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THE EFFECTS OF FORCED ACTIVITY ON CIRCULATING CATECHOLAMINES AND pH AND WATER CONTENT OF ERYTHROCYTES IN THE TOAD

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SUMMARY

In vivo experiments were carried out to determine the effect of forced activity on circulating catecholamine levels, haematocrit, and the pH and water content of erythrocytes in the toad, *Bufo marinus*. In addition, the effect of the beta-adrenergic agonist isoproterenol on erythrocte pH and water content was examined *in vitro*.

Forced activity caused a significant decrease in both whole blood and erythrocyte pH, while haematocrit and circulating adrenaline and noradrenaline levels increased. Erythrocyte water content did not change following forced activity. Addition of isoproterenol to toad blood *in vitro* had no effect on either erythrocyte pH or water content. The apparent absence of beta-adrenergic effects on erythrocyte pH and water content in the toad is in sharp contrast to the response of teleost fish erythrocytes to beta-adrenergic stimulation. The significance of these differences is discussed.

INTRODUCTION

The effects of beta-adrenergic stimulation of teleost erythrocytes have been well documented. Addition of beta-adrenergic agonists to teleost erythrocytes *in vitro* results in an increase in erythrocyte pH and water content (Nikinmaa, 1982; Nikinmaa & Huestis, 1984; Cossins & Richardson, 1985; Heming, Randall & Mazeaud, 1986*b*). It has also been demonstrated *in vivo* that an increase in circulating catecholamine levels results in an increase in erythrocyte pH and water content (Nikinmaa, Cech & McEnroe, 1984; Primmett, Randall, Mazeaud & Boutilier, 1986).

There are, however, only a very limited number of studies that document the effect of beta-adrenergic stimulation on both the pH and water content of nucleated erythrocytes from other species of vertebrates. Nikinmaa & Huestis (1984) have shown that addition of isoproterenol to avian erythrocytes *in vitro* causes an increase in cellular water content only at very high extracellular potassium concentration and does not affect erythrocyte pH. Rudolph & Greengard (1980) have documented cell swelling in amphibian erythrocytes *in vitro* after addition of isoproterenol, but only

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in the presence of a phosphodiesterase inhibitor. To our knowledge, the effect of beta-adrenergic stimulation on erythrocyte pH has not been documented in amphibians. We have examined, therefore, the effects of catecholamines on the pH and water content in erythrocytes from the toad, *Bufo marinus*, both *in vivo* and *in vitro*. In the *in vivo* experiments, we exercised the toads in order to determine if erythrocyte pH and, therefore, the characteristics of haemoglobin:oxygen binding, are modulated during an exercise-induced acidosis in an amphibian, as in teleost fish.

MATERIALS AND METHODS

Adult *Bufo marinus* of both sexes (250–500 g) obtained from Charles D. Sullivan Co. Inc. (Nashville, TN, USA) were used for all experiments. The animals were kept in large fibreglass aquaria with 2–4 cm dechlorinated tap water at the experimental temperature (22°C) for at least a week prior to the experiments.

Toads were anaesthetized in neutralized MS-222 ($1{\cdot}5\ g\ l^{-1}$) and chronically cannulated in the femoral artery (using the method of Boutilier, Randall, Shelton & Toews, 1979). Following surgery, the animals were transferred to light-proof boxes with 500 ml of dechlorinated tap water and allowed to recover overnight.

In vivo *experiments*

Following overnight recovery, a 1-ml control blood sample was removed from the femoral cannula and immediately analysed for pH and haematocrit. The sample was then centrifuged and the plasma was removed for catecholamine analysis. The packed red cells were immediately frozen in liquid nitrogen for later analysis of intracellular pH. The animal was then subjected to 30 min of vigorous exercise. This involved the manual manipulation of the animal by the experimenter so that continuous righting movements were elicited. The goal of this procedure was to induce catecholamine release. Upon completion of this exercise period, the animal was returned to the chamber and another blood sample was immediately taken. Blood samples were also taken 0·5, 1 and 4 h after exercise and analysed as described above for the control sample.

