J. exp. Biol. 155, 549–566 (1991)
Printed in Great Britain © The Company of Biologists Limited 1991

CONTROL OF CATECHOLAMINE RELEASE *IN VIVO* AND *IN SITU* IN THE ATLANTIC COD (*GADUS MORHUA*) DURING HYPOXIA

BY STEVE F. PERRY*, REGINA FRITSCHE, RICHARD KINKEAD* AND STEFAN NILSSON

Department of Zoophysiology, Zoological Institute, University of Göteborg, Box 250 59, S-400 31 Göteborg, Sweden

Accepted 13 September 1990

Summary

We have characterized the elevation of circulating catecholamines in the intact Atlantic cod (*Gadus morhua*) during graded acute (30 min) hypoxia. The potential mechanisms contributing to the mobilization of catecholamines during hypoxia were then assessed *in vivo* using nerve sectioning and pharmacological techniques and *in situ* using a perfused head kidney preparation.

Pre-branchial plasma adrenaline concentrations were significantly elevated at all levels of aquatic hypoxia utilised [water P_{O_2} (Pw_{O_2})=10 kPa (75 mmHg), 7.3 kPa (55 mmHg) or 5.3 kPa (40 mmHg)], whereas noradrenaline levels did not increase significantly in these particular experiments in which Pw_{O_2} was lowered gradually over a 30 min period. All subsequent experiments were performed using a more rapid induction of hypoxia to reach a final Pw_{O_2} of 5.3 kPa within the first 5–10 min of exposure. Blood withdrawn from pre-branchial (ventral aortic) and post-branchial (dorsal aortic) cannulae after 30 min revealed pronounced reductions in P_{O_2} and O_2 content (C_{O_2}) as well as elevated pH. These data support the notion that blood acidosis is not a prerequisite for catecholamine mobilization during hypoxia. Bilateral sectioning of spinal nerves 1–4 innervating the head kidney prevented the elevation of noradrenaline during rapidly induced hypoxia, but had no effect on the rise in plasma adrenaline concentration. After each experiment, fish were exposed to air for 3 min to induce severe stress. Plasma catecholamine levels were significantly reduced during stress, suggesting that the sectioning of the spinal nerves to the head kidney was indeed effective. These results indicated that mechanisms other than neural stimulation of head kidney chromaffin tissue were contributing to the rise in plasma adrenaline level during hypoxia. Neuronal overflow into the circulation, however, was an unlikely possibility since the increase of adrenaline could not be prevented by treating denervated fish with bretylium (an inhibitor of catecholamine release from adrenergic nerve terminals). These data suggested a local direct stimulatory effect

* Present address: Department of Biology, University of Ottawa, 30 George Glinski, Ottawa, Ontario, Canada K1N 6N5.

Key words: *Gadus morhua*, adrenaline, noradrenaline, hypoxia, chromaffin tissue, head kidney.

of blood hypoxaemia on adrenaline release from chromaffin tissue. This hypothesis was confirmed using a blood-perfused head kidney preparation in which hypoxaemia markedly stimulated adrenaline overflow into the effluent blood. Further experiments using a Ringer-perfused head kidney preparation were designed to test the hypothesis that blood catecholamine levels *in vivo* are, in part, controlled by the concentration of catecholamines in the blood entering the head kidney. The results show conclusively that overflow of a particular catecholamine during cholinergic stimulation of the head kidney is controlled independently by the inflowing concentration of that catecholamine. We suggest that this mechanism of 'auto-inhibition' of catecholamine overflow is a functional negative feedback mechanism involved in the control of plasma catecholamine levels in the cod.

Introduction

Aquatic hypoxia is a potent stimulus for elevating blood catecholamine levels in hypoxia-intolerant teleosts including rainbow trout *Oncorhynchus mykiss* (Fievet *et al.* 1987, 1990; Tetens and Christensen, 1987; Boutilier *et al.* 1988) and Atlantic cod *Gadus morhua* L. (Fritsche and Nilsson, 1990). It is believed that the mobilization of catecholamines during hypoxia is important for optimizing gill gas transfer, blood gas transport and regional blood flow distribution (see review by Perry and Wood, 1989). Less well understood, however, are the precise source(s) and mechanism(s) contributing to the hypoxia-mediated rise in levels of circulating catecholamines. The two most likely sources are (i) the catecholamine-containing chromaffin cells (Grove *et al.* 1972; Abrahamsson and Nilsson, 1976) interspersed in the walls of the posterior cardinal vein within the anterior or head kidney (see Nilsson, 1983) and (ii) peripheral adrenergic nerve terminals. The posterior cardinal veins are innervated by sympathetic pre-ganglionic nerve fibres (Nilsson, 1976). Electrical stimulation of these fibres or application of acetylcholine in an *in situ* Ringer-perfused head kidney preparation causes significant release of both adrenaline and noradrenaline (Nilsson *et al.* 1976; Wahlqvist, 1981), indicating the cholinergic nature of these fibres. Thus, increased sympathetic cholinergic discharge to the chromaffin tissue may be an important mechanism contributing to the rise of plasma catecholamines during hypoxia *in vivo*. It is unlikely, however, that this is the sole mechanism of catecholamine mobilization, because bilateral sectioning of nerves to the head kidney did not entirely abolish the elevation of plasma catecholamines in Atlantic cod during 10 min of air-exposure (Wahlqvist and Nilsson, 1980). These authors suggested that this could reflect overflow from adrenergic neurones. Finally, it is possible that lowered blood O_2 tension or content could directly stimulate the chromaffin cells to release catecholamines without involving a neural pathway. The idea that altered blood chemistry may influence catecholamine release from chromaffin tissue is supported by several studies including: (i) stimulation of catecholamine release from non-innervated cardiovascular chromaffin cells of the sea lamprey by CO_2 (Dashow and Epple, 1985); (ii) catecholamine-induced ('catecholaminotropic') release of

catecholamines from chromaffin tissue of denervated American eels (Hathaway and Epple, 1989); and (iii) potassium-induced release of catecholamines from chromaffin cells of dogfish after ganglionic blockade (Opdyke *et al.* 1983). In the present study, we have first characterized in detail the release pattern of catecholamines in Atlantic cod during hypoxia and then assessed the contribution of three potential mechanisms to the overall response: (i) neural stimulation of chromaffin tissue; (ii) adrenergic neuronal overflow; and (iii) direct stimulation of chromaffin tissue by hypoxaemia. This was accomplished *in vivo* using nerve sectioning and pharmacological techniques and *in situ* using a blood-perfused head kidney preparation.

Materials and methods

Experimental animals

Atlantic cod (*Gadus morhua*) of either sex, weighing between 400 and 1000 g, were obtained from a local supplier. Fish were maintained indoors in large aquaria supplied with aerated recirculated sea water at 10–12 °C. Fish were not fed and were used within 2 weeks of capture. Photoperiod was maintained at 12 h light/12 h dark. Experiments were performed between September and December.

