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OXYGEN AND CARBON DIOXIDE TRANSPORT IN VERTEBRATE ERYTHROCYTES: AN EVOLUTIONARY CHANGE IN THE ROLE OF MEMBRANE TRANSPORT

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Summary

Two major strategies are apparent for the regulation of gas transport by vertebrate blood except in the myxinoids, which seem to have little scope for such regulation. In lampreys and teleost fish, haemoglobins have low buffering capacities and large Bohr/Haldane effects. Na^+/H^+ exchange plays an important role in the control of haemoglobin oxygen-affinity in these vertebrate groups. The large Bohr/Haldane effect also facilitates carbon dioxide transport: the blood (or erythrocyte) pH increases upon deoxygenation, thus increasing the concentration of bicarbonate formed at a given carbon dioxide tension. In lampreys, the bicarbonate permeability of the erythrocyte membrane is low. As a consequence, extracellular acid loads cannot be buffered by haemoglobin. In contrast, teleost erythrocytes possess a functional anion exchange, allowing extracellular proton loads to be buffered by haemoglobin. However, because the buffering capacity of teleost haemoglobins is low, buffering of extracellular acid loads is less effective in teleost fish than in elasmobranch fish and in air-breathing vertebrates whose haemoglobins have a high buffering capacity. However, the high buffering capacity of the haemoglobins diminishes the possibility of regulating haemoglobin oxygen-affinity *via* secondarily active Na^+/H^+ exchange, because intracellular pH changes, caused by proton efflux, remain small.

Key words: Bohr effect, Haldane effect, Na^+/H^+ exchange, anion exchange, intracellular pH, oxygen equilibrium curve, agnathan, fish.

Introduction

The amount of oxygen transported per unit volume of blood depends on the oxygen tension, on the number of red cells, on the amount of haemoglobin within the cell and on the oxygen affinity of haemoglobin. The oxygen affinity of haemoglobin plays a dual role: haemoglobin must load oxygen effectively in the capillaries of the gas-exchange organs and unload oxygen in the tissue capillaries at as high a partial pressure of oxygen as possible in order to maintain a large diffusion gradient between the blood and the oxygen-consuming structures. Regulation of the haemoglobin oxygen-affinity has been a subject of many reviews (e.g. Bauer, 1974; Weber and Jensen, 1988; Nikinmaa, 1992). Generally, the major erythrocytic factors controlling the oxygen binding properties of haemoglobin are protons and organic phosphates. Thus, the control of erythrocyte pH and intracellular organic phosphate concentration are of major importance in the regulation of oxygen transport by erythrocytes.

With regard to carbon dioxide transport, the basic vertebrate pattern is the following (for reviews, see Roughton, 1964; Perry, 1986; Klocke, 1987, 1988): carbon dioxide produced in the tissues diffuses down its partial pressure gradient into the erythrocyte, where carbonic anhydrase catalyzes the hydration of carbon dioxide to bicarbonate and protons. The protons formed are largely taken up by the major intracellular buffer, haemoglobin. The amount of bicarbonate formed at any given carbon dioxide tension increases with increasing intracellular pH. Furthermore, depending upon the permeability of the red blood cell membrane to bicarbonate, the bicarbonate formed can be transported from the erythrocyte to the plasma. In the respiratory epithelium, the sequence of events is reversed. Carbon dioxide diffuses down its partial pressure gradient from the respiratory surfaces to the environment. This diffusion and the consequent decrease in the intracellular carbon dioxide tension generate a disequilibrium for the reaction between bicarbonate and carbon dioxide, and thus bicarbonate is dehydrated to carbon dioxide. The protons required for this reaction are given up by the haemoglobin. In the absence of erythrocytic catalysis, very little plasma bicarbonate could be dehydrated to carbon dioxide during the residence time of blood in contact with the respiratory epithelium (0.3–6 s depending on the species, see Hughes *et al.* 1981; Klocke, 1988; Bhargava *et al.* 1992) because of the slow rate of uncatalyzed hydration/dehydration reactions between carbon dioxide and carbonic acid in the plasma. Furthermore, with erythrocytic carbonic anhydrase present, the efficiency of the dehydration of plasma bicarbonate depends on the permeability of the erythrocyte membrane to

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bicarbonate. Although there are quite pronounced differences in the activity of erythrocytic carbonic anhydrase among species (Maren *et al.* 1980; Henry *et al.* 1993), the intracellularly catalysed hydration/dehydration reactions are not considered to be a rate-limiting step in carbon dioxide excretion.

Thus, there is an intimate interaction between the intracellular pH, the properties of haemoglobin, and oxygen and carbon dioxide transport (e.g. Jensen, 1991; Brauner and Randall, 1996). Owing to the predominant role of the anion exchanger in acid equilibration and in the transport of bicarbonate across the erythrocyte membrane of mammals (e.g. Hladky and Rink, 1977), it was, until recently, considered that other membrane transport pathways need not be taken into account when describing how oxygen and carbon dioxide are transported by vertebrate blood. However, studies on non-mammalian vertebrates have shown that, in addition to the anion exchange, the Na^+/H^+ exchanger may also influence intracellular pH (Nikinmaa and Huestis, 1984; Cossins and Richardson, 1985; Nikinmaa *et al.* 1986). Furthermore, the anion exchange pathway is not universally present in erythrocytes (Ohnishi and Asai, 1985; Ellory *et al.* 1987; Nikinmaa and Railo, 1987) and, even when present, the rate of anion exchange varies among species (Jensen and Brahm, 1995). Clearly, differences in the ion transport properties of erythrocyte membranes among species and vertebrate groups will affect both oxygen and carbon dioxide transport (see Nikinmaa, 1992; Nikinmaa *et al.* 1995). As data are now available for various vertebrate groups from agnathans to mammals, this review examines how membrane transport interacts with the functional properties of haemoglobin to control oxygen uptake and carbon dioxide removal in these different groups and focuses on the role of protons in the regulation of gas transport, although it is clear that organic phosphates also play an important role (Bauer, 1974; Nikinmaa, 1990).

