J. exp. Biol. **173**, 25–41 (1992)
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IN VITRO INTERACTIONS BETWEEN OXYGEN AND CARBON DIOXIDE TRANSPORT IN THE BLOOD OF THE SEA LAMPREY (*PETROMYZON MARINUS*)

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Accepted 28 July 1992

Summary

In vitro experiments were carried out to examine the interactions between oxygen and carbon dioxide transport in the blood of the sea lamprey. Oxygen dissociation curves for whole blood obtained from quiescent lampreys had Hill numbers (n_H) ranging from 1.52 to 1.89. The Bohr coefficient for whole blood was −0.17 when extracellular pH (pHe) was considered, but was much greater (−0.63) when red blood cell pH (pHi) was considered. The pHi was largely dependent on haemoglobin oxygen-saturation (S_{O_2}) and the pH gradient across the red blood cell membrane was often reversed when P_{CO_2} was increased and/or S_{O_2} was lowered. The magnitude of the increase in pHi associated with the Haldane effect ranged from 0.169 pH units at 2.9 kPa P_{CO_2} to 0.453 pH units at a P_{CO_2} of 0.2 kPa. Deoxygenated red blood cells had a much greater total CO_2 concentration (C_{CO_2}) than oxygenated red blood cells, but the nonbicarbonate buffer value for the red blood cells was unaffected by oxygenation. Plasma C_{CO_2} was not significantly different under oxygenated or deoxygenated conditions. Partitioning of CO_2 carriage in oxygenated and deoxygenated blood supports recent *in vivo* observations that red blood cell CO_2 carriage can account for much of the C_{CO_2} difference between arterial and venous blood. Together, the results also suggest that oxygen and carbon dioxide transport may not be tightly coupled in the blood of these primitive vertebrates. Finally, red cell sodium concentrations were dependent on oxygen and carbon dioxide tensions in the blood, suggesting that sodium-dependent ion transport processes may contribute to the unique strategy for gas transport in sea lamprey blood.

Introduction

There is growing evidence indicating that the strategy for gas transport in the blood of lampreys may be very different from that in most other vertebrates (Nikinmaa and Tufts, 1989; Tufts and Boutilier, 1989, 1990; Nikinmaa and Mattsoff, 1992; Tufts *et al.* 1992). Lampreys are extant members of a phylogenetically primitive group of vertebrates, the agnathans. Thus, a thorough understanding of the unique strategy for blood gas transport

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Key words: lamprey, blood, erythrocyte, oxygen, carbon dioxide, buffering, pH, Bohr effect, Haldane effect, *Petromyzon marinus*.

in these primitive animals may provide valuable insights into the evolution of respiratory systems in vertebrates.

In most vertebrates, the majority of CO_2 added to the blood by the tissues is transported in the plasma as HCO_3^- to the respiratory organ (Roughton, 1964; Boutilier *et al.* 1979; Boutilier and Toews, 1981; Perry, 1986; Heming, 1984; Klocke, 1987). In the blood of lampreys, however, CO_2 transport is largely dependent on erythrocyte CO_2 carriage (Tufts and Boutilier, 1989, 1990; Nikinmaa and Mattsoff, 1992; Tufts *et al.* 1992). Indeed, Tufts *et al.* (1992) have recently demonstrated that changes in erythrocyte CO_2 concentrations account for as much as 78 % of the total CO_2 difference between arterial and venous blood in the sea lamprey after exercise *in vivo*. Several factors may contribute to the unique distribution of CO_2 transport observed in the blood of lampreys. Chloride/bicarbonate exchange across the red blood cell membrane is an integral component of the CO_2 transport process in the blood of most vertebrates, but appears to be functionally limited or absent in the blood of agnathans (Nikinmaa and Railo, 1977; Ellory *et al.* 1977). Sodium-dependent movements of acid–base equivalents may also affect the distribution of CO_2 across the red blood cell membrane in the sea lamprey (Tufts, 1992). Finally, Nikinmaa and Mattsoff (1992) have recently demonstrated that the blood of the river lamprey shows a relatively large Haldane effect. At present, however, there is a paucity of information on the relative importance of these factors under conditions resembling those occurring in arterial and venous blood *in vivo*.

The unique pattern of carbon dioxide transport may also have critical implications for our understanding of oxygen transport in lampreys. As suggested by Heisler (1986), chloride/bicarbonate exchange across the red blood cell membrane will have an important effect on the red blood cell pH at any given P_{CO_2} (Tufts and Boutilier, 1990). Red blood cell pH in lampreys is often considerably higher than in other vertebrates and is well maintained during relatively large fluctuations in extracellular pH either *in vitro* or *in vivo* (Nikinmaa and Weber, 1985; Mattsoff and Nikinmaa, 1988; Tufts and Boutilier, 1989; Tufts, 1991; Tufts *et al.* 1992). Previous investigations of the oxygen transport properties of lamprey blood, however, have not considered the importance of erythrocyte pH. Thus, as suggested by Nikinmaa and Mattsoff (1992), the magnitude of the Bohr coefficient in lamprey whole blood has probably been largely underestimated. Moreover, the functional significance of the Bohr effect in lampreys may not be fully appreciated (Lapennas, 1983).

The purpose of the present study is, therefore, to elucidate further the strategy for gas transport in the blood of the sea lamprey *Petromyzon marinus*. In these experiments, particular emphasis was placed on the interactions between oxygen and carbon dioxide transport in an attempt to account for the unique relationships recently documented in the arterial and venous blood of these primitive vertebrates *in vivo* (Tufts *et al.* 1992).

Materials and methods

Experimental animals

Adult sea lampreys (*Petromyzon marinus*) weighing approximately 300 g each were collected from the Shelter Valley River, Ontario, during the annual spawning migration

(May–June). The animals were transported to the Biology Department at Queen's University where they were held in circular (1.5 m×1.5 m) tanks containing aerated dechlorinated Kingston tapwater (pH 7.4, 8–10 °C). At least 2 weeks were allowed for acclimation prior to experimentation. The ionic compositon of the water, in mequiv l^{-1}, was as follows: Na^+=2.1, K^+=0.05, Ca^{2+}=2.2, Cl^-=1.3, HCO_3^-=1.5.

The dorsal aorta of anaesthetized lampreys was cannulated with PE50 tubing as described earlier (Tufts, 1991). The animals were permitted to recover in darkened Perspex boxes with flowing aerated fresh water for at least 24 h prior to blood collection. Following the recovery period, blood was drawn from each animal using 1 ml syringes and placed in ice-chilled heparinized glass vessels.

Experimental protocol

6 ml of blood was placed into each of two heparinized glass tonometry vessels and equilibrated in intermittently rotating tonometer vessels against air containing 0.2, 1.0, 3.0 or 5.0 % CO_2 at 10 °C (Wöstoff gas-mixing pump). Following equilibration, 1 ml of blood was removed in an air-tight Hamilton syringe for the analysis of total oxygen content (T_{O_2}), haematocrit, haemoglobin, plasma pH (pHe) and red cell pH (pHi). At this time, the partial pressure of oxygen (P_{O_2}) of the equilibration gas was decreased and that of nitrogen increased. Equilibration and sampling was performed at six different P_{O_2} levels such that oxygen saturation curves were constructed at the following carbon dioxide partial pressures (kPa): 0.20, 0.99, 2.84 and 4.65. Hill analysis of those data values lying between 25 and 90 % oxygenation was performed for each oxygen dissociation curve.

