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**CONTROL OF CATECHOLAMINE STORAGE
AND RELEASE IN TELEOST FISH**

By

Steve G. Reid

A thesis Submitted to the School of Graduate Studies and Research, University of Ottawa,
in Partial Fulfilment of the Requirements for the Degree of Doctor of Philosophy in the
Ottawa-Carleton Institute of Biology

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UNIVERSITÉ D'OTTAWA
UNIVERSITY OF OTTAWA

**CONTROL OF CATECHOLAMINE STORAGE
AND RELEASE IN TELEOST FISH**

Doctor of Philosophy (1995), University of Ottawa
(Biology)

Title: Control of Catecholamine Storage and Release in Teleost Fish

Author: Steve G. Reid, B.Sc. (University of Ottawa)

Supervisor: Dr. Steve F. Perry, Professor, Department of Biology, University of Ottawa

ABSTRACT

This thesis examined a variety of factors controlling the storage and release of the catecholamine hormones, adrenaline and noradrenaline, from chromaffin cells in teleost fish. In these fish, chromaffin cells line the walls of the posterior cardinal vein (PCV) in the anterior kidney region (head kidney). To examine the process of catecholamine release, an *in situ* saline-perfused PCV preparation first was developed and validated in the rainbow trout (*Oncorhynchus mykiss*). This preparation released catecholamines in a dose-dependent manner in response to the cholinceptor agonist carbamylcholine (carbachol). Noradrenaline release was inhibited by pre-perfusion with the ganglion blocker hexamethonium while adrenaline release was attenuated. The removal of calcium from the perfusion fluid prevented the release of both catecholamines. These validation experiments confirm that this preparation is a suitable tool to examine catecholamine secretion in teleost fish.

The mechanisms responsible for the different plasma catecholamine levels observed during exposure to acute hypoxia in rainbow trout and American eel (*Anguilla rostrata*) were examined by assessing both catecholamine storage and *in situ* catecholamine release in these two species. The levels of stored catecholamines were approximately 4X greater in trout, than in eel, and trout chromaffin cells released greater quantities of catecholamines in response to both 60 mmol l⁻¹ K⁺ and carbachol administration, *in situ*. Thus trout chromaffin cells appear to exhibit a greater capacity to release catecholamines than do eel chromaffin cells which may explain the different plasma catecholamine levels during acute hypoxia.

In rainbow trout, cortisol is released from interrenal cells, within the head kidney, during a variety of stressful situations. An experimental elevation of plasma cortisol levels caused an increase in the concentration of catecholamines stored within the PCV and kidney tissue. Additionally, the responsiveness of carbachol-evoked catecholamine release was enhanced, at lower doses of carbachol, in cortisol-treated fish. Cortisol treatment did not alter the *in vitro* activity of phenylethanolamine-N-methyltransferase (PNMT), the enzyme which converts noradrenaline to adrenaline. Thus interactions between cortisol and the chromaffin cells potentially can modify the acute adrenergic stress response in rainbow trout.

The cholinceptor mediating catecholamine release in teleost fish is considered to be of the nicotinic subtype. To investigate a possible involvement of the muscarinic cholinceptor on catecholamine secretion in the American eel, the effects of several cholinergic agonists were examined. The nicotinic receptor agonist, 1, 1-dimethyl-4 - phenylpiperazinium iodide, evoked catecholamine release which was abolished by pre-treatment with hexamethonium while a muscarinic receptor agonist, pilocarpine, did not elicit catecholamine release. The mixed nicotinic/muscarinic cholinceptor agonist, carbachol, caused the release of catecholamines which was abolished by pre-treatment with hexamethonium but not by the muscarinic receptor antagonist, atropine. Thus cholinergic-induced catecholamine release in the American eel appears to be mediated exclusively through the nicotinic receptor.

The proximate stimulus for catecholamine release in rainbow trout, during exposure to acute hypoxia, was examined by acclimating trout to either 5 or 15 °C in

order to modify haemoglobin oxygen-affinity. Acclimation temperature had an obvious effect on haemoglobin oxygen-affinity (P_{50} values of 26.7 and 14.0 torr at 15 °C and 5 °C respectively). At 15 °C, catecholamines were released into the circulation at a PaO_2 threshold of 34.5 torr while at 5 °C the threshold was 24.0 torr. Release thresholds, calculated on the basis of arterial blood oxygen-saturation were similar at both temperatures (approximately 60% Hb O_2 -saturation). Thus the lowering of blood oxygen content, rather than PO_2 , appears to be the proximate stimulus causing catecholamine release in rainbow trout during acute hypoxia.

In addition to the primary mechanism of cholinceptor-induced catecholamine secretion, a variety of non-cholinergic substances may also exert an effect on catecholamine release. Immunohistochemical techniques, in combination with fluorescence microscopy, were used to identify bioactive peptides and amines associated with the chromaffin tissue of five species of fish. Nerve fibres displaying vasoactive intestinal polypeptide-like and pituitary adenylyl cyclase activating polypeptide-like immunoreactivity innervated Atlantic cod (*Gadus morhua*), rainbow trout and European eel (*Anguilla anguilla*) chromaffin cells. In eel, neuropeptide Y-like and peptide YY-like immunoreactivity were located within chromaffin cells. Serotonin-like immunoreactivity was observed within eel and cod chromaffin cells but not in trout where it was localised to celis within the kidney tissue. Further physiological experiments will be required to elucidate the role, if any, of these substances on catecholamine release.

In the rainbow trout, intra-arterial injections of serotonin *in vivo* caused an increase in both plasma adrenaline and noradrenaline levels. *In situ*, bolus injections of

serotonin into the perfusion fluid caused an increase in the level of perfusate catecholamines which was attenuated by pre-treatment with the serotonergic receptor antagonist, methysergide but unaffected by hexamethonium. Thus serotonin is capable of causing the release of catecholamines by interacting with serotonergic receptors on the chromaffin cells.

The effect of adrenocorticotrophic hormone (ACTH), the normal secretagogue of cortisol, on catecholamine release, was investigated in the rainbow trout. Intra-arterial injections of ACTH, *in vivo*, caused an elevation of plasma adrenaline levels. *In situ*, adrenocorticotrophic hormone (ACTH) caused the release of both catecholamines. ACTH-induced release was unaffected by pre-treatment with hexamethonium or methysergide but abolished in Ca^{2+} -free media. Injections of cortisol *in situ* did not elicit catecholamine release. ACTH, therefore, is capable of eliciting catecholamine secretion from the chromaffin cells in rainbow trout.

This thesis has examined aspects of both cholinergic and non-cholinergic control of catecholamine release in several species of teleost fish and, as such, has enhanced our understanding of the various mechanisms leading to the release of catecholamines from the chromaffin tissue into the circulation.

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LIST OF ABBREVIATIONS

α , Greek letter alpha
ACH, acetylcholine
ACTH, adrenocorticotrophic hormone
ak, anterior third of the kidney
apcv, anterior third of the posterior cardinal vein
 β , Greek letter beta
CaO₂, arterial oxygen content
CCH, carbamylcholine (carbachol)
COMT, catechol-O-methyltransferase
CPM, counts per minute
DBH, dopamine- β -hydroxylase
DHBA, 3, 4-dihydroxybenzylamine
DMPP, 1, 1-dimethyl-4-phenylpiperazinium iodide
DPM, disintegrations per minute
EDTA, ethylenediaminetetraacetic acid
FITC, fluorescein isothiocyanate
h, hours
Hb, haemoglobin
HCT, haematocrit
HPLC, high performance liquid chromatography
5-HT, 5-hydroxytryptamine
M, maximal value
MAO, monoamine oxidase
mpcv, middle third of the posterior cardinal vein
mk, middle third of the kidney
MS222, ethyl *m*-aminobenzoate
mU, milliunits
N, number of samples
NPY, neuropeptide tyrosine
OCS, organic counting scintillant
P, pre-sample
PACAP, pituitary adenylyl cyclase activating polypeptide
PBS, phosphate buffered saline
PCA, perchloric acid
PCV, posterior cardinal vein
PO₂, partial pressure of oxygen
PaO₂, arterial partial pressure of oxygen
PwO₂, water partial pressure of oxygen
pHa, arterial pH
pk, posterior third of the kidney
PNMT, phenylethanolamine-N-methyltransferase
ppcv, posterior third of the posterior cardinal vein
PYY, peptide tyrosine tyrosine

R, rhodamine
SEM, standard error of the mean
TH, tyrosine hydroxylase
tk, total kidney
tpcv, total posterior cardinal vein
VIP, vasoactive intestinal polypeptide

Note: In chapter 7 there are numerous abbreviations for antisera which do not appear elsewhere in the thesis. As such, they are not listed here but are described, where appropriate, in that chapter.

CHAPTER 1.

GENERAL INTRODUCTION

Overall Rational

The physiological and biochemical consequences of an elevation in plasma catecholamine levels is a field of extensive study by fish physiologists and biochemists. Indeed, given the multitude of effects exerted by these hormones, on a variety of target organs, it is reasonable to expect that their actions are the focus of extensive study. However, equally important to the general understanding of the physiology of catecholamines are the mechanisms underlying their release from chromaffin cells, into the circulation. In comparison with the literature regarding the consequences of catecholamine release, less is known about the release process of these hormones in fish. On the other hand, the control of catecholamine release is an area of extensive study in mammalian physiology, due in a large part to the ease of obtaining individual, isolated chromaffin cells. Indeed, in mammals, the chromaffin cell is an important model used in the study of secretion and exocytosis. Given the potentially important physiological consequences of an elevation in circulating catecholamines in teleost fish, this thesis has examined a variety of aspects regarding the control of catecholamine release from, and storage within, teleost fish chromaffin cells (see below for specific goals). The results of this thesis will hopefully lead to a greater understanding of the complex pathways initiating the acute adrenergic response in teleost fish.

The Physiology and Biochemistry of Catecholamines

1. The Biosynthesis of Catecholamines

The catecholamine hormones, adrenaline and noradrenaline, are synthesized and stored both within adrenergic neurons and chromaffin cells where they function as neurotransmitters and circulating hormones, respectively. This thesis focuses on catecholamines within chromaffin cells. The biosynthesis of catecholamines occurs via the Blaschko pathway beginning with the amino acid precursor, tyrosine (Blaschko 1939). Tyrosine is hydroxylated by the enzyme tyrosine hydroxylase (TH), which requires both tetrahydropteridine and molecular oxygen as cofactors, to form dihydroxyphenylalanine (DOPA) in the rate-limiting step in the synthesis of both catecholamines. A variety of factors appear capable of regulating the activity of TH. First, TH activity is under negative feedback control from dopamine and noradrenaline (Spector *et al.* 1967), both of which are formed within the Blaschko pathway. Additionally, a variety of neuronal and hormonal factors are capable of increasing the activity of TH. Some of these include: adrenocorticotrophic hormone (ACTH) within the blood (Mueller *et al.* 1970), nicotine (Stachowiak *et al.* 1990a; Hiremagalur *et al.* 1993), glucocorticoids (Stachowiak *et al.* 1990a). Additionally, a variety of neuropeptides including vasoactive intestinal polypeptide (VIP), pituitary adenylyl cyclase activating polypeptide (PACAP), secretin and angiotensin (Zigmond 1985; Stachowiak *et al.* 1990b; Wessels-Reiker *et al.* 1991; Watanabe *et al.* 1992) have all been demonstrated to influence TH activity.

DOPA is hydroxylated by amino acid decarboxylase (AADC, or DOPA decarboxylase) to form dopamine. This enzyme has a broad substrate affinity and

specificity and is present in sufficient quantities that DOPA does not accumulate to any substantial extent. These first two steps within the pathway (TH and AADC) both occur within the cytosol. Dopamine is then transported into secretory vesicles (granules) where it is hydroxylated by dopamine- β -hydroxylase (DBH) to form noradrenaline. DBH requires both molecular oxygen and ascorbic acid as cofactors.

In some chromaffin cells, noradrenaline is transported from the secretory vesicle, back into the cytoplasm where it is methylated to form adrenaline by the enzyme phenylethanolamine-N-methyltransferase (PNMT). As with TH, both cholinergic stimulation of chromaffin cells (Evinger *et al.* 1994) and glucocorticoids (e.g. Wurtman and Axelrod 1966; Wurtman *et al.* 1967) have been demonstrated to influence PNMT activity in mammals. Alternately, once synthesised, noradrenaline may remain stored within the secretory granules. Once synthesized within the cytosol, adrenaline is transported into storage vesicles prior to release.

2. *Storage of Catecholamines*

The arrangement of chromaffin cells differs greatly amongst species (Coupland 1972; Nilsson 1983). In mammals, chromaffin cells are found in a discrete organ, the adrenal medulla (Nilsson 1983) surrounded by the steroidogenic interrenal cells which form the adrenal cortex. In birds, chromaffin cells are found closely intermingled with interrenal cells within an adrenal gland (Coupland 1965). In urodele amphibians, chromaffin cells are located within segmented bodies associated with sympathetic nerves on the ventral side of the kidney whilst, in anurans, chromaffin cells form a distinct mass on the surface of the kidney (Coupland 1965, 1972).

Amongst different classes of fish, the arrangement of chromaffin cells also exhibits substantial diversity. In evolutionary terms, the cyclostomes/agnathans represent the most primitive group of fish. In these fish, the chromaffin cells are distributed within the systemic and portal hearts and in large veins and arteries (e.g. Augustinsson *et al.* 1956). Elasmobranchs represent a more advanced evolutionary stage than the cyclostomes and, in this group, the chromaffin cells are associated with paravertebral autonomic ganglia (e.g. von Euler and Fänge 1961). The axillary bodies, comprising chromaffin cells in association with the gastric ganglia, are the primary source of circulating catecholamines in these fish (Nilsson 1983). The most evolutionarily advanced fish are represented by the teleosts. In teleosts, such as the rainbow trout (*Oncorhynchus mykiss*) and the American eel (*Anguilla rostrata*) the chromaffin cells are located within the walls of the posterior cardinal vein (PCV) and its branches within the kidney tissue in the anterior regions of the kidney ("head kidney") (Nandi 1961; Mastrolia *et al.* 1981, 1984; Nilsson 1983; Gallo *et al.* 1993).

3. Situations Eliciting Catecholamine Secretion into the Circulation

In teleost fish, particularly the rainbow trout (*Oncorhynchus mykiss*), a variety of stressful situations may result in the release of catecholamines from chromaffin tissue and result in an elevation of plasma adrenaline and noradrenaline levels. A potent stimulus for catecholamine release in rainbow trout is exposure to environmental hypoxia (Boutilier *et al.* 1988; Ristori and Laurent 1989; Kinkead *et al.* 1991; Perry *et al.* 1991a; Thomas *et al.* 1991; Perry and Reid 1992, see review by Randall and Perry 1992). During hypoxia, the

oxygen content of the blood must decrease by approximately 50% of its maximal value to trigger the secretion of catecholamines (Perry and Reid 1992).

Trout exposed to environmental hypercapnia (Perry and Kinkead 1989; Hyde and Perry 1990) or metabolic acidosis (Boutilier *et al.* 1986; Aota *et al.* 1990) exhibit elevations in their levels of circulating catecholamines. Hypercapnia causes a respiratory acidosis as carbon dioxide is hydrated to form protons and bicarbonate. This reaction can occur in the plasma at an uncatalysed rate or within the red blood cell (rbc) where the reaction is catalysed by the enzyme carbonic anhydrase. The production of protons from the CO₂ hydration reaction can have effects on the oxygen content of the blood as a reduction in intracellular pH can decrease both the oxygen carrying capacity of haemoglobin (Root effect) and the affinity of haemoglobin for oxygen (Bohr effect). Similar effects can also arise from metabolic protons produced, for instance, during anaerobic metabolism.

Exhaustive exercise (Nakano and Tomlinson 1967; Ristori and Laurent 1985), air exposure (Nilsson *et al.* 1976; Fuchs and Albers 1988), and anaemia (Iwama *et al.* 1987) are all situations during which plasma catecholamine levels are elevated. A unifying theme often arising during periods of catecholamine mobilization is a decrease in blood oxygen content.

4. *Mechanisms of Catecholamine Release*

In teleost fish, chromaffin cells are innervated by pre-ganglionic, cholinergic fibers of the sympathetic nervous system (Nilsson *et al.* 1976; Nilsson 1983; Hathaway and Epple 1989; Perry *et al.* 1991a) which, upon appropriate stimulation, release the

neurotransmitter acetylcholine (ACh) onto the chromaffin cells. The interaction of ACh with nicotinic cholinergic receptors on the chromaffin cell membrane opens voltage-dependent ion channels and results in an influx of sodium ions. Ultimately, the Na^+ influx results in membrane depolarization and the opening of voltage-dependent calcium channels which leads to an influx of extracellular Ca^{2+} into the chromaffin cell (Burgoyne 1991).

Although the exact mechanism by which exocytosis occurs is unknown, a rise in intracellular calcium is a necessary prerequisite for catecholamine release to occur.

Following the opening of calcium channels, the ensuing influx of calcium into the cell and the subsequent rise in intracellular calcium concentration ($[\text{Ca}^{2+}]_i$) initiates a series of intracellular events culminating in the exocytotic release of catecholamines.

Rearrangement of the cytoskeleton is considered to be a key event in the process of exocytosis (Burgoyne 1991). Under “resting” conditions, a network of actin filaments separates catecholamine-containing secretory granules from the plasma membrane preventing release from occurring. The influx of extracellular calcium ions into the cell causes the actin network to disassemble allowing for secretory vesicles to move to the plasma membrane, fuse and release their contents by exocytosis. Microtubules may also play a role in the translocation of secretory granules from their location in the cytosol to the plasma membrane.

Although stimulation of nicotinic cholinergic receptors by acetylcholine is presumably the predominant mechanism leading to the release of catecholamines *in vivo*, recent experimental evidence, both in fish and in a variety of mammalian species, suggests that other factors may play a role in the secretion of catecholamines from chromaffin cells.

When spinal nerves 1 - 4, which innervate the head kidney, are sectioned in the Atlantic cod (*Gadus morhua*), the increase in plasma catecholamine levels observed during air exposure (Wahlqvist and Nilsson 1980), after exhaustive exercise (Butler *et al.* 1989), or in response to environmental hypoxia (Perry *et al.* 1991) was reduced, but not entirely abolished. Wahlqvist and Nilsson (1980) and Butler *et al.* (1989) hypothesized that the failure of the nerve sectioning to eliminate increases in plasma catecholamine levels suggests that overflow from adrenergic neurons can contribute to the elevation of plasma catecholamines during stress. However, Perry *et al.* (1991) blocked the release of catecholamines from adrenergic nerves using bretylium and observed, in spinal nerve-sectioned cod, that a slight elevation of plasma catecholamines still occurred during hypoxia. Thus it appears as if other mechanisms (non-cholinergic) can contribute to the release of catecholamines from the chromaffin cells independent of cholinceptor stimulation.

Recent work has revealed some non-cholinergic mechanisms, in a variety of fish species, that can elicit catecholamine release from chromaffin cells. In the American eel (*Anguilla rostrata*) catecholamine-induced release of catecholamines has been demonstrated (Hathaway *et al.* 1989). This release was unaffected by removal of the sympathetic innervation of the chromaffin cells. In spiny dogfish (*Squalus acanthias*), plasma catecholamine levels rise in response to intra-arterial injections of K^+ even during ganglionic blockade with hexamethonium (Opdyke *et al.* 1983). These authors suggest that the release of potassium ions from skeletal muscle may play a secondary role in releasing catecholamines during strenuous exercise. In sea lamprey, the anneural

chromaffin cells release catecholamines in response to carbon dioxide (Dashow and Epple 1985). In the Atlantic hagfish (*Myxine glutinosa*), a pituitary extract was capable of initiating release *in situ* from the aneural chromaffin cells (Perry *et al.* 1993). Perry *et al.* (1991) demonstrated a direct local stimulatory effect of hypoxemia, *in situ*, on catecholamine secretion in the Atlantic cod. Additionally in cod, high concentrations of catecholamines flowing past the chromaffin cells caused a reduction in the net outflow of catecholamines from the chromaffin tissue. Apparently then, a variety of factors are capable of influencing the release of catecholamines from chromaffin tissue in fish.

In addition to a variety of potential mechanisms influencing catecholamine release from chromaffin cells in fish, assorted bioactive substances have been demonstrated to elicit or modulate the secretion of catecholamines from mammalian chromaffin cells (Burgoyne 1991; Livett and Marley 1993). Receptors for a variety of hormones (adenosine, angiotensin II, ATP, ANP, bradykinin, chromostatin, dopamine D₁ & D₂, endothelin, GABA_A and B, histamine H₁, IGF1, muscarinic, nicotinic, opioid, PGE₂, somatostatin, substance P, VIP) have been identified on bovine adrenal chromaffin cells and these hormones have been implicated as either secretagogues of catecholamines or modulators of the secretory process (see table 1 in Burgoyne 1991).

Two peptide hormones often co-stored in cholinergic nerves with acetylcholine are vasoactive intestinal polypeptide (VIP) and its homologue, pituitary adenylyl cyclase activating polypeptide (PACAP). Both peptides have been implicated in the process of catecholamine release in mammals (e.g. Wakade *et al.* 1991; Watanabe *et al.* 1992; Isobe *et al.* 1993; Yamaguchi 1993). Additionally, a variety of other bioactive substances have

been implicated, in assorted species, in catecholamine release. Some of these substances include: serotonin (Chaouloff *et al.* 1991, 1992), atrial natriuretic factor (ANF; Fernandez *et al.* 1992; Vatta *et al.* 1992a; Vatta *et al.* 1994a, b), adenosine (Casadó *et al.* 1992; Chern *et al.* 1992; Kügelgen 1994), angiotensin (Göthert and Fink 1991; Vatta *et al.* 1992b), histamine (Choi *et al.* 1993; Firestone and Browning 1992; Borges 1994), nitric oxide (Halbrügge *et al.* 1991a; Nagashima *et al.* 1993; Thatikunta *et al.* 1993; Oset-Gasque *et al.* 1994; Torres *et al.* 1994; Uchiyama *et al.* 1994), and endothelins (Halbrügge *et al.* 1991b; Ohara-Imaizumi and Kumakura 1991; Wilson 1991). Additionally, enkephalins (Dillen *et al.* 1993; Dorez *et al.* 1993; Damase-Michel *et al.* 1994) and chromagranins (O'Connor *et al.* 1991; Galindo *et al.* 1991; Wilson *et al.* 1991; Helle *et al.* 1993; Angeletti *et al.* 1994; Helle and Angelettii 1994) often are co-stored with catecholamines in mammalian chromaffin cells. These substances may also exert a modulatory role in catecholamine secretion. Therefore, it is apparent that the process of catecholamine secretion from chromaffin cells, both in fish and mammals, involves multiple bioactive substances and exhibits a greater level of complexity than mere activation of the nicotinic cholinergic pathway of release.

5. Metabolism of Catecholamines

Once released into the circulation, several mechanisms are involved in the clearance and metabolic degradation of catecholamines. Accumulation of catecholamines can occur in both adrenergic neurons/chromaffin cells (uptake 1) as well as other non-neuronal tissues (uptake 2) (see review by Randall and Perry 1992). In fish, a primary site of catecholamine uptake is the gills which, given their location almost immediately

“downstream” of the chromaffin tissue, are exposed to very large quantities of circulating catecholamines. Nekvasil and Olson (1985) demonstrated that, within the gills, pillar cells appear to be the primary site of catecholamine uptake. Additionally, the gill extracts greater quantities of catecholamines from the blood when the proportion of blood flowing through the arterio-venous, opposed to arterio-arterial circulation, increases (Nekvasil and Olson 1986b). Given that catecholamines can decrease blood flow through the arterio-venous pathway, circulating catecholamines can, in effect, attenuate the process of their own degradation. In addition to the gills, almost all tissues, with the exception of the brain, are capable of removing catecholamines from the blood (Nekvasil and Olson 1986a). The relative importance of a particular tissue in this process is dependent upon both the tissue mass and the efficiency of uptake into that tissue (Randall and Perry 1992).

Once uptake (1 or 2) into tissues has occurred, the catecholamines may be repackaged into secretory vesicles (uptake 1 only) or metabolically degraded (uptake 1 and 2). The two primary enzymes involved in catecholamine degradation are monoamine oxidase (MAO) and catechol-O-methyltransferase (COMT) which produce deaminated and O-methylated products, respectively. MAO, a mitochondrial enzyme, is primarily involved in the neuronal degradation of catecholamines whereas COMT, a cytoplasmic enzyme, is found predominantly in aneural tissue. Both enzymes are capable of utilising either noradrenaline or adrenaline as substrates, although noradrenaline appears to be the preferred substrate in the trout gill (Nekvasil and Olson 1986b; Colleti and Olson 1988). Additionally, the product of one enzymatic reaction (e.g. COMT) may be acted upon by the other enzyme (e.g. MAO). In both trout (Nekvasil and Olson 1986a), and cod (Ungell

and Nilsson 1979) O-methylated derivatives begin to appear before deaminated products, an observation consistent with the cellular compartmentalization of these enzymes.

6. *Consequences of Catecholamine Release*

Circulating catecholamines, both adrenaline and noradrenaline, can exert a wide range of physiological and biochemical effects on a variety of target organs. The generalized role of circulating catecholamines is to ameliorate detrimental effects associated with “stress” by optimizing cardiovascular and respiratory functions as well as mobilizing energy stores to provide for the increased energy demands that often accompany stress (see reviews by Perry and Wood 1989; Randall and Perry 1992).

Physiological actions of catecholamines, as with any hormone, initially involve interaction of the catecholamine with an adrenergic receptor (adrenoceptor) located on cellular membranes. Adrenoceptors are typically classified as either alpha (α) or beta (β) with further subdivisions to $\alpha_{1a, b, c}$, $\alpha_{2a, b, c}$, β_1 , β_2 and β_3 (Ahlquist 1948; Lefkowitz 1978; Collins *et al.* 1991; Jacobs *et al.* 1991; Nichols and Ruffolo 1991; Strosberg 1991; Wang *et al.* 1991; Minneman and Esbenshade 1994; Ruffolo and Hieble 1994). These classifications and subdivisions are based on different second messenger systems to which the receptors are linked as well as potency orders for stimulation by assorted agonists and blockade by specific receptor antagonists.

α_1 -adrenoceptor activation stimulates the metabolism of membrane phospholipids to produce inositoltriphosphate (IP_3), which in turn liberates Ca^{2+} from internal calcium stores, and diacylglycerol (DG) which activates protein kinase C resulting in altered Ca^{2+}

fluxes. α_2 -adrenoceptor stimulation causes an inhibition of adenylyl cyclase activity and subsequently lowers intracellular levels of cAMP.

Stimulation of β -adrenoceptors activates the enzyme adenylyl cyclase and the subsequent production of the second messenger cyclic AMP (cAMP). cAMP in turn activates protein kinase A (cAMP-dependent protein kinase) which initiates a phosphorylation cascade that ultimately leads to the physiological effect of the catecholamine.

Rainbow trout red blood cells possess Na^+/H^+ exchangers on their plasma membrane which are activated by stimulation of β_1 -adrenoceptors. The exchange of intracellular protons for plasma sodium ions can cause a subsequent rise in intracellular pH (Nikinmaa 1983, 1992; Holk and Lykkeboe 1995) and have substantial effects on blood oxygen levels. As with mammalian haemoglobin, protons can exert a modulatory role in the binding of molecular oxygen to haemoglobin. A lowering of intracellular pH can cause a decrease in the affinity of haemoglobin for oxygen (Bohr effect), which is manifested as a right shift in the oxygen dissociation curve and an increase in the P_{50} (partial pressure of oxygen at which 50% of the haemoglobin is bound with oxygen). Additionally, the capacity of trout blood (haemoglobin) to carry oxygen is also modified by protons (Root effect) with a decrease in pH lowering the maximal carrying capacity of haemoglobin (Root 1931). Clearly then, adrenergically-activated Na^+/H^+ exchange on the red blood cell membrane can exert substantial effects on blood oxygen content. In addition to these effects at the level of the red blood cell, catecholamines, via α_1 -adrenoceptor stimulation, can cause a splenic contraction and the release of erythrocytes, from the spleen (e.g.

Nilsson and Grove 1974; Milligan and Wood 1982), into the circulation thereby increasing the oxygen carrying capacity of the blood (see review by Perry and Wood 1989).

Catecholamines can also markedly affect carbon dioxide transport in the blood and excretion across the gills (Wood and Perry 1991; Perry *et al.* 1991b; see reviews by Perry and Wood 1989; Jensen 1991). Under normal conditions, carbon dioxide is carried in the blood predominantly in the form of bicarbonate (HCO_3^-). Bicarbonate is formed when CO_2 enters the red blood cell and is hydrated, with the assistance of the enzyme carbonic anhydrase, into H^+ and HCO_3^- ions. The bicarbonate then exits the red cell, in exchange for a Cl^- ion (chloride shift), where it is carried in the plasma until the blood reaches the gills. At the gills, the above process is reversed with HCO_3^- entering the rbc where it is dehydrated to form CO_2 which then diffuses across the rbc membrane and gill epithelia out into the water. The adrenergic activation of rbc Na^+/H^+ exchange and the resultant intracellular alkalosis can disrupt the process of bicarbonate dehydration and CO_2 excretion. When the pH of the red cell increases due to Na^+/H^+ exchange, the intracellular $[\text{HCO}_3^-]$ also increases in a manner predicted by the Henderson-Hasselbach equation. As intracellular bicarbonate levels rise, the chemical gradient for the entry of bicarbonate into the red cell is diminished. Thus, plasma bicarbonate no longer has access to the carbonic anhydrase within the red cell and CO_2 production decreases. Also, proton removal from the red cell will cause a decrease in the partial pressure of carbon dioxide (pCO_2) within the cell as carbon dioxide is hydrated to supply protons for the Na^+/H^+ antiporter. Thus the gradient for CO_2 movement out of the red cell is reversed and CO_2 enters the cell (Thomas and Perry 1994). In addition, bicarbonate within the plasma, which can no

longer enter the red cell, will form carbon dioxide at the uncatalysed rate with extruded protons. This CO₂ can then enter the rbc, along its partial pressure gradient, where it will form more bicarbonate. This process of CO₂ "recycling" (Motaïs *et al.* 1989) also hinders CO₂ excretion across the gill. These processes will all inhibit carbon dioxide excretion as the erythrocytic carbonic anhydrase no longer functions in the excretion process.

Catecholamines may exert influences on the cardiovascular system (see reviews by Nilsson 1983, 1984). Although, in teleosts, such effects are primarily induced by neuronal catecholamines, when circulating catecholamines are elevated they may also exert some control. The heart of most teleosts is adrenergically innervated, with catecholamines exerting positive inotropic and chronotropic effects. The elasmobranch heart lacks adrenergic innervation. As with the heart, the vasculature of teleosts, but not elasmobranchs, is adrenergically innervated (Wahlqvist and Nilsson 1977; Smith 1978) and can also be influenced by circulating catecholamines (Wahlqvist 1980) with catecholamines increasing vascular resistance via α -adrenoceptors on the vascular smooth muscle.

Circulating catecholamines may also cause an elevation in gill diffusing capacity during hypoxia or exhaustive exercise. This can be accomplished by a β -adrenoceptor mediated vasodilation of the afferent lamellar arterioles and thus an increase in the surface area of the gills being perfused (Randall and Daxboeck 1984). In addition, an increase in systemic blood pressure, caused by catecholamines, assists in perfusing the gills by allowing blood to flow through distal lamellae which are not perfused under normal

systemic pressure. There is also the possibility that catecholamines may increase the permeability of the gills to oxygen, carbon dioxide and various non-electrolytes.

It has been suggested that catecholamines exert some effects on the control of breathing in fish. However, this issue is still quite controversial with evidence supporting both sides of the argument (e.g. Randall 1990; Randall and Taylor 1991; Perry *et al.* 1992).

Adrenaline and noradrenaline may also regulate energy supplies in fish during stress. Plasma glucose levels have been demonstrated to increase in response to injected catecholamines, a response presumably mediated through β -adrenoceptors (Wright *et al.* 1989). Increases in plasma glucose arise as a result of activation of liver glycogenolysis and gluconeogenesis along with an inhibition of glycolysis (e.g. Moon *et al.* 1985; Ottolenghi *et al.* 1985, 1986; Mommsen *et al.* 1988; Moon and Mommsen 1990).

Goals of the Thesis

The goal of this thesis was to examine aspects of the control of catecholamine release from the chromaffin cells in teleost fish, predominantly the rainbow trout, *Oncorhynchus mykiss* and the American eel, *Anguilla rostrata*. The ultimate aim was to arrive at a greater understanding of the mechanisms promoting the release of these hormones from the chromaffin cells. All of the components of this thesis are encompassed under the general hypothesis that there are multiple mechanisms involved in controlling catecholamine release from the chromaffin tissue of teleost fish and that interspecific differences in these mechanisms exist between various teleost species.

The majority of experiments within this thesis utilized an *in situ* saline-perfused posterior cardinal vein preparation to examine catecholamine release. This preparation is described in chapter 2. The first part of the thesis examined aspects of cholinceptor-mediated control of catecholamine release. Interspecific differences in the release process between rainbow trout and American eel were examined in an effort to elucidate the reasons behind differences in plasma catecholamine levels seen during severe environmental hypoxia (chapter 3). The effects of the corticosteroid cortisol on catecholamine storage and release were examined in the rainbow trout in an effort to elucidate potential interactions within the pituitary - adrenal axis (chapter 4). The pharmacology of cholinceptor-induced catecholamine release was examined in the American eel to determine whether or not the muscarinic cholinceptor exerted any role, along with the nicotinic receptor, in catecholamine release (chapter 5).

The second part of the thesis examined the control of catecholamine release during hypoxia in rainbow trout in order to elucidate the proximate stimulus, in terms of blood oxygen levels, for catecholamine release during hypoxia (chapter 6).

The third part examined potential non-cholinergic secretagogues/modulators of catecholamine release. An immunohistochemical screening of the chromaffin tissue in rainbow trout, European eel, Atlantic cod, Atlantic hagfish and spiny dogfish identified bioactive substances that may exert a role in catecholamine release (chapter 7). Chapter 8 examined the effects of two non-cholinergic substances, the indolamine serotonin and adrenocorticotrophic hormone (ACTH), on catecholamine release.

CHAPTER 2.

THE IN SITU SALINE-PERFUSED POSTERIOR CARDINAL VEIN PREPARATION

I. Description of the Preparation

In order to investigate the direct effects of a variety of secretagogues on the process of catecholamine release from chromaffin cells, an *in situ*, saline-perfused posterior cardinal vein (PCV) preparation was developed. The following is a general description of this preparation.

Fish (rainbow trout, *Oncorhynchus mykiss* or American eels, *Anguilla rostrata*) were killed and placed ventral side up on ice. An incision was made ventrally along the entire length of the animal and the body wall on the left side was removed. The tissue overlying the heart was removed via blunt dissection and an incision was made in the bulbus arteriosus. In the rainbow trout, a cannula (PE 160 tubing) was inserted through the bulbus and into the ventricle where it was secured with a ligature between the two chambers. In the American eel, the cannula remained in the bulbus to which it was secured. This cannula, in both species, served as an outflow for the perfusion. The PCV was cannulated, with PE 160 tubing, in the anterograde direction and this served as the inflow for the perfusion. In the eel which has both a left and right PCV, the right vein was used as it was the larger of the two. The PCV was perfused, at a flow rate of approximately 1 ml min^{-1} , with modified Cortland saline (Wolf 1963) (in mmol l^{-1} , $[\text{NaCl}] = 125.0$, $[\text{KCl}] = 2.0$, $[\text{MgSO}_4] = 2.0$, $[\text{NaHCO}_3] = 5.0$, $[\text{glucose}] = 7.5$, $[\text{CaCl}_2] = 2.0$, $[\text{KH}_2\text{PO}_4] = 1.25$) gassed with air (final pH = 7.8). Perfusion was accomplished by siphon resulting from the positive pressure difference ($\cong 15 \text{ cm}$) between the surface of the saline and the outflow cannula. An injection port, located just prior to the entrance of the cannula into the vein, facilitated the delivery of drugs into the system.

In all experiments, the PCV was perfused for 20 min prior to sample collection. This period allowed the preparation to come to an "equilibrium" with catecholamine levels decreasing to a stable baseline level. Following this stabilization period, the perfusate was collected for a 1 min period (pre-sample) to assess basal levels of adrenaline and noradrenaline within the perfusate. Following collection of the pre-sample, the catecholamine-releasing response of the chromaffin cells to various stimuli was assessed by injecting a bolus dose of the substance, through the injection port, into the inflow cannula or switching to the desired perfusion fluid. Each preparation received only one dose of a particular treatment.

After a one minute period was allowed for the drug to be delivered to the chromaffin cells the outflowing perfusate was collected, in 1.5 ml microcentrifuge tubes, each minute for the following 5 - 10 minutes, depending on the experiment. The samples were immediately frozen in liquid nitrogen and then stored at -80 °C prior to the analysis of catecholamines. Perfusate adrenaline and noradrenaline levels were determined using high performance liquid chromatography (HPLC) and electrochemical detection (Woodward 1982).

II. Validation of the *In Situ* Preparation

Prior to using the *in situ* saline-perfused PCV preparation to investigate various aspects of catecholamine release from the chromaffin tissue, several basic pharmacological experiments were performed in order to assess the validity of this preparation to examine catecholamine release. Although these initial validation experiments were performed on the rainbow trout, it was reasonable to assume that an eel preparation would respond in a

similar manner. Indeed, with one exception (discussed later), the results of chapters 3 and 5 confirm this assumption.

The initial validation examined the effects of constant perfusion with $60 \text{ mmol l}^{-1} \text{ K}^{+}$ on catecholamine secretion. $60 \text{ mmol l}^{-1} \text{ K}^{+}$ would be expected to depolarise cell membranes independently of receptor activation and second messenger production. As such, release in response to this chemical stimuli would theoretically be the maximum attainable level. Figure 2-1 illustrates the effects of $60 \text{ mmol l}^{-1} \text{ K}^{+}$ on catecholamine release *in situ* in the rainbow trout.

The next stage of the validation process examined the effects of the acetylcholine analogue, carbamylcholine (carbachol). Carbachol is a mixed nicotinic and muscarinic cholinergic agonist which exhibits greater stability than acetylcholine towards degradation by cholinesterases. Following collection of the pre-sample, a dose of carbachol (2.5×10^{-8} , 2.5×10^{-7} , 1.25×10^{-6} , 2.5×10^{-6} or 2.5×10^{-5} moles; $N = 6-10$ for each dose) or saline (control) was injected as a bolus (0.3 ml) into the PCV. After one minute was allowed for the carbachol to be delivered to the chromaffin cells, the perfusate was collected each minute for the next 6 min. Each preparation received only one dose of carbachol. Given the potential for the dilution of agonists within the perfusion, as well as the possibility that some of the drug may flow past the chromaffin cells without a chance to interact with the receptors, it is not possible to determine the exact concentration of agonist which bathes the cells. As such, the doses of carbachol are expressed as the number of moles administered rather than as mol l^{-1} .

Figure 2.1 Perfusate (a) noradrenaline and (b) adrenaline levels (nmol l^{-1}) in response to constant perfusion with $60 \text{ mmol l}^{-1} \text{ K}^+$. The pre-sample represents perfusion with normal saline. The dotted line represents a switch to perfusion with saline containing $60 \text{ mmol l}^{-1} \text{ K}^+$. The data are shown as the mean $\pm$ 1 standard error of the mean. An asterisk (*) indicates a significant difference (one way analysis of variance followed by Fisher's least significant difference test for multiple comparisons or a paired t-test for the maximum value; $p < 0.05$) from the pre-value.

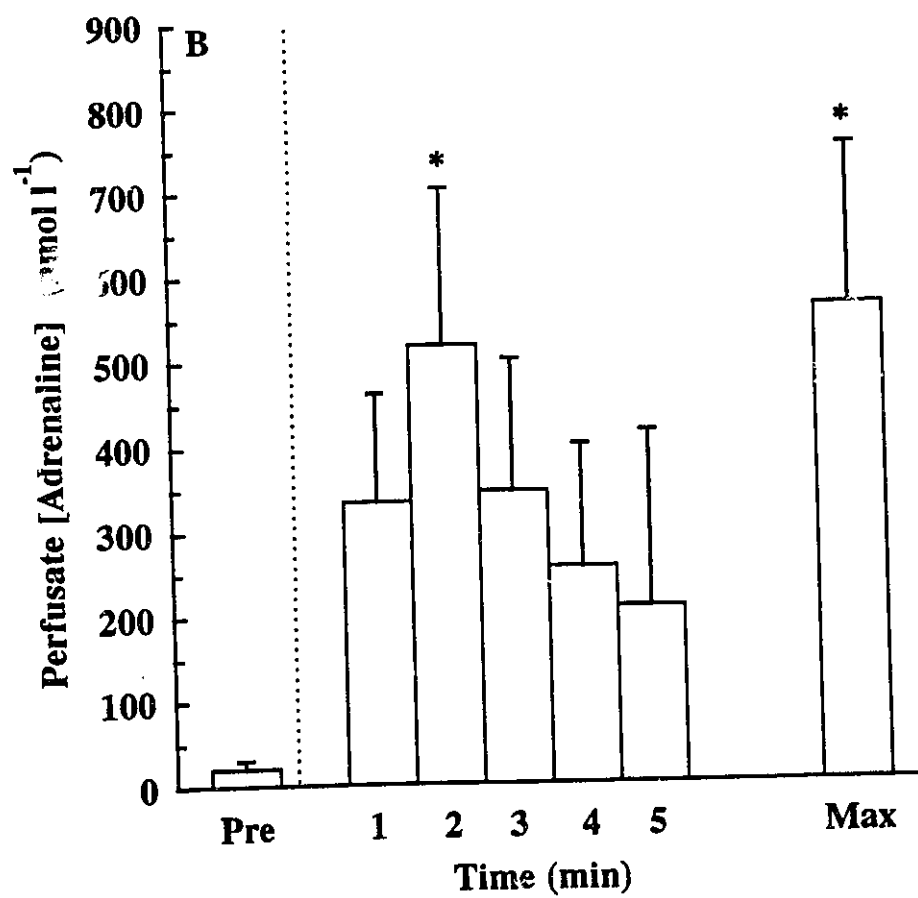
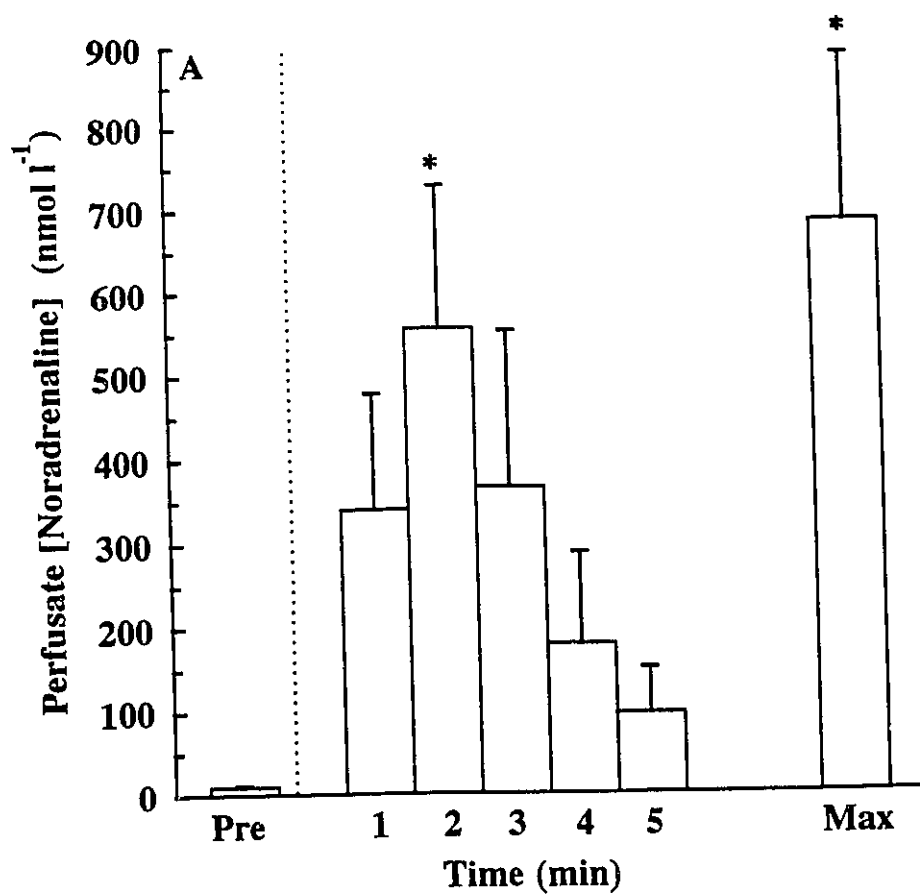


Figure 2-1

These experiments allowed for the determination of a dose-response relationship, to cholinceptor stimulation with carbachol, for catecholamine release *in situ*.

Figure 2-2 illustrates the time course effects of carbachol on the release of noradrenaline into the perfusate. Neither an injection of saline (fig 2-2a) nor the three lowest doses of carbachol (fig 2-2b, c, d) caused an increase in perfusate noradrenaline levels. 2.5×10^{-6} mol carbachol (fig 2-2e) caused an elevation of perfusate noradrenaline levels 2 - 6 min post-injection. Noradrenaline levels ranged between 50 and 80 nmol l⁻¹ during this period. 2.5×10^{-5} mol carbachol (fig 2-2f) caused an increase in perfusate noradrenaline levels in the second minute post-injection.

Figure 2-3 illustrates the time course effects of carbachol on the release of adrenaline into the perfusate. Neither an injection of saline (fig 2-3a) nor the three lowest doses of carbachol (fig 2-3b, c, d) caused a statistically significant increase in perfusate adrenaline levels. 2.5×10^{-6} mol carbachol (fig 2-3e) caused an elevation of perfusate adrenaline levels 2 - 6 min post-injection with values ranging between 75 and 150 nmol l⁻¹ during this period. 2.5×10^{-5} mol carbachol (fig 2-3f) caused an increase in perfusate adrenaline levels in the second and third minutes post-injection.

In order to account for temporal differences in the release process between preparations (i.e. the maximal level of release occurred at differing times in different preparations), the maximum values observed within the sample collection period are presented and statistically compared with the maximal value for the control (saline) group as well as with the pre-values for the given dose (fig 2-4).

Figure 2.2 Perfusate noradrenaline levels in response to an injection of either (a) saline or (b -f) various doses of the cholinceptor agonist carbachol (2.5×10^{-8} , 2.5×10^{-7} , 1.25×10^{-6} , 2.5×10^{-6} and 2.5×10^{-5} mol). The pre-value represents perfusate noradrenaline levels prior to the injection of saline or carbachol. The dotted line represents either the injection of saline or carbachol. Following collection of the pre-sample, delivery of the agonist and a 1 min period to allow delivery of the drug, samples were collected each minute for the next 6 min. The data are shown as the mean $\pm$ 1 standard error of the mean. An asterisk (*) represents a significant difference (one way analysis of variance followed by Fisher's least significant difference test for multiple comparisons; $p < 0.05$) from the pre-sample.

