

POTENCY OF ADRENALINE AND NORADRENALINE FOR β -ADRENERGIC PROTON EXTRUSION FROM RED CELLS OF RAINBOW TROUT, *SALMO GAIARDNERI*

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SUMMARY

The red cell adrenoceptor affinity for the unspecific agonists adrenaline and noradrenaline and the specific β -agonist isoprenaline was studied *in vitro* on whole blood of rainbow trout, *Salmo gairdneri* at 15°C. The erythrocytic adrenoceptors could be pharmacologically characterized as β -receptors of the 'noradrenaline'-type (β_1 -type), with an order of potency of isoprenaline > noradrenaline \gg adrenaline. The adrenoceptor affinities, expressed as agonist concentrations for 50% response (EC_{50}), were 1.3×10^{-8} and 7.6×10^{-7} mol l⁻¹ for noradrenaline and adrenaline, respectively. Winter fish showed a red cell adrenergic response identical to that of summer-acclimated fish. It is concluded that most red cell β -adrenergic responses *in vivo* are exclusively elicited by noradrenaline.

INTRODUCTION

Erythrocytes of rainbow trout, *Salmo gairdneri*, respond to adrenergic stimulation by swelling and cytoplasmic alkalinization (Nikinmaa, 1982, 1983). The increase in intracellular pH has been shown *in vitro* to be due to an adrenergic extrusion of protons against the electrochemical gradient by a stimulated Na⁺/H⁺ counterport (Baroin, Garcia-Romeu, Lamarre & Motais, 1984; Nikinmaa & Huestis, 1984; Cossins & Richardson, 1985; Borgese, Garcia-Romeu & Motais, 1986).

Several studies have pointed to a role of catecholamines in the regulation of red cell pH during stressful conditions such as severe exercise or environmental hypoxia in fish. Burst swimming results in significantly increased plasma adrenaline and noradrenaline concentrations in spotted dogfish, *Scyliorhinus canicula*, and rainbow trout (Butler, Metcalfe & Ginley, 1986; Primmatt, Randall, Mazeaud & Boutilier, 1986). Experiments with the β -receptors blocked by a propranolol injection have demonstrated a functional role for catecholamines by safeguarding the red cell pH and the blood O₂ content, in spite of a lactacidotic condition (Nikinmaa, Cech &

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McEnroe, 1984; Primmitt *et al.* 1986). Acute exposure of rainbow trout to hypoxic water has also been shown to elicit a β -adrenergic Na^+/H^+ exchange of the red cells (Fievet, Motais & Thomas, 1987; Tetens & Christensen, 1987) resulting in increased blood O_2 affinity and O_2 loading in the gills (Tetens & Christensen, 1987).

Few data exist on the red cell receptor affinity for catecholamines. Available data indicate a concentration for half-maximum stimulation of 10^{-7} – 10^{-6} mol l^{-1} , when determined as K^+ -influx (Bourne & Cossins, 1982), red cell volume increase (Nikinmaa, 1982) or rate of acidification of the incubation medium (Cossins & Richardson, 1985). These concentrations are only reached *in vivo* following repeated burst swimming (Butler *et al.* 1986) and are 1–3 orders of magnitude higher than those at less stressful, but physiologically more interesting, conditions, where a specific adrenergic effect on the red cells has been documented (Primmitt *et al.* 1986; Fievet *et al.* 1987; Tetens & Christensen, 1987).

The present determination of concentration–response curves for adrenaline and noradrenaline of rainbow trout whole blood was undertaken to evaluate and characterize further the physiological role of adrenergic stimulation of red cells.

MATERIALS AND METHODS

Animal maintenance and surgery

Rainbow trout, *Salmo gairdneri* Richardson, weighing 1.0–1.4 kg, were obtained from a commercial trout farm and acclimated to $15 \pm 1^\circ\text{C}$ and a 12 h:12 h light:dark photoperiod for 6 months. Winter trout were obtained in early January and kept for 2 weeks at the prevailing winter conditions of 2°C and 8 h:16 h photoperiod. The fish were kept in aerated water in large tanks with a flow-through of tap water. Cannulation of the dorsal aorta was performed under benzocaine anaesthesia as described by Tetens & Christensen (1987). The fish were subsequently enclosed in individual opaque perforated restrainers. A recovery period of at least 2 days was allowed, during which care was taken to minimize mechanical and visual disturbance.

Experimental protocol

Blood samples were slowly taken *via* the catheter into heparinized syringes. All tonometry of blood was performed at $15.0 \pm 0.1^\circ\text{C}$ with water-saturated gas mixtures supplied by Wösthoff gas mixing pumps.