In a second series of experiments, pooled blood was divided into two 6 ml portions which were then transferred to heparinized tonometer vessels. The blood was equilibrated for 1 h at 10 °C against 0.2 % CO_2 in either air or nitrogen. Following equilibration, two 1 ml blood samples were removed from each vessel using 1 ml air-tight Hamilton syringes. The first 1 ml blood sample was analyzed for total CO_2 content of whole blood (C_{CO_2wb}) and true plasma (C_{CO_2e}), pHe, pHi, whole-blood pH (pH_{wb}) and haematocrit (Hct). The second 1 ml blood sample was dispensed into two 0.5 ml Eppendorf tubes and centrifuged. Following centrifugation, the plasma from each of these tubes was discarded and the red cell pellets were saved for analysis of red blood cell water content and sodium concentration respectively. A similar sampling procedure was performed after the blood had been equilibrated at 1.0 (0.96 kPa) and 3.0 % (2.9 kPa) CO_2.

In the final series of experiments, blood was collected from resting lampreys and divided into two 4 ml portions which were then transferred to intermittently rotating tonometers. In each experiment, ouabain (final concentration in blood 10^{-4} mol l^{-1}) was added to the blood in both tonometer vessels. The blood was then equilibrated at 10 °C against a gas mix of 0.2 % CO_2 balance air. Following this initial equilibration period, a 1 ml sample was taken from each tonometer vessel for the determination of red cell water and sodium ion content. In one of the tonometers, the equilibration gas mixture was then switched to a mix of 0.2 % CO_2 balance nitrogen. The other tonometer served as a control and remained at 0.2 % CO_2 in air. The sampling procedure was then repeated for both vessels after an additional 10, 30 and 60 min of equilibration.

Analyses

A PHM 73 pH meter and associated micro-pH unit (Radiometer, Copenhagen) thermostatted at 10 °C was used to measure pH. The pHi was determined on freeze–thawed haemolysates according to the method of Zeidler and Kim (1977).

Haematocrit (Hct) was measured in triplicate using a Clay-Adams microhaematocrit centrifuge. The method of Drabkin and Austin (1935) was used to determine haemoglobin concentration using the procedure and reagents supplied by Sigma Chemical Co. (St Louis).

The total oxygen content (T_{O_2}) of whole blood was obtained by the method of Tucker (1967) using an apparatus similar to that described by Hughes *et al.* (1982).

Total CO_2 content of whole blood (C_{CO_2wb}) and true plasma (C_{CO_2e}) were determined using a Corning model 965 CO_2 analyzer (Ciba Corning Canada Inc.). Red blood cell C_{CO_2} (C_{CO_2i}, in mmol l^{-1} cell water) was subsequently calculated from the following formula:

$$C_{CO_2i} = [C_{CO_2wb} - C_{CO_2e} \times (1 - \text{Hct})]/\text{Hct}$$

and corrected according to the intracellular water value.

The bicarbonate ion concentrations (mmol l^{-1}) of whole blood and plasma were obtained using the following formula:

$$[HCO_3^-] = C_{CO_2} - (P_{CO_2} \times \delta CO_2),$$

where δCO_2 is the solubility of CO_2 in whole blood or plasma, respectively (Boutilier *et al.* 1984). Erythrocyte bicarbonate concentration was determined with the use of the following formula:

$$[HCO_3^-]_i = \{[HCO_3^-]_{wb} - [HCO_3^-]_e \times (1 - \text{Hct})\}/\text{Hct},$$

where $[HCO_3^-]_i$, $[HCO_3^-]_{wb}$ and $[HCO_3^-]_e$ represent the concentration of bicarbonate in the red cell, whole blood and true plasma, respectively, and Hct is the haematocrit. $[HCO_3^-]_i$ was corrected for cell water content.

Red blood cell water content and sodium concentration were determined by first dispensing two 300 μl portions of whole blood into two dried and tared Eppendorf tubes. The water content of the red cells was then obtained by centrifuging (Eppendorf microfuge) the whole blood for 4 min, removing the plasma supernatant, and drying the red cell pellet to a constant weight (2–3 days at 80 °C). For red blood cell sodium analysis, the other red blood cell pellet was agitated to a homogeneous slurry after addition of 300 μl of 8.0 % perchloric acid and then allowed to digest for 12 h. Sodium composition was determined on the supernatant of this acid-extracted slurry using a Corning 410 flame photometer (Ciba Corning Canada). Plasma sodium and water contents were used to correct the red blood cell values for trapped plasma (2.5 %, Houston, 1985). Only such corrected values are reported.

Statistics

Unless stated otherwise, all data are presented as means ± 1 standard error of the mean (S.E.M.). Statistical analysis by two-tailed paired or unpaired *t*-tests, where appropriate,

was used to determine the significance of differences. In the comparison of values obtained through repetitive sampling, the significance of differences was obtained by first employing an analysis of variance followed by Dunnett's two-tailed *t*-test for repeated measures. Lines derived from linear regressions were compared for significant differences in slope by an analysis of covariance. A fiduciary limit of $P<0.05$ was employed throughout.

Results

Whole blood used in the determination of oxygen transport characteristics had a haematocrit of 16.9±0.7 % and a maximal O_2-carrying capacity of 1.01±0.05 mol O_2 mol^{-1} Hb at 100 % oxygenation, N=28. Haematocrit (Hct) and haemoglobin content (Hb) of the whole blood were related by the following linear regression equation: Hb ($g\,dl^{-1}$)=0.26×Hct(%)+0.319, P=0.0001, r=0.96, N=28. Sea lamprey Hb exhibited sigmoidal (n_H=1.52–1.89) O_2 dissociation relationships (Table 1). There was no significant difference between the n_H at 0.20 kPa P_{CO_2} and the n_H values obtained at the higher P_{CO_2} tensions (Table 1). An increase in the ambient P_{CO_2} did result in a rightward shift in the O_2 dissociation curves. Hill analysis of the data indicated a progressive increase (reaching 1.7-fold) in the P_{50} with increasing CO_2 tension. An additional set of experiments confirmed that full saturation of haemoglobin was reached over the entire range of CO_2 tensions used in these experiments.

The Bohr effect, indicated by the data in Table 1, was determined for both plasma pH (pHe) and red blood cell pH (pHi; Fig. 1). It is evident that haemoglobin oxygen-carriage was more strongly dependent on pHi than on pHe. Thus, this inequality resulted in a significantly lower Bohr coefficient calculated by linear regression analysis of pHe data ($\Delta\log P_{50}/\Delta pH=-0.173\pm0.029$) compared with that obtained when the pHi data are considered ($\Delta\log P_{50}/\Delta pH=-0.631\pm0.081$; Fig. 1).

Fig. 2 illustrates that haemoglobin oxygen-saturation ($\%S_{O_2}$) has a considerable effect on the pHi of sea lamprey blood, but not on the pHe. Red blood cell pH is inversely related to $\%S_{O_2}$ and significant increases in pHi occur as S_{O_2} falls below 80 % at all experimental P_{CO_2} levels. The magnitude of the increase in pHi observed with decreasing $\%S_{O_2}$ (from 100 to approximately 10–20 %) appears to be smaller at 4.65 kPa P_{CO_2} than

Table 1. *Hill analysis of sea lamprey whole blood at 10 °C*

	P_{CO_2}			
	0.20	0.99	2.84	4.65
n_H	1.89±0.15	1.59±0.10	1.53±0.15	1.52±0.13
P_{50}	3.14±0.11	4.06±0.10*	4.56±0.25*	5.44±0.37*
pHe at P_{50}	8.073±0.027	7.493±0.020*	7.056±0.034*	6.920±0.020*
pHi at P_{50}	7.640±0.030	7.471±0.015*	7.347±0.007*	7.315±0.010*
N	9	6	6	7

The P_{CO_2} and P_{50} are presented in kPa. n_H, Hill number. N, number of independent experiments. An asterisk denotes a significant ($P<0.05$) difference from the value at 0.20 kPa CO_2 (unpaired *t*-test).

