HAGFISH (MYXINE GLUTINOSA) RED CELL MEMBRANE EXHIBITS NO BICARBONATE PERMEABILITY AS DETECTED BY 18O EXCHANGE

THOMAS PETERS1,*, ROBERT E. FORSTER2 AND GEROLF GROS1
1Abteilung Vegetative Physiologie, Zentrum Physiologie, Medizinische Hochschule, 30623 Hannover, Germany and
2Department of Physiology, School of Medicine, University of Pennsylvania, Philadelphia, PA 19174, USA
*e-mail: Peters.Thomas@MH-Hannover.de

Accepted 29 February; published on WWW 18 April 2000

Summary

The bicarbonate permeability of the plasma membrane of intact hagfish (Myxine glutinosa) red blood cells and the intracellular carbonic anhydrase activity of these cells were determined by applying the 18O exchange reaction using a special mass spectrometric technique. When the macromolecular carbonic anhydrase inhibitor Prontosil–Dextran was used to suppress any extracellular carbonic anhydrase activity, the mean intracellular acceleration of the CO2 hydration/HCO3− dehydration reaction over the uncatalyzed reaction (referred to as intracellular carbonic anhydrase activity A1) was 21320±3000 at 10 °C (mean ± S.d., N=9). The mean bicarbonate permeability of the red blood cell membrane (P HCO3−) was indistinguishable from zero. It can be concluded that CO2 transport within hagfish blood does not follow the classical scheme of CO2 transport in vertebrate blood. It is suggested that the combination of considerable intraerythrocytic carbonic anhydrase activity and low P HCO3− may serve to enhance O2 delivery to the tissue in the exceptionally hypoxia-tolerant hagfish.

Key words: blood, bicarbonate permeability, carbonic anhydrase activity, red blood cell, hagfish, Myxine glutinosa, mass spectrometry, carbon dioxide transport, 18O exchange reaction.

Introduction

The classical scheme of CO2 uptake by the blood of vertebrates involves (i) the diffusion of CO2 from the tissue to the red blood cells via the plasma, (ii) intracellular hydration of CO2 catalyzed by carbonic anhydrase to form bicarbonate (HCO3−) and protons, (iii) intracellular buffering of the protons by haemoglobin and (iv) transfer of the majority of the HCO3− to the plasma via the band 3 protein (also called anion exchanger 1, AE1) in exchange for Cl−. This process is reversed in the CO2-excreting organs such as the lungs, gills and skin. In the case of the hagfishes, this scheme does not seem to hold, because the red blood cells of these extant agnathans, while containing haemoglobin and carbonic anhydrase activity of cells in suspension and the HCO3− permeability of these cells.

4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid (DIDS), at a concentration of 10−4mmol l−1, had no influence on the HCO3− distribution between the red blood cells and the true plasma of M. glutinosa in their test system, although Brill et al. (1992) reported binding of the 3H-labelled AE1 inhibitor dihydro-4,4′-disothiocyanatostilbene-2,2′-disulphonic acid (H2DIDS) to red blood cell membranes of Atlantic hagfish (Myxine glutinosa). This binding, however, was poor.

These contradictory results led us to raise the question of whether any HCO3− transport (not necessarily Cl−/HCO3− exchange) across the red blood cell membrane of the Atlantic hagfish M. glutinosa exists at all. To answer this question, we have applied a special mass spectrometric technique described previously by Itada and Forster (1977). This method allows us to determine simultaneously the intracellular carbonic anhydrase activity of cells in suspension and the HCO3− permeability of the plasma membranes of these cells.

Materials and methods

Animals

Myxine glutinosa Linné were trapped in the Oslo fjord (Norway) and transported to Hannover. Thereafter, the hagfishes were kept in a dark aquarium, filled with artificial sea water with an osmolality of approximately 975mosmol kg−1 at a temperature of 8 °C. The water was constantly filtered and
oxygenated. Every fortnight, the hagfishes were fed fillet of fresh fish *ad libitum* for 1 day.

**Solutions and chemicals**

Hagfish red cells were suspended in a solution of 460 mmol l⁻¹ NaCl, 8 mmol l⁻¹ KCl, 10 mmol l⁻¹ CaCl₂, 20 mmol l⁻¹ MgSO₄, 50 mmol l⁻¹ Hepes; adjusted to pH 7.4 at 10 °C with 1 mmol l⁻¹ NaOH and to an osmolality of 975 mosmol kg⁻¹ using NaCl.

A similar but unbuffered solution was produced, the same osmolality being obtained by the addition of more NaCl. Osmolality was controlled using a digital micro-osmometer (type 5R, Roebling Meßtechnik, Berlin, Germany).

Labelling of NaHCO₃ with ¹⁸O was performed as described by Itada and Forster (1977) employing H₂¹⁸O (Euriso-top, Gif-Sur-Yvette, France). ¹⁸O-labelled solid NaHCO₃ (degree of labelling 6 %) was dissolved to a concentration of 25 mmol l⁻¹ in an unbuffered hagfish saline giving a total osmolality of 950 mosmol kg⁻¹. All solutions were kept at 10 °C.

