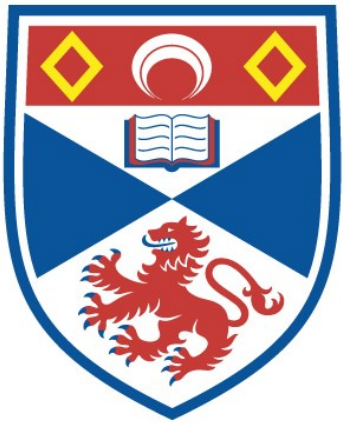
HORMONAL CONTROL OF RECTAL GLAND SECRETION IN THE EUROPEAN LESSER SPOTTED DOGFISH 'SCYLIORHINUS CANICULA'

W. Gary Anderson

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



1995

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Doctor of Philosophy
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April 1995

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ii) I was admitted as a research student under Ordinance No. 12 in October, 1991 as a candidate for the degree of Ph.D. in October, 1991; the higher study for which this is a record was carried out in the University of St. Andrews between 1991 and 1995.

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This thesis is dedicated to my family, particularly my mother and father. I am glad that you decided to hang around in time for the completion dad.

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Finally, these acknowledgements would not be complete if Toni were not included. I have far too much to thank her for, suffice to say, I love her very much.

Abstract

1) Using the corrosion casting technique, which utilises a fast polymerising monomer resin, the vasculature of the rectal gland in *Scyliorhinus canicula* was studied using ight and scanning electron microscopy. Possible control sites for altering blood flow through the rectal gland were identified.

2) A viable and reliable isolated perfused preparation of the rectal gland of *S. canicula* was developed by optimising flow rate, time of perfusion and viability of individual preparations. The combination of dibutryl cyclic adenosine monophosphate plus the potent phosphodiesterase inhibitor Isobutyl-methyl xanthine , was used as a test of viability for individual preparations. This combination potently stimulated chloride clearance rates from the isolated perfused rectal gland.

3) The isolated perfused preparation was then used to test a variety of hormones that could be involved in the control of rectal gland activity. The hormones, vasoactive intestinal peptide and urotensin II did not stimulate chloride clearance rates from the isolated perfused gland. However, the endogenous natriuretic peptide, C-type natriuretic peptide did stimulate chloride clearance rate from the perfused rectal gland in a dose dependant manner. Endogenous glucagon also stimulated chloride clearance rate.

4) Using cannulated fish, administration of angiotensin II and C-type natriuretic peptide *in vivo*, produced vasopressor and vasodepressor responses respectively and C-type natriuretic peptide inhibited the vasopressor action of angiotensin II when the two peptides were administered simultaneously. Administration of angiotensin II to the isolated perfused rectal gland produced a vasoconstrictor effect, increasing perfusion pressure. C-type natriuretic peptide did not produce any significant effect. A combination of the two peptides *in vitro* appeared to reduce the vasoconstrictor effect of angiotensin II. In addition this hormonal combination *in vitro* appeared to exert a synergistic effect increasing chloride clearance rates from the isolated perfused rectal gland.

5) The lack of a VIP stimulatory effect on the isolated perfused rectal gland in the present study is in agreement with previous reports. Using protein purification techniques including, gel-permeation, and reverse-phase high performance liquid chromatography an endogenous gut peptide was obtained that stimulated rectal gland activity in *S. canicula*. This peptide was subsequently characterised as scyliorhinin II and it is proposed that the previously identified rectin and scyliorhinin II are the same peptide.

Table of Contents

List of Figures

List of Tables

List of Plates

Abbreviations

1αOH-B	1α hydroxycorticosterone
AI	Angiotensin I
AII	Angiotensin II
ACTH	Adrenocorticotrophic hormone
ACE	Angiotensin converting enzyme
ANP	Atrial natriuretic peptide
BNP	B-type natriuretic peptide
CNP	C-type natriuretic peptide
VNP	V-type natriuretic peptide
AVA	Arterio-venous anastamoses
AVP	Arginine vasopressin
AVT	Arginine vasotocin
cAMP	Cyclic adenosine monophosphate
CFTR	Cystic fibrosis transmembrane conductance regulator
sCFTR	Shark cystic fibrosis transmembrane conductance regulator
cGMP	Cyclic guanosine monophosphate
8-Br-cGMP	8-Bromo cyclic guanosine monophosphate
DB cAMP	Dibutryl cyclic adenosine monophosphate
HPLC	High performance liquid chromatography
GFR	Glomerular filtration rate
SNGFR	Single nephron glomerular filtration rate
IP_3	inositol 1,4,5-triphosphate
IBMX	Isobutyl methyl xanthine
MS222	Tricaine metanosulphonate

Na^+K^+ATPase	Sodium potassium adenosine triphosphatase
NH_4^+	Ammonium
NKA	Neurokinin A
NKB	Neurokinin B
NPY	Neuro-peptide Y
PKA	Protein Kinase A
PKC	Protein kinase C
Pth-Xaa	Phenylthiohydantoin coupled amino acids
RAS	Renin angiotensin system
S.E.M	Scanning electron microscope
TMAO	Trimethlyamine oxide
scy II	Scyliorhinin II
UI	Urotensin I
UII	Urotensin II
VIP	Vasoactive intestinal peptide

CHAPTER 1
GENERAL INTRODUCTION

1.1 Osmoregulation.

Claude Bernard (1865) regarded osmoregulation of vertebrates as the maintenance of a constant internal "milieu" by the regulation of internal plasma ion concentration and water content in the face of a variable external environment. This regulation involves the complex process of hormone-receptor mediated action at specific "target" organs in the body which homeostatically control the internal composition of the body fluids. Body fluids can be divided into intracellular (rich in protein and potassium ions) and extracellular fluids (low in protein and rich in sodium and chloride ions), and the chemical composition of these two compartments are relatively uniform throughout the vertebrates.

The principle factor effecting salt and water balance in terrestrial vertebrates is dehydration. Water is lost to the environment as a result of evaporation from respiratory surfaces, through sweating and panting, and the production of faeces and urine. Prevention of excessive water loss is aided by the production of a hyperosmotic urine in mammals, and an almost water-free urine in terrestrial birds and reptiles.

Aquatic vertebrates however face very different osmotic problems. Plasma composition of fresh water vertebrates is hyperosmotic to the surrounding medium, therefore water is gained across semi-permeable membranes, such as the gills of fresh water teleosts and the skin of amphibians. These animals tend to drink very little and produce copious amounts of urine to combat the continuous influx of water. Plasma ion concentrations are maintained through the active absorption of ions across the gills of fresh water teleosts (Maetz, 1971) and the skin of amphibians (Middler, Kleeman & Edwards, 1968).

Marine vertebrates exhibit one of three possible osmoregulatory strategies.

1) Maintenance of a plasma osmolality that is 25-30 % of sea water (SW) i.e.; hyposmotic to the surrounding media. These vertebrates are faced with osmotic water loss and salt gain across semi-permeable membranes. Marine mammals, like their terrestrial counterparts, possess the physiological capabilities to produce a highly concentrated urine. Marine birds and reptiles possess highly specialised salt glands which actively secrete a fluid that is almost entirely composed of sodium chloride (Schmidt-Nielsen, 1960). Marine teleosts counteract these ionic and water movements by drinking large amounts of seawater, production of a scant amount of isosmotic urine, and

active excretion of excess plasma sodium and chloride ions across the gill epithelia (Keys & Willmer, 1932).

2) Maintenance of a plasma osmolality that is effectively isosmotic to the external medium. Hagfish belong to the class Agnatha (considered the most ancient of the vertebrate phyla), and their blood plasma composition is almost identical to sea water. They appear to osmoregulate in a similar fashion to marine invertebrates which are thought to osmoconform with limited but specific ionic regulation (Stolte & Schmidt-Nielsen, 1978).

3) The remaining aquatic vertebrate sub-classes, elasmobranchs, holocephalans and coelacanths minimise water loss by elevating their plasma osmolality so that it is hyperosmotic to the surrounding media. Elevation of blood plasma is essentially maintained by the retention of urea and methylamine compounds. However, an osmotic gradient from sea water to blood plasma of sodium and chloride ions is still present and therefore considerable ionic regulation occurs at the gills and kidneys of these fishes. Furthermore, elasmobranchs are equipped with a specialised salt regulating organ, the rectal gland.

The kidneys or renal tissue play a central role in successful osmoregulation and are found in various forms throughout the vertebrate phyla. With the exception of aglomerular teleost fish, the composition of final urine in all other vertebrates can be determined by the regulation of glomerular filtration rate (GFR). This is simply the rate at which blood is filtered at the glomerulus into the tubular lumen of the nephron which is the functional unit of the kidney and is morphologically different in each vertebrate class. Stenohaline marine aglomerular teleosts such as the midshipman, *Poricthhys notatus*, and the toadfish, *Opsanus tau*, rely principally on secretory mechanism to produce a final urine. The nephron of these fish consists of an initial segment with brush border and a terminal collecting duct system (Bulger, 1965; Bulger & Trump, 1965). Mammalian nephrons have particularly long loops of Henle, it is this morphological adaptation that provides mammals with the capacity of producing a concentrated urine through countercurrent multiplication mechanisms and variations in tubular permeability. Correlation can be drawn to the length of the loop of Henle and the concentrating ability of the renal tissue. Indeed some arid dwelling mammals with very long loops of Henle can concentrate their urine up to 25 times that of plasma osmolality (Schmidt-Nielsen, 1979). However, not all vertebrates are equipped with such a well developed

FIGURE 1.1

Figure 1.1 Diagrammatic representation of glomerular nephrons from the major vertebrate classes.

N = neck segment

PT = proximal tubule

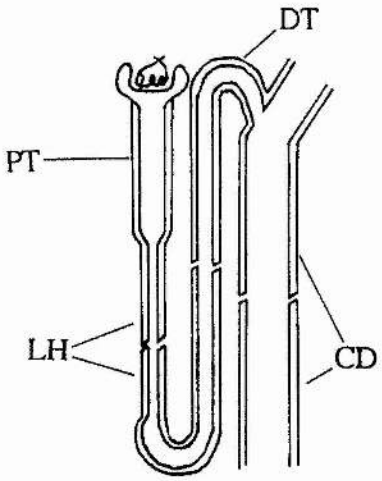
LH = loop of Henle

INT = intermediate segment

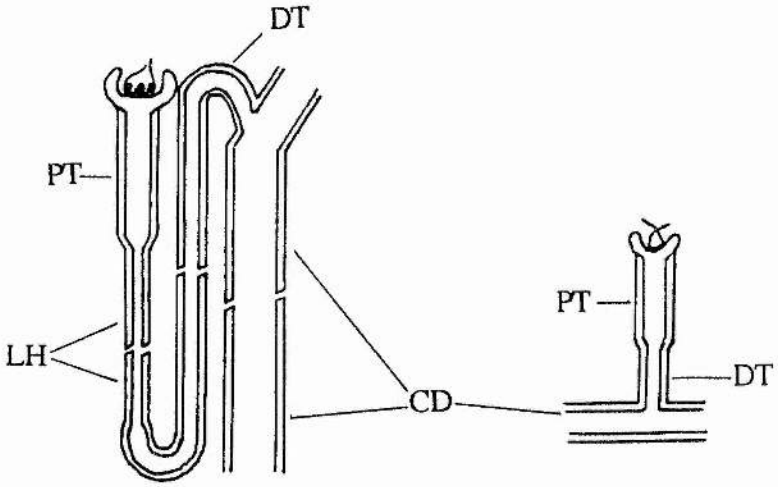
DT = distal tubule

CD = collecting duct

(Adapted from Dantzler, 1992).

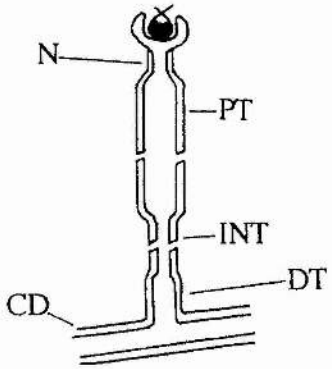


MAMMALIAN

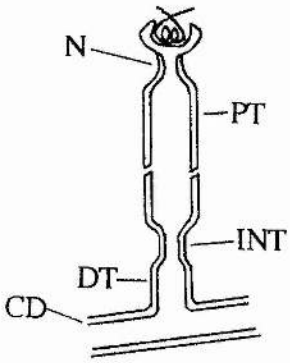


AVIAN
(Mammalian type)

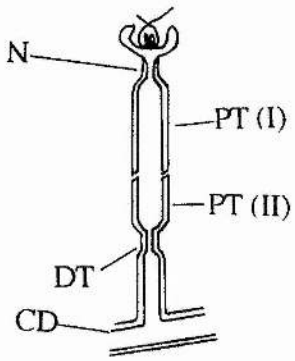
AVIAN
(Reptilian type)



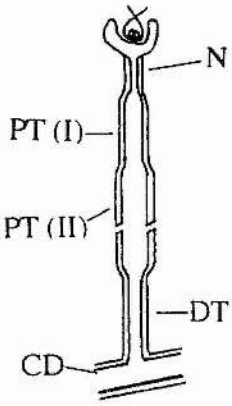
REPTILIAN



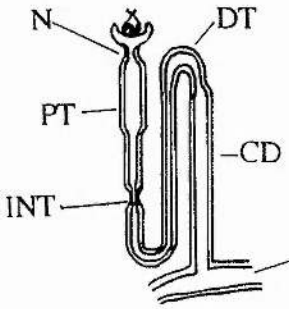
AMPHIBIAN



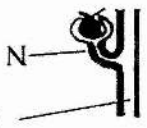
EURYHALINE
TELEOST



ELASMOBRANCH



LAMPREY



HAGFISH

countercurrent multiplying intermediary segment (Figure 1.1) and consequently they rely on a combination of renal and extra-renal functions to maintain plasma salt and water balance. The tissues involved in extra-renal regulation will now be discussed.

1.1a Branchial tissue in fish.

Fish gills are semipermeable membranes covering a large surface area maximising gaseous exchange, via the pavement cells, and are the most obvious site for extra-renal transfer of ions and fluid between plasma and external medium. Studying the common eel, Keys and Willmer (1932) discovered a large cuboidal cell at the base of the secondary lamellae (Figure 1.2a) which they aptly named the "chloride cell" (Figure 1.2b). Such cells are also found in very high density on the opercular membrane of some teleosts such as *Fundulus heteroclitus* and some *Tilapia* species. The definitive identification of the chloride cell as an active salt secreting cell in sea water teleost gills came from Foskett and Scheffey (1982). Utilising the opercular membrane from the sea water adapted *Tilapia mossambicus*, chloride ion fluxes were localised specifically to the chloride cell. Transference of teleosts from fresh water to sea water induces a number of changes in chloride cell morphology which include; 1) an increase in the tubular arrangement in the basolateral membrane; 2) an increase in mitochondria around these tubules; 3) an increase in Na^+K^+ATPase activity, 4) a deepening of the apical pits; 5) an increase in chloride cell numbers per crypt, and; 6) an increase in leakiness of intercellular junctions (Maetz & Bornancin, 1975). Pisam, Caroff and Rambourg (1987) reported that fresh water chloride cells were morphologically different to sea water adapted chloride cells and these were subsequently labelled as A and B Chloride cells respectively, perhaps relating to different functions in fresh and sea water.

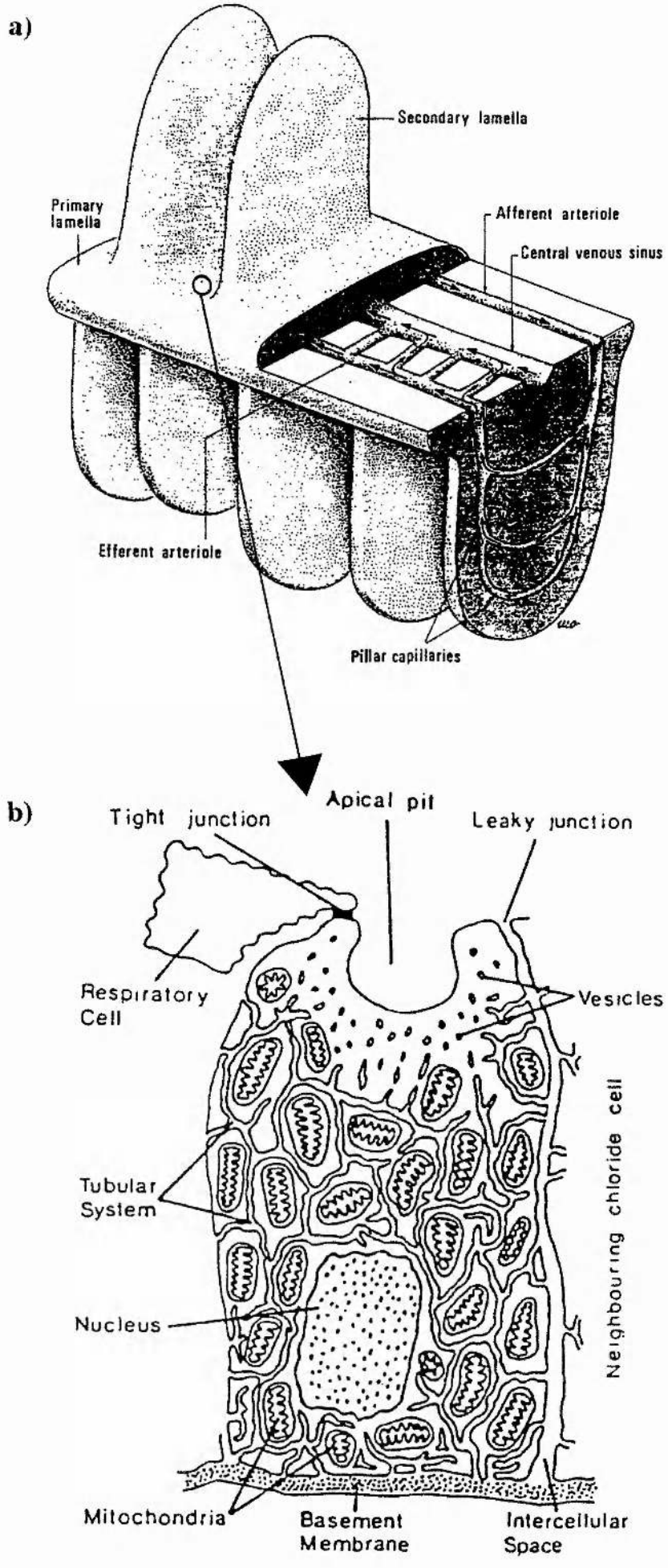
1.1b Amphibian skin.

The majority of amphibian species are faced with excessive amounts of water gain as they inhabit fresh water. Amphibian skin is quite permeable to water and solutes compared to other vertebrates. The control of sodium flux across amphibian skin was first demonstrated by Ussing and Zerahn (1951) in the now famous Ussing chamber. Freshwater amphibians can freely exchange ions and fluid with their surrounding media, indeed sodium and chloride ions are actively transported across amphibian skin (Kirschner, 1983) to maintain plasma ion concentrations. Furthermore, compared to other vertebrate classes

FIGURE 1.2 a & b

Figure 1.2a Illustration of gill lamellae.
(Adapted form Pisam, Caroff & Rambourg, 1987).

Figure 1.2b Diagrammatic representation of a chloride cell from the gill of a marine teleost.
(Adapted from Rankin & Davenport, 1981).


a)
Secondary lamella
Primary lamella
Afferent arteriole
Central venous sinus
Efferent arteriole
Pillar capillaries
b)
Tight junction
Apical pit
Leaky junction
Respiratory Cell
Vesicles
Tubular System
Neighbouring chloride cell
Nucleus
Mitochondria
Basement Membrane
Intercellular Space

amphibians exhibit incredible physiological tolerance, with-standing huge changes in plasma osmotic concentrations (Schmidt-Nielsen, 1979).

1.1c Salt Glands in birds and reptiles.

Avian renal tissue exhibits a proportion of reptilian and mammalian-like nephrons and the presence of a mammalian type nephron in birds would lead one to expect the production of a concentrated urine. Birds do produce such a fluid, but cannot concentrate their urine to the same degree as mammals. This is perhaps the principle reason why marine birds, with a high dietary sodium intake, require an efficient tissue specifically designed to excrete excess sodium chloride with minimal water loss. The avian nasal salt gland is an example of a highly specialised extra-renal gland (Schmidt-Nielsen & Sladen, 1958). Likewise all estuarine and a few arid dwelling reptilian species have developed a salt gland specific in the secretion of sodium chloride (Taplin, Grigg, Harlow, Ellis & Dunson, 1982; Schmidt-Nielsen & Fange, 1958).

1.2 Hormones and receptors.

The effectiveness of any physiological homeostatic mechanism relies on a constant supply of information concerning the state of a particular variable. By the very nature of homeostasis a change in a particular variable must be detected, an effector is then released from endocrine and/or nervous systems, which acts at receptors to bring about cellular changes and return the initial variable back to normal levels.

Endocrine glands are ductless organs or tissues, located throughout the body, which secrete a variety of chemically distinct hormones. Hormones can be broadly categorised as lipophilic or hydrophilic. The majority of steroids are lipophilic hormones which are cell permeable molecules that bind to specific receptors within the nucleus of target cells. Their mechanism of action is at the level of gene regulation where they modulate the rate of transcription (Miesfield, 1989). Hydrophilic hormones are larger, water soluble molecules, that bind to receptors on the membrane of the target cell, which triggers an intracellular signal or second messenger system which alters the behaviour of the cell (Catt & Dufau, 1977). Examples of hydrophilic hormones include angiotensin II and the natriuretic peptides. Receptors are protein macromolecules located either as an integral part of the plasma membrane or within the cell. Hormones bind to receptors with high affinity

and specificity. If hormone structure and therefore shape is altered it is accepted that the receptor must also change in order to remain specific to the homologous hormone. For the purpose of this discussion a brief review of the major hormonal controls of osmoregulation in vertebrates will follow.

1.2a Adenohypophysis.

Of all the hormones secreted by the adenohypophysis only the prolactins have been extensively reported as having a direct role in osmoregulation, in amphibians and fish (particularly teleosts) (Hirano, 1986) and a possible indirect role in birds (Holmes & Phillips, 1985). Adrenocorticotrophic hormone (ACTH) one of the trophic hormones (released to act specifically on other endocrine organs) produced in the adenohypophysis is found throughout the vertebrate phyla. Similarly its action of stimulating the release of corticosteroids from adrenocortical tissue is ubiquitous throughout the vertebrates. Consequently ACTH can be considered to have an indirect osmoregulatory effect as corticosteroids are directly concerned with osmoregulation in all vertebrates (Section 1.2c).

Osmoregulatory influences of prolactin in amphibians include, maintenance of high water content in larvae and breeding conditioned adults (Brown & Brown, 1987). In addition prolactin appears to retain sodium in a variety of aquatic amphibians by reducing skin permeability to sodium and water (Toledo & Jared, 1993), possibly by increasing production of the cutaneous mucous secretion which limits penetration to water (Nakashima & Kamishina, 1990).

Prolactin has been implicated as essential to fresh water survival in euryhaline teleosts since Pickford and Phillips (1959) first illustrated the survival of hypophysectomised killifish, *Fundulus heteroclitus*, in fresh water only after ovine prolactin administration. Reported actions of prolactin in fresh water teleosts include; induction of diuresis (Lam, 1972; Hickman, 1965); reduction of sodium excretion rate across gill epithelia (Dharmamba & Maetz, 1976); dedifferentiation of chloride cell population (Foskett, Bern, Machen & Conner, 1983); and increase in sodium epithelial transport of sodium from mucosa to serosa in isolated urinary bladders (Johnson, Hirano, Bern & Conte, 1972; Hirano, Johnson, Bern & Utida, 1973). Furthermore, in sea water adapted fish ovine prolactin administration has been shown to reduce intestinal water intake in sea water adapted trout, *Onchorhynchus mykiss* (Morely, Chadwick & El Tounsy, 1981) and sea water adapted eel,

Anguilla japonica (Utida, Hirano, Oide, Ando, Johnson & Bern, 1972) although it had no apparent effect on the fresh water eel (Hirano & Utida, 1968).

Other osmoregulatory peptides implicated in teleost osmoregulation include the growth hormones and the recently identified somatolactin (Clarke, Farmer & Hartwell, 1977; Rand-Weaver, Noso, Muramoto & Kawauchi, 1991). There is evidence in several salmonid species that growth hormones may facilitate sea water adaptation. Clarke *et al.* (1977) found that bovine growth hormone decreased plasma sodium concentration in juvenile sockeye salmon after a sea water challenge test. Furthermore plasma growth hormone concentration was seen to peak two days after sea water transfer followed by a decrease to the pre-transfer values in rainbow trout, *O. mykiss* (Sakamoto, Ogasawara & Hirano, 1990). The full extent of growth hormone and osmoregulation in teleosts has yet to be determined. Somatolactin has only recently been identified in teleosts (Rand-Weaver *et al.*, 1991) however given its structural similarity with prolactin and growth hormone it may have a part to play in ionic regulation of teleosts.

1.2b Neurohypophysis.

All Neurohypophysial peptides consist of a sequence of nine amino acids, with residues in positions 1, 5, 6, 7 and 9 being highly conserved (Table 1.1). Residues 1 and 6 are cysteine which are linked by a disulphide bridge in all neurohypophysial peptides characterised to date and variation occurs only in the amino acids 3, 4 and 8 (Acher, 1974 & 1988).

The typical vertebrate neurohypophysis secretes active peptides of two types:

1) Basic peptides which contain a basic amino acid (lysine or arginine) at position 8, these include arginine vasopressin (AVP) in mammals or arginine vasotocin (AVT) in other vertebrates.

2) Neutral peptides, which contain neutral amino acids (leucine, isoleucine, valine or glutamine) at position 8, and these are termed oxytocin like peptides. The oxytocin like peptides do not appear to be involved in osmoregulation, however, the basic peptides, arginine vasopressin (AVP), and arginine vasotocin (AVT) are directly involved in osmoregulation.

In mammals an increase in plasma osmolality leads to an increased secretion of AVP from the neurohypophysis. AVP increases the reabsorption

TABLE 1.1

Table 1.1 Summary of the vertebrate neurohypophysial peptides characterised to date.
(Adapted from Batten & Ingleton, 1987).

	Neutral hormone	Basic hormone
Mammals *Placentals* All except pigs	1 2 3 4 5 6 7 8 9 Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_3 Oxytocin	1 2 3 4 5 6 7 8 9 Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH_3 Arginine vasopressin
Domestic pigs	Oxytocin	1 2 3 4 5 6 7 Lys 9 Lysine vasopressin
Marsupials Macropodidae	Oxytocin	Lysine vasopressin+ 1 Phe 3 4 5 6 7 8 9 Phenypressin
Birds, Reptiles, Amphibians and Lungfishes	Mesotocin	1 2 Ile 4 5 6 7 Lys 9 Arginine vasotocin
Teleostean and Ganoid Fishes	1 2 3 Ser 5 6 7 Ile 9 Isotocin	Arginine vasotocin
Elasmobranchs *Selachii* Skates and Rays	1 2 3 Ser 5 6 7 Ile 9 Glumitocin	Arginine vasotocin
Sharks and Dogfishes	1 2 3 4 5 6 7 Val 9 Valitocin 1 2 3 Asn 5 6 7 Gln 9 Aspartocin 1 2 3 Asn 5 6 7 Val 9 Asvatocin 1 2 Phe Asn 5 6 7 Val 9 Phasvatocin	Arginine vasotocin
Holocephali	Oxytocin	Arginine vasotocin
Cyclostomes	----------	Arginine vasotocin

of water by increasing tubule permeability at the distal convoluted tubule and collecting duct of the nephron, thus concentrating the urine. It is through this process that AVP is very often referred to as anti-diuretic hormone (ADH). A similar effect is seen in birds (Ames, Steven & Skadhauge, 1971) and reptiles (Dantzler & Schmidt-Nielsen, 1966), although in these classes AVT appears to act at both tubular and less potently at pre-glomerular blood vessels to induce anti-diuresis by reduction of glomerular filtration rate. The pre-glomerular blood vessels were thought to be the principle site of action in amphibians (Sawyer & Sawyer, 1952). However, AVT receptors have recently been localised on the collecting tubule (Uchiyama, 1994) as well as the glomerulus (Kloas & Hanke, 1990) of the amphibian nephron. One would anticipate AVT, as an anti-diuretic hormone, to be most appropriate in sea water adapted teleosts. Recently a specific radioimmunoassay was developed for measurement of plasma AVT in euryhaline fish adapted to fresh water and sea water. AVT levels reported for the three species investigated were not significantly different between the two environments (Warne, Hazon, Rankin & Balment, 1994). The role of AVT as a antidiuretic or diuretic and hence a sea or fresh water adaptational hormone in teleosts is as yet undecided. Although the inhibitory effects of both isotocin and AVT on cAMP production were found to be greater in sea water adapted isolated gill cells than in fresh water adapted isolated gill cells (Guibbolini & Lahlou, 1990). The neurohypophysial peptides in elasmobranch fish will be discussed in section 1.6.

1.2c Adrenocorticosteroids.

The adrenal gland or its homologue occurs throughout the vertebrate phyla, from cyclostomes to mammals. It is an endocrine gland which manufactures and secretes chemicals essential for life. As the name suggests the gland lies in close proximity to renal tissue in all vertebrates. The adrenal gland is comprised of two principle tissue types; adrenocortical or interrenal tissue which secretes several steroids; and adrenomedullary or chromaffin tissue (so-called because of the dark brown staining of chromic acid) (Chester Jones, 1976) which secretes catecholamines. Throughout the phyla there is close association between adrenocortical and chromaffin tissue, only in mammals and elasmobranchs are the tissue types distinctly separate.

Corticosteroids in mammals include mineralocorticoids and glucocorticoids. The principle mineralocorticoid in mammals is aldosterone and it is concerned with electrolyte and water balance, primarily by promoting

TABLE 1.2

Table 1.2 Summary of the major circulating adrenocorticosteroids in vertebrates.
(Adapted from Balment & Henderson, 1987).

Group	Corticosteroids in Blood
Mammals	Cortisol/corticosterone, aldosterone, corticosterone, 18-hydroxycorticosterone, 11-deoxycorticosterone
Birds	Corticosterone, aldosterone, 11-deoxycorticosterone
Reptiles	Corticosterone, 18-hydroxycorticosterone, aldosterone
Amphibians	Corticosterone, 18-hydroxycorticosterone, aldosterone, 11-deoxycorticosterone, cortisol
Lungfish	Cortisol, aldosterone, 11-deoxycortisol, 11-deoxycorticosterone, corticosterone
Bony Fish	Cortisol, cortisone, 11-deoxycortisol, corticosterone
Elasmobranchs	1α-hydroxycorticosterone, corticosterone, 11-deoxycorticosterone, 11-deoxycortisol

* Underlined steroids are the major secretory products.

sodium retention in the distal region of the nephron. The principle glucocorticoids in mammals are cortisol and corticosterone (depending on the species) and are concerned with carbohydrate, protein and fat metabolism and with an additional anti-inflammatory effect. For other vertebrates the terms mineralocorticoid and glucocorticoid can be considered as arbitrary as they are based on our knowledge of mammalian corticosteroids. For example, what is considered a glucocorticoid in mammals may well produce mineralocorticoid responses in teleosts (Sandor, Fazekas & Robinson, 1976). Table 1.2 illustrates the major corticosteroids in the different vertebrate classes.

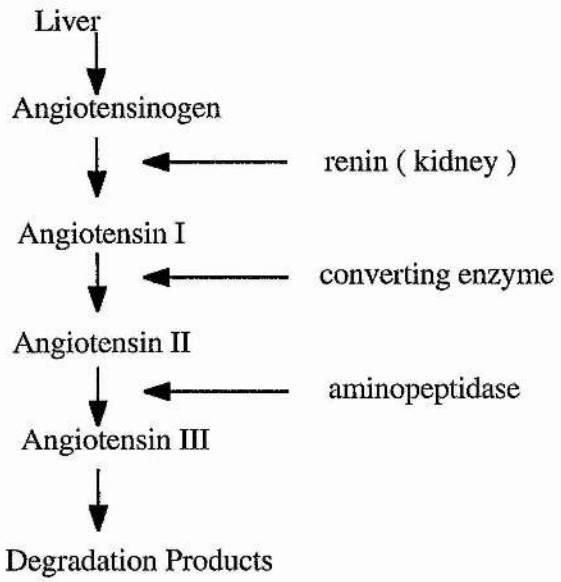
Both aldosterone and corticosterone appear to cause sodium reabsorption in renal tubules in birds (Holmes & Phillips, 1976), but corticosterone administration to *in vitro* slices of avian salt glands did not effect secretory output (Wilson & Butler, 1980), which is similar to the role of these hormones in mammals. In the lizard, *Varanus gouldii*, aldosterone was shown to limit sodium excretion (Rice, Bradshaw & Prendergast, 1982), however, Callard and Callard (1978) demonstrated stimulation of the reptilian salt gland secretion upon administration of corticosterone and aldosterone. In frogs corticosterone administration stimulated sodium influx in the skin (Maetz, Jard & Morel, 1958) urinary bladder and colon (Crabbe & DeWeer, 1964), however, a consistent effect on renal function was not produced (Henderson & Kime, 1987).

Cortisol has been considered as the sea water adaptational hormone in euryhaline teleosts. An increase in the osmotic pressure of the perfusion medium was shown to stimulate the release of cortisol from the *in vitro* perifused interrenal tissue of the rainbow trout, *Onchorhynchus mykiss* (Decourt & Lahlou, 1986). Thus suggesting that cortisol release in teleosts is directly affected by changes in extracellular electrolyte concentrations. Plasma cortisol concentrations of long term sea water and fresh water euryhaline fish are similar (Arnold-Reed & Balment, 1991). However, after transferral from sea water to fresh water there is a marked decrease in plasma levels which recover after 2 days (Arnold-Reed & Balment, 1991). Conversely, after transferral from fresh water to sea water there is a marked increase in plasma cortisol concentrations (Ball, Chester Jones, Forster, Hargreaves, Hawkins & Milne, 1971; Kenyon, McKeever, Oliver & Henderson, 1985) which decline to basal levels after 2 days (Arnold-Reed & Balment, 1991). Furthermore the characteristic changes of the chloride cell in euryhaline teleosts migrating from fresh water to sea water have been induced

FIGURE 1.3

Figure 1.3 Summary of the renin-angiotensin system in mammals.
(Adapted from Balment & Henderson, 1987).

The Mammalian Renin-Angiotensin System



Position of Peptide Cleavage

(Renin Substrate)

	1	2	3	4	5	6	7	8	9	10	11	12
Angiotensinogen	Asp-	Arg-	Val-	Tyr-	Ile-	His-	Pro-	Phe-	His-	Leu-	Leu-	Val

↑ renin

Angiotensin I: Asp- Arg- Val- Tyr- Ile- His- Pro- Phe- His- Leu

↑ converting enzyme

Angiotensin II: Asp- Arg- Val- Tyr- Ile- His- Pro- Phe

↑ aminopeptidase

Angiotensin III: Arg- Val- Tyr- Ile- His- Pro- Phe

Angiotensin IV: Val- Tyr- Ile- His- Pro- Phe

(3-8 AII)

in the trout, *Onchorhynchus mykiss*, by intramuscular injection of cortisol (Laurent & Perry, 1990). In conclusion, in higher vertebrates aldosterone and corticosterone are the principle regulators of salt and water balance. However, cortisol, a glucocorticoid in mammals, regulates salt and water balance in teleosts. 1α hydroxycorticosterone is the principle circulating adrenocorticosteroid in elasmobranchs and its physiological role is further discussed in section 1.7.

1.2d Renin Angiotensin System.

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 most extensively studied although the elements of the RAS have been identified in all vertebrate classes (Takei, Hasegawa, Watanabe, Nakajima & Hazon, 1993) with controversy surrounding the physiological role in the more ancient classes (Nishimura, Oguri, Ogawa, Sokabe & Imai, 1970; Henderson, Oliver, McKeever & Hazon, 1980).

The RAS consists of a variety of peptides with angiotensin II (AII) being the principle biological effector (Figure 1.3), and angiotensin III (AIII) having limited biological activity. The basic structure of AII is highly conserved with substitutions at positions 1 and 5. AI is structurally more diverse with a number of changes occurring at position 9 (Table 1.3).

The RAS is primarily concerned with the regulation of blood volume and has four principle effects;

1) Dipsogen, stimulation of higher brain centres to induce drinking.
2) Vasopressor, AII induces potent constriction of systemic smooth muscle.
3) Conservation of fluid and electrolytes.
4) Close interaction with other hormonal systems involved in osmoregulation.

These effects will now be briefly discussed for all vertebrate classes although the elasmobranch RAS will be discussed in detail in section 1.8.

1.2d.i Cardiovascular actions of AII.

The pressor response of AII appears to be universal throughout the vertebrate phyla (Table 1.4). Abolition of the pressor response by adrenergic receptor blockers such as phentolamine, indicate that the pressor responses are to a varying extent catecholamine mediated in response to AII. Also ACE hydrolysis of AI to AII is necessary for pressor activity as captopril inhibition

TABLE 1.3

Table 1.3 Structures of vertebrate Angiotensin I characterised to date.
(Adapted from Tierney, 1993).

	Amino acid sequence									
Common Structure	1 -	2 Arg	3 Val	4 Tyr	5 -	6 His	7 Pro	8 Phe	9 -	10 Leu
Species variation										
Human, pig, rabbit 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	
Elasmobranch	Asn		Pro		Ile				Gln	

TABLE 1.4

Table 1.4 Summary of the cardiovascular actions of Angiotensin II in some non-mammalian vertebrates investigated to date.

RE, renal extract; RE + plasma, product of homologous renal extract and plasma. (Adapted from Tierney, 1993).

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

TABLE 1.5

Table 1.5 Summary of the dipsogenic actions of Angiotensin II in some non-mammalian vertebrates investigated to date.

* denotes species capable of surviving brackish water or dilute sea water. (Adapted from Tierney, 1993).

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

of ACE blocks the AI but not the AII-induced pressor response (Galardy, Podhasky & Olson, 1984).

1.2d.ii Dipsogenic actions of AII.

Administration of exogenous AII and its effects on drinking rate has been examined in a variety of species from all vertebrate classes (Table 1.5). Interestingly in birds AII administration to the central nervous system was most potent in inducing a drinking response (Takei, Kobayashi, Yanagisawa & Bando, 1979). The advent of the sensitive isotope dilution method, has made drinking rate detection in fish a much more reliable technique (Balment & Carrick, 1985; Hazon, Balment, Perrott & O'Toole, 1989). AII was found to stimulate drinking in both fresh water and sea water stenohaline teleosts (Perrott, Grierson, Hazon & Balment, 1992), an increase that could be inhibited by captopril. Administration of the smooth muscle relaxant papaverine, which is thought to stimulate endogenous renin release, also stimulated drinking which could again be inhibited by captopril (Perrott, *et al.* 1992).

1.2d.iii Renal actions of AII.

AII has been shown to exert a variety of effects on renal function in vertebrates (Table 1.6). The best described vascular effects of AII on renal function can be divided into 2 parts; 1) An indirect effect on glomerular filtration rate (GFR) due to the total systemic vasopressor effect, 2) a local effect on GFR by constriction of afferent and efferent vessels. AII has also been shown to exert a direct effect on renal proximal tubular ion transport. The tubular effect of AII has been reported as bi-phasic, with AII inducing anti-natriuresis at low concentration but diuresis at higher concentrations in mammals (Harris & Young, 1977) and toads (Galli-Gallardo & Gallardo, 1981). AII however, has been found to inhibit tubular sodium reabsorption in the fowl (Stallone & Nishimura, 1985) and the fresh water turtle (Stephens, 1987 cited in, Nishimura, 1987). In fish (Brown, Jackson, Oliver & Henderson, 1978; Brown, Oliver, Henderson & Jackson, 1980) amphibians (Galli-Gallardo & Pang, 1978) and reptiles (Brown, Stephens & Todt, 1983) exogenous administration of non-pressor doses of AII reduced GFR and caused sustained anti-diuresis perhaps by constriction of pre-glomerular blood vessels. Indeed Brown *et al.* (1978; 1980) reported the presence of 3 types of nephrons in the trout; filtering, perfused but not filtering, and not perfused. AII administration was found to effect the individual status of the nephrons or

TABLE 1.6

Table 1.6 Summary of the renal actions of Angiotensin II in some non-mammalian vertebrates investigated to date.

The effects of the ACE inhibitor captopril are also demonstrated in *S. gairdneri*. Inf, infusion; inj, injection; PD, pressor dose; N, natriuresis; D, diuresis; AN, antinatriuresis; AD, antidiuresis; TMG, renal tubular maxima for glucose. (Adapted from Tierney, 1993).

Species	Experimental Conditions	Glomerular Effect	Tubular Effect	Reference
Aves				
Gallus domesticus	AII-amide	-	D + N	Langford and Fallis (1966)
Gallus domesticus	Homologous AII (Renal portal infusion)		D + N	Stallone and Nishimura (1985)
Reptilia				
Pseudemys scripta	Asn^1-Val^5-AII infusion(PD)	↓ GFR	-	Brown *et al.* (1983)
Amphibia				
Xenopus laveis	AII-amide AII infusion	GFR constant	D + N	Henderson and Edwards (1969)
Osteichthyes				
Teleostei				
(Aglomerular)				
Lophius americanus	synthetic AII-amide infusion	-	N + D	Churchill *et al.* (1979)
(Glomerular)				
Anguilla rostrata	AII infusion (PD)	↑GFR , N + D	-	Nishimura *et al.* (1976)
Salmo gairdneri (FW)	capt. infusion	↑ GFR , D	-	Henderson *et al.* (1980)
Salmo gairdneri (SW)	capt. infusion	↑GFR , D	-	Kenyon *et al.* (1985)
Salmo gairdneri (FW)	Asn^1-Val^5-AII	↓ GFR TMG		Brown *et al.* (1980)
Salmo gairdneri (SW)	Asn^1-Val^5-AII	↓GFR TMG		Brown *et al.* (1980)
Dipnoi				
Protopterus aethopius	Val^5-AII-amide infusion.	moderate N + D	-	Sawyer (1970)
Neoceratodus forsteri	Val^5-AII-amide infusion.	D	-	Sawyer *et al.* (1976)

populations of the nephrons, altering the single nephron glomerular filtration rate (SNGFR) (Brown *et al*, 1978 & 1980). It was postulated that total GFR may be the result of a balance between SNGFR and the number of filtering nephrons and hence a change in glomerular recruitment (Gray & Brown, 1985).

1.2d.iv Extra-renal actions of AII.

Angiotensin II appears to inhibit salt gland secretion in birds which is consistent with its generally accepted role of salt and water conservation.

Elevation of circulating AII has been shown to reduce the salt and water output of active glands (Gray, Hammel & Simon, 1986; Gray & Erasmus, 1989). There is, to date, no reported evidence of direct or indirect action of the RAS on salt gland function in reptiles. However one could anticipate a similar response as observed in birds given the general role of salt and water conservation the RAS plays. AII was shown to increase sodium transport in frog skin and the skin of four species of toad from external to internal environments (Coviello, Elso & Fernandez, 1976). Again given the role of the RAS controlling salt and water retention these results are perhaps not surprising. In teleosts ACE activity has been recorded in gill tissue of the rainbow trout, *S. gairdneri*, and fresh water carp, *Cyprinus carpio* (Olson, Lipke, Kullman, Evan & Ryan, 1989; Polanco, Mata, Agapito & Recio, 1990). The gills, like mammalian lungs (where the majority of ACE activity occurs) are the only tissues to receive the entire cardiac output and also have extensive vascular surface area. Thus gill tissue could play an important role in the activation of AI to AII.

1.2d.v Interaction of the RAS and other hormones involved in salt and water balance.

Exogenous administration of AII has been shown to increase corticosteroid production in; mammals (Nishimura, 1987), birds (Klingbeil, 1985; Kocsis, McIlroy, Chiu, Schimmel & Carsia, 1994), amphibians (Dupont, Leboulenger, Vaudry & Valliant, 1976), teleosts (Perrot & Balment, 1990; Henderson, Jotisankasa, Mosely & Oguri, 1976; Perrott & Balment, 1990), and elasmobranchs (Hazon & Henderson, 1985), but not in reptiles (Callard, 1975). In mammals AVP has been reported to inhibit renin release (Reid, 1985). However, AII has been reported to increase plasma AVT levels in the Japanese quail (Kobayashi, Uemura & Takei, 1980). Similar exogenous administration of AII in fresh water eels increases plasma AVT levels however

AVT was shown to decrease plasma renin activity (Henderson, Hughes & Hazon, 1985). In conclusion, it is apparent that the RAS is involved in a variety of osmoregulatory control systems, and the role of the RAS as a hormonal system advocating conservation of salts in blood plasma appears to be prevalent in the majority of vertebrates investigated to date.

1.2e Natriuretic peptides.

Mammalian research into the effects of natriuretic peptides has been extensive since DeBold first described the natriuresis induced in the rat kidney following injection of atrial extracts (DeBold, Borenstein, Veress, & Sonnenberg, 1981). However, following the recent discovery of the natriuretic peptide family in mammals there is an increasing number of natriuretic peptides being discovered in non-mammalian vertebrates indicating that this peptide family has an ancient evolutionary history (Figure 1.4). ANP was the first member of the natriuretic peptide family to be discovered and therefore has been the most extensively studied. Initially, due to degradation during purification processes, peptides of various lengths were isolated, including atriopeptin which has become a frequently used synthetic analogue. Although it is not the naturally occurring peptide it appears to exhibit similar actions as ANP (Genest & Cantin, 1988).

Distension of the atrium follows vascular volume expansion which leads to release of natriuretic factors from the heart. In this regard the heart is recognised as an endocrine gland (Peterson & Benjamin, 1992). Following release, ANP has a variety of physiological functions which reduce blood volume and blood pressure. Blood pressure reduction is principally due to reduced peripheral vascular resistance (partially mediated by direct relaxation of vascular smooth muscle cells), diminished cardiac output, and decreased intravascular volume as a product of diuresis (Genest & Cantin, 1988). Furthermore ANP has been shown to inhibit aldosterone secretion directly, by acting on adrenal glomerulosa cells, and indirectly, by inhibition of renin release (Brenner, Ballerman, Gunning & Zeidel, 1990; Ganguly, 1992).