Experimental protocol

In vivo *experiments*

Fish were anaesthetized in a seawater solution of 100 mg l^{-1} ethyl-*m*-aminobenzoate (MS 222). After cessation of breathing movements, the animal was placed onto an operating table which permitted continuous irrigation of the gills with chilled (10 °C), aerated sea water containing 50 mg l^{-1} MS 222. In all fish, a polyethylene cannula (Clay–Adams PE 50) was inserted occlusively into the third afferent branchial artery to permit periodic withdrawal of ventral aortic (pre-branchial) blood or infusion of bretylium (see below). In one series of experiments, a second cannula (PE 50) was inserted occlusively into the efferent branchial artery of the same gill arch for periodic withdrawal of post-branchial blood. All cannulae were pre-filled with heparinized (50 i.u. ml^{-1}) 0.9 % (w/v) NaCl and secured to the skin with sutures after implantation.

After surgery, fish were placed into opaque acrylic (Perspex) holding boxes supplied with recirculated, aerated sea water. Fish were allowed to recover from the effects of surgery for at least 24 h before experiments commenced (Smith *et al.* 1985). Cannulae were flushed daily with 0.2–0.3 ml of heparinized 0.9 % NaCl.

Series 1. Characterization of catecholamine release during acute graded hypoxia. Separate groups of fish (N=8 in each group) were first exposed to normoxia [Pw_{O_2}=20.7 kPa (155 mmHg)] and then either maintained further under normoxia (controls) or exposed to one of three levels of hypoxia [Pw_{O_2}=10 kPa (75 mmHg), 7.3 kPa (55 mmHg) or 5.3 kPa (40 mmHg)] for a 30 min period. The fish were then returned to normoxia for a further 60 min. Pre-branchial blood samples (0.6 ml) were withdrawn from the cannulae immediately prior to hypoxic exposure (termed

Pre), at 5 min intervals during the hypoxic period, and after 60 min of recovery from hypoxia (termed Post). Blood samples were immediately centrifuged (12 000 *g* for 2 min) and the plasma added to microcentrifuge tubes containing 20 μl of 0.2 mol l^{-1} glutathione and 0.2 mol l^{-1} EGTA. The plasma samples were kept frozen at −80°C for no longer than 2 months before analysis of catecholamine levels. The red blood cells were resuspended in 0.9 % NaCl and then reinjected into the circulation.

Hypoxia was induced by recirculating a static volume (30 l) of chilled (10°C) sea water, gassed appropriately with N_2, to the holding boxes. The Pw_{O_2} of the water within the holding boxes was monitored continuously and, if necessary, the rate of N_2 delivery to the system was adjusted accordingly. Owing to the relatively large volumes of the holding boxes and the slow rate of delivery of hypoxic water, the desired final Pw_{O_2} was obtained within 15–20 min after the initiation of hypoxia.

In a separate experimental series, fish fitted with both pre- and post-branchial cannulae were exposed to hypoxia (5.3 kPa) as above. Pre- and post-branchial blood samples (0.3 ml each) were withdrawn at identical times (see above) and analyzed immediately for P_{O_2}, O_2 content (C_{O_2}) and pH.

Series 2. Effect of head kidney denervation on catecholamine release during severe acute hypoxia. After cannulating the afferent branchial artery, the head kidney was denervated by bilateral section of the first four spinal nerves as described previously (Nilsson *et al.* 1976; Wahlqvist and Nilsson, 1980; Butler *et al.* 1989). Briefly, this involves making incisions dorsally on both sides of the midline and severing spinal nerves 1–4 near the cranium or vertebral column. This procedure is known to prevent, or significantly reduce, the elevation of plasma catecholamines associated with severe 'stress' (15 min air-exposure, Nilsson *et al.* 1976; 10 min air-exposure, Wahlqvist and Nilsson, 1980) or exhaustive exercise (Butler *et al.* 1989). In a sham-operated group of fish, the spinal nerves were exposed and identified but not sectioned. Fish were allowed to recover for at least 24 h from surgery.

Fish were exposed to hypoxia (5.3 kPa) using a slightly modified protocol which permitted the desired Pw_{O_2} to be attained within 5–10 min after the onset of hypoxia. Blood samples (0.3 ml) were removed prior to hypoxia, 30 min after the initiation of hypoxia and 60 min after return to normoxic conditions. In this series, red blood cells were not reinjected into the animals. In all cases, a final blood sample was taken after exposing the fish to air for 3 min.

Series 3. Effect of head kidney denervation plus additional bretylium treatment on catecholamine release during severe hypoxia. After 24 h of recovery from surgery, denervated fish were infused slowly (0.03 ml min^{-1}) *via* the afferent branchial artery cannula with 10 mg ml^{-1} bretylium tosylate (Wellcome Foundation Ltd) at a dose of 10 mg kg^{-1} body mass. The fish were allowed to recuperate for an additional 24 h to dissipate the non-specific effects of bretylium (Smith *et al.* 1985). This protocol has been shown to prevent the release of catecholamines from peripheral adrenergic nerve terminals in this species (Smith *et al.* 1985). In this experiment, therefore, neural stimulation of the head kidney and overflow from

adrenergic nerve terminals can be eliminated as variables contributing to the rise of plasma catecholamines during hypoxia.

In situ *experiments*

Fish were injected *via* the caudal vessels with 1500 i.u. of heparin (0.3 ml) and then killed by a sharp blow to the head. An incision was made on the left side to expose the anterior portion of the swimbladder and the head kidney with surrounding vessels (see Fig. 1). The left posterior cardinal vein and left duct of Cuvier were cannulated for the inflow and outflow of perfusion fluid, respectively (Nilsson *et al.* 1976). The left anterior cardinal vein and gonadal vein were ligated to prevent leakage. In addition, a ligature was secured around the entire vertebral

