PURIFICATION AND CHARACTERISATION OF TWO FORMS OF RENIN FROM HUMAN RENAL CORTEX.

by

GORDON DOUGLAS McINTYRE

Thesis submitted to the University of Glasgow
for the degree of Doctor of Philosophy.

M.R.C. Blood Pressure Unit,
Western Infirmary, Glasgow,
and
Department of Biochemistry,
University of Glasgow.

November, 1983

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

Dedication

To my mother and father

Acknowledgements

I am deeply indebted to my supervisors, Dr B.J. Leckie and Dr J.R. Coggins, for their encouragement and for advice given to me throughout the course of my studies, and to Dr R.Y. Thompson, who in his capacity as my 'auditor', has taken a keen interest in my welfare and progress.

My thanks to Dr A.F. Lever for making available to me the facilities of the MRC Blood Pressure Unit and to Professor R.M.S. Smellie for making available to me the facilities of the Department of Biochemistry.

I wish to thank Imperial Chemical Industries plc for financial support throughout my three years of study.

My thanks also go to the following persons for their assistance in various aspects of this work:

Dr G. Lindop of the Department of Pathology, Western Infirmary for his assistance in obtaining human kidneys.

Dr A.D. Weaver of the University of Glasgow Veterinary Hospital for nephrectomising oxen for the preparation of ox renin substrate.

Dr S. Chaudhuri of the Department of Biochemistry for assistance with peptide separations by HPLC.

Mr J. Jardine of the Department of Biochemistry for carrying out the amino acid analyses.

Mrs M. Hughes of the MRC Blood Pressure Unit for the routine preparation of (^{125}I)angiotensin I.

I also express my gratitude to Miss Ann Matheson for expertly typing this thesis and to Mr I. Ramsden for his excellent illustrations.

Finally, I wish to thank my family and friends for their support during the writing of this thesis.

Statement of Originality

All the experimental work reported in this thesis is my own.

Abbreviations

Abbreviations used are as recommended in the Biochemical Journal Instructions to Authors, 1981, with the following additions:

AI:	angiotensin I
BAEE:	benzoyl arginine ethyl ester
SBTI:	soybean trypsin inhibitor
SDS:	sodium dodecyl sulphate
TEMED	N,N,N',N'-tetramethylethylenediamine

H.77: D-His-Pro-Phe-His-Leu^{R}Leu-Val-Tyr

H.77 is an analogue of the His^{6}-Tyr^{13} octapeptide sequence of equine angiotensinogen in which the N-terminal histidine residue has been replaced with D-histidine and the cleavage site has been modified by the introduction of a reduced peptide bond (R: -CH_2-NH-) in place of the peptide bond normally cleaved by renin.

In the description of ^{125}I-labelled compounds, the use of circular brackets does not imply that all the iodine was the radioactive isotope.

Definition of enzyme units

Acid proteinase: one unit of activity was defined as the activity exhibited by 1µg of pure human spleen cathepsin D in the assay system described in Section 2.4(c).

Human renin: one unit of human renin (MRC reagent 68/356) generated angiotensin I at a rate of 182.8µg/h in the assay system described in Section 2.4(a).

Pig renin: one unit of pig renin (MRC reagent 65/119) generated angiotensin I at a rate of 96.2µg/h in the assay system described in Section 2.4(a).

Trypsin: one BAEE unit of trypsin activity was defined as the activity required to produce an increase in extinction at 253nm of 0.001/min under the assay conditions described in Section 2.4(d).

CONTENTS

INDEX OF FIGURES

INDEX OF TABLES

SUMMARY

1. A new affinity column for renin was prepared by coupling the isosteric peptide inhibitor of renin, H.77 (D-His-Pro-Phe-His-Leu^{R}Leu-Val-Tyr, where R is a reduced peptide bond, -CH_2-NH-), to activated 6-aminohexanoic acid-Sepharose 4B. Chromatography of a crude extract of human kidney cortex on this material resulted in a 5,500-fold purification of renin in 76% yield. The purified enzyme (specific activity 871 units/mg) was free of non-specific acid proteinase activity and was stable at pH 6.8 and -20°C over a period of several months. Examination of this preparation by electrophoresis in the presence of 0.1% (w/v) SDS showed that it contained both renin (M_r 40,000), and an unidentified protein of M_r 17,000.

2. An inactive form of renin was purified from human renal cortex using a combination of chromatography on H.77-Sepharose (to remove active renin), ammonium sulphate fractionation, hydrophobic interaction chromatography and affinity chromatography using a monoclonal immunoadsorbent. This procedure resulted in a 14,750-fold purification of inactive renin in 27% yield. The purified protein was stable at pH 7.4 and -20°C over a period of several months. Examination of this preparation by electrophoresis in the presence of 0.1% (w/v) SDS showed that it consisted of a single polypeptide chain of M_r 48,000. An M_r value of 51,000 was obtained by gel filtration.

The purified inactive renin was completely inactive against ox angiotensinogen, but could be activated by limited proteolysis. Trypsin treatment of inactive renin produced an active form of renin (M_r 47,000 by gel filtration) with a specific activity of 708 units/mg inactive renin. Inactive renin could also be activated by dialysis to pH 3.3. However, acidified inactive renin was re-inactivated by dialysis back to neutral pH.

3. A structural comparison of active renin and inactive renin was carried

out by peptide 'mapping' of ^{125}I-labelled samples of the purified proteins. One dimensional peptide mapping of the labelled proteins in polyacrylamide gels (Cleveland mapping) suggested a structural similarity between the two forms of renin. Analysis of V8 proteinase digests of the labelled proteins by reverse phase high performance liquid chormatography indicated that there are extensive regions of common sequence in the active and inactive forms of renin.

4. The results obtained in the present study indicate that inactive renin is a renin zymogen.

CHAPTER 1

INTRODUCTION

Renin (EC 3.4.99.19) is a highly specific endoproteinase which is synthesised mainly in the juxtaglomerular cells of the afferent glomerular arteriole of the kidney. It is released from the kidney into the circulation where it cleaves the α_2-globulin angiotensinogen to produce the inactive decapeptide, angiotensin I. Subsequently, angiotensin I is cleaved by a peptidyl dipeptide hydrolase (converting enzyme) to give the vasoactive octapeptide, angiotensin II, which has numerous actions concerned with sodium and fluid homeostasis and with the maintenance of blood pressure (Figure 1.1) (Brown et al., 1983; Leckie and Semple, 1983).

Prior to 1970, renin was thought to be an acid-stable enzyme of M_r 40,000. Although there were suggestions that an enzymatically inactive precursor of renin might exist (Meyer et al., 1965; de Vito et al., 1970) direct evidence was lacking. The only variants seen were forms differing slightly in their isoelectric points (Skeggs et al., 1967). The discovery by Lumbers (1971) of an inactive form of renin which could be activated by acidification was therefore of major interest. Since then, high molecular weight forms of renin which can be activated by acidification, limited proteolysis, or exposure to low temperatures, have been found in the kidneys and plasma of several species, including man. The literature concerning the subject has become extensive and reviews have been published by Inagami and Murakami (1980), by Sealey et al. (1980) and by Leckie (1981). However, despite a decade of intensive research, neither the precise chemical nature of the possible precursor forms of renin nor their physiological importance are clearly understood.

