INACTIVE RENIN: INVESTIGATIONS OF A NOVEL PHYSIOLOGICAL CONTROL STEP FOR THE RENIN SYSTEM USING CONSCIOUS SHEEP

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TO MY DAUGHTER, ALYA

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ABSTRACT

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INACTIVE RENIN: INVESTIGATIONS OF A NOVEL PHYSIOLOGICAL CONTROL STEP FOR THE RENIN SYSTEM USING CONSCIOUS SHEEP

by Abdul-Hussain Kareem Mzail

The renin-angiotensin system is involved in maintaining blood pressure and in the regulation of salt and water balance. Overall regulation of the renin system could partially be effected through alterations in the relative rates of secretion of active and inactive forms of renin from the kidney. This thesis describes work which investigated this hypothesis, using conscious volume replete sheep with implanted artery, vein and bladder catheters.

It was found that plasma levels of the two forms of renin did not necessarily change in parallel. Infusion of the β-adrenoceptor agonist, isoprenaline, increased plasma active renin, a response which could be suppressed with the β blocker propranolol. Isoprenaline, initially, had no effect on inactive renin but, subsequently, a decrease was recorded. Propranolol selectively suppressed basal plasma inactive renin.

The calcium antagonist, verapamil, increased both active and inactive renins in plasma but a peak response was attained after 45 min and, despite continued infusion of verapamil, both forms of renin had returned almost to control levels 2½ hr after starting drug administration.

Other studies using two converting-enzyme inhibitors, captopril and enalapril (MK. 421), demonstrated that these drugs increased active and inactive renin in parallel. It is concluded that the feedback inhibition of renin release by angiotensin II suppresses both forms of renin equivalently.

Anaesthesia alters the response of the kidney to experimental manipulations. In conscious sheep, haemorrhage (10% of calculated total blood volume) increased both active and inactive renin. A maximum mean increase of 72% for active and 86% for inactive renin was recorded. However, in pentobarbitone-anaesthetized sheep the same level of haemorrhage increased plasma active renin by 303% and inactive renin by 299%.

Acidification was used to activate inactive renin so that it could be assayed. An attempt was made to identify conditions for reproducible activation of sheep inactive renin by trypsin. But these studies failed to provide any evidence that this would be a viable alternative to acid activation.

The major conclusion from this thesis is that differential secretion of active and an inactive, but activatable, form of renin forms the basis of a new control step in the renin angiotensin system.

GENERAL INTRODUCTION

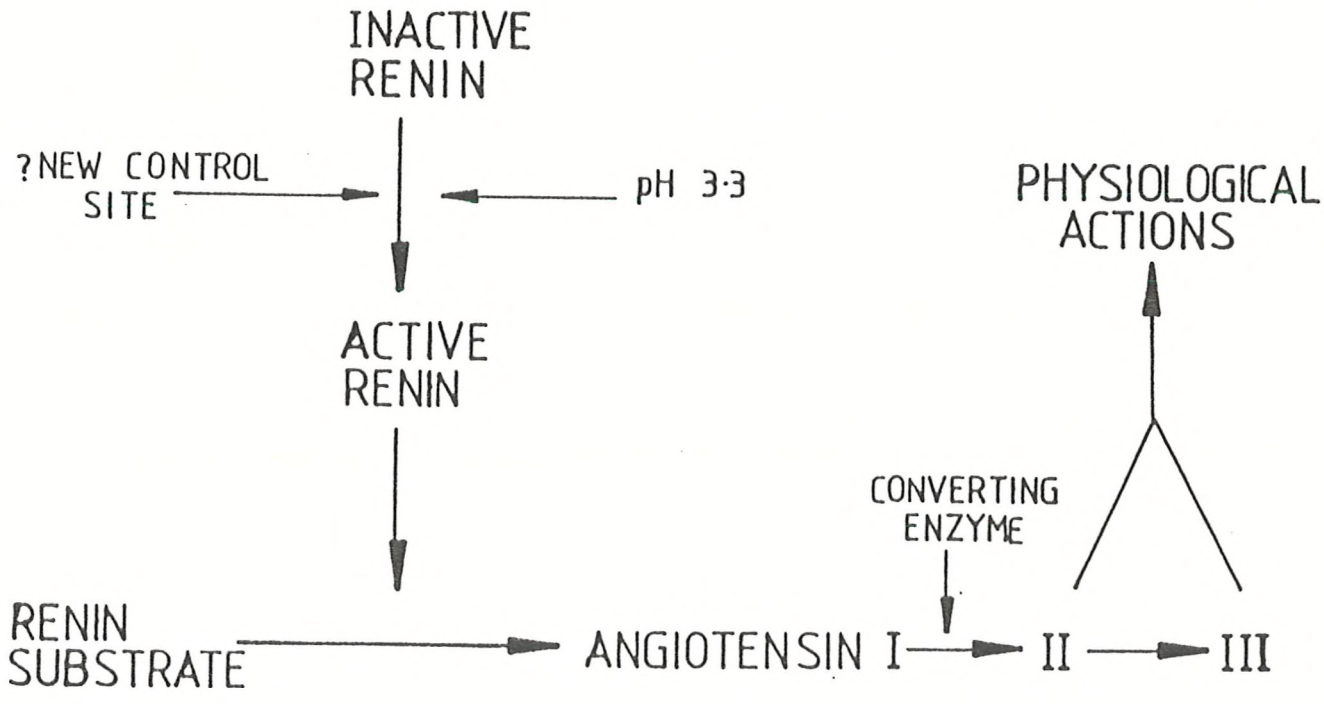
The primary function of the kidney is to regulate the composition and volume of the body fluids. Partly to subserve this role the kidney acts as an endocrine organ secreting renin. The renin-angiotensin system exerts a controlling influence on water and electrolyte homeostasis and on the peripheral resistance to blood flow.

Renin is an enzyme which initiates the sequence of reactions leading to the production of the physiologically active peptides angiotensin II and III (Ang. II, III) (See Fig. 1.1). It is synthesized and stored by specialized cells in the afferent arteriole, the Juxtaglomerular (JG) cells. In response to certain physiological stimuli it is released into the blood stream where it acts on a substrate, angiotensinogen, a glycoprotein synthesized in the liver. Renin splits the Leu^{10}-Leu^{11} bond of angiotensinogen to release the decapeptide angiotensin I (Ang. I) (Fig. 1.2).

Converting enzyme (Kininase II), removes a His^{9}-Leu^{10} fragment from the carboxy terminal portion of Ang. I to release Ang. II. An aminopeptidase in the plasma, removes an aspartate residue from the NH_2-end of Ang. II to produce another biologically active compound, the heptapeptide angiotensin III (Ang. III). It is generally considered that Ang. II is the main effector hormone, but evidence now exists for biological activity associated with the other angiotensins. The actions of these peptides include vasoconstriction, control of aldosterone synthesis, a pressor role within the central nervous system, stimulation of sodium and hence fluid transport in the kidney and gut and a central dypsogenic action.

FIG. 1.1

THE RENIN ANGIOTENSIN SYSTEM



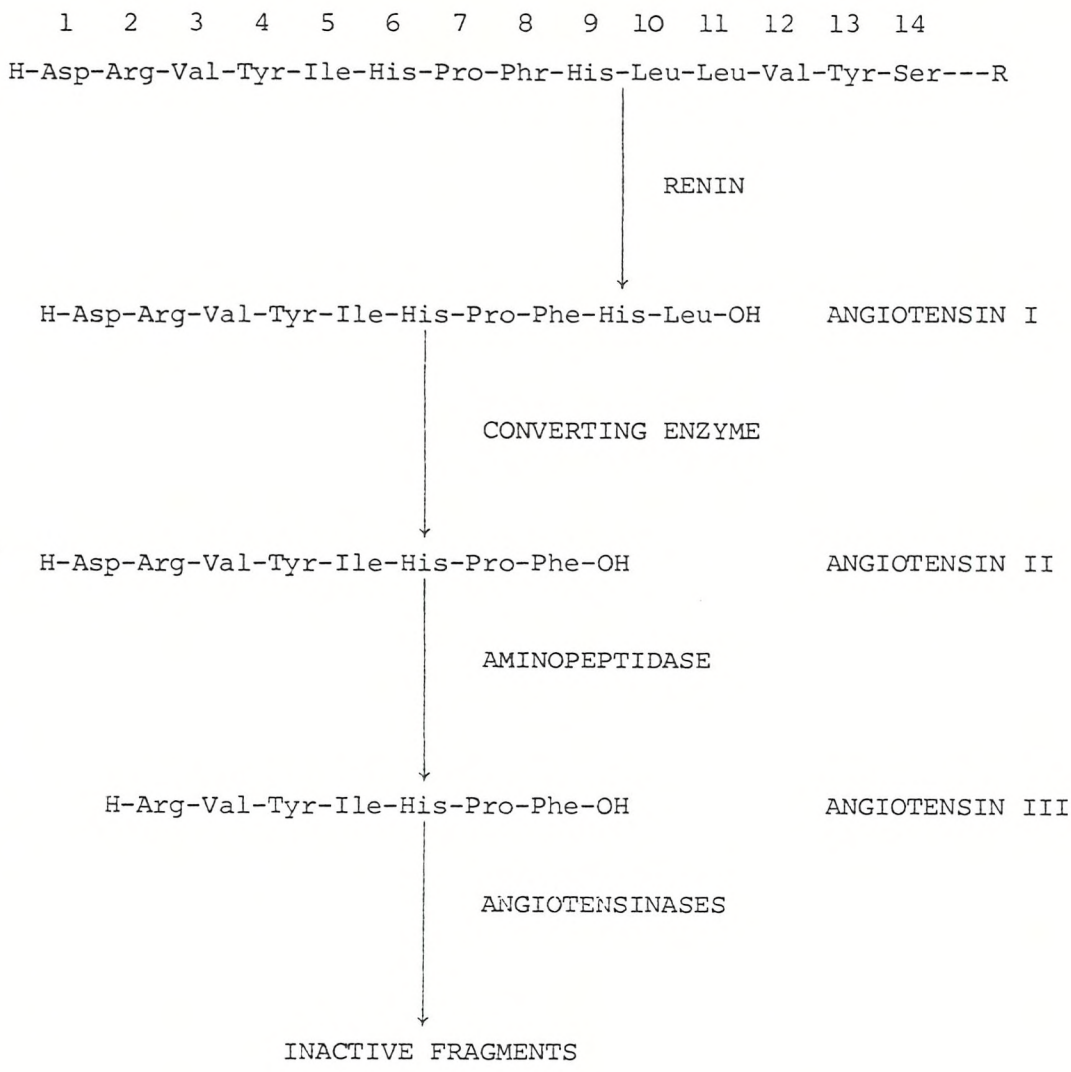


Fig. 1.2 The Enzymatic Conversion of Angiotensinogen into Peptides and Formation of the Different Angiotensins (I, II, III)

The control of renin secretion, primarily by intrarenal mechanisms, is complex and the relatively recent discovery of inactive but activatable forms of renin in plasma and kidney has added a new dimension. It has raised the possibility that inter-conversion of active and inactive renins, or their differential secretion from the kidney may be an important novel site for control of the renin-angiotensin system. This hypothesis is investigated in the succeeding chapters of this thesis. Conscious sheep with chronically implanted artery, vein and bladder catheters have been used so that interference by anaesthetic agents, which may modulate renin release in response to altered physiological situations, is avoided.

The renal sympathetic nerves play an important role in the stimulation of renin release, and this response is mediated via beta-adrenoceptor agonists. An investigation into β-adrenoceptor mediated secretion of active and inactive forms of renin, will be described in Chapter 4. This study used the agonist, isoprenaline and the β-antagonist, propranolol. In Chapter 5 of this thesis, the role of the calcium antagonist, verapamil in altering active and inactive renin secretion, is described. There has been increased interest in the possible antihypertensive actions of such drugs. In addition, as will be seen, calcium ions have a highly unusual role in secretion control for renin. The renin response to angiotensin-converting enzyme inhibitors (ACEI) is of great interest in both basic and clinical research. Captopril is, to date, the most widely used example of ACEI. The effect of captopril and another ACEI on plasma renin activity is thought to mainly result from inhibition of the negative feedback of Ang. II on the JG cells. Some studies on this form Chapter 6.

Haemorrhage has long been recognized as a potent stimulus for renin release. This is mainly based on studies using anaesthetised animals. As anaesthetic agents potentially modify renin secretion, the effect on active and inactive renin secretion of haemorrhage was investigated with and without pentobarbitone anaesthesia. The results of this study are described in Chapter 7.

Finally, activation of the inactive renin is one of the major *in vitro* methods for the evaluation of the inactive renin especially in human plasma. We have therefore investigated this for sheep inactive renin. These studies are presented in Chapter 8 of this thesis.

CHAPTER I

LITERATURE REVIEW

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LITERATURE REVIEW

Renin (E.C.3:4.99.19)

Renin is an endopeptidase, a highly specific proteolytic enzyme which is synthesized and stored by the JG cells in the afferent arterioles. Its function is uniquely limited to cleavage of the decapeptide Ang. I from the NH_2-terminal end of its substrate, an α_2-globulin. Almost all biochemical studies have been done on kidney renin, it remains to be fully determined whether this is the same as the physiologically more important renin in plasma.

Renin was originally described by *Tigerstedt and Bergmann (1898)* as a pressor agent of renal origin. Later, in 1934, *Goldblatt* and his co-workers reported that constriction of the renal artery could produce a persistent arterial hypertension. Despite the fact that renin was detected and named eighty-five years ago, it is only recently that it has been fully purified and characterized. *Haas et al* (1953a) achieved 56,000-fold purification of renin by salt and solvent precipitation. The same authors a year later, reported a method for the isolation of highly purified renin and they suggested that renin has an unusually high stability towards acidic conditions compared to other renal proteins (*Haas et al*. 1954). Before the mid-seventies there were no further substantial advances in the purification of renin; this can be attributed to the relatively small amount of renin present in the kidney, to the instability of the purified enzyme and to confusion with other enzymes which have similar properties. However, recent developments in affinity chromatographic techniques

and the use of protease inhibitors have made it possible to purify renin in stable preparations from kidneys of the pig *(Corvol et al., 1977; Inagami & Murakami, 1977a)*, rat *(Matoba, Murakami & Inagami, 1978)* dog *(Dzau et al., 1979)* and human *(Yokosawa et al., 1980)*.

Renin is considered to be an acid protease and it is inhibited by the potent acid protease inhibitor, pepstatin *(Gross, Lazar, Orth, 1972; Corvol, Devaux, Menard, 1973; McKown, Workman & Gregerman, 1974)*. Also, certain aliphatic diazo compounds inactive mouse submaxillary gland renin *(Inagami, et al., 1977)* together with pig *(Inagami & Murakami, 1977b)* and partially purified human *(McKown, Gregerman, 1975)* kidney renins. This suggests the functional importance of at least one free carboxyl group in or near the active site of the enzyme.

The enzymological and molecular properties of human renin are generally analogous to those of renins purified from the kidney of other species. However, the human enzyme does show some properties which distinguish it *(Yokosawa et al., 1980)*.

Renins isolated from the kidney of rat, dog and pig have a molecular weight between 36000 - 37000, while human renin has a molecular weight of 40000. It has a higher isoelectric point (pH 5.7) than renin from other species (pH 4.5 - 5.4) and only cross-reacts weakly, if at all, with antibodies raised against renin from other species. Renins from other mammalian species do appear to cross-react with each other *(Haas, Goldblatt & Gipson, 1965; Yokosawa et al., 1980)*.

The enzymatic action of renin from all species is not affected by heavy metal ions, dimercaprol, EDTA, or diisopropyl fluorosphosphate (DFP), indicating that disulphide bridges are not essential to the active site and that renin is not a metalloenzyme *(Lee, 1969; Skeggs et al., 1977)*.

Renin Substrate

Renin substrate (angiotensinogen) was first identified as an α_2-globulin by *Plentl, Page & Davis* in 1943. It was found to be a glycoprotein with an approximate molecular weight of 55000 - 60000 which may exist in multiple forms *(Skeggs et al., 1963)*.

Renin substrate is believed to be synthesized by the liver, since it is released from perfused livers *(Nasjletti & Masson, 1971)* from liver slices *(Freeman & Rostorfer, 1972)* and from isolated hepatocytes *(Weigard, Werze & Flage, 1977)*.

Various groups have purified and characterized renin substrate from different species. *Tewksbury et al. (1977)* reported a four-step method for the preparation of human renin substrate which appeared to be homogeneous as assessed by immunochemical and ultracentrifugation techniques. Hog renin substrate was isolated and five different forms characterized in 1963 by *Skeggs et al.* Sheep plasma renin substrate was purified 12000-fold and found to have a molecular weight of about 52000. Multiple forms of renin substrate have been reported from various species *(Skeggs et al., 1963; Printz et al., 1977; Lentz et al., 1978)*. It is possible that this heterogenicity can be accounted for by a difference in carbohydrate content *(Hillgenfelot & Hackenthal, 1982)*. *Tewksbury (1978)* reported that human renin substrate was a glycoprotein with a molecular weight of about 56800 and carbohydrate content of 14%, while *Kokubu et al. (1980)* found that electrophoretically pure human renin substrate had 13% carbohydrate and a molecular weight of 60900.

A 14 amino acid polypeptide chain which functions as a substrate for renin to release Ang. I (Fig.1.2) has been synthesized

(Skeggs et al., 1957). This tetradecapeptide substrate is linked to the remainder of renin substrate by an ester linkage *(Lee & Wilson, 1971)*. The synthetic substrate for renin has not been widely used in renin assay methods for renin because it cross-reacts with antisera to Ang. I.

The rate of renin substrate production can be influenced by a variety of factors. Plasma substrate concentrations are increased by administration of glucocorticoids *(Reid et al., 1973)*, oestrogen *(Menard et al., 1973)* and angiotensins *(Blair-West et al., 1974)*. *Reid et al., 1974*, reported that renin substrate levels in dogs can be increased by haemodilution. It has also been reported that nephrectomy, ureteric ligation and thyroidectomy will increase plasma renin substrate concentration *(Reid et al., 1974; Hiwada et al., 1976; Bouhick et al., 1981)*. Administration of furosemide to hypertensive patients *(Kato et al., 1979)* also has a similar effect.

In most species the concentration of renin substrate in plasma is considerably less than the Km for the renin/homologous substrate reaction. Substrate concentration is therefore considered to affect Ang. I production rate in vivo *(Skinner et al., 1975a)*.

In man it has been established that renin substrate levels increase in severe hypertension *(Gould & Green, 1971)*, during pregnancy *(Helmer & Judson, 1967)* and with oral contraceptive use *(Skinner et al., 1969)*.

Angiotensin Converting Enzyme (E.C. 3.4.15.1)

Angiotensin converting enzyme (ACE) is a general term covering a group of dipeptidyl carboxypeptidase enzymes which cleave Ang. I to form Ang. II. Such an enzyme was first detected in equine plasma by *Skeggs et al. (1954)* who found that, provided Cl^- ions were present, incubation of porcine renin with a crude equine angiotensinogen resulted in the formation of two hypertensive peptides. Subsequently, it was established *(Skeggs et al., 1956)* that this was due to contamination of the angiotensinogen preparation by an enzyme which catalysed the removal of his-leu from the carboxy-terminus of Ang. I to yield Ang. II. The vasopressor response to Ang. I was assumed to be due to its conversion to Ang. II, mediated by the plasma-converting enzyme. In 1967, *Ng & Vane* recognized that this enzyme activity in plasma was insufficient to account for the rapidity of conversion in vivo. Ang. I given intravenously was much more potent than the same dose given intra-arterially. This led *Ng & Vane (1968)* to suggest that during passage through the pulmonary circulation, Ang. I was converted to Ang. II. Since then it has become clear that converting enzyme is a component of the pulmonary vascular endothelium *(Horky et al., 1971)* but that it is also present in many other organs *(Caldwell et al., 1976)*. Electron microscopic studies, with micro-peroxidase labelled antibody *(Ryan et al., 1975)* have shown that, in the lung, converting enzyme is located on the luminal side of the endothelial plasma membrane. Similar data has been published for other tissues including the testis *(Cushman & Cheung, 1971)*, kidney *(Hall et al., 1976)* and some parts of the brain and pituitary gland *(Yang & Neff, 1972; Poth et al., 1975)*.

Angiotensin I conversion in the lung is important due to the large vascular bed, strategic anatomic location and the fact that enzymatically generated Ang. II is not significantly metabolized within the pulmonary vascular bed *(Ng & Vane, 1968; Hodge et al., 1967)*.

Converting enzyme is a zinc metalloprotease and its physicochemical characteristics have been extensively studied by *Harris & Wilson (1982)*. They purified the enzyme and found that the amino-acid composition and carbohydrate content of the bovine lung enzyme are all similar to the values obtained for the human serum enzyme. Another group of workers have purified the enzyme from human kidney by using reverse immuno-adsorption chromatography *(Weare et al., 1982)*.

Concurrent with its action on Ang. I, ACE inactivates bradykinin, a vasodepressor peptide. It catalyses the sequential removal of Phe-Arg and Ser-Pro from the -COOH terminus of bradykinin *(Soffer, Reza & Caldwell, 1974; Soffer & Sonnenblick, 1978)*. Therefore, inhibition of the enzyme, in addition to reducing the vasopressor response to Ang. I, potentiates the vasodepressor effect of bradykinin. It is now widely known as kininase II *(Soffer & Sonnenblick, 1978; Harris & Wilson, 1982)*.

Bakhle (1968) first observed that a bradykinin-potentiating factor, in a mixture of peptides from the venom of Bothrops jararaca, inhibited the conversion of Ang. I by dog lung preparation to Ang. II. Then, in 1970, *Ferreira et al.* found that peptides from the venom (Bothrops) could inhibit both ACE and bradykininase activity in pulmonary extracts. ACE inhibitors have since become increasingly important therapeutically and as tools for examining the contribution of the renin-angiotensin system to the maintenance of blood pressure.

Angiotensin I Converting Enzyme Inhibitors (A.C.E.I.)

Although the ACEI were discovered in the early seventies *(Ondetti et al., 1971)*, until recently the only pharmacologically characterized inhibitors of ACE have been the nonapeptide, SQ 20, 881 and some related peptides isolated from snake venom *(Cushman et al., 1978)*. These compounds have had very limited application because of the need to use them intravenously. Development of new, nonpeptide, orally active inhibitors of ACE, has been based on some studies of the active site of the enzyme *(Ondetti et al., 1977)*.

The similarity between ACE and the digestive enzyme carboxypeptidase A led some researchers to develop a hypothetical model for the binding of substrates to the active site of the enzyme *(Cushman et al., 1971; Das & Soffer, 1975)*. Converting enzyme is a zinc-metallo protease *(Soffer & Sonnenblick, 1978)* and the most important moiety for binding of substrates to its active site is the zinc ion of the enzyme. With the discovery of D.2-Benzyl succinic acid as a potent inhibitor of carboxypeptidase A by *Byers & Wolffender (1973), Ondetti et al., (1977)* proceded to synthesize succinyl-L-proline (SQ 13, 745) as a potential ACEI. It was a moderately potent ACEI and so a large number of compounds were synthesized with the aim of improving the inhibiting potency. More potent orally active inhibitors such as D.2-methylsuccinyl-L-proline (SQ 13, 297) and D.2-methylglutaryl-L-proline (SQ 14, 102) were synthesized *(Cushman et al., 1977)*. Attempts were made to replace the zinc-binding carboxyl group of these compounds with groups with greater affinity for zinc. *Rubin et al. (1978)* synthesized an extremely potent inhibitor designated captopril (SQ 14, 225). Captopril is active both orally and parenterally and

has become the first ACEI to be used therapeutically in the treatment of hypertension *(Keim, 1980)*.

A new class of oral ACEIs was reported by *Patchett et al. (1980)*. These drugs are now being investigated experimentally and are undergoing clinical trials. They are substituted N-carboxymethyl dipeptides which can inhibit ACE without Zn^{++} sulphydryl interaction. This was previously considered to be an essential element for this function *(Ondetti et al., 1977)*.

One of these compounds, MK-421 (ENALAPRIL MALEATE), is the ethyl ester of N- [(S)-1-(ethoxy carbonyl)-3-phenyl propyl]-L-ALa-L-proline *(Patchett et al., 1980)*. MK-421 is considered to be hydrolysed, mainly by a liver esterase, to become an active diacid metabolite which has potent angiotensin-converting enzyme inhibitory activity *(Nakashima & Nishijima, 1982)*. It has a higher potency and a much longer duration of action than captopril but slower onset of action. It appears currently that MK-421 is a well-tolerated, long-acting ACEI which has considerable potential for clinical use *(Biollaz et al., 1981; MacGregor et al., 1981; Brunner et al., 1981; Gavras et al., 1981; Nakashima & Nishijima, 1982)*.

Sites of Renin Storage and Synthesis

Evidence that renin is located in the juxtaglomerular apparatus of the kidney has been reviewed elsewhere *(Tobian, 1962; Peart, 1965; Hartroft, 1966)*. The structure known as the juxtaglomerular apparatus includes both afferent and efferent arterioles of the renal glomerulus, the macula densa region of the distal tubule and the lacis (Fig. 1.3) *(Barajas & Latta, 1967; Barajas, 1979)*.

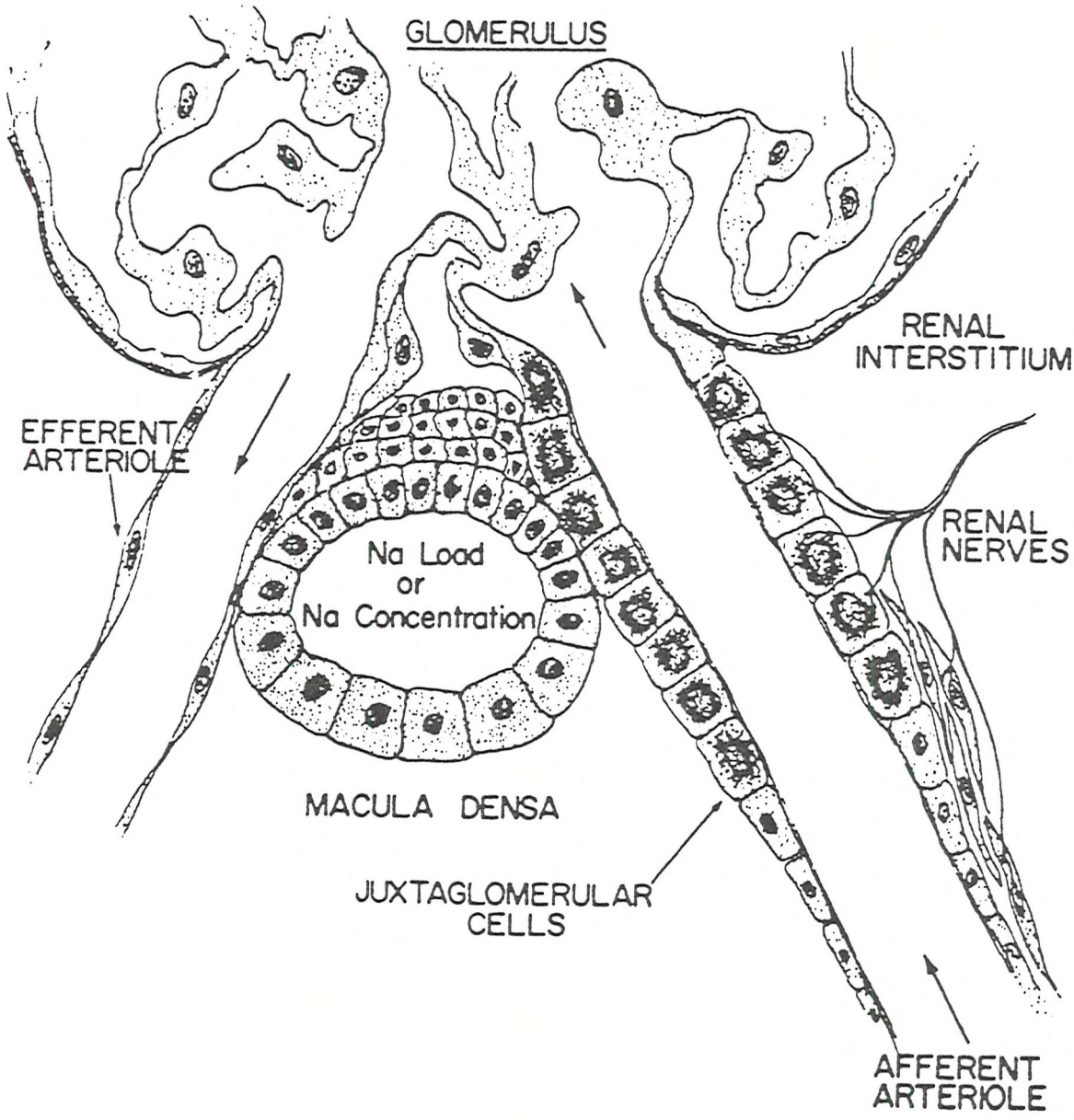
The afferent arteriolar wall contains cells, the juxtaglomerula-granular cells, which exhibit features of endocrine and smooth muscle cells, possessing both secretory granules and myofilaments *(Barajas, 1979)*. They were first observed by *Ruyter (1925)* who suggested that these granular cells are modified smooth muscle cells.

The granular JG cells are distinguished from smooth muscle cells in the afferent arterioles by the presence of numerous cyto-plasmic processes and unfoldings of the plasma membrane *(Gorgas, 1978)*. In 1939, *Goormaghtigh* found that the number and the size of granular cells was increased following renal artery constriction. *Tobian et al. (1959)* suggested an inverse relationship between renal perfusion pressure and the granulation of the juxtaglomerular (JG) cells. By using an immunofluorescent antibody technique *Edelman & Hartroft (1961)* found that labelled renin antibodies were bound mainly in the granular cells. In retrospect this may have been fortuitous, given the lack of purity of the 'renin' preparation used to raise antisera. Other lines of evidence come from microdissection studies on the kidney. *Cook & Pickering (1959)* separated glomeruli from their tubules and found renin only in those glomeruli to which the vascular pole was still attached. Within the JG apparatus, renin has been shown to be contained in the specific granules of the epithelioid (or juxtaglomerular)

FIG. 1.3

THE JUXTAGLOMERULAR APPARATUS

(Taken from Davis, 1971)



cells *(Cook, 1968)*. Under different experimental conditions, such as bilateral adrenalectomy and salt-depletion, electromicroscopic studies by *Barajas (1966)* and by *De Senarclens et al. (1977)* demonstrated the existence of two types of granules. One type contains a crystalloid matrix, is found in close association with the Golgi apparatus and called a protogranule. The second type contains a homogeneous matrix, is larger than the protogranules and is frequently seen fused with the membrane *(Barajas, 1979)*. They were called secretory granules. The authors *(Barajas, 1966; De Senarclens et al., 1977)* concluded that renin is synthesized in the endoplasmic reticulum and stored in the protogranules which are then converted into secretory granules.

It has been shown that the morphological appearance of the JG cells may change in different ways in response to stimuli such as salt-free diet or bilateral adrenalectomy *(Dauda & Devenyi, 1971; Szabo & Devenyi, 1972)*. *Peter et al. (1974)* studied the response of the JG apparatus to bilateral adrenalectomy in rats. They found in their microscopic studies that in the JG cell the volume density of the secretory granules decreased from 32% to 16% and suggested that renin is stored in the granules and released from them in response to adrenalectomy.

The cells of the macula densa form part of the early distal tubule but differ from those of the adjacent portions of the tubule by being narrow columnar cells with basal nuclei. They do not show the characteristics of secretory tissues and have fewer mitochondria than the adjacent tubular epithelial cells *(Barajas & Latta, 1967)*. In 1939, *Goormaghtigh* recognized the possibility that some functional relationship might exist between the macula densa and the JG cells

of the renal afferent arteriole. Later, in the 1960s it was reported that the macula densa cells are highly differentiated cells with characteristics which suggested a specialized function. The cells of the macula densa are separated from granular JG cells by an incomplete basement membrane. The location of the Golgi apparatus in the basal rather than the lumenal side of the macula densa cells is another indication of specialisation *(Hartroft & Newmark, 1961; Barajas & Latta, 1967; Barajas, 1971)*. This histological evidence has formed the basis for a theory for the control of renin secretion. The theory links the macula densa as a sensor for tubular $[Na^+]$ with renin secretion. (See page 37).

The space between the afferent and efferent arterioles and the macula densa contains agranular or lacis cells. These cells are of uncertain function, but may become secretory cells in some pathological conditions.

The presence of nerve fibers in association with the JG apparatus has been established using light and electron microscopy and also by more recently developed histochemical methods. *Muller (1972)* reported adrenergic innervation of both JG and smooth muscle cells of the afferent arterioles. This suggested a functional relationship between the sympathetic nervous system and renin release *(Barajas, 1979)*. (See page 42). There is no significant evidence in support of a role for the parasympathetic nerves in the control of renin release *(Keeton & Cambell, 1981; Torretti, 1982)*.

Extrarenal Sources of Renin

Although the kidney has general been considered to be the primary source of renin, considerable quantities of a 'renin-like' enzyme are found in the plasma of anephric humans *(Capelli, et al., 1968;*

McKenzie & Montgomerie, 1969; Medina et al., 1972; Yu et al., 1972; Weinberger et al., 1977). This raises the possibility of an extra-renal source of renin.

Studies in animals have demonstrated the presence of 'renin-like' enzymes in rabbit uterus *(Ferris, Gorden & Mulrow, 1967)* and in mouse submaxillary gland *(Takeda, De Busk & Grollman, 1969).*

Similar data has been published for various tissues in the dog such as blood vessels, lung, liver, spleen, skeletal muscle and adrenal gland *(Hayduk, Boucher & Genest, 1970)* and brain *(Ganten et al., 1976).* These forms of renin persisted after removal of the kidney. It has been reported that the mouse submaxillary gland *(Cohen et al., 1972)* and rabbit uterus *(Johnson, Ryan & Reyes-Rodriquez, 1971)* have the highest concentration of 'renin-like' enzyme.

Considerable interest has also been shown in the presence of inactive forms of renin in anephric man *(Leckie et al., 1977b; Sealey et al., 1977).* Inactive renin levels in anephric subjects remain fairly constant and are not altered by circumstances which change normal plasma active renin levels *(Sealey et al., 1977).*

Inactive renin has been reported in human amniotic fluid *(Lumbers, 1971; Day et al., 1975),* in rabbit uterus *(Jorgensen, 1976)* and in mouse submaxillary gland *(Poulsen et al., 1981).*

One of the first changes in the renin system that occurs during pregnancy is the appearance of a very high concentration of inactive renin in the circulation *(Skinner et al., 1975)* and these high levels are sustained relatively unchanged throughout pregnancy *(Sealey et al., 1982).* The chorionic cells might be the source of this circulating inactive renin *(Poisner et al., 1981b).* Plasma inactive renin in normal

pregnant *(Hsueh et al., 1982)* and in hypertensive pregnant women *(Sealey et al., 1982)* increased in response to sodium depletion. It was therefore proposed that, during pregnancy, inactive renin may be regulated by the same mechanisms that affect the release of active renal renin. The response to dietary salt would also suggest that the role of inactive renin may be related to maintenance of fluid volume and electrolyte balance.

The concept of a brain renin-angiotensin system originated with the observation by *Fisher-Ferraro et al. (1971)* and *Ganten et al. (1971)* that the components necessary for the formation of Ang. II are present in the CNS. Evidence supporting the existence of a brain renin-angiotensin system has been summarized in recent reviews *(Reid, 1979; Phillips et al., 1979; Ganten et al., 1981)*. Some aspects of these studies are, however, controversial.

It has been proposed that brain renin is actually the acid protease cathepsin D and that this protease is localized intracellularly and cannot have effects outside the cell. It has a low optimum pH, unlike renal renin *(Day & Reid, 1976; Hackenthal, Hackenthal & Hilgenfeldt, 1978a; Hackenthal et al., 1978b)*. However, *Hirose, Yokosawa & Inagami (1978)* demonstrated the presence of a renin in rat brain that is distinguishable from cathepsin D on the basis of its proteolytic activity and neutral pH optimum. It is also similar to renal renin in its response to antirenin antibodies and when injected into the brain induces drinking behaviour. This drinking was blocked by the angiotensin antagonist saralasin *(Reid & Ramsay, 1975)*.

The existence of a brain renin system may ultimately prove to be of primary importance in relation to thirst mechanisms and also to the central control of blood pressure.

Multiple Forms and High Mol. Wt. Inactive Forms of Renin

There is considerable interest in the existence of multiple forms of renin, some of which may have higher mol. wt. than 'ordinary renin' and are enzymatically inactive. Multiple forms of hog kidney renin were reported as early as 1953 by *Haas, Lamfrom & Goldblatt* and in 1967 by *Skeggs et al.* The latter authors also identified several different forms of human kidney renin *(Skeggs et al., 1972)* and suggested that they were probably isoenzymes. *Boyd (1972)* found two forms of both rat and pig renal renin and estimated their mol. wts. using Sephadex G-100 column chromatography. The two rat renins appeared to be isoenzymes and had similar mol. wts. (39000 and 34000), but the two pig renins had different characteristics. Since the mol. wts. of the two pig renins were widely separated (38000 and 60000) *Boyd* suggested that the larger form of renin was a protein-bound form of the smaller one. A binding protein was isolated *(Boyd, 1973, 1974)*. Using a direct rat blood pressure bioassay, *Boyd (1974)* reported that the high mol. wt. form was less active than the smaller one, and also that it could be converted to the smaller, more active, renin by acidification to pH 2.5.

Leckie (1973) identified active and inactive renins in extracts of rabbit kidney using Sephadex G-100 chromatography. She suggested that the high mol. wt. renin (54000) was a zymogen of active rabbit renin (mol. wt. 37000) which was activated by acidification to pH 2.5 and back to pH 7.4. It was later shown that renin exists in rabbit kidney in two forms, one active and one inactive, with mol. wts. 37000 and 55000 respectively *(Leckie & McConnell, 1975b)*.

In 1977, *Inagami & Murakami* purified three high mol. wt. forms of renin from pig kidney 'big renin' (mol. wt. 61000) had a specific

activity 21% of that of active renin and two forms of 'big big renin' (mol. wt. 140000) had specific activity 0.19% and 0.05% respectively when compared with the fully active form. A renin of mol. wt. 62000 had also been purified from pig kidney by *Overturf, Druilhet & Fitz (1979)* and *Slater & Haber (1979)* found a renin of mol. wt. 58000 in a purified preparation of human kidney renin. Other groups reported high mol. wt. forms of renin from dog kidney with mol. wt. 60000 *(Potter, et al., 1978)* and rabbit with mol. wt. 55000 *(Leckie & McConnell, 1975b)*. *Murakami et al. (1981)* purified an inactive form of renin from hog kidney with mol. wt. 51000 and reported that it could be activated by treatment with trypsin or pepsin. The same authors *(Inagami et al. 1982)* have reported that an inactive renin in hog kidney could be purified by affigel-blue affinity chromatography. A 3 million-fold purification of electrophoretically homogenous inactive renin (50000) was achieved. During treatment with proteases this inactive renin underwent reduction in mol. wt. from 50000 to 38000.

There is, therefore, a great deal of information suggesting the presence of high mol. wt. (approximately, 60000) forms of renin in the kidney of many species and furthermore the presence of even higher mol. wt. (110000 and 140000) renins has been detected in the kidney extracts of pig *(Inagami & Murakami, 1977b; Inagami et al., 1982)* and man *(Barrett, Eggena & Sambhi, 1977)*. There is a possible link between changes in renin activity and changes in mol. wt. High mol. wt. inactive forms of renin may be converted, on acidification, to a lower mol. wt. active renin. This could represent either destruction of a binding protein or cleavage of a precursor molecule.

Morris & Johnson (1976) found that the increase in renin activity of rat kidney extracts following acidification was accompanied by 25% decrease in mol. wt. *Rubin (1972)* also reported an increase in activity and the presence of low mol. wt. (43000) renin after acidification of water/acetone extracts of hog kidney. Acidification of inactive kidney renin is not, however, always accompanied by a reduction of mol. wt. *Day & Luetscher (1974)* reported renin of mol. wt. 60000 ('Big' renin) in a patient with Wilm's tumour. On exposure to acid pH there was an increase in renin activity but no change in mol. wt. In extracts of normal kidney they could find only renin of mol. wt. 40000. *Levine et al. (1976)* reported that high mol. wt. hog kidney renin (57000 - 59000) is not activated by acidification to pH 3.3 nor by proteolysis with trypsin or pepsin and they have been unable to change its mol. wt.

The situation has been further complicated by the discovery of high mol. wt. and inactive forms of renin in plasma. Again there is disagreement concerning their mol. wt. In the plasma of normal subjects, inactive renin of mol. wt. 50000 - 58000 has been reported *(Atlas et al., 1981; Yokosawa et al., 1979)*. Similar data has appeared for plasma from pregnant women *(Leckie et al., 1977b)*. On the other hand, *Boyd (1977), Shulkes, Gibson, Skinner (1978)* and *Eggena et al. (1979)* suggested that the mol. wt. of inactive renin in normal plasma may be about the same as the active renin. *Nielsen, Malling & Poulsen (1978)* found that in mouse plasma, there are two high mol. wt. forms of renin. One with a mol. wt. of 800000 which by acidification or proteolysis was converted to more active renin of mol. wt. 40000. A second renin with a mol. wt. 70000 showed an increase in enzyme activity after treatment with acid or proteolytic enzymes

without any change in mol. wt. *Day & Luetscher (1975)* identified a 'big renin' (mol. wt. 63000) in plasma which could be activated by acidification or treatment with pepsin, but this did not involve any change in mol. wt. Similar data was published by *Weinberger et al. (1977)* who found that plasma from normal subjects has a mol. wt. of 42000 before and after acidification. The same authors demonstrated that a high mol. wt. (61000) renin existed in the plasma from anephric subjects after acidification. This suggested an extrarenal source of a renin-like enzyme.

The existence of high mol. wt. and inactive forms of renin has been demonstrated by many groups. Major recent reviews include *(Sealey, Atlas & Laragh, 1980; Inagami & Murakami, 1980; Leckie, 1981 and Sealey et al., 1982)*. However, there is still only a confused picture of the relationships between forms of renin active, inactive and activatable. Perhaps a comparison of the biochemical and molecular properties of the biosynthetic precursor renin and 'inactive renin' may provide some clarification.

Biosynthesis of Renin

The first evidence clearly supporting the existence of a biosynthetic precursor for renin was obtained by *Poulsen et al. (1979)*. They used a cell free translation system, derived from submaxillary glands of mice. They concluded that renin is initially synthesized as a single-chain precursor polypeptide, a prorenin or maybe a preprorenin, with a mol. wt. of 50000. The same authors suggested that prorenin is converted inside the cell to enzymatically-active renin of mol. wt. 40000 and is released into the blood as the latter form.

Carlson et al. (1981) examined the biosynthesis of renin in

canine glomeruli and reported that renin is synthesized in the JG cells of the kidney as a higher mol. wt. (55000) prorenin. They suggested that prorenin is packaged into the protogranules and a crystalline matrix (*Barajas, 1966; de Senarclens et al., 1977).* (See page 13). The prorenin in the protogranules, it was considered, is then converted to active renin (38000 mol. wt.) in a soluble form and is no longer bound in the matrix. The granular matrix becomes a secretory granule which, in response to some particular stimulus, fuses with the cell membrane and spills its contents into the extracellular space. *Carlson et al. (1981)*, therefore concluded that in canine glomeruli renin is present in both the 55000 form and the 38000 form and that the conversion from high to low mol. wt. renin takes place intracellularly.

Dzau, Tanaka & Pratt (1982) investigated the biosynthesis of renin in the mouse submaxillary gland using both cell-free translation and pulse-labelling methods. They found that renin is synthesized as prorenin (mol. wt. 46000) which is processed intracellularly into an intermediate form (mol. wt. 41000). This is then converted by a slow intracellular process to a final storage form (mol. wt. 37000) ready for immediate secretion when appropriate. In the same study, an inactive form of renin (mol. wt. 48000) was identified in the mouse submaxillary gland. The biosynthetic precursor and inactive form of renin do not bind to pepstatin-aminohexyl Sepharose but do bind to affi-gel blue. For active renin, or the activated form, the reverse is true *(Murakami et al., 1981)*. The similarity in the properties of inactive renin and the biosynthetic renin precursor led *Dzau et al. (1982)* to support the suggestion that tissue inactive renin may be the renin precursor. Essentially similar conclusions

were reached by *Catanzaro & Morris (1982)*.

From all these papers it appears that there is substantial evidence in favour of the hypothesis that the biosynthesis of renin involves the formation of a prorenin. It is not yet completely clear whether the inactive renin detected in plasma and kidney extracts is identical to the renin precursor (prorenin) or is a renin-inhibitor complex.

Mechanisms for Activation of Inactive Renin

Inactive renin must be converted to the active form prior to measurement. Experimentally, this can be achieved by acidification, by treatment with proteolytic enzymes or by cryoactivation. This work will now be reviewed and this naturally leads on to the question of the possible physiological mechanisms for renin activation. The in vitro techniques by which inactive renin can be converted to an enzymatically active form have been reviewed by *Leckie (1981)* and by *Sealey et al. (1982)*.

The first evidence for the existence of inactive renin was reported by *Lumbers (1971)* who found that amniotic fluid dialysed to a pH of 3.3 and then back to pH 7.4 had a higher renin concentration than amniotic fluid dialysed to a pH above 4.0. She suggested that this effect was due to activation of an inactive form of renin by acid. Since then many groups have reported an increase in the renin concentration of human amniotic fluid, kidney extracts and human plasma after exposure to a pH between 3.0 - 3.3 followed by dialysis to neutral pH or above *(Skinner, Lumbers & Symonds, 1972; Day & Luetscher, 1974; Leckie & McConnell, 1975a; Boyd, 1977; Shulkes, Gibson & Skinner, 1978)*.

Atlas, Laragh & Sealey (1978) found that acid-activation of inactive human plasma renin is a two-phase process. About 30% of activation occurs during dialysis to pH 3.3 and the remaining 70% occurs at neutral or alkaline pH. It was suggested that endogenous neutral serine proteases participate in the activation of inactive plasma renin in vitro. Their action in vivo, it was suggested, is prevented by inhibitors which can be inactivated by acid or cold.

If inhibitors of serine proteases are added to plasma, little increase in renin concentration occurs after the plasma has been acidified and neutralized by dialysis *(Atlas et al., 1978; Derkx, Tan-Tjiong & Schalekamp, 1978a; Leckie, 1978)*. This is controversial however and other authors have found that almost complete activation of plasma inactive renin occurs in the presence of soya bean trypsin inhibitor (SBTI). This was taken to indicate that activation had taken place during the acidification and was not the result of the action of serine proteases after neutralization *(Hsueh et al., 1980b)*. More recently, *Leckie & McGhee (1980)* and *Hsueh, Carlson & Israel-Hagman (1981)* reported that dialysis of plasma to pH 3.3 at 4°C causes complete activation of inactive renin and that this is reversed during subsequent incubation at higher pH and temperature. Furthermore, *Leckie & McGhee (1980)* postulated that this reversible acid-activation is due to the dissociation and re-association of a renin-inhibitor complex. However, *Atlas et al. (1982)* indicated that reversible acid-activation of inactive renin is a unimolecular reaction and suggested that acid induces conformational changes in a single polypeptide chain. The phenomenon of reversible activation has been discussed by *Sealey et al.* in their review (1982) and they suggested that the presence of a plasma kallikrein and Hageman factor prevent the reversal of acid activation.