Bovine carbonic anhydrase II was obtained from Serva (Heidelberg, Germany). Benzolamide was provided by Lederle (Pearl River, NJ, USA). Unless stated otherwise, all other chemicals used were of analytical grade and were obtained from Merck, Darmstadt, Germany.

**Blood samples**

For blood sampling, animals were decapitated and suspended in an upright position for approximately 1 min. The caudal subcutaneous sinus was then punctured with a sterile 20 gauge hypodermic needle to extract as much blood as possible into a heparin-containing 2 ml disposable plastic syringe. The blood samples from four or five specimens were subsequently pooled.

**Preparation of red cell suspensions**

On sampling, the blood was immediately centrifuged for 5 min at 10 °C and 1000 g (Hettich EBA 8S centrifuge, Hettich, Tuttingen, Germany). The plasma and the buffy coat were discarded. The red blood cells were then washed four times; first twice with a buffered hagfish saline and then twice with a similar but unbuffered solution. The washed erythrocytes were resuspended to a haematocrit (Hct; %) of approximately 25 % in the unbuffered solution supplemented with 5 mmol l⁻¹ glucose. Cell counts were performed microscopically using a Neubauer counting chamber. All measurements were made in duplicate. The respective mean values were used for later calculations.

**Characterization of hagfish red cells**

A diluted red cell suspension was placed in a Neubauer counting chamber, and the length and width of 20 randomly chosen erythrocytes were determined by light microscopy employing a scaled ocular. Mean values were used for later calculations.

The relatively large, oval, flat nucleated red blood cells of *M. glutinosa* (Glomski et al., 1992) were assumed to be elliptical discs. Total surface area (*A*) was calculated using the measured length and width and the mean volume of the red blood cells (*V*). Cell surface area per intracelluar water space (*a*) was determined as:

\[
a = \frac{A_T}{V_{MC} \times F_W},
\]

where *V*<sub>MC</sub> is the mean corpuscular volume and *F*<sub>W</sub> is the cell’s water fraction. By freeze-drying a pellet of washed hagfish red blood cells to constant mass, *F*<sub>W</sub> was found to be 0.745. Mean corpuscular haemoglobin concentration (*MCHC*) was determined as:

\[
MCHC = \frac{[\text{Hb}] \times 1000}{\text{Hct}}.
\]

Haemoglobin concentration ([Hb] g l⁻¹) was measured applying the photometric method of Kleihauer and Betke (1957).

**The ¹⁸O exchange method**

The HCO₃⁻ permeability and the intracellular carbonic anhydrase activity of intact red blood cells were measured using the ¹⁸O exchange reaction (Mills and Urey, 1940) (Fig. 1). Itada and Forster (1977) adapted the method to measure simultaneously the intracellular carbonic anhydrase activity (*A<sub>i</sub>*) of red blood cells in suspension and the HCO₃⁻ permeability (*P<sub>HCO₃⁻</sub>*) of the plasma membranes of these cells. They applied their method with some slight modifications.

**Apparatus**

Our experiments were performed using a small water-jacketed glass reaction vessel with a volume of approximately 1.5 ml (Fig. 2). The mass spectrometric recordings were carried out using an isotope ratio mass spectrometer (Tracermass 20-20, Europa Scientific Ltd, Crewe, UK). For our evaluations, we used the ratio of the mass/charge signal 46 (due to C₁₈O₁₆O) and the mass/charge signal 44 (due to C₁₆O₂), which eliminates any instability in the signal caused by electromagnetic disturbances or drift phenomena.

**Experimental procedure**

Upon dissolving the ¹⁸O-labelled HCO₃⁻, the solution was immediately poured into the reaction vessel. Using a microsyringe, small amounts of hydrochloric acid and aqueous sodium hydroxide were added to adjust the solution to a pH of 7.4 as rapidly as possible. The elapsed time between dissolving
The 18O-labelled HCO3− and the start of the mass spectrometric recording was 10–20 min. The temperature of the reaction solution in the glass vessel was maintained at 10 °C. In each experiment, the total period of mass spectrometric data sampling was 20 min. To determine the rate of the 18O exchange reaction taking place in the pure reaction solution, the signals were first recorded for several minutes prior to the addition of the red blood cell suspension. The suspension was then quickly added by micropipette. Sample volume was 10–50 μl. The Hct of the final red blood cell suspension was calculated as the product of the ratio of the volume of the added red blood cell suspension to the volume of the reaction vessel and the Hct of the added suspension. v, the fraction of the intracellular water space of the erythrocytes in the final reaction solution, was calculated as final Hct×0.745. v ranged from 0.68×10⁻³ to 6.1×10⁻³.

Because the baseline signal was required for the evaluation of the recorded kinetics (Itada and Forster, 1977), isotopic equilibrium in the reaction solution was created by adding 20 μl of a carbonic anhydrase solution (bovine carbonic anhydrase II, 1 mg ml⁻¹) to the reaction mixture in each experiment several minutes before the recording period ended. When a Prontosil–Dextran containing reaction solution was used, addition of the carbonic anhydrase solution served to test the effectiveness of the inhibition of extracellular carbonic anhydrase. Subsequently, 20 μl of washed human red blood cells (Hct 57 %) was added. This caused a reduction in the mass spectrometric signal to the baseline level. After each measurement, the reaction vessel was rinsed at least four times with distilled water.