Natriuretic peptides are considered to act through 2 separate classes of receptors. A guanylate cyclase linked receptor class which is thought to activate many of the natriuretic peptide mediated effects (Levin, 1993; Okolicany, McEnroe, Gregory, Lewicki & Maack, 1991). In this receptor class there is a further subdivision, type A or (ANP-A) exhibits a greater affinity to ANP and BNP; and type B or (ANP-B) which exhibits a greater affinity for CNP (Takei, 1994). The second class was named a clearance

FIGURE 1.4

Figure 1.4 Amino acid sequence of some of the vertebrate natriuretic peptides characterised to date.
(Adapted from Takei, 1994).

Atrial (A-type) natriuretic peptide

eel SK SS P CFG GKL DRIG SY SG L GC NS - R K

bullfrog SS D CFG SRL DRIG AQ SG M GC -G - R RF

Rat SLRR SS -- CFG GRM DRIG AQ SG L GC NSF R --Y

Man SLRR SS -- CFG GRI DRIG AQ SG L GC NSF R --Y

Brain (B-type) natriuretic peptide

fowl M MRD S G -- CFG RRI DRI GSL S GM GC NGS R KN

Dog SPK - M MHK S G -- CFG RRL DRI GSL S GL GC NVL R KY

Rat S - K - M AHS S S -- CFG QKI DRI GAV S RL GC DGL R LF

Man SPK - M VQG S G -- CFG PRM DRI SSS S GL GC KVL R KH

C-type natriuretic peptide

dogfish G P SRG -- CFG V KLDRIG AM SGLGC

eel G WNRG -- CFG L KLDRIG S L SGLGC

killifish G WNRG -- CFG L KLDRIG SM SGLGC

bullfrog G YSR G -- CFG V KLDRIG A F SGLGC

fowl G LSR S -- CFG V KLDRIG SM SGLGC

man G WNRG -- CFG L KLDRIG SM SGLGC

Ventricular natriuretic peptide

trout SFNSC FGNRIERIGSWSGLGC NNVKTGNKKRIFGN

eel KSFNSC FGTRMDRIGSWSGLGC NSL-KNGTKKKIFGN

D-type natriuretic peptide

snake EVKYDPC FGHKIDRINHVSNLGC PSL - RDPRPNAPSTSA
(Dendroaspis auguticeps)

receptor (Maack, Suzuki, Almeida, Nussenzveig, Scarborough, McEnroe & Lewicki, 1987) or type C (ANP-C) receptor, and appears to be independent of guanylate cyclase. It is thought to be involved in clearance of circulating natriuretic peptides as it appears to bind all three with similar affinity (Takei, 1994). In the majority of the mammalian tissues studied ANP-C receptors constituted the overwhelming majority of ANP receptors with the ANP-A and ANP-B receptor ratio varying among tissues (Maack, 1992). A similar picture was observed in the branchial tissue of the Japanese eel (Sakaguchi, Katafuchi, Hagiwara, Takei & Hirose, 1993). Two receptor types were found, the majority of which were of ANP-C type receptors and the remainder having closest homology with ANP-B type receptors.

1.2e.i Cardiovascular actions of natriuretic peptides.

Natriuretic factors have been shown to produce a hypotensive effect in the hen (Anthony, Sima, Satnick & Wideman, 1990), but not in the duck (Gray, Schutz & Gerstberger, 1991). A vasodepressor response following exogenous administration of natriuretic factors has also been observed in the fresh water turtle, *Amyda japonica* (Cho, Kim, Koh & Seul, 1988), two species of frog, *Rana catesbeiana* and *Rana tigrini* (Genest & Cantin, 1988), and the toad, *Bufo arenarum* (De Bruno & Coviello, 1992).

Initial reports using heterologous ANP failed to produce hypotension in the rainbow trout, *Onchorhynchus mykiss* (Duff & Olson, 1986) however, upon isolation of arterial vessels and subsequent administration of heterologous ANP after pre-constriction with AII a marked vasodilation was observed (Olson & Meisheri, 1989). More recent reports have demonstrated the vasodepressor effects of natriuretic factors on sea water adapted flounder, *Platichthys flesus* (Takei, Takahashi, Nishida, & Ando, 1989a; Takei, Takahashi, Watanabe, Nakajima, & Sakakibara, 1989b; Arnold-Reed, Hazon & Balment, 1991), and rainbow trout (Conklin & Olson, 1994). Furthermore, the vasodepressor response to homologous ANP in the eel was found to be 100 times more potent than human ANP (Takei, Takahashi, Watanabe, Nakjima, Sakakibara, Takao & Shimonishi, 1990a; Takei, Y., Tamaki, H. & Ando, K. (1990b). Homologous CNP has also been shown to produce a vasodepressor response in the European dogfish, *Scyliorhinus canicula* (Bjenning, Takei, Watanabe, Nakajima, Sakakibara & Hazon, 1992).

1.2e.ii Renal actions of natriuretic peptides.

Natriuretic peptides have been shown to produce direct diuretic and natriuretic effect on avian renal tubules in the domestic fowl, *Gallus domesticus* (Gregg & Wideman, 1986), and Pekin duck, *A. platyrhynchos* (Schutz, Gray & Gerstberger, 1992). Furthermore radio labelled ANP binding was localised in the reptilian type nephrons and collecting ducts throughout the kidney (Schutz *et al.*, 1992). A similar effect was observed in the domestic fowl, *Gallus domesticus* (Gray, 1993). Natriuretic peptides appear to have no function on renal secretion in the fresh water turtle, *Amyda japonica* (Cho *et al.*, 1988) or frog, *R. esculenta* (Frick & Toygar, 1988).

In the rainbow trout, *O. mykiss*, rat ANP caused a marked diuresis and natriuresis. This was irrespective of the cardiovascular actions of ANP which have been reported as either hypertensive (Duff & Olson, 1986) or hypotensive (Olson & Duff, 1992). However, injection of eel ANP to fresh water eels produced an anti-diuresis (Takei & Balment, 1993) although whether these effects were direct or indirect is not known. Interestingly with the establishment of a homologous radioimmunoassay Takei and Balment (1993) found that following transferral of eels from fresh water to sea water plasma ANP markedly decreased. It is conceivable therefore that ANP is a fresh water adapting hormone in teleosts, which would be consistent with the role of ANP in mammals as a hormone advocating a reduction in plasma volume increase.

1.2e.iii Extra-renal actions of natriuretic peptides.

ANP has been shown to stimulate secretion from the avian salt gland (Schutz & Gerstberger, 1990) and to inhibit AII-stimulated increases in sodium conductance across toad skin (Coviello, Soria, Proto, Berman, Gamundi, Alonso & De Bold, 1989). O'Grady (1989) reported that ANP inhibited sodium uptake of the gut of the sea water adapted flounder. Serosal addition of ANP to the opercular epithelia of *F. heteroclitus* stimulated sodium and chloride secretion (Scheide & Zadunaisky, 1988). Administration of human ANP in marine flatfish *in vivo*, significantly promoted radio labelled $^{22}Na^{+}$ efflux from the animal (Arnold-Reed *et al.*, 1991). Interestingly it was found that ANP produced a greater increase in intracellular levels of cyclic guanosine monophosphate (cGMP) in sea water adapted trout gill cells than fresh water adapted trout gill cells (Balment & Lahlou, 1987). The data suggests that ANP could be considered a sea water adapting hormone which is in marked contrast to the observed renal effects of ANP.

1.2e.iv Interaction of natriuretic peptides with the RAS.

Antagonism between angiotensin II and ANP in mammalian tissue has been observed in; the kidney (Harris, Thomas & Morgan, 1987); vascular smooth muscle cells (Laragh, 1986), adrenal cortex (Antures-Rodriguez, McCann, Rogers & Samson, 1985) and dipsogenic receptors in higher neural brain centres (Nakamura, *et al.*, 1985). Generally the RAS promotes antinatriuretic, vasopressor and water conserving mechanisms while ANP promotes diuretic, natriuretic and vasodilator mechanisms (Struthers, 1986). Direct inhibition of RAS by ANP was observed in mammals (Burnett, Granger & Opgenorth, 1984) an effect apparently mediated by intracellular cGMP (Kurtz, Della Bruna, Pfeilschifter, Taughner & Bauer, 1986).

In Pekin ducks ANP inhibited the RAS only in animals where the RAS activity was previously stimulated by dehydration (Gray *et al.*, 1991). Basal plasma renin concentration in the fresh water turtle, *Amyda japonica*, was not altered by mammalian ANP (Cho *et al.*, 1988) unfortunately possible inhibition of stimulated renin activity was not measured. Antagonistic effects between AII and ANP were also observed in isolated toad skin where ANP inhibited AII stimulated short circuit current (Coviello *et al.*, 1989). The antidiuretic effect of AII was also inhibited by ANP administration in the isolated perifused kidney of *O. mykiss* (Dunne & Rankin, 1992).

1.2e.v Interaction of natriuretic peptides with other osmoregulatory hormones.

ANP binding sites have been illustrated in the adrenal gland of the duck, *A. platyrhynchos* (Schutz, *et al.*, 1992) and furthermore administration of ANP *in vivo* caused a decrease in plasma aldosterone levels but had no effect on corticosterone levels (Gray *et al.*, 1991). Mammalian ANP was also found to have a direct effect on aldosterone production in turtles (Cho *et al.*, 1988), and inhibited AII and ACTH stimulated steroidogenesis in perifused frog interrenal slices (Lihrman, Netchitailo, Feulloley, Cantin, Delarue, Leboulenger, deLean & Vaudry, 1988). Interestingly ANP has been shown to increase cortisol secretion *in vivo* and *in vitro* in sea water adapted flounders, but not in fresh water adapted fish (Arnold-Reed & Balment, 1994). ANP was also found to stimulate cortisol release from *in vitro* perifused adrenal tissue from the fresh water carp, *Cyprinus carpio* (Kloas, Reinecke & Hanke, 1994).

In conclusion, natriuretic peptides appear to exert similar biological effects in all vertebrates investigated to date. There is however confusion of the role of ANP in euryhaline teleost osmoregulation. There is the possibility that ANP in euryhaline teleosts could induce a rapid response in gill tissue to aid sodium extrusion and also induce cortisol secretion for the long term adaptation to a hyperosmotic medium.

1.3 Other hormones involved in osmoregulation.

The thyroid gland secretes two principle hormonal effectors, L-thyroxine (T_4) and L-triiodothyronine (T_3) in response to a variety of physiological changes. However, a specific osmoregulatory role for these hormones in vertebrates is not yet established. In amphibians both T_3 and T_4 increase sodium transport across the skin and urinary bladder (Gorbman, Dickhoff, Vigna, Clark & Ralph, 1983). In euryhaline teleosts T_3 and T_4 are thought to be vital in the hormonal development of sea water osmoregulatory mechanisms (Barron, 1986). A key hormonal event in the transformation of migratory salmonids from parr to smolt is a peak in T_3 and T_4 which declines to basal levels post-migration (Dickhoff, Folmar & Gorbman, 1978).

The caudal neurosecretory system (NS) has been identified in the spinal columns of teleosts (as a dense highly vascularised nervous plexus in the tail known as the urophysis) and elasmobranchs (where the aggregation is more diffuse and forms a neurohaemal area in the tail). The two biologically active components so far isolated and characterised from the caudal NS of teleosts and elasmobranchs are, the CRF-like peptide urotensin I (UI) and the much smaller somatostatin-like peptide urotensin II (UII). Recently, UI content in the urophysis has been shown to increase in the sea water adapted flounder, *P. flesus* (Arnold-Reed, Balment, McCrohan & Hackney, 1991). Furthermore, perifusion of interrenal tissue of the sea water adapted trout, *O. mykiss*, with a combination of UI and ACTH stimulated cortisol secretion rates 100-200% greater than perifuson of UI or ACTH alone (Arnold-Reed & Balment, 1994). Urotensin II has been shown to stimulate sodium chloride and water absorption in the intestine of fresh water fish (Loretz, 1983) and to inhibit active chloride efflux from secretory surfaces such as the skin and operculum in fishes which facilitates fresh water adaptation (Larson & Bern, 1987). It could be postulated that UI acts as a sea water adapting hormone and UII a fresh water adapting hormone (Loretz & Bern, 1981). However, further evidence is required to prove this, particularly as UII appears to inhibit prolactin secretion in fresh water tilapia (Grau, Nishioka & Bern, 1982).

Adrenaline (epinephrine) and noradrenaline (norephinephrine) are the catecholamines released into the blood stream from chromaffin tissue in all vertebrates. The two hormones exert similar physiological functions throughout the vertebrate phyla. The effects of catecholamines are very general and include; stimulation of the heart, changes in vascular smooth muscle contraction, changes in ion transport across some epithelia and stimulation/inhibition of glandular tissue (Bentley, 1987).

1.4 Osmoregulation in Elasmobranchii.

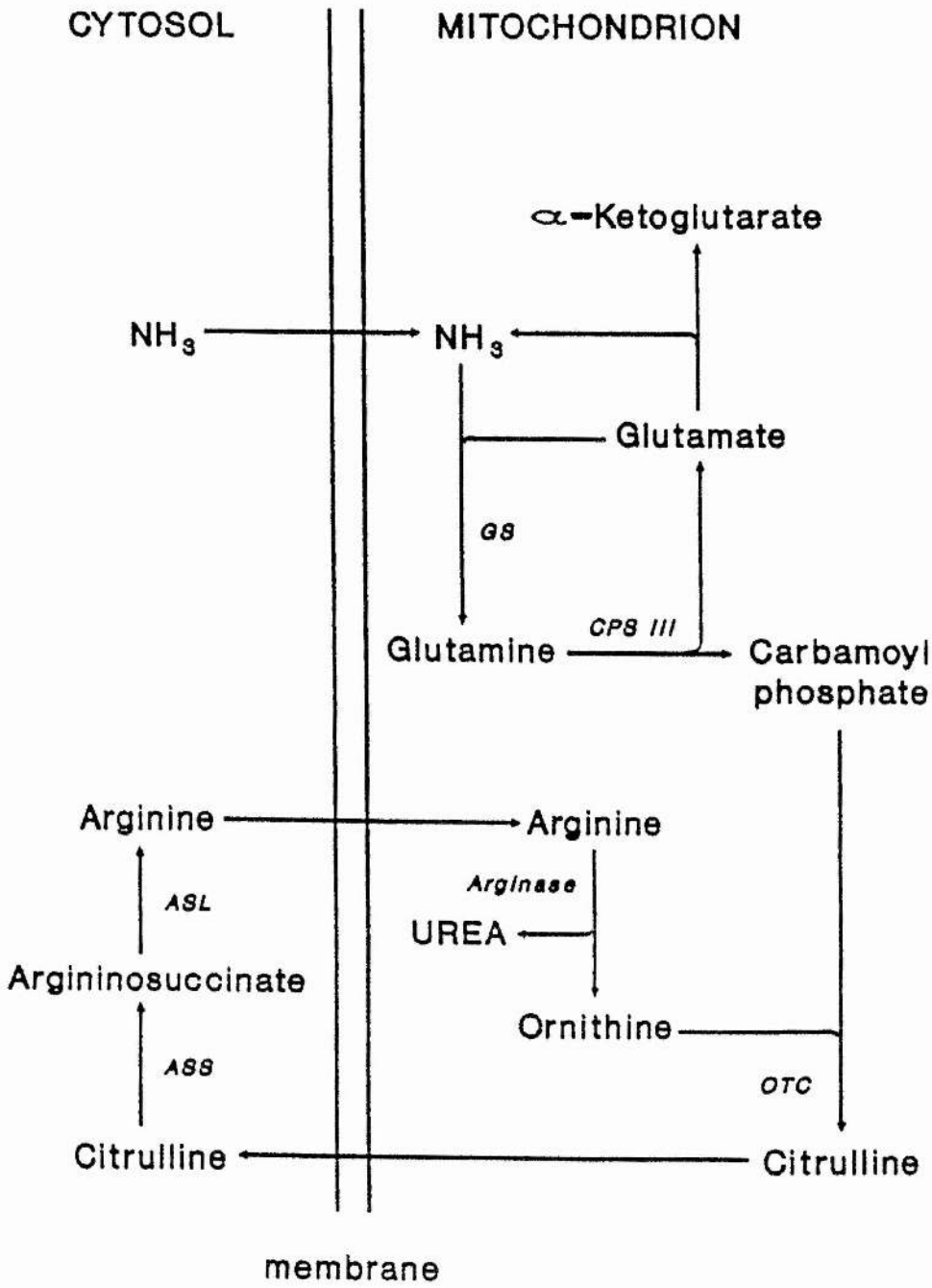
The elasmobranchs and holocephalons are the two subclasses of Chondrichthyes. Elasmobranchs can be separated into two distinct groups; the selachii, which include sharks and dogfish; and the batodei, which include skates and rays. There are far fewer species of holocephalons than the elasmobranchs, with ratfish and rabbitfish the most widely recognised. Consequently research on holocephalons has not been extensive. The two subclasses do have common characteristics such as: a cartilaginous skeleton, unusual endocrine profiles, and a high concentration of urea in the plasma. This introduction will be concerned only with the osmoregulatory processes of elasmobranch fish.

Marine elasmobranchs maintain their plasma slightly hyperosmotic to the surrounding medium (Smith 1936). This is achieved by the retention of urea in high concentrations, which constitutes approximately 35 % of the total plasma osmolality. Therefore, nitrogenous compounds produced from protein catabolism, which are considered waste products in higher vertebrates, are in fact retained by the elasmobranchs. Ammonia accounts for at least 90% of the total nitrogenous waste from teleosts, whereas only 10-30% of nitrogenous waste is excreted as ammonia in elasmobranchs (Smith, 1929). However, reference to ammonia as waste in elasmobranchs is perhaps not justifiable as this compounds is required for the formation of urea in the hepatic ornithine urea cycle (Figure 1.5) (Anderson & Casey, 1984). Despite the requirement of the elasmobranch to retain urea there is still a measurable loss of urea and ammonia through renal and gill tissue. This is not surprising as the chemical concentration gradient of these compounds across semipermeable membranes from plasma to sea water is very high (Table 1. 7).

The toxic effect of urea in higher vertebrates is due to strong perturbing effects on the structure and function of proteins and enzymes (Yancey & Somero, 1978; Yancey, Clark, Hand, Bowlus & Somero, 1982). Interestingly however some elasmobranch proteins, like elasmobranch haemoglobin (Martin, Bonaventura, Fyhn, Fyhn, Garlick & Powers, 1979) have adapted to this perturbing effect of urea, and some even require the presence of urea for optimal structure and function, for example M_4 lactate dehydrogenase (Yancey & Somero, 1978). However many elasmobranch enzymes do not function optimally in the presence of urea and these enzymes rely on the additional presence of methylamine compounds to counteract the disrupting effects of urea (Yancey & Somero, 1980). Methylamines have

FIGURE 1.5

Figure 1.5 Summary of the principle steps of the elasmobranch hepatic ornithine urea cycle.
(Adapted from Anderson & Casey, 1984).


CYTOSOL
MITOCHONDRION
α-Ketoglutarate
NH3
NH3
Glutamate
GS
CPS III
Glutamine
Carbamoyl phosphate
Arginine
Arginine
Arginase
ASL
UREA
Argininosuccinate
Ornithine
ASS
OTC
Citrulline
Citrulline
membrane

been reported to counteract the perturbing effects of urea on sensitive enzymes optimally at intracellular concentration ratios of 2:1 urea:methylamines (Yancey & Somero, 1980). Trimethylamine oxide (TMAO) constitutes approximately 90 % of all the methylamine compounds (Vyncke, 1970) found in elasmobranch plasma and TMAO is maintained within a narrow range of 60-80 mmol/L (Cohen, Krup & Chidsey, 1958). Furthermore TMAO excretion rates were reported to be similar to those of urea (Goldstein & Palatt, 1974). It was postulated that methylamines were selected as osmolytes due to their stabilising ability on urea sensitive enzymes (Yancey & Somero, 1980).

Free amino acids are a third organic osmolyte utilised by elasmobranchs and are used to regulate intracellular osmolality (Perlman & Goldstein, 1988). Urea and TMAO are both freely diffusable between intracellular and extracellular fluid (Fenstermacher, Sheldon, Ratner & Roomet, 1972). Consequently these molecules are in equilibrium between intracellular and extracellular compartments. However, an investigation of free amino acid concentrations in the intracellular and extracellular compartments of the little skate, *Raja erinacea*, demonstrated that free amino acids constituted 20% and 1% of the total osmolyte concentrations of these two fluids respectively (King & Goldstein, 1983). Furthermore, adaptation to dilute sea water led to a marked decrease in intracellular concentration of free amino acids, suggesting some form of adaptive regulation (Forster & Goldstein, 1976; Goldstein, 1981). More recently it has been shown that erythrocytes isolated from the skate swell in a hyptonic medium and then reduce their volume by releasing the beta-amino acids such as taurine and beta-alanine via specific channels. This process is regarded as regulatory volume decrease (RVD) in the cell (Haynes & Goldstein, 1993). It appears therefore that free amino acids are concerned with intracellular osmolality, and methylamines act as functional osmolytes, in that their primary role is counteracting urea toxicity. Urea however, is present primarily as an osmolyte with no particular function other than maintaining an increased plasma osmolality above that of the surrounding medium.

Upon gradual transferral to 50 % sea water the little skate, *R. erinacea*, and the lip shark, *Hemiscyllium plagiosum*, show a decrease in plasma urea, which was attributed to a reduction in urea biosynthesis (Forster & Goldstein, 1976; Wong & Chan, 1977). A detailed study on the European lesser spotted dogfish, *Scyliorhinus canicula*, was carried out by Hazon and Henderson (1984) where plasma urea, sodium and chloride levels were all shown to

decrease following gradual transferral of *S. canicula* to reduced salinity. Plasma urea reduction was attributed to a decrease in blood production rate of urea and a concomitant increase in plasma clearance rates of urea. Interestingly an increase in plasma urea, attributed to a decrease in plasma clearance rates, was observed following transferral of *S. canicula* to 140 % seawater (Hazon & Henderson, 1984). These studies suggest that regulation of plasma urea is a critical factor in the overall homeostasis of elasmobranch plasma.

Plasma levels of sodium and chloride in elasmobranchs are typically between 280 and 300 mmol/L whereas seawater sodium and chloride levels are in excess of 450 mmol/L. Therefore while the marine elasmobranch faces a passive efflux of urea and TMAO, there is also a chemical gradient that favours the influx of sodium and chloride ions (the principle ions in sea water) (Table 1.7). This salt loading is presumably greatest during feeding when the ionic content of the food (principally soft bodied invertebrates for *S. canicula*) plus the inevitable intake of seawater adds to the salt influx, and results in a grossly increased influx of sodium and chloride ions. Typically *S.canicula* tend to gorge food taking in large quantities over a short period of time following periods of starvation. It is possible that in the wild these fish are faced with periods of starvation and therefore the salt load associated with feeding would be intermittent. *S. canicula* must therefore regulate salt and water balance in the face of acute sodium and chloride challenges associated with large changes in dietary intake on an intermittent basis.

Elasmobranchs are equipped with four tissue types directly involved with the regulation of plasma salt and water balance.

1) Renal tissue, as in every other vertebrate class is closely associated with the osmoregulatory control of blood plasma.

2) Gill epithelia, which is in direct contact with the external medium.

3) Elasmobranchs are equipped with a highly specialised salt secreting organ, known as the rectal gland, which excretes an isosmotic solution almost entirely composed of sodium chloride (Burger & Hess, 1960).

4) Gut epithelia, which is in direct contact with ingested fluid and food.

1.4a Gills.

The permeability coefficient for urea in elasmobranch gill epithelia has been reported as one of the lowest in the animal kingdom (Boylan, 1967). The processes involved in creating such an impermeability have not been

TABLE 1.7

Table 1.7 Principle ionic concentrations of elasmobranch plasma, rectal gland fluid, urine and sea water.
(adapted from Silva, Solomon, & Epstein, 1990).

	PLASMA	URINE	RECTAL GLAND FLUID	SEA WATER
SODIUM (mM)	286	240	510	440
POTASSIUM (mM)	7	2	10	10
CHLORIDE (mM)	296	240	510	490
CALCIUM (mM)	2.6	3	1	10
MAGNESIUM (mM)	3.7	40	1	51
UREA (mM)	350	100	10 - 20	0
OSMOLALITY (mOsmols / kg)	1018	800	1018	975 - 1000

extensively studied. To date some sort of "physical barrier" is considered to minimise urea loss across gill epithelia. Nonetheless, despite such an apparently efficient barrier urea loss is still greatest across gill tissue in elasmobranchs (Shuttleworth, 1988), due to the large concentration gradient between plasma and sea water (Smith, 1936).

The elasmobranch gill epithelium has been much less studied with respect to movement of sodium and chloride ions than teleost gill epithelia (Section 1.1a). There is however a large inward concentration gradient for sodium and chloride ions between sea water and elasmobranch plasma. The absence of a substantial electropotential gradient across gill epithelia (Bentley, Maetz & Payan, 1976) led researchers to postulate that elasmobranchs were subject to a constant diffusional uptake of sodium and chloride ions across the gill epithelia (Maetz & Lahlou, 1966; Payan & Maetz, 1973).

Bentley *et al.* (1976) reported active sodium influx across the elasmobranch gill epithelia; while this may seem paradoxical it may be related to acid base balance in elasmobranchs. There are three possible components for the restoration of acid-base balance in the elasmobranch; increased H^+ excretion by kidney and/or gill and passive intervention of blood buffers. Murdaugh and Robin (1967) reported that the buffering capacity of elasmobranch plasma was extremely poor, and the role of the kidney in elasmobranch acid-base regulation was reported as negligible (Cross, Packer, Linta, Murdaugh & Robin, 1969). Consequently, the elasmobranch gill is considered as the primary site for acid-base regulation in elasmobranchs. An active component of sodium influx was proposed where the influx of sodium was coupled to the efflux of H^+ as part of the exchange mechanism regulating acid-base balance (Payan & Maetz, 1973). The active component of this may be related to "facilitated diffusion" involving H^+ and bicarbonate ions (HCO_3^-). A further sodium exchange system with regards to acid-base balance in gill epithelia was postulated by Payan and Maetz (1973) where sodium influx was coupled to ammonium (NH_4^+) efflux. Ammonia is constantly being lost through the gill epithelium so for the elasmobranch to actively extrude this required nitrogenous by-product may appear inappropriate. However, the lack of an efficient buffer system and the possibility of restoring acid-base balance quickly could outweigh the loss of nitrogen through the sodium/ammonium (Na^+/NH_4^+) exchanger in the face of for example, severe hypercapnia (excess blood CO2) (Caliborne & Evans, 1992). Bentley *et al.* (1976) demonstrated that chloride ion influx was passive. However, regulation of chloride efflux has been demonstrated through a chloride/bicarbonate (Cl^-/HCO_3^-) exchange

in elasmobranch gill epithelium where chloride efflux is coupled to bicarbonate influx during periods of hypercapnia (Randall, Heisler & Drees, 1976).

The presence of chloride cells in elasmobranch gill epithelia has also been demonstrated (Doyle & Gorecki, 1961; Wright, 1973). However, elasmobranch branchial Na^+K^+ATPase activity was reported as being 10-15 times below that found in teleosts (Jampol & Epstein, 1970). A specific role for chloride cells in elasmobranch branchial epithelium has yet to be determined (Laurent & Dunel, 1980).

It can be concluded that elasmobranch gill epithelium is the site of greatest urea loss to the external medium despite the apparent "physical barrier" to passive urea diffusion. Furthermore, elasmobranch gill epithelia does not appear to have the capability of producing a greater efflux of sodium and chloride ions than influx. Therefore efficient regulation of sodium and chloride post-feeding through gill epithelia alone is unlikely.

1.4b Kidney.

The kidneys in *S. canicula* are found embedded in the dorsal side of the abdominal cavity as paired elongate organs either side of the dorsal aorta, which eventually fuse below the cloaca. The spiny dogfish, *Squalus acanthias*, has a similar arrangement of kidneys, however the little skate, *R. erinacea*, has a paired lobular kidney with each lobule supplied by a single artery (Deetjan & Antkowiak, 1970). As in most non-mammalian vertebrates elasmobranchs possess a renal portal system. Portal veins are formed from the bifurication of the large caudal vein, and on entry into the kidney divide and anastomose to form a matrix of small vessels. Portal blood mixes freely with blood from the glomerular vasa efferentia before leaving the kidney via the renal veins.

The functional element of elasmobranch renal tissue, the nephron, is a very long and complex tubular system (Hentschel, Mahler, Herter & Elger, 1993; Lacy & Reale, 1985 a & b, 1991 a & b; Stolte, Galaske, Eisenbach, Lechene, Schmidt-Nielsen & Boylan, 1977). The length and complexity of the nephron has undoubtedly contributed to the lack of research into the ultrastructure and mechanisms of tubular epithelia transport in the elasmobranch kidney. The first full ultrastructural analysis of the nephron in elasmobranch fish has only been recently completed on the little skate *R.*

erinacea (Lacy & Reale, 1991 a & b), and *S. canicula* (Hentschel *et al.*, 1993) and provides strong evidence for a countercurrent system involving highly specialised and diverse epithelial transport.

There are two distinct regions of renal tissue in *R. erinacea* and *S. canicula*. 1) A dorsal "bundle" region that is enveloped by a urea impermeable sheath (Figure 1.6) where it is thought that countercurrent exchange occurs (Stolte, *et al.*, 1977); and 2) A ventral "sinus" region which exits from the bundle sheath to form two more distinct loops and a further possible site for countercurrent exchange (Figure 1.6) (Lacy & Reale 1991b). The nephron begins at the urinary pole of the renal corpuscle with a neck segment followed by proximal, intermediate, and distal segments and finally a collecting duct. Each of these segments is further divided into subdivisions based on epithelial morphological differences (Figure 1.6). Epithelial differences within these groups include, presence of flagella, tight or gap junctions, brush borders, mitochondrial density, basolateral invagination and tubular dimension and rigidity. Lacy and Reale (1985 a & b, 1991 a & b) carried out detailed structural analysis of the nephron in the little skate, *R. erinacea*, where 16 morphologically different segments were assigned to the nephron tubule. However for the purpose of this discussion reference shall be made to the less complex model of the *S. canicula* nephron produced by Hentschel *et al.* (1993). The legend accompanying figure 1.6 provides a more detailed description of the epithelial cells lining the tubule.

Elasmobranchs produce a urine that is typically hypo-osmotic to blood plasma. The heterogeneity of tubular epithelial cells may be an adaptation for the retention of urea which is in marked contrast to tubule cells in higher vertebrates, functioning primarily in salt transport mechanisms. Metabolic inhibitors such as phloretin and chromate have been shown to inhibit urea reabsorption, indicating active urea transport in the elasmobranch nephron (Hays, Levine, Myer, Heinemann, Kaplan, Franki & Berliner, 1977). Boylan (1967) demonstrated that only 35% of thiourea (a urea analogue) was reabsorbed in the elasmobranch nephron indicating extreme specificity for urea reabsorption. This led Forster (1970) to suggest an active urea reabsorption mechanism, and micropuncture studies have implicated the second proximal segment (PII) (Figure 1.6) as a possible site of sodium-linked urea reabsorption (Stolte *et al.*, 1977). However, recently a tentative model for passive urea reabsorption was proposed by (Freidman & Hebert, 1990; Hebert & Freidman, 1990) based on the model put forward by Boylan (1972) and, Hentschel, Elger and Schmidt-Nielsen (1986). Their model required; 1) a

relatively proximal segment exhibiting high osmotic water permeability; 2) a relatively distal segment capable of high rates of active salt transport but effectively impermeable to water and urea; 3) a loop through the sinus zone having high water but low urea permeability, thereby permitting osmotic equilibration of the water leading to increased tubular concentration of urea; 4) a relatively terminal tubular segment within the bundle zone exhibiting high urea but low osmotic water permeability, thereby permitting passive diffusion of urea from tubular fluid to interstitium within the bundle zone. This model was however based on little experimental evidence particularly for some of the tubular permeabilities proposed. Detailed analysis of urea permeabilities, and sites of transport along the elasmobranch nephron, is therefore required to ascertain whether urea reabsorption is either active, passive or both.

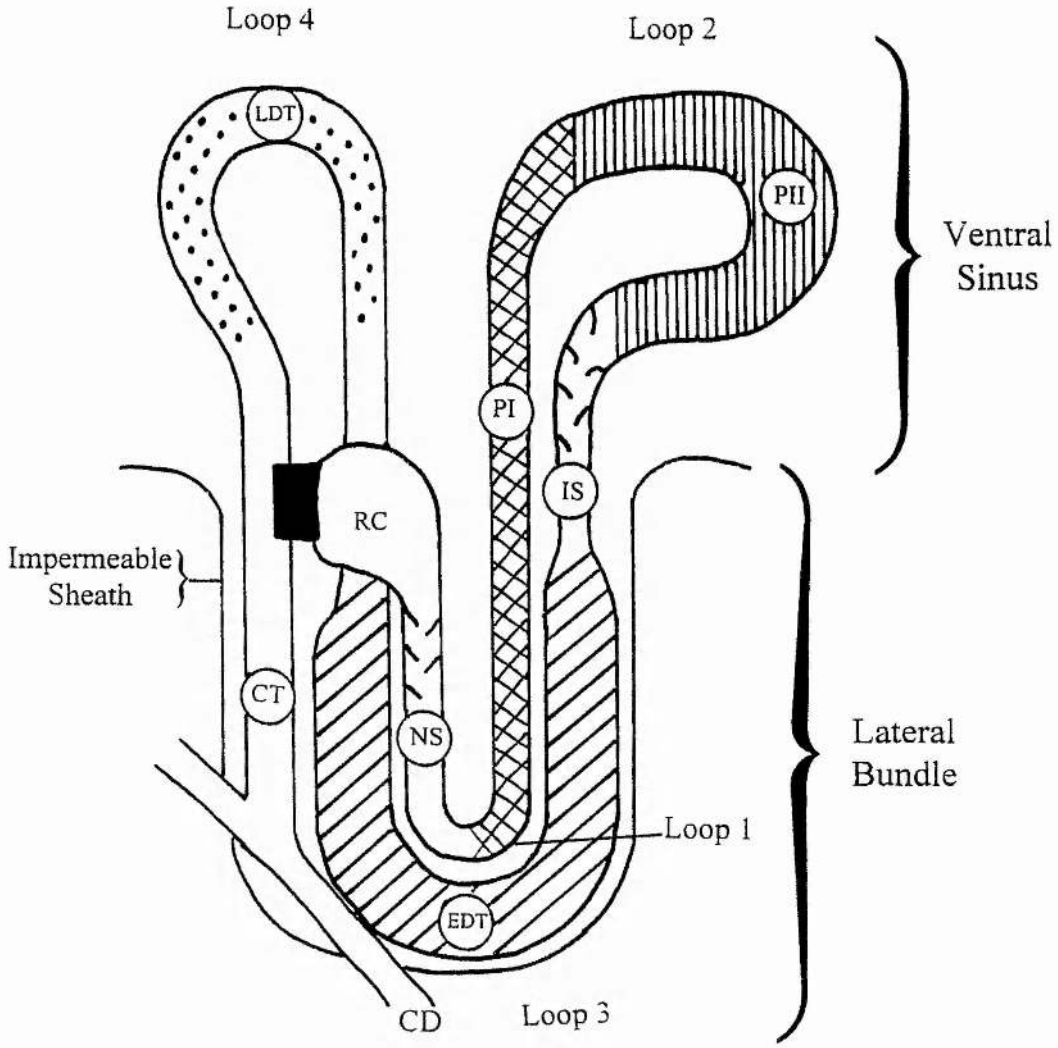
Urea transport in the elasmobranch kidney may certainly assist in regulation of urea plasma concentrations however the kidney also plays a role in the regulation of sodium and chloride plasma ion concentration. Ultrastructural studies have demonstrated that tubular cells in the early distal tubule (EDT) (Figure 1.6) have characteristics very similar to cells that are known to actively transport sodium (Lacy & Reale, 1991b). Furthermore, Freidman and Hebert (1990), and Hebert and Freidman (1990) demonstrated that loop 3 in the EDT exhibits similar characteristics of active sodium and chloride absorption associated with a renal diluting segment in mammals and amphibians. Reversible inhibition of sodium and chloride absorption with loop diuretics such as furosemide, low trans-epithelial resistance ("leakiness"), and negligible water permeability have also been shown (Figure 1.6). In addition the presence of an ouabain sensitive Na^+K^+ATPase system was demonstrated (Hebert & Freidman, 1990).

Active sodium reabsorption has also been demonstrated in elasmobranch nephrons (Boylan, 1967; Stolte *et al.*, 1977) utilising renal micropuncture, which implicated loop 2 in the second proximal region (PII) (Figure 1.6) of *R. erinacea* to be the site of both sodium and chloride reabsorption (Stolte *et al.*, 1977). Furthermore isolated perfused PII tubules from *S. acanthias* have also been shown to actively secrete sodium and chloride ions (Beyenbach & Fromter, 1985) and this appears to drive net fluid secretion (Sawyer, Cliff, Wilhelm, Fromter & Beyenbach, 1985 a & b). What must be remembered is that the ultrastructural evidence for active tubular reabsorption of sodium and chloride ions in the PII segment (Lacy, Schmidt-Nielsen, Galaske & Stolte, 1975; Endo 1984) can also imply active tubular secretion of sodium and chloride ions (Henderson, O'Toole & Hazon, 1988).

FIGURE 1.6

Figure 1.6 Schematic of a single nephron from the elasmobranch, *Scyliorhinus canicula*. Adapted from Hentschel *et al.*, (1993).

As in the majority of vertebrate nephrons studied to date, the elasmobranch nephron consists of both proximal and distal segments. The tubule begins at the renal corpuscle (RC), has a short neck segment (NS) which is lined with epithelial cells possessing long flagellar ribbons, possibly for lumen fluid propulsion. Following the neck segment is the proximal segment which is split into two subdivisions, PI and PII. PI epithelial cells demonstrate morphological features which correlate to tubular reabsorption. PII epithelial cells exhibit dense basolateral invaginations and considerable numbers of large mitochondria indicating active epithelial transport. An intermediate segment (IS) separates the proximal from the distal segments. In *R. erinacea* this segment has been interpreted as being the longest portion of the tubule (Lacy & Reale, 1991b). However, the intermediate section of the nephron in *S. canicula* is considered to be relatively shorter (Hentschel *et al.*, 1993). The distal segment of the nephron has been separated into an early distal tubule (EDT) and a late distal tubule (LDT). EDT epithelial cells have a high concentration of Na^+K^+ATPase localised on the basolateral membranes, a morphological characteristic similar to salt secreting epithelia. The morphology of LDT epithelial cells is strikingly different to those lining the EDT. The final part of the nephron tubule is the collecting tubule (CT) leading to the collecting duct (CD). It is at these segments that passive urea reabsorption is thought to occur.


Loop 4
Loop 2
LDT
PII
Ventral
Sinus
PI
IS
RC
Impermeable
Sheath
CT
NS
Lateral
Bundle
Loop 1
EDT
CD
Loop 3

However histochemical studies have only isolated Na^+K^+ATPase activity (an enzyme closely related to active salt secretion/absorption) in the early and late distal tubules (Figure 1.6) and the collecting duct (Endo 1984; Hebert & Freidman 1990). Closer scrutiny as to the positions of the various salt transporters with relation to apical or basolateral membranes is required to determine whether the tubular epithelia in the PII segment is reabsorbing or secreting.

In conclusion, the elasmobranch nephron appears to be highly complex which could possibly be attributed to urea retention, and the kidney does not appear to be capable of excreting a hyperosmotic urine with respect to blood plasma.

1.4c Rectal Gland.

The elasmobranch rectal gland is a small digitiform gland found at the posterior end of the abdominal cavity (Chapter 2). The gland consists of salt secreting epithelia (Chapter 3) and excretes a fluid that is isomotic to plasma but is almost entirely composed of sodium chloride (Table 1.7). As study of the control of rectal gland secretion forms the major part of this thesis the mechanisms involved will be further discussed in chapters 4-6.

1.4d Gut.

The elasmobranch gut is densely innervated and many neuropeptides involved in controlling gut motility in mammals have been identified along the intestinal tract of the spiny dogfish *S. acanthias* (Holmgren & Nilsson, 1983). However there is no reported evidence in the literature of the elasmobranch gut playing an osmoregulatory role. This is perhaps due to the fact that there is apparent lack of drinking in elasmobranchs (Smith 1936). However, pharmacological manipulation of the endogenous renin angiotensin system in elasmobranchs was shown to induce drinking (Hazon *et al.*, 1989). In view of this, it is therefore possible that the gut in elasmobranchs plays a more significant role in osmoregulation than first thought.

For efficient osmoregulation of plasma sodium and chloride levels, particularly post-feeding, it appears that the elasmobranch requires the combined action gills, kidney and rectal gland. Such integrated function would undoubtedly require hormone receptor mediated control. Endogenous hormone/receptor systems are only just being identified in elasmobranch fish,

hence our knowledge of the control of osmoregulation in elasmobranchs is limited. A brief overview of our current understanding of hormonal control of osmoregulation in elasmobranchs now follows.

1.5 Adenohypophysial peptides involved in elasmobranch osmoregulation.

The presence of prolactin in the elasmobranch pituitary has been demonstrated by cytological (Della Corte & Chieffi, 1961) and immunological studies (Lewis, Singh, Seavey, Lasker & Pickford, 1972). Replacement therapy experiments have provided our limited knowledge of the role prolactin could play in elasmobranch osmoregulation. Removal of the rostral lobe of the pituitary (site of prolactin production) in *Dasyatis sabina*, increased plasma urea and sodium concentrations which were reversed following prolactin injection (de Vlaming, Sage & Beitz, 1975). Hypophysectomised *S. canicula* showed a 50 % decrease in branchial water permeability which was also reversed following prolactin administration (Payan & Maetz, 1970)

Presence of growth hormone in the elasmobranch pituitary has been demonstrated by immunological (Lewis *et al.*, 1972) and cytochemical studies (Dela Corte & Chieffi, 1961) and the amino acid sequence has since been reported for the blue shark, *Prionace glauca* (Yamaguchi, Yasuda, Lewis, Yokoa & Kawauchi, 1989). To date there has been no reported work on the effect of growth hormone on elasmobranch osmoregulation.

1.6 Neurohypophysial peptides involved in elasmobranch osmoregulation.

Elasmobranchs are particular in that they possess an unusual range of neutral neurohypophysial hormones. There have been five oxytocin-like hormones identified in sharks, skates and rays. Valitocin and aspartocin have been identified in *S. acanthias* (Acher, Chauvet & Chauvet, 1972), asvatocin and phasvatocin in *S. canicula* (Acher, Chauvet, Chauvet & Rouille, 1992), and glumitocin in the ray, *Raja clavata* (Acher, Chauvet, Chauvet & Crepy, 1965) (Table 1.1). Neutral neurohypophysial hormones in elasmobranchs therefore appear to be very species specific, the reason for this and their physiological roles is not understood. However, the basic neurohypophysial hormone arginine vasotocin (AVT) has been reported in all species of elasmobranch investigated to date (Table 1.1).

Little is known about the biological functions of AVT in elasmobranchs, although the ability of elasmobranchs to alter renal tubular water permeability (Henderson *et al.*, 1988) suggests that an antidiuretic-like

factor may be present. However, physiological characterisation of endogenous elasmobranch AVT as an anti-diuretic factor has never been investigated.

1.7 Corticosteroids involved in elasmobranch osmoregulation.

The elasmobranch interrenal gland (adrenocortical tissue) lies dorsally between the two posterior lobes of the kidney. The chromaffin (catecholamine secreting) tissue lies along the inner borders of the dorsal kidney surface in discrete islets, close to but distinct from, the adrenocortical tissue (Chester Jones & Mosely, 1980). Elasmobranch plasma concentrations of corticosterone, 11-deoxyhydrocorticosterone and cortisol are very low (Kime, 1977) if not absent (Hazon & Henderson, 1984). Idler and Truscott (1966) identified a novel adrenocorticosteroid circulating in the plasma of the skate, *Raja rhina*. It is an hydroxylated derivative of corticosterone, 1α hydroxycorticosterone (1αOH-B) (Table 1.2). The enzyme producing this derivative has been reported as inactive at 37°C, therefore 1αOH-B is not normally found in mammalian cells. Interestingly, following hypophysectomy and thus removal of the ACTH stimulus, elasmobranchs have been reported to survive for at least 12 months (Hazon and Henderson, 1984). This is a surprising anomaly as most other vertebrate classes survive for only a few days following such an operation. There must therefore be other powerful stimulants, in addition to ACTH, which induce interrenal gland secretion of corticosteroids. Angiotensin II (AII) analogues [Val^5] and [Ile^5] were found to stimulate 1αOH-B secretion from the isolated perifused interrenal gland of *S. canicula*, with the [Ile^5] analogue being the more potent (Hazon & Henderson, 1985; Armour, O'Toole & Hazon, 1993a). Furthermore atrial natriuretic peptide was also seen to produce a similar increase in secretion of 1αOH-B from the isolated perifused interrenal gland of *S. canicula* (Hazon, Decourt, O'Toole, Lahlou & Henderson, 1987).

Receptors for 1αOH-B have been demonstrated in the gills, liver, kidney and rectal gland of the skate, *Raja ocellata* (Burton & Idler 1986). More specifically the majority of 1αOH-B binding sites have been associated with the limited number of chloride cells in the gills of *R. ocellata* and to a proximal portion of the kidney tubule in *R. ocellata* (Burton & Idler, 1986). Inter-renalectomy of *D. sabina* was reported to reduce plasma sodium, chloride, calcium and urea (de Vlaming *et al.*, 1975). However this study had no true control (i.e.. sham operated fish) so the validity of the results is in question.