Stability of catecholamines

The blood sample was divided into 3.0-ml subsamples in Esweiler glass tonometers and equilibrated for 45 min with 0.2% CO_2 , 2.1 or 14.7% O_2 , balance N_2 . Adrenaline or noradrenaline was added and 500- μl blood samples were removed at specified intervals. Plasma was separated by centrifugation and frozen at -70°C for later determination of catecholamines by a radioenzymatic assay (Christensen, Vestergaard, Sørensen & Rafaelsen, 1980).

Concentration-response curves

The blood was kept equilibrated with 0.2% CO₂, 2.1% O₂ remainder N₂ in an Instrumentation Laboratory 237 rotating tonometer. From this blood stock, 490- μ l samples were transferred to Esweiler glass tonometers supplied with the same gas mixture as the IL tonometer.

Fifteen minutes later, 10 μ l of saline (control) or catecholamine solution was added. After a further 3 min the blood was quickly removed and immediately centrifuged for 30 s at 10 000 rev. min⁻¹ in an Eppendorf tube to separate the plasma. Plasma pH, pHe, was measured directly on the supernatant using a Radiometer G299 electrode on a BMS2 unit, thermostatted at 15°C. The change in plasma pH, Δ pHe, elicited by addition of hormone was determined as the difference in pHe between the unstimulated sample (control) and the catecholamine-stimulated one.

In a separate experiment, the changes in pHe and total plasma CO₂ concentration over time were determined. Plasma pH was measured as described above, whereas the total plasma CO₂ content was determined by the method used by Cameron (1971).

To test the efficiency of the β -blockade, propranolol hydrochloride was added to give a final concentration of 2×10^{-4} mol l⁻¹. Noradrenaline (10^{-7} mol l⁻¹) was added 5 min later, and the pHe response measured as described above.

All the drugs used were supplied by the central laboratory of Aarhus Hospital. The pure solutions of L-adrenaline and L-noradrenaline were checked by radioenzymatic assay and found to conform to the specified concentrations. Dilutions were made immediately prior to use with N₂-equilibrated saline.

Data handling

Each concentration-response curve obtained from a given specimen was log transformed according to the method of Ariëns *et al.* (1964), and the following linear relationship was derived:

$$\log \frac{\% \Delta \text{pHe}}{100 - \% \Delta \text{pHe}} = n \log C + k .$$

The mean concentration-response curve for a given drug was then obtained from such separately determined regression lines by calculating the mean concentration necessary to elicit a certain response. The potency of the drug was expressed as the concentration, EC₅₀, necessary to give 50% of the maximal effect.

All values are expressed as mean \pm S.D., except in Table 1, where data taken from the literature are expressed as mean \pm S.E.M. Differences were statistically evaluated by Student's *t*-test for two means (two-tailed test).

RESULTS

Stability of catecholamines

The decrease in concentration of plasma catecholamine during the incubation of whole blood was unaffected by the levels of oxygenation. Incubation at 2.1 and

14.7% O₂ gave identical results. The data could be described as a first-order reaction (an exponential decay). The half-time, $t_{1/2}$, of the reaction was similar for both hormones, about 24 min (Fig. 1). The same $t_{1/2}$ value was measured on blood taken straight from the fish (i.e. at a starting concentration of about 10^{-9} mol l⁻¹) and stored in the syringe at 15°C.

Change of pHe and total CO₂ over time

The change in pHe following addition of hormone peaked at 2–3 min (Fig. 2). The absolute change in pHe differed from specimen to specimen, but the timing of the change in pHe was identical for all blood samples.

Total CO₂ concentration of the plasma, C_{tCO_2} , decreased following the addition of catecholamine (Fig. 3). No change in C_{tCO_2} could, however, be detected during the first 3 min, indicating a delayed washout of CO₂.

