

ENDOCRINE CONTROL OF OSMOREGULATION IN  
THE EURYHALINE EEL, 'ANGUILLA ANGUILLA'

Mary Louise Tierney

A Thesis Submitted for the Degree of PhD  
at the  
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euryhaline eel, *Anguilla anguilla*.

by

Mary Louise Tierney

Thesis submitted for the degree of  
Doctor of Philosophy  
in the University of St. Andrews.

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This thesis is dedicated to my parents and brothers.

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## Abstract

1. Groups of eels, *Anguilla anguilla*, were adapted from freshwater ( FW ) to seawater ( SW ) for periods of 90 - 300 mins. maximum ( acute transfer ), 0 - 7 days ( chronic transfer ), or for more than 14 days ( long-term seawater transfer ).
2. Acute SW transfer led to a decline in blood pressure, an elevation in plasma osmolality and chloride concentration, an immediate "reflex" drinking response and a non-significant increase in plasma angiotensin II ( AII ) concentration.
3. Administration of papaverine to FW adapted eel caused hypotension, with subsequent recovery of blood pressure, elevation in the drinking rate and plasma AII concentration, and a decline in plasma osmolality. Captopril alone had no effect on blood pressure, drinking rate, osmolality or AII concentration, but was successful in partially blocking the papaverine-induced blood pressure recovery and increase in AII concentration, with complete inhibition of the drinking.
4. Administration of papaverine to SW adapted eel caused hypotension, with partial recovery of blood pressure, increased drinking rate, plasma AII concentration and plasma osmolality. Captopril alone caused a sustained decrease in blood pressure, inhibition of basal SW drinking and a reduction in plasma AII concentration, with change in plasma osmolality. Administration of captopril prior to papaverine was successful in partially blocking the papaverine-induced recovery in blood pressure, increase in drinking, plasma AII concentration, and plasma osmolality.

5. Chronic SW transfer led to a general decline in blood pressure, increase in plasma electrolyte concentration, elevation in drinking rate after 4 - 5 days, an increase in plasma AII concentration, and a rise in  $\text{Na}^+\text{-K}^+\text{-ATPase}$ , all leading to long term SW values.
6. Plasma arginine vasotocin concentrations were unchanged in long term-FW and SW adapted fish, with a small transitory rise after 4 days in SW.
7. Cortisol plasma concentrations were similar in both long term- FW and SW- adapted fish, with a rise observed 1 day after transfer to SW.
8. Metabolic clearance rates ( MCR ) and blood production rates ( BPR ) were significantly elevated in long term SW adapted fish and during chronic SW adaptation, compared to the FW levels.
9. Binding of  $^{125}\text{I}\text{-AII}$  to gill ( filaments and lamellae ), brain ( cerebellum and medulla oblongata ), kidney ( head and caudal ), and liver was observed in long term FW and SW adapted fish and 6 day SW transfer animals, with significant increase observed in binding in the caudal kidney and cerebellum and medulla oblongata between the FW group and 6 day SW transfer group.

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## Table of Contents

|         |                              | page no. |
|---------|------------------------------|----------|
| 1.0     | <u>Introduction</u>          |          |
| 1.1     | Osmoregulation               | 1        |
| 1.1.1   | Terrestrial environment      | 1        |
| 1.1.2   | Freshwater environment       | 1        |
| 1.1.3   | Seawater environment         | 2        |
| 1.2     | Teleost skin                 | 3        |
| 1.3     | Teleost gills                | 4        |
| 1.3.1   | Gill structure               | 4        |
| 1.3.2   | Chloride cell                | 6        |
| 1.3.3   | Transport protein            | 9        |
| 1.3.4   | Transport mechanisms         | 11       |
| 1.4     | Teleost gut                  | 16       |
| 1.5     | Kidney and urinary bladder   | 20       |
| 1.5.1   | Teleost kidney               | 22       |
| 1.5.1a  | Morphology                   | 24       |
| 1.5.1b  | Function                     | 25       |
| 1.5.2   | Teleost urinary bladder      | 29       |
| 1.6     | Hormones                     | 31       |
| 1.7     | Receptors                    | 33       |
| 1.8     | Pituitary gland              | 35       |
| 1.8.1   | Structure of adenohypophysis | 35       |
| 1.8.2   | Adenohypophysial secretions  | 37       |
| 1.8.2a  | Prolactin                    | 38       |
| 1.8.2ai | Prolactin in fish            | 39       |
| 1.8.2b  | Growth hormone               | 43       |
| 1.8.2c  | Somatolactin                 | 45       |
| 1.8.3   | Structure of neurohypophysis | 45       |

|           |  |    |
|-----------|--|----|
| 1.8.4     | Neurohypophysial secretions  | 47 |
| 1.8.4a    | Effect on gill function  | 49 |
| 1.8.4b    | Effect on renal function   | 50 |
| 1.9       | Adrenocortical homologue   | 52 |
| 1.9.1     | Structure of adrenocortical homologues   | 52 |
| 1.9.2     | Structure of steroids  | 54 |
| 1.9.3     | Structure of corticosteroids   | 58 |
| 1.9.4     | Cortisol and fish osmoregulation   | 59 |
| 1.9.5     | Steroid dynamics   | 62 |
| 1.9.5a    | Determination of secretory dynamics  | 63 |
| 1.9.5ai   | Blood production rate  | 63 |
|           | (i) Single injection   | 63 |
|           | (ii) Constant infusion   | 64 |
| 1.9.5aaii | Urinary production   | 65 |
| 1.9.5b    | Cortisol secretory dynamics in teleosts  | 66 |
| 1.9.6     | Catecholamines   | 66 |
| 1.10      | The renin-angiotensin system   | 69 |
| 1.10.1    | Introduction   | 69 |
| 1.10.2    | Effect of the RAS on cardiovascular system   | 73 |
| 1.10.3    | Effect of the RAS on drinking in fish  | 75 |
| 1.10.4    | Effect of the RAS on renal action  | 79 |
| 1.10.5    | Interaction between the RAS and other<br>hormones in control of water and electrolytes | 82 |
| 1.10.6    | Angiotensin receptor studies   | 84 |
| 1.11      | Natriuretic peptides   | 85 |
| 1.11a     | Atrial natriuretic peptide in fish   | 89 |
| 1.11ai    | Heterologous studies   | 90 |
| 1.11aaii  | Homologous studies   | 92 |
| 1.11b     | Interaction between atrial natriuretic peptide<br>and other hormones in fish           | 93 |

|        |   |     |
|--------|---|-----|
| 1.11c  | Brain, C-type and ventricular natriuretic peptides                | 93  |
| 1.12   | Caudal neurosecretory system in fish                              | 94  |
| 1.13   | Thyroid hormones  | 97  |
| 1.14   | Objectives  | 99  |
| 2.0    | <u>Material and Methods</u>                                       |     |
| 2.1    | General chemicals   | 101 |
| 2.2    | Radioimmunoassay of angiotensin II                                | 101 |
| 2.2.1  | Standard assay procedure  | 101 |
| 2.2.2  | Inter- and intra-assay variation                                  | 102 |
| 2.2.3  | Extraction of angiotensin II                                      | 102 |
| 2.3    | Radioimmunoassay of arginine vasotocin                            | 103 |
| 2.4    | Radioimmunoassay of cortisol                                      | 103 |
| 2.4.1  | Standard assay procedure  | 104 |
| 2.4.2  | Extraction of cortisol  | 105 |
| 2.5    | Animals   | 105 |
| 2.6    | Cannulations of blood vessels                                     | 103 |
| 2.7    | Osmotic adaptation terminology                                    | 106 |
| 2.8    | <i>In vivo</i> studies  | 106 |
| 2.8.1  | Acute studies   | 106 |
| 2.8.2  | Chronic studies   | 108 |
| 2.9    | Blood pressure studies  | 108 |
| 2.10   | Pharmacological manipulation of the RAS                           | 109 |
| 2.11   | Determination of drinking rate                                    | 109 |
| 2.12   | Determination of cortisol secretory dynamics                      | 110 |
| 2.12.1 | High performance liquid chromatography                            | 111 |
| 2.13   | Na <sup>+</sup> -K <sup>+</sup> -ATPase activity assay            | 112 |
| 2.13.1 | Membrane isolation  | 112 |
| 2.13.2 | Determination of Na <sup>+</sup> -K <sup>+</sup> -ATPase activity | 113 |

|         |   |     |
|---------|---|-----|
| 2.13.3  | Determination of inorganic phosphate    | 113 |
| 2.14    | Angiotensin II receptor studies         | 114 |
| 2.14.1a | Isolation of hepatocytes                | 114 |
| 2.14.1b | <sup>125</sup> I-AII binding studies    | 116 |
| 2.14.2  | Autoradiography                         | 118 |
| 2.15    | General plasma electrolyte analysis     | 121 |
| 2.16    | Determination of protein                | 121 |
| 2.17    | Statistical analysis                    | 122 |
| 3.0     | <u>Results</u>                          |     |
| 3.1     | Hormone radioimmunoassay                | 123 |
| 3.1.1   | Radioimmunoassay of angiotensin II      | 123 |
| 3.1.2   | Radioimmunoassay of cortisol            | 123 |
| 3.2     | Acute studies                           | 128 |
| 3.2.1   | Blood pressure                          | 128 |
| 3.2.2   | Plasma electrolyte composition          | 128 |
| 3.2.3   | Drinking rate                           | 128 |
| 3.2.4   | Angiotensin II concentration            | 134 |
| 3.3     | Pharmacological manipulation of the RAS | 134 |
| 3.3.1   | Freshwater-adapted fish                 | 134 |
| 3.3.1a  | Blood pressure                          | 134 |
| 3.3.1b  | Plasma electrolyte analysis             | 137 |
| 3.3.1c  | Drinking rate                           | 137 |
| 3.3.1d  | Angiotensin II concentration            | 140 |
| 3.3.2   | Seawater-adapted fish                   | 142 |
| 3.3.2a  | Blood pressure                          | 142 |
| 3.3.2b  | Plasma electrolyte analysis             | 145 |
| 3.3.2c  | Drinking rate                           | 145 |
| 3.3.2d  | Angiotensin II concentration            | 145 |
| 3.4     | Chronic studies                         | 150 |

|        |   |     |
|--------|---|-----|
| 3.4.1  | Blood pressure  | 150 |
| 3.4.2  | Plasma electrolyte analysis                           | 150 |
| 3.4.3  | Drinking rate   | 154 |
| 3.4.4  | Angiotensin II concentration                          | 154 |
| 3.4.5  | Arginine vasotocin concentration                      | 154 |
| 3.4.6  | Cortisol  | 158 |
| 3.4.6a | Cortisol concentration                                | 158 |
| 3.4.6b | Cortisol dynamics                                     | 158 |
| 3.4.6c | Metabolism of cortisol                                | 158 |
| 3.4.6d | Metabolic clearance rate<br>and blood production rate | 162 |
| 3.4.7  | Na <sup>+</sup> -K <sup>+</sup> -ATPase activity      | 165 |
| 3.4.8  | Receptor studies                                      | 170 |
| 3.4.9  | Protein determination                                 | 175 |
| 4.0    | <u>Discussion</u>                                     | 176 |
| 5.0    | <u>References</u>                                     | 209 |
| 6.0    | <u>Appendix</u>                                       | 247 |

## List of Figures

|  | Page |
|--|------|
| Figure 1.1 Schematic representation of gill lamella  | 5    |
| Figure 1.2 Schematic representation of choride cell  | 7    |
| Figure 1.3 Schematic representation of Na <sup>+</sup> -K <sup>+</sup> -ATPase enzyme  | 10   |
| Figure 1.4 Possible mechanisms of ion uptake in FW fish gill   | 13   |
| Figure 1.5 Route and mechanism of salt extrusion across SW fish gill   | 15   |
| Figure 1.6 Model of water and ion distribution in the oesophageal mucus  | 17   |
| Figure 1.7 Possible mechanisms of ion transport in the teleost intestine   | 21   |
| Figure 1.8 Five configurational types of marine teleostean kidneys   | 23   |
| Figure 1.9 Postulated mechanisms of NaCl in SW teleost proximal tubule   | 28   |
| Figure 1.10 Phylogentic tree of vertebrate groups with superimposed diagrams of sagittal sections of the typical pituitary gland in each group | 36   |
| Figure 1.11 Amino acid sequences of the prolactin family   | 40   |
| Figure 1.12 Basic steroid structure  | 55   |
| Figure 1.13 Interrelationships and formation of the steroid hormones   | 56   |
| Figure 1.14 The mammalian renin angiotensin system   | 70   |
| Figure 1.15 Amino acid sequence of selected members of the natriuretic peptide   | 86   |
| Figure 1.16 Transcription and translation of the atrial natriuretic peptide gene   | 87   |

|             |   |     |
|-------------|---|-----|
| Figure 1.7  | Possible pathways of urotensin action on teleost osmoregulation   | 98  |
| Figure 2.1  | Schematic diagram of the holding tank for the acute transfer of eel from FW to SW                                 | 107 |
| Figure 2.2  | Diagram of closed recirculating system for the perfusion of isolated liver  | 117 |
| Figure 2.3  | Method for embedding of eel tissue in wax   | 119 |
| Figure 2.4  | Method for rehydration and dewaxing of tissue sections prior for use in autoradiography                           | 119 |
| Figure 2.5  | Method for the photographic development of slides   | 120 |
| Figure 2.6  | Method for the histological staining of slides  | 120 |
| Figure 3.1  | Standard curve for radioimmunoassay of Angiotensin II   | 124 |
| Figure 3.2  | Standard curve for radioimmunoassay of cortisol   | 126 |
| Figure 3.3  | Typical blood pressure trace after acute SW adaptation  | 129 |
| Figure 3.4  | Effect of acute SW adaptation on mean arterial blood pressure   | 130 |
| Figure 3.5  | Effect of acute SW adaptation on drinking rate  | 132 |
| Figure 3.6  | Effect of acute SW adaptation on AII concentration  | 133 |
| Figure 3.7  | Typical blood pressure traces after administration of papaverine and/or captopril to FW-adapted eel               | 135 |
| Figure 3.8a | Effect of administration of papaverine on mean arterial blood pressure of FW eels                                 | 136 |
| Figure 3.8b | Effect of administration of papaverine after an injection of captopril on mean arterial blood pressure of FW eels | 136 |
| Figure 3.9  | Effect of administration of papaverine and/or captopril on drinking rate of FW eels                               | 139 |

|              |  |     |
|--------------|--|-----|
| Figure 3.10  | Effect of administration of papaverine and/or captopril on Angiotensin II concentration of FW eels                 | 141 |
| Figure 3.11  | Typical blood pressure traces after administration of papaverine and/or captopril to SW-adapted eel                | 143 |
| Figure 3.12a | Effect of administration of papaverine on mean arterial blood pressure of SW eels.                                 | 144 |
| Figure 3.12b | Effect of administration of captopril on mean arterial blood pressure of SW eels.                                  | 144 |
| Figure 3.12c | Effect of administration of papaverine after an injection of captopril on mean arterial blood pressure of SW eels. | 144 |
| Figure 3.13  | Effect of administration of papaverine and/or captopril on drinking rate of SW eels.                               | 147 |
| Figure 3.14a | Effect of administration of papaverine on Angiotensin II concentration of SW eel.                                  | 148 |
| Figure 3.14b | Effect of administration of papaverine and/or captopril on Angiotensin II concentration of SW eels.                | 148 |
| Figure 3.15  | Typical blood pressure traces after chronic- and long-term SW-adaptation   | 151 |
| Figure 3.16  | Effect of chronic- and long term- SW adaptation on mean arterial blood pressure                                    | 152 |
| Figure 3.17a | Effects of chronic- and long term- SW adaptation on plasma osmolality  | 153 |
| Figure 3.17a | Effects of chronic- and long term- SW adaptation on plasma chloride concentration                                  | 153 |
| Figure 3.17a | Effects of chronic- and long term- SW adaptation on plasma sodium concentration                                    | 153 |
| Figure 3.18  | Effects of chronic- and long term SW- adaptation on drinking rate  | 155 |

|              |   |     |
|--------------|---|-----|
| Figure 3.19  | Effects of chronic- and long term SW- adaptation on Angiotensin II concentration  | 156 |
| Figure 3.20  | Effects of chronic- and long term SW- adaptation on plasma Arginine Vasotocin concentration                                       | 157 |
| Figure 3.21  | Effects of chronic- and long term SW- adaptation on plasma cortisol concentration   | 159 |
| Figure 3.22  | Constant isotopic infusion  | 160 |
| Figure 3.23a | HPLC profile of plasma spiked steroid standards   | 161 |
| Figure 3.23b | HPLC profile of plasma spiked with tritiated cortisol   | 161 |
| Figure 3.23c | HPLC profile of plasma from SW eel previously infused with tritiated cortisol   | 161 |
| Figure 3.24a | Effect of chronic- and long term- SW adaptation on cortisol concentration   | 164 |
| Figure 3.24b | Effect of chronic- and long term- SW adaptation on Metabolic Clearance Rate (MCR) of cortisol                                     | 164 |
| Figure 3.24c | Effect of chronic- and long term- SW adaptation on Blood Production Rate (BPR) of cortisol  | 164 |
| Figure 3.25  | Standard curve for the determination of inorganic phosphate   | 166 |
| Figure 3.26a | Effect of temperature on $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity in gill membranes from long term- SW adapted eels.       | 167 |
| Figure 3.26b | Effect of membrane dilution on $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity in gill membranes from long term- SW adapted eels. | 167 |
| Figure 3.27  | Effect of chronic- and long term- SW adaptation on $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity in gill membranes              | 168 |
| Figure 3.28  | Effect of chronic- and long term- SW adaptation on $\text{K}^+$ stimulated activity in gill membranes                             | 169 |

|              |   |     |
|--------------|---|-----|
| Figure 3.29  | Percentage specific binding of $^{125}\text{I}$ -AII to tissues of eel during FW to SW transfer                       | 174 |
| Figure 3.30a | Transverse section of eel brain   | 176 |
| Figure 3.30b | Background binding of AII to cerebellum of FW adapted eel   | 177 |
| Figure 3.30c | Non specific binding of AII to cerebellum of FW adapted eel   | 177 |
| Figure 3.30d | Total binding of AII to cerebellum of FW adapted eel  | 178 |
| Figure 3.30e | Total binding of AII to cerebellum of 6 day SW transfer eel   | 178 |
| Figure 3.30f | Total binding of AII to medulla oblongata of FW adapted eel   | 179 |
| Figure 3.30g | Total binding of AII to medulla oblongata of 6 day SW transfer eel  | 179 |
| Figure 3.30h | Total binding of AII to gill of FW adapted eel  | 180 |
| Figure 3.30i | Total binding of AII to head kidney of FW adapted eel   | 180 |
| Figure 3.30j | Total binding of AII to caudal kidney of FW adapted eel   | 181 |
| Figure 3.30k | Total binding of AII to caudal kidney of 6 day SW transfer eel  | 181 |
| Figure 3.30l | Total binding of AII to liver of FW adapted eel   | 182 |
| Figure 3.30m | Total binding of AII to liver of 6 day SW transfer eel  | 182 |
| Figure 3.31  | Typical standard curve for the measurement of protein   | 183 |
| Figure 4.1   | Relationship between drinking rate and angiotensin II plasma concentration during chronic and long term SW adaptation | 194 |
| Figure 4.2   | Relationship between cortisol BPR and angiotensin II plasma concentration during chronic and long term SW adaptation  | 199 |

List of tables

|           |   |     |
|-----------|---|-----|
| Table 1.1 | Structure and phylogeny of vertebrate neurohypophysial hormones                                   | 48  |
| Table 1.2 | Major corticosteroids produced in vertebrate groups,  | 57  |
| Table 1.3 | Angiotensin I amino acid sequence   | 72  |
| Table 1.4 | Vascular effects of angiotensin in non-mammalian vertebrates                                      | 74  |
| Table 1.5 | Dipsogenic effects of angiotensin in non-mammalian vertebrates                                    | 76  |
| Table 1.6 | Renal effects of angiotensin in non-mammalian vertebrates   | 81  |
| Table 1.7 | Effects of atrial natriuretic peptide in fishes   | 91  |
| Table 2.1 | Modified buffers used for the isolation of hepatocytes from long term FW- and SW-adapted eels     | 115 |
| Table 3.1 | Extraction of angiotensin II from eel plasma  | 125 |
| Table 3.2 | Extraction of cortisol from eel plasma  | 127 |
| Table 3.3 | Effect of acute SW adaptation on plasma composition   | 131 |
| Table 3.4 | Effect of administration of papaverine and/or captopril on plasma osmolality of FW eels           | 138 |
| Table 3.5 | Effect of administration of papaverine and/or captopril on plasma osmolality of SW eels           | 146 |
| Table 3.6 | Percentage metabolism of infused tritiated-cortisol during constant isotopic infusion             | 163 |
| Table 3.7 | Variations to <sup>125</sup> I-AII binding assay  | 171 |
| Table 3.8 | Specific grain density of <sup>125</sup> I-AII binding to tissues of eel during FW to SW transfer | 172 |
| Table 4.1 | Na <sup>+</sup> -K <sup>+</sup> -ATPase activity in euryhaline teleosts                           | 201 |

## Abbreviation

|                  |   |
|------------------|---|
| AI               | angiotensin I                             |
| AII              | angiotensin II                            |
| ACE              | angiotensin converting enzyme             |
| AHP              | adenohypophysis                           |
| ACTH             | adrenocorticotropin                       |
| ANP              | atrial natriuretic peptide                |
| ANPir            | immunoreactive atrial natriuretic peptide |
| ATP              | adenosine trisphosphate                   |
| ATPase           | adenosine trisphosphatase                 |
| AVP              | arginine vasopressin                      |
| AVT              | arginine vasotocin                        |
| B <sub>max</sub> | maximal binding capacity                  |
| BPR              | blood production rate                     |
| BNP              | brain natriuretic peptide                 |
| BSA              | bovine serum albumin                      |
| C                | concentration                             |
| C-ANP            | C-type natriuretic peptide                |
| Caudal- NS       | caudal neurosecretory system              |
| cAMP             | cyclic adenosine 3', 5'-monophosphate     |
| CRF              | corticotropin releasing factor            |
| c.p.m.           | counts per minute                         |
| d.p.m.           | disintegrations per minute                |
| EDTA             | ethylenediamine tetra-acetic acid         |
| EGM              | extraglomerular mesangium                 |
| FSH              | follicle stimulating hormone              |
| FW               | freshwater                                |
| GH               | growth hormone                            |

|        |   |
|--------|---|
| GFR    | glomerular filtration rate  |
| I      | constant state of infusion  |
| IT     | isotocin  |
| i.v.   | intravenous   |
| JGA    | juxtaglomerular apparatus   |
| $K_d$  | dissociation constant   |
| LPH    | lipotropin  |
| LH     | leutinising hormone   |
| MD     | macula densa  |
| MCR    | metabolic clearance rate  |
| MSH    | melanotropin  |
| MS222  | tricaine methanosulphate  |
| NHP    | neurohypophysis   |
| OT     | oxytocin  |
| PD     | pars distalis   |
| PI     | pars intermedia   |
| PT     | pars tuberalis  |
| PRA    | plasma renin activity   |
| PRL    | prolactin   |
| RAS    | renin angiotensin system  |
| RIA    | radioimmunoassay  |
| $R_i$  | amount of radioactivity injected  |
| S.D.   | standard deviation  |
| S.E.M. | standard error of the mean  |
| SEM    | scanning electron microscopy  |
| SNGFR  | single nephron glomerular filtration rate                                       |
| SL     | somatolactin  |
| SA     | amount of radioactivity or unique hormone in urine, that is, specific activity. |

|       |   |
|-------|---|
| SW    | seawater  |
| T     | time of urine collection                          |
| TEM   | transmission electron microscopy                  |
| temp  | temperature                                       |
| TMAO  | trimethylamine N-oxide                            |
| TMG   | renal tubular maxima for glucose                  |
| TSH   | thyrotropin                                       |
| UI    | urotensin I                                       |
| UII   | urotensin II                                      |
| VNP   | ventricular natriuretic peptide                   |
| $X_i$ | steady state isotopic concentration in the plasma |

## 1.0 Introduction

## 1.1 Osmoregulation

In order to survive, vertebrates must maintain both the volume of their extracellular fluid and the concentration of the solutes within rather narrow limits. Osmoregulation is the term applied to the homeostatic mechanism involved in regulating the concentrations of body fluids. The underlying mechanisms of vertebrate osmoregulatory homeostasis are similar throughout the group. The kidney, the organ of volume regulation, is ubiquitous and, depending on the class, also works in association with extra-renal regulators such as gills, gut, urinary bladder, skin, cloaca and salt glands to maintain body fluid volume and composition. The problems of water balance encountered by organisms vary greatly and primarily depend upon the environment in which they live.

### 1.1.1 Terrestrial environment

The greatest physiological threat to life on the land is the risk of dehydration. Terrestrial vertebrates may lose water by evaporation across skin and respiratory surfaces, excretion in the urine and faeces and through sweating and panting. Water loss can be minimised by decreasing skin permeability and reducing respiratory evaporation by nasal exhalation. Urinary loss is reduced in mammals by the production of urine hyperosmotic to the plasma, and in birds by the production of an almost water-free urine. Reptiles are incapable of producing a hypertonic urine.

### 1.1.2 Freshwater environment

Freshwater (FW) is hyposmotic to the internal body fluids of the vertebrates which inhabit this environment, and, therefore, these animals face water gain across permeable membranes. This situation is remedied by the copious production of urine which is dilute with respect to the plasma and active reabsorption of sodium and chloride ions across the gills of fish

( Maetz, 1971 ) and by the skin of amphibians ( Middler *et al.*, 1968; Bentley, 1973 ).

### 1.1.3 Seawater environment

Marine vertebrates osmoregulate in one of two ways. They either maintain their body fluids iso- or hyper-osmotic to the environment, or regulate hyposmotically to the environment with plasma osmolality 25 - 30% that of seawater ( SW ). The latter gain solutes and lose water across permeable membranes. In marine teleosts this is compensated for by drinking large volumes of SW and excreting excess absorbed sodium and chloride across the gills and excess magnesium via the kidneys ( Keys and Wilmer, 1932 ). Marine reptiles and birds possess cephalic salt glands for the excretion of excess sodium and chloride ( Schmidt-Nielsen, 1960 ) and marine mammals produce a hyperosmotic urine.

Marine vertebrates that maintain an iso- or hyper-osmotic plasma include some cyclostomes, elasmobranchs, holocephalans, coelacanth and a single amphibian. The cyclostomes are divided into two distinct groups; lampreys, some of which are euryhaline and apparently osmoregulate like euryhaline teleosts; and the stenohaline marine hagfish, which maintain body sodium and chloride concentrations similar ( but not identical ) to SW. Osmotically, hagfish behave like many marine invertebrates and are considered as osmoconformers with limited but specific ionic regulation ( Stolte and Schmidt-Nielsen, 1978 ).

In all other classes that maintain their extracellular fluid iso- or hyper-osmotic to SW there is urea retention. Elasmobranchs, holocephalans and the coelacanth, *Latimeria*, maintain plasma sodium concentration higher relative to marine teleosts but body fluids are rendered hyperosmotic to SW by the retention of organic compounds, primarily urea and trimethylamine oxide. This hyperosmolality of extracellular fluid minimises osmotic water

loss but considerable ionic regulation occurs at the gills and kidney and, in addition, in elasmobranchs, the rectal gland.

The following sections will deal with the organs involved in the osmoregulatory process of euryhaline teleosts, with particular attention to changes that occur in these organs concomitant with movement of the fish from FW to SW.

### 1.2 Teleost skin

The skin of fish forms the interface between the body fluids and the aqueous environment in which they live. The skin of fish is quite impermeable to water and solutes and thereby limits exchanges with the environment, FW or SW ( Fromm, 1968 ). Studies on isolated fish skin are complicated by its adherence to the underlying muscle, and it is difficult to remove without causing damage. In marine fish, the skin is often thicker than that observed in FW species and is supplied with mucus glands and protected by scales. Mucous itself is not impermeable but it may form a layer of reduced exchange of salt and water close to the skin surface ( Parry, 1966 ). Mitochondrial-rich cells have been shown to be present in the flat opercular epithelium of teleost head regions. These cells have been identified as anatomically resembling the chloride cells found in the gill epithelium (see below) ( Karnarky and Kinter, 1977 ).

Chloride cells have been found in the skin of some fish larvae and were first reported in the skin of sardine ( *Sardinops caerulea* ) ( Lasker and Threadgold, 1968 ). Hwang ( 1990 ) demonstrated that, upon SW adaptation, chloride cells, present in the skin of the larvae of ayu ( *Plecoglossus altivelis* ), undergo structural modifications which include the development of interdigitations and numerous leaky junctions, as observed in the adult. During larval development, the density of chloride cells was also seen to change. Most of the lateral skin contained chloride cells immediately after

hatching but after larval development there was an apparent concentration of the cells in the central region of the lateral skin ( Hwang, 1990 )

### 1.3 Teleost Gills

#### 1.3.1 Gill structure

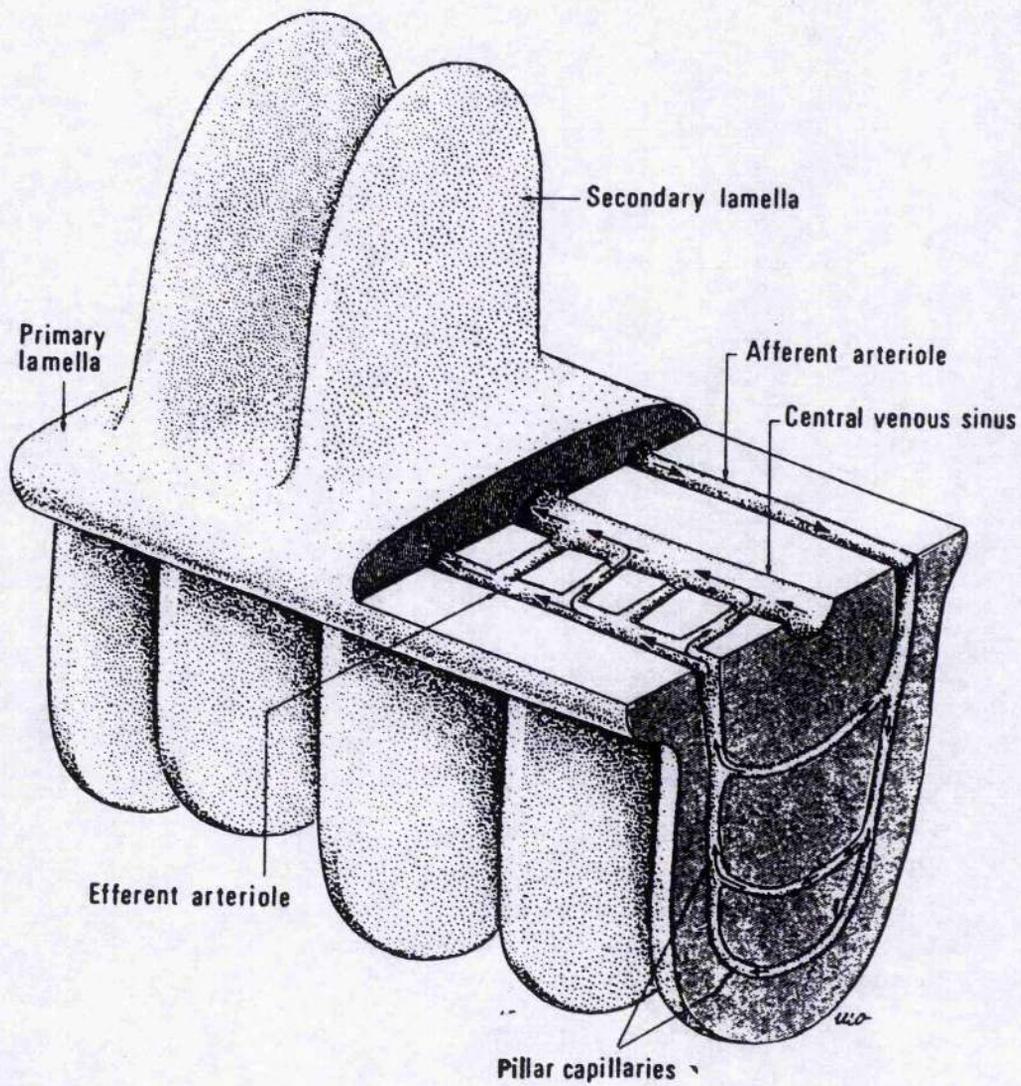
The basic teleost gill consists of four branchial arches, extending from either side of the pharynx, and which lie beneath the operculum of the fish. A double row of gill filaments, or primary lamellae, extend at right angles from the gill arch. The primary lamellae bear a row of respiratory lamellae on each side. The gill is comprised of two types of epithelial; the primary epithelium which covers the primary lamellae including the interlamellar region, and the secondary epithelium which covers the free part of the respiratory lamellae ( see Figure 1.1 ) ( Laurent and Dunel, 1980 ).

The secondary epithelium is generally composed of an outer mucosal layer of flat respiratory cells, also called pavement cells and an inner serosal layer of nondifferentiated cells ( Laurent and Dunel, 1980 ). The respiratory cells are 3 - 5  $\mu\text{m}$  thick, contain relatively few mitochondria, a prominent Golgi apparatus and an abundant rough endoplasmic reticulum ( Maetz, 1971 ). These cells are linked by tight junctions and desmosomes with no gap junctions observed ( Sardet *et al.*, 1979 ). The structure of the respiratory cells appear to be unaffected by environmental salinity. Chloride cells ( see below ) have, however, been shown to be present in the secondary epithelium and their proliferation may be in response to a specific ion deficiency ( Keys and Willmer, 1932; Avella *et al.*, 1987 ).

The basic characteristic of the multilayered primary lamellae are the mitochondrial-rich chloride cells interspaced between the respiratory cells. The primary difference between the epithelium of FW-adapted and SW-adapted fish concerns the chloride cell ( see below ), which are bound to their neighbouring respiratory cells by tight junctions ( Sardet *et al.*, 1979 )

Figure 1.1

Figure 1.1 Schematic representation of gill lamellae  
( Pisam *et al.*, 1987 )



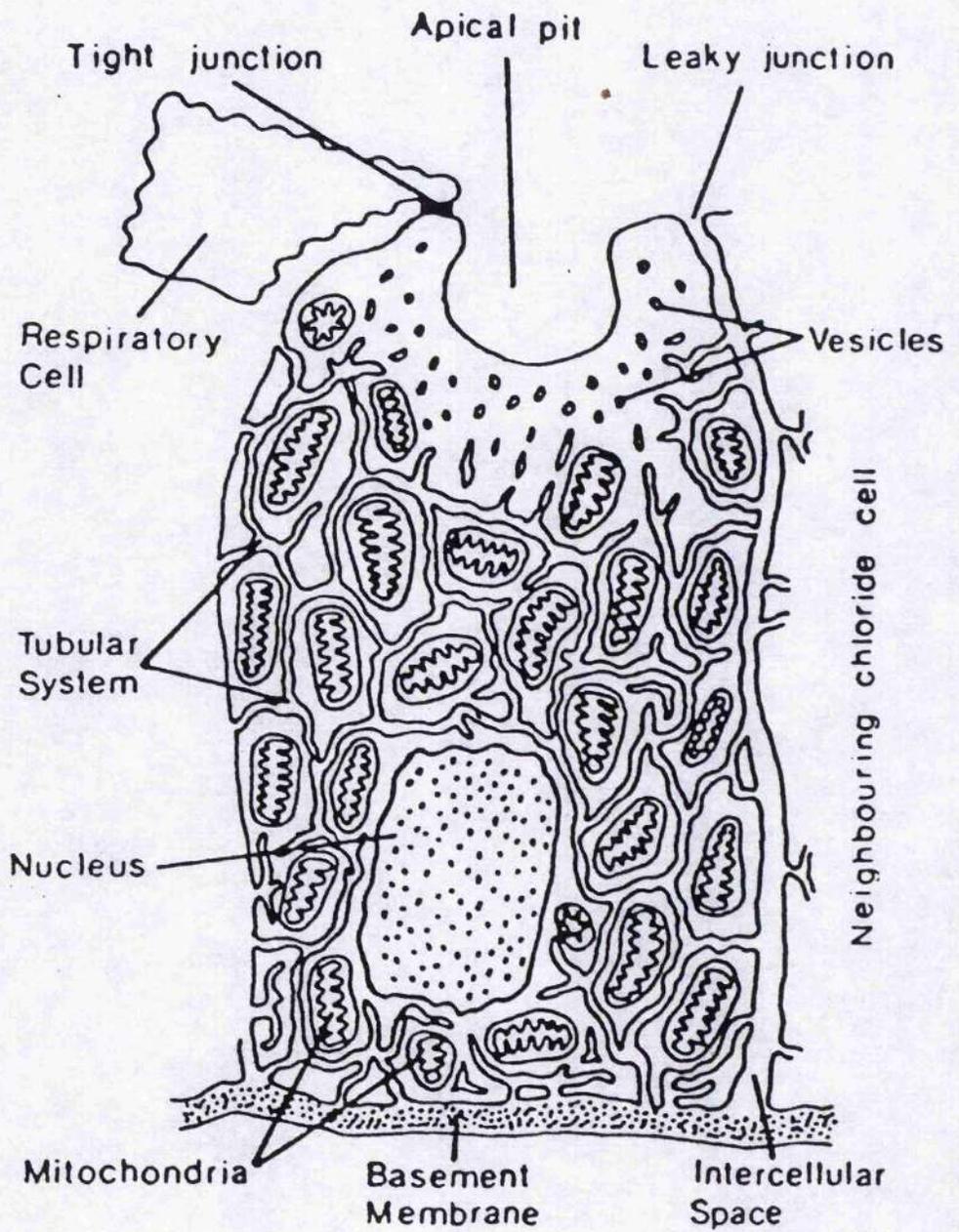
Two blood pathways exist in each gill arch, the arterioarterial and arteriovenous pathways, as shown in Figure 1.1. The arterioarterial pathway supplies blood to the secondary epithelium ( Laurent and Dunel, 1980 ) and consists of the afferent branchial artery, the afferent primary artery situated in each primary lamellae, the respiratory lamellae capillaries and the primary and branchial efferent arteries ( Laurent and Dunel, 1976 ). Innervated sphincters located in the primary lamellae control the arterioarterial vasculature, with perhaps a contribution from the pillar cells as a result of their contraction. The arteriovenous pathway supplies blood to the primary lamellae ( Laurent and Dunel, 1980 ). This vasculature originates from arterioarterial anastomoses which open directly into the central venous sinus ( CVS ). In the eel and Holostei and Chondrostei groups of fish these anastomoses are located on both the afferent and efferent parts of the vasculature, while in the trout they are only on the efferent section. The CVS lies in the core and along the length of the primary lamellae and drains into the branchial veins ( Laurent and Dunel, 1976 ). The primary lamellae blood flow is controlled by a sphincter located around the efferent artery close to its branchial artery junction ( Laurent and Dunel, 1980 ). Thus there is a distinction in the irrigation of the two epithelia ( Girard and Payan, 1980 ).

### 1.3.2 Chloride cell

The site of ion transport across the gill epithelium is generally accepted to be the chloride cell ( Motais and Garcia-Romeu, 1972; Zadunaisky, 1984 ). Chloride cells ( Figure 1.2 ) are large, acidophilic, non-mucus, granular cells containing many mitochondria and were first described by Keys and Wilmer ( 1932 ). They are present in both euryhaline and stenohaline species of FW or SW teleosts. These cells are predominantly situated in the primary epithelial interlamellar region although their presence has been cited as also occurring in the secondary epithelium ( Avella *et al.*, 1987 ). They are

Figure 1.2

Figure 1.2 Schematic representation of a chloride cell from the gill of a marine teleost  
( Rankin and Davenport, 1981 )



characterised by depressions known as apical crypts and a dense tubular system that opens on the basolateral plasma membrane. The apex of each cell has microvilli and is firmly bound to the neighbouring pavement cells by a long and tight junctional apparatus ( Laurent and Dunel, 1980 ).

It is reported that chloride cells undergo characteristic changes in relation to the salinity of the external environment. Two types of chloride cell,  $\alpha$ - and  $\beta$ -chloride cells, have been reported to be present in the gill epithelium of the FW-adapted teleosts, such as the euryhaline guppy, *Lebistes reticulatus*, and the stenohaline gudgeon ( *Gobio gobio* ) and loach ( *Cobitis taenia* ) ( Pisam *et al.*, 1987; 1990 ). Transfer from FW to SW apparently induced the degeneration of the  $\beta$ -chloride cell and the transformation of the  $\alpha$ -chloride cell to the single chloride cell type present in fully adapted SW fish ( Pisam *et al.*, 1987 )

The number of chloride cells is reported to increase upon transfer from FW to SW ( Shirai and Utida, 1970; Utida *et al.*, 1971; Thomson and Sargent, 1977 ) and also undergo structural reorganisation. Acclimation of euryhaline teleosts to SW is accompanied by enlargement of the chloride cell and a deepening of the apical crypt ( Olivereau, 1970; Hossler, 1980 ). In general the other epithelial cells entirely cap the distal portion of the chloride cell apart from the apical crypt ( Philpott and Copeland, 1963 ), and therefore it is the apical crypt which provides the chloride cell with direct contact with the external medium ( Maetz, 1971 ). These pits can be shared by more than one chloride cell ( Sardet *et al.*, 1979 ). With transfer to SW, the proliferation of chloride cells leads to the formation of a complex of two or more interdigitations of these cells, joined together by shallow gap junction, while retaining the tight junction with the pavement cells ( Sardet *et al.*, 1979 ).

A specific structural change observed in the gill upon SW transfer is the development of accessory cells along side the chloride cells. These accessory cells are smaller than the chloride cells and are usually located

between the chloride cell and the pavement cell layers ( Laurent, 1984 ). They are linked to the chloride cells via single strand short junctions. These junctions are thought to be leaky and, therefore, Sardet *et al.* ( 1979 ) hypothesised that transepithelial movement of sodium ions (  $\text{Na}^+$  ) occurs via intercellular junctions, whereas chloride ions (  $\text{Cl}^-$  ) are actively transported across the chloride cells.

### 1.3.3 Transport protein

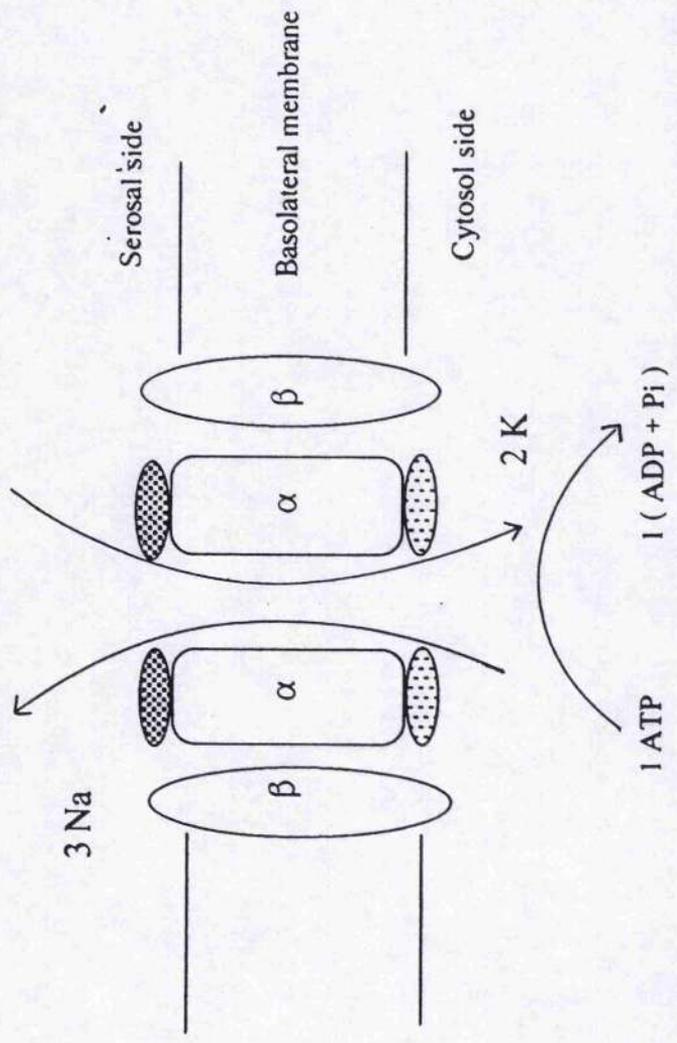
The enzyme,  $\text{Na}^+\text{-K}^+\text{-ATPase}$ , is found in association with the teleost chloride cell. Autoradiographical studies have revealed that  $\text{Na}^+\text{-K}^+\text{-ATPase}$  is located on the serosal rather than the mucosal side of the chloride ( Karnaky *et al.*, 1976 ). In this study tritiated ouabain, perfused via an intracardiac catheter into *Fundulus heteroclitus*, adapted to different salinities, was demonstrated to bind to the entire chloride cell surface apart from the apical crypt, indicating enzyme localisation on the basolateral membrane. A further indication of the location of  $\text{Na}^+\text{-K}^+\text{-ATPase}$  arises from a greater inhibition in activity by ouabain administered in the blood rather than to the gill exterior ( Silva *et al.*, 1977 ; Epstein *et al.*, 1973 ).

$\text{Na}^+\text{-K}^+\text{-ATPase}$  is a ubiquitous integral protein of the plasma membrane and is responsible for the active transport of  $\text{K}^+$  into and  $\text{Na}^+$  out of cells against their concentration gradients ( Jørgensen, 1980 ). The enzyme acts in a cyclic fashion. The basic  $\text{Na}^+\text{-K}^+\text{-ATPase}$  reaction leads to the hydrolysis of ATP to ADP and inorganic phosphate, with the translocation of three Na ions to the extracellular compartment coupled to the transport of two K ions to the intracellular compartment, per molecule of ATP split ( Schuurmans Stekhoven and Bonting, 1981 ) ( Figure 1.3 ).

The  $\text{Na}^+\text{-K}^+\text{-ATPase}$  is composed of two heterologous polypeptide subunits, a catalytic  $\alpha$ -subunit and a glycosylated  $\beta$ -subunit. All known functions of the  $\text{Na}^+\text{-K}^+\text{-ATPase}$  have so far been assigned to the  $\alpha$ -subunit.

Figure 1.3

Figure 1.3 Schematic representation of Na<sup>+</sup>-K<sup>+</sup>-ATPase enzyme



-  Represents cardiac glycoside inhibitor sites.
-  Represents ATP binding sites.

This subunit possesses an ATP-binding site and a phosphorylation site on the cytoplasmic side, and a binding site for cardiac glycosides, such as ouabain, on the extracellular side, as shown in Figure 1.3. The cardiac glycosides inhibit the activity of the enzyme, a feature which is utilised in the determination of enzyme activity. Although to date the  $\beta$ -subunit has not been demonstrated to be involved in the functional aspect of the enzyme, its association with the  $\alpha$ -subunit appears essential for the activity of the enzyme ( Sweadner, 1989 ). Both subunits span the entire membrane ( Cantley, 1981 ).

In teleosts,  $\text{Na}^+$ - $\text{K}^+$ -ATPase activity has been shown to vary in proportion to the external salinity, with the highest level in stenohaline marine species and euryhaline fish adapted to SW ( Epstein *et al.*, 1967; Kamiya and Utida, 1968; Jampol and Epstein, 1970; Zaugg and McLain, 1970; Utida *et al.*, 1971; Forrest *et al.*, 1973; Ho and Chan, 1980 ). The reported values of  $\text{Na}^+$ - $\text{K}^+$ -ATPase activity vary and are dependent partly upon the membrane extraction procedure, subsequent purification of the preparation, and the temperature at which the biochemical assay is carried out ( Kamiya and Utida, 1968; Sargent *et al.*, 1975; Pfeiler, 1978; Busacker and Chavin, 1981 ). An increase in the level of  $\text{Na}^+$ - $\text{K}^+$ -ATPase activity was observed with transfer of euryhaline teleosts such as the killifish ( Epstein *et al.*, 1967), coho salmon, *Oncorhynchus kisutch* ( Zaugg and McLain, 1970 ), the Japanese eel ( Kamiya and Utida, 1968; Utida *et al.*, 1971; Ho and Chan, 1980 ), American eel ( Forrest *et al.*, 1973 ) European eel ( Bornancin and De Renzis, 1972 ) and *Dormitator maculatus* ( Evans and Mallery, 1975 ) from FW to SW.

#### 1.3.4 Transport mechanisms

The precise site of ion exchange in FW teleosts is not clear, although the chloride cells and the respiratory pavement cells have both been implicated ( Pisam *et al.*, 1989 ). The nature of ion exchange in FW-adapted teleosts is also unclear although the active uptake of the major ions,  $\text{Na}^+$  and

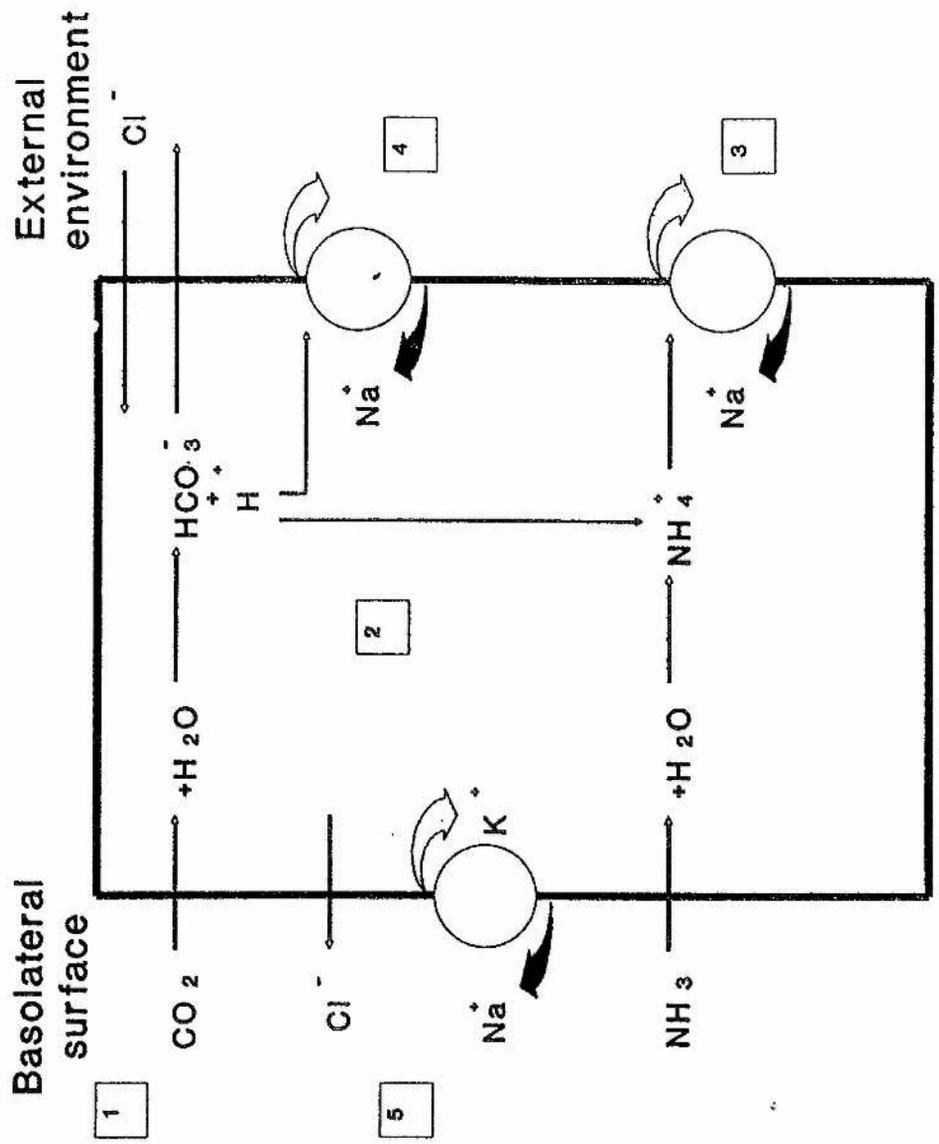
$K^+$ , have been demonstrated to be independent of each other ( Krogh, 1938 ). Ion exchange is reported to occur with  $HCO_3^-$  being exchanged for  $Cl^-$  ( Kerstetter and Kirschner, 1972 ).  $Na^+$  uptake had been linked to both  $H^+$  and  $NH_4^+$  exchange ( Wright *et al.*, 1989 ). Figure 1.4 demonstrates some of the possible routes of ion uptake in FW teleosts.

The short-circuit current technique has been utilised in the study of the mechanism of SW ion transport. This technique requires sections of epithelium, containing particular transport cells, to be laid out flat in Ussing chambers, hence the unsuitability of the complex gill tissue and the suitability of the flat opercular epithelium. The opercular skin of the killifish *Fundulus heteroclitus* consists of a stratified epithelium, composed of four major cell types, mucus cells, pavement cells, non-differentiated cells and chloride cells, with an underlying connective tissue layer ( Degan *et al.*, 1977 ). The morphology of the opercular chloride cell resembles the SW-adapted teleost gill chloride cell. The similar nature of the chloride cell of the isolated operculum to the gill chloride cell means that the opercular chloride cell can be used in the study of SW ion transport and the results extrapolated to the functions of the branchial chloride cell. In addition the opercular epithelium is not complicated by the presence of respiratory cells ( Karnaky and Kinter, 1977 ).

The conditions employed in the short-circuit current technique means that any net movement recorded across the membrane must occur by an active process. Using this technique it has been demonstrated that the efflux of  $Cl^-$  is seven times greater than influx, indicating the presence of a  $Cl^-$  secretory mechanism in the opercular membrane of the tilapia, *Sarotherdon mossambicus*, with similar responses observed in the killifish and the goby, *Gillichthys mirabilis* ( Foskett *et al.*, 1983 ). Foskett and Scheffey ( 1982 ) using the vibrating probe technique, which permits the measurement of extracellular ion density immediately above the epithelial surface, obtained

Figure 1.4

Figure 1.4 Possible mechanisms of ion uptake in FW fish gill  
CO<sub>2</sub> degradation by carbonic anhydrase provides bicarbonate ions for exchange with Cl<sup>-</sup> (1). The H<sup>+</sup> from CO<sub>2</sub> breakdown may combine with NH<sub>3</sub> ions (2) to produce NH<sub>4</sub><sup>+</sup> ions which can be exchanged with Na<sup>+</sup> (3). The alternatives are Na<sup>+</sup>-H<sup>+</sup> exchange (4) or electrogenic H<sup>+</sup> transport with passive flux of Na<sup>+</sup> (not shown). The Na<sup>+</sup> crosses the basolateral membrane via a Na<sup>+</sup>-K<sup>+</sup> exchange with accompanying passive Cl<sup>-</sup> transport.  
( From Sainsbury, 1992 )



direct evidence of the localisation of  $\text{Cl}^-$  secretion in the chloride cell of the operculum and, by inference, the gill as well. The  $\text{Cl}^-$  secretory mechanism of the goby was shown to be sensitive to ouabain, a response which is indicative of a dependence on the  $\text{Na}^+\text{-K}^+\text{-ATPase}$  ( Marshall, 1981 ), and was inhibited by the absence of  $\text{Na}^+$ , indicating the presence of a Na-Cl co-transporter ( Degan and Zadunaisky, 1980 ). The unidirectional sodium fluxes have been shown to be passive and to follow existing electrochemical gradients ( Degan and Zadunaisky, 1980 ).

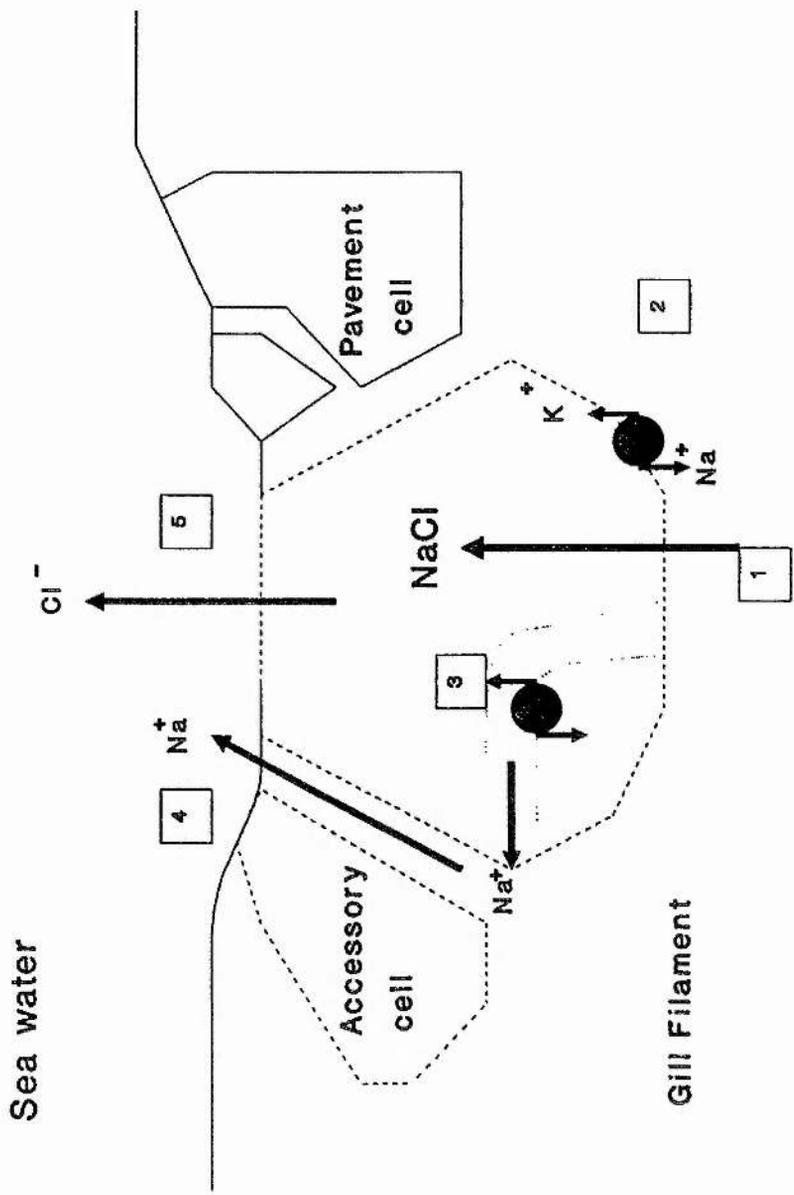
In SW eels, where  $\text{Na}^+$  efflux was measured in parallel with gill  $\text{Na}^+\text{-K}^+\text{-ATPase}$ , the rate of efflux is closely correlated with the level of enzyme activity ( Forrest *et al.*, 1973 ). Ouabain administered in the circulation of SW-adapted American eels inhibited gill  $\text{Na}^+\text{-K}^+\text{-ATPase}$  with a corresponding reduction in both  $\text{Na}^+$  and  $\text{Cl}^-$  efflux ( Silva *et al.*, 1977 ). Active transport of chloride was also suggested by the ability of thiocyanate to inhibit chloride efflux and produce hypernatremia in SW-adapted eels ( Epstein *et al.*, 1973 ). The  $\text{Cl}^-$  transport was inhibited in the killifish by the removal of  $\text{Na}^+$  ( Degan and Zadunaisky, 1980 ) and by the addition of bumetanide ( Foskett *et al.*, 1983 ), indicating the presence of a Na-Cl cotransporter. When the above results are considered in conjunction with observations from studies using vibrating probes, they point to an active  $\text{Cl}^-$  transport system coupled to  $\text{Na}^+\text{-K}^+\text{-ATPase}$ , with a neutral NaCl carrier that passively moves  $\text{Na}^+$  into the chloride cell.  $\text{Na}^+\text{-K}^+\text{-ATPase}$  will maintain low intracellular  $\text{Na}^+$ , which would, therefore, provide the driving force for coupled Na-Cl transport into the cell. It has been proposed that  $\text{Cl}^-$  leaves the cell via an apical electrogenic pump, while  $\text{Na}^+$  is recycled and eventually extruded by a paracellular mechanism ( Foskett *et al.*, 1983; Karnaky, 1986 ). Figure 1.5 shows a model for salt secretion across the chloride cell in SW-adapted teleosts.

Figure 1.5.

**Figure 1.5** Route and mechanism of salt extrusion across SW fish gill

NaCl enters the chloride cell (1) down a concentration gradient maintained by basolateral  $\text{Na}^+\text{-K}^+$  exchange (2). The  $\text{Na}^+$  is either recycled or enters the tubular system (3). From here the  $\text{Na}^+$  is extruded via a paracellular route (4). The  $\text{Cl}^-$  is removed apically by an electrogenic pump (5).

( From Sainsbury, 1992 )



#### 1.4 Teleost gut

In SW, teleosts drink large quantities of water in order to replace osmotic water losses which occur primarily across the gills. Therefore, in SW the gut is an important route of water gain. In order for the ingested seawater to be taken up from the intestinal lumen, it must first undergo desalination, which is reported to occur in the oesophagus of SW-adapted teleosts ( Hirano and Mayer-Gostan, 1976; Parmelee and Renfro, 1983 ).

The oesophagus of FW-adapted teleosts is almost impermeable to both ions and water ( Kirsch *et al* .1975 ). The oesophagus of the FW-adapted eel, *Anguilla japonica*, is lined by a stratified epithelium consisting of three main cell types, numerous mucus cells occupying the middle area of the epithelium, filament-rich cells forming the outermost and basal layers and ribosome-rich cells . The filament-rich cells, which possess many microridges arranged in a finger-like pattern, appear to serve as an effective barrier to the passive diffusion of ions and water through the FW-adapted eel oesophagus ( Yamamoto and Hirano, 1978 ).