There is evidence to support mineralocorticoid activity of 1αOH-B in elasmobranchs. Application of 1αOH-B to the isolated toad skin produced a sodium flux that was 80% of that produced by aldosterone (Grimm, O'Halloran & Idler, 1969) but produced no glucocorticoid activity in adrenalectomised mice (Idler, Freeman & Truscott, 1967). Hazon and Henderson (1984) adapted *S. canicula* to a range of salinities and monitored plasma urea and electrolyte concentration, and 1αOH-B metabolic clearance rate and blood production rate. In 50% sea water plasma urea and electrolyte concentrations fell. However, plasma concentration, metabolic clearance rate and blood production rate of 1αOH-B increased. It was concluded that 1αOH-B was in part, possibly concerned with the regulation of plasma osmolality with particular respect to urea. Dietary restriction has been reported to reduce hepatic production rate and plasma levels of urea (Armour, 1990). Utilising this metabolic manipulation (Armour, O'Toole, & Hazon, 1993b) demonstrated that *S. canicula* with restricted protein intake, adapted to 130% sea water, had equivalent plasma urea concentrations to 100% adapted fish, but had significantly increased plasma sodium and chloride concentrations. Concomitant to this was a greatly increased level of plasma 1αOH-B. Such an increase in plasma 1αOH-B was not observed in high protein diet fish adapted to 130% sea water. This perhaps provides evidence that 1αOH-B is in fact primarily concerned with regulating plasma sodium and electrolyte levels in elasmobranchs (Armour *et al.*, 1993b).

1.8 The renin angiotensin system and elasmobranch osmoregulation.

It was postulated that elasmobranchs did not possess a renin angiotensin system (RAS) (Nishimura *et al.*, 1970). However, responses to components of the RAS have been demonstrated in elasmobranchs. Mammalian AI and AII produced a pressor response when injected into *S. acanthias* (Opdyke & Holcombe, 1976), and *S. canicula* (Hazon *et al.*, 1987). Furthermore, the AI response in *S. canicula* was blocked by the angiotensin converting enzyme (ACE) inhibitor captopril (Hazon *et al.*, 1987). In *S. acanthias* the AII-induced vasopressor response was completely blocked by the α adrenergic antagonist phentolamine, which suggests that the pressor response to AII is mediated 100% by catecholamines (Opdyke & Holcombe, 1976). However, these vasopressor effects of AII in *S. acanthias* could not be reproduced by Churchill, Malvin & Churchill (1985). Further evidence for an endogenous RAS in elasmobranchs was produced by Henderson *et al.* (1980) when elasmobranch renal extracts incubated with rat renin substrate generated

an AII-like pressor factor after injection in the rat bioassay, and furthermore the renal extracts also produced a pressor response on their own. Studies utilising papaverine, a potent vasodilator of vascular smooth muscle have provided further support for the presence of a RAS in elasmobranchs. After injection of papaverine *in vivo* mean arterial blood pressure fell and recovered approximately 30 min later (Hazon *et al.*, 1989). However upon addition of captopril (an ACE inhibitor) shortly after administration of papaverine the vasodepressor response lasted for over 2 hours. This was interpreted as papaverine stimulation of the elasmobranch endogenous RAS which could be subsequently inhibited by the ACE inhibitor captopril. A dipsogenic response was also observed in the elasmobranch *S. canicula* following AII and papaverine administration, furthermore captopril blocked the papaverine-induced dipsogenic response (Hazon *et al.*, 1989). In addition to the vascular and dipsogenic action of AII analogues on the elasmobranchs, infusion of renal extracts and heterologous AII markedly increased plasma concentrations of 1αOH-B (Hazon & Henderson, 1985).

Recently renin like activity was displayed in renal extracts of *S. canicula*, where the extracts acted on synthetic and porcine angiotensinogen to produce AI (Masini, Henderson & Ghiani, 1990). However definitive proof of an endogenous elasmobranch RAS was provided by Takei, *et al.* (1993) who sequenced elasmobranch angiotensin I from plasma taken from the elasmobranch *Triakis scyllia* (Takei *et al.*, 1993) (Table 1.3). Furthermore a 20 fold increase in arterial blood pressure, above that produced by rat A I, was seen in *T. scyllia* following injection of the endogenous AI *in vivo*. This vasopressor action of AII was supported as captopril was found to inhibit the endogenous AI-induced increase in arterial pressure of *T. scyllia in vivo*, but had no effect on the AII-induced increase in blood pressure (Hazon, Tierney, Hamano, Ashida, & Takei, 1995 in press).

1.9 Natriuretic peptides and elasmobranch osmoregulation.

A C-type natriuretic peptide was isolated and sequenced from heart extracts of two species of elasmobranch; the European dogfish, *S. canicula* (Suzuki, Takahashi, Hazon & Takei, 1991); and the spiny dogfish *S. acanthias* (Schofield, Jones & Forrest, 1991) (Figure 1.4). In addition CNP has been found in the heart, brain and plasma of the Japanese dogfish, *T. scyllia* (Suzuki, Takahashi & Takei, 1992; Suzuki, Togashi, Ando & Takei, 1994). These data suggest that CNP is the principal natriuretic peptide in elasmobranchs .

Using an antibody raised against human ANP it was found that following volume expansion of the spiny dogfish, plasma ANP levels were significantly elevated (Epstein, Clark, Taylor, Silva, Dick, Silva & Solomon, 1987). This led these researchers to postulate that ANP was released in response to an increase in intravascular volume expansion and therefore was principally concerned with regulating volume expansion in *S. acanthias.* Administration of ANP to sea water adapted *S. acanthias* induced no renal effects (Yokota & Benyajati, 1986), however following injection of ANP to sea water adapted *S. acanthias*, Benyajati and Yokota (1990) demonstrated a marked decrease in GFR, urine flow and total osmolyte excretion. These authors have suggested that the volume status of the shark may modulate the effects of ANP on renal function. However, injection of a similar dose into *S. acanthias* adapted to 70% sea water significantly stimulated chloride excretion (Solomon, Dubey, Silva & Epstein, 1988).

It was found that synthetic mammalian ANP produced a decrease in pressure in the dorsal aorta of the spiny dogfish *S. acanthias* (Benyajati & Yokota, 1990), a response repeated in ventral aortic rings of *S. acanthias* following addition of rat ANP to the bathing medium (Evans, 1991). Following *in vivo* perfusion of *S. canicula* with human ANP, systemic blood pressure decreased markedly, and the same peptide also induced relaxation of isolated arterial rings from the branchial artery of *S. canicula* (Bjenning, *et al.*, 1992). However, this decrease in systemic blood pressure and aterial relaxation was doubled, when the same preparations were perfused with endogenous sCNP (Bjenning *et al.*, 1992). A similar increase in relaxation was observed in ventral aortic rings isolated from *S. acanthias* following addition of endogenous sCNP to the bathing medium (Evans, Toop, Donald, & Forrest, 1993).

In addition mammalian ANP administration to the *in vitro* perifusion of the isolated interrenal gland of *S. canicula* induced a significant increase in 1αOH-B secretion from the gland (Hazon, *et al.*, 1987). This stimulation of corticosteroid production by ANP is consistent with the natriuretic peptide-stimulation of cortisol production in teleosts (Arnold-Reed & Balment, 1991).

1.10 Other peptides involved in elasmobranch osmoregulation.

Very little research has been carried out on the possible osmoregulatory role of thyroid hormones in elasmobranchs. Following thyroidectomy plasma urea levels were seen to rise (de Vlaming *et al.*, 1975).

These levels were subsequently reduced following replacement therapy with thyroxine, thus implicating the thyroid gland in the regulation of plasma urea levels (de Vlaming *et al.*, 1975).

The vast majority of physiological studies involving the role of the urotensins has been carried out in teleosts (Section 1.3) despite the fact that neurosecretory granules were first observed in the skate caudal spinal cord (Speidel, 1919). In the elasmobranch the neurosecretory area is a rather diffuse neurohaemal area on the ventral surface of the posterior spinal cord (Fridberg, 1962) unlike the compact urophysis in the teleosts.

Urotensin II has been isolated from extracts of the caudal spinal column of *S. canicula* (Conlon, O'Harte, Smith, Tonon & Vaudry, 1992) and recently UI has also been isolated and characterised from caudal extracts of *S. canicula* (M. Conlon, pers. comm.). In addition urotensin II activity has been isolated in the brain of the skate *Raja rhina* (Waugh & Conlon, 1993). Elasmobranch UI and UII are considered structurally similar to the teleost urotensins, thus possibly providing evidence for similar biological roles of these peptides.

Cardiovascular effects of UII have been illustrated in *S. canicula*, and following UII injection *in vivo* a sustained and dose dependent increase in mean arterial blood pressure was observed (Hazon, Bjenning & Conlon, 1993). The administration of the α adrenergic blocker, phentolamine completely abolished the UII effect suggesting that the UII effect was at least in part mediated by catecholamines (Hazon *et al.*, 1993). Similar vasopressor effects produced by AII (which are reported to be entirely catecholamine mediated) (Opdyke & Holcombe, 1976) last for 6-8 minutes whereas the UII vasopressor initiated effect lasted for more than 30 minutes. This could perhaps indicate a slow direct action of UII on systemic blood pressure.

The majority of catecholamine production takes place in the chromaffin tissue as described in section 1.3. However in addition to this granular bodies containing catecholamines are found lining arterial vessels throughout the elasmobranch (Holmgren & Nilsson, 1983). The release of these catecholamines following an AII-induced increase in blood pressure implicates catecholamines in the control of blood pressure in elasmobranchs (Hazon *et al.*, 1993). In addition to their possible vascular function catecholamine plasma concentrations increase in response to hypoxia and stress (Butler, Taylor, Capra & Davison, 1978; Opdyke, Carroll & Keller,

1982) and may control gas exchange, either by altering gill blood flow (Davies & Rankin, 1973) or via a direct effect on oxygen permeability of the gill (Isaia, 1984). Osmoregulatory studies of catecholaminergic effects are concerned principally with the kidney. Pharmacological doses of catecholamines have produced conflicting reports on elasmobranch glomerular filtration rate (Deetjan & Boylan, 1968; Forster, Goldstein & Rosen, 1972). However more recently Brown and Green (1987) demonstrated glomerular diuresis following administration of adrenaline. Catecholamines have also been observed in nerve fibres of the rectal gland of *S. acanthias* (Holmgren & Nilsson, 1983), although a particular function of catecholamines in the rectal gland has not yet been reported.

The following chapters will describe the function of the rectal gland of elasmobranchs and the known mechanisms controlling secretion from the rectal gland. Vasculature of the gland will be described in detail and the development of an isolated perfused preparation will also be described. The isolated perfused preparation was the principle tool used to investigate hormonal influences on rectal gland secretion rate of the European lesser spotted dogfish *Scyliorhinus canicula*.

CHAPTER 2
RECTAL GLAND VASCULATURE

2.1 - Introduction

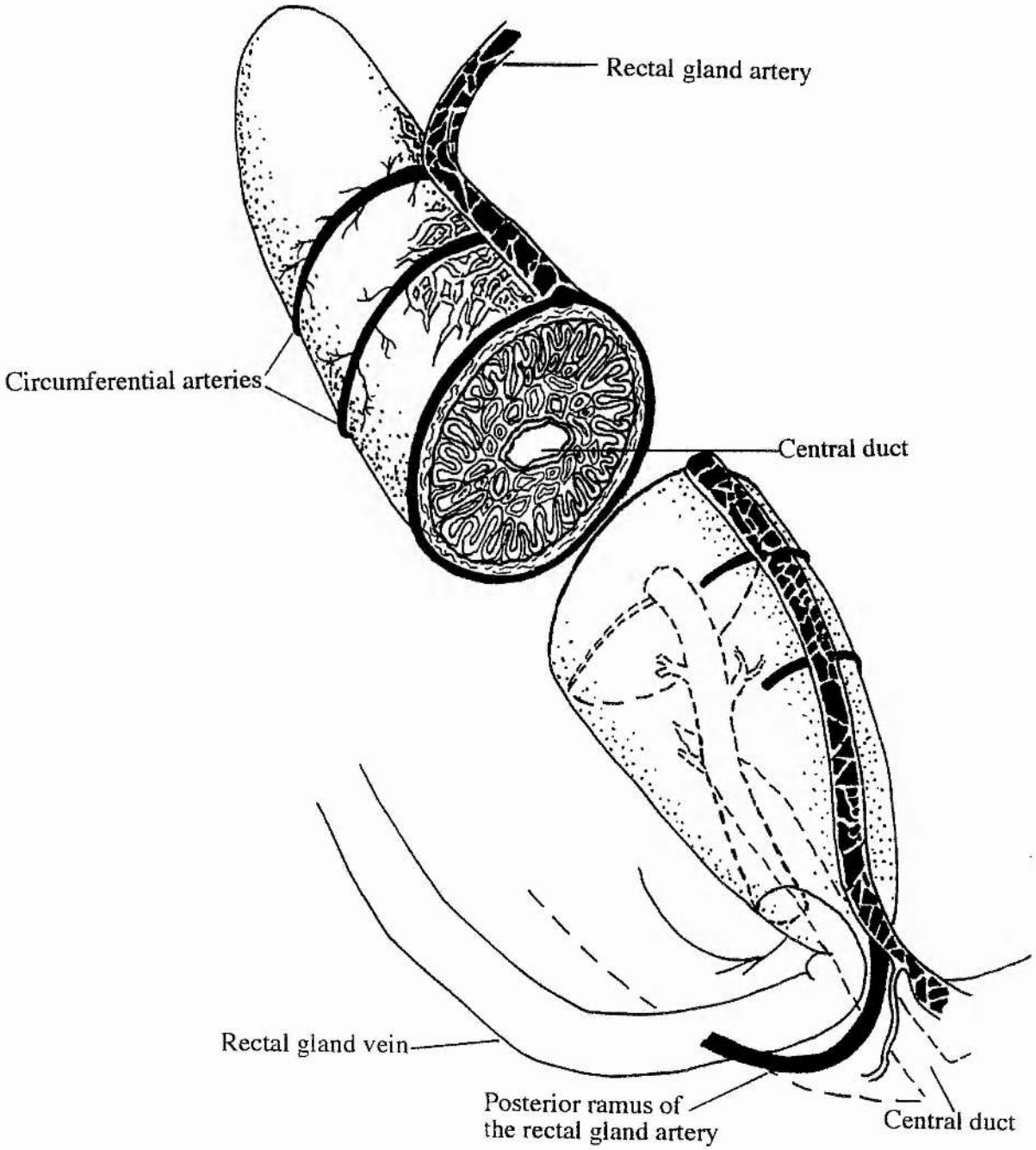
The rectal gland in elasmobranchs is a small digitiform gland situated at the caudal end of the peritoneal cavity and has been shown to secrete a fluid that is isosmotic to blood plasma but is almost entirely composed of sodium chloride (Burger & Hess, 1960). The gland is supplied by a single vessel, the rectal gland artery, which arises from the dorsal aorta. Vascular drainage from the gland is via a central venous sinus which, upon exiting the gland, follows the intestine anteriorally. The central duct of the gland opens into the lower intestine, posterior to the spiral valve and anterior to the rectum. The exiting duct and vein are closely linked, separated only by connective tissue and vessel walls (Figure 2.1). The anatomy of the rectal gland, in terms of blood supply and shape (tapered cylinder), appears to apply to the majority of elasmobranchs species investigated to date (Hoskins, 1917; Doyle, 1962; Bonting, 1966; Haywood, 1974; Kent & Olson, 1982; Shuttleworth, 1988).

The structure of the rectal gland in sharks has been divided into 4 regions: 1) the outer fibro-muscular or capsular area with the principle arterial and venous systems: 2) the narrow sub-capsular layer consisting of smaller arterial and venous vessels, closely connected through arterio-venous anastomoses: 3) a middle glandular layer consisting of radially arranged tubules; and 4) a central region of longitudinally organised ducts arranged around a central canal and venous sinus (Bonting 1966; Kent & Olson, 1982; Masini, Uva, Devecchi & Napoli, 1994) (Plate 2.6).

In order to study the macro- and micro-vasculature and determine possible sites of vascular control in the rectal gland of the European lesser spotted dogfish, *Scyliorhinus canicula*, the technique of corrosion casting was employed (Lametschwandtner, Lametschwandtner & Weiger, 1990), utilising a fast polymerising fluid monomer resin (Mercox CL-2B, Mercox, Japan Vilene, Tokyo, Japan).

FIGURE 2.1

Figure 2.1 Illustration of the principle vascular vessels supplying and draining the secretory epithelia of the rectal gland
Drawn by Dr. T. Beddow, adapted from Kent and Olson, (1982), and Masini *et al*, (1994).


Rectal gland artery
Circumferential arteries
Central duct
Rectal gland vein
Posterior ramus of
the rectal gland artery
Central duct

2.2 - Materials and Methods.

European lesser spotted dogfish, *Scyliorhinus canicula*, of mixed sex and body weight (0.5-1.3 kg) were obtained from waters around Millport on the west coast of Scotland, or from waters around Bangor on the north west coast of Wales. The fish were transported to the Gatty Marine Laboratory aquarium where they were held in 150 L flow-through circular tanks at ambient temperature (3-16°C), under a 12 h light:12 h dark photo period and were not fed. Sacrifice was by a sharp blow to the head followed immediately by transection of the spinal column directly behind the cranium, and destruction of the central nervous system. The abdominal cavity was opened to expose the rectal gland at the posterior end.

Fish were heparinised (approximately 1000 units $500g^{-1}$, with sodium heparin) via the caudal sinus, 1 hour prior to sacrifice, which assisted in preventing blood clotting during the washing phase. Six different specimens of *S. canicula* were used; 2 females and 4 males. Pulled out PE 10 and PE 50 cannula (Portex tubing, Hythe, Kent, England) were prepared previous to fish sacrifice. Approximately 1.5 cm of PE 10 cannula was inserted into a short length of PE 50 cannula (10 cm). This ensured that the narrow bore of the PE 10 cannula was as short as possible. The length of the arterial cannula was important as if it were too long adequate filling of the gland would not occur in the time allowed. The rectal gland artery was cannulated *in situ* as far away from the gland as possible. The gland was then excised, including a large portion of intestine posterior to the spiral valve to ensure that sufficient casting of the rectal gland vein occurred; care was taken not to cut the mesentery too close to the gland as this would provide a site for efflux of the casting media. The preparation was then immediately submerged in, and perfused with elasmobranch Ringer solution (NaCl, 240mM; KCl, 7mM; $CaCl_2$ 10mM; $MgCl_2.6H_2O$, 4.9mM; $NaHCO_3$, 23mM; $Na_2HPO_4.2H_2O$, 0.5mM; Na_2SO_4, 0.5mM; Urea, 360mM; Trimethlyamineoxide (TMAO), 60mM; glucose, 1%; gassed with 95% O_2 and 5% CO_2, pH 7.6;) for a minimum of 30 min at 16°C. It was important to ensure that all blood had been removed from the rectal gland to avoid coagulation which could cause insufficient filling with the resin (Mercox CL-2B).

The ratio of resin to catalyst was 5 ml : 120 mg respectively and the two components were mixed immediately prior to filling the gland as polymerisation of the resin began 15 min after the time of mixing. Ensuring the absence of air bubbles, the syringe containing the resin was connected to the cannula and the resin perfused through the gland in a pulsatile fashion by hand. Perfusion of the casting media was continued until the gland was completely filled (visible by eye). The cannula was then tied in a knot in order to seal the system. The resin was

allowed to polymerise overnight in distilled water at room temperature. Once hardened the casts were submerged in a solution of 10% KOH to dissolve any remaining tissue. This process required 3-4 changes of KOH at 2 hourly intervals. Following maceration the casts were washed 2-3 times in distilled water and stored in an evacuated desiccator for drying. Some casts were further submerged in distilled water, frozen, and longitudinal or transverse cleavages made using a scalpel (Lametschwandtner *et al.*, 1990).

For macrovascular examination a binocular microscope was used (Plate 2.1), and for microvascular examination casts were mounted on SEM stubs using double sided sticky tape, sputter-coated with 15 nm of gold and viewed under a scanning electron microscope (Jeol, JSM, 35CF) at 12 kv (Plates 2.2-2.6).

2.3 - Results

The macrovasculature of the rectal gland can be divided into two distinct regions; the supply on the surface of the gland and blood sinusoids that follow the tubular secretory parenchyma of the gland (Plate 2.2). As the rectal gland artery approaches the anterior third of the gland it bifurcates sending anterior and posterior branches to either end of the outer capsule. These branches give rise to paired or single circumferential arteries which encircle the gland. It is these circumferential arteries that ramify freely to form a dense arterial web in the capsular and sub-capsular regions of the gland and supply the sinus vessels of the secretory parenchyma (Plates 2.1 & 2.2). Sphincter-like control sites of arterial blood flow can be observed on this network (Plates 2.3 & 2.4)

In close association with this arterial network there is also a paired venous system which can be identified by the random arrangement of nuclear imprints (Plate 2.5). This venous system runs in parallel to the arterial system (Plates 2.3) and eventually joins together at the point of entry of the rectal gland artery where two veins run on either side of the artery in the dorsal mesentery (Plate 2.1) and ultimately empty into the posterior cardinal vein (Kent & Olson, 1982). Thus, the arterial supply also has a venous network running in parallel to it carrying blood in the opposite direction.

From the evidence produced there are three possible routes for blood flow through the rectal gland:

1) The posterior end of the rectal gland artery which enters directly into intestinal tissue could be one avenue for blood flow. This route provides the possibility of almost complete arterial bypass of the secretory parenchyma (Plate 2.1 & Figure 2.1).

2) The capsular and sub-capsular venous network which is closely associated with the arterial network (Plate 2.3), provides a second route for blood flow through the rectal gland, and may possibly be an avenue for complete or incomplete perfusion of the secretory parenchyma.

3) The dense capillary network, draining into the large central vein reflecting anteriorally into the intestine, provides a third avenue for blood flow (Plates 2.2 and 2.6). This vessel subsequently runs on the surface of the intestine where it ultimately joins the hepatic portal system (Kent & Olson, 1982).

PLATE 2.1

Plate 2.1 Light microscopy photograph of a vascular corrosion cast of the rectal gland of *Scyliorhinus canicula.*

A (white) illustrates the rectal gland artery approaching the anterior third of the gland, which then bifurcates to either end of the gland (a, white). b (white) demonstrate the circumferential arteries enveloping the gland. The rectal gland artery is flanked on either side by the paired venous system C (white). D (white) shows the position of the rectal gland vein exiting the secretory tissue on the ventral side of the gland. E (black) illustrates the posterior ramus of the rectal gland artery, providing evidence for an almost complete bypass of the secretory parenchyma. Arrows indicate the direction of blood flow.

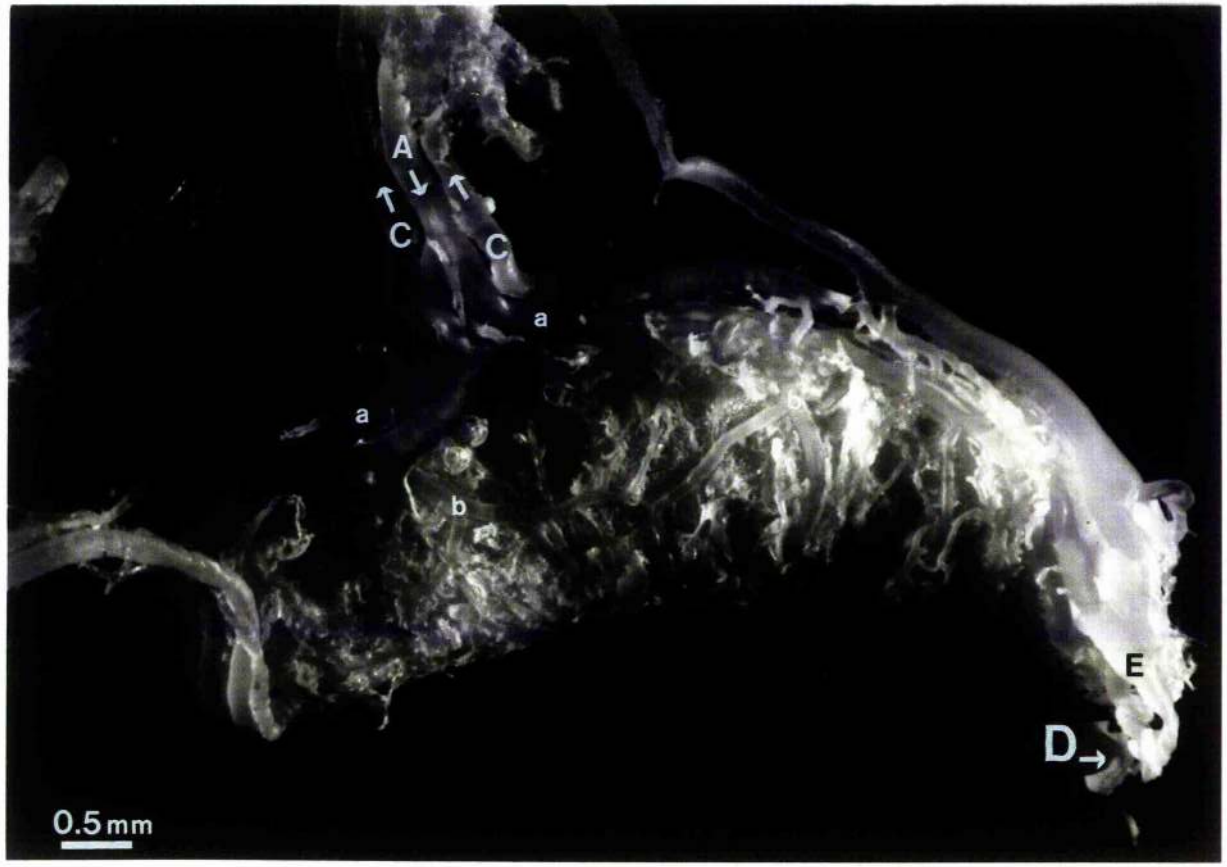
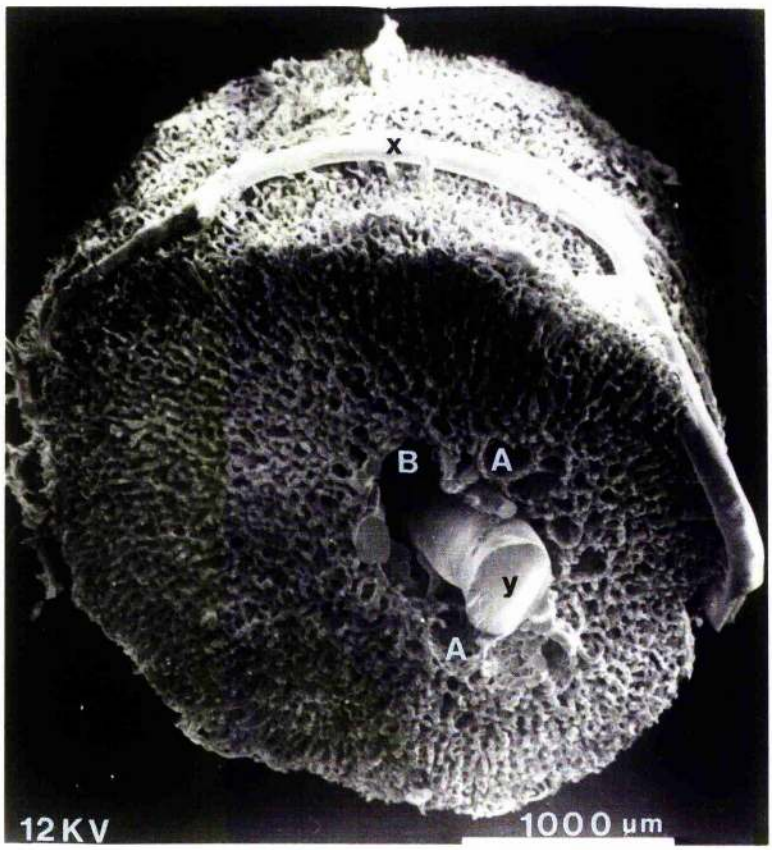

A
C
C
a
a
b
E
D
0.5 mm

PLATE 2.2

Plate 2.2 Cross section of the rectal gland viewed under the S.E.M.
x (black) illustrates a single circumferential artery supplying blood vessels in the secretory parenchyma. y (black) shows the large central vein draining blood from the perfused secretory tissue. A (white) demonstrates smaller ducts, running in parallel to, and which will eventually drain into the central duct B (white).


x
B
A
y
A
12KV
1000 µm

PLATES 2.3, 2.4 & 2.5

Plate 2.3 Paired arterio-venous system on the capsular region of the rectal gland, viewed under the S.E.M.

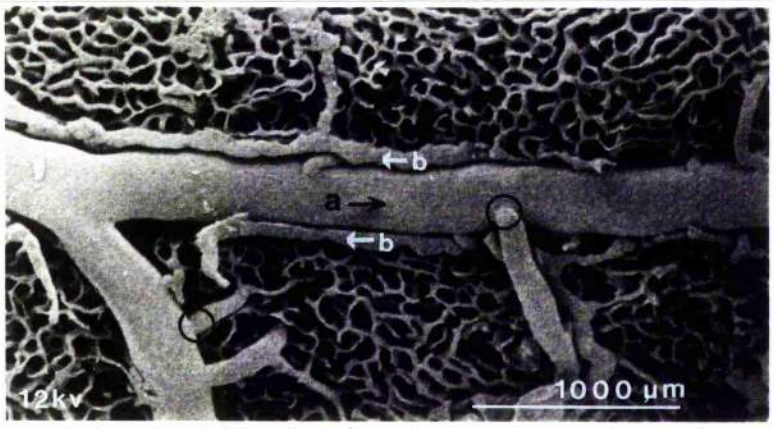
a (black) illustrates a single artery on the capsular region of the gland. b (white) demonstrates the paired venous system and ◯ shows sphincter-like collars on the arterial branches. This provides evidence for possible vascular control sites. Arrows indicate the possible direction of blood flow.

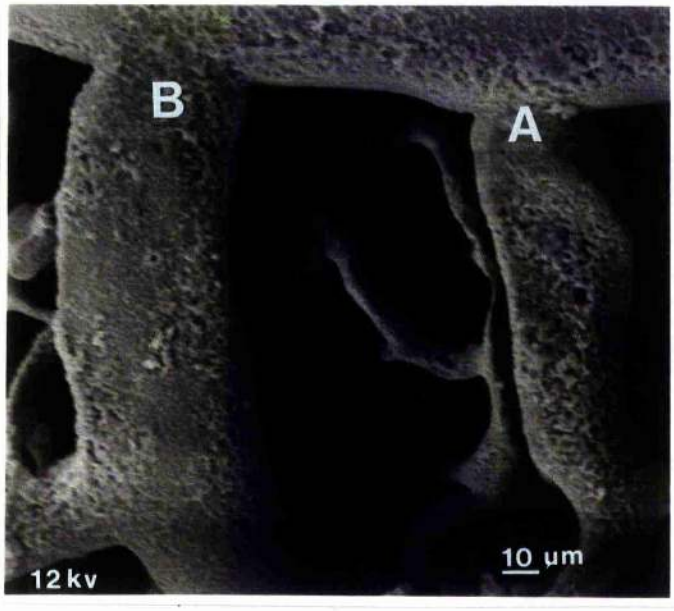
Plate 2.4 Constricted arterial branch on the capsular region of the rectal gland.

This plate demonstrates a slightly constricted sphincter-like collar on vessel A, whereas vessel B does not appear to exhibit any evidence of a sphincter-like collar. Vessel B is possibly fully dilated. This provides evidence for local control of the direction of blood flow.

Plate 2.5 Illustration of the characteristic endothelial imprints of venous vessels viewed under the S.E.M.

X illustrates the characteristic oval shape and random arrangement of venous nuclear endothelial imprints. This allows for identification of vessels as arterial nuclear endothelial imprints are characteristically ordered on the longitudinal axis of the vessel.


b
a
b
12kv
1000 µm


B
A
12 kv
10 µm

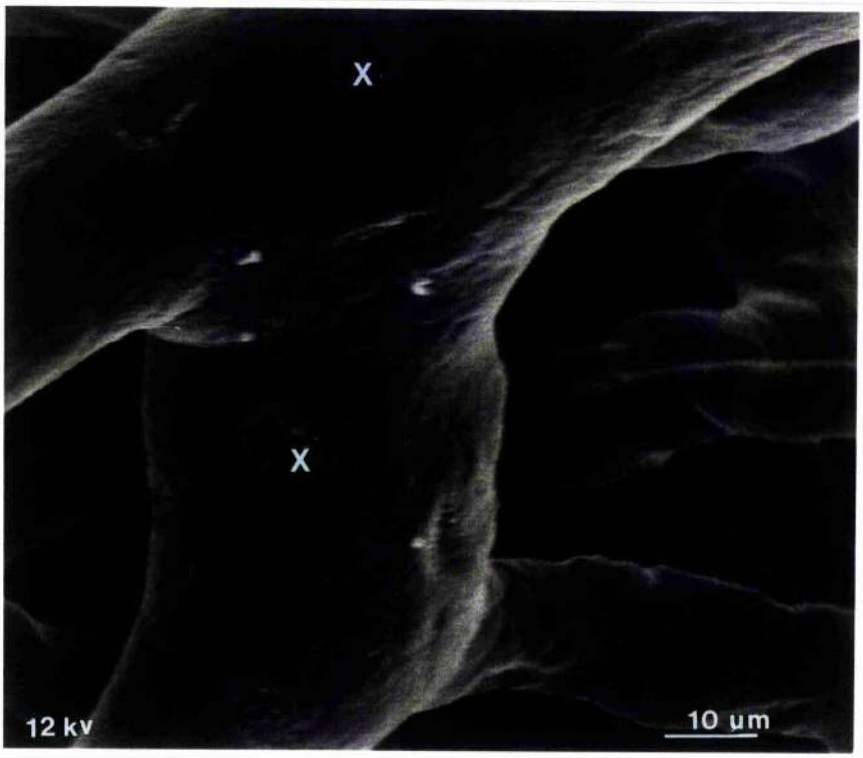
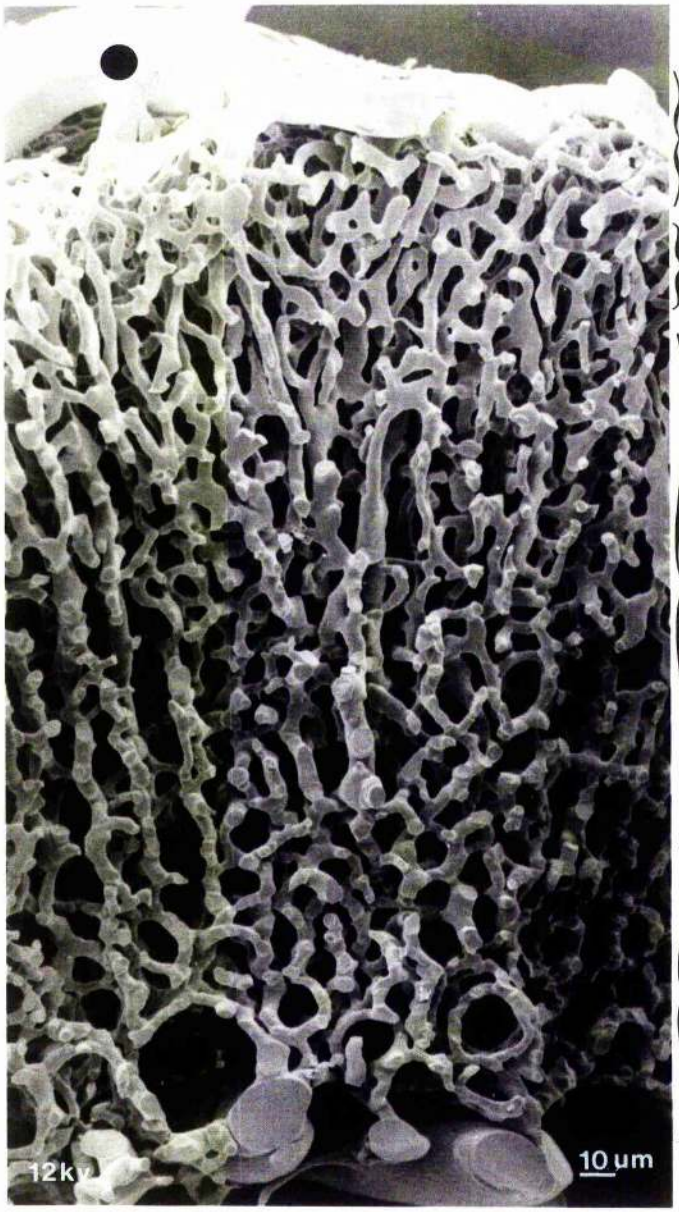

X
X
12 kv
10 µm

PLATE 2.6

Plate 2.6 Cross section of the rectal gland at a higher magnification viewed under the S.E.M.

● shows a single circumferential artery supplying blood vessels in the A, capsular, B, sub-capsular, C, middle glandular, and D, central region of the gland. 12kv, 4cm = 100 µm.


A
B
C
D
12kv
10 µm

2.4 - Discussion

Although there was no pictorial evidence produced for the arterio-venous anastomoses (AVA), described by Kent & Olson (1982) in *S. acanthias*, the macro and micro-vasculature of the rectal gland in *S. canicula* strongly resembles that reported by Kent and Olson (1982) and given that the glands play the same role in the two species, one would anticipate the vasculature to be similar. Furthermore, plates 2.1 and 2.3 do provide evidence for a paired arterio-venous system on the capsular region of the gland.

It is evident from the vasculature of the rectal gland in *S. canicula* and *S. acanthias* that blood flow through the secretory parenchyma of the gland is very complex. Control of blood flow/secretion possibly mediated by peptidergic influence may occur at three different levels.

1) The first is a shunting of the majority of blood through the posterior branch of the rectal gland artery. This would in effect bypass the entire secretory parenchyma of the gland thus possibly providing the gland with a simple "on-off" secretory switch. The activation/deactivation of this switch could explain the observed intermittent behaviour of rectal gland secretion (Burger, 1962; Stoff, Rosa, Hallac, Silva & Epstein, 1979; Kent & Olson, 1982; Shuttleworth 1988).
2) The sphincter-like "collars" on arterial branches (plates 2.3 & 2.4) illustrate a second possible site for the control of blood flow. It is at these sites that possible vasoconstrictors/vasodilators may act in directing blood to or from the secretory parenchyma, thus providing the possibility for selective perfusion of secretory epithelial tissue.
3) The third level of control is due to the intimate contact of the capsular and sub-capsular arterial and venous systems via the AVA, described in detail by Kent & Olson (1982) for *S. acanthias*. The controlling influences on this particular pathway of blood flow depend on a variety of parameters including the outflow pressure of the central vein and interstitial pressure of the gland. If the anastomoses were dilated the AVA could return a proportion of blood entering the gland via the rectal gland artery away from the gland via this closely linked venous system. Control of blood flow through such a system could again be influenced by vasoconstrictors/vasodilators.

It is possible, therefore, that the capsular and sub-capsular regions of the rectal gland are the major sites of vascular control, for the entire secretory parenchyma, and subsequently regulate the volume of blood and therefore the rate at which sodium and chloride reach the secretory epithelia (Kent & Olson, 1982). When considering peptides involved in modulating vasoactivity of blood vessels,

possible candidates include, the potent vasoconstrictor angiotensin II (AII) and vasodilator shark C-type natriuretic peptide, which may work in antagonistic fashion. Galli and Cook (1993) demonstrated the presence of AII receptors in the rectal gland of the Nurse shark. Masini *et al.* (1994) also demonstrated AII-like activity and natriuretic peptide-like activity in the rectal gland of *S. canicula.* Interestingly these areas of activity were found in highest concentration around the capsular and sub-capsular regions of the gland (Plate 2.6). Furthermore, the postvalvular intestinal arteries lie in close proximity to the posterior ramus of the rectal gland artery (Kent & Olson, 1982). Therefore, intestinal arterial blood supply could run over into the secretory parenchyma of the rectal gland, possibly providing a route for gastro-intestinal peptides effecting rectal gland function directly at the secretory epithelia (Kent & Olson 1982). The possibility of the above controlling mechanism are discussed further in chapters 4-6.

It is evident from the vascular pathways described in the present study, and blood flow patterns illustrated by Kent & Olson (1982) that perfusion of the rectal gland's vascular network can be complete, partial or almost absent. The phenomenon of selective blood perfusion to certain regions of electrolyte transporting epithelia is not unusual. Brown, *et al.* (1978) observed the effects of transferral from fresh water to sea water on single nephron glomerular filtration rates (SNGFR's) in the trout, *Salmo gairdneri.* Upon transferral to sea water there was a cessation of filtration in large populations of nephroi. However, the SNGFR's of the remaining perfused nephroi were higher. A selection, therefore, had been made to direct blood flow only to particular populations of nephroi that were appropriate to the homeostatic demands of the fish in terms of renal absorption and secretion and production of a final urine.

Franklin and Grigg (1993) observed a significant increase in vascular volume of the salt gland in 20‰ salt water raised crocodiles, *Crocodylus porosus,* compared to fresh water raised crocodiles. Using the corrosion casting technique, control sites in the form of sphincter-like and valve-like markings were observed in the vast majority of arteries supplying individual lobules in the salt gland of *C. porosus* (Franklin & Grigg, 1993). Hossler and Olson (1990) observed similar changes in vascular volume of the salt gland in 1% sodium chloride fed ducklings, *Anas platyrynchos,* compared to fresh water fed ducklings. Using the corrosion casting technique possible sites of control were also observed on the arteries supplying the capillaries surrounding the pores that lead to the secretory ducts (Hossler & Olson, 1990).

Kent and Olson (1982) argue the possibility of counter-current flow between the arterial and venous systems in the capsular region of the gland. From this, one would anticipate that the returning venous blood would be concentrated by the entering arterial blood supply. This appears peculiar as the function of the rectal gland is to excrete salt, not conserve it. The only possible advantage for such a system could be the conservation of salts in a euryhaline elasmobranch moving from dilute sea water to full strength sea water. The presence of counter-current flow is evident in the vast majority of glandular secretory epithelia, although this is between arterial blood and epithelial secretion. In both the salt glands of *C. porosus* and *A. platyrhynchos*, evidence for counter-current arterial blood flow to epithelial secretion was very strong (Franklin & Grigg, 1993; and Hossler & Olson, 1990). One would predict therefore that if the elasmobranch rectal gland had a counter-current system it would occur between these two fluids. However, evidence for such a system in the elasmobranch rectal gland is regarded as weak (Kent & Olson, 1982; Shuttleworth, 1988). Clearly more research is required to elucidate fully the distribution of blood flow in the elasmobranch rectal gland and consequently support or disprove the argument presented for a counter-current system.

This study has indicated the possibility of control of rectal gland secretion by modulation of blood flow in the gland perhaps by vasoactive peptides acting on AVA. Evidence also exists for vasoactive binding sites in the capsular and sub-capsular region of the gland where the majority of AVA's are situated (Masini *et al.*, 1994). However a second site of control of rectal gland secretion at the level of secretory epithelia has also been implicated. The mechanisms involved in the control of salt secretion at the cellular level will be discussed in the next chapter.

CHAPTER 3
IN VITRO PERFUSION PROTOCOL

3.1 - Introduction

Burger and Hess (1960) were the first to observe the ability of the rectal gland in the spiny dogfish, *Squalus acanthias*, to secrete a fluid that was isosmotic to blood plasma but almost entirely composed of sodium chloride (Table 1.7). Furthermore, the rectal gland of *S. acanthias* was able to maintain high secretion rates over a long periods (12 h) thus having a significant effect on plasma sodium and chloride concentration (Burger & Hess, 1960). Subsequently, the rectal gland in elasmobranchs was characterised as a salt gland.

The accessibility of the rectal gland and its ability to secrete such a high concentration of sodium chloride has led researchers to use this organ as a model tissue to investigate transepithelial sodium chloride transport. A variety of preparations have been developed, including; *in vitro* perfusion of the isolated rectal gland (Stoff *et al.*, 1979); perfusion of isolated rectal gland tubules (Greger & Schlatter, 1984, a & b); epithelial cell cultures (Lear, Cohen, Silva, Lechene & Epstein, 1992); and sub-cellular fractions of epithelial cells (Riordan, Forbrush & Hanrahan, 1994).

In early perfusion studies of the rectal gland of *Squalus acanthias*, variation of sodium chloride concentrations in the perfusate solution, showed that chloride ions were being transported from cell to duct lumen, against a prevailing electrochemical gradient (Hayslett, Schon, Epstein, & Hogben, 1974; Siegel, Schon & Hayslett, 1976). The morphological evidence for active salt transport in rectal gland epithelia is overwhelming and is characteristic of many other salt secreting epithelia such as; the salt gland of marine birds (Riddle & Ernst, 1979), and reptiles (Ellis & Abel, 1964) and the chloride cells of sea water teleosts (Keys & Willmer, 1932). These include, deep lateral intercellular interdigitations, and a highly invaginated basolateral membrane, as found in the stingray, *Urolophus jamaicensis*, and spiny dogfish, *Squalus acanthias* (Doyle, 1962). Freeze fracture experiments reveal the so-called tight junctions between epithelial cells to be relatively shallow (Forrest, Boyer, Ardito, Murdaugh & Wade, 1982), a feature typical of salt secreting epithelia.