An increase in renin concentration occurs if certain proteolytic enzymes are added to plasma, amniotic fluid or kidney extracts. These include trypsin *(Cooper et al., 1974; Leckie, Jordan & McConnell, 1977a)*, pepsin *(Shulkes et al., 1978)*, Cathepsin D *(Morris, 1978)* and plasmin *(Osmond et al., 1978)*.

Sealey, Atlas & Laragh (1978) reported that trypsin treatment results in a 39% increase in plasma renin activity and urinary kallikrein was found to increase plasma renin activity by 66%. Benzamidine appears to protect renin from destruction by trypsin *(Sealey et al., 1982; Sealey, Wilson & Morganti, 1982)*. *Derkx et al. (1979a)* suggested that purified pancreatic kallikrein was one hundred fold more potent than trypsin in activating inactive renin. The same authors and others have also reported that plasma kallikrein may be necessary for the activation of plasma inactive renin since plasma deficient in prekallikrein (Fletcher factor) did not show acid activation *(Derkx et al., 1979b; Sealey et al., 1979a)*. On the other hand, *Millar, Ciappison & Johnston (1978a)* found that acid activation was normal in Fletcher factor deficient plasma. Plasma deficient in factor XII (Hageman factor) also failed to show acid activation. This was attributed to a requirement for Hageman factor for the conversion of plasma prekallikrein to kallikrein *(Derkx et al., 1979a; Sealey et al. 1979a)*.

There are conflicting reports whether kallikrein can activate unacidified inactive renin. *Derkx et al. (1982)* found that plasma kallikrein can activate inactive renin without prior acidification; on the other hand, *Sealey et al. (1982)* showed that plasma kallikrein does not activate unacidified inactive renin, but it does cause irreversible activation of inactive renin in acidified plasma by maintaining acid-exposed renin in an active conformation. *Hsueh, Carlson & Dzau (1983)* reported that reversible activation of inactive renin from human kidney and plasma by acid dialysis occurred on return to pH 7.4, 37^{o}C. However, activation of plasma and kidney inactive renin by acid plus renal kallikrein was not accompanied

by a change in gel filtration elution patterns.

Inactive plasma renin can also be activated in vitro by cold storage. *Osmond et al. (1978)* found that an increase in renin activity takes place following pre-incubation of human plasma samples for three days at + 4°C. The process of cold activation of inactive plasma renin (prorenin) was termed cryoactivation *(Sealey et al., 1976)*. The same authors reported that cryoactivation occurs most rapidly at the lowest temperature than can be maintained without freezing, i.e. - 4°C and a plateau was reached after 4 days at - 4°C when cryoactivation of inactive renin resulted in only about one-third of the increase in plasma renin which could be produced with trypsin *(Sealey et al., 1976, 1979b)*.

The enzyme or enzymes responsible for cryoactivation of inactive renin have not been identified but the evidence suggests that they are probably part of the coagulation or fibrinolytic cascades *(Sealey et al., 1982)*.

Inactive renins from plasma and the kidney have similarities. Both renal and plasma inactive renin bind to Cibacron agarose and do not bind to pepstatin-aminohexyl-agarose (*Inagami et al., 1982; Sealey et al. 1982)*. Both of them are activated by acidification to pH 3.3 and have characteristics similar to those of endogenous active renin. This activation is completely reversed by warming the sample at a higher pH *(Leckie & McGhee, 1980; Hsueh et al., 1981)*. The only distinguishable character between the plasma and kidney inactive renin is that the former has a higher mol. wt. by gel filtration on Sephadex G-100 *(Atlas et al., 1981)*. These results

suggest that inactive renin from plasma and kidney may be the same substance and that their activated forms are similar to the endogenous active renin. This is consistent with the proposal that inactive renin is a precursor of circulating active renin *(Sealey et al., 1982)*.

Although the present state of knowledge of inactive renin is far from complete, a reasonably clear picture is emerging concerning the origin, molecular properties and activation of inactive form of renin. These pictures are much less confusing than they were a few years ago.

Physiological and Pathological Importance of Inactive Renin

Since inactive renin is reduced after nephrectomy *(Derkx et al., 1978b; Richards et al., 1979)*, it is thought to primarily originate from the kidney. Furthermore, the observation by *Millar et al. (1978b)* that the concentration of inactive renin in renal venous blood is higher than in arterial blood strongly suggests a renal origin for inactive renin. Until recently, very few studies concerning inactive renin have been published and its physiological role has yet to be determined.

There has been considerable interest in the possible role played by inactive renin in normal human subjects and in disease mechanisms.

Inactive renin in plasma increases during pregnancy *(Skinner et al., 1975; Sealey, Wilson & Morganti, 1982)*. The last authors found that by 12 weeks of pregnancy, inactive renin was elevated 14-fold. It fell slightly thereafter suggesting that, like active renin, an elevated level of inactive renin is required from the earliest days of gestation to maintain fluid and electrolyte balance in the face of a rapidly changing hormonal and vascular system.

It has been suggested that the inactive renin which is found in amniotic fluid *(Lumbers, 1971)* and in plasma during pregnancy *(Skinner et al., 1975; Sealey et al., 1982)* is synthesized by chorionic cells. *(Skinner, Lumbers & Symonds, 1972; Poisner et al., 1981a)*. The latter authors also studied the cellular sources of renin in the human chorion. By indirect immunofluorescent analysis, and using antisera against pure human kidney renin, they showed that the renin-containing cells in the chorion are restricted to the trophoblast layer. This contains cytotrophoblast cells and atrophic

chorionic villi. Most of the renin in isolated chorionic cells and extracts of intact chorionic laeve is inactive renin *(Poisner et al., 1981b)*. When isolated amniotic and chorionic cells were grown in tissue culture, inactive renin was released continuously from chorionic cells but not from amniotic cells. Exposure to low calcium media decreased inactive renin release *(Poisner et al., 1982)*.

Inactive renin in plasma is increased in patients with renal carcinoma *(Day et al., 1975)*.

Several authors have been interested in the possible role of inactive renin in hypertensive subjects *(Sealey et al., 1977)*. In low renin hypertensives a distinction has been made between patients with primary hyperaldosteronism, in which plasma levels of both active and inactive renin are suppressed and patients who have low renin essential hypertension. The latter group have low active renin but normal or raised inactive renin. This might be associated with impaired conversion of inactive renin to the active form *(Antonipillai et al., 1981)*. Patients with diabetic nephropathy *(Leckie et al., 1978; Hsueh et al., 1980a; Bryer-Ash et al., 1983)* and anephric patients *(Sealey et al., 1979b)* also have raised plasma inactive renin.

Derkx et al. (1976) reported that acute stimulation of renin release by isoprenaline, tilting or diazoxide in normotensive individuals and in patients with essential hypertension increased plasma active renin and reduced inactive renin. The β-blocker propranolol, in patients with essential hypertension increased plasma inactive and decreased active renin. They suggested that inactive renin is physiologically related to active renin and also proposed

that inactive renin can be activated not only in vitro, but also in vivo. Two years later, *Derkx et al. (1978b)* studied the pathophysiological significance of the enzymatically inactive renin in man. They found that peripheral venous values for active and inactive renin in essential hypertension, renovascular hypertension, primary aldosteronism, adrenal insufficiency and in control subjects were all directly correlated. They also measured the renal vein to peripheral vein ratio of both active and inactive renin in patients with essential and renovascular hypertension. The existence of a renal mechanism not only for controlling the total quantity of circulating renin but also for modulating its degree of activation was proposed. They concluded that the control of inactive renin might be linked to active renin in a physiological homeostatic mechanism.

Leckie et al. (1977b) found that saralasin caused a marked increase in active renin but did not affect inactive renin concentration. *Millar et al. (1978c)* and *Millar et al. (1978b)* found that while active renin is increased by sodium deprivation, by upright tilting or by furosemide, inactive renin was not increased by tilting or sodium deprivation but was increased by furosemide. *Atlas et al. (1977)* and *Sealey et al. (1977)* showed that in human subjects sodium deprivation or diuretic therapy caused parallel increases in both active and inactive renin levels and the β-blocker, propranolol, caused either no change or a decrease in inactive renin whereas active renin fell in all subjects. *Hsueh et al. (1978)* found that sodium deprivation caused an increase in both active and inactive renin in man.

Recently, *Morganti et al. (1982)* found that in hypertensive patients the rise in active renin induced by ambulation and captopril

administration is associated, both before and after diuretic therapy with unchanged levels of the inactive renin. *Millar (1982)* showed that in six salt replete normal subjects, during stepwise infusion of Ang. II there was a significant decrease in plasma active renin. No change in inactive renin was observed.

Responses of active and inactive renin in experimental animals to some physiological manipulations have also been reported.

In the anaesthetised rabbit, furosemide diuresis increased plasma active renin but inactive renin disappeared from plasma *Richards et al. (1981a)*. A similar pattern of change was reported from the initial response to dietary sodium depletion, while the animals were in negative sodium balance. Following prolonged feeding of the sodium diet, when sodium excretion in the kidney and gut had adapted to match the reduced intake, plasma levels of both forms of renin were raised *(Grace et al., 1979b)*. Active renin release from rabbit kidney cortex slices in vitro increased, in response to reduced buffer $[Na^+]$ was also accompanied by a decrease in inactive renin *(Munday et al., 1982)*.

In a subsequent study, *Lush, Munday & Noble (1983)* reported that furosemide diuresis in the anaesthetized sheep increased active renin but inactive renin was undetectable in plasma. No change in either form of renin occurred during furosemide diuresis in conscious sheep. Furthermore, when papaverine was infused together with furosemide into conscious animals, an increase in active renin was accompanied by a decrease in plasma inactive renin. These results suggest that the difference between the responses of renin secretion to furosemide diuresis in the conscious and anaesthetized

sheep may be related to differences in renal prostaglandin synthesis.

In summary, where these animal studies involving primary changes in sodium handling by the kidney have resulted in an increase in plasma active renin this has been accompanied by a decrease in plasma inactive renin. In vivo there seems to be a requirement for some other concurrent influence, such as anaesthesia or a vasodilator drug, before changes in sodium handling by the kidney alter renin secretion at all.

In contrast to the effects of changing sodium, *Richards et al. (1979)* have studied the responses of circulating concentrations of active and inactive renin in anaesthetized rabbits to two levels of haemorrhage and found that both active and inactive renin in plasma increased after haemorrhage with no change in the relative proportions of the two forms. In the group of rabbits subjected to severe haemorrhage after ligation of the renal vessels, neither form of renin increased in response to haemorrhage. It was not possible to detect inactive renin in the plasma of rabbits 24 hours after bilateral nephrectomy suggesting that, in the rabbit at least, inactive renin is of renal rather than extrarenal origin.

The β-adrenoceptor agonist, isoprenaline, produced a similar effect to haemorrhage on both active and inactive renin release in anaesthetized rabbits *(Richards, Noble & Munday, 1981b)*. Both forms of renin increased after isoprenaline infusion and then returned to basal values after ceasing the infusion. Addition of isoprenaline to rabbit kidney cortex slices caused an increase in active renin release but inactive renin remained unchanged. This is in contrast to the previous study where reducing $[Na^+]$ increased active and

inhibited inactive renin output *(Munday et al., 1982)*. These authors suggested the existence of an independent sodium sensitive mechanism regulating secretion of inactive renin.

Active and inactive renin might be secreted from two separate pools within the JG apparatus *(Sealey et al., 1980; Noble et al. 1981)*. It appears that plasma active and inactive renin levels are probably determined intrarenally *(Munday et al., 1982)*.

Work on the role of Ca^{++} ions in active and inactive renin secretion has been reviewed elsewhere. (See page 55).

As a result of the work reviewed in this section, one could say that the changes in plasma active renin due to some specific stimuli may be accompanied with an increased, decreased or unchanged plasma inactive renin concentration. It is not yet fully confirmed whether changes in plasma inactive renin are the result of an independent effect of these stimuli on inactive renin release from the kidney or the result of conversion between plasma active and inactive renin in the circulation.

Control of Renin Secretion

During the past 20 years, five basic mechanisms controlling renin release have been described; these are:

1) An intrarenal baroreceptor located in the afferent arterioles of the glomerulus
2) A sodium sensitive receptor associated with the macula densa
3) The sympathetic nervous system mediated by β-adrenoceptors
4) Hormonal factors e.g. angiotensin II, prostaglandins and catecholamines
5) Plasma electrolytes e.g. potassium, sodium and calcium

Major reviews of these mechanisms include those by *Vander, 1967; Davis & Freeman, 1976; Peart, 1978a;Fray, 1980; Keeton & Campbell 1980; Torretti, 1982.* These reviews and many other studies which will be discussed later in this section of this thesis were concerned only with the active form of renin. Some other newer reports which consider inactive renin secretion as well will also be discussed.

Intrarenal Baroreceptor

In 1934 *Goldblatt et al.* discovered that hypertension could be induced by constricting the renal arteries. The release of a circulating pressor substance was initially thought to be a response to renal ischaemia. But, some years later, it was suggested that a haemodynamic factor, such as a pulse pressure, was the stimulus for renin release *(Kohlstaedt & Page, 1940).* This was subsequently rejected when, in 1958, *Kolff* reported that renin or a renin-like substance was released in response to renal artery constriction irrespective of pulsatile or non-pulsatile flow.

Tobian et al., in 1959 proposed the existence of an intrarenal baroreceptor which increased renin secretion in response to a decrease in mean renal perfusion pressure. They noted that, in an isolated kidney, the granulation of the JG cells decreased with an increase in renal perfusion pressure. This observation led *Tobian (1962)* to report that renin release from the granular cells of the JG apparatus was inversely related to the degree of stretch of the renal afferent arterioles and that the amount of stretch was in turn determined by mean renal perfusion pressure. These suggestions have become the basis for the so-called baroreceptor or stretch hypothesis. Consistent with *Tobian's* hypothesis, *McCubbin & Page (1963)* found that renin increased in response to suprarenal aortic constriction in anaesthetized dogs. One of the difficulties in evaluating *Tobian's* hypothesis is that most of the experimental manoeuvres change glomerular filtration rate (GFR). This in turn could alter the composition of the fluid in the nephron, stimulate the macula densa and change renin release. A new approach for testing the baroreceptor hypothesis independent of the effect of renal sodium handling was needed. In consequence, the non-filtering dog kidney model was developed by *Blaine, Davis & Witty (1970)*. They found that, in conscious dogs, both haemorrhage and suprarenal aortic constriction increased renin release in the absence of a functional macula densa. Subsequently they attempted to further isolate the baroreceptor mechanism by sectioning the renal sympathetic nerves and by adrenalectomy. Haemorrhage and suprarenal aortic constriction still produced a significant increase in plasma renin in conscious dogs *(Blaine et al., 1971)*.

The location of the renal baroreceptor was thought to be the

afferent arterioles since papaverine, which prevents renal auto-regulation by dilating the renal afferent arterioles, blocks haemorrhage induced renin secretion from denervated non-filtering kidneys *(Witty et al., 1971)*. Some of the major criticisms of *Tobian's* hypothesis come from observations by various investigators that either systemic or renal arterial hypotension-induced renin release was usually associated with renal vasodilation *(Cowley & Guyton, 1972; Eide, Loynining & Kiil, 1973; Harris & Ayers, 1972 and Eide et al., 1977)*.

It is clear that the proposed intrarenal baroreceptor is more complicated than was originally suggested by *Tobian et al. (1959)*. However, the current concept is that the intrarenal baroreceptor consists of the highly differentiated JG cells, and that the signal for stimulation of renin release is a decrease in stretch. It is not yet known how stretch activates the JG cells *(Davis et al., 1981)*. Perhaps *Fray (1980)* has the answer - he proposed that hyperpolarization might mediate the stimulation of renin release from the JG cells. It was suggested that renal arterial hypotension or renal vaso-constriction stimulated renin secretion by hyperpolarizing the JG cell membrane and decreasing the permeability to Ca^{++} ions *(Fray, 1980)*.

Studies are needed to measure the transmembrane potential of JG cells to determine whether they do become hyperpolarized in association with increased renin secretion *(Davis et al., 1981)*. This is technically difficult because of the problem of getting access to the relatively few JG cells buried within the kidney. Only one paper describing micro-electrode recording from JG cells has been published *(Fishman, 1976)*.

Macula Densa

The macula densa is at the boundary of the ascending loop of Henle and the distal tubule. More than three decades ago, *Goormaghtigh (1945)* proposed the existence of a functional relationship between the granular and the macula densa cells.

The first physiological evidence concerning the macula densa and its ability to regulate renin release from JG cells was provided by *Vander & Miller* in 1964. They showed that the usual increase in renin secretion that occurs during aortic constriction could be prevented by administration of diuretics. They therefore proposed that renin release mediated by the macula densa is inversely related to the macula densa sodium load. They suggested that the diuretic increased the sodium load passing the macula densa and hence blocked the increase in renin release. In contrast to this concept, *Thurau et al. (1967)* proposed that increased rather than decreased macula densa sodium concentration, leads to renin release. In their micropuncture studies, *Thurau et al. (1967)* found that retrograde injections of hypertonic sodium chloride solutions into the distal tubules were associated with proximal tubule collapse. No change in the proximal tubule diameter was observed in control experiments with hypotonic sodium chloride or hypertonic or isotonic solutions of choline chloride or mannitol. They proposed the following sequence of events: increased sodium concentration in the tubule perfusing the macula densa, renin release increased, local Ang. II formation in the JG cells, afferent arteriolar constriction, decreased GFR, proximal-tubule collapse. *Thurau et al. (1967)* concluded that the sodium ion per se is necessary for the response,

and that renin release is directly proportional to the sodium concentration at the macula densa. Their theory was consistent with the suggestion by *Goormaghtigh (1945)* that renin may in some way control single nephron GFR.

Support for the *Thurau et al. (1967)* theory was produced by *Meyer et al. (1968)*. They found that in anaesthetized rabbits furosemide increased renin release. This was attributed to increased sodium concentration at the macula densa. *Cooke et al. (1970)* also used the *Thurau* theory to explain their observation that ethacrynic acid diuresis increased plasma renin activity while chlorthiazide diuresis did not. They suggested that because ethacrynic acid acts on the loop of Henle, whereas chlorothiazide acts on the distal tubule, renin release increased with ethacrynic acid as a result of increased sodium concentration at the macula densa.

There are therefore two opposing theories regarding macula densa mediated renin release which have not yet been fully reconciled. However, the more recent diuretic studies *(Churchill, 1979; Davis et al., 1981)* tend to support *Vander's* concept, that is that the macula densa responds to a decrease in NaCl load by increasing renin release. *Vander's* theory appears to be logical in view of the fact that dietary sodium depletion is associated with increased plasma renin activity in man *(Brown, et al., 1963)*, in the rabbit *(Grace, Munday, Noble & Richards, 1979)* and in the rat *(Keeton, Pettinger & Campbell, 1976)*.

The contribution of chloride ions to the control of plasma renin has been evaluated by *Kotchen, Galla & Luke (1978)*. They demonstrated that, in the NaCl deprived rat, plasma renin activity (PRA)

was not inhibited by dietary-loading with sodium acetate, sodium nitrate, or sodium thiocyanate whereas PRA was inhibited by NaCl and NaBr. *Kotchen et al. (1976)* in a previous study reported that, unlike NaCl, $NaHCO_3$ does not inhibit PRA in the sodium deprived rat. These results suggested a role for chloride ions in regulating the renin responses to sodium loading in the rat. *Abboud, Luke & Galla (1979)* produced selective chloride depletion without sodium depletion in the rat by peritoneal dialysis and concluded that acute selective chloride depletion per se is a potent stimulus for renin release.

The discovery of an active chloride pump in the ascending limb of the loop of Henle by *Kokko (1974)* together with the observations by *Kotchen et al. (1976, 1978)* and *Abboud et al. (1979)* support the view that chloride may be an important element for renin secretion by activating the macula densa. *Sen, Acker & Smeby (1980)* reported that in rats a sodium-free diet with chloride present was as effective in increasing PRA as a chloride-free diet and suggested that both the sodium and chloride must continue to be considered as contributing to the signal for renin secretion via the macula densa.

The concept of the macula densa receptor is partly based on experiments using furosemide *(Vander & Carlson, 1969)*. Studies in which furosemide was associated with increased plasma active renin have usually been performed either on anaesthetized animals or in animals which have been allowed to suffer volume depletion as a result of the diuresis *(Johns & Singer, 1973; Richards, Lush, Noble & Munday, 1981a)*. Recent studies using conscious sheep, in which volume depletion was avoided, have provided some surprising results *(Lush, Munday &*

Noble, 1983). Despite a massive diuresis, furosemide did not cause changes in plasma active or inactive renin unless papaverine was included in the infusion. The basis for the difference between these observations and other published data related to the presence or absence of anaesthetic agents. These may interact with the renal prostaglandin system.

The precise role of the macula densa in the control of renin release has proved difficult to establish although it may possibly become clearer following the recognition of the existence of inactive renin. Altered sodium handling by the rabbit kidney in three quite different situations, the initial response to dietary sodium depletion, furosemide diuresis in anaesthetized animals and reducing $[Na^+]$ bathing in vitro kidney slices, have each produced a similar pattern of response with increased active and decreased inactive renin secretion *(Noble, Richards, Grace & Munday, 1981).* The same authors suggested the existence of an independent sodium sensitive mechanism regulating the secretion of inactive renin.

A new conflict has been raised over secretion control for inactive renin. This is related to species variation. For example, the rat, which is the most widely used species for studies of renin release, may not secrete inactive renin at all *(Nakane et al., 1980a;de Keijzer, Provoost & Derkx, 1982).* In studies using rat kidney slice preparations it has been found that the secretion of renin is inhibited by reducing $[Na^+]$ *(Oelkers et al., 1970; Capponi & Vallotton, 1976; Lyons & Churchill, 1975b).* On the other hand renin secretion from dog kidney slices is increased by decreasing $[Na^+]$ *(Michelakis, 1971a).*

Recently, *Munday et al. (1982)* used a rabbit kidney cortex slice preparation and found that decreasing $[Na^+]$ stimulated release of active renin but reduced inactive renin secretion. The same authors *Noble et al. (1982)* have been unable to detect acid-activation of renin in media bathing rat kidney cortex slices or in extracts of rat kidney and suggested that the characteristics of a single renin release mechanism in the rat kidney resemble those for secretion of inactive, rather than active, renin in other species.

Sympathetic Nervous System and the β-Adrenoceptor

The morphological evidence for a functional relationship between the sympathetic nervous system and renin release has been reviewed by *Barajas, 1979*.

Vander (1965) first reported that electrical stimulation of renal nerves in anaesthetized dogs increased renin release. GFR, renal blood flow (RBF) and sodium excretion were all decreased by renal nerve stimulation. Since mannitol diuresis prevented the nerve-stimulated release of renin, it was possible that the increased release might be secondary to changes at the macula densa.

Assaykeen & Ganong (1971) found that stimulation of the renal nerves led to a two-fold increase in PRA but, unfortunately, the changes in renal function were not measured. Pretreatment with propranolol completely blocked the effect but still the increase in renin release might be attributable to a decrease in stretch in the granular JG cells as a result of constriction of the afferent arterioles. Alternative explanations of the results include a decrease in GFR and consequent stimulation of the macula densa or a direct effect of propranolol on the granular JG cells. It is clear that these previous studies did not discriminate between direct and indirect effects of renal sympathetic nerves on renin release. A direct action of sympathetic nerves on the JG cells was investigated by *Johnson, Davis & Witty (1971)*. In a non-filtering kidney preparation, papaverine was used to block any vasoconstriction response. Activation of the intrarenal baroreceptor and macula densa were thus excluded. They observed a rise in renin release in response to stimulation of the renal nerves and concluded that

this was due to a direct action on the granular JG cells. *Loeffer, Stockigt & Ganong (1972)* found that stimulation of renal nerves in anaesthetized dogs increased PRA and this response was completely blocked by propranolol. However, pretreatment with phenoxybenzamine, an alpha, adrenoceptor antagonist, failed to alter the increase in PRA in response to renal nerve stimulation. They concluded that both renal nerve stimulation and alpha-adrenoceptor antagonist induced renin release act through β-adrenergic receptors. In 1974, *Johns & Singer* discovered that nerve stimulation-induced renin release was not antagonised by d-propranolol, which has only 1% of the β-antagonist effect of ℓ-propranolol in anaesthetized cats, while a racemic mixture of the two forms blocked the increase in renin release but did not prevent stimulation-induced vasoconstriction of the renal vessels. *Taher et al. (1976)* increased renin release by stimulating renal nerves but without changing GFR, RBF or urinary sodium output. The same authors also found that the increase in renin secretion after nerve stimulation was blocked completely by ℓ- and d,ℓ-propranolol and concluded that the increase in renin secretion could not have occurred via the renal baroreceptor or macula densa mechanism.

The general conclusion from all these studies is that renin release is regulated by a β-adrenoceptor located on the granular JG cells. Other reports support the idea that the rise in renin release following haemorrhage *(Blaine, Davis & Witty, 1970)*, hypoglycemia *(Assaykeen et al., 1970)* and pharmacological vasodilation with antihypertensive drugs *(Pettinger, Campbell & Keeton, 1973)*, are all at least partly mediated by an increase in sympathetic outflow to the kidney.

Since physiological activation of the renal nerves originates within the central nervous system (CNS) several studies of the effect of electrical stimulation of parts of the CNS on renin release have been reported. Stimulation of the central grey stratum of midbrain in dogs *(Ueda et al., 1967)*, the medulla oblongata in the pressor area in dogs *(Passo et al., 1971)*, the dorsal pons in cat *(Richardson et al., 1974)* and the lateral hypothalamus in the cat *(Zanchetti & Stella, 1975)* are all associated with altered renin release.

Evidence for a physiological role of the CNS, and specifically of the hypothalamus, in the control of renin secretion was provided by *Zehr & Feigl (1973)* who showed that after electrical stimulation of the hypothalamus in conscious dogs, plasma renin activity was reduced by 50%. The response was abolished by renal denervation.

During the past decade, evidence suggests that the aortic, cardiopulmonary and carotid receptors are involved in the pathways controlling renin release via the renal sympathetic nerves. A hypothesis is that stimulation of the vagally innervated receptors in the cardiopulmonary region acts via tonic discharge in the renal sympathetic nerves to decrease renin release. Inhibiting these receptors results in increased renin secretion *(Davis et al., 1981)*. In support of this hypothesis, vagal cooling in the dog increased renin release during perfusion of the carotid sinus at a constant pressure *(Mancia, Romero & Shepard, 1975)*. *Zehr, Hasbargen & Kurz (1976)* increased left atrial pressure in dogs by placing a balloon in the left atrium in order to stimulate left atrial receptors. This inhibited renin secretion.

Future studies should clarify which areas of the brain are involved in sympathetically mediated renin release. Linked with these renal pathways is the possibility that circulating catecholamines may act on the β-adrenoceptors on the JG cells and modulate renin secretion.

Circulating Catecholamines

Intrarenal artery or intravenous infusion of catecholamines increases renin secretion *(Vander, 1965; Johnson, Davis & Witty, 1971; Ueda et al., 1970; Winer, Chokshi & Walkenhorst, 1971)*.

Vander, 1965, first reported that i.v. infusion of adrenaline or noradrenaline in anaesthetized dogs increased renin secretion. Arterial blood pressure was maintained constant by means of supra-renal aortic constriction but the increase in renin secretion could have been an indirect result of altered sodium excretion. In human subjects, i.v. infusion of catecholamines was also found to increase PRA *(De Champlain et al., 1966)*.

In 1967, *Vander* identified possible mechanisms by which catecholamines might stimulate renin secretion:

1) Direct effect on the granular or macula densa cells
2) Stimulation of JG stretch receptors by inducing arteriolar constriction
3) Stimulation of the macula densa receptor by reducing GFR
4) Stimulation of extrarenal receptors.

Assaykeen et al. (1970) observed an increase in adrenaline and renin secretion during insulin-induced hypoglycemia in anaesthetized dogs. The increase was not blocked by denervation and so they suggested that the hypothalamus responds to changes in the plasma levels of glucose, which then increase adrenaline secretion. This, in turn, leads to increased renin release. Using the non-filtering dog kidney preparation, *Johnson et al. (1971)* showed that intra-renal artery infusion of adrenaline or noradrenaline increased renin secretion. Although adrenaline induced renin secretion was blocked

by papaverine, the response to noradrenaline was sustained during papaverine infusion. It was suggested that noradrenaline acts directly on the JG cells to stimulate renin secretion while adrenaline stimulated renin through activation of the intrarenal baroreceptor.

Michelakis, Caudle & Liddle (1969) reported that renin release was increased by adrenaline, noradrenaline and cyclic adenosine monophosphate (AMP) in dog renal cell suspensions. In an isolated perfused rat kidney preparation *Vandongen, Peart & Boyd (1973)* found that noradrenaline increased renin release even when renal vasoconstriction was blocked with phenoxybenzamine.

It has become generally accepted that the endogenous catecholamines stimulate renin release by activation of the renal β-adrenoceptors *(Davis & Freeman, 1976; Reid, Morris & Ganong, 1978)*. β-agonists stimulate renin release by the isolated kidney *(Vandongen & Greenwood, 1975)*, by renal cortical slices *(Michelakis et al., 1969, Richards et al., 1981b)* and by renal cortical cells *(Johns, Richards & Singer, 1975)*. These observations strongly support the idea of a direct independent action of the β-adrenoceptor influencing renin release.

β-adrenoceptors have been subdivided into two types, β_1 and β_2. It has been proposed that the β-receptors involved in the control of renin secretion are of the β_2 type *(Reid et al., 1978)*. In hypertensive humans practolol, a β-blocker with predominantly β_1 receptor affinity, had little effect on plasma renin levels. On the other hand, propranolol, which blocks both β_1 and β_2 receptors, decreased plasma renin levels *(Esler & Nestel, 1973)*. *Johns & Singer (1974b)*

reported that ICI 66082, a β-blocker, similar to practolol in its action is much less potent than propranolol in blocking the increase in renin secretion produced by stimulation of the renal nerves of cats.

The β-adrenoceptor agonist, isoprenaline has been shown to stimulate renin release when given by intrarenal artery infusion to anaesthetized dogs *(Assaykeen et al., 1974; Winer et al., 1971)* and anaesthetized rabbits *(Richards et al., 1981b)*. Similar results were obtained with isolated, perfused rat kidneys *(Ettienne & Fray, 1979; Nakane et al., 1980)*, rat kidney cortex slices *(Desaulles, Miesch & Schwartz, 1978)* and rabbit kidney cortical slices *(Richards et al., 1981)*. Isolated renal cortical cells of the cat also respond to isoprenaline *(Johns et al., 1975)*. These observations further support the hypothesis that a β-adrenoceptor located on the JG cells is involved in renin secretion.

Some observations have suggested that the stimulation of renin secretion by catecholamines is mediated via cyclic AMP *(Reid et al., 1978)*. Other evidence indicates that calcium flux *(Harada & Rubin, 1978; Peart, 1978)* or prostaglandins *(Berl et al., 1979)* may be involved in the β-adrenoceptor response. Quite recently, *Olson et al. (1983)* reported that noradrenaline and adrenaline stimulated renin release by activation of both the renal α and β-adrenoceptors. The β-adrenoceptor-stimulated renin release appeared to be a direct action independent of the PG system. Conversely the α-adrenoceptor-stimulated renin release appeared to be prostaglandin dependent.

Later in this thesis (see page 106) some work is presented in which the effect of isoprenaline and propranolol on active and inactive renin secretion in conscious sheep was investigated.

Plasma Angiotensins and Renin Secretion

In 1965 *Vander & Geelhoed* proposed a pressure-independent mechanism by which circulating levels of Ang. II inhibited renin secretion through a direct intrarenal action. They observed that i.v. administration of Ang. II suppressed renin release in anaesthetized dogs even when renin secretion was stimulated by renal arterial hypotension or elevation of ureteral pressure. This effect of Ang. II was not mediated by increased aldosterone secretion as infusion of aldosterone for 3 hours did not lower plasma renin activity *(De Champlain et al., 1966)*. Renin suppression by Ang. II was also independent of any change in total RBF or renal perfusion pressure produced by the peptide *(Bunag, Page & McCubbin,1970)*. In anaesthetized dogs with a single non-filtering kidney, *Shade, Davis & Johnson (1973)* found that Ang. II mediated suppression of renin secretion did not depend on the presence of a functional macula densa.

All these observations support the concept that Ang. II inhibits renin release by a negative feedback loop mechanism in which Ang. II acts directly on the JG cells. Consistent with this concept, *Michelakis (1971b)* reported that Ang. II inhibited renin release from dog kidney cell suspensions.

Keeton, Pettinger & Campbell (1976) showed that the competitive antagonist of Ang. II, saralasin, stimulated renin release in conscious normal and sodium depleted rats. This appeared to be caused by blockade of the angiotensin negative feedback mechanism. In sodium loaded animals, however, propranolol reduced saralasin-induced renin release. *Keeton et al. (1976)* therefore suggested that this was associated with an intrarenal β-adrenoceptor.

The negative feedback inhibition by Ang. II is sometimes referred to as the "short loop" mechanism in order to differentiate it from the indirect inhibitory effect of an increase in aldosterone, the "long loop" mechanism *(Keeton et al., 1976)*.

Ang. II also inhibited renin release by an isolated, perfused rat kidney *(Van Dongen, Peart & Boyd, 1974)* and reduced renin release in vitro from rat kidney cortex slices *(Capponi, Gourjon & Vallotton, 1977; Park, Han & Fray, 1981)*. Furthermore, *Van Dongen & Peart (1974)* and *Park et al. (1981)* suggested that the inhibition of renin secretion produced by Ang. II could be due to an increased intracellular $[Ca^{++}]$ of JG cells as a result of increased Ca^{++} ion influx. It has also been reported that Ang. III inhibited renin release in dog *(Freeman, Davis & Lohmeirer, 1975)* and from rat kidney slices *(Naftilan & Oparil, 1978)*.

Later in this thesis (see page 150) some work is described in which the negative feedback effect of Ang. II was blocked by using converting enzyme inhibitors. Effects on active and inactive renin are reported.

Prostaglandins and Renin Secretion

In recent years, there have been many reports suggesting that prostaglandins may play a role in the control of renin secretion and these have been the subject of several reviews *(Werning et al., 1971; Yun et al., 1977; Yun, 1979; Imanishi et al., 1980; Gerber, Olson & Nies, 1981)*.

Vander (1968) examined the effect of PGE_1 and PGE_2 on renin release and suggested that PGs could participate in the control of renal function but probably not in renin release. Three years later *Vane (1971)* discovered that nonsteroidal anti-inflamatory drugs inhibit PG synthesis. The prostaglandin synthetase inhibitor, indomethacin, was found to lower plasma renin activity in dogs *(Data et al., 1978)*, in rabbits *(Larsson, Weber & Anggard, 1974)* and in man *(Frölich et al., 1972)*.

Further evidence confirming the interrelationship between PGs and renin release was that infusion of arachidonic acid, the precursor of the PGs of the "2" series, into the renal artery or the aorta in the rabbit and dog, increased plasma renin activity *(Larsson et al., 1974; Bolger et al., 1976)*. Also, when arachidonic acid was added to the incubation media of rabbit renal cortical slices this increased the release of renin, an effect which was inhibited by the addition of indomethacin *(Weber, Larsson, Anggard et al., 1976)*.

It has been suggested that PGs modify the functioning of the other mechanisms which regulate renin secretion. These include the baroreceptor, *(Data et al., 1978; Gerber et al., 1981; Henrich, 1981)*, the macula densa *(Olson et al., 1980; Gerber et al., 1981)* and the β-adrenoceptor *(Feuerstein & Feuerstein, 1980)*.

In relation to the baroreceptor mechanism, *Data et al. (1978)*

and *Gerber et al. (1981)* found that reducing renal perfusion pressure in non-filtering, denervated, β-blocked kidneys increased renin release. This increase was totally abolished by indomethacin. It therefore appeared that the renal baroreceptor-mediated increase in renin release was PG dependent.

In order to evaluate the relationship between macula densa-stimulated renin release and PG synthesis, *Gerber et al. (1981)* tried to exclude sympathetic influences by renal sympathectomy, adrenalectomy and administration of propranolol. The intrarenal baroreceptor was inactivated by giving the animals a constant intrarenal infusion of papaverine. The macula densa mechanism was stimulated by aortic clamping; renin release increased but, in the presence of indomethacin, plasma renin activity remained unchanged. It was suggested that macula densa mediated renin release required PG synthesis.

Prostaglandins are also thought to be involved in the negative feedback inhibition of renin release by Ang. II. This conclusion was reached because indomethacin inhibits the increase in renin release mediated by administration of the CEI, Captopril (*ABE et al., 1980*).

Berl et al. (1979) examined the relationship of β-adrenoceptor-stimulated renin release and PGs in dogs. Isoprenaline produced an increase in renin release and this increase was not blunted by prior indomethacin administration. It was concluded that β-adrenoceptor induced renin release is PG independent in the dog. A similar conclusion was reached by *Seymour & Zehr (1979), Henrich (1981), Gerber et al. (1981)* and *Olson, Nies & Gerber (1983)*. This is not, however, without dispute.

Feuerstein & Feuerstein (1980), from studies in anaesthetized

cats, reported that isoprenaline induced renin release can be blocked by indomethacin and suggested an involvement of PGs with β-adrenoceptor-mediated renin release. Also, intra-arterial infusion of the α-adrenoceptor agonist phenylephrine in dogs increased renin release, an effect which was severely attenuated by indomethacin. *Gerber et al. (1981)* thus suggested that phenylephrine induced renin release may be due to the generation of a vasodilatory PG. This would counteract vasoconstriction and release renin. However, when the same authors *(Gerber et al. 1981)* infused phenylephrine into the renal artery of dogs with a single non-filtering kidney it did not stimulate renin release, possibly suggesting that α-adrenoceptor agonist stimulated renin release resulted from activation of the macula densa.

In vitro data demonstrate a direct effect of several PGs on the JG cells to stimulate renin release. Using superfused rat kidney slices, *Franco-Saenz et al. (1980)* reported that PGE_2 caused a dose-dependant stimulation of renin secretion which was not inhibited by propranolol, but may be mediated in part by cyclic AMP. *Weber et al. (1976)* showed that in rabbit renal cortical slices, PGEs had no effect, whereas $PGF_{2\alpha}$ caused a decrease in renin release. On the contrary, *Whorton et al. (1977)* and *Whorton, Lazar & Smigel (1980)* reported that PGE_2 stimulated renin release from rabbit renal cortex slices and that prostacyclin (PGI_2) is three times more potent than PGE_2. PGD_2 is inactive in this preparation. It is not yet clear which of the renal PGs is responsible for renin release. It appears that PGI_2 is the leading candidate since it is three times more potent than PGE_2 in releasing renin from rabbit renal cortex slices

(Whorton et al., 1977, 1980). In dogs with non-filtering kidneys which were indomethacin-treated and β-blocked, PGI_2 was more potent than PGE_2 in causing renin release *(Gerber, Keller, Nies, 1979)*.

All of these previous studies were concerned with PGs and the active form of renin. Since inactive forms of renin also exist in the plasma and kidney it will be interesting to find out whether PGs mediate inactive renin secretion. There is a single report which shows that PGs are involved in regulation of active and inactive forms of renin in man *(Salvetti et al., 1982)*.

The effects of prostaglandins modifying renin release may be important in the interpretation of some of the work described in this thesis. This is particularly in relation to the effects of anaesthetic agents on haemorrhage-induced active and inactive renin secretion (see page 175).

Plasma Electrolytes

Calcium plays a key role in stimulus-secretion coupling for hormones *(Rubin, 1970)* and in excitation-contraction coupling in muscles *(Bohr, 1973)*. Since the JG cells are modified smooth muscle cells which contain renin secretory granules *(Barajas & Latta, 1967; Barajas, 1979)*, Ca^{++} ions may also play a role in the stimulus-secretion coupling of renin release. Unlike its stimulatory role in secretory processes in many other tissues *(Rubin, 1970)*, several lines of evidence from in vitro studies support the concept that influx of Ca^{++} ions inhibits the secretion of renin whereas Ca^{++} efflux stimulates renin secretion.

Removal of Ca^{++} ions from the medium bathing various in vitro preparations stimulates renin release *(Van Dongen & Peart, 1974; Fray, 1977; Harada & Rubin, 1978; Park & Malvin, 1978; Ettienne & Fray, 1979; Churchill, 1981; Park, Han & Fray, 1981 and Ginesi, Munday & Noble, 1983)*.

Calcium plays a central role in the mechanisms of renin secretion described previously. The renin stimulatory effect of low perfusion pressure *(Fray, 1977)* and the inhibitory effect of Ang. II *(Van Dongen & Peart, 1974)* require calcium. It has also been reported that renin release induced by adrenaline and the suppression of its release by ouabain were abolished after calcium removal *(Park & Malvin, 1978)*. Isoprenaline is less effective in stimulating renin release when calcium is removed from the perfusion medium *(Fray & Park, 1979)*. *Ginesi et al. (1983)* reported that release of both active and inactive renin from rabbit kidney cortex slices is inhibited by raised $[Ca^{++}]$ and stimulated by buffers with zero $[Ca^{++}]$ and 5 mM-EGTA. Addition of

verapamil or flunarizine to the control media increased both active and inactive renin. But in contrast to the calcium antagonists, verapamil and flunarizine, the calcium ionophore A23187 failed to alter secretion of either form of renin in the presence of 2.3 mM-Ca^{++} *(Ginesi, Munday & Noble, 1982)*.

Churchill & Churchill (1979), using an in vitro rat kidney cortex slice preparation, showed that a decrease in extracellular $[K^+]$ inhibits renin release and that this inhibitory effect can be antagonized by incubating the slices in a calcium-free medium. These results are consistent with other papers which show that a high $[K^+]$ inhibits renin release from pig *(Park & Malvin, 1978)*, rat *(Churchill, 1980)* rabbit *(Ginesi et al., 1983)* and dog *(Park et al., 1981)* renal cortex slices. The effect is probably due to depolarization of the JG cells and consequent influx of Ca^{++} ions. This potassium inhibitory effect on renin was abolished by calcium antagonists such as verapamil *(Churchill, 1980; Park et al., 1981)*.

Ginesi et al. (1983) reported that depolarization with high $[K^+]$ media suppressed not only active but also inactive renin secretion from rabbit renal cortex slices.

In vivo studies by *Vander (1970)* showed that PRA was suppressed in anaesthetized dogs after an intrarenal artery infusion of a potassium salt. *Shade et al. (1972)* demonstrated that intrarenal arterial infusion of KCl and NaCl failed to decrease renin secretion in dogs with non-filtering kidneys. Since no haemodynamic changes were observed, the authors *(Shade et al., 1972)* suggested that the renin response to NaCl and KCl resulted from a renal tubular mechanism. It has been shown in several animal studies that an acute increase

in plasma $[K^+]$ causes an immediate decrease in tubular reabsorption of sodium. These studies were carried out in anaesthetized dogs *(Kahn & Bohrer, 1967; Vander, 1970)*, anaesthetized rats *(Brandis, Keys & Windhager, 1972)* and conscious dogs *(Youn et al., 1976)*. Decreased renin secretion was observed in anaesthetized dogs *(Vander, 1970; Flamenbaum et al., 1975; Stephen et al., 1978)* and conscious dogs *(Young et al., 1976; McCaa, McCaa & Guyton, 1975)*.

Hartroft & Hartroft (1961) proposed an inverse relationship between plasma $[Na^+]$ and the granulation of the JG cells. In 1965, *Brown et al.* observed an inverse correlation between plasma $[Na^+]$ and PRA in hypertensive patients. *Yamamoto et al. (1969)* found that the low plasma $[Na^+]$ resulting from peritoneal dialysis of anaesthetized dogs was associated with marked increase in renin secretion, and administration of hypertonic saline decreased renin secretion. *Shade et al. (1972)* studied anaesthetized thoracic caval-constricted dogs with either a single filtering or non-filtering kidney. Intrarenal infusion of hypertonic saline in dogs with a filtering kidney decreased renin secretion, whereas a similar infusion into dogs with a non-filtering kidney had no effect on renin release. The authors suggested that the inhibition of renin secretion by NaCl was mediated by a renal tubular mechanism and eliminated the possibility of a vascular action or a direct effect of NaCl on the granular cells of the JG apparatus.

Grace et al. (1979a, 1979b) studied the relationship between dietary sodium and plasma active and inactive renin levels in rabbits kept in metabolism cages. They found that active renin increased by 97% during sodium depletion and returned to control level on repletion. Both changes were completed within one day of changing the diet. Inactive renin fell to zero during sodium depletion and

returned to control levels over about 13 days during sodium repletion.

From in vitro studies rather conflicting results have been published - *Michelakis (1971)* found an inverse relationship between renin release and the $[Na^+]$ of the incubation medium when the osmolality was maintained constant in the dog renal cortex slices. In contrast, *Braverman, Freeman & Rostorfer (1971)* showed that renin secretion from rat kidney slices decreased as $[Na^+]$ decreased. *Blendstrup et al. (1975)* found a similar direct relationship between $[Na^+]$ and renin release from isolated superfused rat glomeruli in vitro. *Lyons & Churchill (1975b)* reported that changes in $[Na^+]$ had opposite effects on renin secretion from rat renal cortex slices and from cell suspensions. With kidney slices, increasing $[Na^+]$ caused increase in renin secretion, while increasing $[Na^+]$ in the medium suppressed renin secretion from the renal cortex cell suspension. The same authors *(Lyons & Churchill, 1975a)*, in other study, also found that ouabain decreased renin secretion in kidney cortex slices while $[Na^+]$ in the incubated medium increased.

Recently, *Munday, et al. (1982)* investigated the relationship of $[Na^+]$ and renin secretion not only on the active form of renin, but also the effect of changing $[Na^+]$ on inactive renin release. They found that, in rabbit kidney cortex slices preparations, active renin secretion was increased when incubation medium $[Na^+]$ was reduced, while inactive renin release decreased when $[Na^+]$ was reduced. Addition of ouabain abolished the changes due to low $[Na^+]$ and release of both forms of renin was similar to control rates *(Ginesi & Noble, 1983)*.

Studies involving changes in $[Na^+]$, $[K^+]$ and $[Ca^{++}]$ in media

bathing various *in vitro* kidney preparations have provided useful clues about intracellular events controlling renin secretion. Whether changes in plasma concentration of these ions contribute primarily to the regulation of renin secretion is open to considerable doubt.

Anaesthetic Agents and Renin

Anaesthetic agents have complex actions on vascular smooth muscle which have been reviewed by *Altura et al. (1980)*. General anaesthetic agents have been variously reported to produce increased, decreased or unchanged total peripheral vascular resistance, with corresponding effects on systemic arterial blood pressure *(Horowitz, 1977; Altura & Altura, 1975; Altura & Weinberg, 1979; McGrath & MacKenzie, 1977)*.

It is generally agreed that deep surgical anaesthesia will result in dilatation and hypotension *(Parker & Adams, 1978; Altura & Winberg, 1979 and Hug, 1979)*, due to actions on the central and peripheral nervous system and on the myocardium *(Horowitz, 1977; Dundee & Wyant, 1974; Smith & Smith, 1972)*. Central mechanisms of general anaesthesia were reviewed by *Richards, 1978*. More recently, *Altura et al. (1980)* proposed that the dilatory actions of anaesthetic agents are causally related to interference with the movement of Ca^{++} ions across cell membranes.