1.1 Purification and properties of renin

Although renin was discovered by Tigerstedt and Bergman in 1898, it has

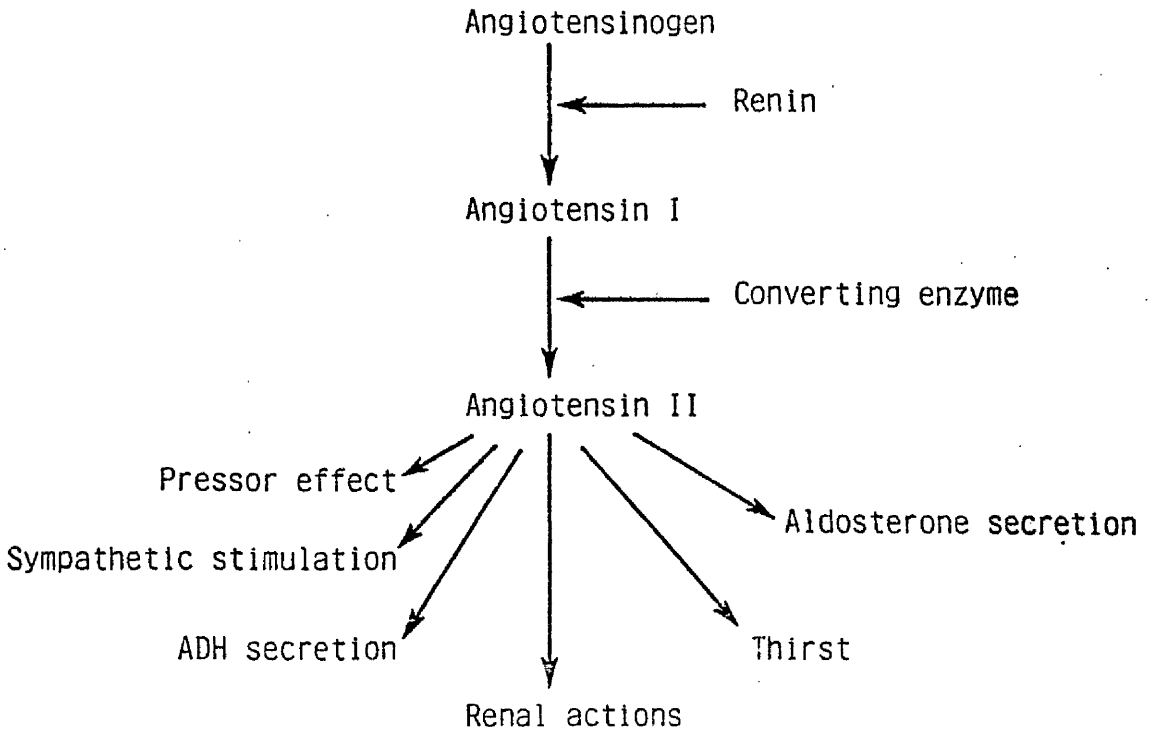

Figure 1.1 **Outline of the renin-angiotensin system and some of the main actions of angiotensin II.**

been purified to homogeneity only recently. Early attempts at renin purification were hindered by the extremely low concentrations of renin in the kidney, and by the instability of the enzyme during purification (Peart et al., 1966). However, the application of affinity chromatography to this problem, and in particular the use of the acylated pentapeptide pepstatin as an affinity ligand, has resulted in the complete purification of renin from hog (Murakami and Inagami, 1975; Corvol et al., 1977; Inagami and Murakami, 1977), rat (Matoba et al., 1978), dog (Dzau et al., 1979) and human (Yokosawa et al., 1978; Slater et al., 1978; Yokosawa et al., 1980) kidney.

Renin or renin-like enzymes are present in a variety of extrarenal tissues including the uterus and placenta (Symonds et al., 1968), amniotic fluid (Brown et al., 1964), adrenal gland (Naruse et al., 1983), vascular walls (Re et al., 1982) and brain (Inagami et al., 1982a). Renin is also present in the submaxillary gland (Bing et al., 1980). In the mouse, the synthesis of submaxillary gland renin is under both genetic and hormonal control and in certain strains of mice the enzyme may represent up to 5% of the total protein content of the gland (Wilson et al., 1977; Wilson et al., 1981; Wilson et al., 1982). Although the physiological relevance of this phenomenon is unknown, it has allowed the purification of renin in sufficient quantities to allow the determination of its primary structure (Misono et al., 1982).

The properties of purified submaxillary gland renin and those of the purified renal enzymes are summarised in Table 1.1.

Renin is a member of the aspartic proteinase group of enzymes which also includes pepsin, chymosin, cathepsin D and various microbial proteinases. Like these enzymes, renin possesses two carboxyl groups which are essential for catalysis. The chemical modification of either of these groups leads to an irreversible loss of enzyme activity. Thus, renin is inactivated by 1,2-epoxy-3-(p-nitrophenoxy)propane and by active site directed diazo compounds (McKown

Table 1.1

Properties of purified renins from various species

Properties	Species					
	Human[1]	Hog[2]	Rat[3,a]		Dog[4]	Mouse (submaxillary gland)[5]
M_r value						
a) Gel filtration	42,000	42,000	I,II,III:	41,000	42,000	43,000
b) Electrophoresis in presence of SDS	40,000	42,500	I: II: III:	37,000 36,000 35,000	36,000	30,000 (chain A) 5,500 (chain B)
c) Sedimentation equilibration	40,000	36,400	ND		ND	36,000-37,000
pH optimum	6.0[d] 8.0[e]	5.5-7.0[b,e]	4.0-4.5[b] 5.5-6.5[e] 6.0-6.5[f]		ND	6.5[c] 8.3[g]
Glycoprotein	Yes	Yes	Yes		Yes	No

Data taken from:

1. Yokosawa et al. (1980), Higaki et al. (1983)
2. Inagami and Murakami (1977)
3. Matoba et al. (1978)
4. Dzau et al. (1979)
5. Cohen et al. (1972), Inagami et al. (1977) Misono and Inagami (1982).

a. three isoenzymes, I,II and III have been reported
b. with octapeptide substrate (Reinharz and Roth, 1969)
c. with tetradecapeptide substrate (Skeggs et al., 1957)
d. with human angiotensinogen
e. with hog angiotensinogen
f. with rat angiotensinogen
g. with sheep angiotensinogen

ND = no data

and Gregerman, 1975; Misono and Inagami, 1980).

The primary structure of mouse submaxillary gland renin has been determined both by the analysis of the purified enzyme (Misono et al., 1982) and by the sequencing of the renin gene (Panthier et al., 1982a). The amino acid sequence of this enzyme shows considerable homology with the sequences of other aspartic proteinases. The sequence identity with porcine pepsin is 43%. Renin is also homologous with chymosin (34% identity) and with penicillopepsin (22% identity).

Compared with other aspartic proteinases, renin shows a highly restricted substrate specificity. Although renin will cleave a number of synthetic peptide substrates (Skeggs et al., 1957; Reinharz and Roth, 1969; Murakami et al., 1981), the only known naturally occurring renin substrate is angiotensinogen. The amino terminal sequences of equine, human and rat angiotensinogens are shown in Table 1.2. The minimum peptide sequence required for substrate activity appears to be the 6-13 octapeptide (Skeggs et al., 1957). Renin specifically cleaves the peptide bond between the amino acid residues at positions 10 and 11. In the horse and the rat, this is a leucyl-leucine bond. Human angiotensinogen has a leucyl-valine scissile bond. These differences are reflected in the species specificity of the renin-substrate reaction. Human angiotensinogen is cleaved only by human renin, whereas other mammalian angiotensinogens are cleaved by both human and animal renins.

The purified animal renins have a broad pH optimum in the range pH 5.5 to pH 7.0 when tested with homologous renin substrates, although both higher and lower pH optima can occur with heterologous substrates or synthetic peptide substrates (Inagami et al., 1977b). Human kidney renin has a somewhat narrower pH optimum with its homologous substrate at about pH 6.0 (Yokosawa et al., 1980).