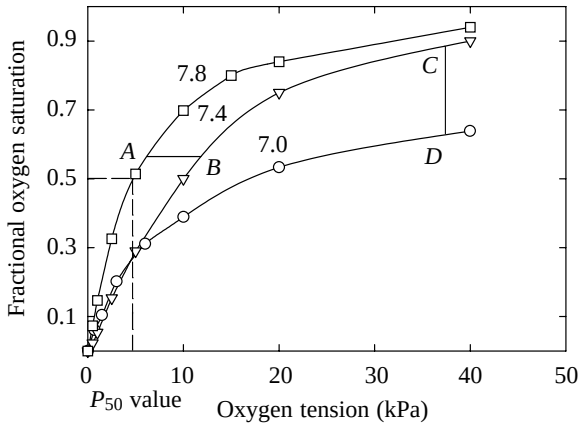

Fig. 1. Hypothetical oxygen equilibrium curves indicating the decrease in the oxygen affinity, i.e. the Bohr effect (distance *A*–*B*), and the decrease in the maximal oxygen saturation reached, i.e. the Root effect (distance *C*–*D*), induced by a decrease in the pH in the environment of the haemoglobin. The P_{50} value indicates the oxygen tension at which haemoglobin is 50 % saturated with oxygen. The curves are often described using the empirical equation: $y=K_A P_{O_2}{}^n/(1+K_A P_{O_2}{}^n)$, in which y is the the fractional oxygen saturation of haemoglobin, K_A is the equilibrium association constant for the overall haemoglobin–oxygen reaction, P_{O_2} is the oxygen tension and n is Hill's coefficient; the n value describes the interaction in oxygen binding between different haemoglobin subunits (globin chains).

Interactions between protons and haemoglobin molecules

Three types of interaction between protons and haemoglobin influence gas transport. First, the buffering capacity of the haemoglobin molecule is the major determinant of the buffering power of vertebrate erythrocytes and, in most vertebrates, the buffering power of blood. Second, protons influence the oxygen affinity of most vertebrate haemoglobins (Bohr and Root effects; see Fig. 1). Third, oxygenation influences the proton binding of haemoglobin.

The major determinant of the buffering capacity of haemoglobins at physiological pH values is the number of histidine residues in the haemoglobin chains, because the imidazole group of histidine has a pK value within the physiological range of 6–8 (the actual pK value varies greatly depending on the environment surrounding the imidazole group). The number of histidine residues per haemoglobin chain is only two in the lampreys (*Lampetra* and *Petromyzon*), 4–5 in teleost fish (Jensen, 1989), 4–8 in the hagfish (*Myxine glutinosa*; Paléus and Liljeqvist, 1972) and much higher, 7–14, in other vertebrate groups (see Jensen, 1989; Nikinmaa, 1990). In addition, the N-terminal amino acids of both lamprey and teleost haemoglobins are usually acetylated, and thus the amino group cannot take up protons at physiological pH values. Correspondingly, the buffering capacity of haemoglobin molecules is much lower in lamprey and teleost fish than in other vertebrate groups, as measured by direct titration (Jensen, 1989; F. B. Jensen, unpublished data).

In contrast, the Haldane effect (the effect of oxygenation on the proton binding properties of haemoglobin, which is manifested as a deoxygenation-induced increase in erythrocytic pH or in the pH of haemoglobin solution) of lamprey and teleost haemoglobins is often very large – the intraerythrocytic pH at a constant extracellular pH increases by up to 0.3–0.4 units when haemoglobin is deoxygenated (Jensen, 1986, 1989; Nikinmaa and Mattsoff, 1992; Ferguson *et al.* 1992). When the Haldane effect is measured by direct titration, the maximal proton uptake of carp haemoglobin upon deoxygenation is 0.95 protons per haemoglobin chain; that of dogfish (*Squalus acanthias*) haemoglobin is only 0.19 proton per chain (Jensen, 1989). The proton uptake upon deoxygenation of lamprey (*Lampetra fluviatilis*) haemoglobin appears to be one proton per haemoglobin chain (Nikinmaa, 1993).

As is obvious from the classical linkage equations (Wyman, 1964), the existence of a large effect of oxygenation on proton binding by haemoglobin predicts that there must also be a large

effect of protons on oxygen binding by haemoglobin (i.e. a large Bohr effect). This is clearly the case: pronounced Bohr factors have been described for lamprey (Ferguson *et al.* 1992; Nikinmaa, 1993) and many teleost fish haemoglobins (e.g. Weber and Lykkeboe, 1978; Jensen and Weber, 1982; Table 1). In addition, in both lampreys and teleost fish, low pH values reduce the oxygen saturation of haemoglobin markedly even at very high oxygen tensions, up to 140 atmospheres (101.3 kPa) of pure oxygen (Root effect; Scholander and van Dam, 1954; Fig. 1). In teleost fish, the Root effect is due to an extreme stabilization of the deoxy conformation of the tetrameric haemoglobin (for a review, see Pelster and Weber, 1991), whereas in lamprey it results from the stabilization of the low-affinity aggregated form of the haemoglobin (Perutz, 1990; Nikinmaa, 1993). Other vertebrate groups, including myxinoids (Manwell, 1958; Bauer *et al.* 1975), generally have smaller Bohr factors than lampreys and teleost fish, although diving mammals, for example, generally exhibit larger Bohr effects than non-diving species of similar size (Hilpert *et al.* 1963; Horvath *et al.* 1968; Lenfant *et al.* 1968, 1970; Table 1). The molecular basis of the Bohr effect and its variations have been reviewed (Riggs, 1988).

The Bohr (and the Haldane) effect is increased by the binding of organic phosphates to haemoglobin (Jensen and Weber, 1985; Jensen, 1989). Organic phosphates also decrease erythrocyte pH when the anion exchanger is functioning, but there is no significant secondarily active transport of protons, since they alter the distribution ratio for permeable anions and protons (Duhm, 1972; Hladky and Rink, 1977; Nikinmaa, 1992).