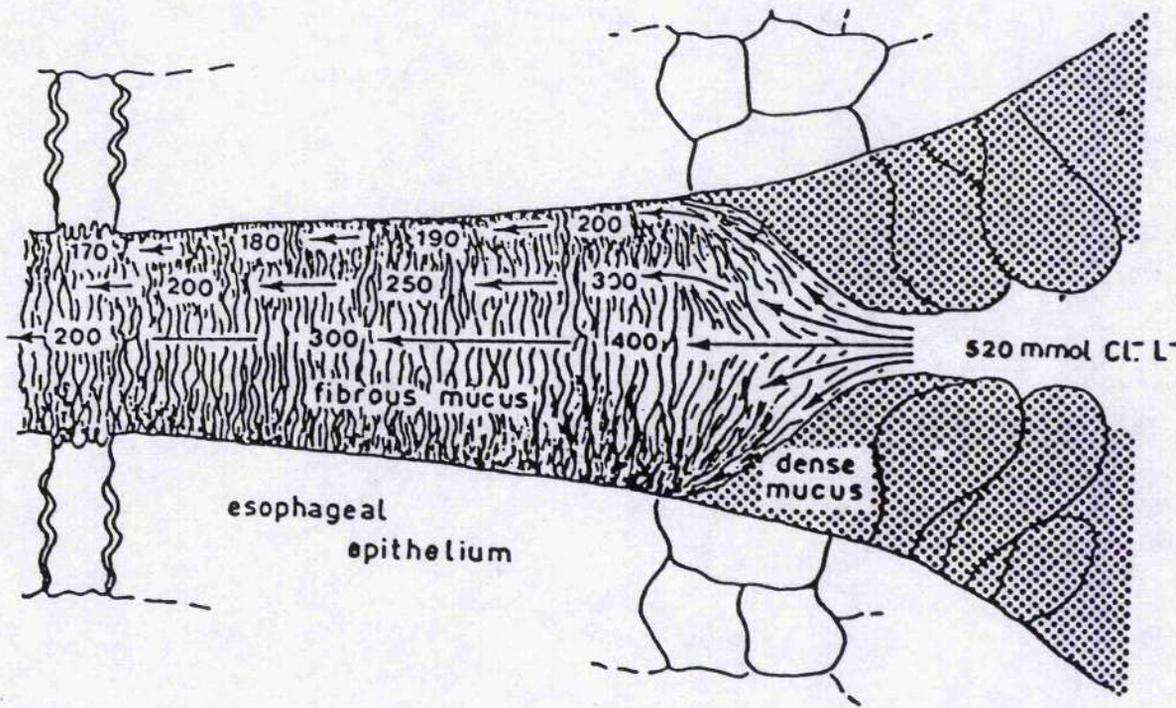
With transfer of the eel to SW a progressive change in the predominant cell type of the oesophagus occurs. The stratified FW-adapted epithelium is present at the beginning of the oesophagus, but is progressively replaced by a simple columnar epithelium relatively free from mucus cells, and highly vascularised beneath the mitochondrial-rich columnar cells. Important foldings increase the epithelial surface area ( Yamamoto and Hirano, 1978 ).

The oesophageal mucus plays an important role in the maintenance of mucosa-to-serosa net ion fluxes. The mucus forms a diffusion barrier and becomes more fibrous closer to the epithelial membrane ( Figure 1.6 ). Therefore, it is likely that physical resistance to longitudinal water flow increases in the mucus layer from lumen to cell contact, and ions move down the concentration gradient towards the epithelium and are absorbed ( Kirsch

Figure 1.6

Figure 1.6 Model of water and ion distribution in the oesophageal mucus

Numbers refer to local concentrations in Cl<sup>-</sup> ions ( mmol/l):  
arrows have a length proportional to the local water flow.  
( From Kirsch, 1978 )



*et al*, 1975; Hirano and Mayer-Gostan, 1976 ). If the thickness of the mucus layer decreases from the beginning to the end of the oesophagus, then SW could flow down the oesophagus through the mucus layer at different speeds and thus allow a progressive desalination to occur ( Kirsch *et al*,1983 ).

As already stated the oesophagus of the FW-adapted eel is almost impermeable to both  $\text{Na}^+$  and  $\text{Cl}^-$  ions and to water ( Hirano and Mayer-Gostan, 1976 ). A distinctive characteristic of the SW-adapted teleost oesophagus is its selective permeability to Na and Cl independent of water permeability. When the isolated oesophagus from the SW-adapted eel was filled with SW and incubated in Ringer solution, a large quantity of  $\text{Na}^+$  and  $\text{Cl}^-$  ions moved out of the lumen, with negligible water movement ( Hirano and Mayer-Gostan, 1976). The oesophageal osmoregulatory function of the sculpin was assessed by *in vivo* perfusion experiments ( Sleet and Weber, 1982 ) and in agreement with the *in vitro* studies in the FW fish, the oesophagus was impermeable to water and ions, and had very low serosa-to-mucosa net ion fluxes, which may be related to mucus secretions. In the SW fish as a result of changes in Na and Cl permeability there was a rapid absorption down the electrochemical gradient between the lumen and serosa of approximately 50 - 70% of the ions ingested with SW.

The stomach has little role to play in osmoregulation, although, it was previously considered to play a part in the dilution of the lumen fluid as a result of gastric acid secretion ( Holstein, 1979a, b ). The stomach receives water that has already undergone partial desalination. In the isolated eel stomach ( Hirano and Mayer-Gostan, 1976 ) there was no movement of Na or water, whereas some secretion of  $\text{Cl}^-$  ion coupled to  $\text{H}^+$  ion secretion into the lumen occurred. No difference in this secretion was observed between the FW- and SW-adapted eel.

The intestine, which receives partially desalinated water, plays an active part in gut osmoregulation. Histological studies on the epithelial

lining structure of the SW-adapted goby, *Gillichthys mirabilis*, revealed numerous folds consisting of three cell types, absorptive cells, mucus cells and basal cells (Loretz, 1983a). The columnar cells which are rich in mitochondria appear to have greater conductance of chloride than sodium ions across the intestinal apical membrane of the goby. The number of mucosal cells in the intestine of the American eel increased with transfer from FW to SW and as a consequence the weight of intestinal mucus increased 32% during the adaptation (MacKay and Janicki, 1979).

Isolated intestines from SW-adapted cultured Japanese eels showed active primary transport of sodium and passive water transport from mucosa to serosa, with the molar ratio of water to sodium lost from the intestinal sac greater than in FW-adapted eels (Oide and Utida, 1967). Skadhauge (1969) and Hirano (1967) reported a similar augmentation in intestinal absorption of water and NaCl with transfer of the eel from FW to SW. Using phenol red as an indicator of water ingestion and intestinal absorption in the Japanese eel, Oide and Utida (1968) demonstrated that 60 - 75% of SW ingested is absorbed in the intestine. In the posterior intestine sodium and chloride are actively reabsorbed and  $\text{CaCO}_3$  precipitates out of solution, thereby creating osmotically "free water", which can be reabsorbed.

$\text{Na}^+\text{-K}^+\text{-ATPase}$  in the gut was seen to increase with transfer of the American eel from FW to SW (Jampol and Epstein, 1970). The increased water uptake in the SW-adapted eel intestine was observed to be ouabain sensitive, indicating a link with  $\text{Na}^+\text{-K}^+\text{-ATPase}$  (Huang and Chen, 1971). The SW eel intestine also possesses a  $\text{Na}^+\text{-K}^+\text{-Cl}^-$  cotransport system, a  $\text{K}^+$  leak system and a  $\text{Cl}^-$  leak system (Ando, 1992). During  $\text{Na-K-Cl}$  cotransport there is active transport of NaCl to the serosal side via  $\text{Na}^+\text{-K}^+\text{-ATPase}$  and apical  $\text{Cl}^-$  channels respectively while  $\text{K}^+$  is passively transported across the luminal membrane via a " $\text{K}^+$  leak system" (Ando, 1981; Ando, 1985). This creates a local osmotic gradient for passive water reabsorption. The flounder

intestine also appears to possess the  $\text{Na}^+\text{-K}^+\text{-Cl}^-$  cotransport system, which seems to be a characteristic feature of salt absorbing epithelia ( Musch *et al*, 1982 ). A coupling of  $\text{Na}^+$  transport with amino acid and sugar transport is also observed ( Collie and Hirano, 1987 ). Figure 1.7 shows the possible transport pathways in fish intestine.

### 1.5 Kidney and urinary bladder

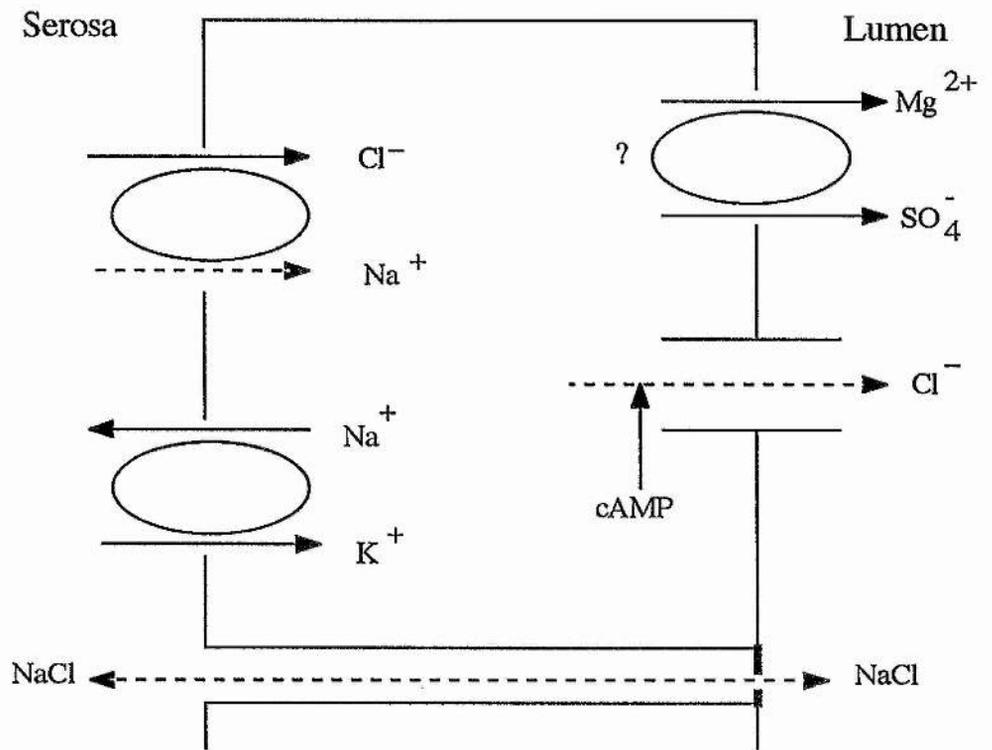
Vertebrate kidneys function on the filtration-reabsorption principle, with the addition of tubular secretion. A few teleostean species are found to differ from this general pattern, in that they lack the ultrafiltration mechanism and depend entirely upon a secretory-type of kidney.

All vertebrate kidneys consist of units, called nephrons, the number involved depending on species and size. The first section of the nephron is the Bowman's capsule where ultrafiltration of the blood plasma occurs. Each Bowman's capsule is fed by a small artery which divides into a tuft of capillaries, the glomerulus. Together, the Bowman's capsule and glomerulus constitute a Malpighian or renal corpuscle. Fluid, termed the glomerular filtrate, is forced out through the walls of the glomerular capillaries into the tubule which leads from the Bowman's capsule. Within the tubule the fluid is modified, both by tubular reabsorption and secretion, to form the final urine. This tubule can be generally divided into two parts: the proximal tubule in which water and many solutes, e.g. salts and glucose are reabsorbed, and a distal tubule that continues the process of changing the tubular fluid into urine. The distal tubules join to form collecting ducts, and these empty the urine into the renal pelvis. From here, the urine passes through the ureters to the urinary bladder, from which, at intervals, it is discharged to the exterior ( Schmidt-Neilson, 1986 )

The mammalian nephron consists of proximal and distal segments separated by a characteristic thin loop-forming segment, known as Henle's

Figure 1.7

Figure 1.7 Possible mechanisms of ion transport in the teleost intestine  
( Adapted from Trischitta *et al.*, 1992 )



loop. A similar structure, though not as well developed, is found in the bird kidney. This loop permits the formation of urine more concentrated than blood plasma. The loop is missing in almost all fish ( Rankin and Davenport, 1981 ), amphibians, and reptiles, which are unable to produce urine of a higher concentration than the blood plasma ( Schmidt-Neilson, 1986 ).

A key measurement of renal function is the glomerular filtration rate ( GFR ), which may be determined by the clearance of a marker molecule, such as [<sup>3</sup>H] inulin, in the urine, after injection of the substance into the fish. Similarly the single nephron glomerular filtration rate ( SNGFR ) may be determined by micropuncture of an individual nephron.

The teleost kidney will now be discussed in greater detail.

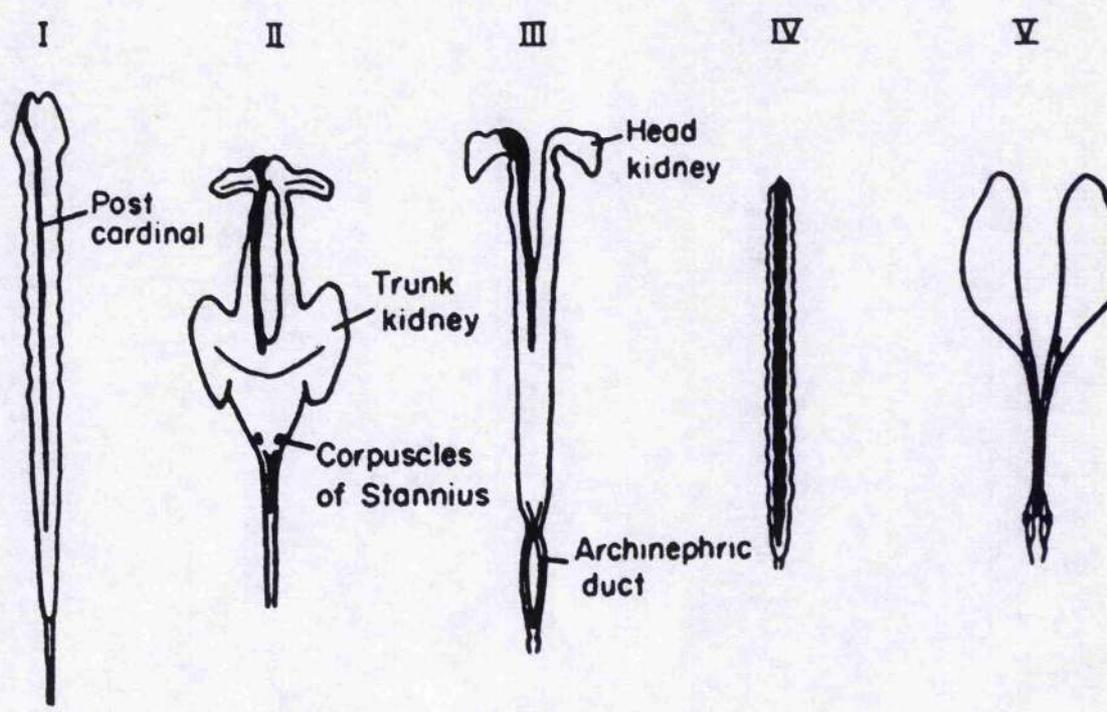
#### 1.5.1 Teleost kidney

The teleost kidney is usually divided into two portions, the head kidney and the caudal kidney. The head kidney generally consists of lymphoid, hematopoietic, interrenal, and chromaffin ( suprarenal ) tissue. The amount of hematopoietic and pigment cells distributed among the tubules and vascular spaces in the caudal kidney is variable. The corpuscles of Stannius are normally located on the dorsal surface from the middle to the posterior region of the kidney. Fusion of the two archinephric ducts may occur at the posterior end of the kidney or between the kidney and urinary papilla. Dilations of the archinephric duct may form a urinary bladder, where storage and modification of the urine occurs ( Hickman and Trump, 1969 ).

The teleostean kidney can be divided into five configurational classes as shown in Figure 1.8. depending on the degree of fusion between the two kidneys and the distinction between caudal and head kidney ( Hickman and Trump, 1969 ), (I) the sides of the two kidneys are totally fused with no clear distinction between body and head kidney, eg herrings; (II) only the anterior parts of the two kidneys are not fused, and there is a clear distinction between

Figure 1.8

Figure 1.8 Five configurational types of marine teleostean kidneys  
( From Hickman and Trump, 1969 )



caudal and head kidney, eg eels; (III) only the posterior parts are fused, with the anterior portion represented by two slender branches and there is a clear distinction between the caudal and head kidney, eg mullets and flounders; (IV) only the extreme posterior portion is fused and the head kidney is unrecognisable, eg pipefish and; (V) complete separation of the two kidneys, eg anglerfish.

There is a tendency for the kidney of the euryhaline fish to resemble that of stenohaline fish of the primary environment, e.g. the nephrons of the European and American eels are grouped with the typical FW teleosts ( Grafflin, 1937 ).

This section will primarily describe the morphological and functional adaptability of the teleost kidney with respect to the transfer of fish from FW to SW.

#### 1.5.1a Morphology

The structure of a typical FW teleost nephron is a renal corpuscle, containing a well-vascularised glomerulus, linked by a ciliated neck segment to an initial proximal segment followed by a second proximal segment, both possessing a variable brush border, intermediate and distal segments and a collecting duct system. A typical marine teleost nephron consists of a glomerulus-containing renal corpuscle, a neck segment of variable length, two or three proximal segments, and a collecting tubule and collecting duct system. An intermediate segment is sometimes present between proximal segment 1 and 2.

Using scanning electron microscopy ( SEM ) and transmission electron microscopy ( TEM ) the epithelial ultrastructure of the glomerulus of the FW-adapted rainbow trout, *Onchorynchus mykiss* ( = *Salmo gairdneri*, ) is seen to comply with the general vertebrate pattern ( Brown *et al.*, 1983 ). Large, rounded podocytes with processes terminating in interdigitating pedicles and

few cytoplasmic microprojections, were present. Slit pores lay between the interdigitating pedicles. After transfer to SW for at least three weeks, the podocytes were considerably flattened and close together, and, when visible, the pedicles were usually broader with little visible interdigitation. There appeared to be a reduction in the number of pedicles and slit pores and an increase in the number of cytoplasmic microprojections.

The size of the glomerulus of the European eel, *Anguilla anguilla*, decreased 10 days after transfer from FW to SW ( Olivereau and Olivereau, 1977 ). Modification of glomerular size after FW to SW transfer of the trout seemed to take longer to achieve, with little change in size of the glomeruli after nine weeks in SW compared to the FW-adapted trout, however, there was a 40% reduction in size after one year in SW ( Brown *et al.*, 1983 ).

In the eel the brush borders of the proximal tubules became thinner after transfer to SW. The distal and collecting tubules of FW-adapted fish had a highly folded basal plasmalemma, which was less evident in SW ( Olivereau and Olivereau, 1977 ). Therefore, it is clear, that, morphological changes occur in the kidney of teleosts during adaptation from a hyposmotic to a hyperosmotic environment.

The kidneys of some teleosts are aglomerular and work on a secretory principle. The marine aglomerular nephron of the midshipman, *Porichthys notatus*, and the toadfish, *Opsanus tau*, consist of an initial segment with brush border and a terminal collecting duct system ( Bulger, 1965 ; Bulger and Trump, 1965 ).

#### 1.5.1b Function

Utilising marker molecules, such as [<sup>3</sup>H]methoxy inulin and [<sup>14</sup>C]polyethylene glycol, originally designed for the measurement of GFRs in mammalian studies, SW-adapted fish are found to have lower GFR and urine flow compared to FW-adapted animals ( Brown *et al.*, 1978 : Brown *et*

*al.*, 1980 : Chester-Jones, *et al.*, 1969; Sharratt *et al.*, 1964 a,b ). There may be some instances where urine production rates are changed by regulation of tubular water reabsorption rather than by GFR ( Schmidt-Neilson and Renfro, 1975; Oide and Utida, 1968 ). The methods used for determining GFR may, however, result in an underestimation of the GFR as it appears the marker molecules can penetrate the bladder and possibly the ureters and renal tubules ( Beyenbach and Kirschner, 1976 ).

SNGFRs were higher in SW-adapted trout compared to FW-adapted trout ( Brown *et al.*, 1978 ). Due to the inaccessibility of rainbow trout nephrons for micropuncture an indirect technique was used in this study.  $^{14}\text{C}$ -ferrocyanide was infused into the animal as the renal clearance of this marker was found to be indicative of glomerular filtration. As previously mentioned, the GFR in the FW-adapted animal is higher than that in SW-adapted fish. Therefore, whilst SNGFR of SW animals are higher than those in FW, it may be that filtration is distributed to nephron populations selected to meet the homeostatic demands of the fish.

Three population types of nephron have been identified based on perfusion patterns; filtering ( F ), non-filtering but perfused ( NF ), and non-perfused ( NP ) ( Henderson and Brown, 1980; Brown *et al.*, 1980 ). The proportion of each type of nephron in FW-adapted and SW-adapted rainbow trout ( Brown *et al.*, 1980 ) was as follows:

F = FW 45% - SW 5%

NF = FW 42% - SW 44%

NP = FW 12% - SW 50%

The higher SNGFRs in the filtering glomeruli of SW trout ( Brown *et al.*, 1978 ) may result from blood flow being directed towards certain populations of nephrons.

The renal tubular transport maxima for glucose (TMG), i.e. the glucose reabsorptive capacity, decreased with transfer from FW to SW ( Hickman,

1968 ). The TMG in SW-adapted teleosts was about one tenth that of FW kidneys and the renal blood flow approximately half ( Jackson *et al*, 1977 ). This decrease in TMG was considered to reflect a reduction in the number of filtering nephrons observed after SW transfer.

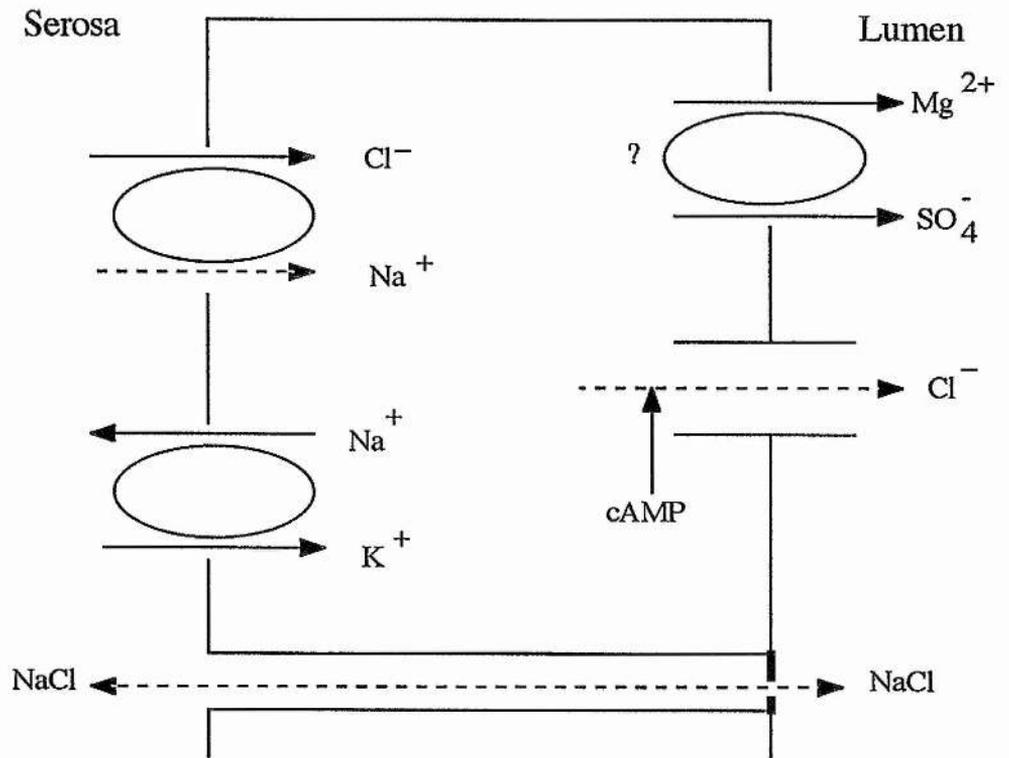
Both proximal tubules participate in the reabsorption of  $\text{Na}^+$  and  $\text{Cl}^-$  with a simultaneous secretion of divalent ions (  $\text{Mg}^{2+}$ ,  $\text{SO}_4^{2-}$  ) occurring. Reabsorption of  $\text{Na}^+$  and  $\text{Cl}^-$  was reduced in SW ( Olivereau and Olivereau, 1977 ). A NaCl-linked fluid secretion in proximal tubules has been reported in a diverse group of marine fish, the dogfish shark, the winter flounder and the SW-adapted euryhaline killifish ( Beyenbach, 1986; Beyenbach, 1982 ) and also in the FW-adapted killifish, albeit in a low number of tubules ( Cliff and Beyenbach, 1988 ). Tubular secretion is used by aglomerular fish, such as the marine toadfish, *Opsanus tau*, to produce urine. In the winter flounder, it was reported that fluid secretion in the proximal tubules was driven by both  $\text{MgCl}_2$  and NaCl, with NaCl secretion quantitatively more important and providing the basal fluid secretion rate ( Beyenbach *et al.*, 1986; Cliff *et al.*, 1986a ). The fluid-secreting proximal tubule has a leaky epithelia, which apparently arises from the paracellular pathway, which is permeable to both  $\text{Na}^+$  and  $\text{Cl}^-$ , with slightly more permaselectivity to  $\text{Na}^+$  ( Beyenbach *et al*, 1986 ). Cyclic adenosine monophosphate ( cAMP ) stimulated fluid and Na and Cl secretion. In the FW fish this secretion of fluid and solutes may initially appear surprising, however, this may form an additional renal mechanism for the production of a dilute urine ( Cliff and Beyenbach, 1988 ). Figure 1.9 shows the possible transport pathways for the secretion of  $\text{Na}^+$  and  $\text{Cl}^-$  in the shark renal tubule modified from Beyenbach ( 1986 ), which is postulated to be similar for the SW-adapted teleost.

In the kidney of FW teleosts  $\text{Na}^+$ - $\text{K}^+$ -ATPase is present for the conservation of sodium and the enzyme activity was reported to be higher for marine teleosts compared to FW-adapted fish ( Jampol and Epstein, 1970 ),

Figure 1.9

Figure 1.9 Postulated mechanisms of NaCl secretion in SW teleost proximal tubule as modified from mechanism of NaCl secretion in shark renal proximal tubule ( Beyenbach, 1986 ).

Solid arrows, movement against the electrochemical gradient;  
broken arrows, movement down electrochemical gradient.



however, activity was seen to increase with the transfer of *Platichthys stellatus* from SW to FW ( Utida *et al*, 1974 ).

### 1.5.2 Teleost urinary bladder

The urinary bladder of terrestrial vertebrates is derived from the rectum, but that of fishes is of the same embryological origin as the kidney. The urinary bladder modifies urine and has a role to play in teleost osmoregulation.

The urinary bladder of fishes is a thin walled sac, and is the result of the fusion and dilation of the two archinephric ducts of the kidney ( Hickman and Trump, 1969 ). Three layers are distinguishable in the teleost bladder: (a) an inner layer lined by one or two regionally segmented cell types, mitochondrial-rich columnar cells and cuboidal cells; (b) a thin middle layer consisting of connective tissue called the tunica propria, and containing some smooth muscle fibers; (c) a thin outer layer composed of connective tissue.

The inner layer of the urinary bladder of the euryhaline goby, *Gillichthys mirabilis*, is lined with both columnar and cuboidal cells, maintained in distinct regions ( Loretz and Bern, 1980 ). In 5 % SW-acclimated fish and full SW-adapted fish active reabsorption of Na and Cl from the lumen occurs across the columnar cell region via a neutral cotransport mechanism. In full SW-adapted goby bladders there is also present in addition an electrogenic Na reabsorption component. Cuboidal cell regions from both 5%- and full SW-acclimated fish produced little mucosal to serosal NaCl transport ( Loretz and Bern, 1980 ).

Generally, urinary bladders from SW-adapted fish have higher water absorption and osmotic permeability and lower or equal NaCl absorption rates, compared to those in FW ( Hirano *et al*, 1973; Johnson *et al*, 1972; Doneen, 1976; Utida *et al*, 1974 ). Marine fish appear to have a more variable rate of water movement and osmotic permeability, which may be related to

the degree of development of the bladder and the efficiency of the kidney tubular function ( Hirano *et al*, 1973 ). Bladders of stenohaline FW fish, such as the carp ( *Cyprinus carpio* ), Japanese catfish ( *Parasilurus asotus* ) and white catfish ( *Ictalurus catus* ), are relatively impermeable to water ( Hirano *et al.*, 1973 ). This impermeability may be necessary to prevent osmotic water inflow from the dilute urine.

FW-adapted *Gillichthys mirabilis* had lower water transport and osmotic permeability compared to SW-adapted fish ( Doneen, 1976 ) and three species of euryhaline flounder ( *Paralichthys olivaceus*, *Platichthys stellatus* and *Kareius bicoloratus* ) demonstrated a reduced osmotic permeability to water and increased permeability to sodium and chloride ions on adaptation to hypotonic media ( Hirano *et al.*, 1973 ).

It seems that the origin of the euryhaline fish may have some effect on the ability of the urinary bladder to modify its permeability when adapting to an osmotically different environment. Bladders of euryhaline fish of FW origin, such as trout and *Tilapia*, appear relatively impermeable to water irrespective of environmental salinity, while those of SW origin, such as the flounders and *Gillichthys*, may change from permeable in SW to relatively impermeable in FW ( Hirano *et al.*, 1973 ).

The urinary bladder actively takes up  $\text{Na}^+$  and  $\text{Cl}^-$  ( Loretz and Bern, 1980 ) with the apparent involvement of a ouabain sensitive,  $\text{Cl}^-$ - $\text{Na}^+$ - $\text{K}^+$  cotransport system ( Renfro, 1975 ).  $\text{Na}^+$ - $\text{K}^+$ -ATPase is present in the urinary bladder. FW-adapted fish were found to have a higher activity compared to SW fish ( Fossat *et al.*, 1974 ) which may be indicative of active  $\text{Na}^+$  and  $\text{Cl}^-$  absorption from the urine.

## 1.6 Hormones

The effectiveness of any biological homeostatic mechanism depends upon a constant supply of information concerning the state of a particular controlled variable. This information may be conveyed by the nervous or endocrine systems. These systems are intimately involved in the overall regulation of homeostasis. In addition, the nervous system is itself affected by hormones and, conversely, endocrine glands are partly dependent on nervous control ( Baulieu, 1990 ).

Classically endocrine glands are ductless organs or tissues, located throughout the body, which secrete a variety of chemically distinct hormones. A hormone may be defined as a signal molecule secreted into the internal milieu, most frequently, into the blood perfusing the gland. The internal milieu is considered to be the extracellular fluid which bathes all the tissues and provides the medium for communication and exchange between the cells and external environment. The blood delivers hormones to target cells, where they exert their physiological effect. The receptor is the chemical structure required by the target cell to recognise and receive the hormone ( Baulieu, 1990 ).

In addition to their transport throughout the body to target cells, by the vascular system, some hormones act locally, influencing the activity of neighbouring cells, a phenomenon defined as a paracrine effect. Examples include neurotransmitters, neurohormones, chemical agents of the immune system such as interferons and interleukins, and nitrous oxide, the endothelium-derived relaxation factor ( Änggård, 1990 ). If the cell itself utilises the hormone it produces ( and naturally also provides the hormone to identical neighbouring cells ) the hormone is termed autocrine. Cultured cells often respond to growth factors they secrete and many tumour cells over-produce and release growth factors that stimulate inappropriate and unregulated growth of the tumour itself.

Hormones can be broadly classified on the basis of their chemical nature, as lipophilic or hydrophilic hormones, the former being predominantly soluble in lipid and the latter predominantly soluble in water. Lipophilic hormones include the steroid and thyroid hormones, whereas hydrophilic hormones include large polypeptides such as insulin, small peptides such as angiotensin II ( AII ) and small charged molecules such as adrenaline.

In general, vertebrate endocrine glands show anatomical and embryological homologies, but there may be considerable diversity in their morphological arrangements, secretions and apparent physiological roles. Some endocrine glands, such as the adrenomedullary homologue, are ubiquitous among vertebrates, secreting chemically similar hormones with comparable actions. Other endocrine glands are not universally present. The parathyroid glands are observed only in tetrapods, and the caudal neurosecretory system appears to be present only in teleosts and elasmobranchs while cyclostomes lack ultimobranchial bodies. Some endocrine glands, the adenohypophysis for example, although ubiquitous among vertebrates, may produce individual hormones which have different functions in various groups.

The differences in physiological function and sites of action observed with some hormones are often associated with a change in the chemical structure of the hormone. There is considerable species variation in the amino acid substitution in pituitary peptide hormones, prolactin for example ( Section 1.8.2a ). Similarly, the neurohypophysial hormones ( Section 1.8.4 ) and angiotensins ( Section 1.10.1 ) vary in amino acid substitutions from group to group.



hormone, and binding sites with high affinity. Receptor binding has to be specific both in terms that it can be blocked by addition of unlabelled hormone and that it cannot be prevented by the addition of any hormone not known to interact with the particular receptor.

Hormone-receptor interaction may be investigated by radioreceptor assays or tissue section autoradiography. One of the aims of carrying out binding studies is to determine both affinity and abundance of specific sites in a tissue or membrane, that is, the steady state kinetics of the binding process.

Radioreceptor assays measure displacement of the specific binding of tracer amounts of labelled hormone from receptor sites by unlabelled hormone, during an incubation period long enough to establish equilibrium binding. This type of assay was first introduced by Lin and Goodfriend ( 1970 ) and Lefkowitz *et al.* ( 1970 ) to determine the specific binding of  $^{125}\text{I}$ -angiotensin and  $^{125}\text{I}$ -ACTH to appropriate target tissues. A number of features of specific binding are similar for different hormones. The rate and extent of binding is elevated with an increase in either labelled hormone or receptors. Maximum binding is generally observed between 25 - 37 $^{\circ}\text{C}$ , with the optimum pH usually found to be 7.0 - 7.4 ( Posner, 1975 ).

Autoradiographic studies provide information on the location of hormone - receptor binding. To localise hormone receptors, fixed tissue slices are incubated with labelled hormone, the slices are washed, coated with a thin layer of photographic emulsion and developed. The developed grains appear as silver grey dots in the light microscope. It is assumed that the localisation of the hormone defines the receptor site ( Salih *et al*, 1979 ).

The major endocrine systems implicated in fish osmoregulation will now be considered in terms of the morphological changes and alterations in secretions of hormones that occur with transfer from FW to SW.

## 1.8 Pituitary gland

In all vertebrates, the pituitary gland consists of two parts, the adenohypophysis ( AHP ) and the neurohypophysis ( NHP ). Both portions are derived from embryonic ectoderm, the AHP as an upgrowth from Rathke's pouch, a dorsal outpushing of buccal ectoderm and the NHP as a downgrowth of neural ectoderm ( the infundibular sac ) from the floor of the diencephalon ( Batten and Ingleton, 1987 ). The two parts form a discrete and composite organ which has many different endocrine functions ( Ball and Baker, 1969 ). Figure 1.10 show schematic diagrams of the anatomical relationship between the AHP and the NHP of different vertebrate classes. The AHP and NHP will be considered separately in the following sections, along with the hormones they secrete which are considered to have an osmoregulatory function.

### 1.8.1 Structure of adenohypophysis

The AHP is essentially an aggregation of epithelial, peptide-secreting cells, which can usually be further divided into characteristic regions: the pars distalis ( PD ), pars intermedia ( PI ), and pars tuberalis ( PT ) ( Figure 1.10 ).

In the majority of mammals the AHP cells occur in cords or small groups and in close proximity to blood vessels. Birds are the only class of vertebrate in which the AHP is without a PI , but well-developed PT and PD are present. The PD appears as two distinct regions, the rostral or cephalic lobe and the caudal lobe ( Batten and Ingleton, 1987 ).

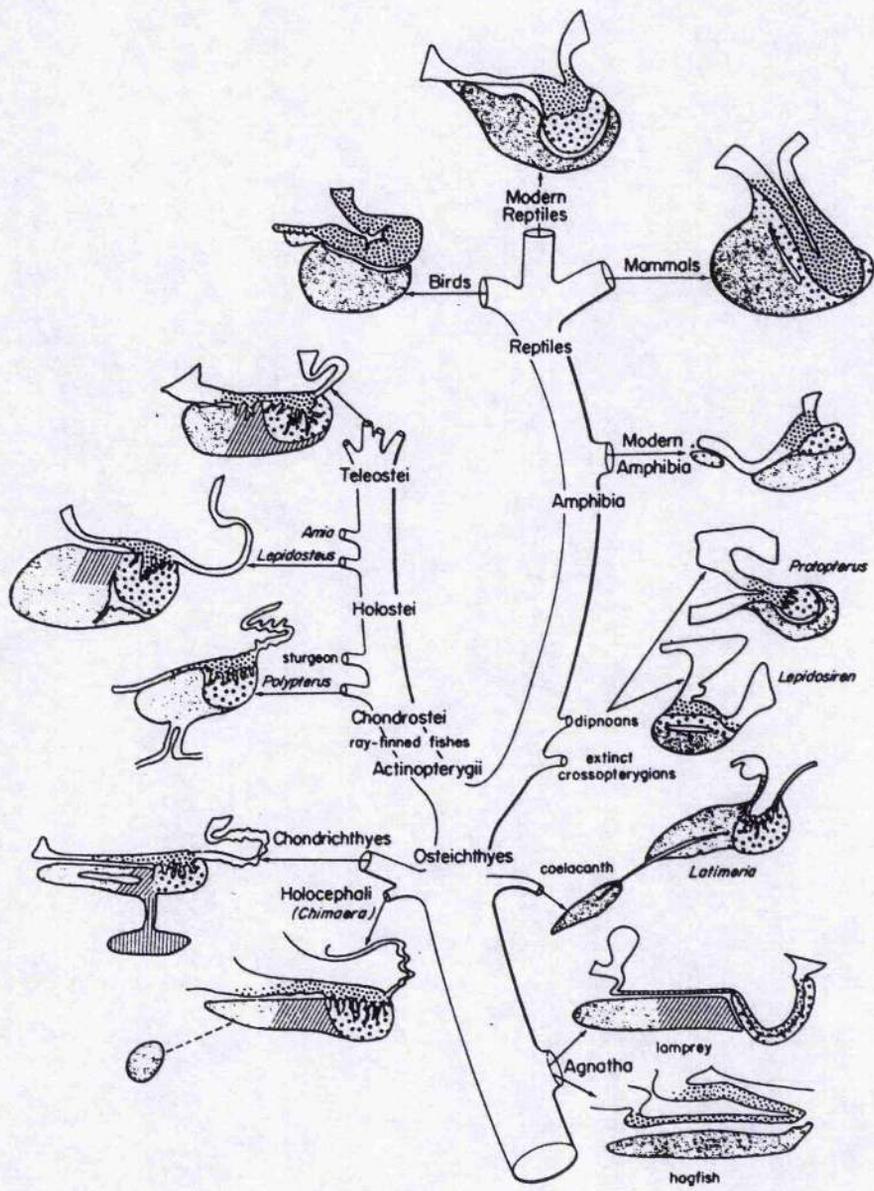
The AHP is well evolved in reptiles, and it is composed of all three regions, PD, PI and PT. The PD is also composed of a cephalic and caudal lobe, as found in birds. The PT is found in varying degrees of development depending upon the species ( Norris, 1985 ). A unique feature of the reptilian AHP is the total lack of innervation of the PI. The vascular system between

Figure 1.10

**Figure 1.10** Phylogenetic tree of vertebrate groups with superimposed diagrams of sagittal sections of the "typical" pituitary gland in each group.

The parts of the pituitary are shaded to show equivalence of structures; fine stippling and cross-hatching, zones of the pars distalis; open circles, pars intermedia; plus signs, pars tuberalis; coarse dots, neurohypophysis.

( From Gorbman, 1983 )



the pars nervosa of the NHP and the PI is continuous, so the control of the PI cells must be via this neurovascular link ( Batten and Ingleton, 1987 ).

The AHP of amphibians consists of PT, PI and PD. The PI has a poor vascular supply, but it is directly innervated by aminergic neurons thought to originate in the hypothalamus. The PD is not separable into discrete regions although there is a tendency for some regionalisation of cellular types ( Norris, 1985 ).

All fish, except the hagfish, possess a differentiated AHP. There are differences between fish and other vertebrates with respect to the morphology of the AHP. The PT is absent in all classes of fish, although there is perhaps a homologous structure in the elasmobranchs, the pars ventralis, which is formed from lateral outgrowths of Rathke's pouch and is generally attached to the PD by a short stalk. A blood supply is shared between the PD and the median eminence of the NHP. The PD is differentiated into two zones, the rostral and proximal pars distalis. The PI is intimately connected with the pars nervosa of the NHP to form a neurointermediate lobe, a structure common to all fish apart from the hagfishes and the lungfishes. Posterior to this lobe, in elasmobranchs and most bony fishes, is a unique structure, called the saccus vasculosus. This structure is formed from the floor of the diencephalon ( Norris, 1985; Batten and Ingleton, 1987 ).

### 1.8.2 Adenohypophysial secretions

The AHP hormones are divided into three categories: (1) the growth hormone/prolactin family ( Wallis, 1992 ) which includes prolactin ( PRL ), growth hormone ( GH ) and the recently discovered somatolactin ( SL ); (2) the glycoprotein hormones, thyrotropin ( TSH ) and gonadotropin. of which there are two distinct forms in most vertebrates, follicle stimulating hormone ( FSH ) and luteinising hormone ( LH ) and (3) the corticomelanotropins,  $\alpha$ -

and  $\beta$ -melanotropin (MSH), adrenocorticotropin (ACTH) and  $\beta$ -lipotropin (LPH).

The secretion of GH, PRL, TSH, FSH and LH occurs from distinct cells in the pars distalis, whereas ACTH is secreted by both the pars distalis and the pars intermedia. Alpha-MSH,  $\beta$ -MSH and  $\alpha$ -LPH are produced by cells of the pars intermedia by cleavage of the large precursor molecule, pro-opiomelanocortin. Somatolactin is synthesised in the PI (Rand-Weaver *et al*, 1991)

Of the hormones secreted by the AHP, only PRL has been extensively reported as having a role in osmoregulation, although there is some data suggesting an osmoregulatory function for GH. Prolactin and GH are thought to have evolved from a common ancestral origin by gene duplication and subsequent divergence (Kawauchi, 1992). The biological function of SL is as yet unknown. There is little evidence for any direct osmoregulatory effects for any of the other AHP hormones (Brown and Brown, 1987). Although PRL, GH and SL belong to the same family of proteins they will be discussed individually in the following sections.

#### 1.8.2a Prolactin

Prolactin is a protein hormone released by the PD. Prolactin is extremely versatile and is reported to have at least 82 different biological functions in vertebrates, involving reproduction, osmoregulation, growth and metabolism (Hirano, 1986). The effects of PRL are numerous and include a role in mammary gland development and function. The secretory action of the mammary gland is also dependant upon the actions of PRL in synergism with corticosteroids to initiate, maintain and augment milk secretion (Nicoll and Bern, 1968). Prolactin also has a role in the production of crop "milk" in a variety of bird species (Nicoll and Bern, 1968) and restores the secretion of the salt gland in the hypophysectomised duck.

Administration of PRL to adult male lizard, *Anolis carolinensis*, caused growth with seasonal variation, but its effect was greater in the winter than in the spring. Prolactin does, however, exert a distinct somatotropic ( nonfat growth ) action regardless of season ( Licht and Jones, 1967 ). Injection of PRL induces "water-drive" in the amphibian salamanders, which normally return to water for reproductive purposes during their life cycle.

#### 1.8.2ai Prolactin in fish

The effects of PRL on teleost osmoregulation are manifold and PRL has been observed to act on osmoregulatory surfaces, including the gills, gut, kidney and urinary bladder ( Hirano, 1986 ). A recent study by Arakawa *et al.* ( 1992 ) examined the occurrence and development of PRL in the Japanese eel using leptocephali, glass eels and sexually, immature, cultured eels. Prolactin cells, detected in the pituitary of the leptocephali, decreased as the fish grew larger, although Oriyasa *et al.*( 1992 ) found no significant changes in the number of PRL-ir cells in the leptocephali. Oriyasa *et al.* ( 1992 ) reported an apparent increase in the staining intensity and cells sizes for PRL-ir cells during growth, which they suggested was indicative of storage rather than active secretion of PRL by these cells, until metamorphosis to the glass eel stage. The percentage of PRL cells in glass eels and cultured eels was similar, and twice that in the leptocephalus ( Arakawa *et al.*, 1992 ). The increase of PRL cells in the glass eel appears to be preparation for FW adaptation or migration to rivers.

The majority of experiments on the actions of PRL in teleosts have utilised heterologous PRL, although homologous studies may now be undertaken with the recent isolation and sequencing of some teleost PRLs. Teleostean PRLs have 60 - 80 % sequence identity with each other and 20 - 30 % with mammalian PRLs ( Figure 1.11 ). Prolactins isolated from teleosts differ from mammalian PRL in that they are missing one disulphide loop

Figure 1.11

Figure 1.11 Alignment of the partial amino acid sequences of ePRL with the corresponding N-termini of PRLs from rat, carp and chum salmon

( Modified from Suzuki et al, 1991c )

rat

V G L N D L L E R A S E L S D K L H S L S T S L T N D L D S H F P P V G R V M M P R P S M C H T S S L Q V P N D K

10

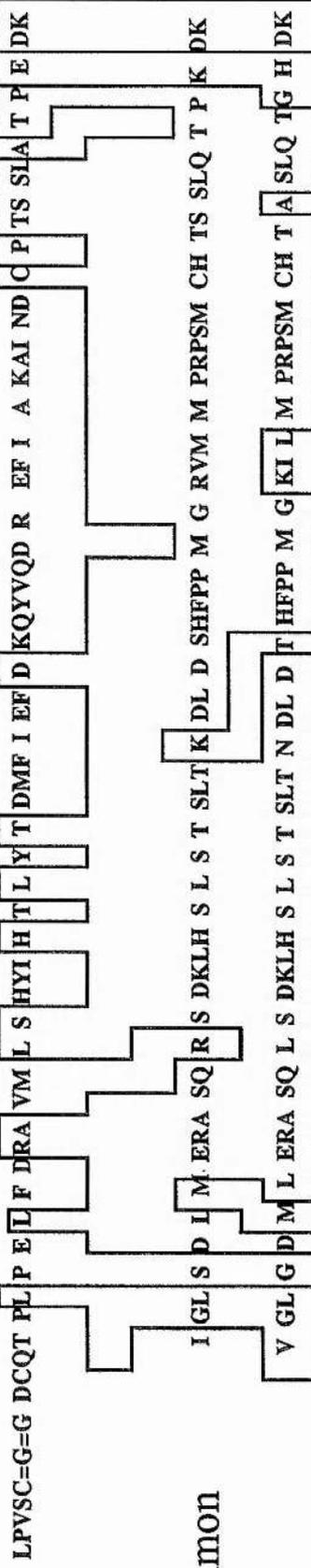
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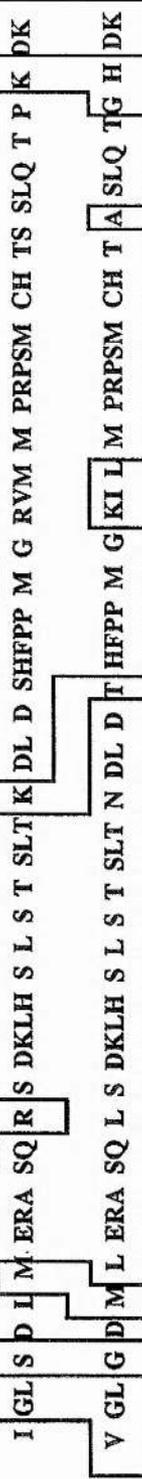
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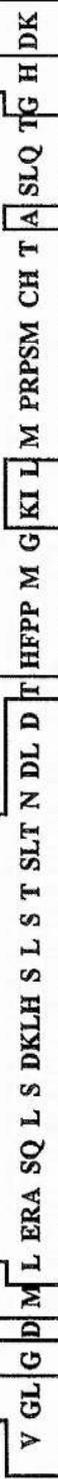
carp



chum salmon



eel



from the N-terminal region ( Suzuki *et al.*, 1991c ). Two highly homologous forms of PRLs have been found in salmon ( Yasuda *et al.*, 1986 ), tilapia ( Specker *et al.*, 1985 ) and eel ( Suzuki *et al.*, 1991c ). One cell type of the rostral lobe was demonstrated to synthesise both tilapia PRLs which appeared also to be stored in the same secretory granule ( Specker *et al.*, 1993 ).

The role of PRL in teleost osmoregulation was initially recognised by Pickford and Phillips ( 1959 ) who discovered that ovine PRL was necessary to promote the survival of hypophysectomised killifish, *Fundulus heteroclitus*, in FW, and it is now generally accepted that PRL is essential for the survival of euryhaline teleosts in FW. Prolactin plasma concentrations are seen to be greater in FW-adapted teleosts compared to SW- adapted animals, in tilapia, *Oreochromis mossambicus* ( Nicoll *et al.*, 1981) coho salmon, *Oncorhynchus kisutch* ( Avella *et al.*, 1991 ), Atlantic salmon, *Salmo salar* ( Prunet and Boeuf, 1985 ), chum salmon, *Oncorhynchus kiewi* , ( Hasegawa *et al.*, 1987; Hirano *et al.*, 1990 ) and Japanese eel, *Anguilla japonica* ( Suzuki and Hirano, 1991 ).

It appears that the principal role of PRL in ion and water transport in the gills is a reduction in permeability. Hypophysectomy in FW-adapted *Fundulus heteroclitus* resulted in a marked increase in sodium outflux which was corrected by prolactin ( Maetz *et al.*, 1967 ). Ovine PRL increased plasma Na<sup>+</sup> and Cl<sup>-</sup> concentrations and reduced net Na<sup>+</sup> excretion rate across the gill of SW-adapted tilapia ( *Tilapia mossambicus* ) ( Dharmamba *et al.*, 1973; Dharmamba and Maetz, 1976 ). Prolactin reduced the permeability of the branchial epithelium to water and ions by reducing Na<sup>+</sup>-K<sup>+</sup>-ATPase activity in FW adapted teleosts ( Bern, 1975 ). Administration of PRL appeared to cause a dedifferentiation of the chloride cell population ( Foskett *et al.*, 1983 ).

SW-adapted teleosts have a higher rate of water influx across the intestine compared to FW-adapted fish. This increase in water influx was

blocked in the SW-transferred Japanese eel, *Anguilla japonica*, after hypophysectomy ( Hirano, 1967 ). The intestinal water intake was reduced in SW-adapted trout ( *Onchorynchus mykiss* ) ( Morely *et al.*, 1981 ) and SW-adapted eel ( *Anguilla japonica* ) ( Utida *et al.*, 1972 ) by treatment with ovine-PRL, although it had no effect on the FW eel ( Hirano and Utida, 1968 ).

A characteristic feature of FW fish is a copious urine production. The reduction in urine flow of FW teleosts caused by hypophysectomy was restored by ovine-PRL ( Stanley and Fleming, 1967 ). Ovine PRL also reduced water reabsorption in the kidney of the FW- adapted starry flounder, *Platichthys stellatus*, ( Foster, 1975 ). The diuretic action of PRL may be due to increased GFR, increased number or functionality of glomeruli ( Lam, 1972 ) and/or reduction in tubular reabsorption of water ( Hickman, 1965 ). Prolactin is also known to decrease the sodium content of urine in FW goldfish, *Carassius auratus*, suggesting stimulation of tubular reabsorption of the ion ( Lahlou and Giordan, 1970 ), and  $\text{Na}^+\text{-K}^+\text{-ATPase}$  was stimulated in the kidney of hypophysectomised *Fundulus* in FW ( Pickford *et al.*, 1970a ).

Urinary bladders isolated from FW-adapted and PRL- treated SW-adapted *Platichthys* show greater sodium transport from mucosa to serosa compared to the control SW-adapted fish ( Johnson *et al.*, 1972; Hirano *et al.*, 1973 ). Transfer from SW to FW stimulated an increase in  $\text{Na}^+\text{-K}^+\text{-ATPase}$  activity in the urinary bladder of the euryhaline flounder, *Platichthys stellatus*, an increase which was mimicked in the SW flounder by the administration of ovine PRL ( Utida *et al.*, 1974 ). Prolactin was without effect on bladder  $\text{Na}^+\text{-K}^+\text{-ATPase}$  activity of the SW flounder, *Kereius bicoloratus* ( Utida *et al.*, 1974 ). This fish does not survive in FW and exhibits a high basal level of  $\text{Na}^+\text{-K}^+\text{-ATPase}$  activity in normal bladder. Adaptation to SW stimulated an increase in ion absorption by the goby bladder, an effect at least partly caused by cortisol ( Loretz, 1980, see page 60 ). Prolactin and cortisol appear to act in conjunction since cortisol is necessary to show the effects of

PRL ( Clarke and Bern, 1980 ), although the relationship between these two hormones is still unclear.

The presence of PRL has been demonstrated in the rostral lobe of the elasmobranchs *Triakis* and *Myliobatis* pituitary ( Sage and Bern, 1970 ). Removal of the rostral lobe of the PD in the euryhaline stingray, *Dasyatis sabina*, caused an increase in plasma osmolarity, urea and sodium concentrations, an effect that was reversed by PRL injections ( De Valming *et al.*, 1975 ). Prolactin injections also reversed the 50% reduction in branchial water permeability induced by hypophysectomy in *Scyliorhinus canicula* ( Payan and Maetz, 1973 ).

#### 1.8.2b Growth hormone

Growth hormone is a protein hormone released by GH-specific cells in the rostral PD of the pituitary. As its name suggests GH is important in the stimulation of somatic growth of all tissue types in all classes of vertebrates. It is released episodically, but in vertebrates with seasonal growth, release is greater during the growing period ( Batten and Ingleton, 1987 ). Body growth of young rats ceases after hypophysectomy, but is resumed to a limited degree upon GH injection. Growth hormone stimulates the transport of amino acids into cells and the incorporation of these amino acids into proteins ( Gorbman *et al.*, 1983 ).

Growth hormone cells have been immunocytochemically detected in the Japanese eel at all stages of development ( Arakawa *et al.*, 1992 ). In this study the percentage of GH-cell area to the whole pituitary area ( %GH ) was similar in leptocephali and glass eels, with a tendency to decrease as leptocephali became larger. The %GH was further increased in the cultured eel, although they were being held in FW. However, Orikasa *et al* (1992) reported an increase in cell size in leptocephali of the Japanese eel with the growth of the animal, and postulated that GH may be stored but not actively

secreted from these cells until metamorphosis to the glass eel stage. Arakawa *et al.* ( 1992 ) suggested that GH may be necessary for SW adaptation as well as for growth. However, in organ-cultured eel pituitary GH release was not affected by changes in the medium osmolarity, although residual GH contents in pituitaries in hyposmotic media were significantly less than those in hypertonic media ( Suzuki *et al.*, 1991a ). The GH cells of *Anguilla anguilla* were more degranulated in hyposmotic medium than in hyperosmotic medium ( Baker and Ingleton, 1975; Benjamin and Baker, 1978 ). It appears, however, that osmolality of the medium does not affect the GH synthesis in the eel since there was no significant difference in total amounts of GH produced by the cultured eel pituitary under various osmotic conditions ( Suzuki *et al.*, 1991b ).

Growth hormone has been isolated from teleosts, both FW-adapted species, such as the chum salmon, *Oncorhynchus keta* ( Kawauchi *et al.*, 1986 ) and SW-adapted fish, for e.g. the Atlantic cod, *Gadus morhua* ( Rand-Weaver *et al.*, 1989 ). Two forms of GH have been isolated from Japanese eel pituitaries *in vitro* ( Kishida *et al.*, 1987; Yamaguchi *et al.*, 1987 ) and from salmon ( Kawauchi *et al.*, 1986 ). Eel GH1 consists of 190 residues with two disulfide bridges formed between residues 52 - 163 and 180 - 188, and was found to have to greater identity with avian and mammalian GHs than with salmon GH, 55% compared to 48% identity, respectively ( Yamaguchi *et al.*, 1987 ). It appears that the sequence homology between teleost species is less than expected.

All the teleost GHs so far isolated are found to be potent growth stimulators in juvenile rainbow trout ( Kawauchi *et al.*, 1986; Rand-Weaver *et al.*, 1989; Kishida *et al.*, 1987 ).

There is much evidence supporting a role for GH in SW adaptation in salmonids. An increase in GH was observed during the first 12 hours after SW transfer of the chum salmon, *Oncorhynchus keta*, with a tendency

towards a decrease when the transfer was SW to FW ( Hasegawa *et al.*, 1987 ). Administration of GH increased  $\text{Na}^+\text{-K}^+\text{-ATPase}$  activity and chloride cell density in FW sea trout parr, *Salmo trutta trutta*, and led to 100% survival of the parr after transfer to SW ( Madsen, 1990 ). Plasma GH concentration was seen to peak two days after SW transfer followed by a decrease to the pre-transfer values in rainbow trout, *Oncorhynchus mykiss* ( Sakamoto *et al.*, 1990 ). The secretory dynamics of GH were determined in this study. Metabolic clearance rates of GH increased significantly four days after transfer from FW to 75% SW and returned to the FW level after 3 - 4 weeks in 75% SW. This increase was associated with an elevation in the calculated secretion rate.

#### 1.8.2c Somatolactin

Somatolactin is a recently discovered hormone, isolated from the pituitary glands of teleost fish. Somatolactin belongs to the PRL/GH family and is synthesised by the PI of teleosts adapted to both FW and SW, such as FW rainbow trout, green mollies, catfish, killifish and eel and SW cod and flounder ( Rand-Weaver *et al.*, 1991a ). The sequence of cod SL has been determined ( Rand-Weaver *et al.*, 1991b ). Somatolactin consists of 209 amino acid. Three disulfide bonds are found between residues  $\text{Cys}^5 - \text{Cys}^{15}$ ,  $\text{Cys}^{65} - \text{Cys}^{181}$ ,  $\text{Cys}^{198} - \text{Cys}^{206}$ , which are homologous to those of tetrapod PRLs. The identity of the SL amino acid sequences isolated from different teleosts was between 73% and 81% ( Takayama *et al.*, 1991 ). Teleost SL show slightly higher homology to the tetrapod GHs and PRLs ( average 29% identity ) than to the teleost hormones ( average 24% identity ) ( Kawauchi, 1992 ). SL may play a role in reproduction ( Planas *et al.*, 1992 ) and given its structural identity with PRL and GH may have a part in ion control in teleost fish, although more research is required to elucidate the true physiological function.

### 1.8.3 Structure of neurohypophysis

The NHP can basically be divided into three regions, distally the pars nervosa ( or neural lobe ), the neural stem, and proximally the median eminence ( Batten and Ingleton, 1987 ).

The mammalian NHP is well differentiated and two distinct subregions can be identified. The median eminence , which is the more anterior portion of the NHP, consists mainly of aminergic axonal endings and the pars nervosa, which is in contact with the PI, is composed of peptidergic axonal endings. The median eminence has a blood supply in common with the AHP, specifically called the hypothalamo-hypophysial portal system. An abundant but separate blood supply characterises the pars nervosa ( Norris, 1985 ).

In birds the median eminence is different to that found in mammals. The primary capillaries are more superficial and do not form a complex vascular bed. The median eminence is differentiated into a rostral portal system and a caudal area. The different regions of the avian NHP receive separate innervation ( Schreibman, 1986 ).

The reptilian NHP is composed of a globular pars nervosa and a median eminence situated anterior to the PD. The median eminence contains a plexus of capillaries derived from a branch of the carotid artery, which enters the pars distalis ( Norris, 1985 ).

In amphibians the median eminence forms in the ventral part of the NHP, anterior to the pars nervosa. The pars nervosa is generally larger in the more terrestrial amphibians. The PI of the AHP receives blood from all sections of the NHP and general circulation, while only the median eminence supplies blood to the PD ( Schreibman, 1986 )

In the fishes, the agnathans have a well developed NHP innervated from the anterior hypothalamus, which is considered the equivalent of a pars

nervosa, but the median eminence is absent. Elasmobranchs have a well developed median eminence and pars nervosa, the latter which is interdigitated with the PI of the AHP. The teleost NHP can usually be subdivided into an anterior part, which branches into the PD, and posterior section, which branches into the PI ( Batten and Ingleton, 1987 ). In general the teleost NHP does not appear to possess a true median eminence or portal system, though there is some debate over the presence of a median eminence in teleosts. There is a complete interdigitation of the NHP with the PD as well as with the PI.

#### 1.8.4 Neurohypophysis secretions

All the NHP peptides consist of nine amino acids, but distinct structural variations occur between different vertebrates ( Table 1.1 ). The first and sixth residues are cysteines, which are linked by a disulphide bridge to form a cystine residue. In all NHP peptides, characterised to date, the amino acids at position 1, 5, 6, 7 and 9 are conserved, whereas those at positions 3, 4 and 8 are variable ( Archer, 1974, 1988; Archer and Chauvet, 1988 ).

The typical vertebrate neurohypophysis contains active peptides of two types: (1) basic peptides, for example arginine vasopressin ( AVP ) and arginine vasotocin ( AVT ), which contain a basic amino acid ( lysine or arginine ) at position 8, and (2) neutral peptides, oxytocin-like peptides which contain neutral amino acids ( leucine, isoleucine, valine or glutamine ) at position 8 ( Table 1.1 )

In vertebrates the main hormonal activity of the NHP hormones is in the control of water retention. The main NHP peptides found in mammals are AVP and oxytocin ( OT ), with AVT and isotocin ( IT ) being the major NHP peptides in teleosts. The osmoregulatory role of NHP hormones is not yet clear in teleosts, although effects in the kidney have been reported in the eel, *Anguilla anguilla*, which affects urine flow rates ( see below ).

