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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*

BY B. L. TUFTS AND R. G. BOUTILIER

Biology Department, Dalhousie University, Halifax, Nova Scotia, Canada B3H 4J1

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Summary

Carbon dioxide transport and ion distributions were examined in the blood of the lamprey, *Petromyzon marinus*. Over the P_{CO_2} range studied, the erythrocytes had the highest total CO_2 content, followed by whole blood and true plasma. The nonbicarbonate buffer values were $-37{\cdot}0\,\mathrm{mequiv\,l^{-1}\,pH\,unit^{-1}}$ for erythrocytes, $-3{\cdot}3\,\mathrm{mequiv\,l^{-1}\,pH\,unit^{-1}}$ for whole blood and $-0{\cdot}1\,\mathrm{mequiv\,l^{-1}\,pH\,unit^{-1}}$ for true plasma. These results are in sharp contrast to the models of carbon dioxide transport in the blood of other vertebrates and are consistent with the view that chloride/bicarbonate exchange is virtually absent in agnathan erythrocytes. Protons are passively distributed in *Petromyzon* blood. However, the distribution ratio for chloride between plasma and erythrocytes was strikingly different from the distribution ratio for protons. In the absence of rapid chloride/bicarbonate exchange, the erythrocyte volume is relatively constant over the physiological pH range. A model is presented to explain carbon dioxide transport in lamprey blood which does not involve a rapid chloride/bicarbonate exchange mechanism on the erythrocyte membrane.

Introduction

In mammals, as blood passes through the tissues, CO_2 diffuses into the erythrocyte where it combines with water to form bicarbonate and a proton in the presence of erythrocytic carbonic anhydrase. The bicarbonate formed by the CO_2 hydration reaction is rapidly exchanged for plasma chloride and the bulk of CO_2 transported in the blood is in the form of plasma bicarbonate (Roughton, 1964). At the lung, the bicarbonate re-enters the erythrocyte where it combines with a proton to form water and CO_2, which is excreted. Rapid chloride/bicarbonate exchange is an integral part of this CO_2 transport system. In this system, the chloride/bicarbonate exchange and the acid–base reactions within the erythrocyte

Key words: lamprey, chloride/bicarbonate exchange, erythrocytes, CO_2 transport, ion distributions, buffering.

also link the movements of protons, bicarbonate and chloride, and these ions are distributed in a Donnan equilibrium between the plasma and the erythrocyte (Hladky & Rink, 1977). Rapid, passive anion exchange has also been demonstrated in nucleated erythrocytes (Cameron, 1978; Obaid *et al.* 1979; Romano & Passow, 1984). Thus, the CO_2 transport properties of the blood of the lower vertebrates are basically similar to those of mammalian blood (Boutilier *et al.* 1979; Boutilier & Toews, 1981; Randall & Daxboeck, 1984). As in mammals, the presence of rapid anion exchange on the erythrocyte membrane also contributes to the similar distribution ratio for protons, bicarbonate and chloride ions in the blood of these animals (McDonald *et al.* 1980; Albers & Goetz, 1985; Heming *et al.* 1986; Tufts & Randall, 1988).

Recent evidence suggests that chloride/bicarbonate exchange may be absent in *Lampetra fluviatilis* erythrocytes (Nikinmaa & Railo, 1987). In addition, Ellory *et al.* (1987) have demonstrated that chloride/bicarbonate exchange activity is extremely low in erythrocytes of the hagfish, *Eptatretus stouti*. Clearly, the low activity or absence of this exchanger would have a large influence on the CO_2 transport properties and distributions of ions in the blood of these animals. The purpose of these experiments was, therefore, to determine whether the CO_2 transport properties and distributions of ions in the blood of the lamprey *Petromyzon marinus* support the view that the anion exchanger is virtually absent in agnathan erythrocytes.

Materials and methods

Animals

Adult lampreys, *Petromyzon marinus* Linnaeus, were collected during their spawning migration from a fishladder on the Lahave River in Nova Scotia, Canada. The animals were transported to the Aquatron facility at Dalhousie University where they were held in freshwater aquaria at 10°C for at least 1 week prior to the experiment.

Blood sampling

The blood used in these experiments was obtained from anaesthetized lampreys *via* caudal vessel puncture or from resting lampreys *via* a dorsal aortic cannula which had been surgically implanted at least 24 h prior to the experiment.