With the help of the Bohr (and the Root) effect, oxygen delivery in the tissues can be accomplished while maintaining a large oxygen partial pressure gradient between capillary blood and the working tissue. The pH in capillaries of rapidly metabolizing tissues tends to decrease owing to the production of metabolic acids and carbon dioxide. Consequently, haemoglobin oxygen-affinity will be reduced and more oxygen will be given up at a given partial pressure of oxygen. The greater the Bohr effect, and the decrease of pH in the capillary blood, the more oxygen will be delivered. The Root effect can be used to increase the partial pressure of oxygen to very high levels in the swimbladder and in the poorly vascularized fish eye (for a review, see Pelster and Weber, 1991).

The facilitation of oxygen delivery *via* the Bohr effect requires that the pH of capillary blood should decrease. However, in tissue capillaries, haemoglobin is deoxygenated and the blood pH tends to increase owing to the Haldane effect. As a consequence, the arterio-venous pH changes are reduced and, in species with a large Haldane effect, even reversed (Milligan and Wood, 1987; Tufts *et al.* 1992). Because the Haldane effect increases the venous pH in relation to arterial pH, it has been suggested that the oxygen delivery in tissues could be compromised by a large Haldane effect (e.g. Lapennas, 1983; Nikinmaa, 1990). However, there is a profound difference between the acidification caused by carbon dioxide and metabolic protons, and the alkalization caused by deoxygenation. Whereas carbon dioxide and metabolic protons will acidify blood at a constant oxygen saturation, the protons taken up by haemoglobin upon deoxygenation are part of the haemoglobin mechanism and will be released as soon as the oxygen saturation of haemoglobin increases to its original level. Consequently, pH at a constant oxygen saturation is not affected by the oxygenation-dependent proton uptake or release and, therefore, the haemoglobin oxygen-affinity cannot be affected either. On the basis of these considerations, although it is quite clear that a large Haldane effect is required for efficient carbon dioxide transport in species whose haemoglobins have a low buffering capacity, it is unlikely that the facilitation of carbon dioxide

Table 1. *Examples of Bohr factors in the blood or in haemoglobin solutions of different vertebrates*

Group/species	Bohr factor	Conditions	Source
Myxinoids			
Myxine glutinosa	–0.07	Haemolysate	1
Eptatretus stouti	None	Hb solution	2
Eptatretus cirrhatus	–0.43	Whole blood	3
Lampreys			
Lampetra fluviatilis	–1.03	Erythrocytes	4
Petromyzon marinus	–0.63	Erythrocytes	5
Elasmobranch fish			
Rhinobatos batillum	–0.08	Red cell	6
Hemiscyllium oscellatum	–0.29	suspension	6
Carcharhinus melanopterus	–0.35		6
Negaprion acutidens	–0.24		6
Teleost fish			
Oncorhynchus mykiss	–0.50	Whole blood	7
Tinca tinca	–1.27	Hb solution + GTP	8
Cyprinus carpio	–0.98	Whole blood	9
Amphibians			
Bufo marinus	–0.19	Whole blood	10
Birds			
Antarctic penguins (*Pygoscelis adeliae, P. papua, P. antarctica)*	–0.5	Whole blood	11
Catharacta skua	–0.35		11
Mammals			
Orcinus orca	–0.6	Whole blood	12
Elephant	–0.36	Whole blood	13
Dog	–0.49	Whole blood	14

Sources: 1, Bauer *et al.* (1975); 2, Manwell (1958); 3, Wells *et al.* (1986); 4, Nikinmaa (1993); 5, Ferguson *et al.* (1992); 6, Wells *et al.* (1992); 7, Tetens and Lykkeboe (1981); 8, Jensen and Weber (1982); 9, Weber and Lykkeboe (1978); 10, Wells *et al.* (1989); 11, Milsom *et al.* (1973); 12, Lenfant *et al.* (1968); 13, Hilpert *et al.* (1963); 13, Lapennas (1983).

Hb, haemoglobin.

transport would take place at the expense of oxygen delivery to tissues.

Role of membrane transport in the cellular control of gas transport

Myxinoid erythrocytes

The intraerythrocytic pH of hagfish in the physiological pH range is much lower than the extracellular pH (Fig. 2): Tufts and Boutilier (1990) measured the erythrocyte pH (pHi) and extracellular pH (pHe) of *Myxine glutinosa* after equilibration of blood at different carbon dioxide tensions. The relationship between pHi and pHe fitted a straight line with the equation:

$$\mathrm{pHi} = 0.775\mathrm{pHe} + 1.104\,. \qquad (1)$$

Neither pH nor volume disturbances activate ion transport pathways in the red blood cell membrane (Nikinmaa *et al.* 1993). Thus, it appears that the oxygen equilibrium curve cannot be regulated by membrane transport. Furthermore, since organic phosphates do not exert a specific influence on haemoglobin oxygen-affinity in hagfish (Bauer *et al.* 1975), it appears that there is little scope for regulating haemoglobin oxygen-affinity to respond to changes in oxygen availability or oxygen demand. Indeed, there appear to be no erythrocytic responses to hypoxia in the Pacific hagfish *Eptatretus stouti* (Bernier *et al.* 1996).

Efficient oxygen loading by hagfish erythrocytes in the gills can be ensured, as the intrinsic haemoglobin-oxygen affinities appear to be quite high (Manwell, 1958; Wells *et al.* 1986). In contrast, oxygen unloading in the tissues takes place at low oxygen tensions and, therefore, the diffusion of oxygen to the sites of consumption is slowed because of the high oxygen affinity and the small Bohr effect of haemoglobin. Even at rest, the unloading partial pressures of oxygen are relatively low: the mixed venous oxygen tension of the hagfish (*Eptatretus cirrhatus*) is 2.3 kPa (Wells *et al.* 1986), much lower than that of *Oncorhynchus mykiss*, which may be as high as 6 kPa (Soivio *et al.* 1981). Furthermore, there is a pronounced reduction in the mixed venous oxygen tension of exercised hagfish to 0.4 kPa (Wells *et al.* 1986), which markedly decreases the rate of oxygen diffusion from blood to tissues and reduces the aerobic scope for activity. To some extent, oxygen delivery to the tissues of hagfish (*Myxine glutinosa*) can be facilitated by the carbon dioxide sensitivity of the oxygen affinity of the haemoglobin (Bauer *et al.* 1975): the carbon dioxide produced in the tissues will shift the oxygen equilibrium curve to the right, facilitating oxygen delivery.