Table 1.1

Table 1.1    Structure and phylogeny of vertebrates  
                  neurohypophysial hormones  
( From Batten and Ingleton, 1987 ).

|   | Neutral hormone  | Basic hormone  |
|---|--|--|
| Mammals<br><i>Placentals</i><br>All except pigs     | 1 2 3 4 5 6 7 8 9<br>Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH <sub>3</sub><br>Oxytocin | 1 2 3 4 5 6 7 8 9<br>Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH <sub>3</sub><br>Arginine vasopressin |
| Domestic pigs                                       | Oxytocin   | 1 2 3 4 5 6 7 Lys 9<br>Lysine vasopressin  |
| <i>Marsupials</i><br>Macropodidae                   | Oxytocin   | 1 Phe 3 4 5 6 7 8 9<br>Lysine vasopressin+   |
| Phalangeridae                                       | 1 2 3 4 5 6 7 Ile 9<br>Mesotocin   | Arginine vasopressin   |
| Birds, Reptiles,<br>Amphibians and<br>Lungfishes    | Mesotocin  | 1 2 Ile 4 5 6 7 Lys 9<br>Arginine vasotocin  |
| Teleostean and Ganoid<br>Fishes                     | 1 2 3 Ser 5 6 7 Ile 9<br>Isotocin  | Arginine vasotocin   |
| Elasmobranchs<br><i>Selachii</i><br>Skates and Rays | 1 2 3 Ser 5 6 7 Ile 9<br>Glumitocin  | Arginine vasotocin   |
| Sharks and Dogfishes                                | 1 2 3 4 5 6 7 Val 9<br>Valitocin +<br>1 2 3 Asn 5 6 7 Gln 9<br>Aspar(g)itocin        | Arginine vasotocin   |
| <i>Holocephali</i>                                  | Oxytocin   | Arginine vasotocin   |
| Cyclostomes   | -----  | Arginine vasotocin   |

In order to assess the physiological significance of the effects of administered doses of AVT in teleosts it is important to establish the normal range of plasma AVT concentrations in these fish. Using a rat diuretic bioassay Perrott *et al.* ( 1991 ) found a higher pituitary AVT content in the euryhaline flounder, *Platichthys flesus*, acclimated to FW compared to SW adapted fish and a corresponding pattern in plasma AVT concentration. Warne and Balment ( 1991 ) also reported a significantly higher plasma AVT concentration in the FW-adapted compared to the SW-adapted flounder with values in the same range as reported for tetrapods. Using an eel ventral aorta bioassay to measure AVT levels in extracted eel plasma, Holder *et al.* ( 1982 ) found a concentration of 10.9 pg/ml in the FW eel, but did not measure the hormone in the SW-adapted eel. The effect of altered environmental salinity in the expression of vasotocin precursor genes in trout using *in situ* hybridisation techniques was investigated by Hyodo and Urano ( 1991 ). The pro AVT hybridisation signals in magnocellular neurones decreased upon transfer from FW to 80% SW. Holder ( 1968 ) however, showed a higher pituitary content in long term SW adapted eel compared to FW adapted fish, which perhaps reflects a lower plasma concentration in SW fish as a result of slower release of the hormone by the pituitary. Henderson *et al.* ( 1985 ) did, however, report a considerable elevation in plasma AVT concentration in long-term SW adapted eels (  $778 \pm 95.8$  pg/ml ) compared to eels held in distilled water (  $28.2 \pm 3.8$  pg/ml ), although Bentley ( 1971 ) found no change in plasma AVT in eels transferred from FW to SW. It appears that the physiological range of plasma AVT concentrations falls between  $10^{-12}$  -  $10^{-11}$ M, a level comparable to that of tetrapods.

#### 1.8.4a Effect on gill function

The physiological function of NHP hormones at the gill is still unclear. AVT caused an increase in resistance to blood flow across the gill ( Bennett

and Rankin, 1986 ), which may result in a diversion of blood flow through the gill.

Guibbolini *et al.* ( 1988 ) demonstrated the presence of AVT receptors on the gill and subsequently designated this receptor a new type 'NH<sub>f</sub>' as a result of further characterisation ( Guibbolini and Lahlou, 1990 ). They observed that the AVT and IT peptide receptors involved in trout gill were closer to V<sub>1</sub> than to the V<sub>2</sub> type with respect to the non-stimulation of adenylate cyclase. The receptors did not respond to a V<sub>2</sub> agonist, but were blocked by a V<sub>1</sub> antagonist. However, these researchers also reported that the receptors differed from mammalian V<sub>1</sub> receptors in other ways, such as the direct inhibition of cAMP by AVT and IT in gill membrane preparations. The inhibition of cAMP production by AVT was also reported for dispersed gill cell preparations ( Sainsbury and Balment, 1991 ), with a greater effect in gill tissue from SW-adapted fish rather than FW adapted fish.

A higher maximal binding capacity ( B<sub>max</sub> ) and lower dissociation constant ( K<sub>d</sub> ) was reported for isolated gill cells from SW- adapted eels compared to FW-adapted eels ( Guibbolini *et al.*, 1988 ). Gill morphology was also examined in this study and a higher number of chloride cells was found in SW adapted animals. It was suggested that the observed variations in B<sub>max</sub> and K<sub>d</sub> of AVT may be associated with the changes in chloride cell population.

#### 1.8.4b Effect on renal function

At high doses of AVT a diuretic action has been described in several species of teleost, trout ( Pang *et al.*, 1983 ), goldfish ( Maetz *et al.*, 1964 ), eels ( Chester Jones *et al.*, 1969 ) and lungfish ( Sawyer and Pang, 1975 ). Henderson and Wales ( 1974 ), however, observed different effects on kidney function with respect to the dose of AVT administered to the FW-adapted eel. High doses ( 10-100ng/kg ) which were sufficient to produce a pressor effect

caused a diuresis, while lower doses ( 0.001-0.01 ng/kg ) induced a glomerular antidiuresis in the FW eel without affecting the cardiovascular system. The diuretic actions of the higher doses seem likely to be pharmacological with the lower doses inducing physiological effects, when considered in relation to the physiological range of plasma AVT concentration since established in teleosts ( Balment *et al.*, 1993 ). In FW-adapted eels IT produced a glomerular diuresis without a sustained blood pressure increase and, like AVT, Babiker and Rankin ( 1978) found low doses of this hormone to be antidiuretic and high doses to be diuretic.

The effects of AVT may involve regulation of blood flow in specific capillary beds. Sawyer ( 1972 ) suggested that AVT causes diuresis by the vasoconstriction of efferent glomerular arterioles, and hence affects GFR. When the trout trunk preparation of Pang *et al.* ( 1983 ) was perfused under a constant rate, AVT produced both pressor and diuretic responses. These responses were abolished by the pretreatment of the preparation with KBIV 24, an analogue of AVT which is a specific antagonist to the vascular action of AVT. The diuretic response may be the result of peripheral vasoconstriction and elevated systemic blood pressure overriding glomerular vasoconstriction. An antidiuretic response was obtained in this preparation when the perfusion pressure was kept constant, which suggests this response may be due to glomerular vasoconstriction without a change in systemic blood pressure ( Pang *et al.*, 1983 ).

The renal mechanism involved in AVT-induced changes in urine flow appears to derive largely from a change in the numbers of functioning nephrons, since the TMG increased concomitantly with urine flow and GFR ( Henderson and Wales, 1974; Babiker and Rankin, 1978 ). During AVT-induced antidiuresis there was a reduction in the number of filtering nephrons, which led to a decrease in GFR and hence a decline in urine flow. For the mediation of this response there must be a vascular AVT receptor

within the kidney. The successful blocking of the AVT-induced antidiuresis in the trout trunk, perfused under constant pressure, by the  $V_1$  receptor antagonist KBIV 24 ( Pang *et al.*, 1983 ) suggests that this receptor is similar to the tetrapod phospholipase C-linked  $V_1$  type. When high doses of AVT were administered to SW adapted eels a diuresis was seen, as in FW adapted eels. The lower doses that caused an antidiuretic response in the FW eel had no effect in the SW eel ( Babiker and Rankin, 1978 ). Long-term SW-adapted eels already exhibit a marked antidiuresis and, therefore, a further increase in that activity would be illogical as divalent ion regulation and excretion of some nitrogenous compounds would be impaired ( Maetz and Lahlou, 1974 ).

Although it is possible that the antidiuretic activity observed in some teleosts may be mediated by vascular  $V_1$  type receptors antidiuresis in other vertebrates is usually associated with tubular  $V_2$  receptors coupled to cAMP generation. Sainsbury and Balment ( 1991 ) recently demonstrated the presence of a receptor similar to the adenylate cyclase-linked  $V_2$  type receptor of higher vertebrates, within the kidney. In isolated renal tubules, AVT (  $10^{-11}$ - $10^{-7}$ M ) evoked a dose-dependent increase in cAMP production. The effect was apparently similar in both FW and SW trout. These results are in contrast to the findings of Lahlou *et al.* ( 1988 ) who reported an AVT-dependent inhibition of glucagon-induced cAMP increase in hepatocytes isolated from rainbow trout ( *Onchorynchus mykiss* ) maintained in FW.

## 1.9 Adrenocortical homologue

### 1.9.1 Structure of adrenocortical homologue

An adrenocortical homologue occurs in all vertebrate groups from cyclostomes to mammals. The adrenal gland, as the name suggests, is situated adjacent to the kidneys, a situation that is ubiquitous throughout the vertebrates. In mammals the gland is composed of two tissues arranged in zones, an outer cortex surrounding an inner medulla. The cortex is of

mesodermal origin, called the interrenal or adrenocortical tissue and secretes several steroids. The medulla is composed of neural tissue which is termed chromaffin tissue due to the dark brown staining action of chromic acid, and secretes catecholamines ( Chester Jones, 1976 ). There is a close association between adrenocortical and chromaffin tissue throughout the vertebrates, except in the Elasmobranchii where a complete separation is found.

In birds, the adrenal gland is a discrete organ lying anterior to the kidneys, often wholly or partly covered by the gonads. The two component tissues are intermingled to a varying degree without the formation of a sharply defined cortex and medulla ( Holmes and Phillips, 1976 ).

The reptilian adrenal gland is usually a discrete encapsulated body, situated near the kidney and often adjoining the gonads. A variable degree of intermingling of the adrenocortical and chromaffin tissue is observed in reptiles ( Lofts, 1978 ).

The amphibian adrenocortical homologue is not a discrete gland separate from the kidney. The gland is situated in the ventral surface of the kidney with intermingling of the two tissue types ( Hanke, 1978 ).

In teleosts, holosteans and the coelacanth, the two tissues form a diffuse organ at the anterior part of the kidney ( the head kidney ), situated around the posterior cardinal veins and their branches, while the dipnoans possess an even more dispersed structure ( Chester-Jones and Mosley, 1980 ).

In elasmobranch fish the two tissue components are completely separated from each other. The adrenocortical tissue comprises a discrete gland, the interrenal gland, which lies dorsally between the two posterior lobes of the kidney. The chromaffin tissue lies along the inner borders of the dorsal kidney surface in discrete islets, close to, but distinct from, the adrenocortical tissue ( Chester-Jones and Mosley, 1980 ).

The ultrastructure of adrenocortical tissue is essentially similar throughout the vertebrates ( Lofts and Bern, 1972 ). Characteristic features

include large mitochondria packed with tubular cristae and dense matrix, extensive smooth endoplasmic reticulum and numerous electron dense droplets, containing precursors of steroid synthesis. Throughout the vertebrates the adrenocortical homologue secretes a number of steroid hormones, known as the corticosteroids.

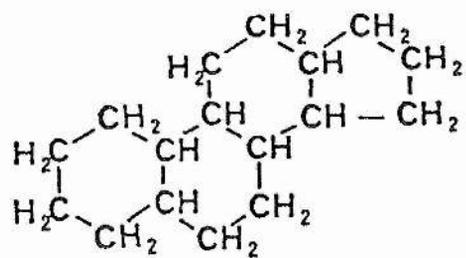
### 1.9.2 Structure of steroids

The steroids are a large class of lipids with a common ring structure consisting of three six-membered rings and one five-membered ring joined to each other by common sides. Figure 1.12 shows the basic unit, the cycloperhydrophenanthrene nucleus. Several different types of steroids arise as a result of the variety of enzymes involved in steroid biosynthesis, and may be classified according to the number of carbon atoms they contain, (a) the corticosteroids consisting of 21 carbon atoms ( C<sub>21</sub> ); (b) the androgens with 19 carbon atoms ( C<sub>19</sub> ) and; (c) the oestrogens containing 18 carbon atoms ( C<sub>18</sub> ). The androgens and oestrogens are known as the sex steroids due to their effects on reproductive processes and sexual characteristics. The corticosteroids primarily affect hydromineral balance and intermediary metabolism.

The corticosteroids are secreted by adrenocortical tissue. The pathway of corticosteroid synthesis in mammals is well documented and generally holds for lower vertebrates. All hormones from the adrenal cortex are derived from cholesterol compounds. Figure 1.13 summarises the chemical structures of the various corticosteroids and the routes for their biosynthesis. Table 1.2 lists the major corticosteroids produced by the vertebrate groups. The main corticosteroids synthesised by mammals are aldosterone and cortisol, though some rodents may predominately secrete corticosterone ( Sandor *et al.*, 1976 ); for amphibians, reptiles and birds, aldosterone and

**Figure 1.12**

Figure 1.12 Basic steroid structure



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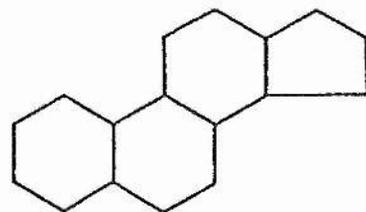


Figure 1.13

Figure 1.13 Interrelationships and formation of the steroid hormones  
( Modified from Bentley, 1982 )



Table 1.2

Table 2      Major corticosteroids produced in vertebrate groups  
( From Balment and Henderson, 1987 )

| Group         | Corticosteroids in Blood   |
|---------------|--|
| Mammals       | <u>Cortisol/corticosterone</u> , aldosterone, corticosterone, 18-hydroxycorticosterone, 11-deoxycorticosterone |
| Birds         | <u>Corticosterone</u> , aldosterone, 11-deoxycorticosterone  |
| Reptiles      | <u>Corticosterone</u> , 18-hydroxycorticosterone, aldosterone  |
| Amphibians    | <u>Corticosterone</u> , 18-hydroxycorticosterone, aldosterone, 11-deoxycorticosterone, cortisol                |
| Lungfish      | <u>Cortisol</u> , aldosterone, 11-deoxycortisol, 11-deoxycorticosterone, corticosterone                        |
| Bony Fish     | <u>Cortisol</u> , cortisone, 11-deoxycortisol, corticosterone  |
| Elasmobranchs | <u>1<math>\alpha</math>-hydroxycorticosterone</u> , corticosterone, 11-deoxycorticosterone, 11-deoxycortisol   |

\* Underlined steroids are the major secretory products.

corticosterone; for teleosts, cortisol, and for elasmobranchs, a unique steroid,  $1\alpha$ -hydroxycorticosterone.

### 1.9.3 Structure of corticosteroids

Corticosteroids have been classified into two main categories, the glucocorticoids ( e.g. cortisol and corticosterone ) which regulate carbohydrate metabolism, and mineralocorticoids ( e.g. aldosterone ) which maintain salt balance. However, this distinction, which is based on mammalian models, is somewhat arbitrary as their physiological effects often overlap ( Sandor *et al.*, 1976 ), for instance cortisol which is considered as a glucocorticoid in mammals may act as a mineralocorticoid in fish.

In mammals, aldosterone acts mainly to promote sodium reabsorption in a variety of target organs, including the kidney, sweat glands, salivary glands, urinary bladder, intestine and mammary glands. Cortisol and corticosterone have lesser effects on osmoregulation and are predominately involved in the control of intermediary metabolism in this group.

There is evidence for mineralocorticoid actions in birds and reptiles, a greater amount of information having accrued for birds than for reptiles. Corticosteroids have been reported to both increase and decrease salt gland secretion in reptiles ( Holmes and McBean, 1964; Bradshaw, 1975 ), while in birds they increase nasal gland secretion and increase sodium ion reabsorption ( Holmes and Phillips, 1976 ).

In amphibians, aldosterone promotes sodium flux across the skin of saline adapted frogs ( Maetz *et al.*, 1958 ) and may also promote renal natriuresis, possibly as a secondary effect to the skin flux ( Henderson *et al.*, 1972 ). Aldosterone increases sodium transport across the skin, urinary bladder and colon ( Crabbe and De Weer, 1964; Cofre and Crabbe, 1967 ), and in high doses glucocorticoid-like activity has been demonstrated ( Hanke and Newman, 1972 ).

In teleosts cortisol is the major corticosteroid released by the interrenal gland of both FW and SW teleosts. The following section deals with the osmoregulatory function and synthesis of corticosteroid in fish in greater detail

#### 1.9.4 Cortisol and teleost osmoregulation

Cortisol is the major corticosteroid released by the interrenal gland of both FW and SW teleosts ( Sandor *et al.*, 1967 ) and has a well established effect on osmoregulation. It is generally accepted that cortisol is involved in water and electrolyte balance, particularly during migration from FW to SW in euryhaline species when its effects closely interact with those of prolactin ( Specker and Schreck, 1982 ). Most of the data on the effects of cortisol on sodium movements have been gathered from European, North American and Asian eels ( *Anguilla anguilla*, *A. rostrata*, *A. japonica*, respectively ), but similar actions have been observed in other species.

In the gill osmotic and diffusional permeabilities are greater in FW teleosts than those in marine teleosts ( Isaia, 1984 ). The gills are an important target organ for cortisol, which stimulates active ion transport in FW and SW fishes. Cortisol increased the influx of water into Japanese eel gills incubated in FW ( Ogawa, 1975 ), and SW mullet, *Chelon labrosus*, gills incubated in deionised water, but not those of mullet adapted for 7 to 30 days to FW ( Gallis *et al.*, 1979 ). Corticosteroid receptors have been reported to be present in gill cytosol preparations of brook trout, *Salvelinus fontinalis* ( Chakraborti *et al.*, 1987), and of the American eel and trout ( Sandor *et al.*, 1984 ).

Administration of cortisol has been shown to increase  $\text{Na}^+\text{-K}^+\text{-ATPase}$  activity in several FW-adapted species including eel, mullet and tilapia ( Epstein *et al.*, 1971; Kamiya, 1972; Forrest *et al.*, 1973; Gallis *et al.*, 1979; Balm, 1986 ). In FW-adapted American eel, cortisol administration induced a rise in the specific activity of  $\text{Na}^+\text{-K}^+\text{-ATPase}$  in gill filaments and intestinal mucosa,

to a level comparable to that elicited by transfer to salt water ( Epstein *et al.*, 1971 ). In cultured isolated primary gill filaments from both FW-adapted pre- and postsmolt coho salmon, addition of cortisol to the culture media resulted in increases in Na<sup>+</sup>-K<sup>+</sup>-ATPase activity relative to control ( McCormick and Bern, 1989). In this study it was necessary to utilise cortisol concentrations equal to and greater than the normal circulating levels, due to the absence of a blood system which would ensure efficient delivery of the hormone *in vivo*. McCormick *et al.* ( 1991 ) demonstrated that the ability of cortisol to increase Na<sup>+</sup>-K<sup>+</sup>-ATPase activity in salmonids is, partly at least, influenced by the developmental stage of the fish. Cortisol has the greatest stimulatory effect on gill Na<sup>+</sup>-K<sup>+</sup>-ATPase when the fish are almost ready to undergo seaward migration. As previously described in Section 1.3.3 Na<sup>+</sup>-K<sup>+</sup>-ATPase is localised chiefly to the basolateral membrane of the chloride cell ( Karnaky *et al.*, 1976 ). The chloride cell undergoes characteristic changes with transfer from FW to SW and these changes can be induced in FW trout, *Onchorynchus mykiss*, by intramuscular injection of cortisol ( Laurent and Perry, 1990 ). The increases in gill Na<sup>+</sup>-K<sup>+</sup>-ATPase activity induced by cortisol were found to be a result of increases in the number of sodium pumps ( McCormick and Bern, 1989 ), as observed in SW adaptation ( Sargent and Thompson, 1974 ). Cortisol treatment increased gill Na<sup>+</sup>-K<sup>+</sup>-ATPase activity in hypophysectomised ( Bjornsson *et al.*, 1987; Butler *et al.*, 1972; Milne *et al.*, 1971; Pickford *et al.*, 1970 ) and intact teleosts ( Dange, 1986; Epstein *et al.*, 1971; Richman and Zaugg, 1987 )

SW adapted teleosts have a higher sodium turn-over rate than FW-adapted fish ( Maetz *et al.*, 1967). Increased sodium turnover, perhaps a reflection of renal sodium retention, was demonstrated in the kidney of SW-adapted *Fundulus heteroclitus* treated with cortisol ( Pickford *et al.*, 1970 ). The urinary bladder of SW-adapted fishes have a higher water permeability compared to FW-adapted fishes ( Hirano *et al.*, 1973 ). Doneen ( 1976 )

observed that cortisol elevated the water permeability and stimulated sodium and chloride reabsorption of the bladder of the gobiid teleost *Gillichthys mirabilis* adapted to SW, but was without effect on fish adapted to only 5% SW. However, Johnson *et al.*, ( 1972 ) reported urinary bladder water movement to be unaffected by cortisol in both FW and SW- adapted starry flounder, *Platichthys stellatus*.

Adrenalectomy was initially carried out in the European eel by Chester Jones *et al.* ( 1964 ). Adrenalectomy of FW eels reduced urine flow and sodium reabsorption while cortisol injection restored urine flow to normal ( Chan *et al.*, 1969 ; Chester Jones *et al.*, 1969 ). Adrenalectomy of SW-adapted eels caused an increase in plasma Na<sup>+</sup> concentration and a decrease in branchial Na<sup>+</sup> extrusion ( Mayer *et al.*, 1967 ). These effects of adrenalectomy may be overcome by cortisol injections.

Hypophysectomy of FW-adapted eels lowers plasma Na<sup>+</sup> slowly, but when the eels are placed in SW they accumulate Na<sup>+</sup>. Plasma cortisol is also reduced following hypophysectomy but can be restored to normal by treatment with ACTH. Neither cortisol nor ACTH can completely restore normal ion balance which suggests prolactin or another factor may also be necessary. Mammalian ACTH may maintain interrenal structure in hypophysectomised fish, but PRL has no effect, suggesting that ACTH interacts directly with the interrenal gland while PRL operates at a different level, but both are required for normal osmoregulatory balance.

Circulating cortisol levels are similar in FW- adapted and SW- adapted eels ( Henderson *et al.*, 1974; Henderson *et al.*, 1976; Ball *et al.*, 1971, Forrest *et al.*, 1973 ). Cortisol levels, however, are elevated upon FW to SW transfer, with a peak in concentration 1-3 days or 3-7 days after transfer according to the season and age of animals ( Ball *et al.*, 1971 ). Kenyon *et al.* ( 1985 ) reported a threefold increase in the plasma cortisol concentration of the eel, *Anguilla anguilla*, within five hours of transfer to SW. Elevation of corticosteroids in

FW salmonids prior to their seaward migration has been associated with changing osmoregulatory physiology. Arnold-Reed and Balment ( 1989 ) observed an increase in plasma cortisol levels upon transfer from FW to SW within 24 hours in the flounder, although concentrations of long term FW and SW fish were similar. Two days after SW transfer, cortisol concentration had decreased again to that in FW fish. Cortisol levels in SW fish, by contrast, showed a significant decrease 24 hours after transfer to FW although they recovered by 2 days.

#### 1.9.5 Steroid dynamics

The peripheral concentration of "free" hormone is a direct measure of its availability for its metabolic role but gives no measure of the precise hormonal production rate. Hormones may be taken up rapidly at their target organ or may be quickly inactivated or excreted. It is therefore of great relevance to measure the secretory dynamics of hormones.

Many hormones, steroids in particular, are transported in the blood, closely associated with, or directly bound to plasma proteins. Corticosteroids bind to plasma proteins of either low affinity - high capacity, or high affinity - low capacity. Binding proteins create a "buffered pool" and the chemical nature of this relationship is such that an equilibrium exists between the "bound" and the "free" hormone. A substantial proportion of steroid hormone is in the bound form but it is thought that only the minor unbound fraction is biologically active. There are several suggested reasons as to why hormones bind to proteins in the plasma:

- (1) to delay the inactivation of the hormone by enzymes present in the blood;
- (2) to contribute to the specificity of hormonal action;
- (3) to act as a circulating pool of the hormone which may extend or moderate the action of the hormone.

The blood hormone concentration is a reflection of both production and catabolism of the hormone. The production and excretion of a steroid hormone is in kinetic equilibrium with the concentration of hormone circulating in the blood. When the hormone concentration in the blood is in a "steady state" ( i.e. constant ), the amount of hormone entering, and irreversibly removed, from the blood must be equal. This leads to the concept of metabolic clearance rate ( MCR ) which is defined as the volume of blood irreversibly " cleared " of a steroid per unit time ( Tait *et al.*, 1962 ).

Thus: 
$$\text{MCR} = \frac{\text{BPR}}{\text{C}}$$

where MCR = metabolic clearance rate (ml/hr)

BPR = blood production rate ( $\mu\text{g/hr}$ )

C = endogenous steroid concentration ( $\mu\text{g/ml}$ )

#### 1.9.5a Determination of secretory dynamics

The methods that have generally been employed in the determination of steroid secretory dynamics, are based on the principle of isotope dilution: blood production rate and urinary production rate.

##### 1.9.5ai Blood production rate

This method relates the MCR to the endogenous concentration of the hormone and may be determined either by a single injection or constant infusion procedures.

##### ( i ) Single injection

This method involves administering a single dose of a tracer hormone and sequentially measuring the decreasing concentration of the label in the plasma. The declining concentration of radioactivity reflects its clearance from circulation. The radioactivity concentration in the blood is plotted against time and MCR is calculated as follows:

$$\text{MCR} = \frac{\text{Ri ( dpm )}}{\text{area under radioactivity-time curve (dpm/ml/hr)}} \text{ (ml/hr)}$$

where, Ri = amount of radioactivity injected into the plasma.

BPR may then be determined from the MCR:

$$\text{BPR} = \text{MCR} \times \text{C}$$

The radioactivity-time curve obtained with this method is a reflection of hormonal distribution within the various body compartments, and so is complex as a result. The curve represents a series of linear portions, each portion corresponding to hormonal distribution within a particular compartment.

#### ( ii ) Constant infusion

This procedure involves infusing the radioactive tracer into the blood at a constant rate, in order to achieve a steady state at which the concentration of the radioactive hormone in plasma is stable. In the steady state the distribution of the hormone between the plasma compartment and all other body compartments is in equilibrium and therefore these different rates of distribution do not affect the calculation of BPR. In the steady state, the amount of steroid secreted will also be equal to that entering the circulation. This means the production rate will equal the secretion rate. It is assumed that the continuous infusion of a radioactive compound does not stimulate the secretion of the gland. The ratio of the rate of infusion of radioactive steroid to the final concentration of radioactive hormone will then be equal to the ratio of the hormonal secretion rate of the non-isotopic steroid plasma concentration. This ratio in both cases is the MCR ( Schulster *et al.*, 1976 ). The MCR can be calculated as follows:

$$\text{MCR} = \frac{\text{I.}}{\text{Xc}}$$

where MCR = metabolic clearance rate (ml/hr)

- I = constant state of infusion ( dpm/hr)  
 Xc = steady-state isotopic concentration in plasma  
 (dpm/ml)

It is important to know whether the constantly infused tritiated hormone undergoes substantial metabolism as this technique depends upon maintaining a constant plasma level of the tracer ( Henderson *et al.*, 1976 ). Any metabolism of the labelled hormone must be taken into account in the calculation of the MCR.

The BPR is determined from the MCR, as previously described, with the calculation:

$$\text{BPR} = \text{MCR} \times C$$

There are a number of conditions associated with the determination of MCRs and BPRs using radioactively labelled hormones:

- (a) the radioactive label must behave chemically in the same manner as the endogenous hormone;
- (b) the label must be radioactively pure and stable *in vivo*;
- (c) the tracer must have a very high specific activity and, therefore, not have any pharmacological effects on the animal.

#### 1.9.5aii Urinary production rate

In this method a single injection of tracer hormone is administered with the production rate being calculated by monitoring the urinary excretion of the traces using the following equation:

$$\text{PR urinary} = \frac{R_i \times T}{SA}$$

where PR urinary = urinary production rate (  $\mu\text{g/hr}$  )

Ri = amount of radioactivity injected ( dpm )

SA = amount of radioactive hormone or unique metabolite in urine ( dpm/ $\mu\text{g}$  ), i.e. specific activity.

T = time of urine collection ( hr )

This method, however, assumes that the kidney is the only route of removal of the hormone and that there is a unique urinary metabolite. It does not take account of any quantity of hormone that has not been irreversibly removed from circulation

#### 1.9.5b Cortisol secretory dynamics in teleosts

Using the constant infusion technique it has been demonstrated that, despite a similar plasma cortisol concentration in long term FW- and long term SW-adapted eel, both the MCR and BPR were significantly elevated in the SW animal compared to the FW eel ( Henderson *et al.*, 1974; Leloup-Hatey, 1974 ). The MCR was significantly higher 24 hours after SW transfer of the Atlantic salmon, *Salmo salar*, although the cortisol protein binding was significantly lower and BPR was unchanged ( Nichols and Weisbart, 1985 ). The effect of SW-adaptation on the secretory dynamics of cortisol, during the initial period of SW transfer in the eel has not been determined to date.

#### 1.9.6 Catecholamines

Catecholamines, such as epinephrine ( adrenaline ), norepinephrine ( noradrenaline ) and dopamine, are derivatives of catechol, which in turn is a derivative of the amino acids phenylalanine and tryosine. Catecholamines are stored in membrane-bound granules in adrenal medullary cells. It has been shown that epinephrine and norepinephrine are stored in separate adrenal medullary cells ( Coupland, 1972 ), even though norepinephrine is a biosynthetic precursor of epinephrine.

The general pattern of action of the catecholamines in the tetrapods appear to be similar. They seem to carry out cardiac-stimulating and hyperglycemic actions in all vertebrates.

Burnstock ( 1969 ) has suggested that circulating catecholamines, released from diffusely distributed catecholaminergic cells, appear to play a more significant role in lower vertebrates than in higher vertebrates, in which direct nervous control has been developed to a more extensive degree. This section will deal exclusively with the actions of catecholamines in teleosts.

Under resting conditions the plasma levels of epinephrine and norepinephrine in teleosts amount to about 1nM, with dopamine apparently present in only very small amounts. Catecholamines are known to exert effects on blood flow through the gills, on active ion transport mechanisms and on branchial permeability to water, ions and organic substances.

The catecholamines, epinephrine, norepinephrine, and dopamine have been found to widely occur in the tissues of the American eel, *Anguilla rostrata*, with the bulk of the catecholamines in the systemic blood originating from chromaffin cells in the wall of the posterior cardinal veins ( presumed adrenal medulla equivalent of the eel ) ( Epple *et al.*, 1989 ).

A transient increase in catecholamines was observed after transfer from FW to SW of carp, tilapia and trout ( Hegbag and Hanke, 1984 ). Studies utilising isolated gill preparations, perfused under constant pressure, have reported vasodilation of the branchial circulation after administration of epinephrine ( Keys and Bateman, 1932; Richards and Fromm, 1969; Rankin and Maetz, 1971 ) which may be preceded by a transient vasoconstriction ( Wood, 1975 ). This response occurs in both FW-adapted fish ( Payan and Girard, 1977 ) and SW-adapted fish ( Claiborne and Evans, 1980 ). Vasoconstriction appears to be mediated by  $\alpha$ -adrenergic receptors and vasodilation by  $\beta$ -adrenergic receptors ( Wood, 1975). Alpha-receptors situated on the arterio-venous anastomosis could be responsible for the decrease in blood flow in the central sinus compartment, favouring an increase in blood flow toward the lamellae ( Girard and Payan, 1976; Claiborne

and Evans, 1980 ). The main effect of catecholamines appears to be a greater blood flow through the lamellae ( Steen and Kruyssen, 1964; Richards and Fromm, 1969 ) and enlargement of the functional respiratory surface ( Haywood *et al.*, 1977; Nilsson, 1984 ).

A  $\beta$ -mediated stimulation of  $\text{Na}^+$  influx was observed after administration of catecholamines to the perfused FW-adapted trout head, whereas  $\text{Na}^+$  efflux was unaffected ( Payan *et al.*, 1975 ). However, in the free swimming trout the opposite effect was found ( Wood and Randall, 1973 ). This contrasting action of epinephrine has also been reported for  $\text{Cl}^-$  uptake. Epinephrine stimulated  $\text{Cl}^-$  influx via  $\alpha$ -adrenergic receptors in the isolated FW-adapted trout head, while *in vivo* infusion of epinephrine inhibited  $\text{Cl}^-$  uptake ( Perry *et al.*, 1984 ). An increase in the branchial permeability to small molecules such as water and urea was observed after administration of epinephrine and these increases are more effective in FW than in SW ( Isaia, 1979 ). These effects may be mediated via  $\beta$ -adrenergic receptors ( Haywood *et al.*, 1977 ).

In the gill perfusion of the SW-adapted eel epinephrine increased the chloride concentration in the perfusion fluid ( Keys and Bateman, 1932 ) and increased water loss in the SW-adapted mullet ( Pic *et al.*, 1974 ). Sodium influx through the gill is stimulated via  $\beta$ -receptors and is thought to be a result of the stimulation of the  $\text{Na}^+/\text{NH}_4^+$  and  $\text{Na}^+/\text{H}^+$  exchanges in SW adapted fish ( Payan and Girard, 1978 ) while chloride entry is inhibited by  $\alpha$ -receptor blockade ( Mayer-Gostan and Maetz, 1980 ).

Catecholamines seem to be involved in kidney function, since on removal of innervation, renal tubular sodium and water reabsorption were decreased ( Di Bona, 1983 ). Upon SW transfer of trout the normal reduction in GFR may be prevented by the administration of adrenergic blockers. This implies a role for  $\alpha$ -adrenergic nerves in the reduction of GFR on SW adaptation ( Elger and Hentschel, 1983 ). There have been few studies on the

osmoregulatory effects of catecholamines in elasmobranchs and these have been restricted to the kidney. The initial studies employed pharmacological doses and produced conflicting results on GFR ( Deetjen and Boylan, 1968; Forster *et al.*, 1972 ) but Brown and Green ( 1987 ) demonstrated a clear glomerular diuresis, in response to a pressor dose of adrenaline ( Brown, pers. comm. ).

## 1.10 The renin-angiotensin system

### 1.10.1 Introduction

The renin-angiotensin system ( RAS ) is an enzyme-activated peptide mediated effector of extracellular electrolyte and fluid balance in many vertebrates ( Olson, 1992 ). To date the mammalian RAS has been more extensively investigated than that of lower vertebrates. However, elements of the RAS have been identified in all vertebrate groups, although controversy surrounds the physiological role of the RAS in cyclostomes and elasmobranchs ( Nishimura *et al.*, 1970; Henderson *et al.*, 1980; Hazon and Henderson, 1985; Hazon *et al.*, 1989 ). Recently this controversy has been resolved, at least for elasmobranchs, with the isolation and sequencing of a homologous Angiotensin I ( AI ) ( Takei and Hazon, pers. comm. ).

The RAS consists of a number of angiotensins and their metabolising enzymes. The sequence of events in the RAS is shown in Figure 1.14. Renin, an aspartyl proteinase stored in the juxtaglomerular granules of mammalian kidney, hydrolyses angiotensinogen, a glycoprotein, to form the decapeptide AI. Renin, a rate-limiting component of the RAS ( Baxter *et al.*, 1984 ), is released in an inactive form and therefore must itself be activated.

Angiotensin I, which appears to be almost biologically inactive, is hydrolysed at the carboxyl terminal end, principally by angiotensin converting enzyme ( ACE ). Angiotensin converting enzyme is a peptidyl-dipeptide hydrolase and is the final enzyme involved in the generation of the biologically active

**Figure 1.14**

**Figure 1.14 The Mammalian Renin-Angiotensin System  
( From Balment and Henderson, 1987 )**



octapeptide Angiotensin II ( AII ) ( Wallace *et al.*, 1978 ). Angiotensin converting enzyme activity, along with renin, is considered one of the rate limiting steps determining the formation of AII ( Henderson *et al.*, 1993 ). Inhibitors of ACE, such as captopril, have been widely used to investigate the effects and functions of the RAS. In some instances AII may be hydrolysed to the less active heptapeptide, Angiotensin III ( AIII ) ( Chiu *et al.*, 1976 )

The basic structure of AII is conserved throughout the vertebrate groups with interchange of asparagine ( Asn ) and aspartic acid ( Asp ) occurring at position 1 and valine ( Val ) and isoleucine ( Ile ) at position 5. Angiotensin I is structurally more diverse with a number of different amino acids occupying position 9 ( Table 1.3 ). The newly isolated elasmobranch AI is unique in possessing a proline residue in position 3 ( Takei and Hazon, pers. comm. )

The RAS is probably most highly developed in the kidney of mammals where it is associated with the juxtaglomerular apparatus ( JGA ). The specific components of the mammalian JGA are juxtaglomerular ( JG ) cells containing renin-forming granules, the macula densa ( MD ) and the extraglomerular mesangium ( EGM ). No other vertebrate group demonstrates the same degree of organisation of the juxtaglomerular apparatus. The MD and EGM are absent in birds, reptiles, amphibians and teleosts. JG granules are present in tetrapods, glomerular and aglomerular teleosts, and holocephalians. Some sarcopterygians and primitive bony fishes also possess JG granules. A complete JG apparatus, including granulated afferent arteriolar smooth muscle cells, close apposition of the distal tubules with glomerular mesangial cells, has been observed in elasmobranchs ( Lacy and Reale, 1990 ). The evidence to date seems to suggest that all vertebrate classes, apart from the cyclostomes, possess a RAS. It is highly unlikely, however, that the RAS should be lacking in just a single vertebrate group. The reported absence of a RAS in this group may be more a reflection of a lack

Table 1.3

Table 1.3 Angiotensin I amino acid sequences from various vertebrates

| Amino acid sequence                   |         |     |     |     |     |     |     |     |     |     |
|---------------------------------------|---------|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Common Structure                      | 1       | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   | 10  |
| Species variation                     | -       | Arg | Val | Tyr | -   | His | Pro | Phe | -   | Leu |
| Human, pig, rabbit<br>rat, dog, horse | Asp     |     |     |     | Ile |     |     |     | His |     |
| Bovine, turtle                        | Asp     |     |     |     | Val |     |     |     | His |     |
| Fowl                                  | Asp     |     |     |     | Val |     |     |     | Ser |     |
| Snake                                 | Asp/Asn |     |     |     | Val |     |     |     | Tyr |     |
| Bullfrog                              | Asp     |     |     |     | Val |     |     |     | Asn |     |
| Goosefish                             | Asn     |     |     |     | Val |     |     |     | His |     |
| Salmon                                | Asn/Asp |     |     |     | Val |     |     |     | Asn |     |
| Eel                                   | Asp/Asn |     |     |     | Val |     |     |     | Gly |     |
| Eelasmobranch                         | Asn     |     | Pro |     | Ile |     |     |     | Gln |     |

of sensitivity of the methods employed in investigating the presence of the RAS with this particular group.

Classically, the RAS has been perceived as a blood-borne circulating system, however, there is now evidence of the existence of endogenous RASs in tissues, for example in brain and heart ( Campbell, 1987 ). AII produced by these local systems may exert an autocrine influence on the cells in which it is formed or a paracrine influence on neighbouring tissues ( Dzau, 1989 ). There have also been reports of the presence of a local renal RAS in mammalian kidneys ( Celio *et al.*, 1981 ), although the relative contribution of the systemic and tissue systems to local angiotensin production is not clear ( Campbell, 1987 ). It may be, therefore, that local renal angiotensin production plays a greater role in the initial adjustment of kidney function, compared to the circulating system, although this situation is far from clear.

In mammals, the RAS functions in the regulation of blood pressure, salt and water balance, renal and adrenocortical activity and therefore may play a central integrative role in body fluid homeostasis. In non-mammalian vertebrates the roles for AII remains to be fully elucidated. Tables 1.4, 1.5 and 1.6 lists some of the reported effects of AII on the vascular system, drinking and renal activity, respectively, in a variety of non-mammalian vertebrates.

#### 1.10.2. Effect of the RAS on cardiovascular system

It is important to maintain arterial blood pressure at a level sufficient to perfuse tissues. Administration of exogenous AII increases blood pressure in selected species of all vertebrate classes ( see Table 1.4 ). AII is found to be pressor in the hagfish ( *Myxine glutinosa* ) ( Carroll and Opdyke, 1982 ) and in the dogfish ( Opdyke and Holcombe, 1976; Hazon *et al.*, 1989 ). These pressor responses are abolished by the use of phentolamine, an  $\alpha$ -adrenergic receptor blocker, indicating these vertebrates depend on a catecholamine-mediated response to AII. Partially purified eel and rat renins produced prolonged

Table 1.4

Table 1.4     Vascular effects of Angiotensin in non-mammalian vertebrates

Summary of the effect of synthetic AII and homologous renal products on blood pressure in some representative non-mammalian vertebrates. RE, renal extract; RE + plasma, product of homologous renal extracts and plasma.  
( Modified from Armour, 1990 )

| Species   | Treatment  | Response        | -adrenergic blockade      | Reference                      |
|---|--|-----------------|---------------------------|--------------------------------|
| <u>Aves</u><br><i>Gallus domesticus</i>           | RE + plasma  | pressor         | -                         | Taylor <i>et al.</i> (1970)    |
| <i>Gallus domesticus</i>                          | AII  | pressor         | 100 % phentolamine        | Carroll and Opdyke (1982)      |
| <i>Gallus gallus</i>                              | Asp <sup>1</sup> -Val <sup>5</sup> -AI/AII               | pressor         | 60-70 % phenoxybenzamine  | Nishimura <i>et al.</i> (1982) |
| <u>Reptilia</u><br><i>Pseudemys swanniensis</i>   | RE, RE + plasma  | pressor         | -                         | Nothstine <i>et al.</i> (1971) |
| <i>P. scripta elegans</i>                         | synthetic Asp <sup>1</sup> -Ile <sup>5</sup> -AII        | pressor         | 50 % phenoxybenzamine     | Zehr <i>et al.</i> (1981)      |
| <i>Chrysemys scripta elegans</i>                  | AII  | pressor         | 80 % phentolamine         | Carroll and Opdyke (1982)      |
| <i>Caiman sclerops</i>                            | RE, RE + plasma  | RE only pressor | -                         | Nothstine <i>et al.</i> (1971) |
| <i>Ptyas koros</i>                                | RE, synthetic Asp <sup>4</sup> -Ile <sup>5</sup> -AI/AII | pressor         | AII, 50 % by phentolamine | Ho <i>et al.</i> (1984)        |
| <i>Alligator mississippiensis</i>                 | Asp <sup>1</sup> -Val <sup>5</sup> -Ser <sup>9</sup> -AI | pressor         | -                         | Silldorff and Stephens (1992)  |
| <u>Amphibia</u><br><i>Rana catesbiana</i>         | AII  | pressor         | 40 % phentolamine         | Carroll and Opdyke (1982)      |
| <i>R. catesbiana</i>                              | RE + substrate   | pressor         | -                         | Johnston <i>et al.</i> (1967)  |
| <u>Osteichthyes</u><br><i>Anguilla rostrata</i>   | RE + plasma, Asp <sup>1</sup> -Ile <sup>5</sup> -AI/AII  | pressor         | 30-40 % phentolamine      | Nishimura <i>et al.</i> (1978) |
| <i>Cyclopterus lumpus</i>                         | AII  | pressor         | 10 % phentolamine         | Carroll and Opdyke (1982)      |
| <i>Salmo gairdneri</i>                            | Asn <sup>1</sup> -Val <sup>5</sup> -AII                  | pressor         | -                         | Gray and Brown (1985)          |
| <u>Chondrichthyes</u><br><i>Squalus acanthias</i> | Goosefish AII  | pressor         | 100 % phentolamine        | Carroll (1981)                 |
| <i>Scyliorhinus canicula</i>                      | Asp <sup>1</sup> -Ile <sup>5</sup> -AII                  | pressor         | -                         | Hazon <i>et al.</i> (1989)     |
| <u>Agnatha</u><br><i>Myxine glutinosa</i>         | AII  | pressor         | 100 % phentolamine        | Carroll and Opdyke (1982)      |

pressor responses in intact and hypophysectomised European eel and in nephrectomised rats ( Henderson *et al.*, 1976 ) and synthetic Asn<sup>1</sup>, Val<sup>5</sup>- AII and semi-purified eel angiotensin increased blood pressure in FW American eel ( Nishimura and Sawyer, 1976 ). (Asp<sup>1</sup>-Val<sup>5</sup>)-AII also produced a pressor response in intact, hypophysectomised and stanniectomised eel ( *Anguilla anguilla* L. ) (Henderson *et al.*, 1976 ) and Gray and Brown ( 1985 ) observed a pressor response in (Asn<sup>1</sup>-Val<sup>5</sup>)-AII infused rainbow trout. Sar-AII produced neither agonistic or antagonistic response on blood pressor and an ACE inhibitor inhibited vasopressor responses to eel angiotensin in the American eel ( Nishimura *et al.*, 1978 ).

ACE hydrolysis of AI to AII is necessary for pressor activity ( Galardy *et al.*, 1984 ). ACE-like activity has been demonstrated in a wide variety of vertebrate organs, predominately respiratory and kidney ( Lipke and Olson, 1988 ). In rainbow trout ACE activity was observed in gill tissue and the Corpuscles of Stannius ( Olson *et al.*, 1986 ), and Polanco *et al.* (1990 ) demonstrated ACE activity in gill, heart and spleen of the carp ( *Cyprinus carpio* ). The gills, like mammalian lungs, are the only tissues to receive the entire cardiac output and also have an extensive vascular surface area. Thus gills could play an important role in the activation of AI. Recently ACE-like activity has been demonstrated in tissues of the elasmobranch, *Scyliorhinus canicula*, with the highest activity recorded in the gills ( Uva *et al.*, 1992 )

### 1.10.3 Effect of the RAS on drinking

Stimulation of water intake by exogenous AII has been examined in a variety of species from all classes of vertebrates ( see Table 1.5 ). The FW cyclostome *Lampetra japonica* does not drink water and (Asn<sup>1</sup>-Val<sup>5</sup>)-AII failed to induce drinking ( Kobayashi *et al.*, 1983 ), however, (Asp<sup>1</sup>-Ile<sup>5</sup>)-AII induced drinking in the dogfish, *Scyliorhinus canicula* ( Hazon *et al.*, 1989 ). Marine teleosts are faced with osmotic water loss, and thus they drink SW to compensate for their osmotic water loss. There are conflicting reports on the

Table 1.5

Table 1.5    Dipsogenic effects of Angiotensin in non-mammalian vertebrates

Summary of the effects of sythetic angiotensins and the converting enzyme inhibitor, captopril on drinking behaviour in some representative non-mammalian vertebrates.

\* denotes species capable of surviving in brackish.

( Modified from O'Toole, 1987 ).

| Species                                  | Habitat | Treatment                                      | Response    | Reference                      |
|--|---------|--|-------------|--------------------------------|
| <u>Aves</u>                              |         |  |             |                                |
| <i>Zunotrichia leucophrys gambelli</i>   |         | Val <sup>5</sup> -AII amide                    | drinking    | Wada <i>et al.</i> (1975)      |
| <i>Coturnix coturnix japonica</i>        |         | Val <sup>5</sup> -AII                          | drinking    | Takei (1977 a,b)               |
| <u>Reptilia</u>                          |         |  |             |                                |
| <i>Calotes versicolor</i>                |         | Asn <sup>1</sup> -Val <sup>5</sup> AII amide   | no drinking | Kobayashi <i>et al.</i> (1979) |
| <i>Kinosternon subrubrum</i>             |         | Asn <sup>1</sup> -Val <sup>5</sup> AII         | drinking    | Kobayashi <i>et al.</i> (1979) |
| <i>Elaphe quadrivirigata</i>             |         | Asn <sup>1</sup> -Val <sup>5</sup> AII         | drinking    | Kobayashi <i>et al.</i> (1979) |
| <u>Amphibia</u>                          |         |  |             |                                |
| <i>Rana brevipoda</i>                    | FW      | AII  | no drinking | Hirano <i>et al.</i> (1978)    |
| <i>Rana brevipoda</i>                    | FW      | dehydration                                    | no drinking | Hirano <i>et al.</i> (1978)    |
| <i>Rana temporaria</i>                   |         | captopril injection                            | drinking    | Bolton and Henderson (1987)    |
| <u>Teleostei</u><br>(Euryhaline species) |         |  |             |                                |
| <i>Anguilla japonica</i>                 | FW      | Asn <sup>1</sup> -Val <sup>5</sup> -AII        | drinking    | Takei <i>et al.</i> (1979)     |
| <i>A. japonica</i>                       | SW      | Asn <sup>1</sup> -Val <sup>5</sup> -AII        | drinking    | Takei <i>et al.</i> (1979)     |
| <i>A. japonica</i>                       | FW      | pithed/Asn <sup>1</sup> -Val <sup>5</sup> -AII | drinking    | Takei <i>et al.</i> (1979)     |
| <i>Platichthys flesus</i>                | FW      | AI/AII   | drinking    | Carrick and Balment (1983)     |
| <i>P. flesus</i>                         | SW      | AI/AII   | drinking    | Carrick and Balment (1983)     |
| seven brackish water species             | BW      | Asn <sup>1</sup> -Val <sup>5</sup> -AII        | drinking    | Kobayashi <i>et al.</i> (1983) |
| <i>Anguilla anguilla</i>                 | FW      | Asp <sup>1</sup> -Val <sup>5</sup> -AII        | drinking    | Perrott <i>et al.</i> (1992)   |
| (Freshwater species)                     |         |  |             |                                |
| <i>Cottus bairdi</i>                     | FW      | Asp <sup>1</sup> -Ile <sup>5</sup> -AII        | no drinking | Beasley <i>et al.</i> (1986)   |
| <i>Carassius auratus</i>                 | FW*     | Asp <sup>1</sup> -Ile-AII                      | no drinking | Beasley <i>et al.</i> (1986)   |
| <i>Carassius auratus</i>                 | FW      | Asn <sup>1</sup> -Val <sup>5</sup> -AII        | drinking    | Kobayashi <i>et al.</i> (1983) |
| (Seawater species)                       |         |  |             |                                |
| <i>Pleuronectes americanus</i>           | SW      | Asp <sup>1</sup> -Ile <sup>5</sup> -AII        | drinking    | Beasley <i>et al.</i> (1986)   |
| 13 species SW fish                       | SW      | Asn <sup>1</sup> -Val <sup>5</sup> -AII        | no drinking | Kobayashi <i>et al.</i> (1983) |
| <i>Mugil cephalus</i>                    | SW*     | Asn <sup>1</sup> -Val <sup>5</sup> -AII        | drinking    | Kobayashi <i>et al.</i> (1983) |
| <u>Elasmobranchii</u>                    |         |  |             |                                |
| <i>Scylorhinus canicula</i>              | SW*     | Asp <sup>1</sup> -Ile <sup>5</sup> -AII        | drinking    | Hazon <i>et al.</i> (1989)     |

action of exogenously administered AII in stenohaline marine and FW species. Kobayashi *et al.* ( 1983 ) stimulated drinking in the FW goldfish ( *Carassius auratus* ) by injecting (Asn<sup>1</sup>-Val<sup>5</sup>)-AII, while Beasley *et al.* ( 1986 ) obtained contrasting results after injection of (Asp<sup>1</sup>-Ile<sup>5</sup>)-AII. Some stenohaline marine species such as the winter flounder ( *Pseudopleuronectes americanus* ) and the longhorn sculpin ( *Myoxocephalus octodecemspinosus* ) demonstrated a dipsogenic response after injection of (Asp<sup>1</sup>-Ile<sup>5</sup>)-AII ( Beasley *et al.*, 1986 ) while others such as the grass puffer ( *Fugu niphobles* ) and the black rockfish ( *Sebastes inermis* ) apparently showed no response to Asn<sup>1</sup>-Val<sup>5</sup>-AII ( Kobayashi *et al.*, 1983 ). These differences may partly be a result of differences in technique and type of AII used. Using the sensitive isotope dilution method ( Balment and Carrick, 1985; Hazon *et al.*, 1989 ) Asp<sup>1</sup>-Val<sup>5</sup>-His<sup>9</sup>-AI or Asp<sup>1</sup>-Val<sup>5</sup>-AII was seen to be effective in stimulating drinking in the stenohaline FW carp, stenohaline marine fish, apart from the sea scorpion which has an extremely high basal rate of drinking, and euryhaline species such as the European eel ( *Anguilla anguilla* ) ( Perrott *et al.*, 1992 ). Intraarterial injection of (Asn<sup>1</sup>-Val<sup>5</sup>)- AII and semi-purified eel angiotensin accelerated drinking in water replete FW eels and dehydrated SW eels ( Takei *et al.*, 1979).

Although there remains some doubt concerning the exact role of endogenous renin and angiotensin in the control of drinking the dipsogenic response appears to rely on an intact RAS ( Carrick and Balment, 1983 ). Administration of papaverine ( smooth muscle relaxant ) or haemorrhage, may be used to manipulate the endogenous RAS through the stimulation of renin release. In the dogfish, *Scyliorhynchus canicula*, papaverine induced an increase in the low basal levels of drinking, an elevation which was reduced by the co-administration of captopril ( Balment *et al.*, 1987, Hazon *et al.*, 1989). In FW flounders papaverine lowered plasma osmolarity and chloride concentration, due to the stimulation of drinking and hence ingestion of FW

( Balment and Carrick, 1985 ). In FW-adapted flounder the administration of captopril alone had no effect on ingestion of water but it prevented the dipsogenic response to papaverine and decreased the normal high rate of drinking in the SW- adapted fish ( Balment and Carrick, 1985; Carrick and Balment, 1983 ). In the killifish, *Fundulus heteroclitus*, P-113 ( competitive inhibitor of AII ) and the ACE-inhibitor ( SQ 20881 ) caused a reduction in the drinking rate of the long-term SW-adapted animal and the acute SW-adapted animal and prevented AII-stimulated drinking ( Malvin *et al.*, 1980 ).

In order to determine renin release, plasma renin activity ( PRA ) is usually used as an index. The PRA is measured by the rate of AI formation after incubation of plasma under inhibition of the converting enzyme and angiotensinases ( Boucher *et al.*, 1967 ). The PRA of the SW-adapted European eel was double that found in the FW-adapted fish ( Henderson *et al.*, 1976 ). PRA was reported to increase within 0.5 - 8 hours after transfer of the Japanese eel from FW to SW ( Sokabe *et al.*, 1973 ), and reached SW levels 3 - 5 days after SW transfer of the European eel ( Henderson *et al.*, 1976 ). When the converse transfer was undertaken, PRA decreased in *Anguilla rostrata* after three days in FW ( Nishimura *et al.*, 1976 ) and within one day in the European eel ( Henderson *et al.*, 1976 ). Nishimura *et al.* ( 1976 ) found no change in the plasma renin concentration of the aglomerular toadfish, *Opsanus tau*, with transfer from 50 - 5% SW, although Capelli *et al.* ( 1970 ) reported an elevation in renin activity with decreased salinity, in the same species.

Bailey and Randall ( 1981 ) showed that in the rainbow trout, PRA was linearly correlated to the amount of haemorrhage, and cumulative haemorrhage or a single massive bleed from toadfish kept in 50% SW induced a stepwise increase in PRA ( Nishimura *et al.*, 1976 ). In the FW eel, *Anguilla japonica*, which had undergone oesophageal cannulation, drinking was induced by haemorrhage ( Hirano, 1974 ). Upon withdrawal of

approximately 30% of the total blood, the FW eel immediately drank for the next 15 hours with a very gradual return to the original drinking rate after 40 hours. Plasma AII concentration increased after acute haemorrhage which amounted to 14.3% blood volume in the rainbow trout ( Bailey and Randall, 1981 ). Furthermore, using the isolated nonfiltering perfused kidney preparation these authors found that a decrease in renal perfusion pressure resulted in an increase in renin release and postulated, therefore, that a baroreceptor mechanism may function in this fish.

AII effects on drinking appear to be most pronounced in euryhaline fish adapted to SW. Henderson *et al.* ( 1985 ) found a higher concentration of AII in the plasma of SW-adapted eel compared to the FW-adapted eel. When *Anguilla japonica* was transferred from FW to SW drinking was induced after 15 min, and AII concentration increased after two hours ( Okawara *et al.*, 1987 ).

The site of action for AII stimulated drinking is not clear. Evidence has been accumulated indicating osmoreceptors mediating drinking are found in the hypothalamus ( Peck and Blass, 1975 ). Hirano *et al.* ( 1972 ) demonstrated that in the " decerebrated " eel adapted to both FW and SW the water intake was at almost the same rate as in the intact eel during the course of SW-adaptation. ( Asn<sup>1</sup>-Val<sup>5</sup> )-AII induced drinking in eel in which forebrain and midbrain regions were destroyed, whereas vagotomized eels failed to respond to the administration of AII ( Takei *et al.*, 1979 ). These studies suggest that drinking in the SW eel is regulated at a lower level in the central nervous system, perhaps the medulla oblongata.

#### 1.10.4 Effect of the RAS on renal function

In non-mammalian vertebrates renal responses vary, as renal function is very sensitive to differences in GFR due to changes in systemic blood

pressure. Therefore, in these groups the wider systemic effects of AII may also affect renal excretory patterns ( Balment and Henderson, 1987 ) ( Table 1.6 ).

When investigating the biological significance of the RAS, renal renin content seems to be a better indicator of functionality than PRA, on the basis of reports in the literature which show wide variability in values of PRA, and which are therefore difficult to compare with each other ( Henderson *et al.*, 1976; Sokabe *et al.*, 1975 ). Renal renin content is generally higher in FW-adapted than in SW-adapted teleosts ( Capelli *et al.*, 1970; Mizogami *et al.*, 1968 ), perhaps a reflection of the lower PRA as previously discussed. It has been suggested that the decreased renal renin content of SW- adapted fish may be involved in the decrease of GFR and urine volume ( UV ) that occurs with transfer from FW to SW of euryhaline teleosts. Bailey and Randall ( 1981 ) showed that in the rainbow trout, PRA was linearly correlated to the amount of haemorrhage. Furthermore, using the isolated nonfiltering perfused kidney preparation, these researchers observed that a decrease in renal perfusion pressure resulted in an increase in renin release. They postulated therefore that an intrarenal baroreceptor mechanism may function in this fish ( Bailey and Randall, 1981 ). Pressor and relatively high doses of [Asp<sup>1</sup>-Val<sup>5</sup>]-AII produced glomerular diuresis and natriuresis in FW-adapted eels ( Nishimura and Sawyer, 1976 ) and lungfishes ( Sawyer *et al.*, 1976 ).

The physical changes observed upon injection of AII are similar to those observed in SW. As previously described, Brown *et al.* ( 1978, 1980 ) reported the presence of three types of nephrons, filtering, perfused but not filtering and non-perfused. FW trout had a greater percentage of filtering nephrons ( also indicated by elevated TMG ) and higher GFR, but a lower SNGFR compared to SW-adapted fish ( Section 1.5.1b ). Infusion of [Asn<sup>1</sup>-Val<sup>5</sup>]-AII caused a sustained antidiuresis and a shift in emphasis from filtering nephrons to non-perfused nephrons in both FW-adapted and SW-adapted trout. SNGFR was unaffected in FW trout, but was reduced to the

Table 1.6

Table 1.6     Renal effects of Angiotensin in non-mammalian vertebrates

Summary of the effects of synthetic angiotensin II (AII) and the converting enzyme inhibitor on renal function in some representative non-mammalian vertebrates. Inf, infusion; inj, injection; PD, pressor dose; N, natriuresis; D diuresis; AN, anti-natriuresis; AD, antidiuresis.

( Modified from O'Toole, 1987 )

Table 1.6 Renal effects of Angiotensin in Non-mammalian Vertebrates

| Species  | Experimental Conditions                            | Glomerular Effect | Tubular Effect | Reference                      |
|--|--|-------------------|----------------|--------------------------------|
| <u>Aves</u><br><i>Gallus domesticus</i>  | AII-amide  | -                 | D + N          | Langford and Fallis (1966)     |
| <u>Reptilia</u><br><i>Pseudemys scripta</i>  | Asn <sup>1</sup> -Val <sup>5</sup> -AII infus (PD) | ↓GFR              | -              | Brown <i>et al.</i> (1983)     |
| <u>Amphibia</u><br><i>Xenopus laevis</i>   | AII-amide AII inf                                  | GFR constant      | D + N          | Henderson and Edwards (1969)   |
| <u>Osteichthyes</u><br><u>Teleostei</u><br>(Agglomerular)<br><i>Lophius americanus</i> | synthetic AII-amide infus                          | -                 | N + D          | Churchill <i>et al.</i> (1979) |
| (Glomerular)<br><i>Anguilla rostrata</i>   | AII infus (PD)                                     | ↑ GFR , N + D     | -              | Nishimura <i>et al.</i> (1976) |
| <i>Salmo gairdneri</i> (FW)  | capt. infus  | ↑ GFR , D         | -              | Henderson <i>et al.</i> (1980) |
| <i>Salmo gairdneri</i> (SW)  | capt. infus  | ↑ GFR ; D         | -              | Kenyon <i>et al.</i> (1985)    |
| <i>Salmo gairdneri</i> (FW)  | Asn <sup>1</sup> -Val <sup>5</sup> -AII            | ↓ GFR<br>TMG      |                | Brown <i>et al.</i> (1980)     |
| <i>Salmo gairdneri</i> (SW)  | Asn <sup>1</sup> -Val <sup>5</sup> -AII            | ↓ GFR<br>TMG      |                | Brown <i>et al.</i> (1980)     |
| <u>Dipnoi</u><br><i>Protopterus aethopius</i>  | Val <sup>5</sup> -AII-amide inf.                   | moderate N + D    | -              | Sawyer (1970)                  |
| <i>Neoceratodus forsteri</i>   | Val <sup>5</sup> -AII-amide inf.                   | D                 | -              | Sawyer <i>et al.</i> (1976)    |

FW level in SW trout ( Brown *et al.*, 1980 ). These experiments, however, involved the addition of norepinephrine to the infusate in order to maintain blood pressure and urine production rates in the anaesthetised fish. [Asn<sup>1</sup>-Val<sup>5</sup>]-AII infusions did not cause a pressor response in either the FW- or SW-adapted trout and so any systemic effect may have been masked by a near maximal norepinephrine-induced vasoconstriction. Gray and Brown ( 1985 ) subsequently investigated renal effects of [Asn<sup>1</sup>-Val<sup>5</sup>]-AII in the absence of norepinephrine. An initial antidiuresis and reduction in GFR was observed, although these were not sustained for the duration of the experiment. The TMG also showed an initial decrease, with only a partial recovery to control levels observed. Since GFR fully recovered and TMG only partially recovered this study suggests an increase in SNGFR in order to compensate for the reduced number of filtering nephrons. The implied increase in SNGFR may be a result of a systemic pressor response to AII previously unseen due to the presence of norepinephrine. GFR may be the result of a balance between SNGFR and the number of filtering nephrons, one compensating for the other and so GFR recovered.

