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

THOMAS PETERS[1,*], ROBERT E. FORSTER[2] AND GEROLF GROS[1]

[1]*Abteilung Vegetative Physiologie, Zentrum Physiologie, Medizinische Hochschule, 30623 Hannover, Germany* and [2]*Department of Physiology, School of Medicine, University of Pennsylvania, Philadelphia, PA 19174, USA*

*e-mail: Peters.Thomas@MH-Hannover.de

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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 ^{18}O 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 CO_2 hydration/HCO_3^- dehydration reaction over the uncatalyzed reaction (referred to as intracellular carbonic anhydrase activity A_i) was 21 320±3000 at 10 °C (mean ± S.D., N=9). The mean bicarbonate permeability of the red blood cell membrane ($P_{HCO_3^-}$) was indistinguishable from zero. It can be concluded that CO_2 transport within hagfish blood does not follow the classical scheme of CO_2 transport in vertebrate blood. It is suggested that the combination of considerable intraerythrocytic carbonic anhydrase activity and low $P_{HCO_3^-}$ may serve to enhance O_2 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, ^{18}O exchange reaction.

Introduction

The classical scheme of CO_2 uptake by the blood of vertebrates involves (i) the diffusion of CO_2 from the tissue to the red blood cells *via* the plasma, (ii) intracellular hydration of CO_2 catalyzed by carbonic anhydrase to form bicarbonate (HCO_3^-) and protons, (iii) intracellular buffering of the protons by haemoglobin and (iv) transfer of the majority of the HCO_3^- to the plasma *via* the band 3 protein (also called anion exchanger 1, AE1) in exchange for Cl^-. This process is reversed in the CO_2-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 (Carlsson et al., 1980; Maren et al., 1980), lack an electrophoretically clearly identifiable band 3 protein (Ellory et al., 1987). A very low influx of $^{36}Cl^-$ into Pacific hagfish (*Eptatretus stouti*) red blood cells (Ellory et al., 1987) and the very poor buffering of KOH added to a suspension of hagfish red blood cells (Ellory et al., 1987) suggest the lack of a functionally significant AE1 in red blood cell membranes of hagfishes. However, Tufts and Boutilier (1990b) found total CO_2 and apparent HCO_3^- (total CO_2 minus dissolved CO_2) concentrations to be higher in true plasma than in separated plasma of *M. glutinosa*. Their observation can only be understood in terms of some shift of acid or base equivalents occurring across the red blood cell membrane. Nevertheless, Tufts and Boutilier (1990b) reported that the AE1 inhibitor 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid (DIDS), at a concentration of 10^{-4} mmol l^{-1}, had no influence on the HCO_3^- 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′-diisothiocyanatostilbene-2,2′-disulphonic acid (H_2DIDS) 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 HCO_3^- transport (not necessarily Cl^-/HCO_3^- 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 HCO_3^- 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 975 mosmol 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} $CaCl_2$, 20 mmol l^{-1} $MgSO_4$, 50 mmol l^{-1} Hepes; adjusted to pH 7.4 at 10 °C with 1 mol 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 $NaHCO_3$ with ^{18}O was performed as described by Itada and Forster (1977) employing $H_2{}^{18}O$ (Euriso-top, Gif-Sur-Yvette, France). ^{18}O-labelled solid $NaHCO_3$ (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, Tuttlingen, 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 was used for later calculations.

The suspensions of washed red blood cells added to the HCO_3^- solution had a mean Hct of 22.4±2.5 % and a mean cell count of $219.4\times10^3\pm9.0\times10^3$ mm^{-3} (means ± S.D., N=4). After preparation, the red blood cell suspensions were stored in a refrigerator at 6 °C and used on the same day.

Characterization of hagfish red cells

A diluted red blood 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_T) was calculated using the measured length and width and the mean volume of the red blood cells (V_{MC}).