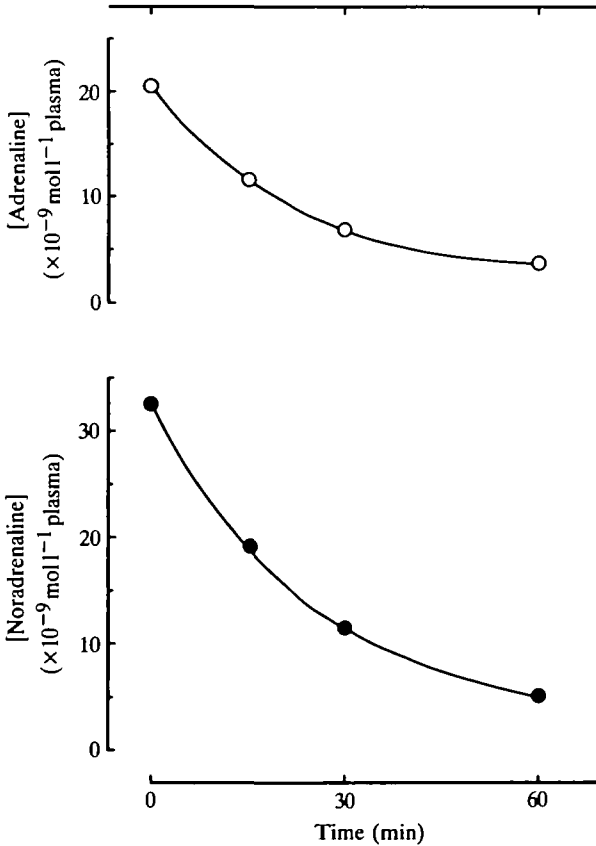


Fig. 1. Course of degradation over time of added adrenaline (○) and noradrenaline (●) in whole blood equilibrated with 0.2% CO₂, 2.1% O₂, remainder N₂. The half-time, $t_{1/2}$, for the degradation was 22 min for adrenaline and 25 min for noradrenaline. Average values of two determinations.

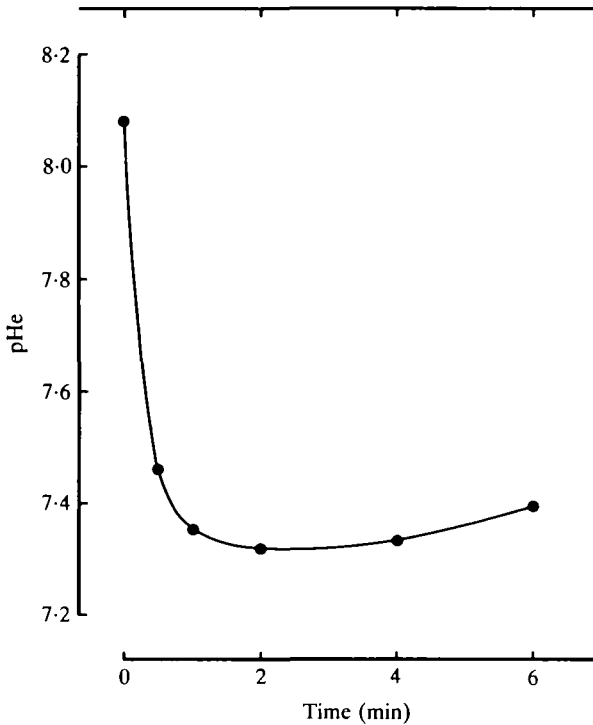


Fig. 2. An example of the change over time in plasma pH, pHe, upon the addition of $10^{-5} \text{ mol l}^{-1}$ noradrenaline. Equilibration conditions as described for Fig. 1.

Concentration-response curves

The raw data for summer-acclimated trout are presented in Fig. 4. The maximal effect (at $C > 10^{-5} \text{ mol l}^{-1}$) evoked by adrenaline was slightly lower than for noradrenaline, indicating a somewhat lower intrinsic activity of adrenaline relative to noradrenaline. Saturating concentrations of isoprenaline ($C > 10^{-5} \text{ mol l}^{-1}$) caused the same effect as that elicited by noradrenaline (not shown). Incubation of blood with $10^{-4} \text{ mol l}^{-1}$ propranolol prior to the addition of $10^{-7} \text{ mol l}^{-1}$ noradrenaline diminished the response to $4.5 \pm 2.1\%$, reflecting a virtually complete blocking of the adrenoceptors.

Fig. 4 shows a clear difference between the two catecholamines in the plasma concentrations necessary to elicit a certain drop in pHe. This difference in affinity was further shown with the log transformation of all data (six specimens) covering responses between 5 and 95%, as shown in Fig. 5. Transformation of the data for each curve (one specimen) resulted in highly linear relationships with correlation coefficients ranging from $r = 0.92$ to $r = 0.99$. The mean individual concentration-response curves for the two hormones, derived from these linear relationships, are depicted in Fig. 6. The adrenoceptor affinity, expressed as the concentration for 50% of the maximal effect, EC_{50} , was $1.30 \pm 0.59 \times 10^{-8} \text{ mol l}^{-1}$ for noradrenaline and $7.58 \pm 7.83 \times 10^{-7} \text{ mol l}^{-1}$ for adrenaline. The difference in EC_{50} values was

