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SHORT COMMUNICATION

AN *IN VITRO* AND *IN VIVO* STUDY OF THE DISTRIBUTION OF AMMONIA BETWEEN PLASMA AND RED CELLS OF RAINBOW TROUT (*SALMO GAIRDNERI*)

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Fish are ammoniotelic animals and therefore produce ammonia as the end-product of protein metabolism. (The term ammonia or T_{amm} will be used to indicate the total ammonia concentration, while NH_4^+ and NH_3 will refer to ammonium ion and ammonia, respectively.) Ammonia is a weak base that is produced as NH_3 or NH_4^+ depending on the biochemical reaction and exists in solution as NH_3 and NH_4^+. Owing to the relatively high pK of ammonia (pK = 9·7 at 10°C) and the physiological pH of body fluids, the predominant form of ammonia in tissue compartments is the ionic form, NH_4^+. Biological membranes are highly permeable to NH_3 and much less permeable to NH_4^+ (Klocke, Andersson, Rotman & Forster, 1972; Castell & Moore, 1971; Bown *et al.* 1975; Boron, 1980; Lockwood, Finn, Campbell & Richman, 1980), which requires ion carriers for transport. Thus, the extent of movement of NH_4^+ between tissue compartments depends on the availability of these carriers and their affinity for NH_4^+. Transfer of ammonia between tissue compartments is largely determined by NH_3 gradients (see Randall & Wright, 1987), but NH_4^+ electrochemical gradients may also be important (Thomas, 1974; Boron & DeWeer, 1976). The purpose of this study was to determine whether ammonia was passively distributed between red cells and plasma at rest and during an extracellular acidosis. Protons are passively distributed across red cell membranes over a range of pH values in trout (Heming *et al.* 1986). Thus, if ammonia is passively distributed, the distribution will be determined by red cell-to-plasma pH gradients.

In the *in vitro* experiments blood was collected from the dorsal aortic catheter (for technique, see Soivio, Westman & Nyholm, 1972) of donor fish, pooled, and then divided between tonometers (4 ml per tonometer). Each tonometer received either a 0·2 % CO_2 (control) or a 1·0 % CO_2 (hypercapnia) humidified gas mixture in air and

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was shaken for 90 min in a 9°C water bath before measurements were taken. Blood was analysed for whole blood pH (pH_e), red cell pH (pH_i), whole blood and plasma ammonia concentrations (T_{amm}), plasma and red cell water content, and haematocrit (Hct). In the *in vivo* experiment, dorsal aortic cannulated fish were placed in individual, low volume (2 l) flow-through chambers to recover for 48 h. In the 30 min prior to sampling, inflow water was turned off and fish chambers were aerated with either 100 % air (water pH = 7·0, control) or switched to 1 % CO_2 in air (water pH = 5·6, hypercapnia). A 2 ml blood sample was withdrawn from each fish at the end of 30 min and analysed for pH_e, pH_i, whole blood and plasma T_{amm}, plasma and red cell water content, and Hct.

Arterial pH_e was measured immediately after blood had been collected using a Radiometer microelectrode (E5021) and acid–base analyser (PHM72), maintained at 9°C. Red cell pH_i was directly measured by the freeze–thaw technique (Zeidler & Kim, 1977). Plasma and red cell water content were calculated from initial wet weights and final dry weights after samples had been dried to constant weight in an oven (100°C). Whole blood and plasma samples (250 μl) were assayed for T_{amm} by the glutamate dehydrogenase enzymatic assay (Kun & Kearney, 1971). Red cell T_{amm} was calculated as follows:

$$\text{red cell } T_{amm} = \frac{\{(\text{whole blood } T_{amm}) - ([1 - \text{Hct}/100]\ [\text{plasma } T_{amm}])\}}{\text{Hct}/100} .$$

Red cell T_{amm} levels calculated above were then corrected for water content and the final concentration was expressed as $\mu mol\,l^{-1}$ cell water. Data are expressed as mean ± 1 S.E.M. (*N*), where *N* equals the number of animals sampled (*in vivo*) or the number of tonometers containing blood (*in vitro*). Student's paired and unpaired *t*-tests have been used to compare the significance ($P < 0·05$) between mean values.