#### 1.10.5 Interaction between the RAS and other hormones in control of water and electrolytes

The RAS can influence the secretion or synthesis of other hormones that are important for hydromineral homeostasis. In mammals the RAS appears to influence aldosterone production via a feedback loop, whereby renin secretion is altered as a result of information obtained from the JG apparatus or through the MD and /or sympathetic nerve. Resultant changes in angiotensin formation alter aldosterone secretion, which then influences renal Na retention and K excretion ( Nishimura, 1987).

In non-mammalian vertebrates the role of the RAS in the stimulation of corticosteroid production is not as clear as in mammals. Both Val<sup>5</sup>-AII and

Ile<sup>5</sup>-AII stimulated steroidogenesis in the isolated perfused interrenal gland preparation of the dogfish ( O'Toole *et al.*, 1990 ). AII did not stimulate corticosteroid release in *in vitro* preparations of interrenal tissue in trout, but it did act in synergy with other secretagogues ( Decourt and Lahlou, 1987 ). Injection of [Asp<sup>1</sup>-Val<sup>5</sup>]-AII or eel renin preparation into intact or hypophysectomised eels, *Anguilla anguilla*, increased plasma cortisol concentration (Henderson *et al.*, 1976; Perrott and Balment, 1990 ). However, SW adaptation only transiently ( 24-48 hours ) increased plasma cortisol in eels ( Ball *et al.*, 1971 ) , whereas PRA increased slowly and steadily over 3 - 5 days ( Henderson *et al.*, 1976 ). The effects of the administration of the ACE inhibitor, captopril, on plasma cortisol concentrations are not clear. In the European eel, captopril was demonstrated to augment rather than hinder the initial cortisol increase obtained with FW-SW transfer ( Kenyon *et al.*, 1985 ), while in the euryhaline flounder, *Platichthys flesus*, maintained in 50% SW, captopril was successful in blocking the papaverine-induced plasma cortisol concentration increase ( Perrott and Balment, 1990 ). Plasma renin activity in eels fully adapted to SW was greater than that in eels fully adapted to FW ( Henderson *et al.*, 1976 ). When eels were transferred from SW to FW the plasma cortisol levels were unchanged while PRA decreased ( Nishimura *et al.*, 1976 ). Angiotensin administration produced a transient increase in plasma cortisol concentration in free swimming flounder ( Perrott and Balment, 1990 ). However, it is possible that the time course of changes in RAS activity may not directly correlate with changes in corticosteroid concentration. To date the possible relationship between RAS activity and cortisol secretory dynamics has not been investigated.

Exogenous AVT reduced PRA activity by 60% in FW eel ( Henderson *et al.*, 1985 ) and so inhibition of the RAS in this fish may be partly mediated by AVT. The effect of ANP on RAS is as yet unclear. In the SW-adapted flounder, ANP administration caused hypotension and stimulation of

cortisol secretion ( Arnold-Reed *et al.*, 1991 ). The hypotension and subsequent recovery in blood pressure observed in that study is likely to have stimulated the RAS as well as cortisol secretion.

#### 1.10.6 Angiotensin receptor studies

Angiotensin II binding sites have been identified by radioreceptor assay or autoradiography in almost all of the vertebrate tissues on which AII actions have been reported. For instance AII receptors have been characterised in mammalian vascular tissues by ligand binding studies ( Regoli *et al.*, 1974 ; McQueen *et al.*, 1984 ) and in rat glomeruli ( Sraer *et al.*, 1974). Radioligand studies using  $^{125}\text{I}$ -AI identified specific binding sites for AI with low affinity in several target organs of AII ( Goodfriend *et al.*, 1972 ), but it is not yet determined whether AI and AII bind to the same receptor. The presence of AIII binding sites have been demonstrated in the gerbil brain where a lack of AII binding was observed ( Harding *et al.*, 1981 ). Two distinct AII receptor subtypes have been characterised by utilising recently discovered structurally dissimilar, nonpeptide receptor AII antagonists. AII receptors may be divided into two types according to the affinity to different antagonists; type 1 receptors (  $\text{AT}_1$  ) which have high affinity to DuP 753 and type 2 (  $\text{AT}_2$  ) which have high affinity to EXP655 or PD 123319 ( Chiu *et al.*, 1989; Pucell *et al.*, 1991 ).

The information regarding AII receptors in non-mammalian species is scarce, despite numerous studies on its physiological actions. There seems to have been an evolution of receptor sites for AII. Mammals, birds and reptiles, but not amphibians ( Hirano *et al.*, 1978 ), have dipsogenic receptors in the brain responsive to AII ( Fitzsimmons, 1980 ). It has been suggested that dipsogenic receptors for angiotensins appeared at a later evolutionary stage than the amphibia. However, euryhaline teleosts, such as the eel, do respond to the hormone by drinking ( Hirano *et al.*, 1978; Perrott *et al.*, 1992 ).

Moreover, the dipsogenic receptors of teleost fish may occur in a different part of the brain, the medulla oblongata, from terrestrial vertebrates. Thus receptors for angiotensin have evolved independently at least twice during the course of vertebrate evolution.

In the rainbow trout, [Asn<sup>1</sup>-Val<sup>5</sup>]-AII decreased GFR by decreasing the number of filtering glomerulus when acclimated in FW, and by decreasing SNGFR when acclimated in SW ( Brown *et al.*, 1980; Gray and Brown, 1985 ). Specific [Asn<sup>1</sup>-Val<sup>5</sup>]-AII binding was observed in the glomerulus of rainbow trout by autoradiography ( Brown *et al.*, 1990 ). Other binding sites for AII were distributed in a variety of tissues including aorta, heart, brain, gills, digestive tract, liver, renal proximal tubules, urinary bladder, and adrenocortical tissue ( Cobb and Brown, 1992 ).

### 1.11 Natriuretic peptides

Since the first discovery of a natriuretic factor in the rat atrium ( De Bold *et al.*, 1981), there has been considerable interest in what is now known to be a family of natriuretic peptide hormones. To date, this family consists of atrial natriuretic peptide ( ANP ), brain natriuretic peptide ( BNP ) ( Needleman *et al.*, 1989 ), C-type natriuretic peptide ( CNP ) (Suzuki *et al.*, 1991b ), and ventricular natriuretic peptide ( VNP ) ( Takei *et al.*, 1991 ) ( see Figure 1.15 ). Atrial natriuretic peptide was the first of the family to be isolated and therefore the vast majority of research into the natriuretic peptides has been carried out with ANP.

In mammals ANP is synthesised as a prehormone which consists of 151/152 amino acids, in atrial myocytes. Cleavage of the amino-terminal "signal" sequence yields pro-ANP-( 1-126 ), the major storage form of the peptide. Bioactive peptides are derived from the carboxy-terminus, with the predominant circulating form being ANP-( 99-126 ), more commonly known as ANP-( 1-28 ) ( Figure 1.16 ) ( Brenner *et al.*, 1990 ).

Figure 1.15

Figure 1.15 Amino acid sequence of selected members of the Natriuretic peptide  
( Modified from Takei and Balment 1993)

Atrial ( A- type ) natriuretic peptide

|          |      |      |       |     |      |    |      |          |       |
|----------|------|------|-------|-----|------|----|------|----------|-------|
| eel      | SKS  | SS P | CFG   | GKL | DRIG | SY | SG L | GCNS     | - R K |
| bullfrog |      | SS D | CFG   | SRL | DRIG | AC | SG M | GC - G - | R RF  |
| man      | SLRR | SS   | - CFG | GRM | DRIG | AC | SG L | GCNSFR   | - Y   |
| rat      | SLRR | SS   | - CFG | GRI | DRIG | AC | SG L | GCNSFR   | - Y   |

Brain ( B- type ) natriuretic peptide

|      |        |      |     |       |     |      |        |      |    |     |      |
|------|--------|------|-----|-------|-----|------|--------|------|----|-----|------|
| fowl | M      | MRD  | S G | - CFG | RRI | DRIG | S L S  | GM   | GC | NGS | R KN |
| dog  | SPK-M  | MHK  | S G | - CFG | RRL | DRI  | S L S  | GL   | GC | NVL | R KY |
| man  | SPK-M  | V QG | S G | - CFG | RRM | DRI  | SS S S | GL   | GC | KVL | R KH |
| rat  | S- K-M | A HS | S S | - CFG | QKI | DRI  | GAV    | S RL | GC | DGL | R LF |

C-type natriuretic peptide

|           |             |     |   |    |      |     |       |
|-----------|-------------|-----|---|----|------|-----|-------|
| dogfish   | G P S R G - | CFG | V | KL | DRIG | AM  | SGLGC |
| eel       | G W N R G - | CFG | L | KL | DRIG | S L | SGLGC |
| killifish | G W N R G - | CFG | L | KL | DRIG | SM  | SGLGC |
| bullfrog  | G Y S R G - | CFG | V | KL | DRIG | AF  | SGLGC |
| fowl      | G L S R S - | CFG | V | KL | DRIG | SM  | SGLGC |
| man       | G L S K G - | CFG | L | KL | DRIG | SM  | SGLGC |

Ventricular natriuretic peptide

|     |             |     |       |      |             |      |         |
|-----|-------------|-----|-------|------|-------------|------|---------|
| eel | K S F N S - | CFG | T R M | DRIG | SWSGLGCNSL- | KNGT | KKKIFGN |
|-----|-------------|-----|-------|------|-------------|------|---------|

Figure 1.16

Figure 1.16 Transcription and translation of the atrial natriuretic peptide gene  
( From Brenner *et al.*, 1990 )



Although it is generally accepted that atrial distension is a major stimulus of ANP release, presumably due to elevation of venous blood pressure, the mechanisms that regulate ANP release are poorly understood. In mammals ANP affects blood pressure and volume, kidney function and smooth muscle relaxation ( De Bold *et al.*, 1981 ). The combined effects of ANP on vasculature, kidneys and adrenals serve both acutely and chronically to reduce systemic blood pressure as well as intravascular volume. The reduction in blood pressure is the consequence of reduced peripheral vascular resistance (partly mediated by direct relaxation of vascular smooth muscle ), diminished cardiac output, and decreased intravascular volume. In the kidney, ANP acts on specific receptors in renal vasculature and tubule epithelium to induce hyperfiltration, inhibition of Na<sup>+</sup> transport and suppression of renin release, all of which are effects responsible for natriuresis, diuresis, and produce a subsequent reduction in blood volume and blood pressure. Atrial natriuretic peptide also acts to lower blood pressure and blood volume by inhibiting aldosterone secretion, both indirectly by inhibiting renin secretion and directly by acting on adrenal glomerulosa cells ( Genest and Cantin, 1988; Brenner *et al.*, 1990 ).

Atrial natriuretic peptide often acts in association with other hormones in mammals and has been shown to inhibit basal aldosterone secretion, and aldosterone secretion stimulated by a variety of agents, including AII, ACTH, dibutyryl cAMP and potassium ( Elliott and Goodfriend 1986; Atarashi *et al.*, 1984; Chartier *et al.*, 1984; De Lean *et al.*, 1984; Metzler and Ramsay, 1989; Campbell *et al.*, 1985 ). Atrial natriuretic peptide also inhibited basal, as well as KCl or AII- stimulated, immunoreactive AVP secretion from rat posterior pituitary ( Obana *et al.*, 1985 ). However, Baylis and Burrell ( 1991 ) have questioned the physiological significance of numerous studies which indicate that high doses of atrial peptides administered either peripherally or intracerebroventricularly appear to reduce osmoregulated vasopressin

secretion and drinking. Their studies show that a moderate elevation of plasma ANP has no effect on osmoregulated ANP but may blunt thirst appreciation.

In contrast to the mammalian situation, ANP studies on lower vertebrates have been less extensive. Binding sites for ANP were reported in the bladder and kidney of the toad, *Xenopus laevis* ( Kloas and Hanke, 1992 ). In this study specific binding sites were observed only in the glomeruli and in the adrenal tissue of the kidney, and osmotic stress of acclimation to 1.5% salt water increased renal but not adrenal ANP binding.

#### 1.11a Atrial natriuretic peptide in fish

The majority of studies on ANP effects in fish have been carried out using heterologous peptides or antibodies raised against mammalian ANP or BNP, however the sequences of some teleost and elasmobranch species have recently become known ( Figure 1.15 ). Using antibodies raised against human ANP ( hANP ) ( Evans *et al.*, 1989 ), or rabbit ANP ( Chapeau *et al.*, 1985 ), immunoreactive ANP ( ANP<sub>IR</sub> ) has been found in plasma, atria and ventricles of a variety of teleost, chondrichthyan and agnathan fishes. In the teleost *Gila atraria* ( Westenfelder *et al.*, 1988 ), *Squalus acanthias*, *Scyliorhinus canicula*, *Raja clavata* and *Chimaera monstrosa* ( Reinecke *et al.*, 1987 ), electron micrographs of atrial and ventricular cardiocytes demonstrated many perinuclear granules, resembling mammalian atrioocytes ANP- containing secretory granules. Binding sites for mammalian ANP have been demonstrated in heart, renal system and aorta of hagfish, *Myxine glutinosa* ( Kloas *et al.*, 1988 ).

The sequence of eel ANP is shown in Figure 1.15. Eel ANP consists of 27 amino acid residues and has a 59% sequence homology to  $\alpha$ -hANP. In terms of the potency of eANP compared to  $\alpha$ -hANP, eANP is 110 times more potent in eels, nearly equipotent in quail and 20 times less potent in rats

( Takei *et al.*, 1989 ).

Results obtained using heterologous ANP will be reported first, with homologous studies discussed thereafter. Table 1.7 lists some of the reported effects of ANP in fishes.

#### 1.11ai Heterologous studies

Using heterologous RIAs, teleost plasma ANP concentrations are reported to be greater in SW- adapted compared to FW- adapted animals and to decrease after SW fish were transferred to hypertonic media ( Westenfelder *et al.*, 1988; Evans *et al.*, 1989; Smith *et al.*, 1992 ) and so it appears that salt loading rather than volume expansion may be the primary stimulus for ANP

Intraarterial injection of synthetic ANP or trout heart extract may produce a bi-phasic pressor-depressor response ( Duff and Olson, 1986 ), no response at all ( Eddy *et al.*, 1990 ), or a depressor response ( Olson and Duff, 1992 ). The variability of this response may be due to the rate of ANP administration as a rapid ANP injection produced pressor responses ( Duff and Olson, 1986 ), slower injection of approx 10 min ( Eddy *et al.*, 1990 ) had no effect, and low level, constant infusion of more than one hour ( Olson and Duff, 1992 ) caused a delayed but steady decline in arterial pressure. The reason for this variability is unknown but may be due to differences in ANP plasma concentrations caused by the different injection techniques. The administration of hANP to flounder adapted to SW causes hypotension ( Arnold-Reed *et al.*, 1991a )

Ando *et al.*, ( 1992 ) proposed that ANP may act to lower plasma NaCl level in marine teleost by increasing NaCl extrusion through the gills and inhibiting NaCl uptake through the intestine, since Scheide and Zadunaisky ( 1988 ) reported that rat ANP ( 3-28 ) stimulated the short-circuit current through modulation of Cl<sup>-</sup> secretion across opercular epithelium of SW killifish. However, in flounder epithelium, rat ANP ( 5-28 ) had no effect on

Table 1.7

Table 1.7 Effects of atrial natriuretic peptide in fishes  
Summary of the effects of atrial natriuretic peptides and homologous heart extracts in some teleosts and elasmobranchs.

AP, atriopeptin; ANP, atrial natriuretic peptide; HE, homologous heart extract; GFR, glomerular filtration rate; UFR, urine flow rate.

( Modified from Armour, 1990 ).

| Species                                  | Test substance                        | Response  | Reference                           |
|--|---------------------------------------|---|-------------------------------------|
| <u>Teleostei</u>                         |                                       |   |                                     |
| <i>Salmo gairdneri</i><br>(FW)           | synthetic ANP, HE                     | vasopressor   | Duff and Olson<br>(1986)            |
| <i>Opsanus tau</i>                       | APIII, HE                             | vasopressor<br>(HE only)                                  | Lee and Malvin<br>(1987)            |
| <i>S. gairdneri</i> (FW)                 | human ANP                             | ↓ pulse pressure  | Eddy <i>et al</i> (1990).           |
| <i>S. gairdneri</i> (FW)                 | rat ANP                               | vasodilation, arterial<br>rings                           | Olson and Meisheri<br>(1989)        |
| <i>Opsanus beta</i>                      | rat ANP                               | vasodilation, arterial<br>rings                           | Evans <i>et al.</i> (1989).         |
| <i>S. gairdneri</i> (FW)                 | synthetic ANP, HE                     | diuresis, natriuresis                                     | Duff and Olson<br>(1986)            |
| <i>Pseudopleuronectes<br/>americanus</i> | API, APIII                            | ↓ intestinal Na<br>absorption                             | O'Grady <i>et al</i> (1985)         |
| <i>Fundulus<br/>heteroclitus</i>         | rat ANP                               | ↑ opercular Cl<br>secretion                               | Scheide and<br>Zadunaisky (1988)    |
| <i>S. gairdneri</i><br>(FW, SW)          | eel ANP(infus)and<br>rat ANP          | vasodepressor   | Olson and Duff<br>(1992)            |
| <i>S. gairdneri</i><br>(FW, SW)          | eel ANP, rat ANP<br>(bolus injection) | biphasic<br>pressor/depressor.<br>diuresis<br>(eANP only) | Olson and Duff<br>(1992)            |
| <i>Platichthys flesus</i>                | hANF                                  | ↑ cortisol secretion                                      | Arnold-Reed and<br>Balment (1991)   |
| <i>Platichthys flesus</i>                | hANF                                  | vasopressor, Na <sup>+</sup><br>efflux ↑                  | Arnold-Reed <i>et al.</i><br>(1991) |
| <i>Anguilla japonica</i>                 | eANP                                  | ↓ intestinal NaCl and<br>water absorption                 | Ando <i>et al.</i> (1992).          |
| <u>Elasmobranchii</u>                    |                                       |   |                                     |
| <i>Scyliorhinus<br/>canicula</i>         | human ANP                             | vasodepressor   | Hazon <i>et al.</i> (1987)          |
| <i>Squalus acanthias</i>                 | APII                                  | vasodepressor   | Benyajati and<br>Yokota (1990).     |
| <i>S. acanthias</i>                      | APII                                  | ↓ GFR and UFR<br>100% SW                                  | Benyajati and<br>Yokota (1988)      |
| <i>S. acanthia</i>                       | APII                                  | ↑ GFR and UFR 70%<br>SW                                   | Solomon <i>et<br/>al.</i> (1988)    |
| <i>S. canicula</i>                       | human ANP                             | 1α -OH-B secretion  | Hazon <i>et al.</i> (1987)          |

the potential difference ( O'Grady *et al.*, 1985 ). Gill permeability to water of trout *in vitro* was unaffected by ANP ( Olson and Meisheri, 1989 ), but binding to chloride cells has been demonstrated in both FW and SW eel ( Broadhead *et al.*, 1989 ).

A decrease in NaCl and water absorption across the SW eel intestine was observed after perfusion of hANP and rat ANP (rANP) through isolated intestine mounted in a Ussing chamber ( Ando *et al.*, 1992 ). In the rainbow trout ( *Oncorhynchus mykiss* ), toadfish ( *Opsanus tau* ) and *Gilia atraria*, mammalian ANP or homologous heart extract caused diuresis and natriuresis ( Duff and Olson, 1986; Lee and Malvin, 1987; Westenfelder *et al.*, 1988 ). ANP-mediated diuresis, therefore, is found in teleosts with or without glomeruli. In trout, rat ANP increased urine flow and electrolyte excretion irrespective of a hypertensive ( Duff and Olson, 1986 ) or hypotensive ( Olson and Duff, 1992 ) cardiovascular response.

ANP resulted in vasodilation of trout, *Oncorhynchus mykiss*, gill, muscle-kidney, and splanchnic bed precontracted with epinephrine ( Olson and Meisheri, 1989) and the isolated head of *Opsanus beta* precontracted with carbachol ( Evans *et al.*, 1989 ). Tissues which were not precontracted in the study of Olson and Meisheri ( 1989 ), and the rectal gland of *Squalus acanthias* ( Solomon *et al.*, 1985 ) did not respond to ANP.

#### 1.11aii Homologous studies

In contrast to the studies utilising heterologous ANP for the determination of plasma ANP levels, Takei and Balment ( 1993 ) using a homologous antibody for eel ANP reported a decrease in eel plasma ANP concentration with FW to SW transfer. Since the discovery that ANP belongs to a family of natriuretic peptides it is unclear whether the heterologous RIAs previously employed measured ANP, VNP or CNP in the fishes, but it seems

clear that plasma levels of natriuretic peptides are affected by changes in salinity ( Takei, 1992 ).

Injection of eel ANP was observed to produce systemic hypotension in the eel, with greater efficacy than hANP ( Arnold-Reed *et al.*, 1991 ). Injecting eel ANP to FW eels did not produce the diuresis previously reported to occur after administration of heterologous ANP, but caused antidiuresis ( Takei and Balment, 1993 ), an effect which became evident at 2 nmol/kg eel ANP. Eel ANP was more potent than hANP or rANP in producing a decrease in NaCl and water absorption across SW eel intestine ( Ando *et al.*, 1992 ) and was found to be a more potent inhibitor than acetylcholine, serotonin and histamine.

#### 1.11b Interaction between atrial natriuretic peptide and other hormones in fish

The interaction of ANP with other endocrine systems in fish is just beginning to be evaluated. In the perfused dogfish, *Scyliorhinus canicula*, interrenal preparation ANP stimulated the release of 1- $\alpha$ -hydroxycorticosterone ( O'Toole *et al.*, 1990 ). In SW-adapted flounder hANP increased plasma cortisol concentration ( Arnold-Reed *et al.*, 1991a; Arnold-Reed and Balment, 1991 ), and stimulated cortisol release by perfused interrenal tissue from SW but not FW trout ( Arnold-Reed and Balment, 1991 ). This apparent unresponsiveness of FW interrenal tissue is interesting as Balment and Lahlou ( 1987 ) had previously observed that ANP had no consistent effect on nucleotide production in isolated trout gill cells derived from FW fish, but caused a response in SW gill cells.

#### 1.11c Brain, C-type and ventricular natriuretic peptides

ANP belongs to a family of natriuretic peptides. BNP was initially isolated from porcine brain ( Sudoh *et al.*, 1988 ), although it is now thought

not to be primarily of brain origin, as the content of BNP in porcine brain is less than that in atria. CNP was isolated from the killifish and eel brains ( Price *et al.*, 1990; Takei *et al.*, 1990 ) and appears to be the major peptide in the brain, although a substantial amount was isolated from both cardiac atria and ventricles of the dogfish, *Scyliorhinus canicula* ( Suzuki *et al.*, 1991b ) indicating that it may also be a circulating peptide ( Evans *et al.*, 1993). The C type natriuretic peptides isolated from both *Scyliorhinus canicula* and *Squalus acanthias* are reported to produce vasodilatory effects *in vivo* and *in vitro* in the respective dogfish ( Bjenning *et al.*, 1992; Evans *et al.*, 1993 ). The homologous CNP for *Squalus acanthias* stimulated chloride secretion in the rectal gland ( Solomon *et al.*, 1992 ). Takei *et al.* ( 1991 ) isolated a new eel natriuretic peptide, which they call ventricular natriuretic peptide ( VNP ). However, there is the possibility that eel VNP belongs to the B-type natriuretic peptide, as the B-type natriuretic peptide in eel and the presence of a VNP type in mammals have yet to be verified. Specific radioimmunoassays for eel ANP, VNP and CNP have been utilised by Takei and Balment ( 1993 ). They found that plasma ANP, VNP and CNP levels declined two weeks post-SW transfer. Eel ANP and VNP inhibited drinking in both FW and SW eels, and both peptides produced an antidiuresis in FW eels. Plasma cortisol concentrations increased after administration of eel ANP to SW- adapted eels, but not to FW-adapted eels. Eel VNP had no effect on cortisol levels. It appears that each of the eel natriuretic peptides is involved in osmoregulation in the eel.

### 1.12 Caudal neurosecretory system in fishes

Teleosts and elasmobranchs possess a caudal neurosecretory system ( caudal-NS ), which is located in the terminal spinal cord segments. There is no evidence for the presence of caudal neurosecretory cells in tetrapod spinal cords ( Bern, 1969 ). The caudal-NS consists of an array of large peptide-

producing neurones which in teleosts aggregate into an organ, the urophysis, at the level of the last vertebral unit ( Larson and Bern, 1987 ).

The caudal-NS synthesises and releases at least two peptide hormones, urotensins I and II ( UI and UII, respectively ). Urotensin I and UII have been chemically characterised and show some homology with corticotrophin releasing factor ( CRF ) and somatostatin, respectively ( Kobayashi *et al.*, 1986 ). Urotensin I belongs to a peptide family which is cysteine free and is composed of 41 amino acid residues. Urotensin II consists of 12 amino acids residues and contains two hemicystinyl residues ( Bern, 1985 ). The urophysis of teleosts is richly vascularised ( Kobayashi *et al.*, 1986 ).

There is some support for the involvement of the caudal-NS in osmoregulation of fish. Various studies report the sensitivity of the caudal-NS to changes in the external environment. Differences in the neurosecretory granule appearance and activity after osmotic challenge have been observed in various teleosts, such as flounder, *Platichthys flesus*, ( Arnold-Reed and Balment, 1991a,b ), trout, *Salvelinus fontinalis*, ( Chevalier, 1976, 1978 ) the black molly, *Mollienesia sphenops*, ( Kriebel, 1980 ). There is some confusion over the response of the caudal-NS to osmotic challenge in these studies. The urophysis appeared depleted of neurosecretory material in the black molly ( Kriebel, 1980 ) and the trout ( Chevalier, 1976, 1978 ) transferred from FW to SW, whereas in the flounder ( Arnold-Reed and Balment, 1991a ) an elevated peptide content in SW compared to FW adapted fish was demonstrated by morphological, gel electrophoresis and bioassay techniques. The significance of these apparently conflicting reports is as yet unknown. These studies were limited, however, in that they did not distinguish between the UI and UII components of the system

Using electrodes to record extracellular impulses of the caudal neurosecretory units in *Tilapia mossambicus*, during intravenous ( i.v. )

injection of hypotonic (  $\text{Na}^+$ -free ) and hypertonic solutions, it has been found that the caudal-NS is responsive to the concentration of sodium salts and not to osmotic pressure itself ( Yagi and Bern, 1965 ). This study suggests the presence of at least two types of neurosecretory units, one activated by high  $\text{Na}^+$  concentration and a second activated by a  $\text{Na}^+$ -free solution.

Bern ( 1985 ) postulated that the urotensins may influence the osmoregulatory capabilities of teleosts by vascular effects. Both peptides are vasopressor in teleosts, with UII being approximately ten times more potent than UI in increasing fish blood pressure ( Chan and Bern, 1976 ). UII stimulates caudal venous pressure in the eel ( Chan, 1975 ). Thus UII in particular could affect ion and water transport rates in various osmoregulatory organs such as gill, kidney, intestine and skin by modulation of blood flow ( Larson and Bern, 1987 ).

In FW-adapted fish, UI inhibited anterior intestinal NaCl and water absorption ( Mainoya and Bern, 1984 ) and UII stimulated posterior intestinal NaCl absorption ( Loretz, 1983 ). Urotensin II stimulated sodium and chloride absorption by the posterior intestine of the goby, *Gillichthys mirabilis*, adapted to 5% SW ( Loretz and Bern, 1981 ). This increase was thought to be a result of excess apical ion influx rather than increased  $\text{Na}^+$ - $\text{K}^+$ -ATPase activity. In SW-adapted fish UII stimulated anterior intestinal water and NaCl absorption ( Mainoya and Bern, 1984 ), UI stimulated and UII inhibited active chloride efflux from the skin of the goby.

In general urophysial extracts increased urine flow and excretion of at least sodium ( Maetz *et al.*, 1964 ; Bern *et al.*, 1967; Chester Jones *et al.*, 1969 ). These effects may be explained by the vasopressor activities of UII ( and of higher doses of UI ) ( Chan, 1975 ). Both urotensins stimulated active sodium absorption in the urinary bladder, although UII was by far the more potent ( Loretz and Bern, 1981 ).

The urotensins may also influence the osmoregulatory functions by regulating the secretion of other osmoregulatory hormones, such as cortisol and/or prolactin ( Bern, 1985 ). In the euryhaline flounder a role for the caudal-NS in the regulation of the interrenal secretion of cortisol has been demonstrated by Arnold-Reed and Balment ( 1989 ). In the FW tilapia, *Sarotherdon mossambicus*, UII inhibited prolactin release ( Grau *et al.*, 1982 ). Thus, there appears to be many ways in which the caudal-NS could affect osmoregulation and be affected by osmotic conditions as postulated in Figure 1.17.

Loretz and Bern ( 1981 ) have suggested that the activities of UI would be consistent with SW adaptation and those of UII with FW adaptation. It may be that the caudal-NS is concerned with only fine osmoregulatory adjustments, and thus respond only to slight fluctuations in environmental salinity ( Larson and Bern, 1987 ). This might explain the reported inconsistent actions of these peptides in various species.

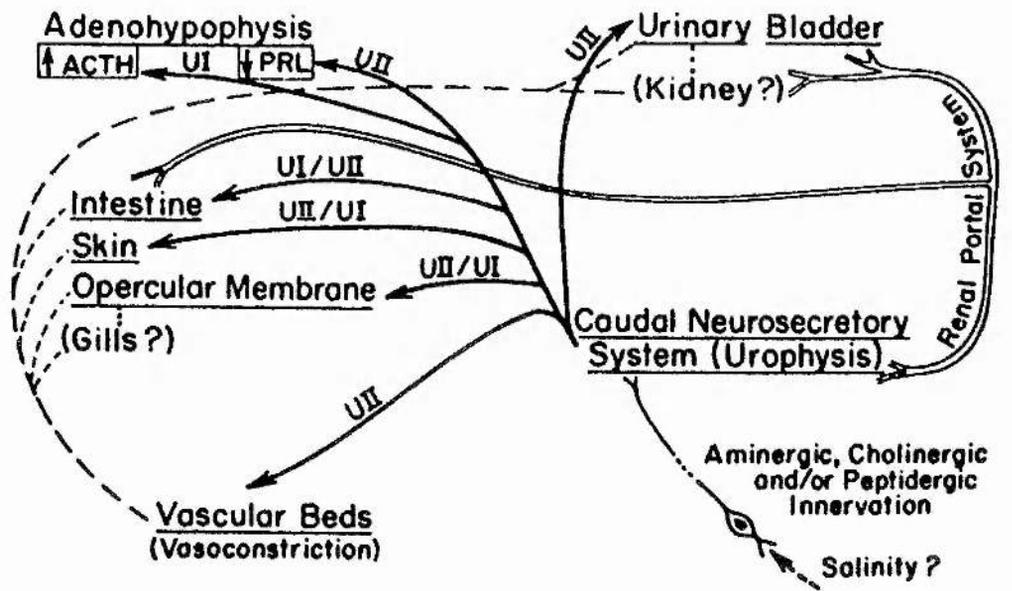
### 1.13 Thyroid hormones

The secretions of the thyroid gland, L-thyroxine ( $T_4$ ) and L-triiodothyronine ( $T_3$ ), affect a number of organs and play a role in many biological functions. The nature of these functions is quite diverse. Some of these include a role in the control of oxygen consumption in mammals, birds and amphibians, increasing territoriality and general activity in the lizard, *Anolis carolinensis*, initiating amphibian metamorphosis and the application of  $T_3$  and  $T_4$  to amphibian skin or to the urinary bladder causes an increase in  $Na^+$  transport ( Gorbman *et al.*, 1983 ).

The association between the thyroid hormones and osmoregulation in fish is as yet unclear. It is thought that rather than having a direct effect on osmoregulation the thyroid hormones may be vital to the development of seawater osmoregulatory mechanisms ( Barron, 1986 ). The relationship between smoltification and thyroid hormones has been investigated. A

Figure 1.17

Figure 1.17 Possible pathways of urotensin action on teleost osmoregulation  
Direct (——) and indirect (----- ) routes of action are shown.  
( Larson and Bern, 1987 )



major attribute of the transformation from parr to smolt is a rise of the thyroid hormones to a peak before returning to the baseline levels, concomitant with morphological changes in coho salmon that were indicative of the smoltification process ( Dickhoff *et al.*, 1978 ). When the secretory dynamics of this surge in  $T_4$  concentration were investigated, it was seen to be the result of an initial elevation in secretion rate and a lowered rate of  $T_4$  clearance ( Specker *et al.*, 1984 ). Treatment of salmonids with thyroid hormones prior to transfer to SW appeared to induce greater adaptability in treated fish ( Refstie, 1982 ). When thiourea, a substance that blocks the production of thyroid hormones, was administered to *Fundulus heteroclitus*, the animal was unable to survive in SW for more than three weeks. The mortality induced by this treatment and SW transfer was caused by elevations in serum osmolality and  $Na^+$  concentration. Thiourea had no effect in FW ( Knoepfel *et al.*, 1982 ).

There appears to be an interaction between thyroid hormone,  $T_4$ , and cortisol, in the stimulation of  $Na^+$ - $K^+$ -ATPase activity. Studies by Dange *et al.* ( 1986 ) on the SW-adapted tilapia reported that, while cortisol, but not  $T_4$ , administered alone, stimulated the enzyme activity, dual administration of the hormones under hypo- and hyperosmotic conditions augmented the cortisol effect.

#### 1.14 Objectives

The main objectives of this study were to investigate the endocrine responses that occur during the acute and chronic adaptation of the eel from FW to SW, by means of an integrative approach. The circulating plasma hormonal profiles of AII, AVT and cortisol during transfer from FW to SW were to be determined, in conjunction with the effects of SW transfer on the mean arterial blood pressure, drinking rate and plasma electrolyte levels

The actions of the RAS on a variety of physiological functions during long term- FW and -SW adaptation were to be examined in closer detail through the pharmacological manipulation of the endogenous system. The receptor population characteristics of AII in relation to changes that may occur during chronic adaptation to SW were to be investigated in a variety of osmoregulatory tissues. The secretory dynamics of cortisol during chronic SW adaptation were to be elucidated.

## 2.0 Materials and methods

## 2.1 General chemicals

All chemicals were obtained from Sigma Chemical Co. ( Poole, Dorset, U.K. ) unless otherwise stated.

## 2.2 Radioimmunoassay of Angiotensin II

Plasma AII concentration was determined by a modified procedure of Nussberger *et al.* ( 1985, 1986 ). Human AII ( Ile<sup>5</sup> ) standard was kept stored at a concentration of 400  $\mu$ M and diluted with radioimmunoassay ( RIA ) buffer containing 0.1 M Tris, 0.5% ( w/v ) RIA grade bovine serum albumin ( pH 7.8 at 4°C ). <sup>125</sup>I-Tyr<sup>4</sup>, Ile<sup>5</sup>-AII was obtained from Du Pont ( Du-Pont Straße 1, D-6380 Bad Homburg, Germany ), and had a specific activity of 2200 Ci/mmol. The label was reconstituted in the RIA buffer and used at a concentration of 0.6 fmol/50  $\mu$ l ( approximately 2,000 c.p.m./50  $\mu$ l ). The AII antibody was kindly supplied by Prof. Nussberger, ( Centre Hospitalier, Lausanne, Switzerland ) and was stored in a -80°C freezer at 1: 100 dilution, until required.

### 2.2.1 Standard assay procedure

0.5 ml of AII standard were added to triplicate assay tubes and double diluted to form a standard curve of 0.625 - 80 pM, with 4 nM being used to determine non-specific binding ( NSB ). Extracted samples were reconstituted in RIA buffer and 0.5 ml pipetted in duplicate into assay tubes. 50  $\mu$ l of label containing 2,000 c.p.m. were added to each tube. 0.5 ml of antiserum at 1:20,000 dilution were added to each tube, the mixture vortexed and the tubes allowed to equilibrate for 48 h at 4°C. 0.3 ml of 2% ( w/v ) charcoal solution containing 0.2 % dextran T70 were added to each tube and spun at 1,500 g in an MSE Mistral 3000 at 4°C for 15 min to achieve separation. The resulting supernatant containing bound radioactivity was counted for 5 min in a gamma counter ( Minaxi Auto Gamma R5000 series counter, Canberra-

Packard, Pangbourne, Berks., U.K. ). The total counts tube consisted of radioactive ligand and RIA buffer, made up to 1 ml without the antiserum.

Results are expressed as % Bound where,

$$\% \text{ Bound} = \frac{\text{c.p.m. bound in sample tube}}{\text{c.p.m. in total counts tube}} \times 100$$

A standard curve of % Bound against AII ( fmol/ml ) was then plotted and values for unknown samples read off the curve and corrected for recovery and volume.

### 2.2.2 Inter- and intra-assay variation

Extracted samples from a plasma pool were assayed with each set of unknowns and their mean and standard deviation ( S.D. ) calculated. The inter-assay coefficient of variation was calculated using

$$\frac{\text{S.D.}}{\text{Mean}} \times 100$$

In the same way repeat samples of the plasma pool were assayed within each individual RIA and their mean and S.D. calculated. The intra-assay coefficient of variation was then calculated using the above equation.

### 2.2.3 Extraction of angiotensin II

Angiotensin II was extracted from plasma samples using SEP PAK C18 cartridges ( Waters Associates, Millipore Corp., Northwich, Cheshire ). The SEP PAKs were primed with 5 ml methanol and washed with 20 ml distilled water. Samples were then applied to the columns at a rate of 0.5 - 0.25 ml/min using an infusion pump ( Harvard Apparatus, Massachusetts, U.S.A. ). The SEP PAKs were washed in 20 ml acetic acid and the peptide eluted at a constant rate in 5ml methanol using the infusion pump. Using plasma "spiked" with iodinated AII as an indicator, the rate of methanol elution was varied in order to find the optimal for maximal recovery of the peptide. After use each SEP PAK was reconstituted using 5ml of 8M urea and then washed with 20 ml of distilled water. Each SEP PAK was used four times

when regenerated in this way without any appreciable decrease in peptide recovery.

### 2.3 Radioimmunoassay of arginine vasotocin

Arginine vasotocin concentrations were measured by J. Warne ( University of Manchester, U.K. ) using the antiserum and protocol previously developed by Gray and Simon ( 1983 ) for measurement of AVT in birds. The assay buffer consisted of 0.1M Tris-( hydroxy-methyl )-methylamine ( pH. 7.4 ) containing 3% BSA and 2% neomycin sulphate. Unlabelled AVT and antiserum were incubated for 48 h. at 4°C prior to the addition of <sup>125</sup>I-AVP. The antiserum recognised both AVT and AVP and this allowed the use of commercially available <sup>125</sup>I-AVP as the radioisotope marker. The standard curve was obtained by adding 200 µl of doubling dilutions of standard ( 0.1 - 100 pg ) to 200 µl of antiserum giving a final antiserum dilution of 1:200,000. 200 µl of samples were assayed in duplicate. 50 µl ( 2,000 - 3,000 c.p.m. ) of <sup>125</sup>I-AVP was added after 48 h and the incubation continued for another 24 h. Separation was achieved by the rapid addition of 900 µl absolute ethanol, followed by mixing and centrifugation at 4,000 g for 10 min at 4°C. The supernatants were removed by aspiration and radioactivity in the pellets estimated in a gamma counter. AVT was extracted from blood samples using SEP PAK C18 cartridges prior to assay.

### 2.4 Radioimmunoassay of cortisol

The concentration of cortisol in eel plasma was measured using a radioimmunoassay based upon that of Waring ( 1990 ). Cortisol standard was kept stored at 2 mg/ml in ethyl acetate at -20°C. The ethyl acetate was evaporated off and the assay standards made up in RIA phosphosaline buffer containing sodium dihydrogen phosphate.2H<sub>2</sub>O ( 39 mM ) , sodium hydrogen phosphate ( 61.3 mM ), sodium chloride ( 154 mM ) and sodium azide (15.4 mM ). Tritiated cortisol was obtained from Amersham International

( Buckinghamshire, U.K. ) and had a specific activity of 92.7 Ci/mmol. The label was stored at -20°C in 9:1 toluene/ethanol. An aliquot of radioactive solution was removed, the solvents evaporated off and resuspended in RIA buffer to give a concentration of 20,000 d.p.m./100µl. The cortisol antiserum was obtained from the Scottish Antibody Production Unit ( S.A.P.U., Carlisle, Lanarkshire, Scotland ) and was raised from sheep ( specificity being: cortisol 100%, corticosterone 0.18%, cortisone 0.07%, 11-deoxycortisol 0.58%, 11-deoxycorticosterone 0.03% ; manufacturers data ). It was stored at -20°C at a 1:20 dilution.

#### 2.4.1 Standard Assay Procedure

250 µl cortisol standards ranging from 20 - 0.156 ng/ml were added to triplicate assay tubes, with 20 µg/ml being used to determine NSB. 250 µl of reconstituted extracted plasma samples were pipetted in duplicate into assay tubes. 0.5 ml of antibody at 1:1000 dilution were added to each tube. The mixture was vortexed and allowed to stand at room temperature for 30 min. 100 µl of phosphosaline buffer containing 20,000 d.p.m. <sup>3</sup>H-cortisol were then added to each tube. The tubes were incubated at 37°C for one hour and then left at 4°C overnight. 200 µl of the charcoal mixture ( as per Section 2.2.1 ) were added to each tube and left standing on ice for 15 min. Each sample was then centrifuged at 1500 g at 4°C for 15 min to separate the bound and the free radioactivity. The supernatant containing the bound counts was decanted and 3 ml of scintillation fluid added ( Emulsifier Scintillant 299TM, Packard Instrument Co. Inc., Illinois, U.S.A. ). Radioactivity was counted for 5 min on a liquid scintillation counter ( 2000CA Tri-Carb Scintillation Analyser, Canberra-Packard, Pangbourne, Berks. ).

Inter- and intra-assay variation were also determined for cortisol by using the procedure and calculations previously described for AII in Section 2.2.2

#### 2.4.2 Extraction of cortisol

Cortisol was extracted from plasma samples by means of SEP PAK C18 cartridges using a modified version of that employed by Hofreiter *et al.* ( 1983 ). The SEP PAKs were primed for use as previously described in Section 2.2.3. Samples were applied at 0.5 ml/min using an infusion pump. The column was washed first with 2 ml of 20% acetone, followed by 2 ml of distilled water. Cortisol was eluted at a constant rate in 5 ml methanol. The methanol was evaporated and the steroid reconstituted in 1 ml RIA buffer. The percentage recovery and maximal elution rate was defined by the application of <sup>3</sup>H Cortisol to the column as in Section 2.2.3. The SEP PAK columns were regenerated with methanol for re-use four times after washing with 8 M urea and distilled water.

#### 2.5 Animals

Eels, *Anguilla anguilla* , ( 250-800g ), were supplied by Mr. Hamish Wilson, Kirkbank, Kelso. They were maintained in aquaria at the Gatty Marine Laboratory either in aerated running freshwater ( FW eels ) or in free-flowing, aerated sea-water ( SW eels ) at 8 - 14°C. The lighting regime was 12 light:12 dark.

#### 2.6 Cannulation of Blood Vessels

Eels were anaesthetised by immersion in FW or SW containing 0.5% 3-Aminobenzoic Acid Ethyl Ester ( Methanesulfonate salt, MS222 ) until cessation of breathing and loss of muscular tone. During surgery the fish were kept moist but the gills were not irrigated. A ventral incision was made in the tail region to expose the caudal artery and caudal vein. The aorta and vein were cannulated using portex tubing cannulae ( internal diameter 0.58 mm, outside diameter 0.96 mm ) ( Portex Ltd., Kent, U.K. ), in the direction of the flow of blood, and secured to the body wall. The incision was closed by a purse string suture and the fish allowed 48 h to recover prior to use in any

experiment. The caudal aortic cannula permitted blood sampling and monitoring of blood pressure, while the caudal vein cannula allowed i.v. injections and constant infusions to be carried out.

### 2.7 Osmotic adaptation terminology

The following terminology was employed in this study for the adaptation of eels to SW:

- (a) Acute adaptation involved transition from FW to SW over a period of 90 min. Some individuals were maintained in SW for up to 300 min:
- (b) Chronic adaptation of eels utilised a FW to SW transfer period of 7 days.
- (c) Long-term adaptation to FW or SW entailed a period of at least 14 days in the appropriate environment.

### 2.8 *In vivo* studies

*In vivo* studies were performed following two protocols:

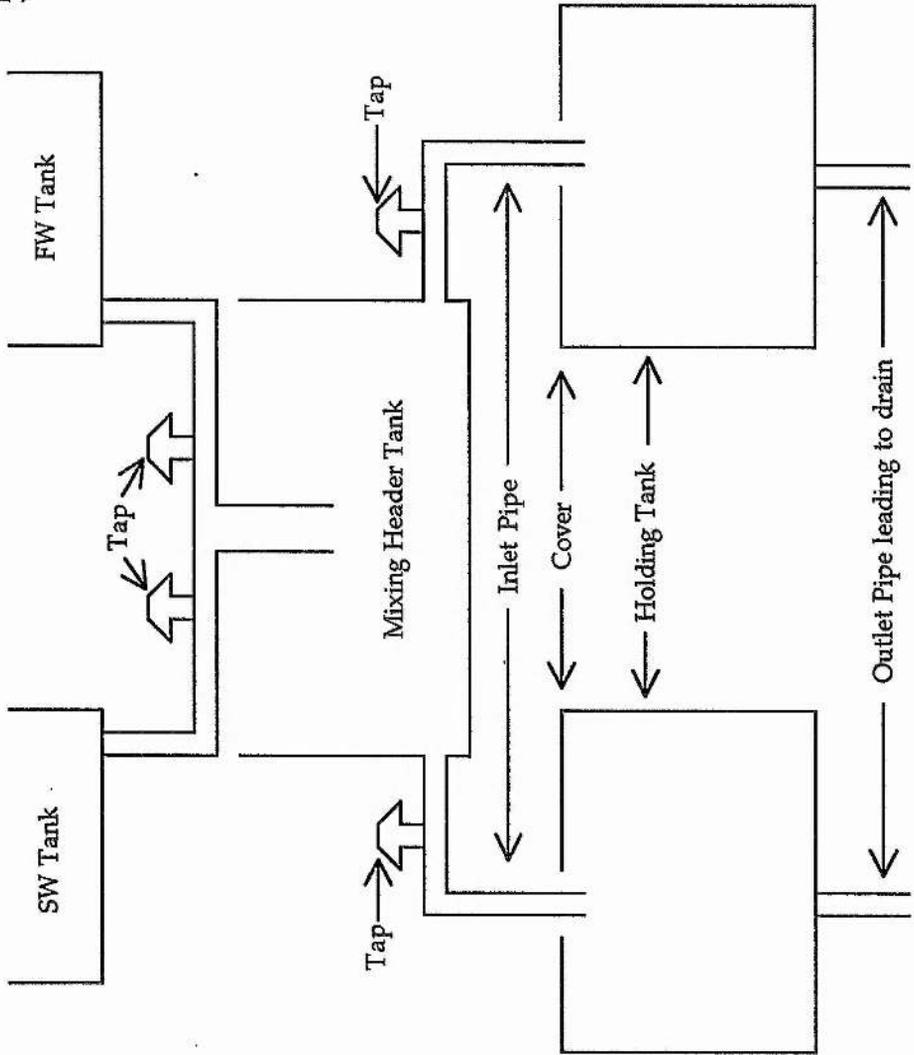
#### 2.8.1 Acute studies

The acute studies utilised chronically cannulated freshwater eels. Individual eels were held separately in a tank as shown in Figure 2.1. The holding tank was fed running water by an inlet pipe from a header tank, and drained by an outlet pipe. The flow of water was regulated by a series of taps. In order to acutely adapt the fish to SW, the FW flow to the mixing tank was switched off and the tank allowed to empty of FW. Simultaneously the SW pipe was opened leading to the mixing tank and SW was therefore permitted

Figure 2.1

Figure 2.1. Schematic diagram of the holding tank for the acute transfer of eel from FW to SW.

Direction of  
Water Flow



to flow into the holding tank. This meant that the proportion of FW and SW entering the holding tanks was easily altered with no disturbance to the fish, or change in the total volume of water in which the fish was held. The fish were adapted to SW over a 90 min period. Manipulation of the catheters was accomplished without disturbance to the fish as the tanks were covered and the cannulae were led outside the tanks.

This transfer procedure and method for manipulating the fish without disturbance was also used for chronically adapting fish for the measurement of blood pressure ( see Section 2.9 )and for the determination of cortisol secretory dynamics ( see Section 2.12 ). The stress to which the fish were subjected during transfer from FW to SW was therefore kept to a minimum, an important consideration to take into account in the measurement of plasma cortisol levels.

### 2.8.2 Chronic studies

Eels were adapted from FW and held in SW for a period of 7 days or long term-adapted for 14 or more days in SW. These fish were kept in large holding tanks which permitted the change from FW to SW to be made by switching the inflow from one medium to the other with no disturbance to the eels.

### 2.9 Blood pressure studies

Blood pressure was monitored via the caudal aorta in both FW and SW- chronically adapted eels, using an Elcomatic EM750 pressure transducer attached to a George Washington 400 MD/4 pen recorder. Mean arterial blood pressure was calculated as the arithmetic average of systolic and diastolic pressures.

## 2.10 Pharmacological manipulation of the RAS

The endogenous RAS was manipulated through the use of papaverine ( smooth muscle relaxant ) and captopril ( inhibitor of ACE ). These substances were administered to both FW and SW chronically adapted eels. For the determination of drinking rates the substances were administered intraperitoneal with all other parameters determined by injection via the indwelling caudal vein cannula. The following doses in equal volumes of saline ( 200  $\mu$ l ) were injected into the fish, as appropriate according to the experimental design:

- ( a ) 0.9 % saline as control
- ( b ) Papaverine at 15 mg/kg dissolved in 0.9 % saline.
- ( c ) Captopril ( SQ14225, E.R. Squibb and Sons Inc., New Jersey ) at 72 mg/kg dissolved in 0.9 % saline.
- ( d ) Captopril at 72mg/kg, followed by an injection of papaverine at 15 mg/kg after 15 min

A period of 20 h was permitted between each experimental manipulation so as to ensure complete recovery. A number of parameters were determined:

- ( 1 ) blood pressure ( see Section 2.9 )
- ( 2 ) plasma osmolality ( see Section 2.15 )
- ( 3 ) drinking rate (see Section 2.11 )
- ( 4 ) plasma AII concentration ( see Section 2.2 )

When serial blood samples were obtained from an individual fish, a volume of 0.9% saline, equal to that of the blood removed, was returned to the fish immediately.

## 2.11 Determination of drinking rate

The drinking rate was determined by measuring the gut accumulation of <sup>51</sup>Cr-EDTA ( Amersham International ) ( Hazon *et al.*, 1989 ). Eels were placed in groups of three in 20 liters of the appropriate medium ( i.e. FW or

SW ) which contained 10  $\mu\text{Ci/l}$   $^{51}\text{Cr-EDTA}$  for 1 - 4 h for the acute studies, and for 6 h for the chronic and long term SW determinations. At the end of the appropriate period the eels were sacrificed by being immersed in phenoxyethanol ( 5 ml/l ) and subsequent cutting of the spinal cord. Blood samples were removed from the caudal sinus and the gut removed after ligation at both ends. Gut contents were counted for  $^{51}\text{Cr}$  activity on a gamma counter. Duplicate water samples were also counted. Drinking rates were estimated using the following calculation modified from Carrick and Balment ( 1983 ):

$$\text{Drinking Rate} = \frac{C}{M \times T}$$

where C= total counts incorporated into gut  
M= concentration of bathing medium  
T= time in tracer containing medium

All values are corrected for the weight of the fish and expressed as ml/kg/h  $\pm$  standard error of the mean ( S.E.M. )

Due to the necessity of maintaining the radioactive label within the tank, it was not possible to switch the flow of water from FW to SW, for the acute adaptation studies, by using the through flow system. Groups of fish were physically transferred to tanks, but preliminary studies have shown that this method of transfer does not affect the drinking rate.

### 2.12 Determination of cortisol secretory dynamics

Following cannulation fish were allowed 48 h to recover, before commencement of the isotopic infusion studies. A priming dose of 10  $\mu\text{Ci}$  of  $^3\text{H-cortisol}$  was followed by a constant infusion of 1  $\mu\text{Ci/h}$ . at a rate of 6  $\mu\text{l/min}$  using a Harvard infusion pump. This method led to equilibration of the label within the plasma from 4 - 7 h. Blood samples were taken at time zero and thereafter hourly samples were obtained. The plasma was counted for 5 min for tritium on the liquid scintillation counter. In addition cortisol

concentrations were determined for the same plasma samples and the plasma was also checked for metabolism of the tracer by high performance liquid chromatography ( HPLC ) analysis ( see Section 2.12.1 ).

The constant infusion of tracer molecules allows the determination of the metabolic clearance rate ( MCR ) of the endogenous material ( Tait *et al.*, 1962 ). The calculations for MCR and BPR have been previously discussed in Section 1.9.5.

### 2.12.1 High performance liquid chromatography

It is important to know the percentage of infused tritiated cortisol metabolised during the time course of the experiment since this must be taken into account in the calculation of the MCR. This analysis was carried out by means of HPLC. Components of the HPLC system included a 410LC pump ( Perkin Elmer Ltd., Buckinghamshire, U.K. ) fitted with a 175  $\mu$ l ( Rheodyne, Cotati, California, U.S.A. ) 7125 syringe loading sample injector. The sample was injected into the HPLC column using a Hamilton syringe ( Hamilton Co., Reno, Nevada, U.S.A. ). A LC1-100 integrator ( Perkin Elmer.Ltd. ) was used to record UV detection at 254 nm., with analytical chromatographies performed on a C-18 column.

The mobile phase used in the HPLC analysis was an isocratic 60/40 ( by volume ) methanol/water with 0.1 % trifluoroacetic acid ( TFA ) gradient at a rate of 1 ml/min as described by Hofreiter *et al.* ( 1983 ). All eluants were HPLC grade and degassed prior to use for 15 min with helium. The cortisol standard solution was stored at a concentration of 19.4 mg/ml, dissolved in methanol. Corticosterone in methanol was used as the internal standard at a working concentration of 328 ng/350  $\mu$ l. Both these standards were kept at 4°C. The retention times of the standards were elucidated by their addition to plasma and injecting 175  $\mu$ l of the resulting extracted mixture onto the HPLC column. Since this technique was being utilised to examine the percentage

metabolism of tritiated cortisol, plasma was also spiked with  $^3\text{H}$ -cortisol and treated in the same manner as the cold standards. The resulting fractions were collected at a rate of 1 ml/min and counted for radioactivity on the scintillation counter.

Cortisol was extracted from the infused samples as in Section 2.4.2 after pre-treatment of the plasma. 50  $\mu\text{l}$  of plasma were made up to 1 ml by the addition of 0.9 % saline. 350  $\mu\text{l}$  corticosterone were mixed with the plasma and centrifuged for 5 min at 700 g. The resulting supernatant was applied to the preconditioned SEP PAK and extracted for cortisol. The eluted methanol was evaporated off and the sample reconstituted in 1 ml methanol. 175  $\mu\text{l}$  of the sample were applied to the HPLC column. Fractions were collected each minute using a fraction collector ( Ultrorac 7000, L.K.B., Bromma, Sweden ) and counted for radioactivity. The metabolism was calculated from the summation of the radioactivity in the non-cortisol peaks divided by the total radioactivity in the trace.

### 2.13 $\text{Na}^+$ - $\text{K}^+$ -ATPase activity assay

The preparation of gill membrane and subsequent determination of  $\text{Na}^+$ - $\text{K}^+$ -ATPase activity in the membrane was carried out according to a modified procedure of Mayer-Gostan and Lemaire ( 1991 ).

#### 2.13.1 Membrane isolation

The fish were killed by decapitation and exsanguination. The gill arches were excised and the epithelium scraped from the underlying cartilage into a hypotonic saline solution ( 25 mM NaCl, 1 mM dithiothreitol, 1 mM hepes-tris, pH 7.4 ) . All isolation procedures were carried out on ice. The scraping was disrupted by 20 strokes in a dounce homogeniser ( clearance space of 13  $\mu\text{m}$  ) and subsequently filtered through four layers of gauze. The homogenate was centrifuged at 1,000 g for 15 min at 4°C. The resulting pellet was resuspended in a sucrose buffer ( 0.3 M sucrose, 1 mM disodium EDTA,

0.1% ( w/v ) sodium deoxycholate, 5 mM tris-buffer, pH7.4 ) and held at -20°C until assayed for Na<sup>+</sup>-K<sup>+</sup>-ATPase activity. An aliquot of membrane preparation was kept for protein determination.

#### 2.13.2 Determination of Na<sup>+</sup>-K<sup>+</sup>-ATPase activity

200 µl of the enzyme fraction were added to 800 µl of incubation medium containing 120 mM NaCl, 50 mM tris, 5 mM NaN<sub>3</sub>, 3.5 mM MgCl<sub>2</sub>·6H<sub>2</sub>O and either 20 mM KCl ( to measure K<sup>+</sup>-stimulated-ATPase activity ) or 20 mM KCl and 1 mM ouabain ( to measure the ouabain-sensitive component of the ATPase activity ). The tubes were incubated at 37°C for 5 min. Disodium ATP was also incubated at 37°C. Then 50 µl from the incubated tubes were added in duplicate to a 96 microwell plate ( Dynatech Laboratories Ltd., West Sussex, U.K. ). The reaction was started by the addition of 50 µl of ATP ( final concentration 3 mM, pH 7.4 ) to each well, except for the first two wells of each sample under going assay. These two wells were used to calculate the background hydrolysis of the ATP. 150 µl of the dye reagent were added to these two wells prior to the addition of ATP. The plate was shaken and incubated at 37°C for 15 min and the enzyme reaction was stopped by the addition of 150 µl of Malachite Green Reagent ( see Section 2.13.3 ). The same orientation of the plate was used to initiate and terminate the reaction. All solutions were added to the microplate using a multichannel pipette ( Dynatech ).

#### 2.13.3 Determination of inorganic phosphate

The colour reagent was prepared from stock solutions of ammonium molybdate ( 5.72% w/v in 6 N HCl ), polyvinyl alcohol ( 2.32% w/v ), malachite green ( 0.0812% ) and distilled water mixed in the ratio of 5:5:10:5 for one microplate. 150 µl of this reagent were added to the 100µl of either standard or test sample in each well of the microplate using the multichannel pipette. Absorbance was read on a Dynatech MR5000 plate reader at 630 nm.

A standard curve was constructed by preparing a series of phosphate dilutions in assay buffer. The standard curve ranged from 0.156 nmol/100 $\mu$ l to 10 nmol/100 $\mu$ l and was linear over these values.

## 2.14 Angiotensin II receptor studies

### 2.14.1a Isolation of hepatocytes

This technique was based on the method by Walsh and Moon ( 1983 ), with all steps being carried out using plastic ware to avoid cell adhesion.

The fish were taken from the appropriate osmotic environment and anaesthetized for 10-20 min in either FW or SW containing 1.5 g/l MS-222, buffered to pH 7.4 with 0.75 g/l tromethamine base.

A ventral incision was made to expose the liver and the hepatic portal artery was cannulated. If the cannulation was successful then the liver started to clear upon commencement of the perfusion. The hepatic portal vein was cut to allow the blood to escape. The liver was perfused with either a modified FW or SW teleost Ringer A ( Table 2.1a ) at a rate of 3 ml/min for 5 min. At this point the liver was gently massaged as suggested by Moon *et al.* ( 1985 ) to minimise red blood cell contamination and to increase the cell yield. The liver was detached from its adhesions, with the gall bladder still intact, and placed in a funnel on a pierced petri dish. It was then perfused for 45 min with 30 ml of the appropriate Ringer B ( Table 2.1b ) in a closed recirculating system ( Figure 2.2 ). The final perfusion was with Ringer A for 5 min. The liver was placed into a plastic petri-dish and doused with ice-cold Ca<sup>+</sup>-free modified Hanks medium ( Table 2.1c ). The cell suspension was filtered through three layers of gauze, centrifuged at 1,000 g for 2 min, washed once with 5 ml of Ca<sup>+</sup>-free modified Hanks medium, followed by four washes in the final suspension medium consisting of the modified Hanks medium

Table 2.1

Table 2.1. Modified buffers used for the isolation of hepatocytes from long term FW- and SW- adapted eels

Table 2.1a. Modified Ringer A.

Table 2.1.b Modified Ringer B.

Collagenase type IV ( for use with hepatocyte preparations ) was obtained from Sigma Chemical Co.

Table 2.1.c. Modified Hanks Medium.

\*  $\text{NaHCO}_3$  was added after gassing the medium with 99.5 %  $\text{O}_2$  - 0.5 %  $\text{CO}_2$  for 30 min; pH is adjusted to 7.8 at 20°C.

