STUDIES ON ATRIAL NATRIURETIC PEPTIDE.

A thesis submitted for the degree of

Doctor of Medicine

to the University of Glasgow.

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ACKNOWLEDGEMENTS AND DECLARATION OF ORIGINALITY.

The work in this thesis was performed in the Medical Research Council Blood Pressure Unit, Western Infirmary, Glasgow between August 1987 and July 1990. I am grateful to the Director, Dr A F Lever for the opportunity to work in his Unit and for introducing me to ANP. Dr. J M C Connell supervised many of the projects and I am indebted to him for many helpful discussions and criticism.

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Excluding the collection of some samples from patients (above) and many of the routine assays - the work contained within this thesis is my own. The exceptions to this are the studies in Chapters 7 & 8. The study in Chapter 7 was the first study of NEP inhibition in man and was planned with Dr. Stephen Dilly of Pfizer. I subsequently designed and planned the study in Chapter 8 - in May 1988, based on the findings in Chapter 7 - and later analysed the results. However, the day-to-day running of this study was performed by Dr. Janice O'Connell - following her appointment (supported by Pfizer (UK)) in August 1988. I am especially grateful to Janice for permitting me to use data which she collected.

Abbreviations.

AA	Amino acids.
ACE	Angiotensin Converting Enzyme (EC 3.4.15.4).
Aldo	Aldosterone.
Ang II	Angiotensin II.
ANOVAR	Analysis of variance.
ANP	Atrial Natriuretic Peptide.
Bmax	Receptor density (maximal binding).
BNP	Brain Natriuretic Peptide.
cGMP	Cyclic guanosine monophosphate.
DOCA	Deoxycorticosterone acetate.
EDRF	Endothlium derived relaxing factor.
ERPF	Estimated renal plasma flow.
FeNa(Li)	Fractional excretion of sodium (lithium).
FF	Filtration fraction.
Kd	Receptor dissociation constant (50% binding).
GFR	Glomerular Filtration Rate.
NEP	Neutral Endopeptidase (EC 3.4.24.11).
SE/SEM	Standard error of the mean.
SHR	Spontaneously Hypertensive Rat.

List of Figures and Tables.

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Publications and Presentations to Learned Societies.

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Presentations:

Abstracts of the work in this thesis were presented at the following meetings (Reference Journal).

Society for Endocrinology 1988, 1989, 1990 (Journal of Endocrinology 117, 121, 124, 127(supplements)).

Medical Research Society, London 1989 (Clinical Science 74(Suppt 20)).

American College of Cardiology, Los Angeles 1989 (Journal of the American College of Cardiology 13(2))

American Society for Hypertension, New York 1989 (American Journal of Hypertension 2(5)).

Geschellschaft fur Nephrologie, Bern 1989 (Klinische Wochenschrift 67).

Scottish Society of Physicians, Dunfermline 1989.

International Society of Hypertension, Montreal 1990 (Journal of Hypertension 8(Suppt 5)).

Symposium on Atrial Natriuretic Factor, Ottowa 1990.

Scottish Renal Association, Inverness 1991 (Kidney International [In press]).

SUMMARY: Studies on Atrial Natriuretic Peptide.

Atrial Natriuretic Peptide (ANP) is a hormone secreted by the heart with a unique spectrum of actions: natriuresis, diuresis, vasorelaxation and suppression of the renin-angiotensin system. This thesis is divided into four sections each of which examines a different aspect of ANP. The common theme is the question of how the actions of ANP may be "harnessed" therapeutically, either directly using novel inhibitors of the enzyme Neutral Endopeptidase (NEP; EC 3.4.24.11) which degrades ANP, or indirectly, by investigating potential interactions with available therapeutic agents including Angiotensin Converting Enzyme (ACE) inhibitors, and calcium channel blockers.

1. The first section describes plasma levels of ANP in a variety of human conditions including hypertensive disorders, pregnancy, and in the human fetus. Data are presented to support the presence of a functional ANP hormonal system in the human fetus. Samples obtained by cordocentesis demonstrate that plasma levels are higher in rhesus disease, and are related to the severity of the anaemia in this condition. Furthermore - like the adult - the fetus can be shown to release ANP in response to volume expansion (blood transfusion).

In adults ANP levels are also elevated in volume expanded states such as mineralocorticoid hypertension and pregnancy; and are lower in volume depleted states such as adrenocortical insufficiency. Although the major influence on the release of ANP is thought to be intravascular volume, ANP levels are also very high in patients with pre-eclampsia (PET) and malignant phase hypertension (where

intravascular volume is not expanded) indicating that cardiac afterload is also a determinant of ANP release.

2. The second section examines the renal actions of ANP and the interaction with the renin-angiotensin system in dogs. Infusion of 5 pmol/kg/min ANP is shown to cause a four to five-fold increase in plasma ANP, with a two to three-fold increase in urine volume and sodium excretion, a minor fall in blood pressure and a 50% reduction in renin.

Inhibition of the renin-angiotensin system by acute administration of enalaprilat caused a three-fold increase, whilst infusion of low dose Ang II virtually abolished, the natriuretic and diuretic effects of ANP. Circulating (and intrarenal) Ang II levels are thus proposed to exert a major modulatory role on the renal actions of ANP, and that combination with ACE inhibitors may potentiate the possible therapeutic effects of ANP. The interaction between ANP and Ang II is proposed to occur largely at the level of the renal. In the simplest model the proximal tubular effects of Ang II on sodium reabsorption regulate the delivery of sodium to the distal tubule where ANP has its major effects. However, interactions at other sites within the nephron cannot be excluded. Plasma renin was suppressed by ANP to a similar extent regardless of changes in sodium excretion (when given alone or combined with Ang II or ACE inhibition) with the inference that ANP suppresses renin secretion by a direct effect on the juxtaglomerular apparatus, rather than indirectly via delivery of sodium to the macula densa. Finally, from the relationship between ANP, Ang II and sodium excretion it is possible to estimate that physiological levels of ANP achieve approximately 15% of their natriuretic effects by suppression of endogenous Ang II.

3. The third experimental section examines the effects of NEP inhibition (NEPi) on ANP. In anaesthetised rats acute administration of the NEPi UK 69,578 caused a rise in plasma ANP, associated with a natriuresis, diuresis and kaliuresis which paralleled the response to 5 pmol/kg/min ANP in the same preparation.

In a subsequent study in man, acute administration of UK 69,578 caused a dose-related increase in plasma ANP, associated with a rise in urine volume and sodium excretion and suppression of renin consistent with the known actions of ANP, and the predicted effects of inhibition of ANP degradation. However, although 10 day administration of the oral NEP inhibitor UK 79,300 produced an upward trend in plasma ANP and down regulation of platelet ANP receptors consistent with sustained inhibition of ANP degradation, and was associated with a natriuresis on the first day of treatment, there were no sustained effects on sodium balance. Furthermore, although treatment with UK 79,300 blunted the pressor response to phenylephrine (on the final day of treatment), the response - both pressor and on aldosterone release - to Ang II infusion was enhanced. This unexpected effect is likely to be due to the effects of NEPi of Ang II degradation and will restrict the therapeutic application of NEP inhibitors.

Finally, treatment with the NEPi SCH 34,826 inhibited the development of hypertension in DOCA salt hypertensive rats, but was without effect on renal function, sodium excretion or exchangeable body sodium. Although modest, this study demonstrates the therapeutic potential of NEPi - at least in the restricted setting of a renin-independent model of hypertension.

4. The final section investigates the possible role of Calcium on the release of ANP in the isolated superfused rat atrium. Reduction of

extracellular Calcium - either by omission from the bathing medium, by the use of EGTA - or treatment with the calcium channel blocker Verapamil, resulted in the dissociation of developed tension and ANP release but had no direct effect on the release of ANP. It is concluded that ANP release is, at least partially, independent of Calcium and that the therapeutic use of calcium channel blockers is unlikely to impair the release of ANP.

This theme is extended in the subsequent chapter where it is shown that acute administration of Nifedipine has no effect on the release of ANP in response to a saline infusion in normal volunteers. Indeed, the saline load is cleared more rapidly in the presence of Nifedipine.

The general conclusions are that ANP has therapeutic potential and, although NEP inhibitors represent a useful first attempt to harness this potential they are limited by their modest effects on plasma ANP and their lack of specificity (particularly with respect to Ang II degradation). The studies with ACE inhibitors and, to a lesser extent, calcium channel blockers suggest potential therapeutic combinations with NEP which require further investigation. However, it is likely that the development of alternative, more potent and more specific agents will be needed to harness the therapeutic potential of ANP.

SECTION 1.

INTRODUCTION AND LITERATURE REVIEW.

Chapter 1.

Introduction.

The concept of a natriuretic hormone has been the subject of debate for many years (see deWardener & Clarkson (1985)). However, the clear identification of such a hormone eluded discovery until the seminal observation of deBold (deBold et al., (1981)) that extracts of atrial tissue cause a natriuresis and diuresis when infused into an anaesthetised rat. These findings permitted the re-interpretation of two much older observations: firstly that the cardiac atria contain granules on both light and electron microscopy which are similar to granules in which peptide hormones are stored in other tissues (Kirsch (1955)) and, secondly, that distension of the atria by balloon catheter resulted in a natriuresis in experimental animals (Henry et al., (1956)). From these observations deBold deduced that a peptide hormone (now known as Atrial Natriuretic Peptide or Factor, ANP) was synthesised by the heart and acted on the kidney to cause a

natriuresis and diuresis. Since then thousands of reports[1] have been published confirming and extending these observations. It is now known that ANP is indeed synthesised and secreted by the cardiac atria (and is also present in other tissues), that the principal stimulus to release is atrial distension and that the peptide acts not only to cause natriuresis and diuresis, but as a vasodilator, an antagonist of the renin-angiotensin-aldosterone system and as a neurohormone, together with a number of other actions. The major actions of ANP are summarised in Figure 1.1 and Chapter 2 reviews more fully the known physiological and pathophysiological actions of ANP.

The work in this thesis covers several different areas relating to ANP. It is divided into four experimental sections (2-5), each of which is self-contained. Following the introduction and review (**Section 1**), **Section 2** (Chapter 3) describes a number of small studies of plasma ANP levels in humans of relevance to the development and regulation of the ANP hormonal system. **Section 3** (Chapter 4) examines the renal actions of physiologically relevant infusions of ANP in the conscious dog and, by the use of inhibitors of Angiotensin Converting Enzyme (ACE) and angiotensin II infusion, examines the importance of the interaction between ANP and this major antinatriuretic hormonal system. In **Section 4** (Chapters 5-10) the possibility of "harnessing" the actions of ANP to therapeutic advantage is examined using inhibitors of Neutral Endopeptidase (NEP, EC 3.4.24.11) to inhibit hormone degradation in both man and experimental animals. In the final

[1]The literature on ANP is beyond the scope of this review and is covered comprehensively in two recent monographs (Brenner and Stein (1989), Struthers (1990)).

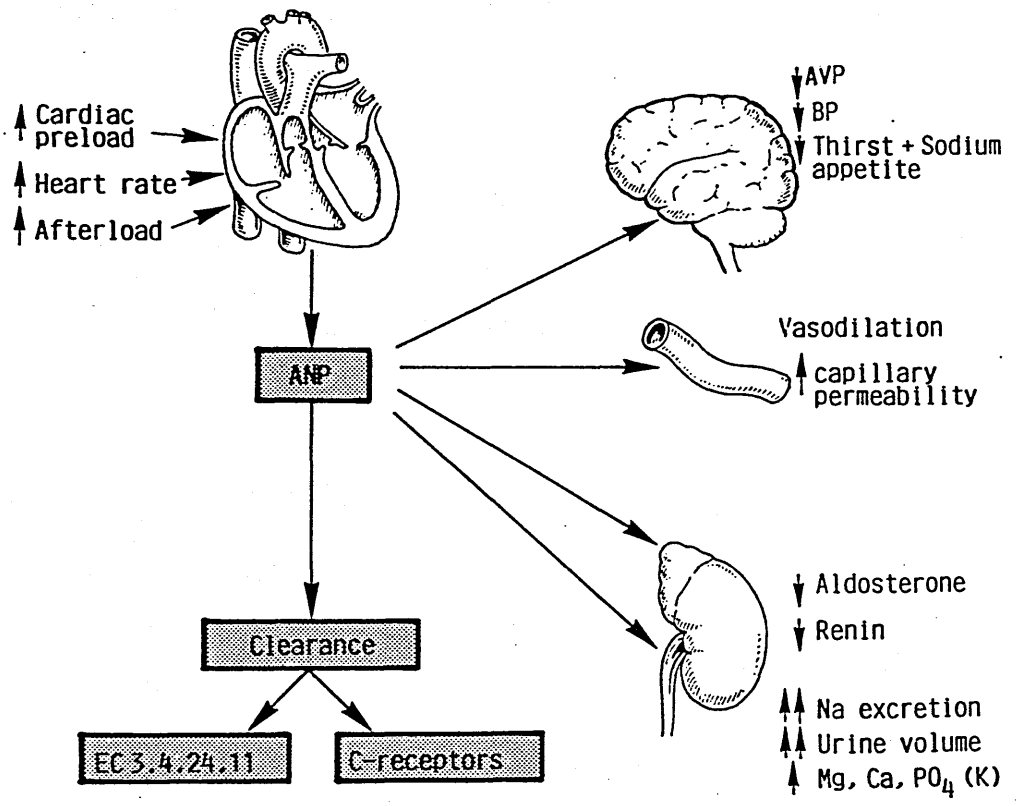

FIGURE 1.1.

PHYSIOLOGY OF ANP.

Outline of the major actions and regulation of Atrial Natriuretic Peptide (ANP).

experimental section (**Section 5**, Chapters 11&12) the influence of calcium in the regulation of ANP release is examined in vitro and in man. **Section 6** (Chapter 13) is a short general discussion of the findings and draws together the main theme of the thesis which is the possible therapeutic actions of ANP and potential interactions with other pharmacological agents in the treatment of a variety of common conditions including hypertension and heart failure.[2]

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[2]**Terminology.** Many names have been proposed for the atrial hormone based on its chemical nature, site of production and actions. Atrial Natriuretic Factor (ANF) was the term first used by deBold (deBold et al., (1981)) at a time when the nature of the hormone was unknown. With the finding that the factor was a peptide most workers adopted the name Atrial Natriuretic Peptide (ANP). However, recently the working party of the World Health Organisation and International Society for Hypertension have suggested that the term ANF should be adopted but this advice has not been universally adopted and in this thesis in view of the peptide nature of the hormone I have used the term ANP. Most of the other names (including cardionatrin, auriculin, atriopeptin and many others) have fallen from use. Dzau (1987) also suggested that the amino acid (AA) numbering of ANP should be from the amino terminal of the precursor molecule (also termed pro-ANP) rather than the carboxyl terminus of the active peptide, such that the active circulating form previously termed $_{1-28}$ANP should now be termed $_{99-126}$ANP (see Chapter 2, Figure 2.2). This has been followed as far as possible, as has the use of prefixes (h, human; r, rat) to indicate the species of origin. Previously the prefixes α, ß and y were used to indicate the circulating 28 amino acid form (α), a dimer of α (ß) and the prohormone (y). These prefixes have been dropped, although still used for commercial ANP preparations (e.g. Bachem UK). Finally, many individualised terms were used for purified peptide fractions or synthetic derivations of ANP (e.g. the 15 AA analogue of ANP synthesised by Maack et al,.(1987)) was termed C-ANP). This terminology has also been abandonned in favour of the numerical system with AA labelled numerically from the amino end of the prohormone.

Chapter 2.

Atrial Natriuretic Peptide: Review of the literature.

2.1 Historical Background.

The cardiac atria have been implicated in sodium and volume homeostasis for many years, the prevailing theory being that neural input from receptors in the atria produced alterations in the activity of the autonomic nervous system and vasopressin (ADH). This view was based on the results of a variety of different experiments. In 1956, Henry et al., observed that balloon distension of the atrium in anaesthetised dogs caused a diuresis, which was abolished by cardiac denervation. The renal effects of intravascular volume expansion were also shown to be absent if atrial volume was kept constant by selective atrial tamponade (Goetz et al,.(1970)). Natriuretic effects of atrial distension were later demonstrated by Reinhardt et al., (1977) who found that left atrial distension caused a natriuresis in conscious dogs. The ability of cardiac denervation to abolish these effects suggested that neural input from volume receptors in the heart in some way regulated renal function probably through ADH, the renin-

angiotensin system (RAAS) and sympathethic nervous activity. However, in a series of experiments deWardener provided evidence that a circulating factor was involved in the natriuresis of volume expansion and that this factor was separate from the RAAS and ADH. Infusion of plasma from a volume expanded dog caused natriuresis in a recipient animal (deWardener et al.,(1961)), implicating a circulating factor, and these renal effects occur in the presence of elevated levels of ADH and aldosterone. These results suggested the presence of another natriuretic substance which deWardener termed "third factor". At the same time, histological investigation of mammalian atria also revealed the presence of dense granules (Kisch (1956), Jamieson and Palade (1964), Figures 2.1a & 2.1b) with characteristics of secretory granules. Although the density of granules was later found to change under certain conditions -notably alterations in sodium balance (deBold (1979)) - their contents remained uncertain until the publication of deBold et al., (1981) showing that extracts of atria, but not ventricle (which do not contain granules (Jamieson and Palade (1964)) caused a 30 fold increase in sodium excretion in a bio-assay rat. From these observations deBold hypothesised that the atria produce a natriuretic hormone (termed "atrial natriuretic factor"), in response to changes in intravascular volume and sodium balance. This theory drew together the above observations by explaining the presence of secretory granules in the atria, the diuretic and natriuretic effects of atrial distension and the involvement of a circulating hormone in the response to atrial distension. An atrial hormone may, in fact, only explain part of the story. The cardiac nerves also influence renal function by other mechanisms and the hormone subsequently purified from the heart (atrial natriuretic

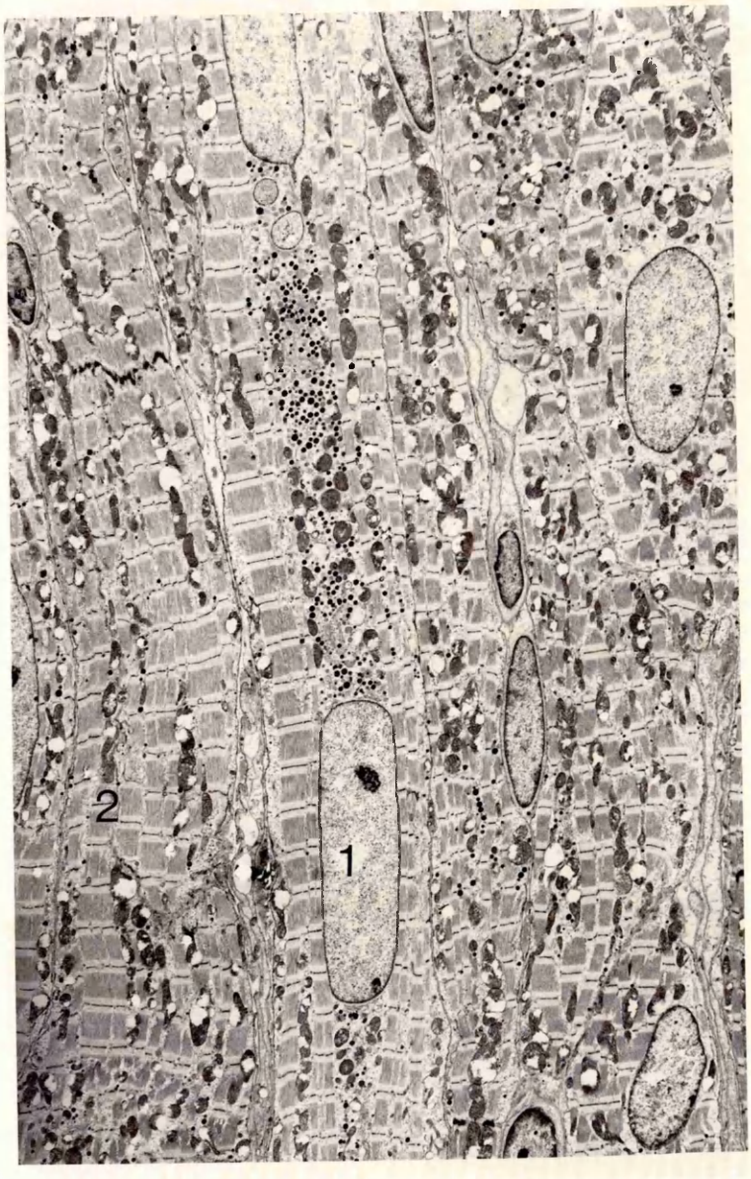


FIGURE 2.1(A):

ATRIAL MYOCYTE AND STORAGE GRANULES.

(A) Electron micrograph of an atrial myocyte showing electron dense ANP storage granules in a typical perinuclear distribution (Magnification x 2,600).
1. Nucleus. 2. Myofibrils.

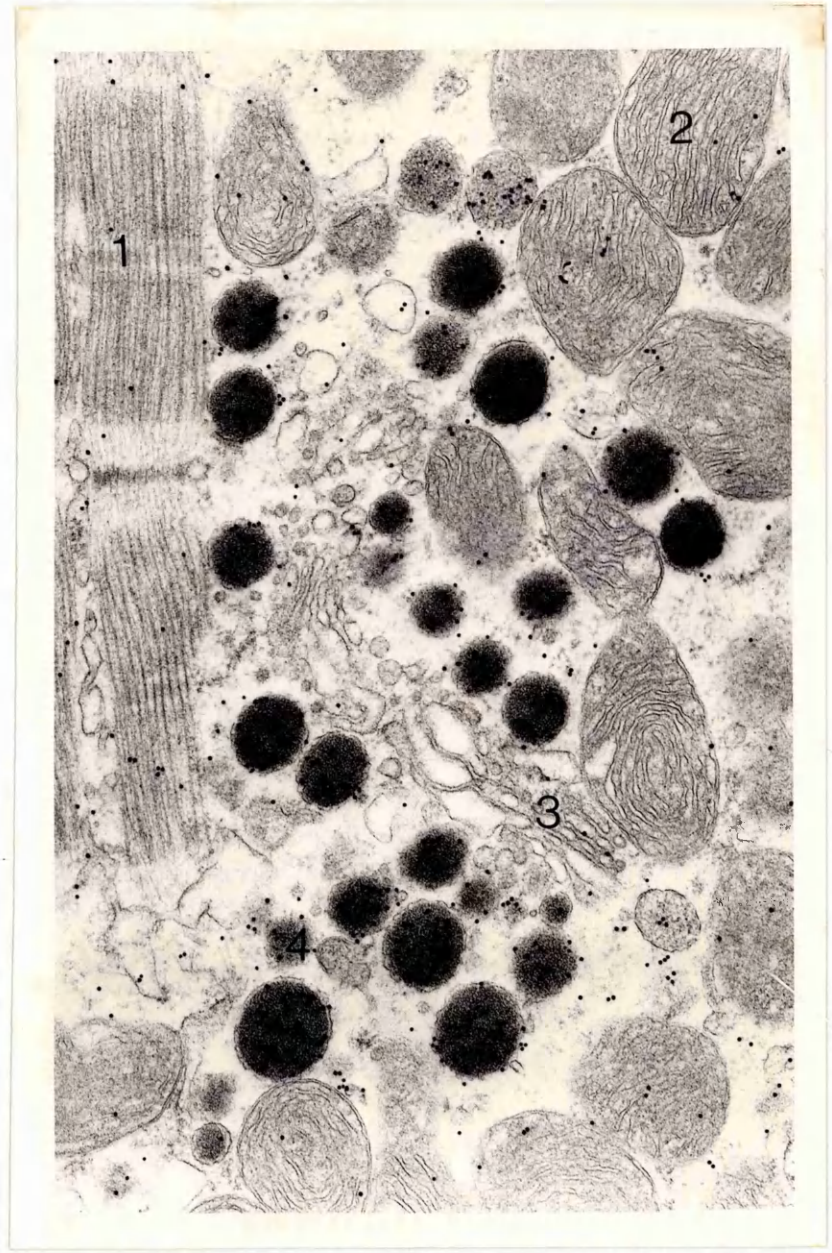


FIGURE 2.1(B):

ATRIAL MYOCYTE AND STORAGE GRANULES.

(B) Localisation of ANP to the storage granules using immunogold labelling with an antibody to $hANP_{99-126}$ (Magnification x 36,000; after Lindop et al.,(1987)).
1. Myofibrils. 2. Mitochondria. 3. Smooth Endoplasmic Reticulum. 4. ANP Storage Granules, dotted with immunogold label.

Photomicrographs courtesy of Ms. Jane Hair, Department of Pathology, Western Infirmary, Glasgow.

peptide or factor, ANP or ANF) does not fulfil all the functions of deWardener's third factor since it does not inhibit Na/K ATPase activity. It seems likely that a separate low molecular weight Na/K ATPase which exhibits digoxin-like immunoreactivity (DLIF) may also circulate (deWardener and Clarkson (1985), Kelly and Smith (1989)). The role of this digoxin-like substance and of the cardiac nerves is beyond the scope of this review and I will concentrate on the specific role of ANP.

2.2. Structure and synthesis of ANP.

Initial attempts to sequence ANP from plasma and atrial extracts resulted in the identification of many structurally related peptides (for review see Inagami et al.,(1989)) many of which were artefacts of the extraction process, differing only in the length of the peptide recovered. However, the synthesis of oligonucleotide probes from these peptides allowed several groups to virtually simultaneously identify the gene for ANP in 1984 in human and rat (Nakayama et al.,(1984), Oikawa et al.,(1984), Flynn et al.,(1985)) atrial tissue. From the gene sequence the structure of peptide itself was confirmed.

The gene for ANP in man is located on the short arm of chromosome 1 (Dracopoli et al.,(1988)). It contains 3 exons and two introns and encodes a peptide of 151 AA in man - and of similar length in other species (see Jarcho et al.,(1989), Gardner et al.,(1990) for reviews of the molecular biology of ANP). This 151 AA peptide (preproANP) has a 25 AA "signal peptide" at its amino terminal which contains a high percetage of hydrophobic amino acids and is thought to be involved in the processing of the hormone by the endoplasmic reticulum. Removal of this "signal peptide" leaves the 126 AA peptide

(proANP, previously termed gamma ANP (Kangawa and Matsuo (1984), Figure 2.2.), which is the major intracellular form of ANP (Kangawa and Matsuo (1984)) and which can be shown immunohistochemically within the storage granules in the atria (Lindop et al.,(1987)). The further processing of ANP is less clear. Stimuli which cause ANP release cause centrifugal movement of the storage granules which are largely found in the perinuclear distribution (Jamieson and Palade (1964), Agnoletti et al.,(1989), Figures 2.1a and 2.1b) and these granules subsequently release their contents by exocytosis (Severs (1989)). The peptide which is present in the peripheral circulation is the 28 AA peptide (99-126 ANP, αANP) cleaved from the carboxy terminal of the prohormone (Yamaji et al.,(1985), Figure 2.2.). This low molecular weight peptide is similarly found in coronary sinus plasma in man (Espiner et al.,(1986), Yandle et al.,(1987)) and in the isolated perfused heart or atrium (Lang et al.,(1985), Dietz (1987), Schiebinger et al.,(1987)) suggesting that cleavage of proANP to ANP does not occur in plasma. However, cultured atrial cells have been reported either to secrete low or high molecular weight ANP (Shields and Glembotski (1988), Ito et al.,(1988), Greenwald et al.,(1989)) and there is therefore still debate as to whether the final processing of ANP occurs within the atrial granule or is mediated by an enzyme within the atrial interstitium as has been proposed by Needleman (Ito et al.,(1988)). Wherever the site of cleavage, the N-terminal fragment also circulates with a much longer plasma half life than the smaller peptide, plasma levels are 10 times higher than $_{99-126}$ANP (Buckley et al.,(1989) and may have some renal effects (Martin et al.,(1990)) although the biological role is not known. An outline of the synthesis and forms of ANP is shown in Figure 2.2. There is a high degree of

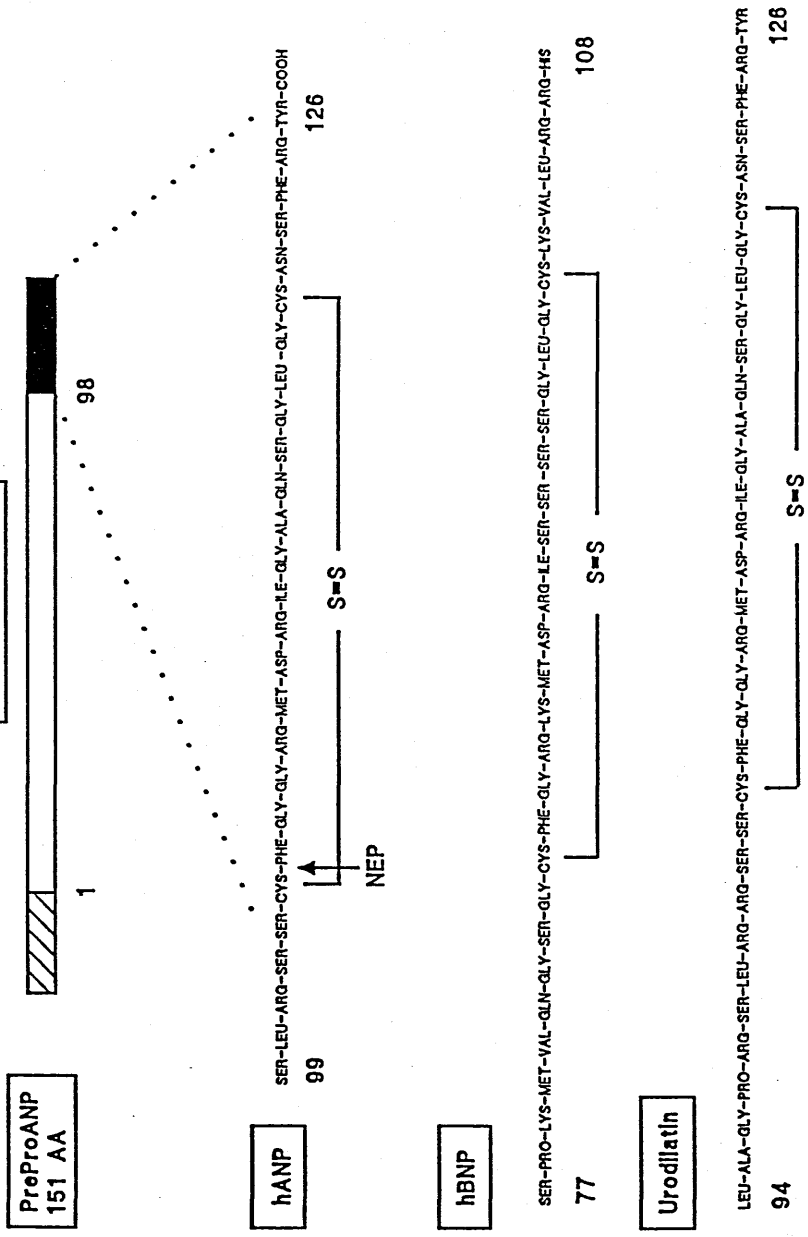


FIGURE 2.2:

STRUCTURE OF ANP.

Structure of Atrial Natriuretic Peptide and related peptide hormones using standard amino acid nomenclature. NEP indicates the major site of enzymatic cleavage by Neutral Endopeptidase; S=S indicates the position of the disulphide bond forming the loop of ANP (see text for details).

homology between the structure of ANP in different species. The biologically active, circulating peptide is virtually identical between species and, for example, rat and man differ only in the substitution of Methionine for Isoleucine at position 110 (Figures 2.2, 2.3.). Similarly, there is approximately 85% homology in the overall sequences of proANP between rat and man.

Regulation of atrial gene expression: Synthesis of ANP appears to be a major role of the atria and, quantitatively, messenger RNA (mRNA) for ANP has been reported to account for as much as 3% of the total mRNA. Changes in mRNA have been studied in a variety of conditions and experimental situations and a general pattern has emerged. ANP mRNA levels tend to follow long term changes in plasma ANP levels, with reciprocal changes in atrial immunoreactive ANP content i.e. if synthesis is increased, plasma levels are increased but stores are reduced indicating more rapid turnover within the atria. For example in two studies of the effect of prolonged water deprivation (Nakayama et al.,(1984), Takayanagi et al.,(1985)), ANP mRNA levels were reduced by more than 70%, with a rise in intracellular immunoreactive ANP (two-fold). Changes in salt intake have beeen found to have more variable effects, although Takayanagi et al.,(1985) found that a low salt diet reduces ANP mRNA. Chronic volume and sodium expansion in the DOCA salt rat (Ballerman et al.,(1986)) also increases mRNA and plasma ANP. In another hypertensive model, the SHR, Arai et al.,(1987) found an increase in plasma ANP and in ANP mRNA, with reduced atrial ANP content. However, these changes were more marked in the left atrium and provide evidence for a differential role of the atria in response to changes in pre- and after-load. This is explored in more detail in chapter 3, but it appears that the right atrium is perhaps more

important in the regulation of ANP release in response to changes in preload (volume expansion) while the left atrium is more importnant in the response to changes in afterload (hypertension). Other factors also regulate ANP gene expression. In rats (Gardner et al.,(1986,1987), Matsubara et al.,(1987)) glucocorticoids and thyroid hormone increase in vitro atrial mRNA expression; with similar effects on atrial cells in culture (Matsubara et al.,(1987)). Thyroid hormones have a non-specific effect on gene expression but the effect of glucocorticoids may be due to the association of a glucocorticoid-like receptor close to the ANP gene (Jarcho and Seidman (1989)). Finally, the expression of ANP mRNA is also dependent on the concentration of calcium in the bathing medium (Lapointe et al.,(1990)) and can be increased by calcium, calcium agonists or phorbol ester. The potential role of calcium is considered further below (2.4).

Ventricular ANP gene expression: Immunoreactive ANP and mRNA are present in cardiac tissue in humans, and in other species, from the earliest stages of development. There is now abundant evidence (Chapter 3, Kikuchi et al.,(1988), Wu et al.,(1988)) that a functional ANP hormonal system exists in fetal life (Robillard and Weiner (1987); Chapter 3). What appears to differ between the fetus and the adult is that the ventricular and atrial expression of the ANP gene are very similar in the fetus, atrial levels prodominate in the neonate and normal adult. However, in the adult with heart failure (Yasue et al.,(1989), Saito et al.,(1989)) or in experimental models of heart failure (Thibault et al.,(1989)) there is renewed ventricular expression of ANP. The stimulus to this "return" to the fetal state appears to be ventricular dilatation and it may well contribute to the raised plasma levels of ANP which are seen in heart failure.

The gene for ANP is also expressed in other tissues including the aortic arch (Gardner et al.,(1987)) and the nervous system (see below, 2.10). Plasma levels appear to be almost entirely of atrial origin except in heart failure when the ventricles (Saito et al.,(1989), Yasue et al.,(1989)) and possibly the lung (Gutkowska et al.,(1989)) may contribute.

2.3. Secretion of ANP:

Th release of ANP and plasma hormone levels are inextricably linked; plasma levels being dependent on secretion rate, in the absence of changes in clearance. However, for clarity, it is simplest to deal with them individually; this section includes the experimental control of ANP release and the section deals principally with normal and pathophysiological levels.

Manipulation of ANP release in vivo: The earliest study of plasma ANP in man (Lang et al.,(1985)) found that plasma hormone levels were elevated in response to acute volume expansion, as one might predict for a natriuretic and diuretic hormone. Subsequent studies have gone on to assess the dynamic regulation of plasma ANP, predominantly in man. Short-term intravenous infusions of 1-2 litres of saline increase circulating plasma ANP 2-3 fold (Anderson et al.,(1986), Richards et al.,(1988)). Head out water immersion, which causes a redistribution of intra - vascular fluid to the thorax, also increases ANP (Epstein et al.,(1987)) as does head down tilt (Hodsman et al.,(1987)) and plasma volume expansion following treatment with adrenocortical steroids (Connell et al.,(1987), Weidmann et al.,(1988)). Conversely, decreases in atrial volume due to haemorrhage, water deprivation, frusemide or head up tilt tend to lower plasma ANP (Gauquelin et

al.,(1988), Kimura et al.,(1986), Hodsman et al.,(1987), Haller et al.,(1987), Cameron et al.,(1988), Hollister et al.,(1986)). Similar volume-dependent changes in plasma hormone levels are seen in pathological conditions and these are discussed below (2.4). These observations suggest that the atria "sense" intravascular volume and release hormone appropriately. Although these studies involve manipulation of intravascular, and hence atrial, volume there was for some time debate as to whether atrial volume, or pressure, was the final common stimulus to the release of ANP. Studies such as those of Richards et al.,(1986) and Anderson et al.,(1986) showed a close correlation between atrial pressure and plasma ANP in patients undergoing cardiac catheterisation and normal subjects infused with saline. However, if pressure and volume are dissociated, for example in cardiac tamponade (where the collection of pericardial fluid results in increased atrial pressure but reduced volume) plasma ANP is not elevated and studies in man (Northridge et al.,(1989)) and in experimental animals (Koller et al.,(1987), Mancini et al.,(1987), Edwards et al.,(1988), Stone et al.,(1990)) have shown that hormone levels rise if the tamponade is released (despite the fall in atrial pressure). Thus, atrial distension is the final common stimulus to ANP release - although under most circumstances pressure and volume will tend to change in parallel.

Chronic regulation of plasma ANP follows a similar pattern to the acute regulation and to the regulation of the ANP gene (2.2), in both experimental and pathophysiological conditions. For example, higher levels are found in normal subjects with experimental steroid induced hypertension (Connell et al.,(1987), Weidmann et al.,(1988)) and a similar pattern in animals treated with deoxycorticosterone (DOCA;

Ballerman et al.,(1985), Grekin et al.,(1986)). However, debate surrounds the role of dietary salt intake in regulation of plasma hormone levels. Some (Sagnella et al.,(1987), Richards et al.,(1986a)) have found a positive correlation between plasma ANP and sodium intake, however others disagree (Weidmann et al.,(1986)), and, in view of the small changes in ANP produced by large differences in sodium intake, it is quite likely that ANP responses are secondary to vascular volume changes. Other factors have been shown to elevate plasma ANP. Levels are higher in paroxysmal tachyarrhythmias (Espiner et al.,(1986)), and in experimental pacing-induced increases in heart rate suggesting that ANP release may be heart rate dependent and increases in cardiac afterload by infusion of pressor substances (Uelinger et al.,(1987), Christensen et al.,(1989)) increases plasma ANP indicating that increased blood pressure can independently stimulate ANP release (as it can in the isolated heart (Dietz (1987))); probably due to a relative increase in left atrial secretion (Arai et al.,(1987)).

In vitro release: From the in vivo studies above it is clear that ANP is released in response to changes in atrial volume. The classical explanation for this would involve stimulation of atrial "stretch" receptors and, presumably, reflexly mediated release. However, the stimulus secretion coupling mechanism appears to lie within the individual atrial myocyte, since the isolated working heart (Dietz (1987)), isolated atria (Bilder et al.,(1986), Agnoletti et al.,(1987), Cho et al.,(1988)) and even isolated myocytes (Gibbs (1987b)) release ANP when stretched. Isolated atrial preparations also release ANP in response to increases in heart rate (Schiebinger and Linden (1986)) and in response to a variety of hormonal stimuli

including α- and ß-adrenoceptor agonists, acetylcholine, angiotensin II and endothelin (Rankin (1987), Wong et al.,(1988b), Gibbs (1987a), Schiebinger et al.,(1987,1988,1990), Schiebinger (1989)). Furthermore direct activation of the second messenger systems using the calcium ionophore A23187, forskolin, dibutyryl cAMP or activators of Protein Kinase C (Iida and Page (1989), Matsubara et al.,(1988), Hirata et al.,(1988), Saito et al.,(1986)) have also been reported to increase ANP release. These divergent in vitro stimuli all potentially involve intracellular calcium by different mechanisms and, taken together with the suggestion (Allen et al.,(1989)) that stretch-sensitive calcium channels may exist within the heart, this has led to the theory that an increase in intracellular calcium is the final common stimulus to ANP release. This is discussed more fully in chapters 11 and 12. Briefly, in support of this theory, Schiebinger has shown (Schiebinger (1989), Schiebinger and Gomez-Sanchez (1990)) that the effects of endothelin and adrenoceptor agonists on ANP release are blocked by calcium channel blockers and others have shown that treatment of isolated cell or the whole animal with ouabain (to increase intracellular calcium) increases the release of ANP (Bloch et al.,(1988), Yamamoto et al.,(1988)). Similarly, infusion of sub-pressor doses of calcium (Finn et al.,(1988), Fujimura et al.,(1989)) increases plasma hormone levels. However, the release mechanism does not appear to be this simple. Reduction of extracellular calcium using EGTA has been shown to increase ANP release (deBold and deBold (1989), Ito et al.,(1989)) and at present the role of calcium remains uncertain. It seems likely that intracellular calcium should play a role, but perhaps different intracellular calcium pools are involved in control of tension-development and hormone release (Chapter 11).

Extra-atrial ANP: The contribution of non-atrial tissues to circulating levels of ANP is also uncertain. In the fetal heart both atria and ventricular cells synthesise ANP (Kikuchi et al.,(1987), Wu et al.,(1987)) but by the time of birth the ventricular component becomes insignificant and remains dormant in normal adults. Recent studies in animals and man have demonstrated that ventricular ANP production may be "switched on" in cardiac failure (Saito et al., (1989), Franch et al.,(1988)). During cardiac catheterisation in patients with heart failure, Yasue et al.,(1989) found a rise in plasma ANP between the atria and aorta suggesting active secretion by the ventricle. The relationships between mRNA, stored ANP, secretion and the stimuli to secretion in the ventricle are still to be fully elucidated, but peptide appears to be synthesised on demand, with no intermediate storage step.

2.4. Plasma levels of ANP.

Normal levels: With the advent of radio-immunoassays for ANP there have been many reports on plasma hormone levels. Richards et al.,(1987a) were amongst the first to report on the effects of pre-extraction of plasma which, in addition to removing high molecular weight species, removes interfering substances in plasma resulting in lower, more consistent plasma ANP values. Results from direct RIA therefore must be viewed with caution. The levels of ANP now reported by most groups are in the range 5-50 pg/ml (2-15 pmol/l), in normal man. Similar or slightly higher plasma levels have been reported in other species (e.g rat (Soejima et al.,(1988), and dog (Cernacek et al.,(1987), O'Hanian et al.,(1987), Bie et al.,(1988)).

Circulating ANP is detectable during fetal life (Chapter 3,

Robillard and Weiner (1987), Kingdom et al.,(1989)), but its role and normal range unknown. Levels are high at birth and in the early neonatal period (Yamaji et al.,(1987), Ito et al.,(1988)) but fall rapidly in the puerperium. Thereafter, there is a slow age-related increase in plasma ANP (Chapter 3, Ohashi et al.,(1987), Haller et al.,(1987)). Careful diurnal measurement of plasma ANP has revealed no significant rhythm in normal subjects (Richards et al.,(1987c)), although one may emerge under certain pathological circumstances (eg Pre-eclampsia; Miyamoto et al.,(1988a,b)). Plasma levels are also determined by changes in posture (Hodsman et al.,(1987)), levels falling in the upright position, and by exercise (Richards et al.,(1986), Hodsman et al.,(1987)).

Pathophysiological levels: Following on from the list of factors which stimulate the experimental release of ANP (2.3) there is general agreement that circulating levels are elevated in pathological conditions where plasma volume is expanded such as chronic renal failure, congestive heart failure, and Conn's syndrome (Tonolo et al.,(1988, 1989a), Walker et al.,(1987), Cody et al.,(1987), Raine et al.,(1986), Richards et al.,(1986), Yamaji et al.,(1986, 1988), Ogawa et al.,(1987), Raine et al.,(1986), Chapter 3) and that in these syndromes plasma levels fall in response to appropriate therapy: dialysis, diuretic therapy and amiloride respectively. In cardiac failure the levels are related to right atrial pressure and hence the degree of plasma volume expansion (Richards et al.,(1986)) and may be increased as much as 10-20 times the upper limit of normal. A similar pattern is seen in animal models of heart failure (Riegger et al.,(1987), Franch et al.,(1988)). In essential hypertension the general consensus appears to be that levels are only modestly elevated

(< 50%, Sagnella et al.,(1988), Montorsi et al.,(1988)) on average and are often still within the "normal" plasma range. In the absence of increased intravascular volume in this condition elevated levels are probably due to increased cardiac afterload and in experimental models ANP mRNA is higher in the left rather than the right atrium (Arai et al.,(1987)). However, Ferrier et al.,(1989) have reported that ANP levels may be low in the offspring of hypertensive parents, and thus proposed ANP deficiency as the cause of hypertension. However, this has not been confirmed. In animal models of essential hypertension reported levels are variable although the consensus is for an increase in the SHR (Arai et al.,(1987), Gutkowska et al.,(1986)). Normal pregnancy is accompanied by an expansion of plasma volume of up to 50%. There is an appropriate increase in circulating levels of ANP which correlates with the gestational age (Jackson et al.,(1988)) in some series, but not in others (Steegers et al.,(1990), Chapter 3). In pre-eclampsia ANP is further increased despite the relative volume contraction in this condition (Otsuki et al.,(1988), Miyamoto et al.,(1989a,b), Chapter 3). The reason for this is not known but again presumably relates to increased cardiac afterload and, in keeping with this, plasma hormone levels closely follow the abnormal diurnal rhythm in arterial pressure in pre-eclampsia (Miyamoto et al.,(1989a,b)). Whether elevated ANP contributes to the reduction in plasma volume, proteinuria, oedema and suppression of the renin-aldosterone system which characterise pre-eclampsia is not yet clear. Even in the fetus there is evidence of a pathophysiological role for ANP. The peptide is synthesised by the fetal heart (Kikuchi et al.,(1987)) from the earliest periods of development and circulates in human fetal blood. Plasma ANP concentrations rise in response to intravascular

transfusion and are relatively increased in Rhesus iso-immunisation (Robillard and Weiner (1987), Weiner and Robillard (1988), Kingdom et al.,(1989)), where they are related to the severity of the anaemia (Chapter 3).

Other factors which are reported to increase plasma ANP include spontaneous tachyarrhythmias (Espiner et al.,(1986)) and exercise, where levels may rise up to three-fold at peak exercise (Richards et al.,(1987), Hodsman et al.,(1987)). The changes in both these conditions may be related to heart rate and have been proposed to contribute to the diuresis which follows paroxysmal tacharrhythmias, in particular. Elevated levels have also been reported in Bartter's syndrome (Gordon et al.,(1986), where they are inappropriate for the blood pressure and plasma volume; and poorly controlled diabetes (Bell et al.,(1988)) where they have been proposed to contribute to the development of albuminuria. Neither the mechanism, nor the the significance of these observations is currently understood.

2.5. Degradation and clearance of ANP.

In man ANP has a very short plasma half life, as was initially suggested by the short duration of the biological effects of bolus administration (Richards et al.,(1985)). The disappearance of the peptide from the circulation is best fitted by a biexponential decay with half-life values of between 1 and 2 minutes for the fast component and around 15 minutes for the slow component of the decay curve in man (Ohashi et al.,(1987), Nakao et al.,(1986)). Other workers have fitted a single exponential curve and the values obtained relect the fast component only with values of 1.3 minutes in the rat (Almeida et al.,(1988)) and 3.1 minutes in man (Yandle et al.,

(1986)). The volume of distribution in man is around 200 ml/kg, and the calculated metabolic clearance rate 20-25 ml/min/kg (Nakao et al., (1986), Ohashi et al.,(1987)). These data suggest rapid clearance of the peptide by tissues.

The sites of ANP clearance have been determined by a variety of methods. When radiolabelled ANP is injected into rats and the peptide distribution determined radiographically (Condra et al.,(1987)), most activity develops in the kidney and liver over the first few minutes. Radioactivity persists at these sites before appearing in the urine and thyroid (free iodine). Although this suggests that the kidney is a major site of degradation radioactivity was diffusely located in other vascular beds. Further assessment of the role of other tissues and vascular beds has been made by the measurement of arterio-venous gradients in hormone levels and by the measurement of degradtion in isolated perfused organs. Approximatley 80 % of ANP is removed by a single passage through the kidneys in dogs (Weselcouch et al.,(1987)), with similar falls in man (Richards et al.,(1986b), Schutten et al.,(1987)). Nephrectomy has been reported to prolong the half life of plasma ANP approximately two-fold (Luft et al.,(1987). In the isolated kidney (Brier et al.,(1988)) radiolabelled ANP is rapidly removed, even in the non-filtering kidney by a process that is saturable and with a reported clearance rate (Brier et al.,(1988)) six times the GFR. Thus, the balance of evidence suggests the kidney to be a major site of ANP clearance. Plasma hormone levels also fall across other vascular beds (Richards et al.,(1986b)), including the liver, lung (Northridge et al.,(1990b)) and limbs (Yandle et al.,(1986), Schutten et al.,(1987)), and in the mesenteric circulation in vitro (Murthy et al.,(1986)).

Two processes appear to be involved in the clearance of ANP of ANP at these sites; both are dealt with elsewhere. First, in 1977, Kenny demonstrated that neutral endopeptidase (NEP, EC 3.4.24.11), an enzyme found principally in the kidney, specifically degrades ANP (Stephenson and Kenny (1987)). This has subsequently been confirmed and the role of NEP is discussed in Section 4 (and specifically reviewed in Chapter 5). Secondly, also in 1977, Maack prpoposed the existance of a clearance receptor (Maack et al.,(1987)) which binds circulating peptide, followed by internalisation and degradation (see below). Both these mechanisms appear to operate and inhibition of both produces greater effects than either alone (Koepke et al.,(1988)), but nothing is known about their relative importance. Based on studies with NEP inhibitors (Section 4) Sybertz has proposed that enzymatic degradation of the peptide corresponds to the fast component of the plasma decay curve, and receptor mediated uptake the slow component (EJ Sybertz, personal communication).

2.6. ANP receptors and mechanism of hormone action.

Cyclic GMP and mechanism of action:

Shortly after the discovery of ANP it was noted that the actions of ANP in vascular smooth muscle were associated with the generation of cyclic GMP (cGMP, Winquist et al.,(1984)). This relationship has subsequently been confirmed in all the major target tissues for ANP, including kidney, adrenal, lung, liver, brain (see Lewicki (1989) for review) and in isolated cell lines (Lyall et al.,(1987), Lewicki (1989)). Cyclic GMP has thus been proposed to act as the intracellular "nd messenger for ANP and recently it has been shown that the ANP-B

receptor is a single transmembrane protein containing an ANP binding site and guanylate cyclase (below).

In many tissues our knowledge of the mechanism of action of ANP goes no further than generation of cGMP. In some early studies there were reports that cAMP was also reduced, but this is not a consistent finding and may be secondary to changes in cGMP (Lewicki (1989)). In vascular smooth muscle, the mechanism of ANP-dependent vasodilatation is better understood. In addition to the generation of cGMP, there is a fall in intracellular calcium concentration or inhibition of agonist-stimulated increases in intracellular calcium (Meyer-Lehnert et al.,(1986), Appel et al.,(1987), Hassid (1986), Lewicki (1989)). The contraction of the smooth muscle cell is dependent on intracellular calcium and the state of phosphorylation of myosin light chain which is in turn dependent on the calcium and a cGMP-dependent enzyme - myosin light chain kinase. In keeping with the suggestion that ANP may cause vasorelaxation by this mechanism, Paglin et al.,(1988) have shown that ANP reduces the phosphorylation of myosin light chain in aortic rings. In other tissues the subcellular mechanism of action is not known. In the kidney, at least in the distal tubule and in the suppression of renin, cGMP is involved; but in the adrenal cGMP does not appear to be involved (see below).

In vivo, cGMP in plasma and urine correlates well with the biological actions of infused ANP (Wong et al.,(1988)), even in the lowest dose studies (Richards et al.,(1988b) and to follow, for example, diurnal changes in plasma ANP (Miyamoto et al.,(1989)).

ANP Receptors:

Membrane receptors for ANP were identified shortly after the

discovery of the peptide and, following the experimental work of Maack (Maack et al.,(1987)), it is now known that these can be classified into two sub-groups: a guanylate cyclase (GC) linked receptor (or ANP-B receptor), which mediates the biological actions of ANP through the second messenger cGMP; and a smaller, non-GC linked receptor (the ANP-C or "clearance" receptor) which is involved in removing ANP from the circulation.

The presence of a guanylate cyclase linked receptor was suggested initially by the observations that cGMP levels follow plasma ANP (above). The existence of a second receptor subtype was initially suggested by Maack et al.,(1987)) who found that a 15 AA ring contracted analogue of ANP (4-23 ANP, C-ANP, dANP or 102-121 ANP; see Figure 2.3) bound with high affinity in the isolated rat kidney but was devoid of demonstrable effects. However, it did cause natriuresis and diuresis in the intact animal and, if co-infused with full-length ANP in the isolated kidney, the renal effects of the latter were augmented. From these observations Maack proposed that the truncated ANP bound to a receptor involved in the clearance of ANP. Similar effects have since been reported for related truncated ANP analogues (Koepke et al.,(1989)) and Almeida et al.,(1989) have shown that co-infusion of $_{4-23}$ANP and radiolabelled 28 AA ANP results in prolongation of the half-life of the intact peptide, reduced volume of distribution and delayed appearance of degradation products.

ANP-B Receptor: Initial attempts to characterise the ANP receptor using standard cross-linking and purification techniques demonstrated a 130 kDa protein which co-purified with guanylate cyclase activity (Paul et al.,(1987)). The reason for this difficulty in separating the ANP receptor and guanylate cyclase was subsequently demonstrated

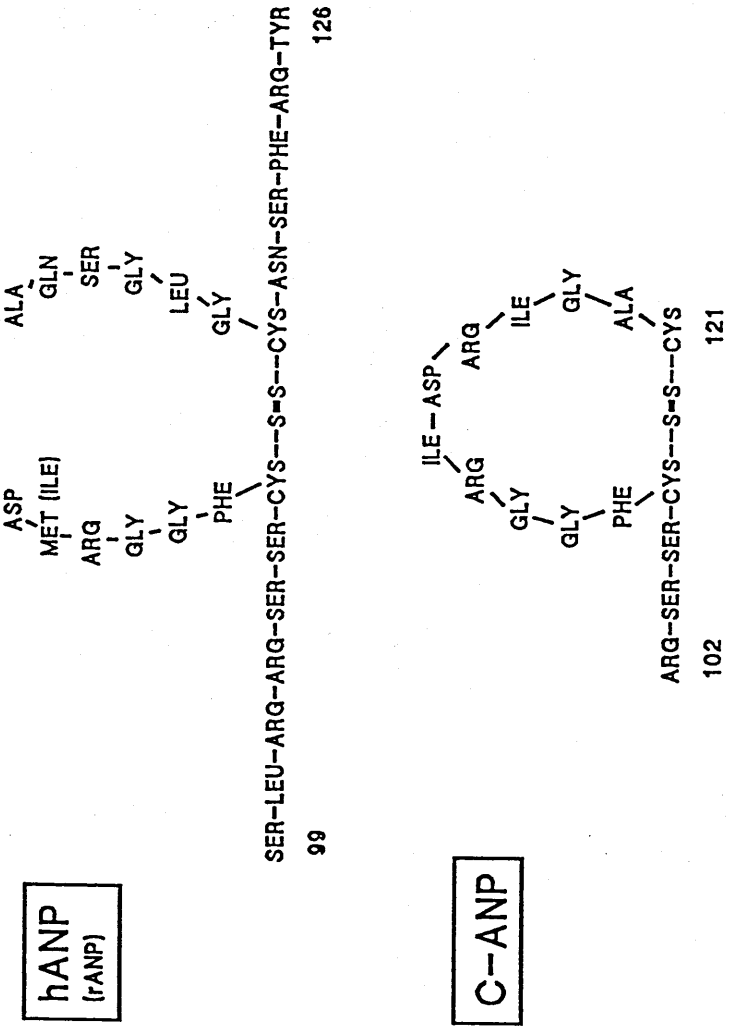


FIGURE 2.3:

STRUCTURE OF ANP AND C-ANP:

Structure of the circulating form of ANP in man, showing the disulphide-linked ring, and of the truncated C-ANP receptor analogue $_{4-23}$ANP (Maack et al.,(1987)).

by Lowe (Lowe et al.,(1989), Schulz et al.,(1989a)). Using the gene sequence from a known (sea urchin) membrane form of guanylate cyclase (GC) to probe a human cDNA library. They identified partial cDNA clones for membrane GC and these were then used to prepare full length DNA sequences of rat and human DNA (Chinkers et al.,(1989), Lowe et al.,(1989)). Expression of these genes in cell culture conferred specific high affinity ANP binding sites and ANP stimulated GC activity (Lowe et al.,(1989), Chinkers et al.,(1989)). The gene encodes a protein of 130 kDa identical to that found with standard biochemical methods. Analysis of the protein found that it contained a short extracellular domain with a high degree of homology with the ANP binding site of the C-ANP receptor (below, Fuller et al.,(1988), Porter et al.,(1989)), a short single transmembrane domain and an intracellular component with two domains showing homology to the catalytic sites of protein kinases and soluble guanylate cyclase. Thus a single molecule contains both an extracellular ANP "receptor" and the enzyme GC. The GC activity is found at the COOH terminal and deletion of the sequence coding for the protein kinase domain (Chinkers and Garbers (1989)) produces a receptor with high constitutive GC activity which was unresponnsive to ANP; implying that the GC activity is normally suppressed by a protein kinase element and this suppression is released by ANP binding.

Recently the terminology of GC-linked receptors has become slightly confused by their subdivision into A-ANP and B-ANP subtypes following the isolation of a gene coding for a similar, but non-identical, GC-linked receptor from rat brain (Schultz et al.,(1989)). This receptor was labelled B-ANP and the original GC-ANP receptor re-labelled A-ANP. The major difference between the coded proteins lies

in the extracellular "binding" site and the "new" receptor appears to have a higher affinity for BNP (2.10), although its functional role remains to be established.

ANP-C Receptor: This receptor is smaller and the basic subunit is a 65-70 kDa protein which may exist as a homodimer of 120-130 kDa (Fuller et al.,(1988), Porter et al.,(1989)). Analysis of the gene for this receptor (Fuller et al.,(1988), Porter et al.,(1989)) has shown that the gene encodes a protien which, by hydrophobic analysis, is predicted to to have a 436 amino acid extracellular domain, a short single transmembrane domain of 23 AA and a short intracellular tail of 37 AA. The protein binds ANP equally well in the mono or dimeric forms and does not contain guanylate cyclase by either structural analysis or functional studies.

Localisation: Receptors have been localised by in vitro autoradiography and analysis of isolated tissues. For the most part their distribution coincides with the known functions of ANP (Chai et al.,(1986), see Martin and Ballerman (1989) for review). For example, using autoradiography (Chai et al.,(1986), Mendelsohn et al.,(1987)) ANP specific binding is found in the kidney (principally the glomerulus, intra-renal vasculature and inner medulla), in the vasculature (of the kidney, aorta, adrenal cortex, lung, liver and isolated smooth muscle cells) and in the adrenal gland and brain (particularly in the areas involved in volume homeostasis). Interestingly, the pattern of distribution closely parallels that of Angiotensin II (Mendelsohn et al.,(1987)). The affinity and density of these sites are reviewed by Martin and Ballerman (1989). Using standard ligand binding techniques (which do not discriminate between ANP-B and ANP-C receptors) the Kd values obtained (i.e. concentrations

at which 50% binding is achieved) are in the range of 10^{-9}-10^{-11}M which are obviously consistent with biological activity. The reported density of receptors varies from 10 sites per cell (platelet) to values in the range of 100,000 sites per cell in cultured smooth muscle cells with similar values in membrane preparations of 10 to greater than 1000 fmol/mg membrane protein in vascular tissue (see Martin and Ballerman (1989) for review). The density of receptors and their Kd values (which are close to the observed plasma levels of ANP - in the pM range) are consistent with a physiological role for ANP.

Little is known of the relative density of B-ANP and C-ANP receptors. Maack et al.,(1987) initially suggested that 99% of the receptors in the kidney were of the clearance type. However, this seems likely to be an overestimation based on the failure to allow for ligand degradation and more recent estimates (using in vitro autoradiography and the ability of excess $_{4-23}$ANP to displace ^{125}I-ANP as a measure of C-ANP receptor binding) suggest more equal numbers of receptor subtypes (Brown et al.,(1990)) in the kidney. Brown et al.,(1990) have also shown that the C-receptor is limited to the renal cortex (glomeruli) but the B-receptor is present throughout the kidney. However, the relative density of receptor subtype is likely to vary between tissues (Martin and Ballerman (1989)).