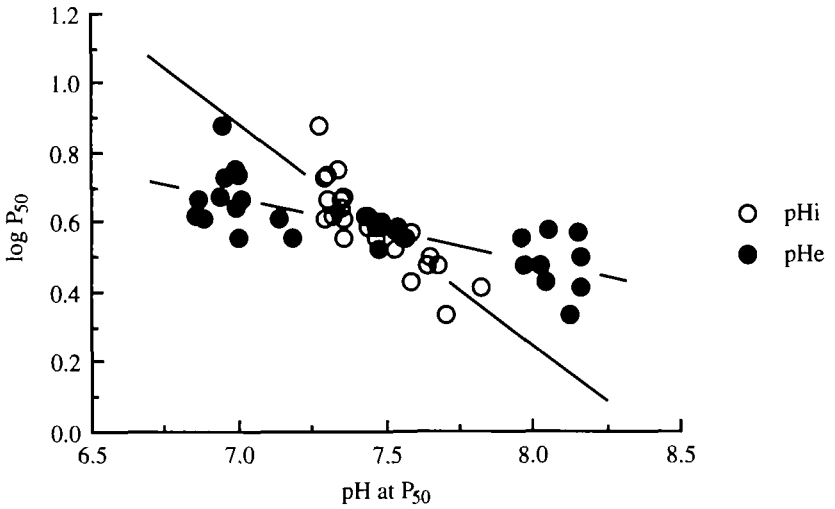

Fig. 1. The CO_2 Bohr effect of sea lamprey whole blood determined for both red blood cell (pHi) and plasma (pHe) pH of the sea lamprey. The linear regression relationships are as follows: $P_{50}=(-0.173\pm0.029)$pHe$+1.881$ ($r=0.76$, $N=28$) and $P_{50}=(-0.631\pm0.081)$pHi$+5.297$ ($r=0.84$, $N=28$). The slopes of the lines are significantly ($P<0.05$) different (ANCOVA).

at 0.20 kPa. This may be due, however, to the nature of the logarithmic pH scale, since the calculated changes in actual proton concentration are approximately similar.

The correlation between pHi, pHe and blood oxygenation was further examined using air-equilibrated and nitrogen-equilibrated blood (Hct=17.6±1.8) over a range of CO_2 tensions to determine the magnitude of the increase in pHi associated with the Haldane effect (Fig. 3). In these experiments, significantly greater pHi values were obtained in deoxygenated blood over the entire pHe range. The increase in pHi associated with the Haldane effect ranged from 0.453 pH units at 0.2 kPa P_{CO_2} to 0.169 pH units at 2.9 kPa P_{CO_2}.

Deoxygenated blood also had a significantly greater CO_2-carrying capacity than oxygenated blood in the sea lamprey (Fig. 4A). At a P_{CO_2} of 2.9 kPa, the C_{CO_2} of deoxygenated blood was 2.95±0.43 mmol l^{-1} higher than that of oxygenated blood. The molar concentration of Hb for the blood used in these experiments can be calculated on the basis of a monomeric relative molecular mass for lamprey Hb of 18 200 (Lenhert *et al.* 1956) and the previously described relationship between Hct and Hb content of lamprey whole blood. At a P_{CO_2} of 2.9 kPa, the Haldane coefficient, which can then be calculated from the present data, is 1.1 mmol CO_2 mmol^{-1} monomer Hb or 4.4 mmol CO_2 mmol^{-1} tetrameric Hb. This Haldane effect can be attributed to a large increase in the C_{CO_2} of the sea lamprey red blood cells (Fig. 4B). Indeed, at 2.9 kPa, oxygenated red blood cells contain 21.85±1.96 mmol CO_2 l^{-1} cell water, or only 55 % of the C_{CO_2} of deoxygenated red blood cells (39.91±1.09 mmol CO_2 l^{-1}) at the same P_{CO_2}. There were no significant differences between the C_{CO_2} of oxygenated and deoxygenated plasma (Fig. 4C).

Non-bicarbonate buffer relationships for oxygenated and deoxygenated lamprey blood are presented in Fig. 5. Whole-blood bicarbonate concentration is significantly greater in deoxygenated blood over the entire pH range. Again, this is largely attributable to

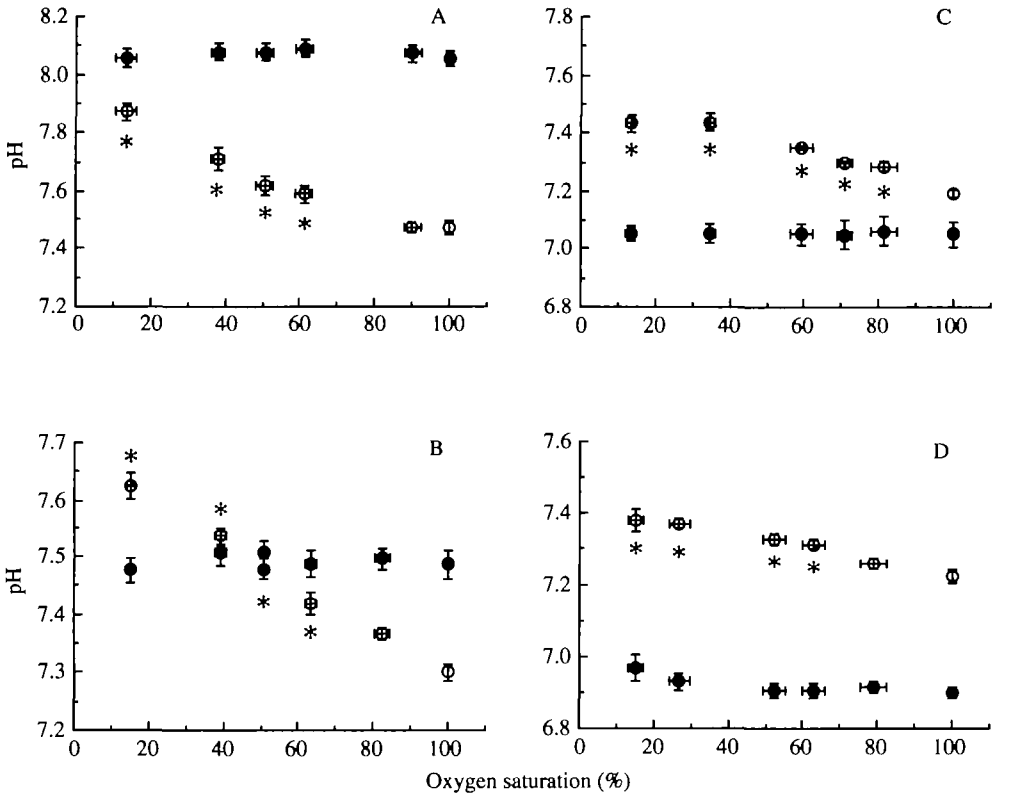

Fig. 2. Relationship between the pH of the red blood cell (○, pHi) and of the plasma (●, pHe) and oxygen saturation of sea lamprey haemoglobin at (A) 0.2, (B) 0.99, (C) 2.84 and (D) 4.65 kPa P_{CO_2}. An asterisk denotes a significant ($P<0.05$) difference compared to the pH value obtained at 100 % oxygen saturation (ANOVA, Dunnett's *t*-test). *N* values as in Fig. 1.

differences between oxygenated and deoxygenated red blood cells (Fig. 5B). Only minor differences are observed in the plasma concentration of bicarbonate under these conditions (Fig. 5C). There was a significant difference in the nonbicarbonate buffer value ($\beta=\Delta HCO_3^-/\Delta pH$) between oxygenated and deoxygenated blood (Table 2). However, there was no significant effect of oxygenation on the nonbicarbonate buffer values determined for red blood cells or plasma (Table 2).