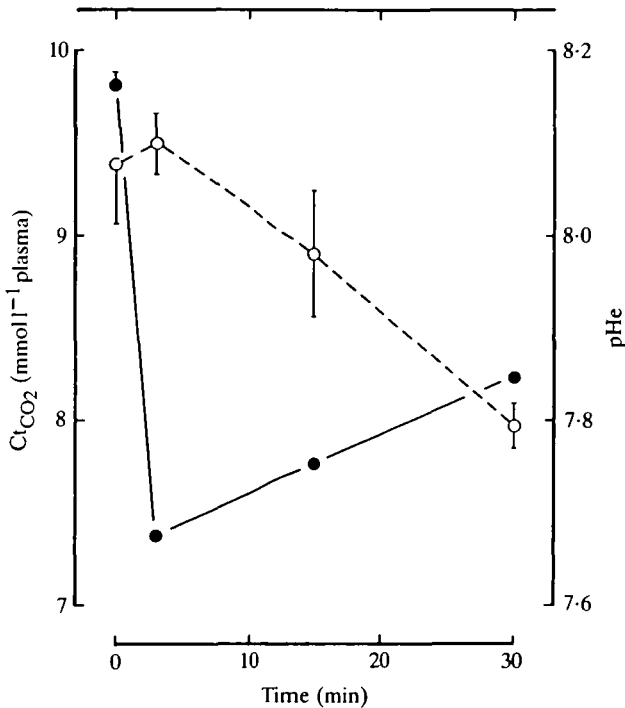


Fig. 3. Changes over time in plasma pH, pHe (●) and total plasma CO₂ concentration, CtCO₂ (○) following the addition of 10⁻⁵ mol l⁻¹ noradrenaline. Equilibration conditions as described for Fig. 1. Mean ± s.d., N = 4.

statistically significant ($P < 0.05$). The EC₅₀ value for isoprenaline was approximately 9×10^{-9} mol l⁻¹, only slightly lower than for noradrenaline (not shown).

The red cell adrenoceptor affinities for adrenaline and noradrenaline in winter trout did not differ significantly ($P > 0.2$) from those of summer-acclimated trout. The EC₅₀ values were $7.93 \pm 1.32 \times 10^{-9}$ mol l⁻¹ for noradrenaline and $3.33 \pm 1.56 \times 10^{-7}$ mol l⁻¹ for adrenaline (Fig. 7).

DISCUSSION

Comments on methods

The resting levels of plasma catecholamines in rainbow trout have been shown to be 0.63 ± 0.09 and $1.23 \pm 0.09 \times 10^{-9}$ mol l⁻¹ for adrenaline and noradrenaline, respectively, under identical fish-holding and blood-sampling conditions (Tetens & Christensen, 1987). The pre-incubation of blood prior to the addition of catecholamine predictably ($t_{1/2} = 24$ min; Fig. 1) diminished these catecholamine concentrations to levels so low that hardly any adrenergic stimulation remained. The measured Δ pHe values thus fully reflected a stimulation by added catecholamine of cells with quiescent Na⁺/H⁺ exchangers (Cossins & Richardson, 1985). The actual concentration of catecholamine in the blood at the measuring time ($t = 3$ min) was,

owing to the $t_{1/2}$ value of 24 min (Fig. 1), very close to that calculated from the amount added.

The adrenergic effect measured in this study (ΔpHe) is directly proportional to the quantity of protons transferred from the red cells to the plasma if the buffer value of the plasma is constant (i.e. independent of pH). Such a condition does not exist in an open system with free exchange of molecular CO_2 between blood and gas. The blood in the tonometer, however, appeared to behave as a closed buffer system during the first 3 min (Fig. 3), possibly because of diffusion limitations. During this period, about 0.14 mequiv of protons were buffered per litre of plasma by bicarbonate, if calculated using the pK' and αCO_2 values determined for rainbow trout plasma by Boutilier, Iwama, Heming & Randall (1985), resulting in an estimated increase in P_{CO_2} of 2.9 mmHg ($= -\Delta\text{C}_{\text{HCO}_3^-}/\alpha\text{CO}_2$). The non-bicarbonate buffering amounted to 1.22 mequiv l^{-1} plasma, when based on a non-bicarbonate buffer value for separated plasma of 2.59 mequiv l^{-1} pH^{-1} (Milligan & Wood, 1986). Hence, about 90% of the protons extruded from the red cells during the initial 3 min were buffered by the non-bicarbonate buffers of the plasma, i.e. the plasma proteins. In the present study, it can thus be assumed that the measured ΔpHe is directly proportional to the adrenergic Na^+/H^+ exchange throughout the range of pHe values measured.