Inhibition of extracellular carbonic anhydrase activity

To inhibit extracellular carbonic anhydrase activity, we applied the carbonic anhydrase inhibitor Prontosil–Dextran at a concentration of 10⁻⁴ mol l⁻¹ (concentration refers to Prontosil residues not to dextran molecules) in the reaction solution in some experiments. The action of this sulfonamide was restricted to the extracellular space, because it was coupled to dextran with a molecular mass of 5000 Da.

**Bicarbonate permeability of hagfish red cells**

\[
\text{COO} + \text{H}_2\text{O} \leftrightarrow \text{H}_2\text{CO}_2 \leftrightarrow \text{HCO}_2^- + \text{H}^+ \\
\text{CO}_2 + \text{H}_2\text{O} \\
\text{CO}_2 = M_t 44 \quad \text{COO} = M_t 46
\]

Fig. 1. Scheme of the 18O exchange reaction. The stable oxygen isotope 18O (O) introduced into the solution by adding 18O-labelled bicarbonate is transferred to CO2 and H2O in exchange for the naturally most abundant 16O (O). Since the water pool of the solution is approximately three orders of magnitude larger than the pool of CO2, H2CO3 and HCO3−, the 18O added eventually ends up almost completely in the water. Unlabelled and labelled CO2 molecules have different relative molecular masses (Mt 44 and Mt 46, respectively).

\[\text{CO}_2 = 44, \quad \text{COO} = 46\]

Fig. 2. Schematic drawing of the inlet system of the mass spectrometer allowing the continuous detection of the concentrations of C18O16O (Mt 46) and C16O2 (Mt 44) in the solution in which the 18O exchange reaction takes place. Gases dissolved in the solution diffuse across a thin Teflon membrane, which is supported mechanically by a sintered glass disc and separates the solution from the high vacuum of the mass spectrometer.
Prontosil–Dextran was produced in our own laboratory following the procedure described by Geers et al. (1985), giving approximately 0.01 Prontosil residues per dextran molecule. To test for the effects of the dextran per se, control experiments were carried out in which a reaction solution containing pure dextran was used. The dextran concentration was varied between $0.5 \times 10^{-2}$ and $2.0 \times 10^{-2}$ moles$^{-1}$.

**Evaluation of the kinetics**

The mass spectrometric recordings were stored as electromagnetic data files and subsequently analyzed applying the equations describing the $^{18}$O exchange process in a suspension of carbonic-anhydrase-enclosing cells, as described by Itada and Forster (1977). The set of equations used to estimate $A_i$ and $P_{HCO_3} \cdot$ from the disappearance curve of $^{18}$O$^{16}$O using a curve-fitting procedure is documented in Böllert et al. (1997). The evaluation requires values for the reaction constants for the uncatalyzed hydration of CO$_2$ ($k_u$) and the dehydration of HCO$_3$− ($k_d$), the first apparent dissociation constant of H$_2$CO$_3$ ($K_1'$), the first true dissociation constant of H$_2$CO$_3$ ($K_{HA}$), the extracellular pH, the intracellular water space as a fraction of the whole suspension ($v$), the surface area to intracellular water volume of the red blood cells ($a$) and two measures of the shape of the curve, the fractional drop in the first rapid phase (SR) and the slope of the second slower phase ($n$) (Fig. 3).

Calculations were carried out using a personal computer and custom-designed software which used the Matlab v. 4 software package (MathWork Inc., MA, USA). For the least-squares fit, we employed the Matlab procedure ‘fmins’, which applies the Simplex algorithm and is based on the work of Nelder and Mead (1965). Although a negative value of $P_{HCO_3} \cdot$ does not make physical sense, the algorithm used allowed such a value to result from the fitting procedure.

**Constants used for the calculations**

Both the CO$_2$ hydration rate constant ($k_u$) and the H$_2$CO$_3$ dehydration constant ($k_d$), were calculated using the half-time of the exponential decay of the $^{18}$O$^{16}$O concentration in the reaction mixture prior to the addition of cells, according to the $^{18}$O exchange theory of Mills and Urey (1940).

Because all measurements were performed at 10°C (a typical temperature in the cold sea habitat of poikilothermic hagfishes), values of the first apparent dissociation constant of carbonic acid ($K_{1'}$) and the first true dissociation constant of carbonic acid ($K_{HA}$) for this temperature have been used in the calculations. The value used for $K_{1'}$, 8.356×$10^{-7}$ moles$^{-1}$, holds for an ionic strength ($\mu$) of 0.63 and a temperature of 10°C and was deduced from a value of 3.389×$10^{-7}$ moles$^{-1}$ for the same temperature at $\mu$=0 (Landolt and Börnstein, 1962) and the equation:

$$pK_{1'}(\mu=0) = pK_{1'}(\mu=0) - 0.495\sqrt{\mu} \quad (3)$$

(Forst, 1925). The deduced value matches the values listed by Harned and Bonner (1945). To calculate $K_{HA}$, we started out from the value of 1.698×$10^{-4}$ moles$^{-1}$ that holds for 25°C and $\mu=0$ (Wissbrun et al., 1954). We corrected the value to $\mu=0.63$ using the equation:

$$pK_{HA}(\mu=0) = pK_{HA}(\mu=0) - 0.495\sqrt{\mu} \quad (4)$$

In addition, the value of $K_{HA}$ was also corrected for 10°C using the relationship:

$$d\log_{e}K_{HA} = \frac{\Delta H}{R} \frac{d}{T} \quad (5)$$

(Moore, 1962), where $\Delta H$ is the change in enthalpy, $R$ is the universal gas constant and $T$ is the absolute temperature; $\Delta H$=4226.85 J moles$^{-1}$ (Edsall, 1969). This gives $K_{HA}$=3.828×$10^{-4}$ moles$^{-1}$ at 10°C and $\mu=0.63$.

For our calculations, two additional parameters had to be determined. First, the water volume of the red blood cells expressed as the fraction of the total solution volume in the reaction vessel ($v$) and, second, the surface-to-volume ratio ($a$) of the cells under investigation (see above).

To allow a comparison of $A_i$ values calculated from different experiments, we had to account for differences in extracellular reaction velocities because, despite extensive rinsing of the vessel after each experiment and the use of a carbonic anhydrase inhibitor in the reaction solution, the extracellular reaction velocity differed slightly from experiment to experiment. Therefore, we calculated the factor ($A_e$) by which the reaction had already been accelerated in the solution prior to the addition of the sample. The value of $A_e$ was calculated by dividing the CO$_2$ hydration velocity constant ($k_u$)

![Diagram](image-url)
determined experimentally \((k_{u, \text{expt}})\) by the value holding for the prevailing experimental conditions according to the literature \((k_{u, \text{lit}})\):

\[
A_e = \frac{k_{u, \text{expt}}}{k_{u, \text{lit}}}. \tag{6}
\]

\(k_{u, \text{lit}}\) was estimated to be \(7.2 \times 10^{-3}\) s\(^{-1}\) at 10°C from the value of \(3.58 \times 10^{-2}\) s\(^{-1}\) reported by Ho and Sturtevant (1963) to hold for 25°C, applying the Arrhenius equation as follows:

\[
\frac{k_{u, T_1}}{k_{u, T_2}} = e^{-\frac{E_a}{R} \left( \frac{1}{T_1} - \frac{1}{T_2} \right)} \tag{7}
\]

where \(k_{u, T_1}\) and \(k_{u, T_2}\) are CO\(_2\) hydration constants at different absolute temperatures, \(E_a\) is the activation energy (74910 J mol\(^{-1}\); Sanyal and Maren, 1981) and \(R\) is the universal gas constant (8.31 J K\(^{-1}\) mol\(^{-1}\)).

Each calculated value of \(A_i\) was corrected by multiplying it by the value of \(A_e\) for the same experiment.

**Results**

**Hagfish red cells**

The morphological variables determined for washed hagfish red blood cells contained in the five pooled red blood cell suspensions investigated are listed in Table 1.

**Time course of M1,46/M44 when working with hagfish red blood cells**

Using a suspension of washed hagfish red blood cells, we recorded a clearly biphasic time course for the disappearance of \(^{18}\)O-labelled CO\(_2\) from the HCO\(_3^-\) solution (Fig. 3), as reported by Itada and Forster (1977) for experiments with human red cells. The signal of the unlabelled CO\(_2\) species C\(_{16}\)O\(_2\), immediately after the addition of a red blood cell sample, was changed by only a negligible amount.

The first phase of the biphasic kinetic pattern, recorded immediately after the addition of a red blood cell sample, was characterized by a rapid decay of the signal. During the subsequent second phase, the slope of the logarithm of the signal *versus* time curve was small. The absolute sizes of the decay during the first phase and of the slope during the second phase depended on the sample volume employed. The large reduction shown in Fig. 3 is indicative of significant intracellular carbonic anhydrase activity. The small slope of the signal during the second phase indicates at best, low HCO\(_3^-\) permeability of the red blood cell membrane.

**Calculated values for \(A_i\) and \(P_{HCO_3^-}\)**

The results of evaluation of a number of experiments, in which no carbonic anhydrase inhibitor was present in the reaction solution, are given in Table 2. The evaluation of every experiment of this set led to a positive value for the HCO\(_3^-\) permeability of the red blood cell membrane. The mean calculated \(P_{HCO_3^-}\) is \(2 \times 10^{-5}\) cm s\(^{-1}\).

Calculated values of \(P_{HCO_3^-}\) and \(A_i\) obtained from an analysis of nine experiments in which a Prontosil–Dextran-containing reaction solution was used are presented in Table 3, which shows an approximately equal number of positive and negative values for \(P_{HCO_3^-}\), all with an absolute value of \(\approx 10^{-5}\) cm s\(^{-1}\). In this case, the mean calculated \(P_{HCO_3^-}\) is \(-4 \times 10^{-7}\) cm s\(^{-1}\).

