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UNIVERSITY OF SOUTHAMPTON

ACTIVATION AND SECRETION OF INACTIVE RENIN

A thesis presented for the degree of
Doctor of Philosophy

by

Laura Mary Ginesi



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UNIVERSITY OF SOUTHAMPTON
ABSTRACT
FACULTY OF MEDICINE
PHYSIOLOGY AND PHARMACOLOGY
Doctor of Philosophy
ACTIVATION AND SECRETION OF INACTIVE RENIN
by Laura Mary Ginesi

The renin-angiotensin system is important in the regulation of extracellular fluid volume and the maintenance of arterial blood pressure. The control of this system is complex, but a major determinant of its activity is the secretion of renin by granular cells in the kidney cortex. In recent years, an inactive, but activatable form of this enzyme has been identified in the kidneys of most species, and it is thought to be secreted into plasma. A potential site for control of the renin-angiotensin system could involve differential secretion of these two forms of renin, and this may involve a sodium-sensitive mechanism.

A recent hypothesis suggests that a kallikrein could be the physiological activator of inactive renin. In accordance with this proposal, the serine protease inhibitor, Trasylol, was found to inhibit acid-activation of inactive renin in rabbit plasma. However, in urethane-anaesthetised rabbits, the inhibition caused no immediate change in circulating levels of either form of renin. In contrast, both forms of renin were reduced after a delay which appeared to be related to the accumulation of Trasylol within renal tissue. Furthermore, high concentrations of the inhibitor reduced the release of both forms of renin by rabbit kidney cortex slices in vitro.

It is concluded that plasma kallikrein is not the primary factor which determines the relative amounts of circulating active and inactive renin.

Calcium is important for stimulus-secretion coupling in glandular and muscle cells, where it is thought to play a stimulatory role. In contrast, recent work has shown that active renin secretion by the kidney is inhibited by calcium. Studies using the rabbit kidney cortex slice preparation confirmed these findings, and demonstrated that the secretion of inactive renin was also reduced by calcium. The effects of Na, K and Mg ions on active and inactive renin secretion in vitro appeared to depend on the presence of external calcium. However, studies with the two calcium antagonist drugs, verapamil and flunarizine, and the calcium ionophore A 23187, indicated that renin secretion may depend not only on a change in the intracellular concentration of calcium, but also on consequent changes in intracellular sodium.

No other studies of stimulus-secretion coupling for inactive renin have been published. The work provides considerable clarification of existing literature on active renin secretion. This stems from the realization that the single renin secreting mechanism in the rat, by far the most widely studied species, corresponds more closely to the inactive rather than the active renin release mechanism in other animals.

GENERAL INTRODUCTION

The proteolytic enzyme renin plays an important part in the maintenance of arterial blood pressure and extracellular fluid volume. It initiates the sequence of reactions which leads to the production of the physiologically active peptides, angiotensins II and III (See Fig. 1.1).

Renin is mainly synthesised and stored in modified smooth muscle cells (granular cells) in the wall of the afferent arteriole as it enters the glomerulus of the kidney. After it is released into the circulation, renin acts on its endogenous substrate, angiotensinogen, to liberate the decapeptide angiotensin I. Converting enzyme (Kininase II), which has been identified in the vascular endothelium of many tissues, removes a His¹⁰ - Leu¹¹ fragment from the carboxy terminal portion of angiotensin I to release angiotensin II.

By means of its potent pressor activity, angiotensin II participates in the regulation of systemic arterial blood pressure. The peptide also promotes the conservation of salt and water, both directly and indirectly through its action in the adrenal cortex. It is also a potent dipsogen. Amino peptidases in plasma produce angiotensin III, the des-1-aspartyl heptapeptide, which has recently been shown to have biological activity. Further proteolysis of angiotensin is thought to produce only inactive fragments.

The control of renin secretion from the kidney is complex, and involves changes in arterial blood pressure, sodium transport, sympathetic nerves and a variety of humoral factors. A new site for control of the renin-angiotensin system may involve an inactive form of renin which has been shown to exist in kidney and plasma. Acid, cold storage and protease enzymes have all been shown to activate these forms of renin in vitro. A recent hypothesis suggests that the serine protease enzyme kallikrein may take part in the physiological activation of inactive renin. This possibility has been investigated in the rabbit in the experiments which will be described in Chapter 3 of this thesis, using a commercially-available protease inhibitor known as Trasylol (aprotinin, Bayer).

In Chapter 4 of this thesis, the role of calcium ions in regulating renin secretion has been investigated. Calcium plays an important part in stimulus-secretion coupling in granular cells and in excitation-contraction coupling of muscle cells. However, in

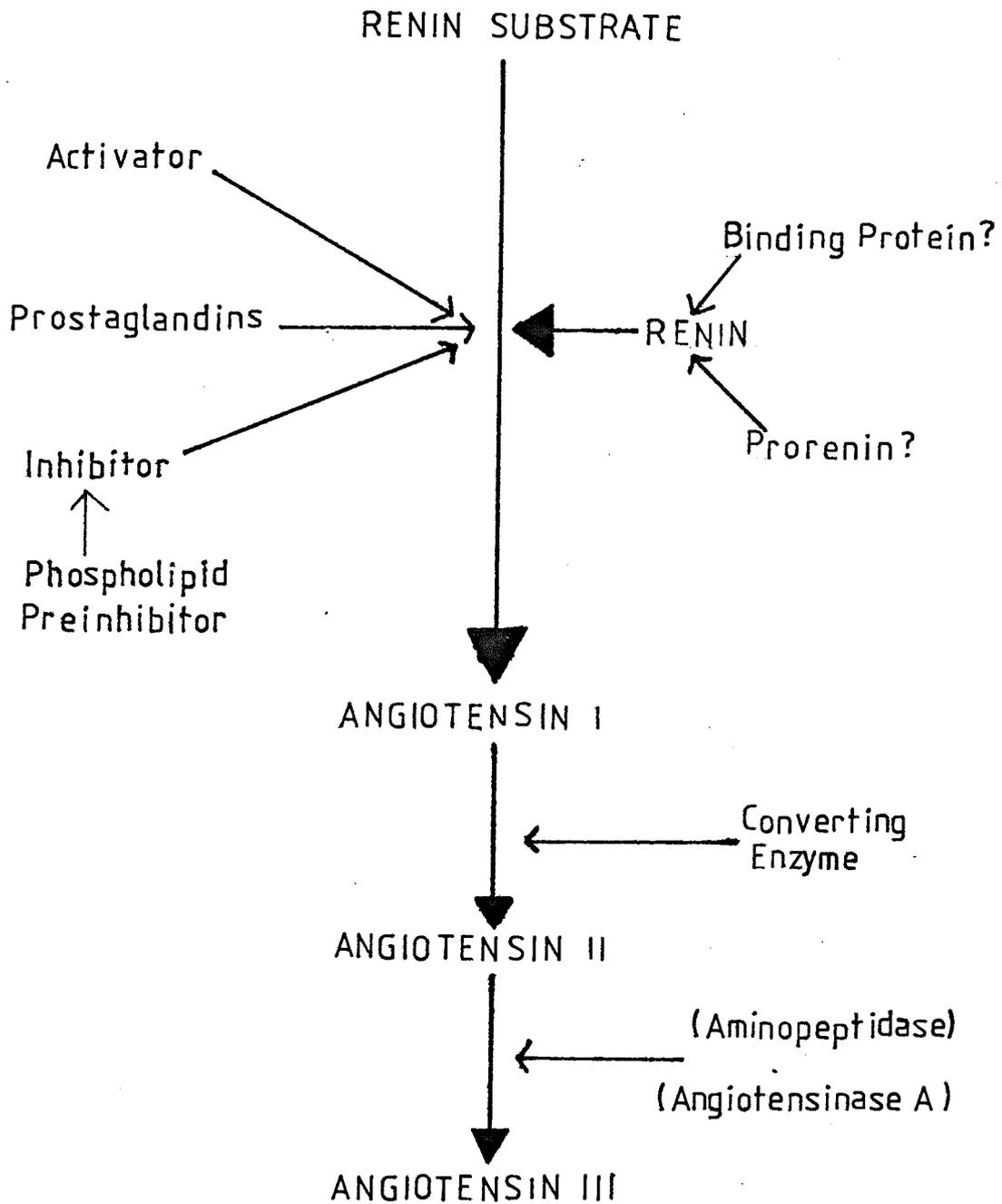


Figure 1:1

The Renin-Angiotensin System

contrast to their stimulatory role in other secretory tissues, calcium ions inhibit the release of active renin from the kidney. Since differential release of inactive renin from juxtaglomerular cells could be a new site for control of the renin-angiotensin system, the effect of changing the concentration of calcium and other ions in the incubation medium on the release of both active and inactive renin has been investigated using rabbit kidney cortex slices. Stimulation of renin secretion by calcium deprivation was further investigated in the studies which will be described in Chapter 5. In these studies, the effects of calcium antagonist drugs and the calcium ionophore on renin release was determined.

CHAPTER I

LITERATURE REVIEW

RENIN (EC 3.4.99.19)

Renin is a highly specific proteolytic enzyme which is synthesised, stored and secreted by the granular cells of the juxtaglomerular apparatus of the kidney. Although it has no physiological activity of its own, renin cleaves the Leu¹⁰-Leu¹¹ bond of both natural and synthetic forms of its substrate to liberate angiotensin I (Ang I). Its catalytic activity is not affected by heavy metal ions dimercaprol, EDTA or by diisopropyl fluorophosphate (DFP) which shows that disulphide bridges are not an essential part of its active site, and it is not a metalloenzyme. (Lee, 1969; Skeggs *et al*, 1977) Renin binds to concanavalin A (Printz & Dworschack, 1977) and therefore is a glycoprotein.

Although it was first discovered in 1898 (Tigerstedt & Bergman, 1898), renin has only recently been completely purified. Haas *et al*, (1953a) achieved a 56,000-fold purification of renin by salt and solvent precipitation. This preparation had a specific activity of 780 GU/ mg protein. Before 1977, many groups had tried to improve the purification of renin, but were unable to prepare renin with significantly greater specific activity. This can be attributed to the very low concentration of renin in the kidney, to confusion with other enzymes which have similar properties, and to the instability of renin which appeared to increase as purification progressed. However, the development of affinity chromatography and the use of protease inhibitors have now allowed complete purification of hog (Inagami & Murakami, 1977b; Corvol *et al*, 1977), rat (Matoba, Murakami & Inagami, 1977), dog (Dzau *et al*, 1979) and human (Yokosawa *et al*, 1980) renins.

Human renin appears to have a number of properties which distinguish it from kidney renins of other species (Yokosawa *et al*, 1980) The molecular weights of pure pig, rat and dog renins are in the range 35-37,000 daltons while human kidney renin has molecular weight of 40,000. It also has a higher isoelectric point (pH 5.7) than renin from other species (pH 4.5-5.4). In addition, antibodies raised against human renin had negligible cross-reactivity with other preparations of renin, while other renins appear to have similar antigenic properties.

Now that purification of renin with high specific activities has been achieved, complete characterisation of the enzyme may be

possible in the future. Currently there is also a great deal of interest in the high molecular weight and enzymatically inactive forms of renin which are present in the kidneys and plasma of most species (see page 8).

RENIN SUBSTRATE.

The presence of a substrate for renin in plasma was first reported by Plentl, Page & Davis (1943) who found that "renin activator" was a component of the α_2 -globulin fraction of plasma. In recent years, purification and partial characterisation of angiotensinogen has been achieved. Tewksbury et al (1977) prepared homogeneous human renin substrate and later (Tewksbury et al, 1978) reported that it was a glycoprotein with molecular weight 56,800 daltons and carbohydrate content of 14 %. Kokubu et al (1980) found that electrophoretically pure human angiotensinogen had a carbohydrate content of 13 per cent and molecular weight of 60,900. Skeggs et al (1957) isolated and sequenced a 14 amino acid polypeptide chain which functioned as a substrate for renin (see Fig.1.3) to release angiotensin I and a 4 amino acid fragment. The tetradecapeptide substrate is linked to the remainder of the angiotensinogen molecule by an ester linkage (Lee & Wilson, 1971).

The existence of several forms of renin substrate in plasma was first suggested by Skeggs et al in 1963 and has been confirmed by other groups. Printz et al (1977) found 4 major and 2 minor peaks of substrate activity in human plasma by isoelectric focussing and Lentz et al (1978) resolved 2 major peaks which accounted for 40-50 % of the recovered substrate activity, and 5-6 minor peaks using a descending pH gradient elution from a DEAE cellulose column. However, using a direct radioimmunoassay for human renin substrate, Eggena et al (1977) found only one form of angiotensinogen in normal subjects and in patients with essential hypertension, although 3 forms were present in women taking oral contraceptives. Gordon & Sachin (1977) reported that plasma of non-pregnant women and women taking the contraceptive pill had two forms of renin substrate (MW 65,000 and 450,000-500,000 respectively) and both were present in increased amounts in the latter group. A third form (MW 350,000) was present in the plasma of pregnant women.

The functional significance of the various forms of angiotensinogen in plasma remains to be clarified. Eggena *et al* (1978) suggested that heterogeneity may account for the altered kinetics of the renin-substrate reaction which is observed in some hypertensive states.

It is now known that angiotensinogen is synthesised in the liver and disappears from the circulation after hepatectomy (Tateishi & Masson, 1972). Furthermore, renin substrate is released by perfused livers (Masjletti & Masson, 1971), liver slices (Freeman & Rostorfer, 1972) and by isolated liver cells (Weigard *et al*, 1977). The rate of renin substrate release can be influenced by a variety of factors. Both glucocorticoids and oestrogens increase angiotensinogen levels in rats (Menard *et al*, 1973) dogs (Reid *et al*, 1973) and humans (Eggena *et al*, 1973). The effect of oestrogens may be mediated by mechanisms that are controlled by hormone receptors since immature rats did not respond to ethinyl oestradiol, but gave an adult response to dexamethasone (Krakoff & Eisenfeld, 1977).

Haemodilution (Reid *et al*, 1974) and angiotensin II (Blair-West *et al*, 1974) increase renin substrate levels in dogs. Nephrectomy and ureteric ligation (Hiwada *et al*, 1976) and thyroidectomy (Bouhick *et al*, 1981) also increase its production. Kato *et al* (1979) found that furosemide increased angiotensinogen levels in hypertensive patients.

ANGIOTENSIN I CONVERTING ENZYME (EC 3.4.15.1)

In 1954, Skeggs *et al* found that two forms of the hypertensive peptide hypertensin (angiotensin) were present after incubation of partially purified pig renin and horse angiotensinogen. They later (Lentz *et al*, 1956) established that the two forms were due to contamination of the angiotensinogen preparation by an enzyme which catalyzed the removal of His - Leu from the carboxy terminus of angiotensin I. During the following decade, it was assumed that the pressor action of angiotensin I was the result of its conversion in plasma, until Ng and Vane (1967) found that plasma converting enzyme activity was insufficient to explain the rapidity of conversion *in vivo*. Subsequently, (Ng & Vane, 1968) these authors reported that angiotensin I (Ang I) was converted to angiotensin II (Ang II) during a single passage through the pulmonary circulation. The

presence of converting enzyme in the lung was later confirmed (Horky et al, 1971) and it is now known to be present in lymph (Horky et al, 1971), kidney (Hall et al, 1976), testes (Cushman & Cheung, 1971) and parts of the brain and pituitary gland (Yang & Neff, 1972; Poth et al, 1975).

Converting enzyme is a membrane-bound glycoprotein with molecular weight of 129,000-480,000 daltons (Oshima, Gesce & Erdos, 1974; Das & Soffer, 1975). It is a metalloenzyme which requires one molar equivalent of zinc for its catalytic activity. In 1968, Bakhle reported that conversion of angiotensin I by canine pulmonary particles was inhibited by a bradykinin-potentiating factor (BPF) which had been extracted from the venom of a pit viper, Bothrops jaracara (Ferreira, 1965). It is now clear that converting enzyme, which is also known as Kininase II, catalyses the sequential removal of Phe-Arg and Ser-Pro from the carboxy terminus of bradykinin in addition to removing His-Leu from Ang I (Soffer, Reza & Caldwell, 1974; Dorer et al, 1974). Thus the net physiological effect of increasing converting enzyme activity is vasopressor since it inactivates bradykinin and increases circulating levels of angiotensin II. By means of antibodies raised against pure preparations of pulmonary converting enzyme, it has been established that the enzyme is located on the luminal surface of the pulmonary endothelium (Ryan et al, 1975), vascular endothelium and proximal tubular cells (Caldwell, Seegal & Hsu, 1976; Soffer & Case, 1978).

In recent years, interest in converting enzyme has been renewed with the development of synthetic inhibitors of converting enzyme, some of which are based on the peptides present in snake venoms. These drugs are now being evaluated widely and in addition to their role as anti-hypertensives, may prove useful in clarifying some of the relationships between the renin-angiotensin and kallikrein-kinin systems. Orally-active inhibitors are now available for clinical use in the treatment of hypertension (Ferguson et al, 1977; Patchett et al, 1980).

INACTIVE RENIN

The earliest evidence that more than one form of renin existed was obtained by Haas et al (1953b) who found three interconvertible forms of the enzyme in a partially purified preparation of hog kidney renin. Skeggs et al (1967) reported that the specific activity of hog renin which had been prepared at pH 7.0 was increased by exposure to acid. A similar increase in renin activity of human amniotic fluid after acidification was also observed by Lumbers (1971) who suggested that this effect could be due to activation of an inactive form of renin. Since then, many groups of workers have confirmed that acid-activatable inactive renin is present in human amniotic fluid and plasma (Skinner et al, 1972; Day & Leutscher, 1974; Leckie & McConnell, 1975a; Derkx et al, 1976). Inactive renin is also present in plasma of the pig (Bailie et al, 1979; Boyd, 1974), the rabbit (Leckie et al, 1973; Richards et al, 1979), the dog (Funakawa, Funae & Yamamoto, 1978), the mouse (Neilsen, Malling & Poulsen, 1979) and the sheep (Lush et al, 1980). Although Nakane et al (1979) found no evidence for inactive renin in the rat, other workers (Vandongen et al, 1977) reported that 15 % of rat renin was in an inactive form.

Renin activity in human plasma also increases when plasma is stored at -5°C for 4 days (Osmond, Ross & Scaiff, 1973; Sealey et al, 1976) but cryo-activation is not observed at 4°C (Hseuh et al, 1978; Millar et al, 1980).

In recent years it has become apparent that both acid- and cryo-activation of inactive plasma renin depends on the presence of endogenous serine protease enzymes. Indeed, a wide variety of proteolytic enzymes are powerful activators of inactive renin in vitro. Urinary kallikrein has been reported to be more potent than trypsin in activating inactive renin in plasma (Sealey, Atlas & Laragh, 1978). Pancreatic kallikrein is similarly effective (Derkx et al, 1979c). Since acid-activation of inactive renin seems to depend on conversion of prekallikrein to kallikrein in plasma, Sealey et al (1979a) have suggested that kallikrein may be a physiological activator of renin. However, many proteolytic enzymes such as plasmin (Osmond et al, 1978), pepsin and cathepsin D (Morris, 1978), thrombin (Overturf, Druilhet & Fitz, 1979), an enzyme similar to cathepsin B (Slater, Gounaris & Haber, 1979) and a metalloproteinase from puff adder venom (Lawrence

& Morris, 1981) can also activate inactive renin. This suggests that renin activation is not a unique property of kallikreins.

The role of serine proteases in activation of inactive renin is an interesting area for study and there is currently a great deal of interest in this subject. It will be discussed in greater detail in Chapter 3 of this thesis.

MULTIPLE MOLECULAR WEIGHT FORMS OF RENIN

The biochemical nature of inactive renin remains to be clarified. Multiple forms of the enzyme have been identified and many of these appear to have low enzymatic activity. From a purified preparation of porcine kidney renin, Inagami & Murakami (1977) isolated three forms of renin when acidification had been avoided during the purification. "Big renin" (molecular weight (MW) 61,000 daltons) had specific activity which was 21 % of that of fully active renin (MW 42,000). Two forms of "big big renin" (MW 140,000) were also found and had specific activities of 0.19 % and 0.05 % respectively when compared with the fully active form. Overturf *et al* (1979) have also purified renin with molecular weight 62,000 from pig kidney, and Slater & Haber (1979) found a renin with molecular weight of 58,000 in a purified preparation of human kidney renin. In a very recent abstract, (Takii, Murakami & Inagami, 1981) complete purification of inactive renin from hog kidney was reported. A 1.2 million-fold purification yielded a protein with a molecular weight of 51,000 which could be activated by treatment with trypsin or pepsin. The effect of acidification or cold exposure on the activity of this protein was not mentioned.

Other groups have found high molecular weight forms of renin by gel filtration of impure kidney extracts. Boyd (1972) found two forms of pig renal renin with molecular weights of 38,000 and 60,000. He later (Boyd, 1973; Boyd, 1974) isolated a binding protein which was associated with the high molecular weight form and which was capable of converting the small, fast-acting form to the larger, slow-acting renin. A similar binding protein (MW 13,000) was isolated from rabbit kidney extracts (Leckie & McConnell, 1975b). The inhibitor was destroyed by exposure to pH 2.5 while the inactive form (MW 55,000) was converted to active renin (MW 37,000). A binding protein may also exist in the dog since acidification of a cytosol fraction from renin granules prevented the formation of a high molecular weight

renin (60,000 daltons) which occurred when it was incubated with a 40,000 dalton form (Kawamura et al, 1979).

In contrast to these findings, Day & Leutscher (1974) reported that acid-activation of "big renin" (MW 60,000) from kidneys of patients with renal carcinoma was not associated with a change in its molecular weight. Levine et al (1976) found a high molecular weight form of renin (MW 57,000-59,000) in purified pig kidney extracts despite an acidification step during the preparation. Further acidification did not result in increased renin activity. Similarly, a 65,000 dalton form of renin was present in an acid extract of dog kidney (Potter et al, 1978).

Thus there appears to be general agreement that a renin with molecular weight of approximately 60,000 daltons is present in the kidney of many species. An even larger form (MW greater than 140,000) has also been reported in the pig (Inagami & Murakami, 1977) and human (Barrett et al, 1977) kidney extracts. By gel filtration, the molecular weight of pure, active renin is about 42,500 (Inagami & Murakami, 1977). The relationship between changes in molecular weight and activation of the enzyme is far from clear.

High molecular weight forms of renin are also present in plasma. However, there appears to be greater controversy over the molecular weight. Day & Leutscher (1975a) found varying proportions of "big renin" (MW 63,000) and normal renin (MW 43,000) in the plasma of patients with diabetic neuropathy and renal carcinoma, while normal plasma contained only the low molecular weight form. "Big renin" could be activated by acid and protease enzymes but this did not involve any change in molecular weight (Day & Leutscher, 1975b). Hseuh et al (1978) found both "big renin" (MW 60,000) and normal renin (MW 40,000) in plasma of normal men taking a high salt diet. Both active and inactive renin were present in both peaks. Only the low molecular weight form was present in subjects taking a low salt diet. Recently, Nubuo et al (1981) reported that two high molecular weight forms (MW 52,000 and 60,000) which could be activated by acidification were present in normal plasma in addition to a 40,000 dalton form of renin.

Some groups have reported that the molecular weight of plasma inactive renin is close to that of active kidney renin (Boyd, 1977; Eggena et al, 1979; Schulkes, Gibson & Skinner, 1978). Others have

reported that the molecular weight is approximately 55,000 daltons (Atlas et al, 1979, Leckie et al, 1977a; Leckie et al, 1977b; Yokosawa et al, 1979). Thus, there is no general concensus as to the molecular weight of inactive plasma renin. Most of the reports suggest that activation of plasma inactive renin, at least in humans, does not involve any change in its molecular weight (Day & Leutscher, 1975; Eggena et al, 1979; Hseuh et al, 1978; Schulkes et al, 1978). In the mouse, there are two high molecular weight forms of plasma renin (MW 70,000 and 800,000). Both forms are activated by acidification, but this is only accompanied by a reduction in molecular weight in the larger form (Neilsen, Malling & Poulsen, 1978).

The possibility that inactive renin is either a true zymogen of renin or an inhibitor-bound form of the enzyme, and the different physiological implications of these two hypotheses have recently been reviewed by Leckie (1981).

THE PHYSIOLOGICAL ROLE OF INACTIVE RENIN

Until recently, relatively few studies concerning inactive renin had been published, and so its physiological role is rather unclear at present. Inactive renin in plasma increases during pregnancy (Skinner et al, 1975) and in patients with renal carcinoma (Day et al, 1975). Inactive renin forms a large proportion of total renin in the plasma of hypertensive patients (Sealey et al, 1977), of patients with diabetic neuropathy (Hseuh et al, 1980; Leckie et al, 1978) and in anephric patients (Sealey et al, 1977). This suggest that inactive renin could be a potentially important site for control of the renin system.

Inactive renin in plasma is thought to be primarily derived from the kidney since it is reduced after nephrectomy (Derkx et al, 1978; Richards et al, 1979). The responses of active and inactive renin to various acute stimuli have been measured. With some experimental manipulations, stimulation of active renin is accompanied by a decrease in circulating inactive renin. An inverse relationship between active and inactive renin was observed by Derkx et al (1976) in human subjects during upright tilting, acute vasodilatation by diazoxide and isoprenaline infusion. Dietary sodium depletion, a powerful stimulus for active renin release, was associated with reduced plasma inactive renin in human subjects (Millar et al, 1978)

and in rabbits (Grace et al, 1979). During furosemide treatment, inactive renin disappeared from the circulation of rabbits (Richards et al, 1981) and dogs (James & Hall, 1974). These findings have led to the suggestion that a sodium-sensitive mechanism could control activation or release of inactive renin from the kidney (Richards et al, 1981). This concept is supported by studies using kidney cortex slices. Reducing the concentration of sodium bathing the slices was found to increase active, but reduce inactive renin secretion (Munday, Noble & Richards, 1980).

Inactive renin has also been reported to increase while active renin is suppressed by propranolol in both hypertensive (Atlas et al, 1977) and normotensive (Derkx et al, 1976) subjects, and after clonidine treatment in hypertensive patients (Atlas et al, 1977).

In contrast to the above studies, where there was a degree of independence between the changes in active and inactive renin, the two forms increased in parallel during haemorrhage in dogs (James & Hall, 1974) and rabbits (Richards et al, 1979). In the pig, both forms of renin were increased by isoprenaline, furosemide, saline and propranolol (Baillie et al, 1979). Isoprenaline infusion into the rabbit caused an increase in both forms of renin (Richards et al - in press). However, the same authors found that in vitro, isoprenaline increased active renin secretion in a dose-related fashion, whilst inactive renin remained unchanged. An increase in both active and inactive renin was observed in hypertensive patients during dietary sodium restriction (Atlas et al, 1977).

Despite the many reports where changes have been observed, other groups have failed to record any alteration in inactive renin during stimulation or suppression of the renin-angiotensin system. No change in circulating inactive renin was observed after furosemide (Rumpf et al, 1978), saralasin infusion (Kapelgaard et al, 1978; Leckie et al, 1977b) or captopril administration (Millar et al, 1980), although active renin increased. Similarly, saline loading (Weinberger et al, 1977), indomethacin (Rumpf et al, 1978) and propranolol (Birkenhager et al, 1978) suppressed the renin-angiotensin system without a change in inactive renin.

From these findings, it appears that the nature of the stimulus could be important in determining the relative amounts of active and inactive renin secreted by the kidney, but this can only partially explain the discrepancies among the findings. These could also be

related to differences in species and methodologies. Further investigation of activation and release of inactive renin are clearly needed to clarify the role that it plays within the renin system.

THE CONTROL OF RENIN SECRETION.

Before the control mechanisms which are thought to regulate the release of renin can be discussed, it is necessary to describe briefly the structure of the juxtaglomerular apparatus (see Fig. 1.2). The close anatomical relationship between the cells of the afferent and efferent arterioles, the macula densa cells of the distal tubule and the juxtaglomerular granular cells is an important factor in renin secretion.

Granular cells are usually found in the media of the afferent arteriole and synthesise, store and secrete renin. These cells are differentiated vascular smooth muscle cells. They contain myofibrils, but are distinguished from typical smooth muscle cells in the afferent arteriole by the presence of numerous cytoplasmic processes and infoldings of the plasma membrane (Gorgas, 1978). The endoplasmic reticulum and Golgi membranes are well-developed in granular cells, a characteristic of secretory function. The dense, membrane-bound granules of juxtaglomerular cells contain renin (Morris & Johnstone, 1976; Gross & Barajas, 1978).

In 1952, De Meylder identified sympathetic nerves which travelled to the afferent arterioles, and the presence of non-myelinated, noradrenergic nerve fibres which terminate close to the granular cells has been confirmed in most species (Barajas, 1964; Biava & West, 1966; Nilson, 1965; Wagermark *et al*, 1968). These sympathetic fibres terminate in varicosities which are separated from the cells by 1200-2000 Å, with a basement membrane intervening between the two structures (Barajas *et al*, 1977; Barajas & Muller, 1973). Thus there is morphological evidence for a functional relationship between the sympathetic nervous system and renin release. Cholinergic innervation of granular cells is less well-defined. McKenna & Angelakos (1968) found that the afferent arterioles of the dog receive cholinergic innervation from ganglion cells in the hilar region. However, the distribution of renal nerves which contain acetylcholinesterase appears to be similar to that of adrenergic fibres (Barajas, 1979).

(Taken from Davis, 1971)

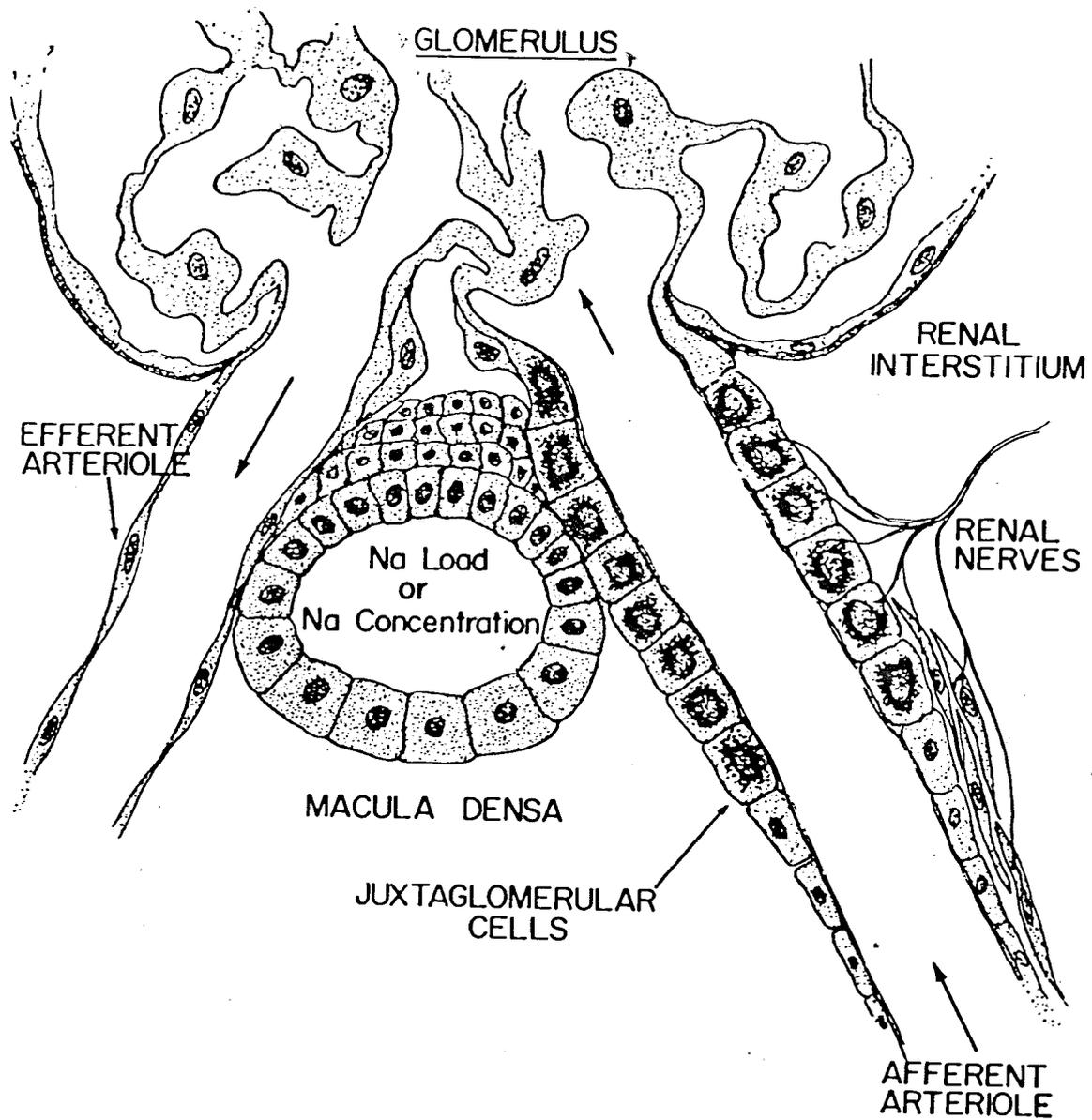


Figure 1.2 The Juxtaglomerular Apparatus

The macula densa marks the transition between the ascending limb of the loop of Henle and the distal convoluted tubule. The cells on the glomerular side of the tubule in this segment are heavily nucleated and lie in close contact with granular cells in the vascular pole of the tubule of origin. Macula densa cells may be columnar or cuboidal depending on the species which is studied. The Polkissen, or polar mass, consists of mostly agranular cells which lie in the angle which is formed between the afferent and efferent arteriole. These cells are in close contact with the macula densa and are continuous with cells from the arteriolar walls, where they may replace vascular smooth muscle cells.

During the past two decades, five basic mechanisms for the control of renin secretion have been generally agreed:-

- 1) An intrarenal baroreceptor.
- 2) A macula densa receptor which responds to changes in sodium (or chloride) load in tubular fluid.
- 3) Renal sympathetic nerves and circulating catecholamines.
- 4) Humoral factors including angiotensin II and prostaglandins.
- 5) Plasma electrolytes.

These control mechanisms and the pharmacological alteration of renin release have recently been thoroughly reviewed by Fray (1980) and by Keeton & Campbell (1980). Earlier major reviews include those by Vander (1967), Davis & Freeman (1976) and Peart (1978). These reviews, and many of the studies which will be discussed in the following section of this thesis, have been concerned only with the release of active renin. Where possible, studies which include inactive renin will also be included.

INTRARENAL BARORECEPTOR

In 1934, Goldblatt and his colleagues induced hypertension in dogs by constriction of the renal arteries and suggested that the subsequent ischaemia led to the release of a circulating pressor substance (renin) which mediated the hypertension. Later (Kohlstaedt & Page, 1940) it was proposed that the reduction of pulse amplitude could be responsible for increased renin release following reduction

in renal blood flow, but Kolff (1958) reported that renin was released under these circumstances whether flow was pulsatile or non-pulsatile. The presence of an intrarenal "stretch" receptor was proposed by Tobian (1962) who suggested that renin release was inversely proportional to the degree of stretch of renal afferent arteriolar walls. This, in turn, is determined by renal perfusion pressure.

Some of the most convincing evidence that an intrarenal vascular receptor exists and determines renin release from granular cells has been obtained by Blaine and his co-workers. This group developed the non-filtering dog kidney preparation (Blaine, Davis and Witty, 1970) to investigate the baroreceptor hypothesis and found that haemorrhage and constriction of the aorta above the renal arteries caused a two-fold increase in plasma renin activity (PRA) in conscious dogs even after the influence of the macula densa (see page 17), renal sympathetic nerves and circulating catecholamines (see page 18) had been removed (Blaine, Davis & Prewitt, 1971). The intrarenal baroreceptor appeared to be located at the level of the afferent arterioles since papaverine, which dilates these vessels and therefore prevents renal autoregulation, abolished the increase in renin secretion (Witty et al, 1971). However, it must be noted that papaverine, which is a phosphodiesterase inhibitor, itself reduces basal renin secretion from rat kidney slices (Churchill, McDonald & Churchill, 1980).

The nature of the stimulus detected by the baroreceptor is not clear. According to Tobian's hypothesis, the degree of stretch of the afferent arterioles regulates renin secretion. However, hypotension-induced renin release is usually associated with renal vasodilatation and a reduction in renal vascular resistance (Cowley & Guyton, 1972; Eide et al, 1973; Gotshall et al, 1974; Schmidt et al, 1972; Skinner et al, 1964). In a review, Davis & Freeman (1976) proposed that the intrarenal baroreceptor could respond to a change in wall tension of the afferent arteriole. According to Laplace's Law, wall tension is the product of the transmural pressure gradient (difference between intra-luminal pressure and renal interstitial pressure) and the radius of the vessel. They stated that arteriolar wall tension could be altered by 1) changes in transmural pressure gradient, 2) changes in afferent arteriolar diameter, 3) sympathetic nerve activity 4) intrinsic myogenic factors and 5) changes

in elastic components of the vessel wall.

MACULA DENSA RECEPTOR

The " macula densa hypothesis " can be summarised as follows:- a decrease in tubular concentration of sodium in the region of the macula densa stimulates renin release from granular cells. It was first proposed by Vander in 1967 who also reviewed the anatomical basis for such a relationship. At first sight this theory appears logical in view of the fact that dietary sodium depletion is associated with increased plasma renin activity in man (Brown et al, 1963), dog (Brubacher & Vander, 1968; Mogil et al, 1969) rats (Keeton, Pettinger & Campbell, 1976) and rabbits (Grace et al, 1979).

Early studies in which " loop diuretics " were used to investigate the macula densa hypothesis provided conflicting results. Ethacrynic acid increased both renin release and sodium excretion in anaesthetised dogs (Cooke et al, 1970; Birbari et al, 1972) even when volume depletion was prevented. Meyer et al (1972) found that furosemide produced a rapid release and sodium excretion in rabbits. Thus, it appeared that a direct relationship between tubular sodium excretion and renin secretion existed. However, in the light of more recent findings, these results can be reconciled with Vander's hypothesis since furosemide and ethacrynic acid themselves stimulate renin release (Bailie, Crosslan & Hooke, 1976; Eide et al, 1975; Osborn, Hooke & Bailie, 1977). These diuretics also appear to inhibit sodium transport at the macula densa and therefore prevent the cells from sensing the increase in sodium load (Schnermann, Ploth & Hermle, 1976).

A criticism which can be levelled at almost all studies of furosemide-induced changes in renin release is that they have been carried out using either anaesthetised animal preparations or that volume depletion has occurred as a result of the diuresis. In a recent series of experiments using conscious sheep in which volume depletion was avoided some surprising results were obtained (Lush, Munday & Noble, 1980a). A massive diuresis was not accompanied by changes in either active or inactive renin in plasma unless papaverine infusion was simultaneously included in the protocol (Lush, Munday & Noble, 1980b). The difference between these observations and other published data was attributed to the absence of anaesthetic agents.

Furosemide diuresis in pentobarbitone-anaesthetised, volume-replete sheep maintained in an upright posture was accompanied by an increase in plasma active renin and concurrent decrease in the inactive form. These observations throw some doubt on the existence of a macula densa receptor at all.

In anaesthetised dogs, volume expansion with isotonic saline was found to increase the fractional excretion of sodium and suppress renin release (Nash *et al*, 1968) while infusion of hypertonic saline reduced fractional sodium excretion and increased renin secretion. Further evidence in support of the macula densa hypothesis has been provided by Churchill and his co-workers. They studied early distal tubular fluid which had been obtained by micropuncture of cortical distal tubules of anaesthetised rats. They (Churchill, Churchill & McDonald, 1978) found that early distal tubular sodium load and not sodium concentration was directly related to dietary sodium intake. The rate of renin secretion was inversely related to sodium intake. More recently Churchill *et al* (1979) reported that both saline- and mannitol-induced diuresis increased sodium load in the distal tubule and reduced renin release. However, the concentration of sodium in tubular fluid was increased by saline and reduced by mannitol. They concluded therefore, that a change in sodium load, rather than its concentration regulates renin release .

Although much evidence suggests that renin release is inversely related to sodium at the macula densa, some workers maintain that the converse hypothesis is true (Thurau *et al*, 1967; Thurau *et al*, 1972). From studies using a micrograde injection technique, they conclude that an increase in sodium load as a result of increased glomerular filtration (GFR) stimulates renin release and that this system is involved in autoregulation of the blood flow to individual nephrons. This controversy remains to be clarified.

RENAL SYMPATHETIC NERVES

The role played by the autonomic nervous system in the control of renin release was reviewed by Zanchetti *et al* (1976). Vander

(1965) was the first to report the effect of electrical stimulation of the afferent arteriole and its surrounding sympathetic fibres. Renin release increased and GFR, RBF and sodium excretion were all reduced. Since the increase in renin secretion was prevented by mannitol diuresis, he proposed that the fall in sodium excretion after nerve stimulation had caused the increase in renin release. This interpretation was in accordance with the macula densa hypothesis.

Hoeffler et al (1972) also reported that stimulation of the renal artery and nerves of anaesthetized dogs increased plasma renin activity. However, this response was completely abolished by the β -adrenergic antagonist, propranolol. Pretreatment with phenoxybenzamine, an α -adrenoreceptor antagonist, had no effect on the increase in renin release in response to renal nerve stimulation. The authors therefore proposed that renal nerve stimulation altered renin release by way of a β -adrenergic receptor. Nerve stimulation-induced renin release was not antagonised by d propranolol, which has only 1% of the β -antagonistic effect of the l isomer, in anaesthetized dogs, (Taher et al, 1976) and cats, (Johns & Singer, 1974) while a racemic mixture of the two forms prevented the increased renin release, but not the vasoconstriction of renal vessels.

In most of the above studies, changes in renal sodium handling were not measured, and so renin release via the macula densa receptor could not be excluded. When renal nerves of a non-filtering kidney were stimulated in anaesthetized dogs pretreated with papaverine, (Johnson, Davis & Witty, 1971) plasma renin activity still increased. Similarly, Taher et al (1976) stimulated the renal nerves of anaesthetized dogs at a frequency which did not change RBF, GFR or sodium excretion, but produced a consistent rise in PRA. Thus, neurally-induced changes in renin release were confirmed as being mediated by a β -adrenoreceptor and were considered independent of the intrarenal baroreceptor and macula densa receptor.

In 1967, Ueda et al reported that stimulation of the mesencephalic pressor area of the brain produced a rise in renal venous PRA and a fall in RBF and MAP. Renal denervation prevented the response of renin to stimulation. Similar increase in PRA have been observed after stimulation of the superior colliculus, (Blair & Feigl, 1968) the medulla oblongata (Passo et al, 1971a and 1971b), the hypothalamus, (Natcheff, Hogofetov & Tzaneva, 1977; Zehr & Feigl, 1973)

and the dorsolateral pons (Richardson et al, 1974). When it was reported, renal denervation and propranolol, but not phenoxybenzamine abolished the increase in renin release (Natcheff et al, 1977; Passo et al, 1971a, 1971b; Richardson et al, 1974; Zehr & Feigl, 1973).

Thus, plasma renin activity can be altered by neural activity in higher centres of the central nervous system via changes in the activity of renal sympathetic β -adrenergic nerve tracts.

CIRCULATING CATECHOLAMINES

The first evidence that suggested that catecholamines could be involved in regulating the secretion of renin was obtained by Vander (1965), who reported that both adrenaline and noradrenaline increased renin activity in anaesthetized dogs when perfusion pressure was held constant. Since GFR and sodium excretion were reduced, he concluded that the renin response was mediated by activation of the macula densa. However, Nash et al (1968) found that noradrenaline still induced renin release after saline infusion had restored sodium excretion to normal levels.

In the non-filtering dog kidney preparation, pretreatment with papaverine abolished the increase in renin release after adrenaline, but not after noradrenaline (Johnson, Davis & Witty, 1971). Since the response to both catecholamines was accompanied by reduced renal blood flow, these workers concluded that adrenaline stimulated renin through activation of the intrarenal baroreceptor, while noradrenaline exerted a direct effect on juxtaglomerular cells.

Otsuka et al (1970) induced hypoglycaemia with insulin in anaesthetized dogs and thus increased circulating levels of adrenaline. Plasma renin activity also increased, although arterial blood pressure was unchanged. Propranolol antagonised hypoglycaemia-induced renin release, (Assaykeen et al, 1970) while phenoxybenzamine produced a small potentiation of the response. Intravenous infusion of noradrenaline increased both plasma renin activity and systemic blood pressure and reduced renal blood flow, GFR and urinary sodium

excretion in anaesthetized dogs (Ueda et al, 1970). The α -adren-
 ergic antagonist, dibenamine, reduced the effect of noradrenaline on
 renal function, but did not affect the renin response. In contrast,
 propranolol antagonised noradrenaline-induced renin secretion. Thus,
 these results suggest that both adrenaline and noradrenaline increase
 renin release via a β -adrenoreceptor on juxtaglomerular cells.

Although some early results suggested that isoprenaline did
 not change renin secretion, (Ayers, Harris & Hefer, 1969; Bragg, Page
 & McCubbin, 1966), subsequent investigators have reported that iso-
 prenaline stimulates renin release when infused intravenously in
 anaesthetized dogs, (Assaykeen, Tanigawa & Allison, 1974; Chokslit,
 Yeh & Samet, 1972; Tanigawa, Allison & Assaykeen, 1972), isolated
 perfused rat kidneys, (VanDongen, Peart & Boyd, 1973; Viskoper et al,
 1977), and when added to rat (Weinberger, Aoi & Henry, 1975), and
 rabbit (Richards et al, In press) kidney cortex slices in vitro.
 Recently, Himori, Hayagawa & Ishimori (1979) tried to classify the
 β -adrenoreceptor involved in isoprenaline-induced renin release.
 Since the β_1 -antagonist, atenolol, suppressed both the hypotension
 and increased renin release, which were induced by isoprenaline,
 while the β_2 -antagonist, IPS-399 reduced only the hypotensive res-
 ponse, these workers concluded that isoprenaline acted at a β_2 -
 adrenoreceptor on granular cells to stimulate renin secretion.

The involvement of dopamine in the control of renin release
 remains to be clarified. Specific dopamine receptors appear to be
 present in the kidney of the dog, since the increase in both renin
 activity and renal blood flow after dopamine infusion into denerv-
 ated kidneys of anaesthetized dogs were reduced by the dopamine anta-
 gonist, haloperidol (Ueda et al, 1975). Propranolol, in contrast,
 did not significantly alter the response. In human subjects, how-
 ever, dopamine either did not change, (Atuk, Ayers & Westfall, 1968),
 or increased (Wilcox et al, 1974) plasma renin activity in normal
 subjects, but reduced renin levels in cirrhotic patients with various
 degrees of renal impairment (Barnado et al, 1970). A dopaminergic
 inhibitory control mechanism for renin release which functions diff-
 erently in normotensive and spontaneously hypertensive rats, has
 been reported (Sowers et al, 1981). Increased interest in dopamine
 as a drug and developments in the pharmacology of blocking agents
 for dopamine will surely lead to clarification of dopamine-induced
 renin release.

HUMORAL FACTORS

A variety of blood-bone factors are known to influence renin secretion. Angiotensin II appears to suppress the release of renin and thus form part of a negative feedback loop. Intravenous infusion of ang II reduced plasma renin activity in anaesthetized dogs when renal perfusion pressure was maintained by a suprarenal aortic clamp (McDonald et al, 1975; Vander & Geelhoed, 1965), and when the renin-angiotensin system had been stimulated by sodium depletion in dogs, (Bunag, Page & McCubbin, 1967) sheep, (Blair-West et al, 1971) and human subjects (De Champlain et al, 1966). Furthermore, plasma renin activity increased in rabbits which had been immunized against ang II (Stokes, Oates & Weber, 1975)

Angiotensin II suppressed renin release in the isolated, perfused rat kidney, (Hofbauer et al, 1974; Van Dongen, Peart & Boyd, 1974) and reduced renin release in vitro from kidney cortex slices of the rat (Capponi et al, 1977; Michelakis, 1971). In contrast, Morris, Nixon & Johnston (1976) have reported that renin release by isolated glomeruli was unchanged by ang II. The inhibitory effect of ang II on renin release from rat renal cortex slices in vitro was abolished by the specific antagonist 1-Sar-S-Ala-ang II (Capponi et al, 1977; Naftilan & Oparil, 1978). It now appears that the inhibitory response to ang II depends on the presence of calcium in the incubation medium, (Van Dongen & Peart, 1974) and is abolished by the Ca^{2+} -antagonist, verapamil (Park, Han & Fray, 1981), but not by its methoxy derivative, D 600 (Churchill, 1980).

In recent years, the role of prostaglandins in the regulation of renin secretion has been the subject of numerous investigations. In 1971, Werning et al reported that plasma renin activity increased after PGE_1 was injected into the aorta of anaesthetized dogs. Arterial blood pressure fell, while heart rate, urinary volume and urinary excretion of sodium and potassium were increased. This group therefore concluded that PGE_1 stimulated renin release through changes in renal sodium handling. However, when urinary losses were replaced, intrarenal infusion of PGE_1 still increased renin activity (Riley, 1974). In sodium-depleted dogs which had been pretreated with the prostaglandin synthesis inhibitor, indomethacin, in order to eliminate the effects of endogenous prostaglandins, and with renal

perfusion maintained by a clamp on the aorta, infusion of PGE₂ increased PRA as well as increasing urine flow, sodium excretion and renal blood flow (Yun *et al*, 1977).

In order to eliminate the effects of changes in sodium handling at the macula densa, the renal sympathetic nerves and endogenous prostaglandins, Gerber *et al* (1978) studied the response to intravenous infusion of PGE₂ in anaesthetized dogs with a single, denervated non-filtering kidney. The animals were pretreated with indomethacin and given an infusion of propranolol. Since both renal blood flow and renin release increased, this group suggested that the effect of PGE₂ was mediated by activation of the renal baroreceptor or by a direct effect of PGE₂ on granular cells. *In vitro* PGE₁ and PGE₂ did not change renin secretion from rat (Corsini, Crosslan & Bailie, 1974) or rabbit, (Weber *et al*, 1976 a & 1976 b) renal cortex slices. These results support the concept that the release of renin by PGE is due to the activation of the intrarenal baroreceptor.

In contrast to PGE₁, the prostaglandin precursor arachidonic acid appears to stimulate renin release by a direct action on granular cells, since it increases renin secretion from rabbit renal cortex slices *in vitro*, (Weber *et al*, 1976; Whorton *et al*, 1978) and from isolated rat glomeruli (Beierwaltes *et al*, 1980). These effects are abolished by inhibitors of prostaglandin synthesis. When infused into the aorta of saline-loaded anaesthetized rats, arachidonic acid caused an increase in plasma renin activity, (Weber *et al*, 1975) even though urine volume, sodium excretion and mean arterial blood pressure were unchanged. Similar findings have been reported after intrarenal infusion of arachidonic acid in intact dogs, (Bolger *et al*, 1976) and in dogs with a single, denervated, non-filtering kidney (Data *et al*, 1978).

It is not yet clear which prostaglandin is the renin-releasing metabolite of arachidonic acid, but prostacyclin (PGI₂) is emerging as a possible candidate. Bolger *et al* (1978) reported that renal blood flow, urinary volume and sodium excretion increased together with renal venous PRA after intrarenal infusion of PGI₂ in anaesthetized dogs. Gerber *et al* (1978) found that prostacyclin was more potent in stimulating renin release in anaesthetized dogs with a denervated, non-filtering kidney than were PGE₂ or D₂. PGI₂ caused a dose-related increase in renin secretion from rabbit renal cortex slices *in vitro* (Whorton *et al*, 1977). More recently, these

workers have found that both basal and arachidonic acid-stimulated renin release were abolished by 9, 11-azoprosta-5, 13-denoic acid. This compound inhibits the synthesis of prostacyclin without affecting PGE₂ or PGF_{2α}. In a recent paper, Jackson *et al* (1981) found that an active metabolite of PGI₂, 6-keto-PGE₁ was about five times more potent than prostacyclin in causing renin secretion from non-filtering, β-blocked kidneys of anaesthetized dogs.

In contrast to other prostaglandins, PGF_{2α} inhibits renin secretion from rabbit renal cortex slices *in vitro* (Weber *et al*, 1976). Furthermore, PRA is suppressed in women receiving PGF_{2α} intra-amniotically to induce abortion (Fyhrquist, Soveri & Widholm, 1976). The enzyme PGE₂-9-ketoreductase converts PGE₂ to PGF_{2α}. Weber, Larson & Sherer (1977) found that its activity was increased in rats taking a high salt diet, and suggested that activation of the enzyme could be involved in adjusting PRA during periods of altered sodium intake. However, Lusman *et al* (1973) found no changes in the plasma concentrations of PGE₂ and PGF_{2α} in normal subjects during changes in sodium intake. Similarly, the relative excretion of PGE₂ and PGF_{2α} in urine was unchanged during alteration of dietary sodium intake (Campbell *et al*, 1981). There have been no reports to date of the effect of prostaglandins on the secretion of inactive forms of renin. *In vitro* separations would appear to be particularly appropriate for such an investigation.

Variations in the plasma concentration of antidiuretic hormone, (ADH) within the physiological range, also appear to be involved in the regulation of renin release. Vasopressin reduced PRA in anaesthetized dogs, (Shade *et al*, 1973) even in the absence of a functional macula densa, and in anaesthetized rats (Churchill, Churchill & McDonald, 1973). Exogenous ADH also suppressed PRA after it had been increased in dogs by dietary sodium depletion, (Johnson, Kinter & Beeuwkes, 1979; Tagawa *et al*, 1971) aortic constriction, (Bunag, Page & McCubbin, 1967) or ureteral occlusion (Vander, 1968). Furthermore, plasma renin is elevated in rats with hereditary diabetes insipidus, (Henderson *et al*, 1978) and is very low in human subjects with the syndrome of inappropriate secretion of ADH (Fichman, Michelakis & Horton, 1974). These patients have high levels of vasopressin.

In the isolated rat kidney, ADH reversed the stimulation of

renin release by isoprenaline (Konrads *et al*, 1978; Van Dongen, 1975) but did not itself change renin release. Similarly, vasopressin did not alter renin secretion from rat renal cortex slices *in vitro* (De Vito *et al*, 1970). In human subjects, the reduction in PRA which was observed after the administration of vasopressin could be due to expansion of the extracellular fluid volume, (Annat *et al*, 1976; Newsome & Bartter, 1968) or to a direct intrarenal effect of ADH (Hesse & Nielsen, 1977). However, Goodwin, Ledingham & Laragh (1970) observed only slight changes in PRA of human subjects after administration of vasopressin both in the presence and absence of fluid expansion. The physiological relevance of interactions between ADH and renin release mechanisms remains to be clearly demonstrated.

In summary, there are complex interactions between renin release mechanisms and plasma or local tissue levels of other hormones, particularly ang II, prostaglandins and antidiuretic hormone. The evidence elicited from *in vivo* experiments, while clearly important to the overall picture, is often difficult to interpret. Secondary changes in salt and water homeostasis will themselves affect renin release mechanisms. Studies carried out *in vivo* often provide more direct evidence of the site of action of the hormones. They have, however, sometimes failed to provide consistent results from different laboratories. A possible source of such confusion could be the way in which samples have been processed for assay. In retrospect, we can see that some studies were made of active renin only and others of total renin. Secretion control for inactive renin in this context is almost entirely unexplored territory.

PLASMA ELECTROLYTES

The effects of various ions on the intake of renin secretion have been studied extensively using *in vitro* techniques in order to eliminate the influence of the intrarenal baroreceptor, macula densa, sympathetic nervous system and circulating hormones.

The role of sodium ions in the regulation of renin release is controversial. Some investigators have found that increasing the

concentration of sodium in the medium increased renin release from rat kidney cortex slices (Braverman et al, 1971; Hammerson et al, 1971; Lyons & Churchill, 1975a; Oelkers et al, 1970). Similarly, reducing the concentration of sodium suppressed renin release (Capponi & Valloton, 1976). One group of workers reported that when the osmolality of the medium was unchanged, increasing the concentration of sodium stimulated the release of renin (Blendstrup et al, 1975). However, they also found that this effect of sodium was reversed (Frederiksen, Leyssac & Skinner, 1975) when the osmolality increased. They concluded therefore that sodium elicited renin release by virtue of its osmotic effect on water movement rather than by a direct effect on granular cells. In contrast to these findings, Weinberger & Rosner (1971) found no change in renin release from rat kidney cortex slices when the concentration of sodium was increased, provided osmolality was constant. In contrast to the investigators described above, other studies have shown that increasing the concentration of sodium suppressed renin release from dog, (Michelakis, 1971) and rabbit, (Richards et al, 1980) renal cortex slices, and from suspensions of rat renal cortical cells (Lyons & Churchill, 1975b)

Unlike its stimulatory role in secretory processes in many other tissues, (Rubin, 1970) there is a growing body of evidence that calcium ions inhibit the secretion of renin. Studies with isolated, perfused kidneys show that hypercalcaemia inhibits renin release (Kotchen et al, 1974; Watkins et al, 1976). Removal of calcium from the medium, and complete depletion of calcium by the addition of chelating agents such as EDTA and EGTA are powerful stimulators of renin release (Fray, 1977; Fray & Park, 1979; Harada & Rubin, 1978; Van Dongen & Peart, 1974).

Calcium may play a central role in the regulation of renin secretion. The inhibitory effect of ang II requires the presence of calcium in the medium (Van Dongen & Peart, 1974). Similarly, low perfusion pressure stimulates renin release only when calcium is present in the medium, and becomes less effective as the concentration of calcium is raised (Fray & Park, 1979). It has also been reported (Park & Malvin, 1977) that renin release induced by adrenaline, and the suppression of its release by ouabain were abolished after calcium removal.

More recently, experiments using drugs which antagonise the

influx of Ca^{2+} into cells have been described. Angiotensin II, ADH, ouabain and high concentrations of potassium ions all inhibited the release of renin by dog renal cortex slices (Park, Han & Fray, 1981). All of these effects were abolished by the calcium antagonist, verapamil. The methoxy-derivative of verapamil, D 600, abolished the inhibitory effect of potassium ions, (Churchill, 1980) but did not antagonise the effect of ang II, even at doses 2 - 6-fold greater than had been required to antagonise the effect of potassium.

Thus, according to these studies, an increase in the intracellular concentration of calcium, which is achieved by ang II, ouabain, or depolarization by high extracellular concentrations of potassium, inhibits the secretion of renin. On the other hand, calcium chelators, isoprenaline or low extracellular concentrations of Ca reduce intracellular levels of calcium and appear to stimulate renin secretion.

The juxtaglomerular granular cells are modified smooth muscle cells. Normal smooth muscle contraction is associated with influx of Ca^{2+} ions, and Peart (1978) drew analogies between situations which cause smooth muscle contraction, and control of renin release. A vasoconstrictive agent such as angiotensin is associated with increased influx of Ca^{2+} into smooth muscle and inhibition of renin release. Isoprenaline, a vasodilator, stimulates renin release. When our understanding of the intracellular control of secretion of renin eventually approaches the degree of sophistication currently available for other secretory ^{tissues} such as the gastric mucosa and the pancreatic islet tissues, intracellular interactions between Ca ions and renin secretory mechanisms for the two forms of renin will probably prove crucially important.

Small changes in the local concentration of potassium ions can result in large changes in renin release. Vander (1970) found that PRA was suppressed in anaesthetized dogs after an intrarenal artery infusion of a potassium salt. Urinary excretion of both sodium and potassium was increased and no change in the renal blood flow, GFR or arterial blood pressure was observed. Similar results were obtained in sodium depleted dogs. This suppression of renin activity in anaesthetized dogs has been confirmed by other groups (Flamenbaum et al, 1975; Schneider et al, 1972; Shade et al, 1972; Stephens et al, 1978). In conscious dogs, chronic K^+ -depletion for 5 - 7 weeks increased plasma renin activity (Abbrecht & Vander, 1970).

During this period, the plasma concentration of potassium fell, but plasma sodium was unchanged. Potassium repletion restored renin to control levels. Similar inverse relationships between potassium and plasma renin have been reported in conscious dogs, (McCaa, McCaa & Gayton, 1975; Young et al, 1976) conscious rats, (Sealey et al, 1970) and normal human subjects (Brunner et al, 1970; Hollenberg et al, 1975).

Miller and his co-workers (1975) studied the effects of potassium loading in adrenalectomized patients and in patients with primary adrenal insufficiency, all of whom were taking a fixed daily dose of mineralocorticoids. Potassium loading was associated with negative sodium balance and increased plasma renin in these subjects. This was in contrast to the suppression of PRA which had been observed in normal subjects. When sodium losses were replaced in Addisonian patients, potassium loading did not change plasma renin activity. It was concluded that the suppression of PRA by potassium required high circulating levels of mineralocorticoids, which were absent in Addisonian patients.

Kotchen, Galla & Luke (1976) studied the effects of oral ingestion of KCl and KHCO_3 in conscious sodium-depleted rats. They found that KCl reduced plasma renin; HCO_3 did not. They later (Kotchen, Galla & Luke, 1978) reported that the chloride ion may itself inhibit renin release, and concluded that the anion which accompanies potassium may modify the signal that is delivered to the macula densa.

In vitro, increasing the concentration of potassium in the incubation medium reduces basal renin secretion from renal cortex slices of the rat, (Churchill & Churchill, 1980) pig, (Park & Malvin, 1978) dog, (Park, Han & Fray, 1981) and from the isolated, perfused rat kidney, (Fray 1978). Depolarization by high concentrations of potassium is thought to increase the permeability of the cell membrane to other ions and thus causes an influx of calcium into the juxtaglomerular cell, which in turn inhibits the release of renin. Park & Malvin (1978) reported that high concentrations of potassium stimulated renin release when calcium was absent. Calcium antagonists verapamil, (Park, Han & Fray, 1981) and D 600, (Churchill & Churchill, 1980; Churchill, 1980) restore renin secretion to normal levels in the presence of high concentrations of potassium. Thus, high concentrations of potassium inhibit renin release, both by a Ca

linked action on granular cells and by altering the delivery of sodium at the macula densa.