The total quantity of protons buffered in the first 3 min thus is about 1.4 mequiv l^{-1} plasma, which in these conditions (haematocrit $\approx 19\%$) equals an extrusion from

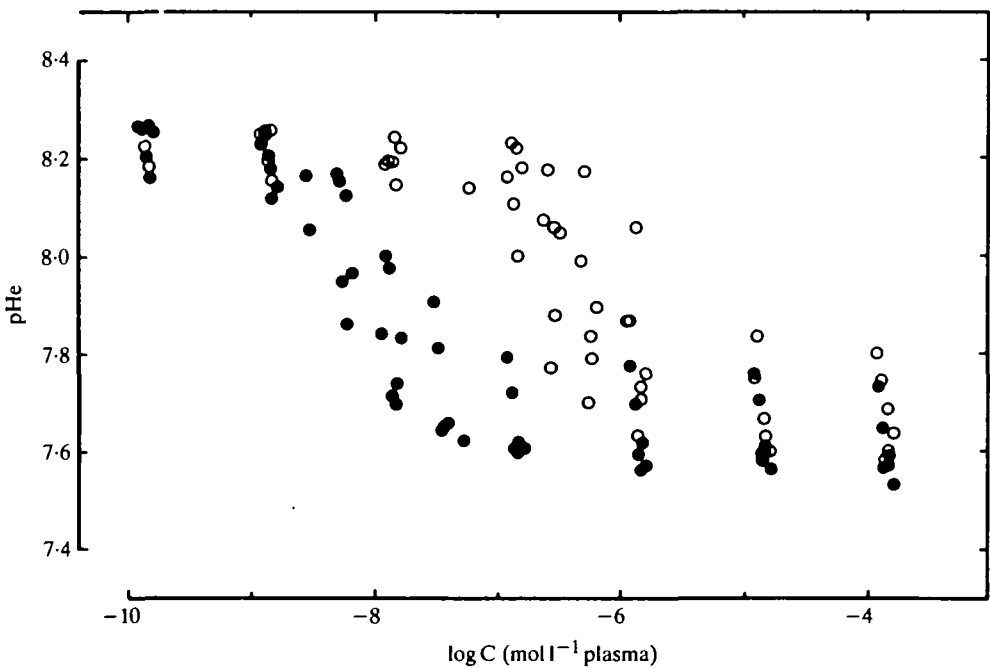


Fig. 4. Measured pHe values of six concentration-response curves for adrenaline (○) and noradrenaline (●). Summer-acclimated trout.

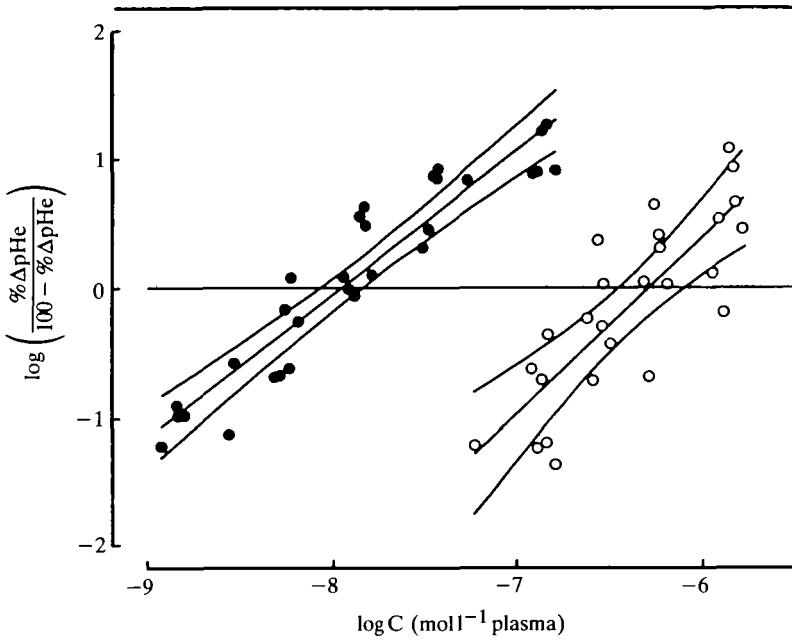


Fig. 5. Log transformation of the raw data of Fig. 4. Only responses between 5 and 95 % are included. Regression lines with 95 % confidence limits.