Control experiments in which we used reaction solutions containing pure dextran up to a maximum concentration of 20 mmol l\(^{-1}\), which is twice the concentration of dextran present when we used Prontosil–Dextran, revealed that pure dextran does not reduce the calculated HCO\(_3^-\) permeability of the red blood cell membrane.

| Table 2. Results of mass spectrometric experiments with Myxine glutinosa red cells |
|----------------|-------------------|--------|
| Experiment | \(P_{HCO_3^-}\) (cm s\(^{-1}\)) | \(A_i\) |
| 1 | 7.430 \times 10^{-6} | 27 760 |
| 2 | 2.602 \times 10^{-5} | 22 140 |
| 3 | 2.623 \times 10^{-5} | 29 300 |
| 4 | 3.310 \times 10^{-5} | 24 610 |
| 5 | 3.365 \times 10^{-6} | 26 550 |
| 6 | 9.208 \times 10^{-6} | 31 340 |
| 7 | 1.277 \times 10^{-5} | 31 330 |
| 8 | 2.603 \times 10^{-5} | 47 200 |
| 9 | 1.520 \times 10^{-5} | 21 330 |
| 10 | 9.900 \times 10^{-6} | 20 160 |
| 11 | 2.701 \times 10^{-5} | 37 630 |
| Mean | 2.060 \times 10^{-5} | 29 030 |
| S.D. | 9.373 \times 10^{-6} | 75 50 |

The reaction solution contained no carbonic anhydrase inhibitor. \(P_{HCO_3^-}\), bicarbonate permeability of the red cell plasma membrane; \(A_i\), intracellular carbonic anhydrase activity defined as the factor by which CO\(_2\) hydration is accelerated compared with uncatalyzed CO\(_2\) hydration.

Experiments were performed at 10°C.
To inhibit extracellular carbonic anhydrase activity, measurements were performed using a reaction solution containing the macromolecular carbonic anhydrase inhibitor Prontosil–Dextran. $P_{\text{HCO}_3^-}$, bicarbonate permeability of the red cell plasma membrane; $A_i$, intracellular carbonic anhydrase activity defined as the factor by which CO$_2$ hydration is accelerated compared with uncatalyzed CO$_2$ hydration.

The blood of four *Myxine glutinosa* specimens was pooled, and samples of the combined suspension of washed red cells were used in the nine experiments.

### Discussion

#### Characteristics of hagfish red blood cells

The mean length (28 μm) and width (18 μm) of washed red blood cells from *Myxine glutinosa* measured here agree roughly with the values of Dohn and Malte (1998), who report the major axes of the cell to be 25 μm and 20 μm, respectively. The $V_{MC}$ of Atlantic hagfish red cells measured in the present study (1020 μm$^3$) is close to that of Dohn and Malte (1998; 1160 μm$^3$). Glomski et al. (1992) reported a somewhat higher $V_{MC}$ of 1530 μm$^3$ for red blood cells of the same species. Wells et al. (1986) published a $V_{MC}$ of red blood cells of the Pacific hagfish *Eptatretus cirrhatus* of 507 μm$^3$. The $MCHC$ values reported by Glomski et al. (1992) (21 g dl$^{-1}$) and Wells et al. (1986) (19 g dl$^{-1}$) agree with our value (20 g dl$^{-1}$), indicating that swelling or shrinkage of the cells is not responsible for the differences in $V_{MC}$. We conclude that the present mean ratio of cell surface area to cell volume of hagfish red blood cells appears to represent a reasonable estimate for cells under physiological conditions.

#### Necessity of extracellular carbonic anhydrase inhibition

Even slight lysis of the hagfish red blood cells, which contain a high level of carbonic anhydrase activity, will cause a considerable acceleration of extracellular $^{18}$O exchange and will increase the slope of the second phase of the kinetic pattern significantly. In this case, our interpretation of the kinetics will falsely attribute the greater slope to a higher $P_{\text{HCO}_3^-}$ of the red blood cell membrane. The appearance of carbonic anhydrase in the reaction solution will also to some extent affect the estimate of $A_i$. For this reason, we decided to add a powerful extracellular carbonic anhydrase inhibitor to the reaction solution. The difference between the results obtained from the experiments performed in the presence (Table 3) and those performed in the absence (Table 2) of the extracellular carbonic anhydrase inhibitor illustrates the need for this measure. When working without Prontosil–Dextran in the reaction solution, or with pure dextran in it, we obtained only positive results for $P_{\text{HCO}_3^-}$, and the mean value of $P_{\text{HCO}_3^-}$ was 2×10$^{-5}$ cm s$^{-1}$ rather than the value of $-4\times10^{-7}$ cm s$^{-1}$ obtained when working with a reaction solution containing Prontosil–Dextran. This indicates that some degree of red blood cell lysis had occurred, resulting in substantial carbonic anhydrase activity in the reaction solution when the latter did not contain Prontosil–Dextran.

Prontosil–Dextran appears to be an appropriate carbonic anhydrase inhibitor for use in these experiments. Effective inhibition of extracellular carbonic anhydrase by Prontosil–Dextran at the concentration applied was substantiated by the observation of the inhibition of the enzymatic activity of the bovine carbonic anhydrase added to the reaction solution at the end of each experiment. Control experiments, in which a reaction solution containing pure dextran was used, revealed that dextran does not influence the result of the calculation of $P_{\text{HCO}_3^-}$.