An additional set of experiments was conducted to determine any relationship between changes in haematocrit and changes in the erythrocyte water content. These two parameters were measured in five animals subjected to the previously described protocol with the exception that only two samples (control and 0 h post-exercise) were taken.

In vitro *experiments*

In this series of experiments, toads were cannulated as described in the previous section and allowed to recover overnight. The next day, blood was collected from several cannulated toads and pooled. Samples (2·5 ml) of blood were then transferred to glass tonometers and equilibrated with a humidified 5 % CO_2/95 % air mixture delivered by Wösthoff gas mixing pumps. This gas mixture resulted in blood pH values close to those of animals immediately following exhaustive exercise (se

Fig. 1). Following a 90-min equilibration period, a 1-ml blood sample was taken from each tonometer and centrifuged; 0·7 ml in one tube for the determination of plasma and erythrocyte pH and the remaining 0·3 ml in another tube for the determination of cell water content. The pH of the plasma (pH_e) was measured immediately and the remaining plasma from both sample tubes was discarded. The packed erythrocytes from the first sample tube were then frozen in liquid nitrogen for later determination of erythrocyte pH while those from the second sample tube were saved for the determination of cell water content. Baroin, Garcia-Romeu, Lamarre & Motais (1984) have demonstrated that rainbow trout erythrocytes exhibit significant beta-adrenergic sensitivity following 3 h of equilibration *in vitro* at 15 °C and therefore it was assumed that the equilibration time (90 min) would not alter the beta-adrenergic sensitivity of the cells. At this point, each tonometer received either 10^{-5} mol l^{-1} isoproterenol (final concentration) or an equivalent volume (50 μl) of Mackenzie's amphibian saline. Isoproterenol was chosen since the regulation of pH in teleost erythrocytes appears to be a beta-adrenergic effect (Nikinmaa *et al.* 1984; Primmett *et al.* 1986) and beta-adrenergic effects are most potently stimulated by isoproterenol (Lefkowitz, 1976). The concentration of 10^{-5} mol l^{-1} was used in order to saturate the beta-adrenergic receptors. The sampling procedure was then repeated following another 30 min of equilibration. It should be noted that in representative cases ($N = 3$), plasma catecholamine measurements were made at this point in the experiment. In these cases, plasma adrenaline and noradrenaline levels from the control tonometers were very similar to those of resting animals *in vivo*.

Analytical procedures

Measurements of pH were made using a Radiometer PHM 72 acid–base analyser and associated micro-pH unit (Radiometer, Copenhagen, Denmark). Erythrocyte pH measurements were made using the freeze–thaw method of Zeidler & Kim (1977). Plasma adrenaline and noradrenaline levels were determined by high pressure liquid chromatography (Spectra Physics, model SP8700) with electrochemical detection (Woodward, 1982; Primmett *et al.* 1986). The water content of the erythrocytes was determined from the wet and dry mass of the packed erythrocytes according to the method of Nikinmaa & Huestis (1984).

The significance of the results was assessed using a paired Student's *t*-test. Differences were accepted as being significantly different at the $P < 0{\cdot}05$ level.

RESULTS AND DISCUSSION

Control (resting) levels of adrenaline and noradrenaline for *Bufo marinus* measured in the present study were slightly lower than resting levels recorded for *Bufo arenarum* (Donoso & Segura, 1965). Exhaustive exercise in the toad was associated with large increases in circulating levels of adrenaline and noradrenaline (Table 1). While there are no values available from the literature for catecholamine levels following activity in amphibians, the magnitude of these increases was similar

Table 1. *Effect of forced activity on the circulating adrenaline and noradrenaline levels (nmol l^{-1}) in the toad*

	Time (h)				
	C	0	0·5	1	4
Adrenaline ($N = 11$)	2·2 ± 0·4	36·5 ± 3·2*	1·7 ± 0·2	1·7 ± 0·2	1·4 ± 0·3
Noradrenaline ($N = 6$)	0·3 ± 0·1	2·7 ± 0·5*	0·2 ± 0·1	0·1 ± 0·1	0·1 ± 0·1

C = control; 0, 0·5, 1 and 4 = hours following 30 min of forced activity.
Values are means ± 1 S.E.M.
Asterisks denote significant ($P < 0·05$) difference from the control.