† Solutions used for SW hepatocytes were adapted from Lockwood (1961)

Ringer A

| Reagent              | Concentration (mM) |              |
|----------------------|--------------------|--------------|
|                      | FW                 | †S W         |
| NaCl                 | 110                | 200          |
| KCl                  | 3.0                | 3.5          |
| Na <sub>2</sub> EDTA | 5.0                | 5.0          |
| Na Heparin           | 1.5 Units/ml       | 1.5 Units/ml |
| Hepes                | 10                 | 10           |

Ringer B

| Reagent           | Concentration (mM) |            |
|-------------------|--------------------|------------|
|                   | FW                 | †S W       |
| NaCl              | 110                | 200        |
| KCl               | 3                  | 3.5        |
| CaCl <sub>2</sub> | 5                  | 0.45       |
| Glucose           | 10                 | 10         |
| Hepes             | 10                 | 10         |
| Na Heparin        | 1.5 U/ml           | 1.5 U/ml   |
| Collagenase       | 0.35 mg/ml         | 0.35 mg/ml |

Hanks buffer

| Reagent                         | Concentration (mM) |      |
|---------------------------------|--------------------|------|
|                                 | FW                 | †S W |
| NaCl                            | 110                | 200  |
| KCl                             | 3.0                | 3.5  |
| KH <sub>2</sub> PO <sub>4</sub> | 1.25               | 0.2  |
| MgSO <sub>4</sub>               | 0.6                | \    |
| MgCl <sub>2</sub>               | 1.0                | 3.0  |
| NaHCO <sub>3</sub> *            | 5.0                | 5.0  |
| Hepes                           | 10                 | 10   |

supplemented by 10 mM CaCl<sub>2</sub> and 1 % (w/v)-defatted bovine serum albumin.

Cell number was counted using a haemocytometer and viability was checked using the 1 % Trypan Blue exclusion test at regular intervals. Preparations containing initially more than 5 % stained cells were discarded.

#### 2.14.1b<sup>125</sup>I-AIIBinding Studies

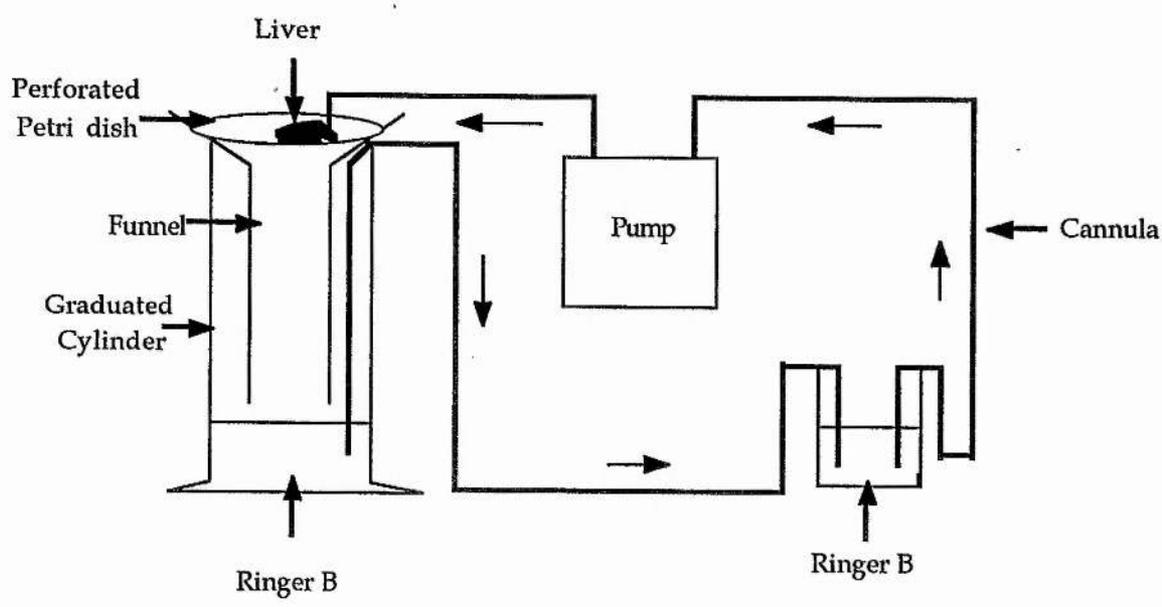
Binding assays were performed using a modified method of that utilised by Guibbolini *et al.* ( 1988 ) for AVT binding to gill cells. Isolated hepatocytes were incubated in modified Hanks medium ( final volume 500 µl ) containing CaCl<sub>2</sub> and BSA in the presence of 19 µl <sup>125</sup>I-AII ( 28 pM or approximately 50,000 c.p.m.) and 20 µl unlabelled AII in the concentration range 1-200 nM. Binding was initiated by the addition of cells to the AII solution. Following 20 min incubation at 20°C the bound AII was separated by rapid centrifugation of the cells ( 50 g, 30 sec ). The pellet was washed twice with 500 µl of ice-cold Hanks medium and counted for radioactivity in the gamma counter. Non-specific binding was assessed by the addition of 1000 fold excess unlabelled AII ( 1-200 µM). Specific binding was defined as the difference between total binding to the cells minus radioactivity bound in the presence of excess AII.

In order to differentiate between radioactivity bound to the cell surface and that internalized, cells were washed with an acid-saline buffer ( 0.2 M acetic acid containing 0.5 M NaCl, pH 2.5 ) according to the procedure of Nau *et al.* ( 1987 ). 500 µl of incubation mixtures were removed at appropriate time points, centrifuged as before, washed once with the acid-saline buffer and again with the Hanks medium. The resultant pellet was counted for radioactivity in the gamma counter.

Figure 2.2

Figure 2.2. Diagram of closed recirculating system for the perfusion of isolated liver.

The liver is perfused for 45 min with Ringer B which contains collagenase.



### 2.14.2 Autoradiography

Tissue sections were prepared for autoradiography by the protocol listed in Figure 2.3. 10  $\mu\text{m}$  sections were cut and applied to gelatinised slides with 3 sections per slide. Prior to use in autoradiography the tissue sections were dewaxed and rehydrated by the method listed in Figure 2.4.

The slides were then immersed in Na phosphate buffer ( Na Phosphate 10 mM, pH 7.4 , NaCl 120 mM, disodium EDTA 5 mM and BSA 0.2% ) and left for 15 min without stirring. The slides were dried around each section. All AII solutions were made up in Na phosphate buffer. Incubations were carried out with ( a )  $^{125}\text{I}$ -AII ( 1nM ) to determine total binding ( TB ): ( b ) labelled AII as in ( a ) plus a 1000 fold excess of unlabelled AII to evaluate non-specific binding ( NSB ); and ( c ) buffer only to assess the development of background silver grains. 20  $\mu\text{l}$  of appropriate AII solution were added by pipette to each individual section and incubated in a moist box for one hour at room temperature. After this period the slides were washed in Tris-HCl ( 0.2 M Tris, 0.1 N HCl, pH 7.5 ) for five min ( x 3 times ) and dried.

The next stage in the procedure involved coating the slides with photographic emulsion in the dark room. Due to supply problems two different types of emulsion were subsequently used in this study, LM-1 ( Amersham International ) and G 5 ( Ilford Ltd. ). Spacers were placed between each slide in order to avoid cross-contamination. The slide box was wrapped securely in a black bag and left in a fridge for 3 days. Then the slides were removed from the fridge and allowed to equilibrate to room temperature ( i.e. temperature of the solutions ) within the darkened bag. The slides were removed in the Dark Room and developed as in Figure 2.5.

The developed slides were stained using haematoxylin and eosin as described in Figure 2.6. The slides were mounted using DPX and allowed to dry. Grain counts ( per  $350\mu\text{m}^2$  grid area ) were made on five random tissue sections for each tissue type from each fish to establish ( a ) TB, ( b ) NSB and

Figure 2.3

Figure 2.4

Figure 2.3. Method for embedding of eel tissue in wax.

Figure 2.4. Method for rehydration and dewaxing of tissue sections prior for use in autoradiography.

### Preparation of samples for wax sectioning

- (1) Place each tissue in 80% ethanol for 3 h with hourly changes of solvent.
- (2) Place each in 90% ethanol for 1 hour.
- (3) Place in 95% ethanol overnight
- (4) Place tissues in absolute ethanol for 1 hour. Change the solvent and leave for a further 30 min.
- (5) Place tissues in 50% ethanol/50% xylene for 5 min, making one change after 2 min and 30 s.
- (6) Place in xylene for 5 min.
- (7) Mix xylene and wax ( TAAB Laboratories Rquipment Ltd., Reading, Berks.) ( 50/50 ) in the oven and allow the mixture to come up to temperature to avoid solidification of the wax.
- (8) Place tissues in the xylene/wax mixture for 15 min at approx. 50°C.
- (9) Place in wax at 50°C for 45 min. Change the wax and leave for a further 45 min to allow wax to penetrate the tissue.
- (10) Pour into moulds and allow the wax to harden overnight.

### Rehydration of wax sections

- (1) Wax sections are dried overnight and then put into xylene for 40 min with a change of solvent after 20 min.
- (2) Then the sections are put into a series of alcohol as follows:
  - (a) Absolute alcohol for 1 min
  - (b) Absolute alcohol for 30 s
  - (c) 95% alcohol for 15 s ( x2 ).
  - (d) 90% alcohol for 15 s
  - (e) 80% alcohol for 15 s
  - (f) Deionised water for 15 s

Figure 2.5

Figure 2.6

Figure 2.5. Method for the photographic development of slides.

Figure 2.6. Method for the histological staining of slides.

#### Development of incubated slides

- 1) Remove slides from fridge and allow to equilibrate to room temp. (i.e. temp of solution).
- 2) Place slides in developer ( Ilford Phenisol, Ilford Ltd., Mobberley, Cheshire ) diluted 1:4 with distilled water for 1 min.
- 3) Place slides in stop solution of 0.5 % acetic acid for 1 min.
- 4) Place slides in fix solution of 30 % sodium thiosulphate for 4-6 mins.
- 5) Wash slides in running tap water for 15 min.
- 6) Final wash in distilled water.

#### Histological staining of slides

- 1) Place slides in filtered Haematoxylin stain for 1 min.
- 2) Wash in tap water for 15 min with a final wash in distilled water.
- 3) Place slide in 1% Eosin solution (quick dip and remove).
- 4) Place directly into 70% Alcohol to remove excess stain.
- 5) Dip for only a few seconds in 80%, 90%, 95%I, 95%II.alcohol
- 6) Dip for 1 min in Absolute Alcohol I and II for 30 s.
- 7) Place in Xylene I and II for total of 5 min.

( c ) background. Specific binding ( SB ) was taken as the difference in grain counts per  $350\mu\text{m}^2$  for tissues incubated with  $^{125}\text{I}$ -AII alone ( TB ) and those with  $^{125}\text{I}$ -AII in the presence of excess unlabelled AII ( NSB ), with deduction of mean background counts for particular tissues and fish. Percentage specific binding was obtained from the following equation;

$$\% \text{ SB} = \frac{\text{TB} - \text{NSB}}{\text{TB}} \times 100$$

The densities of silver grains were determined at a magnification of 1000x for gill ( lamellae and filament ), kidney ( head and body ), liver and brain ( cerebellum and medulla oblongata), and were counted "blindly".

### 2.15 General plasma analysis

Plasma osmolality was measured by freezing point depression ( Robling Automatic, Camlab, Cambridge ). Plasma chloride concentrations was measured using an automatic chloride titrator ( Chloride Analyser 925, Corning Ltd., Essex, U.K. ) and plasma sodium concentration by flame emission spectrophotometry ( Corning 480 Flame Photometer, Corning Ltd., Essex, U.K. ).

### 2.16 Determination of protein

Protein concentrations were measured using the method of Bradford ( 1976 ). One hundred mg of Coomassie Brilliant Blue G250 dye were dissolved in 50 ml 95 % ethanol and 100 ml 85 % w/v orthophosphoric acid and the volume made up to one litre using distilled water. Three ml of a 1:2 dilution of this stock were then added to 100  $\mu\text{l}$  samples and BSA standards ranging from 0-100 $\mu\text{g}$ . The blue colour was stable for 10-60 min and samples and standards were measured spectrophotometrically at 595 nm. A standard curve of  $\mu\text{g}$  BSA against absorbance at 595 nm was plotted and samples for absorbance calculated to give protein content.

### 2.17 Statistical analysis and data presentation

Comparisons were made between the FW-adapted values and the various time periods after transfer to SW by means of the Bonferroni protected t-test ( INSTAT, computer statistical package ). For comparison of blood pressure levels obtained after the pharmacological manipulation of the RAS, each data point was compared to the corresponding time point after the injection of 0.9% saline as the control, by means of the paired t-test (INSTAT).

In the autoradiography experiments, comparisons of grain densities for NSB and TB sections were compared by ANOVA. Percentage specific binding was compared for each tissue type by analysis of variance ( ANOVA ) and any statistical difference was subsequently determined by the protected Bonferroni post t-test.

The following criteria was employed to demonstrate statistical significance;

- \* for  $p < 0.05$
- \*\* for  $p < 0.01$
- \*\*\* for  $p < 0.005$

Where it was deemed necessary, sequential time points which had the same level of significance were enclosed by brackets positioned above the relevant graph, inclusive of the points on the outer sides.

### 3.0 Results

### 3.1 Hormone radioimmunoassays

#### 3.1.1 Radioimmunoassay of angiotensin II

Figure 3.1 shows a typical standard curve for angiotensin II. The intra-assay variation was  $7.38 \pm 2.19\%$  ( mean  $\pm$  S.E.M., n= 8 ) and the inter-assay variation was calculated to be  $6.37 \pm 1.76\%$  ( mean  $\pm$  S.E.M., n= 8 ). The effect of sample dilution is shown, superimposed on the standard curve in Figure 3.1. The efficiency of SEP PAK C18 cartridges in extracting AII from eel plasma is shown in Table 3.1a. The plasma was applied at a rate of 0.5 ml/min and the various wash phases checked for their percentage recovery. The majority of AII retrieved was eluted in the first 2 ml of methanol, with maximal recovery achieved by using 5 ml of methanol. The effect of elution rate on the percentage recovery is shown in Table 3.1b. Maximum recovery was accomplished at a rate of 0.25 ml/min, and this rate was therefore used in all subsequent extractions of AII from plasma samples.

#### 3.1.2 Radioimmunoassay of cortisol

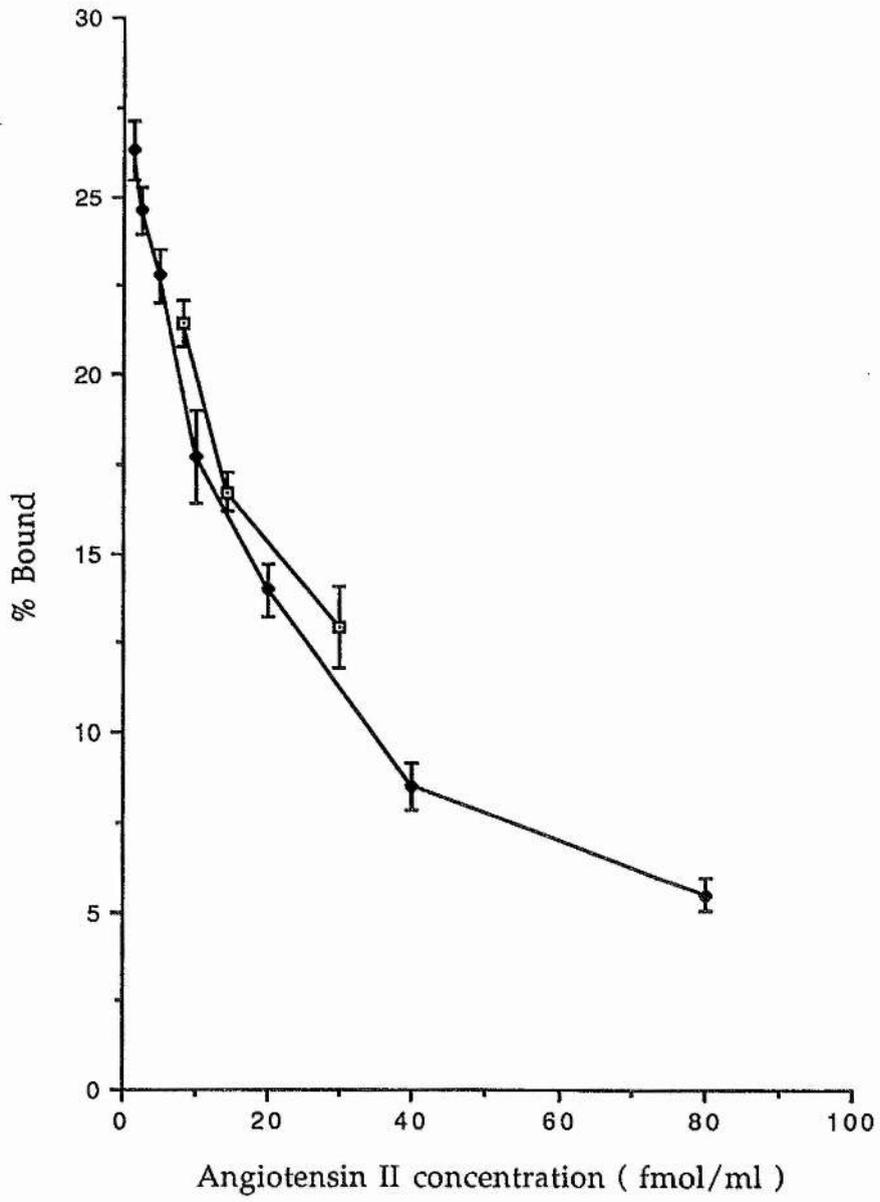
A typical standard curve for the radioimmunoassay of cortisol is shown in Figure 3.2. The intra-assay variation was  $2.45 \pm 0.69\%$  ( mean  $\pm$  S.E.M., n=12 ) and the inter-assay variation was  $4.23 \pm 0.25\%$  ( mean  $\pm$  S.E.M., n=12 ). Table 3.2a shows the percentage recovery of cortisol from eel plasma using SEP PAK C18 cartridges. Negligible amount of steroid was lost during the water wash. In the first 2 ml of methanol the majority of cortisol was eluted, with quantitatively all of the extractable steroid retrieved by eluting with 5 ml of methanol. The effect that the elution rate has on the percentage recovery of cortisol is shown in Table 3.2b. An extremely high rate of recovery is achieved with an elution rate of 0.25 ml/min. This rate was therefore utilised in the extraction of cortisol from plasma samples.

Figure 3.1

Figure 3.1. Standard curve for the radioimmunoassay of Angiotensin II.

The amount of AII ( fmol/ml ) is plotted on the abscissa against % binding on the ordinate. Each point represents the mean  $\pm$  S.E.M. of three replicates.

The effect of plasma dilution on AII concentration is shown by the open boxes. Each point represents the mean  $\pm$  S.E.M. of four replicates.



**Table 3.1**

Table 3.1 Extraction of Angiotensin II from eel plasma.

Table 3.1.a Extraction of plasma samples

Extraction of  $^{125}\text{I}$ -AII from eel plasma using SEP PAK C18 cartridges with an elution rate of 0.5 ml/min. Values are means  $\pm$  S.E.M. of eight replicates

Table 3.1.b Effect of elution rate on the extraction of plasma samples.

Values are means  $\pm$  S.E.M. for eight replicates.

| % Recovery          | Mean $\pm$ s.e.m. |
|---------------------|-------------------|
| In water phase      | 0.7 $\pm$ 0.2     |
| In 1st 2ml methanol | 78.3 $\pm$ 1.3    |
| In 2nd 2ml methanol | 3.0 $\pm$ 0.6     |
| In 5th 1ml methanol | Not detectable    |
| Total Recovery (%)  | 82.0 $\pm$ 2.1    |

| Rate of Elution (ml/min) | % Recovery (mean $\pm$ s.e.m.) |
|--------------------------|--------------------------------|
| 0.25                     | 84.4 $\pm$ 0.7                 |
| 0.5                      | 80.0 $\pm$ 2.5                 |

Figure 3.2

Figure 3.2. Standard curve for the radioimmunoassay of cortisol.  
The amount of cortisol ( ng/ml ) is plotted on the abscissa against %  
binding on the ordinate. Each point represents the mean  $\pm$  S.E.M. of  
four replicates.

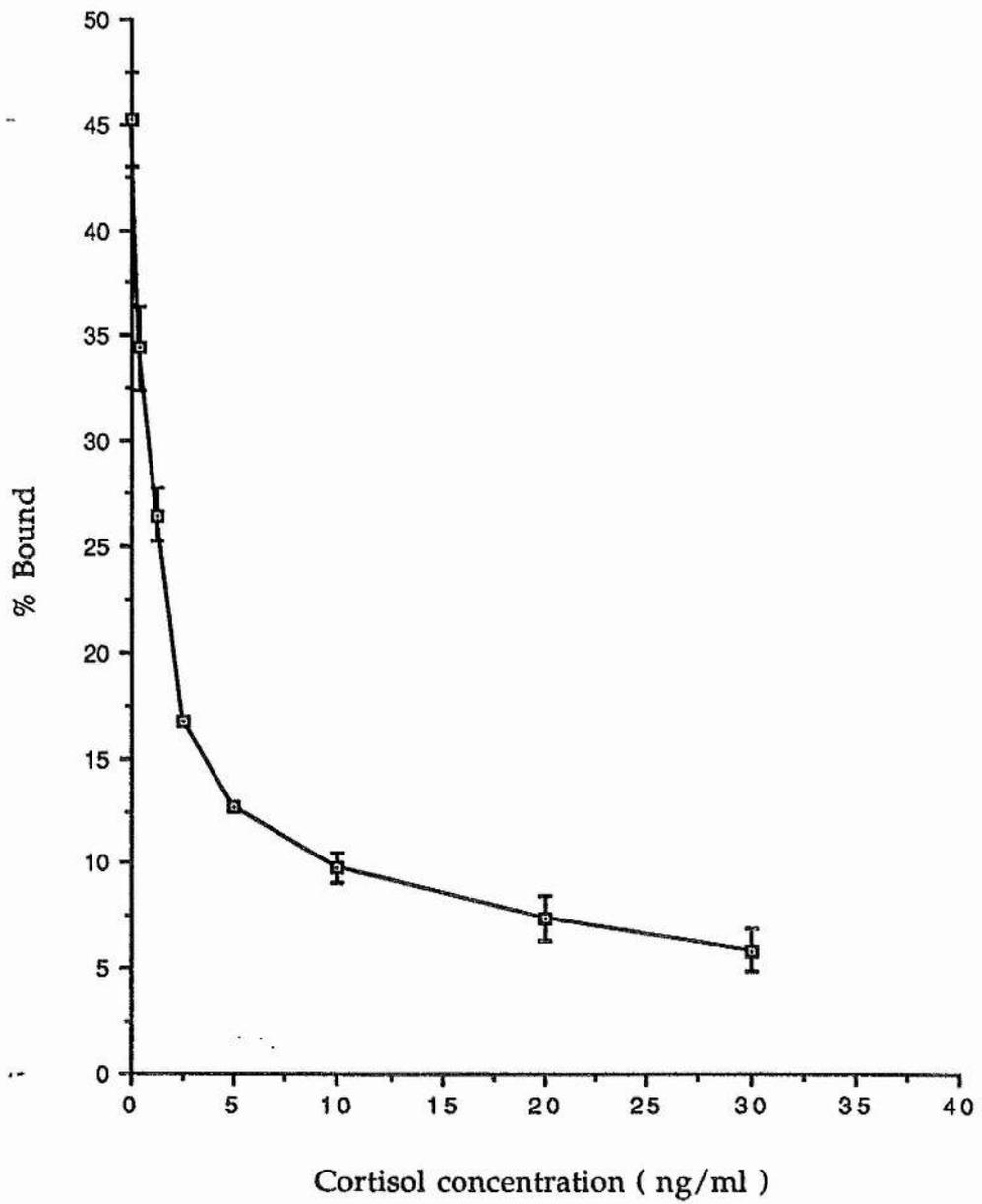


Table 3.2

Table 3.2. Extraction of cortisol from eel plasma.

Table 3.2.a Extraction of plasma samples

Extraction of <sup>3</sup>H-Cortisol from eel plasma using SEP PAK C18 cartridges with an elution rate of 0.25 ml/min. Values are means  $\pm$  S.E.M. of eight replicates

Table 3.2.b Effect of elution rate on the extraction of plasma samples.

Values are means  $\pm$  S.E.M. for eight replicates.

| % Recovery  | Mean $\pm$ s.e.m.  |
|---|--|
| In water phase<br>In 1st 2ml methanol<br>In 2nd 2ml methanol<br>In 5th 1ml methanol | $1.5 \pm 0.2$<br>$99.6 \pm 5.8$<br>$1.4 \pm 0.2$<br>Not detectable |
| Total Recovery  | $102.3 \pm 6.1$  |

| Rate of Elution (ml/min) | % Recovery (mean $\pm$ s.e.m.) |
|--------------------------|--------------------------------|
| 0.25                     | $108.9 \pm 7.4$                |
| 0.5                      | $101.2 \pm 9.4$                |
| 1.0                      | $77.4 \pm 2.2$                 |

## 3.2 Acute studies

### 3.2.1 Blood pressure

Figure 3.3 demonstrates the effect of acute SW transfer on a typical arterial blood pressure trace. The blood pressure was allowed to stabilise prior to manipulation. Figure 3.4 shows the response of mean blood pressure after acute transfer to SW. Preliminary experiments run over the 90 min transfer period showed an initial increase in blood pressure. After 90 min, the mean pressure was still higher ( $30.5 \pm 1.5$  mm Hg) than the starting FW value of  $28.3 \pm 1.5$  mm Hg, although the trend was to a decline in blood pressure. Therefore the experimental period was extended and the blood pressure recorded for 5 h. It can be seen in Figure 3.4, that upon transfer to SW there was an immediate increase in the mean arterial pressure from the resting FW level of  $28.3 \pm 1.5$  mm Hg, which was sustained over a 50 minute period. Thereafter the blood pressure declined, and within 5 h was at a significantly lower level,  $24.1 \pm 1.5$  mm Hg ( $p < 0.01$ ), compared to the FW value.

### 3.2.2 Plasma electrolyte analysis

Plasma osmolality and chloride concentration were measured in FW prior to the change over to SW and after 90 min when SW transfer had been achieved. As seen in Table 3.3 the plasma osmolality was higher after acute transfer to SW at  $335.1 \pm 4.2$  mOsmol/kg from an initial FW level of  $312.0 \pm 4.1$  mOsmol/kg ( $p < 0.05$ ). A similar pattern was seen for plasma chloride concentration which showed a concomitant increase from  $78.3 \pm 4.0$  mMol/l to  $94.1 \pm 4.3$  mMol/l ( $p < 0.05$ ) (Table 3.3).

### 3.2.3. Drinking rate

The effect of acute SW transfer on drinking in eel was determined. An increase in drinking from a basal FW value of  $0.02 \pm 0.01$  ml/kg/h to  $0.25 \pm 0.07$  ml/kg/h ( $p < 0.005$ ) after one hour acute SW transfer was observed

Figure 3.3

Figure 3.3 Typical blood pressure trace after acute SW adaptation

Arrow indicates the start of the transition period from FW to SW.  
90 mins later from this arrow the eel is in 100 % SW

Fig. 3.3

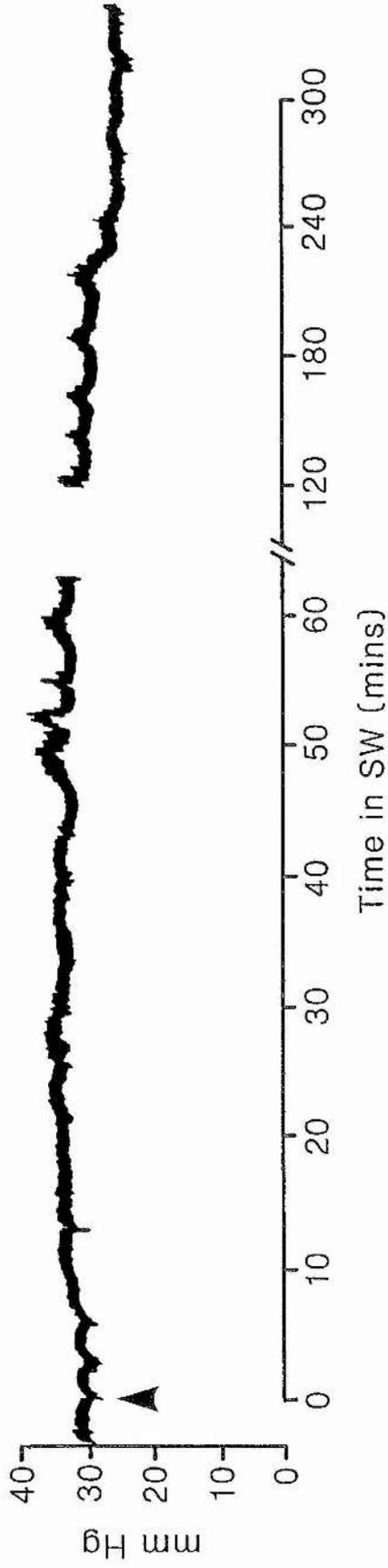


Figure 3.4

Figure 3.4 Effect of acute SW adaptation on mean arterial blood pressure

Results are means  $\pm$  S.E.M. of 11 animals.

\* indicates statistically significant differences at  $p < 0.05$  ( at 10, 20, 270 and 300 min ) compared to basal FW mean arterial blood pressure ( paired t-test ).

\*\* indicates statistically significant differences  $p < 0.01$  ( at 30 - 50 min and 240 min ) compared to basal FW mean arterial blood pressure ( paired t-test ).

Blood Pressure ( mm. Hg )

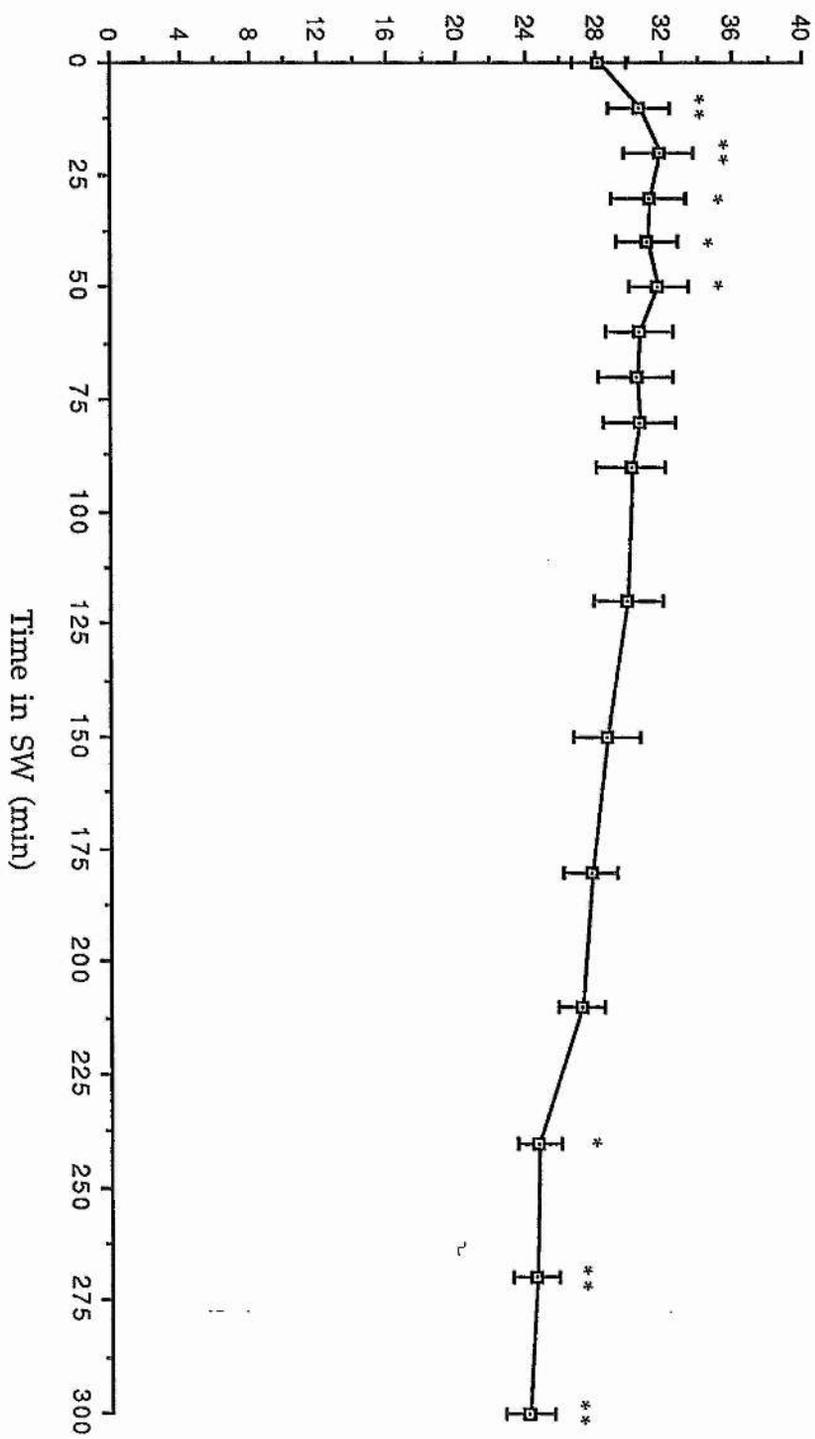


Table 3.3

Table 3.3. Effect of acute SW adaptation on plasma composition.

Samples were obtained in FW and after 90 min when SW transfer had been achieved. Results are means  $\pm$  S.E.M. of 10 animals.

\* indicates statistically significant differences at  $p < 0.05$  from FW group ( 0 min in SW ) ( paired t-test ).

|                           | Time in SW<br>(mins) |           |
|---------------------------|----------------------|-----------|
|                           | 0                    | 90        |
| Osmolality<br>(mOsmol/kg) | 312 ± 4              | 335 ± 4 * |
| Chloride<br>(mmol/l)      | 78 ± 4               | 94 ± 4 *  |

Figure 3.5

Figure 3.5. Effect of acute SW adaptation on drinking rate.

Results are means  $\pm$  S.E.M. of six animals.

FW group is indicated as time 0 min in SW on the graph. All other time points represent groups of eels that have been held in 100 % SW for the corresponding time interval.

\*\*\* indicates statistically significant differences at  $p < 0.005$  from FW control group ( 0 min in SW ) ( unpaired t-test ).

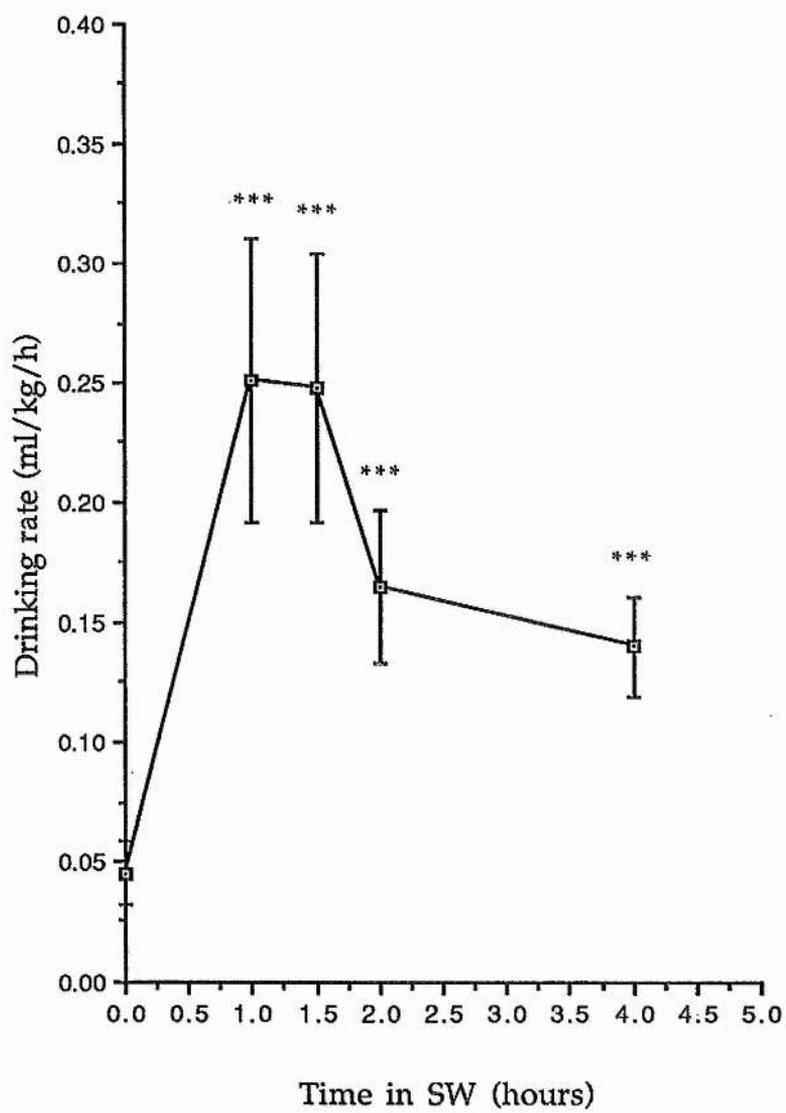
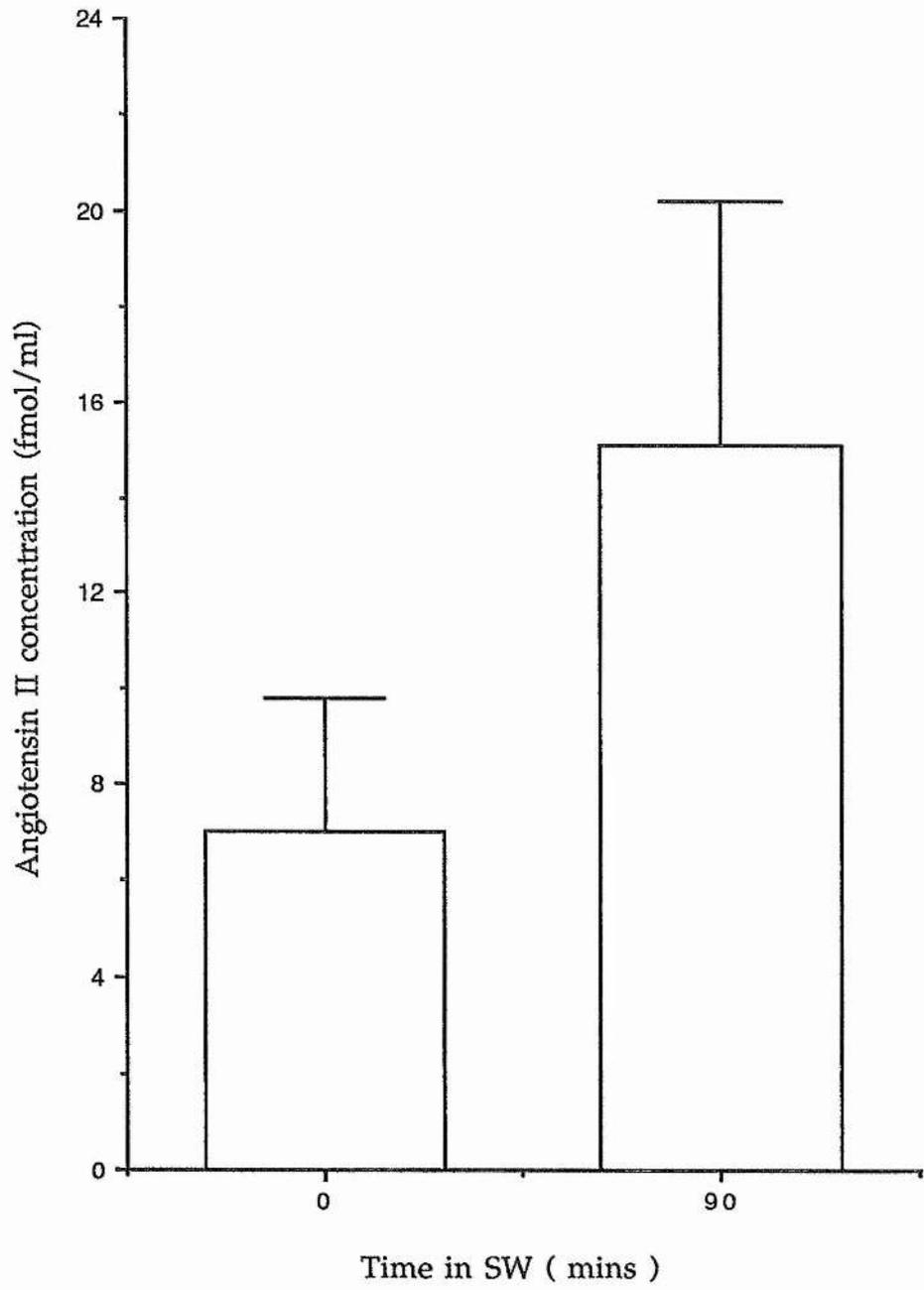


Figure 3.6

Figure 3.6. Effect of acute SW adaptation on plasma AII concentration.

Results are means  $\pm$  S.E.M. of six animals. Plasma AII concentrations were determined prior to the changeover to SW ( at time zero ) and after 90 min when SW transfer had been achieved.



( Figure 3.5 ). This increase was maintained for 90 min after SW transfer, and thereafter started to decline, although the drinking rate after 4 h (  $0.14 \pm 0.02$  ml/kg/h ) was still significantly higher than the FW basal rate (  $p < 0.001$  ).

#### 3.2.4. Angiotensin II

Figure 3.6 shows the plasma AII levels during the initial acute transfer period from FW to SW. Plasma AII levels were augmented after the 90 min transfer period into full SW, with the concentration rising from  $7.02 \pm 2.79$  fmol/ml initially to  $15.07 \pm 5.14$  fmol/ml after 90 min, although this increase was not statistically significant.

### 3.3. Pharmacological manipulation of the RAS

#### 3.3.1 Freshwater-adapted eel.

##### 3.3.1a Blood pressure

Injection of 0.9 % saline, as control, into a FW-adapted eel had no effect on the mean arterial blood pressure. Figure 3.7a shows a typical trace obtained after injection of saline. For an individual fish the blood pressure values recorded during this manipulation were used as a comparison with the corresponding blood pressure trace obtained after the injection of papaverine and/or captopril. Figure 3.7b shows a typical trace of the blood pressure response after the administration of papaverine. Injection of papaverine into FW eels caused an initial decrease in mean arterial blood pressure. The blood pressure declined from  $33.8 \pm 2.9$  mmHg to  $22.8 \pm 3.5$  mmHg (  $p < 0.005$  ) within the first twenty min ( Figure 3.8a ). The blood pressure started to recover after 20 min and within 70 min it was similar to the control pressure of  $34.3 \pm 2.1$  mm Hg. A typical blood pressure response to the administration of captopril is seen in Figure 3.7c. When captopril only was administered the blood pressure was unaffected, with values of  $33.5 \pm 2.5$  mm Hg,  $32.8, \pm 1.9$  mm Hg,  $32.8 \pm 2.3$  mm Hg corresponding to levels of  $34.1 \pm$

Figure 3.7

Figure 3.7 Typical blood pressure traces after the administration of papaverine and/or captopril to FW-adapted eel

Figure 3.7a Typical response after injection of saline

The arrow indicates the timing of the injection of 0.9 % saline.

Figure 3.7b Typical response after injection of papaverine

The arrow indicates the timing of the injection of papaverine.

Figure 3.7c Typical response after injection of captopril

The arrow indicates the timing of the injection of captopril

Figure 3.7d Typical response after injection of captopril 15 min prior to papaverine

The first arrow indicates the timing of the captopril injection, followed 15 min later by an injection of papaverine, as shown by the second arrow on the trace.

Fig. 3.7

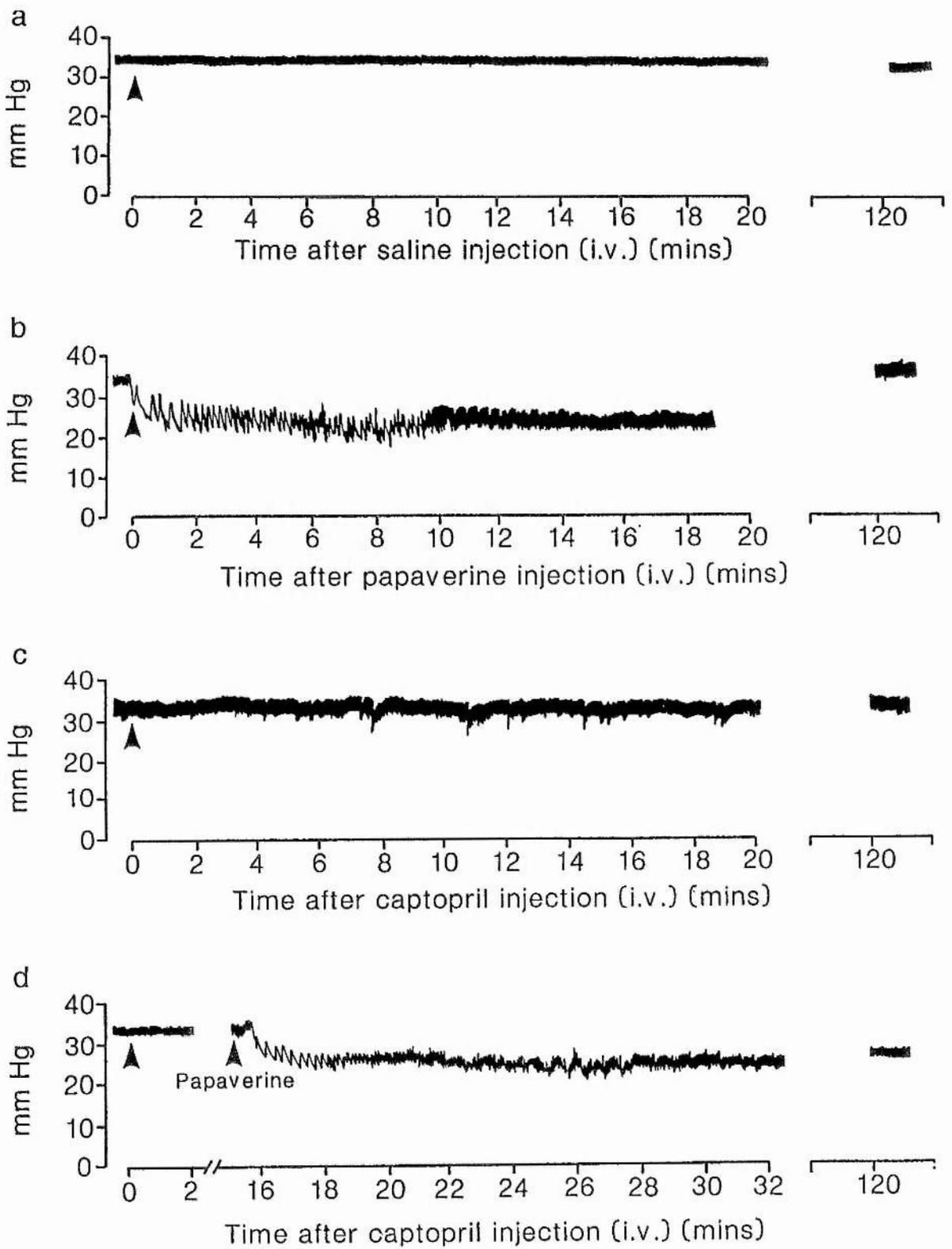


Figure 3.8

Figure 3.8.a. Effect of administration of papaverine on mean arterial blood pressure of FW eels.

Papaverine was administered at a dose of 10 mg/kg. Results shown are means  $\pm$  S.E.M. of six animals.

\* indicates statistically significant differences at  $p < 0.05$  compared to the corresponding time interval of the control arterial pressure recorded after an injection of 0.9% saline ( paired t-test ).

The open boxes illustrate the effect of papaverine on mean arterial blood pressure, with the filled boxes representing the control values.

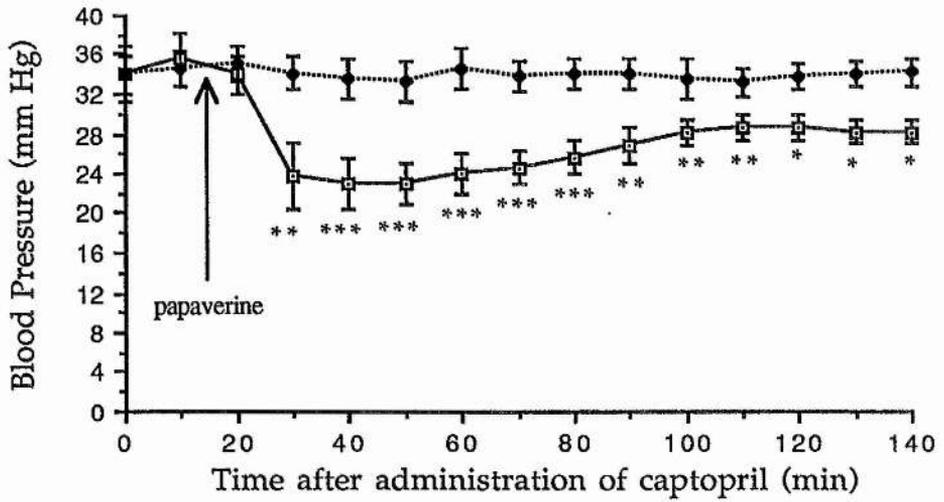
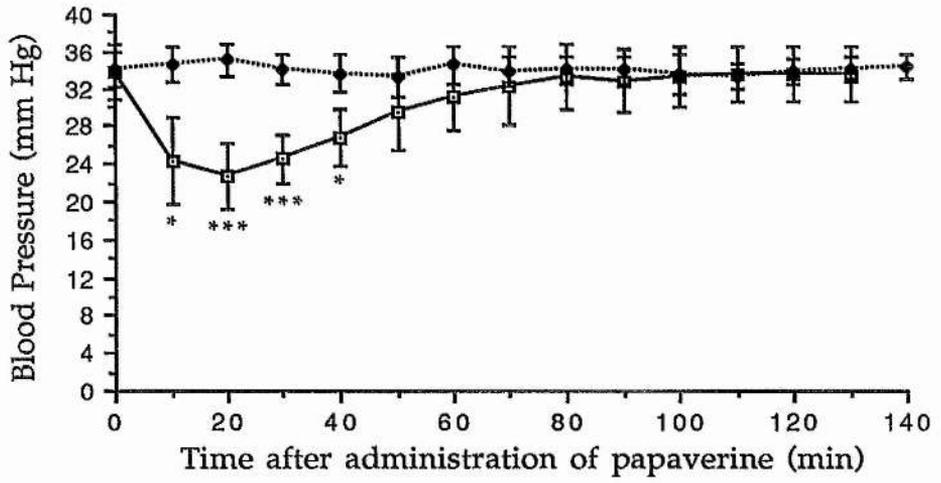
\*\*\* indicates statistically significant differences at  $p < 0.005$  compared to the corresponding control arterial pressure ( paired t-test ).

Figure 3.8.b Effect of administration of papaverine after an injection of captopril, on mean arterial blood pressure of FW eels.

Single injections of captopril ( 72 mg/kg ) and papaverine ( 10 mg/kg ) were administered to FW cannulated eels. Captopril was administered 15 minutes prior to papaverine. Time zero commences with the captopril injection and administration of papaverine is indicated by the arrow. Results shown are means  $\pm$  S.E.M. of six animals.

The open boxes illustrate the effect of papaverine, after the prior administration of captopril, on mean arterial blood pressure, with the filled boxes representing the control values.

\*, \*\* and \*\*\* indicates statistically significant differences at  $p < 0.05$ ,  $p < 0.01$  and  $p < 0.005$ , respectively, compared to the corresponding control arterial pressure recorded after an injection of 0.9% saline ( paired t-test ).



min, respectively. Figure 3.7d shows a typical blood pressure trace obtained after the injection of captopril 15 min prior to papaverine. Administering papaverine 15 min after an injection of captopril led to a decrease in the mean arterial blood pressure, from  $34.0 \pm 1.9$  mmHg to  $23.1 \pm 2.5$  mmHg ( $p < 0.05$ ) within twenty min. In all experiments involving the administration of papaverine 15 min post- captopril, time zero was taken to commence with the captopril injection. A complete recovery of blood pressure to the starting levels did not occur with the pressure remaining at  $28.2 \pm 1.2$  mmHg after 100 min ( Figure 3.8b ) compared to the control value of  $33.5 \pm 2.1$  mm Hg (  $p < 0.01$  ).

#### 3.3.1.b, Plasma electrolyte analysis

Plasma samples were analysed for osmolality prior to injection of either papaverine or captopril, and then again once it had been established that the mean arterial blood pressure was starting to recover. In FW eels administration of papaverine led to a significant decrease in plasma osmolality which dropped from  $285.0 \pm 4.8$  mOsmol/kg to  $261.4 \pm 4.9$  mOsmol/kg (  $p < 0.01$  ) after 60 min when the mean blood pressure was  $31.0 \pm 3.5$  mm Hg ( Table 3.4 ). The mean arterial blood pressure of FW eels was unaffected by the injection of captopril and neither did this group of eels show any significant alteration to osmolality with a value of  $281.6 \pm 5.8$  mOsmol/kg 60 min post-injection. The injection of papaverine 15 min after the administration of captopril to FW eels gave a slight but non-significant decrease in osmolality from  $270.2 \pm 5.8$  mOsmol/kg, prior to injection, to  $261.2 \pm 5.5$  mOsmol/kg after 80 min ( Table 3.4 ).

#### 3.3.1c Drinking rate

Administration of papaverine caused a significant increase in the ingestion of fluid of FW eels ( Figure 3.9 ). The drinking rate was elevated from a basal rate of  $0.06 \pm 0.02$  ml/kg/h in the control group to  $0.29 \pm 0.07$

Table 3.4

Table 3.4. Effect of administration of papaverine and/or captopril on plasma osmolality of FW eels.

Single injections of papaverine and/or captopril were given. The dose of papaverine and/or captopril administered is given. When both substances were given in a single manipulation captopril was administered 15 min prior to papaverine and timing commenced upon injection of captopril. Results are means  $\pm$  S.E.M. for five animals.

† indicates osmolality measured 80 min after injection of captopril.

\*\* indicates statistically significant differences at  $p < 0.01$  compared to the corresponding time interval of the control arterial pressure recorded after an injection of 0.9 % saline ( paired t-test ).

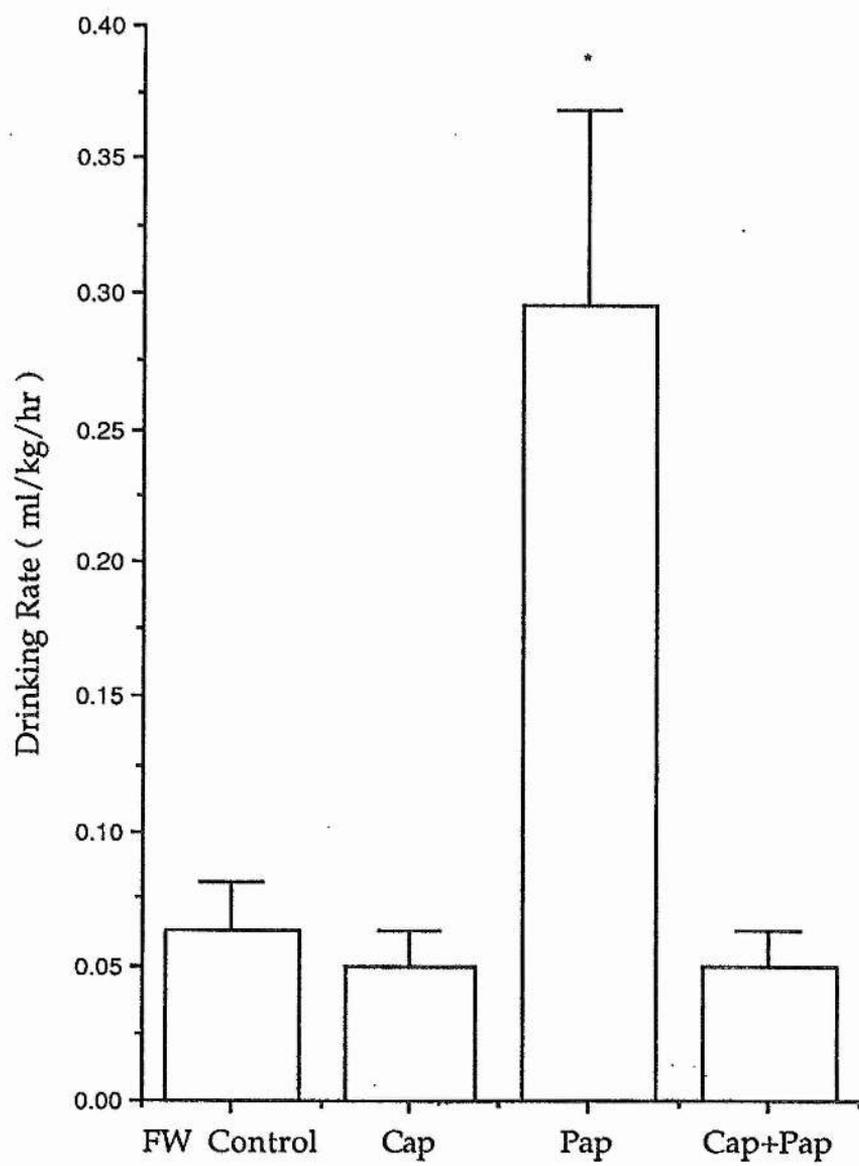
|   | Time after administration<br>( mins ) |               | Change in<br>Osmolality<br>( mOsmol/kg ) |
|---|---------------------------------------|---------------|--|
|   | 0                                     | 60            |  |
| Papaverine<br>( 10mg/kg )                               | 285.0 ± 4.8                           | 261.4 ± 4.9   | -13.6 ± 4.4 <sup>**</sup>                |
| Captopril<br>( 72mg/kg )                                | 284.8 ± 6.0                           | 281.6 ± 5.8   | -3.2 ± 4.1                               |
| Captopril<br>( 72mg/kg ) +<br>Papaverine<br>( 10mg/kg ) | 270.2 ± 5.8                           | † 261.2 ± 5.5 | -9.0 ± 3.4                               |

Figure 3.9

Figure 3.9 Effect of administration of papaverine and/or captopril on drinking rate of FW eels.

Individual injections of papaverine (10 mg/kg ) and/or captopril ( 72 mg/kg ) were given. When both substances were given in a single manipulation captopril was administered 15 min prior to papaverine and timing commenced upon injection of captopril. Results are means  $\pm$  S.E.M. for five animals.

\* indicates statistically significant differences at  $p < 0.05$  from values in the FW control group ( paired t-test ).



ml/kg/h in the experimental group (  $p < 0.05$  ). Administration of captopril had no discernible effect on the low rate of drinking in FW eels. When captopril was injected 15 min before papaverine the previously observed response to papaverine was blocked and a drinking rate of  $0.05 \pm 0.01$  ml/kg/h was recorded (Figure 3.9).

#### 3.3.1.d, Angiotensin II concentrations

AII concentrations were measured in samples obtained on the recovery part of the blood pressure traces as for the plasma osmolality. When papaverine was administered to FW eels a highly significant increase in AII concentration was observed with values rising from  $8.09 \pm 0.43$  fmol/ml to  $29.18 \pm 1.91$  fmol/ml (  $p < 0.001$  ) after 60 min ( Figure 3.10 ). A small but non-significant decrease in plasma AII concentration was observed 60 min after an injection of captopril. Upon administration of papaverine 15 min after captopril to FW eels a significant increase in AII was found. Values were elevated from  $8.42 \pm 0.58$  fmol/ml to  $13.28 \pm 0.89$  fmol/ml (  $p < 0.001$  ) after 80 min ( Figure 3.10 ). This increase was, however, not as great as that observed when papaverine alone was injected into the animal with percentage increases of 57% and 260% respectively.

In summary the hypotension, and subsequent recovery in blood pressure, observed with papaverine administration gave rise to greatly elevated rates of drinking. This physiologically inappropriate increase in fluid ingestion for a FW eel occurred when AII concentration was increased and was accordingly associated with dilution of body fluids as indicated by a decrease in plasma osmolality.

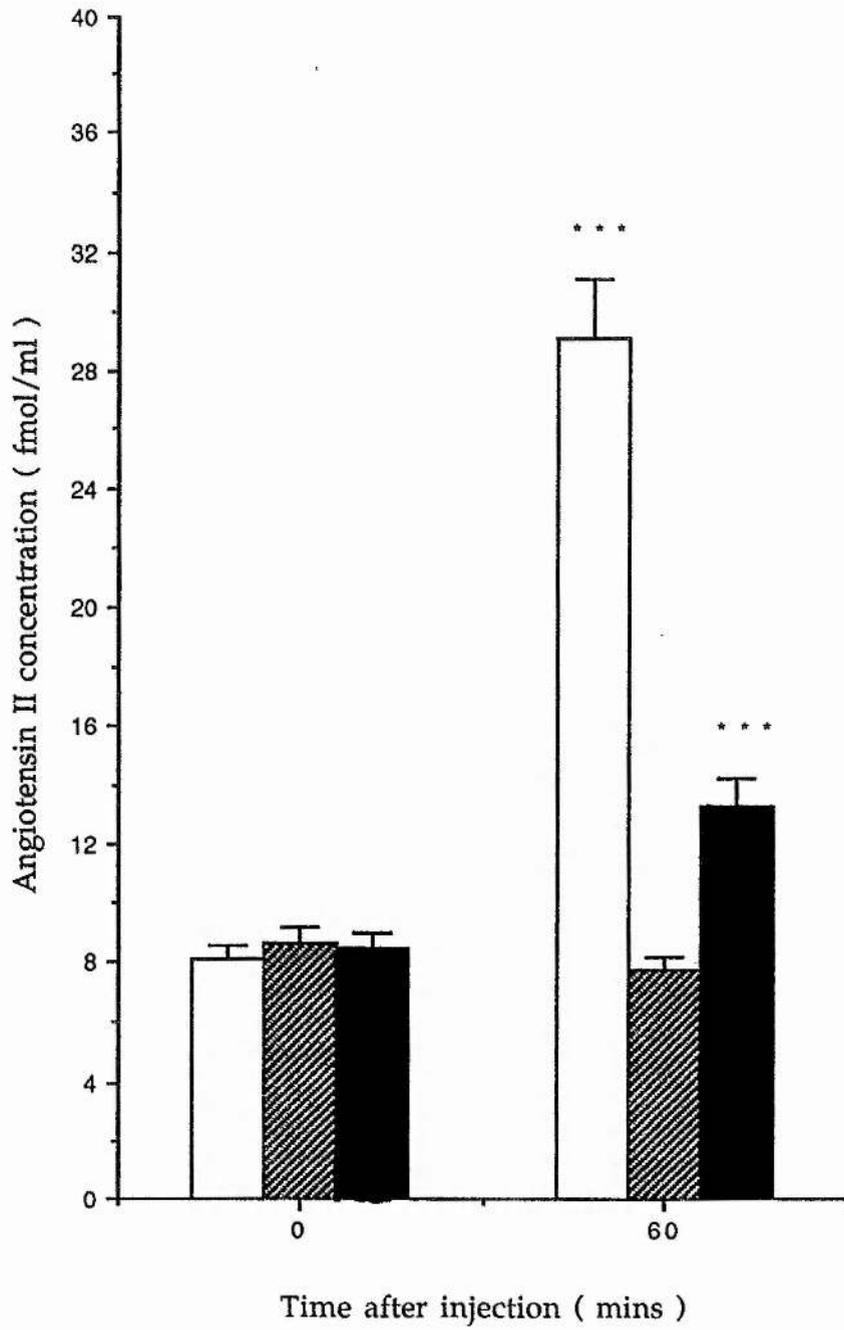
With the injection of captopril alone no change in mean arterial blood pressure, osmolality or drinking rate was measured. There was a slight but non-significant decrease in plasma AII concentration.