Many general anaesthetics are capable of altering tissue and plasma concentrations of cyclic nucleotides and neurohumoral substances including catecholamines *(Altura, 1980)*. Furthermore, anaesthetic agents may play an important role in determining the response of renin secretory mechanisms to experimental stimuli *(Pettinger, 1978)*. However, the mechanisms by which anaesthetic agents release renin appear to be as diverse as the pharmacological properties of the anaesthetics themselves *(Keeton & Campbell, 1981)*.

Pentobarbital is widely used as an anaesthetic agent in many physiological and pharmacological studies, but it does represent an

abnormal physiological situation. It is not surprising then that there are conflicting reports about the effect of pentobarbital on PRA. These have been reviewed by *Pettinger, (1978)*.

Yun et al. (1979) studied the effect of sodium pentobarbital and laparotomy on PRA in dogs. They showed that PRA was increased by pentobarbital anaesthesia and that laparotomy does not appear to cause any further increase in PRA. On the other hand, *Bailie et al. (1979)* in their studies on the effect of anaesthesia on cardiovascular and renal function in the newborn piglet found that pentobarbital causes an increase in systemic blood pressure and a decrease in PRA. A third group of researchers, including *Fray et al. (1974)*, reported that although pentobarbital anaesthesia decreased the blood pressure of trained dogs, this was not accompanied by any change in PRA. *Yun et al. (1978)* reported that the increase in PRA by pentobarbital anaesthesia could not be prevented by indomethacin. In contrast, *Montgomery et al. (1977)* showed that indomethacin did prevent the rise in PRA produced by pentobarbital anaesthesia.

Recently, *Lush et al. (1983)* showed that in sheep, pentobarbital anaesthesia per se did not alter either active or inactive renin concentration in plasma. There were, however, important differences in the responses of active and inactive renin secretion to furosemide diuresis between conscious and anaesthetized sheep. (See page 39). It was suggested that induction of anaesthesia is accompanied by potentiation of PG synthesis within the kidney and that this altered the response of the juxtaglomerular cells to physiological stimuli.

The lack of consistency in response of physiological systems to anaesthetic agents reinforces the need to avoid their use wherever possible.

Physiological Actions of Angiotensins - Introduction

Until recently, Ang. II was considered as the only biologically important effector hormone of the renin-angiotensin system but it is now clear that Ang. I and Ang. III also have some activity. All of these effects of angiotensins help to restore and maintain blood pressure when the body is threatened by hypovolemia and/or hypotension.

The actions of Ang. I and Ang. III are discussed in the latter part of this section, but first the specific physiological roles for Ang. II will be discussed. These are summarized in Fig. 4.1.

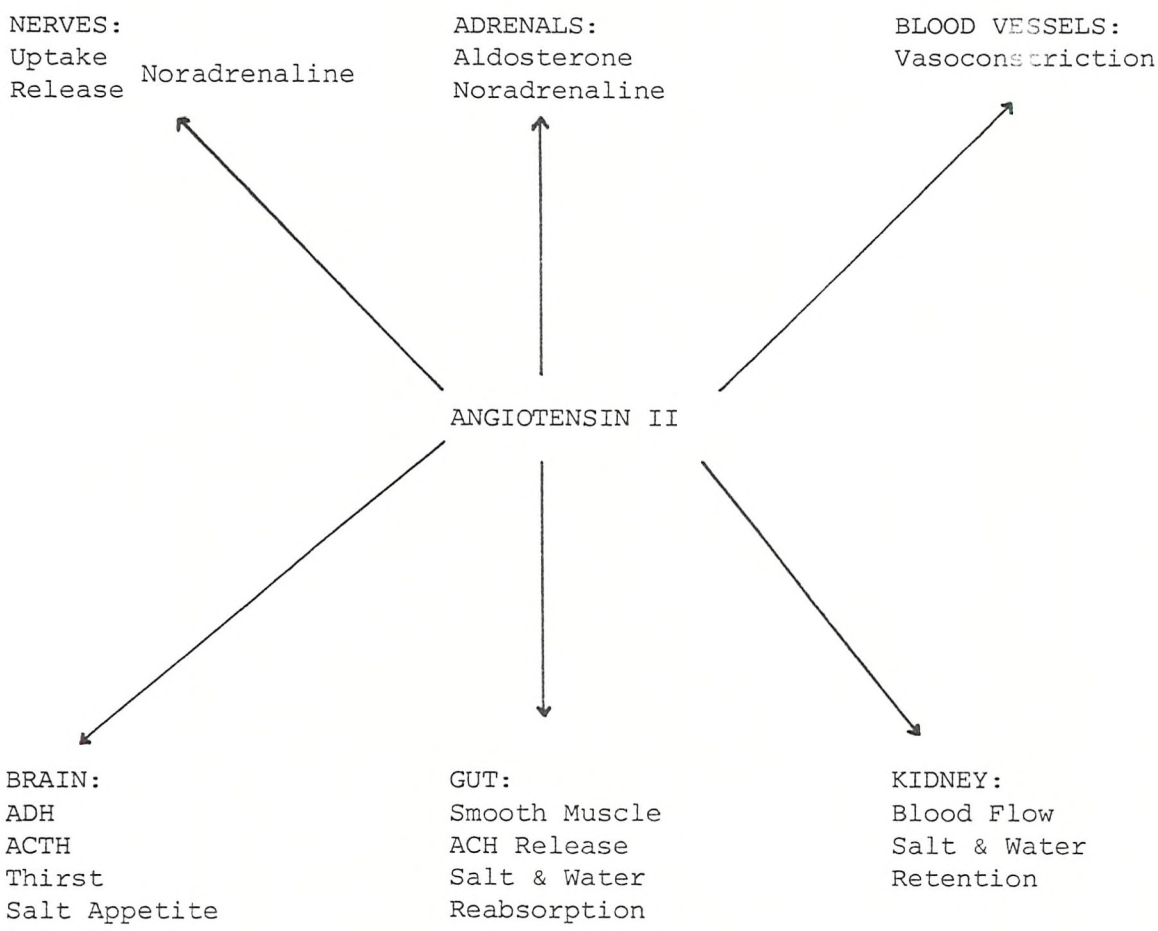


FIG. 1.4

PHYSIOLOGICAL ACTION OF ANG. II

Pressor Actions of Ang. II

There are several components of the acute pressor response to Ang. II including a direct stimulatory effect on vascular smooth muscle and indirect actions mediated via the central and sympathetic nervous systems. The direct effect of Ang. II on smooth muscle is independent of changes in transmembrane potential *(Shibata & Briggs, 1966)*, but dependent rather on the release of membrane bound calcium to initiate contraction of the myofilament *(Baudouin et al., 1972)*. Other evidence suggests that the induction of smooth muscle contraction by Ang. II involves the mobilisation of intracellular calcium stores *(Altura & Altura, 1971; Deth & Van Breemen, 1974)*.

An indirect pressor action of Ang. II was first reported by *Bickerton & Buckley, 1961)*. The response was inhibited by α-adrenergic blocking agents and potentiated by β-blockers *(Bickerton & Buckley, 1961; Vogin & Buckley, 1964)*. The centrally-mediated pressor response is well documented and has been reviewed by *Ganong (1977)* and by *Phillips (1983)*.

Augmentation of the pressor response to Ang. II is also enhanced by interaction with the sympathetic nervous system. Ang. II facilitates neurotransmission, both at ganglia *(Kaneko, McCubbin & Page, 1961)* and at nerve terminals *(Benelli, Della Bella & Gardini, 1964)*. The potentiating effect of Ang. II on neurotransmission has been attributed to inhibition of neuronal re-uptake of noradrenaline *(Khairallah, 1972)*, enhanced transmitter release *(Hughes & Roth, 1971)* and a stimulatory effect on noradrenaline synthesis by an action on dopamine β-hydroxylase *(Ackerly, Blumberg & Peach, 1976)*. For further discussion of work in this area see the reviews by *Starke (1977) and*

Zimmerman (1978). Recently, *Lewis, Hoffman & Phillips (1983)* reported that injection of Ang. II into the brain ventricles of conscious rats induced pressor, antidiuretic and drinking responses. Bradykinin may inhibit drinking induced by Ang. II.

Ang. II and Sodium and Water Transport

Sodium and fluid transport is stimulated by Ang. II through both direct and indirect mechanisms. In vitro, Ang. II stimulates sodium transport in frog skin *(McAffee & Locke, 1967)*, isolated rat jejunum *(Crocker & Munday, 1970)*, rat colon *(Davis, Munday & Parsons, 1970)* and rat kidney cortex slices *(Munday, Parsons & Poat, 1971).* In vivo, it has been shown by *Bolton et al. (1975)* that i.v. injection of Ang. II stimulated fluid transport by rat jejunum. It is thought that many of these actions of Ang. II on transport mechanisms are mediated by noradrenaline *(Brunton, Parsons & Poat, 1978; Levens et al., 1979).*

Ang. II also stimulates sodium and water transport indirectly by promoting the secretion of aldosterone and possibly ADH *(Malvin, 1971).* The role of angiotensin in the control of aldosterone secretion has been reviewed by *Reid & Ganong (1977).* Ang. II mediated aldosterone secretion may not involve cyclic AMP *(Shima, Kawashima & Hirai, 1978)* and is probably linked to changes in calcium fluxes *(Fakundig, Chow & Catt, 1979).*

Intrarenal Effect of Angiotensins

The role of the intrarenal renin-angiotensin system in the control of renal function has recently been reviewed by *Levens, Peach & Carey (1981).* Although angiotensin has been shown to have a direct tubular effect on sodium transport *(Munday et al., 1971)*, some other authors

have suggested that the antinatriuretic and antidiuretic effects of angiotensins in vivo are secondary to renal vasoconstriction and reduced GFR *(Malvin & Vander, 1967; Bonjour & Malvin, 1969; Itskovitz & McGiff, 1974; and Blumberg et al., 1977)*. Furthermore, Ang. II may act in conjunction with renal PGs. The interactions between Ang. II and PGs have been reviewed by *Baer & McGiff (1980)*.

Physiological Actions of Ang. I and Ang. III

Ang. I and Ang. III are potent agonists for some of the functions of the renin-angiotensin system *(Goodfriend & Peach, 1975; Davis & Freeman, 1977)*. The existence of Ang. I as a precursor to Ang. II was first discovered by *Skeggs et al. (1954)*, but the recent development of ACEI has facilitated investigations of the actions of Ang. I. The decapeptide, Ang. I, is equipotent with Ang. II in stimulating the release of adrenaline and noradrenaline from the adrenal medulla *(Peach, 1971)*. This appeared to be a direct effect of Ang. I, since no conversion of Ang. I to Ang. II could be detected. Ang. I may have an important role in the centrally mediated pressor response *(Solomon, Cavero & Buckley, 1974)*. In the rat, Ang. I may induce thirst when administered centrally *(Fitzsimons, 1971)*. Inhibition of ACE had no effect on the response to Ang. I *(Bryant & Flak, 1973; Casner, Goldman & Lehr, 1976)*, but *Casner et al. (1976)* added that there is no certainty that this is truly an Ang. I response because of the difficulty of ensuring complete converting enzyme blockade.

Ang. III appears to be inactive in nervous tissue, but extremely potent in the stimulation of aldosterone secretion from the adrenal cortex *(Peach & Chiu, 1974; Goodfriend & Peach, 1975 and Douglas et al., 1976)*. *Bumpus et al. (1961)* and *Khosla, Smeby & Bumpus (1974)* have reported that Ang. III has about 40% of the pressor activity of Ang. II. *Bravo, Khosla & Bumpus (1976)* have suggested that the heptapeptide, Ang. III, may be formed without prior production of Ang. II and this involves the formation of an intermediate, the nonapeptide, des-Asp'-Ang-I, *(Schmitz, Campbell & Itskovitz, 1976)*.

The actions of the three angiotensins have been reviewed by *Goodfriend & Peach (1977)*.

CHAPTER II

RENIN ASSAY METHODS

CHAPTER II

RENIN ASSAY METHODS

Renin is usually assayed indirectly by its ability to generate angiotensins from endogenous or exogenous substrate. The angiotensins generated were originally assayed biologically using in vitro colon *(Regoli & Vane, 1964)* and rat blood pressure preparations. These bioassays are well documented and have been reviewed by *Boucher & Genest (1974)*.

Goldblatt et al. (1943) injected renin-containing samples into conscious dogs and measured the changes in arterial blood pressure. This method was suitable for assay of kidney extracts, but was not sufficiently sensitive for the assay of plasma renin. However, *Helmer & Judson (1963)* found that the angiotensins generated by renin in plasma samples could be measured by bioassay after an in vitro incubation step. Bioassay techniques are very laborious and several problems arise with this type of assay. They are not now normally used.

In the last 12 years methods have been developed to measure generated angiotensin by radioimmunoassay (RIA) techniques. Although some renin assays have relied on measurement of Ang II *(Poulsen, 1969)*, RIA of generated Ang I is now normally used as the basis for the assay of renin. It is much easier to use inhibitors to block ACE than to guarantee complete conversion of Ang I to Ang II. Converting enzyme inhibition can be achieved by using inhibitors such as 8-hydroxy-quinoline *(Haber et al., 1969)*, EDTA and BAL *(Boyd et al., 1969)* or phenyl-methyl-sulphonyl fluoride (PMSF) *(Eley & Kelly, 1977)*. A high recovery rate for generated Ang I can be obtained. Furthermore, Ang I is the first product of the enzymatic activity of renin and therefore there is no need to use highly sophisticated enzyme kinetic analysis methods to analyse results.

In some laboratories protection of the generated angiotensin is achieved by adding specific antibodies to the incubation mixture and capturing angiotensin as it is formed. There is no need therefore for angiotensinase inhibitors to be added *(Poulsen, 1971)*.

The rate of production of Ang I during incubation of renin samples can be affected by various things. Plasma substrate concentration may be rate limiting *(Gould & Green, 1971; Reid et al., 1978)* and, therefore, renin samples are often incubated in the presence of excess exogenous substrate. The value obtained from this type of assay is called "plasma-renin concentration" (PRC). When endogenous plasma renin substrate is used to generate angiotensin, the value obtained is referred to as "plasma-renin activity" (PRA).

The kinetics of the renin-substrate reaction were reviewed by *Poulsen (1973)*. In the case of PRA, the reaction does not follow zero-order (substrate concentration independent) kinetics, since the rate of Ang I production depends both on the amount of renin and the amount of substrate. Therefore, when substrate levels are reduced, as in liver diseases, or increased, as in pregnancy or contraceptive pill administration (see page 7), assay results for renin may be falsely low or high. PRA therefore, reflects more than one component of the renin-angiotensin system *(see Swales, 1979a)*. Such confusion can be avoided by adding excess exogenous substrate *(Stockigt, Collins & Biglieri, 1971)*. In this case the rate of Ang I production is independent of the substrate concentration, and the reaction follows zero-order kinetics.

There is no general agreement concerning the pH to use for renin assay. *Osmond, McFadzean & Scaiff (1974)* reported marked fluctuations in pH during incubations and added that the pH of incubated plasma may rise to pH 8.0 within a few hours, even in the presence of buffer.

Cohen et al. (1971) reported that the optimum pH for Ang I generation is pH 5.5 - 6.5. Other groups have described the incubation of non-buffered, neat serum *(Boyd et al., 1969)* and serum buffered at pH 7.4 *(Stockigt et al., 1971)*.

In plasma there may also be other factors which interfere with the antigen-antibody reaction. For example, the presence of non-specific interference by immuno-reactive Ang I like substances may complicate the assay for Ang I. Several groups have tried to extract Ang I before assay in order to prevent such non-specific interference. *Boyd et al. (1969)* and *Boyd, Jones & Peart (1972)* used Fullers earth to adsorb angiotensin. *Drayer & Benraad (1975)* and *Lijnen, Amery & Fagard (1978)* added Dowex resin to the incubation mixture at the beginning to protect the angiotensin as it is generated. The extraction of generated angiotensin is unnecessary when a specific antiserum is available as, by including a non-incubated blank, non-specific-interference can be calculated and corrected for *(Stockigt et al., 1971)*.

The principle of RIA is competitive inhibition by unlabelled Ang I of the binding of radioactively labelled Ang I to a specific antibody. The unknown concentration of Ang I in plasma samples is determined from the degree of binding of labelled Ang I in unknown samples compared with that observed in standard solutions which contain known amounts of added unlabelled Ang I. The three reagents needed for RIA of Ang I are:

1) Pure hormone (Ang I) as a standard
2) A radioactively labelled hormone
3) A suitable antibody

Standard Ang I is commercially available. The radioactively labelled Ang I is usually prepared by incorporating I^{125} into the Ang I molecule by the chloramine-T-reaction *(Hunter & Greenwood, 1962)*. Since the

concentration of unlabelled Ang I modifies the binding of labelled Ang I, techniques are therefore required for separating bound and free labelled Ang I. The most commonly used method of separation was described by *Herber et al. (1965)*, in which the free label is adsorbed by dextran-coated charcoal. After centrifugation the supernatant will contain antibody-bound I^{125} Ang I. Good separation has also been reported by using a second antibody raised against the Ang I antibody *(Cohen et al., 1971)*.

Antisera are prepared by conjugating Ang I to rabbit serum albumin *(Goodfriend, Levine & Fassman, 1964)*. Since Ang I is a small molecule (mol. wt., 1280) it is not very antigenic. Antibodies are therefore raised by injecting coupled Ang I, emulsified with Freund's complete adjurant, into rabbits (see page 74 for a full description of antisera preparation).

Other techniques for renin assay, including fluorimetric methods, have been reported by *Galen et al. (1978) and Murakami et al. (1981)*. A synthetic tetradecapeptide substrate has been used but this substrate is not specific for renin alone and can be hydrolyzed by other proteases. The synthetic substrate also cross-reacts with antisera for Ang I.

Direct measurement of renin has had only limited success. The isolation of very pure renin preparations *(Yokosawa, Inagami & Haas, 1978; Yokosawa, 1980)* raised the possibility of producing specific antibodies which would enable direct measurement of renin by RIA. The development of such assays has been reported *(Galen et al., 1979; Malling & Poulsen, 1977)*. However, the antibody preparations also bind high mol. wt., inactive forms of renin (see page17). As a consequence it may therefore prove difficult to identify renin from different tissues and to distinguish between active and inactive forms of renin.

The following sections in this chapter describe the evaluation of a conventional radioimmunoassay method for PRC using commercially available I^{125} Ang I and laboratory raised antiserum. This RIA method measures Ang I generated from Sheep substrate (see page 84).

Inactive forms of renin must be converted to the active form before they can be assayed. Several methods have been reported for in vitro activation of inactive renin (see page 23). Acid activation was used routinely for the experiments described in this thesis although some work on tryptic activation of inactive renin is also described in Chapter VIII. Measurement of active and inactive renin will be discussed as follows:

1) RIA of Ang I

2) Generation of Ang I

3) Activation of inactive renin

1) Radioimmunoassay (RIA) of Ang I

The basic principles of the RIA of peptide hormones, particularly insulin, were established by *Berson & Yalow (1959, 1960)*. These principles have been applied to the deter-ination of Ang I *(Stockigt et al., 1971)*. RIA is based on the principle that the binding of I^{125} Ang I tracer to a specific antiserum is modified by the presence of unlabelled Ang I (either standard Ang I or Ang I in the unknown samples). In order to determine the concentration of Ang I in unknown samples, a standard curve is first prepared. Known amounts of standard Ang I are added to assay tubes containing diluted antiserum and tracer and the proportion of label which is bound at known concentrations of standard Ang I is then calculated. From the standard curve, the concentration of Ang I in unknown samples can be derived.

Protocol for RIA of Ang I

All solutions for the assay were prepared in 0.05 m Tris-HCI buffer (pH 7.4) containing 0.36% w/v. bovine serum albumin (Fraction V, RIA grade, Sigma) and 0.2% w/v neomycin sulphate (Sigma). Ang I standard (as 5-iso leucine Ang I diacetate) was obtained from Bachem. Ang I tracer (5-iso leucine Ang I, iodinated with I^{125} on tyrosine at position 4) was obtained from CNTS, Orsay, France, and had a minimum specific activity of 1 mCi/µg Ang I.

The antiserum to Ang I was prepared as described on page 74

Assays were prepared in a cold tray at 4°C using plastic LP3 tubes since Ang I is adsorbed onto glass. Assay tubes were allowed to equilibrate for 18 h at 4°C. For the standard curve the tubes contained the following:

Standard Ang I	250 µl
Diluted antiserum	200 µl
Labelled Ang I	50 µl (approx. 12000 cpm)

In order to assay unknown samples, Ang I standard was replaced with 50 µl of unknown samples and 200 µl of Tris-HCI buffer.

The next step was the separation of bound and free labelled Ang I. This was achieved by adding to each tube 1 ml of Tris-HCI buffer containing 1% dextran (Grade C, BDH) and 1% charcoal (Norit GSX, Hopkin & Williams). The tubes were then vortex mixed and centrifuged. Bound tracer was determined by counting 0.5 ml of the supernatant for 2 minutes in a Beckman Biogamma Counter. In order to minimise pipetting errors each estimation was done in triplicate.

Preparation of Antiserum

Antisera for Ang I were prepared as follows:

Ang I (I leu 5) diacetate was first conjugated to rabbit serum albumin *(Goodfriend et al., 1964)*. Ang I (4 mg) was dissolved in 2 ml sterile distilled water containing 0.2% neomycin sulphate (Sigma). Then, 60 mg 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide (Emanuel) and 2 mg rabbit serum albumin (Fraction V Powder, Sigma) were added. The mixture was incubated for 2 hr at room temperature and dialysed against 4 changes of 2 l distilled water containing 0.2% neomycin over 48 hrs at 4°C. The conjugated Ang I was diluted with 0.9% sterile saline to a total volume of 8 ml and stored at -20°C.

An aliquot (1.5 ml) of conjugated Ang I solution was emulsified with an equal volume of Freund's complete adjuvant (DIFCO) and 1 ml of the emulsion was given by multi-site intradermal injection on the back of each of three sandy-lop rabbits. After 4 and 8 weeks, boosting doses of the immunogen emulsion (1 ml) were given subcutaneously to each of the three rabbits. After 12 weeks, the rabbits were exsanguinated following cannulation of the abdominal aorta under thiopentone anaesthesia. About 100 - 120 ml of blood was collected from each rabbit.

Testing of antisera

After separating the sera, the affinity and specificity of antisera for Ang I were determined. Serial dilutions of the antisera were made in a range between 1:100 and 1:25,600, using 50 mM Tris-HCI buffer (ph 7.4) containing 0.36% w/v. bovine serum albumin and 0.2% w/v neomycin. Standard amounts (50 µl) of labelled Ang I (approx. 12,000 cpm) were added to the diluted antisera so that the final volume in each tube was 0.5 ml. The samples were then incubated

overnight at 4°C. Bound and free labelled Ang I were separated by adding 1 ml of dextran/charcoal solution to each tube (see page 73). Supernatant (0.5 ml) containing the bound label, was counted on a Beckman gamma counter for 2 min and the total percentage of labelled Ang I bound was calculated.

It can be seen from Table 2.1 that antisera I and III bound just over 55 and 50% respectively of added labelled Ang I at a dilution of 1:200. This percentage bound dropped to just 12 and 18% at a dilution of 1 in 25,600. On the other hand, antiserum II bound over 88% of added label at a dilution of 1:2000 (Table 2.2). A dilution curve from 1:1000 to 1:25,600 for antiserum II was therefore prepared (Table 2.2).

Figure 2.1 shows the dilution curves for all three antisera in which the values are presented graphically by plotting the percentage of label bound against the logarithm of the dilution factor.

A wide range of standard curves for antiserum II was constructed. Fixed amounts of labelled Ang I were added to five dilutions of (Table 2.3) antiserum II (1:2000, 1:3000, 1:6000, 1:12000 and 1:24000), in the presence of Ang I in a range of concentrations between 5 and 2500 pg. The final volume of the mixture in each tube was 500 µl. The antiserum dilution quoted is with respect to the final volume. The tubes contained the following:

Standard Ang I	250 µl
Antiserum	200 µl
Labelled Ang I	50 µl (approx. 12,000 cpm)

Table 2.3 shows results for these five dilutions of antiserum II and results are also expressed graphically in Figure 2.2. The percentage label bound is plotted against the logarithm of the amount of Ang I. From Table 2.3 and Figure 2.2, it can be clearly seen that

Dilution Factor	Percentage Label Bound	
	R. No. I	R. No. III
100	57.6	60.1
200	55.7	50.4
400	50.9	48.2
800	44.4	45.8
1600	40.3	35.6
3200	20.0	32.8
6400	18.9	22.9
12800	13.5	21.4
25600	12.1	18.4

TABLE 2.1

Antibody Dilution Curves - Antisera I and III

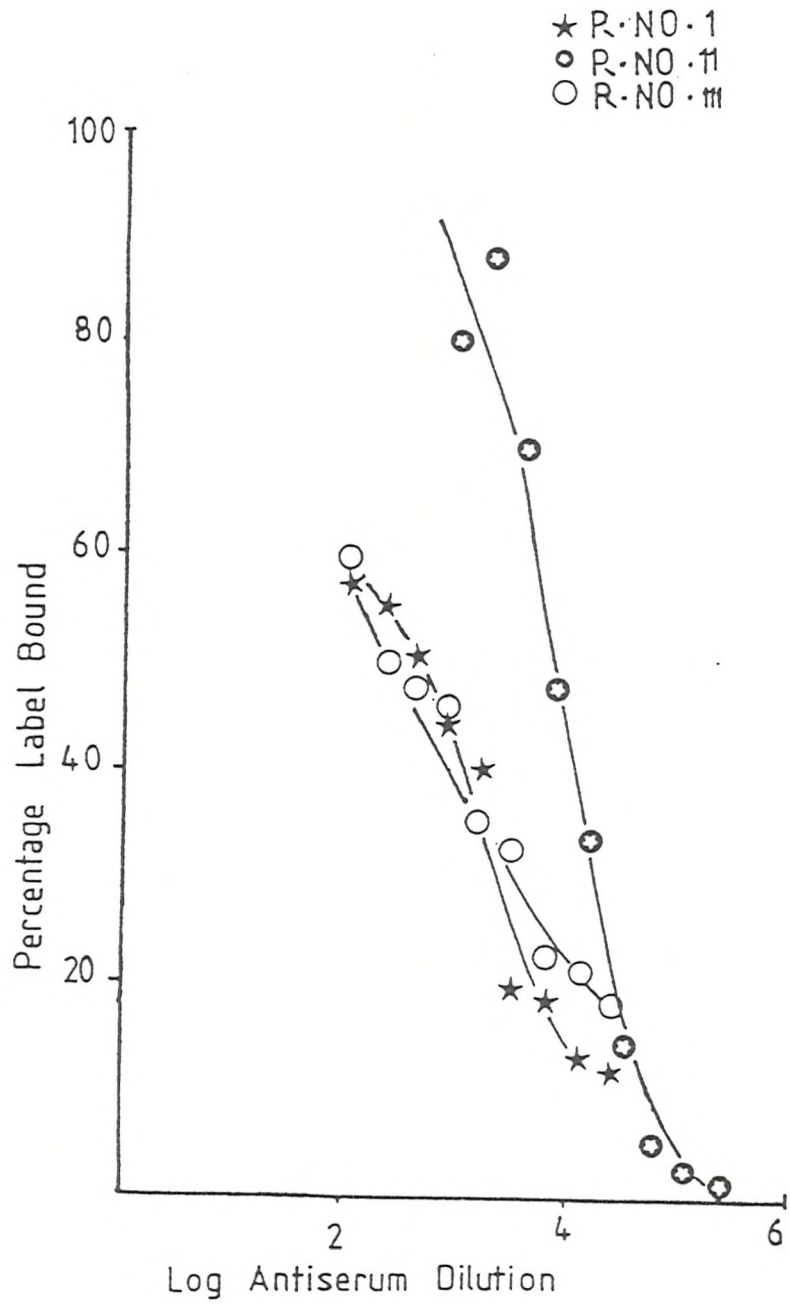
Dilution Factor	Percentage Label Bound
	R. No. II
1000	80.4
2000	88.6
4000	70.3
8000	48.1
16000	33.8
32000	14.5
64000	5.6
128000	3.2
256000	2.1

TABLE 2.2

Antibody Dilution Curve - Antiserum II.

FIG. 2.1

DILUTION CURVE OF THREE ANTISERA (I, II, III)



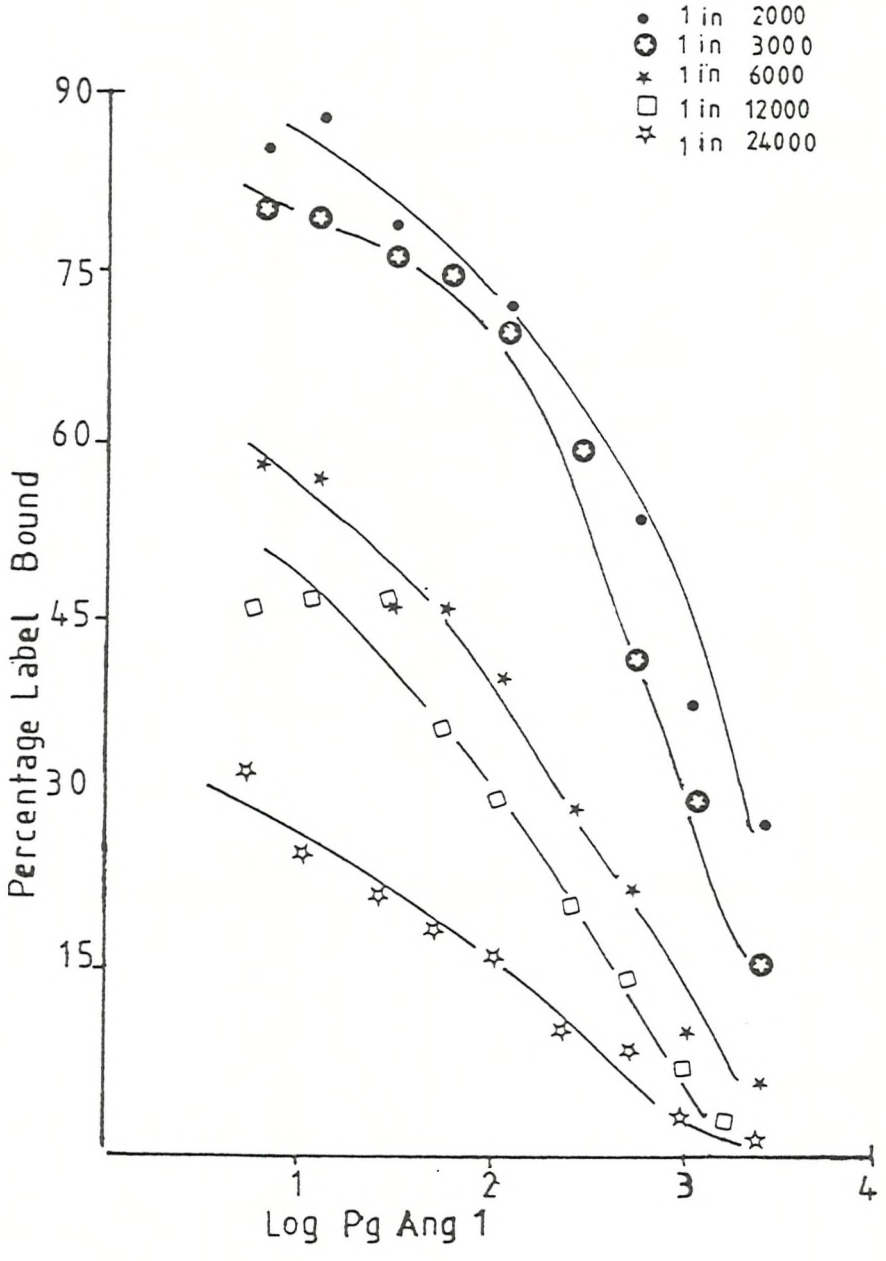
Angiotensin I	Percentage Label Bound				
	Antiserum Dilution				
	2000	3000	6000	12000	24000
5 pg	85	80	58	46	32
10 pg	88	80	57	47	25
25 pg	79	76	46	47	22
50 pg	72	75	46	36	19
100 pg	72	70	40	30	17
250 pg	61	60	29	21	10
500 pg	54	42	22	15	9
1000 pg	38	30	10	7	3
2500 pg	28	16	6	5	1

TABLE 2.3

Standard Curves in Dilutions of Antiserum II.

FIG. 2.2

STANDARD CURVES AT FIVE DILUTIONS OF ANTISERUM II



the maximum binding falls as the antiserum becomes more dilute. It can also been seen that, as expected, the slope of the standard curves for antiserum II over the range 5 to 2500 pg Ang I differs according to the dilution of the antibody.

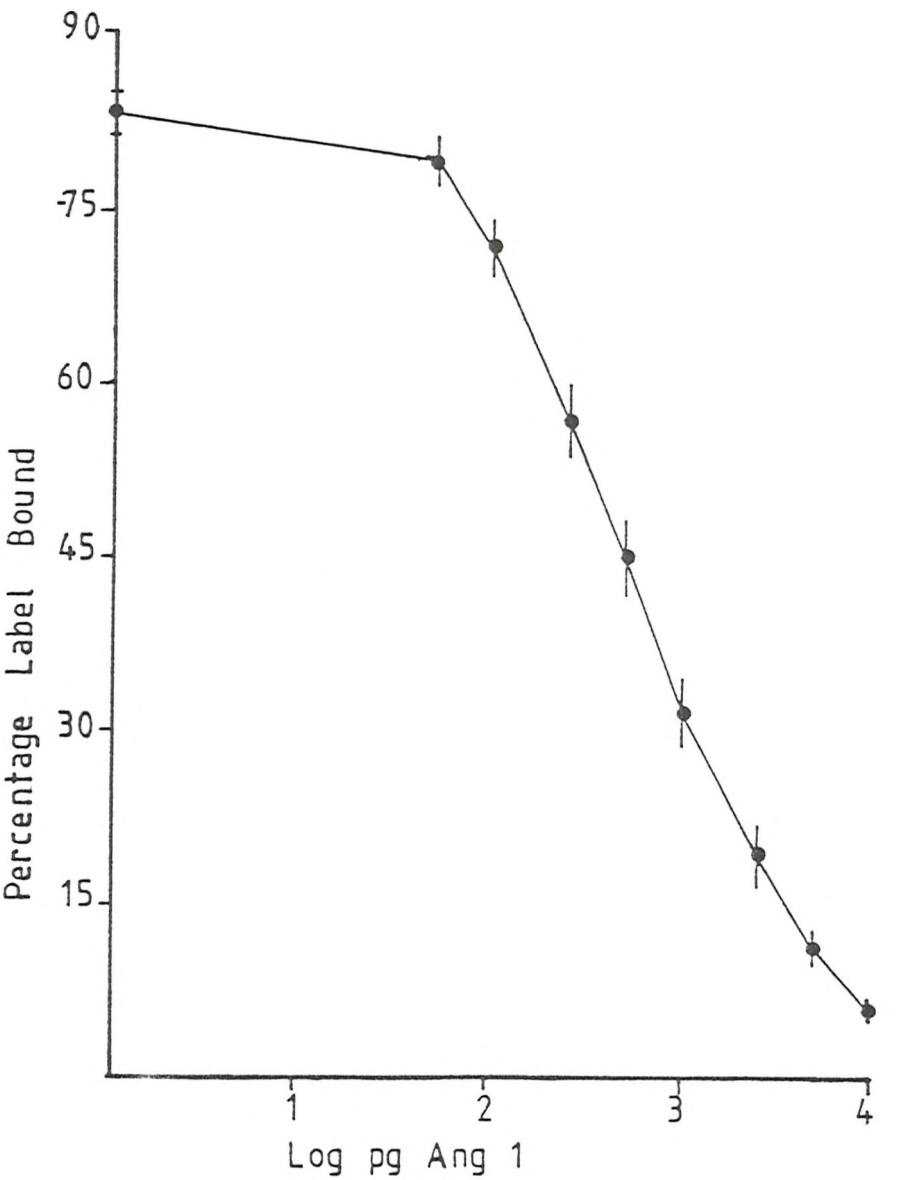
For any usable RIA the standard curve must be steep and linear over the whole range of *[Ang I]* used. However, absolute linearity is usually impossible to obtain, since antisera contain immunoglobulins with a range of binding affinities. However, usable linearity for a reasonably wide range of *[Ang I]* is possible. Table 2.3 and Fig. 2.2 show that the slope of the most important part of the curve (100 to 2500 pg) was steep and linear using antiserum II in a dilution of 1:3000. This dilution was selected for the assays of unknown samples for all the experimental work described in this thesis.

From the pooled data for 16 individual assays (Fig. 2.3), antiserum II, in a dilution of 1 in 3000, gave a maximal binding of 79.1 ± 1.9% in the presence of 50 pg Ang I. This fell to 5.8 ± 0.9% in the presence of 10000 pg Ang I. This slope and range was regarded as quite satisfactory for routine use.

The specificity of the antiserum was checked. Cross-reaction with several peptides including Ang II, III and saralasin was negligible (Table 2.4). With synthetic tetradecaptide renin substrate cross reactivity was found to be 16.1%. This did not pose a problem as only natural renin substrate was to be used for renin measurement. Furthermore, the cross-reaction of antiserum II with natural renin substrate was negligible in the presence of 50 µl of 1:1 natural renin substrate/inhibitor solution (Table 2.4). Antiserum II did cross-react with partially purified sheep renin which had been prepared by Dr. D. Lush. This preparation had a renin activity of 2.75×10^6 ng Ang I/ml. hr. as determined by RIA. The cross-reaction was found to be about 10%.

FIG. 2.3

CUMULATIVE STANDARD CURVE FROM 16 ASSAYS



Peptide	% Cross-Reaction
Ang. II	2.2
Ang. III	1.7
Saralasin	2.8
Synthetic Renin Substrate (Tetradecapeptide)	16.1
Natural Renin Substrate	0.35

TABLE 2.4

The Cross-reaction of Antiserum II with Different Peptides

However, this renin activity is about 3 x 10^5 times higher than the plasma renin activity of normal sheep and the cross reaction is irrelevant to the work described in this thesis.

To conclude this section, it appears that the RIA for Ang I as described here was quite satisfactory for the measurement of Ang I in the work described in the following chapters of this thesis.

2) Incubation of renin samples with substrate to generate Ang I:

Renin substrate preparation

The sheep renin substrate used for the experimental work of this thesis was prepared in this laboratory using methods described by *Skinner (1967)*. Under thiopentone, halothane/nitrous oxide anaesthesia sheep were subjected to bilateral nephrectomy. Six days later the animals were exsanguinated and blood was collected into chilled containers which contained 2 mg/ml EDTA - Na_2 as anticoagulant. Plasma was separated by centrifugation and then dialysed at 4°C for 48 hr against isotonic citric acid/phosphate buffer (pH 4.5). Ths plasma was heated to 32°C for 45 min and then dialysed at 4°C for 48 hr against isotonic phosphate/saline buffer (pH 7.5). Before use, the substrate preparations were checked for endogenous renin activity and angiotensinase activity. If the endogenous renin activity was less than 0.05 ng Ang I/ml/24 hr incubation at 37°C and Ang I(50-500 ng/ml) recovery values more than 85% during 24 hr incubation at 37°C, the renin substrate preparations were considered acceptable. Trasylol (FBA) (100 U/ml) and neomycin sulphate (0.2%) were added to the substrate preparation before storage at -20°C.

In order to avoid any possible changes in renin activity as a result of using different substrate preparations, only one substrate preparation was used for the assay of samples from one particular

set of experiments.

As the assay is based on measurement of *in vitro* generated Ang I it is essential to prevent the breakdown of Ang I, once formed, either by ACE or by non-specific proteases. This was achieved by incubating the samples in a solution containing converting enzyme and angiotensinase inhibitors. The inhibitor solution, which was prepared in 0.1 M phosphate buffer (pH 7.0), contained the following:

0.1 m-mol/l dimercaprol (Sigma)

2.68 m-mol/l EDTA-Na_2 (BDH)

3.44 m-mol/l 8-hydroxyquinoline (BDH)

22.0 m-mol/l neomycin sulphate (Sigma).

In order to make Ang I production substrate concentration independent (zero order kinetics) and to minimise changes in Ang I production due to differences in the amount of endogenous substrate present, the unknown renin samples were incubated in the presence of exogenous sheep renin substrate. The substrate and inhibitor solution were mixed in a ratio of 1:1. Aliquots (500µl) of this mixture were transferred to assay tubes and 50 µl of the unknown sample was added. Duplicates were prepared, one tube being stored at -20°C (non-incubated blank), the other being incubated at 37°C for 3 hr. After the incubation, samples were assayed for Ang I.

Characteristics of renin-substrate reaction

A standard, partially purified, sheep renin preparation produced by Dr. D. Lush in our laboratory was used. The renin was prepared using methods based on those described by *Deodhar, Haas & Goldblatt (1964)* and *Haas et al. (1972)*. Since the partial purification of renin included an acidification step, only active renin should be present.

During incubation of unknown renin samples with the substrate/inhibitor mixture, the rate of Ang I production should be directly related to renin activity. This was investigated as following:

a) Time-course of Ang I production

Aliquots (50 µl) of $\frac{1}{1000}$ and $\frac{1}{2000}$ dilution of partially purified sheep renin were incubated at 37°C for 0,2,4 and 8 h with 500 µl of substrate/inhibitor mixture (1:1). The samples were then assayed for Ang I content. Figure 2.4 shows the results. The correlation coefficients (r^2) were found to be 0.99 ($P < 0.05$) for $\frac{1}{1000}$ and 0.98 ($P < 0.05$) for $\frac{1}{2000}$ dilutions. These results indicate that Ang I production is directly related to the length of the incubation periods.

b) Ang I production and renin concentration

From the sheep renin preparation, serial dilutions were made and aliquots (50 µl) of each were incubated with sheep renin substrate/inhibitor mixture for 1 hr at 37°C. Generated Ang I was then measured. Results in Fig. 2.5 demonstrate that Ang I production was linear (r^2 = 0.97, $P < 0.05$) and directly related to the concentration of renin present.

c) Effect of sheep renin substrate on Ang I production rate

An equal volume of the inhibitor solution was added to serial dilutions of sheep renin substrate. Aliquots (50 µl) of $\frac{1}{1000}$ dilution of the sheep renin preparation were then incubated for 1 hr at 37°C with 500 µl aliquots of the diluted substrate/inhibitor mixture and assayed for Ang I. Fig. 2.6 shows that the rate of Ang I production was *[substrate]* independent until the dilution of substrate was greater than 1 in 8. In relation to the renin activity found in plasma samples, this suggests that the amount of substrate added to

FIG. 2.4

EFFECT OF INCUBATION TIME ON ANG I CONTENT (N = 4)

Sheep Renin Dilution: $a = \frac{1}{1000}$, $b = \frac{1}{2000}$

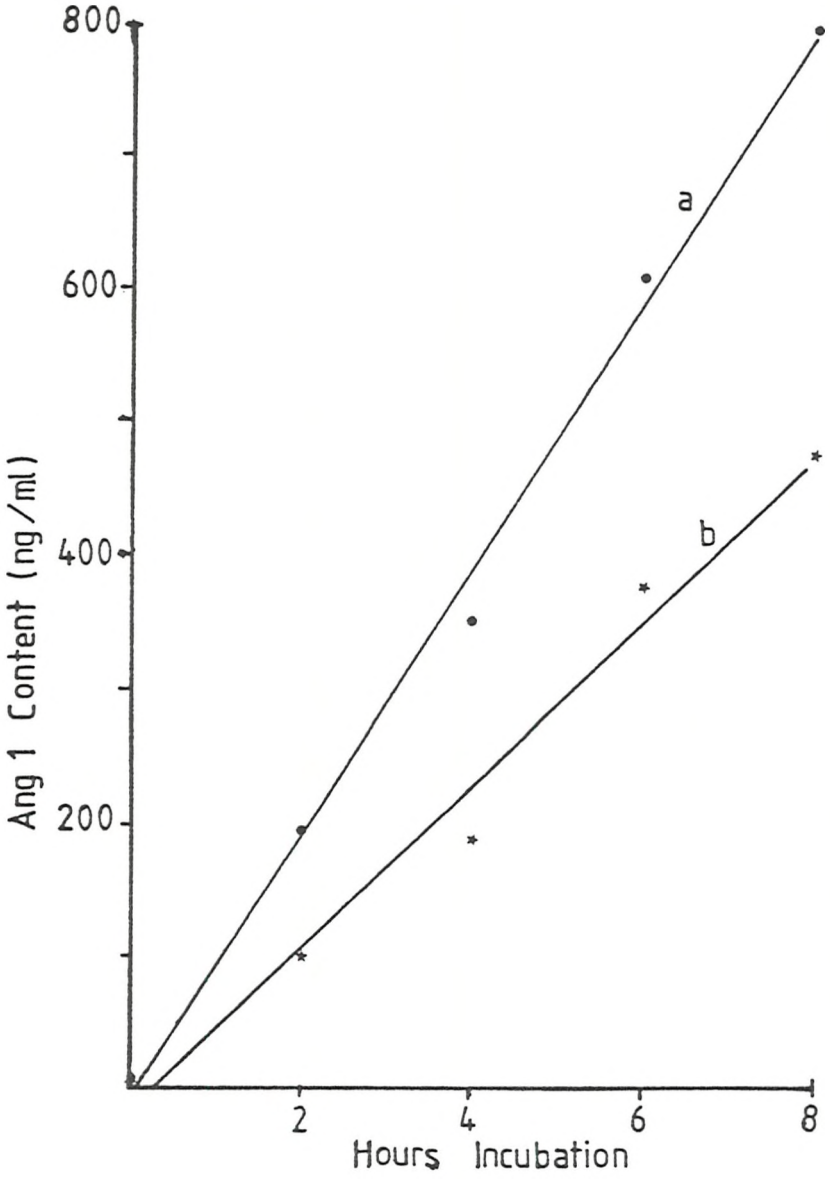


FIG. 2.5

EFFECT OF RENIN DILUTION ON ANG I PRODUCTION RATE (N = 5)

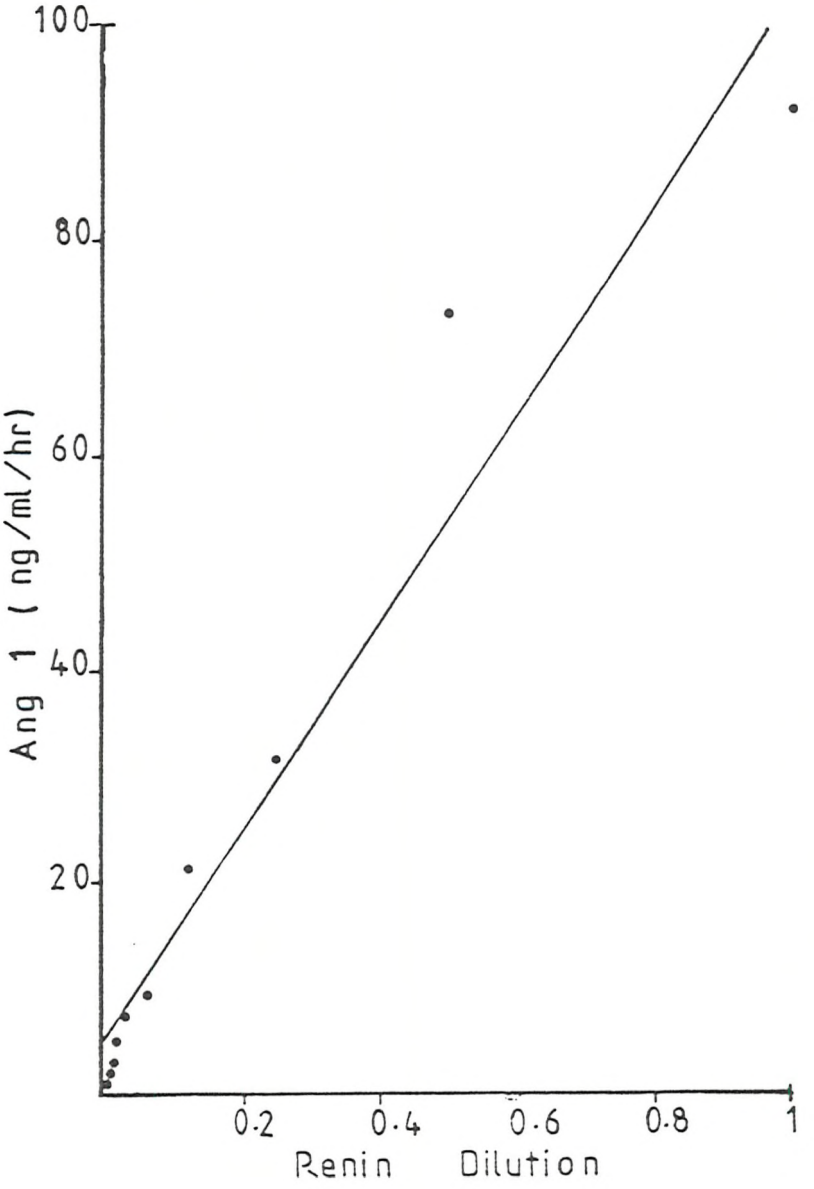
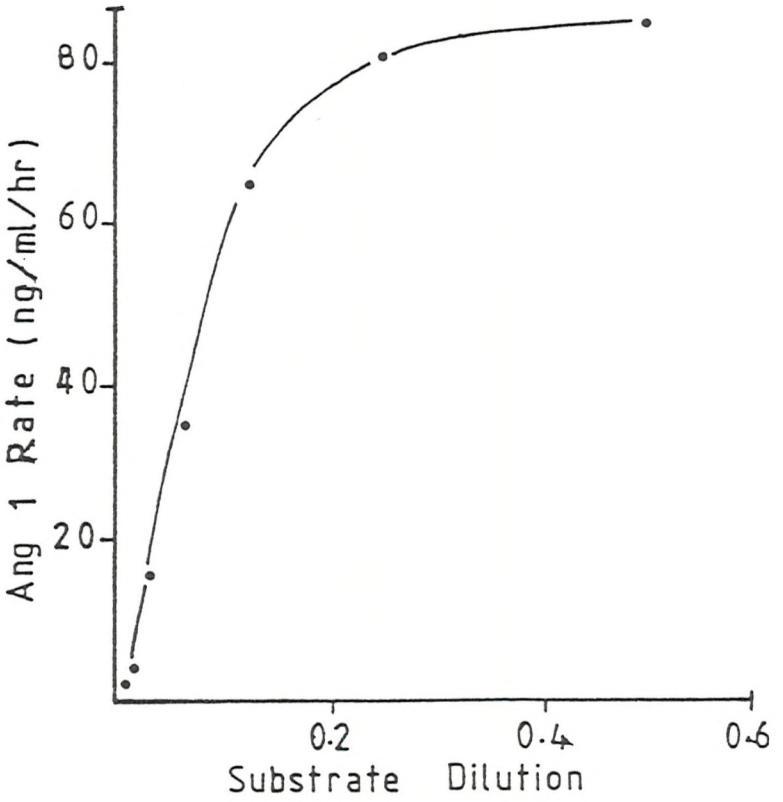


FIG. 2.6

EFFECT OF SUBSTRATE DILUTION ON ANG I PRODUCTION RATE (N =5)



plasma samples constituted a considerable excess. It is very unlikely that substrate concentrations become rate limiting for plasma renin estimations. This is important as zero order kinetic conditions were assumed when calculating the results.