Table 2. *Effect of forced activity on the haematocrit (%) and erythrocyte water content (%) in the toad*

	Time (h)				
	C	0	0·5	1	4
Haematocrit ($N = 10$)	22·3 ± 1·4	33·5 ± 1·4*	26·2 ± 1·8*	22·1 ± 1·4	18·4 ± 1·2*
Cell water ($N = 5$)	68·4 ± 1·0	68·4 ± 0·8	—	—	—

C = control; 0, 0·5, 1 and 4 = hours following 30 min of forced activity.
Values are means ± 1 S.E.M.
Asterisks denote significant ($P < 0·05$) difference from the control.

to that found in the rainbow trout (Primmett *et al.* 1986) following exhaustive exercise.

The effect of the exercise period on the haematocrit of the toad is shown in Table 2. The haematocrit was significantly elevated following exercise and did not return to control values until 1 h into the recovery period. McDonald, Boutilier & Toews (1980) documented similar increases in haematocrit in *Bufo marinus* following forced activity. In *Rana catesbeiana*, it has also been shown that infusion of adrenaline or noradrenaline (Mbangkollo & deRoos, 1983; Herman, 1977) or a period of handling (Mbangkollo & deRoos, 1983) will cause an increase in the haematocrit.

There are several factors which may have contributed to this increase in haematocrit. In teleosts, increased haematocrit levels following exhaustive exercise are due to erythrocyte swelling as well as an increase in the circulating number of erythrocytes relative to the plasma volume (Nikinmaa *et al.* 1984; Primmett *et al.* 1986). In *Bufo*, the absence of erythrocyte swelling in the presence of increased catecholamine levels *in vivo* (Table 2) is interesting in view of the study of Rudolph & Greengard (1980) in which catecholamines were found to stimulate swelling in frog erythrocytes *in vitro*. The ion exchanges involved in the adrenergic swelling response in frog erythrocytes (Palfrey & Greengard, 1981) resemble those in teleosts (Baroin *et al.* 1984; Nikinmaa & Huestis, 1984; Heming *et al.* 1986*b*). However, swelling has only been documented in frog erythrocytes *in vitro* if the cells are adrenergically

stimulated in the presence of a phosphodiesterase inhibitor. The present experiments indicate that these ion exchange processes are not capable of raising the erythrocyte water content in the toad during beta-adrenergic stimulation *in vivo*. This may be due to changes in other factors that are capable *in vivo* of influencing erythrocyte water content, such as plasma osmotic pressure. However, Table 3 shows that the beta-adrenergic agonist isoproterenol caused no increase in the cell water content of toad erythrocytes *in vitro*. In fact, there was a small decrease in cell water content *in vitro* at 120 min both in erythrocytes treated with saline and with isoproterenol. Together these results seem to indicate that beta-adrenergic stimulation of toad erythrocytes does not result in changes in water content under physiological conditions. The increase in haematocrit in the present experiments is, therefore, probably due to an increase in the circulating number of erythrocytes relative to the plasma volume. According to Boutilier, Emilio & Shelton (1986), uptake of plasma water by osmotically enriched muscle cells may account for a portion of the haematocrit increase. Erythrocyte recruitment may also have been a contributing factor, since Nilsson & Grove (1974) have demonstrated that constriction of the spleen may be induced in teleosts by perfusion of the splenic artery with adrenaline or noradrenaline.

The effect of forced activity on the whole blood and erythrocyte pH is illustrated in Fig. 1. The acidosis in whole blood following the activity period is similar to that found by McDonald *et al.* (1980) for *Bufo marinus* after forced activity. The erthrocyte pH also falls during this period, but the magnitude of this drop (0·270 pH units) is less than that of the extracellullar pH (0·429 pH units). Thus, the pH difference across the erythrocyte membrane is 0·443 pH units at rest and is reduced to 0·284 pH units after exercise. Hladky & Rink (1977) explain that a reduction in pH reduces the net charge on the haemoglobin inside the erythrocyte. This would result in a reduction in the erythrocyte proton concentration (relative to the extracellular