Intrarenal infusion of magnesium chloride in dogs, at a level which caused a four-fold increment in the concentration of magnesium in plasma, resulted in an increase in plasma renin activity (Churchill & Lyons, 1976). Mean arterial blood pressure was reduced and renal blood flow and glomerular filtration rate were unchanged. Since urine volume and sodium excretion increased, which would be expected to suppress renin release, it was concluded that magnesium influenced plasma renin activity, either by means of renal sympathetic nerves or by acting directly on granular cells. Wilcox (1978) investigated these possibilities by auto-transplanting a kidney to the neck of dogs. Intravenous infusions of $MgCl_2$ increased the release of renin into plasma and increased renal blood flow. GFR, sodium excretion and systemic arterial pressure were unchanged. In addition, the stimulation of renin release by magnesium was antagonised by concurrent hypercalcaemia and saline infusion.

In isolated rat kidney, increasing the concentration of magnesium in the perfusate, stimulated renin release (Fray, 1977). This effect was suppressed by increasing the concentration of potassium, or by an increase in renal perfusion pressure (Ettienne & Fray, 1979). Since magnesium did not cause a further increase in the rate of renin release after removal of calcium from the medium, (Fray, 1978) it was concluded that magnesium acted directly on the granular cell, to hyperpolarize the cell membrane. It was suggested that this resulted in an increase in the rate of renin release by lowering the intracellular concentration of calcium.

Morimoto *et al* (1970) reported that reducing the concentration of magnesium caused a small fall in renin released by dog renal cortex slices. A further reduction in renin release was observed when both calcium and magnesium were removed. In contrast to this, no significant change in the release of renin from isolated, superfused glomeruli was observed when magnesium concentration was reduced (Baumbach & Heyssac, 1977). Furthermore, plasma renin activity in normal human subjects taking a low-sodium diet did not change (Kisch *et al*, 1976) when $Mg SO_4$ was infused intravenously. Equimolar concentrations of calcium chloride and saline were both found to reduce renin levels.

From these findings, it therefore appears that magnesium stimulates renin release, while low concentrations of magnesium inhibit its secretion. However, the mechanism by which it alters the rate of secretion from granular cells is unclear.

Lithium ions may also stimulate the release of renin. Lithium therapy is often used in the treatment of manic states, and such patients have been reported to have a concentrating defect and primary polydipsia (Forrest *et al*, 1974; Singer *et al*, 1972). Acute infusion of lithium causes natriuresis and diuresis (Cox & Singer, 1978; Vander, 1968). Using toad bladder *in vitro*, lithium ions have been found to antagonise the hydro-osmotic effect of ADH and the increase in sodium transport that is induced by aldosterone (Cox & Singer, 1978; Singer & Franko, 1973).

In 1973, Gutman, Jamir & Benzakein, reported that PRA of rats was increased after lithium infusion. They suggested that this effect could be due to the natriuresis, since lithium did not increase PRA in rats taking a high dietary sodium intake. More recently, Nally, Rutecki & Ferris (1980) have studied the effects of Li in anaesthetised dogs. Acute infusion of lithium caused a rise in mean arterial pressure, PRA and urinary prostaglandin E excretion, which was accompanied by natriuresis and diuresis. The converting enzyme inhibitor SQ 20881 (teprotide) prevented the increase in both arterial blood pressure and prostaglandin E excretion, which suggests that the enhanced excretion of PGE after lithium infusion was secondary to the production of ang II. They concluded therefore, that the increase in PRA after Li was the result of a direct effect of Li at the juxtaglomerular cells.

EXTRARENAL SOURCES OF RENIN

Although plasma levels of renin are reduced after nephrectomy, several groups have measured both active, (Yu et al, 1972; Medina et al, 1972) and inactive (Leckie et al, 1977b; Sealey et al, 1977a and 1977b) renin activity in the plasma of anephric patients. Weinberger et al (1977) found that the plasma of anephric patients contained renin which had a molecular weight of 61,000 daltons. A similar value was obtained for the molecular weight of "renin-like activity" from salivary glands. However, the molecular weight of renin in normal plasma was 42,000 daltons. As a result of these findings, it was suggested that renin in the anephric patients was derived from the submaxillary salivary gland. Furthermore, renin from the submaxillary gland of the mouse is immunologically cross-reactive with kidney renin from the same species (Menzie, Hoffman & Michelakis, 1978). Purified salivary gland renin (molecular weight, 37 - 43,000 daltons) cleaves the Leu¹⁰ - Leu¹¹ bond of synthetic tetradecapeptide substrate to yield angiotensin I and a tetrapeptide fragment (Cohen et al, 1972). This suggests that, in some situations, extrarenal renin could contribute to total plasma renin

Renin-like activity has been measured in many other tissues, including brain, (Fischer-Ferraro et al, 1971; Ganten et al, 1971a), uterus, (Gordon, Ferris & Mulrow, 1967; Hodari, Carretero & Hodgkinson, 1969) and spleen (Johnson & Poisner, 1977). Renin activity can also be measured in human amniotic fluid, and increases after acidification, (Lumbers, 1971; Skinner et al, 1975) and cold storage (Craven & Symonds, 1978). Both uterine and placental renin activity increase during pregnancy in humans, (Carretero et al, 1972) and may contribute to the increase in plasma renin activity which is observed in pregnant women, (Weir et al, 1975) and in the luteal phase of the menstrual cycle (Uezano et al, 1981).

The brain renin-angiotensin system has aroused a great deal of interest in recent years, and could be involved in central regulation of thirst (see page 41). Ganten et al (1977) localised iso-renin in highly purified synaptosomes obtained from mitochondrial fractions of dog brain cells. Renin which had been separated from other acid proteases in extracts of rat brain (Hirose, Yokosawa & Inagami, 1978) appeared to be a true renin and was inhibited by antibodies which had been raised against pure porcine kidney renin. However,

in contrast to these findings, Hackenthal, Hackenthal & Hilgenfeldt (1978) concluded that isorenin from rat brain and pseudo-renin from hog spleen were closely related or identical to cathepsin D from bovine spleen, and had enzymatic properties which were distinct from semi-purified rat kidney renin. Similarly, Johnson & Poisner (1977) found that pseudo-renin from bovine spleen behaved identically to cathepsin D. Only further, careful study of renin-like activity in tissues will clarify this controversy.

The walls of the aorta and large blood vessels contain a renin-like enzyme (Barrett et al, 1978; Gould, Skeggs & Kahn, 1964; Thurston et al, 1978) which generates ang I within the physiological pH range. Vascular renin has also been reported to have a longer half-life than plasma renin. Although it is not entirely clear whether the presence of this enzyme in the walls of blood vessels is due to local synthesis or to selective uptake of renin from plasma, it may prove to be an important regulator of tissue perfusion.

Swales (1976) suggested that arterial pressure could be maintained locally by production of ang II within the vessel walls. In support of this concept, Barrett, Eggena & Sambhi (1978) demonstrated divergence between plasma and aortic renin in spontaneously hypertensive rats during treatment with hydralazine or propranolol. However, Thurston & Swales (1978) could not detect a similar relationship between these two forms of renin except during a short period after bilateral nephrectomy.

HALF-LIFE AND INHIBITORS AND ACTIVATORS OF RENIN

Plasma renin activity at a given time depends both on its rate of secretion from the kidney, and the rate at which it is degraded. In addition, the presence of endogenous inhibitors and activators of renin may also affect its measurement. At present, this remains a very neglected area of study.

The half-life of renin has been estimated, but values which have been obtained are at variance. In the dog, two components in the decay of renin have been reported with time constants of six and twenty minutes, (Assaykeen, Otsuka & Ganong, 1968) or ten minutes and ninety-three minutes (Michelakis & Misukoshi, 1971). Similarly, two components of renin decay have been observed in man, (Michelakis & Misukoshi, 1971) with time constants of thirteen and two hundred and eighty minutes respectively. In contrast to these findings, the half-life of renin has been estimated at three minutes, (Al-Merani et al, 1978) and sixteen minutes (Peters-Haefeli, 1971) in the rat. The major site of metabolism of renin is in the liver, (Heacox, Harvey & Vander, 1967; Schneider et al, 1970) although the kidney may also play a part in removing renin from the circulation (Houssay, Braun-Menendez & Dexter, 1942).

A variety of compounds have been found to inhibit the renin-
renin substrate interaction both in vitro and in vivo. In 1967, Sen, Smeby & Bumpus isolated a phospholipid fraction from the kidney and from blood that inhibited the in vitro reaction after a preliminary incubation period, and which reduced the blood pressure of rats with renal hypertension. They later reported, (Smeby, Sen & Bumpus, 1967) that the phospholipid pre-inhibitor was hydrolysed to a lysophospholipid during the incubation. The kidney has been proposed to play a part in converting the pre-inhibitor to the inhibitor phospholipid (Baggio et al, 1975). The initial step in the synthesis of prostaglandins is the activation of acylhydrolase, such as phospholipase A, (Kunze & Vogt, 1971) and the release of prostaglandin precursors and a lysophospholipid (McMurray & Magee, 1972). Thus, although the physiological significance of the lysophospholipid inhibitor of renin remains to be clarified, it is interesting that the by-product of the intrarenal synthesis of prostaglandins may be an endogenous inhibitor of renin. In support of this hypothesis,

Zachariah et al (1975) have reported that the activity of phospholipase₂ was reduced in hypertensive states where plasma renin activity was high. Interest in the phospholipid inhibitor system however, seems to have waned in recent years.

In 1973, Barrett, Eggena & Sambhi studied the progressive decline in the rate of angiotensin generation when human plasma was added to renin, and concluded that the accumulation of des-angiotensin I substrate could inhibit the renin substrate reaction. However, Hackenthal, Hackenthal & Hofbauer (1977) found no evidence for product inhibition using des-angiotensin I substrate which had been prepared from purified rat angiotensinogen. In a later attempt to identify a renin inhibiting factor in plasma, Kotchen, Talwalkar & Welch (1977) extracted neutral lipid fractions from the plasma of normotensive subjects, and found that these inhibited the renin-substrate interaction in vitro. These workers have also reported (Kotchen, Welch & Talwalkar, 1978) that the pressor response of anephric rats to renin, but not to angiotensin II, was inhibited by linoleic acid and have suggested that fatty acids may modify the measurement of plasma renin activity, and may affect the production of angiotensin I in vivo.

The presence of a renin activator in plasma has also been proposed (Sambhi & Wiedman, 1972; Sambhi et al, 1975) to account for the accelerated rate of production of ang I by plasma by hypertensive patients. This group found that adding normal human plasma did not change the rate of ang I generation by semi-purified renin from a homologous substrate, while plasma from hypertensive subjects caused a two-fold increase in V_{max} . The activator has not been chemically characterised as yet.

ANGIOTENSINS

Renin per se has no known physiological action other than to catalyse the production of angiotensin I. Until recently, ang II was considered to be the main effector peptide of the renin-angiotensin system, but it is now clear that ang I and (des-Asp¹) ang II (which is also known as angiotensin III) also have biological activity. Recent developments in the synthesis of ang II analogues, which have antagonistic properties, have been important in clarifying the vast spectrum of physiological actions of ang II. These actions will be described in the first part of this review. Ang I and ang III will be discussed in a later section.

PRESSOR ACTION OF ANGIOTENSIN II

Angiotensin II is the most potent, naturally occurring vasoconstrictor agent known. Despite this apparently simple statement, the physiological importance of the pressor action remains highly contentious. Studies with the converting enzyme inhibitor, captopril have, in some cases, provided evidence that the pressor actions of ang II are important in blood pressure homeostasis in normal subjects (MacGregor et al, 1980). There has been enormous interest in the role played by the renin system in the pathogenesis of high blood pressure. Consideration of the extensive literature is outside the scope of this thesis. The current position would seem to be as follows. Certain authors, (see Laragh, 1980) feel that the renin system has a major role in maintaining or causing elevated blood pressure in most forms of human hypertension. Other authors, (see Swales, 1979) favour a rather more limited view and consider that the changes in the renin-angiotensin system in essential hypertension are secondary to renal damage rather than a primary cause of the hypertension.

Part of the confusion over the involvement of the renin system in blood pressure control may be related to the multiplicity of

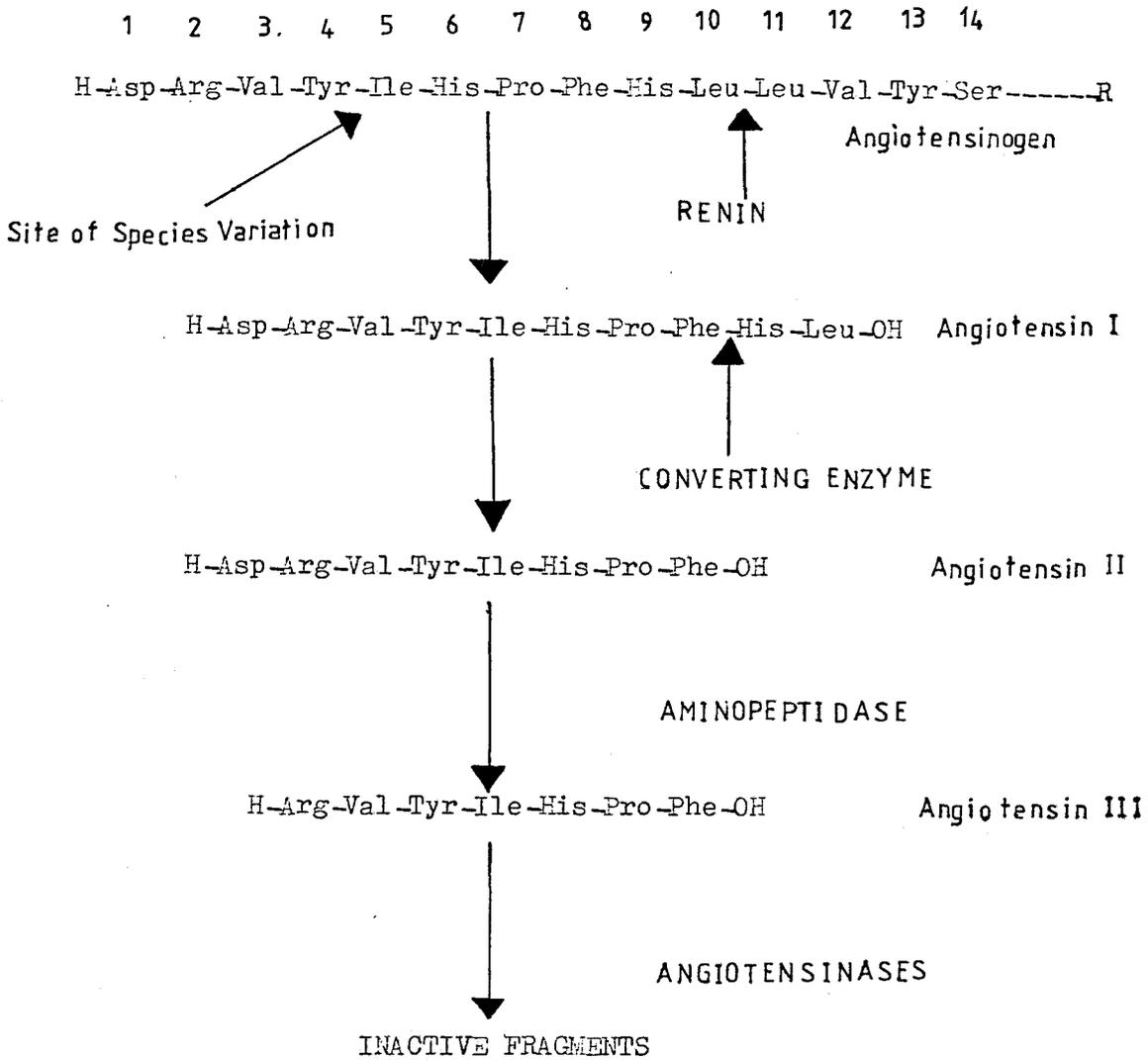


Fig. 1:3

The Enzymatic Conversion of Horse Angiotensinogen Into Peptides.

physiological activities of ang II, which contribute to the overall pressor response.

Peripheral vascular resistance is influenced not only by plasma renin activity and circulating levels of ang II, but also by the responsiveness of vascular smooth muscle to ang II. This has been reported to vary during alterations in dietary sodium intake in man, (Hollenberg *et al*, 1975; Reid & Laragh, 1965) and rats, (Douglas, 1979) and is increased during the development of hypertension in these species (Collis & Alps, 1975; Kisch, Dluhy & Williams, 1976). In contrast to these findings, Cowley & Lohmeier (1978) reported that short-term alterations in salt and water balance did not change the vascular sensitivity to ang II.

Specific binding sites for ang II have been identified in the intact rabbit aorta, (Lin & Goodfriend, 1970) and in microsomal preparations of rabbit, (Badouin *et al*, 1972; Devynck *et al*, 1973) and guinea pig aorta (Le Morvan & Palaic, 1975). However, these receptors appear to have a low affinity for ang II, compared with circulating levels. In addition, large vessels such as the aorta may not contribute significantly to peripheral vascular resistance. More recently, a single class of angiotensin receptors were identified in a particulate fraction prepared from rat mesenteric arteries (Gunter, Gimborne & Alexander, 1980). These binding sites were saturable at physiological concentrations of ang II, and were of high affinity.

The direct effect of ang II on vascular smooth muscle is independent of any change in transmembrane potential, (Shibata & Briggs, 1966) and depends on the presence of extracellular calcium ions (Ackerley, Moore & Peach, 1977; St Louis *et al*, 1977). However, in addition to its effects on vascular cells, ang II also interacts with the central and peripheral nervous systems, and therefore exerts an indirect pressor effect in the circulation.

When injected into vertebral arteries of conscious dogs, subpressor doses of ang II elicit an increase in blood pressure (Fukiyama, McCubbin & Page, 1971). In rats, injection of ang II into the anterior hypothalamic/preoptic region of the brain causes an increase in both arterial blood pressure and in heart rate (Benarroch *et al*, 1981). This effect depends on the presence of a serotonergic and noradrenergic pathways in the fore-brain and brain-stem. Actions of angiotensin on structures within the central nervous

have been reviewed by Fitzsimmons (1980). These are of particular interest in view of the demonstration of the brain-isoenzyme system (reviewed by Ganten, 1981).

Angiotensin II also interacts with peripheral nervous pathways by potentiating transmission from ganglia (Kaneko, McCubbin & Page, 1961) and nerve terminals (Benelli, Della Bella & Gandini, 1964). This effect is inhibited by saralasin (Zimmermann, 1973) and is therefore mediated by specific angiotensin receptors on nerve endings. Ang II also stimulates noradrenaline biosynthesis by an action on dopamine β hydroxylase (Ackerley, Elenberg & Peach, 1976) and inhibits re-uptake of noradrenaline after stimulation of sympathetic nerves (Khairallah, 1972). This may account for the facilitation of neural transmission. The importance of these effects of ang II on adrenergic nerve endings has been reviewed by Zimmermann (1978).

ANGIOTENSIN II IN THE HEART

When ang II is infused into animals with intact baroreceptor reflexes, reflex bradycardia is observed (Page & Olmstead, 1961). However, when baroreceptor reflexes are suppressed by barbiturate anaesthesia, or vagal tone is inhibited by atropine or vagotomy, ang II causes cardiac acceleration. Similarly, in isolated heart preparations, ang II can elicit positive inotropic responses (increase in myocardial contractility) and increase heart rate (Mishith, Davis & Youmans, 1962; Bonnardeaux & Regoli, 1974). The presence of specific ang II receptors in myocardial cells has been implicated since the effects of ang II in isolated canine heart preparations are suppressed by saralasin (Kobayashi, Furukawa & Chiba, 1978).

Recently, Lumbers and her co-workers have studied the effect of ang II on baroreceptor reflexes in conscious dogs (Lumbers, McCloskey & Potter, 1979) and sheep (Lee, Ismay & Lumbers, 1980). By recording from vagal efferents in dogs they found that ang II had

an inhibitory action on the vagus and this prevented cardiac slowing in response to an increase in systolic pressure induced by injection of ang II. When the pressor action of ang II was antagonised by simultaneous infusion of sodium nitroprusside in sheep, ang II produced tachycardia. They concluded that ang II causes a central, dose-dependent reduction in vagal tone that is normally antagonised by the baroreceptor reflex response to an increase in arterial pressure.

ANGIOTENSIN II AND FLUID AND ELECTROLYTE BALANCE

The renin-angiotensin system is involved in maintaining the constancy of the extracellular medium in several ways. Angiotensin II exerts a direct action on transporting epithelia and influences sodium transport indirectly by stimulating the secretion of aldosterone and possibly that of antidiuretic hormone (ADH). In addition, angiotensin influences salt and electrolyte balance by its effect on renal haemodynamics, (see page 42) and thirst mechanisms, (see page 41).

In rat renal cortex slices, (Munday, Parsons & Poat, 1971) ang II stimulated salt and water transport by means of a ouabain-insensitive, potassium-independent sodium pump. The effect of ang II does not involve cyclic AMP, (Munday, Parsons & Poat, 1972) and is blocked by inhibiting protein synthesis at the level of translation. The effect of ang II on sodium transport has also been investigated in other transporting epithelia such as frog skin, (McAlfee & Locke, 1967) isolated rat jejunum, (Crocker & Munday, 1970) and rat colon, (Davies, Munday & Parsons, 1970) tissues which have structural and functional properties similar to those of the nephron. The jejunum responds to ang II with uptake of sodium and water at low doses, and inhibition of transport at high doses (Bolton et al, 1975). Recently, it has been found that stimulation of sodium transport by ang II in rat jejunum is mediated by noradrenaline, (Levens et al, 1979) and is inhibited by reserpine and phentolamine.

Angiotensin II also exerts a biphasic action in the kidney. Using a micropuncture technique, Harris & Young (1977) found that low doses of ang II caused sodium retention, while high doses resulted

in natriuresis and diuresis in the rat. Similarly, ang II bound to isolated proximal tubule cells and exerted a biphasic effect on the rate of sodium extrusion (Freedlander & Goodfriend, 1977). Two classes of binding sites for ang II were later (Freedlander et al, 1980) identified using ^{125}I -labelled tracers. One class of receptor had a high affinity for the peptide and were located on the basolateral membrane; low affinity sites were associated with brush-border epithelium. However, the physiological significance of these receptors remains to be clarified. Angiotensin II may also stimulate sodium transport in the loop of Henle. Ploth & Navar (1979) found that inhibition of the intrarenal renin-angiotensin system by the converting enzyme inhibitor, teprotide, or with saralasin caused a reduction in sodium transport, as measured by micropuncture, between the proximal and distal tubules.

In man, (Laragh et al, 1960) and rats, (Hauger, Aguilera & Catt, 1978) ang II stimulates the release of aldosterone from the zona glomerulosa of the adrenal cortex. Similar findings have been reported from experiments using isolated, perfused adrenal glands, (Ganong et al, 1962) and adrenal slices (Kaplan & Bartter, 1962). Aldosterone promotes sodium re-absorption and enhances the excretion of potassium. Receptor sites for ang II have been identified in isolated zona glomerulosa cells; and their number and affinity increases in rats taking low sodium, (Hauger et al, 1978) or high potassium diets, (Douglas, 1979)

Unlike the stimulation of aldosterone secretion by ACTH, the response to ang II does not involve cyclic AMP (Shima, Kawashima & Hirai, 1978). Fakundig, Chow & Catt (1979) found that ang II increases aldosterone production by increasing the rate of calcium flux, which in turn results in an increase in the intracellular calcium concentration. Angiotensin II appears to increase the rate of conversion of cholesterol to pregnenolone (Kaplan & Bartter, 1962; Muller, 1966) which is the first stage in the biosynthetic pathway for aldosterone. Prolonged stimulation by ang II also increases the production of aldosterone from corticosterone (Komer & Muller, 1979).

Angiotensin II may act centrally to inhibit aldosterone secretion (Brooks & Malvin, 1980). Intra-cranial administration of ang II in anaesthetized sodium-depleted dogs reduced plasma aldosterone, PRA and potassium concentration. However, the fall in plasma aldosterone could be secondary to changes in plasma ang II or potassium

levels. Intraventricular injection of ang II in conscious rats increased the secretion of corticosterone from the adrenal cortex (Daniels-Severs *et al*, 1971). It has been suggested that ang II acts directly on the anterior pituitary to enhance the secretion of ACTH. This would in turn alter adrenal cortical function, (Maran & Yates, 1977). In addition, ang II may influence the secretion of ADH from the posterior pituitary. The significance of this relationship is unclear, and is based on the finding by Mouw *et al* (1971) who showed that ang II induced the secretion of ADH. High, pressor doses, however, were required to elicit this response.

It seems likely that the physiologically important site for ang II to stimulate the secretion of ADH will be located within the central nervous system and again the brain renin system may prove to be important for this function (see review by Fitzsimmons, 1980).

ANGIOTENSIN II AND THIRST

That there is a brain renin-angiotensin system which is involved in the regulation of thirst, is now quite well established. When injected intra-cranially, ang II is a potent dipsogen in many species (Fitzsimmons, 1972). Very low (10^{-12} M) concentrations of ang II elicited drinking in dogs when injected into the preoptic nucleus and sub-fornical organ. Specific binding sites for ang II have been identified in rat brain, (Snyder, 1978) and the drinking response is suppressed by the ang II inhibitor, saralasin (Cooling & Day, 1973). By means of immunofluorescence techniques, ang II has been localised in nerve terminals in some regions of rat brain (Ganten *et al*, 1977). These findings suggest that endogenous angiotensin could play a role in the regulation of thirst mechanisms.

The effectiveness of systemic ang II in eliciting drinking responses is more controversial, however. Elghozi *et al* (1977) noted that the regions of the brain which are most sensitive to the action of both blood-borne and intra-cranial ang II are vascularized, periventricular structures, which lie outside the blood-brain-barrier. These include the sub-fornical organ and organum vasculosum of the

lamina terminalis (OVLT). It was later reported, (Fitzsimmons, Kucharczyk & Richards, 1978) that systemic administration of ang II could elicit a drinking response. Thus angiotensin may cross the blood-brain-barrier. A similar conclusion was reached by Nemeroff & Prange (1978). Angiotensin-induced thirst has recently been reviewed by Fitzsimmons (1979; 1980).

INTRARENAL EFFECTS OF ANGIOTENSIN II

The direct effects of ang II on sodium transport in the nephron have already been described (see page 39).

When subpressor doses of ang II are infused directly into the kidney, both renal blood flow (RBF) and glomerular filtration (GFR) are reduced (Bock et al, 1968). As the reduction in RBF is greater than that of GFR (Navar & Langford, 1974), filtration fraction increases. Since infusion of ang II may not accurately mimic the effect of intrarenally-formed peptide, several investigators have used inhibitors of the renin-angiotensin system in their investigations.

In early studies, saralasin was found to reduce renal vascular resistance, and thereby increase renal blood flow, after renin levels had been increased by sodium depletion, (Mimran et al, 1974) renal artery occlusion, (Satoh & Zimmerman, 1975) and high output cardiac failure (Freeman et al, 1975). These authors observed no changes in GFR or in water and electrolyte excretion. However, infusion of saralasin was associated with a fall in mean arterial pressure, which in turn could mask any changes in electrolyte excretion. A further difficulty in interpreting data obtained with saralasin is that the blocker itself has some antagonist affinity.

More recently, intrarenal infusions of angiotensin antagonists in doses that did not significantly change mean arterial pressure, increased renal blood flow, GFR and sodium and electrolyte excretion Hall et al, 1977; Trippodo et al, 1977). Similarly, the converting enzyme inhibitor, SQ 20881, (Hall et al, 1979; Kimbrough et al, 1977) increased GFR and sodium and potassium excretion in normal and sodium-depleted dogs.

Angiotensin II decreases blood flow to the outer kidney cortex, (La Grange & Schmidt, 1975) but has a variable effect on inner cortical flow, where flow is dependent on prostaglandin synthesis. Ang II reduces cortical and medullary blood flow when prostaglandin synthesis is inhibited by indomethacin (Itskovitz & McGiff, 1974). When it is infused directly into peritubular capillaries, (Jensen & Steven, 1977) ang II reduces capillary diameter as a result of active contraction, and may therefore be a physiological regulator of hydrostatic pressure in peritubular capillaries (Steven & Thorpe, 1977).

The role of the intrarenal renin-angiotensin system in the control of renal function has recently been reviewed by Levens, Peach & Carey (1981) and interactions between ang II and prostaglandins in the regulation of renal haemodynamics has been reviewed by Baer & McGiff (1980).

ACTIONS OF ANGIOTENSIN I AND ANGIOTENSIN II

The first evidence which suggested that ang I possessed any physiological activity was obtained by Peach (1971), who showed that ang I was equipotent with ang II in stimulating the release of catecholamines from the isolated, perfused adrenal medulla. No evidence for the conversion of ang I to ang II could be detected, (Peach, Bumpus & Kairallah, 1971) and the intact decapeptide is required for this response (Ackerley, Felger & Peach, 1976). Intra-cranial injection of ang I evokes a drinking response (Fitzsimmons, 1971), but it is a less potent dipsogen than ang II (Fitzsimmons & Kucharczyk, 1978). Ang I also facilitates the release of noradrenaline from peripheral sympathetic nerves (Johnson, Marshall & Needleman, 1974). However, it is important to remember that any action of ang I depends on the fact that it is not converted to ang II. Converting enzyme inhibitors may not completely prevent the conversion from taking place.

The pressor response to (des-¹asp) ang II, (angiotensin III) has been measured in several species. When compared with ang II, the pressor potency of ang III is 30 - 50% in rats, (Spielman, Davis & Freeman, 1976; Vaughan et al, 1977) 24% in conscious rabbits (Rowe,

Noble & Munday, 1979) and 11 - 36% in humans (Carey et al, 1978). Intrarenal infusions of ang II and ang III in sodium-depleted dogs were found to cause a comparable reduction in both renal blood flow and renin release (Freeman, Davis & Lohmeier, 1975). The effect of ang III on renal blood flow has been confirmed by Freeman, Davis & Khosla (1978) and Britton, Beierwaltes & Fiksen-Olsen (1979). However, ang III may only play a minor role in the regulation of renal blood flow, (Hall et al, 1979) since infusion of (des¹-asp, ile⁸) ang II, which antagonises ang III, had no effect on renal haemodynamics in dogs. A similar conclusion was reached by Taub, Caldicott & Hollenberg (1977).

Angiotensin III appears to be at least as effective as ang II in stimulating aldosterone synthesis in the rat, (Campbell, Brooks & Pettinger, 1974; Spielman et al, 1976) but possesses only 15 - 30% of the steroidogenic activity of ang II in human subjects taking a normal sodium diet (Carey et al, 1978). However, when subjects took a low sodium intake, the response of aldosterone synthesis to ang III was greater than the response to ang II. A single class of receptors for both peptides has been identified in bovine adrenal cells, (Vallotton et al, 1981) but a second class of receptors with very high affinity for ang III have been found in rat adrenals Devynck et al, 1977). The physiological actions of "other" angiotensins were reviewed by Peach, (1977) and by Davis & Freeman, (1977).

THE KALLIKREIN-KININ SYSTEM

Kallikreins are a group of serine protease enzymes which release vaso-active peptides, known as kinins, from endogenous substrates which are present in plasma, lymph and interstitial fluid. Kallikrein was first discovered in 1926 by Frey, who demonstrated that a thermo-labile, non-dialyzable substance from urine produced arterial hypotension when injected intravenously in dogs. In 1934, Werle described a similar substance from the pancreas and blood, and noted that it was inactive but could be readily activated. Many of the early conceptions of kallikreins have been changed. It is now clear that two classes of kallikreins exist: plasma kallikrein and glandular kallikreins. These two forms of the enzyme have distinct properties, which are summarised in Table 1.1. There are also two different forms of the endogenous kallikrein substrate. These are known as, low molecular weight (LMW) and high molecular weight (HMW) kininogen. The kallikrein-kinin system has been recently reviewed by Schacter (1980).

PLASMA KALLIKREIN

Kallikrein in plasma was once thought to be a circulating form of pancreatic kallikrein, but it is now accepted that the plasma enzyme is different from all other forms. The kallikrein-kinin system in plasma is much better characterized than, for example, the renal system. The enzyme exists only in an inactive form, prekallikrein, in plasma. Plasma prekallikrein is a basic protein which has a molecular weight of approximately 107,000 daltons and pI of 8.5-8.9 (Spragg & Austen, 1977). For activation, prekallikrein is enzymatically cleaved by Hageman factor or trypsin (Wuepper, 1977). Hageman factor (factor XII) is important in the intrinsic pathway of the cascade which leads to the formation of fibrinogen, as it activates plasma thromboplastin antecedent (PTA). Hageman factor itself

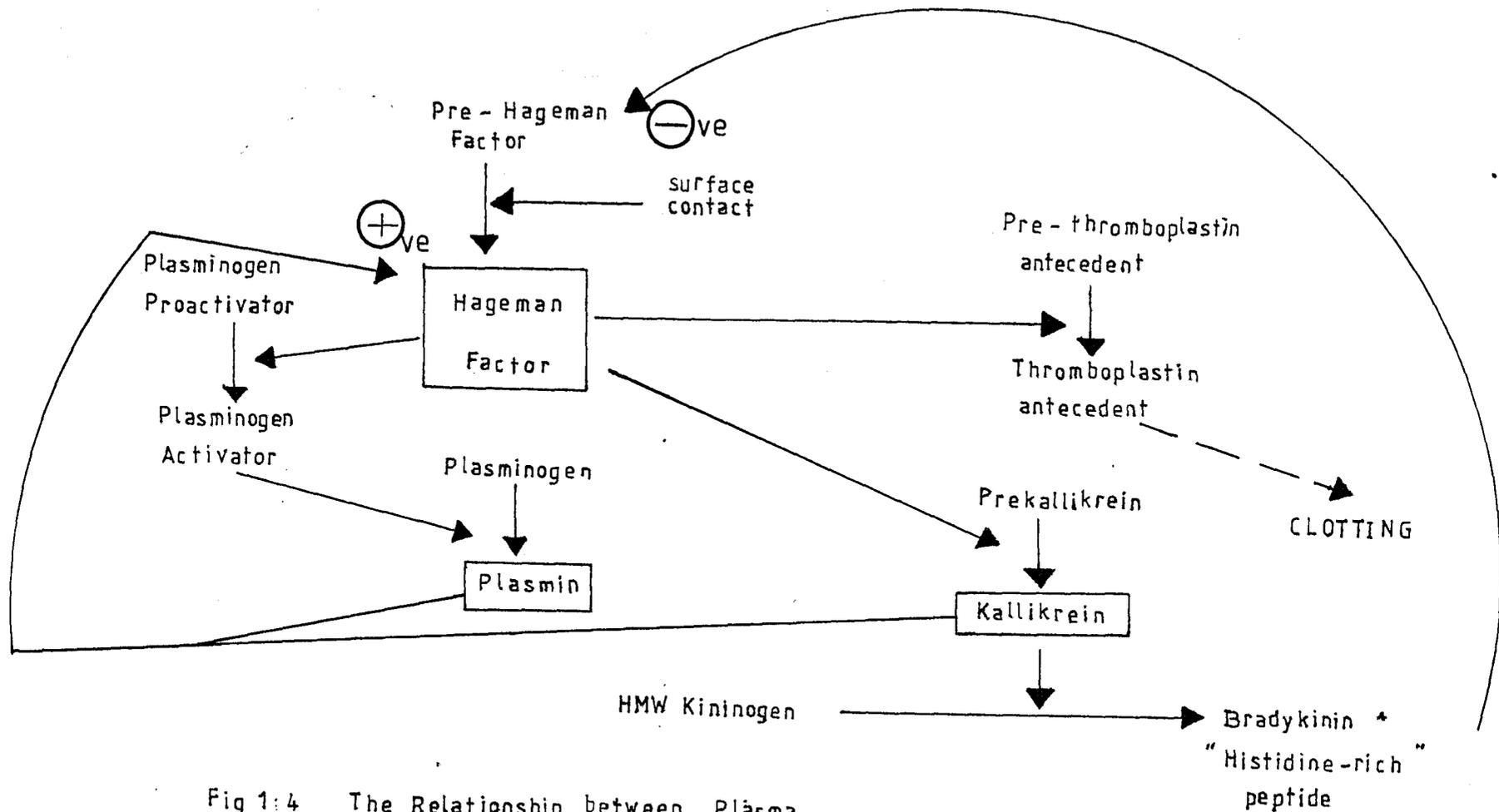


Fig 1:4 The Relationship between Plasma Kallikrein and Clotting Mechanisms (from Katori et al, 1975)

is activated by contact with negatively charged surfaces, including glass, silica and collagen (Webster & Oh-Ishi, 1977) in the presence of prekallikrein and other plasma factors. Kallikrein may further activate Hageman factor (see Fig 1.4).

After it has been activated, kallikrein acts on high molecular weight kininogen, which is an α_2 -globulin of molecular weight 80,000 - 200,000 daltons, to release bradykinin. The molecular weight of prekallikrein may change on activation. In the rabbit, prekallikrein has MW of 100,000 daltons, while the MW of active kallikrein is 80,000. In contrast, the molecular weight of human kallikrein is the same as that of prekallikrein (Colman & Bagdasarian, 1976). Four inhibitors of kallikrein have been identified in plasma, (Heinberger, Haupt & Schmidt, 1971) C \bar{I} esterase inhibitor, α_2 -macroglobulin, α_1 -antitrypsin and antithrombin III. However, these are not specific for kallikrein alone, but inhibit a range of serine proteases which all have similar active sites.

Bradykinin is a basic nonapeptide, (pI=10) by virtue of the arginine residues at either end of the molecule. Its primary effect is to increase vascular permeability at the site of injury, but it is also a potent vasodilator, and induces contraction of bronchial and intestinal smooth muscle. The half-life of injected bradykinin is about fifteen seconds. In plasma, bradykinin is inactivated mainly by angiotensin I converting enzyme, (kininase II) which is located in vascular endothelium of most tissues, (see page 6).

GLANDULAR KALLIKREINS

Kallikrein-like enzymes have been identified in a number of tissues which produce fluid secretions, including pancreas, salivary glands, gut, reproductive tract and sweat glands (Nustad, Gautvik & Pierce, 1977). The complete amino acid sequence of porcine pancreatic kallikrein has been established (Tschesche et al, 1979). This enzyme exists in two forms, A and B, which differ only in their carbohydrate content. Both consist of two peptide chains which are linked by disulphide bridges, have 252 amino acid residues, and molecular

TABLE 1.1 Characteristics of Plasma & Glandular Kallikreins
(after Carretero & Scili, 1980)

	<u>Plasma Kallikrein</u>	<u>Glandular Kallikreins</u>
Molecular Weight	100,000	24,000 - 44,000
Substrate	HMW kininogen	HMW & LMW kininogen
Kinin liberated	Bradykinin	Lys-bradykinin (kallidin)
Inhibition by		
Soya Bean Trypsin Inhibitor	Yes	No
Function	<ul style="list-style-type: none"> a) Coagulation b) Fibrinolysis c) Inflammation d) complement activation e) Blood pressure homeostasis? 	<ul style="list-style-type: none"> a) Regulation of local blood flow b) Water and electrolyte excretion? c) Blood pressure homeostasis?

weights of 23,600 daltons. Pancreatic, submandibular and urinary kallikrein of the pig have similar amino acid compositions and are immunologically cross-reactive (Fritz et al, 1977). By immunofluorescence, Orstavick et al (1980) localized kallikrein in the granular portion of acinar cells in the human pancreas. Kallikrein was absent from acinar cells in salivary glands, but was present in the striated duct cells.

Glandular kallikreins are rarely released into the circulation, and therefore do not contribute significantly to the regulation of systemic pressure. Kallikrein-like enzymes are released from rat jejunum when it is perfused with hyperosmolar glucose (Worthington & Cuschieri, 1977). A similar situation occurs in the "dumping syndrome" which is sometimes observed in patients after partial gastrectomy (Hinslaw, Joergenson & Davies, 1957). This syndrome is caused by the passage of hyperosmolar material directly into the jejunum. High levels of kallikrein in plasma are also associated with acute pancreatitis, where kallikrein is released into the blood from the pancreas (Popieraitis & Thompson, 1969). Both of these syndromes are associated with hypotension and increased levels of circulating kinins.

RENAL KALLIKREIN-KININ SYSTEM

Renal and urinary kallikrein, like other glandular kallikreins, are acidic glycoproteins with molecular weight of 27,000 - 43,000 daltons (Pisano, 1975). Urine and kidney of the rat contain four forms of kallikrein, and those in urine are identical to those in the kidney (Kustad et al, 1974; Nustad et al, 1975). Most (90%) of renal kallikrein is found in the cortex, and its concentration is greater in the outer cortex than in the inner cortex (Sciqli, Carretero & Oza, 1976). In the rat, kallikrein has been localized in the cells of the distal tubule, (Orstavick et al, 1976) between the macula densa and the collecting duct. Furthermore, stop-flow experiments have shown that kallikrein is released into tubular fluid from more distal parts of the nephron (Sciqli et al, 1978). Within

the cell, the localization of renal kallikrein is uncertain. Some investigators have reported that kallikrein is associated with plasma membranes and endoplasmic reticulum, (Heidrich & Geiger, 1980; Mustad, 1970; Ward, 1975) but others have found that it is associated with lysosomes (Baggio *et al*, 1975; Carvallo & Diniz, 1966).

Bradykinin, lys-bradykinin (kallidin) and met-lys-bradykinin have all been identified in urine (Hial, Kaiser & Pisano, 1976). These appear to be formed in the kidney or urine, since kinins which are injected into the renal artery fail to appear in urine (Nasjletti, Colina-Chourio & McGiff, 1975). Hall *et al* (1976) and Carone *et al* (1976) reported that the brush border of the proximal tubule is rich in kinases, particularly kininase II. Thus, filtered kinins are destroyed before reaching the more distal parts of the nephron. Similarly, radiolabelled pancreatic kallikrein injected into the dog is destroyed before reaching the urine (Mills, Paterson & Ward, 1975). However, radiolabelled kinins which are injected into the distal tubule appear intact in urine (Carone *et al*, 1976). Thus, kallikreins and kinins in urine are derived from the kidney and are not related to plasma kallikrein.

Recent reviews of the renal kallikrein-kinin system include those by Carretero & Scicli (1980) and Nasjletti & Malik (1981).

Since kallikrein excretion in urine is thought to reflect intrarenal turnover of the enzyme, and therefore of kinins, many workers have studied the effects of a wide variety of physiological and pharmacological stimuli on kallikrein excretion in urine. A variety of renal vasodilators including kinins, (Barraclough & Mills, 1965; Webster & Gilmore, 1964) acetylcholine, (Mills *et al*, 1976; Vander, 1964) prostaglandin E₁, (Johnston, Herzog & Laufer, 1967; Mills & Obika, 1977) and dopamine (Goldberg, McDonald & Zimmerman, 1963; Mills & Obika, 1976) increase both sodium and kallikrein excretory rates. Kallikrein excretion is directly related to renal artery pressure distal to a clamp, (Bevan *et al*, 1974) and it is possible that these vasodilators could be acting to increase renal kallikrein excretion via a change in renal artery pressure. The effect of intrarenal infusion of bradykinin is abolished by reducing renal artery pressure (Mills & Obika, 1977).

Paradoxically, noradrenaline (Mills & Obika, 1977) and angiotensin II (MacFarlane, Adetuyibi & Mills, 1974) also increase kallikrein excretion in urine. These effects appear to be mediated, at

least in part, by prostaglandins, since they were reduced by indomethacin. Recently, Maier et al (1981) studied the effect of changing renal artery pressure on kallikrein excretion during haemorrhagic hypotension in the pig. They concluded that the kallikrein-kinin system was activated as pressure was reduced within the autoregulatory range. On the other hand, the activity of the renin-angiotensin system increased most profoundly after pressure had fallen below 70mm Hg. In contrast to these findings, Johnston et al (1980b) reported that both systems were activated throughout the pressure range, and concluded that the vasodilator action of kinins modulated the effect of angiotensin,

Many of the observed effects of renal kallikreins are mediated by the kinins which they liberate. Infusion of kallidin (Webster, 1964) and bradykinin (Barraclough & Mills, 1965; Stein et al, 1972; Willis et al, 1969) increases renal blood flow and sodium excretion without altering GFR. Most of the decrease in renal vascular resistance occurs in the afferent arteriole (Baylis et al, 1976). More recently, Johnston et al (1981a) found that kinins increased renal blood flow and reduced renal vascular resistance in rats receiving a high sodium diet, but not those taking a low sodium diet. However, it is probable that kinins administered in this way do not mimic the effect of intrarenally-produced kinins, since filtered kinins are destroyed in the proximal tubule, while kallikrein liberates kinins in the distal part of the nephron. Moreover, the majority of studies which have been reported have been performed with bradykinin rather than kallidin. Bradykinin and kallidin have different potencies, (Reis et al, 1971) and may be hydrolysed at a different rate (Johnston & Erdos, 1977).

With the development of converting enzyme inhibitors (CEI), these drugs have been used to study the effects of increased levels of kinins in kidney tissue. Bailie & Barbour (1975) found that juxtamedullary blood flow and sodium excretion increased. Similarly, Johnston et al (1981b) observed an increase in renal blood flow after captopril treatment in both sodium-loaded and sodium-depleted rats. They noted that infusion of kinins only increased RBF in rats taking the high sodium diet. The problem with this type of study is that it is difficult to differentiate between the effects of inhibition of ang II production and potentiation of kinins.

Other experimenters have used the serine protease inhibitor, aprotinin (Trasylol), which inhibits kallikrein and other protease

enzymes. Kramer et al (1979) found that renal blood flow, GFR, urine volume and excretion of prostaglandin E_2 were all reduced after administration of Trasylol in volume-expanded rats. A fall in renal blood flow and an increase in renal vascular resistance has been reported by Johnston and his co-workers (1981a and b) in sodium-depleted rats given Trasylol.

Sodium-depletion increases kallikrein excretion in man, (Levy, Frigon & Stone, 1978; Margolius et al, 1974 a and b) and rats (Geller et al, 1972; Johnston et al, 1981a; Johnston, Matthews & Dax, 1976; Mimram et al, 1977). This may be related to the increase in plasma renin which is also observed during dietary sodium-depletion, and will be discussed in more detail in a later part of this thesis (see page 116).

There is greater controversy surrounding the relationship between kallikrein excretion and increased sodium intake. Antibodies to bradykinin decreased sodium excretion in saline-infused rats (Marin-Grez, 1974), which suggests that release of kinins may mediate the natriuresis that occurs during volume expansion. Furthermore, the concentration of kinins in renal venous blood increased during saline infusion in anaesthetized dogs (Marin-Grez et al, 1972). A similar increase in kallikrein in renal lymph has also been reported (De Bono & Mills, 1974). Consistent with this view are reports that kallikrein excretion in urine is directly related to dietary sodium intake in man, (Adetuyibi & Mills, 1972) and increases during saline infusion in dogs, (De Bono & Mills, 1974) and rats (Croxatto et al, 1975 and 1976; Godon & Damas, 1974).

In man, saline infusion did not change kallikrein excretion (Levy et al, 1978; Margolius et al, 1974a). It may be that some critical fluid volume must be exceeded before increased sodium intake can stimulate kallikrein excretion. When rats (Johnston et al, 1976; Marin-Grez & Carretero, 1973) and rabbits (Mills & Ward, 1975) were denied free access to water, increasing dietary sodium intake did not cause an increase in the excretion of kallikrein. Water loading itself appears to increase kallikrein excretion in man, (Levy et al, 1978) rats, (Croxatto et al, 1975 and 1976) and rabbits (Mills et al, 1976; Mills & Ward, 1975). This may be related to the prostaglandin-stimulatory effect of kinins, (McGiff et al, 1976; Nasjletti & Colina-Chourio, 1976; Terragno et al, 1972) since pretreatment with indomethacin blocks the increase in solute-free water in response to bradykinin infusion (McGiff, Itskovitz & Terragno, 1975).

The physiological role of the kallikrein-kinin system in the kidney has been reviewed by Baer & McGiff (1980), by Levinsky (1979) and by Nasjletti & Malik (1981).

THE KALLIKREIN-KININ SYSTEM IN HYPERTENSION

Over forty years ago, Elliot & Nuzum (1934) found that hypertensive patients excrete subnormal amounts of kallikrein in urine. In recent years, this has been confirmed by several groups of investigators (Levy et al, 1977; Margolius et al, 1971; Miyashita, 1971; Seino et al, 1975). In contrast to these findings, Lawton & Fitz (1977) found no reduction in kallikrein excretion in patients with "normal renin" essential hypertension. These authors suggested that kallikrein excretion could be correlated with plasma renin in hypertensive states. Thus, the reduced excretion of kallikrein that was observed in the previous studies of unclassified hypertensive patients could be accounted for by the presence of "low renin" hypertensives. Kallikrein excretion is also high in patients with primary aldosteronism (Abe et al, 1977; Lechi et al, 1976; Margolius et al, 1974b; Seino et al, 1975 and 1977). These subjects have low plasma renin. In addition, treatment with aldosterone increased urinary kallikrein excretion, (Nasjletti, McGiff & Colina-Chourio, 1978) and kallikrein activity in renal extracts (Nishimura et al, 1980) from rats. Spironolactone treatment reduces kallikrein excretion in primary aldosteronism, (Margolius et al, 1974b) but not in essential hypertension (Seino et al, 1977).

There appears to be an important racial difference between negro and caucasian subjects in relation to the role of the renal kallikrein-kinin system in the pathogenesis of hypertension. Epidemiological studies have shown that black children (Zimmer et al, 1976) and adult black males (Levy et al, 1977 and 1978) have lower kallikrein concentrations in urine than age-matched white subjects. Upright posture and sodium restriction increased both plasma renin and urinary kallikrein excretion in all groups except black hypertensives.

A variety of animal models have been used to investigate the

role of kallikrein in hypertension. Goldblatt one-kidney hypertension is induced by placing a clip on one renal artery and removing the contralateral kidney. This model has been associated with decreased urinary kallikrein excretion (Croxatto et al, 1976; Geller et al, 1975; Lechi et al, 1976b; Pisano et al, 1974) and reduced kallikrein activity in renal tissue (Albertini et al, 1979; Favaro et al, 1975). Conflicting results have been obtained using the two-kidney Goldblatt hypertension model, where the contralateral kidney remains functional. Kallikrein excretion has been found to be unchanged (Albertini et al, 1979) reduced, (Keiser et al, 1976) or increased (Johnston et al, 1976). It is interesting that kallikrein excretion is within the normal range in patients with renovascular hypertension (Abe et al, 1977; Margolius et al, 1971; Seino et al, 1975).

Studies with spontaneously hypertensive (SHT) strains of rats have yielded conflicting results concerning kallikrein excretion. Favaro et al (1975) found reduced kallikrein excretion in rats of this strain, compared to normotensive Wistar rats; but Porcelli, Bianchi & Croxatto (1975) reported that it was only reduced in SHT rats which developed hypertension. Both normotensive and hypertensive Wistar-Kyoto rats had similar kallikrein excretion at 16 weeks, (Geller et al, 1975) but it was reduced in the hypertensive group at 23 weeks. Kallikrein excretion was also reduced in rats which were bred to be susceptible to the hypertensive effect of sodium (Carettero, 1978).

In summary, many studies suggest that the activity of the kallikrein-kinin system may be suppressed in some hypertensive states. However, this effect may be limited to some forms only of essential hypertension in man, and to some animal models of hypertensive states. Mills (1979) has reviewed the role of kallikrein in the control of blood pressure.

CHAPTER 2

ASSAY METHODS AND IN VITRO TECHNIQUES.

RENIN ASSAY METHODS.INTRODUCTION

The vast majority of techniques which have been described for the measurement of renin are "indirect" assays. They are based on the ability of renin to generate angiotensins from endogenous and exogenous substrates. Goldblatt et al (1943) measured the change in arterial blood pressure in dogs after injection of kidney extracts. This method, which relies on the conversion of ang I to ang II in the circulation, was sufficient to estimate renin activity in this instance, but was not sensitive to the lower levels of renin in plasma samples. Later experimenters (Helmer & Judson, 1963) included an in vitro incubation step, and found that the angiotensin generated by renin in plasma samples could then be measured by bioassay. The most commonly-used preparation for measuring renin by bioassay was the anaesthetized, ganglion-blocked rat, but an in vitro rat colon preparation has also been described (Regoli & Vane, 1964).

There are several problems which are encountered in the use of bioassay techniques. Generally, incubation of renin with angiotensinogen results in a mixture of angiotensins which are then assayed against a single standard ang II. Most bioassays depend on the ability of ang II to cause vasoconstriction, and therefore increase arterial blood pressure. Thus, in order to measure ang II, converting enzyme must be present, and must not impose a rate-limiting step. Furthermore, breakdown of ang II by angiotensinases must be prevented. However, it is not generally possible specifically to inhibit angiotensinases, without also inhibiting converting enzyme. For example, the chelating agent, EDTA, which blocks the activity of angiotensinases, also inhibits conversion of ang I to ang II (Skeggs et al, 1956). The presence of other vasoactive peptides in the incubated, unknown sample can cause renin activity to be overestimated. This problem can be overcome by dialysing plasma before it is incubated (Helmer & Judson, 1963) or by separating angiotensin from other plasma components by Dowex resin (Boucher, Menard & Genest, 1967)

Most of the studies which have been performed during the last decade, have involved the measurement of renin by radioimmuno-assay. This technique was first described by Berson & Yalow, and these authors have reviewed the principles upon which radioimmuno-assay is based (Berson & Yalow, 1968). The earliest radioimmuno-assay systems for renin measured ang II (Boyd, Landon & Peart, 1967) that was produced during the incubation step. However, as with bioassay, the incomplete conversion of ang I to ang II remained a problem. Poulsen (1971) described an "antibody trapping" technique to overcome these difficulties. Unknown samples were incubated in the presence of antibody raised against ang II. Thus, ang II was effectively "trapped" as it was formed, and angiotensinase inhibitors were unnecessary.

There are many advantages in measuring renin by generation of ang I. First, its conversion to ang II can be readily inhibited. By including inhibitors of converting enzyme 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 achieved. Since ang I is the first product of the renin/substrate interaction, the kinetic analysis is relatively simple.

Poulsen, (1973) has reviewed the kinetics of the renin-substrate reaction. Plasma renin activity (PRA) is generally accepted as a measurement of renin activity in vivo. However, PRA is usually measured in samples which contain endogenous renin substrate. This 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 which is present. Thus, when the circulating level of angiotensinogen is increased, as for example, in women taking oral contraceptives (see page 5), PRA could appear to increase, although the actual concentration of renin may be unchanged. This problem can be avoided by adding exogenous renin substrate during the incubation (Stockigt, Collins & Biglieri, 1971) and measuring plasma renin concentration (PRC). In this case, the rate of ang I production is independent of the substrate concentration, and the reaction follows zero order kinetics.

The amount of ang I produced during an in vitro incubation step can be influenced by a variety of factors. The presence of non-specific interference by immuno-reactive ang I-like material in plasma and cross-reactivity between renin substrate and antisera can

complicate assays for ang I. This may be overcome by extracting ang I on Dowex resin (Hollemaans, Van der Meer & Cloosterziel, 1969; Lijnen, Amery & Fagard, 1978) or Fullers earth (Boyd et al, 1969). However, extraction is unnecessary if non-incubated samples are included in the radioimmuno-assay system (Stockigt et al, 1971). There is no general agreement concerning the pH to use for renin assay. Methods have been described for incubating neat, unbuffered serum (Boyd et al, 1969) and serum buffered at pH 7.4 (Stockigt et al, 1971). The optimum pH for ang I generation is pH 5.5 - 6.5, and this range is often preferred (Cohen et al, 1971).

The molecular weight of ang I is low, (1280) and therefore it is not highly antigenic. Antisera are usually prepared by conjugating ang I to rabbit serum albumin by the carbodi-imide reaction described by Goodfriend, Levine & Fassman (1964). Although there is no general agreement about the best immunization site, an emulsion of the conjugate with Freund's complete adjuvant is often injected intradermally into multiple sites in the back of the rabbit. This is followed by subcutaneous booster injections after a delay of several weeks.

Radioimmuno-assay (RIA) of ang I is based on the principle that the presence of unlabelled ang I in the assay mixture modifies the binding of a radio-labelled tracer peptide. The tracer is usually prepared by incorporating ^{125}I into the ang I molecule by the chloramine-T reaction (Hunter & Greenwood, 1962). In order to measure the proportion of bound tracer, the unbound label must be separated from it. The simplest, and most commonly used method of separation is by activated charcoal. Free label is adsorbed onto dextran-coated charcoal leaving antibody-bound ^{125}I -ang I in the supernatant. Good separation of bound and free radio-labelled tracer has also been reported by Cohen et al (1971), who raised a second antiserum to anti-ang I. This was used to coat assay tubes.

Sealey et al (1972) compared values for PRA of human plasma, which had been obtained both by RIA and by bioassay. They found a high degree of correlation between the two groups of values. However, the values obtained by RIA were 2.2 times greater than those measured by bioassay. They concluded that this was due to the following:

- 1) Ang I generated during an in vitro incubation was injected and compared with an ang II standard for bioassay.

- 2) When equal weights of ang I and ang II are compared, ang II has 1.7 times greater pressor activity.
- 3) Plasma samples must be incubated for a longer period before bioassay than for RIA.

The recent purification of renin (see page 4) may enable direct measurement of renin by RIA. Indeed, such assays have already been reported (Galen et al, 1979; Malling & Poulson, 1977; Michelakis et al, 1974). However, renins derived from different sources have partial amino acid sequences in common, and are immunologically cross-reactive. It may therefore prove difficult to identify renins from different tissues, and to distinguish between active and inactive renins.

Fluorimetric assays for renin have recently been described (Galen et al, 1978; Murakami et al, 1981). However, these substrates are not specific for renin alone, and are susceptible to hydrolysis by other proteases. Such assays have been used to measure renin during the various stages of purification. Assay methods which are based on immuno-electrophoresis of renin (Harris, Munday & Noble, 1975) and inhibition of haemagglutination (Fukuchi, 1974) have also been described.

MEASUREMENT OF RENIN CONCENTRATION

The work which will be described in the later chapters of this thesis has involved the measurement of both active and inactive renins. Specific assays for inactive renin are, as yet, unavailable. In order to measure this form of renin, it must first be activated, and, in the experiments described here, this has been achieved by in vitro acidification. The difference between active renin concentration, and total renin concentration after acidification, is taken to represent the amount of inactive renin that is present in the samples. In the following section of this chapter, the methods which were used will be described. The three stages in the measurement of renin, activation of inactive renin, generation of ang I and radio-immunoassay of ang I are shown in Fig. 2.1.

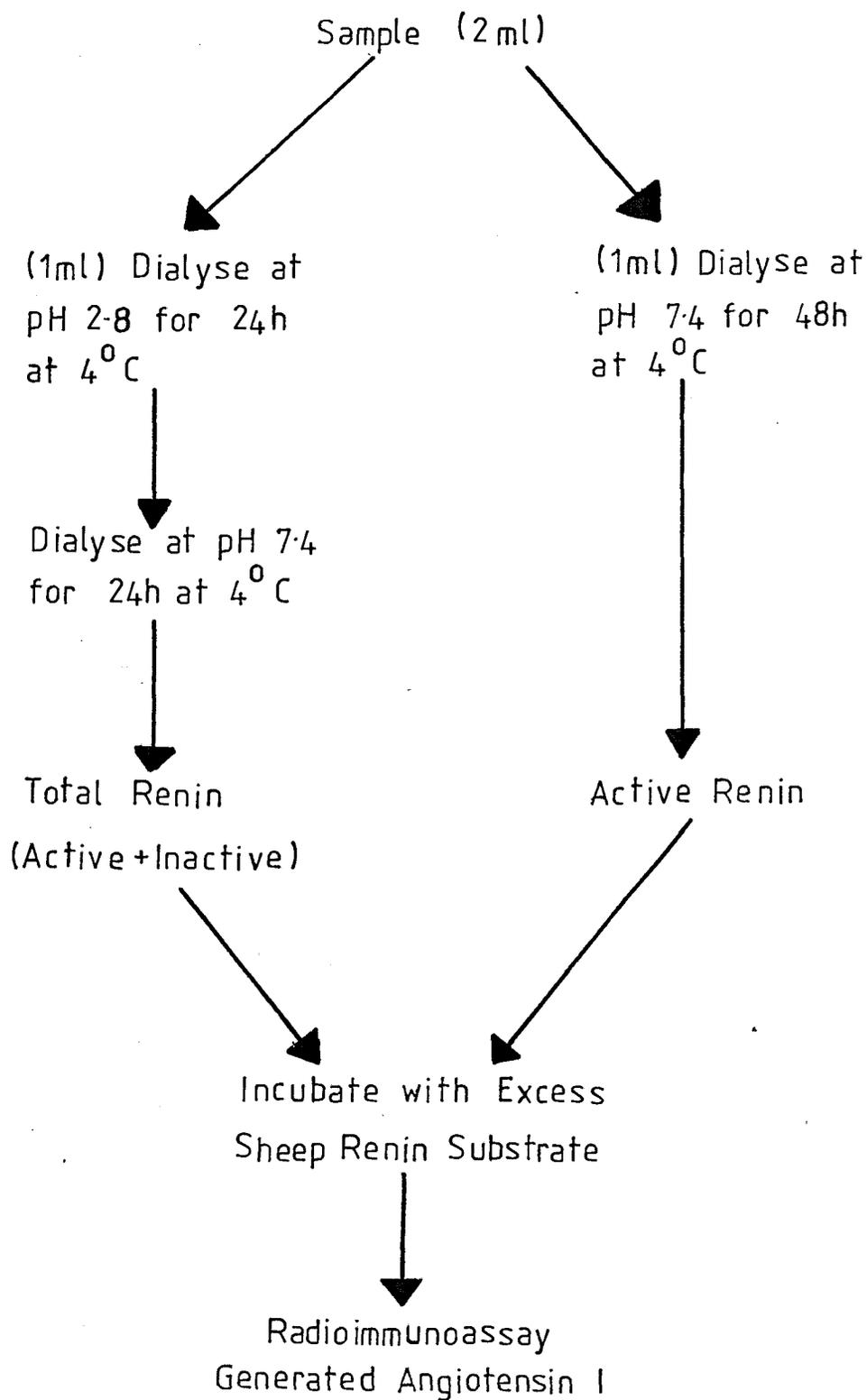
RADIO-IMMUNOASSAY OF ANGIOTENSIN I

Radio-immunoassay of ang I is, in fact, the final stage of the measurement of renin concentration. However, in order to consider the evaluation of the in vitro incubation step and the dialysis procedure, the radio-immunoassay step must first be described.