Na^+K^+ATPase is responsible for the energy dependent exchange of cytoplasmic sodium for extracellular potassium and was first discovered by Skou in crab neurones (1957). This enzyme is found in high concentration on the basolateral membrane of rectal gland epithelia where the mitochondria also appears to be found in highest concentration (Goertemiller & Ellis 1976; Eveloff, Karnaky, Silva, Epstein & Kinter, 1979). Bonting (1966) reported that after perfusion of 10^{-4} M ouabain (a specific inhibitor to Na^+K^+ATPase) secretion from the rectal gland of *S. acanthias* was completely blocked, which suggested

that the enzyme played a primary and rate limiting step in the secretory mechanism. This argument was supported further when increased chloride secretion from the isolated perfused rectal gland of *S. acanthias* was found to be significantly reduced following addition of ouabain to the perfusate (Silva, Stoff, Field, Fine, Forrest & Epstein 1977). Ouabain inhibition of salt secretion and chloride transport was also found to be dependent on sodium concentration in the perfusate, thus supporting the proposed model of active sodium transport via Na^+K^+ATPase in the basolateral membrane of the secretory epithelia (Silva *et al.*, 1977; Eveloff, Kinne, Kinne-Saffran, Murer, Silva, Epstein, Stoff & Kinter, 1978). Further work on the effect of ouabain on the rectal gland of *S. acanthias* was carried out by Silva, Epstein, Stevens, Spokes & Epstein (1983). These researchers reported that stimulation of the isolated perfused rectal gland of *S. acanthias* significantly altered ouabain binding characteristics. Two classes of ouabain binding sites were suggested; one of high and one of low affinity. Stimulation appeared to increase the affinity of the high affinity binding sites, suggesting there was an immediate change in Na^+K^+ATPase configuration/activity/number upon stimulation, that regulated the overall secretory mechanism (Silva *et al.*, 1983).

As well as the enzyme Na^+K^+ATPase there are a number of other transporting mechanisms involved in the secretion of sodium chloride from plasma to tubular lumen. Silva *et al.* (1977) postulated that a neutral sodium chloride carrier effected movement of chloride ions into the cell powered by the downhill movement of sodium ions into the cell. Low intracellular sodium concentrations and a large downhill electrochemical gradient for sodium would be maintained by the Na^+K^+ATPase actively extruding sodium from the cell. The high concentrations of chloride found in the cell would then diffuse down the electrochemical gradient into the lumen of the duct, and sodium ions pumped out of the cell would move down an electrochemical gradient into the lumen via a paracellular route. Chloride secretion was therefore considered a secondary active transport driven by the sodium gradient across the basolateral membrane, created by the active Na^+K^+ATPase pump and a sodium-chloride (Na-Cl) co-transporter (Silva *et al.*, 1977; Eveloff *et al.*, 1978).

Loop-diuretics such as bumetanide, piretamide and furosemide, named for their ability to decrease water absorption into the tubular lumen of the loop of Henle by inhibition of a Na-Cl co-transporter, were found to inhibit secretion in *in vitro* preparations of the rectal gland of *S. canicula* (Kinne & Kinne-Saffran, 1979). Further studies suggested that the Na-Cl co-transporter in the rectal gland of *S. acanthias* involved a sodium-potassium-chloride (Na-K-Cl) co-transporting

system (Palfrey, Silva & Epstein, 1984). Using rubidium (^{86}Rb) as a potassium substitute, potassium flux properties in the isolated epithelia were investigated with respect to chloride dependence, sodium dependence and the action of bumetanide (Hannafin, Kinne-Saffran, Freidman & Kinne, 1983). It was concluded that, the passage of chloride ions into the lumen of the duct was in fact secondary to the action of a Na-K-2Cl co-transporter (Hannafin *et al*, 1983). Furthermore the presence of a Na-K-2Cl co-transporter in the rectal gland tubule of *S. acanthias* was demonstrated using a series of inhibitors which illustrated that the Na-K-2Cl co-transporter was the only avenue for controlled sodium entry into the cell (Greger & Schlatter, 1984a & b). Immunofluorescent studies have also shown the Na-K-2Cl co-transporter to be distributed almost exclusively along the basolateral membrane and excluded from the apical side of the cell (Lytle, Xu, Biemesderfer, Haas & Forbrush, 1992).

A third ion channel was subsequently discovered on the basolateral membrane of the rectal gland epithelia (Greger and Schlatter, 1984a). Through the use of patch clamp techniques a potassium conductance channel was shown to extrude potassium into the extracellular fluid, thus providing a reservoir for the continuous recirculation of potassium through the cell, via the Na^+K^+ATPase pump and the Na-K-2Cl co-transporter (Greger, Gogelein & Schlatter, 1987). Known secretagogues were found to increase the binding of the radio-labelled loop-diuretic [^3H] benzmetanide to the Na-K-2Cl co-transporter in the perfused rectal gland and isolated tubules of *S. acanthias* (Forbrush, Haas & Lytle, 1992) an increase which was subsequently blocked by specific potassium channel blockers such as Barium (Ba^+).

Further work using patch clamping also identified two chloride channels on the apical membrane of the rectal gland cell, a large conductance channel considered only to be open when the rectal gland was stimulated (Greger, Schlatter & Gogelein, 1987) and a small conductance channel perhaps accounting for low chloride conductance in the unstimulated rectal gland (Gogelein, Schlatter & Greger, 1987). A third chloride channel with close homology to human CFTR (Cystic fibrosis transmembrane conductance regulator) was also identified and localised in the elasmobranch rectal gland of *S. acanthias* (Marshall, Martin, Picciotto, Hockfield, Nairn & Kaczmarak, 1991). Investigation using sub-cellular fractions found this third, and smallest chloride channel, to be present in the highest frequency in epithelial cells of stimulated tubules isolated from the rectal gland of *S. acanthias* (Hanrahan, Duguay, Samson, Alon, Jensen, Riordan & Grzelczak, 1993). The mammalian CFTR apical chloride channel, was cloned by Riordan, Alon, Grzelczak, Dubel and Sun

(1991). It is considered to be a finely regulated, low conductance channel (Bear, Kartner, Bridges, Jensen, Ramjeesingh & Riordan, 1992). Whether this channel (sCFTR) is the principle regulator of chloride secretion in rectal gland secretory epithelia as it is in apical membranes of salt secreting cells in mammals, is not known. It appears therefore that the stimulated gland has three types of chloride channels on the apical membrane that differ markedly in their characteristics, two of which are considered CFTR-like channels (Riordan *et al.*, 1994). The CFTR channel is phosphorylated/activated by the intracellular enzyme protein kinase A (PKA), which binds to a large hydrophilic central domain (R domain) (Riordan *et al*, 1994). It has been reported that phosphorylation of multiple R domain sites activates the CFTR channel (Dulhanty & Riordan, 1994 a & b). This gives rise to the possibility of various activated stages of the CFTR channel. It is therefore possible that the two previously reported CFTR-like channels are both CFTR-like channels at various stages of activation.

In summary, present evidence suggests that the net secretion of sodium chloride by rectal gland secretory epithelia is brought about through the combined action of four transport pathways, which together mediate secondary active transcellular chloride transport accompanied by paracellular sodium movement.

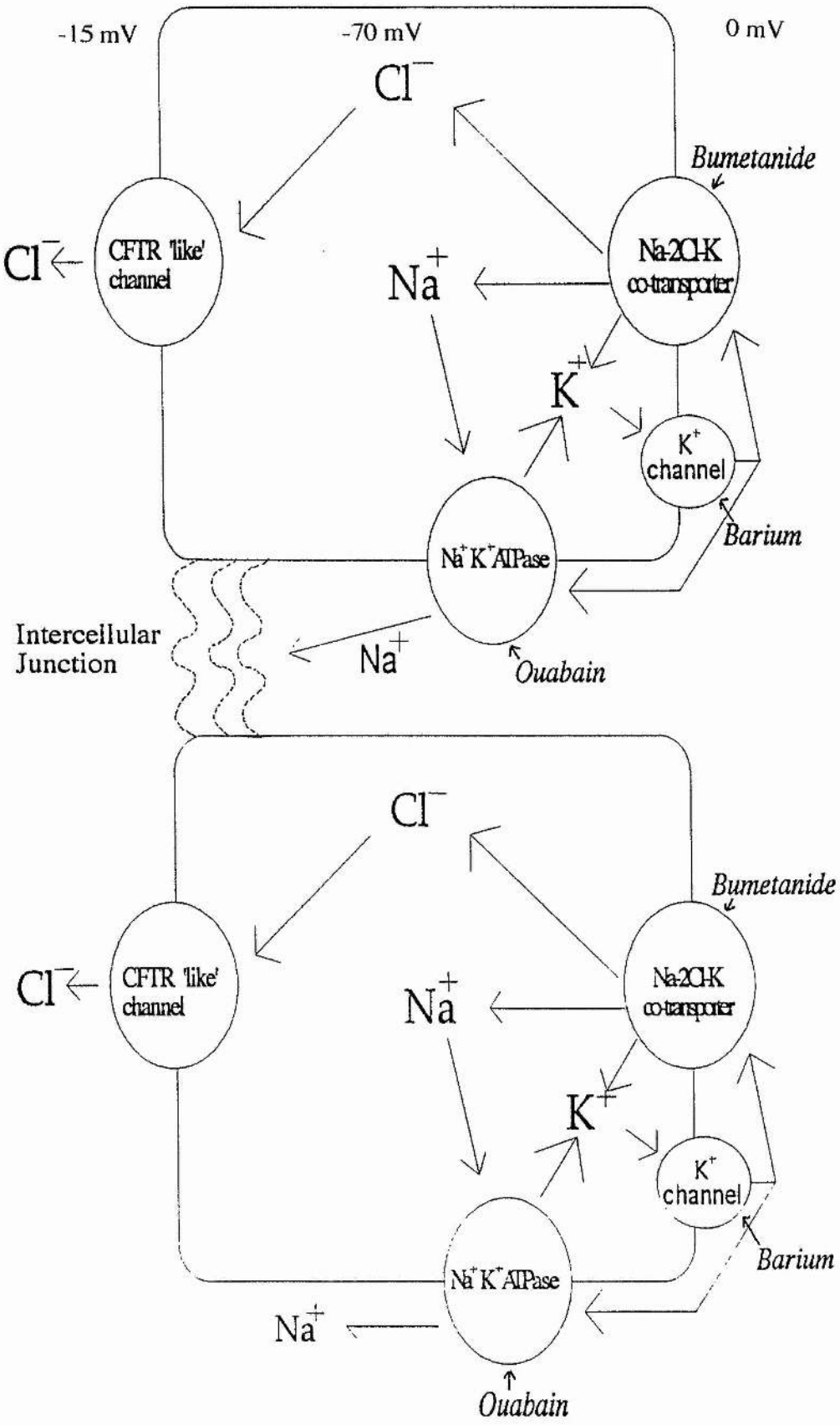
1) The basolateral Na^+K^+ATPase pump which creates the inwardly directed sodium gradient.
2) Potassium channels in the basolateral membrane which mediate potassium recycling.
3) The basolateral Na-K-2Cl co-transporter which utilises the sodium gradient to drive chloride ions above their electrochemical equilibrium.
4) Chloride channels in the apical membrane which permit passive loss of chloride ions into duct lumen.

The primary and rate limiting step in salt secretion could occur at any one or more of the transport pathways described. Figure 3.1 illustrates the currently accepted pathways for sodium chloride secretion in rectal gland epithelia. This model is also the accepted model for salt transport in other salt secreting epithelia such as the avian and reptilian salt glands and chloride cells in teleosts.

The second messenger cyclic adenosine monophosphate (cAMP) has been implicated in the stimulation of rectal gland secretion (Stoff, Silva,

FIGURE 3.1

Figure 3.1 Schematic illustrating the currently accepted pathways and membrane proteins involved in the active secretion of sodium chloride from rectal gland epithelia.
Adapted from Riordan *et al.*, (1994).


Lumen
Bloodstream
-15 mV
-70 mV
0 mV
Cl⁻
Bumetanide
Cl⁻
CFTR 'like' channel
Na⁺
Na-2Cl-K co-transporter
K⁺
K⁺ channel
Barium
Na⁺K⁺ATPase
Intercellular Junction
Na⁺
Ouabain
Cl⁻
Bumetanide
Cl⁻
CFTR 'like' channel
Na⁺
Na-2Cl-K co-transporter
K⁺
K⁺ channel
Barium
Na⁺K⁺ATPase
Na⁺
Ouabain

Field, Forrest, Stevens & Epstein, 1977). The initial site of action of cAMP, and subsequent cascade of events leading to salt secretion has been the subject of much debate in the literature. Consequently cell permeable analogues of cAMP, such as dibutryl cAMP in association with theophylline, which inhibits the breakdown of intrinsic cAMP by phosphodiesterases, have been used as potent tools to investigate the intracellular mechanisms involved in salt secretion in rectal gland epithelia of elasmobranchs.

It was found that following addition of cAMP plus theophylline to the perfusate, chloride secretion of the isolated perfused rectal gland of *S. acanthias* significantly increased above basal levels (Silva *et al*, 1977). Shuttleworth and Thompson (1980a) reported that the increased oxygen consumption of rectal gland slices from *S. canicula*, resulting from the addition of dibutryl cAMP and theophylline, measured by percentage binding of tritiated ouabain, was entirely abolished by ouabain. They suggested that cAMP stimulated the existing Na^+K^+ATPase pumps rather than recruitment of latent pumps. However no evidence for direct stimulation of Na^+K^+ATPase by cAMP was elucidated rather it was proposed that cAMP was acting on what was then presumed to be a Na-Cl co-transporter. As a result of the action of cAMP on the Na-Cl co-transporter, intracellular sodium concentration would increase leading to the recruitment of latent Na^+K^+ATPase pumps on the basolateral membrane, which in turn would produce an increase in sodium efflux. Intracellular sodium concentration would then be restored at the cost of extra recruitment of latent sites and therefore increased transcellular sodium flux (Shuttleworth & Thompson 1980b). Using electron probe analysis on single cell preparations of the rectal gland of *S. acanthias*, Lear *et al.* (1992) showed a 30% drop in intracellular sodium 8 minutes after stimulation by cAMP; this was equated to a direct stimulatory effect of cAMP on the Na^+K^+ATPase activity. However, a time lag of 8 mins between stimulation and the first measurement of a change in intracellular sodium concentration could be considered too long to define the immediate target of cAMP.

By manipulation of intracellular chloride and patch clamping Greger *et al.* (1984 a & b; 1987) maintained that the primary event in cAMP mediated stimulation of sodium chloride secretion was an increase in apical chloride conductance. When stimulated by cAMP the chloride conductance of the apical cell membrane increased by a factor of 10, and in a non-stimulated state the Na-K-2Cl co-transporter was triggered by lowering intracellular chloride. This mode of action by cAMP was supported by Hanrahan *et al.* (1993) in rectal gland

tubules of *S. acanthias* where elevation in the intracellular level of cAMP activated the apical sCFTR channel. Indeed Riordan *et al.* (1994) postulated that the primary and rate-limiting regulatory step resides at the sCFTR chloride channel, which in humans is considered to be optimally designed to respond to hormones employing cAMP as a second messenger. It appears therefore that the initial site of action of cAMP following stimulus of rectal gland epithelia occurs at a chloride channel on the apical membrane.

As can be seen cellular regulation of ionic movement in sodium chloride secretory epithelia in the rectal gland is the subject of intense debate in the literature. The current study was concerned with regulation of rectal gland secretion as a complete organ, consequently, the activities of individual protein transport mechanisms involved in ionic regulation of sodium chloride secretion were not investigated.

In order to investigate factors controlling rectal gland function in *S. canicula* it was necessary to develop an isolated perfused preparation. *S. acanthias* is approximately 4-5 times larger than *S. canicula* thus the surgical procedures involved in isolation and perfusion are relatively straightforward. The technical difficulties involved in isolating the rectal gland from *S. canicula* are perhaps indicative of the lack of *in vitro* studies carried out on this species of elasmobranch. The isolated perfused preparation is particularly useful when investigating mechanisms of peptidergic control of secretion in *S. canicula*, especially when discrepancies have been reported between the effects of peptides on the rectal gland of *S. acanthias* and *S. canicula* (Shuttleworth & Thorndyke, 1984). In developing this technique it was necessary to determine the optimal perfusion conditions and develop a standard method of stimulation of the rectal gland to monitor short and long term viability.

3.2 - Materials and Methods

Fish were caught, stored and sacrificed as described in section 2.2. A cannula was inserted into the rectal gland artery, as close to the gland as anatomy allowed, using PE 10 cannula (1.5cm) inserted into PE 50 cannula (15cm) and tied in place with EP3 surgical silk suture thread (Davis & Geck, Cyanamid of Great Britain Ltd, Hampshire). The rectal gland vein and duct were then cannulated using pulled out PE 50 cannula. Due to the proximity of these two vessels a single ligature of EP3 thread was used to tie off both the venous and duct cannulae. Care was taken to ensure that there was no damage done to either vessel walls. A very large secretion rate at the onset of perfusion usually meant that perfusion fluid was leaking from the vein into the duct, and abnormally low chloride concentrations of the secretory fluid helped confirm this. Connective tissue around the gland was cut away and the prepared gland transferred to a specially designed perspex chamber containing elasmobranch Ringer (Section 2.2). The final pH of the Ringer was corrected to 7.6 immediately prior to all perfusion experiments. The chamber was then transferred to a constant temperature bath which was held at 16°C for the duration of the experiment at which time perfusion with elasmobranch Ringer was initiated. The maximum time from cannulation to perfusion was 15 min. Rectal glands were perfused at a constant rate using an infusion pump (Harvard apparatus (935), Massechusetts, U.S.A). The pressure transducer (Elcomatic EM750 pressure transducer) attached to a George Washington 400 MD/4 pen recorder, was used to measure changes in perfusion pressure whilst maintaining a constant perfusion flow rate. This value will be subsequently referred to as "perfusion pressure". Figure 3.2 illustrates the experimental set-up.

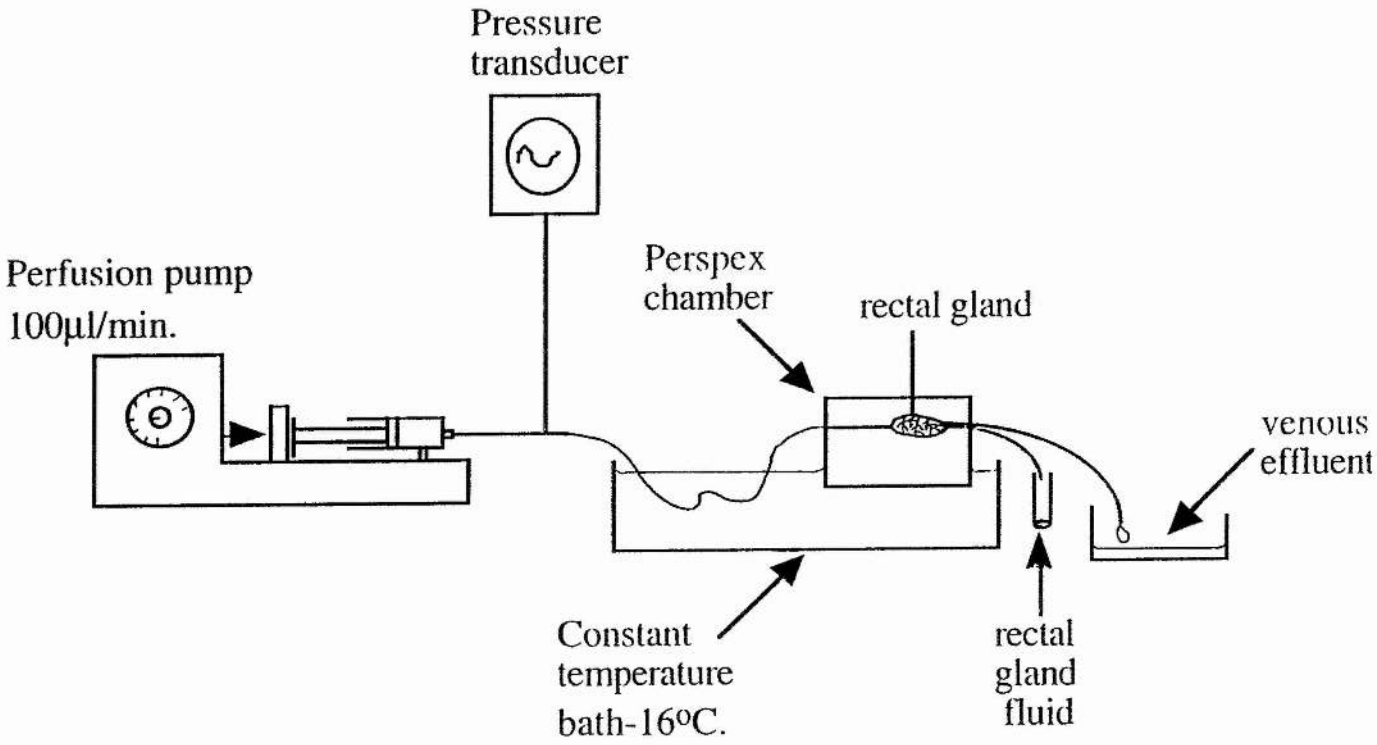
Rectal glands were weighed immediately after the experiment and wet weights ranged from 50 - 260 mg. Timed samples of secreted fluid were collected in previously weighed eppendorf tubes then re-weighed to determine secretory volume assuming a fluid specific gravity of 1. Average basal volume of secretory fluid was 28.36 +/- 3.42 µl/hr. Chloride concentration of the secretory fluid was measured using a chloride analyser (Corning 925) and ranged from 400-600 mmol/L. Secretion rate of chloride was expressed as chloride clearance rate in units of µmol of Cl^-/h/ gram of wet weight.

In developing the isolated perfused preparation a number of criteria were assessed in the initial stages of the perfusion experiments, in order ensure the viability and reliability of the experimental set-up.

1) *Assessment of a suitable perfusion flow rate.* Glands were perfused with elasmobranch Ringer for 15min at each flow rate and the effect of increasing

FIGURE 3.2

Figure 3.2 Experimental set up for the *in vitro* perfusion of the isolated rectal gland of *Scyliorhinus canicula.*


Pressure
transducer
Perfusion pump
100µl/min.
Perspex
chamber
rectal gland
venous
effluent
Constant
temperature
bath-16°C.
rectal
gland
fluid

perfusion flow rate on the perfusion pressure and chloride clearance rate of *in vitro* perfused rectal glands was monitored. Secretory fluid was collected over a 15 min period at each perfusion flow rate and calculation of chloride clearance rates were made as described above.

2) *Collection of basal clearance rates.* Collection of basal chloride clearance rates had to be when rectal glands were quiescent, so secretory fluid was collected immediately after the onset of perfusion and every following 15min for 3 hr in order to assess if the initial period of perfusion affected rectal gland chloride clearance rates, and if the gland continued to be viable after a long period of perfusion.

3) *Establishment of standard stimulatory test for viability.* Following the establishment of a basal collection period the most potent stimulant for rectal gland chloride clearance rate was determined. Dibutryl cAMP (0.25 mM) plus theophylline (1 mM) (Section 3.1) or iso-butyl methylxanthine (1 mM) (IBMX) (a specific cAMP phosphodiesterase inhibitor) were all tested on the isolated perfused preparations. Chloride clearance rate was monitored over a 75 min period. The first 15 min involved perfusion with dibutryl cAMP and/or the phosphodiesterase inhibitors, which was followed by 60 min of Ringer perfusion. During the transfer from treatment to Ringer and vice-versa the perfusion line was disconnected for a maximum of 10 seconds.

4) *Determination of recovery period following stimulation.* It was important to ascertain how long the stimulatory effect of a particular treatment lasted on the isolated perfused rectal gland, so as to ensure complete recovery to basal chloride clearance rates before administration of the next treatment. Therefore a short time course collection of every 15 min following stimulation was carried out.

5) *Determination of maximal experimental time period.* To determine if preparations were still viable a similar stimulatory dose (and time course collection) was perfused at the end of the experimental period, which was a maximum of 7 hours.

3.2a - Statistical analysis

Paired analysis of variance was carried out to ascertain the effect of the different secretagogues in comparison to basal values. This was carried out in conjunction with a Tukey's post-hoc test. If variation was observed an alternative Welch t-test was used to calculate the final measure of significance, whereby Gausian distribution was assumed with unequal standard deviations.

3.3 - Results

Figure 3.3a illustrates the effect of increasing perfusion flow rate on the perfusion pressure created by the *in vitro* perfused rectal glands. From 50 µl/min to 300 µl/min the mean perfusion pressure increase was approximately 2.5 fold, although not statistically different there was an obvious trend. Figure 3.3b illustrates the effect of increasing perfusion rates on the chloride clearance rates from the *in vitro* perfused rectal glands. The mean chloride clearance rate increased by approximately 4.5 fold as perfusion rate increased from 50 µl/min to 300 µl/min; although this was not statistically different there was an obvious trend. A perfusion rate of 100 µl/min was established as the constant perfusion rate for any further perfusion experiments.

At the beginning of perfusion it was evident (Figure 3.4) that chloride clearance rates were relatively high but declined to constant basal levels approximately 30-45 min after the perfusion began. Consequently a 'washout period' was established of a minimum of 30 min before basal secretion rate samples were collected over a period of 60 min. Figure 3.4 also illustrates that the perfused gland was still responsive to a stimulant (0.25 mM dibutryl cAMP plus 1 mM IBMX) after 180 min of Ringer perfusion.

Figure 3.5 illustrates the singular effects of 0.25 mM dibutryl cAMP, 1 mM theophylline and 1 mM IBMX, and the effects of dibutryl cAMP plus the phosphodiesterase inhibitors administered at the same concentrations. Dibutryl cAMP (0.25 mM) on its own had no stimulatory effect on rectal gland chloride clearance rates yet in the presence of 1 mM theophylline or 1 mM IBMX there was a significant increase in chloride clearance rates of 1.8 and 2.9 fold respectively. However these increases were of similar magnitude when rectal glands were perfused with theophylline or IBMX alone (1.9 and 2.9 fold, respectively). The combination of 0.25 mM dibutryl cAMP plus 1 mM IBMX was used as a standard stimulant for each preparation thereafter. If a gland did not exhibit stimulated chloride clearance rates above basal levels after this treatment the preparation was discarded.

Figure 3.6a compares the short term effect of dibutryl cAMP plus IBMX on chloride clearance rates and figure 3.6b compares the stimulated chloride clearance rates against basal chloride clearance rates collected over the same time periods. The mean chloride clearance rate of the rectal glands were maximal during perfusion of dibutryl cAMP plus IBMX and remained significantly higher than basal levels in the first 15 min period following

FIGURE 3.3 a & b.

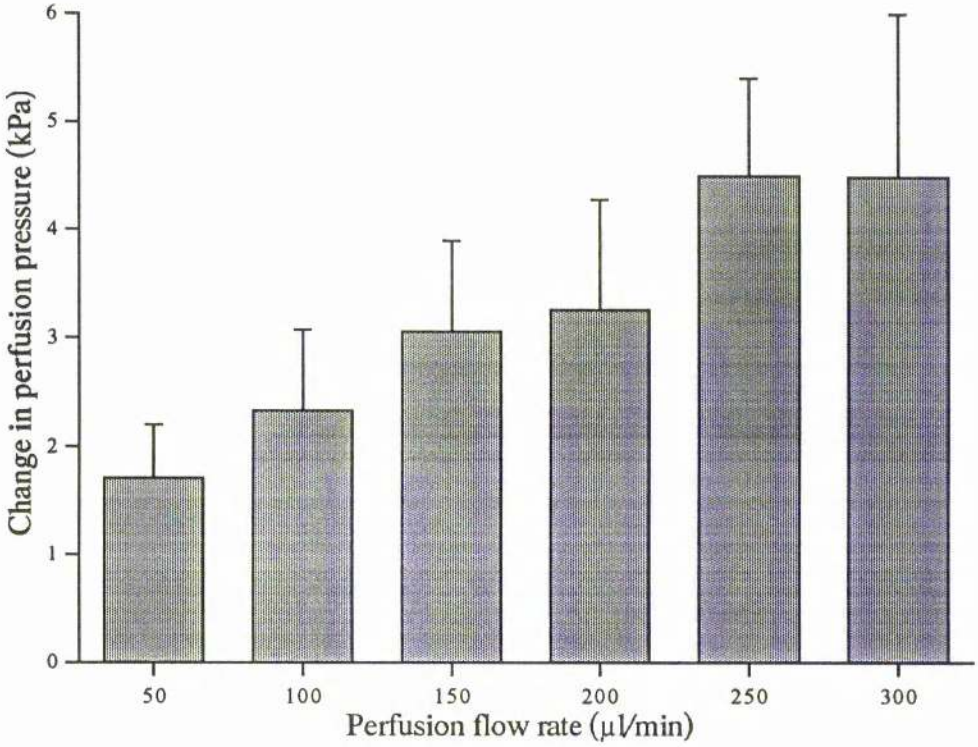
Figure 3.3 a Effect of increasing perfusion flow rate on the perfusion pressure created by the isolated perfused rectal gland.

Change in perfusion pressure (kPa) (ordinate) against increasing perfusion flow rates (µl/min) (abscissa). Values are expressed as a mean kPa ± SEM; 1.71 ± .0.49 for 50 µl/min; 2.33 ± 0.74 for 100 µl/min; 3.05 ± 0.84 for 150 µl/min; 3.25 ± 1.02 for 200 µl/min; 4.49 ± 0.91 for 250 µl/min; and 4.48 ± 1.51 for 300 µl/min (n=7, 7, 6, 7, 5 & 4 respectively) (alternative Welch t-test).

Figure 3.3b Effect of increasing perfusion flow rate on clearance rates of the isolated perfused rectal gland.

Clearance rate (µmol/h/g) (ordinate) against increasing perfusion flow rates (µl/min) (abscissa). Values are expressed as a mean µmol/h/g ± SEM; 929 ± 240 for 50 µl/min; 768 ± 146 for 100 µl/min; 1566 ± 545 for 150 µl/min; 2557 ± 925 for 200 µl/min; 2578 ± 886 for 250 µl/min; and 3141 ± 1130 for 300 µl/min (n=6) (alternative Welch t-test).

a)


Change in perfusion pressure (kPa)
0
1
2
3
4
5
6
50
100
150
200
250
300
Perfusion flow rate (µl/min)

b)

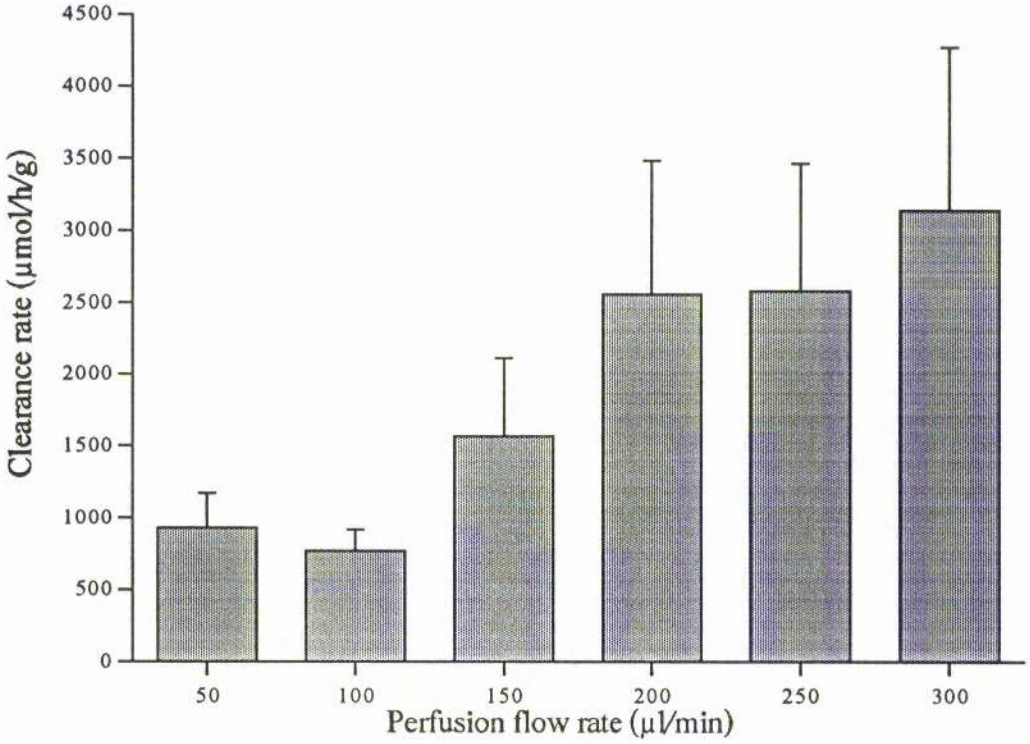

Clearance rate (µmol/h/g)
0
500
1000
1500
2000
2500
3000
3500
4000
4500
50
100
150
200
250
300
Perfusion flow rate (µl/min)

FIGURE 3.4

Figure 3.4 Long term collection of basal clearance rates followed by a single dose of dibutryl cAMP plus IBMX.

Clearance rates (µmol/h/g) (ordinate) against time (min) (abscissa). Values are expressed as a mean µmol/h/g ± SEM. 4161 ± 910 for 0-15 min; 2269 ± 511 for 15-30 min; 1240 ± 363 for 30-45 min; 625 ± 357 for 45-60 min; 206 ± 107 for 60-75 min; 312 ± 235 for 75-90 min; 374 ± 245 for 90-105 min; 283 ± 170 for 105-120 min; 193 ± 193 for 120-135 min; 154 ± 127 for 135-150 min; 107 ± 107 for 150-165 min; 139 ± 84 for 165-180 min; 64 ± 64 for 180-195 min; 21 ± 21 for 195-210 min; 4221 ± 740 for 210-225 min; 2409 ± 291 for 225-240 min; 329 ± 137 for 240-255 min; 350 ± 190 for 255-270 min; 181 ± 71 for 270-285 min; 232 ± 122 for 285-300 min (n = 7) (alternative Welch t-test).

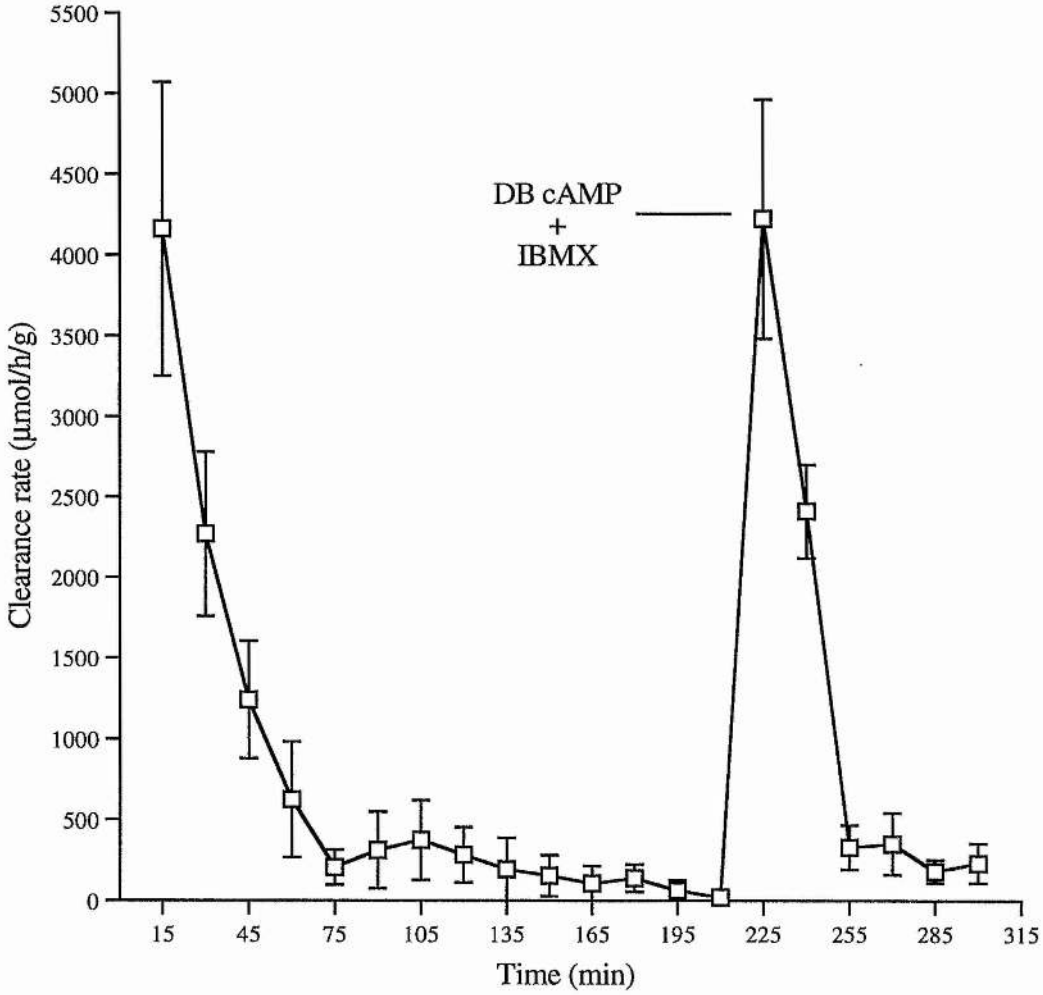

5500
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
Clearance rate (µmol/h/g)
DB cAMP
+
IBMX
15
45
75
105
135
165
195
225
255
285
315
Time (min)

FIGURE 3.5

Figure 3.5 Effect of dibutryl cAMP plus phosphodiesterase inhibitors on the clearance rates of the isolated perfused rectal gland.

Clearance rates (µmol/h/g) (ordinate) against treatment (abscissa). Values are expressed as a mean µmol/h/g ± SEM; 813 ± 205 for basal; 732 ± 245 for dibutryl cAMP; 1594 ± 146 for theophylline; 1433 ± 223 for dibutryl cAMP plus theophylline; 2367 ± 140 for IBMX; and 2382 ± 362 for dibutryl cAMP plus IBMX (n= 18, 8, 17, 16, 16, & 14 respectively). *$p<0.05$, ***$p<0.001$ compared with basal clearance rates (alternative Welch t-test).

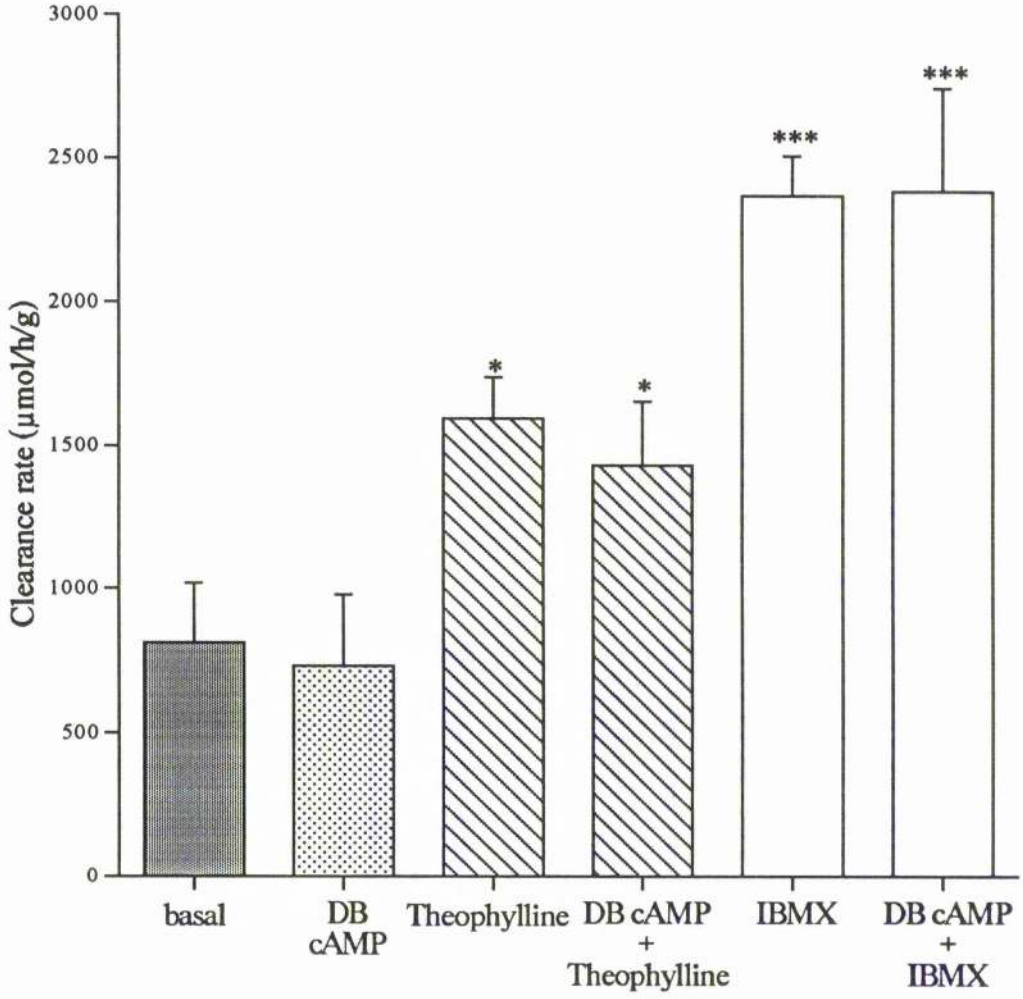

3000
2500
2000
1500
1000
500
0
Clearance rate (µmol/h/g)
*
*
***
***
basal
DB cAMP
Theophylline
DB cAMP + Theophylline
IBMX
DB cAMP + IBMX

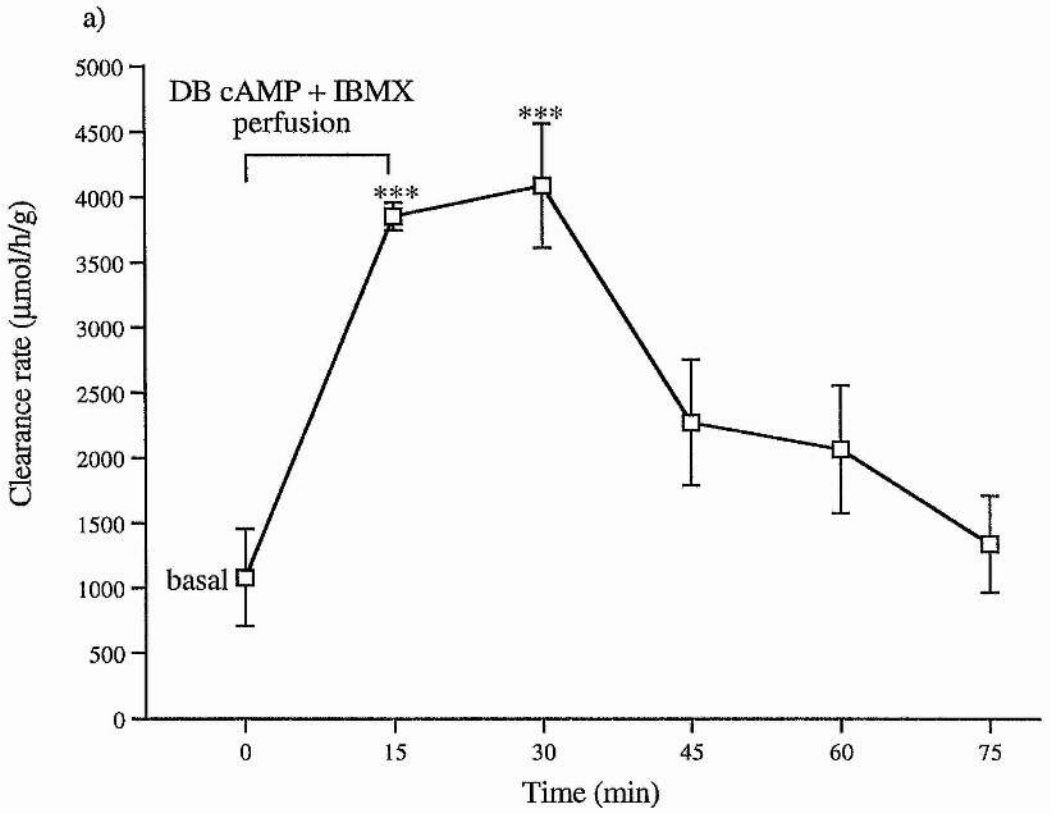
FIGURE 3.6 a & b

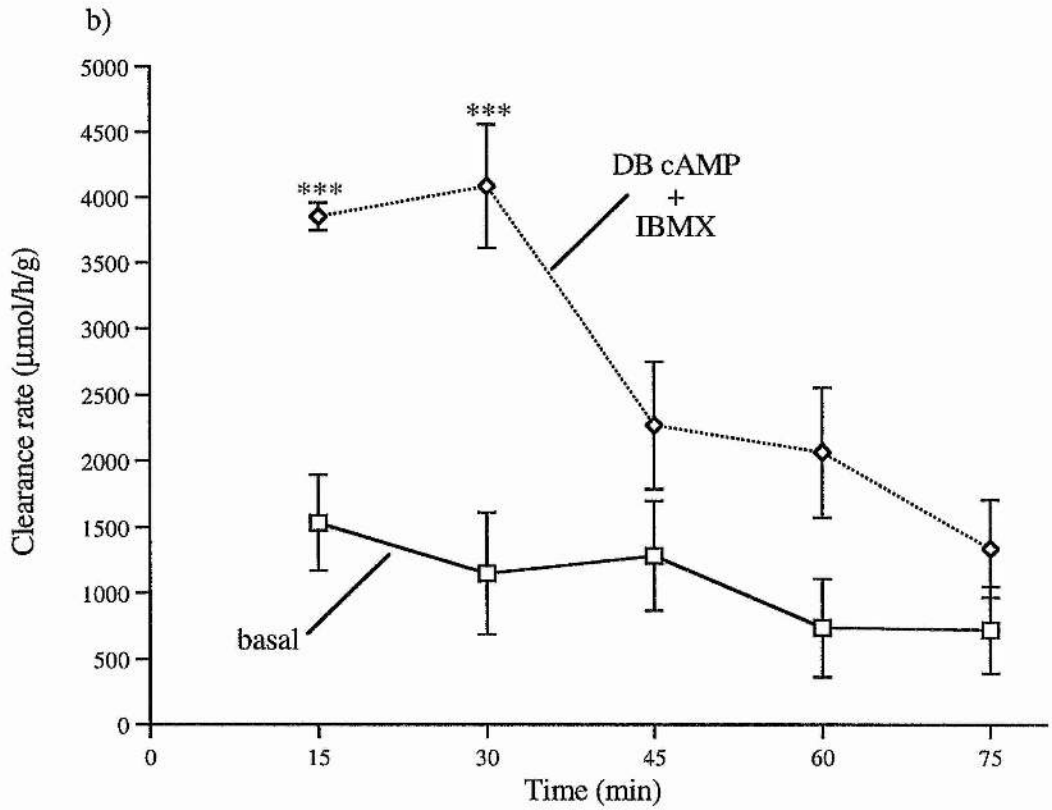
Figure 3.6a Time course of dibutyrl cAMP plus IBMX on clearance rates of the isolated perfused rectal gland compared to total basal values.