It appears likely that complete inhibition of carbonic anhydrase released from hagfish red blood cells was accomplished by Prontosil at 10$^{-4}$ mol l$^{-1}$. The inhibition constant $K_i$ of Prontosil coupled to dextran molecules with a relative molecular mass of 5000 is 1.1×10$^{-7}$ mol l$^{-1}$ when bovine red blood cell carbonic anhydrase II is studied at 25°C (Geers et al., 1985).

#### Accuracy of the $P_{\text{HCO}_3^-}$ determination

The evaluation of the kinetics obtained when Prontosil–Dextran was used in the reaction solution gave values for the HCO$_3^-$ permeability of the hagfish red blood cell membrane that were scattered very closely around zero (Table 3). How well does the present method differentiate between a total lack of HCO$_3^-$ permeability of the red blood cell membrane and merely a low HCO$_3^-$ permeability? To answer this question, we used a newly developed and more comprehensive method to evaluate the kinetics of the extracellular C$^{18}$O$^{16}$O concentration determined by mass spectrometer. The more comprehensive mathematical model (Wunder et al., 1997) is based on six linear differential equations each describing the time course of the concentration of singly-$^{18}$O-labelled CO$_2$, HCO$_3^-$ or H$_2$O in the extracellular and intracellular compartments. All six equations contain a term describing the rates of the chemical reactions affecting the concentration of the respective $^{18}$O-labelled molecular species (CO$_2$ or HCO$_3^-$ or H$_2$O) in the intra- or extracellular compartment and a term that describes the flux of the
respective molecular species across the red blood cell membrane. In addition to the variables used in the model of Itada and Forster (1977), we had to insert in the new method a value for the water permeability of the red blood cell membrane and a value for the CO₂ permeability of this membrane. Since these values are not known for hagfish red blood cell membrane, we used published values for red blood cell membranes from other species. Benga et al. (1993) determined a water permeability of the membranes of cow and sheep red blood cells of approximately $3 \times 10^{-3}$ cm s⁻¹ at 20 °C and of approximately $5 \times 10^{-3}$ cm s⁻¹ at 37 °C, so it appeared plausible to assume a water permeability of the hagfish red blood cell membrane of $2 \times 10^{-3}$ cm s⁻¹ at 10 °C. Since Gros and Moll (1971) reported the CO₂ permeability of the membranes of bovine red blood cells at 22 °C to be 3 cm s⁻¹, we used a CO₂ permeability for the hagfish red blood cell membrane of 2 cm s⁻¹ at 10 °C.

The best fit of the numerical solution of the system of the six differential equations to the recorded kinetics gave values of the intraerythrocytic carbonic anhydrase activity and the HCO₃⁻ permeability of the red blood cell membrane that were similar to the results obtained when the model of Itada and Forster (1977) was applied in the evaluation. In both cases, the value of $P_{\text{HCO}_3^-}$ was very near to zero. Once the best fit had been obtained, we held the calculated value for the intraerythrocytic carbonic anhydrase activity and all other variables constant except the value for the HCO₃⁻ permeability of the red blood cell membrane. For the latter quantity, we entered a new value. We then recalculated the theoretical course of the kinetics (Fig. 4). Knowing the concentration of dissolved bicarbonate in the reaction solution, the pH of this solution and the pK of the bicarbonate buffer system at the given ionic strength and temperature, the concentration of $^{18}$O-labelled CO₂ in the reaction solution can be calculated from the mass spectrometric signal. This allowed us to show, in Fig. 4, the concentration of $\text{C}^{18}\text{O}^{16}\text{O}$ versus time.

Subsequently, we determined how well the calculated curve fitted the trace of the recorded kinetics. By entering various values for the HCO₃⁻ permeability of the red blood cell membrane, we determined how the quality of the fit depended on the value used for $P_{\text{HCO}_3^-}$ (Fig. 5). Fig. 5 shows that values of $P_{\text{HCO}_3^-}$ of $10^{-6}$ cm s⁻¹ or below do not differ significantly in their effect on the goodness of the fit. The single values of $P_{\text{HCO}_3^-}$ in Table 3 and the mean value of $-4 \times 10^{-7}$ cm s⁻¹ emphasise the conclusion that the $P_{\text{HCO}_3^-}$ of hagfish red blood cell is indistinguishable from zero. Thus, our data provide no evidence for the presence of a HCO₃⁻ pathway in hagfish red blood cell membrane.