The present data can be used to predict the actual distribution of CO_2 between plasma and red blood cells in oxygenated and deoxygenated blood (Fig. 6). In these experiments, the haematocrit was somewhat lower than that observed *in vivo* (Tufts, 1991; Tufts *et al.* 1992). This difference is probably because some animals were used in two 'separate' series of experiments and considerable volumes of blood were removed from each animal. Thus, to facilitate comparisons with previous *in vivo* studies, a resting haematocrit of 27.4 %, as observed *in vivo* (Tufts *et al.* 1992), was used to predict the distribution of CO_2 between plasma and red blood cells in oxygenated and deoxygenated blood (Fig. 6). For both oxygenated and deoxygenated blood, the importance of the red blood cell in CO_2 carriage increases as the P_{CO_2} increases. In oxygenated whole blood,

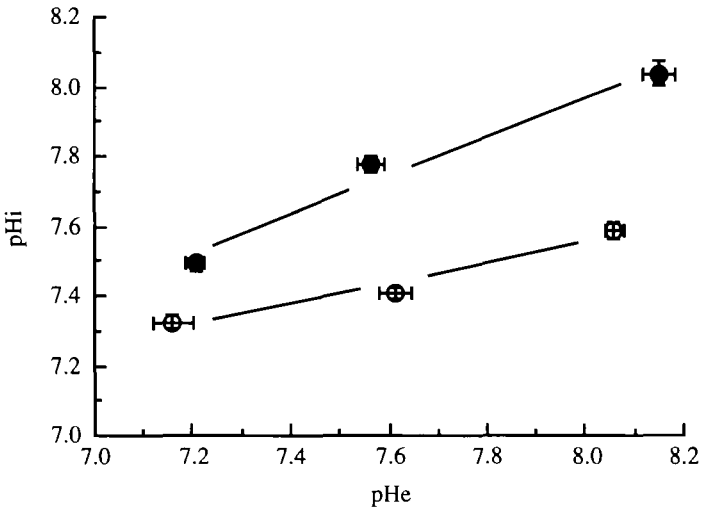

Fig. 3. Relationship between the plasma pH (pHe) and the red blood cell pH (pHi) of oxygenated (○) and deoxygenated (●) sea lamprey whole blood. N=6 independent experiments. The linear regression relationship for oxygenated blood was pHi=0.291pHe+5.220, r=0.98 and that for deoxygenated blood was pHi=0.565pHe+3.455, r=0.99.

the CO_2 attributable to the red blood cells increases from 17 % to 35 % over the experimental P_{CO_2} range. Similarly, in deoxygenated blood, the increase is from 26 % to as much as 48 % at a P_{CO_2} of 2.90 kPa.

Sodium-dependent pH regulation has been demonstrated in lamprey red blood cells (Nikinmaa, 1986; Nikinmaa *et al.* 1986; Tufts, 1992). Thus, in order to characterize further the factor(s) associated with the observed relationships between red blood cell pH, C_{CO_2}, P_{CO_2} and oxygenation, changes in erythrocyte sodium concentration ($[Na^+]$) were examined. It was found that $[Na^+]$ increased with deoxygenation and/or decreased pH (increased P_{CO_2}; Fig. 7). A final series of experiments was therefore carried out in which ouabain (10^{-4} mol l^{-1}) was added to block the Na^+/K^+-ATPase. The purpose of these experiments was to eliminate the potential impact of reduced ATP concentrations on the Na^+ distribution across the erythrocyte membrane. In the presence of ouabain, red blood cell $[Na^+]$ became significantly elevated in deoxygenated blood compared to blood that remained under oxygenated conditions (Fig. 8). Thus, the observed increases in red blood cell $[Na^+]$ under deoxygenated conditions in the previous experiments (Fig. 7) are apparently not attributable to a reduction in Na^+/K^+-ATPase activity.

Discussion

As in other vertebrates, the oxygen transport functions of lamprey blood are largely determined by the intrinsic affinity of haemoglobin for oxygen, the cooperativity of haemoglobin oxygen-binding and the effects of pH and CO_2 on haemoglobin function. To our knowledge, the present experiments are the first to examine these characteristics in lampreys using intact red blood cells collected from quiescent cannulated animals at a

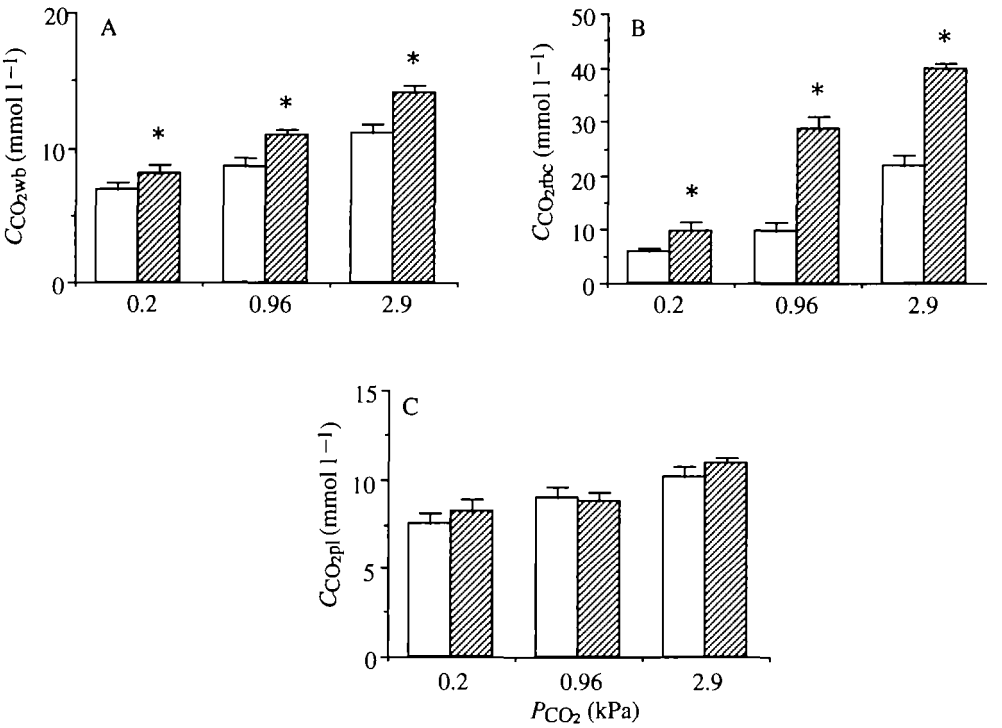

Fig. 4. Carbon dioxide concentrations for (A) whole blood, (B) red blood cells and (C) true plasma of the sea lamprey equilibrated under oxygenated (open columns) and deoxygenated (hatched columns) conditions. An asterisk denotes a significant (P<0.05) difference between oxygenated and deoxygenated conditions (paired t-test, N=6).

temperature (10 °C) typical of their environment. Unlike previous studies, we have also demonstrated the critical importance of intracellular pH measurements in these experiments.

The P_{50} values presented in Table 1 indicate that the affinity of sea lamprey haemoglobin for oxygen is much lower in the present study than previously reported by Manwell (1963) using intact red blood cells. This is somewhat surprising considering that the previous investigation was carried out at a much higher temperature (25 °C). However, the difference may be related to the fact that the present experiments use blood collected from relatively unstressed sea lampreys. In this regard, it is noteworthy that river lampreys exposed to hypoxic stress have a higher blood oxygen-affinity than that of normoxic animals (Nikinmaa and Weber, 1984). Furthermore, stress responses in many species of fish have a significant impact on blood oxygen-affinity (Nikinmaa and Tufts, 1989; Nikinmaa, 1990).