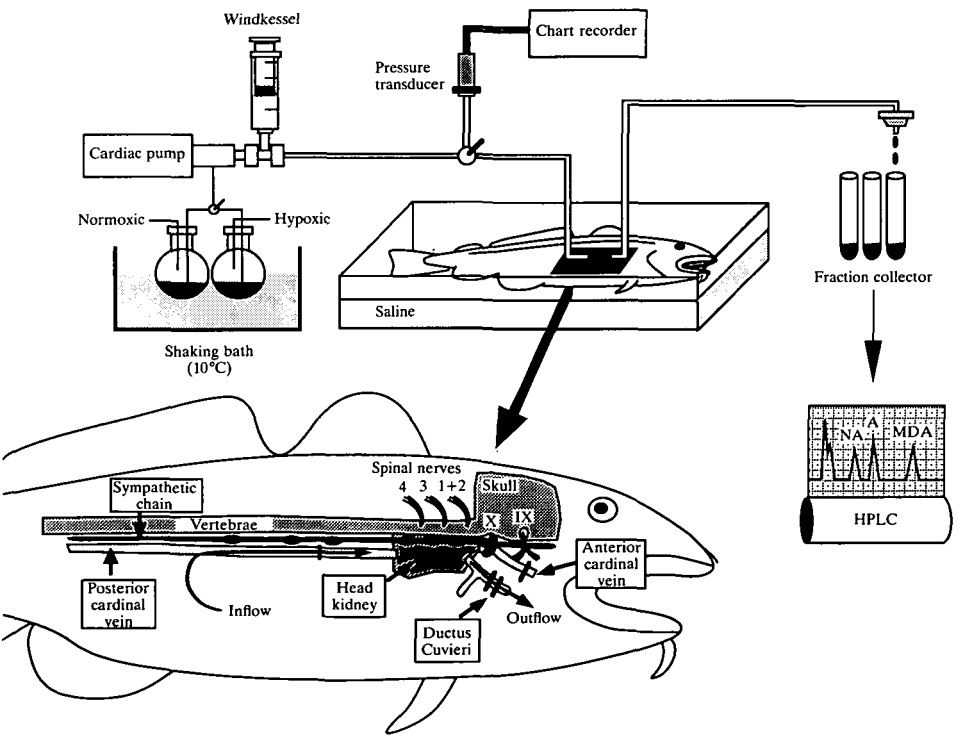

Fig. 1. A schematic diagram of the *in situ* blood/Ringer-perfused cod head kidney preparation. The anatomical arrangement of the head kidney, inflowing and outflowing blood vessels, and the placement of cannulae, are illustrated in magnified form in the lower left portion of the diagram. The preparation was perfused with either normoxic or hypoxic perfusion fluid using a cardiac pump in conjunction with a pressure-pulse dampening Windkessel. Perfusion pressure was monitored continuously and displayed on a chart recorder. Effluent from the head kidney was collected using a fraction collector and subsequently assessed for catecholamine [NA, noradrenaline; AD, adrenaline; MDA, methyldopamine (internal HPLC standard)] content using HPLC.

column at the level of the inflow cannula to prevent backflow of perfusion fluid in the posterior cardinal vein. The fish was immersed in 0.9 % NaCl (10°C) within an organ bath and the *in situ* head kidney was perfused with either seawater-adapted trout blood or modified (pH=7.8–7.9 using 0.3 % CO_2) cod Ringer (Holmgren and Nilsson, 1974) at a constant flow of 1.0 ml min^{-1} using a customized cardiac perfusion pump (pump frequency=35 strokes min^{-1}). The pressure pulse was dampened by installing a Windkessel on the perfusion pump outflow (Fig. 1). Perfusion pressure was continuously monitored by connecting the inflow catheter to a pressure transducer (Statham P23) in conjunction with a chart recorder (Grass model 70).

Effluent perfusion fluid was collected in microcentrifuge tubes containing 20 μl of glutathione/EGTA at 1 min intervals using a fraction collector. These samples were then stored at −80°C prior to analysis of catecholamine content.

Series 1. In situ *blood-perfused head kidney.* Donor blood was obtained from seawater-adapted rainbow trout (*Oncorhynchus mykiss*; 3–5 kg) by slow withdrawal from a dorsal aortic cannula that had been implanted 24 h previously according to the method of Soivio *et al.* (1975). Blood sampling was terminated when the fish showed signs of struggling or became agitated. Using this protocol, it was possible to obtain 75–125 ml of blood from a single fish, depending on its mass. The blood was washed by centrifugation (500 *g* for 10 min), removal of the supernatant and resuspension in cod Ringer. This procedure was repeated twice. Finally, the blood cells were resuspended in cod Ringer to a final haematocrit of 15–20 %. This pool of blood was divided into equal samples in round-bottomed tonometry flasks and immersed in a constant temperature bath (10°C). One flask was gassed with a normoxic gas mixture (0.3 % CO_2 in air) while the other was gassed with a hypoxic gas mixture (0.3 % CO_2, 2.0 % O_2, remainder N_2).

Once perfusion pressure had stabilized (usually 2–3 min), the head kidney was perfused with normoxic blood for 5 min (mean P_{O_2}=18 kPa; mean C_{O_2}=4.1 vol%; mean pH=8.07). In the control group, the head kidney was perfused for a further 5 min with normoxic blood while, in the experimental group, the perfusion fluid was switched to hypoxic blood (mean P_{O_2}=3.1 kPa; mean C_{O_2}=1.7 vol%; mean pH=8.10). In both the control and experimental groups, a bolus injection of acetylcholine (10^{-6} mol in 0.2 ml) was administered to the preparation immediately after the second 5 min period. At the end of each 5 min period and 2 min after the injection of acetylcholine, a blood sample was withdrawn from a T-connection in the input catheter to the head kidney, and processed for catecholamine analysis. Catecholamine overflow into the blood from the chromaffin tissue of the head kidney was determined from the difference in catecholamine concentrations between the inflowing and outflowing blood at each of these sample times. Since this dose of acetylcholine has been shown to stimulate markedly catecholamine overflow in a previous study (Nilsson *et al.* 1976), any preparations that did not display an increase in catecholamine overflow were not considered to be viable and were therefore rejected (less than 5 % of all preparations).

Series 2. In situ *Ringer-perfused head kidney preparation.* Preparations were

perfused with cod Ringer gassed with 0.3 % CO_2 in air. In all experiments, the head kidney was perfused for a 6 min control period followed by a further 6 min after a bolus injection of 10^{-6} mol acetylcholine to the inflowing Ringer. Four separate experimental series were performed in which the concentrations of catecholamines in the inflowing Ringer were varied. In the first, both adrenaline and noradrenaline were nominally zero; in the second, only the adrenaline concentration was elevated ([adrenaline]=235.4±30.7 nmol l^{-1}; N=8); in the third, only noradrenaline was elevated ([noradrenaline]=192.7±28.2 nmol l^{-1}; N=8); in the fourth, both adrenaline and noradrenaline were elevated ([adrenaline]=164.7±32.1 nmol l^{-1}, [noradrenaline]=178.0±38.1 nmol l^{-1}; N=8). The overflow of each catecholamine was calculated as above.

Analytical procedures

Blood/water/Ringer P_{O_2} and pH were measured using Radiometer P_{O_2} and micro-capillary pH electrodes in conjunction with Radiometer PHM-73 acid–base analyzers and BMS3 Mk2 blood micro-systems. All electrodes were maintained at ambient water temperature. Blood C_{O_2} was measured on 20 μl samples according to the method of Tucker (1967) using a Radiometer P_{O_2} electrode in a sealed chamber maintained at 37 °C.

Plasma noradrenaline and adrenaline levels were determined on alumina-extracted plasma or Ringer samples using high performance liquid chromatography (HPLC) with electrochemical detection according to the basic method of Woodward (1982) as modified and described in detail by Fritsche and Nilsson (1990).