Table 3. *Effect of isoproterenol on the water content (%), extracellular pH (pH_e), intracellular pH (pH_i) and on the pH gradient (ΔpH) of toad erythrocytes equilibrated with 5 % CO_2/95 % air*

	90 min	Treatment	120 min
Control ($N = 9$)			
cell water	69·1 ± 0·4	saline	67·9 ± 1·4*
pH_e	7·516 ± 0·017		7·511 ± 0·016
pH_i	7·124 ± 0·010		7·134 ± 0·017
ΔpH	+0·392		+0·377
Experimental ($N = 9$)			
cell water	68·5 ± 0·52	isoproterenol	67·9 ± 0·4*
pH	7·514 ± 0·016	(10^{-5} mol l^{-1})	7·511 ± 0·015
pH_i	7·128 ± 0·011		7·140 ± 0·018
ΔpH	+0·386		+0·371

Values are means ± 1 S.E.M.
Asterisks denote significant ($P < 0·05$) difference from the 90-min value.

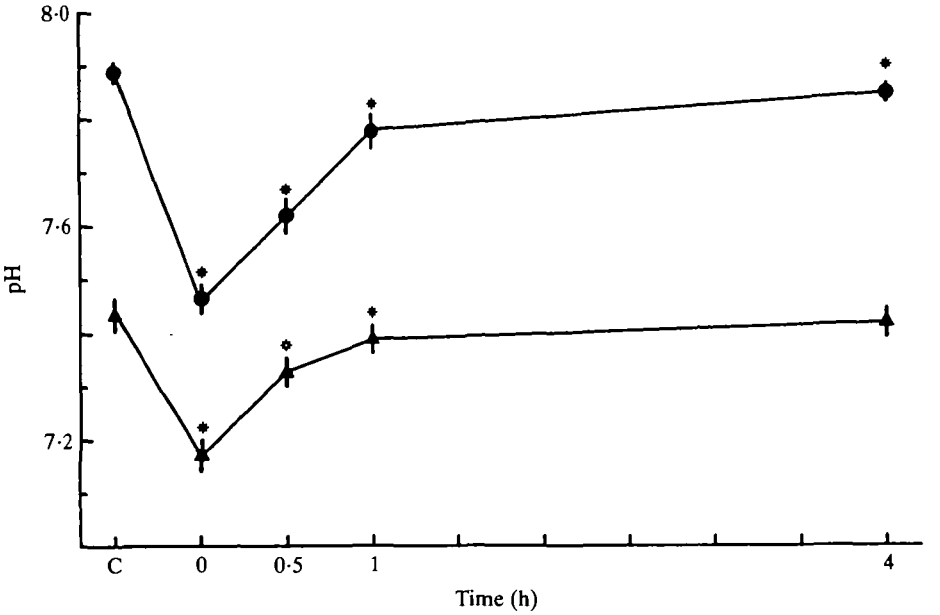

Fig. 1. Whole blood (●) and erythrocyte (▲) pH in 11 toads before (C) forced activity and at 0, 0·5, 1 and 4 h following forced activity. Asterisks indicate values significantly ($P < 0·05$) different from the control (C) values. All values are given as means ± S.E.M.

concentration) and, therefore, in the erythrocyte transmembrane pH gradient. The erythrocyte transmembrane pH gradient also decreases with decreasing pH in rainbow trout erythrocytes equilibrated *in vitro* in the absence of catecholamines (Heming *et al.* 1986*a*).