Radio-immunoassay of ang I is based on the principle that the binding of ^{125}I -ang I tracer to a specific antiserum is modified by the presence of unlabelled peptide. 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 is then determined. From the Standard Curve, the concentration of ang I in unknowns can be calculated.

For the RIA system described here, all solutions were prepared 0.05 M Tris-HCL buffer (pH 7.4) which contained 0.36% (w/v) bovine serum albumin (Fraction V, RIA grade, Sigma) and 0.2% neomycin sulfate (Sigma). The tracer which was used, 5-isoleucine-angiotensin I iodinated with ^{125}I at Tyr⁴ had specific activity of at least 1mCi/ μg ang I (CNTS, Orsay, France). The antiserum which was used through-

Fig.2:1 Estimation of Active and Inactive Renin Concentration



out the work had previously been prepared in our laboratory by Dr. H. K. Richards, and had negligible cross-reactivity with ang II and ang III (Richards, 1980). In addition, the antiserum did not cross-react with the 3 - 8 hexapeptide or 4 - 8 pentapeptide fragments of ang II. There was significant cross-reaction with the tetradecapeptide synthetic renin substrate, but this was not used in any subsequent experimental work. There was no detectable cross-reaction with the natural substrate preparations which were used, (see page 64).

The assay protocol was carried out in a cold tray at 4°C, using plastic tubes, since ang I is adsorbed by glass. For the Standard Curve, assay tubes contained a range (0 - 2500 pg) of ang I standard (5-isoleucine ang I diacetate, Bachem). The tubes contained the following mixture:

Ang I standard	250 μ l
Diluted antiserum	200 μ l
¹²⁵ I-ang I	50 μ l (approx 12,000 dpm).

In order to assay unknown samples, ang I standard was replaced with 50 μ l of unknown sample and 200 μ l of Tris-HCL buffer. The assay tubes were allowed to equilibrate overnight.

After the equilibration, antibody bound and free labelled ang I were separated. A solution containing 1% dextran (Grade C, BDH) and 1% charcoal (Norit GSX, Hopkin & Williams) was prepared in Tris-HCL buffer. An aliquot (1ml) of this solution was added to each assay tube, which was then rapidly mixed and centrifuged. The amount of bound tracer in the supernatant was then determined by counting the radioactivity of 500 μ l in a Beckman Biogamma Counter for two minutes. In order to minimise the error of pipetting solutions, the procedure was carried out in duplicate, or in triplicate if the number of unknown samples was small.

For any radio-immunoassay to be viable, the Standard Curve must be steep and linear over the working range. Absolute linearity is usually impossible to obtain, since antisera contain immunoglobulins with a range of binding affinities. Therefore, estimation of the amount of antigen bound to antibody depends on the concentration and affinities of the antibodies. The optimum dilution of the antiserum was determined in the following experiment.

Serial dilutions of the antiserum were prepared. A known amount of tracer and 0, 50 or 2000 pg of standard ang I were added to the assay tubes. The binding of ^{125}I -ang I was then determined, (Table 2.1). The results which were obtained show that the sensitivity of the antiserum to ang I depends on its dilution, (Fig. 2.2). When the concentration of the antiserum was high, a large proportion of the tracer was bound at all concentrations of standard ang II. In contrast, little of the tracer was bound at high dilutions of the antiserum, and binding was not sensitive to changes in ang I concentration. However, in a dilution of 1/5000, the antibody was sensitive to a wide range (50 - 2000 pg) of ang I concentrations.

TABLE 2.1 Antibody Dilution Curves

Antibody Dilution	% Label Bound		
	0	50	2000 pg Ang I
1 in 100	84.5	83.4	81.5
1 in 500	78.2	74.5	71.9
1 in 1,000	71.5	67.8	61.2
1 in 5,000	69.4	52.1	20.8
1 in 10,000	57.8	45.2	12.7
1 in 20,000	34.2	24.7	9.5
1 in 50,000	12.1	9.4	1.8

In order to compile a composite Standard Curve (Table 2.2), the binding of radio-labelled ang I was determined in ten separate assays, when the antiserum was diluted 1/4000. This dilution was selected for assay of unknown samples.

The results in Table 2.2 indicate that the Standard Curve is linear over a range of 100 - 2000 pg of ang I. A small change in the concentration of ang I results in a relatively larger change in the binding of the tracer (Fig. 2.3).

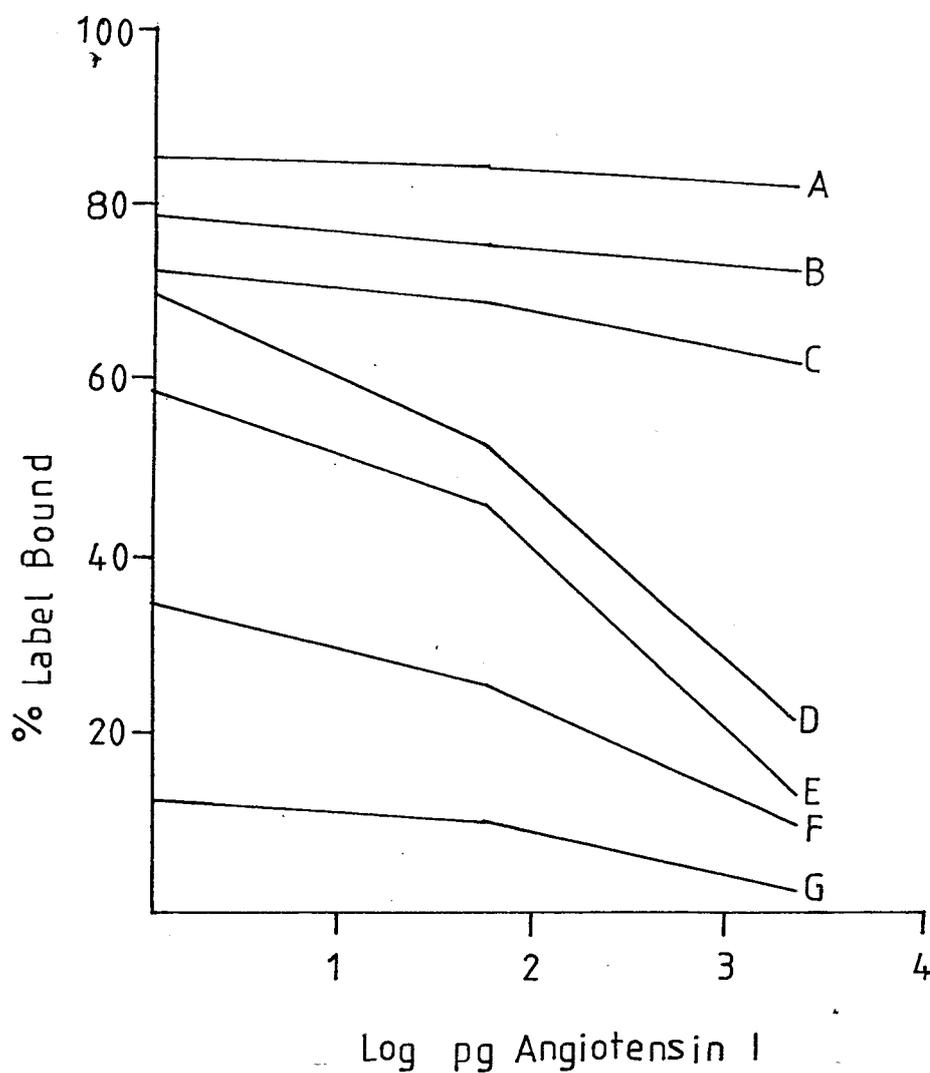


Fig 2:2 Antibody Dilution Curves

A	1/100	B	1/500
C	1/1,000	D	1/5,000
E	1/10,000	F	1/20,000
G	1/50,000		

In order to assay unknown samples, it is important that other factors in plasma do not interfere in the binding of ang I. Inclusion of 50 μ l of plasma from a nephrectomised sheep produced an identical Standard Curve to that obtained in the absence of the sample. Thus, there are no plasma factors, other than ang I itself, which modify the binding of the tracer.

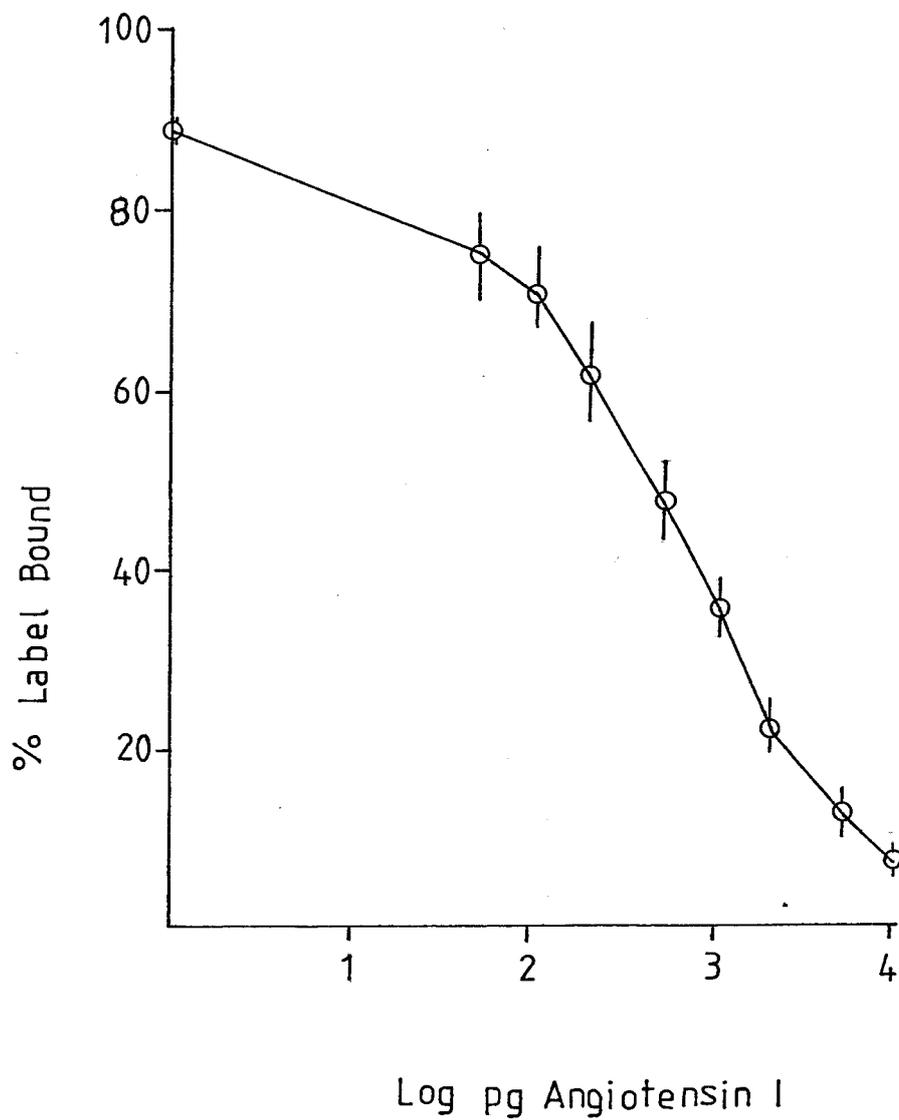
TABLE 2.2 Standard Curve for angiotensin I

Ang I (pg)	% Label Bound
0	88.1 \pm 2.7
50	74.7 \pm 5.3
100	70.8 \pm 4.8
200	61.6 \pm 5.5
500	49.0 \pm 4.7
1,000	35.6 \pm 3.3
2,000	22.4 \pm 3.1
5,000	12.6 \pm 2.9
10,000	7.7 \pm 2.1

Data shown as mean \pm s.e.m. of binding in 10 separate Standard Curves.

The coefficient of replicate variation (mean \pm s.e.m. of the % deviation of replicates) was calculated. For acidified plasma samples, the coefficient was determined as 4.58 \pm 0.43, while for samples which had been dialysed at neutral pH, the coefficient was 4.28 \pm 0.45, in twenty-four separate assays of plasma samples. The RIA procedure was therefore considered quite adequate for the work described subsequently.

Fig. 2-3 Standard Curve
(Antibody Diluted 1 in 4000)



GENERATION OF ANGIOTENSIN I

Sheep renin substrate was prepared according to the method of Skinner (1967). Sheep were anaesthetised with thiopentone and maintained on halothane/nitrous oxide/oxygen. They were bilaterally nephrectomised using flank incisions. After six days, the sheep were again anaesthetised and one carotid artery was cannulated. Blood was collected in chilled containers, which contained EDTA Na₂ (1 mg/ml of plasma). After centrifugation, plasma was collected and dialysed to pH 4.5 at 4°C for forty-eight hours, using isotonic citrate /phosphate buffer. This was followed by heating to 32°C for forty-five minutes, and a second dialysis to pH 7.5 using isotonic saline/phosphate buffer for a further forty-eight hours at 4°C. Aprotinin (Trasylol, 100 KIU/ml) was added before storage at -20°C until use.

Before it could be used for the incubation of plasma samples, the endogenous renin activity and angiotensinase activity of substrate preparations was determined. Aliquots of substrate were incubated for twenty-four hours at 37°C in the presence and absence of standard ang I. Preparations were considered to be acceptable if endogenous renin activity was less than 0.05 ng ang I/24 h, and the recovery of added ang I (100 & 1000 pg) was greater than 85%.

The incubation of renin-containing samples and substrate to generate ang I was carried out in the presence of converting enzyme and angiotensinase inhibitors. An inhibitor solution, which was prepared in 0.1 M phosphate buffer (pH 7.0) contained the following:

dimercaprol (Sigma)	0.1 m Moles/l
EDTA Na ₂ (BDH)	2.68
8-hydroxyquinoline (BDH)	3.44
Neomycin Sulphate (Sigma)	22

For the incubation step, sheep renin substrate and the inhibitor solution were mixed in the ratio 1 : 1. Aliquots (500 µl) of this mixture were transferred to assay tubes and 50 µl of the unknown sample was added. This procedure was carried out in duplicate, one tube being stored at -20°C (non-incubated blank), the other being incubated at 37°C for three hours. After the incubation, samples were assayed for ang I. The validity of this step was determined in

in the following experiments.

A standard rabbit renin preparation was used to measure the rate at which ang I was produced during the incubation step. This was prepared according to the method of Deodhar, Haas & Goldblatt (1964) and Haas *et al* (1972) from an aqueous extract of frozen rabbit kidneys. After acidification to pH 2.5, the precipitate which was produced when the pH was increased to 6.5 was removed by centrifugation. Ammonium sulphate was then added to the supernatant to give a final concentration of 2.4 M at pH 4.5. After overnight sedimentation, the precipitate was dialysed against distilled water for twenty-four hours at 4°C to bring the renin-containing fraction into solution. The extract was acidified to pH 2.8, and the supernatant was separated from denatured proteins. Ethanol (95%) was then added to the supernatant to give a final concentration of 10% and the precipitate formed during overnight sedimentation was discarded. The pH of the supernatant was adjusted to 4.5, and renin was precipitated by addition of ammonium sulphate (2.4 M). This was dissolved in distilled water and centrifuged. The pH of the supernatant was adjusted to 7.5 and it was made isotonic with sodium chloride.

Since the partial purification of renin included acidification steps, only active renin should be present. The protein concentration of the solution was determined by absorption at 280 nm and was 7.1 mg/ml. By radio-immunoassay, renin activity was 4.8×10^5 ng ang I/ml/h. Thus, the specific activity of the preparation was 6.8×10^4 ng ang I/mg/h.

TIME COURSE OF ANG I PRODUCTION

Partially-purified renin was diluted (1 : 1000 and 1 : 2000) and aliquots (50 μ l) were incubated with 500 μ l of substrate/inhibitor mixture at 37°C for 0, 2, 4 and 8 hours. The samples were then assayed for ang I content (see Table 2.3). Angiotensin I production was linear with respect to time (see Fig. 2.4), the correlation coefficients (r^2) being 0.97 and 0.95 for the 1 : 1000 and 1 : 2000 dilutions of renin respectively.

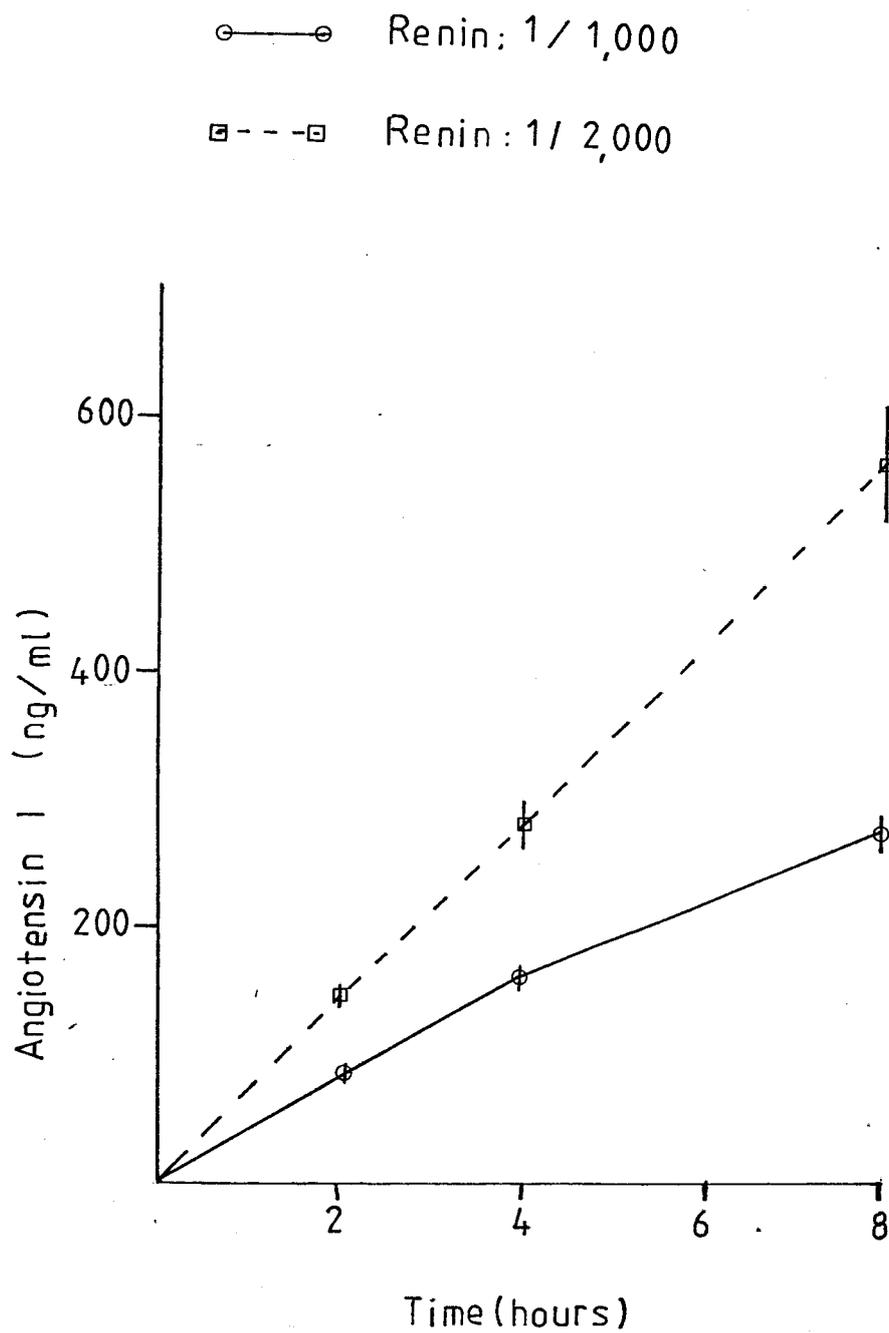


Fig 2.4 Angiotensin I Production vs Time

TABLE 2.3 Angiotensin I Production During Incubation of Rabbit Renin with Sheep Renin Substrate

Incubation Time (h)	Ang I (ng/ml)	
	Renin Dilution 1/1000	1/2000
0	2.45 ± 0.25	2.92 ± 1.11
2	145.7 ± 5.4	84.6 ± 3.9
4	276.9 ± 18.5	159.7 ± 9.4
8	559.2 ± 43.7	274.1 ± 13.9

Results are shown as mean ± s.e.m. of data from four experiments.

These results indicate that ang I production is directly related to the length of the incubation period.

ANGIOTENSIN I PRODUCTION AND RENIN CONCENTRATION

Partially purified standard rabbit renin (diluted 1 ; 1000) was serially diluted, and aliquots (50 μ l) of each were incubated with sheep renin substrate/inhibitor mixture for 0 and 3 hours at 37°C. After the incubation, the rate at which ang I was produced was determined by radio-immunoassay (see Table 2.4). The results show that ang I generation was linear ($r^2 = 0.97$) and directly related to the concentration of renin which was present (Fig. 2.5).

Fig.2: 5 The Effect of Renin Concentration on Angiotensin I Production.

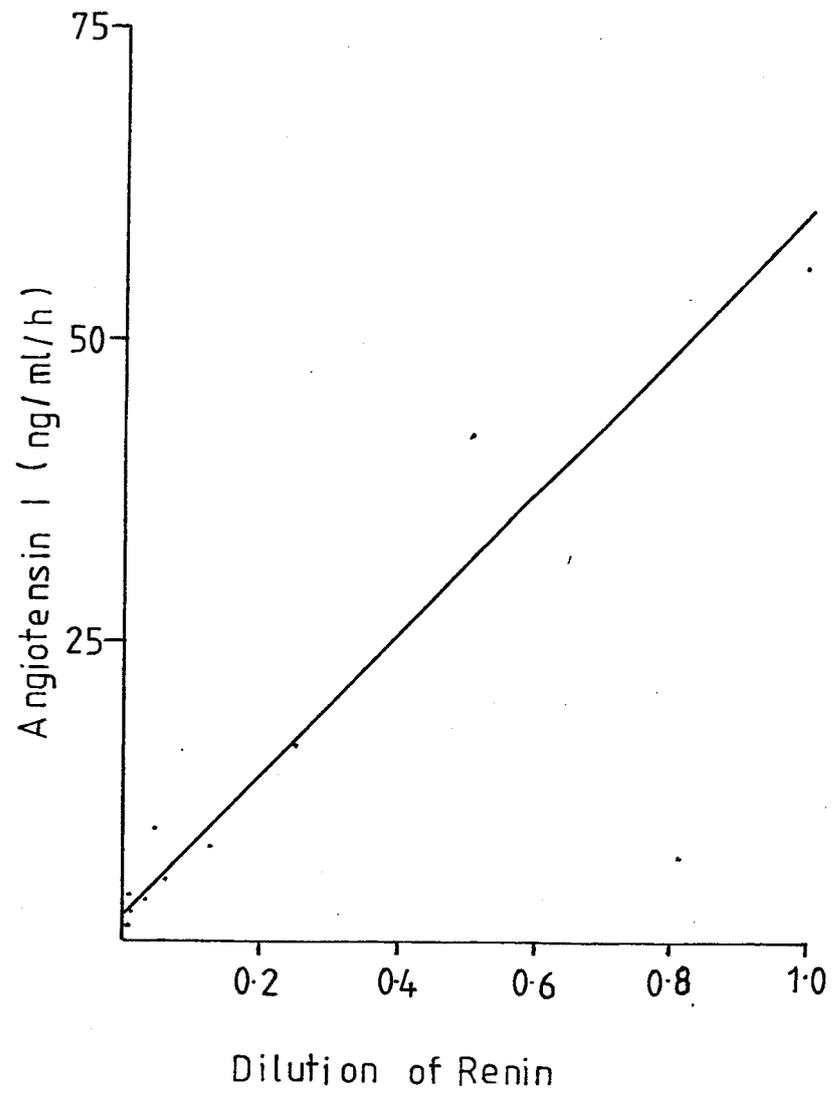


TABLE 2.4 The Effect of Renin Concentration on Angiotensin I Production During the Incubation

Renin Dilution	Ang I production (ng ang I/ml/h)
1	55.4 ± 3.1
1 in 2	41.7 ± 2.2
1 in 4	15.9 ± 0.8
1 in 8	7.5 ± 0.4
1 in 16	4.9 ± 0.2
1 in 32	3.3 ± 0.2
1 in 64	2.9 ± 0.2
1 in 128	2.0 ± 0.1
1 in 256	1.5 ± 0.1
1 in 512	0.8 ± 0.2

Results shown as mean ± s.e.m. of data from four experiments.

ANGIOTENSIN I PRODUCTION AND SHEEP SUBSTRATE CONCENTRATION

Serial dilutions of sheep renin substrate were prepared, and an equal volume of the inhibitor solution was added to each. Aliquots (50 μ l) of the partially-purified rabbit renin (1 : 1000 dilution) were added and tubes were incubated for 0 and 3 hours before assay of ang I (Table 2.5).

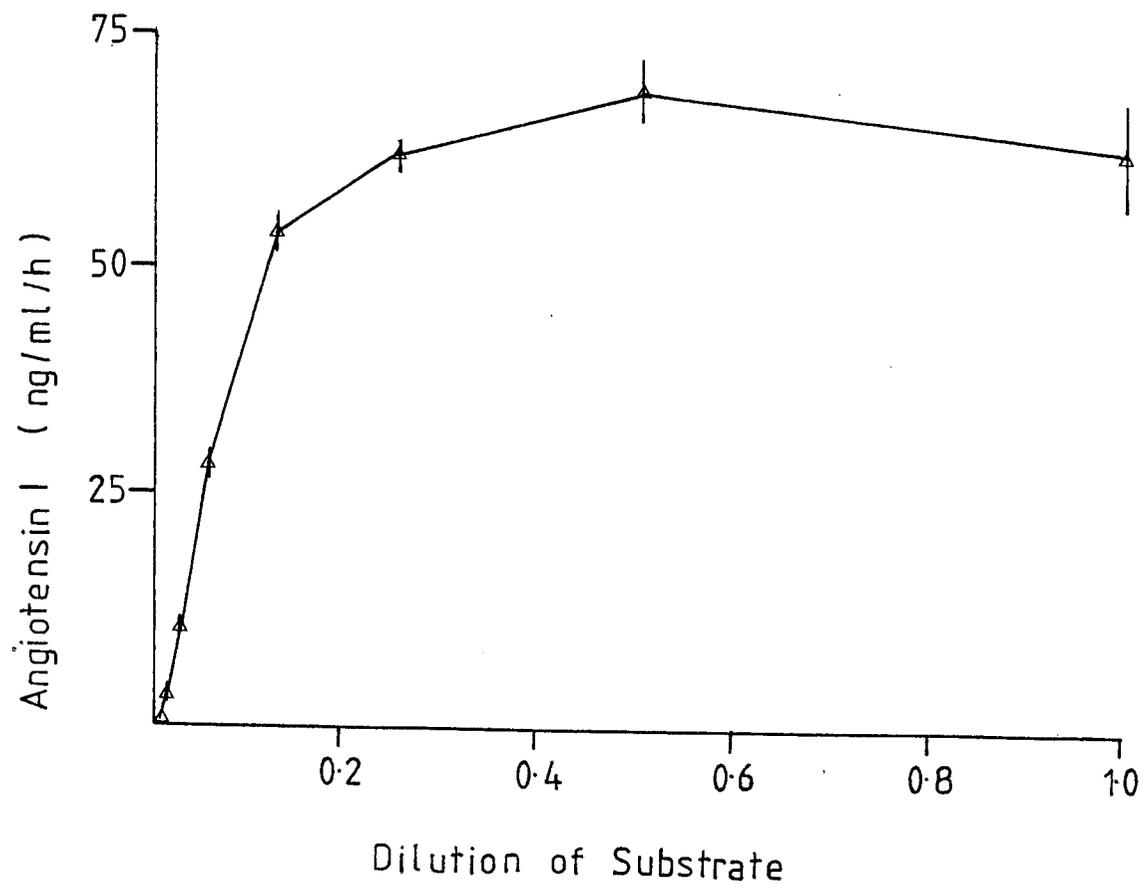
TABLE 2.5 The Effect of Substrate Concentration on Angiotensin I Production

Renin Dilution	Ang I production (ng ang I/ml/h)
1	62.4 ± 5.9
1 in 2	63.5 ± 3.4
1 in 4	61.7 ± 2.2
1 in 8	52.9 ± 1.9
1 in 16	27.4 ± 1.2
1 in 32	10.6 ± 0.4
1 in 64	3.2 ± 0.4
1 in 138	0.3 ± 0.3

Results shown as mean ± s.e.m. of data from four experiments.

The results (see Fig. 2.6) indicate that it was only when the dilution of substrate was beyond 1 in 8 that the substrate concentration had a substantial effect on the rate of ang I generation. Plasma renin activity is usually in the range of 0 - 20 ng ang/ml/h, and the equivalent range for in vitro kidney slice experiments is 0 - 20 ng ang I/mg wet weight of tissue/h. When using the exogenous source of renin substrate undiluted, it seems unlikely that substrate concentration would have a significant influence on the results which are obtained. The use of zero order kinetics in the analysis of data is also validated. The sheep renin preparation was therefore considered to be suitable for use in the work which will subsequently be described.

Fig.2:6 The Effect of Substrate Concentration on Angiotensin I Production.



ACTIVATION OF INACTIVE RENIN

Activation of inactive renin can be achieved by acidification or cold storage of samples, or by the action of protease enzymes. Throughout the work described in this thesis, inactive renin was activated by an acidification step. Half of each sample was dialysed against 170 mM glycine/HCL buffer, (pH 2.8) containing 0.005 M EDTA Na₂ for twenty-four hours at 4°C, and then back to pH 7.4 in 175 mM phosphate buffer containing 0.005 M EDTA Na₂ for a further twenty-four hours. The remainder of each sample was dialysed only to pH 7.4 for forty-eight hours at 4°C.

After the acidification step, a significant increase, ($p < 0.01$) in the renin concentration of plasma collected from normal rabbits was observed. Inactive renin comprised 15 - 20% of the total renin concentration of rabbit plasma. The possibility that this increase could be due to factors other than the activation of inactive renin was investigated in the following experiments.

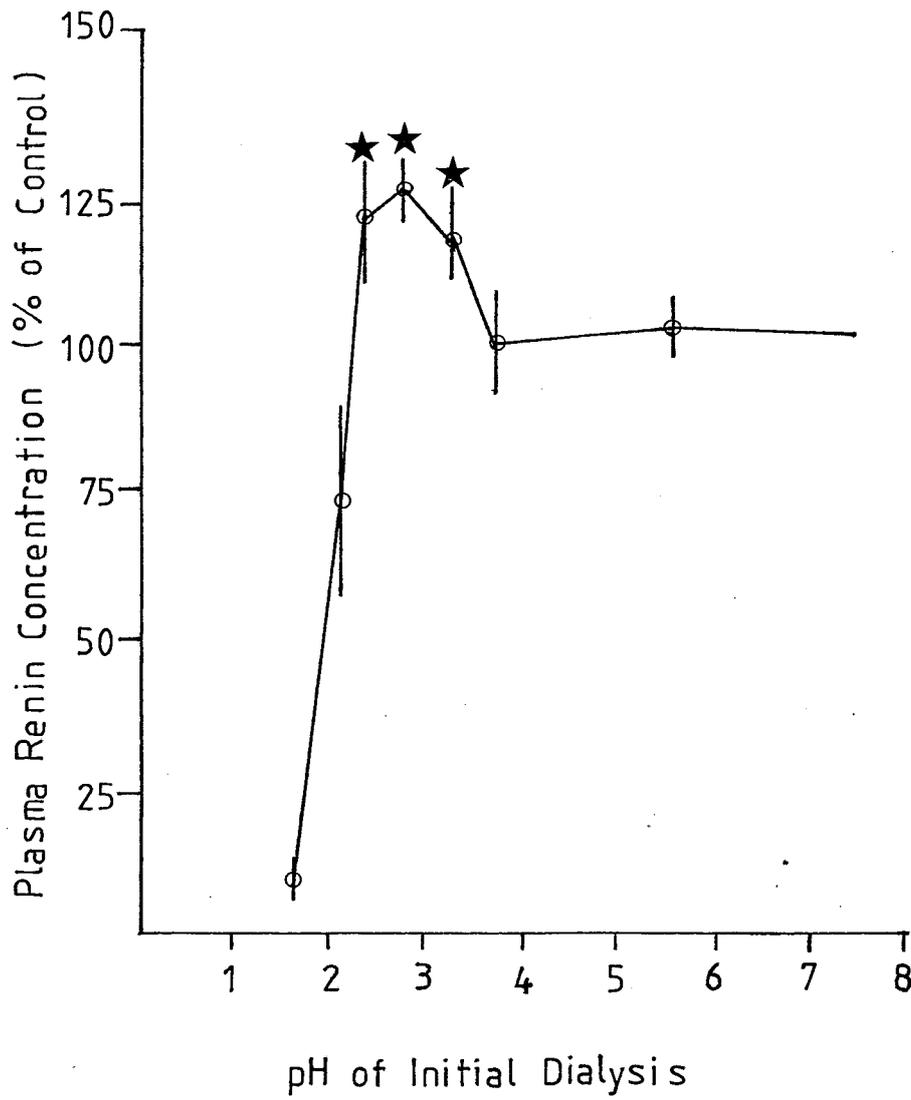
pH OPTIMUM OF ACTIVATION

In order to determine the pH at which maximum activation of inactive renin takes place, glycine/HCL and citric acid/phosphate buffers, (170 mM) with a range of pH values from 1.6 - 7.4 were prepared, (Table 2.6). Plasma collected from five rabbits was divided into aliquots, (1 ml) each of which was dialysed against one of these buffers for twenty-four hours at 4°C. Samples were then dialysed back to pH 7.4 over a further twenty-four hours at 4°C. Renin concentration, (PRC) was measured by radio-immunoassay of ang I after incubation with sheep renin substrate. The change in renin activity is shown in Fig. 2.7, where the results have been expressed as a % of renin activity in samples dialysed to pH 7.4 only, (active renin).

The results indicate that the maximum increase in renin activity occurred when plasma was dialysed to pH 2.8. Renin concentration did not increase significantly when the initial dialysis was carried

Fig.2:7 The Effect of pH on Plasma Renin

★ $p < 0.01$ Increase on
Acidification



out above pH 3.2, and renin was destroyed during dialysis at pH less than 2.3. For the experimental work described in this thesis, pH 2.8 dialysis was adopted for routine activation of inactive renin.

TABLE 2.6 The effect of pH on rabbit plasma renin concentration

pH of 1st Dialysis	P.R.C (ng Ang I/ml/h)	Renin Activity (% of pH 7.4).
7.4	13.5 ± 2.5	100
5.5	13.8 ± 3.2	101.6 ± 6.5
3.7	12.9 ± 5.4	98.7 ± 8.2
3.2	15.7 ± 3.2	116.8 ± 7.4
2.7	16.1 ± 3.0	124.5 ± 5.7
2.3	15.2 ± 2.1	119.2 ± 9.8
2.1	10.2 ± 4.0	72.4 ± 15.6
1.6	1.7 ± 0.5	9.1 ± 3.4

EFFECT OF DIALYSIS ON RECOVERY OF RENIN

The effect of the dialysis procedure on the recovery of renin was investigated. It is possible that the dialysis protocol which was adopted resulted in some loss of renin activity. If this effect were greater in the non-acidified samples, this could explain the apparent increase in renin concentration after acidification, which we attribute to the activation of inactive renin.

A 50 μ l aliquot of standard rabbit renin, (1/1000) was added to samples, (1 ml) of plasma from five rabbits. They were then subjected to one of the following procedures.

- a) Frozen at -20°C for 48 hours.
- b) Acid dialysis (pH 2.8) followed by dialysis back to pH 7.4.
- c) Neutral dialysis (pH 7.4 only).

In order to determine the amount of renin which had been added, plasma samples to which no renin had been added were subjected to the same protocol.

Renin recovery was calculated by taking the difference in renin concentration of the samples which had been stored at -20°C as 100%, ie, no loss of added renin, (see Table 2.7). The loss of renin during dialysis was $8.5 \pm 4.3\%$ at pH 7.4 and $11.7 \pm 5.5\%$ at pH 2.8. These values are not significantly different.

TABLE 2.7 The Effect of Dialysis on Recovery of Renin

Protocol	PRC (ng ang I/ml/h)		% Recovery
	Plasma + Renin	Plasma	
Storage at -20°C for 48 h	76.6 ± 13.4	14.5 ± 6.2	100
Dialysis at pH 7.4	71.5 ± 9.5	12.8 ± 7.2	92.0 ± 5.6
Dialysis at pH 2.8 then pH 7.4	70.9 ± 6.7	15.1 ± 5.4	90.4 ± 7.2

Results are shown as mean \pm s.e.m. of data from five experiments.

These data are very similar to those published by Richards et al, (1981) from our laboratory. The results may indicate a small degree of underestimation of inactive renin in studies discussed later in this thesis. This would, however, be a uniform loss, and would not affect the pattern of changes which were observed. From this experiment, it was concluded that the increase in renin activity after acidification of plasma is not caused by differential loss of renin during dialysis.

EFFECT OF DIALYSIS ON ANGIOTENSINASES

If angiotensinases in plasma were destroyed during the acidification step, then a reduced rate of breakdown of ang I produced during the incubation step could explain the apparent increase in renin concentration.

This possibility was investigated by preparing a substrate/inhibitor mixture, to which 25 ng/ml standard ang I had been added, (A) and a mixture which contained substrate and inhibitor solution only, (B). Aliquots, (50 μ l) of acidified and non-acidified plasma from four rabbits were incubated with the mixtures for eight hours at 37°C. The percentage recovery of ang I was then calculated by subtracting the ang I content of B from that of A.

It was found that $85.7 \pm 4.2\%$ of the ang I which was initially added, (25 ng/ml) remained after incubation of plasma samples which had been subjected to dialysis at pH 2.8. A similar value, ($82.7 \pm 3.1\%$) was calculated for samples which had been dialysed to pH 7.4 only. Thus, a reduction in angiotensinase activity cannot account for the increase in PRC after acidification.

EFFECT OF DIALYSIS ON ENDOGENOUS SUBSTRATE

It is possible that changes in endogenous renin substrate, and therefore in the subsequent production of ang I during the incubation step, could occur during dialysis of plasma samples. Although it is reasonable to suppose that any such change should have only a very small effect on the final measurement of PRC, since excess sheep renin substrate is added for the incubation step, the possibility that endogenous substrate is affected by dialysis was investigated in the following experiment.

Aliquots, (1 ml) of plasma from five rabbits were divided into three groups. One group of samples were stored at -20°C for forty-eight hours, the other two groups being dialysed at either acid or neutral pH. After dialysis, $50\ \mu\text{l}$ of each sample was incubated with inhibitor/buffer, (1:1) solution, to which $50\ \mu\text{l}$ of a 1:5 dilution of rabbit renin preparation, (see page 67) had been added. Thus, only endogenous renin substrate was available during the incubation step. Furthermore, complete utilisation of the substrate was ensured by the presence of excess renin. After eight hours incubation at 37°C , ang I production was measured.

In plasma samples which had been subjected to dialysis at pH 2.8, production of ang I during the incubation was very low, (46.2 ± 9.3 ng ang I/ml). This value was significantly less, ($p < 0.001$) than ang I generation by samples stored at -20°C , (572.8 ± 41.3 ng ang I/ml). This finding shows that endogenous renin substrate in rabbit plasma is destroyed by acidification.

Dialysis at neutral pH, (7.4) for forty-eight hours also reduced, ($p < 0.05$ compared with frozen samples) at which ang I was produced, (410.4 ± 28.5 ng ang I/ml). However, this value was significantly greater, ($p < 0.001$) than that obtained for acidified plasma samples.

These results suggest therefore, that endogenous substrate is reduced in both acidified and non-acidified samples. The reduction in substrate is greater however, in samples which have been subjected to an initial dialysis at pH 2.8. Thus, changes in endogenous substrate cannot explain the apparent increase in plasma renin concentration after acidification.

DISCUSSION

In the work which will be described in subsequent chapters of this thesis, factors affecting the level of plasma renin, and in particular, the inactive form of the enzyme, have been investigated. In general, renin has to be measured by means of indirect assays which are based on its ability to liberate ang I from renin substrate. Furthermore, in order to determine the amount of inactive renin that is present in a given unknown sample, the inactive renin must first be activated in vitro. It is then possible to measure this form of renin in the same way as active renin.

Radio-immunoassay is now a popular means of detecting small concentrations of peptides and other hormones in plasma, and is the method which is used routinely to determine the rate at which ang I is produced by active and inactive renin. This type of assay is based on the competitive reaction between labelled and unlabelled ang I and the antiserum. Application of RIA techniques to the measurement of ang I depends, therefore, upon obtaining an antiserum which has high specificity and high affinity for the peptide.

The antibody which was used throughout the experiments described in this thesis was raised by Dr. H. K. Richards, who found it to be highly specific for ang I, and found it did not cross-react with angiotensins II and III or their hexapeptide and pentapeptide fragments. Synthetic tetradecapeptide renin substrate did cross-react with the antibody, but this substance was not used for the incubation of renin-containing samples. By including non-incubated blanks in the assay system, the problem of non-specific interference from plasma substrate and other factors in plasma was avoided. These blanks usually contributed only 1 - 10 pg ang I, and renin activity was measured as the difference between this and the ang I content of incubated samples. The dilution of antiserum which was routinely used, (1/4000) gave a Standard Curve which was steep and linear within a range of 50 - 2000 pg ang I. However, the working range could be extended by increasing the antiserum dilution, (Fig 2.2).

Measuring the production of ang I during the incubation step was found to be a reliable indication of plasma renin concentration. The reaction follows zero order kinetics, and ang I production in vitro is independent of the substrate concentration. Furthermore, the rate at which ang I is released is linear with respect to time,

(Fig. 2.4) and is directly related to the concentration of renin that is present, (Fig. 2.5)

By dialysing samples of rabbit plasma against buffers with a range of pH values, (1.6 - 7.4) the pH optimum for activation of inactive renin was found to be pH 2.8. A similar value for the maximal activation of inactive renin in both rabbit and sheep plasma was found by Dr. D. J. Lush, (1981). The increase in renin concentration after acidification is of the order of 15 - 20% in the plasma of the normal rabbit, and is statistically significant, ($p < 0.01$). This phenomenon cannot be explained by differences in the recovery of renin after dialysis in angiotensinase activity, or in endogenous substrate levels. It is probable that the increase in renin activity is not due to the activation of a renin-like enzyme such as cathepsin D or pseudorenin. Although all of these enzymes can produce ang I from both natural and synthetic renin substrate, the rate at which they do so, and the pH optima for their reactions are lower than that of kidney renin, (Hackenthal, Hackenthal & Hilgenfeldt, 1978). However, the pH optimum and K_m of "activated" renin in plasma is similar to active renin, (Day & Leutscher, 1975; Derkx et al, 1978b). In addition to this, the pH optima of high molecular weight renins in man, (Slater & Haber, 1979) dog, (Potter et al, 1978) and pig, (Levine et al, 1978) are similar to the pH optimum of fully active kidney renin. Thus, the increase in renin activity after acidification appears to reflect the activation of an inactive form.

Throughout this thesis, active plus "activated" renin will be referred to as the total renin concentration that is present. This term is also used by workers in other laboratories, (Derkx et al, 1976; Leckie, McConnell & Jordan, 1977). Terms such as "prorenin" have also been used to describe inactive renin, (Sealey et al, 1977; Rumpf et al, 1980) but this term is rather ambiguous since it suggests that inactive renin is a true proenzyme of renin, similar to the insulin precursor, proinsulin. The biochemical nature of inactive renin has not been sufficiently well defined to make this assumption.

Acidification may not necessarily activate all of the inactive renin that is present. This could only be verified by a direct assay for this form of renin. However, as will be seen in a later part of this thesis, (Chapters 3 and 4), higher levels of inactive renin, (30 - 35% of the total renin) can be measured in kidney extracts and in the supernatant collected from kidney cortex slices which have

been incubated in vitro in the absence of extracellular calcium. There is therefore no obvious reason why plasma levels of inactive renin greater than 15 - 20% of total renin concentration would not be measured if they were present. There is thus no evidence that the difference between the levels of inactive renin in the rabbit and the higher proportion of inactive renin found in human plasma, (up to 90 per cent of total renin activity) can be attributed to methodological artefacts.

ASSAY OF KALLIKREINSINTRODUCTION

Kallikreins, like renin, are usually measured by means of indirect assay techniques, based on the rate at which they hydrolyse a substrate. Since plasma and glandular kallikreins possess different properties, (see page 48) the methods which are used for their measurement, and the problems which arise, are different. In this section of the thesis, the measurement of kallikrein in urine and in plasma will be discussed. In general, other glandular kallikreins are measured using similar techniques to those used to assay urokallikrein.

The most commonly-used assays for kallikrein in urine (urokallikrein) are based on the kininogenase and esterolytic properties of the enzyme. Kininogenic assays depend on the ability of kallikreins to liberate kinins from their endogenous substrates. In many ways, this type of assay is similar to those used for renin estimation. Kininogen is prepared from heat-treated plasma (Spragg & Austen, 1974). Unknown samples are then incubated in vitro with kininogen in the presence of kininase inhibitors such as EDTA and 1, 10 phenanthroline (Marin-Grez & Carretero, 1972). These prevent degradation of the kinins released. Care must be taken with the kininogen selected for this type of assay, since the rate of production of kinins often varies with the substrate used. For example, cat salivary gland kallikrein cannot utilise horse kininogen as a substrate (Bhoola et al, 1965). On the other hand, kallikrein from the guinea pig coagulating gland fails to release kinin from rat plasma (Bhoola et al, 1962; Shacter, 1963) but can hydrolyse kininogens from other species, including man, dog and rabbit.

After the incubation step, the rate of kinin production is measured by bioassay or radio-immunoassay. Several smooth muscle preparations which are sensitive to kinins have been described. The most commonly-used preparation for in vitro bioassay of kinins is the terminal portion of the guinea pig ileum (Rocha E Silva, 1952) but

the rat uterus also responds to bradykinin (Erspaner, 1948). The problem of non-specific interference by other plasma components has been partially overcome with the use of strips of cat jejunum for bioassay of kinins. Margolius et al (1971) reported that pretreatment with chymotrypsin renders this preparation more sensitive to bradykinin, but not to serotonin or acetylcholine. In vivo, the femoral artery of the anaesthetized dog, pretreated with an ang I converting enzyme inhibitor, has been used to estimate the vasodilator action of kinins (Colina-Chourio & McGiff, 1975; Marin-Grez & Carretero, 1972). The hypotensive activity of kinins has also been measured after intravenous or intra-arterial injection in the anaesthetized dog (Roblero, Ryan & Stewart, 1973).

Although radio-immunoassay of kallikrein can be used to measure the kininogenase activity of urinary kallikrein, (Carretero et al, 1976) several problems are encountered. Like ang I, bradykinin and kallidin have relatively low molecular weights, (1240 and 1384 respectively) and only have weak antigenic properties. Therefore, they must first be conjugated to larger protein haptens, such as rabbit serum albumin, (Goodfriend et al, 1964) before an antiserum can be obtained. Secondly, it is difficult to obtain radiolabelled kinin which has high specific activity. Therefore, ^{125}I is usually incorporated into a tyrosine-containing analogue of the kinin, such as Tyr⁸-bradykinin. Antibodies to bradykinin often cross-react with kininogen, (Edery & Abraham, 1976) and therefore the concentration of kinins in an unknown sample could be overestimated. Carretero et al (1976) have reported however, that a minimum of 10 pg of kallidin could be measured in this way.

In order to overcome some of these difficulties, esterolytic assays for urokallikrein have been widely used. These techniques are based on the ability of kallikreins to hydrolyse synthetic aromatic esters of arginine, for example, p-tosyl-L-arginine methyl ester, (TAME), alpha-N-benzoyl-L-arginine methyl ester, (BAME) and alpha-N-carbobenzyl-L-arginine methyl ester, (ZAME). On hydrolysis, these substrates yield the acyl derivative of arginine and methanol. Kallikrein activity is then estimated by determining the amount of methanol, (Beavan et al, 1971; Matsuda et al, 1976; Moriya et al, 1971) or residual ester, (Nustad & Pierce, 1974; Webster & Pierce, 1961) that is present.

With esterase assays, it is important to remember that there

may be some interference from non-kallikrein esterases in urine, (Nustad & Pierce, 1974; Hial et al, 1974) although it has been reported that kallikrein is the only measurable alkaline esterase in human and rat urine (Pierce, 1970). Pancreatic kallikrein, and possibly some other tissue kallikreins, are present in an inactive form, (see Schacter, 1980) and must first be activated before their esterolytic activity can be measured. However, kallikrein in urine is generally present in an active form. In addition, esterase assays of kallikrein can only be used to evaluate the activity of the enzyme rather than its concentration, as there is no way to determine whether kallikrein in samples is completely active or not.

In recent years, direct radio-immunoassays for kallikrein in human and rat urine have been described (Carretero et al, 1978; Chung et al, 1979; Goodfriend & Oday, 1979; Shimamoto, Chao & Margolius, 1980). However, antibodies to kallikrein will also bind to prekallikreins and inhibitor-complexed kallikreins, and may not accurately reflect, therefore, kallikrein activity in vivo.

There are many problems encountered in measuring the activity of the kallikrein-kinin system in plasma. Traditionally, serine proteases which take part in clotting and fibrinolytic processes were measured by estimating the rates of formation or dissolution of fibrin. Later, Sherry & Troll (1954) described lysine and arginine esters, including TAME, which were sensitive to hydrolysis by thrombin, plasmin and other serine protease enzymes in plasma. Although a degree of specificity was reported, (Sherry et al, 1965) these substrates are hydrolysed by many serine protease enzymes.

During the last decade, the active sites of some of these proteases, and the sites of their catalytic activity have been partially characterised. Relatively specific chromogenic substrates have also been introduced. Chromogenic substrates for measuring thrombin, (S-2238, Chromozym TH and S-2160) plasmin, (S2257) and kallikrein, (Chromozym PK) are now commercially available (Mattler & Bang, 1977). These substrates are all peptide p-nitroanilides with the chromophore p-nitroaniline, (pNA) linked to the remaining peptide by an amide linkage. Chromogenic substrates are themselves colourless, but yield pNA on hydrolysis. This can be measured by the change in absorbance at 405 nm.

Chromozym PK represents the carboxy terminal portion of the bradykinin molecule which is released from HMW kininogen by the action

of plasma kallikrein. It has the structure Bz-Fro-Phe-Arg-pNA. Although this substrate can be cleaved by plasma kallikrein, it is also subject to hydrolysis by plasmin and trypsin, (Bang & Mattler, 1978; Mattler & Bang, 1977) but not by thrombin or factor Xa. Plasmin contributes about 4% of the kallikrein activity measured with this substrate (Stormorken *et al* 1978). Another complication with this method of assay is that plasma which is deficient in Hageman factor (factor XII) and Fletcher factor will not generate kallikrein activity. The reason for this is that prekallikrein must first be activated by Hageman factor before it can be measured. However, Claeson *et al*. (1978) reported that if Fletcher factor is present in concentrations which are only 25% of normal values, kallikrein activity can be measured.

Some groups of workers have measured the activity of the kallikrein-kinin system in plasma by radio-immunoassay of circulating bradykinin. Recent review articles by Goodfriend & Ody (1979) and by Talamo & Goodfriend (1979) have discussed the problems associated with this technique. Some of these have already been mentioned, since similar problems are encountered when bradykinin or kallidin liberated during kininogenic assays of urinary or other glandular kallikreins are measured by radio-immunoassay. In addition, bradykinin formation must be avoided during the collection of blood samples. Inhibitors must therefore be included with plasma samples, and the effect of these on the bradykinin - antibody interaction has not been reported. In addition to its cross-reactivity with kininogen, antisera to bradykinin may also bind to larger kinins such as cholecystokinin, which are present in plasma. Recently, Saito (1979) described a direct radio-immunoassay for plasma kallikrein, which was sensitive to 2 ug/ml and values obtained by this method correlated closely with determinations of plasma kallikrein activity made using a Fletcher factor clotting assay.

In the experiments which will be described in chapter three of this thesis, some of the relationships between active and inactive renin, and kallikreins have been investigated. In these experiments, urinary kallikrein was measured by hydrolysis of TAME, while kallikrein activity in plasma was estimated by using chromozym PK, which is available in a commercial kit, (Boehringer). The validation of these assays will now be discussed.

ASSAY OF KALLIKREIN IN URINE

In the experiments which will be described in Chapter Three of this thesis, kallikrein activity in urine samples was estimated by means of an esterase assay in which p-tosyl-L-arginine methyl ester (TAME) was used as the substrate. For routine measurements, 200 μ l of urine was incubated with 2 ml of TAME (Sigma) solution (0.055 M in 0.1M phosphate buffer, pH 7.95) in an oscillating water bath at 37°C. After incubation for one minute (blanks) and thirty-one minutes, an aliquot (1 ml) of this mixture was removed. The reaction was terminated by addition of 500 μ l of 15% trichloroacetic acid (TCA) and tubes were centrifuged for five minutes. Methanol liberated during the hydrolysis was then estimated by oxidation to formaldehyde. An aliquot (500 μ l) of supernatant was removed to tubes containing 100 μ l of 2% potassium permanganate. After removal of excess KMnO_4 with 100 μ l of 10% sodium sulphite, the solution was mixed with 4 ml chromotropic acid. This was prepared by dissolving 400 mg of 4, 5-dihydroxy-2, 7-naphthalene-disulphonic acid in 20 ml of distilled water, and adding 180 ml of 65% sulphuric acid. The chromotropic acid solution is yellow in colour, but after addition of the unknown sample and incubation in a boiling water bath for fifteen minutes, a purple solution was obtained. Adsorbance at 580 nm was measured.

The assay was standardised using a stock solution of methanol (0.24 M) which was diluted with buffer to obtain a Standard Curve. A unit of kallikrein activity (U) is the amount of kallikrein required to hydrolyse 1 μ mole of TAME in one minute at 37°C and pH 7.95.

The validity of this method was investigated in the following experiments.

THE STANDARD CURVE

A cumulative Standard Curve was prepared from data obtained in six separate assays (Table 2.8). Methanol (0.24 M) was diluted with buffer to give a range of concentrations from 0.1 - 2.4 μ moles.

These were then oxidised to formaldehyde and incubated with chromotropic acid for fifteen minutes. The Standard Curve obtained in this way (see Fig. 2.8) is linear and has a correlation coefficient (r^2) of 0.99. Thus, the absorbance at 580 nm is directly related to the amount of methanol which is present.

TABLE 2.8 Standard Curve for methanol

Methanol (μ moles/tube)	Absorbance at 580 nm
2.4	0.207 \pm 0.004
1.2	0.128 \pm 0.007
1.0	0.099 \pm 0.008
0.8	0.084 \pm 0.004
0.6	0.064 \pm 0.006
0.4	0.057 \pm 0.006
0.3	0.033 \pm 0.007
0.2	0.015 \pm 0.003
0.1	0.003 \pm 0.001

Results are shown as mean \pm s.e.m. of six Standard Curves.

THE EFFECT OF KALLIKREIN CONCENTRATION ON METHANOL PRODUCTION

The dependence of methanol production from TAME during the incubation step on the concentration of kallikrein which is present in the unknown sample was investigated in the following experiment. A standard solution of pancreatic kallikrein (Sigma, 0.2 mg/ml) was serially diluted. Aliquots (200 μ l) of these solutions were incubated for one and thirty-one minutes with 2 ml of 0.055M TAME.

Fig. 2:8 The Standard Curve for Methanol.

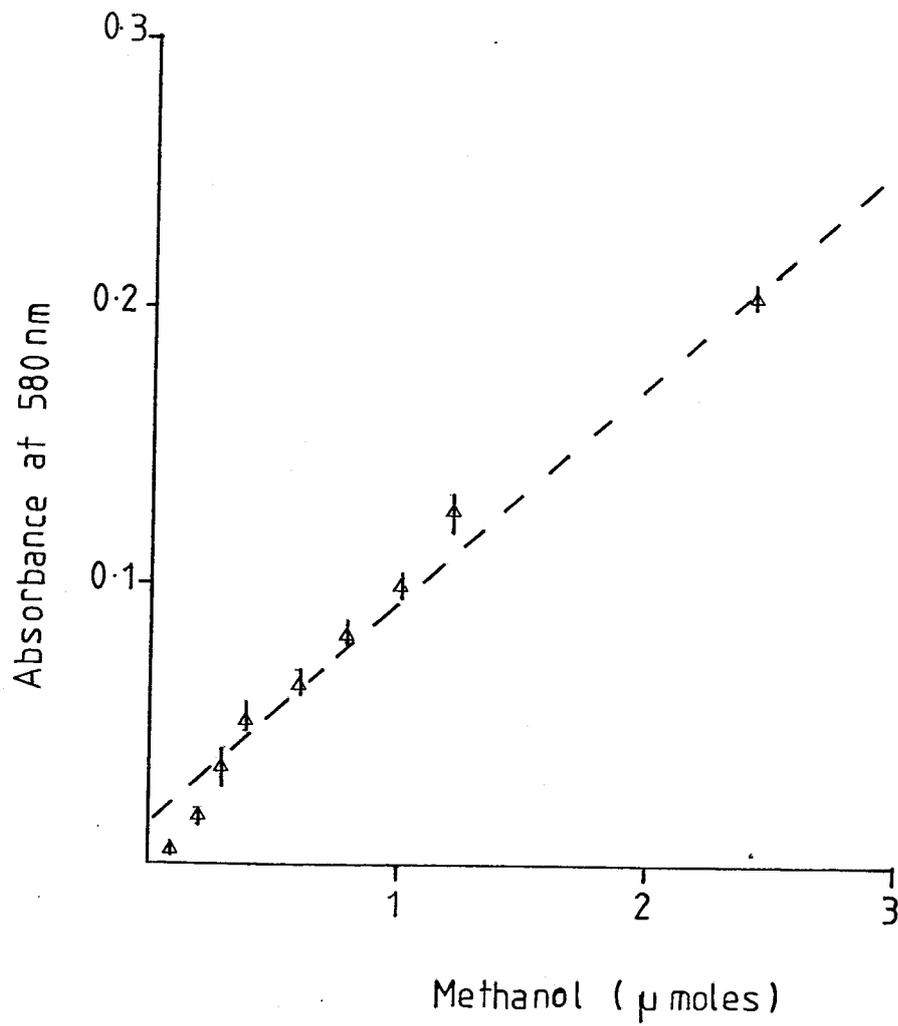
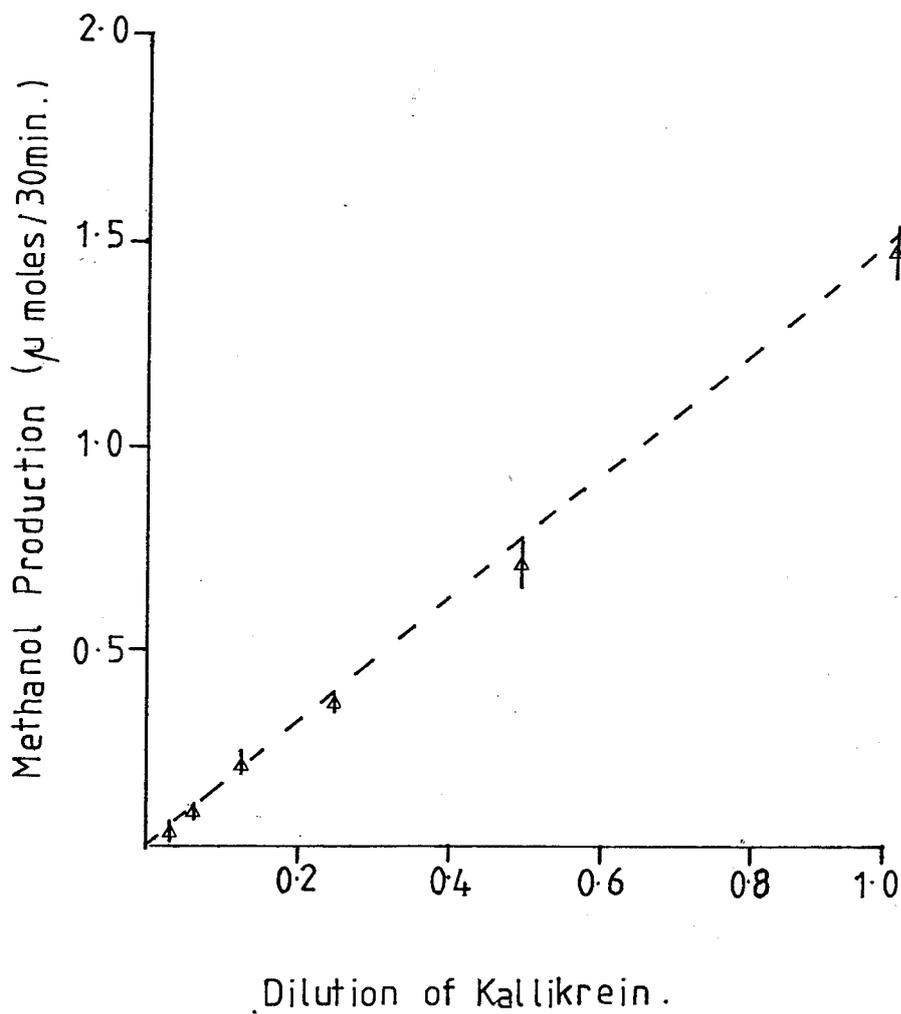


Fig. 2:9 The Effect of Kallikrein Concentration on Methanol Production.



Methanol production was then estimated as previously described, (see page 87). Table 2.9 shows the results which were obtained. Methanol production during the incubation was linear ($r^2 = 0.97$) and directly related to the concentration of kallikrein which was present (see Fig. 2.9). This result shows that hydrolysis of TAME is directly proportional to kallikrein concentration.