Figure 3.10

Figure 3.10 Effect of administration of papaverine and/or captopril on Angiotensin II concentration of FW eels.

Individual injections of papaverine (10 mg/kg ) and/or captopril ( 72 mg/kg ) were given. Blood samples were taken at time 0 min and 60 min post injection for papaverine only and captopril only, and at 80 min when papaverine was administered 15 min after captopril. The open bars demonstrate the effect of papaverine only; the hatched bars, captopril alone; the closed bars show the effect of papaverine after the administration of captopril.

\*\*\* indicates statistically significant differences at  $p < 0.005$  from initial concentrations at time 0 min ( paired t-test ).



When captopril was injected 15 min prior to papaverine, mean arterial blood pressure declined with only partial recovery achieved. Osmolality was not significantly altered and the dipsogenic response to papaverine was completely abolished by captopril. Plasma AII concentration was elevated but this increase was smaller than that observed when only papaverine was injected. These results suggest a threshold level for the dipsogenic action of AII since the increase in concentration did not result in a concomitant elevation in drinking rate.

### 3.3.2 Seawater-adapted eel

#### 3.3.2a Blood pressure

Injection of 0.9 % saline, into a SW-adapted eel had no effect on the mean arterial blood pressure. Figure 3.11a shows a typical trace obtained after injection of saline. Figure 3.11b shows a typical trace of the blood pressure response after the administration of papaverine. In SW fish when papaverine alone was administered the blood pressure was reduced from  $24.2 \pm 1.4$  to  $17.0 \pm 1.3$  mmHg (  $p < 0.001$  ) within ten min and started to recover after 30 min ( Figure 3.12a ). However this recovery was incomplete and blood pressure was still significantly reduced after 2 h (  $23.2 \pm 1.6$  mmHg,  $p < 0.05$  ) compared to the control blood pressure of  $24.8 \pm 0.9$  mm Hg. A typical blood pressure response to the administration of captopril is seen in Figure 3.11c. Administration of captopril alone caused a significant decrease in mean blood pressure with the pressure declining from  $24.2 \pm 1.3$  mm Hg to  $17.0 \pm 0.3$  mm Hg within 20 min of the injection ( Figure 3.12 c ). This hypotension was sustained for the duration of the experiment without showing any signs of recovery. Figure 3.11d shows a typical blood pressure trace obtained after the injection of captopril 15 min prior to papaverine. When captopril was administered 15 min before an injection of papaverine to SW eels a decline from  $23.0 \pm 1.3$  mm Hg to  $16.8 \pm 1.3$  mm Hg was observed within 10 min of

Figure 3.11

**Figure 3.11 Typical blood pressure traces after the administration of papaverine and/or captopril to SW-adapted eel**

**Figure 3.11a Typical response after injection of saline**

The arrow indicates the timing of the injection of 0.9 % saline

**Figure 3.11b Typical response after injection of papaverine**

The arrow indicates the timing of the injection of papaverine

**Figure 3.11c Typical response after injection of captopril**

The arrow indicates the timing of the injection of captopril.

**Figure 3.11d Typical response after injection of captopril 15 min prior to papaverine**

The first arrow indicated the timing of the captopril injection, followed 15 min later by an injection of papaverine, as shown by the second arrow on the trace.

Fig. 3.11

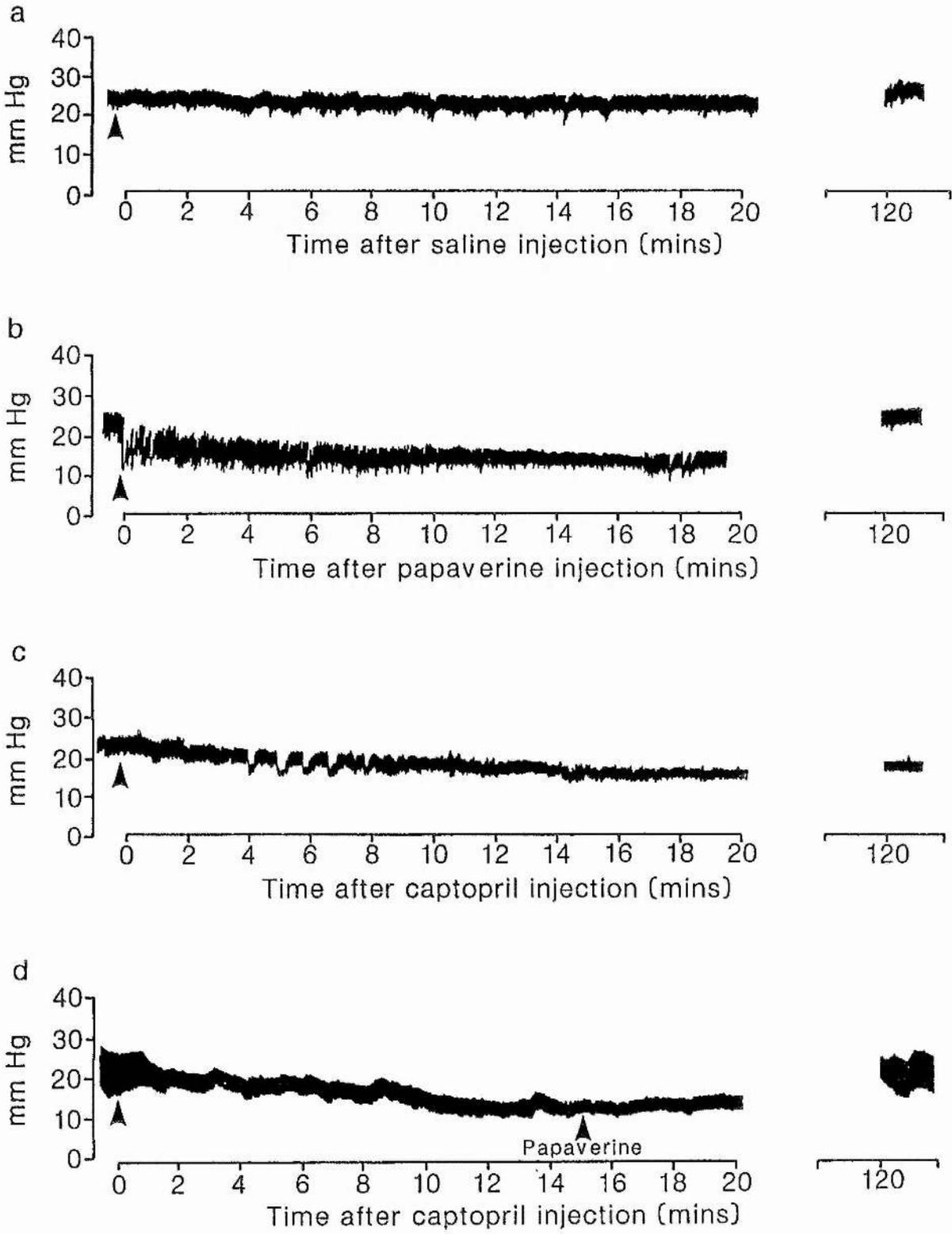


Figure 3.12

Figure 3.12.a Effect of administration of papaverine on mean arterial blood pressure of SW eels.

Papaverine was administered at a dose of 10 mg/kg. Results shown are means  $\pm$  S.E.M. of six animals.

\* indicates statistically significant differences at  $p < 0.05$  compared to the corresponding time interval of the control arterial pressure recorded after an injection of 0.9 % saline ( paired t-test ).

\*\* indicates statistically significant differences at  $p < 0.01$  compared to the corresponding control arterial pressure ( paired t-test ).

\*\*\* indicates statistically significant differences at  $p < 0.005$  compared to the corresponding control arterial pressure ( paired t-test ).

Figure 3.12b Effect of administration of captopril on mean arterial blood pressure of SW eels.

Captopril was administered at a dose of 72 mg/kg. Results shown are means  $\pm$  S.E.M. of six animals.

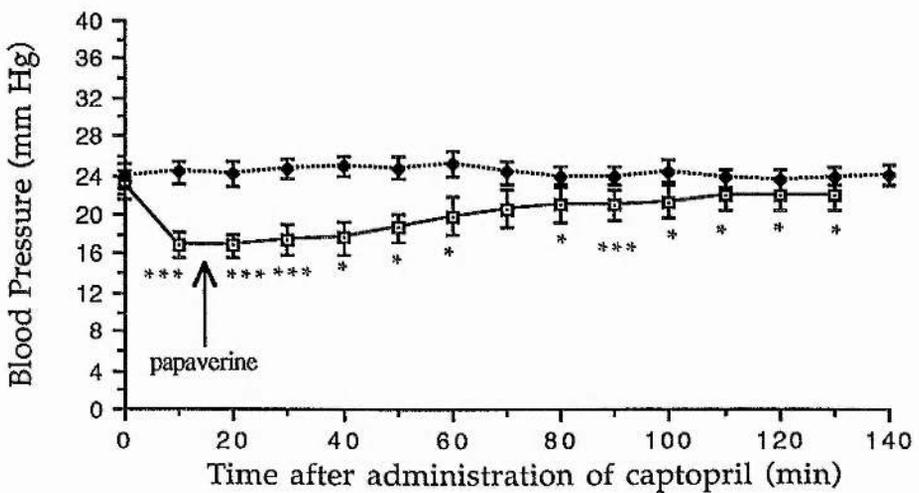
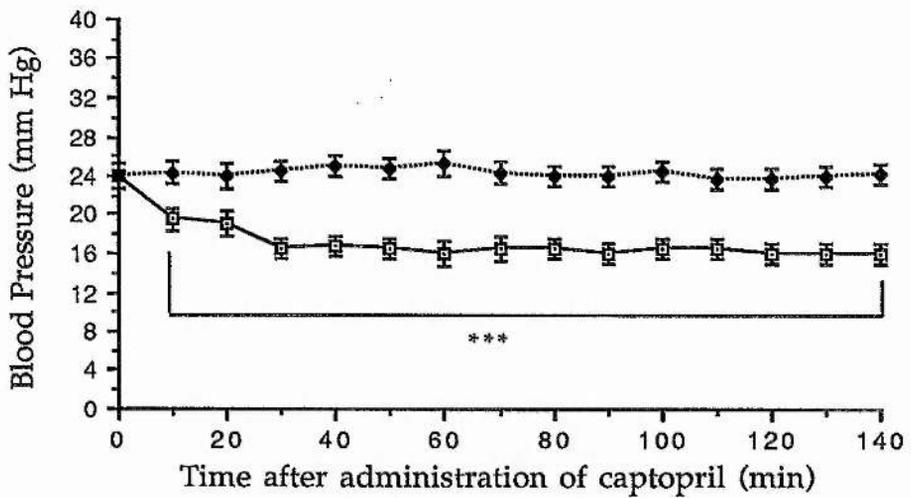
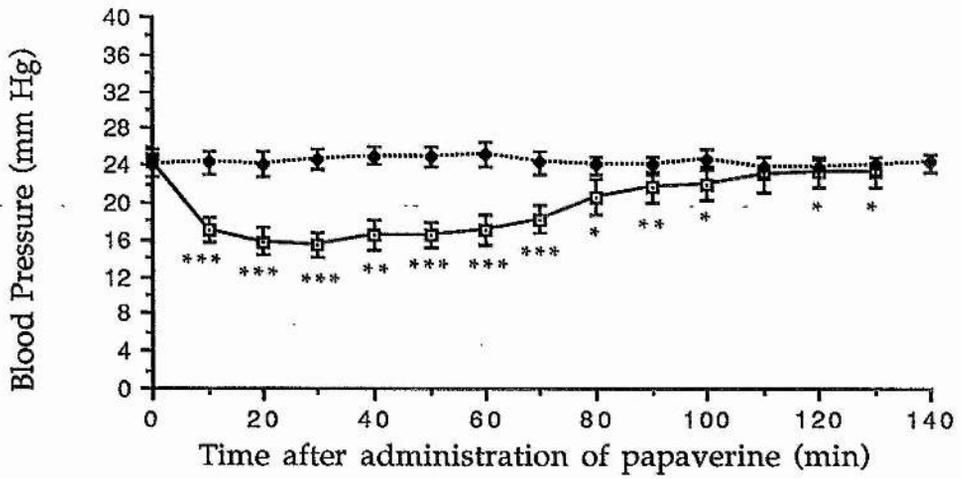
\*\*\* indicates statistically significant differences at  $p < 0.005$  compared to the corresponding control arterial pressure ( paired t-test ).

Figure 3.12.c Effect of administration of papaverine after an injection of captopril on mean arterial blood pressure of SW eels.

Single injections of captopril ( 72mg/kg ) and papaverine ( 10 mg/kg ) were administered to SW cannulated eels. Captopril was administered 15 min prior to papaverine. Time zero commences captopril injection and administration of papaverine is indicated by the arrow. Results shown are means  $\pm$  S.E.M. of six animals.

\* indicates statistically significant differences at  $p < 0.05$  compared to the corresponding control arterial pressure ( paired t-test ).

\*\*\* indicates statistically significant differences at  $p < 0.005$  compared to the corresponding control arterial pressure ( paired t-test ).



the administration of captopril ( Figure 3.12d ). A partial recovery in mean arterial blood pressure (  $21.0 \pm 1.5$  mm Hg ) was obtained after approximately 70 min, but the pressure was sustained at a lower rate thereafter (  $22.2 \pm 1.6$  mm Hg after 120 min ) compared to the SW control arterial blood pressure of  $23.6 \pm 1.1$  mm Hg.

### 3.3.2 b Plasma electrolyte analysis

In the SW group an injection of papaverine led to a significant increase in plasma osmolality with values increasing from  $352.8 \pm 11.63$  mOsmol/kg to  $399.4 \pm 7.2$  mOsmol/kg after 60 min (  $p < 0.01$  ) ( Table 3.5 ). Injection of captopril alone did not have any effect on the plasma osmolality after 60 min ( Table 3.5 ) while administering captopril 15 min prior to papaverine caused a significant increase in osmolality with values increasing from  $372.5$  mOsmol/kg to  $400.8$  mOsmol/kg (  $p < 0.05$  ) after 80 min. This rise in osmolality (  $28.3 \pm 7.0$  mOsmol/kg ) was however smaller than that obtained with an injection of papaverine alone (  $46.6 \pm 9.4$  mOsmol/kg ).

### 3.3.2c Drinking rate

Administration of papaverine resulted in a significant increase in the basal drinking rate of SW eel from  $0.51 \pm 0.07$  ml/kg/h to  $1.58 \pm 0.28$  ml/kg/h (  $p < 0.005$  ) ( Figure 3.13 ). Captopril was effective in reducing the basal drinking rate in SW eels to  $0.09 \pm 0.02$  ml/kg/h (  $p < 0.005$  ) ( Figure 3.13 ). The injection of captopril 15 min prior to papaverine blocked the papaverine-induced increase in drinking, with a rate of  $0.63 \pm 0.09$  ml/kg/h being recorded. This rate was similar to the normal SW drinking rate of the eel.

### 3.3.2d Angiotensin II concentrations

Plasma samples were taken at intervals over a four hour period after injection of papaverine into SW fish and the AII concentration determined.

Table 3.5

Table 3.5. Effect of administration of papaverine and/or captopril on plasma osmolality of SW eels.

Single injections of papaverine and/or captopril were given. The dose of papaverine and/or captopril administered is given. When both substances were given in a single manipulation captopril was administered 15 min prior to papaverine and timing commenced upon injection of captopril. Results are means  $\pm$  S.E.M. for five animals.

† indicates osmolality measured 80 min after injection of captopril.  
\* and \*\*\* indicates statistically significant differences at  $p < 0.05$  and  $p < 0.005$ , respectively, compared to values prior to injection at 0 min (paired t-test).

|   | Time after administration<br>(mins) |               | Change in<br>Osmolality<br>(mOsmol/kg) |
|---|-------------------------------------|---------------|--|
|   | 0                                   | 60            |  |
| Papaverine<br>(10mg/kg)                             | 352.8 ± 11.6                        | 399.4 ± 7.2   | 46.6 ± 9.4 <sup>***</sup>              |
| Captopril<br>(72mg/kg)                              | 373.8 ± 3.1                         | 369.8 ± 5.2   | -4 ± 5.2                               |
| Captopril<br>(72mg/kg) +<br>Papaverine<br>(10mg/kg) | 372.5 ± 4.9                         | † 400.7 ± 6.4 | 28.3 ± 7.0 <sup>*</sup>                |

Figure 3.13

Figure 3.13. Effect of administration of papaverine and/or captopril on drinking rate of SW eels.

Individual injections of papaverine (10 mg/kg ) and/or captopril (72 mg/kg ) were given. When both substances were given in a single manipulation captopril was administered 15 minutes prior to papaverine and timing commenced upon injection of captopril. Results are means  $\pm$  S.E.M. for five animals.

\*\*\* indicates statistically significant differences at  $p < 0.005$  compared to values in the SW control group ( unpaired t-test ).

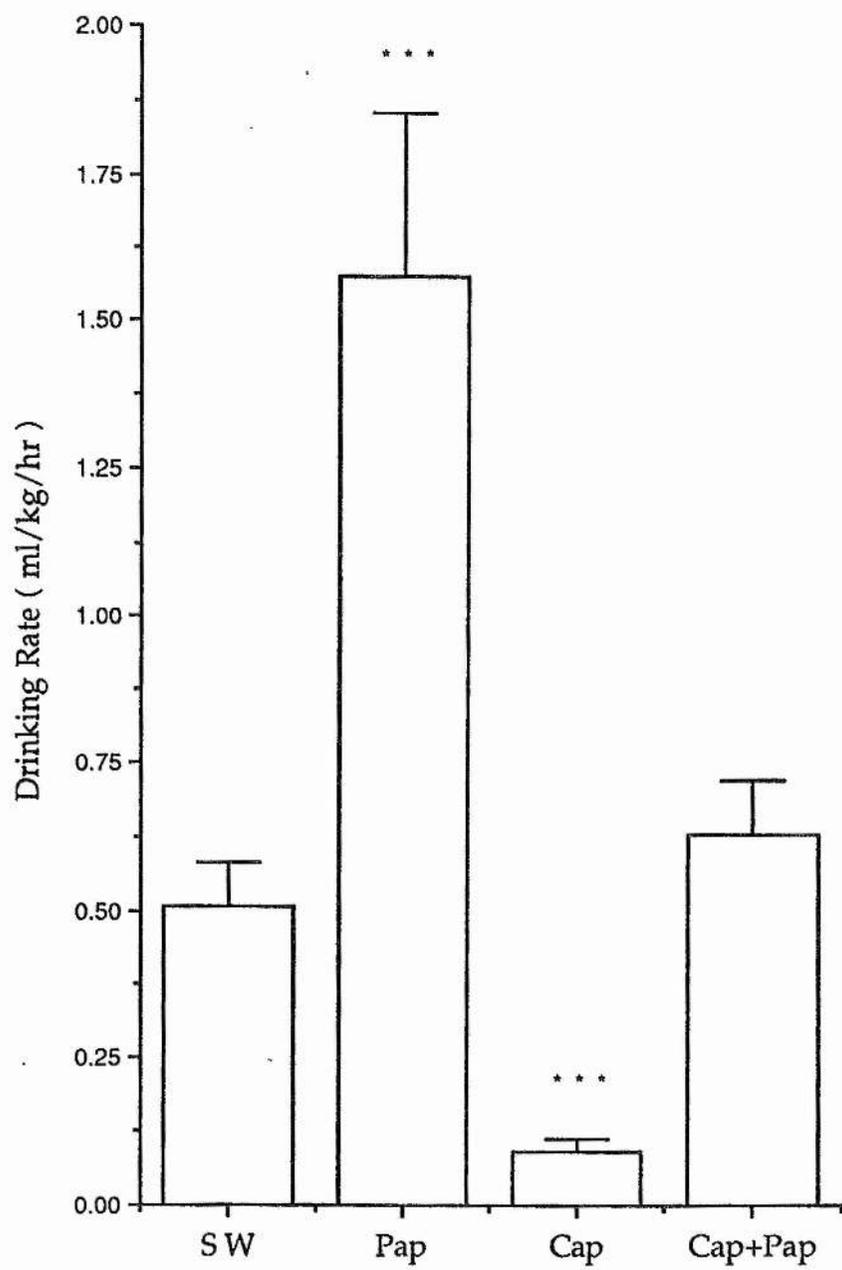


Figure 3.14

Figure 3.14.a Effect of administration of papaverine on plasma angiotensin II concentration of SW eels.

Blood samples were taken at intervals over four hours. Results are means  $\pm$  S.E.M. of six animals.

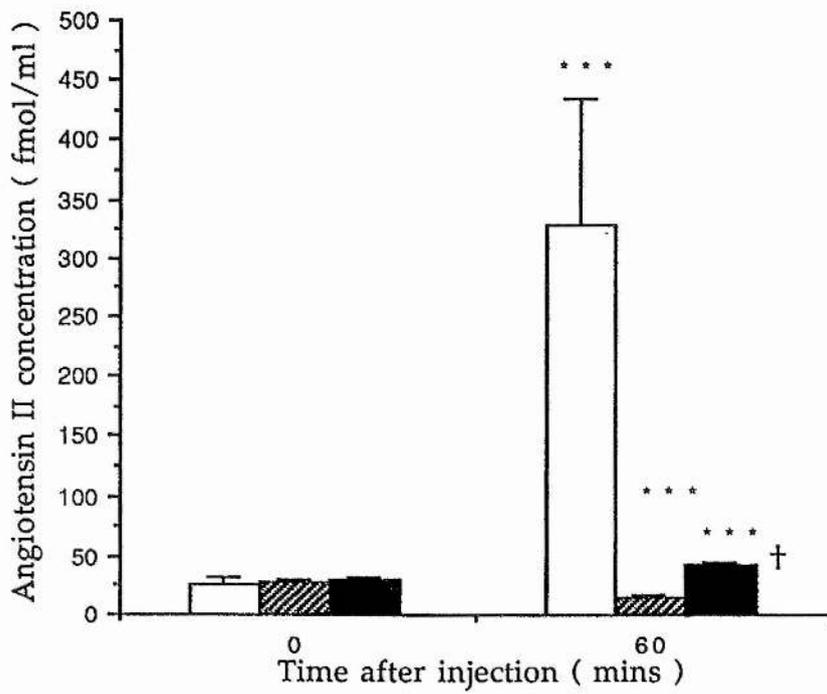
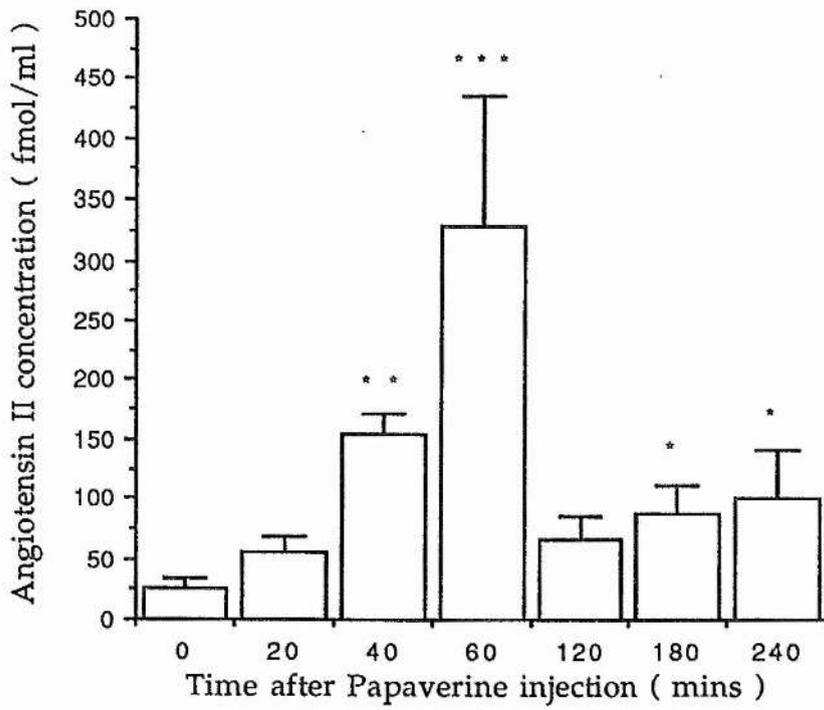
\*, \*\* and \*\*\* indicates statistically significant differences at  $p < 0.05$ ,  $p < 0.01$  and  $p < 0.005$  from initial concentrations at time 0 min (paired t-test).

Figure 3.14.b Effect of administration of papaverine and/or captopril on plasma angiotensin II concentration of SW eels.

Individual injections of papaverine (10 mg/kg) and/or captopril (72 mg/kg) were given. When both substances were given in a single manipulation, captopril was administered 15 min prior to papaverine and timing commenced upon injection of captopril. Results are means  $\pm$  S.E.M. of six animals.

Clear bars indicate papaverine only given; hatched bars, captopril only; solid bars show both papaverine and captopril administration. † indicates plasma AII concentration measured 80 min after injection of captopril.

\*\*\* indicates statistically significant differences at  $p < 0.005$  from initial concentrations at time 0 min (paired t-test).



It was clearly seen that papaverine administration led to a large increase in plasma AII which was maximal at  $329.15 \pm 106.67$  fmol/ml ( $p < 0.005$ ) after 60 min ( Figure 3.14a ). This rapidly decreased but remained significantly increased compared to the initial plasma AII concentration.

Giving captopril alone to SW fish showed a significant decrease in plasma AII after 60 min with a drop from  $27.72 \pm 1.96$  fmol/ml to  $14.64 \pm 2.44$  fmol/ml ( $p < 0.005$ ) ( Figure 3.14b ). When both captopril and papaverine were given together an elevation in plasma AII levels was observed after 60 min, rising from  $29.67 \pm 1.92$  fmol/ml to  $42.68 \pm 1.67$  fmol/ml ( $p < 0.005$ ) ( Figure 3.15c ). This increase was, however, much smaller than that obtained when papaverine only was administered to the animals.

In summary, upon injection of papaverine hypotension was seen to occur immediately. Almost a complete recovery of blood pressure was achieved after 100 min although the blood pressure was still significantly different to the resting level. This recovery in blood pressure gave rise to an increase in drinking rate which was associated with an elevation in plasma AII concentration and plasma osmolality.

Captopril caused a sustained decrease in mean arterial blood pressure. Osmolality was unaffected, but the basal drinking rate of SW eels was significantly reduced by captopril. Plasma AII concentration also decreased.

When captopril was injected prior to papaverine the mean arterial blood pressure was initially reduced with only partial recovery achieved. The dipsogenic response to papaverine was blocked, with an increase in both plasma AII concentration and plasma osmolality being observed. As with the FW eel the changes in plasma osmolality and AII concentration obtained after the administration of both captopril and papaverine were less than those found when papaverine alone was injected. The response of the eel to the presence of a hyperosmotic environment, i.e. SW, apparently relied on the

activation of the endogenous RAS as seen by the almost total blockade of the drinking and blood pressure responses by the pre-administration of captopril.

### 3.4 Chronic studies

#### 3.4.1, Blood pressure

Figure 3.15 shows a representative trace of arterial blood pressure in an individual fish, recorded at daily intervals after chronic transfer from FW to SW. A statistically significant difference was found between the mean arterial blood pressure of long term FW- and long term SW- adapted eels ( that is, eels held in either FW or SW for more than 14 days ), with resting levels of  $31.8 \pm 2.8$  mm Hg and  $22.1 \pm 0.9$  mm Hg.(  $p < 0.01$  ) respectively. When eels were chronically transferred from FW to SW and the mean blood pressure monitored daily during this period of adaptation, a general decline in the pressure was observed ( Fig 3.16 ). On the second day post- SW transfer the blood pressure was significantly reduced to  $28.6 \pm 2.2$  mm Hg (  $p < 0.05$  ), and, thereafter, the mean pressure continued to decrease. After three days SW transfer the mean arterial blood pressure was at a level comparable to that found in long term SW adapted animals and no significance differences were observed thereafter, between the long term SW group and the chronic SW adaptation group.

#### 3.4.2 Plasma electrolyte analysis

Plasma osmolality was shown to be significantly different between long term FW-adapted animals (  $294.3 \pm 33$  mOsmol/kg ) and long term SW-adapted eels (  $372.0 \pm 7.9$  mOsmol/kg,  $p < 0.05$  ). A corresponding difference was also seen in plasma chloride and sodium concentrations ( Figures 3.17a, b, c ). Sodium concentrations increased from  $168.0 \pm 2.9$  mMol/l to  $190.1 \pm 7.9$  mMol/l (  $p < 0.05$  ) and chloride concentrations from  $82.6 \pm 5.1$  mMol/l to  $149.0 \pm 5.1$  mMol/l (  $p < 0.005$  ).

Figure 3.15

Figure 3.15 Typical blood pressure trace after chronic- and long-term- SW adaptation

Chronic SW adaptation entailed transferring groups of eel from FW to SW for a period of 7 days. Long term- SW adaptation meant that the eel had been held in SW for a period of at least 14 days prior to the experiment.

Fig. 3.15

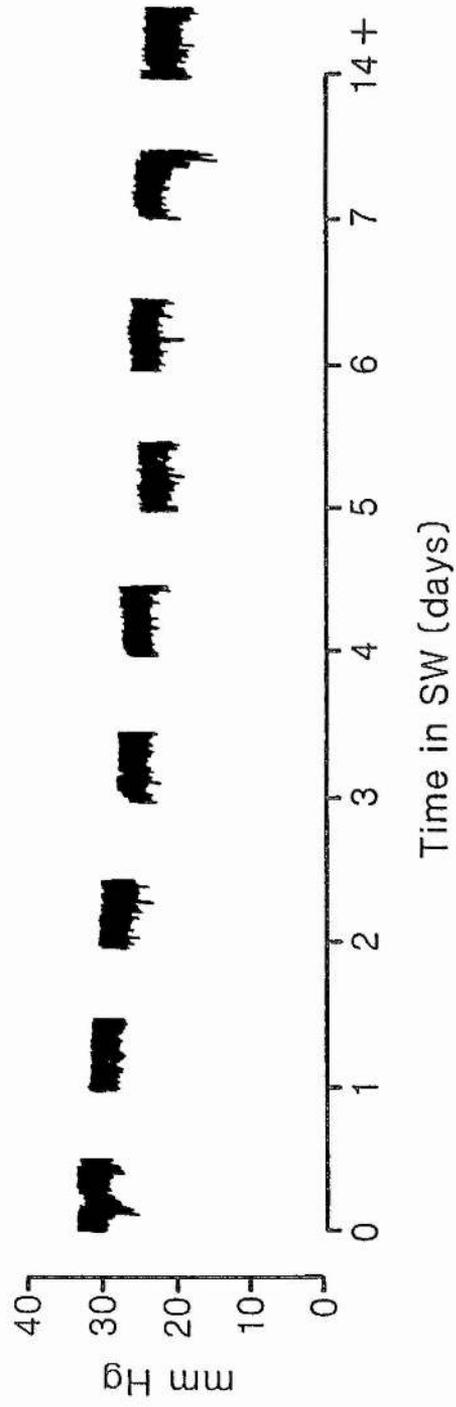


Figure 3.16

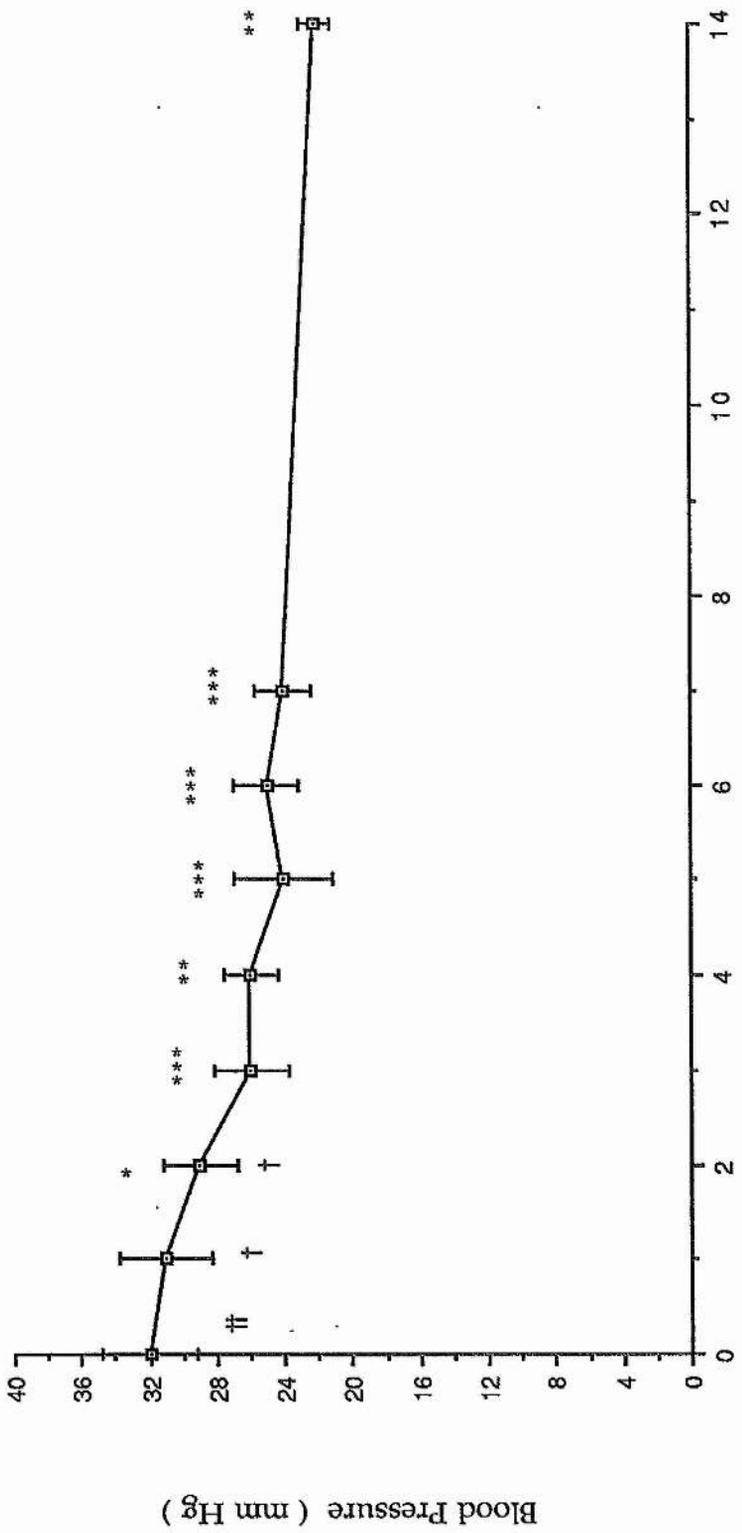
Figure 3.16 Effect of chronic- and long term- SW adaptation on mean arterial blood pressure.

Results are means  $\pm$  S.E.M. of seven animals.

Arterial blood pressure was measured daily for a period of 1 h in individual fish, for a period of 7 days after transfer from FW to SW. Eels that had been maintained in SW for at least 14 days, prior to cannulation, were utilised for the long term SW studies

\*, \*\* and \*\*\* indicate statistically significant differences at  $p < 0.05$ ,  $p < 0.01$  and  $p < 0.005$ , respectively, from FW basal pressure values ( paired t-test for chronically adapted SW animals, unpaired t-test for long-term SW- adapted animals ).

† and †† indicate statistically significant differences at  $p < 0.05$  and  $p < 0.01$ , respectively from values in long term SW adapted animals ( unpaired t-test ).



Time in SW ( days )

Figure 3.17

**Figure 3.17. Effect of chronic- and long term- SW adaptation on plasma electrolyte composition.**

**Figure 3.17.a Effect of chronic- and long term- SW adaptation on plasma osmolality.**

Results are means  $\pm$  S.E.M. of sixteen animals.

\* and \*\* indicate statistically significant differences at  $p < 0.05$  and  $p < 0.01$ , respectively, from values in FW adapted animals ( unpaired t-test ).

**Figure 3.17.b Effect of chronic- and long term- SW adaptation on plasma sodium concentration.**

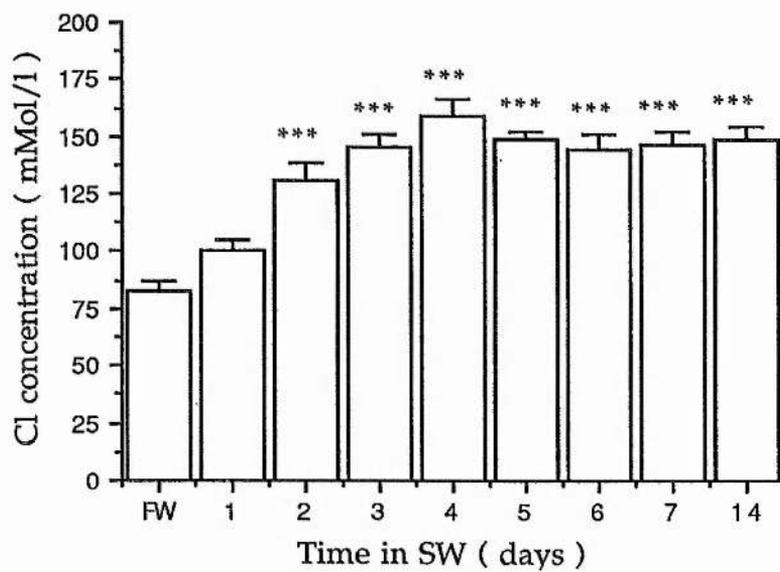
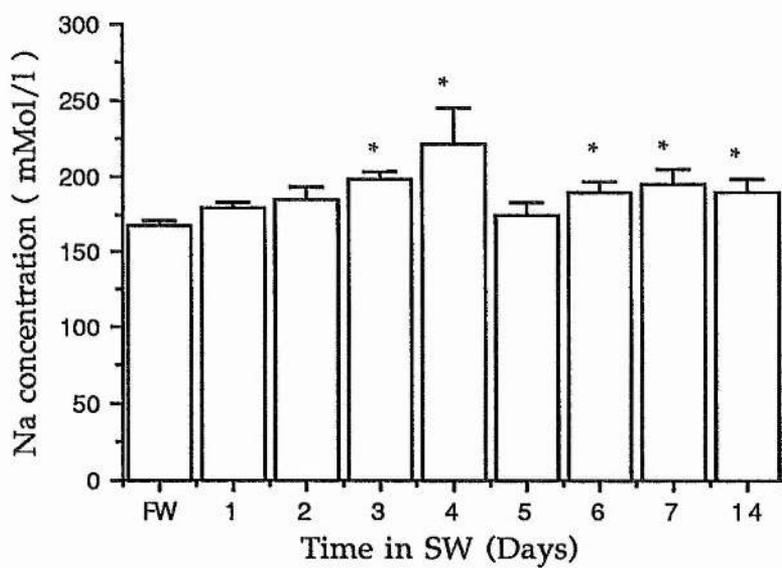
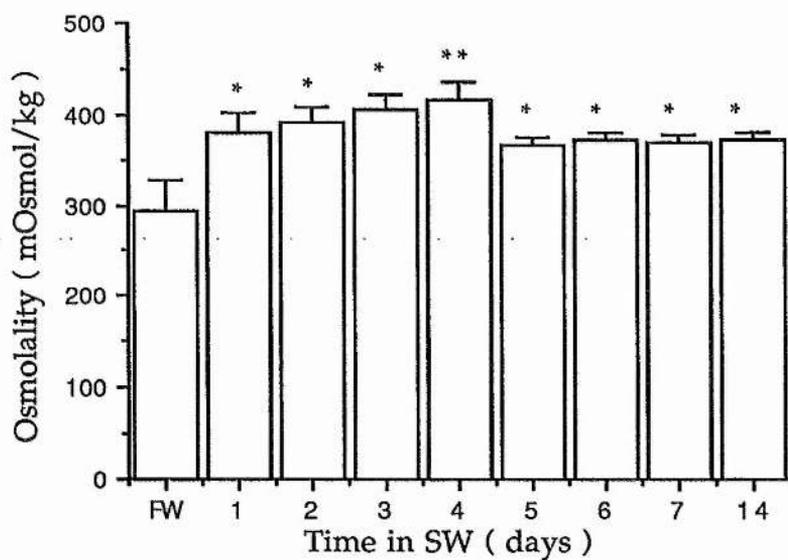
Results are means  $\pm$  S.E.M. of sixteen animals.

\* indicates statistically significant differences at  $p < 0.05$  from values in FW adapted animals ( unpaired t-test ).

**Figure 3.17.c Effect of chronic- and long term- SW adaptation on plasma chloride concentration.**

Results are means  $\pm$  S.E.M. of sixteen animals.

\*\*\* indicate statistically significant differences at  $p < 0.005$  from values in FW adapted animals ( unpaired t-test ).



When eels were transferred from FW to SW an increase in osmolality was observed by the first day of transfer ( Fig 3.17a ). Osmolality peaked four days post-transfer at  $416.1 \pm 20.2$  mOsmol/kg (  $p < 0.01$  ). Thereafter the osmolality declined to that seen in the long term SW-adapted fish. A concomitant increase in plasma sodium and chloride concentrations was also observed, with levels of  $220.5 \pm 23.5$  mMol/l (  $p < 0.05$  ) and  $159.1 \pm 7.2$  mMol/l (  $p < 0.005$  ) respectively, after four days in SW ( Figure 3.17 b,c ).

#### 3.4.3 Drinking rate

Long term FW-adapted eels had a very low rate of imbibition (  $0.06 \pm 0.02$  ml/kg/h ). Figure 3.18 shows that the drinking rate was significantly elevated in long term SW-adapted eels (  $0.49 \pm 0.12$  ml/kg/h ) compared to the FW rate. During chronic transfer from FW to SW, the rate of drinking did not become significantly elevated until 6 days post SW transfer when a value of  $0.25 \pm 0.04$  ml/kg/h was recorded (  $p < 0.01$  ). Thereafter the rate of fluid ingestion increased to  $0.54 \pm 0.07$  ml/kg/h, on day 7, a level comparable to the long term SW level.

#### 3.4.4 Angiotensin II concentration

Fresh-water adapted fish were observed to have an AII concentration of  $8.32 \pm 1.17$  fmol/ml. The plasma AII concentration was seen to increase upon SW adaptation to a maximum six days after SW transfer with a value of  $69.84 \pm 4.88$  fmol/ml (  $p < 0.005$  ) (Figure 3.19 ). The long-term SW adapted fish were shown to maintain their plasma AII levels at an elevated concentration of  $28.24 \pm 2.80$  fmol/ml (  $p < 0.005$  ) to that of FW eels, but without sustaining the six day peak.

#### 3.4.5 Arginine vasotocin concentration

Plasma AVT concentrations were measured in long term- FW and SW adapted eels. AVT levels after 14+ days in SW were comparable with those in

Figure 3.18

Figure 3.18 Effect of chronic- and long term- SW adaptation on drinking rate.

Results are means  $\pm$  S.E.M. of six animals.

\*\* and \*\*\* indicate statistically significant differences at  $p < 0.01$  and  $p < 0.005$ , respectively, compared to values in FW adapted animals ( unpaired t-test ).

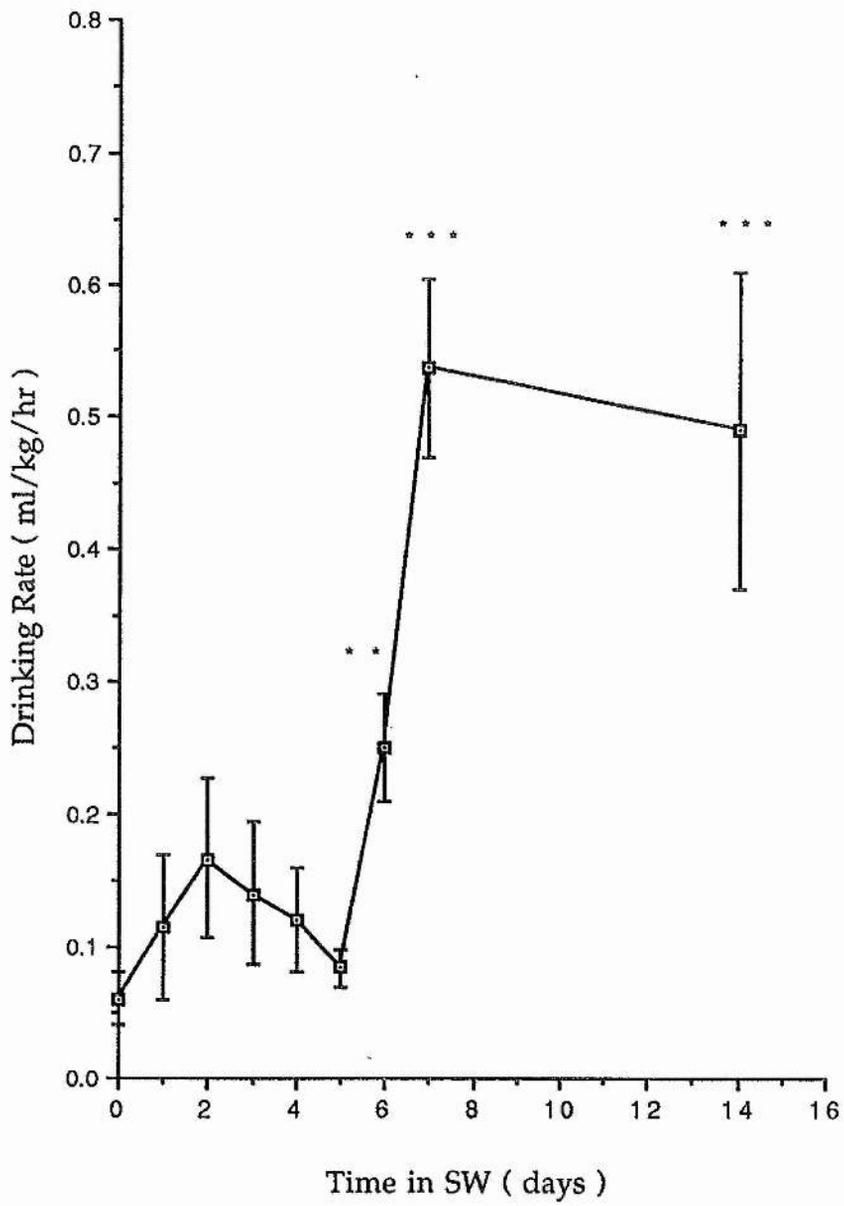


Figure 3.19

Figure 3.19 Effect of chronic- and long term- SW adaptation on plasma angiotensin II concentration.

Results are means  $\pm$  S.E.M. of; eight animals for 6-Day transfer group; seven animals for FW, 3-Day transfer and long term SW adapted groups; six animals for 1-, 2-, 4- and 7-Day transfer groups, and five animals for 5-Day transfer group.

\*\* \*indicates statistically significant differences at  $p < 0.005$  from values in FW adapted animals ( unpaired t-test ).

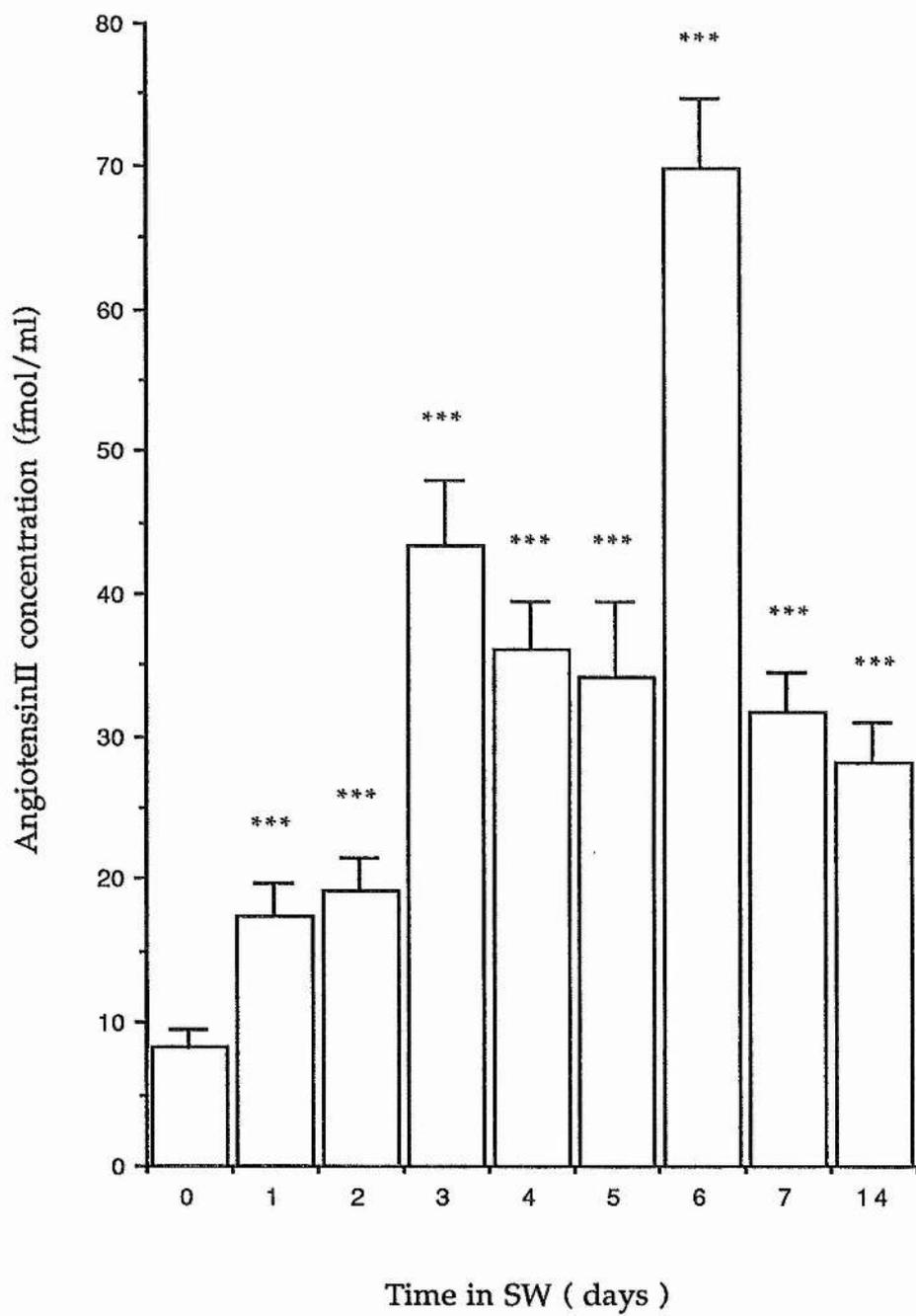
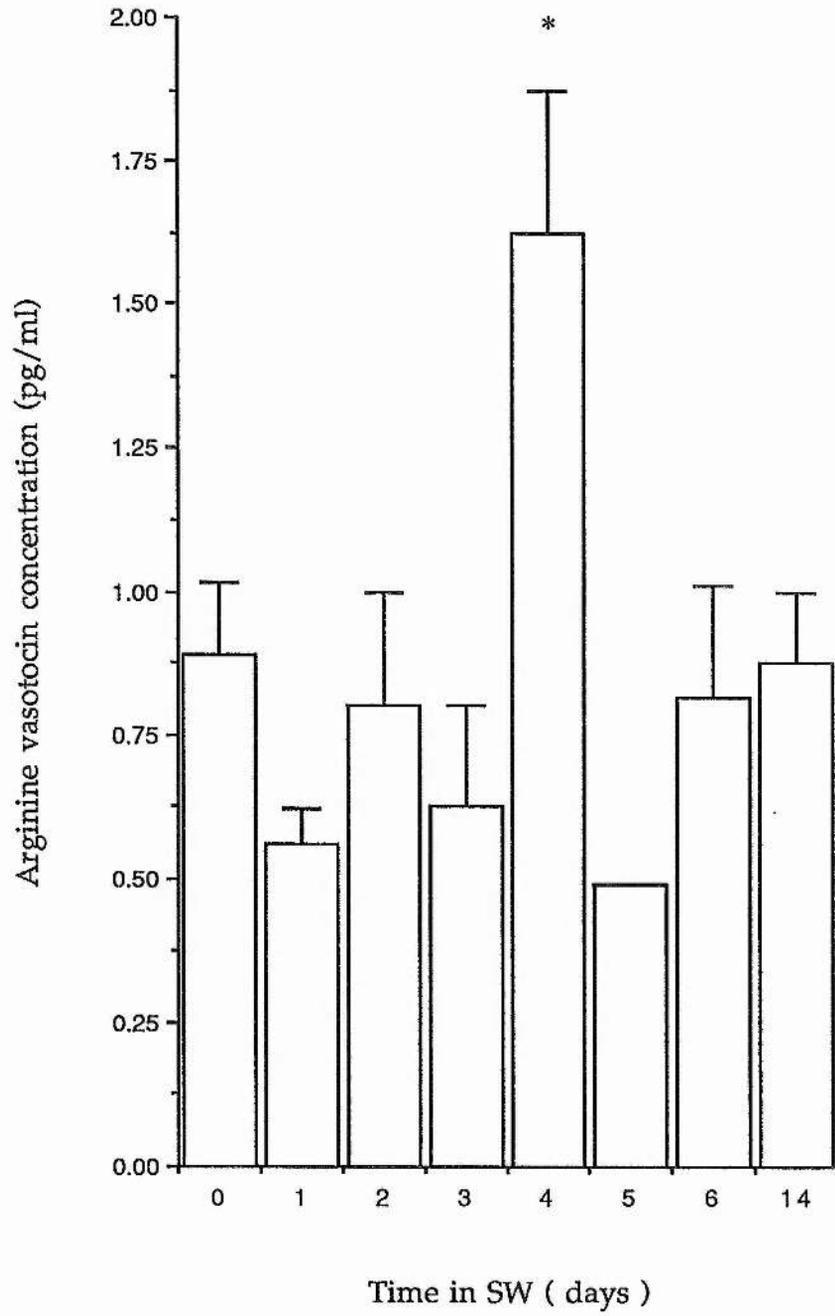


Figure 3.20

Figure 3.20. Effect of chronic- and long term- SW adaptation on plasma arginine vasotocin ( AVT ) concentration

Results are means  $\pm$  S.E.M. of six animals.

\* indicates statistically significant differences at  $p < 0.05$  compared to values in FW group ( unpaired t-test ).



FW animals ( FW  $0.90 \pm 0.12$  pg/ml and SW  $0.89 \pm 0.14$  pg/ml ) as is shown in Figure 3.20. When plasma AVT concentrations were measured in eels during the process of SW acclimation a small transitory rise in concentration around the fourth day after transfer was measured (  $1.64 \pm 0.21$  pg/ml,  $p < 0.05$  ) compared to FW levels.

### 3.4.6 Cortisol

#### 3.4.6a Cortisol concentration

Plasma cortisol concentrations were measured in long-term FW and SW adapted eels and chronically transfer eels. No significant difference was found between the long-term FW- and SW-adapted groups which had concentrations of  $10.1 \pm 0.9$  ng/ml and  $9.6 \pm 1.0$  ng/ml respectively. After transfer from FW to SW an immediate rise in the plasma cortisol concentration was observed giving a peak of  $23.2 \pm 4.3$  ng/ml ( $p < 0.005$ ) after one day in sea-water ( Figure 3.21 ). Thereafter, the plasma concentration showed a general decline towards the long term FW and SW values.

#### 3.4.6b Cortisol dynamics

With the administration of a priming dose of  $^3\text{H}$ -cortisol prior to the commencement of the constant infusion of label, a steady state of radioactivity in the blood plasma was achieved from four to seven hours. Figure 3.22 shows a typical example. Blood samples were taken at hourly intervals for the determination of cortisol production rates ( see Section 3.4.6 d) . The endogenous plasma cortisol concentration did not change during the infusion.

#### 3.4.6c Metabolism of cortisol

The percentage metabolism of the tritiated cortisol was determined by using a 60/40 Methanol/Water isocratic gradient on a HPLC column. The elution profiles of plasma spiked with cortisol and the internal standard,

Figure 3.21

Figure 3.21 .Effect of chronic- and long term- SW adaptation on plasma cortisol concentration.

Results are means  $\pm$  S.E.M. of; 14 animals for 2-Day transfer group; 12 animals for 3-Day transfer group; 11 animals for FW group; nine animals for 1-, 5- and 7-Day transfer and long term SW-adapted groups; six animals for 6-Day transfer group.

\*, \*\* and \*\*\* indicates statistically significant differences at  $p < 0.05$ ,  $p < 0.01$  and  $p < 0.005$ , respectively, compared to values in FW group ( unpaired t-test ).

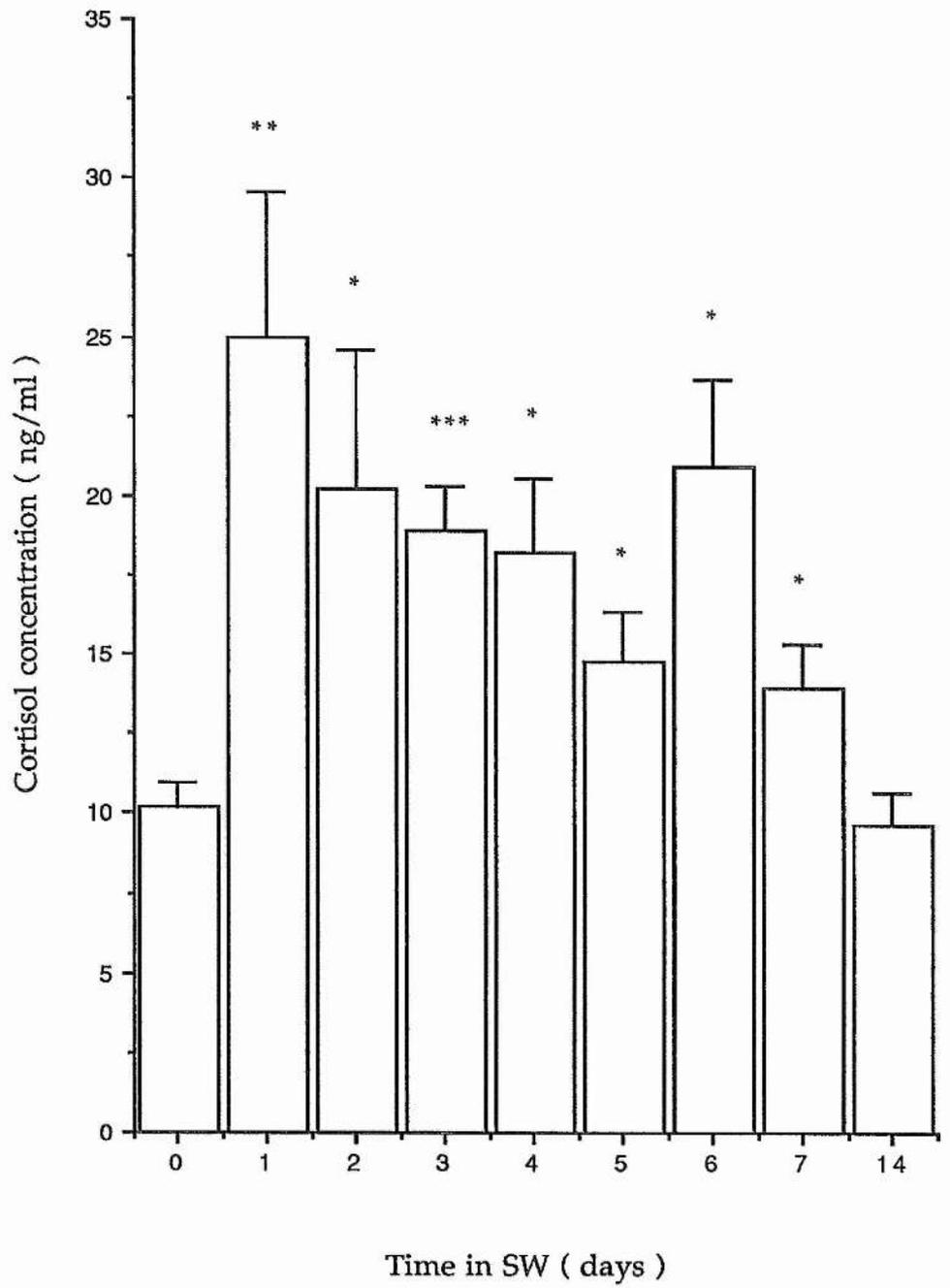


Figure 3.22

Figure 3.22 Constant isotopic infusion

Typical experiment to determine the time to achieve a " steady state " of radioactivity in plasma during continuous infusion of tritiated cortisol ( $^3\text{H}$ -Cortisol). Following a single injection of  $10\mu\text{Ci}$ , an infusion of  $1\mu\text{Ci/hr}$  was begun at time zero, and blood samples were taken at intervals for eight hours.

Open squares show the plasma concentration of tritium ( dpm/ml ) and solid diamonds show endogenous cortisol concentration ( ng/ml ).