When a Hill plot was applied to the results by plotting log *[substrate]* against log $(\frac{v}{V-v})$ (Fig. 2.7), a linear relationship was obtained. This indicates that the two components are directly related to each other and that Ang I production was a result of the reaction between the renin and its substrate since the rate of Ang I production was decreased as *[substrate]* decreased.

From the results described in this section, it was clearly indicated that Ang I production was directly related to the incubation period and the amount of renin present, but independent of substrate concentration in the presence of excess exogenous substrate.

3) Activation of inactive renin

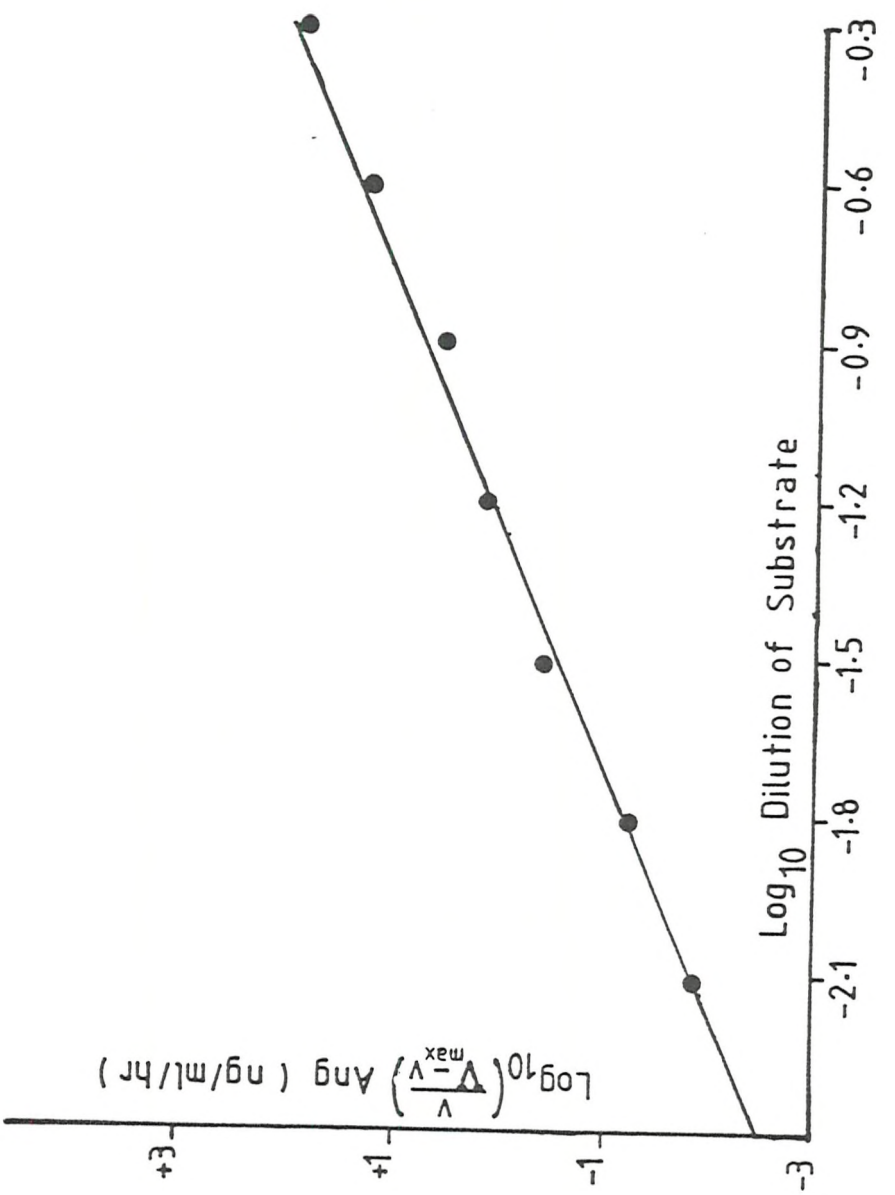
Inactive renin can only be measured after conversion to the enzymatically active form. Several techniques have been used for this purpose (see page 23).

The method chosen for routine use for the experiments described in this thesis is the most widely used method for activating inactive renin, acid-activation. It involves dialysis of plasma samples against a low pH buffer. One half of each plasma sample was dialysed to pH 3.3 over 24 hr at 4°C against glycine/HCl (170 mM) buffer and then dialysed to a pH 7.4 over a further 24 hr period against a phosphate buffer (175 mM). The other half of each sample was dialysed at pH 7.4 for 48 hr at 4°C.

After incubation with substrate and RIA of generated Ang I, acidified aliquots will yield a value for active plus activated renin

FIG. 2.7

APPLICATION OF HILL PLOT FOR THE EFFECT OF SUBSTRATE DILUTION ON ANG I PRODUCTION RATE (N = 5)



whilst non-acidified aliquots will be assayed for active renin only. The dialysis procedure is summarized in Figure 2.8.

pH Dependence of activation

Different groups of researchers have used different pHs for acid activation of inactive renin. For example, *James & Hall (1974)* used pH 4.5 for dog plasma. *Derkx et al. (1976)* and *Boyd (1977)* used pH 3.3 and *Leckie, McConnell & Jordan (1977a)* pH 3.0 for human plasma. *Richards et al. (1981)* and *Ginesi et al. (1983)* used pH 2.8 to activate rabbit plasma inactive renin.

The optimum pH for activation of sheep inactive renin was investigated. Plasma samples were collected from five different animals and aliquots were each dialysed for 24 hr at 4°C against one of a series of ten 170 mM glycine/Hcl or citric acid/phosphate buffer- which covered the pH range 1.6 - 7.4 phosphate buffer. Renin concentrations were measured by RIA of Ang I after incubation with sheep renin substrate.

From Figure 2.9 it can be clearly seen that the maximum increase in renin activity occurred when plasma was dialysed to pH 3.3 and this was therefore adopted for the routine activation of inactive renin for the experimental work described in this thesis.

There is a possibility that the increase in plasma renin activity after acidification might be related to factors other than the activation of inactive renin. To exclude such a possibility the activation method was evaluated in the following experiments.

a) The effect of dialysis procedure on renin recovery

It was conceivable that the dialysis procedure resulted in some loss of renin activity. If this loss was greater in non-acidified samples, this could explain the apparent increase in renin activity

FIG. 2.8

DIALYSIS PROCEDURE

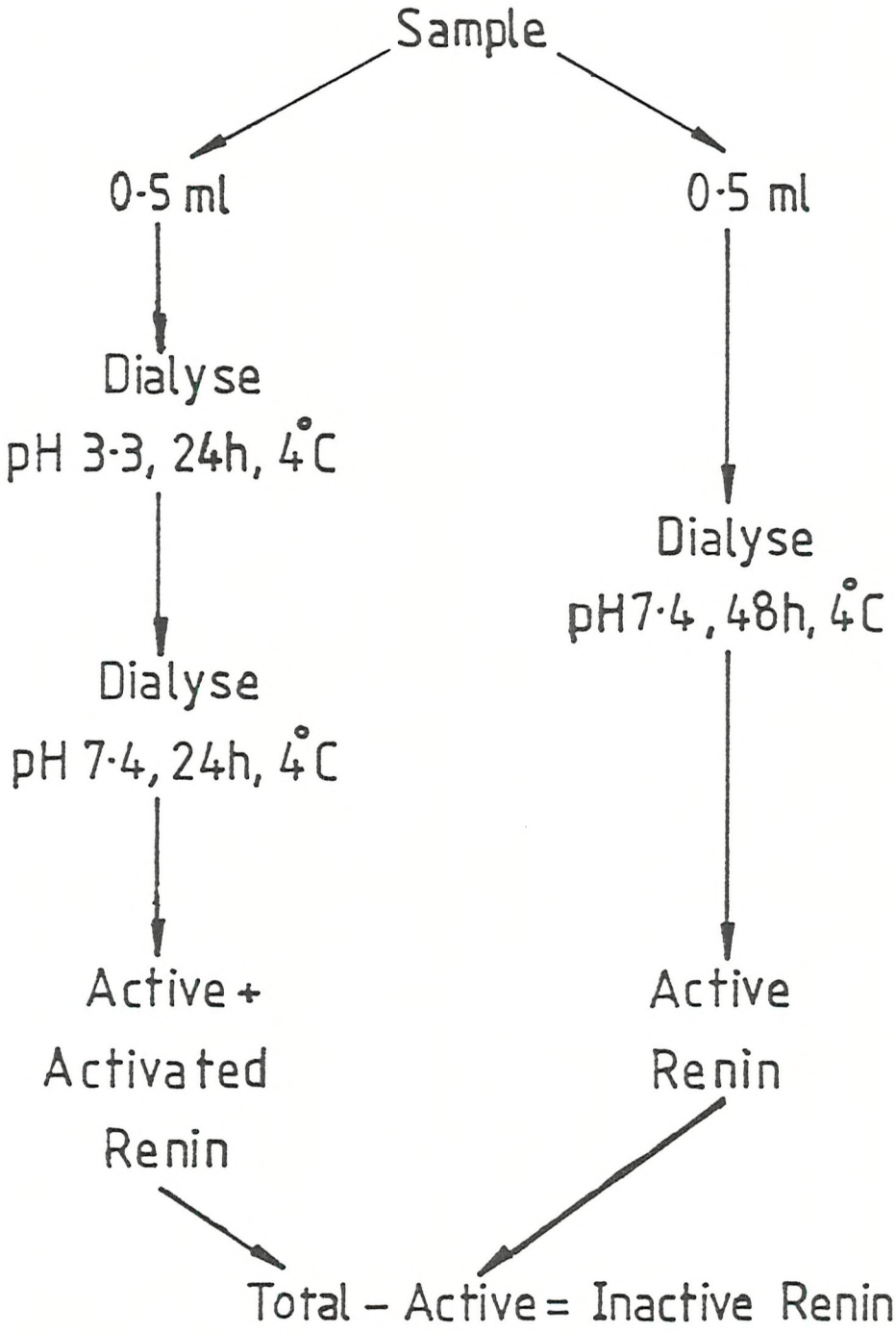
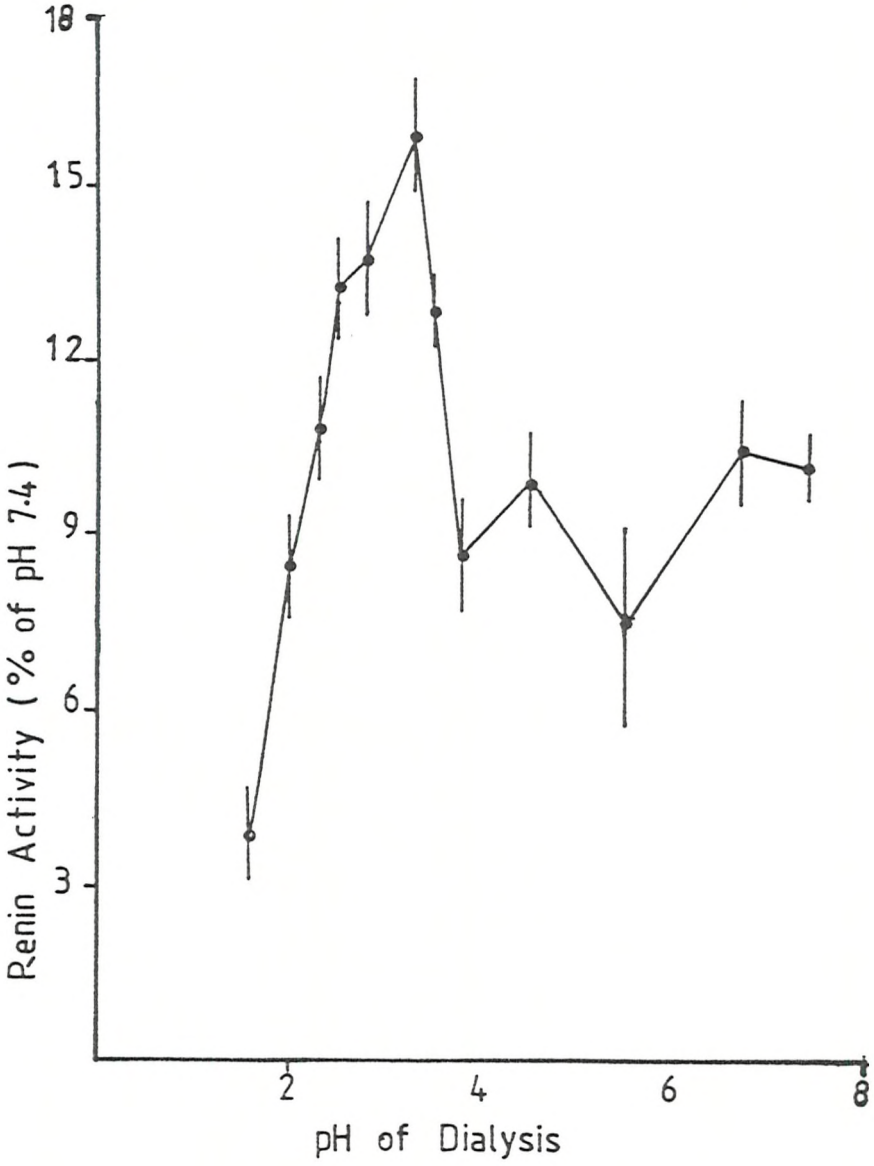


FIG. 2.9

EFFECT OF INITIAL DIALYSIS PH ON SHEEP PLASMA RENIN ACTIVITY (N = 5)



after acidification. Therefore, the effect of the dialysis protocol on the recovery of exogenous renin added to plasma samples was investigated. Plasma samples were collected from 5 different sheep and divided into two halves. Aliquots (50 µl) of a 1:1000 dilution of the sheep renin preparation previously described (see page 85) were added to one half of the plasma samples and they were then treated as follows:

i) Frozen at -20°C for 48 hr Or

ii) Dialysed to pH 3.3 for 24 hr and then dialysed to pH 7.4 for further 24 hr Or

iii) Just subjected to pH 7.4 dialysis for 48 hr.

The remaining halves of the original samples, to which no renin had been added, were subjected to the same procedures. The amount of renin which had been added to the first half of the samples could therefore be determined. Renin recovery was calculated by taking the difference in renin concentration of the samples which had been stored at -20°C (assuming no loss of added renin) as 100%. Comparison of groups (i) and (ii) showed a 13.2 $\pm$ 2.4% loss in renin activity during dialysis to pH 3.3. Comparison of groups (i) and (iii) showed a loss of 11.8 $\pm$ 2.1% during dialysis to pH 7.4. Since a similar loss in renin activity occurred during both dialysis procedures, the increase in renin activity following acidification cannot be attributed to differential loss of renin during dialysis.

b) Effect of the dialysis procedure on plasma antiotensinase activity

The possibility existed that destruction of angiotensinases in plasma provided the basis for the rise in renin activity during acid dialysis. This was investigated as follows:

Aliquots (2 ml) from each of five pools of sheep plasma were first dialysed either to pH 7.4 for 48 hr at 4°C or to pH 3.3 for 24 hr at 4°C and then to pH 7.4 for a further 24 hr at 4°C. qliquots (50 µl) from those dialysed samples were incubated at 37°C for 8 hr with 500 µl of either the substrate/inhibitor mixture to which 25 ng/ml Ang I standard was added (A), or the substrate/ inhibitor mixture alone (B). Subtracting the Ang I content of B from that of A gave the amount of added Ang I remaining after the incubation. This remaining Ang I was expressed as a percentage of the amount initially present (25 ng/ml). This was the percentage recovery. It was found that after the incubation of plasma samples which had been dialysed to pH 7.4, the mean recovery of added Ang I was 84.5 ±3.4%. For those samples which were dialysed to pH 3.3 and pH 7.4 mean recovery was 88.1 ± 3.7%. Since the results were not significantly different, it was suggested that the increase in renin activity observed after acid dialysis was not related to the reduction in angiotensinase activity.

Effect of the dialysis procedure on endogenous substrate

Changes in endogenous renin substrate during the two dialysis procedures would in any case have only a very small effect on the final measurement of plasma renin concentration as dialysed samples were incubated with an excess of exogenous substrate (see page 84). Nevertheless, changes in endogenous substrate concentration were investigated in the following way:

Aliquots of plasma samples from five sheep were divided into three groups. The first two groups of samples were either dialysed to pH 3.3 for 24 hr and pH 7.4 for a further 24 hr at 4°C or dialysed

to pH 7.4 only for 48 hr at 4°C. The third group of samples was stored at -20°C. Aliquots (50 µl) of all groups of samples were incubated for 8 hr at 37°C with 500 µl of inhibitor/buffer mixture (1:1) containing excess exogenous renin (25 µl of 1:10 dilution of sheep renin preparation). Therefore, only endogenous substrate was available to generate Ang I and complete utilisation of substrate was ensured by the presence of excess renin.

The rate of production of Ang I from the plasma which had been stored at -20°C was 778.6 ± 66.5 ng/ml, whereas equivalent data for plasma which had been dialysed to pH 3.3 was 65.4 ± 4.6 ng Ang I/ml. This was significantly less ($P < 0.001$) than Ang I generated by samples stored at -20°C. These results indicate that endogenous renin substrate in sheep plasma is destroyed by acidification. The amount of Ang I generated from the plasma which had been dialysed to pH 7.4 was 575.6 ± 37.6 ng Ang I/ml. This was reduced significantly ($P < 0.05$) compared with frozen samples, but was significantly greate- ($P < 0.001$) than for the acidified samples.

These results indicate that endogenous substrate decreased significantly during dialysis, but this decrease was found to be much greater in samples subjected to acid dialysis. Therefore, the increase in renin activity observed after acid dialysis cannot be related to the changes in endogenous substrate.

Coefficient of replicate variation for renin assays

From triplicate estimations of 25 separate assays of plasma samples, the coefficient of replicate variation (mean ± S.E. of the standard deviation of replicates was calculated, i.e. coefficient of variation = (standard deviation ÷ mean) x 100. This was found to be 4.2 ± 0.7%

for the acidified samples and 6.0 ± 0.7% for non-acidified samples. Since the values of this coefficient were low this indicates good reproducability for the assays as a whole and provides further evidence to support the conclusion that the renin assay methods employed were sufficiently accurate for use in the experimental work described in this thesis.

The coefficient of replicate variation for assays reported here is very similar to data obtained by other people working in our laboratory. Coefficient of 4.6 ± 0.9% for active renin and 5.9 ± 1.4% for total sheep renin was reported by *Lush et al. (1983)*. A coefficient of 4.0% for rabbit active renin and 5.2% for total renin was reported by *Richards et al. (1981a)*. This data can be compared with a coefficient of variation of 9.4% for human inactive renin published by *Sealey et al. (1979b)*.

CHAPTER III

MATERIALS AND METHODS

CHAPTER III

MATERIAL AND METHOD

Introduction

Conscious, chronically catheterised volume replete sheep were used as experimental models for the in vivo studies described in this thesis. The reasons why such an animal model was chosen are as follows:

1) It is desirable to use an animal preparation free from the side effect of anaesthetic agents. Many general anaesthetics are known to alter tissue and plasma concentrations of cyclic nucleotides and neurohumoral substances such as catecholamines *(Altura, 1980)*. Furthermore, anaesthetic agents may play an important role in determining the response of renin secretion mechanisms to experimental stimuli *(Pettinger, 1978; Keeton & Campbell, 1981)*. (See page 60).

2) This animal model allowed urine to be collected continuously via a balloon catheter sutured into the bladder and exteriorised through the abdominal wall. There was no difficulty in ensuring complete voiding of the bladder and dead space volume was minimal. Collection of blood samples, infusion of drugs and monitoring of blood pressure were achieved using the implanted femoral artery and vein cannulae while the sheep were conscious and standing unrestrained in a pen.

3) Since we managed to keep the catheters patent for up to about 5 weeks, several experiments were performed on each animal. They also acted as their own controls. A three day recovery period was left between experiments.

4) The large body size of the sheep allowed serial collection of blood samples without interference of a renin response to haemorrhage.

5) Sheep will stand unrestrained in a pen throughout a day and do not need to be trained to remain quiet. Unlike dogs, they do not chew their cannulae.

Surgical Preparation

Sheep (28 - 48 kg body weight) of either sex were maintained on a diet of hay and concentrates with tap water ad libitum. Prior to surgery, sheep were starved for 48 hours to help prevent regurgitation of rumen and stomach contents during anaesthesia. Anaesthesia was induced with sodium thiopentone (May & Baker) given i.v. via the jugular vein in a 5% solution. Most sheep required between 0.5 - 1.0 g thiopentone for satisfactory induction. After intubation anaesthesia was maintained with 0.5 - 1.0% halothane (I.C.I.) in N_2O/O_2 (2:1) carrier gas.

Under sterile conditions, the bladder was exposed using a supra-pubic abdominal incision and a 16 FG Foley balloon catheter (Warne Surgical Products Ltd.) inserted into the bladder through a small incision on the ventral aspect. The edges of the incision and the surrounding portion of the bladder wall were sutured to provide a firm collar around the catheter. The balloon at the tip of the catheter was then inflated. The urethral canal was tied off (below the bladder in females and half-way down the visible external urethral passage in males) in order to ensure that the catheter was the only possible exit for urine.

The right femoral artery and vein were cannulated with 2m lengths of Tygon tubing (formulation S54-HL: 3.2 mm O.d. x 1.6 mm i.d.). A 15 cm length of tubing was inserted approximately 10 cm from the iliac bifurcation. The ends of the cannulae were therefore in the aorta or inferior vena cava but below the level of the renal blood vessels. Cannulae were secured in place with seven silk (size 0) ties placed around the vessel. After tightening the ties around the cannulae, starting from the distal end, all the silk threads were tied together. The free ends of the cannulae were led to the back of the animal, exteriorized between the scapulae and sealed with stainless-steel plugs. Cannulae were flushed daily with sterile saline and refilled with heparinized saline (100 u/ml). This methodology is based on techniques developed for use in dogs at the Cleveland Clinic, U.S.A., by Dr. C. M. Ferrario. In the sheep cannulae remained patent for up to 5 weeks.

Experimental Protocol

At least three days were allowed between the preparative surgery and subsequent experiments. Only one experiment was performed on each animal in any three-day period and results were always compared with data from control experiments performed in the same animals on a different day. Experimental studies and controls were carried out in randomized order.

During experiments in which sheep were studied while conscious, the animals were minimally restrained in a pen with free access to food and water. The bladder catheter was connected to a wide bore tube leading outside the pen to enable collection of urine (see Fig.3.1). The femoral artery cannula was used to record blood pressure continuously

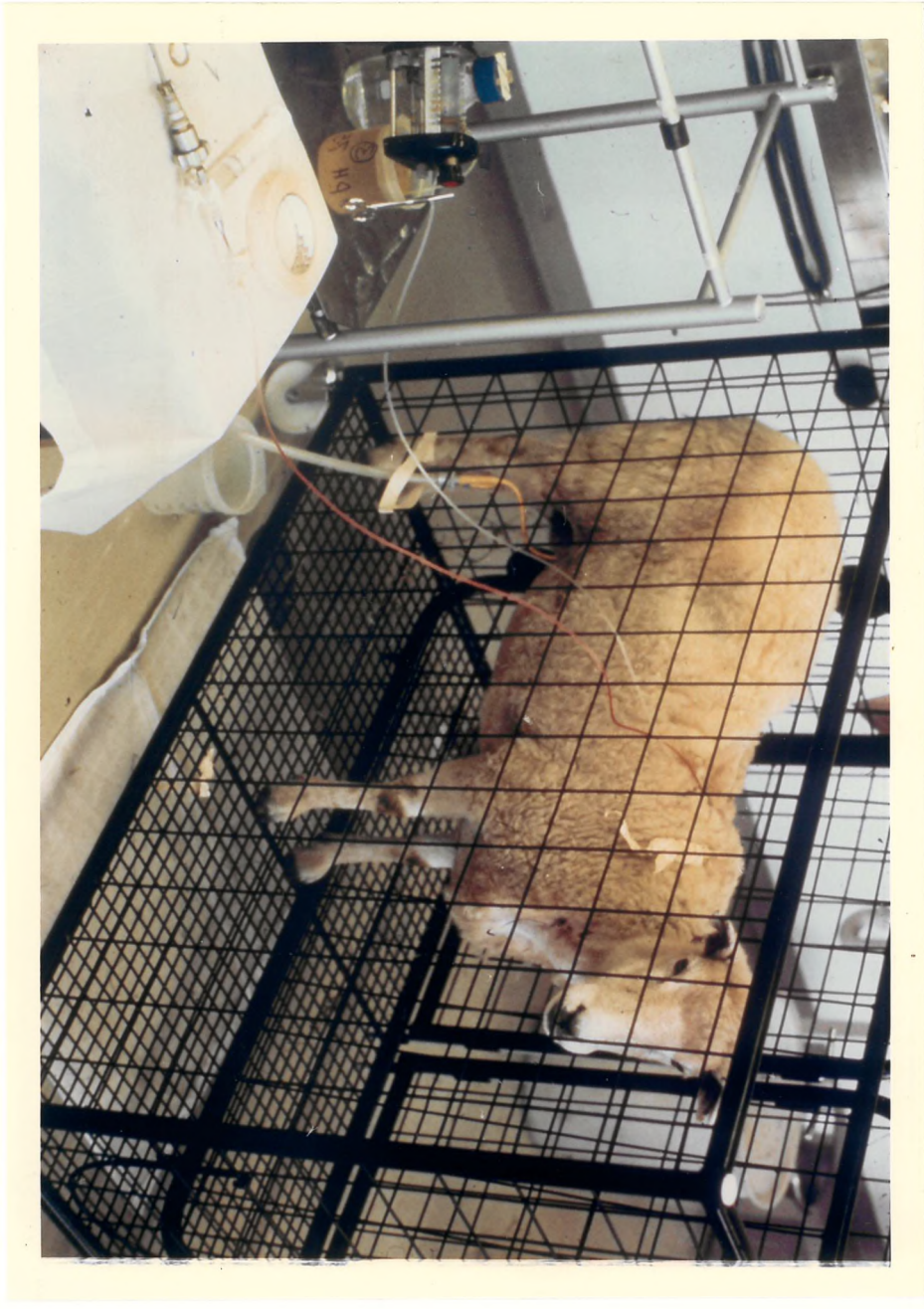


FIG. 3.1

PHOTOGRAPH OF CONSCIOUS SHEEP DURING AN EXPERIMENT

and to collect arterial blood samples. As the animals were prepared with at least 1 m of external tubing it was not normally necessary to approach close to the animals when collecting a blood sample. The femoral vein cannula was used to give an infusion of 0.9% sterile saline (150 mM) containing 2.5 g/ℓ creatinine (Sigma) and 2.0 g/ℓ p-aminohippuric acid (PAH) (Sigma). This solution was given in a priming dose of 3 ml/kg and then as a continuous infusion at a rate of 3 ml/kg./hr., for the duration of the experiment. The experimental protocol is outlined diagramatically in Fig. 3.2.

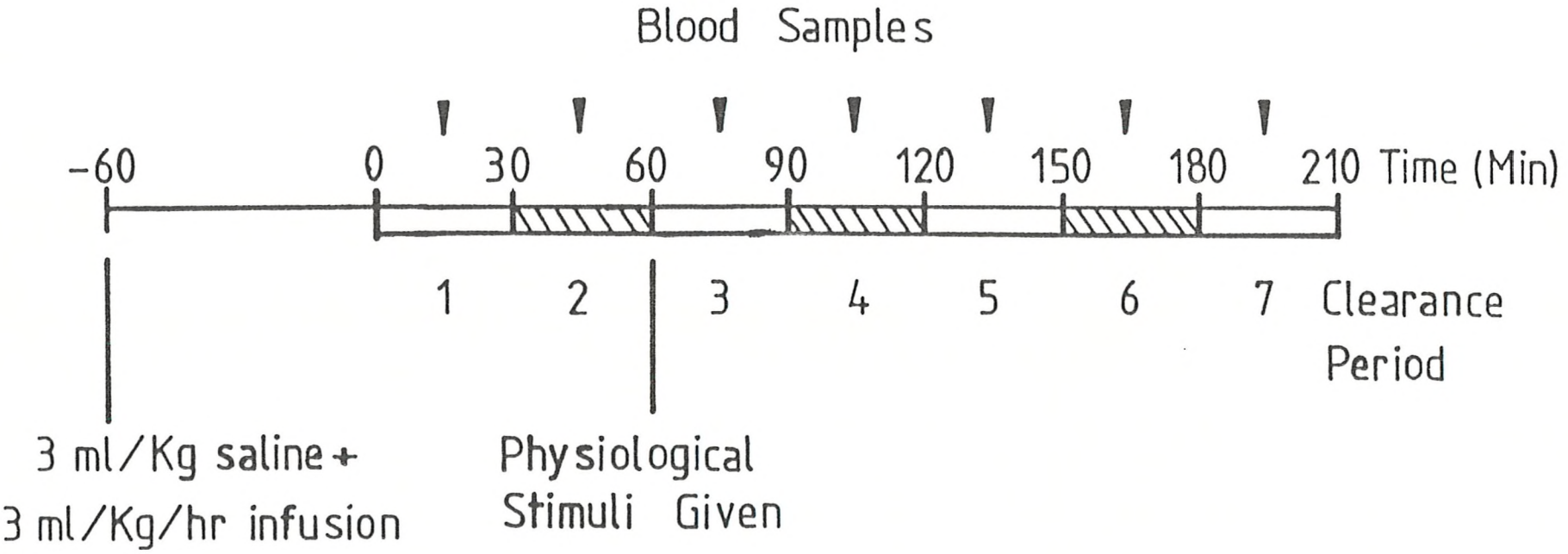
One hour after the priming dose, the first of seven consecutive 30 min. clearance periods was started. At the end of each clearance period the volume of urine collected was measured and 6 ml retained for analysis. At the midpoint of each clearance period an 8 ml arterial blood sample was withdrawn into a chilled centrifuge tube containing 100 µℓ of anticoagulant (100 mg/ml EDTA-Na_2). A further 0.5 ml blood sample was used for serum $[Na^+]$ and $[K^+]$ measurements. Blood samples were cooled on ice for 5 min., centrifuged for 15 min. and then retained on ice until the end of experiment when plasma, serum and urine samples were stored at -20°C to await analysis.

Throughout each clearance period, fluid loss was regulated to within 50 ml by replacing urinary losses with 0.9% sterile saline given i.v. This is the basic procedure under which control experiments were carried out. Variations and specific details of the experimental manipulations used will be described in the subsequent chapters of this thesis where appropriate.

Active and total plasma renin concentration were measured in the arterial blood sample following dialysis at pH 3.3 and pH 7.4 (Fig.2.8), by radioimmunoassay of generated Ang. I (See Chapter II).

FIG. 3.2

EXPERIMENTAL PROTOCOL



Plasma and urinary concentrations of creatinine and PAH were assayed by autoanalysis methods (Technicon). Plasma and urinary concentration of sodium and potassium were measured with an integrating flame photometer (Evans Electroselenium).

Statistical Analysis of Data

The results of all data are presented as mean $\pm$ standard error of the mean (M $\pm$ s.e.m.). Statistical analysis of results was by paired or unpaired Student's t-test as appropriate. Since plasma concentrations of hormones do not follow a normal distribution pattern, this precludes the use of Student's paired or unpaired t-test for the analysis of raw data for plasma renin levels. As changes in renin levels are of more interest than the absolute value, a percentage transformation was applied to the data taking the initial plasma active renin measurement in each individual animal as 100%. This transformed data is normally distributed and can therefore be analysed with Student's paired or unpaired t-test. The expression of inactive renin as a percentage of total renin allows the same tests to be used. Presentation of data in this form was used to illustrate situations associated with changes in the relative, as well as the absolute amounts of the two forms of renin.

CHAPTER IV

ACTIVE AND INACTIVE RENIN IN CONSCIOUS SHEEP: EFFECT OF ISOPRENALINE AND PROPRANOLOL

CHAPTER IV

ACTIVE AND INACTIVE RENIN IN CONSCIOUS SHEEP: EFFECT OF ISOPRENALINE AND PROPRANOLOL

Introduction

A number of stimuli have been reported to alter the release of renin in vivo, these include changes in renal haemodynamics, changes in the rate of sodium transport in the renal tubules and actions of catecholamines either locally released or carried to the JG cells via the circulation *(Vander, 1967; Davis & Freeman, 1976; Keeton & Campbell, 1981)*.

There are several mechanisms by which catecholamines could increase renin secretion. Firstly, they could exert a direct action on the renin-secreting cells of the JG apparatus. Second, by constricting the afferent arterioles, catecholamines could activate the renal vascular receptor and hence alter renin secretion. Third, catecholamines could reduce GFR by constricting the afferent arteriole and stimulate renin release through the macula densa receptor mechanism *(Reid, Morris & Ganong, 1978)*. Fourth, catecholamines could increase renin secretion by promoting the reabsorption of Na^+ and Cl^- ions in the proximal tubules and consequently decreasing their delivery to the macula densa *(Reid et al., 1978)*.

In vivo, it is difficult to separate the various actions of catecholamines on the JG cells *(Davis & Freeman, 1976; Peart, 1978)*. In vitro experiments have demonstrated that catecholamines can increase renin secretion through activation of β-adrenergic receptors on the JG cells *(Katz & Lindheimer, 1977; Morris et al., 1979; Peart, 1978b)*. There is disagreement concerning the pharmacological

characteristics of these receptors. Data from different sources suggest that the receptors behave as β_1 *(Himori, Izumi & Ishimori, 1980)*, β_2 *(Davis & Freeman, 1976)* or "β-nonselective" *(Nakane et al., 1980b)* types.

β-agonist stimulated renin release may be mediated by a rise in cAMP *(Hofbauer et al., 1978; Reid et al., 1978)*. Other evidence indicates that PGs *(Berl et al., 1979)* and calcium flux may participate in the β-adrenoceptor mediated renin release *(Harada & Rubin, 1978; Peart, 1978a; Peart, 1978b)*.

The β-adrenoceptor agonist, isoprenaline, stimulates renin release when given by i.v., i.a. or by intrarenal artery infusion to anaesthetized dogs *(Assaykeen, Tanigawa & Allison, 1974)*, anaesthetized cats *(Johns & Singer, 1973)* and anaesthetized rabbits *(Weber et al., 1977; Richards et al., 1981b)*. Isoprenaline induced-renin release in vivo may in part be secondary to intrarenal vasodilation, but work done in vitro also indicates a direct action. *Johns, Richards & Singer (1975)* reported that isoprenaline stimulates renin secretion by isolated kidney cortex cells. It also promotes renin release from the isolated perfused rat kidney *(Fray & Park, 1979)* and renal cortex slices *(Richards et al., 1981)*. This in vitro work supports the hypothesis that a β-adrenoceptor, located on the JG cells is involved in renin release. What of the secretion control for inactive forms of renin?

The renin response to β-adrenoceptor antagonists (β-blockers) is of great interest in both basic and clinical research. Propranolol is usually used as the standard for comparison of other β-adrenoceptor antagonists even though it is a non-selective antagonist. Propranolol has been extensively studied as far as the active form of renin is

concerned (See review, *Keeton & Campbell, 1981)*. The limited studies carried out on human subjects suggest that β-blockers, while decreasing active renin produce no change, or even a rise, in inactive renin *(Derkx et al., 1976; Atlas et al., 1977)*. Infusion of isoprenaline into urethane-anaesthetized rabbits caused an immediate increase in both active and inactive renin secretion *(Richards et al., 1981b)*. Interpretation of this data has been complicated by the subsequent demonstration that anaesthetic agents have a very pronounced effect on renin secretory mechanisms. It was decided therefore, to study responses to the β-adrenoceptor agonist, isoprenaline, and the β-blocker propranolol in three separate series of experiments, using conscious volume replete sheep.

Aortic blood pressure was monitored throughout the entire time course of each experiment. Other aspects of renal function, such as GFR, ERPF, sodium and potassium excretion, urine flow rate and $[Na^+]$ and $[K^+]$ in the serum, were also assessed. Control experiments were carried out in the same animals, but on different days. A preliminary report of this work has been published as an abstract *(Munday, Mzail & Noble, 1983)*.

Methods

Sheep were prepared under thiopentone/nitrous oxide/halothane anaesthesia with indwelling artery, vein and bladder catheters (See Chapter III). The basic design of the experiments in this study was the same as previously described (Fig. 3.2). The variations to the basic procedure were that the β-adrenoceptor agonist isoprenaline and the β-blocker propranolol were used in three series of experiments.

1) In the first group, isoprenaline (0.05 mg/kg) was given i.v. to six conscious volume replete sheep at the beginning of clearance period three, after one hour of internal control measurements. Isoprenaline administration then continued as an i.v. infusion at a rate of 0.5 µg/kg/min until the end of the experiment.

2) In the second group, propranolol (4.0 mg/kg) was given i.v. to six conscious sheep after one hour of control measurements and then continued as an infusion at a rate of 40 µg/kg/min until the end of the experiment.

3) In the third group of six animals, the effect of propranolol was also investigated in isoprenaline-treated animals using the dose levels outlined above. Isoprenaline infusion started after the first two (internal control) clearance periods. After three further 30 min clearance periods propranolol infusion commenced at the beginning of clearance period six. The isoprenaline infusion was maintained during the propranolol administration.

Throughout each clearance period fluid lost as urine was replaced with 0.9% sterile saline. Plasma renins and renal function were monitored while the animals were conscious and only minimally restrained. Active and total plasma renin levels were measured as described in Chapter II. In each clearance period $[Na^+]$ and $[K^+]$, in serum and urine, creatinine and PAH clearances and urine flow were measured. In control experiments, which were carried out in the same animals, the above protocol was followed exactly except that isoprenaline and/or propranolol were not given and were replaced with saline vehicle.

Results

Table 4.1 shows data for plasma active, inactive and total renin for the isoprenaline treated animals and the corresponding control experiments in the same animals. Active renin as a percentage of the initial value, and inactive renin as a percentage of the total renin, are also shown in the same table. Plasma active and inactive renin concentrations and inactive renin as a percentage of total renin are presented diagramatically in Fig. 4.1.

During the first hour of control measurements (internal controls), plasma active and inactive renin levels were stable (Fig. 4.1). This remained true in the control group for the entire time course of the experiment.

Isoprenaline increased plasma active renin concentration from 11.2 $\pm$ 1.6 ng Ang I/ml. hr (control) to 25.1 $\pm$ 2.2 ng Ang I/ml.hr ($P < 0.02$) just 15 min after the initial dose of isoprenaline. After 135 min of isoprenaline infusion (clearance period 7) active renin had increased to 49.8 $\pm$ 2.1 ng Ang I/ml.hr ($P < 0.001$), i.e. active renin increased by 305.9% compared to the control group.

For the first 75 min after isoprenaline administration, plasma inactive renin concentration did not alter. Subsequently, a decrease from 8.4 $\pm$ 2.6 ng Ang I/ml.hr to 3.8 $\pm$ 1.8 ng Ang I/ml.hr ($P < 0.02$) occurred after 105 min (clearance period 6) of isoprenaline infusion (Fig. 4.1). The proportion of the total renin which was inactive tended to decrease and this decrease became statistically significant in the last two samples (clearance period, 6, 7, Fig. 4.1).