Statistical analysis

All data are presented as means±1 standard error of the mean (S.E.M.). The results have been statistically analyzed using a factorial analysis of variance followed by Fisher's LSD multiple comparison test; 5 % was taken as the fiducial limit of significance.

Results

In vivo *experiments*

During normoxia, noradrenaline was the predominant catecholamine in the plasma, although not all comparisons were statistically significant (Fig. 2). Exposure of fish to gradual hypoxia, in which the desired Pw_{O_2} was reached within 15–20 min, caused significant elevation of plasma adrenaline levels. Since plasma noradrenaline levels did not vary, there was frequently a change in the noradrenaline/adrenaline ratio such that adrenaline became the predominant plasma catecholamine during hypoxia (Fig. 2). The pattern of catecholamine release into the plasma did not coincide with the severity of the imposed hypoxia. Indeed, the consistently highest values of plasma adrenaline were measured after 30 min of exposure to the mildest degree of hypoxia utilized (10 kPa; Fig. 2B). All fish

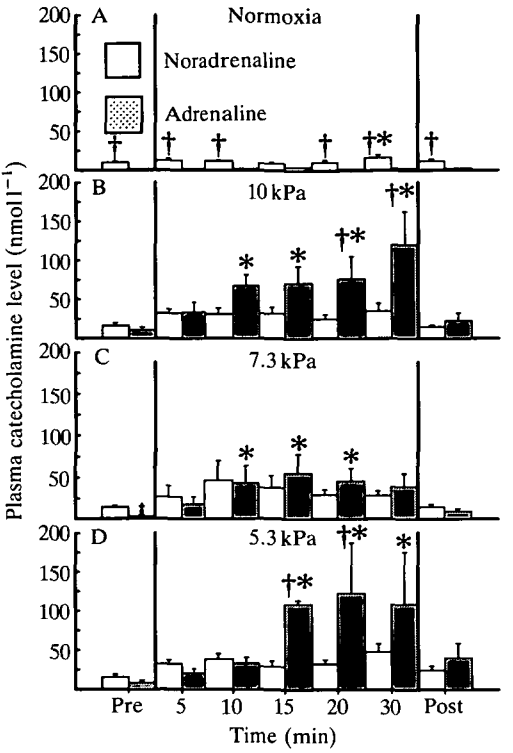

Fig. 2. Pre-branchial plasma catecholamine levels in Atlantic cod before (Pre), during and after (Post) a 30 min period of (A) normoxia (controls; N=8) or three levels of acute hypoxia yielding water P_{O_2} values of (B) 10 kPa (75 mmHg; N=8), (C) 7.3 kPa (55 mmHg; N=8) and (D) 5.3 kPa (40 mmHg; N=8). Noradrenaline values are represented by the open boxes and adrenaline by the shaded boxes. * indicates a statistically significant difference compared to the corresponding Pre value; † indicates a significant difference between the levels of adrenaline and noradrenaline at corresponding sample times.

survived the hypoxia and, after 60 min of recovery under normoxic conditions, plasma adrenaline concentration had returned to pre-hypoxic levels. Blood sampling itself was without marked effect because plasma catecholamine levels in the normoxic control group remained low and virtually constant (Fig. 2A).

The respiratory status of pre- and post-branchial blood samples was evaluated in a separate experimental series on fish exposed to the most severe level of hypoxia (Pw_{O_2}=5.3 kPa). There was a pronounced hypoxaemia in both blood compartments (Fig. 3A,B) and a gradual rise in blood pH that could be attributed to a pronounced hyperventilation (Kinkead *et al.* 1990) and resultant lowering of CO_2 partial pressure.

All subsequent experiments were performed using a Pw_{O_2} of 5.3 kPa in which the target Pw_{O_2} was reached within 5–10 min; a single blood sample was withdrawn after 30 min of hypoxia instead of the multiple samples as taken in

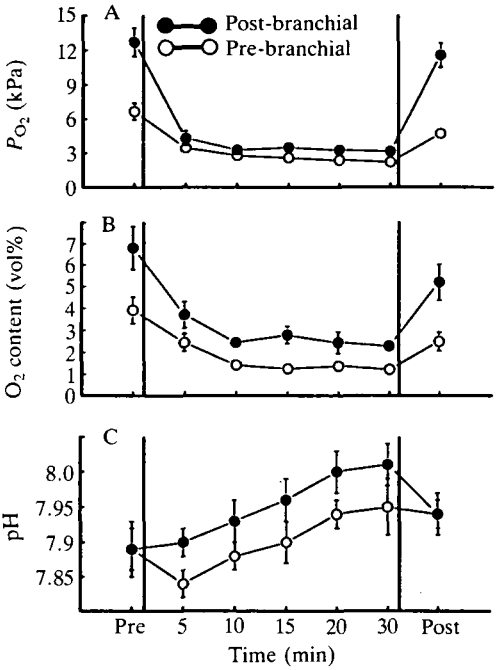

Fig. 3. The effects of severe acute hypoxia (Pw_{O_2}=5.3 kPa) on (A) oxygen partial pressure (P_{O_2}), (B) oxygen content and (C) pH in pre-branchial (○) and post-branchial (●) blood samples withdrawn from indwelling cannulae. All other details as in Fig. 1.

previous experiments. Under these conditions, sham-operated fish displayed similar increases in the levels of both catecholamines (Fig. 4). Denervation of the head kidney entirely prevented the hypoxia-induced rise in plasma noradrenaline concentration, but was without effect on plasma adrenaline levels (Fig. 4). In contrast, denervation was successful in preventing the large rise in the levels of both plasma catecholamines during 3 min of air-exposure. Treating denervated fish with bretylium did not further alter the pattern of catecholamine release observed in denervated fish except that this group displayed the lowest plasma noradrenaline levels after air-exposure, indicating a neuronal component to the rise in plasma noradrenaline at this time.