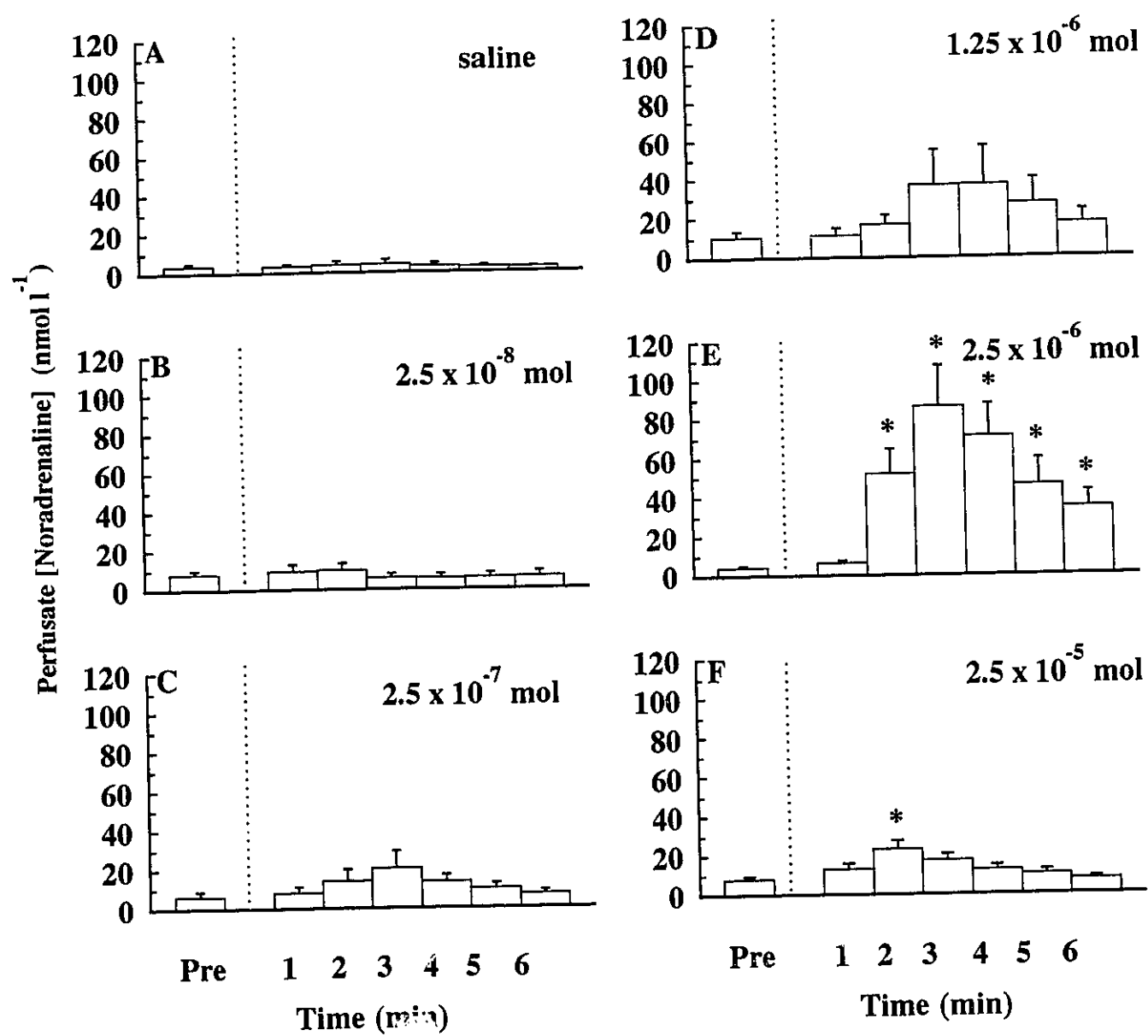


Figure 2-2

Figure 2-3 Perfusate adrenaline levels in response to an injection of either (a) saline or (b-f) various doses of the cholinceptor agonist carbachol (2.5×10^{-8} , 2.5×10^{-7} , 1.25×10^{-6} , 2.5×10^{-6} and 2.5×10^{-5} mol). The pre-value represents perfusate adrenaline levels prior to the injection of saline or carbachol. The dotted line represents either the injection of saline or carbachol. Following collection of the pre-sample, delivery of the agonist and a 1 min period to allow delivery of the drug, samples were collected each minute for the next 6 min. The data are shown as the mean $\pm$ 1 standard error of the mean. An asterisk (*) represents a significant difference (one way analysis of variance followed by Fisher's least significant difference test for multiple comparisons; $p < 0.05$) from the pre-sample.

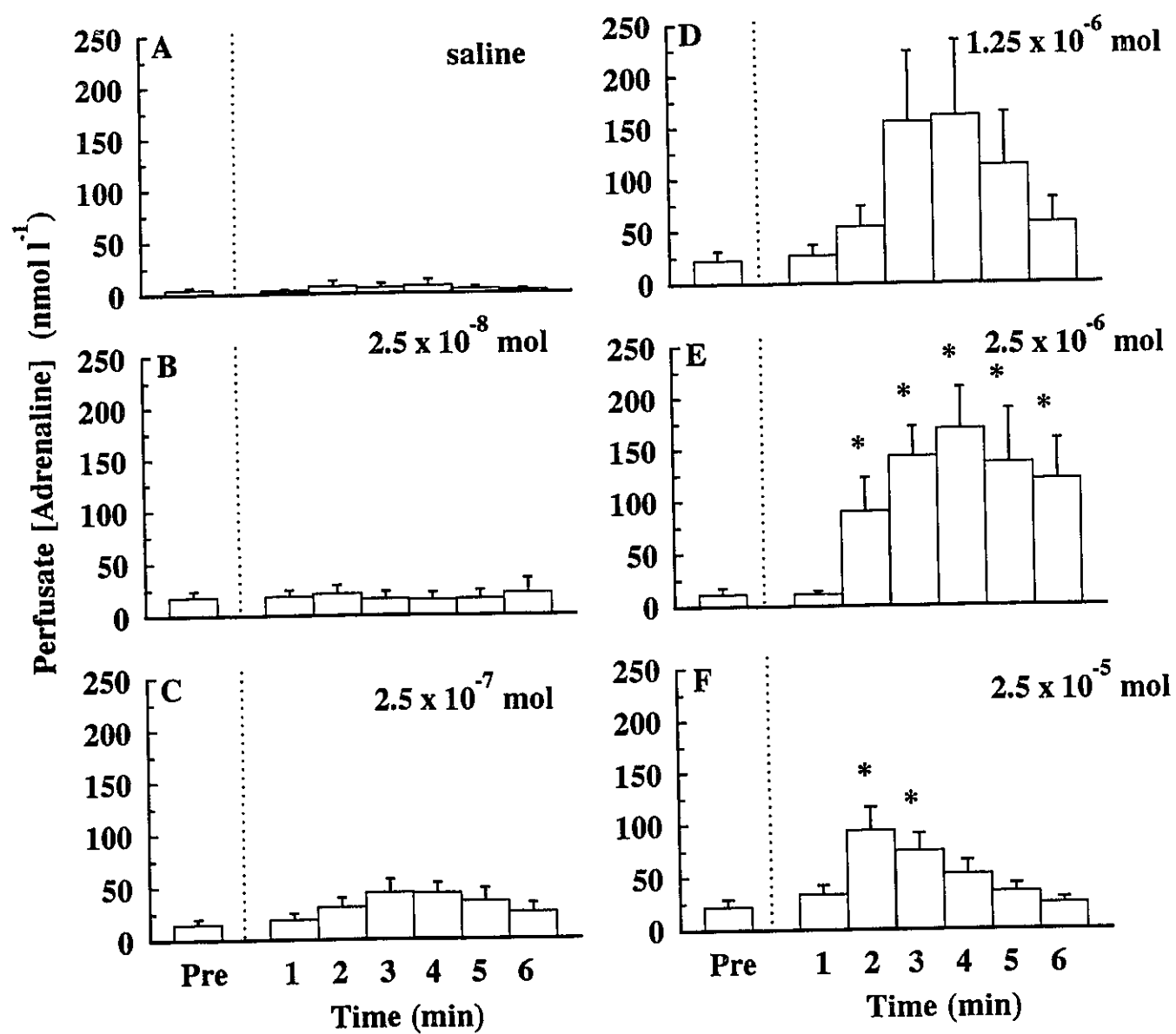


Figure 2-3

Figure 2-4 Perfusate (a) noradrenaline and (b) adrenaline levels in response to an injection of saline or a variety of doses of carbachol. The open circles represent pre-values whereas the closed circles represent the maximal perfusate catecholamine level following the injection of saline or carbachol. The data are shown as the mean $\pm$ 1 standard error of the mean. An asterisk (*) represents a significant difference (one way analysis of variance followed by Fisher's least significant difference test for multiple comparisons; $p < 0.05$) from the maximum value in response to the saline injection. A plus sign (+) represents a significant difference between the pre-value and the maximal value for any given dose of carbachol (paired t-test).

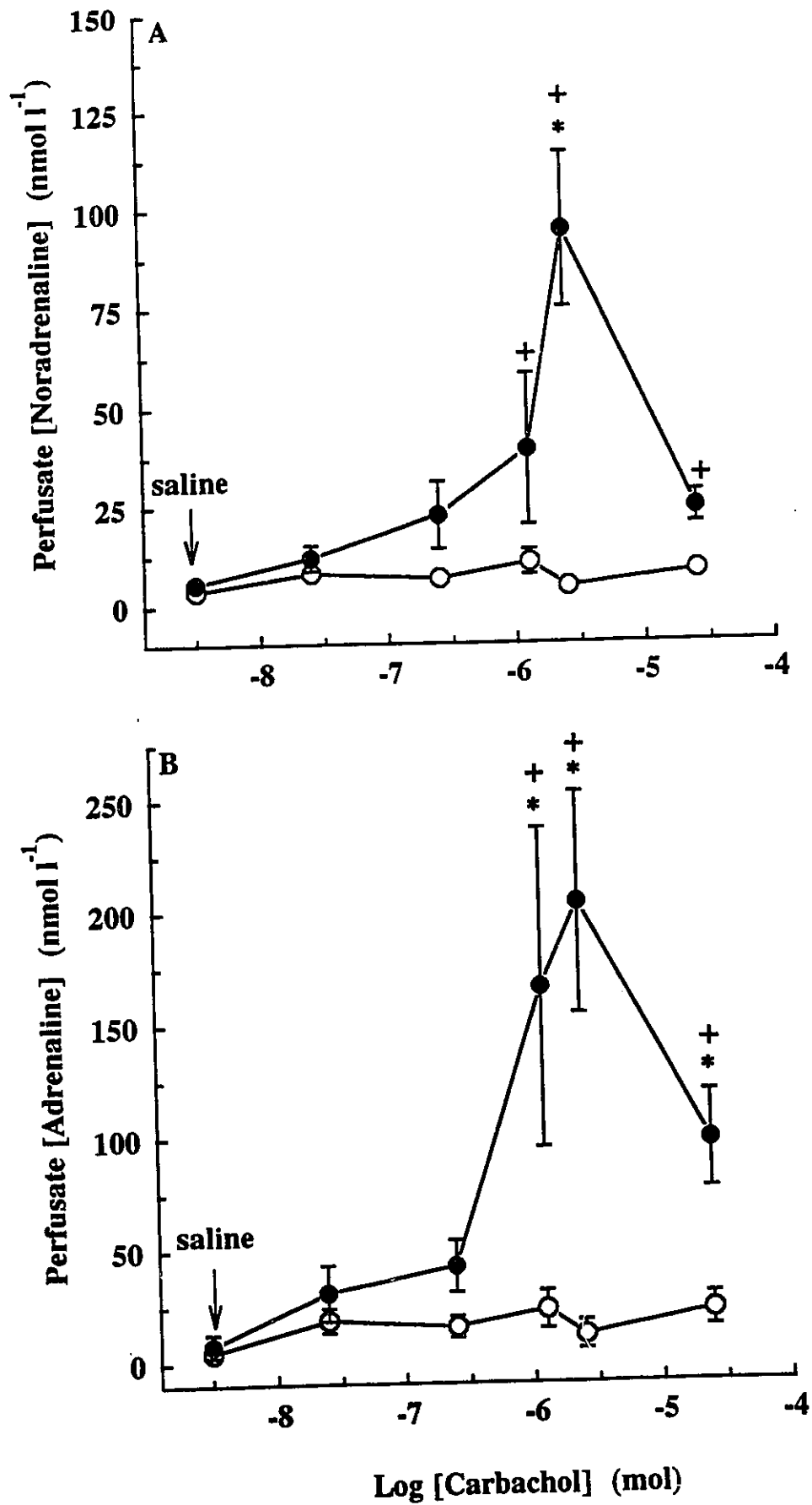


Figure 2-4

Figure 2-4a illustrates maximal perfusate noradrenaline levels in response to the various doses of carbachol and the control injection of saline. The maximal value in response to 2.5×10^{-6} mol carbachol was greater than the maximal value of saline. The maximal values in response to the three highest doses of carbachol were greater than the pre-values in those groups.

Figure 2-4b illustrates the maximal perfusate adrenaline levels in response to carbachol and saline. The three highest doses of carbachol elicited maximal adrenaline release which was greater than the maximal value for saline as well as being greater than the pre-values within those groups. In subsequent chapters, the data are predominantly presented in a form which compares the maximum value with the pre-value. In some cases, sequential histograms like figures 2-1 to 2-3 are presented.

In another set of experiments, the ganglion blocker hexamethonium (to inhibit nicotinic cholinergic-evoked catecholamine release) was added to the perfusion saline (final concentration 10^{-4} mol l⁻¹) during the 20 minute stabilization period, carbachol delivery and sample collection. After collection of the pre-sample, a bolus injection of carbachol (2.5×10^{-6} mol) was delivered and the perfusate collected for the next 6 min. The presence of hexamethonium in the perfusion fluid abolished the carbachol-induced release of noradrenaline (figure 2-5a) but not adrenaline (figure 2-5b). However, maximal adrenaline release in response to carbachol in the presence of hexamethonium was significantly less than the response to carbachol alone (compare figures 2-4 and 2-5). Thus, although the release of adrenaline was not inhibited completely by hexamethonium, it was attenuated.

Figure 2-5. Perfusate (a) noradrenaline and (b) adrenaline levels in response to an injection of carbachol (2.5×10^{-6} mol) following 20 min pre-treatment with the ganglion blocker, hexamethonium (10^{-4} mol l⁻¹). The pre-value represents perfusate catecholamine levels prior to the injection of carbachol. The dotted line represents the injection of carbachol. Following collection of the pre-sample and the 1 min drug delivery period, the samples were collected each minute for the next 6 min. In addition to the time course of release, the maximal release value (Max) is also reported on the right of the histogram. The data are shown as the mean $\pm$ 1 standard error of the mean. An asterisk (*) represents a significant difference from the pre-value.

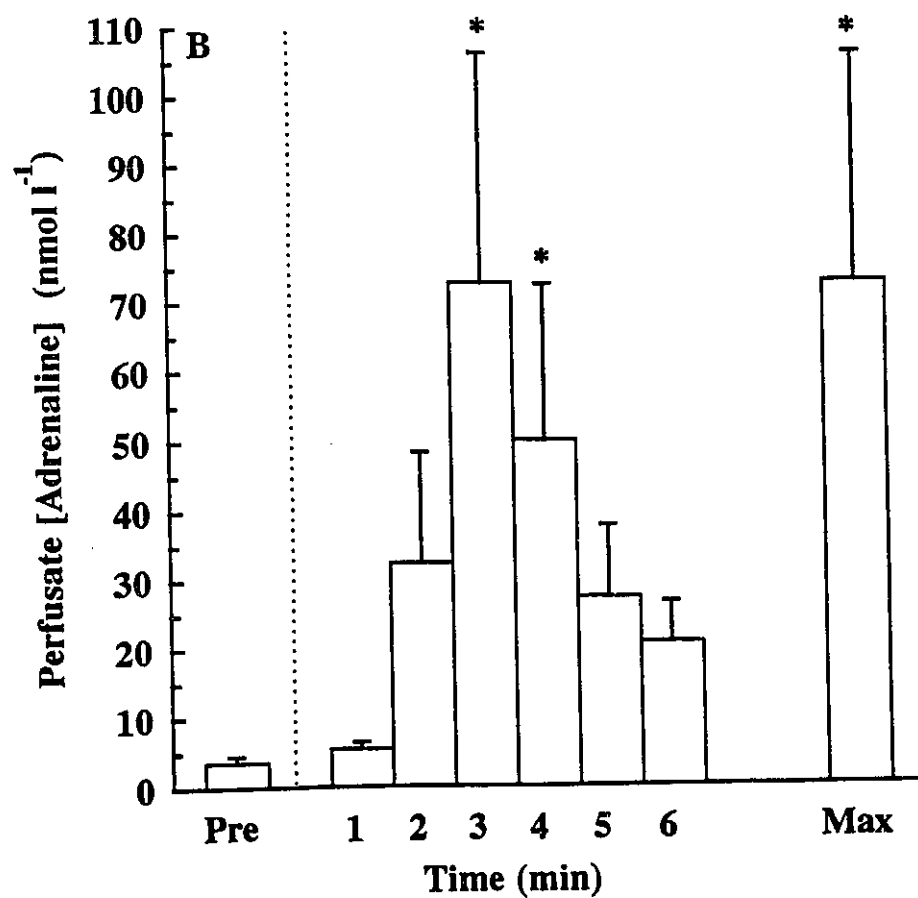
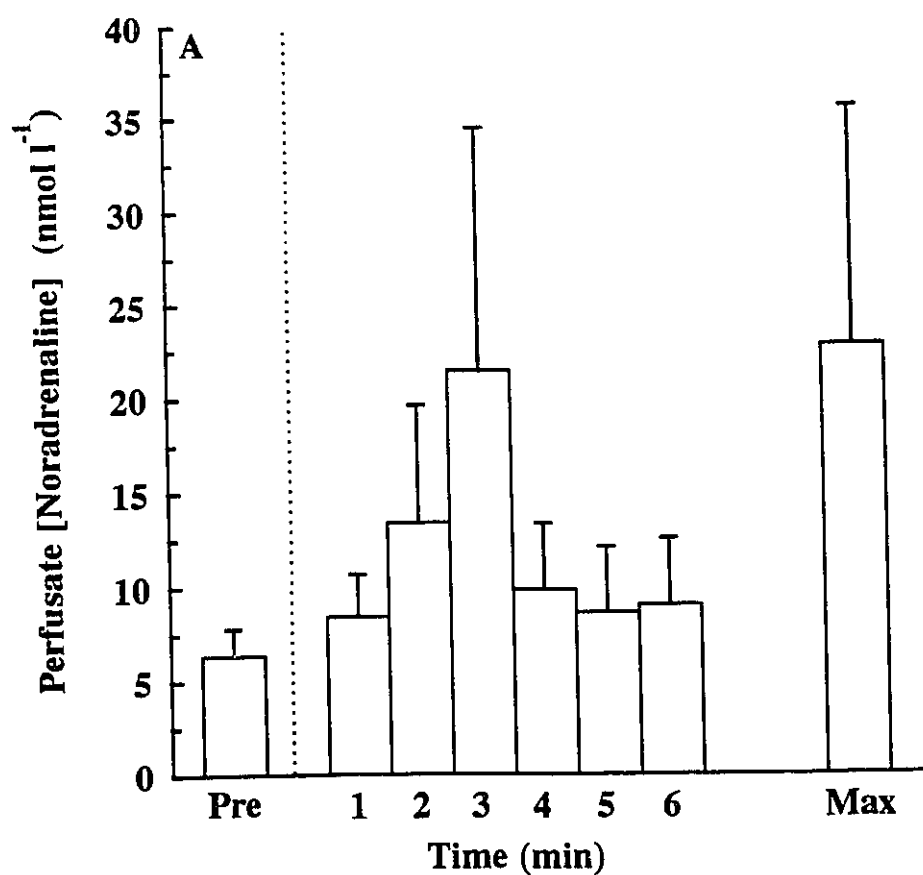


Figure 2-5

The inability of hexamethonium to completely inhibit the carbachol-induced release of adrenaline suggests the involvement of muscarinic cholinceptors in the process of catecholamine release. Chapter 5 addresses the question of muscarinic versus nicotinic receptor-evoked release of catecholamines in the American eel. However, in trout, muscarinic receptors may be involved only in the release of adrenaline since hexamethonium prevented the release of noradrenaline. This suggests the presence of two different chromaffin cell types, those containing adrenaline and those containing noradrenaline, a conclusion further supported by results within chapters 3 and 7.

Nicotinic receptor-evoked catecholamine secretion from chromaffin cells requires the presence of extracellular calcium ions as an influx of calcium into the cells initiates a series of intracellular events leading to the exocytotic release of catecholamines (Burgoyne 1991). The Ca^{2+} dependence of catecholamine release, from the trout chromaffin tissue, was tested by perfusing a set of preparations with Ca^{2+} -free saline prior to, during and after an injection of 2.5×10^{-6} mol carbachol. Under these conditions, the injection of carbachol did not elicit the release of either catecholamine (figure 2-6) suggesting that the presence of extracellular calcium is an absolute requirement for catecholamine secretion.

Muscarinic receptor stimulation is associated with a mobilization of calcium from intracellular stores in contrast to nicotinic receptor stimulation which causes an influx of calcium into the cell through ion channels (Burgoyne 1991). The observation that hexamethonium does not completely prevent carbachol-induced adrenaline release (figure 2-5b), suggesting involvement of the muscarinic receptor, seems to be at odds with the requirement for extracellular calcium in the process of release (figure 2-6). It is possible

that the mobilization of calcium from intracellular stores may, in itself, be insufficient to evoke release. However, a rise in intracellular calcium due to muscarinic receptor stimulation may subsequently cause the opening of voltage-dependent calcium channels and permit an influx of extracellular calcium. The influx of extracellular calcium would then evoke the release of catecholamines. Indeed, the delayed rise in perfusate adrenaline following carbachol administration in the presence of hexamethonium (figure 2-5b) is consistent with this suggestion.

The results of these validation experiments demonstrate that the release of stored catecholamines from the chromaffin tissue occurs in response to cholinergic agonist stimulation and requires the presence of extracellular calcium. Thus the chromaffin cells in this preparation are responding to pharmacological manipulation in a manner expected based on available information regarding the secretory response from vertebrate chromaffin cells (Burgoyne 1991). As such, this preparation appears to be a valid tool with which to examine various aspects of catecholamine release from the chromaffin cells of teleost fish.

Figure 2-6. Perfusate (a) noradrenaline and (b) adrenaline levels in response to an injection of carbachol (2.5×10^{-6} mol) following 20 min pre-perfusion with Ca^{2+} -free media (with EDTA). The pre-value represents perfusate catecholamine levels prior to the injection of carbachol. The dotted line represents the injection of carbachol. Following collection of the pre-sample and the 1 min drug delivery period, the samples were collected each minute for the next 6 min. In addition to the time course of release, the maximal release value (Max) is also reported on the right of the histogram. The data are shown as the mean $\pm$ 1 standard error of the mean.

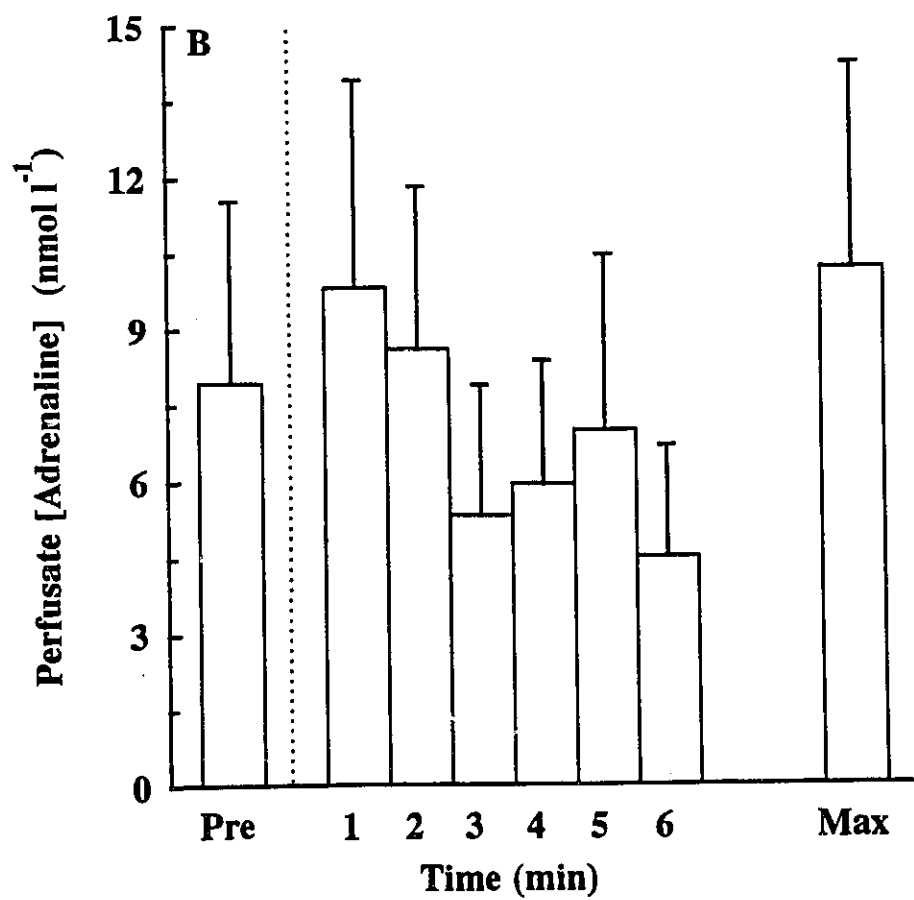
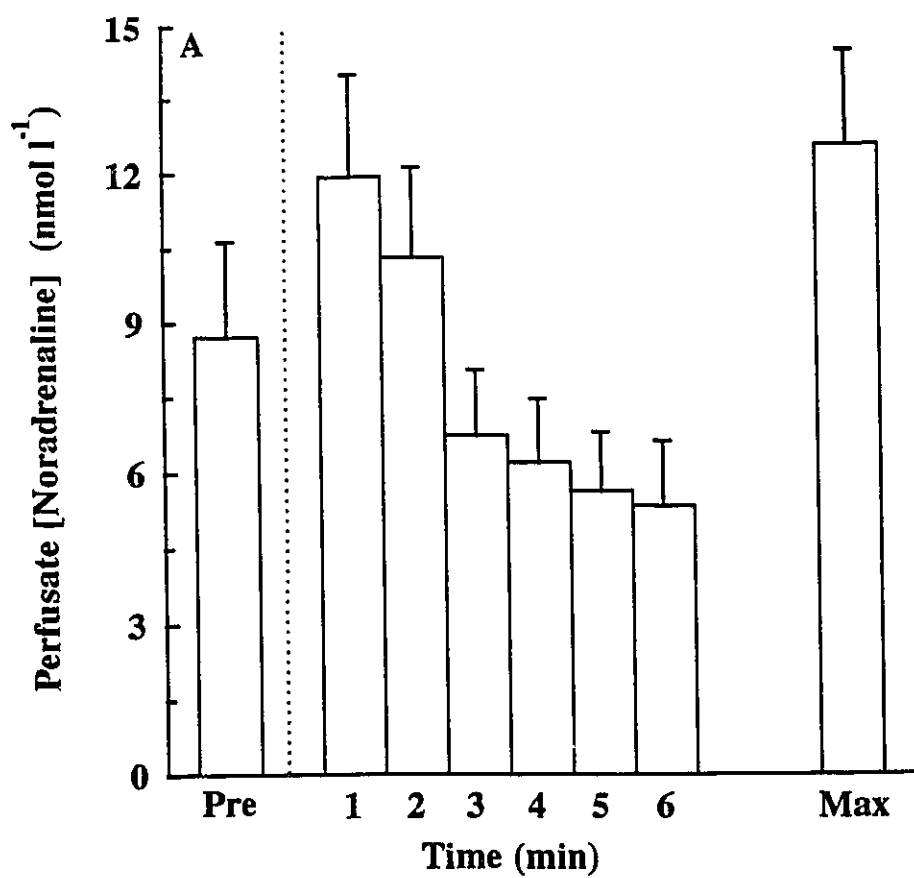


Figure 2-6

CHAPTER 3.

STORAGE AND DIFFERENTIAL RELEASE OF CATECHOLAMINES IN RAINBOW TROUT (*Oncorhynchus mykiss*) AND AMERICAN EEL (*Anguilla rostrata*)

INTRODUCTION

In response to severe environmental hypoxia, teleost fish release the catecholamine hormones, adrenaline and noradrenaline, into their circulation (Boutilier *et al.* 1988; Ristori and Laurent 1989; Perry *et al.* 1991; Kinkead *et al.* 1991; Thomas *et al.* 1991; Perry and Reid 1992; see review by Randall and Perry 1992). Although relatively few species have been examined, it is nevertheless apparent that plasma catecholamine levels during hypoxia can be quite varied among different species (see Randall and Perry 1992). Perry and Reid (1992) exposed rainbow trout (*Oncorhynchus mykiss*) and American eels (*Anguilla rostrata*) to varying levels of environmental hypoxia reaching as low as 35 torr water PO_2 (P_wO_2) for trout and 20 torr P_wO_2 for eel. In both species, defined arterial PO_2 (P_aO_2) and arterial oxygen content (C_aO_2) thresholds for the release of catecholamines were observed with the partial pressure thresholds corresponding approximately to the P_{50} values of haemoglobin. Despite these similarities, plasma adrenaline levels in trout reached a concentration of approximately 300 nmol l^{-1} whereas the levels in the eel were only $4\text{-}5 \text{ nmol l}^{-1}$. The situation was similar for noradrenaline with levels of approximately 100 nmol l^{-1} and 3 nmol l^{-1} reported for the trout and eel, respectively.

The goal of this chapter was to investigate the underlying reasons as to why plasma catecholamine levels are lower in the eel, compared to trout, given an equal or even more severe level of environmental hypoxia. This was accomplished by i) measuring the levels of stored catecholamines in tissues expected to make a major contribution to circulating levels and by ii) examining the ability of chromaffin cells within the posterior cardinal vein (PCV) to release

catecholamines in response to stimuli that are expected to cause release. The latter was accomplished using an *in situ* perfused PCV preparation (chapter 2).

MATERIALS AND METHODS

Experimental Animals

Rainbow trout (*Oncorhynchus mykiss*) weighing between 200 and 300 g (experimental N = 61) were obtained from Linwood Acres Trout Farm (Cambellcroft, Ontario) and were transported to the University of Ottawa in oxygenated water.

American eels (*Anguilla rostrata*) weighing approximately 100 g (experimental N = 89) were obtained from an eel ladder (Saunders Hydroelectric Dam, Cornwall, Ontario) and were transported on ice to the University of Ottawa.

Both trout and eels were maintained in large fibreglass tanks supplied with flowing, aerated, dechlorinated and dechloraminated City of Ottawa tapwater (see Perry *et al.* 1989 for water chemical composition). Water temperature was 12 °C. The photoperiod was maintained at 12L:12D. Trout were fed a commercial diet every second day until 48 h prior to experimentation. Eels were not fed. All fish were allowed to acclimate to the aquaria conditions for at least a period of three weeks before experimentation.

Environmental Hypoxia

In order to ensure that the fish utilized in this study responded to hypoxia in a similar manner as those used by Perry and Reid (1992), both trout and eels were exposed to two levels of environmental hypoxia, corresponding to the most severe levels used in the previous study.

Animal Preparation

Rainbow trout were anaesthetized in a solution (0.1 g l^{-1}) of ethyl-aminobenzoate (MS 222, Sigma) adjusted to pH 7.5 with sodium bicarbonate and were placed onto an operating table where the gills were continuously irrigated with a solution of the anaesthetic. An indwelling polyethylene cannula (Clay-Adams PE 50; internal diameter = 0.580 mm, outer diameter = 0.965 mm) was implanted into the dorsal aorta (Soivio *et al.* 1975) to permit periodic blood sampling. The trout were then placed into individual opaque Perspex boxes supplied with aerated flowing water and allowed to recover for 48 h prior to experimentation.

American eels were anaesthetized in a solution of MS 222 (2 g l^{-1} ; adjusted to pH 7.5 with sodium bicarbonate) and transferred to a dissecting tray. The eels were kept out of water without gill irrigation for the duration of the surgery. The pneumogastric artery was exposed, isolated and cannulated according to Hyde and Perry (1990). The eels were allowed to recover for 48 h in individual Perspex boxes supplied with flowing, aerated water prior to experimentation.

Experimental Protocol

Trout and eels were exposed to two levels of environmental hypoxia. For trout the P_{wO_2} was either 30 or 40 torr and for eels either 20 or 30 torr. The levels of hypoxia chosen were based on the study of Perry and Reid (1992) and were expected to yield arterial oxygen partial pressure levels around and below the P_{50} (the PO_2 of the blood yielding 50% haemoglobin-oxygen binding) value for each species.

The fish were subjected to hypoxia by halting their normal aerated water flow and replacing it with hypoxic water. The hypoxic water was derived from a water/gas equilibration column pre-set to a desired PO_2 by adjusting both the rate of water and nitrogen flow through the column. The water flow through the column was sufficient to allow the water within the experimental box to reach the desired final PO_2 within 5 min. Both the water flowing from the column and water within the box were continuously monitored for PO_2 (see below). The P_wO_2 within the box never varied more than 2 torr from the desired target P_wO_2 and it was this value that was used to calculate the mean P_wO_2 for the various groups.

A blood sample (0.65 ml) was withdrawn from the arterial cannula in each fish prior to, and 15 min after, the desired degree of hypoxia was reached. The blood was analyzed immediately for arterial oxygen partial pressure (P_aO_2), oxygen content (C_aO_2), and haemoglobin concentration ([Hb]). The remaining blood was centrifuged (30 s, X 12 000 g) and the plasma stored at -80 °C for subsequent analysis of catecholamines. The red blood cells from the pre-hypoxia sample were suspended in Cortland saline (Wolf 1963) and re-injected into the dorsal aorta or pneumogastric artery.

Determination of catecholamine storage levels

Rainbow trout (N = 6) and American eels (N = 6 for PCV levels; N = 9 for kidney and heart levels) were killed by a blow to the head or anaesthetic overdose (10 g l⁻¹ MS 222), respectively (see below for comment on the differing methods of killing). In all cases, the kidney and the

posterior cardinal vein (PCV) were divided into thirds (anterior, middle, and posterior) based on their total length. Each of these regions were removed from the animal and placed into pre-weighed microcentrifuge tubes. In addition to the kidney and PCV, the ventricle and the bulbus arteriosus also were removed. Once the tissue wet mass was determined, 1 ml of 4% perchloric acid (PCA) containing 2 mg ml⁻¹ of EDTA and 0.5 mg ml⁻¹ of sodium bisulphate was added to each tube and the samples homogenized (Nilsson 1989). The supernatants were then diluted 100 X in the same PCA solution, stored at -80 °C and subsequently analyzed for catecholamines (see below).

Storage levels were measured in eels within one week of their arrival in the aquatic facility and six months later. The levels of stored catecholamines were identical in each group indicating that the lack of feeding in the eels was not affecting storage levels and therefore the comparison with fed trout was valid.

***In situ* experiments**

The *in situ* saline-perfused PCV preparation (chapter 2) was used to assess the ability of the chromaffin cells in each species to respond to two different stimuli expected to cause membrane depolarization and thus the exocytotic release of catecholamines. In the first series of experiments the vein was perfused with Cortland saline containing 60 mmol l⁻¹ K⁺ in order to elicit catecholamine release via direct membrane depolarization. A second series involved a bolus injection of the acetylcholine analogue carbachol (carbamylcholine; 10⁻⁸ to 10⁻⁵ mols in 0.3 ml) in order to evaluate cholinergic-induced catecholamine release.

Rainbow trout were killed by a blow to the head whilst American eels were killed by overdose with anaesthetic (10 g l^{-1} MS 222). The experimental protocol initially called for both species to be killed by a blow to the head. Whilst this technique was suitable for trout it was not for eels because the body of an eel will continue to move owing to nervous reflexes in a manner which is highly disruptive to the surgery and perfusion procedure. To eliminate this problem the eels were killed with an anaesthetic overdose, a method which eliminates entirely the reflex movements. To ensure that the two methods of killing were comparable, and would not jeopardise the interspecific comparison, levels of stored catecholamines were measured in a separate group of trout killed either by an anaesthetic overdose (10 g l^{-1} MS 222; $N = 8$) or by a blow to the head ($N = 8$). Also, the *in situ* release of catecholamines in response to injections of 10^{-6} or 10^{-7} mols carbachol ($N = 6$ for each dose) was measured in trout killed by an overdose of anaesthetic. Neither the levels of stored catecholamines nor the measured perfusate levels in response to carbachol injections differed in trout killed by anaesthetic overdose from those killed by a blow to the head. Thus, with respect to catecholamine storage and *in situ* release, any differences observed between the two species **cannot** be explained by the different methods used to kill the animals. Moreover, it was shown previously (Reid *et al.* 1994) that long term food deprivation (2 months) in rainbow trout was without effect on catecholamine release *in situ*. Thus it is also unlikely that the absence of feeding in eels could contribute to differences in catecholamine release between the two species.

Analytical Techniques

During the *in vivo* experiments, blood and water PO₂ were measured using PO₂ electrodes (Radiometer E5046) housed in thermostatted (12 °C) cuvettes. P_wO₂ from the water/gas equilibration column and the experimental boxes was monitored continuously by allowing the water to flow by siphon across the electrodes. The PO₂ electrodes were used in conjunction with Radiometer PHM-71 acid-base analyzers and, in the case of blood samples, with BMS3 MK2 blood micro-systems.

Whole blood pH was determined with a micro-capillary pH electrode (Radiometer G299A) in conjunction with a Radiometer PHM-71 acid-base analyzer and BMS3 MK2 blood micro-system. Oxygen content (CaO₂) was measured on 20 µl samples according to an established method (Tucker 1967) using a Radiometer PO₂ electrode in a sealed chamber maintained at 37 °C. Haemoglobin measurements were performed in duplicate on 20 µl blood samples using a commercial spectrophotometric haemoglobin assay kit (Sigma Chemical Company).

Statistical Analysis

The data are presented as the mean $\pm$ 1 standard error of the mean (SEM). Where appropriate, the data have been statistically analyzed either by a two sample t-test or a one way analysis of variance followed by Fishers least significant difference test for multiple comparisons. *In vivo* data were analyzed using a paired t-test. The fiducial limits of significance were 5%.

RESULTS

In vivo Hypoxia

The results of this experiment are consistent with those of Reid and Perry (1992). In both groups of trout ($P_{wO_2} = 40$ or 30 torr) the P_{aO_2} fell to 13.5 - 15.9 torr (Table 3-1). In the eel ($P_{wO_2} = 30$ or 20 torr), oxygen partial pressure fell to 7.8 - 10.3 torr (Table 3-2). In trout, both adrenaline and noradrenaline increased significantly in the two groups whilst plasma catecholamines were unchanged in hypoxic eels (Fig 3-1).

Catecholamine storage

The storage of catecholamines within the various tissues is expressed on a concentration basis (μg of catecholamine g^{-1} tissue wet weight). Figure 3-2 illustrates the storage concentrations of adrenaline and noradrenaline in the trout and eel. This figure illustrates several points. First, the primary site of both adrenaline and noradrenaline storage in both species was the anterior two thirds of the posterior cardinal vein. Second, in these regions, the storage levels in trout were greater than those of the eels.

Figure 3-3 illustrates the tissue [adrenaline]/[noradrenaline] storage ratios for trout and eels. In all regions of the posterior cardinal vein and the heart the ratio was greater in eels than in trout; the ratios were similar in the regions of the kidney.

Table 1. Arterial blood respiratory variables in rainbow trout (*Oncorhynchus mykiss*) before and after 15 min exposure to external hypoxia (either 40 or 30 torr final P_wO_2). Values are shown as means $\pm$ 1 SEM. N = 6 - 7 in each group. All hypoxic values are significantly different from the pre-values with the exception of [Hb].

	40 torr		30 torr	
	Pre	15 min	Pre	15 min
P_wO_2 (torr)	158.9 $\pm$ 1.0	40.5 $\pm$ 0.4	157.6 $\pm$ 0.1	31.2 $\pm$ 0.8
P_aO_2 (torr)	72.4 $\pm$ 7.3	15.9 $\pm$ 1.8	102.4 $\pm$ 9.9	13.5 $\pm$ 2.2
C_aO_2 (vol%)	8.88 $\pm$ 0.49	3.43 $\pm$ 0.51	11.57 $\pm$ 1.29	2.97 $\pm$ 0.83
[Hb] (g/dl)	7.60 $\pm$ 0.64	8.28 $\pm$ 0.41	7.50 $\pm$ 0.89	6.63 $\pm$ 0.46
O_2/Hb (ml/g)	1.20 $\pm$ 0.10	0.46 $\pm$ 0.09	1.56 $\pm$ 0.08	0.45 $\pm$ 0.12

Table 2. Arterial blood respiratory variables in American eel (*Anguilla rostrata*) before and after 15 min exposure to external hypoxia (either 30 or 20 torr final P_wO_2). Values are shown as means $\pm$ 1 SEM. N = 6-7 in each group. All hypoxic values are different from the pre-values with the exception of [Hb].

	30 torr		20 torr	
	Pre	15 min	Pre	15 min
P_wO_2 (torr)	157.9 $\pm$ 1.5	29.0 $\pm$ 0.5	156.5 $\pm$ 1.6	20.8 $\pm$ 0.5
P_aO_2 (torr)	90.3 $\pm$ 13.9	10.3 $\pm$ 0.5	107.0 $\pm$ 9.5	7.8 $\pm$ 0.5
C_aO_2 (vol %)	6.80 $\pm$ 1.06	3.02 $\pm$ 0.51	8.30 $\pm$ 1.22	3.27 $\pm$ 0.49
[Hb] (g/dl)	7.22 $\pm$ 1.14	7.50 $\pm$ 1.15	6.44 $\pm$ 0.60	6.74 $\pm$ 0.07
O_2/Hb (ml/g)	0.96 $\pm$ 0.07	0.53 $\pm$ 0.13	1.28 $\pm$ 0.11	0.07 $\pm$ 0.04

Figure 3-1. The effects of acute (15 min) external hypoxia on (a) plasma [adrenaline] and (b) plasma [noradrenaline] in rainbow trout (open bars) and American eel (closed bars). Trout were exposed to 40 or 30 torr water PO_2 while eels were exposed to 30 or 20 torr. Values are shown as means $\pm$ 1 SEM (N = 6-7 for each group). An asterisk (*) denotes that the hypoxic (H) value was significantly different from the corresponding pre-hypoxia (P) value. The inserted panels are enlargements of the catecholamine values in the eel.

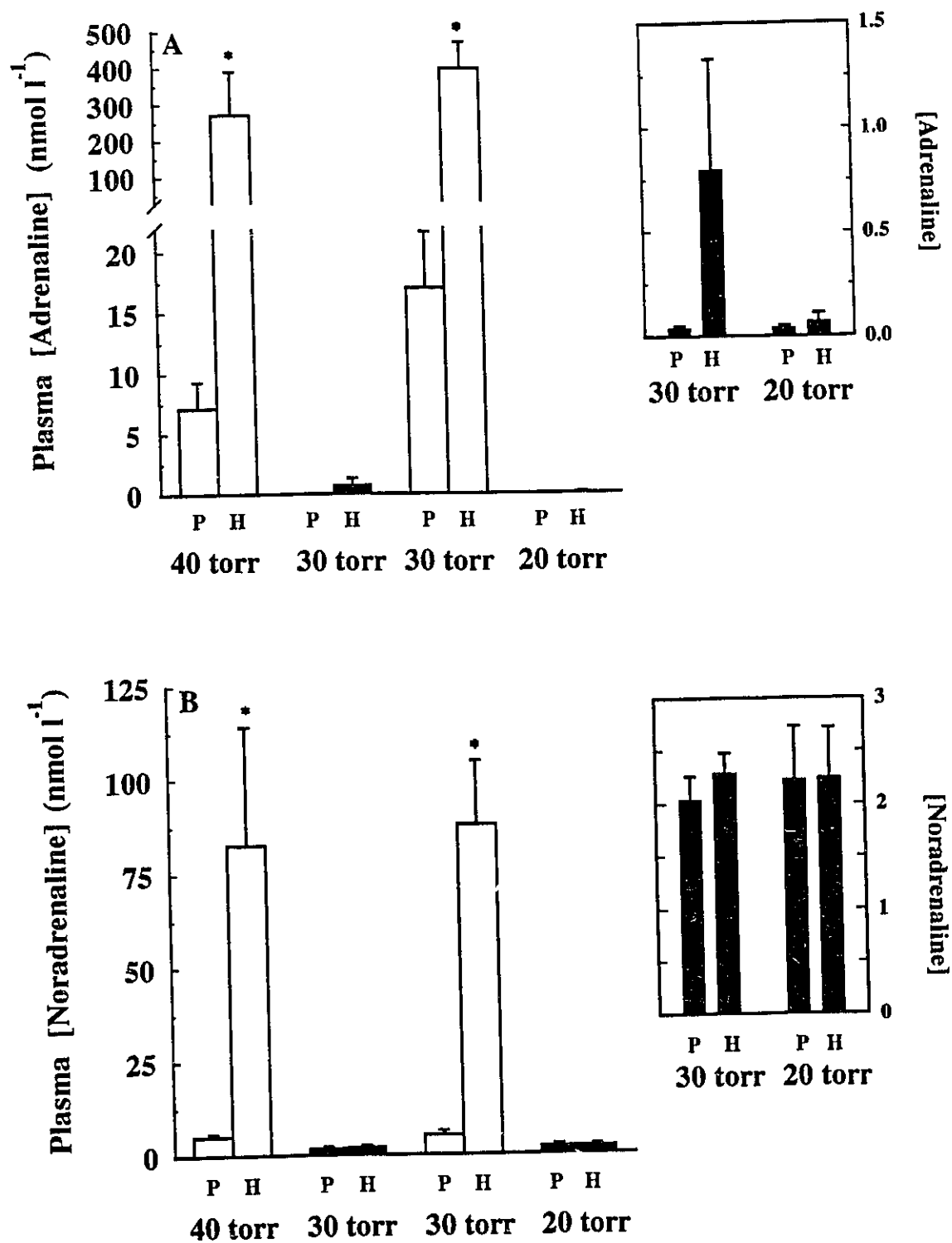


Figure 3-1

Figure 3-2. Tissue concentrations of (a) adrenaline and (b) noradrenaline (expressed as $\mu\text{g g}^{-1}$) in rainbow trout (open bars) and American eels (closed bars). Values are shown as means $\pm$ 1 SEM (N = 6 for trout; N = 6-9 for eels). + denotes a significant difference in the tissue concentration between the two species. Abbreviations: **ak**, anterior third of the kidney; **mk**, middle third of the kidney; **pk**, posterior third of the kidney; **apcv**, anterior third of the posterior cardinal vein (PCV); **mpcv**, middle third of the PCV; **ppcv**, posterior third of the PCV; **v**, ventricle; **b**, bulbus arteriosus.