Hog renin contains glucosamine and, with the exception of submaxillary

Table 1.2

Comparison of the N-terminal sequences of equine, human and rat angiotensinogens

	1	2	3	4	5	6	7	8	9	10	Renin ↓ 11	12	13	14
Horse [a]	asp	arg	val	tyr	ile	his	pro	phe	his	leu	leu	val	tyr	ser
Human [b]	asp	arg	val	tyr	ile	his	pro	phe	his	leu	val	ile	his	thr
Rat [c]	asp	arg	val	tyr	ile	his	prr	phe	his	leu	leu	tyr	tyr	ser

Key

a. Data from Skeggs et al. (1957)

b. Data from Tewksbury et al. (1981)

c. Data from Bouhnik et al. (1981)

gland renin, all mammalian renins appear to be glycoproteins based on their ability to bind to concanavalin A-Sepharose (Matoba et al., 1979; Yokosawa et al., 1980) or on a positive Schiff reaction (Corvol et al., 1977). The purified renal renins consist of a single polypeptide chain and have M_r values in the range 38,000-42,000 when measured by gel filtration. Slightly lower M_r values (35,000-37,000) have been obtained for hog, rat and dog renál renins using SDS/polyacrylamide gel electrophoresis or by sedimentation equilibrium analysis (Inagami and Murakami, 1977; Matoba et al., 1979; Dzau et al., 1979). Mouse submaxillary gland renin consists of two polypeptide chains (A,M_r 30,000 and B,M_r 5,500) linked by a disulphide bond (Misono and Inagami, 1982).

1.2 Biosynthetic precursors of renin

Many proteins are synthesized as inactive precursors or zymogens which are subsequently converted to active forms by limited proteolysis. This is true for a large number of extracellular proteinases including pepsin and chymosin, the best characterised of the aspartic proteinases. Pepsin, for example, is secreted by the chief cells of the gastric mucosa as the zymogen pepsinogen. In the acid environment of the stomach, pepsinogen is converted to pepsin both by an intramolecular reaction and by an intermolecular reaction involving newly formed pepsin (Hartsuck et al., 1977). During this conversion, several peptides containing a total of 44 amino acid residues are removed from the amino terminal end of pepsinogen.

In recent years it has become apparent that renin is also synthesized as a precursor.

Most eukaryote secretory proteins possess, during translation, an amino terminal extension of about 16 to 25 amino acid residues. This 'pre-' or 'signal' sequence directs the nascent polypeptide through the membrane of the endoplasmic reticulum into the cisternal space for eventual secretion of the protein from the cell (Blobel and Dobberstein, 1975). A membrane bound signal

peptidase cleaves the signal peptide from the growing polypeptide chain shortly after it enters the lumen of the endoplasmic reticulum and thus 'preproteins' rarely exist *in vivo*. They can, nevertheless, be demonstrated using cell-free translation systems, which are devoid of hydrolytic enzymes.

Poulsen *et al.* (1979b), using renin-specific antibodies to examine the products of cell-free translation of mouse submaxillary gland mRNA, have demonstrated the existence of a renin precursor of M_r 50,000. More recently, Lund *et al.* (1982) have found that a slightly smaller renin precursor of M_r 48,000 is produced by the translation of submaxillary gland mRNA microinjected into *Xenopus laevis* oocytes. Since *Xenopus* oocytes can remove signal peptides from the translation products of non-*Xenopus* mRNAs (Jilka *et al.*, 1979), these results indicate that the primary translation product of the renin gene contains a signal sequence of M_r 2,000.

A similar finding has been reported by Dzau *et al.* (1982). These authors found that the translation of mouse submaxillary gland mRNA by a rabbit reticulocyte lysate yielded an M_r 48,000 'preprorenin'. The addition of dog pancreatic microsomes, which contain signal peptidase activity, resulted in the conversion of the preprorenin to a renin precursor of M_r 46,000. The same authors carried out pulse labelling experiments in which the biosynthetic incorporation of ^{35}S-methionine into renin was studied using cultured mouse submaxillary gland tissue. In these experiments, the renin precursor of M_r 46,000 or 'prorenin' was formed within minutes. This was slowly converted to a renin of M_r 37,000 which was the sole form secreted into the culture medium.

The existence of 'pre' and 'pro' sequences in the primary translation product of mRNA coding for renin has been confirmed by examination of the gene for renin from the submaxillary gland of the mouse (Panthier *et al.*, 1982a). A model for the post-translational processing of renin based on this sequence and on the structure of pure submaxillary gland renin is shown in Figure 1.2.

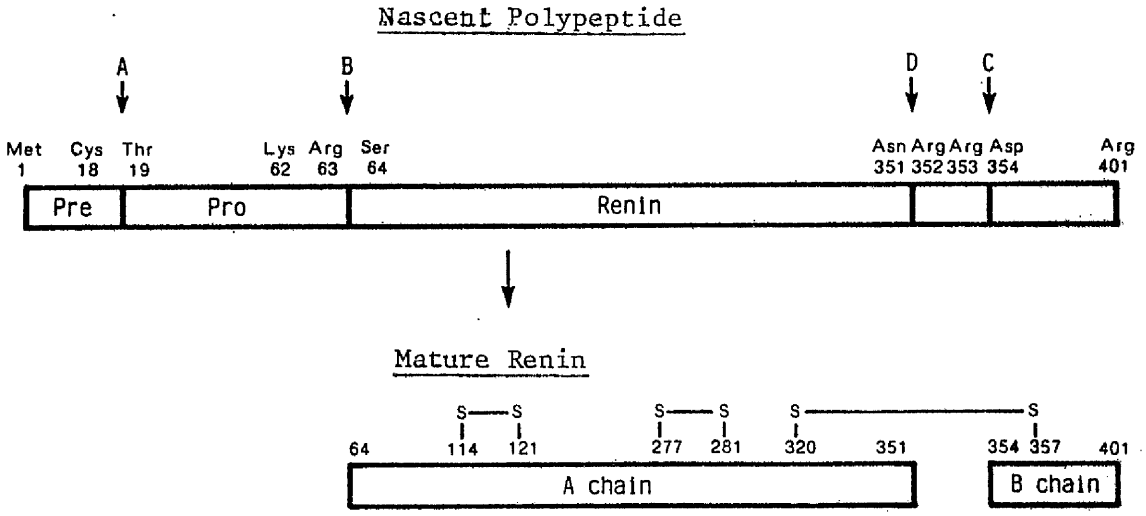

Figure 1.2 Schematic representation of the possible maturation process of mouse submaxillary gland renin [a]

Submaxillary gland renin is synthesized initially as preprorenin which is rapidly converted to prorenin by removal of the signal peptide (cleavage A). Prorenin is converted to mature renin by two proteolytic cleavages following dibasic peptides (cleavages B and C) and by removal of the Arg^{352} - Arg^{353} dipeptide (cleavage D). This produces the A and B renin chains which are linked by a disulphide bond.

[a] data taken from Misono et al. (1982), Panthier et al. (1982a) and Corvol et al. (1983).

This model involves the cleavage of the 'pre' and 'pro' sequences from the nascent renin polypeptide and the further cleavage of renin to produce the A and B chains of mouse submaxillary gland renin.

There is some evidence to suggest that precursors of renin are also formed during its biosynthesis in the kidney. The gene coding for mouse submaxillary gland renin has arisen by duplication of the gene coding for the kidney enzyme (Panthier et al., 1982b). This appears to be a fairly recent event in evolutionary terms and thus it is likely that the primary transcripts of the two genes are very similar in structure. Poulsen et al. (1980) have shown that, as in the case of mouse submaxillary gland renin, a 'preprorenin' of M_r 50,000 is formed by the cell-free translation of mouse kidney mRNA. More recently, Parmentier et al. (1983) have obtained a 'preprorenin' of M_r 45,000 by the cell-free translation of human kidney mRNA. A biosynthetic precursor of kidney renin (M_r 55,000) has also been detected by Carlson et al. (1981), using isolated canine glomeruli in culture. Pulse-chase studies showed that this precursor was converted into mature renin of M_r 38,000 over a period of hours.

The post-translational processing of kidney renin must differ slightly from that of the mouse submaxillary gland enzyme, both because the renal enzyme is glycosylated, and because it consists of a single polypeptide chain rather than two disulphide bonded chains. Nevertheless, it seems probable that 'prepro' and 'pro' forms of the enzyme exist, at least transiently, during its biosynthesis.