Function of the C-ANP receptor: In contrast to the B-ANP receptor, where ligand binding stimulates production of cGMP, the precise function of the C-ANP receptor is uncertain. The most likely function remains that initially proposed by Maack, based on his physiological studies in the isolated kidney, and subsequently confirmed by Koepke et al.,(1988) and Almeida et al.,(1989). Further evidence to support receptor-mediated clearance of ANP comes from studies in cultured

cells (Morel et al.,(1987)), where there is rapid internalisation of ANP. This process appears to be specific to the low molecular weight receptor (Murthy et al.,(1989)). The suggestion (Morel et al.,(1987)) that internalised peptide may exert a physiological role has yet to be explored.

Receptor regulation: When ligand concentrations are increased receptor numbers are usually reduced (down-regulation). ANP receptors appear to behave in this way. For example, in man, platelet ANP binding is reduced in heart failure and hypertension, where plasma ANP is elevated (Schiffrin (1987), Schiffrin et al.,(1988)). Similarly, in experimental animal models with elevated plasma ANP, such as the DOCA-salt hypertensive rat (Schiffrin and St. Louis (1987)) ANP binding is reduced. There is also evidence of heterologous ANP receptor regulation by ligands other than ANP. In circumstances where Angiotensin II is elevated ANP binding is also reduced. However, in contrast to the effects of ANP where down-regulation is associated with loss of biological activity, the effects of exogenous ANP are enhanced. This is interpreted as suggesting that Ang II causes reduction of the C-ANP receptor, thus increasing the availability of the peptide at the guanylate cyclase linked receptor (Chabrier et al.,(1988)).

In summary, two types of ANP membrane receptors have been described: one linked to guanylate cyclase (and hence the biological actions of ANP, through the generation of cGMP) and the other not linked to any second messenger system, apparently involved in receptor-mediated removal of ANP from the circulation.

2.7. Renal actions of ANP.

The first demonstrated effects of atrial extracts were on the kidney (deBold et al (1981)) and, since then, studies have consistently reported that infusion of synthetic ANP causes natriuresis, diuresis and suppression of renin release (considered below, 2.9.). The mechanisms underlying these effects are still unresolved despite attempts to dissect out the intrarenal mechanisms using either infusion of exogenous ANP in the whole animal (the clearance of markers, such as PAH and Inulin, being used to infer sites of action within the kidney) or in vitro investigation of isolated nephron segments.

Renal effects of ANP infusion:

Early studies of the renal effects of ANP used large supraphysiological doses of the peptide and, although these produced dramatic increases in urine volume and electrolyte excretion, their physiological role is difficult to interpret (eg Richards et al.,(1985), Weidman et al.,(1986) in man; Burnett et al.,(1984) in dogs). However, several recent studies have examined the effects of low dose (≤2 pmol/kg/min; Anderson et al.,(1987), Richards et al.,(1988a,b), Cottier et al.,(1988), Morice et al.,(1988), Solomon et al.,(1988)) ANP infusion - which raise plasma ANP less than two-fold. The renal response to this physiologically relevant increase in plasma ANP comprises a 50-100% increase in sodium excretion, a lesser increase in urine volume, no change in potassium excretion and, where measured (Richards et al.,(1988a,b), Solomon et al.,(1988)), small increases in the excretion of magnesium and calcium. These studies have also addressed the question of how these effects arise. The

consensus is for no change in glomerular filtration rate (GFR); a downward trend in renal plasma flow, with an upward trend in filtration fraction and thus the rise in sodium excretion is either demonstrated (or inferred) to result from reduced tubular reabsorption of sodium (FeNa, Cottier et al.,(1988), Anderson et al.,(1987)). Only Solomon et al.,(1988) have attempted, by the use of lithium clearance as a marker of proximal tubular function, to indentify the segment of the tubule involved; but found only an upward trend in lithium clearance. In dogs and rats similar overall patterns are observed in urine volume and electrolyte excretion (eg Soejima et al.,(1988), Zimmerman et al.,(1989), Pichet et al.,(1988), Seymour et al.,(1986), Dunn et al.,(1986), Harris et al.,(1989), Chapter 4). Caution is required, however, in interpreting these data. As a marker of GFR, inulin claerance may only be accurate to within 10% (Maack (1986)) and may thus conceal changes of a few percent which may be enough to account for the overall changes in sodium excretion. With higher levels of ANP this becomes evident. In rats and dogs further increases in the infused dose of ANP result in increased sodium excretion and the emergence of increasing potassium excretion and GFR (Soejima et al.,(1988), Bie et al.,(1988), Burnett et al.,(1986), Dunn et al.,(1986), Seymour et al.,(1986), Harris et al., (1989)). In man, higher infusion rates (15 pmol/kg/min, Brown and Corr (1987); 30 pmol/kg/min, Weidmann et al.,(1986)) result in greater increases in sodium excretion and urine volume (3-6 fold) and also to a demonstrable rise in GFR (about 15%), but with additional increases in total and proximal tubular (FeNa & FeLi; Brown and Corr (1987)) excretion of sodium. Thus, from these studies it appears that a tubular component is essential and it is not clear whether a change in

GFR only appears at high doses, or is always present and only detected at high doses - which seems most likely. In support of this, ANP (McMurray et al.,(1988d)) causes an increase in excretion of albumin (filtered by the glomerulus) but not ß2-microglobulin (a tubular protein), implying a glomerular action.

The other information which infusion studies have given us is the long term response to ANP. Low doses in man (Janssen et al.,(1989)) or dogs (Kivlign et al.,(1988), Mizelle et al.,(1990)) infused for several days cause an increase in sodium excretion only over the first day or so, but slightly higher doses (around 10 pmol/kg/min; Mizelle et al.,(1988)) cause a sustained rise in sodium excretion. The reasons for these differences are unclear but they are important when it comes to assessing the therapeutic potential of ANP (see below, Section 4) and where higher plasma levels may be required to produce sustained effects.

Intrarenal Mechanisms:

More detailed intrarenal investigations - both structural and physiological - have attempted to unravel the site of action of ANP. **Receptor Localisation:** Specific binding sites for ANP have been localised by autoradiography and are found in the glomeruli and in the medulla, where binding sites are distributed along the vasa recta and collecting ducts (Chai et al.,(1986), Koseki et al.,(1986), Brown et al.,(1990)). Receptors have also been characterised in isolated rat glomeruli (eg Ballerman et al.,(1986); Kd 0.46 nM), rat mesangial cells (Ballerman et al.,(1986), Kd 0.22 nM), and inner medullary collecting duct (Gunning et al.,(1987), Kd 0.07 nM), but are apparently absent from proximal tubule. Thus, at all these sites ANP receptors are present with physiologically relevant binding

coefficients, at which ANP might act. The evidence for and against activity at individual intrarenal sites is as follows:

Glomerulus: In addition to the data from infusion studies other observations point to a role for the glomerulus. Mesangial ANP receptors are present (Ballerman et al.,(1986) and, in vitro, ANP antagonises angiotensin II-stimulated mesangial cell contraction (Appell et al.,(1986)). In vivo, this would increase glomerular surface area and glomerular filtration coefficient, and thus glomerular filtration rate. In the isolated perfused rat kidney ANP (although only pharmacological doses have been studied) causes an increase in GFR (eg Murray et al.,(1985), Firth et al.,(1986)), although a tubular component also contributes towards the diuresis especially with increasing dose.

Tubule: The proximal convoluted tubule is apparently devoid of ANP receptors and it is therefore surprising that some infusion studies have suggested a proximal tubular effect based on the results of studies using lithium clearance (FeLi; Brown and Corr (1987), Solomon et al.,(1988)). In vitro, in the isolated perfused proximal tubule segment ANP has no direct effect (Baum and Toto (1986)). It does, however, inhibit angiotensin II stimulated sodium reabsorption in the proximal tubule (Harris et al.,(1987)) at physiologically relevent levels. In the absence of ANP binding sites at this site this effect may be mediated by changes in local blood flow. A further mechanism by which ANP may indirectly alter function in the proximal tubule is by interfering with glomerulo-tubular feedback, through which proximal tubular reabsorption is altered in response to changes in GFR (Harris et al.,(1988, 1989), Zhuo et al.,(1989), Harris and Skinner (1989)). Such an effect has been proposed in the whole animal (Marguiles and

Burnett (1990), Huang and Cogan (1987)).

Perfusion of the loop of Henle in vitro is similarly without effect (Kondo et al.,(1986)) and Vinay et al.,(1987)) found no effects on the metabolism of the thick ascending limb. However, the loop of Henle may be indirectly involved in the actions of ANP since some workers have suggested that ANP may increase medullary washout, thereby reducing the concentrating ability of the loop of Henle. This effect might also be mediated via changes in blood flow, specifically in the vasa recta (see below).

There is no evidence of a direct effect of ANP in the distal convoluted tubules. However, since ANP impairs the synthesis and secretion of aldosterone by the adrenal (see below) it will have an indirect effect on distal tubular Na/K exchange. The extent to which this is involved in the response to ANP is not known but, since aldosterone levels change relatively slowly (Hall (1986), Mitchell and Navar (1989)), it is unlikely to contribute to the acute effects of ANP.

Collecting Duct: Surprisingly, it is in the collecting duct that most direct evidence points to a site of action for ANP. As noted above, membrane receptors are present at this site. Microperfusion and micropuncture of the collecting duct (Sonnenberg et al.,(1986), Briggs et al,(1982), van de Stolpe and Jamieson (1988)) have shown reduced reabsorption of sodium in the collecting duct due to ANP. Sonnenberg (1990) has recently confirmed this by demonstrating in the intact nephron that retrograde perfusion of ANP into the collecting duct causes a natriuresis, presumably by interaction with luminal receptors. In the inner medullary collecting duct Ziedel has shown that ANP increases cyclic GMP production, inhibits oxygen consumption

and reduces sodium reabsorption (Ziedel et al.,(1987, 1988, 1989)), thereby confirming that ANP can exert direct effects at this site. In a mathematical modelling study of the effects of ANP in the rat kidney, Meija et al.,(1990) found that a 50% reduction in sodium transport in sodium transport in the collecting duct matched experimentally obtained values and could theoretically account for all the observed effects of ANP in the tubule.

Vasculature: The intra-renal vasculature has many binding sites for ANP and isolated preconstricted intra-renal vessels dilate in response to ANP (Aalkjaer et al.,(1985)). Using micropuncture (Dunn et al.,(1987)) in the rat 500 ng/kg/min ANP (a pharmacological dose) appears to selectively dilate the afferent arteriole while constricting the efferent arteriole, thus increasing intraglomerular capillary pressure. Marin-Grez et al.,(1987) have further studied this using video microscopy to show that ANP caused dilation of pre-glomerular and constriction of post-glomerular vessels. A similar study of in vitro perfused glomeruli (Veldkamp et al.,(1988)) reported a similar pattern and theoretically such changes might contribute to increases in GFR.

In the whole animal, however, although some high dose studies reported a transient (Burnett et al.,(1984) or sustained increase in ERPF (Dunn et al.,(1986), Hintze et al.,(1984)), the general consensus from pfysiological studies in man is for a fall (or downward trend) in ERPF (Biollaz et al.,(1986), Cottier et al.,(1988), Richards et al.,(1988a,b), Solomon et al.,(1988), Anderson et al.,(1987)), even when correction is made for extraction of PAH (Janssen et al.,(1987)). However, as with inulin clearance (above), PAH clearance is a relatively insensitive method which may not detect small changes. Even

in the absence of changes in overall renal plasma flow re-distribution of flow may take place within the kidney. Diverting blood from the cortex to the medulla would theoretically reduce the role of the glomeruli and also increase medullary washout, reducing concentrating capacity. Kiberd et al.,(1987) demonstrated that 67pmol/kg/min ANP caused an immediate increase in sodium excretion but found that increased vasa recta blood flow took approximately 45 minutes to develop, Davies and Briggs (1987) similarly reported that the medullary gradient was reduced. However, the question arises of whether changes in medullary concentration are primary or secondary to the natriuresis, since the natriuretic actions of ANP precede changes in solute gradient (Kiberd et al.,(1987)). Other factors, such as the renal interstitial pressure have been proposed to contribute to the effects of ANP but their role is unknown (Garcia-Estan and Roman (1990)).

Overall, the balance of evidence fails to localise the effects of ANP to one single nephron segment and it seems more likely to involve the interaction of effects at several different nephron sites. The situation is further complicated by the existence of factors which modulate the effects of ANP on the kidney.

Factors modulating the renal effects of ANP: When the renal perfusion pressure is reduced in either the intact animal (Seymour et al.,(1987)) or in the isolated perfused rat kidney (Firth et al.,(1987)) then the natriuretic effects of ANP are blunted. The mechanisms underlying this are unclear. Redfield et al.,(1989) infused saralasin but failed to produce any improvement, infering from this observation that activation of the intra-renal renin angiotensin

system is not responsible. Furthermore when Ang II was used to restore perfusion pressure the natriuretic response was enhanced. However, saralasin is a partial agonist and the results are therefore difficult to interpret. Secondly, the renal sympathetic nerves are important Simmonetta et al.,(1990)). In experimental cirrhosis in the rat (Koepke et al.,(1987)) the natriuretic actions of ANP are similarly blunted. Denervation of the kidney restores the effect of ANP. Again, the underlying mechanism is unclear. A direct effect on renal blood flow may be important, but alternatively this may simply reflect a physiological antagonism since sympathetic activation causes sodium retention (Koepke and diBona (1987)). A further alternative involves activation of the renin-angiotensin system by sympathetic nerves. Once more further investigation is required to dissect out the relative importance of these actions. Finally, the renin-angiotensin system appears to have a central role in modulating the actions of ANP. When Ang II is infused (McMurray and Struthers (1988b), Seymour and Mazack (1988)) or the renin-angiotensin system activated by diuretics, prostaglandins or other exogenous means (McMurray and Struthers (1988a,b), Lang and Struthers (1990) the natriuretic actions of ANP are blunted or abolished. Interuption of the renin-angiotensin system by ACE inhibition (Bie et al.,(1990), Chapter 4) can enhance the actions of ANP. This latter observation is the subject of debate (see Chapter 4) and ACE inhibitors have also been reported to have either no effect or to lessen the natriuretic action of ANP (Gaillard et al.,(1988,1989), Richards et al.,(1989a)), presumably reflecting the dual effects of suppression of Ang II and reduction in renal perfusion pressure (Polonia et al.,(1990)). The site of this interaction within the kidney is also uncertain and is discussed in more detail in

chapter 4 but, briefly, may involve either a specific interaction between ANP and Ang II at a number of sites within the nephron, with the simplest model invisaging Ang II (by its proximal tubular effects on sodium reabsorption) regulating the delivery of sodium to the distal tubule where ANP then acts. Furthermore, suppression of renin, and thus intrarenal Ang II, may partly contribute to the observed natriuretic effects of ANP.

In summary the renal effects of ANP are exerted at three levels: **(i)** direct actions on the collecting tubule and possibly other nephron segments; **(ii)** inhibition of the synthesis of other renal hormones (aldosterone, renin, AVP); **(iii)** indirect effects by interference with the vascular (angiotensin) and tubular (AVP) actions of other hormones; and glomerulotubular feedback.

2.8. Cardiovascular actions of ANP.

The first studies with atrial extracts (deBold et al.,(1981)) and synthetic ANP (Burnett et al.,(1984), Maack et al.,(1984)) demonstrated hypotensive effects. However, these early studies employed pharmacological doses of ANP; more subtle vasodilator and hypotensive effects have been found with physiologically relevant doses, together with other effects including increased capillary permeability and impaired baroreceptor sensitivity. As with the renal actions of the peptide, investigators have studied the integrated action of ANP in the whole animal and the effects on its isolated components.

Actions of ANP on isolated vessels. In vitro, isolated segments of large capacitance vessels (such as the aorta), can be made to relax

with ANP if preconstricted (Winquist et al.,(1984), Garcia et al., (1984)). This effect is not endothelium dependant (Winquist et al.,(1984)) but nanomolar concentrations of ANP are generally required to produce effects. Similar effects have been confirmed in human capacitance vessels (Hughes et al.,(1988)). In resistance vessels, however, ANP appears to have little in vitro effect. Aalkjaer et al., (1985) studied the effects of ANP on cardiac, mesenteric, cerebral and peripheral resistance vessels, using the myograph technique, and found no effect at micromolar concentrations of ANP. These findings have been confirmed in the rat (deMay et al.,(1987)) and in human tissue (Hughes et al.,(1988), (1989)). One exception is the renal circulation (Aalkjaer et al.,(1985), deMay et al.,(1987)) where ANP does cause vasorelaxation in isolated resistance vessels, and in the isolated perfused kidney (Garcia et al.,(1984)), although whether this occurs in vivo is still debated (2.7). There are conflicting reports on the effects of ANP on veins. The rabbit facial vein dilates with ANP (Winquist et al.,(1984), although other preparations including the human saphenous vein (Hughes et al.,(1988)) do not. In fact the in vitro assessment of vasodilator substances - such as ANP - are difficult to interpret because of the need to preconstrict the vessels. Proctor and Bealer (1987) found that ANP was more effective at dilating skeletal muscle vessels preconstricted with Ang II rather than vasopressin or noradrenaline. The vasoconstrictor used is therefore important. Furthermore, the dose-relationships between the vasoconstrictor and vasodilator agents are seldom investigated and, the finding that ANP does not dilate a vessel maximally constricted with micromolar concentrations of Ang II means only that ANP is not an effective vasodilator in the presence of high concentrations of a

potent constrictor. Clearer results come from isolated tissue preparations such as the "isolated" human forearm (Webb et al.,(1988)), Hughes et al.,(1988)). Infusion of doses as low as 10 ng/min produced increases in forearm blood flow, although the plasma levels of ANP were increased above the physiological range. The only other site where human vessels are accessible in vivo is in the retinal circulation. Using fluorescein angiography Mann et al.,(1987) have shown that large doses (1μg/kg) dilate large retinal arteries. Although an elegant technique demonstrating vasodilator effects of ANP in vivo, pharmacological doses of ANP were employed and the resistance vasculature is probably beyond the resolution of this method.

Although ANP can dilate vessels from different tissues qualitative and quantitative differences in the vasodilator effects of physiologically relevant doses of ANP have not been fully assessed. Furthermore, attempts to examine overall changes in blood flow are limited by the fact that changes in blood pressure or flow in one organ may activate counter-regulatory influences which further alter regional blood flow. It is clear that muscle and skin blood flow is increased by low dose ANP infusion (Hughes et al.,(1988), Webb et al.,(1988)). Garcia et al.,(1985) investigated the regional effects of ANP (1μg/kg) using radiolabelled microspheres in the rat and found that high dose ANP increased skin and renal blood flow but had no effect on other organ flow. However, before we can draw conclusions on the differential effects of ANP on organ blood flow, these studies will need to be repeated with low dose infusions of the peptide.

Finally, ANP can reduce cardiac output (see below), one possible explanation for which would involve a direct negative inotropic effect on the heart. Although there is conflicting data, the balance of

evidence favours no effect with studies such as that of Criscione et al.,(1987)) unable to demonstrate any direct effects on contractility in the isolated heart.

Infusion of ANP: In man, the history of the cardiovascular actions of ANP follows the same pattern as the renal effects, with large reductions in blood pressure being seen in the early studies (eg Richards et al.,(1985), Weidmann et al.,(1986)) but not in later studies using physiologically relevant doses of ANP (Richards et al.,(1988a,b), Solomon et al.,(1988), Cottier et al.,(1988), Anderson et al.,(1987)). Part of the reason for this apparent absence of effect is methodological. Richards et al.,(1988c) have recently shown that infusion of as little as 0.75 pmol/kg/min ANP can reduce blood pressure measured intra-arterially, but these changes cannot be detected by non-invasive measurement of blood pressure in the other low dose studies (above). If infusion is carried on beyond a few hours then reduction in blood pressure can be detected (1 pmol/kg/min; Janssen et al.,(1989)) non-invasively after approximately 24 hours. There are no other invasive studies in normal man, and we have instead to rely on data from patients with heart failure (e.g Cody et al.,(1987), Goy et al.,(1988)) and hypertension (Tonolo et al.,(1989)). The consensus from studies in heart failure (reviewed by Northridge et al.,(1991)) is for cardiac output to increase and right atrial, pulmonary capillary wedge, systemic vascular resistance and arterial pressure to fall, with infusions rates of greater than 10 pmol/kg/min (eg Cody et al.,(1987)). With lower dose infusions (less than 10 pmol/kg/min; eg Goy et al.,(1988)) arterial pressure does not change, cardiac filling pressures fall and there is a trend towards an increase in cardiac output and reduced systemic resistance. In

hypertensive patients (Tonolo et al.,(1989)) short term administration of 1 or 2 pmol/kg/min ANP caused a 10 and 20 mmHg fall in blood pressure respectively, associated with a reduced haematocrit, a downward trend in cardiac output and in cardiac filling pressures. Interestingly, a significant rise in sodium excretion was only observed with the higher dose.

These studies indicate that ANP can lower blood pressure (most clearly at high doses) and cardiac filling pressures, possibly by a combined arteriovenous dilatation but also by contraction of the intravascular volume (see below). Cardiac output is generally maintained, and there is little change in heart rate, making ANP an attractive therapeutic agent in heart failure (see later; Section 4).

Studies in experimental animals permit more detailed characterisation of these effects by invasive measurement of cardiovascular function. The earliest studies of high dose ANP (Maack et al.,(1984), Burnett et al.,(1984) showed clear reductions in arterial pressure. Of later studies on the dose-response relationship of the haemodynamic effects of ANP that of Bie et al.,(1988) is possibly the most complete, involving infusion of 4, 8 and 16 pmol/kg/min ANP, each for 20 minutes. Sodium excretion only increased in the higher dosage groups, whilst 25-50% reductions in right and left atrial pressures were evident from the lowest dosage group. There were modest reductions in arterial pressure (approximately 5 mmHg) in the two higher dose groups and similar, modest reduction in cardiac output with high doses. Heart rate did not increase, nor was there any dramatic change in calculated total peripheral resistance. Zimmerman et al.,(1987b) observed a similar pattern with reduction of cardiac filling pressure with low doses of ANP. Such studies tell us that very

low doses of ANP can cause acute reduction in cardiac filling pressures but the reasons are still not clear. In many studies changes in filling pressure were detected before changes in haematocrit and urinary changes, and are thus unlikely to be dependent on changes in intravascular volume. Furthermore veins (above) appear to be relatively insensitive to the effects of ANP in vitro and in vivo (Webb et al.,(1988)). An alternative suggestion is that venous resistance - rather than venous capacitance - is increased (Lee and Goldman (1988)), however, this has not been confirmed.

The precise mechanism of these vascular effects still awaits elucidation, as does the question of whether these (potentially therapeutic) effects are sustained during prolonged administration.

Although ANP dilates arteries in vitro this is not necessarily the mechanism by which it lowers blood pressure. Several studies have been designed to look specifically at the short and long-term mechanism of reduction of blood pressure. Acute falls in blood pressure appear to be dependent on reduced cardiac output. For example, in sheep, Parkes et al.,(1988) infused ANP for five days, producing a sustained reduction in blood pressure. On the first day a fall in blood pressure was accompanied by reduced cardiac output and intravascular volume. However, by the final day, the pattern had changed with cardiac output returning to normal, the reduced blood pressure at this stage being secondary to a reduction in calculated peripheral vascular resistance.

Whether reduced whole body sodium contribtes to the fall in blood pressure is still unclear; Janssen et al.,(1989) in their study of low dose ANP in essential hypertension found that the hypotensive effects of ANP were associated with a persistent negative sodium balance of

approximately 50-100 mmoles. However, Garcia et al.,(1985) demonstrated in the SHR that low dose ANP (in the range 1-2 pmol/kg/min) caused a fall in blood pressure without any effect on sodium excretion and, in a similar series of experiments, Garcia et al.,(1987) were unable to demonstrate any changes in plasma or extracellular volume accompanying the reduction in blood pressure with ANP.

In man, the only study to examine the blood pressure lowering effects of ANP with simultaneous invasive cardiac measurements is that of Tonolo et al.,(1989). Infusion of ANP (1, then 2 pmol/kg/min for 2 hours) caused a 10 mmHg reduction in systolic pressure; associated with a natriuresis, increased haematocrit and reduced cardiac filling pressures but no change in total peripheral resistance. The inference from these studies is that <u>when</u> infusion of ANP causes a reduction in blood pressure, then the initial decrease is due to reduced cardiac output secondary to reduced filling pressures as a consequence of a combination of natriuresis-diuresis and increased capillary permeability. The longer term changes in blood pressure appear to be mediated by reduced vascular resistance. Whether this is a direct effect of ANP or indirectly mediated by suppression of pressor systems is still not known. Similarly, the contribution of natriuresis-diuresis to the intermediate and long-term hypotensive effects of ANP is also uncertain. In even the best documented study (Janssen et al.,(1989)) the hypotensive effects of low dose infusion and the return to baseline after cessation of ANP lagged 12-24 behind the changes in renal function.

Capillary permeability: During infusion of ANP many studies have shown

a rise in haematocrit (eg Richards et al.,(1988a,b), Weidmann et al.,(1986), Solomon et al.,(1988), Trippodo et al.,(1986)). This effect is due to contraction of the intravascular volume and can occur in the absence of changes in urine volume, for example in nephrectomised animals and in man (Fluckinger et al.,(1986), Tonolo et al.,(1988)). The reason for this being that ANP can directly increase capillary permeability (Huxley et al.,(1987)).

Baroreceptor function: As a general rule, although not without exception, infusion of ANP is not associated with an increase in heart rate, even in the presence of a significant fall in blood pressure (eg Zimmerman et al.,(1987)). This has been interpreted as indicating that ANP inhibits the baroreceptor-mediated reflex tachycardia under these circumstances. ANP attenuates the heart rate response to externally applied neck pressure (Elbert and Cowley (1988)) in man; and has been shown to reduce the reflex increase in forearm vascular resistance in response to lower body negative pressure (Takeshita et al.,(1987), although others disagree (Volpe et al.,(1988)). The precise role of the effects of ANP are still unknown, as is the site of action. As noted above, ANP is present within the brainstem where it may interfere with cardiovascular reflexes. However, mRNA for ANP is also expressed in the aortic arch (Gardner et al.,(1988)) where it may have a local effect on the afferent limb of the reflex. Similarly, ANP has been shown to impair ganglionic transmission and to reduce sympathetic nerve activity measured directly in anaesthetised rats (despite reduced blood pressure; Thoren et al.,(1986). A final note of caution concerns the infrequent reports of vasovagal syncope with ANP infusion (eg Biollaz et al.,(1987)). This is only seen with high doses, rapidly recovers and is likely to be an idiosyncratic reaction.

In summary ANP exerts a variety of vascular effects including increases in capillary permeability, direct vasodilator effects, inhibition of baroreceptor function and effects on the brain stem cardiovascular centres which together result in reduced arterial and cardiac filling pressures.

2.9. Endocrine actions of ANP.

In addition to its vascular and renal effects, ANP exerts a spectrum of endocrine effects the best documented of which are suppression of renin release and inhibition of the synthesis and secretion of aldosterone. Other interactions with neuroendocrine systems are covered later.

Renin: Suppression of circulating renin was one of the first effects of ANP to be demonstrated (Maack et al.,(1984), Burnett et al.,(1984)), setting it apart from other natriuretic agents where stimulation of renin is the rule. In experimental animals, subsequent studies employing low dose infusion of ANP have shown similar suppression of basal renin levels (e.g. Seymour et al.,(1986), Zimmerman et al.,(1987), Chapter 4, Figure 4.3.) or the renin response to, for example, reduced renal perfusion pressure (Scheuer et al.,(1987), or ACE inhibition (Chapter 4, Table 4.1.). In man, the earliest studies (e.g. Richards et al.,(1985)) found that renin did not rise during the hypotensive response to high dose ANP. The recent series of low dose infusion studies (Richards et al.,(1988a,b), Cottier et al.,(1988), Anderson et al.,(1987)) have confirmed the ability of very modest increments in plasma ANP to suppress renin release. Others have demonstrated the ability of ANP to inhibit the

release of renin in response to a variety of stimuli including ß-agonists, diuretics and ACE inhibition (McMurray and Struthers (1988a,b; Lang and Struthers (1990). The mechanism through which ANP achieves these effects is still unresolved. Theoretically, it may involve a direct effect on the juxtaglomerular apparatus -which secretes renin - or be mediated indirectly by alterations in the delivery of sodium to the macula densa, or via baroreceptor-regulated renin release. The precise mechanism is difficult to resolve for the simple reason that under most circumstances infusion of ANP causes parallel increases in sodium excretion (and hence delivery of sodium to the macula densa) and suppression of renin. In support of a macula densa mechanism, in the non-filtering kidney (Opgenorth et al.,(1986) and in functionally anephric humans with chronic renal failure (Richards et al.,(1988d)) infusion of ANP has no effect on either basal or stimulated renin release. However, in the study in Chapter 4, ANP causes a similar fractional reduction in plasma renin regardless of changes in overall sodium excretion (manipulated by infusion of Ang II or an ACE inhibitor (Figure 4.8.,Table 4.1.)). Attempts to unravel this problem in vitro have similarly given conflicting results. In isolated juxtaglomerular preparations (Antonipallai et al.,(1986), Kurtz et al.,(1986)) ANP inhibits hormone release; an effect mediated by cGMP. However, in isolated segments of renal microvasculature, Itoh et al.,(1987) found that ANP stimulated renin release. A further potential mechanism is on baroreceptor mediated renin release, since it has been shown (Marin-Grez et al.,(1986), Veldkamp et al.,(1988)) that ANP can dilate the afferent arteriole.

Overall, although there are conflicing reports these mostly come from either in vitro studies or non-physiological preparations - like

the non-filtering kidney. It seems likely, based on studies such as that in Chapter 4, where physiological increments of ANP are assessed in conscious animals, that in a physiological setting ANP has a direct effect on renin release. However, like the renal effects of ANP in general, suppression of renin may involve the balance of several different regulatory influences.

Adrenocorticosteroids: In contrast with renin there is no doubt that ANP directly inhibits aldosterone synthesis and secretion. However, the effects on cortisol secretion are uncertain.

In infusion studies, short term administration of ANP has a variable effect on plasma aldosterone with either no effect (eg Weidmann et al.,(1986), Richards et al.,(1985), Cottier et al.,(1988), Morice et al.,(1988), Anderson et al.,(1987)) or a reduction of about 50% (Maack et al.,(1984), Zimmerman et al.,(1987); Solomon et al.,(1988), Richards et al.,(1988a,b)). The absence of effects in some studies probably reflects the slow time course of changes in aldosterone (and the short duration of ANP infusion) and the superimposed diurnal reduction in aldosterone during the period of study. When aldosterone is stimulated, then ANP blunts the rise in plasma levels in response to infusion of Ang II or ACTH (Cuneo et al.,(1987), McMurray et al.,(1988c)). In contrast ANP has no effect on the cortisol response to ACTH (Cuneo et al.,(1987), McMurray et al.,(1988c)). This ability of ANP to inhibit stimulated aldosterone production indicates a direct action on the adrenal (confirmed in vitro, below), although under normal circumstances the ANP-dependent reduction in renin (Maack et al.,(1984), Richards et al.,(1988a,b)) would be expected to cause a secondary reduction in aldosterone.

In addition long term exposure of the adrenal to ANP has been

shown to result in down-regulation of zona glomerulosa receptors and to inhibition of the growth and steroidogenic capacity of the zona glomerulosa by a process which is independent of angiotensin II or ACTH (Rebuffat et al.,(1988), Mazzochi et al.,(1987)).

In vitro, ANP inhibits the synthesis and secretion of aldosterone by cultured adrenal glomerulosa cells from a variety of species (Kudo and Baird (1986), Goodfriend et al.,(1984), Lang and Struthers (1990) - for review) in response to many agonists including Ang II, potassium, ACTH, forskolin and dibutyryl cAMP. The mechanism by which ANP produces this reduction in aldosterone is unclear. Stimulation of adrenocortical cyclic GMP synthesis by ANP has been described but this appears not to be involved (Matsuoka et al,, (1987)) in the steroid response since cyclic GMP by itself will tend to stimulate rather than inhibit steroidogenesis (Nambi et al.,(1982), Ganguly et al.,(1989)). A common factor in the mechanism of all adrenal secretagogues is the change in cell calcium metabolism (Kojima et al, 1986). However, ANP appears to reduce aldosterone secretion independently of changes in intracellular calcium (Capponi et al.,(1986)) or activation of the phosphotidylinositol pathway (Ganguly et al.,(1989)).

In summary, although ANP directly inhibits aldosterone secretion, the mechanisms are unknown (see Lang and Struthers (1990)) for more comprehensive review.

ANP also has effects on other hormonal systems. The suppression of ADH and of the pituitary hormones are the most completely described (see below, 2.10) but receptors for ANP are also present in the ovary (Kim et al.,(1987)), where ANP has been shown to stimulate luteal guanylate cyclase (Budnik et al.,(1987)) and in the placenta (Sen (1986), Hatjis and Grogan (1988)) where ANP may potentially exert

other endocrine effects.

2.10. Actions of ANP in the nervous system.

Shortly after its identification in the heart ANP was demonstrated in the central nervous system (Morii et al.,(1985)). Immunoreactive ANP is present in high concentration in a number of areas of rat brain including the subfornical organ, area postrema and choroid plexus and to a lesser extent in other areas (Quirion et al., (1984)). ANP is also present in the adrenal medulla (Ong et al., (1988)). There is now considerable evidence to show that ANP is locally produced in the brain not least because ANP does not readily cross the blood-brain barrier (Levin et al.,(1988)). Neurones with cell bodies which stain for ANP are present and project largely to regions in the lateral wall of the third ventricle known to be involved in osmoregulation (Standaert et al.,(1985)). Messenger RNA for ANP is present in neural tissue (Glembotski et al.,(1985), Gardner et al.,(1987)), but unlike the heart the major storage form of ANP appears to be the biologically active form (Shiono et al.,(1986)). Low doses of ANP administered into the cerebral ventricles inhibit the release of vasopressin in normally hydrated rats (Samson et al.,(1987)) and antagonise the stimulatory action of co-administered angiotensin II on AVP release in the rat (Yamada et al.,(1986)). Central ANP also inhibits dehydration- and angiotensin-stimulated water drinking in rats (Antunes-Rodriguez et al.,(1985)). Recent studies have shown that central administration of ANP can lower reduce sympathetic outflow (Schultz et al.,(1990)) and lower blood pressure via an α_2 adrenergic mechanism (Levin et al.,(1988)). Within the central nervous system ANP also inhibits the pressor effects of

centrally administered angiotensin (Itoh et al.,(1986), Casto et al.,(1987)). The role of ANP at other sites within the brain are not as clearly defined. Although ANP receptors are present in the pituitary and ACTH and LH are increased and decreased respectively by ANP (Horvath et al.,(1986), Samson et al.,(1988)) these effects are not apparent in isolated pituitary cells. They may be due to a hypothalamic action, inhibiting the release of LHRH and prolactin perhaps through an effect on dopaminergic pathways (Samson (1988)).

2.11. Related natriuretic peptides.

The search to identify the distribution of ANP led not only to the identification of ANP in other tissues but also in the identification of closely related peptide hormones. The most studied is Brain Natriuretic Peptide (BNP, Sudoh et al.,(1988), Figure 2.2), which in man is a 32 AA peptide (Kambayashi et al.,(1990)). Paradoxically, although it was discovered in the brain BNP is widely distributed and is also present in atrial tissue (Kambayashi et al.,(1990).

Brain Natriuretic Peptide.

Sudoh et al.,(1988) initially identified a 26 AA peptide from porcine brain with approximately 50% homology with ANP, and containing a 17 AA disulphide linked ring structure. In the same year a larger 32 AA peptide (BNP-32, Sudoh et al.,(1988)) was discovered, which is the same size as human BNP, subsequntly identified in atrial tissue (Kambayashi et al.,(1990)).

The processing of BNP is similar to that of ANP, and has been best characterised in porcine heart where two large forms of the

peptide are found: a 131 AA peptide (preproBNP, Maekawa et al.,(1988)) and a 106 AA peptide (y-BNP, Minamino et al.,(1988)). At the carboxyl end of both these peptides is the 32 AA BNP and it seems likely that removal of a 25 AA "signal peptide" from preproBNP produces yBNP, which is then cleaved to leave the active 32 AA peptide (Porter et al.,(1989)). As with ANP the predominant storage form in the heart is the propeptide (yBNP) but in the brain the 26 and 32 AA forms predominate. Although there is strong homology between BNP and ANP, this is less marked for the precursors which show little homology and, unlike ANP, there are apparently large species differences and in man, for example, preproBNP has been shown to contain 134 AA (Sudoh et al.,(1989)).

In the brain concentrations of immunoreactive BNP are at least ten times those of ANP, but show a similar distribution through the areas involved in cardiovascular homeostasis (2.10). BNP is also present in the atria where the concentration of immunoreactive hormone is about 1% of the level of ANP, although the absolute cardiac concentrations of BNP are much greater than those in the brain. Circulating hormone (Mukoyama et al.,(1990, 1991)) plasma levels of approximately 1 pmol/l, and appear to behave like ANP being increased increased in heart failure.

Finally, the functions of BNP closely parallel those of ANP. In most tissues ANP and BNP have been shown to bind to the same receptor sites with equal affinity, with a similar autoradiographic distribution, and to stimulate the production of cGMP (Iwata et al.,(1989), Brown and Czarnecki (1989), Gunning et al.,(1990)). In fact two subtypes of guanylate cyclase linked ANP receptor are now recognised based on their differential affinity for ANP and BNP (Chang

et al.,(1989); section 2.7). The GC-A subtype recognises both while the GC-B subtype shows preferential affinity for BNP. The spectrum of biological effects of BNP is identical to that of ANP; Sudoh et al.,(1988) demonstrated natriuretic, diuretic and hypotensive effects; in the adrenal gland BNP inhibits aldosterone secretion (Higuchi et al.,(1989)); and in the brain intraventricular administrtion of BNP has a similar spectrum of effects to ANP and, for example, inhibits the pressor response to Ang II (Shirakami et al.,(1988)).

Urodilatin.

Urodilatin is a 32 AA peptide identical to the 32 AA carboxy terminal of the ANP precursor (i.e. 95-126 hANP, Figure 2.2.) identified in human urine (for review see Feller et al.,(1990)). This peptide does not appear to circulate in the blood and, unlike ANP, it is not cleaved by NEP (Gagelmann et al.,(1988)). Preliminary reports, using immunohistochemical techniques (Feller et al.,(1990)) suggest that urodilatin immunoreactivity can be localised in the tubular cells of the distal nephron, the suggestion being that it is produced at this site and acts as a local hormone within the kidney, with its proposed site of action being on luminal ANP receptors in the collecting duct. The biological effects of infused urodilatin are also similar to ANP and are mediated by cGMP (Riegger et al.,(1988)) although it may not cause suppression of renin and aldosterone (Feller et al.,(1990)).

Recently, more related natriuretic peptides have been discovered including Iso-ANP and CNP (Sudoh et al.,(1990)). The roles of these are yet to be established but it seems probable that more peptides related to ANP will be discovered which form part of the ANP "hormonal

system".

2.12. Therapeutic effects of ANP.

Obviously the spectrum of actions of ANP (Figure 1.1) indicate that the hormone may be useful in the treatment of a variety of conditions including hypertension and heart failure, and in other oedematous states.

The effects of ANP in hypertension and heart failure are described in some detail in the section on the cardiovascular effects of ANP and are reviewed in detail by Richards (1990) and Northridge et al.,(1991). Studies in heart failure are limited to the acute effects of ANP but show the potentially beneficial combination of diuresis and natriuresis, renal effects, suppression of the renin-angiotensin system but more importantly a clear reduction in cardiac pre-load at low doses of ANP, without any significant impairment of cardiac output. The results of long term studies are therefore awaited with interest. In hypertension, acute administration of ANP has similarly been shown to reduce blood pressure. However, there are now several studies of prolonged low dose infusion of ANP in hypertensive animal models including DOCA salt, Goldblatt and Spontaneously (SHR) hypertensive rats. The models have been studied by Garcia and colleagues (eg Garcia et al.,(1985, 1987) who have found that infusion of 1-2 pmol/kg/day produces a fall in blood pressure within 24 hours, with maximal effects at the end of a six day infusion protocol. Reductions of up to 30 % were observed, and such reductions have been confirmed in the SHR over a 7 day infusion by deMay et al.,(1987)). Confirmation that these effects might by translated into an effective antihypertensive effect in man have recently been provided by Janssen

et al.,(1989). Infusion of approximately 1 pmol/kg/min ANP for five days into six mild hypertensive patients resulted in a 10% fall in blood pressure. Blood pressure reduction was not evident until 12 hours after the start of infusion and maximal, sustained effects were seen after 36 hours. Further assessment of the antihypertensive effects of ANP are limited by the need for intravenous administration of the peptide; possible ways to circumvent this problem are discussed in Section 4. The fact that such a low dose of ANP lowers blood pressure is reassuring since ANP might be predicted to be less effective in hypertensives than normal subjects for two reasons. Firstly, plasma levels of ANP are already elevated (2.3) and, secondly, ANP receptors are down-regulated (eg Schiffrin et al.,(1987)). However, to the contrary evidence in man (Weidmann et al.,(1986), Cusson et al.,(1987)) and in experimental animals (Onwochei et al.,(1987), Pollock and Arendshorst (1990)) suggests that there is an enhanced natriuresis in response to ANP, and enhanced release of ANP in response to atrial distension.

Other conditions: Other areas where ANP appears to have therapeutic potential include acute renal failure and asthma. In experimental ischaemic acute renal failure (Nakamoto et al.,(1987)) and Uranyl nitrate induced renal failure (Heidgreder et al.,(1988) in rats, pharmacological infusion of ANP improved glomerular filtration rate. In dogs infusion of 0.3 μg/kg/min ANP improved immediate and 24 hour function of autotransplanted kidneys, subjected to 24 hours cold ischaemia (Lewis et al.,(1989)). However, a controlled trial in human cadaveric transplants (Ratcliffe et al.,(1991)) failed to show any beneficial effects, reflecting the multitude of factors determining outcome in human transplantation.

Recently, Hulks et al.,(1990a,b) have shown that ANP causes bronchodilation in the normal human airway at pharmacological concentrations and in asthmatic subjects at pathophysiological levels. The mechanism underlying this effect awaits further investigation and could conceivably be a direct action on bronchial smooth muscle or an indirect effect via vasodilation and the "washout" of bronchoconstrictor substances. Nevertheless, it does suggest that either ANP or stimulation of cGMP, may offer an alternative therapeutic approach to the treatment of asthma.

In summary, ANP has a unique and potentially beneficial spectrum of therapeutic effects, the use of which is limited by the need to administer the hormone parenterally.

2.13. Physiological Role of ANP.

Despite the considerable literature on the actions of ANP there is still debate as to whether ANP has any physiological role (Goetz (1990)). This suggestion has emerged for two main reasons. Firstly, as noted above, many of the early studies with ANP employed doses which we now know to be within the pharmacological range, and subsequent studies with physiological infusion have shown very modest increases in sodium excretion and urine volume (Richards et al.,(1988a,b), Cottier et al.,(1988), Anderson et al.,(1987), Morice et al.,(1988)). Secondly, classical physiological and pharmacological techniques involve infusion of compounds and hormones in isolation without taking into account the physiological circumstances in which these chnages would normally occur. For example, with regard to ANP, volume expansion would normally cause not only an increase in plasma hormone levels but also suppression of a number of systems which oppose the

actions of ANP (2.7) notably the renin-angiotensin system, and (via centrally-mediated reflexes) vasopressin and the sympathetic nervous system. Hence, if exogenous ANP is infused to produce levels similar to those achieved by volume expansion then a similar natriuresis would not necessarily be expected. This is important when we consider the experimental evidence which has been used to support the claim that ANP has no physiological role. Goetz et al.,(1986a,b) found that balloon distension of the left atrium in dogs caused a four-fold increase in plasma ANP and a natriuresis. When the experiment was repeated in dogs with denervated hearts the same increase in plasma ANP was seen but the natriuresis abolished. The authors suggest that this implies the absence of a role for ANP in response to volume denervation but, since it is known that cardiac denervation by disrupting vagal inhibitory input to the central circulatory control centres results in activation of the renin-angiotensin system and sympathetic nervous system both of which can inhibit ANP action then it is clear that this conclusion is unfounded. In a similar type of experiment Richards et al.,(1989b) examined the renal effects of a saline load and compared them with the effects of ANP infused to similar levels. ANP infusion was less effective than the rise of ANP produced by saline infusion. This is interpreted, correctly, as indicating that ANP is not the only factor involved in the clearance of a saline load, where there will be suppression of renin, ADH and the sympathetic nervous system. Goetz (Goetz et al.,(1988)) has also studied the clearance of a saline load in dogs. The saline was infused following a high dose ANP infusion - when plasma ANP was falling from 1500 to 300 pg/ml - and caused an increase in urinary sodium excretion. Goetz has implied that increased sodium excretion in the

presence of falling plasma ANP means that ANP has no role in sodium excretiom. However, once again this phenomenon can be explained by the potentiation of the renal effects of ANP by the other effects of atrial and intravascular volume expansion (Metzler and Ramsay (1988).

Two methods have been attempted to study the effects of "taking out" the ANP system. The simplest involves removal of the atrial appendages (Benjamin et al.,(1988)) as a result of which excretion of a saline load is impaired. However, this experiment may also remove the influence of the cardiac nerves and so is non-specific. In the absence of specific antagonists of ANP rats immunised against ANP have been studied (eg Greenwald et al.,(1988)). These models have problems in that the presence of ANP antibodies make measurement of circulating hormone levels impossible and the antisera used are often polyclonal and relatively non-specific. In the chronically immunised rat Greenwald et al.,(1988) found no difference in the adaptation to mineralocorticoid administration or changes in dietary sodium intake, although the ability to excrete a sodium load was impaired. These data have been interpreted as demonstrating that ANP has no effect on long term sodium balance. However, in the SHR (Itoh et al.,(1989)) immunisation against ANP increases the development of hypertension, suggesting long term effects are important. Using an alternative approach, transgenic mice with two copies of the ANP gene are hypotensive and show increased sodium excretion (Steinhelper et al.,(1990), suggesting that ANP plays an essential role in cardiovascular homeostasis. Presumably the development of mice deficient in the ANP gene, and the development of specific ANP receptor antagonists will finally settle this argument.

The argument for a physiological role for ANP is, on the other

hand, almost overwhelming. Gene expression, hormone release and plasma ANP levels are altered, appropriately for a natriuretic hormone, by changes in sodium balance and intravascular volume (see earlier in this chapter). Membrane receptors are present in all target tissues where effects are seen and have Kd values appropriate for an interaction to occur at "normal" plasma ANP levels. Finally, low dose infusion of ANP causes (albeit modest and slowly developing) increases in urine volume and sodium excretion, blood pressure, renin and aldosterone levels. Overall, the prevailing view of the role of ANP is moving away from that of a very powerful natriuretic hormone to that of a more modest hormone which does not simply have direct natriuretic effects on the kidney but has a spectrum of influences on the vasculature, the renin-angiotensin system and other hormonal systems giving it a more subtle modulatory role as a counterweight to the powerful antidiuretic, antinatriuretic and pressor systems.

The concept of ANP "balancing" the effects of the renin-angiotensin system is particularly attractive since ANP interacts with the RAAS at so many different sites. In addition to the physiological interaction between a natriuretic and antinatriuretic system, and the general overlap of ANP and Ang II receptors (Mendelsohn et al.,(1987)) there are several specific interactions in (a) the nervous system, (b) the vasculature and (c) the kidney. Furthermore, ANP inhibits the secretion of (d) renin and (e) aldosterone. Finally, as if to complete the interaction at virtually level of the renin-angiotensin system it has recently been shown that ANP may have ACE inhibitory potential (Kawaguchi et al.,(1990)). Thus, ANP is ideally placed to counter-regulate the major antinatriuretic hormonal system.

Finally, although the balance of evidence over the last few years

has moved away from the concept of ANP as a powerful hormone to one with a more subtle modulatory role, it should be borne in mind that this chapter has dealt with ANP alone. However, ANP now appears to be one of a family of related peptide hormones which includes BNP, CNP, urodilatin, the N-terminal fragments of pro-ANP, iso-ANP and possibly many more. Not until the complete family are identified, and their individual and additive effects studied, will we understand the complex role of this natriuretic peptide hormonal system.

SECTION 2.

PLASMA LEVELS OF ATRIAL NATRIURETIC PEPTIDE.

Chapter 3.

Circulating ANP in man: Pathophysiological regulation of plasma levels and development of the hormonal system.

Introduction:

In order to establish a physiological role for a putative hormone - such as ANP - it is essential to have a method for detecting circulating hormone. For ANP, such methodology lagged several years behind the discovery, sequencing and synthesis of the peptide (Chapter 2). Early radio-immunoassays (RIA) became available in the mid-1980's but gave highly variable results (possibly due to an interfering substance in plasma (Richards et al., (1987a)) and subsequently, pre-extraction of plasma using reverse phase (SEP-PAK) columns resulted in lower, but more consistent values. Since 1987 most groups have used these methods (Richards et al., (1987a), Appendix 1) resulting in hundreds of published reports on plasma ANP and a general consensus that plasma levels are principally regulated by changes in intravascular volume (Chapter 2).

It seems appropriate to begin this thesis by reviewing my own

experience with measurement of plasma ANP using the RIA (with pre-extraction) developed by Mark Richards and Brenda Leckie in the MRC Blood Pressure Unit (Richards et al., (1987), Appendix 1). Four groups of patients or studies are included in this chapter. The first includes levels in normal subjects and a mixture of patients, reflecting the spectrum of pathology admitted to the MRC Blood Pressure Unit, and included primarily for descriptive purposes. The remaining three sections cover specific studies: in **(a)** the human fetus, **(b)** pregnancy and **(c)** malignant hypertension. The individual relevance of these studies is discussed but together they provide evidence of a physiological role for ANP in man from the stage of the developing fetus and demonstrate that plasma ANP in humans is regulated not only by cardiac pre-load, but also cardiac after-load.

Methods:

Normal Subjects and Patients:

The "normal" subjects comprise people admitted to the MRC Blood Pressure Unit (between 1987 and 1989) for assessment of suitability as kidney donors for relatives on renal replacement therapy. These 46 "patients" (14 female, 32 male; mean age 32 [range 21-62] years) were assessed according to a standard protocol involving a three day admission to the Unit. None had any significant past medical history, all were normal on full clinical examination (blood pressure 123±2/76±1, mean±SEM) and none were on regular drug therapy. During the admission all subjects had serial blood pressure measurements performed by the nursing staff; two 24 hour urine collections for

creatinine clearance; two blood samples for biochemical profile, renin, aldosterone and ANP; chest X-Ray, ECG and renal arteriography. The blood samples were taken between 8 and 9 a.m. with the subjects supine, through an in-dwelling catheter (Venflon, Viggo, Sweden), after a period of overnight rest. Assay methodology and collection procedure are described in Appendix 1.

The remaining samples from patients admitted electively for investigation (e.g. Conn's syndrome, dexamethasone suppressible hyperaldosteronism [DSH], essential hypertension [EH], acromegally, hypothyroidism) were taken as above; whilst those for emergency admissions (e.g. malignant phase hypertension [MP], phaeochromocytoma, adrenal insufficiency) were taken after 30 minutes supine rest through an indwelling catheter according to established protocol in the MRC Blood Pressure Unit. No patients were on any drug treatment at the time of sampling unless stated and in some the response to treatment is documented (Conn's syndrome, malignant hypertension, adrenal insufficiency).

Fetal Samples:

Samples were obtained from patients at the Queen Mother's Hospital, Glasgow undergoing cordocentesis (umbilical cord sampling under ultrasound guidance) for routine obstetric indications. The protocol was approved by the Hospital Ethical Committee and patients gave informed written consent. Samples were obtained from 13 patients with Rhesus iso-immunisation in whom 20 procedures were performed (3 patients had three procedures, one had two); and in 12 patients where sampling was performed for karyotype determination. Mean gestational age was 27.5 weeks (range 23-32 weeks) in the Rhesus group and 25.8

(17-34) in the karyotype group. Indications for karyotype determination were: abdominal wall, intestinal or diaphragmatic defect on ultrasound scan (four), urinary tract obstruction (three), previous child with genetic abnormality (two), pre-eclampsia (PET, one), idiopathic growth retardation (IUGR, one) and unexplained polyhydramnios (one). One fetus with polyhydramnios and one fetus in the Rhesus group had abnormal karyotypes (trisomy 21).

Sampling: Cordocentesis was performed with the mother sedated using lorazepam, papaveretum and perphenazine. Maternal blood samples were taken with the mother recumbent, through an indwelling cannula, immediately before fetal sampling. Fetal samples were obtained through a 20G needle inserted into the umbilical vein under ultrasound guidance. In two of the karyotype group where this was not possible samples were taken from the heart. Fetal blood was confirmed by estimation of mean red cell volume and haematocrit. Fetal blood samples (1-2 mls) and maternal samples (10 mls) were collected and assayed for ANP by RIA as previously described (Richards et al.,(1987a), Appendix 1).

Transfusion: In 12 of the rhesus cases blood samples were obtained before and after intravascular transfusion of 32-105 (mean 63.5) mls of washed resuspended red cells (haematocrit 70-80%). ANP was undetectable in donor blood. The mean pretransfusion haematocrit was 27.1% (range 9-39%), compared with 43.5% (35-57) in the karyotype group.

Statistical Analysis: Data is shown in figure 3.3. For the purpose of statistical comparison the results of intracardiac samples were excluded and where cordocentesis was repeated in the sample patient only the first sampling is included (leaving a total of 10 in the

karyotype group and 12 in the rhesus group, with 9 pre- and post-transfusion samples). Comparison between groups is by Wilcoxon rank test or Students t-test as appropriate and relationships between variables by correlation coefficient.

Normal and Hypertensive Pregnancy:

A cross-sectional study was performed in women attending the ante-natal clinic at the Queen Mothers Hospital, Glasgow. Normal subjects of comparable age, with normal menstrual histories, were recruited from the staff of the Western Infirmary. The protocol was approved by the Queen Mothers Hospital Ethical Committee, and all subjects gave informed consent. The subjects were divided into five groups, each of 15 subjects: **1.** Non-pregnant menstruating women, not on hormonal contraception. Normal pregnancies, gestation **2.** 8-12 weeks; **3.** 16-28 weeks; and **4.** 36-40 weeks. **5.** Patients (primigravida, 36-40 weeks gestation) with pre-eclampsia, defined as a rise in blood pressure to >140/90, with a rise in diastolic of >15 mmHg during pregnancy. None of these patients had significant proteinuria at the time of sampling (i.e. $\geq$ 0.3g/24 hours) although this subsequently developed in two. Blood was collected with the subjects in a semi-recumbent position after 15 minutes rest. Renin was determined by the method of Millar et al.,(1981) and samples for ANP collected and assayed as described above (Richards et al.,(1987), Appendix 1).

Statistical analysis is by non-paired T-test between non-pregnant and pregnant groups and between third trimester groups.

Malignant Phase Hypertension:

The case records of 15 patients presenting with untreated

malignant hypertension (defined by grade IV fundal changes) between 1987 and 1989 were examined. All patients admitted during this period had blood pressure and blood samples taken according to a standard protocol. Blood samples were taken through an indwelling catheter after 30 minutes recumbency, and blood pressure was the mean of three recordings taken, using a standard mercury manometer, by the MRC Blood Pressure Unit nursing staff. Blood samples were analysed for urea and electrolytes, components of the renin-angiotensin system and ANP. The sampling techniques and assay methodology were as previously described (Appendix 1; Richards et al.,(1987)). Data are presented as mean±SEM or mean (range) where distribution was not normal. The relationship between variables was determined by coefficient of correlation; where appropriate data were log-transformed (e.g. ANP, Figure 3.1) before analysis.

Results:

Normal subjects and patients:

The results are shown in Figures 3.1 and 3.2. In the normal group plasma ANP levels were normally distributed with levels similar to those reported by other groups using pre-extracted radio-immunoassays for ANP (mean±SEM 26.7±6.3 pg/ml, [8.1±2.0 pmol/ml]). In this group there was no relationship between ANP and blood pressure; there was a negative relationship with active renin concentration (23.8±1.9 [mean±SEM] μU/ml) which failed to achieve significance (P=0.2) and a strong correlation with age (r=0.47, P<0.001, Figure 3.2). There were no differences between ANP levels between men and women. In the various pathological groups: ANP levels congregated towards the upper end of the "normal" group in patients with untreated essential

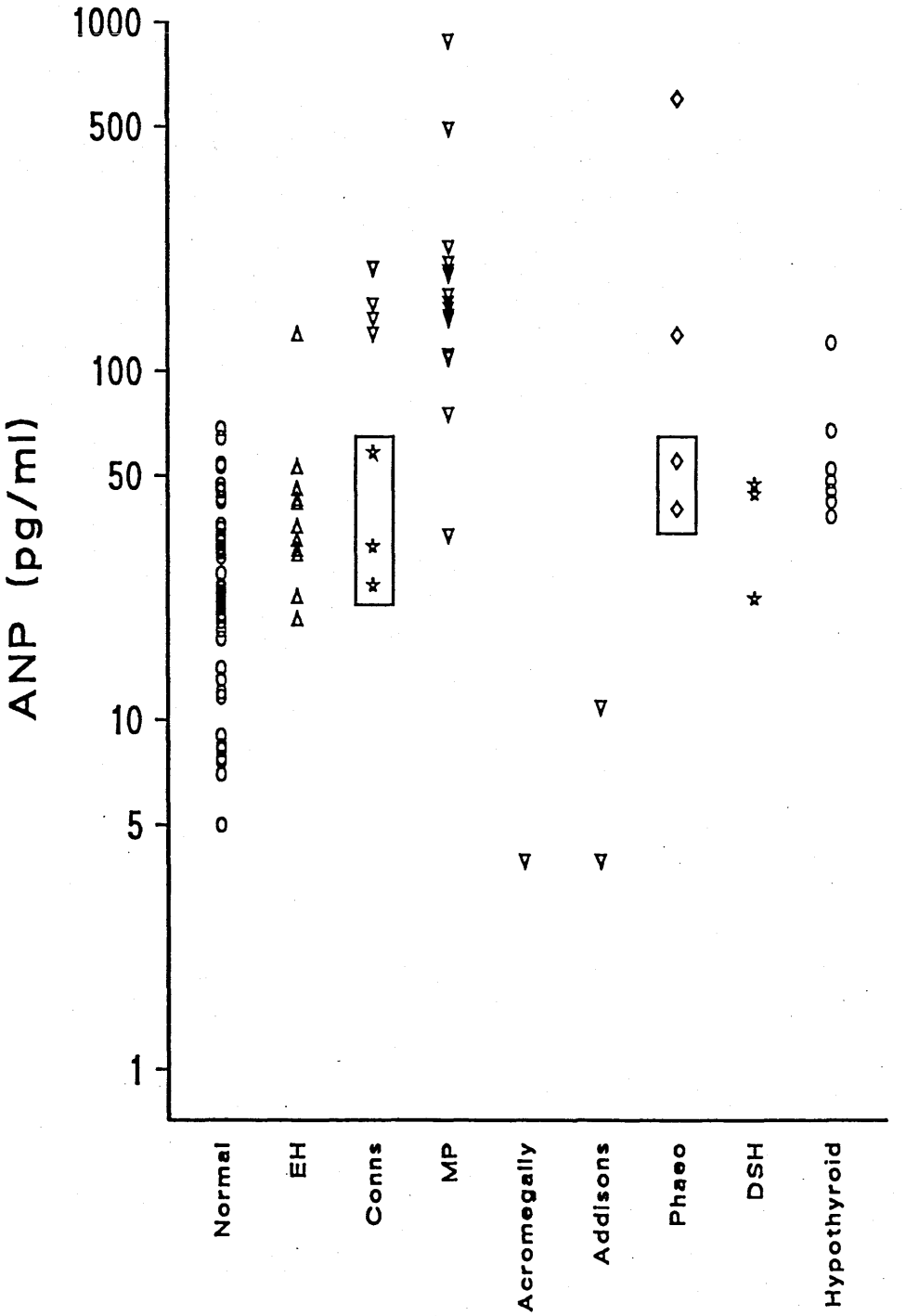
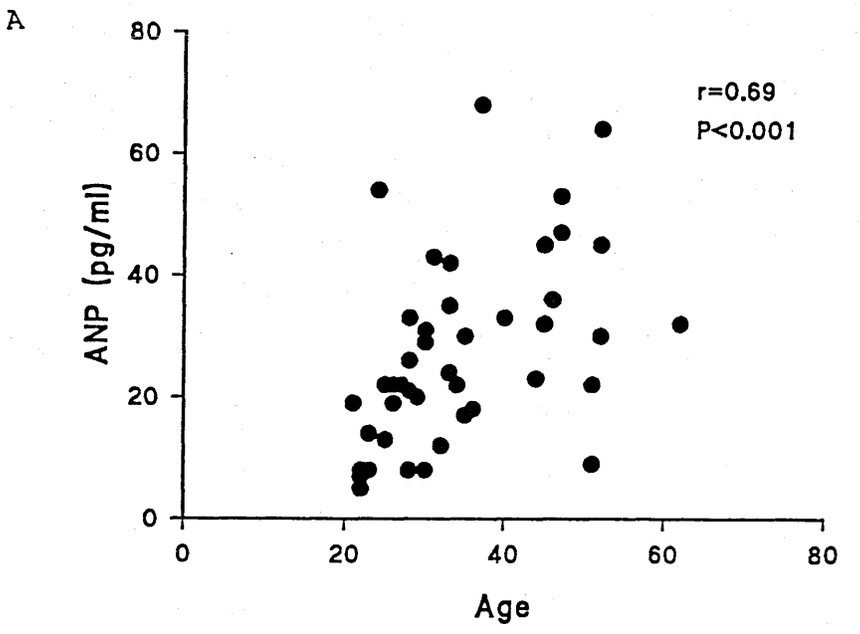


FIGURE 3.1:

NORMAL AND PATHOLOGICAL HUMAN ANP LEVELS.