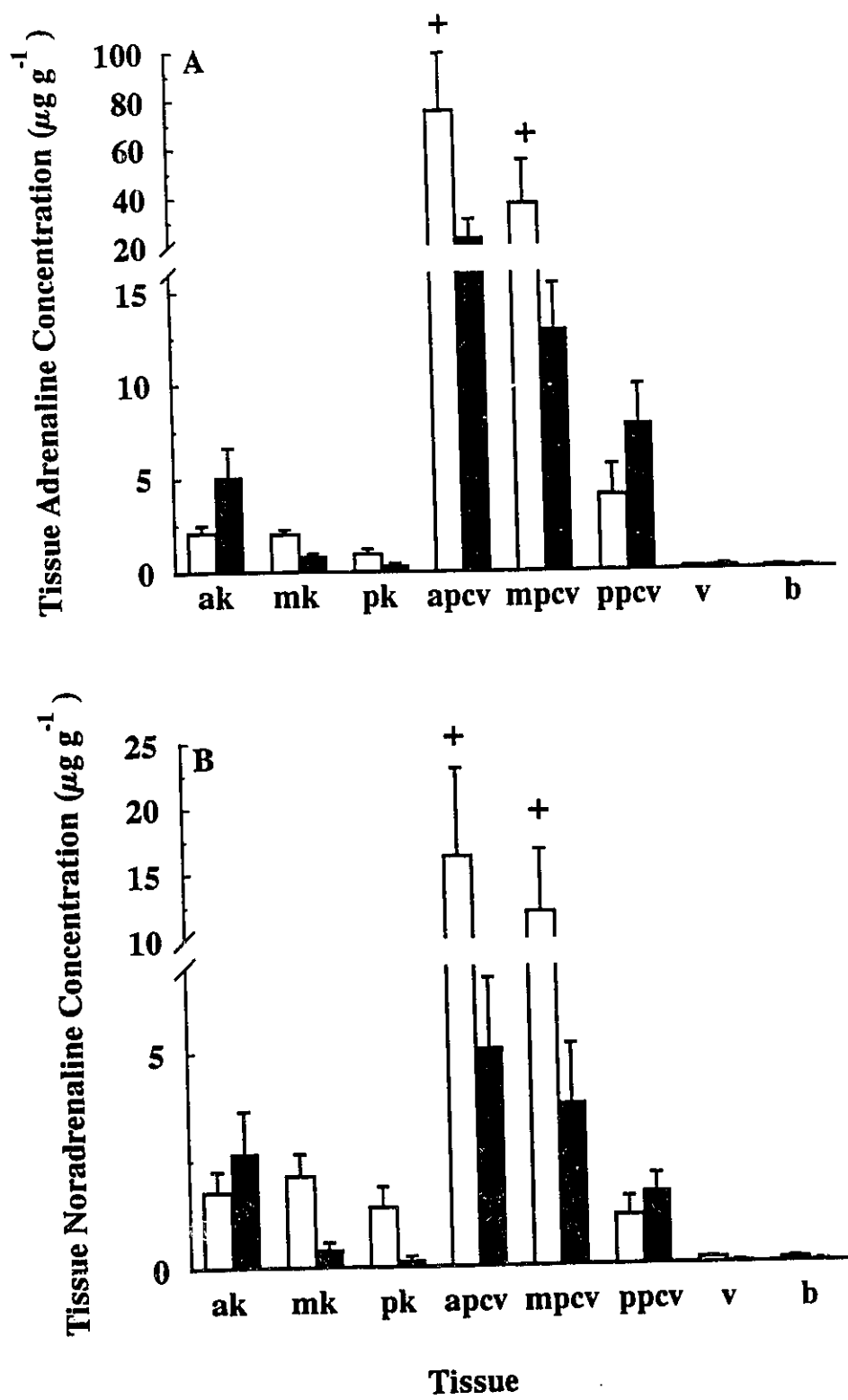


Figure 3-2

Figure 3-3. Tissue [adrenaline]/[noradrenaline] storage ratios in rainbow trout (open bars) and American eels (closed bars). Values are shown as means $\pm$ 1 SEM (N = 6 for trout; 6-9 for eels). + denotes a significant difference in the storage ratio between the two species. Abbreviations: The same as for figure 3-2.

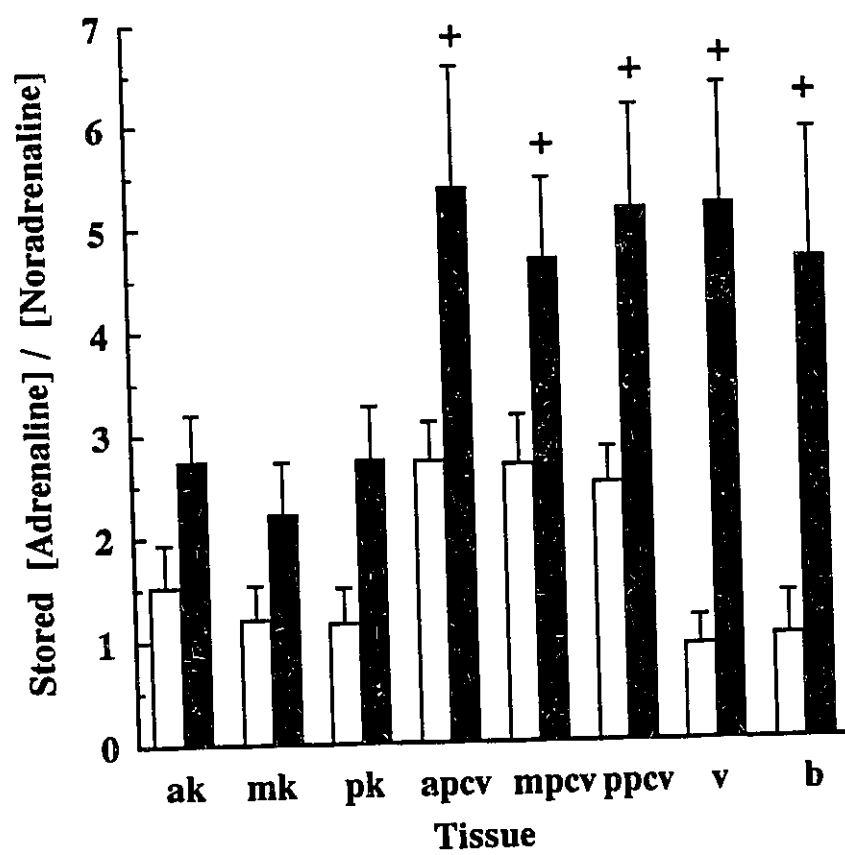


Figure 3-3

***In situ* Experiments**

Figure 3-4 shows the results of constant perfusion with $60 \text{ mmol l}^{-1} \text{ K}^{+}$ through the PCV. In trout, $60 \text{ mmol l}^{-1} \text{ K}^{+}$ caused a marked release of both adrenaline (fig 3-4a) and noradrenaline (fig 3-4b) into the perfusate with levels reaching upwards of 600 nmol l^{-1} in the fifth min. In eel, the response to $60 \text{ mmol l}^{-1} \text{ K}^{+}$ was less pronounced; adrenaline levels reached approximately 25 nmol l^{-1} and were significantly elevated from the pre- K^{+} values only in the seventh min. Similarly, noradrenaline levels increased significantly only in the fourth min and the levels throughout were approximately 5 nmol l^{-1} .

Figure 3-5 illustrates the time course of both adrenaline (a, b and c) and noradrenaline (d, e and f) release in response to a bolus injection of saline (a and d), 2×10^{-7} mols carbachol (b and e) and 10^{-6} mols carbachol (c and f). These are representative doses to illustrate the pattern of catecholamine release following carbachol administration. The values for the other doses of carbachol (10^{-8} , 2×10^{-8} , 10^{-7} and 10^{-5} mols) are not shown in this figure. The perfusate catecholamine levels in the fifth through eighth min post-injection also are not shown as they had, in all cases, returned to pre-injection levels. All of the doses greater than 2×10^{-8} mols caused the release of adrenaline in both trout and eel. The release was short-lived and occurred in the first several min after the bolus injection. At all of the doses of carbachol where both species released adrenaline, the levels attained in the trout were significantly greater than in the eel. Injections of saline did not elicit adrenaline or noradrenaline release in either species. The two lowest carbachol doses did not evoke noradrenaline release in either species. The four highest doses produced

noradrenaline release in both species with the levels attained in the trout always being greater than those in the eel. In summary, Fig 3-5 and the data mentioned above demonstrate that at all levels of carbachol eliciting a response, trout released greater quantities of catecholamines than did eels.

Figure 3-6 depicts dose-response curves for carbachol-evoked catecholamine release in the two species. The two inserts show the dose response curves for eel on a different scale to more clearly illustrate that catecholamines levels do indeed increase in the eel in response to carbachol. This analysis also indicates a greater responsiveness, to carbachol, in the trout than in the eel. In both species, adrenaline and noradrenaline release was seen to occur in response to the four highest doses of carbachol with the release always being greater in the trout than in the eel. In all cases, the peak value in each animal occurred within the first 3 min following the carbachol injection.

Figure 3-7 depicts the changes in the [adrenaline]/[noradrenaline] ratio in the trout in response to a saline injection, three representative doses of carbachol and 60 mmol l⁻¹ K⁺. The saline injection produced no change in the [adrenaline]/[noradrenaline] ratio. 10⁻⁸ mols of carbachol caused no change whereas 2 x 10⁻⁷ mols produced a transient increase. 10⁻⁶ mols of carbachol resulted in an increase for the first 4 min after injection before the levels returned to pre levels. 60 mmol l⁻¹ K⁺ produced a delayed response with the ratio remaining constant during the initial stages of the perfusion and increasing during the last three min.

Figure 3-4. Perfusate (a) [adrenaline] and (b) [noradrenaline] in rainbow trout (open bars) and American eels (closed bars) in response to perfusion with Cortland saline containing 60 mmol l⁻¹ K⁺. The first two min represent perfusion with normal Cortland saline. The dotted line represents a switch to perfusion with the high K⁺. Values are shown as means $\pm$ 1 SEM (N = 6 for trout; 12 for eels). An asterisk (*) denotes a significant difference from the combined mean of the two pre samples (min 1-2) within an individual species; + denotes a significant difference in catecholamine levels between the two species at corresponding times.

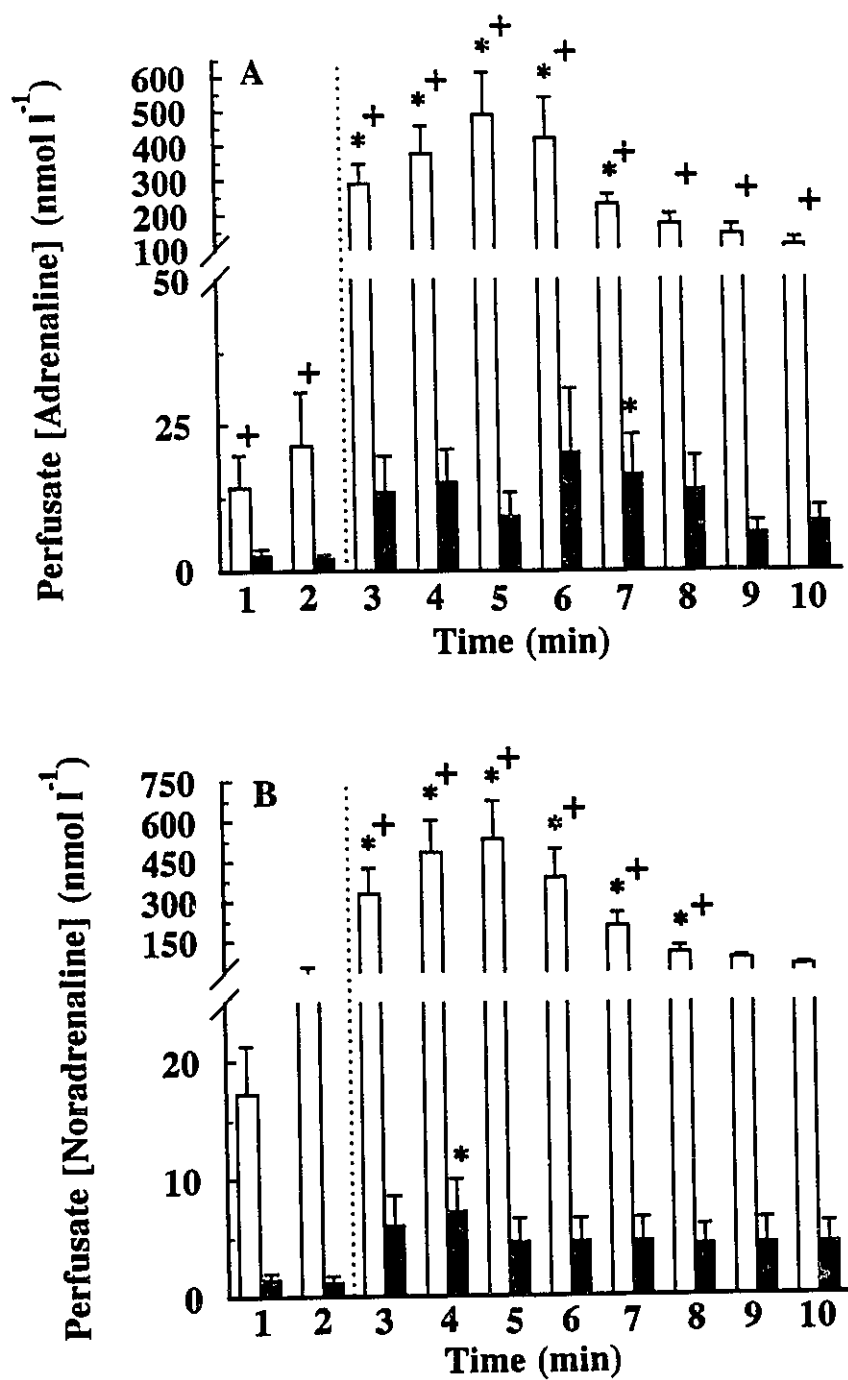


Figure 3-4

Figure 3-5. Perfusate adrenaline concentration ([adrenaline]) (a, b and c) and perfusate noradrenaline concentration ([noradrenaline]) (d, e and f) in rainbow trout (open bars) and American eels (closed bars) in response to injections of saline (a and d), 2×10^{-7} mols carbachol (b and e) and 10^{-6} mols carbachol (c and f). Values are shown as means $\pm$ 1 SEM (N = 6 for trout; 6-12 for eels). The first two min represent perfusion with normal Cortland saline. The dotted line represents the time at which the bolus injection of saline or carbachol was administered. An asterisk (*) denotes a significant difference from the combined mean of the two pre values (1-2 min); + denotes a significant difference in perfusate [adrenaline] or [noradrenaline] between the two species at corresponding times.

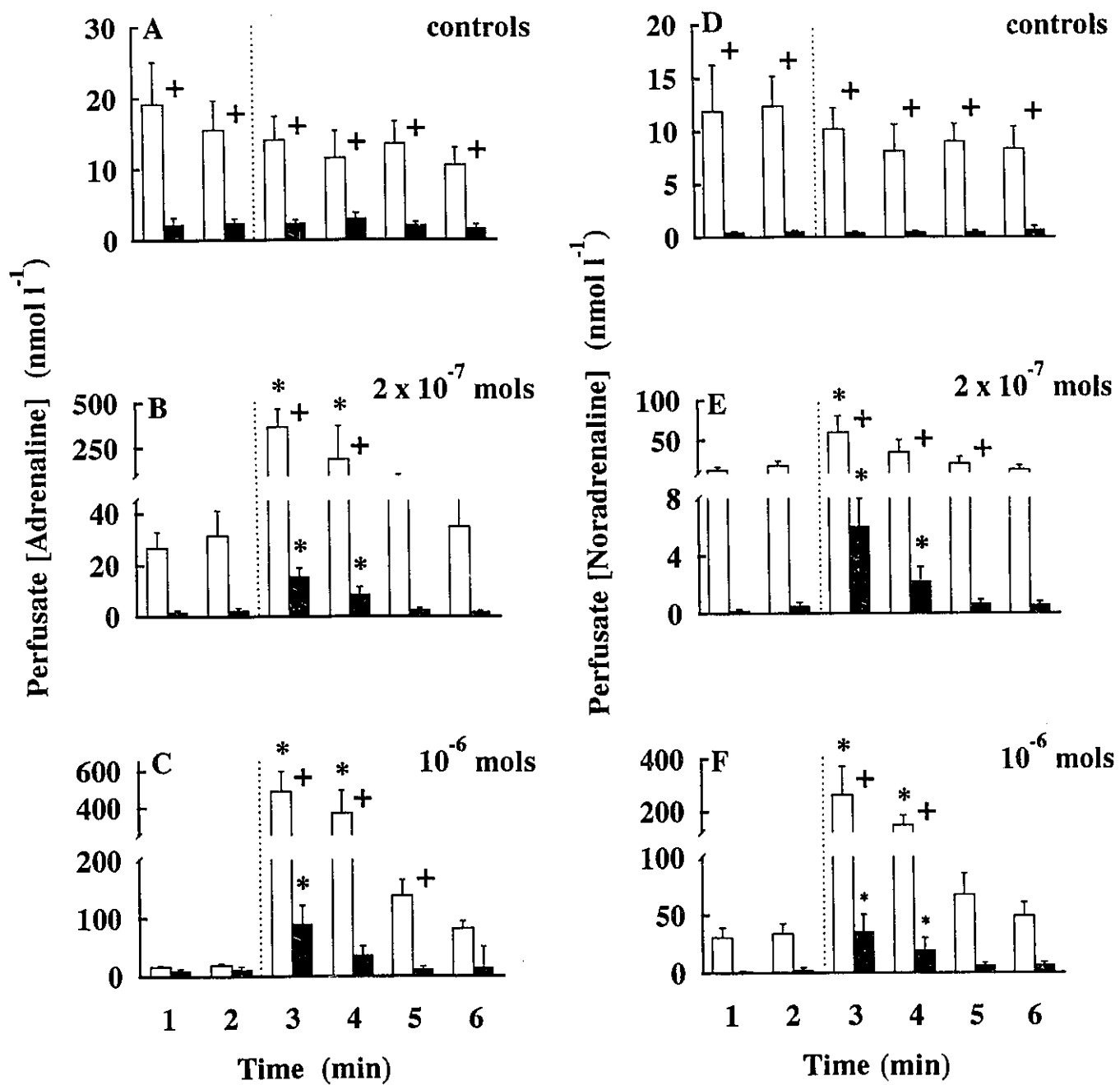


Figure 3-5

Figure 3-6. Dose response curves for carbachol depicting (a) maximum perfusate [adrenaline], and (b) maximum perfusate [noradrenaline], as a function of log carbachol dose in rainbow trout (open circles) and American eels (closed circles). Values are shown as means $\pm$ 1 SEM (N = 6 for trout; 6-12 for eels). The arrow points to the [catecholamine] observed in response to an injection of saline. An asterisk (*) denotes a significant difference in the [catecholamine] in response to a given dose of carbachol compared to the saline injection. + denotes a significant difference in levels between the two species for a given carbachol dose. The two inserts depict the dose response curves for the eel on a different scale in order to demonstrate that catecholamine levels did increase in response to several doses of carbachol.

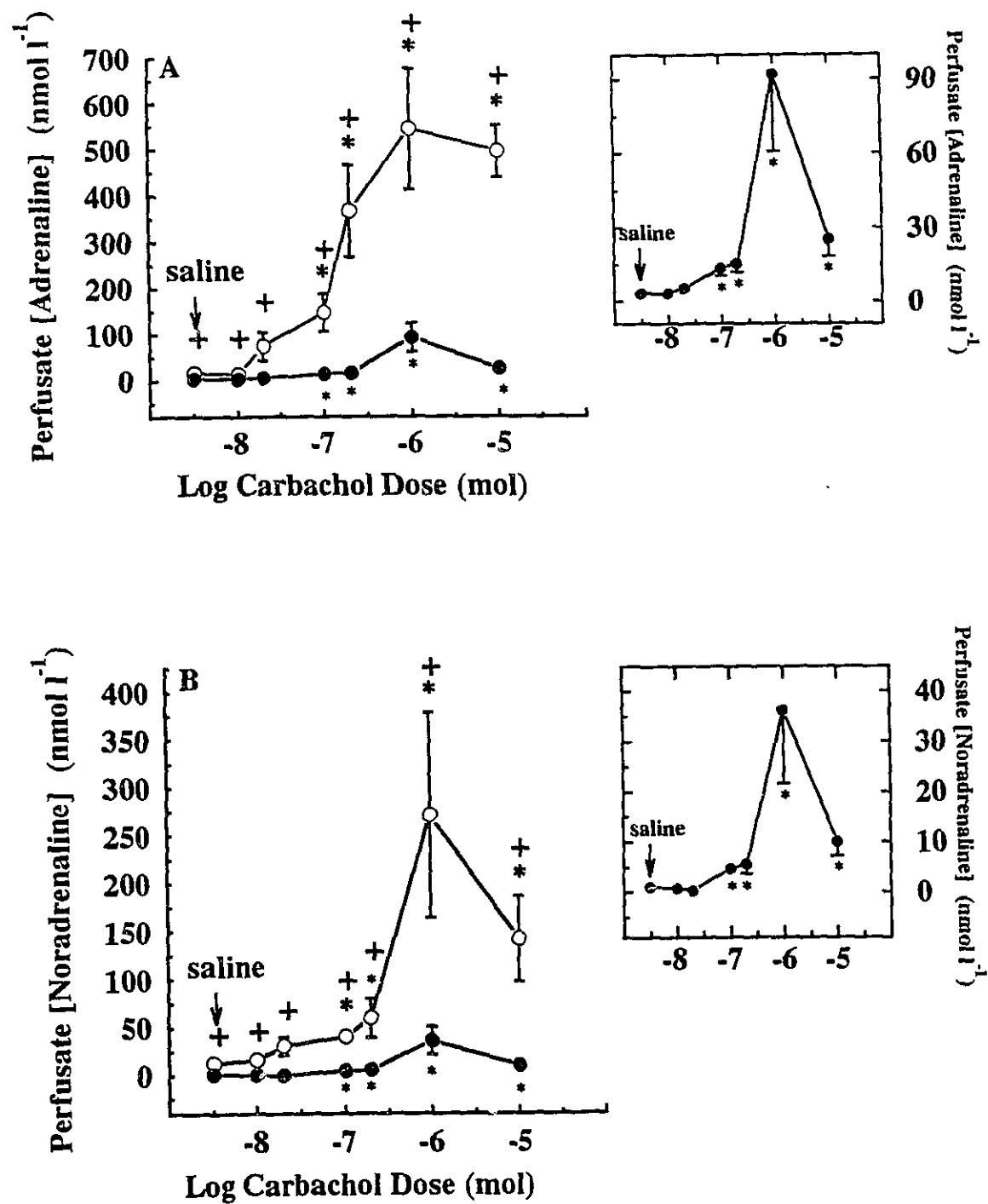


Figure 3-6

Figure 3-7. Perfusate [adrenaline]/[noradrenaline] ratios in response to (a) saline, (b-d) three doses of carbachol and (e) perfusion with $60 \text{ mmol l}^{-1} \text{ K}^{+}$ in rainbow trout. Values are shown as means $\pm$ 1 SEM (N = 6). An asterisk (*) denotes a significant difference from the combined mean of the two pre (1-2 min) values.

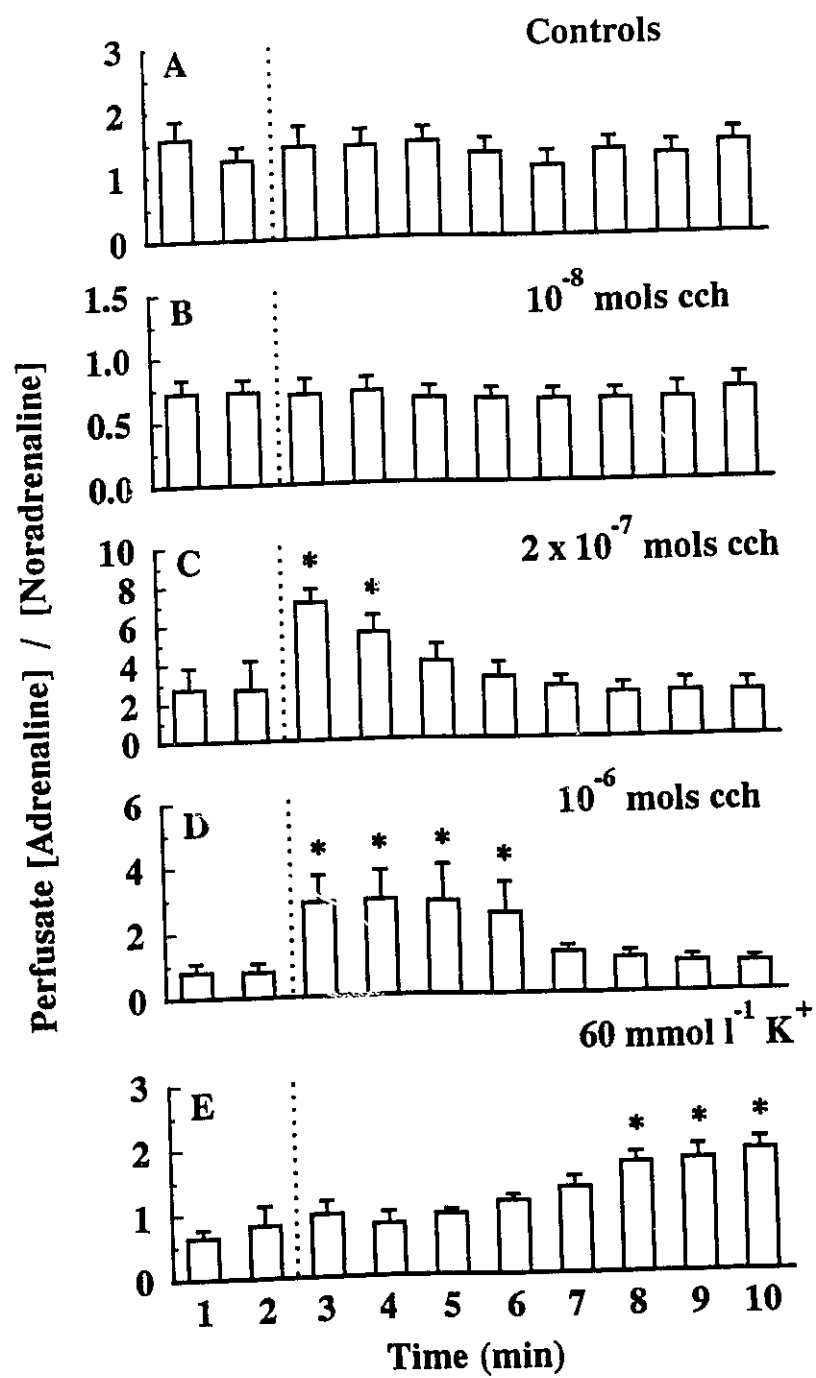


Figure 3-7

DISCUSSION

In vivo Hypoxia

The primary goal of this chapter was to evaluate the underlying mechanisms responsible for the widely differing plasma catecholamine levels observed during environmental hypoxia (Perry and Reid 1992) in two species, rainbow trout and American eels that exhibit widely different tolerances to hypoxia. The results of the initial component of this chapter confirm, in part, those of Perry and Reid (1992).

Trout exposed to 40 or 30 torr hypoxia and eels exposed to 30 or 20 torr hypoxia displayed P_aO_2 values consistent with those of the previous study. Plasma adrenaline or noradrenaline levels did not increase significantly in either group of eels whereas both catecholamines were greatly elevated in trout.

Catecholamine Storage

There are several hypotheses which may explain the differences in catecholamine levels seen during hypoxia in trout and eel. First, it is conceivable that differing levels of stored catecholamines may contribute to the different plasma levels. This could be a plausible explanation if trout stored large quantities of catecholamines which would be available for mobilization into the circulation whilst eel stored practically none, relying primarily on *de novo* synthesis to provide catecholamines for release into the circulation. However as was demonstrated in this study, this is probably not an

underlying cause as both species stored catecholamines in sufficient quantities to elevate plasma levels under appropriate releasing stimuli.

It is apparent from this study that the anterior region of the PCV is the primary storage site for both adrenaline and noradrenaline with the middle region also storing considerable quantities. The kidney tissue itself contains less catecholamines compared to the PCV. The anterior and middle regions of the trout PCV contain more catecholamines than the corresponding regions in the eel. One could suggest, as was mentioned above, that these differences in storage may contribute to the differences in plasma catecholamines during hypoxia with the higher storage levels in the trout resulting in higher plasma levels. However, since the eel showed **no** significant increase in plasma catecholamines during hypoxia it is unlikely that differences in storage could contribute to this phenomenon as there are still large quantities of adrenaline and noradrenaline present in the eel PCV and kidney chromaffin cells and are therefore theoretically available for release. Moreover, the amount of catecholamines released into the circulation during hypoxia is only a small percentage ($\approx 2\text{-}3\%$) of the total level of stored catecholamines, further indicating that differential storage cannot be contributing to the differing plasma levels seen during hypoxia.

Stimulation of the Chromaffin Cells with a Cholinergic Agonist

At the level of cholinergic receptor stimulation of the chromaffin cells there are several possible differences that might explain the discrepancies in plasma catecholamine levels observed during hypoxia. The chromaffin cells of the eel may be less densely innervated by cholinergic fibres

in comparison to the trout. Another possibility is that the eel chromaffin cells have fewer cell surface cholinergic receptors than the trout, either in addition to or independent of the amount of cholinergic innervation. Also, the affinity of these receptors for acetylcholine and cholinergic agonists such as carbachol may differ in the two species. The results of this study indicate that the chromaffin cells of the trout respond to cholinergic agonist stimulation to a greater extent than those of the eel during *in situ* perfusion. As demonstrated in figure 3-6, both species begin to release catecholamines at the same dose of carbachol (10^{-7} mols). This suggests that the difference in the extent of the response to carbachol cannot likely be explained by a difference in the affinity of the cholinergic receptors for carbachol. The fact that the maximal catecholamine levels observed in trout were greater than those in eel suggests that trout chromaffin cells have greater numbers of cholinergic receptors than do the chromaffin cells in the eel or that one or more post-receptor events is amplified in the trout compared to eel. This differential ability to respond to stimulation with a cholinergic agonist may explain the differences observed in plasma levels during hypoxia.

An examination of the adrenaline/noradrenaline ratios in Fig 3-7 suggests the existence of two different types of chromaffin cells in trout. In the trout, perfusion with $60 \text{ mmol l}^{-1} \text{ K}^{+}$ caused no initial increase in the [adrenaline]/[noradrenaline] ratio whereas stimulation with carbachol, at higher doses, caused an immediate increase in the ratio. This suggests that carbachol selectively caused the release of adrenaline while K^{+} was non-selective. The delayed rise in the ratio observed with $60 \text{ mmol l}^{-1} \text{ K}^{+}$ was most likely the result of a depletion of noradrenaline, but not adrenaline, stores during the first several min of the K^{+} perfusion. Potassium causes the exocytotic release of catecholamines by directly altering the membrane potential without the intervention of receptors or

receptor-coupled second messenger systems (Burgoyne 1991). For this reason, K^+ would be expected to cause the maximal possible release of catecholamines in a non-selective manner. Acetylcholine/carbachol on the other hand, rely on their interaction with physiological receptors and the subsequent activation of an intracellular second messenger system to stimulate exocytotic release. Thus the fact that carbachol caused a preferential release of adrenaline whereas K^+ did not, suggests the existence of both adrenaline and noradrenaline containing chromaffin cells. The existence of two different types of chromaffin cells has been demonstrated previously in teleost fish (Mastrolia *et al.* 1981, 1984; Gallo *et al.* 1993; see chapter 2), urodele amphibians (Accordi 1991; Laforgia and Capaldo 1991) and mammals (Coupland 1984; Chritton *et al.* 1991). Also, chapter 7 reports histochemical evidence for the presence of two such cell types in teleost fish. The [adrenaline]/[noradrenaline] ratios in eel exhibited more variability and did not show an obvious trend for a differential release of adrenaline over noradrenaline.

Although the chromaffin cells within the posterior cardinal vein of the eel store less catecholamines than those of the trout they still store substantial quantities. It is thus somewhat surprising that $60 \text{ mmol l}^{-1} K^+$ did not cause a larger level of release than was observed. In this instance, one cannot implicate low affinity cholinergic receptors or a lack of receptors. Presumably there is some aspect of the exocytotic process which differs fundamentally between trout and eel and prevents the non-specific depolarising stimulus of K^+ from eliciting a larger response than was observed.

In summary, plasma catecholamines levels during hypoxia rise to a much greater extent in trout than in eels. These differences may be explained by differences in the responsiveness of the

chromaffin cells to cholinergic stimulation but probably not by differences in levels of stored catecholamines. Additionally, in the trout there appear to be two different populations of chromaffin cells, ones containing predominantly adrenaline and others, noradrenaline.

CHAPTER 4.

THE EFFECTS OF CORTISOL ON CATECHOLAMINE STORAGE AND RELEASE IN THE RAINBOW TROUT, *ONCORHYNCHUS MYKISS*

INTRODUCTION

A common response in the rainbow trout, *Oncorhynchus mykiss*, to a variety of stressors (see chapter 1) is the mobilisation into the circulation, of stress hormones such as catecholamines (i.e. adrenaline and noradrenaline; see review by Randall and Perry 1992) and cortisol (e.g. Donaldson 1981; Pickering *et al.* 1991; Vijayan and Moon 1992). Cortisol, the major corticosteroid produced by the interrenal cells (which like the chromaffin cells, are also located within the head kidney in teleost fish; Nandi 1961) is released into the circulation upon stimulation of these cells by adrenocorticotrophic hormone (ACTH) released from the pituitary (see review by Axelrod and Reisine 1984).

Given that catecholamines and cortisol are released into the circulation under stressful conditions, and that chromaffin and interrenal cells are in close physical proximity, the potential exists for both endocrine and paracrine interactions between the two cell types. Investigations on mammalian adrenal glands have revealed that corticosteroids are capable of increasing the activity of phenylethanolamine-N-methyltransferase (PNMT), the enzyme which catalyses the synthesis of adrenaline from its immediate precursor, noradrenaline (Wurtman and Axelrod 1966; Wurtman *et al.* 1967; Jiang *et al.* 1989; Wan and Livett 1989; Ross *et al.* 1990; Evinger *et al.* 1992; Bebito *et al.* 1993). Corticosteroids, such as cortisol, have been shown to influence gene transcription, messenger RNA stability, translation, and enzyme activity and stability (see review by Yamamoto 1985).

Whilst numerous studies have examined the effects of corticosteroids on catecholamine biosynthesis in mammalian species, few studies (Mazeaud 1972; Jönsson *et al.* 1983) have investigated possible interactions in fish. These studies revealed that, unlike in mammals, cortisol does not appear to increase *in vitro* PNMT activity in rainbow trout.

In addition to possible effects on catecholamine biosynthesis, cortisol may also exert a modulatory role on the process of catecholamine release. In rainbow trout,

chronic cortisol treatment increases the number of internalized β -adrenoceptors in the red blood cell (rbc) (Reid and Perry 1991; Reid *et al.* 1991; see review by Perry and Reid 1993) and upon appropriate stimulation (e.g. *in vitro* hypoxia; Reid *et al.* 1993) these receptors are translocated to the plasma membrane potentially sensitising the cells to β -adrenergic stimulation. Additionally, cortisol treatment can increase the number of cell surface β_2 -adrenoceptors on trout hepatocytes, thereby increasing hepatocyte catecholamine responsiveness (Reid *et al.* 1992; see review by Perry and Reid 1993). By analogy to the rbc and hepatocyte, cortisol may influence cholinceptor numbers both within and on the surface of the chromaffin cell, potentially increasing the capacity of these cells to release catecholamines in response to cholinergic stimulation.

Given the effect of cortisol on catecholamine biosynthesis/PNMT activity in mammals (e.g. Wurtman and Axelrod 1966; Wurtman *et al.* 1967), the apparent lack of an effect in rainbow trout (Mazeaud 1972; Jönsson *et al.* 1983), and the potential for cortisol to influence receptor numbers on the chromaffin cells (e.g. Reid and Perry 1991), the goal of this chapter was to examine the effects of chronic cortisol treatment on 1) the levels of catecholamines stored within various regions of the kidney and posterior cardinal vein, 2) the ability of the chromaffin cells to release catecholamines in response to cholinergic stimulation, and 3) *in vitro* phenylethanolamine N-methyltransferase (PNMT) activity.

MATERIALS AND METHODS

Experimental Animals

Rainbow trout (*Oncorhynchus mykiss*) weighing 229 ± 2 g (experimental N = 205) were obtained from Linwood Acres Trout Farm. Holding conditions were as outlined in chapter 3 with the exception of the water temperature which was 10 °C.

Cortisol-Treatment

Rainbow trout were given an intraperitoneal injection (0.35 ml) of either coconut oil alone (control) or containing 50 mg kg⁻¹ cortisol (hydrocortisone 21-hemisuccinate, Sigma). This method of implantation has been shown to release cortisol over a long period of time (Vijayan and Leatherland 1989; Perry and Reid 1994). The plasma cortisol concentration was significantly higher in the cortisol-treated group (31.1 ± 7.3 ng ml⁻¹) compared to the control group (2.4 ± 0.9 ng ml⁻¹) after 7 days of treatment.

Determination of Catecholamine Storage Levels Following Cortisol-Treatment

Following 1, 3 or 7 days of either cortisol or control treatment, rainbow trout were killed by a blow to the head and the kidney and posterior cardinal vein (PCV) were divided into thirds (anterior, middle and posterior) on the basis of their total length. The levels of catecholamines within these tissue regions were determined as outlined in chapter 3.

In Situ Catecholamine Release Following Cortisol-Treatment

Following 7 days of cortisol treatment, the catecholamine-releasing response of the chromaffin cells to cholinergic stimulation was assessed by injecting a bolus dose (10^{-8} , 2.5×10^{-8} , 5.0×10^{-8} , 10^{-7} , 5.0×10^{-7} , 10^{-6} , 5.0×10^{-6} , or 10^{-5} mols) of the cholinceptor agonist carbamylcholine (carbachol), through the injection port, into the inflow cannula.

Each preparation received only one dose of carbachol. The doses were chosen in accordance with previous studies utilising this preparation (Reid *et al.* 1994; see also chapter 3).

Phenylethanolamine-N-methyltransferase (PNMT) Activity Following Cortisol

-Treatment

Phenylethanolamine-N-methyltransferase (PNMT) activity was measured using an established assay protocol (Wurtman and Axelrod 1965; Wurtman *et al.* 1966; Abrahamson and Nilsson 1976; Abrahamson 1979, 1980; Jönsson 1983; Jönsson *et al.* 1983) on the third and seventh days of treatment (cortisol-treated and oil-treated). Rainbow trout were killed by a blow to the head and the anterior third of the kidney and posterior cardinal vein (head kidney region) was removed and placed into 4 ml of ice-cold phosphate buffered saline (0.1 mol l^{-1} , pH = 7.9) containing 0.3 mol l^{-1} sucrose. After determining wet mass, the tissue was homogenized using a pestle and centrifuged for 15 min at $10\,500 \text{ g}$. The supernatant was removed and centrifuged for 60 min at $105\,000 \text{ g}$. All centrifugations were performed at $4\text{ }^{\circ}\text{C}$. An aliquot of the supernatant ($100\text{ }\mu\text{l}$) was incubated, in 1.5 ml microcentrifuge tubes, for 30 min at $37\text{ }^{\circ}\text{C}$ with $150\text{ }\mu\text{l}$ of 1 mmol l^{-1} phenylethanolamine and $50\text{ }\mu\text{l}$ of ^{14}C -S-adenosylmethionine (dissolved in 0.1 mol l^{-1} phosphate buffer; specific activity = 58 mCi mmol^{-1}). In this reaction, phenylethanolamine serves as the substrate for methylation; ^{14}C -S-adenosylmethionine as the methyl group donor.

The reaction was terminated by the addition of 0.5 ml of 0.5 mol l^{-1} borate buffer (boric acid; pH = 10). The reaction mixture was then added to 6 ml of toluene:isoamylalcohol (97:3) where the radioactive product was extracted into the organic (toluene) phase. The mixture was shaken for 15 min and then centrifuged at 3000 g for 10 min. 4 ml of the toluene was added to a scintillation vial containing 10 ml of an organic

scintillation fluor (OCS, Amersham). Radioactivity was determined using a Canberra Packard (2500 TR) liquid scintillation counter, with CPM converted to DPM using an external standard technique.

Analytical Techniques

Plasma cortisol concentrations were determined using a radioimmunoassay kit (ICN Biomedicals, Carson, CA). The protein content of the standards was adjusted to reflect values found in trout plasma and increase assay sensitivity (Anderson *et al.* 1991).

Statistical Analysis

The data are presented as the mean $\pm$ 1 standard error of the mean (SEM). All statistical analysis, including determinations of normality and variance, was performed with a statistical software package (SigmaStat., Jandel Scientific). Paired data were analysed using either a paired t-test or a signed rank test. Non-paired data were analysed using a two sample t-test or a rank sum test. 5% was taken as the level of significance.

RESULTS

Catecholamine Storage Levels Following Cortisol Treatment

Figure 4-1 illustrates the effects of 1, 3 and 7 days of cortisol-treatment on the levels of noradrenaline stored within the kidney and posterior cardinal vein (PCV). On each day, the greatest concentrations of noradrenaline were located within the PCV. One day of cortisol treatment (fig 4-1a, d) had no effect on the levels of stored noradrenaline. On the third day of treatment (fig 4-1b, e) there was an increase in [noradrenaline] within the anterior and posterior thirds of the kidney as well as in the anterior and posterior thirds of the PCV (ppcv). Total kidney (tk) and total PCV (tpcv) [noradrenaline] were also greater than in control fish. Following seven days of cortisol-treatment (fig 4-1c, f), the posterior third of the kidney (pk) exhibited increased levels of noradrenaline. Within the cortisol-treated group, on the third day the [noradrenaline] within the middle third of the kidney was greater than on day 1. Additionally, on the seventh day the [noradrenaline] within the anterior, posterior and total kidney were greater than on day 1.

The effects of cortisol-treatment on stored adrenaline levels, within the kidney and PCV, are illustrated in figure 4-2. As with noradrenaline, the greatest concentrations of adrenaline occurred within the PCV. One day of cortisol-treatment did not influence the levels of adrenaline stored in the kidney or PCV (fig 4-2a, d). On the third day of treatment the [adrenaline] within the anterior, posterior and total kidney (ak, pk and tk) was elevated above the levels observed in control fish (fig 4-2b). Within the posterior cardinal vein the [adrenaline] was greater in all regions (apcv, mpcv, ppcv and tpcv) (fig 4-2e). Following seven days of treatment there were increases in all of the kidney regions (ak, mk, pk and tk) (fig 4-2c) as well as in the middle third of the PCV (mpcv) [total PCV, tpcv, $p = 0.06$] (fig 4-2f). On the seventh day, within the cortisol-treated group, the [adrenaline] in the anterior, posterior and total kidney (ak, pk, tk) was greater than on day 1.

Figure 4-1. The effects of 1 (a and d), 3 (b and e) and 7 (c and f) days of cortisol-treatment on levels of stored noradrenaline (μg noradrenaline g^{-1} tissue wet weight) within various regions of the kidney (a, b, and c) and posterior cardinal vein (PCV) (d, e, and f) in rainbow trout. In all cases, the open bar represents control fish (N = 6 on days 1 & 3; N = 4 on day 7); the dark bar represents cortisol-treated fish (N = 6 on days 1 & 3; N = 8 on day 7). The data are shown as the mean $\pm$ 1 SEM. An asterisk (*) denotes a significant difference between the control and cortisol-treated fish on any given day. A plus sign (+) denotes a significant difference between the value on day 3 or 7 with the value on day 1. Abbreviations: **ak**, anterior third of the kidney; **mk**, middle third of the kidney; **pk**, posterior third of the kidney; **tk**, total kidney (ie, the sum of the ak, mk and pk); **PCV**, posterior cardinal vein; **apcv**, anterior third of the PCV; **mpcv**, middle third of the PCV; **ppcv**, posterior third of the PCV; **tpcv**, total PCV (i.e. the sum of the apcv, mpcv and ppcv).

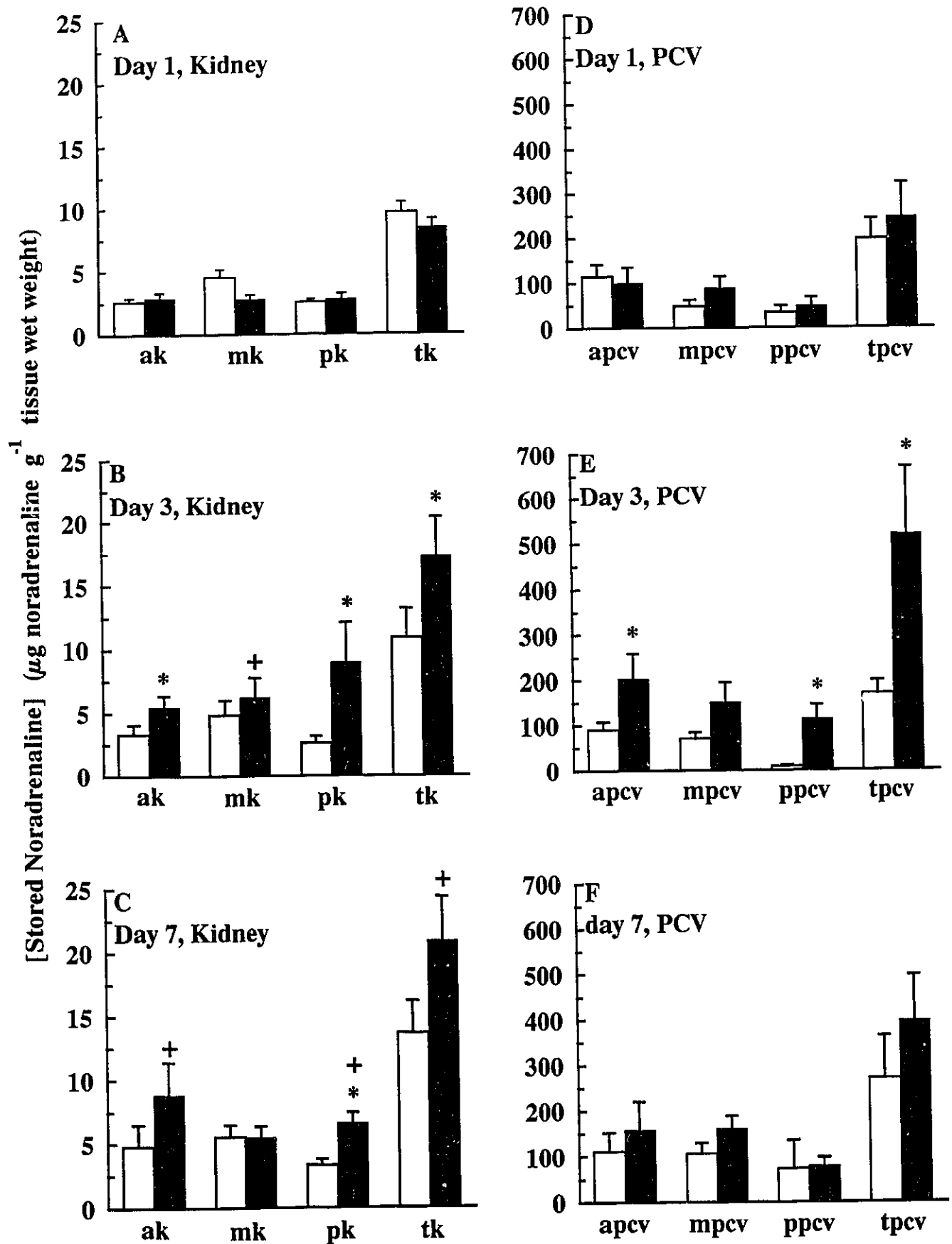


Figure 4-1

Figure 4-2. The effects of 1 (a and d), 3 (b and e) and 7 (c and f) days of cortisol-treatment on levels of stored adrenaline (μg adrenaline g^{-1} tissue wet weight) within various regions of the kidney (a, b, and c) and posterior cardinal vein (PCV) (d, e, and f) in rainbow trout. In all cases the open bar represents control fish ($N = 6$ on days 1 & 3; $N = 4$ on day 7); the dark bar represents cortisol-treated fish ($N = 6$ on days 1 & 3; $N = 8$ on day 7). The data are shown as the mean $\pm$ 1 SEM. An asterisk (*) denotes a significant difference between the control and cortisol-treated fish on any given day. A plus sign (+) denotes a significant difference between values on day 7 and day 1. Abbreviations: The same as for figure 4-1.