It has been demonstrated that while plasma pH falls after exhaustive exercise in teleosts, an increase in circulating catecholamine levels causes the erythrocyte pH to be maintained (Nikinmaa *et al.* 1984) or increased (Primmett *et al.* 1986). Beta-adrenergic agonists also cause an increase in the pH of teleost erythrocytes *in vitro* (Nikinmaa & Huestis, 1984; Cossins & Richardson, 1985; Heming *et al.* 1986*b*). Nevertheless, the erythrocyte pH fell significantly following activity (0·270 pH units; Fig. 1) in the present experiments, even though circulating catecholamine levels increased (Table 1). In addition, isoproterenol had no effect on the pH of erythrocytes equilibrated *in vitro* (Table 3). The effect of catecholamines on the pH of toad erythrocytes is therefore quite different from the effects which have been documented in teleosts. Note, however, that at the same extracellular pH, there is a difference in the magnitude of the pH gradient between the *in vivo* experiment at 0 h (Fig. 1) and the *in vitro* experiment (Table 3). This difference in the pH gradient could reflect some degree of intracellular pH regulation *in vivo* which is not observed *in vitro*. However, the difference can also be explained as a haemolysis effect on the liquid junction potential of the pH apparatus (Siggaard-Andersen, 1961; Boutilier, Iwama, Heming & Randall, 1985), since whole blood pH was measured in Fig. 1 whereas true plasma pH was measured in Table 3.

It is possible that the regulation of erythrocyte pH *via* adrenergic mechanisms is an adaptation which may be specific to water-breathing vertebrates. However, the difference in the adrenergic effects on the erythrocyte between the toad and teleost fish may also be explained if the subsequent effects on the oxygen-carrying capacity in the blood of each of these animals are considered. In teleost fish, a fall in the erythrocyte pH lowers the oxygen-carrying capacity of the blood because of the Root shift (Cameron, 1971; Nikinmaa *et al.* 1984; Boutilier, Iwama & Randall, 1986). However, anuran amphibians do not possess a Root shift (Bridges, Pelster & Sheid, 1985) and a fall in the erythrocyte pH would not lower the oxygen-carrying capacity in the blood of *Bufo marinus* as it does in teleosts. The functional significance of the adrenergic response in teleost erythrocytes may, therefore, be to offset the Root shift.

In conclusion, circulating catecholamine levels in the toad increase following forced activity. The activity period also caused an increase in haematocrit and plasma acidosis which have previously been described for *Bufo marinus*. There was an apparent absence, however, of any beta-adrenergic regulation of pH or water content of erythrocytes in the toad, *Bufo marinus*. This is in sharp contrast to the response of teleost fish erythrocytes to beta-adrenergic stimulation and may be due to the fact that amphibians do not have a Root shift.

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REFERENCES

BAROIN, A., GARCIA-ROMEU, F., LAMARRE, T. & MOTAIS, R. (1984). Hormone-induced co-transport with specific pharmacological properties in erythrocytes of rainbow trout, *Salmo gairdneri*. *J. Physiol., Lond.* **350**, 137–157.

BOUTILIER, R. G., EMILIO, M. G. & SHELTON, G. (1986). The effects of mechanical work on electrolyte and water distribution in amphibian skeletal muscle. *J. exp. Biol.* **120**, 333–350.

BOUTILIER, R. G., IWAMA, G. K., HEMING, T. A. & RANDALL, D. J. (1985). The apparent pK of carbonic acid in rainbow trout blood plasma between 5 and 15°C. *Respir. Physiol.* **61**, 237–254.

BOUTILIER, R. G., IWAMA, G. K. & RANDALL, D. J. (1986). The promotion of catecholamine release in rainbow trout (*Salmo gairdneri*) by acute acidosis: interactions between red cell pH and haemoglobin oxygen-carrying capacity. *J. exp. Biol.* **123**, 145–157.

BOUTILIER, R. G., RANDALL, D. J., SHELTON, G. & TOEWS, D. P. (1979). Acid–base relationships in the blood of the toad, *Bufo marinus*. I. The effects of environmental CO_2. *J. exp. Biol.* **82**, 331–334.

BRIDGES, C. R., PELSTER, B. & SHEID, P. (1985). Oxygen binding in the blood of *Xenopus laevis* (Amphibia) and evidence against Root effect. *Respir. Physiol.* **61**, 125–136.

CAMERON, J. N. (1971). Oxygen dissociation characteristics of the blood of the rainbow trout, *Salmo gairdneri*. *Comp. Biochem. Physiol.* **38**A, 699–704.

COSSINS, A. R. & RICHARDSON, P. A. (1985). Adrenaline-induced Na^+/H^+ exchange in trout erythrocytes and its effects upon oxygen-carrying capacity. *J. exp. Biol.* **118**, 229–246.