Although hagfish (*Myxine glutinosa*) erythrocytes contain carbonic anhydrase (Maren *et al.* 1980), several factors limit the efficiency of their carbon dioxide excretion compared with other vertebrates. First, owing to the low intraerythrocytic pH, the intracellular bicarbonate concentration at any given carbon dioxide tension is low (Tufts and Boutilier, 1990; Fig. 3). Second, the buffering capacity and the Bohr/Haldane effect of haemoglobin are small so that the removal of protons produced by the hydration of carbon dioxide is inefficient. Third, anion transport across the erythrocyte membrane is slow (Ellory *et al.* 1987), which precludes the utilization of plasma bicarbonate in carbon dioxide excretion on a physiological time scale. Fourth, the haematocrit values of hagfish are low, only 8–15 % (Hardisty, 1979; Wells *et al.* 1986; Davison *et al.* 1990), reducing the total amount of carbon dioxide carried in the blood. To some extent, carbon dioxide excretion may be facilitated by the specific binding of molecular carbon dioxide to haemoglobin. However, even this effect is small because of

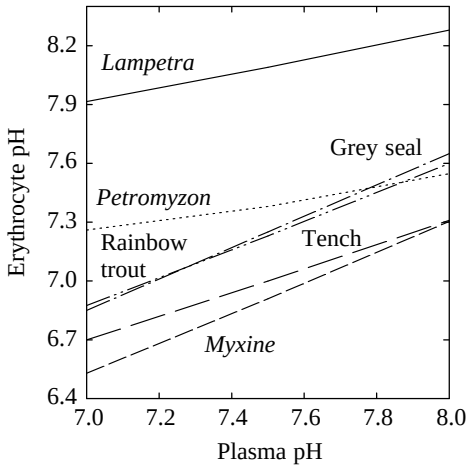

Fig. 2. Erythrocyte *versus* plasma pH for various vertebrates. Data on hagfish *Myxine glutinosa* from Tufts and Boutilier (1990), on tench *Tinca tinca* from Jensen and Weber (1982), on rainbow trout *Oncorhynchus mykiss* from Nikinmaa *et al.* (1987*b*), on grey seal *Halichoerus grypus* from Boutilier *et al.* (1993), on *Petromyzon marinus* from Ferguson *et al.* (1992) and on *Lampetra fluviatilis* from Nikinmaa (1986).

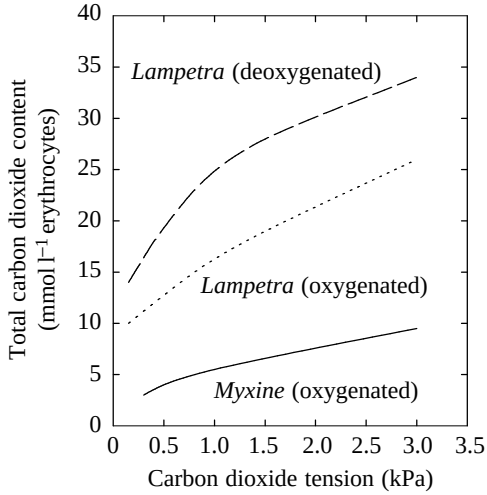

Fig. 3. Carbon dioxide dissociation curves for the erythrocytes of *Myxine glutinosa* (data from Tufts and Boutilier, 1990) and of *Lampetra fluviatilis* (data from Nikinmaa and Mattsoff, 1992), indicating the large difference in the intraerythrocytic carbon dioxide stores and the pronounce effect of deoxygenation on the carbon dioxide content of *Lampetra* erythrocytes.

the low haematocrit value and mean cellular haemoglobin concentration (Wells *et al.* 1986).

In summary, inefficient oxygen and carbon dioxide transport may be major factors in limiting the scope for activity of hagfish (Hardisty, 1979). However, there are some uncertainties when the present data on hagfish erythrocytes are related to their physiological function. First, all the studies on haemoglobin and membrane function in hagfish have been carried out at atmospheric pressures. Hagfish normally live at depths greater than 30 m, and quite often at depths between 500 and 1000 m (see Hardisty, 1979). Thus, they are exposed to high hydrostatic pressures which are known to affect both protein function and membrane transport pathways (e.g. Cossins and Macdonald, 1989; Gibbs and Somero, 1989). Second, although the anion exchanger is virtually absent in hagfish erythrocytes, as indicated both by electrophoretic data and the virtual lack of 4,4′-diisothiocyanostilbene-2,2′-disulphonic acid (DIDS)-sensitive chloride or bicarbonate transport across the erythrocyte membrane (Ellory *et al.* 1987; Tufts and Boutilier, 1990), it appears that bicarbonate equilibration between plasma and erythrocytes occurs (Wells *et al.* 1986; Tufts and Boutilier, 1990). One possible route for bicarbonate transport is the unique Na^+-dependent carboxylic acid transport pathway of hagfish erythrocytes (Tiihonen, 1995).

Lamprey erythrocytes

Regulation of gas transport in lampreys has been reviewed recently (Nikinmaa *et al.* 1995). Lamprey erythrocytes exhibit major functional differences in comparison with those of hagfish. First, they maintain a much higher erythrocyte pH (Nikinmaa, 1986; Tufts, 1992; Fig. 2) by secondarily active Na^+/H^+ exchange. Second, the erythrocyte membrane has a very low permeability to bicarbonate and acid equivalents (Nikinmaa and Railo, 1987; Tufts and Boutilier, 1989). Third, the haemoglobin within intact erythrocytes has a low buffering capacity but exhibits pronounced Bohr and Haldane effects: the intraerythrocytic pH of lamprey erythrocytes increases by 0.3–0.4 units upon deoxygenation (Ferguson *et al.* 1992; Nikinmaa and Mattsoff, 1992). Fourth, oxygenation markedly affects the apparent cooperativity of lamprey haemoglobins within the cells (Bird *et al.* 1976; Nikinmaa, 1993). Fifth, changes in the intracellular haemoglobin concentration affect the haemoglobin oxygen-affinity by influencing the aggregation state of haemoglobin (Airaksinen and Nikinmaa, 1995). The haematocrit value is also much higher in lampreys (25–40 %; Mattsoff and Nikinmaa, 1988; Tufts, 1991) than in hagfish.