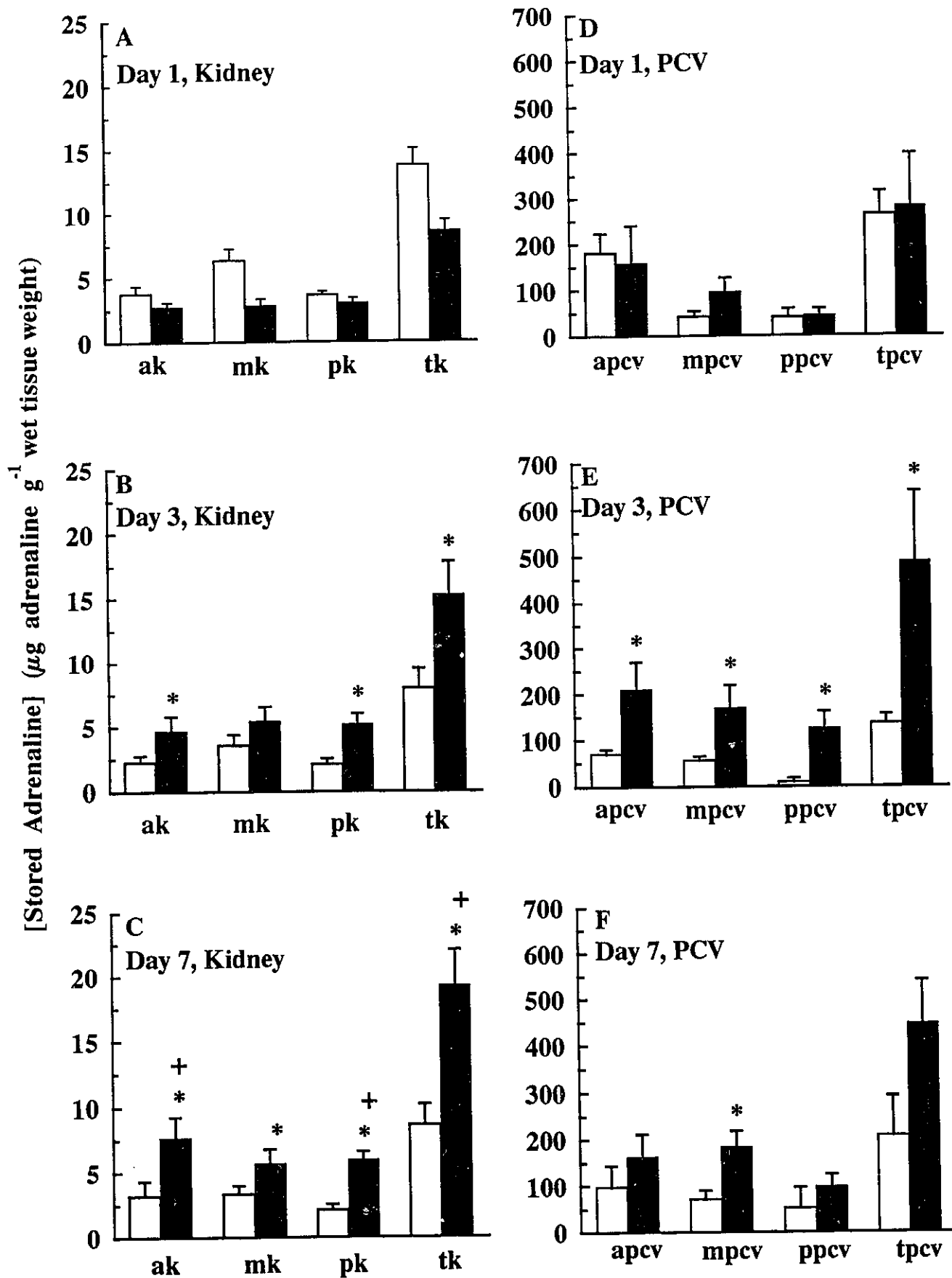


Figure 4-2

The ratios of stored adrenaline to noradrenaline within both the kidney and PCV are illustrated in figure 4-3. Throughout the seven day period the ratio of adrenaline:noradrenaline remained constant within the various tissue regions in the cortisol-treated fish. In the control fish however, the adrenaline:noradrenaline ratio decreased over the seven day period. As such, the ratios in the cortisol-treated fish were greater than the control fish on the seventh day (figure 4-3e, f).

***In Situ* Catecholamine Release Following Cortisol Treatment**

The basal levels (i.e. non-stimulated levels) of noradrenaline and adrenaline in the outflowing perfusate, prior to the addition of carbachol, are reported in table 4-1. Basal levels of noradrenaline were similar within the two groups whereas basal adrenaline levels were significantly greater in the cortisol-treated group.

The effects of the cholinceptor agonist, carbachol on the *in situ* release of noradrenaline are illustrated in figure 4-4. In the control group (fig 4-4a), significant release occurred at doses of 5×10^{-7} mols and higher. In the cortisol-treated preparations, all of the doses of carbachol elicited a significant release of noradrenaline (fig 4-4b). Although statistical analysis revealed these differences in noradrenaline release, between the two groups, the trends were not immediately obvious. On the other hand, such a trend for carbachol-evoked adrenaline release (see below) was more apparent. The levels of perfusate noradrenaline, at the higher doses of carbachol, were similar in the two groups. An injection of saline (fig 4-4a, b) did not alter perfusate noradrenaline levels.

The effects of carbachol on the *in situ* release of adrenaline are illustrated in figure 4-5. In the control group (fig 4-5a), doses of carbachol between 10^{-7} and 10^{-5} mols caused the release of adrenaline. In the cortisol-treated fish (fig 4-5b), with the exception of 10^{-8} mol, all of the carbachol doses administered were able to elicit adrenaline release.

Figure 4-3. The effects of 1 (a and b), 3 (c and d) and 7 (e and f) days of cortisol-treatment on the ratio of stored adrenaline:noradrenaline within various regions of the kidney and posterior cardinal vein in rainbow trout. In all cases, the open bar represents control fish (N = 6 on days 1 & 3; N = 4 on day 7); the dark bar represents cortisol-treated fish (N = 6 on days 1 & 3; N = 8 on day 7). The data are shown as the mean $\pm$ 1 SEM. An asterisk (*) denotes a significant difference between the control and cortisol-treated fish on any given day. Abbreviations: The same as for figure 4-1.

Stored Adrenaline:Noradrenaline Ratio

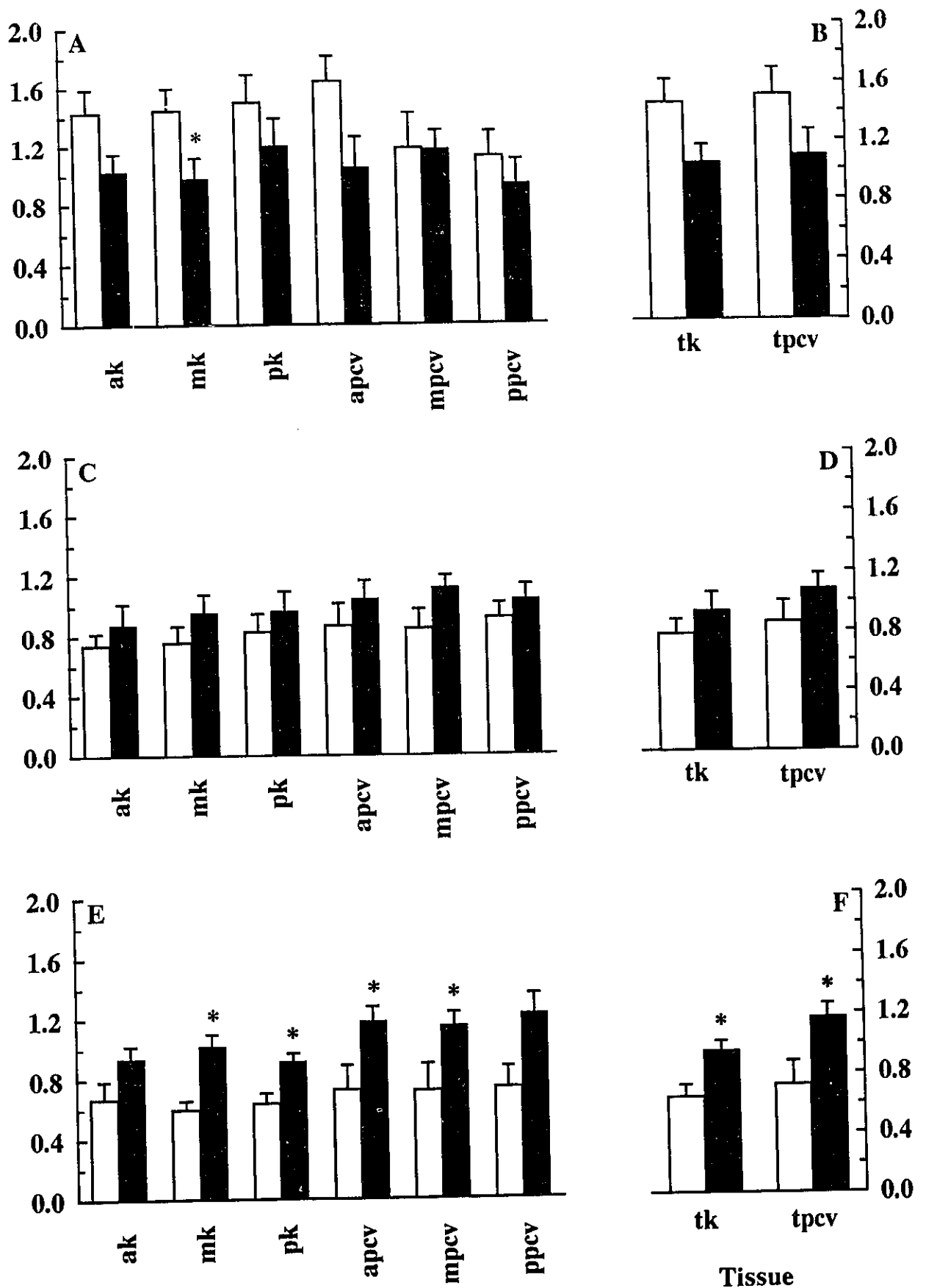


Figure 4-3

Table 4-1. Basal levels (nmol l^{-1}) of noradrenaline and adrenaline within the perfusate (i.e. levels prior to the administration of saline or carbachol) in rainbow trout following 7 days of cortisol ($N = 71$) or oil implantation ($N = 70$). The data are shown as the mean $\pm$ 1 SEM. An asterisk (*) denotes a significant difference between the control and cortisol-treated fish.

Treatment	[Noradrenaline]	[Adrenaline]
Control (oil implant only)	7.85 ± 0.83	17.95 ± 1.97
Cortisol-Treated	9.92 ± 0.83	$24.45 \pm 2.52^*$

Figure 4-4. Maximum perfusate noradrenaline levels (nmol l^{-1}) in response to injections of a variety of doses (10^{-8} , 2.5×10^{-8} , 5.0×10^{-8} , 10^{-7} , 5.0×10^{-7} , 10^{-6} , 5.0×10^{-6} , and 10^{-5} moles; $N = 6$ for each dose) of the cholinceptor agonist carbachol in rainbow trout following 7 days of cortisol-treatment. The open bars (figure a) represent control fish whereas the dark bars (figure b) represent the cortisol-treated fish. **P** refers to the pre (prior to carbachol or saline injection) level of noradrenaline within the perfusate for a given group. **M** is the maximum level of noradrenaline within the perfusate in response to a given injection of carbachol. The data are shown as the mean $\pm$ 1 SEM. An asterisk (*) represents a significant difference between the pre (P) value for any given treatment and the maximum (M) level of noradrenaline within the perfusate following the treatment.

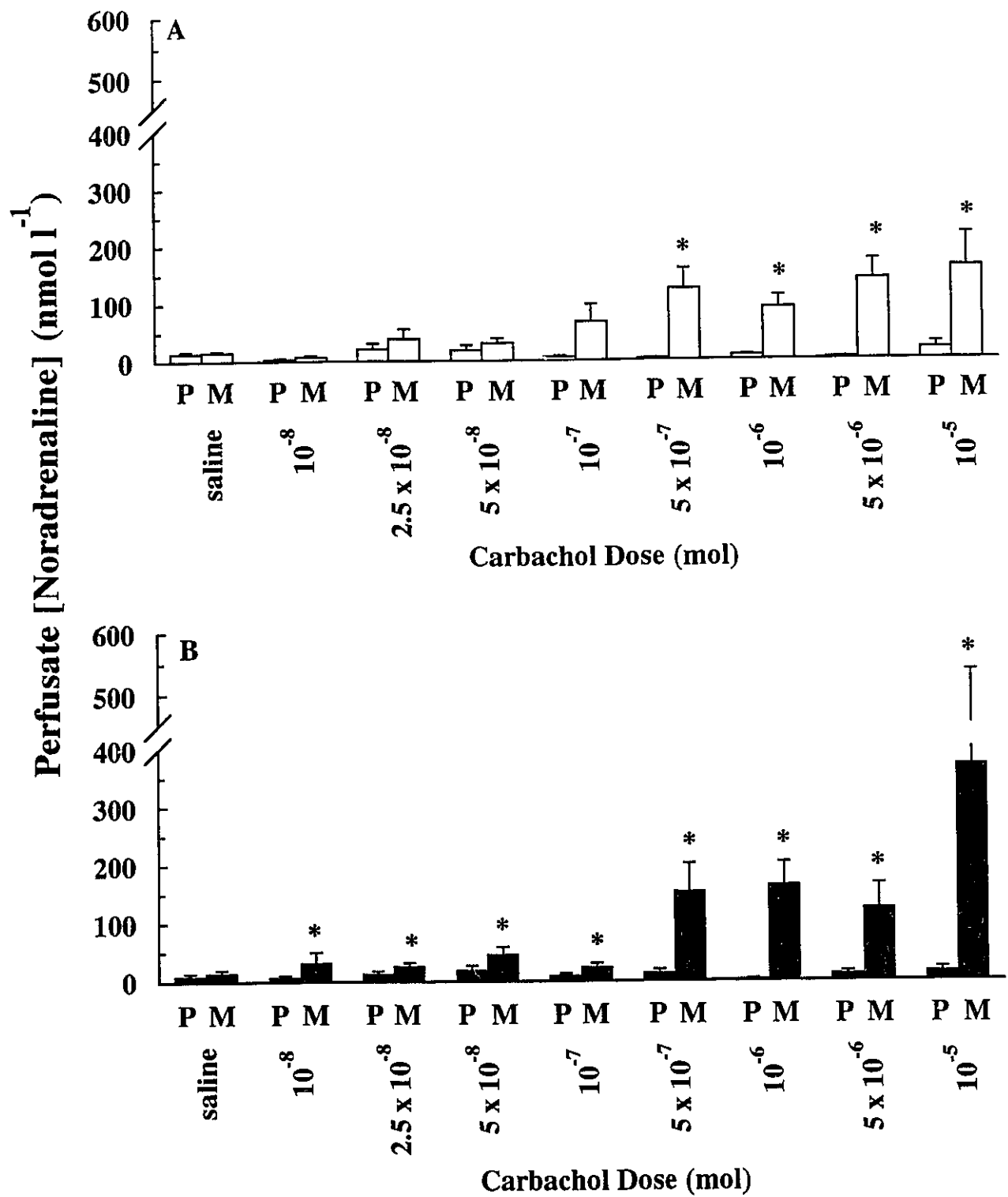


Figure 4-4

Figure 4-5. Maximum perfusate adrenaline levels (nmol l^{-1}) in response to injections of a variety of doses (10^{-8} , 2.5×10^{-8} , 5.0×10^{-8} , 10^{-7} , 5.0×10^{-7} , 10^{-6} , 5.0×10^{-6} , and 10^{-5} moles; $N = 6$ for each dose) of the cholinceptor agonist carbachol in rainbow trout following 7 days of cortisol-treatment. The open bars (figure a) represent control fish whereas the dark bars (figure b) represent the cortisol-treated fish. The data are shown as the mean $\pm$ 1 SEM. An asterisk (*) represents a significant difference between the pre (P) value for any given treatment and the maximum (M) level of adrenaline within the perfusate following the treatment. Abbreviations are the same as for fig. 4-4.

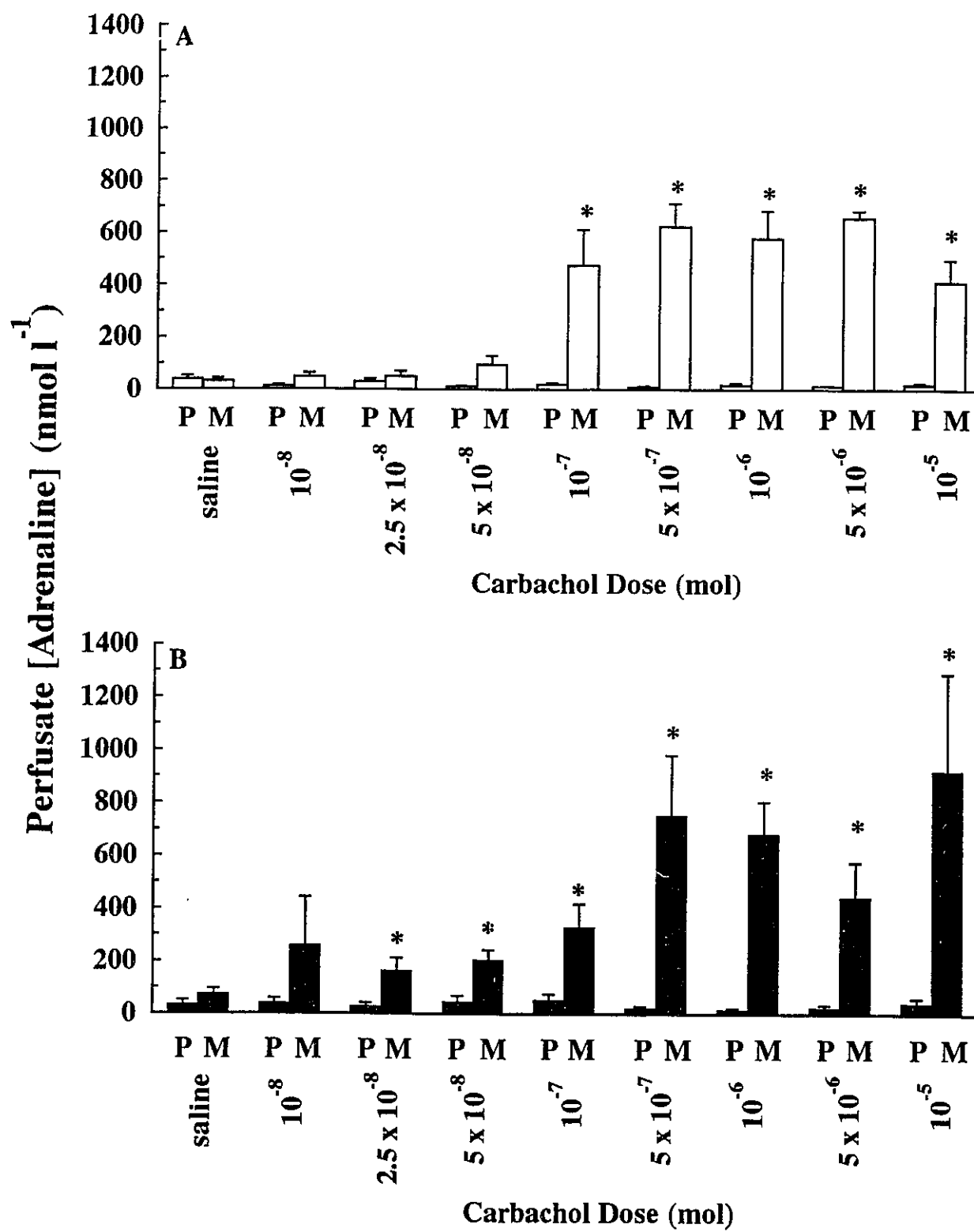


Figure 4-5

At the higher doses of carbachol, the levels of perfusate adrenaline were similar in the two groups. An injection of saline (fig 4-5a, b) did not alter perfusate adrenaline levels.

The perfusate adrenaline:noradrenaline ratios following carbachol administration are illustrated in figure 4-6. In the control group (fig 4-6a) the ratio was increased over the pre-level in response to 10^{-8} , 5×10^{-8} , 10^{-6} and 5×10^{-6} mols carbachol while in the cortisol-treated fish the ratio increased in response to 2.5×10^{-8} , 5×10^{-8} , 10^{-7} and 5×10^{-7} mols carbachol.

Phenylethanolamine-N-Methyltransferase (PNMT) Activity

The maximal activities of phenylethanolamine N-methyltransferase (PNMT) following 3 and 7 days of cortisol-treatment are reported in table 4-2. On both days, there was no difference in the enzyme activity between the control and cortisol-treated groups. In addition, the activity in both groups was greater on the seventh day than the third day.

Figure 4-6. Maximum perfusate adrenaline:noradrenaline ratios in response to injections of a variety of doses (10^{-8} , 2.5×10^{-8} , 5.0×10^{-8} , 10^{-7} , 5.0×10^{-7} , 10^{-6} , 5.0×10^{-6} , and 10^{-5} mols; $N = 6$ for each dose) of the cholinceptor agonist carbachol in rainbow trout following 7 days of cortisol-treatment. The open bars (figure a) represent control fish whereas the dark bars (figure b) represent the cortisol-treated fish. The data are shown as the mean $\pm$ 1 SEM. An asterisk (*) represents a significant difference between the pre (P) value for any given treatment and the maximum (M) level of adrenaline within the perfusate following the treatment. Abbreviations are the same as for fig. 4-4.

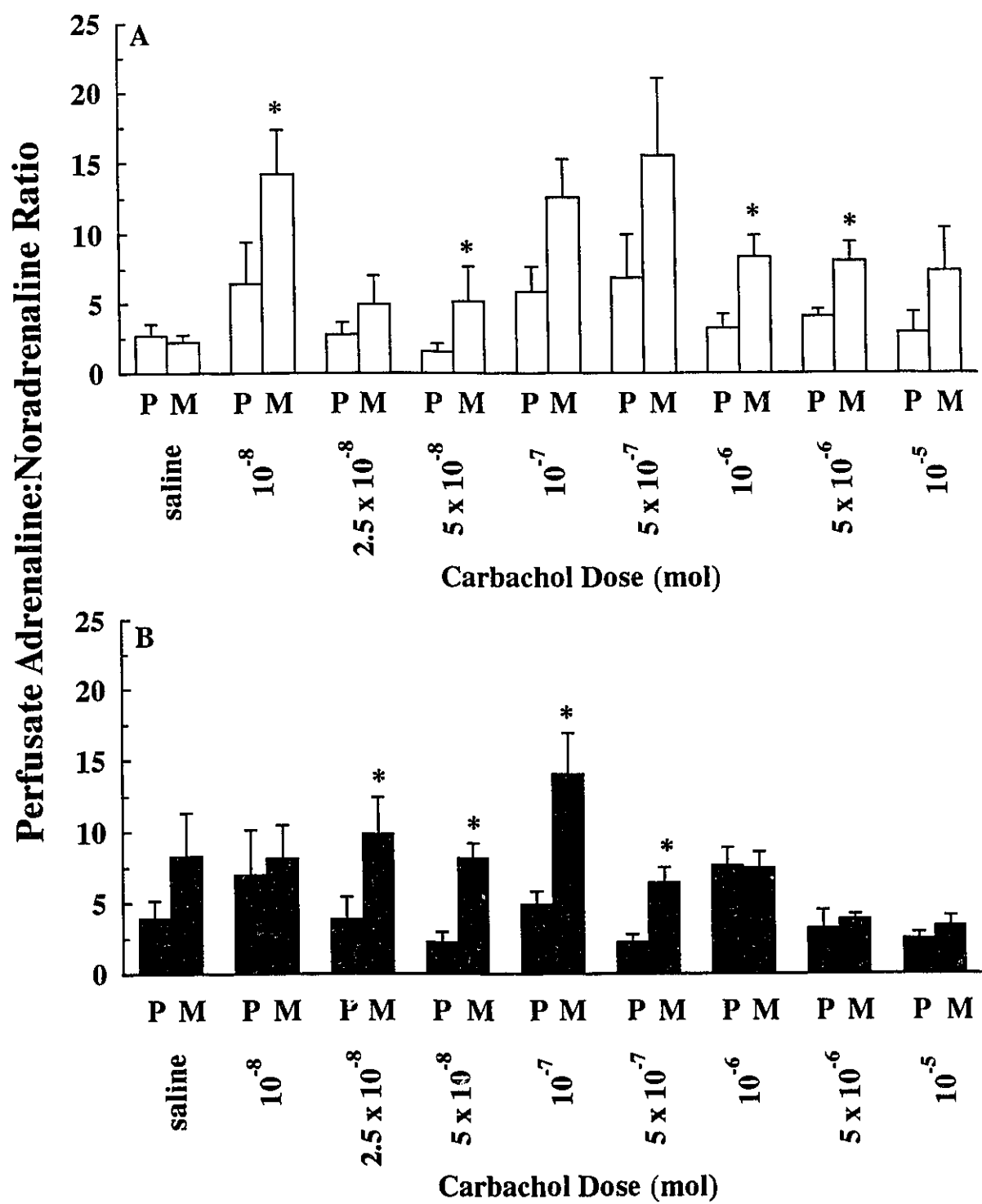


Figure 4-6

Table 4-2: Phenylethanolamine-N-methyltransferase (PNMT) activities (nmol product formed $\text{h}^{-1} \text{g}^{-1}$ tissue wet weight) in the anterior head kidney region of rainbow trout given either an implant of coconut oil (controls) or cortisol suspended in coconut oil. Activities were determined after 3 (N = 6 for both groups) and 7 (N = 8 for both groups) days of treatment. The data are shown as the mean $\pm$ 1 SEM. No significant differences were detected on any given day.

	Day 3	Day 7
Controls (oil implant only)	0.47 ± 0.06	0.97 ± 0.09
Cortisol-treated	0.52 ± 0.10	0.97 ± 0.20

DISCUSSION

Catecholamine Storage and Phenylethanolamine N-methyltransferase Activity

The results of this study demonstrate that an elevation of circulating cortisol levels is capable of increasing the levels of both adrenaline and noradrenaline stored within the kidney and posterior cardinal vein of rainbow trout. The effects of cortisol on catecholamine storage levels were not manifested in the short term as the levels of stored catecholamines were unaltered after one day of treatment. The increase in stored catecholamines observed on both the third and seventh day implies that modulation of catecholamine storage by cortisol is, temporally, a longer term phenomenon and is consistent with the delayed effects of steroid hormones, including cortisol, in fish (e.g. Vijayan *et al.* 1991).

The increase in the levels of stored adrenaline and noradrenaline, on the third and seventh days of treatment, may be explained by an increase in the synthesis of catecholamines. In mammals, several factors, including cortisol, regulate the activity of phenylethanolamine N-methyltransferase (PNMT), the enzyme which catalyses the methylation of noradrenaline to adrenaline. Cortisol, in mammals, has been shown to increase the transcription of the gene encoding for PNMT often resulting in an increase in the activity of the enzyme. However, in other cases, an increase in transcriptional activity does not necessarily cause an increase in enzyme activity (Wong *et al.* 1993). In lower vertebrates (Wurtman *et al.* 1968; Mazeaud 1972; Jönsson *et al.* 1983) cortisol administration appears to have little or no effect on PNMT activity. However, the possibility existed that a different regimen of cortisol treatment (such as in this study) may have revealed an effect of cortisol on the activity of this enzyme.

However, in the current study, in accordance with the previous studies (e.g. Jönsson *et al.* 1983), the *in vitro* activity of PNMT was unaltered after three and seven days of cortisol treatment. In this case, the PNMT activity was determined under " $V_{\max}$ " conditions (Abrahamsson 1980). As such, although the maximal activity of the enzyme

was unaffected by cortisol treatment, the possibility exists that other aspects of enzyme activity, such as affinity for the substrate, may have been altered (i.e. increased) by cortisol-treatment *in vivo*. Clearly the increased storage levels observed in the cortisol-treated fish cannot be explained by an increase in the maximal activity of this enzyme. In both groups of fish, PNMT activity was greater on the seventh day than on the third day. Although there is no obvious explanation for these differences, the important comparison is between the control and cortisol-treated fish, on any given day.

The increase in stored noradrenaline, in addition to adrenaline, suggests that cortisol may exert an effect on the biosynthesis of catecholamines prior to the conversion of noradrenaline to adrenaline. Thus, other enzymes within the Blaschko pathway prior to PNMT (i.e. tyrosine hydroxylase, amino acid decarboxylase or dopamine- β -hydroxylase) may have been altered by cortisol-treatment. Indeed, Jönsson *et al.* (1983) demonstrated a tendency, in rainbow trout, for cortisol to increase the activity of dopamine- β -hydroxylase (D β H), the enzyme which converts dopamine to noradrenaline. Ciaranello *et al.* (1975) demonstrated, in rat adrenals, that treatment with ACTH inhibits degradation of D β H suggesting the maintenance of steady state levels of this enzyme by endogenous corticosteroids.

In addition to potential influences on the biosynthesis of catecholamines, cortisol may alter the process of catecholamine degradation (Axelrod and Reisine 1984). The degradation of catecholamines involves deamination via the enzyme monoamine oxidase (MAO) and O-methylation with catechol-O-methyltransferase (COMT) (see review by Randall and Perry 1992). Cortisol may influence the activity of these enzymes by altering stages from gene transcription through enzyme degradation. Any decrease in the rate of degradation would, presumably, affect both adrenaline and noradrenaline to a similar degree as both COMT and MAO can utilise either catecholamine as a substrate.

In Situ Catecholamine Release Following Cortisol-Treatment

Cortisol treatment increased the basal levels of adrenaline, but not noradrenaline within the perfusate of the *in situ* preparation. The increased basal secretion of adrenaline may reflect the greater levels of adrenaline stored within the chromaffin cells of the cortisol-treated fish. Alternatively, the exocytotic release process leading to the fusion of secretory granules with the plasma membrane may have been modulated by cortisol-treatment.

In addition to affecting the basal release of adrenaline, cortisol treatment altered the release response to cholinergic stimulation. Lower doses of carbachol (10^{-8} to 10^{-7} moles) caused the release of catecholamines in the cortisol treated fish but not in the control fish, whilst the higher doses resulted in similar release in both groups. This phenomenon was more obvious for adrenaline than noradrenaline. A greater enhancement of carbachol-evoked adrenaline release, compared with noradrenaline release, is consistent with the effects of cortisol treatment on the basal release of these catecholamines. Thus the chromaffin cells in the cortisol treated fish were able to respond to cholinergic stimulation with an increased sensitivity. This suggests that cortisol may have altered the affinity (K_m) of the cholinceptor for carbachol allowing for release to occur at lower levels of stimulation. Alternately, cortisol may have induced the synthesis of cholinceptors which possess a greater affinity for carbachol than receptors already present on the chromaffin cell membrane. Additionally, any post-receptor modifications within the signal transduction pathway could account for the enhanced ability to release catecholamines.

Previous studies have demonstrated that chronic stress can alter both catecholamine storage and the process of cholinergic-induced catecholamine release (Nilsson 1990; Nilsson and Block 1991; Reid *et al.* 1994). Chronic stress also results in increased plasma cortisol concentrations and/or cortisol-mediated physiological effects in fish (Vijayan and Leatherland 1990). Indeed, the increase in plasma cortisol levels in this

study (approximately 30 ng ml⁻¹) was probably lower than levels observed during a variety of stressful situations (e.g. acute handling stress, Vijayan and Moon 1992). As such, it is possible that the influence of chronically elevated plasma cortisol levels on catecholamine storage and cholinceptor-induced release is being underestimated. Given the effects of cortisol on catecholamine storage and release in this study, it is likely that elevated cortisol associated with a chronic stress situation has a modulatory effect on the adrenergic response to an acute stress in rainbow trout.

Summary

In conclusion, cortisol is capable of increasing levels of stored catecholamines and sensitising the process of catecholamine release to cholinergic stimulation suggesting a potential interactive and synergistic response to stress within the trout pituitary-adrenal axis (also see chapter 8).

CHAPTER 5.

**CHOLINOCEPTOR-MEDIATED RELEASE OF
CATECHOLAMINES IN THE AMERICAN EEL, *ANGUILLA ROSTRATA***

INTRODUCTION

Stimulation of cholinceptors on the chromaffin cell membrane is considered to be the primary mechanism initiating the release of catecholamines in teleost fish (Nilsson 1983; Perry *et al.* 1991). Cholinergic receptors are pharmacologically classified as either nicotinic or muscarinic cholinceptors based on differential activation by either nicotine or muscarine (Burgoyne 1991). Although little attention has been focused on the pharmacology of the cholinceptor on fish chromaffin cells, cholinceptor-induced release of catecholamines from mammalian chromaffin cells is an area of extensive study. Bovine adrenal chromaffin cells, which serve as a model system for studying catecholamine secretion (see reviews by Ungar and Phillips 1983; Burgoyne 1991), appear to release catecholamines in response to nicotinic, but not muscarinic, receptor stimulation. A variety of muscarinic receptor sub-types have been identified on bovine adrenal chromaffin cells (Barron *et al.* 1986; Yamanaka *et al.* 1986; Aguilar *et al.* 1992), yet their role, if any, in the process of catecholamine release, is unclear. Forsberg *et al.* (1986) demonstrated that muscarinic receptor stimulation of bovine adrenal chromaffin cells enhances nicotinic receptor-induced catecholamine secretion. Conversely, other studies (Derome *et al.* 1981; Swilem and Hawthorne 1983; Cheek and Burgoyne 1985) suggested that muscarinic receptor agonists could inhibit nicotinic agonist-evoked release either directly via muscarinic receptor stimulation or indirectly, at high concentrations, by interacting with nicotinic receptors.

The presence of muscarinic receptors on chromaffin cells and their involvement in catecholamine release are highly variable amongst species. Canine chromaffin cells possess muscarinic receptors (Tobin *et al.* 1992) which do not induce secretion in response to endogenous neuronal acetylcholine yet a low level of release occurs in response to infused muscarinic agonists (Kennedy *et al.* 1991). Perfused feline adrenal glands secrete catecholamines when stimulated with both muscarinic (Ballesta *et al.* 1989; Uceda *et al.* 1992) and nicotinic agonists (Ballesta *et al.* 1989). In addition, Ballesta *et al.*

(1989) demonstrated that muscarinic agonists potentiate the release of catecholamines in response to nicotine or high levels of K^+ . Cultured chicken chromaffin cells secrete catecholamines in response to muscarinic receptor, but not nicotinic receptor, stimulation (Ledbetter and Kirshner 1975; Knight and Baker 1986). Hamster chromaffin cells (Liang and Perlman 1979) release catecholamines in response to nicotinic but not muscarinic agonists whilst porcine chromaffin cells secrete catecholamines in response to both types of agonists (Xu *et al.* 1991). Certainly then, amongst vertebrate species so far studied, cholinceptor-induced secretion of catecholamines from chromaffin cells is far from uniform.

Cholinoceptor-induced release of catecholamines from teleost chromaffin cells is considered to be mediated by the nicotinic cholinoceptor (Nilsson *et al.* 1976; Nilsson 1983). However any involvement of the muscarinic receptor in this process has not been examined. As such, the goal of this study was to determine whether cholinergic agonist-induced release of catecholamines from American eel chromaffin cells involves muscarinic, in addition to nicotinic, receptor stimulation.

MATERIALS AND METHODS

Experimental Animals

American eels (*Anguilla rostrata*), weighing 98.0 ± 1.5 g (experimental N = 198) were obtained from an eel ladder (Saunders Hydroelectric Dam, Cornwall, Ontario) and transported on ice to the University of Ottawa. Holding conditions were as described in chapter 3 with the exception that water temperature was 4 °C and fish were acclimated to the aquarium conditions for a period of 5 months prior to experimentation.

Experimental Protocol

The ability of the chromaffin cells to release catecholamines in response to cholinergic agonist stimulation, was assessed using the *in situ*, saline-perfused PCV preparation.

Direct Membrane Depolarization

The catecholamine-releasing response of the chromaffin cells to direct membrane depolarization was assessed by perfusing the PCV with Cortland saline containing 60 mmol l⁻¹ K⁺ (KCl; as described in chapters 2 and 3). To ensure that the ganglion blocker hexamethonium (which will block the response to nicotinic receptor agonists, see below) did not inhibit the action of 60 mmol l⁻¹ K⁺, the preparation was pre-treated for 20 min with 10⁻³ mol l⁻¹ hexamethonium prior to switching the perfusion to the high K⁺ saline (which also contained hexamethonium).

Stimulation of the Chromaffin Cells with Cholinergic Agonists

The effects of cholinergic stimulation on catecholamine release were assessed with the nicotinic receptor agonist 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP), the muscarinic receptor agonist pilocarpine (hydrochloride salt) and the joint

nicotinic/muscarinic receptor agonist carbachol. Following the 20 min stabilization period and the collection of the pre-sample, a bolus dose of either DMPP (10^{-8} , 10^{-7} , 5×10^{-7} , 10^{-6} , 2×10^{-6} , 5×10^{-6} , 10^{-5} , 5×10^{-5} mol), pilocarpine (10^{-8} , 10^{-7} , 5×10^{-7} , 10^{-6} , 5×10^{-6} , 10^{-5} , 10^{-4} mol), carbachol (10^{-5} mol), or saline (control) was injected into the infusion cannula. The doses of both DMPP and pilocarpine were chosen in accordance with doses of carbachol that elicited release in a dose-dependent manner (see chapters 2 & 3). During certain experiments, the ganglion blocker hexamethonium (chloride salt; 10^{-3} mol l^{-1} , to block nicotinic agonist-evoked release) or the muscarinic receptor antagonist atropine (sulphate salt; 10^{-5} mol l^{-1}) were present in the perfusion saline during the 20 min stabilization period, drug injection and sample collection.

Data Presentation and Statistical Analysis

In all figures the data [mean $\pm$ 1 standard error of the mean (SEM)] are presented as bar graphs. The entire bar indicates the total concentration of catecholamines (adrenaline + noradrenaline) in the perfusate. The darkened and light areas of the bar represent the noradrenaline and adrenaline components, respectively. The error bars are indicative of the variability associated with the individual catecholamines however an asterisk (*) indicates a significant difference in the total catecholamine level, compared with the corresponding pre-value for total catecholamines.

Where appropriate, the data were statistically analyzed using either a one way analysis of variance followed by Fisher's least significant difference test for multiple comparisons or a two sample t-test. The fiducial limit of significance was 5%.

RESULTS AND DISCUSSION

Catecholamine Secretion in Response to $60 \text{ mmol l}^{-1} \text{ K}^{+}$

Figure 5-1 illustrates the effect of stimulation with $60 \text{ mmol l}^{-1} \text{ K}^{+}$ on the release of catecholamines from chromaffin cells. High concentrations of K^{+} directly depolarize cell membranes independent of receptor activation and second messenger production. Thus the amount of catecholamines released in response to this chemical stimulus would, theoretically, be the maximum attainable level.

Catecholamine levels remained unchanged from pre-values following an injection of saline (controls) (figure 5-1a). In response to $60 \text{ mmol l}^{-1} \text{ K}^{+}$ (figure 5-1b), perfusate catecholamine levels increased from the pre-value to approximately 100 nmol l^{-1} adrenaline + noradrenaline in the second min after its addition. This level persisted throughout the remainder of the post-injection period. Adrenaline was the predominant catecholamine released into the perfusate. The addition of $10^{-3} \text{ mol l}^{-1}$ hexamethonium (a ganglion blocker inhibiting the response to nicotinic receptor agonists) did not alter the pattern of catecholamine release evoked by K^{+} (figure 5-1c). Thus, this concentration of hexamethonium would be expected to specifically block nicotinic-receptor evoked catecholamine release without exerting any non-specific effects on the release process (see below).

The perfusate [adrenaline]/[noradrenaline] ratio following $60 \text{ mmol l}^{-1} \text{ K}^{+}$ was not different from the pre-value ratio suggesting that K^{+} does not cause the preferential release of one catecholamine over the other, a similar situation to K^{+} -induced catecholamine secretion in the trout (chapters 2 & 3). Therefore, the predominance of adrenaline within the perfusate, under both basal conditions (pre) and following $60 \text{ mmol l}^{-1} \text{ K}^{+}$, presumably reflects differences in storage levels of the two catecholamines. Indeed, in both the American eel and rainbow trout, the concentration of adrenaline within the posterior cardinal vein is approximately 4 X greater than that of noradrenaline (see chapter 3).

Figure 5-1. Total catecholamine levels (adrenaline + noradrenaline) (nmol l^{-1}) within the perfusate in response to (a) saline, (b) $60 \text{ mmol l}^{-1} \text{ K}^{+}$ and (c) $60 \text{ mmol l}^{-1} \text{ K}^{+}$ preceded by 20 min perfusion with $10^{-3} \text{ mol l}^{-1}$ hexamethonium. In all cases, the darkened portion of the bar represents the noradrenaline component of the total catecholamines, the clear portion, adrenaline. The pre-value represents levels during perfusion with normal Cortland saline prior to K^{+} administration or the injection of saline. The dotted line represents either an injection of saline (a) ($N = 12$) or a switch to perfusion with $60 \text{ mmol l}^{-1} \text{ K}^{+}$ (b & c; $N = 8$ & 6 , respectively). Following the injection/perfusion switch and a 1 min delivery period, the perfusate was collected each min for 4 min. Values are shown as means $\pm$ SEM for the individual catecholamines. An asterisk (*) denotes a significant difference in the total catecholamine level from the pre-total catecholamine level.

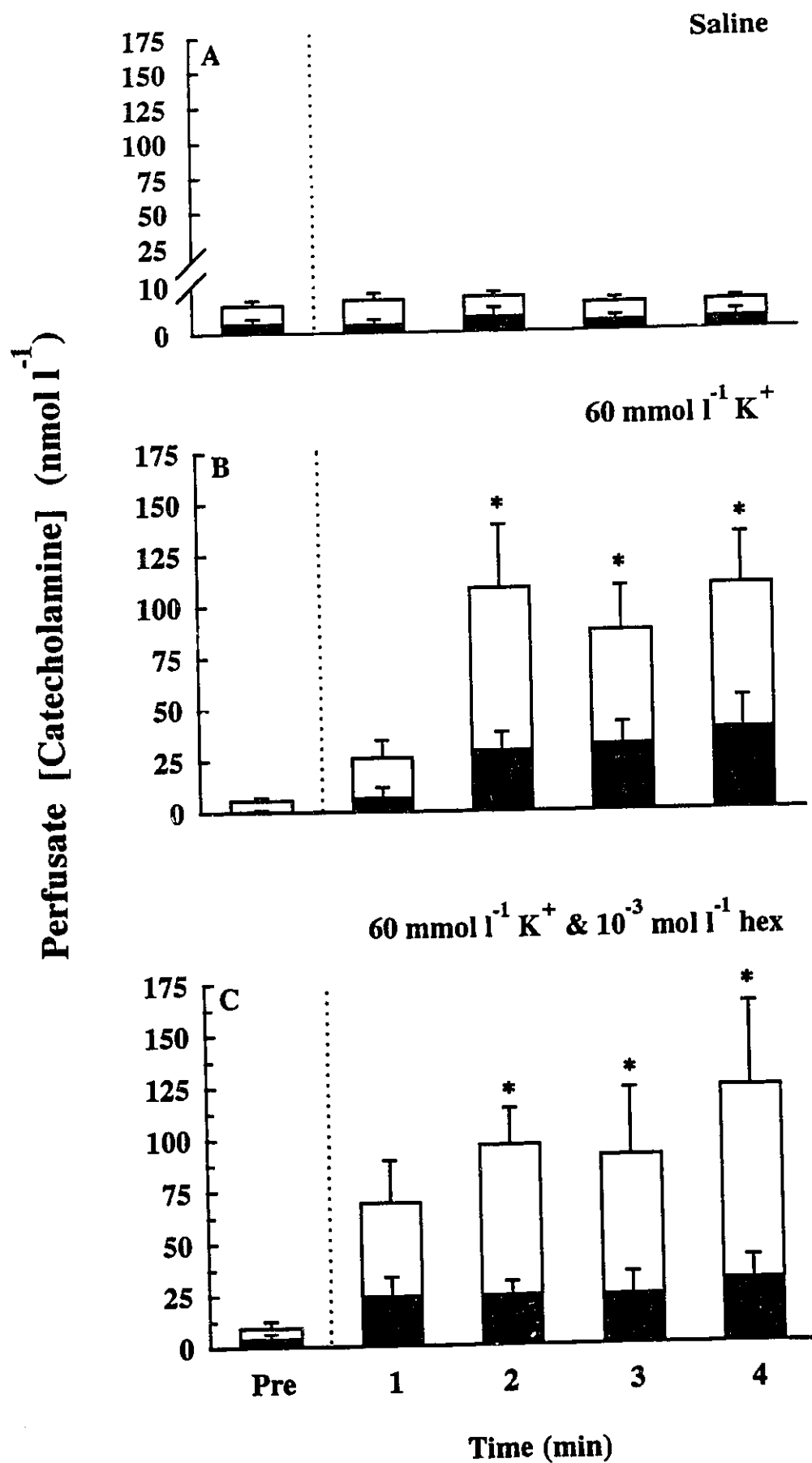


Figure 5-1

The persistent secretion of catecholamines following the application of K^+ demonstrates that the chromaffin cells within the posterior cardinal vein are viable and capable of releasing catecholamines. Release in response to non-specific stimuli, such as K^+ , confirm the validity of the preparation to investigate physiological effects such as cholinergic-receptor activation (also see chapter 2 for validation of the preparation).