Arterial blood pressure, GFR, and ERPF, are shown in Fig. 4.2 Isoprenaline administration reduced arterial blood pressure by a mean of 10 mm Hg compared to the control experiments in the same group

TABLE 4.1

Active and Inactive Renin in Conscious Sheep: Effect of Isoprenaline

Clearance Period	Plasma Renin Activity (ng Ang I/ml/hr)		Inactive Renin (% of Total	Plasma Active Renin (% of Period 1)
	Active Renin	Inactive Renin		
1	11.6 ± 1.4	8.7 ± 1.4	42.9 ± 4.4	100
2	10.8 ± 1.6	9.8 ± 1.1	48.7 ± 3.5	92.3 ± 4.2
3	12.6 ± 2.3	6.3 ± 0.6	36.8 ± 7.6	103.5 ± 10.5
4	10.2 ± 0.8	6.0 ± 2.1	32.3 ± 7.3	92.9 ± 8.4
5	11.2 ± 1.5	6.9 ± 1.8	36.3 ± 8.8	95.9 ± 7.7
6	11.7 ± 1.7	8.4 ± 2.6	38.5 ± 7.6	103.8 ± 10.8
7	11.3 ± 1.2	8.9 ± 2.7	40.2 ± 6.8	98.6 ± 9.8
1	11.2 ± 1.6	10.6 ± 2.7	46.3 ± 6.6	100
2	10.4 ± 1.0	11.4 ± 2.8	47.9 ± 6.4	97.2 ± 6.3
⟶	Isoprenaline priming dose and start of infusion			
3	25.1 ± 2.2	11.2 ± 3.6	25.2 ± 4.9	264.1 ± 43.9
4	38.0 ± 1.6	9.1 ± 4.6	14.2 ± 8.3	375.9 ± 51.2
5	40.0 ± 1.1	10.8 ± 4.2	14.5 ± 7.2	391.5 ± 60.8
6	45.7 ± 3.3	3.8 ± 1.8	6.2 ± 3.4	422.6 ± 44.2
7	45.9 ± 2.1	3.5 ± 3.0	4.3 ± 5.7	433.7 ± 54.3

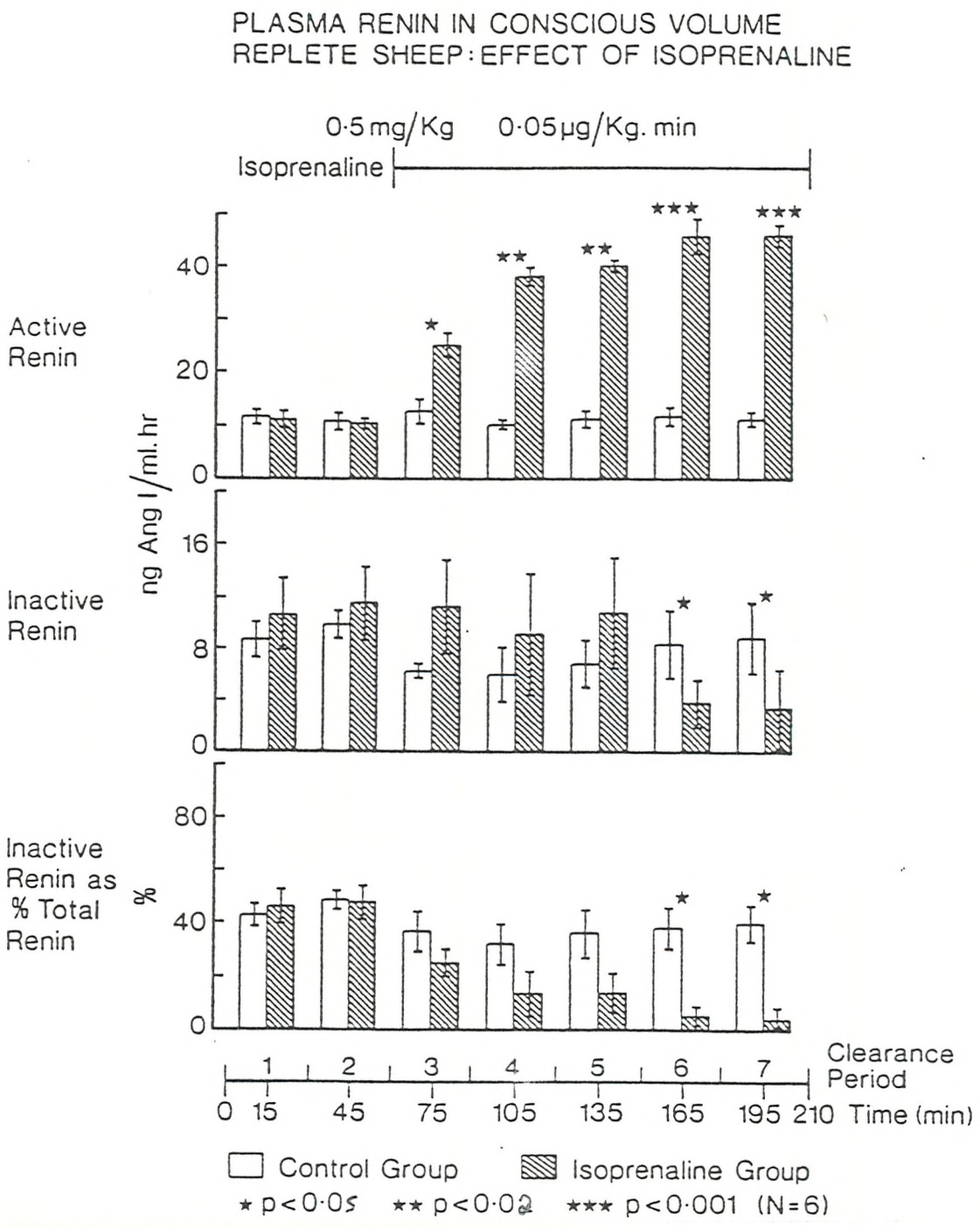
Results are shown as mean ± s.e.m. (n = 6)

Top = Controls

Bottom = Isoprenaline given as priming dose (0.5 mg/kg) and as infusio n (0.05 µg/kg/min)

For the statistical analysis of the results, see Fig. 4.1

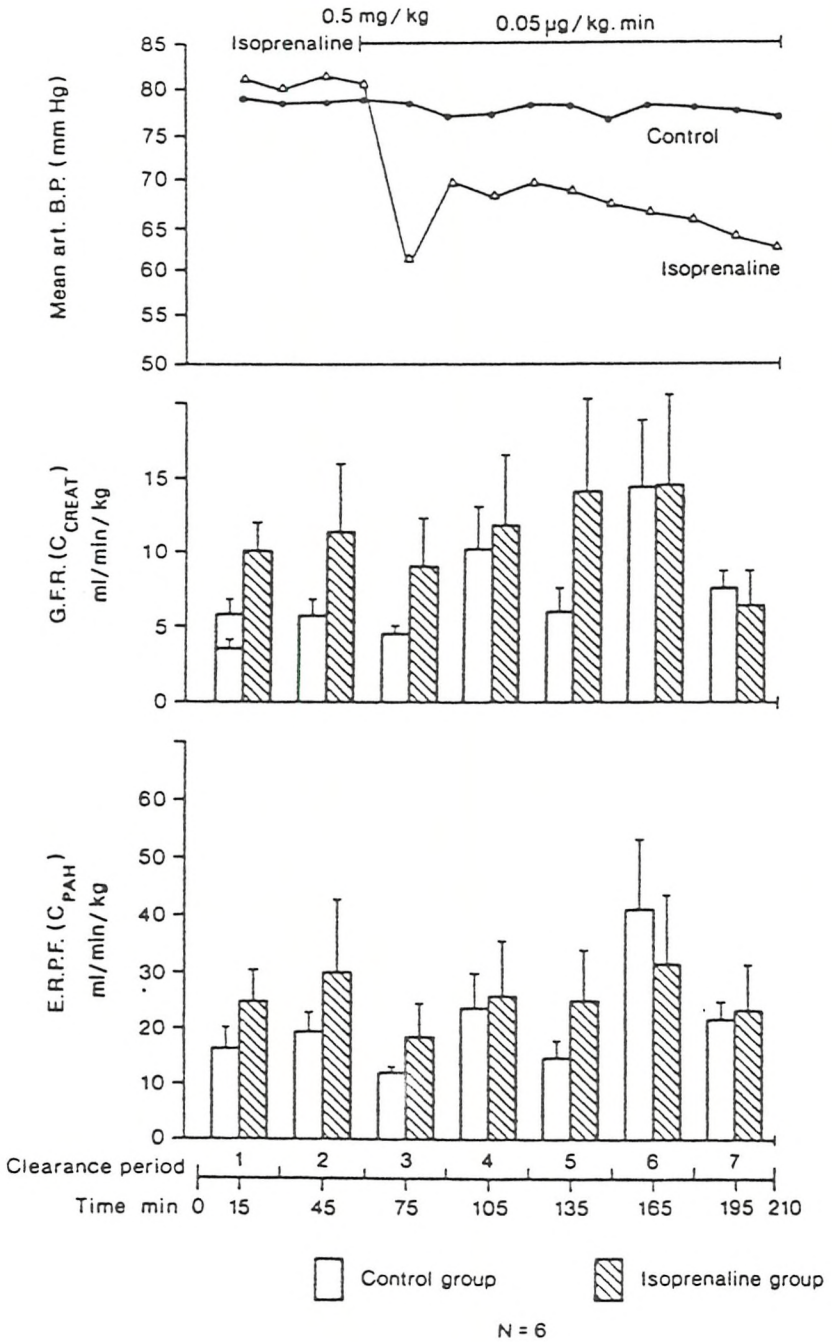
FIG. 4.1



Data shown for active and inactive renin concentrations on this figure and all subsequent equivalent figures has been subjected to a percentage transformation. This was done to normalize the data so that parametric test could be applied, see page 105.

FIG. 4.2

EFFECT OF ISOPRENALINE ON ARTERIAL BLOOD PRESSURE, GFR AND ERPF



of animals. Blood pressure remained depressed until the end of the experiments. No significant changes were observed in either GFR or in ERPF.

Heart rate was also measured in two sheep during isoprenaline and propranolol infusion. Only about 10% increase in heart rate occurred during isoprenaline infusion and 10% decrease with propranolol (Table 4.2). The changes in heart rate were therefore considered to be only minor.

Table 4.3 shows data for sodium and potassium excretion and urine flow rates, together with $[Na^+]$ and $[K^+]$ in serum and urine in isoprenaline treated and control animals. No significant changes were observed in any of these parameters with the exception of urinary $[K^+]$ which was significantly increased and almost doubled during the first 75 min of isoprenaline infusion.

In the second group of six conscious sheep propranolol was given after two 30 min control clearance periods. Plasma active, inactive and total renin concentration in this group and the corresponding controls are shown in Table 4.4. Active renin as a percentage of the initial value and inactive renin as a percentage of total renin are also shown in the same table. Active and inactive renin concentrations and inactive renin as a percentage of total renin are also presented diagramatically in Fig. 4.3.

Propranolol did not affect plasma active renin concentration but reduced inactive renin. Plasma inactive renin decreased from 3.2 ± 0.2 ng Ang I/ml.hr (period 1) to 1.0 ± 0.4 ng Ang I/ml.hr (period 4), ($P < 0.01$), 45 min after the initial dose of propranolol. Comparing controls to the experimental groups (period 4) plasma inactive renin decreased by almost 65.5%, ($P < 0.05$). Plasma inactive

TABLE 4.2

EFFECT OF ISOPRENALINE AND PROPRANOLOL ON HEART RATE IN CONSCIOUS SHEEP

	Isoprenaline		Propranolol	
	I	II	I	II
Control	60	70	70	74
⟶	Isoprenaline administration		Propranolol administration	
+ 15 min	74	76	64	72
+ 30 min	70	80	60	64
+ 45 min	80	72	64	66
+ 60 min	68	74	64	66
+ 75 min	66	76	62	68
+ 90 min	64	78	60	70
+ 105 min	66	74	66	64
+ 120 min			64	70
+ 135 min			66	70

Isoprenaline was given as priming dose (0.5 mg/Kg) and as infusion (0.5 µg/Kg/min), n = 2

Propranolol was given as priming dose (4 mg/Kg) and as infusion (40 µg/Kg/min), n = 2

TABLE 4.3

EFFECT OF ISOPRENALINE ON RENAL FUNCTION IN CONSCIOUS SHEEP

Clearance period	1	2	3	4	5	6	7
Time (min)	0 30	60	90	120	150	180	210
Isoprenaline bolus (0.5 mg/kg)		↓					
Isoprenaline infusion (0.05 µg/kg/min)		———	———	———	———	———	———
Urine flow (ml/hr/kg)							
Exp.	2.1 ± 0.5	3.4 ± 1.1	1.2 ± 0.4	1.6 ± 0.5	2.0 ± 0.7	2.6 ± 0.8	2.5 ± 0.8
Control	3.5 ± 0.2 *	2.6 ± 0.5	3.0 ± 0.4 **	2.5 ± 0.6	1.8 ± 0.2	2.4 ± 0.2	2.3 ± 0.2
Na^+ excretion (µmol/min/kg)							
Exp.	3.1 ± 0.8	5.6 ± 2.0	1.5 ± 0.4	2.2 ± 0.8	3.7 ± 1.6	4.8 ± 2.0	5.0 ± 2.2
Control	4.4 ± 0.9	4.6 ± 1.2	4.8 ± 0.9 **	2.6 ± 0.6	3.1 ± 0.3	3.8 ± 0.5	3.4 ± 0.5
K^+ excretion (µmol/min/kg)							
Exp.	4.8 ± 1.4	6.2 ± 1.9	4.1 ± 1.4	4.1 ± 1.2	4.5 ± 1.3	5.3 ± 1.4	4.0 ± 1.1
Control	5.4 ± 0.6	3.9 ± 0.7	4.1 ± 0.7	2.7 ± 0.6	2.6 ± 0.5	3.4 ± 0.5	3.2 ± 0.6

Results are shown as the mean ± S.E. of mean, and are compared to control experiments performed in the same animals (n = 6)

* Denotes $P < 0.05$; ** Denotes $P < 0.01$ for comparison of control and isoprenaline experiments

(Table 4.3 is continued on the next page)

TABLE 4.3

EFFECT OF ISOPRENALINE ON RENAL FUNCTION IN CONSCIOUS SHEEP

Clearance period	1	2	3	4	5	6	7
Time (min)	0	30	60	90	120	150	180	210
Isoprenaline bolus (0.5 mg/kg)			↓				
Isoprenaline infusion (0.05 µg/kg/min)			———	———	———	———	———
Urine $[Na^+]$ (m.mol/l)							
Exp.	85.8±15.8	100.3±15.1	84.2±14.2	82.8±14.1	114.2±24.3	110.5±22.5	120.8±25.6
Control	78.0±13.1	102.2±14.2	94.3± 7.0	95.2±17.0	103.7± 9.4	102.8±17.0	92.7±15.2
Plasma $[Na^+]$ (m.mol/l)							
Exp.	141.0 ±1.5	138.8 ± 1.1	140.8 ± 1.4	137.3 ± 1.8	142.0 ± 1.6	139.5 ± 1.3	140.3 ± 1.7
Control	140.5 ± 0.9	142.2 ± 2.1	147.5 ± 2.2	147.8 ± 1.0	143.0 ± 2.2	139.2 ± 1.6	143.8 ± 1.5
% of filtered Na^+ excreted							
Exp.	0.32 ± 0.1	0.64 ± 0.3	0.22 ± 0.1	0.19 ± 0.05	0.25 ± 0.05	0.32 ± 0.1	0.53 ± 0.1
Control	0.61 ± 0.1	0.62 ± 0.2	0.70 ± 0.1	0.35 ± 0.2	0.46 ± 0.1	0.35 ± 0.1	0.53 ± 0.1
			**				

Results are shown as the mean ± S.E. of mean, and are compared to control experiments performed in the same animals (n = 6)

** Denotes $P < 0.01$ for comparison of control and isoprenaline experiments

(Table 4.3 is continued on the next page)

TABLE 4.3

EFFECT OF ISOPRENALINE ON RENAL FUNCTION IN CONSCIOUS SHEEP

Clearance period	1	2	3	4	5	6	7
Time (min)	0 30	60	90	120	150	180	210
Isoprenaline bolus (0.5 mg/kg)		↓					
Isoprenaline infusion 0.05 µg/kg/min)			———	———	———	———	———
Urine $[K^+]$ (m.mol/l)							
Exp.	134.7±21.0	128.8±25.8	197.7±24.8	168.0±16.6	161.0±15.9	134.0± 9.9	111.7±19.5
Control	95.0±10.2	93.7±13.4	80.7±12.4 **	92.7±16.5 **	88.8±15.5 **	94.5±20.3	93.5±21.8
Plasma $[K^+]$ (m.mol/l)							
Exp.	4.5 ± 0.2	4.0 ± 0.2	4.1 ± 0.2	3.7 ± 0.3	3.8 ± 0.2	3.6 ± 0.2	3.8 ± 0.4
Control	4.0 ± 0.1	4.0 ± 0.2	4.1 ± 0.3	3.8 ± 0.3	4.3 ± 0.4	3.8 ± 0.3	4.0 ± 0.3
% of filtered K^+ excreted							
Exp.	28.6 ± 8.4	58.6 ± 25.8	30.0 ± 7.7	33.1 ± 14.9	31.8 ± 15.9	25.9 ± 9.5	29.4 ± 11.4
Control	24.8 ± 3.4	20.0 ± 3.8	21.9 ± 2.7	13.2 ± 6.2	13.2 ± 3.1	11.7 ± 4.5	12.1 ± 2.3

Results are shown as the mean ± S.E. of mean, and are compared to control experiments performed in the same animals (n = 6)

** Denotes $P < 0.01$ for comparison of control and isoprenaline experiments

TABLE 4.4

Active and Inactive Renin in Conscious Sheep: Effect of Propranolol

Clearance Period	Plasma Renin Activity (ng Ang I/ml/hr)		Inactive Renin (% of of Total)	Plasma Active Renin (% of Period 1)
	Active Renin	Inactive Renin		
1	8.2 ± 1.3	2.8 ± 0.1	26.5 ± 2.9	100
2	8.5 ± 1.4	2.4 ± 0.3	23.0 ± 2.5	104.7 ± 4.6
3	8.7 ± 1.5	3.1 ± 0.5	26.7 ± 0.6	108.0 ± 11.7
4	8.0 ± 1.5	2.9 ± 0.9	25.7 ± 3.6	96.8 ± 7.1
5	7.9 ± 1.4	2.9 ± 0.4	29.6 ± 4.2	95.9 ± 4.8
6	8.7 ± 1.7	3.2 ± 0.2	30.8 ± 3.6	105.2 ± 6.8
7	9.0 ± 1.5	3.8 ± 0.3	27.4 ± 3.2	111.0 ± 11.2
1	9.5 ± 1.0	3.2 ± 0.2	25.9 ± 2.0	100
2	10.1 ± 1.4	2.9 ± 0.2	24.5 ± 3.6	111.0 ± 10.4
——→	Propranolol priming dose and start of infusion			
3	9.8 ± 1.3	2.5 ± 0.5	19.6 ± 2.9	108.3 ± 7.6
4	8.3 ± 1.1	1.0 ± 0.4	10.4 ± 4.6	91.2 ± 6.6
5	8.0 ± 1.1	1.7 ± 0.3	18.4 ± 3.3	88.4 ± 7.6
6	6.9 ± 1.2	1.5 ± 0.2	19.2 ± 3.1	83.1 ± 8.1
7	7.0 ± 0.8	1.4 ± 0.1	18.2 ± 2.5	84.5 ± 4.2

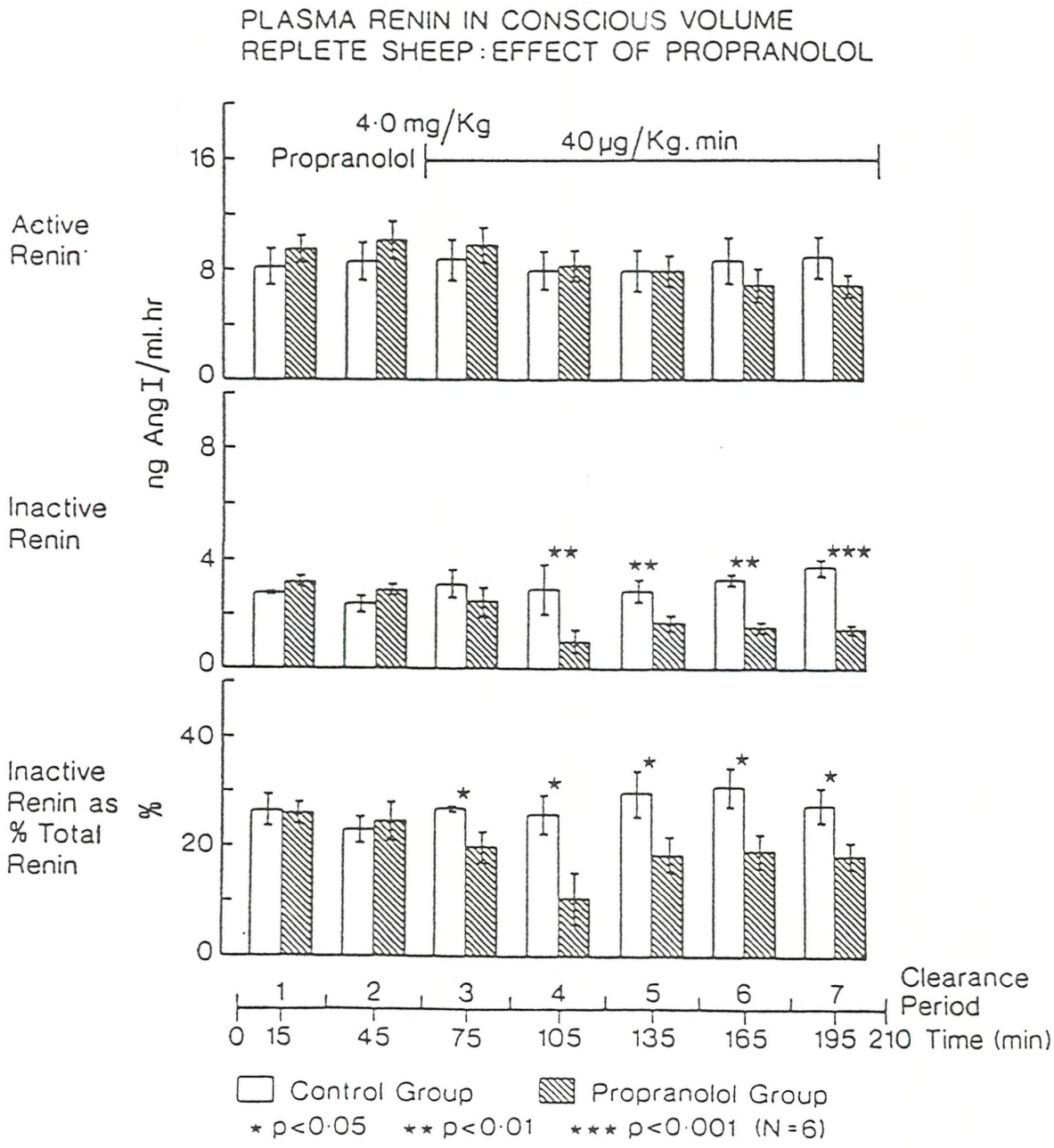
Results are shown as mean ± s.e.m. (n = 6).

Top = Controls

Bottom = Propranolol as priming dose (4 mg/kg) and as infusion (40 µg/kg/min)

For the statistical analysis of the results, see Fig. 4.3

FIG. 4.3


PLASMA RENIN IN CONSCIOUS VOLUME REPLETE SHEEP: EFFECT OF PROPRANOLOL
4·0 mg/Kg Propranolol
40 µg/Kg. min
Active Renin
Inactive Renin
Inactive Renin as % Total Renin
ng Ang I/ml. hr
%
16
8
0
8
4
0
40
20
0
**
***
*
1 2 3 4 5 6 7
Clearance Period
0 15 45 75 105 135 165 195 210 Time (min)
Control Group
Propranolol Group
* p<0·05 ** p<0·01 *** p<0·001 (N=6)

renin remained suppressed for the duration of the experiment. The proportion of the total renin which was inactive decreased significantly 15 min after propranolol infusion and also remained below the control levels till the end of the experiments (Table 4.4, Fig. 4.3).

From Fig. 4.4 it can be seen that propranolol administration did not alter either creatinine or PAH clearances, in other words propranolol did not have any significant effect on GFR or ERPF. It can also be seen from the same figure that propranolol infusion decreased systemic arterial blood pressure. The blood pressure dropped by a mean of 6 mm Hg compared to the corresponsing controls and this hypotensive action of propranolol remained effective throughout the entire time course of the experiments. The data for sodium and potassium excretion and urine flow rates together with $[Na^+]$, $[K^+]$ in serum and urine are shown in Table 4.5. With the exception of $[K^+]$ in urine, propranolol had no consistent effect on any of these parameters. Urinary $[K^+]$ tended to decrease after propranolol infusion and this decrease became statistically significant in the last three clearance periods (period 5, 6 and 7), when the $[K^+]$ values decreased by half ($P < 0.05$), compared to their controls (Table 4.5).

In the third group of experiments the effect of propranolol was investigated in isoprenaline-treated animals. Results for plasma active, inactive and total renin concentrations and the percentage of total renin which was inactive are shown in Table 4.6. As in the first group, it can be seen from this Table and Fig. 4.5 that isoprenaline increased plasma active renin from 9.2 $\pm$ 2.4 ng Ang I/ml.hr (control, period 1), to 26.2 $\pm$ 5.3 ng Ang I/ml.hr

FIG. 4.4

EFFECT OF PROPRANOLOL ON ARTERIAL BLOOD PRESSURE, GFR AND ERPF

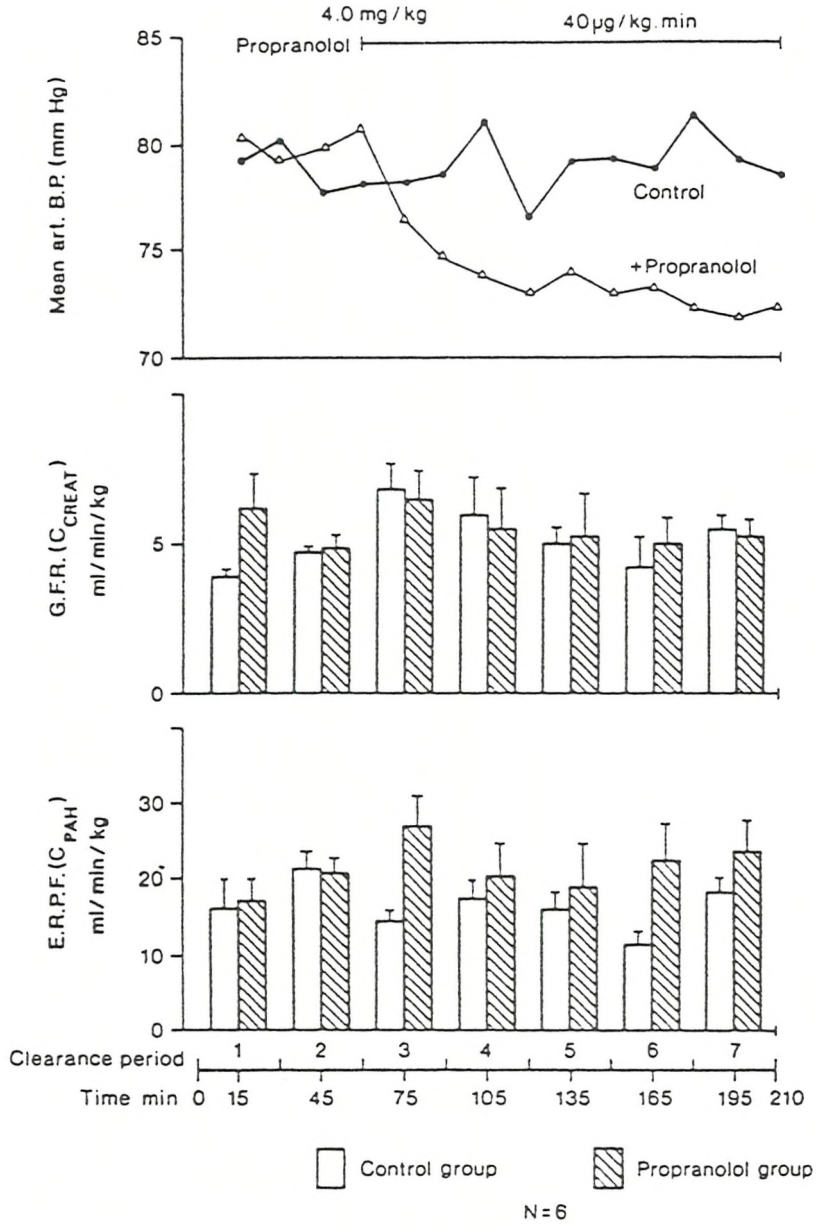


TABLE 4.5

EFFECT OF PROPRANOLOL ON RENAL FUNCTION IN CONSCIOUS SHEEP

Clearance period	1	2	3	4	5	6	7
Time (min)	0	30	60	90	120	150	180	210
Propranolol bolus (4 mg/kg)			↓					
Propranolol infusion (40 µg/kg/min)			———	———	———	———	———	———
Urine flow (ml/kg/hr)							
Exp.	1.9 ± 0.8	1.6 ± 0.5	2.8 ± 0.8	2.8 ± 0.9	4.1 ± 1.7	2.3 ± 0.7	2.2 ± 0.8
Control	2.4 ± 1.0	1.7 ± 0.6	1.7 ± 0.6	2.6 ± 0.7	1.6 ± 0.3	1.3 ± 0.4	1.6 ± 0.3
Na^+ excretion (µmol/kg/min)							
Exp.	1.6 ± 0.5	1.7 ± 0.3	2.5 ± 0.6	2.4 ± 0.5	2.2 ± 0.3	2.1 ± 0.4	2.6 ± 0.5
Control	2.3 ± 0.8	2.3 ± 0.9	2.7 ± 0.8	4.9 ± 1.4	2.0 ± 0.3	1.8 ± 0.5	2.0 ± 0.4
K^+ excretion (µmol/kg/min)							
Exp.	2.7 ± 0.5	2.4 ± 0.4	2.7 ± 0.3	2.2 ± 0.4	1.8 ± 0.3	1.5 ± 0.3	1.7 ± 0.4
Control	3.7 ± 1.4	3.2 ± 0.8	2.6 ± 0.8	4.0 ± 1.1	2.3 ± 0.5	1.8 ± 0.2	2.9 ± 0.7

Results are shown as the mean ± S.E. of mean and are compared to control experiments performed in the same animals (n = 6). There are no statistically significant changes.

(Table 4.5 is continued on the next page)

TABLE 4.5

EFFECT OF PROPRANOLOL ON RENAL FUNCTION IN CONSCIOUS SHEEP

Clearance period	1	2	3	4	5	6	7
Time (min)	0 30		60 90	120	150	180	210
Propranolol bolus (4 mg/kg)			↓				
Propranolol infusion (40 μg/kg/min)			———	———	———	———	———
Urine $[Na^+]$ (m.mol/l)							
Exp.	66.1±13.4	77.6±11.2	67.0±14.0	71.1±20.0	53.8±15.4	69.5±13.4	94.9±16.5
Control	71.3±13.6	77.0±23.4	107.7±12.2	130.7±13.2	86.5±13.5*	85.8±11.1	84.5±7.9
Plasma $[Na^+]$ (m.mol/l)							
Exp.	137.3±1.0	140.4±2.8	143.7±2.8	142.0±2.2	138.0±2.8	142.0±2.0	144.0±2.9
Control	139.0±1.6	138.3±2.5	145.7±4.0	144.7±1.7	144.0±2.4	142.3±3.5	143.0±3.3
% of filtered Na^+ excreted							
Exp.	0.38±0.2	0.47±0.2	0.47±0.1	0.47±0.2	0.70±0.2	0.80±0.3	0.61±0.2
Control	0.41±0.2	0.37±0.1	0.32±0.1	0.55±0.1	0.33±0.1	0.31±0.1	0.26±0.1

Results are shown as the mean ± S.E. of mean and are compared to control experiments performed in the same animals (n = 6).

* Denotes $P < 0.05$ for comparison of control and propranolol experiments.

(Table 4.5 is continued on the next page)

TABLE 4.5

EFFECT OF PROPRANOLOL ON RENAL FUNCTION IN CONSCIOUS SHEEP

Clearance period	1	2	3	4	5	6	7
Time (min)	0	30	60	90	120	150	180	210
Propranolol bolus (4 mg/kg)			↓				
Propranolol infusion (40 µg/kg/min)							
Urine $[K^+]$ (m.mol/l)							
Exp.	131.9±20.9	115.6±20.5	69.8±16.0	72.2±23.5	48.8±14.7	46.0±10.4	58.0±15.8
Control	119.7±13.6	157.8±23.9	110.2±20.2	114.3±23.1	95.5±11.0 *	112.0±20.2 **	117.8±17.1 *
Plasma $[K^+]$ (m.mol/l)							
Exp.	3.8 ± 0.2	3.6 ± 0.4	4.1 ± 0.3	4.1 ± 0.3	3.8 ± 0.2	3.8 ± 0.2	4.0 ± 0.3
Control	3.8 ± 0.4	3.5 ± 0.1	3.5 ± 0.2	3.6 ± 0.2	3.6 ± 0.3	3.8 ± 0.3	3.7 ± 0.3
% of filtered K^+ excreted							
Exp.	15.4 ± 3.5	18.8 ± 5.7	15.1 ± 4.1	13.4 ± 3.0	21.9 ± 6.2	19.2 ± 8.5	9.8 ± 2.6
Control	28.5 ± 11.1	19.2 ± 5.0	13.4 ± 4.8	21.3 ± 6.9	13.8 ± 3.1	15.5 ± 2.8	17.3 ± 4.6

Results are shown as the mean ± S.E. of mean and are compared to control experiments performed in the same animals (n = 6).

* Denotes $P < 0.05$; ** Denotes $P < 0.02$ for comparison of control and propranolol experiments.

TABLE 4.6

Active and Inactive Renin in Conscious Sheep: Effect of Isoprenaline and Propranolol

Clearance Period	Plasma Renin Activity (ng Ang I/ml/hr)		Inactive Renin (% of of Total)	Plasma Active Renin (% of Period 1)
	Active Renin	Inactive Renin		
1	8.7 ± 1.8	4.2 ± 1.0	32.2 ± 1.5	100
2	8.3 ± 1.7	4.3 ± 1.3	33.2 ± 4.3	97.5 ± 6.6
3	8.9 ± 2.6	3.6 ± 0.7	31.9 ± 5.3	93.9 ± 11.9
4	7.8 ± 1.8	3.2 ± 0.7	29.8 ± 3.0	87.6 ± 7.9
5	9.5 ± 1.8	4.5 ± 1.2	30.4 ± 3.0	113.5 ± 11.9
6	9.4 ± 1.7	4.3 ± 1.0	31.9 ± 4.9	114.2 ± 14.2
7	8.8 ± 1.4	3.7 ± 0.8	28.6 ± 3.4	108.5 ± 9.1
8	10.2 ± 2.2	4.0 ± 0.9	28.1 ± 2.7	119.5 ± 5.7
1	9.2 ± 2.4	3.8 ± 0.9	31.0 ± 1.9	100
2	8.9 ± 2.7	4.0 ± 1.1	34.5 ± 4.7	96.6 ± 11.7
——→	Isoprenaline priming dose and start of infusion			
3	15.9 ± 3.3	3.6 ± 1.0	18.9 ± 2.7	196.9 ± 20.9
4	23.1 ± 4.3	3.8 ± 0.8	15.9 ± 4.1	292.9 ± 32.2
5	26.2 ± 5.3	4.1 ± 1.2	14.4 ± 3.9	330.9 ± 45.1
——→	Propranolol priming dose and start of infusion			
6	14.6 ± 3.2	3.5 ± 1.0	19.8 ± 3.2	181.2 ± 20.6
7	9.8 ± 2.0	2.4 ± 0.7	17.5 ± 6.0	130.0 ± 22.7
8	11.4 ± 2.9	2.3 ± 0.6	18.7 ± 4.7	129.9 ± 8.2

Results are shown as mean ± s.e.m. (n = 6)

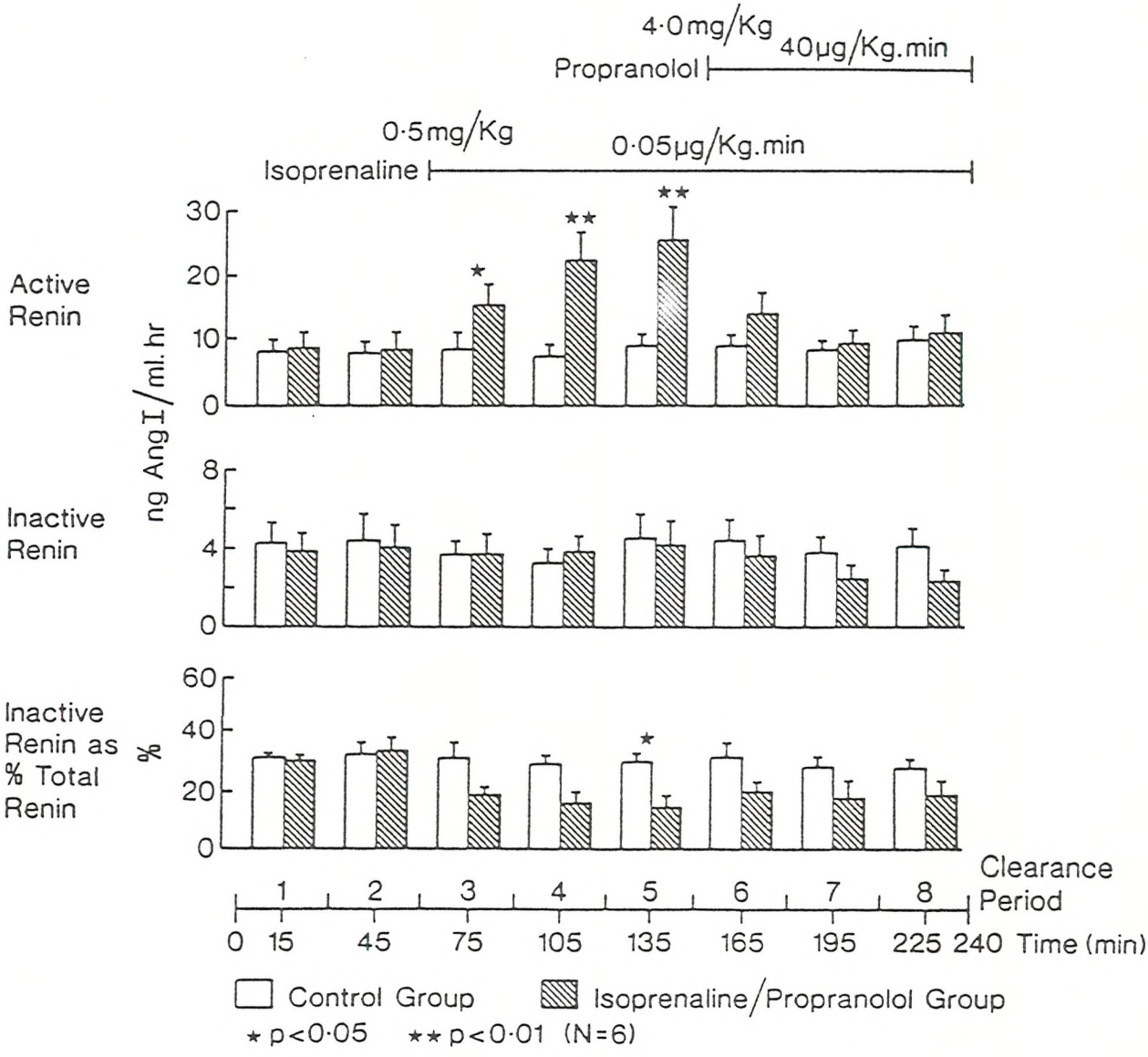
Top = Controls

Bottom = Isoprenaline and propranolol given as priming dose (0.5, 4 mg/kg) and as infusion (0.05, 40 µg/kg/min) respectively

For the statistical analysis of the results, see Fig. 4.5

FIG. 4.5

PLASMA RENIN IN CONSCIOUS VOLUME REPLETE SHEEP: EFFECT OF ISOPRENALINE + PROPRANOLOL



(clearance period 5) ($P < 0.01$). Isoprenaline therefore increased plasma active renin concentration by up to 175.8%, ($P < 0.01$), (clearance period 5) compared to the control group (Fig.4.5). Plasma inactive renin remained unchanged. This data is very similar to the first group of animals, described in this chapter. After 90 min of isoprenaline infusion, at the beginning of clearance period 6 (Fig.4.6) propranolol administration commenced whilst continuing the infusion of isoprenaline. Plasma active renin concentration, which had initially been increased by isoprenaline, was reduced to control levels. No significant changes occurred in inactive renin. Propranolol therefore inhibited the isoprenaline-induced increase in plasma active renin without any effect on plasma inactive renin.

Fig.4.6 shows arterial blood pressure, GFR and ERPF. Once again, as in the first group, isoprenaline infusion reduced arterial blood pressure by a mean of 11 mm Hg and this hypotensive effect was sustained until the end of the experiments.

Table 4.7 shows the data for sodium and potassium excretion and urine flow rates plus $[Na^+]$ and $[K^+]$ in serum and urine. There were no substantial changes in any of these variables.

Discussion

The responses of plasma active and inactive renin concentration to the β-adrenoceptor agonist isoprenaline and the β-blocker, propranolol were investigated in three separate series of experiments.

In conscious sheep, i.v. isoprenaline increased plasma active renin by up to 306% compared to the controls (clearance period 7), (Table 4.1, Fig. 4.1). Isoprenaline infusion, for the first 75 min did not alter plasma inactive renin and even then a decrease occurred (Fig. 4.1).

FIG. 4.6

EFFECT OF ISOPRENALINE AND PROPRANOLOL ON ARTERIAL BLOOD PRESSURE, GFR AND ERPF

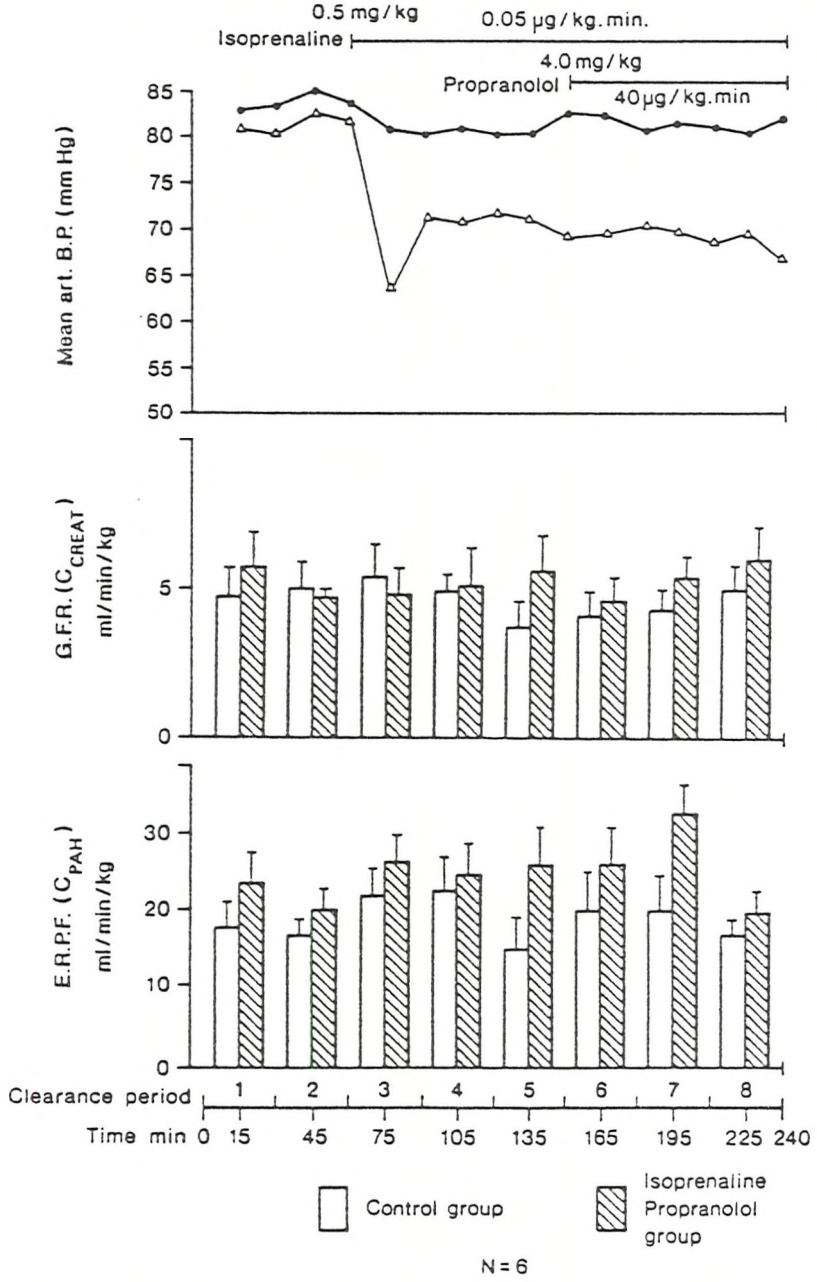


TABLE 4.7

EFFECT OF ISOPRENALINE AND PROPRANOLOL ON RENAL FUNCTION IN CONSCIOUS SHEEP

Clearance period	1	2	3	4	5	6	7	8
Time (min)	0 30		60 90	120	150	180	210	240
Propranolol as per Table 4.5					↓	Propranolol		
Isoprenaline as per Table 4.3			↓ Isoprenaline					
Urine flow (ml/hr/kg)								
Exp.	2.5 ± 0.8	2.2 ± 0.5	2.3 ± 0.8	1.9 ± 0.6	3.4 ± 1.8	2.3 ± 0.6	3.0 ± 0.9	1.8 ± 0.5
Control	2.2 ± 0.6	1.8 ± 0.6	2.3 ± 0.6	2.3 ± 0.5	2.3 ± 0.5	1.7 ± 0.3	1.6 ± 0.4	0.8 ± 0.1
Na^+ excretion (μmol/min/kg)								
Exp.	2.8 ± 1.0	3.1 ± 0.1	2.2 ± 0.5	2.3 ± 0.5	2.5 ± 0.4	2.1 ± 0.3	2.9 ± 0.5	2.1 ± 0.4
Control	2.3 ± 0.7	2.1 ± 0.7	2.7 ± 0.7	2.5 ± 0.6	2.6 ± 0.6	2.6 ± 0.7	3.2 ± 1.0	1.1 ± 0.3
K^+ excretion (μmol/min/kg)								
Exp.	3.3 ± 0.8	3.3 ± 0.7	2.1 ± 0.4	2.0 ± 0.4	1.9 ± 0.2	2.2 ± 0.7	3.0 ± 0.8	3.2 ± 0.4
Control	2.6 ± 0.3	2.0 ± 0.3	2.2 ± 0.4	2.1 ± 0.3	1.7 ± 0.2	1.6 ± 0.3	1.7 ± 0.3	1.9 ± 0.3 *

Results are shown as the mean ± S.E. of mean and are compared to control experiments performed in the same animals (n = 6)

* Denotes $p < 0.05$ for comparison of control and isoprenaline and propranolol experiments

(Table 4.7 is continued on the next page)

TABLE 4.7

EFFECT OF ISOPRENALINE AND PROPRANOLOL ON RENAL FUNCTION IN CONSCIOUS SHEEP

Clearance period	1	2	3	4	5	6	7	8
Time (min)	0 30		60	90	120	150	180	210 240
Propranolol as per Table 4.5						↓ Propranolol		
Isoprenaline as per Table 4.3			↓ Isoprenaline					
Urine $[Na^+]$ (m.mol/l)								
Exp.	76.0±13.3	87.1±13.0	71.7±10.5	109.8±22.7	109.3±30.2	94.0±25.0	96.1±25.9	96.3±30.1
Control	53.9± 5.8	78.3± 8.2	80.3±14.6	84.2±22.0	79.7±22.6	88.9±16.7	111.7±15.4	80.8±12.9
Plasma $[Na^+]$ (m.mol/l)								
Exp.	136.2±1.8	138.5±1.7	143.2±2.8	148.7±3.1	140.2±2.9	134.3±5.0	139.8±4.4	135.8±1.1
Control	138.3±1.2	145.2±3.1	140.8±3.8	143.7±2.2	141.0±2.7	137.5±1.5	144.7±3.2	142.5±2.7
% of filtered Na^+ excreted								
Exp.	0.57±0.2	0.63±0.2	0.52±0.1	0.50±0.1	1.10±0.5	0.71±0.2	1.10±0.3	0.70±0.2
Control	0.70±0.4	0.32±0.1	0.38±0.1	0.40±0.1	0.70±0.1	0.90±0.3	0.53±0.2	0.20±0.3

Results are shown as the mean ± S.E. of mean and are compared to control experiments performed in the same animals (n = 6).

There are no statistically significant changes

(Table 4.7 is continued on the next page)

TABLE 4.7

EFFECT OF ISOPRENALINE AND PROPRANOLOL ON RENAL FUNCTION IN CONSCIOUS SHEEP

Clearance period	1	2	3	4	5	6	7	8
Time (min)	0 30		60	90 120		150 180	210	240
Propranolol as per Table 4.5						↓ Propranolol		
Isoprenaline as per Table 4.3			↓ Isoprenaline					
Urine $[K^+]$ (m.mol/l)								
Exp.	97.8±17.7	98.3±11.2	72.3±11.7	107.7±25.3	87.7±25.4	92.5±29.5	76.7±22.1	156.5±47.6
Control	106.2±22.5	102.8±23.7	77.8±17.1	76.3±24.0	54.2±9.6	61.0±9.8	73.7±15.4	145.8±14.9
Plasma $[K^+]$ (m.mol/l)								
Exp.	3.9 ± 0.3	3.7 ± 0.3	3.7 ± 0.2	4.0 ± 0.2	3.7 ± 0.1	3.8 ± 0.2	3.7 ± 0.1	3.9 ± 0.4
Control	3.5 ± 0.1	3.7 ± 0.4	4.1 ± 0.4	4.1 ± 0.4	3.6 ± 0.2	3.7 ± 0.1	4.2 ± 0.5	3.5 ± 0.2
% of filtered K^+ excreted								
Exp.	18.3±4.2	22.4±5.5	17.9±3.3	14.1±2.9	14.1±2.1	15.5±3.6	17.4±3.6	23.0±5.4
Control	13.4±2.9	12.6±2.1	10.3±1.5	11.0±1.5	20.7±6.2	20.3±8.1	11.1±2.3	11.2±1.3

Results are shown as the mean ± S.E. of mean and are compared to control experiments performed in the same animals (n = 6)

There are no statistically significant changes

Systemic arterial blood pressure decreased by a mean of 10 mm Hg throughout the isoprenaline infusion (2½ hr), Fig. 4.2). However, isoprenaline did not produce a significant intrarenal vasodilator action, since no significant changes were observed in either GFR or ERPF (Fig. 4.2).

Results obtained with the β-blocker drug propranolol were not merely the converse of those obtained with the β-agonist isoprenaline. Propranolol did not change plasma active renin concentration but selectively suppressed inactive renin.

We do not have any immediate complete explanation for these results but we can say that the absence of an effect of propranolol on plasma active renin was not attributable to an inadequate dose of propranolol. In isoprenaline treated animals propranolol inhibited the isoprenaline-induced increase in plasma active renin (Table 4.6, Fig. 4.5). In addition, propranolol reduced arterial blood pressure by a mean of 6 mm Hg throughout the entire time course of the experiments.

