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**REVIEW**

# Red blood cell pH, the Bohr effect, and other oxygenation-linked phenomena in blood $O_2$ and $CO_2$ transport

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**Abstract**

The discovery of the S-shaped $O_2$ equilibrium curve and the Bohr effect in 1904 stimulated a fertile and continued research into respiratory functions of blood and allosteric mechanisms in haemoglobin (Hb). The Bohr effect (influence of pH/$CO_2$ on Hb $O_2$ affinity) and the reciprocal Haldane effect (influence of $HbO_2$ saturation on $H^+/CO_2$ binding) originate in the Hb oxy–deoxy conformational change and allosteric interactions between $O_2$ and $H^+/CO_2$ binding sites. In steady state, $H^+$ is passively distributed across the vertebrate red blood cell (RBC) membrane, and intracellular pH ($pH_i$) changes are related to changes in extracellular pH, Hb-$O_2$ saturation and RBC organic phosphate content. As the Hb molecule shifts between the oxy and deoxy conformation in arterial-venous gas transport, it delivers $O_2$ and takes up $CO_2$ and $H^+$ in tissue capillaries (elegantly aided by the Bohr effect). Concomitantly, the RBC may sense local $O_2$ demand via the degree of Hb deoxygenation and release vasodilatory agents to match local blood flow with requirements. Three recent hypotheses suggest (1) release of NO from *S*-nitroso-Hb upon deoxygenation, (2) reduction of nitrite to vasoactive NO by deoxy haems, and (3) release of ATP. Inside RBCs, carbonic anhydrase (CA) provides fast hydration of metabolic $CO_2$ and ensures that the Bohr shift occurs during capillary transit. The formed $H^+$ is bound to Hb (Haldane effect) while $HCO_3^-$ is shifted to plasma via the anion exchanger (AE1). The magnitude of the oxylabile $H^+$ binding shows characteristic differences among vertebrates. Alternative strategies for $CO_2$ transport include direct $HCO_3^-$ binding to deoxyHb in crocodilians, and high intracellular free [$HCO_3^-$] (due to high $pH_i$) in lampreys. At the RBC membrane, CA, AE1 and other proteins may associate into what appears to be an integrated gas exchange metabolon. Oxygenation-linked binding of Hb to the membrane may regulate glycolysis in mammals and perhaps also oxygen-sensitive ion transport involved in RBC volume and $pH_i$ regulation. Blood $O_2$ transport shows several adaptive changes during exposure to environmental hypoxia. The Bohr effect is involved via the respiratory alkalosis induced by hyperventilation, and also via the $pH_i$ change that results from modulation of RBC organic phosphate content. In teleost fish, $\beta$-adrenergic activation of $Na^+/H^+$ exchange rapidly elevates $pH_i$ and $O_2$ affinity, particularly under low $O_2$ conditions.

*Keywords* blood gas transport, Bohr–Haldane effect, erythrocyte, haemoglobin, membrane interaction, oxygen-sensitive ion transport.

 

A century ago, in 1904, Christian Bohr discovered the S-shaped form of the whole blood $O_2$ dissociation curve (Astrup & Severinghaus 1985), and the famous paper describing the influence of $CO_2$ on blood $O_2$ equilibria was published (Bohr *et al.* 1904). Both discoveries were of fundamental importance for the understanding of the respiratory functions of blood and had tremendous impact on research into haemoglobin (Hb) function and blood gas transport for decades to come. Christian Bohr was primus motor in the study, but the two co-authors (both assistants in Bohr's laboratory in Copenhagen at that time), are also worth mentioning: K.A. Hasselbalch became a pioneer in acid–base physiology, and August Krogh became one of greatest physiologist in the 20th century. In fact, it was the talent of Krogh in designing apparatus and methods that made possible the investigation of the influence of $CO_2$ on blood oxygen binding (Schmidt-Nielsen 1984, Edsall 1986). In the original study, increased $CO_2$ tensions were shown to decrease $O_2$ affinity of dog whole blood (Fig. 1; Bohr *et al.* 1904). A few years later (1910), studies from Joseph Barcroft's laboratory in Cambridge showed that addition of acid (lowering of pH) caused a similar displacement of the $O_2$ dissociation curve as an elevation of $Pco_2$ (Astrup & Severinghaus 1985). Indeed, it is the simultaneous change in pH that is the main explanation for the influence of $CO_2$ on $O_2$ affinity, although $CO_2$ also exerts a specific effect on $O_2$ affinity at constant pH. The influence of $CO_2$ (pH) on blood/Hb $O_2$ binding was assigned 'the Bohr effect' by Haldane (Astrup & Severinghaus 1985). From the influence of $CO_2$ on Hb $O_2$ binding it follows logically that a reciprocal effect (i.e. influence of Hb $O_2$ saturation on $CO_2$ binding) must exist. Bohr and co-workers addressed the issue in their original paper by stating 'in our experiments no certain effect of $O_2$ tension on the simultaneous $CO_2$ absorption was observed'. In fact, it took 10 years following the discovery of the Bohr effect before a higher binding of $CO_2/H^+$ in deoxygenated than oxygenated blood became firmly established through the classical publication by Haldane and co-workers (Christiansen *et al.* 1914).

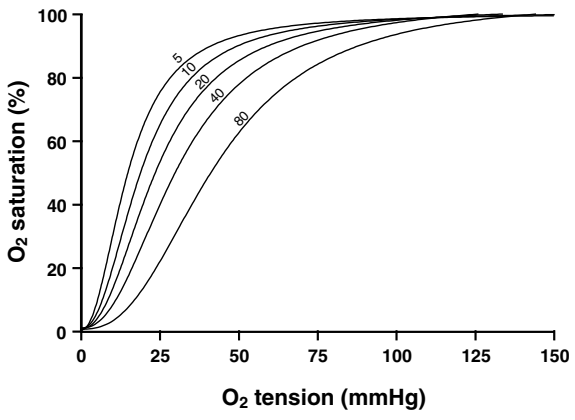


**Figure 1** Influence of $CO_2$ on $O_2$ equilibrium curves of dog blood at 38 °C. Numbers at each curve show the $CO_2$ tension in mmHg. The equilibrium curves were drawn using the data listed in Table IV of the paper by Bohr *et al.* (1904).