V_{MC} was calculated using the haematocrit and the cell count. Cell surface area per intracellular water space (a) was determined as:

$$a = \frac{A_T}{V_{MC} \times F_W} , \quad (1)$$

where V_{MC} is the mean corpuscular volume and F_W is the cell's water fraction. By freeze-drying a pellet of washed hagfish red blood cells to constant mass, F_W was found to be 0.745. Mean corpuscular haemoglobin concentration (*MCHC*) was determined as:

$$MCHC = \frac{[Hb] \times 1000}{Hct} . \quad (2)$$

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

The ^{18}O exchange method

The HCO_3^- permeability and the intracellular carbonic anhydrase activity of intact red blood cells were measured using the ^{18}O exchange reaction (Mills and Urey, 1940) (Fig. 1). Itada and Forster (1977) adapted the method to measure simultaneously the intracellular carbonic anhydrase activity (A_i) of red blood cells in suspension and the HCO_3^- permeability ($P_{HCO_3^-}$) of the plasma membranes of these cells. We 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 $C^{18}O^{16}O$) and the mass/charge signal 44 (due to $C^{16}O_2$), which eliminates any instability in the signal caused by electromagnetic disturbances or drift phenomena.

Experimental procedure

Upon dissolving the ^{18}O-labelled HCO_3^-, 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

$$CO\mathbf{O} + H_2O \rightleftharpoons H_2C\mathbf{O}O_2 \rightleftharpoons HC\mathbf{O}O_2^- + H^+$$
$$\Downarrow\Uparrow$$
$$CO_2 + H_2\mathbf{O}$$

$CO_2 = M_r\,44$ $\qquad$ $CO\mathbf{O} = M_r\,46$

Fig. 1. Scheme of the ^{18}O exchange reaction. The stable oxygen isotope ^{18}O (**O**) introduced into the solution by adding ^{18}O-labelled bicarbonate is transferred to CO_2 and H_2O in exchange for the naturally most abundant ^{16}O (O). Since the water pool of the solution is approximately three orders of magnitude larger than the pool of CO_2, H_2CO_3 and HCO_3^-, the ^{18}O added eventually ends up almost completely in the water. Unlabelled and labelled CO_2 molecules have different relative molecular masses (M_r 44 and M_r 46, respectively).

the ^{18}O-labelled HCO_3^- 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 ^{18}O 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.