Plasma levels of Atrial Natriuretic Peptide in normal subjects and patients with a variety of clinical conditions - see text for details. EH=Essential Hypertension, MP=Malignant phase, Phaeo=Phaeochromocytoma, DSH=Dexamethasone Suppressible Hyperaldosteronism. Enclosed points represent treated subjects.



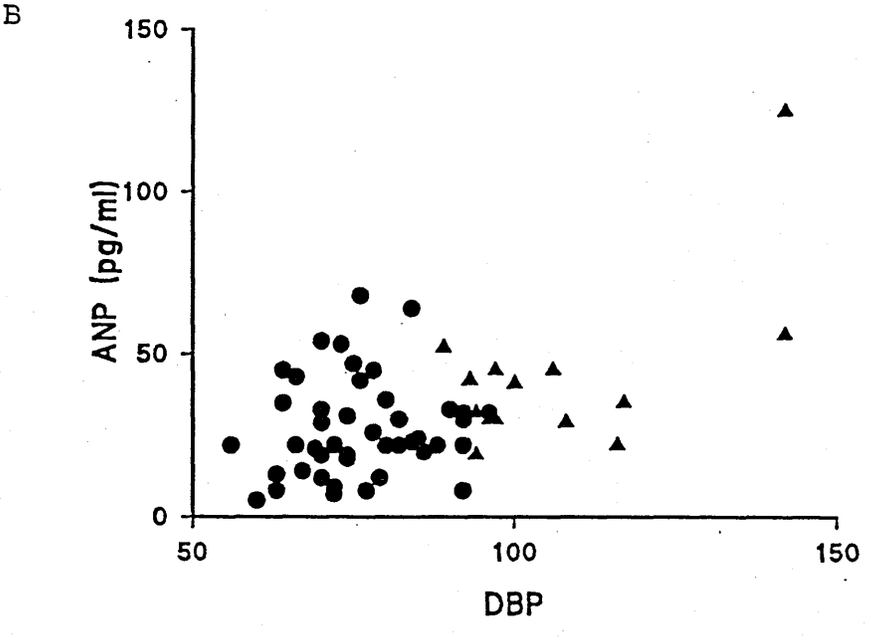


FIGURE 3.2:

PLASMA ANP, AGE AND BLOOD PRESSURE:

Relationship between plasma ANP, age (years, A) and diastolic blood pressure (DBP, mmHg, B). Data from the "normal" subjects in Figure 3.1 (●) and patients with Essential Hypertension (▲). r=correlation coefficient.

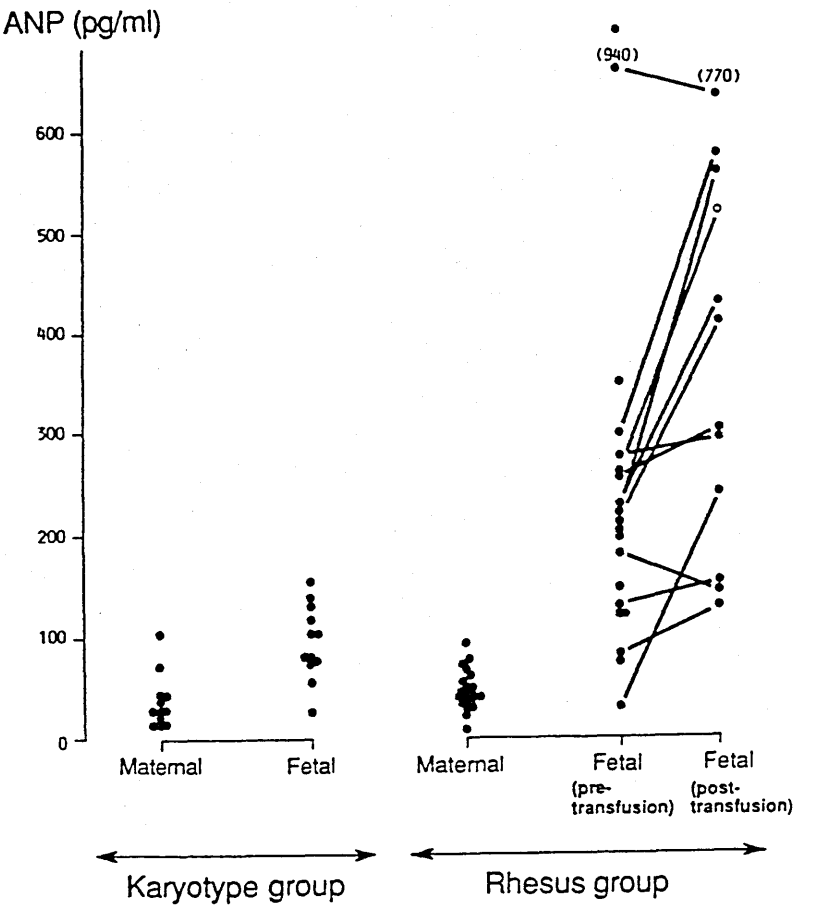
hypertension, but were not statistically higher than levels in patients matched for age and sex in the normal potential renal donor group. Plasma levels were increased in untreated Conn's syndrome (where intravascular volume and body sodium content are increased) but were normalised in patients with Conn's syndrome successfully treated with amiloride (Figure 3.1). Low ANP values were predictably found in two patients with acute adrenocortical insufficiency, where intravascular volume is depleted. The relationship between volume and sodium status in this condition is illustrated in Table 3.1 which shows the time course of the response to fluid and steroid replacement in a young man with acute adrenocortical insufficiency. ANP levels are low at the time of presentation, in contrast to the greatly elevated renin levels reflecting intravascular volume contraction; ANP rose, with appropriate reciprocal changes in renin levels during treatment.

All of the above findings are consistent with the view that ANP levels are determined principally by cardiac preload. However, some plasma ANP levels are not consistent with this view. In a single patient with untreated acromegally - where total body sodium and blood pressure (McLellan et al.,(1988)) are generally increased - one of the lowest recorded ANP levels was seen (4 pg/ml). Paradoxically, high values were seen in two other conditions - phaeochromocytoma and malignant phase hypertension - where hypertension is generally associated with reduced intravascular volume and sodium content (Genest et al.,(1983), Ball (1983)).

Fetal samples:

The full results are shown in Figures 3.3a and 3.3b. Mean plasma ANP (187.0±37.3 pg/ml) were significantly higher than maternal levels

A



B

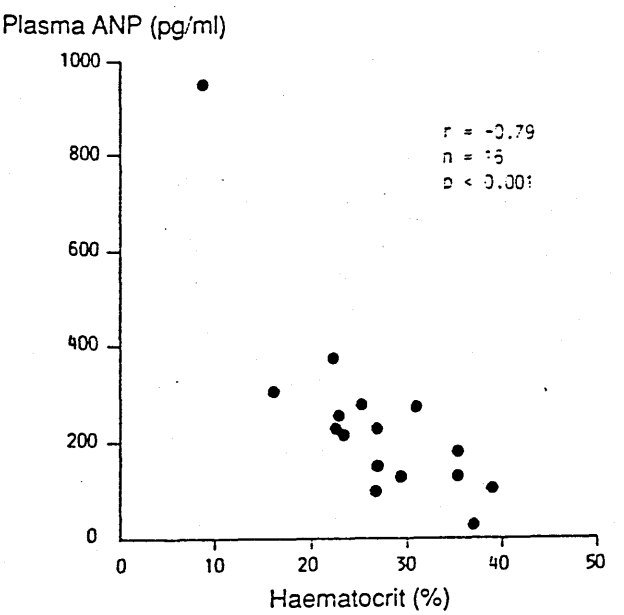


FIGURE 3.3:

HUMAN FETAL ANP.

(a) Plasma levels of Atrial Natriuretic Peptide (ANP, pg/ml) in human fetal samples and the response to intravascular transfusion.
(b) Relationship between ANP and haematocrit in Rhesus disease. See text for details (r=correlation coefficient).

(37.3±4.9 pg/ml); mean±SEM; $p<0.01$, n=22). There was no significant correlation between maternal and fetal ANP ($r=-0.25$, $p=0.25$). Plasma ANP was significantly higher in the fetuses with rhesus iso-immunisation compared with the karyotype group (262.3±61.7 [n=12] vs 98.0±11.5 [n=10], $p<0.05$). The pre-transfusion fetal ANP showed a strong inverse relationship with haematocrit ($r=-0.73$, $p<0.01$, n=22) for the whole group and ($r=-0.83$, $p<0.01$, n=12) for the rhesus group (Figure 3.3b). Following transfusion, ANP rose from 264.2±64.4 pg/ml ($p=0.02$, n=12, Wilcoxon test; $p=0.02$, n=9 - excluding duplicate transfusions). The rise with transfusion was evident despite the dilutional effects of transfusion of large volumes of blood containing negligible ANP.

There was no significant relationship between gestational age and ANP, or the rise in ANP and the volume of transfusion; nor were any trends evident in the four subjects who underwent repeated sampling. In the two intracardiac samples in the karyotype group excluded from statistical analysis, ANP was 311 and 388 pg/ml, higher than the values from the umbilical vein samples.

Normal and hypertensive pregnancy:

The results are shown in Figure 3.4. ANP and renin were significantly increased during pregnancy as compared with normal controls but showed no trend with gestation. In the pre-eclampsia group (5) there was a further significant ($P=0.04$) rise in ANP and a non-significant fall in renin compared with the normal third trimester group.

There was an inverse correlation between ANP and renin in the third trimester pregnancies (groups 4 and 5; $r=-0.4$, $p=0.027$, n=30).

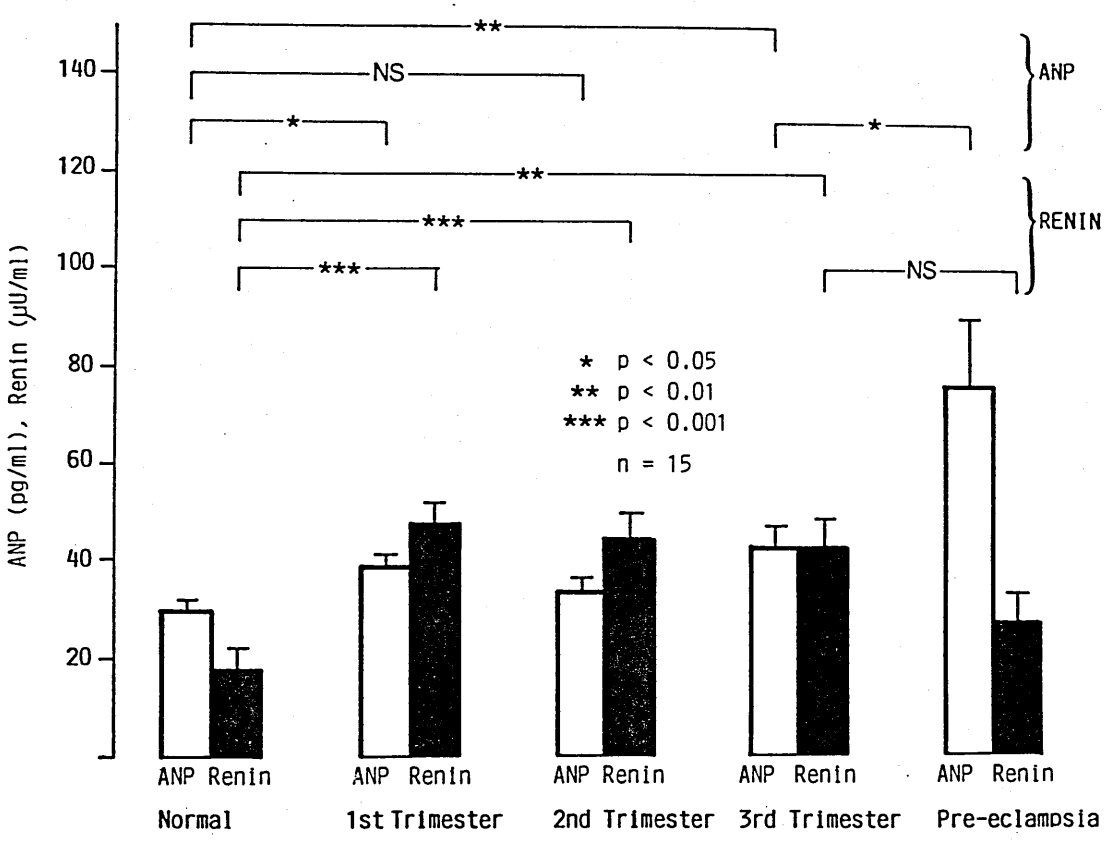


FIGURE 3.4:

ANP IN HUMAN PREGNANCY.

Plasma Atrial Natriuretic Peptide (ANP, pg/ml) and active renin concentration (Renin, μU/ml) in normal and hypertensive pregnancy (Pre-eclampsia). * indicate level of significance by T-test.

Although weak, this suggests that there is reciprocal relationship between ANP and renin, compatible with the suggestion that low renin levels may be a consequence of increased ANP. In addition in the hypertensive group (mean gestation at delivery 39 weeks) the ANP/renin ratio was inversely correlated with birthweight ($r=-0.67$, $p<0.01$, $n=15$). Since birthweight is a crude indicator of the severity of pre-eclampsia in this group with similar gestational age, it may be that the ANP/renin ratio is related to the severity of pre-eclampsia.

Malignant phase hypertension:

The results are shown in Table 3.2. In keeping with a diagnosis of malignant phase hypertension blood pressure was elevated and there was biochemical evidence of activation of the renin-angiotensin system. Renal function was moderately impaired and plasma levels of ANP elevated.

There was an inverse relationship between ANP and plasma Na ($r=-0.56$, $P<0.05$) and a positive relationship between ANP and active renin concentration ($r=0.85$, $P<0.01$).

In four patients where sampling was repeated within 48 hours of starting treatment, ANP levels fell in all cases by a mean of 66 (range 10-128 pg/ml) without alteration in plasma creatinine concentration.

Discussion:

Normal Subjects and Patients:

The plasma levels of ANP reported here are similar to those reported by most other groups (e.g. Lang et al.,(1985), Richards et

TIME:	0	6	12	24 (HOURS)							
				(DAYS)- 2		3	4	5	7	14	28
Na	119	123	125	127	129	132	130	138	136	138	139
K	7.3	7.2	5.3	5.2	4.9	4.9	4.5	5.4	4.3	4.8	4.7
U	16.2	15.5	12.2	9.1	6.5	5.4	4.1	4.3	4.7	5.6	7.9
Renin	1209	---	757	---	---	---	---	195	---	5	---
AII	---	---	65	---	---	---	---	38	---	---	---
Aldo	6	---	6	---	---	---	---	1	---	---	---
ANP	11	---	14	---	---	---	---	---	---	85	---
BP (Supine)	120	110	114	122	122	124	120	112	126	---	---
	72	70	64	68	92	64	68	74	68	---	---
BP (Standing)	90	---	128	110	142	116	128	112	116	---	---
	-	---	70	94	88	74	78	82	60	---	---
T4	85	----------							76	80	104
TSH	26.7	----------							10.7	8.6	6.3

TABLE 3.1:

ANP IN ADDISONIAN CRISIS.

Sequence of changes in plasma electrolytes, ANP, the renin-angiotensin system, blood pressure and thyroid function in a 43 year old man presenting with an Addisonian crisis. Treatment was commenced with intravenous fluids and hydrocortisone at time 0, and replaced with oral hydrocortisone and fludrocortisone at 48 hours. Normal ranges: Na (135-145 mmol/l), K (3.5-5.1 mmol/l), U (Urea, 4-11 mmol/l), Renin (5-50 μU/ml), AII (Ang II, 3-12 pg/ml), Aldo (<18 ng/dl), ANP (5-55 pg/ml), T4 (Thyroxine, 55-156 nmol/l), TSH (Thyroid Stimulating Hormone, <5.0 U/ml).

A typical pattern of electrolytes, blood presure, thyroid hormones and markers of the renin-angiotensin system are shown on admission; with appropriate responses to treatment.

Variable	Units(NR)	Number	Value	Correlation with ANP
SBP	mmHg	15	232±7	0.21
DBP	mmHg	15	138±5	0.18
Na	mmol/l	15	139.3±0.1	-0.56 *
K	mmol/l	15	3.6±0.1	-0.40
Ct	µmol/l	15	199.5±28.8 (80-525)	0.35
Renin	µU/ml(5-50)	13	298±116 (18-1420)	0.85 **
AngII	pg/ml(3-12)	15	93.7±23.4 (6-240)	0.45
Aldo	ng/dl(<18)	14	13.1±2.4 (1-38)	-0.15
ANP	pg/ml(5-35)	15	220.5±55.2. (34-896)	-

TABLE 3.2:

ANP IN MALIGNANT PHASE HYPERTENSION.

Data form 15 subjects with Malignant Phase Hypertension. Data are shown as mean±SEM if normally distributed and mean (range) if non-normal. Non-normal data were log-transformed before correlation.

al.,(1987), Espiner et al.,(1986)) in normal subjects. Previous studies have demonstrated the relationship between ANP and age (Haller et al.,(1987), Ohashi et al.,(1987), Montorsi et al.,(1987)) shown in Figure 3.2. However, the mechanism of this increase with age is not known and may theoretically be due to either increased secretion or impaired clearance of the peptide. The finding that ANP was not related to blood pressure in this small group confirms the findings of Montorsi et al.,(1987) who found no relationship in normal subjects but a strong positive correlation in subjects with essential hypertension; the relationship with age, however, was absent in the hypertensive group. In normal subjects plasma renin activity has usually been inversely correlated with ANP (Richards et al.,(1988d), Montorsi et al.,(1987)) as one would predict for a hormone with an opposing physiological role and in view of the known inhibition of renin release by ANP (Chapter 2).

There has been debate over reported plasma levels of ANP in essential hypertension. Although several groups have reported no change in essential hypertension the larger studies using careful age-sex matching have revealled small increases in ANP in hypertension (Montorsi et al.,(1987), Sagnella et al., (1988)). The present study is probably representative in that levels were slightly high but many remained within the "normal" range and the finndings failed to achieve statistical significance. The mechanisms responsible for the observed increases in plasma ANP in essential hypertension are unknown but seem likely to be related to increased cardiac afterload, mediated by left atrial distension (below).

The first physiological stimulus to release ANP was intravascular volume expansion (Lang et al.,(1985), Chapter 2) leading to the view

that the major regulatory influence on ANP release is intravascular volume (via atrial distension). The finding that levels are elevated in volume expanded states such as hyperaldosteronism or hypothyroidism and are reduced in volume deplete states such as adrenal insufficiency (and respond appropriately to treatment; Figure 3.1, Table 3.1) support this view. There is universal agreement that ANP is elevated in Conn's syndrome (Yamaji et al.,(1986), Espiner et al.,(1987)), and in the present series the levels were three to four times the upper limit of normal. In the milder condition - DSH - where hypertension, electrolyte and hormonal disturbances are often less marked than in Conn's syndrome, the changes in ANP were also less marked and within the "normal" range. Reports differ, however, on whether levels are normal (Ladenson et al.,(1987)) or even reduced in hypothyroidism (Zimmerman et al.,(1987)), and it is known that levels are also elevated in thyrotoxicosis (Woolf and Moult (1987)). This may reflect the fact the pathological changes in thyroid hormone status are dependent not only on intravascular volume but also heart rate (Bilder et al.,(1986), Schiebinger and Linden (1986)); tachycardia in thyrotoxicosis, bradycardia in hypothyroidism) which will tend to oppose the blood volume changes in thyroid disease. Furthermore, expression of the gene for ANP is dependent on thyroid hormone (Gardner et al.,(1987), Matsubara et al.,(1987)).

The levels in the other groups do not fit with volume dependant regulation. In both malignant phase hypertension and pre-eclampsia hypertension is associated with reduced intravascular volume; both are considered below. In acromegally, hypertension is associated with expanded intravascular volume (McKnight et al.,(1988)); paradoxically the levels of ANP are low and fail to rise following saline infusion

(McKnight et al.,(1988)). The level in a single patient in the present series confirms this finding, and although the reason for the low ANP is not known it may be mechanical reflecting the inability of the hypertrophied heart to distend in this condition. Finally, there were four measurements from patients with a phaeochromocytoma, one of whom presented in a hypertensive crisis. In this condition intravascular volume is reduced, and the stimulus to release must come from either increased heart rate or afterload.

Fetal Samples:

Messenger RNA for ANP and immunoreactive peptide are known to be present in the fetal heart of many species including man (Wu et al.,(1988), Mercadier et al.,(1989)). The present observations confirm that ANP is actively secreted by the human fetal heart, at least in the third trimester, and that the regulation of plasma levels is dependent on intravascular volume.

The evidence for physiological secretion of ANP by the fetus comes from three pieces of evidence. First, that the fetal hormone levels are higher than simultaneous maternal values. It is likely that this is the low molecular weight form of ANP (99-126 ANP), since the samples were pre-extracted and 28 AA ANP is the form present in cord blood (Ito et al.,(1988), Yamaji et al.,(1986)). Secondly, fetal plasma ANP levels rose in response to blood transfusion (i.e. intravascular volume expansion), confirming findings in the sheep (Ross et al.,(1987)) and earlier studies in the human fetus (Robillard & Weiner, (1987), Kingdom et al.,(1989)). This occurs despite the dilutional effects of transfusion (with blood containing no ANP), although this may explain the fall observed in two cases (Figure 3.3).

Finally, the observation that the highest levels were found in intracardiac samples suggests that the source of circulating ANP in the fetus - like the adult - is the heart.

Further evidence that the regulation of fetal ANP release is similar to the adult comes from the pathophysiological changes that are seen in rhesus iso-immunisation. Levels of ANP were higher in fetuses with this condition compared with karyotype "control" group. This confirms the findings of Weiner and Robillard (1988), and presumably reflects intravascular volume expansion due to anaemia. In keeping with this plasma ANP levels were, for the first time, noted to be related to the severity of rhesus disease, with a strong inverse correlation between fetal haematocrit and ANP. This contrasts with the findings of Robillard and Weiner (1988) but may reflect the small numbers in their study since the fetus with the lowest haematocrit had the highest ANP. Thus the long term regulation of ANP in the fetus - like the adult - is likely to be dependent on intravascular volume.

The precise role of ANP in the fetus is not known but there are several potential physiological and pathophysiological roles. Firstly, membrane receptors are present for ANP in the placenta (Sen (1986), McQueen et al.,(1990a,b)) and changes in receptor affinity and concentration have been noted in pre-eclampsia and IUGR respectively (Hatjis and Grogan (1989), McQueen et al.,(1990b)) suggesting some involvement in these pathophysiological processes. The localisation of receptors on vascular tissue suggests that ANP might act as a placental vasodilator and it can certainly attenuate the vasoconstrictor response to infused Ang II in the isolated perfused human placenta (McQueen et al.,(1990a)). The elevated levels of ANP in Rhesus disease may therefore have a role in improving placental

perfusion in this condition. Elevated levels of ANP may also increase vascular permeability (Chapter 2, Huxley et al.,(1987)) and contribute to oedema formation in rhesus disease. There is also some evidence for a potential renal effect in the fetus. When ANP is infused into the ovine fetus, albeit at pharmacological concentrations, there is an increase in urine volume and sodium excretion (Brace and Cheung (1987)). Since fetal urine output is the major source of amniotic fluid in the latter part of pregnancy, ANP may regulate amniotic fluid volume.

In summary, these results confirm the presence of an active ANP hormonal system in the human fetus, which appears to be regulated in the same way as the adult but the precise role of which will require further study.

Pregnancy:

Intravascular volume increases by about 1.5 litres in normal pregnancy, the largest increase occuring within the first trimester. In pre-eclampsia, however, there is a relative contraction of the plasma volume (for review, Lindheimer and Katz (1986), Brown (1988)). Pregnancy is therefore a reproducible model in which to study the effects of long term changes in volume status on plasma ANP. The findings in normal pregnancy, with a sustained rise in ANP from the first trimester onwards, suggest volume dependent regulation and are a consistent finding (Otsuki et al.,(1987), Jackson et al.,(1988), Bond et al., (1989)). In pre-eclampsia, however, despite the relative contraction of intravascular volume (Brown (1988)), plasma ANP is further increased - again a consistent finding. Unlike phaeochromocytoma (above) or malignant phase (below) PET is not

generally associated with significant tachycardia or renal impairment, and increased afterload seems the most likely stimulus to ANP release. Miyamoto et al., (1989a,b) have confirmed this observation and extended it to show that there is a marked diurnal rhythm of ANP in PET - virtually absent in normals (Richards et al.,(1987)) - but which follows the diurnal rhythm of blood pressure (BP) and cGMP in PET. The superimposable rhythms of ANP, cGMP and blood pressure suggest that ANP is dependent upon BP rather than influencing it - where BP would be expected to be low in the presence of elevated ANP. Thus, PET appears to be another condition where ANP levels are regulated by cardiac afterload.

The other side to this study is the measurement of renin, which also rises in normal pregnancy but is then - paradoxically - suppressed in PET, despite volume contraction (Brown (1988), Lindheimer and Katz (1987)). These observations are well established, although they remain unexplained. The finding that ANP is elevated in PET offers one potential explanation for the reduced renin levels, since ANP is known to suppress renin release (Chapter 2). The inverse relationship between renin and ANP observed in the present series of measurements would support this suggestion. Furthermore, the severity of PET is difficult to quantify clinically but there was a relationship between the ratio of ANP/renin (the ratio multiplying the individual changes in these variables) and birthweight (a crude indicator of severity - since the gestational ages were similar). Thus, identification of these abnormal humoral patterns may be useful in identifying patients with PET, and a longitudinal study is required to see if such changes precede the clinical presentation.

Malignant Hypertension:

Malignant phase hypertension is not a discrete diagnosis but rather a condition which may develop in patients with hypertension (Genest et al.,(1983)). In contrast to hypertension in general, the presence of an underlying cause for hypertension (secondary hypertension) is high - perhaps as high as 50%. The patients also have a mixture of end-organ damage, and particularly a variable degree of renal impairment. I have not given the underlying diagnosis, often because it was not clearly established, but none of the patients required dialysis. Renal impairment might be predicted to raise plasma ANP - as the kidneys are involved in clearance (Chapters 2, 5 - but the plasma levels were still higher than those reported in renal failure (Tonolo et al.,(1988,1989a)) where intravascular volume would also be increased. In support of this plasma levels fell after treatment in four subjects despite the absence of significant improvement in renal function. Intravscular volume is reduced in malignant phase and although there is likely to be an increased heart rate (sympathetic activation) it seems likely that the major stimulus to hormone release is increased afterload.

ANP levels were significantly correlated with plasma active renin concentration in contrast to the findings in normal subjects (e.g. Montorsi et al.,(1987)) but similar to the pattern seen in heart failure (Richards et al.,(1986b), Espiner et al.,(1986)) where overactivation of the renin-angiotensin system is similarly associated with loss of the normal inter-relationships between renin and ANP. In addition there was a negative correlation between ANP and plasma sodium. Whether this merely reflects the severity of malignant phase or whether ANP may contribute to the reduced body sodium content which

is a feature of this condition is not clear. Elevated levels of ANP may also contribute, with reduced plasma potassium, to the absence of a significant increase in aldosterone despite high circulating Ang II levels, since ANP can directly inhibit aldosterone synthesis and secretion (Chapter 2).

Conclusions:

The studies in this chapter were largely performed through accidents of circumstance. However, they each describe measurements of plasma ANP in man and many of the observations are unique.

Taken together they show that a functional ANP hormonal system is present even in the developing human fetus. In the adult they reveal that plasma hormone levels are dependent on intravascular volume and blood pressure (pre-load and after-load) and that the relative contributions from these stimuli may change (for example in pregnancy/ PET). These observations confirm experimental observations that short term changes in intravascular volume (e.g. saline infusion; Hodsman et al.,(1986), Chapter 12, Figure 12.1) or blood pressure (infusion of pressor agents; Uelinger et al.,(1987), Chapter 8, Figures 8.3 and 8.4) increase plasma ANP. The mechanism underlying the differential release is not known but it appears from animal studies that afterload dependent release is primarily from the left atrium (Arai et al.,(1987)). This effect is likely to be mediated by atrial distension and, in this respect, Syndhorst and Gutkowska (1988) have shown that the left atrium is more sensitive to stretch than the right.

Overall, the findings that plasma ANP follow changes in blood volume, sodium balance and blood pressure further supports the theory

that ANP is likely to play a physiological role in the regulation of these parameters (Chapter 2).

SECTION 3.

RENAL ACTIONS OF ATRIAL NATRIURETIC PEPTIDE: INTERACTION WITH THE RENIN ANGIOTENSIN SYSTEM.

Chapter 4.

Renal actions of Atrial Natriuretic Peptide: Effects of Angiotensin II and ACE inhibition.

Introduction:

Although the kidney appears to be the target of the major actions of ANP including natriuresis, diuresis and suppression of renin release, the underlying mechanisms are incompletely understood (Chapter 2, Ziedel (1989); Raine et al.,(1989)). There is still debate over the relative importance of direct glomerulo-tubular effects (i.e. mediated by ANP receptors in the kidney) and indirect effects (e.g. those mediated indirectly by suppression of other hormonal systems such as the renin-angiotensin system and vasopressin), and secondly, the importance of factors which are known to modulate the renal actions of ANP. For example, it is known that increased renal sympathetic nerve activity (Koepke and diBona (1987), Koepke et al.,(1987)), reduced renal perfusion pressure (Seymour et al.,(1987), Davies and Briggs (1987a), Firth et al.,(1987)) and increased Angiotensin II (Ang II; McMurray and Struthers (1988b), Showalter et al.,(1988)) levels all reduce the effect of ANP, but the physiological

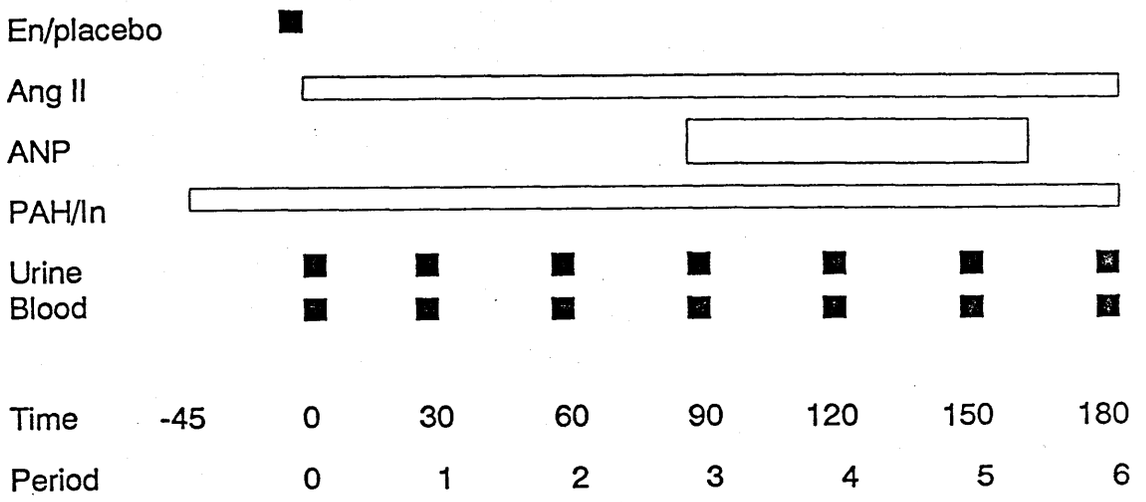
relevance of these observations is not clear.

The study in the present chapter was designed to examine the renal effects of low dose ANP infusion in the dog and the role of the interaction between the renin-angiotensin system and ANP. The interaction was examined by infusing ANP together with either Ang II or an ACE inhibitor (to raise and lower plasma Ang II). The ACE inhibitor was given both acutely and chronically, since ACE inhibition has been reported to augment, attenuate or have no effect on the response to ANP (Wang and Gilmore (1985), Bie et al.,(1990), Hirata et al.,(1987), Richards et al.,(1989), diNicolantonio and Morgan (1987), Seymour and Mazack (1988), Gaillard et al.,(1988,1989), Hansell and Ulfendahl (1987), Salazar et al.,(1987))), despite the consensus that Ang II inhibits the actions of ANP. In addition an attempt was made to assess the contribution which reduced Ang II levels (via suppression of renin release) make to the renal effects of ANP, by "clamping" plasma Ang II levels by co-infusion of Ang II and an ACE inhibitor (Mizelle et al.,(1989)), thereby removing any ANP dependent changes in endogenous Ang II (via suppression of renin, Chapter 2).

Methods:

These experiments were performed in the Wellcome Surgical Institute at the University of Glasgow Veterinary School.

Techniques: Eight beagle dogs with externalised carotid artery loops were studied on seven separate occasions at least four weeks apart. On each occasion they received a diet containing 30mmoles of sodium and 30 mmoles of potassium per day for 3 days before each experimental day and were fasted on the morning of the study. Cannulae were inserted into the bladder (6 FG dog catheter, Portex), externalised carotid



Group 1. Placebo.

Group 2. ANP infusion alone.

Group 3. ANP infusion plus Enalaprilat (En).

Group 4. ANP infusion on the 4th day of Enalapril treatment.

Group 5. (Supplementary group), Enalaprilat alone.

Group 6. ANP infusion plus Enalaprilat plus Ang II infusion (1 pmol/kg/min).

Group 7. ANP infusion plus Enalaprilat plus Ang II infusion (5 pmol/kg/min).

FIGURE 4.1:

OUTLINE OF PROTOCOL: INTERACTION OF ANP AND ANG II.

A. Study protocol for this series of experiments, indicating the timing of infusions, blood sampling and urine collections. Time in minutes.
B. Outline of the experimental groups.

artery loop (20g teflon, "Intraflon 2", Vygon, Sweden) for arterial pressure measurements and blood sampling, and in a foreleg vein (30 cm, 3 FG, Portex, UK) for infusion. The arterial line was heparinised continuously by 5% Dextrose containing 10 i.u./ml heparin (Heparin Sodium, Evans, UK) per ml at a flow rate of 3 ml/hour, and connected to a Strathan P23 ID pressure transducer (Gould Electronics, UK). This was connected to a Gould Model 8188-812 Amplifier and data digitalised using the Gould DASA 4600 analog-digital data aquisition system on an IBM PC AT computer. Blood pressure was measured intermittently and averaged over the final 150 seconds of each collection period (see below). The dogs stood throughout the experimental period supported in a hip sling.

Protocol: After cannulation 0.9% NaCl was infused at 0.83ml/min via the peripheral vein to replace blood and urine losses. Para amino hippuric acid (PAH, MSD, Rahway, NJ) and Inulin (Gesselbad, Germany) were administered as bolus injections (0.1mg/0.5ml saline PAH, 6.5 ml 10% Inulin in 5% Dextrose) and were therafter added to the saline infusion to give a final infusion rate of 8.3mg/min Inulin, 6mg/min PAH. After a 30 minute equilibration period the bladder was emptied and seven 30 minute urine collections made, the urine volume was measured volumetrically and urine stored for determination of PAH, Inulin, Na and K. At the end of each collection period samples were taken (14ml blood) for measurement of ANP, renin, Ang II, aldosterone, Na, K, PAH and inulin. Following the first collection period the dogs were given either Enalaprilat (3mg/kg in 10 ml 0.9% NaCl) or placebo (0.9% NaCl, 10ml). Thereafter the 0.9% NaCl infusion was either continued unaltered or Ang II added to given a final infusion rate of 1 or 5 pmol/kg/min. Finally during the 4th-6th collection periods ANP

was infused by separate pump at 5pmol/kg/min in 0.9% NaCl (0.5ml/min). For this ANP (αhANP, Bachem, UK) was prepared as previously descibed (Richards et al.,(1988a)) by dissolving the peptide in Haemaccel (Polygeline 3.5%, Hoechst, UK) containing glacial acetic acid and bovine serum albumin. Aliquots (2ml) were stored at minus 20°C until use, being diluted in 50ml 0.9% NaCl for infusion.

The protocol was subdivided into three major steps to examine firstly the effects of ANP alone (compared with placebo); then the effects of ACE inhibition or Ang II on the response to ANP were compared with the response to ANP alone. Finally, an additional step was included to look at the effects of acute ACE inhibition alone.

In total, therefore, there were seven separate study limbs for each animal (see Figure 4.1): **(1)** Placebo, **(2)** ANP alone, **(3)** ANP + acute Enalapril (3mg/kg), **(4)** ANP + chronic Enalapril (5mg b.d. for three days), **(5)** ANP + Enalaprilat + Ang II ("pressor", 5pmol/kg/min), **(6)** ANP + Enalaprilat + Ang II ("non-pressor", 1pmol/kg/min), and finally, **(7)** Enalaprilat alone.

Samples and Assays: Samples for ANP were collected into tubes containing EDTA and aprotonin (Trasylol, 100 k.i.u./ml, Bayer, UK) on ice. The samples were rapidly separated at 4°C and stored at -20°C for assay. ANP was determined on pre-extracted samples by RIA as previously described for hANP (Richards et al.,(1987a), Appendix 1). Renin (Millar et al.,(1981)), Ang II (Morton and Webb (1983)) and Aldosterone (Fraser et al.,(1973)) were measured by standard methods; Na and K were measured by ion electrode (Beckman model 2A, Beckman Instruments, Ireland). ERPF and GFR were determined by the urinary clearances of PAH and inulin using the standard clearance equation.

Statistics: Statistical Analysis was performed using the SPSS/PC+

package (SPSS Inc. Il, USA). Overall analysis was by MANOVA with repeated measures and where significant post-hoc analysis at individual time points was by paired T-test. Three effects were studied in a stepwise manner: (a) the effect of ANP alone, compared with placebo; (b) the effects of ACE inhibitor + ANP, compared with ANP alone (the Enalaprilat alone limb was included after the initial design and was included only to detail the pattern of effects of ACE inhibition and was not included in the statistical analysis); (c) the effects of Ang II + ANP, compared with ANP alone. Comparisons between groups for single variables (e.g Area Under the Curve, AUC) was by ANOVA (ONEWAY, SPSS) with post hoc analysis using Student's T-test with the Bonferoni correction (Wallenstein et al.,(1980), Godfrey (1981)). Where necessary (eg active renin concentration) large variations in baseline values were corrected for by analysis of transformed data. Full MANOVA analysis is shown in Appendix 2.

Results:

Effects of ANP alone:

Plasma ANP: ANP infusion caused an increase in circulating levels of ANP from around 40 pg/ml to around 200 pg/ml (Figure 4.3, Table 4.1).

Urinary effects: The renal effects of ANP are summarised in Figure 4.2 (and Figure 4.9). There was a gradual increase in urinary sodium excretion over the 90 minutes of the infusion from a basal level of 16.8±3.7 μmol/min (period 2, P2) to a peak value of 52.8±10.1 μmol/min; sodium excretion remained unchanged in the placebo limb. Urine volume showed a similar increase from 344±36 μl/min (P2) to a peak level of 543±68 μl/min. In contrast urine volume fell from 346±44

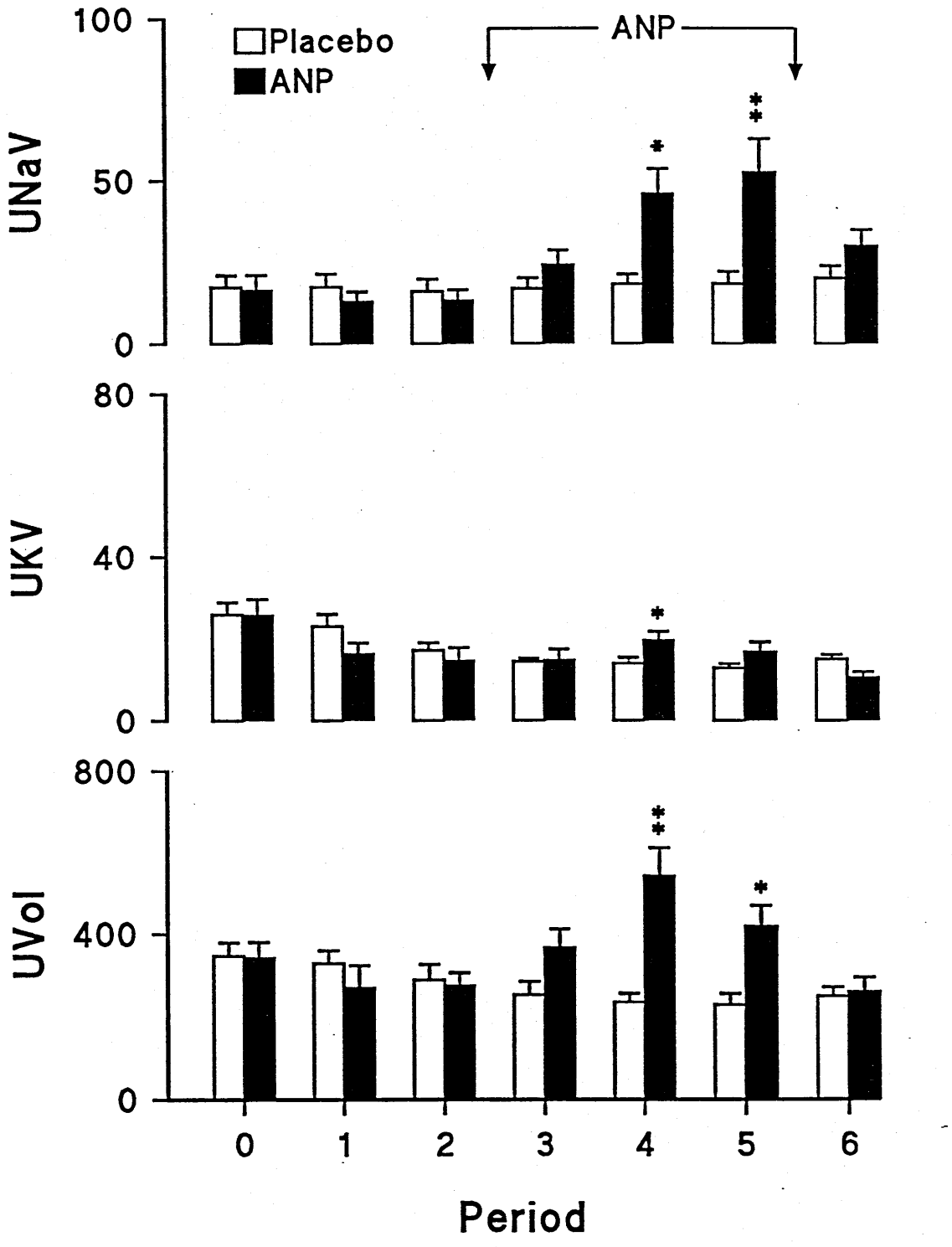


FIGURE 4.2:

RENAL EFFECTS OF LOW DOSE ANP IN DOGS.

Effects of infusion of ANP (5pmol/kg/min) on urinary sodium (UNaV, μmol/min) and potassium (UKV, μmol/min) excretion, and urine volume (UVol, μl/min). Each period represents a 30 minute collection period; ANP was infused during periods 3-5. Asterixes indicate significance levels (* $P<0.05$, ** $P<0.01$ by paired T-test where overall differences were established by MANOVA).

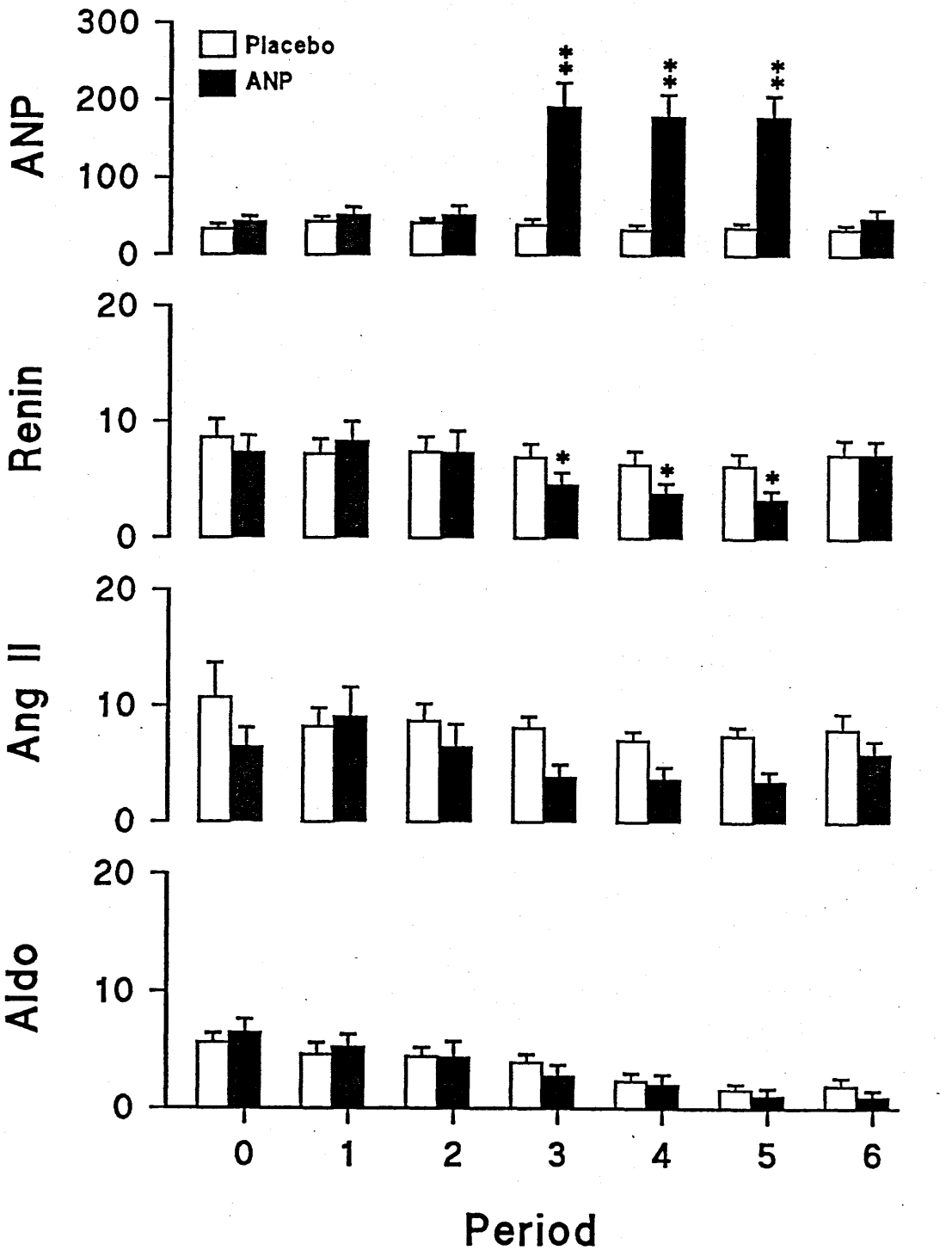


FIGURE 4.3:

HORMONAL RESPONSE TO ANP INFUSION IN DOGS.

Changes in plasma hormone levels with infusion of 5pmol/kg/min ANP or placebo during periods 3-5. Each period represents a 30 minute time interval. ANP (pg/ml), Renin (active renin concentration, μU/ml), Ang II (pg/ml), Aldo (ng/dl). Asterixes indicate significance level (*P<0.05, **P<0.01, by paired T-test where overall differences between groups present by MANOVA - see Appendix 2).

to a nadir of 228±26 µl/min in the placebo limb. Potassium excretion showed a downward trend in both groups but rose again during ANP infusion from 14.7±3.0 µmol/min (period 2) to 19.7±1.9 µmol/min, while continuing to fall on placebo.

Clearance Data: Changes in GFR and ERPF are shown in Figure 4.5 and in Table 4.3. The ANP-induced natriuresis was associated with a rise in GFR but this was highly variable and, as a consequence, non-significant. ERPF showed a downward trend in both the active and placebo groups, and filtration fraction (FF) an upward trend, but again with no significant differences. However, fractional sodium excretion, which gives some indication of tubular function, was significantly increased by ANP infusion.

Haemodynamic Effects: Blood pressure and pulse were stable during placebo infusion and showed a small downward trend during ANP (Figure 4.4, Table 4.2). Neither effect was significant and there was no observed change in haematocrit.

Hormonal Effects: The hormonal effects of ANP infusion and placebo are shown in Figures 4.3, 4.8 and in Table 4.1. Basal plasma ANP levels were around 40 pg/ml (14 pmol/l) and infusion caused an increase in plasma levels to between 140 and 220 pg/ml. In these normal dogs plasma active renin concentration (Table 4.2) was approximately 8 µU/ml and fell during ANP infusion by about 50% reaching a nadir of 3.4±0.7 µU/ml at the end of the infusion. Ang II showed a similar reduction during ANP infusion but, due largely to baseline variability, this failed to achieve statistical significance. No effect of ANP on aldosterone was evident. However, plasma aldosterone levels fell during both placebo and ANP treatment presumably reflecting the superimposed diurnal rhythm (since the studies were

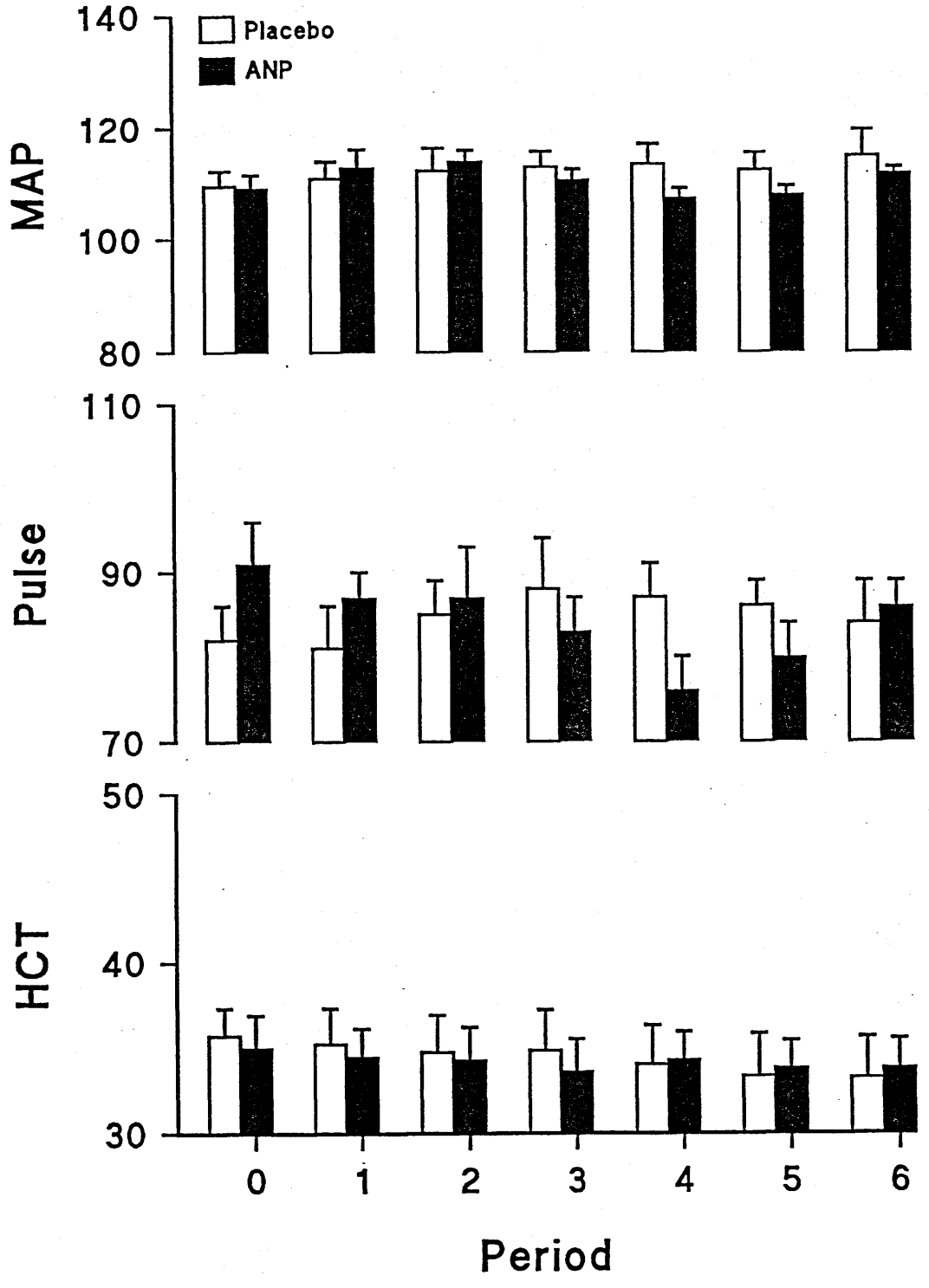


FIGURE 4.4:

BLOOD PRESSURE, PULSE AND HAEMATOCRIT CHANGES DURING LOW DOSE ANP INFUSION IN DOGS.

Effects of 5pmol/kg/min ANP or placebo on MAP (mean arterial pressure, mmHg), pulse rate (/min) and haematocrit (HCT, %). ANP was infused during periods 3-5, each period representing 30 minutes. There were no statistically significant effects.

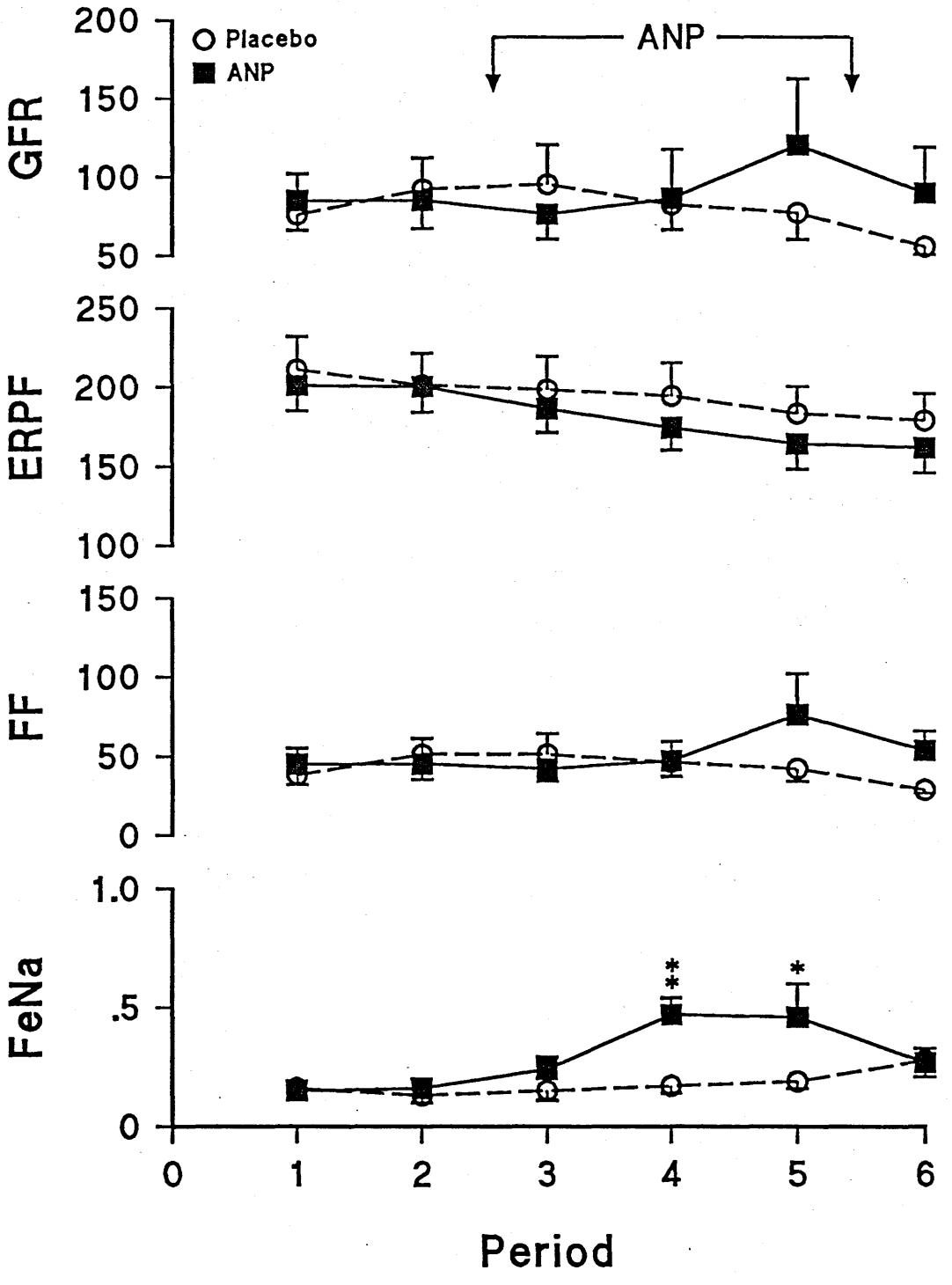


FIGURE 4.5:

RENAL FUNCTION INDICES DURING ANP INFUSION IN DOGS.

Renal function data during infusion of ANP or placebo. Data are given as mean±SEM, the time scale represents 30 minute collection periods, and ANP was infused during periods 3-5. GFR (glomerular filtration rate, ml/min), ERPF (estimated renal plasma flow, ml/min), FF (filtration fraction, %) and FeNa (fractional excretion of sodium, %). Asterixes indicate significance by paired t-test where overall differences established by MANOVA; * P<0.05, ** P<0.01).

carried out in the morning).

ACE Inhibition:

Plasma ANP: Basal plasma levels of ANP were not altered by pre-treatment with ACE inhibitor, either acutely or chronically, and the levels achieved during infusion of ANP were similar to those achieved by infusion of the peptide alone - in the range of 150-200 pg/ml (Table 4.1).

Renal Effects: Acute administration of Enalaprilat (3 mg/kg, Figures 4.6, 4.9) caused an increase in sodium excretion from 14.7±2.9 (basal, period 0) μmol/min to 35.9±10.1 μmol/min (P2); infusion of ANP caused a further increase to a peak of 118.3±25.4 μmol/min, more than double the increase produced by ANP alone. Urine volume showed a similar pattern increasing from a basal value of 364±33 to 675±141 μl/min after En (P2), with a further rise to 1104±158 μl/min during ANP infusion. Potassium excretion showed a downward trend across the protocol with no effect of ANP infusion. After pre-treatment with Enalapril (5mg b.d.) for three days basal levels of urine volume, potassium and sodium excretion were marginally higher than the placebo limb. ANP infusion caused a similar natriuresis to that of ANP alone, reaching a peak value 67.8±15.3; the diuresis was larger than that due to ANP alone (peak urine flow 888±267 μl/min). Although urine flow and, to a lesser degree sodium excretion, were greater than ANP alone at all times this failed to achieve statistical significance (Figures 4.5 & 4.9).