TABLE 2.9 The Effect of Kallikrein Dilution on Methanol Production

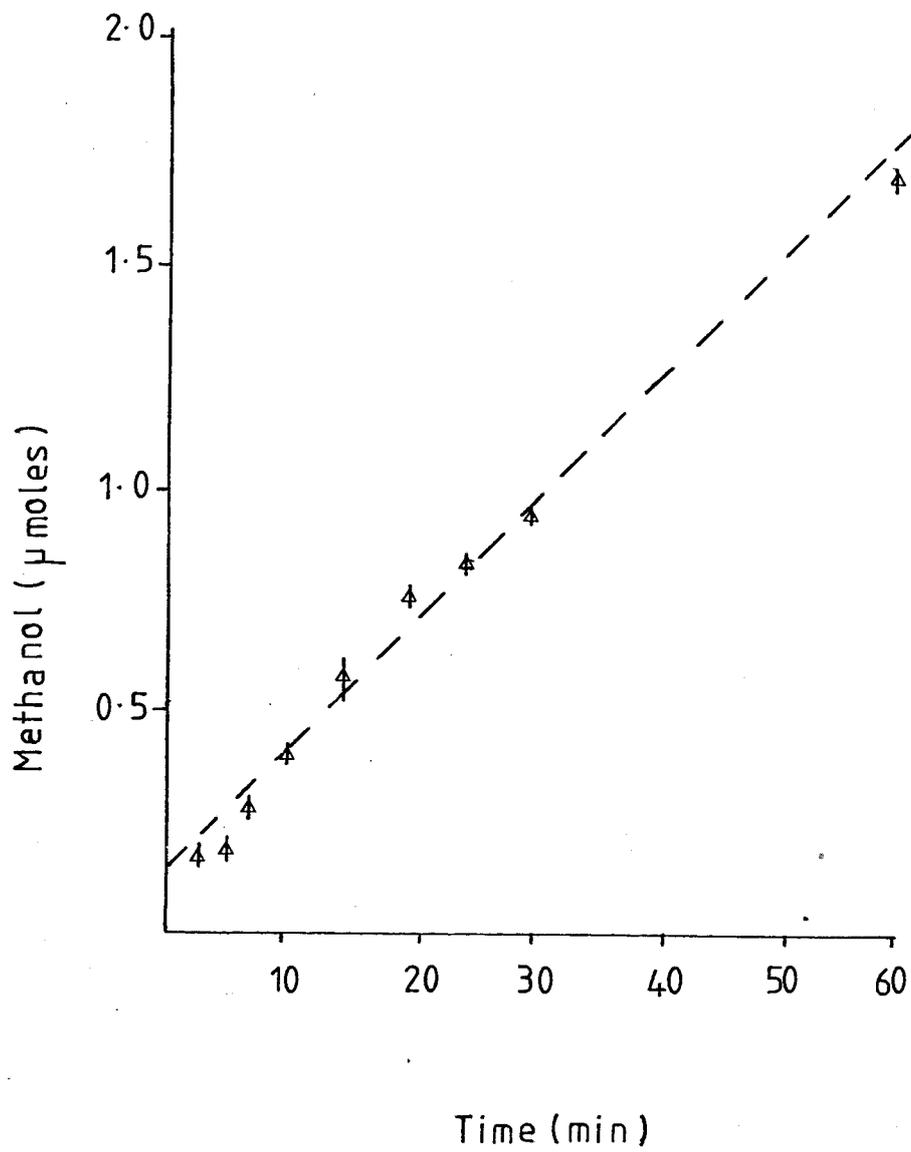
Dilution of Kallikrein (0.2 mg/ml)	Methanol Production (μ mol/30 min)
x 1	1.46 \pm 0.06
1 in 2	0.69 \pm 0.05
1 in 4	0.36 \pm 0.02
1 in 8	0.20 \pm 0.03
1 in 16	0.09 \pm 0.01
1 in 32	0.02 \pm 0.02

Results are shown as mean \pm s.e.m. of data from four experiments.

TIME COURSE OF METHANOL PRODUCTION

Aliquots (200 μ l) of a standard kallikrein solution (0.2 mg/ml) were incubated with 2.0 ml TAME (0.055M) as previously described (see page 87), for varying periods of time, up to sixty minutes. Methanol generated was assayed (Table 2.10). The rate at which methanol was produced was linear with respect to time (see Fig. 2.10) the

Fig. 2:10 Methanol Production vs Time



correlation coefficient (r^2) being 0.98.

TABLE 2.10 Methanol Production During Incubation of Kallikrein with TAME

Incubation Time (mins)	Methanol (μ moles/ml)
0	0.12 \pm 0.01
3	0.18 \pm 0.02
5	0.19 \pm 0.01
7	0.27 \pm 0.01
10	0.41 \pm 0.02
15	0.54 \pm 0.04
20	0.77 \pm 0.03
25	0.87 \pm 0.02
30	0.92 \pm 0.02
60	1.68 \pm 0.06

Results are shown as mean \pm s.e.m. of data from three experiments.

This indicates that methanol production is directly related to the incubation time.

EFFECT OF SUBSTRATE CONCENTRATION ON METHANOL PRODUCTION

In order to investigate the effect of substrate concentration on the rate of methanol production, an 0.11M solution of TAME was prepared. This was serially diluted with phosphate buffer to give a range of concentrations (see Table 2.11). Aliquots (200 μ l) of a standard kallikrein solution (0.2 mg/ml) were then incubated for zero and thirty minutes at 37°C with 2 ml of each substrate dilution. The

methanol generated was then assayed.

TABLE 2.11 The Effect of Substrate Concentration on Methanol Production

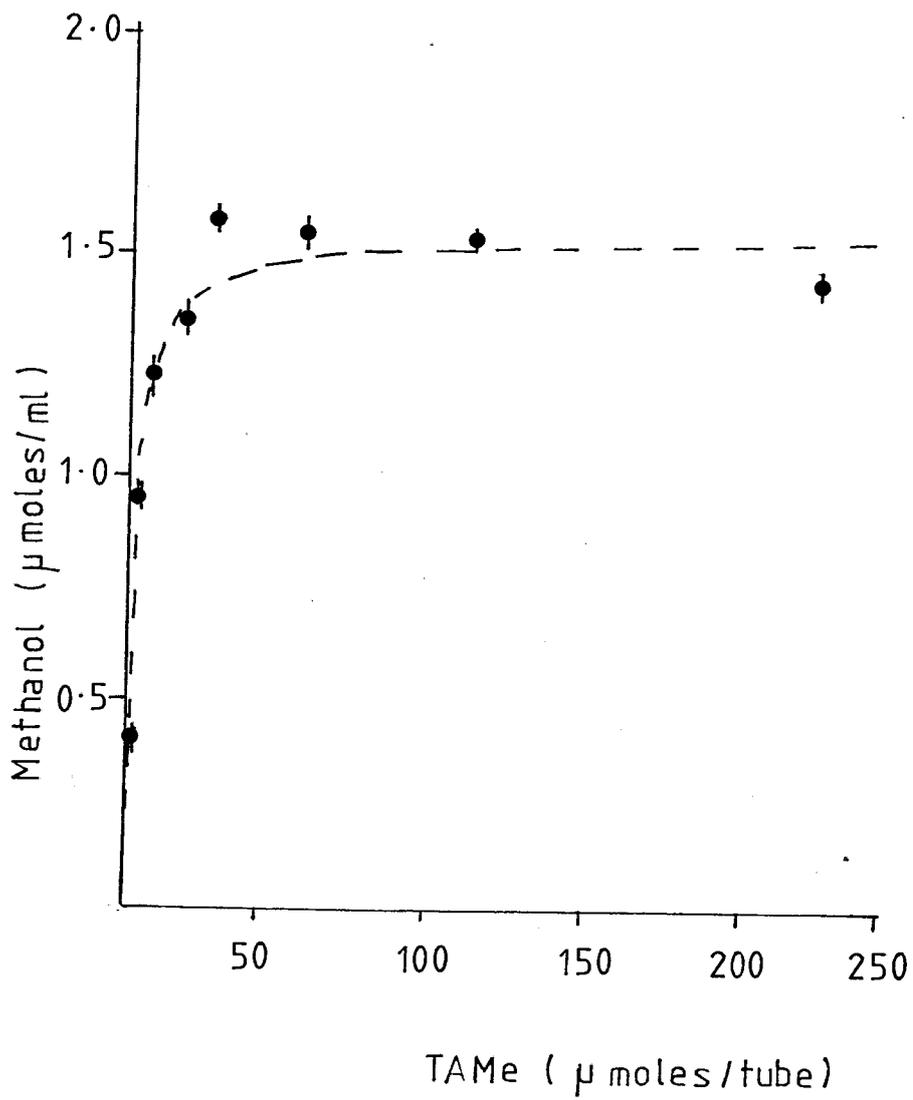
TAMe (μ moles/ml)	Methanol (μ moles/ml)
220	1.406 \pm 0.04
110	1.462 \pm 0.14
55	1.54 \pm 0.13
27.5	1.58 \pm 0.01
13.8	1.32 \pm 0.09
6.4	1.11 \pm 0.15
3.4	0.90 \pm 0.03
1.7	0.72 \pm 0.13
0.86	0.39 \pm 0.05

Results are shown as mean \pm s.e.m. of data from three experiments.

These results indicate that, at the concentration of kallikrein which was used, methanol production was independent of the concentration of TAMe when it was greater than 13.8 μ moles/ml. The data obtained in this experiment were subjected to a hyperbolic analysis, and the reaction was found to have K_m of 2.18 μ moles/ml and V_{max} of 1.54 μ moles methanol/ml/h.. Thus the cleavage of TAMe by kallikrein appears to follow zero order kinetics (see Fig. 2.11).

The concentration of TAMe which was selected for routine determinations of kallikrein activity was 0.055M. The concentration of the standard kallikrein solution (0.2 mg/ml) is equivalent to kallikrein activity of 4.0 U/ml. In the experiments which will be described in Chapter 3 of this thesis, kallikrein activity of rabbit urine was in the range 0.4 - 2.5 U/ml. This suggests that zero order kinetics apply under the conditions used for routine handling of urine samples.

Fig. 2:11 The Effect of TAME Concentration on Methanol Production



Taken together, these results show that hydrolysis of TAME by kallikrein can be used to determine the concentration of the purified enzyme. However, the major problem with esterase assays is that TAME and other arginine esters are not specific for kallikrein alone. This is demonstrated in the following experiment.

Solutions containing a variety of proteolytic enzymes were prepared (see Table 2.12). All of the enzymes were used at a concentration of 0.2 mg/ml. Aliquots (200 μ l) of these were incubated with 2 ml 0.055M TAME, as previously described (see page 87) and the methanol produced was measured.

The results confirm that TAME is susceptible to hydrolysis by a variety of proteolytic enzymes. Methanol was liberated from the substrate by trypsin, plasmin and lysozyme in addition to kallikrein. In contrast, neither thrombin nor urokinase could significantly utilise TAME as a substrate. Thus, although kallikrein is reported to be the major alkaline esterase in human and rat urine (Pierce, 1970), it must be borne in mind that the presence of other protease enzymes in urine could lead to an over-estimation of kallikrein activity.

TABLE 2.12 Hydrolysis of TAME by Protease Enzymes

Enzyme	Methanol (μ moles/ml)
Pancreatic Kallikrein (Sigma)	0.91 \pm 0.01
Trypsin (BDH)	0.99 \pm 0.14
Porcine Plasmin (Sigma)	0.84 \pm 0.02
Thrombin (Sigma)	0.16 \pm 0.003
Human Urokinase (Sigma)	0.14 \pm 0.01
Pancreatic Lysozyme (BDH)	1.00 \pm 0.13

Results are shown as mean \pm s.e.m. of data from three experiments.

ASSAY OF PLASMA KALLIKREIN

The chromogenic substrate, Chromozym PK (Boehringer) was used to measure plasma kallikrein in the experiments which will be described in Chapter 3 of this thesis. This substrate was obtained in a commercial kit which also provided the other reagents required for the assay.

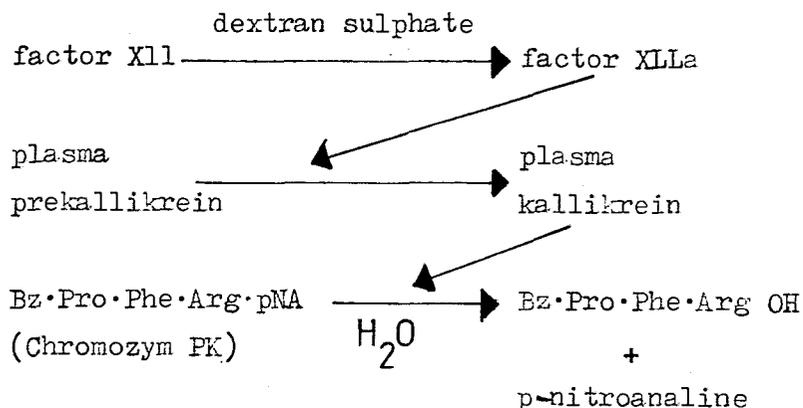
Since kallikrein is usually present in plasma in an inactive form, prekallikrein, the first step in the assay protocol involves its activation (see Fig. 2.12). For this assay, blood samples were collected using tri-sodium citrate (0.11M) as anti-coagulant (9 parts blood : 1 part citrate). In order to achieve activation of pre-kallikrein, aliquots (100 μ l) of plasma were incubated with an equal volume of dextran sulphate solution (0.025 g/l) for seven minutes at 0°C. These samples were immediately assayed.

A buffer concentrate, (pH 7.9, ionic strength 0.15) was provided with the assay kit. This was diluted 1 in 10 with distilled water. For the incubation step, the tube contained:

1.5 ml buffer solution
0.5 ml Chromozym PK (1.5 mM)
20 μ l activated plasma

Tubes were incubated for three minutes at 37°C and the reaction was terminated by the addition of 400 μ l of 10% acetic acid. The absorbance of the solution at 405 nm was determined. Reagent blanks contained 1.5 ml buffer, 0.5 ml Chromozym PK, 20 μ l of dextran sulphate and 400 μ l of acetic acid.

FIG. 2.12 Assay of Kallikrein in Plasma



Plasma kallikrein activity (U/ml) was then calculated according to the formula:

$$\text{U/ml} = \frac{\Delta A/\text{min} \times V \times F}{E \times d \times v}$$

Where V = volume of reaction mixture

F = dilution factor

E = molar extinction coefficient of p-nitroaniline
 $(10.4 \times 10^{-3} \text{ l cm}^{-1} \text{ mol}^{-1})$

v = volume of diluted plasma

d = light path

The validity of this assay system was investigated. However, since purified plasma kallikrein was not available, this evaluation is limited.

PRODUCTION OF P-NITROANILINE DURING THE INCUBATION STEP

Aliquots (20 μl) of pooled, activated rabbit plasma were incu-

bated at 37°C with Chromozym PK for 0 - 10 minutes, and the change in absorbance at 405 nm was determined.

TABLE 2.13 Change in Absorbance at 405 nm

Time (minutes)	Δ Optical Density $\times 10^{-3}$
0	0
2	0.04 \pm 0.01
3	2.02 \pm 0.01
4	2.05 \pm 0.01
5	2.08 \pm 0.01
10	2.04 \pm 0.01

Results are shown as mean \pm s.e.m. of three experiments

The absorbance at 405 nm increased during the first three minutes of the incubation (see Table 2.13) but there was no further change in optical density when samples were incubated for a longer period of time. Thus, a three minute incubation was adequate for measuring the maximum kallikrein activity in plasma samples (see Fig. 2.13).

EFFECT OF CHROMOZYM PK CONCENTRATION ON PLASMA KALLIKREIN ACTIVITY

For routine measurements of plasma kallikrein, Chromozym PK was prepared in a concentration of 1.5 mM. Thus, each assay tube contained 0.7 μ mole of the substrate. The effect of changing its concentration on the measurement of plasma kallikrein activity was

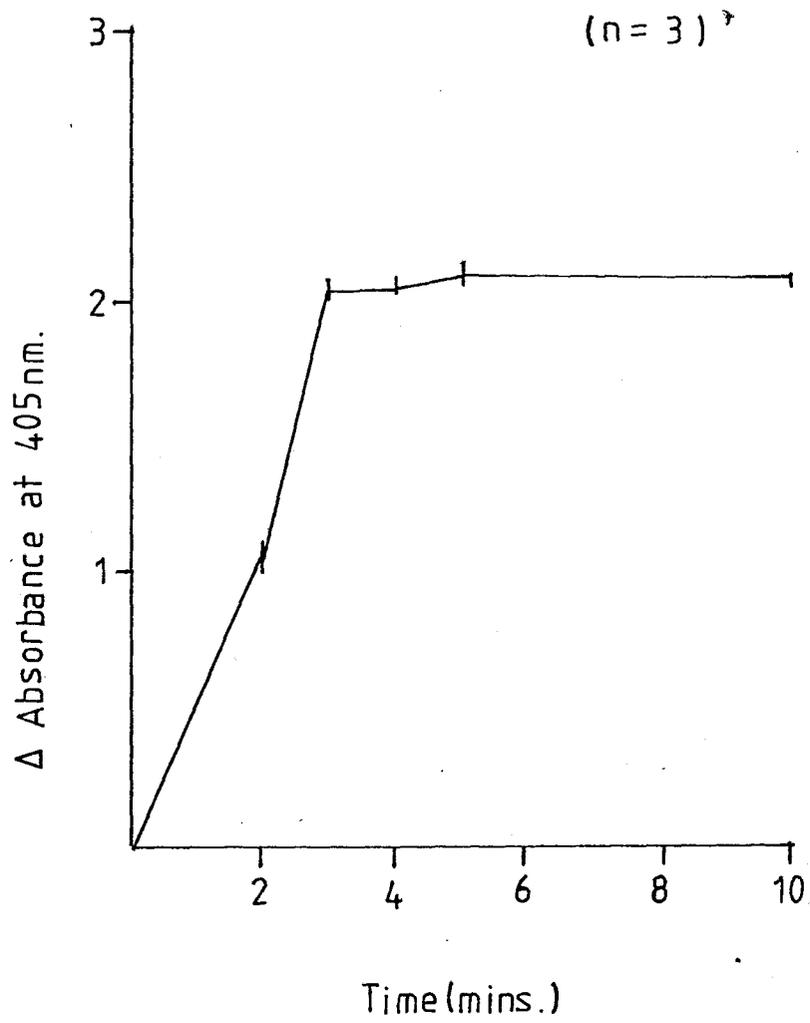


Fig.2:13 Production of p-Nitroaniline
with Time.

determined in the following experiment. A solution of Chromozym PK (3.0 μ M) was serially diluted with buffer and aliquots of activated rabbit plasma were incubated at 37°C for three minutes with 500 μ l of each dilution and 1.5 ml of buffer. The change in absorbance was then determined (Table 2.14).

TABLE 2.14 Effect of Substrate Dilution on Absorbance at 405 nm

Chromozym PK (μ moles/tube)	Δ Optical Density $\times 10^{-3}$
1.5	2.04 \pm 0.01
0.7	2.04 \pm 0.01
0.35	2.02 \pm 0.01
0.125	0.98 \pm 0.01

Results are shown as mean s.e.m. of three experiments.

The results (see Fig. 2.14) indicate that there is no significant difference in kallikrein activity as determined by this method, until the concentration of Chromozym PK is less than 0.35 μ moles/tube. Thus, the hydrolysis of Chromozym PK by plasma kallikrein appears to follow zero order kinetics.

HYDROLYSIS OF CHROMOZYM PK BY PROTEASE ENZYMES

It is likely that Chromozym PK, as well as TAME, is not a specific substrate for kallikreins alone. It may be susceptible to hydrolysis by other proteolytic enzymes. This possibility was investigated in the following experiment. A series of solutions of commercially available proteolytic enzymes (0.2 mg/ml) were prepared

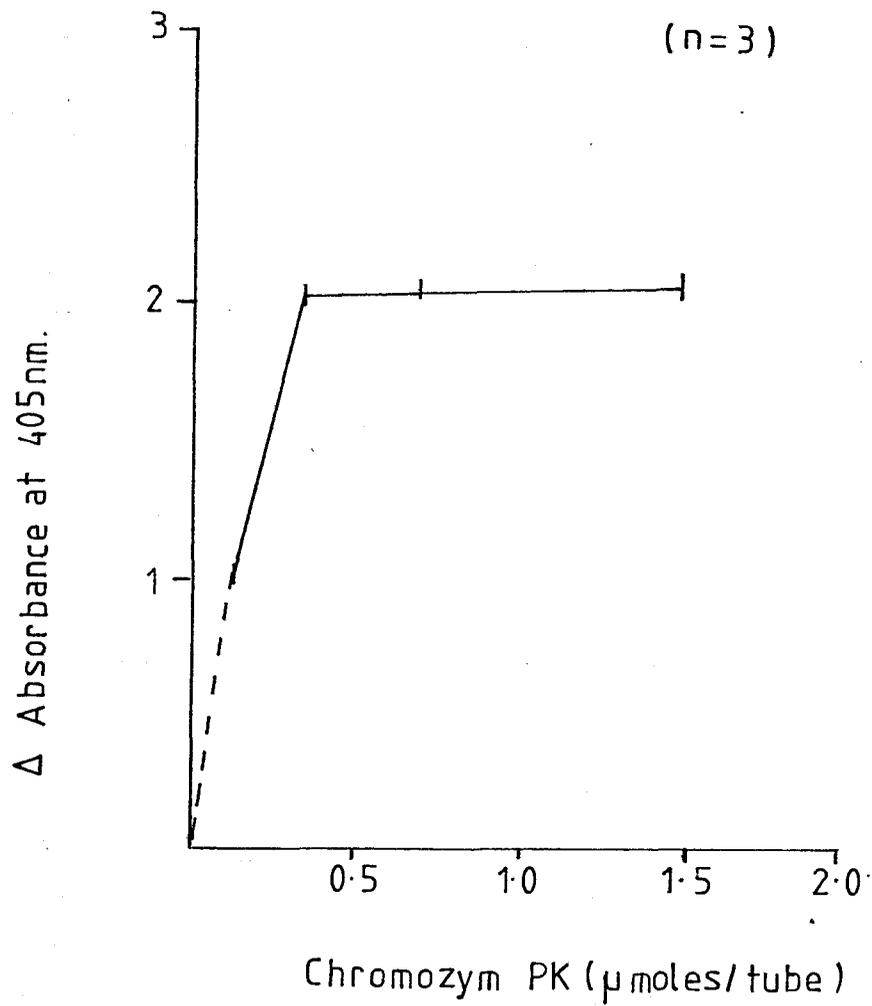


Fig. 2:14 Effect of Chromozym PK Dilution on Absorbance at 405nm.

(see Table 2.15). Aliquots (20 μ l) of each of these solutions were incubated with Chromozym PK (see page 97) and the change in absorbance at 405 nm was determined. The results were compared with those obtained using rabbit plasma alone.

TABLE 2.15 Hydrolysis of Chromozym PK by Protease Enzymes

Enzyme	D Optical Density $\times 10^{-3}$
Plasma	2.01 \pm 0.01
Plasmin (Sigma)	1.89 \pm 0.01
Trypsin (BDH)	0.91 \pm 0.01
Pancreatic Kallikrein (Sigma)	0.52 \pm 0.01
Thrombin (Sigma)	0.03 \pm 0.01

Results are shown as mean \pm s.e.m. of data from three experiments.

Although Chromozym PK is sensitive to kallikrein in plasma, the results show that it is also cleaved by plasmin. Trypsin and pancreatic kallikrein also liberated p-nitroanaline from this substrate. However, like TAME, Chromozym PK was not hydrolysed by thrombin.

The effect of omitting the activation step on plasma kallikrein activity was also determined. p-Nitroanaline was not liberated from Chromozym PK if plasma had not been previously incubated with dextran sulphate. Furthermore, kallikrein activity was not present in serum, and was reduced when blood samples were collected in glass tubes, and when heparin and EDTA, rather than citrate were used as anticoagulants. Both of these agents inhibit clotting mechanisms at a later stage in the cascade of events which leads to activation of fibrinogen. Thus, when these are used for blood sampling, plasma prekallikrein is activated before the assay can be carried out.

DISCUSSION .

In the experiments which will be described in Chapter 3 of this thesis, the activity of kallikrein, rather than its absolute concentration in plasma and urine were measured. TAME was considered to be a suitable substrate for urinary kallikrein, since the reaction followed zero order kinetics, and was dependent on the amount of kallikrein which was present and on the length of the incubation period. The evaluation of Chromozym PK as a substrate for plasma kallikrein was more limited, since a purified preparation of this enzyme was not available. However, hydrolysis of the substrate by plasma appeared to be independent of substrate concentration. This suggests that the reaction follows zero order kinetics. In addition, an incubation period of three minutes was found to give a maximal value for kallikrein activity in rabbit plasma.

The major problem which is encountered with esterolytic assays of kallikrein is that the substrates which are in general use are not specific for this enzyme alone. The results which were obtained in these experiments confirm the findings of other workers using human material, who have found that both TAME and Chromozym PK can be hydrolysed by enzymes other than kallikrein (Fassler, Duckert & Marbet, 1978; Hial et al, 1974; Matler & Bang, 1977; Nustad & Pierce, 1974). It has been reported that plasmin hydrolysis comprises about 4% of kallikrein activity measured with Chromozym PK (Stormorken et al, 1978). In view of these findings, it is important to remember therefore, that hydrolysis of both TAME and Chromozym PK may reflect changes in the activity of kallikrein and non-kallikrein esterases in both plasma and urine.

In the assay of plasma kallikrein, prekallikrein must be activated in vitro before its activity can be estimated. This is achieved by incubation with dextran sulphate in the experiments which are described in this thesis. However, plasma which is deficient in Hageman factor and Fletcher factor will not generate kallikrein activity. These clotting factor deficiencies are relatively rare in human subjects, and have not been reported to exist in rabbits. It is unlikely that they will affect the results of the experiments described in Chapter 3. Furthermore, Claeson et al (1978) have reported that if Fletcher factor is present in concentrations which are

only 25% of normal levels, then plasma will generate full kallikrein activity as measured by Chromozym PK.

Despite the reservations outlined above, the two forms of kallikrein assay were considered suitable for use in the later experimental studies.

IN VITRO INCUBATION OF KIDNEY CORTEX SLICESINTRODUCTION

In vitro kidney preparations have been widely used to study renin secretion. Renin is released into the perfusion medium by isolated kidneys (Fray, 1978) and by isolated glomeruli (Baumbach & Leyssac, 1977; Harada, Lester & Rubin, 1979). Other examples of in vitro kidney preparations include kidney cortex slices (De Vito et al, 1970; Michelakis, 1971) and isolated, renin-containing granules, (Funakawa, Higashio & Yamamoto, 1978). In the isolated tissue, the effects of arterial blood pressure and renal sympathetic nerves, which often complicate the interpretation of in vivo investigations, are avoided. The rate at which renin is released into the incubation medium is determined by its composition, and therefore the direct effect of a given stimulus on juxtaglomerular cells can be studied.

Early work suggested that renin release from kidney cortex slices is an active process (Bozovic & Efendic, 1969; Braverman, Freeman & Rostorfer, 1971) which is independent of the rate of renin synthesis (De Vito et al, 1970). However, in a recent study, Park, Han & Fray (1981) found that renin release by dog kidney cortex slices was increased when they were incubated at 4°C, and they suggested therefore, that it was ^{not} an energy-dependent process. Other workers have found that renin release from cortex slices is uncontrolled (Braverman et al, 1971; Corsini, Hook & Bailie, 1974) and is unresponsive to external stimuli (De Vito et al, 1970; Corsini et al, 1974).

Nevertheless, many investigators have found that the rate of renin release in vitro changes in response to sodium (Michelakis, 1971, Munday, Noble & Richards, 1980a) calcium (Baumbach & Leyssac, 1976; Fray, 1977) angiotensin (Naftilan & Oparil, 1978) catecholamines (Fray & Park, 1979; Harada & Rubin, 1978; Munday, Noble & Richards, 1980b) and to a variety of other physiological and pharmacological stimuli.

Park et al (1978) measured the rate at which renin was released in vitro in the dog and suggested that there are two pools of renin. One of these appeared to release renin at a rate of about 1.5% of

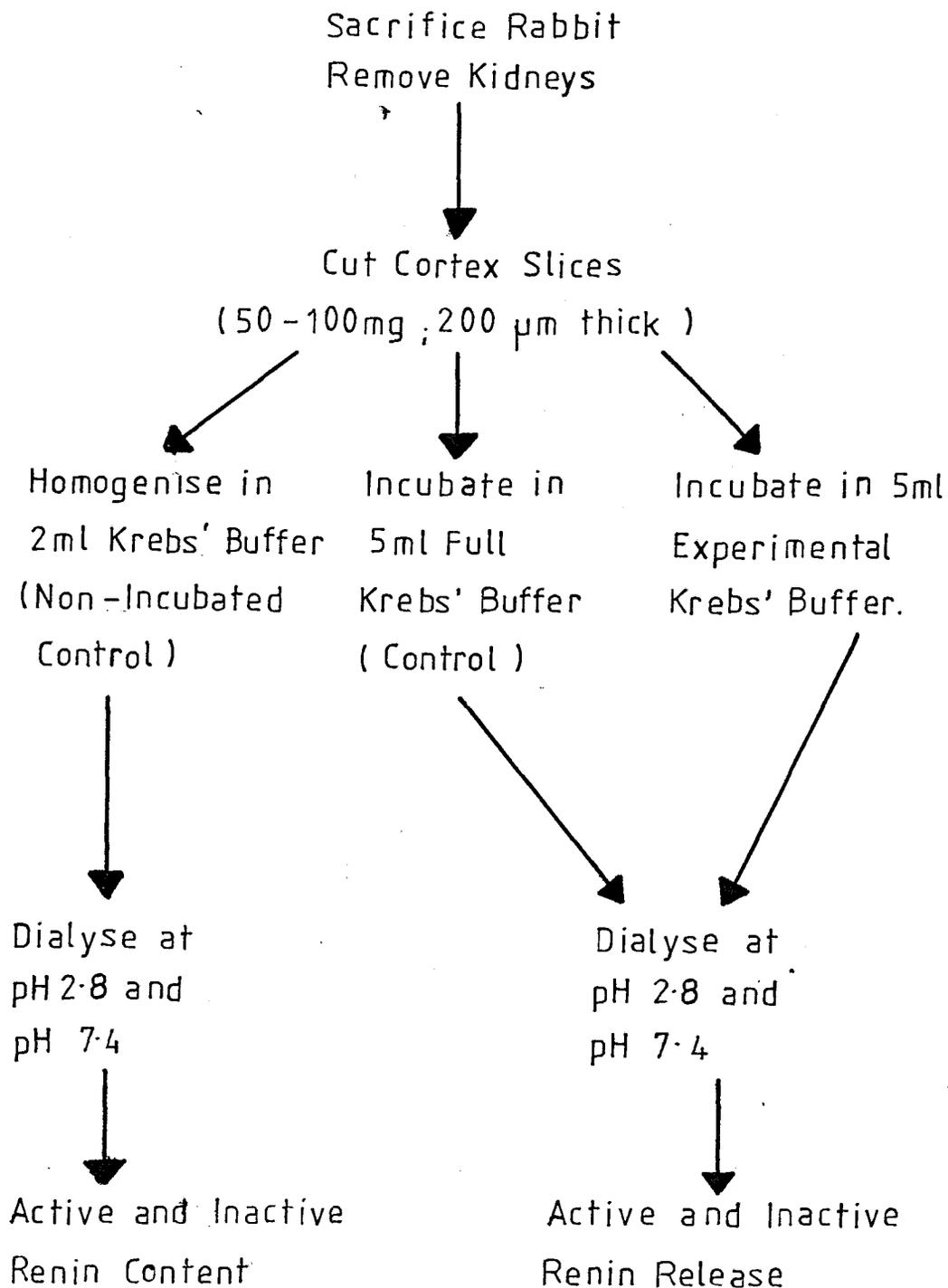
the total renin content per hour, while the rate of secretion from the other pool depended on the magnitude of the stimulus. In contrast, Funakawa, Higashio & Yamamoto (1978) concluded from their study with isolated, renin-containing granules, that there are three storage forms of the enzyme. One of these pools, about 20% of the total, was soluble and readily released into the incubation medium. The second, hard-to-release, insoluble form, amounted to about 70% of the stored renin, and appeared in the supernatant when the granules were destroyed by osmotic lysis. A further 8% of the stored renin remained in the membrane fraction of the granules.

Rabbit renal cortex slices have been used during part of the work which will be described in Chapter 3 of this thesis, and throughout the experiments concerning the role of calcium in the control of renin secretion (Chapters 4 & 5). Although there have been many studies describing active renin release in vitro, very few have been reported concerning inactive renin (Bailie, Derkx & Schalekamp, 1979; Munday, Noble & Richards, 1980a and b). If the physiological role of inactive renin is to be clarified, it is important that mechanisms controlling its release from the kidney should be studied.

MATERIALS AND METHODS

New Zealand White rabbits were sacrificed by neck fracture and both kidneys were removed and kept on ice. Cortex slices which weighed 50 - 100 mg and were approximately 200 μ m thick, were cut by hand with a razor blade. All experiments were carried out in duplicate using one slice from each kidney of the rabbit. One pair of slices was retained to be homogenised with Krebs'-Ringer bicarbonate (see Table 2.15) as a non-incubated control. These were subsequently homogenised and centrifuged in order to measure the total renin content of slices. For each experiment, at least one pair of slices were incubated in unmodified Krebs'-Ringer bicarbonate (pH 7.4). Flasks were incubated at 37°C in a slowly-oscillating water bath for ninety minutes. The incubation media were bubbled with 95% O₂/5% CO₂ gas mixture at ten minute intervals. At the end of the incubation,

Fig. 2:15 Experimental Protocol for In Vitro Investigations.



aliquots (2 - 3 ml) of the supernatant were collected and centrifuged for five minutes to remove all cell debris. After dialysis (see page 74), the active and inactive renin released by the slices was determined as ang I generated/h/mg wet weight of tissue.

STATISTICAL ANALYSIS OF DATA

Values for renin concentration, both in plasma and in supernatant collected after in vitro incubation of kidney cortex slices, are not normally distributed. In consequence, non-parametric statistics must be applied during analysis of data. Throughout the work described in this thesis, the Wilcoxon Signed Rank Test has been used for statistical analysis of paired data. In most of the experiments which will be described, values for active renin have been subjected to a percentage transformation, and have also been expressed as a percentage of the control value. Students' paired t-tests have been applied to data obtained in this way. Statistical significance has only been shown in tables of data when this has been revealed by both analyses.

In addition, values obtained for inactive renin (total - active renin concentration) have been expressed as a percentage of total renin concentration. Students' paired t-tests have been applied to data obtained in this way.

TABLE 2-16 . The Composition of Krebs'-Ringer Bicarbonate

	Stock Solutions	Proportions
A	0.9% NaCl	100
B	1.15% KCl	4
C	1.22% CaCl ₂	3
D	2.11 KH ₂ PO ₄	1
E	3.82% MgSO ₄ · 7H ₂ O	1
F	1.3% NaHCO ₂	21
G	2.6% Glucose	10

Final Molarities

Na	133 m Moles/l
K	5.7 m Moles/l
Cl	116.6 m Moles/l
Ca ²	2.3 m Moles/l
HCO ₃	23 m Moles/l
H ₂ PO ₄	1.1 m Moles/l
SO ₄ ²⁻	1.1 m Moles/l
Mg ²⁺	1.1 m Moles/l
Glucose	10.3 m Moles/l

This basic medium was used for control incubations, and could be modified as appropriate to the design of the experiments.

DISCUSSION

Active renin released by rabbit kidney cortex slices incubated in complete Krebs' buffer is in the range 5-7 ng ang I/h/mg wet weight of tissue. Renin activity in the supernatant increases ($p < 0.01$) after acid dialysis, and inactive renin comprises some 15-20% of the total renin released (see Chapters 3, 4 and 5). By measuring the renin content of non-incubated control slices, it is found that 8-10 % of stored renin is released into the incubation medium during the ninety-minute experimental period.

Dr. H.K. Richards (unpublished data), using an exactly equivalent protocol in this laboratory, studied the time course of renin release into the supernatant during the incubation period. He found that it was linear for up to two hours. He also found that the rate of renin release was not significantly different if the incubation media were not bubbled with 95% O₂/5% CO₂ intermittently during the incubation. It is conceivable that bubbling gas through the buffer could damage kidney cortex slices. However, in the in vitro investigations described here, the incubation media are bubbled with gas at ten minute intervals, and this does not appear to alter renin release. It is feasible that continuous bubbling might damage the slices. Subsequent release of prostaglandins could alter the rate of renin release (see page 22). However, there is no evidence to suggest that this occurs when the protocol which has been described here is followed.



CHAPTER 3.

DOES KALLIKREIN ACTIVATE INACTIVE RENIN ?

INTRODUCTION

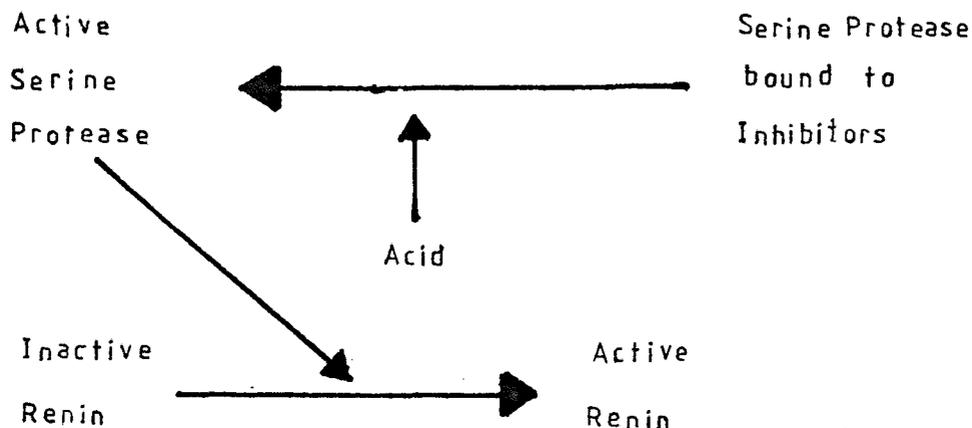
The existence of inactive forms of renin in plasma and in kidney extracts is now well established. If activation of inactive renin is indeed a new site for control of the renin - angiotensin system, then questions are raised concerning the nature of the activation process. Although it appears likely that activation of inactive renin is determined enzymatically, the biochemical nature and anatomical localisation of the activating enzyme(s) remains a matter for contention. Potential renin activators have been evaluated almost entirely by means of in vitro techniques, using selective blocking agents. However, such studies can be misleading and it is clearly important that an alternative approach to the problem is devised in order to provide physiologically relevant, rather than pharmacologically interesting answers to the questions which are posed.

Experimentally, inactive renin can be activated, and therefore measured, by acidification followed by dialysis back to neutral pH, by exposure to low temperature or by treatment with protease enzymes (see page 8). The presence of serine protease enzymes appears to be necessary for acid-activation of inactive renin. If inhibitors of serine proteases, such as aprotinin and soya bean trypsin inhibitor, are added to plasma before it is dialysed, the degree of acid activation is reduced (Leckie, 1978; Atlas et al, 1978a; Derkx et al, 1978a). Cryoactivation of inactive renin in human plasma also depends on the presence of a serine protease (Atlas et al, 1978b; Osmond & Loh, 1978).

Derkx et al (1979b) found that "acid activation" of inactive renin does not take place during the acidification step itself, but occurs during the subsequent "alkaline phase" when plasma is dialysed back to pH 7.4. It has been suggested that endogenous protease inhibitors which are present in plasma are destroyed by low pH (Atlas et al, 1978a). This was thought to allow serine proteases to activate inactive renin during the second stage of dialysis (see Fig 3.1).

However, there is some recent evidence that suggests that complete but reversible activation of inactive renin may take place at low pH. Hseuh et al (1980) found that kallikrein was only effective in activating inactive renin in plasma which had been pretreated with acid. It was ineffective in activating inactive renin in untreated plasma. When such untreated plasma was combined with acidified plasma, the percentage activation by renal kallikrein was related to the proportion of acid-

FIG 3.1 Proposed mechanism whereby acidification of plasma leads to activation of inactive renin.



dialysed plasma which was present. They therefore suggested that renin could require some structural alteration, presumably achieved by acidification, prior to activation by kallikrein.

Leckie (1981) has also reported that complete activation of inactive renin in human plasma takes place at low pH. The increase in renin activity was reversed by rapid neutralisation and warming of plasma. The activation at acid pH was rendered irreversible by dialysis back to neutral pH by a process which is sensitive to protease inhibition. She suggested that rapid neutralisation could cause a reassociation of renin and an "inhibitor protein" which had become dissociated at low pH. During the slow dialysis back to neutral pH, the "inhibitor protein" could be inactivated by serine proteases, thereby rendering the activation irreversible.

These recent findings may explain the observation made by other authors that kallikrein did not activate inactive renin at neutral pH unless prior acidification of plasma had taken place (Derkx, Tan-Tjiong & Schalekamp, 1978a). However, the biochemical nature of inactive renin remains to be clarified (see page 9) and although some groups have found that activation of inactive renin is associated with a change in its molecular weight, others have found that no such change can be measured, which suggests that activation involves only a conformational change in the enzyme rather than dissociation of the enzyme from a binding protein.

Many different protease enzymes, including trypsin, plasmin, pepsin and cathepsin D have been reported to activate inactive renin in plasma, in kidney extracts and in amniotic fluid. This indicates that renin activation in plasma is not carried out by one specific protease. Kallikreins have been subject to much attention in recent years and Sealey et al (1978) have proposed that plasma kallikrein may be the physiological activator of inactive renin. The evidence to support this hypothesis will now be reviewed. It must be remembered that "kallikrein" is the generic term for a series of enzymes and that the enzymes are derived from various sources and will therefore have different properties (see page 45). Which form of kallikrein, if any, is important will relate to questions concerning the anatomical location of the renin-activating process.

Osmond et al (1978) reported that there was a high proportion of inactive renin in plasma from patients who were deficient in the kallikrein precursor, prekallikrein (Fletcher factor). Fletcher factor-deficient plasma has lower renin activity after acidification (Derckx et al, 1979b) and cold treatment (Sealey et al, 1979b) than after trypsin activation. However, in contrast to these findings, Miller et al (1978) found that acid activation of inactive renin in Fletcher factor - deficient plasma was normal.

Similarly, the "alkaline phase" of acid activation was almost completely absent in plasma which was deficient in Hageman factor (Tatemichi & Osmond, 1978; Sealy et al, 1979a). However, when plasma kallikrein was added to such samples, activation of inactive renin was normal. Since Hageman factor is necessary for activation of prekallikrein in plasma, it was concluded that acid activation of inactive renin is initiated by Hageman factor-dependent conversion of prekallikrein to kallikrein. There is a great deal of evidence to support this possibility. It has now been confirmed by several groups that kallikrein added to plasma samples is a powerful activator of inactive renin (see page 8). Therefore the possibility that there could be a link between the renin and kallikrein ^{systems} in plasma, where kallikreins are involved in coagulation and fibrinolysis is an interesting one. However, it is important to note that kallikreins, and indeed other candidates for the role of renin activator, for example, plasmin, are normally present in plasma as inactive precursors. Experimental methods for their activation (acidification and cold treatment) have no apparent physiological relevance. In addition, there remains the

possibility that activation of inactive renin does not actually take place in plasma at all. A potentially more interesting area to search for a renin activating system may therefore be within the kidney.

There are many similarities between renin and renal kallikreins. The zonal distribution of the two enzymes is similar: their concentrations within the kidney being greater in the cortex than in the medulla. Renin and kallikrein have been found in the same microsomal fraction of kidney extracts (Wilson *et al*, 1976). After they are released, renin and plasma kallikrein act on plasma globulins to liberate angiotensin I and lysyl-bradykinin (kallidin) respectively. Angiotensin I converting enzyme (kininase II) acts on both of these peptides. It promotes cleavage of angiotensin I to the more active peptide angiotensin II and mediates the breakdown of kinins. Thus, converting enzyme can augment the activity of the renin-angiotensin system and suppress the kallikrein-kinin system. These two systems contribute to regulation of the renal circulation and extracellular fluid volume. With prostaglandins, which may exert a partial control over both renin and kallikrein (Data *et al*, 1978; Olson, 1977) the interactions between the renin-angiotensin and kallikrein kinin system can result in major modifications of renal haemodynamics and excretion of salt and water. This subject has recently been reviewed by Baer & McGiff (1980).

Urinary kallikrein is capable of activating inactive renin *in vitro* and is more potent than trypsin in this respect (Sealey, Atlas & Laragh, 1978). Activation of inactive renin by kallikrein may occur as blood flows through the kidney. Local production of bradykinin, which is a powerful vasodilator, could be antagonised by activation of inactive renin and the subsequent production of angiotensin II. Although the simultaneous release of two vasoactive peptides with opposing actions may, at first sight, seem inefficient, they could act at different sites to regulate systemic arterial pressure whilst maintaining renal perfusion. Bradykinin formed locally within the kidney would probably exert its major physiological action intrarenally. Kinins are unlikely to have any significant systemic activity as they are inactivated in the pulmonary circulation. Angiotensin II, however, does have systemic pressor activity (see page 35).

Flamenbaum, Gagnon & Ramwell (1979) found that infusion of kinins into the renal artery stimulated renin release. This response was not mediated by prostaglandins, unlike many of the effects of kinins in the kidney, since it was not abolished by indomethacin. Vinci *et al* (1979)

have also demonstrated a significant correlation between circulating levels of kinins and plasma renin activity. Purified rat urinary kallikrein stimulated renin release from superfused rat renal cortex slices in a dose-related manner (Suzuki, 1980). The stimulation was inhibited by Trasylol but did not appear to be mediated by kinins, since bradykinin did not increase renin release.

The renin-angiotensin system may also stimulate the kallikrein-kinin system. Infusion of angiotensin II into the renal artery increases kallikrein excretion (MacFarlane, Adetuyibi & Mills, 1974) but if the kidney is protected from the increase in renal artery pressure by a clamp, angiotensin II no longer increases kallikrein excretion (Mills, Newport & Obika, 1978). The latter authors also found that the increase in kallikrein excretion was reduced by indomethacin, suggesting that angiotensin-induced release of kallikrein requires prostaglandins. Wong *et al* (1975) showed that when PRA was high in salt-depleted normal subjects, plasma free bradykinin levels were also high. Both were reduced by saline infusion. Similarly, they found that renin and bradykinin levels increased when subjects on a normal salt intake adopted an upright posture.

Margolius *et al* (1974) reported that the rise in kallikrein excretion in normotensive subjects given furosemide and a low salt diet was related to the increase in PRA. A similar relationship between plasma renin activity and kallikrein excretion has been found in rats during furosemide administration (Johnstone, Matthews & Dax, 1976) and during bumetanide, bendroflumethiazide and hydrallazine treatment (Nielsen, & Arrigoni-Martel, 1977).

In contrast to this, Abe *et al* (1978) concluded that the time courses of changes in kallikrein and kinin excretion and the increase in plasma renin activity after furosemide were independent, and that the augmentation of the kallikrein-kinin system was directly dependent on the effects of furosemide. In addition to this, it has been reported (Croxatto *et al*, 1978; Croxatto *et al*, 1979) that semi-purified rat and hog renin preparations almost completely abolished kallikrein excretion and increased sodium excretion in hyper-hydrated rats.

Augmentation of the kallikrein-kinin system antagonises stimulation of the renin-angiotensin system and subsequent vasoconstriction. The response of the kallikrein-kinin system to changes in renal artery pressure is the converse of that for renin. Bevan *et al* (1974) found that kallikrein excretion is directly related to renal artery pressure

distal to a clamp applied to the renal artery of the dog; kallikrein excretion in urine was reduced when the clamp was applied and increased when it was released. Kaiser et al (1976) reported a similar relationship in dogs with a chronic renal artery constriction. Kallikrein excretion has also been negatively correlated with renal vascular resistance during renal artery constriction (Mills et al, 1976) and during intrarenal infusion of prostaglandin E₂ (Mills & Obika, 1977b).

Prostaglandins appear to play a central role in mediating the complex interactions between the renin-angiotensin and kallikrein-kinin systems. Angiotensin II increases prostaglandin synthesis (Terragno et al, 1976; McGiff, 1977). Prostaglandins appear to stimulate renin release (see page 22). Kinins increase prostaglandin release from the perfused kidney (Colina-Chourio et al, 1976; McGiff et al, 1972) and renal medulla (Zusman & Kaiser, 1977) by activating phospholipases which supply arachidonic acid for prostaglandin synthesis (Hong & Levine, 1976; McGiff, 1977; Terragno et al, 1976). In turn, prostaglandin infusion increases kallikrein excretion (Obika & Mills, 1976).

Thus, since prostaglandins can stimulate renin release, activation of the kallikrein system can influence the renin-angiotensin system both directly and indirectly. Similarly, the renin-angiotensin system can alter the activity of the renal kallikrein-kinin system through prostaglandins. A further link between the two systems may be aldosterone. Angiotensin II increases aldosterone secretion from the zona glomerulosa of the adrenal cortex (see page 39) and aldosterone stimulates kallikrein synthesis or secretion (Margolius et al, 1974; Margolius et al, 1976).

Although it is interesting to speculate on the existence of yet another link between these two systems which is mediated through the activation of inactive renin, there is as yet little evidence that such a relationship exists in vivo. In the experiments which will be described in the remainder of this chapter, the possibility that kallikrein takes part in the physiological activation of inactive renin has been investigated using the serine protease inhibitor, aprotinin, which is more commonly known as Trasylol (Bayer). This compound was selected for the following studies because, unlike many of the protease inhibitors which have been used for in vitro studies, Trasylol can be used for in vivo suppression of protease activity. It has in fact been used in the treatment of pancreatitis for many years.

Trasylol is one of a group of protease inhibitors which are of

both animal and plant origin. In 1930, Kraut, Frey & Werle studied kallikrein inhibitors which they had extracted from bovine pancreas, parotid glands and liver. Kunitz & Nothrop (1936) independently prepared a trypsin inhibitor from bovine pancreas. More recently it has been shown that these inhibitors are identical. Trasylol has 16 different amino acids in a chain which consists of 58 residues (Kassell et al, 1965a) and the molecule is stabilised by 3 disulphide bridges (Kassell & Laskowski, 1965b).

Trasylol has a broad spectrum of action and inhibits several proteolytic enzymes, including kallikreins, plasmin, trypsin, chymotrypsin and proteases from leucocytes and damaged cells (Schultze, 1979). However, the inhibitor exerts a degree of specificity in its activity and many proteases including thrombin, pepsin and cathepsin are unaffected by Trasylol.

The experiments which will be described have been divided into four sections:

- I. The in vitro effect of Trasylol on acid-activation of inactive renin in rabbit plasma.
- II. The effect of Trasylol in vivo on plasma active and inactive renin in the rabbit.
- III. The effect of Trasylol on in vitro release of renin from rabbit renal cortex slices.
- IV. The effect of Trasylol on acid-activation of inactive renin in the supernatant from rabbit kidney cortex slices.

SECTION I.The In Vitro Effect of Trasylol on Acid-Activation of Inactive Renin in Rabbit Plasma.

In view of the reports that have been published suggesting that serine protease enzymes are necessary for acid activation of inactive renin in human plasma, the effect of the protease inhibitor was investigated in rabbit plasma.

MATERIALS & METHODS.

Six New Zealand White rabbits (2.5 - 3.0 kg) were anaesthetised with 25% urethane (1.25g/kg b.w.) administered intraperitoneally. A cannula was inserted in the abdominal aorta. Blood was collected using Na₂EDTA (1mg/ml of plasma) as anticoagulant and kept on ice. After centrifugation in a bench centrifuge for 5 minutes, the plasma was separated. Trasylol, in a range of concentrations, was added to aliquots (2ml) of plasma. Sets of plasma samples were then subjected to each of the dialysis protocols outlined on page 74 so that active and total renin activity could be measured.

RESULTS

The absolute values for active and total renin are expressed in ng AI generated/ml of plasma/h of incubation and are shown in table 3.1. Renin concentration has also been expressed as a percentage of the control value (no Trasylol added) for active renin for each experiment (see Fig. 3.2). Table 3.2 shows the absolute values calculated for inactive renin (difference between active and total renin concentration) and these have also been expressed as a proportion of the total renin concentration of each sample (see Fig. 3.3).

TABLE 3.1 The Effect of Trasylol on Active and Total Plasma Renin Concentration (PRC).

Trasylol Concentration	PRC (ngAI/ml/h).		Plasma Active Renin (%)
	Active	Total	
0 (Control)	9.1±2.9	11.0±3.4 *	100
25 KIU/ml	9.4±3.0	11.2±2.3 *	98.4±3.8
50	8.9±2.8	10.7±3.4 *	101.5±6.4
75	8.9±2.8	10.3±3.3 ns	97.0±6.4
100	10.2±3.1	11.0±3.4 ns	109.4±8.9
200	9.3±2.8	9.7±2.9 ns	110.6±7.4
300	9.6±3.0	10.1±3.2 ns	110.0±5.4
500	9.5±2.9	10.0±3.0 ns	108.0±8.9

Results are shown as mean ± s.e.m. of data obtained from 6 rabbits. Statistical analysis by Wilcoxon Signed Rank Test.

KIU = kallikrein
inhibitory unit

* $p < 0.01$

Increase in renin activity
on acidification.

Active Renin (%) =
active renin concentration
as % of control value.

No significant changes in active renin concentration were observed when Trasylol was added to plasma samples before they were dialysed. This shows that the protease inhibitor itself does not interfere with renin measurement using the methods already described, (see page 59).

However, inactive renin was significantly reduced ($p = 0.05$) from 1.74 ± 0.42 in control samples to 0.65 ± 0.29 ngAI/ml/h (Table 3.2). This represents a 57% inhibition of acid activation when the concentration of Trasylol was 100 KIU/ml. As the amount of the inhibitor was increased, acid activation of inactive renin was progressively reduced and there was an 81% inhibition of activation when the concentration of Trasylol was 500 KIU/ml. An inverse linear relationship was observed between inactive renin measured in samples after the

Fig. 3:2 The Effect of Trasylol (Aprotinin) during Dialysis of Plasma

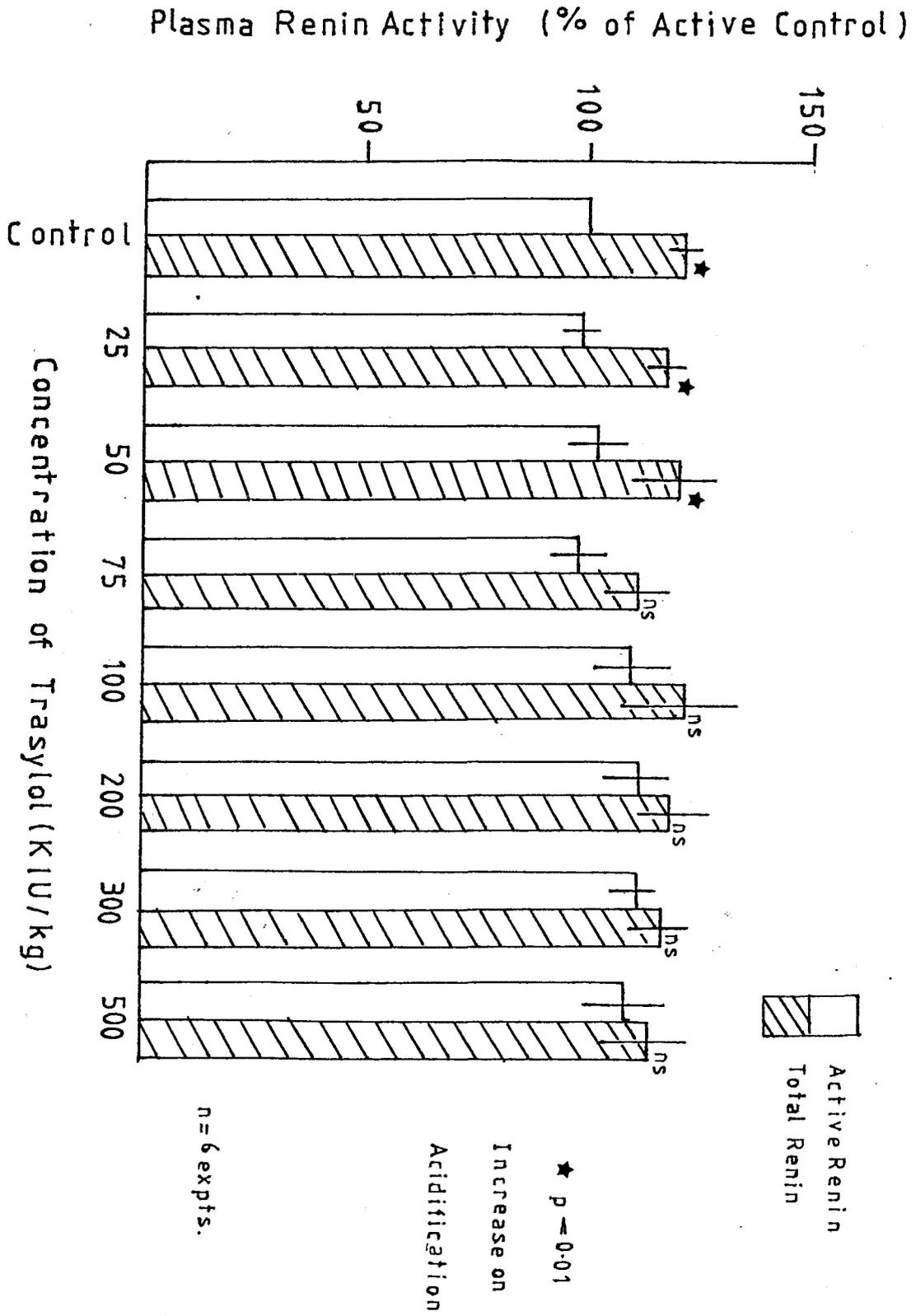
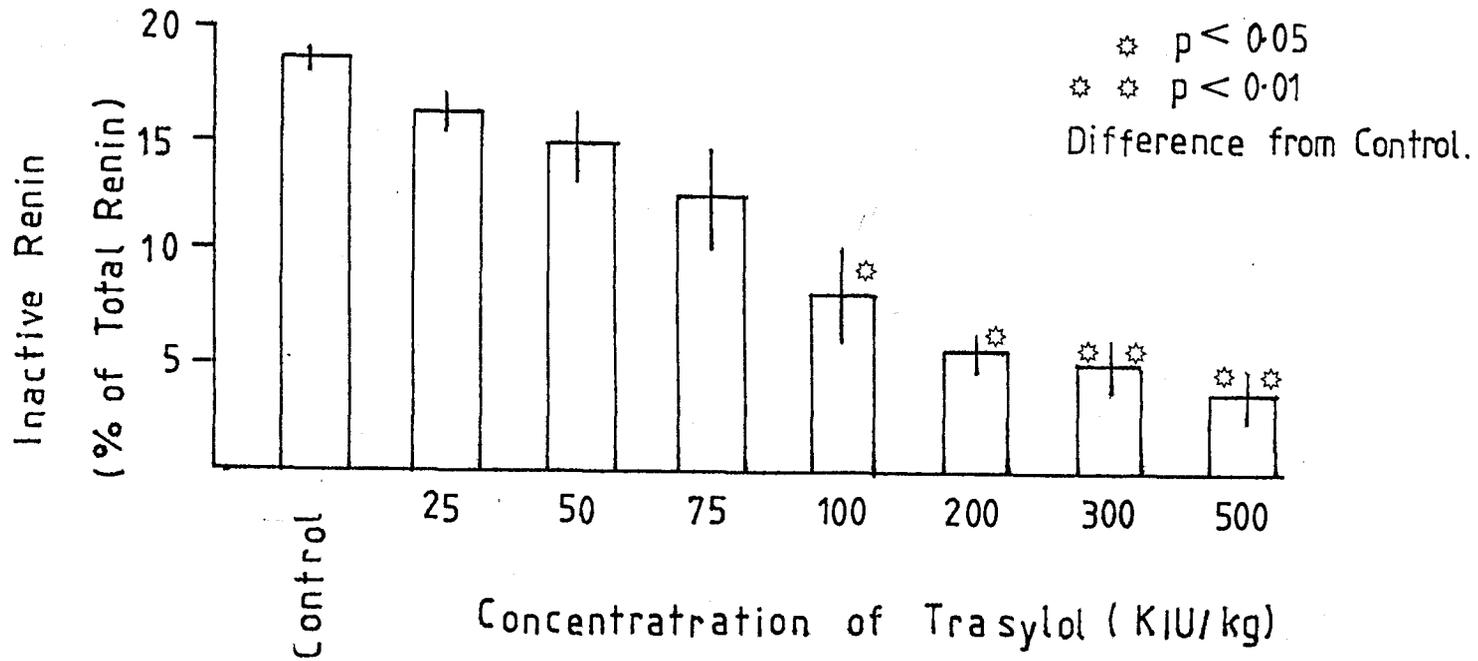


Fig. 3:3

The Effect of Trasylol (Aprotinin) on Acid

Activation of Inactive Plasma Renin



n=6 experiments

To date, however, patients with these deficiencies have not been available for study in the Southampton area.

SECTION II The Effect of Trasylol (in vivo) on Plasma Renin in the Rabbit.