Experimental protocol

In the first series of experiments, the collected blood was equilibrated in an intermittently rotating glass tonometer at 10°C with a humidified gas mixture of 0·2 %, 1 % or 3 % CO_2 in air (Wösthoff gas-mixing pumps, Bochum, FRG). Following a 30 min equilibration period, a 1 ml sample was removed from the tonometer using a 1 ml Hamilton gas-tight syringe. A 50 μl sample of this blood was immediately dispensed into another Hamilton syringe for the analysis of total CO_2 content. Another 100 μl portion of the original sample was used for duplicate

hematocrit measurements. Following the determination of the hematocrit, 50 μl of the plasma fraction from the hematocrit tubes was analyzed for total CO_2 content. The remainder of the original sample was divided equally between two 0·5 ml Eppendorf tubes and centrifuged. After centrifugation, the plasma pH of the first tube was measured and the remaining plasma from this tube was discarded. The cell pellet was frozen in liquid nitrogen for later analysis of erythrocyte pH. The plasma from the second tube was removed, frozen in liquid nitrogen and stored for subsequent ion analyses. The erythrocyte pellet from this tube was saved for the determination of both erythrocyte water content and ion concentrations. This equilibration and sampling procedure was repeated at each of the specified gas concentrations.

Another series of experiments examined the effects of the protonophore 2,4-dinitrophenol (2,4-DNP) on the distribution of ions across the lamprey erythrocyte membrane. In these experiments, the collected blood was divided equally between two tonometers. The blood from the first tonometer was incubated in the presence of 10^{-4} mol l^{-1} 2,4-DNP. An equivalent volume (50 μl) of saline was added to the second tonometer which served as a control. The equilibration and sampling procedure were similar to the first series of experiments. In this series, however, the total CO_2 content determinations were omitted and the humidified gas mixtures also included 0·6 % and 2 % CO_2 in air.

Analyses

Plasma and erythrocyte pH determinations were made with a Radiometer PHM 84 pH meter and associated micro-pH unit (Radiometer, Copenhagen, Denmark). The freeze–thaw method of Zeidler & Kim (1977) was used to prepare the erythrocyte pellet for the determination of pH. Erythrocyte water content was determined by weighing the wet cell pellet, drying it to a constant mass at 80 °C and reweighing it (Nikinmaa & Huestis, 1984). The dried erythrocyte pellet was then dissolved in 200 μl of 5·5 mol l^{-1} HNO_3 for the analysis of erythrocyte ions. Chloride concentrations were measured with a Buchler–Cotlove chloride titrator (Buchler Instruments Inc., USA). Sodium and potassium determinations were made with a Corning 410 flame photometer (Ciba Corning Dianostics Corp., Canada). Total CO_2 contents of whole blood and plasma were measured with a Carle Series 100 gas chromatograph (Carle Instruments Inc., USA), as described by Boutilier *et al.* (1985). Bicarbonate concentrations of whole blood and plasma were calculated using the following equation:

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

where C_{CO_2} is the measured total CO_2 content of whole blood or plasma, P_{CO_2} is the partial pressure of CO_2 in the delivered gas, determined from the barometric pressure and the water vapour pressure, and αCO_2 is the solubility of CO_2 taken from Boutilier *et al.* (1984). The erythrocyte bicarbonate concentration was calculated as:

$$[HCO_3^-]_i = \{[HCO_3^-]_{bl} - [HCO_3^-]_{pl} \times (1 - Hct)\}/Hct,$$

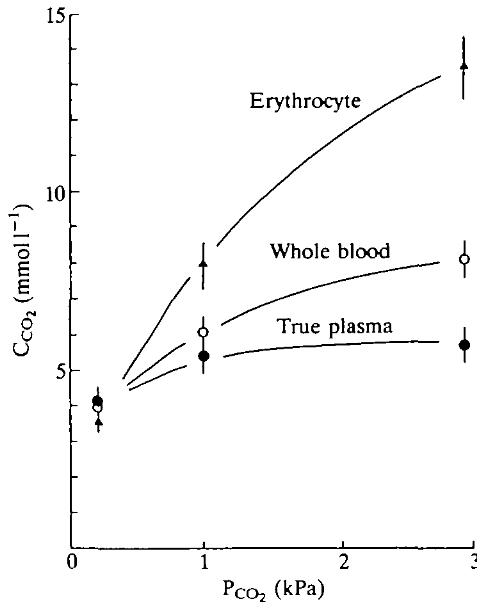

Fig. 1. Carbon dioxide dissociation curves for erythrocytes (▲), whole blood (○) and true plasma (●) of the lamprey *Petromyzon marinus*. All values are means ± S.E.M. ($N=8$). 1 kPa = 7·5 mmHg.