In the additional limb where Enalaprilat was given alone there was a sustained increase in sodium excretion to around 50 μmol/min, lasting for 3 hours after treatment. This observation is important

since it confirms the original assumption that Enalaprilat pre-treatment has a stable effect on sodium excretion. Furthermore it can be seen (Figures 4.6, 4.9) that the total increase in sodium excretion during combined treatment with ANP and Enalaprilat at each time point (Periods 3-5) is greater than the sum of the individual contributions of ANP and Enalaprilat; this finding is more clearly shown in the summary diagram (Figure 4.9).

Clearance data: Renal plasma flow (ERPF) was marginally higher during ACE inhibition, but there was a large degree of variability and no statistically significant effects. Glomerular filtration rate (GFR), tended to be lower but was similarly variable and did not achieve statistical significance. Fractional excretion of sodium was, however, greatly increased during pre-treatment with ACE inhibitor and mean levels were twice as high during co-administration of ANP and Enalaprilat (or Enalapril) as they were during infusion of ANP alone (Table 4.3). However, once more because of the large SEM these effects failed to achieve significance by repeated measures analysis. The FeNa during treatment with Enalaprilat alone increased by approximatley 50% and remained at this level for three hours, once again re-inforcing the original assumption that acute ACE inhibition causes a sustained and constant effect.

Haemodynamic Effects: Enalaprilat pre-treatment caused a small reduction in mean arterial pressure (Table 4.2, 5.5 mmHg), with a further marginal reduction occuring with the addition of ANP (2 mmHg). After 3 days of treatment with Enalapril mean arterial pressure tended to be slightly lower, but only by a few mmHg, and in the additional limb Enalaprilat alone caused a sustained fall in blood pressure of about 5 mmHg. None of these effects achieved statistical significance,

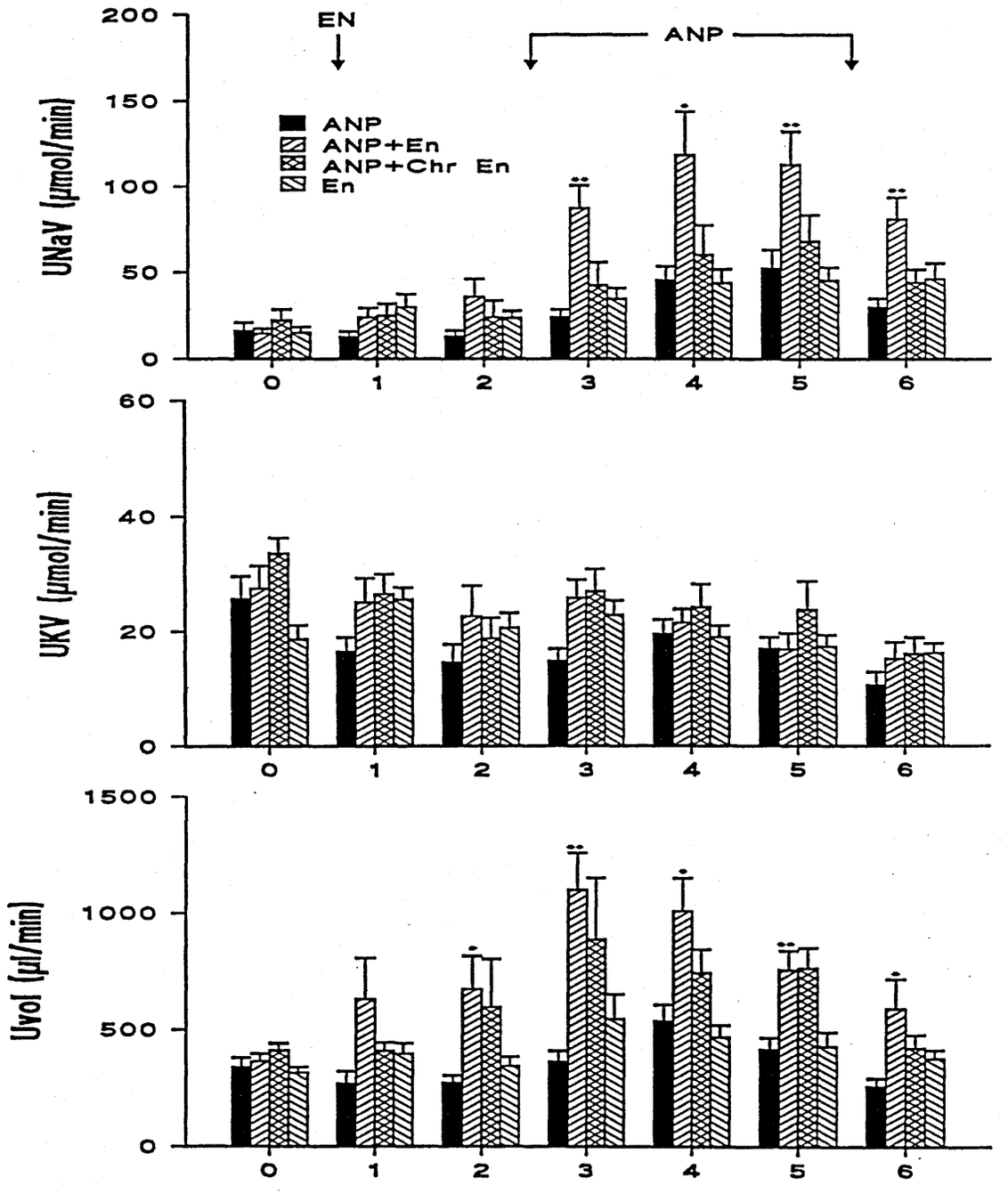


FIGURE 4.6:

INFLUENCE OF ACE INHIBITION ON RENAL EFFECTS OF ANP.

Effects of infusion of ANP 5pmol/kg/min on sodium and potassium excretion (UNaV, UKV, µmoles/min) and urine volume (UVol, µl/min) either given alone, in the presence of Enalaprilat (3mg/kg), or on the fourth day of treatment with Enalapril 5mg b.d. The effects of Enalaprilat alone are also shown but were not subject to statistical analysis (see text). Treatments were compared with ANP alone by MANOVA, with post hoc analysis by paired T-test *P<0.05, ** P<0.01. X axis shows 30 minute time periods, ANP was given during periods 3-5.

suggesting that blood pressure is relatively independent of renin in these salt replete animals.

Hormonal Effects: Renin concentration responded appropriately to pretreatment with ACE inhibitors. The basal level was three times higher at 25.7±4.7 after chronic Enalapril and rose sharply following intravenous Enalaprilat to a similar level (P2) - 24.1±7.1 μU/ml. Levels fell during ANP infusion to about one third pre-infusion values, and rebounded quickly to pre-infusion levels during the recovery period (Table 4.1, Figures 4.8, 4.9). In the additional limb Enalaprilat alone caused a sustained increase in renin concentration.

Angiotensin II levels are not shown. In a series of preliminary experiments in four dogs (data not shown) Enalaprilat caused complete suppression of circulating Ang II for three hours to levels below the limit of detection of the radio-immunoassay (0.5-1.0 pg/ml). It was not possible to repeat these measurements here because the volume of blood taken did not allow for measurement of Ang I, which is greatly elevated by ACE inhibition and which interferes with Ang II estimation. However, one would predict that Ang II would be suppressed below the limits of detection for the full 3 hour period. Aldosterone levels fell over the three hour period during which samples were taken. These changes were similar to those seen in the placebo limb and were not significantly different from levels seen during ANP infusion. The pattern of aldosterone levels again presumably reflects diurnal variation in hormone levels and the absence of a significant reduction during ACE inhibition suggests that aldosterone levels are only partially dependent on Ang II in these animals.

Effects of Angiotensin II:

Plasma ANP: Plasma ANP levels during peptide infusion were again similar in this limb of the protocol (Table 4.1).

Renal Effects: In this part of the study (Figures 4.7, 4.9) Enalaprilat was followed by infusion of two doses of Ang II: a "non-pressor" dose designed to restore plasma levels close to normal (1pmol/kg/min) and a "pressor" dose (5pmol/kg/min). Basal sodium excretion was lower in the limb receiving 1 pmol/kg/min and showed a small increase from 9.0±1.9 (period 2) to 16.3±3.4 μmol/l during ANP infusion. Overall sodium excretion, even when corrected for baseline variability, was significantly lower than than ANP alone ($P<0.001$, MANOVA). 5 pmol/kg/min Ang II caused a marked antinatriuresis, UNaV falling from 18.7±4.5 (P0) to 10.0±2.3 (P2) μmol/min and remaining between 8 and 10 μmol/min despite subsequent infusion of ANP, potassium excretion showed a similar trend and although there was no evidence of antidiuresis, there was also no evidence of a diuretic response to ANP.

Clearance data: ERPF was significantly reduced during high dose Ang II infusion. GFR values were surprisingly (for "low" dose Ang II) also reduced (Table 4.3) but this failed to achieve statistical significance. However, there was no suggestion of an increase in GFR during infusion of ANP in either of the Ang II "clamp" groups. Basal levels of FeNa were not significantly lower in the Ang II limbs than in the placebo limb but values were much lower during ANP infusion, reaching statistical significance (by repeated measures analysis) with high dose Ang II. Furthermore, infusion of ANP was associated with a small rise in FeNa in the low dose Ang II group, albeit non-significant, but no change was evident in the high dose group.

Haemodynamic Effects: Mean arterial pressure was almost identical in

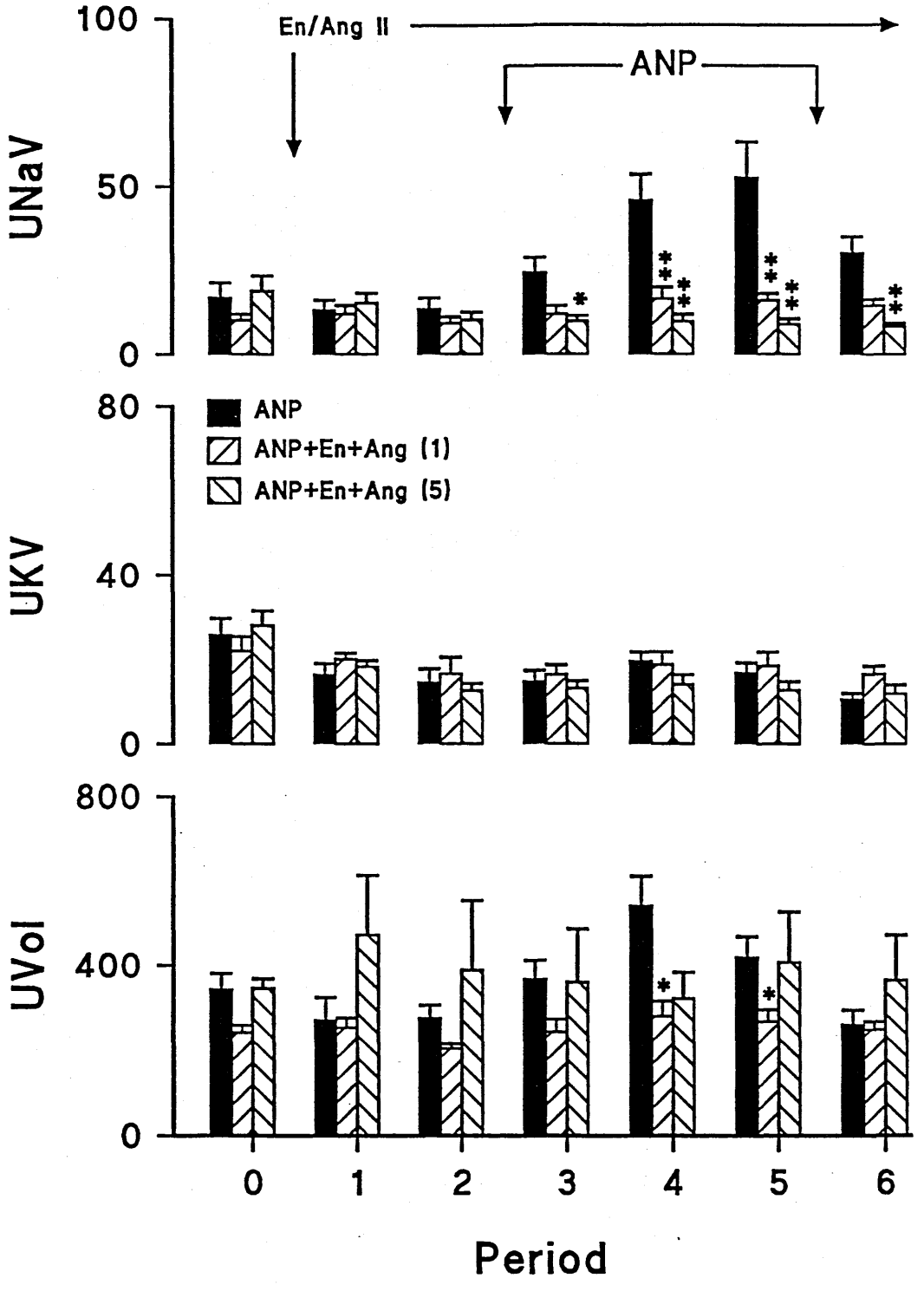


FIGURE 4.7:

INFLUENCE OF ANG II ON RENAL EFFECTS OF ANP.

Effects of ANP (5pmol/kg/min) on sodium excretion (UNaV, μmoles/min), potassium excretion (UKV, μmoles/min) and urine volume (UVol, μl/min), given alone or with plasma Ang II "clamped" by co-infusion of 1pmol or 5pmol/kg/min Ang II (periods 1-6) after the administration of Enalaprilat (3mg/kg) following period 0. Treatments were compared with ANP alone by MANOVA, with post hoc analysis by paired T-test *$P<0.05$, ** $P<0.01$. Periods represent 30 minute collections, ANP infused during periods 3-5.

the low dose Ang II and placebo groups, suggesting that Ang II had been clamped within the physiological range (although plasma levels were subsequently found to be slightly higher than basal - see below). High dose Ang II caused a modest, sustained rise in MAP of about 10 mmHg. In neither group was there any suggestion of a reduction in blood pressure during ANP infusion, in contrast to the trend towards blood pressure reduction in the group receiving ANP alone.

Hormonal Effects: When Ang II was infused following Enalaprilat renin levels did not rise but remained "normal" with the low dose infusion and suppressed (from 8.9±1.8 [P0] to 4.5±1.1 [P2] μU/ml) during the high dose infusion. When ANP was then infused there was a similar fall in renin - to that observed with ANP alone - during low dose Ang II infusion, and a small but perceptible decrease during high dose infusion., with a rebound rise in both Ang II limbs after discontinuing ANP. When the renin levels are expressed as a percentage of pre-ANP infusion levels (i.e. P2) the percentage fall in renin was similar in all limbs (Figure 4.8), with a significant reduction in the corrected area under the curve for renin during ANP infusion (Figure 4.9). The raw data are given in Table 4.1.

Ang II and aldosterone results are given in Table 4.1. Plasma Ang II levels achieved during low dose Ang II infusion were slightly higher than baseline (circa 14 pg/ml) and levels of around 60pg/ml were achieved during high dose infusion. There were no changes during the ANP infusion confirming that co-administration of En and Ang II produced an effective "clamp". Aldosterone levels were similar in all except the high dose Ang II group where they were approximately twice basal. Infusion of ANP caused a small reduction in these high aldosterone levels although they remained much higher than those seen

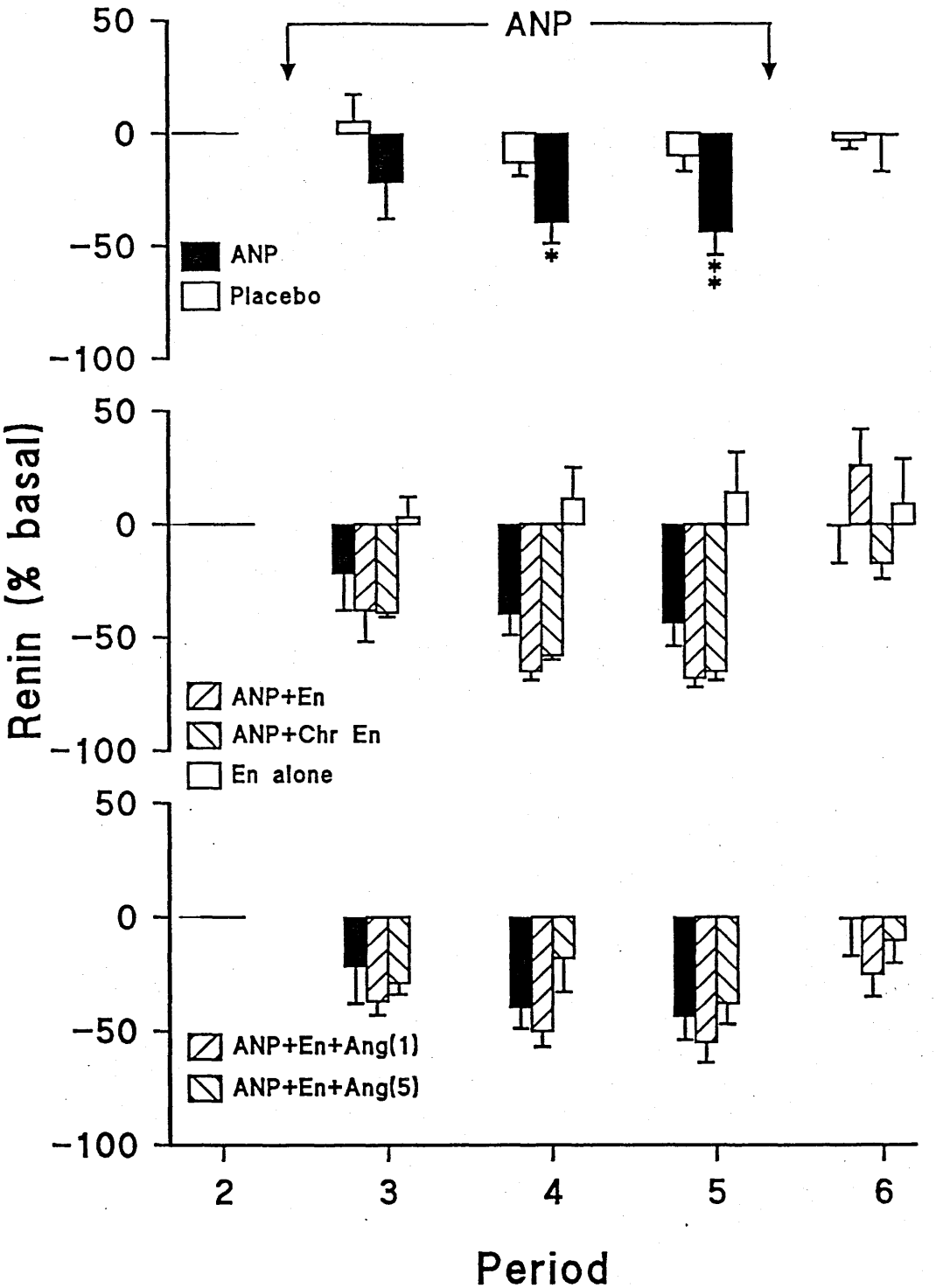


FIGURE 4.8:

RENIN RESPONSE TO ANP INFUSION: INFLUENCE OF ANG II AND ACE INHIBITION.

Plasma active renin concentration expressed as a percentage of the level at the end of period 2 to compensate for the differing effects of Ang II and ACE inhibition on basal levels (see text for details). Absolute values are given in Table 4.1. Renin was significantly suppressed by ANP (vs Placebo, * $P<0.05$, ** $P<0.01$), but in none of the other treatment groups was there any difference compared with the effects of ANP alone. The effects of Enalaprilat alone are also shown.

during infusion of ANP alone and the absence of an ideal control group (i.e. Ang II without ANP) makes the significance of this observation uncertain.

Discussion:

Several previous reports (Wang and Gilmore (1985), Bie et al.,(1990), Hirata et al.,(1987), Richards et al.,(1989a), diNicolantonio and Morgan (1987), Seymour and Mazack (1988), Gaillard et al.,(1988,1989), Hansell and Ulfendahl (1987), Salazar et al.,(1987)) have examined the interaction between ANP and Ang II on renal function in intact animals and man. Although there is almost universal agreement that Ang II can attenuate the natriuretic and diuretic actions of ANP, removal of circulating Ang II by administration of an ACE inhibitor has also been reported to attenuate these actions of ANP. The present experiments address some of the inconsistencies in previous studies by using conscious animals, physiological doses of ANP, modest doses of converting enzyme inhibitor and attempting to establish the animals on a "normal" sodium diet. Under these circumstances ACE inhibition clearly enhances the actions of ANP and Ang II attenuates it, suggesting a modulatory role for renal Ang II on the renal actions of ANP.

Actions of ANP.

Basal plasma levels of ANP were around 40 pg/ml, similar to most previous reports in dogs (Mizelle et al., (1990), O'Hanian et al., (1988), Zimmerman et al.,(1987). Infusion of 5pmol/kg/min ANP caused plasma levels to rise 4-5 fold, achieving plasma levels close to previous reports using similar dose infusions (OHanian et al., (1988), Pichet et al. (1987), Cernacek et al., (1988), Bie et al., (1988)).

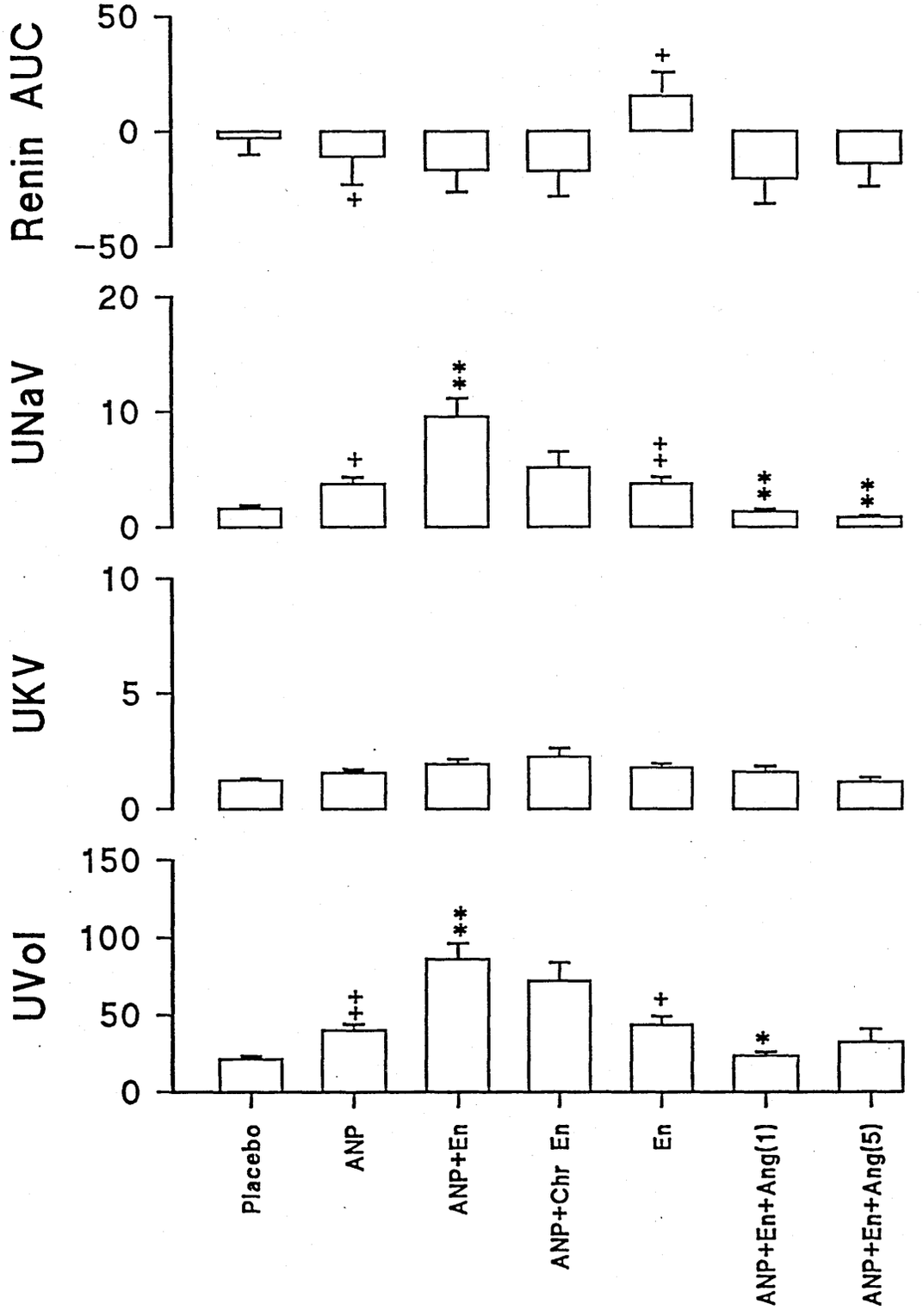


FIGURE 4.9:

SUMMARY OF EFFECTS ON RENIN AND RENAL FUNCTION OF ANP INFUSION AND INFLUENCE OF ANG II AND ACE INHIBITION.

Summary of the effects of ANP in each of the different limbs of the protocol (see Figure 4.1 and text for details). AUC=calculated area under the curve for renin, corrected for baseline variation (see text for details), UNaV (sodium excretion, mmoles), UKV (potassium excretion, mmoles), UVol (urine volume, ml) for the 90 minute period during which ANP was infused.

* indicate significance vs ANP alone and + vs placebo (*

There was marked variability in the final plasma levels achieved (140-220 pg/ml) despite identical infusion rates (Table 4.1), presumably reflecting interindividual differences in clearance rate. However, there was no evidence of higher plasma levels in the presence of Enalaprilat (as others have suggested, Wilkins et al.,(1987)). Overall, the differences in effect between increments in plasma levels of four or five-fold are likely to be slight (Cernacek et al., (1988)). ANP caused sodium excretion to rise two to three fold, urine volume doubled and there was a minor increase in potassium excretion. These observations are consistent with similar findings in other species infused with physiologically relevant doses of ANP (Human: Richards et al, (1988a,b), Soloman et al.,(1989), Anderson et al.,(1987), Morice et al.,(1988), Cottier et al.,(1988)); Rat: Soejima et al.,(1987) & Chapter 6) and a similar study in conscious dogs infused with approximately 3.3 pmol/kg/min ANP (Pichet et al.,(1989)), but conflicts with the findings of O'Hanian (O'Hanian et al., (1988)) who failed to show any renal effects during infusion of 3.3 pmol/kg/Min ANP in conscious dogs. There is no obvious explanation for these differences but the overall balance of evidence confirms a role for ANP at physiological/ pathophysiological levels. In the study of Pichet et al., (1989) absolute values for sodium excretion were substantially higher than the values reported here (basal levels 50 vs. 20 μEq/min) probably reflecting the higher dietary intake of their animals (110 vs. 30 mmoles Na/ day). There was a modest, non-significant, fall in mean arterial pressure and heart rate (table 4.2) consistent with previous observations that the hypotensive effects of ANP are only clearly seen at higher doses (Pichet et al.,(1989), O'Hanian et al.,(1988), Cernacek et al.,(1988), Bie et al.,(1988)) or

during chronic administration of ANP (Janssen et al.,(1989), Mizelle et al.,(1990)). Active renin concentration fell during ANP infusion (consistent with the known actions of ANP in man (e.g. Richards et al.,(1988)) and in dogs (Richards et al.,(1988), Burnett et al.,(1984)) and although there was a similar trend in Ang II (presumably secondary to the changes in plasma renin) this failed to achieve statistical significance due to baseline variability (Figure 4.3). Aldosterone fell in both active and placebo limbs, the fall in the placebo limb presumably reflecting diurnal variation; there was no significant effect of ANP over the short time interval studied.

This mechanism underlying the renal actions in this study, with an upward trend in GFR, a downward trend in renal plasma flow and a significant increase in FeNa almost exactly parallel the consensus view from low dose studies in man (Richards et al, (1988a,b), Soloman et al.,(1989), Anderson et al.,(1987), Morice et al.,(1988), Cottier et al.,(1988))). From these data one would predict that the observed natriuresis is due to inhibition of tubular sodium reabsorption (Chapter 2) but, since no attempt was made to dissect out contributions from the proximal and distal tubules (for example by lithium clearance), it is not possible to say where in the tubule these effects occur. Furthermore, measurement of GFR by inulin clearance (Maack (1986)) is relatively inaccurate with up to 10% error. The upward trend seen in the present study may thus mask a true small effect on GFR, and increased GFR is certainly seen with higher infusion rates of ANP (Chapter 2; 2.7). Thus from this data the natriuresis due to low dose infusion of ANP appears to be mediated predominantly by reduced tubular reabsorption of sodium.

Effects of ACE inhibition:

Acute administration of Enalaprilat caused a small, sustained natriuresis and diuresis with small reduction in mean arterial pressure, reflecting the tonic basal activation of the renin-angiotensin system in these salt-replete animals (Hall (1986), Mitchell and Navar (1989)). Furthermore, the natriuresis - in the absence of a change in plasma aldosterone - is consistent with the modern view that many of the effects of the renin-angiotensin-aldosterone system are mediated by the direct actions of Ang II on the renal tubule rather than through aldosterone (Hall (1986), Mitchell and Navar (1989)). Subsequent administration of ANP resulted in a greatly increased diuresis and natriuresis, when compared with the actions of ANP alone. There was no significant alteration in potassium excretion. However, in the group pre-treated with Enalapril (5mg b.d.), basal urine volume and sodium excretion were increased and although ANP administration caused sodium excretion and urine volume to rise the effect was not significantly greater than ANP alone. These findings contrast with previous studies where ACE inhibition blunted the effects of ANP (e.g. Gaillard et al.,(1988, 1989), Seymour and Mazack (1988), Wilkins et al.,(1987)), but only acute ACE inhibition significantly increased the actions of ANP. There are several possible reasons for this, the most likely being that ACE inhibition causes a fall in arterial pressure. It is known from studies on the isolated kidney (Firth et al.,(1987) and experimental animals (Seymour et al.,(1987), Davies and Briggs (1987)) that reductions in renal perfusion pressure attenuate the natriuretic actions of ANP. The mechanism of this effect is not understood but does not appear to involve activation of the intra-renal renin-angiotensin system, since

infusion of saralasin does not restore the natriuresis (Redfield et al., (1989)). In fact, restoration of perfusion pressure by infusion of Ang II can restore the natriuretic effects of ANP (Redfield et al.,(1989), Davies and Briggs (1987)). Activation of the renal sympathetic nervous system can also attenuate the natriuretic effects of ANP (Koepke and diBona (1988)) and will tend to be activated during systemic hypotension and in anaesthetised preparations. The use of conscious animals in the present study should avoid sympathetic activation and although circulating catecholamines were not measured there was no change in heart rate. Richards et al.,(1989) also reported an increase in cortisol during ACE inhibition which may indicate activation of a "stress" response perhaps associated with activation of the sympathetic nervous system. If we accept that hypotension (with associated reduction in renal perfusion pressure and sympathetic activation) is the factor most likely to inhibit the natriuretic response to ANP (above) then it is obvious that the dose of ANP used is important - since hypotension is a dose dependent effect (Bie et al.,(1988)) in dogs. The use of low dose ANP in the current study - in contrast to previous reports (above) - removes some of this effect, since only a small fall in blood pressure was produced. The dose of ACE inhibitor is also important. Polonia et al.,(1990) found that the natriuretic effects of 50pmol/kg/min ANP were enhanced by a low dose of Captopril but when combined with high dose Captopril (where BP fell) the effects were blunted. Sodium balance is also important, and was not controlled in many studies. Richards et al.,(1989) studied volunteers on a high salt diet (300 mmol/day) which will tend to suppress renin activity and thereby make ACE inhibition superfluous. The fact that these authors found any

enhancement of the natriuretic actions of ANP (albeit minor, and non-significant) is surprising. A greater enhancement might have been seen had ACE inhibitor been administered against a background of an active renin-angiotensin system. Finally, there are clear differences between the effects of acute administration and chronic administration of ACE inhibitor on the actions of ANP. In the present study acute administration caused a marked enhancement whilst "chronic" administration caused only a minor non-significant enhancement. The reasons for this are not clear. There were no differences in BP between the groups, nor was there any evidence of adaptation within the circulating renin-angiotensin system since renin activity and aldosterone levels were similar before administration of ANP (i.e. P2) in both ACE inhibitor limbs. It may be that the intra-renal renin angiotensin system "escapes" from ACE inhibition during chronic administration or that these animals are in relative negative sodium balance having presumably undergone a natriuresis on the first day of treatment. Whatever the expalnation, it it may explain some of the observed differences between acute studies and studies of chronic pre-treatment with ACE inhibitors (Wilkins et al.,(1987), Gaillard et al.,(1988,1989)).

Recently, Bie et al.,(1990) have published findings in conscious dogs infused with ANP in the presence of ACE inhibition plus aldosterone blockade with Canrenoate showing potentiation of the actions of ANP. The present findings suggest that ACE inhibitors would explain the major part of this potentiation. The observation that ACE inhibition enhances the renal effects of ANP fits well with observations made during non-pharmacological manipulation of the renin-angiotensin system. For example when renin is suppressed by

volume expansion (Metzler and Ramsay (1989)) or mineralocorticoid excess (Gaillard et al.,(1988), Parkes et al.,(1988)) then ANP is more potent. The overall consensus from the present study and previous reports must be that ACE inhibition potentiates the actions of ANP as long as antinatriuretic mechanisms are simultaneously activated.

Interaction with angiotensin II.

Having established the effects of ACE inhibition (above), Ang II should inhibit the renal effects of ANP and this is well established (Salazar et al.,(1987), Showalter et al.,(1987), McMurray and Struthers (1988a,b), Lang and Struthers (1990)). The purpose of the present study was to examine the interaction between ANP and Ang II using physiologically relevant doses of both peptides, and to remove the effects of ANP on endogenous Ang II by the combination of Ang II and ACE inhibitor (Mizelle et al.,(1989)). In the present experiments high dose Ang II (which increased plasma Ang II ten-fold (Table 4.1) and increased blood pressure (about 10 mmHg, Table 4.2). There was no effect on baseline sodium excretion. Renin levels were suppressed (Figures 4.8, 4.9, Table 4.3) and aldosterone increased approximately three-fold (although due to SEM this did not achieve statistical significance, Table 4.1, Appendix 2). The effects of ANP on the kidney and on arterial pressure were completely abolished, although the effect on renin suppression remained (Figure 4.8, see below). In the low dose Ang II group, despite the fact that blood pressure was controlled at "normal" levels as intended, plasma Ang II subsequently turned out to be approximately twice normal (Table 4.1). The pattern of renal effects is virtually identical to that of high dose Ang II except that blood pressure remained "normal" and during infusion of ANP there was a small upward trend in sodium excretion which, although

not statistically significant suggests that the response to ANP is not completely abolished. These data confirm that Ang II opposes the renal actions of ANP and it is apparent that the effects of the antinatriuretic hormone are more potent since doubling plasma levels virtually abolishes the response to a five-fold increase in ANP.

Site of the ANP-Ang II interaction:

Having established that increased Ang II attenuates, and ACEi (reduced Ang II) augments the effects of ANP, the measurements made in this study allow us to make limited judgements regarding the intra-renal site of this ANP-Ang II interaction. There is little evidence of an interaction in renal blood flow. Although ERPF is lower during Ang II infusion, it also falls during treatment with ANP alone and the small increase seen during treatment with ACE inhibitor is not statistically significant. The data on glomerular blood flow is similarly difficult to interpret due to the large variability. ANP caused an increase in mean GFR, although this failed to achieve statistical significance. Ang II infusion caused a reduction in GFR which would not be predicted to occur, at least at this dose of Ang II, which is usually reported to increase or preserve GFR (see Hall (1986), Mitchell and Navar (1989) - for review). When infused together, GFR remained low and there was no evidence of an upward trend in GFR during ANP infusion. However, the greater natriuresis during treatment with Enalaprilat and ANP was not accompanied by increases in GFR, suggesting that an interaction at the level of the glomerulus is not of major importance. Fractional excretion of sodium gives some indication about tubular function, although it does not localise the effect to any particular tubule section. For this variable the pattern of change does follow the observed changes in,

for example, total sodium excretion. ANP alone caused a rise in FeNa; the greater natriuresis seen with combined ACE inhibition and ANP was associated with mean values of FeNa twice those seen with ANP alone (although once more not statistically significant); and the reduced natriuresis during Ang II treatment was associated with a reduced FeNa. The balance of evidence therefore points to an interaction at the level of the renal tubule, although one can only speculate on the tubule segments involved. The main antinatriuretic effects of Ang II occur in the proximal tubule (Hall (1986)), those of ANP seem best characterised in the distal tubule and collecting duct (Chapter 2). Therefore the simplest model might involve Ang II acting as a "gate", regulating the delivery of sodium to the distal tubule where ANP can then act (Mizelle et al.,(1989), Bie et al.,(1990)). This simple model alone might account for all the observed effects. However, it is more likely to involve a mixture of effects at different sites in the nephron. For example ANP and Ang II have opposing effects in the isolated glomerulus (Appel et al.,(1986)) in vitro and, since glomerular receptors for both are present (Martin and Ballerman (1989)), such an effect may well occur in vivo. Minor changes in GFR may be missed by the relatively imprecise measurement of inulin clearance (Maack (1986)). A trend towards increased GFR with ANP was evident in the present study and was absent when Ang II was infused. Other workers have suggested that the effects of ANP in the distal tubule are quantitatively inadequate to account for the observed effects of ANP (Harris and Skinner (1990)) and suggest instead that there is an interaction between ANP and Ang II within the proximal tubule - where Harris et al.,(1987, 1988) have shown in careful micropuncture studies that ANP opposes the effects of Ang II on sodium

reabsorption through disruption of glomerulotubular balance.

Overall, the data points to a physiologically relevant interaction between ANP and Ang II which is predominantly tubular and may simply involve Ang II regulating the passage of sodium to the distal tubule where ANP is effective, but it is not possible to exclude effects at other sites within the nephron.

Renin release:

There is abundant evidence that ANP suppresses basal and stimulated renin release in vivo (Chapter 2, Burnett et al., (1984), Maack et al.,(1984), Scheuer et al.,(1987), Richards et al.,(1988)) and in vitro (Deray et al.,(1987)). The current findings confirm that physiological levels of ANP are effective in the dog, and cause approximately 50% reduction in circulating active renin. Debate continues as to whether the effects of ANP on renin are direct or indirect (i.e. acting directly on the renin-secreting cells or indirectly through a macula densa mechanism) and, for example, ANP has been shown to have no effect in t he non-filtering kidney (Chapter 2, Opgenorth et al.,(1987)). However, in this study the percentage reduction in renin is similar in all the limbs of the protocol even when the natriuresis is abolished by Ang II, suggesting a direct action, independent of the distal delivery of sodium. Many of the actions of ANP persist after infusion has stopped implying continuation of action at a post-receptor level (eg Singer et al.,(1987)), since the plasma half-life of ANP is only a few minutes (Chapter 5). For example the natriuresis caused by ANP alone (Figure 4.2) persisted into the recovery period. However, in all limbs of the study renin activity had returned to the appropriate baseline (period 2) with 30 minutes of cessation of the ANP infusion, again suggesting

a mechanism of action independent of sodium delivery to the macula densa and possibly acting through an short-lived intracellular post-receptor mechanism.

Contribution of renin suppression to the natriuretic effects of ANP: One of the original aims of this study was to assess the role of suppression of the renin-angiotensin system to the natriuretic actions of ANP. To some extent it is not possible to answer this question for the simple reason that despite normal arterial pressure the low dose Ang II clamp resulted in twice normal plasma levels of Ang II. However, some information can be salvaged from the data. Figure 4.10 shows the relationship between the ratio of plasma ANP/Ang II and UNaV. Individually Ang II is inversely related to sodium excretion and, under circumstances where Ang II suppressed (ACE inhibition) ANP is directly related to UNaV. Overall, the relationship between log (ANP/Ang II) and log UNaV is linear:

$$\textbf{Log UNaV = 0.27 Log [ANP/ Ang II] + 1.01.}$$

From this simple relationship it is possible to predict the natriuretic response to ANP from the plasma levels of ANP and Ang II (Table 4.1). With infusion of ANP alone the estimated sodium excretion during ANP infusion is 2.8 mmoles/90 minutes; compared with an actual value of 3.7, and a calculated "placebo" value of 1.2 mmoles/90 minutes. When a constant value of Ang II (equal to basal levels) is put into the equation then a value of 2.4 mmoles/ 90 minutes is obtained, thus suppression of Ang II would be predicted to contribute approximately 15% to the natriuresis during ANP infusion, with the major actions of ANP being due to direct effects on the kidney (Bie et

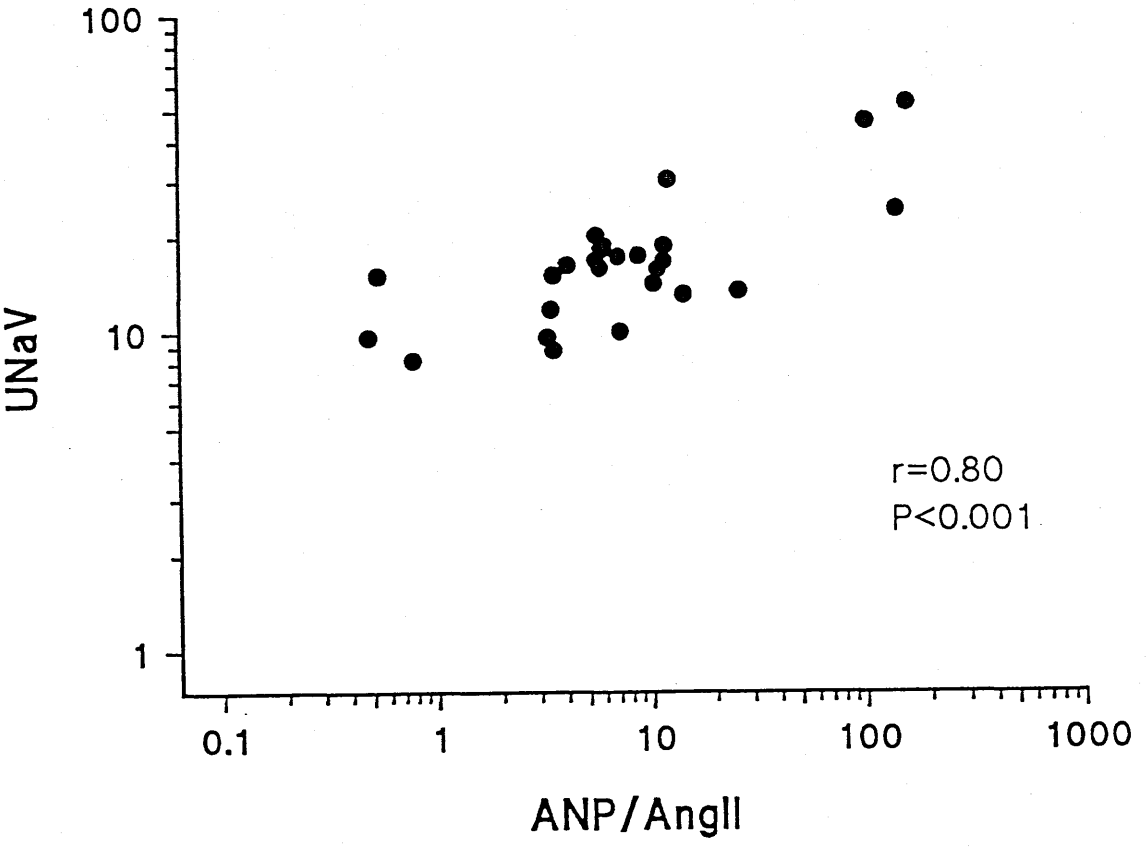


FIGURE 4.10:

RELATIONSHIP BETWEEN ANG II AND ANP CONCENTRATIONS AND SODIUM EXCRETION IN DOGS.

Relationship between the ratio of ANP/Ang II (both pg/ml) and sodium excretion (μmol/min). Data from 32 dog studies, from the placebo, ANP alone, and high and low dose ANG II limbs of the study (n=8 for each). Each point represents the mean value for 8 dogs at a single time point, with 7 time points from each experimental limb. r=linear correlation coefficient.

al.,(1990). In order to confirm this estimate it would be necessary to repeat the experiments with different levels of Ang II "clamp". However, without an instantaneous measurement of Ang II (other than blood pressure) this ideal may be difficult to achieve.

In conclusion this series of experiments demonstrates that modest increments in Ang II can attenuate or abolish the renal effects of ANP and, as one would predict (but in contrast to much of the published literature), that inhibition of the renin-angiotensin system by administration of Enalaprilat augments the action of ANP. The failure of other workers to demonstrate this is due in part to the confounding effects of changes in renal perfusion pressure produced by ACE inhibitors but mainly to the differences between acute and chronic administration of ACE inhibitors on the subsequent actions of ANP. These findings are consistent with a modulatory role for Ang II on the actions of ANP in the kidney, with the large ANP-induced natriuresis in the presence of Enalaprilat reflecting the maximum "potential" ANP effect. Since ANP can reduce renin release and, as a consequence, reduces circulating and intrarenal Ang II, the question arises (Bie et al.,(1990)) of the extent to which this reduction in Ang II contributes to the natriuresis of ANP. In the present series of experiments "clamping" Ang II by co-infusion of ANP and Ang II was able to blunt the effects of ANP suggesting that reduction in Ang II is a necessary component of the natriuresis. However, it was clear that modest increments of Ang II have more potent antinatriuretic actions than larger increments in ANP have natriuretic. Due to difficulty in "clamping" plasma Ang II at absolute levels it was only possible to estimate the contribution of suppressed Ang II to the

natriuretic actions of ANP - at 15%. With respect to renin, debate has surrounded the mechanism by which ANP inhibits renin release. The ability of ANP to produce a fall in renin in the face of a reduction in urinary sodium excretion favours a direct rather than a macula densa mediated mechanism.

Finally, from a therapeutic point of view (Section 4) these studies suggest that suppression of the renin-angiotensin system may offer a mechanism for enhancing the effects of ANP.

ANP

Time	0	1	2	3	4	5	6
Placebo	33.4±6.9	43.6±5.9	42.0±5.3	39.1±7.6	33.0±6.6	36.0±6.2	34.3±6.0
ANP alone**	44.0±6.0	52.9±9.1	52.3±11.7	192.3±30.1**	179.9±27.5**	180.6±26.1**	49.7±10.8
ANP+EN	38±6.4	31.3±5.2	45.2±20.1	147.2±29.1	190.3±33.8	180.5±34.5	53.0±15.4
ANP+Chr EN	35.6±6.1	34.8±7.1	33.6±6.6	153.7±14.9	136.7±9.9	158.3±27.7	30.8±23.6
EN alone	32.4±3.8	30.9±3.9	34.3±5.7	37.4±11.4	32.7±6.9	35.6±9.1	37.9±10.3
ANP+EN+Ang 1	53.8±8.6	51.3±8.1	54.6±8.1	144.3±11.1	137.8±4.7	150.0±9.0	46.4±5.9
ANP+EN+Ang 5	34.9±6.7	32.3±7.2	29.5±4.1	180.1±18.7	197.7±15.3	204.2±23.7	45.0±11.0

RENIN

Time	0	1	2	3	4	5	6
Placebo	8.6±1.6	7.2±1.3	7.4±1.3	6.9±1.2	6.3±1.2	6.2±1.1	7.2±1.3
ANP alone**	7.4±1.4	8.4±1.6	7.4±1.8	4.6±1.0*	3.9±0.8*	3.4±0.7*	7.3±1.1
ANP+EN***	8.5±2.3	17.4±6.1	24.1±7.1*	13.5±3.6*	8.0±2.2	6.9±1.9	22.8±4.2*
ANP+Chr EN***	25.7±4.7*	25.4±4.0**	28.6±5.3**	17.1±3.0**	11.8±2.1*	9.6±1.5*	20.6±3.1*
EN alone***	6.0±1.1*	13.0±3.9	19.1±5.3*	18.7±5.0*	20.3±5.7*	19.0±4.5*	21.7±5.6*
ANP+EN+Ang 1	9.0±2.2	10.1±3.3	12.2±3.9	7.2±2.5	5.2±1.7	3.9±1.0	8.2±2.9
ANP+EN+Ang 5	8.9±1.8	8.3±2.6	4.5±1.1	3.0±0.5	3.3±0.4	2.7±0.3	3.8±0.6

ANG 2

Time	0	1	2	3	4	5	6
Placebo	10.7±3.0	8.2±1.6	8.7±1.5	8.1±1.0	7.0±0.8	7.4±0.8	8.0±1.4
ANP alone	6.5±1.6	9.1±2.5	6.5±1.9	3.9±1.0	3.7±1.0	3.5±0.8	5.9±1.1
ANP+EN+Ang 1**	9.2±1.4	15.9±2.1	14.8±2.5	14.3±2.3**	14.4±2.5*	13.1±1.3**	15.5±2.4**
ANP+EN+Ang 5***	6.8±1.3	60.6±4.6***	64.3±5.5***	58.9±3.9***	61.5±5.7***	65.4±5.5***	58.3±3.8

ALDO

Time	0	1	2	3	4	5	6
Placebo	5.6±0.8	4.6±1.0	4.4±0.8	3.9±0.7	2.3±0.7	1.6±0.5	1.9±0.7
ANP alone	6.5±1.1	5.3±1.0	4.4±1.3	2.8±0.9	2.1±0.8	1.1±0.6	1.0±0.5
ANP+EN*	3.8±0.9*	2.5±0.4*	2.8±0.7	2.1±0.6	1.3±0.4	0.9±0.4	0.5±0.3
ANP+Chr EN	3.8±0.9	4.5±1.0	3.4±0.9	2.8±1.0	2.4±0.6	1.4±0.7	1.3±0.7
EN alone	4.6±0.8	4.9±1.2	2.0±0.6	1.4±0.6	0.1±0.1	0.1±0.1	0.3±0.2
ANP+EN+Ang 1	6.0±0.9	6.6±1.01	6.4±1.7	4.0±0.9	4.5±1.6	2.4±0.8	2.4±0.7
ANP+EN+Ang 5	14.0±8.6	15.5±6.6	17.6±5.4	14.6±4.6	12.4±3.6	8.6±2.5	11.6±3.1

TABLE 4.1:

PLASMA HORMONE LEVELS.

Levels of ANP (pg/ml), Ang 2 (pg/ml), Renin (μU/ml), Aldo (ng/dl) in each of the treatment groups against time interval. Data are shown as mean±SEM. Significance by MANOVA of ANP vs Placebo and of all other groups against ANP illustrated by asterixes on label. Where significant, individual time points compared by T-test. * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

BP (MAP)

Time	0	1	2	3	4	5	6
Placebo	109.5±2.8	110.9±3.1	112.3±4.1	112.9±2.8	113.4±3.6	112.4±3.1	114.9±4.7
ANP alone	109.3±2.3	113.0±3.2	114.1±1.9	110.6±1.9	107.3±1.7	108.0±1.5	111.9±1.0
ANP+EN**	114.0±4.5	108.5±3.8	108.5±3.5	106.4±3.3	106.3±2.7	105.9±4.1	104.9±3.7
ANP+Chr EN	107.4±3.1	108.4±2.6	109.4±2.7	107.5±2.8	104.6±2.2	103.1±2.2	107.9±4.7
EN alone	113.1±2.3	108.5±3.4	108.1±2.8	107.4±3.5	110.9±3.6	110.0±3.9	108.9±4.5
ANP+EN+Ang 1	111.0±5.3	113.4±3.1	114.8±3.5	112.8±2.8	114.3±2.7*	118.1±2.8**	116.5±3.4
ANP+EN+Ang 5**	112.4±3.0	118.4±2.8	124.5±3.5**	123.5±3.1**	121.8±2.7***	122.6±2.7***	123.6±2.6*

PULSE

Time	0	1	2	3	4	5	6
Placebo	82±4	81±5	85±4	88±6	87±4	86±3	84±5
ANP alone	91±5	87±3	87±6	83±4	76±4	80±4	86±3
ANP+EN	89±7	90±6	85±6	80±4	85±4	83±6	87±4
ANP+Chr EN	90±5	93±6	90±4	91±6	84±5	87±5	90±
EN alone	72±11	88±3	85±5	85±4	92±4	86±5	84±4
ANP+EN+Ang 1	89±9	96±7	94±7	84±6	92±7	91±5	93±5
ANP+EN+Ang 5	91±6	90±6	91±6	91±4	88±5	91±6	94±5

HCT

Time	0	1	2	3	4	5	6
Placebo	35.7±1.6	35.2±2.1	34.7±2.2	34.8±2.4	34.0±2.3	33.3±2.5	33.2±2.4
ANP alone	35.0±1.9	34.5±1.6	34.3±1.9	33.6±1.9	34.3±1.6	33.8±1.6	33.8±1.7
ANP+EN	35.3±1.5	36.5±1.5	36.5±1.6	37.0±1.9	37.5±1.6	37.9±1.9	36.0±1.5
ANP+Chr EN	37.1±2.2	37.1±2.4	36.6±2.0	36.6±2.0	36.6±2.0	35.7±2.1	36.0±2.1
EN alone	35.5±1.4	35.5±1.5	35.4±1.2	35.5±1.6	35.5±1.4	35.3±1.1	35.3±1.1
ANP+EN+Ang 1	35.5±2.2	35.3±1.0	35.5±5.1	35.5±1.8	35.3±1.8	35.8±1.8	35.8±1.8
ANP+EN+Ang 5	35.0±1.8	36.3±1.8	35.8±1.5	35.3±1.9	35.5±1.8	34.3±1.5	34.3±1.8

TABLE 4.2:

PULSE, BLOOD PRESSURE AND HAEMATOCRIT:

Mean arterial pressure (MAP), pulse rate (beats per minute) and haematocrit (HCT, %) in each of the treatment groups against time interval. Statistical significance indicated by asterixes as in Table 4.1 (Data are shown as mean±SEM).

Time	0	1	2	3	4	5	6
				ERPF			
Placebo	-	211±21	201±20	198±21	194±21	183±17	179±17
ANP alone	-	201±16	200±16	186±15	174±14	164±16	162±16
ANP+EN	-	254±21	228±23	214±24	203±22	204±20	209±21
ANP+Chr EN	-	248±12	235±13	213±14	219±16	202±14	204±13
EN alone	-	227±22	235±24	239±24	235±23	223±21	218±19
ANP+EN+Ang 1	-	211±13	202±15	184±15	163±12	159±12	179±22
ANP+EN+Ang 5**	-	221±15	192±15	170±13	159±11*	150±11	147±11
				GFR			
Placebo	-	76±10	92±20	95±25	82±16	77±17	56±5
ANP alone	-	85±17	85±18	76±16	86±31	120±42	90±29
ANP+EN	-	74±12	84±14	82±12	83±10	84±7	87±8
EN+Chr EN	-	44±5	51±8	63±15	62±15	77±17	80±17
EN alone*	-	51±5	48±4	54±9	59±12	69±16	67±17
ANP+EN+Ang 1	-	52±4	57±7	58±6	54±5	53±5	54±6
ANP+EN+Ang 5	-	57±7	62±7	60±7	56±8	62±8	65±7
				FF			
Placebo	-	38±6	51±10	51±13	46±9	42±8	29±2
ANP alone	-	45±10	45±10	42±8	47±12	76±26	54±12
ANP+EN	-	34±8	39±8	38±3	42±5	42±3	43±4
ANP+Chr EN	-	18±3	22±4	31±9	30±9	39±10	40±9
EN alone	-	23±4	20±2	26±6	29±8	36±10	34±8
ANP+EN+Ang 1	-	24±3	28±4	32±4	34±4	33±5	31±7
ANP+EN+Ang 5	-	27±3	34±4	36±3	36±5	42±5	45±4
				FeNa			
Placebo	-	0.16±0.03	0.13±0.03	0.15±0.04	0.17±0.03	0.19±0.03	0.28±0.07
ANP alone**	-	0.15±0.02	0.16±0.04	0.24±0.05	0.47±0.07**	0.46±0.14*	0.27±0.06
ANP+EN	-	0.30±0.14	0.39±0.16	0.77±0.21	1.07±0.38	0.97±0.30	0.94±0.36
ANP+Chr EN	-	0.46±0.16	0.43±0.22	0.67±0.26	0.94±0.33	0.91±0.31	0.58±0.16
EN alone	-	0.46±0.17	0.37±0.08	0.49±0.10	0.68±0.17	0.66±0.19	0.66±0.21
ANP+EN+Ang 1	-	0.17±0.04	0.13±0.05	0.15±0.04	0.22±0.04	0.21±0.03	0.19±0.03
ANP+EN+Ang 5**	-	0.18±0.03*	0.12±0.02	0.12±0.03	0.15±0.05*	0.11±0.03*	0.09±0.02*

TABLE 4.3:

RENAL FUNCTION INDICES:

Estimated renal plasma flow (ERPF, ml/min), Glomerular filtration rate (GFR, ml/min), Filtration fraction (FF, %), Fractional excretion of sodium (FeNa, %) in each of the treatment groups against time interval. Statistical significance indicated by asterixes as in Table 4.1 (Data are shown as mean±SEM).

Section 4.

NEUTRAL ENDOPEPTIDASE AND ATRIAL NATRIURETIC PEPTIDE.

Chapter 5.

Harnessing the Therapeutic Potential of ANP: Neutral Endopeptidase and NEP Inhibitors.

The unique combination of actions produced by infusion of ANP - natriuresis, diuresis, vasorelaxation and suppression of the renin-angiotensin system (Chapter 2) - quickly aroused interest as a potential therapeutic combination in hypertension, heart failure and other oedematous states (Jardine et al., (1989), Fried (1989). However, since ANP is not orally available, investigation of this therapeutic potential was limited by the need for parenteral infusion. In theory, there are several ways to circumvent this problem. Firstly, it may be possible to synthesise ANP analogues which are stable to enzymatic degradation, by modifying the parent ANP molecule. This approach was partially responsible for identifying the C-ANP receptor (Maack et al., (1987), Scarborough (1989), Koepke et al.,(1989) and, although C-receptor analogues are available (eg $_{4-23}$ANP, see Chapter 2), which potentiate the effects of ANP by blocking receptor mediated clearance, they are mostly large peptides (Okolicany et al.,(1990)),

and to date no specific analogues to the guanylate cyclase-linked receptor have been identified. An alternative approach is to increase production of ANP and, although some agents such as dexamethasone (Shields et al.,(1987)), α and ß-adrenoceptor agonists (Schiebinger & Linden (1987); Schiebinger et al.,(1987)) stimulate the release of ANP in vitro, no agents which specifically release ANP have been identified. A further alternative is at the subcellular level by the inhibition of cGMP degradation. Recent reports suggest that cGMP phosphodiesterase inhibitors are potent antihypertensive agents (Wilkins et al.,(1990)) but the actions of these compounds is not specific to ANP and may potentiate the effects of agents acting through soluble guanylate cyclase (eg EDRF). Although this is an interesting and potentially important antihypertensive mechanism it will not be considered further here.

Finally, ANP's actions may be potentiated by the inhibition of enzymatic degradation of the peptide. Although it has been known for some time that ANP is rapidly cleared from the circulation (Espiner et al., (1986), Richards et al.,(1986)) it was not until 1987 that an enzyme responsible for ANP degradation was identified. This enzyme, Neutral Endopeptidase (NEP, EC 3.4.24.11) is found principally in the renal brush border. Almost simultaneously in 1987 a number of groups (Stephenson & Kenny (1987), Olins et al (1987), Sonnenberg et al (1987)) reported that ANP was degraded by preparations of renal brush border membrane which are rich in peptidases, and that the principal site of attack is at the Cys-Phe bond of ANP (Figures 2.2 & 2.3). This enzymatic degradation co-purifies with purification of NEP from renal brush border membranes (Sonnenberg et al.,(1987)), and is blocked by specific enzyme inhibitors (Stephenson & Kenny (1987), Olins et

al,(1987), Sonnenberg et al., (1987), Bertrand and Doble (1988)). The identification of a specific enzyme degrading ANP stimulated the development of inhibitors of NEP, and the re-evaluation of earlier enzyme inhibitors, drawing on the technology used in the design of ACE inhibitors. In 1987, when the present studies were started, this appeared to be the most reproducible way of elevating plasma ANP, and the most promising line of research. The remainder of this chapter describes the characteristics of NEP, and the development of NEP inhibitors.