The biological function of the $CO_2$ effect was clearly recognized by Bohr and colleagues: $CO_2$ entering the tissue capillaries promotes the release of oxygen by decreasing the blood $O_2$ affinity, and in the lung capillaries the efflux of $CO_2$ to the alveoli increases blood $O_2$ affinity and improves Hb $O_2$ binding. Also, it is now a well established fact that the sigmoid $O_2$ equilibrium curve means that more oxygen can be given off to the tissues for a given decrease in $Po_2$ than if the curve had been hyperbolic. With current terminology one can say that the Bohr *et al.* (1904) paper revealed both the homotropic interaction in Hb oxygen binding (i.e. cooperativity as reflected by the sigmoid curve form) and the heterotropic interaction between binding of different ligands (i.e. the decrease in $O_2$ affinity upon binding of $H^+/CO_2$) (Edsall 1986).

The effect of pH on Hb $O_2$ binding made it clear that the microenvironment inside the red blood cells (RBCs) is of profound importance for Hb function. Notably, since blood is a two compartment system with Hb located inside the RBCs, it is the erythrocyte pH rather than plasma pH that is the true influential factor. It also soon became obvious that species differences in $O_2$ affinity exists, and that this can be seen as an adaptation to environment, as exemplified by the finding of higher blood $O_2$ affinity in hypoxia-tolerant fish than in fish living in well-aerated waters (Krogh & Leitch 1919). Interestingly, it was speculated that some unknown factors could play a role in this adaptation, as evident from the following citation from the Krogh & Leitch (1919) paper:

> 'We believe that the adaptation of the fish blood must be brought about by some substance or substances present along with the hæmoglobin within the corpuscles, and we wish to point out the general significance of the hæmoglobin being enclosed in corpuscles surrounded by semipermeable membranes. By this arrangement just that chemical environment can be secured which is most suitable for the respiratory function of the hæmoglobin in that particular organism, while at the same time the chemical composition of the blood plasm can be adapted, as it must needs be, to the general requirements of the body cells'.

This statement reflects genuine insight, and, with today's knowledge in mind, it is almost as if the influence of organic phosphates on $O_2$ affinity (not to be discovered until the late 1960s) was foreseen.

The Bohr effect (influence of $pH/CO_2$ on $O_2$ binding) and the reciprocal Haldane effect (influence of $O_2$ binding on $H^+/CO_2$ binding) have the same molecular

 

origin and can be viewed as two sides of the same coin. The Bohr–Haldane effect has become the classical example of the appropriate allosteric interaction between ligand binding sites that makes Hb elegantly constructed to fulfil the $O_2$ and $CO_2$ transport needs in the blood. Recent research has suggested that linking of RBC/Hb oxygenation with physiological needs extends even further. Thus, changes in RBC oxygenation may mediate appropriate release of effectors that control local blood flow, and cause modulation of ion transport mechanisms that are involved in the control of RBC pH and volume. Another intriguing recent advance is that proteins involved in various aspects of respiratory gas transport and red cell acid–base status are structurally associated in an appropriate fashion at the cellular level. The present contribution to the hundred year anniversary for the publication of the Bohr *et al.* (1904) paper in this journal has the purpose to briefly review the Bohr effect and other oxygenation-linked phenomena in the control of red cell pH and blood $O_2$ and $CO_2$ transport. The treatise will be comparative, primarily with examples from the two most intensively studied vertebrate groups: mammals and fish.

## Molecular mechanisms of the Bohr effect and the basic linkage equation

The molecular origin of the Bohr effect was not dealt with in the Bohr *et al.* (1904) paper, but Haldane and colleagues (Christiansen *et al.* 1914) mentioned two possible mechanisms for the influence of $HbO_2$ saturation on blood $CO_2$ binding:

> 'It may be that the oxy-hæmoglobin molecule is more acid. Another possible explanation is that when oxy-hæmoglobin in blood is reduced the molecules aggregate to a greater extent'.

The first of these two possibilities explains the alkaline Bohr effect (at acid pH some vertebrate haemoglobins show an acid or reversed Bohr effect, which will not be addressed in the present paper) at physiological pH of most vertebrates. The latter possibility is, however, also valid in some species. Thus, pH- and deoxygenation-dependent association of tetramers occurs in some frogs and birds (Riggs 1988), and hagfish and lamprey Hbs are monomeric in the oxygenated state and aggregate to oligomers upon deoxygenation (Fago & Weber 1995, Nikinmaa *et al.* 1995).

The normal alkaline Bohr effect originates in the conformational change of tetrameric Hb between the R (oxy) and T (deoxy) structure, which changes the molecular surroundings of specific amino acid residues (the Bohr groups) in the molecule, altering their $pK_a$. Oxygenation (binding of $O_2$ to the haem groups) decreases the $pK_a$ of the Bohr groups (Hb becomes more acid) and protons are released:

$$\text{H-Hb} + \text{O}_2 \leftrightarrow \text{Hb}^- - \text{O}_2 + \text{H}^+$$

Reciprocally, preferential binding of Bohr protons to the low $O_2$ affinity T structure stabilizes this structure by introducing extra bonds, delaying the transition to the high $O_2$ affinity R structure, whereby the $O_2$ affinity of the haem groups is decreased. Two major Bohr groups are the C-terminal histidine of $\beta$-chains (accounting for up to 50% of the Bohr effect in vertebrate Hbs) and the N-terminus of $\alpha$-chains, but additional groups contribute (Riggs 1988, Lukin & Ho 2004).

The tight relationship between the Bohr and Haldane effects is illustrated by the linkage equation that reveals identity between the Bohr and Haldane coefficients (Wyman 1964).

For a symmetrical $O_2$ equilibrium curve, this relation can be simplified to:

$$-(\Delta \log P_{50}/\Delta \text{pH}) = 1/4\Delta Z_{\text{H}},$$

where $P_{50}$ is the $O_2$ tension at half saturation, and $Z_H$ is the number of protons taken up per tetramer upon a full change from oxy- to deoxyHb. The Bohr effect can accordingly be studied both by measurements of $O_2$ equilibria and by $H^+$ titration, and both methods have been widely used (Kilmartin & Rossi-Bernardi 1973, Jensen *et al.* 1998a). The allosteric interaction between $O_2$ and $H^+$ binding sites is further modulated by organic phosphates. Organic phosphates bind preferentially to the T structure in the central cavity between the two $\beta$-chains and decrease $O_2$ affinity (Perutz 1983). Presence of organic phosphates at physiological concentrations additionally increases the Bohr effect (Kilmartin & Rossi-Bernardi 1973, Jensen *et al.* 1998a).