Propranolol administration was not associated with any statistically significant changes in GFR, ERPF, electrolyte excretion, urine flow rate or serum and urine electrolyte concentrations with the exception of urinary $[K^+]$ which dropped by almost half.

The importance of the sympathetic nervous system and circulating catecholamines in controlling renin secretion has been discussed in Chapter I (Page 42). As far as active renin is concerned, the β-adrenoceptor agonist, isoprenaline has been previously shown to be a potent stimulus for renin secretion (*Ueda et al., 1970; Winer, Chokshi & Walkenhorst, 1971; Assaykeen et al., 1974*). These authors and others found that the rise in plasma renin activity during

isoprenaline infusion could be blocked by the β-adrenoceptor antagonist propranolol *(Meyer et al., 1971)*. Results presented here are consistent with these reports.

Michelakis & McAllister (1972) found that multiple small doses of propranolol suppressed both supine and upright PRA in hypertensive and normotensive humans without affecting blood pressure. It has also been observed that propranolol lowered PRA by 60% to 70% in salt-deprived and normal anaesthetized dogs *(Zehr & Feigl, 1972)*. *Buhler et al. (1972)* suggested that there is a relationship between the ability of propranolol to lower renin release and its ability to lower blood pressure. This is certainly not the case in this study, as far as active renin is concerned. During propranolol infusion blood pressure dropped by a mean of 6 mm Hg. plasma active renin did not change. It is interesting that the generalisation by *Buhler et al. (1972)* would apply as far as inactive renin and propranolol are concerned. There are some reports that propranolol does not affect plasma renin in normal subjects. For example, *Winer et al., (1969)* found that propranolol did not change supine PRA in normal humans. Results presented here from studies on normal sheep are consistent with this as far as the effect of propranolol on plasma active renin is concerned.

Of the other studies done in this laboratory using conscious sheep as an experimental animal, surprisingly, the diuretic furosemide did not alter plasma active or inactive renin in conscious volume replete sheep *(Lush et al., 1983)*. This conscious animal preparation is, however, clearly able to respond to β-adrenoceptor agonists and antagonists. Although the previous report *(Lush et al., 1983)* raised the possibility that the macula densa Na^+ receptor system

reported to be involved in the regulation of renin secretion does not function unless intrarenal prostaglandin synthesis is primed by anaesthesia, this does not appear to be the case with the β-adrenoceptor mechanism. Another previous report from our laboratory, of the effect of isoprenaline on active and inactive renin secretion in anaesthetized rabbits, showed that isoprenaline increased both forms of plasma renin in parallel *(Richards et al., 1981b)*. The discrepancy between these observations and the present study could relate to the species used but, perhaps more likely, is that it illustrates a further example of interference by anaesthetic agents in experiments of this nature.

From the results described in this chapter of this thesis, we can conclude that in conscious volume replete sheep active and inactive renin secretion mechanisms can respond independently to some physiological stimuli. This concept was developed by studying other stimuli for renin secretion. In the next chapter some work with the calcium antagonist, verapamil, is described.

CHAPTER V

ACTIVE AND INACTIVE RENIN IN CONSCIOUS SHEEP: EFFECT OF VERAPAMIL

CHAPTER V

ACTIVE AND INACTIVE RENIN IN CONSCIOUS SHEEP: EFFECT OF VERAPAMIL

The calcium antagonists are a group of drugs characterized by their ability to influence the transmembrane flux of Ca^{++} ions *(Fleckenstein et al., 1969)*. Verapamil, nifedipine and diltiazem are examples of inhibitors of calcium dependent excitation-contraction coupling *(Fleckenstein, 1981)*.

There has been increasing interest in the possible antihypertensive action of calcium antagonists (see *Bowman, 1980)*, a role probably associated with the peripheral vasodilator action of these drugs *(Klein et al., 1983; Smith, 1983)*. The effects of calcium antagonists on renin secretion are therefore of great interest, but rather complex to investigate. Reduction in systemic arterial blood pressure, intrarenal vasodilation, changes in renal sodium handling and direct actions of calcium antagonists on renin secretory cells may all influence renin secretion.

The relationship between the renin secretory mechanism and Ca^{++} ions is rather unusual since increased intracellular free $[Ca^{++}]$ inhibits renin secretion *(Park et al., 1981; Ginesi et al., 1983)* and low $[Ca^{++}]$ stimulates renin secretion *(Van Dongen & Peart, 1974)*. Other secretory tissues, with the exception of the parathyroid gland, are stimulated by increased intracellular $[Ca^{++}]$ *(Douglas, 1968; Rubin 1970)*.

Most previous studies have only been concerned with the active form of renin but it is now clear that secretion of inactive renin must also be considered in relation to calcium antagonists. Verapamil has peripheral and intrarenal vasodilator actions *(Fleckenstein, 1977;*

Zelis & Flaim, 1982; Millward et al., 1983), but it is not clear whether this vasodilator action influences renin secretion. Oral administration of verapamil to patients with mild, uncomplicated hypertension did not change plasma renin despite reducing arterial blood pressure *(Leonetti, 1980)*. This is in contrast to other vasodilator drugs such as hydrallazine and minoxidil which are associated with increased plasma renin activity *(Keeton & Campbell, 1981)*. Furosemide, which also produced intrarenal vasodilation, failed to alter either plasma active or inactive renin in conscious sheep but, in the same study, furosemide given to anaesthetized sheep increased plasma active renin whilst inactive renin decreased to zero *(Lush et al., 1983)*.

In vitro, the inhibitory effect of depolarizing (high $[K^+]$) conditions on active renin release are blocked by calcium antagonist drugs including verapamil *(Park et al., 1981)*, D600 *(Churchill, 1980)* and diltiazem *(Churchill, McDonald & Churchill, 1981)*. Calcium antagonists do not necessarily have identical effects on both active and inactive renins. *Ginesi, Munday & Noble (1983)* showed that, in otherwise unmodified incubation buffers, verapamil and flunarizine did not change active renin secretion although inactive renin release was increased by both antagonists.

In this thesis section the acute effects of the calcium antagonist verapamil on active and inactive renin secretion are reported. Conscious, chronically catheterized, volume replete sheep were used for this study. Concurrent changes in other aspects of renal function were also monitored and arterial blood pressure was continuously recorded. All experimental data is compared to control experiments performed in the same group of animals on a different day. A

report of this work has been published as an abstract *(Munday, Mzail & Noble, 1983)*.

Methods

Sheep were prepared with indwelling femoral artery and vein cannulae and a bladder catheter as described in Chapter III. The basic design of the experiment was the same as that shown in Fig. 3.2 with the following modifications. After two 30 min clearance periods, which served as internal controls, six conscious animals were given a priming dose of verapamil (Abbott) (0.5 mg/Kg) followed by addition of verapamil to the i.v. saline infusion to provide dose of 0.1 mg/Kg/hr until the end of the experiment. GFR and effective renal plasma flow (ERPF) were measured as clearance of creatinine and PAH. Plasma and urinary $[Na^+]$, $[K^+]$ and urine flow rate were also assessed. During each clearance period, fluid lost as urine was replaced with 0.9% sterile saline given intravenously.

Active and total plasma renin levels were measured as described previously (See Chapter II). For control experiments exactly the same protocol was followed except that verapamil was omitted.

Results

Plasma active and inactive renin concentration for verapamil-infused animals and for control experiments in the same animals, are shown in Table 5.1. Active renin expressed as a percentage of the initial sample taken in each animal and inactive renin as a percentage of the total renin, are also shown in this table. Active and inactive renin concentrations and inactive renin as a percentage of total renin are presented diagramatically in Fig. 5.1.

TABLE 5.1

Active and Inactive Renin in Conscious Sheep: Effect of Verapamil

Clearance Period	Plasma Renin Activity (ng Ang I/ml/hr)		Inactive Renin (% of Total)	Plasma Active Renin (% of Period 1)
	Active Renin	Inactive Renin		
1	8.2 ± 1.3	2.8 ± 0.1	26.5 ± 2.9	100
2	8.5 ± 1.4	2.4 ± 0.3	23.0 ± 2.5	104.7 ± 4.6
3	8.7 ± 1.5	3.1 ± 0.5	26.7 ± 0.6	108.0 ± 11.7
4	8.0 ± 1.5	2.9 ± 0.9	25.7 ± 3.6	96.8 ± 7.1
5	7.9 ± 1.4	2.9 ± 0.4	29.6 ± 4.2	95.9 ± 4.8
6	8.7 ± 1.7	3.2 ± 0.2	30.8 ± 3.6	105.2 ± 6.8
7	9.0 ± 1.5	3.8 ± 0.3	27.4 ± 3.2	111.0 ± 11.2
1	7.8 ± 1.5	2.9 ± 0.2	23.6 ± 2.8	100
2	8.4 ± 1.3	2.4 ± 0.2	23.1 ± 1.4	110.0 ± 5.6
⟶	Verapamil priming dose and start of infusion			
3	11.0 ± 1.9	3.8 ± 0.4	27.1 ± 3.5	145.3 ± 7.7
4	16.2 ± 2.3	5.5 ± 0.6	27.0 ± 2.8	223.1 ± 16.5
5	15.3 ± 2.2	4.2 ± 0.7	22.7 ± 4.4	209.7 ± 13.9
6	12.0 ± 2.0	4.4 ± 0.6	26.4 ± 2.7	163.9 ± 13.1
7	9.4 ± 1.7	4.5 ± 0.7	33.3 ± 3.5	129.1 ± 11.8

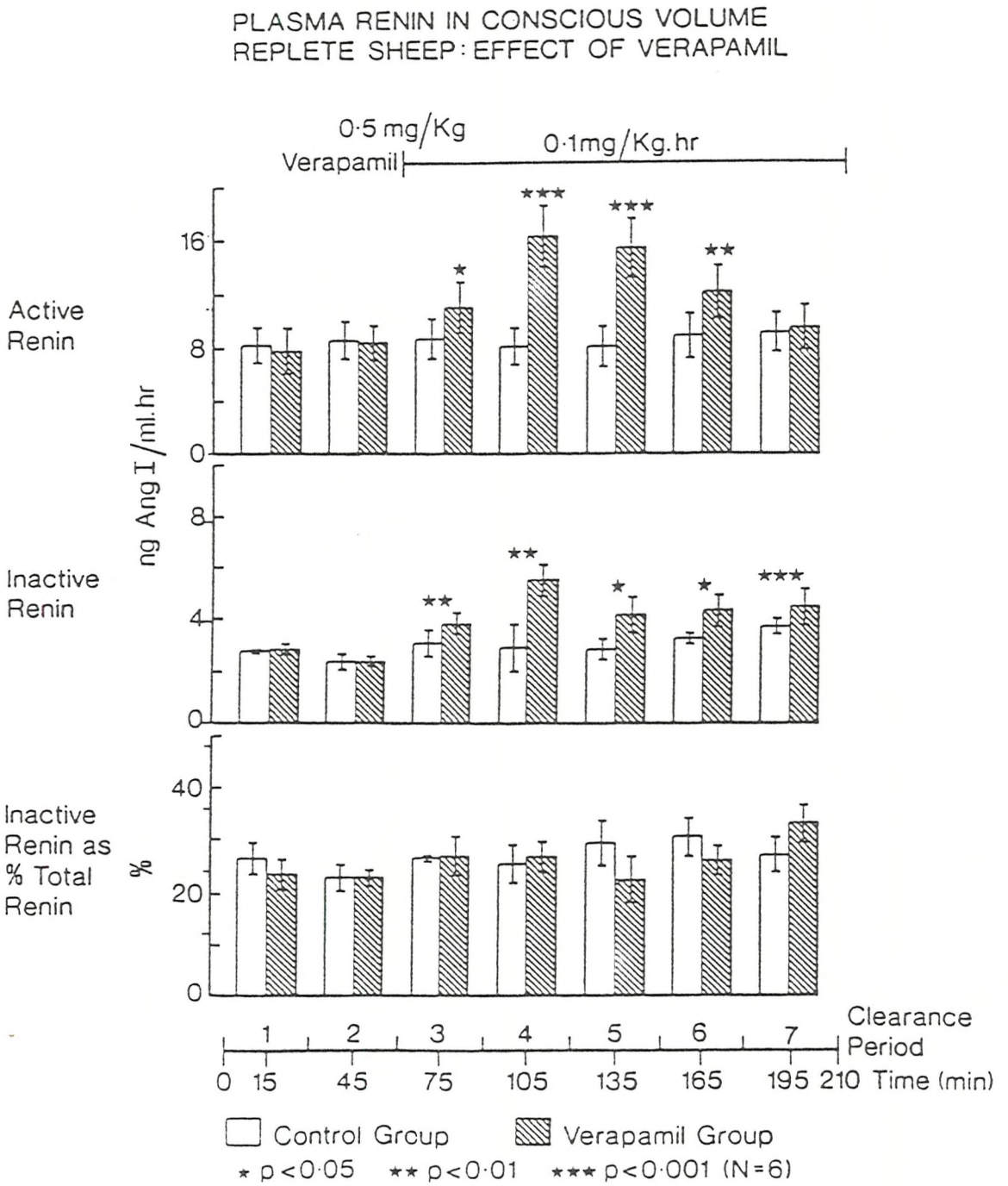
Results are shown as mean ± s.e.m. (n = 6)

Top = Controls

Bottom = Verapamil given as priming dose (0.5 mg/kg) and as infusion (0.1 mg/kg/hr)

For the statistical analysis of the results, see Fig. 5.1

FIG. 5.1


PLASMA RENIN IN CONSCIOUS VOLUME REPLETE SHEEP: EFFECT OF VERAPAMIL
0·5 mg/Kg Verapamil
0·1mg/Kg.hr
Active Renin
Inactive Renin
Inactive Renin as % Total Renin
ng Ang I/ml.hr
%
Clearance Period
0 15 45 75 105 135 165 195 210 Time (min)
Control Group
Verapamil Group
* p<0·05 ** p<0·01 *** p<0·001 (N=6)

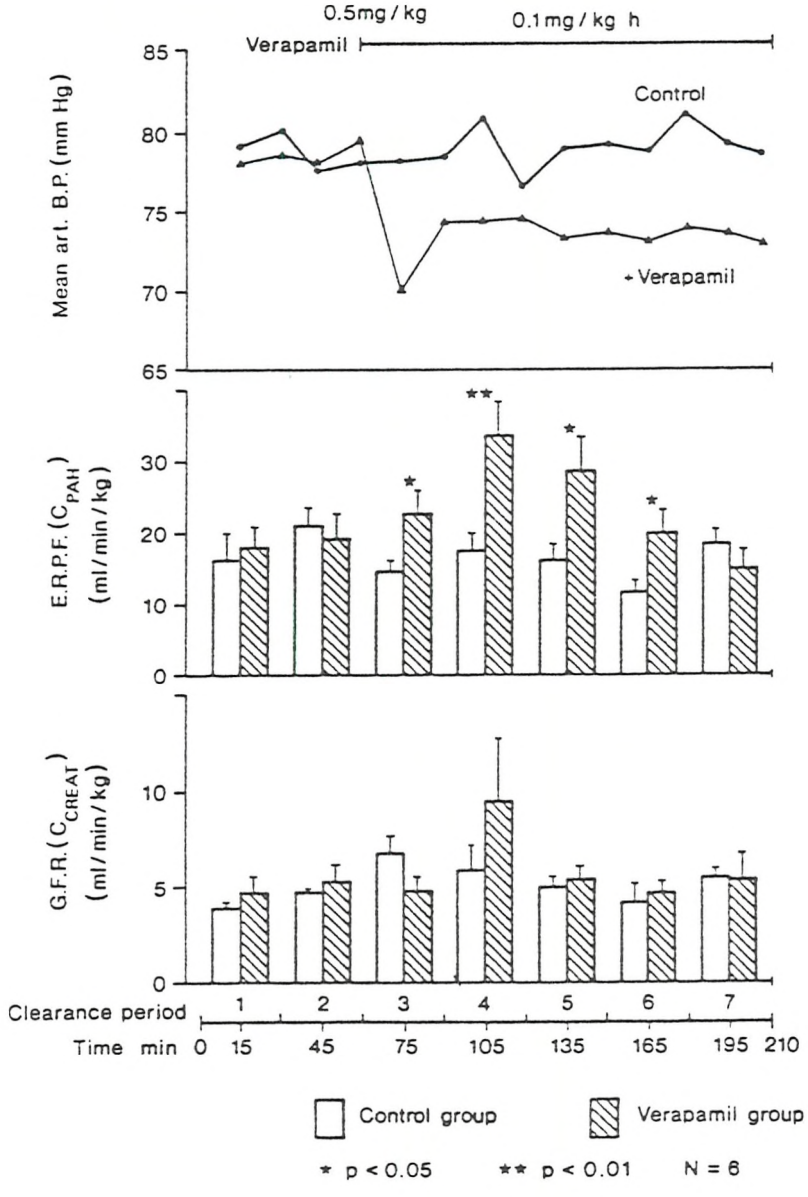
During the first two 30 min clearance periods before verapamil was given, plasma active and inactive renin concentrations were stable. In the control group this remained true for the duration of the study. In the group receiving verapamil, plasma active renin was increased ($P < 0.05$) 15 min after the initial bolus injection of the calcium antagonist (Fig. 5.1). A peak response was attained 45 min after giving verapamil (clearance period 4). At this time plasma active renin had increased by $123 \pm 16.5\%$, ($P < 0.02$). Despite continued infusion of the drug, plasma active renin returned almost to control levels within the 2½ hr, post-drug time course of the experiment.

Changes in plasma inactive renin followed a similar pattern and time course to those for the active form of renin (Fig. 5.1). A peak response was achieved after 45 min when plasma inactive renin had increased by 168%. There were therefore no significant changes in the proportion of total renin which was inactive, i.e., there was no change in the relative amount of active and inactive renin in plasma, Fig. 5.1.

Data for arterial blood pressure, GFR, and effective renal plasma flow (ERPF) are shown in Fig. 5.2. Verapamil administration produced a mean decrease in arterial pressure of 5.0 mm Hg compared to the controls in the same animals and this hypotensive effect was sustained throughout the verapamil infusion. Administration of verapamil was also initially associated with an intrarenal vasodilation, as shown by the increase in ERPF by up to 90.2% ($P < 0.02$) compared to the control experiments (clearance period 4), but this did not persist until the end of the experiment. The changes in intrarenal blood flow thus followed a similar time course to the changes in plasma

FIG. 5.2

EFFECT OF VERAPAMIL ON ARTERIAL BLOOD PRESSURE, GFR AND ERPF



renin while the systemic-hypotensive effect was sustained throughout the verapamil infusion. Although a peak increase in GFR of 59.7% was reached in clearance period 4, this was not statistically significantly different to the control group.

The data for the sodium and potassium excretion and urine flow rates are shown in Table 5.2. There was no consistent pattern of change in any of these variables in comparison to the control data. Serum $[Na^+]$ and $[K^+]$ were also unchanged. The percentage of filtered Na^+ and K^+ which was excreted was calculated and again there were no significant changes in these variables (Table 5.2).

In summary, plasma levels of both active and inactive renin were significantly increased 15 min after the initial dose of verapamil and a peak response in both forms was attained after 45 min. However, plasma active and inactive renin concentration returned towards the control levels within 2½ hr, despite continued infusion of verapamil. ERPF and GFR, as assessed by PAH and creatinine clearances, were increased, but again only transiently. Sodium and potassium excretion and urine flow rates and plasma $[Na^+]$ and $[K^+]$ were all unchanged by verapamil.

Discussion

Conditions associated with increased renin secretion are analogous to those producing vasodilation. A decrease in cytoplasmic $[Ca^{++}]$ stimulates both the secretion of renin from the JG cells and relaxation of vascular smooth muscle cells *(Van Dongen & Peart, 1974; Fray & Park, 1979)*. The basal tone of vascular smooth muscle depends primarily on receptor-mediated activation of intracellular and

TABLE 5.2

EFFECT OF VERAPAMIL ON RENAL FUNCTION IN CONSCIOUS SHEEP

Clearance period	1	2	3	4	5	6	7
Time (min)	0	30	60	90	120	150	180	210
Verapamil bolus (0.5 mg/kg)			↓					
Verapamil infusion (0.1 mg/kg/hr)			———	———	———	———	———	———
Urine $[Na^+]$ (m.mol/l)							
Exp.	59.7±17.6	73.8±23.6	92.5±24.4	75.0±20.6	97.7±21.8	92.0±19.1	82.2±19.1
Control	71.3±13.6	77.0±23.4	107.7±12.2	130.7±13.2*	86.5±13.5	85.8±11.1	84.5±7.9
Plasma $[Na^+]$ (m.mol/l)							
Exp.	140.5±2.0	137.0±2.7	141.3±3.1	139.8±1.9	138.7±4.1	173.8±4.0	139.5±1.5
Control	139.0±1.6	138.3±2.5	145.7±4.0	144.7±1.7	144.0±2.4	142.3±3.5	143.0±3.3
% of filtered Na^+ excreted							
Exp.	0.23±0.1	0.14±0.04	0.65±0.2	0.42±0.2	0.56±0.2	0.43±0.2	0.31±0.2
Control	0.41±0.2	0.37±0.1	0.32±0.1	0.55±0.1	0.33±0.1	0.36±0.1	0.26±0.1

Results are shown as the mean ± S.E. of mean and are compared to control experiments performed in the same animals (n = 6).

* Denotes $P < 0.05$ for comparison of control and verapamil experiments.

(Table 5.2 is continued on the next page)

TABLE 5.2

EFFECT OF VERAPAMIL ON RENAL FUNCTION IN CONSCIOUS SHEEP

Clearance period	1	2	3	4	5	6	7
Time (min)	0 30	60	90	120	150	180	210
Verapamil bolus (0.5 mg/kg)	↓						
Verapamil infusion (0.1 mg/kg/hr)	———	———	———	———	———	———	———
Urine flow (ml/hr/kg)							
Exp.	1.3 ± 0.4	1.9 ± 0.4	2.5 ± 0.6	2.9 ± 0.6	2.6 ± 0.2	1.8 ± 0.4	1.5 ± 0.4
Control	2.4 ± 1.0	1.7 ± 0.6	1.7 ± 0.6	2.6 ± 0.7	1.6 ± 0.3 *	1.3 ± 0.4	1.6 ± 0.3
Na^+ excretion (μmol/min/kg)							
Exp.	1.5 ± 0.8	2.4 ± 1.1	3.9 ± 1.3	4.0 ± 1.4	3.4 ± 0.9	2.3 ± 0.7	1.9 ± 0.9
Control	2.3 ± 0.8	2.3 ± 0.9	2.7 ± 0.8	4.9 ± 1.4	2.0 ± 0.3	1.8 ± 0.5	2.0 ± 0.4
K^+ excretion (μmol/min/kg)							
Exp.	2.6 ± 1.1	2.8 ± 0.8	2.9 ± 0.7	3.5 ± 0.8	2.8 ± 0.7	2.5 ± 0.7	1.9 ± 0.6
Control	3.7 ± 1.4	3.2 ± 0.8	2.6 ± 0.8	4.0 ± 1.1	2.3 ± 0.5	1.8 ± 0.2	2.9 ± 0.7

Results are shown as the mean ± S.E. of mean and are compared to control experiments performed in the same animals (n = 6).

* Denotes $P < 0.05$ for comparison of control and verapamil experiments.

(Table 5.2 is continued on the next page)

TABLE 5.2

EFFECT OF VERAPAMIL ON RENAL FUNCTION IN CONSCIOUS SHEEP

Clearance period	1	2	3	4	5	6	7
Time (min)	0	30	60	90	120	150	180	210
Verapamil bolus (0.5 mg/kg)			↓				
Verapamil infusion (0.1 mg/kg/hr)			———	———	———	———	———
Urine $[K^+]$ (m.mol/l)							
Exp.	124.7±27.3	112.5±29.8	127.8±38.1	109.3±32.0	76.8±17.3	96.7±19.2	86.5±15.1
Control	119.7±13.6	157.8±23.9	110.2±20.3	114.3±23.2	95.5±11.0	112.0±20.3	117.8±17.1
Plasma $[K^+]$ (m.mol/l)							
Exp.	3.8 ± 0.1	3.7 ± 0.3	3.6 ± 0.4	3.4 ± 0.3	3.2 ± 0.3	3.2 ± 0.3	3.2 ± 0.4
Control	3.8 ± 0.4	3.5 ± 0.1	3.5 ± 0.2	3.6 ± 0.2	3.6 ± 0.3	3.8 ± 0.3	3.7 ± 0.3
% of filtered K^+ excreted							
Exp.	15.7±5.7	23.0±11.1	24.0±8.0	13.9±3.0	19.0±4.5	19.8±6.7	14.9±5.7
Control	28.5±11.1	19.2±5.0	13.4±4.8	21.3±6.9	13.8±3.1	15.5±2.8	17.3±4.6

Results are shown as the mean ± S.E. of mean and are compared to control experiments performed in the same animals (n = 6). There are no statistically significant changes.

extracellular Ca^{++} pools *(Zelis & Flaim, 1981)*. The primary mechanism for the vasodilator effects of calcium antagonists, including verapamil, is blockade of Ca^{++} ion influx *(Smith, 1983)*. The peripheral and intrarenal vasodilator effects of verapamil have been well documented *(Fleckenstein, 1977; Zelis & Flaim, 1982; Millward et al., 1983)*. In the present study these actions are illustrated by a decrease in systemic arterial blood pressure and an increase in ERPF (Fig. 5.2). It is possible that such vasodilator actions of verapamil are responsible for the increase in plasma active and inactive renin (Fig. 5.1), but this seems unlikely, since quantitatively greater furosemide-induced changes in PAH and creatinine clearance *(Lush et al., 1983)* were not associated with any change in active or inactive renin secretion in conscious sheep.

These observations on verapamil and its effects on active and inactive renin secretion in conscious sheep have several implications.

Firstly, this study has confirmed that i.v. administration of verapamil does increase plasma active renin. However, despite continued infusion of verapamil, plasma active renin returned almost to control levels after 2½ hr. This may explain the existence of conflicting reports in the literature which fail to agree on whether verapamil does or does not increase plasma renin levels *(Lester & Rubin, 1977; Leonetti et al., 1980; Logan & Chatzitias, 1980)*.

Secondly, plasma inactive renin changes followed a similar time course to changes in plasma active renin. This is consistent with previous in vitro studies done in this laboratory which showed that verapamil increased both active and inactive renin secretion by rabbit kidney cortex slices *(Ginesi, 1982)*.

Thirdly, verapamil infusion in conscious sheep was associated

with an increase in ERPF. This vasodilator action of verapamil followed a similar time course to the changes in plasma renin. There is no clear explanation for the transient nature of the effect of verapamil on the renal vasculature. It is possible that either the kidney became refractory to verapamil or, alternatively, a physiological autoregulatory mechanism may have opposed the verapamil-induced intrarenal vasodilation. It has been suggested that the autoregulatory mechanism for renal blood flow is Ca^{++} ion dependent *(Cohen & Fray, 1982)*.

The transient change in plasma renin cannot readily be related to a decrease in the plasma concentration of the drug during the time course of the experiment. Verapamil was administered as an initial bolus injection (0.5 mg/Kg) and then continued as an i.v. infusion at a rate of 0.1 mg/Kg/hr until the end of the experiment. The pharmacokinetic characteristics of verapamil have been investigated after both i.v. and oral administration. The concentration of the drug in plasma exhibits a bi-exponential decay, an initial rapid distribution phase, lasting for about 35 min, is followed by a much slower elimination phase with a half-life ranging from 3 to 7 hr *(Schomerus et al., 1976)*. This suggests that, in the present study, the plasma levels of verapamil would not have fallen in the latter part of the experiment.

The change in plasma active and inactive renins after verapamil infusion in this present study does not appear to be linked to a decrease in arterial blood pressure. The hypotensive effect of verapamil was sustained throughout the verapamil infusion (Fig. 5.2), whereas the change in renin was transient (Fig. 5.1). Change in renin secretion cannot be related to sodium and potassium excretion

as there were no discernable changes in these variables (Table 5.2).

The question remains then, what is the trigger for the increase in plasma active and inactive renin after verapamil administration? There is a general support for the concept that calcium antagonists, including verapamil, inhibit membrane transport of calcium and block the inward current of Ca^{++} ions into the smooth muscle cells. Such an action may also occur in the JG cells, since they are considered to be modified smooth muscle cells. In vitro, altered calcium flux does affect renin release *(Park et al., 1981; Churchill, 1980; Churchill et al., 1981)*. *Van Dongen & Peart (1974)* suggested that decreasing intracellular $[Ca^{++}]$ stimulated renin release by the isolated perfused rat kidney. It has also been shown that decreasing extracellular $[Ca^{++}]$ and therefore presumably intracellular $[Ca^{++}]$ as well, stimulated both active and inactive renin secretion by kidney cortex slices *(Ginesi et al., 1983)*. It is clear from in vitro studies that verapamil could influence renin secretion by a direct action on the JG cells and this appears to be the most likely mechanism for the increase in active and inactive renin secretion in this study.

CHAPTER VI

ACTIVE AND INACTIVE RENIN IN CONSCIOUS SHEEP: EFFECT OF TWO ANGIOTENSIN CONVERTING-ENZYME INHIBITORS (ACE I), CAPTOPRIL AND ENALAPRIL (MK 421)

CHAPTER VI

ACTIVE AND INACTIVE RENIN SECRETION IN CONSCIOUS VOLUME REPLETE SHEEP: EFFECT OF TWO ANGIOTENSIN CONVERTING-ENZYME INHIBITORS (ACEI), CAPTOPRIL AND ENALAPRIL (MK421)

Ang II acts directly on the JG cells to inhibit renin release. This feedback inhibition of renin secretion contributes to overall blood pressure homeostasis *(Haber, 1978; Swales, 1979a; Heel et al., 1980; Sullivan, 1983)*. The feedback loop can be interrupted by inhibiting angiotensin converting enzyme (ACE) and therefore preventing the formation of Ang. II.

Inhibition of ACE is a novel approach to the treatment of several cardiovascular diseases including hypertension and congestive cardiac failure *(Rubin & Rein, 1983)*. The earliest ACEI were derived from venom of the Brazilian pit viper. Later, synthetic peptide analogues were developed (for more details, see page 10) , but these synthetic peptides were only active when given intravenously.

Captopril is the only orally active ACEI currently available for general clinical use and it is now becoming a first choice antihypertensive drug. Other compounds are in various developmental stages and one of these, enalapril (MK 421) is undergoing clinical trials. From studies in both animals and man, it is known that captopril induces an increase in PRA primarily by inhibition of the negative feedback of Ang. II on renin release *(Case et al., 1978; Asaad & Antonaccio, 1979; Millar & Johnston, 1979; Heel et al., 1980; Keim et al., 1980; Luderer et al., 1980; Mikami et al., 1982; and Fallo et al., 1983)*.

Most of these studies with ACE inhibitors were just concerned with the active form of renin. Some studies have been performed on human subjects in which both active and inactive renin were measured but

these have failed to give consistent results. *Mantero et al. (1982)* found that in essential hypertensive patients, a single dose of captopril induced a rapid and persistent increase in active renin, whereas inactive renin tended to decrease. On the other hand, *Sealey et al., (1981)* studied the responses of active and inactive renin to captopril in a group of untreated hypertensive patients and found that, during chronic captopril treatment (4 weeks), both active and inactive renin increased. After acute administration, captopril had no effect on inactive renin, but active renin increased markedly.

We therefore decided to investigate the acute effects of captopril and enalapril on plasma active and inactive renin in two groups of conscious volume replete sheep. Control experiments were performed in the same animals on different days. Arterial blood pressure was recorded continuously during each individual experiment and other aspects of renal function were also assessed. The efficacy of the captopril and enalapril doses used in these studies was evaluated by monitoring the suppression by the inhibitors of the pressor response to Ang I in conscious sheep. A preliminary report as an abstract in press *(Mzail & Noble, 1984)*.

Methods

A group of conscious sheep were prepared with indwelling femoral artery and vein cannulae and a bladder catheter as previously described in Chapter III. The basic design of the experiments was the same as that shown in Figure 3.2(page 104) with the following exceptions: Two ACEI, captopril and enalapril, were used in two separate series of experiments.

1) In the first series, captopril (1 mg/kg, i.v.) was given as a bolus injection to six conscious sheep at the beginning of clearance period three.

2) In the second series of experiments, an identical protocol was followed except that the dose of enalapril used was 0.5 mg/kg.

Existing reports from studies on human subjects suggested that these doses of the two inhibitors would be approximately equipotent. In control experiments in the same animals, the above protocol was followed exactly except that an ACEI was not given. A minimum 3 day recovery period was allowed between randomly ordered captopril, enalapril and control studies.

As in previous investigations, volume depletion as a result of urinary loss was prevented by replacement with sterile saline. Active and inactive plasma renin concentrations were measured as described in Chapter II (page 72). Arterial blood pressure was recorded in every animal for the entire time course of the experiment. In each clearance period $[Na^+]$ and $[K^+]$ in serum and urine, creatinine and PAH clearances, Na^+ and K^+ excretion and urine flow rates were measured.

In order to check on the efficacy and duration of converting enzyme blockade sheep were given i.v. doses of Ang I (0.3, 1, 3, 10, 30 µg). The pressor response to Ang I was recorded and then captopril (1 mg/kg) or enalapril (0.5 mg/kg) was given as a bolus i.v. injection. The ability of both ACEIs to block the pressor response of Ang I was monitored immediately after giving the inhibitor and subsequently at 30 min intervals for 2 hr. (captopril) or 2½ hr. (enalapril). At each time interval a minimum of 3 different doses of Ang I was used to provide a limited dose-response relationship. Four sheep were used for each inhibitor.

Results

It can be clearly seen from Figure 6.1, 6.2 that the doses of the two ACEI, captopril (1 mg/kg) and enalapril (0.5 mg/kg) were sufficient to provide substantial blockade of the pressor response to Ang I. In order to quantify the blockade, the dose of Ang I needed to give 25 mm Hg rise in blood pressure was calculated and is now referred to as the D_{25} value (Fig. 6.3). Comparing the inhibitory effect of the two ACEI, captopril was rapid in onset and decayed fairly quickly with time. Blockade with enalapril was slowed in onset and was maintained for a much longer time.

Values for active and inactive plasma renin, in captopril treated animals, together with their corresponding controls are shown in Table 6.1. Active renin as a percentage of the initial control sample (clearance period 1) and inactive renin as a percentage of total renin are also shown in the same table. Data for active and inactive renin and inactive renin as a percentage of total are presented diagramatically in Fig. 6.4. Equivalent data for the enalapril treated animals and the corresponding controls are shown in Table 6.2 and Fig. 6.5.

There was a predictable progressive rise in plasma active renin after captopril administration. Captopril increased plasma active renin from 9.3 $\pm$ 1.9 ng Ang I/ml.hr (control, period 1) to 19.4 $\pm$ 2.6 ng Ang I/ml.hr (period 3, $P<0.02$), 15 min after administration of captopril. In clearance period 6, 105 min after the bolus injection of captopril was given, plasma active renin had increased to 67.5 $\pm$ 15.3 ng Ang I/ml.hr ($P < 0.01$), an increase of 812% compared to the control group ($P < 0.001$). During the next clearance period (period 7), however, the increase in plasma active renin was only 687% ($P < 0.001$) compared to the corresponding controls. This suggests that the maximum increase in plasma active renin

FIG. 6.1

DOSE RESPONSE CURVES TO ANG I BEFORE AND AFTER ADMINISTRATION OF THE ACEI, CAPTOPRIL (N , = 4)

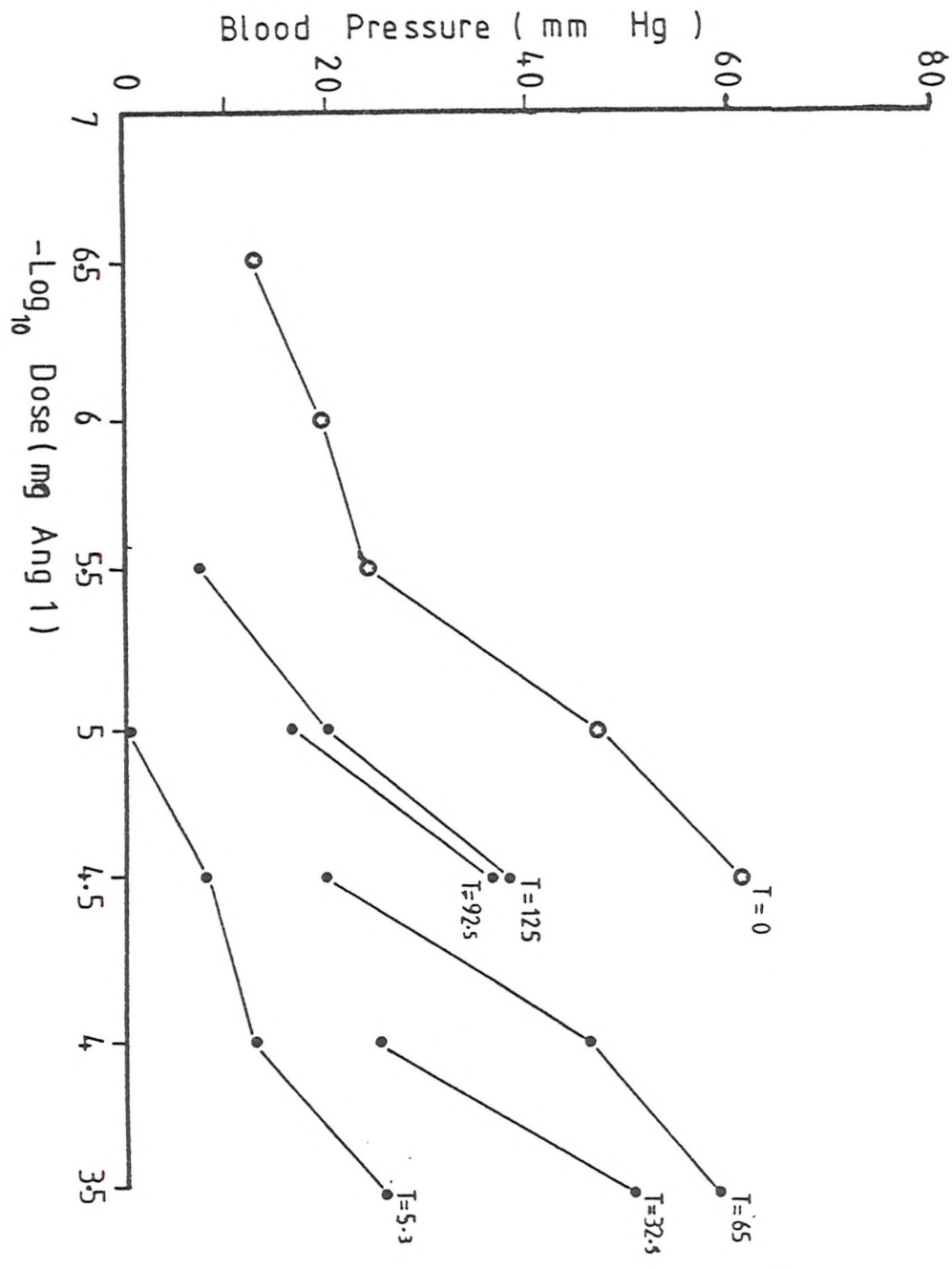


FIG. 6.2

DOSE RESPONSE CURVES TO ANG I BEFORE AND AFTER ADMINISTRATION OF THE ACEI, ENALAPRIL (MK 421) (N = 4)

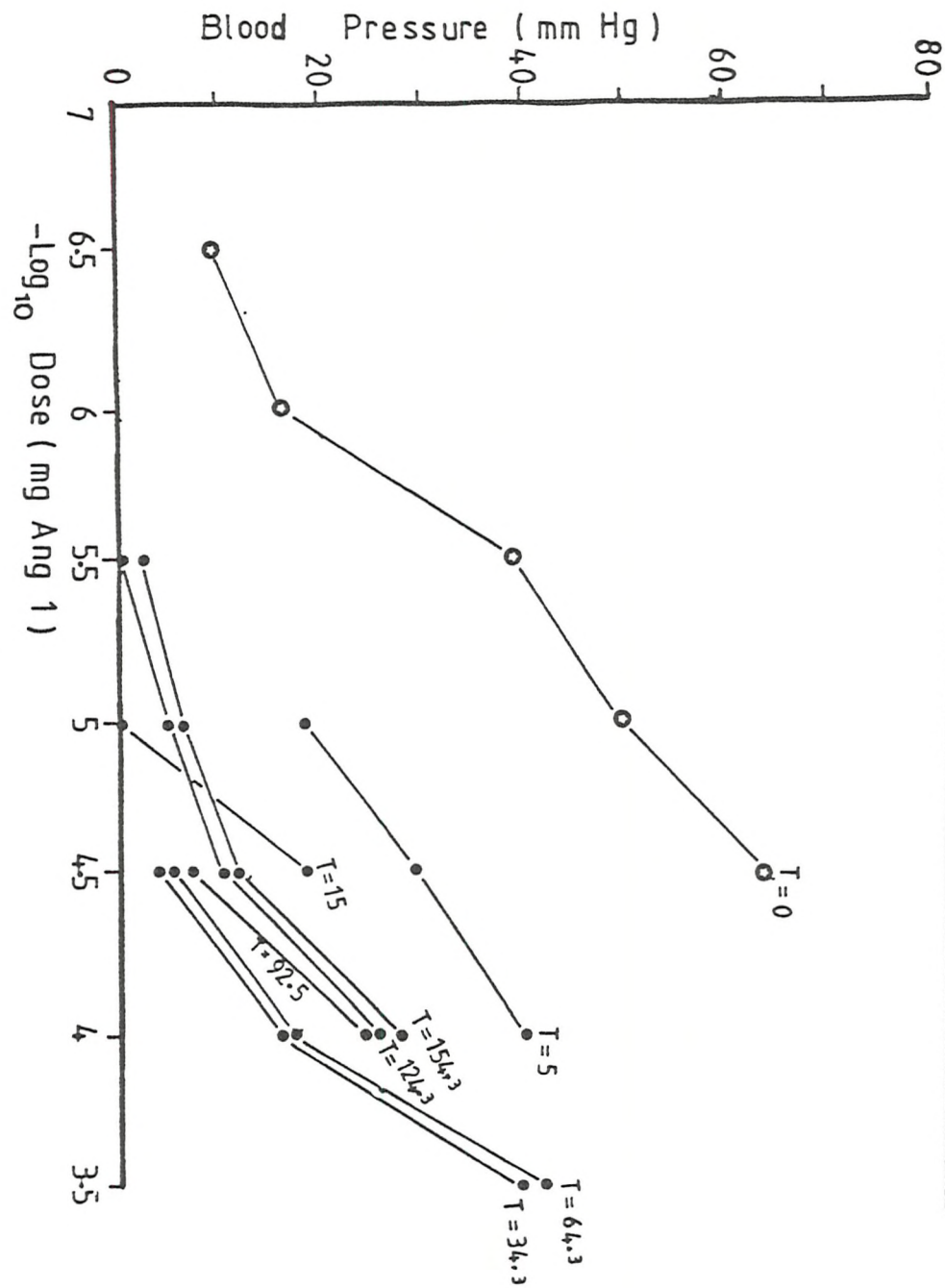


FIG. 6.3

CAPTOPRIL AND ENALAPRIL (MK 421) DECAY OF INHIBITION OF ANG I PRESSOR RESPONSE - DOSE NEEDED TO GIVE 25 mm Hg RISE IN BLOOD PRESSURE (D_{25}) (No.= 4)

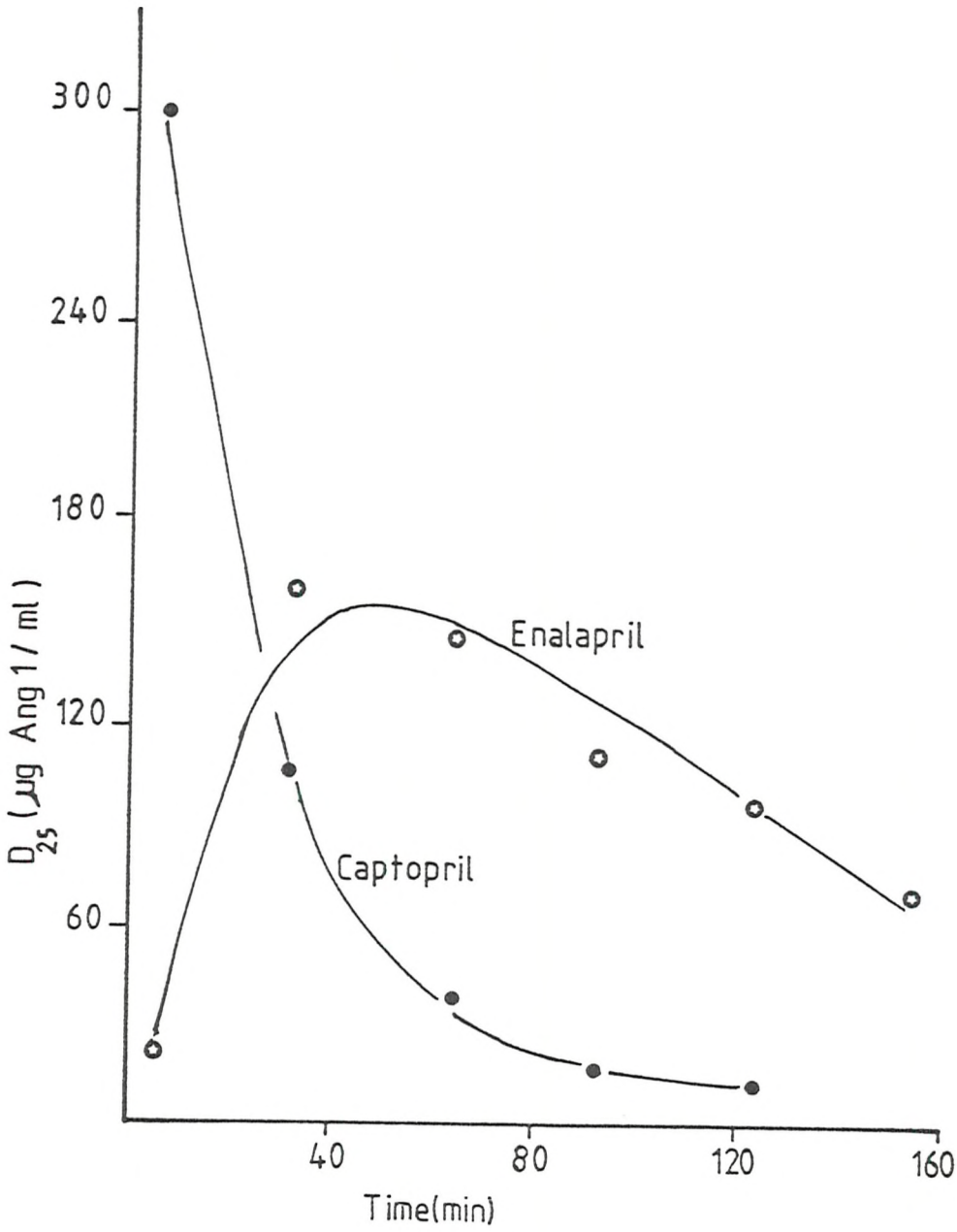


TABLE 6.1

Active and Inactive Renin in Conscious Sheep: Effect of Captopril

Clearance Period	Plasma Renin Activity (ng Ang I/ml/hr)		Inactive Renin (% of Total)	Plasma Active Renin (% of Period 1)
	Active Renin	Inactive Renin		
1	8.4 ± 1.4	3.0 ± 0.4	27.0 ± 2.8	100
2	8.1 ± 1.7	2.7 ± 0.2	28.6 ± 5.2	102.7 ± 22.6
3	7.9 ± 1.3	2.7 ± 0.4	25.7 ± 2.2	100.5 ± 15.7
4	7.7 ± 1.3	2.5 ± 0.6	24.2 ± 6.1	93.8 ± 10.3
5	8.1 ± 1.4	3.0 ± 1.0	25.2 ± 3.6	114.8 ± 29.9
6	7.4 ± 1.4	2.6 ± 0.4	26.2 ± 3.4	102.9 ± 30.2
7	7.0 ± 1.1	2.7 ± 0.5	28.5 ± 4.3	91.5 ± 16.8
1	9.3 ± 1.9	2.7 ± 0.5	23.8 ± 2.9	100
2	9.1 ± 1.4	2.8 ± 0.4	24.5 ± 3.3	106.5 ± 13.2
→	Captopril as a bolus injection			
3	19.4 ± 2.6	4.7 ± 1.4	18.3 ± 5.1	247.0 ± 42.9
4	40.1 ±10.1	8.4 ± 3.8	15.4 ± 4.5	423.7 ± 51.7
5	54.1 ±12.8	14.7 ± 8.0	14.6 ± 5.4	590.0 ± 67.8
6	67.5 ±15.3	12.5 ± 4.6	14.3 ± 4.4	758.5 ±101.8
7	55.1 ±10.2	21.8 ± 9.5	23.3 ± 4.4	617.8 ± 46.0