In situ *experiments*

Blood-perfused head kidney preparation

The washing and resuspension of trout blood in cod Ringer eliminated endogenous adrenaline, but noradrenaline levels were greatly elevated (60–100 nmol l^{-1}) for unknown reasons. This procedure was repeated several times with identical results. Thus, the blood-perfused head kidney preparation was used solely to assess the direct effects of hypoxaemia on adrenaline overflow since it became apparent that the elevated level of inflowing noradrenaline was

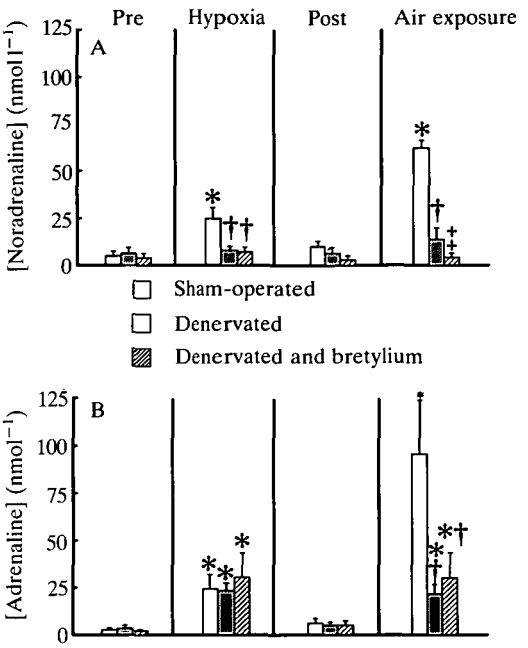

Fig. 4. The effects of severe hypoxia (Pw_{O_2}=5.3 kPa) on plasma levels of (A) noradrenaline and (B) adrenaline in sham-operated (open boxes; N=7), head-kidney denervated (stippled boxes; N=7) denervated plus bretylium-treated (cross-hatched boxes; N=6) Atlantic cod. In each group, fish were severely stressed by exposure to air for 3 min immediately after the post-hypoxic blood sample was removed. * indicates a statistically significant difference compared to the corresponding pre-hypoxic value; † indicates a statistically significant difference compared to the sham-operated group; ‡ indicates a statistically significant difference compared to the denervated group.

impairing the ability of the chromaffin tissue to release this catecholamine into the effluent blood in response to cholinergic stimulation (see below).

Perfusing the head kidney under normoxic conditions caused a slight, but significant, overflow of adrenaline into the blood (Fig. 5). Acutely switching the perfusion fluid to hypoxic blood, to simulate a reduction of O_2 content by approximately 50% (see Fig. 3), caused a pronounced increase in adrenaline overflow after 5 min of perfusion, while overflow in the normoxic control group remained constant. The bolus addition of 10^{-6} mol acetylcholine caused large increases in adrenaline overflow that were similar in the normoxic and hypoxic preparations. Neither hypoxia nor acetylcholine affected the perfusion pressure, which usually remained constant throughout the duration of the experiment. In a few experiments, perfusion pressure increased gradually throughout the experiment, but this was unrelated to the status of the blood.

Ringer-perfused head kidney preparation

Preparations perfused with Ringer containing nominally zero catecholamine

levels displayed equivalent basal overflow of adrenaline and noradrenaline at a rate of 15–20 pmol min^{-1} (Fig. 6). The bolus addition of 10^{-6} mol acetylcholine caused an immediate, but transient, stimulation of catecholamine overflow into

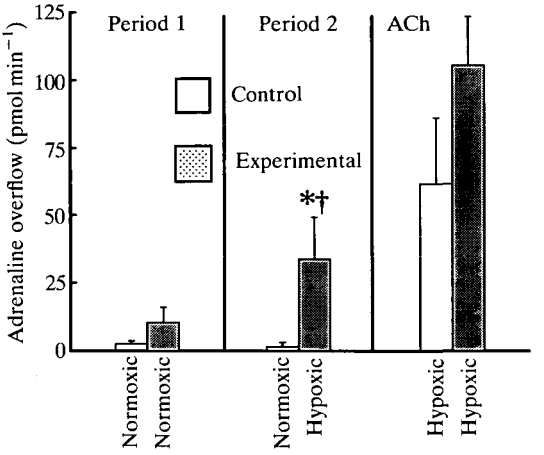

Fig. 5. The effects of hypoxaemia on adrenaline overflow in the *in situ* blood-perfused head kidney preparation. Control preparations (open boxes; $N=8$) were perfused with normoxic blood for two successive 5 min periods, whereas the experimental group was perfused with normoxic blood during period 1 and then hypoxic blood during period 2. The effects of acetylcholine (ACh) were then immediately assessed in each group without altering the oxygen status of the blood. * indicates a statistically significant difference compared to the value during period 1; † indicates a statistically significant difference compared to the corresponding control value. See text for further details.

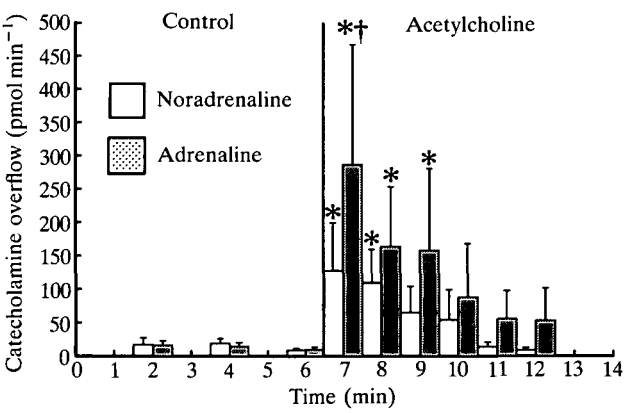

Fig. 6. Catecholamine overflow in the *in situ* Ringer-perfused head kidney preparation during a control period and immediately after bolus administration of 10^{-6} mol acetylcholine. Noradrenaline overflow is represented by the open boxes and adrenaline overflow is represented by the shaded boxes. * indicates a statistically significant difference compared to the corresponding catecholamine overflow value immediately before addition of acetylcholine (6 min); † indicates a statistically significant difference compared to the overflow value of other catecholamines.

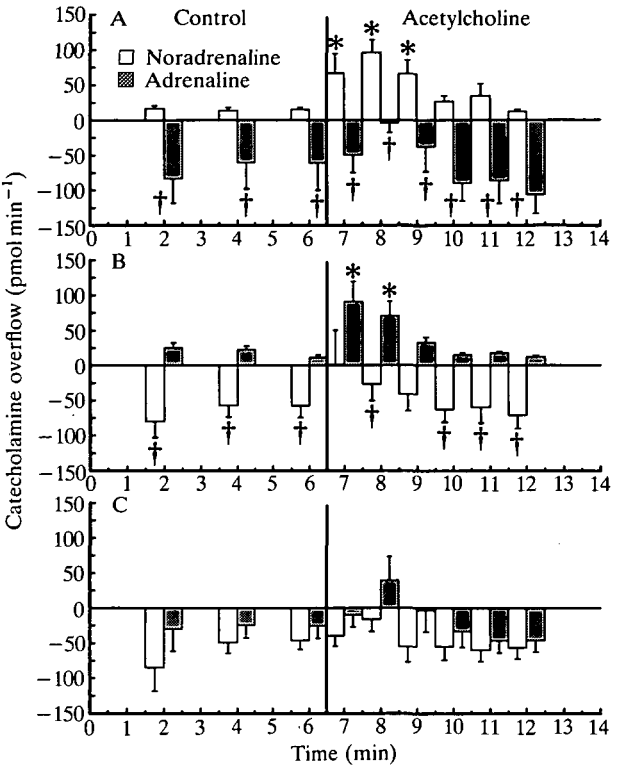

Fig. 7. Catecholamine overflow in the *in situ* Ringer-perfused head kidney preparation during a control period and immediately after bolus addition of 10^{-6} mol acetylcholine under conditions of (A) high inflowing adrenaline levels, (B) high inflowing noradrenaline levels and (C) high inflowing levels of both adrenaline and noradrenaline. * indicates a statistically significant difference compared to the corresponding catecholamine overflow value immediately before addition of acetylcholine (6 min); † indicates a statistically significant difference compared to the overflow value of other catecholamines.

the effluent (Fig. 6). The stimulation was greatest in the first minute after addition and then gradually dissipated over the remaining 5 min period; noradrenaline overflow returned to control levels more rapidly than adrenaline overflow. The application of acetylcholine stimulated adrenaline overflow to a greater extent than it did noradrenaline overflow, although this difference was statistically significant only transiently (Fig. 6) owing to the large variability of the data.