The magnitude of the Bohr effect varies between vertebrate classes. In general, elasmobranch fishes have low or insignificant Bohr effects, mammals and birds have intermediate Bohr effects and teleost fishes and lampreys have high Bohr effects (Jensen 1989, Nikinmaa 1997). The Bohr factor can, however, vary considerably between species within classes, as, e.g. in the diverse group of teleost fishes (Brauner & Randall 1998). Many teleosts have multiple Hb components in their RBCs. Some of these (anodic Hbs) have high Bohr effects and some (cathodic Hbs) show no (or reverse) pH sensitivity (Weber 2000). Another unique feature in teleosts is the Root effect, which is an extreme pH sensitivity, where the Hb not only shows a strong decrease in $O_2$ affinity at low pH (Bohr effect) but also loses its cooperativity (Brittain 1987, Jensen *et al.* 1998a). The physiological role of the Root effect is to drive oxygen into the swim bladder and to supply $O_2$ to the avascularized retina (Pelster 2001).

 

The specific effect of $CO_2$ is due to oxygenation-dependent binding of $CO_2$ to the α-amino groups of the α and β chains, forming carbamate (Kilmartin & Rossi-Bernardi 1973):

$$\text{Hb-NH}_3^+ \leftrightarrow \text{Hb-NH}_2 + \text{H}^+,$$

$$\text{Hb-NH}_2 + \text{CO}_2 \leftrightarrow \text{Hb-NHCOOH},$$

$$\text{Hb-NHCOOH} \leftrightarrow \text{Hb-NHCOO}^- + \text{H}^+$$

Interestingly, since carbamate formation preferentially occurs in deoxy-Hb, and the α-$NH_3^+$ group of the N-terminal amino group (α1 Val in many Hbs) is one of the Bohr groups (Riggs 1988), the release of $H^+$ associated with oxylabile carbamate formation will decrease the alkaline Bohr effect (i.e. $H^+$ uptake upon deoxygenation). In teleost Hbs the α-amino groups of α-chains are acetylated and those of the β-chains are involved in organic phosphate binding, which may explain the small specific $CO_2$ effect in these Hbs (Weber & Lykkeboe 1978, Jensen & Weber 1982). Limited oxylabile carbamate formation in teleosts accordingly helps preserving their high alkaline Bohr effect (Jensen *et al.* 1998a).

## Steady state red blood cell pH

With erythrocyte pH being the influential factor in the Bohr effect, it is important to overview its control. In the basic steady state, secondary active transport pathways (e.g. $Na^+/H^+$ exchange and $K^+/Cl^-$ cotransport) are silent and do not play a role in the control of RBC volume and pH, and the RBC resembles a Donnan system (Hladky & Rink 1977, Nikinmaa 1990). The RBC cation content stays constant, and the membrane is permeable to $Cl^-$ and $HCO_3^-$ (via the anion exchanger, AE1) as well as to water and $CO_2$, whereas it is impermeable to large intracellular anions (Hb and organic phosphates). In this Donnan-like system, $Cl^-$, $HCO_3^-$ and $H^+$ become passively distributed with a distribution ratio ($r$) between the intracellular (i) and extracellular (e) spaces:

$$r = [\text{Cl}^-]_\text{i}/[\text{Cl}^-]_\text{e} = [\text{HCO}_3^-]_\text{i}/[\text{HCO}_3^-]_\text{e} = [\text{H}^+]_\text{e}/[\text{H}^+]_\text{i}$$

A Donnan-like distribution of protons across the membrane has been reported for both anucleated mammalian RBCs (e.g. Funder & Wieth 1966, Hladky & Rink 1977) and nucleated RBCs of lower vertebrates (e.g. Heming *et al.* 1986). The distribution ratio, and thus RBC $pH_i$, is controlled by the amount and net charge carried by the RBC impermeable anions. Their negative charge at physiological pH means that $r < 1$, whereby $[H^+]_i > [H^+]_e$ and $pH_i < pH_e$ (Hladky & Rink 1977).

The net charge of the impermeable anions is physiologically changed through changes in $pH_e$, changes in $HbO_2$ saturation and changes in the cellular organic phosphate content. A decrease in $pH_e$ causes a decrease in $pH_i$. The intracellular $H^+$-buffering by Hb and organic phosphates decreases their negative charge, which increases $r$ and thereby the intracellular $[Cl^-]$ and $[HCO_3^-]$, causing water influx and cell swelling. The relationship between $pH_i$ and $pH_e$ is well-described by linear regression, and in oxygenated blood the slope ($\Delta pH_i/\Delta pH_e$) of the line typically is 0.6–0.75 (Fig. 2). The $\Delta pH_i/\Delta pH_e$ value is important for estimating the intracellular Bohr factor from the extracellular Bohr factor (Duhm 1976). For example, in tench the extracellular Bohr coefficient ($-\Delta\log P_{50}/\Delta pH_e$) is 0.72 and $\Delta pH_i/\Delta pH_e$ is 0.61, resulting in an intracellular (true) Bohr factor ($-\Delta\log P_{50}/\Delta pH_i$) of: 0.72/0.61 = 1.18 (Jensen & Weber 1982). The position of the $pH_i$ vs. $pH_e$ relationship varies between species, reflecting large species differences in the transmembrane pH gradient (Fig. 2). In oxygenated human RBCs, $pH_i$ is 7.2 at the physiological $pH_e$ of 7.4 (Hladky & Rink 1977), giving a transmembrane gradient of 0.2 pH units, whereas in the fish tench (physiological $pH_e$ of 8.05) the gradient is 0.7 pH units (Jensen & Weber 1982).

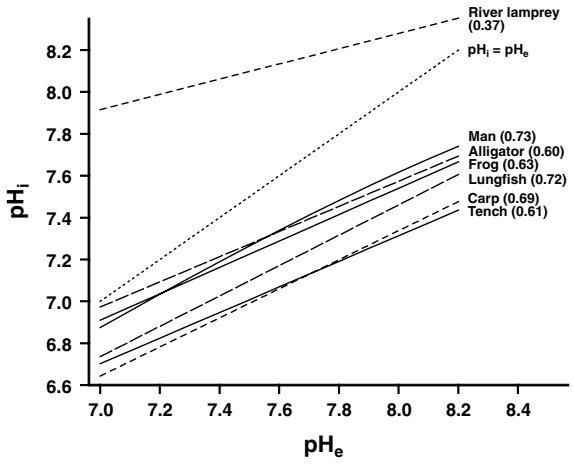


**Figure 2** Relationship between red blood cell $pH_i$ and extracellular $pH_e$ in oxygenated blood of selected vertebrates. In steady state, $H^+$ is passively distributed across the RBC membrane with $pH_i < pH_e$. Species and $\Delta pH_i/\Delta pH_e$ values are depicted to the right. River lamprey makes a special case. In this species $pH_i$ is maintained higher than $pH_e$ by secondarily active $Na^+/H^+$ exchange. Data: man (Funder & Wieth 1966), alligator *Alligator mississippiensis* (Jensen *et al.* 1998c), frog *Rana temporaria* (Wells & Weber 1985), African lungfish *Protopterus aethiopicus* (Jensen *et al.* 2003), carp *Cyprinus carpio* (Albers *et al.* 1983), tench *Tinca tinca* (Jensen & Weber 1982), river lamprey *Lampetra fluviatilis* (Nikinmaa 1986).