Results are shown as mean ± s.e.m. (n = 6)

Top = Controls

Bottom = Captopril given as a bolus injection (0.5 mg/kg)

For the statistical analysis of the results, see Fig. 6.4

FIG. 6.4

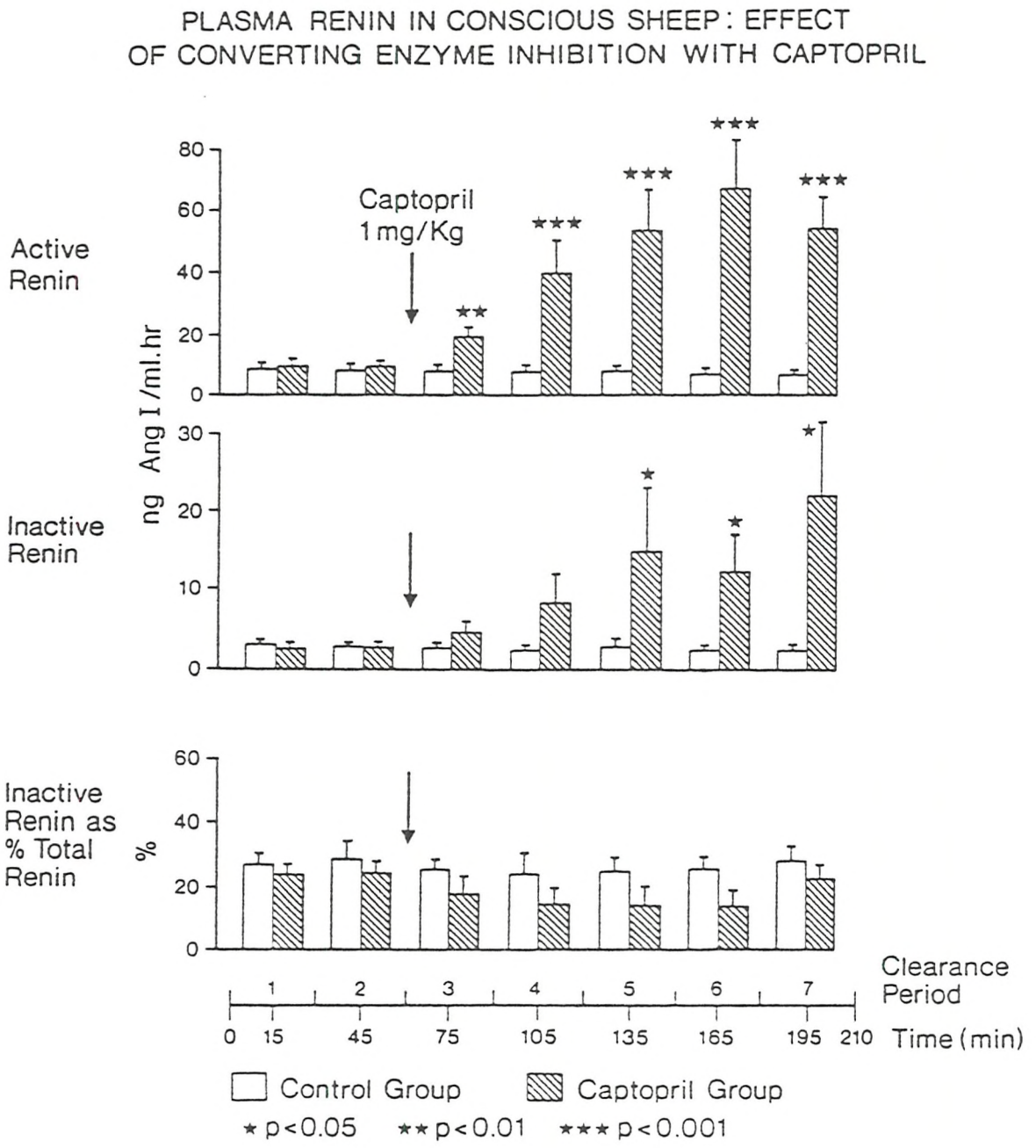

PLASMA RENIN IN CONSCIOUS SHEEP: EFFECT OF CONVERTING ENZYME INHIBITION WITH CAPTOPRIL
Active Renin
Inactive Renin
Inactive Renin as % Total Renin
ng Ang I /ml.hr
%
Captopril 1 mg/Kg
Clearance Period
Time (min)
Control Group
Captopril Group
★ p<0.05 ★★ p<0.01 ★★★ p<0.001

TABLE 6.2

Active and Inactive Renin in Conscious Sheep: Effect of Enalapril (MK.421)

Clearance Period	Plasma Renin Activity (ng Ang I/ml/hr)		Inactive Renin (% of Total)	Plasma Active Renin (% of Period 1)
	Active Renin	Inactive Renin		
1	8.4 ± 1.4	3.0 ± 0.4	27.0 ± 2.8	100
2	8.1 ± 1.7	2.7 ± 0.2	28.6 ± 5.2	102.7 ± 22.6
3	7.9 ± 1.3	2.7 ± 0.4	25.7 ± 2.2	100.5 ± 15.7
4	7.7 ± 1.3	2.5 ± 0.6	24.2 ± 6.1	93.8 ± 10.3
5	8.1 ± 1.4	3.0 ± 1.0	25.2 ± 3.6	114.8 ± 29.9
6	7.4 ± 1.4	2.6 ± 0.4	26.2 ± 3.4	102.9 ± 30.2
7	7.0 ± 1.1	2.7 ± 0.5	28.5 ± 4.3	91.5 ± 16.8
1	7.9 ± 0.8	2.4 ± 0.3	23.6 ± 3.5	100
2	8.4 ± 1.1	2.3 ± 0.3	21.7 ± 1.2	105.5 ± 7.8
——→	Enalapril (Mk.241) as a bolus injection			
3	12.1 ± 1.0	4.1 ± 1.4	22.5 ± 5.6	153.8 ± 7.3
4	25.6 ± 5.6	7.0 ± 1.1	23.1 ± 38.	316.3 ± 56.0
5	35.5 ± 5.0	9.8 ± 1.8	21.6 ± 3.3	440.7 ± 28.0
6	49.1 ± 8.7	12.8 ± 3.5	19.3 ± 1.8	615.7 ± 81.3
7	58.3 ±10.2	18.7 ± 5.6	22.5 ± 2.3	729.1 ± 97.9

Results are shown as mean ± s.e.m. (n = 6)

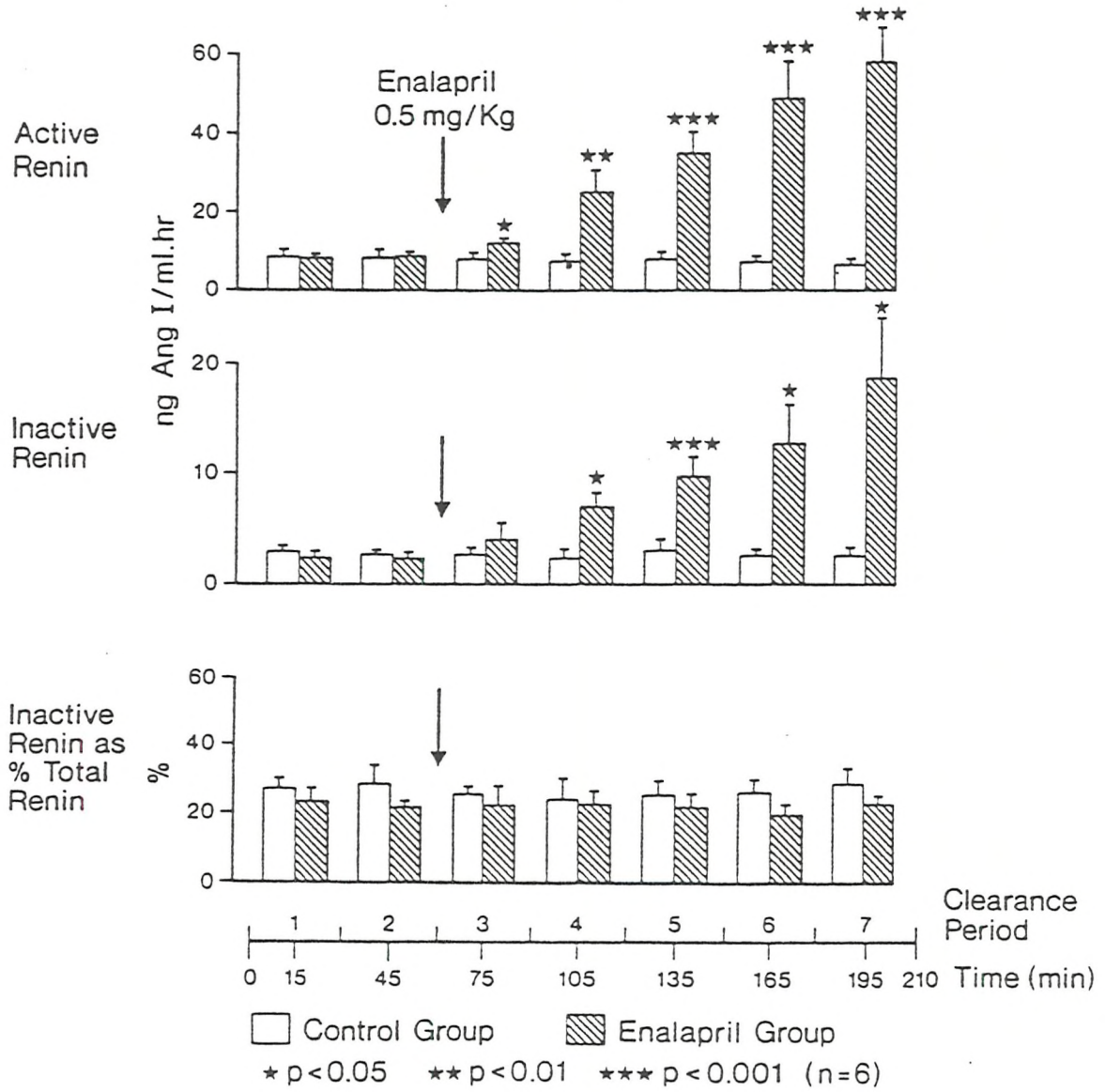
Top = Controls

Bottom = Enalapril given as a bolus injection (0.5 mg/kg)

For the statistical analysis of the results, see Fig. 6.5

FIG. 6.5

PLASMA RENIN IN CONSCIOUS SHEEP : EFFECT OF CONVERTING ENZYME INHIBITION WITH ENALAPRIL



may have been reached 105 min after administration of the drug and thereafter active renin values started to return towards the control levels. A further series of blood samples taken over a longer time course would have provided confirmation of this trend but this data is not available.

Changes in plasma inactive renin followed a basically similar time course and pattern to the changes in plasma active renin. Inactive renin concentration increased from 2.7 $\pm$ 0.5 ng Ang I/ml.hr (control) to 4.7 $\pm$ 1.4 ng Ang I/ml. hr (period 3), but this was not statistically significant. Later, 105 min after captopril injection (period 6), plasma inactive renin increased by 380% ($P < 0.05$) compared to the corresponding controls. With the next clearance period (period 7), unlike active renin, inactive renin continued to rise and increased from 2.7 $\pm$ 0.5 ng Ang I/ml. hr (period 1) to 21.8 $\pm$ 9.5 ng Ang I/ml.hr (period 7) ($P < 0.05$), an increase of 707% ($P < 0.02$) in comparison to the equivalent controls. The relative amount of active to inactive renin, as shown by calculation of the percentage of total renin which was inactive, did not change significantly (Fig.6.4).

In the second series, enalapril (0.5 mg/kg) was given exactly as in the captopril study. Both active and inactive renins steadily increased after enalapril (Table 6.2, Fig.6.5). In the sample taken 15 min after enalapril was given active and inactive renins increased from 7.9 $\pm$ 0.8 ng Ang I/ml. hr and 2.4 $\pm$ 0.3 ng Ang I/ml. hr respectively, (control, period 1) to 12.1 $\pm$ 1.0 ng Ang I/ml.hr and 4.1 $\pm$ 1.4 ng Ang I/ml.hr (period 3). Later, 105 min after injection of enalapril (period 6), plasma active renin further increased to 564% ($P < 0.001$) and inactive renin to 392% ($P < 0.02$) above control values. In plasma samples retained 135 min (period 7) after enalapril administration both active

and inactive renins had still not reached peak values. Plasma active renin concentration increased to 58.3 ± 10.2 Ang I/ml hr and inactive renin to 18.7 ± 5.6 ng Ang I/ml hr, rises of 733% for active and 592% for the inactive renin compared to the controls. As active and inactive renin increased approximately in parallel there were no significant changes in their relative proportions (Fig. 6.5). Results for enalapril were then essentially similar to those for captopril.

Figs. 6.6, 6.7 show mean arterial blood pressure, GFR, and ERPF. Both captopril and enalapril reduced arterial blood pressure by a mean of 7 mm Hg compared to the controls. This depressor effect was maintained for both inhibitors for the duration of the experiments. No substantial variations in GFR, or ERPF were obserfed.

Data for Na^+ and K^+ excretion, urine flow rates $[Na^+]$, $[K^+]$ in serum and urine are shown in Table 6.3 for the captopril group and in Table 6.4 for the enalapril group. Neither inhibitor produced significant changes in any of these variables.

Discussion

The effects of ACEI, on renin secretion and on other aspects of renal function, are of great interest, from both a therapeutic standpoint in the treatment of hypertension and from the point of view of their use as physiological tools for studying the renin-angiotensin system.

Captopril (1 mg/kg) and enalapril (0.5 mg/kg) were both given as a single i.v. dose. The effect of these drugs on plasma renin activity is thought to result mainly from inhibition of the negative feedback of Ang II on the JG cells *(Bernard et al., 1980)*. The results obtained in conscious sheep (Figs. 6.4, 6.5) showing an increase in plasma active renin

FIG. 6.6

EFFECT OF CAPTOPRIL ON ARTERIAL BLOOD PRESSURE, GFR AND ERPF

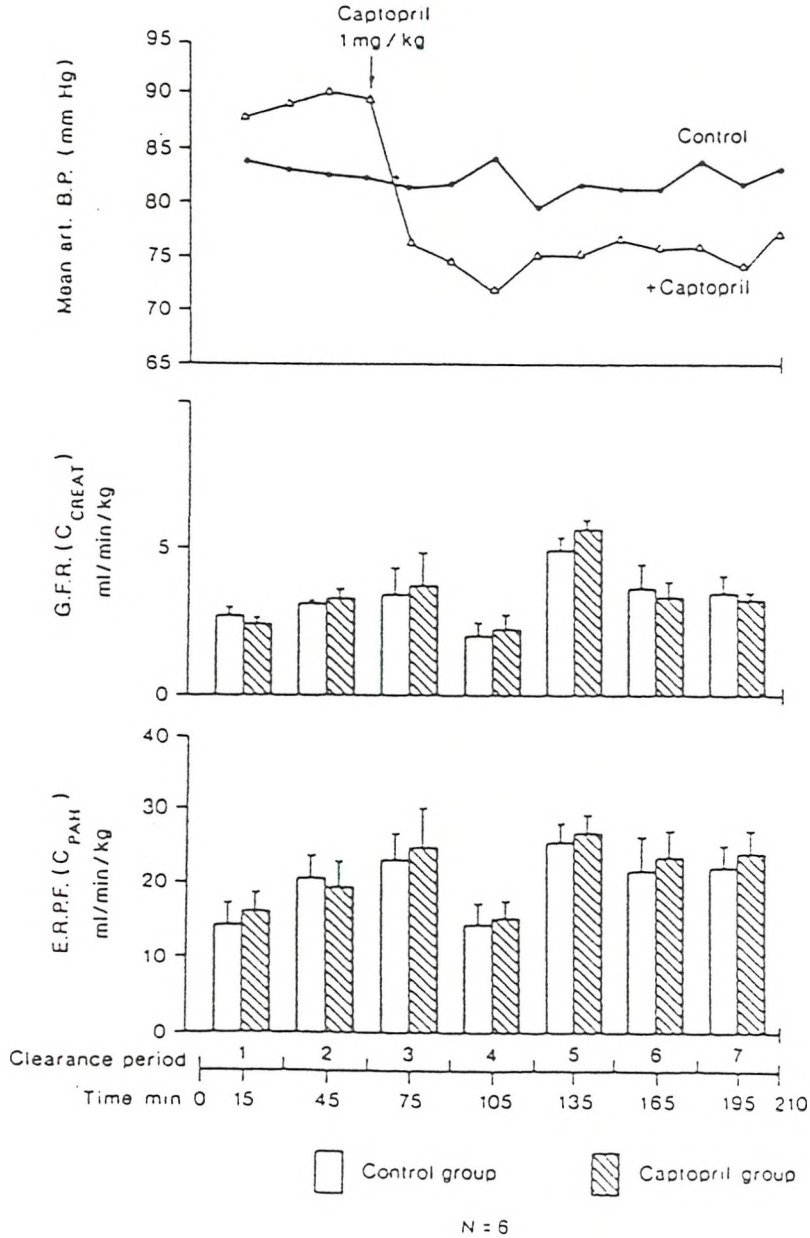


FIG. 6.7

EFFECT OF ENALAPRIL (MK 421) ON ARTERIAL BLOOD PRESSURE, GFR AND ERPF

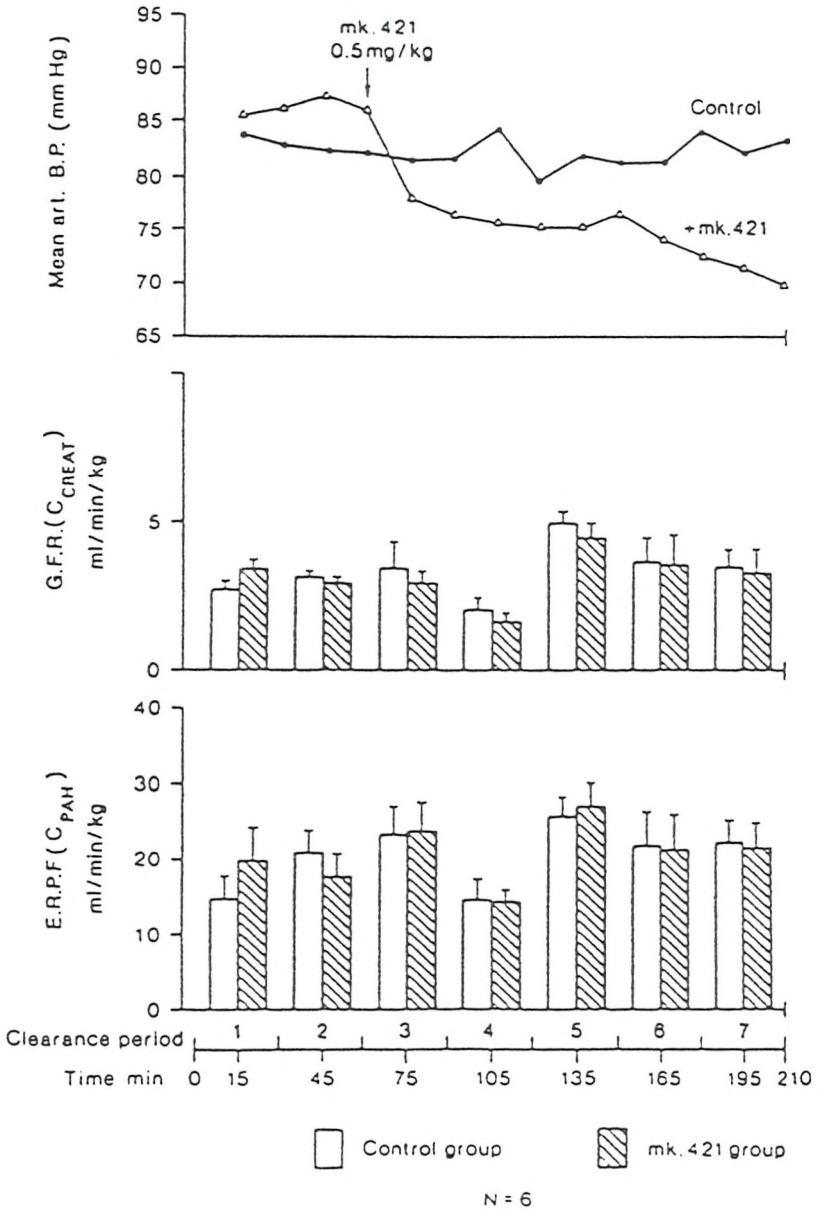


TABLE 6.3

EFFECT OF CAPTOPRIL ON RENAL FUNCTION IN CONSCIOUS SHEEP

Clearance period	1	2	3	4	5	6	7
Time (min)	0	30	60	90	120	150	180	210
Captopril bolus (1 mg/kg)			↓				
Urine flow (ml/hr/kg)							
Exp.	1.8 ± 0.2	2.4 ± 0.6	3.6 ± 0.6	1.8 ± 0.6	2.4 ± 0.1	1.8 ± 0.6	2.4 ± 0.7
Control	3.0 ± 1.2	1.8 ± 0.6	3.0 ± 0.7	1.2 ± 0.5	1.8 ± 0.2	1.8 ± 0.7	1.8 ± 0.6
Na^+ excretion (µmol/min/kg)							
Exp.	3.6 ± 1.1	4.5 ± 1.4	5.9 ± 0.3	3.0 ± 0.3	3.9 ± 0.6	3.7 ± 0.2	3.2 ± 0.4
Control	4.6 ± 1.5	3.2 ± 0.7	4.8 ± 0.7	2.0 ± 0.5	3.3 ± 0.9	2.9 ± 0.8	2.9 ± 0.9
K^+ excretion (µmol/min/kg)							
Exp.	4.1 ± 0.6	5.9 ± 1.2	4.6 ± 0.6	2.8 ± 0.1	3.7 ± 0.4	2.9 ± 0.3	2.3 ± 0.1
Control	4.7 ± 0.9	5.0 ± 0.9	3.4 ± 0.9	2.0 ± 0.4	5.0 ± 0.4	3.6 ± 0.8	2.5 ± 0.4

Results are shown as the mean ± S.E. of mean and are compared to control experiments performed in the same animals (n = 6). There are no statistically significant changes.

(Table 6.3is continued on the next page)

TABLE 6.3

EFFECT OF CAPTOPRIL ON RENAL FUNCTION IN CONSCIOUS SHEEP

Clearance period	1	2	3	4	5	6	7
Time (min)	0	30	60	90	120	150	180	210
Captopril bolus (1 mg/kg)			↓				
Urine $[Na^+]$ (m.mol/l)							
Exp.	99.9±22.6	112.6±15.8	101.8±5.6	111.6±21.1	104.6±12.7	128.0±21.0	111.7±24.6
Control	105.5±23.5	106.5±17.1	104.8±8.9	109.5±18.2	91.3±18.1	108.0±30.7	114.3±33.6
Plasma $[Na^+]$ (m.mol/l)							
Exp.	139.7±3.7	148.3±2.4	152.8±2.8	147.7±3.6	154.3±5.4	148.3±5.3	144.7±5.4
Control	139.2±3.0	146.2±2.9	151.0±2.7	146.3±4.1	153.8±4.3	146.5±6.1	145.3±4.4
% of filtered Na^+ excreted							
Exp.	1.0 ± 0.3	1.4 ± 0.3	1.5 ± 0.4	1.1 ± 0.2	0.4 ± 0.1	0.8 ± 0.1	0.7 ± 0.1
Control	1.1 ± 0.3	1.1 ± 0.2	1.2 ± 0.3	0.7 ± 0.1	0.4 ± 0.1	0.6 ± 0.2	0.6 ± 0.2

Results are shown as the mean ± S.E. of mean and are compared to control experiments performed in the same animals (n = 6). There are no statistically significant changes.

(Table 6.3 is continued on the next page)

TABLE 6.3

EFFECT OF CAPTOPRIL ON RENAL FUNCTION IN CONSCIOUS SHEEP

Clearance period	1	2	3	4	5	6	7
Time (min)	0	30	60	90	120	150	180	210
Captopril bolus (1 mg/kg)			↓				
Urine $[K^+]$ (m.mol/l)							
Exp.	127.0±10.1	193.1±59.4	75.7±6.4	111.7±21.9	103.2±12.6	96.6±13.8	86.3±15.2
Control	123.8±14.5	200.3±59.2	98.8±14.7	149.7±28.2	118.0±14.1	97.0±10.8	111.0±19.3
Plasma $[K^+]$ (m.mol/l)							
Exp.	4.1 ± 0.3	4.1 ± 0.3	4.0 ± 0.1	3.9 ± 0.1	3.8 ± 0.2	3.9 ± 0.4	4.0 ± 0.2
Control	4.1 ± 0.2	3.9 ± 0.3	4.1 ± 0.2	4.1 ± 0.3	4.1 ± 0.3	3.9 ± 0.2	4.2 ± 0.3
% of filtered K^+ excreted							
Exp.	46.1±5.8	47.9±11.1	40.5±10.5	43.0±12.0	16.3±1.0	22.4±1.0	19.8±3.0
Control	44.2±5.4	43.1±7.5	39.9±7.8	38.2±6.9	19.1±1.5	21.5±2.7	22.5±5.7

Results are shown as the mean ± S.E. of mean and are compared to control experiments performed in the same animals (n = 6). There are no statistically significant changes.

TABLE 6.4

EFFECT OF ENALAPRIL (MK 421) ON RENAL FUNCTION IN CONSCIOUS SHEEP

Clearance period	1	2	3	4	5	6	7
Time (min)	0 30	60	90	120	150	180	210
Enalapril bolus (0.5 mg/kg)		↓					
Urine flow (ml/hr/kg)							
Exp.	3.6 ± 1.2	2.4 ± 0.7	18. ± 0.2	1.2 ± 0.6	1.8 ± 0.3	1.2 ± 0.3	1.2 ± 0.2
Control	3.0 ± 0.6	1.8 ± 0.6	3.0 ± 0.7	1.2 ± 0.5	1.8 ± 0.2	1.8 ± 0.7	1.8 ± 0.6
Na^+ excretion (µmol/min/kg)							
Exp.	5.9 ± 1.9	3.9 ± 0.8	3.6 ± 0.6	1.1 ± 0.2	2.5 ± 0.9	2.3 ± 1.0	2.5 ± 1.1
Control	4.6 ± 1.5	3.2 ± 0.7	4.8 ± 0.7	2.0 ± 0.5	3.3 ± 0.9	2.9 ± 0.8	2.9 ± 0.9
K^+ excretion (µmol/min/kg)							
Exp.	5.6 ± 1.3	4.0 ± 0.5	3.8 ± 0.2	1.9 ± 0.2	3.8 ± 0.2	2.3 ± 0.4	2.7 ± 0.5
Control	4.7 ± 0.9	5.0 ± 0.9	3.4 ± 0.9	2.0 ± 0.4	5.0 ± 0.4	3.6 ± 0.8	2.5 ± 0.4

Results are shown as the mean ± S.E. of mean and are compared to control experiments performed in the same animals (n = 6). There are no statistically significant changes.

(Table 6.4is continued on the next page)

TABLE 6.4

EFFECT OF ENALAPRIL (MK 421) ON RENAL FUNCTION IN CONSCIOUS SHEEP

Clearance period	1	2	3	4	5	6	7
Time (min)	0	30	60	90	120	150	180	210
Enalapril bolus (0.5 mg/kg)			↓				
Urine $[Na^+]$ (m.mol/l)							
Exp.	122.0±28.6	102.9±17.2	113.9±14.6	91.7±14.9	137.8±74.0	81.7±27.2	96.7±31.2
Control	105.5±23.4	106.5±17.1	104.8±8.9	109.5±18.2	91.3±18.1	108.0±30.7	114.3±33.6
Plasma $[Na^+]$ (m.mol/l)							
Exp.	138.7±2.0	144.0±3.2	148.7±2.4	144.5±4.2	150.1±2.7	140.3±6.0	146.0±2.4
Control	139.2±3.0	146.2±2.9	151.0±2.7	146.3±4.1	153.8±4.3	146.5±6.1	145.3±4.4
% of filtered Na^+ excreted							
Exp.	1.0 ± 0.4	0.9 ± 0.2	0.9 ± 0.1	0.5 ± 0.4	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.1
Control	1.1 ± 0.3	1.1 ± 0.2	1.2 ± 0.3	0.7 ± 0.1	0.4 ± 0.1	0.6 ± 0.2	0.6 ± 0.2

Results are shown as the mean ± S.E. of mean and are compared to control experiments performed in the same animals (n = 6). There are no statistically significant changes.

(Table 6.4 is continued on the next page)

TABLE 6.4

EFFECT OF ENALAPRIL (MK 421) ON RENAL FUNCTION IN CONSCIOUS SHEEP

Clearance period	1	2	3	4	5	6	7
Time (min)	0	30	60	90	120	150	180	210
Enalapril (0.5 mg/kg)			↓					
Urine $[K^+]$ (m.mol/l)								
Exp.	134.8±18.9	119.5±22.0	113.2±13.3	159.7±26.7	184.6±46.0	112.5±13.5	135.5±16.0
Control	123.8±14.5	200.3±59.0	98.8±14.7	149.7±28.2	118.0±14.1	97.0±10.8	11.0 ±9.3
Plasma $[K^+]$ (m.mol/l)								
Exp.	3.9 ± 0.3	3.8 ± 0.3	3.5 ± 0.2	3.8 ± 0.4	3.5 ± 0.2	3.4 ± 0.2	3.6 ± 0.3
Control	4.2 ± 0.3	4.3 ± 0.4	3.7 ± 0.2	3.5 ± 0.2	3.7 ± 0.2	3.3 ± 0.3	3.9 ± 0.3
% of filtered K^+ excreted								
Exp.	44.2±6.9	36.6±3.5	39.3±2.5	35.7±4.3	22.5±2.6	23.8±3.8	30.4±5.6
Control	44.2±5.4	43.1±7.5	39.9±7.8	38.2±6.9	19.1±1.5	21.5±2.7	22.5±5.7

Results are shown as the mean ± S.E. of mean and are compared to control experiments performed in the same animals (n = 6). There are no statistically significant changes

are consistent with reports from other laboratories for studies on human subjects *(Bernard et al., 1980; Heel et al., 1980; Mikami et al., 1982; Fallo et al., 1983; Sullivan, 1983).*

It has been shown in this study that, for example, 135 min after administration of captopril and enalapril, plasma active renin was increased by 687% and 733% respectively (control vs experimental, clearance period 7)(Fig.6.4&6.5). Furthermore, both captopril and enalapril also increased inactive renin concentration in plasma by 707% and 592%. *Millar, Hammat & Johnston (1980)* studied the effect of a single dose captopril on plasma levels of active and inactive renin in salt-replete and salt-deplete normal subjects and found that inactive renin remained constant in both groups. However, plasma active renin and Ang I were increased after treatment with captopril. Reports from other laboratories on the effect of captopril on plasma inactive renin have not generally followed a pattern consistent with the results in this thesis.

Sealey et al. (1981), from a study in previously untreated hypertensive patients, found that acute administration of captopril increased active but not inactive renin. Similar data for anaesthetized rats was published by *Barrett, Eggena & Sambhi (1982)* and for human subjects by *Morganti et al. (1982).* We have no explanation for the apparent discrepancy between these results and our own but suggest that the following aspects of the experimental design employed might be important: species used, the fact that the animals were studied in the absence of anaesthetic interference and were not allowed to become volume depleted, and the time course of the study. In human subjects there is a vast excess of inactive renin in plasma compared to the active form. It is, therefore, more difficult to measure changes in

inactive renin in human subjects than a species such as the sheep which has more directly equivalent concentrations of the two forms of renin in plasma.

A report by *Goto et al. (1983)*, suggested that the increase in plasma renin activity following captopril administration to hypertensive patients may be due to the conversion of inactive renin to the active form. This certainly did not appear to be the case in this study since both active and inactive renin increased in parallel (Fig.6.4).

There were no substantial differences between the effects of the two ACEI, captopril and enalapril, either on active or inactive renin secretion or the other aspects of the renal function monitored. However, it is worthwhile to note that there were differences in the effects of the two inhibitors on the pressor responses to Ang I. The dose response curve (Fig. 6.1, 6.2) and D_{25} data (Fig.6.3), show that the inhibitory effect of enalapril started slower than that of captopril and the duration of action was longer than for captopril. The curve (Fig.6.2) was still substantially shifted to the right even 155 min after enalapril administration, while with captopril recovery was much quicker. Our results in this respect are consistent with *Takata, Nicolantonio & Hutchinson (1982)*. It is interesting that the differences in time course for recovery from the inhibition of the Ang I pressor response by the two drugs was not mimiced in their effects on renin secretion. This aspect of the study may warrant further investigation, perhaps over a more extended time course.

This study has also confirmed that enalapril is more potent than captopril. The dose of enalapril used (0.5 mg/kg) was only half of the dose of captopril (1.0 mg/kg), but produced comparable effects on

renin secretion. Captopril lowers blood pressure in hypertensive patients and normotensive subjects for six to eight hours. This effect depends on the Na balance of the subjects and the activity of the renin-angiotensin system *(Rubin & Reid, 1983)*. These effects of captopril and other ACEI on PRA and mean arterial blood pressure support the hypothesis that the renin-angiotensin system contributes to maintaining elevated blood pressure in some forms of hypertension *(Heel et al., 1980; Fallo et al., 1983)*.

Captopril was suspected to have other hypotensive actions in addition to effects on the renin-angiotensin system. These relate to the kallikrein-kinin and prostaglandin systems *(Heel et al., 1980)*, since ACE is the same as kininase II (one of the enzymes responsible for the degradation of bradykinin into inactive fragments), some effects of captopril on the kallikrein-kinin and prostaglandin systems could be anticipated *(Heel et al., 1980)*. *Rubin et al., (1981)* and *Mikami et al. (1982)* were however unable to demonstrate participation of either the kallikrein-kinin system or prostaglandins in the hypotensive response to captopril. This thesis does not contribute to this area of study as it proved impossible to measure sheep urinary kallikrein using conventional techniques.

Generally speaking, neither captopril nor enalapril produced any significant acute changes in GFR, ERPF, Na^+ and K^+ excretion, urine flow rates or $[Na^+]$ and $[K^+]$ in serum and urine. Although $[K^+]$ in urine tended to increase in the enalapril treated group, mainly in the first three clearance periods (period 3, 4, 5) after inhibitor administration (Table 6.4). These changes were not statistically significant. Our results in this respect are in contrast to those reported for studies in dogs by *Zimmerman & Wong (1981)*, who found that ACEI produced renal

vasodilation and natriuresis. Results presented here are in agreement with a report by *Nelson et al. (1982)*, who found that i.v. infusion of captopril and enalapril in conscious sheep, decreased arterial blood pressure and increased plasma renin concentration, but did not alter urinary Na^+ excretion. They suggested that urinary Na^+ excretion in the sheep is not as dependent on Ang II as has been reported in other species. There may be other aspects of the methodology used which could affect the results obtained.

CHAPTER VII

ACTIVE AND INACTIVE RENIN IN CONSCIOUS AND ANAESTHETIZED SHEEP: EFFECT OF HAEMORRHAGE

CHAPTER VII

ACTIVE AND INACTIVE RENIN IN CONSCIOUS AND ANAESTHETIZED SHEEP: EFFECT OF HAEMORRHAGE

Haemorrhage-induced renin secretion was first described by *Hamilton & Collins (1942)*. Since then haemorrhage has been recognized as a potent, but complex, stimulus for renin release and has been widely studied in so far as the active form of renin is concerned.

Haemorrhage may increase renin secretion through activation of arterial stretch receptors (intrarenal baroreceptors) *(Skinner, McCubbin & Page, 1964; Blaine & Davis, 1971)*, by an increase in renal sympathetic nerve activity *(Taher et al., 1976; Bunag, Page & McCubbin, 1966)*, or by an increase in circulating catecholamines *(Vander, 1965; Ueda et al., 1970; Reid, Schrier & Earley, 1972)*. Haemorrhage may also stimulate renin secretion via the macula densa by a decrease in sodium delivery to the distal nephron *(Vander Miller, 1964; Witty et al., 1971)*. Furthermore, haemorrhage may promote renin secretion through the renal PG system *(Henrich et al., 1978)*.

Quite recently, the role played by the renal perfusion pressure, the sympathetic β-adrenoceptor and the renal PGs, on renin release during haemorrhage was investigated in anaesthetized dogs by *Henrich, Schrier & Berl (1979)*. They also concluded that mechanisms regulating renin secretion following haemorrhage are multifactorial.

Both active and inactive renin in plasma are increased after haemorrhage in anaesthetized rabbits *(Richards et al., 1979)*. No investigation of the effect of haemorrhage on both forms of renin has, however, been reported from studies using conscious animals. Anaesthetics have a marked effect on renin secretion mechanisms. Furosemide diuresis in anaesthetized sheep increased

plasma active renin and inactive renin become undetectable. No change in either form of renin occurred during furosemide diuresis in conscious sheep *(Lush et al., 1983)*. Therefore, the effect of haemorrhage on plasma active and inactive renin was investigated using both conscious and anaesthetized sheep. An abstract describing this work has been published *(Mzail & Noble, 1983)*.

Methods

Animals were prepared under thiopentone/halothane/nitrous oxide anaesthesia with indwelling artery, vein and bladder catheters, as previously described (See Chapter III, Page 100).

Two series of experiments were carried out in this study. In the first group, six sheep were studied while conscious and only minimally restrained. A control blood sample was taken and then the animals were haemorrhaged by withdrawing 10% of calculated blood volume through the arterial catheter, over a period not exceeding 5 min. It was assumed that total blood volume was equivalent to 8% of the body weight. A further series of six blood samples was collected at 30 min intervals. Three successive daily samples were also obtained (See Fig. 7.1).

In the second group of four animals a similar protocol was followed except that, after two initial control blood samples had been taken while the animals were conscious, anaesthesia was induced with an i.v. injection of 25 mg/Kg sodium pentobarbitone (Sagatal; May & Baker). Anaesthesia was maintained during the experiment with 120 - 150 mg supplements of pentobarbitone. A level of anaesthesia was maintained in which the corneal reflex was suppressed.

In order to avoid changes in renin secretion due to the changes

in posture, the sheep were suspended in an upright posture during anaesthesia with padded slings placed under the four limbs and neck (See Fig. 7.2). Two further control samples were taken at 30 min intervals after induction of anaesthesia and then the animals were haemorrhaged exactly as for the first group. A further series of six blood samples was then collected at 30 min intervals (See Fig. 7.3).

Active and total plasma renin concentrations were measured by radioimmunoassay of generated Ang. I as described (See Chapter II, Page 72).

Results

For the first group of 6 conscious animals, data for active and inactive renin are shown in Table 7.1. Active renin as a percentage of the initial control sample (clearance period 1) and inactive renin as a percentage of total renin are also shown in the same table. In Fig. 7.1, the results for plasma active and inactive renin concentration and for inactive renin as a percentage of tota- renin are presented diagramatically.

Both active and inactive renin increased following haemorrhage. Active renin increased from 11.1 $\pm$ 2.2 ng Ang. I/ml. hr (before haemorrhage) to 14.2 $\pm$ 2.4 ng Ang. I/ml. hr ($P < 0.01$), 30 min after haemorrhage (Fig. 7.1). Inactive renin increased from 3.3 $\pm$ 0.5 ng Ang. I/ml. hr (before haemorrhage) to 5.4 $\pm$ 0.6 ng Ang. I/ml. hr ($P < 0.01$), 60 min after haemorrhage. Although both active and inactive renin in plasma increased significantly after haemorrhage, changes were unexpectedly small, a maximum mean increase of 72% for active renin and 86% for inactive renin. In addition, a plateau

TABLE 7.1

Active and Inactive Renin in Conscious Sheep:

Effect of Haemorrhage (10% of Calculated Total Blood Volume)

Collection Time (min)	Plasma Renin Activity (ng Ang I/ml/hr)		Inactive Renin (% of Total	Plasma Active Renin (% of Period 1)
	Active Renin	Inactive Renin		
0 →	11.1 ± 2.2	3.3 ± 0.5	24.0 ± 2.3	100
	Start of haemorrhage (10% of calculated total blood volume			
30	14.2 ± 2.4	3.5 ± 0.5	20.3 ± 2.0	132.1 ± 4.1
60	16.8 ± 2.7	5.4 ± 0.6	25.1 ± 1.3	158.9 ± 15.3
90	16.4 ± 3.0	6.0 ± 0.8	27.6 ± 1.6	154.8 ± 18.9
120	17.3 ± 2.2	5.6 ± 0.8	24.3 ± 2.0	172.1 ± 23.8
150	15.9 ± 1.8	5.4 ± 0.7	25.4 ± 1.8	161.2 ± 24.1
180	15.4 ± 2.0	4.7 ± 0.7	24.0 ± 2.3	154.4 ± 23.3
24 (hr)	9.7 ± 2.0	3.6 ± 0.9	26.4 ± 2.2	96.6 ± 17.8
48 (hr)	9.2 ± 1.7	2.4 ± 0.6	21.0 ± 1.5	87.4 ± 12.7
72 (hr)	10.6 ± 2.0	3.2 ± 0.5	23.3 ± 2.2	97.5 ± 5.8

Results are shown as mean ± s.e.m. (n = 6)

For the statistical analysis of the results, see Fig. 7.1

FIG. 7.1

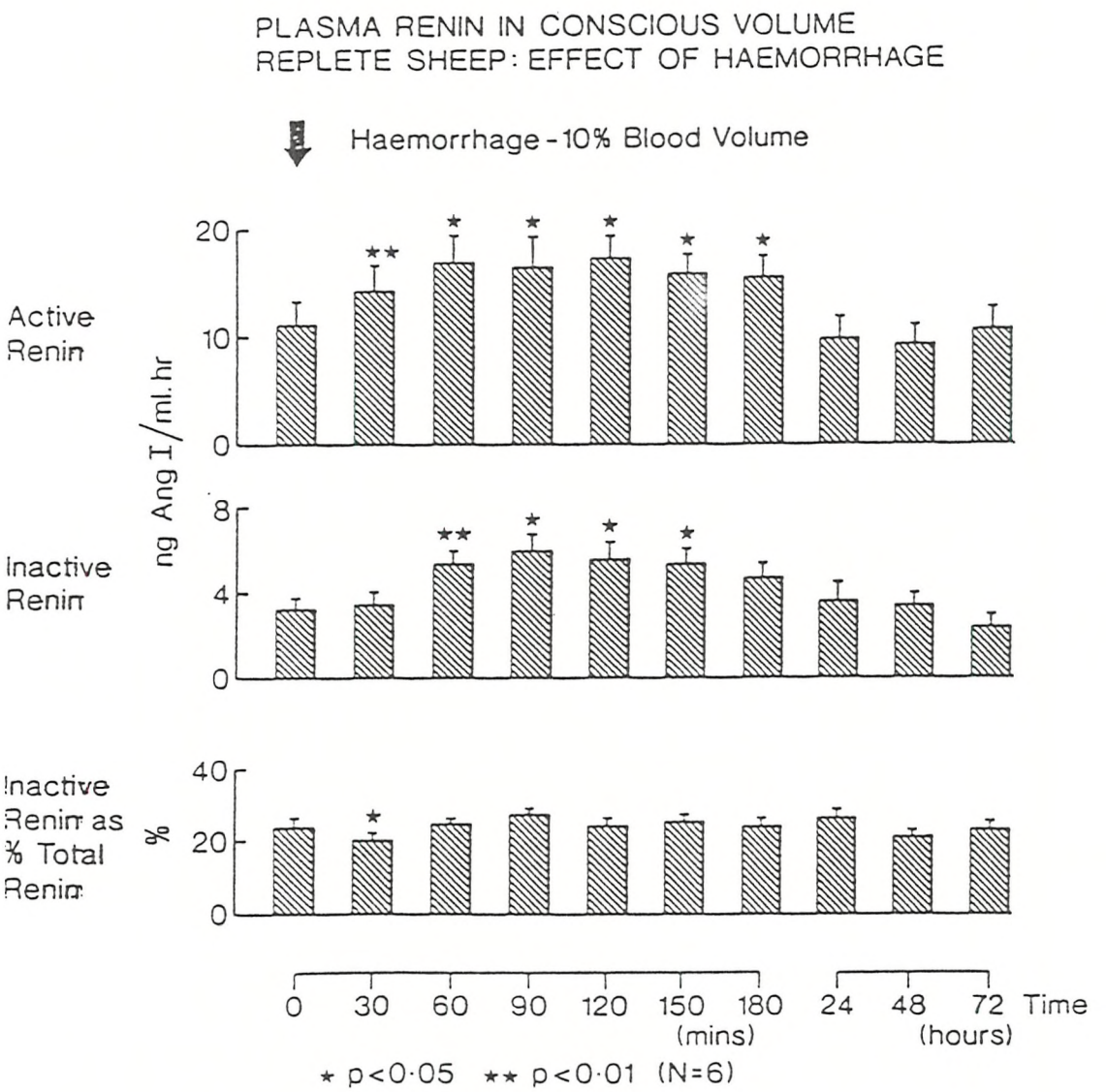

PLASMA RENIN IN CONSCIOUS VOLUME REPLETE SHEEP: EFFECT OF HAEMORRHAGE
Haemorrhage - 10% Blood Volume
Active Renin
Inactive Renin
Inactive Renin as % Total Renin
ng Ang I/ml.hr
%
20
10
0
8
4
0
40
20
0
0 30 60 90 120 150 180 (mins)
24 48 72 (hours)
Time
* p<0·05 ** p<0·01 (N=6)

FIG. 7.2

PHOTOGRAPH OF ANAESTHETIZED SHEEP DURING AN EXPERIMENT

TABLE 7.2

Active and Inactive Renin in Anaesthetized Sheep:

Effect of Haemorrhage (10% of Calculated Total Blood Volume)

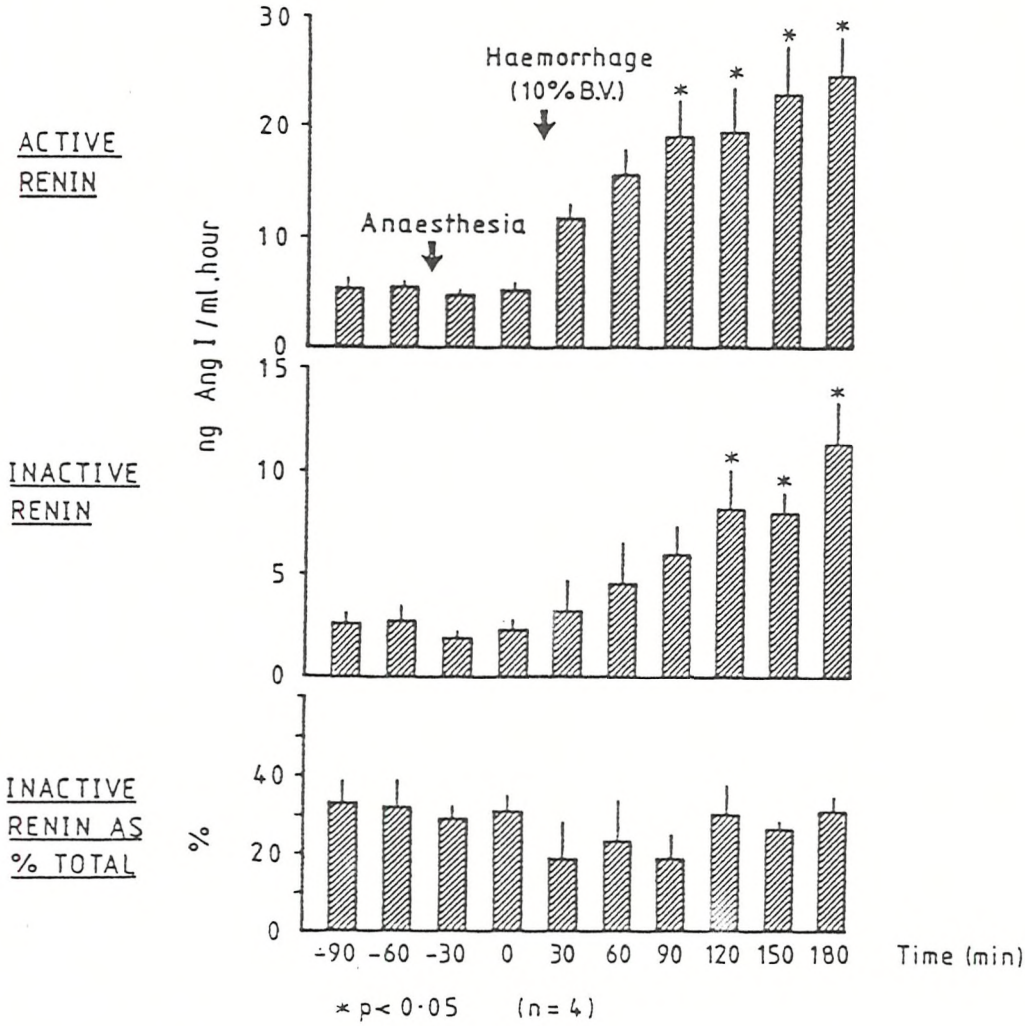
Collection Time (min)	Plasma Renin Activity (ng Ang I/ml/hr)		Inactive Renin (% of Total)	Plasma Active Renin (% of Period 1)
	Active Renin	Inactive Renin		
0	5.4 ± 1.0	2.6 ± 0.5	33.5 ± 5.4	100
30	5.5 ± 0.5	2.7 ± 0.7	32.4 ± 6.7	118.4 ± 32.0
	→ Induction of anaesthesia			
60	4.8 ± 0.5	1.9 ± 0.2	29.2 ± 3.0	99.1 ± 24.5
90	5.2 ± 0.8	2.3 ± 0.4	31.3 ± 4.0	105.1 ± 20.2
	→ Start of haemorrhage (10% of calculated total blood volume)			
120	11.6 ± 1.3	3.2 ± 1.5	18.8 ± 9.7	239.4 ± 49.0
150	15.4 ± 2.3	4.5 ± 2.0	23.5 ±10.6	300.0 ± 84.8
180	18.8 ± 3.3	5.9 ± 1.4	18.6 ± 6.2	378.5 ± 79.0
210	19.2 ± 4.0	8.1 ± 1.9	30.5 ± 7.4	402.4 ±108.1
240	22.6 ± 4.4	7.9 ± 1.0	26.7 ± 1.8	467.9 ±113.0
270	24.3 ± 3.5	11.3 ± 2.0	31.6 ± 4.0	503.0 ±111.4

Results are shown as mean ± s.e.m. (n = 4)

For the statistical analysis of the results, see Fig. 7.3

FIG. 7.3

PLASMA RENIN IN PENTOBARBITONE-ANAESTHETISED SHEEP: EFFECT OF HAEMORRHAGE



response was achieved after 60 min for both active and inactive renins. At 180 min, post-haemorrhage plasma active and inactive renin concentrations were 15.4 ± 2.0 ng Ang. I/ml. hr and 4.7 ± 0.7 ng Ang. I/ml. hr respectively (seventh blood sample). This suggests that plasma active and inactive renins were starting to return to control levels only 3 hr after the haemorrhage. Both forms of renin had returned to baseline 24 hr after haemorrhage and remained so 48 hr and 72 hr post-haemorrhage (Fig.7.1).