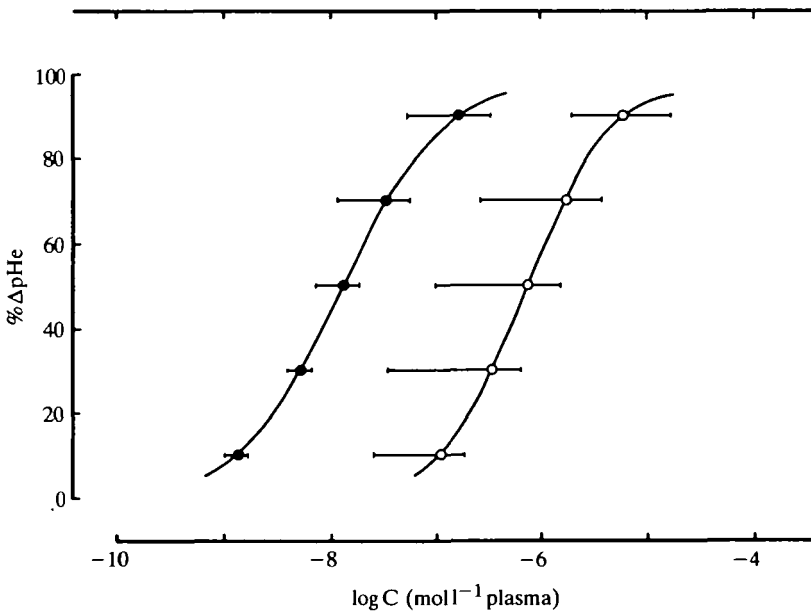


Fig. 6. Concentration-response curves for adrenaline (O) and noradrenaline (●). Summer-acclimated trout. The curves represent mean individual sensitivity (Ariëns, Simonis & Rossum, 1964) of six specimens. Horizontal bars indicate $\pm S.D.$

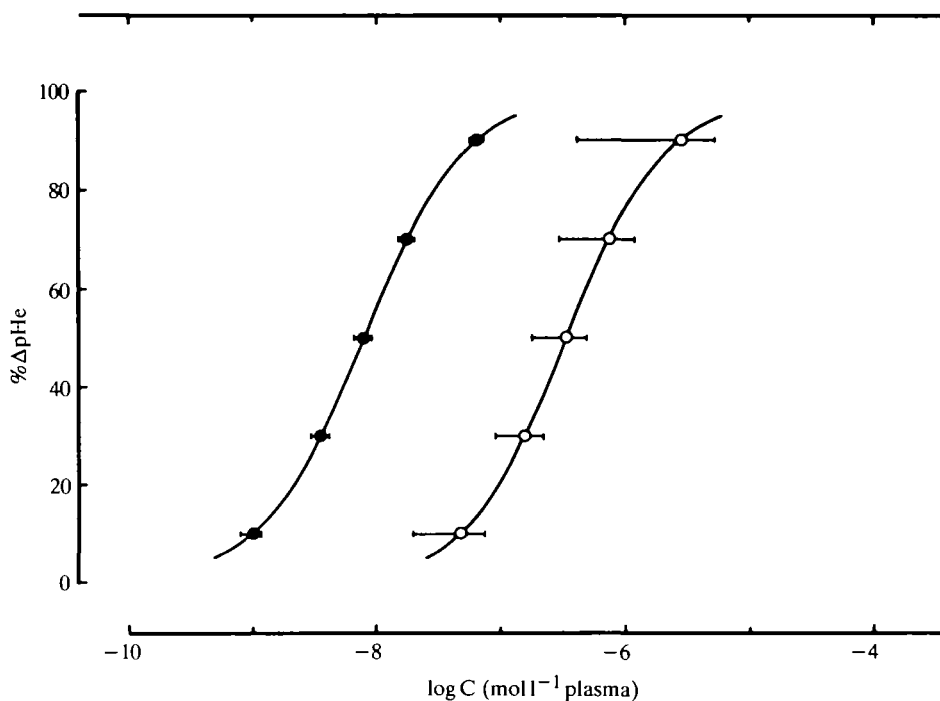


Fig. 7. Concentration-response curves for winter trout ($N = 3$). Details as in Fig. 6.

the red cells of about 6 mequiv l⁻¹ red cells. This is in line with the 6–7 mequiv l⁻¹ red cells of protons extruded in the first 2–3 min of an incubation with saturating concentrations of either adrenaline (Cossins & Richardson, 1985) or isoprenaline (Baroin *et al.* 1984).

Stability of catecholamines in whole blood

The present study demonstrates that the degradation of adrenaline and noradrenaline in the blood is a much slower process than their disappearance from the plasma in intact fish. Nekvasil & Olson (1986) report a 50% removal from the circulation and an impressive 80% enzymatic inactivation of the remaining circulating catecholamines within 10 min for rainbow trout, leaving only 10% of the injected dose in the active form in the circulation. No study has to our knowledge documented the existence of the catabolizing enzymes monoamine oxidase and catechol-*O*-methyltransferase in fish blood. Our data, however, indicate that their catabolic capacity in trout blood (if they exist in this tissue) is much lower than in other fish tissues, particularly the kidney and liver (Mazeaud, 1974).