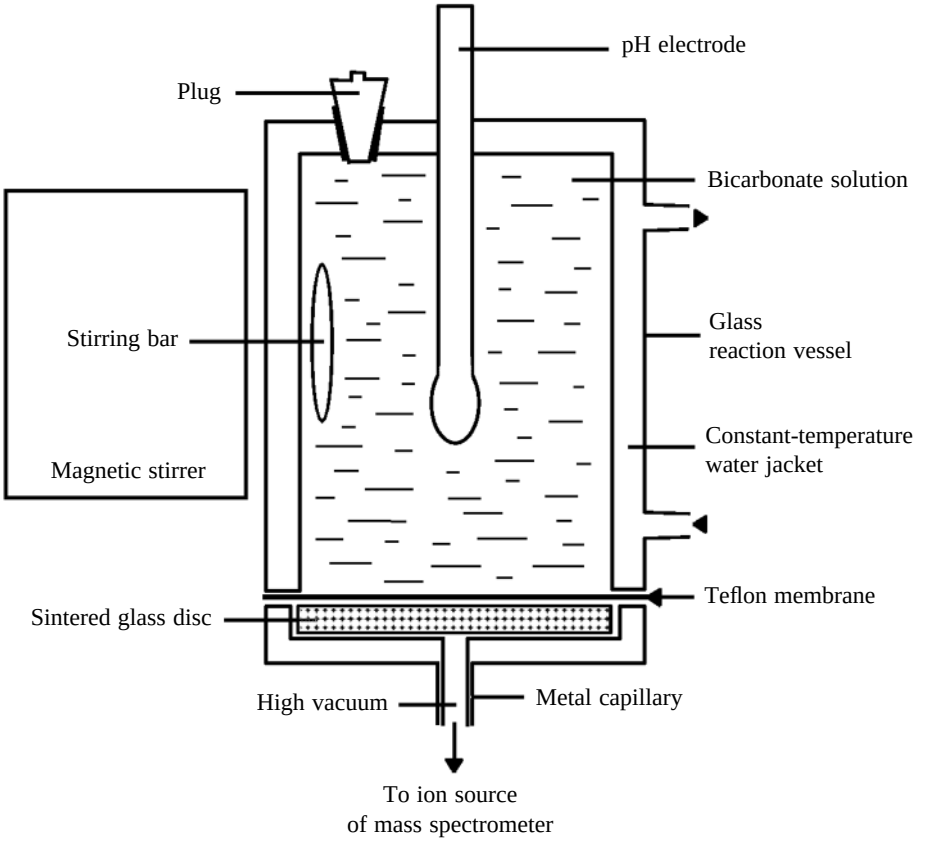

Fig. 2. Schematic drawing of the inlet system of the mass spectrometer allowing the continuous detection of the concentrations of $C^{18}O^{16}O$ (M_r 46) and $C^{16}O_2$ (M_r 44) in the solution in which the ^{18}O exchange reaction takes place. Gases dissolved in the solution diffuse across a thin 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 0.5×10^{-2} and 2.0×10^{-2} mol l^{-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 $P_{HCO_3^-}$ from the disappearance curve of $C^{18}O^{16}O$ using a curve-fitting procedure is documented in Böllert et al. (1997). The evaluation requires values for the reaction contants for the uncatalyzed hydration of CO_2 (k_u) and the dehydration of HCO_3^- (k_v), the first apparent dissociation constant of H_2CO_3 (K_1'), the first true dissociation constant of H_2CO_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 $P_{HCO_3^-}$ does not make physical sense, the algorithm used allowed such a value to result from the fitting procedure.

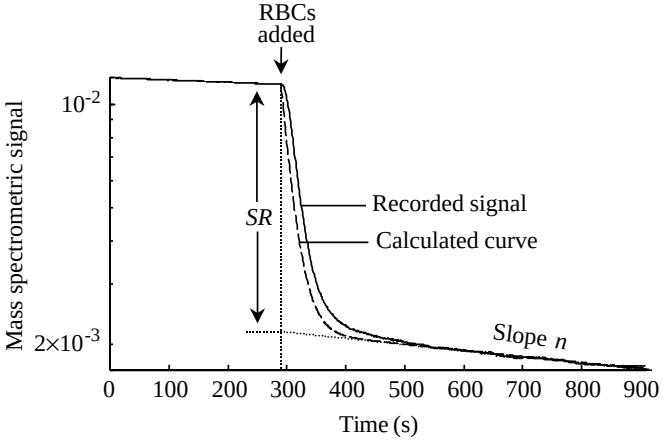

Fig. 3. Typical recording of the ratio M_r 46/M_r 44 signal *versus* time plotted semilogarithmically. The baseline signal representing isotopic equilibrium has been subtracted from all data. Hagfish red blood cells (RBCs) were added after 290 s. The so-called step ratio (SR), which gives the amplitude of the rapid first phase of the reaction, and the slope n, which gives the slope of the logarithmic signal *versus* time curve during the second phase of the reaction, are used to calculate $P_{HCO_3^-}$ and A_i (the intracellular carbonic anhydrase activity) applying the set of equations of Itada and Forster (1977). The graph also illustrates the result of the curve-fitting procedure. Solid line, recorded signal; dashed line, fitted calculated curve.

Constants used for the calculations

Both the CO_2 hydration rate constant (k_u) and the H_2CO_3 dehydration constant (k_v), were calculated using the half-time of the exponential decay of the $C^{18}O^{16}O$ 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} mol l^{-1}, holds for an ionic strength (μ) of 0.63 and a temperature of 10 °C and was deduced from a value of 3.389×10^{-7} mol l^{-1} for the same temperature at μ=0 (Landolt and Börnstein, 1962) and the equation:

$$pK_1'_{(\mu\neq0)} = pK_1'_{(\mu=0)} - 0.495\sqrt{\mu} \quad (3)$$

(Hastings and Sendroy, 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} mol l^{-1} that holds for 25 °C and μ=0 (Wissbrun et al., 1954). We corrected the value to μ=0.63 using the equation:

$$pK_{HA(\mu\neq0)} = pK_{HA(\mu=0)} - 0.495\sqrt{\mu}. \quad (4)$$

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

$$d\log_e K_{HA} = -\frac{\Delta H}{\boldsymbol{R}}\, d\,\frac{1}{T} \quad (5)$$

(Moore, 1962), where ΔH is the change in enthalpy, $\boldsymbol{R}$ is the universal gas constant and T is the absolute temperature; ΔH=4226.85 J mol^{-1} (Edsall, 1969). This gives $K_{HA}=3.828\times10^{-4}$ mol l^{-1} at 10 °C and μ=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)

determined experimentally ($k_{u,exptl}$) by the value holding for the prevailing experimental conditions according to the literature ($k_{u,lit}$):

$$A_e = \frac{k_{u,exptl}}{k_{u,lit}} . \quad (6)$$

$k_{u,lit}$ was estimated to be $7.2\times10^{-3}\,s^{-1}$ at 10 °C from the value of $3.58\times10^{-2}\,s^{-1}$ reported by Ho and Sturtevant (1963) to hold for 25 °C, applying the Arrhenius equation as follows:

$$\frac{k_{u,T1}}{e^{-\frac{E_a}{\boldsymbol{R}}\left(\frac{1}{T_1}-\frac{1}{T_2}\right)}} = k_{u,T2} , \quad (7)$$

where $k_{u,T1}$ and $k_{u,T2}$ are CO_2 hydration constants at different absolute temperatures, E_a is the activation energy (74 910 J mol^{-1}; Sanyal and Maren, 1981) and $\boldsymbol{R}$ is the universal gas constant (8.31 J K^{-1} mol^{-1}).

Each calculated value of A_i was corrected by multipying 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 M_r*46/* M_r*44 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 $C^{16}O_2$ 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.

Table 1. *Morphological characteristics of the* Myxine glutinosa *red blood cells*

V_{MC} (μm^3)	*MCHC* (g dl^{-1})	Cell width (μm)	Cell length (μm)	Surface-to-volume ratio
1020±80	20±3	18±3	28±3	12 790±820

V_{MC}, mean corpuscular volume; *MCHC*, mean corpuscular haemoglobin concentration.

All values given are means ± S.D.

V_{MC}, *MCHC* and surface-to-volume ratio data are results from five suspensions of washed hagfish red blood cells.

The blood of four or five hagfishes was used to produce each suspension.

To determine the cell width and length, 20 hagfish red cells chosen at random from each of the five red blood cell suspensions were studied microscopically; thus, for these two parameters, N=100.

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 2×10^{-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 $\ll10^{-5}$ cm s^{-1}. In this case, the mean calculated $P_{HCO_3^-}$ is -4×10^{-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	7.430×10^{-6}	27 760
2	2.602×10^{-5}	22 140
3	2.623×10^{-5}	29 300
4	3.310×10^{-5}	24 610
5	3.365×10^{-6}	26 550
6	9.208×10^{-6}	31 340
7	1.277×10^{-5}	31 330
8	2.603×10^{-5}	47 200
9	1.520×10^{-5}	21 330
10	9.900×10^{-6}	20 160
11	2.701×10^{-5}	37 630
Mean	2.060×10^{-5}	29 030
S.D.	9.373×10^{-6}	7550

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.

Table 3. *Results of mass spectrometric measurements of the bicarbonate permeability of the* Myxine glutinosa *red cell membrane and the carbonic anhydrase activity within* Myxine glutinosa *red cells at 10 °C*

Experiment	$P_{HCO_3^-}$ (cm s^{-1})	A_i
1	9.931×10^{-8}	25 710
2	-3.123×10^{-6}	22 530
3	4.213×10^{-6}	20 720
4	-3.592×10^{-6}	21 610
5	1.248×10^{-6}	22 760
6	1.981×10^{-6}	25 000
7	-3.158×10^{-7}	19 880
8	-4.829×10^{-6}	17 130
9	6.797×10^{-7}	16 510
Mean	-4.043×10^{-7}	21 320
S.D.	2.755×10^{-6}	2970

To inhibit extracellular carbonic anhydrase activity, measurements were performed using a reaction solution containing the macromolecular carbonic anhydrase inhibitor Prontosil–Dextran.

$P_{HCO_3^-}$, 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.

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 blood 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 $P_{HCO_3^-}$ 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 $P_{HCO_3^-}$, and the mean value of $P_{HCO_3^-}$ was 2×10^{-5} cm s^{-1} rather than the value of -4×10^{-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 $P_{HCO_3^-}$.

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 $P_{HCO_3^-}$ *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_2O 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_2O) 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 CO_2 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 3×10^{-3} cm s^{-1} at 20 °C and of approximately 5×10^{-3} cm s^{-1} at 37 °C, so it appeared plausible to assume a water permeability of the hagfish red blood cell membrane of 2×10^{-3} cm s^{-1} at 10 °C. Since Gros and Moll (1971) reported the CO_2 permeability of the membranes of bovine red blood cells at 22 °C to be 3 cm s^{-1}, we used a CO_2 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 HCO_3^- 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 $P_{HCO_3^-}$ 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 HCO_3^- 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 CO_2 in the reaction solution can be calculated from the mass spectrometric signal. This allowed us to show, in Fig. 4, the concentration of $C^{18}O^{16}O$ *versus* time.