1.3 High molecular weight and inactive forms of renin

The in vitro demonstration of a biosynthetic precursor of renin does not necessarily imply that such a precursor exists in vivo as an inactive zymogen.

Renin is stored within juxtaglomerular cells in granules (Cook and Pickering, 1962). These granules sediment with the mitochrondrial and lysosomal fractions during the differential centrifugation of kidney cortex

extracts (Ogino et al., 1967; Nustad and Rubin, 1970), but can be separated from other subcellular organelles by density gradient centrifugation (Sagnella and Peart, 1979). Granules prepared using this method (Sagnella et al., 1980; Murakami et al., 1980b), or by ultrafiltration of crude kidney cortex extracts (Funakawa et al., 1978) contain a single form of renin of M_r 40,000. However, many investigators have shown that crude extracts of kidney cortex contain, in addition to the form of renin of M_r 40,000, one or more high molecular weight forms of renin. Some of the high molecular weight forms of renin described in the literature appear to be complexes of renin with a renin binding protein, while others may be biosynthetic precursors of renin analogous to zymogens such as pepsinogen or trypsinogen.

(a) Protein-bound forms of renin

The M_r 40,000 form of renin in dog (Funakawa et al., 1978), pig (Murakami et al., 1980b), rat (Sagnella et al., 1980) and mouse (Iwao et al., 1982) kidney cortex can be converted to a higher molecular weight form of the enzyme by interaction with an acid-labile binding protein. The formation of the renin-renin binding protein complex occurs spontaneously in crude kidney extracts, even at low temperatures (Kawamura et al., 1980). However, it is greatly enhanced by reagents which either oxidise or alkylate sulphydryl groups (Funakawa et al., 1978). Thus, renin can be extracted almost entirely in the high molecular weight form if N-ethyl maleimide is included in the extraction mixture (Inagami et al., 1977a).

Neither the true size of the renin-renin binding protein complex nor its stoichiometry are clear at present. The complex dissociates during gel filtration giving rise to an elution peak which corresponds to an M_r value of around 60,000 (Ueno et al., 1981). An M_r value of 113,000 has been obtained by sedimentation equilibrium analysis of a crude preparation of the pig kidney renin-renin binding protein complex (Ueno et al., 1981) and this is consistent

with the finding that the renin binding protein has an M_r value in excess of 47,000 by gel filtration (Kawamura et al., 1979; Yamamoto et al., 1980; Sagnella et al., 1980; Ueno et al., 1981). Recently, however, Takahashi et al. (1983) have obtained an M_r value of 42,000 by SDS/polyacrylamide gel electrophoresis for purified pig renin binding protein. These authors suggested that the renin-renin binding protein complex consists of equimolar amounts of renin and the binding protein, giving an M_r value of around 80,000 for the complex. Alternatively, it is possible that two molecules of binding protein associate with each renin molecule to give a complex of M_r 120,000.

Although renin binding proteins have been found in the renal cortex of all animal species tested, their physiological function remains obscure. The binding protein isolated from pig renal cortex is an inhibitor of the renin-renin substrate reaction (Ueno et al., 1981; Takahashi et al., 1983). However, there is no evidence that it has a direct inhibitory function in vivo. Takaori et al. (1982) have suggested that the renin binding protein may play a role in the reabsorption of renin filtered from the circulation by the glomerulus.

(b) Possible precursor forms of renin

Evidence that an inactive precursor of renin might exist was first obtained during studies on renin synthesis and release by the rat kidney (de Vito et al., 1970). In these experiments, kidney slices were incubated in culture medium in the presence of the protein synthesis inhibitors, actinomycin D and puromycin. Over a 7 hour incubation period, renin was released into the medium in an amount which represented about 75% of the original activity present in the slices. However, although protein synthesis was inhibited, there was no detectable decrease in the renin content of the tissue. De Vito suggested that this result was due to the activation of an inactive 'prerenin'.

Direct evidence for the existence of an activatable form of renin was provided by Lumbers (1971) who showed that amniotic fluid dialysed to a pH of

3.3 and then back to pH 7.4 had a higher renin concentration than amniotic fluid dialysed to a pH above 4.0. Since then, many workers have reported that an increase in the measured renin concentration of human amniotic fluid and plasma occurs after exposure to a pH of between 3.0 and 3.3, followed by dialysis to neutral pH (Skinner et al., 1972; Day and Luetscher, 1974; Leckie et al., 1977; Derkx et al., 1976; Boyd, 1977). The amount by which the measured plasma renin concentration increases, and by inference, the amount of inactive renin present, depends on the source of the plasma. In man, between 70% and 90% of the total circulating renin is present as an inactive form of the enzyme (Cooper et al., 1976; Derkx et al., 1976). Values of 15% for rat plasma (Vandongen et al., 1977), 20% for rabbit plasma (Richards et al., 1981), 50% for sheep plasma (Lush et al., 1983), 80% for pig plasma (Bailie et al., 1979), and 33% for dog plasma (James and Hall, 1974) have been reported although, in the latter case, it is not clear that the increase in angiotensin I generating activity observed after acidification is due solely to the activation of inactive renin (Sealey et al., 1980).

Plasma inactive renin can also be activated by exposure to low temperatures. Cold treatment of human plasma at -5°C for 4 days results in an increase in renin activity (Osmond et al., 1973; Sealey et al., 1976), but to a lesser extent than after acid treatment (Atlas et al., 1978; Hsueh et al., 1978). Little or no increase in the renin activity of plasma occurs at 4°C (Hsueh et al., 1978; Millar et al., 1980).

The increase in plasma renin activity resulting from the above manipulations is irreversible and has been shown to depend on the presence of endogenous serine proteinases (Atlas et al., 1978; Derkx et al., 1978a; Leckie, 1978). Plasma serine proteinase activity is normally maintained at low levels by circulating proteinase inhibitors. However, many of these inhibitors are inactivated by acidification. The dialysis of plasma to acid pH, therefore,

results in an increase in serine proteinase activity which activates inactive renin when the plasma is returned to neutral pH (Derkx et al., 1982; Sealey et al., 1982a). Cold treatment of plasma is thought to reduce the potency of plasma proteinase inhibitors, thus allowing endogenous serine proteinases to slowly activate inactive renin (Sealey et al., 1982a).

Plasma inactive renin can be activated in vitro by a variety of proteinases (Leckie, 1981; Inagami et al., 1982b), including certain enzymes of the coagulation and fibrinolytic systems, as well as a number of tissue proteinases. An inactive form of renin which can be activated by limited proteolysis is also present in hog and human renal cortex, and in the perfusate from human kidneys (Murakami et al., 1980c; Atlas et al., 1980). This has led some investigators to suggest that inactive renin is a renin zymogen that is activated either within the circulation, or at sites distal to its site of synthesis (Schalekamp and Derkx, 1981; Sealey et al., 1982a). Alternative possibilities that must be considered, however, are that inactive renin is a protein bound form of renin or that it is a renin-like enzyme which is activated by limited proteolysis.

Angiotensinogen can act as a substrate for enzymes other than renin, such as pepsin (De Fernandez et al., 1965) and cathepsin D (Hackenthal et al., 1978). Angiotensin I generating activities which are not inhibited by anti-renin antibody, and which differ from renin in their pH optima, or reaction kinetics, have been found in the brain (Day and Reid, 1976; Dzau et al., 1980), kidney (Hackenthal et al., 1978), blood vessel walls (Thurston et al., 1978) and adrenal gland (Naruse et al., 1983). Trypsin-activated renins from human plasma and kidney, however, are similar to purified renal renin with respect to their pH optima, reaction kinetics, specificity, and inhibition by pepstatin. Moreover, trypsin-activated inactive renin and endogenous active renin are inhibited identically by monospecific anti-renin antibodies (Atlas et al., 1982b; Hsueh et

al., 1983). It seems unlikely, therefore, that the activity produced by limited proteolysis of inactive renin is due to a non-renin proteinase.