Nicotinic Agonist Administration

Figure 5-2 illustrates perfusate catecholamine levels in response to a variety of doses of the nicotinic receptor agonist DMPP. Perfusate catecholamine levels were elevated in response to injections of 10^{-7} (1 min), 5×10^{-7} (1-2 min), 5×10^{-6} (2-3 min), 10^{-5} (1 min) and 5×10^{-5} (1 min) mol DMPP. The decrease in perfusate catecholamine levels seen in the third to fourth minutes probably reflects washout of DMPP from the perfusion. However, cholinceptor desensitization may also contribute to the decrease in catecholamine levels. In all cases, adrenaline was the predominant catecholamine within the perfusate. No statistically significant increase in perfusate catecholamine levels was observed in response to 10^{-8} , 10^{-6} and 2×10^{-6} mol DMPP. The low level of release observed with the highest dose of DMPP may reflect a toxic effect at the higher concentration. Such a decrease in perfusate catecholamine levels at the highest dose of agonist is not uncommon in this preparation (e.g. chapter 2). As was the case with the K^+ treatment, the perfusate [adrenaline]/[noradrenaline] ratio following the DMPP injection was not different from the pre-value ratio. Thus nicotinic-receptor activation with DMPP does not cause a preferential release of either adrenaline or noradrenaline in eel (see below).

Pre-treatment (20 min) with hexamethonium abolished the DMPP-induced release of catecholamines (figure 5-3). This inhibition confirms that DMPP is indeed acting via

Figure 5-2. Total catecholamine levels (adrenaline + noradrenaline) (nmol l^{-1}) within the perfusate in response to the nicotinic receptor agonist 1, 1 - dimethyl -4-phenylpiperazinium iodide (DMPP). In all cases, the darkened portion of the bar represents the noradrenaline component of the total catecholamines, the clear portion, adrenaline. The pre-value represents levels during perfusion with normal Cortland saline prior to injection. The dotted line represents an injection of (a) 10^{-8} mol DMPP (N=8), (b) 10^{-7} mol DMPP (N=8), (c) 5×10^{-7} mol DMPP (N=8), (d) 10^{-6} mol DMPP (N=7), (e) 2×10^{-6} mol DMPP (N=6), (f) 5×10^{-6} mol DMPP (N=5), (g) 10^{-5} mol DMPP (N=8), (h) 5×10^{-5} mol DMPP (N=7). Following the injection and a 1 min delivery period, the perfusate was collected each min for 4 min. Values are shown as means $\pm$ SEM for the individual catecholamines. An asterisk (*) denotes a significant difference in the total catecholamine level from the pre-total catecholamine level.

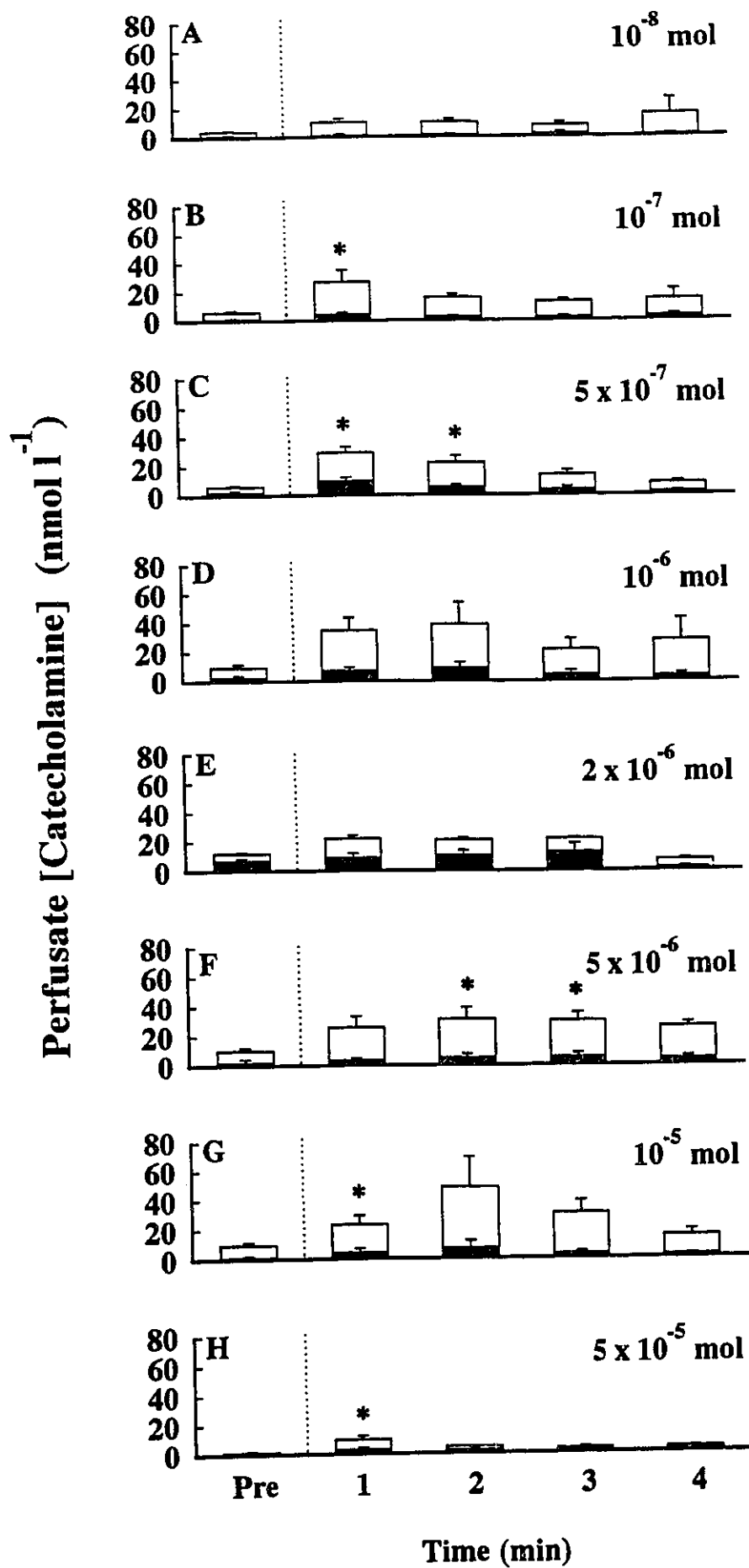


Figure 5-2

Figure 5-3. Total catecholamine levels (adrenaline + noradrenaline) within the perfusate in response to (a) 10^{-5} mol DMPP and (b) 10^{-5} mol DMPP preceded by 20 min pre-perfusion with the ganglion blocker hexamethonium (10^{-3} mol l^{-1}). The darkened portion of the bar represents the noradrenaline component of the total catecholamines, the clear portion, adrenaline. The pre - value represents levels during perfusion with normal Cortland saline prior to injection. The dotted line represents an injection of 10^{-5} mol DMPP. Following the injection and a 1 min delivery period, the perfusate was collected each min for 4 min. Values are shown as means $\pm$ SEM for the individual catecholamines. An asterisk (*) denotes a significant difference in the total catecholamine level from the pre-total catecholamine level.

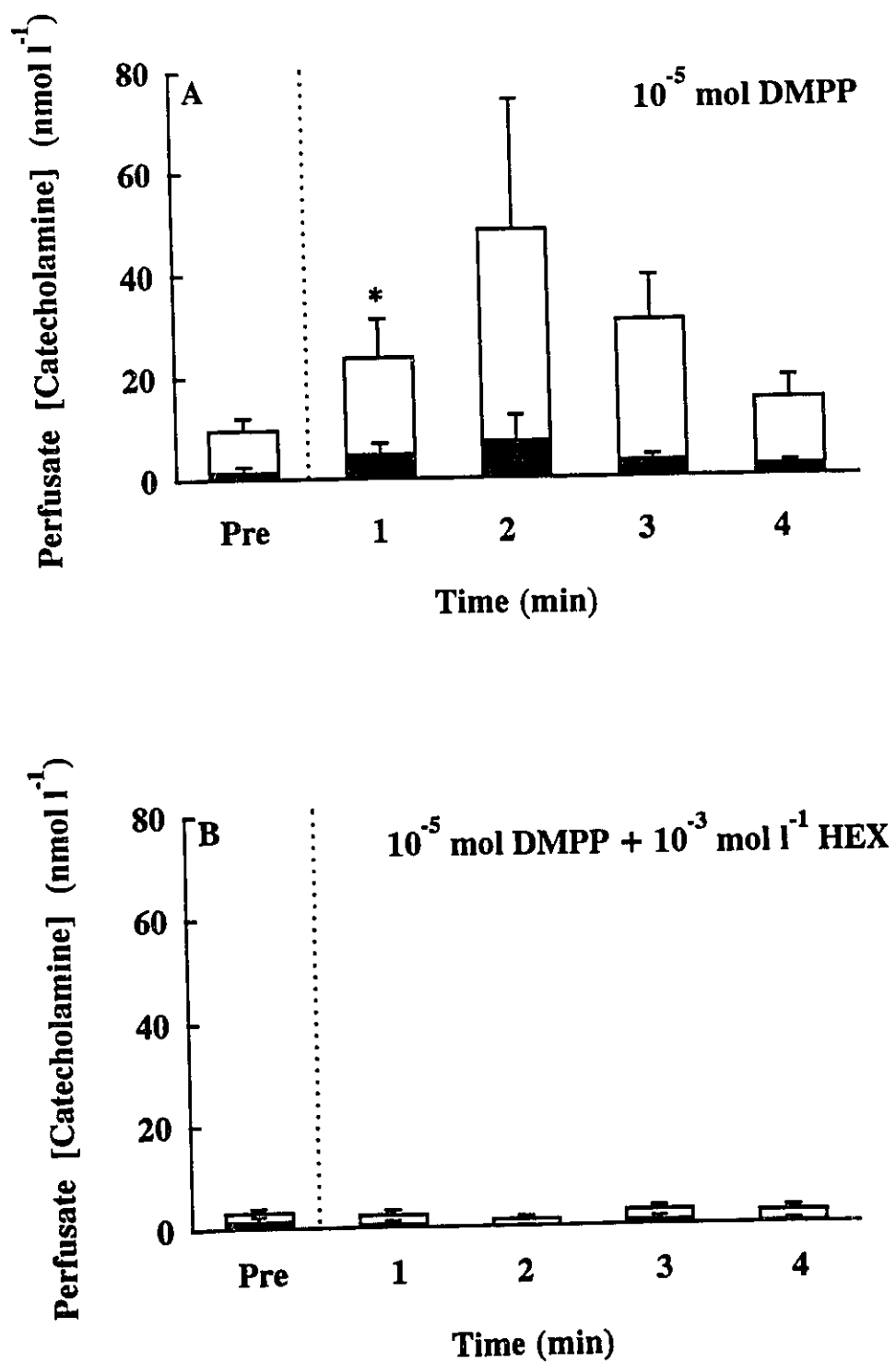


Figure 5-3

nicotinic receptors to evoke the release of catecholamines rather than interacting with other hormonal receptors that may be present on the chromaffin cell membrane.

Nicotinic receptor activation is associated with the entry of extracellular Ca^{2+} into the chromaffin cells through voltage-dependent channels (Burgoyne 1991). The ensuing elevation of intracellular free Ca^{2+} levels is a necessary requirement for exocytosis. The release of catecholamines in response to DMPP supports the contention that stimulation of the nicotinic receptor is sufficient, in itself, to promote the secretion of catecholamines from chromaffin cells in the American eel.

Muscarinic Agonist Administration

Pilocarpine failed to elicit the release of catecholamines at any of the 7 doses administered (10^{-8} - 10^{-4} mol; figure 5-4) and 20 min pre-treatment with atropine did not alter the lack of response to pilocarpine (data not shown). The perfusate

- [adrenaline]/[noradrenaline] ratio was unaltered by injections of pilocarpine. The inability of pilocarpine to evoke catecholamine secretion demonstrates that stimulation of muscarinic receptors if they are present, in the absence of other stimuli, is insufficient to evoke the secretion of catecholamines from eel chromaffin cells.

The stimulation of muscarinic receptors can activate phospholipase C thereby causing production of inositol 1, 4, 5 - triphosphate and liberation of calcium from intracellular stores (Unsicker 1993). However, the rise in intracellular free Ca^{2+} associated with the mobilization of intracellular Ca^{2+} reserves is, in some species, not sufficient to evoke catecholamine secretion (Burgoyne *et al.* 1993). In other species, muscarinic receptor stimulation can cause the release of calcium from intracellular stores in sufficient quantities to elicit the exocytotic release of catecholamines from the chromaffin cell (see above).

Figure 5-4. Total catecholamine levels (adrenaline + noradrenaline) (nmol l^{-1}) within the perfusate in response to the muscarinic receptor agonist pilocarpine. In all cases, the darkened portion of the bar represents the noradrenaline component of the total catecholamines, the clear portion, adrenaline. The pre-value represents levels during perfusion with normal Cortland saline prior to injection. The dotted line represents an injection of pilocarpine (a) 10^{-8} mol, (b) 10^{-7} mol, (c) 5×10^{-7} mol, (d) 10^{-6} mol, (e) 5×10^{-6} mol, (f) 10^{-5} mol (g) 10^{-4} mol ($N = 7-8$ for each dose). Following the injection and a 1 min delivery period, the perfusate was collected each min for 4 min. Values are shown as means $\pm$ SEM for the individual catecholamines.

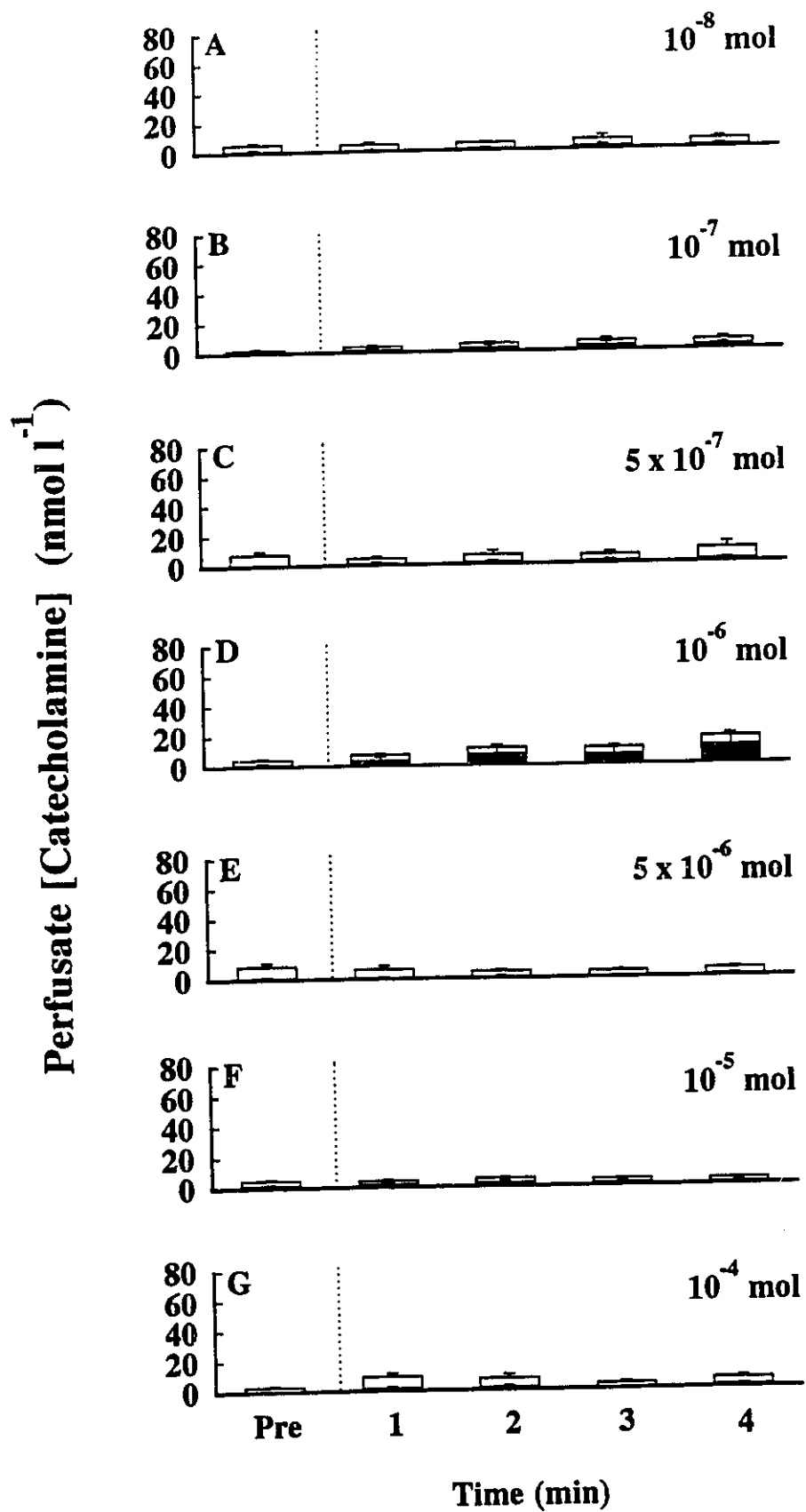


Figure 5-4

Stimulation with a Mixed Nicotinic/Muscarinic Agonist

Figure 5-5 demonstrates the effects of carbachol (a mixed nicotinic/muscarinic receptor agonist), in the presence or absence of the ganglion blocker hexamethonium (to block nicotinic receptor-induced release) or the muscarinic receptor antagonist atropine. An injection of saline (figure 5-5a) did not evoke catecholamine release whereas carbachol administration (figure 5-5b) caused perfusate catecholamine levels to increase (30-50 nmol l⁻¹) for the first 2 min post-injection. Catecholamine levels returned to pre-values for the remainder of the perfusion (see above). As was the case with K⁺ and DMPP, the predominant catecholamine released into the perfusate was adrenaline. The [adrenaline]/[noradrenaline] ratio did not change with the addition of carbachol which is unlike the situation in rainbow trout where carbachol caused a preferential release of adrenaline (see chapter 3).

Both carbachol and 60 mmol l⁻¹ K⁺ produced an increase in perfusate catecholamines at 2 min post-treatment. The perfusate catecholamine level in response to 60 mmol l⁻¹ K⁺ was significantly greater than in response to carbachol. Thus direct membrane depolarization (K⁺) is capable of evoking a greater secretory response than activation of cholinergic receptors (carbachol). Possibly "limitations" in the cholinergic receptor/second messenger systems may be responsible for this difference.

When the preparation was pre-treated for 20 min with the ganglion blocker hexamethonium, no increase in perfusate catecholamines occurred in response to the carbachol injection with levels remaining below 3 nmol l⁻¹ for the duration of the perfusion. Under these conditions (where hexamethonium blocks nicotinic agonist-evoked responses), presumably only muscarinic receptors, if present, were available to undergo activation by carbachol. The ability of hexamethonium to completely abolish the secretory response to carbachol suggests that nicotinic-receptor activation is an absolute requirement for cholinergic-induced catecholamine secretion in the American eel.

Figure 5-5. Total catecholamine levels (adrenaline + noradrenaline) within the perfusate in response to (a) saline (N=12), (b) carbachol (10^{-5} mol) (N=8), (c) carbachol (10^{-5} mol) following pre-treatment (20 min) with hexamethonium (10^{-3} mol l^{-1}) (N=6), and (d) carbachol (10^{-5} mol) following pre-treatment (20 min) with atropine (10^{-5} mol l^{-1}) (N=7). In all cases, the darkened portion of the bar represents the noradrenaline component of the total catecholamines, the clear portion, adrenaline. The pre-value represents levels during perfusion with normal Cortland saline prior to injection. The dotted line represents an injection of either saline (a) or carbachol (b, c & d). Following the injection and a 1 min delivery period, the perfusate was collected each min for 4 min. Values are shown as means $\pm$ SEM for the individual catecholamines. An asterisk (*) denotes a significant difference in the total catecholamine level from the pre-total catecholamine level.

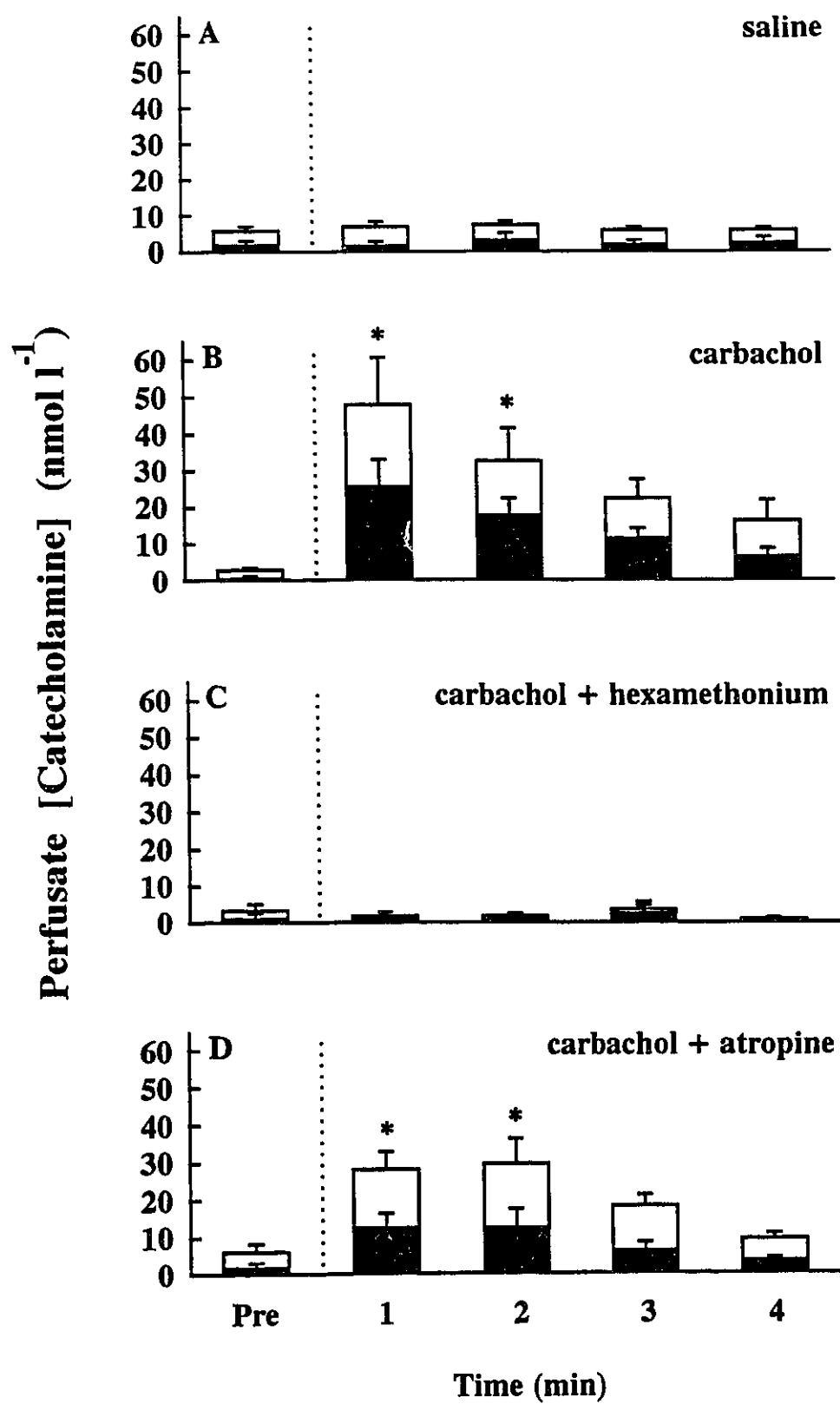


Figure 5-5

Pre-treatment with the muscarinic receptor antagonist, atropine, did not abolish the carbachol-induced release of catecholamines. Indeed, the catecholamine levels 1 and 2 min post-injection in response to carbachol alone and carbachol + atropine were not significantly different from one another. This indicates that stimulation of muscarinic receptors, if indeed they are present, does not contribute to the cholinergic agonist-induced release of catecholamines, a situation in apparent contrast to that in the rainbow trout (see chapter 2).

Summary

In conclusion, this chapter demonstrates that the process of cholinergic agonist-induced catecholamine secretion from the chromaffin cells in the American eel does not involve the muscarinic cholinergic receptor whereas the activation of nicotinic receptors is sufficient, in itself, to induce catecholamine release. Given the results of this chapter together with those of chapters 2 and 3, it appears as if there are interspecific differences, between rainbow trout and American eel, with respect to the pharmacology of cholinergic-induced catecholamine release from the chromaffin cells. These differences are discussed in detail in chapter 9 (general discussion).

CHAPTER 6.

**THE EFFECTS OF ACCLIMATION TEMPERATURE ON
THE DYNAMICS OF CATECHOLAMINE RELEASE DURING
ACUTE HYPOXIA IN THE RAINBOW TROUT**

INTRODUCTION

In rainbow trout, a potent stimulus for the release of catecholamines into the circulation in exposure to severe environmental hypoxia (e.g. Boutilier *et al.* 1988; see chapter 3). Given the sigmoidal nature of the oxygen dissociation curve (e.g. fig 6-1), both blood oxygen tension (PO_2) and oxygen content decrease simultaneously at moderate to severe levels of hypoxia. Owing to this obligate relationship between PO_2 and oxygen content, it has proved difficult to distinguish between the effects of these two variables on promoting catecholamine release during hypoxia.

Recently, Perry and Reid (1992) proposed a mechanism to explain the abrupt release of catecholamines into the circulation during exposure of both rainbow trout and American eels to various levels of environmental hypoxia. According to this theory, catecholamines are released when the blood oxygen content is lowered to a critical catecholamine release threshold. Further, this theory predicts that the blood PO_2 at which this threshold is reached will vary according to the affinity of Hb O_2 -binding. Thus, fish possessing blood of high Hb O_2 -affinity (low P_{50} , the PaO_2 at which oxygen content is 50% of the maximal value) would be expected to release catecholamines at considerably lower blood PO_2 values than fish with low Hb O_2 -affinity (high P_{50}). In each instance, catecholamine release would occur at a uniform value of Hb O_2 -saturation (oxygen content) corresponding to approximately 45-60 % Hb O_2 -saturation. However, this model was based on data collected from two species (trout and eel) possessing widely different Hb O_2 -affinities. Therefore, it was conceivable that the differences in the catecholamine release profiles simply reflected intrinsic interspecific differences in PO_2

release thresholds and that the uniformity of the O₂ content release thresholds may have been coincidental.

The goal of this chapter was to determine the proximate stimulus, in terms of blood oxygen status, causing catecholamine release during hypoxia. The experimental design was to manipulate the intrinsic properties of Hb O₂-binding in trout and to assess the impact on the dynamics of catecholamine release during acute hypoxia. Hb O₂-binding affinity was altered by acclimating fish to either 5 or 15 °C. If catecholamines are indeed released into the circulation at a particular and variable arterial PO₂ (PaO₂) threshold corresponding to a critical and uniform O₂ content threshold (Perry and Reid 1992), then the predicted effect of acclimating fish to lower water temperature would be a lowering of the PaO₂ threshold at which catecholamines are mobilized, in agreement with the increase in Hb O₂-affinity induced by low temperature.

MATERIALS AND METHODS

Experimental Animals

Rainbow trout (*Oncorhynchus mykiss*) weighing between 200 and 300g (experimental N = 116) were maintained (conditions as previously described) at 12 °C for approximately 2 weeks before being separated into two different temperature acclimation groups; one group was acclimated to 5 °C and the other to 15 °C. The temperature was varied by 0.5 °C per day until the desired final temperature was reached. Fish were maintained under the final acclimation conditions for at least 2 months before experimentation.

Animal Preparation

As described in chapter 3, an indwelling polyethylene cannula was implanted into the dorsal aorta to permit periodic blood sampling.

Experimental Protocol

Individual groups of fish (N = 5 - 8 in each group) were acutely exposed to levels of hypoxia ranging between 30 - 80 torr. Specifically, the levels of hypoxia utilized were 80, 70, 60, 50, 45, 40, 35 and 30 torr. The lower limits of 30 torr (5 °C) and 35 torr (15 °C) were selected on the basis of the results of preliminary experiments showing marked mortality at more severe levels of hypoxia (especially at the higher temperature). In the present study, mortalities were observed at 35 torr (1 fish) and at 30 torr (2 fish) in the fish acclimated to 15 °C and 5 °C, respectively; the results from these animals have not been incorporated. The upper level of 80 torr was selected on the basis of preliminary

results showing a lack of catecholamine mobilization at this level of hypoxia and the level immediately preceding it (70 torr).

Hypoxia was initiated by first stopping the air and water flow to the experimental chamber and then quickly re-establishing water flow at the same rate using hypoxic water exiting a water/gas equilibration column previously set to the target PwO_2 . The PwO_2 was adjusted by manipulating the rates of nitrogen and water flow through the column. The water flow rate into the experimental chamber was always in excess of 5 l min^{-1} and was sufficient to achieve the desired degree of hypoxia in the chamber within 5 min. Fish were returned to normoxic conditions by re-establishing normal (normoxic) water flow and aeration.

The inflowing water and the water within the experimental chamber were continuously monitored for PO_2 . Usually, the PwO_2 of the inflow and chamber did not vary by more than 1 - 2 torr and the PwO_2 of the experimental chamber, rather than the column, was used in calculating the mean PwO_2 of the various hypoxic groups.

Blood samples (0.6 ml) were withdrawn from the dorsal aortic cannula pre-hypoxia, at 5 and 15 min after reaching the desired degree of hypoxia and 15 min after return to normoxic conditions. The arterial blood was analyzed immediately after sampling to determine PaO_2 , oxygen content (CaO_2), whole blood pH (pH_a) and haemoglobin concentration ($[Hb]$). The remaining blood was centrifuged and the plasma (200-250 μl) stored at -80°C prior to determination of catecholamine levels. The red cell pellet was resuspended in teleost saline and reinjected into the dorsal aorta.

Analytical Techniques

Whole-blood pH was determined with a microcapillary pH electrode (Radiometer G299A). Blood or water PO₂ was measured using PO₂ electrodes (Radiometer E5046) housed in thermostatted cuvettes (ambient water temperature 5 or 15 °C). PwO₂ from the equilibration column or the experimental chamber was monitored continuously by allowing the water to flow by siphon through the measuring chambers of the electrodes. The pH and PO₂ electrodes were maintained at the appropriate acclimation water temperature and used in conjunction with Radiometer PHM-71 acid-base analyzers and BMS3 MK2 blood micro-systems. The PO₂ electrodes were calibrated with water equilibrated to known PO₂ values using air-saturated water. The pH electrode was calibrated using precision buffers (Radiometer). CaO₂ was measured on 20 µl samples according to an established method (Tucker 1967). [Hb] measurements were performed in duplicate on 20 µl blood samples using a commercial spectrophotometric haemoglobin assay kit (Sigma).

In Vivo Oxygen Dissociation Curves

Oxygen dissociation curves were constructed using the measured values of PaO₂, CaO₂, and [Hb] from samples withdrawn pre-hypoxia and at 5 and 15 min of hypoxia. The values obtained after the return to normoxia were not utilized because of the possibility of persistent alterations of Hb O₂-affinity initiated by catecholamines. To adjust for differences in [Hb] and variable quantities of physically dissolved O₂ in the plasma, the amount of haemoglobin-bound O₂ per unit haemoglobin ([O₂]/[Hb]) was calculated using O₂ solubility coefficients for trout plasma (Boutilier *et al.* 1984). Oxygen dissociation curves were constructed by plotting [O₂]/[Hb] as a function of PaO₂ and fitting the data to

a sigmoidal function using an iterative curve-fitting program in a commercial graphics software package (Sigmaplot 5.0; Jandel Scientific). Blood oxygen-affinity (P_{50} ; the PaO_2 at half maximal Hb O_2 -saturation) and Hill coefficients were derived from the Hill plot.

Catecholamine Release Thresholds

The PaO_2 and $[\text{O}_2]/[\text{Hb}]$ thresholds for catecholamine release were calculated as described by Perry and Reid (1992). The technique is used to estimate the point at which plasma catecholamine levels rise significantly above baseline values. First, the mean baseline catecholamine levels were calculated by incorporating all values above a critical PaO_2 , the criterion for which was that plasma catecholamine levels were stable for at least 10 torr below this value. Next, the highest individual PaO_2 value with a catecholamine level statistically higher than baseline (outside the 95% confidence interval) was determined and the mean PaO_2 was calculated for all PaO_2 values below that value. The PaO_2 threshold was then calculated as that mean PaO_2 plus its 95% confidence interval. $[\text{O}_2]/[\text{Hb}]$ thresholds were calculated in a similar fashion.

Statistical Analysis

Where appropriate, the data are presented as the mean values ± 1 standard error of the mean. The results have been statistically analyzed by analysis of variance followed by Fisher's least significant difference test for multiple comparisons. 5% was taken as the level of significance.

RESULTS

Blood Acid-Base/Respiratory Variables

The effects of acute graded hypoxia on arterial blood respiratory variables at the two acclimation temperatures are shown in tables 6-1 (5 °C) and 6-2 (15 °C). For clarity, only the pre-hypoxia and the 15 min hypoxia data are presented as there were no significant differences in any of the measured variables between 5 and 15 min of hypoxia. After 15 min of recovery from hypoxia, all of the measured blood respiratory/acid-base variables had returned to pre-hypoxia levels except for whole blood pH (pHa) at the two severest levels of hypoxia (35 and 40 torr PwO₂) in the 15 °C acclimated fish.

Exposure of fish to hypoxia caused reductions in PaO₂, CaO₂, and O₂ bound to haemoglobin ([O₂]/[Hb]) that were roughly proportional to the severity of the hypoxia. The changes in CaO₂ and [O₂]/[Hb] were more pronounced in the fish acclimated to 15 °C (compare tables 6-1 and 6-2). Blood [Hb] was essentially unaltered during hypoxia; the decreases in [Hb] that were occasionally observed were probably attributable to the blood sampling rather than being a consequence of the hypoxia, *per se*. Whole-blood pH remained constant at PwO₂ values above 40 torr and 35 torr in the 15 and 5 °C fish, respectively. Below these levels of PwO₂, whole blood pH declined significantly and the reduction in pHa was more pronounced in the 15 °C acclimated fish.

Whole-blood pH was consistently (although not always) higher in the 5 °C acclimated fish in accordance with the predicted inverse relationship between blood temperature and pHa (see Heisler 1984). Occasionally, the pre-hypoxia values of PaO₂,

Table 6-1. Arterial blood respiratory variables in rainbow trout (*Oncorhynchus mykiss*) acclimated to 5 °C before and after 15 min of exposure to graded levels of external hypoxia ranging between 30 and 80 torr nominal final PwO₂. Values are shown as means $\pm$ 1 SEM (in parentheses); N = 5 - 8 in each group. An asterisk (*) indicates a significant difference from the pre-hypoxia value. A plus sign (+) indicates a significant difference from the corresponding value at 15 °C.

	Normoxia		80 torr		70 torr		60 torr		50 torr	
	Pre	15 min	Pre	15 min	Pre	15 min	Pre	15 min	Pre	15 min
PwO₂	160.1	157.8	160.0	83.4 *	160.6	69.3 *	160.3	60.2 *	159.5	49.7 *
(torr)	(0.3)	(1.7)	(0.4)	(2.0)	(0.3)	(0.5)	(0.2)	(0.7)	(0.3)	(0.3)
PaO₂	118.9 +	112.5	97.1 +	51.2 *	120.8 +	49.1 *	124.7 +	33.7 *	123.5 +	26.7 **
(torr)	(5.4)	(8.2)	(6.3)	(8.2)	(4.5)	(6.0)	(1.8)	(6.42)	(4.8)	(4.6)
CaO₂	7.6	7.3	6.9	6.6	7.5 +	5.7 **	6.8	5.1 **	9.1 +	5.1 **
(vol%)	(0.5)	(0.6)	(0.5)	(0.2)	(0.4)	(0.4)	(0.4)	(0.7)	(0.4)	(0.4)
[Hb]	7.4	6.7	7.2	6.8	7.4	6.7	6.5	5.9	9.2 +	8.1 *
(g dl ⁻¹)	(0.8)	(0.6)	(0.5)	(0.3)	(0.5)	(0.3)	(0.6)	(0.5)	(0.5)	(0.5)
O₂/Hb	1.06	1.10	0.98	0.95	1.03	0.86 **	1.09	0.90 **	1.00	0.65 **
(ml g ⁻¹)	(0.08)	(0.04)	(0.05)	(0.05)	(0.05)	(0.04)	(0.08)	(0.10)	(0.03)	(0.08)
pHa	7.94 +	7.91	7.93	8.00 +	8.09 +	8.14 +	7.96	7.93	8.06 +	7.95
	(0.02)	(0.01)	(0.08)	(0.04)	(0.05)	(0.05)	(0.04)	(0.09)	(0.03)	(0.10)

Table 6-1 continued

	45 torr		40 torr		35 torr		30 torr	
	Pre	15 min	Pre	15 min	Pre	15 min	Pre	15 min
PwO₂	156.6	43.4 *	155.6	39.9 *	160.0 +	34.3 *	160.6	31.9 *
(torr)	(1.3)	(0.3)	(3.0)	(1.7)	(1.0)	(0.3)	(0.3)	(1.4)
PaO₂	111.5	18.0 *	115.6	16.5 *	113.8	12.8 *+	104.6	13.9 *
(torr)	(5.7)	(2.4)	(6.4)	(1.7)	(8.9)	(0.8)	(17.1)	(1.1)
CaO₂	8.1	4.0 *	7.6	4.8 *+	7.2	3.1 *+	7.7	3.1 *
(vol%)	(0.6)	(0.4)	(0.7)	(0.5)	(0.3)	(0.4)	(0.4)	(0.2)
[Hb]	7.1	6.2	6.7	6.5	6.8	7.0 *	7.0	6.0 *
(g dl ⁻¹)	(0.6)	(0.4)	(0.8)	(0.7)	(0.4)	(0.4)	(0.5)	(0.2)
O₂/Hb	1.16 +	0.73 *+	1.17	0.72 *+	1.07	0.48 *+	1.12	0.51 *
(ml g ⁻¹)	(0.05)	(0.10)	(0.06)	(0.09)	(0.07)	(0.07)	(0.06)	(0.03)
pHa	7.91	7.9	8.09 +	8.05 +	8.00 +	7.82 *+	8.04	7.72 *
	(0.03)	(0.06)	(0.05)	(0.08)	(0.06)	(0.06)	(0.04)	(0.07)

Table 6-2. Arterial blood respiratory variables in rainbow trout (*Oncorhynchus mykiss*) acclimated to 15 °C before and after 15 min of exposure to graded levels of external hypoxia ranging between 35 and 80 torr nominal final PwO₂. Values are shown as means $\pm$ 1 SEM (in parentheses); N = 5 - 8 in each group. An asterisk (*) indicates a significant difference from the pre-hypoxia value. A plus sign (+) indicates a significant difference from the corresponding value at 5 °C.

	Normoxia		80 torr		70 torr		60 torr		50 torr	
	Pre	15 min	Pre	15 min	Pre	15 min	Pre	15 min	Pre	15 min
PwO₂	148.4	149.4	156.0	79.5 *	156.1	71.1 *	150.0	53.6 *	151.5	48.8 *
(torr)	(1.3)	(0.4)	(1.4)	(1.4)	(1.4)	(1.1)	(1.5)	(1.9)	(1.8)	(2.8)
PaO₂	85.4 +	99.8	113.5 +	50.6 *	105.1 +	45.6 *	106.3	29.3 **	98.2 +	17.7 **
(torr)	(7.8)	(6.5)	(2.9)	(4.3)	(5.8)	(7.1)	(5.6)	(2.1)	(7.6)	(1.5)
CaO₂	6.7	6.8	7.2	6.1 *	6.1 +	4.4 **	6.0	3.4 **	7.3 +	2.7 **
(vol%)	(0.4)	(0.6)	(0.2)	(0.5)	(0.5)	(0.5)	(0.4)	(0.2)	(0.6)	(0.5)
[Hb]	7.7	6.6	7.6	6.9 *	6.6	6.7	5.9	5.1	7.3 +	7.0
(g dl ⁻¹)	(0.5)	(0.5)	(0.2)	(0.2)	(0.4)	(0.3)	(0.6)	(0.6)	(0.5)	(0.4)
O₂/Hb	0.92	0.91	0.95	0.88	0.92	0.66**	1.05	0.62**	0.99	0.40**
(ml g ⁻¹)	(0.07)	(0.06)	(0.03)	(0.05)	(0.05)	(0.07)	(0.06)	(0.03)	(0.02)	(0.07)
pHa	7.81+	7.85	7.93	7.89 +	7.85	7.91 +	7.88	7.88	7.85 +	7.89
	(0.02)	(0.02)	(0.02)	(0.02)	(0.02)	(0.03)	(0.02)	(0.03)	(0.02)	(0.03)

Table 6-2 continued.

	45 torr		40 torr		35 torr	
	Pre	15 min	Pre	15 min	Pre	15 min
PwO₂	153.1	43.2 *	150.0	36.8 *	146.3+	33.4 *
(torr)	(2.5)	(1.6)	(1.8)	(1.1)	(3.2)	(0.9)
PaO₂	92.1	19.1 *	100.6	17.9 *	91.9	17.9 *
(torr)	(10.0)	(1.1)	(5.5)	(1.7)	(9.1)	(1.8)
CaO₂	7.5	3.9 *	7.2	2.3 *	6.6	1.6 *+
(vol%)	(0.5)	(0.4)	(0.5)	(0.3)	(0.4)	(0.1)
[Hb]	8.2	8.4 +	7.1	7.1	7.4	6.5
(g dl ⁻¹)	(0.3)	(0.2)	(0.5)	(0.5)	(0.4)	(0.6)
O₂/Hb	0.92 +	0.47 *+	1.05	0.32 *+	0.86	0.26 *+
(ml g ⁻¹)	(0.04)	(0.05)	(0.06)	(0.03)	(0.08)	(0.03)
pHa	7.92	7.86	7.83 +	7.57 *+	7.78	7.43 *+
	(0.03)	(0.04)	(0.02)	(0.07)	(0.03)	(0.06)

[Hb] or $[O_2]/[Hb]$ were significantly different between the two acclimation groups, although no obvious pattern was evident.

Blood Oxygen Dissociation Curves

In vivo blood oxygen dissociation curves (fig 6-1) were constructed for the 5 and 15 °C acclimated fish using the blood respiratory data gathered during the acute hypoxia experiments (tables 6-1, 6-2); the recovery data were not used. In theory, the $[O_2]/[Hb]$ of fully saturated Hb is 1.34 ml O_2 g⁻¹ Hb. In the present study, the $[O_2]/[Hb]$ at maximal binding was 1.1 ml g⁻¹, approximately 80 % of the theoretical maximum. This value is somewhat lower than those reported for trout haemoglobin in previous studies and indicates a significant fraction of non-functional haemoglobin of unknown origin. The $[O_2]/[Hb]$ at maximal binding was identical at both temperatures, thereby allowing valid comparisons between the two experimental groups. The apparent P_{50} values were determined assuming that maximal binding occurred at a $[O_2]/[Hb]$ value of approximately 1.1 ml g⁻¹.

At 5 °C, the *in vivo* P_{50} value was 14.0 torr whereas at 15 °C the P_{50} value was increased to 26.7 torr. The Hill coefficients were 1.29 and 1.76 at 5 and 15 °C, respectively.

Figure 6-1. *In vivo* blood oxygen dissociation curves of rainbow trout acclimated to (a) 5 °C or (b) 15 °C. The curves were constructed by sampling dorsal aortic blood from normoxic and acutely hypoxic fish. For comparison, the dashed lines represent the oxygen dissociation curve for the other temperature. The inserts in each panel are Hill plots from which the P_{50} values were calculated.

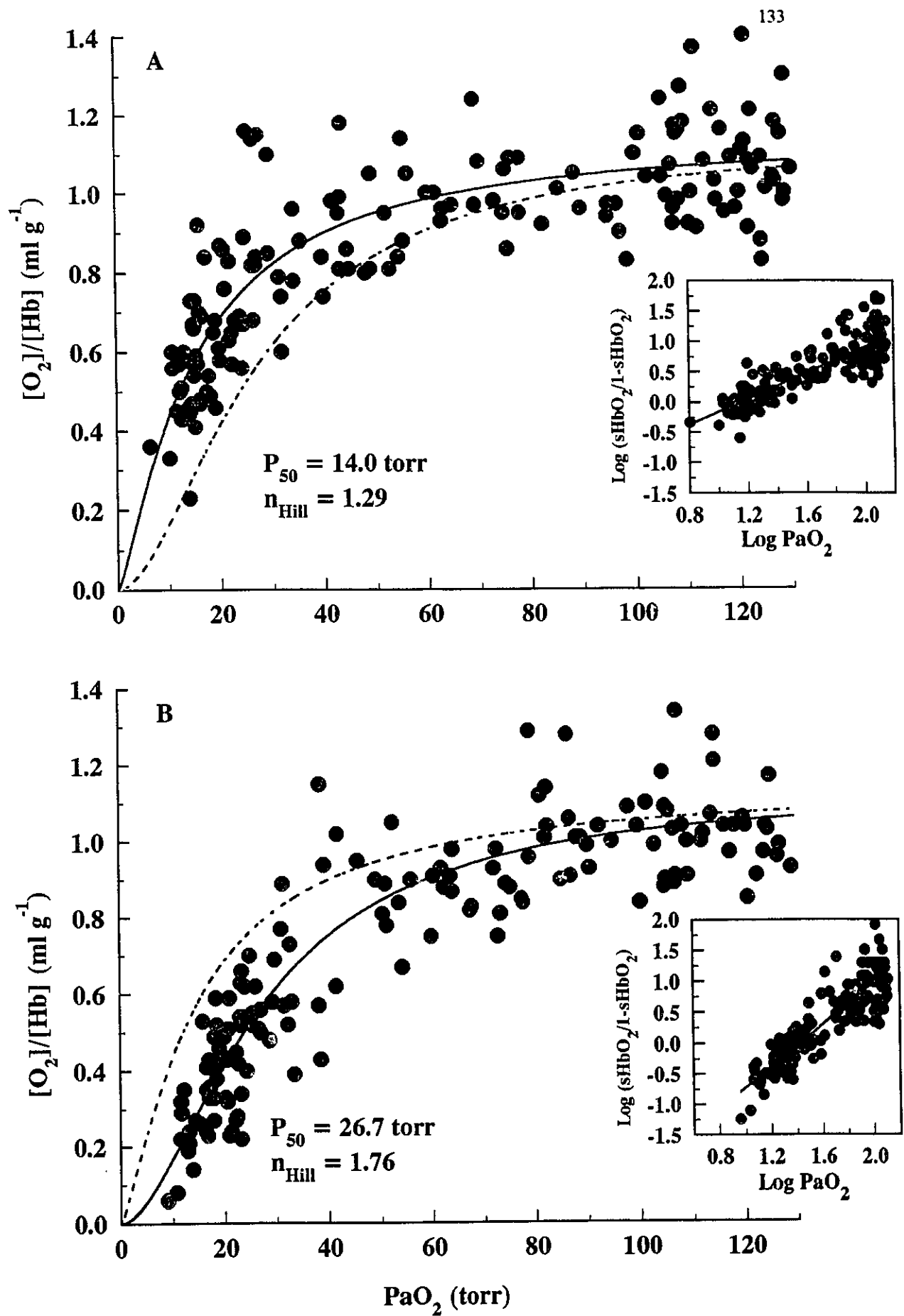


Figure 6-1

Plasma Catecholamine Levels

Regardless of the acclimation temperature, plasma catecholamine levels were elevated at PwO_2 values less than or equal to 60 torr (fig. 6-2). Adrenaline was the sole catecholamine released into the circulation at $PwO_2 = 60$ torr. Below 60 torr both adrenaline and noradrenaline levels were elevated, with adrenaline being the prevalent circulating catecholamine in most instances (fig 6-2). At PwO_2 values of 40 torr and 35 torr, plasma adrenaline levels were significantly greater in the 15 °C acclimated fish (fig 6-2a). At PwO_2 values of 50 torr and below, plasma noradrenaline levels were always higher (with the exception of 35 torr) in the 15 °C fish.