Subsequently, we determined how well the calculated curve fitted the trace of the recorded kinetics. By entering various values for the HCO_3^- permeability of the red blood cell membrane, we determined how the quality of the fit depended on the value used for $P_{HCO_3^-}$ (Fig. 5). Fig. 5 shows that values of $P_{HCO_3^-}$ 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 $P_{HCO_3^-}$ in Table 3 and the mean value of -4×10^{-7} cm s^{-1} emphasise the conclusion that the $P_{HCO_3^-}$ of hagfish red blood cell is indistinguishable from zero. Thus, our data provide no evidence for the presence of a HCO_3^- pathway in hagfish red blood cell membrane.

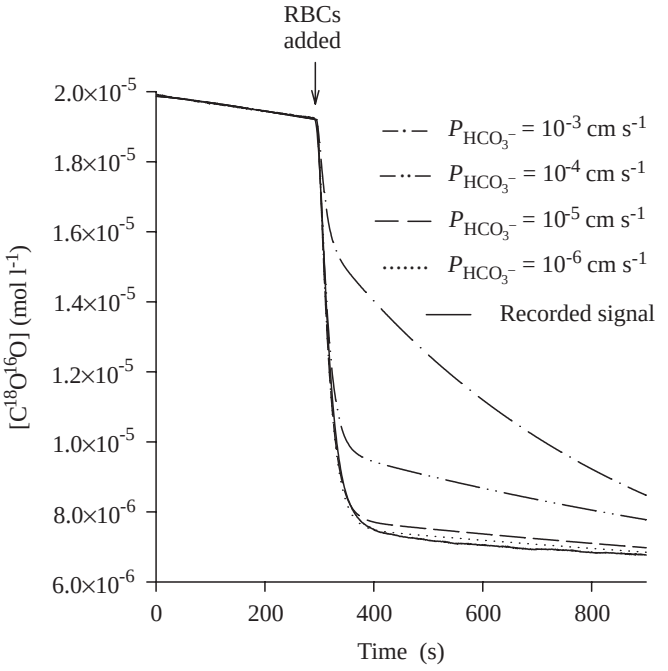

Fig. 4. Decay of the concentration of ^{18}O-labelled carbon dioxide in the reaction solution (solid line). The curve comes from an experiment (listed as no. 1 in Table 3), in which a suspension of hagfish red blood cells (RBCs) was added to a Prontosil–Dextran-containing reaction solution. Evaluation 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 ($P_{HCO_3^-}$) of 6.828×10^{-13} cm s^{-1} (best fit). The four upper traces represent curves calculated employing the indicated value of $P_{HCO_3^-}$. 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.

$P_{HCO_3^-}$ *and* A_i *of hagfish red blood cell*

The present results indicate that the HCO_3^- permeability of the hagfish red blood cell membrane is clearly much lower than that of most vertebrate red blood cell membranes. The mean

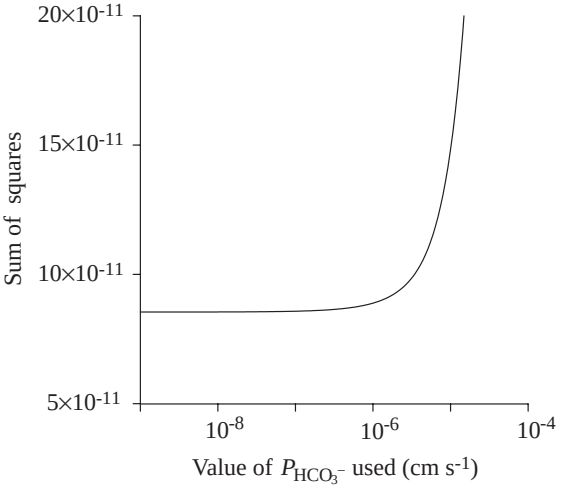

Fig. 5. Illustration of the dependence of the quality of fit on the value used for the HCO_3^- permeability of the hagfish red blood cell membrane ($P_{HCO_3^-}$). The goodness of the fit is expressed by the sum of squares of deviations (*y*-axis). The calculations are based on the recorded signal from experiment 1 (Table 3; Fig. 4).

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*

Temperature (°C)	Species	$P_{HCO_3^-}$ (cm s^{-1})	A_i	N
10	Hagfish	≈0	21 000	9
10	Man	2.5×10^{-4}	43 000	10
15	Flounder	5.5×10^{-4}	187 000	10
37	Man	9.0×10^{-4}	13 000	8

$P_{HCO_3^-}$, 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 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.