Deoxygenation increases *r* due to the uptake of Bohr protons by the Hb. The increase in $pH_i$ upon deoxygenation reaches 0.3–0.4 pH units in teleost RBCs (Albers *et al.* 1983, Jensen 1986, Brauner *et al.* 1996), which exceed mammalian standards by a factor of 10. Lampreys (that actively maintain high RBC $pH_i$ – cf. Fig. 2 and below) show similar large $pH_i$ changes with $O_2$ saturation (Nikinmaa *et al.* 1995). The large $\Delta pH_i$ (deoxy-oxy) in teleosts and lampreys is caused by high oxygenation-linked $H^+$ binding coupled with low buffer values at fixed Hb conformation (Jensen 1986, 1989, Nikinmaa 1997). Furthermore, as conclusively shown for teleosts, the $pH_i$ rise with deoxygenation may not be linearly related to $O_2$ saturation. The major change occurs between 100 and 40% saturation in tench (Fig. 3; Jensen 1986), rainbow trout (Brauner *et al.* 1996) and tuna (Lowe *et al.* 1998), suggesting almost full exploitation of the oxygenation-linked $H^+$ exchange within resting arterial-venous $O_2$ saturation differences.

An increase in the RBC content of organic phosphates, as seen with 2,3-DPG in human RBCs at high altitude (Lenfant *et al.* 1968), affects the Donnan distribution ratio in the expected manner, leading to a decrease in $pH_i$ (Duhm 1972). In teleost fish, hypoxia decreases the RBC organic phosphate (ATP and/or GTP) content, elevating steady state $pH_i$ (Wood & Johansen 1973). In both cases the Bohr shift associated with the $pH_i$ change makes a contribution to the blood $O_2$ affinity change during hypoxia that is equal to or even higher than the direct allosteric effect of the phosphates on $HbO_2$ binding (Duhm 1972, Wood & Johansen 1973, Jensen & Weber 1982).

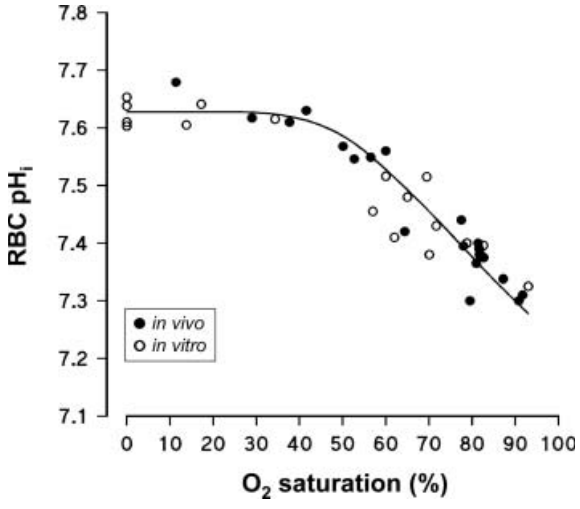


**Figure 3** Non-linear relation between RBC $pH_i$ and blood $O_2$ saturation in tench *Tinca tinca* at $pH_e$ 7.9 (from Jensen 1986).

## Arterio-venous transport of $O_2$

The high $Po_2$ normally encountered at the respiratory surfaces (lungs/gills) causes close to full $O_2$ saturation of the blood. As the blood subsequently is circulated to the tissues, it gives off $O_2$ while passing the capillary beds, where $Po_2$ decreases. At rest, perhaps some 25% of the $O_2$ is offloaded, placing the venous point 'on the shoulder' of the $O_2$ equilibrium curve. Any further $O_2$ offloading will accordingly proceed on the steep portion of the curve. It is important that the $O_2$ delivery satisfies the tissue $O_2$ need. When tissue $O_2$ consumption increases, capillary $Po_2$ decreases further, and more $O_2$ is extracted from the blood. The Bohr effect significantly augments the $O_2$ delivery, because metabolically produced $CO_2$ acidify the RBCs and drives off more $O_2$ at any given capillary $Po_2$. The presence of carbonic anhydrase (CA) in vertebrate RBCs is essential for the Bohr effect. CA secures rapid $CO_2$ hydration and RBC acidification in tissue capillaries (and the reverse in lung/gill capillaries), thus allowing the Bohr shift to occur during capillary transit (Maren & Swenson 1980). In heavy exercise, when a critical low capillary $Po_2$ is reached, lactic acidosis in muscle capillary blood further increases $O_2$ dissociation and facilitates $O_2$ delivery (Stringer *et al.* 1994).

Local vasodilation in the metabolically active tissues secures an adequate blood supply, and the recruitment of more capillaries improves tissue $O_2$ diffusion conductance. Many mechanisms, both neural and humoral, are involved in the regulation of the microcirculation. Nitric oxide produced in vascular endothelial cells relaxes nearby vascular smooth muscle and thereby causes local vasodilation. Interestingly, the RBCs themselves may play a key role in local blood flow regulation by sensing local $O_2$ demand through Hb deoxygenation and matching the release of vasodilatory compounds to the degree of deoxygenation. Three attractive hypotheses have been proposed in recent years:

The NO radical is effectively scavenged by Hb (e.g. via reaction with oxyHb to form metHb and nitrate), but the biological activity of NO is not completely lost in blood vessels. It has been hypothesized that NO can bind to the minor fraction (1%) of deoxygenated haems in oxygenated blood, become transferred to Cys$\beta$93 to form *S*-nitroso-Hb, and subsequently be released as NO when the blood is deoxygenated by declining $O_2$ tension in the microcirculation (Jia *et al.* 1996, Stamler *et al.* 1997, Pawloski *et al.* 2001). The ensuing vasodilation would accordingly grade blood flow to local $O_2$ requirements. The mechanism is currently debated and has been questioned for various reasons (Fago *et al.* 2003, Gladwin *et al.* 2003, Herold 2003).