Figure 6-3 illustrates the relationship between PaO_2 and total plasma catecholamine levels (adrenaline + noradrenaline). At each acclimation temperature, plasma catecholamine levels remained remarkably constant over a wide range of PaO_2 but then increased abruptly when a critical PaO_2 threshold was reached. The PaO_2 thresholds were widely different at the two acclimation temperatures. At 5 °C, the calculated PaO_2 threshold for catecholamine release was 24 torr (fig 6-3a), whereas at 15 °C the corresponding PaO_2 threshold was 34.5 torr (fig 6-3b). The difference in these catecholamine release PaO_2 thresholds (10.5 torr) was approximately equal to the difference in the *in vivo* P_{50} values (12.7 torr) at the two distinct temperatures.

The relationships between $[O_2]/[Hb]$ and plasma catecholamine levels are shown in fig 6-4. The calculated catecholamine release threshold was essentially identical at each acclimation temperature, corresponding to approximately 60% Hb O_2 -saturation.

Figure 6-2. The effects of graded acute (15 min) external hypoxia on (a) plasma adrenaline and (b) plasma noradrenaline levels in rainbow trout acclimated to either 5 °C (open bars) or 15 °C (closed bars). Plasma catecholamine levels are expressed as a function of the nominal values for the partial pressure of oxygen in the water (PwO_2). The actual mean measured PwO_2 values did not differ significantly from the nominal values and are shown in tables 6-1 and 6-2 for 5 °C and 15 °C, respectively. Values are shown as means $\pm$ 1 SEM (N = 5 - 8 for each group). An asterisk (*) indicates a significant difference from the pre-hypoxia value. A plus sign (+) indicates a significant difference from the corresponding value at the other acclimation temperature.

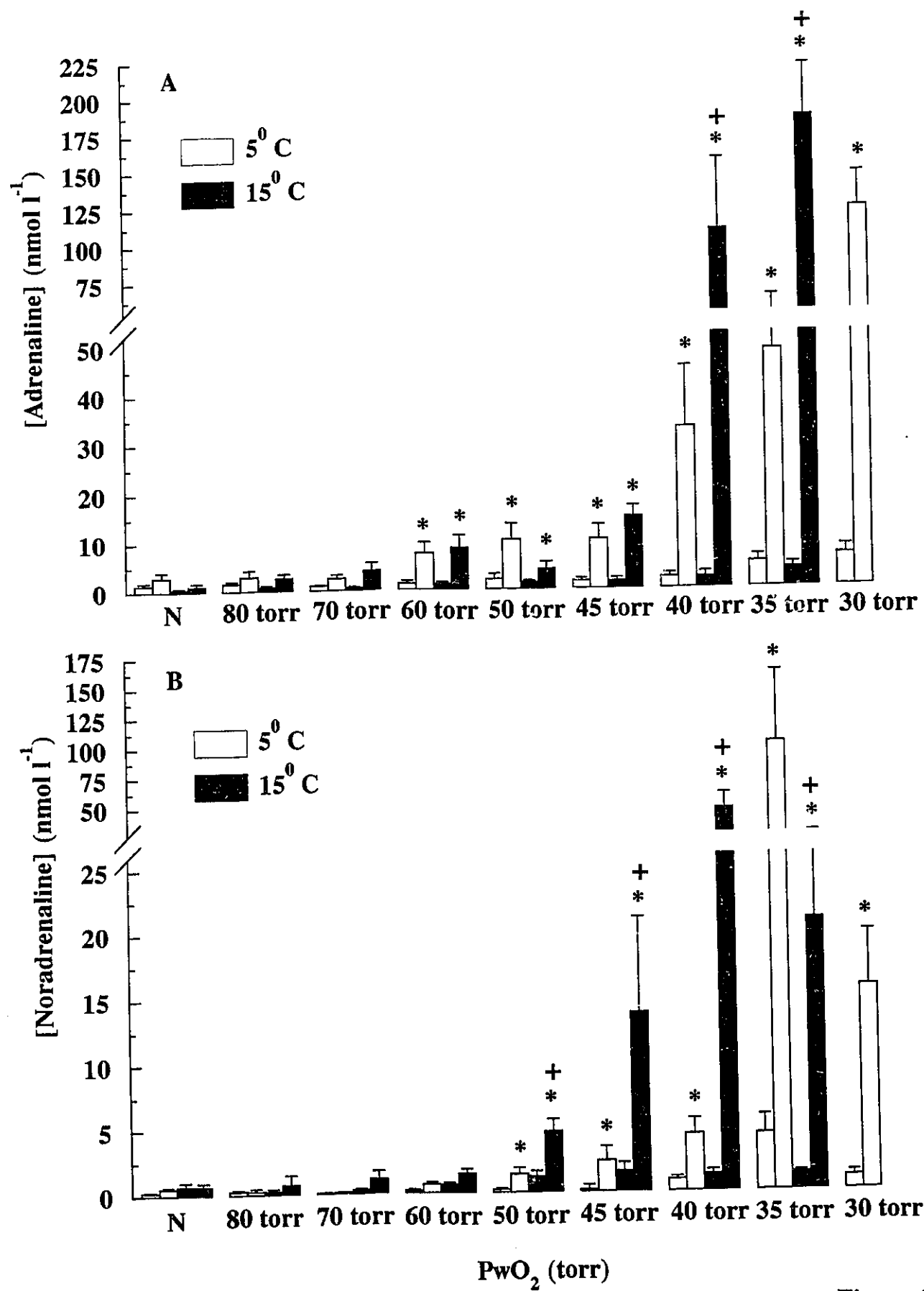


Figure 6-2

Figure 6-3. The relationship between arterial PO_2 (PaO_2) and total plasma catecholamine levels ([adrenaline + noradrenaline]) in rainbow trout acclimated to (a) 5 °C or (b) 15 °C and subjected to acute (15 min) graded hypoxia. The areas within the boxes represent the zones of catecholamine release which were determined by calculating the PaO_2 thresholds for release with the right-hand edge of the box representing the PaO_2 threshold of release. At each acclimation temperature, the catecholamine data have also been expressed using a logarithmic scale (inserts) to show more clearly the scatter about the baseline values.

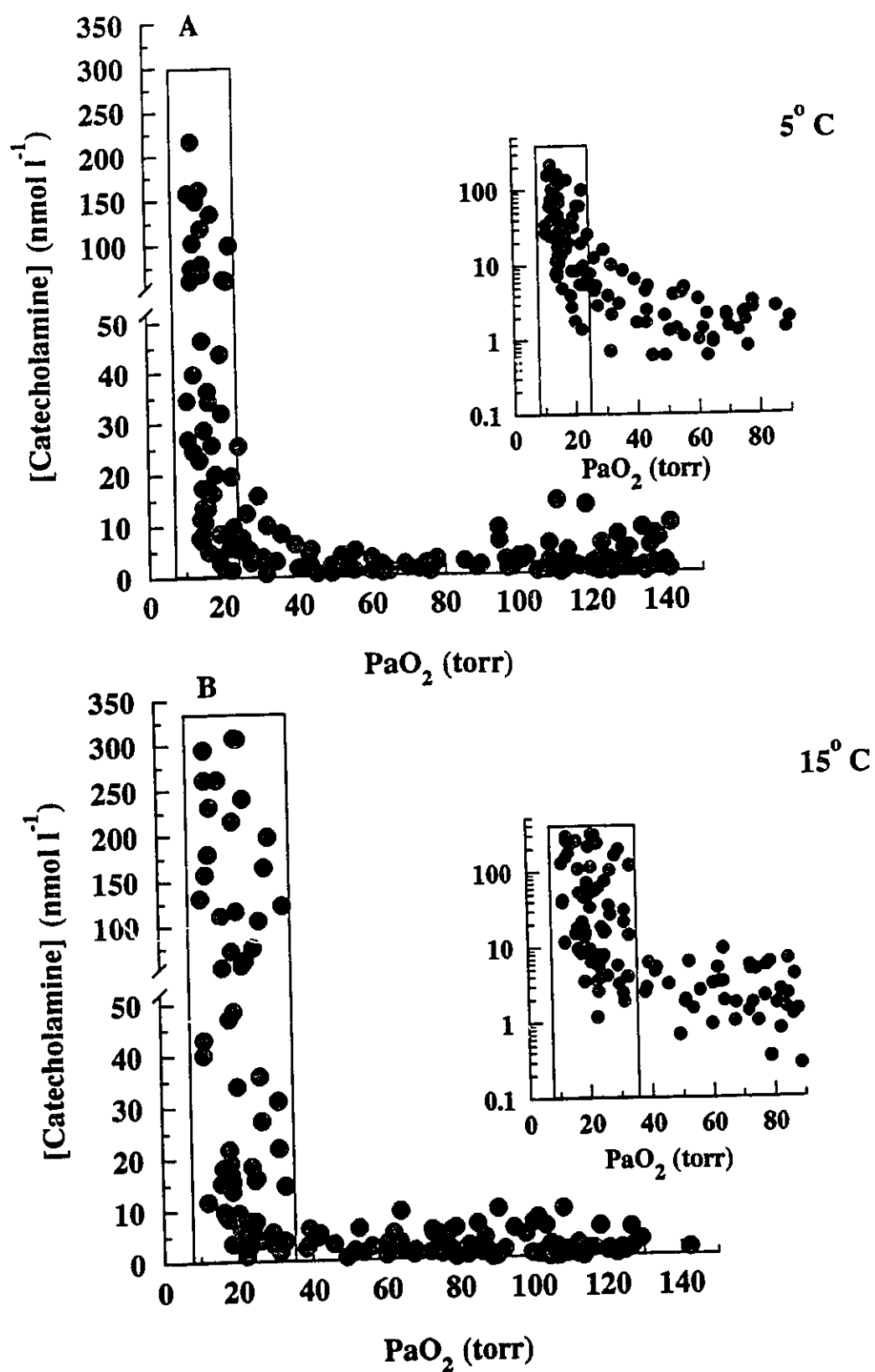


Figure 6-3

Figure 6-4. The relationship between arterial blood oxygen content (expressed as $[O_2]/[Hb]$) and total plasma catecholamine levels ([adrenaline + noradrenaline]) in rainbow trout acclimated to (a) 5 °C or (b) 15 °C and subjected to acute (15 min) graded hypoxia. The areas within the boxes represent the zones of catecholamine release which were determined by calculating the $[O_2]/[Hb]$ thresholds for release with the right-hand edge of the box representing the $[O_2]/[Hb]$ threshold of release. At each acclimation temperature, the catecholamine data have also been expressed using a logarithmic scale (inserts) to show more clearly the scatter about the baseline values.

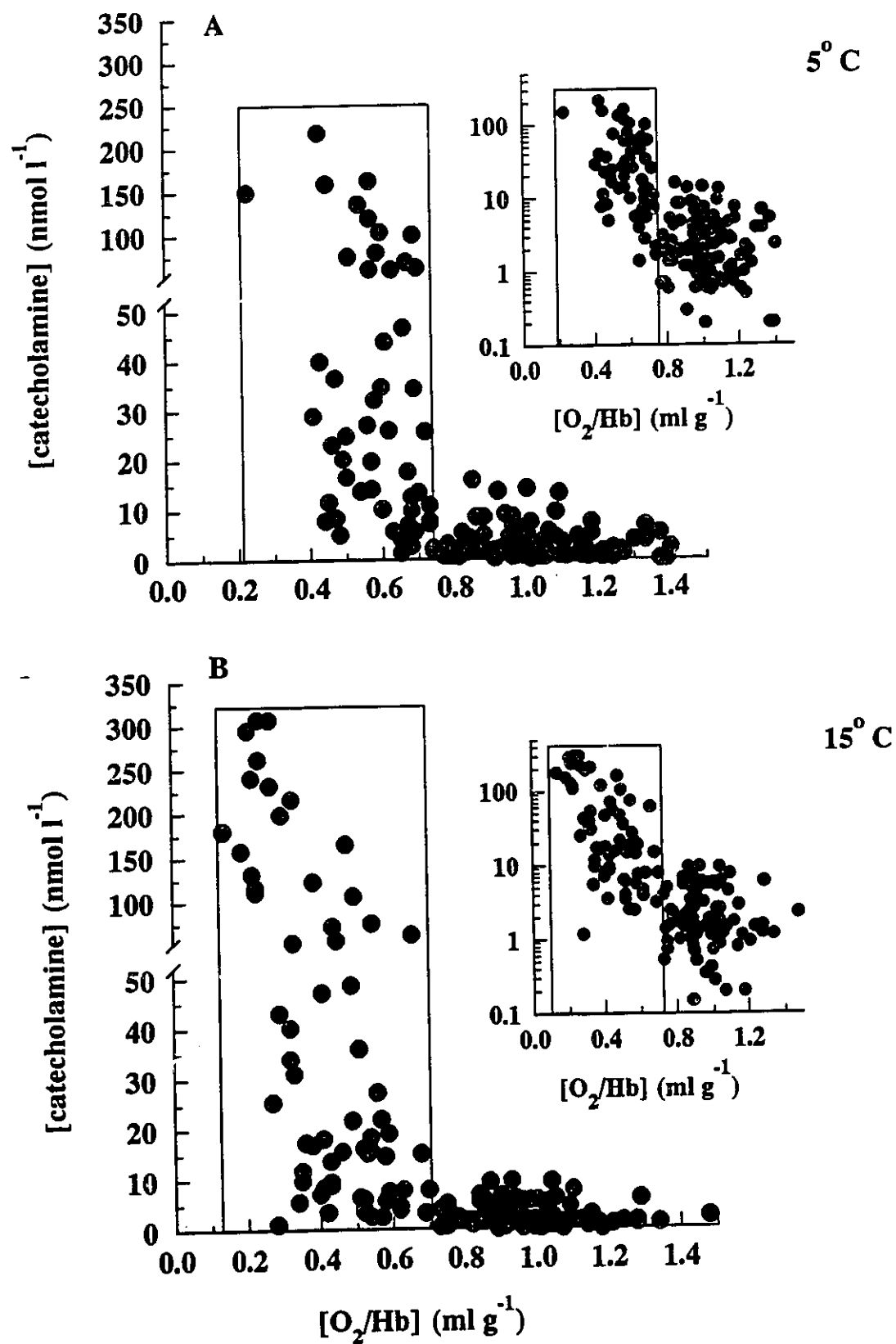
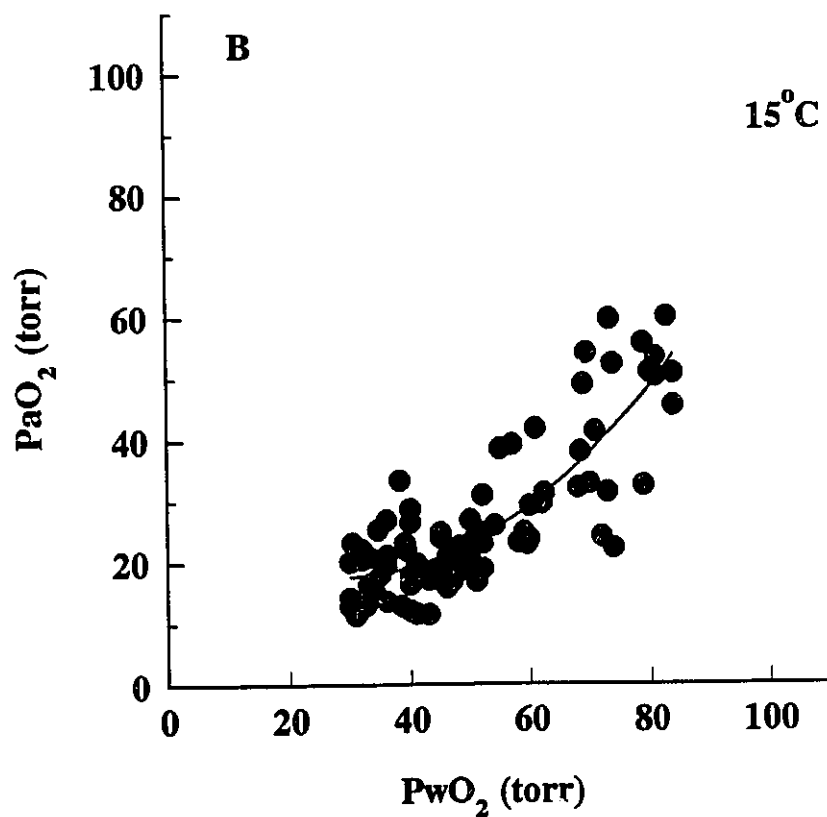
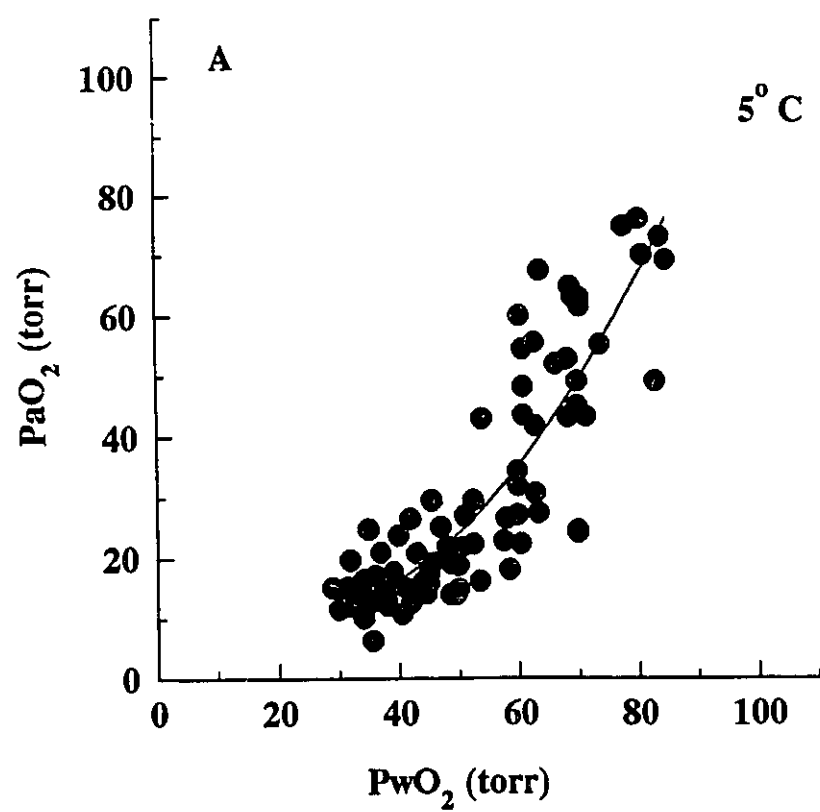


Figure 6-4

Water-Blood PO₂ Relationship

The relationships between PwO₂ and PaO₂ during normoxia and hypoxia are illustrated in fig 6-5. PaO₂ declined with decreasing PwO₂ in an essentially similar manner in both acclimation groups. During moderate hypoxia (PwO₂ > 60 torr), for any given value of PwO₂, PaO₂ at 5 °C was generally higher than at 15 °C. These differences were not apparent at the more severe levels of hypoxia.

Figure 6-5. The relationship between water PO_2 (PwO_2) and arterial blood PO_2 (PaO_2) during normoxia or graded acute (15 min) hypoxia in rainbow trout acclimated to either (a) 5 °C or (b) 15 °C. The curves were fitted using a commercial graphics software package (Sigmaplot 5.0; Jandel Scientific).

**Figure 6-5**

DISCUSSION

Effects of Acclimation Temperature on Catecholamine Release

The results of this chapter support the theory (see introduction) proposed by Perry and Reid (1992). At each acclimation temperature, catecholamines were released into the circulation as the blood oxygen status traversed a critical PO_2 threshold corresponding to a uniform value of approximately 60% Hb O_2 -saturation. This catecholamine release threshold was similar to the release threshold (45 - 60 % Hb O_2 -saturation) reported by Perry and Reid (1992). Owing to the decrease in P_{50} in the 5 °C acclimated fish, this threshold was reached at a lower PaO_2 than in the 15 °C acclimated fish. In other words, the difference in the PaO_2 thresholds at the two temperatures was essentially equal to the difference in the P_{50} values. The simplest explanation for these data is that the lowering of Hb O_2 -saturation (or a closely related variable such as blood oxygen content) is the proximate signal causing the release of catecholamines rather than a lowering of the blood PO_2 *per se*.

A depression of oxygen content, to a critical release threshold, presumably initiates the release of catecholamines from chromaffin cells via neural (sympathetic) pathways. Although speculative, within the arterial circulation there probably exist chemoreceptors sensitive to changes in the oxygen content of the blood (or extracellular fluid) surrounding them. Upon a decrease of blood oxygen content to a critical threshold, these receptors would initiate a neural reflex which ultimately results in the stimulation of the sympathetic nerves innervating the chromaffin cells. Conceptually it is perhaps simpler to envision the existence of a chemoreceptor sensitive to changes in PO_2 rather than oxygen content.

However, oxygen content receptors are most likely responding to changes in the amount of oxygen delivered to the receptor cell per unit time. Therefore changes in the rate of oxygen delivery to these putative chemoreceptors is a possible explanation for the control of catecholamine release via depressions in blood oxygen content.

In addition to oxygen content depression initiating catecholamine release via neural stimulation of the chromaffin cells, Perry *et al.* (1991) demonstrated a direct effect of blood hypoxemia, *in situ*, on catecholamine release from the chromaffin cells in Atlantic cod. Presumably therefore, there is an oxygen content/delivery- sensitive chemoreceptive system present in/on the chromaffin cells. Such a mechanism could possibly take the form of oxygen sensitive K^+ channels which close upon exposure to a critical level of blood oxygen content. Closure of K^+ channels would result in membrane depolarization and ultimately release of catecholamines via exocytosis.

Regardless of the mechanism underlying the relationship between blood oxygen content and catecholamine release, the obvious and important consequence of this relationship is that catecholamines are released into the circulation only upon marked impairment of blood O_2 transport. Unlike the cardiovascular and ventilatory adjustments to hypoxia (see reviews by Perry *et al.* 1992; Fritsche and Nilsson 1993), which often begin with only slight reductions in PwO_2 , the release of catecholamines is not initiated until PwO_2 is lowered to very low levels (usually below 60 torr; see review by Randall and Perry 1992). Given that the predominant effect of elevated plasma catecholamine levels is to enhance branchial O_2 transfer and blood O_2 transport, the physiological significance of their delayed release into the circulation after only a severe reduction of blood O_2 content

is evident. From a design viewpoint, it would be impractical to link changes in PO_2 *per se* to catecholamine mobilization because PO_2 is not a reliable indicator of blood oxygen content. This reflects the non-linear relationship between PO_2 and Hb O_2 -saturation as well as the interactive effects of numerous allosteric modifiers of Hb O_2 -binding, such as carbon dioxide, protons and organic phosphates (Weber and Jensen 1988; Jensen 1991). Thus, the reliance of catecholamine release on a critical lowering of blood O_2 content may have evolved so as to improve O_2 delivery when it is compromised by a developing hypoxemia.

A linkage between blood O_2 content/Hb O_2 -saturation and plasma catecholamine levels has been suggested by the results of previous studies in addition to the aforementioned comparison of trout and eel (see above). First, anaemic fish release catecholamines into the circulation (Iwama *et al.* 1987; Perry *et al.* 1989) even under hyperoxic conditions (Perry *et al.* 1989). In anaemic fish, Hb O_2 -saturation is not lowered, which suggests that there is a specific role in the lowering of blood oxygen content in causing release. Second, it was shown that the cause of catecholamine release during hypercapnic acidosis in trout is the associated hypoxemia (owing to the Root effect) rather than the acidosis itself (Perry *et al.* 1989). Third, Fievet *et al.* (1990) reported that the PaO_2 threshold for catecholamine release was substantially lowered after repeated episodes of acute hypoxia. An interpretation of these data is that Hb O_2 -affinity was raised after the initial episode of hypoxia [a result, at least in part, of catecholamine release (Nikinmaa 1983)] and thus led to a lowering of the PaO_2 threshold.

Effects of Acclimation Temperature on Hb O₂-Affinity

The merit of the *in vivo* O₂ dissociation curve is that it yields functional P₅₀ values that encompass the net effects of potential curve modifiers, including changes in blood acid-base status and elevated catecholamine levels. The P₅₀ values reported in this chapter are similar to those previously reported *in vitro* (Milligan and Wood 1987; Soivio *et al.* 1980; Vorger 1986) or *in vivo* (Tetens and Christensen 1987). The reasons for the significant amount of non-functional haemoglobin (maximal Hb O₂-saturation was only approximately 80% of the theoretical maximum) in these fish are unknown but it presumably reflects an unusually large fraction of methaemoglobin.

The results of this chapter demonstrate an increase in Hb O₂-affinity as temperature was lowered from 15 to 5 °C (P₅₀ decreased from 26.7 torr to 14 torr). Although the inverse relationship between temperature and Hb O₂-affinity is well documented following acute changes in blood temperature (e.g. Vorger 1986; see also reviews by Weber and Jensen 1988; Jensen 1991), considerably less is known of the chronic effects of acclimation to different temperatures. It is generally believed, however, that chronic temperature changes elicit smaller effects on Hb O₂-affinity than do acute changes (see review by Wood 1980). For example, Weber *et al.* (1976) demonstrated that acclimation of rainbow trout to temperatures varying between 5 and 22 °C for as long as 4 months was without effect on the Hb O₂-affinity of whole blood, assessed *in vitro*. Clearly the results of this chapter showing an effect of chronic temperature acclimation on Hb O₂-affinity are in marked contrast to the study of Weber *et al.* (1976). Further, the changes in P₅₀ were essentially similar to the changes that accompany acute temperature changes *in*

vitro (e.g. Vorger 1986). Red blood cell intracellular pH or levels of red blood cell organic phosphates were not measured in this study, making it difficult to compare these results with those of previous studies. Although not always statistically significant, whole blood pH (pHa) was generally elevated in the fish acclimated to 5 °C in accordance with the usual inverse relationship between pHa and temperature (Heisler 1984). Thus it is also likely that red blood cell pH was elevated in the 5 °C acclimated fish given that hydrogen ions are passively distributed across the red blood cell membrane (see Nikinmaa 1992).

Other Potential Causes/Correlates of Catecholamine Release

Exposure of fish to the more severe levels of hypoxia (30-40 torr) elicited marked acidosis of the blood (see tables 6-1, 6-2). It is tempting to speculate therefore, that the blood acidosis is a cause (or one of the causes) of the greatly elevated plasma catecholamine levels during severe hypoxia. Although several studies have reported significant correlations (Tang and Boutilier 1988; Perry and Reid 1992) or relationships (Boutilier *et al.* 1986; this chapter) between the extent of blood acidosis and circulating catecholamine levels, it is difficult to ascribe a direct role to blood acidosis in causing catecholamine release because of the hypoxemia that normally accompanies acidosis in teleost fish. Furthermore, it has been demonstrated (Perry *et al.* 1989; Aota *et al.* 1990) that acidosis itself does not initiate catecholamine release in trout unless it is associated with blood hypoxemia. An additional problem in assigning a role for blood pH changes in the control of catecholamine release is the inherent difficulty in separating the cause of release from the consequences of release. Catecholamines, when released into the blood, cause acidification of the plasma as a result of the stimulation of red blood cell Na^+/H^+

exchange (see reviews by Nikinmaa 1992; Thomas and Perry 1992) and thus high levels of catecholamines would normally accompany blood acidification even if acidosis itself were not a cause of catecholamine release. Finally, at moderate levels of hypoxia (> 40 torr), blood acidosis is clearly not a factor in triggering release because blood pH is either unaltered at such times (this chapter) or even elevated owing to hyperventilation. Variations in the PaO_2 versus PwO_2 relationship also cannot explain the differing patterns of release at the different temperatures, as these relationships were essentially indistinguishable at the PwO_2 levels at which catecholamines are released (fig 6-5).

The results of this chapter and other studies (Perry *et al.* 1989; Thomas *et al.* 1992; Perry and Reid 1992) provide compelling evidence that lowering of blood Hb O_2 -saturation and/or blood oxygen content is the factor that signals catecholamine release during hypoxia. However, owing to the nature of this study, in which acclimation to different temperatures was used as a tool to modify Hb O_2 -affinity, the possibility of involvement of other temperature-dependent factors, such as thermal modulation of a PO_2 receptor, cannot be excluded. The important theme emerging from these studies is that catecholamines are released into the circulation of teleost fish only when a critical threshold of blood O_2 content is reached. Presumably, the lowering of oxygen content to a critical threshold would stimulate both neural and local (if present on the chromaffin cells in trout) oxygen chemoreceptors at a similar threshold allowing for a synergistic initiation of catecholamine release in response to severe hypoxia.

CHAPTER 7.

**IMMUNOHISTOCHEMICAL LOCALIZATION OF BIOACTIVE
PEPTIDES AND AMINES ASSOCIATED WITH THE CHROMAFFIN TISSUE OF
FIVE SPECIES OF FISH**

INTRODUCTION

In contrast to teleost fish, the chromaffin cells in cyclostomes/agnathans, such as the Atlantic hagfish (*Myxine glutinosa*), are distributed within the systemic and portal hearts and in large veins and arteries (Augustinsson *et al.* 1956; Johnels and Palmgren 1960; Östlund *et al.* 1960; Bloom *et al.* 1961; von Euler and Fänge 1961) whereas in elasmobranchs (e.g. spiny dogfish, *Squalus acanthias*) chromaffin cells are associated with paravertebral autonomic ganglia (von Euler and Fänge 1961). The axillary bodies, comprising chromaffin cells in association with the gastric ganglia, are the primary source of circulating catecholamines in elasmobranchs (Nilsson 1983).

Numerous studies, both immunohistochemical and biochemical, have revealed the presence of bioactive peptides and amines, including vasoactive intestinal peptide (VIP) (Leboulenger *et al.* 1983a, b), neuropeptide Y (NPY) (Majane *et al.* 1985; Kuramoto 1987; Steiner *et al.* 1989; Fried *et al.* 1991; Fernandez-Vivero *et al.* 1993), somatostatin (Lundberg *et al.* 1979), substance P (Kuramoto *et al.* 1985; Kuramoto 1987), enkephalins (Viveros *et al.* 1980; Leboulenger *et al.* 1983a, b, 1986; Viveros and Wilson 1983; Kuramoto 1987; Reinecke *et al.* 1992) and the biogenic amine, serotonin (Holtzworth *et al.* 1984; Kuramoto 1987; Delarue *et al.* 1988, 1992; Fernandez-Vivero *et al.* 1993), within vertebrate chromaffin cells. Thus these cells may function physiologically as more than a mere source of circulating catecholamines. Additionally, these bioactive substances may serve as modulators of catecholamine secretion from the chromaffin cells.

In contrast to the extensive literature available regarding the non-catecholamine contents of chromaffin cells in many vertebrate (mammalian, amphibian) species, virtually nothing is known about the presence of peptides or biogenic amines in the chromaffin cells of fish. By analogy with other vertebrates, the chromaffin cells in fish and the nerves supplying them may also contain bioactive substances similar to those identified in other vertebrate species. The goal of this chapter was to identify and localize, using immunohistochemical techniques, biogenic peptides and amines associated with the chromaffin cells in rainbow trout (*Oncorhynchus mykiss*), Atlantic

cod (*Gadus morhua*), European eel (*Anguilla anguilla*), spiny dogfish (*Squalus acanthias*), and Atlantic hagfish (*Myxine glutinosa*).

MATERIALS AND METHODS

Experimental animals

Atlantic cod, Atlantic hagfish, European eels and spiny dogfish were captured by local fishermen along the west coast of Sweden and transported to the University of Göteborg in aerated seawater. Rainbow trout were obtained from local fish farms and transported to the University of Göteborg in aerated freshwater.

Fish were maintained indoors in large fibreglass aquaria supplied with aerated recirculated (10 °C) seawater (Atlantic cod, Atlantic hagfish, spiny dogfish and European eels) or freshwater (rainbow trout). The photoperiod was maintained at 12L:12D. All fish were allowed to acclimatize to the aquarium conditions for a minimum of 1 week prior to experimentation.

Tissue fixation

Atlantic cod, rainbow trout and spiny dogfish were killed by a sharp blow to the head, whereas European eels and Atlantic hagfish were killed by anaesthetic overdose (10 g l⁻¹ 3-ethyl-m-aminobenzoate, MS 222). The PCV from Atlantic cod, rainbow trout and European eels were removed. Both left and right axillary bodies were taken from spiny dogfish whereas both the systemic and the portal hearts were removed from hagfish. All tissues were fixed by immersion in Zamboni fixative (15% picric acid, 2% formaldehyde in 0.1 mol l⁻¹ phosphate buffer, pH 7.2) for 16 h. The fixative was washed from the tissue with 80% ethanol (3 X 15 min), dehydrated (95% and 100% ethanol; 30 min each), xylene-treated (30 min) and rehydrated (100%, 95%, 80% and 50% ethanol). Separate aliquots of tissue were fixed in 4% formaldehyde for 4 h and were not dehydrated. The tissue, regardless of the fixative, was then left for 16 h in phosphate buffer with 30% sucrose.

The tissue was quick frozen in mounting medium (Tissue-Tek) by immersion in liquid nitrogen. Sections (10 µm) were cut on a cryostat and thaw-mounted on poly-L-lysine coated slides.

Immunohistochemistry

Sections were incubated for 16 h in a sealed moist chamber with primary antisera (see Table 7-1 for a description of the antisera). The sections were then washed (3 X 10 min) in phosphate buffered saline (PBS, 0.1 mol l⁻¹, pH 7.2, containing 0.5 mol l⁻¹ NaCl), incubated in a moist sealed chamber for 1 h with the relevant secondary antiserum conjugated with fluorescein isothiocyanate (FITC) or rhodamine (R), and then washed (3 X 10 min) with PBS. Coverslips were mounted onto the slides with bicarbonate-buffered glycerol (1:1, pH 8.5) and the slides were viewed under a fluorescence microscope equipped with filters suitable for the excitation of FITC (green fluorescence, 495 nm) or R (red fluorescence, 515 nm). Photographs of sections were taken with a camera (Tmax film) mounted on the microscope.

Double labelling

In order to determine more precisely the location of serotonin, VIP and pituitary adenylyl cyclase activating peptide (PACAP) with respect to the chromaffin cells (see Results) and to ascertain the possible differential distribution of tyrosine hydroxylase (TH), dopamine- β -hydroxylase (DBH) and phenylethanolamine-N-methyltransferase (PNMT) amongst different chromaffin cells, sections were incubated, as described above, with the following combinations of antisera: Ga-DBH (produced in goat) and either TE-101 (TH), TE-103 (DBH), TE-104 (PNMT), AES-308 (serotonin), Mi-VIP, PACAP 27 or PACAP 38 (all produced in rabbits). The sections were washed and incubated with swine-anti-rabbit antisera conjugated with R (Swar-R) for 16 h, washed (3X with PBS) and incubated with non-conjugated rabbit antisera for a further 2 h. Following this incubation, the sections were not washed but were immediately incubated (2 h) with rabbit-anti-goat antisera conjugated with FITC (Rag-F). The sections were then washed and mounted with coverslips as described above. Chromaffin cells were visualized either under green (Ga-DBH) or red (TE 101, 103 and 104) fluorescence whereas serotonin, VIP and PACAP were also viewed under red fluorescence and compared with the green fluorescence for Ga-DBH.

In the eel, it was also necessary to determine the location of the peptides NPY and PYY with respect to the chromaffin cells (see Results). In addition to the double labelling mentioned above, sections were incubated (16 h) with a mixture of primary antisera against PYY (produced in guinea pig) and antisera against either TH, DBH, PNMT, serotonin, VIP, PACAP or NPY (all produced in rabbits). Sections were then washed and incubated (1 h) with a mixture of goat-anti-guinea pig (gagp-R; red fluorescence) and swine-anti-rabbit (swar-FITC; green fluorescence) antisera, washed and viewed.

Specificity controls

The specificity of labelling reactions was determined by preincubating (96 h) the antisera with the substances that they were raised against and with other substances that stained immunopositively in similar locations or with substances having similar sequences/structures (see Table 7-2 for a description of the antigens used to preadsorb the various antisera). Following the 96 h incubation, the antisera/antigen solution was applied to the tissue sections as described above. In all cases, the labelling observed was specific and no cross-reactivity with other substances occurred. The application of secondary antisera alone produced no specific labelling.

Table 7-1. Details of the antisera used in this study including name or code, working dilution (antisera were diluted in 0.9 % PBS containing 0.05 mg ml⁻¹ bovine serum albumin, 0.05 mg ml⁻¹ thyroglobulin, 2.5 mg ml⁻¹ Na azide, 20 mg ml⁻¹ EDTA), source and host. Abbreviations: TH tyrosine hydroxylase, DBH dopamine- β -hydroxylase, PNMT phenylethanolamine-N-methyltransferase, VIP vasoactive intestinal peptide, PACAP pituitary adenylyl cyclase activating peptide, NPY neuropeptide Y, PYY peptide tyrosine tyrosine, ME met-enkephalin, MERF met-enkephalin-arg⁶-phe⁷, MERGL met-enkephalin-arg⁶-gly⁷-leu⁸, LPLRFamide leucine-proline-leucine-arginine-phenylalanine-NH₂, FMRFamide phenylalanine-methionine-arginine-phenylalanine-NH₂, FITC fluorescein isothiocyanate, R rhodamine, CRB Cambridge Research Biochemicals

Substance	Antisera code	Dilution	Source	Host
TH	TE-101	1:100	Eugene Tech, Eugene, USA	Rabbit
	BO-TH	1:100	Boehringer, Mannheim, Ger	Rabbit
DBH	TE-103	1:100	Eugene Tech, Eugene, USA	Rabbit
	Ga-DBH	1:200	R. A. Rush, Adelaide, Aust	Goat
	Ra-DBH	1:200	R. A. Rush, Adelaide, Aust	Rabbit
PNMT	TE-104	1:100	Eugene Tech, Eugene, USA	Rabbit
	Mi-PNMT	1:50	Milab, Malmo, Sweden	Rabbit
Serotonin	AES-308	1:100	Sera-lab, Sussex, UK	Rabbit
	0601	1:100	Immunotech, Marseille, Fra	Rabbit
	SER-ICM	1:100	Immunochem, Carson, USA	Rabbit
	CRB-SER	1:100	CRB, Cambridge, UK	Rabbit
VIP	Mi-VIP	1:1000	Milab, Malmo, Sweden	Rabbit
	MAR-VIP	1:500	Milab, Malmo, Sweden	Guinea pig
PACAP	PACAP 27	1:400	Peninsula Labs, Belmont, USA	Rabbit
	PACAP 38	1:400	Peninsula Labs, Belmont, USA	Rabbit
NPY	NPY-A	1:100	Amersham, Little Chalfont, UK	Rabbit
	NPY-ab22	1:100	Peninsula, Belmont, USA	Rabbit
PYY	MAR-PYY	1:100	CRB, Cambridge, U.K.	Guinea pig
Enkephalins				
ME	Mi-enk	1:100	Milab, Malmo, Sweden	Rabbit
MERF	L189	1:100	G.J. Dockray, Liverpool, UK	Rabbit
MERGL	L150	1:100	G.J. Dockray, Liverpool, UK	Rabbit
LPLRFamide	L197	1:100	G.J. Dockray, Liverpool, UK	Rabbit
FMRFamide	L155	1:100	G.J. Dockray, Liverpool, UK	Rabbit

Galanin	P-GAL	1:100	Perinsula, Belmont, USA	Rabbit
	CRB-GAL	1:100	CRB, Cambridge, UK	Rabbit
Histamine	Mi-HIST	1:100	Milab, Malmo, Sweden	Rabbit
Substance P	CRB-SP	1:100	CRB, Cambridge, UK	Rabbit
	G10	1:100	A-C Jönsson, Sweden	Rabbit
Dopamine	DA	1:200	Immunotech, Marseille, Fra	Rabbit
Somatostatin	INC-SST	1:100	Milab, Malmo, Sweden	Rabbit
Chromogranin	Mi-CAB	1:100	Milab, Malmo, Sweden	Rabbit
Chromogranin A	L300	1:50	G.J. Dockray, Liverpool, UK	Rabbit
	L340	1:50	G.J. Dockray, Liverpool, UK	Rabbit
Chromogranin B	L339	1:50	G.J. Dockray, Liverpool, UK	Rabbit
Swine-anti-rabbit (FITC or R)	Swar-FITC	1:20	Dakopatts, Glostrup, Denmark	Swine
	Swar-Rhodamine	1:20		Swine
rabbit-anti-goat (FITC)	Rag-FITC	1:10	Dakopatts, Glostrup, Denmark	Rabbit
goat-anti-guinea pig (R)	gagp-Rhodamine	1:10	Cappel Res. Rgts., Turnhout, Belgium	Goat

Table 7-2. Details of the antigens used in the preadsorption specificity controls. For any given substance, each antisera listed was preincubated with each of the antigens listed for 96 h prior to application to tissue sections. Abbreviations and antisera codes/dilutions are the same as in Table 7-1.

Substance	Antisera	Antigen	[Antigen]	Source
TH	TE-101	TH	10^9 units l^{-1}	Sigma, St. Louis, USA
		DBH	10^9 units l^{-1}	Sigma, St. Louis, USA
		NPY (eel only)	10^{-4} mol l^{-1}	CRB, Cambridge, UK
		PYY (eel only)	10^{-4} mol l^{-1}	CRB, Cambridge, UK
		Serotonin	10^{-3} mol l^{-1}	Sigma, St. Louis, USA
DBH	TE-103 Ga-DBH Ra-DBH	DBH	10^9 units l^{-1}	Sigma, St. Louis, USA
		TH	10^9 units l^{-1}	Sigma, St. Louis, USA
		NPY (eel only)	10^{-4} mol l^{-1}	CRB, Cambridge, UK
		PYY (eel only)	10^{-4} mol l^{-1}	CRB, Cambridge, UK
		Serotonin	10^{-3} mol l^{-1}	Sigma, St. Louis, USA
PNMT	TE-104	PNMT	10^9 units l^{-1}	Sigma, St. Louis, USA
		DBH	10^9 units l^{-1}	Sigma, St. Louis, USA
		NPY (eel only)	10^{-4} mol l^{-1}	CRB, Cambridge, UK
Serotonin	AES-308 0601 SER-ICM CRB-SER	Serotonin	10^{-3} mol l^{-1}	Sigma, St. Louis, USA
		DBH	10^9 units l^{-1}	Sigma, St. Louis, USA
		NPY (eel only)	10^{-4} mol l^{-1}	CRB, Cambridge, UK
		PYY (eel only)	10^{-4} mol l^{-1}	CRB, Cambridge, UK
VIP	Mi-VIP	VIP	10^{-4} mol l^{-1}	CRB, Cambridge, UK
		PACAP 38	10^{-4} mol l^{-1}	CRB, Cambridge, UK
PACAP	PACAP 27 PACAP 38	PACAP 38	10^{-4} mol l^{-1}	CRB, Cambridge, UK
		VIP	10^{-4} mol l^{-1}	CRB, Cambridge, UK
NPY (eel only)	NPY-A NPY-ab22	NPY	10^{-4} mol l^{-1}	CRB, Cambridge, UK
		PYY	10^{-4} mol l^{-1}	CRB, Cambridge, UK
		serotonin	10^{-3} mol l^{-1}	Sigma, St. Louis, USA
		DBH	10^9 units l^{-1}	Sigma, St. Louis, USA
PYY (eel only)	MAR-PYY	PYY	10^{-4} mol l^{-1}	CRB, Cambridge, UK
		NPY	10^{-4} mol l^{-1}	CRB, Cambridge, UK
		DBH	10^9 units l^{-1}	Sigma, St. Louis, USA
		serotonin	10^{-3} mol l^{-1}	Sigma, St. Louis, USA
Substance P	CRB-SP G10	substance P	10^{-3} mol l^{-1}	Bachem Feinkemikalien AG, Bubendorf, Switz.
Galanin	P-GAL CRB-GAL	galanin	10^{-3} mol l^{-1}	CRB, Cambridge, UK

RESULTS

Immunohistochemical identification of chromaffin cells

The identification and localization of the chromaffin cells was accomplished by examining the pattern of labelling obtained with antisera raised against the Blaschko pathway enzymes involved in catecholamine biosynthesis (TH, D β H, PNMT) known to take place within these cells. In the teleost fish (Atlantic cod, rainbow trout and European eel), labelling for these enzymes (see Table 7-3) was observed in cells (chromaffin cells) within the walls of the PCV [see Figs. 7-1 (TH), 7-2 (D β H), 7-3 (PNMT) for cod; Figs. 7-10, 7-11 (both D β H) for trout; Figs. 7-17 (TH), 7-18 (PNMT), 7-19 (D β H) for eel]. The pattern of labelling suggested that the majority of the chromaffin cells formed aggregates, however, individual cells [e.g. Fig. 7-11, D β H in trout] could often be observed independent of the larger groups of cells. The intensity of labelling within the chromaffin cells was similar in all three teleost species and, occasionally, processes were observed extruding from the chromaffin cells (e.g. Fig. 7-11). In the European eel, labelling for these enzymes was also observed within nerve fibres and ganglia (Fig. 7-22, PNMT) within the vein wall. No neuronal labelling with enzyme antisera was evident in trout or cod PCV.

In all three teleosts, double labelling experiments revealed that the distribution of TH and D β H was identical [see Fig. 7-7a (D β H), b (TH), cod; Fig. 7-31a (D β H), b (TH), eel]. Two different antisera for D β H (TE 103, Ga-D β H) also produced identical labelling in all three teleosts [e.g. Fig. 7-6a (Ga-D β H), b (TE-103) in cod]. Double labelling revealed that the distribution of D β H was more extensive than that of PNMT, with some D β H-labelled cells showing no immunopositive reaction for PNMT. This phenomenon was most apparent in the cod [Fig. 7-8a (D β H), b (PNMT)] and less so in the trout [Fig. 7-15a (D β H), b (PNMT)]. In the eel, the labelling patterns for the two enzymes were more similar than in the other species [Fig. 7-28a (PNMT), b (D β H)]; however, some D β H-positive cells did not label for PNMT [Fig. 7-33a (D β H), b (PNMT)].