Neutral Endopeptidase:

First discovered by Kenny in Leeds, NEP is a Zinc containing metallo-endopeptidase localised the brush border of the proximal tubule (Kerr & Kenny (1974), Booth & Kenny (1974)), a site rich in peptidase enzymes. At the time of its discovery no specific role for NEP was apparent. However, the discovery of endogenous opiates or enkephalins in the late 1970's was followed by the demonstration of a specific enzyme "enkephalinase" (Malfroy et al (1978)), which degraded these peptides at the Gly^3-Phe^4 amide bond (Figure 5.1). Later enkephalinase activity was detected in other organs including the kidney (Llorens & Schwartz (1981)) and was subsequently demonstrated to be identical to NEP (Malfroy & Schwartz (1984)). The enzyme has since been shown to be present to a varying extent in most tissues (Table 5.1) and to have a bewildering collection of substrates (Table 5.1) to which ANP was added in 1987 (Stephenson & Kenny (1987)).

In their initial study these authors found that ANP was degraded by kidney micro-villous membrane in vitro and the pattern of hydrolysis was similar with purified NEP. A number of degradation

products were identified with several potential sites for hydrolysis within the ANP structure, the most important site appearing to be within the 17 AA ring of ANP at the Cys-Phe bond (Cys_{105}, Phe_{106}; Figures 2.2, 2.3). Phosphoramidon, an inhibitor of NEP caused 80-100% suppression of degradation, with no affect of either ACE or aminopeptidase inhibitors (Stevenson & Kenny (1987). Other groups subsequently published similar findings (Sonnenberg et al., (1987), Olins et al., (1987), Koehn et al., (1987), Bertrand & Doble (1988)), that kidney membrane preparations degraded ANP at the Cys-Phe bond, that ANP degrading activity co-purified with NEP, was pH sensitive and was inhibited by the existing NEP inhibitors Thiorphan and Phosphoramidon.

Properties of NEP:

The gene for NEP has recently been cloned (Malfroy et al.,(1988)). The gene sequence shows a high degree of conservation (with 93% homology between rat and rabbit, NEP's and a similar degree of homology with human enzyme) and encodes for a protein of around 62,000 daltons. By hydrophobic analysis this can be shown to have a small hydrophobic membrane spanning region near the amino terminus, and a large hydrophillic region, containing the active site, projecting from the cell surface (Devault et al.,(1987), Malfroy et al.,(1987)). The enzyme contains 5 or 6 potential N-linked glycosylation sites, and estimates for the molecular weight of the glycosylated enzyme vary around 87-95,000 (Devault et al.,(1987)). NEP shares a highly conserved nucleotide consensus sequence with other metallo-endopeptidases including Thermolysin (Schwartz (1989), Devault et al., (1987), Erdos & Skidgell (1989)), particularly in the residues co-

ordinating the position of the zinc atom in the catalytic site.

Distribution of NEP:

NEP is a widely distributed enzyme (Table 5.1; Erdos and Skidgell (1989)). In the brain it is found in the globus pallidus and substantia nigra and in centres responsible for pain perception, the distribution being similar to that of endogenous opiate receptors (Malfroy et al., (1978), Llorens et al., (1982). The density of enkephalinase in the brain is appropriately regulated by exogenous opiates, and inhibition of NEP activity using specific inhibitors potentiates the effects of endogenous opiates. In the kidney small amounts of NEP are found in the glomerulus, but the densest concentration appears in the brush border of the proximal tubule, where its presumed role - like that of other peptidase enzymes - is to conserve the protein content of peptides filtered by the glomerulus (Kenny and Stephenson (1988)). The enzyme is also present in other brush border membranes including the gastrointestinal system and in the placenta (Erdos and Skidgell (1989)), and in the male in seminiferous tubules and spermatozoa (Erdos et al., (1985)). In pulmonary tissue (Johnstone et al.,(1985)), NEP is found in the alveoli and in the airways, but there is little enzyme in vascular endothelium. Recent work has demonstrated that NEP is identical to a cell surface marker expressed on lymphocytes - Calla - and neutral endopeptidase at this site may be involved in the degradation, and the regulation of the local micro environment of chemotactic peptides (Kenny et al.,(1989)).

Substrates for NEP and Design of Enzyme Inhibitors:

A **Distribution of NEP.**

Kidney (Brush border of proximal tubule).

Central Nervous System (Neuronal & synaptic membranes).

Lung (Epithelial cells).

Male Genital Tract (Testis, epididymus, prostate).

Human Neutrophils and Fibroblasts.

Thyroid.

Adrenal.

Placenta.

Gut.

B **Hormones Cleaved by NEP.**

Atrial Natriuretic Peptide.

Insulin ß chain.

Enkephalins.

Endorphins.

Bradykinin.

Substance P.

Oxytocin.

LHRH.

Angiotensins I & II.

Gastrin.

Cholecystokinin.

TABLE 5.1:

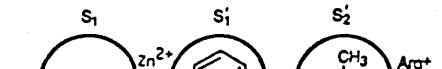
SUBSTRATES AND DISTRIBUTION OF NEP.

(A) Distribution and **(B)** Substrate specificity of NEP.
(After Erdos & Skidgell (1989), Connell & Jardine (1990).

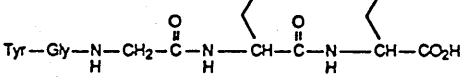
In addition to enkephalins, endorphins and a number of other neurotransmitter substances have been shown to be substrates for NEP. The distribution of the enzyme at nerve terminals and on axonal membranes suggest it has a functional role in the regulation of neurotransmitter substances. Other substrates have also been identified, and these are listed in Table 5.1 (Erdos and Skidgell (1989)). Most peptides have a molecular weight of less than 3000 daltons, similar to ANP (approximately 3100), although NEP may degrade larger peptides, such as Interleukin I. The enzyme cleaves peptides at the amino side of hydrophobic amino acids. Table 5.2 shows diagrammatically, the predicted active site of neutral endopeptidase. Using the terminology of (Schechter and Berger (1967)), where binding sites (S) and amino acid substrates (P) are labelled by their position relative to the cissile bond with e.g. S_1, S_2.... towards the amino terminal end of the molecule and S_1', S_2'.... towards the carboxyl terminal end of the molecule, NEP requires a hydrophobic amino acid at the S_1' position (Schwartz (1989)). This knowledge about subsite specificity for neutral endopeptidase has been used to design specific inhibitors. Since it is known that the natural substrate Met-enkephalin has the amino acid composition tyr-gly-gly-phe-met and is cleaved at the gly-phe bond one can see that phenylalanine fits into the hydrophobic subsite (Figure 5.1), and it seems unlikely (since glycine does not possess a side chain) that there is any specificity likely to be attributed to the P_1 and P_2 amino acids. Llorens et al.,(1980) used this rationale to explore the requirement for inhibitory dipeptide on NEP. They demonstrated that peptides the with sequence X-Ala were most potent inhibitors when X-Phe, and when dipeptides of the form Phe-X were introduced into their system then

those with a short side chain, and with a potentially charged group (carboxylate) were the most potent. Phe-Ala was the most potent inhibitor with an IC_{50} of 1μM. Similar technology led to the design of Thiorphan (Roques et al.,(1980)) and a number of other agents (Erdos & Skidgell (1989), Schwartz (1989)). With the discovery of ANP as a substrate there has been renewed interest in NEP inhibitors, and a number of newer agents are now available including SQ 29,072 (Seymour et al.,(1989a,b)), SCH 34,086 and SCH 39,370 (Sybertz et al.,(1989), Sybertz et al.,(1988)) UK 69,578 (Danilewicz et al.,(1989), Northridge et al.,(1989)). The structures of the available NEP inhibitors are shown in Figure 5.1. These molecules share a hydrophobic side chain corresponding to the hydrophobic S_1' pocket of neutral endopeptidase, and a carboxyl molecule to bind to the positively charged arginine in NEP. The P_2'-amino acid seems to be of lesser importance, and to date there has been little work on exploring the importance of peptides or the structure of the inhibitor towards the amino terminal binding regions of NEP. Danilewich et al.,(1989) describing the development of UK 69,578 (UK 73,967), noted that lengthening the molecule towards the amino terminal probably had little effect in terms of specificity other than affecting the stereo chemistry of the S_1' and S_2' binding sites. The relative potency of the available neutral endopeptidase inhibitors is given in Figure 5.1. These values have been obtained in vitro and can only be interpreted in broad terms as a guide to the possible inhibitory potency of these agents. It is known that neutral endopeptidase is pH sensitive (Schwartz (1989), Bertrand and Doble (1988)), and similarly the actions of Thiorphan as an inhibitor shows a large pH difference. The inhibitors were all tested in separate systems (with widely different

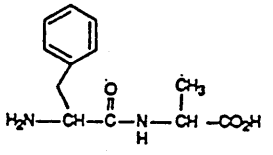
Neutral Endopeptidase



(Met) Enkephalin



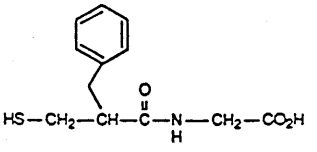
Phe-Ala



1.0±0.2μM

(Llorens et al.,(1980))

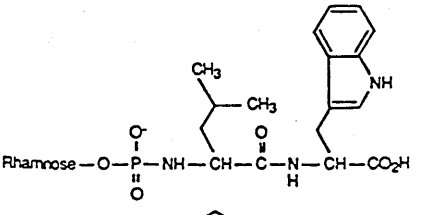
Thiorphan



2 nM

(Schwartz (1989).

Phosphoramidon



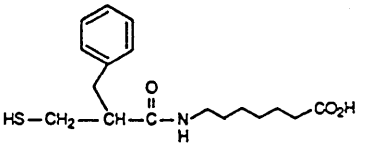
13 μM.

(Danilewicz et al.,(1990))

2 nM

(Schwartz (1989),

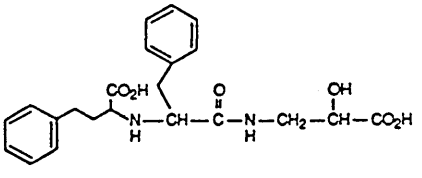
SQ 29,072



26 nM

(Seymour et al.,(1989))

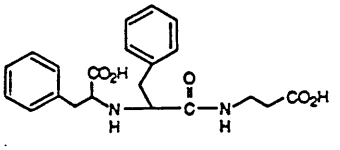
SCH 39370



11.2 nM

(Sybertz et al.,(1989))

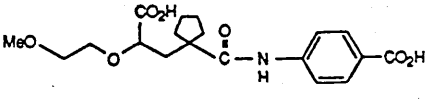
SCH 32615



19 nM

(Sybertz et al.,(1990))

UK 73,967



14 nM

(Danilewicz et al.,(1990))

(Shepperson et al.,(1991))

FIGURE 5.1:

STRUCTURE OF NEP AND NEP INHIBITORS.

Diagrammatic representation of the active site of Neutral Endopeptidase and some of the available NEP inhibitors, with estimates of Ki.

results e.g. Thiorphan - Figure 5.1), and until they are compared one against the other in a standardised preparation with careful attention to pH and buffering changes then one cannot compare these values absolutely.

Studies with NEP inhibitors on ANP:

Following the in vitro work described above (Sonnenberg et al., (1988), Olins et al.,(1987, 1989); Stephenson & Kenny (1987)) showing that NEP inhibitors prevent the degradation of ANP in vitro, a number of groups have gone on to study these compounds in vivo, with the aim of elevating endogenous ANP in the hope of finding an effective agent for hypertension and heart failure. The following four chapters describe work with UK 69,578 in rats and human, the effects of prolonged oral administration of an orally active derivative of UK 69,578 (UK 79,300) and the antihypertensive effects of NEPi using SCH 34,826. The results of in vivo studies with these agents and recent work with novel NEP inhibitors are discussed in Chapter 10.

Chapter 6.

Acute Inhibition of Neutral Endopeptidase in Rats.

Introduction.

The demonstration (Chapter 5) that NEP degrades ANP led to the development of specific NEP inhibitors (NEPi). In 1987 one such NEPi (UK 69,578, developed by Pfizer Central Research, UK) became available for human studies. This inhibitor prevents the degradation of ANP in vitro (Danilewicz et al., (1989), Northridge et al., (1989), Samuels et al., (1989)) but, at that time, although sufficient data was available for approval of the drug for human studies, there was no published data on UK 69,578. The purpose of the present study was to assess the effect of UK 69,578 using the anaesthetised rat as a simple experimental model. The effects on urine volume, electrolyte excretion and plasma ANP were assessed and compared with low-dose infusion of ANP (5 pmol/Kg/min).

Methods.

Male Sprague-Dawley rats (350-420 g, Olac, UK) were anaesthetised

with Pentobarbitone Sodium (60 mg/ml, Sagital, RMB Animal Health Ltd., UK.) 0.1 ml/kg intraperitoneally and anaesthesia maintained by intravenous administration of 0.05 ml as required (approximately 0.2ml/hr). The left carotid artery and jugular vein were cannulated using PE 30 tubing, the bladder with PE 50 tubing (10 cm, and the trachea with PE 240 tubing (3cm). 0.45% sodium chloride solution (2ml/hr) was infused into the jugular vein by infusion pump (Vial Medical, France), and drugs administered through a 3-way tap in the tubing. The arterial cannula was connected to a pressure transducer (Stratham PD 23, Gould Electronics, UK) and intra-arterial pressure monitored continuously on a chart recorder (Elcomatic 720A, Elcomatic Ltd., UK.). Urine was collected into pre-weighed 1ml tubes and the experiment abandoned if there was blood staining. The cannulation procedure took approximately 30 minutes after which the rat was allowed to equilibrate for 60 minutes. Thereafter, the following protocol was followed. After the 60 minute run-in period ten 15 minute urine collections were made. Following the first two (basal) a blood sample was taken (0.5 ml from the arterial line, replaced with an equal volume of 0.45% saline) and drug/ placebo (in 0.5 ml 0.45% saline) administered via the venous line. Over the next eight urine collections 0.45% saline was administered as before or replaced with ANP (rANP, Bachem Ltd., UK,[5 pmol/Kg/min]; prepared as in Chapter 4, Richards et al.,(1988a)) dissolved in 0.45% saline. At the end of the sixth collection period a 0.5 ml blood sample was taken and replaced with an equal volume of 0.45% saline, and at the end of the tenth period 3-5 mls of blood were withdrawn through the arterial line and the animal killed by injection of 1.0 ml Sagital. The blood samples were collected into tubes containing 100µl 50mM EGTA + Trasylol 100

i.u. on ice. Blood was separated at 4°C and plasma stored at -20°C until assay for ANP (Appendix 1). Urine volume was calculated by weighing tubes and electrolytes (Na,K) measured using a Beckman model 2A analyzer (Beckman Instruments, Ireland).

Overall there were five treatment groups: Placebo (n=6), ANP alone (n=6), and UK 69,578 alone (1,10,100 mg/Kg; n=5 in each group).

Statistical analysis. Urinary data were analyzed as area under the curve (periods 3-10) and compared against placebo by t-test with the Bonferroni correction for repeated comparisons. For the plasma ANP data, overall treatment effects were compared with placebo using ANOVAR with repeated measures and subsequent comparison by dose at individual time points by t-test with the Bonferroni correction (Godfrey (1981), Wallenstein et al.,(1981)).

Results:

The results are summarised in Figure 6.1. Urine volume, sodium and potassium excretion were stable during the placebo limb, and there were no differences in these parameters during the baseline periods (1-2) between experimental groups. During infusion of 5 pmol/kg/min ANP there was a gradual increase in sodium excretion which reached a peak after 60 minutes and which persisted, declining slowly, thereafter. Urine volume and potassium excretion showed similar trends. After administration of 1mg/kg UK 69,578 there was a gradual increase in urine volume and sodium excretion which failed to achieve statistical significance, but there was a rise in potassium excretion. Sodium and potassium excretion and urine volume were all increased following 10 and 100 mg/kg UK 69,578. The temporal pattern of these changes and their magnitude was very similar to that produced by

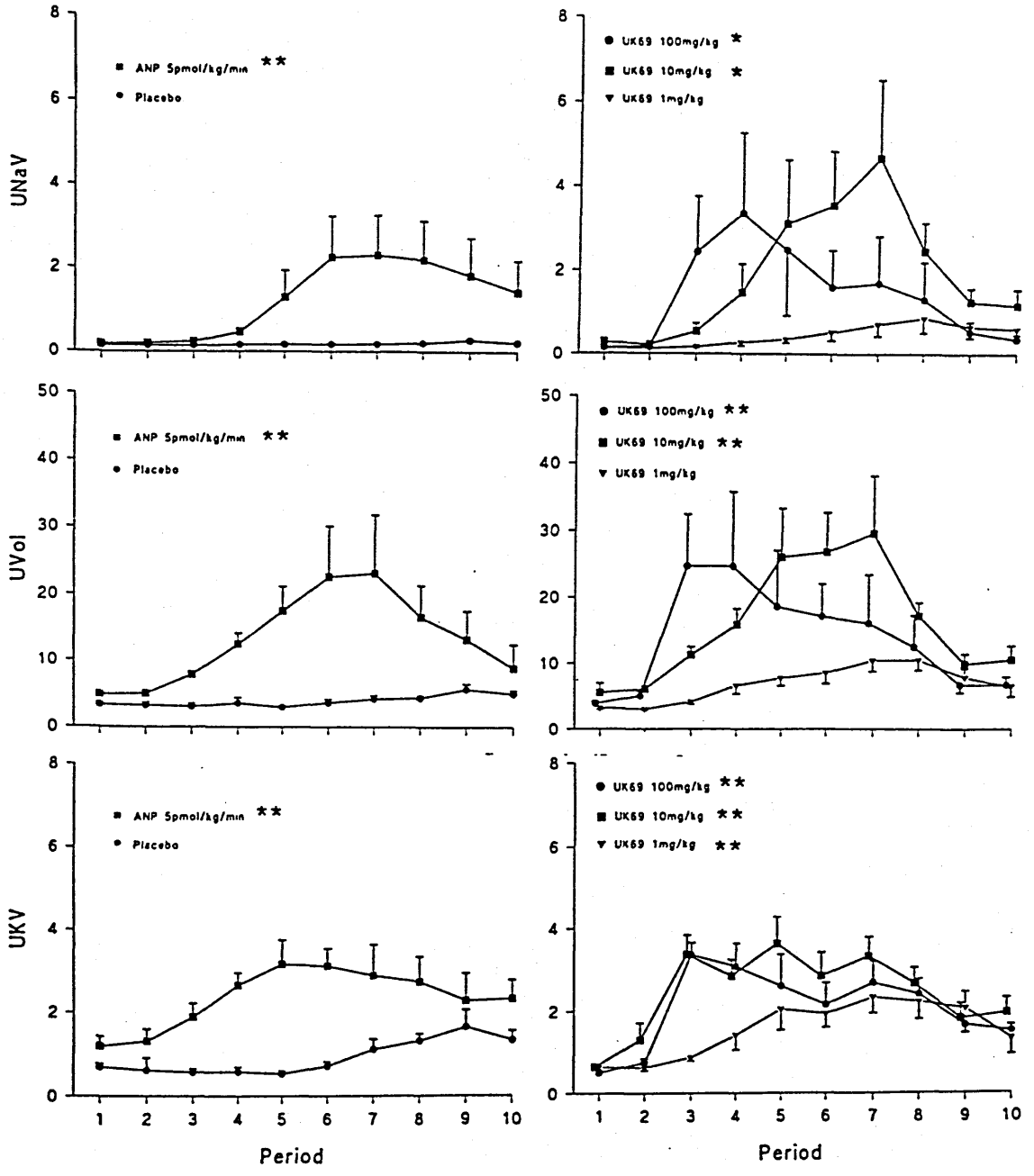


FIGURE 6.1:

EFFECTS ON RENAL FUNCTION OF ANP AND UK 69,578 IN THE ANAESTHETISED RAT.

Urinary sodium and potassium excretion (UNaV, UKV, µmol/min) and urine volume (UVol, µl/min) in response to treatment with ANP 5 pmol/kg/min given during periods 3-10 (left panel), or UK 69,578 (UK 69, 1, 10 or 100 mg/kg) given as a bolus after period 2. Data are shown as meanSEM. Statistical analysis is by comparison of area under the curve (Periods 3-10) by T-test, * $P<0.05$, ** $P<0.01$ vs. Placebo.

infusion of ANP in the 10 mg/kg group. However, the highest dose of UK 69,578 produced a more rapid rise in urine volume and sodium excretion, which began to decline after 30 minutes. Arterial pressure (not shown) was not significantly altered by any treatment. Plasma ANP remained stable in the placebo group with levels similar to those reported by other workers in rats (see chapter 9) between 50-100 pg/ml. Only in the group receiving 100 mg/kg UK 69,578 was there a significant effect of treatment with UK 69,578 with time (ANOVAR P=0.042), but no significant effect of ANP or individual doses of UK 69,578 at individual time points (Figure 6.2).

Discussion.

This was a preliminary study aimed at addressing the question of whether NEP inhibition, and specifically UK 69,578, has any in vivo effects on renal function and plasma ANP. Only a small group of animals was studied and this is reflected by the large variability shown in Figure 6.1. Simple statistical methods (analysis of area under the curve) were employed, with the aim of establishing the presence or absence of effect, and the time course data are essentially descriptive. Even with these reservations it is clear that NEP inhibition by UK 69,578 can increase plasma ANP and that there is an increase in urine volume and sodium excretion which, especially in the mid-dose group, closely parallels the effects of ANP infusion.

Effects of ANP: The plasma levels of ANP in the control periods of each experimental group are broadly similar to those reported in other studies in normal rats (Soejima et al.,(1988)). Infusion of 5pmol/kg/min ANP produced a modest, two to three-fold increase in circulating hormone levels well within the reported pathophysiological

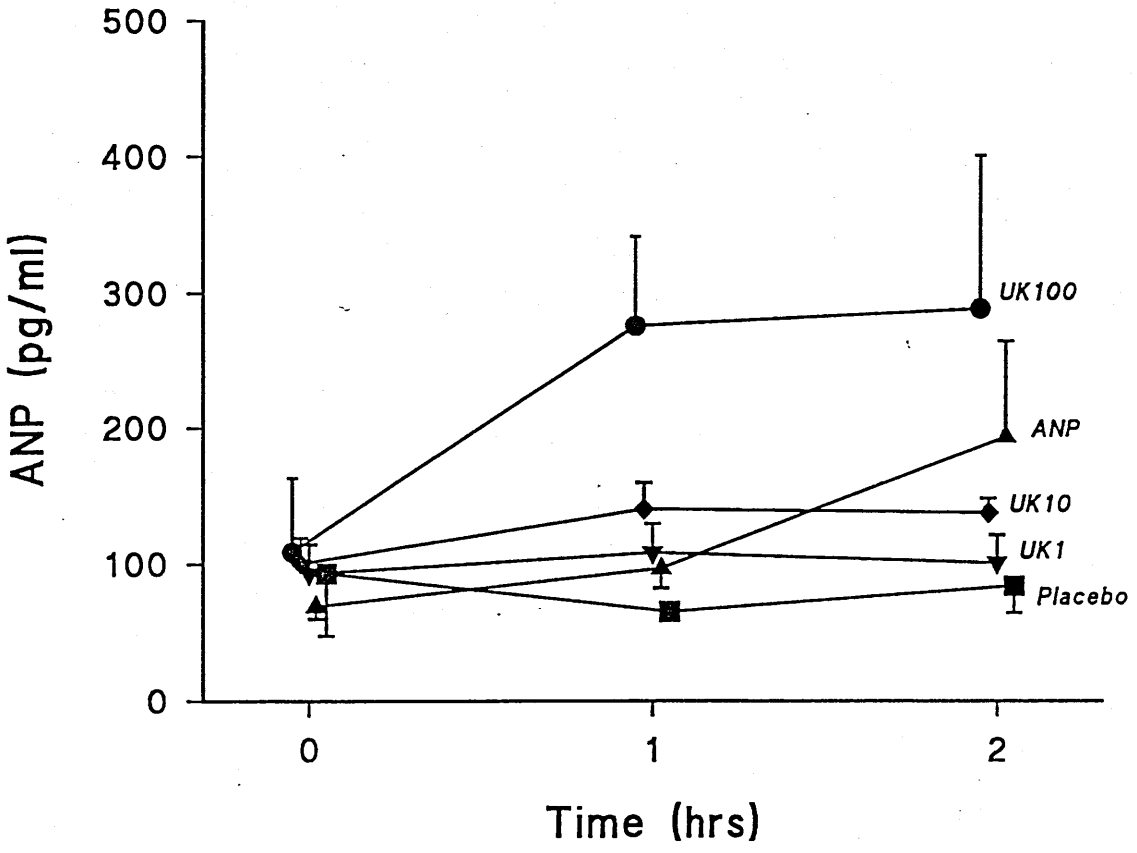


FIGURE 6.2:

EFFECTS ON PLASMA ANP OF ANP INFUSION AND UK 69,578 IN THE ANAESTHETISED RAT.

Plasma ANP (pg/ml) before and during infusion of ANP (5pmol/kg/min, ▲ , periods 3-10), UK 69,578 (1, ▼ ,10, ◆ ,100, ● mg/kg , given as a bolus after period 2) or placebo (■). All treatments started at time 0. Data are shown as mean±SEM, n=5 for each of the UK 69,578 limbs and n=6 for the ANP and placebo limbs.

range in the rat (Soejima et al.,(1988), chapter 9). The response seen with a slowly rising natriuresis and diuresis is similar to that reported by other workers in the rat (Soejima et al.,(1988)), dog (Chapter 4, Pichet et al.,(1988), Cernacek et al.,(1987)) and in man (Richards et al.,(1988a,b), Morice et al.,(1988), Anderson et al.,(1987), Cottier et al.,(1987), Solomon et al.,(1988)). The increase in potassium excretion is greater than predicted from these studies but has been well documented in all species with high dose infusion (Chapter 2, eg deBold et al.,(1981), Richards et al.,(1986)), and in the rat is known to be dose related (Soejima et al.,(1986)). In the present series of experiments dietary electrolyte intake was not controlled and may contribute to the kaliuresis.

Effects of UK 69,578: There is a striking similarity in the pattern of urine volume and electrolyte excretion produced by bolus administration of UK 69,578 and the infusion of ANP in the present series of experiments. The sequence of changes at 10 mg/kg/min almost paralleled the response to ANP infusion. At 1 mg/kg little effect was apparent except on potassium excretion and 100mg/kg there was a more rapid onset of effect. While it is difficult to infer much from such a small series, it may potentially reflect a more rapid rise in plasma ANP, or a local effect at extravascular sites perhaps in the kidney. Alternatively, higher doses may conceivably be less specific and the different time course may reflect the involvement of, for example, intrarenal kinins (Ura et al.,(1987), Scicli and Carretero (1989)) in the natriuresis. Although there was a significant treatment effect on plasma ANP, hormone levels were not significantly elevated by individual doses. This absence of a significant dose effect is likely to reflect the small sample size and the large variability in ANP

levels in this study (Figure 6.2) rather than a genuine absence of effect. There was no hypotensive effect of NEPi in the present study, but this is not surprising in view of the modest increment in circulating ANP, significant effects on blood pressure having been reported only with higher plasma levels in the rat (Soejima et al.,(1988)).

In conclusion, two findings emerge from this study. Firstly, that NEP inhibition causes renal effects similar to ANP and secondly, there is a strong suggestion that plasma ANP is increased, providing circumstantial evidence that NEP inhibition acts via ANP. Similar findings have since been reported by other workers in experimental animals (Chapter 10) and justify the investigation of the effects, and potential therapeutic actions, of these agents in man (Chapters 7 & 8).

Chapter 7.

Acute administration of UK 69,578 in normal man.

Introduction.

The demonstration that NEP inhibitors reduce the degradation of ANP in vitro (Chapter 5) and in experimental animals (Chapter 6) raises the question of whether similar (potentially therapeutic) effects occur in man. UK 69,578 (Pfizer UK) was approved for experimental studies in man in 1987. This chapter describes the first study of this compound, and of the effects of NEP inhibition, in human subjects.

With a novel type of compound such as UK 69,578 there are particular problems in study design. First, the list of substrates for NEP is large, and inhibition of degradation of (for example) neuropeptides and kinins might be predicted to cause haemodynamic and psychotropic effects not detectable in experimental animals. For this reason this study was designed to cautiously explore the dose-response relationship, the main objective being to establish the safety of this compound in man. The other aims were to examine the effects of NEP

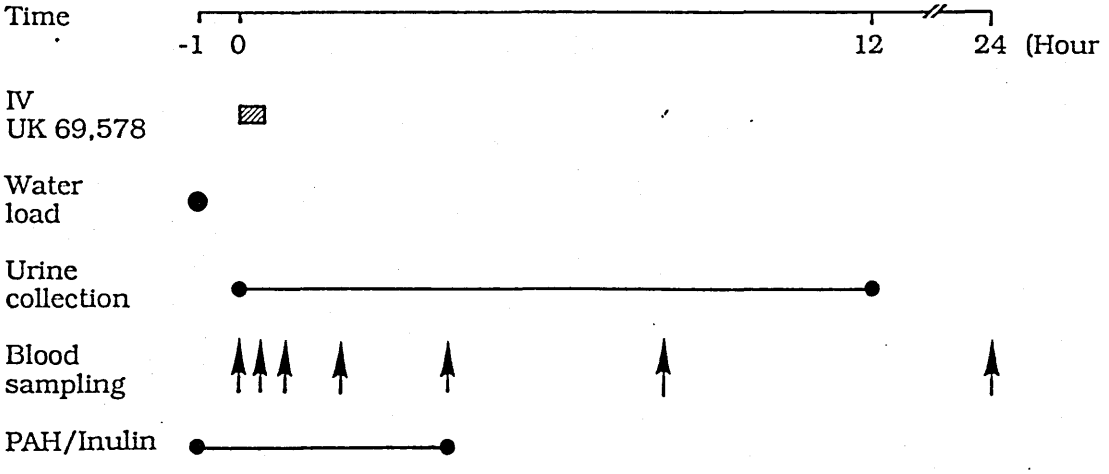
inhibition on plasma ANP and the components of the RAAS; effects on renal function and arterial blood pressure. At the outset it was intended to examine renal function in detail using PAH and inulin clearance methods. However, it was not possible to establish that either inulin or PAH did not interfere with infusion or assay of UK 69,578 until after the lower dosage groups were completed. For this reason studies with PAH and inulin were only included in the higher dosage groups, and the analysis of these data is viewed as a separate study within the context of the large dose ranging study (see below, Figure 7.1).

Methods.

Sixteen normal male volunteers were studied. The subjects were between 21 and 49 years, were within 10% of the predicted normal body weight for height, and had no abnormality in medical history, clinical examination, biochemical profile, full blood count, ECG and dipstick urinalysis. All subjects gave informed written consent prior to involvement in the study, which was approved by the hospital Ethical Committee.

Each subject was studied on four separate occasions at least one week apart. The subjects were divided into four groups of four and each subject received three doses of the compound plus a placebo. Within each group the doses were arranged in ascending order, but the placebo was randomly inserted. The four dosage groups were also studied in ascending order, and the doses were arranged so that the highest dose in one group overlapped with the lowest dose in the subsequent group, providing data on eight subjects at these dosage levels for subsequent statistical analysis. The dose-range of 0.025

A



B

Group 1.	0.025	0.05	0.1						P
Group 2.			0.1	0.2	0.5				P
Group 3.					0.5	1.0	2.0		P
Group 4.							2.0	5.0	10.0	P

FIGURE 7.1:

PROTOCOL FOR ADMINISTRATION OF UK 69,578 IN MAN.

Outline of protocol (A) and dosage groups (B). Dose of UK 69,578 in mg/kg, P=placebo. Inulin and PAH clearance were studied in groups 3 & 4 (see text for details).

to 10.0 mg/kg was studied, and the dosage schedule is illustrated in Figure 7.1.

On each occasion the subjects were studied on the 4th day of a diet containing 150 mmols of sodium and 70 mmols of potassium per day, provided by the hospital dietician. They continued on this diet until the morning following the study day. On the day of study the subjects were admitted to the Investigation Ward at 6am; two heparinised cannulae (venflon, Viggo, Sweden) were inserted into opposite forearm veins, one for blood sampling, the other for infusion, and following this subjects remained supine (except to pass urine) for the duration of the study. After a short period of acclimatization, they received an oral loading dose of 350 mls tap water, and thereafter hourly urine collections were made for 13 hours, the urine volume being replaced by an equal volume of drinking water. Urine volume was measured, and aliquots taken for measurements of sodium, potassium and ANP. After one hour, basal blood samples were taken for measurement of electrolytes, haematocrit, active renin concentration, angiotensin II, aldosterone and ANP. UK 69,578 or placebo (normal saline) were then administered by a 20 minute intravenous infusion and blood sampling was repeated at 2, 8 and 24 hrs post-dosing. In addition, blood samples were taken for pharmacokinetic analysis at 0.3, 0.5, 1, 1.5 and 4 hrs post-drug administration, and for ANP at 0.5, 1 and 4 hrs. The electrocardiogram was monitored continuously during the first 4 hrs, and blood pressure measured using a digital semi-automatic sphygmomanometer (Copal, Takeda, Japan). The subjects were allowed home 12 hours after dosing, and returned to the Unit at 24 and 96 hrs when after a period of supine rest for 30 mins, blood pressure was recorded and blood samples repeated.

In the two higher dosing groups PAH (MSD, Rahway, NJ, USA), 0.5g and inulin (Laevosan-Gesellschaft, Linz, Germany), 3.0 g were given by bolus injection 1 hr before administration of the drug and followed by a constant infusion of PAH (0.5 g/h and inulin 1.0 g/h) for 5 hrs (i.e. until 4 hrs after administration of the compound.) Renal plasma flow (ERPF) and glomerular filtration rate (GFR) were calculated from the plasma levels of PAH and inulin respectively according to the method of Schnurr et al.,(1980)). In these dosage groups the subjects received both placebo and 2mg/kg UK 69,578 and this subset of the study was analysed separately as a simple comparison between 2mg/kg UK 69,578 and placebo in eight subjects.

Assays: Active renin concentration (Millar et al.,(1981)), angiotensin II (Morton & Webb (1985)), and aldosterone (Fraser, Guest & Young (1973)), Inulin (Varley et al.,(1980), PAH (Waugh and Beall (1974)) were measured by standard methods. Samples for ANP were collected into chilled tubes containing aprotonin (Trasylol, Bayer UK 100 IU/ml) and were measured by radioimmunoassay with pre-extraction of plasma samples as described in Appendix 1 (Richards et al.,(1987)). Full blood count and haematocrit were measured by coulter counter (Coulter Electronics, Miami, USA), plasma electrolytes and liver enzymes by autoanalyser. Urea and electrolytes were measured by ion sensitive electrode (Beckman Model 2A, Beckman Inc., Ireland) and urine ANP was measured by a modified version of the plasma method (Richard et al.,(1987), Appendix 1). This involved extraction of 10ml of urine (mean recovery 84%, n=16), with a lower limit of detection of 2pg/ml urine.

Pharmacokinetics: These estimations were performed by Dr. NJ Cussans of Pfizer (UK)). Plasma concentrations of UK 69,578 were assayed using

EC 3.4.24.11 prepared from rat kidney as standard. Unknown drug concentrations in diluted plasma and urine were calculated from the inhibition of hydrolysis by the endopeptidase of ^{14}C-labelled Hippuryl-L-phenylalanyl-L-arginine. Hydrolysis was quantified by the release of labelled uric hippuric acid, determined by liquid scintillation counting. Plasma and urine samples were assayed against known concentrations of UK 69,578. The area under the plasma concentrations versus time curve (AUC) was calculated using the trapezoidal rule for measured plasma levels up to 8 hrs post-dose and the term in elimination rate concentration (ß) calculated from the slope of the curve at 8 hrs.

Statistical Analysis: Statistical analysis for the dose ranging study is by crossover analysis of variance (ANOVAR), using the GENSTAT 5 computer package (GENSTAT (1984)) and was performed by Dr. Glen Andrews of the Statistics Department at Pfizer (UK). Subject, period and treatment where included as factors in the model. In addition to compensate for baseline variation the baseline measurements for each subject were used as covariates in the analysis and treatment means adjusted for baseline variation in the subsequent analysis. Where significant differences were apparent by Anovar, individual timepoint comparisons were made using Dunnet's procedure (Dunnett (1955)).

In the subset where inulin and PAH clearance were measured comparison between 2mg/kg UK 69,578 and placebo is by repeated measures analysis of variance (MANOVAR, SPSS/PC+, SPSS Inc, USA), with post-hoc analysis by paired t-test.

Results:

Dose Ranging Study:

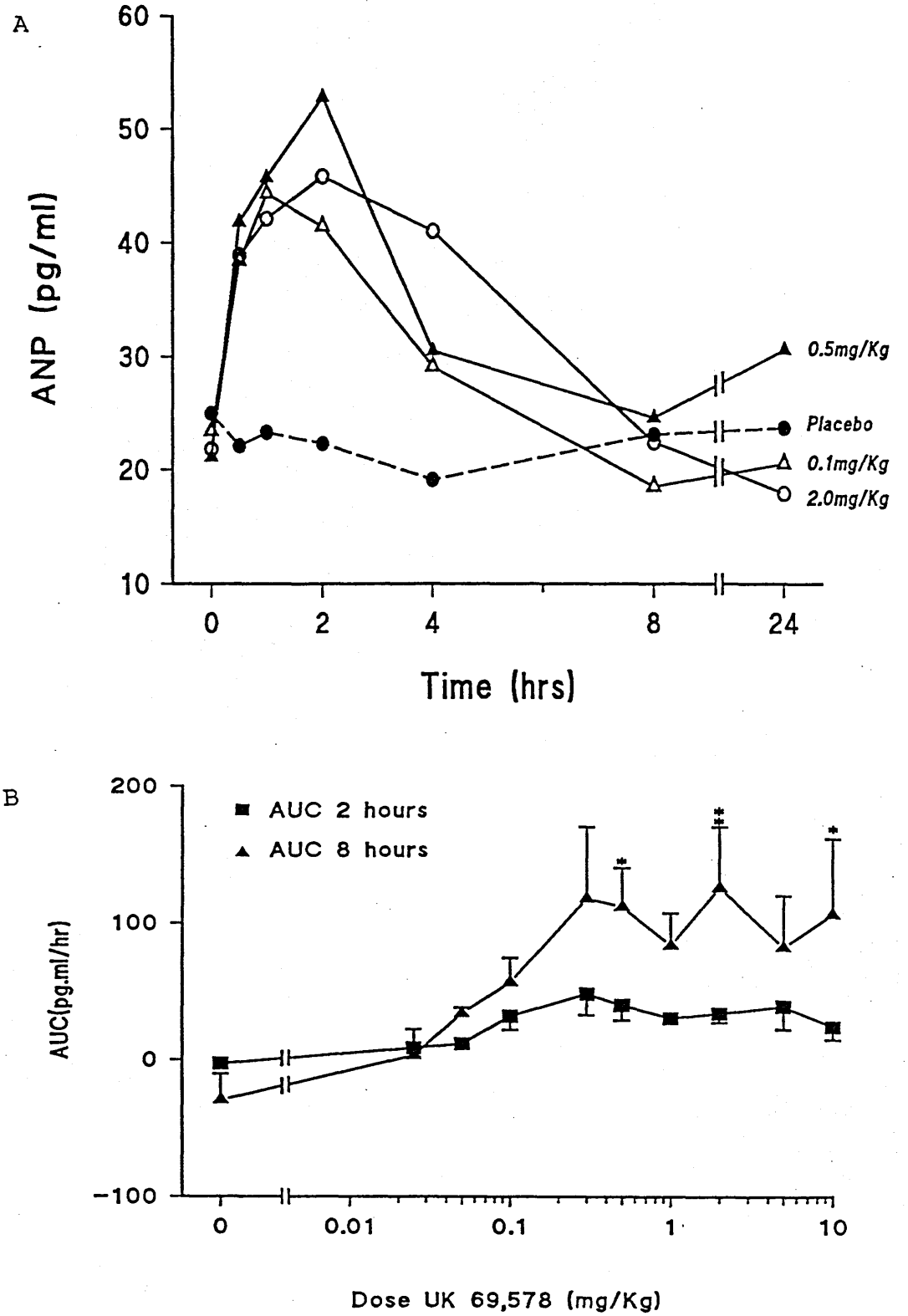


FIGURE 7.2:

PLASMA ANP RESPONSE TO UK 69,578 IN MAN.

(A) Mean plasma levels of ANP (pg/ml) following infusion of UK 69,578 at 0.1 (△), 0.5 (▲), and 2.0 (○) mg/Kg or placebo (●). N=16 (placebo) and n=8 (all other groups). See Table 7.1 for complete data.

(B) Area under the plasma ANP curve (pg.hr/ml) against dose of UK 69,578 (mg/kg). N=16 (placebo), n=8 (0.1, 0.5, 2.0 mg/Kg) and n=4 (all other groups). * P<0.05, ** P<0.01 by ANOVAR; data shown as mean+SEM

The administration of UK 69,578 was well tolerated in all subjects, and there were no reported side effects attributable to the compound. There were no significant changes in plasma electrolytes, liver transaminases, blood count, haematocrit, ECG or blood pressure at anytime after administration of UK 69,578.

Plasma ANP:

Plasma ANP rose rapidly after administration of the compound reaching peak levels of between two and three times basal value within 120 mins of drug administration. The levels then slowly returned to normal within 8 hrs (Figure 7.2). There were no changes in plasma ANP following placebo. A clear effect of the compound on plasma ANP was evident at doses of 0.1 mg/kg and above. Above this dose there was little effect on the peak levels of plasma ANP achieved although there is an impression that the effect may have been prolonged. Analysis of the area under the curve (AUC) for plasma ANP (0-8 hrs) revealed a significant increase in AUC at 0.5 mg/kg and above (Figure 7.2, Table 7.1). However, at doses above 0.5 mg/kg there was no evidence of further significant increase in AUC, suggesting that a near maximal effect had been achieved at this dose, although the true dose-response relationship may be obscured by the differing numbers of subjects in each dosage group, interindividual variation in response to UK 69,578, and the limited number of sampling times.

Renal function:

Sodium excretion: Compared with the placebo group, urinary sodium excretion increased following UK 69,578 with maximal changes in the slope with a cumulative sodium curve over the first 6hrs post-dose, and the curves tending to run parallel thereafter (Figure 7.3). The

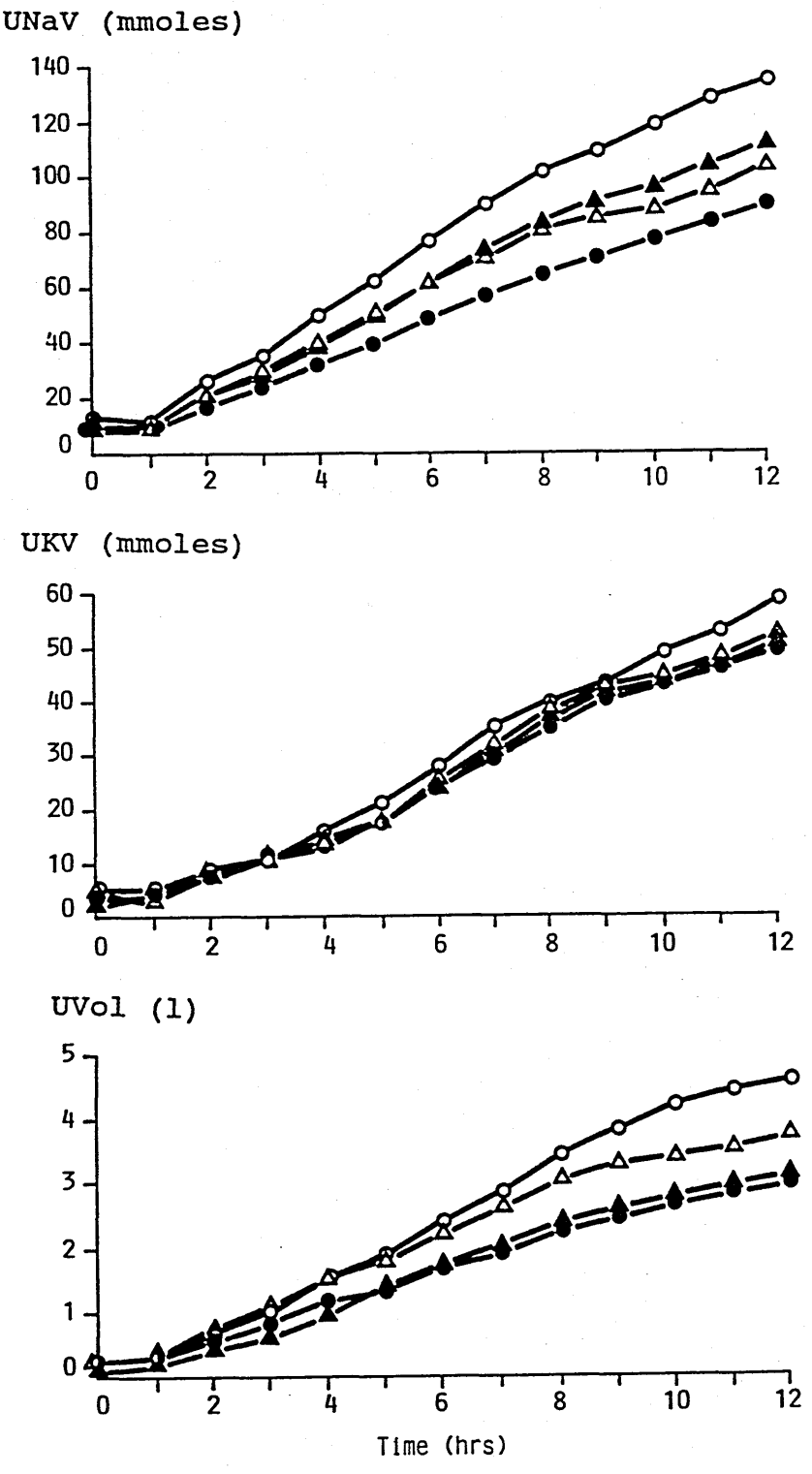


FIGURE 7.3:

RENAL EFFECTS OF UK 69,578 IN MAN.

Mean cumulative urine volume (UVol), sodium (UNaV) and potassium (UKV) excretion for 12 hours following administration of UK 69,578 0.1 (△), 0.5 (▲), and 2.0 (○) mg/Kg or placebo ● (n=16, placebo; n=8 all other groups). See Figure 7.5 for full data.

mean cumulative 8 hour excretion of sodium rose from 64.9 mmols in placebo (appropriate to a dietary intake of 150 mmols/day) to 116.1 mmols on a maximum dose (Figure 7.5). Statistically significant increases were evident to doses above 0.3 mg/kg, and the pattern of increase with dose was similar to that for ANP with near maximal increases being achieved at 0.3 mg/kg and only a small upward trend thereafter.

Figure 7.6 shows a relationship between area under the curve for plasma ANP and cumulative urine sodium excretion over 8 hrs. It is clear from this that there is an approximately linear relationship between the area under the curve for ANP and urinary sodium excretion, between doses of 0.025 mg/kg and 0.5 mg/kg, but there is little further increase in either area under the curve for ANP or urinary sodium excretion at doses above this.

Potassium excretion: There were no significant alterations in potassium excretion following UK 69,578 (Figures 7.3, 7.5).

Urine volume: increased following UK 69,578 with doses above 2mg/kg (Figure 7.3). Although there is a clear increase in a cumulative urine volume (Figure 7.5), it is difficult to quantify beyond the first part of the curve since oral water loading will have reinforced the prolonged the early diuresis (this is more clearly shown in Figure 7.8).

Urinary ANP excretion: The limit of the detection of the urinary ANP assay was 2 pg/ml. A high urinary output caused by waterloading and UK 69,578 diluted ANP to below the limit of detection in some patients, particularly in the lower dose groups. However, despite the increased urine flow with higher doses of UK 69,578, ANP was detectable in the urine and at doses of 2mg/kg and above urinary ANP

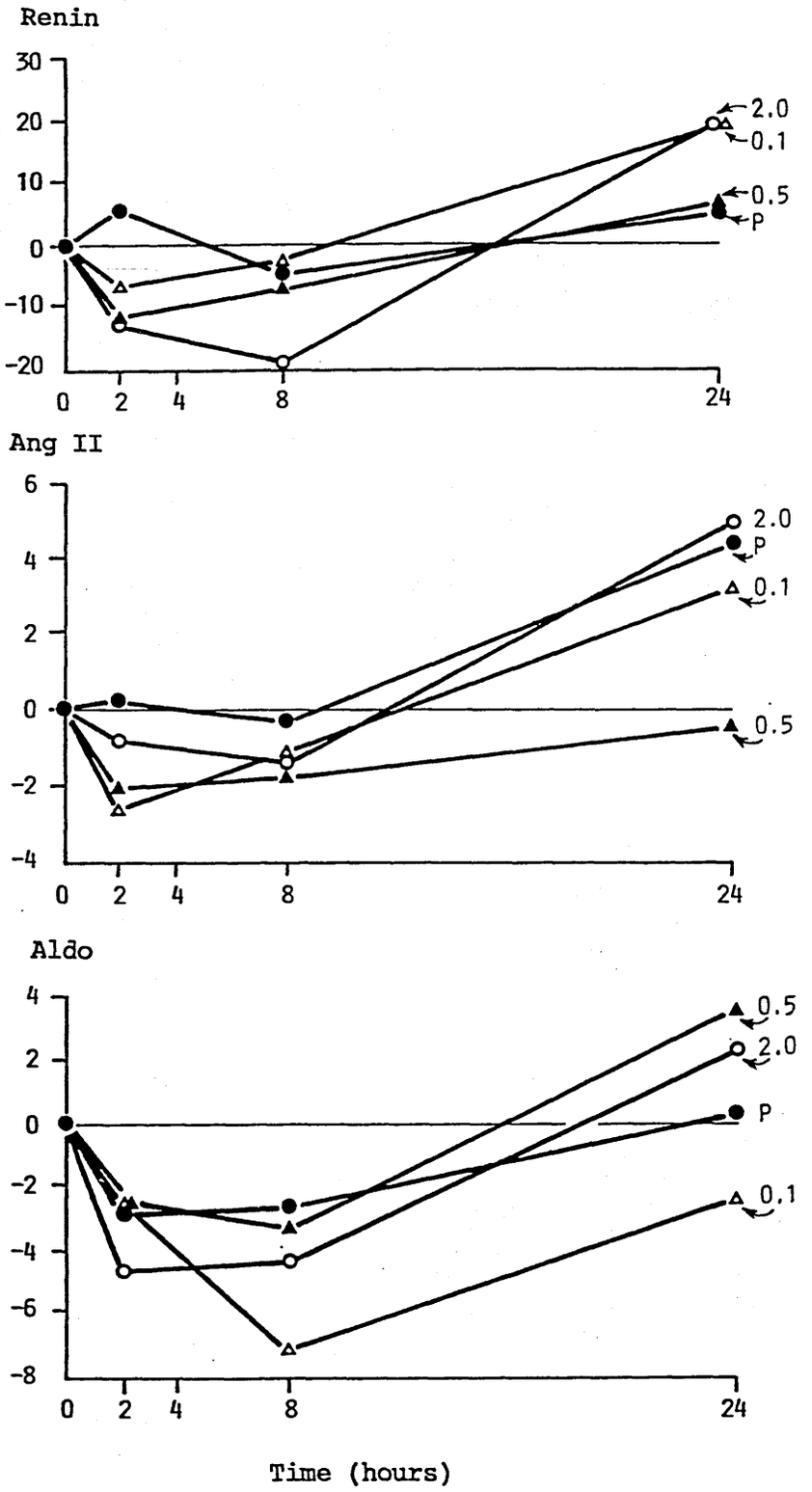


FIGURE 7.4:

HORMONAL EFFECTS OF UK 69,578 IN MAN.

Changes in mean plasma active renin concentration (μU/ml), Ang II (pg/ml), and aldosterone (ng/dl). Data are shown as change from basal following 0.1 (△),0.5 (▲) and 2.0 (o) mg/Kg UK 69,578 or placebo (●). N=16 for placebo group and 8 for the other groups. See Table 7.1.

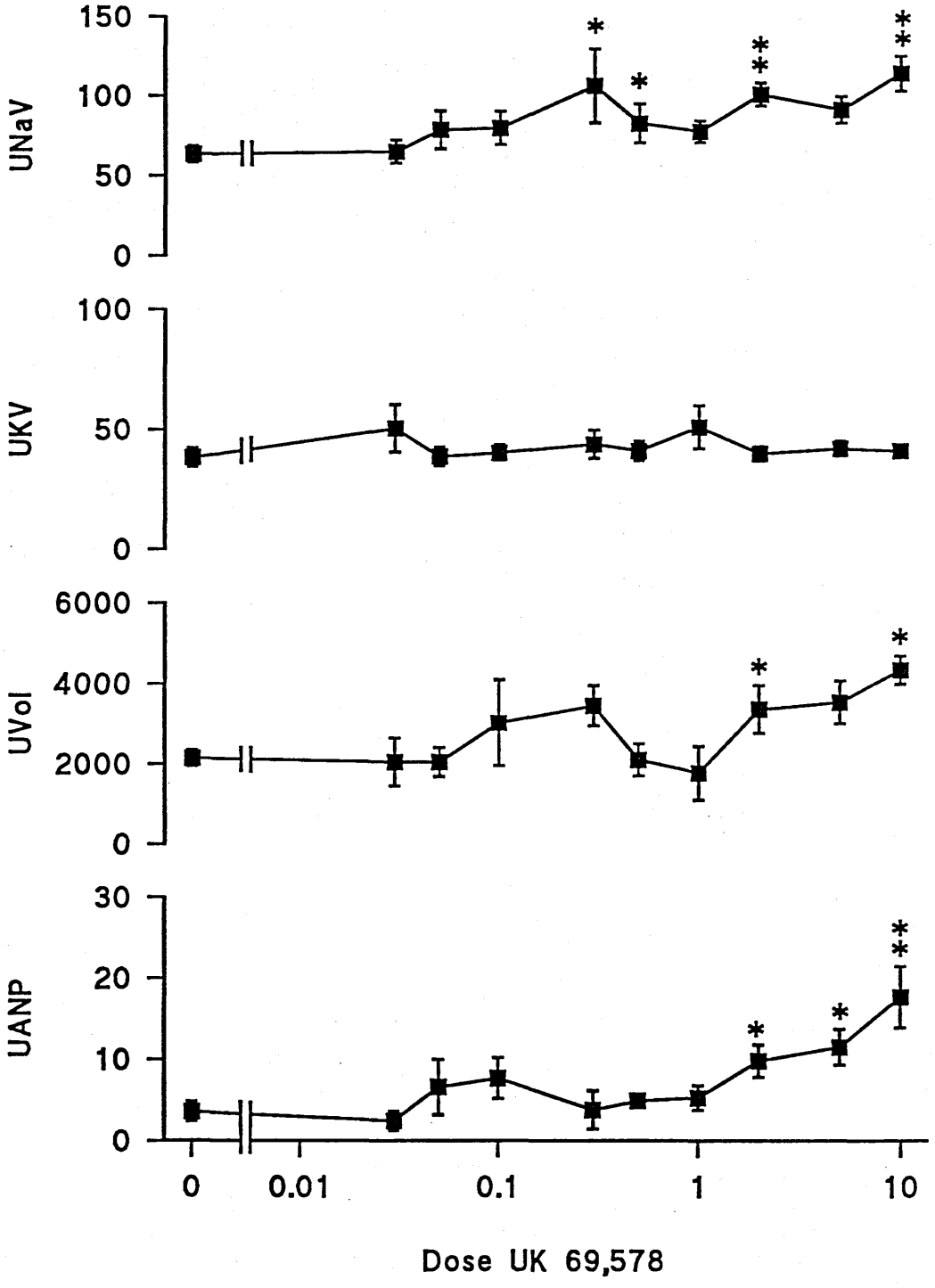


FIGURE 7.5:

DOSE RESPONSE RELATIONSHIP BETWEEN UK 69,578 AND URINE VOLUME, ANP AND ELECTROLYTE EXCRETION.

Summary of the dose-response relationship between dose of UK 69,578 and Cumulative urine volume (UVol, ml/8hrs), sodium (UNaV, mmoles/8hrs), potassium (UKV, mmoles/8hrs) and ANP (UANP, ng/8hrs). Data are given as Mean±SEM, n=16 (placebo, 0), n=8 (0.1, 0.5, 2.0 mg/kg), n=4 (all other doses). * $P<0.05$, ** $P<0.01$ by ANOVAR against paired placebo values.

excretion was higher than placebo (Figure 7.5; the timing of the changes is more clearly shown in Figure 7.8).

Plasma renin, angiotensin and aldosterone (Figure 7.4, Table 7.1):

Plasma concentration of active renin was relatively unchanged after placebo, but showed a downward trend following UK 69,578 for up to 8 hrs post-dose in the higher dosage groups. The suppression of plasma renin at 2 hrs was statistically significant at the 2mg/kg dose ($p<0.01$) and there was a rebound rise in renin at 24hrs, which was statistically significant in the 0.3 and 1 mg/kg dosage groups. Plasma angiotensin II and aldosterone showed similar trends, although neither reached statistical significance.

Pharmacokinetics:

Pharmacokinetic data for UK 69,578 were derived by fitting the data to a one compartment model. The elimination half-life of the compound was 1.1±0.2 hrs (mean±SD). The volume of distribution was 0.25±0.05 kg with 56±8.2 % of the excretion being renal within the first 12 hrs. The plasma area under the curve (µg.hr/ml; 0-8 hrs) was 6.1±1.0 µg/ml/mg/kg. Full pharmacokinetic data is given in Appendix 3.

Renal Function Study (2 mg/kg subgroup):

The data presented in Figure 9-12 comes from the subset of subjects in whom renal function was studied using inulin and PAH clearance. I have taken the opportunity to present illustrative data on blood pressure, urine volume, ANP and electrolyte excretion in addition to GFR, ERPF and fractional excretion of sodium.

Figure 7.7 shows systolic blood pressure, diastolic blood

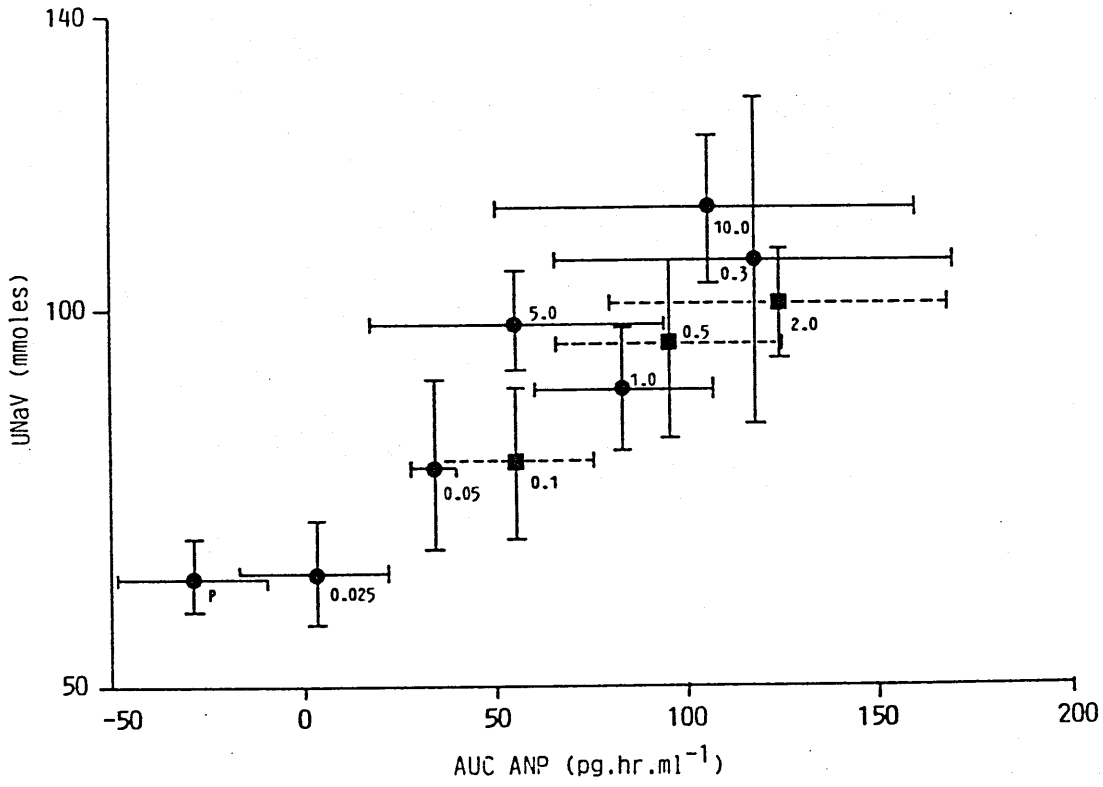


FIGURE 7.6:

RELATIONSHIP BETWEEN DOSE OF UK 69,578, PLASMA ANP AND SODIUM EXCRETION.