These differences between lampreys and hagfish markedly affect both oxygen and carbon dioxide transport. It is obvious that the oxygen-carrying capacity of the blood is higher in lampreys than in hagfish because of the larger number of erythrocytes per unit volume. It is also obvious that the intrinsic haemoglobin oxygen-affinities of lampreys are much lower than those of hagfish. Since the haemoglobins exhibit a large Bohr factor and a low intrinsic oxygen affinity, the high intracellular pH is required to achieve effective oxygen loading at the gills (Nikinmaa *et al.* 1995). Even though the erythrocyte pH values of lampreys are 0.5–1.0 units higher than those of hagfish erythrocytes (Fig. 2), the whole-blood oxygen affinities of lampreys are lower (Ferguson *et al.* 1992; Nikinmaa, 1993). The oxygen affinities of lamprey erythrocytes are in the range expected for active teleost fish such as salmonids (Nikinmaa *et al.* 1995).

The high intracellular pH is also required to ensure that the dissociation/association reactions of haemoglobin occur in intact erythrocytes, since these reactions are the basis of any cooperative phenomena and of the Bohr and Haldane effects of lamprey haemoglobins (see Perutz, 1990; Nikinmaa *et al.* 1995). Given that protons have a marked effect on the oxygen affinity of haemoglobin, and that the concentration of haemoglobin within the cell also influences its oxygen affinity, physiological regulation of oxygen transport could involve either volume or pH changes. The haemoglobin oxygen-affinity is, indeed, increased in hypoxic lampreys, and this increase is associated with both red cell swelling and an increase in erythrocyte pH (Nikinmaa and Weber, 1984).

Because of the virtual impermeability of the lamprey red blood cell membrane to protons (and bicarbonate), only carbon-dioxide-induced acid loads will enter the erythrocyte. Thus, metabolic proton production, as takes place in exhausting exercise, will only acidify the plasma compartment. Exhausting exercise causes a pronounced reduction in both arterial and venous plasma pH in *Petromyzon marinus* (Tufts *et al.* 1992), by 0.36 and 0.46 units, respectively. Exhausting exercise also causes a pronounced carbon dioxide load, as shown by the increase of carbon dioxide tension by 0.39 kPa in venous blood and by 0.17 kPa in arterial blood (Tufts *et al.* 1992). Despite this load, the red blood cell pH is maintained at 7.5 in the arterial and 7.65 in the venous blood. The maintenance of red cell pH is, however, due to the large Haldane effect. The data of Tufts *et al.* (1992) show that the arterial oxygen saturation decreased from approximately 95 % to approximately 75 % and the venous saturation from approximately 75 % to approximately 18 % owing to the exhausting exercise. In the absence of a carbon dioxide load, these decreases of oxygen saturation would have caused approximately 0.1 and 0.25 unit increases in the pH of arterial and venous erythrocytes, respectively (Fig. 4). Thus, it is clear that the erythrocytic environment is influenced by the carbon dioxide load. Unfortunately, published data on the oxygen binding properties of haemoglobin within intact erythrocytes of *Petromyzon marinus* do not enable accurate estimates of how this carbon dioxide acid load affects the oxygen equilibrium curves *in vivo*. Calculations based on the oxygen saturations and oxygen tensions given by Tufts *et al.* (1992), and the *n* values (at P_{50} value; see Fig. 1) given by Ferguson *et al.* (1992), however, suggest that the oxygen affinity of haemoglobin may have decreased. The rightward shift may be larger than calculated on the basis of constant *n* value, since studies on other lamprey species (Bird *et al.* 1976;

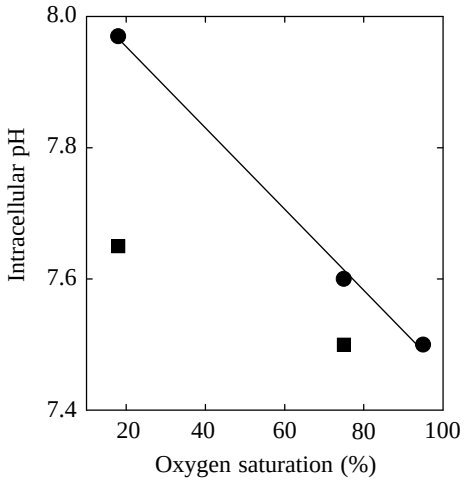

Fig. 4. The effect of oxygen saturation and carbon dioxide load on the erythrocyte pH of *Petromyzon marinus*. The line was obtained by taking the *in vitro* dependence of erythrocyte pH on oxygen saturation (Ferguson *et al.* 1992) and shifting it to the *in vivo* erythrocyte pH of resting animals (Tufts *et al.* 1992). Circles indicate the erythrocyte pH values in arterial blood of resting animals (95 % saturation) and values which would result from the exercise-induced decrease in haemoglobin oxygen-saturation in venous (18 % oxygen saturation) and in arterial (75 % oxygen saturation) blood without the carbon dioxide load. Squares show the actual values measured after exercise in venous (18 % saturation) and arterial (75 % saturation) blood (Tufts *et al.* 1992), demonstrating the pronounced effect of carbon dioxide load on the erythrocyte pH.

Nikinmaa, 1993) have indicated that the apparent n value decreases with decreasing oxygen saturation.