The oxygen dissociation characteristics of sea lamprey whole blood indicate haemoglobin cooperativity and could be described as sigmoidal (n_H=1.52–1.89; Table 1). These n_H values are higher than the values (n_H=1.0–1.2) originally determined by Wald and Riggs (1951) for dialyzed sea lamprey haemoglobins. Under conditions of increased concentration, deoxygenation or reduced pH, the predominantly monomeric lamprey haemoglobins will interact in dimer and tetramer aggregations (Riggs, 1972; Hardisty,

1979). Thus, the greater concentrations present in intact erythrocytes in the present study have probably resulted in some aggregation and positive cooperativity (i.e. $n_H>1$). The present values are also somewhat greater than the value of 1.2 reported by Manwell (1963) for intact erythrocytes of adult *P. marinus*. However, it should be noted that direct comparisons are difficult since our study was conducted at a temperature normally experienced by sea lampreys (10 °C) whereas the experiments of Manwell (1963) were carried out at 25 °C, quite close to the upper limit of their temperature range. We found no evidence of a relationship between the n_H values and pH; the n_H values were not

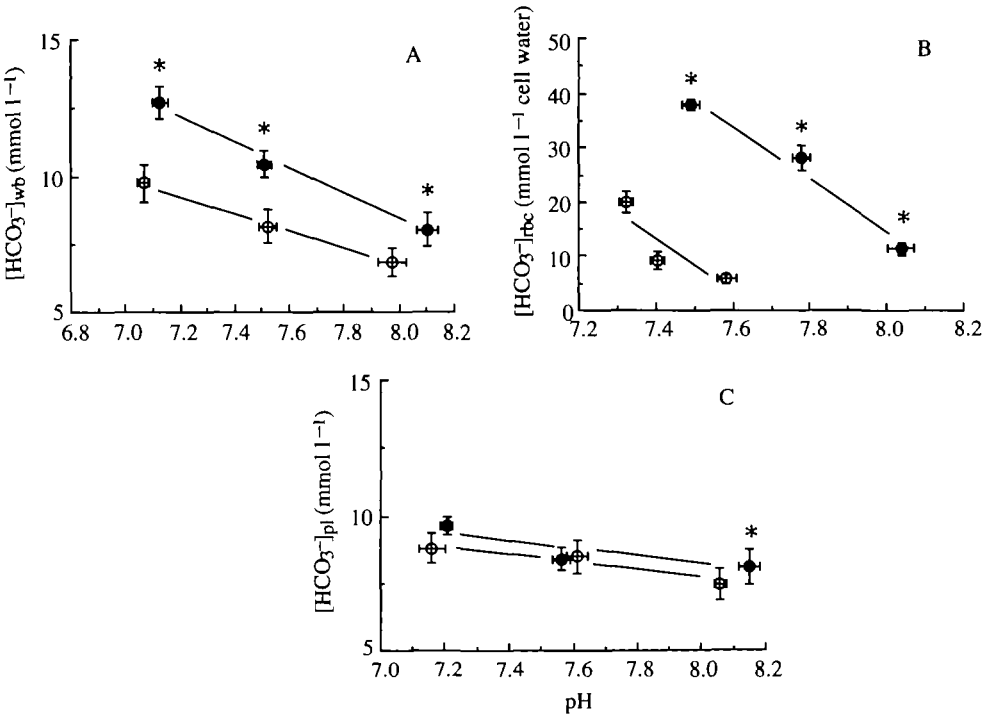

Fig. 5. The relationship between pH and bicarbonate concentration ($[HCO_3^-]$) for (A) whole blood, (B) red blood cells and (C) true plasma of the sea lamprey equilibrated under oxygenated (○) and deoxygenated (●) conditions. An asterisk denotes a significant ($P<0.05$) difference between oxygenated and deoxygenated conditions (paired *t*-test, $N=6$). Nonbicarbonate buffer values are presented in Table 2.

Table 2. *Non-bicarbonate buffer values ($\beta=\Delta HCO_3^- \Delta pH^{-1}$) of oxygenated and deoxygenated sea lamprey blood*

β	Oxygenated	Deoxygenated
Red cell	−48.28±8.30	−51.05±7.10
Whole blood	−3.19±0.56	−4.78±0.36*
True plasma	−1.60±0.78	−1.51±0.54

An asterisk denotes a significant ($P<0.05$) difference from the corresponding oxygenated value (paired *t*-test, $N=6$).

significantly different over the range of P_{CO_2}. This may be explained by the fact that relatively large changes in P_{CO_2} and/or pHe of lamprey blood are associated with much smaller changes in pHi of the intact erythrocytes (Tufts and Boutilier, 1989, 1990; Tufts, 1991; Fig. 3). The described relationship between n_H and pH for lamprey haemoglobin may, therefore, be obscured or perhaps not relevant under more physiological conditions using intact cells. Nevertheless, our results do indicate that haemoglobin cooperativity is present in intact sea lamprey erythrocytes at a temperature (10 °C) compatible with their natural environment.

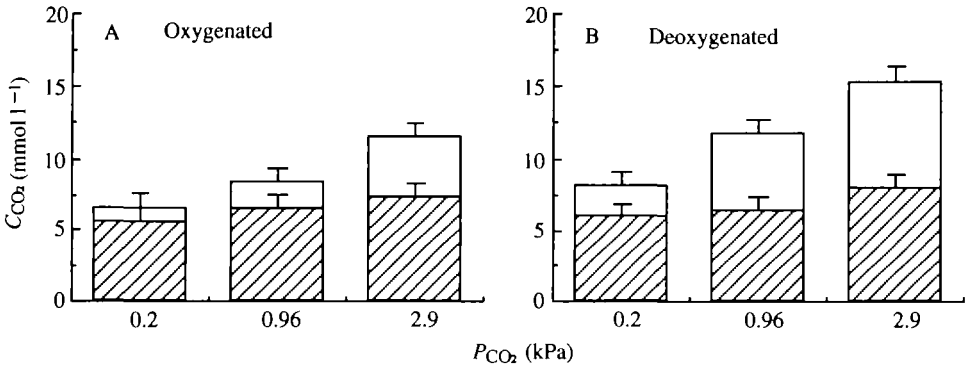

Fig. 6. Relative contribution of red blood cells (open columns) and true plasma (hatched columns) towards total carbon dioxide carriage in sea lamprey whole blood under (A) oxygenated and (B) deoxygenated conditions assuming an *in vivo* haematocrit of 27.4 % (Tufts *et al.* 1992).

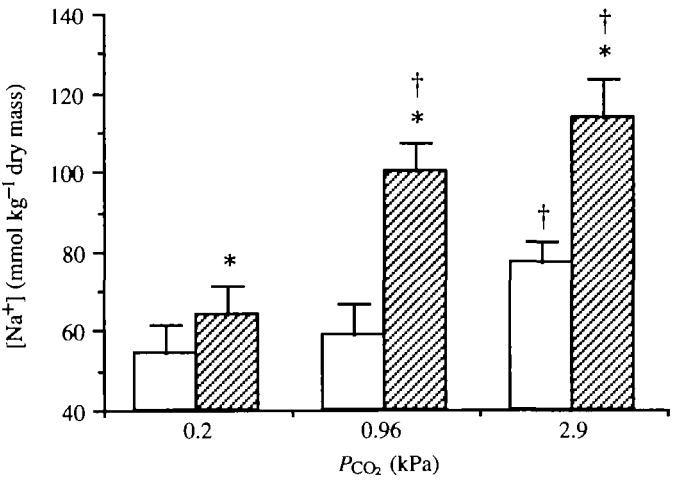

Fig. 7. Sodium concentration ([Na$^+$]) in sea lamprey red blood cells equilibrated under oxygenated (open columns) or deoxygenated (hatched columns) conditions at 0.2, 0.96 and 2.9 kPa P_{CO_2}. An asterisk denotes a significant (P<0.05) difference compared to the corresponding oxygenated value (paired t-test). A dagger indicates a significant (P<0.05) difference compared with the value observed at 0.2 kPa P_{CO_2} (ANOVA, Dunnett's t-test). N=6 for all values.