Clearance rates (µmol/h/g) (ordinate) against time (15 min intervals) (abscissa). Values are expressed as a mean µmol/h/g ± SEM; 1080 ± 375 for basal total; 3854 ± 105 for 0-15 min; 4086 ± 473 for 15-30 min; 2270 ± 483 for 30-45 min; 2036 ± 490 for 45-60 min; and 1338 ± 371 for 60-75 min (n = 6 for each point). ***p<0.001 compared with total basal clearance rates (alternative Welch t-test).

Figure 3.6b Time course of dibutryl cAMP plus IBMX on clearance rates of the isolated perfused rectal gland compared to time course of basal values.

Clearance rates (µmol/h/g) (ordinate) against time (15 min intervals) (abscissa). Values are expressed as a mean µmol/h/g ± SEM. Basal values were: 1534 ± 362 for 0-15 min; 1148 ± 465 for 15-30 min; 1283 ± 417 for 30-45 min; 735 ± 372 for 45-60 min; 719 ± 330 for 60-75 min. Dibutryl cAMP plus IBMX were as figure 3.6a (n = 6 for each point). ***p<0.001 compared with total basal clearance rates (alternative Welch t-test).


a)
DB cAMP + IBMX
perfusion
***
***
basal
Clearance rate (µmol/h/g)
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
0
15
30
45
60
75
Time (min)


b)
***
***
DB cAMP
+
IBMX
basal
Clearance rate (µmol/h/g)
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
0
15
30
45
60
75
Time (min)

stimulation. However during the following 45 min, chloride clearance rates decreased to basal levels. Thereafter a standard experimental protocol was established which included, a 15 min treatment perfusion period followed by 60 min of ringer perfusion during which time basal chloride clearance rates were re-established.

A dose of dibutryl cAMP plus IBMX similar to that at the start of an experiment was administered at the end of each experiment to check for the viability of individual preparations (Figures 3.7 a & b). It was found that preparations were still viable after six and a half hours of perfusion with 80 % of the glands producing an increase in chloride clearance rate that was 80 % or more than chloride clearance rates achieved following perfusion of the initial dibutryl cAMP plus IBMX dose. However if a preparation did not produce an increase in chloride clearance rate after the final dose of dibutryl cAMP plus IBMX that was more than 60 % of the chloride clearance rate produced by the initial dose, the results obtained with this gland were discarded.

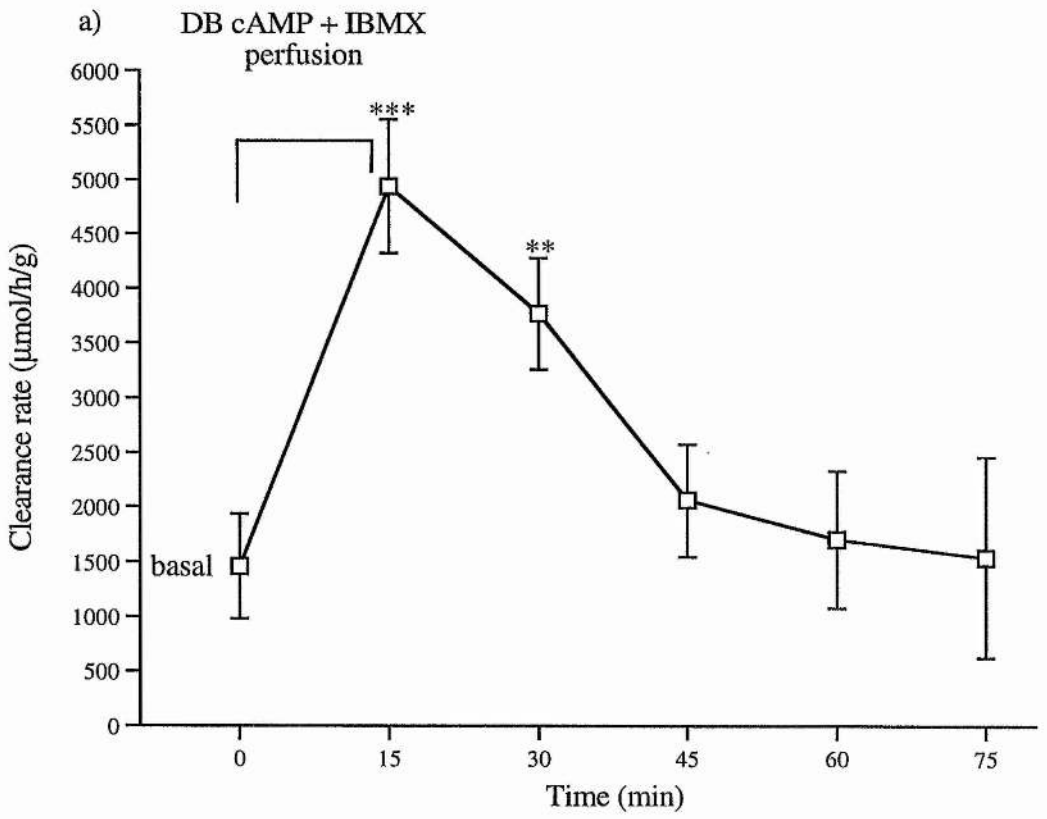
FIGURE 3.7 a & b

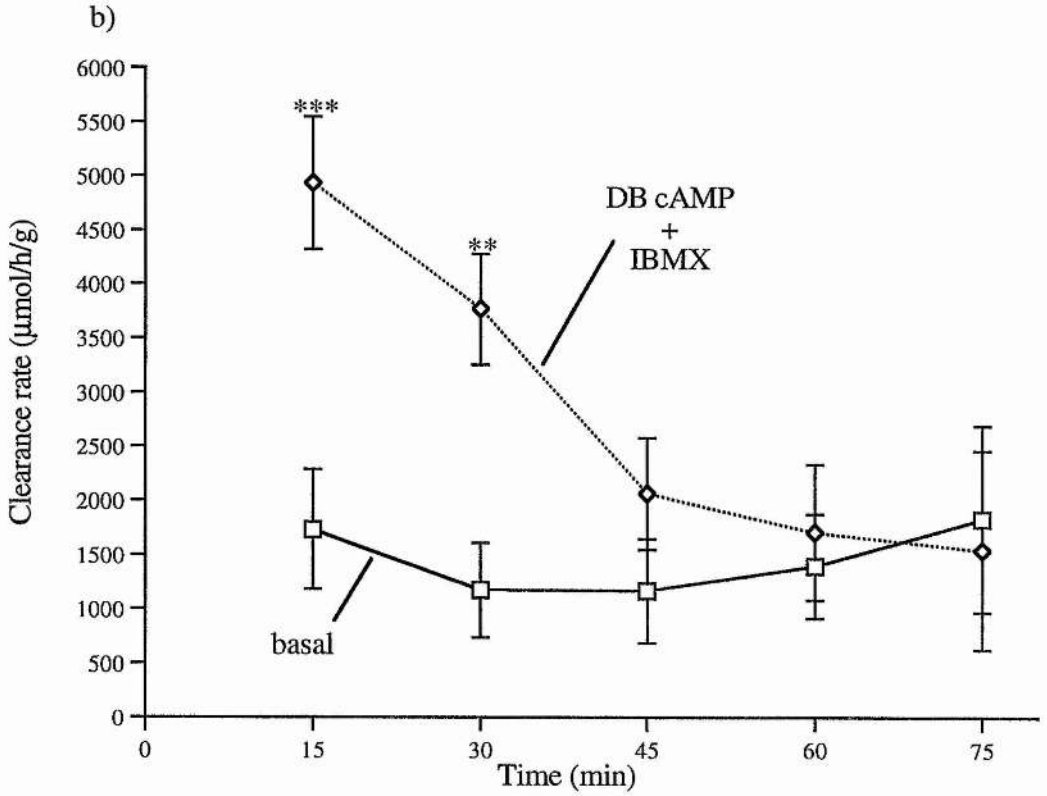
Figure 3.7a Time course of dibutryl cAMP plus IBMX on clearance rates of the isolated perfused rectal gland at the end of an experimental period compared to total basal values.

Clearance rates (µmol/h/g) (ordinate) against time (15 min intervals) (abscissa). Values are expressed as a mean µmol/h/g ± SEM; 1577 ± 477 for basal total; 4935 ± 615 for 0-15 min; 3766 ± 510 for 15-30 min; 2061 ± 515 for 30-45 min; 1703 ± 625 for 45-60 min; and 1539 ± 917 for 60-75 min (n = 6). **p<0.01, ***p<0.001 compared with basal clearance rate (alternative Welch t-test).

Figure 3.7b Time course of dibutryl cAMP plus IBMX on clearance rates of the isolated perfused rectal gland at the end of an experimental period compared to timed collection of basal values.

Clearance rates (µmol/h/g) (ordinate) against time (15 min intervals) (abscissa). Values are expressed as a mean µmol/h/g ± SEM. Basal values were: 1731 ± 550 for 0-15 min; 1171 ± 436 for 15-30 min; 1163 ± 481 for 30-45 min; 1391 ± 480 for 45-60 min; 1827 ± 861 for 60-75 min. Dibutryl cAMP plus IBMX were as figure 3.7 a (n = 6). **p<0.01, ***p<0.01 compared with total basal clearance rate (alternative Welch t-test).


a)
DB cAMP + IBMX
perfusion
***
**
basal
Clearance rate (µmol/h/g)
6000
5500
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
0
15
30
45
60
75
Time (min)


b)
***
**
DB cAMP
+
IBMX
basal
Clearance rate (µmol/h/g)
6000
5500
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
0
15
30
45
60
75
Time (min)

3.4 - Discussion

The aim of this chapter was to establish a standard experimental protocol for isolated perfusion of the rectal gland in *S. canicula*. Two of the principle features of isolated perfusion regardless of physical parameters are: 1) a preparation that produces a near constant basal secretion rate throughout the experimental period: and 2) a preparation that is capable of responding to secretagogues by increasing chloride clearance rates above the basal levels. Furthermore, in achieving these aims the physical parameters established should be representative of *in vivo* physiological conditions. It is clear from the results produced in this chapter that the experimental design facilitates the maintenance of a steady basal chloride clearance rate and a possible increase in chloride clearance rates from the isolated perfused rectal gland of *S. canicula*.

Dorsal aortic blood pressure of *S. canicula* at 15°C was calculated as 3.9kPa (Short, Taylor & Butler, 1979) and 3.8 kPa at 17°C (Butler & Taylor, 1975). The perfusion pressure produced by the rectal gland during perfusion at 100 µl/min in this study was 2.33 ± 0.74 kilo Pascal's (kPa). This however was considerably less than the perfusion flow rate used by Kinne and Kinne-Saffran (1979) of 1-2 ml/min, equating to a pressure of approximately 38 mmHg (5.1 kPa), a value above that of dorsal aortic blood pressure in *S. canicula*. However afferent perfusion pressure in the perfused rectal gland of *S. canicula* was maintained at 17 mmHg (2.28 kPa) by Shuttleworth (1983b). One can assume therefore that a perfusion rate of 100 µl/min was well within normal physiological values with respect to pressure of rectal gland vasculature in *S. canicula*.

Shuttleworth and Thompson (1986) indicated that perfusion flow rate was the important parameter determining secretion rate in isolated perfused rectal glands of *S. acanthias*. Sodium clearance rates from the isolated perfused rectal gland of *S. canicula* were found to progressively decline, below perfusion flow rates of 500 µl/min (Shuttleworth & Thompson, 1986). Although perfusion flow rate in this study was limited to below 300 µl/min (Figure 3.3) it was evident that even at a perfusion rate of 100 µl/min the gland was capable of maintaining basal secretion rate. Rectal glands of *S. acanthias* perfused at 4-9 ml/min, an approximate pressure of 4.89 kPa, had basal chloride clearance rates of 105 ± 15 µmol/h/g (Stoff *et al.*, 1977). Compared to the present study (813 ± 205 µmol/h/g) this seems very low. However these investigators were able to stimulate the rectal gland secretion rate of *S. acanthias* approximately 10 fold upon the addition of 1mM theophylline to the perfusate.

In response to dibutryl cAMP plus IBMX secretion rates for rectal gland fluid collected were elevated above basal levels, chloride concentrations of this fluid did not vary within or between preparations. A similar situation was reported by Shuttleworth & Thompson (1986) when investigating the effect of increasing perfusion flow rate on sodium concentration of secretory fluid from the isolated rectal gland of *S. canicula*. One could assume therefore that the principle variable effecting rectal gland secretion rate was a change in secretory volume rather than changes in chloride concentration of rectal gland fluid.

It is well documented in the literature that an increase in chloride secretion from the rectal gland acts via a cAMP pathway (Shuttleworth & Thompson, 1980b; Stoff *et al.*, 1977). It would appear from the present study that cAMP had no effect on chloride clearance rate of the isolated perfused rectal gland of *S. canicula*. However, addition of phosphodiesterase inhibitors to the perfusion media increased secretion significantly above basal levels. The increased potency of IBMX on chloride clearance rates compared to theophylline, was perhaps due to the fact that IBMX is a more lypophilic compound than theophylline and therefore at similar concentrations a reduced response to theophylline was observed. A dose response curve for theophylline on the isolated perfused preparation of *S. acanthias* (Silva *et al.*, 1977) showed an increase in theophylline concentration in the perfusion media, produced a concomitant increase in chloride clearance rates from the rectal gland.

If increased secretory activity is mediated by a cAMP pathway one would anticipate that a combination of cAMP plus phosphodiesterase inhibitors (preventing the breakdown of intrinsic and extrinsic cAMP) would produce an increase in chloride clearance rates above that produced by the perfusion of the inhibitor alone, however, this was not the case. A similar effect was described by Shuttleworth and Thompson (1980b) where cAMP did not significantly stimulate ouabain inhibited oxygen consumption from rectal gland slices of *S. canicula*. The addition of theophylline to the media caused a significant rise in oxygen consumption, however this increase was no greater when cAMP and theophylline were added together (Shuttleworth & Thompson, 1980 b). However a synergistic stimulatory effect of theophylline and cAMP was observed in the isolated perfused rectal gland of *S. acanthias* (Silva *et al.*, 1977).

The apparent discrepancy between these two studies is difficut to explain. It is possible that there is sufficient intrinsic phosphodiesterase activity to degrade completely any cAMP perfused, and therefore, basal chloride clearance rates would be maintained. Hence the subsequent addition of phosphodiesterase

inhibitors alone would increase intrinsic cAMP concentration to cause maximal stimulation. However the concentration of dibutryl cAMP (0.25 mM) perfused in this study, would be expected to cause an increase in chloride clearance rates unless intrinsic phosphodiesterase activity was remarkably high.

Another possible explanation is that IBMX has also been reported to displace bound ATP from the first nucleotide binding domain of mammalian CFTR, and thus IBMX or related compounds may directly influence activation of the CFTR channel (Logan, Hiestand, Huang, Muccio, Haley, & Sorcher, 1993). This is however a highly controversial concept and requires further research to confirm the initial report.

The apparent absence of cAMP stimulation in the isolated perfused rectal gland of *S. canicula* in this study remains problematical although it is in agreement with previous studies (Shuttleworth & Thompson, 1980a). One possible experimental approach which may in the future be assessed is the perfusion of the adenylate cyclase stimulant, forskolin, which would confirm or disprove the concept of cAMP stimulation in rectal gland epithelia of *S. canicula*. This would be an immediate requirement for any further perfusion studies in this species of elasmobranch, particularly when perfusion of forskolin in the isolated perfused rectal gland of *S. acanthias* produces increases in chloride secretion rate approximately 10 times above basal (Silva, Stoff, Leone & Epstein, 1985). Forskolin was also shown to stimulate isolated cultured epithelial cells of the rectal gland of *S. acanthias* (Karnaky, Valentich, Currie, Oehlienschlager & Kennedy, 1991).

The standard protocol developed in this chapter was used to test the effects of various peptides on the chloride clearance rates of *in vitro* perfused rectal gland of *S. canicula*. The results and possible modes of action of some of these peptides are illustrated in the following chapters.

CHAPTER 4
HORMONAL CONTROL OF RECTAL GLAND SECRETION RATE

4.1 - Introduction

Burger (1962) first observed an intermittent pattern of secretion from the rectal gland of *S. acanthias* which was attributed to a "daily rhythm". Subsequent investigations on rectal gland function in *S. acanthias* and *S. canicula* have reported a similar pattern of intermittent activity (Burger, 1962; Stoff, *et al.*, 1979; Kent & Olson, 1982; Shuttleworth 1988).

One aspect of the intermittent behaviour of rectal gland activity is rapid activation of secretion, leading one to suggest the influence of neuroendocrine and/or autocrine factor/s on rectal gland secretion. Initial histological investigations did not find significant amounts of neuronal tissue in the rectal gland (Chan, Phillips & Chester Jones, 1967). However this study was limited in its investigation. More recently vasoactive intestinal peptide-like (VIP) and bombesin-like (neurotransmitter) immunoreactive nerve fibres have been observed, following the radially arranged secretory tubules and also around the central excretory duct of the rectal gland of *S. acanthias* (Holmgren & Nilsson, 1983).

Since the rectal gland is an appendage of the elasmobranch hindgut, a number of intestinal peptides that stimulate gastrointestinal activity were tested as possible activators in the isolated perfused rectal gland of *S. acanthias* (Stoff *et al.*, 1979). The only significant secretagogue was a porcine analogue of VIP, a stimulatory effect which was also observed in isolated perfused tubules of *S. acanthias* (Forrest, Wang, & Beyenbach, 1983). Although there is little doubt that VIP induces an increase in secretion of the isolated perfused rectal gland and rectal gland tubules of *S. acanthias*, the argument for VIP as a universal stimulant for rectal gland secretion in elasmobranchs is not convincing. In the crossed perfused studies carried out by Solomon, Taylor, Dorsey, Silva and Epstein (1985a) and Solomon, Taylor, Sheth, Silva and Epstein (1985b), where the isolated gland of *S. acanthias* was perfused via a donor fish, VIP did not elucidate an increase in secretion rate from the rectal gland of *S. acanthias* (Solomon *et al.*, 1985a). Studies by Shuttleworth and Thorndyke (1984) on rectal gland slices of the lesser spotted dogfish, *Scyliorhinus canicula*, and the ray, *Raja clavata*, indicated that porcine VIP was without effect even at high concentrations. Furthermore, even endogenous VIP isolated from *S. canicula* (Dimaline & Thorndyke, 1985) did not stimulate isolated perfused rectal glands of *S. canicula* (Thorndyke & Shuttleworth, 1985).

In vivo studies illustrated that rectal glands maintained increased secretory activity for more than 2-3 hours (Burger, 1962). Such a lengthy period of activity suggests hormonal influence on long-term increased rectal gland secretion. In relation to this, the role of natriuretic factors in rectal gland function have been extensively studied. Using the isolated *in vitro* preparation it was found that serum taken from *S. acanthias* following

extracellular volume expansion stimulated secretion from the isolated perfused rectal gland, whereas serum taken before expansion did not (Solomon *et al.*, 1985a). Furthermore, atrial and ventricle extracts from *S. acanthias* also stimulated rectal gland secretion (Solomon *et al.*, 1985a). Atriopeptin, a synthetic analogue of mammalian atrial natriuretic peptide (ANP) was tested on the same preparation and was found to significantly increase chloride secretion rate (Solomon *et al.*, 1985a). Similar results were obtained from the isolated perfused rectal gland of *S. acanthias* following perfusion of rat atrial natriuretic peptide and endogenous heart extracts (Silva *et al.*, 1985). The endogenous C-type natriuretic peptide or sCNP was found to stimulate secretion rate 7 to 8 times above basal levels in the isolated perfused rectal gland of *S. acanthias* (Solomon, Protter, McEnroe, Porter, & Silva, 1992).

Although the stimulatory effect of sCNP on secretion of the isolated perfused rectal gland of *S. acanthias* is in no doubt, the mode of action is somewhat unknown. It was found that mammalian ANP had no effect on isolated tubules or single cells suggesting that a whole gland preparation was required for heterologous natriuretic peptide stimulation of secretion (Silva, Stoff, Solomon, Lear, Kniaz, Greger & Epstein, 1987). These workers argued that mammalian ANP-stimulated chloride secretion from the isolated rectal gland of *S. acanthias* was secondary to the action of mammalian ANP in releasing endogenous VIP, which then triggered an increase in secretion. However, the addition of atriopeptin to cell cultures increased chloride dependent, bumetanide sensitive, short-circuit current, thus suggesting that atriopeptin directly stimulated secretion in isolated cells of the rectal gland of *S. acanthias* (Karnaky *et al.*, 1991). This was further supported when endogenous sCNP was found to directly stimulate rectal gland cells of *S. acanthias* (Silva, Epstein & Solomon, 1993).

Recently two types of CNP receptors have been identified on plasma membrane preparations of the rectal gland of *S. acanthias*, one which is linked to a guanylate cyclase pathway (therefore considered to be an active receptor), and the other, a low molecular weight receptor with no known links to a second messenger system (therefore considered to be inactive, possibly a 'clearance receptor') (Gunning, Cuero, Solomon & Silva, 1993). sCNP has been linked to a guanylate cyclase pathway and therefore one would anticipate the second messenger cyclic guanosine monophosphate (cGMP) to increase secretion from rectal gland epithelia. However, this was not observed following perfusion of the cell permeable analogue 8-Br cGMP in the isolated perfused preparation of *S. acanthias* (Silva *et al.*, 1987), or upon the addition of 8-Br cGMP to cell cultures of *S. acanthias* (Silva *et al.*, 1993). However, the addition of 10^{-7} M atriopeptin to cell cultures of *S. acanthias* was found to increase intracellular levels of cGMP 10 fold (Karnaky *et al.*, 1991).

Glucagon, a peptide involved in metabolism in mammals, has been characterised in the elasmobranch *S. canicula* (Conlon, O'Toole, & Thim, 1987). Plasma levels of glucagon may increase following feeding in mammals, although, whether the same response occurs in elasmobranchs is as yet unknown. From the assumption that plasma glucagon levels could rise post-feeding it is possible that glucagon has an effect on rectal gland secretion when the need for rectal gland activity is highest. However, heterologous glucagon was tested as a possible secretagogue on the isolated perfused rectal gland of *S. acanthias* (Stoff *et al.*, 1979) and did not influence secretion in the rectal gland.

A caudal neurosecretory system has been identified in elasmobranchs by Fridberg (1962) with subsequent identification of the peptide Urotensin II (U II) from *S. canicula* (Conlon, O'Harte, Smith, Balment, & Hazon, 1992). In teleosts it is evident that the urotensins (I & II) play a major role in osmoregulation. It is a possibility therefore that the endogenous elasmobranch U II could have an effect on rectal gland secretion particularly in the light of the cardiovascular effects of U II in *S. canicula* (Hazon, *et al.*, 1993).

Given the central role of the rectal gland in maintaining ionic balance and volume regulation it is possible that numerous peptides may be involved in the hormonal control of rectal gland secretion. The effects of a variety of peptides on the secretion rate from the *in vitro* perfused rectal gland preparation of *S. canicula* were observed and the results are presented in section 4.3.

4.2 - Materials and Methods

4.2a - *In vitro* cannulation and perfusion.

Dogfish were caught and stored as described in section 2.2. Cannulation and perfusion was carried out as described in section 3.2, with a dose of dibutryl cAMP (0.25 mM) plus IBMX (1mM) administered at the beginning and end of the experiment to ensure the viability of the preparations. The following peptides were administered in a random dose on the isolated preparation in the same protocol as for the dibutryl cAMP plus IBMX dose.

1) Endogenous *Scyliorhinus* urotensin II (U II) supplied by Professor M. Conlon, Creighton University, Omaha, Nebraska (Conlon *et al.*, 1992)
2) Endogenous *Scyliorhinus* glucagon obtained from Peninsula laboratories, St. Helens, England (Conlon *et al.*, 1987).
3) Porcine vasoactive intestinal peptide (VIP) obtained from Sigma Chemicals Co, Poole, Dorset, England (Bodansky, Klausner & Said, 1973).
4) Endogenous *Scyliorhinus* C-type natriuretic peptide (sCNP) supplied by Dr. Y. Takei, Ocean Research Institute, Tokyo, Japan (Suzuki *et al.*, 1991).
5) 8-Bromo cyclic guanosine monophosphate (8-Br cGMP) obtained from Sigma Chemicals Co.

4.2b - *In vivo* cannulation and perfusion.

S. canicula of mixed sex ranging from 0.5 to 1.3 kg, were anaesthetised in tricaine methanosulphonate (Sigma) (MS222; 1:10,000). A ventro-lateral incision was made posterior to the pectoral fin to expose the coeliac vein and artery. Cannula (PE 50) filled with elasmobranch Ringer were introduced into these two vessels with the coeliac arterial cannula inserted as close to the dorsal aorta as possible. The cannulae were then tied off with surgical silk (EP4) and sodium Heparin was introduced to prevent blood clotting in the cannulae. The free ends of the cannulae were blocked with pins and the incision sutured. A ventrolateral incision was then made at the hind part of the abdominal cavity to expose the rectal gland and posterior intestine. Pulled out cannulae (PE 50), filled with elasmbranch Ringer were used to cannulate the rectal gland duct and vein. The rectal gland vein was effectively bridged with approximately 1.5 cm of cannula as tying off the duct cannula on its own would have occluded the venous return. The incision was then sutured and the fish placed back in the water to recover. The duct cannula was left open ended during a 48 hr recovery period prior to further experimentation.

Fish were perfused with elasmobranch Ringer at 100 µl/min via the coeliac artery to establish a basal chloride clearance rate from the rectal gland.

Secretory fluid was collected in the "finger tips" cut from surgical gloves, over hourly periods. Following the initial basal period, 10^{-7} M sCNP was perfused for the following hour, after which Ringer was perfused for the next 2 hours to monitor changes in rectal gland chloride clearance rates.

4.3 - Results

Figure 4.1 illustrates the effects of homologous urotensin II on the isolated perfused rectal gland. Dibutryl cAMP plus IBMX stimulated mean chloride clearance rates almost 3 fold above basal levels, both pre- and post-experimental period. Urotensin II at 10^{-12} M and 10^{-9} M did not effect chloride clearance rates and although a concentration of 10^{-6} M did cause an increase, it was not significantly greater than basal chloride clearance rates.

Figure 4.2 illustrates the effects of homologous glucagon on the isolated perfused rectal gland. Dibutryl cAMP plus IBMX stimulated these preparations almost 2 fold above basal levels, both pre- and post-experimental period. Glucagon at 10^{-12} M and 10^{-9} M did not effect chloride clearance rates and although a concentration of 10^{-6} M did cause an increase it was not significantly greater than basal chloride clearance rates over a period of 75 min. However, figures 4.3 a & b illustrate a time course of secretion rate over 15 min periods during and after perfusion of 10^{-6} M glucagon, compared with a time course of basal secretion rate. During the 15 min period of 10^{-6} M glucagon perfusion there was a significant increase in chloride clearance rates above basal chloride clearance rates from the perfused rectal gland (Figure 4.3 a & b).

Figure 4.4 illustrates the effects of porcine VIP on the isolated perfused rectal gland. Dibutryl cAMP plus IBMX stimulated these preparations almost 4.5 fold above basal levels, both pre- and post-experimental period. At concentrations of 10^{-12}, 10^{-9}, and 10^{-6} M porcine VIP failed to produce a significant increase above basal chloride clearance rates. Although at 10^{-12} M porcine VIP chloride clearance rates were higher than basal values this was not a significant difference.

Figure 4.5 illustrates the effects of homologous sCNP on the isolated perfused rectal gland. Dibutryl cAMP plus IBMX stimulated these preparations almost 2 fold above basal levels, both pre- and post-experimental period. sCNP administered via the perfusate at concentrations of 10^{-12} and 10^{-9} M, did not stimulate chloride clearance rates. However, at 10^{-8}, 10^{-7}, and 10^{-6} M sCNP significantly increased chloride clearance rates from the preparations, which was maximal at 10^{-7} M sCNP. Figures 4.6 a, b, & c illustrate the time course of secretion rate over 15 min periods both during and after perfusion of sCNP. Basal values, however, were obtained over a period of 75 min prior to perfusion of peptide. Interestingly, stimulatory effects of sCNP at 10^{-8}, 10^{-7}, and 10^{-6} M are all significant only during the period of peptide perfusion. At 10^{-7} M sCNP there was almost a 4 fold increase in chloride clearance rate; 10^{-6} M sCNP

FIGURE 4.1

Figure 4.1 Effect of increasing doses of homologous urotensin II together with the effects of dibutryl cAMP plus IBMX before and after peptide perfusion on the clearance rate of the isolated perfused rectal gland. Clearance rates (μmol/h/g) (ordinate) against treatment (abscissa). Values are expressed as a mean μmol/h/g, ± SEM; 1103 ± 245 for basal; 2955 ± 264 for pre-cAMP plus IBMX; 833 ± 260 for 10^{-12} U II; 642 ± 218 for 10^{-9} U II; 1692 ± 224 for 10^{-6} U II; and 2724 ± 374 for post-cAMP plus IBMX (n= 14, 14, 14, 8, 11, & 6 respectively). **p<0.01, ***p<0.001 compared to total basal clearance rate (alternative Welch t-test).

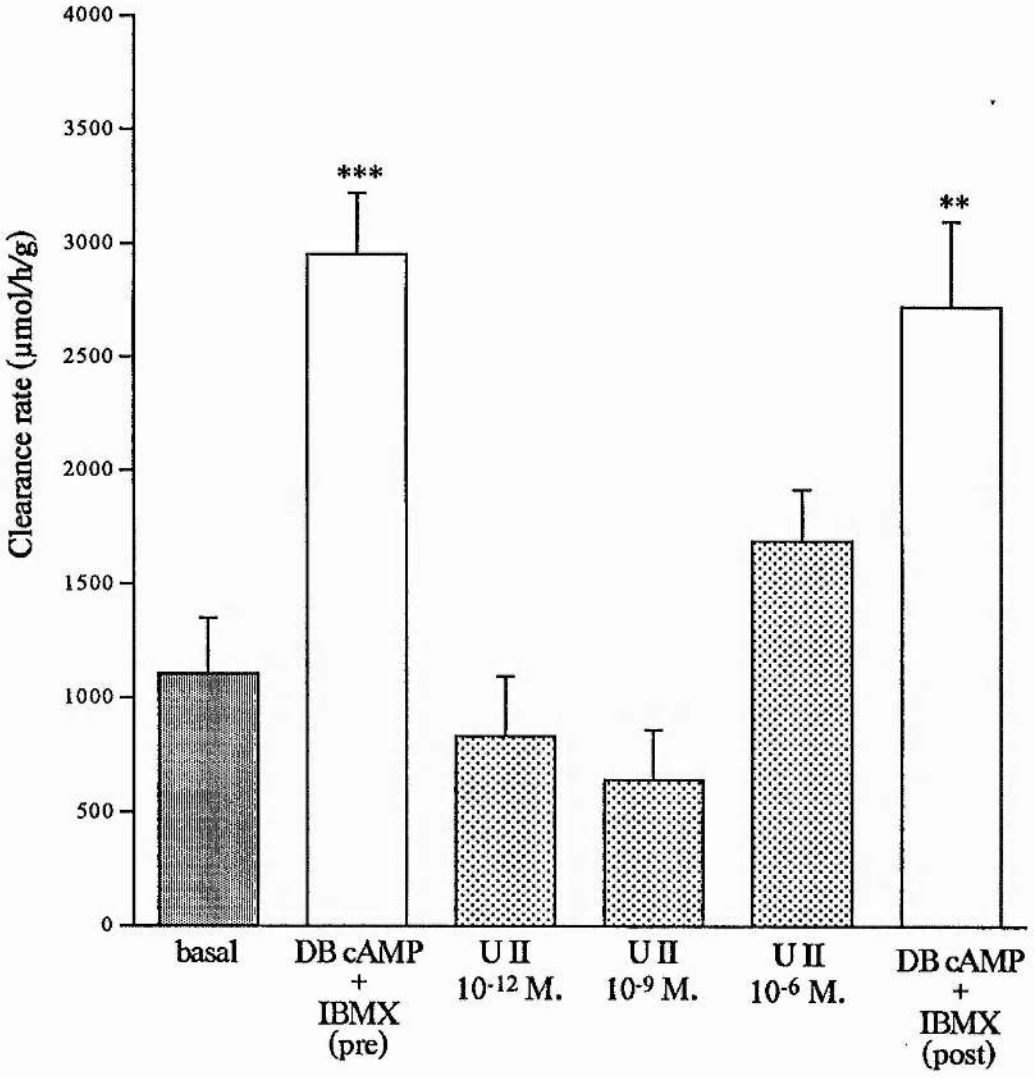

Clearance rate (µmol/h/g)
4000
3500
3000
2500
2000
1500
1000
500
0
***
**
basal
DB cAMP + IBMX (pre)
U II 10-12 M.
U II 10-9 M.
U II 10-6 M.
DB cAMP + IBMX (post)

FIGURE 4.2

Figure 4.2 Effect of increasing doses of homologous glucagon together with the effects of dibutryl cAMP plus IBMX before and after peptide perfusion on the clearance rate of the isolated perfused rectal gland.
Clearance rates (µmol/h/g) (ordinate) against treatment, (abscissa). Values are expressed as a mean µmol/h/g, ± SEM; 1457 ± 477 for basal; 3033 ± 533 for pre-cAMP plus IBMX; 1198 ± 273 for 10^{-12} glucagon; 1258 ± 564 for 10^{-9} glucagon; 2616 ± 648 for 10^{-6} glucagon; and 2801 ± 594 for post-cAMP plus IBMX (n= 6). **p<0.01, ***p<0.001 compared to basal clearance rates (alternative Welch t-test).

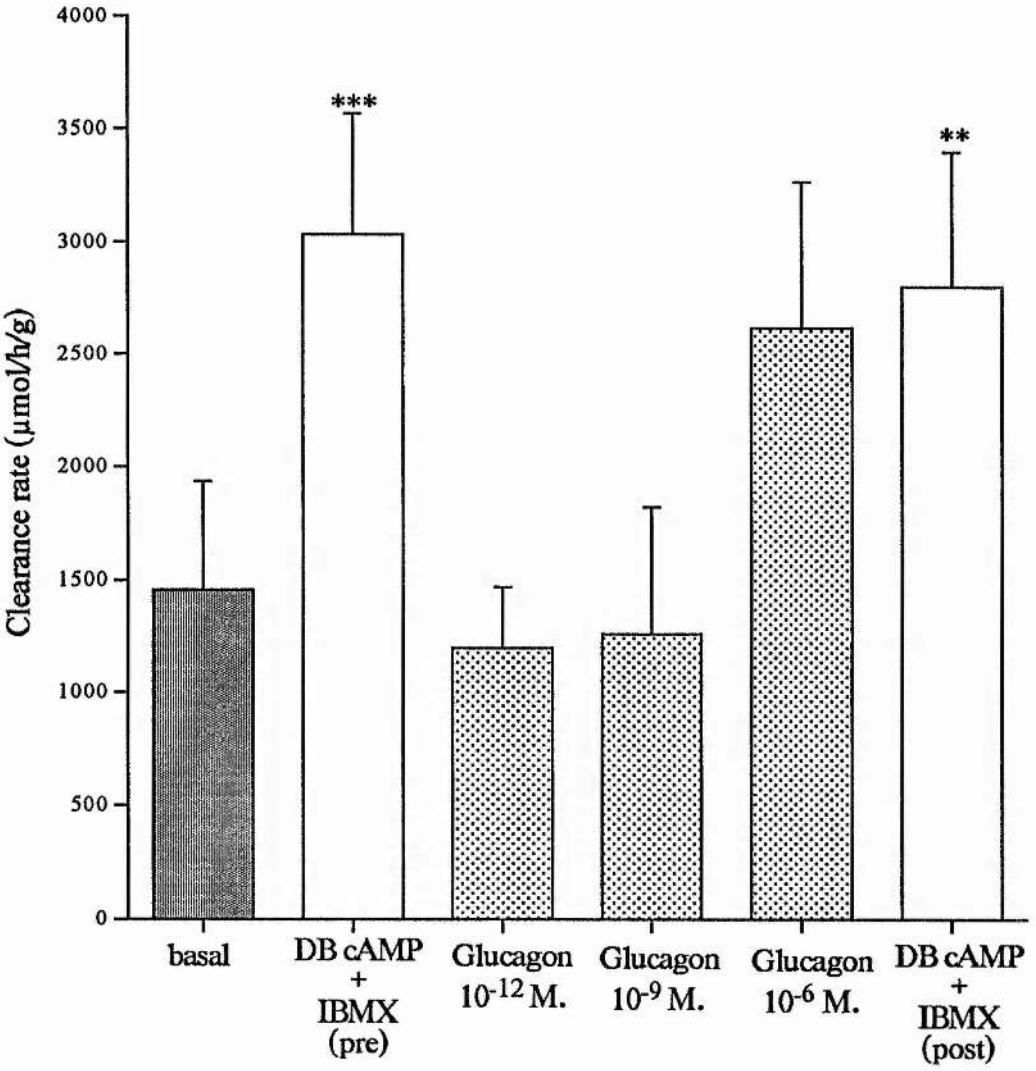

Clearance rate (µmol/h/g)
4000
3500
3000
2500
2000
1500
1000
500
0
***
**
basal
DB cAMP + IBMX (pre)
Glucagon 10-12 M.
Glucagon 10-9 M.
Glucagon 10-6 M.
DB cAMP + IBMX (post)

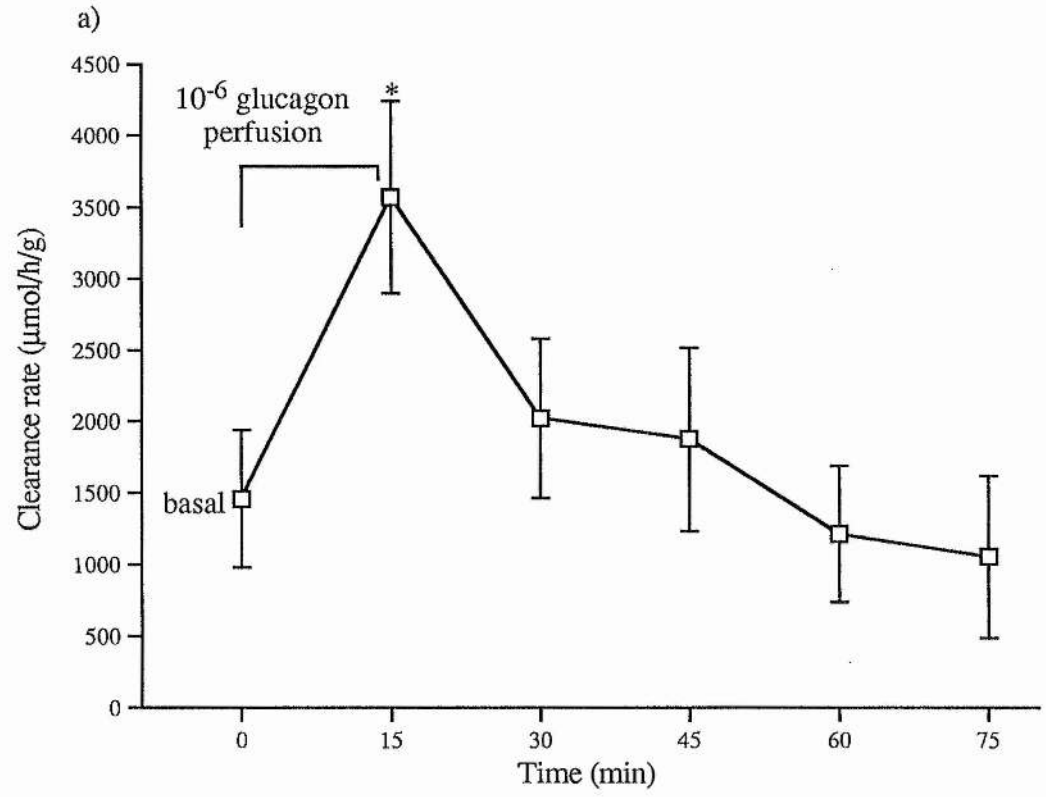
FIGURE 4.3 a & b

Figure 4.3a Time course of 10^{-6} M homologous glucagon on clearance rates of the isolated perfused rectal gland compared to total basal values.

Clearance rates (µmol/h/g) (ordinate) against time (15 min intervals) (abscissa). Values are expressed as a mean µmol/h/g ± SEM; 1457 ± 477 for basal total; 3569 ± 672 for 0-15 min; 2020 ± 560 for 15-30 min; 1872 ± 642 for 30-45 min; 1211± 473 for 45-60 min; and 1052 ± 565 for 60-75 min (n = 6). *$p<0.05$ compared with basal clearance rates (alternative Welch t-test).

Figure 4.3b Time course of 10^{-6} M homologous glucagon on clearance rates of the isolated perfused rectal gland compared to time course of basal values.

Clearance rates (µmol/h/g) (ordinate) against time (15 min intervals) (abscissa). Values are expressed as a mean µmol/h/g ± SEM. Basal values were; 1731 ± 550 for 0-15 min; 1171 ± 436 for 15-30 min; 1163 ± 481 for 30-45 min; 1391 ± 480 for 45-60 min; and 1827 ± 481 for 60-75 min. 10^{-6} M Glucagon values are as above, (n = 6). *$p<0.05$ compared with total basal clearance rates (alternative Welch t-test).


a)
4500
4000
3500
3000
2500
2000
1500
1000
500
0
Clearance rate (µmol/h/g)
10^{-6} glucagon perfusion
*
basal
0
15
30
45
60
75
Time (min)

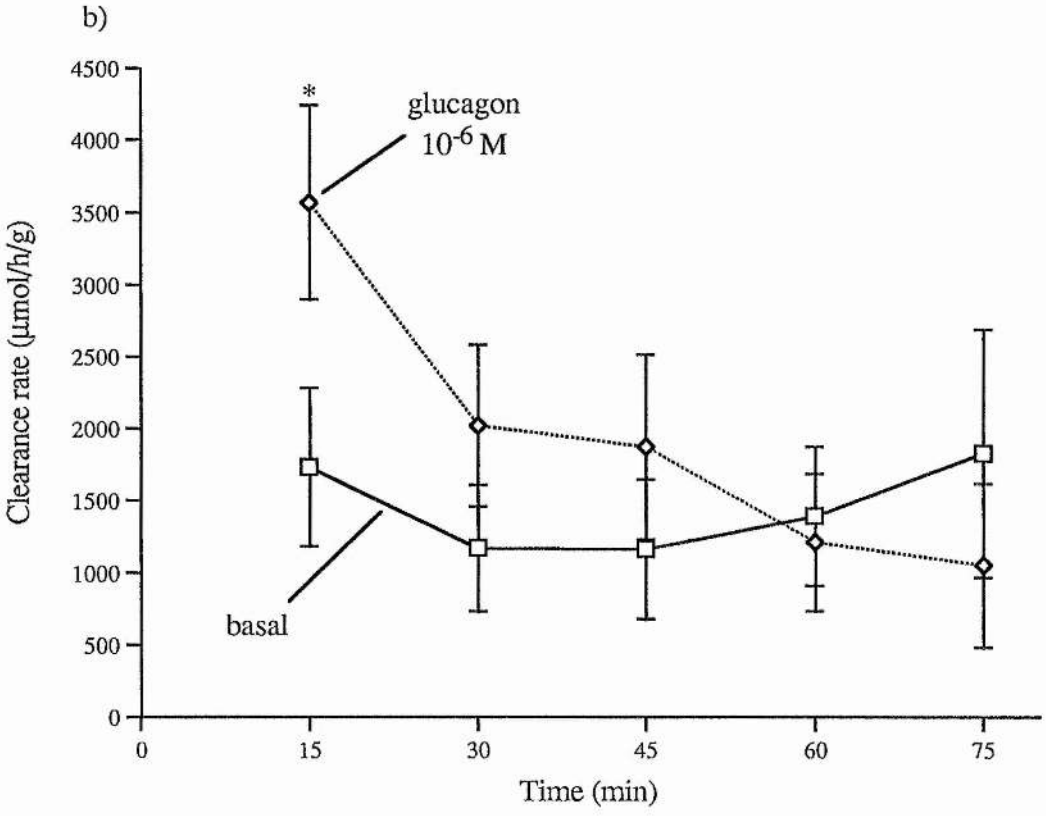

b)
4500
4000
3500
3000
2500
2000
1500
1000
500
0
Clearance rate (µmol/h/g)
*
glucagon
10^{-6} M
basal
0
15
30
45
60
75
Time (min)

FIGURE 4.4

Figure 4.4 Effect of increasing doses of porcine VIP together with the effects of dibutryl cAMP plus IBMX before and after peptide perfusion on the clearance rate of the isolated perfused rectal gland.

Clearance rates (µmol/h/g) (ordinate) against treatment (abscissa). Values are expressed as a mean µmol/h/g, ± SEM; 462 ± 112 for basal; 2320 ± 352 for pre-cAMP plus IBMX; 1040 ± 335 for 10^{-12} VIP; 698 ± 258 for 10^{-9} VIP; 927 ± 279 for 10^{-6} VIP; and 2321 ± 359 for post-cAMP plus IBMX (n=12, 12, 9, 8, 10, & 6 respectively). ***$p<0.001$ compared to basal clearance rates (alternative Welch t-test).