Relationship between area under the plasma ANP curve and cumulative urinary sodium excretion in the 8 hours following administration of UK 69,578 or placebo. Doses of UK 69,578 as marked; n=16 placebo, solid squares represent n=8, solid circles n=4. Data are given as mean±SEM.

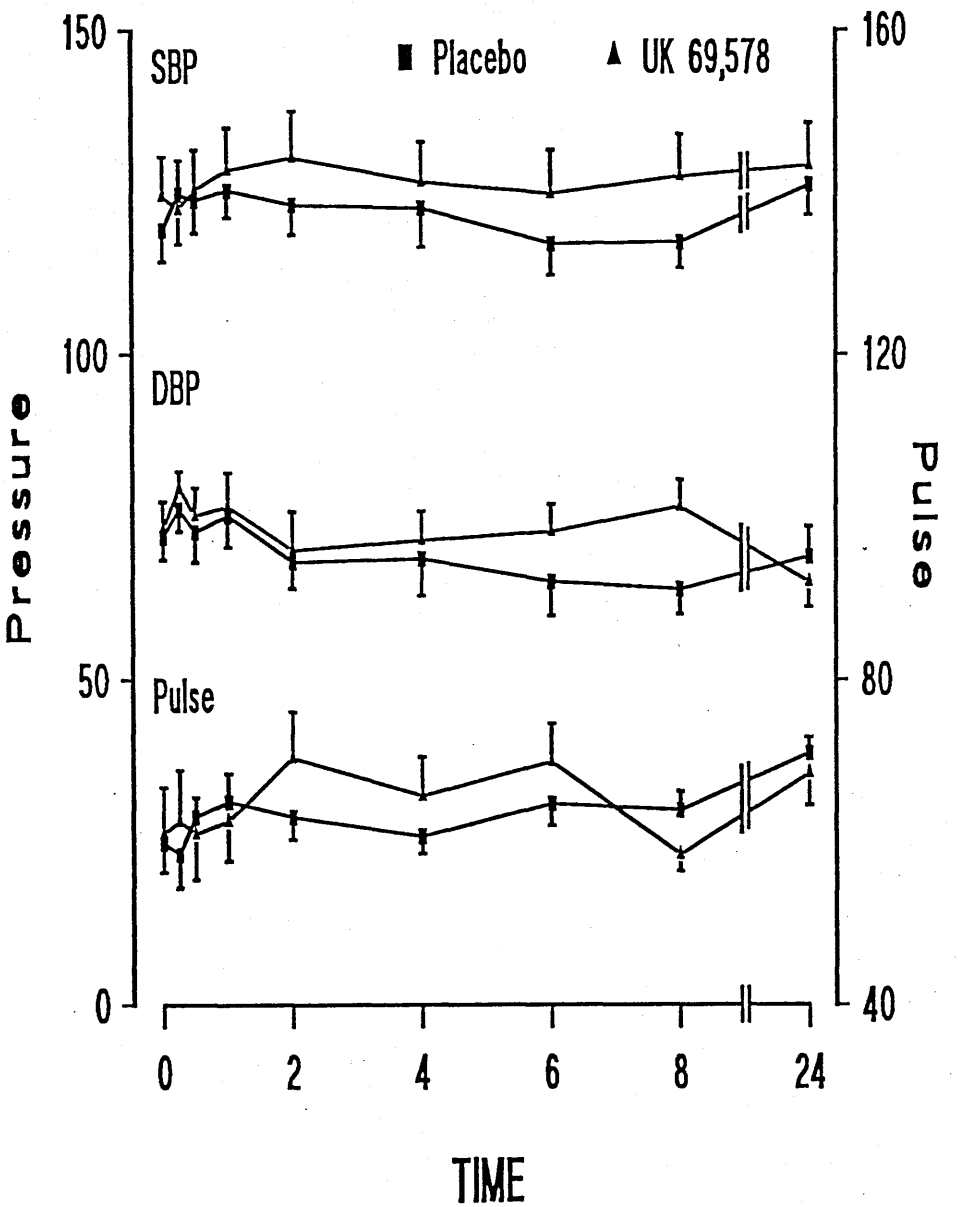


FIGURE 7.7:

CHANGES IN BLOOD PRESSURE AND PULSE IN RESPONSE TO UK 69,578 (2 mg/Kg).

Systolic blood pressure (SBP, mmHg), diastolic blood pressure (DBP, mmHg) and pulse (beats per minute) for 2mg/kg UK 69,578 vs matched placebo (n=8). Data are given as mean±SEM. There were no statistically significant effects.

pressure and pulse for the 24hrs following administration of 2mg/kg UK 69,578 or placebo. Although blood pressure and pulse were slightly lower on the active limb this was clearly not significant. Figure 7.8 demonstrates the time course of urinary electrolyte excretion in this group of subjects, more clearly than the cumulative graphs demonstrated in Figures 7.3 and 7.5. Urinary sodium excretion increased by 2hr post-administration of UK 69,578, and remained elevated until 7hrs post-dose. Potassium excretion was unchanged, and the increase in urine volume was more variable. The time course of urinary ANP excretion is shown and clearly parallels the changes in plasma hormone levels (Figure 7.2, Table 7.1.).

Renal Function Indices: Glomerular filtration increased in both active and placebo limbs. Analysis of the absolute GFR data was non-significant, although the active limb was slightly higher when percentage increases in GFR were studied (Connell and Jardine (1990)). ERPF showed a similar rise in both groups which failed to reach statistical significance. There was no difference in filtration fraction, and although fractional excretion of sodium was higher in the active group, by analysis of variance, this reached significance only at the 2hrs post-dose time point.

Discussion:

At the time of publication (Jardine et al.,(1989,1990)) this was the first study to report the effects of NEP inhibition in normal subjects. It demonstrates that acute administration of NEP in man causes a rise in plasma ANP, in a dose-related manner, associated with diuresis and natriuresis and a pattern of renal function in neurohumoral effects characteristic of ANP. Plasma ANP concentrations

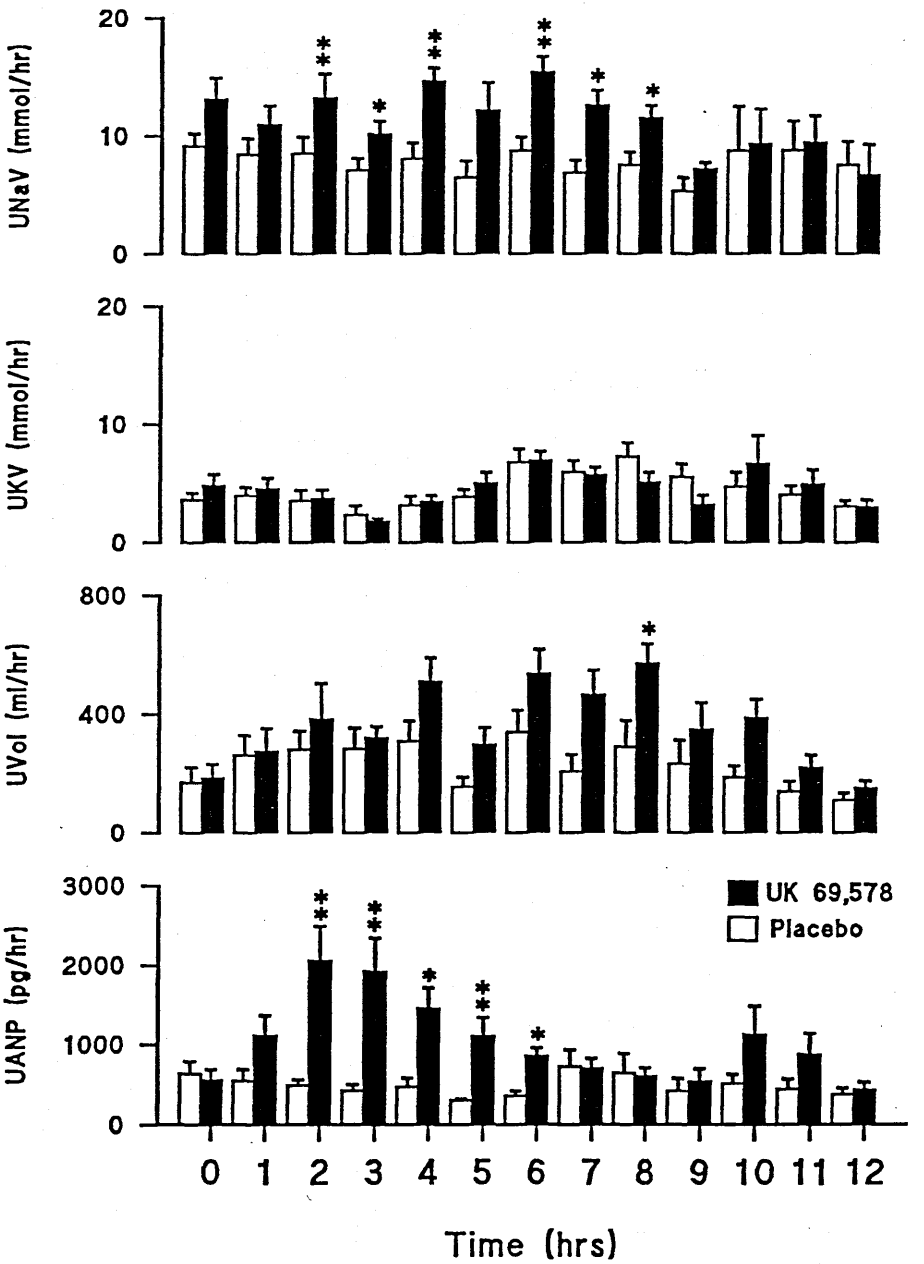


FIGURE 7.8:

TIME COURSE OF CHANGES IN RENAL FUNCTION IN RESPONSE TO UK 69,578 (2mg/Kg).

Time course of changes in urinary sodium excretion (UNaV, mmols/hr), potassium excretion (UKV, mmols/hr), urine volume (UVol, mls/hr) and urinary ANP (UANP, pg/hr) against time for 12hrs post-administration of UK 69,578 or placebo. Data shown as mean±SEM, * $P<0.05$, ** $P<0.01$ (paired T-Test after MANOVA).

were increased for more than 4hrs in the high-dose group, with peak levels 2-3 times basal values being achieved. These data confirm the findings in animals (Chapter 6) and suggest that degradation of ANP by NEP is a major route of elimination of circulating ANP. Although the circulating half-life of UK 69,578 is only 1.1 hours, plasma ANP levels were elevated for more than 4 hrs (Figure 7.2), and urinary ANP excretion prolonged for a similar period (Figure 7.8). This may be interpreted in a number of ways. Firstly, plasma levels of UK 69,578 may not reflect the levels of drug at the active site, either in the renal tubule or at an extravascular site. Alternatively, only low plasma levels may be required to inhibit the enzyme completely, and the dose-response relationship, with near maximal effects (on plasma ANP and renal function, Figure 7.5) being achieved with intermediate doses would support this suggestion.

Renal Actions: Urinary sodium excretion and volume rose in parallel in with the changes in circulating ANP (Figures 7.3, 7.6). While the extent of the increase in circulating levels of ANP produced by UK 69,578 were relatively modest, there is evidence from a number of recent studies that similar small increases in circulating ANP (of between 50-100 pg/ml (18-30 pmol/l)) during low-dose infusion are similarly associated with significant natriuresis and diuresis (Richards et al., (1988a,b), Cottier et al.,(1987), Anderson et al.,(1987), Solomon et al.,(1988), Morice et al.,(1988)). Both the magnitude of the natriuresis and diuresis achieved by UK 69,578, and the absence of an effect on potassium excretion are consistent with the effects of ANP. The confirmation of the findings in Chapter 6, of the similar pattern of renal effects produced by ANP or NEPi provides further evidence that the effects of NEP inhibition are likely to be

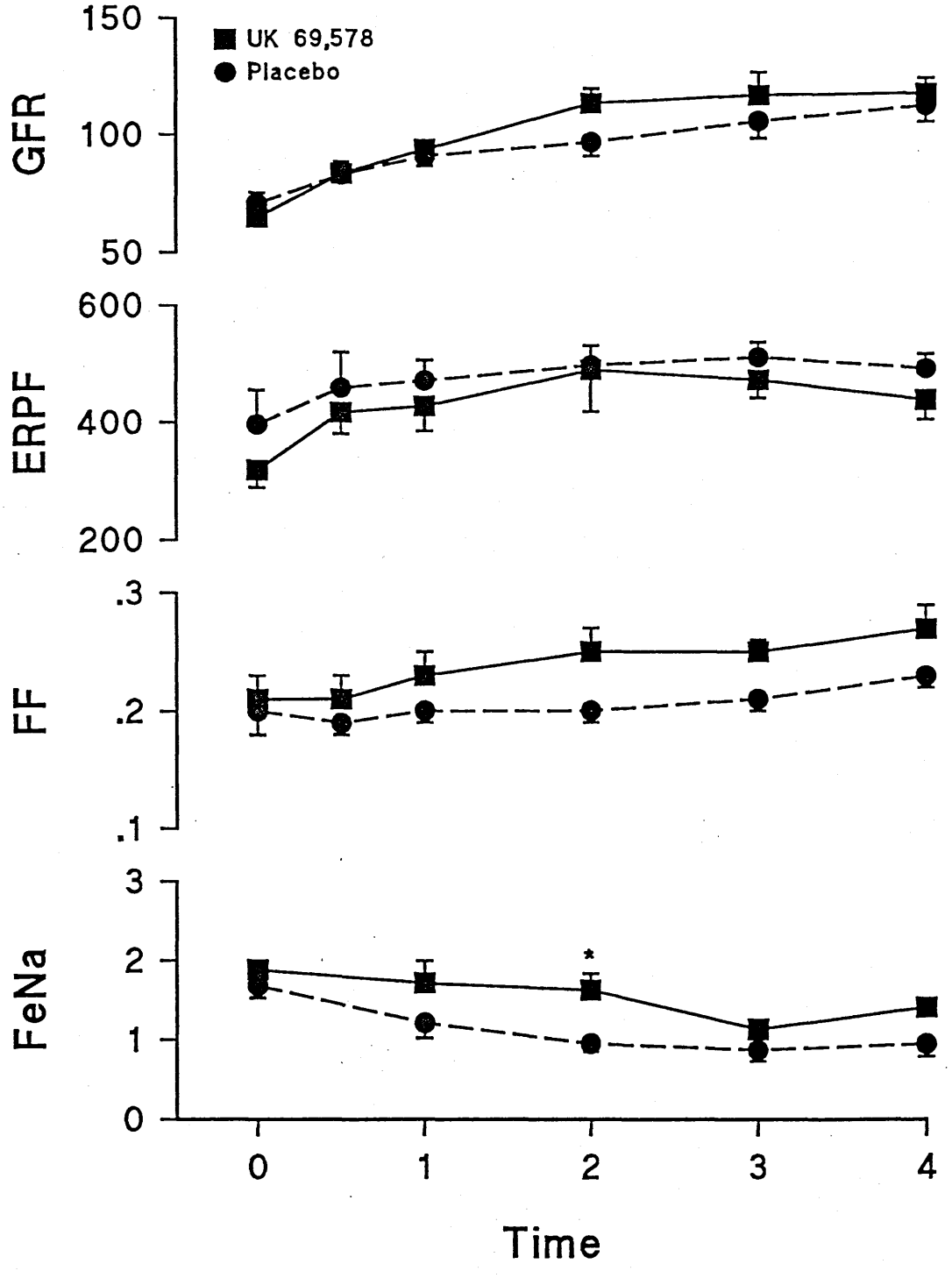


FIGURE 7.9:

RENAL FUNCTION INDICES AFTER TREATMENT WITH UK 69,578 (2 mg/Kg).

Urine function data during treatment with UK 69,578 (2 mg/Kg). Glomerular filtration rate (GFR, mls/min), estimated renal plasma flow (ERPF, mls/min), filtration fraction (FF), fractional excretion of sodium (FeNa, %). * $P<0.05$, ** $P<0.01$ by paired T-test where overall differences identified by repeated measures MANOVA.

mediated by ANP. The relationship between ANP and sodium excretion provides further evidence for this (Figure 7.6). Although such evidence is circumstantial, more direct evidence comes from the observation that, in animal studies, the effects of UK 69,578 are greatly attenuated by co-administration of ANP antisera ((Samuels et al.,(1989), Shepperson et al.,(1991), Northridge et al.,(1989), Chapter 10).

From our knowledge of the renal mechanisms of action of ANP (Chp. 2), it is possible that NEP inhibition may act on the kidney either by increases in plasma ANP or, because NEP is a tubular ectoenzyme, on ANP levels within the tubule. In fact both urinary and plasma hormone levels were increased, indicating that both mechanisms may operate. There was no evidence of either mechanism predominating, the differences in the dose-response relationships between plasma and urinary ANP being largely technical, due to the difficulty in measuring urinary hormone (see Methods, Figure 7.5.).

Renal function indices: In the subset of patients in whom PAH and inulin clearances were performed, there were no demonstrable changes in glomerular filtration, renal plasma flow or filtration fraction but the fractional excretion of sodium was increased. However, GFR and ERPF both rose in both the active and placebo limbs of the protocol. There are several explanations for this. It may reflect the timing of the study, with a 6am start, and a diurnal variation in GFR and ERPF, or, alternatively, it may reflect the water replacement and initial water loading in the experimental protocol. Alternatively, although a standard regime was employed, and the samples taken 5 mins prior to the commencement of UK 69,578/placebo infusion suggested that steady-state levels of inulin and PAH were achieved, this may not have been

the case. Even if this were the case, since the same regime was used in each part of the study, it is still possible to compare the effects of active and placebo treatment.

In fact, just as the overall pattern of renal effects is similar to the effects of low dose ANP, so are the more detailed measurements of renal function. The consensus from the studies of ANP infusion rates less than 2 pmol/kg/min (Richards et al., (1988a,b), Cottier et al.,(1987), Anderson et al.,(1987), Solomon et al.,(1988), Morice et al.,(1988)) is for no change in GFR, either a downward trend or no change in ERPF, an upward trend in filtration fraction and a rise in FeNa. The results of the present study with NEP inhibition are therefore consistent with the effects of ANP.

Dose-response relationship: There was little change in plasma ANP (either peak levels, 2hr levels or area under the curve), urine volume of sodium excretion and doses below 0.1 mg/kg, but clear increases in ANP and urinary sodium excretion at 0.5 mg/kg, and only modest changes thereafter (Figures 7.2, 7.3 and 7.5). These findings suggest that maximal inhibition of the enzyme may be achieved at quite low doses with little effect on further increases.

Hormonal Effects: Despite the natriuresis observed following administration of UK 69,578 there was no activation of the renin-angiotensin system, consistent with the known physiology of ANP, (Chapters 2&4, Figures 1.1, 4.3.), and the results of low-dose infusion studies (Richards et al., (1988a,b), Cottier et al.,(1987), Anderson et al.,(1987), Solomon et al.,(1988), Morice et al.,(1988)). Indeed plasma active renin concentration fell significantly in the 8 hrs after administration of the compound, and there was a rebound activation of renin at 24 hrs which could be explained by the effects

of the preceding natriuresis, but (at 24 hrs) in the absence of sustained elevation in ANP. Indeed, a virtually identical pattern - with a dramatic rebound rise in renin - following ANP infusion, has previously been described (Bie et al.,(1988)). Angiotensin II and aldosterone levels showed a similar trends which failed to achieve statistical significance. Although the Ang II levels were stable in the placebo limb, aldosterone levels fell during the day, presumably reflecting the diurnal variation in aldosterone levels (Richards et al.,(1987)), and the greater fall in aldosterone levels at 8 hours in the active limbs was not statistically significant. It is therefore not possible to comment further on the changes in aldosterone other than to suggest that a number of influences might be contributing to the downward trend, including diurnal rhythm, elevated ANP (Chapter 2, Cuneo et al.,(1987), McMurray and Struthers (1988c)) and indirectly via reduced plasma Ang II.

Overall, the pattern of changes in the renin-angiotensin system is similar to those achieved in low dose infusion studies with ANP, where renin is generally reduced but the effects on Ang II (where measured) and aldosterone more variable, but showing a downward trend.

General Effects: The remaining effects of treatment with UK 69,578 were essentially negative, confirming the safety of this compound at least for acute administration. The were no effects on mood or conscious level, despite the fact that a number of neuropeptides are metabolised by NEP. This may be due to failure of UK 69,578 to pass the blood brain barrier as has been suggested for other NEPi (Schwartz (1989)). No changes were observed in the heart rate or blood pressure (illustrated in Figure 7.7). Infusion of ANP is generally reported not

to alter blood pressure - measured non-invasively (e.g. Richards et al.,(1988a)) - but more recent work (Richards et al., (1988c)) suggests that intra-arterial pressure in seated subjects may fall during ANP infusion at physiological doses. Such an effect would have been missed by the present study, and therefore a modest hypotensive effects cannot be excluded.

There were also no changes in plasma electrolytes, liver enzymes, full blood count or haematocrit. The latter finding conflicts with studies of ANP infusion (e.g. Richards et al.,(1988), Tonolo et al.,(1989)), where haematocrit is usually increased, and may reflect the automated measurement of haematocrit in the present study or the waterloading protocol which may mask a genuine rise in haematocrit.

In conclusion, this study demonstrates that acute administration of a neutral endopeptidase inhibitor in man is safe. Infusion of UK 69,578 elevates circulating levels of ANP, presumably through inhibition of degradation of the peptide by NEP, and produces a combination of diuresis, natriuresis, suppression of the renin-angiotensin system, all of which are consistent with an action of NEPI through ANP. Furthermore, the changes in plasma ANP and sodium excretion were related to the dose of UK 69,578 and there was a clear relationship between the effects of NEPi on plasma ANP and urinary sodium excretion.

In man, as in the rat (Chapter 6), acute NEP inhibition therefore appears to fulfil its potential by increasing plasma ANP, and justifying the investigation of the effects of prolonged treatment (Chapter 8).

HORMONE	DOSE (mg/kg)	N	TIME (hrs) 0	2	4	8	24
ANP	Placebo	16	25.0±3.5	22.3±3.1	19.1±2.1	23.1±3.3	23.7±3.7
Renin			33.9±5.2	38.6±6.6		29.6±5.4	39.1±5.6
Ang II			8.2±1.5	8.4±1.5		7.9±1.2	12.5±1.5
Aldo			11.3±2.5	8.5±1.4		8.6±1.9	11.8±1.1
ANP	0.025	4	11.5±1.3	19.5±2.7	12.5±2.5	11.0±1.5	15.0±2.4
Renin			30.0±7.3	29.3±4.6		28.3±7.0	31.3±4.3
Ang II			21.1±6.9	18.0±8.3		9.1±1.6	15.6±6.8
Aldo			12.0±4.0	5.3±0.5		7.5±0.7	11.5±4.5
ANP	0.05	4	18.5±3.4	23.0±4.7	21.8±4.7	17.5±2.9	15.0±2.5
Renin			25.0±7.5	32.3±5.7		22.0±7.7	34.8±11.8
Ang II			4.8±1.4	10.3±3.9		13.1±9.0	9.2±4.4
Aldo			8.3±1.5	5.3±0.6		4.5±0.3	6.8±0.5
ANP	0.1	8	23.4±4.6	41.4±7.3	29.1±4.6	18.5±2.5	20.5±3.4
Renin			25.6±7.6	18.8±5.7		28.3±7.6	45.1±11.9
Ang II			8.7±6.1	6.1±1.2		7.6±1.2	11.8±3.3
Aldo			16.0±3.8	14.4±3.0		8.9±2.0	14.6±3.2
ANP	0.3	4	29.0±6.9	70.0±14.8	31.5±5.5	34.8±11.6	25.8±6.7
Renin			33.3±14.6	13.0±4.9		32.5±21.9	92.8±24.1 **
Ang II			8.6±3.8	5.1±1.6		9.1±5.1	23.3±6.7
Aldo			17.8±3.5	25.0±6.5		13.0±0.6	23.3±6.7
ANP	0.5	8	21.1±2.1	52.8±9.8 **	30.5±4.7	24.6±4.6	30.6±2.4
Renin			40.5±13.0	28.6±8.0		34.3±11.0	47.4±7.0
Ang II			10.3±2.5	8.2±1.8		8.6±2.8	9.9±1.2
Aldo			14.1±3.4	12.6±3.5		10.8±3.9	17.6±3.0
ANP	1.0	4	28.6±11.6	35.5±4.7	24.5±4.9	21.8±3.8	15.4±2.1
Renin			49.4±5.0	40.4±4.8		39.6±1.6	73.2±9.2 *
Ang II			9.6±2.1	10.6±2.5		5.9±1.4	13.5±3.1
Aldo			14.4±1.8	8.6±1.4		6.8±0.8	14.6±2.8
ANP	2.0	8	21.8±2.7	45.8±6.2 **	41.0±7.5 **	22.4±3.0	17.9±2.1
Renin			35.6±6.4	23.0±2.7 *		16.8±2.8	55.1±7.9
Ang II			6.9±1.5	6.1±0.7		5.6±0.7	11.7±1.3
Aldo			11.0±2.0	6.3±1.5		6.6±1.4	13.3±1.9
ANP	5.0	4	23.0±6.5	41.0±15.8	37.0±8.2	19.5±6.2	18.3±6.2
Renin			24.5±6.5	23.5±7.2		19.3±16.9	42.8±12.7
Ang II			6.9±2.8	6.9±0.9		4.5±1.0	10.9±0.5
Aldo			8.5±2.3	4.0±2.7		4.0±0.4	13.0±2.1
ANP	10.0	4	14.5±4.2	31.5±13.8	32.3±11.5	22.0±8.1	18.5±3.2
Renin			24.3±4.8	19.5±5.3		9.8±0.9	49.3±12.0
Ang II			7.5±1.8	5.9±1.0		4.8±0.8	9.5±1.4
Aldo			8.0±1.8	4.5±1.0		4.3±1.0	12.3±2.1

TABLE 7.1:

Concentrations of active renin (μU/ml), angiotensin II (pg/ml), aldosterone (ng/dL) and ANP (pg/ml) for all dosage groups. Values given as mean±SEM, pre-dose and 2, 8 and 24hrs post-dose. Data from groups where n>4 are shown in Figures 7.2-7.4. Statistical analysis by ANOVAR with individual time points being compared with matched placebo by Dunnett's procedure (*

Chapter 8.

Effects of prolonged NEP inhibition: 10 day administration of UK 79,300 in normal subjects.

Introduction.

The early problem in determining the effect of long term changes in plasma ANP was that sustained elevation in plasma ANP could only be achieved by infusion of exogenous peptide. However, the demonstration that acute NEP inhibition increases plasma ANP (Chapters 6 & 7) offered a potential mechanism for producing sustained increases in endogenous ANP. In 1988, UK 79,300 an orally bioavailable prodrug of the active enantiomer of UK 69,578 became available for human use (Danilewicz et al.,(1989)) and was subsequently shown to cause a pattern of changes in plasma ANP similar to that of UK 69,578 (O'Connell et al.,(1989)) and to offer a mechanism for chronically elevating endogenous ANP by oral drug therapy.

The study described in this chapter was designed to look at the effects of administration of UK 79,300 (Candoxatril, Pfizer UK) in normal subjects. This compound is a prodrug of the active enantiomer of UK 69,578 (i.e. UK 73,967) and has an oral bioavailability of

approximately 40% in animal studies (NJ Cussans, personal communication). Based on this, a dose of 200mg UK 79,300 (which is equivalent to 2-3mg/kg UK 69,578 i.v. at which dose clear effects are seen, Chapter 7) twice daily was used. The primary aim of this study, as in the previous one (Chapter 7), was to establish the safety of UK 79,300 and to measure a number of simple parameters including blood pressure, urine volume and electrolyte excretion and plasma hormone levels. In addition investigations were performed to look for specific effects of NEP inhibition. First, since it was assumed that an effect through ANP would involve changes in sodium balance, this was examined in two ways - by a metabolic balance study and the measurement of exchangeable sodium. Secondly, on the final day of each limb of the protocol subjects were infused with graded doses of phenylephrine and Ang II to look for potential antipressor effects of NEP inhibition and, by the measurement of hormone levels during Ang II infusion, to examine the interaction with the renin-angiotensin system and effects on aldosterone synthesis. Finally, the effects of treatment on platelet ANP receptors was measured both as a marker of effect and since changes in receptor affinity or number may potentially alter the effectiveness of chronic therapy.

Methods:

Eight normal human volunteers were studied. Subjects were aged 21-49 years, had no abnormality on clinical examination, ECG, biochemical profile, full blood count or urinalysis and gave informed written consent before participating in the study. The study lasted six weeks and consisted of two two-week experimental periods separated by a two week "washout period". On one limb of the study the subjects

received active treatment (UK 79,300 - 200 mg b.d. p.o.) and on the other placebo (one capsule b.d.); the design being randomised and double-blind. The study limbs were identical and lasted from a Sunday (9 a.m.) until the following Friday (2 p.m.). During this period the subjects took a diet containing 200 mmoles sodium and 80 mmoles potassium per day. Urine was collected throughout each 24 hour period from the start (9 a.m. - 9 a.m.) in thymol containers and urine volume, Na and K measured. Each morning between 9 and 9.30 a.m., after a 15-30 minute period of rest blood pressure and pulse were measured (in duplicate, lying and standing) using a semi-automatic sphygmomanometer (Copal, Takeda, Japan). After a three day equilibration period, during which the subjects received only placebo capsules, drug/placebo were given for the following ten days (Wednesday a.m. - Friday a.m. inclusive). Immediately before the first active/placebo dose, and after one, three, five, seven and nine days of active/placebo treatment blood samples were taken for measurement of biochemical profile, full blood count, renin, angiotensin II, aldosterone and ANP after 30 minutes rest and before the morning dose. Sampling technique (Chapter 7), collection and assay methodology were as previously described (Appendix 1, Richards et al., (1987), Millar et al., (1981), Morton and Webb (1983), Fraser, Guest and Young (1973), Chapter 7). On the eighth day of active/placebo treatment an extra 50 ml of blood was collected into sodium citrate and platelet ANP receptors measured (see below). The subjects then received Na-22 and exchangeable body sodium measured by the method of Davies and Robertson (1973)) from a plasma sample taken after 18 hours equilibration and urinary Na-22 excretion.

On the final day of each limb of the study subjects were admitted

to the investigation ward at 7.30 a.m. Heparinised cannulae (Viggo, Venflon) were inserted into opposite forearm veins for infusion and sampling and the subjects remained supine thereafter. Fasting, pre-dose samples were taken (as above) and the subjects received UK 79,300 or placebo. After 45 minutes a saline infusion was begun and changed at 45 minute intervals to contain incremental doses of Ang II (Ciba-Geigy; final dose: 2, 4 and 8 ng/Kg/min Ang II). The total volume of infusion was less than 50mls. At the end of each phase of the infusion blood was taken (as above) for measurement of renin, Ang II, aldosterone and ANP and supine blood pressure recorded. At the end of the Ang II infusion subjects were given lunch and allowed a 90 minute "washout" before receiving an incremental infusion of Phenylephrine (Boots Pharmaceuticals, UK). Saline alone was infused for 15 minutes followed by 6 five minute infusions of Phenylephrine at 0.3, 0.6, 0.9, 1.35, 2.0, 3.0 μg/Kg/min. Blood pressure was recorded at the end of each dose and the ECG monitored continuously. The infusion was stopped if the heart rate fell below 40/min.

Platelet Receptor Assay: Platelet ANP receptors were assayed using a modification of the method of McQueen et al.,(1990 a,b) and Schiffrin (Schiffrin (1988), Schiffrin et al.,(1989,1991)). 45mls of blood were taken into tubes containing 5 mls 3.8% sodium citrate, and platelets obtained by differential centrifugation (Dominiczak et al.,(1990)) but centrifugation for 5 mins at 1000 g, the supernatant removed, EGTA added to a final concentration of 5 mM and a platelet pellet obtained by centrifugation for 10 mins at 1000 g, the pellet was then resuspended in 10 mls Medium 199^{+} (Gibco Ltd., UK). All steps were performed at room temperature. The platelet concentration was then measured (Coulter Counter, Coulter USA) and the sample diluted in

Medium 199^{+} to give a final concentration of 500 x 10^{9}/l. ANP binding was then determined by displacement of radio-labelled ANP (^{125}I-ANP, Amersham, UK) in incubation volumes of 1 ml containing 100 x 10^{6} platelets, 17 pM ^{125}I-ANP and increasing concentrations of unlabelled ANP (0-800 pM). Specific binding was determined after incubation for 1 hr at 22°C. The incubation was terminated by diluting the incubating mixture with 7 ml 50 mM TRIS/120 mM NaCl/0.05% BSA, pH 7.4 and filtered under vacuum through Whatman GS/F glass fibre filters prewet with 1% NaCl/1% BSA/5% Tween 20/1% phenylethanylimine. The filters were dried, removed and trapped radioactivity determined. Under these conditions there was no detectable ligand degradation under standard assay conditions, in contrast to findings in other tissues (McQueen et al., (1990 a,b)), presumably reflecting the absence of peptidase enzymes in this assay system. ANP binding increased linearly up to 1.5 x 10^{8} platelets/tube and in time course studies the plateau was reached at 40 mins. Receptor affinity (Kd) and density of binding sites (Bmax) were determined using a weighted least squares non-linear regression method (McQueen et al.,(1984). In the absence of detectable ligand degradation by HPLC (in contrast to other tissues - McQueen et al., (1990b)) equilibrium conditions were assumed. Statistical comparison of binding parameters and determination of the number of binding sites was as previously described (McQueen et al., (1984), McQueen et al., (1990a,b)).

Materials: ^{125}I-αh-ANP was obtained from Amersham UK, unlabelled αh-ANP from Penninsula Laboratories, UK, Medium 199 plus (containing 25 mM HEPES, 0.4% BSA, 0.1% bacitracin, 0.5 mM phenylmethylsulphonyl fluoride (PMS), 100 KIU/ml Trasylol) from Gibco, UK, and all other chemicals from Sigma (UK).

Statistical analysis is by repeated measures analysis of variance (MANOVA, SPSS/PC+, IBM) and where significant by MANOVA, post hoc analysis is by paired t-test at individual time points.

Results:

Prolonged administration of UK 79,300 was well tolerated by all subjects with no reported side effects, and in keeping with the previous findings with acute administration of UK 69,578 (Chapter 7). There were no observed changes in blood pressure, plasma electrolytes or liver enzymes (see Table 8.1 for summary data). The only significant effects were that there was a fall in haemoglobin and in body weight in both limbs of the study, reflecting venesection and dietary restriction.

Urinary sodium and potassium excretion and urine volume are shown in Figure 8.1. There were no significant changes in potassium excretion, but both urinary sodium excretion and volume were significantly different in the active limb by repeated measures MANOVA. Post-hoc analysis revealed that this was due to a natriuresis and diuresis in the first 24 hours after starting treatment (Day 1) and that there was a subsequent antinatriuresis and diuresis on Day 3, with variable changes in each thereafter. Cumulative measurements of urinary sodium excretion, based on the balance study were not significantly different at the end of the study (1687±56 [UK] vs. 1703±83 [Pl] mmoles/9 days, P=NS). Measurement of exchangeable sodium similarly showed no difference in exchangeable body sodium, measured between the 7th and 8th day of drug/placebo administration (3043±107 [UK] vs. 3068±119 [Pl] mmoles, P=NS).

In contrast to the findings with acute NEP inhibition there were

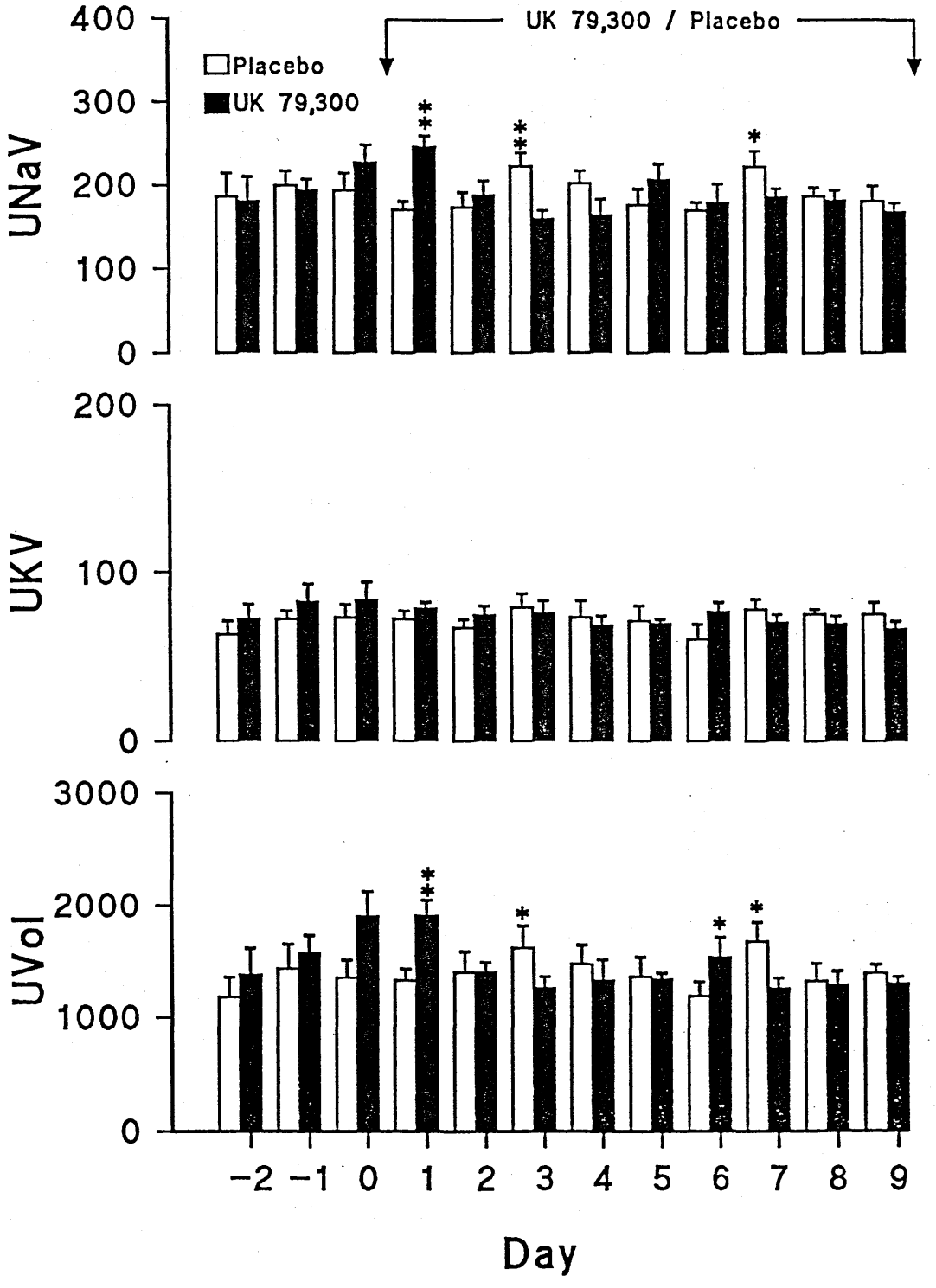


FIGURE 8.1:

RENAL EFFECTS OF UK 79,300 IN MAN.

Urine volume (ml/24 hours), sodium and potassium excretion (UNaV, UKV mmoles/24 hours) during treatment with UK 79,300 (200mg b.d., days 1-9) after a three day control period (days -2 - 0). Data are shown as mean±SEM. * $P<0.05$, ** $P<0.01$ by paired T-test where overall difference between active and placebo established by MANOVA.

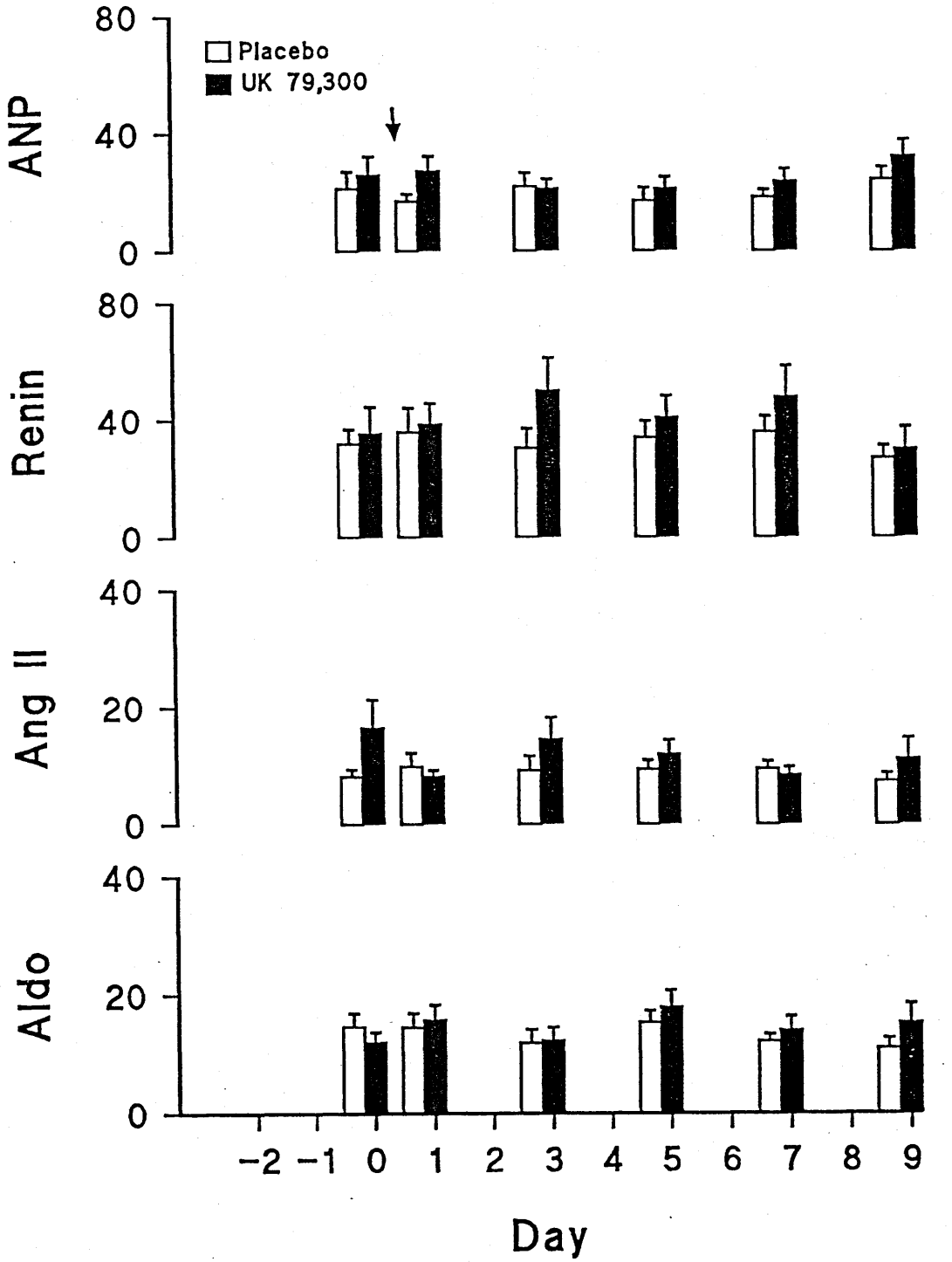


FIGURE 8.2:

HORMONAL EFFECTS OF UK 79,300.

Plasma ANP (pg/ml), active renin concentration (Renin, μU/ml), angiotensin II (Ang II, pg/ml) and aldosterone (Aldo, ng/dl). UK 79,300 was administered (200mg b.d.) from day 0 onwards. Data are shown as mean±SEM, there were no statistically significant effects.

only minor alterations in plasma hormone levels shown in Figure 8.2. Plasma ANP was marginally higher in the first day of administration, and on days 5,7 and 9 but this failed to achieve statistical significance. Plasma levels of active renin concentration, angiotensin II and aldosterone were also marginally higher during active treatment, but again there were no significant statistical changes.

Figure 8.3 shows the hormonal data from the final day of the study. Basal ANP levels were slightly higher in the active limb and showed a greater increase during infusion of the pressor agent Ang II, although again this was not statistically significant. Plasma ANP was also higher before infusion of phenylephrine and, although ANP increased during Phe infusion, there was no significant difference in the response during active or placebo treatment. There were unexpected changes in the other hormonal measurements during Ang II infusion. Active renin concentration was suppressed in both phases of the study. However, Ang II levels were significantly higher when infused in the presence of Uk 79,300, although post-hoc analysis did not identify a specific time point. Repeated measures MANOVA also identified a significantly greater rise in plasma aldosterone during Ang II infusion in the active limb of the study; with paired t-tests being significant in the highest two dosage groups (Figure 8.4). Blood pressure increased to a similar extent in both limbs of the study during Ang II infusion but there was a significantly reduced pressor response to Phenylephrine infusion in the active group ($P<0.001$, MANOVA; Figure 8.3).

Platelet binding data is shown in Figure 8.5. There was no difference in the affinity of ANP receptor (Kd=55.6±12.3 [placebo], 42.4±14.1 [UK 79,300], pM) but there was a significant reduction in

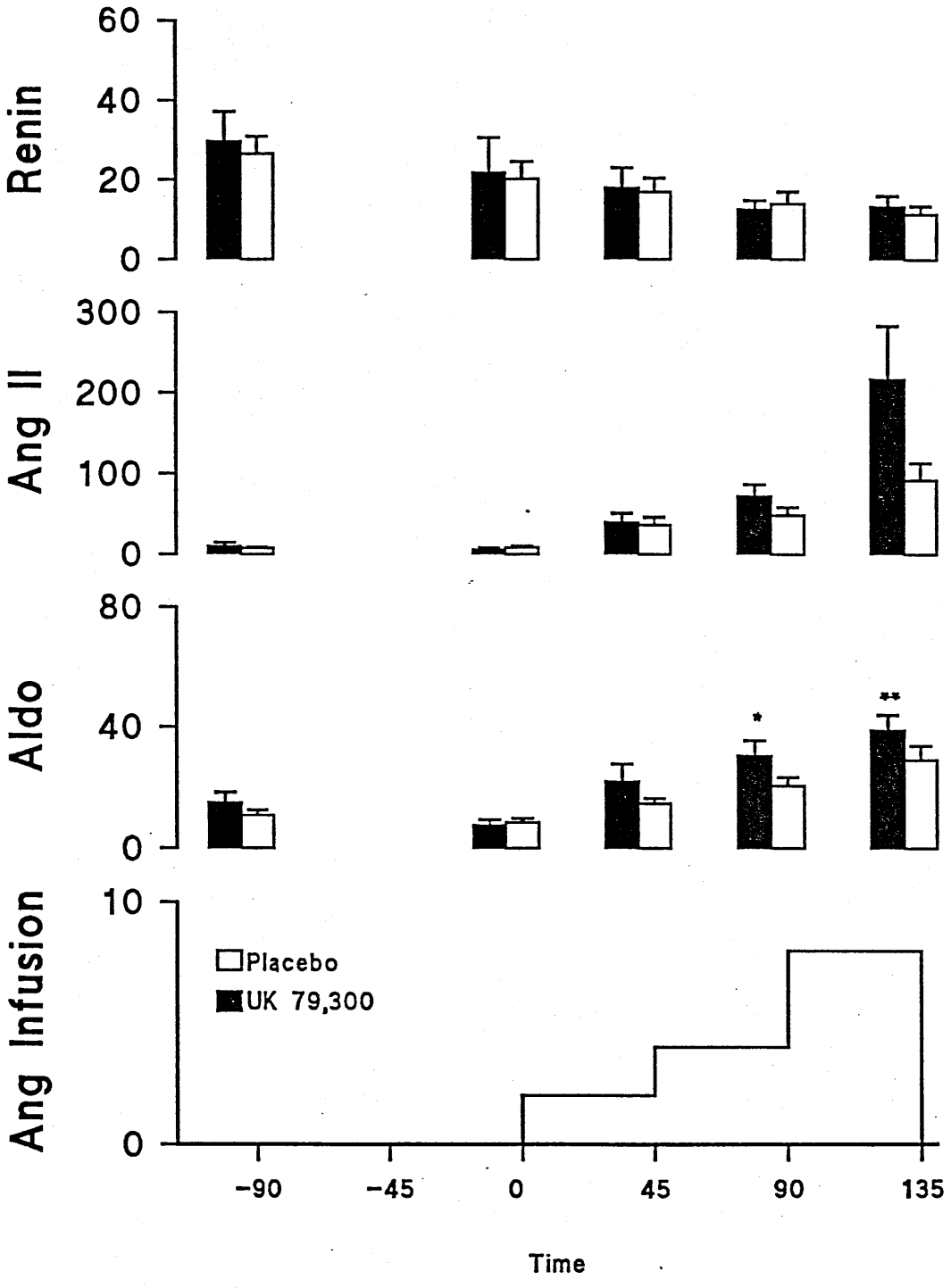


FIGURE 8.3:

HORMONAL RESPONSE TO ANG II INFUSION DURING TREATMENT WITH UK 79,300 IN MAN.

Changes in active renin concentration (Renin, μU/ml), angiotensin II (Ang II, pg/ml) and aldosterone (Aldo, ng/dl) with infusion of angiotensin II (0,2,4 and 8 ng/kg/min) on the tenth day of treatment with UK 79,300 or placebo. Plasma Ang II levels were significantly higher in the active limb by repeated measures MANOVA ($P=0.009$), but there was no significance at individual time points. * $P<0.05$ ** $P<0.01$ indicate significance by T-test where overall significance first established by MANOVA.

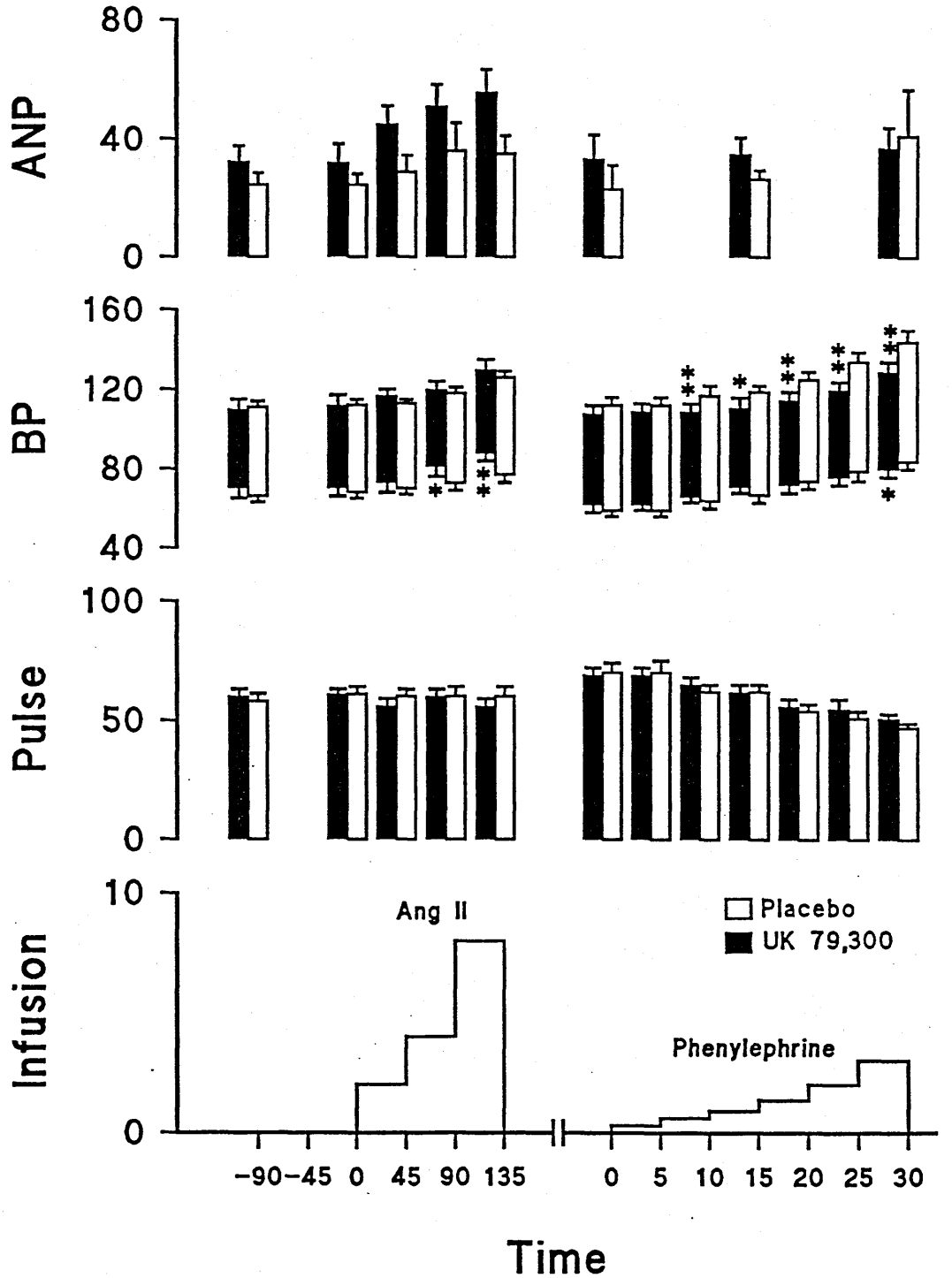


FIGURE 8.4:

ANP AND BLOOD PRESSURE RESPONSE TO ANG II AND PHENYLEPHRINE INFUSION DURING TREATMENT WITH UK 79,300.

Plasma ANP (pg/ml), blood pressure (BP, mmHg) and pulse rate (Pulse, beats per minute) during graded infusion of Ang II (ng/kg/min) and Phenylephrine (μg/kg/min) on the tenth day of treatment with UK 79,300 or placebo. * $P<0.05$, ** $P<0.01$ indicate significance by paired t-test where overall significance identified by repeated measures MANOVAR.

the number of binding sites in the active limb of the study (39±4 vs 22±3 fmol/10^9 platelets, p=0.0034; t-test).

Discussion.

This study was the first to examine the effects of prolonged administration of NEPi in normal man. As was seen with acute administration of the related compound UK 69,578 there were no adverse effects and no changes in resting blood pressure, plasma electrolytes, liver enzymes or full blood count (other than a fall in haemoglobin due to blood sampling and weight loss due to dietary restriction).

Renal Effects:

Overall, there was a signficant difference in sodium excretion during active treatment. The pattern of natriuresis - with an effect only on the first day of treatment - is similar to that seen during low dose infusion of ANP in man (Janssen et al., (1989)) and animals (Kivlign et al., (1988), Mizelle et al.,(1988)) where a natriuresis was only evident during the first 24 hours of ANP infusion. In contrast to these studies however, there was no evidence that the subjects remained in negative sodium balance. From the metabolic balance data, there was a rebound antinatriuresis on day three of the active limb and, overall, there was no significant difference in total calculated sodium balance over nine days of treatment with UK 79,300 or placebo. Exchangeable body sodium was lower in the active limb but the difference was not statistically significant, and the magnitude of the difference found is at the limit of detection for this method (Davies and Robertson (1973)) and is much less than would be seen with diuretic agents such as frusemide.

Urine volume showed a similar pattern to sodium excretion. MANOVA demonstrated a significantly higher urine volume on the active limb, and post hoc analysis revealed that there was a significant diuresis only on day one, with a relative antidiuresis on day three. Overall total (9 day) urine volume was not different in the active limb. In keeping with the findings from acute administration of the related NEP-I UK 69,578 there was no change in potassium excretion (Chapter 6).

These results demonstrate that in the first 24 hours after the start of treatment UK 79,300 has similar effects to UK 69,578 and acute administration of ANP. However, these effects are not sustained during prolonged treatment. Possible reasons for this are discussed below.

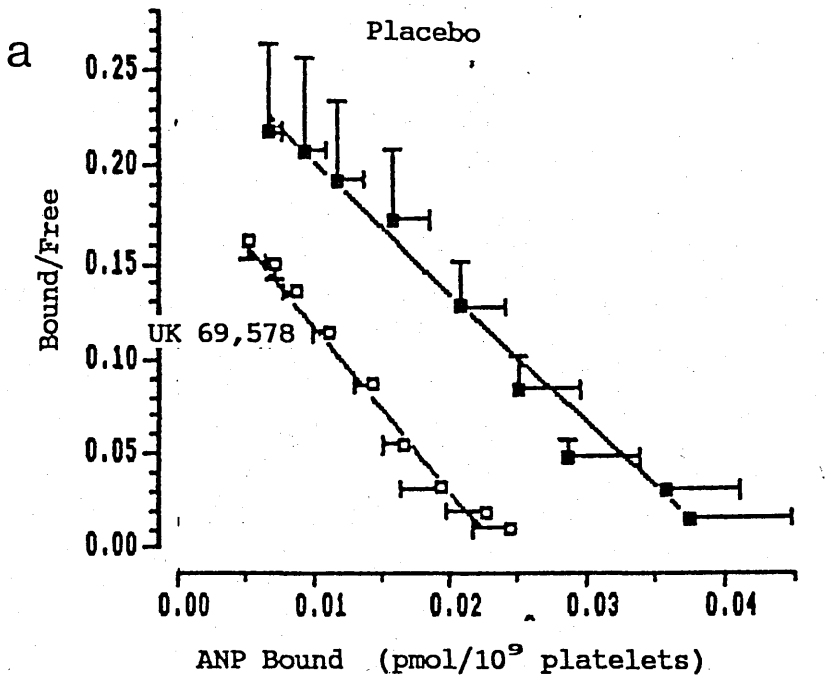
Hormonal Effects:

Although plasma levels of ANP were slightly higher in the active limb, this failed to reach statistical significance. There are several possible reasons for this. From the earlier study (Chapter 7), and the findings of O'Connell et al., (1989) that UK 79,300 has similar acute effects to UK 69,578 and the finding of a natriuresis during the first 24 hours of active treatment it is surprising that ANP was not elevated. From a statistical viewpoint this is at least partly due to the fact that baseline (Day 0) levels of ANP were inexplicably higher in the active limb. From a methodological point of view, limiting blood sampling to the pre-dose period may have missed a rise in ANP since plasma levels had returned to normal or near-normal levels 8 hours after administration of UK 69,578. There is some evidence to support this. Firstly, plasma levels were higher on the final day of the study 90 minutes after administration of UK 79,300. During

infusion of Ang II there was the expected rise in plasma ANP (presumably as a result of increased arterial pressure [Chapter 3, Uelinger et al.,(1987)]). In the presence of UK 79,300, there is an exaggerated rise in plasma ANP, implying at least some inhibition of ANP degradation. Secondly, the down-regulation in platelet ANP receptors is consistent with a sustained increase in plasma ANP. Furthermore, two recent preliminary reports of long term NEP inhibition (with UK 79,300) in man have found a sustained increase in urinary cGMP (O'Connell et al.,(1990)) or persistent increase in plasma ANP (Bevan et al.,(1991)) suggesting that the failure to find an elevated ANP in the current study may be methodological.

The tendency for plasma active renin concentration to be higher in the active limb would be consistent with the findings from the acute dosage (Chapter 7) where there was a rebound rise in renin following an initial suppression, after administration of UK 69,578. The predose sampling in this study with UK 79,300 would tend to pick up these rebound rises in hormone. The trend for angiotensin II and aldosterone levels to be higher could be explained in a similar way, although the effect of NEPi on Ang II degradation (see below) will also increase Ang II.