Little is known about the structural relationship between the active and inactive forms of renin. As expected for a renin zymogen, human inactive renin is completely inactive against renin substrate and is slightly larger than its active counterpart (Sealey et al., 1982a). M_r values of around 50,000 and 56,000 have been obtained by gel filtration for kidney and plasma inactive renin, respectively. However, the extremely low concentration of inactive renin both within human renal cortex and in human plasma (Sealey et al., 1980) has, to date, prevented its purification and detailed characterisation. It is therefore not clear whether human inactive renin is indeed a renin zymogen or whether it is simply a protein bound form of the active enzyme analogous to the forms of renin described in Section 1.3(a).

At present, the best candidate for a purified renin zymogen is an inactive renin of M_r 50,000 isolated from pig renin cortex (Inagami et al., 1982c; Takii and Inagami, 1982). The activity and size of this protein were unaffected by agents known to dissociate protein complexes, such as SDS, urea, or guanidine hydrochloride. In contrast, it was fully activated after treatment with either plasmin or trypsin and a concomitant reduction in size to an M_r value close to that of active renin (38,000) was observed. It is highly likely, though not yet proven, that this protein corresponds to the prorenin demonstrated during the in vitro translation of mRNA coding for renin (Section 1.2).

1.4 The present study

The renin-angiotensin system plays a central role in the regulation of blood pressure and participates in many forms of hypertension. The possibility that inactive renin is a renin zymogen and that the activation of inactive renin is a control point for the regulation of circulating renin activity is therefore of

great interest. However, as discussed in the preceding section, little is known about human inactive renin at the molecular level. Most attempts to characterise this form of renin have involved studies of its properties in either whole or fractionated plasma, or in crude kidney extracts. The aim of the present study was to isolate small quantities of both active renin and inactive renin from human renal cortex in order to allow a more detailed structural characterisation of the two forms of the enzyme.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

(a) Biochemicals

All reagents were of the best grade available commercially. With the exceptions of the compounds listed below, the reagents were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K. Ammonium sulphate was 'AnalaR' grade rather than 'Enzyme' grade throughout.

Benzamidine hydrochloride, bovine serum albumin (Fraction V), Coomassie Brilliant Blue G, haemoglobin (type I: bovine, crystallised), haemoglobin (type II: bovine, substrate grade), β-Lactoglobulin B (from bovine milk), ovalbumin (electrophoretically pure), phenylmethanesulphonyl fluoride, polyoxyethylenesorbitan monolaurate (Tween 20), soybean trypsin inhibitor (SBTI), and N-succinimidyl-3-(4-hydroxyphenyl)propionate were obtained from Sigma (London) Chemical Company Ltd., Poole, Dorset, U.K.

Blue Dextran and Dextran T10 were purchased from Pharmacia (Great Britain) Ltd., Hounslow, Middlesex.

Bovine serum albumin (electrophoretically pure) was purchased from Boehringer Corporation (London) Ltd., Lewes, Sussex, U.K.

Human serum albumin was obtained from A.B. Kabi, Stockholm, Sweden.

(Ile^5)-angiotensin I was obtained from Schwartz Bioresearch, Orangeburg, New York.

(b) Reagents for polyacrylamide gel electrophoresis

Acrylamide and N,N'-methylene-bis-acrylamide (specially purified for gel electrophoresis), ammonium persulphate, sodium dodecyl sulphate and N,N,N',N' tetramethylenediamine (TEMED) were 'AnalaR' grade reagents which were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K.

(c) Enzymes

Aldolase (EC 4.1.2.13) from rabbit muscle, carbonic anhydrase (EC 4.2.1.1) from bovine erythrocytes and pyruvate kinase (EC 2.7.1.40) from rabbit muscle were purchased from Boehringer Corporation (London) Ltd., Lewes, Sussex, U.K. Lysozyme (EC 3.2.1.17) from chicken egg white (electrophoretically pure) and trypsin (EC 3.4.21.4) from bovine pancreas (type III) were purchased from Sigma (London) Chemical Company Ltd., Poole, Dorset, U.K. V8 proteinase from Staphylococcus aureus was obtained from Miles Laboratories Ltd., Stoke Poges, Slough, U.K.

Pig renin (MRC reagent 65/119) and International Reference Standard Human Renin (MRC reagent 68/356) were obtained from the National Institute for Biological Standards and Control, Hampstead, London, U.K.

Cathepsin D (3.4.23.5) from human spleen was a gift from Dr A.J. Barrett, Strangeways Research Laboratory, Cambridge, U.K.

(d) Radiochemicals

(^{3}H)acetic anhydride (500mCi/mmole) and N-succinimidyl-3-(4-hydroxy, 5-(^{125}I)iodophenyl)propionate (2000Ci/mmole) were purchased from Amersham International PLC, Bucks, U.K. ^{14}C-methylated proteins were obtained from New England Nuclear, Southampton, U.K., and were as follows:

^{14}C-methylated bovine serum albumin (0.017mCi/mg)

^{14}C-methylated ovalbumin (0.012mCi/mg)

^{14}C-methylated carbonic anhydrase (0.011mCi/mg)

(^{125}I)angiotensin I (circa 500mCi/mg) was prepared in the Blood Pressure Unit Laboratories by Mrs M. Hughes. The radioiodination of angiotensin I and the purification of the labelled peptide were carried out as described by Morton et al., (1976).

(e) Chromatographic materials

Octyl-Sepharose CL-4B, Sephadex G50 (medium grade) and Sephadex

G100 (superfine grade) were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Activated 6-aminohexanoic acid Sepharose 4B (Cat. No. A9019) and cyanogen bromide activated-Sepharose 4B were obtained from Sigma (London) Chemical Co. Ltd., Poole, Dorset, U.K.

(f) Miscellaneous materials

Scintillator 299 was obtained from Packard-Becker BV Chemical Operations, The Netherlands. The peptide H.77 was kindly supplied by Dr M. Szelke, Department of Chemical Pathology, Royal Postgraduate Medical School, Hammersmith Hospital, London. F15 monoclonal antibody was kindly supplied by Dr B. Pau, Centre de Recherches Clin-Midy, Montpellier, France. Human kidneys were obtained from individuals examined post mortem in the Department of Pathology, Western Infirmary, Glasgow.

2.2 General methods

(a) Glassware

All items of glassware were washed in solutions of the detergent Decon 75 (Decon Laboratories Ltd., Hove, U.K.), rinsed thoroughly with tap water, then distilled water, and dried in an oven at 60°C. For amino acid analysis, glassware was additionally treated by immersion in concentrated nitric acid for 1h followed by distilled water for 1h. Pipettes were soaked in 2% (v/v) Decon 75 for 10 days and were thoroughly rinsed using an automatic pipette washer. Finally, the pipettes were rinsed with distilled water and dried in an oven at 60°C.

(b) Micropipetting

Solution volumes in the range 10µl to 1ml were transferred reproducibly using either 'Pipetman Digital Pipettes' (Anachem Ltd., Luton, U.K.) or adjustable 'Finnpipettes' (Jencons (Scientific) Ltd., Leighton Buzzard, Bedfordshire, U.K.). Volumes in the range 0.5µl to 50µl were transferred accurately using either a SMI Digital Adjust Micro/Pettor (Alpha Laboratories,

Eastleigh, Hampshire, U.K.), or microsyringes.

(c) pH measurement

Measurements of pH were carried out using an Elcomatic Instruments Ltd., model 7065 digital pH meter fitted with a combination electrode (Gallenkamp & Co. Ltd., East Kilbride, Glasgow, U.K.). This apparatus was standardised regularly using solutions of pH 7.0 and pH 4.0 prepared using buffer tablets (BDH Chemicals Ltd., Poole, Dorset, U.K.).

