 1984). In both cases the red cell pH can be maintained despite the marked extracellular acidification induced by the stress. As a result, oxygen transport can be maintained. When striped bass were treated with beta-adrenergic antagonist, both the arterial oxygen concentration and intracellular pH were lower than in 'control' chased animals (Nikinmaa et al. 1984).

However, the adrenergic response is not always present. Jensen et al. (1983) did not observe any cellular swelling in response to chasing in tench. Nikinmaa & Jensen (1986) moreover found that 'winter' rainbow trout were completely devoid of the adrenergic responses to chasing: their red cell pH decreased, and red cell volume apparently remained unchanged. Thus, there may be differences in the adrenergic responses to stress between species, and the acclimation history of the animals may be an important determinant of the adrenergic response. Additionally, the changes in adrenaline concentration are dependent on how the animals are exercised: when rainbow trout swim to exhaustion against a current, the blood catecholamine concentration increases less than when the fish are physically disturbed e.g. by tail-grabbing (Butler & Metcalfe 1985).

Acknowledgements. The author's original research reviewed in this article has been supported by grants from the University of Helsinki and the Finnish National Research Council.

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Received 13.IX.1985
Printed 22.IV.1986