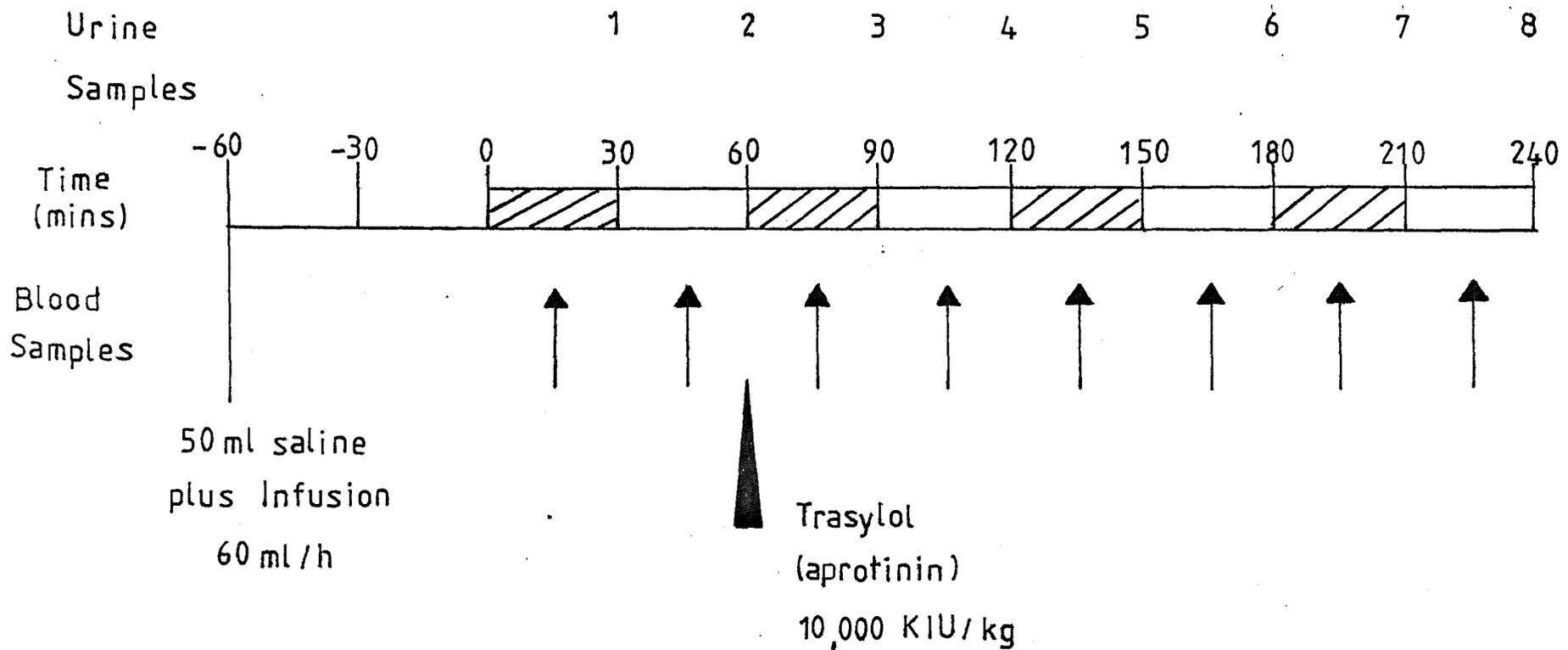
In the previous experiment it was confirmed that Trasylol will substantially inhibit acid activation of inactive renin in rabbit plasma as well as in human plasma. The recent hypothesis that kallikreins may be responsible for activating inactive renin in vivo was investigated in the following experiment. Kallikrein activity in urine and plasma was measured when Trasylol was administered intravenously to rabbits. If kallikrein plays an "activator" role, then a change in its activity ought to be reflected by changes in the relative amounts of active and inactive renin in plasma.

MATERIALS AND METHODS

Male New Zealand White rabbits (2.5 - 3.0 kg) were anaesthetised with 25% urethane, 1.25 g/kg b.w.) intraperitoneally. A tracheostomy was performed, and the right common carotid artery was cannulated to monitor arterial blood pressure and to collect blood samples. The right jugular vein was cannulated for drug and saline infusion and a balloon catheter was passed into the bladder for urine collection. The experimental protocol is shown in Fig. 3.4. Both the control (n = 8) and experimental (n = 10) groups of samples received 50 ml of 0.9% saline intravenously at the start of the experiment and were infused thereafter at a rate of 60 ml/h. After a 60 minute stabilisation period, sample collection was begun. Urine was collected for each 30 minute period, its volume recorded, and it was then stored at -20°C. Mid-way through each urine collection, a 4 ml sample of arterial blood was taken. Part of each sample (1 ml) was collected into trisodium citrate (0.11M; citrate: blood = 1:9) and kept on ice. This sample was used for assay of plasma kallikrein. The remainder of each sample was collected into Na₂EDTA (1 mg/ml of plasma) as anticoagulant, for renin measurement. After centrifugation, plasma was separated and stored at -20°C until assay.

After two control periods, Trasylol (10,000 KIU/kg b.w.) was administered to one group of rabbits, and blood and urine samples were

Fig. 3:4 Experimental Protocol



collected for a further 180 minutes. The same time course, omitting administration of Trasylol, was followed in the control group of animals.

The concentrations of sodium and potassium in urine were measured in an integrating flame photometer (Evans Electroelenium) and urinary kallikrein was estimated by hydrolysis of TAME (see page 87). Kallikrein activity in plasma was measured by hydrolysis of Chromozym PK (see page 97) and after dialysis, active and total renin were measured by generation of angiotensin I from sheep renin substrate (see page 66).

RESULTS

After intravenous injection, Trasylol is distributed throughout the extracellular space (Kaller *et al*, 1978). Taking this distribution volume into account and assuming that extracellular fluid represents about 20% of body weight, then the plasma concentration of the protease inhibitor should be of the order of 50 KIU/ml, following administration of Trasylol as described.

From the results obtained in the previous experiment, (see page 119) the proportion of inactive renin in plasma would be underestimated by only 1 - 2% at this concentration of Trasylol.

Data for plasma active, and total renin are shown as both raw data and in a percentage transformation, taking active renin in the initial sample collected from each rabbit as 100%. Similarly, inactive renin in each sample has been represented as a proportion of the total renin concentration.

No significant changes were observed in either active (Table 3.3) or inactive (Table 3.5) renin in the group of control animals.

TABLE 3.3 Plasma Active and Total Renin in 8 Control Animals (no Trasylol).

Collection Period	PRC (ngAI/ml/h)		Plasma Active Renin (%)
	Active	Total	
1	15.6±1.7	17.3±2.1 *	100
2	16.5±3.5	18.2±3.2 *	101.1±13.9
3	16.8±3.7	18.7±1.9 *	101.4±14.8
4	15.7±4.6	17.0±2.9 *	91.9±18.3
5	17.1±3.7	18.6±2.7 *	103.3±11.7
6	16.9±1.6	18.6±3.0 *	110.2±7.7
7	15.1±1.9	16.4±2.2 *	106.7±5.8
8	16.2±2.2	18.0±2.1 *	109.2±4.6

Results are expressed as mean ± s.e.m. Statistical analysis by Wilcoxon Signed Rank Test.

★ $p < 0.01$ Increase in Renin activity after acidification.

TABLE 3.4 Plasma Active and Total Renin in 10 Experimental animals given i.v. Trasylol.

Collection Period	PRC (ngAI/ml/h)		Plasma Active Renin (%)
	Active	Total	
1	14.9±2.6	16.2±2.4 *	100
2	14.8±1.6	16.2±2.2 *	98.6±6.7
3	15.3±1.6	16.7±1.9 *	103.4±6.6
4	12.7±1.6	13.9±2.5 *	84.8±6.4
5	11.7±2.0	12.1±1.5ns	77.2±7.0
6	10.5±1.5	11.2±1.3ns	70.9±6.3 ☆
7	10.0±1.6	10.5±1.6ns	69.2±6.0 ☆
8	9.8±1.8	11.0±1.7 *	68.2±6.4 ☆

► Intravenous injection of Trasylol (10,000 KIU/kg b.w.).

☆ $p < 0.001$ Difference from control.

There was no immediate change in active (Table 3.4) or inactive (Table 3.5) renin in the animals which were given Trasylol intravenously. In the fourth collection period, however, when the blood sample was taken 105 minutes after intravenous injection of protease inhibitor, active renin concentration was reduced by 30% ($p < 0.001$) from 14.9 ± 2.6 to 10.5 ± 1.5 ng AI/ml/h, (see Fig 3.5). This was accompanied by a fall ($p < 0.05$) in inactive renin to only $2.5 \pm 4.7\%$ of the total renin concentration, (see Fig. 3.6 & Table 3.5).

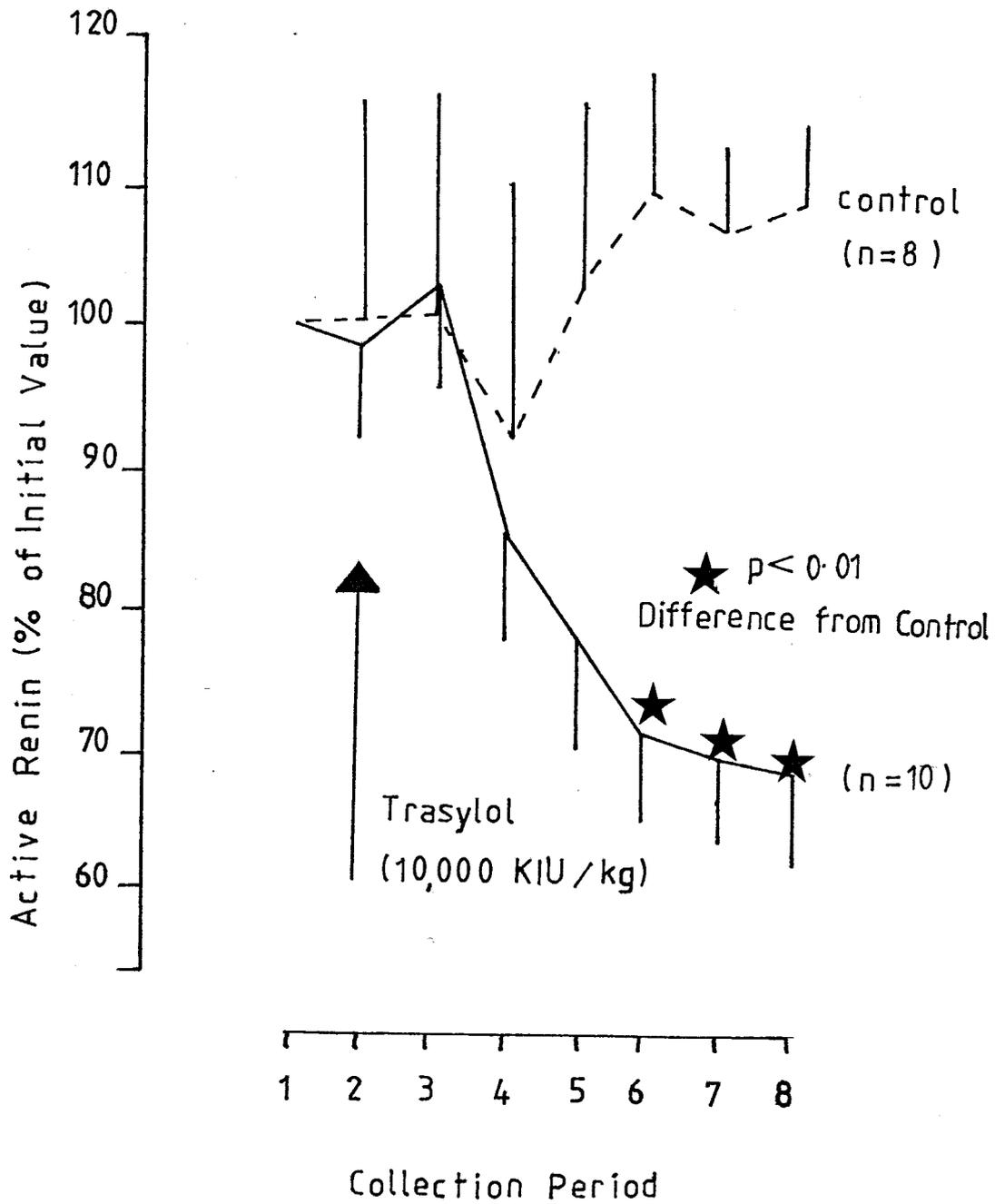
Plasma kallikrein activity, measured by hydrolysis of Chromozym PK, is shown in Table 3.6 and the results have been illustrated in Fig. 3.7. Plasma kallikrein activity did not change significantly in the control group, but was reduced ($p < 0.01$) to 0.15 ± 0.06 U/ml from control levels (0.47 ± 0.04 U/ml) after Trasylol administration. Thus plasma kallikrein was inhibited by up to 70% following protease inhibition.

Although plasma kallikrein activity was immediately reduced by Trasylol, no corresponding change in the amount of inactive renin or in the relative proportions of active and inactive renin were observed. If kallikrein were playing a major role in the activation of inactive renin in plasma, any change in kallikrein activity of plasma ought to be reflected by an opposite change in plasma inactive renin concentration. The results which have been obtained show that this relationship does not occur in rabbit plasma.

Immediately after intravenous injection of Trasylol, urine flow was reduced ($p < 0.001$) and fell from 1.4 ± 0.4 ml/30 minutes to 0.5 ± 0.2 ml/30 minutes. By the time the final collection was made, three hours after Trasylol administration, urine flow had returned towards control values, (see Table 3.7 & Fig. 3.8).

This finding supports the work of other groups who have shown that kallikrein plays an important role in promoting the excretion of water by the kidney, (see page 49).

Fig. 3.5 The Effect of Trasylol on Active Plasma Renin.



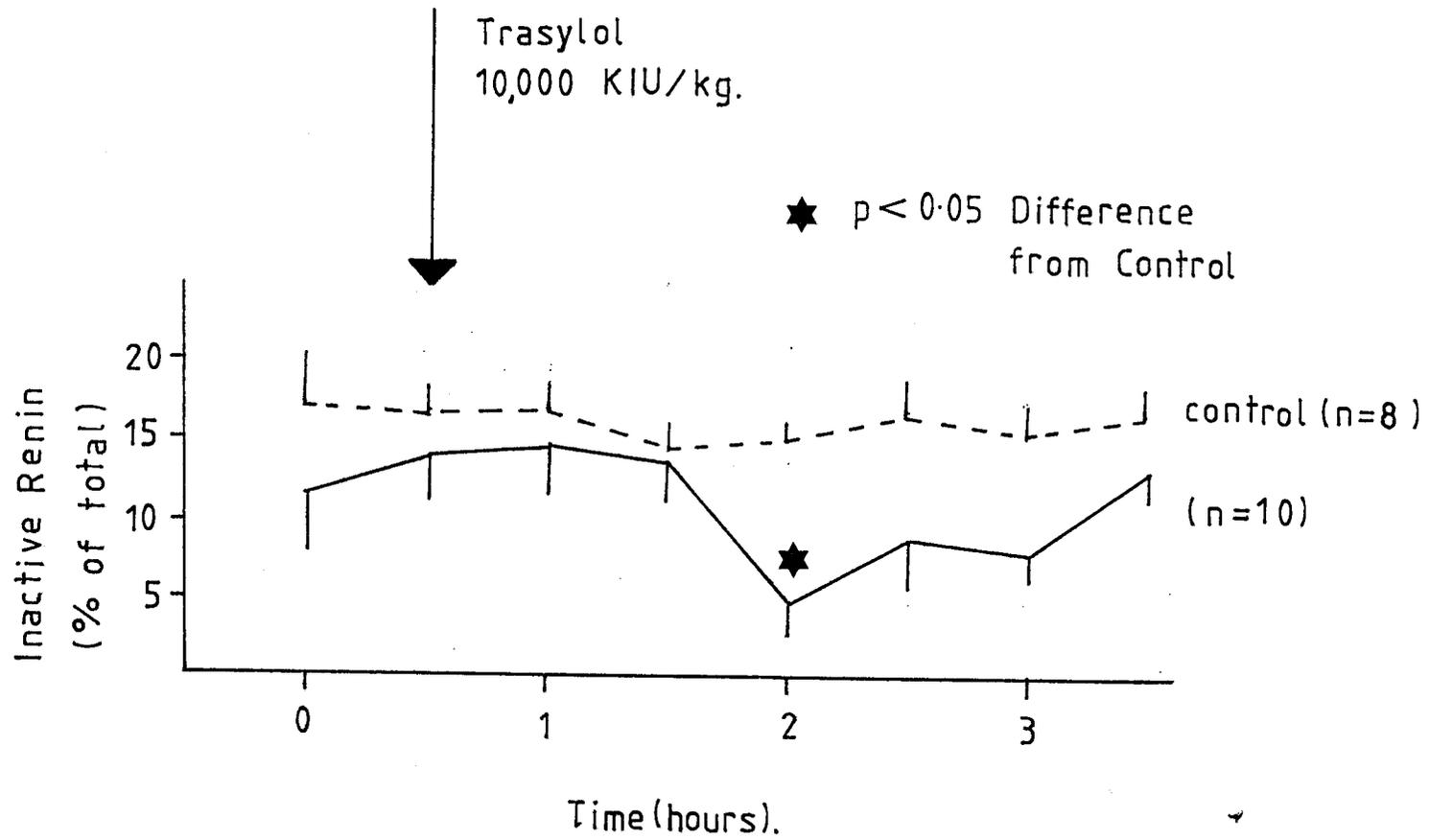


Fig. 3-6 The Effect of Trasylol on Inactive Renin In Vivo

TABLE 3.5 Inactive Renin in Control (Group 1) and Experimental (Group 2) Animals Given Trasylol i.v.

Collection Period	Group 1 (Control) Inactive Renin (% of Total)	Group 2 (Trasylol) Inactive Renin (%)
1	17.1±3.4	13.2±3.6
2	16.6±1.8	13.8±2.8
3	16.8±1.9	▶ 14.3±3.0
4	14.3±2.0	13.5±2.5
5	14.8±1.2	2.5±4.7 ★
6	16.4±2.6	8.7±3.2
7	15.2±2.2	7.7±1.5
8	16.9±1.5	12.5±1.9

Statistical analysis of data for inactive renin and plasma kallikrein by Wilcoxon Signed Rank Test. Results shown as mean ± s.e.m.

★ p=0.05; ★ ★ p<0.01; Difference from control

▶ Trasylol (10,000 KIU/kg) intravenously.

TABLE 3.6 Plasma Kallikrein (Pk) Activity in Group 1 and Group 2 Rabbits (U/ml).

Collection Period	Group 1 Pk	Group 2 Pk
1	0.47±0.07	0.47±0.04
2	0.51±0.05	▶ 0.58±0.11
3	0.61±0.15	0.35±0.05
4	0.58±0.14	0.22±0.06 ★
5	0.53±0.09	0.15±0.06 ★★
6	0.36±0.04	0.20±0.06 ★★
7	0.32±0.07	0.17±0.06 ★★
8	0.42±0.11	0.32±0.08

1 unit (U) of kallikrein activity is equivalent to the amount of plasma kallikrein which is required to hydrolyse 1 μ mol of Chromozym PK per minute at 37°C.

Fig. 3:7 The Effect of Trasylol on Plasma Kallikrein

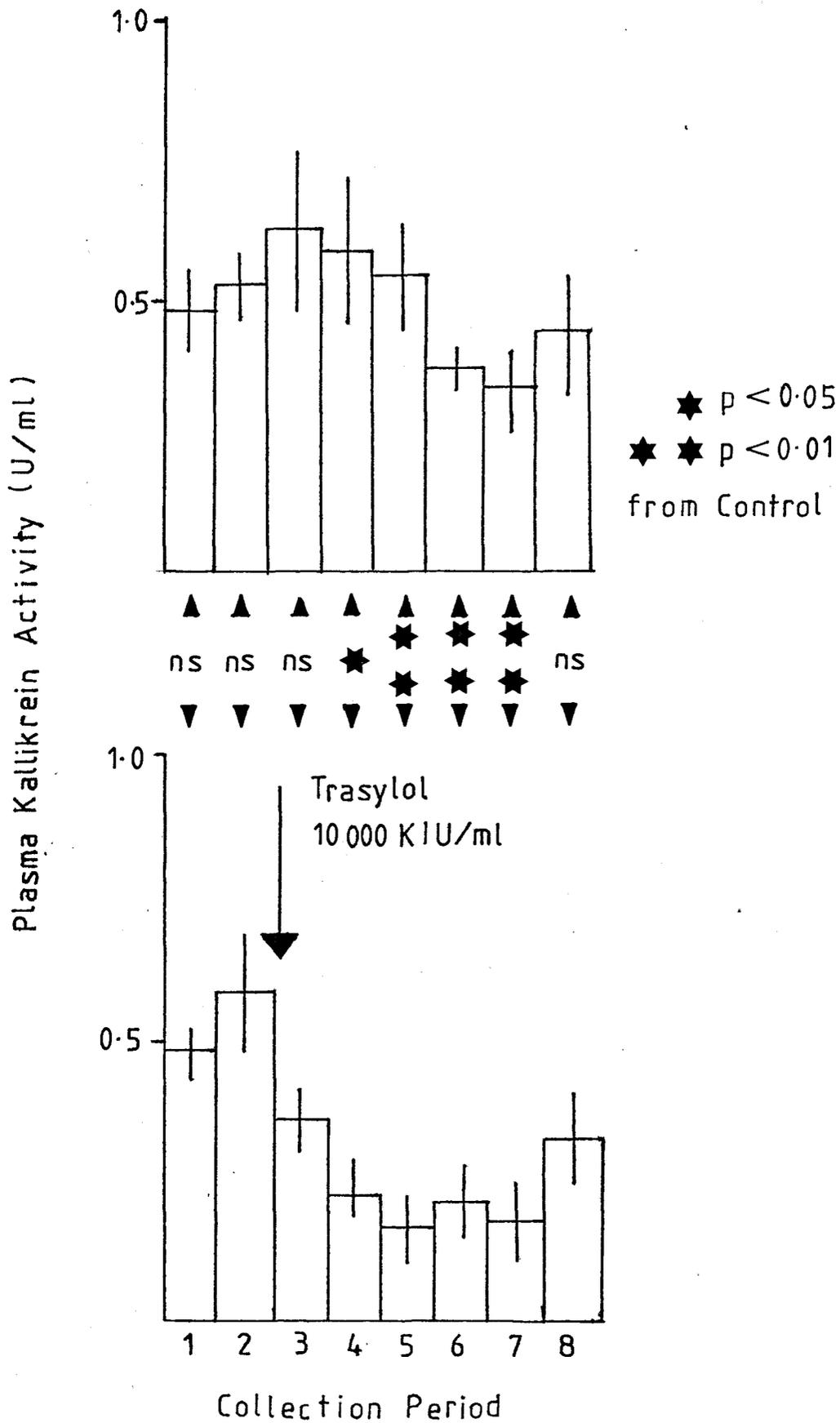


TABLE 3.7 Urine Flow (vol./30 minutes) in Group 1 and Group 2 Rabbits.

Collection Period	Group 1 Urine Flow (U/30 min)	Group 2 Urine Flow (U/30 min)
1	1.3±0.2	1.4±0.4
2	1.2±0.1	1.2±0.3
3	1.5±0.2	0.5±0.2 ★
4	1.1±0.1	0.3±0.2 ★
5	1.2±0.1	0.4±0.2 ★
6	1.1±0.2	0.3±0.2 ★
7	0.8±0.1	0.3±0.1 ★
8	1.4±0.2	0.8±0.3

Statistical analysis of data for urine flow and urinary kallikrein by Students' t-test. Results expressed as mean ± s.e.m. for both groups. Group 1: n = 8; Group 2: n = 10.

★ p < 0.001. Difference from control urine flow.

★ p < 0.001. Difference from initial Uk. ▶ 10,000 KIU/kg Trasylol i.v.

TABLE 3.8 The Effect of Intravenous Trasylol Administration on the Excretion of Kallikrein in Urine (Uk).

Collection Period	Group 1 (Control) Uk (U/minute).	Group 2 (Trasylol) Uk (U/minute.).
1	0.58±0.15	0.44±0.19
2	0.49±0.14	0.32±0.19
3	1.10±0.23 ★	0.56±0.24
4	1.29±0.34 ★	1.29±0.26
5	1.48±0.26 ★	1.20±0.25
6	1.98±0.11 ★	0.79±0.30 ☆
7	2.36±0.14 ★	0.68±0.12 ☆
8	2.29±0.32 ★	0.52±0.16 ☆

1 unit (U) of kallikrein activity ≡ the amount of kallikrein in urine which is required to hydrolyse 1 umole of TAME per minute at 37°C.

☆ p < 0.01 Difference from control group

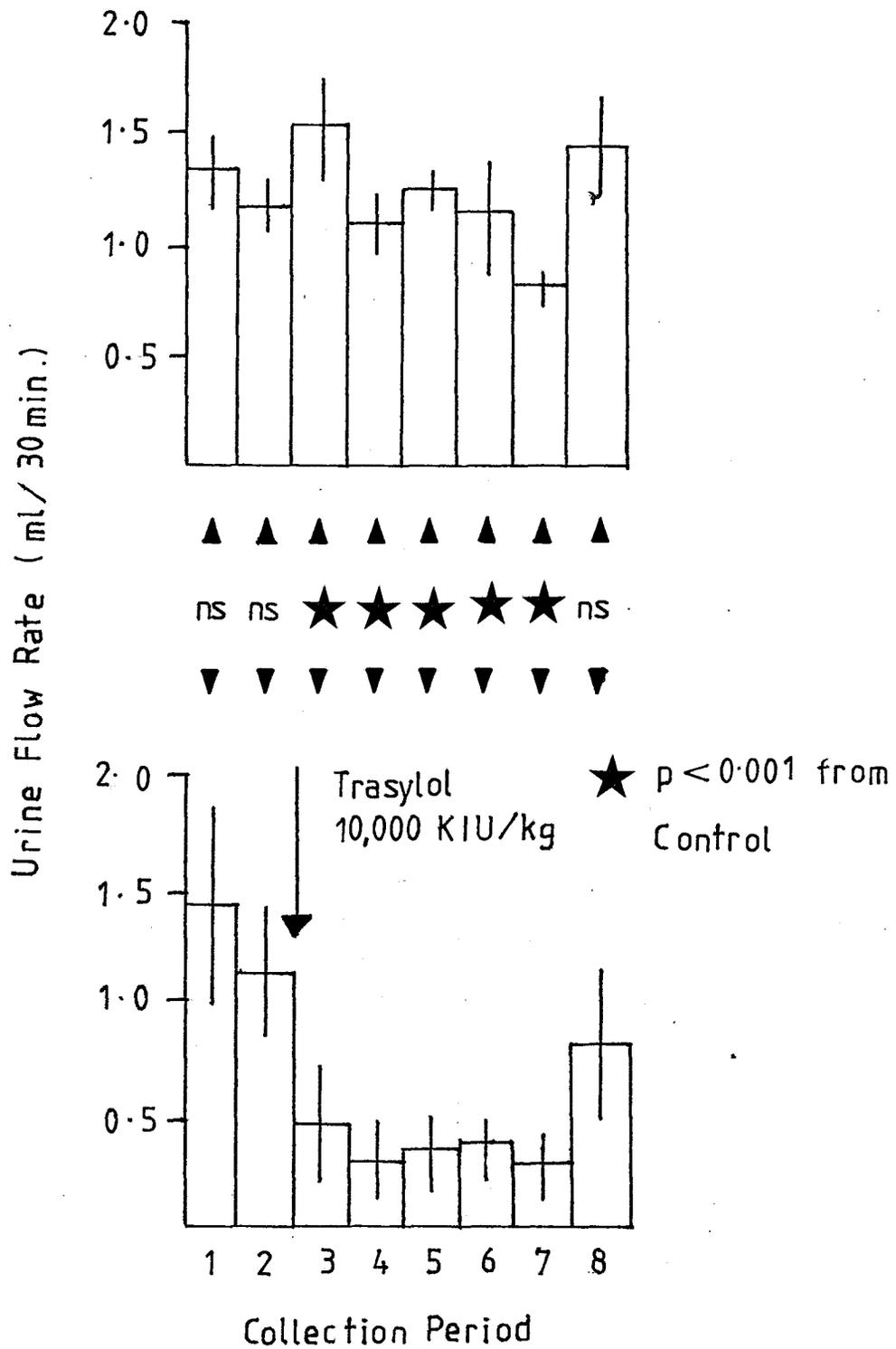
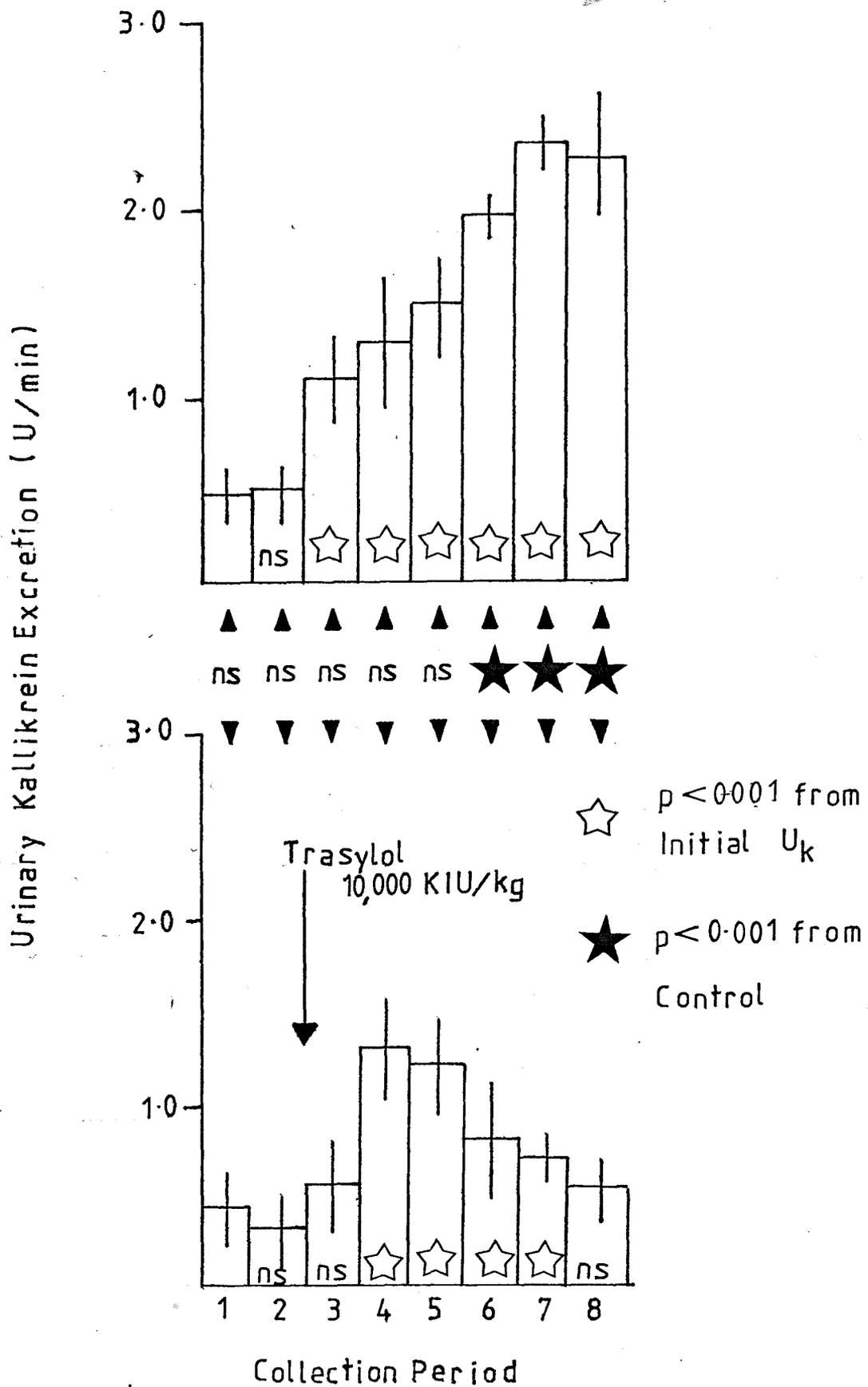


Fig 3:8 The Effect of Trasyolol on Urine Flow

Fig. 3:9 The Effect of Trasylol on Urinary Kallikrein



Kallikrein excretion in urine (Uk) for both groups of rabbits is shown in Table 3.8. Kallikrein activity in urine, and thus kallikrein excretion, increased four-fold ($p < 0.001$) in the control group of animals which were given a saline infusion only, (see Fig. 3.9).

Initially, kallikrein excretion in the animals which were given Trasylol, followed a similar pattern to that observed in the control group. It reached a maximum value of 1.3 ± 0.3 U/minutes in the fourth collection period. However, for the remainder of the experiment, kallikrein activity in urine was significantly less ($p < 0.001$) than in the untreated group.

These results demonstrate that kallikrein excretion in the rabbit progressively increases during saline infusion. Although this effect appeared to be abolished after Trasylol administration, the reduction in kallikrein activity of urine which was observed is possibly the result of inability to measure enzymic activity since some Trasylol is excreted in urine. Thus, although the observed kallikrein excretion was lower than that in the control group, this does not necessarily reflect any change in the absolute concentration of the enzyme in urine. This possibility could be confirmed by using a direct assay for urinary kallikrein.

Trasylol administration had an immediate effect on both sodium and potassium excretion (see Table 3.9) and this is shown graphically in Fig. 3.10. There were no significant changes in either sodium or potassium excretion in the control group, although both tended to fall over the period of the experiment. However, both sodium and potassium excretion were reduced ($p < 0.001$ and $p < 0.001$ respectively) immediately after protease inhibition. These results suggest that kallikrein promotes electrolyte excretion, in addition to water excretion, in the kidney.

TABLE 3.9 The Effect of Trasylol Administration on Sodium and Potassium Excretion

Collection Period		Control (mmol/30 minutes).	Trasylol (mmol/30 minutes)
1	Sodium Excretion	0.92±0.14	1.09±0.22
2		0.82±0.22	0.94±0.16
3		1.11±0.12	0.39±0.09 ★
4		0.77±0.19	0.20±0.07 ★ ★
5		0.77±0.23	0.27±0.06 ★ ★ ★
6		0.73±0.26	0.18±0.07 ★ ★ ★
7		0.76±0.18	0.20±0.05 ★ ★ ★
8		0.79±0.22	0.44±0.05
1	Potassium Excretion	0.055±0.008	0.054±0.009
2		0.052±0.009	0.049±0.010
3		0.064±0.008	0.013±0.003 ★ ★ ★
4		0.045±0.011	0.007±0.002 ★ ★ ★
5		0.049±0.008	0.012±0.004 ★ ★ ★
6		0.048±0.008	0.013±0.003 ★ ★ ★
7		0.032±0.017	0.014±0.003 ★ ★ ★
8		0.056±0.009	0.034±0.011

Results are expressed as mean ± s.e.m. Statistical analysis by Students' t-test.

★ p < 0.05; ★★ p < 0.01; ★★★ p < 0.001: difference from control.

☆ p < 0.05 Difference from control Na⁺ excretion.
 ☆ ☆ p < 0.01
 ☆ ☆ ☆ p < 0.001

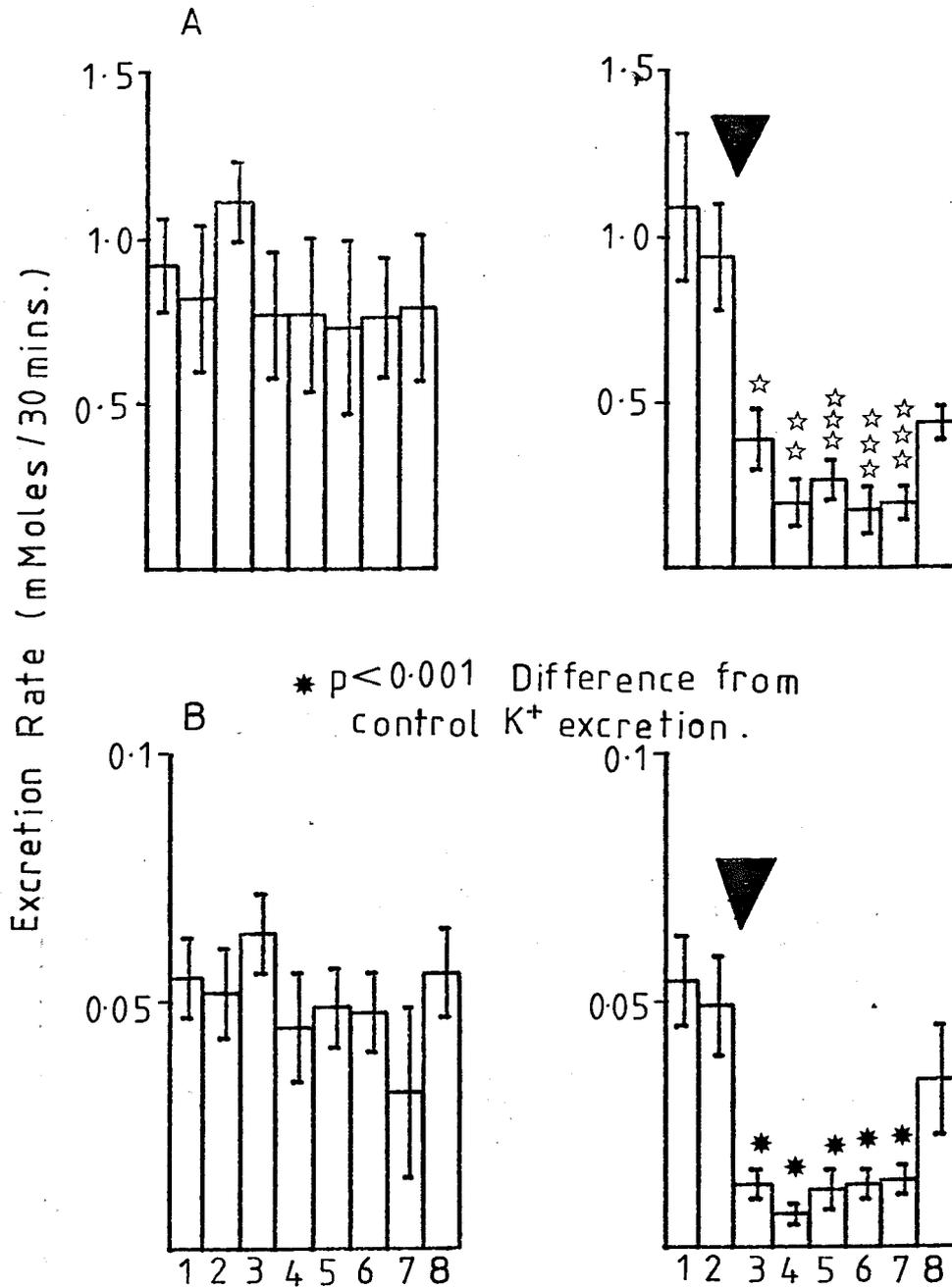


Fig.3:10 The Effect of Trasylol (▲ : 10,000 KIU/kg) on Electrolyte (A=Na⁺ ; B=K⁺) Excretion.

DISCUSSION

Plasma kallikrein activity after intravenous Trasylol administration was only 34% of that in the control group, (see Table 3.6). If plasma kallikrein had been the primary activating enzyme for inactive renin, one could have expected to see an increase in inactive renin and a corresponding decrease in active renin in plasma. No change was observed in either form of renin, (see Tables 3.4 and 3.5). This suggests that plasma kallikrein is not the primary factor which controls the relative proportions of active and inactive renin in plasma. Indeed, after a delay of about 90 minutes, a 30% reduction in plasma active renin was accompanied by a fall in the proportion of inactive from about 15% to 2% of the total renin concentration.

There is evidence to suggest that Trasylol is accompanied in renal tissue before it is excreted (Kaller, 1970). The later changes in plasma renin could therefore be related to such an event, (see Table 3.10).

TABLE 3.10 ¹³¹I-Trasylol Concentrations in Various Tissues in Mice 30 Minutes After Intravenous Injection, (400,000 KIU/kg b.w.).

Organ	Trasylol Concentration (KIU/g Fresh Tissue)	Distribution Quotient Organ Conc./Serum Conc.
Serum	1,139	1.00
Kidney	10,284	9.03
Liver	338	0.30
Muscle	300	0.26
Brain	50	0.04

After Kaller, (1970).

The inhibitor itself, or some of its metabolites, could reduce the rate of renin secretion from juxtaglomerular cells and thereby reduce

plasma active renin. On the other hand, the observed changes in plasma renin could be the result of concurrent changes in renal electrolyte handling. Before further evaluation of the changes in renin activity in plasma could be made, it was necessary to investigate the direct effects of Trasylol on renin release from the kidney. Experimental work in which this was investigated ^{is described} in Section III of this chapter, (see page 143). Some other aspects of the work described in the present chapter will first be discussed.

Immediate and substantial changes in urine flow and sodium and potassium excretion were observed after Trasylol injection, (see Tables 3.7 & 3.9; and Figs. 8 & 10). Similar findings were reported recently by Kramer et al., (1979) from studies in volume expanded rats receiving isotonic saline. They found that Trasylol caused a reduction in GFR. This was accompanied by reductions in urine volume, sodium and potassium excretion and excretion of PGE₂. The inhibitor had no effect in non-volume-expanded rats.

Many of the effects of renal kallikreins appear to be mediated by kinins. Antibodies to bradykinin reduced sodium excretion after saline infusion by 50%, (Marin-Grez, 1972). Consistent with this view is the demonstration that kallikrein excretion varies directly with dietary salt intake in man, (Adetuyibi & Mills, 1972) and increases during saline infusion in dogs, (DeBono & Mills, 1974; Mills et al., 1976) and in rats, (Croxatto et al., 1975; Croxatto et al., 1976; Godon & Damas, 1974).

Kallikrein excretion increased four-fold when rabbits in the control group were receiving a saline infusion at the rate of 1 ml/minute in the work described here. During the course of the experiments, the animals became progressively saline-loaded. They were receiving saline at the rate of 60 ml/h and urine flow rate was only about 3-4ml/h.

Although excretion of kallikrein in urine initially tended to follow the same pattern in the group of animals which received Trasylol, it was reduced in the later stages of the experiment. This could suggest that some Trasylol is excreted in an active form in urine. However, at least part of the Trasylol appears to be broken down in the cells of the proximal renal tubules (Just, 1973; Just & Haberman, 1973). The metabolic products have not yet been identified, but are thought to be low molecular weight fragments which are excreted in urine.

The finding that urine flow and sodium and potassium excretion were reduced after intravenous injection of Trasylol is consistent with the

role kinins are thought to play in regulating sodium excretion. It has frequently been shown, (see page 49) that kinins promote salt and water excretion in the kidney. Thus, administration of Trasylol would be expected to inhibit liberation of kinins by renal kallikrein. Urine flow and electrolyte excretion are consequently reduced.

There remains some controversy about kallikrein excretion during saline infusion. In man, kallikrein excretion remained unchanged during saline infusion, (Margolius, 1974; Levy, Frigon & Stone, 1978). It is not clear whether this may be due to a species difference or to some other factor. Kallikrein excretion was unchanged in rabbits, (Mills & Ward, 1975) and rats, (Johnston, Matthews & Dax, 1976; Marin-Grez & Carretero, 1973) when dietary sodium intake was increased without free access to water. This suggests that the ^{intracellular} fluid volume must be expanded before kallikrein excretion increases in response to sodium loading.

As was discussed previously, on the basis of the results obtained in vivo from the experiments reported in this section of the thesis, it is not possible to explain the mechanism of the delayed changes in active and inactive plasma renin following Trasylol injection. One possibility is that Trasylol has a direct action on renin-secreting cells. This was investigated in the next series of experiments, in which renin release in response to the addition of Trasylol in vitro to kidney cortex slices was studied.

SECTION III.The In Vitro Effect of Trasylol on Renin Release.

Kidney slices and other in vitro preparations have been widely used to study renin release mechanisms and have been discussed in detail in another section of this thesis (see page 106). There are several advantages in studying renin secretion in this way. As was found in the previous experiment, results of in vivo investigations are often difficult to interpret. In some cases, this is due to the influence of extrarenal factors such as blood pressure, anaesthesia and circulating catecholamines which themselves can alter renin secretion. In the experiment described in section II, it was not possible to determine whether changes in plasma renin occurred in response to changes in renal handling of sodium after kallikrein inhibition, or were the result of a direct effect of Trasylol or its metabolites on renin-secreting cells. With the use of an in vitro technique, some of these problems can be avoided.

MATERIALS AND METHODS.

The preparation and incubation of rabbit kidney cortex slices has already been described (see page 107). In the following experiment, four pairs of cortical slices were incubated in Krebs'-Ringer bicarbonate media to which 50, 100, 200 or 400 KIU/ml Trasylol had been added. A further pair of slices were incubated in unmodified Krebs' as an Incubated Control. As a Non-Incubated Control, a pair of kidney cortex slices were homogenised with Krebs' buffer (2ml), centrifuged in a bench centrifuge and stored at -20°C until assay to measure the renin content of slices.

RESULTS.

Active and total renin release by kidney cortex slices is shown in Table 3.11. Raw data were subjected to a percentage transformation, taking active renin release by slices which were incubated in unmodified buffer as 100%. This data is also shown in Table 3.11,

Table 3.11. The Effect of Trasylol on Renin Release (ng AI/mg wet wt. of tissue/h) from Rabbit Kidney Cortex Slices.

Trasylol Concentration	Renin Release (ngAI/mg/h)		Active Renin Release (%)
	Active	Total	
0	6.3±1.6	8.2±1.7	100
50 KIU/ml	7.9±2.2	9.1±1.8	128.0±22.7
100	6.3±1.8	7.9±1.5	102.4±23.7
200	6.7±1.6	7.6±1.2	105.1±19.4
400	4.6±1.3	6.1±1.7	69.7±9.2 ★
Non-Incubated Control	52.2±8.7	64.5±9.2	

Results are shown as mean ± sem of data from 9 experiments. Statistical analysis of data by Wilcoxon Signed Rank Test.

★ p < 0.05 Difference from Incubated Control

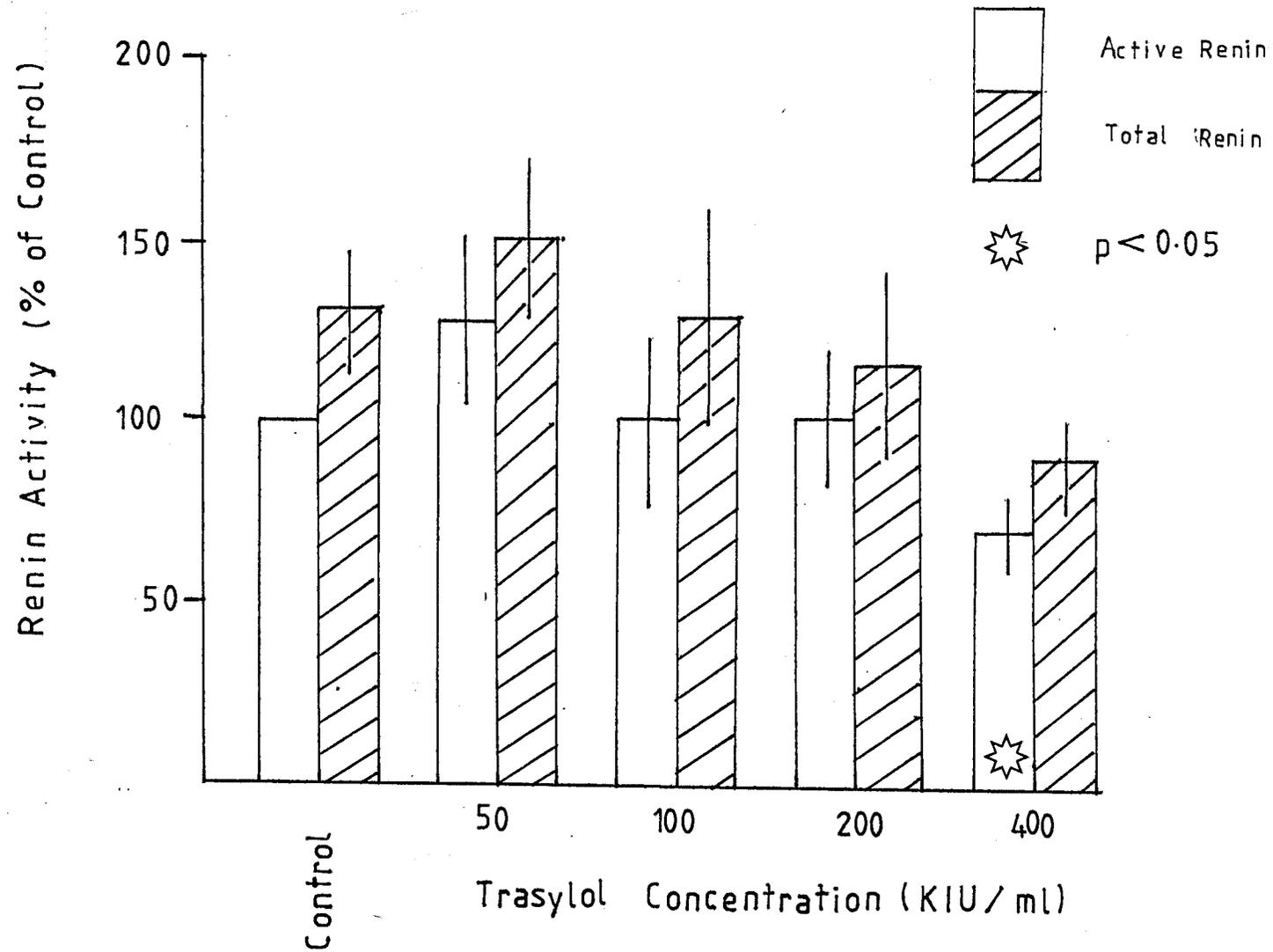
and results have been illustrated graphically in Fig. 3.11.

There were no significant changes in active renin released from kidney cortex slices except at the highest concentration of Trasylol which was used (400 KIU/ml). At this dose level, active renin release was reduced by 30%. This is a very high concentration of Trasylol and it is probable that it has little relevance to any in vivo situation in which Trasylol was used.

Inactive renin was apparently reduced in a manner which was related to the concentration of Trasylol which was present in the incubation medium. The percentage of the total renin released which was inactive was reduced from 22.1 % in the Incubated Control slices to 5.4 % when 400 KIU/ml was present (Table 3.12 and Fig. 3.12).

However, before further conclusions can be drawn from these results, it will be necessary to investigate the effect of protease inhibition on acid-activation of slice supernatants. These results could represent merely a failure to measure inactive renin, rather than an actual change in secretion of inactive renin. In experiment I (see page 119). it was found that high concentrations of Trasylol inhibited acid-activation of inactive plasma renin by up to 80 %.

Fig. 3:11 The Effect of Trasylol on Renin Release In Vitro



In the current experiment, inactive renin appeared to be reduced in slice supernatants even when low concentrations (50 and 100 KIU/ml) of Trasylol had been added to the incubation medium, although the changes were not statistically significant. Therefore, the possibility that inactive renin activation was inhibited in slice supernatant was investigated and the results from that series of experiments are described in the next section of this thesis.

Table 3.12. The Effect of Trasylol on Inactive Renin Released by Rabbit Kidney Cortex Slices.

Trasylol Concentration	Inactive Renin ng AI/mg/h	Inactive Renin (% of Total Renin)
0	2.1 ± 0.4	22.1 ± 2.9
50 KIU/ml	1.5 ± 0.6	17.8 ± 2.8
100	1.4 ± 0.6	12.4 ± 3.2
200	0.8 ± 0.3 ★	6.1 ± 4.0
400	0.8 ± 0.2 ★★	5.4 ± 3.5
Non-Incubated Control	24.4 ± 6.3	36.5 ± 4.2

Results are expressed as mean ± s.e.m. of data obtained from 9 experiments.

Statistical analysis by Wilcoxon Signed Rank Test.

★ p < 0.05 } Difference from Inactive Renin Released by
 ★★ p < 0.01 } Incubated Control slices.

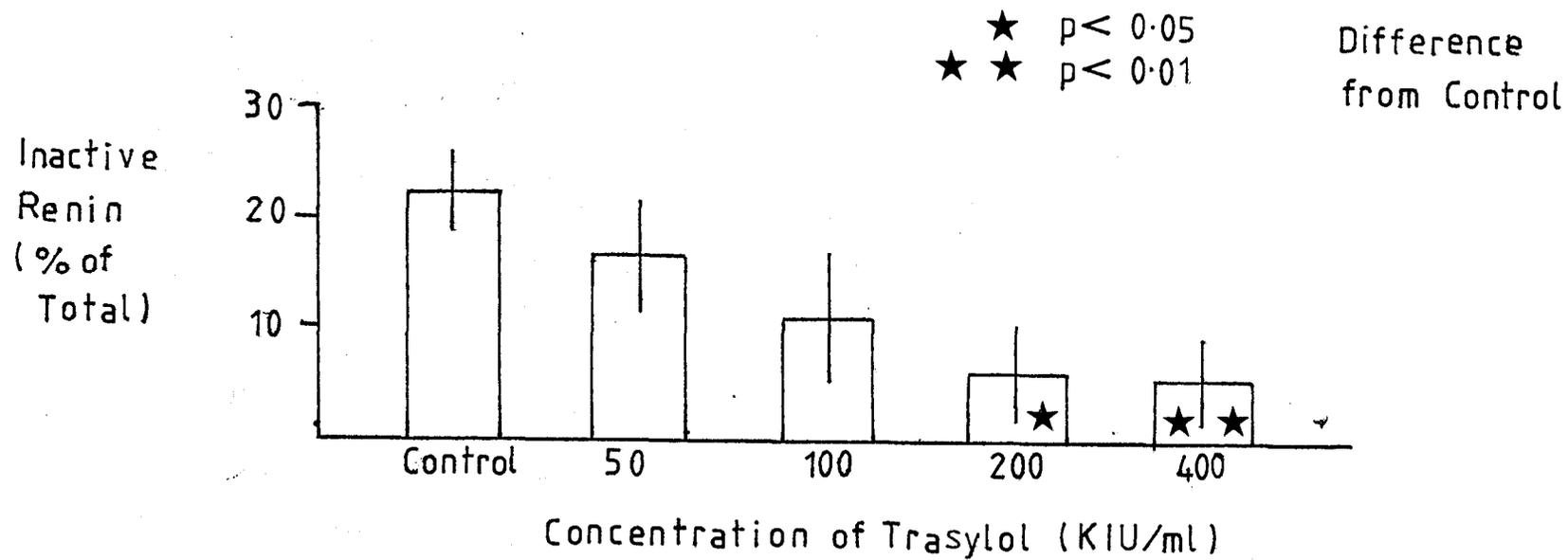


Fig.3:12 The Effect of Trasylol on Inactive Renin Release In Vitro (n=6 expts.)

SECTION IV.

The Effect of Trasylol on Acid-Activation of Inactive Renin from Kidney Cortex Slice Supernatant.

MATERIALS AND METHODS.

Kidney cortex slices were prepared as previously described (see page 106) from 6 New Zealand White rabbits. The slices were incubated for 90 minutes in unmodified Krebs'-Ringer bicarbonate buffer. At the end of the incubation period, the supernatants were pooled. Trasylol (50-100 KIU/ml) was added to 2ml aliquots of supernatant before they were dialysed. At least one sample from each experiment did not have Trasylol added to it, and this served as a control. After dialysis, samples were incubated with excess sheep renin substrate and then assayed for active and total renin concentration. (see page 59).

RESULTS.

The results which were obtained for active and total renin concentration in slice supernatants are shown in Table 3.13. There was some indication that adding high concentrations of Trasylol (200 and 400 KIU/ml) to supernatant samples resulted in an increase in active renin measured. This increase was of the order of 20-30% (see Fig. 3.13), but was not statistically significant.

Although there was a tendency for the proportion of inactive renin which could be measured to fall (see Fig. 3.14), the apparent amount of inactive renin present in samples of supernatant remained constant (see Table 3.14). At this point, it is important to note that the actual amount of active and inactive renin in each aliquot of supernatant must be the same. Therefore any changes which are observed represent the effect of Trasylol on the measurement of renin.

Table 3.13. The Effect of Trasylol on Renin Concentration in Pooled Kidney Cortex Slice Supernatants.

Trasylol Concentration	Renin Concentration (ng AI/ml/h)		Active Renin (% of Control)
	Active	Total	
0 (Control)	84.2±17.9	100.2±18.4	100
50 KIU/ml	92.6±16.8	105.2±17.1	107.7±8.6
100	87.6±15.4	110.5±14.1	108.1±9.1
200	113.7±21.4	125.5±16.1	130.8±9.4
400	108.3±19.7	119.4±18.2	121.2±8.9

Results are shown as mean ± sem of data obtained from 6 experiments.

★ p < 0.01 Increase after acidification.

KIU = Kallikrein inhibitory units.

Table 3.14. Inactive Renin in Pooled Rabbit Kidney Cortex Slice Supernatants:- Effect of Trasylol.

Trasylol Concentration	Inactive Renin	
	ng AI/ml/h	% of Total Renin
0 (Control)	16.6 ± 5.2	17.0 ± 2.8
50 KIU/ml	21.0 ± 3.6	19.1 ± 3.0
100	13.3 ± 3.7	14.8 ± 1.3
200	18.8 ± 2.6	12.6 ± 2.2
400	14.3 ± 2.8	10.2 ± 2.0

Results are shown as mean ± sem of data obtained from 6 experiments.

KIU = Kallikrein Inhibitory Unit.

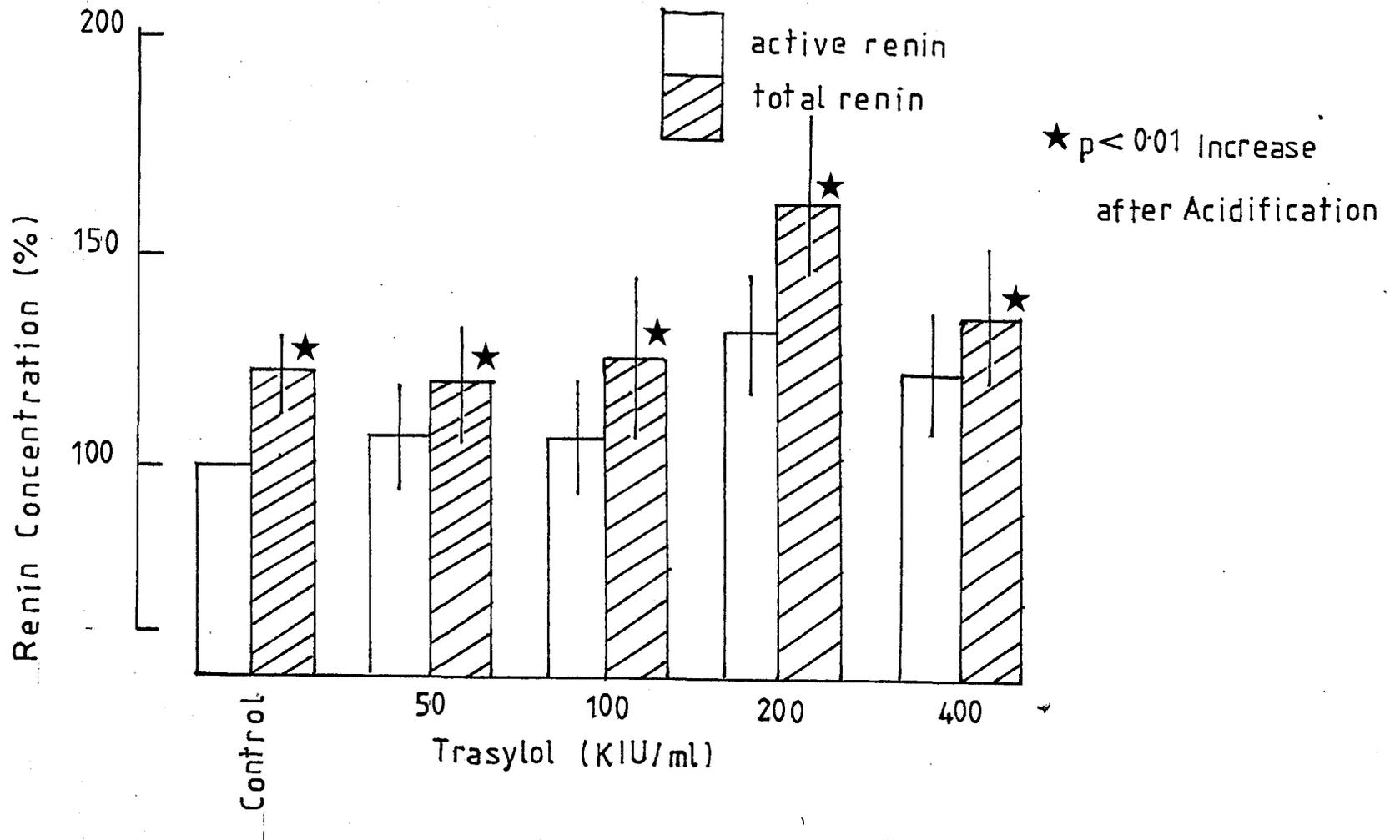
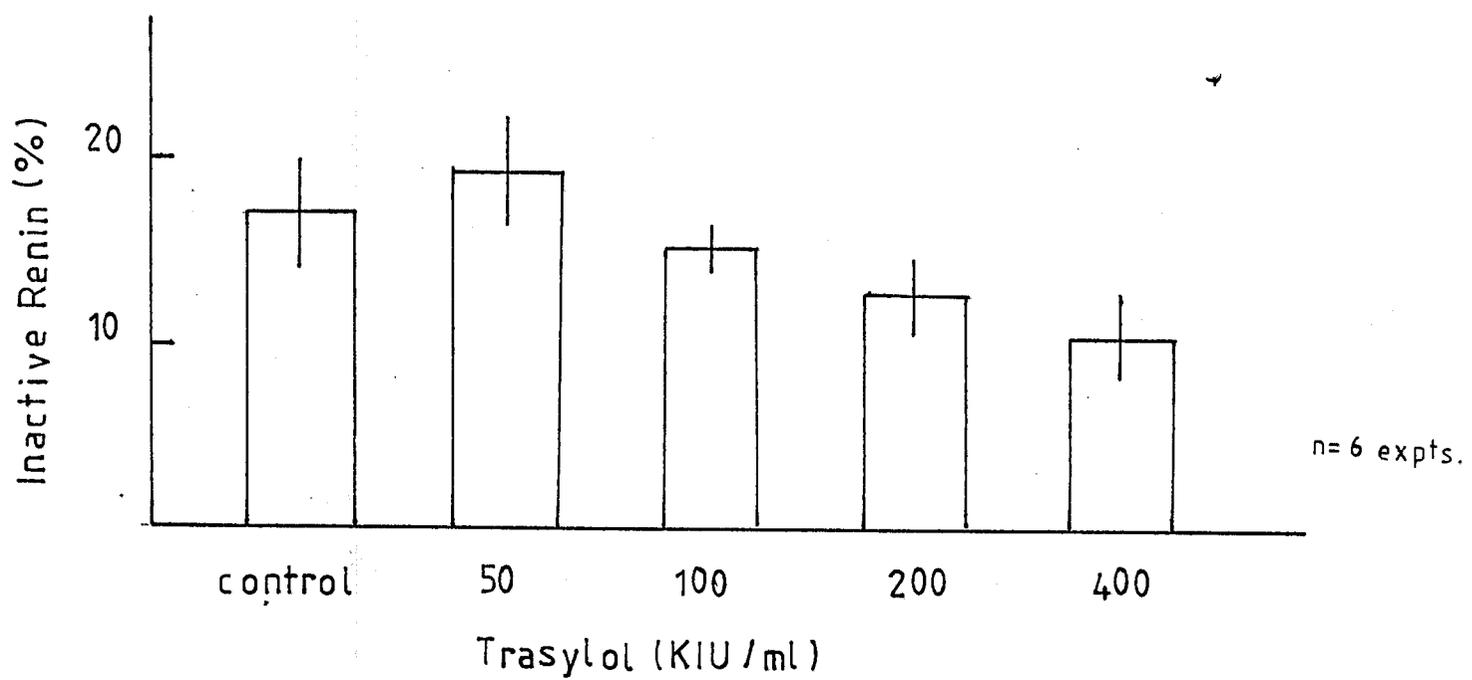


Fig 3.13 The Effect of Trasyol During Dialysis of Slice Supernatant

Fig 3-14 The Effect of Trasylol on Inactive Renin
in Slice Supernatant



These results show that Trasylol does not inhibit acid-activation of inactive renin that is released by rabbit renal cortex slices in vitro, and this finding is in marked contrast to the results that were obtained for plasma inactive renin (see Section I). In plasma samples, it was found that Trasylol inhibited acid activation of inactive renin by up to 80 %.