The high erythrocyte pH, and its large oxygenation-dependent changes (Nikinmaa, 1986; Nikinmaa and Mattsoff, 1992; Ferguson *et al.* 1992), which are manifested as the higher erythrocyte pH in venous than in arterial blood (Tufts *et al.* 1992), are very important for effective carbon dioxide transport. Because the transport of bicarbonate across the erythrocyte membrane of lampreys is very slow [the half-time for equilibration is, most likely, similar to that of chloride equilibration, i.e. more than 2 h in *Lampetra fluviatilis* erythrocytes (Nikinmaa and Railo, 1987; Tufts and Boutilier, 1989)] and there is no carbonic anhydrase accessible to plasma in the respiratory epithelium, most of the carbon dioxide excreted in the gills is carried within the erythrocytes as bicarbonate (see Tufts and Boutilier, 1989; Tufts *et al.* 1992; Nikinmaa *et al.* 1995). The erythrocyte bicarbonate stores are increased by the high erythrocyte pH, as dictated by the Henderson–Hasselbalch equation:

$$\mathrm{pH} = \mathrm{pKa} + \log[\mathrm{HCO_3^-}]/\alpha P_{\mathrm{CO_2}}, \quad (2)$$

in which pKa is the apparent dissociation constant for carbon dioxide; α is the solubility coefficient for carbon dioxide in water, and $P_{\mathrm{CO_2}}$ is the carbon dioxide tension. The equation can be converted to:

$$[\mathrm{HCO_3^-}]/\alpha P_{\mathrm{CO_2}} = 10^{(\mathrm{pH}-\mathrm{pKa})}, \quad (3)$$

which shows that, at a given carbon dioxide tension, the bicarbonate concentration of the solution increases with increasing pH. Furthermore, the marked Haldane effect will increase the difference in the total carbon dioxide content of venous and arterial blood (see Fig. 3). Nikinmaa *et al.* (1995) estimated that more than 80 % of the maximal decrease in the total carbon dioxide content of the erythrocytes can be attributed to the oxygenation-dependent pH changes and consecutive changes in the bicarbonate concentration. With the help of these erythrocyte properties, carbon dioxide transport potential in lampreys is as great as in active teleosts such as rainbow trout despite the fact that very little plasma bicarbonate can be utilized in carbon dioxide transport.

There is, however, one disadvantage of the gas transport strategy utilized by lampreys. The intracellular buffering capacity is isolated from the extracellular compartment. As a consequence, extracellular non-carbon-dioxide acid loads cause marked fluctuations in plasma pH of lampreys (Mattsoff and Nikinmaa, 1988). Thus, the major advantage gained by the evolution of rapid anion exchange appears to be related to extracellular buffering rather than to gas transport which, as the lamprey strategy testifies, can be effective even without rapid bicarbonate movements.

Elasmobranch erythrocytes

In contrast to lamprey erythrocytes, which do not possess a functional anion exchange pathway, the erythrocytes of elasmobranch fish exhibit very rapid anion exchange (Obaid *et al.* 1979). Also, in contrast to lampreys, elasmobranch haemoglobins exhibit high buffering capacities (Jensen, 1989). Furthermore, whereas lamprey haemoglobin is insensitive to organic phosphates, elasmobranch haemoglobins are sensitive to both ATP and GTP – and possibly other cofactors such as urea – which are present in the erythrocytes (Weber *et al.* 1983; Wells and Weber, 1983; Wells *et al.* 1992). In view of these differences, the regulatory mechanisms involved in oxygen and carbon dioxide transport are also markedly different in the two groups of animals.

It appears that the oxygen and carbon dioxide transport properties of the blood of elasmobranch fish show relatively little variations. Elasmobranchs seem to lack the pronounced adjustments observed in, for example, teleost fish as a response to different activity levels or different habitats (Wells *et al.* 1992): the oxygen affinities of all species are relatively high and the Bohr and Haldane factors small (see Table 1). Oxygen transport by elasmobranch erythrocytes appears to be mainly modulated by organic phosphates (Wells *et al.* 1992). Even if changes in intracellular pH play a role, they are, most likely, brought about by changes in the organic phosphate concentrations within the erythrocyte and consecutive changes in the anion and proton distribution ratio brought about by the anion exchanger (see Nikinmaa, 1992), since no published data are available indicating a role for secondarily active transport in the control of intracellular pH.

In elasmobranchs, plasma bicarbonate is available for carbon dioxide excretion owing to the presence of the rapid

anion exchange. The bicarbonate produced in the hydration of carbon dioxide in the tissues can be exchanged for chloride, thus removing one of the end products. Furthermore, the high buffering capacity of haemoglobin will effectively take up the protons produced. In the gills, the sequence of events is reversed. In addition to the role of erythrocytic carbonic anhydrase in carbon dioxide excretion, recent data suggest that carbon dioxide excretion could be facilitated by the presence of carbonic anhydrase in the plasma (Wood *et al.* 1994) or in the gill epithelium accessible to the plasma (see Brauner and Randall, 1996). However, earlier studies on the dogfish *Squalus acanthias* (Swenson and Maren, 1987) were unable to demonstrate an effect of gill carbonic anhydrase on carbon dioxide excretion.

The presence of an anion exchange and the large buffering capacity of haemoglobin are of major importance in the buffering of extracellular acid loads. Consequently, extracellular acid–base disturbances generated by metabolic acid loads are reduced, and different cell types need to expend less energy for intracellular pH regulation than would be required to respond to large variations in extracellular pH.

Teleost erythrocytes

In contrast to elasmobranch haemoglobins, the buffering capacity of teleost haemoglobins is generally small and the Bohr and Haldane effects large (see e.g. Jensen, 1991; Table 1; there are, however, haemoglobin components of teleost fish which show either no or reversed Bohr/Haldane effect, see e.g. Gillen and Riggs, 1973). Furthermore, catecholamine-activated Na^+/H^+ exchange influences the erythrocyte pH of many teleost fish even though the anion exchange pathway is present (Nikinmaa and Huestis, 1984; Cossins and Richardson, 1985; for a review, see Nikinmaa, 1992). However, there are relatively large differences in the turnover rate of both the catecholamine-activated Na^+/H^+ exchange (Salama and Nikinmaa, 1989) and the anion exchange (Jensen and Brahm, 1995) between species. Also, both the intrinsic haemoglobin oxygen-affinity and the whole-blood oxygen-affinity vary markedly between teleost species according to the widely varying oxygen availability in the environment and oxygen demand by the animals.