(d) Centrifugation

Preparative centrifugation was routinely performed at 4°C. Accelerations of up to 1000g were obtained using a 'Centra-7R' benchtop centrifuge (Damon/IEC (U.K.) Ltd., Dunstable, Beds., U.K.). Accelerations of up to 100,000g were obtained using a Beckman L2-65B centrifuge fitted with a titanium Ti-60 rotor (Beckman RIIC Ltd., Glenrothes, Fife, U.K.).

(e) Dialysis and ultrafiltration

The dialysis of samples was carried out using Visking Tubing (Scientific Instrument Centre Ltd., 1 Leeke Street, London) which had been immersed in boiling lmM-EDTA, pH 7.0 for 15 minutes and then washed in distilled water. Sample volumes of less that 500µl were dialysed using Schleicher & Schull type UH 100/10 Ultra Thimbles (Anderman & Co. Ltd., East Molesey, Surrey, U.K.). The Ultra Thimbles were sealed using small glass stoppers in order to minimise the increase in sample volume during dialysis.

The concentration of protein samples was carried out by ultrafiltration using a vacuum dialysis apparatus (Schleicher & Schull) fitted with a type UH 100/10 ultra thimble.

(f) Measurement of protein concentration

Protein concentration was measured using the dye-binding method of Bradford (1976) as modified by Macart & Gerbaut (1982).

Dye reagent Coomassie Brilliant Blue G (100mg) and SDS

(30mg) were dissolved in 50ml of 95% (w/v) ethanol. 85% (w/v) phosphoric acid (100ml) was added to this solution and the mixture was then diluted to a final volume of 1 litre.

1ml of the dye reagent was added to 50µl of each sample to be assayed. The absorbance at 595nm was measured after 15 min against a reagent blank prepared from 50µl of the appropriate buffer and 1ml of the dye reagent. Standard curves were prepared from protein solutions containing 0.5µg to 10µg of bovine serum albumin in 50µl of 50mM-sodium phosphate, pH 7.0.

(g) Distilled water

Glass distilled water stored in either glass or polythene containers was used in all experiments.

2.3 Preparation of chromatographic media

(a) Sephadex Gels

Sephadex gels were swollen and poured according to the manufacturer's instructions and were stored at 4°C in 0.02% (w/v) sodium azide.

(b) H.77-Sepharose

H.77 was coupled to Sepharose through the N-terminal amino group by reaction with the N-hydroxysuccinimide ester of 6-aminohexanoic acid-Sepharose 4B (activated 6-aminohexanoic acid - Sepharose 4B). Activated Sepharose (10g) was swollen and washed at room temperature with 2 litres of 1mM-HCl on a sintered-glass funnel. H.77 (100mg) was dissolved in 30ml of 0.1M-$NaHCO_3$ (pH 8.0)/0.5M-NaCl (coupling buffer) and mixed with the washed gel. The gel suspension was gently shaken for 16h at 4°C, after which excess ligand was washed from the gel with coupling buffer (500ml). The remaining reactive groups were blocked by treatment of the gel in 100ml of 1M-ethanolamine/HCl, pH 9.0, for 1h at 20°C. The H.77-Sepharose was washed with five cycles of coupling buffer (200ml) followed by 0.1M-sodium

acetate (pH 4.0)/0.5M-NaCl (200ml) and finally equilibrated with 50mM-Tris/HCl, pH 7.4.

The amount of peptide coupled to the gel was determined by amino acid analysis after hydrolysis of 0.5ml of H.77-Sepharose in 2ml of 6N-HCl for 20h at 110°C. 0.5µmoles of DL-norleucine was included in the hydrolysis mixture as an internal standard. Analysis of the hydrolysate was carried out using a model 4400 amino acid analyser (LKB Instruments Ltd., S. Croydon, Surrey), and the peptide content was calculated from the recovery of phenylalanine. 0.9µmoles of H.77 were coupled per ml of gel.

(c) F15-Sepharose

F15 monoclonal antibody was coupled to Sepharose by reaction with cyanogen bromide activated Sepharose 4B. Activated Sepharose (1g) was swollen and washed at room temperature with 200ml of 1mM-HCl on a sintered glass funnel. F15 monoclonal antibody (12mg) dissolved in 6ml of 0.2M-$NaHCO_3$ (pH 8.5)/0.5M-NaCl (coupling buffer) was mixed with the washed gel and the gel suspension was then gently shaken for 20h at 4°C. The remaining reactive groups were blocked by treatment of the gel with 20ml of 1M-ethanolamine/HCl, pH 9.0 for 1h at 20°C. The F15-Sepharose was then washed with five cycles of coupling buffer (20ml) followed by 0.2M-sodium acetate (pH 4.0)/0.5M-NaCl (20ml) and finally equilibrated with 50mM-sodium phosphate, pH 7.4.

2.4 Enzyme assays

(a) Renin

Renin concentration was measured using the method of Millar et al., (1980). This method is based on the radioimmunoassay of the angiotensin I generated during the incubation of renin with excess ox renin substrate.

The following stock reagents were prepared:

Buffer A: 3M-Tris/HCl (pH 6.9)/0.2M-EDTA

Buffer B: 50mM-Tris/HCl (pH 7.4)/5mM-EDTA/0.5% (w/v) bovine serum albumin (Fraction V)

Dextran coated charcoal: 6.3g of charcoal and 0.125g of Dextran T70 were suspended in 100ml of buffer B.

Assays were carried out by incubating 35µl of enzyme solution at 37°C with 55µl of a pre-mixed solution consisting of ox renin substrate (see Section 2.5), buffer A, and antiserum to angiotensin I diluted in buffer B (1:68 v/v). The ratio of the pre-mixed components was 8:2:1 by volume. The enzyme reaction was terminated by adding 1.4ml of ice-cold buffer B. 10pg (5,000-10,000cpm) of (^{125}I)angiotensin I in 50µl of buffer B was added to the mixture and the radioimmunoassay was completed by further incubation at 4°C for 48h. The separation of free and bound ligand was achieved by adding 150µl of Dextran coated charcoal and immediately centrifuging the mixture for 10 min at 1,000g and 4°C. The supernatant solution was removed by suction and the (^{125}I)angiotensin I adsorbed to the charcoal pellet was then measured using a model NE 1612 gamma counter (Nuclear Enterprises Ltd., Sighthill, Edinburgh, U.K.).

Standard curves for angiotensin I were prepared by serial dilution of (Ile ^{5}) angiotensin I in 35µl of 7% (w/v) human serum albumin from 1000pg to 8pg angiotensin I per tube in duplicate. Tubes containing no angiotensin I were also included. 55µl of the pre-mixed substrate solution was added to each tube and the standards were then treated in an identical manner to the unknown assay samples.

For each enzyme sample the velocity of angiotensin I generation was determined using one timed incubation in duplicate and a zero time measurement. The assay results were expressed in International Units of renin by comparison with the International Reference Standard Preparation of

Human Renin (MRC reagent 68/356). One unit of renin generated angiotensin I at a rate of 182.8µg/h in the above assay system (Figure 2.1).

(b) Inactive renin

Inactive renin was assayed by measuring the increase in renin activity produced by the treatment of samples with trypsin. The activation of inactive renin was carried out using a modification of the method described by Atlas et al. (1980). The following stock solutions were prepared:

Buffer A: 50mM-Tris/HCl (pH 7.4) /20mM-benzamidine/ 5mM-EDTA/1% (w/v) bovine serum albumin (Fraction V).

Trypsin solution: Crystalline trypsin (12,000 BAEE units/mg see Section 2.4(d)) was dissolved in 1mM-HCl to a final concentration of 1mg/ml. Fresh solutions of the enzyme were prepared each day. The crystalline enzyme was stored at -20°C in a desiccator. Under these conditions, no loss in the activity of the enzyme was observed over a period of 2 years.