Fig. 7 illustrates the effects of adding high levels of adrenaline and/or noradrenaline to the inflowing perfusion fluid. The high concentrations of adrenaline used (178–234 nmol l^{-1}) were selected to simulate the peak levels of plasma [adrenaline] after 30 min of exposure to severe hypoxia ($P_{W_{O_2}}$=5.3 kPa; Fig. 2). A similar high concentration of noradrenaline was used to allow effective comparison. High levels of inflowing adrenaline (Fig. 7A) did not affect the basal

or acetylcholine-stimulated overflow of noradrenaline. In contrast, adrenaline overflow was negative even after addition of acetylcholine, indicating that clearance from the inflowing Ringer was exceeding rate of release. Although there was a trend towards increased (more positive) overflow after acetylcholine addition, the changes were not statistically significant (Fig. 7A). Essentially identical results were obtained using high levels of inflowing noradrenaline (Fig. 7B); adrenaline overflow was unaffected but noradrenaline overflow was negative throughout the experiment, although again there was a trend towards increased overflow after acetylcholine addition (Fig. 7B). Overflow of both catecholamines was prevented (aside from a non-significant positive overflow at 8 min) when both adrenaline and noradrenaline levels were experimentally elevated (Fig. 7C).

Discussion

In this study, we have (i) characterized the pattern of plasma catecholamine elevation in cod during acute hypoxia, (ii) evaluated the importance of several mechanisms causing this elevation of catecholamines, and (iii) uncovered a previously undescribed process that may significantly affect the levels of circulating adrenaline and noradrenaline during hypoxia. Each of these topics is discussed in detail below.

Pattern of plasma catecholamine elevation during hypoxia

The results presented here demonstrate two distinctly different patterns of catecholamine release in cod during hypoxia, depending upon how rapidly the final $P_{W_{O_2}}$ is attained. A gradual reduction of $P_{W_{O_2}}$ caused significant elevations of plasma adrenaline levels without affecting noradrenaline levels. These findings are in general agreement with previous studies on another species (*Oncorhynchus mykiss*) that have also shown adrenaline to be the predominant catecholamine during aquatic hypoxia (Fievet *et al.* 1987, 1990; Boutilier *et al.* 1988; S. Thomas, R. Kinkead, P. J. Walsh, C. M. Wood and S. F. Perry, in preparation). In contrast, a more rapid induction of hypoxia in the present study caused similar increases in the plasma levels of both catecholamines. In the only other study that has addressed the pattern of catecholamine release during hypoxia in cod (Fritsche and Nilsson, 1990), it was shown that a very rapid induction of severe hypoxia ($P_{W_{O_2}}$=5.3 kPa reached within 2 min) initially caused an increase only in plasma noradrenaline concentration, whereas the rise in plasma adrenaline concentration was delayed by approximately 6 min. The reasons for these temporal and methodology-dependent differences in the pattern of catecholamine release into the plasma during hypoxia are unknown. On the basis of the catecholamine ratio of the cod head kidney chromaffin tissue (86 % adrenaline, 14 % noradrenaline; Abrahamsson and Nilsson, 1976), one would predict plasma adrenaline levels always greatly to exceed noradrenaline levels during hypoxia if the two catecholamines were stored in the same cells. Thus, we suggest that adrenaline and

noradrenaline may be stored in different chromaffin cell types, as demonstrated in amphibians (Coupland, 1971; Mastrolia *et al.* 1976). Further, these cell types may respond differently to potential catecholamine-releasing stimuli such as increased cholinergic activity and hypoxaemia. In support of this idea, we have shown that the ratio of adrenaline to noradrenaline overflow in response to addition of acetylcholine in the Ringer-perfused head-kidney preparation significantly deviated from the overflow ratio based on the head kidney catecholamine content. In addition, Nilsson *et al.* (1976) demonstrated variable adrenaline/noradrenaline overflow ratios in a perfused cod head kidney preparation depending upon whether the preparation was stimulated electrically or with acetylcholine.

Factors other than the nature of catecholamine release from the chromaffin tissue (or peripheral adrenergic neurones) affect the pattern of elevation of plasma catecholamines during hypoxia. Ultimately, the catecholamine levels achieved in the plasma are the net result of several opposing phenomena, including release from storage sites, neural and extra-neural re-uptake (Busacker and Chavin, 1977; Ungell, 1985*a*,*b*; Nekvasil and Olson, 1986*a*) and metabolic degradation (Nekvasil and Olson, 1986*b*; Colletti and Olson, 1988). In both trout (Nekvasil and Olson, 1986*a*,*b*) and cod (Ungell, 1985*a*,*b*), noradrenaline is the preferred substrate for uptake into most tissues that have been examined (cod cardiac tissue is a notable exception; Ungell, 1985*a*), resulting in a more rapid plasma clearance of this catecholamine. Thus, the lower plasma concentration of noradrenaline often observed during hypoxia can reflect both the relatively low concentration in the chromaffin tissue (the presumed major site of storage and release) and the relatively rapid clearance from the plasma.

Mechanism(s) of plasma catecholamine elevation during hypoxia

In the present study, fish exposed to severe hypoxia (Pw_{O_2}=5.3 kPa) displayed an elevation of blood pH, presumably caused by the pronounced hyperventilation (R. Kinkead, R. Fritsche, S. F. Perry and S. Nilsson, in preparation) and consequent lowering of blood P_{CO_2}. The elevation of plasma catecholamine levels under these conditions reinforces the notion that blood acidosis, *per se*, is not a prerequisite for catecholamine mobilization (Perry and Wood, 1989). The results of several studies (e.g. Boutilier *et al.* 1986; Tang and Boutilier, 1988) have demonstrated an elevation of plasma catecholamine levels during blood acidosis in rainbow trout, yet the obligatory direct relationship between red blood cell pH and oxygen content makes it difficult to differentiate specific effects of the acidosis from the effects of acidosis-induced hypoxaemia. It was recently demonstrated that, during hypercapnic acidosis in rainbow trout, hypoxaemia rather than blood acidosis is the proximate stimulus for catecholamine mobilization (Perry *et al.* 1989). Further experiments are required to extend this conclusion to other physiological disturbances which simultaneously induce acidosis and hypoxaemia.