DISCUSSION.

Trasylol did not significantly inhibit acid activation of inactive renin released by kidney cortex slices into the incubation medium. It is interesting that when an attempt was made to measure kallikrein activity released from slices, no kallikrein activity could be measured. This has also been the experience of workers in another laboratory (I.H. Mills- personal communication). Failure to detect kallikrein could, of course be the result of several factors. This could have been caused by inactivation or breakdown of the enzyme after its release into the incubation medium. Kallikrein may be released from the kidney slices in an inactive form. On the other hand, the method which was used to measure kallikrein may not have been adequately sensitive. Perhaps the most likely possibility is that the kidney simply does not secrete kallikrein. If this is the case, how does inactive renin that is released by kidney cortex slices become measurable?

The finding that Trasylol does not inhibit acid activation of inactive renin in slice supernatants does not exclude the possibility that a serine protease which is unaffected by Trasylol could be involved. Activation of inactive renin in amniotic fluid does not require the presence of kallikrein, since prekallikrein is absent, and very little kallikrein is present in this fluid (Miller et al, 1978). Different tissues appear to contain different renin-activating enzymes. Morris & Lumbers (1972) suggested that a pepsin-like protease acts on inactive renin in amniotic fluid. A similar enzyme could be involved in activating uterine inactive renin (Jorgensen, 1979). A thiol protease is present in renal tissue which may activate inactive renin in the kidney (Inagami et al, 1977; Slater et al, 1979).

Since Trasylol did not significantly inhibit acid activation of

inactive renin in slice supernatant, the reduction in inactive renin which was observed in vitro (Section III) when high concentrations of Trasylol were present in the incubation medium can only, to a very limited extent, be explained by artificial effects on its activation. Thus inactive renin secretion in vitro is reduced by high concentrations of the protease inhibitor. This again is perhaps a surprising observation. If we imagine inter-conversion of active and inactive renin stored in the kidney, then addition of the protease inhibitor might be expected to increase the amount of inactive renin relative to the active form. This could be the case, but the mixture of renins which is released by kidney cortex slices does not reflect the relative proportions in which they are stored. As the data from in vitro experiments has shown, about 15-20 % of the total renin released by incubated control slices is in the inactive form. However, inactive renin forms about 35 % of the renin which can be extracted from non-incubated control slices (Table 3.12).

In vivo, both active and inactive renin in plasma were reduced about 90 minutes after Trasylol had been administered intravenously (Section II). The results which have been obtained in vitro in experiments III and IV suggest that the change in plasma renin could be related to accumulation of the protease inhibitor in renal tissue.

GENERAL DISCUSSION.

In the first series of experiments described in this chapter of the thesis, it was found that the protease inhibitor Trasylol, reduced the acid-activation of rabbit plasma inactive renin by up to 80 %. These results confirmed the findings of other groups who have reported that inhibitors such as Trasylol, soya bean trypsin inhibitor and benzamidine-HCl reduce acid- and cryo-activation of inactive renin in human plasma (see page 123). Previous work had suggested that a serine protease enzyme, and in particular, kallikrein, could be involved in activating renin physiologically. This was investigated by giving Trasylol intravenously to urethane-anaesthetised, saline-infused, male rabbits.

Despite a 70 % reduction in plasma kallikrein activity, plasma active and inactive renin were both unchanged. This suggests that activation of inactive renin by kallikrein is not normally taking place in plasma. Plasmin is also inhibited by Trasylol and has been reported to activate inactive renin in vitro (Osmond et al, 1978). It can also apparently be ruled out as a physiological activator of renin. Some of the less widely studied proteases which are able to activate inactive renin in vitro eg cathepsin B, cathepsin D, pepsin and thrombin (see page 8) are all unaffected by Trasylol. Thus, the possibility remains that one or more of these enzymes could be taking part in the conversion of inactive renin in plasma.

Rumpf et al (1980) have recently found that a close relationship exists between plasma levels of prekallikrein and the amount of "pro-renin" relative to active renin present in plasma during sodium loading and sodium restriction in human volunteers. Sealey et al (1977b) reported that kallikrein excretion was positively correlated with the percentage of total renin which was inactive. No such relationship was observed in the experiments which have been reported here, or in those of Rumpf et al (1980). However, although the results obtained using Trasylol lead to the conclusion that kallikrein is not the primary factor regulating the relative proportions of active and inactive renin in rabbit plasma, the possibility that local activation of inactive renin by kallikrein, or plasmin, may take place in blood vessels cannot be excluded. Such activation could take part in regulating tissue perfusion, but would not necessarily influence

significantly the amount of circulating inactive renin.

In the experiments described in this chapter of the thesis, changes in both active and inactive renin which were observed in vivo appear to be the result of changes in the rate of renin secretion from juxtaglomerular cells after accumulation of Trasylol in renal tissue. High concentrations of Trasylol reduced both active and inactive renin secretion in vitro from rabbit kidney cortex slices. It is difficult to assess whether inhibition of renin secretion was due to a direct effect of Trasylol, or its metabolites, on juxtaglomerular granular cells, or was related to the inhibition of intrarenal activity of kallikreins and kinins. This aspect of the project in particular merits further investigation.

Another interesting aspect of the work described in this chapter, is the difference between activation mechanisms for inactive renin found in plasma, and inactive renin released by kidney cortex slices. The plasma mechanism was inhibited substantially by Trasylol (see Table 3.2), whereas activation of inactive renin in the supernatant was relatively unaffected by the presence of Trasylol (see Table 3.14). This observation, which has not previously been reported, may provide a further clue to unravel the complex interactions between the renin-angiotensin system and the kallikrein-kinin system.

CHAPTER 4.

THE ROLE OF CALCIUM IN REGULATION OF RENIN SECRETION

In the following chapter of this thesis, experiments are described which form an investigation into stimulus-secretion coupling for active and inactive renin. In vivo, the two forms of renin respond independently to differing physiological stimuli (see page 11). This raises the possibility that control of secretion at the intracellular level may not be the same for the two forms of renin. This was therefore investigated by changing the ionic composition of media bathing rabbit kidney cortex slices.

It is well known that calcium plays an important part in stimulus-secretion coupling in glandular cells, and in excitation-contraction coupling in skeletal and smooth muscle cells. Granular cells in the juxtaglomerular apparatus are modified vascular muscle cells, and thus it is conceivable that calcium ions are involved in regulating the secretion of renin. It is only during the last five years or so that this aspect of the control of renin secretion has been studied. For this reason, the involvement of calcium is not nearly so well defined as, for example, the fundamental role that this cation plays in regulating pancreatic exocrine secretory activity. However, there is a growing body of evidence which suggests that calcium plays an inhibitory role in the secretion of renin (see page 26) and may be of central importance in renin release. This is particularly interesting as it appears to be one of the few known situations where calcium ions have an inhibitory action.

A mechanism analogous to renin secretion might have been the secretion of parathyroid hormone (PTH). This is controlled by a negative feedback inhibition by the level of ionic calcium in blood (Arnaud, 1978). PTH increases the renal tubular absorption of Ca and increases the rate of Ca efflux from bone. In addition, PTH reduces the level of inorganic phosphate (Pi) in plasma by enhancing its excretion in urine. The initial stage in the production of PTH is the synthesis of a polypeptide, Pre-Pro-PTH, of 115 amino acids. Either during or after synthesis, a peptide (23 amino acids) is cleaved by enzymic activity located in the reticular membranes. Pro-PTH is then transported to the Golgi apparatus, where it is converted to PTH by proteolytic removal of the amino terminal sequence of six amino acids by trypsin-like or carboxypeptidase B-like enzymes. The

calcium ionophore (A23187) selectively inhibits the conversion of Pro-PTH to PTH (Habener & Kronenberg, 1978). It is thought that the presence of calcium enhances the degradation of Pro-PTH to inactive fragments, while a reduction in the concentration of calcium increases the efficiency of conversion of Pro-PTH to PTH (Chu *et al*, 1973; Habener *et al*, 1975).

However, only 7% of the content of PTH in parathyroid cells is in the Pro-PTH form. This amount has been attributed to precursor which is in transit within the membrane systems. Furthermore, Pro-PTH cannot be detected either in plasma or in the supernatant collected after *in vitro* incubation of parathyroid gland slices. This suggests that the precursor is not normally secreted (Habener & Kronenberg, 1978). In contrast, inactive renin is present in plasma and is released into the bathing fluid during *in vitro* incubation of kidney cortex slices. The inhibition of renin secretion by calcium may thus involve a mechanism different to that in the parathyroid gland. It must be stressed however, that only active renin secretion has been previously studied. Most investigations have used the rat as an experimental model, and it is a matter for debate whether the rat kidney does in fact secrete inactive renin (see page 8).

Interconversion between active and inactive renin, or differential secretion from two separate storage pools, could provide a new explanation for previously published data. Several of the experiments reported in this section of the thesis are concerned with aspects of the involvement of calcium ions in modifying active and inactive renin secretion. Previously reported studies have shown that alterations in the concentrations of potassium and magnesium ions change the rate at which renin is released from isolated, perfused kidneys. These effects are modified by removal of calcium from the medium.

The increase in renin release which had been induced by calcium deprivation was inhibited by increasing the concentration of potassium in the perfusion fluid (Fray & Park, 1979). Park & Malvin (1978) reported that depolarization by high concentrations of extracellular potassium inhibited renin release from pig kidney cortex slices *in vitro* when calcium was present in the medium. The same concentration of potassium stimulated renin release when calcium ions were absent. Hypermagnesaemia stimulated renin release from isolated rat kidneys (Churchill & Lyons, 1976; Fray, 1977) and from the autotransplanted denervated dog kidney (Wilcox, 1978). This stimulation was

antagonised by hypercalcaemia or high concentrations of sodium in the perfusion medium.

In the experiments which are to be described, the effects of altering the concentrations of calcium, potassium, magnesium and sodium ions in the incubation media were studied. In addition, the effects of potassium, sodium and magnesium were investigated in the absence of calcium. This was done in order to determine whether the response of the renin-secreting cells to these ions is modified in the absence of calcium.

In all these experiments, rabbit renal cortex slices were prepared and incubated as described in Chapter 2 (page 106). All experiments were performed in duplicate, and a mean value for renin release (ng ang I/mg wet weight of tissue/hour of incubation) was obtained at every concentration of the ion under study, and for each animal. Data were then pooled and statistically analysed using the Wilcoxon Signed Rank Test (see page 109). Renin release was also calculated as a percentage of the control rate, that is, renin release in unmodified Krebs'-Ringer bicarbonate buffer. In order to determine the proportional changes in secretion, inactive renin has been expressed as a percentage of the total renin concentration released into the supernatant. Pooled data in this case were statistically analysed using a paired Students' t-test.

I THE EFFECT OF CALCIUM ION CONCENTRATION ON RENIN RELEASE IN VITRO

For this experiment, incubation media were prepared in which the concentration of calcium was altered. The concentration of other ions in the final solution was close to, or unchanged from, that in full Krebs'-Ringer bicarbonate buffer. In some experiments, the chelating agent ethyleneglycol-bis-(aminoethylether)-N-N¹-tetraacetic acid (EGTA, 5 m Moles/l) was added to a Ca²⁺-free medium, in order to ensure that the extracellular space was completely free of calcium. To check that EGTA per se had no effect on renin release, EGTA (5 m Moles/l) was also added to unmodified Krebs'-Ringer bicarbonate. Where necessary, one part of an 0.4% mannitol solution was

added to ensure that the osmolarity of the medium was in the range, 245 - 260 m Osm/l.

The media used in the experiment were as follows:

- 1) Ca^{2+} -free Krebs'-Ringer bicarbonate buffer containing EGTA (5 m Moles/l)
- 2) Ca^{2+} -free Krebs'-Ringer bicarbonate buffer
- 3) Krebs'-Ringer bicarbonate buffer containing 1.2 m Moles/1 Ca^{2+}
- 4) Krebs'-Ringer bicarbonate buffer containing 2.3 m Moles/1 Ca^{2+} (Control)
- 5) Krebs'-Ringer bicarbonate buffer containing 4.6 m Moles/1 Ca^{2+}
- 6) Krebs'-Ringer bicarbonate buffer containing 9.2 m Moles/1 Ca^{2+}

RESULTS

Active renin which could be extracted from homogenised kidney cortex slices (Non-Incubated Control) was 71.5 ± 17.2 ng ang I/mg/h (see Table 4.1). After acidification, renin concentration increased to 117.1 ± 25.1 ng ang I/mg/h, and thus inactive renin comprised $38.1 \pm 3.9\%$ of the total renin stored in cortex slices (see Table 4.2). In unmodified Krebs'-Ringer bicarbonate buffer (Incubated Control) where $[\text{Ca}^{2+}]$ was 2.3 m Moles/l, active renin released during the ninety-minute incubation was 8.2 ± 3.0 ng ang I/mg/h. This represents approximately 8% of the extractable renin in cortex slices. Inactive renin formed $17.4 \pm 2.8\%$ of the total renin activity released by the control slices.

TABLE 4.1 The Effect of Calcium Concentration on Renin Release
In Vitro

Incubation Medium	Renin Release (ng ang I/mg/h)		
	Active	Total	Inactive
Ca ²⁺ -depleted	23.5 ± 7.2☆☆☆	33.8 ± 10.3 *	9.8 ± 1.7 ☆
Ca ²⁺ -free	9.9 ± 2.7	15.2 ± 3.9 *	5.3 ± 0.7 ☆
1.2 m Moles/l Ca ²⁺	9.0 ± 1.3	11.8 ± 2.3 *	2.5 ± 0.6
2.3 m Moles/l ◀	8.2 ± 2.9	9.3 ± 3.3 *	0.9 ± 0.4
4.6 m Moles/l	4.4 ± 1.4	5.0 ± 1.8 ns	0.7 ± 0.5
9.2 m Moles/l	1.6 ± 0.6☆☆☆	1.8 ± 0.4 ns	0.3 ± 0.1 ☆
Non-Incubated Control	71.5 ± 17.3	117.1 ± 25.2 *	45.3 ± 7.4

Results are shown as mean ± s.e.m. for ten experiments.

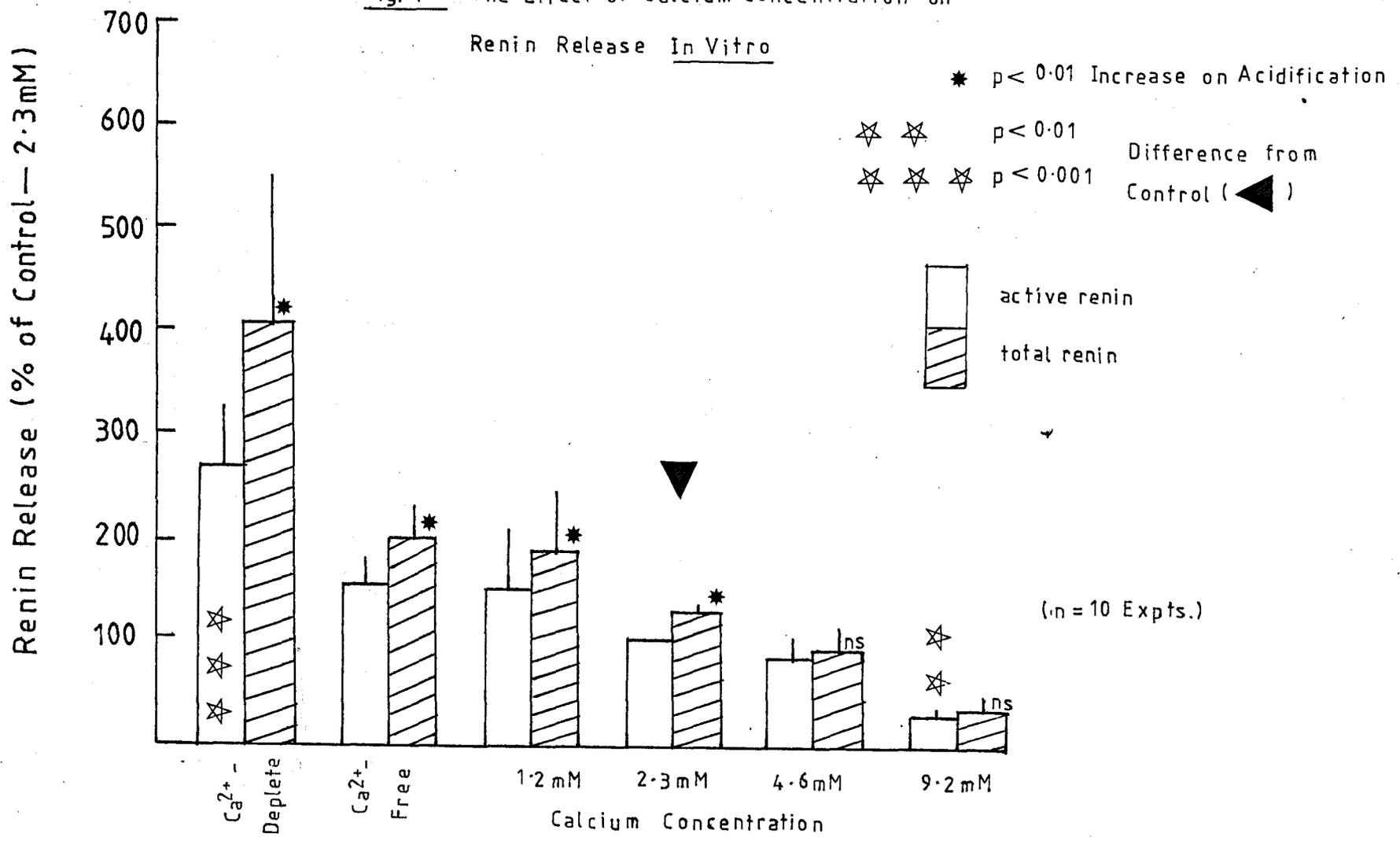
◀ Unmodified Krebs'-Ringer bicarbonate buffer.

☆☆☆ p < 0.001 Difference from * p < 0.01 Increase on
 ☆☆ p < 0.01 Control Acidification
 ☆ p < 0.05

TABLE 4.2 Changes in Renin Secretion in Response to Calcium

Incubation Medium	Active Renin (% of Control)	Inactive Renin (% of Total)
Ca ²⁺ -depleted	267.0 ± 58.4	34.9 ± 6.0
Ca ²⁺ -free	156.1 ± 23.8	34.5 ± 6.2
Ca ²⁺ 1.2 m Moles/l	149.0 ± 58.7	27.7 ± 6.5
2.3 m Moles/l ◀	100	17.4 ± 2.8
4.6 m Moles/l	82.8 ± 21.7	9.6 ± 1.5
9.2 m Moles/l	28.4 ± 5.0	8.3 ± 1.3
Non-Incubated Control		38.1 ± 3.9

Fig. 4.1 The Effect of Calcium Concentration on Renin Release In Vitro



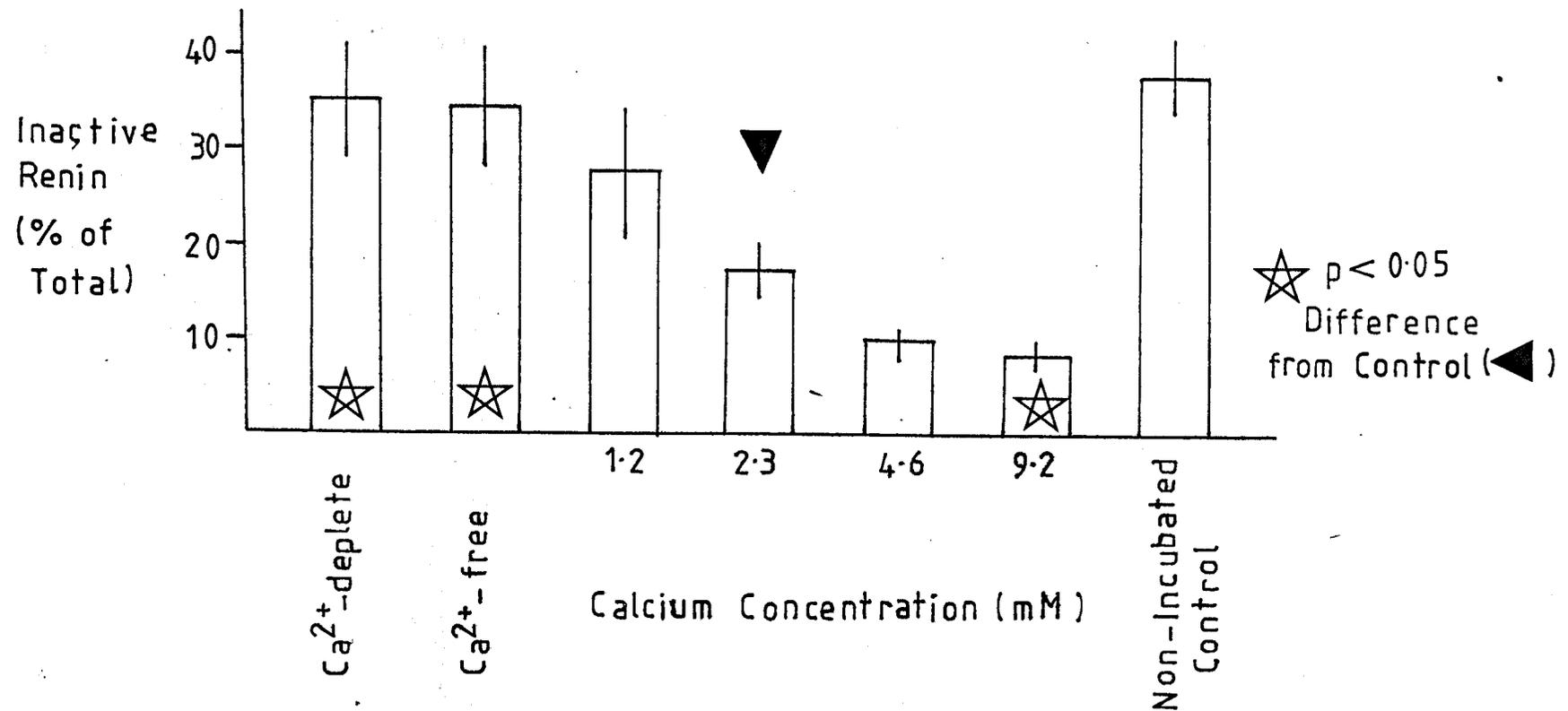


Fig. 4.2 The Effect of Calcium Concentration on Inactive Renin Release In Vitro

Active renin release increased by 50% when $[Ca^{2+}]$ was reduced to 1.2 m Moles/l. Renin secretion was further stimulated when slices were incubated for ninety minutes in Ca^{2+} -free and Ca^{2+} -depleted media. In the Ca^{2+} -free medium which contained EGTA (Ca^{2+} -depleted medium) active renin release was 23.5 ± 7.1 ng ang I/mg/h, which represents an increase of 167% compared with renin released by control slices ($p < 0.001$).

When $[Ca^{2+}]$ was increased from 2.3 to 4.6 and 9.2 m Moles/l, active renin was reduced to 4.4 ± 1.4 and 1.6 ± 0.6 ng ang I/mg/h respectively. Thus, there is an inverse linear relationship between $[Ca^{2+}]$ in the incubation medium and active renin release from kidney cortex slices ($r^2 = 0.88$). These results are shown in Fig. 4.1.

The changes in the inactive renin released by cortex slices were relatively greater than for the active form. Table 4.1 shows that a ten-fold increase in inactive renin occurred in calcium-depleted media. Fig. 4.2 shows that there is also an inverse relationship ($r^2 = 0.93$) between $[Ca^{2+}]$ and the proportion of total renin that is inactive. Inactive renin (9.8 ± 1.7 ng ang I/mg/h) formed $34.9 \pm 6.0\%$ of the total renin released by slices which had been incubated in Ca^{2+} -depleted media. This proportion is not significantly different from the proportion of inactive renin that can be extracted from non-incubated slices ($38.1 \pm 3.9\%$) and is significantly greater ($p < 0.05$) than the amount of inactive renin that was released by control slices which were incubated in unmodified Krebs'-Ringer bicarbonate buffer.

In contrast, inactive renin formed only $8.2 \pm 1.3\%$ of the total renin activity after acidification of supernatant collected from slices that had been incubated in 9.2 m Moles/l Ca^{2+} . This represents a significant reduction in the amount of inactive renin that was released ($p < 0.05$).

Addition of the chelating agent EGTA (5 m Moles/l) did not change renin release. After acidification, renin activity in the supernatant increased from 6.8 ± 3.2 to 13.3 ± 5.4 ng ang I/mg/h ($p < 0.01$). Neither active nor inactive renin ($19.8 \pm 5.5\%$ of total renin) were significantly different from control values. Thus, addition of EGTA in the presence of calcium did not itself stimulate renin secretion. Diffusion studies using sheep carotid arteries have shown that free extracellular $[Ca^{2+}]$ and $[Mg^{2+}]$ are below 10^{-11} Moles/l when EGTA is added to Ca^{2+} -free media (Keatinge, 1980). It is this very low concentration of calcium which stimulates the release of renin.

DISCUSSION .

In this experiment, high concentrations of calcium in the bathing fluid inhibited the release of active renin in vitro by rabbit kidney cortex slices. In contrast, secretion of renin was stimulated by low concentrations of Ca^{2+} , and by Ca^{2+} depletion. These findings are in agreement with those of other workers, who have reported that hypercalcaemia inhibits renin secretion (Kotchen *et al.*, 1978; Fray & Park, 1979). In addition, these results show that a similar relationship exists between calcium and inactive renin. This has not previously been reported. The amount of inactive renin which was released by slices increased when calcium was absent, and was reduced in the presence of high concentrations of calcium. Thus, high $[\text{Ca}^{2+}]$ inhibits the release of both active and inactive renin. This is in contrast to the stimulatory role of this ion in other secretory tissues.

Peart (1977) suggested that an alteration in the calcium flux across the juxtaglomerular cell membrane is the final common pathway for the control of renin secretion. In the experiments which will be described now, cell membrane characteristics have been altered by changing the extracellular concentrations of potassium, sodium and magnesium ions. These experiments were performed with concurrent changes in the calcium content of the incubation media.

II THE EFFECT OF POTASSIUM ION CONCENTRATION ON RENIN RELEASE IN VITRO

High extracellular concentrations of potassium ions tend to depolarize cell membranes. Since the external concentration of calcium is greater than that inside the cell, depolarization should cause an increase in intracellular $[\text{Ca}]$. This is because the permeability of membranes to all ions increases following depolarization.

For the following experiment, a series of incubation media, each with a different $[\text{K}^+]$ was prepared. As before, all other ions

were kept as close as possible to their concentrations in unmodified Krebs' buffer, and the osmolarity was kept near to 250 m Osm/l by the addition of 0.4% mannitol as appropriate. The range of $[K^+]$ used was as follows:

- a) 1 m Mole/l
- b) 5.7 m Moles/l (Incubated Control)
- c) 11 m Moles/l
- d) 20 m Moles/l

In the first series, $[Ca^{2+}]$ was 2.3 m Moles/l throughout. The same experiment was also carried out in Ca-depleted media, which were prepared by omitting calcium and adding EGTA (5 m Moles/l) to the buffer.

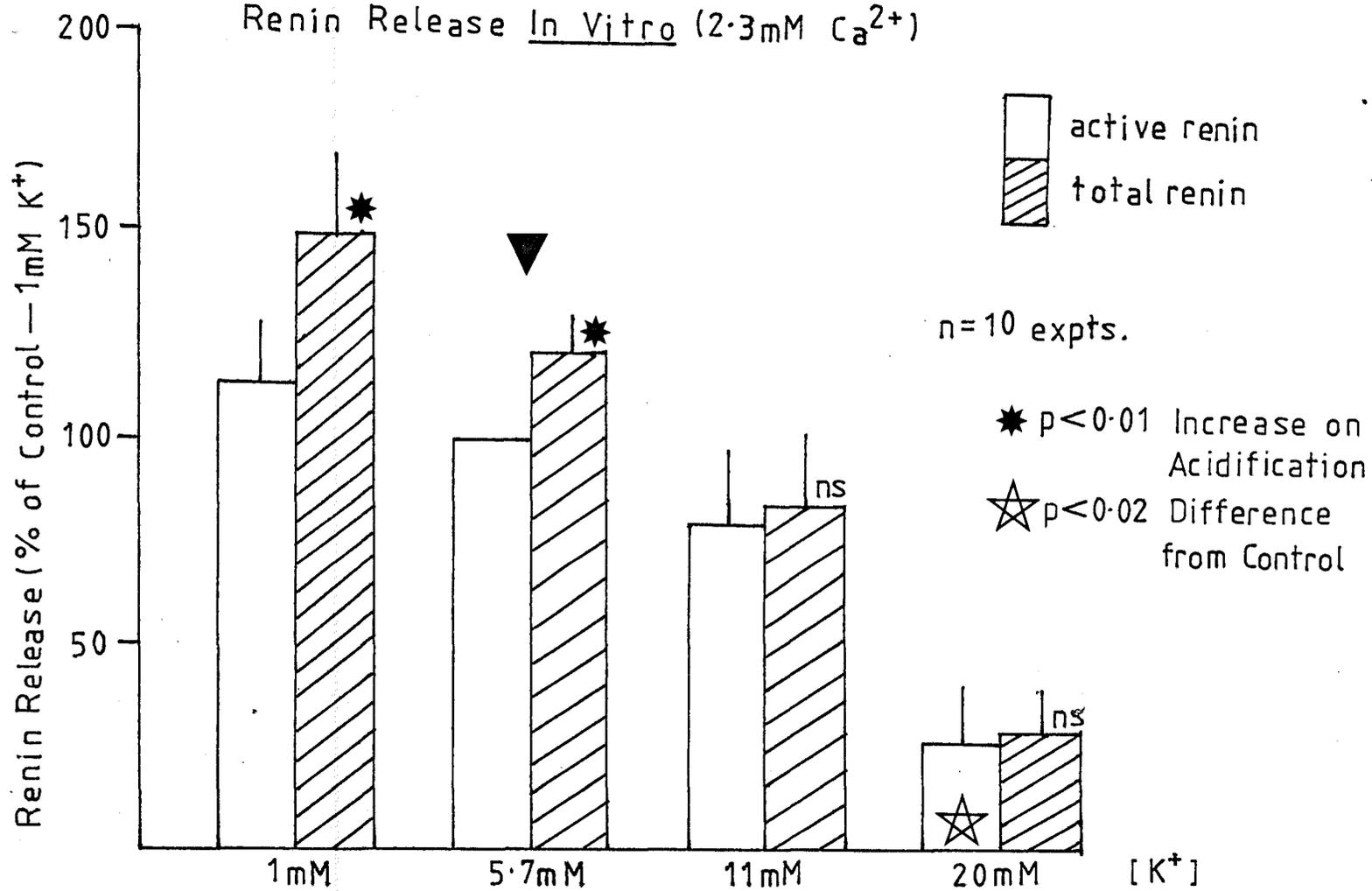
RESULTS

In incubated control slices ($[K^+] = 5.7$ m Moles/l; $[Ca^{2+}] = 2.3$ m Moles/l) active renin release in vitro was 7.9 ± 1.7 ng ang I/mg/h. After acidification, renin concentration in the supernatant increased to 9.2 ± 2.1 ng ang I/mg/h, and thus inactive renin comprised $16.5 \pm 2.0\%$ of the total renin which was released (see Tables 4.3 and 4.4).

Renin release in vitro was progressively inhibited by increasing $[K^+]$ in the incubation medium. When $[K^+]$ reached 20 m Moles/l, active renin was reduced ($p < 0.02$) to 2.1 ± 0.6 ng ang I/mg/h. This represents a 73% reduction when compared with the control rate (see Fig 4.3). Similarly, inactive renin was reduced from 1.1 ± 0.4 to 0.2 ± 0.1 ng ang I/mg/h ($p < 0.02$). This represents only $6.2 \pm 2.2\%$ of the total renin concentration in the supernatant (see Fig. 4.4).

When the concentration of potassium was reduced to 1 m Mole/l in order to hyperpolarize the granular cell membranes, active renin concentration in the supernatant did not change. However, inactive renin was increased to 2.6 ± 0.5 ng ang I/mg/h ($p < 0.02$) which represents $35.4 \pm 6.4\%$ of the total renin released by the slices. This is not significantly different from the proportion of renin that is inactive which can be extracted from non-incubated control slices.

Fig. 4.3 The Effect of Potassium Concentration on Renin Release In Vitro (2.3mM Ca²⁺)



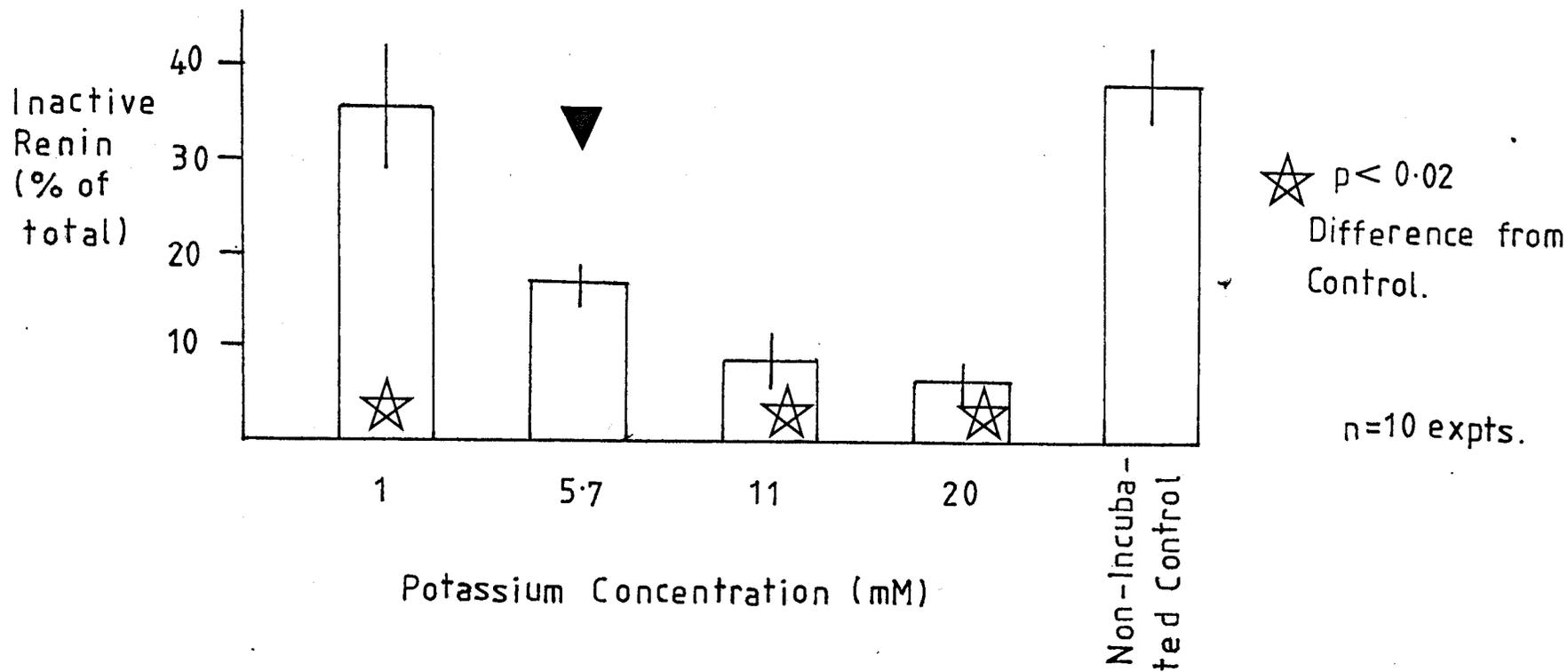


Fig. 4.4 The Effect of K^+ on Inactive Renin Release
In Vitro ($2.3 \text{ mM } Ca^{2+}$)

TABLE 4.3 The Effect of Potassium Concentration on Renin Release
In Vitro (Ca^{2+} present)

Incubation Medium	Renin Release (ng ang I/mg/h)		
	Active	Total	Inactive
[K^+] 1 m Mole/l	8.7 \pm 1.4	11.3 \pm 1.4 *	2.6 \pm 0.5 ☆
▶ 5.7 m Moles/l	7.9 \pm 1.7	9.2 \pm 2.1 *	1.1 \pm 0.4
11 m Moles/l	5.5 \pm 0.9	5.9 \pm 0.9 ns	0.3 \pm 0.1 ☆
20 m Moles/l	2.1 \pm 0.6 ☆	2.2 \pm 0.6 ns	0.2 \pm 0.1 ☆
Non-Incubated Control	71.5 \pm 17.3	117.1 \pm 25.2 *	45.3 \pm 7.4

Results are shown as mean \pm s.e.m. of data from ten experiments.

* $p < 0.01$ Increase after acidification ☆ $p < 0.02$ Difference from Control

◀ Unmodified Krebs'-Ringer bicarbonate buffer.

TABLE 4.4 Changes in Renin Secretion in Response to Potassium
(Ca^{2+} present).

Incubation Medium	Active Renin (% of Control)	Inactive Renin (% of Total)
[K^+] 1 m Mole/l	113.4 \pm 14.8	35.4 \pm 6.4
▶ 5.7 m Moles/l	100	16.5 \pm 2.0
11 m Moles/l	79.3 \pm 18.1	8.5 \pm 2.9
20 m Moles/l	26.7 \pm 28.7	6.2 \pm 2.2

Thus, when calcium ions are present (2.3 m Moles/l), high extra-cellular concentrations of potassium would tend to depolarize the granular cells, and result in a influx of calcium ions. This inhibits the secretion of both active and inactive renin. However, hyperpolarization, which could be expected to reduce the influx of calcium

stimulated the release of inactive renin only.

The results which were obtained when this experiment was repeated using Ca^{2+} -deprived media are shown in Tables 4.5 and 4.6. The inhibitory effect of high $[\text{K}^+]$ on renin release in vitro is abolished when calcium is removed. The kidney no longer responds to changes in $[\text{K}^+]$ in a dose-related manner.

TABLE 4.5 The Effect of Potassium Concentration on Renin Release in Calcium-Depleted Media (☆)

Incubation Medium	Renin Release (ng ang I/mg/h)		
	Active	Total	Inactive
2.3 m Moles/l Ca^{2+} ▶ 5.7 m Moles/l K^+	7.9 ± 1.7	9.2 ± 2.1*	1.1 ± 0.4
☆ 1 m Mole/l K^+	2.8 ± 0.8 ☆	3.5 ± 0.9*	0.8 ± 0.3
☆ 5.7 m Moles/l K^+	23.6 ± 5.7☆☆	34.1 ± 9.9*	10.1 ± 2.3 ☆
☆ 11 m Moles/l K^+	20.0 ± 4.0☆☆	34.1 ± 4.7*	12.7 ± 2.4 ☆☆
☆ 20 m Moles/l K^+	24.7 ± 2.7☆☆	38.1 ± 3.1*	13.3 ± 2.8☆☆

Results are shown as mean ± s.e.m. from ten experiments.

* $p < 0.01$ Increase after ☆ $p < 0.01$

Acidification ☆☆ $p < 0.02$

☆☆☆ $p < 0.05$

▶ Unmodified Krebs'-Ringer bicarbonate buffer

* $p < 0.05$ Difference
 * * * $p < 0.01$ from Control

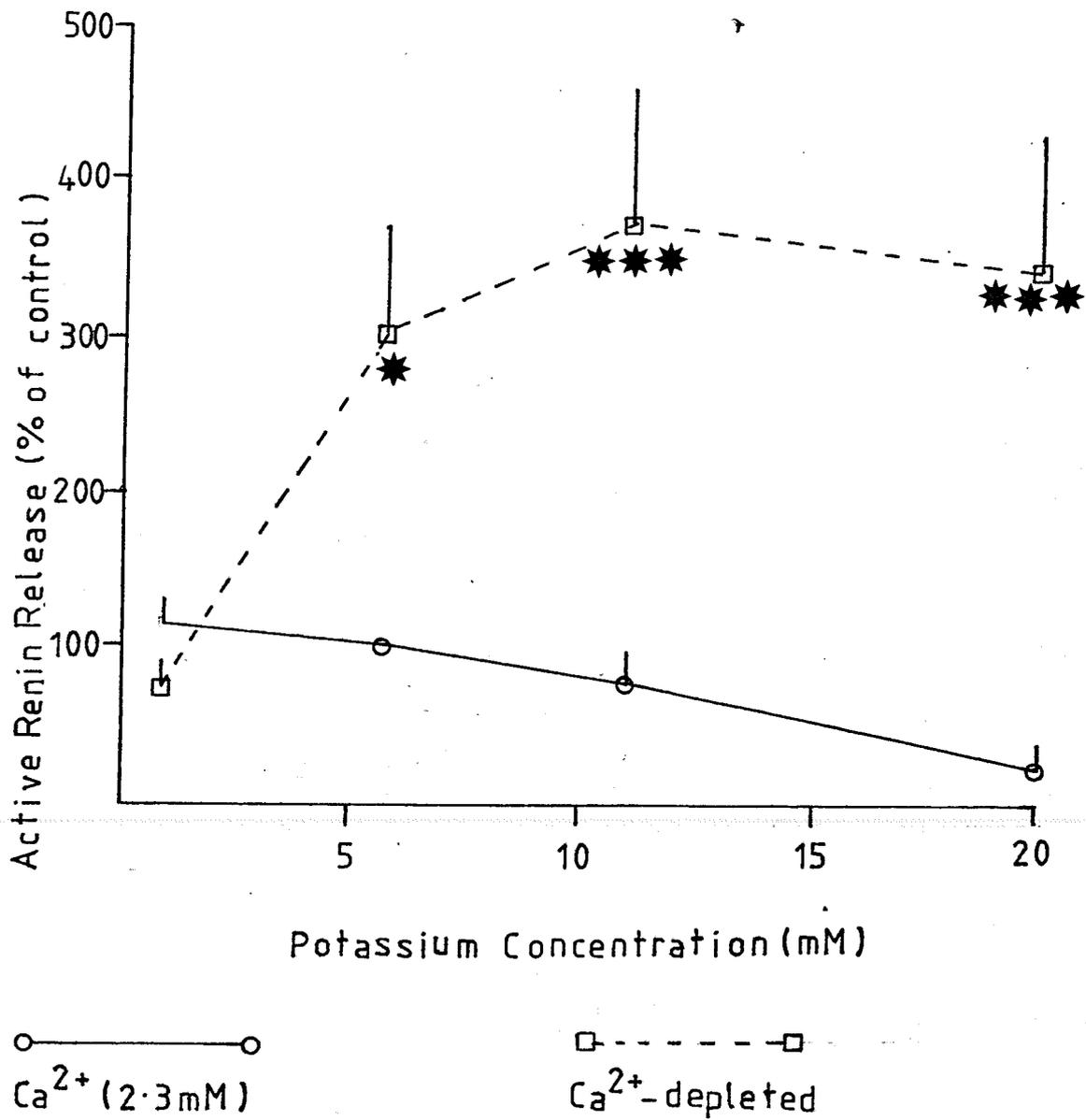


Fig. 4.5 The Effect of Extracellular K⁺ on Renin Release in the Presence and Absence of Ca²⁺

TABLE 4.6 Changes in Renin Release in Vitro in Response to Changes in Potassium (Ca^{2+} -Deprived-☆)

Incubation Medium	Active Renin (% of Control)	Inactive Renin (% of Total)
2.3 m Moles/l Ca^{2+} ▶ 5.7 m Moles/l K^+ ☆ 1 m Mole/l K^+	100	16.5 ± 2.0
☆ 5.7 m Moles/l K^+	74.1 ± 14.2	29.3 ± 3.6
☆ 11 m Moles/l K^+	300.8 ± 65.4	24.8 ± 3.1
☆ 20 m Moles/l K^+	373.7 ± 83.5	30.3 ± 4.8
☆ 20 m Moles/l K^+	341.0 ± 82.6	39.2 ± 4.8

When both normal (5.7 m Moles/l) and high concentrations (11 and 20 m Moles/l) of potassium were present in the incubation medium, active renin release was more than three-fold greater than from control kidney cortex slices (see Fig. 4.5). The stimulation of active renin secretion was accompanied by large increases in the amount of inactive renin that was released (see Fig. 4.6). Inactive renin formed 24.8 ± 3.1, 30.3 ± 4.8 and 39.2 ± 4.8% of the total renin concentration which was measured after acidification of the supernatant. These values are significantly greater ($p < 0.05$; $p < 0.02$ and $p < 0.01$ respectively) than inactive renin measured in control samples. They are not significantly different from the proportion of total extractable renin that is inactive in Non-Incubated Control slices.

In contrast to this, hyperpolarization by reducing $[\text{K}^+]$ partially antagonised the effect of calcium depletion on renin release in vitro. Active renin release from slices which were incubated in Krebs' buffer which contained 1 m Mole/l K^+ and 5 m Moles/l EGTA was significantly less ($p < 0.05$) than from control slices in vitro. The amount of inactive renin (0.8 ± 0.3 ng and 1/ng/h) which was released

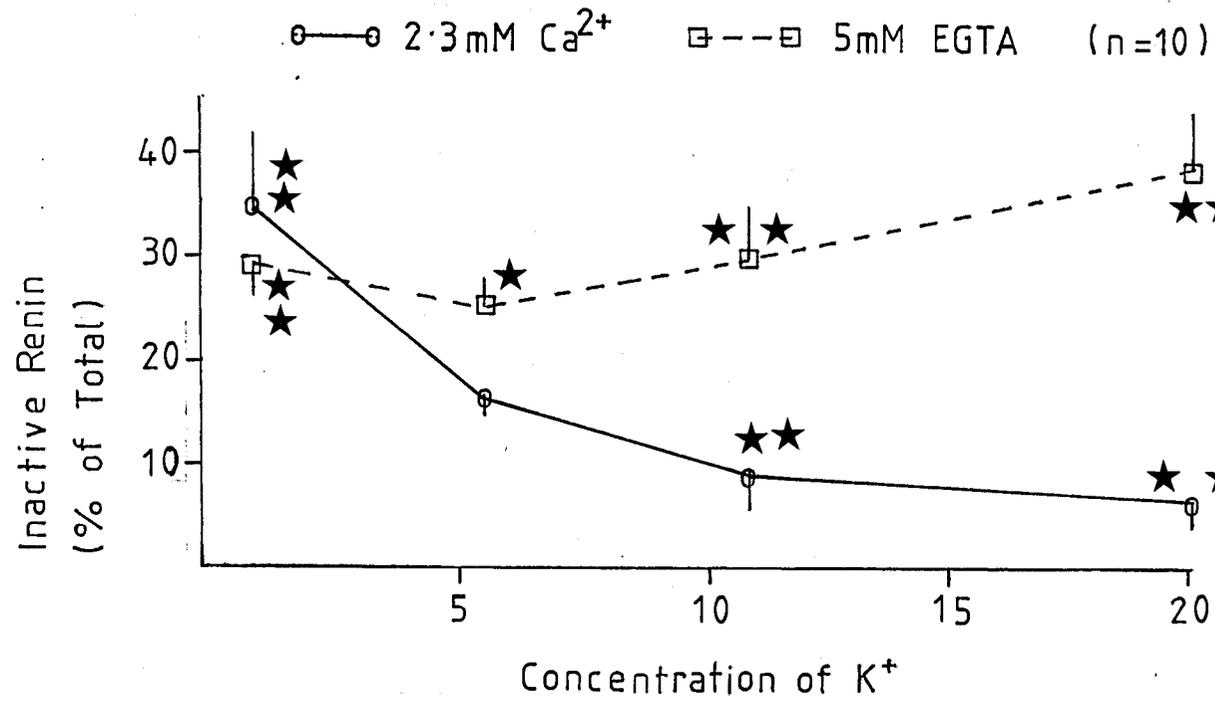


Fig. 4:6. The Effect of Extracellular [K⁺] on Inactive Renin in the Presence and Absence of Ca²⁺

was not significantly different from that released by control slices, but it formed a significantly greater ($p < 0.02$) proportion of the total renin concentration ($29.3 \pm 3.6\%$) than that released by control slices ($16.5 \pm 2.0\%$).

DISCUSSION

The results of this experiment indicate that high $[K^+]$ inhibits renin release when calcium is present in the extracellular medium, but stimulate the secretion of renin when calcium is absent. Thus, the response of renin-secreting cells to depolarization depends on the presence of calcium ions, and is modified when they are removed. Similar findings for active renin alone have been reported by Park & Malvin (1978) who used pig renal cortical slices in their experiments. Recently, Churchill (1980) and Park, Han & Fray (1981) reported that the calcium antagonists verapamil and its methoxy derivative D600, abolished the inhibitory effect of high $[K^+]$ on active renin release by rat and kidney cortex slices in vitro. A new calcium antagonist, diltiazem, which is reported to be more selective for voltage-sensitive calcium channels, also blocked the inhibitory effect of high $[K^+]$ in rat kidney cortex slices (Churchill, McDonald & Churchill, 1981). The efficiency of this blockade was enhanced by lowering the concentration of calcium in the external medium.

The results suggest, therefore, that raising $[K^+]$ inhibits renin release by increasing the intracellular concentration of calcium. When calcium is removed from the incubation medium however, depolarization results in efflux of calcium, since the intracellular concentration is greater than that in the extracellular fluid when the chelating agent, EGTA, is present. Thus, the findings for high $[K^+]$ are in agreement with the current hypothesis. The effect of hyperpolarization, however, is rather surprising.

Hyperpolarization could be expected to reduce the permeability of the cell membrane, and Ca^{2+} influx would therefore be reduced. Accordingly, this should cause an increase in the secretion of both forms of renin. In fact, only inactive renin was increased when the

concentration of potassium was reduced. When the extracellular medium was deprived of calcium, hyperpolarization did not completely restore renin secretion to control values, as might have been expected. In this case, inactive renin still formed a large proportion of the total renin concentration of the supernatant.

In a recent paper, Churchill & Churchill (1981) reported that lowering $[K^+]$ to 1 m Mole/l inhibited both basal and isoprenaline-induced renin release. They found that this effect was not antagonised by D600, and suggested that hyperpolarization reduced renin secretion by reducing the rate of Na^+/Ca^{2+} diffusion exchange, which results subsequently in an increase in the intracellular concentration of calcium. The response of renin release mechanisms in kidney cortex slices to changes in $[Na^+]$ in the presence and absence of calcium was therefore investigated.

II THE EFFECT OF SODIUM ION CONCENTRATION ON RENIN RELEASE IN VITRO

The role of sodium ions in the control of renin release is controversial (see page 25). In the rabbit, dietary sodium depletion stimulates the release of active renin, and inhibits inactive renin (Grace et al, 1979). A similar pattern is seen during furosemide diuresis (Richards et al, 1981). In vitro, rabbit kidney cortex slices respond to reduction in $[Na^+]$ by increasing active renin, and reducing inactive renin secretion (Munday et al, 1980). In the following experiment using kidney slices, the extracellular concentration of sodium was altered by replacing NaCl in Krebs'-Ringer bicarbonate buffer with an equivalent concentration of Choline Chloride (2.16%). As in the previous experiment, the protocol was repeated using Ca^{2+} -depleted media in order to determine whether the effect of low $[Na^+]$ on renin release was modified in the absence of calcium. The incubation media which were used in the initial experiment were as follows:

- 1) Krebs'-Ringer bicarbonate buffer containing 133 m Moles/l Na^+ (Incubated Control).
- 2) Krebs'-Ringer bicarbonate buffer containing 106 m Moles/l Na^+
- 3) Krebs'-Ringer bicarbonate buffer containing 78 m Moles/l Na^+
- 4) Krebs'-Ringer bicarbonate buffer containing 57 m Moles/l Na^+
- 5) Krebs'-Ringer bicarbonate buffer containing 23 m Moles/l Na^+

RESULTS

Renin release by incubated control slices ($[\text{Na}^+] = 133$ m Moles/l) was 7.3 ± 1.8 ng ang I/mg/h during the ninety minute incubation (see Table 4.7). After the acidification step, renin concentration increased to 10.4 ± 2.8 ng ang I/mg/h, and so inactive renin comprised $18.7 \pm 3.9\%$ of the total renin released into the supernatant (Table 4.8). When $[\text{Na}^+]$ was reduced, active renin release was significantly increased ($p < 0.01$) to 14.2 ± 4.4 ng ang I/mg/h. This represents an increase of nearly 100%, when compared with incubated control slices (see Fig. 4.7). The relationship between sodium concentration and active renin release is linear, and has a correlation coefficient (r^2) of 0.81.

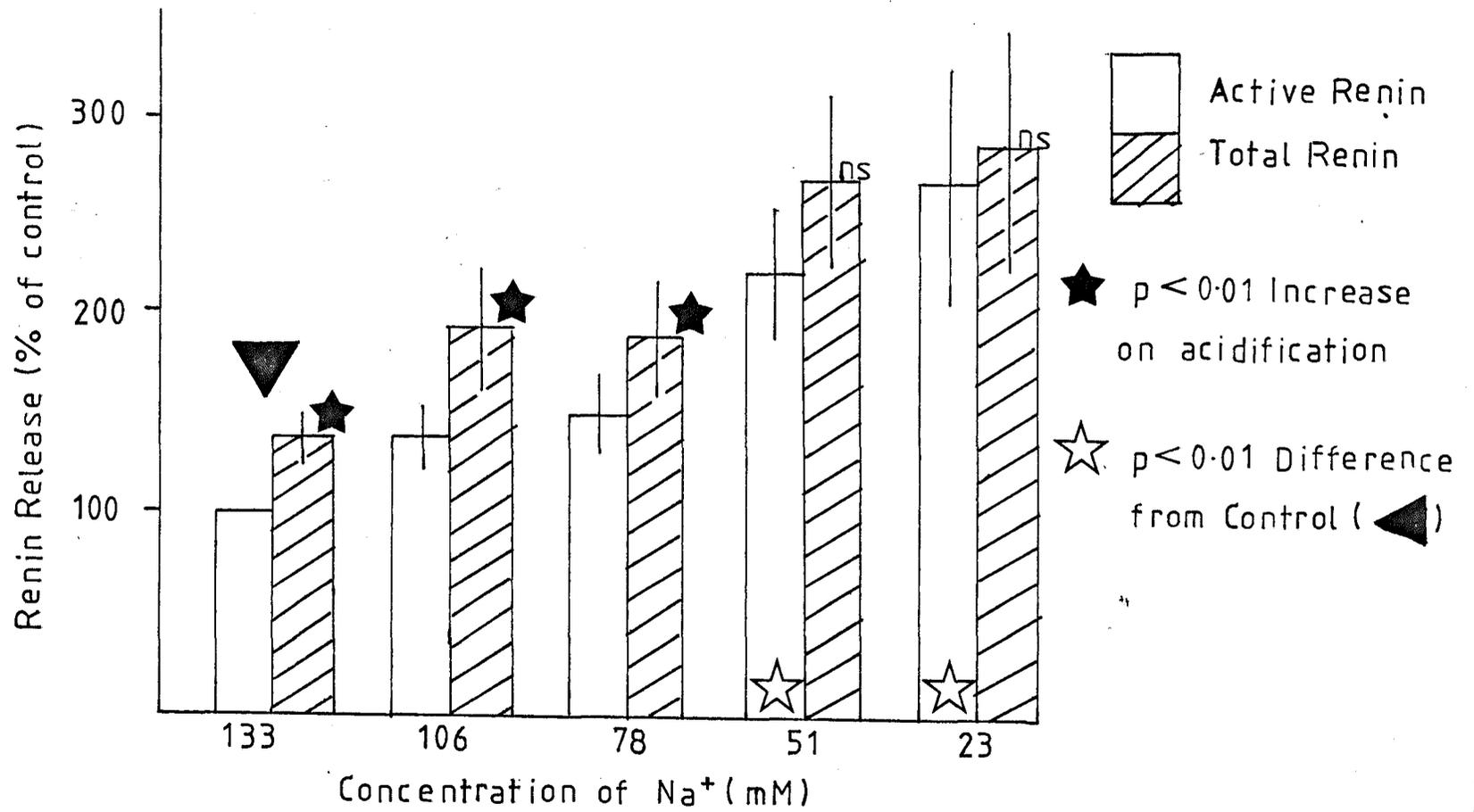


Fig. 4:7 The Effect of Changing [Na⁺] on Renin Release.

TABLE 4.7 The Effect of Sodium Concentration on Renin Release
In Vitro (Ca^{2+} present).

Incubation Medium [Na^+]	Renin Release (ng ang I/mg/h)		
	Active	Total	Inactive
▶ 133 m Moles/l	7.3 ± 1.8	10.4 ± 2.8 *	2.5 ± 0.8
106 m Moles/l	11.0 ± 3.7	15.1 ± 6.0 *	2.9 ± 0.9
78 m Moles/l	11.2 ± 2.5	13.2 ± 2.9 *	1.8 ± 0.6
57 m Moles/l	14.2 ± 4.4 ☆	14.9 ± 2.5 ns	0.9 ± 0.3 ☆
23 m Moles/l	13.3 ± 2.3 ☆	14.0 ± 2.5 ns	0.5 ± 0.2 ☆☆

Results shown as mean ± s.e.m. of data from eight experiments.

* $p < 0.01$ Increase on Acidification ☆ $p < 0.01$ Difference
 ☆ ☆ $p < 0.001$ from Control

▶ Unmodified Krebs'-Ringer bicarbonate buffer.

The amount of inactive renin which was released by the slices into the incubation medium was significantly reduced ($p < 0.001$) when [Na^+] was reduced from 133 to 23 m Moles/l (see Table 4.7). There was no longer a significant increase in the renin concentration after acid dialysis, when the slices had been incubated in low Na buffer, since inactive renin formed only $3.9 \pm 2.9\%$ of the total renin that was released (Fig. 4.6). Thus, a positive linear relationship ($r^2 = 0.92$) exists between sodium concentration, and inactive renin released into the bathing fluid during the in vitro incubation of rabbit kidney cortex slices.

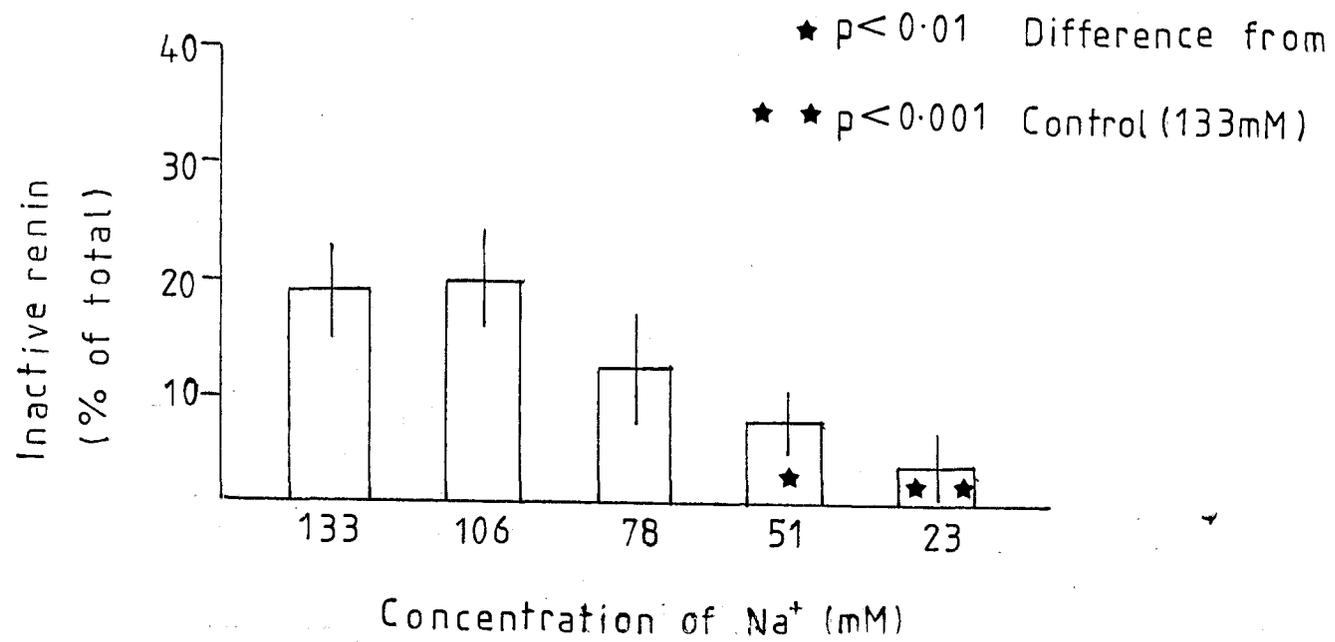


Fig.4:8 · The Effect of Changing $[\text{Na}^+]$ on Inactive Renin Release.

TABLE 4.8 Changes in Renin Secretion in Response to changing
 $[Na^+]$ (Ca^{2+} present)

Incubation Medium $[Na^+]$	Active Renin (% of Control)	Inactive Renin (% of Total)
▶ 133 m Moles/l	100	18.7 ± 3.9
106 m Moles/l	138.3 ± 14.8	19.8 ± 4.3
78 m Moles/l	150.5 ± 17.8	12.3 ± 4.8
51 m Moles/l	220.2 ± 32.4	7.7 ± 3.0
23 m Moles/l	186.4 ± 57.9	3.9 ± 2.9

Results expressed as mean ± s.e.m. of eight experiments.