where $[HCO_3^-]_i$, $[HCO_3^-]_{bl}$ and $[HCO_3^-]_{pl}$ are the erythrocyte, whole blood and plasma bicarbonate concentrations, respectively, and Hct is the hematocrit.

Results

CO_2 dissociation curves of whole blood, true plasma (plasma equilibrated with erythrocytes) and erythrocytes are compared in Fig. 1. At a P_{CO_2} of 0·2 kPa (=1·5 mmHg), C_{CO_2} values for the three compartments were similar. At elevated P_{CO_2} values, however, the erythrocytes had the highest C_{CO_2}, followed by whole blood and true plasma. At the highest experimental P_{CO_2} (3·01 kPa), the C_{CO_2} of the erythrocytes reached 13·4 ± 1·0 mmol l^{-1} whereas the C_{CO_2} of whole blood at this P_{CO_2} was 8·1 ± 0·5 mmol l^{-1} and that of true plasma was 5·7 ± 0·5 mmol l^{-1}. The erythrocytes also had the highest buffering capacity of the three compartments examined (Fig. 2). The erythrocyte buffer value ($\Delta HCO_3^-/\Delta pH$) was −37·0 mequiv l^{-1} pH unit^{-1}, whereas the buffer values of whole blood and true plasma were only −3·3 and −0·1 mequiv l^{-1} pH unit^{-1}, respectively.

The consequences of the large buffering capacity of the erythrocyte and the low buffering capacity of the plasma were demonstrated when erythrocyte pH was plotted against plasma pH in lamprey blood (Fig. 3). The slope of the regression line generated for this relationship was only 0·27 and, therefore, a plasma pH change of 0·368 units was required to cause an erythrocyte pH change of 0·1 unit. The relationship between pHe and pHi was not significantly different when

lamprey blood was equilibrated in the presence of the protonophore 2,4-DNP. Thus, the protons appeared to be passively distributed across the erythrocyte membrane in lamprey blood in the present experiments. It should also be noted that the relationship between pHe and pHi for blood obtained *via* caudal vessel

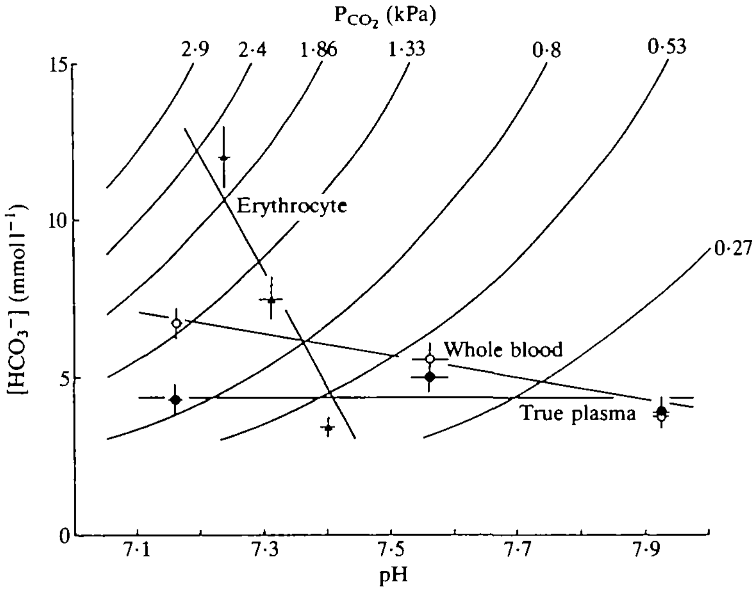

Fig. 2. Nonbicarbonate buffer lines for erythrocytes (▲), whole blood (○) and true plasma (●) of the lamprey, *Petromyzon marinus*. All values are means ± S.E.M. ($N = 8$). The nonbicarbonate buffer values ($\Delta HCO_3^-/\Delta pH$) for erythrocytes, whole blood and true plasma are −37·0, −3·3 and −0·1 mequiv l^{-1} pH unit^{-1}, respectively. 1 kPa = 7·5 mmHg.