$P_{\text{HCO}_3^-}$ and $A_i$ of hagfish red blood cell

The present results indicate that the HCO₃⁻ permeability of the hagfish red blood cell membrane is clearly much lower than that of most vertebrate red blood cell membranes. The mean

![Fig. 4. Decay of the concentration of $^{18}$O-labelled carbon dioxide in the reaction solution (solid line). The curve comes from an experiment (listed as no. 1 in Table 3), in which a suspension of hagfish red blood cells (RBCs) was added to a Prontosil–Dextran-containing reaction solution. Evaluation of the recording on the basis of the extended theoretical model (see text for details) gave an intracellular carbonic anhydrase activity of 28 800 and a bicarbonate permeability of the red blood cell plasma membrane ($P_{\text{HCO}_3^-}$) of $6.828 \times 10^{-13}$ cm s⁻¹ (best fit). The four upper traces represent curves calculated employing the indicated value of $P_{\text{HCO}_3^-}$. Values used for all other variables, including the intraerythrocytic carbonic anhydrase activity, were the same in all four calculations. These values were the same as those used in the determination of the best fit. The applied intracellular carbonic anhydrase activity was 28 800.](image)

![Fig. 5. Illustration of the dependence of the quality of fit on the value used for the HCO₃⁻ permeability of the hagfish red blood cell membrane ($P_{\text{HCO}_3^-}$). The goodness of the fit is expressed by the sum of squares of deviations ($y$-axis). The calculations are based on the recorded signal from experiment 1 (Table 3; Fig. 4).](image)
intracellular carbonic anhydrase activity, in contrast, is rather high, albeit only half that of human red blood cells at the same temperature. For comparison, values of $P_{\text{HCO}_3^-}$ and $A_i$ determined using the same mass spectrometric method for red blood cells of hagfish, human and European flounder, a teleost, are given in Table 4. The flounders were caught in the Baltic sea. At similar temperatures, human and flounder red blood cell membranes exhibit HCO$_3^-$ permeabilities much higher than those of hagfish red blood cell membranes. As expected, the HCO$_3^-$ permeability of a given red blood cell membrane increases with temperature (compare human values at 10 and 37 °C). In contrast, the intracellular carbonic anhydrase activity of a given red blood cell decreases with increasing temperature because of the more pronounced rise in the reaction rate of the uncatalyzed CO$_2$ hydration than of the carbonic-anhydrase-catalyzed reaction at increasing temperature (Sanayal and Maren, 1981).

The results of the present mass spectrometric investigations of hagfish red blood cells are consistent with the findings of Ellory et al. (1987) and with some of the findings of Tufts and Boutilier (1990b) suggesting the absence of significant anion exchange across hagfish red blood cell membranes. Even if, in hagfish red blood cell membranes, a HCO$_3^-$ permeability of $10^{9-6} \text{cm s}^{-1}$ or less exists, this is no evidence for the presence of a proteinaceous HCO$_3^-$ transport system in these membranes. Norris and Powell (1992) estimated the HCO$_3^-$ permeability of phospholipid vesicles at 25 °C to be $3.3 \times 10^{-6} \text{cm s}^{-1}$, and it can be assumed that a somewhat smaller value will hold at 10 °C. Thus, it remains unclear which component of the hagfish red blood cell membrane was responsible for the low but detectable binding of H$_2$DIDS observed by Brill et al. (1992) and how H$_2$DIDS reduced the already extremely low Cl$^-$ transport rate across this membrane in the experiments of Ellory et al. (1987). Most notable is the apparent contradiction between the majority of the findings of Tufts and Boutilier (1990a,b) and the present findings. The results of the aforementioned authors indicate acid–base exchange across hagfish red blood cell membranes at a rate sufficient to produce higher total CO$_2$ and HCO$_3^-$ concentrations in the true plasma than in the red blood cells of $M$. glutinosa when blood of this species is equilibrated with CO$_2$ for 30 min. One may conclude that the rate of HCO$_3^-$ transport across the red blood cell membrane of $M$. glutinosa is either too low to be detected using the $^{18}$O exchange method but large enough to be of significance in the in vitro CO$_2$ equilibration lasting for 30 min; alternatively, an acid–base exchange system may operate in the hagfish red blood cell membrane that does not involve the transport of HCO$_3^-$. 

**Physiological significance of the lack of a fast HCO$_3^-$-transporting protein**

Since carbonic anhydrase activity is absent from the plasma of most vertebrate species and the capillary transit time of the blood is too short to allow a significant contribution from uncatalyzed CO$_2$ hydration within the plasma to CO$_2$ transport by the blood, the high carbonic anhydrase activity and the large non-bicarbonate buffer capacity within the red blood cells are in general of great importance for CO$_2$ transport by vertebrate blood. Because of the rapid anion exchange across the red blood cell membrane, the majority of the HCO$_3^-$ generated within the red blood cells is transported into the plasma. In $M$. glutinosa, the situation is obviously different because the red blood cells of this species exhibit a high carbonic anhydrase activity, but the red blood cell membranes possess a very low HCO$_3^-$ permeability or none at all. In consequence, most of the HCO$_3^-$ formed inside the red blood cells remains confined to the intraerythrocytic space during transit of the red blood cells through the capillaries because, although the cardiovascular system of hagfishes is complicated, including more than one heart and exhibiting distinct partitioning (Forster, 1998), it seems very unlikely that capillary transit times in $M$. glutinosa are several orders of magnitude longer than in other vertebrates. Thus, at first glance, compared with other vertebrate species, including fish, $M$. glutinosa seems to be at a disadvantage in removing CO$_2$ from the tissues and passing it on to the water through the gills.