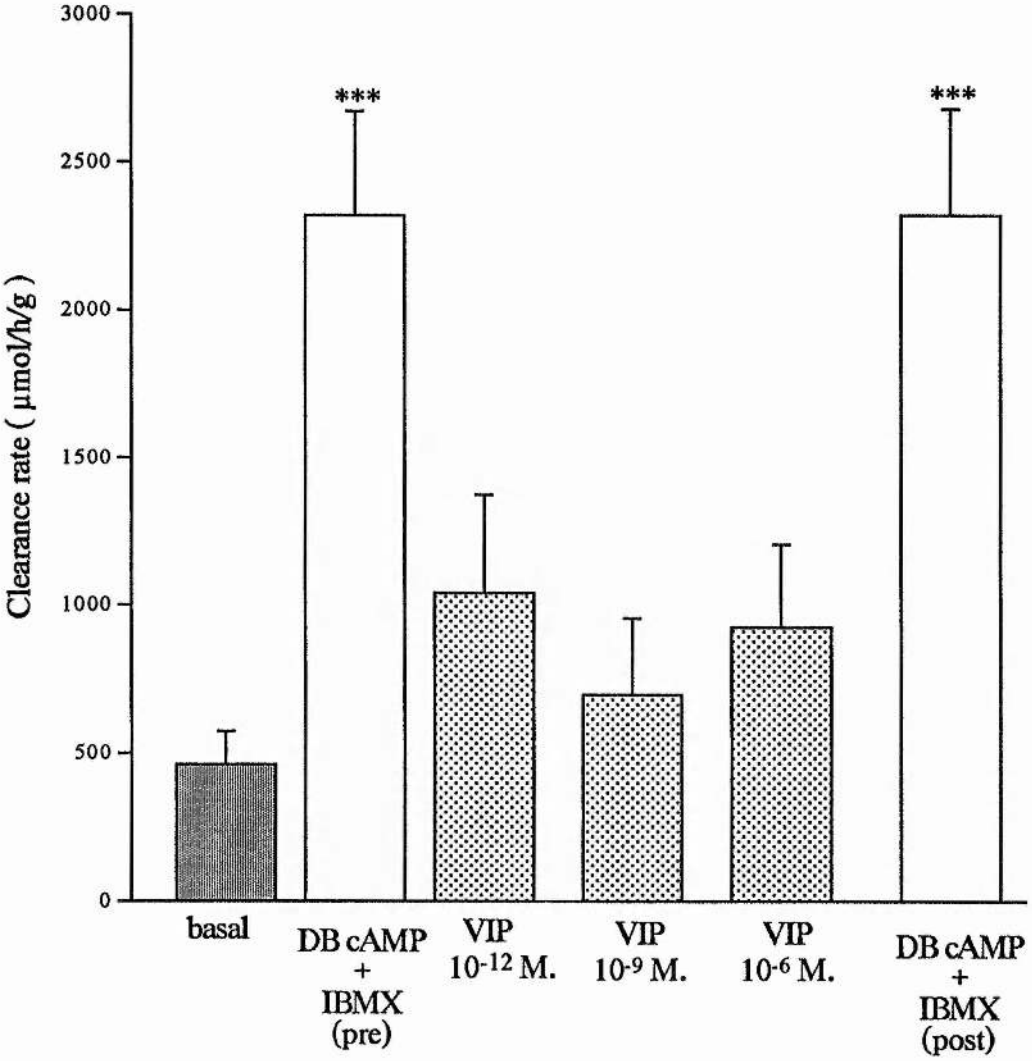

Clearance rate (µmol/h/g)
3000
2500
2000
1500
1000
500
0
***
***
basal
DB cAMP + IBMX (pre)
VIP 10-12 M.
VIP 10-9 M.
VIP 10-6 M.
DB cAMP + IBMX (post)

FIGURE 4.5

Figure 4.5 Effect of increasing doses of homologous sCNP together with the effects of dibutryl cAMP plus IBMX before and after peptide perfusion on the clearance rate of the isolated perfused rectal gland.
Clearance rates (µmol/h/g) (ordinate) against treatment (abscissa). Values are expressed as a mean µmol/h/g, ± SEM; 1445 ± 291 for basal; 2897 ± 255 for pre-cAMP plus IBMX; 1513 ± 315 for 10^{-12} sCNP; 1191 ± 579 for 10^{-9}sCNP; 2454 ± 360 for 10^{-8} sCNP; 3131 ± 471 for 10^{-7} sCNP, 2518 ± 470 for 10^{-6} sCNP; and 2522 ± 254 for post-cAMP plus IBMX (n= 22, 22, 5, 9, 11, 9, 9, & 12 respectively). *p<0.05, **p<0.01, ***p<0.001 compared to basal clearance rates, (alternative Welch t-test).

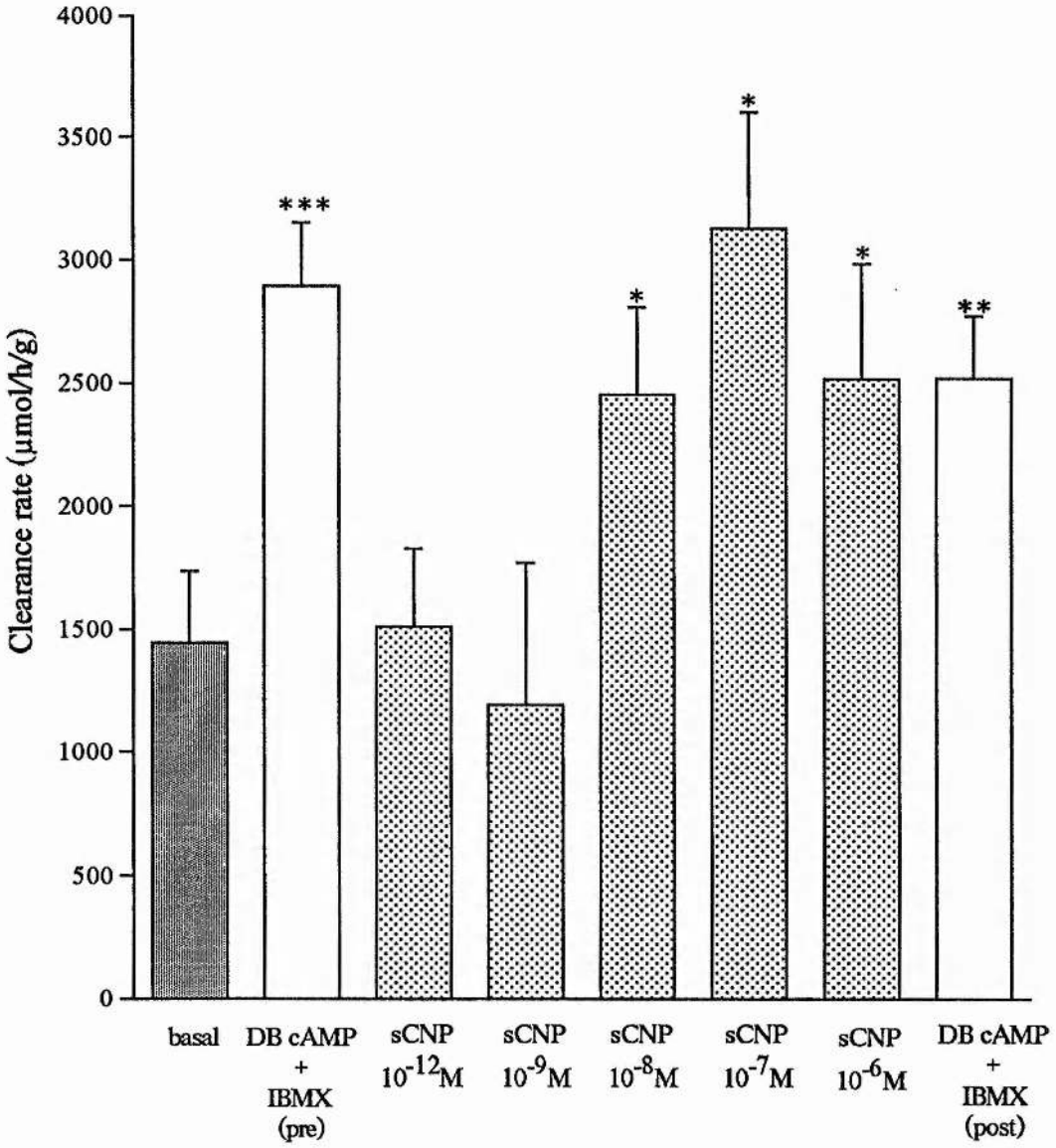
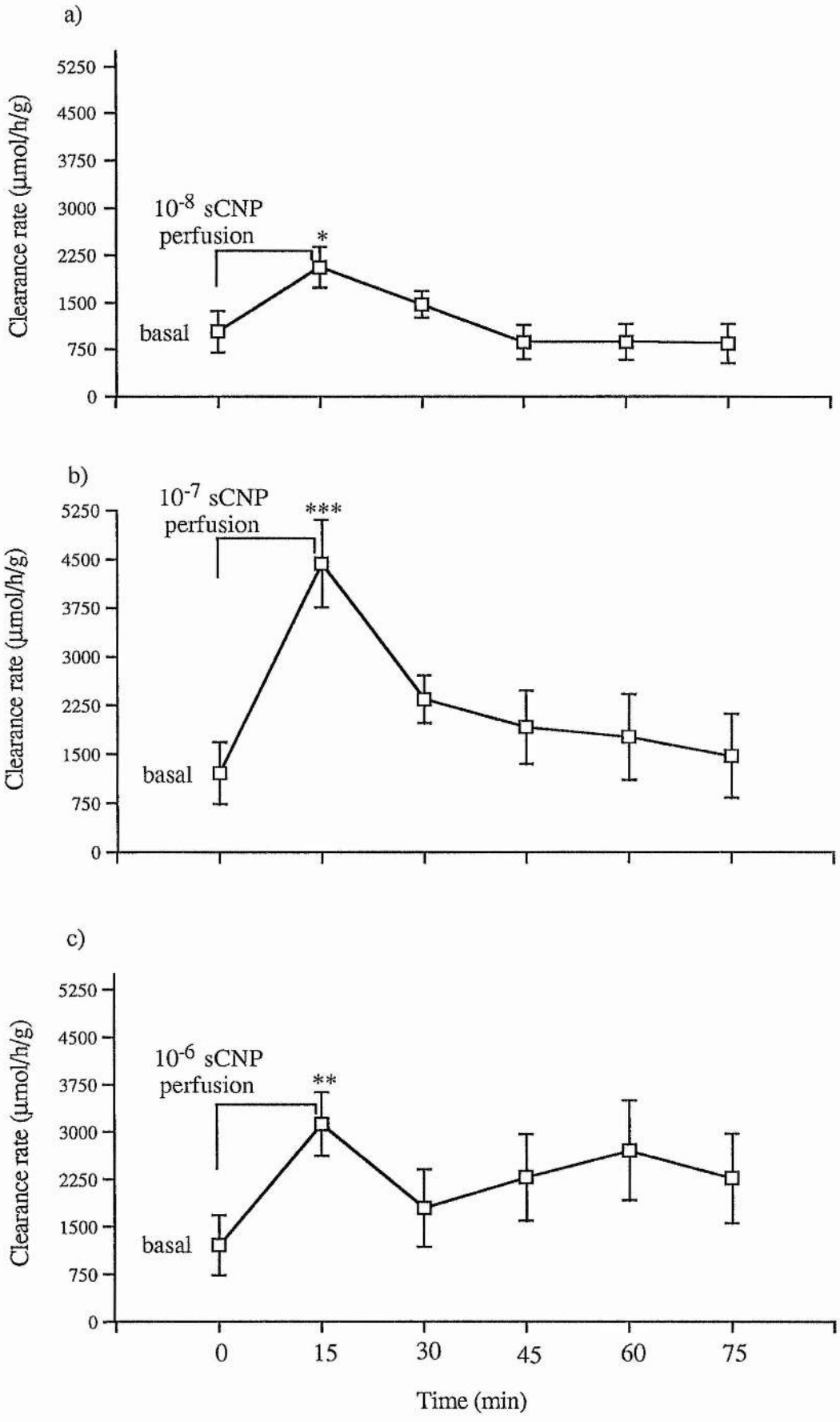

4000
3500
3000
2500
2000
1500
1000
500
0
Clearance rate (μmol/h/g)
***
*
*
*
*
**
basal
DB cAMP + IBMX (pre)
sCNP 10^{-12}M
sCNP 10^{-9}M
sCNP 10^{-8}M
sCNP 10^{-7}M
sCNP 10^{-6}M
DB cAMP + IBMX (post)

FIGURE 4.6 a, b & c.

Figure 4.6a Time course of 10^{-8} M homologous sCNP on clearance rates of the isolated perfused rectal gland compared to total basal value.
Clearance rates (µmol/h/g) (ordinate) against time (15 min intervals) (abscissa). Values are expressed as a mean µmol/h/g ± SEM; 1033 ± 333 for basal total; 2061 ± 324 for 0-15 min; 1470 ± 214 for 15-30 min; 864 ± 273 for 30-45 min; 868 ± 286 for 45-60 min; and 844 ± 313 for 60-75 min (n =12). *p<0.05 compared with total basal clearance rates (alternative Welch t-test).

Figure 4.6b Time course of 10^{-7} M homologous sCNP on clearance rates of the isolated perfused rectal gland compared to total basal value.
Clearance rates (µmol/h/g) (ordinate) against time (15 min intervals) (abscissa). Values are expressed as a mean µmol/h/g ± SEM; 1210 ± 474 for basal total; 4435 ± 675 for 0-15 min; 2347 ± 372 for 15-30 min; 1915 ± 563 for 30-45 min; 1765 ± 663 for 45-60 min; and 1475 ± 641 for 60-75 min (n =8). ***P<0.001 compared with total basal clearance rates (alternative Welch t-test).

Figure 4.6c Time course of 10^{-6} M homologous sCNP on clearance rates of the isolated perfused rectal gland compared to total basal value.
Clearance rates (µmol/h/g) (ordinate) against time (15 min intervals) (abscissa). Values are expressed as a mean µmol/h/g ± SEM; 1210 ± 474 for basal total; 3122 ± 502 for 0-15 min; 1795 ± 612 for 15-30 min; 2280 ± 686 for 30-45 min; 2700 ± 788 for 45-60 min; and 2264 ± 706 for 60-75 min (n =8). **P<0.01 compared with total basal clearance rates (alternative Welch t-test).


a)
Clearance rate (µmol/h/g)
5250
4500
3750
3000
2250
1500
750
0
10^{-8} sCNP perfusion
*
basal
b)
Clearance rate (µmol/h/g)
10^{-7} sCNP perfusion
***
basal
c)
Clearance rate (µmol/h/g)
10^{-6} sCNP perfusion
**
basal
0
15
30
45
60
75
Time (min)

produced an approximate 3 fold increase in chloride clearance rate; and at 10^{-8} M sCNP an approximate 2 fold increase in chloride clearance rate above basal levels.

It was evident in the present study that sCNP stimulated chloride clearance rates in the isolated perfused rectal gland of *S. canicula*. Natriuretic peptides are in general thought to act via the second messenger system guanylate cyclase thus altering intracellular concentrations of cGMP (Brenner *et al.*, 1990). Consequently the cell permeable analogue of cGMP, 8-Bromo-cGMP was tested on the *in vitro* rectal gland preparations. Figure 4.7 illustrates the effects of 8 Br-cGMP on the isolated perfused rectal gland. Dibutryl cAMP plus IBMX stimulated these preparations over 2 fold above basal chloride clearance rates both pre- and post-experimental period. Although 10^{-8} M cGMP did not significantly increase chloride clearance rates above basal levels, values were approximately 60% higher than basal.

Having observed a stimulatory effect of sCNP *in vitro* the peptide was used to try and stimulate secretion *in vivo*. Figure 4.8 illustrates the effect of 10^{-7} M sCNP on chloride clearance rates of the rectal gland *in vivo*. Due to the intermittent nature of the gland, basal secretion rates were highly variable and were therefore collected over a 2 hour period. Figure 4.8 shows that sCNP at 10^{-7} M did have a significant effect on rectal gland chloride clearance rates although this effect was somewhat delayed following perfusion of the peptide for 1 hour (section 5.1).

FIGURE 4.7

Figure 4.7 Effect of increasing doses of 8-Bromo cyclic guanosine monophosphate together with the effects of dibutryl cAMP plus IBMX before and after peptide perfusion on the clearance rate of the isolated perfused rectal gland.

Clearance rates (µmol/h/g), (ordinate) against treatment, (abscissa). Values are expressed as a mean µmol/h/g, ± SEM; 1428± 419 for basal; 3592 ± 714 for pre-cAMP plus IBMX; 2692 ± 1174 for 10^{-8} cGMP; 2323 ± 761 for 10^{-6} cGMP; 2142 ± 877 for 10^{-4} cGMP; and 2724 ± 916 for post-cAMP plus IBMX (n = 9, 9, 5, 7, 8, & 6, respectively). *$p<0.05$, compared to basal clearance rates (alternative Welch t-test).

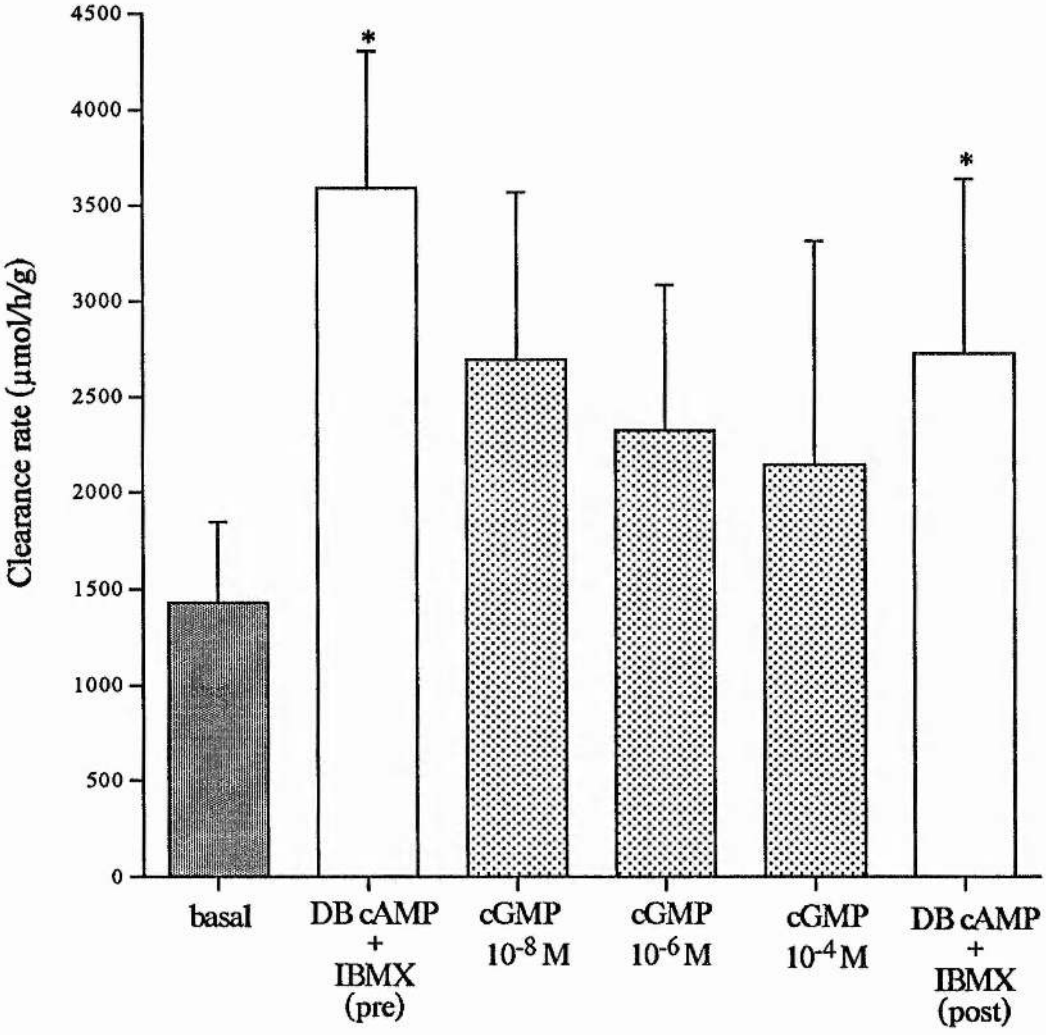
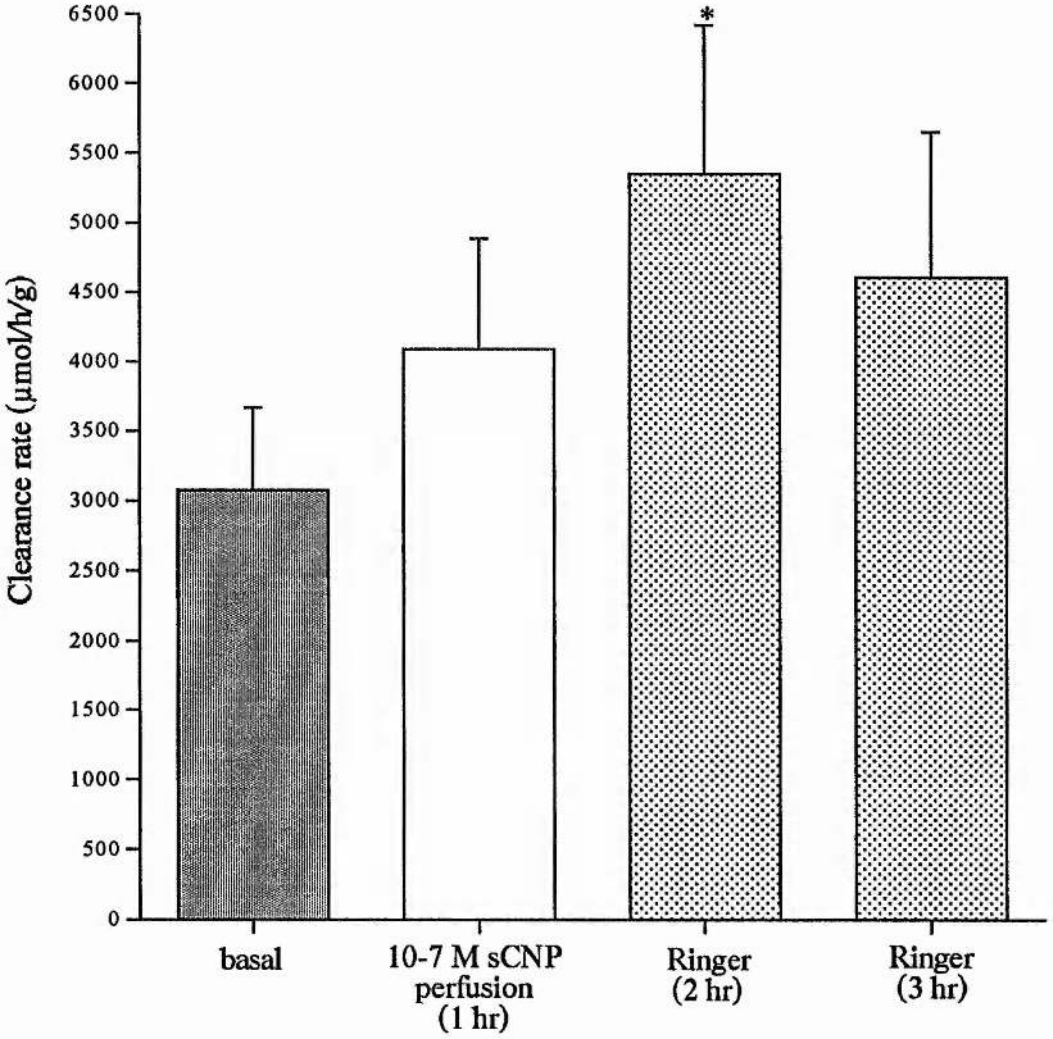

Clearance rate (µmol/h/g)
4500
4000
3500
3000
2500
2000
1500
1000
500
0
*
*
basal
DB cAMP + IBMX (pre)
cGMP 10-8 M
cGMP 10-6 M
cGMP 10-4 M
DB cAMP + IBMX (post)

FIGURE 4.8

Figure 4.8 Effect of homologous sCNP (10^{-7} M) on rectal gland clearance rates *in vivo*.

Clearance rates (µmol/h/g) (ordinate) against time (h) (abscissa). Values are expressed as a mean µmol/h/g, ± SEM; 3076 ± 591 for basal; 4090 ± 795 for 1 hour of sCNP perfusion; 5349 ± 1072 for the first hour following sCNP perfusion; 4610 ± 1043 for the second hour following sCNP perfusion (n = 32). *$p<0.05$, compared to basal clearance rates (paired student t-test).


Clearance rate (μmol/h/g)
6500
6000
5500
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
*
basal
10-7 M sCNP perfusion (1 hr)
Ringer (2 hr)
Ringer (3 hr)

4.4 - Discussion

Figure 4.5 demonstrates that porcine VIP has no effect on the chloride clearance rates of the isolated perfused rectal gland of *S. canicula*. This supports the suggestion of a non-VIP homologous intestinal peptide possibly being involved in rectal gland control (Shuttleworth & Thorndyke, 1984), although such a peptide has never been characterised (Thorndyke & Shuttleworth, 1985). The mode of action of VIP on the rectal gland in *S. acanthias* has been extensively studied (Section 4.1), but a stimulatory effect on the rectal gland of *S. canicula* was never observed. Further investigation and identification of an homologous intestinal peptide stimulating rectal gland secretion is discussed in chapter 6.

Urotensin II was only recently characterised in elasmobranchs (Conlon *et al.*, 1992). However despite the reported osmoregulatory effects this peptide induces in teleosts (Larson & Bern, 1987) very little work has been carried out on the possible osmoregulatory function of U II in elasmobranchs. Figure 4.1 demonstrates that homologous U II has no significant effect on rectal gland chloride clearance rates in *S. canicula*. U II has been seen to increase arterial blood pressure in isolated preparations of the ceoliac artery (Hazon *et al*, 1993). However, this action is possibly secondary to the release of the catecholamine noradrenaline from regions of chromaffin tissue lining arterial vessels. More recently, intravenous injection of U II was shown to cause an approximate 5 fold increase in circulating noradrenaline levels in *S. canicula* (Hazon & Conlon, unpublished data). Concentrations of noradrenaline markedly reduced efferent perfusion flow in the isolated perfused rectal gland of *S. canicula*, possibly by constriction of the vessels within the secretory parenchyma (Shuttleworth, 1983 b). In light of the evidence from the literature, the reason for a slight increase in chloride clearance rate following perfusion of the rectal gland with 10^{-6} M U II can only be described as artifactual.

Figure 4.3a illustrates that 10^{-6} M glucagon significantly increased chloride clearance rates from the gland. Very little research has been carried out on post-feeding circulating levels of homologous glucagon in elasmobranchs. The reason for choosing this particular peptide as a possible effector was principally due to the intermittent behaviour of the rectal gland and the possibility that rectal gland secretion is maximal following feeding which is when one could predict circulating levels of glucagon to be maximal. In addition, elasmobranch glucagon is produced in islet cells of pancreatic tissue (Conlon *et al.*, 1987). The blood systems of the pancreas and intestine are closely linked (Shuttleworth, 1988), and there is also close communication between intestinal vasculature and

rectal gland vasculature (Section 2.4). Thus, these closely linked vascular systems could allow for rapid glucagon transportation to the rectal gland. Although the mechanisms behind the action of glucagon are unknown, it is possible that high concentrations of glucagon are released post-feeding which could be linked to the increased chloride clearance rate of the rectal gland. Interestingly, adenylate cyclase activity and hence cAMP production in plasma membrane preparations of trout gills have been shown to be sensitive to hormonal agents such as mammalian glucagon (Guibbolini & Lahlou, 1987 a & b). More specifically the observed increase in adenylate cyclase activity was attributed, in the majority, to chloride cells, the salt transporting cells of teleost gill epithelia (Guibbolini & Lahlou, 1987a). This indicates the possibility of glucagon acting via a cAMP pathway in salt secreting epithelia of teleosts.

It appears that in *S. acanthias* VIP and sCNP are the major regulators in the model proposed for control of rectal gland secretion (Silva, Lear, Reichlin & Epstein, 1990). Considering that VIP does not appear to influence the rectal gland in *S. canicula* it was important to assess whether homologous sCNP had a similar effect in *S. canicula* as in *S. acanthias*. Figures 4.5 and 4.6 illustrate the stimulatory effect of sCNP on the *in vitro* perfused rectal gland of *S. canicula*. It is evident that sCNP has a similar effect on rectal gland chloride clearance rates in *S. canicula* as in *S. acanthias*. Solomon *et al* (1992) found that maximal stimulation of the rectal gland occurred at 10^{-6} M sCNP and produced secretion rates approximately 8 fold above basal levels. In the present study sCNP maximally stimulated the *in vitro* gland of *S. canicula* at 10^{-7} M, approximately 4.5 fold above basal levels (Figure 4.6 b). These concentrations are however, not directly comparable as perfusion flow rates between the two studies differ markedly.

The site of action of sCNP however, is less well understood and the possible avenues are discussed in section 4.1. In view of this, the cell permeable analogue of the second messenger cGMP (8-Br-cGMP) was tested on the isolated perfused preparation. There was no stimulation of rectal gland chloride clearance rates seen during perfusion of 8-Br-cGMP which was in agreement with the findings of Silva *et al* (1993). Further research is therefore required to ascertain the pathway by which this potent secretagogue (sCNP) acts.

As well as *in vitro* effects of sCNP *in vivo* effects of sCNP were also observed. The intermittent nature of the rectal gland provided inherent difficulties in the *in vivo* study. Maintaining constant basal levels was very difficult and so detection of a peptide derived response from the rectal gland to

sCNP was problematical, although an increase in chloride clearance rates was observed. Interestingly a delay in sCNP-induced increase in rectal gland secretion to the *in vivo* perfusion of sCNP was observed. Such a delay has also been reported in the vasodepressor effects of sCNP on vascular smooth muscle of *S. canicula* (Bjenning *et al*, 1992). The possible vascular effects of sCNP in conjunction with the potent vasoconstrictor, angiotensin II, are reported both *in vivo* and *in vitro* in the following chapter.

CHAPTER 5
VASCULAR EFFECTS OF sCNP AND AII

5.1 - Introduction.

Although the vasodilatory effects of sCNP on smooth muscle *in vivo* and *in vitro* in elasmobranchs appear conclusive (Section 1.9) local effects of natriuretic peptides on the vasculature of the rectal gland have not been well studied. A single report illustrates the effect of atriopeptin on the vasculature of the rectal gland in *S. acanthias* (Solomon *et al.*, 1985a). The *in situ* effect of an intra-arterial injection of synthetic atriopeptin was an immediate decrease in systemic blood pressure, a concomitant decline in perfusion pressure, with an increase in blood flow to the rectal gland, indicating a reduction in vascular resistance within the gland itself (Solomon *et al*, 1985a). Duct flow was also found to increase in association with the increase in blood flow to the gland (Solomon *et al.*, 1985a). The *in vitro* preparation sustained a similar increase in duct flow and chloride secretion rate. However, with a constant rectal gland perfusion pressure, no change in perfusate flow rate was observed and therefore, it was considered that atriopeptin had no haemodynamic effect on the *in vitro* perfused rectal gland of *S. acanthias* (Solomon *et al.*, 1985a). Thus the single study on the haemodynamic effects of natriuretic peptides on the vasculature of the rectal gland produces conflicting results from the *in vivo* and *in vitro* preparations utilising a heterologous ANP analogue.

The antagonistic effects of AII to natriuretic peptides have been well documented in the literature and have been previously described in section 1.2e iv. Chapter 2 describes the identification of AII-like receptors in the rectal gland of the nurse shark (Galli & Cook, 1994), and AII-like and ANP-like receptors in the capsular and sub-capsular regions of the rectal gland of *S. canicula* (Masini *et al.*, 1994). The fact that these two peptides were found localised in close association near the potential vascular control sites of the capsular and sub-capsular regions of the gland strongly suggests an antagonistic mechanism. The initial aim of this chapter was to describe *in vivo* the effects of sCNP, AII and a combination of the two peptides on arterial blood pressure. The second objective was to ascertain if there was any vascular effect detectable in the isolated perfused preparation following perfusion of AII, sCNP, singly and in combination.

5.2 - Materials and Methods.

In vivo cannulation and measurement of blood pressure.

Dogfish were anaesthetised in a 1:10,000 solution (w/v) of MS222 (tricaine methanosulphate). During surgery the fish were kept under moist and cool conditions but the gills were not irrigated. The coeliac artery and vein were cannulated as described in section 4.2.2. An incision was also made immediately behind and in line with the lower jaw to expose the first afferent branchial artery which was cannulated with PE 50 cannula, and the incision was closed with silk sutures. All fish were allowed a 48 hr recovery period prior to any subsequent manipulation. Blood pressure was monitored via the branchial artery 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.

Endogenous angiotensin II (AII) and C-type natriuretic peptide (sCNP) were supplied by Dr. Y. Takei, Japan, and reconstituted in dogfish Ringer. Peptides were injected via the splenic vein, with a maximum volume of 200 µl being administered as a single dose. Observation of a steady basal blood pressure was followed by a control injection of 200 µl of dogfish Ringer. Dogfish AII (0.02 nmol/kg) was injected causing an increase in blood pressure, which was allowed to return to basal levels. AII (0.02 nmol/kg) plus sCNP (5 nmol/kg) were then administered as a single injection and subsequent changes in blood pressure were recorded for the following hour. A final dose of AII (0.02 nmol/kg) was then administered, as a comparison to the initial AII response. Peak responses were compared to mean basal levels calculated during a 10 min period before injection. Statistical tests were not carried out due to the low number of experiments. *In vitro* measures of perfusion pressure from the rectal gland were carried out as described in chapter 3.

5.3 - Results.

Figures 5.1 a, b & c illustrate typical blood pressure traces following injections of AII, sCNP and the combination of AII plus sCNP on *in vivo S. canicula* blood pressure. A classic vasopressor response was observed following injection of AII to the cannulated dogfish (Figure 5.1a). The increase began almost immediately post-injection, peaked approximately 2 min later and returned to basal levels approximately 12 min post-AII injection. A combined injection of AII plus sCNP resulted in a delay in the AII-induced response by approximately 1.45 min (Figure 5.1a). Furthermore, the combination of AII plus sCNP initially caused a reduction of the maximal AII-induced response by approximately 20% (Figure 5.2) which recovered after 1 hour (Figure 5.2) although there was still a small delay in the AII induced response (30 seconds) (Figure 5.1b). A single injection of sCNP (5 nmol/kg) produced a slow steady decline reaching a maximal of 1.83 kPa below basal mean arterial blood pressure. In addition pulse pressure was reduced by approximately 30 % 10 min after injection of sCNP (Figure 5.1c). Such a decline in blood pressure and pulse pressure was also evident following the AII-induced response after joint administration of AII and sCNP (Figure 5.1a). These experiments were only carried out in three fish but the blood pressure responses are comparable to those reported by Bjenning *et al.* (1992).Furthermore, the reduction in pulse pressure was also reported in rainbow trout, *Onchorhynchus mykiss* (Eddy, Smith, Hazon & Grierson, 1990).

Figure 5.3 illustrates the effect of Ringer perfusion on perfusion pressure of the isolated perfused rectal gland. It was necessary to carry out this experiment as a control to ensure that any changes in perfusion pressure observed following perfusion of 10^{-9} M AII, 10^{-7} M sCNP or both were independent of flow rate. Perfusion of homologous sCNP of the isolated perfused rectal gland caused a slight decrease in perfusion pressure observed from 5 to 23 min although this was not significantly different from the initial basal value (Figure 5.4a). Perfusion of homologous AII on the isolated perfused rectal gland caused a significant increase in perfusion pressure which occurred after 9 mins and reached a maximum of 5.24 ± 1.26 kPa above basal after 29 min (Figure 5.4b). Combined perfusion of sCNP and AII on the isolated perfused preparation (Figure 5.4c) caused an increase in the perfusion pressure which was not evident during perfusion of sCNP alone (Figure 5.4a). A steady increase in perfusion pressure was observed, which was significantly greater than basal levels after 29 min (3.39 ± 1.23 kPa) of perfusion in comparison to the AII-induced increase which was significantly greater after 9 min and reached a maximum after 30 mins (Figure 5.4b).

FIGURE 5.1 a, b & c.

Figure 5.1a Typical blood pressure trace of changes induced in the *in vivo* mean arterial blood pressure following injection of Angiotensin II (AII) and shark C-type natriuretic peptide (sCNP).

The initial peak illustrates the immediate vasopressor effect of Angiotensin II (0.02 nmol/kg). The second peak illustrates the change in mean arterial blood pressure following a combined injection of AII plus sCNP (0.5 nmol/kg). sCNP can be seen to delay the AII-induced increase by approximately 1.45 min and also reduce the maximal AII response by approximately 20%.

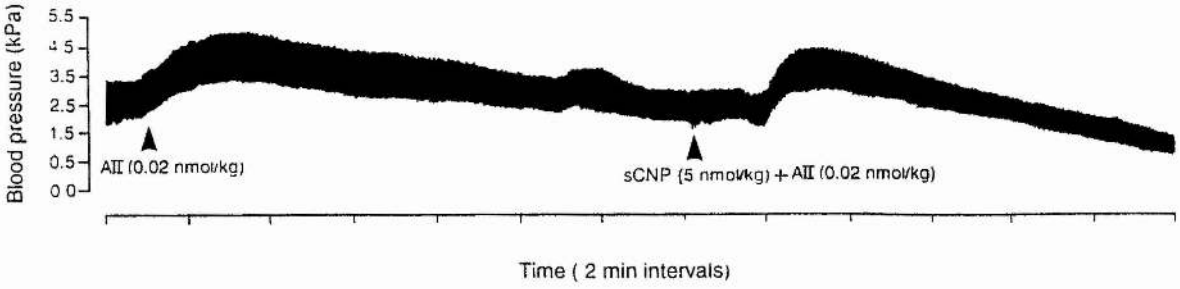
Figure 5.1b Typical blood pressure trace of changes induced in the *in vivo* mean arterial blood pressure following injection of AII one hour after the combined injection of AII and sCNP.

Following injection of AII (0.02 nmol/kg) one hour after the combined injection of AII and sCNP (0.5 nmol/kg) the maximal AII response appears to have recovered. However a small delay in the AII-induced increase in blood pressure, of approximately 30 seconds is still evident.

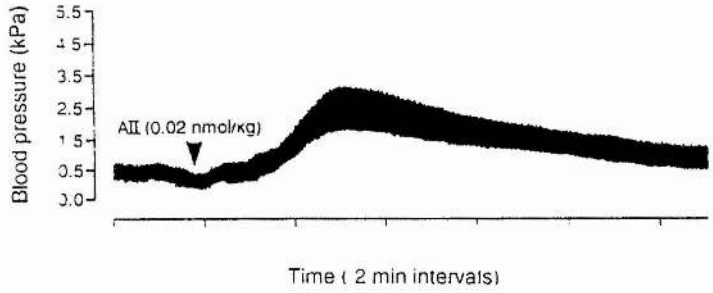
Figure 5.1c Typical blood pressure trace of changes induced in the *in vivo* mean arterial blood pressure following injection of sCNP.

Following injection of sCNP (0.5 nmol/kg) mean arterial blood pressure fell below basal levels, reaching a maximal decrease of 1.83 kPa approximately 25 min post-injection, and remained below basal levels even after 45 min

a


Blood pressure (kPa)
5.5
4.5
3.5
2.5
1.5
0.5
0.0
AII (0.02 nmol/kg)
sCNP (5 nmol/kg) + AII (0.02 nmol/kg)
Time (2 min intervals)

b


Blood pressure (kPa)
5.5
4.5
3.5
2.5
1.5
0.5
0.0
AII (0.02 nmol/kg)
Time (2 min intervals)

c

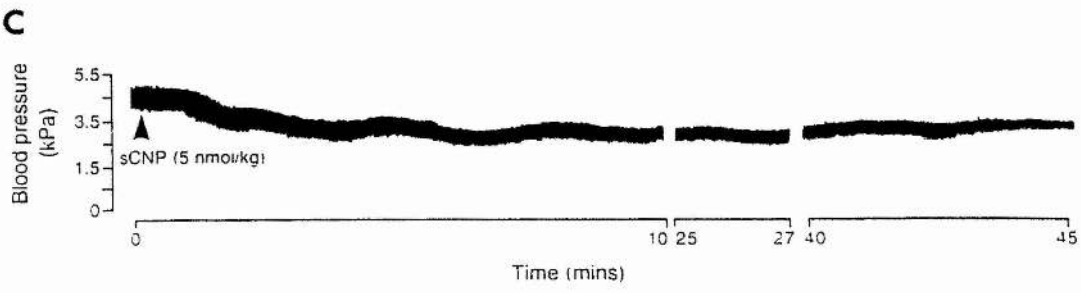

Blood pressure (kPa)
5.5
3.5
1.5
0
sCNP (5 nmol/kg)
0
10
25
27
40
45
Time (mins)

FIGURE 5.2

Figure 5.2 Illustration of the effect of sCNP on the vasoconstrictive effects of AII in the cannulated dogfish.

Change in mean arterial blood pressure (kPa) (ordinate) against treatment (nmol/kg) (abscissa). Values are expressed as a mean kPa ± S.E.M (n=3). Statistical tests were not done due to the small number of experiments carried out.

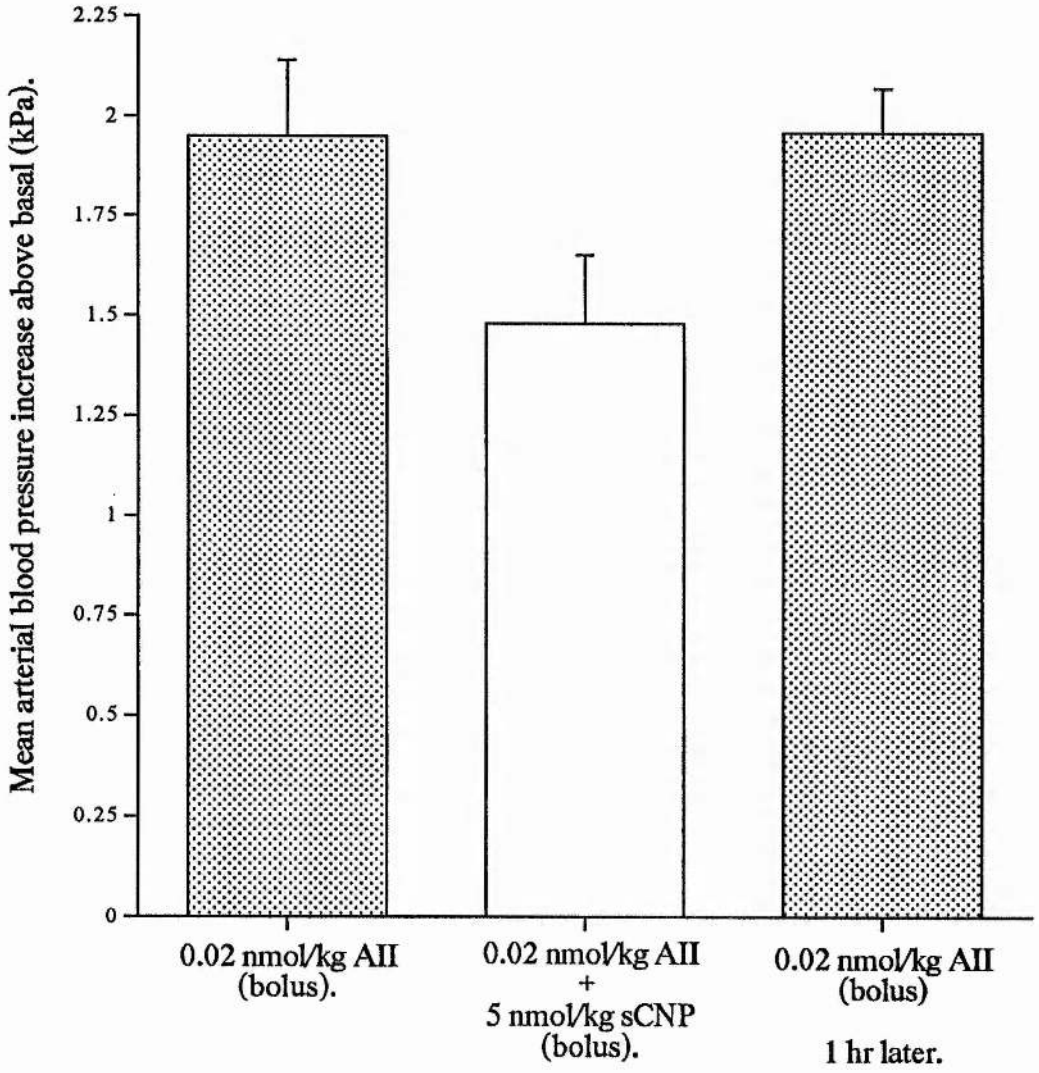

Mean arterial blood pressure increase above basal (kPa).
2.25
2
1.75
1.5
1.25
1
0.75
0.5
0.25
0
0.02 nmol/kg AII (bolus).
0.02 nmol/kg AII + 5 nmol/kg sCNP (bolus).
0.02 nmol/kg AII (bolus) 1 hr later.

FIGURE 5.3

Figure 5.3 Illustration of the effect of Ringer perfusion on perfusion pressure from the isolated perfused rectal gland.

Perfusion pressure was taken as a measure of the change in pressure whilst maintaining a constant perfusion flow from the isolated perfused rectal gland vasculature. Perfusion pressure (kPa) (ordinate) against time (min) (abscissa). Values are expressed as a mean kPa ± SEM. Zero time = 0.1 ± 0.18 kPa. (n=9) (alternative Welch t-test).