Adrenoceptor type

The virtually complete blocking by propranolol of any plasma acidification following the addition of a saturating concentration of noradrenaline indicates that the adrenoceptors of rainbow trout red cells consist exclusively of the β -type,

confirming the results of Nikinmaa (1982) and Mahé, Garcia-Romeu & Motais (1985). The adrenergic responses with a potency order of isoprenaline $>$ noradrenaline \gg adrenaline best fit the criterion set for the β_1 -type in mammalian tissues (Lands *et al.* 1967), more correctly described as a 'noradrenaline'-receptor (Stene-Larsen, 1981). The adrenoceptors of rainbow trout are thus of the same type as those found by radioligand binding studies on turkey red cells, in contrast to the β_2 -type of frog red cells (De Lean, Hancock & Lefkowitz, 1982) and human erythrocytes (Sager, 1983).

Potency of adrenaline and noradrenaline

The present study could not document a diminished β -adrenergic response of the red cells of winter-adapted fish, as suggested by Nikinmaa & Jensen (1986). Furthermore, neither intrinsic activity nor affinity was significantly different in winter-adapted trout compared with the summer-acclimated fish, reflecting an unchanged binding characteristic of the red cell adrenoceptors.

The affinity for adrenaline of the rainbow trout red cell β -adrenoceptors was similar to that reported by Bourne & Cossins (1982), Nikinmaa (1982) and Cossins & Richardson (1985), when determined on the basis of various different red cell responses. The affinity for noradrenaline was only slightly higher than the value for adrenaline in the study by Bourne & Cossins (1982), with EC_{50} values of 8×10^{-7} and $2 \times 10^{-6} \text{ mol l}^{-1}$, respectively. We found, however, that the receptor affinity for noradrenaline was significantly higher, with a mean EC_{50} value nearly 60 times lower than for adrenaline (Fig. 6). Although there might be differences between stocks of rainbow trout, the most probable explanation is a use by Bourne & Cossins (1982) of racemic noradrenaline, which has a significantly lower potency than the L-isomer (Bowman & Rand, 1980).

Our results show that nearly all of the β -adrenergic red cell responses observed under various physiological conditions in rainbow trout can be ascribed to an adrenoceptor binding exclusively of noradrenaline. This is illustrated in Table 1, where the red cell adrenergic response is calculated from the mean concentration-response curves of Fig. 6, using plasma catecholamine concentrations cited in the literature. It is clear from Table 1 that a response based on receptor binding of adrenaline can only occur under very stressful conditions. Repeated burst swimming (Butler *et al.* 1986) or the unnatural situation of lifting the fish out of the water for 'grab and stab' percutaneous blood sampling (Tetens & Christensen, 1987) induces a significant release of adrenaline into the circulation, causing a specific adrenaline-related adrenergic response. The extremely high plasma adrenaline concentrations of $6.6 \times 10^{-7} \text{ mol l}^{-1}$ (Tetens & Christensen, 1987) and $1.2 \times 10^{-6} \text{ mol l}^{-1}$ (Butler *et al.* 1986) can, provided competitive receptor binding to noradrenaline is disregarded, elicit a red cell response of 45% and 62%, respectively. It is thus clear that adrenaline can only play a minor role in *in vivo* β -adrenergic stimulation of rainbow trout red cells. Circulating adrenaline might, however, have a physiological

Table 1. Concentrations of plasma adrenaline and noradrenaline and the resulting red cell response of rainbow trout, *Salmo gairdneri*, subjected to various experimental conditions