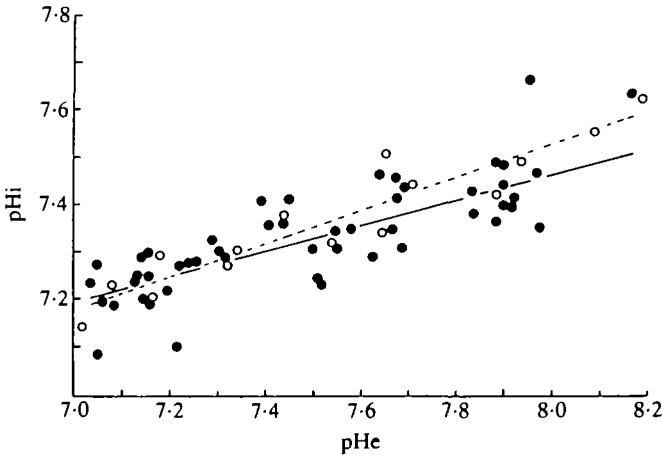

Fig. 3. Erythrocyte pH (pHi) *versus* extracellular pH (pHe) of lamprey, *Petromyzon marinus*, blood. Solid circles represent control values and open circles represent values in the presence of 10^{-4} mol l^{-1} 2,4-dinitrophenol.

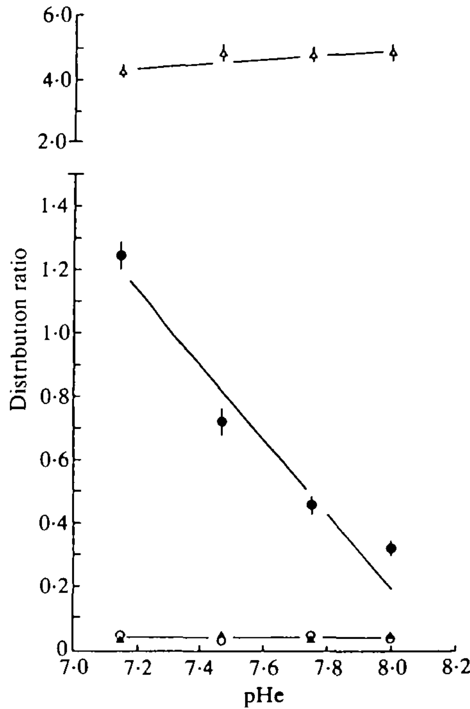

Fig. 4. Distribution ratios *versus* extracellular pH (pHe) for sodium (△), potassium (▲) and chloride (○) ions and protons (●) in the blood of the lamprey *Petromyzon marinus*. All values are means ± S.E.M.

puncture was not significantly different from that for blood obtained *via* the dorsal aortic cannula in resting animals.

The distribution ratios of sodium, potassium, chloride and protons across the erythrocyte membrane in lamprey blood are plotted against pHe in Fig. 4. Of these, only the distribution ratio for protons (rH^+) changed according to the extracellular pH. It is noteworthy that the distribution ratio for chloride was not only independent of pHe, but was far removed from rH^+. Indeed, the overall mean rCl^- in the present experiments was only 0·04. The low rCl^- values are a reflection of the extremely low erythrocyte concentrations of chloride measured in these experiments (Table 1). It is unlikely that these values represent an error in analysis since simultaneous analysis of trout blood ($N=6$) resulted in an erythrocyte chloride concentration of 33·6 mequiv l^{-1} and an rCl^- of 0·25. These trout values are similar to results obtained by other researchers (Ferguson & Boutilier, 1988; Heming *et al.* 1986). It should also be pointed out that any error caused by trapped plasma in the erythrocyte pellet would result in an overestimate of the erythrocyte chloride concentration. Thus, the actual erythrocyte chloride concentration in *Petromyzon marinus* may be slightly lower than the value of 4·3 ± 0·3 mequiv l^{-1} obtained in these experiments.