We have identified two mechanisms of catecholamine elevation during hypoxia in cod and eliminated another. Based on the results of catecholamine measurements during hypoxia after head kidney denervation with or without bretylium

treatment, it is clear that (i) neural innervation of the chromaffin tissue is a requirement for elevation of plasma noradrenaline, but not adrenaline, and (ii) overflow from peripheral adrenergic nerve terminals does not significantly contribute to the elevation of either catecholamine. The fact that adrenaline, but not noradrenaline, release continues after head kidney denervation further supports our contention that these catecholamines are stored in different chromaffin cell types responsive to different release stimuli. The lack of an effect of head kidney denervation on plasma adrenaline levels during hypoxia does not exclude neural stimulation of chromaffin tissue as a mechanism of adrenaline release in intact animals. It does, however, suggest the existence of an alternative mechanism of release, which we believe is mediated by a direct local effect of blood hypoxaemia on the chromaffin cells.

The possible stimulatory nature of hypoxaemia on catecholamine release was tested using a perfused head kidney preparation. Since a lowering of C_{O_2} and/or P_{O_2} could account for the direct effect of hypoxaemia on adrenaline release from chromaffin tissue, it was necessary to use blood, rather than conventional cod Ringer, as a perfusion fluid. In the absence of sufficient quantities of homologous blood, we chose to use seawater-adapted rainbow trout blood that had been washed and re-suspended in cod Ringer. The only apparent ill-effect on the preparation was a gradual increase in perfusion pressure, which is common in most blood-perfused fish organ preparations (Perry and Farrell, 1989). Nonetheless, the preparation responded to cholinergic stimulation (acetylcholine) by releasing catecholamines in a similar fashion to the Ringer-perfused head kidney preparation, indicating that this organ was still functional. The results of experiments in which the head kidney was exposed to an acute change in blood oxygen status, such as might occur in venous blood *in vivo*, confirm that hypoxaemia alone can stimulate the chromaffin tissue to release adrenaline and thus provides a mechanism to explain the elevation of plasma [adrenaline] in head-kidney-denervated hypoxic cod. Unfortunately, we were unable to confirm that noradrenaline release is unresponsive to hypoxaemia (as indicated *in vivo*) because the inflowing blood contained 60–100 $nmol\,l^{-1}$ noradrenaline, despite the double washing and re-suspension protocol. We can offer no explanation for this persistent elevation of noradrenaline in the blood except uptake by erythrocytes and subsequent release into the plasma.

Other studies have also reported elevated levels (although significantly reduced compared to controls) of adrenaline in head-kidney-denervated cod during air-exposure (Wahlqvist and Nilsson, 1980) or exhaustive aerobic exercise (Butler *et al.* 1989). This may also reflect local direct effects of hypoxaemia or, as suggested by the authors, release from adrenergic nerve terminals. In agreement with the results of Wahlqvist and Nilsson (1980), only plasma [adrenaline] increased significantly during air-exposure in denervated fish. Since this increase was not affected by bretylium treatment in the present study, we suggest that local hypoxaemia was the most likely cause. These data also are consistent with the idea of differential storage and release of the two catecholamines.

Control of catecholamine release from chromaffin tissue

In preliminary experiments we observed that noradrenaline overflow from the head kidney preparation perfused with blood containing high levels of noradrenaline was unresponsive to acetylcholine. This led us to the idea that release of either catecholamine into the blood could be controlled independently as a function of the inflowing levels of that particular catecholamine. This phenomenon of negative feedback of head kidney catecholamine overflow was confirmed by manipulating the inflowing concentrations of one or both catecholamines in the Ringer. This inhibitory effect is based upon the fact that the overflow into the circulation is the net result of two opposing processes: clearance of inflowing catecholamines and release of stored catecholamines. In turn, clearance is the summed effect of re-uptake, metabolism and tissue binding. It is apparent that, given a constant catecholamine-releasing stimulus, catecholamine overflow from the head kidney will cease when the inflowing levels rise to an extent at which catecholamine clearance exceeds catecholamine release. We cannot predict what circulating levels would be required *in vivo* to evoke this negative feedback, although they presumably would be in the physiological range since overflow was abolished in the perfused preparation at catecholamine inflow levels of 165–235 nmol l^{-1} using a very potent releasing stimulus (10^{-6} mol acetylcholine). We suggest that this mechanism, acting in conjunction with neural and extraneural re-uptake and metabolism (see above), serves to prevent unnecessary elevation of plasma catecholamine levels above those required to elicit physiological effects. Furthermore, this mechanism permits independent control of the plasma levels of each catecholamine.

In summary, we have shown that the pattern of plasma catecholamine elevation during hypoxia in Atlantic cod reflects both neural stimulation of the head kidney and a direct stimulatory action of hypoxaemia on the chromaffin tissue. It would appear that the release of each catecholamine can be controlled independently by several mechanisms including differential responses to potential releasing stimuli and the circulating level of the catecholamine. Clearly, the chromaffin tissue within the head kidney does not simply release catecholamines at a constant ratio into the circulation, but instead releases variable quantities of adrenaline and noradrenaline depending upon the nature of the stimuli and the existing levels in the blood.

This work was supported by a Swedish Natural Science Research Grant to SN and a Natural Sciences and Engineering Research Council of Canada Grant to SFP. RK was the recipient of a Scholarship from The Swedish Institute. We wish to thank Ms Gunilla Rydgren for skilful technical assistance with the analyses of catecholamines and Mr Uno Larsson for supplying the fish. We are grateful for the advice and assistance of Dr Michael Axelsson throughout these experiments.

References

ABRAHAMSSON, T. AND NILSSON, S. (1976). Phenylethanolamine-*N*-methyl transferase (PNMT)

activity and catecholamine content in chromaffin tissue and sympathetic neurons in the cod, *Gadus morhua*. *Acta physiol. scand.* **96**, 94–99.

BOUTILIER, R. G., DOBSON, G., HOEGER, U. AND RANDALL, D. J. (1988). Acute exposure to graded levels of hypoxia in rainbow trout (*Salmo gairdneri*): metabolic and respiratory adaptations. *Respir. Physiol.* **71**, 69–82.

BOUTILIER, R. G., HEMING, T. A. AND IWAMA, G. K. (1986). Acute extracellular acidosis promotes catecholamine release in rainbow trout (*Salmo gairdneri*): interactions between red cell pH and Hb-O_2 carrying capacity. *J. exp. Biol.* **123**, 145–157.