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 $P_{HCO_3^-}$ 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 (Sanyal 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^{-6} 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 4.3×10^{-6} 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_2DIDS observed by Brill et al. (1992) and how H_2DIDS 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 (Gallaugher 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

of CO_2. 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 CO_2-transporting capacity of the blood, but the Haldane effect was found to be of minor importance for CO_2 transport by the blood of *M. glutinosa* (Tufts et al., 1998). Carbonic anhydrase activity in the plasma could give rise to fast conversion of CO_2 to HCO_3^- 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 CO_2 transport by hagfish blood caused by the absence of fast HCO_3^- transport across the red blood cell membrane. However, the small CO_2 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 CO_2 transport in *M. glutinosa* may, however, favourably affect the oxygen transport properties of the blood of this species. HCO_3^- has been shown to act as a potent allosteric effector on hagfish haemoglobin. In red blood cell lysates, an increasing HCO_3^- concentration causes a significant decrease in the oxygen affinity of the haemoglobin (Fago et al., 1999). Thus, an exceptionally large increase in the intraerythrocytic HCO_3^- 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 HCO_3^- 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 O_2 release by the blood may be very important in hagfishes. Thus, the absence of fast HCO_3^- 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).

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References

Benga, G., Borza, T., Popescu, O., Porutiu, D. and Matei, H. (1993). Comparative nuclear magnetic resonance studies of diffusional water permeability of red blood cells from sheep and cow. *Comp. Biochem. Physiol.* **104**B, 589–594.

Böllert, P., Peters, T., Engelhardt, W. v. and Gros, G. (1997). Mass spectrometric determination of HCO_3^- permeability and carbonic anhydrase activity in intact guinea-pig colon epithelium. *J. Physiol., Lond.* **502**, 679–691.

Brill, S. R., Musch, M. W. and Goldstein, L. (1992). Taurine efflux, band 3 and erythrocyte volume of the hagfish (*Myxine glutinosa*) and lamprey (*Petromyzon marinus*). *J. Exp. Zool.* **264**, 19–25.

Carlsson, U., Kjellström, B. and Antonsson, B. (1980). Purification and properties of cyclostome carbonic anhydrase from erythrocytes of hagfish. *Biochim. Biophys. Acta* **612**, 160–170.

Dohn, N. and Malte, H. (1998). Volume regulation in red blood cells. In *The Biology of Hagfishes* (ed. J. M. Jørgensen, J. P. Lomholt, R. E. Weber and H. Malte), pp. 300–306. London: Chapman & Hall.

Edsall, J. T. (1969). Carbon dioxide, carbonic acid, and bicarbonate ion: Physical properties and kinetics of interconversion. In *CO_2: Chemical, Biochemical and Physiological Aspects* (ed. R. E. Forster, J. T. Edsall and F. J. W. Roughton), pp. 15-17. Washington: NASA.

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.

Fago, A., Malte, H. and Dohn, N. (1999). Bicarbonate binding to hemoglobin links oxygen and carbon dioxide transport in hagfish. *Respir. Physiol.* **115**, 309–315.

Forster, M. E. (1990). Confirmation of the low metabolic rate of hagfish. *Comp. Biochem. Physiol.* **96**A, 113–116.

Forster, M. E. (1998). Cardiovascular function in hagfishes. In *The Biology of Hagfishes* (ed. J. M. Jørgensen, J. P. Lomholt, R. E. Weber and H. Malte), pp. 236–258. London: Chapman & Hall.

Gallaugher, P. and Farrell, A. P. (1998). Hematocrit and blood oxygen-carrying capacity. In *Fish Respiration* (ed. S. F. Perry and B. L. Tufts), pp. 185–227. London: Academic Press.

Geers, C., Gros, G. and Gärtner, A. (1985). Extracellular carbonic anhydrase of skeletal muscle associated sarcolemma. *J. Appl. Physiol.* **59**, 548–558.

Glomski, C. A., Tamburlin, J. and Chainani, M. (1992). The phylogenetic odyssey of the erythrocyte. III. Fish, the lower vertebrate experience. *Histol. Histopath.* **7**, 501–528.

Gros, G. and Moll, W. (1971). The diffusion of carbon dioxide in erythrocytes and hemoglobin solutions. *Pflügers Arch.* **324**, 249–266.