Regulation of haemoglobin oxygen-affinity in teleost fish in response to environmental changes involves two temporally separated systems: an initial, rapid adjustment by a catecholamine-induced increase in intracellular pH (Nikinmaa, 1982, 1983) and a slower, but more permanent, increase caused by a decrease in the cellular NTP concentration and a consecutive, passive increase in erythrocyte pH, as first observed by Wood and Johansen (1972, 1973). Although the reduction of erythrocyte NTP concentration as a means of regulating haemoglobin oxygen-affinity was described approximately 25 years ago, the regulatory pathway involved in the reduction is still not known (see Nikinmaa and Boutilier, 1995).

In contrast, intensive investigations during the 1980s on the catecholamine-sensitive Na^+/H^+ exchange have elucidated the regulatory pathways involved in some detail. First, a reduction in the ratio between oxygen availability (normally a reduction in the arterial oxygen content; see Perry *et al.* 1989) and oxygen demand at the level of chromaffin tissue causes liberation of catecholamines to the bloodstream (Perry *et al.* 1991*a*). Catecholamines bind to the beta-adrenergic receptors on the red blood cell membrane. The number of adrenergic receptors available to plasma catecholamines increases in hypoxic conditions (Marttila and Nikinmaa, 1988): internalized receptors are probably recycled to the cell membrane (Reid *et al.* 1991; Reid and Perry, 1991). Binding of catecholamines to the receptors causes an accumulation of cyclic AMP (Mahé *et al.* 1985) and activation of the Na^+/H^+ 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, 1992). However, recent data (Berenbrink and Bridges, 1994) indicate that, at least in cod, adrenaline is more potent than noradrenaline. Activation of the Na^+/H^+ exchange is able to increase the intracellular pH, if its turnover rate approaches the uncatalyzed rate of dehydration of bicarbonate and protons to carbon dioxide (i.e. the speed at which protons are buffered extracellularly; Motais *et al.* 1989; Nikinmaa *et al.* 1990; Nikinmaa and Boutilier, 1995). The low buffering capacity of teleost haemoglobins also contributes to the observed adrenergic pH changes: if the buffering capacity were greater, much more pronounced proton fluxes would be required for a similar change in intracellular pH. The activity of the exchanger is increased at low oxygen tensions (Motais *et al.* 1987). Thus, in normoxic tench and carp erythrocytes, catecholamines do not cause an increase in intracellular pH either *in vivo* or *in vitro* (Jensen, 1987; Nikinmaa *et al.* 1987*a*; Salama and Nikinmaa, 1988). However, when the arterial oxygen tension of carp erythrocytes was reduced below 1.2 kPa, there was a pronounced increase in intracellular pH. The increase was associated with a propranolol-inhibitable increase in intracellular Na^+ concentration and water content (Nikinmaa *et al.* 1987*a*). All these properties make the adrenergic response uniquely suited to control haemoglobin function in acute hypoxia (Tetens and Christensen, 1987; Nikinmaa *et al.* 1987*a*; Claireaux *et al.* 1988). However, the response is also important in physically disturbed normoxic animals whenever the arterial oxygen saturation is reduced because of the Root effect, as in rainbow trout and striped bass (Primmett *et al.* 1986; Nikinmaa *et al.* 1984). In contrast, oxygen transport in exercised normoxic tench is facilitated by a pronounced increase in the arterial oxygen tension of the blood (Jensen *et al.* 1983).

With regard to carbon dioxide transport, teleost fish have adopted a completely different strategy from that of elasmobranch fish: although both have a functional anion exchange, the haemoglobins of teleost fish have a low buffering capacity and often show a large Haldane effect (Jensen, 1991). The low buffering capacity will reduce the efficiency of carbon dioxide hydration in the tissues and the

efficiency of bicarbonate dehydration in the gills because the number of protons that can be taken up or released by haemoglobin will be limited. However, when combined with a large Haldane effect, the hydration and dehydration reactions are driven in the forward direction because, in the tissues, the deoxygenation-induced increase in erythrocyte pH will increase the amount of bicarbonate that can be formed at a given carbon dioxide tension and, in the gills, the oxygenation-induced acidification will favour the dehydration of bicarbonate to carbon dioxide. Thus, the situation in teleost fish resembles that in lampreys, with the exceptions that the erythrocyte pH is generally lower than in lampreys (Fig. 2; which tends to decrease the carbon dioxide excretion potential) and that plasma bicarbonate is available for carbon dioxide excretion (which increases the carbon dioxide excretion potential). On the basis of the data of Perry *et al.* (1996), it appears that, in terms of carbon dioxide excretion, the strategy of a low buffering capacity but a large Haldane effect is more effective than the strategy of a high buffering capacity but a small Haldane effect: the conversion of radioactive plasma bicarbonate to carbon dioxide was much slower in the dogfish (*Scyliorhinus canicula*) than in the teleost fishes studied.

The extent to which plasma bicarbonate can be utilized in carbon dioxide excretion depends on the residence time of blood in the gills and the rate of anion exchange. The residence time of blood in the gills varies between 0.5 and 6 s. The half-times for chloride equilibration in various teleost fish are approximately 0.1 s for rainbow trout and cod, 0.2 s for carp and 0.3 s for eel at 15 °C (Jensen and Brahm, 1995). On the basis of these values, nearly full equilibration (which requires 5–6 half-times) is possible for cod and rainbow trout during the residence time of blood in the gills [although earlier data on rainbow trout suggest that, in this species, the anion exchange would also be slower (Romano and Passow, 1984). Romano and Passow (1984) obtained a half-time of approximately 0.8 s for chloride equilibration, in which case equilibration would be incomplete]. For carp and eel, a significant disequilibrium will remain at the end of the passage of blood through the gill capillaries. Thus, in these species, the role of erythrocytic bicarbonate in carbon dioxide excretion is increased and this is favoured by a very large Haldane effect, as exemplified by the lamprey (see also Nikinmaa, 1993). Accordingly, the Haldane effect of carp is large (Jensen, 1989).