A number of small labelled cells were observed showing TH-like immunoreactivity (IR) within both the systemic and portal hearts of the hagfish (see Table 7-3). These chromaffin cells,

Table 7-3. Summary of the immunohistochemical screening for Blaschko pathway enzymes, peptides and biogenic amines in the Atlantic cod, rainbow trout, European eel, Atlantic hagfish and spiny dogfish. A positive sign (+) indicates positive immunostaining for a substance whereas a negative sign (-) indicates a lack of staining. The location of the staining is given in brackets. Abbreviations as in Table 7-1 and including: cc chromaffin cell (as identified by labelling for TH, DBH and PNMT), n nerve fibres, g ganglia, x cell-type other than the chromaffin cells, y not possible to determine whether the cells are chromaffin cells or otherwise.

Substance	Atlantic cod	Rainbow trout	European eel	Atlantic hagfish	Spiny dogfish
TH	+(cc)	+(cc)	+(cc, n, g)	+(cc)	+(n)
DBH	+(cc)	+(cc)	+(cc, n, g)	-	-
PNMT	+(cc)	+(cc)	+(cc, n, g)	-	-
Serotonin	+(cc)	+(x)	+(cc)	+(y)	-
VIP	+(n)	+(n)	+(n, g)	-	+(n)
PACAP	+(n)	+(n)	+(n, g)	-	+(n)
NPY	-	-	+(cc, n, g)	-	-
PYY	-	-	+(cc, x)	-	-
Enkephalins	-	-	-	-	-
LPLRFamide	-	-	-	-	-
FMRFamide	-	-	-	-	-
Galanin	-	-	-	-	+(n)
Histamine	-	-	-	-	-
Substance P	-	-	-	-	+(n)
Dopamine	-	-	-	-	-
Chromogranins	-	-	-	-	-
Somatostatin	-	-	-	-	-

although few in number, often appeared in close proximity to one another and were observed to have processes extending from them and forming a cellular network (see Figs. 7-40, 7-41, 7-42). No labelling was observed within the cardiac muscle fibers. None of the antisera raised against the other two enzymes (DBH and PNMT) showed any labelling.

In the dogfish axillary bodies, none of the enzyme antisera produced any labelling within cells of the type observed in the other four species. However, positive labelling for TH was observed in nerve fibres (Fig. 7-46).

Identification and localization of peptides and amines associated with the chromaffin cells

As summarized in Table 7-3, no labelling was observed with antisera raised against the enkephalins [met-enkephalin (ME), met-enkephalin-arg⁶-phe⁷ (MERF), met-enkephalin-arg⁶-gly⁷-leu⁸ (MERGL)], leu-pro-leu-arg-phe-NH₂ (LPLRFamide), phe-met-arg-phe-NH₂ (FMRFamide), histamine, dopamine, somatostatin or chromogranins. Galanin-like and substance P-like IR (Figs. 7-44, 7-47, respectively) were observed in nerves within the dogfish axillary bodies but no labelling for these two peptides was observed in the other species. Nerves containing VIP were observed within the walls of the PCV in the cod (Fig. 7-4), trout (Fig. 7-14) and eel (Fig. 7-36). Double labelling experiments revealed that these nerve fibres were located in the immediate vicinity of the chromaffin cells [e.g. Figs. 7-16a (DBH), b (VIP) in trout]. The VIP-like peptide, PACAP, was also observed within nerve fibres (e.g. Fig 7-24 in eel) located around the chromaffin cells [e.g. Figs. 7-13a (DBH), b (PACAP 27)]. In addition to nerve fibres amongst the chromaffin cells, PACAP- and VIP- containing ganglia (aggregates of nerve fibres) were identified within the walls of the PCV (Fig. 7-21, PACAP; Fig. 7-23, VIP) in the eel, but not in the other two teleosts. Neither VIP nor PACAP were observed within the hagfish hearts. In the dogfish axillary bodies, both of these peptides were seen within nerve fibres (Figs. 7-43, 7-45, PACAP; Fig 7-48, VIP).

Antisera raised against both NPY and PYY produced positive labelling within the PCV of the eel, with NPY being located both in chromaffin cells, nerves and ganglia [Fig. 7-25 (NPY, ganglia); Fig. 7-27 (NPY, chromaffin cells); Fig 7-26, PYY]. Double labelling techniques demonstrated that the pattern of NPY labelling (apart from that within the ganglia) was identical to that for D β H [Fig. 7-34a (D β H), b (NPY) and Fig. 7-35a (NPY), b (D β H)]. Labelling for PYY was more extensive than for the enzymes [e.g. Fig. 7-32a (PYY), b (TH)]. In some cases enzyme-positive cells did not label for PYY. A population of PYY-positive cells did not label for the enzymes. The patterns of NPY and PYY labelling were very similar but not identical in all cases [Fig. 7-29a (PYY), b (NPY)]. Neither NPY nor PYY were seen in the other four species.

Positive labelling for the indolamine serotonin was observed within the PCV of cod (Fig. 7-5) and eel (Fig. 7-20) with double labelling experiments demonstrating that these serotonin-containing cells were analogous to the D β H-positive cells, although not all of the chromaffin cells contained serotonin [e.g. Fig. 7-9a (D β H), b (serotonin) in cod]. In addition, in the eel, the distribution of PYY and serotonin was not identical [Fig. 7-30a (PYY), b (serotonin)]. In the trout, positive labelling for serotonin was observed in a separate population of cells that were distinct from the chromaffin cells and that were located within the kidney tissue and not in the walls of the PCV (Fig. 7-12). These serotonin-like cells in rainbow trout were mostly spherical in shape and always appeared individually, never in aggregates.

In both the systemic and portal hearts of the hagfish, positive serotonin labelling was observed in a small number of cells (Figs. 7-37 to 7-39) with processes similar to those seen in the TH-positive cells. The application of TH and serotonin antisera to serial sections of the hagfish hearts was unable to confirm whether the immunoreactivity occurred in the same cell type. Unfortunately, the antibodies at our disposal did not permit the double labelling experiments necessary to investigate this possibility more thoroughly. No labelling for serotonin was observed in the dogfish axillary bodies.

Figs. 7-1 to 7-9 Cod, PCV

Fig. 7-1 TH-positive (TE-101) cells (chromaffin cells). An asterisk is within the lumen of the PCV. x83

Fig. 7-2 DBH-positive (Ra-DBH) cells (chromaffin cells). An asterisk is within the lumen of the PCV. x92

Fig. 7-3 PNMT-positive (TE-104) cells (chromaffin cells). An asterisk is within the lumen of the PCV. x318

Fig. 7-4 VIP-like (Mi-VIP) immunoreactive nerves. x509

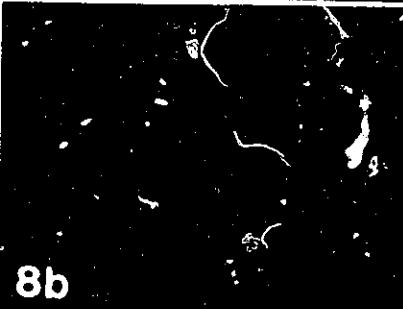
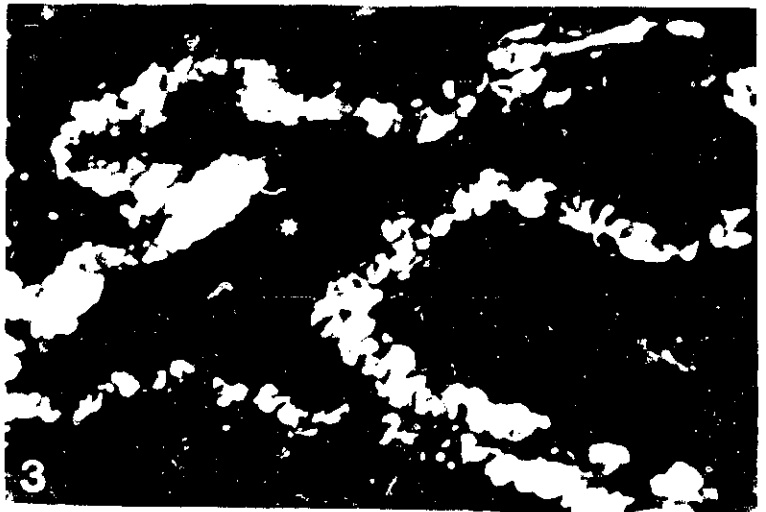
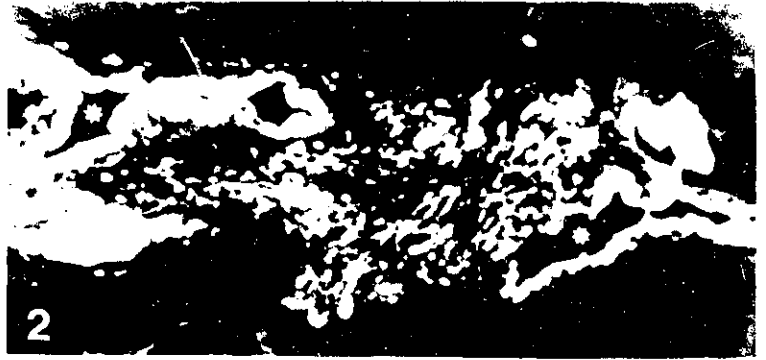
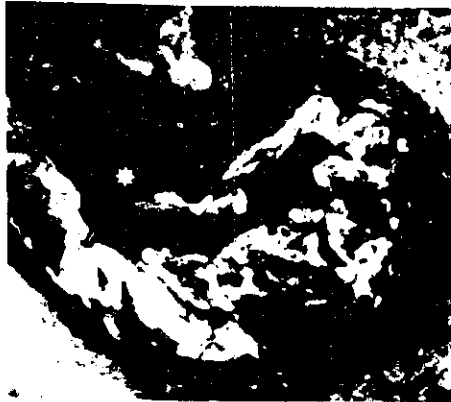
Fig. 7-5 Serotonin-like (AES-308) immunoreactive cells. Kidney tubules (arrowhead) are visible on both sides of the vein. x83

Fig. 7-6 Double labelling for DBH with (a) Ga-DBH and (b) TE-103. The two different DBH antisera produce identical labelling patterns. x83

Fig. 7-7 Double labelling for (a) DBH (Ga-DBH) and (b) TH (TE-101). Note that the labelling pattern for TH and DBH are identical. x83

Fig. 7-8 Double labelling for (a) DBH (Ga-DBH) and (b) PNMT (TE-104). Note that not all of the DBH-labelled cells are labelled for PNMT. Arrowheads denote DBH-labelled cells that do not label for PNMT. x83

Fig. 7-9 Double labelling for (a) DBH (Ga-DBH) and (b) serotonin (AES-308). Note that not all of the DBH-labelled cells are labelled for serotonin. An arrowhead denotes a DBH-labelled cell that does not label for serotonin. x83



Figures 7-10 to 7-16 Trout PCV

Fig. 7-10 D β H-positive (TE-103) cells (chromaffin cells). x83

Fig. 7-11 D β H-positive (Ra-D β H) cells (chromaffin cells). Arrowheads illustrate processes extruding from a chromaffin cell. Note the large nuclei within the individual cells. x165

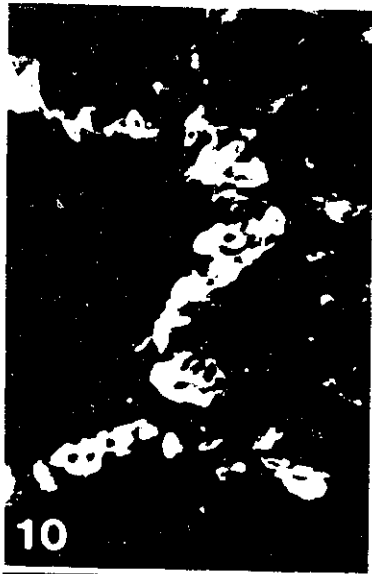
Fig. 7-12 Serotonin-positive (AES-308) cells (eg, arrowheads) within the trout kidney (note the presence of the kidney tubules, asterisk). x83

Fig. 7-13 Double labelling for (a) D β H (Ga-D β H) (chromaffin cells) and (b) PACAP 27 (nerve fibers). x83

Fig. 7-14 VIP-like (Mi-VIP) immunoreactive nerves. Arrowheads illustrate several of the small nerve fibers. x83

Fig. 7-15 Double labelling for (a) D β H (Ga-D β H) and (b) PNMT (TE-104). x83

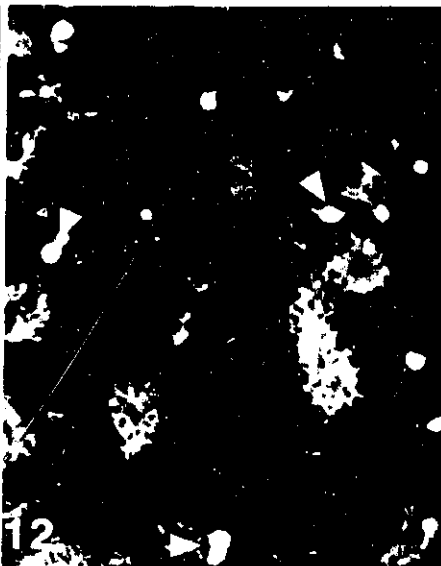
Fig. 7-16 Double labelling for (a) D β H (GaD β H) illustrating chromaffin cells and (b) VIP (Mi-VIP) illustrating nerves (arrows). x83



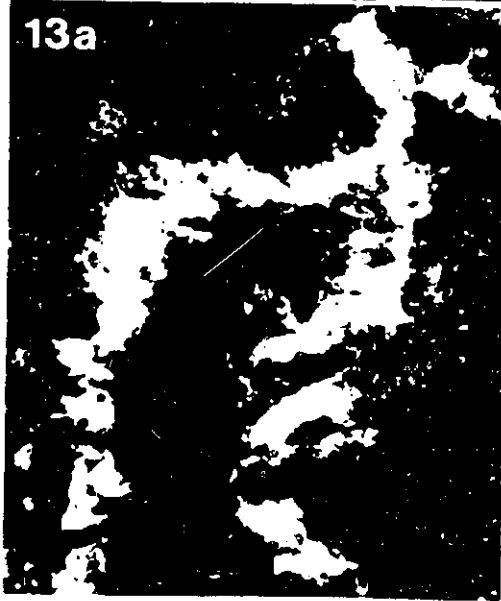
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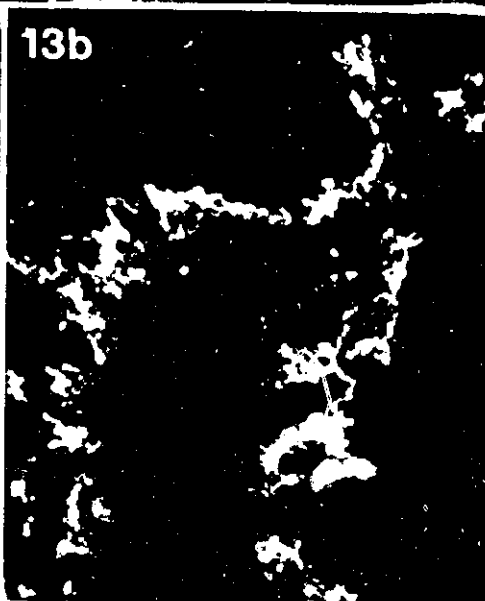
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12



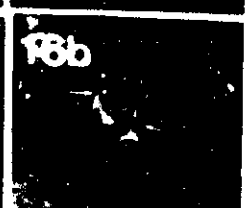
13a



13b



15b



16a

16b

Figs. 7-17 to 7-36 eel PCV

Fig. 7-17 TH-positive (TE-101) cells (chromaffin cells). x83

Fig. 7-18 PNMT-positive (TE-104) cells (chromaffin cells). x83

Fig. 7-19 DBH-positive (Ga-DBH) cells (chromaffin cells). x83

Fig. 7-20 Serotonin-positive (AES-308) cells (chromaffin cells). x83

Fig. 7-21 PACAP 27-positive nerve fibres (ganglion). Arrowheads illustrate nerve fibers connected with the ganglia. x83

Fig. 7-22 PNMT-positive (TE-104) nerve fibre (arrowhead) within a ganglion. x83

Fig. 7-23 VIP-like (Mi-VIP) nerve fibres within a ganglion. x83

Fig. 7-24 PACAP 38-like immunoreactive nerves (e.g. arrowheads). x83

Fig. 7-25 NPY-positive (NPY-A) cells within a ganglia (arrowhead) and a nerve (small arrow). x83

Fig. 7-26 PYY-positive (Mar-PYY) cells. x83

Fig. 7-27 NPY-positive (NPY-ab22) cells. x41

Fig. 7-28 Double labelling for (a) PNMT (TE-104) and (b) DBH (Ga-DBH). Note that the labelling pattern for the two antisera are identical. x83

Fig. 7-29 Double labelling for (a) PYY (Mar-PYY) and (b) NPY (NPY-ab22). x83

Fig. 7-30 Double labelling for (a) PYY (Mar-PYY) and (b) serotonin (AES-308). Note the PYY-labelled cell in (a) (arrowhead) that does not label for serotonin (b). x83

Fig. 7-31 Double labelling for (a) DBH (Ga-DBH) and (b) TH (TE-101). Note that the labelling patterns in these chromaffin cells are identical. x165

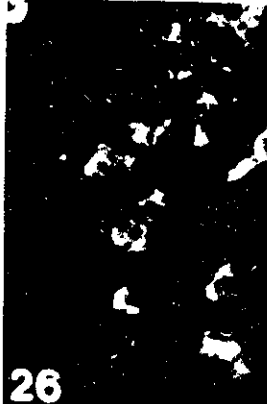
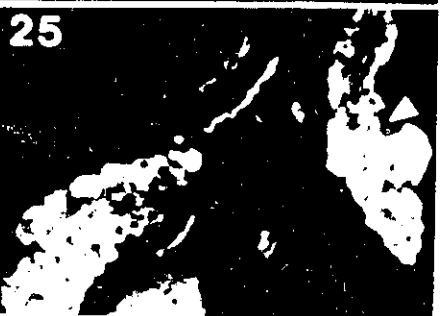
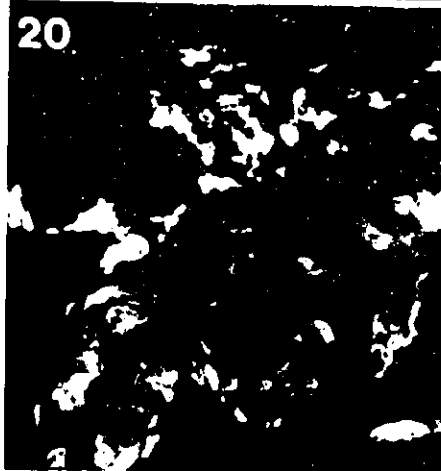
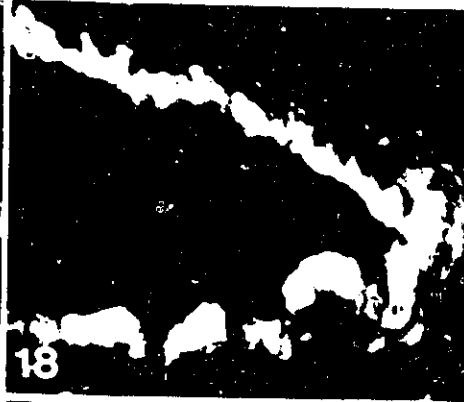
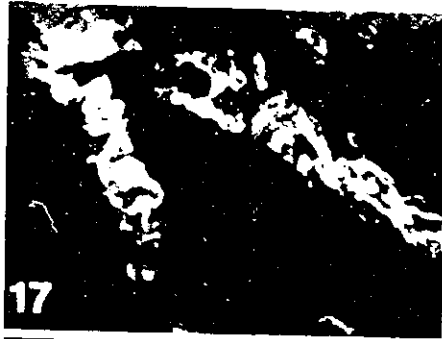
Fig. 7-32 Double labelling for (a) PYY (MAR-PYY) and (b) TH (TE-101). x83

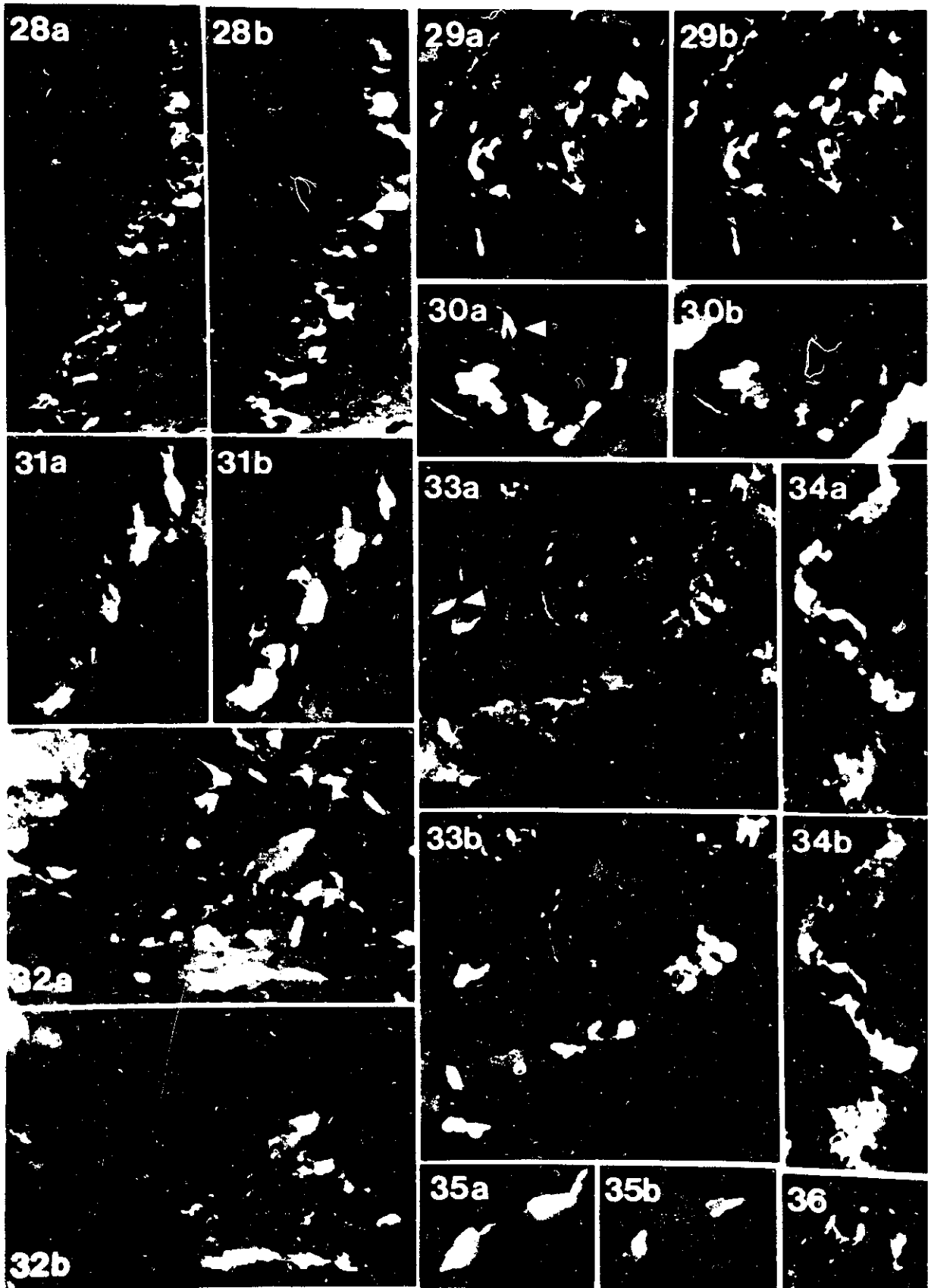
Fig. 7-33 Double labelling for (a) DBH (Ga-DBH) and (b) PNMT (TE-104). Note the DBH-labelled cell in (a) (arrowhead) that does not label for PNMT in (b). x41

Fig. 7-34 Double labelling for (a) DBH (Ga-DBH) and (b) NPY (NPY-A). x83

Fig. 7-35 Double labelling for (a) NPY (NPY-A) and (b) DBH (Ga-DBH). x165

Fig. 7-36 VIP-like (Mi-VIP) immunoreactive nerve fibres. x83





Figs. 7-38 to 7-42 Hagfish hearts

Fig. 7-37 Serotonin-like (AES-308) immunoreactive cells (e.g. arrowhead) within the hagfish systemic heart. x83

Fig. 7-38 Serotonin-like (SER-ICM) immunoreactive cells (arrowheads) within the hagfish systemic heart. Note the long thin processes (small arrows) that appear to extend from the cells. x83

Fig. 7-39 Serotonin-like (0601) immunoreactive cells within the hagfish portal heart. x165

Fig. 7-40 TH-like (TE-101) immunoreactive cells within the hagfish systemic heart. x83

Fig. 7-41 TH-like (TE-101) immunoreactive cells within the hagfish portal heart. x83

Fig. 7-42 TH-like (TE-101) immunoreactive cells within the hagfish systemic heart. x83

Figs. 7-43 to 7-48 Dogfish axillary bodies.

Fig. 7-43 PACAP 27-like immunoreactive nerve fibres. x83

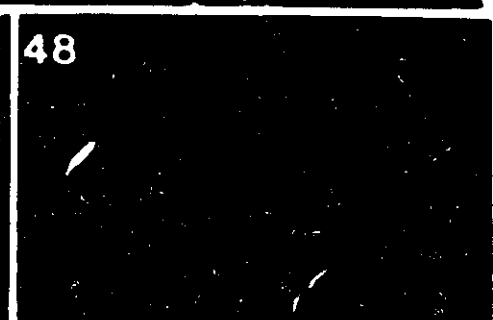
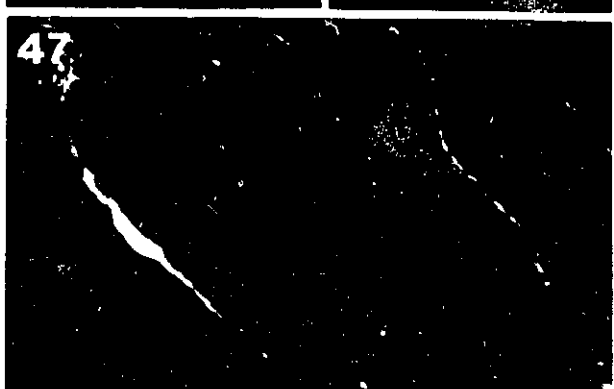
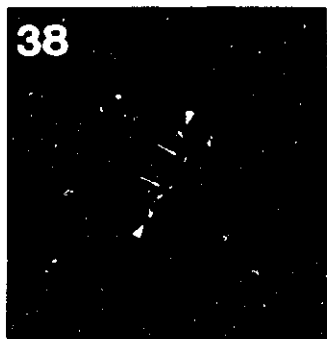
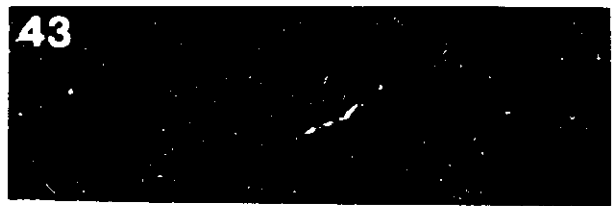
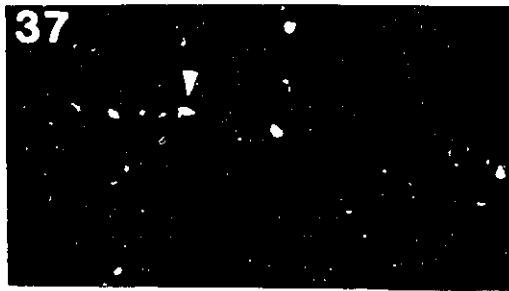
Fig. 7-44 Galanin-like (P-GAL) immunoreactive nerve fibres. x83

Fig. 7-45 PACAP 38-like immunoreactive nerve fibres. x83

Fig. 7-46 TH-like (TE-101) immunoreactive nerve fibres. x83

Fig. 7-47 Substance P-like (MI-SP) immunoreactive nerve fibres. x83

Fig. 7-48 VIP-like (MI-VIP) immunoreactive nerve fibres. x83



DISCUSSION

Comparison with other vertebrates

The results of this chapter support the contention that, in fish, as in other vertebrates, chromaffin cells and associated nerve fibres contain bioactive peptides and amines. The presence of the biogenic amine serotonin within the chromaffin cells of cod and eel is analogous to other vertebrates. However, in rainbow trout, the absence of serotonin in the chromaffin cells is unique amongst the three teleosts studied. Indeed, whereas the results of this study suggest that fish chromaffin cells are more complex than previously thought, the inability to detect peptides within these cells, with the exception of NPY and PYY in the eel, may reflect a less complex chromaffin cell than that found in other vertebrates. The apparent absence of enkephalins from teleost chromaffin cells is particularly interesting given that these substances are found in a wide range of vertebrate chromaffin cells. Thus, although the chromaffin cell of non-fish vertebrates may have evolved into a multi-substance secretory cell, the role of the chromaffin cell in fish may be confined, primarily, to the storage and secretion of catecholamines and other amines, such as serotonin (and NPY/PYY in the eel).

The observation, in the three teleosts, that some of the chromaffin cells are positive for DBH but not for PNMT suggests the presence of a sub-population of chromaffin cells that do not contain adrenaline. The existence of separate adrenaline-containing and noradrenaline-containing cells has previously been documented in teleost fish; *Scardinius erythrophthalmus* (Mastrolia *et al.* 1981), rainbow trout (Mastrolia *et al.* 1984) and *Gasterosteus aculeatus* (Gallo *et al.* 1993). Indeed the results of chapters 2 and 3 also provide indirect evidence for the existence of two types of chromaffin cells.

Potential physiological roles of serotonin, VIP, PACAP and NPY/PYY

In trout, serotonin was not detected within the chromaffin cells but in a separate population of cells within the kidney tissue. The results of chapter 8 demonstrate that serotonin is

capable of eliciting catecholamine release from trout chromaffin cells, *in situ*. This population of serotonin-containing cells is a possible source of serotonin that can act on chromaffin cells to initiate catecholamine release. In eel and cod, if serotonin is released from the chromaffin cells (where it is stored), either in conjunction with or independently of catecholamines, it may act in an autocrine fashion to stimulate the release of catecholamines. Regardless of the source of serotonin, a mechanism is presumably present to cause the release of serotonin from its storage site.

Given the ability of serotonin to cause the release of catecholamines from trout chromaffin cells (see chapter 8), this indolamine (identified within the hagfish hearts) may exert a similar effect on hagfish chromaffin cells. Unlike teleost and elasmobranch chromaffin cells, these cells lack extrinsic innervation in the hagfish. Therefore, cholinergic stimulation of catecholamine mobilization is unlikely to be a physiologically relevant stimulus for release, although Perry *et al.* (1993) have demonstrated catecholamine release *in situ* in response to the cholinergic agonist carbachol. Clearly other factors, possibly serotonin, are involved in the secretion process within this species (see chapter 8).

The close association of chromaffin and interrenal cells in teleost fish (Nandi 1961) provides an environment for potential paracrine interactions between catecholamines, steroids and serotonin (and NPY and PYY in the eel). In both humans (Lefebvre *et al.* 1992) and frogs (Idres *et al.* 1991), serotonin has been demonstrated to evoke the secretion of steroids from adrenocortical tissue *in vitro*. Possible interactions within the pituitary-adrenal axis have been discussed in chapters 4 and 8 (also see chapter 9, general discussion).

In the three teleosts, the VIP- and PACAP- containing nerves in the vicinity of the chromaffin cells may be involved in the process of catecholamine release. Indeed, Wakade *et al.* (1991) and Yamaguchi (1993) demonstrated that VIP is a neurotransmitter in rat and dog adrenals, respectively, capable of eliciting catecholamine secretion. PACAP exerts a similar role in rat (Watanabe *et al.* 1992) and sheep (Isobe *et al.* 1993) adrenals. In addition, pre-synaptic modulation of cholinergic nerve activity may affect the release of acetylcholine onto the

chromaffin cells (Masuo 1993). The possibility that VIP and/or PACAP are co-stored with acetylcholine within cholinergic nerves and co-released in response to appropriate stimuli should also not be overlooked.

Within the chromaffin cells of the European eel, NPY and PYY may act in an autocrine fashion to influence catecholamine release. In addition, PYY in the population of non-chromaffin cells may exert a paracrine influence over the chromaffin cells. The presence of ganglia containing VIP/PACAP/TH/DBH/PNMT/NPY within the eel PCV but not in cod or trout PCV is an interesting morphological observation suggesting interspecific differences in the pattern of innervation, not only of the chromaffin cells, but also of the entire PCV.

These bioactive substances (serotonin, VIP, PACAP, NPY and PYY), either within the chromaffin cells or in neurons innervating them, may have a physiological role in the process of catecholamine release above and beyond the basic process of cholinergic stimulated secretion.

Enzyme immunoreactivity in the hagfish hearts and dogfish axillary bodies

The presence of both DBH- and PNMT-activities within dogfish axillary bodies (Jönsson 1982; Abrahamsson 1979) and hagfish hearts (Jönsson 1983) has been demonstrated previously using **biochemical** techniques. The inability of the antisera raised against DBH or PNMT to produce a labelling reaction in chromaffin cells of these species suggests an evolutionary divergence in the protein structure of these enzymes. The mammalian antigens (DBH and PNMT) used to produce the antisera probably exhibit enough differences from the hagfish and dogfish proteins to prevent these antisera from detecting the enzymes in these two species. In evolutionary terms, cyclostomes and elasmobranchs are much more primitive than teleost fish. This is reflected, in this study, by the recognition of the teleost enzymes but not hagfish or dogfish enzymes (with the exception of TH) by the antibodies against mammalian DBH and PNMT.

In dogfish axillary bodies, nerves, but not chromaffin cells, exhibit positive labelling for TH. This suggests the presence of two TH isozymes in the axillary body. Multiple TH isozymes

with different characteristics have previously been identified in, for example, the human nervous system (Grima *et al.* 1987). DBH, PNMT and chromaffin cell TH need to be isolated and purified from the axillary bodies in order to localize immunohistochemically the chromaffin cells in the dogfish and their spatial orientation with respect to nerves with TH-, VIP-, PACAP-, galanin- and substance P-like immunoreactivity.

In conclusion, this chapter demonstrates the presence of bioactive peptides and amines in the chromaffin cells and associated nerves in fish and, as such, establishes a basis for future physiological and morphological studies designed to elucidate the role of these substances in the control of catecholamine release. The following chapter examines the effects of two non-cholinergic substances (serotonin and adrenocorticotrophic hormone) on catecholamine secretion in the rainbow trout.

CHAPTER 8.

**SEROTONIN AND ADRENOCORTICOTROPIC
HORMONE (ACTH) MEDIATED-RELEASE OF CATECHOLAMINES
IN THE RAINBOW TROUT, *ONCORHYNCHUS MYKISS***

INTRODUCTION

In addition to cholinceptor-induced catecholamine release in teleost fish, the results of the previous chapter, and indeed studies on fish (e.g. Perry *et al.* 1993) and other vertebrates (e.g. see review by Livett and Marley 1993), suggest that a variety of non-cholinergic bioactive substances may exert a role on the release of catecholamines from chromaffin cells. In rainbow trout, intra-arterial injections of the biogenic amine serotonin (5-hydroxytryptamine) have been demonstrated to cause an increase in the levels of circulating catecholamines (Fritsche *et al.* 1992). In this species, serotonin is located, amongst other places, within cells in the kidney tissue in the vicinity of the posterior cardinal vein (chapter 7). These serotonin-containing cells may function as a local source of serotonin which, if released, may be capable of exerting direct paracrine or endocrine effects on the chromaffin cells to initiate or modulate catecholamine secretion.

Intra-arterial injections of serotonin may also indirectly result in catecholamine mobilization via vasoconstrictive effects on the gills resulting in an impairment of gas transfer and subsequently blood hypoxaemia, which, in itself is a stimulus for catecholamine release in fish (Perry *et al.* 1991). Thus serotonin may exert a stimulatory effect on catecholamine release either directly by interacting with the chromaffin cells or indirectly by causing blood hypoxemia as a result of branchial vasoconstriction. As such, one of the goals of this chapter was to determine whether serotonin exerts a direct effect on rainbow trout chromaffin cells to initiate catecholamine release.

In addition to the possibility of non-cholinergic control of catecholamine release by serotonin, adrenocorticotrophic hormone (ACTH), the normal secretagogue of cortisol (see

chapter 4) has also been implicated as a possible secretagogue for catecholamines (Ottaviani *et al.* 1992). Perry *et al.* (1993) demonstrated, using an *in situ* preparation, that a pituitary extract was capable of eliciting catecholamine release from Atlantic hagfish (*Myxine glutinosa*) chromaffin cells. Since ACTH is released, from the pituitary, in response to stress in vertebrates, these authors suggested it was a possible constituent of the pituitary extract which evoked catecholamine release. Thus, the second goal of this chapter was to investigate any direct effects of ACTH on catecholamine release from trout chromaffin cells.

MATERIALS AND METHODS

Experimental Animals

Rainbow trout weighing between 250 and 350g (N = 189) were maintained under conditions outlined in chapter 3.

Serotonin

The Effects of Serotonin on Catecholamine Release in Vivo

An indwelling polyethylene cannula was implanted into the dorsal aorta to permit periodic blood sampling. An initial (pre) blood sample (0.35 ml) was withdrawn prior to administering a bolus injection of either saline or serotonin (50 or 250 nmol kg⁻¹). Following the injection, the cannula was flushed with 0.15 ml of saline to ensure that all of the drug was delivered to the fish. Blood (0.35 ml) was sampled 2, 5, and 10 min after the injection, centrifuged (10 000 g, 30s) and the plasma removed, frozen in liquid nitrogen and stored at -80 °C until the determination of catecholamine levels. The red blood cells were resuspended in Cortland saline (Wolf, 1963) and re-injected into the dorsal aorta.

To account for temporal differences in the release process between fish, the maximum value from either the 2, 5 or 10 minute sample was taken as the maximal acute response and statistically compared with the pre-values.

The Effects of Serotonin on Catecholamine Release in Situ

Following collection of the pre-sample (see chapter 2) a bolus dose of serotonin (250 nmol kg^{-1}) was injected into the inflow cannula. In other experiments, the preparation was pre-perfused for 20 min with either the ganglion blocker hexamethonium ($10^{-4} \text{ mol l}^{-1}$; to inhibit nicotinic cholinceptors) or the serotonergic receptor antagonist methysergide ($10^{-5} \text{ mol kg}^{-1}$) prior to sample collection and serotonin administration.

Storage of Serotonin Within the Posterior Cardinal Vein

Trout ($N = 6$) were killed by a sharp blow to the head and the posterior cardinal vein was removed and placed into a pre-weighed microcentrifuge tube. The tissue was treated in the same manner as described for determination of catecholamine analysis (chapters 3 and 4) prior to the analysis of serotonin levels.

Effects of Cholinergic Stimulation on Serotonin Release In Situ

Following the collection of the pre-sample, a dose of carbachol ($2.5 \times 10^{-6} \text{ mol}$) was injected into the inflow cannula ($N = 6$). Sample collection was as described in chapter 2. Serotonin levels (either in tissue or perfusate) were determined using HPLC with electrochemical detection, however the samples were not subjected to alumina extraction.

ACTH

The Effects of ACTH on Catecholamine Release In Vivo

An initial (pre) blood sample (0.35 ml) was withdrawn, from a dorsal aorta cannula, prior to administering a bolus (0.3 ml) of ACTH. The doses of ACTH were selected to provide nominal levels of 20, 40 or 200 mU ml⁻¹ in the blood volume of the fish (blood volume was taken to be 5% of the body weight). Control fish were injected with saline. Following the injection, the cannula was flushed with 0.15 ml of saline to ensure that all of the drug was delivered to the fish. Sampling was as described above for serotonin.

The Effects of ACTH on Catecholamine Release In Situ

Following collection of the pre-sample, a bolus dose (0.4 ml) of ACTH (2, 20, 40, 100, 160, 400, or 2000 mU; Sigma ACTH 1-39, porcine, 89 U mg⁻¹) was administered into the PCV. Doses of ACTH were selected based on previous studies that showed enhanced cortisol release in head kidney preparations with ACTH (Vijayan and Leatherland 1990).

In three separate series of perfusions, the preparation was pre-treated (20 min) with either the ganglion blocker hexamethonium (10⁻³ mol l⁻¹; N = 12), the serotonergic receptor antagonist, methysergide (10⁻⁴ mol l⁻¹; N = 8) or Ca²⁺-free saline (with 2 mmol l⁻¹ EDTA; N = 6) prior to an injection of 200 mU ACTH. The ACTH for these perfusions

was prepared in saline containing either hexamethonium or methysergide, or in a Ca^{2+} -free medium with EDTA.

The Direct Effects of Cortisol on Catecholamine Release In Situ

In order to investigate the possibility that the *in situ* release of catecholamines in response to ACTH, (see results) may have been mediated by cortisol, injections of 40 and 200 ng cortisol (hydrocortisone 21-hemisuccinate, Sigma; delivered in 0.4 ml) were administered into the perfusion preparation. These doses were selected to approximate levels of cortisol in the arterial blood (100 ng ml^{-1}) and PCV (estimate 500 ng ml^{-1}) observed during stressful situations.

Statistical Analysis

The data are presented as the mean $\pm$ 1 standard error of the mean (SEM). All statistical analysis, including determinations of normality and variance, was performed with a statistical software package (SigmaStat., Jandel Scientific). Paired data were analysed using either a paired t-test or a signed rank test. Non-paired data were analysed using a two sample t-test or a rank sum test. 5% was taken as the level of significance.

RESULTS

Serotonin

The Effects of Serotonin on Catecholamine Release In Vivo

Figure 8-1 illustrates the effects of intra-arterial injections of serotonin on plasma noradrenaline (fig 8-1a) and adrenaline (fig 8-1b) levels. Neither an injection of saline nor 50 nmol kg^{-1} serotonin caused an increase in either plasma [catecholamine]. 250 nmol kg^{-1} serotonin caused the plasma [noradrenaline] and [adrenaline] to increase to approximately 75 nmol l^{-1} and 80 nmol l^{-1} , respectively.

The Effects of Serotonin on Catecholamine Release In Situ

The effects of serotonin on the release of noradrenaline *in situ* are illustrated in figure 8-2. An injection of serotonin (250 nmol kg^{-1}) caused an elevation of perfusate noradrenaline levels to approximately 20 nmol l^{-1} . Pre-treatment with the serotonergic receptor antagonist methysergide did not inhibit the release of noradrenaline into the perfusate in response to an injection of serotonin (perfusate noradrenaline approximately 7 nmol l^{-1}), however the maximal level of release was significantly less than in response to serotonin in the absence of methysergide. An injection of serotonin following pre-treatment with the ganglion blocker hexamethonium caused an elevation of perfusate noradrenaline levels similar to serotonin alone.

The effects of serotonin on the release of adrenaline *in situ* are illustrated in figure 8-3. An injection of serotonin (250 nmol kg^{-1}) caused an elevation of perfusate adrenaline

Figure 8-1. Plasma (a) [noradrenaline] and (b) [adrenaline] (nmol l^{-1}) in rainbow trout prior to (light bars) and following (dark bars) an intra-arterial injection of either saline or serotonin (50 or 250 nmol kg^{-1}) ($N = 6-8$ for each group). The data are shown as the mean $\pm$ SEM. An asterisk denotes a significant difference between the pre (P) value and the maximum level (M) following serotonin administration.

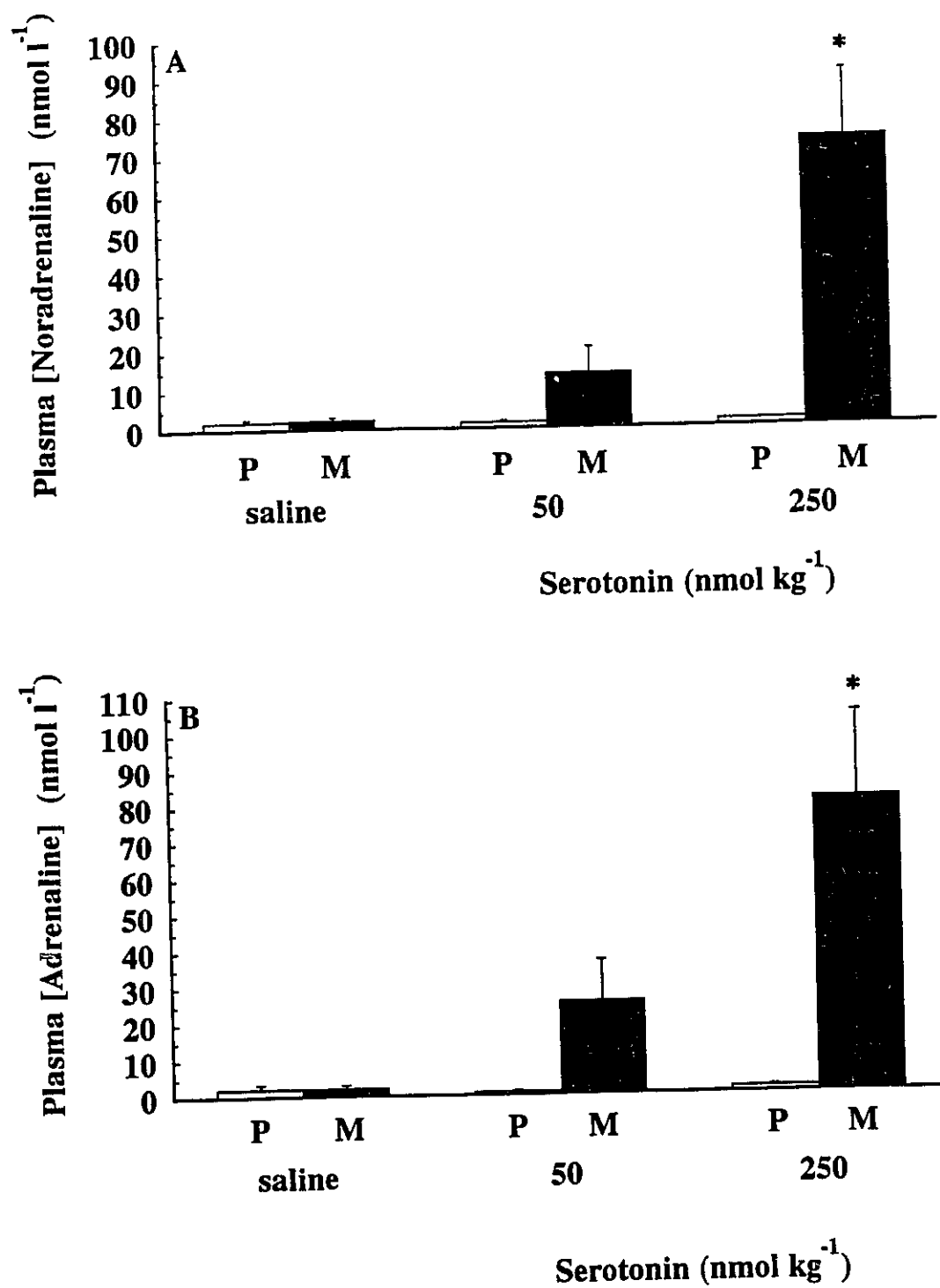


Figure 8-1

Figure 8-2. Maximum perfusate [noradrenaline] (nmol l^{-1}) prior to (light bars) and following (dark bars) an injection of either saline ($N = 7$), serotonin (250 nmol kg^{-1} , $N = 10$), serotonin (250 nmol kg^{-1}) following pre-treatment with the serotonergic receptor antagonist methysergide ($10^{-5} \text{ mol l}^{-1}$, $N = 7$) or serotonin (250 nmol kg^{-1}) following pre-treatment with the ganglion blocker hexamethonium ($10^{-4} \text{ mol l}^{-1}$, $N = 6$). The data are shown as the mean $\pm$ SEM. An asterisk denotes a significant difference between the pre (P) value and the maximal (M) value following serotonin administration. A plus sign (+) indicates a significant difference from the maximal value in response to serotonin alone.