Condition	Adrenaline		Noradrenaline		Reference
	Concentration ($\times 10^{-9}$ mol l $^{-1}$)	Response (%)*	Concentration ($\times 10^{-9}$ mol l $^{-1}$)	Response (%)*	
Resting (catheter)	8.95 \pm 4.94 (53)	1	1.83 \pm 0.97 (56)	15	Woodward (1982)
	1.4 \pm 0.5 (20)	0	10.2 \pm 2.4 (20)	45	Butler, Metcalfe & Gimley (1986)
	0.91 \pm 0.13 (12)	0	0.74 \pm 0.10 (12)	7	Primmitt, Randall, Mazcaud & Boutlier (1986)
'Resting' (grab and stab)	4.97 \pm 0.92 (4)	1	11.64 \pm 2.41 (4)	48	Fievet, Motans & Thomas (1987)
	0.63 \pm 0.09 (7)	0	1.23 \pm 0.09 (7)	10	Tetens & Christensen (1987)
Swimming (catheter)	27	3	18	57	Nakano & Tomlinson (1967)
	2-655	0-46	2-207	16-92	V. Tetens & N. J. Christensen (unpublished)
Steady swimming (1 length s $^{-1}$) to apparent exhaustion (2 length s $^{-1}$)	0.3 \pm 0.7 (13)	0	2.5 \pm 2.0 (13)	18	Butler <i>et al.</i> (1986)
	14.4 \pm 3.3 (7)	2	22.8 \pm 6.1 (7)	62	Butler <i>et al.</i> (1986)
Repeated burst swimming burst swimming (120% U _{crit})	212 \pm 89 (17)	21	85 \pm 46 (17)	85	Butler <i>et al.</i> (1986)
	37	4	27	66	Primmitt <i>et al.</i> (1986)
Acute hypoxia (catheter)	5 min, P _{wO₂} = 40 mmHg, 15°C	3	13.7 \pm 2.1 (4)	51	Fievet <i>et al.</i> (1987)
	18 min, P _{wO₂} = 40 mmHg, 15°C	1	13.3 \pm 4.6 (4)	51	Fievet <i>et al.</i> (1987)
	5 min, P _{wO₂} = 40 mmHg, 15°C	0	3.5 \pm 0.7 (7)	23	Tetens & Christensen (1987)
	30 min, P _{wO₂} = 40 mmHg, 15°C	0	7.0 \pm 1.7 (7)	36	Tetens & Christensen (1987)
	120 min, P _{wO₂} = 40 mmHg, 15°C	1	14.5 \pm 4.1 (7)	53	Tetens & Christensen (1987)
	5 min, P _{wO₂} = 35 mmHg, 15°C	3	28 (2)	67	V. Tetens & N. J. Christensen (unpublished)
	20 min, P _{wO₂} = 35 mmHg, 15°C	1	21 (2)	61	V. Tetens & N. J. Christensen (unpublished)
60 min, P _{wO₂} = 35 mmHg, 15°C	1	20 (2)	60	V. Tetens & N. J. Christensen (unpublished)	

Mean \pm S.E.M., with number of animals given in brackets.

* Calculated from the log transformed mean concentration-response curve for adrenaline ($EC_{50} = 7.58 \times 10^{-7}$ mol l $^{-1}$; $n = 1.035$) and noradrenaline ($EC_{50} = 1.30 \times 10^{-8}$ mol l $^{-1}$; $n = 0.904$) and expressed as percentage of the maximal response. Only responses calculated from mean catecholamine concentrations are shown. n is a Hill coefficient.

significance in regulating the function of other tissues and organs such as the gill vasculature (Wood, 1974; Butler *et al.* 1986) or in regulating cardiac function (Stene-Larsen, 1981).

The mean concentration–response curve for noradrenaline is, in contrast to the one for adrenaline, so positioned (Fig. 6) that a well-regulated red cell β -adrenergic response is possible, as illustrated by the range of responses elicited by reported *in vivo* concentrations of plasma noradrenaline (Table 1). Our findings thus confirm that the physiological effects of blood O₂ binding reported in various studies (Nikinmaa *et al.* 1984; Primmitt *et al.* 1986; Fievet *et al.* 1987; Tetens & Christensen, 1987) can indeed be explained by a β -adrenergic alkalization of the red cells. All these *in vivo* findings must, however, be ascribed to an adrenoceptor binding exclusively of noradrenaline. The red cell adrenoceptor affinity for noradrenaline is so high that truly stress-free experimental conditions must be established to secure unstimulated red cells *in vivo*. The resting conditions referred to in Table 1 do not necessarily fulfil this requirement.

The very different potencies of adrenaline and noradrenaline for the β -adrenergic red cell response make it tempting to speculate on a differential stimulation of various tissues by a controlled release into the circulation of adrenaline and noradrenaline as proposed by Capra & Satchell (1977). Table 1 shows that adrenaline is mainly released during severe physiological strain (burst swimming, emersion) or during initial exposure to severe hypoxia (Fievet *et al.* 1987; V. Tetens & N. J. Christensen, unpublished results). However, it appears from this study that noradrenaline, rather than adrenaline, is the predominant circulating catecholamine in rainbow trout under natural conditions, in contrast to the conclusions of Mazeaud, Mazeaud & Donaldson (1977). A differential catecholamine release could possibly result in humoral regulation of various tissues, tuned to the kind and degree of physiological strain.

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