DONOSO, A. O. & SEGURA, E. T. (1965). Seasonal variation of plasma adrenaline and noradrenaline in toads. *Gen. comp. Endocr.* **5**, 440–443.

HEMING, T. A., RANDALL, D. J., BOUTILIER, R. G., IWAMA, G. K. & PRIMMETT, D. (1986*a*). Ionic equilibria in red blood cells of rainbow trout (*Salmo gairdneri*): Cl^-, HCO_3^- and H^+. *Respir. Physiol.* (in press).

HEMING, T. A., RANDALL, D. J. & MAZEAUD, M. M. (1985*b*). Effects of adrenaline on ionic equilibria in red blood cells of rainbow trout (*Salmo gairdneri*). *Respir. Physiol.* (in press).

HERMAN, C. A. (1977). Comparative effects of epinephrine and norepinephrine on plasma glucose and haematocrit levels in the American bullfrog (*Rana catesbeiana*). *Gen. comp. Endocr.* **32**, 321–329.

HLADKY, S. B. & RINK, T. J. (1977). pH equilibrium across the red cell membrane. In *Membrane Transport in Red Cells* (ed. J. C. Ellory & V. L. Lew), pp. 115–135. London: Academic Press.

LEFKOWITZ, M. D. (1976). β-Adrenergic receptors: recognition and regulation. *New Engl. J. Med.* **295**, 323–328.

MBANGKOLLO, D. & DEROOS, R. (1983). Comparative effects of epinephrine, norepinephrine, and a gentle handling stress on plasma lactate, glucose, and haematocrit levels in the American bullfrog (*Rana catesbeiana*). *Gen. comp. Endocr.* **49**, 167–175.

MCDONALD, D. G., BOUTILIER, R. G. & TOEWS, D. P. (1980). The effects of enforced activity on ventilation, circulation and blood acid–base status in the semi-terrestrial anuran, *Bufo marinus*. *J. exp. Biol.* **84**, 273–287.

NIKINMAA, M. (1982). Effects of adrenaline on red cell volume and concentration gradient of protons across the red cell membrane in the rainbow trout, *Salmo gairdneri*. *Molec. Physiol.* **2**, 287–297.

NIKINMAA, M., CECH, J. J., JR & MCENROE, M. A. (1984). Blood oxygen transport in stressed striped bass (*Morone saxatilis*): role of beta-adrenergic responses. *J. comp. Physiol.* **154**B, 365–369.

NIKINMAA, M. & HUESTIS, W. H. (1984). Adrenergic swelling of nucleated erythrocytes: cellular mechanisms in a bird, domestic goose, and two teleosts, striped bass and rainbow trout. *J. exp. Biol.* **113**, 215–224.

NILSSON, S. & GROVE, D. J. (1974). Adrenergic and cholinergic innervation of the spleen of the cod *Gadus morhua*. *Eur. J. Pharmac.* **28**, 135–143.

PALFREY, C. H. & GREENGARD, P. (1981). Hormone-sensitive ion transport systems in erythrocytes as models for epithelial ion pathways. *Ann. N.Y. Acad. Sci.* **372**, 291–308.

PRIMMETT, D. R. N., RANDALL, D. J., MAZEAUD, M. & BOUTILIER, R. G. (1986). The role of catecholamines in erythrocyte pH regulation and oxygen transport in rainbow trout (*Salmo gairdneri*) during exercise. *J. exp. Biol.* **122**, 139–148.

RUDOLPH, S. A. & GREENGARD, P. (1980). Effects of catecholamines and prostaglandin E_2 on cyclic AMP, cation fluxes and protein phosphorylation in the frog erythrocyte. *J. biol. Chem.* **255**, 8534–8540.

SIGGAARD-ANDERSEN, O. (1961). Factors affecting the liquid-junction potential in electrometric blood pH measurement. *Scand. J. clin. lab. Invest.* **13**, 205–211.

WOODWARD, J. J. (1982). Plasma catecholamines in resting trout, *Salmo gairdneri* Richardson, by high pressure liquid chromatography. *J. Fish Biol.* **21**, 429–432.

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