ANP binding: showed a significant reduction in the number of binding sites on the active limb of the study, similar to the pattern seen in patients with elevated plasma ANP due to heart failure (Schiffrin (1988) or hypertension (Schiffrin et al.,(1988)). The nature of platelet receptors has recently been clarified (Schiffrin et al.,(1991)). Human platelets contain a mixture of high and low molecular weight receptors. The low molecular weight receptor appears to be the C-ANP receptor (Chapter 2) but the high molecular weight



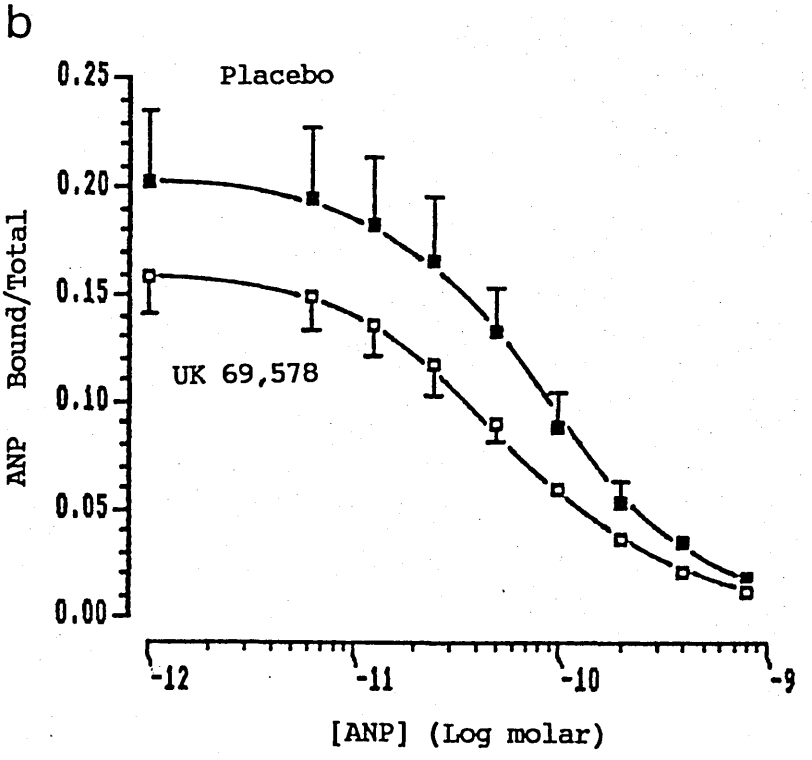


FIGURE 8.5:

PLATELET ANP RECEPTORS - DURING UK 79,300 TREATMENT.

A. Scatchard analysis and B. binding curves for platelet ANP binding from samples taken on the 8th day of treatment with UK 79,300 or placebo. Data are shown as mean±SD, see text for details.

form, although similar to the B-ANP receptor is not functionally linked to guanylate cyclase. However, regardless of their nature these receptors do behave appropriately in a number of conditions, such as heart failure (Schiffrin (1987)). The down regulation of platelet ANP receptors in the current study is therefore consistent with a sustained rise in plasma ANP during treatment with UK 79,300. The balance of evidence from platelet binding and the initial natriuresis and diuresis would suggest that plasma ANP levels are elevated, but that increased plasma levels have been missed by sampling prior to administration of the drug (and plasma hormone levels have returned to normal. Although platelets receptors have no proven biological significance they are likely to act as markers for the biologically active ANP receptor, and one would therefore predict down regulation of this receptor subtype in other tissues. Such an effect may partially account for the failure of sustained hormone elevation to produce a sustained natriuresis and diuresis.

Infusion data: The infusions on the final day had two aims. Firstly to identify possible antihypertensive effects in normal subjects in case there were no changes in blood pressure during the study (as was found, Table 8.1) and secondly to examine the effects of NEP inhibition on the adrenal response to Ang II since ANP has been shown to inhibit adrenal function both in vitro (Chapter 2) and in vivo (Cuneo et al.,(1987)). The Ang II infusion protocol was similar to that employed by Cuneo et al.,(1987) where ANP was found to inhibit the aldosterone response to Ang II infusion. In contrast to the clear inhibition of aldosterone production in these studies UK 79,300 resulted in an increased aldosterone response to Ang II. The reason for this is clear, if unexpected, in that Ang II levels were also

increased presumably reflecting the fact that NEP degrades Ang II (Chapter 5). Even when the elevated levels of Ang II were taken into account by examining the ratio of Aldo/Ang II there was no evidence of any inhibition of aldosterone production (data not shown). This disappointing observation suggests that the non-specific actions of NEPi may render them ineffective as antihypertensive agents at least in circumstances where Ang II is elevated. The blood pressure response to infusion confirms this with an exaggerated pressor response to Ang II in the presence of UK 79,300. However, the pressor response to Phenylephrine was reduced confirming that NEPi may have antihypertensive actions under some circumstances. It should be noted that this pattern of effects - with a greater inhibition of Phe than Ang II - contrasts with previous reports that ANP specifically inhibits the pressor effects of Ang II (Proctor and Bealer (1987)).

The effects of NEP inhibition on Ang II may explain the limited renal effects of NEP inhibition. If we accept that plasma ANP is raised during prolonged treatment with UK 79,300 (above) then the transient natriuresis, rebound antinatriuresis and absence of any effect on overall sodium balance must be due either to activation of antinatriuretic mechanisms or of factors which modulate the renal actions of ANP (Chapter 4). Catecholamines were not measured in this study, and there was no increase in heart rate to suggest sympathetic activation (Koepke and diBona (1987)); nor was there a significant fall in blood pressure (Seymour et al.,(1987)). Activation of the renin-angiotensin system therefore seems the most likely mechanism. In keeping with this, Ang II levels were higher during active treatment and we have established that Ang II degradation was inhibited, which will magnify any Ang II-mediated effects. In fact, although the early,

transient natriuresis (Figute 8.1) is similar to the effects of ANP (Janssen et al.,(1989), Kivlign et al.,(1988)), the rebound antinatriuresis and lack of overall effects on sodium balance is not. Inhibition of Ang II degradation may explain this, by amplifying the effects of activation of the renin-angiotensin system.

A further relevant question is whether or not NEP inhibition has any effect on baroreceptor function, since ANP is thought to attenuate the baroreceptor response to a variety of stimuli (Chapter 2). Baroreceptor function was not formally assessed in the present study, although the heart rate/systolic pressure relationship did not differ between active and placebo treatment groups for either Ang II or phenylephrine Figure 8.4). Further studies are required to assess any effect of NEPi on baroreceptor function.

In conclusion, this study confirms the safety of NEP inhibition in man, but fails to achieve the initial potential of these agents (Chapter 7). The renal effects are modest, but consistent with low dose infusion studies of ANP in man and animals (Janssen et al.,, (1989), Kivlign et al.,(1989)) with a diuresis and natriuresis only on the first day. Plasma ANP was probably elevated, although this was not clearly established from the plasma measurements, but the finding of most concern from the present study is that NEP inhibition is non-specific and can also inhibit Ang II degradation. As a result of this, although there appeared to be some anti-pressor potential against the effects of phenylephrine infusions, the pressor response to Ang II was increased. Although this does not completely exclude a therapeutic role for NEPi, it is likely to limit its use to situations where the renin-angiotensin system is not activated.

Day		0	1	2	3	4	5	6	7	8	9
SBP	P	119±5	115±3	126±5	117±4	119±5	116±3	117±5	116±5	121±5	114±4
	UK79	117±3	115±3	116±5	118±5	118±5	112±4	120±4	111±5	120±3	114±4
SBP (S)	P	106±5	110±4	113±4	105±4	107±6	108±3	109±5	101±3	110±5	108±5
	UK79	116±5	106±4	110±3	103±4	109±4	105±3	106±5	107±4	108±3	107±3
DBP	P	70±4	67±4	69±2	67±3	67±3	62±3	63±3	64±5	68±4	68±4
	UK79	69±3	69±4	69±3	66±4	67±4	67±3	68±4	63±3	62±4	72±4
DBP (S)	P	76±4	72±5	66±6	67±5	67±5	68±4	66±5	69±3	69±5	75±4
	UK79	78±3	76±5	73±5	73±3	66±4	70±4	67±4	69±3	70±4	75±3
WT	P	73.4 ±3.1	73.4 ±3.0		73.3 ±2.9		73.0 ±3.0		72.7 ±2.9		72.9 ±2.9
	UK79	74.2 ±3.1	73.8 ±3.1		73.6 ±3.2		73.4 ±3.2		72.9 ±2.9		72.9 ±2.9
HCT	P	42±1	41±1		41±1		41±1		41±1		39±1
	UK79	43±1	43±1		42±1		42±1		42±1		40±1
Na	P	140.3 ±0.6	140.4 ±0.8		140.0 ±0.6		140.6 ±0.8		140.9 ±0.5		140.9 ±0.6
	UK79	140.4 ±0.4	139.8 ±0.5		139.8 ±0.4		139.9 ±0.5		140.8 ±0.3		140.9 ±0.5
K	P	4.1±0.1	4.0±0.1		4.0±0.1		4.1±0.1		4.1±0.1		4.0±0.1
	UK79	4.2±0.1	4.2±0.1		4.2±0.1		4.2±0.1		4.2±0.1		4.1±0.1
U	P	5.7±0.3	6.1±0.5		5.5±0.3		6.0±0.3		5.4±0.3		6.2±0.4
	UK79	5.6±0.3	5.3±0.3		5.8±0.3		6.0±0.3		5.4±0.3		5.5±0.3
Ct	P	96.0 ±3.7	95.0 ±5.1		91.9 ±4.0		98.8 ±4.3		100.6 ±5.4		93.8 ±5.7
	UK79	99.4 ±5.8	89.9 ±5.5		94.7 ±2.7		91.8 ±5.4		91.1 ±7.0		94.4 ±4.4

TABLE 8.1

BLOOD PRESSURE, WEIGHT AND BIOCHEMICAL DATA.

Blood Pressure (systolic supine (SBP) and standing SBP(S); diastolic supine (DBP) and standing DBP(S); mmHg, mean±SEM). Weight in Kg (Wt), Haematocrit (Hct, %), and plasma sodium, potassium and urea (Na, K, U, mmol/l) and creatinine (μmol/l). There were no differences between active (UK79) and placebo (P) for any of these variables. Data shown as mean±SEM (n=8) against time in days; UK 79,300 started after day 0.

Chapter 9.

Antihypertensive Effects of NEP inhibition: Studies with SCH 34826 in normal and DOCA-salt rats.

Introduction.

The findings in the previous chapter (Chapter 8) suggest that chronic administration of NEPi may inhibit ANP degradation but that the effects on sodium homeostasis are short lived and modest. Despite the absence of a fall in resting blood pressure, this does not exclude a potential antihypertensive effect of NEP inhibition, particularly as ANP is reported to cause more pronounced effects in hypertensive subjects (Richards et al., (1989), Cusson et al., (1987)). The attenuation of the pressor response to phenylephrine (Chapter 8) during NEPi treatment suggests some potential antihypertensive effect (albeit with the caveat that NEPi are unlikely to be effective in situations where the RAAS is activated (Chapter 8)). The study described in this chapter was therefore undertaken to examine the effects of SCH 34826 (see chapter 5, a NEP inhibitor of comparable potency to UK 69,578) on plasma ANP, sodium balance and blood pressure in normal and DOCA salt hypertensive rats.

Methods.

Twenty four male Sprague Dawley rats (weight 200-250g, OLAC Ltd., UK) were used. Two separate studies were performed in normal rats and in rats made DOCA-salt hypertensive by unilateral nephrectomy and the implantation of 25mg DOCA pellet subcutaneously (Russo et al.,(1990)). The animals were housed singly in metabolic cages allowing quantification of dietary intake and urine output. They received a "sodium free" diet (Special Diet Services, Essex, UK., containing approximately 6mmoles Na/Kg) and 1% NaCl containing 74 μBq/l Na-22 to drink (Harrap (1986), Brown (1981)). After a four week equilibration period during which blood pressure was measured twice weekly by the tail-cuff method (BP recorder 8005, W&W Electronics, Basel, Switzerland), the experimental phase of the study began. Urine volume was measured daily and Na and K concentration measured (Beckman model 2A analyzer, Beckman Inc., Ireland). Urinary excretion of cGMP was determined by radioimmunoassay (Lyall et al.,(1987)), using a commercially available kit (Amersham, UK); the inter and intra-assay coefficients of variation were 1.4 and 0.8% respectively. Intake of of food and saline was measured. The experimental part of the study lasted 14 days. The first 3 days were a control period, the next three days the rats received either 10 mg/Kg SCH 34826 or placebo 12 hourly by subcutaneous injection, then 30mg/kg SCH 34826 or placebo and finally 90 mg/kg SCH 34826 for five days (after Sybertz et al.,(1989), Janssen et al., (1989), Kivlign et al.,(1988)) to allow time for a maximal effect to appear. Blood pressure was determined at the end of each phase by the tail cuff method and exchangeable body sodium measured by the method of Brown (1981). Briefly the rats were placed

in a perspex frame and radioactivity measured in an ARMAC counter. Exchangeable body sodium (NaE) was estimated by comparison with a standard "ghost" containing 150 mls of Na-22/1% NaCl using the equation.....

$$\text{NaE(mmol/Kg)} = \text{(Counts per rat)} \times \frac{26.7}{\text{Standard Counts}} \times \text{Weight of rat}$$

On the final day the rats were sacrificed by guillotine exactly two hours after the final dose and trunk blood collected in chilled tubes containing EDTA/Trasylol for determination of plasma ANP (Appendix 1).

Statistical analysis is by repeated measures ANOVAR of active versus placebo within normal and hypertensive groups (SPSS/PC+, SPSS Inc., USA). Where significant ($P<0.05$) post hoc analysis at individual dose or time points was by Student's t-test.

Results.

Metabolic Balance. The normal rats had stable 24 hour urine volume, sodium and potassium excretion throughout the study period, without evidence of any changes at any dose of SCH 34,826 (Figure 9.1). The DOCA salt rats (Figure 9.2) had slightly higher baseline levels of Na and K excretion and, in particular, urine volume, but again there was no difference between active and placebo during treatment with SCH 34826. In the DOCA salt rats there was a downward trend in Na excretion in both active and placebo groups during the 14 day experimental period perhaps because these animals had not achieved a stable equilibrium (i.e. intravascular volume and total body sodium

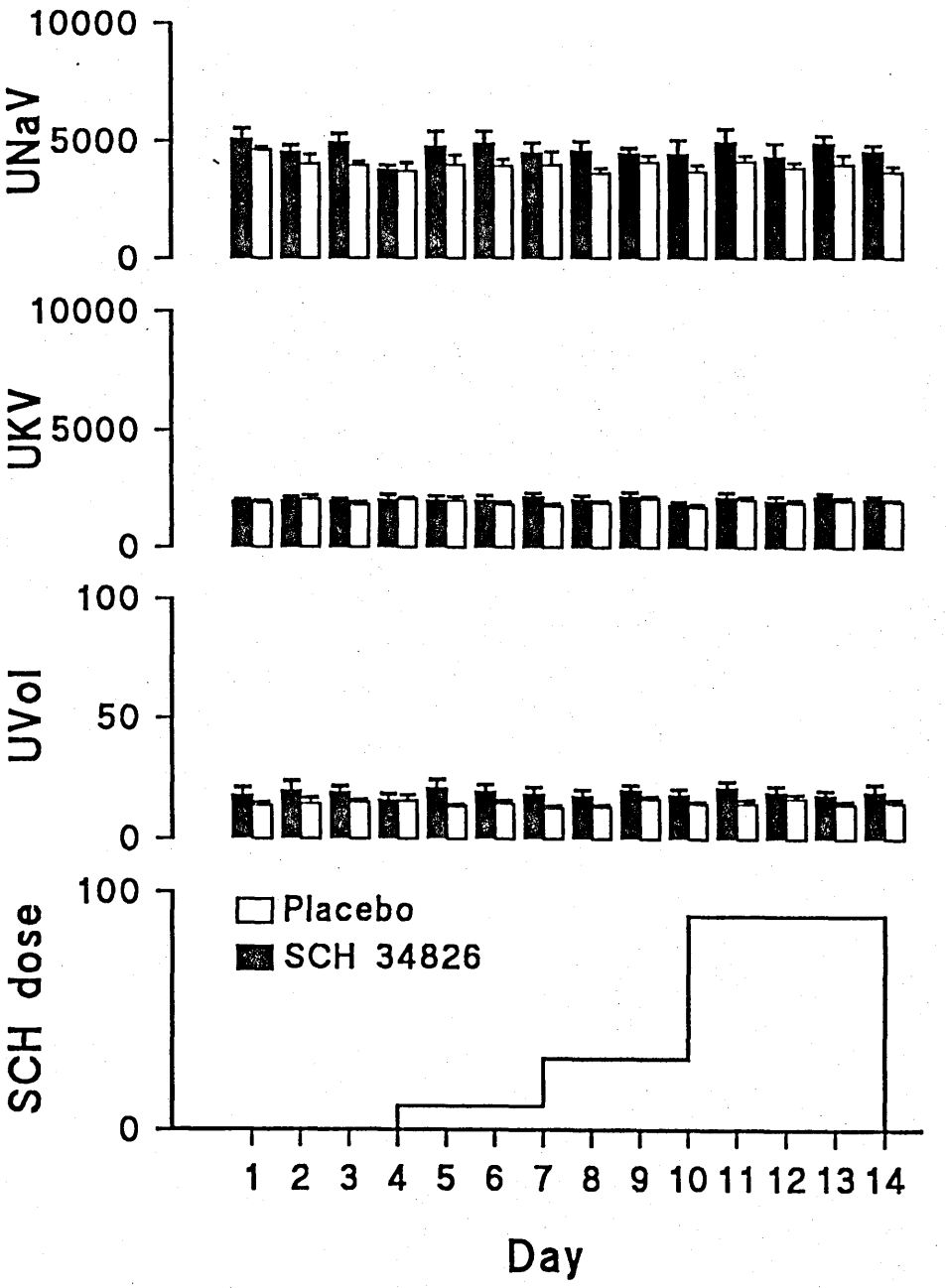


FIGURE 9.1:

RENAL EFFECTS OF SCH 34,826 IN NORMAL RATS.

Urinary sodium excretion (UNaV, μmoles/day), potassium excretion (UKV, μmoles/day), urine volume (UVol, ml/day) and dose of SCH 34,826 (mg/kg b.d.) against time in days. Normal SD rats, n=6 in each group. Data are given as mean±SEM and there were no statistically different effects.

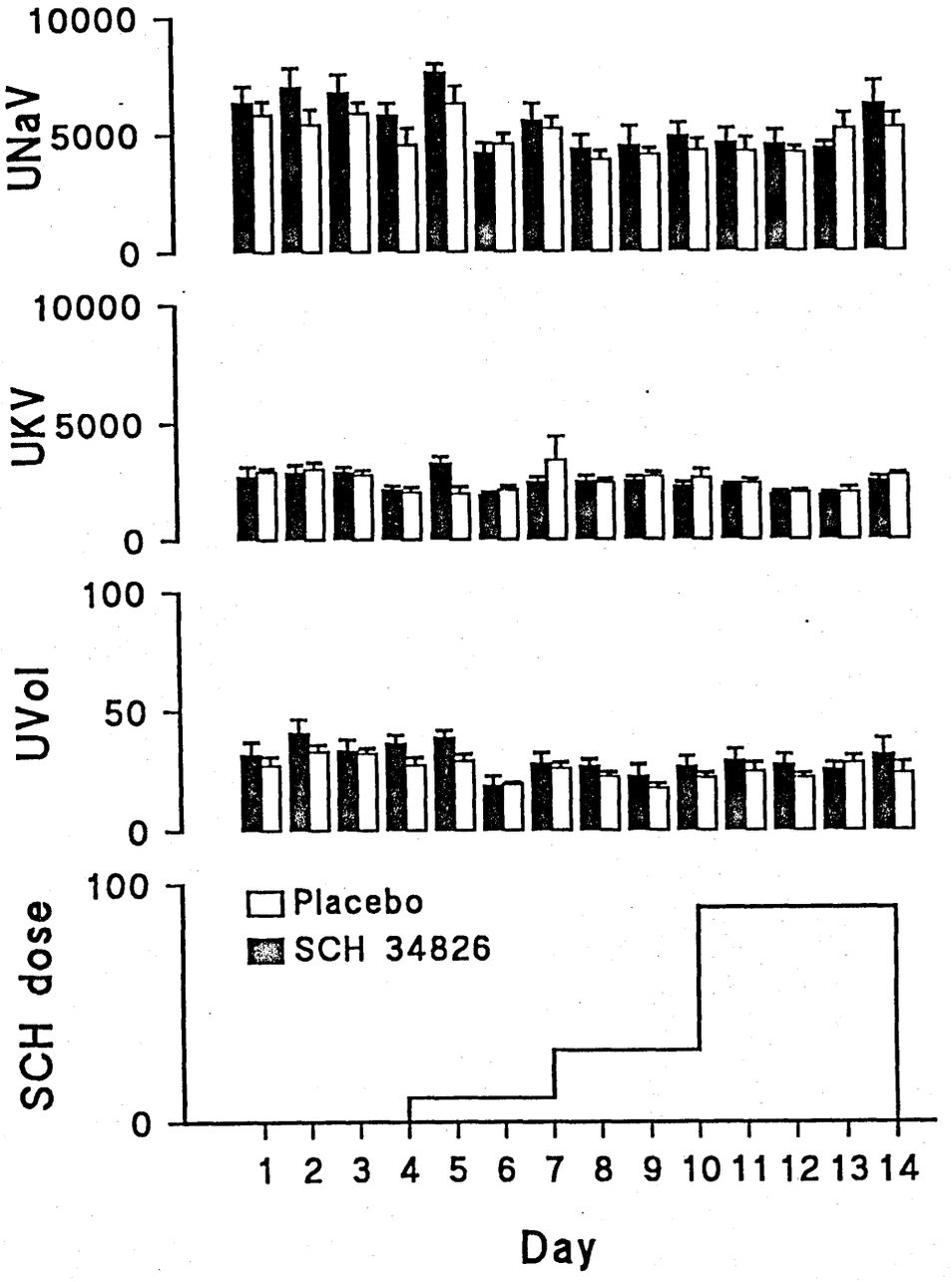


FIGURE 9.2:

RENAL EFFECTS OF SCH 34,826 IN HYPERTENSIVE RATS.

Urinary sodium excretion (UNaV, μmoles/day), potassium excretion (UKV, μmoles/day), urine volume (UVol, ml/day) and dose of SCH 34,826 (mg/kg b.d.) against time in days. DOCA salt hypertensive rats, n=6 in each group. Data are given as mean±SEM and there were no statistically different effects.

were still expanding) during the first few days of the experimental period. In both the normal and DOCA salt rats there was a non-significant tendency for Na excretion and urine volume to be higher in the rats treated with SCH 34826 but this was also evident in the control period, and there is therefore no evidence that NEPi had any effect. In all groups urinary Na excretion approximated to 70-80% of the calculated Na intake, a finding similar to that observed by others using this method.

Exchangeable Sodium. In the normal rats exchangeable body sodium levels of between 35 and 40 mmoles/kg were found, similar to previous reports in normal animals (Mannheim et al.,(1986), McAreavey et al.,(1984)). However, measurement of exchangeable sodium gave no further indication of activity of the NEPi (Figure 9.3). Indeed, NaE tended to be higher during active treatment in both normal and DOCA salt groups with the DOCA salt rats showing approximately 20% expansion of NaE (Simpson et al.,(1986), McAreavey et al.,(1984)).

Blood Pressure. The normal rats had high baseline readings but showed a downward trend with repeated measurements. As in humans (Chapter 8) there was no evidence of a fall in blood pressure in normal rats (Figure 9.3) during treatment and, in fact, the mean SBP was lower in the placebo group. The baseline SBP was about 20 mmHg higher in the DOCA salt hypertensive rats and continued to rise in the placebo group. In contrast, those DOCA salt rats receiving SCH 34826 showed a downward trend in SBP which was maximal at the highest dose, where there was a 40 mmHg difference between the active and placebo groups.

Plasma ANP. This was determined in trunk blood obtained two hours after administration of 90mg/Kg SCH 34826 on the final day of the study. ANP was elevated six-fold in the DOCA salt rat compared to the

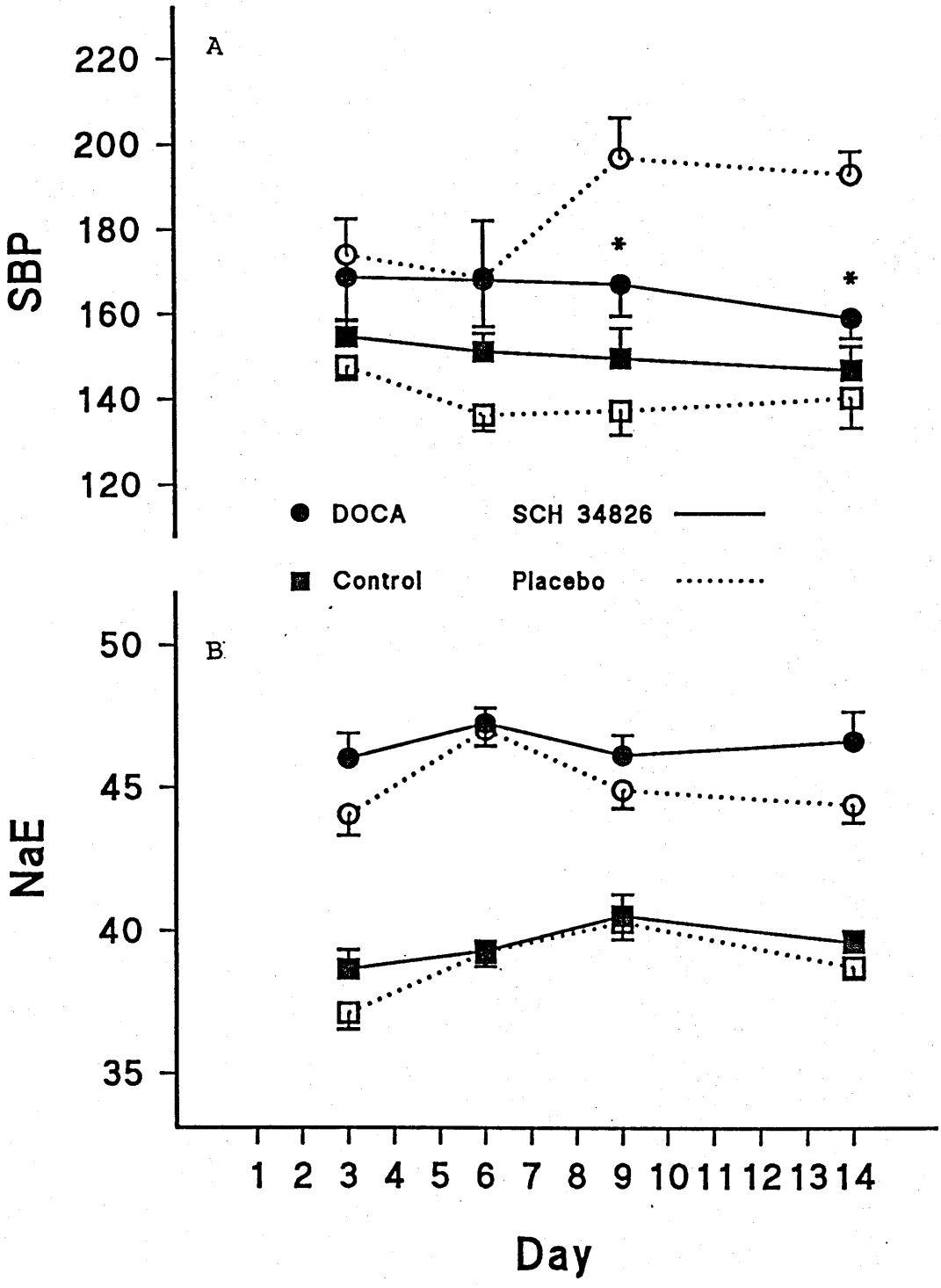


FIGURE 9.3:

BLOOD PRESSURE AND EXCHANGEABLE SODIUM IN NORMAL AND HYPERTENSIVE RATS DURING TREATMENT WITH SCH 34,826.

(A) Systolic blood pressure (SBP, mmHg) measured by the tail cuff method in conscious rats in each of the four experimental groups as indicated (n=6 in each group). * $P<0.05$ SCH 34,826 vs. placebo within the DOCA salt hypertensive rats by T-test with the Bonferoni correction.
(B) Exchangeable sodium within the same groups as labelled.

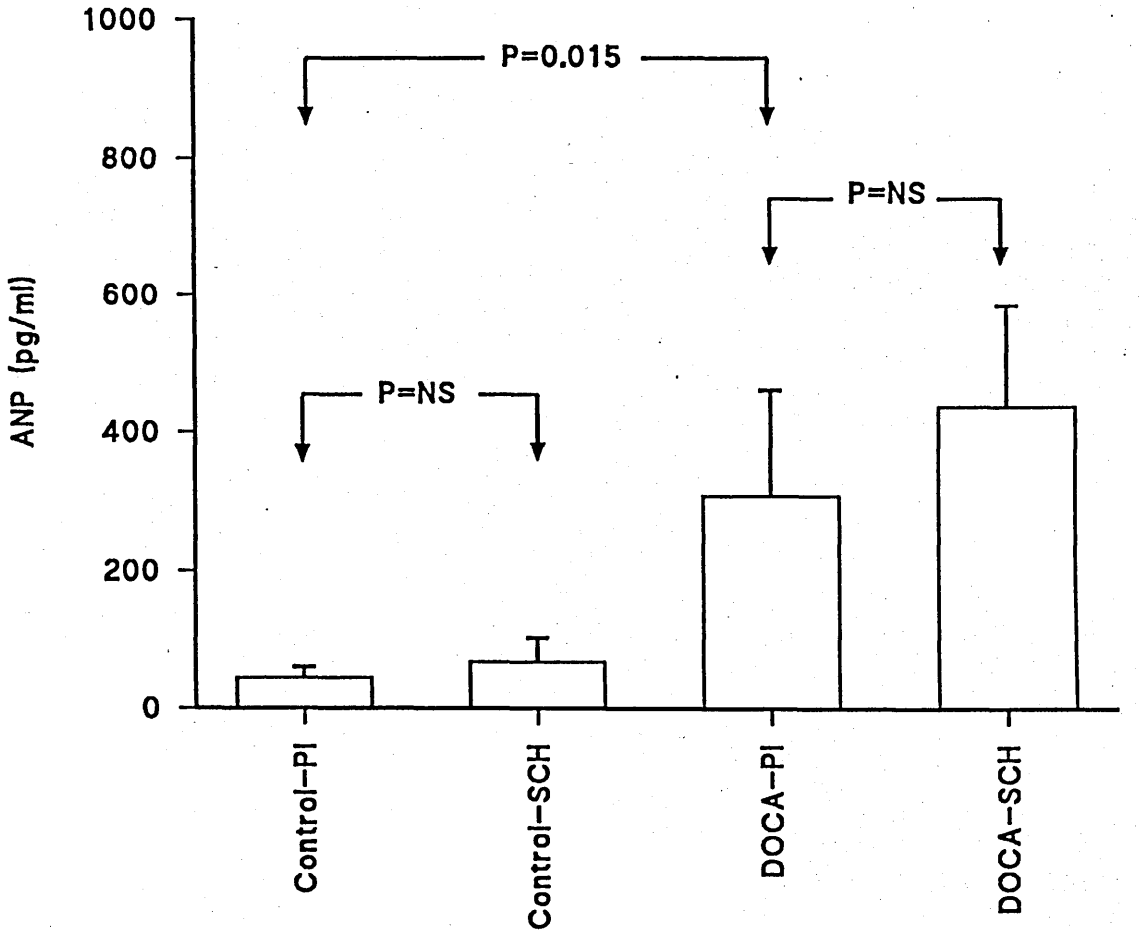


FIGURE 9.4:

PLASMA ANP AFTER TREATMENT WITH SCH 34,826 IN RATS.

Plasma ANP (pg/ml) measured two hours after the final dose of SCH 34,826 (90mg). Significance as indicated by T-test.

normal animal, where levels of around 50-100 pg/ml were seen. Hormone levels were 50% higher in the active treatment groups of both normal and DOCA salt animals but, due to small numbers of animal and the large SEM, these observations were not significant.

Cyclic GMP excretion, which is used as a marker of ANP activity, was increased in DOCA salt rats, paralleling the rise in plasma ANP, compared with the normal group. However, although there was a small increase in plasma ANP during NEP inhibition there was no eveidence of a similar trend in either the normal or the DOCA salt hypertensive groups.

Discussion.

The majority of findings in this study simply confirm those in the previous study (Chapter 8) in normal humans in that there was no significant effect on exchangeable sodium during prolonged treatment with NEPi (in this case SCH 34826 in rats). Like the preceding study it seems likely that ANP degradation was inhibited, although the higher values found in both normal and hypertensive rats were limited to a single measurement and failed to reach statistical significance. The most important finding in the present study was the significant antihypertensive effect of SCH 34826 in DOCA salt hypertensive rats.

Plasma ANP:

Plasma ANP was greatly elevated in DOCA salt hypertension as one would predict for a volume expanded state and as has previously been shown in this hypertensive strain (e.g Schiffrin and St Louis (1987)) and in other volume expanded states (Chapter 2, Chapter 3). The blood samples were taken without anaesthetic and were necessarily limited to a single time point. However, plasma ANP levels were higher two hours

after the final dose in both normal and hypertensive rats. The absence of statistical significance may reflect the small sample size and the magnitude of the rise is similar to that seen after acute administration of UK 69,578 in rats (Chapter 6) and to the non-significant increase seen on the final day of treatment with UK 79,300 in man (Chapter 8). However, it may be that SCH 34,826 - despite the in vitro similarity (Figure 5.1) - is a less potent NEP inhibitor than UK 69,578 (Chapter 10).

The pattern of changes in cGMP is interesting, but similarly fails to produce any evidence for an ANP-mediated effect. It is known that ANP receptors are down-regulated in DOCA hypertension (Schiffrin and St-Louis (1987)). In the present study, urinary excretion of cyclic GMP was greater in the DOCA salt rats, but only about three times higher than in normal rats, compared with a ten-fold increase in plasma ANP. The differing increases in plasma ANP and urinary cGMP in the hypertensive group are consistent with a reduction in ANP receptors. Despite the trend towards an increase in plasma ANP during NEP inhibition in each group there was no evidence for an increase in cGMP. In recent studies of NEP inhibitors in rats (see Chapter 10 for review) acute treatment with NEP inhibitors is almost universally accompanied by an increase in cGMP. It is not clear whether the findings in the present study mean that NEP inhibition does not act via ANP or whether the relationship between ANP and cGMP changes with prolonged treatment - perhaps due to a down-regulation of receptor numbers as was shown in the human subjects receiving UK 79,300 (Chapter 8).

Urine volume and electrolyte excretion:

Despite the findings in earlier chapters (Chapters 6-8) there

were no observed changes in urine volume, sodium or potassium excretion. This may be due to the difficulty in quantifying urine volume and electrolyte excretion in rats, rather than a difference in potency between NEPi, and the small number of rats in each group. In a previous study of prolonged treatment with Phosphoramidon and Captopril in DOCA salt rats (Nakagawa and Nasjletti (1987)), where ACE inhibition should have minimal effects, urinary sodium excretion was increased on the last day of a two-week treatment. It is possible therefore that a minor effect on renal function may have been below the limit of detection of the present experimental protocol.

Exchangeable Sodium: The failure of exchangeable sodium to show any change during NEPi confirms the findings in man (Chapter 8) and, as with the metabolic measurements, only excludes a large effect such as the differences between normal and DOCA salt animals (Brown (1981), Figure 9.3). A minor effect may still be present.

Blood Pressure: The absence of a significant change in sodium balance in this study, despite the convincing fall in blood pressure might suggest that the actions of these agents is independent of any effect on ANP. However, the mechanism by which ANP causes hypotension is not clear. Acute reductions in blood pressure, and those caused by administration of high dose infusion appear to be mediated by a reduction in cardiac output due to reduced filling pressures (Tonolo et al.,(1989), Parkes et al.,(1988)), due to a combination of the renal actions of the peptide and increased vascular permeability (increased haematocrit). The later hypotensive actions of the peptide may be independent of any change in sodium balance, the implication being that a direct vascular effect is present. For example administration of 2 pmol/kg/min ANP to the SHR for 7 days (Garcia et

al., (1985) produced a 20% fall in BP but no effect on sodium balance, and in a subsequent study these authors found no changes in intravascular volume during chronic treatment with ANP (Garcia et al.,(1985)). ANP can therefore produce antihypertensive effects in the absence of changes in sodium balance and the findings in the present study are therefore still consistent with a mechanism of action through ANP.

In conclusion, prolonged administration of NEPi in the rat - as in the human (Chapter 8) - has no demonstrable effects on sodium balance. However, treatment with SCH 34,826 (90 mg/kg) for 5 days did result in a significant reduction in blood pressure in a renin-independent model of hypertension - the DOCA salt rat. These observations confirm the possible therapeutic use of NEPi - at least in this model of hypertension.

GROUP	NORMAL		DOCA	
	PLACEBO	SCH 34826	PLACEBO	SCH 34826
UNaV				
0	4.02±0.18	4.88±0.36	5.69±0.55	6.73±0.54
10	3.88±0.29	4.54±0.29	5.12±0.50	5.89±0.35
30	3.90±0.17	4.55±0.17	4.39±0.33	4.81±0.65
100	3.91±0.27	4.66±0.27	4.60±0.45	4.99±0.55
UKV				
0	1.92±0.12	2.02±0.06	2.88±0.18	2.84±0.24
10	1.96±0.11	2.05±0.11	2.34±0.18	2.45±0.14
30	1.96±0.12	2.15±0.07	2.81±0.35	2.50±0.12
100	1.96±0.09	2.06±0.11	2.32±0.18	2.17±0.08
UVol				
0	14.4±1.4	19.2±2.8	31.0±2.4	35.7±4.5
10	14.5±1.4	19.4±2.4	25.4±1.9	31.8±3.0
30	14.0±0.9	19.0±2.0	22.1±0.8	26.2±3.6
100	15.0±1.3	19.4±1.9	24.3±2.8	28.4±4.0
UcGMP				
0	10.8±1.3	10.2±1.3	24.0±3.5	19.6±5.6
10	7.3±0.5	7.5±0.8	19.8±4.4	19.0±4.4
30	10.5±0.9	10.4±0.9	33.3±5.6	26.6±7.2
100	14.7±1.5	12.4±1.6	29.1±7.3	23.8±4.5

TABLE 9.1:

SUMMARY OF RENAL EFFECTS OF SCH 34,826 IN RATS.

Summary figure of the mean values of urinary sodium excretion (UNaV, mmoles/day), potassium excretion (UKV, mmoles/day), urine volume (UVol, ml/day) and cyclic GMP excretion (UcGMP, nmoles/day) by treatment (Dose of SCH 34,826 in mg/kg b.d). All data shown as mean±SEM, n=6. There were no significant effects of treatment of any variable either in normal or DOCA salt rats.

Chapter 10.

NEP inhibitors in perspective.

Introduction:

In 1987 when first reports emerged that NEP degrades ANP in vitro, NEP inhibitors appeared to be a logical means of elevating plasma ANP to possible therapeutic benefit (Chapter 5). The studies in the last four chapters were designed to examine this hypothesis and clearly show that acute NEP inhibition can cause an increase in plasma ANP and natriuresis, diuresis and suppression of renin (Chapters 6&7). This pattern of effects is exactly what one would predict for an inhibitor of ANP degradation. However, although chronic NEP inhibition lowers blood pressure in the DOCA salt rat (Chapter 9) there was no evidence of a sustained effect on sodium balance (Chapters 8&9), despite a probable sustained elevation in plasma ANP. The reasons for this appear to be that NEP is non-specific and NEP inhibition may potentiate both natriuretic (ANP) and antinatriuretic (e.g. Ang II) mechanisms.

In this chapter the results of other studies with NEP inhibitors,

published since 1987, are reviewed and the relevance of the work in this thesis is discussed. The overall aim is to discuss the actions, mechanisms of action and therapeutic potential of NEP inhibitors.

Actions of NEPi

Animal Studies:

In chapter 6 acute NEP inhibition was shown to cause elevation of plasma ANP and natriuresis and diuresis. The effects of several other NEPi have now been investigated by other workers with similar findings.

Thiorphan/ Phosphoramidon: Following the demonstration that Thiorphan inhibits ANP degradation in vitro (Olins et al.,(1987)), Olins et al.,(1989) have studied the effects of Thiorphan in conscious rats. Treatment with Thiorphan, during an ANP infusion, produced a 2-3 fold increase in plasma ANP for up to 60 minutes (at a dose of 30mg/kg, with doses as low as 1mg/kg showing effect). The infusion of high doses of ANP was required to show changes in plasma hormone levels, and changes in urinary function were not studied. In contrast, Koepke et al.(1989) studied the effect of Thiorphan (30mg/kg i.v.), during infusion of ANP, but were unable to demonstrate any potentiation over the effect of ANP alone at a dose of 50ng/kg/min (approx. 16 pmol/kg/min of ANP). However, in a further study Smits et al.(1989) examined the same dose of Thiorphan 30mg/kg in conscious rats. In this case the inhibitor caused non-significant increases in plasma ANP levels and those achieved by infusion of the peptide and although Thiorphan alone had no effect on renal function NEP inhibition

greatly augmented the natriuresis and diuresis of infused ANP (67ng/kg/min). None of the above studies (in the rat) were able to demonstrate a significant rise in endogenous ANP with Thiorphan, although the renal and haemodynamic changes are consistent with t he actions of ANP. Increased plasma levels have since been shown in the rabbit (Marleau et al., (1990)) where Thiorphan (25 μg/kg/min for 90 minutes) increased plasma ANP from 65±26 to 163±52 pg/ml, lending support to the theory that the renal actions of Thiorphan are likely to be secondary to inhibition of ANP degradation.

Similar findings have been made with Phosphoramidon (Figure 5.1). Ura et al. (1987), in a study designed primarily to examine kinin metabolism, noted that Phosphoramidon (110 or 330 μg/hr/kg) increased urine volume by 15%, and urine sodium excretion by 37%, but without change in renal blood flow, GFR or potassium excretion; a pattern of changes consistent with the known effects of ANP. Lafferty et al. (1989) compared the effects of Phosphoramidon in normal rats and rats with 5/6 nephrectomy, a volume expanded state. While Phosphoramidon had no effect on plasma ANP in the normal rat, it did double fractional excretion of sodium, and in the 5/6 nephrectomy rat it caused a 5-fold increase in sodium and fractional sodium excretion, with an increase in GFR and a 3-fold rise in plasma ANP. Thiorphan had a similar effect, which was also more marked in the rats with reduced renal mass. Webb et al.,(1989) have also compared the effects of Phosphoramidon and Thiorphan on the renal and cardiovascular response to bolus administration of ANP (0.5 nmol/kg) in the anaesthetised rat. Thiorphan (0.01, 0.1 and 1 mg/kg/min) and Phosphoramidon (0.001, 0.05 and 0.01 mg/kg/min) increased urinary cGMP excretion (approximately two-fold). Sodium excretion was augmented

only in the low dose groups and blood pressure (20 mmHg) in the high dose groups; the fall in blood pressure presumably attenuating the natriuretic effect (Seymour et al.,(1987)).

New NEPi: A number of newly designed NEP inhibitors (see Figure 5.1) have also been assessed in experimental animals.

In mice UK 69,578 (3mg/kg; Danilewicz et al., (1989)) increased urine volume and sodium excretion, in response to infusion of 600 µl saline, two-fold (from 136±11 to 249±15 µl/min and 10.8±1.1 to 21.8±1.5 µEq/min). In this study the rise in plasma ANP was also augmented - the peak level being doubled, and greatly prolonged. A doubling of endogenous plasma ANP has also been reported in dogs with experimental heart failure (Alabaster et al., (1989) treated with UK 69,578. Shepperson et al.,(1991) have recently confirmed these findings and shown that UK 69,578 (1-10 mg/kg) augments the natriuresis and diuresis due to saline infusion (20 ml/hr) in anaesthetised rats. The natriuretic effects of UK 69,578 were abolished by co-administration of a polyclonal ANP antisera in contrast to the effects on a quantitatively similar natriuresis produced by hydrochlorthiazide; suggesting that the observed effects of NEPi are mediated by ANP. Furthermore, acute administration of this NEP inhibitor (3-30 mg/kg) caused a dose dependent fall in BP in the DOCA salt rat of up to 30 mmHg for more than 5 hours after a single dose.

Another recently developed NEPi SQ 29,072 (Seymour et al., (1989a)) has also been shown to prolong the half-life of exogenous ANP in the spontaneously hypertensive rat (SHR) and to augment the hypotensive effects of ANP (3nmol/kg). In a more detailed study with

this compound (Seymour et al.,(1989b)), SQ 29,072 (100 μmol/kg) prolonged the depressor response to ANP for up to two hours and increased the natriuretic response to 10 and 30 nmol/kg ANP, approximately five-fold in the first hour. The increase in natriuretic response was dependent on the dose of SQ 29,072, as was augmentation of the hypotensive effect of ANP. Urinary cGMP excretion was also increased, and it was noted that the effects of NEPi were greater in volume expanded rats, presumably due either to increased endogenous ANP or suppression of antinatriuretic mechanisms.

In a more recent paper (Seymour et al.,(1991)) combined inhibition of ACE and NEP, either by the use of Captopril and SQ 29,072, or by SQ 28,133 - a compound which inhibits both NEP and ACE - resulted in a reduction in blood pressure in the SHR (-62±9 [both] vs -26±5 [SQ 29,072] or -40±10 [Captopril]) which was greater than the effect of either agent alone. In the DOCA salt rat - a non-renin dependent model of hypertension, ACE inhibition did not enhance the natriuretic effects of NEPi. Overall, these suggest that the effects of NEPi, like those of ANP (Chapter 4), are potentiated by ACE inhibition.

Two studies have examined the effects of acute NEP inhibition with the related compound SQ 28,603 (Seymour et al., (1991a)) in dogs with experimental heart failure (Marguiles et al.,(1990), Cavero et al.,(1990)). Treatment with 30-60 mg SQ 28,603 doubled plasma ANP and increased urine volume, and the excretion of sodium and cGMP to a greater extent than the infusion of exogenous ANP (to achieve similar plasma levels, Cavero et al.,(1990)) suggesting either that an alternative mechanism to ANP is involved or the plasma ANP level does not reflect levels of ANP at the active site - perhaps within the

kidney. Infusion of low dose ANP into one renal artery of dogs (Marguiles et al.,(1990)), in the presence or absence of SQ 28,603 given intravenously, again doubled the effects of ANP on urine volume and sodium excretion. The NEPi alone also caused a natriuresis in the control kidney, due to an increase in FeNa but not GFR, while the natriuresis in the supplemented kidney was associated with a rise in GFR, FeNa and FeLi (a marker of proximal tubular function). This pattern of effects is consistent with the renal mechanism of ANP, where GFR is increased only by high doses (Chapters 2,4) and FeNa by low doses.

Schering have also developed a series of NEP inhibitors (Chipkin et al., (1988), which prevent the in vitro degradation of ANP (Sybertz et al., (1989)). In normal rats SCH 39,370 (30 mg/kg i.v.) caused a five-fold increase in plasma ANP during infusion of the peptide at 1 µg/kg/min for 30 minutes, with an associated increase in the natriuresis and diuresis approximately 50% (Sybertz et al., (1989)). SCH 34,826 augmented the rise in plasma ANP following ANP injection (30 µg/kg), with increased natriuresis and diuresis in both the SHR and DOCA salt rat (Sybertz et al., (1990)). There was an associated increase in urinary ANP excretion (100 fold/3hrs) and cGMP excretion (3 fold/ 3 hours) in the DOCA salt rat. SCH 34,826 only reduced blood pressure acutely in the DOCA salt rat, and had no effect in the SHR. However, after 5 days of treatment with 90 mg/kg SCH 34,826 there was a 20 mmHg reduction in blood pressure in the SHR.

From these studies in experimental animals it is possible to draw a series of conclusions on the actions of NEPi. Although this pattern of effects is consistent with an action through ANP and urinary cGMP - a marker of ANP action - is generally reported to be increased,

plasma levels are not invariably elevated. It is not clear whether this is due to difficulty in sampling from small animals, genuine species differences or whether there are differences in potency between the agents (Figure 5.1). Plasma ANP may be little more than a marker for tissue levels of ANP - perhaps within the kidney - which may be functionally more important. In the study in chapter 6, plasma levels were increased by treatment, and significant increases have been seen in other species and agents (Cavero et al.,(1990), Marguiles et al.,(1990), Marleau et al.,(1990), Lafferty et al.,(1989)). The demonstration of an increase in ANP in these studies may reflect the fact that baseline ANP levels would be elevated by volume expansion in the rats (Chapter 6) and heart failure in the dogs (Cavero et al.,(1990), Marguiles et al.,(1990). There is also a suggestion that the response to NEPi is enhanced in these conditions, although whether this is due to higher ANP levels is not known (Seymour et al.,(1989a,b), see below). However, these data do suggest that the renal response to NEPi is governed by the same factors as ANP (Chapter 4) being reduced by a fall in blood pressure (Webb et al.,(1989)), and increased by volume expansion (Lafferty et al.,(1989)) and ACE inhibition (Seymour et al.,(1991b)).

All the above studies have examined the acute effects of NEPi and apart from the observation that blood pressure falls with chronic treatment (Sybertz et al.,(1990)) there is no published data on the prolonged renal or hormonal effects. The study in chapter 9 failed to show any effects on urine volume, electrolyte or cGMP excretion, or body sodium of prolonged treatment with SCH 34,826 in either normal or DOCA salt rats, despite an upward trend in plasma ANP and a significant reduction in blood pressure in the hypertensive animals.

Why the haemodynamic effects of NEPi should be evident, but the renal effects disappear with prolonged is not clear. Similar effects are apparent with prolonged dosing in man (see below) and may be due to the activation of antinatriuretic mechanisms and the non-specific effects of NEP inhibition.

Human Studies:

More recently NEPi have been investigated in man (Chapters 7&8). The study described in chapter 7 confirmed that UK 69,578 inhibits the degradation of ANP in man, raising plasma hormone levels two-three fold and with an associated diuresis, natriuresis and suppression of plasma renin. This confirms the renal effects of NEPi in animals but the clearer increase in plasma ANP, and the observed suppression of renin provide more substantial evidence that these agents work - as predicted - through inhibition of ANP degradation. The close relationship between changes in plasma ANP and sodium excretion (Figure 7.8) following treatment with UK 69,578 supports this hypothesis. In patients with heart failure this compound (UK 69,578 (Northridge et al., (1990); Figure 10.1) and in patients with essential hypertension its oral prodrug (UK 79,300 (O'Connell et al.,(1990)) have almost identical acute effects on plasma ANP, urine volume and sodium excretion and renin concentration. Richards et al.,(1990) have recently confirmed this pattern of effects in normal subjects given UK 79,300 orally, and shown parallel increases in plasma ANP, and urinary sodium and cGMP excretion, with reduced plasma renin and aldosterone.

The only other reported studies in man are with Sinorphan (an oral prodrug of Thiorphan; Gros et al.,(1989), Kahn et al.,(1990),

Schwartz et al.,(1990), Dussaule et al.,(1991)). In normal subjects and in patients with heart failure or ascites this agent inhibits plasma NEP and causes short term increases in plasma ANP. The pattern being similar to that of UK 69,578, and is associated with a transient increase in sodium excretion. In patients with ascites the natriuresis was less marked, but again there was a close association between the elevation in plasma ANP and cGMP (Dussaule et al.,(1991)), both of whcih were inversely related to NEP activity.

There have been no published reports on the effects of prolonged NEPi. The study in chapter 8 confirms the findings with acute NEP inhibition, in that a natriuresis and diuresis were seen on the first day of treatment. However, in contrast to the effects of prolonged low dose ANP infusion (Janssen et al.,(1989), MIzelle et al.,(1988), Kivlign et al.,(1988)) this was not maintained for the duration of treatment; instead there was an antinatriuresis on third day of treatment and overall sodium balance was not altered. Theoretically, this may be due either to activation of antinatriuretic "defence" mechanism or failure to produced a sustained rise in ANP. In fact, although pre-dose plasma ANP levels were not elevated, overall plasma levels probably were elevated as shown by the down-regulation of platelet ANP receptors (Figure 8.5). It is likely that these effects on ANP were offset by the activation of antinatriuretic mechanisms. One of the most significant findings in this study was that Ang II levels were elevated by NEP inhibition - at least during infusion of Ang II - thus antinatriuretic mechanisms may be potentiated by this treatment. In these normal subjects there was also no evidence of any antihypertensive effects. A preliminary report in hypertensive subjects treated with UK 79,300 for 28 days (Bevan et al.,(1991)) has

reported a sustained increase in ANP (approximately two-fold) supporting the view that plasma ANP is likely to be elevated by prolonged treatment. Renal function data have not yet been reported from this study but a very limited reduction in blood pressure was achieved by prolonged treatment.

These studies in man confirm the acute effects of NEPi in animals, but the more complex studies have allowed the time course in plasma ANP, and other hormones such as renin, to be well characterised. The pattern of hormonal and renal changes provide further evidence that NEPi act via ANP. Despite the promise of the results of acute NEP inhibition, the effects of chronic therapy are short-lived at least partly due to the non-specific effects of NEPi on Ang II degradation.

Therapeutic effects of NEP inhibition:

Hypertension:

Many of the animal studies (above) have examined the acute effects of NEP inhibition in hypertensive strains, and the consensus from these is that acute NEPi enhances the hypotensive response to pharmacological doses of exogenous ANP (in both normal and hypotensive rats; Sybertz et al.,(1989, 1990), Seymour et al.,(1989)). Given alone, the antihypertensive effects of NEPi are less clear. In renin-independent models such as the DOCA-salt rat (Sybertz et al.,(1989), Seymour et al.,(1989, 1990), Shepperson et al.,(1991)) acute NEP inhibition lowers blood pressure by up to 40 mmHg. In the SHR model of essential hypertension however, there are differing reports on whether acute NEP inhibition is equally (Seymour et al., (1991)) or less

(Sybertz et al.,(1990) effective. As noted above there are few studies of prolonged NEP inhibition. Sybertz et al.,(1990) found a 20 mmHg reduction in blood pressure compared with placebo in rats treated with SCH 34,826 (90 mg b.d. for 5 days), which is less impressive than the acute actions. A similar antihypertensive effect is seen in DOCA salt rats in Chapter 9. Further studies are required to assess whether or not NEPi are more (or only) effective in low renin forms of hypertension such as the DOCA salt rat (as suggested in chapter 8) and whether high absolute levels of ANP must be achieved (either by infusion or in situations where endogenous ANP is elevated, eg DOCA-salt rat).

In humans there is only a little preliminary work on the antihypertensive effects of NEPi. In normal subjects and patients with heart failure, acute or chronic treatment has no effect on blood pressure (Chapters 7 & 8, Northridge et al.,(1990), Richards et al.,(1990)). In patients with mild essential hypertension, acute administration of UK 79,300 (O'Connell et al.,(1991)) increased plasma ANP and sodium excretion but had no effects on blood pressure. The only "positive" study of NEPi in humans with hypertension is the uncontrolled and therefore uninterpretable study of Lefrancois et al.,(1990) where treatment with increasing doses of Sinorphan resulted in a 15 mmHg fall in mean blood pressure over a four week period. A more recent study (Bevan et al.,(1991)) in which 40 patients were randomised to receive either UK 79,300 or placebo for 28 days, found a 10/3 mmHg reduction in supine blood pressure in the active group compared with 5/4 mmHg in placebo (P=NS). The only significant differences were in the standing systolic pressure which fell by 10 mmHg compared with 1 mmHg in the placebo group; despite a sustained rise in basal ANP from 37 to 56 mmHg.

The mechanism of blood pressure reduction cannot be resolved from

these studies. However, in the absence of any renal effects of prolonged treatment it seems most likely that observed antihypertensive effects of chronic treatment are due to direct vascular effects of ANP, whilst the acute effects may involve changes in intravascular volume. This pattern is also seen during infusion of the exogenous peptide (Chapter 9 - Discussion).

Overall, although there are only limited data on the antihypertensive effects of prolonged NEPi, these appear modest and may be restricted to low renin forms of hypertension.

Heart Failure:

Despite the high circulating levels of ANP in heart failure (Cody et al.,(1987)), with down-regulation of ANP receptors (Schiffrin (1988)), and the associated neurohumoral activation, both of which might be expected to offset the effects of ANP/NEP, NEP inhibition appears to be at least as effective as in normal subjects. In their preliminary study of UK 69,578 in six patients with mild chronic heart failure, Northridge et al.,(1989,1990; Figure 10.1) found that NEP inhibition caused a two to five fold increase in plasma ANP (with levels of over 1000 pg/ml in some patients), associated with a two to three-fold increase in urine volume and sodium excretion for approximately four hours. Furthermore, there was a significant reduction in both right atrial and pulmonary capillary wedge pressures, sustained for up to eight hours (see Figure 10.1). This pattern of changes has since been confirmed, albeit in a very preliminary form, (Kahn et al.,(1990)) using the oral NEPi Sinorphan. In patients with more severe heart failure this compound is reported to cause a similar rise in ANP over 2-3 hours, associated with a 20%

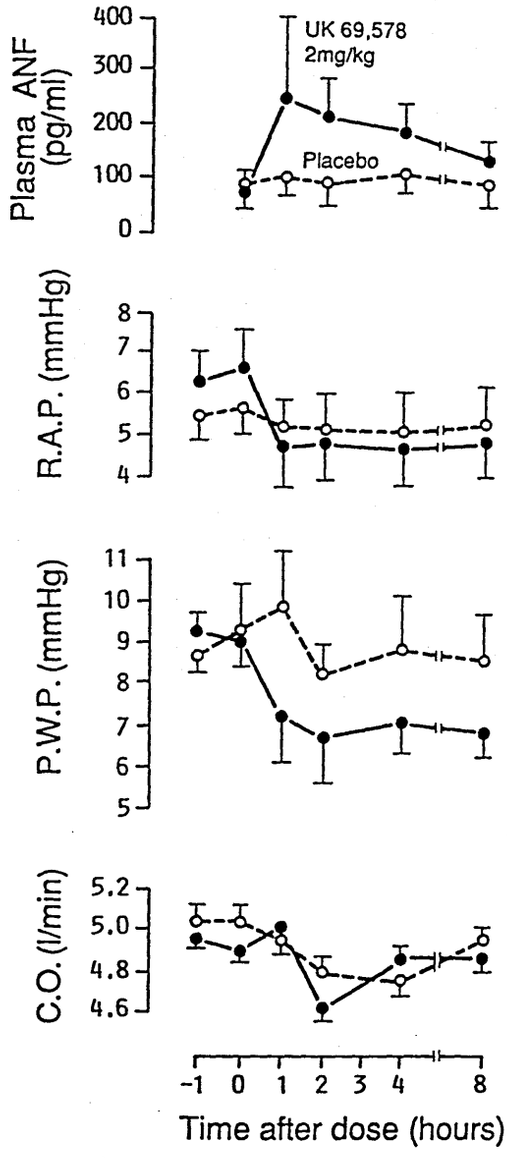


FIGURE 10.1:

EFFECT OF NEP INHIBITION IN HEART FAILURE.

Summary of the effects of UK 69,578 in six patients with mild (NYHA grade 1/2) heart failure on plasma ANP (ANF, pg/ml), right atrial (RAP) and pulmonary capillary wedge pressure (PWP, mmHg) and cardiac output (CO, l/min) against time in hours. UK 69,578 was administered over 20 minutes at time 0. (after Northridge, Jardine et al., (1990) with permission).

reduction in renin and in cardiac filling pressures.

There have been no detailed studies of the renal actions of NEP inhibition in heart failure in humans. However, the findings of Cavero et al.,(1990), in dogs with experimental heart failure (due to rapid ventricular pacing), suggest that the renal effects are maintained or enhanced (compared to either normal dogs or the infusion of ANP in dogs with heart failure). Why this should be the case when one might expect the opposite in a condition where there is activation of the renin-angiotensin system and down regulation of ANP receptors is unknown and although Marguiles et al.,(1990) have suggested that increased degradation of ANP in heart failure may be responsible, the involvement of other substrates for NEP must be excluded. A further alternative is that the threshold for the venodilator actions of ANP is lower than that for the other actions of the peptide (Bie et al.,(1988)) and it is these effects which may be particularly useful in the treatment of heart failure. The question of whether or not these potentially beneficial effects are present during chronic treatment are awaited with interest.

How do NEP inhibitors work?:

Having reviewed the effects of NEP inhibition, the questions remain of how these effects are achieved and whether the original assumption that inhibition of ANP degradation is the likely mechanism is correct. In fact, NEP has many substrates (Figure 5.1) and there is evidence both for and against an ANP-mediated effect.

Much of the evidence supporting an ANP-mediated effect is circumstantial:

1. Acute NEP inhibition raises plasma ANP, approximately 2-3 fold for several hours (Chapters 6 & 7, Kahn et al.,(1990), Northridge et al.,(1990), Lafferty et al.,(1989), Marleau et al.,(1990), Danilewicz et al.,(1990), Shepperson et al.,(1991), Marguiles et al.,(1990), Cavero et al.,(1990), Sybertz et al.,(1989)). The urinary excretion of ANP is also enhanced.