▶ Unmodified Krebs'-Ringer bicarbonate buffer.

These results show that when calcium is present in the incubation medium, lowering $[Na^+]$ stimulates the release of active renin, but reduces inactive renin. They provide further evidence to suggest that active and inactive renin release may be independently controlled. However, from the data which are shown in Tables 4.9 and 4.10, it is clear that, like the effect of potassium, the response of renin-secreting cells to low $[Na^+]$ is modified when they are deprived of calcium.

TABLE 4.9 The Effect of changing [Na⁺] on Renin Release in Ca²⁺-depleted Media (☆)

Incubation Medium	Renin Release (ng ang I/ng/h)		
	Active	Total	Inactive
133 m Moles/l Na ⁺ 2.3 m Moles/l Ca ²⁺ ◀	7.3 ± 1.8	10.4 ± 2.8 ★	2.5 ± 0.8
133 m Moles/l Na ⁺ ☆	15.2 ± 1.3 ☆	20.8 ± 2.6 ★	5.4 ± 1.2 ☆
106 m Moles/l Na ⁺ ☆	14.7 ± 1.9 ☆	18.8 ± 2.5 ★	4.2 ± 0.7 ☆
78 m Moles/l Na ⁺ ☆	10.2 ± 2.2 ☆	11.8 ± 2.3 ★	1.9 ± 1.4
51 m Moles/l Na ⁺ ☆	9.0 ± 2.0	11.8 ± 2.3 ★	2.7 ± 0.3
23 m Moles/l Na ⁺ ☆	9.9 ± 2.9	13.9 ± 4.7 ★	2.6 ± 1.3

Results are shown as mean ± s.e.m. of data from eight experiments.

☆ p < 0.01

Difference
from Control

★ p < 0.01

Increase on
Acidification

◀ Unmodified Krebs' Ringer bicarbonate buffer.

TABLE 4.10 Changes in Renin Secretion in Response to changing $[Na^+]$ (Ca^{2+} -deprived ★)

Incubation Medium Na	Active Renin (% of Control)	Inactive Renin (% of Total)
133 m Moles/l Na^+ 2.3 m Moles/l Ca^{2+})	100	18.7 ± 3.9
133 m Moles/l Na^+ ★	190.0 ± 12.4	34.3 ± 5.8
106 m Moles/l Na^+ ★	199.5 ± 38.6	25.3 ± 6.5
78 m Moles/l Na^+ ★	171.6 ± 49.2	25.1 ± 8.0
51 m Moles/l Na^+ ★	166.1 ± 49.2	24.4 ± 6.0
23 m Moles/l Na^+ ★	171.2 ± 37.2	16.2 ± 7.3 ★

Data expressed as mean ± s.e.m. of results from eight experiments.

★ $p < 0.05$ Difference from Response to Ca^{2+} -depletion.

As observed in previous experiments, calcium depletion, which is achieved by incubating the cortex slices in Ca^{2+} -free media, to which 5 m Moles/l EGTA was added, caused a significant increase in the release of active ($p < 0.01$) and inactive renin ($p < 0.01$) into the bathing fluid (see Figs. 4.9 and 4.10).

Low $[Na^+]$ partially antagonised this stimulation. When $[Na^+]$ was 51 and 23 m Moles/l in the Ca^{2+} -depleted medium, active renin release was 9.0 ± 2.0 and 9.9 ± 2.9 ng ang I/mg/h during the incubation period. These values are not significantly different from active renin released by slices in unmodified Krebs'-Ringer buffer (7.3 ± 1.8 ng ang I/mg/h). However, this reduction did not represent a significant change in the response to calcium depletion. An alternative way of viewing these data, is to say that in the absence of calcium, the correlation between active renin release and $[Na^+]$ is reversed. That is, to reduce $[Na^+]$ inhibits active renin release in

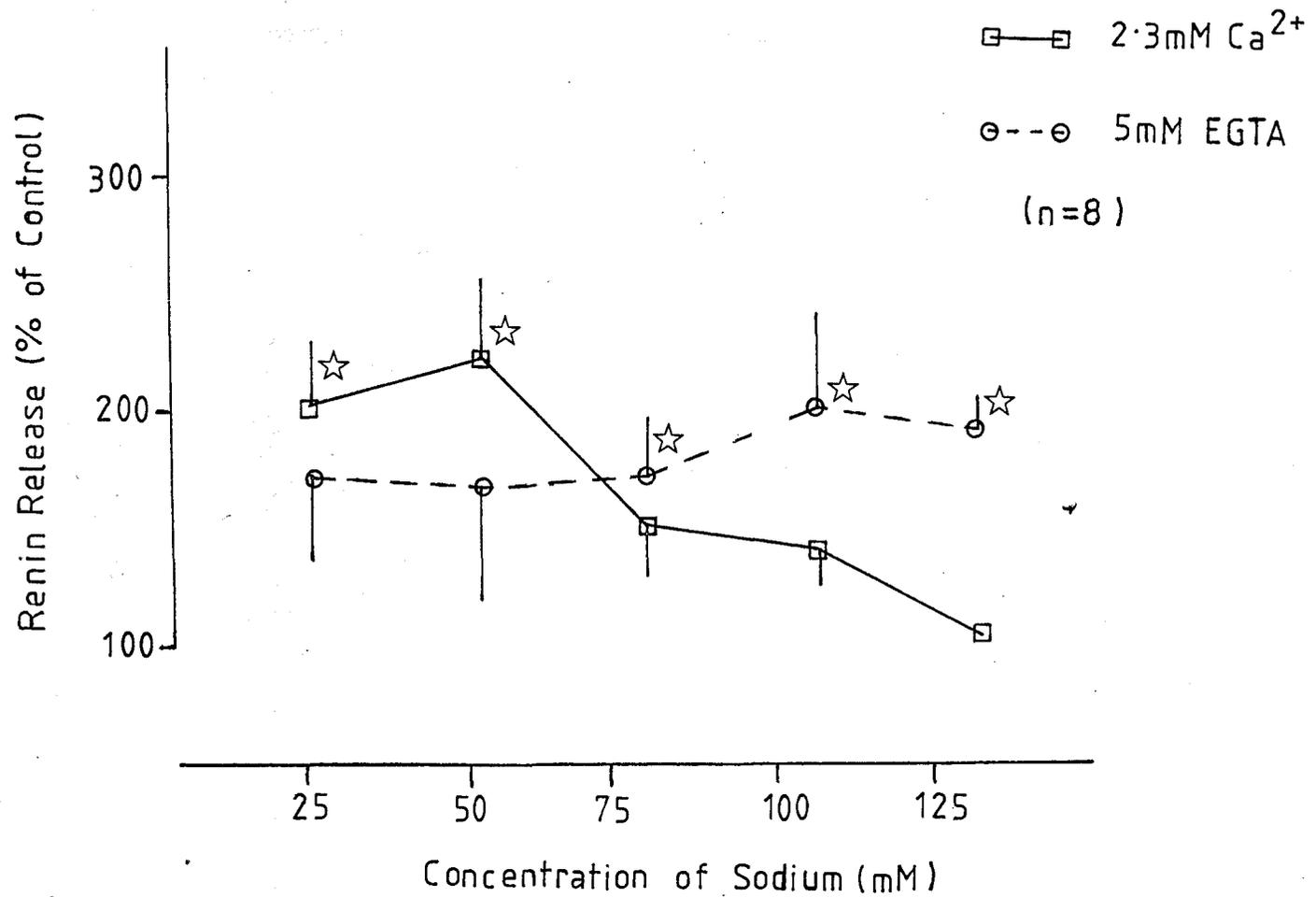


Fig. 4:9 The Effect of Changing [Na⁺] on Active Renin Release in the Presence and Absence of Ca²⁺

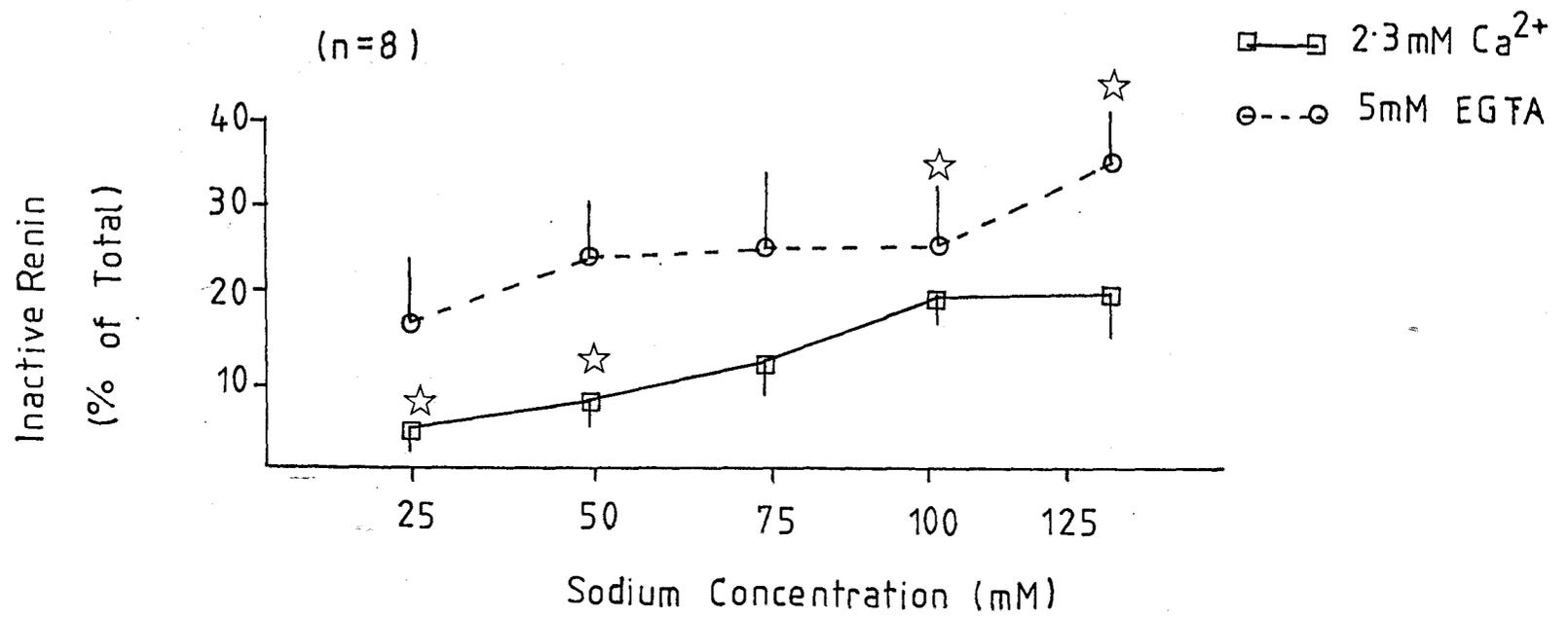


Fig. 4:10 The Effect of Changing $[\text{Na}^+]$ on Inactive Renin Release in the Presence and Absence of Ca^{2+}

the absence of calcium.

Reducing $[Na^+]$ restored inactive renin release to control values in the Ca^{2+} -depleted medium. Inactive renin formed $34.3 \pm 5.8\%$ of total renin in the supernatant collected after slices had been incubated in the Ca^{2+} -depleted medium (133 m Moles/l Na^+) but comprised only $16.2 \pm 7.3\%$ of the total renin activity when $[Na^+]$ was 23 m Moles/l. This represents a significant reduction ($p < 0.05$) in the response to calcium deprivation. The correlation between $[Na^+]$ and inactive renin release was not reversed by Ca^{2+} -depletion.

DISCUSSION

The results of this experiment indicate that the effect of reducing $[Na^+]$ on renin release from rabbit kidney cortex slices, is modified by removal of calcium. A similar change in the response to high potassium concentrations was also observed in the previous experiment (see page 164).

Reducing $[Na^+]$ in those media in which slices were incubated antagonised the stimulatory effect of low $[Ca^{2+}]$ on active renin secretion. Thus, in contrast to its stimulatory role when calcium was present, low $[Na^+]$ now inhibited the release of renin. Lowering $[Na^+]$ tended to reduce inactive renin secretion, both in the presence and absence of calcium. However, while inactive renin was reduced from 19 to 4% of total renin when calcium present, it fell from 34% to 16% in its absence.

The significance of these different responses to sodium is not clear. Reduced $[Na^+]$ inhibits the release of active renin from rat kidney cortex slices (Capponi & Valloton, 1976; Lyons & Churchill, 1974 & 1975). In contrast, the results reported here and those of Munday *et al* (1980) show that low $[Na^+]$ stimulates active renin release from rabbit kidney cortex slices. A similar relationship has been reported by Michelakis (1971) who used canine kidney cortex slices for his experiments.

Cuabain inhibits the membrane Na^+/K^+ ATP-ase, and tends therefore, to equalise the intracellular and extracellular $[Na^+]$. This

drug inhibits the release of renin from rat (Lyons, 1980; Park & Malvin, 1978) and dog (Park et al, 1981) kidney cortex slices. Some of these findings appear to be contradictory. When the extracellular $[Na^+]$ is reduced, the intracellular $[Na^+]$ would also be expected to fall, since the electrochemical gradient for sodium is diminished. On the other hand, the intracellular $[Na^+]$ should increase after treatment with ouabain. Active renin release from rat kidney cortex slices in vitro is reduced under both of these conditions. In smooth muscle, a Na^+/Ca^{2+} exchange mechanism may exist (Reuter & Seitz, 1968). Increasing the activity of the membrane Na^+/K^+ ATP-ase in turn appears to enhance the efflux of calcium in exchange for sodium influx. Although it is not yet clear whether a similar exchange takes place across the JG cell membrane, it is possible that some of the effects of sodium on renin secretion in the rat could be explained by alterations in these transmembrane fluxes. When $[Na^+]$ in the extracellular fluid is reduced, the rate of influx of this ion is also reduced. This in turn reduces the rate of Ca^{2+} efflux, and the subsequent increase in intracellular $[Ca^{2+}]$ inhibits the secretion of renin. Similarly, ouabain reduces the rate of sodium influx, leading therefore to an increase in the level of intracellular calcium.

A Na^+/Ca^{2+} exchange mechanism, however, cannot fully explain the effect of low $[Na^+]$ in the rabbit, where only inactive renin secretion is reduced. Indeed, a recent paper by Aaronson & Van Breemen (1981) casts some doubt on the existence of this exchange mechanism. They reported that although $[Ca^{2+}]$ in smooth muscle cells was increased by removal of sodium, this increase did not occur when the electrochemical gradient for sodium was diminished by ouabain. These workers concluded that the Na gradient was not per se responsible for controlling the cellular Ca content. Clearly, there is much that is still not understood regarding the relationship between movements of sodium and calcium across plasma membranes.

Unfortunately, there have to date been no reports concerning the effect of ouabain on renin release from the rabbit kidney. When calcium is present, lowering $[Na^+]$ stimulates the release of the active form of renin in this species. This suggests that a different mechanism may mediate the effect of sodium in the rabbit. In addition, reducing $[Na^+]$ inhibits the secretion of inactive renin,

which suggests that the release of this form of renin may be controlled by a sodium-sensitive step.

The secretion of both forms of renin is enhanced by calcium deprivation. The results of this experiment suggest that lowering $[Na^+]$ reduces this response. Removal of calcium from the extracellular fluid may itself alter the permeability of the granular cell membrane, since calcium ions are thought to stabilise the plasma membrane by increasing the electrical potential gradient for sodium (Constantin, 1968). Thus, when calcium is removed, cells tend to become loaded with sodium. This effect will be diminished when $[Na^+]$ in the extracellular fluid is reduced. The possibility remains, therefore, that the stimulatory effect of calcium deprivation on renin secretion could be mediated by the subsequent changes in intracellular sodium concentration.

How can the conflicting reports in the literature concerning inhibition or stimulation of renin secretion by reduced $[Na^+]$ be reconciled? Two observations may be pertinent. First, in the rabbit, the relation between $[Na^+]$ and active renin secretion can be reversed by removal of Ca^{2+} from the incubation medium. The second observation concerns the species used in previous studies. In the rat, which may not secrete any inactive renin (see page 8), reduction of $[Na^+]$ in the medium bathing *in vitro* preparations results in an inhibition of renin release. In species which do secrete inactive renin however, the dog and the rabbit, the reverse correlation is found for active renin release. However, the pattern of response for inactive renin secretion in the only species which has been studied, the rabbit, corresponds to that of the single form of renin in the rat. This poses many questions which will need to be investigated in the future.

IV THE EFFECT OF MAGNESIUM ION CONCENTRATION ON RENIN RELEASE IN VITRO

Interactions between calcium and magnesium ions have been reported in many different circumstances. Magnesium ions can antag-

onise the action of calcium ions within the cell (Rahwan, Barowitz & Miya, 1973) and can inhibit calcium induced degranulation of mast cells (Kanno *et al.*, 1973). In addition, magnesium ions are also able to carry inward current during slow discharges of vascular smooth muscle (Keatinge & Harman, 1980). Furthermore, high extracellular concentrations of magnesium hyperpolarize cell membranes, and inhibit calcium influx (Altura & Altura, 1971). Therefore, the effect of magnesium ions on renin secretion *in vitro* was investigated, both in the presence and absence of extracellular Ca^{2+} .

Pairs of rabbit kidney cortex slices (see page 106) were incubated in one of the following media for ninety minutes:

- 1) Mg^{2+} -free Krebs'-Ringer bicarbonate buffer.
- 2) Krebs'-Ringer bicarbonate buffer containing Mg^{2+} 1.1 m Moles/l. (Incubated Control).
- 3) Krebs'-Ringer bicarbonate buffer containing Mg^{2+} 2.2 m Moles/l.
- 4) Krebs'-Ringer bicarbonate buffer containing Mg^{2+} 4.4 m Moles/l.
- 5) Krebs'-Ringer bicarbonate buffer containing Mg^{2+} 8.8 m Moles/l.

Those media which contained high concentrations of Mg^{2+} were prepared using a stock solution of MgCl_2 (1.56% w/v) in order that the concentration of SO_4^{2-} remained unchanged (1.1 m Moles/l) throughout the experiment. The osmolality of the media was maintained by the addition of 0.4% mannitol solution as appropriate. As in previous experiments, the protocol was repeated using Ca^{2+} -free media to which EGTA (5 m Moles/l) had been added.

RESULTS

Renin concentration in the supernatant collected from incubated control slices ($[\text{Mg}^{2+}] = 1.1$ m Moles/l) was 8.8 ± 1.2 ng ang I/mg of tissue/h, and this increased ($p < 0.01$) to 11.4 ± 1.6 ng ang I/mg/h after acidification.

Active renin release was reduced to 6.2 ± 1.8 ng ang I/mg/h when magnesium ions were absent from the incubation medium. This fall was of the order of 30% (see Table 4.12) and was statistically significant ($p < 0.01$). Fig. 4.11 shows that there was a small increase in active renin released by the slices which had been incubated in 4.4 or 8.8 m Moles/l Mg^{2+} . However, the increases were not statistically significant.

At the suggestion of Dr. J. C. S. Fray, a later experiment included flasks containing Krebs' buffer with 20 m Moles/l Mg^{2+} . Dr. Fray had found that in his experiments with the isolated, perfused rat kidney, renin secretion did not respond to Mg^{2+} until its concentration was greater than 10 m Moles/l. This high concentration of magnesium had not been included in the original series of experiments as it was considered to be very unphysiological. However, active renin release was not significantly altered, even when the incubation medium contained 20 m Moles/l Mg^{2+} .

Inactive renin formed $21.6 \pm 3.1\%$ of the total renin activity released by incubated control slices (see Fig. 4.12). No statistically significant changes were observed either in the absolute amount of inactive renin that was released (Table 4.11) or in the proportion of total renin which was inactive.

TABLE 4.11 The Effect of Magnesium Concentration on Renin Release In Vitro (Ca^{2+} present).

Incubation Medium	Renin Release (ng ang I/mg/h)		
	Active	Total	Inactive
Mg^{2+} -free	6.2 ± 1.8 ★	8.2 ± 1.2 *	2.1 ± 0.6
Mg^{2+} 1.1 m Moles/l ◀	8.8 ± 1.2	11.4 ± 1.6 *	2.4 ± 0.5
Mg^{2+} 2.2 m Moles/l	6.9 ± 1.0	8.6 ± 1.2 *	1.9 ± 0.4
Mg^{2+} 4.4 m Moles/l	9.2 ± 1.2	12.1 ± 1.6 *	2.7 ± 0.8
Mg^{2+} 8.8 m Moles/l	9.2 ± 1.3	11.7 ± 1.7 *	2.4 ± 0.6
Mg^{2+} 20 m Moles/l	8.6 ± 2.1	10.3 ± 2.4 *	1.8 ± 0.5
Non-Incubated Control	48.3 ± 8.8	76.7 ± 13.8 *	31.5 ± 4.2

Table 4.11 Key

- ▶ Unmodified Krebs'-Ringer bicarbonate buffer.
 ☆ $p < 0.01$ Difference from Control
 * $p < 0.01$ Increase after Acidification.

TABLE 4.12 The Response of Renin Release in vitro to Changes in $[Mg^{2+}]$ (Ca^{2+} present).

Incubation Medium	Active Renin (% of Control)	Inactive Renin (% of Total)
Mg^{2+} -free	76.8 ± 5.0	21.4 ± 3.7
Mg^{2+} 1.1 m Moles/l ▶	100	21.6 ± 3.1
Mg^{2+} 2.2 m Moles/l	97.1 ± 1.8	26.5 ± 4.4
Mg^{2+} 4.4 m Moles/l	106.5 ± 15.5	19.4 ± 5.1
Mg^{2+} 8.8 m Moles/l	107.4 ± 11.5	24.4 ± 5.4
Mg^{2+} 20 m Moles/l	106.2 ± 14.2	14.9 ± 4.2

The results of this experiment are in contrast to the findings of other workers, who have reported that magnesium ions stimulate the release of active renin from dog (Churchill & Lyons, 1976; Wilcox, 1978) and rat kidneys (Etienne & Fray, 1979) by hyperpolarizing the JG cell membrane.

Table 4.13 shows the effect of changing $[Mg^{2+}]$ in a calcium deprived medium. As was found in previous experiments, active renin release increased ($p < 0.02$) to 10.8 ± 0.5 ng ang I/mg/h in the absence of calcium (1.1 m Moles/l Mg^{2+} ; EGTA 5 m Moles/l). Inactive renin also increased ($p < 0.01$) to 3.9 ± 0.4 ng ang I/mg/h, which represents $30.8 \pm 4.7\%$ of the total renin that was released (Table 4.14).

Active renin release remained significantly greater ($p < 0.01$ and $p < 0.05$ respectively) than incubated control values when $[Mg^{2+}]$ was 2.2 and 4.4 m Moles/l in the calcium-deprived media. However, when $[Mg^{2+}]$ was 8.8 m Moles/l, active renin concentration in the

TABLE 4.13 . The Effect of $[Mg^{2+}]$ on Renin Release in Ca^{2+} -Deprived Media (★)

Incubation Medium	Renin Release (ng ang I/mg/h)		
	Active	Total	Inactive
1.1 m Moles/l Mg^{2+} (★)	8.8 ± 1.2	11.4 ± 1.6 ★	2.4 ± 0.5
2.3 m Moles/l Ca^{2+} Mg^{2+} -free (★)	5.5 ± 1.2	7.8 ± 1.4 ★	2.3 ± 0.4
1.1 m Moles/l Mg^{2+} (★)	10.8 ± 0.5 ☆☆	14.5 ± 0.8 ★	3.9 ± 0.4 ☆☆
2.2 m Moles/l Mg^{2+} (★)	11.1 ± 1.7 ☆☆	14.3 ± 1.7 ★	3.3 ± 0.2 ☆☆
4.4 m Moles/l Mg^{2+} (★)	9.1 ± 1.0 ☆	15.1 ± 1.9 ★	4.9 ± 1.0 ☆☆
8.8 m Moles/l Mg^{2+} (★)	7.7 ± 0.9	12.8 ± 1.5 ★	5.1 ± 0.6 ☆☆

◀ Unmodified Krebs' -Ringer bicarbonate buffer.

☆ $p < 0.05$

☆☆ $p < 0.02$

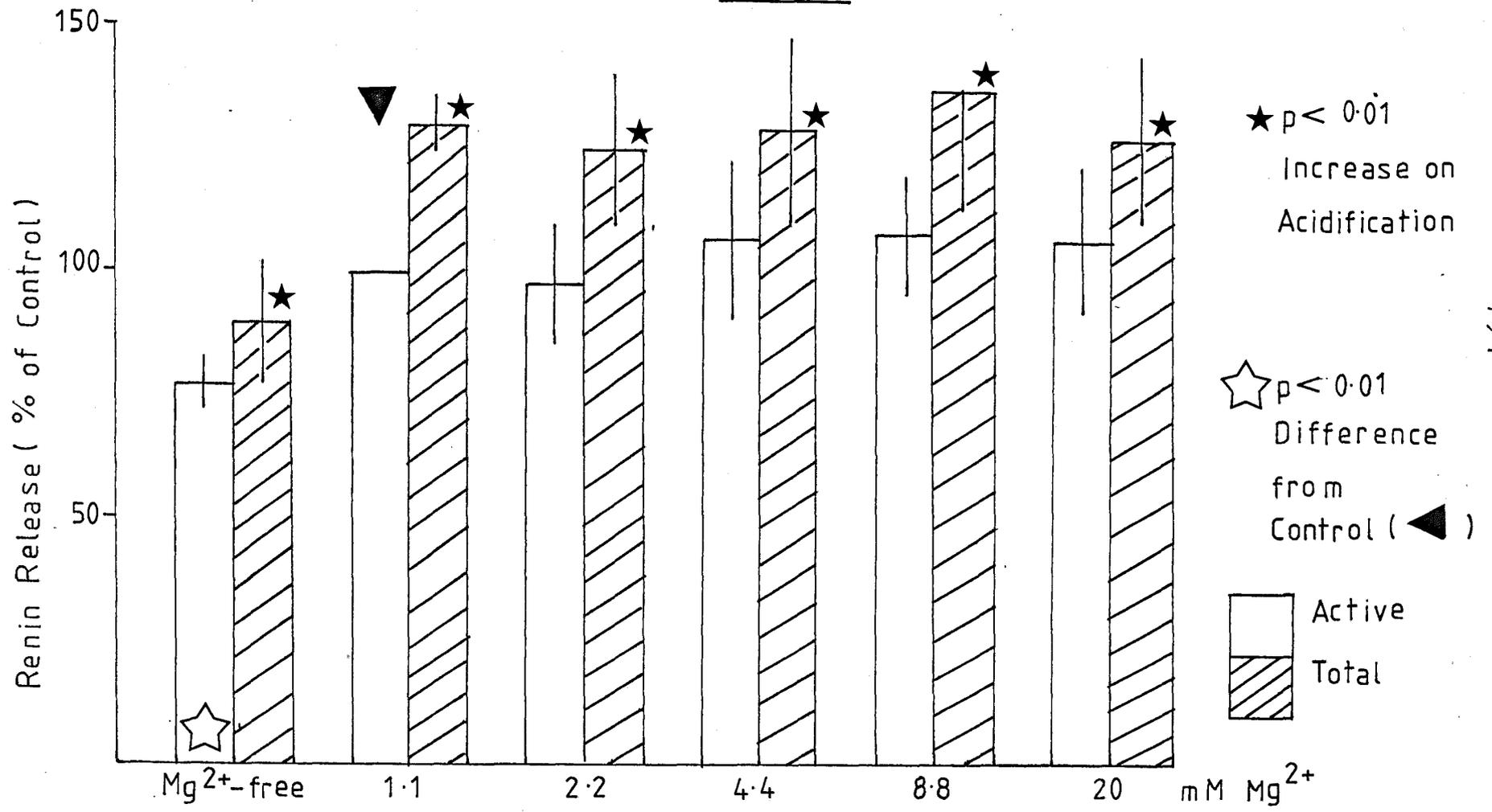
☆☆☆ $p < 0.01$

Difference from Control

★ $p < 0.01$ Increase after Acidification.

supernatant was not significantly different from that released by control slices (Fig. 4.13). When magnesium and calcium were both absent from the incubation medium, active renin release was also restored to control values.

Fig. 4:11 The Effect of Mg^{2+} on Renin Release In Vitro ($2.3 \text{ mM } Ca^{2+}$)



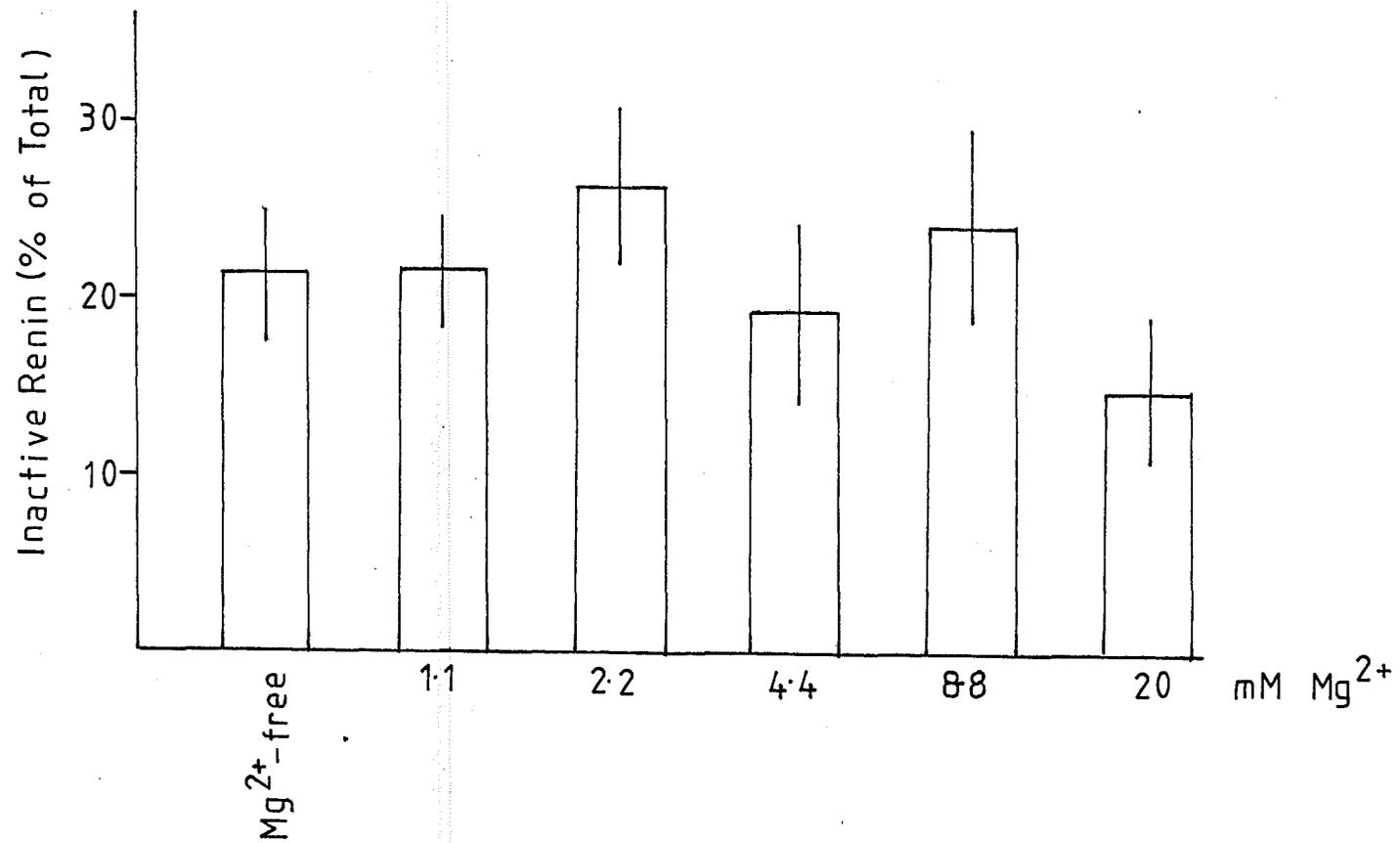


Fig. 4:12 The Effect of Mg²⁺ on Inactive Renin Release In Vitro (2.3mM Ca²⁺)

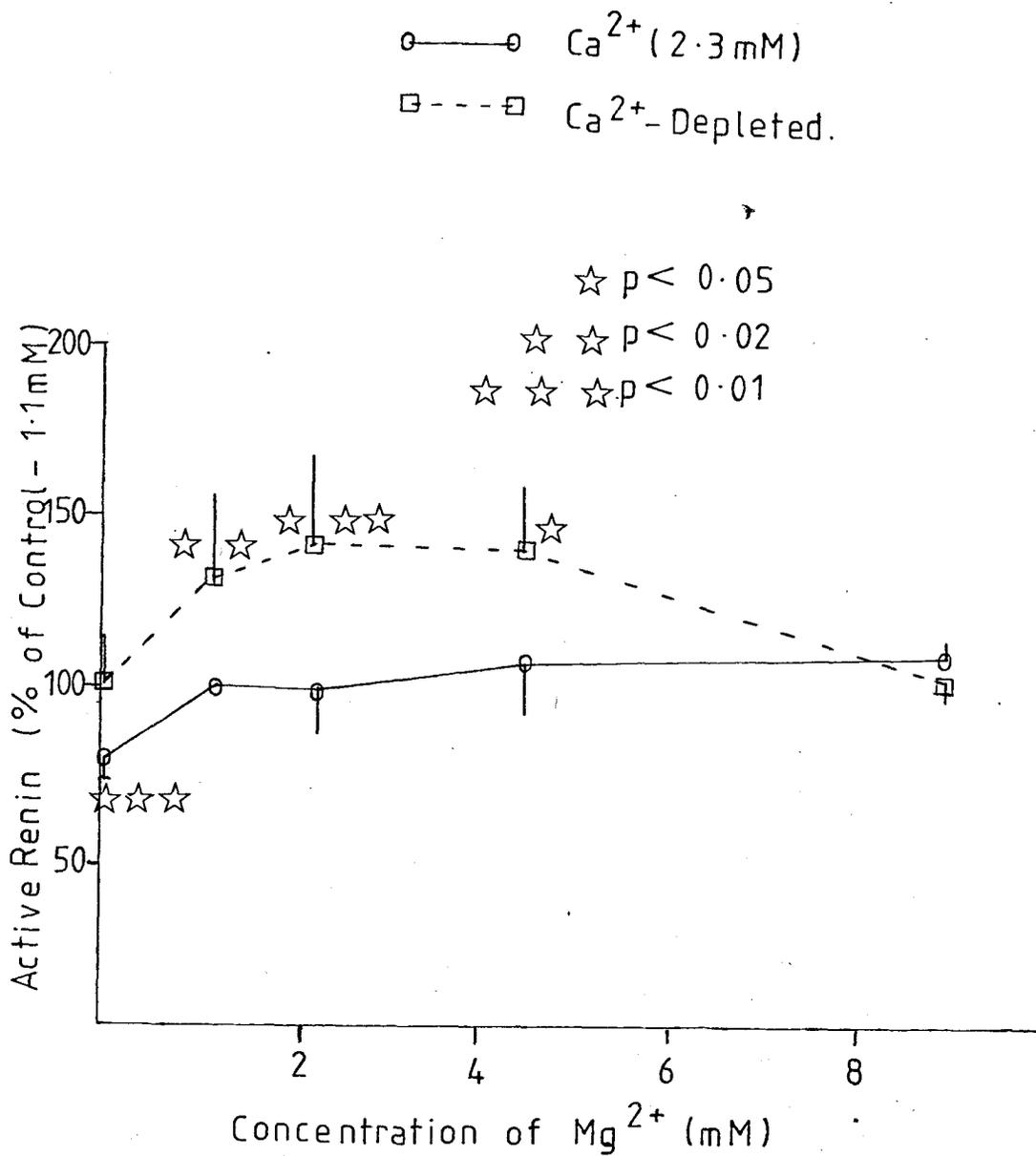


Fig. 4:13 The Effect of $[\text{Mg}^{2+}]$ on Active Renin in the Presence and Absence of Ca^{2+}

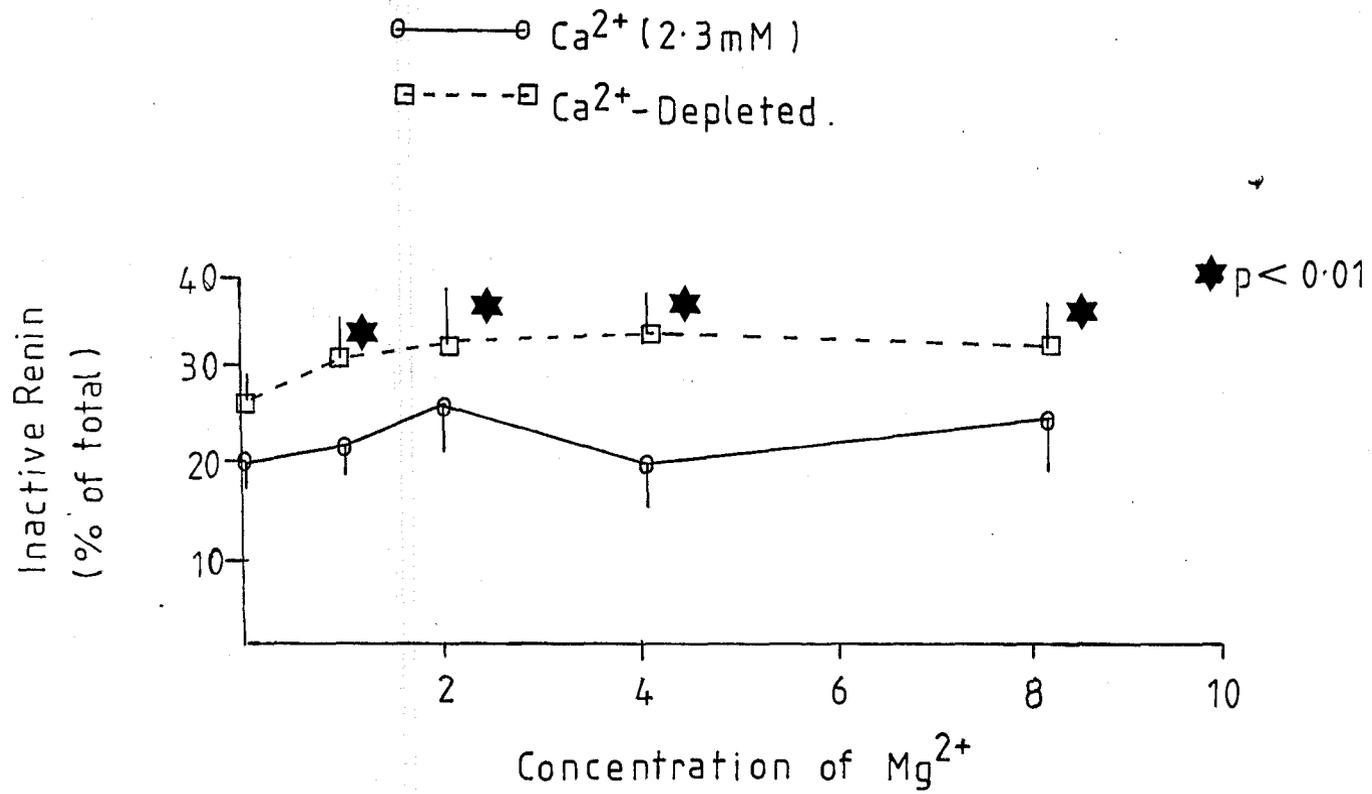


Fig.4:14 The Effect of $[\text{Mg}^{2+}]$ on Inactive Renin in the Presence and Absence of Ca^{2+}

TABLE 4.14 The Effect of Magnesium Ions on the Response to Calcium Deprivation (★)

Incubation Medium	Active Renin (% of Control)	Inactive Renin (% of Total)
1.1 m Mole/l Mg^{2+} ◀	100	21.6 ± 3.1
Mg^{2+} -free ★	99.1 ± 13.9 ☆	25.5 ± 3.6 ☆
1.1 m Moles/l Mg^{2+} ★	131.6 ± 24.5	30.8 ± 4.7
2.2 m Moles/l Mg^{2+} ★	141.3 ± 24.0	33.1 ± 5.5
4.4 m Moles/l Mg^{2+} ★	139.3 ± 15.7	34.3 ± 4.2
8.8 m Moles/l Mg^{2+} ★	101.1 ± 11.1 ☆	33.3 ± 4.2

◀ Unmodified Krebs' -Ringer bicarbonate buffer.

☆ $p < 0.01$ Difference from Ca^{2+} depleted (1.1 m Mole/l Mg^{2+})

Removal of Mg^{2+} from the incubation medium also antagonised the effect of calcium deprivation on inactive renin release. However, although increasing $[Mg^{2+}]$ to 8.8 m Moles/l restored active renin to control values in the Ca^{2+} -deprived medium, the absolute amount of inactive renin that was released tended to increase even further, and formed 33% of total renin released (Fig. 4.14).

DISCUSSION

In these experiments, high $[Mg^{2+}]$ did not significantly change the release of either active or inactive renin in vitro. This is in contrast with the reports of other groups (Churchill & Lyons, 1976; Fray, 1977; Wilcox, 1978). It is not clear whether this is due to differences between species, or to some other factor. Previous studies concerning the effect of magnesium ions on renin secretion have used infusion of $MgCl_2$ intrarenally (Churchill & Lyons, 1976; Fray, 1977; Wilcox, 1978). However, Baumbach & Leyssac (1977) reported that altering $[Mg^{2+}]$ caused no significant change in renin release from isolated, perfused rat glomeruli. A similar result has been obtained in the experiments reported here. This suggests that the stimulatory effect of magnesium ions on renin released by perfused kidneys could be mediated by changes in afferent arteriolar tone. Removal of magnesium from the bathing fluid caused a reduction in active renin concentration in the supernatant. The amount of inactive renin that was released was unchanged.

In common with the effects of sodium and potassium ions, the effect of magnesium ions on renin release in vitro was modified by removal of calcium from the incubation medium. Under these conditions, removing magnesium restored both active and inactive renin to control values, and thus antagonised the effect of Ca^{2+} -depletion. A partial antagonism of the stimulation of renin release achieved by removal of calcium was observed when $[Mg^{2+}]$ was increased to 8.8 m Moles/l. Although active renin secretion was similar to control values under these conditions, inactive renin secretion was stimulated, and this form still comprised a large (33%) proportion of the total renin concentration.

Early work by Douglas & Rubin (1963 and 1964) suggested that raising $[Mg^{2+}]$ reduced calcium influx into stimulated cells. More recent studies have revealed however, that magnesium interferes with the action of calcium at some intracellular site (Rahwan et al 1973) in adrenal medullary chromaffin cells. Kanno et al (1973) also reported that intracellular injection of Mg^{2+} could inhibit calcium-induced degranulation of mast cells. A similar intracellular compe-

tition between calcium and magnesium was proposed by Turlapaty & Carrier (1973) for vascular smooth muscle. In the experiments reported here, Mg^{2+} partially inhibited renin release in response to low calcium. This suggests that a similar type of competition may exist in JG cells for the release of active renin.

V THE EFFECT OF LITHIUM IONS ON RENIN RELEASE IN VITRO

Recent reports have suggested that plasma renin activity is elevated in patients receiving lithium therapy (Murphy *et al*, 1969) and in dogs (Nally, Rutecki & Ferris, 1980) and rats (Gutman *et al*, 1973) during acute infusion of lithium chloride (see page 30). This effect is accompanied by natriuresis, diuresis and an increase in urinary PGE excretion. It is not yet clear if the increase in PRA after lithium occurs in response to a change in renal handling of sodium, or is due to direct, or indirect, effects of lithium on juxta-glomerular cells.

This was investigated in the following experiment by incubating rabbit kidney cortex slices in media which contained a range of $[Li^+]$ (0 - 20 m Moles/l). The effect of lithium ions in the absence of calcium was not investigated.

MATERIALS AND METHODS

Pairs of rabbit kidney cortex slices were prepared as previously described (see page 106) and were incubated in modified Krebs'-Ringer bicarbonate buffer, in which NaCl was partially replaced by an equimolar concentration of LiCl (0.6% w/w). One pair of slices was incubated in unmodified Krebs' buffer as a control. A further pair of slices was incubated in Krebs's buffer in which 20 m Moles/l of the NaCl had been replaced by 20 m Moles/l choline chloride (2.16%).

This ensured that any effect of lithium was due directly to this ion and could not be attributed to removal of NaCl. The incubation media were as follows:

- 1) Unmodified Krebs'-Ringer bicarbonate buffer (Incubated Control).
- 2) Krebs'-Ringer bicarbonate buffer containing 1 m Mole/l Li^+
- 3) Krebs'-Ringer bicarbonate buffer containing 5 m Moles/l Li^+
- 4) Krebs'-Ringer bicarbonate buffer containing 10 m Moles/l Li^+
- 5) Krebs'-Ringer bicarbonate buffer containing 20 m Moles/l Li^+
- 6) Krebs'-Ringer bicarbonate buffer containing 20 m Moles/l choline chloride.

RESULTS

TABLE 4.15 The Effect of Lithium Ions on Renin Release In Vitro

Incubation Medium	Renin Release (ng ang I/mg/h)		
	Active	Total	Inactive
Li^+ 0 ◀	6.8 ± 1.7	8.7 ± 1.6 *	2.0 ± 0.3
Li^+ 1 m Mole/l	8.4 ± 1.5	11.1 ± 1.8 *	2.6 ± 0.5
Li^+ 5 m Moles/l	8.9 ± 2.1	10.9 ± 2.2 *	2.2 ± 0.3
Li^+ 10 m Moles/l	10.5 ± 2.2 ☆	13.3 ± 2.4 *	2.8 ± 0.4
Li^+ 20 m Moles/l	13.9 ± 3.5 ☆☆	17.8 ± 2.9 *	3.1 ± 1.2
20 m Moles/l chol.	7.6 ± 1.6	9.9 ± 1.8 *	2.2 ± 0.2

◀ Unmodified Krebs'-Ringer bicarbonate buffer

* $p < 0.01$ Increase after
Acidification

☆ $p < 0.05$
☆☆ $p < 0.01$

Difference
from Control

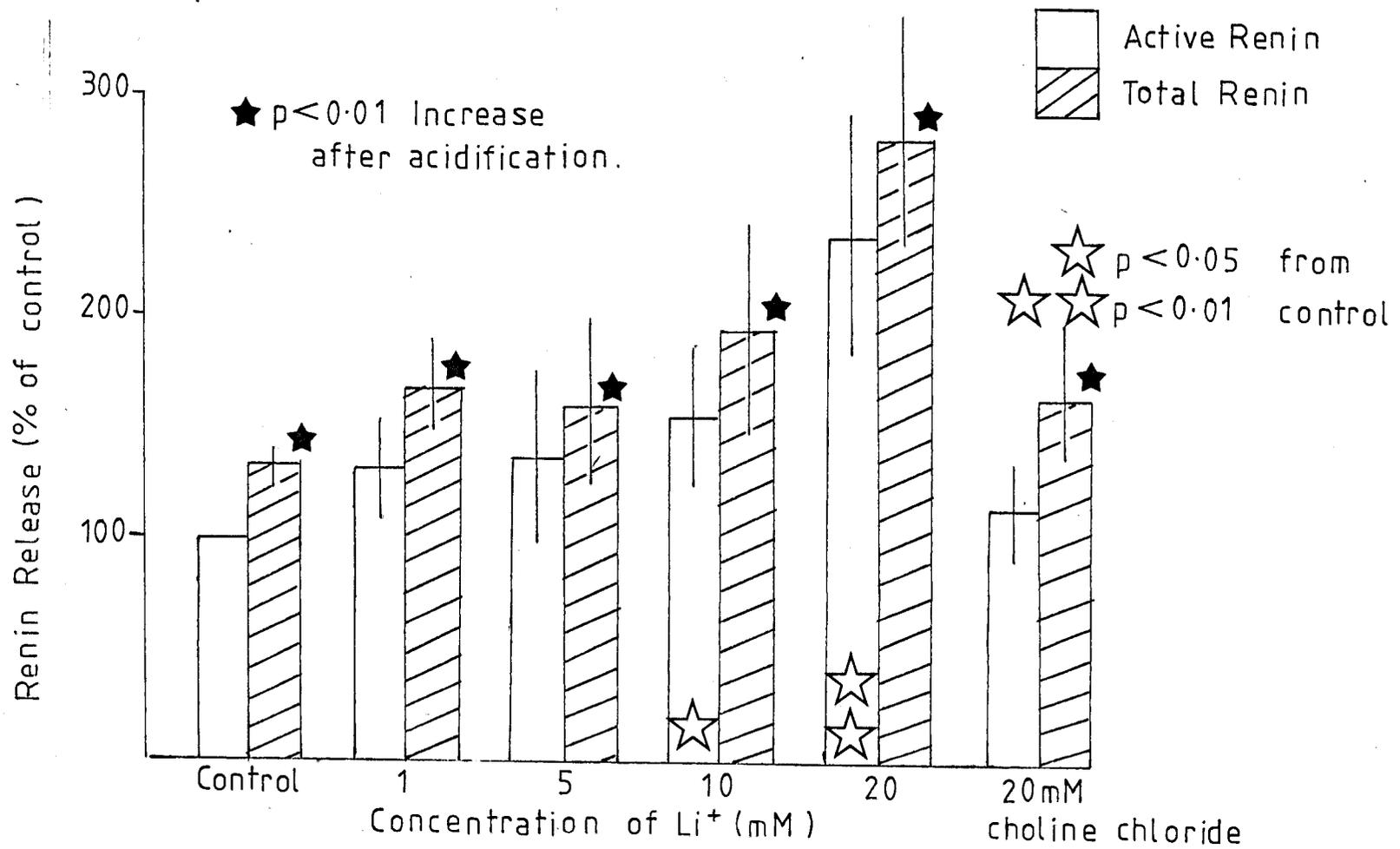


Fig. 4:15 The Effect of lithium on Renin Release.

As the concentration of lithium ions in the incubation medium increased from 0 - 20 m Moles/l, a progressive increase was observed in the release of active renin by the slices (Table 4.15). This increase was not due to the removal of sodium ions from the incubation medium, since replacement with 20 m Moles/l choline chloride caused no significant change in active renin concentration in the supernatant (see Fig. 4.15).

Although the proportion of total renin that was inactive was reduced from 24.8 ± 8.9 to $11.1 \pm 3.9\%$ (Table 4.15) when lithium was added to the Krebs' buffer, the absolute concentration of inactive renin in the supernatant was unchanged (Fig. 4.16). Indeed, the concentration of inactive renin tended to increase, but the change was not statistically significant.

These results suggest that lithium ions stimulate the release of renin from kidney cortex slices in vitro. This increase was not accompanied by a significant change in the release of inactive renin.

TABLE 4.16 The Response of Renin Secretion In Vitro to Lithium Ions

Incubation Medium	Active Renin (% of Control)	Inactive Renin (% of Total)
Li + 0	100	24.8 ± 8.9
Li + 1 m Mole/l	130.3 ± 21.2	22.3 ± 6.1
Li + 5 m Moles/l	136.4 ± 36.4	14.4 ± 4.4
Li + 10 m Moles/l	154.5 ± 31.8	15.6 ± 3.9
Li + 20 m Moles/l	233.3 ± 54.5	11.1 ± 3.9
20 m Moles/l choline	112.1 ± 20.6	22.8 ± 5.1

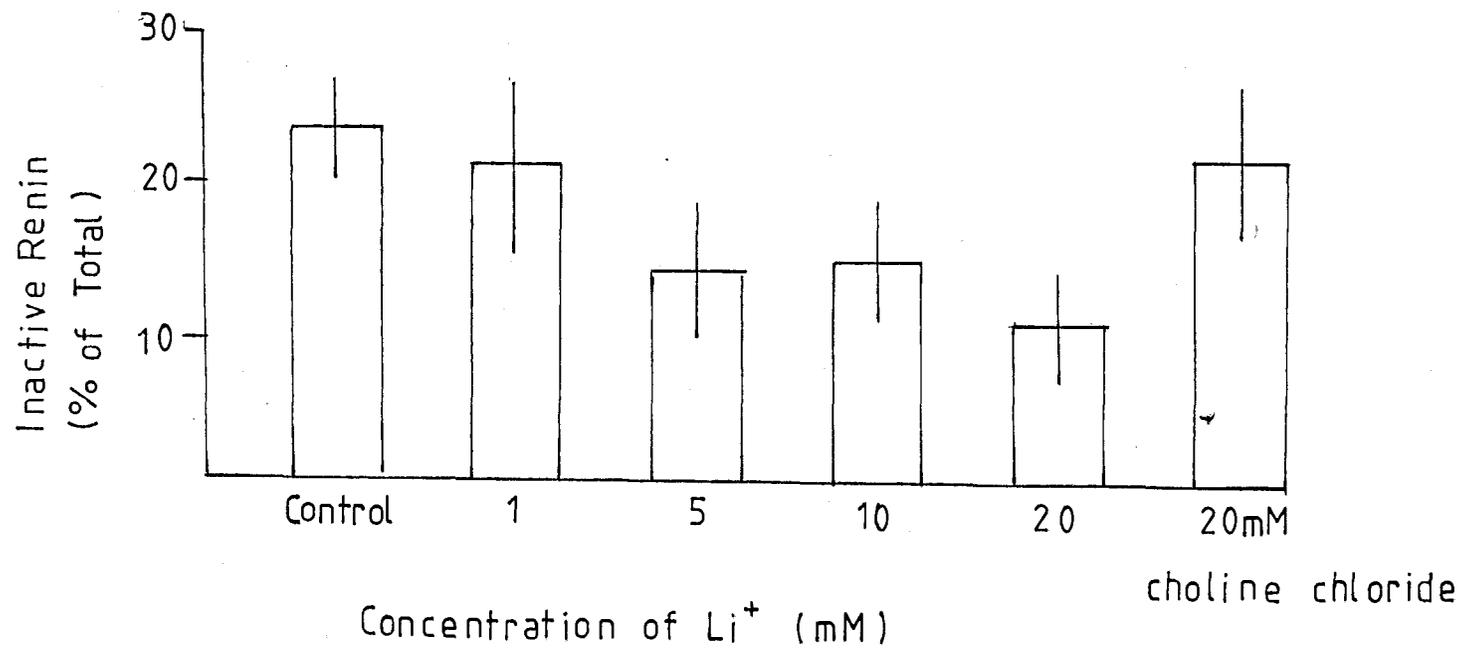


Fig. 4:10 The Effect of [Li⁺] on Inactive Renin Release.

DISCUSSION

The results which have been obtained in this experiment indicate that lithium ions stimulate active renin release by rabbit kidney cortex slices in vitro by a direct action at the juxtaglomerular cell. The pattern of response to lithium is similar to the response to removing sodium from the medium. However, the effect of lithium ions is not due to a reduction in the extracellular concentration of sodium from 133 to 113 m Moles/l, since replacement with 20 m Moles/l choline chloride did not significantly change renin release. This finding suggests that lithium ions may compete with extracellular sodium ions and thereby cause an increase in active renin release. Inactive renin however, was relatively unchanged by the presence of lithium ions in the bathing fluid.

Gutman et al (1973) reported that infusion of lithium salts caused PRA to increase in rats. This effect appeared to be the result of natriuresis which accompanied the response to lithium, since the increase in renin was prevented by increasing the dietary intake of sodium. However, Nally et al (1980) concluded that the increase in PRA in dogs after acute infusion of lithium chloride, was not due to a change in sodium delivery at the macula densa, to a change in renal vascular resistance, or to activation of the sympathetic nervous system. They explained their data in terms of a direct effect of lithium on JG cells. The results which are reported here confirm that lithium acts at granular cells to increase active renin release. Inactive renin, in contrast, did not change in response to lithium ions.

GENERAL DISCUSSION

The work reported in this section of the thesis represents the first investigation into the secretion of both active and inactive renin from the kidney in response to changes in the ionic composition of the extracellular medium. At the present time, there is a limited amount of published data concerning the control of plasma levels of inactive renin from studies of human subjects, dogs, pigs and rabbits (see page 11). During the last five years, stimulus-secretion coupling for renin has aroused a great deal of interest. In particular, the role played by calcium ions has received much attention. In this respect, the renin-angiotensin system differs from the vast majority of other secretory systems since increasing intracellular $[Ca^{2+}]$ appears to inhibit renin secretion rather than stimulate it.

A large proportion of the renin that is stored within the renal cortex is present in an inactive but activatable form. It is also widely reported that inactive forms of renin are present in plasma (see page 8) and these are thought to be secreted by the kidney. However, stimulus-secretion coupling for this form of the enzyme has not been investigated. Indeed, most studies using in vitro techniques to investigate the effects of electrolytes on renin secretion have used the rat as an experimental model. This is the one species for which there is doubt concerning the existence of inactive renin (see page 8).

Before discussing specific aspects of the work, there are some general points that should be made. Under the experimental conditions used in this laboratory, inactive renin represents 15 - 20% of the total renin found in the plasma of normal rabbits (Richards et al, 1979; Richards et al, 1981a and b). Rabbit kidney cortex slices incubated in complete Krebs'-Ringer bicarbonate buffer secrete the two forms of renin in the same relative proportions as are present in plasma. This has been illustrated in all of the five sections of this thesis chapter. However, the mixture of renins that can be extracted after homogenisation of the tissue is rather different. In this case, inactive renin forms about 36% of the total renin that can be measured after acidification. Thus, stimulus-secretion coupling within the granular cells cannot be simply a matter of releasing

renin from a single, stored pool. Moreover, the experiments reported in this thesis show that the relative proportions of active and inactive renin that are secreted in response to different experimental manipulation can vary widely.

This poses many questions concerning how the selectivity of the response is determined within the renin-secreting cells. Two broad possibilities arise:

- I:- Two separate pools of renin within the JG cell are subject to independent, or partially independent control processes.
- II:- A single secretion control mechanism for both forms of renin exists together with a mechanism for interconversion of the two forms of renin. Variations in the activity of this activating system would be reflected in changes in the mixture of renin secreted.

At this stage, it is not possible to distinguish between these two hypotheses, but they do provide a basis for further investigation. However, an important clue might be available. In several circumstances studied, the mixture of renins that was normally secreted altered to reflect the mixture that was stored within the renal cortex. For example, in response to calcium deprivation (see page 158) or to hyperpolarization by low $[K^+]$ (see page 164), the relative amount of inactive renin that was secreted increased to about 35%. Under no experimental conditions studied so far did inactive renin form a greater proportion than this value. This could be taken as evidence in favour of the second hypothesis outlined above. If interconversion is a unidirectional process, ie inactive \rightarrow active renin, and the secretion of renin is regulated by one set of mechanisms, then secretion of inactive renin in greater proportions than are stored in the kidney would not be possible. On the other hand, the first hypothesis, that secretion of the two forms of renin is independently controlled, would suggest that there is no reason why a mixture containing more than 35% inactive renin could not be secreted.

Stimulus-secretion coupling for renin is clearly very much more complex than was hitherto anticipated. In situations where total renin (ie active and inactive) secretion either increased or decreased, there are few examples reported in this thesis where the two forms of renin change in parallel and maintain the same relative pro-

portions.

Total renin release increased during calcium deprivation. Both active and inactive renin increased under these conditions, but the effect of low $[Ca^{2+}]$ on inactive renin was more pronounced than its effect on active renin. Furthermore, the effect of calcium depletion on both forms of renin was greater when the medium contained a high concentration of K^+ .

The changes in active renin that were observed under these conditions provide further evidence in support of the current hypothesis that a reduction in cytoplasmic $[Ca^{2+}]$ stimulates the secretion of renin. Depolarization, in the absence of external calcium, will tend to increase the rate of calcium efflux from the cell, and therefore stimulate the secretion of active renin. The results of these experiments also suggested that the secretion of inactive renin could be regulated by a similar mechanism. Release of this form of renin was inhibited by high $[Ca^{2+}]$ and by depolarization in the presence of Ca^{2+} , but was increased in the absence of calcium.

However, the response to hyperpolarization achieved by incubating the slices in low $[K^+]$, casts some doubt on this relationship. As might have been expected, hyperpolarization increased total renin release when calcium was present. However, this stimulation represented an increase in only inactive renin, while active renin was unchanged. On the other hand, total renin release was reduced when slices were incubated in Mg^{2+} -free media. In this case, active renin release was inhibited, while inactive renin release was unchanged.

Earlier work by Dr. H. K. Richards in this laboratory suggested that the release of inactive renin was regulated by a sodium-sensitive mechanism. Dietary sodium depletion when the animals are in negative sodium balance, reduces inactive renin and increases active renin. Similar changes are observed during furosemide diuresis and when kidney cortex slices are incubated in Krebs'-Ringer bicarbonate buffer, where the concentration of sodium is reduced. These findings provide evidence in favour of the second hypothesis outlined above, that interconversion of the two forms of renin takes place prior to secretion. Thus, the effect of reducing $[Na^+]$ could be explained by enhanced conversion of inactive renin to the active form. In contrast, an increase in Na^+ transport could be expected to inhibit conversion of inactive renin and cause its relative proportion to increase.

Can a sodium-sensitive mechanism such as this be reconciled with the results obtained in the experiments reported here? During calcium depletion, the proportion of total renin that was inactive increased to about 35%. Ca^{2+} ions are thought to be important in regulating the permeability of the cell membrane. Thus, removal of calcium could cause the plasma membrane to become leaky and the cells to become loaded with sodium. If a sodium-sensitive step regulates inactive renin, then conversion of inactive renin would be inhibited. Ca^{2+} -depletion stimulates the secretion of renin, thus both active and inactive renin in the supernatant increase.

However, when Ca^{2+} -depletion is accompanied by low $[\text{Na}^+]$ the tendency for cells to become sodium loaded would be diminished. Indeed, reducing $[\text{Na}^+]$ partially antagonised the effect of low $[\text{Ca}^{2+}]$ and the effect on inactive renin was more pronounced than with the active form. Clearly, it may prove important to consider changes in Na^+ transport and intracellular $[\text{Na}^+]$ in addition to intracellular $[\text{Ca}^{2+}]$ when investigating stimulus-secretion coupling for renin.