Are there features of hagfish blood that compensate for the seeming disadvantage? A high haematocrit would favour the transport of HCO$_3^-$ within the red blood cells. However, in comparison with the blood of many teleosts, hagfish blood exhibits a low Hct (Gallaher and Farrell, 1998). In $M$. glutinosa, most of the bicarbonate formed within the red cells is confined to the intraerythrocytic space during capillary transit of the blood, so a high non-bicarbonate buffer capacity of the red blood cells would be advantageous for the transport

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Species</th>
<th>$P_{\text{HCO}_3^-}$ (cm$^2$ s$^{-1}$)</th>
<th>$A_i$</th>
<th>$N$</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>Hagfish</td>
<td>0</td>
<td>21000</td>
<td>9</td>
</tr>
<tr>
<td>10</td>
<td>Man</td>
<td>2.5 x 10^{-4}</td>
<td>43000</td>
<td>10</td>
</tr>
<tr>
<td>15</td>
<td>Flounder</td>
<td>5.5 x 10^{-4}</td>
<td>187000</td>
<td>10</td>
</tr>
<tr>
<td>37</td>
<td>Man</td>
<td>9.0 x 10^{-4}</td>
<td>13000</td>
<td>8</td>
</tr>
</tbody>
</table>

$P_{\text{HCO}_3^-}$, bicarbonate permeability of the red cell plasma membrane; $A_i$, intracellular carbonic anhydrase activity defined as the factor by which CO$_2$ hydration is accelerated by carbonic anhydrase compared with uncatalyzed CO$_2$ hydration.

Flounder and human red blood cells were washed in 150 mmol l$^{-1}$ NaCl. For the mass spectrometric experiment, these red blood cells were suspended in 125 mmol l$^{-1}$ NaCl + 25 mmol l$^{-1}$ $^{18}$O-labelled NaHCO$_3$. The reaction rate and dissociation constants used for the evaluation of the recordings were corrected to the chosen experimental conditions (temperature and ionic strength). Temperatures of 10 °C and 15 °C are typical of the habitats of Atlantic hagfish and European flounder, respectively.

[Table 4. Comparison of the mass spectrometrically determined bicarbonate permeabilities of the plasma membranes of intact red cells of the Atlantic hagfish (Myxine glutinosa), the European flounder (Platichthys flesus) and man and the intracellular carbonic anhydrase activities of these cells]

---

**Table 4. Comparison of the mass spectrometrically determined bicarbonate permeabilities of the plasma membranes of intact red cells of the Atlantic hagfish (Myxine glutinosa), the European flounder (Platichthys flesus) and man and the intracellular carbonic anhydrase activities of these cells**
of CO₂. But the red blood cells of the hagfishes do not show a particularly high non-bicarbonate buffer capacity (Wells et al., 1986; Tufts and Boutilier, 1990b; Tufts and Perry, 1998). A distinct Haldane effect could increase the CO₂-transporting capacity of the blood, but the Haldane effect was found to be of minor importance for CO₂ transport by the blood of M. glutinosa (Tufts et al., 1998). Carbonic anhydrase activity in the plasma could give rise to fast conversion of CO₂ to HCO₃⁻ and vice versa in the plasma, but the plasma of M. glutinosa does not exhibit significant carbonic anhydrase activity (T. Peters, unpublished result).

One may conclude that neither the plasma nor the red blood cell of M. glutinosa possesses any features that might compensate for a limitation of CO₂ transport by hagfish blood caused by the absence of fast HCO₃⁻ transport across the red blood cell membrane. However, the small CO₂ transport capacity of hagfish blood might be sufficient at the low mass-specific metabolic rate reported for hagfishes by several authors (Smith and Hessler, 1974; Munz and Morris, 1965; Forster, 1990).

The particular features of CO₂ transport in M. glutinosa may, however, favourably affect the oxygen transport properties of the blood of this species. HCO₃⁻ has been shown to act as a potent allosteric effector on hagfish haemoglobin. In red blood cell lysates, an increasing HCO₃⁻ concentration causes a significant decrease in the oxygen affinity of the haemoglobin (Fago et al., 1999). Thus, an exceptionally large increase in the intraerythrocytic HCO₃⁻ concentration during the passage of the red blood cells through the tissue capillaries, caused by the high intraerythrocytic carbonic anhydrase activity and the very low HCO₃⁻ permeability of the red blood cell membrane, may facilitate the delivery of oxygen to the tissue. The very low oxygen partial pressure in the venous blood of Eptatretus cirrhatus, a Pacific hagfish species, observed when the animal exercised under normoxic conditions (Wells et al., 1986) may indicate that a mechanism supporting O₂ release by the blood may be very important in hagfishes. Thus, the absence of fast HCO₃⁻ transport across the red blood cell membrane may represent a special evolutionary adaptation of hagfishes and may contribute to the extraordinary hypoxia-tolerance which has been reported for M. glutinosa (Malte and Lomholt, 1998).

We very much appreciate the donation of hagfishes by the biological station at Drøbak (Norway), an Oslo University utility. We thank Dr M. Wunder for programming the software for the evaluation of the mass spectrometric recordings. We appreciate the help of V. Endeward in assessing the precision of our determinations of bicarbonate permeabilities. Skilful technical assistance by S. Bauer is gratefully acknowledged.

References

Benga, G., Borza, T., Popescu, O., Poprutiu, D. and Matei, H. (1993). Comparative nuclear magnetic resonance studies of