Slight quantitative differences between *in vitro* and *in vivo* data are shown in Table 1, but the overall results and conclusions are the same whether blood was held in tonometers (*in vitro*) or in live animals (*in vivo*) prior to analysis. Red cell ammonia levels are consistently higher than plasma levels, resulting in ammonia concentration ratios (plasma-to-red cell) of between 0·3 and 0·4 (Table 1). Control red cell pH_i predicted from the plasma-to-red cell ammonia distribution was not significantly different from measured pH_i and there was no difference between calculated plasma and red cell NH_3 tensions (P_{NH_3}) in the control experiment (*in vitro* and *in vivo*, Table 2). The same was not true for the hypercapnia experiment (*in vitro* and *in vivo*), where predicted red cell pH_i was significantly different from measured pH_i and calculated red cell P_{NH_3} was greater than plasma P_{NH_3} (Table 2). Our calculations of P_{NH_3} levels assume an equilibrium between NH_3 and NH_4^+ in each compartment. Thus, when there is an NH_3 gradient from red cell to plasma there will also be an electrochemical gradient for NH_4^+. In hypercapnia, therefore, there is a net diffusion gradient for both NH_3 and NH_4^+ out of the red cell.

Ammonia gradients during hypercapnia may develop between intra- and extracellular compartments because of high rates of ammonia production. We tested this possibility in trout blood *in vitro*, by following whole blood T_{amm} levels over time

during hypercapnic exposure, and found that ammonia levels did not change. Thus, intracellular ammoniagenesis is not a factor in the development of P_{NH_3} gradients during hypercapnia. Ammonia accumulation in the red cell, therefore, can only be maintained by the active uptake of NH_4^+ in the face of NH_3 diffusion out of the red cell down the P_{NH_3} gradient and NH_4^+ electrochemical gradient. Secondary active transport of NH_4^+ is linked to the energetically favourable movement of Na^+ in

Table 1. *Ammonia distribution between red cells and plasma in trout,* in vitro *and* in vivo, *under control and hypercapnic conditions*

Treatment	pH_e	pH_i (freeze–thaw method)	Plasma T_{amm} ($\mu mol\,l^{-1}$)	Red cell T_{amm} ($\mu mol\,l^{-1}$)	Ammonia concentration ratio†
Control					
in vitro ($N = 6$)	8.03 ± 0.05	7.48 ± 0.02	304 ± 8	1048 ± 81	0·29
in vivo ($N = 7$)	8.02 ± 0.03	7.50 ± 0.01	311 ± 19	782 ± 55	0·40
Hypercapnia					
in vitro ($N = 7$)	7.63 ± 0.02*	7.25 ± 0.01*	318 ± 9	969 ± 52	0·33
in vivo ($N = 7$)	7.55 ± 0.02**	7.28 ± 0.01**	323 ± 3	872 ± 30	0·37

Red cell pH_i was measured directly by the freeze–thaw method.
* Significantly different from *in vitro* control, paired *t*-test, $P < 0.05$.
** Significantly different from *in vivo* control, unpaired *t*-test, $P < 0.05$.
† Ratio of plasma T_{amm} to red cell T_{amm}.

Table 2. *Measured red cell pH_i from Table 1 compared with red cell pH_i calculated from the ratio NH_3:NH_4^+ in the red cell using the Henderson–Hasselbalch equation*

Treatment	pH_i (freeze–thaw technique)	pH_i (ammonia)	Plasma P_{NH_3} (μTorr)	Red cell P_{NH_3} (μTorr)
Control				
in vitro ($N = 6$)	7.48 ± 0.02	7.46 ± 0.05	103.2 ± 13.4	106.7 ± 10.5
in vivo ($N = 7$)	7.50 ± 0.01	7.51 ± 0.04	97.2 ± 8.5	93.5 ± 6.0
Hypercapnia				
in vitro ($N = 7$)	7.25 ± 0.01	7.11 ± 0.04*	43.3 ± 2.7	59.9 ± 4.2**
in vivo ($N = 7$)	7.28 ± 0.01	7.09 ± 0.01*	36.2 ± 1.7	56.6 ± 2.0**

This calculation assumes that plasma NH_3 = red cell NH_3 and red cell NH_4^+ = red cell ($T_{amm} - NH_3$).
Plasma P_{NH_3} calculated with plasma T_{amm} and pH_e is compared with red cell P_{NH_3} calculated with red cell T_{amm} and red cell pH_i, values taken from Table 1.
* Significantly different from measured pH_i, unpaired *t*-test, $P < 0.05$.
** Significantly different from plasma P_{NH_3}, unpaired *t*-test, $P < 0.05$.
1 Torr = 133·3 Pa.