An alternative idea is that nitrite can be a vascular storage pool of NO. Nitrite (present at approx 0.5 $\mu$M

 

in human plasma) is an oxidative metabolite of NO, but NO can be regenerated from $NO_2^-$. Arterial-venous [$NO_2^-$] differences (Gladwin *et al.* 2000) and nitrite infusion-caused vasodilation (Cosby *et al.* 2003) support the idea that nitrite acts as NO donor in humans. Fish exposed to nitrite similarly show cardiovascular changes that support NO generation from nitrite and its potent vasoactivity (Aggergaard & Jensen 2001, Jensen 2003). It was recently found that nitrite is reduced to NO by deoxyHb (that thus functions as a nitrite reductase), so that nitrite entering the RBCs is converted to NO to an extent that depends upon the degree of deoxygenation of blood (Cosby *et al.* 2003). The ensuing vasodilation may therefore potentially increase blood flow to a level that matches local $O_2$ needs (Fig. 4). The mode of nitrite entry into erythrocytes is an essential part of the mechanism. In fish, nitrite preferentially permeates the membrane at low $O_2$ saturation (Jensen 1990, 1992, 2003), thus apparently supplying nitrite for intracellular NO generation in an appropriate way. Information on mammalian RBCs is limited, but in pig the oxygenation-dependency of nitrite entry seems smaller than in fish (Jensen 2003).

A third mechanism by which RBCs can act as a sensor and effector of local $O_2$ delivery is through release of ATP. Mammalian RBCs release ATP in response to low $O_2$ levels (Ellsworth *et al.* 1995, Jagger *et al.* 2001). The extracellular ATP subsequently binds to purinergic receptors and elicits vasodilation, partly via stimulation of NO synthesis and release from vascular endothelial cells. The ATP release is linked to the degree of Hb deoxygenation, apparently providing a mechanism for matching local blood flow to $O_2$ demand (Ellsworth *et al.* 1995, Ellsworth 2000, Jagger *et al.* 2001, González-Alonso *et al.* 2002).

All the above three hypotheses have the R $\rightarrow$ T conformational change as an essential event for sensing $O_2$ conditions and mediating an appropriate response. Thus, the role of the Hb oxy-deoxy conformational change in securing adequate $O_2$ delivery may extend far beyond the classical Bohr effect. The relative importance of each mechanism and their distribution among species (e.g. in lower vertebrates) is, however, not known.

## Blood $CO_2$ transport

The $CO_2$ produced in metabolism diffuses into the tissue capillary blood. Due to the absence of CA in plasma (except in some elasmobranchs – cf. Gilmour *et al.* 2001), $CO_2$ is not hydrated here to any appreciable extent, and most $CO_2$ enters the RBCs. As a lipophilic molecule, $CO_2$ rapidly diffuses through the lipid bilayer of the RBC membrane (Klocke 1988, Geers & Gros 2000). Recent research points to an additional entry of $CO_2$ via aquaporin-1 (AQP1) and the anion exchanger AE1 (Forster *et al.* 1998, Cooper *et al.* 2002, Blank & Ehmke 2003), but this is debated (Verkman 2002). Inside the RBC, diffusion of $CO_2$ is slowed down by the high concentration of Hb, and facilitated $CO_2$ diffusion (diffusion of $HCO_3^-$ and simultaneous buffer-facilitated $H^+$ diffusion) may be more important (Geers & Gros 2000). Diffusion in RBCs does not limit overall $CO_2$ exchange, because diffusion distances generally are in the order of one micrometer or less (Klocke 1988).

Carbamate has been estimated to contribute some 13% to $CO_2$ exchange in man (Klocke 1988), but recently an estimate of 5% was found more reasonable at rest (Geers & Gros 2000). In teleost fish, low specific $CO_2$ effects (cf. above) and low physiological $Pco_2$ render oxylabile carbamate formation of insignificant importance for $CO_2$ transport. Some elasmobranch Hbs do not have acetylated α-amino groups of α-chains, and oxylabile carbamino formation may be more significant in these.

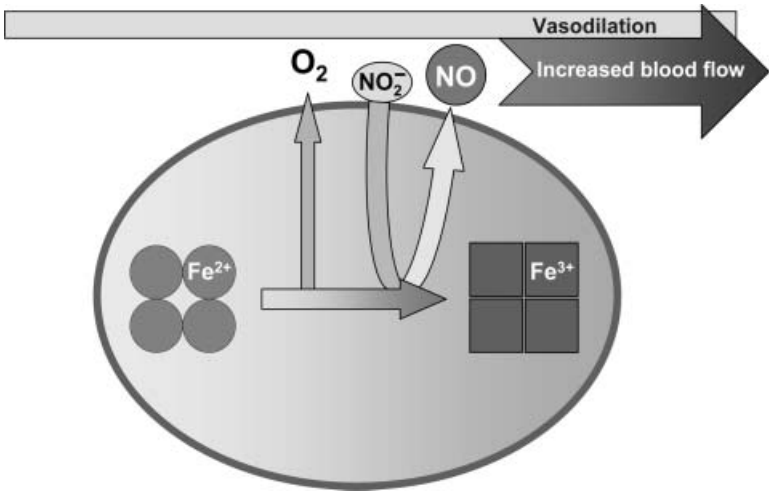


**Figure 4** One of three recent hypotheses (see text) in which the degree of RBC deoxygenation may be involved in adjusting blood flow to tissues. Nitrite (an oxidative product of NO production in vascular endothelial cells) enters the RBC when $Po_2$ and $O_2$ saturation decreases, and is reduced to NO by a fraction of the deoxy haems. Release of NO from RBCs subsequently vasodilates vessels and increases blood flow (Cosby *et al.* 2003). Oxidized haem groups ($Fe^{3+}$) can be reduced to $Fe^{2+}$ by afterwards RBC metHb reductase activity.

Inside the RBC, CA catalyses $CO_2$ hydration, and most $CO_2$ is converted to $HCO_3^-$ and $H^+$. Many vertebrates have two erythrocytic CA isozymes: CA I (with relatively low turnover rate) and isozyme CA II (high turnover rate), but agnathans and elasmobranchs have only a CA I-like isozyme and teleost fishes have CA II (Henry & Heming 1998). The acceleration of $CO_2$ hydration/dehydration by erythrocytic CA is essential for an efficient $CO_2$ transport, and the venous-arterial difference in total $CO_2$ would decrease drastically in its absence (Geers & Gros 2000).