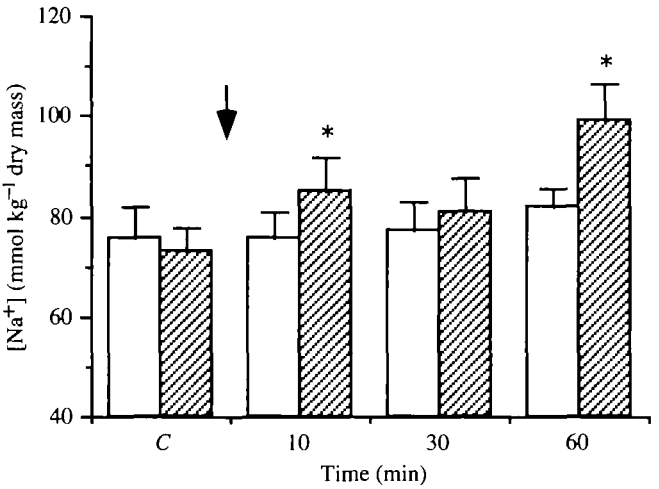

Fig. 8. Sodium concentration ([Na^+]) in sea lamprey red blood cells equilibrated in the presence of ouabain (10^{-4} mol l^{-1}). Control (*C*) samples were taken after 1 h of equilibration in a 0.2 % CO_2/air mixture. Thereafter, samples were taken 10, 30 and 60 min after further equilibration in either a 0.2 % CO_2/air mixture (open columns) or a 0.2 % CO_2/N_2 mixture (hatched columns) (arrow designates change in equilibration gas). An asterisk denotes a significant difference between oxygenated and deoxygenated blood (paired *t*-test; N=6).

The present study is the first to determine experimentally the Bohr coefficient of lamprey whole blood using both the extracellular and the erythrocyte pH (Fig. 1). To date, extracellular pH has been used exclusively for such determinations and investigators have reported relatively small Bohr coefficients between −0.14 and −0.34 in lamprey whole blood (Bird *et al.* 1976; Nikinmaa and Weber, 1984). It has been demonstrated, however, that the erythrocyte pH is largely maintained over a broad range of extracellular pH values in the blood of these animals (Nikinmaa, 1986; Tufts and Boutilier, 1989, 1990; Tufts, 1991). As suggested by Nikinmaa and Mattsoff (1992), consideration of only the extracellular pH would therefore largely underestimate the Bohr coefficients. Indeed, in the present study, the Bohr coefficient obtained with respect to the erythrocyte pH is much larger than the value obtained when the extracellular pH values are considered (Fig. 1). Moreover, the Bohr coefficient based on the erythrocyte pH (−0.63) is much closer to that obtained for sea lamprey haemoglobin solutions (−0.7; Manwell, 1963). In the river lamprey, Nikinmaa and Mattsoff (1992) have also estimated a Bohr coefficient of −0.93 based on the erythrocyte pH. Thus, in whole blood, it is clear that erythrocyte pH values must be considered in order to obtain a reliable measure of the pH sensitivity of lamprey haemoglobin. These results may provide important insights into gas transport in these primitive vertebrates. According to the analysis of Lapennas (1983), oxygen delivery at the tissues will be optimized in mammals when the magnitude of the Bohr effect is about half of the respiratory quotient (i.e. −0.35 to −0.50). This analysis also indicates that pH compensation and CO_2 transport will be optimized when the Bohr effect approaches the respiratory quotient. In lampreys, therefore, Bohr effects previously determined on intact cells using pHe suggested that the system was oriented

towards oxygen delivery. However, the present experiments using pHi to determine the Bohr factor clearly suggest that the system may be oriented more towards CO_2 transport, or the functional equivalent of the Bohr effect, the Haldane effect. Indeed, a large Haldane effect may be essential for CO_2 transport in lampreys since an integral component of CO_2 transport in higher vertebrates, erythrocyte chloride/bicarbonate exchange, appears to be limited or absent in agnathans (Ellory *et al.* 1977; Nikinmaa and Railo, 1977).

Several *in vivo* studies have demonstrated that the erythrocyte pH in lampreys is considerably higher than in most other vertebrates and negative transmembrane pH gradients have even been reported under certain conditions (Nikinmaa and Weber, 1984; Mattsoff and Nikinmaa, 1988; Tufts *et al.* 1992). In the river lamprey, *L. fluviatilis*, the oxygenation state of haemoglobin has a large impact on the erythrocyte pH (Nikinmaa and Mattsoff, 1992). Our results indicate that this is also the case in the sea lamprey (Fig. 2). In the present study, we have further examined this relationship over a range of P_{CO_2} levels in an attempt to explain the unique relationship between pHe and pHi often observed *in vivo*. This analysis confirms that very small or negative transmembrane pH gradients may occur under a number of circumstances in sea lampreys *in vivo* (Fig. 2). Indeed, the negative transmembrane pH gradients recently observed *in vivo* by Tufts *et al.* (1992) would be expected in venous blood after exercise as a result of the elevation in P_{CO_2} and simultaneous reduction in P_{O_2}. Thus, the unique relationship between pHe and pHi which has been documented in lampreys *in vivo* may be largely explained by the effects of oxygen and carbon dioxide on the pH gradient in these animals.

The large dependence of red blood cell pH on haemoglobin oxygenation is not surprising considering both the large intracellular Bohr effect (Fig. 2) and the fact that the Bohr and Haldane effects in vertebrate haemoglobin are interrelated (Wyman, 1964; Jensen, 1989). In the sea lamprey, the difference in erythrocyte pH between fully oxygenated and deoxygenated blood is greater than in most vertebrates. At an extracellular pH of 7.9, our data indicate a pHi difference of 0.40 pH units (Fig. 3). In comparison, the pHi difference at the same extracellular pH is 0.35 pH units in the tench (Jensen, 1986) and about 0.27 pH units in the carp (Albers *et al.* 1983); two animals generally considered to have large Haldane-mediated increases in red blood cell pH. Nikinmaa and Mattsoff (1992) have recently demonstrated that pHi increases by 0.24 pH units at a pHe of 7.5 in another agnathan, the river lamprey, *Lampetra fluviatilis*, when the oxygen saturation of the blood is reduced from 100 % to 7 %. This value approaches the 0.29 pH unit difference obtained in the present experiments for fully oxygenated and deoxygenated sea lamprey blood at the same extracellular pH. Thus, the relatively large dependence of red blood cell pH upon oxygenation in comparison with most other vertebrates may be a common feature of the blood of lampreys.