The erythrocyte water content is plotted against extracellular pH in Fig. 5.

Table 1. *Overall mean concentrations of sodium, potassium and chloride in plasma and erythrocytes of the lamprey* Petromyzon marinus

	Concentration (mequiv l^{-1})	
Ion	Plasma	Erythrocyte
Sodium ($N = 47$)	122·7 ± 1·9	27·5 ± 0·7
Potassium ($N = 47$)	3·6 ± 0·1	94·6 ± 0·8
Chloride ($N = 47$)	112·0 ± 1·2	4·3 ± 0·3

Values are means ± S.E.M.

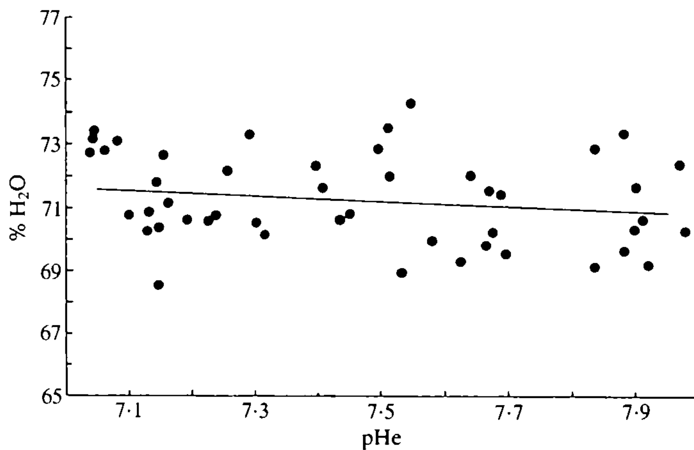

Fig. 5. Erythrocyte water content (% H_2O) *versus* extracellular pH in the lamprey *Petromyzon marinus*.

Water content increased as pHe decreased, but the magnitude of the increase was only 0·07 %/0·1 pH unit. Thus, the erythrocyte water content varied less than 1 % over the entire pH range studied.

Discussion

Nikinmaa & Railo (1987) have demonstrated that the apparent chloride permeability of *Lampetra fluviatilis* erythrocytes is similar to the apparent chloride permeability of lipid bilayers at the same temperature. In the same study, these authors found that chloride movements across the erythrocyte membrane were not affected by the anion exchange inhibitor 4,4-diisothiocyanostilbene 2,2-disulphonic acid (DIDS). Ellory *et al.* (1987) have also demonstrated extremely low chloride/bicarbonate exchange activity in erythrocytes from the hagfish, *Eptatretus stouti*. In the present study, the CO_2 transport properties of the blood from another agnathan species, *Petromyzon marinus*, clearly support the growing view

that erythrocytic chloride/bicarbonate exchange is virtually absent in agnathan erythrocytes.

The CO_2 transport properties of mammalian blood have been extensively described by Roughton (1964). Similar models of CO_2 transport have been described in the blood of other vertebrates (Boutilier *et al.* 1979; Boutilier & Toews, 1981; Randall & Daxboeck, 1984). In these systems, the majority of the bicarbonate formed by the CO_2 hydration reaction within the erythrocyte is exchanged for plasma chloride *via* the anion exchange mechanism on the erythrocyte membrane. Thus, at any given P_{CO_2}, the total CO_2 content of true plasma is greater than that of either whole blood or erythrocytes (Roughton, 1964; Boutilier *et al.* 1979; Boutilier & Toews, 1981). In the present study, the total CO_2 content of true plasma was lower than that of either whole blood or erythrocytes (Fig. 1). Indeed, in *Petromyzon marinus* blood, the erythrocytes had the highest C_{CO_2} content over the majority of the P_{CO_2} range studied. Carbamino formation could contribute somewhat to the high erythrocyte C_{CO_2} levels. These results suggest, however, that the bicarbonate formed within the erythrocytes of *Petromyzon marinus* is not transferred to the plasma, but remains within the erythrocyte.