In most vertebrates, both endproducts of the CA-catalysed equilibrium reaction are removed, because $H^+$ is bound to Hb, whereas $HCO_3^-$ is transported to plasma in exchange for $Cl^-$ via AE1. Thus, the reaction can proceed further towards bicarbonate formation, increasing the $CO_2$ carrying capacity of the blood, with most $CO_2$ being carried as $HCO_3^-$ in plasma. In man, the relative contributions of the Bohr–Haldane effect and the anion exchange to $HCO_3^-$ formation are about equal (Wieth *et al.* 1982).

The Hb-$H^+$ equilibria consist of basic $H^+$ buffering and oxygenation-linked $H^+$ binding (fixed acid Bohr–Haldane effect). The contribution of these two mechanisms in binding $H^+$ and aiding $CO_2$ uptake in blood varies between species. Teleost and lamprey Hbs have low buffer values at a constant oxygenation degree and high Bohr–Haldane effects, whereas elasmobranchs have high buffer values and insignificant Bohr–Haldane effects, and many other vertebrates (including mammals) have an intermediate situation (Jensen 1989, Nikinmaa 1997). The low buffer values of teleost and lamprey Hbs is due to a lower number of both total and titratable histidine residues than in other vertebrate Hbs (Jensen 1989), and in teleosts α-amino groups of α-chains are additionally acetylated. The large oxygenation-linked $H^+$ uptake and $pH_i$ increase (Fig. 3) upon deoxygenation in teleosts and lampreys increases the amount of $HCO_3^-$ formed inside the RBC at a given $Pco_2$, which is beneficial for $CO_2$ transport and $CO_2$ excretion in situations where anion exchange is rate-limiting. This strategy reaches its extreme in lampreys that are devoid of functional AE (see below). Another interesting aspect is the non-linearity of the Bohr proton exchange in teleosts (Fig. 3), which potentially changes the degree of linkage between $O_2$ and $CO_2$ exchange according to situation (Brauner & Randall 1998). Thus, whereas the full oxygenation-linked $H^+$ exchange may be exploited at resting arterial-venous $O_2$ saturation differences (Jensen 1986), the Bohr effect may almost vanish if the arterial $O_2$ saturation during hypoxia drops towards 50%; and in exercise the linkage will change as venous $O_2$ saturation decreases below 50% saturation (Brauner & Randall 1998).

A functional RBC anion exchanger that carries out facilitated diffusion of $Cl^-$ and $HCO_3^-$ has been found in all species tested, except for lampreys (Ohnishi & Asai 1985, Nikinmaa & Railo 1987) and hagfish (Ellory *et al.* 1987). Although anion exchange via AE1 is fast, it is the slowest step in $CO_2$ transport and excretion, and it is therefore considered rate-limiting in both mammals and lower vertebrates (Wieth *et al.* 1982, Tufts & Perry 1998). Anion transport rates vary between species and with temperature, but it appears that the temperature sensitivity of RBC anion exchange matches that of $CO_2$ production in ectotherms, and that the RBC anion permeability is rather similar among species when compared at their preferred temperature (Jensen *et al.* 2001). Presumably, anion exchange is completed during capillary transit at rest, but at high blood flow rates (e.g. during exercise), where transit times decrease, it may proceed after capillary passage and be involved in post-capillary $Pco_2$ and pH changes.

Lampreys that are functionally devoid of $HCO_3^-/Cl^-$ exchange carry $CO_2$ as free $HCO_3^-$ inside the RBCs (Tufts & Boutilier 1989). Due to their actively maintained high RBC pHi and large pHi increase upon deoxygenation, high intracellular [$HCO_3^-$] values can be upheld (Nikinmaa 1986, 1997). Crocodiles also carry most $CO_2$ taken up in tissue capillaries within the RBCs, but here it is as Hb-bound $HCO_3^-$, as result of allosteric $HCO_3^-$ binding to deoxyHb (Jensen *et al.* 1998c). Direct binding of $HCO_3^-$ to the Hb may also occur in hagfish (Fago *et al.* 1999).

## Interaction of soluble proteins with membrane proteins

Interestingly, CA II binds to the C-terminal cytoplasmic domain of AE1 in mammalian RBCs, where it is ideally placed to catalyse hydration of incoming $CO_2$ and to deliver $HCO_3^-$ to the membrane spanning domain of AE1 that carries out $HCO_3^-/Cl^-$ exchange (Reithmeier 2001). The metabolon may additionally involve close association with AQ1 that transports $H_2O$ (and perhaps $CO_2$) (Reithmeier 2001) and other membrane proteins that might channel $CO_2$ and $O_2$ through the membrane, thus forming an integrated $CO_2/O_2$ gas exchange unit at the RBC membrane (Bruce *et al.* 2003). Similar structural integrity may apply to lower vertebrates, as a linkage of some CA to the membrane also occurs in fish RBCs (Henry & Heming 1998).

AE1 in mammalian RBCs also contains a large N-terminal cytoplasmic domain (cdb3) that is anchored to the cytoskeleton and additionally contains binding sites for Hb and several glycolytic enzymes (Low 1986, Zhang *et al.* 2000). The interaction with Hb is oxygenation-dependent, with preferential binding of deoxyHb. The ultimate end of cdb3 contains several negatively

 

charged amino acid residues that correspond stereochemically to the positive charges of the 2,3-DPG binding site on deoxyHb (Walder *et al.* 1984). The interaction accordingly leads to a decrease in Hb-$O_2$ affinity (Walder *et al.* 1984) and an increase in the Bohr factor (Jensen *et al.* 1998b) as is the case for the interaction between Hb and organic phosphates. Hb binding to cdb3 sets $O_2$ free close to the membrane, but the affinity effect is of minimal importance for bulk $O_2$ transport. Thus, whereas the RBC membrane contains 1 million copies of AE1 (Low 1986), a single human RBC with mean cellular Hb content of 0.45 fmol has 271 million Hb tetramers (obtained by multiplication with Avogadros number), and only a minute fraction of the total Hb will be bound. The binding of Hb to the membrane may have other functions. Competition between Hb and glycolytic enzymes for common binding sites on cdb3 appears to be involved in the regulation of glycolysis in mammalian RBCs, so that binding of deoxyHb under low $O_2$ conditions displaces glycolytic enzymes and accelerates glycolysis (Walder *et al.* 1984, Messana *et al.* 1996). The interaction of Hb with cdb3 has also been suggested to mediate oxygen-sensitive ion transport involved in RBC volume and $pH_i$ regulation, possibly via conformational changes that are transmitted to other transporters via the cytoskeleton (see Motais *et al.* 1987, Gibson *et al.* 2000, and below).