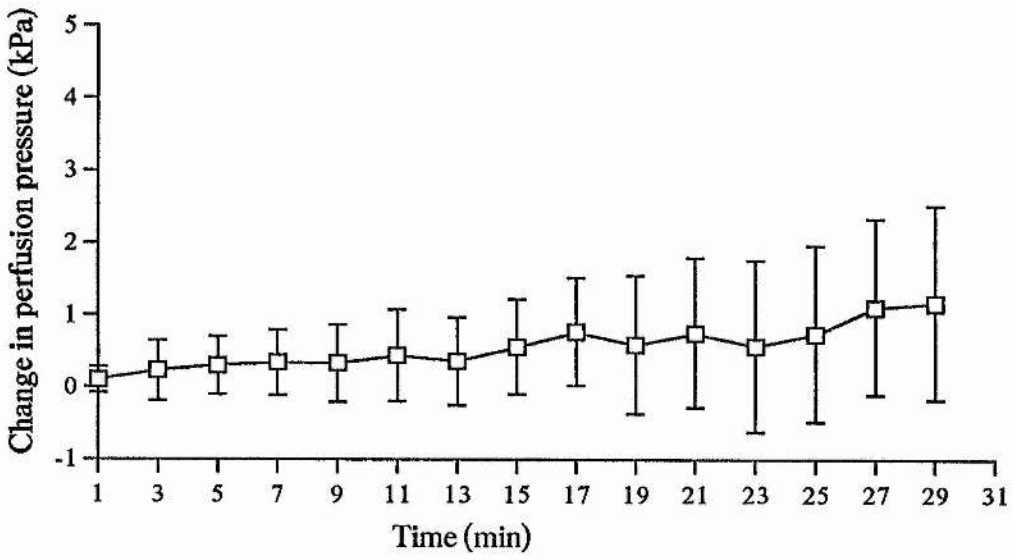

Change in perfusion pressure (kPa)
5
4
3
2
1
0
-1
1
3
5
7
9
11
13
15
17
19
21
23
25
27
29
31
Time (min)

FIGURE 5.4 a, b & c.

Figure 5.4a Illustration of the effect of 10^{-7} M endogenous sCNP perfusion on Perfusion pressure from the isolated perfused rectal gland.
Perfusion pressure (kPa) (ordinate) against time (min) (abscissa). Values are expressed as a mean kPa ± SEM. Zero time = 0.25 ± 0.19 kPa. (n=7) (alternative Welch t-test).

Figure 5.4b Illustration of the effect of 10^{-9} M endogenous A II perfusion on perfusion pressure from the isolated perfused rectal gland.
Perfusion pressure (kPa) (ordinate) against time (min) (abscissa). Values are expressed as a mean kPa ± SEM. Zero time = 0.54 ± 0.33 kPa. (n=7). *$p<0.05$ compared with basal levels (alternative Welch t-test).

Figure 5.4c Illustration of the combined effects of 10^{-9} M and 10^{-7} M endogenous A II and sCNP perfusion on perfusion pressure from the isolated perfused rectal gland.
Perfusion pressure (kPa) (ordinate) against time (min) (abscissa). Values are expressed as a mean kPa ± SEM. Zero time = 0.13 ± 0.23 kPa. (n=6). *$p<0.05$ compared with basal levels (alternative Welch t-test).

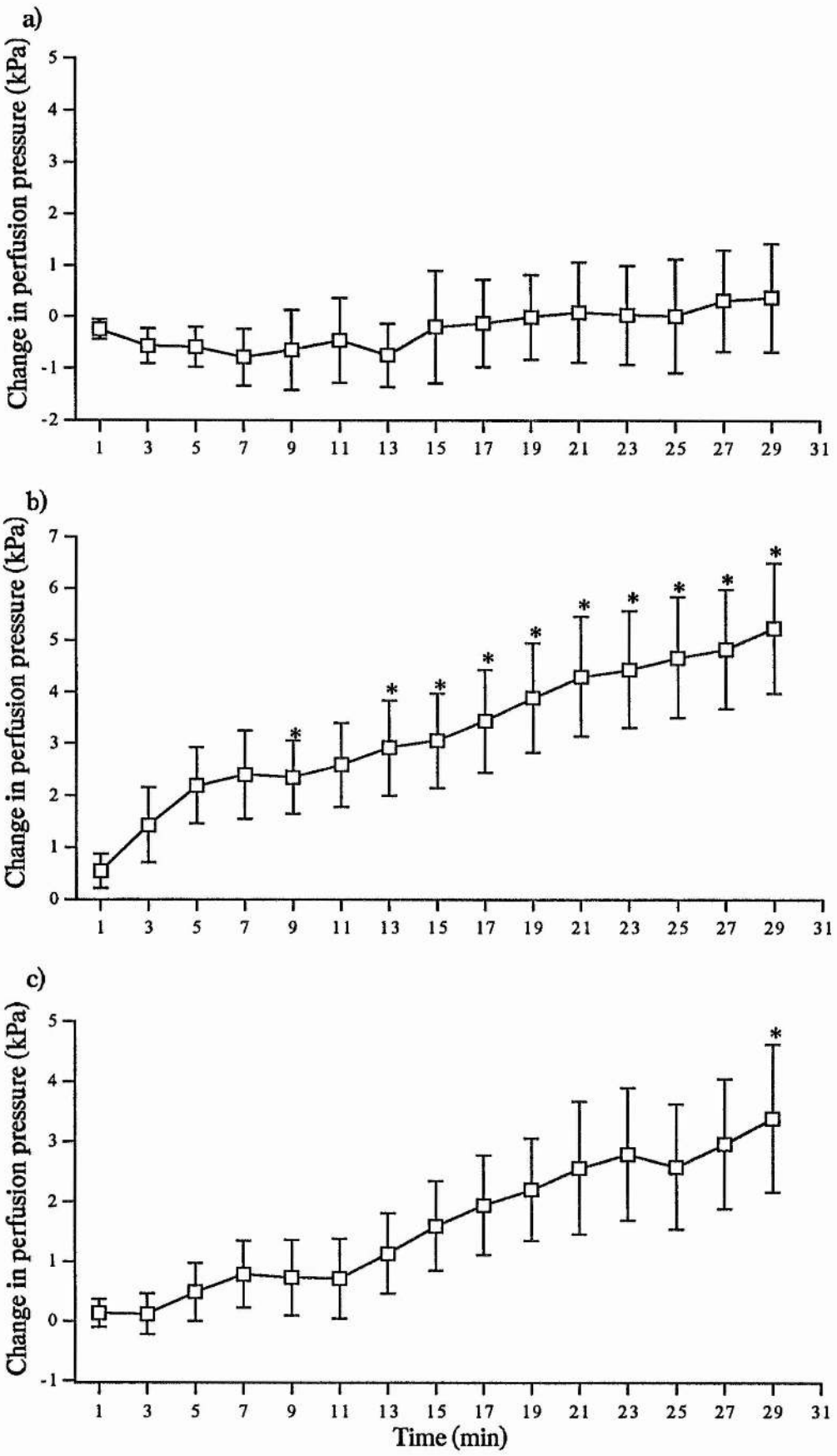

a)
Change in perfusion pressure (kPa)
5
4
3
2
1
0
-1
-2
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31
b)
Change in perfusion pressure (kPa)
7
6
5
4
3
2
1
0
*
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31
c)
Change in perfusion pressure (kPa)
5
4
3
2
1
0
-1
*
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31
Time (min)

Figure 5.5a illustrates the effect of 10^{-7} M sCNP on chloride clearance rates of the isolated perfused rectal gland. A significant increase (approximately 2 fold above basal) in chloride clearance rates was observed during the first 10 mins of sCNP perfusion, after which an increase above basal chloride clearance rates was maintained but not at a significant level. 10^{-9} M AII appeared to have no significant effect on chloride clearance rates from the isolated perfused rectal gland although a slight decrease below basal level was observed between 10 and 20 mins (Figure 5.5b). There was an initial delay in the sCNP-induced increase in chloride clearance rate following perfusion of 10^{-9} M AII and 10^{-7} M sCNP. This was followed by a marked increase, approximately 6 fold above basal levels, in chloride clearance rates which then declined but remained significantly higher than basal for the following 25 min (Figure 5.5c).

FIGURE 5.5 a, b & c.

Figure 5.5a Time course of 10^{-7} M sCNP on clearance rates of the isolated perfused rectal gland compared to total basal levels.

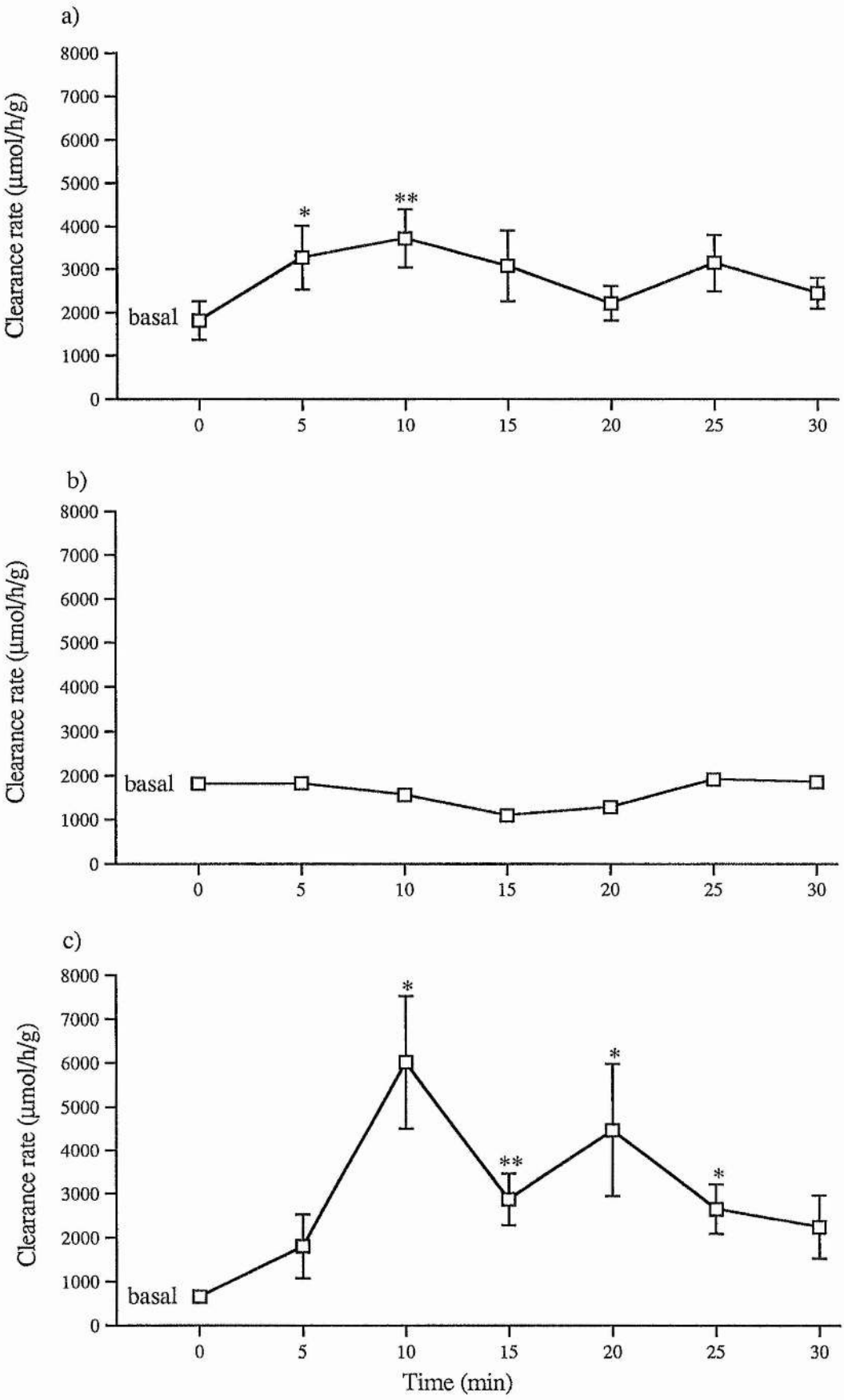
Clearance rates (µmol/h/g) (ordinate) against time (5 min intervals) (abscissa). Values are expressed as a mean µmol/h/g ± SEM; 1813 +/. 446 for basal total; 3273 ± 741 for 0-5 min; 3711 ± 676 for 5-10 min; 3074 ± 816 for 10-15 min; 2205 ± 399 for 15-20 min; 3140 ± 655 for 20-25 min, and 2447 ± 354 for 25-30 min (n=15). *p<0.05, **p<0.01 compared with basal clearance rates (alternative Welch t-test).

Figure 5.5b Time course of 10^{-9} M A II on clearance rates of the isolated perfused rectal gland compared to total basal levels.

Clearance rates (µmol/h/g) (ordinate) against time (5 min intervals) (abscissa). Values are expressed as a mean µmol/h/g ± SEM; 1813 ± 446 for basal total; 1823 ± 585 for 0-5 min; 1559 ± 395 for 5-10 min; 1093 ± 419 for 10-15 min; 1284 ± 352 for 15-20 min; 1917 ± 510 for 20-25 min and 1862 ± 648 for 25-30 min (n=12) (alternative Welch t-test).

Figure 5.5c Time course of the combined effect of 10^{-9} M A II and 10^{-7} M s CNP on clearance rates of the isolated perfused rectal gland compared to total basal levels.

Clearance rates (µmol/h/g) (ordinate) against time (5 min intervals) (abscissa). Values are expressed as a mean µmol/h/g ± SEM; 652 ± 133 for basal total; 1804 ± 732 for 0-5 min; 6018 ± 1508 for 5-10 min; 2873 ± 589 for 10-15 min; 4469 ± 1516 for 15-20 min; 2655 ± 564 for 20-25 min and 2251 ± 718 for 25-30 min (n=7). *p<0.05, **p<0.01 compared with basal clearance rates (alternative Welch t-test).


a)
Clearance rate (µmol/h/g)
8000
7000
6000
5000
4000
3000
2000
1000
0
basal
*
**
0
5
10
15
20
25
30
b)
Clearance rate (µmol/h/g)
basal
c)
Clearance rate (µmol/h/g)
basal
Time (min)

5.4 - Discussion.

The effects of AII *in vivo* as a vasopressor hormone are in agreement with the findings of Hazon *et al.* (in press) and the vasodepressor effects of sCNP *in vivo* are in agreement with Bjenning *et al.* (1992). The observed decrease in pulse pressure following injection of sCNP is possibly due to increased compliance of arterial circulation (Eddy, *et al*, 1990). The effects of sCNP on the AII-induced increase in blood pressure were two fold, a delay in increase and a reduction in the maximal response, thus suggesting that sCNP may indeed being acting in an antagonistic fashion to AII in the control of arterial blood pressure.

On a local vascular level it is evident from the present study that AII has increased the perfusion pressure during constant perfusion flow leading one to suspect that the AII has acted as a vasoconstrictor on smooth muscle in blood vessels at the capsular and sub-capsular levels (Chapter 2). If sCNP was acting as a vasodilator on the vasculature of the rectal gland one would predict a steady decline in perfusion pressure from the rectal gland. This however was not apparent, rather there was no significant effect on perfusion pressure in the isolated perfused rectal gland. Perfusion of AII plus sCNP produced an *in vitro* vasoconstrictor effect on the vasculature of the rectal gland. However, the increase in perfusion pressure was less than that recorded following perfusion of AII alone. Although these two sets of data were not significantly different it is possible that sCNP was exerting an antagonistic effect on the vascular effect of AII in the rectal gland. As previously stated the capsular and sub-capsular regions possibly provide the major site for vascular control of the rectal gland. AII and sCNP receptors are found in close association in the rectal gland capsular and sub-capsular regions (Masini *et al.*, 1994). Undoubtedly AII and sCNP exert potent vasoconstrictor (Hazon *et al.*, in press) and vasodilator effects (Bjenning *et al.*, 1992) respectively.

It is evident that sCNP significantly stimulated chloride clearance rates from the isolated perfused rectal gland. This stimulation was maintained above basal secretory levels but only significantly during the initial 10 min of sCNP perfusion. The lack of inhibition of rectal gland secretion *in vitro* using AII was most probably due to the basal level of rectal gland secretion. It may be difficult to inhibit secretion from glands that are already secreting at such low basal levels.

A combination of AII and sCNP was perfused through the isolated rectal gland to examine any possible inhibitory effect AII may have on chloride clearance rates. Interestingly, a delay in the sCNP-induced increase in chloride clearance rate was observed following the combined perfusion of AII and sCNP.

However, following this delay, there was a marked increase in maximal chloride clearance rates, above that produced by sCNP alone, which was maintained over a longer period of time. This reported increase in maximal stimulation is somewhat difficult to explain. Certainly *in vivo* the vasculature of the rectal gland would most likely be influenced by a combination of AII and sCNP, coupled to this could be an additional direct effect of sCNP on secretory epithelia thus producing a maximal response (Karnaky *et al*, 1991). However, this hypothesis is difficult to prove from the results presented in the this study, and subsequent in depth research is required to ascertain if these two peptides do in fact exert a synergistic effect on rectal gland chloride clearance rate. Furthermore, the demonstration of renin-like and angiotensin converting enzyme-like activity in the rectal gland tissue of the elasmobranch *S. canicula* (Masini *et al*, 1994), suggests the presence of a local endogenous rectal gland renin angiotensin system (RAS). This indicates that a local RAS could play a role in the perfusion of secretory epithelia, in addition to the possible systemic effects of circulating angiotensin II.

Comparison of the results in this chapter concerning perfusion pressure from the rectal gland, to those produced by Solomon *et al.* (1985a) is unavoidable but not particularly valid as the current study was essentially concerned with the effects of homologous peptides as opposed to heterologous peptides. There are inherent dangers in using heterologous peptides when investigating peptidergic influences on secretory tissue function, and this will be further discussed in chapter 6.

CHAPTER 6
CHARACTERISATION OF AN ENDOGENOUS STIMULATORY GUT PEPTIDE

6.1 - Introduction

The effects of gastrointestinal peptides have been studied extensively on the isolated perfused rectal gland preparation of *Squalus acanthias*. The reason for this focus on the effect of gut peptides is related to the intermittent feeding behaviour of many elasmobranchs, leading to high salt loading and hence maximal requirement for rectal gland activity (Stoff *et al.*, 1979). A pathway of transport for such factors was postulated by Kent & Olson (1982) and was described in section 2.4. Certainly in *Scyliorhinus canicula*, the largely invertebrate diet and tendency to gorge food coupled to ingestion of seawater while feeding, will exacerbate salt loading and lead to a requirement for increased rectal gland secretion.

Following investigation of the effects of peptides on the isolated perfused rectal gland preparation of *S. acanthias* (Stoff *et al.*, 1979) it was found that porcine VIP was the only peptide that significantly stimulated rectal gland activity. Since then VIP has been used extensively as a stimulator of the isolated perfused preparation of *S. acanthias* (Silva, Epstein, Karnaky, Reichlin, & Forrest, 1993a; Silva *et al.*, 1985; Silva *et al.*, 1987). Furthermore, VIP-like immunoreactivity has been found in the secretory parenchyma of the rectal gland of *S. acanthias* (Holmgren & Nilsson, 1983). There is, therefore, strong evidence for the involvement of VIP as a regulatory peptide in rectal gland activity of *S. acanthias*, especially with the report that VIP directly stimulated short-circuit current in rectal gland cell cultures of *S. acanthias* (Karnaky *et al.*, 1991).

In contrast to the *in vitro* studies in *S. acanthias*, isolated perfusion of both whole gland and rectal gland slices of *S. canicula* and *Raja Clavata* with porcine VIP (Shuttleworth, 1983b) did not stimulate secretion even at high concentrations of 10^{-6} M. The present study has confirmed the lack of a stimulatory effect of VIP on rectal gland chloride clearance rates in *S. canicula* (Section 4.3). In the search for an homologous gut peptide that stimulated rectal gland secretion, Shuttleworth and Thorndyke (1984) partially purified a single fraction from the intestine of *S. canicula* that stimulated oxygen consumption in rectal gland slices of *S. canicula* and *R. clavata* and increased secretory flow rate in the perfused rectal gland of *S. acanthias*. The partially purified fraction, although never fully characterised, was considered the principle intestinal factor controlling rectal gland secretion in elasmobranchs and was named "rectin". The "purified" rectin was found to be distinct from elasmobranch VIP, and it was postulated that rectin may be related to the urotensins (Thorndyke and Shuttleworth, 1985). Although it was known that elasmobranchs possess a neurosecretory area in the tail (Fridberg, 1962), evidence for endocrine cells containing urotensins in the intestine was non-existent. Also at

this time neither the structure of rectin or elasmobranch urotensins was known so comparison of two non-identified peptides was difficult.

The potential dangers of using non-homologous peptides in comparative studies of peptide/hormone action are highlighted by the essential differences in the effects of porcine VIP on the rectal gland of these two closely related species of dogfish. The sequence of homologous *Scyliorhinus* VIP varies appreciably from porcine VIP, particularly at the C-terminus (Figure 7.2) although this does not appear to affect potency of binding to porcine VIP receptors (Dimaline, Young, Lee, Shuttleworth & Thorndyke, 1987). However in the case of *S. canicula*, not even the homologous VIP stimulated rectal gland activity (Thorndyke & Shuttleworth, 1985).

The aim of this chapter was to use the techniques of peptide purification (gel permeation, chromatography, and reverse phase HPLC) to isolate and sequence biologically active material extracted from *S. canicula* intestine that stimulated the secretory activity of the isolated perfused rectal gland of the dogfish.

6.2 - Materials and Methods

6.2a - Tissue extraction.

Small intestine was taken from 45 adult specimens of both sexes and the tissue was immediately frozen. Tissues were stored at -20°C until time of extraction. Tissue (524 g) was homogenised at 0°C in 4 litres of the following solution: (3:1 EtOH : H_2O mixture) 250 ml distilled water, 750 ml absolute alcohol, and 18 ml concentrated HCl ; and centrifuged (4000 g for 30 min). The ethanol was removed from the supernatant under reduced pressure and, after further centrifugation (400 g for 30 min), the extract was pumped (100 ml/h) onto 12 Sep-Pak C18 cartridges (Waters Associates, Milford, MA) connected in series. Bound material was eluted with acetonitrile / water / trifluoroacetic acid (70:29.9:0.1, by vol.) and lyophilised.

6.2b - Purification of dogfish gut extracts.

The dogfish gut extract, after partial purification on Sep-Pak cartridges, was redissolved in 20 ml of 1% trifluoroacetic acid and chromatographed on a 90-cm x 5-cm Biogel P-10 (fine) column (Bio-Rad, Richmond, CA) equilibrated with 1 M acetic acid at a flow rate of 72 ml/h. Fractions (12 ml) were collected, freeze dried and resuspended in 1 ml of Ringer. The ability of aliquots (100 µl) of the fraction to stimulate the activity of the rectal gland was determined by bioassay (see below).

The fractions, showing activity in this assay, denoted by the bars in figure 6.1 were pooled (total volume = 60 ml) and pumped at a flow rate of 2 ml/min onto a 25 x 1 cm Vydac 218TP510 (C_{18}) reverse-phase column (Separations Group, Hesperia, CA), equilibrated with 0.1% (by vol) trifluoroacetic acid/water at a flow rate of 2 ml/min. The concentration of acetonitrile in the eluting solvent was raised to 21% (by vol.) over 10 min, held at this concentration for 30 min and raised to 49% over 60 min using linear gradients. Absorbance was measured at 214 and 280 nm. Fractions (1 min) were collected and assayed for bioactivity at appropriate dilution. The fractions producing the greatest response (denoted by the bar in figure 6.2a) were rechromatographed on a 0.46 x 25 cm Vydac 214TP54 (C_4) reverse-phase column equilibrated with acetonitrile / water / trifluoroacetic acid (7.0:92.9:0.1 by vol) at a flow rate of 1.5 ml/min. The concentration of acetonitrile in the eluting solvent was raised to 28% (by vol.) over 40 min using a linear gradient. The peak producing the greatest response (denoted by the bar in figure 6.2b) was chromatographed on a 0.46 x 25 cm Vydac 218TP54 (C_{18}) column equilibrated with acetonitrile / water / trifluoroacetic acid (14.0:85.9:0.1 by vol) at a flow rate of 1.5 ml/min. The concentration of acetonitrile in the eluting solvent was raised to 35% over 40 min using a linear gradient.

6.2c - Structural analysis.

The primary amino acid sequence of the dogfish gut peptide (approximately 3 nmol) was determined by automated Edman degradation using Applied Biosystems model 471A sequenator modified for on-line detection of phenylthiohydantoin-coupled amino acids (Pth-Xaa) under gradient elution conditions. Standard operating procedures were used and the detection limit for Pth-Xaa was 0.5 pmol. Hydrolysis (24 h at 110°C in 5.7 M HCl) of approximately 1 nmol peptide was carried out and amino composition was determined by pre-column derivatization with phenylisothiocyanate using an Applied Biosystems model 420A derivatizer and 130A separation system. The detection limit for phenylthiocarbamyl-labelled acids was 1 pmol.

6.2d - Bioassay technique.

The stimulatory activity of each fraction (100 µl aliquots) in figure 6.1, was tested using the *in vivo* perfusion technique, as described in section 4.2.b, although fractions were perfused for 30 min only with secretory fluid collected over 60 min periods. The ability of aliquots (100µl) of each fraction produced in subsequent purification steps (Figures 6.2 a, b, & c) to stimulate the activity of the rectal gland was determined using the *in vitro* perfusion technique as described in section 4.2.1.

6.3 - Results

6.3a - Purification of the dogfish gut peptide.

The elution profile of the dogfish gut extract, after partial purification on Sep-Pak cartridges, on a Biogel P-10 gel permeation column is shown in figure 6.1. Material that stimulated the activity of the rectal gland was eluted in two zones: weakly active fractions at void volume of the column (molecular mass > 10,000 daltons) and strongly active fractions with an elution volume between that of dogfish neuropeptide Y and mammalian neurokinin A indicating a molecular mass in the region of 2000 - 3000. The earlier eluting fractions were not investigated further. The fractions denoted by the bar in figure 6.1 were pooled and chromatographed on a semipreparative Vydac C_{18} column (Figure 6.2a). Bioactivity was associated with three fractions (48, 49, & 50). These fractions were pooled and chromatographed on an analytical Vydac C_4 column (Figure 6.2b) and bioactivity was associated with the major peak in the chromatogram denoted by the bar. After rechromatgraphy on an analytical Vydac C_{18} column (Figure 6.2c), the dogfish gut peptide was eluted as a sharp symmetrical peak. The peak was devoid of absorbance at 280 nm indicating the absence of tryptophan and tyrosine residues in the peptide. The final yield of pure material was approximately 14 nmol.

6.3b - Structural characterisation of dogfish gut peptide.

The primary structures of the dogfish gut peptide were determined by automated Edman degradation (Table 6.1). It was possible to assign, without ambiguity, phenylthiohydantoin-coupled amino acid residues for 18 cycles of operation of the sequenator, except that no signals were detected during cycles 7 and 13. The amino acid sequence of the isolated dogfish gut peptide was identical to that of scyliorhinin II, previously isolated from dogfish gut (Conlon, Deacon, O'Toole, & Thim, 1986) and which contains a cystine bridge between residues 7 and 13. The amino acid composition of the dogfish gut peptide [Found: Asx 3.3 (3), Ser 2.7 (3), Gly 1.8 (2), Pro 2.7 (3), Val 1.0 (1), Met 0.8 (1), Leu 1.0 (1), Phe 1.0 (1), Lys 2.0 (2) residues/mol peptide] is consistent with the results of Edman degradation and demonstrates that the full sequence of the peptide had been obtained. The values in parentheses are the number of residues predicted from sequence analysis. Cysteine was detected in the peptide hydrolyzate but not quantified. Identity of the dogfish gut peptide with scyliorhinin II was confirmed by the fact that a mixture of homologous gut peptide (1 nmol) and synthetic scyliorhinin II (1 nmol) was eluted from an analytical Vydac C18 column under the elution conditions illustrated in Figure 6.2c, as a single peak.

TABLE 6.1

Table 6.1 Automated Edman degradation of *Scyliorhinus* gastrointestinal peptide which stimulated rectal gland secretion from the isolated perfused rectal gland.

Cycle no.	Residue	Yield (pmol)
1	Ser	478
2	Pro	1874
3	Ser	355
4	Asn	1327
5	Ser	299
6	Lys	1180
7	(Cys)	N.D.
8	Pro	844
9	Asp	931
10	Gly	713
11	Pro	575
12	Asp	612
13	(Cys)	N.D.
14	Phe	203
15	Val	121
16	Gly	102
17	Leu	23
18	Met	9

N.D., no phenylthiohydantoin-coupled amino acid detected.

FIGURE 6.1

Figure 6.1 Gel-permeation chromatography (Biogel P-10) of an extract of dogfish intestine after partial purification on Sep-Pak cartridges.
Fractions denoted by the bar contained material that stimulated the activity of the rectal gland *in vivo* and were pooled for further purification by HPLC. The arrows show the void volume (V_0) of the column and the elution volumes of neuropeptide Y (NPY) and neurokinin A (NKA).

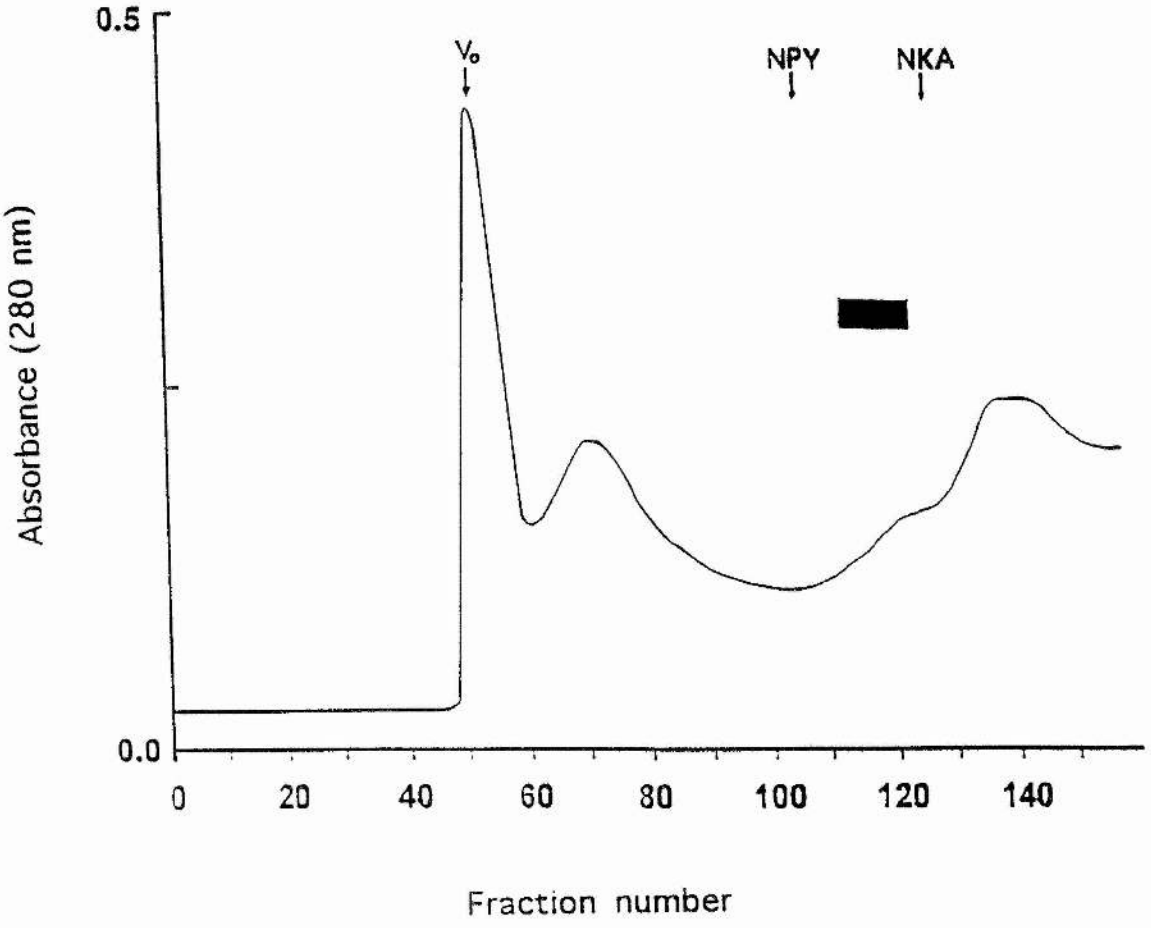

0.5
0.0
Absorbance (280 nm)
V_o
NPY
NKA
0
20
40
60
80
100
120
140
Fraction number

FIGURE 6.2 a, b & c.

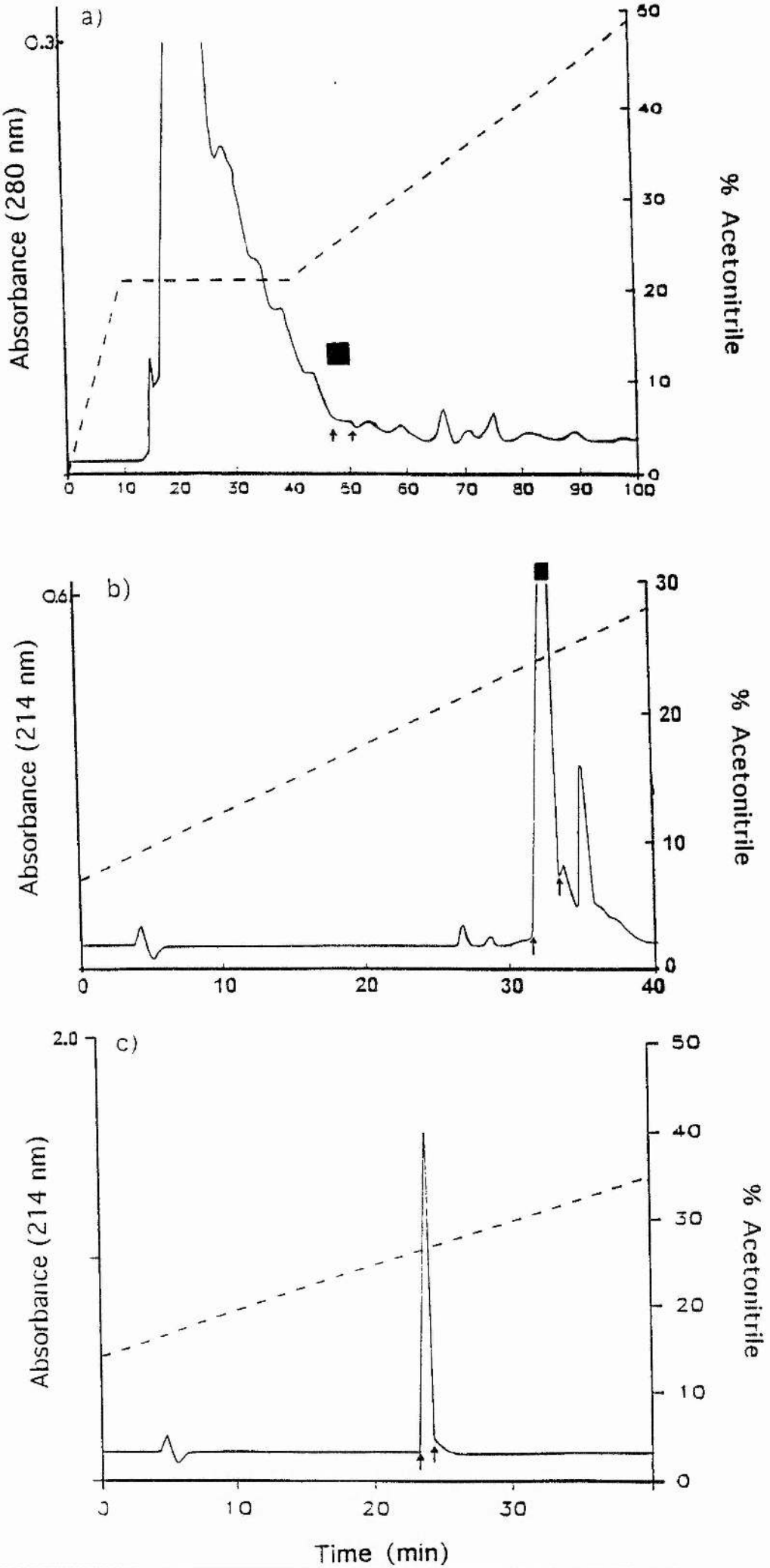
Figure 6.2a, b, & c Purification of a dogfish gut peptide by various stages of reverse-phase HPLC.

(a) a semi-preparative Vydac C18 column.

(b) an analytical Vydac C4 column.

(c) an analytical Vydac C18 column.

The bars denote the fractions containing material that stimulated the activity of the isolated perfused rectal gland. The arrows indicate where peak collection began and ended and the dashed line, the concentration of acetonitrile in the eluting solvent.


a)
0.3
Absorbance (280 nm)
% Acetonitrile
0
10
20
30
40
50
60
70
80
90
100
b)
0.6
Absorbance (214 nm)
% Acetonitrile
0
10
20
30
40
c)
2.0
Absorbance (214 nm)
% Acetonitrile
Time (min)

6.3c - Bioassay results.

Figure 6.3 illustrates the effects of the purest intestinal fraction before identification and sequencing. Dibutryl cAMP plus IBMX both pre- and post-perfusion of fraction 49.9 produced the expected increase in chloride clearance rates. The larger chloride clearance rate at the end of the experimental period is most probably related to the small n number in this study due to the lack of material. However, it is evident that this fraction was extremely effective in stimulating the activity of the isolated perfused rectal gland, almost 4 fold above basal levels.

Following the identification and characterisation of the stimulatory fraction as scyliorhinin II, synthetic scyliorhinin II (Peninsula Laboratories) was tested on the isolated perfused gland. Figure 6.4 illustrates the effect of synthetic scyliorhinin II on the isolated perfused rectal gland. This peptide did not significantly stimulate the isolated perfused preparation. A separate batch of scyliorhinin II was obtained from Professor Conlon (Creighton University, Omaha, Nebraska) and tested on the isolated perfused preparation. Figure 6.5 illustrates the effects of this batch of scyliorhinin II on the isolated perfused rectal gland. The standard doses of dibutryl cAMP and IBMX pre- and post-experiment increased chloride clearance rates 4 fold above basal levels. Scyliorhinin II (10^{-9} M) significantly increased chloride clearance rates approximately 2 fold above basal levels. Figure 6.6 illustrates the timed collection of secretory fluid over 15 min periods during and after perfusion of 10^{-6} M scyliorhinin II (Professor Conlon's synthetic compound) compared to total basal fluid collected over 75 min. It is evident that during perfusion of 10^{-6} M scyliorhinin II, chloride clearance rates from the isolated perfused rectal gland increased almost 4 fold above basal levels and were still significantly higher during the first 15 min of Ringer perfusion.

FIGURE 6.3

Figure 6.3 Effect of the purest intestinal fraction together with the effects of dibutryl cAMP plus IBMX before and after peptide perfusion on the clearance rates of the isolated perfused rectal gland.

Clearance rates (μmol/h/g) (ordinate) against treatment (abscissa). basal was 714 ± 553; pre-dibutryl cAMP plus IBMX was 2383 ± 454; fraction 49.9 was 2152 ± 820, and post-dibutryl cAMP plus IBMX was 5271 ± 838, (n=3). A larger n number was not obtainable due to the lack of material available.

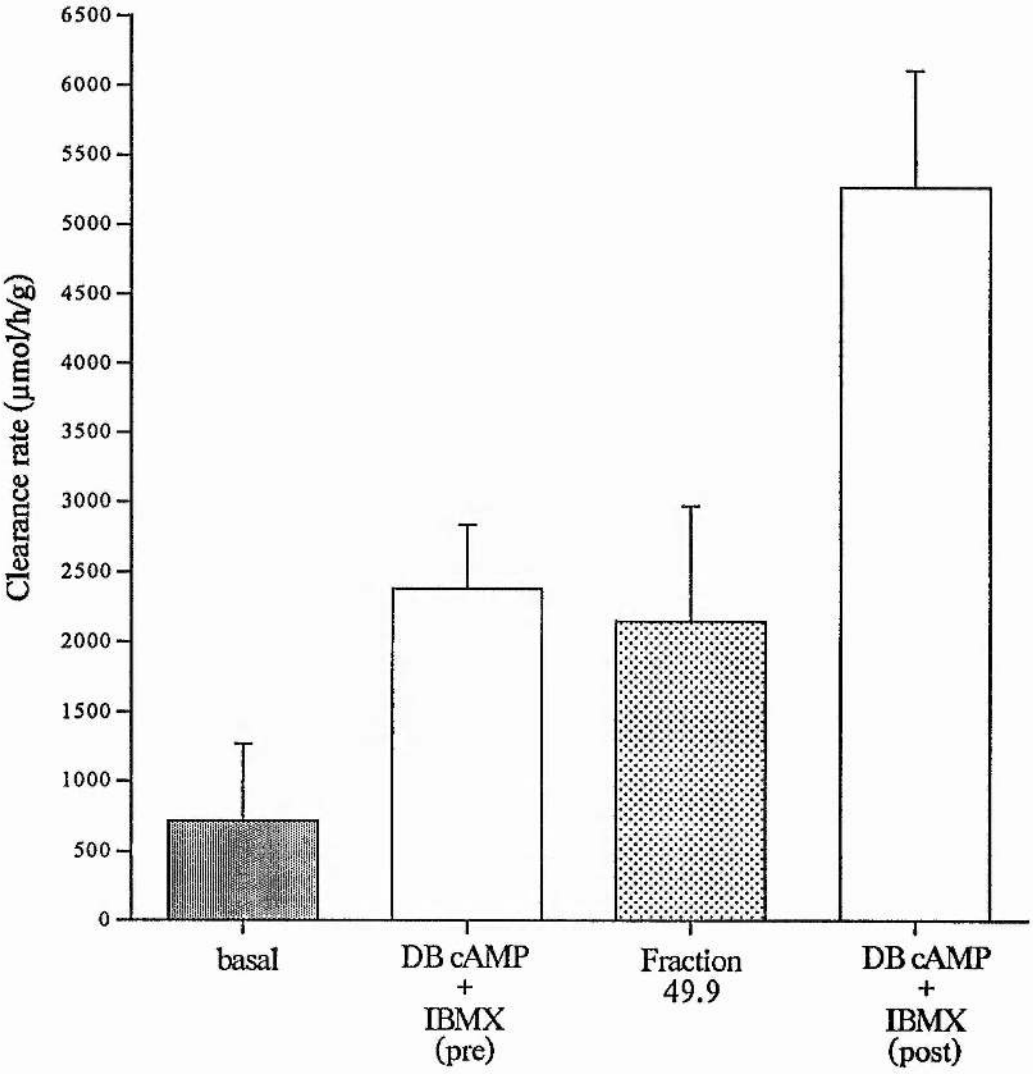

Clearance rate (µmol/h/g)
6500
6000
5500
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
basal
DB cAMP + IBMX (pre)
Fraction 49.9
DB cAMP + IBMX (post)

FIGURE 6.4

Figure 6.4 Effect of increasing doses of synthetic scyliorhinin II (purchased from Peninsula Laboratories) together with the effects of dibutryl cAMP plus IBMX before and after peptide perfusion on the clearance rates of the isolated perfused rectal gland.

Clearance rates (μmol/h/g) (ordinate) against treatment (abscissa). Values are expressed as a mean μmol/h/g ± SEM; 1158 ± 310 for basal; 2667 ± 315 for pre-dibutryl cAMP plus IBMX; 1026 ± 341 for 10^{-12} scyliorhinin II; 1023 ± 247 for 10^{-9} scyliorhinin II; 1611 ± 173 for 10^{-6} scyliorhinin II; and 2598 ± 541 for post-dibutryl cAMP plus IBMX (n=7). *$p<0.05$, **$p<0.01$ compared to basal clearance rate (alternative Welch t-test).

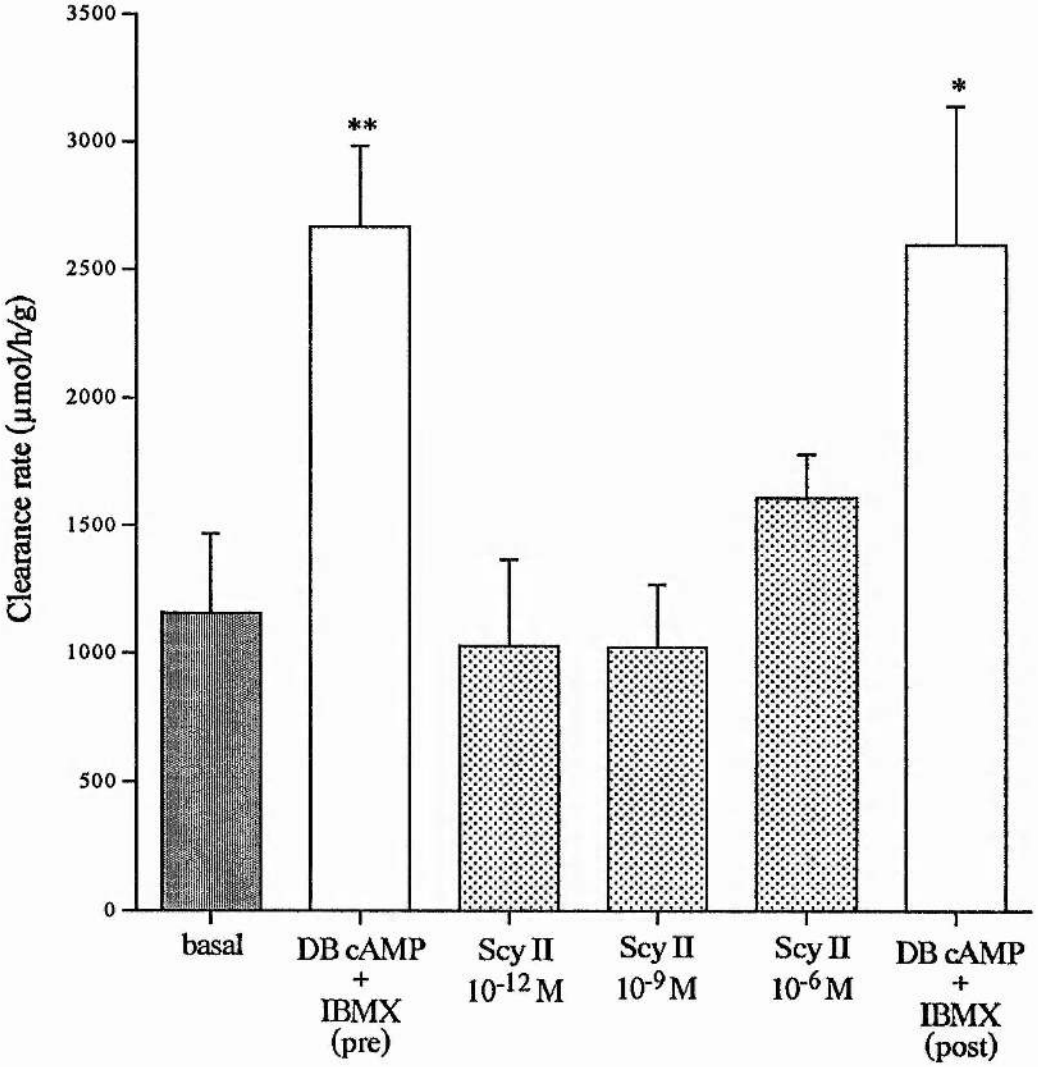

Clearance rate (µmol/h/g)
3500
3000
2500
2000
1500
1000
500
0
**
*
basal
DB cAMP + IBMX (pre)
Scy II 10-12 M
Scy II 10-9 M
Scy II 10-6 M
DB cAMP + IBMX (post)

FIGURE 6.5

Figure 6.5 Effect of increasing doses of synthetic scyliorhinin II (supplied by Professor M. Conlon) together with the effects of dibutryl cAMP plus IBMX before and after peptide perfusion on the clearance rates of the isolated perfused rectal gland.