SBTI solution: Soybean trypsin inhibitor was dissolved in 50mM-Tris/HCl, pH 7.4 to a final concentration of 2mg/ml.

50µl of each sample was added to 450µl of Buffer A. 50µl of trypsin solution was added and the solution was incubated for 5h at 4°C. The reaction was stopped by adding 50µl of SBTI solution and the renin concentration was then measured as described in Section 2.4(a). Zero time samples were included in the renin assay to allow for angiotensin I immunoreactive material generated during the incubation of unknown samples with trypsin.

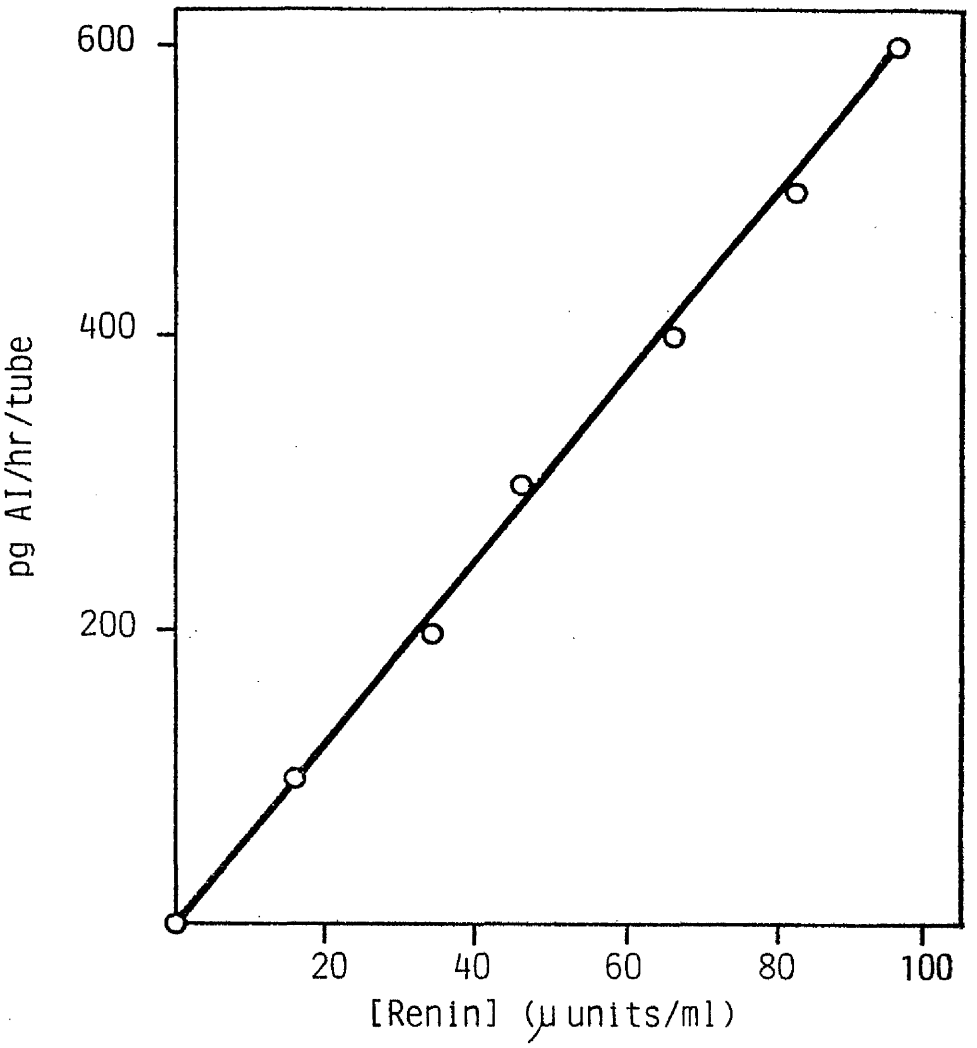

Figure 2.1 **Renin assay calibration curve**

Dilutions of the International Reference Standard Preparation of Human Renin (MRC reagent 68/356) in 50mM-Tris/HCl (pH 7.4)/5mM-EDTA/0.5% (w/v) bovine serum albumin were assayed as described in Section 2.4(a) and the velocity of angiotensin I generation (expressed as pg angiotensin I/hr/tube) was plotted against the enzyme concentration of the sample. Each point is the mean of five measurements.

The renin concentration measured after trypsin-activation was defined as the total renin concentration. The active renin concentration was measured after an identical incubation at 4°C except that the trypsin solution was replaced with 1mM-HCl. The inactive renin concentration was calculated as the difference between the total renin concentration and the active renin concentration.

(c) Acid proteinase

Acid proteinase activity was assayed by measuring the release of ^{3}H-labelled peptides from (^{3}H)acetylhaemoglobin. The labelled substrate (35×10^{6} dpm/mg) was prepared as described by Hille et al. (1970). Crystalline haemoglobin (650mg) was reacted with 0.05mmoles of (^{3}H)acetic anhydride (500mCi/mmole) in 3.5ml of 0.01M-sodium phosphate, pH 7.5, for 1h at 4°C. The product was purified by chromatography on a column (1.6cm x 26cm) of Sephadex G50 equilibrated with the same buffer. The labelled haemoglobin (20ml) was precipitated by adding 100ml of 3%(w/v) trichloroacetic acid, resuspended in 0.1M-sodium tetraborate, and then dialysed against 0.01M-sodium tetraborate followed by 0.01M-sodium phosphate, pH 7.4. The purified (^{3}H)acetylhaemoglobin was stored at -20°C at a concentration of 20mg/ml.

Assays were carried out by incubating 10µl of enzyme solution for 1h at 37°C with 50µl of 1M-sodium formate, pH 3.3 and 40µl of a substrate solution prepared by dissolving 87mg of (^{3}H)acetylhaemoglobin and 1g of unlabelled haemoglobin in water to a final volume of 20ml. The reaction was terminated by adding 0.5ml of 3% (w/v) trichoroacetic acid and precipitated protein was removed by centrifugation at 1000g and 4°C for 30min. A 0.2ml sample of the supernatant solution was then removed for liquid scintillation counting in 8ml of Packard scintillator 299. Assay blanks were prepared by delaying the addition of the enzyme solution until after the introduction of the trichloroacetic acid.