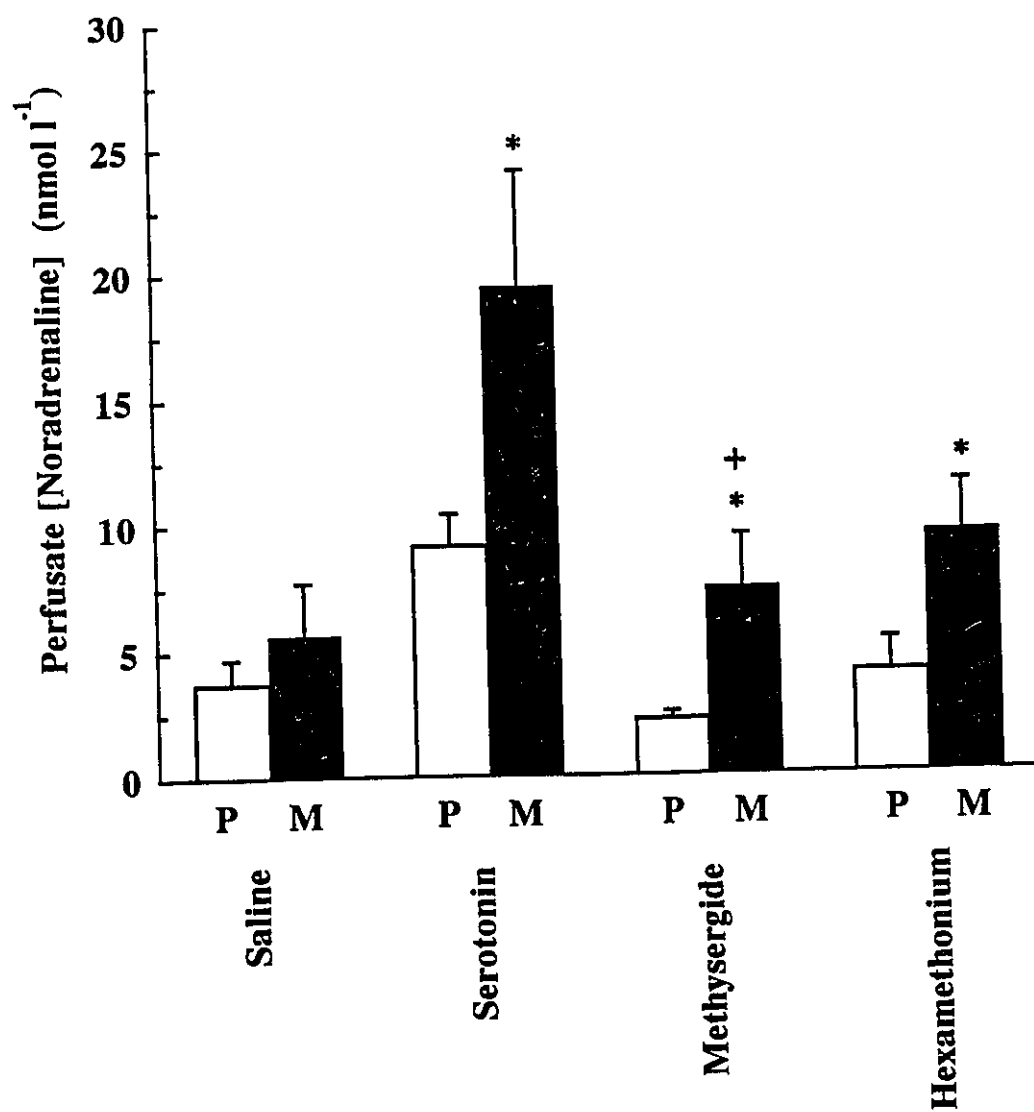


Figure 8-2

Figure 8-3. Maximum perfusate [adrenaline] (nmol l^{-1}) prior to (light bars) and following (dark bars) an injection of either saline ($N = 7$), serotonin (250 nmol kg^{-1} , $N = 10$), serotonin (250 nmol kg^{-1}) following pre-treatment with the serotonergic receptor antagonist methysergide ($10^{-5} \text{ mol l}^{-1}$, $N = 7$) or serotonin (250 nmol kg^{-1}) following pre-treatment with the ganglion blocker hexamethonium ($10^{-4} \text{ mol l}^{-1}$, $N = 6$). The data are shown as the mean $\pm$ SEM. An asterisk denotes a significant difference between the pre (P) value and the maximal (M) value following serotonin administration. A plus sign (+) indicates a significant difference from the maximal value in response to serotonin alone.

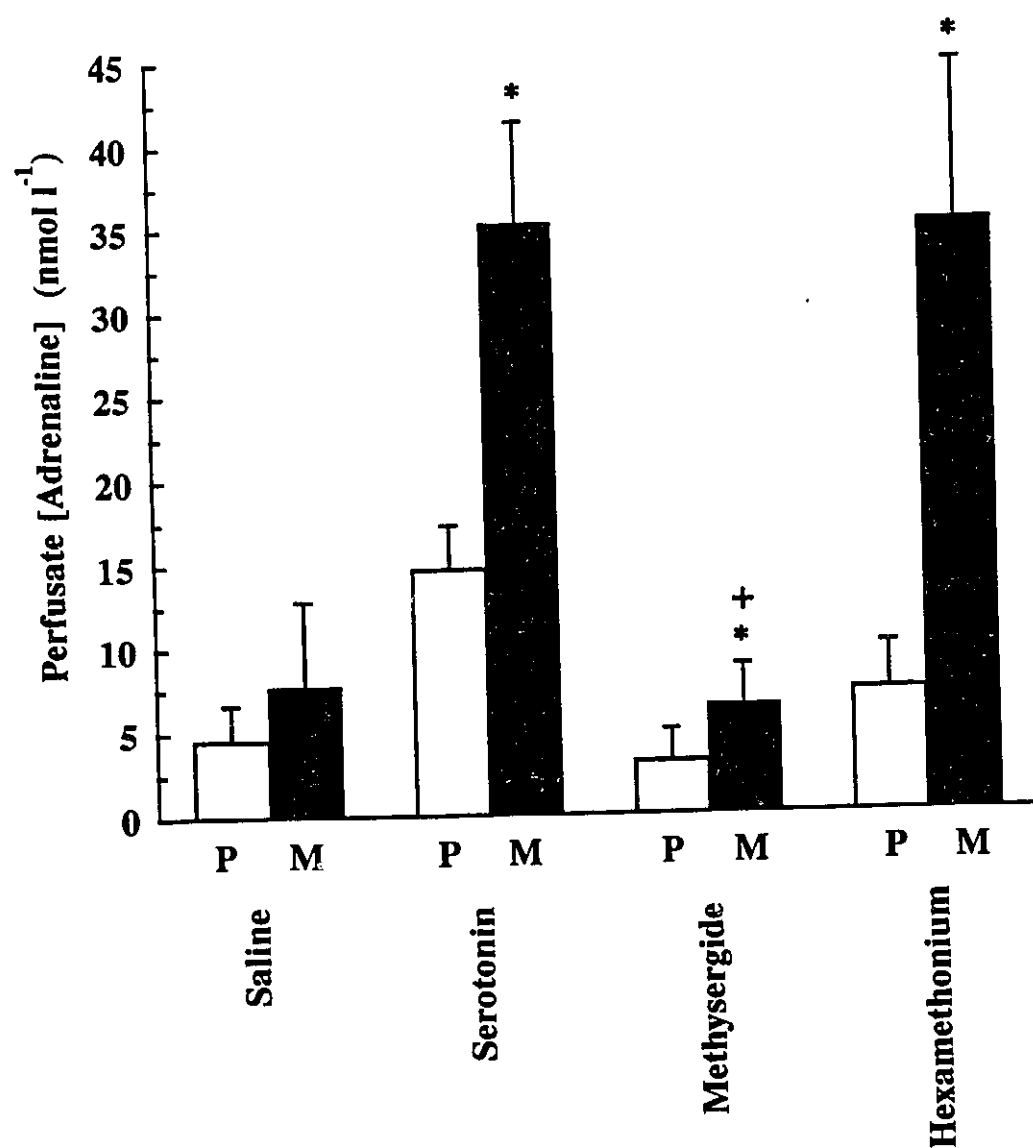


Figure 8-3

levels to approximately 35 nmol l^{-1} . Pre-treatment with the serotonergic receptor antagonist methysergide did not inhibit the release of adrenaline into the perfusate in response to an injection of serotonin (perfusate adrenaline approximately 6 nmol l^{-1}), however the maximal level of release was significantly less than in response to serotonin in the absence of methysergide. An injection of serotonin following pre-treatment with the ganglion blocker hexamethonium caused an elevation of perfusate adrenaline levels (approximately 35 nmol l^{-1}) similar to serotonin alone.

Storage and Release of Serotonin

The concentration of serotonin contained within the posterior cardinal vein was $44.6 \pm 6 \text{ } \mu\text{g g}^{-1}$ tissue wet weight. Bolus injections of carbachol ($2.5 \times 10^{-6} \text{ mol}$) into the inflowing perfusate did not evoke the release of serotonin ($N = 6$).

ACTH

The Effects of ACTH on Catecholamine Release in Vivo

Plasma noradrenaline levels, which ranged between approximately $1\text{-}6 \text{ nmol l}^{-1}$ (fig 8-4a), were not elevated in response to administration of ACTH (20, 40 or 200 mU ml^{-1} , nominal concentration in the blood). Conversely, plasma adrenaline (fig 8-4b) was elevated in response to 40 and 200 mU ml^{-1} ACTH but not 20 mU ml^{-1} .

Figure 8-4. Plasma (a) [noradrenaline] and (b) [adrenaline] (nmol l^{-1}) in rainbow trout ($N = 6$ or 7 in each group) prior to (light bars) and following (dark bars) an injection of ACTH (20, 40 or 200 mU final concentration in the blood) into the dorsal aorta. An asterisk (*) denotes a significant difference between the pre (P) value and the maximum (M) level following the injection.

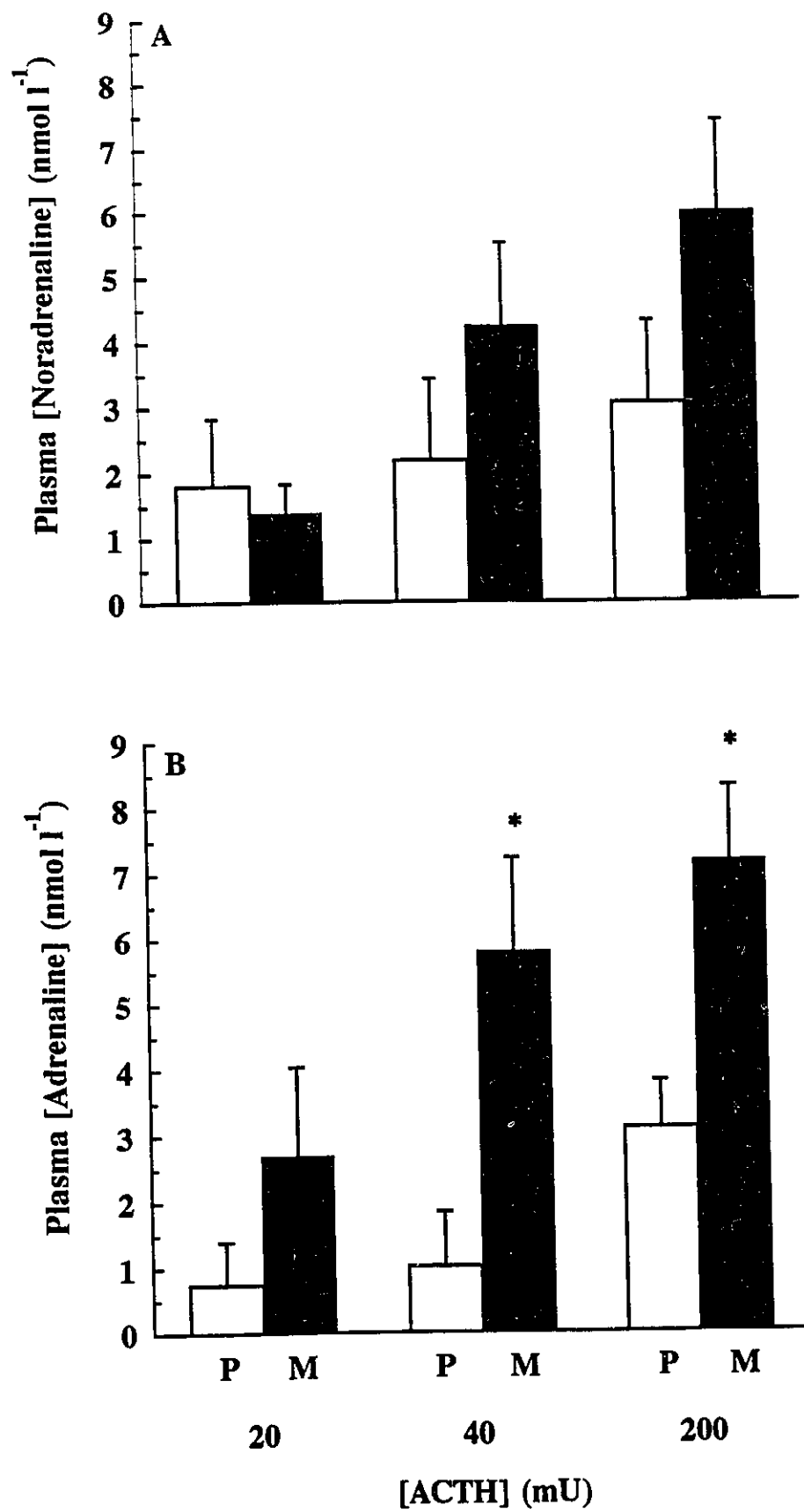


Figure 8-4

The Effects of ACTH on Catecholamine Release In Situ

Figure 8-5a illustrates the effects of *in situ* injections of ACTH on the release of noradrenaline into the perfusate. All of the doses of ACTH elicited the release of noradrenaline, albeit in a non dose-dependent manner. Noradrenaline levels ranged from approximately 12 to 18 nmol l⁻¹. Adrenaline release in response to ACTH (fig 8-5b) occurred at all but the lowest dose. Unlike noradrenaline, the release was dose-dependent with levels ranging from approximately 30 to 150 nmol l⁻¹. Hill plot analysis (fig 8-5c) provided an ED₅₀ value (dose of ACTH which produced 50% of the maximal response) of 83 mU ACTH for *in situ* adrenaline release.

Pre-treatment of the perfusion preparation with the ganglion blocker hexamethonium (N = 12) or the serotonergic receptor antagonist, methysergide (N = 8) did **not** inhibit or attenuate the release of either noradrenaline or adrenaline in response to an injection of 200 mU ACTH. The removal of calcium from the perfusion media (N = 6) prevented the release of both catecholamines when 200 mU ACTH was administered.

The Direct Effects of Cortisol on Catecholamine Release In Situ

Neither a bolus dose of 40 nor 100 ng cortisol elicited the release of either catecholamine into the perfusate (table 8-1). Additionally, an injection of 50 µg of cortisol (N = 4) did not elicit the release of either catecholamine.

Figure 8-5. Maximum perfusate (a) [noradrenaline] and (b) [adrenaline] (nmol l^{-1}) in response to injections of a variety of doses (2, 20, 40, 100, 160, 400 or 2000 mU; $N = 12$ for each dose) of adrenocorticotrophic hormone (ACTH). The open circles represent the pre-value at each dose and the dark circles represent the post-ACTH injection maximum value. The data are shown as the mean $\pm$ 1 standard error of the mean (SEM). An asterisk (*) represents a significant difference between the pre (P) value for any given dose and the maximum (M) level following the treatment. Fig 8-5c is a Hill plot of the data for adrenaline release; $Y = 0.40X - 0.92$, $ED_{50} = 83 \text{ mU ACTH}$, $R = 0.90$.

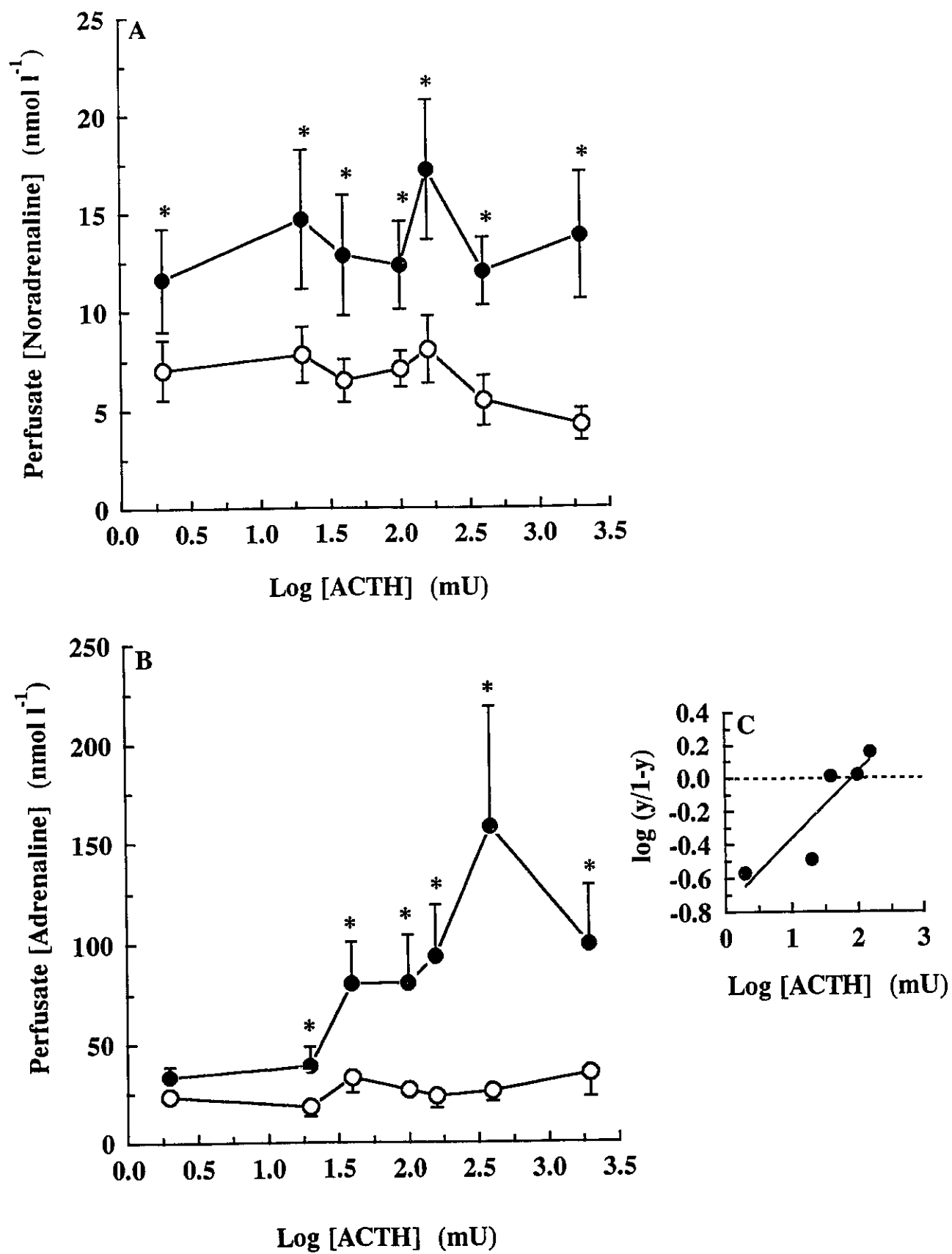


Figure 8-5

Table 8-1. Perfusate noradrenaline and adrenaline concentrations (nmol l^{-1}) prior to (Pre) and following (Max) an injection of either 40 or 200 ng (in 0.4 ml; N = 6 for each dose) cortisol into the perfusion preparation. The data are shown as the mean $\pm$ SEM. Neither dose elicited a significant elevation of perfusate catecholamines.

Cortisol Dose (ng)	Noradrenaline		Adrenaline	
	Pre	Max	Pre	Max
40	2.5 ± 1.2	2.8 ± 1.3	26.4 ± 5.9	26.4 ± 6.8
200	5.3 ± 2.6	6.9 ± 3.7	24.8 ± 9.5	28.6 ± 7.2

DISCUSSION

Serotonin-Mediated Release of Catecholamines

The initial results of this chapter confirm that intra-arterial injections of serotonin can elevate plasma catecholamine (adrenaline and noradrenaline) levels (see Fritsche *et al.* 1992). Serotonin-mediated elevations of plasma catecholamines could occur as a result of serotonin-induced hypoxaemia (Fritsche *et al.* 1992) or by a direct interaction of serotonin with the chromaffin cells.

The ability of serotonin to evoke both noradrenaline and adrenaline release *in situ* indicates that serotonin can exert a direct effect on the chromaffin cells to stimulate catecholamine secretion. Serotonin-induced catecholamine release was attenuated by pre-treatment with the serotonergic receptor antagonist methysergide suggesting that serotonin is exerting its effects via serotonergic receptors on the chromaffin cell membrane. The inability of methysergide to completely abolish the serotonin-induced release of catecholamines may have been due to an insufficient concentration of methysergide in the perfusion fluid. Alternately, there may be multiple types of serotonergic receptors on the chromaffin cells, not all of which are methysergide-sensitive. Pre-treatment with the ganglion blocker hexamethonium did not abolish the serotonin-induced release of either catecholamine, thus serotonin is not exerting its effects through stimulation of nicotinic cholinergic receptors. Together, these results demonstrate that serotonin is interacting with serotonergic receptors on the chromaffin cell membrane to elicit catecholamine release.

Chapter 7 demonstrated, using immunohistochemical techniques, the presence of a local source of serotonin within the kidney tissue surrounding the posterior cardinal vein. However, in this current chapter, serotonin was detected within the posterior cardinal vein using HPLC and electrochemical detection. It is most likely that the serotonin detected within the PCV using the HPLC technique was actually present within a small quantity of kidney tissue that adhered to the vein. Regardless, the important physiological ramification, with respect to catecholamine release, is the presence of a local source of serotonin which may exert effects on the chromaffin cells. Additionally, administration of carbachol did not cause the release of serotonin into the perfusate. It is possible that the carbachol did not come into direct contact with the serotonin-containing cells or alternately, serotonin release from these cells is not under the control of cholinergic nerves. Presumably, however, a mechanism exists to cause the release of serotonin from its storage site.

ACTH-Mediated Release of Catecholamines

This study demonstrated that the pituitary hormone ACTH is capable of eliciting the release of catecholamines in rainbow trout, both *in vivo* and *in situ*. The inability of cortisol to **directly** (table 8-1) cause the release catecholamines *in situ* (as opposed to the longer term modulation of storage and cholinceptor-induce release; see chapter 4) suggests that ACTH is directly interacting with the chromaffin cells to initiate catecholamine release rather than exerting effects through ACTH-induced cortisol release. Thus ACTH may be involved in the immediate release of catecholamines whereas an

elevation of plasma cortisol (caused by ACTH) exerts a longer-term effect on catecholamine storage and release (see chapter 4).

Pre-treatment with a ganglion blocker (hexamethonium) or a serotonergic receptor antagonist (α ethysergide) did not inhibit the ACTH-induced secretion, *in situ*, suggesting that ACTH is not exerting its effects by interacting with either of these receptor types on the chromaffin cell membrane. Like serotonin, ACTH receptor stimulation can activate the enzyme adenylyl cyclase resulting in production of the second messenger cAMP. Presumably, cAMP elicits a series of phosphorylation events which ultimately leads to the exocytotic release of catecholamines.

Although ACTH (and serotonin) is capable of eliciting the release of catecholamines, its role as a physiological secretagogue of catecholamines during stress, *in vivo*, is questionable. Indeed, the release of adrenaline in response to ACTH, *in vivo*, was quite small compared with the large increases in plasma catecholamines observed during acute stress (e.g. hypoxia, Perry and Reid 1992). Given the primary mechanism of cholinergic-induced catecholamine release, any release initiated by other secretagogues (i.e. ACTH, serotonin) is probably minor in nature. Indeed, given the multiple pathways leading to the secretion of catecholamines from trout chromaffin cells, not only does the relative contribution of each pathway come into question, but also the rationale for a variety of mechanisms capable of causing catecholamine secretion. Since catecholamines are released during a wide range of stressful situations, Livett and Marley (1993) suggested that the need to release catecholamines under different circumstances is accomplished by the chromaffin cells being capable of responding to a variety of hormones

and neurotransmitters which are released under differing situations and which possess different receptor-signal transduction systems. Additionally, it is possible that repeated stimulation of the sympathetic nerves, the primary mechanism of catecholamine release, may result in a down-regulation or desensitization of cholinceptors on the chromaffin cells leading to a blunted cholinergic response. Indeed, Reid *et al.* (1994) demonstrated that 5 days of twice daily chasing to exhaustion decreased the sensitivity of *in situ* catecholamine release, to cholinergic stimulation, by approximately an order of magnitude. Under these circumstances, other secretagogues may assume a more important role.

Although the presence of multiple secretagogues may ensure that catecholamines can continue to be released under periods of cholinceptor desensitization or in response to assorted stressors, this redundancy might also reflect evolutionary changes in the process of release from fish chromaffin cells. In the Atlantic hagfish, chromaffin cells lack any form of innervation (Augustinsson *et al.* 1956). Given that plasma catecholamines in this species are elevated during periods of stress, alternate mechanisms of release, other than cholinergic stimulation, must be present. Perry *et al.* (1993) demonstrated that an extract of cod pituitary was capable of eliciting catecholamine release from hagfish chromaffin cells and suggested that ACTH may have been the component of the pituitary which initiated the release. Since both ACTH and serotonin (which is also present in cod and hagfish pituitaries; S.G. Reid and A.-C. Jönsson, unpublished observations) can stimulate the production of cAMP, it is possible that the more primitive system involves blood borne hormones, originating from distant endocrine tissue, which interact with the chromaffin cells to elicit catecholamine release. In more evolutionarily advanced fish (e.g.

rainbow trout) with developed nervous systems and chromaffin cell innervation, these circulating hormones may have lost their importance in the release process, but nevertheless still remain capable of exerting secretory effects.

Summary

This chapter demonstrates that both serotonin and ACTH are capable of eliciting catecholamine release from rainbow trout chromaffin cells. These results, in concert with those of the previous chapter, suggest that catecholamine release from teleost chromaffin cells is under the control of non-cholinergic, in addition to cholinergic, mechanisms.

CHAPTER 9.
GENERAL DISCUSSION

Cholinergic Control of Catecholamine Release

Interspecific Differences Between Rainbow Trout and American Eels

1. *Chromaffin Cell Cholinoceptor Pharmacology*

The pharmacology of the cholinoceptor responsible for initiating catecholamine release from chromaffin cells was examined in both the rainbow trout and American eel (chapters 2 and 5, respectively). Previously, cholinoceptor-induced catecholamine secretion was considered to be mediated through the nicotinic cholinoceptor, as opposed to the muscarinic subtype. The results of this thesis suggest the possible involvement of a muscarinic, in addition to nicotinic, receptor in adrenaline release from trout but not eel, chromaffin cells. Pre-perfusion of the rainbow trout posterior cardinal vein (PCV) with the ganglion blocker hexamethonium (to inhibit nicotinic receptor-evoked release; chapter 2) prevented carbachol-evoked noradrenaline release suggesting that nicotinic receptor stimulation is an absolute requirement for **noradrenaline** release in this species. In this case, if a muscarinic receptor were involved in the release process, hexamethonium would, presumably, not completely inhibit release. On the other hand, the inability of hexamethonium to prevent adrenaline release in response to carbachol supports the contention that muscarinic receptors may be involved in the release of **adrenaline** from chromaffin cells. These results also suggest the presence of two types of chromaffin cells in the rainbow trout, those containing adrenaline and those containing noradrenaline (also see chapters 3 and 7) with muscarinic receptors present only on the adrenaline-containing cells.

When the trout PCV preparation was perfused with a Ca^{2+} -free media, the application of carbachol did not elicit the release of either catecholamine. As such, this implies that an influx of extracellular calcium into the chromaffin cell is an absolute requirement for exocytosis, and hence catecholamine release, to occur. However, the activation of muscarinic receptors (see above) generally is considered to be associated with the mobilization of calcium from intracellular calcium stores such as the endoplasmic reticulum (Burgoyne 1991). If muscarinic receptors indeed are involved in adrenaline release from trout chromaffin cells, presumably their activation must, in some manner result in an influx of calcium into the cell. Possibly muscarinic receptor-induced mobilization of intracellular calcium causes Ca^{2+} channels on the plasma membrane to open allowing for the entry of extracellular calcium which, in turn, stimulates catecholamine release via exocytosis. Indeed, this hypothesis is consistent with the delayed release of adrenaline in response to carbachol during treatment with hexamethonium.

Although the results of the hexamethonium experiment outlined in chapter 2 suggest a role for muscarinic receptors in adrenaline release from trout chromaffin cells, this particular question was not the central focus of that chapter. However, muscarinic versus nicotinic cholinceptor-mediated catecholamine release was examined extensively in the American eel (chapter 5). This study utilized pure nicotinic and muscarinic agonists and antagonists in addition to the mixed nicotinic/muscarinic agonist carbachol which activates both types of receptors, similar to the endogenous neurotransmitter acetylcholine. The results of this chapter did not support the hypothesis of a role for

muscarinic receptors in the release of either catecholamine from eel chromaffin cells. Pilocarpine, a pure muscarinic receptor agonist, did not elicit catecholamine release at any of the doses administered whilst pre-treatment with a muscarinic receptor antagonist (atropine) did not attenuate or inhibit carbachol-evoked release. On the other hand, both a pure nicotinic receptor agonist (DMPP) and the mixed agonist (carbachol) were capable of causing hexamethonium-sensitive catecholamine release, indicative of nicotinic receptor involvement in the release process.

Given the apparent involvement of muscarinic receptors in adrenaline release in trout but not eel, one may speculate that chromaffin cells in trout exhibit a greater degree of "flexibility" with respect to their response to acetylcholine released from sympathetic nerves. Perhaps, as may be the case with non-cholinergic secretagogues (chapter 8; see below), the presence of two types of cholinceptors capable of eliciting catecholamine (in this case, adrenaline) release may allow the chromaffin cells to continue to release catecholamines in the event of a desensitization or down-regulation of one type of (e.g. nicotinic) cholinceptor. Clearly further research is required to elucidate what, if any, role a muscarinic receptor exerts in the control of catecholamine release from trout chromaffin cells.

2. Plasma Catecholamine Levels During Hypoxia

In addition to potential interspecific differences, between trout and eel, with respect to cholinceptor-mediated catecholamine secretion, there are also differences in the "ability" of these two species to elevate plasma catecholamine levels during exposure to acute hypoxia. A previous study (Perry and Reid 1992) demonstrated that during

severe environmental hypoxia rainbow trout elevated their plasma catecholamine levels to approximately 400-500 nmol l⁻¹ whilst in eel, plasma levels rose to less than 10 nmol l⁻¹. The focus of chapter 3 was to investigate potential explanations for these differing levels of plasma catecholamines observed during hypoxia.

The levels of adrenaline and noradrenaline stored within the posterior cardinal vein and kidney were approximately 4X greater in the trout than in the eel. Although not tested in this thesis, these storage differences may be explained by different activities of enzymes involved in the biosynthesis of catecholamines. Alternately, there may be less chromaffin cells in the eel PCV and kidney tissue, compared with the trout. Although a quantitative determination of chromaffin cells may prove to be a difficult task, the immunohistochemical identification of chromaffin cells in the rainbow trout and **European** eel (chapter 7) did not reveal any obvious differences in the number, size or density of chromaffin cells in these two species (and the Atlantic cod). Thus differential enzyme activities within the Blaschko pathway is probably the most plausible explanation for the differences in stored catecholamine concentrations. Given that one of the primary roles of circulating catecholamines is to optimize cardiovascular and respiratory functions such as oxygen transport in the blood, and the observation that eels appear to have a greater tolerance to environmental hypoxia than trout, it is not suprising that eels store less catecholamines than trout. Indeed, given that only a small percentage of stored catecholamines (i.e. 2-3%) are released during a severe acute stress, it is interesting that trout store such large quantities of catecholamines, especially given that neural stimulation of the chromaffin cells by acetylcholine activates several of the enzymes involved in

catecholamine biosynthesis. However, although trout store greater quantities of catecholamines than eels, these storage differences probably cannot account for the differences in plasma levels seen during hypoxia. Indeed, differences in storage levels could account for the differing plasma levels only if eels stored very small amounts of catecholamines (or none at all) but rather relied on *de novo* synthesis to provide catecholamines for release into the circulation upon exposure to a stress.

It is possible that not all stored catecholamines within chromaffin cells are readily available for release during an acute stress such as hypoxia. Indeed, within chromaffin cells, catecholamine-containing storage granules are separated from the plasma membrane by a network of actin filaments which must disassemble in order for secretory granules to move toward, and fuse with, the plasma membrane. Upon disassembly of this network it is possible that, in trout chromaffin cells, more storage vesicles are in “close enough proximity” to the plasma membrane to fuse and release their contents than is the case in eel chromaffin cells. Indeed in both species, given that such a small percentage of stored catecholamines are released, the majority of storage vesicles may not be in a position for immediate exocytosis upon appropriate stimulation of the cells.

Although the levels of stored catecholamines may partially account for the differences in plasma levels observed during hypoxia, other factors clearly are involved. Dose response curves for carbachol-evoked adrenaline and noradrenaline release, *in situ*, revealed that for any given carbachol dose, trout chromaffin cells released greater quantities of catecholamines than eel chromaffin cells. However, in both species catecholamine release occurs at approximately the same dose of carbachol (10^{-7} moles)

suggesting that there are no differences in the affinity of the release process but rather there is a greater maximal response (i.e. " $V_{\max}$ ") for carbachol-evoked catecholamine release in trout. Possible explanations for this phenomenon could include a greater number of cholinceptors on the trout chromaffin cells and/or a greatly amplified post-receptor signal transduction pathway leading to exocytosis of the storage vesicle contents. Given that trout release much greater quantities of catecholamines in response to the non-specific depolarising stimulus of $60 \text{ mmol l}^{-1} \text{ K}^{+}$, this latter possibility appears likely regardless of any differences in the density of cholinceptors on the chromaffin cell membrane. Thus trout chromaffin cells have a greater ability to release catecholamines in response to cholinceptor stimulation than do eel chromaffin cells.

In contrast to these interspecific differences in catecholamine release during hypoxia, both the eel and trout appear to release catecholamines into the circulation in response to a depression of blood oxygen content corresponding to approximately 45-60% of its maximal value (Perry and Reid 1992). The results of chapter 6 demonstrate that, during exposure to acute hypoxia, catecholamines are released into the circulation, at a critical PaO_2 threshold (the value of which depends on the P_{50} value), corresponding to a critical oxygen content threshold of approximately 60% of maximal value. Thus, the proximate stimulus for the release of catecholamines during acute hypoxia is a depression of blood oxygen content rather than the partial pressure of oxygen. Given that circulating catecholamines tend to guard against depressions in oxygen content by optimising oxygen transport across the gills and transport in the blood, linking the release of catecholamines to blood oxygen content levels is an appropriate physiological design.

3. Contents of the Chromaffin Cells

Using immunohistochemical techniques in combination with fluorescence microscopy, differences between the chromaffin cells in the rainbow trout and **European** eel were revealed (chapter 7). Given the close relationship between the European (*Anguilla anguilla*) and American (*Anguilla rostrata*) eel, it is reasonable to extrapolate from one species to the other and speculate that similarities within the chromaffin cells exist. The chromaffin cells of the European eel contained neuropeptide tyrosine (neuropeptide Y; NPY)-like and peptide tyrosine tyrosine (PYY)-like immunoreactivity which was not localised in the chromaffin cells of either the rainbow trout or Atlantic cod. These differences may result from the different stages of teleost evolution represented by these three species. Rainbow trout and American/European eels are considered to be primitive teleosts whereas the Atlantic cod is considered to be an advanced teleost. The presence of these peptides within the eel chromaffin cells may represent a more “primitive” state which has been “selected against” in more advanced species. Interestingly, chromaffin cells in a variety of “higher” vertebrates (i.e. amphibians, birds and mammals) often contain a plethora of bioactive substances, which suggests that the presence of NPY/PYY in the eel chromaffin cells may not be representative of a more “primitive” chromaffin cell. However, until physiological experiments are performed to ascertain whether or not these peptides are released from the chromaffin cells and, what, if any physiological effects they exert, these differences remain interesting morphological curiosities.

Serotonin-like immunoreactivity was identified within the chromaffin cells in eel and cod but not trout. In the trout, serotonin-like immunoreactivity was localised within a population of small spherical cells in the kidney tissue rather than in the walls of the posterior cardinal vein (chapter 7). Interestingly, and perhaps contrary to this observation, was the detection of serotonin within the trout posterior cardinal vein using HPLC with electrochemical detection (see chapter 8). It is difficult to imagine that antisera which recognize serotonin in both the cod and eel would not do so in trout chromaffin cells, if it were there, especially given the obvious labelling of the other cell types within the kidney. Perhaps the easiest explanation for this apparent contradiction is that during the measurement of serotonin by HPLC, some kidney tissue, with the serotonin-containing cells, adhered to the PCV. However, regardless of the exact source of serotonin, the physiologically relevant observation is that serotonin is capable of eliciting the release of catecholamines from trout chromaffin cells (chapter 8). In eel and cod, serotonin may not be capable of eliciting catecholamine release as there is no local source of serotonin (other than the chromaffin cells themselves) to act on the chromaffin cells. Although serotonin originating from neuroepithelial cells of the gills may reach the chromaffin cells via the circulation, this perhaps is unlikely as most of serotonin released from these cells is taken back up into the neuroepithelial cells before exiting into the general systemic circulation. Therefore a local source of serotonin within the vicinity of the chromaffin cells may be required in order for serotonin to exert a physiological role in the release of catecholamines.

Modulation of Catecholamine Storage and Release By The Pituitary-Interrenal Axis

In general, an elevation of plasma catecholamine levels is considered to be a response to acute stress (e.g. hypoxia, chasing) with catecholamine release occurring almost immediately upon exposure to the stressful condition. On the other hand, the mobilization of cortisol into the circulation is, temporally, a longer term response to stress. The fact that both these substances are released in response to stress, in conjunction with the close physical proximity of their storage sites (chromaffin and interrenal cells), suggests that interactions between these two different cell types may occur.

Cortisol is released into the circulation upon stimulation of the interrenal cells by adrenocorticotrophic hormone (ACTH) which, in turn, is released from the pituitary. The results of chapter 8 demonstrate that ACTH is capable of eliciting an elevation of plasma adrenaline levels *in vivo* and perfusate adrenaline and noradrenaline levels *in situ*.

However, although the perfusate catecholamine concentration increased substantially following ACTH administration, the increase in the levels of plasma adrenaline following an intra-arterial injection of ACTH were quite low (less than 10 nmol l^{-1}). This suggests that although ACTH may be capable of eliciting the release of catecholamines from the chromaffin cells, it's physiological role as a secretagogue, *in vivo*, under "normal" conditions is debatable. However, under appropriate conditions, ACTH may exert a more prominent role on catecholamine secretion *in vivo*. For instance, during periods of cholinceptor down-regulation or desensitization, other secretagogues such as ACTH or serotonin (chapter 8) may exert a more important role in the release of catecholamines. Under such circumstances, an up-regulation of receptors for ACTH and serotonin may

occur, thus increasing the sensitivity of the chromaffin cells to stimulation by these hormones.

Elevated plasma cortisol levels can influence the storage of catecholamines within chromaffin cells and both basal and carbachol-evoked catecholamine release from these cells (chapter 4). In mammals, corticosteroids, such as cortisol, have been demonstrated to increase the activity of phenylethanolamine-N-methyltransferase (PNMT), the enzyme which converts noradrenaline to adrenaline. Although an experimental elevation of plasma cortisol levels increased the concentration of stored adrenaline and noradrenaline within various regions of the kidney and PCV, PNMT activity was unaltered by cortisol treatment. Thus the activities of other enzymes within the Blaschko pathway may increase in response to an elevation of plasma cortisol levels.

An increase in basal adrenaline release may have resulted from the elevated levels of stored catecholamines whereas the increased sensitivity of the release process to cholinceptor stimulation may be explained by a cortisol-induced up-regulation of cholinceptors on the chromaffin cell membrane, a situation which would be analogous to the effects of cortisol on trout red blood cell and hepatocyte β -adrenoceptors. Regardless of the mechanism of action by which cortisol is exerting its effects on catecholamine storage and release, it is apparent that the pituitary-interrenal axis is capable of modulating both of these processes. Previous studies have demonstrated that chronic stress can alter both catecholamine storage and the process of cholinergic-induced catecholamine release (e.g. Nilsson 1990; Nilsson and Block 1991; Reid *et al.* 1994). Given the aforementioned effects of cortisol on catecholamine storage and release, it is likely that elevated plasma

cortisol levels, associated with a chronic stress situation, have a modulatory effect on the adrenergic response to an acute stress in rainbow trout.

Non-Cholinergic Control of Catecholamine Release

In addition to the effects of serotonin and ACTH on catecholamine release in the rainbow trout (chapter 8), the presence of both vasoactive intestinal polypeptide (VIP)-like and pituitary adenylyl cyclase activating polypeptide (PACAP)-like immunoreactivity innervating the chromaffin cells of rainbow trout, European eel and Atlantic cod (chapter 7) suggests a possible role for these substances in controlling catecholamine release. Both VIP and PACAP may be co-stored with acetylcholine in the sympathetic nerves where they may function as peptidergic neurotransmitters. Like serotonin and ACTH, PACAP is capable of activating adenylyl cyclase thus producing the second messenger cAMP. Through a series of phosphorylation events, cAMP could possibly mediate the closure of K^+ channels. This, in turn, could lead to a membrane depolarization sufficient to open voltage-dependent Na^+ channels and ultimately Ca^{2+} channels. An ensuing influx of calcium could, conceivably, initiate the exocytotic release of catecholamines.

The co-storage of VIP and acetylcholine in sympathetic nerves innervating chromaffin cells is not without precedent. Guo and Wakade (1994) demonstrated, in the rat adrenal, that low frequency stimulation of the splanchnic nerve caused the neural release of VIP from the nerve which in turn stimulated the secretion of **adrenaline** from the adrenal medulla. Upon high frequency nerve stimulation, acetylcholine was also released and caused the release of both **adrenaline and noradrenaline**.

Thus it is apparent that non-cholinergic substances are capable of eliciting the release of catecholamines from chromaffin cells in teleost fish. Clearly further experiments are required to elucidate the physiological roles, if any, of VIP and PACAP and to further examine the *in vivo* effects of serotonin and ACTH. The presence of non-cholinergic secretagogues, such as serotonin and ACTH, may allow for the release of catecholamines under a variety of stressful situations when different hormones/neurotransmitters are released (Livett and Marley 1993). These different secretory pathways also may serve as “back-up” systems in the event of a down-regulation or desensitization of the primary cholinergic pathway of catecholamine release. Alternately, the ability of ACTH and serotonin to elicit catecholamine release may represent a more primitive system originating from species (e.g. hagfish) whose chromaffin cells lack cholinergic innervation. Clearly non-cholinergic control of catecholamine release in fish has the potential to be a field of extensive investigation in the future.

CONTROL OF CATECHOLAMINE RELEASE AND ITS RELATIONSHIP TO WHOLE ANIMAL PHYSIOLOGY

The differences in the process of catecholamine release between the rainbow trout and the American eel may reflect the different lifestyles of these two species as well as their evolutionary relationship to each other. Species such as the rainbow trout often attempt to regulate the detrimental effects associated with stresses (e.g. environmental hypoxia) whereas attempts at regulation do not occur to such an extent in the American eel. As such, the adrenergic stress response and an elevation of plasma catecholamine levels is most likely of greater importance in the rainbow trout than the American eel (e.g. Perry and Reid 1992). This phenomenon is reflected by the differences in the process of cholinergic control of catecholamine release in these two species (chapters 2 and 5). The implication that the muscarinic cholinergic receptor is involved in the release of catecholamines in the trout but not the eel suggests that trout chromaffin cells possess a greater degree of flexibility with respect to secreting catecholamines. Indeed the presence of two populations of cholinergic receptors (nicotinic and muscarinic) capable of eliciting release may help to guard against a blunted cholinergic response given a stress-induced down-regulation of cholinergic receptors. Such a "safeguard" may not be as important in the eel given the much reduced adrenergic response compared to trout. The greater importance of the adrenergic stress response in trout, compared with the eel, is also reflected by the observation that trout chromaffin cells possess a greater ability to release catecholamines than eel chromaffin cells (chapter 3).

The ability of non-cholinergic substances such as serotonin and ACTH to act as secretagogues of catecholamines in trout also provides a greater degree of flexibility with respect to catecholamine release from chromaffin cells. Although the effects of these substances were not examined in the eel, the suggestion that these substances may have been the primary secretagogue of catecholamines in more primitive fish such as the hagfish (see chapter 8 and above) indicates that these effects may be observed in a variety of vertebrates.

Although cholinergic-induced release is the predominant mechanism leading to the secretion of catecholamines in species such as the rainbow trout and indeed in higher vertebrates (e.g. Burgoyne 1991) the presence of a variety of secretagogues in trout may also reflect a greater reliance on circulating catecholamines in lower, compared with higher, vertebrates. For example, adrenergic control of cardiovascular function in higher vertebrates is predominantly mediated via neuronal, rather than circulating, catecholamines. In lower vertebrates where the situation is reversed, a plethora of mechanisms controlling catecholamine release may ensure that various aspects of the **efferent** adrenergic response are not compromised by a stress-induced blunted **afferent** pathway.

In summary, the differences in the release process observed between the rainbow trout and the American eel, may reflect, in part, the different lifestyles of these two teleost species. Additionally the presence of non-cholinergic pathways of catecholamine release may reflect, not only a greater degree of flexibility with respect to catecholamine

secretion, but also evolutionary differences in the reliance of circulating versus neuronal catecholamines across the vertebrate lineage.

CONCLUSION

This thesis examined aspects of both cholinergic and non-cholinergic control of catecholamine release in teleost fish and hopefully will provide both a physiological and morphological basis for future studies in this field, particularly with respect to the non-cholinergic control of catecholamine release. In conclusion, the results of this thesis have increased our understanding of the complex reflex pathways comprising the afferent limb of the acute adrenergic response to stress in teleost fish.

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