The importance of the Haldane effect for CO_2 carriage in sea lamprey blood is illustrated in Fig. 4A. Over the P_{CO_2} range examined, the total CO_2 content of whole blood was enhanced by 15–27 % after deoxygenation. It is noteworthy that this increase is entirely due to an increase in the total CO_2 content of the red blood cells (Fig. 4B). At a P_{CO_2} of 2.9 kPa, the C_{CO_2} of deoxygenated red blood cells was approximately double that of oxygenated red blood cells. In comparison, plasma C_{CO_2} was not significantly different

between oxygenated and deoxygenated blood (Fig. 4C). The differences in C_{CO_2} between oxygenated and deoxygenated lamprey erythrocytes are not a result of a significant difference in nonbicarbonate buffer values (Table 2), but there is considerably more bicarbonate formed in deoxygenated lamprey red blood cells at any given P_{CO_2} (Fig. 5; Nikinmaa and Mattsoff, 1992). In most vertebrates, a similar increase in red blood cell [HCO_3^-] would be associated with the transfer of bicarbonate to the plasma *via* the anion exchanger within the red blood cell membrane (Roughton, 1964; Cameron, 1979; Perry, 1986; Nikinmaa, 1990). However, there is growing evidence that this process is functionally limited or absent in lamprey blood (Nikinmaa and Railo, 1987; Tufts and Boutilier, 1989, 1990; Nikinmaa, 1990; Nikinmaa and Mattsoff, 1992; Tufts *et al.* 1992). The present results also indicate that anion exchange is at least functionally absent *in vitro* since none of the bicarbonate formed as a result of the Haldane effect is transferred to the plasma.

Assuming an *in vivo* haematocrit of 27.4 % reported by Tufts *et al.* (1992), the present data can be used to partition the distribution of CO_2 in fully oxygenated and deoxygenated whole blood. This distribution can then be compared with the pattern of CO_2 transport observed in sea lampreys *in vivo*. At a P_{CO_2} of 0.2 kPa, the red blood cells carry about 17 and 26 % of the total CO_2 in oxygenated and deoxygenated blood respectively (Fig. 6). These values are quite similar to values of 16 and 22 % determined by Tufts *et al.* (1992) for arterial and venous blood of resting sea lampreys *in vivo*. Moreover, these values are substantially greater than the 8 and 10 % values determined for arterial and venous erythrocytes in the rainbow trout (Heming, 1984). *In vitro*, the contribution of the red blood cell to total CO_2 carriage increases markedly to 21 % and 45 % for oxygenated and deoxygenated blood, respectively, when the P_{CO_2} is increased to 0.96 kPa. Similarly, immediately after exercise *in vivo*, there is an increase in P_{CO_2} in both arterial and venous blood, and the CO_2 attributable to the red blood cells increases to 25 and 38 %, respectively, in these compartments (Tufts *et al.* 1992). The distribution of CO_2 between plasma and red blood cells observed in arterial and venous blood *in vivo* can therefore be largely explained by the present *in vitro* experiments.

Carbon dioxide excretion across the gills will be largely determined by (i) the carbon dioxide tensions in arterial and venous blood and (ii) the carbon dioxide dissociation characteristics of plasma and red blood cells under both oxygenated and deoxygenated conditions. Taking these factors into consideration, the present *in vitro* results indicate that arteriovenous differences in red blood cell C_{CO_2} should account for a large fraction of the CO_2 excreted across the gills of the sea lamprey. For example, when the CO_2 distribution in deoxygenated blood at an elevated CO_2 tension (2.9 kPa) is compared to that of oxygenated blood at a low CO_2 tension (0.2 kPa), it can be determined that 71 % of the difference in whole-blood C_{CO_2} is attributable to changes in C_{CO_2} within the red blood cells (Fig. 6). Again, this correlates well with the recent observations of Tufts *et al.* (1992) indicating that, unlike other vertebrates, the majority of CO_2 excreted across the gills of sea lampreys *in vivo* can be attributed to arteriovenous changes in red blood cell C_{CO_2}. *In vivo*, changes in red blood cell C_{CO_2} account for about 60 % of the arteriovenous difference in lamprey whole blood at rest and almost 80 % after exercise (Tufts *et al.* 1992).

As suggested by Tufts and Boutiler (1990), the absence of net bicarbonate movements across the membrane of lamprey red blood cells may also have a considerable impact on the red blood cell pH in these animals. In most vertebrates, transfer of bicarbonate across the erythrocyte membrane *via* the anion exchanger is associated with a decrease in red blood cell pH at any given P_{CO_2} (Heisler, 1986). In contrast to other vertebrates, however, all the bicarbonate formed by deoxygenated haemoglobin in lampreys appears to be retained within their red blood cells (Nikinmaa and Mattsoff, 1992; Figs 4, 5). Thus, the functional absence of bicarbonate transfer across the red blood cell membrane *in vitro* may be associated with a relatively larger Haldane-mediated increase in the red blood cell pH compared to that in other vertebrates (Figs 2, 3). The relatively high red blood cell pH observed in lampreys *in vivo* may also be related to the absence of bicarbonate movements from the red blood cell to the plasma (Nikinmaa and Weber, 1984; Mattsoff and Nikinmaa, 1988; Tufts, 1991; Tufts *et al.* 1992).

There is a marked elevation in the sodium concentration in sea lamprey red blood cells when the P_{CO_2} of the equilibration gas is increased (Fig. 7). This is also observed in the presence of ouabain after a step increase in P_{CO_2} (Tufts, 1992). The increase in intracellular sodium concentration is therefore probably not due to a reduction in the activity of the Na^+/K^+-ATPase and may indicate activation of ion transport processes across the red blood cell membrane. The present results suggest that sodium-dependent ion transport processes may also be activated in deoxygenated blood (Figs 7, 8). In contrast, Nikinmaa and Mattsoff (1992) recently observed no changes in sodium levels in deoxygenated blood of the river lamprey. Nevertheless, it is noteworthy that the red blood cell pH of both the river lamprey and the sea lamprey are dependent on the extracellular sodium concentration (Nikinmaa, 1986; Nikinmaa *et al.* 1986; Tufts, 1992). Moreover, extracellular sodium concentration also influences the distribution of CO_2 across the red blood cell membrane in the sea lamprey (Tufts, 1992). Thus, the activation of ion transport processes may, in fact, significantly contribute to the respiratory properties of lamprey blood and further experiments to clarify their relative importance are clearly warranted.

In conclusion, the present results confirm the view that the strategy for gas transport in agnathans is markedly different from that in more recent vertebrates. When the red blood cell pH is used to determine the Bohr effect, it is apparent that CO_2 transport may take precedence over oxygen delivery in the blood of these animals. The present *in vitro* results also confirm that the majority of CO_2 added to the blood by the tissues is probably transported within the red blood cell to the gills. This strategy may be necessary for effective CO_2 transport since red blood cell chloride/bicarbonate exchange appears to be functionally limited or absent in agnathans. It is possible, however, that retention of bicarbonate within the red blood cell may also compromise oxygen delivery by limiting red blood cell pH changes as blood passes through the tissues. Thus, oxygen and carbon dioxide transport appear to be largely uncoupled in the blood of these primitive vertebrates. In view of the present results, one can speculate that more effective coupling between oxygen and carbon dioxide transport may have been an important selective pressure in the evolution of rapid chloride/bicarbonate exchange in red blood cells.

This study was supported by an NSERC Operating Grant to B.L.T., an NSERC Postdoctoral Fellowship to R.A.F. and NSERC Summer Awards to N.S. and B.B. The authors would also like to thank the Lamprey Control Center (Department of Fisheries and Oceans) in Sault Ste Marie, Ontario, for their assistance in obtaining the sea lampreys.

References

ALBERS, C., GOETZ, K. H. AND HUGHES, G. M. (1983). Effect of acclimation temperature on intraerythrocytic acid–base balance and nucleotide triphosphates in the carp, *Cyprinus carpio*. *Respir. Physiol.* **54**, 145–159.