The N-terminal part of the cytoplasmic domain of rainbow trout AE1 contains a cluster of negative charges (Hübner *et al.* 1992), suggesting that an interaction between cdb3 and Hb may also occur in fish. Synthetic peptides corresponding to the first 10 and 20 amino acids of the N-terminal fragment of trout cdb3 does, however, not influence $O_2$ affinity of trout Hb in spite of their large influence on human Hb (Jensen *et al.* 1998b, Weber 2000). Thus, it appears that the interaction between Hb and cdb3 in nucleated teleost RBCs (if present) is considerably weaker than in anucleated mammalian RBCs. Teleost Hbs commonly have negatively charged Asp or Glu at position NA2 of the $\beta$-chains (in contrast to positive or neutral residues in mammalian Hbs), which may weaken any interaction with the negatively charged peptides (Jensen *et al.* 1998b).

## Blood $O_2$ transport during environmental hypoxia

Species that are tolerant to hypoxia have higher blood $O_2$ affinities than less tolerant species. This is observed in all vertebrate classes from fish (Krogh & Leitch 1919) to birds (Faraci 1991) and mammals (Bouverot 1985). Often such species differences in blood $O_2$ affinity are paralleled by similar $O_2$ affinity differences for the purified Hbs, reflecting that it originates in species-specific differences in globin structure (amino acid sequence) evolved in the Hbs (Jensen *et al.* 1993). In the hypoxia-tolerant llama, however, the intrinsic $HbO_2$ affinity is low, and a high blood $O_2$ affinity is resulting from a weak Hb interaction with 2,3-DPG (Bauer *et al.* 1980). The obvious advantage of a high blood $O_2$ affinity is that it assures high arterial $O_2$ saturations at low $O_2$ tensions.

On top of the 'evolutionary' coded respiratory properties of Hb, intraspecific physiological mechanisms are needed to fine-tune blood $O_2$ transporting properties. One immediate (i.e. seconds) response to hypoxia is hyperventilation. This limits the arterial $Po_2$ decrease, and it additionally decreases arterial $Pco_2$, which induces a respiratory alkalosis. The rise in arterial pH increases blood $O_2$ affinity via the Bohr effect, thus improving $O_2$ loading at low $Po_2$. Among air-breathing vertebrates, birds are exceptionally tolerant to respiratory alkalosis, because the decrease in $Pco_2$ does not constrict cerebral vessels and oppose hypoxia-induced increases in cerebral blood flow (as is the case in mammals), and the pH-induced rise in $O_2$ affinity is important for their capability to fly at high altitude (Faraci 1991). In severe hypoxia the respiratory alkalosis may be countered by a lactacidosis, and in some aquatic environments hypoxia is accompanied by environmental hypercapnia, which opposes the ventilatory alkalosis by superimposing a respiratory acidosis (Jensen *et al.* 1993). In these situations the Bohr effect decreases $O_2$ affinity, reflecting a need for additional mechanisms to safeguard blood $O_2$ transport.

A change in $O_2$ affinity that proceeds at intermediate speed (hours to days) is modulation of RBC organic phosphate content. Whereas mammals have 2,3-DPG and birds have inositol pentaphosphate (IPP) as main RBC phosphates, ectothermic vertebrates have ATP (Weber & Jensen 1988). In fish, GTP is also of wide occurrence, and a few species contain 2,3-DPG and IPP (Val 2000). Shortly after the discovery of the allosteric influence of organic phosphates on $O_2$ affinity, it was shown that the RBC 2,3-DPG level increases within 1–2 days presence at high altitude in humans, and that this is paralleled by a decrease in blood $O_2$ affinity (Lenfant *et al.* 1968). In fish, hypoxia decreases the ATP/GTP content and increases blood $O_2$ affinity (Wood & Johansen 1973). When both ATP and GTP are present, GTP is the prime modulater, as reflected by larger decreases in GTP than ATP concentration and a greater allosteric effect of GTP than of ATP (Weber & Lykkeboe 1978, Weber & Jensen 1988). The change of $O_2$ affinity in opposite directions in hypoxia-exposed fish and mammals may look like a paradox. However, in order to properly evaluate $O_2$ affinity changes, knowledge of the arterial and venous positions on the $O_2$ equilibrium curve is required. During mild hypoxia,

 

as typically encountered by mammals, a lowered $O_2$ affinity improves $O_2$ unloading without significantly affecting arterial $O_2$ saturation, and blood $O_2$ capacitance is increased, but in severe hypoxia the loss in arterial $O_2$ saturation cancels the advantage for unloading (Turek *et al.* 1973). Consequently, in severe hypoxia, mammals (like fish) would be better suited with an increased $O_2$ affinity, and a high $O_2$ affinity is indeed what is observed in mammals native to high altitudes (see above).

An increase in blood [Hb] via erythropoiesis constitute a slow (weeks) but effective adaptation to hypoxia in mammals and birds that are transferred from sea-level to high altitude (Bouverot 1985, Maginniss *et al.* 1997). Activation of hypoxia-inducible factor-1 (HIF-1) increases the transcription of the erythropoietin gene, leading to increased circulating erythropoietin levels that subsequently stimulate proliferation and differentiation of erythroid progenitor cells into RBCs (Bunn *et al.* 1998, Jelkmann 2003). The ensuing elevated $O_2$ capacity raises $O_2$ capacitance at low $Po_2$, but there is an inherent upper limit to this strategy, as the benefits of elevated haematocrit eventually is countered by increased blood viscosity. Usage of the quantitative strategy is more variable in fish. Some fish (e.g. carp, tench, plaice) show no elevation of blood [Hb] in spite of their exposure to much more severe hypoxia than encountered by mammals or birds, whereas others (e.g. trout, yellowtail, eel) show elevations of [Hb] (Weber & Jensen 1988). In the latter case, the elevation of [Hb] results from mechanisms such as release of RBCs from the spleen rather than stimulated erythropoiesis, although erythropoiesis may be important in sustained hypoxia. The variable and limited use of elevated [Hb] in fish reflect a greater reliance on modulation of $O_2$ affinity via the above mechanism and via $\beta$-adrenergic stimulation of the RBCs.

## The $\beta$-adrenergic response in teleost RBCs

Apart from the already mentioned active maintenance of high $pH_i$ via $Na^+/H^+$ exchange in lamprey RBCs (Nikinmaa 1986, 1997, Tufts & Perry 1998), the most prominent example of secondarily active ion transport influencing $pH_i$ is the $\beta$-adrenergic response in teleost RBCs. In teleosts, the $Na^+/H^+$ exchanger is under $\beta$-adrenergic control, and its activation plays an important role in controlling $pH_i$ and Hb-$O_2$ affinity in stress (reviewed by Nikinmaa 1992, 1997). A significant role of $Na^+/H^+$ exchange for $pH_i$ regulation in teleost and lamprey RBCs is compatible with the low buffer values of their Hbs (Jensen 1989), which ensures that relatively large $pH_i$ changes can be produced by $Na^+/H^+$ exchange activity (Nikinmaa 1997).