BUSACKER, G. P. AND CHAVIN, W. (1977). Uptake, distribution, and turnover of catecholamine radiolabel in the goldfish, *Carassius auratus* L. *Can. J. Zool.* **55**, 1656–1670.

BUTLER, P. J., AXELSSON, M., EHRENSTRÖM, F., METCALFE, J. D. AND NILSSON, S. (1989). Circulating catecholamines and swimming performance in the Atlantic cod, *Gadus morhua*. *J. exp. Biol.* **141**, 377–387.

COLLETTI, A. E. AND OLSON, K. R. (1988). Catecholamine metabolism by the perfused rainbow trout gill. *J. exp. Zool.* **248**, 177–184.

COUPLAND, R. E. (1971). Observations of the form and size distribution of chromaffin granules and on the identity of adrenaline- and noradrenaline-storing chromaffin cells in vertebrates and man. *Mem. Soc. Endocr.* **19**, 611–635.

DASHOW, L. AND EPPLE, A. (1985). Plasma catecholamines in the lamprey: Intrinsic cardiovascular messengers? *Comp. Biochem. Physiol.* **82C**, 119–122.

FIEVET, B., CAROFF, J. AND MOTAIS, R. (1990). Catecholamine release controlled by blood oxygen tension during deep hypoxia in trout: effect on red blood cell Na/H exchanger activity. *Respir. Physiol.* **79**, 81–90.

FIEVET, B., MOTAIS, R. AND THOMAS, S. (1987). Role of adrenergic-dependent H^+ release from red cells in acidosis induced by hypoxia in trout. *Am. J. Physiol.* **252**, R269–R275.

FRITSCHE, R. AND NILSSON, S. (1990). Autonomic nervous control of blood pressure and heart rate during hypoxia in the cod, *Gadus morhua*. *J. comp. Physiol.* (In press).

GROVE, D. J., STARR, C. R., ALLARD, D. D. AND DAVIES, W. (1972). Adrenaline storage in the pronephros of the plaice, *Pleuronectes platessa* L. *Comp. gen. Pharmac.* **3**, 205–212.

HATHAWAY, C. B. AND EPPLE, A. (1989). The sources of plasma catecholamines in the American eel, *Anguilla rostrata*. *Gen. comp. Endocr.* **74**, 418–430.

HOLMGREN, S. AND NILSSON, S. (1974). Drug effects on isolated artery strips from two teleosts, *Gadus morhua* and *Salmo gairdneri*. *Acta physiol. scand.* **90**, 431–437.

MASTROLIA, L., GALLO, V. AND MANELLI, H. (1976). Cytological and histological observations on chromaffin cells of the suprarenal gland of *Triturus cristatus* (urodele amphibian). *Boll. Zool.* **43**, 27–36.

NEKVASIL, N. P. AND OLSON, K. R. (1986*a*). Plasma clearance, metabolism, and tissue accumulation of ^{3}H-labelled catecholamines in trout. *Am. J. Physiol.* **250**, R519–R525.

NEKVASIL, N. P. AND OLSON, K. R. (1986*b*). Extraction and metabolism of circulating catecholamines by the trout gill. *Am. J. Physiol.* **250**, R526–R531.

NILSSON, S. (1976). Fluorescent histochemistry and cholinesterase staining of sympathetic ganglia in a teleost, *Gadus morhua*. *Acta zool., Stockh.* **57**, 69–77.

NILSSON, S. (1983). Autonomic nerve function in the vertebrates. In *Zoophysiology*, vol. 13 (ed. D. S. Farner, B. Heinrich, K. Johansen, H. Langer, G. Neuweiler and D. J. Randall). Berlin: Springer-Verlag. 253pp.

NILSSON, S., ABRAHAMSSON, T. AND GROVE, D. J. (1976). Sympathetic nervous control of adrenaline release from the head kidney of the cod, *Gadus morhua*. *Comp. Biochem. Physiol.* **55C**, 123–127.

OPDYKE, D. F., BULLOCK, J., KELLER, N. E. AND HOLMES, K. (1983). Dual mechanism for catecholamine secretion in the dogfish shark *Squalus acanthias*. *Am. J. Physiol.* **244**, R641–R645.

PERRY, S. F. AND FARRELL, A. P. (1989). Perfused preparations in comparative respiratory physiology. In *Techniques in Comparative Respiratory Physiology, Society for Experimental Biology Seminar Series* **37** (ed. C. R. Bridges and P. J. Butler), pp. 223–257. Cambridge: Cambridge University Press.

PERRY, S. F., KINKEAD, R., GALLAUGHER, P. AND RANDALL, D. J. (1989). Evidence that

hypoxaemia promotes catecholamine release during hypercapnic acidosis in rainbow trout (*Salmo gairdneri*). *Respir. Physiol.* **77**, 351–364.

PERRY, S. F. AND WOOD, C. M. (1989). Control and coordination of gas transfer in fishes. *Can. J. Zool.* **67**, 2961–2970.

SMITH, D. G., NILSSON, S., WAHLQVIST, I. AND ERIKSSON, B. M. (1985). Nervous control of the blood pressure in the Atlantic cod, *Gadus morhua*. *J. exp. Biol.* **117**, 335–347.

SOIVIO, A., NYHOLM, K. AND WESTMAN, K. (1975). A technique for repeated blood sampling of the blood of individual resting fish. *J. exp. Biol.* **62**, 207–217.

TANG, Y. AND BOUTILIER, R. G. (1988). Correlation between catecholamine release and degree of acidotic stress in trout. *Am. J. Physiol.* **255**, R395–R399.

TETENS, V. AND CHRISTENSEN, N. J. (1987). Beta-adrenergic control of blood oxygen affinity in acutely hypoxia exposed rainbow trout. *J. comp. Physiol.* **157**, 667–675.

TUCKER, V. A. (1967). A method for oxygen content and dissociation curves on microliter blood samples. *J. appl. Physiol.* **23**, 407–410.

UNGELL, A-L. (1985*a*). Accumulation of intra-arterially administered [^{3}H]adrenaline and [^{3}H]noradrenaline in various tissues of the Atlantic cod, *Gadus morhua*. *Comp. Biochem. Physiol.* **81**C, 25–28.

UNGELL, A.-L. (1985*b*). Uptake of ^{3}H-adrenaline and ^{14}C-noradrenaline into neuronal and extraneuronal tissue compartments in the perfused gas gland of the swimbladder of the Atlantic cod, *Gadus morhua*. *J. comp. Physiol.* **155**B, 479–483.

WAHLQVIST, I. (1981). Branchial vascular effects of catecholamines released from the head kidney of the Atlantic cod, *Gadus morhua*. *Molec. Physiol.* **1**, 235–241.

WAHLQVIST, I. AND NILSSON, S. (1980). Adrenergic control of the cardio-vascular system of the Atlantic cod, *Gadus morhua*, during 'stress'. *J. comp. Physiol.* **137**B, 145–150.

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