many cells (see Maetz & Garcia-Romeu, 1964; Kinsella & Aronson, 1981; Wright & Wood, 1985). The trout red cell membrane Na^+/H^+ exchange mechanism is known to be active during an acidosis (Nikinmaa, Steffensen, Tufts & Randall, 1987), but if NH_4^+ can replace H^+ in exchange for Na^+, then red cell ammonia stores would be depleted during hypercapnia. Instead, we observed an accumulation of ammonia during hypercapnia, therefore NH_4^+ substitution for H^+ in Na^+/H^+ exchange cannot be involved. The ability of NH_4^+ to replace K^+ in Na^+,K^+-ATPase is well established in many tissues, including red cell membranes (Post & Jolly, 1957; Sorensen, 1981). We tested the possibility that NH_4^+ was replacing K^+ in the Na^+,K^+-ATPase by adding the specific Na^+,K^+-ATPase inhibitor, ouabain, to hypercapnic blood, *in vitro* (Tables 3, 4). The addition of ouabain did not alter the distribution of ammonia between red cells and plasma (Table 3) and red cell-to-plasma P_{NH_3} gradients were not abolished (Table 4). This implies that even if Na^+,K^+-ATPase plays a role in ammonia accumulation within the red cell during hypercapnia, it cannot be a major one.

Table 3. *Ammonia distribution between red cells and plasma* (in vitro) *during hypercapnia, with ouabain (10^{-4} mol l^{-1}) and without (control)*

Treatment	pH_e	pH_i (freeze–thaw method)	Plasma T_{amm} (μmol l^{-1})	Red cell T_{amm} (μmol l^{-1})	Ammonia concentration ratio†
Control ($N = 7$)	7·64 ± 0·03	7·25 ± 0·03	346 ± 17	1016 ± 61	0·34
Ouabain ($N = 7$)	7·64 ± 0·01	7·24 ± 0·01	354 ± 16	1000 ± 82	0·35

† Ratio of plasma T_{amm} to red cell T_{amm}.
No significant difference between control and ouabain values.

Table 4. *Measured red cell pH_i from Table 3 compared with red cell pH_i calculated from the ratio NH_3:NH_4^+ in the red cell using the Henderson–Hasselbalch equation, for control (without ouabain) and ouabain (10^{-4} mol l^{-1}) treatments*

Treatment	pH_i (freeze–thaw technique)	pH_i (ammonia)	Plasma P_{NH_3} (μTorr)	Red cell P_{NH_3} (μTorr)
Control ($N = 7$)	7·25 ± 0·03	7·13 ± 0·03*	53 ± 2	70 ± 4**
Ouabain ($N = 7$)	7·24 ± 0·01	7·16 ± 0·03*	55 ± 3	69 ± 5**

This calculation assumes that plasma NH_3 = red cell NH_3 and that red cell NH_4^+ = red cell ($T_{amm} - NH_3$).
Plasma P_{NH_3} calculated with plasma T_{amm} and pH_e is compared with red cell P_{NH_3} calculated with red cell T_{amm} and red cell pH_i values taken from Table 3.
* Significantly different from measured pH_i, unpaired t-test, $P < 0.05$.
** Significantly different from plasma P_{NH_3}, unpaired t-test, $P < 0.05$.
1 Torr = 133·3 Pa.

It is possible that changes in pH and water content, which will lead to changes in ammonia distribution, may have caused the development of ammonia gradients between red cell and plasma during hypercapnia. Whole blood pH remained stable after 30 min of hypercapnia *in vitro*. Red cell water content increased significantly between control (*in vitro*, $65{\cdot}1 \pm 0{\cdot}4\,\%$; *in vivo*, $65{\cdot}7 \pm 0{\cdot}3\,\%$) and hypercapnia (*in vitro*, $68{\cdot}4 \pm 0{\cdot}3\,\%$; *in vivo*, $68{\cdot}9 \pm 0{\cdot}3\,\%$) experiments. It seems likely that the water content of red cells was stable following 90 min of exposure to hypercapnia *in vitro*. Thus it appears that non-steady states for pH and water content cannot account for the red cell-to-plasma P_{NH_3} gradients *in vitro* during hypercapnia. It also seems to be an unlikely explanation of the *in vivo* results because of the similarity of the *in vitro* and *in vivo* data.

We conclude that ammonia is passively distributed according to the plasma-to-red cell H^+ distribution in blood at resting pH values, but not during hypercapnia. Ammonia accumulation during hypercapnia cannot be accounted for by red cell ammoniagenesis or NH_4^+ substitution for K^+ in the Na^+,K^+-ATPase, but must be due to some other active NH_4^+ uptake process. Whether ammonia is passively distributed between plasma and other intracellular compartments in fish is not known. This question is interesting in light of the fact that the distribution of H^+ across intracellular compartments, other than red cell membranes (Lassen, 1977; Heming *et al.* 1986), is not passive (see Roos & Boron, 1981). Thus, if NH_4^+ is able to move across tissue membranes, then one would predict that the distribution of ammonia would follow the membrane potential and not the H^+ distribution. However, if tissue membranes are essentially impermeable to NH_4^+ then one would expect the distribution of ammonia to follow the H^+ distribution, as do other weak acids and bases with impermeant ion forms (see Randall & Wright, 1987).

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