Since nearly full equilibration of chloride (and bicarbonate) is possible across the erythrocyte membrane of rainbow trout within the residence time of blood in the gills, the contribution of plasma and red cell bicarbonate to carbon dioxide excretion depends on the relative proportions of red cells and plasma and on the pH values of the two compartments. The results of Perry *et al.* (1982) indicate that approximately 70 % of excreted carbon dioxide stems from plasma bicarbonate. Approximately 15 % comes from red cell bicarbonate (Heming, 1984), the rest coming from molecular carbon dioxide, plasma and erythrocyte carbamino compounds, etc. The proportion of carbon dioxide excretion from the erythrocytes increases if the proportion of erythrocytes in the blood and the erythrocyte pH increase, as happens after adrenergic stimulation. Thus, although the conversion of plasma bicarbonate to carbon dioxide by a constant number of erythrocytes is slowed after adrenergic stimulation (Perry *et al.* 1991*b*), the conversion of blood bicarbonate to carbon dioxide appears not to be affected (Nikinmaa and Vihersaari, 1993).

Erythrocytes of air-breathing vertebrates

Apart from the predominant role of chloride/bicarbonate exchange in acid equilibration across the erythrocyte membrane and in carbon dioxide excretion, there is no conclusive evidence that other transport pathways make a significant contribution to the regulation of oxygen and carbon dioxide transport in amphibians, reptiles, birds or mammals. The potential for such effects remains since, in amphibians, the Na^+/H^+ exchange makes a contribution to the steady-state pH of erythrocytes, at least in the salamander *Amphiuma tridactylum* (Tufts *et al.* 1987; Cala *et al.* 1988, 1992). In reptilian and avian erythrocytes, oxygen-sensitive ion transport has been observed (Tosteson and Robertson 1956; Klahr *et al.* 1969). Furthermore, the Na^+/H^+ exchange plays a prominent role in the volume regulation of dog erythrocytes (e.g. Parker, 1988).

As a generalization, the erythrocytes of air-breathing vertebrates have the following characteristics: the anion exchange pathway is functional, the buffering capacity of haemoglobin is high, the Bohr/Haldane effect is generally smaller than in lampreys and teleost fish (see Nikinmaa, 1990; Table 1) and the haemoglobins are sensitive to organic phosphates, although there is marked variation in both the oxygen binding properties of haemoglobin and the effects of organic phosphates on haemoglobin oxygen-affinity among species (see Nikinmaa, 1990). In general terms, the strategy for oxygen and carbon dioxide transport and their interactions in the erythrocytes of air-breathing vertebrates is similar to that of elasmobranchs. Regulation of haemoglobin oxygen-affinity in response to environmental changes is achieved mainly *via* modulation of organic phosphate concentrations (e.g. Wood and Lenfant, 1979, 1987). As in elasmobranchs, plasma bicarbonate in air-breathing vertebrates is available for carbon dioxide excretion owing to the presence of the rapid anion exchange. Furthermore, owing to the high buffering capacity of haemoglobin, it will effectively take up the protons produced in the tissues and release them in the lungs. In addition to the role of erythrocytic carbonic anhydrase, there is carbonic anhydrase in the capillary endothelia of lungs in mammals (Effros *et al.* 1978), which catalyses the extracellular reactions between bicarbonate and carbon dioxide. The extracellular reactions are speeded up by a factor of 100–150 (Effros *et al.* 1978), so that the half-time of the reaction in the extracellular compartment becomes similar to the half-time for chloride/bicarbonate exchange. However, existing data suggest that the contribution of extracellular carbonic anhydrase to carbon dioxide excretion is small, only a few per cent of the total carbon dioxide production (e.g. Klocke, 1987), because the availability of protons for the dehydration reaction in the

lungs is limited owing to the low buffering capacity of plasma. It is possible that this limitation is overcome in seals, since the plasma buffering capacity of the grey seal *Halichoerus grypus* approaches that of the erythrocytes (Boutilier *et al.* 1993).

General conclusions

The buffering properties of haemoglobin and the interactions between protons and oxygen in the haemoglobin reaction contribute significantly to the cellular regulation of gas transport in vertebrates. Lampreys and teleost fish, in which haemoglobins have a low buffering capacity and often a large Bohr/Haldane effect, utilize the secondarily active Na^+/H^+ exchange in the control of haemoglobin oxygen-affinity. Owing to the low buffering capacity, changes in the intracellular pH, caused by the Na^+/H^+ exchange, are relatively large and are manifested as pronounced changes in the haemoglobin oxygen-affinity because of the large Bohr/Haldane effect. The large Bohr/Haldane effect also facilitates carbon dioxide transport: the blood (or erythrocyte) pH increases upon deoxygenation, so that the concentration of bicarbonate formed at a given carbon dioxide tension is increased. The major difference between lampreys and teleost fish is in the bicarbonate permeability of the erythrocyte membrane. Owing to the slow bicarbonate permeation, extracellular acid loads cannot be buffered by haemoglobin in lampreys, whereas in teleost fish, which have a functional erythrocytic anion exchange, extracellular proton loads can be buffered by haemoglobin. However, because of the low buffering capacity of teleost haemoglobins, the buffering is less effective than in elasmobranch fish and air-breathing vertebrates.

The high buffering capacity of the haemoglobins of elasmobranch fish and air-breathing vertebrates results in effective buffering of extracellular proton loads, but diminishes the possibility of regulating haemoglobin oxygen-affinity *via* secondarily active Na^+/H^+ exchange, because changes in the intracellular pH, caused by proton efflux, remain small. Since the Bohr/Haldane factors of the haemoglobins of elasmobranch fish and air-breathing vertebrates are generally smaller than those of lamprey and teleost haemoglobins, haemoglobin oxygen-affinity will be little affected by the small changes in the intracellular pH. Also, although carbon dioxide hydration in the tissues is facilitated by the large buffering capacity of haemoglobin, this strategy appears to be less effective for carbon dioxide excretion than the one utilizing a low buffering capacity but a large Haldane effect.

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