According to Nikinmaa & Railo (1987), erythrocyte buffering is effectively isolated from the extracellular compartment in *Lampetra fluviatilis* since practically no extracellular pH recovery occurred in an unbuffered erythrocyte suspension after acidification or alkalinization. The nonbicarbonate buffer lines of true plasma, whole blood and erythrocytes for *Petromyzon marinus* provide further evidence that erythrocytic bicarbonate does not have access to the plasma in agnathan blood (Fig. 2). The slope of the nonbicarbonate buffer line for true plasma in this study is only $-0{\cdot}1\,\text{mequiv}\,l^{-1}\,\text{pH}\,\text{unit}^{-1}$, whereas that of whole blood is $-3{\cdot}3\,\text{mequiv}\,l^{-1}\,\text{pH}\,\text{unit}^{-1}$. In other species, the slope of the true plasma buffer line is invariably higher than that of whole blood (Woodbury, 1974; Boutilier *et al.* 1979; Boutilier & Toews, 1981; Heisler, 1986). This relationship occurs because bicarbonate formed within the erythrocyte (after nonbicarbonate buffering of protons) is transferred *via* the anion exchanger to the plasma to re-establish the Donnan equilibrium for bicarbonate (Woodbury, 1974; Heisler, 1986). Only the nonbicarbonate buffer lines of separated plasma (i.e. plasma that has not been equilibrated in the presence of erythrocytes) for other species approach the slope of the true plasma buffer line found for *Petromyzon marinus* blood. The explanation for this result can only be that the bicarbonate formed within the erythrocyte does not reach the plasma. This would also explain the unusually high nonbicarbonate buffer value for the erythrocytes ($-37{\cdot}0\,\text{mequiv}\,l^{-1}\,\text{pH}\,\text{unit}^{-1}$). In trout erythrocytes, the nonbicarbonate buffer value at a similar temperature is only $16{\cdot}5\,\text{mequiv}\,l^{-1}\,\text{pH}\,\text{unit}^{-1}$ (calculated from Heming *et al.* 1986). The measured nonbicarbonate buffer values of *Petromyzon* true plasma and erythrocytes do not resemble the 'apparent' buffer values commonly measured for these compartments in other vertebrates. Our buffer values do, however, resemble the actual nonbicarbonate buffer values which would be

expected for these compartments prior to the bicarbonate redistribution process (Heisler, 1986).

The erythrocyte pH in *Lampetra fluviatilis* is actively maintained by a sodium/proton exchange mechanism (Nikinmaa, 1986; Nikinmaa *et al.* 1986). In the present experiments, however, the pH of *Petromyzon* erythrocytes was not affected by the protonophore 2,4-DNP (Fig. 3). These results suggest that protons are passively distributed across the erythrocyte membrane in *Petromyzon*. Thus, in *Petromyzon* the relatively low slope of the pHi *versus* pHe regression line must be due solely to the high buffering capacity of the erythrocytes.

In nucleated erythrocytes, the chloride/bicarbonate exchanger normally increases the permeability of the erythrocyte membrane to chloride ions and links the movements of chloride, bicarbonate and protons (Hladky & Rink, 1977; Fortes, 1977). Thus, in erythrocytes with a rapid anion exchange mechanism, the distribution ratios for protons and chloride ions are similar (Albers & Goetz, 1985; Heming *et al.* 1986; Tufts & Randall, 1988). In *Petromyzon* blood, however, the distribution ratios for protons and chloride ions are strikingly different from each other (Fig. 4). Such differences suggest that either protons or chloride ions are not in Donnan equilibrium. Our data indicate that protons are in electrochemical equilibrium in *Petromyzon* erythrocytes since the protonophore 2,4-DNP did not alter the relationship between pHe and pHi (Fig. 3). Chloride ions are, therefore, probably not distributed according to a Donnan equilibrium in *Petromyzon* erythrocytes. This result is not entirely explained by the absence of the anion exchanger since passive conductive pathways for chloride should bring this ion into electrochemical equilibrium. Ellory *et al.* (1987) have suggested that chloride ion movements may occur across the membrane of *Eptatretus stouti* erythrocytes *via* an unidentified transporter which is not DIDS-sensitive. A similar transport mechanism may be keeping chloride out of electrochemical equilibrium in *Petromyzon* erythrocytes and this possibility clearly warrants further investigation.