Table 7.2 shows the data for plasma active and inactive renin concentrations for the second group of four pentobarbitone-anaesthetized sheep. Active renin as a percentage of the initial sample and inactive renin as a percentage of total renin are also shown in the same table. The results are expressed diagramatically in Fig. 7.3.

Pentobarbitone anaesthesia *per se* did not affect either active or inactive renin in plasma since there were no significant differences between the control blood samples before (blood sample 1, 2) or after (blood sample 3, 4) induction of anaesthesia (Fig.7.3). Both plasma active and inactive renin increased significantly after haemorrhage, active renin increased from 5.2 ± 0.8 ng Ang. I/ml. hr before haemorrhage, (blood sample 4) to 19.2 ± 4.0 ng Ang. I/ml. hr ($P < 0.05$), 2 hr after haemorrhage (blood sample 8). Inactive renin also increased from 2.3 ± 0.4 ng Ang. I/ml. hr before haemorrhage (blood sample 4) to 8.1 ± 1.9 ng Ang. I/ml. hr ($P < 0.05$), after haemorrhage (blood sample 8). Haemorrhage in this group of anaesthetized animals therefore produced much more dramatic changes in plasma renins than in the group of conscious sheep. When the final blood sample was taken, 180 min after the haemorrhage, plasma

active renin had increased by a mean of 303% and inactive renin by 299%. This can be compared with changes of just 72% for active and 86% for inactive renin in the conscious animal group. A further point of interest is that in the anaesthetized sheep a plateau response for plasma renin was not reached within the time course of the study. In both anaesthetized and conscious animals, there were no significant changes in the relative amounts of active and inactive renin (Figs. 7.1, 7.3).

A typical trace of aortic blood pressure in an individual animal before, during, and after induction of pentobarbitone anaesthesia is shown in Fig. 7.4. Blood pressure was stable when the animal was conscious and pentobarbitone anaesthesia initially had a depressor action which lasted less than 10 min. This was followed by a phase of increased blood pressure and then, 20 min after induction of anaesthesia, the blood pressure became stable again. This was, however, at a level higher than when the animal was conscious. There was no correlation between the time course of the increase in the two forms of renin and the changes in blood pressure.

Discussion

Haemorrhage is widely considered to be a very potent stimulus for renin secretion but in this study some doubts are raised about this. When six conscious sheep were subjected to a 10% reduction in their blood volume, although both active and inactive renin increased significantly the response (Fig. 7.1) was less marked than previous reports had suggested *(Scornik & Paladini, 1964; Jakschik et al., 1974; Henrich, et al., 1978; Henrich et al., 1979 and Richards et al., 1979).*

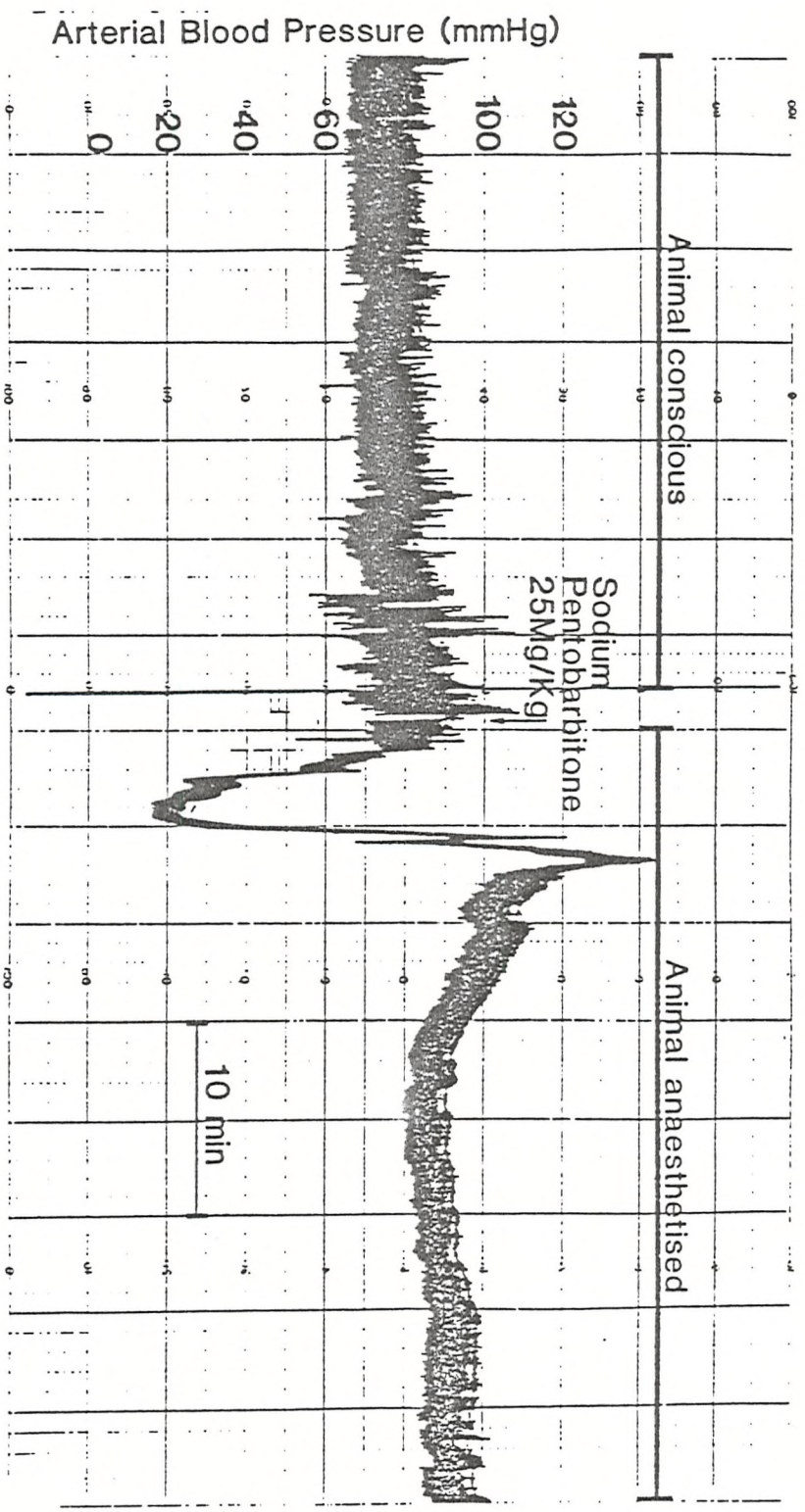


FIG. 7.4

Aortic Blood Pressure in a Sheep before, During and After Induction of Pentobarbitone Anaesthesia

The differences between these studies might be partly attributable to the time course used or to species variations but it was felt that the effect of anaesthetic agents could be the most interesting to pursue. This was indeed the case since, in the second group of this study (anaesthetized animals), haemorrhage produced quantitatively greater changes in both active and inactive renin (Fig. 7.1). Results from this group are more in context with published reports concerning haemorrhage responses in anaesthetized animals.

There are several interesting points which have emerged during this study. First, this is the first report comparing the effect of haemorrhage on plasma renins in anaesthetized and conscious animals. Secondly, although in the two groups of animals the responses of active and inactive renin to haemorrhage are qualitatively the same, quantitatively are very different. Thirdly, in conscious animals, a plateau response was reached after 60 min for both renins with a maximum mean increase of 72% for the active and 86% for the inactive renin (Fig. 7.1). In pentobarbitone anaesthetized sheep a plateau response was not achieved even 3 hr post-haemorrhage when active and inactive renin had increased by a mean of 303% and 299% respectively (Fig. 7.3). Fourthly, haemorrhage did not change the relative proportions of the two forms of renin in either group of animals. This is in contrast to some other experimental manipulations we have studied (See Chapter IV).

Basically, results in this study, are consistent with other published reports of studies of the effect of haemorrhage on active and inactive renin secretion. In a study in anaesthetized dogs, haemorrhage caused a rise in both active and inactive renin

(James & Hall, 1974). It has also been reported that during suppression of renin release by expansion of the extracellular fluid volume in anaesthetized pigs, both forms of renin decreased *(Bailie, Derkx & Schalekamp, 1979)*. *Richards et al. (1979)*, from their study in anaesthetized rabbits, found that both forms of renin in plasma increased after haemorrhage. The same authors *(Richards et al., 1979)* showed that, after ligation of the renal blood vessels, neither form of renin in plasma responded to haemorrhage. Renins released during haemorrhage were therefore of renal origin.

It is not yet fully clear how haemorrhage stimulates renin secretion. Studies using the nonfiltering, denervated dog kidney preparation suggested that an intrarenal baroreceptor, the macula densa and the renal nerves are all participating in the renin response to acute haemorrhage *(Witty et al., 1971)*. A similar conclusion was reached by *Henrich et al. (1979)* on the basis of studies in animals with normally functioning kidneys. *Hodge, Lowe & Vane (1966)* reported that application of local anaesthetics inhibits renin release in response to haemorrhage, thus indicating a role for the renal nerves.

If we assume that stimulation of renin secretion during haemorrhage involves multifactorial mechanisms, the study reported here raises a new question. Why is the increase in both forms of renin relatively small in conscious animals but much higher in pentobarbitone-anaesthetized animals, following exactly the same level of haemorrhage. It is possible that either the primary changes in blood pressure during induction of anaesthesia or the anaesthesia itself might produce the dramatic increase in renin secretion in anaesthetized

animals. From the typical trace of aortic blood pressure of an individual animal (Fig. 7.4) it can be seen that the blood pressure dropped suddenly during induction of anaesthesia. It is unlikely that this was the basis of the difference in the response of plasma renin in conscious and anaesthetized sheep. Anaesthesia per se did not effect PRA either in this study (Fig. 7.3) or in earlier studies from our laboratory in which this was studied over a longer time course *(Lush et al., 1983)*. Although the blood pressure dropped during induction of anaesthesia, within approximately 20 min the blood pressure had stabilised again and at a level higher than when the animal was conscious.

A possible basis for the differences in haemorrhage response in anaesthetized and conscious animals lies with the renal prostaglandin system. Haemorrhage activates PG release in both conscious *(Vatner, 1974)* and anaesthetized *(Henrich et al., 1978)* dogs. Under other circumstances, it has been found that renal PG release is higher in anaesthetized than in conscious animals *(Terragno, 1977)*. Furthermore, *Romero, Dunlap & Strong (1976)* found that inhibition of PG synthesis abolishes the renin response to haemorrhage in conscious rabbits. On the other hand, *Henrich et al. (1979)* reported that, in pentobarbitone anaesthetized dogs, haemorrhage significantly stimulated renin secretion in animals pre-treated with indomethacin.

Although, from this study, we do not present any direct evidence, it seems likely that the enhanced stimulatory effect of haemorrhage on active and inactive renin secretion in anaesthetized animals might be related to increased intrarenal PG synthesis.

CHAPTER VIII

TRYPTIC ACTIVATION OF INACTIVE RENIN IN SHEEP PLASMA

CHAPTER VIII

TRYPTIC ACTIVATION OF INACTIVE RENIN IN SHEEP PLASMA

Introduction

Acidification has been used to activate inactive renin for all the work so far described in this thesis. For human inactive renin activation can also be achieved by using proteolytic enzymes such as trypsin *(Cooper et al., 1974; Leckie et al., 1977; Sealey et al., 1979; Noth, Cariski & Havelick, 1980 and Rappelli et al., 1981)*. Theoretically, trypsin could activate renin via an effect on one of the coagulation or fibrinolytic factors. However, *Sealey et al. (1979)* found that neither Hageman factor nor pre-kallikrein (Fletcher factor) participates in tryptic activation, since activation can take place in plasma deficient in these factors. The same authors reported that activation of human inactive plasma renin by trypsin is more rapid at -4°C than at 6°, 25° or 37°C. On the other hand, *Osmond & Cooper (1978)* concluded that trypsin at near-optimal concentrations consistently activated human plasma inactive renin (prorenin) whether at 4°, 23° or 37°C. This indicated that cold was not an essential concomitant of tryptic activation.

These techniques (cryoactivation, acid and tryptic activation) have been discussed elsewhere (page 32) and have been recently reviewed by *Leckie (1981)* and by *Sealey et al. (1982)*.

As tryptic activation is one of the three common methods used to investigate inactive renin, particularly in human studies, it was decided to evaluate tryptic activation of sheep inactive renin. This was considered important partly because there appear to be species differences in the apparent amount of inactive renin in plasma.

There is disagreement concerning the optimal conditions necessary

to achieve full activation of inactive renin. An attempt was therefore made to identify conditions for reproducible activation by trypsin of sheep inactive renin with a view to possibly using this method routinely as an alternative to acidification.

Materials and Methods

Blood was collected from conscious male and female sheep through jugular vein cannulae into cooled centrifuge tubes containing disodium EDTA as anticoagulant (approximate final concentration = 1 mg/ml plasma). Plasma was either stored at -20°C or processed immediately. Solutions of trypsin and trypsin inhibitors were always freshly prepared. Sources of the chemicals used were as follows:

Trypsin (T-8253, SIGMA). Trypsin inhibitors used were SOYA BEAN (SBTI) TYPE-II-S, 1-S, (SIGMA, T-928), and LIMA BEAN (LBTI), TYPE-1-L, (SIGMA, T-9378).

Five groups of experiments were carried out and details of the experimental designs are included with the results.

For all the groups, inactive renin was estimated as plasma renin concentration in the samples which had been treated with trypsin minus plasma renin concentration in the control samples. Renin concentrations of all samples were determined by RIA of generated Ang I (see page 68), values are presented as a mean ± S.E. of the mean for renin concentration data and also as a percentage, taking control samples as 100%. Student's paired t-test was used for the analysis of the data.

Results

Group 1 The aim of this experiment was to investigate the effect of different trypsin concentrations (0, 0.1, 0.5, 1.0, and 2.0 mg/ml plasma) on the activation of inactive renin in sheep plasma. Trypsin

was added to plasma samples collected from 6 different conscious sheep. The samples were divided into two aliquots and incubated as duplicates for 10 min at 37°C. In one batch of samples (group A), trypsin was then inhibited by the addition of SBTI in a concentration double the original trypsin concentration. In group B, SBTI was used at a concentration four times the original trypsin concentration.

Table 8.1 shows results for plasma renin concentration before (control, no trypsin added) and after treatment with trypsin. Under the conditions used in this experiment there was no evidence of activation of inactive renin by trypsin. On the contrary, plasma active renin concentration decreased as trypsin concentration increased (Table 8.1). This was the case for both concentrations of SBTI and so did not seem to be related to the concentration of protease inhibitor used.

Group 2 Because there was no activation of inactive renin by trypsin in the first group of experiments, it was decided to change the incubation time and temperature, i.e., reduce incubation temperature but use a longer period of time. Plasma samples were obtained from 6 different sheep. Trypsin was added in the same concentrations as in the first group of experiments. The samples were divided into two halves and incubated in duplicates at 23°C for 45 min. As in the first group of experiments, the trypsin was then inhibited by SBTI, again using concentrations double (group A) and four times (group B) higher than the corresponding trypsin concentration.

Table 8.2 shows the results obtained. Once again, as in the first group, trypsin failed to activate inactive renin. An inverse relationship was found between trypsin concentration and plasma active renin, the higher the trypsin concentration added, the lower the renin

TABLE 8.1

The Effect of Trypsin on Sheep Plasma Renin
(Incubated at 37°C for 10 min)

Plasma Renin Activity (ng Ang I/ml/hr)					
No Treatment (control)	Trypsin Concentration (mg/ml)				
	0.1	0.5	1.0	2.0	
15.9 ± 0.7	14.2 ± 0.6	13.3 ± 0.9	10.4 ± 1.1	7.1 ± 0.9	A
100%	89.8 ± 2.0% **	83.3 ± 2.9% **	66.6 ± 8.0% **	45.6 ± 6.8% ***	
14.7 ± 0.8	15.2 ± 1.3	13.9 ± 1.0	11.5 ± 1.7	8.4 ± 1.4	B
100%	104.4 ± 10.0%	94.4 ± 4.2%	77.3 ± 8.5% *	57.8 ± 9.2% **	

Results are shown as mean ± s.e.m. and as a percentage of control (n = 6).

Trypsin reaction was stopped by SBTI double (A) and four times higher (B) than trypsin concentration.

Statistical analysis of results was by paired student's t test.

* Denotes $P < 0.05$; ** Denotes $P < 0.01$; *** Denotes $P < 0.001$

TABLE 8.2

The Effect of Trypsin on Sheep Plasma Renin
(Incubated at 23°C for 45 min)

Plasma Renin Activity (ng Ang I/ml/hr)					
No Treatment (control)	Trypsin Concentration (mg/ml)				
	0.1	0.5	1.0	2.0	
18.2 ± 1.3	16.7 ± 0.6	13.6 ± 1.1	10.0 ± 1.2	7.0 ± 1.1	A
100%	93.3 ± 4.0%	77.3 ± 8.6% *	55.7 ± 6.1% ***	38.5 ± 5.1% ***	
16.9 ± 1.0	15.8 ± 1.3	15.4 ± 1.3	11.6 ± 1.4	6.9 ± 1.2	B
100%	94.2 ± 6.6%	93.1 ±11.4%	68.6 ± 7.1% **	40.6 ± 6.3% ***	

Results are shown as mean ± s.e.m. and as a percentage of control (n = 6).

Trypsin reaction was stopped by SBTI double (A) and four times higher (B) than trypsin concentration.

Statistical analysis of results was by paired Student's t test.

* Denotes $P < 0.05$; ** Denotes $P < 0.01$; *** denotes $P < 0.001$

concentration recorded. Results were again independent of the concentration of SBTI used to stop the reaction.

Group 3 As the results from the second group of experiments were again disappointing, it was decided to try varying the incubation time used. Therefore, a third experiment was carried out using the same incubation temperatures as before (37°C and 23°C) with a shorter incubation time.

Plasma was collected from 6 different sheep and trypsin was added to aliquots in one of the following concentrations: 0, 0.25, 0.5 or 1 mg/ml of plasma. The samples were divided into two batches. In the first batch, the samples were incubated in duplicates for 5 min at 37°C and in the second batch the samples were incubated at 23°C for 10 min. For all samples the trypsin reaction was stopped by adding LBTI in a concentration four times higher than the corresponding trypsin concentration.

The results are shown in Table 8.3. Trypsin still did not activate inactive renin and, as in the first two groups of experiments, there was a reduction in total plasma renin. However, the magnitude of the reduction was smaller than in the first and second groups of experiments.

Group 4 In view of problems experienced particularly with the high trypsin concentrations and the relatively long period of incubation it was decided to concentrate on the lowest trypsin concentration and to use a shorter period of incubation. In addition, we decided to include a third incubation temperature and to increase the trypsin inhibitor concentration to six times the corresponding trypsin concentration for the first time.

Trypsin was added in a concentration of either 0.1 or 0.2 mg/ml

TABLE 8.3

The Effect of Trypsin on Sheep Plasma Renin

(A = Incubated at 37°C for 5 min)
(B = Incubated at 23°C for 10 min)

Plasma Renin Activity (ng Ang I/ml/hr)				
No Treatment (Control)	Trypsin Concentration (mg/ml)			
	0.25	0.5	1.0	
12.2 ± 1.1	11.2 ± 0.9	10.5 ± 1.2	10.6 ± 1.3	A
100%	93.4 ±10.5%	87.5 ±10.0%	87.1 ±11.2%	
11.1 ± 0.5	9.6 ± 1.2	8.4 ± 2.0	9.0 ± 1.3	B
100%	87.0 ±10.4%	77.7 ±17.7%	76.4 ±11.6%	

Results are shown as mean ± s.e.m. and as a percentage of control (n = 6).

Trypsin reaction was stopped by LBTI four times higher than trypsin concentration.

Statistical analysis of results was by paired Student's t test. There are no statistically significant changes.

to plasma collected from 3 different sheep. The samples were each divided into 3 sets of aliquots. In the first set, the samples were incubated in duplicate at 37°C for 2 min and for the second set the samples were incubated at 23°C also for 2 min. The third set of samples was kept in the ice-tray for 5 min, at a temperature of approximately 4°C. Trypsin activity was then inhibited using LBTI in a concentration six times higher than trypsin in the first and second sets of samples and four times in the third.

As can be seen from Table 8.4, under these circumstances, neither activation nor a reduction in total plasma renin was observed.

Group 5 From the results of the fourth group of experiments (Table 8.4), it appears that a low incubation temperature (4°C) and the short period of incubation (5 min) are more effective in conserving plasma renin than the conditions previously used. However, it also appears that the low trypsin concentration used (0.1, 0.2 mg/ml of plasma) was not sufficient to activate inactive renin in such a short incubation period. We therefore wanted to see the effect of higher trypsin concentrations, but using a low temperature and in a short period of incubation. In addition, as both SBTI and LBTI had been used in previous experiments, it was necessary to check whether this affected the results obtained. Therefore, a fifth series of experiments was planned. Trypsin was added in a final concentration of 1mg/ml to plasma collected from 6 different sheep. The samples were divided into two halves and incubated in duplicate at 0°C for 3 min. In one half of each sample, trypsin activity was stopped by the addition of SBTI in a concentration five times higher than trypsin. To the second part of each sample, trypsin was inhibited with LBTI used in the same concentration as for SBTI.

TABLE 8.4

The Effect of Trypsin on Sheep Plasma Renin

(A = Incubated at 37°C for 2 min)
(B = Incubated at 23°C for 5 min)
(C = Incubated at 4°C for 5 min)

Plasma Renin Activity (ng Ang I/ml/hr)			
No Treatment (Control)	Trypsin Concentration (mg/ml)		
	0.1	0.2	
9.7 ± 0.5	8.7 ± 1.2	8.1 ± 1.1	A
100%	89.8 ±11.3%	84.0 ±12.1%	
9.9 ± 1.1	9.0 ± 2.1	9.0 ± 0.8	B
100%	88.9 ±12.0%	95.2 ±19.2%	
9.2 ± 1.1	8.9 ± 1.0	8.9 ± 1.1	C
100%	98.1 ± 9.2%	102.0 ±21.9%	

Results are shown as mean ± s.e.m. and as a percentage of control (n = 3).

Trypsin reaction was stopped by SBTI six times higher than trypsin concentration in A and B and four times higher than trypsin concentration in C.

Statistical analysis of results was by paired Student's t test. There are no statistically significant changes.

Table 8.5 shows the results obtained. There was an approximately 5%, non significant, increase in total plasma renin concentration, under these incubation conditions with no substantial differences between the LBTI and the SBTI treated samples. (Table 8.5).

Discussion

Dialysis to acid pH and back to pH 7.4 has been very widely used to activate inactive renin *(Lumbers, 1971; Day & Luetscher, 1974; Skinner et al., 1975b Derkx et al., 1976)*. Acid activation of inactive renin has also been evaluated in this laboratory and it has been found that inactive renin can be activated by acidification in rabbit plasma *(Grace et al., 1979b;Richards et al., 1979, 1981)*, in supernatant fluid of rabbit renal cortex slice preparations *(Ginesi et al., 1983)* and in sheep plasma *(Lush et al., 1983)*. (See also this thesis, page 90). In some other laboratories, activation of inactive renin is achieved by incubation with trypsin *(Morris & Lumbers, 1972; Osmond & Cooper, 1978; Cooper, Murray & Osmond, 1977; Sealey et al., 1979; Noth et al., 1980)*. These investigations have mainly been concerned with human inactive renin.

In this study, we have tried to define conditions for reproducible activation of sheep plasma inactive renin by trypsin. Sheep plasma renin was subjected at 37°C, 23°C, 4°C and 0°C to various treatments reported elsewhere to convert inactive renin into its active form in human and some animal plasmas. In all these studies trypsin failed to provide evidence of activation of inactive renin. Similar experiences have been reported from some other laboratories *(Sealey et al., 1979; Atlas et al., 1981)*.

The discrepancies between our results and those from some other

TABLE 8.5

The Effect of Trypsin on Sheep Plasma Renin
(Incubated for 3 min. in ice-bath)

Plasma Renin Activity (ng Ang I/ml/hr)		
No Treatment (Control)	Trypsin (1 mg/ml) + LBTI (5 mg/ml)	Trypsin (1 mg/ml) + SBTI (5 mg/ml)
15.1 ± 1.1	15.5 ± 2.3	16.0 ± 2.4
100%	104.2 ±17.5%	105.8 ±19.1%

Results are shown as mean ± s.e.m. and as a percentage of control (n = 6).

Statistical analysis of results was by paired Student's t test.

There are no statistically significant changes.

laboratories cannot be explained in terms of inadequate or inappropriate trypsin or trypsin inhibitor concentrations. In this study trypsin was used in concentrations up to 2 mg/ml of plasma and trypsin inhibitors were used in concentrations up to six times higher than the equivalent trypsin concentration. For human plasma, maximal tryptic activation will occur at a concentration of 0.5 mg/ml, as reported by *Cooper et al. (1977)*, and by *Noth et al. (1980)*, or with a concentration of 1 mg/ml, as reported by *Sealey et al. (1979)*. *Rappelli et al. (1981)* used 2 mg/ml trypsin.

It has been reported by *Sealey et al. (1979)* that the incubation temperature must be maintained below 37°C to avoid destruction of renin. Our results in this aspect are in agreement since a significant reduction in total plasma renin occurred on incubating the samples at 37°C (Table 8.1). However, we also noticed that the period of incubation is extremely important as 10 min incubation at 37°C reduced total plasma renin concentration by 54% (Table 8.1), whereas 5 min incubation at the same temperature produced just 12% reduction (Table 8.3). It was also shown that there were no substantial differences as a consequence of using different trypsin inhibitor concentrations (Tables 8.1, 8.2, 8.4). In other words, there was no correlation between the magnitude of the effect of trypsin and the concentration of trypsin inhibitor. In addition, there were no significant differences when using two different trypsin inhibitors SBTI and LBTI (Table 8.5).

Rappelli et al. (1981) found that trypsin induced activation of inactive renin in human plasma is independent of endogenous antitryptic activity and suggested that trypsin, when added to human plasma, may act rapidly and is not affected by the concentration of trypsin inhibitors. On the other hand, *Sealey et al.(1979)* have shown that activation of

inactive plasma renin by trypsin is more rapid at -4°C than at 6°, 25° or 37°C. It was suggested that this was because certain endogenous trypsin inhibitors are less effective at reduced temperature. Therefore, less trypsin is required to overcome them in cold plasma.

It appears from the current literature that there is no general agreement concerning either the optimal conditions for activation of inactive renin by trypsin or the mechanism by which trypsin activates inactive renin. Furthermore, renin from different animal species can be activated to varying degrees. Tryptic activation is relatively prominent in dogs, very modest in rabbits and absent in rats *(Osmond & Cooper, 1978)*.

Further investigations will indicate whether the existing variations in results represent true species differences or methodological variations. *Noth et al. (1980)* suggested that commercial trypsin preparations vary considerably in their activities. There are probably other factors which have not yet been discovered. Investigation of these possibilities could provide insight into the nature of inactive renin and the mechanisms of its activation in plasma by exogenous and endogenous means.

In relation to the work described elsewhere in this thesis, these studies with tryptic activation of sheep plasma inactive renin have failed to provide any evidence that this would be a preferable technique to acid activation for routine use.

CHAPTER IX

GENERAL DISCUSSION

CHAPTER IX

GENERAL DISCUSSION

Over the last two decades, investigation of the physiological mechanisms controlling renin secretion has been almost entirely concerned with the enzymatically active form of renin (Chapter I, page 34).

The situation became more complicated when, in 1971, *Lumbers* first suggested that there were also inactive but activatable forms of renin (see page 17). In man the concentration of inactive renin in plasma far exceeds that of active renin.

In this thesis, experimental work is described which examines the physiological mechanisms controlling plasma levels of both active and inactive renins. The experimental model used, the chronically catheterised conscious sheep, has a number of significant advantages compared to other preparations available. These are summarised on page 99. It was found that the response of the two forms of renin to physiological manipulations was not necessarily identical and depended on the nature of the stimulus involved.

β-adrenoceptor agonists stimulate active renin release. This concept is based on studies in vivo *(Vander, 1965; Assaykeen et al., 1970; Johnson et al., 1976; Richards et al., 1981b)*, investigations using isolated perfused rat kidneys *(Vandongen & Peart, 1974; Fray & Park, 1979)* and work using rabbit kidney cortex slices *(Richards et al., 1981b)*.

In the studies with isoprenaline and propranolol described in Chapter IV of this thesis and in studies with isoprenaline in vivo and in vitro by *Richards et al. (1981b)*, both active and inactive renin secretion were investigated. In conscious sheep, the effect

of isoprenaline stimulating active renin release was not matched by a corresponding increase in plasma inactive renin (Table 4.1, Fig. 4.1). This is in contrast to data published by *Richards et al. (1981)* from studies using anaesthetised rabbits in which isoprenaline increased both active and inactive renin. Isoprenaline also increased both forms of renin in the plasma of anaesthetized pigs *(Bailie et al., 1979)*. The difference in inactive renin secretion between these studies might be related to the presence of anaesthetic agents, but other possibilities, such as species differences and methodological variations cannot be excluded.

The isoprenaline-stimulated active renin secretion, was blocked by the β-blocker, propranolol (Fig. 4.5) but, strangely, propranolol on its own only suppressed plasma levels of inactive renin (Table 4.4 Fig. 4.3). Neither isoprenaline nor propranolol was associated with any significant change in creatinine or PAH clearances or Na^+ excretion rate. These studies provide further evidence of a dissociation between the responses of active and inactive renin secretion mechanisms to some physiological stimuli. The other work described here shows that this is by no means always the case.

In studies which are described in Chapter V of this thesis, verapamil increased both plasma active and inactive renin in conscious volume replete sheep (Table 5.1, Fig. 5.1). The most likely mechanism for the increased active and inactive renin secretion was considered to be a direct effect of verapamil on calcium fluxes into the JG cells. In vitro studies have shown that incubating rabbit kidney cortex slices in low $[Ca^{++}]$ buffers leads to increased secretion of both active and inactive renin *(Ginesi et al., 1983)*. These studies are not, however, directly analogous to the in vivo studies presented

in Chapter V. The changes in inactive renin in the low $[Ca^{++}]$ kidney slice experiments were quantitatively greater than for active renin. In a different study, *(Ginesi et al., 1982)*, using a similar in vitro preparation, verapamil selectively stimulated inactive renin secretion, both in the presence and absence of Ca^{++} ions. *Churchill (1980)*, *Churchill et al. (1981)* and *Park et al. (1981)* also using in vitro preparations demonstrated that calcium antagonist drugs, including verapamil, block the effects of membrane depolarisation and consequent calcium flux on renin release.

As an alternative interpretation, the effect of verapamil in vivo could be related to its vasodilator action. This was clearly reflected in a reduction in mean arterial blood pressure and an increase in PAH clearance (Fig.5.2). However, quantitatively greater furosemide induced changes in PAH clearances *(Lush et al., 1983)* were not associated with any changes in active or inactive renin secretion in conscious sheep.

Fray & Park (1979) concluded, on the basis of work using the isolated perfused rat kidney, that Ca^{++} ions play a central role in several mechanisms of renin release. They suggested that lowering perfusion pressure hyperpolarizes the JG cells, lowers the calcium permeability and stimulates renin release *(Fray, 1978)*. Furthermore, *Fray & Park (1979)* proposed that isoprenaline, through a β-adrenergic mechanism, inhibits Ca^{++} influx, stimulates Ca^{++} efflux and sequesters Ca^{++} into intracellular stores. All three effects would reduce cytoplasmic $[Ca^{++}]$ and stimulate renin release.

Although the work which has been described in Chapters IV and V in this thesis does not challenge these ideas in so far as the active form of renin is concerned, it is more difficult to rationalise the

results for inactive renin. For instance, if verapamil stimulates inactive renin release in a comparable way to active renin, why does isoprenaline fail to increase inactive renin release (see Chapter IV). Intrarenal control of renin secretion is clearly more complicated than was previously thought to be the case.

Although verapamil is presently used therapeutically mainly as an antiarrhythmic and antianginal agent, recent clinical studies have raised the possibility that calcium antagonists, possibly including verapamil, might also be of value in the management of acute hypertensive crises and in the treatment of essential hypertension *(Smith, 1983)*. The potential anti-hypertensive action of verapamil in relation to the two forms of renin is at present inconclusive. Administration of verapamil increased both active and inactive renin in plasma, although this appeared to be a transient response (Fig.5.1).

The renin-angiotensin system is perhaps the most important of several humoral vasoconstrictor and vasodilator mechanisms implicated in blood pressure regulation. Its end product, the octapeptide Ang II, can modulate vasoconstrictor activity and is also involved in volume homeostasis *(Mendelsohn, 1980)*. Ang II exerts a negative feedback effect on renin secretion from JG cells *(Davis, 1971; Sullivan, 1983)*. In the studies which are presented in Chapter VI of this thesis, this was blocked by inhibiting Ang I converting enzyme. Two ACEI, captopril and enalapril were used.

From the therapeutic point of view, using ACEI as antihypertensive drugs represents a new approach. Most antihypertensive therapy involves either interruption of the sympathetic nervous system, reducing total peripheral resistance by vasodilators or reducing the extracellular volume by diuretics *(Johnston et al., 1982)*. Captopril

is becoming a first choice antihypertensive drug (see page 150).

It is well known that captopril can induce an increase in plasma renin activity by inhibiting the negative feedback of angiotensin II on renin release *(Case et al., 1978; Heel et al., 1980; Mikami et al., 1982* and *Fallo et al., 1983)*. As far as active renin is concerned, the results of the studies discussed in Chapter VI are in agreement with these reports. Both captopril and enalapril increased plasma active renin concentration (Figs. 6.4, 6.5). It was not known what would happen to the inactive form of renin during the administration of ACEI. However, it was clearly demonstrated (Figs. 6.4, 6.5) that inactive renin in plasma also increased in parallel with active renin after administration of both captopril and enalapril. *Goto et al. (1983)* reported that in 9 of 20 hypertensive patients who received captopril, active renin increased while inactive renin decreased. On the other hand, in the remaining 11 patients both active and inactive renin increased slightly. The conflict between our results and the findings of *Goto et al.* could relate to species variations, the time course of the study, the dosage procedure or to differences in the way samples were processed for assay of active and inactive renin. In general it is much easier to exert adequate control over the design of experiments using experimental animals than human subjects.

It has been suggested that the antihypertensive effect of ACEI is not only related to blockade of the renin-angiotensin system. ACEI also potentiate the vasodepressor action of bradykinin *(Murthy et al., 1977; Murthy, Waldron & Goldberg, 1978; Heel et al., 1980)* and increase PG synthesis *(Swartz et al., 1980; Heel et al., 1980)*. *Overlack, Stumpe & Sealey (1980)* found that the hypotensive effect

of captopril was abolished in patients with normal or low-renin essential hypertension by infusion of aprotinin, but not in those with high plasma renin activity. It might be argued that the effects observed after aprotinin administration could be due either to inhibition of kallikreins or other enzymes not involved in the generation of kinins in vivo. Aprotinin is a polyvalent inhibitor which inhibits many other proteolytic enzymes as well *(Vogel & Werle, 1970)*.

Rubin et al. (1981) and *Mikami et al. (1982)* suggested that neither the kallikrein-kinin system nor the prostaglandin system is involved in the hypotensive activity of captopril. But *Carretero, Guillermo-Scicli & Maitra (1981)* proposed a compromise conclusion, that some of the pharmacological effects of ACEIs are due to an increase in kinin concentration either directly or through the release of PGs. Recently, it has been suggested that captopril and other ACEI in doses within the therapeutic range exert their antihypertensive action partly by inhibiting the facilitatory action of Ang II on neurogenic vasoconstriction which lead to a reduction in sympathetic tone. ACEI may also interfere with sympathetic reflexes *(Unger, Ganten & Lang, 1983)*. Whatever the mechanisms prove to be by which ACEI exert their anti-hypertensive action, the major goal of these present studies (Chapter VI) was to evaluate the effect of the two ACEI on inactive renin secretion. Both captopril and enalapril clearly increased inactive renin secretion in conscious sheep. Furthermore, it was interesting that both captopril and enalapril produced qualitatively similar effects on both active and inactive renin and also on the other aspects of the renal function monitored (Fig.6.6,6.7). In terms of the inhibition of the pressor action of Ang I, enalapril was slower in onset and lasted for a longer

period of time, compared to captopril (Fig.6.1,6.2). These findings are consistent with data published by *Takata et al. (1982)*.

In Chapter VII of this thesis, studies designed to investigate the effect of haemorrhage on plasma active and inactive renin are reported. Both conscious and anaesthetized sheep were used. Studies in dogs with non-filtering kidneys *(Witty et al., 1971)* and normal kidneys *(Henrich et al., 1979)* suggest that haemorrhage provides a multifactorial stimulus for renin release. This concept has been reviewed in more detail in Chapter VII (page 175).

Generally speaking, haemorrhage is thought to be a potent stimulus for renin secretion *(James & Hall, 1974; Richards et al., 1979)*. However it was found that the response of the active and inactive renin secretion mechanisms to the same level of haemorrhage in conscious animals (Fig. 7.1) was very much less than in anaesthetized animals (Fig. 7.3). It would, therefore, appear that some primary or secondary effect of anaesthesia enhanced the stimulatory effect of haemorrhage on renin secretion.

Lush et al. (1983) reported that furosemide diuresis at two dose levels did not alter plasma levels of either active or inactive renin in conscious sheep. However, administration of furosemide to anaesthetized sheep *(Lush et al., 1983)* progressively increased plasma active renin whilst inactive renin decreased to zero. Furthermore, it has been shown by the same authors and also in Figure that induction of pentobarbitone anaesthesia per se failed to alter either form of renin in plasma. The question which remains to be answered is, what is the basis for the difference in renin secretion between conscious and anaesthetized sheep.

Anaesthesia has marked effects on renal PG synthesis. *Terragno,*

Terragno & McGiff (1977) showed that the concentration of PGE in renal venous blood of the chloralose-anaesthetized-laparotomized dogs was 8-fold greater than in conscious dogs. Indomethacin reduced renal blood flow by 40% in anaesthetized dogs but had no effect in conscious animals. In another study, *Swain et al. (1975)* concluded that prostaglandins play only a minor role in regulating the renal circulation in conscious animals, in contrast to studies in anaesthetized animals *(Lonigro et al., 1973)*.

The relation between PG and renin secretion is widely reported in the literature and has been discussed in Chapter I (page 51). Prostaglandins appear to increase renin release partly by a direct action on the JG cells *(Misono et al., 1977)*. In addition they may modulate renin release mediated by the intrarenal baroreceptor *(Gerber et al., 1981; Henrich , 1981)* or the macula densa mechanism *(Olson et al., 1980; Gerber et al., 1981)*. β-adrenoceptor mediated renin release is thought to be PG independent *(Seymour & Zehr, 1979; Olson, Nies & Gerber, 1983)*. It is possible therefore, that the difference between the responses of renin secretion to haemorrhage in conscious and anaesthetized sheep may be attributed to potentiation of PG synthesis in anaesthetized animals. On the other hand, anaesthetic agents have complex actions on vascular smooth muscle (see page 60) which have been reviewed by *Altura et al. (1980)*. One of these actions is a reduction in the movement of Ca^{++} ions across cell membranes. Since the JG cells from which renin is secreted are modified vascular smooth muscle cells *(Barajas, 1979)*, anaesthetics may also modify Ca^{++} fluxes in this tissue. The JG cells respond to a reduction in intracellular $[Ca^{++}]$ with an increase in renin secretion *(Fray, 1977; Park & Malvin, 1978; Fig 5.1)*.

Another interesting observation which emerged from these studies is that there was only a transient decrease in arterial blood pressure following induction of pentobarbitone anaesthesia (Fig. 7.4). Before the first blood sample for renin estimation was collected, the blood pressure was above its pre-anaesthetic level (Fig. 7.4). A similar finding was reported by *Lush et al. (1983)* and the elevated blood pressure was sustained for the duration of the experiment. Since a reduction in arterial blood pressure would tend to increase renin secretion by stimulating the intrarenal baroreceptor (see page 34), the elevated blood pressure in anaesthetized animals would be expected to suppress, rather than to potentiate the effect of haemorrhage on renin secretion. It is, therefore, highly unlikely that the transient reduction of arterial blood pressure could explain the difference in plasma renin response to haemorrhage between the conscious (Fig. 7.1) and anaesthetized sheep (Fig.7.3).

The control of renin secretion from the kidney is effected through several mechanisms (see page 34). The concept of different release mechanisms for the two renins has been proposed by *Rumpf et al. (1978)*. In this laboratory, it has also been suggested that differential secretion of active and inactive renin is regulated by an intrarenal sodium sensitive mechanism. This may be located at the macula densa *(Richards et al., 1981; Noble et al., 1981)*. Although other reports from our laboratory suggest that this receptor will only function after it has been primed by other intra- or extra-renal factors, such as prostaglandins *(Lush et al., 1983)*.

The changes in active, inactive and total renin observed in the experimental work described in this thesis are summarized in Table 9.1 From these results, it could be suggested that the β-adrenoceptor

TABLE 9.1

SUMMARY OF THE EFFECT OF VARIOUS STIMULI ON ACTIVE AND INACTIVE RENIN

		ACTIVE RENIN	INACTIVE RENIN
Verapamil	Conscious	↑	↑
Isoprenaline	Conscious	↑	–
Prpranolol	Conscious	–	↓
Isoprenaline+propranolol	Conscious	–	–
Captopril	Conscious	↑	↑
Enalapril	Conscious	↑	↑
Haemorrhage	Conscious	↑	↑
Haemorrhage	Anaesthetised	↑	↑

mechanisms may be involved in the activation of inactive renin, in addition to its role in the control of active renin secretion.

Other mechanisms such as changes in intracellular $[Ca^{++}]$ produced by Ca^{++} channel blocking drugs, the negative feedback mechanism of Ang II on renin secretion and the intrarenal-baroreceptor exert equivalent effects on both active and inactive renin secretion.

In view of the results which have been discussed in the different chapters of this thesis, it appears that a fruitful area for future research would be to define in more detail the physiological mechanisms leading to inactive renin secretion. A preparation free from anaesthetic effects is essential for this work. It may then be confirmed that differential secretion of the two forms of renin is a regulatory step for the whole renin system and is important in controlling blood pressure and electrolyte homeostasis.

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