BIRD, D. J., LUTZ, P. L. AND POTTER, I. C. (1976). Oxygen dissociation curves of the blood of larval and adult lampreys (*Lampetra fluviatilis*). *J. exp. Biol.* **65**, 449–458.

BOUTILIER, R. G., HEMING, T. A. AND IWAMA, G. K. (1984). Physico-chemical parameters for use in fish respiratory physiology. In *Fish Physiology*, vol. XA (ed. W. S. Hoar and D. J. Randall), pp. 401–430. New York: Academic Press.

BOUTILIER, R. G., RANDALL, D. J., SHELTON, G. AND TOEWS, D. P. (1979). Acid–base relationships in the blood of the toad, *Bufo marinus*. I. The effects of environmental CO_2. *J. exp. Biol.* **82**, 331–344.

BOUTILIER, R. G. AND TOEWS, D. P. (1981). Respiratory properties of blood in a strictly aquatic and predominantly skin-breathing urodele, *Cryptobranchus alleganiensis*. *Respir. Physiol.* **46**, 161–176.

CAMERON, J. N. (1979). Excretion of CO_2 in water-breathing animals: a short review. *Mar. biol. Lett.* **1**, 3–13.

DRABKIN, D. L. AND AUSTIN, J. H. (1935). Spectrophotometric studies. II. Preparations from washed blood cells; nitric oxide hemoglobin and sulfhemoglobin. *J. biol. Chem.* **112**, 51–65.

ELLORY, J. C., WOLOWYK, M. W. AND YOUNG, J. D. (1987). Hagfish (*Eptatretus stouti*) erythrocytes show minimal chloride transport activity. *J. exp. Biol.* **129**, 377–383.

HARDISTY, M. W. (1979). *Biology of the Cyclostomes*. Cambridge: Cambridge University Press. 428pp.

HEISLER, N. (1986). Buffering and transmembrane ion transfer processes. In *Acid–Base Regulation in Animals* (ed. N. Heisler), pp. 3–47. Amsterdam: Elsevier.

HEMING, T. A. (1984). The role of fish erythrocytes in transport and excretion of carbon dioxide. PhD thesis, University of British Columbia, Vancouver, BC. 177pp.

HOUSTON, A. H. (1985). Erythrocyte magnesium in freshwater fishes. *Magnesium*, **4**, 106–128.

HUGHES, G. M., BELAUD, A., PEYRAUD, C. AND ADCOCK, P. J. (1982). A comparison of two methods for measurement of O_2 content of small (20 μl) samples of fish blood. *J. exp. Biol.* **96**, 417–420.

JENSEN, F. B. (1986). Pronounced influence of Hb-O_2 saturation on red cell pH in tench blood *in vivo* and *in vitro*. *J. exp. Zool.* **238**, 119–124.

JENSEN, F. B. (1989). Hydrogen ion equilibria in fish haemoglobins. *J. exp. Biol.* **143**, 225–234.

KLOCKE, R. A. (1987). Carbon dioxide transport. In *Handbook of Physiology*, section 3, *The Respiratory System*, vol. 4, *Gas exchange* (ed. L. E. Farhi and S. M. Tenney), pp. 173–197.

LAPENNAS, G. N. (1983). The magnitude of the Bohr coefficient: Optimal for oxygen delivery. *Respir. Physiol.* **54**, 161–172.

LENHERT, P. G., LOVE, W. E. AND CARLSON, F. D. (1956). The molecular weight of haemoglobin from *Petromyzon marinus*. *Biol. Bull. mar. biol. Lab., Woods Hole* **111**, 293–294.

MANWELL, C. (1963). The blood proteins of cyclostomes. A study in phylogenetic and ontogenetic biochemistry. In *The Biology of* Myxine (ed. A. Brodal and R. Fänge), pp. 372–455. Oslo: Universitetsforlaget.

MATTSOFF, L. AND NIKINMAA, M. (1988). Effects of external acidification on the blood acid–base status and ion concentrations of lamprey. *J. exp. Biol.* **136**, 351–361.

NIKINMAA, M. (1986). Red cell pH of lamprey (*Lampetra fluviatilis*) red blood cells is actively regulated. *J. comp. Physiol.* B **156**, 747–750.

NIKINMAA, M. (1990). *Vertebrate Red Blood Cells*. Berlin, Heidelberg: Springer Verlag. 262pp.

NIKINMAA, M., KUNNAMO-OJALA, T. AND RAILO, R. E. (1986). Mechanisms of pH regulation in lamprey (*Lampetra fluviatilis*) red blood cells. *J. exp. Biol.* **122**, 355–367.

NIKINMAA, M. AND MATTSOFF, L. (1992). Effects of oxygen saturation on the CO_2 transport properties of *Lampetra* red cells. *Respir. Physiol.* (in press).

NIKINMAA, M. AND RAILO, R. E. (1987). Anion movements across lamprey (*Lampetra fluviatilis*) red cell membrane. *Biochim. biophys. Acta* **899**, 134–136.

NIKINMAA, M. AND TUFTS, B. L. (1989). Regulation of acid and ion transfer across the membrane of nucleated erythrocytes. *Can. J. Zool.* **67**, 3039–3045.

NIKINMAA, M. AND WEBER, R. E. (1984). Hypoxic acclimation in the lamprey, *Lampetra fluviatilis*: organismic and erythrocytic responses. *J. exp. Biol.* **109**, 109–119.

PERRY, S. F. (1986). Carbon dioxide excretion in fishes. *Can. J. Zool.* **64**, 565–572.

RIGGS, A. (1972). The haemoglobins. In *The Biology of Lampreys*, vol. 2 (ed. M. W. Hardisty and I. C. Potter), pp. 209–252. London: Academic Press.

ROUGHTON, F. J. W. (1964). Transport of oxygen and carbon dioxide. In *Handbook of Physiology*, vol. 1 (ed. W. O. Fenn and H. Rahn), pp. 767–825. Washington, DC: American Physiological Society.

TUCKER, V. A. (1967). Method for oxygen content and dissociation curves on microliter blood samples. *J. appl. Physiol.* **23**, 410–414.

TUFTS, B. L. (1991). Acid–base regulation and blood gas transport following exhaustive exercise in an agnathan, the sea lamprey, *Petromyzon marinus*. *J. exp. Biol.* **159**, 371–385.

TUFTS, B. L. (1992). Sodium-dependent pH regulation in sea lamprey (*Petromyzon marinus*) red blood cells. *Can. J. Zool.* **70**, 411–416.

TUFTS, B. L., BAGATTO, B. AND CAMERON, B. (1992). *In vivo* partitioning of carbon dioxide transport between plasma and red blood cells in the sea lamprey, *Petromyzon marinus*. *J. exp. Biol.* (in press).

TUFTS, B. L. AND BOUTILIER, R. G. (1989). The absence of rapid chloride/bicarbonate exchange in lamprey erythrocytes: implications for CO_2 transport and ion distributions between plasma and erythrocytes in the blood of *Petromyzon marinus*. *J. exp. Biol.* **144**, 565–576.

TUFTS, B. L. AND BOUTILIER, R. G. (1990). CO_2 transport in agnathan blood: evidence of erythrocyte Cl^-/HCO_3^- exchange limitations. *Respir. Physiol.* **80**, 335–348.

WALD, G. AND RIGGS, A. (1951). The haemoglobin of the sea lamprey, *Petromyzon marinus*. *J. gen. Physiol.* **35**, 45–53.

WYMAN, J. (1964). Linked functions and reciprocal effects in haemoglobin: A second look. *Adv. Protein Chem.* **19**, 223–286.

ZEIDLER, R. AND KIM, H. D. (1977). Preferential hemolysis of postnatal calf red cells induced by internal alkalinization. *J. gen. Physiol.* **70**, 385–401.