This possibility is interesting, since granular cells are modified vascular smooth muscle cells. In that tissue, sodium ions appear to be important carriers of inward current during contraction (Keatinge & Harman, 1980). In the following chapter of this thesis, two calcium antagonist drugs, verapamil and flunarizine, and the calcium ionophore, A23187, will be used to investigate further the response of renin-secreting cells to calcium deprivation. These experiments were performed with the aim of clarifying the nature of the renin secreting control mechanism.

One interesting finding in the experiments described in this chapter is that, in almost every case, the response of inactive renin to changes in the ionic composition of the bathing fluid follow a pattern similar to that observed by other workers in the rat. The controversy surrounding the role of sodium in the regulation of the secretion of renin has already been described (see page 26). In the rat, lowering $[\text{Na}^+]$ inhibits renin secretion. In the rabbit, and in other species which secrete inactive renin, low $[\text{Na}^+]$ inhibits the release of inactive renin. Similarly, high $[\text{Ca}^{2+}]$ (see page 158) and depolarization by high $[\text{K}^+]$ (see page 164) inhibit renin secretion in the rat and reduce inactive renin release by rabbit kidney cortex slices. Calcium deprivation (see page 158) and hyperpolarization (see page 164) increase the release of both rat renin

and rabbit inactive renin. In some of these situations, the changes in active renin in the rabbit have been more variable.

These findings are interesting since they suggest that there may be a fundamental difference between the rat, which is often used as an experimental model for investigations into the renin-angiotensin system, and other species such as the rabbit. The rat kidney does not appear to secrete inactive renin. These experiments indicate that the changes in inactive renin secretion by rabbit kidney cortex slices are similar to those reported for active renin in the rat. However, the responses of rabbit active renin are more variable and could reflect differences in activation of inactive renin. Further evidence to support these ideas arises in the succeeding chapter.

CHAPTER 5

THE EFFECTS OF TWO CALCIUM ANTAGONISTS AND

THE CALCIUM IONOPHORE ON RENIN RELEASE

IN VITRO

THE EFFECT OF VERAPAMIL AND FLUNARIZINE ON RENIN RELEASE IN VITRO

A wide variety of pharmacological agents including skeletal muscle relaxants, local anaesthetics, anticonvulsants, antiarrhythmic agents and antihypertensive drugs are now known to possess calcium antagonistic properties. The role of calcium antagonism in the therapeutic action has been reviewed by Rahwan, Piascik & Witiak (1979). Generally, calcium antagonists can be classified into one of two groupings:

- 1) Those which block the influx of extracellular calcium ions (eg D600, verapamil flunarazine, cinnarazine, nifedipine local anaesthetics and methadone).
- 2) Those which interfere with mobilization (or enhance sequestration) of intracellular calcium ions. This group includes magnesium ions, sodium nitroprusside and diazoxide.

The experiments which have been described in Chapter 4 suggest that the responses of rabbit kidney cortex slices to potassium (see page 164) sodium (see page 174) and magnesium (see page 186) depend, at least in part, on the ability of these ions to alter the rate of flux of calcium across the juxtaglomerular cell membrane. In view of these findings, the effect of two calcium antagonists, verapamil and flunarizine, on active and inactive renin release in vitro was investigated.

Verapamil, and its methoxy derivative, D6000, appear to inhibit the augmented Ca^{2+} influx through the activated calcium channel in the cell membrane. In potassium-depolarized coronary vessels, verapamil and D600 produce a slow onset, but complete, relaxation, which is only reversed by increasing the extracellular calcium concentration (Fleckenstein *et al*, 1976).

Flunarizine, and its parent compound, cinnarizine, antagonise the vasoconstrictor actions of many vasoactive substances by inhibiting the influx of extracellular calcium during smooth muscle stimulation. They inhibit the extracellular calcium-dependent component of noradrenaline-induced smooth muscle contraction (tonic contraction), but not the intracellular calcium-dependent component (phasic contraction). Thus, it is believed that flunarizine and cinnarizine

block the slow channel calcium influx through the cell membrane without interfering with mobilization of intracellular calcium pools (Godfriend & Kaba, 1972). These components have no effect at either α - or β -adrenoreceptor sites. Flunarizine was selected for the following experiments since it is reported to be almost three times more potent than cinnarizine, as assessed by inhibition of the pressor response to noradrenaline in the perfused hind limb of the dog (Van Neuten, 1978).

MATERIALS AND METHODS

Rabbit kidney cortex slices were prepared as before (see page 106) and were incubated in one of the following media:

- 1) Unmodified Krebs'-Ringer bicarbonate (Control).
- 2) Ca^{2+} -free Krebs'-Ringer bicarbonate.
- 3) Ca^{2+} -depleted Krebs'-Ringer bicarbonate containing 5 mMoles/l EGTA.

Slices were also incubated in the same media, to which verapamil (50 μ Moles/l) or flunarizine (50 μ Moles/l) had been added. Both verapamil and flunarizine were dissolved in methanol : Krebs' buffer (2 : 1) and were added to the incubation media to give a final methanol concentration of less than 2%. All experiments were carried out in duplicate.

RESULTS

The Effect of Verapamil (50μ Moles/l) and Flunarizine (50μ Moles/l) on Basal Renin Release in vitro.

The results of this experiment are shown in Table 5.1. Values for active renin have also been expressed as a percentage of incubated control values, and inactive renin has been expressed as a percentage of the total renin released (Table 5.2). The results are illustrated in Fig. 5.1.

Addition of Verapamil to the control ($2.3 \text{ m Moles/l Ca}^{2+}$) incubation medium increased active renin release from 7.4 ± 2.5 to $10.3 \pm 3.6 \text{ ng ang I/mg/h}$. Although this change represents an increase of $94.6 \pm 44.4\%$ above control values, it was not statistically significant. Flunarizine also increased active renin release by $70.2 \pm 3.4\%$ to $10.4 \pm 3.8 \text{ ng ang I/mg/h}$. This change also was not statistically significant.

TABLE 5.1 The Effect of Ca^{2+} -antagonists on Basal Renin Release

Incubation Medium	Renin Release (ng ang I/mg/h)		
	Active	Total	Inactive
Control (2.3 m Moles/l Ca^{2+})	7.4 ± 2.5	8.3 ± 3.0 *	0.88 ± 0.39
Control+			
Verapamil (50 μ Moles/l)	10.3 ± 3.6	13.9 ± 4.9 *	3.5 ± 1.4 ☆
Control+			
Flunarizine (50 μ Moles/l)	10.4 ± 3.8	13.4 ± 4.2 *	1.9 ± 0.8 ☆

Results are shown as mean ± s.e.m. of data from ten experiments.

★ p < 0.01 Increase after acidification ☆ p < 0.01 Difference from Control Inactive Renin ☆ p < 0.05

TABLE 5.2 The Effect of Ca^{2+} -antagonists on Basal Renin Release

Incubation Medium	Active Renin (% of Control)	Inactive Renin (% of Total)
Control (2.3 m Moles/l Ca^{2+})	100	12.5 ± 2.5
Control+		
Verapamil (50 μ Moles/l)	194.6 ± 44.4	28.9 ± 5.2 ☆
Control+		
Flunarizine (50 μ Moles/l)	170.2 ± 34.0	18.9 ± 5.5 ☆

Results are shown as mean ± s.e.m. of data from ten experiments.

☆ p < 0.02 Difference from Control % Inactive Renin.

★ $p < 0.01$ Increase after Acidification
 ☆ $p < 0.05$ Difference from Control
 ☆ $p < 0.01$ Difference from Control

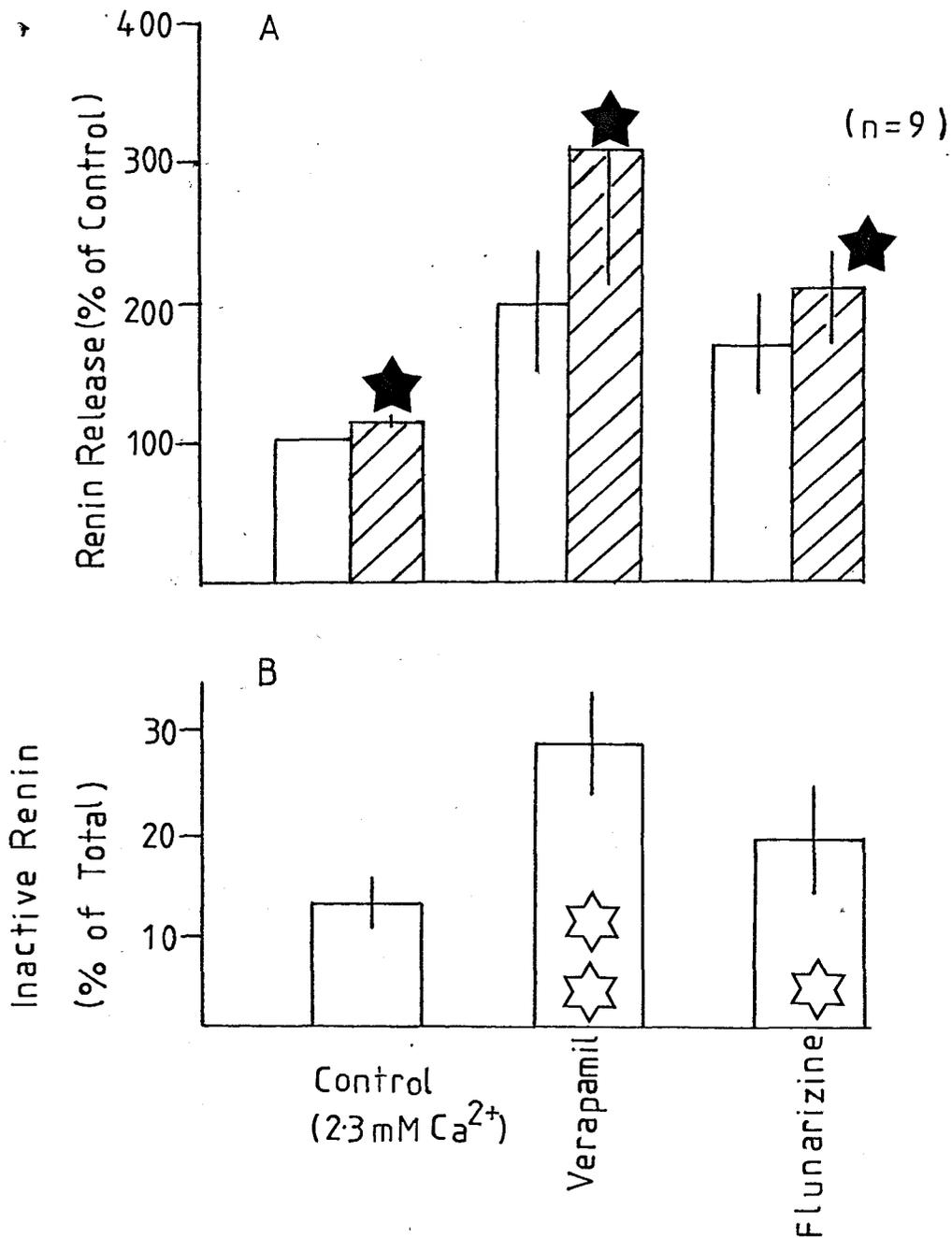


Fig. 5:1 The Effect of Calcium Antagonists on Basal
 A) Active and B) Inactive Renin Release
In Vitro

In contrast to this, both flunarizine and verapamil caused significant increases in the amount of inactive renin which was released into the incubation medium ($p < 0.01$ and $p < 0.05$ respectively). This effect cannot be due to the presence of the methanol/Krebs' vehicle, since active renin (5.5 ± 2.4 ng ang I/mg/h) and inactive renin ($21.3 \pm 7.8\%$ of total renin) were not significantly different from control values when $50 \mu\text{l}$ of this solution was added to the incubation medium.

These findings suggest that the calcium antagonists tend to increase active renin release by rabbit kidney cortex slices in vitro. In addition, they caused a significant increase in inactive renin release. The rate at which renin is released by granular cells is thought to be determined by the intracellular [Ca]. The results of this experiment suggest that the antagonists may in some way reduce the intracellular [Ca] and therefore increase the rate of release of both forms of renin. The changes in inactive renin were relatively greater and statistically significant.

THE EFFECT OF CALCIUM ANTAGONISTS ON RENIN RELEASE In Vitro IN RESPONSE TO A LOW CONCENTRATION OF CALCIUM.

In an attempt to clarify the mechanism whereby low concentrations of calcium stimulate the release of renin, rabbit renal cortex slices were incubated in Ca-free media (Table 5.3) and Ca-depleted media (Table 5.5) to which the calcium antagonists (both $50 \mu\text{Moles/l}$) had been added.

As previously observed (see page 155) active and inactive renin release increased by some 60% ($p < 0.01$) when the slices were incubated in a Ca-free medium (see Fig. 5.2). Inactive renin also increased from $12.5 \pm 2.5\%$ of total renin released by control slices to $26.0 \pm 7.5\%$ of total renin in the Ca-free media (Table 5.4).

TABLE 5.3 The Effect of Calcium Antagonists on Renin Release
in Ca-free Media

Incubation Medium	Renin Release (ng ang I/mg/h)		
	Active	Total	Inactive
Control	7.4 ± 2.5	8.3 ± 3.0 *	0.9 ± 0.4
Ca-free	10.5 ± 4.0 ☆	13.4 ± 4.4 *	2.9 ± 1.1
Ca-free + Verapamil (50 μ Moles/l)	11.3 ± 4.4 ☆	19.4 ± 6.6 *	6.1 ± 2.5 ☆
Ca-free + Flunarizine (50 μ Moles/l)	10.2 ± 5.0	11.2 ± 5.4 *	1.1 ± 0.46

Results are shown as mean ± s.e.m. of data from ten experiments.

☆ $p < 0.01$ Difference from Control * $p < 0.01$ Increase on Acidification

The addition of verapamil and flunarizine did not significantly alter the response of active renin release to low extracellular $[Ca^{2+}]$. However, inactive renin was increased from 2.9 ± 1.1 ng ang I/mg/h in Ca-free media, to 6.1 ± 2.5 ng ang I/mg/h when verapamil was present. In contrast to this, inactive renin was reduced to 1.1 ± 2.5 ng ang I/mg/h when flunarizine was present. Flunarizine therefore tended to restore both active and inactive renin to control values. Thus flunarizine and verapamil appeared to produce opposing actions on the response of inactive renin release in calcium-free media. A further increase in inactive renin ($p < 0.01$ compared to control) was observed when slices were incubated in Ca-free media containing verapamil, while inactive renin approached control values when flunarizine was present.

Fig5:2 The Effect of Calcium Antagonists on Renin Release in Response to Low $[Ca^{2+}]$

★ $p < 0.01$ Increase on Acidification.

☆ $p < 0.01$ Difference from Control

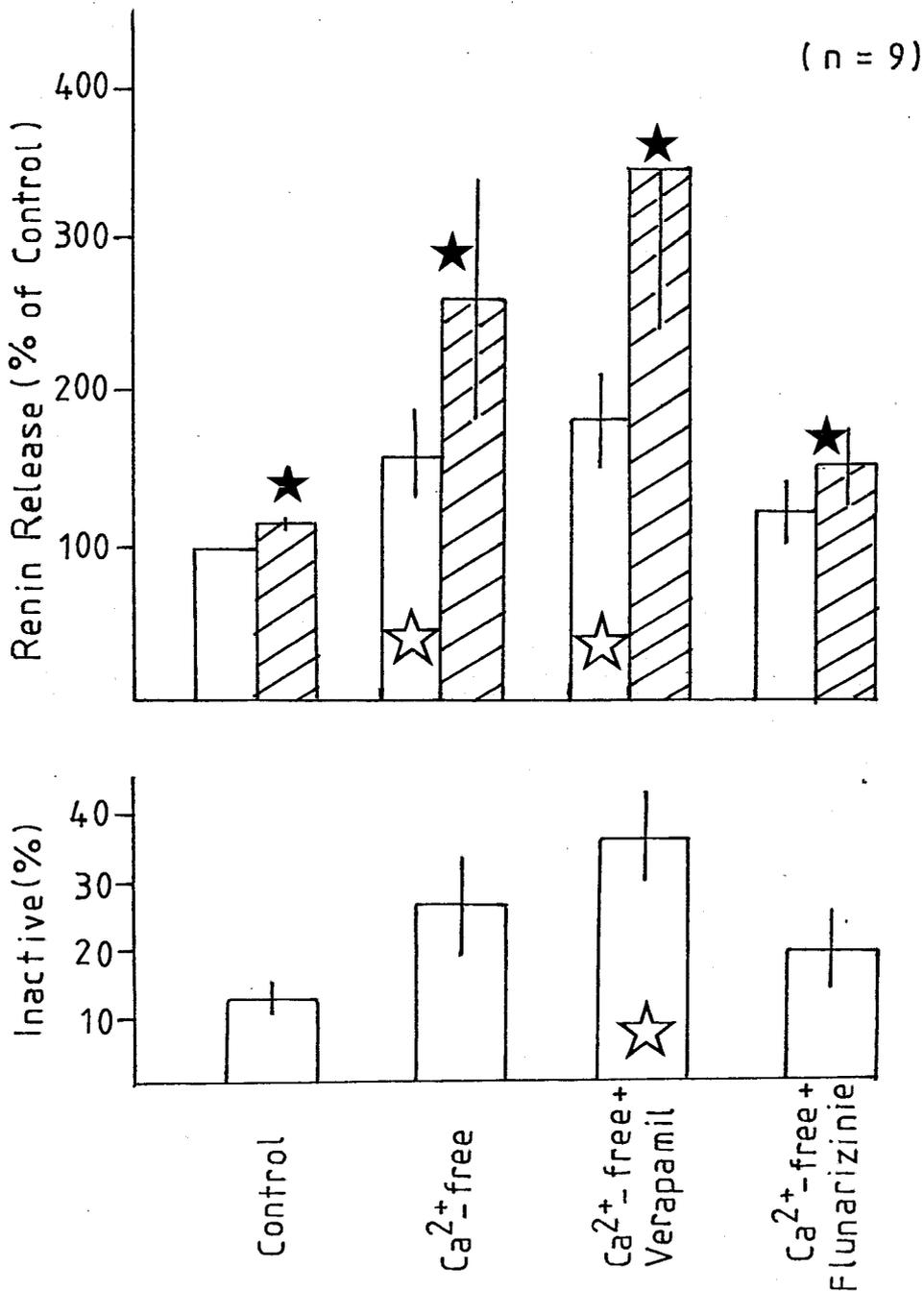


TABLE 5.4 .The Effect of Ca-antagonists on the Response of Renin Release to Low Extracellular Concentrations of Calcium.

Incubation Medium	Active Renin (% of Control)	Inactive Renin (% of Total)
Control (2.3 m Moles/l Ca)	100	12.5 ± 2.5
Ca-free	158.7 ± 30.3	26.0 ± 7.6
Ca-free + Verapamil (50 μ Moles/l)	182.1 ± 26.9	36.5 ± 6.6
Ca-free + Flunarizine (50 μ Moles/l)	122.7 ± 20.4	19.5 ± 5.3

THE EFFECT OF CALCIUM ANTAGONISTS ON THE STIMULATION OF RENIN RELEASE IN VITRO IN RESPONSE TO CALCIUM DEPLETION

The previous experiment was repeated by incubating rabbit kidney cortex slices in a calcium depleted medium (5 m Moles/l EGTA added) in the presence of verapamil or flunarizine (both 50 μ Moles/l). The results obtained are shown in Table 5.5 and the percentage changes in the response to calcium depletion are shown in Table 5.6 and Fig. 5.3.

Calcium depletion increased the release of both active ($p < 0.01$) and inactive ($p < 0.01$) renin by the slices. When verapamil was included in the calcium depleted medium, active renin release was reduced from 13.2 ± 4.8 to 10.9 ± 3.8 ng ang I/mg/h. However, this change does not represent a significant reduction in the response of active renin release to calcium depletion, and both values were significantly greater than active renin released by control slices ($p < 0.01$ and $p < 0.01$ respectively). Similarly, addition of verap-

amil did not cause any further significant increase in the amount of inactive renin released by the slices. This form represented $34.1 \pm 5.0\%$ of total renin released ($p < 0.01$ from control).

TABLE 5.5 The Effect of Calcium Antagonists on Renin Release During Ca-depletion.

Incubation Medium	Renin Release (ng ang I/mg/h)		
	Active	Total	Inactive
Control (2.3m Moles/l Ca^{2+})	7.4 ± 2.5	8.3 ± 3.0 *	0.9 ± 0.4
Ca-depleted (5 m Moles/l EGTA)	13.2 ± 4.8 * *	17.4 ± 6.6 *	4.2 ± 1.9 *
Ca-depleted + Verapamil (50 μ Moles/l)	10.9 ± 3.8 * *	17.8 ± 6.6 *	6.5 ± 2.9 * *
Ca-depleted + Flunarizine (50 μ moles/l)	8.2 ± 3.2	11.1 ± 4.0 *	2.8 ± 1.6

Results are shown as mean \pm s.e.m. of data from ten experiments.

* $p < 0.01$ Increase on Acidification * $p < 0.02$ Difference from Control
* $p < 0.01$

In contrast to verapamil, flunarizine antagonised the effect of calcium depletion on both active ($p < 0.01$) and inactive ($p < 0.02$) renin release in vitro. When flunarizine was present in the Ca^{2+} -depleted medium, active renin release was reduced to 8.2 ± 3.2 ng ang I/mg/h: a value which was not significantly different from that of control slices. A similar reduction in inactive renin from 26.1 ± 3.9 to $19.5 \pm 5.3\%$ of total renin was also observed.

★ $p < 0.01$ Increase on Acidification
 ☆ $p < 0.02$ Difference from Control
 ☆ $p < 0.01$

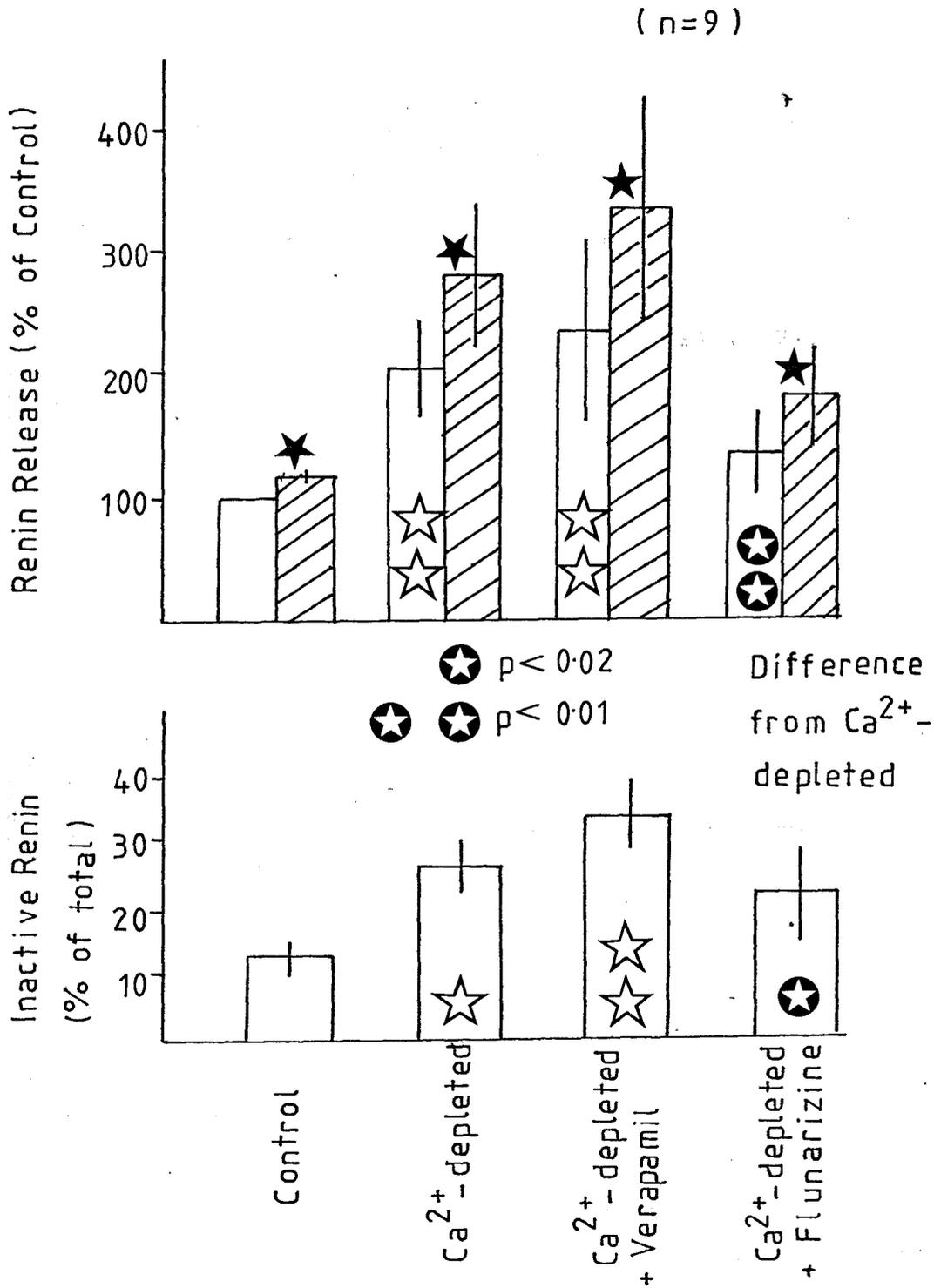


Fig.5:3 The Effect of Calcium Antagonists on Renin Release in Response to Ca²⁺ deprivation

TABLE 5.6 The Effect of Calcium Antagonists on the Response of Renin Release to Ca-depletion.

Incubation Medium	Active Renin (% of Control)	Inactive Renin (% of Total)
Control	100	12.5 ± 2.5
Ca-depleted	203.4 ± 39.2 ☆	26.1 ± 3.9 ☆
Ca-depleted + Verapamil (50 μ Moles/l)	233.8 ± 71.4 ☆	34.1 ± 5.0 ☆
Ca-depleted + Flunarizine (50 μ Moles/l)	135.2 ± 33.4	22.3 ± 7.0

Results shown as mean ± s.e.m of data from ten experiments.
 ☆ p < 0.01 Difference from Control.

The results obtained in this experiment indicate that flunarizine antagonises the stimulation of both active and inactive renin release by calcium depletion. Verapamil, however, did not change this response.

DISCUSSION

Some reports have suggested that verapamil has no effect on basal renin secretion in the rat (Logan & Chatzitis, 1980) and cat (Lester & Rubin, 1977). However, both flunarizine and verapamil tended to increase the rate of active renin release by rabbit kidney cortex slices in vitro, although these changes were not statistically significant. Inactive renin release in vitro was greatly increased by both of the antagonists. This finding provides further evidence that the control of inactive renin release may be partially independent from the active form. Furthermore, if renin release from granular cells is determined by the intracellular concentration of calcium, this observation indicates that flunarizine and verapamil may themselves cause a reduction in some critical calcium pool, and thereby stimulate the release of inactive renin.

Both low concentrations of Ca^{2+} and Ca^{2+} -depletion are thought to increase the release of renin by lowering the intracellular concentration of calcium in juxtaglomerular cells. In the experiments where slices were incubated in Ca^{2+} -free media, flunarizine partially antagonised the stimulatory effect of this manipulation, by restoring the release of active and inactive towards control values. This change, however, did not represent a significant reduction in the response to low extracellular calcium concentration. When the extracellular calcium concentration was further reduced by including 5 mM EGTA in the incubation medium ($[Ca^{2+}] \approx 10^{-11}$ moles/l), flunarizine completely abolished the increase in renin release. This suggests that, in the renal cortex, flunarizine may inhibit the enhanced Ca efflux which occurs during calcium depletion. In other vascular tissues, flunarizine antagonises the increased Ca^{2+} influx which occurs during stimulation. Unlike the situation in other secretory systems however, calcium depletion stimulates the release of renin from granular cells. The finding that flunarizine inhibits this response may prove to be useful in elucidating the mechanism by which calcium regulates the release of renin.

In contrast to flunarizine, verapamil had no inhibitory effect on the response of active or inactive renin to low extracellular Ca or to calcium depletion. Indeed, verapamil tended to increase renin release and, in particular inactive renin, in these experiments.

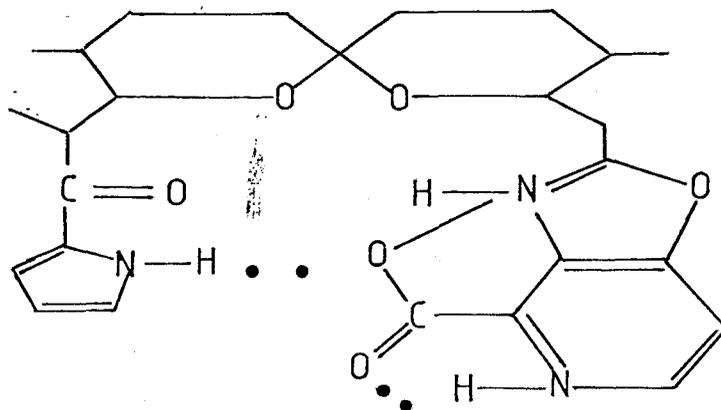
Recently, Baumbach & Skott (1981) reported that the methoxy derivative of verapamil, D600, increased the response of isolated rat glomeruli to calcium deprivation. The difference in effect of the two antagonists used could be related to differences in their tissue selectivity. Using contracted vascular smooth muscle strips and cat papillary muscle, Van Neuten (1978) demonstrated that flunarizine was 150 times more selective for vascular smooth muscle than for cardiac tissues. In the same experiments, verapamil showed no such selectivity. Since JG granular cells are modified vascular smooth muscle cells, flunarizine could be displaying selective antagonism of calcium efflux from these cells.

THE EFFECT OF THE CALCIUM IONOPHORE A23187 ON RENIN RELEASEIn Vitro

Ionophores form lipid-soluble complexes with polar cations and thereby increase the permeability of the cell membrane to these ions. The properties of the earliest ionophores, which were metabolites of micro-organisms, were recognised by virtue of their stimulatory effect on energy-linked transport in mitochondria. Synthetic compounds which share their characteristics are now available. The properties and biological applications of ionophores have been reviewed by Pressman (1976).

A23187 is one of the group of carboxylic ionophores, and has high affinity for divalent cations, and little or no affinity for monovalent ones. Like other ionophores, the backbone of the A23187 molecule assumes a critical conformation around a cavity in space which is surrounded by oxygen atoms (see Fig. 5.4). Ionophores effectively engulf the cation by displacing the water of hydration. The lipid solubility of the complex which is formed, can be explained by effective shielding of the polar interior by the remainder of the ionophore molecule. The selectivity of A23187 for divalent cations has been attributed to the presence of nitrogen atoms within the complexation sphere.

FIG. 5.4 The Structure of A23187.



The calcium ionophore A23187 has been used in many secretory tissues to study stimulus-secretion coupling. In accordance with the stimulatory role that calcium plays in these systems, A23187 has been found to increase the rate of secretion. However, A23187 has been reported to inhibit the release of renin from the isolated, perfused rat kidney (Fynn, Onomakpome & Peart, 1977) and from isolated, superfused rat glomeruli (Baumbach & Leyssac, 1977). In contrast to these findings, Marada, Lester & Rubin (1979) found that the calcium ionophore caused a dose-related increase in renin secretion in the isolated, perfused cat kidney. This effect was abolished by pre-treatment with reserpine, and was partially antagonised by propranolol, which suggests that the stimulation was mediated by the release of catecholamines. However, these authors also reported that A23187 increased renin secretion by perfused cat glomeruli.

The reason for these discrepancies were not clear, but all of the authors agreed that the effects of the ionophore were dependent on the presence of external calcium. In both species, renin secretion was increased by the ionophore in the absence of calcium. However, in only one of the above experiments (Baumbach & Leyssac, 1977) was the effect of calcium depletion alone investigated.

In the previous experiment, both verapamil and flunarizine were found to increase the release of inactive renin in vitro. In addition, these calcium antagonists caused active renin release to rise in the presence of external calcium. To investigate further the role of calcium in renin secretion, the calcium ionophore was included in incubation media.

MATERIALS AND METHODS

Rabbit kidney cortex slices were prepared as previously described (see page 106) and were incubated in flasks which contained 5 ml of one of the following media:

- 1) Unmodified Krebs'-Ringer bicarbonate buffer (Incubated Control).
- 2) Ca^{2+} -free Krebs'-Ringer bicarbonate buffer.
- 3) Ca^{2+} -depleted Krebs'-Ringer bicarbonate buffer.

The calcium ionophore, A 23187 (1.78 mg) was dissolved in 97% methanol, and 50 μl of this was added to the flasks to give a final concentration of 17 μM Moles/l. A second series of flasks to which 50 μl of the methanol vehicle had been added, served as controls. Each incubation was carried out in duplicate, using one slice from each kidney.

RESULTS

Active renin released from control slices which were incubated in flasks which contained 50 μl of 97% methanol was 3.4 ± 0.4 ng ang I/mg/h. Inactive renin comprised $14.6 \pm 3.3\%$ of the total renin released into the supernatant. This shows that renin secretion was unaffected by inclusion of this amount of methanol. When slices were incubated in unmodified Krebs' buffer (no methanol present) active renin release was 4.4 ± 0.4 ng ang I/mg/h, and inactive renin formed $15.0 \pm 3.0\%$ of total renin.

Table 5.7 shows the effect of the calcium ionophore on renin release by cortex slices incubated in the control medium (2.3 m Moles/l Ca). As in previous experiments, the results have been subjected to a percentage transformation (Table 5.8) in order to show any change in the response (Fig. 5.5).

TABLE 5.7 The Effect of the Calcium Ionophore on Basal Renin Release.

Incubation Medium	Renin Release (ng ang I/mg/h)		
	Active	Total	Inactive
Control (2.3 m Moles/l Ca)	3.4 ± 0.4	4.1 ± 0.6 *	0.7 ± 0.2
Control + A 23187 (17 μ Moles/l)	3.7 ± 0.4	4.7 ± 0.6 *	1.0 ± 0.4

Results shown as mean ± s.e.m. of data from nine experiments.

* p < 0.01 Increase after acidification.

TABLE 5.8 Basal Renin Release in the Presence of A 23187

Incubation Medium	Active Renin (% of Control)	Inactive Renin (% of Total)
Control	100	14.6 ± 3.3
Control + A 23187	115.6 ± 14.2	18.7 ± 6.7

Results are shown as mean ± s.e.m. of data from nine experiments.

These results show that the calcium ionophore had no significant effect on the basal rate of renin secretion. This finding is rather surprising, since the ionophore is thought to increase the Ca permeability of the cell membrane and thereby increase the intracellular concentration of calcium. From the results of previous experiments, A 23187 could therefore have been expected to reduce the secretion of both forms of renin.

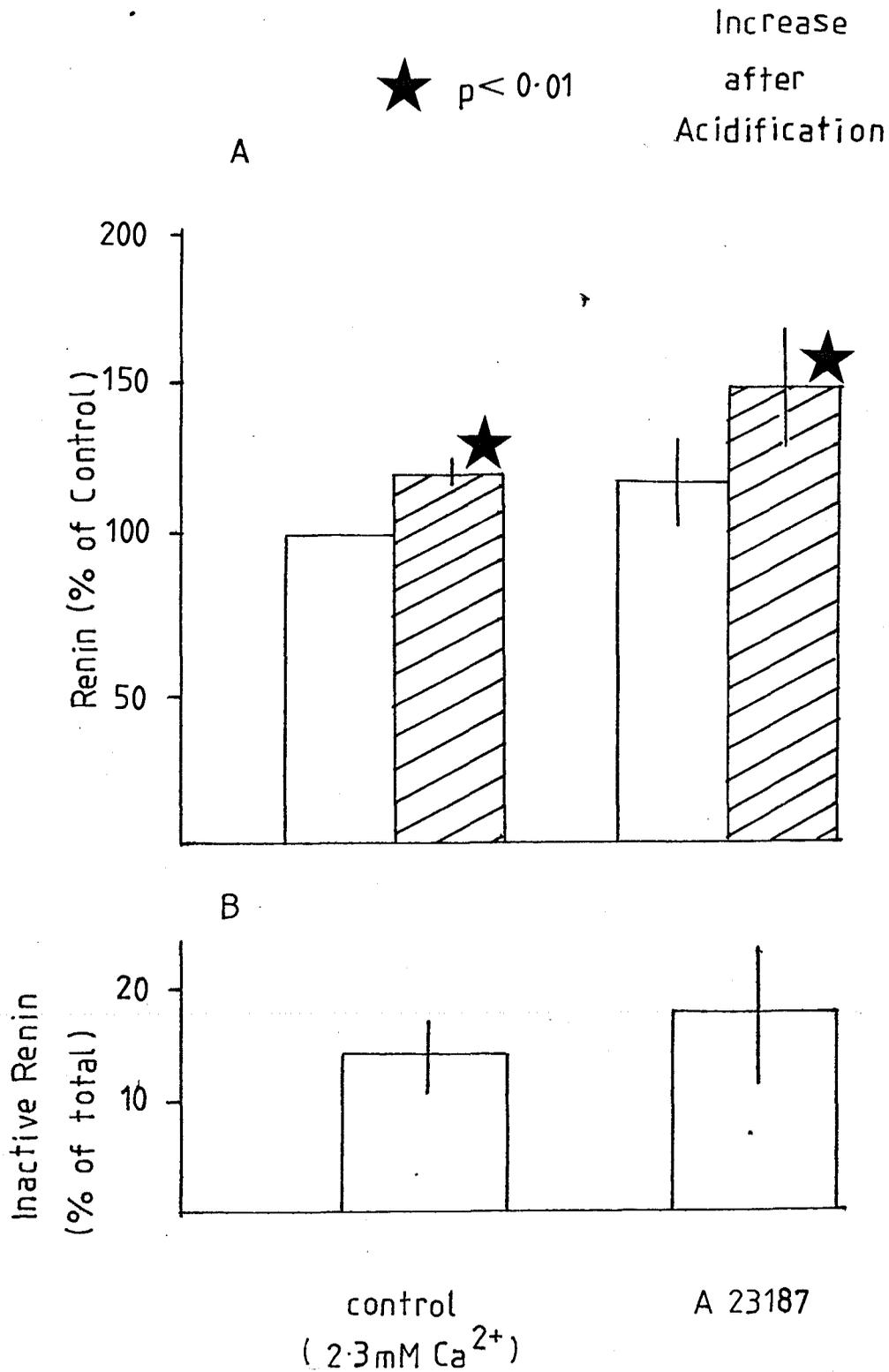


Fig. 5:5 The Effect of the Calcium Ionophore on Basal A) Active and B) Inactive Renin Release In Vitro (n=9)

Active renin release increased to 4.6 ± 0.9 ng ang I/mg/h when the slices were incubated in Ca-free media. A similar increase in the amount of inactive renin from 0.7 ± 0.2 to 1.5 ± 0.5 ng ang I/mg/h was also observed. However, neither of these changes were statistically significant (see Table 5.9). The difference in the response to low extracellular calcium in the presence of A 23187 is shown in Fig. 5.6. Although active renin release did not change significantly when the ionophore was present in the Ca-free medium, inactive renin was significantly reduced ($p < 0.02$) to 0.3 ± 0.2 ng ang I/mg/h and formed only $3.8 \pm 3.3\%$ of total renin activity in the supernatant (Table 5.10).

TABLE 5.9 The Effect of A 23187 (17μ Moles/l) on Renin Release (Ca-free media)

Incubation Medium	Renin Release (ng ang I/mg/h)		
	Active	Total	Inactive
Control ($2.3 \text{ m Moles/l Ca}^{2+}$)	3.4 ± 0.4	4.1 ± 0.6 ★	0.6 ± 0.2
Ca-free	4.6 ± 0.9	6.2 ± 1.1 ★	1.6 ± 0.5
Ca-free + A 23187	4.5 ± 0.6 ☆	4.8 ± 0.7	0.3 ± 0.2

Results shown as mean \pm s.e.m. of data from nine experiments.

☆ $p < 0.02$ Difference from Control ★ $p < 0.01$ Increase on Acidification.

These results show that A 23187 inhibits the increase in inactive renin secretion that occurs when kidney cortex slices are incubated in Ca-free media. In contrast, the response of active renin to low extracellular $[\text{Ca}^{2+}]$. A similar result was obtained using slices which were incubated in Ca-depleted media (see Fig. 5.7).

TABLE 5.10 . The Effect of A 23187 (17 μ Moles/l) on the Response to Low Extracellular $[Ca^{2+}]$

Incubation Medium	Active Renin (% of Control)	Inactive Renin (% of Total)
Control (2.3 m Moles/l Ca)	100	14.6 \pm 3.3
Ca-free	132.7 \pm 15.9	24.2 \pm 5.7
Ca-free + A 23187	133.7 \pm 12.9	3.8 \pm 3.3 ☆

Results are shown as mean \pm s.e.m. of data from nine experiments.

☆ $p < 0.02$ Difference from Ca-free.

Active renin release in vitro increased significantly to 5.2 ± 0.8 ng ang I/mg/h ($p < 0.01$) in slices which were incubated in the presence of EGTA (5 m Moles/l). Ca-depletion also caused a significant increase ($p < 0.05$) in the amount of inactive renin which was released (see Table 5.11). The response of inactive renin to Ca-depletion completely inhibited ($p < 0.01$) when the calcium ionophore was present. Active renin release however, was unchanged.

★ $p < 0.01$. Increase on Acidification.

$p < 0.02$ Difference from Control (active).

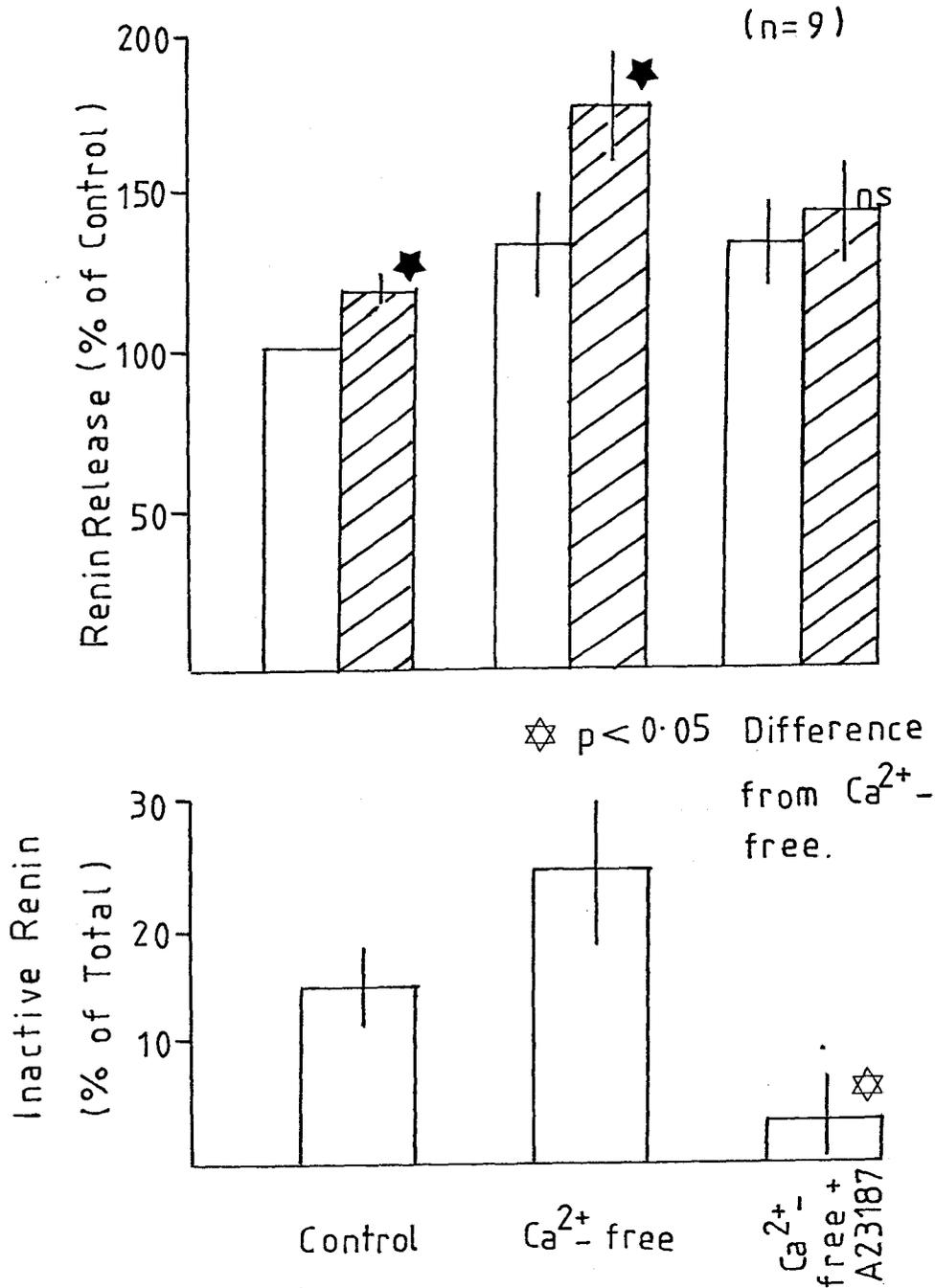


Fig. 5:6 The Effect of the Calcium Ionophore on Renin Release in Response to Low [Ca²⁺]

★ $p < 0.01$ Increase on Acidification

$p < 0.05$ Difference from Control

$p < 0.01$

(n = 9)

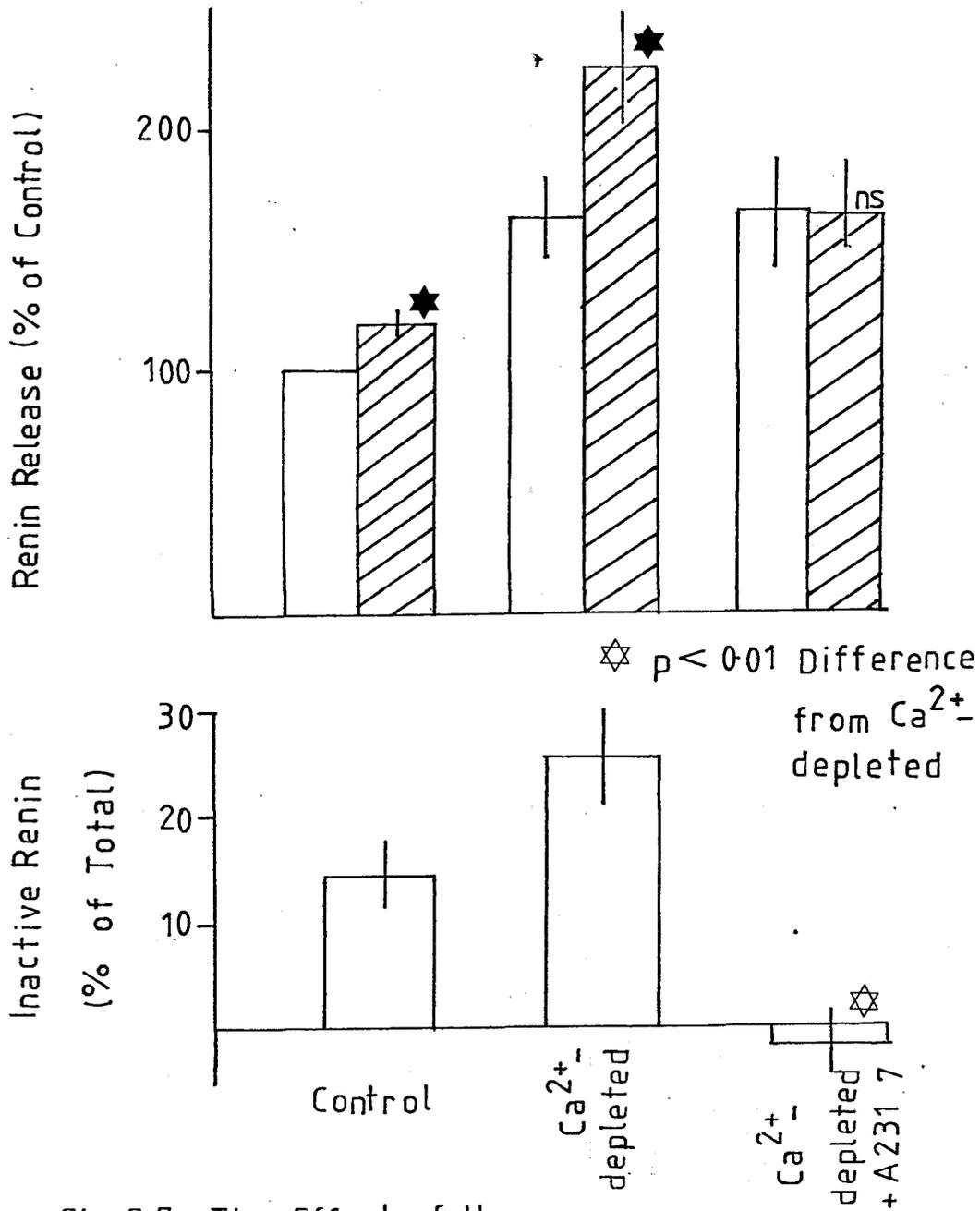


Fig. 5:7 The Effect of the Calcium Ionophore on Renin Release in Response to Ca²⁺-Deprivation

TABLE 5.11 The Effect of A 23187 (17 μ Moles/l) on Renin Release in Ca-depleted Media.

Incubation Medium	Renin Release (ng ang I/mg/h)		
	Active	Total	Inactive
Control (2.3 m Moles/l Ca ²⁺)	3.4 \pm 0.4	4.1 \pm 0.6 *	0.7 \pm 0.2
Ca-depleted	5.2 \pm 0.6 $\star\star$	7.3 \pm 1.0 *	2.1 \pm 0.6 \star
Ca-depleted + A 23187	5.2 \pm 0.8 $\star\star$	5.2 \pm 0.9	0.04 \pm 0.2

Results are shown as mean \pm s.e.m. of data from nine experiments.

\star p < 0.05 Difference \star p < 0.01 Increase after
 $\star\star$ p < 0.01 from Control Acidification

TABLE 5.12 The Effect of the Calcium Ionophore on the Response to Calcium Depletion.

Incubation Medium	Active Renin (% of Control)	Inactive Renin (% of Total)
Control (2.3 m Moles/l Ca)	100	14.6 \pm 3.3
Ca-depleted	161.4 \pm 16.2	25.6 \pm 4.3
Ca-depleted + A 23187	163.3 \pm 22.4	1.7 \pm 3.1 \star

Results are shown as mean \pm s.e.m. of data from nine experiments.

\star p < 0.01 Difference from Ca-depleted.

DISCUSSION

The results of this experiment are rather surprising. If the calcium flux across the granular cell membrane, and, therefore, the intracellular concentration of calcium, is the final determinant of renin release, then it could have been expected that renin release by rabbit kidney cortex slices in vitro would be reduced by A 23187. However, no change in either active or inactive renin could be detected when calcium was present in the incubation medium. Removal of calcium stimulated the secretion of both forms of renin, but when the ionophore was present, inactive renin was virtually absent from the supernatant while active renin remained unchanged. These results suggest that the calcium flux per se may not be the sole determinant of renin secretion. Since granular cells are modified vascular smooth muscle cells, it may be useful to study more closely the ionic basis for contraction of blood vessels.

In that tissue, arterial smooth muscle spike discharges are associated with influx of sodium. The first indication of this was observed by Keatinge (1968a) who showed that spontaneous spike discharges could be obtained when sheep carotid arteries were placed in a Na-based medium which was free of both Ca and Mg. Addition of the divalent cations caused slight repolarization and cessation of spike activity. Spike discharges in Na-free media could also be reduced by replacing external Na with Tris. A later study (Keatinge, 1968b) showed that there was an influx of sodium associated with each spike discharge. In contrast to this, slow waves can only be elicited from arteries if either Ca or Mg is present (Keatinge, 1978a).

It is thought that Ca and Mg stabilize the cell membrane by increasing the electrical potential gradient in the region of the activating gates for the voltage-dependent Na channels. In this respect, vascular smooth muscle cells are similar to those of skeletal muscle, where calcium also stabilises the membrane by determining the conductance of sodium (Constantin, 1968). However, spike discharges did not behave like Na-based action potentials, since they were insensitive to tetrodotoxin (Keatinge, 1968a) and it is now clear that spike discharges are attributable to a channel which is permeable to both Na and Ca (Keatinge, 1978a).

When they are placed in Ca-free media, vascular smooth muscle

cells become loaded with sodium, and it is this influx which elicits spike discharges. By analogy, it is possible that stimulation of renin release in Ca-depleted media could be the result of sodium loading. Such a stimulation could also explain why reducing $[Na^+]$ antagonised the effect of removal of external calcium on renin secretion in vitro (see page 174). When Na is reduced in the absence of calcium, the cells cannot become sodium-loaded, and thus renin secretion is restored to control values. In addition, the effect of the calcium antagonists (see page 210) could also be explained by an alteration in sodium influx. By causing a general inhibition of calcium entry into granular cells, verapamil and flunarizine could increase the influx of sodium, thereby increasing ^{inactive} renin secretion.

Influx of sodium into granular cells could also determine the rate of conversion of inactive renin to the active form. For example, during reduced $[Na^+]$, the influx of extracellular sodium into the cell is reduced. Furthermore, active renin increases while inactive renin is reduced, which suggests that activation of inactive renin is a sodium-sensitive mechanism. The effect of including A 23187 in the Ca-depleted incubation media is similar to the effect of Low $[Na^+]$. By stabilizing the cell membrane, A 23187 reduces the influx of sodium, which in turn could increase the conversion of inactive to active renin. Thus, active renin in the slice supernatant increases, while inactive renin is reduced. In contrast to this, hyperpolarization by reducing extracellular K (see page 168) reduces the activity of the Na/K ATPase pump. This in turn causes an increase in the influx of sodium, and could reduce the conversion of inactive renin. Thus, active renin in the supernatant is unchanged, while inactive renin increases.

A change in the influx of sodium cannot completely explain the results which have been obtained. Depolarization with high K would increase the permeability of the cell membrane to other ions. The resultant Ca influx and increase in intracellular calcium has been proposed to inhibit renin secretion (Fray & Park, 1979). However, depolarization would also increase the intracellular concentration of sodium. This influx could inhibit conversion of inactive renin and therefore increase the amount of inactive renin in the supernatant. It would also tend to increase active renin, according to the above hypothesis.

It is therefore clear that both sodium and calcium may be in-

portant in determining the secretion of renin. The effect of ouabain, which inhibits the Na/K ATPase, on renin release in vitro, and the responses of renin to other stimulatory agents could be useful in elucidating these mechanisms. It is interesting that ouabain blocked the stimulatory effect of noradrenaline on renin secretion from dog kidney cortex slices in vitro (Park, Han & Fray, 1981). Furthermore, the stimulatory effect of isoprenaline on renin release from the isolated perfused rat kidney was reduced by lowering the concentration of Na in the perfusing medium from 145 to 25 m Moles/l (Fray & Park, 1979).

Much of the controversy surrounding the role of sodium in the regulation of renin secretion has arisen as a result of the differing responses to altered $[Na^+]$ which are obtained when the rat has been used as an experimental model, and when other species such as the dog, the rabbit and man have been investigated. The major difference between these species is that the rat kidney does not appear to secrete inactive renin. This observation may prove to be important in clarifying the mechanism that is involved in stimulus-secretion coupling for renin release.

It has been previously suggested (see page 207) that the single renin secreting mechanism present in the rat corresponds to the inactive renin secretion mechanism in animals which secrete both active and inactive renin. This concept produces some clarification of the existing literature concerning the effect of the calcium ionophore on renin secretion. From the data reported here, it would be expected that the ionophore would inhibit active renin secretion in the rat, and stimulate it in other species such as the cat. This has indeed been reported (see page 223).

Rabbit active renin, in many cases was increased or reduced in parallel with inactive renin, eg. when $[Ca^{2+}]$ or $[K^+]$ was increased. However, in other situations such as hyperpolarization by low $[K^+]$, only inactive renin secretion was stimulated. Reducing $[Na^+]$ in incubation media increased active, but decreased inactive renin release. This could suggest that changes in active renin secretion are partially dependent on activation of inactive renin. The results which have been reported here and in Chapter 4 of this thesis provide further evidence to suggest that activation of inactive renin could be determined by a sodium-sensitive mechanism. In addition, the secretion of both forms of renin appears to be regulated by cal-

cium ions which inhibit their release.

Stimulus-secretion coupling for renin is a relatively recent area for research. Clearly, there remain many questions which must be answered before the operation of this mechanism is clarified. There is now a great deal of evidence in the literature to support the hypothesis that calcium plays a central inhibitory role in regulating the secretion of renin from the rat kidney. However, the work which has been described in this thesis indicates that other factors, possibly sodium ions, may be important also for stimulus-secretion coupling of renin release in those species whose kidneys store and secrete inactive renin.

CONCLUSIONS

CONCLUSIONS

The work described in this thesis has been concerned with two broad areas of interest. In Chapter 3, the possibility was investigated that a serine protease enzyme, in particular a kallikrein, could be a physiological activator of inactive renin.

Acid-activation of inactive renin in pooled plasma from normal rabbits was inhibited by the protease inhibitor Trasylol. This suggested that kallikrein, or some other serine protease, was necessary for in vitro activation of inactive renin. However, no initial change in the relative proportions of either active or inactive renin could be detected after intravenous administration of Trasylol to rabbits. This was despite a large reduction of plasma kallikrein activity. Some 90 minutes after giving the inhibitor, both active and inactive renin were reduced. This appeared to be related to accumulation of Trasylol within renal tissue. Indeed, a high concentration of Trasylol reduced the secretion of both forms of renin from rabbit kidney cortex slices in vitro. Since the protease inhibitor only partially inhibited acid-activation of inactive renin in the supernatant collected after an in vitro incubation, it was concluded that Trasylol inhibited release of both forms of renin. Whether this was due to a direct action of Trasylol on renin-secreting cells, or was related to the reduction in activity of the renal kallikrein-kinin system is unclear.

Taken together, these studies indicated that plasma kallikrein is not the major factor which determines the relative proportions of active and inactive renin circulating in plasma. Plasma renin concentration appeared to be determined intrarenally. Therefore, an investigation into the regulation of secretion of active and inactive renin was begun using a rabbit kidney cortex slice preparation.

In recent years, the finding that calcium ions inhibited the release of renin has aroused a great deal of interest. As yet, there is only very limited published data concerning stimulus-secretion coupling for inactive renin. In accordance with the current hypothesis, increasing extracellular $[Ca^{2+}]$ inhibited the secretion of active renin by kidney slices and removal of calcium stimulated its release. A similar relationship between $[Ca^{2+}]$ and inactive renin was also observed.

The inhibitory effect of depolarization by high $[K^+]$ on both active and inactive renin was thought to be mediated by an increase in membrane permeability, and the subsequent rise in intracellular calcium. However, the results which were obtained when slices were incubated in hyperpolarizing media (1 m Mole/l K^+) were rather surprising in relation to the existing hypothesis. Although total renin secretion increased under these conditions, this was the result of an increase in inactive renin secretion only, when calcium was present. Active renin secretion did not change. In the absence of extracellular calcium, hyperpolarization, which could be expected to reduce the rate of calcium efflux from JG cells, restored only active renin to control values.

In common with the effect of changing $[K^+]$, the response of renin-secreting cells to changing $[Na^+]$ was modified in the absence of external Ca^{2+} . In the presence of calcium, reducing $[Na^+]$ caused active renin secretion to rise and reduced inactive renin. However, reducing $[Na^+]$ partially antagonised the stimulation of active renin secretion that had been induced by calcium depletion. Inactive renin, in this case, was not significantly different from control values. These effects can be explained, at least in part, if activation and secretion of inactive renin are regulated by a sodium-sensitive mechanism.

Increasing the concentration of magnesium had little effect on renin release in the presence of calcium. However, both active and inactive renin released were reduced in an Mg^{2+} -free medium. This is thought to increase intracellular free Ca^{2+} , which thus leads to an inhibition of secretion. Accordingly, low $[Mg^{2+}]$ antagonised the effect of calcium depletion. Increasing $[Mg^{2+}]$ also partially antagonised the enhanced secretion of renin after calcium depletion by restoring active renin secretion to control values. Magnesium ions may replace calcium ions, and therefore this effect might be attributed to their stabilizing effect on the cell membrane.

Further evidence to support the concept that a sodium-sensitive mechanism could be important in regulating activation and secretion of inactive renin was obtained from experiments in which the two calcium antagonists, verapamil and flunarizine, and the calcium ionophore A 23187 were added to the incubation medium. Flunarizine, which may be more selective for vascular smooth muscle than verapamil antagonised the effect of calcium depletion on renin release. In

contrast, verapamil tended to increase still further the release of both forms of renin. In the presence of calcium, A 23187 did not cause any significant change in either form of renin. Addition of the ionophore to buffers which contained low $[Ca^{2+}]$ almost completely abolished inactive renin secretion. The presence of the ionophore did not significantly change active renin, which was similar to its value in Ca^{2+} -free and Ca^{2+} -depleted media.

This finding provided evidence that some other factor could be important in determining the secretion of renin in the rabbit. Although changes in intracellular $[Ca^{2+}]$ could account for many of the changes in inactive renin secretion that had been observed, alterations in the secretion of active renin did not necessarily follow a similar pattern. Regulation of the secretion of active renin could be partially dependent on activation of inactive renin.

These experiments have shown that there could be a fundamental difference between the rat, which is often used as an experimental model for studies of the renin-angiotensin system, and other species such as the rabbit, which secrete inactive renin. It is possible to achieve considerable clarification of contentious areas in the existing literature if the single renin secretion mechanism in the rat is correlated with the data for inactive renin secretion in the rabbit. In species which secrete two forms of renin, it may prove important to consider changes in intracellular sodium, in addition to changes in calcium, when studying the secretion of renin. These two ions are not independent of each other, since any change in extracellular or intracellular calcium will subsequently alter the integrity of the plasma membrane. This, in turn, will have an effect on the permeability to sodium.

Whatever the precise details of the mechanisms which regulate inactive renin secretion, it seems clear that interconversion between the two forms of renin, prior to secretion from the kidney, or independent secretion from two stored pools of the hormone, is an important level for control in the renin-angiotensin system.

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