2. Acute (e.g. Chapters 6 & 7) and chronic (Chapter 8) NEP inhibition produce a pattern of changes in urine volume, sodium and potassium excretion which is similar to that caused by low dose infusion of ANP (at plasma levels similar to those achieved by NEP inhibition). The natriuresis is directly related to the rise in plasma ANP (Chapter 7) and when compared directly - one against the other (Chapter 6, Cavero et al.,(1990), Marguiles et al.,(1991)) - NEPi and ANP produce a similar pattern of effects.

3. The actions of NEPi are usually associated with an increase in urinary cGMP excretion (Dussaule et al.,(1990), Richards et al.,(1990), Marguiles et al.,(1990), Sybertz et al.,(1989, 1990) - a marker of ANPs cellular effects (Wong et al.,(1988)), even in the absence of a demonstrable rise in plasma ANP (Sybertz et al., (1989,1990)). Furthermore, the natriuresis is quantitatively related to changes in cGMP excretion (Dussaule et al.,(1991)).

4. Although few studies have examined the mechanisms underlying the renal effects of NEPi and the results are inconsistent, they are similar to those of ANP (Chapter 2). In chapter 7, there was a significant rise in FeNa, but not GFR or ERPF, similar to low dose ANP. This pattern has been confirmed for NEPi given alone (Cavero et al.,(1990), Marguiles et al.,(1990)). However, NEPi given in the presence of elevated exogenous (Marguiles et al.,(1989)) or endogenous

(Lafferty et al.,(1990)) ANP increase GFR, the pattern being similar to that of high dose ANP administration (Chapter 2).

5. The endocrine (suppression of renin, Chapter 7) and vascular (hypotensive, Chapter 9) effects of NEPi are very similar to those of ANP and quite dissimilar to any other diuretic.

6. NEPi potentiate the response to infused ANP (Smits et al.,(1990), Seymour et al.,(1989), Olins et al.,(1989)), although whether or not this is a specific effect remains to be seen.

7. Finally, the renal response to NEPi is modulated in the same way as the response to exogenous ANP by intravascular volume, arterial pressure and the renin-angiotensin system (Seymour et al.,(1989, 1991); Webb et al.,(1988)).

Although the above evidence is circumstantial, taken together it provides strong evidence for an ANP-mediated effect. The only evidence to date which directly implicates ANP is the observation of Samuels (Samuels et al.,(1989), Shepperson et al.,(1991)) confirmed by Schwartz et al.,(1990) that polyclonal ANP antisera block the natriuretic response to NEPi but not to other diuretics.

The evidence against ANP is less convincing and centres on possible alternative mechanisms. Inhibition of kinin degradation is the most likely, since kinins are also natriuretic, diuretic and vasorelaxant (Scicli and Carretero (1986)) and urinary kinin levels are increased by NEP inhibition (Ura et al.,(1987)). Against a role for kinins are the observations that kinins stimulate rather than suppress renin secretion (Carretero et al.,(1989)) and NEPi does not enhance the response to exogenous bradykinin (Sybertz et al.,(1990), Gros et al.,(1991)). However, infusion of a kinin receptor antagonist (Smits et al.,(1990)) has been shown to abolish the natriuretic

response to combined treatment with ANP plus Thiorphan in rats. It remains to be seen whether this is a specific effect on ANP and/or NEPi, or indeed whether this agent is a specific kinin receptor antagonist. A similar blunting of the natriuretic response to phosphoramidon by a kinin antagonist (albeit in the presence of an ACE inhibitor) has been reported by Nakagawa et al.,(1990). However, in a similar experiment (Roman et al.,(1988)) a kinin antagonist failed to alter the natriuretic response to combined NEP and ACE inhibition in rats. Nevertheless, it is impossible to dismiss a contribution from increased circulating or intra-renal kinins on the observed renal effects.

A further, as yet unconfirmed suggestion (Vane (1990)) is that NEPi may inhibit the the conversion of Endothelin to its active form, offering another hypotensive mechanism. The balance of evidence, however, strongly favours the original hypothesis: that NEPi act via inhibition of ANP degradation.

One final problem which is difficult to explain is how inhibition of an enzyme on the luminal aspect of the proximal tubule can have any effect on circulating hormone levels. Two points emerge from this. The first is that NEP is found in other tissues and it has recently been confirmed that NEP inhibition raises plasma ANP even in anephric rat (Barclay et al.,(1991)). In this model UK 69,578 reduces the degradation of exogenous ANP by approximately 40% ($T\ \frac{1}{2}\ [\beta]$ = 140 vs 102 secs). The second point is that of the site of action of NEPi (Granger (1990)). Inhibition of NEP at its major site - in the proximal tubule - should only increase hormone levels more distally within the tubule, where the hormone may act on luminal receptors.

Sonnenberg (1990) has recently confirmed that stimulation of these receptors results in increased sodium excretion and Stephenson and Kenny (1988) have postulated that the role of NEP is to regulate the delivery of ANP to this site, rather than to recover the peptide from the urine. The findings that cGMP excretion may be increased before plasma ANP (Sybertz et al.,(1989,1990)) and that the renal effects of NEPi are greater than can be reproduced by infusing ANP to similar plasma levels (Cavero et al.,(1990)) are consistent with an extravascular action of NEPi -which seems most likely to be within the tubule.

Overall, both increased intra-tubular and plasma ANP are likely to contribute to the renal effects of NEPi.

Conclusions:

This section cover the time-period from the discovery that NEP degrades ANP (1987) to the present. In that short time a variety of NEPi (including older "enkephalinase" inhibitors and newly designed compounds) have been investigated in animals and in man. To some extent these agents have fulfilled their original promise in that they raise endogenous ANP and cause a spectrum of renal, endocrine and vascular effects similar to infused ANP. However, although they appear to have beneficial effects in some forms of hypertension and in heart failure, the effects on long term salt and water balance appear to be limited. This is partly due to lack of enzyme specificity (with inhibition of the degradation of other substrates for NEP including the antinatriuretic hormone Ang II), but also to the very modest effects that ANP has when increased to the levels produced by NEP inhibition in the last four chapters.

Further work is required to assess the effectiveness and acceptability of NEPi in different forms of hypertension (specifically non-renin and renin dependent hypertension) and whether or not it is more effective in combination with other agents (Chapter 13). Although not as potent as was first hoped, the recent work on NEPi has shown that it is possible to harness at least some of the therapeutic potential of ANP with oral drug therapy. Future work should aim at the development of more specific inhibitors of ANP degradation, combination of NEPi with other agents (Chapter 13) with the aim of increasing plasma ANP levels two to four times those achieved by NEP, at which levels sustained haemodynamic and renal effects would be expected (Mizelle et al.,(1990)).

SECTION 5.

CALCIUM AND THE RELEASE OF ANP.

Chapter 11.

Effects of extracellular calcium and calcium antagonists on the release of ANP in vitro.

Introduction.

This final section examines aspects of the role of calcium on the release of ANP in vitro (Chapter 11) and in vivo (Chapter 12). There were two aims behind these studies. Firstly, there are theoretical reasons to believe that atrial stretch-secretion coupling is likely to involve calcium (see below) and, secondly, following the general theme of this thesis - to examine the possible therapeutic interactions between ANP (NEPi, Section 4) and calcium antagonists.

ANP is secreted in response to atrial distension. The stretch-secretion coupling mechanism is self-contained within the atrial myocyte since the isolated heart (Dietz (1987)), atria (Bilder et al.,(1986)) or dispersed myocytes (Gibbs (1987b)) release ANP in response to distension. The nature of this stretch-secretion coupling mechanism is still unknown but, for a number of reasons, may involve intracellular calcium. Stretch-activated ion channels

are present in other tissues, for example in nerve sensory terminals, proximal tubular and choroid plexus epithelial cells (Sackin (1986), Christensen (1987)), and endothelial cells (Lansman et al.,(1987)). Stretch sensitive calcium channels have been proposed in cardiac muscle (Allen et al.,(1988)) to account for part of the classical length-tension relationship in cardiac muscle. The simplest model for ANP release would involve similar stretch activated channels to elevate intracellular calcium which is a central event in exocytosis in other tissues (Rubin (1982)). With regard to ANP a number of stimuli other than stretch have been shown to increase hormone release including increased heart rate (Schiebinger, Baker and Linden (1987)), α- and ß-adrenoceptor agonists (Schiebinger and Linden (1988)), Endothelin (Stasch et al.,(1989)) and cardiac glycosides (Yamamoto et al.,(1988)). In the heart all of these stimuli increase the availability of intracellular calcium by differing mechanisms, although other factors which occur as a consequence of increased calcium - for example, contractility - may indirectly alter secretion (below).

The purpose of this first study was therefore to look at a simple experimental model - the isolated rat atrium - and examine the hypothesis that ANP release is dependent on intracellular calcium (Ca_i), by altering Ca_i indirectly through changes in extracellular calcium (Ca_o) or calcium antagonists.

Methods:

The right atrium was removed from male SD rats (300g) killed by cervical dislocation, and rapidly immersed in ice cold physiological solution (below). The atrium was then suspended between a stainless steel electrode (Figure

11.1) and a tension transducer (Elcomatic EM 750, Elcomatic, UK) and superfused with physiological solution (PS) at 37°C at a rate of 1ml/minute. The superfusion cannula acted as a second electrode and the preparation was electrically paced using an isolated stimulator at 2 Hz using a supramaximal square wave stimulus. The preparation was initially suspended under 0.5g resting tension and developed tension was continuously recorded using a Washington FC100/FC142 amplifier/chart recorder (Washington Ltd., UK). The physiological solution contained 125 mM NaCl, 25 mM NaHC03, 5 mM HEPES, 5 mM Dextrose, 1 mM MgS04, and either 1 mM EGTA or 0 mM CaCl2 or 2 mM CaCl2 ("normal"). In one series of experiments Verapamil (Cordilox, Abbott, UK) was added to "normal" PS to a final solution of 1 μM.

The preparation was allowed 60 minutes to stabilise (after Shiebinger et al.,(1986)), during which it was superfused with "normal" PS, following which three 5 minute control collections of effluent were made. Thereafter there were four experimental limbs: in (a) superfusion was continued with normal PS for a further 12 five minute collection periods; in (b) the superfusate was changed to one containing (nominally) 0 mM calcium for six 5 minute periods before returning to "normal" PS for the remaining six 5 minute collections; in (c) the superfusate was changed to one containing 1 mM EGTA for five minutes before returning to "normal" PS for the remaining 11 periods and in (d) superfusate containing 1μM verapamil was given for six 5 minute periods before returning to "normal" PS for the remaining six periods. Effluent was collected in chilled 5 ml tubes and rapidly frozen at -20°C, being stored at this temperature until assay. ANP was measured by direct radio-immunoassay (Appendix 1). Pre-extraction is used to remove large molecular weight substances from plasma prior to RIA. Isolated atria are reported to release

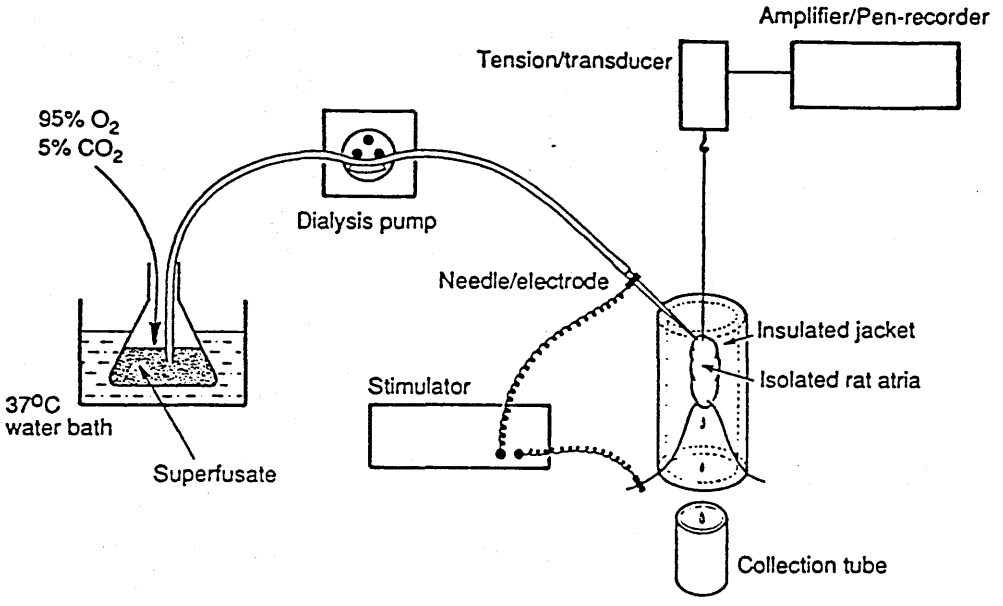
mature (28AA) peptide and there were no plasma components present; pre-extraction was therefore deemed unnecessary.

Results:

Stability of the preparation: Release of ANP was highly variable and fell steadily over the first 45 minutes (Figure 11.1) in normal calcium PS. This presumably represents leakage from damaged cells and after one hour the rate of hormone release was relatively stable.

Manipulation of Calcium: Changing to nominally calcium free PS (0mM Ca) resulted in a decline in developed tension over a period of a few minutes reflecting the depletion of intracellular calcium and the diffusion of calcium from the interstitial fluid into the superfusate (Figure 11.2). When 2 mM calcium was restored the developed tension returned to normal. In contrast the high dose of verapamil resulted in a slow decline in developed tension to about half the basal values, with no evidence of recovery, suggesting irreversible, possibly toxic effects on the myocytes with this concentration of verapamil. In preliminary experiments, 5 mM EGTA for 30 minutes resulted in rapid abolition of tension from which there was no recovery (in contrast to the results of deBold and deBold (1989)). As a compromise 1 mM EGTA was given for 5 minutes. There was a rapid abolition of tension (over a few minutes) representing depletion of intracellular calcium and the diffusion of calcium out of the interstitial spaces.

In contrast to the clear and predictable changes in developed tension which are produced by manipulation of Ca_o, there were no differences in the release of ANP, which gradually declined to between 50 and 70 % of basal levels over the 60 minutes of the interventional part of the study.



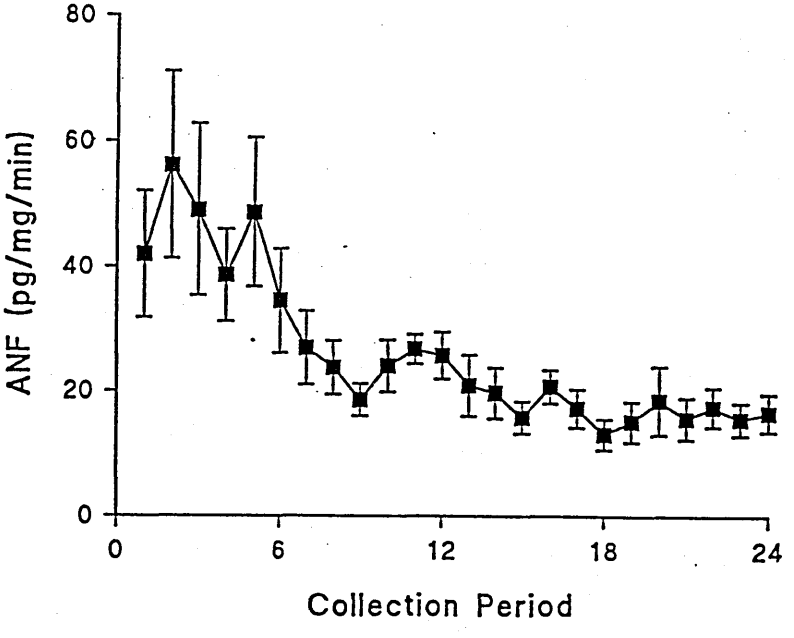


FIGURE 11.1:

RELEASE OF ANP BY THE ISOLATED RAT ATRIUM.

A. Schematic representation of the perfusion apparatus for the study of the release of ANP from the rat atrium in vitro (see text for details).

B. Release of ANP (pg/mg atrial tissue/minute) during 24 5 minute collection periods. n=8, data shown as mean±SEM.

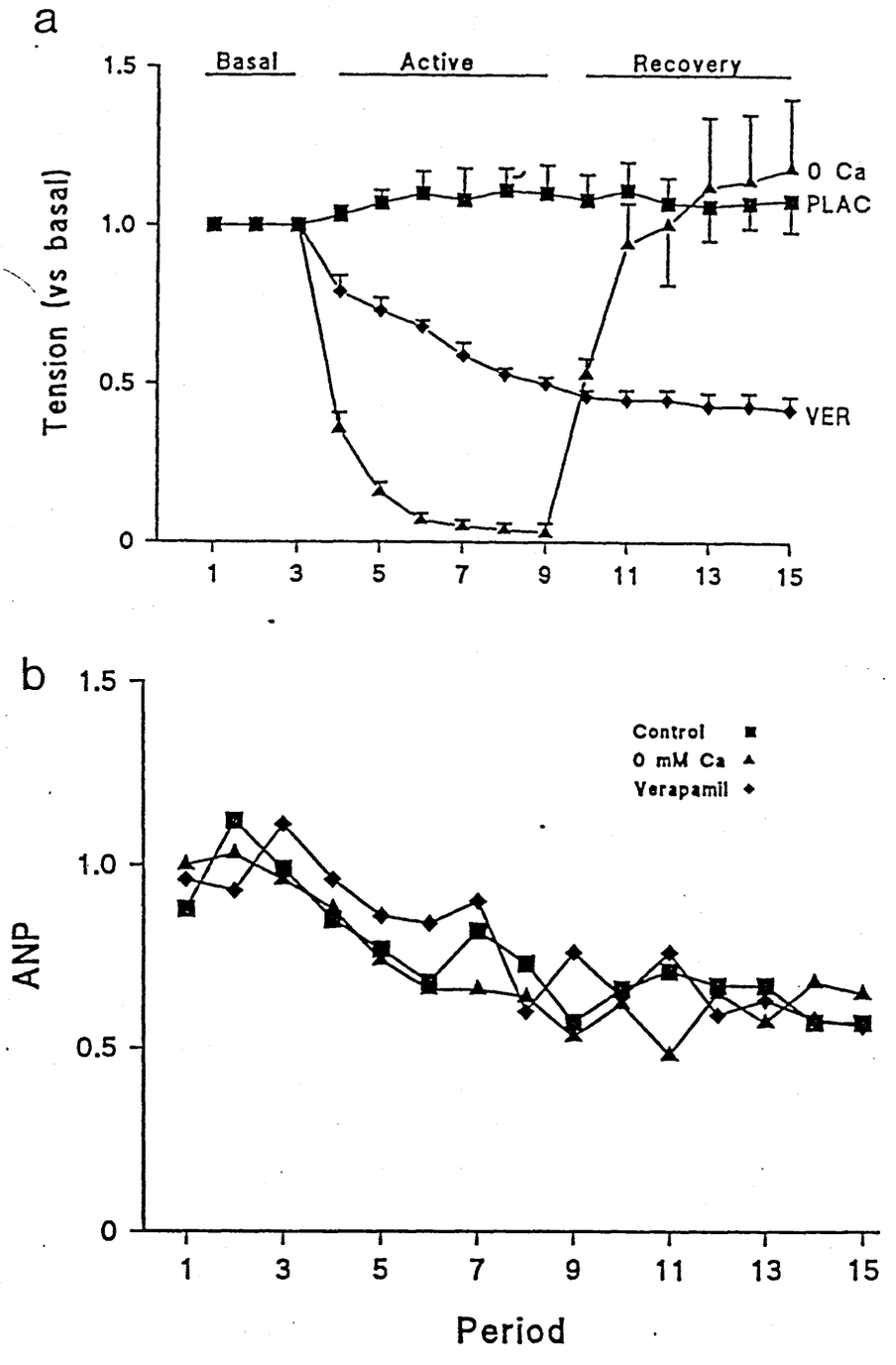


FIGURE 11.2:

EFFECTS OF ZERO CALCIUM AND VERAPAMIL ON ANP RELEASE.

Effects on (A) tension - as a fraction of basal tension - and (B) ANP release - as a fraction of basal release - of changing the superfusate to 0 [Ca] or 10^{-6} M Verapamil. After 3x5 minute control collections in 2mM Ca superfusate, the superfusate was changed for 6x5 minute collections before being restored to normal for the final 6x5 minute collections. n=8 in each group, only mean data points are shown for clarity.

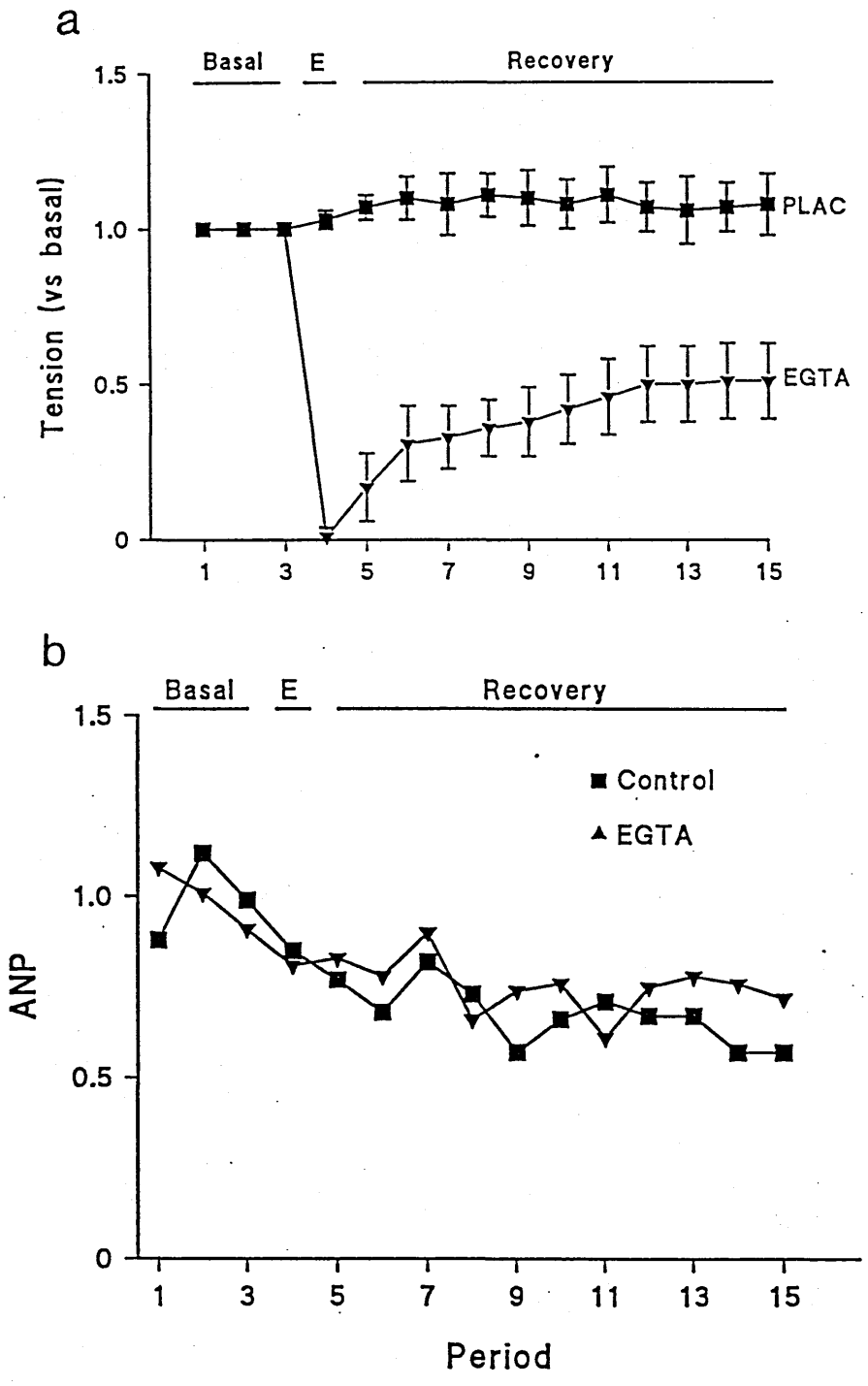


FIGURE 11.3:

EFFECTS OF EGTA ON ANP RELEASE.

Effects on **(A)** tension - as a fraction of basal tension - and **(B)** ANP release - as a fraction of basal release - of changing the superfusate to 1 mM EGTA. After 3x5 minute control collections in 2mM Ca superfusate, the superfusate was changed for 1x5 minute collection before being restored to normal for the final 11x5 minute collections. n=8 in each group, only mean data points are shown for clarity.

Discussion:

This study was limited in its objectives - to examine the potential influence of calcium on unstimulated ANP release in vitro. The results are similarly limited - to showing no effect of calcium on ANP release, but a clear dissociation between ANP release and developed tension. It is easy to criticise the failure to study stimulated ANP release but, in fact, the isolated atrium is probably far from the ideal preparation to study the release of ANP (see below), there was a large inter-preparation variability (necessitating the expression of the data as percentage values - a problem reported by other workers e.g. Schiebinger et al.,(1987,1988)) and in preliminary experiments it was not possible to produce consistent release either by mechanical or hormonal stimulation (not shown). However, the observed findings are entirely consistent with the published reports showing that calcium can stimulate, inhibit or have no effect on the release of ANP in vitro (see below). It does not seem possible to absorb these apparently contradictory observations into a single theory and part of the problem lies in the absence of an ideal preparation for studying ANP release in vitro.

Experimental models: The isolated atrium is composed of multiple cell layers, the innermost cells dependent on diffusion of nutrients and waste products through the extracellular matrix. Thus, these inner layers will be, at best, metabolically compromised. The time scale for diffusion is well illustrated in the present experiments where it can be seen that the time taken for calcium to diffuse out of the preparation (and contraction to cease) is several seconds; for larger molecules such as glucose and especially ANP the time scale would be expected to be much greater. In fact, contraction of the

cells may be expected to be important in the circulation of fluid within the extracellular space and Cho et al.,(1988) found that distension of the isolated atrium only produced an increase in ANP release after distension was released, consistent with this suggestion. Another problem relates to the use of EGTA to lower Ca. EGTA disrupts cell membranes causing "skinning" of the plasma membrane (Miller (1977)) and this disruption is probably important in cell viability since the preparation did not recover full contractile strength even after a short exposure. Increasing the permeability of the membrane in this way may perhaps promote the fusion of vesicles within the cell increasing the release of ANP. A further complicating feature is the suggestion by Needleman that a calcium dependent enzyme may be involved in the processing of pro-ANP to ANP (Ito et al.,(1988)). Finally, many groups have failed to control heart rate. Increases in heart rate are known to stimulate ANP release (Schiebinger and Linden (1986)) and intracellular calcium is similarly dependent on contraction frequency. Schiebinger (1989) found that quiescent atria did not release ANP in response to ß-adrenoceptor stimulation, in contrast to beating atria, therefore the results of studies on unpaced atria must be viewed with caution. What then is the evidence for and against a role for calcium in ANP release ?

1. Calcium-dependent release. At the level of gene expression, in cultured atrial cells, calcium increases ANP mRNA (LaPointe et al.,(1990)). Similarly, in tissue culture experiments increases in extracellular calcium within the physiological range (0-2 mM), or treatment with the calcium ionophore A23187 (Hirata et al.,(1988), LaPointe et al.,(1990)) increase ANP release and these effects can be blocked by the calcium channel blocker verapamil (LaPointe et al.,(1990). Infusion of subpressor doses of calcium into normal animals

(Yamamoto et al.,(1988)) and man (Finn et al.,(1990)) causes an increase in plasma ANP. In man Finn et al., (1990) found that increasing plasma calcium by approximately 0.3 mM caused a 40 % increase in plasma ANP with a 140% increase in urinary sodium excretion; in dogs the rise in calcium was much greater (3-fold) and resulted in a 4-fold increase in plasma ANP (Yamamoto et al.,(1988)). Ouabain, which increases both intracellular calcium and cardiac contractility causes a similar increase in plasma ANP in dogs (Yamamoto et al.,(1988)) and in cultured atrial cells in vitro (Bloch et al.,(1988). In isolated preparations Schiebinger (1989) found that reducing extracellular calcium from 1.8 to 0.2 mM, or the addition of a calcium channel blocker (10μM Nitrendipine), or a blocker of Ca release from sarcoplasmic reticulum (Ryanodine 1μM) inhibited ß-adrenoceptor or adenylate cyclase mediated release. The stimulatory effect of Endothelin, which acts through phosphotidylinositol turnover, on ANP released from paced isolated atria was similarly inhibited by reducing extracellular calcium to 0.2 mM or treatment with the calcium channel blocker Nitrendipine but not by Ryanodine (Schiebinger and Gomez-Sanchez (1990)). One failing of these studies (Schiebinger (1989), Schiebinger and Gomez-Sanchez (1990)) is that contractility changes tended to parallel ANP release and since contractility may be predicted to influence the rate of appearance of ANP in the superfusate (above) this may influence the interpretation of these results.

2. Non-calcium dependent release.: There are few other reports that calcium has no role in the release of ANP. However, Gibbs (1987), found that calcium removal did not inhibit the effect of osmotic stretch on ANP release in isolated perifused atrial cells in keeping with the findings of the present series of experiments.

3. Negative modulation of ANP release by calcium.: Although the rationale for calcium dependent ANP release is strong, and much of the above evidence convincing, there are a number of studies showing the opposite effect of calcium i.e. suppression of release with increased calcium (deBold and deBold (1989), Greenwald et al.,(1989)). In deBold's study 2 mM EGTA was used to remove calcium and caused a sustained increase in hormone release and inhibition of contractility. These effects were fully reversed by restoration of calcium and are therefore inexplicably different from the present study, where the effects of EGTA were irreversible. When nominally Ca free buffer was used there was a transient rise in ANP release which may be due to increased heart rate as a consequence of removal of extracellular calcium. Greenwald et al.,(1989) used cultured myocytes which released only proANP, the release of which was stimulated by addition of EGTA - to lower calcium - and increased by ionomycin - which increases intracellular calcium. Iida and Page (1989) have shown in cultured cells that phorbol ester stimulated ANP release is present even when influx of calcium was reduced by EGTA and sarcoplasmic reticulum release of calcium by ryanodine, implying that calcium is not essential. However, they also found that ANP release was reduced when contraction was inhibited by tetrodotoxin, supporting the theory (above) that contraction may play a role in the migration and release of atrial granules (even in isolated cells).

Overall, these studies have in common the use of EGTA, which may be inappropriate (above) but do confirm that ANP release can be stimulated in the absence of extracellular calcium in some preparations and experimental circumstances.

In conclusion from the present study reduction in extracellular calcium or calcium channel blockers has little effect on ANP release. However, the balance of evidence from studies emerging over the last three to four years is that ANP release is, at least partially, dependent on calcium. The inability of the present study to demonstrate this may be methodological. Removal of calcium would be predicted to reduce ANP release and this may be difficult to see against the background of a falling baseline (Figure 11.1 - 11.3), and changes in contractility since this may affect the rate of appearance of ANP in the superfusate.

Many factors might contribute to the apparently opposite findings of different workers regarding the role of calcium in ANP release. Firstly, there is no ideal preparation in which to study release is unknown. Secondly, it is conceivable (and probably desirable) that contractility and hormone release should be regulated independently, perhaps by different calcium pools within the cell. There appears to be a consensus that release of calcium from sarcoplasmic reticulum is not essential (Schiebinger and Gomez-Sanchez (1990), Iida and Page (1989)). Finally, calcium may be a co-factor in release and other stretch-sensitive factors may be important. For example, it has recently been shown that atrial cell membrane phosphotidylinositol turnover is independently increased by atrial stretch (Harsdorf et al.,(1989) and direct activation of protein kinase C increases the release of ANP (Iida and Page (1989)). However, althoug such a mechanism may be partially calcium-independent it cannot explain the the actions of disparate stimuli, including ß-agonists, which do not act through phosphotidylinositol turnover.

Further work is obviously required to work out the precise mechanism by

which the atrial cell regulates hormone release and future in vitro studies will need to circumvent all the above problems. To remove the problems of cell viability (falling baseline release) and diffusion time isolated or cultured cells would be the best choice, but these will require to be paced in the culture dish, presumably by field stimulation, in order to remove the confounding effects of changes in heart rate. The application of a physiological stimulus to ANP release in isolated cells is difficult since osmotic stretch is non-physiological and may disrupt the cell membrane. However, this may be circumvented by culturing cells on a flexible membrane (Buck (1983)) allowing the study of factors which modulate release. Finally, the use of chelating agents such as EGTA must be used with caution in view of their partially irreversible effects on cell membranes.

Chapter 12.

Effects of Nifedipine on ANP response to saline loading in man.

Introduction:

Although the results of studies on the role of calcium in ANP release in vitro are conflicting (Chapter 11) there is a greater consensus from in vivo studies. Increasing extracellular calcium by infusion (Yamamoto et al.,(1988), Fujimara et al.,(1989), Finn et al.,(1990)) or intracellular calcium using ouabain (Yamamoto et al.,(1988), Bloch et al.,(1989)) increases plasma ANP with an associated increase in sodium excretion. The purpose of the present study was to examine the effects of acute treatment with a calcium channel blocker (Nifedipine, shown to inhibit ANP release in vitro) on the release of ANP in response to saline loading in man, (a) to further investigate the role of calcium in ANP release (Chapter 11) and (b) with the possible therapeutic use of ANP (Chapters 5-10) to assess any possible interactions with calcium channel blockers.

Methods:

The protocol was approved by the Western Infirmary Ethical Committee and all subjects gave written informed consent before participating. Eight normal male subjects were studied (aged 26-49 years) on two separate study days, at least one week apart. No formal dietary restrictions were made but subjects were asked to adhere to their normal diet throughout the study. On each study day subjects were admitted to the Unit at 8a.m. heparinised cannulae were inserted into opposite forearm veins for sampling and infusion. The subjects remained supine for the duration of the study except to pass urine. After a 30 minute rest subjects emptied their bladders and drank 350mls tap water. Thereafter, urine collections were made at hourly intervals for the following six hours. Bolus doses of PAH and inulin (0.5 and 3.0 g respectively) were given at time zero and then infused at 0.5 and 1.0 g/hour respectively for the remaining six hours. After one hour Nifedipine (Adalat, Bayer (UK), 10mg) or identical placebo were given (double blind) with 150 mls tap water. A further hour was allowed for absorption and 1000 ml 0.9% NaCl at room temperature was then administered over 30 minutes by intravenous infusion. Blood samples were taken for measurement of PAH, inulin, electrolytes, FBC, renin, and ANP (Figure 1) at 1, 2, 4, and 6 hours; with additional samples for GFR, ERPF and ANP at 2.5, 3 and 5 hours. ERPF and GFR were calculated from plasma levels of PAH and Inulin assuming steady state conditions (Schnurr et al.,(1980)). Blood pressure, and pulse were measured at 30 minute intervals throughout.

Assays: ANP was measured by radioimmunoassay as previously reported (Richards et al.,(1987)). Renin, PAH and inulin were measured by established methods (Millar et al.,(1980), Richards et al.,(1987)).

Statistical analysis: is by repeated measures analysis of variance between

treatments with post hoc comparison between groups using paired T-test when appropriate (SPSS/PC+, SPSS inc.,USA).

Results:

1. Plasma ANP (Figure 12.1).: Baseline ANP levels were well within the normal range (5-50 pg/ml). Overall there was no statistically significant difference between the plasma ANP levels and their response to saline loading in the presence or absence of Nifedipine. However, the peak level of ANP was slightly higher in the presence of Nifedipine and fell more rapidly than the placebo group.

2. Urine Electrolyte Excretion (Figure 12.1).: In the presence of Nifedipine there was an increase in the diuresis and natriuresis in response to saline loading. Cumulative urine volume following saline infusion was increased from 530±96 to 698±93 mls/4hours (P=NS) and urinary sodium excretion from 54.4±3.5 to 82.6±7.6 mmoles/ 4hours ($P<0.05$). Potassium excretion was not significantly altered. By MANOVAR only urinary Na excretion was significantly increased ($P<0.05$), the increase in UNaV being significant in the two hours following saline infusion (Figure 12.1).

3. Pulse and Blood Pressure. (Table 12.1). There were no significant changes in blood pressure or pulse rate in these normal subjects, although the systolic and diastolic pressures were marginally lower in the active limb (Nifedipine).

4. Hormone levels (Table 12.1). Renin and angiotensin II levels were suppressed by saline infusion in both groups but there was no difference between active and placebo limbs, nor was there any significant effect on aldosterone levels.

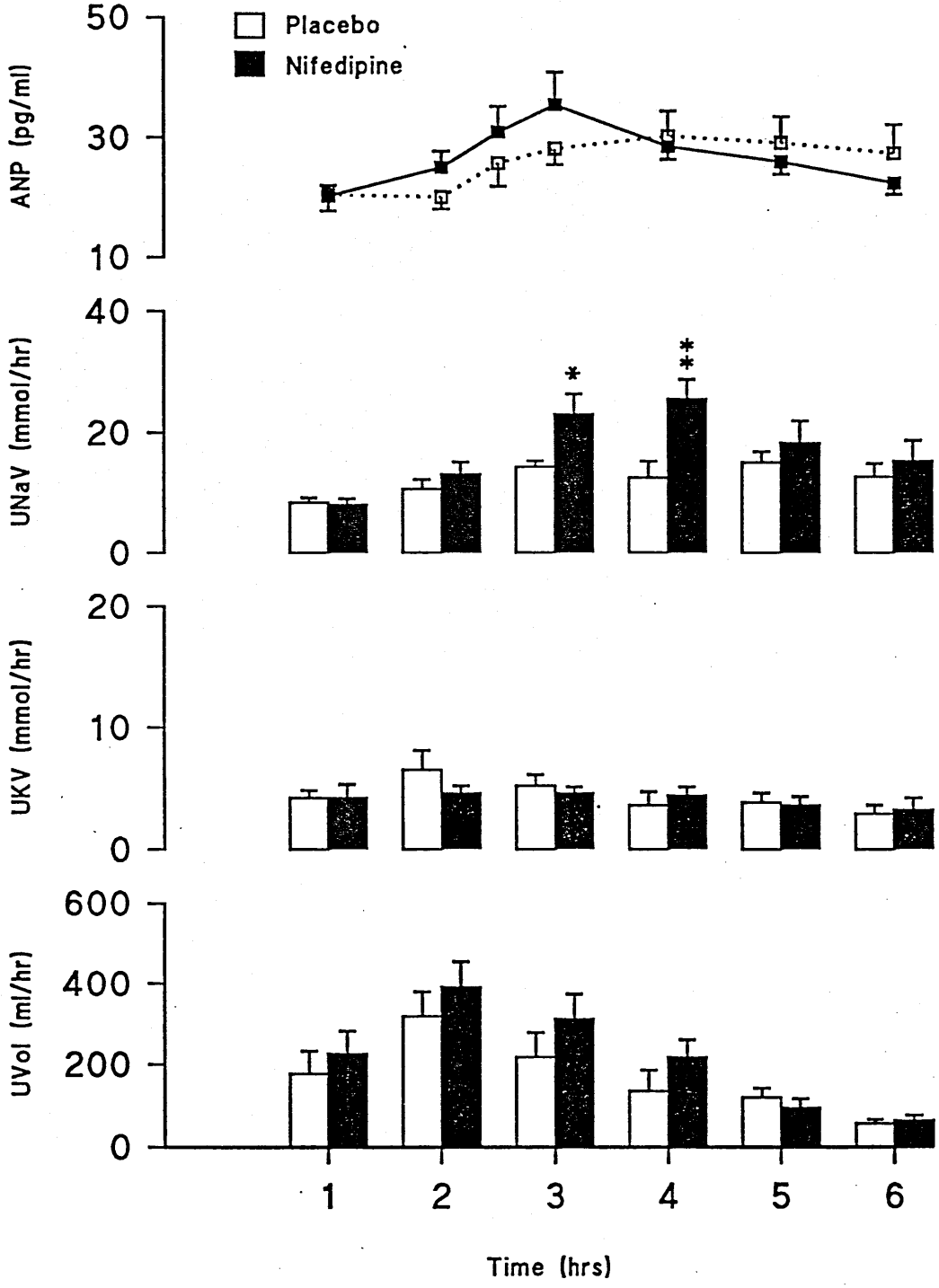


FIGURE 12.1:

EFFECTS OF NIFEDIPINE ON THE ANP RESPONSE TO SALINE INFUSION.

Effects of infusion of 1l of normal saline into 8 normal volunteers on plasma ANP, urine volume and sodium and potassium excretion (UNaV, UKV, UVol). Data are given as mean±SEM. Saline was infused over 30 minutes starting at 2 hours (see text for details).

5. Renal Function Indices (Table 12.1). Infusion of saline was associated with a transient rise in renal plasma flow (ERPF) and a sustained increase in glomerular filtration rate. There was no significant difference in the pattern of these effects between the active and placebo limbs. Fractional excretion of sodium was also transiently increased following infusion and was consistently higher in the active limb, being twice the level in the placebo limb at the 4 hour time point (i.e. two hours after the start of saline infusion). However, due to the baseline differences this failed to achieve statistical significance (P=NS).

Discussion:

The results confirm that saline infusion stimulates an increase in plasma ANP (Anderson et al.,(1986), Richards et al.,(1988), Ohashi et al.,(1987), Hodsman et al.,(1987)). However, despite the original hypothesis (Chapter 11) there was no evidence that Nifedipine inhibits either release or the renal effects of ANP.

Pretreatment with Nifedipine resulted in an upward trend in basal ANP levels and saline infusion resulted in slightly higher peak hormone levels. These observations suggest that there is no impairment of ANP release in vivo despite the in vitro evidence that calcium antagonists can inhibit ANP release (Schiebinger and Gomez-Sanchez (1990), Schiebinger (1989)). The more rapid fall in hormone levels in the Nifedipine group may reflect quicker excretion of the saline load and therefore reduction of the stimulus to ANP release. A rise in plasma ANP following oral Nifedipine has previously been observed (Rappelli et al.,(1987)), but the reason for this is not known. It may perhaps reflect changes in metabolism either directly or by redistribution in regional

blood flow (as has been suggested for other vasodilators (e.g. Wilkins et al.,(1988)). There was no change in heart rate (an independent stimulus to ANP release (Schiebinger, Baker and Linden, 1987)) following Nifedipine, thus the effects are not secondary to reflex changes in heart rate.

In keeping with previous reports Nifedipine caused a small increase in basal urine volume and sodium excretion (Rappelli et al.,(1987) and for reviews, Loutzenhiser and Murray (1989), Chellingsworth and Kendall (1987)), and greatly increased the rate of clearance of the infused saline load. Since plasma ANP levels were similar in both active and control groups it is likely that this exaggerated natriuretic and diuretic effect is either due directly to Nifedipine or possibly through a synergistic effect between ANP and Nifedipine in the kidney. There was no evidence of any differences in glomerular filtration rate or renal plasma flow between active and control groups although both increased release during saline infusion. Fractional excretion of sodium was higher on the active treatment limb and, although this failed to reach statistical significance (P=0.64), the pattern of these effects - i.e. enhancement of fractional sodium excretion (Chapter 4) with Nifedipine - is consistent with potentiation of the effects of ANP in the kidney. Both Gaillard et al.,(1989) and Seino et al., (1988) reported a similar pattern. In these studies when ANP was infused in the presence or absence of a calcium channel blocker, there were no significant changes in GFR, but the ANP-induced rise in FeNa was enhanced. There was no consistent effect on blood flow and the implication is that calcium channel blockers directly enhance the effects of ANP at a tubular level.

Finally, renin levels were suppressed by saline infusion in both limbs of the study. The plasma renin levels were lower, although not significantly

so, in the active limb consistent with the fact that renin release in negatively modulated by calcium in vitro. There were no significant changes in the other components of the renin-angiotensin system.

In conclusion, although the balance of evidence favours some involvement of calcium in the control of ANP release (Chapter 11) and although calcium channel blockers inhibit ANP release in vitro (Schiebinger (1989), Schiebinger and Gomez-Sanchez (1990)) there is no evidence from the present study that ANP release is inhibited. In fact, plasma levels were, if anything, higher during a saline load and calcium channel blockers are therefore unlikely to interfere with the therapeutic usefulness of ANP (or NEPi). Furthermore, sodium and water excretion were enhanced by Nifedipine. Whether this reflects the potentiation of ANP, as it appears to (Gaillard et al.,(1989), Seino et al.,(1988)), merits further investigation. If Nifedipine does potentiate the renal effects of ANP then the combination of ANP (or NEPi) and a calcium channel blocker may be a potent therapeutic combination (Chapter 13).

TIME (hours)	1	2	3	4	5	6
RENIN (μU/ml)	15.7±2.3 13.3±1.8	22.3±3.6 21.2±7.5		14.7±3.5 10.0±0.7		12.7±3.3 9.5±1.0
ANG II (pg/ml)	9.7±2.2 11.9±1.3	14.5±3.1 11.9±2.4		8.8±1.6 5.1±0.64		8.1±2.3 9.5±1.0
ALDO (ng/dl)	4.1±0.8 2.8±0.3	3.1±0.6 6.4±3.0		3.4±0.5 3.3±0.4		2.9±0.6 3.9±1.0
SYSTOLIC (mmHg)	119±5 117±3	116±5 119±3	119±5 124±3	118±4 120±4	116±4 119±4	119±3 119±4
DIASTOLIC (mmHg)	68±4 72±4	66±5 71±5	64±4 74±4	63±3 67±4	65±4 63±4	63±3 66±4
PULSE (/min)	61±3 58±1	63±3 61±2	63±3 59±2	61±4 59±1	64±3 61±2	66±4 62±2
GFR (ml/min)		78.0±4.6 75.0±3.0	99.5±5.9 95.2±4.1	107.6±6.6 103.9±4.6	111.0±6.5 108.7±5.4	111.0±6.0 111.3±5.9
ERPF (ml/min)		481.2±43.7 450.3±18.9	516.1±48.1 502.8±34.6	539.8±50.7 499.0±32.5	483.9±29.9 496.3±26.7	459.4±39.9 465.8±26.2
FeNa (%) (P=0.068)		1.26±0.20 1.05±0.18	1.10±0.11 1.73±0.30	1.76±0.28 0.86±0.20	1.22±0.25 0.98±0.10	1.03±0.25 1.03±0.25

TABLE 12.1:

HORMONAL, BLOOD PRESSURE AND RENAL EFFECTS OF SALINE INFUSION - INFLUENCE OF NIFEDIPINE.

Changes in plasma renin, angiotensin II (Ang II) and aldosterone (aldo); blood pressure (systolic & diastolic) and pulse rate; glomerular filtration rate (GFR), renal plasma flow (ERPF) and fractional excretion of sodium (FeNa) in eight normal volunteers infused with 1l saline over 30 minutes, starting at 2 hours. For each variable the upper value (mean±SEM) is in the Nifedipine limb of the protocol (see text). There were no significant differences between active and placebo limbs.

SECTION 6.

GENERAL DISCUSSION.

Chapter 13:

Conclusions: Possible Clinical Applications.

With the exception of chapter 3 this thesis contains little of direct clinical relevance and it therefore seems appropriate to conclude by assessing the possible clinical importance of ANP. This can be broadly divided into two major limbs: the possible diagnostic use of plasma ANP measurements and the possible therapeutic use of exogenous ANP.

Diagnostic: From the data presented in chapter 3 it is clear that a functional ANP hormonal system is present from at least the third trimester of human fetal life. In both the fetus and the adult intravascular volume status is the most important determinant of plasma ANP concentration, with hormone levels being appropriately elevated in volume expanded states (Rhesus isoimmunisation in the fetus, mineralocorticoid and normal pregnancy in the adult). However, it is also clear that cardiac afterload has an independent stimulatory

effect on ANP release (in pre-eclampsia, malignant phase hypertension and in pheochromocytoma, where elevated arterial pressure is generally associated with reduced intravascular volume). However, it seems highly unlikely that measurement of plasma ANP could contribute anything to the clinical assessment of patients with these conditions where the diagnosis is based on other factors and hormone measurements. Similarly, in the fetus with rhesus isoimmunisation, although the reciprocal relationship between plasma ANP and haematocrit - and hence the severity of rhesus disease - is of interest it is unlikely to contribute to the clinical assessment of these patients.

Where measurement of plasma ANP may be of clinical relevance is in those conditions where paradoxical changes in ANP occur. The clearest example of this is in pre-eclampsia (PET). In normal pregnancy, there is a progressive increase in ANP with gestation and also of renin concentration. In PET, however, this pattern is altered and, despite the relative reduction in intravascular volume, ANP is further increased and renin paradoxically reduced. Using the ratio of ANP/renin as a crude indicator, the ratio increased approximately 1 (pg/ml ANP/ μU/ml Renin) during normal pregnancy to values of > 10 were found in PET and showed a weak inverse relationship with the birthweight. If these neurohumoral changes predate the clinical development of PET they may be a useful indicator of <u>at risk</u> pregnancies worthy, at least, of further investigation.

Therapeutic: The remaining sections of this thesis deal either directly (Section 4) or indirectly (Sections 3 & 5) with factors likely to influence the therapeutic usefulness of ANP.

The pattern of actions of ANP, and the results of preliminary studies of ANP infusion in hypertension and heart failure (Chapter 2), show therapeutic potential - although we have perhaps been misled as to the potency of ANP from the early high dose studies. The main section of this thesis is devoted (Section 4) to an attempt to harness this potential by inhibition of ANP degradation using NEP inhibitors, with the studies in Sections 3 and 5 examining the interactions of ANP, ACE inhibitors and calcium channel blockers (possible agents for combination therapy with ANP or NEPi).

In 1987, (Chapter 5) the use of NEP inhibitors to inhibit the degradation of ANP appeared to be a means of harnessing the therapeutic potential of ANP. To some extent this has been realised with the clear demonstrations, in normal animals (Chapter 6) and normal man (Chapter 7) that NEP inhibition can cause increases in plasma ANP and a natriuresis, diuresis and suppression of the renin-angiotensin system consistent with an ANP-mediated effect. What was subsequently disappointing was the failure of prolonged NEP inhibition to cause a sustained increase in sodium excretion, exchangeable sodium or blood pressure (Chapter 8) but, more importantly, that fact that Ang II levels were increased in the presence of a NEPi. While, in retrospect, this simply reflects that Ang II is a substrate for NEP it is likely to limit the usefulness of NEPi especially in renin-dependent forms of hypertension. In mitigation, the use of NEPi in models of hypertension does lower blood pressure (Chapter 9, 10) albeit by a relatively small amount and in renin-independent models of hypertension. Thus, NEP inhibitors have failed to achieve their theoretical promise but they have fulfilled some of their potential by producing a sustained rise in plasma ANP - a useful first step towards

harnessing the effects of ANP.

The modest effects of NEPi have already prompted the investigation of therapeutic combinations (eg Koepke and diBona (1989)). From the data presented here the combination of ACE inhibitors and ANP (and, by inference NEPi) is likely to produce a potent natriuresis and diuresis with significant antihypertensive effect. This has recently been confirmed by Seymour et al.,(1991b) using both ACEi and NEPi and a novel compound which inhibits both enzymes. The use of calcium antagonists (Chapters 11 & 12) in combination with ANP may also be a useful therapeutic combination with no evidence, at least in acute studies, of the postulated inhibition of ANP release by these agents. However, there are - at least - two caveats. Firstly, the use of ACEi is limited in perhaps 5% of patients by a kinin-induced cough. Exogenous, recombinant NEP (Kiguchi et al.,(1989)) can inhibit this type of cough and, presumably, the combined inhibition of ACE and NEP might be limited by this side effect. Secondly, and most importantly, the major reason for pursuing the therapeutic potential of ANP was the unique spectrum of actions of ANP (Chapters 2, 4 & 5) which cannot be achieved by any single available therapeutic agent. If we now have to look at therapeutic combinations with NEPi then we have to question whether or not these confer any benefit over the currently available, proven therapeutic combinations (e.g. thiazide diuretic + ACEi) in hypertension. Whether or not this is the case remains to be seen and would, at least, merit further investigation of these compounds.

This rather negative view on the likely therapeutic potential of NEPi does not necessarily detract from the attractiveness of ANP as a therapeutic agent. What is required is more potent and specific

stimulators of ANPs actions. Cyclic GMP phosphodiesterase inhibitors (Wilkens et al.,(1990) appear to offer a much more potent enhancement of ANP effects at the post-receptor level. However, these agents will potentiate the effects of, for example, EDRF (Endothelium Derived Relaxing Factor) which are mediated by soluble guanylate cyclase and therefore may be too potent for human use. Nevertheless, these agents also merit further investigation. The ideal compound has yet to be discovered, but perhaps the development of a specific ligand for the B-ANP receptor is the most likely to be attained.

Overall, the progress in research to harness the therapeutic potential of ANP has followed the pattern seen in ANP in general. ANP was initially seen as a very potent natriuretic, vasodilator hormone. However, with time it now appears to have a much more subtle role, perhaps counterbalancing the effects of the renin-angiotensin system (Chapter 2) under normal circumstances, but easily over-ridden by the activated renin-angiotensin (or other antinatriuretic) systems. Similarly, our hopes for the therapeutic potential of ANP may have waned with these initial studies of NEP inhibitors. However, the search for more specific and potent stimulators of ANP receptors or post-receptor mechanisms may yet produce a novel antihypertensive agent.

SECTION 7.

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Appendix 1.

Measurement of plasma ANP.

Sample Collection: Blood samples for ANP were collected through indwelling cannulae (Venflon, Viggo, Sweden) after at least 15 minutes supine rest. The blood (10ml) was collected into EDTA tubes containing 100 k.i.u. aprotinin (Trasylol, Bayer UK) on ice. The sample was separated at 4°C (1500 g x 15 minutes) and the plasma stored until assay at 20°C. In fact, although rapidly separated, the blood samples were stable for up to one hour at room temperature in the presence of Trasylol (data not shown).

Samples from experimental animals were collected as above, but generally smaller volumes (3 ml dog, chapter 4; 0.5-5 ml rat, chapter 6). Urine samples (20ml) were similarly collected in Trasylol, then frozen until assay.

ANP was determined by radio-immunoassay of pre-extracted samples (Richards et al.,(1987)).

Extraction: Plasma (3 ml, human; 200 µl- 2 ml, rat) and urine (10 ml) were extracted on C18 reverse phase columns (SEP-PAK, Waters Associates, UK). The SEP PAKS were pre-activated with Methanol (5 ml) then washed through with 5ml distilled water. Plasma or urine samples were acidified before application to the columns by the addition of 0.25 ml 2M HCl per ml of plasma/urine. The acidified sample was then spun to precipitate large proteins and the supernatant added to the columns and "washed in" with 15 ml (3 x 5 ml) 0.1% trifluroacetic acid (TFA). The samples were then eluted using 2 ml 60% Acetonitrile/ 40% 0.1% TFA. All steps were performed at room temperature, and samples drawn through the SEP-PAK under vacuum if required.

The eluted samples were then dried overnight under compressed air at 37°C and the dried samples stored at -20°C if required. For assay these samples were reconstituted in 0.5 ml assay buffer (below) and dissolved by 3 x 10 minute spells in in an ultrasonic bath (Sonitank), interspersed by 3 x 60 second spells in a vortex mixer.

The recovery of human plasma ANP through the extraction proceduce was invariably greater than 80%, and generally greater than 90%. For urine, the calculated recovery was 84% (n=16).

Assay: The assay buffer contained 100mmol/l sodium phosphate (pH 7.4) containing 50 mmol/l NaCl, 0.1% w/v bovine serum albumen, 0.1% Triton X-100, and 50 kiu/ml Aprotonin; all biochemicals from Sigma or BDS, UK except aprotonin [Trasylol, Bayer UK]).

The assay was performed - in duplicate - in 1.5ml tubes containing 250µl: 100µl sample, 100µl antibody at a dilution of 1:10,000, and 50µl label (2pg ^{125}I-ANP in 50µl buffer). The standard curve was prepared by serial dilution (two-fold dilutions; n=8) from 100pg/tube (400 pg/ml final concentration) to 0.4 pg/tube (1.6 pg/ml final concentration). The assay mixtures were vortexed for 30 seconds then incubated for 24 hours at 4°C. Bound and free radioactivity were

separated using dextran-coated charcoal (1ml of Norixt GSX charcoal 6g, Dextran T70 620 mg in 1l Tris buffer) then centrifuged for 15 minutes at 1500g. The supernatant was then aspirated and free radioactivity measured in a gamma counter. The concentrations of ANP were then determined by comparison with the standard curve.

The limit of detection of the assay is approximately 1 pg per tube. The intra and inter-assay coefficients of variation are consistently less than 10%.

Appendix 2.

Analysis of Variance Table - for Chapter 4.

Full analysis table for chapter 4 "Renal actions of ANP: interactions with Ang II and ACE inhibition". P value represents the significance of each variable analysed by MANOVAR (with repeated measures, SPSS/PC+) against (A) Placebo, (B) ANP alone. Asterixes indicate P values close to significance (*) and NS - non-significant. Full analysis of each limb against both placebo and ANP (A,B) is included for interest, although the study was designed and analysed by comparing the effects of ANP alone against placebo, then the other limbs (3-6) against ANP alone. The Enaliprilat alone limb was added later and not included in the formal analysis.

For abbreviations - see list in Introduction to Thesis.

Group		UNaV	UKV	UVol	Renin	Ang II	Aldo	ANP	BP	P	GFR	ERPF	FeNa	FF
1. Placebo	A vs Plac	-	-	-	-	-	-	-	-	-	-	-	-	-
	B vs ANP	-	-	-	-	-	-	-	-	-	-	-	-	-
2.ANP alone	A	<0.001	0.018	<0.001	0.004	NS	NS	<0.001	NS	NS	NS	0.086(*)	0.003	0.086(*)
	B	-	-	-	-	-	-	-	-	-	-	-	-	-
3.ANP+En	A	<0.001	NS	<0.001	<0.001	NS	NS	<0.001	0.003	NS	NS	NS	0.11(*)	NS
	B	<0.001	NS	0.009	<0.001	0.051(*)	0.003	NS	0.004	NS	NS	NS	NS	NS
4.ANP+En+Ang(1)	A	NS	0.033	0.002	<0.005	0.010	<0.001	NS	NS	NS	NS	NS	NS	NS
	B	<0.001	NS	0.004	NS	0.013	NS	NS	0.001	NS	NS	NS	NS	NS
5.ANP+En+Ang(5)	A	<0.001	0.052	NS	NS	<0.001	<0.001	NS	NS	NS	<0.001	0.001	0.006	NS
	B	<0.001	NS	0.046	NS	<0.001	NS	NS	0.001	NS	NS	<0.001	0.003	NS
6.ANP+Chronic EN	A	<0.001	<0.001	0.003	<0.001	NS	NS	<0.001	NS	NS	0.020	0.061(*)	0.001	0.019
	B	NS	NS	NS	<0.001	NS	0.067(*)	NS	NS	NS	NS	NS	NS	NS
7.En alone	A	<0.001	<0.001	<0.001	<0.001	NS	NS	NS	NS	NS	0.034	NS	NS	0.013
	B	NS	NS	NS	<0.001	0.023	NS	NS	NS	NS	NS	0.004	NS	NS

Appendix 3:

Pharmacokinetic data - Chapter 7.

Summary of the pharmacokinetic data from chapter 7 - "The effects of acute administration of the NEP inhibitor UK 69,578 in normal man". Dose of UK 69,578 in mg/kg, with the number of completed subjects in brackets, variables as labelled (mean±SD). Results courtesy of Dr. NJ Cussans, Pfizer Central Research, UK.

DOSE UK 69,578	0.1(4)	0.3(4)	0.5(7)	1.0(4)	2.0(7)	5.0(4)	10.0(4)	Overall Mean.
Elimination Half-life (h)	0.78±0.13	0.80±0.11	1.00±0.19	1.20±0.10	1.10±0.06	1.30±0.10	1.30±0.10	1.1±0.1
Plasma Clearance (ml/min/kg)	3.2±1.2	2.5±0.5	3.5±0.3	2.5±0.7	2.4±0.3	2.6±0.3	2.5±0.6	2.7±0.1
Volume of Distribution (l/kg)	0.20±0.03	0.17±0.02	0.32±0.05	0.26±0.02	0.23±0.02	0.28±0.02	0.28±0.09	0.25±0.01
Peak Plasma concentration (µg/ml)	0.5±0.1	1.7±0.3	2.1±0.2	4.1±0.1	8.2±0.6	18.0±0.4	43.0±0.9	4.4±0.2 (µg/ml/mg/kg)
Plasma AUC (0-8 hrs, µg.h/ml)	0.62±0.16	2.0±0.4	2.5±0.3	6.3±0.3	15.0±2.0	34.0±3.5	80.0±15.5	6.7±0.3 (µgh/ml/mg/kg)
Renal Elimination (% of dose)	63±9	59±7	59±9	64±1	52±8	40±5	56±16	56±2