The $\beta$-adrenergic response of teleost RBCs (Fig. 5) is elicited when catecholamines are released to the blood in various stress situations. In hypoxia this occurs at a low-$Po_2$ threshold that corresponds to 50–60% $HbO_2$ saturation, as seen in two trout species and eel (Thomas & Perry 1992). Binding of noradrenalin and adrenalin to $\beta$-adrenergic receptors in the membrane leads to accumulation of cAMP and activation of $Na^+/H^+$ exchange, so that $Na^+$ enters the cell along its electrochemical gradient (created by the $Na^+/K^+$ pump) and $H^+$ exits (Nikinmaa 1992). This elevates $pH_i$ and shifts the CA-catalysed $CO_2$ hydration towards $HCO_3^-$ formation. Surplus $HCO_3^-$ is subsequently exchanged with $Cl^-$ via AE1. There is a net uptake of $Na^+$ and $Cl^-$, which is followed by osmotically obligated water, and the RBCs swell. In the extracellular space, $HCO_3^-$ and $H^+$ may

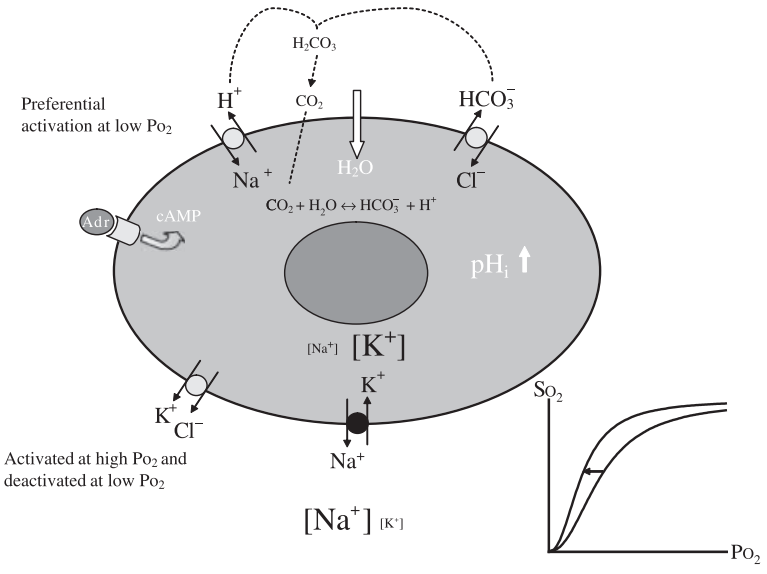


**Figure 5** The $\beta$-adrenergic response in teleost RBCs (see text for details).

 

combine and form $CO_2$, but only at the slow uncatalysed rate (CA is absent), and there is accordingly a net $H^+$ efflux from the RBC for some time after adrenergic stimulation (Motais *et al.* 1989, Nikinmaa 1992). The result of $\beta$-adrenergic stimulation is an increased $pH_i$ and an increased cell volume, which leads to an increased Hb-$O_2$ affinity (Fig. 5).

The $Na^+/H^+$ exchanger is oxygen-sensitive and is preferentially activated at low $Po_2$ (Motais *et al.* 1987, Salama & Nikinmaa 1988), whereby $\beta$-adrenergic regulation of $pH_i$ is particularly potent during hypoxia. Also, the RBCs stay swollen, because the $K^+/Cl^-$ cotransporter that normally induces regulatory volume decrease is reciprocally controlled: it is activated by high $Po_2$ but deactivated at low $Po_2$ (Fig. 5, Jensen 1990, 1992, Borgese *et al.* 1991, Nielsen *et al.* 1992). This reciprocal regulation of the two transporters may be via the same factors (Cossins & Gibson 1997). It has been proposed that oxygenation-dependent interaction of Hb with the membrane (specifically the N-terminal cytoplasmic fragment of AE1) may be involved (Motais *et al.* 1987, Jensen 1990, Borgese *et al.* 1991, Gibson *et al.* 2000), whereas other studies suggest that the oxygen dependency is mediated by reactive oxygen species, notably hydroxyl radicals (Bogdanova & Nikinmaa 2001, Nikinmaa *et al.* 2003). The absent interaction of trout Hb with peptides of the N-terminal cytoplasmic fragment of trout AE1 (as opposed to their interaction with human Hb) (Jensen *et al.* 1998b, Weber 2000), and a marked difference in $O_2$ affinity between the ion transport mechanisms and bulk Hb in trout (Berenbrink *et al.* 2000) seems to argue against the Hb hypothesis. However, Hb may interact directly with the $Na^+/H^+$ and $K^+/Cl^-$ transporters, and only a minute fraction of the Hb (that can have different $O_2$ affinity from the bulk Hb) need be involved, so definite conclusions must await further study.

The $\beta$-adrenergic response provides a mechanism for rapid (minutes) elevation of $pH_i$ and $O_2$ affinity in fish exposed to hypoxia (Tetens & Christiansen 1987), and it supplies selective protection of RBC $pH_i$ during exhausting exercise, so that $pH_i$ does not fall in spite of extracellular lactacidosis (Primmett *et al.* 1986). The Bohr shift associated with the increase in $pH_i$ is the most important factor in increasing $O_2$ affinity in hypoxia (Nikinmaa 1983), but the dilution of Hb and organic phosphates associated with cell swelling also contributes (Holk & Lykkeboe 1995). The $\beta$-adrenergic response shows species differences. In carp, tench and flounder the response is absent under normoxic conditions (e.g. during exercise-stress) but present in hypoxia, whereas salmonids show $\beta$-adrenergic swelling both during normoxic and hypoxic stress (cf. Nikinmaa 1992). In salmonids, the response can also vary with season, which appears explained by seasonal changes in RBC turnover and erythropoiesis, as young RBCs have significantly larger $\beta$-adrenergic response than older RBCs (Lecklin *et al.* 2000, Koldkjær *et al.* 2004).

## Concluding remark

The discovery of the Bohr effect (Bohr *et al.* 1904) inspired intense research into the respiratory functions of blood and Hb, which has covered all vertebrate classes. As evident from the selected examples, the study of blood gas transport continues to disclose intriguing new features, reflecting that the topic remains ripe for fruitful research also for years to come.

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