In erythrocytes with an anion exchanger, there is an increase in erythrocyte chloride concentration, and therefore rCl^-, with decreasing pH, and water is drawn into the cell osmotically (Hladky & Rink, 1977; Heming *et al.* 1986; Nikinmaa *et al.* 1987; Tufts & Randall, 1988). In *Petromyzon* rCl^- did not change significantly and the water content of erythrocytes varied less than 1 % over the pH range studied (Figs 4, 5). A similar situation occurs when trout erythrocytes have been incubated in the presence of the anion exchange blocker DIDS (Nikinmaa *et al.* 1987). Thus, the absence of rapid chloride movements across the erythrocyte membrane also results in a rather constant erythrocyte volume.

The extremely low rCl^- in *Petromyzon* blood results from the unusually low levels of chloride within the erythrocyte (Table 1). It is unlikely that these levels represent a measurement error since the distribution ratios for sodium and potassium in *Petromyzon* blood (Fig. 4) and simultaneously determined chloride distribution ratios for *Salmo gairdneri* blood (see Results section) gave values similar to those in the literature. According to Hladky & Rink (1977), bicarbonate

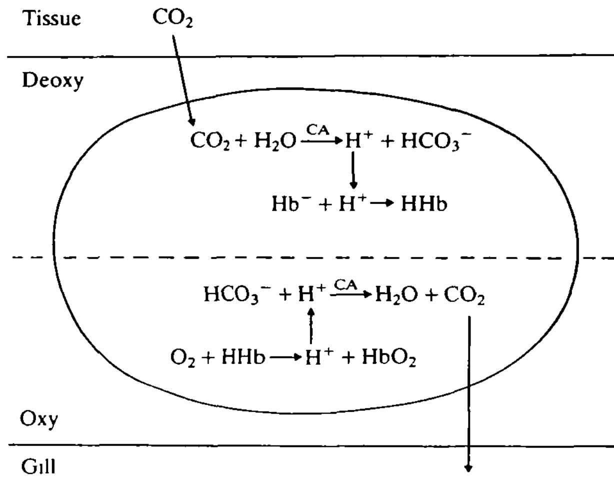

Fig. 6. Model of carbon dioxide transport in the blood of the lamprey *Petromyzon marinus*. In the tissues (Deoxy), CO_2 diffuses into the erythrocyte where it is hydrated to form HCO_3^- and a proton which is bound to hemoglobin (Hb). The HCO_3^- is not transported to the plasma, but is carried within the erythrocyte to the gill. At the gill (Oxy), oxygenation of hemoglobin liberates a proton which combines with the HCO_3^-, and CO_2 is evolved. CA, carbonic anhydrase.

movements *via* the anion exchanger will be greatly influenced by the erythrocyte chloride concentration. Thus, it follows that vertebrate erythrocytes contain ample levels of chloride to meet the requirements of the anion exchanger during the excretion of CO_2. In this regard, it is interesting that the chloride concentrations in *Petromyzon* erythrocytes are extremely low, since the results of this study suggest that the anion exchanger is absent in *Petromyzon* erythrocytes.

Clearly, the transport and excretion of CO_2 in agnathans does not conform to any of the models described for other vertebrates since these models all involve rapid chloride/bicarbonate exchange across the erythrocyte membrane. In agnathan blood, CO_2 still diffuses across the erythrocyte membrane and is hydrated to form bicarbonate and a proton. Nikinmaa *et al.* (1986) have demonstrated that carbonic anhydrase is present in *Lampetra fluviatilis* erythrocytes. As in other vertebrates, therefore, this reaction proceeds at the catalysed rate. In the absence of rapid chloride/bicarbonate exchange, however, the resulting bicarbonate is transported *within the erythrocyte* to the gas exchange organ rather than exported for carriage in the plasma. At the gas exchange organ, oxygenation of hemoglobin presumably liberates the protons that combine with bicarbonate to evolve CO_2. These events are summarized in Fig. 6.

The lamprey is an extant member of a phylogenetically primitive group of vertebrates. These results, therefore, invite speculation about the evolution of gas

transport by the vertebrate erythrocyte. In its early stages, the erythrocyte may have evolved to carry both oxygen *and* carbon dioxide. The importance of the plasma in CO_2 transport and, therefore, of elevated levels of plasma bicarbonate must only have arisen after the evolution of erythrocytic chloride/bicarbonate exchange.

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