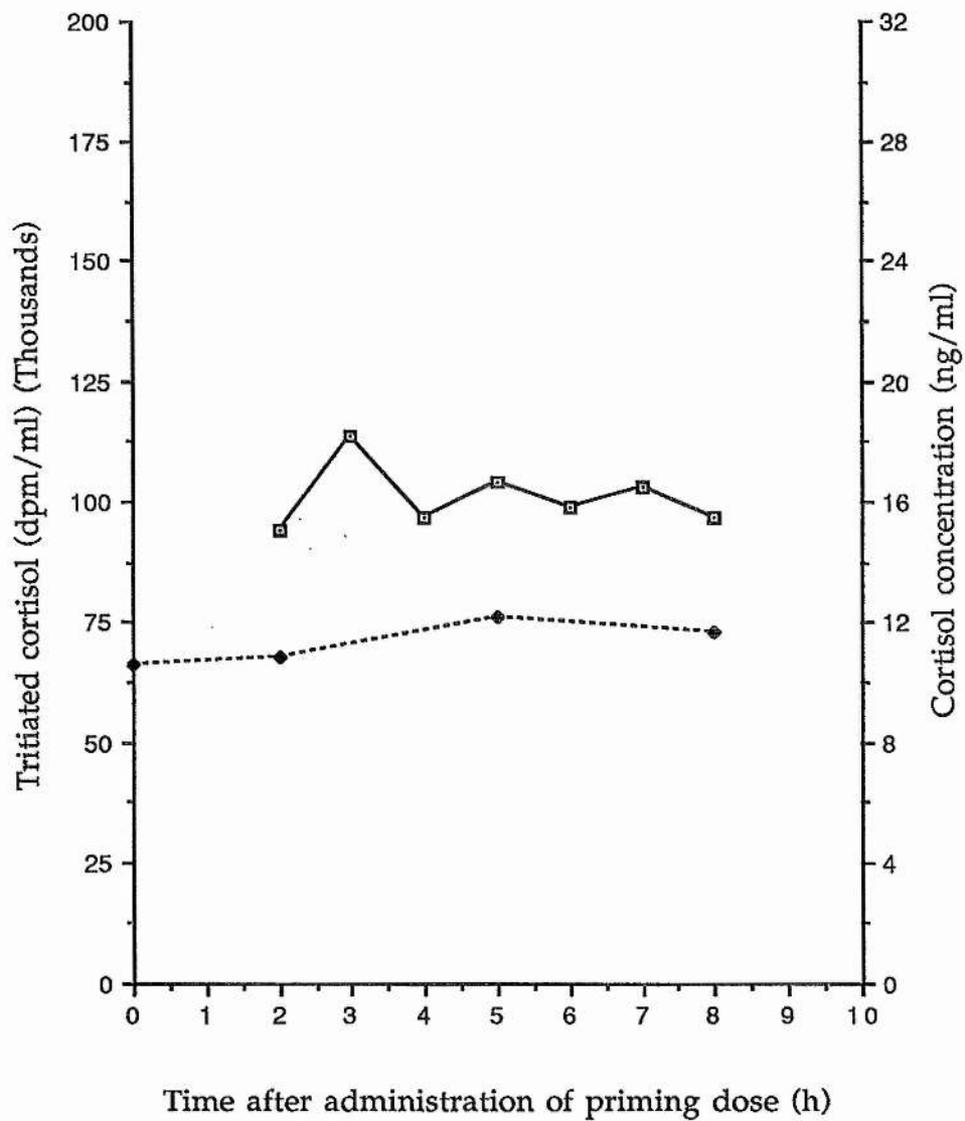


Figure 3.23

Figure 3.23a HPLC profile of plasma spiked steroid standards.

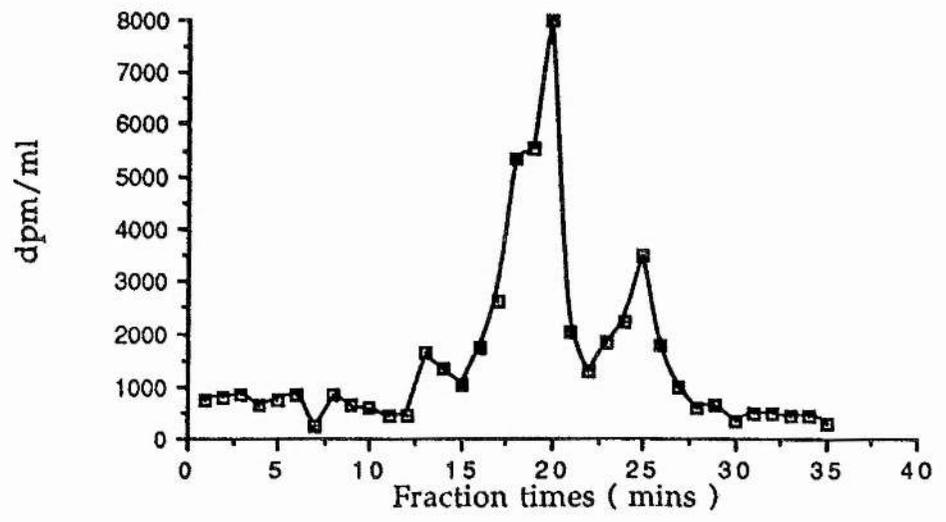
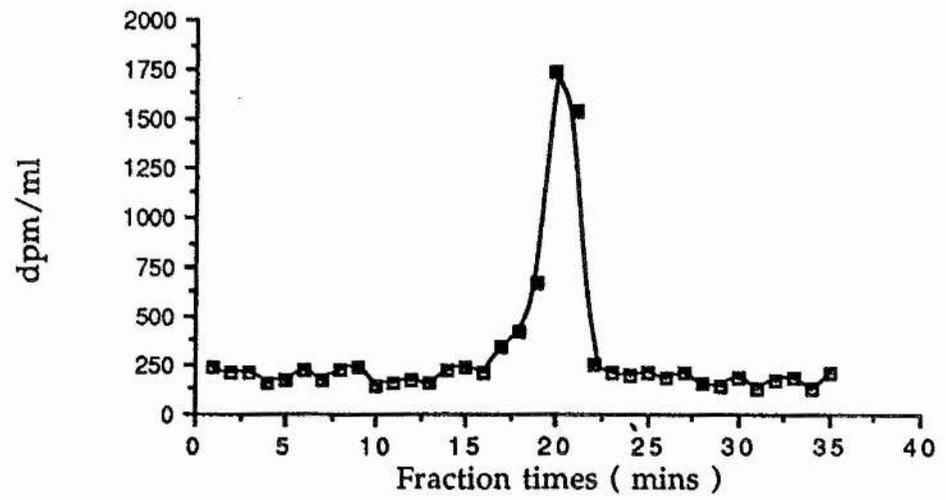
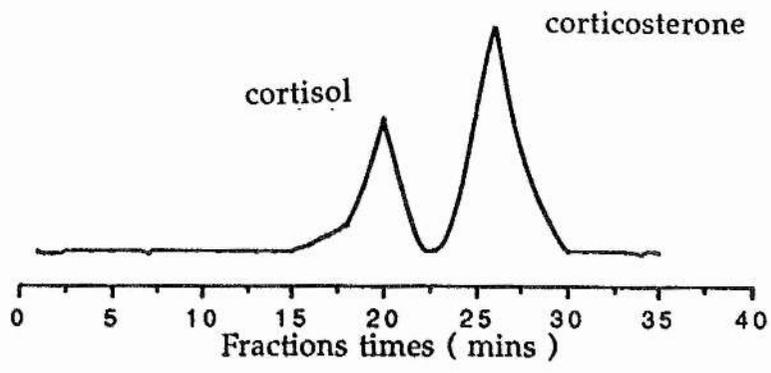
Cortisol ( 19.4 mg/ml ) and corticosterone (working concentration of 328 ng/350 $\mu$ l ) were added to plasma and extracted using SEP PAK C18 cartridges. 175  $\mu$ l of the eluant was applied to a HPLC column and the steroids eluted with an isocratic gradient of 60/40 ( by vol. ) methanol/water containing 0.1% Trifluoroacetic acid.

Figure 3.23b HPLC profile of plasma spiked with tritiated cortisol.

20,000 dpm  $^3$ H-Cortisol is added to plasma, extracted and 175  $\mu$ l applied to HPLC column. Fractions were collected at rate of 1ml/min and counted for radioactivity. The amount of radioactivity in dpm/ml is plotted on the abscissa against time of collection of fraction ( min ) on the ordinate

Figure 3.23c HPLC profile of plasma from SW eel previously infused with tritiated cortisol.

A blood sample was obtained from an eel at the end of constant isotopic infusion experiment. Cortisol was extracted and the eluant applied to a HPLC column. The resulting fractions ( 1ml/min ) were counted for radioactivity. The amount of radioactivity in dpm/ml is plotted on the abscissa against time of collection of fraction ( min ) on the ordinate



corticosterone, are shown in Figure 3.23a, with the retention time of each having been previously confirmed by individual application to the HPLC column. As can be seen, cortisol was eluted after 20 min with corticosterone being retained until approximately 26 min. Figure 3.23b shows the elution profile obtained from plasma which had been spiked with  $^3\text{H}$ -cortisol. The peak from the radioactive labelled steroid corresponded to the cold cortisol peak and so the plasma samples obtained from the infusion of tritiated cortisol were assessed for percentage metabolism by means of this HPLC system.

Figure 3.23c shows a typical radioactive HPLC profile obtained from an infused plasma sample. The  $^3\text{H}$ -cortisol peak can be clearly seen eluting after 20 min, with other smaller peaks in the vicinity which correspond to metabolites of cortisol. These metabolites were not identified in this study.

Each sample was assessed for metabolism of the infused label and this percentage was taken into account in the calculation of the MCR. There was no significant difference in percentage metabolism between the FW eels and any of the other experimental groups as shown in Table 3.6.

#### 3.4.6d Metabolic clearance and blood production rates

As previously seen ( Figure 3.21 ) the plasma concentrations of cortisol in long-term FW and long-term SW adapted fish were similar. There was however a difference in the MCR of cortisol between these two groups of fish. As shown in Figure 3.24b the FW-adapted eels had an average MCR of  $25.21 \pm 1.07$  ml/h/kg, while the SW adapted eels had an MCR of  $37.91 \pm 1.55$  ml/h/kg (  $p < 0.005$  ). The cortisol production rate was similarly lower in the FW-adapted fish,  $0.24 \pm 0.02$  ng/kg/h, compared to  $0.35 \pm 0.03$  ng/kg/h (  $p < 0.005$  ) in SW-adapted fish ( Figure 3.24c ).

Figure 3.24a shows the increase in plasma cortisol concentration upon chronic adaptation from FW to SW. When the cortisol secretory

Table 3.6

Figure 3.23a HPLC profile of plasma spiked steroid standards.

Cortisol ( 19.4 mg/ml ) and corticosterone (working concentration of 328 ng/350 $\mu$ l ) were added to plasma and extracted using SEP PAK C18 cartridges. 175  $\mu$ l of the eluant was applied to a HPLC column and the steroids eluted with an isocratic gradient of 60/40 ( by vol. ) methanol/water containing 0.1% Trifluoroacetic acid.

Figure 3.23b HPLC profile of plasma spiked with tritiated cortisol.

20,000 dpm <sup>3</sup>H-Cortisol is added to plasma, extracted and 175  $\mu$ l applied to HPLC column. Fractions were collected at rate of 1ml/min and counted for radioactivity. The amount of radioactivity in dpm/ml is plotted on the abscissa against time of collection of fraction ( min ) on the ordinate

Figure 3.23c HPLC profile of plasma from SW eel previously infused with tritiated cortisol.

A blood sample was obtained from an eel at the end of constant isotopic infusion experiment. Cortisol was extracted and the eluant applied to a HPLC column. The resulting fractions ( 1ml/min ) were counted for radioactivity. The amount of radioactivity in dpm/ml is plotted on the abscissa against time of collection of fraction ( min ) on the ordinate

| Time in SW (days) | % Metabolism |
|-------------------|--------------|
| long term FW      | 34.5 ± 3.7   |
| 2                 | 42.4 ± 3.0   |
| 4                 | 33.8 ± 2.3   |
| 6                 | 37.2 ± 2.4   |
| long term SW      | 42.8 ± 3.2   |

Figure 3.24

Figure 3.24a Effect of chronic- and long term- SW adaptation on cortisol concentration .

Results are means  $\pm$  S.E.M. for six animals.

\* indicates statistically significant differences at  $p < 0.05$  from values in FW group ( unpaired t-test ).

Figure 3.24b Effect of chronic- and long term- SW adaptation on Metabolic Clearance Rate ( MCR ) of cortisol.

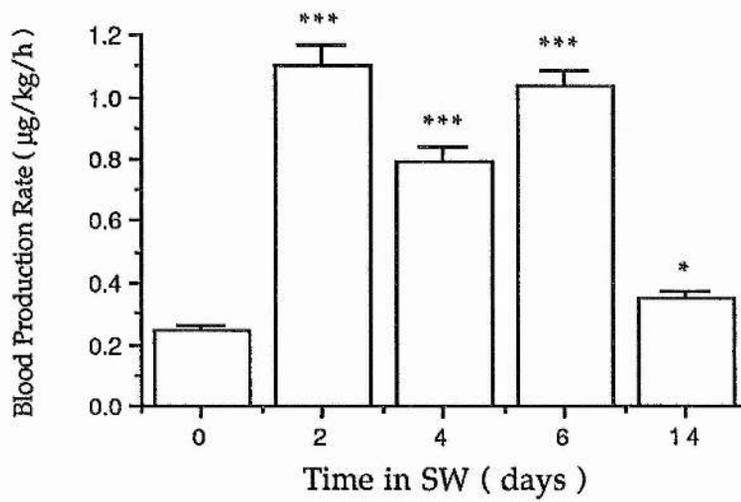
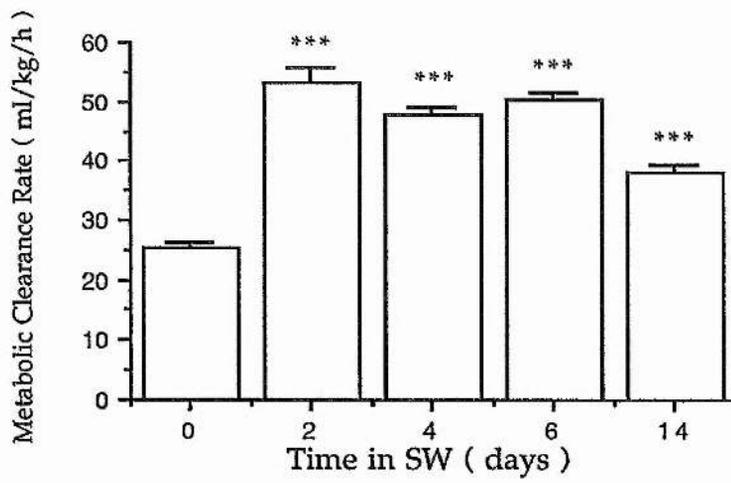
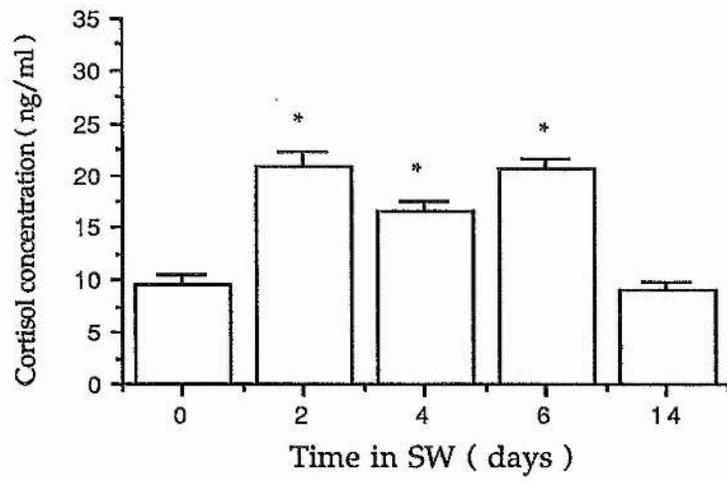
Results are means  $\pm$  S.E.M. for six animals.

\*\*\* indicates statistically significant differences at  $p < 0.005$  from values in FW group ( unpaired t-test ).

Figure 3.24.c Effect of chronic- and long term- SW adaptation on Blood Production Rate ( BPR ) of cortisol.

Results are means  $\pm$  S.E.M. for six animals.

\*\*\* indicates statistically significant differences at  $p < 0.005$  from values in FW group ( unpaired t-test ).



dynamics were investigated 2,4 and 6 days post-SW transfer corresponding and significant increases in MCR and BPR of the steroid were found when compared to the FW rates. The MCR ( Figure 3.24b ) of these three experimental groups was approximately double that of the FW animals with values of  $53.33 \pm 2.27$  ml/kg/h,  $47.74 \pm 1.22$  ml/kg/h and  $50.32 \pm 1.29$  ml/kg/h respectively (  $p < 0.005$  for all groups ). Figure 3.24c shows the rise in the cortisol production rate obtained upon chronic adaptation to SW with mean values of  $1.10 \pm 0.07$   $\mu\text{g}/\text{kg}/\text{h}$ ,  $0.79 \pm 0.05$   $\mu\text{g}/\text{kg}/\text{h}$  and  $1.04 \pm 0.05$   $\mu\text{g}/\text{kg}/\text{h}$  for 2, 4 and 6 days respectively (  $p < 0.005$  for all groups ). The cortisol production rate produces a similar pattern to the cortisol concentration as is clearly seen in Figure 3.24.

#### 3.4.7 Na<sup>+</sup>-K<sup>+</sup>-ATPase activity

A typical standard curve for the measurement of Na<sup>+</sup>-K<sup>+</sup>-ATPase activity is shown in Figure 3.25. The effects of temperature on Na<sup>+</sup>-K<sup>+</sup>-ATPase activity was examined in long term-SW adapted eels ( Figure 3.26a ). The highest activity was obtained at 37°C (  $p < 0.01$  ) and, therefore, all subsequent experiments were carried out at this temperature in order to calculate maximal activity. The effect of membrane dilution was examined and the greatest activity was obtained using a membrane dilution of 1:100, as is seen in Figure 3.26b (  $p < 0.01$  ).

Na<sup>+</sup>-K<sup>+</sup>-ATPase activities for the various experimental groups are shown in Figure 3.27. Long term-FW and long term-SW adapted fish have significantly different activities with values of  $1.48 \pm 0.21$   $\mu\text{mol}/\text{h}/\text{mg}$  protein and  $6.69 \pm 0.54$   $\mu\text{mol}/\text{h}/\text{mg}$  protein (  $p < 0.005$  ), respectively. Upon chronic adaptation to SW maximal activity was observed 3 - 4 days post SW transfer when Na<sup>+</sup>-K<sup>+</sup>-ATPase levels of  $6.35 \pm 1.43$   $\mu\text{mol}/\text{h}/\text{mg}$  protein and  $6.11 \pm 0.83$   $\mu\text{mol}/\text{h}/\text{mg}$  protein (  $p < 0.005$  for both ), respectively, were measured.

Figure 3.25

Figure 3.25 Standard curve for the determination of inorganic phosphate

The amount of inorganic phosphate ( Pi ) is plotted on the abscissa against O.D. measured at 630 nm on the ordinate. Each point represents the mean  $\pm$  S.E.M. of nine replicates.

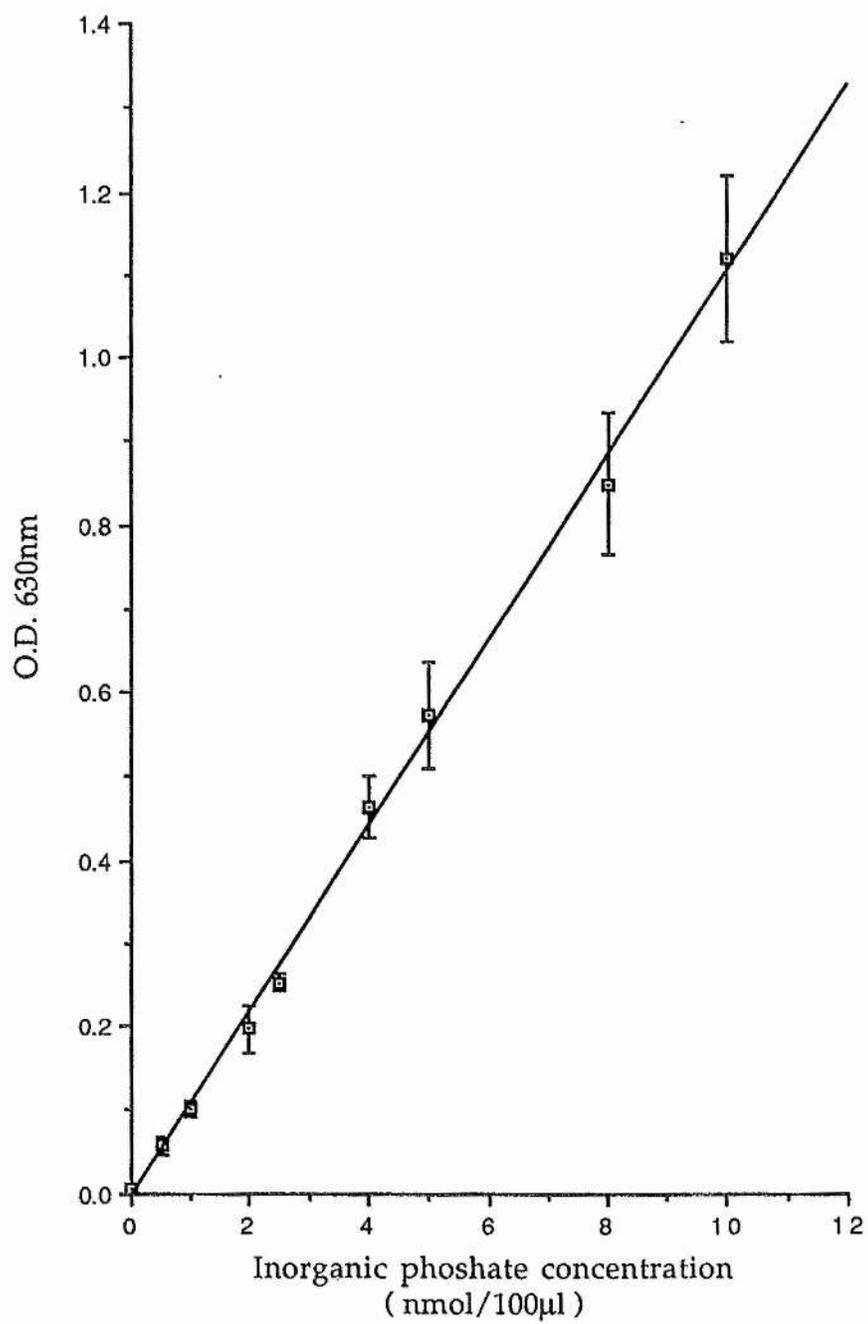


Figure 3.26

Figure 3.26a Effect of temperature on Na<sup>+</sup>-K<sup>+</sup>-ATPase activity in gill membranes from long term- SW adapted eels.

Results are means  $\pm$  S.E.M. for four replicates.  
 $p < 0.01$  for all groups ( ANOVA ).

Figure 3.26b Effect of membrane dilution on Na<sup>+</sup>-K<sup>+</sup>-ATPase activity in gill membranes from long term- SW adapted eels.

Results are means  $\pm$  S.E.M. for three replicates.  
 $p < 0.01$  for all groups ( ANOVA ).

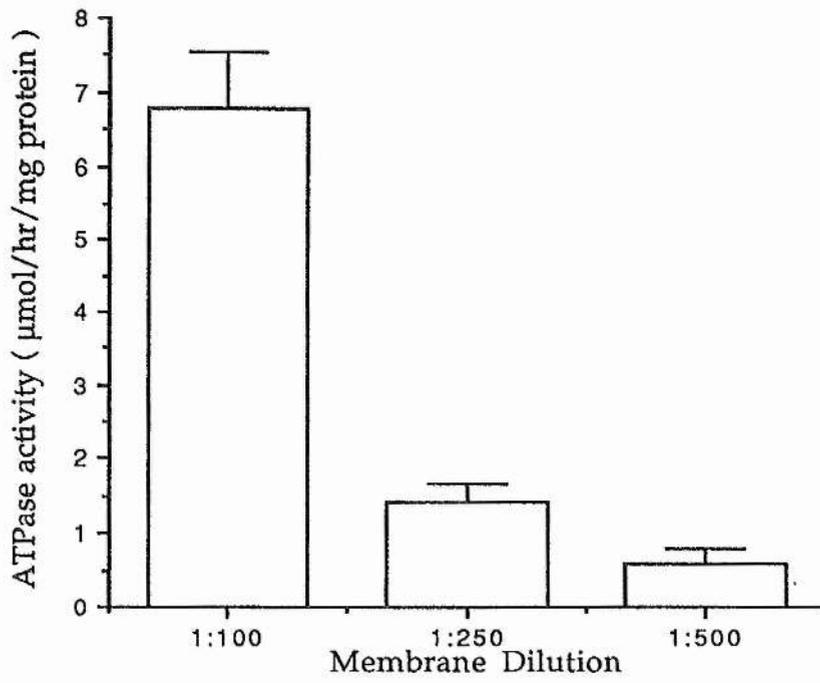
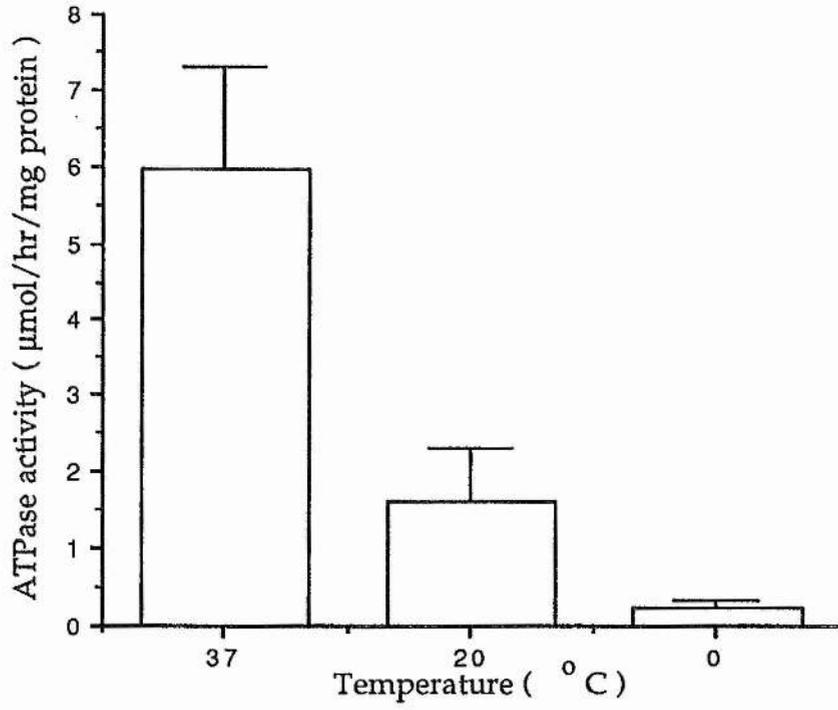


Figure 3.27

Figure 3.27 Effect of chronic- and long term- SW adaptation on the ouabain sensitive Na<sup>+</sup>-K<sup>+</sup>-ATPase activity in gill membranes

Results are means  $\pm$  S.E.M. for ten animals.

\*\*\* indicates statistically significant differences at  $p < 0.005$  from values in FW group ( unpaired t-test ).

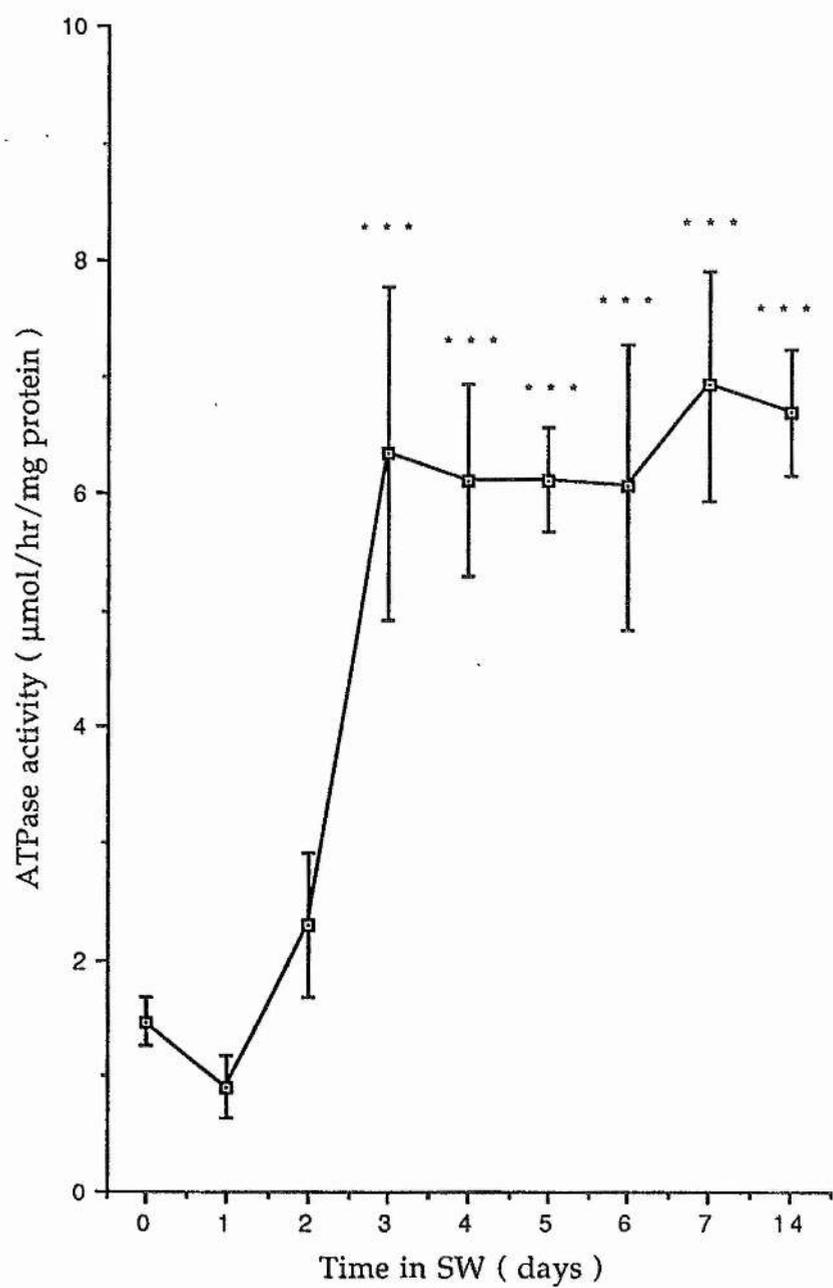
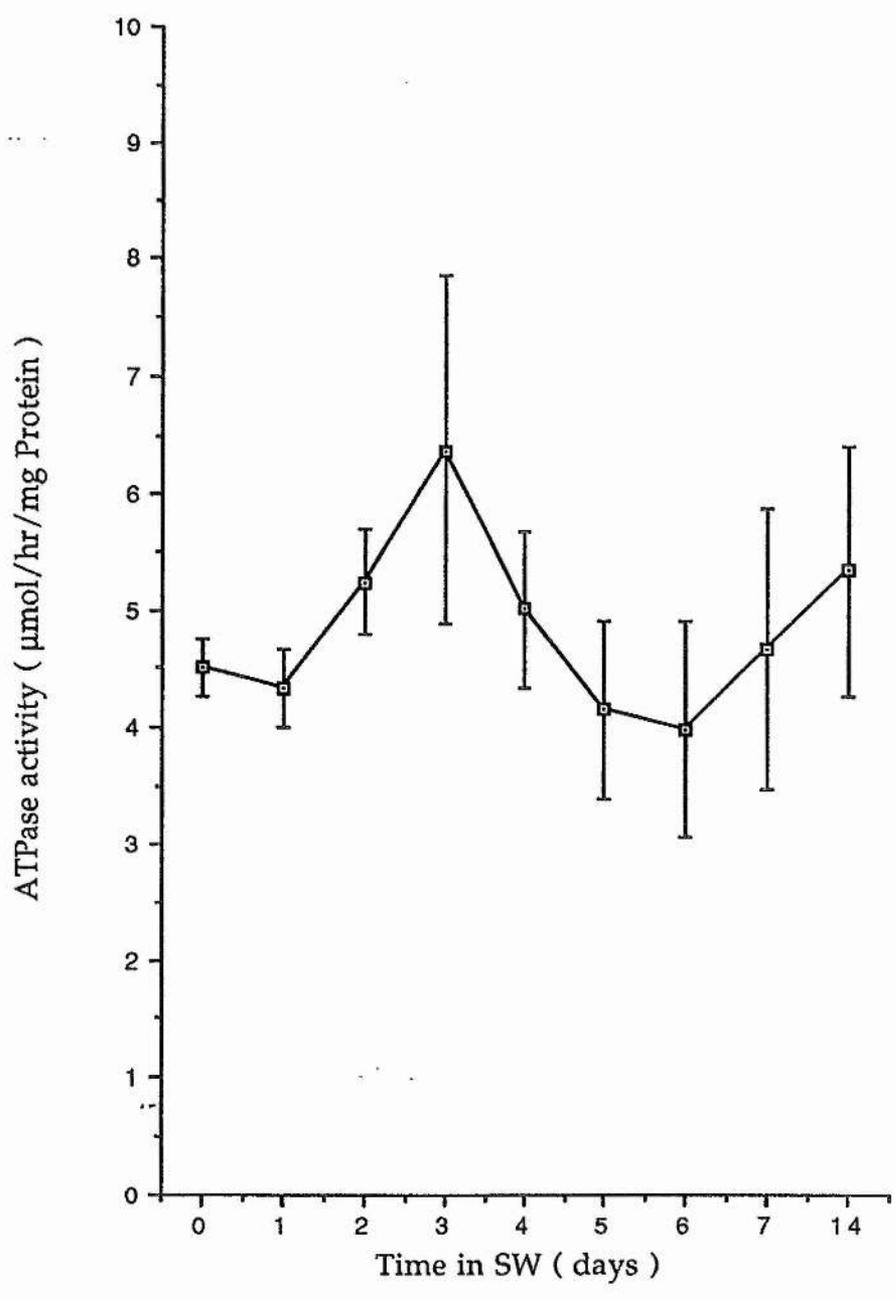


Figure 3.28

Figure 3.28 Effect of chronic- and long term- SW adaptation on  
K<sup>+</sup>stimulated-ATPase activity in gill membranes

Results are means  $\pm$  S.E.M. for ten animals.

Values were compared to FW values and no significant differences were found for any of the groups. ( unpaired t-test ).



Thereafter the activity declines, but nevertheless the level remains significantly higher than that of the FW animals.

K<sup>+</sup>-stimulated ATPase activity was not significantly altered upon either chronic or long term SW adaptation compared to the levels found in FW adapted fish ( 3.28 ).

#### 3.4.8 Receptor studies

Table 3.7 lists changes to the experimental procedure used in the investigation of AII binding to isolated hepatocytes. The percentage specific binding was extremely low in all cases, generally less than 1% of <sup>125</sup>I-AII added. The differential obtained between the TB tubes and the NSB tubes was considered to be too low to warrant any further studies into AII receptor characteristics by means of isolated hepatocytes. Therefore, a qualitative examination of AII binding to various tissues during FW to SW transfer was carried out by means of autoradiography.

Binding was observed as silver grains and showed good penetration of tissues. Grain density was counted over a 350 μm<sup>2</sup> area of five random areas for each tissue section for individual fish. Table 3.8 shows the specific grain densities obtained after deduction of background grain counts from NSB and TB sections. A problem arose with the emulsion used in this study as two types ( LM-1 and G-5 ) had to be used, which resulted in large differences in NSB and TB grain density within experimental groups. Therefore, in order to standardise results the grain densities were expressed as a percentage of total binding ( see Section 2.14 ).

Significant differences were found between TB and NSB grain densities (  $p < 0.05$  to  $p < 0.001$  ), which indicates good displacement of iodinated AII by the excess unlabelled AII. Background counts were subtracted from the TB and NSB sections and SB was determined from the differences between these two values. The % SB was calculated as detailed in section 2.14.2.

**Table 3.7**

Table 3.7 Variations employed in the  $^{125}\text{I}$ -AII binding assay for isolated hepatocytes

|  |
|--|
| (1) Varying concentration of $^{125}\text{I}$ -AII in the range 10 - 50 pM.  |
| (2) Varying number of cells in suspension in the range $0.5 \times 10^6$ cells/500 $\mu\text{l}$ - $1.5 \times 10^6$ cells/500 $\mu\text{l}$ . |
| (3) Varying concentration of $\text{CaCl}_2$ from 1.5 mM - 20 mM   |
| (4) Check for clumping of cells  |
| (5) Check for internalisation of receptors   |

Table 3.8

Table 3.8 Specific grain density of  $^{125}\text{I}$ -AII binding to tissues of eel during FW to SW transfer

Results are mean  $\pm$  S.E.M. of : 5 animals for FW group; 4 animals for 6-Day transfer group; 2 animals for the long term SW-adapted group held in SW for at a minimum period of 14 days..

| Tissue            | FW         | S W        | 6day SW    |
|-------------------|------------|------------|------------|
| Gill filament     | 760 ± 245  | 1129 ± 670 | 854 ± 307  |
| Gill lamellae     | 673 ± 299  | 988 ± 301  | 1203 ± 401 |
| Liver             | 578 ± 155  | 899 ± 343  | 1098 ± 503 |
| Cerebellum        | 648 ± 285  | 868 ± 247  | 940 ± 473  |
| Medulla oblongata | 790 ± 369  | 902 ± 299  | 1034 ± 486 |
| Head kidney       | 1388 ± 598 | 1903 ± 501 | 1688 ± 407 |
| Caudal kidney     | 673 ± 256  | 1048 ± 847 | 1673 ± 365 |

Figure 3.29 shows the % specific binding of AII to various tissues in long-term FW- and SW-adapted eels and 6 day SW transfer fish. Specific binding was seen to occur in all tissues studied and in both long-term FW- and SW-adapted and 6 day SW transfer eels. In the both gill filament and gill lamellae, although overall no significant difference was found between any of the transfer groups, it is interesting to note that there seems to be a trend for increased AII binding in gill tissue with transfer to SW. Binding was observed in both the cerebellum and medulla oblongata regions of the brain, with significant increases after 6 day SW transfer, compared to the FW value ( $p < 0.05$  for both). There appears to be a tendency for a higher percentage of SB in the long-term SW-adapted eels compared to the FW group, although these groups were not statistically significant from each other. A high % SB was observed in the head kidney of all three adaptation groups, with no differences between individual groups. A specific increase in AII binding was observed in the caudal kidney between the FW group and six day SW transfer fish ( $p < 0.01$ ). AII binding in the liver was demonstrated to be significantly different in both the long-term SW-adapted and six day SW transfer groups compared to the FW binding ( $p < 0.01$  and  $p < 0.005$ , respectively).

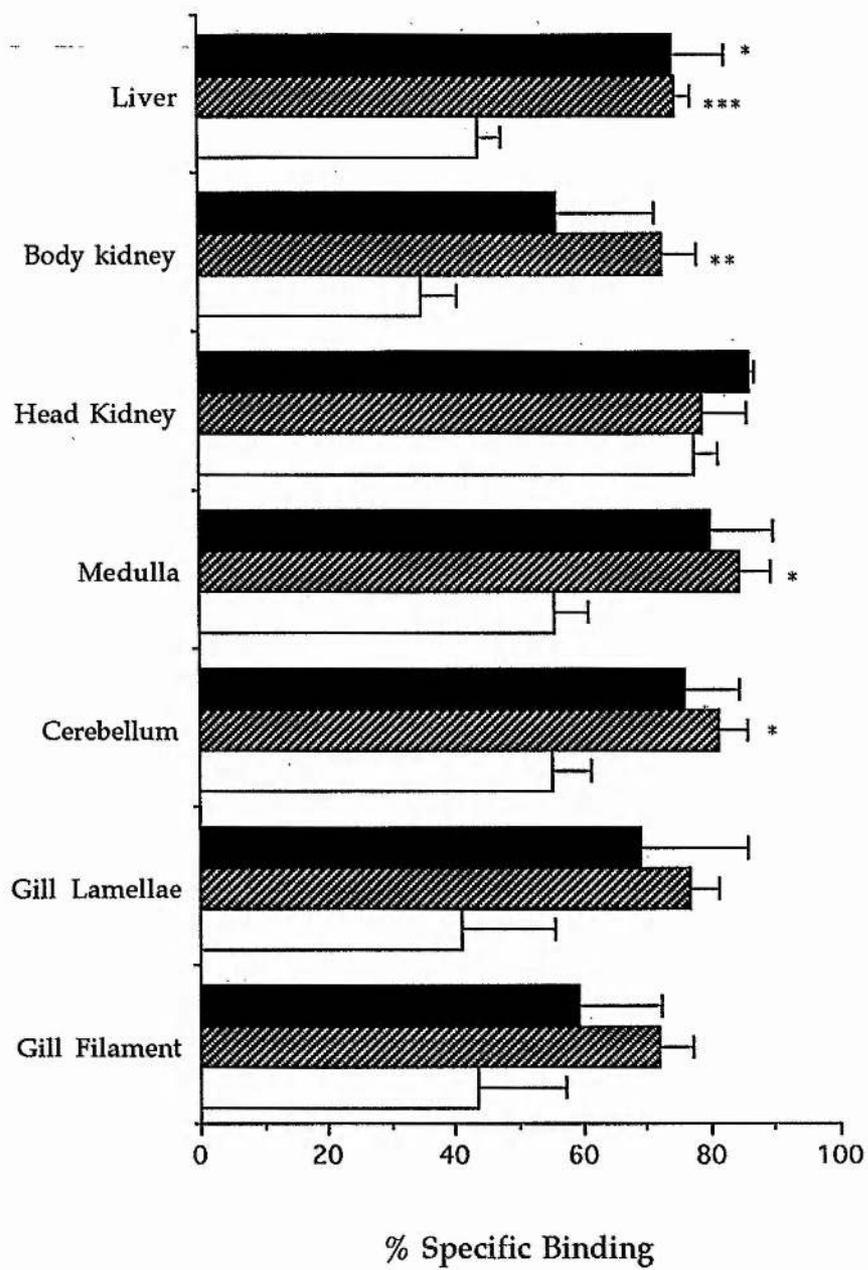
Figures 3.30 a - m show representative photomicrographs of light microscope (LM) autoradiographs. As can be seen in these photomicrographs a particular problem associated with this technique is that objective lenses above 25 x do not have sufficient depth of focus to allow simultaneous sharp imaging of silver grains and tissue sections. For the purpose of manually counting grain density the lenses were adjusted in order to bring the silver grains into focus. The eel brain is shown in Figure 3.30a, with grain densities determined within the boxed areas. Representative background and non-specific binding for the cerebellum region of the brain are shown in Figures 3.30 b and c respectively. Total binding of AII by various tissues in different experimental groups are shown in Figures 3.30 d - m.

Figure 3.29

Figure 3.29 Percentage specific binding of  $^{125}\text{I}$ -AII to tissues of eel during FW to SW transfer

Results are means  $\pm$  S.E.M. of : 5 animals for FW group; 4 animals for 6-day transfer group; 2 animals for the long term SW-adapted group. \*, \*\*, and \*\*\* indicate statistically significant differences at  $p < 0.05$ ,  $p < 0.01$ , and  $p < 0.005$ , respectively, compared to values in FW group ( ANOVA, Bonferroni protected t-test )

The open bars represent long term FW-adapted tissue sections; hatched bars are 6 day SW transfer group; closed bars are the long term- SW adapted animals which have been in SW for at least 14 days.



Specific binding was observed in both the cerebellum (Figure 3.30d, e) and medulla oblongata (Figure 3.30 f,g) regions of the brain, with a significant increase in binding between the FW-adapted groups (Figure 3.30 d and f, respectively) and the six day SW transfer groups (Figure 3.30 e and g, respectively). Figure 3.30h shows AII binding to the FW gill, with binding to the central filament (F) and the gill lamellae (L) observed in all transfer groups. Binding of radioactive AII to the FW head kidney section is shown in Figure 3.30i, with specific binding to adrenocortical tissue not distinguishable at the microscopic magnification available. Figures 3.30 j and k show binding to the caudal kidney in the FW and 6 day SW transfer eel, respectively. Sectioning of the tissues did not allow specific binding of AII to glomeruli and proximal tubules to be identified. Binding of AII to liver sections is shown in Figures 3.30 l and m, for FW and 6 day SW transfer animals, respectively.

#### 3.4.9 Protein determination

Figure 3.31 shows a typical graph obtained by using the Bradford assay for protein determination. This assay was used to measurement the protein content of gill and liver tissues.

Figure 3.30 a

Figure 3.30a Transverse section of eel brain

Grains counted in boxed areas: cerebellum ( C ) and medulla oblongata ( M ).

Bar, 2 mm

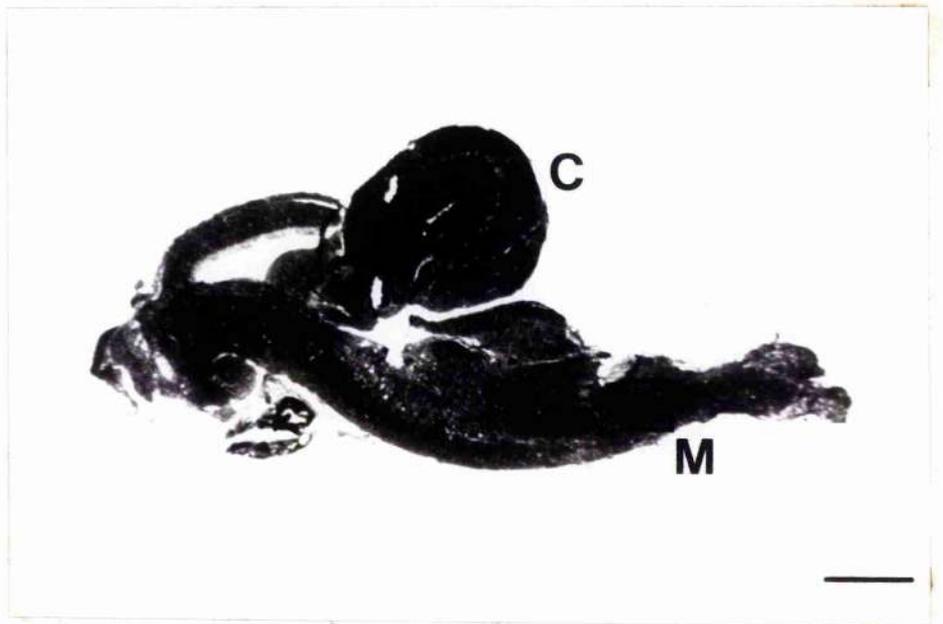


Figure 3.30 b

Figure 3.30c

Figure 3.30b Background binding of AII to cerebellum of FW adapted eel

Bar, 10 $\mu$ m ( scale is the same for all following autoradiographs )

Figure 3.30c Non specific binding of AII to cerebellum of FW adapted eel

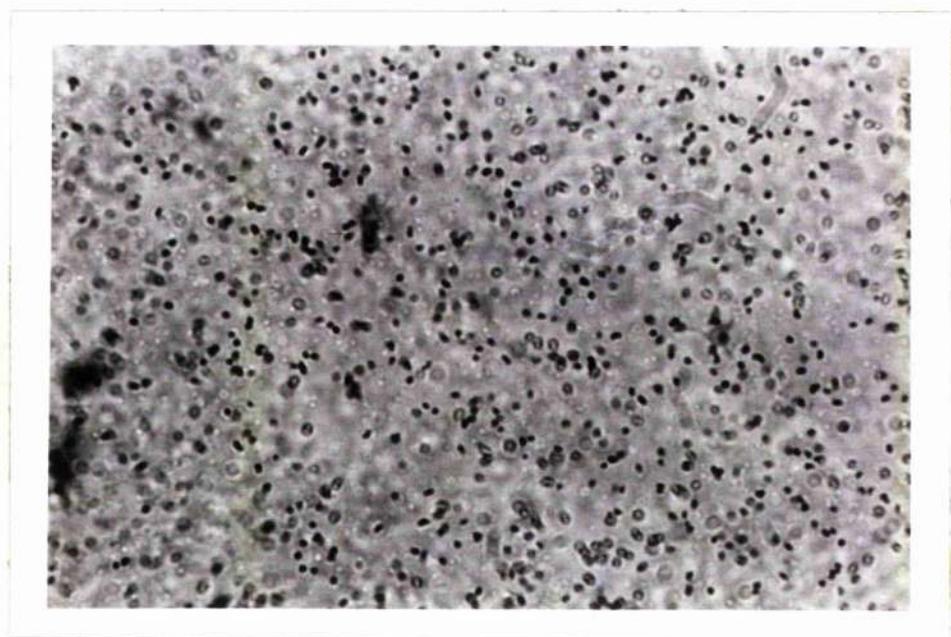
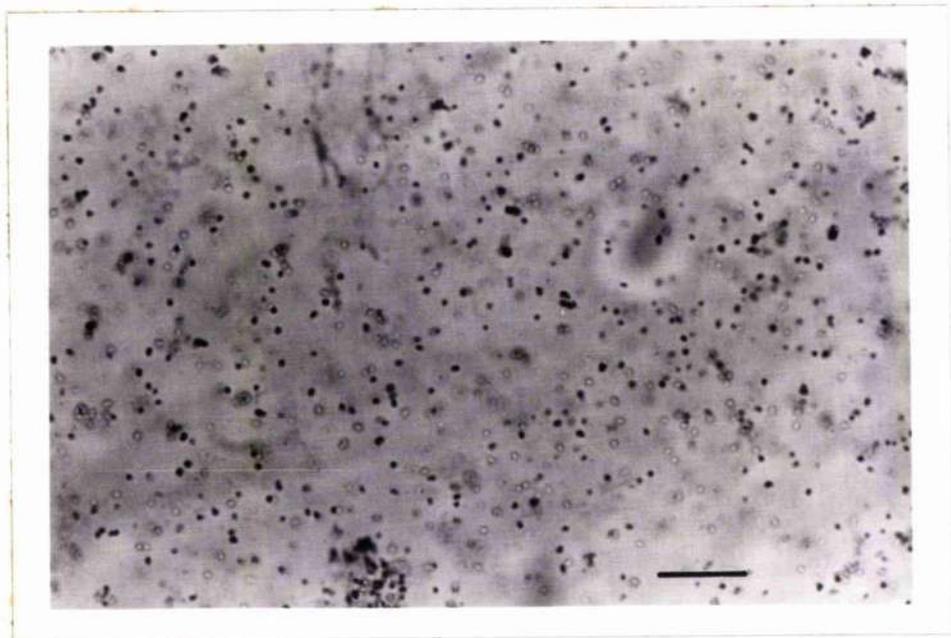


Figure 3.30d

Figure 3.30e

Figure 3.30d Total binding of AII to cerebellum of FW adapted eel

Figure 3.30e Total binding of AII to cerebellum of 6 day SW transfer eel

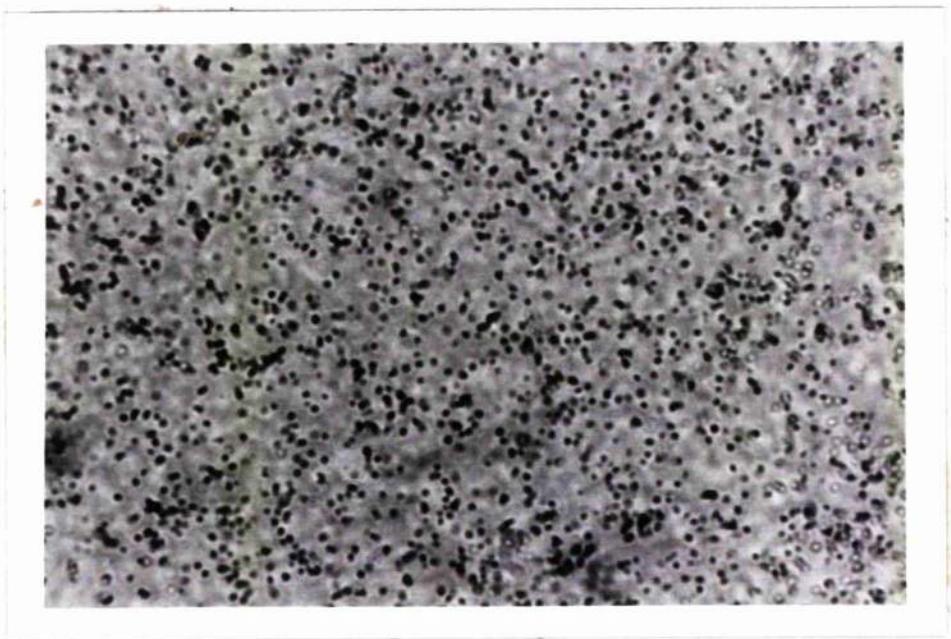
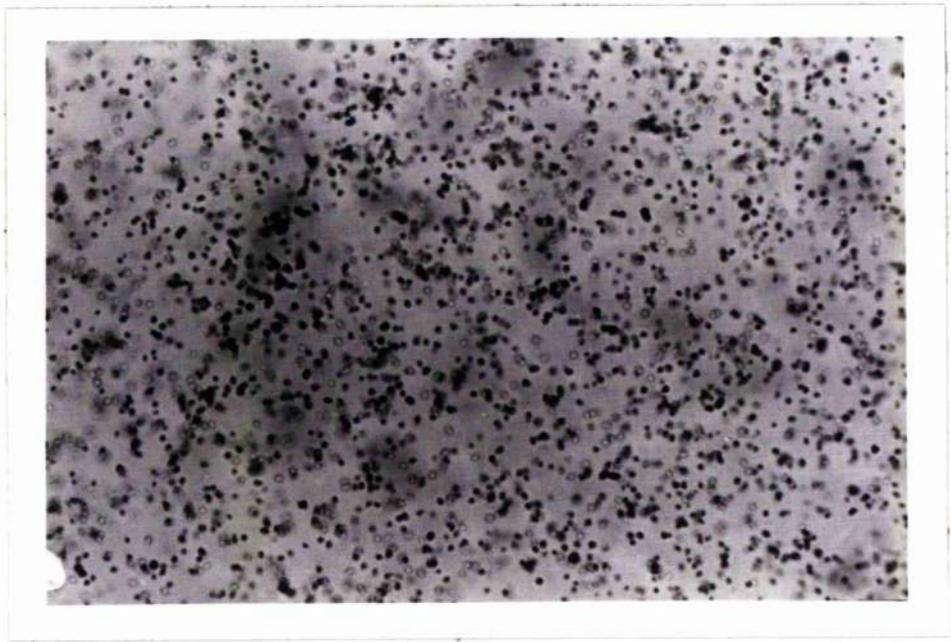


Figure 3.30f

Figure 3.30g

Figure 3.30f Total binding of AII to medulla oblongata of FW adapted eel

Figure 3.30g Total binding of AII to medulla oblongata of 6 day SW transfer eel

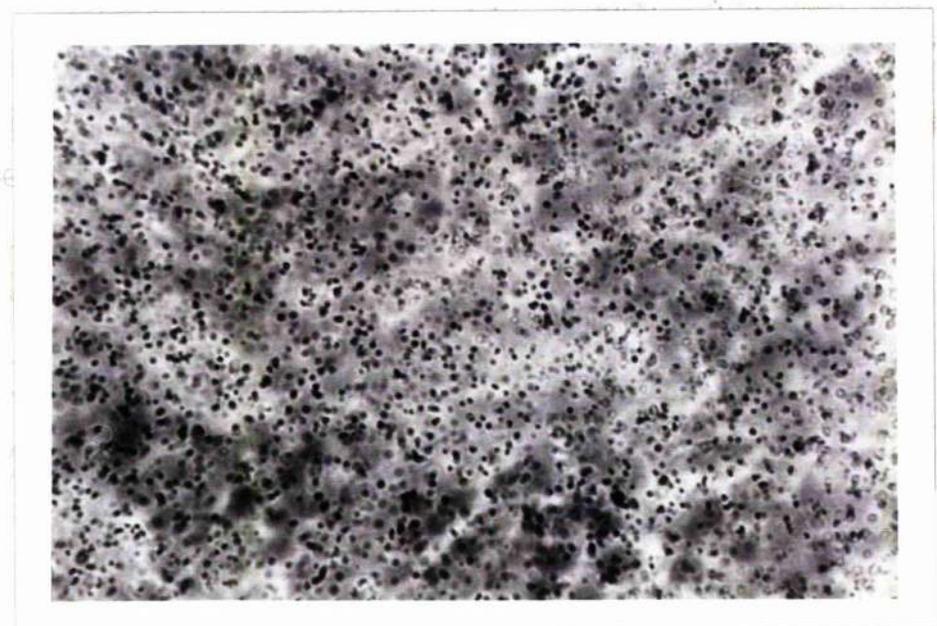
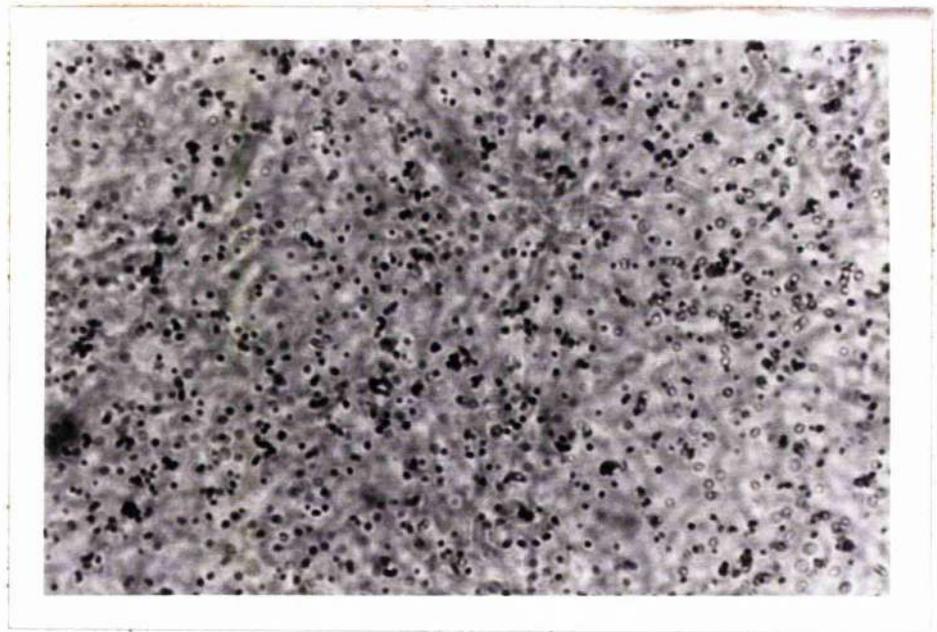


Figure 3.30h

Figure 3.30i

Figure 3.30h Total binding of AII to gill of FW adapted eel  
Binding to the central filament ( F ) and the lamellae ( L ) are  
indicated by arrows.