Harned, H. S. and Bonner, F. T. (1945). The first ionization of carbonic acid in aqueous solution of sodium chloride. *J. Am. Chem. Soc.* **67**, 1026–1031.

Hastings, A. B. and Sendroy, J. (1925). The effect of variation in ionic strength on the apparent first and second dissociation constant of carbonic acid. *J. Biol. Chem.* **65**, 445–455.

Ho, C. and Sturtevant, J. M. (1963). The kinetics of the hydration of carbon dioxide at 25°. *J. Biol. Chem.* **238**, 3499–3501.

Itada, N. and Forster, R. E. (1977). Carbonic anhydrase activity in intact red blood cells measured with ^{18}O exchange. *J. Biol. Chem.* **252**, 3881–3890.

Kleihauer, E. and Betke, K. (1957). Zur Hämoglobinbestimmung mittels Cyanhämoglobin nach Betke und Savelsberg. *Ärztl. Lab.* **3**, 202–205.

Landolt, H. and Börnstein, R. (1962). (eds) *Zahlenwerte und Funktionen aus Physik, Chemie, Astronomie, Geophysik und Technik*. 6th edn, vol. II, part 2, section 22261. Berlin, Heidelberg: Springer.

Malte, H. and Lomholt, J. P. (1998). Ventilation and gas exchange. In *The Biology of Hagfishes* (ed. J. M. Jørgensen, J. P. Lomholt, R. E. Weber and H. Malte), pp. 223–234. London: Chapman & Hall.

Maren, T. H., Friedland, B. R. and Rittmaster, R. S. (1980). Kinetic properties of primitive vertebrate carbonic anhydrases. *Comp. Biochem. Physiol.* **67**B, 69–74.

Mills, G. A. and Urey, H. C. (1940). The kinetics of isotopic exchange between carbon dioxide, bicarbonate ion, carbonate ion and water. *J. Am. Chem. Soc*. **62**, 1019–1026.

Moore, W. J. (1962). *Physical Chemistry*, 3rd edn, pp. 180–182. London: Prentice Hall.

Munz, F. W. and Morris, R. W. (1965). Metabolic rate of the hagfish, *Eptatretus stoutii*. *Comp. Biochem. Physiol*. **16**, 1–6.

Nelder, J. A. and Mead, R. (1965). A simplex method for function minimization. *Comput. J.* **7**, 308–313.

Norris, F. A. and Powell, G. L. (1992). Characterization of CO_2/carbonic acid mediated proton flux through phosphatidylcholine vesicles as model membranes. *Biochim. Biophys. Acta* **1111**, 17–26.

Sanyal, G. and Maren, T. H. (1981). Thermodynamics of carbonic anhydrase catalysis. *J. Biol. Chem*. **256**, 608–612.

Smith, K. L. and Hessler, R. R. (1974). Respiration of benthopelagic fishes: *in situ* measurements at 1230 m. *Science* **184**, 72–73.

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

Tufts, B. L. and Boutilier, R. G. (1990b). CO_2 transport properties of a primitive vertebrate, *Myxine glutinosa* (L.). *Exp. Biol*. **48**, 341–347.

Tufts, B. L. and Perry, S. F. (1998). Carbon dioxide transport and excretion. In *Fish Respiration* (ed. S. F. Perry and B. L. Tufts), pp. 229–281. London: Academic Press.

Tufts, B. L., Vincent, C. J. and Currie, S. (1998). Different red blood cell characteristics in a primitive agnathan (*M. glutinosa*) and a more recent teleost (*O. mykiss*) influence their strategies for blood CO_2 transport. *Comp. Biochem. Physiol*. **119**A, 533–541.

Wells, R. M. G., Forster, M. E., Davidson, W. and Taylor, H. H. (1986). Blood oxygen transport in the free swimming hagfish, *Eptatretus cirrhatus*. *J. Exp. Biol*. **123**, 43–53.

Wissbrun, K. F., French, D. M. and Patterson, A., Jr (1954). The true ionization constant of carbonic acid in aqueous solution from 5 to 45 °. *J. Phys. Chem*. **58**, 693–695.

Wunder, M., Böllert, P. and Gros, G. (1997). Mathematical modelling of the role of intra- and extracellular activity of carbonic anhydrase and membrane permeabilities of HCO_3^-, H_2O and CO_2 in ^{18}O exchange. *Isotopes Environ. Health Stud.* **33**, 197–205.