Clearance rates (μmol/h/g) (ordinate) against treatment (abscissa). Values are expressed as a mean μmol/h/g ± SEM; 497 ± 146 for basal; 2052 ± 313 for pre-dibutryl cAMP plus IBMX; 345 ± 52 for 10^{-12} scyliorhinin II; 949 ± 151 for 10^{-9} scyliorhinin II; 1193 ± 210 for 10^{-6} scyliorhinin II; and 1860 ± 300 for post-dibutryl cAMP plus IBMX (n=12, 12, 10, 12, 12, & 12 respectively). *$p<0.05$, **$p<0.01$ ***$p<0.001$ compared to basal clearance rates (alternative Welch t-test).

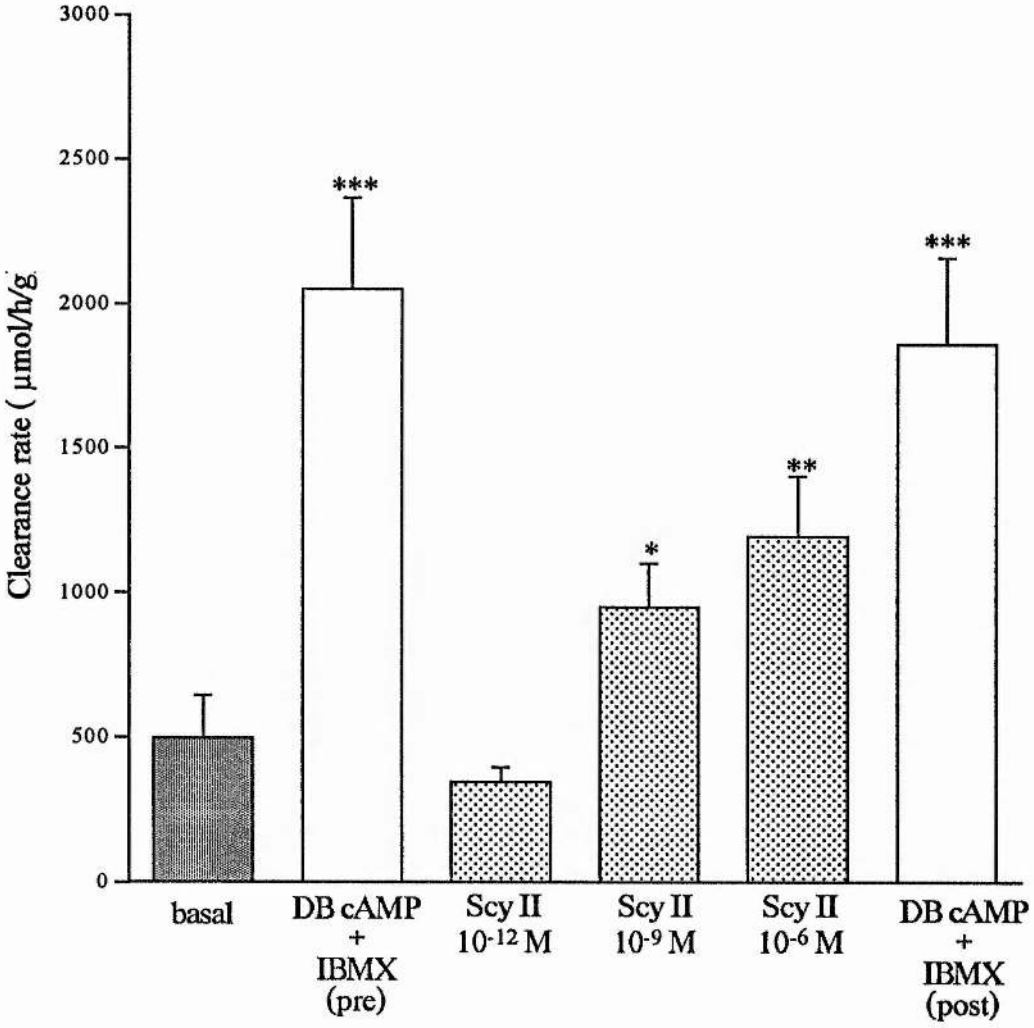
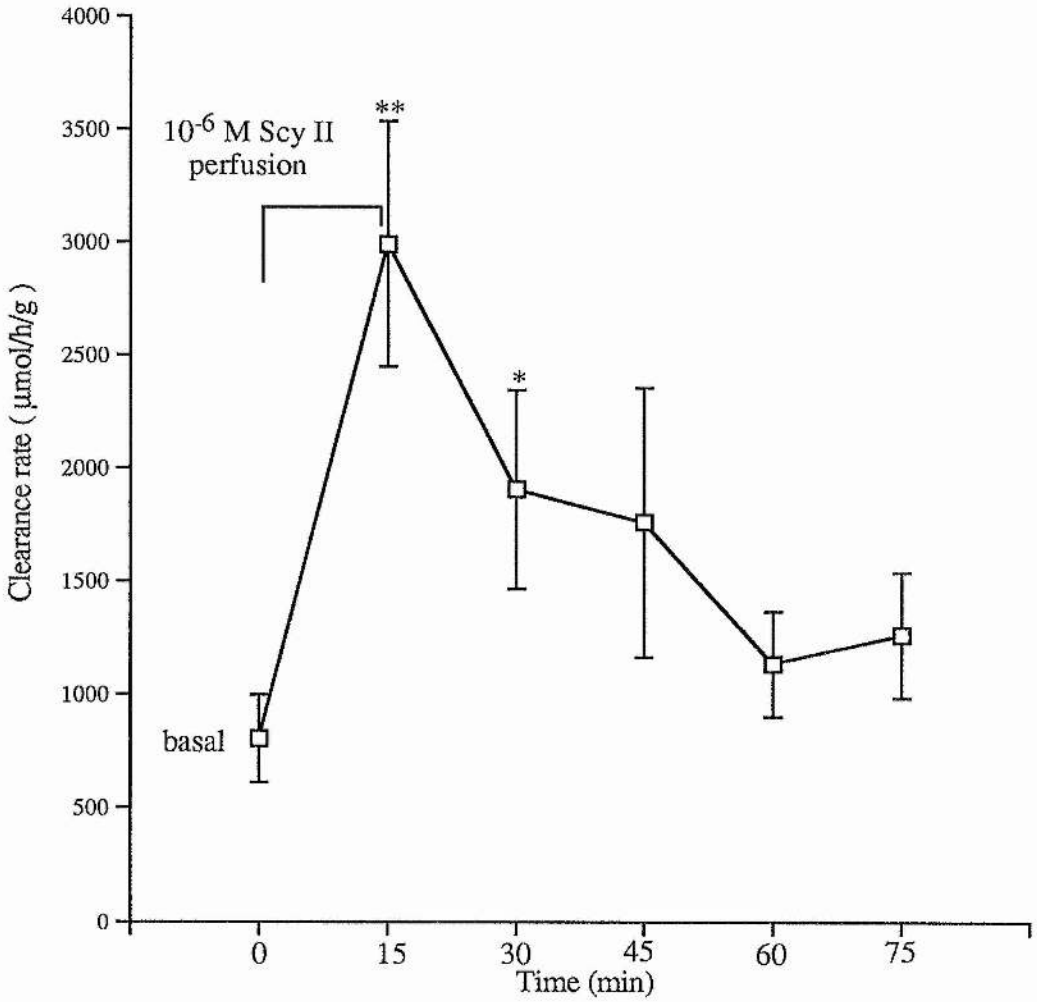

3000
2500
2000
1500
1000
500
0
Clearance rate (μmol/h/g
***
*
**
***
basal
DB cAMP + IBMX (pre)
Scy II 10-12 M
Scy II 10-9 M
Scy II 10-6 M
DB cAMP + IBMX (post)

FIGURE 6.6

Figure 6.6 Time course of 10^{-6} M scyliorhinin II (Scy II) on clearance rates of the isolated perfused rectal gland compared to total basal values. Clearance rates (µmol/h/g) (ordinate) against time (15 min intervals) (abscissa). Values are expressed as a mean µmol/h/g ± SEM; 803 ± 193 for basal total; 2992 ± 542 for 0-15 mins; 1906 ± 439 for 15-30 min; 1760 ± 596 for 30-45 min; 1133 ± 234 for 45-60 min; and 1261 ± 277 for 60-75 min (n = 7, 6, 6, 6, 6, & 6 respectively). *$p<0.05$ compared with total basal clearance rate (alternative Welch t-test).


4000
3500
3000
2500
2000
1500
1000
500
0
Clearance rate (μmol/h/g)
10-6 M Scy II
perfusion
**
*
basal
0
15
30
45
60
75
Time (min)

6.4 - Discussion

In the present study a peptide isolated from dogfish gut extract was a potent stimulator of the isolated perfused rectal gland preparation of *S. canicula* (Figure 6.3) and the sequence of this fraction was found to be identical to that of scyliorhinin II (Conlon *et al.*, 1986). Furthermore, synthetic scyliorhinin II gave a dose dependent stimulation of rectal gland secretion (Figure 6.5) suggesting that it may indeed be the homologous gut peptide modulating rectal gland activity, at least in *S. canicula*. It is proposed therefore that the uncharacterised rectin previously suggested as the gut peptide modulating rectal gland secretion in *S. canicula* (Shuttleworth & Thorndyke, 1984) is in fact scyliorhinin II.

Scyliorhinin II, first isolated from an extract of *S. canicula* intestine on the basis of its ability to contract longitudinal muscle in guinea pig ileum (Conlon, *et al.*, 1986) and subsequently from the intestine of the ray, *Torpedo marmorata* (Conlon & Thim, 1988) is a member of the tachykinin family of regulatory peptides. The tachykinins share a common amino acid sequence (-Phe-X-Gly-Leu-Met. NH_2 ; X=Phe, Tyr, Ile, Val) at the C-terminus of the peptides and a similar spectrum of biological activities. Several tachykinins have been isolated from elasmobranch tissues (Waugh, Sower, Bjenning, & Conlon, 1994) but their biosynthetic relationship to each other and to the mammalian tachykinins, substance P, neurokinin A and neurokinin B is unclear. In addition to scyliorhinin II, the dogfish gut synthesises a second tachykinin, scyliorhinin I (Ala-Lys-Phe-Asp-Lys-Phe-Tyr-Gly-Leu-Met. NH_2) (Conlon, *et al.*, 1986) which was also found in the brain of *S. canicula* (Waugh, Wang, Hazon, Balment, & Conlon, 1993). Scyliorhinin II was not detected in an extract of dogfish brain but this tissue synthesises a tachykinin ([Lys^1, Arg^1, Gly^5] substance P) that is structurally more similar to mammalian substance P (Waugh *et al.*, 1993). A peptide that is structurally related to neurokinin A has not been isolated from dogfish tissues but [Leu^3, Gly^4] neurokinin A has been purified, together with [His^3] scyliorhinin I from the brain of the ray, *Raja rhina* (Waugh *et al.*, 1994). In mammals, neurokinin A and substance P are products of the post-translational processing of the same biosynthetic precursor (preprotachykinin A) (Nakanishi, 1986) whereas neurokinin B is derived from a different precursor (preprotachykinin B). Clearly, further work is required to elucidate the biosynthetic relationship between scyliorhinin I, scyliorhinin II and [Lys^1, Arg^1, Gly^5] substance P in the dogfish.

To date tachykinins in mammals have been localised predominantly to nervous tissue. However, studies carried out on *S. acanthias* (El-Salhy, 1984; Holmgren, 1985) indicate that in elasmobranchs localisation of tachykinins is not restricted to nervous tissue alone but also to gastrointestinal endocrine-like cells.

Similarly immunohistochemical studies, using an antiserum directed against terminal region of neurokinin A, have shown that scyliorhinin II is fou endocrine-like cells in gastric and intestinal mucosa of *S. canicula* (M. Con unpublished data). The presence of tachykinins in endocrine cells of *S. acanthi* and *S. canicula* suggests the possibility of scyliorhinin II having a classical endocrine effect on rectal gland activity. In mammals there is some confusion over the second messenger systems linked to tachykinin receptors. Tachykinins have been shown to stimulate both the inositol phosphate and cAMP pathways (Nakajima, Tsuchida, Negishi, Ito & Nakanishi, 1992) and also inhibit the cAMP pathway (Laniyonu, Sliwinski-Lis & Fleming, 1988). However, in fish there is no reported evidence of tachykinin effects on second messenger systems. Further work is therefore required to elucidate whether scyliorhinin II represents an authentic circulatory hormone in *S. canicula*.

What is apparent from this study is the homogeneity required for certain peptides to have an effect on their target organ. The difference in structure between the two batches of scyliorhinin II was not possible to investigate. However two apparently identical synthetic peptides produced markedly different biological responses in the isolated perfused rectal gland. The presence of a cysteine bridge in the structure of scyliorhinin II is established and is essential for the peptide to exhibit its full biological activity as this has a profound effect on the three dimensional configuration of the peptide. The formation of cysteine bridges in synthetic peptides is notoriously difficult (M. Conlon, pers. comm.), which may explain the difference in biological activity between the two apparently identical peptides.

CHAPTER 7
GENERAL DISCUSSION

7 - General Discussion.

The majority of isolated perfusion studies have concentrated on the spiny dogfish *Squalus acanthias*, with only a few studies carried out on the lesser spotted dogfish *Scyliorhinus canicula*. This may reflect the greater procedural difficulties associated with the smaller species *S. canicula*, in which the cannulation of the rectal gland's vasculature is technically more demanding. The present study has demonstrated however that such a preparation can be adapted to *Scyliorhinus canicula* and produce reliable and consistent results.

The rectal gland of elasmobranchs is thought to be specific in the regulation of sodium and chloride and to uniformly concentrate salt (Burger & Hess, 1960). Furthermore, it is thought to secrete a sufficient amount of salt to exert a significant effect on the total plasma osmolality of *S. acanthias* (Burger & Hess, 1960). The significance of the rectal gland in the overall homeostatic control of elasmobranchs was challenged by Haywood (1974). Following ligation of the rectal gland artery in *Poroderma africanum*, it was demonstrated that the fish was capable of regulating plasma salt and water balance even after a salt load (Haywood, 1974). A similar result was also observed in *S. acanthias* following removal of the rectal gland (Burger, 1965). However, in both studies the return of plasma sodium and chloride levels back to normal plasma concentrations in fish with non-functional glands was considerably slower. In addition Burger (1965) observed that in glandless fish urine chloride loss was approximately 2 to 3 times greater than in control fish. It appeared that the glandless fish would commonly control plasma chloride levels by increasing urine volume (Burger, 1965). Intra-arterial together with intra-muscular sodium loading, and force feeding, of the lip shark, *Hemiscyllium plagiosum*, did not appear to alter plasma sodium levels but did alter sodium content of muscular tissue (Chan *et al*, 1967). Interestingly, similar manipulation of fish that had the rectal gland removed showed a further increase in sodium content of muscular tissue and a slight but significant increase in plasma sodium content (Chan *et al.*, 1967). It is apparent therefore that removal of the rectal gland does reduce the osmoregulatory capabilities of elasmobranchs particularly following sodium and/or volume load.

It has been suggested that the rectal gland was closely involved in plasma sodium and/or volume control (Burger, 1962, 1965). Burger (1962) postulated that there were three main "humoral factors" influencing rectal gland secretion: "1) an osmotic component; 2) a volume component; and 3) a particular effect of sodium chloride". Therefore, subsequent research concentrated on the isolation of an individual humoral factor which effected rectal gland secretion.

Injection of 3 ml of 5% sodium chloride into the stomachs of the stingray, *Urolophus jamaciensis* produced a marked dilation of rectal gland blood vessels within minutes (Doyle, 1962). Intra-arterial and intramuscular sodium loading, also caused a temporary but significant rise in rectal gland fluid excretion in the lip shark, *Hemiscyllium plagiosum* (Chan *et al.*, 1967). Both these early studies demonstrated increased rectal gland activity following volume and salt loading, however neither attempted to isolate the components and investigate their individual effects on rectal gland activity.

A series of unique experiments was carried out by Solomon *et al.* (1984 a & b; 1985, a & b) to investigate the individual effects of volume expansion and salt loading on rectal gland function in *S. acanthias*. The experiments involved three different kinds of preparation:

1) Glands perfused *in situ*, with haemodynamic and pharmacological manipulations delivered via the dorsal aorta.

2) Explanted glands perfused from the blood of a donor fish via the dorsal aorta, with haemodynamic manipulations delivered to the donor fish. Thus removing neural connections to the explanted gland. In addition, an on-line perfusion pump delivered pharmacological manipulations to the explanted gland.

3) *In vitro* perfusion of the rectal gland at constant perfusion pressure.

For the purpose of this discussion the experiments are described as follows, 1) *in situ* preparations, 2) explanted preparations, and 3) *in vitro* preparations.

It was found that infusion of isotonic shark Ringer, isotonic hyponatraemic shark Ringer (10% mannitol to replace the sodium), and hypertonic saline all produced an increase in secretion in the *in situ* preparation (Solomon *et al.*, 1984a). Similar effects were also observed in the explanted preparation (Solomon *et al.*, 1984a). Furthermore, infusion of small amounts of a highly concentrated salt solution, altering the plasma osmolality of the donor fish, but not the volume (as blood was removed previous to infusion), did not effect rectal gland secretion in the explanted preparation (Solomon *et al.*, 1985b). However, infusion of isotonic Ringer, increasing donor fish plasma vol by 50%, produced a 4 fold increase in secretion rate and 3 fold decrease in vascular resistance from the explanted rectal gland preparation.

The ratio of arterial to venous oxygen content was also monitored across the *in situ* preparation (Solomon *et al.*, 1984b). It was found that this ratio did not differ between control and stimulated preparations with approximately 95% of the arterial oxygen extracted. In order to increase secretion an increased supply of

oxygen for active epithelial salt transport was required. Therefore, it was postulated that an increase in blood flow to the rectal gland was necessary for an increase in secretion rate (Solomon *et al*, 1984b). However, using a rotary pump to increase blood flow (and therefore oxygen delivery to the explanted preparation) did not stimulate secretion, although it is possible that this increased blood flow did not perfuse the secretory parenchyma (Solomon *et al*, 1984b). It was postulated that an increase in chloride secretion, inducing tissue hypoxia, caused vasodilation of the rectal gland vasculature and hence an increase in blood flow. However, inhibition of volume loaded-induced secretion in the explanted gland with bumetanide did not alter vasodilation of the gland (Solomon *et al.*, 1984b). From these studies three principle conclusions were reported; 1) volume expansion of the plasma in the elasmobranch appears to be the major factor stimulating rectal gland activity; 2) denervation of the gland in the explanted preparation still allows for an increase in secretion rate; 3) a haemodynamic factor is released following volume expansion, which stimulates secretion from the rectal gland (Solomon *et al.*, 1984 a & b; 1985b). Feeding and the possibility of subsequent sodium chloride reabsorption would lead to the physiological conditions akin to volume expansion. One could therefore postulate that following feeding rectal gland activity would be highest. Indeed it has been shown in this laboratory that rectal gland Na^+K^+ATPase activity peaks approximately 9 hr post-feeding with a 16 fold increase above basal levels, followed by a return to basal levels approximately 48 hr post-feeding (S. MacKenzie pers. comm.).

The role of shark C-type natriuretic peptide (sCNP) as a dose dependent stimulator of rectal gland secretion in the elasmobranchs investigated to date further supports the function of the peptide as a hormone concerned with reducing physiological increases in plasma volume. Such a response was also postulated for the action of ANP on the salt gland of the domestic duck, *Anas platyrhynchos* (Schutz & Gerstberger, 1990).

The dose response curve produced by atriopeptin stimulation of secretion of the isolated rectal gland of *S. acanthias* (Solomon *et al.*, 1985a) was found to be very narrow, indicating an all or nothing response to atriopeptin. If this were the case a threshold of sCNP *in vivo* would have to be reached to counteract a particular inhibitory factor on rectal gland activity. Such a factor could certainly be vasoactive, as the design of rectal gland vasculature (Chapter 2) facilitates complete, partial, or almost absent perfusion of the secretory epithelia.

The intermittent nature of rectal gland secretion suggests that the blood flow through the gland during periods of quiescence is minimal which was indeed demonstrated by Kent and Olson (1982). It is possible therefore *in vivo* that during periods of quiescence the vasoconstrictive effects of local and/or systemic AII may direct blood flow away from the secretory epithelia through the by-pass and shunt vessels described in Chapter 2. The apparent synergistic action of AII and sCNP in increasing secretion rate *in vitro* is more difficult to explain. However, the rectal gland *in vivo* would undoubtedly be confronted with a combination of these two peptides in the blood perfusing the secretory epithelia.

The gastro-intestinal neuropeptide, vasoactive intestinal peptide (VIP) has been extensively used as a stimulator of the isolated perfused preparation of *S. acanthias* (Silva *et al.*, 1985; Silva *et al.*, 1987; Silva *et al.*, 1993a). Furthermore, VIP-like immunoreactivity has been found in the secretory parenchyma of the rectal gland of *S. acanthias* and *S. canicula* (Masini *et al*, 1994) indicating that VIP, as a neuropeptide, could have a direct effect on rectal gland secretion (Holmgren & Nilsson, 1983). However, reports to date on the regulation of rectal gland secretion in *S. canicula* (Shuttleworth, 1983b; Shuttleworth, 1988; Shuttleworth & Thorndyke, 1984) illustrate a major difference in the nature of gut peptides modulating rectal gland activity of these two elasmobranch species. Isolated perfusion of both whole gland and rectal gland slices of *S. canicula* with VIP (Shuttleworth, 1983b) did not stimulate secretion even at concentrations of 10^{-6} M. In view of this Shuttleworth and Thorndyke (1984) attempted to isolate and characterise an homologous gut peptide that regulated rectal gland secretion. A stimulatory fraction was isolated but never characterised and was named rectin. This study has demonstrated that rectin is the tachykinin, Scyliorhinin II, and is a potent stimulator of rectal gland secretion rate in *S. canicula*.

Immunocytological and immunohistological studies the gut of on *S. acanthias* (El-Salhy, 1984; Holmgren, 1985) indicate that in elasmobranchs, localisation of tachykinins is not restricted to nervous tissue alone (as in mammals) but also to gastrointestinal endocrine-like cells. Similarly immunohistochemical studies using an antiserum directed against the C-terminal region of neurokinin A have shown that a scyliorhinin II-like peptide is found in endocrine-like cells in gastric and intestinal mucosa of *S. canicula* (J.M. Conlon pers comm.). Tachykinins in mammals (Substance P, Neurokinin A and neurokinin B) are thought to induce vasodilation through specific neurokinin (NK) receptors (Waugh *et al.*, 1993). Although current knowledge regarding physiological functions of tachykinins in fish is limited, both trout substance P and neurokinin A have been shown to stimulate the motility of isolated trout intestinal

muscle (Jensen, Olson & Conlon, 1993). A cardiovascular effect was also demonstrated in *S. acanthias* where substance P was shown to cause an increase in heart rate, cardiac output and, in particular, coeliac arterial blood flow (Holmgren, Axelsson & Farrell, 1992). It has been reported that scyliorhinin I has high affinity for mammalian NK1 and NK2 receptors and scyliorhinin II for the mammalian NK3 receptor (Buck & Krstenansky, 1987). Scyliorhinin I and synthetic dogfish substance P were shown to decrease arterial blood pressure in the rat. However, this was not repeated in the dogfish suggesting that the scyliorhinin I-NK1 receptor complex is not involved in cardiovascular regulation in elasmobranchs (Waugh *et al.*, 1993). Further research is required to ascertain the physiological relationship between tachykinins in elasmobranchs.

The present study has concentrated on stimulatory factors of rectal gland secretion rate, with perhaps possible inhibition by angiotensin II. However, *in vitro* perfusion studies in *S. acanthias* have identified two neuropeptides involved in the inhibition of rectal gland secretion. Infusion of the hypothalamic peptide, somatostatin in the explanted preparation utilised by Solomon *et al* (1984b) was found to inhibit cAMP and forskolin-induced stimulation (Silva *et al.*, 1985). However, somatostatin did not affect VIP binding but did inhibit VIP stimulated accumulation of cAMP, although the precise site of action remained unclear (Silva *et al.*, 1985). A further study on the action of somatostatin was carried out by Silva, *et al.* (1990) in which bombesin was used to investigate neuronal control of somatostatin on the perfused rectal gland of *S. acanthias.* Addition to the perfusate of bombesin alone produced no effect, however, when bombesin was added to glands previously stimulated with VIP, a reversible inhibition in chloride secretion was observed, in addition to a 10 fold increase in the liberation of somatostatin into the venous effluent. It was therefore postulated that bombesin inhibition of VIP-stimulated chloride secretion was secondary to the release of somatostatin (Silva *et al*, 1990). Neuropeptide Y (NPY) is a highly conserved neuropeptide found throughout the elasmobranchs and produces vasoconstrictor effects in the skate coronary artery and dogfish gill artery (Bjenning, 1992). Synthetic porcine NPY has been shown to inhibit secretion from whole gland, isolated tubules, and isolated cellular preparations of *S. acanthias* rectal gland stimulated by VIP, forskolin and cAMP (Silva *et al.*, 1993). Furthermore, NPY inhibition of rectal gland secretion in *S. acanthias* was thought to be mediated at least in part by intracellular calcium concentration (Silva, Solomon, Landsberg, Hervieux, Emmonds & Epstein, 1991).

The elasmobranch interrenal homologue 1α hydroxycorticosterone (1αOH-B) has also been implicated in the control of rectal gland secretion in

elasmobranchs. Holt and Idler (1975) studied the effect of interrenalectomy and steroid replacement on the output of rectal gland fluid of the skate, *Raja ocellata*. It was found that after interrenalectomy there was a decrease in volume, osmolality, and sodium and chloride concentrations of rectal gland fluid. Furthermore, using 1αOH-B and corticosterone in steroid replacement therapy rectal gland fluid output increased. Holt and Idler (1975) suggested that, 1αOH-B and corticosterone were involved in the regulation of the rectal gland. However Chan *et al* (1967), had previously reported that cortisol and deoxycorticosterone decreased rectal gland secretion in *Hemiscyllium plagiosum* These animals were however intact and the steroids used in the study were heterologous corticosteriods. Clearly further work is required to determine whether 1αOH-B plays a significant role in the regulation of rectal gland secretion. More recent studies have shown that 1αOH-B appears to regulate plasma sodium levels (Armour *et al.*, 1993 a & b) which supports the role of 1αOH-B as a steroid controlling rectal gland secretion rate. However, the highest levels of 1αOH-B were found in protein starved fish where sodium and chloride were retained in order to maintain plasma osmolality in 130% sea water (Armour *et al.*, 1993a). This suggests that 1αOH-B may have an inhibitory effect on rectal gland secretion rate.

Glucagon appears to exert a stimulatory effect on rectal gland secretion in the present study but has no effect on the isolated perfused rectal gland of *S. acanthias* (Stoff, *et al.*, 1979). The principle difference between these two studies is that in the present study homologous glucagon was used. Given the findings of Guibollini and Lahlou (1987 a & b) that glucagon stimulates adenylate cyclase activity in trout gill epithelium, it is possible that glucagon exerts a similar effect on rectal gland secretory epithelia. However, a concentration of 10^{-6} M glucagon was required to stimulate secretion from the isolated perfused rectal gland and 5.7 times 10^{-10} M glucagon was sufficient to stimulate cAMP production in isolated trout gill epithelium (Guibollini and Lahlou 1987b).

In the present study investigation of primary controlling factors in rectal gland activity has led to a brief examination of the second messenger systems mediating the secretory process. The second messenger cyclic-guanosine monophosphate (cGMP) has been implicated as the mediator of natriuretic peptide-induced cellular changes in a variety of epithelia, and guanylate cyclase linked receptors have also been identified on the secretory epithelia of the rectal gland of *S. acanthias* (Gunning *et al*, 1993). The synthetic analogue atriopeptin has been shown to stimulate short circuit current in isolated rectal gland cell preparations of *S. acanthias* (Karnaky *et al.*, 1991). Concomitant to this

stimulation was an increase in intracellular concentration of cGMP approximately 40 fold above basal levels (Karnaky *et al.*, 1991), thus suggesting a close relationship between increased chloride secretion and increased intracellular cGMP concentration. However, cell permeable analogues of cGMP did not stimulate rectal gland secretion in the present study or confluent monolayers of cultured rectal gland cells from *S. acanthias* (Silva, *et al.*, 1993). This finding led Silva *et al* (1993) to postulate that sCNP stimulated rectal gland secretion by an indirect route (possibly VIP mediated) and a direct route (possibly protein kinase C mediated). Furthermore, using phorbol esters to stimulate protein kinase C (PKC) Feero and Valentich (1991) demonstrated that activation of PKC stimulated activity in cultured rectal gland cells of *S. acanthias*. However, Silva, Solomon, Brignull, Hornung, Landsberg, Solomon, Wolff and Epstein (1992) demonstrated that the activation of PKC did not stimulate secretion from isolated perfused rectal glands of *S. acanthias*. It is evident from the present study that neither cAMP or cGMP appear to regulate secretion from the isolated perfused gland of *S. canicula*. Interestingly, in isolated mammalian smooth muscle cells ANP has been shown to act via the C-type receptor, or clearence receptor (Anad-Srivastava & Trachte, 1993). The ANP-C-type receptor complex in smooth muscle cells is thought to inhibit adenylate cyclase and stimulate production the inositol phosphate, IP_3 (Anad-Srivastava & Trachte, 1993; Smyth & Keenan, 1994). The possibility of the ANP-C-type receptor complex in rectal gland epithelia requires further investigation.

High intracellular levels of the inositol phosphates have been measured in the rectal gland of *S. canicula* (Simpson & Sargent, 1985). Activation of the inositol phosphate pathway has been demonstrated following rat ANP and VIP-induced stimulation of rectal gland tubules of *S. acanthias* (Ecay & Valentich, 1991). However, in rectal gland plasma membrane preparations of *S. acanthias* human ANP did not activate the inositol phosphate pathway whereas VIP did (Brand, Taylor, Spokes & Silva, 1990). Activation of the inositol phosphate pathway enhances intracellular calcium concentration from intracellular stores and extracellular fluid (Darnell, Lodish & Baltimore, 1990). However, the calcium ionophore A23187 produced no effect on ouabain binding and ouabain-sensitive oxygen consumption in rectal gland slices of *S. canicula* (Shuttleworth, 1983a), although verapamil (a calcium channel blocker) did inhibit cAMP-stimulated ouabain sensitive oxygen consumption. This suggested that cAMP-induced stimulation of ouabain sensitive oxygen consumption in the rectal gland was a calcium-dependent rather than calcium-mediated process (Shuttleworth, 1983a). Endogenous adenosine was found to bind to A_1 adenosine receptors on stimulated

secretory cells of rectal gland cell cultures of *S. acanthias* (Kelley, Poeschla, Barron, & Forrest, 1990; Kelley, Aassar, & Forrest, 1991). These researchers postulated that endogenous adenosine was released in response to hormone-stimulated secretion and acted at A_1 adenosine receptors to provide a negative feedback to chloride secretion. It appears that intracellular mediation of secretion in the rectal gland of elasmobranchs involves a complex combination of a variety of second messenger systems. For future research of second messenger systems involved in the control of secretion from rectal gland epithelia an isolated cell preparation would be more appropriate.

Undoubtedly the major proportion of research into the control and action of the rectal gland in elasmobranchs has centred on a single species, *Squalus acanthias*. Furthermore, research into the control of secretion in *S. acanthias* has concentrated on the direct effects on secretory epithelia and a principle factor involved in this appears to be VIP. Figure 7.1 illustrates our current knowledge on the control rectal gland secretion in *S. acanthias* from the evidence presented in the literature.

It is evident from this and other studies investigating the control mechanisms of the rectal gland of *S. canicula* that VIP does not stimulate rectal gland secretion. Indeed the gastro-intestinal stimulatory factor of rectal gland secretion in *S. canicula* identified in this study is scyliorhinin II which is structurally unrelated to homologous VIP (Figure 7.2). Interestingly in *S. acanthias* plasma levels of VIP were not shown to increase following volume load (Solomon *et al*, 1985a). A similar measurement of plasma scyliorhinin II levels post-feeding would determine if this peptide is released from endocrine cells in the stomach in response to feeding and possible subsequent gastro-intestinal distension, and thus determine whether scyliorhinin II represents an authentic circulatory hormone. Furthermore, it would be important to ascertain if the stimulatory effects of scyliorhinin II on rectal gland secretion were universal in the elasmobranchs. In addition somatostatin has been shown to directly inhibit VIP-induced stimulation of secretion in the rectal gland of *S. acanthias*. To determine if VIP and scyliorhinin II are functionally similar in *S. acanthias* and *S. canicula*, respectively possible inhibitory effects of somatostatin on scyliorhinin II-induced stimulation of the rectal gland of *S. canicula* would have to be investigated.

The results presented in this and other studies indicate that the rectal gland is a highly vascular organ and it is evident from the literature that increased secretion rate appears to require an increase in blood flow through the gland (Kent & Olson, 1982; Solomon *et al*, 1984b). Consequently the present study has

FIGURE 7.1

Figure 7.1 Possible hormonal control mechanisms on the rectal gland of *Squalus acanthias* based on our current knowledge in the literature.

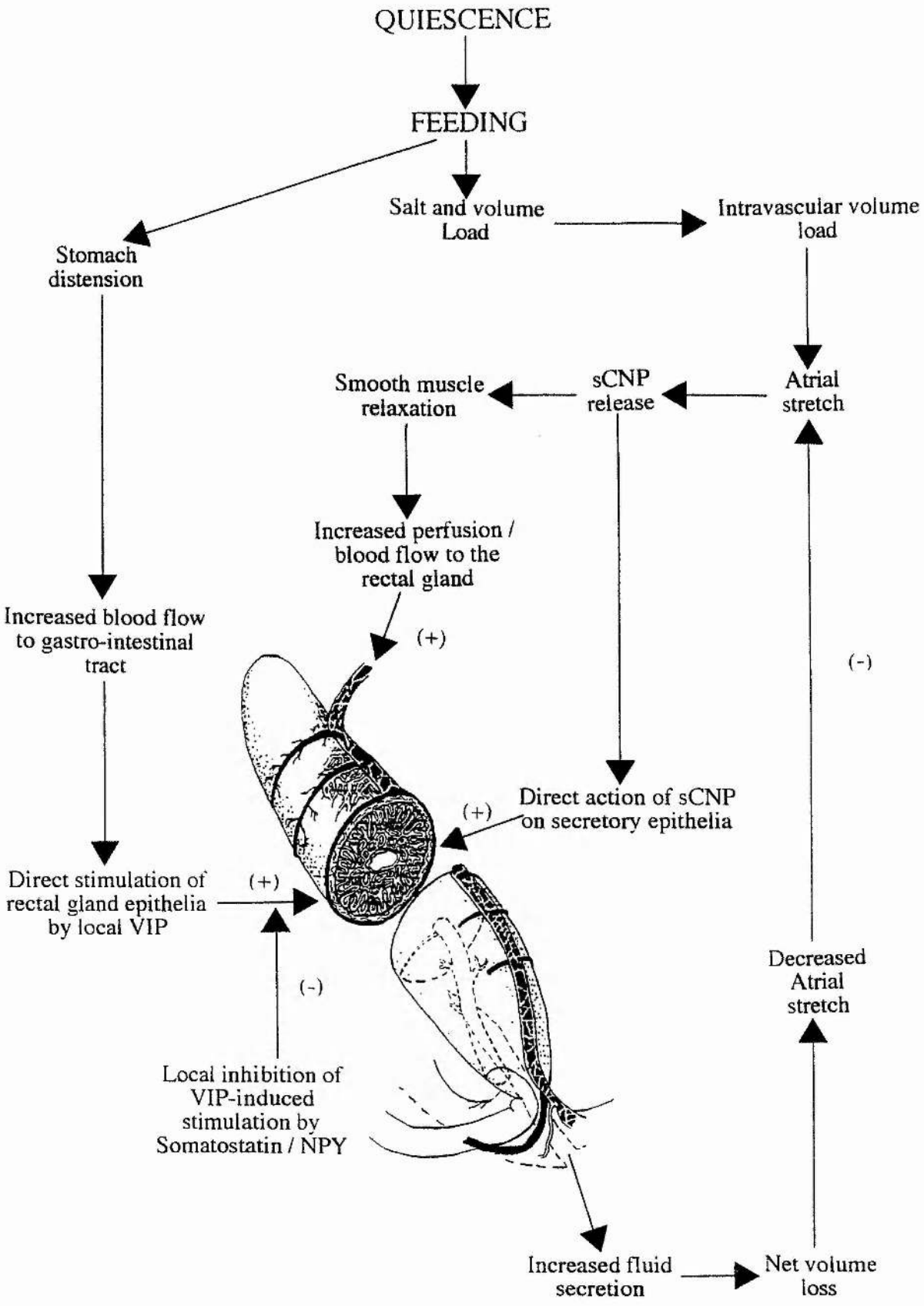

QUIESCENCE
FEEDING
Salt and volume Load
Intravascular volume load
Stomach distension
Atrial stretch
sCNP release
Smooth muscle relaxation
Increased perfusion / blood flow to the rectal gland
Increased blood flow to gastro-intestinal tract
(+)
(-)
Direct action of sCNP on secretory epithelia
(+)
Direct stimulation of rectal gland epithelia by local VIP
(+)
(-)
Local inhibition of VIP-induced stimulation by Somatostatin / NPY
Decreased Atrial stretch
Increased fluid secretion
Net volume loss

FIGURE 7.2

Figure 7.2 Structural comparison of some tachykinins and some vasoactive intestinal peptides characterised to date.

SCYLIORHININ II Ser Pro Ser Asn Ser Lys (Cys) Pro Asp Gly Pro Asp (Cys) Phe Val Gly Leu Met
(Elasmobranch)

NEUROKININ A His Lys Thr Asp Ser Phe Val Gly Leu Met
(Mammal)

NEUROKININ B Asp Met His Asp Phe Phe Val Gly Leu Met
(Mammal)

DOGFISH VIP His Ser Asp Ala Val Phe Thr Asp Asn Tyr Ser Arg Ile Arg Lys Gln Met Ala Val Lys Lys Tyr Ile Asn Ser Leu Leu Ala

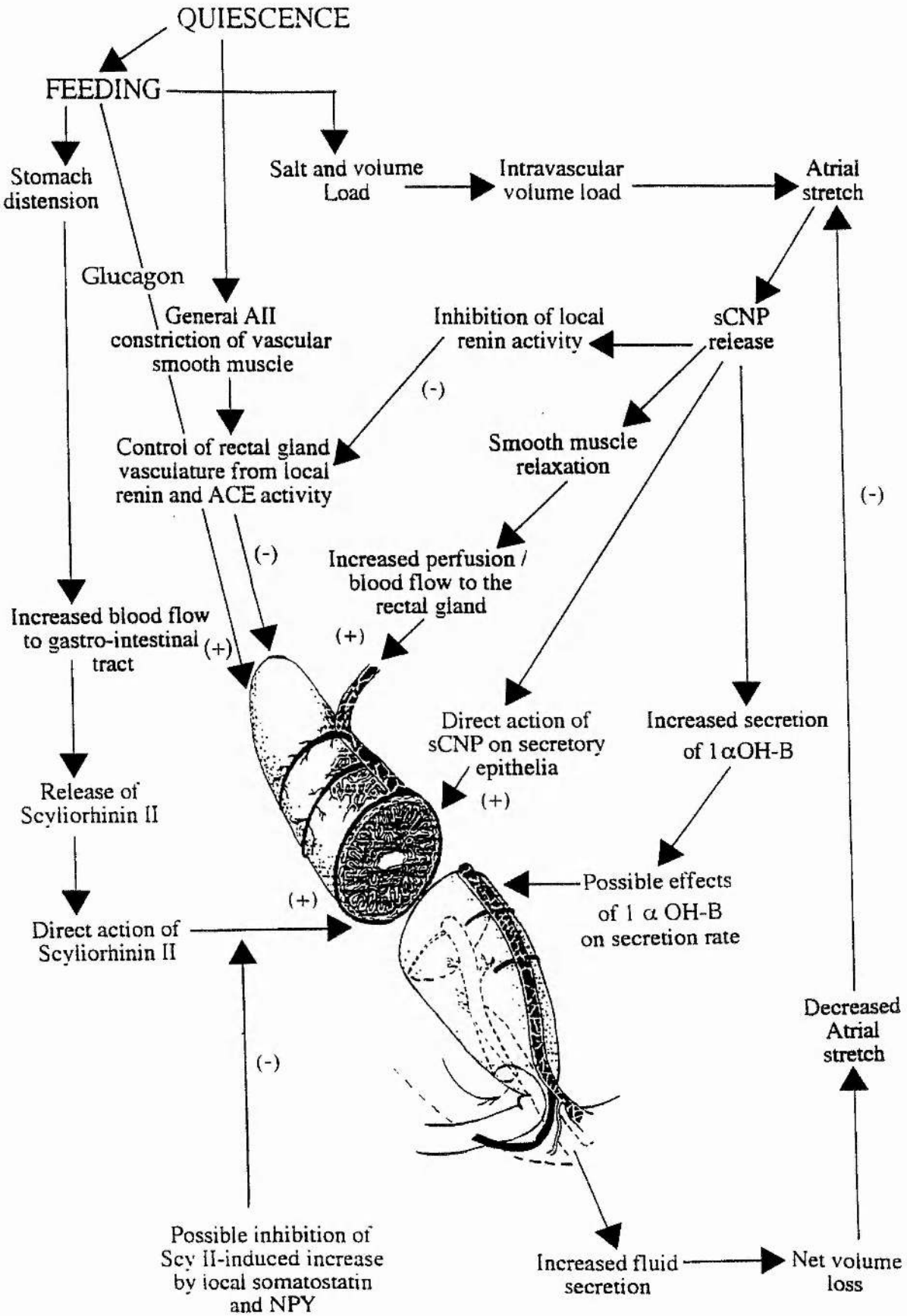
PORCINE VIP His Ser Asp Ala Val Phe Thr Asp Asn Tyr Thr Arg Leu Arg Lys Gln Met Ala Val Lys Lys Tyr Leu Asn Ser Ile Leu Asn

investigated the possible vascular control of rectal gland secretion utilising two apparently antagonistic vasoactive peptides, sCNP and AII. Unexpectedly a combination of these two peptides produced a synergistic effect on secretion rate of the isolated perfused preparation. Further work is required to clarify the mode of action of these two peptides to ascertain the true roles of AII and sCNP in the stimulated rectal gland. Such work could include, monitoring of blood flow rates using microspheres, pharmacological manipulation of the concentrations of AII and sCNP used in the *in vitro* preparation and perhaps measurement of oxygen consumption in tissue slices of the rectal gland following administration of a combination of AII and sCNP. Furthermore, there is a need to address the possibility of a local RAS influencing rectal gland activity.

In conclusion, the present study has highlighted a major difference in hormonal control of rectal gland secretion between two closely related elasmobranch species. VIP thought to be a potent stimulator of rectal gland activity in *S. acanthias* did not stimulate the isolated perfused rectal gland of *S. canicula*. The previously identified, but never characterised, stimulatory gut peptide, rectin, may in fact be scyliorhinin II. Glucagon a hormone that had no effect on rectal gland activity in *S. acanthias* appeared to stimulate rectal gland activity in *S. canicula*, although concentrations used were somewhat supra-physiological. Unlike VIP and glucagon the homologous natriuretic peptide, C-type natriuretic peptide, appears to significantly stimulate rectal gland activity in both *S. acanthias* and *S. canicula*. Figure 7.3 demonstrates the possible hormonal control of rectal gland secretion in *S. canicula* based on the evidence in the literature and the findings of the present study.

FIGURE 7.3

Figure 7.3 Possible hormonal control mechanisms on the rectal gland of *Scyliorhinus canicula* based on our current knowledge in the literature, and the findings of the present study.


QUIESCENCE
FEEDING
Stomach distension
Salt and volume Load
Intravascular volume load
Atrial stretch
Glucagon
General AII constriction of vascular smooth muscle
Inhibition of local renin activity
sCNP release
(-)
Control of rectal gland vasculature from local renin and ACE activity
Smooth muscle relaxation
(-)
Increased perfusion / blood flow to the rectal gland
(-)
Increased blood flow to gastro-intestinal tract
(+)
(+)
Direct action of sCNP on secretory epithelia
(+)
Increased secretion of 1αOH-B
Release of Scyliorhinin II
(+)
Possible effects of 1 α OH-B on secretion rate
Direct action of Scyliorhinin II
(-)
Decreased Atrial stretch
Possible inhibition of Scy II-induced increase by local somatostatin and NPY
Increased fluid secretion
Net volume loss

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APPENDIX

Appendix 1 Amino acid abbrevations

One-letter	Three-letter	Name
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	Tryosine