Figure 3.30i Total binding of AII to head kidney of FW adapted eel

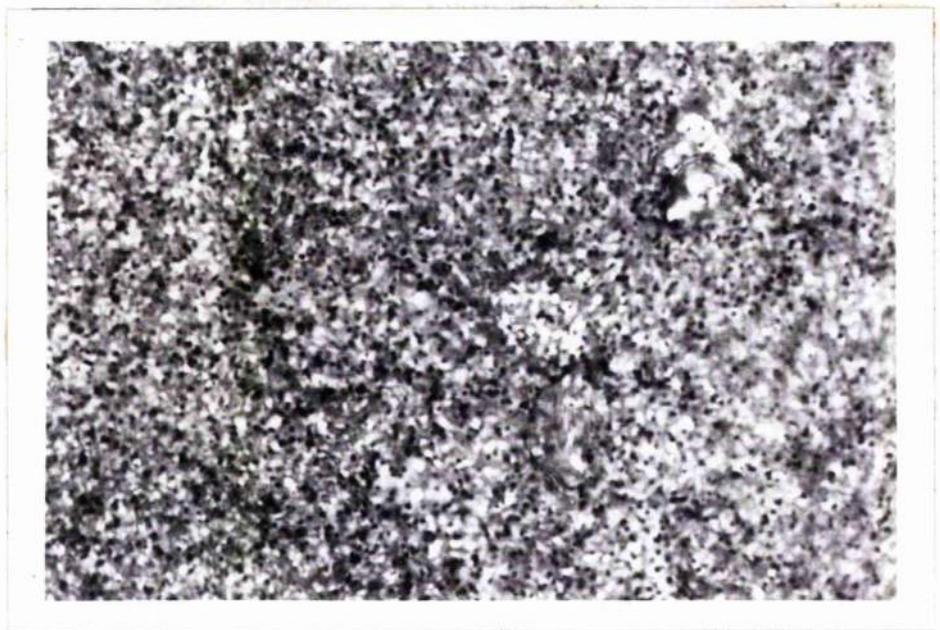
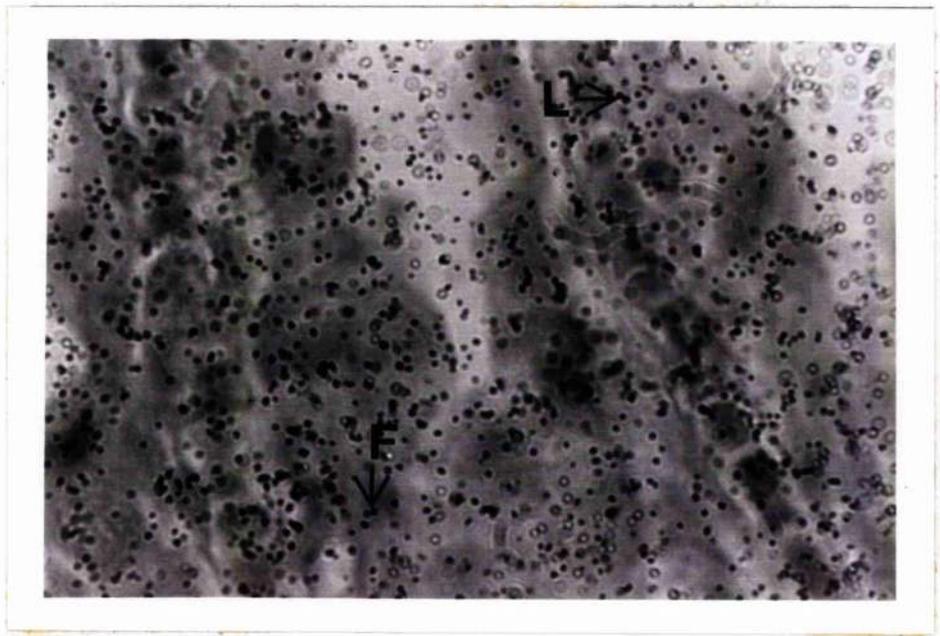


Figure 3.30j

Figure 3.30k

Figure 3.30j Total binding of AII to caudal kidney of FW-adapted eel

Figure 3.30k Total binding of AII to caudal kidney of 6 day SW transfer eel

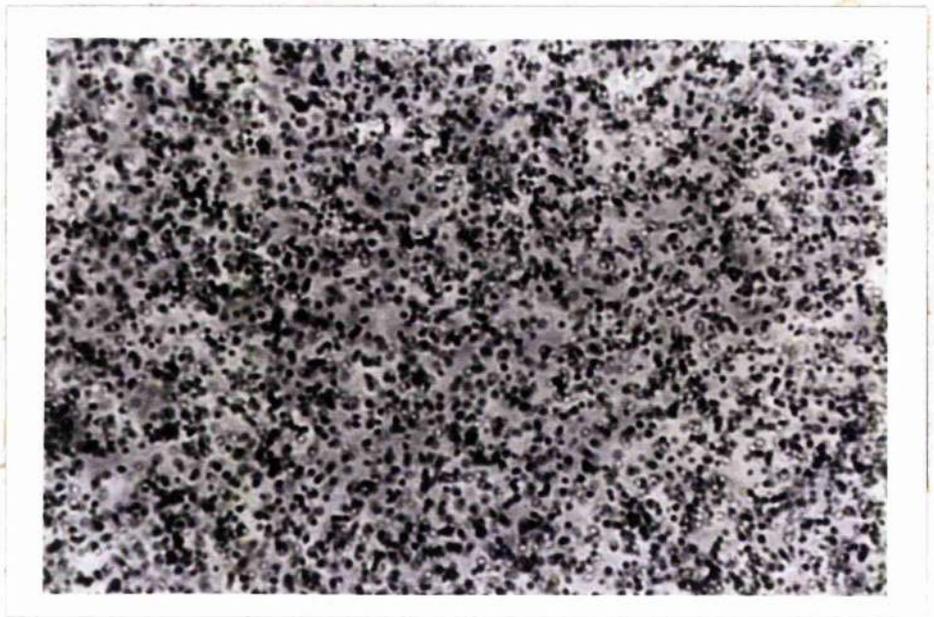
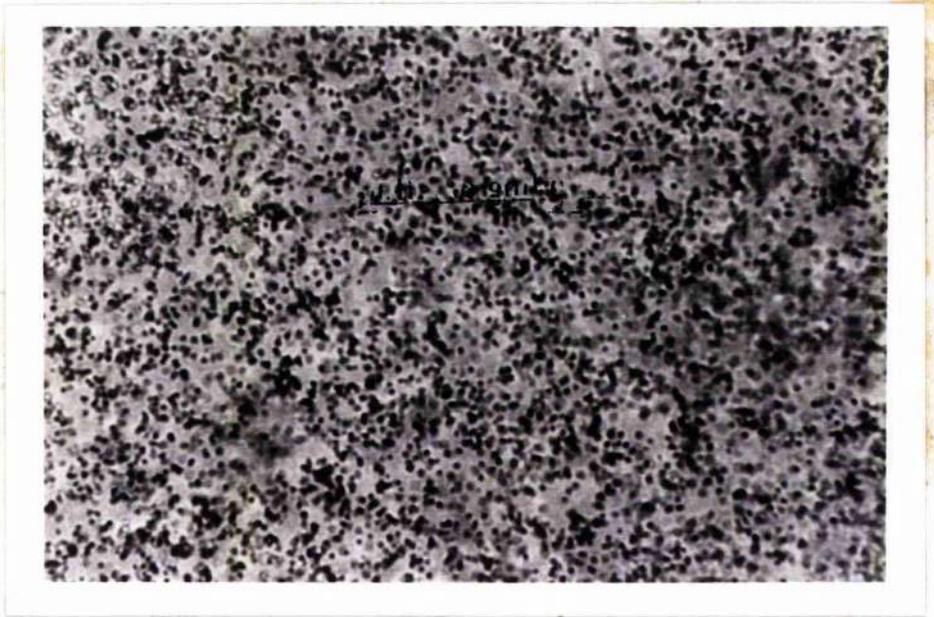


Figure 3.30l

Figure 3.30m

Figure 3.30l Total binding of AII to liver of FW adapted eel

Figure 3.30m Total binding of AII to liver of 6 day SW transfer eel

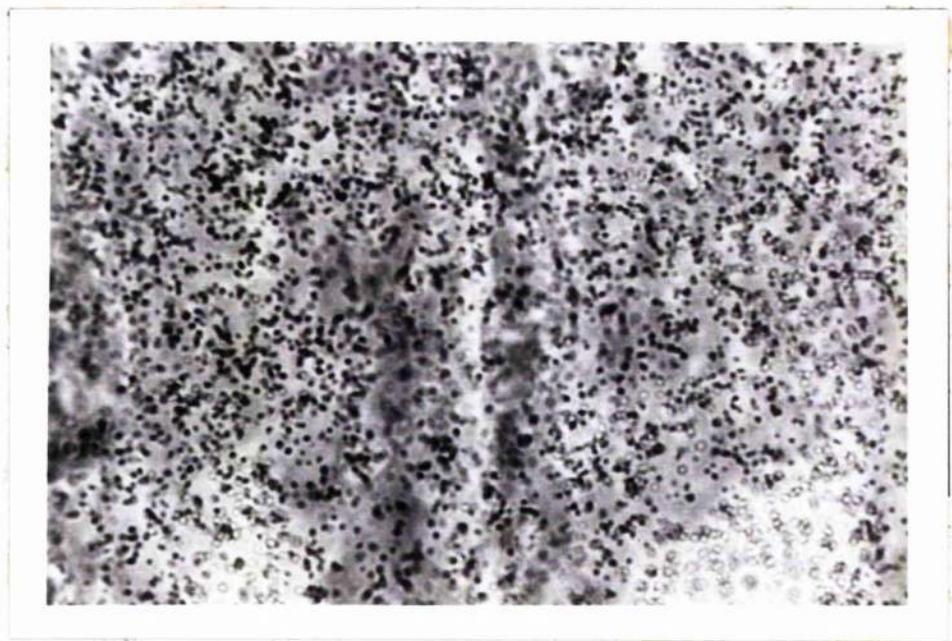
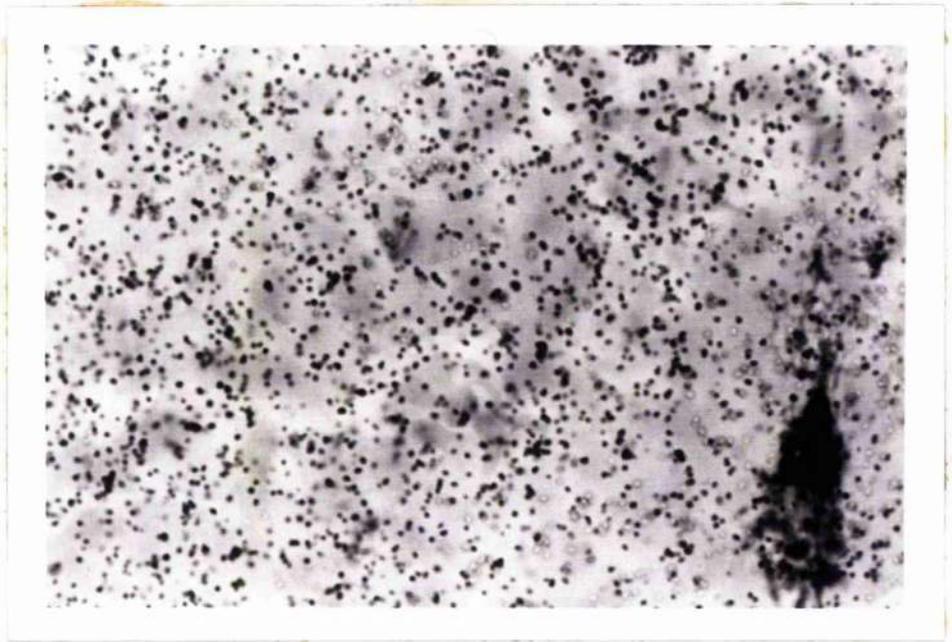
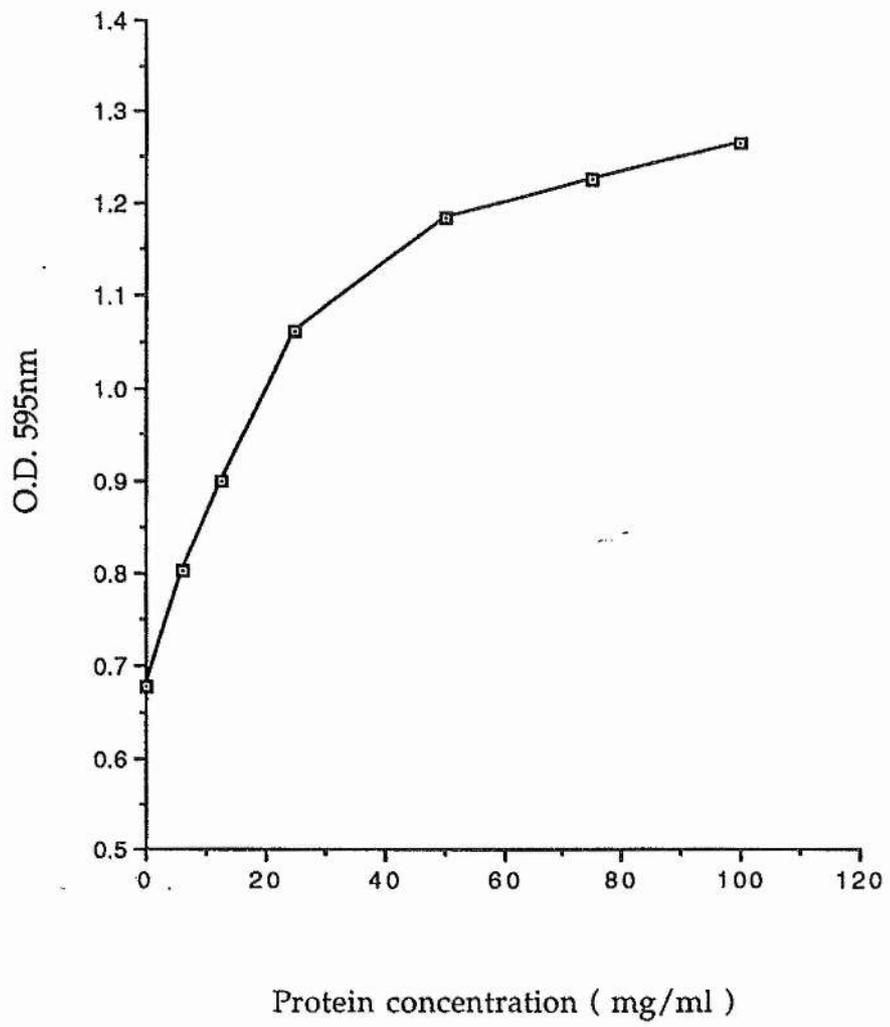


Figure 3.31

Figure 3.31 Typical standard curve for the measurement of protein.

The amount of protein ( mg/ml ) is plotted on the abscissa against O.D. at 595 nm on the ordinate. Each point represents the mean of three determinations.



## 4.0 Discussion

#### 4.0 Discussion

The maintenance of homeostasis during the movement of euryhaline teleosts from a FW environment to a SW environment involves the integration of many factors, ranging from intrinsic properties of epithelia to hormonal action, in response to changes in ionic concentrations. These factors have to be considered together in order to ascertain the overall response of teleosts to changing environmental salinity. The transfer of teleosts from FW to SW is associated with certain physiological changes, including an increase in drinking rate, an increase in the number and size of the chloride cells and a concomitant increase in  $\text{Na}^+\text{-K}^+\text{-ATPase}$  activity, a change in the permeability of the gut, and a reduction in urine flow and GFR. The plasma electrolyte levels are regulated within a certain range irrespective of the environmental salinity. The overall picture obtained with the transfer of the euryhaline eel from FW to SW in this study will now be discussed.

Angiotensin II is known to increase blood pressure in all vertebrates so far studied, including in the teleosts, the FW-adapted trout and FW- and SW-adapted eels ( Gray and Brown, 1985; Nishimura and Sawyer, 1976; Takei *et al.*, 1979 ) and the flounder adapted to 50% SW ( Perrott and Balment, 1990 ). Angiotensin II is a potent vasopressor in teleosts, with renin release occurring after a reduction in aortic or renal perfusion pressure ( Bailey and Randall, 1982; Nishimura *et al.*, 1979 ).

The physiological response to SW transfer appears to be bi-phased with an initial immediate response occurring over minutes and hours ( termed acute adaptation ), and a longer adjustment period over a period of days to one week ( termed chronic adaptation ), eventually leading to the long-term SW regulation of the physiological parameters. The acute and chronic periods are adjustive phases distinguished by marked departures from the established FW physiological parameters, as osmoregulatory mechanisms

adapt to the change in environmental salinity leading to the establishment of the regulative phase of adaptation to SW.

With transfer from FW to SW the passive loss of water to the environment presents one of the major problems for the maintenance of a constant blood pressure, and it has been proposed that the RAS evolved as a mediator of blood pressure control ( Nishimura and Sawyer, 1976 ). After acute SW transfer the blood pressure was initially seen to increase, with some fluctuation occurring over the first 90 min of the transfer period, but subsequently declined to the low levels recorded for the fully adapted SW eel, after a five hour period, in agreement with the study of Chester Jones *et al.* ( 1969 ). The FW resting mean arterial blood pressure in the present study is higher than that recorded by Chester Jones *et al.* ( 1969 ).

Plasma osmolality and chloride concentration increased above the FW levels, and was associated with an immediate increase in drinking and a non-significant increase in plasma AII concentration. The drinking response has been termed a "reflex response" to SW transfer and has previously been reported for the Japanese eel as measured by an invasive technique which utilises a cannulated oesophagus ( Hirano, 1974; Takei *et al.*, 1979 ) and for the European eel ( Kirsch, 1972; Kirsch and Mayer-Gostan, 1973 ). A non-invasive technique for the measurement of the drinking rate was used in this study, and an immediate increase in drinking was demonstrated which was maximal during the first 90 minutes period post SW transfer. However the values obtained in this study were considerably less than those determined via oesophageal cannulation ( Hirano, 1974 ). This difference is a reflection of the fact that water removed via the cannula in the latter study is not returned to the stomach, thus reinforcing the drinking response, and leading to an overestimation of the drinking rate. The isotope dilution method ( Balment and Carrick, 1985; Hazon *et al.*, 1989 ) used in the present method is a sensitive technique, suitable for determining low drinking rates without

disturbing the fish. Hirano ( 1974 ) suggested that the chloride ion in SW was responsible for initiation of the reflex drinking action, as demonstrated by the ability of various  $\text{Cl}^-$  salts, but not non- $\text{Cl}^-$  salts, when added to FW, to initiate drinking in the FW-adapted eel. This may be borne out by the concomitant increase in plasma chloride concentration with the elevation in drinking, in the present study.

The increase in the drinking rate with acute transfer to SW may, be partly associated with a pulse of  $\text{Na}^+$ - $\text{K}^+$ -ATPase activity in the gills seen to occur within three hours of FW to SW transfer ( Dr. G. Luke, pers. comm.). A reserve capacity of  $\text{Na}^+$ - $\text{K}^+$ -ATPase pump activity in rat peritoneal mast cells has been reported ( Knudsen and Johansen, 1990 ), with an increase in intracellular  $\text{Na}^+$  concentration causing recruitment of the latent pool of  $\text{Na}^+$ - $\text{K}^+$ -ATPase in rat kidney cells ( Barlet-Bas *et al.*, 1990 ). The immediate increase in  $\text{Na}^+$ - $\text{K}^+$ -ATPase activity ( Dr. G. Luke, pers. comm. ) precedes the reported increase in chloride cell number ( Shirai and Utida, 1970 ) with transfer to SW and may, therefore, be associated with recruitment of a latent pool of  $\text{Na}^+$ - $\text{K}^+$ -ATPase in gill. Increased drinking in SW requires enhanced  $\text{Na}^+$ - $\text{K}^+$ -ATPase activity in the gut and gills to produce osmotically free water.  $\text{Na}^+$ - $\text{K}^+$ -ATPase is intimately associated with the chloride cells of the gills. The activity of this enzyme is apparently influenced by cortisol ( Epstein *et al.*, 1971; Dange, 1986; Richman and Zaugg, 1987 ), which has also been reported to increase significantly within the first four hours after transfer from FW to SW ( Kenyon *et al.*, 1985 ).

It would be expected, given the dipsogenic action of AII, that the increase in AII during acute SW transfer would be greater to coincide with the immediate reflex drinking, although the involvement of the RAS in the reflex drinking is unclear ( Takei *et al.*, 1979 ). PRA in the European eel has previously been shown to remain at the basal FW level for 24 hours after SW transfer ( Henderson *et al.*, 1976 ). The plasma concentration of AII in the

present study was determined 90 minutes after SW transfer, an interval which corresponded to a period of elevated blood pressure. However, an increase in the renal perfusion pressure did not affect the rate of renin release in the FW-adapted trout ( Bailey and Randall, 1981 ), which may explain the absence of a significant increase in acute plasma AII concentration. The small increase in AII, which provided a plasma AII concentration of approximately 15 fmol/ml, although not statistically significant, may fall within the threshold level of AII required for the stimulation of drinking.

Most studies have examined the physiological role of the RAS by utilising peptides based on mammalian angiotensins which undoubtedly differ in amino acid sequence and, therefore, physiological potency from teleost angiotensins. As the exact amino acid sequence of *Anguilla anguilla* AII has not yet been determined, in this study, the endogenous RAS of the eel was pharmacologically manipulated. Papaverine is classically considered to stimulate the endogenous RAS by causing hypotension which results in an increase in PRA and hence elevates plasma AII concentrations ( Carrick and Balment, 1983 ). Captopril, an ACE inhibitor, binds to the active site of ACE inhibiting the formation of AII from AI, and thus is assumed to block the activation of the RAS.

The RAS has been implicated in the maintenance of blood pressure in higher vertebrates ( Nishimura, 1978 ), although its role in teleosts is more uncertain. In the FW-adapted eel in this study, papaverine caused a lowering of the mean arterial blood pressure with full recovery by the end of the experimental period. The administration of captopril alone had no effect on blood pressure in the FW-adapted eel, suggesting AII does not play a role in maintaining basal blood pressure in the FW-adapted eel, while the administration of both substances in a single experiment caused hypotension with subsequently only partial recovery.

In the SW-adapted eel, papaverine was again effective in causing hypotension, but only partial recovery of blood pressure was achieved. Captopril caused a sustained hypotensive response in the SW-adapted eel, suggesting AII in SW does play a significant role in maintaining basal blood pressure and this was further supported by the observation that the administration of papaverine, 15 minutes after captopril, resulted in hypotension with only partial recovery achieved. Apparently, in SW-adapted eels the partial recovery in blood pressure seen after the papaverine-induced hypotension was not altered by the prior administration of captopril, although captopril was successful in partially blocking the recovery of blood pressure in the FW-adapted eel. This suggests that some other control mechanisms apart from the RAS may be involved in the recovery of blood pressure after papaverine administration. Blood pressure of the flounder (*Platichthys flesus*), adapted either to FW or SW, was found not to be affected by the administration of captopril (Balment and Carrick, 1985). The RAS of the SW-adapted eel was more responsive to pharmacological manipulation than that of the FW-adapted eel. Sustained hypotension and assumed blockage of the system was achieved in the SW-adapted fish by the administration of captopril alone, which was ineffective in the FW eel. This may reflect a greater activity of the RAS in SW-adapted eels than in FW-adapted fish, and this view is supported by the higher PRA in SW-adapted teleosts compared to FW-adapted fish (Sokabe *et al.*, 1973; Henderson *et al.*, 1976), and the lower renal renin content in the SW-adapted Japanese eel.

It is possible that the RAS evolved as a dipsogenic response (Balment and Carrick, 1985). Euryhaline teleosts, including the European eel, and stenohaline FW and marine species have been found to have increased rates of drinking after administration of either Asp<sup>1</sup>-Val<sup>5</sup>-His<sup>9</sup>-AI or Asp<sup>1</sup>-Val<sup>5</sup>-AII (Perrott *et al.*, 1992). Although some uncertainty remains about the exact role of endogenous renin and angiotensin in the control of

drinking the dipsogenic response appears to rely on an intact RAS ( Carrick and Balment, 1983 ). In the present study, in agreement with the results obtained by Perrott *et al.* ( 1992 ), the pharmacological manipulation of the RAS in both the FW- and SW-adapted eel resulted in a similar pattern of action on the rate of drinking, with the exception of the lack of response to captopril in the FW-adapted eel. Papaverine was effective in stimulating drinking by 400% in the FW-adapted eel which had a very low basal rate of drinking. This increase was associated with a concomitant reduction in blood osmolality of 4.7% most probably as a result of the ingestion of FW which is physiologically inappropriate. Captopril alone had no effect on either the rate of fluid ingestion or plasma osmolality, but was capable of blocking 82% of the papaverine-induced increase in FW drinking when administered 15 minutes prior to an injection of papaverine. The rate of drinking was similar to the FW basal level, with no change in plasma osmolality.

In the SW eel, papaverine elevated the high basal drinking rate by 300% , with a concomitant increase in plasma osmolality ( 13.2% ) presumably due to an increase in SW intake. Captopril reduced drinking rates of SW-adapted eels by 83%, a response previously reported in the SW-adapted flounder ( Balment and Carrick, 1985 ), killifish ( *Fundulus heteroclitus* ) ( Malvin *et al.*, 1980 ) and the eel ( Perrott *et al.*, 1992 ). This response provides support for the role of an activated RAS in the increase in drinking observed with the transfer of the euryhaline eel to a hyperosmotic environment as seen in this study and reported by Hirano ( 1974 ). Captopril was also successful in preventing the elevation of drinking previously observed to occur in the SW-adapted eel after the administration of papaverine.

In spite of the successful blockage of basal drinking and the papaverine-induced increase in the SW eel by captopril, plasma osmolality increased when captopril was administered prior to papaverine, although the increase

was less than that observed after the administration of papaverine alone. The cause of this increase in osmolality is not known.

It is generally assumed that papaverine acts to manipulate the endogenous RAS through its hypotensive action, which acts as a stimulus for renin release ( Nishimura *et al.*, 1979 ), and, therefore, in theory should lead to an elevation in plasma AII concentrations and hence restoration of blood pressure. The present study provides the first direct evidence that papaverine does in fact increase plasma AII levels in both FW- and SW-adapted fish. Papaverine caused a greater increase in plasma AII concentration in the SW-adapted fish compared to the FW-adapted eel, where endogenous PRAs has been reported to be low ( Nishimura *et al.*, 1976; Henderson *et al.*, 1986 ). The resultant increase in plasma AII concentration caused by papaverine in both FW- and SW-adapted eels was partially blocked ( 54.5% and 87% inhibition, respectively ) by the prior administration of captopril. In the FW eel captopril alone had no effect on plasma AII concentration, but significantly decreased the plasma AII concentration of the SW-adapted eel. Determination of the time course involved in the increase of AII concentration after injection of papaverine into the SW-adapted eel was undertaken. The increase in AII concentration was seen to occur within the first twenty minutes with the maximum level attained after 60 minutes. Thereafter the concentration rapidly declined but remained significantly elevated above the basal SW plasma AII concentration, even after 4 hours.

Overall these results provide support for a role of an intact, activated RAS in the acclimation of the euryhaline eel from FW to SW. As a consequence of the decreased systemic blood pressure caused by the administration of papaverine and hence activation of the endogenous RAS, plasma AII concentrations were elevated in both FW- and SW-adapted eels, an increase which subsequently led to increased drinking rates. The time course involved in the papaverine-induced elevation of plasma AII

concentration in the SW-adapted eel was reflected in the restoration of the arterial blood pressure and the increased drinking rate during the six hours after the administration of papaverine. Captopril inhibited the papaverine-induced responses to blood pressure and drinking in both groups, although it did not completely block the papaverine-induced formation of AII in either group.

Captopril, however, is a general and not a specific inhibitor of ACE, as is demonstrated by its ability to only partially block the increase in plasma AII concentration caused by papaverine in both long-term FW- and SW-adapted fish ( 54.5% and 87% inhibition, respectively ) in the current study. This perhaps questions the efficacy of captopril as an ACE inhibitor, and the mechanism of action of captopril is unclear ( Dzau, 1989; Campbell, 1987 ). In mammals, captopril binds to the active site of the ACE in a similar way to AII and, therefore, blocks the conversion of AII from AI ( Skeggs *et al.*, 1976 ). Captopril was originally considered to work solely through the prevention of AII formation, however, in man at least, it has also been reported to be partly responsible for the inactivation of the potent vasodilator, bradykinin, a component of the kallikrein-kinin system ( Campbell, 1987; Olson, 1992 ). Inhibition of ACE with a substance such as captopril, would, therefore, result not only in a decrease in plasma AII levels, but also an increase in circulating and renal concentrations of kinins. Bradykinin is a pressor in the Japanese eel ( Chan and Chow, 1976 ), and may account for the partial recovery in blood pressor seen to occur in both the FW- and SW- adapted eel in the present study when captopril was administered prior to papaverine.

Thus it would appear that drinking in SW-adapted eel is at least partly dependent upon an intact RAS. The small increase in AII concentration found after the administration of both captopril and papaverine in the FW-adapted eel was apparently not great enough to cause an elevation of the FW basal drinking rate. However, the decrease in plasma AII concentration, after

the injection of captopril alone, to the SW-adapted eel was associated with a decrease in the drinking rate from the SW basal rate. These results suggest a threshold level for the dipsogenic action of AII, which may vary between FW- and SW-adapted fish.

Partial dehydration of the Japanese eel or *Tilapia mossambicus*, either by FW to SW transfer or exposure to air, caused a decrease in renal renin content and an increase in PRA ( Sokabe *et al.*, 1966, 1973 ). This increase in PRA could be due to a fall in systemic blood pressure caused by dehydration, or perhaps an increase in plasma sodium. In previous studies PRA was reported to progressively increase with transfer to SW, with a maximum level in the European eel, corresponding to the long-term SW-adapted level, after 3 - 5 days ( Henderson *et al.*, 1976 ), and a maximum activity, higher than the SW value, in the Japanese eel four days after SW transfer ( Sokabe *et al.*, 1973 ). The actual values of PRAs reported in these studies are highly variable and are difficult to compare with each other. For instance, the long-term FW value of the Japanese eel was higher than the long-term SW activity demonstrated in the European eel. The chronic transfer of eels from FW to SW resulted in a gradual reduction in mean arterial blood pressure to the long-term SW-adapted level within 3 - 4 days. This was associated with an initial increase in plasma osmolality which was maximal after four days and thereafter regulated at a new level similar to the plasma osmolality of the long-term SW-adapted fish. The decrease in blood pressure would probably cause an increase in PRA as previously reported ( Sokabe *et al.*, 1966, 1973 ), and hence an elevation in plasma AII concentration. In the present study plasma AII concentration was seen to gradually increase to a maximal value six days after SW transfer, with a concomitant increase in the rate of drinking during chronic adaptation to SW. Long-term acclimation to SW was associated with a lower resting mean arterial blood pressure, an elevation in drinking and a higher plasma AII concentration compared to the FW

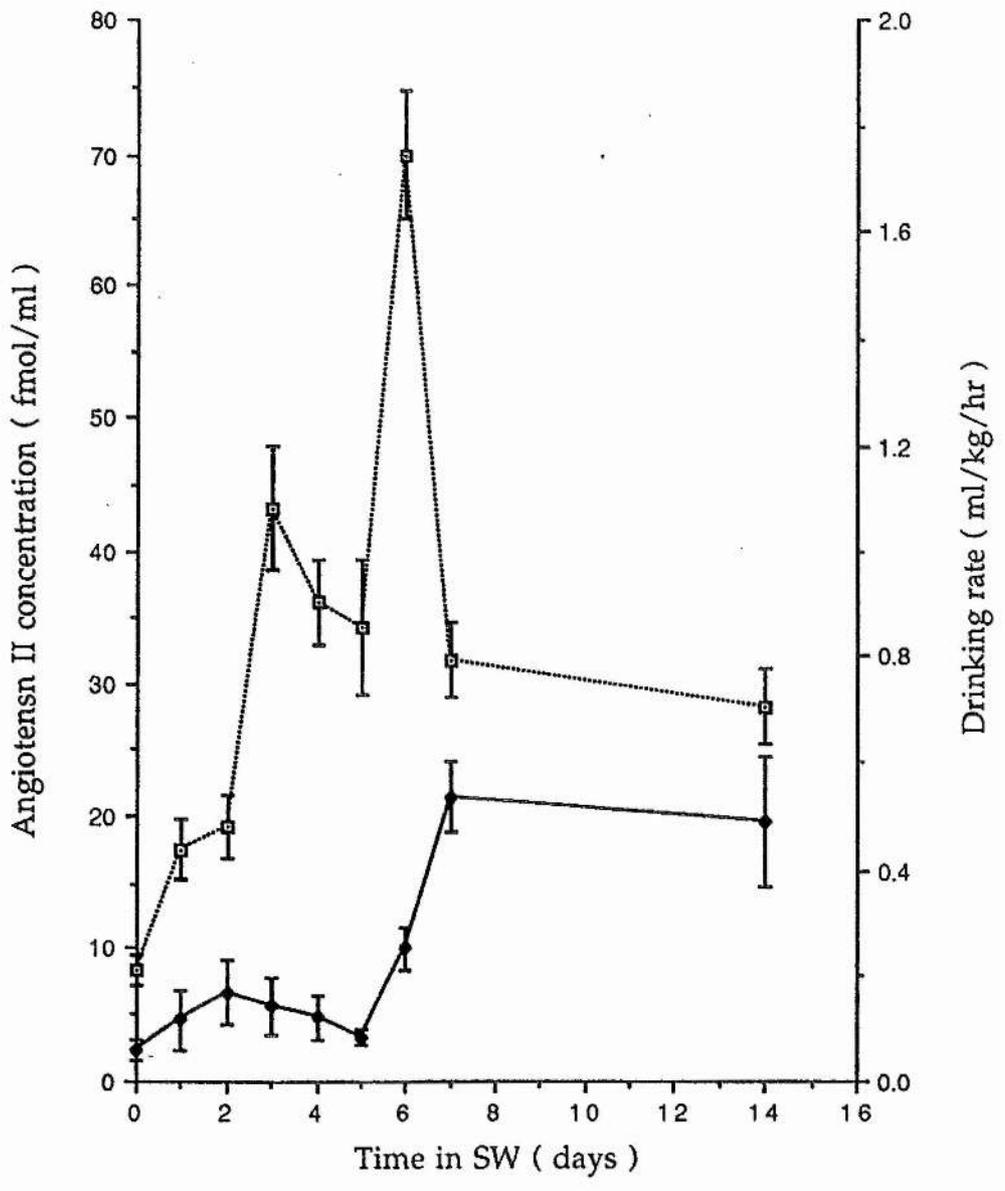
concentration. Drinking is essential in SW-adapted teleosts in order to replace water lost osmotically across the permeable membranes, the gills in particular ( Maetz, 1970; Bentley, 1971 ). Although the drinking rates observed during SW transfer follow the pattern of change in plasma AII concentration ( Figure 4.1 ) the actual elevation of circulating AII during the first five days in SW do not result in the expected higher drinking rates during this initial transfer period. Some other endocrine factor may also be involved in modulating drinking, during this period. The greatly elevated AII concentration seen on day six does, however, correspond to a high rate of drinking. Thereafter the drinking rate is maintained at the high basal SW level and is associated with AII plasma concentration and plasma electrolyte levels regulated at a new higher "SW" values, greater than FW-adapted levels, although lower than the maxima achieved during the initial transfer adjustment period. This study did not confirm the extremely high plasma AII concentration previously reported for SW-adapted eels ( Henderson *et al.*, 1985 ).

AII also has an effect on the renal function of teleosts, which was not directly investigated in the current work. SW adapted teleosts have lower GFR and urine flow compared to FW-adapted fish, but a higher SNGFR, associated with a reduction in the number of filtering nephrons upon SW transfer ( Brown *et al.*, 1978; Brown *et al.*, 1980; Chester Jones *et al.*, 1969; Sharratt *et al.*, 1964a, b ). The RAS appears to act locally in the kidney to regulate the population of filtering nephrons ( Gray and Brown, 1985 ). The reduced urine flow seen in the long-term SW-adapted eel compared to the FW-adapted fish occurs immediately upon transfer to SW ( Chester Jones *et al.*, 1969 ). The time course involved in the reduction of urine flow rate during FW to SW transfer does not appear to correspond to the increase in plasma AII concentration. Therefore, the RAS may be more important in the long term SW regulation of a lower GFR and urine flow rate than during the

Figure 4.1

Figure 4.1 Relationship between drinking rate and plasma angiotensin II concentration during chronic and long term SW adaptation

Drinking rate is denoted by the broken line, plasma AII concentration is represented by the solid line.



acute phase of SW transfer, which may involve other endocrine factors. It may, however, be that the increase in concentration of AII required to bring about these adjustments in renal function is low or related to local renal tissue RAS, as a consequence of the importance of reducing the osmotic loss of water in SW through a reduction in urine volume.

Plasma AVT concentration was not seen to be significantly different between long-term FW- and SW-adapted eels, with only a small transitory rise in concentration observed four days after chronic SW transfer. There was no evidence in this study for the very high plasma concentrations of AVT previously reported in SW eel (Henderson *et al.*, 1985). The physiological role of AVT in teleost osmoregulation is still unclear. Taking the increase in plasma AII concentration that occurs with chronic SW transfer into account and in view of the reported contribution of AII to the stimulation of vasopressin secretion in tetrapods (Ramsay *et al.*, 1978), it may be possible that an interaction between AII and AVT occurs in fish. It appears that the circulating concentration of the hormone in the eel is comparable to that of tetrapods and other teleosts, in the range of  $10^{-12}$  -  $10^{-11}$  M (Bentley, 1971; Perrott *et al.*, 1991). It may be that AVT has a greater effect on the "emergency" responses of the eel after acute transfer to SW. The action of AVT on gill function is not yet clear, but in terms of a renal effect it is known to induce a change in urine flow. Administering lower doses of AVT, which lie within the physiological range, has been reported to cause an antidiuresis, mainly from a change in the number of filtering nephrons (Henderson and Wales, 1974; Babiker and Rankin, 1978; Brown *et al.*, pers. comm., SEB abstract A5, 1993). This reduction in urine flow happens immediately after transfer to SW, and hence any significant effect of AVT may occur during this period. In retrospect, plasma AVT concentrations should have been determined during acute SW transfer. However, at the time these assays were carried out,

in the University of Manchester by Justin Warne, the relevant plasma samples were not available.

The adrenocorticosteroids have been implicated in body fluid homeostasis of teleost fishes. The plasma cortisol concentration in long-term FW- and SW-adapted eels in this study were similar, in agreement with previous studies ( Ball *et al.*, 1971; Henderson *et al.*, 1974 ), although the actual cortisol concentrations were lower in the present study, at approximately 10 ng/ml compared to 40 - 50 ng/ml ( Henderson *et al.*, 1974 ) and 30 mg/ml ( Ball *et al.*, 1971 ), which may reflect differences in RIA sensitivity or in differences in the stress level of the fish. Pickering and Pottinger ( 1989 ) reported that basal plasma cortisol concentrations in unstressed salmonid fish were normally in the range 0 - 5 ng/ml, but that in the literature values in excess of 10 ng/ml were found to occur in "unstressed" fish. In the present study the experimental design was set up so as to cause least stress to the fish as possible. Instead of physically transferring the fish from a FW to a SW tank, the flow of water into an individual tank was changed from fresh- to SW as required, and hence the potential elevation in plasma cortisol levels as a result of handling stress was greatly reduced. The similarity in plasma cortisol between FW- and SW-adapted teleosts in the present study is perhaps surprising when considering the reported increased role for cortisol upon acclimation to SW. In this study there was an increase in plasma cortisol concentration one day after SW transfer with a general decline thereafter to the long-term SW-adapted level.

The circulating cortisol plasma concentrations may not reflect the activity of the interrenal gland and a more accurate indicator may be obtained from the secretory dynamics of the gland. Henderson *et al.* ( 1974 ) found higher MCR and BPR in long term SW-adapted eel compared to long term FW-adapted fish. An elevated MCR associated with a SW transfer period of 24 hours was also reported for the Atlantic salmon ( *Salmo salar* ) ( Nichols

and Weisbart, 1985 ). Secretory dynamics may be assessed by the constant isotopic technique, which not only determines the secretion rate of the steroid, which has been used as an index of the " physiological usage " of the hormone, but also its metabolic clearance rate. The cortisol secretory rates were calculated over the period during which the plasma radioactivity concentration was constant, and a steady state existed between the rate of infusion and the metabolic removal of the tritiated cortisol. The endogenous plasma cortisol concentration was stable during this period and the interrenal was not stimulated during the time course of infusion of the radioactive tracer.

When the secretory dynamics of cortisol were assessed a difference was observed in both the MCR and BPR between long-term FW- and SW-adapted eels, despite similar plasma concentrations of the steroid. This reflects a difference in the activity of the interrenal gland which is greater in the long-term SW-adapted eel. The MCRs for both long term FW- and SW-adapted eel were comparable to those observed by Henderson *et al.* ( 1974 ). The BPRs were lower, a reflection of the correspondingly lower plasma cortisol concentrations. With chronic SW transfer the cortisol secretory dynamics were elevated over the initial transfer period. Both the MCR and BPR of eels transferred to SW for 2, 4, or 6 days were higher than the FW-adapted eel and complied with the higher plasma cortisol concentration. These results suggest that increased interrenal secretory activity observed over the first few days in SW is reflected in increased plasma cortisol levels. However, interrenal secretory activity remained significantly elevated at 6 day and 14+ days post SW transfer, when plasma cortisol concentrations had apparently returned to values similar for the FW-adapted eel. Thus, the role of the interrenal gland in osmoregulation may extend beyond both the "acute" and "chronic" SW transfer period and play a role in long-term SW regulation.

In order to determine the secretory dynamics of cortisol it is important to assess the percentage metabolism of the infused tritiated cortisol. HPLC analysis of blood samples was found to be an appropriate alternative method to the paper chromatography technique previously used by Henderson *et al.* ( 1974 ) to determine metabolism of the labelled steroid. Similar results on the percentage of the tritiated cortisol that undergo metabolism were obtained in the two studies with about 30 % metabolism reported by Henderson *et al.* ( 1974 ), and between 34% - 43% metabolism observed in the current study. No difference in the percentage metabolism between any of the experimental groups was recorded. The metabolites were not identified.

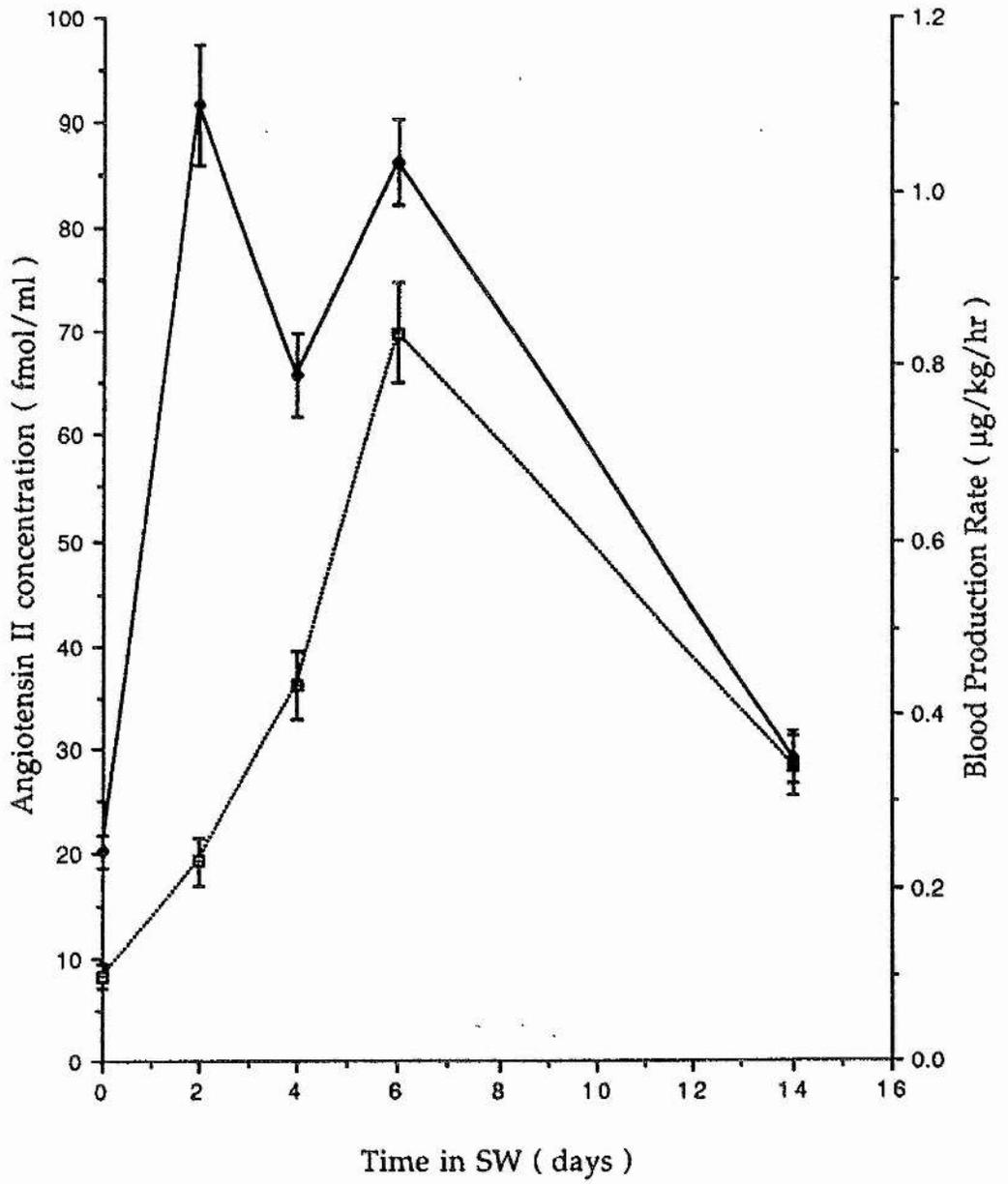
An ability of the RAS to stimulate corticosteroid production has been postulated from the well known stimulatory response of angiotensin on aldosterone secretion in mammals ( Nishimura, 1987 ). However, the role of the RAS in the stimulation of teleostean steroidogenesis is not clear. The similar FW and SW plasma cortisol concentrations as reported in this and other studies do not relate to the elevated PRA ( Henderson *et al.*, 1976 ) and the higher plasma AII concentrations observed for the long term FW- and SW- adapted eel. However, Figure 4.2 shows the relationship between plasma AII concentration and the BPR of cortisol. It appears that the RAS does not have an influence on interrenal activity during the initial adjustment period that occurs with transfer from FW to SW. The RAS may have a greater effect on long-term SW steroidogenesis, although Kenyon *et al.* ( 1985 ) reported an augmentation rather than an inhibition of plasma cortisol concentration in the European eel during SW transfer after the administration of captopril. Perrott and Balment ( 1990 ), however, reported a RAS influence on the control of circulating cortisol levels in flounder ( *Platichthys flesus* ) adapted to 50% SW, with inhibition of cortisol levels by captopril and an increase in concentration after an injection of papaverine.

Cortisol is reported to influence  $\text{Na}^+\text{-K}^+\text{-ATPase}$  activity ( Epstein *et al.*,

Figure 4.2

Figure 4.2 Relationship between cortisol blood production rate and plasma angiotensin II concentration during chronic and long term SW adaptation

Plasma AII concentration is denoted by the broken line, cortisol BPR is represented by the solid line.



1971; Danger, 1986; Richman and Zaug, 1987 ).  $\text{Na}^+\text{-K}^+\text{-ATPase}$  is located basolaterally in the chloride cells, intimately connected with the extensive tubular system. The number and size of chloride cells is known to increase with transfer of teleosts from FW to SW.  $\text{Na}^+\text{-K}^+\text{-ATPase}$  is assayed routinely in many laboratories. Although  $\text{Na}^+\text{-K}^+\text{-ATPase}$  is frequently measured in isolated plasma membranes preparations, as opposed to studying the purified enzyme, the assay may present problems due to the vesicular nature of the membrane.  $\text{Na}^+\text{-K}^+\text{-ATPase}$  requires  $\text{Na}^+$ ,  $\text{Mg}^{2+}$ , and ATP at the cytoplasmic face, and requires  $\text{K}^+$  and is inhibited by ouabain at the extracellular face. Because preparations of isolated cell membranes are usually composed of a combination of more-or-less impermeable vesicles, the measured activity is generally submaximal due to the inaccessibility of the interior of the vesicle ( Forbush III, 1983 ). Differences in activity from one preparation to another may thus reflect differences in the degree of vesiculation rather than in the amount of, or condition of the enzyme. This problem may be overcome by the addition of detergents to the homogenisation medium and in the present study 0.1% sodium deoxycholate was used.

Differences in activity from one preparation to another may also arise as a result of differences in the membrane preparation and the subsequent degree of purification of the preparation, and the temperature at which the enzyme assay is carried out, as shown in Table 4.1. Since the  $\text{Na}^+\text{-K}^+\text{-ATPase}$  activity is calculated on the basis of the protein concentration in the preparation, successive centrifugational procedures purify the membrane homogenate by the removal of inactive protein. In this study only partial purification was undertaken, via the filtering of the homogenate through gauze, followed by centrifugation at 1,000 g, with the  $\text{Na}^+\text{-K}^+\text{-ATPase}$  assay carried out on the resultant pellet. The remainder of the assay followed the procedure of Mayer-Gostan and Lemaire ( 1991 ) and was in the form of a micro-assay. The level of activity reported by Mayer-Gostan and Lemaire

Table 4.1

Table 4.1 Na<sup>+</sup>-K<sup>+</sup>-ATPase activity in euryhaline teleosts

| Species            | Pellet                                   | Assay Temp<br>(°C) | Na <sup>+</sup> -K <sup>+</sup> - ATPase Activity<br>( $\mu$ mol/hr/mg Protein) |         | Reference                     |
|--------------------|--|--------------------|---|---------|-------------------------------|
|                    |  |                    | FW  | SW      |                               |
| <i>A. japonica</i> | microsomal                               | 25                 | 10  | 45      | Kamiya and Utida (1968)       |
| <i>A. rostrata</i> | homogenate                               | 37                 | 6   | 11.4    | Jampol and Epstein (1970)     |
| <i>A. anguilla</i> | homogenate                               | 27                 | 1.131   | 1.26    | Bornancin and deRenzis (1972) |
| <i>A. anguilla</i> | microsomal                               | 35                 | 35  | 45 - 60 | Butler and Carmichael (1972)  |
| <i>A. japonica</i> | isolated chloride cell preparation       | 25                 | not detected  | 6.9     | Kamiya (1972)                 |
| <i>A. anguilla</i> | homogenate                               | 30                 | 3.1   | 7.9     | Sargent and Thompson (1974)   |
| <i>A. anguilla</i> | microsomal                               | 30                 | 16  | 51      | Sargent and Thompson (1974)   |
| <i>A. anguilla</i> | microsomal                               | 10                 | 2*  | 6.25*   | Sargent <i>et al.</i> (1975)  |
| <i>A. anguilla</i> | microsomal                               | 20                 | 4*  | 10*     | Sargent <i>et al.</i> (1975)  |
| <i>A. anguilla</i> | microsomal                               | 35                 | 11*   | 40*     | Sargent <i>et al.</i> (1975)  |
| <i>A. anguilla</i> | isolated chloride cell preparation       | 20                 | 4.92  | 38.9    | Sargent <i>et al.</i> (1975)  |
| <i>A. anguilla</i> | microsomal                               | 30                 | -----   | 0.8     | Bell <i>et al.</i> (1977)     |
| <i>A. anguilla</i> | microsomal (after pretreatment with SDS) | 30                 | -----   | 341     | Bell and Sargent (1979)       |
| <i>A. anguilla</i> | homogenate                               | 30                 | -----   | 10.6    | Bell and Sargent (1979)       |
| <i>A. anguilla</i> | microsomal                               | 30                 | -----   | 54      | Bell and Sargent (1979)       |
| <i>A. rostrata</i> | homogenate                               | 37                 | 3.4*  | 14.2    | Epstein <i>et al.</i> (1980)  |

|                              |            |    |       |       |                                 |
|------------------------------|------------|----|-------|-------|---------------------------------|
| <i>A. japonica</i>           | homogenate | 25 | 2.52  | 6.15  | Ho and Chan (1980)              |
| <i>A. japonica</i>           | microsomal | 25 | 14.37 | 32.33 | Ho and Chan (1980)              |
| <i>Platichthys flesus</i>    | homogenate | 37 | 20    | 14    | Staggs and Shuttleworth (1982)  |
| <i>Salmo gairdneri</i>       | microsomal | 37 | 1*    | 6*    | Madsen and Naarmanse (1989)     |
| <i>A. anguilla</i>           | microsomal | 37 | 11.95 | 38.93 | Mayer-Gostan and Lemaire (1991) |
| <i>Oreochromis niloticus</i> | microsomal | 37 | 10.7  | ----- | Mayer-Gostan and Lemaire (1991) |

\* denotes activity estimated from reference graph.

----- denotes activity not measured for particular adaptation

( 1991 ) was higher than those determined in the present study with values of 11.95  $\mu\text{mol/h/mg}$  protein and 38.7  $\mu\text{mol/h/mg}$  protein, for long-term FW- and SW-adapted eels, respectively. These activities are somewhat higher than those found in the present study, with FW and SW activities of 1.48  $\mu\text{mol/hr/mg}$  protein and 6.69  $\mu\text{mol/h/mg}$  protein, respectively, but the discrepancies are probably mainly a result of the degree of homogenate purification. Mayer-Gostan and Lemaire ( 1991 ) utilised a highly purified microsomal pellet. The present study does, however, corroborate the reported increase in  $\text{Na}^+\text{-K}^+\text{-ATPase}$  activity by a factor of 4 - 5, after adaptation to SW.

Many  $\text{Na}^+\text{-K}^+\text{-ATPase}$  assays on membrane preparations are carried out at what is for fish a relatively high temperature, between 30°C and 37°C. This is because at low assay temperatures Mg-ATPase is apparently the predominant ATPase activity in these preparations and masks any stimulatory effect due to the addition of  $\text{Na}^+$  ( Pfeiler, 1978 ).

$\text{Na}^+\text{-K}^+\text{-ATPase}$  is important in maintaining the intracellular concentration of  $\text{Na}^+$  low and that of  $\text{K}^+$  high, and in previous studies of the SW eel, where the movement of  $\text{Na}^+$  was measured concurrently with gill  $\text{Na}^+\text{-K}^+\text{-ATPase}$ , the rate of  $\text{Na}^+$  efflux was closely correlated with the level of enzyme activity ( Degan and Zadunaisky, 1980 ). During chronic SW adaptation, gill  $\text{Na}^+\text{-K}^+\text{-ATPase}$  activity increased to a new level, comparable to the long-term SW activity, after an initial lag phase of 2 - 3 days, with electrolyte regulation occurring on approximately the same time scale. After the immediate reflex response drinking apparently did not occur above the basal FW level to any great extent during this adjustory period, until at least day six, when the fish has the regulatory ability to excrete excess ions through the gills. So it may be that the eel does not drink SW until the ion excretory mechanisms have adjusted to the long-term SW regulatory levels.

The possible role of ANP in teleost osmoregulation, though far from being clear and not examined in the present study, cannot be overlooked. Conflicting results have been obtained on the circulating concentrations and actions of the peptide, through the use of heterologous and homologous antibodies. Homologous antibodies to teleostean ANP have only recently become available, and, using a specific antibody for eel ANP Takei and Balment ( 1993 ) showed a decrease in plasma concentration with transfer from FW to SW, in contrast to reported increases in studies utilising a heterologous assay ( Westenfelder *et al.*, 1988; Evans *et al.*, 1989; Smith *et al.*, 1992 ). Since it is now clear that ANP belongs to a family of natriuretic peptides, there is uncertainty whether the heterologous assays actually measured the teleost ANP or another members of the peptide family. An apparent stimulatory interaction between ANP and cortisol, has been reported to occur in flounder using heterologous ANP ( Arnold-Reed *et al.*, 1991a; Arnold-Reed and Balment, 1991a ), and in the eel using homologous ANP ( Takei, 1992 ).

Suitable sources of receptor for structure and function studies range from metabolically active whole cells, through several stages of decreasing structural integrity, to solubilized and purified receptors. An investigation into the effects of FW to SW transfer on AII receptor populations was undertaken in the current study. A previous study ( Grierson, 1990 ) indicated the presence of AII receptors in eel liver membrane preparations. Therefore, it was considered feasible to carry out receptor binding studies on isolated hepatocytes with the view to elucidating AII receptor characteristics. Isolated hepatocytes are suitable for carrying out metabolic studies ( Renaud and Moon, 1980a, b; Campbell *et al.*, 1983; Moon and Mommsen, 1990 ). However, difficulties were observed in the present study while investigating receptor binding using the isolated cell approach. The specific binding observed under a variety of different experimental procedures was extremely

low, and generally less than 1% of  $^{125}\text{I}$ -AII added. The form of labelled AII used in the study was not the native eel form, but Grierson ( 1990 ) reported that eel FW and SW liver membrane preparations were apparently as responsive to the Ile<sup>5</sup> sequence of  $^{125}\text{I}$ -AII as to the Val<sup>5</sup>, with the two sequences of AII possessing the same ability to displace each radioactive peptide.

There is some concern about the effects of exposure to digestive enzymes, such as collagenase, on membrane receptor integrity ( Berry *et al.*, 1983 ). For future studies on receptor binding in isolated fish hepatocytes it may be necessary to prepare cells without the use of collagenase, as examined by Berry *et al.*, ( 1983 ) in rat hepatocytes. In this study EDTA was used instead of collagenase to separate the liver cells. Initially, in the resultant preparation, there were a number of trypan blue stained cells. However, after further centrifugation utilising Percoll gradient, a preparation of predominantly living cells was obtained. This method may, therefore, overcome the problems associated with enzymatic degradation of the liver and cell surface receptors in hepatocytes as observed in this study.

In view of the unsuccessful attempts to characterise AII receptors through the use of isolated hepatocytes, the next logical step was to carry out an autoradiographical study on various osmoregulatory tissues of *Anguilla anguilla*. AII binding in long term FW- and SW-adapted eels and six day SW transfer eels was investigated in the present study. The six day SW transfer group were chosen as the highest plasma AII concentration was recorded during this time period.

As a method for elucidating receptor characteristics autoradiography has certain limitations. Detailed quantitative analysis of AII binding would have required the saturation of binding sites in all tissues by appropriate  $^{125}\text{I}$ -AII concentrations. Since the various concentrations for the different tissues were not established in the present study, comparisons of specific binding

percentages should only be taken as a sign of changes in binding trends during SW adaptation.

This study has demonstrated that AII binds to a number of tissues in the euryhaline eel. In view of the results, in the current study, demonstrating an elevation of the circulating plasma AII concentration during the adaptation of eel from FW to SW, it is interesting to note the tendency for a higher specific binding in different tissues during SW transfer. As previously stated AII is known to be dipsogenic in teleosts ( Perrott *et al.*, 1992 ). The binding of AII in the brain in the present study, is in agreement with Cobb and Brown ( 1992 ), and is particularly interesting in view of the report by Takei *et al.* ( 1979 ) that drinking in teleosts may be under the influence of the medulla oblongata. Binding in both the medulla oblongata and the cerebellum increased significantly six days after transfer from FW to SW. This period corresponds to the elevated drinking rate previously seen to occur during FW to SW transfer in this study, and so provides support for central control of drinking.

Specific AII receptors in the gill of the rainbow trout in both FW and SW media have previously been reported ( Cobb and Brown, 1992 ). In the present study, although there was no significant difference between any of the experimental groups, there appeared to be a tendency for higher binding in the 6 day SW transfer group and the long-term SW-adapted group compared to the FW fish. Binding of AII to the central filaments would appear to indicate an association with gill vasculature and, therefore, perhaps a possible role in controlling branchial blood flow. The effects of AII on branchial ion and water fluxes are unknown, although this study suggests the presence of AII receptors in the gill epithelium.

Binding of AII to the head kidney is interesting in view of the presence of adrenocortical tissue in this particular tissue. AII has been reported to stimulate steroidogenesis in *in vitro* interrenal preparations by working in

synergy with other secretagogues ( Decourt and Lahlou, 1987 ), and to increase plasma cortisol concentration after injection into fish ( Perrott and Balment, 1990 ). A high specific binding was seen in all transfer groups with no significant difference occurring between the groups.

The binding of AII to the caudal kidney was not separated into binding to glomeruli and to proximal tubules, due to the sectioning of the tissue. Therefore, the information obtained from this study about AII binding to various sections of the caudal kidney is limited. Glomerular binding of AII has been previously reported in FW- and SW-adapted trout ( Brown *et al.*, 1990; Cobb and Brown, 1992 ). AII produced an antidiuretic action in the trout, reducing the number of filtering nephrons ( Brown *et al.*, 1980; Gray and Brown, 1985 ). AII action on renal tubules has been reported to include a decrease in the net tubular reabsorption of water in trout ( Brown *et al.*, 1980 ). It is impossible to tell from the present study where the AII is binding in the caudal kidney, although there is a significant elevation in binding after six day SW transfer.

The physiological role of AII in teleost liver is not yet known. AII receptors appear to be present in the liver of all transfer groups in the current study, with a significant elevation in the six day SW transfer and the long-term SW transfer groups, compared to the FW group. The significance of AII binding in liver is not clear although it has been previously reported, by autoradiography in the trout ( Cobb and Brown, 1992 ) and by radioreceptor assay in the eel ( Grierson, 1990 ). In mammals AII is thought to be involved in carbohydrate metabolism by the liver ( Campanile *et al.*, 1982 ), stimulating both gluconeogenesis and glycogenolysis ( Sernia *et al.*, 1985 ). Data obtained from the rat indicated that the liver may be a major site of renin substrate biosynthesis ( Freeman and Rostorfer, 1972 ). The liver has a high blood volume and, therefore, AII could potentially play a role in controlling blood flow and blood vessel resistance.

In summary AII binding was observed to significantly increase, 6 days after SW transfer, in the medulla oblongata and cerebellum regions of the brain, the caudal kidney and the liver. The increased binding in these tissues, at least in the brain and caudal kidney, provide further support for the central control of drinking and the role of AII in renal function, respectively, during chronic FW to SW transfer. In the long-term SW-adapted groups, no significant differences were found in any of the tissues, apart from the liver, compared to the FW-adapted group, although the tendency appeared to be for increased binding of AII to various tissues in SW.

This study provides evidence for the existence of specific binding sites for AII in a variety of tissues of the eel during SW adaptation. However, receptor binding studies on membrane preparations from key osmoregulatory tissues such as gill and renal preparations, are required to elucidate the actions and receptor characteristics of AII during SW adaptation. The use of the specific nonpeptide receptor AII agonists, DuP 753, EXP655 and PD 123319 ( Chiu *et al.*, 1989; Pucell *et al.*, 1991 ) may enable the characterisation of AII receptors into subtypes. Further studies would entail cloning the AII receptor, which would allow differential expression of functional protein during SW adaptation to be investigated. Use of the AII receptor probe would also enable specific target sites for AII binding to be confirmed and the up/down regulation of receptors in relation to changes in circulating AII concentrations to be examined.

Classically the RAS has been assigned evolutionary roles in the maintenance of blood pressure ( Nishimura and Sawyer, 1976 ), the control of drinking ( Balment and Carrick, 1985 ), or the control of renal function ( Bailey and Randall, 1981, Henderson *et al.*, 1981 ). The RAS has generally been considered to have evolved with the emergence of the teleosts, but due to recent findings with regards to the isolation of AI from elasmobranchs

( Takei and Hazon, pers. comm. ) and immunocytochemical evidence for the existence of RAS in annelids ( Salzet *et al.*, 1992 ), the evolutionary origin of RAS may have to be re-evaluated, and further research is clearly required.

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## 6.0 Appendix

Appendix 1 Amino acid abbreviations

| <u>One-letter</u> | <u>Three-letter</u> | <u>Name</u>   |
|-------------------|---------------------|---------------|
| A                 | Ala                 | Alanine       |
| C                 | Cys                 | Cysteine      |
| D                 | Asp                 | Aspartic acid |
| E                 | Glu                 | Glutamic acid |
| F                 | Phe                 | Phenylalanine |
| G                 | Gly                 | Glycine       |
| H                 | His                 | Histidine     |
| I                 | Ile                 | Isoleucine    |
| K                 | Lys                 | Lysine        |
| L                 | Leu                 | Leucine       |
| M                 | Met                 | Methionine    |
| N                 | Asn                 | Asparagine    |
| P                 | Pro                 | Proline       |
| Q                 | Gln                 | Glutamine     |
| R                 | Arg                 | Arginine      |
| S                 | Ser                 | Serine        |
| T                 | Thr                 | Threonine     |
| V                 | Val                 | Valine        |
| W                 | Trp                 | Tryptophan    |
| Y                 | Tyr                 | Tyrosine      |