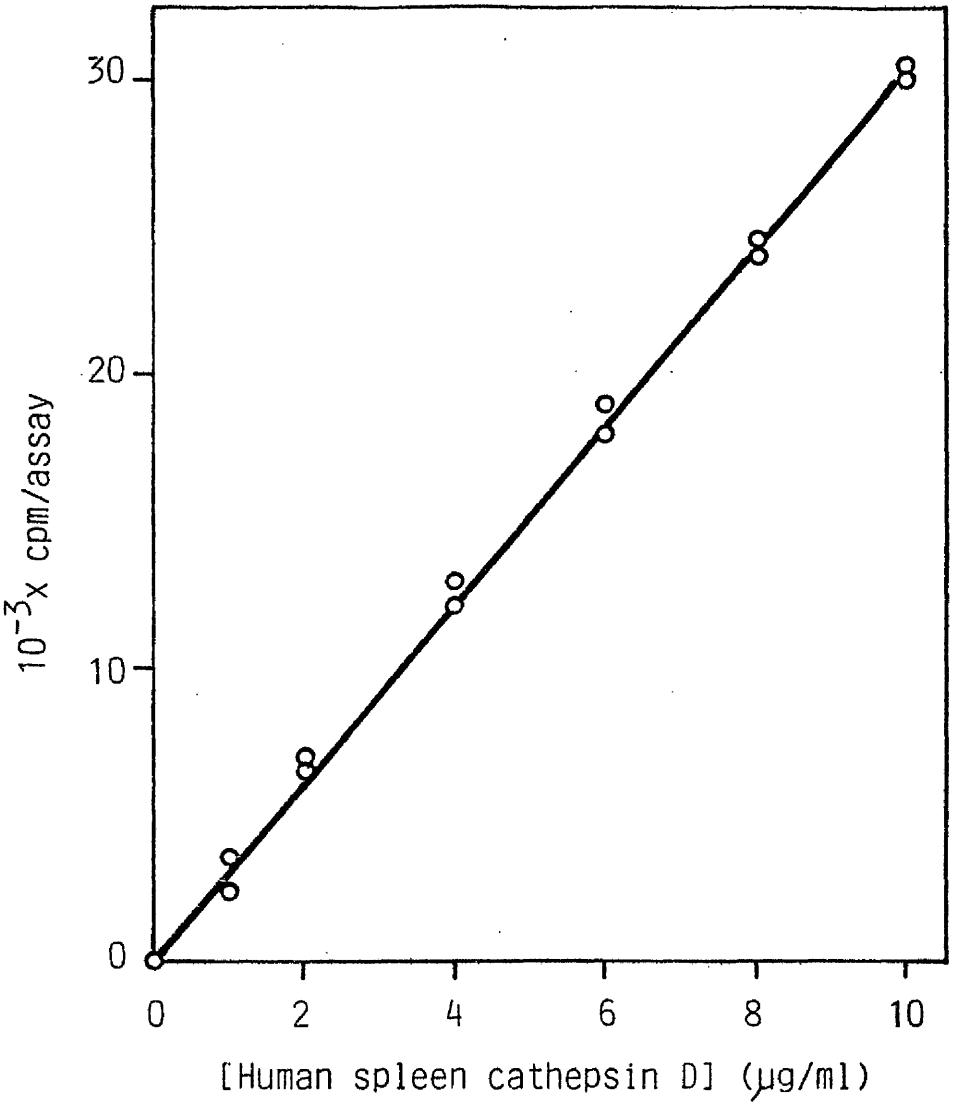

Figure 2.2. Standard curve for acid proteinase assay

Dilutions of pure human spleen cathepsin D in 10mM-sodium phosphate (pH 7.0)/0.005% (w/v) bovine serum albumin were assayed by measuring the release of ^{3}H-labelled peptides from (^{3}H)acetyl haemoglobin as described in Section 2.4(c) and the results (expressed as cpm/assay) were plotted against enzyme concentration.

The assay results were expressed in units of enzyme activity by comparison with human spleen cathepsin D (Figure 2.2). One unit of activity was defined as the activity exhibited by 1µg of pure human spleen cathepsin D in the above assay system.

(d) Trypsin

Trypsin activity was measured using the method of Schwert & Takenaka (1955). Samples (200µl) of trypsin dissolved in 1mM-HCl were incubated at 25°C with 2.8ml of a substrate solution consisting of N-benzoyl-L-arginine ethyl ester (0.96nM) in 50mM Tris/HCl (pH 8.0)/20mM $CaCl_2$. The production of N-benzoyl-L-arginine was monitored spectrophotometrically at 253nm using cells with a 1cm light path. One BAEE unit of trypsin activity was defined as the activity required to produce an increase in extinction at 253nm of 0.001/min under the above conditions.

2.5 Preparation of ox renin substrate

Renin substrate was partially purified from the plasma of bi-nephrectomised oxen by ammonium sulphate fractionation.

Whole blood (39 litres) was collected from three bi-nephrectomised oxen one week after nephrectomy, using EDTA (5mM) as anticoagulant, and was centrifuged for 30 min at 1000g and 4°C. The plasma obtained (18 litres) was made 1.4M with respect to ammonium sulphate by adding 205.6g of crushed ammonium sulphate per litre of plasma over a period of 30 min with constant stirring. The solution obtained was allowed to stand overnight at 4°C and then precipitated protein was removed by centrifugation for 30 min at 1000g and 4°C. The supernatant solution (15.9 litres) was adjusted to 3.0M with respect to ammonium sulphate by adding a further 270.7g of crushed ammonium sulphate per litre of solution over a period of 60 min. The solution was stirred overnight at 4°C and then precipitated protein was collected by centrifugation for 90 min at 1000g and 4°C. The precipitate

was dissolved in 3 litres of distilled water and was then dialysed exhaustively against 50mM-sodium phosphate, pH 6.9. The partially purified ox renin substrate obtained after dialysis (9 litres) was aliquoted into 500ml portions and was stored frozen at -20°C.

The concentration of angiotensinogen in the fractionated ox plasma was measured by radioimmunoassay of the angiotensin I produced after exhaustive incubation with pig renin. This was carried out using a modification of the renin assay method described in Section 2.4(a). Samples (40µl) of the substrate solution diluted in 7% (w/v) human serum albumin were incubated at 37°C with 1 unit of pig renin (MRC reagent 65/119) dissolved in 35µl of 50mM - Tris/HCl (pH 7.4)/0.5% (w/v) bovine serum albumin (Fraction V) and 15µl of a pre-mixed solution consisting of buffer A and antiserum to angiotensin I diluted in buffer B (1:68 v/v). The ratio of the pre-mixed components was 2:1 by volume. The enzyme reaction was terminated by adding 1.4ml of ice-cold buffer B and the radioimmunoassay of angiotensin I was completed as previously described in Section 2.4(a). Standard curves for angiotensin I were prepared by serial dilution of (Ile^5) angiotensin I in 35µl of 7% (w/v) human serum albumin from 1000pg to 8pg angiotensin I per tube in duplicate. Tubes containing no angiotensin I were also included. 40µl of 7% (w/v) human serum albumin and 15µl of the pre-mixed antibody solution were added to each tube and the standards were then treated in an identical manner to the unknown assay samples.

Under the above conditions, the amount of angiotensin I released from ox angiotensinogen reached a plateau after 8h, corresponding to 4.2µg angiotensin I released per ml of stock substrate solution.

2.6 Polyacrylamide gel electrophoresis

(a) SDS/polyacrylamide gel electrophoresis

Electrophoresis in the presence of 0.1% (w/v) SDS was carried out using

the discontinuous system described by Laemmli (1970).

The following stock solutions were prepared:

Solution A: 30% (w/v) acrylamide/0.8% (w/v) bisacrylamide (stored at 4°C).

Solution B: 1.5M-Tris/HCl, pH 6.8 (stored at 4°C).

Solution C: 1.5M-Tris/HCl, pH 8.8 (stored at 4°C).

Solution D: 10% (w/v) SDS.

Solution E: 1.5% (w/v) ammonium persulphate (freshly prepared before use).

Reservoir buffer: 0.025M-Tris/0.192M-glycine (pH 8.3)/0.1% (w/v) SDS.

Sample buffer: 72.5mM-Tris/HCl (pH 6.8)/3% (w/v) SDS/10% (v/v) glycerol/5% (v/v) 2-mercaptoethanol/0.002% (w/v) bromophenol blue.

The gel mixtures were prepared using the volumes indicated in Table 2.1. In each case, the gel mixture, except for TEMED, was prepared at 4°C in a small flask and degassed for 2 min using a water suction pump. Polymerisation was initiated immediately before pouring the gel by adding the correct volume of TEMED to the mixture.

Slab gels were cast between glass plates separated by 1mm spacers. The resolving gel mixture was poured between the glass plates leaving sufficient space for a stacking gel to be poured later. Gel buffer of the same composition as in the resolving gel was then gently layered onto the gel surface. After polymerisation was complete the overlay was removed and the resolving gel surface was rinsed with a small volume of stacking gel mixture. This was discarded and the remaining space between the glass plates was filled with the stacking gel mixture. A comb for forming sample wells was immediately inserted into the gel mixture and the stacking gel was

Table 2.1

Recipe for gel preparation using the SDS-discontinuous buffer system of Laemmli (1970)

Stock solution	Final acrylamide concentration (%)[a]		
	Stacking gel	Resolving gel	
	3	10	12.5
Solution A	3.0	10.0	12.5
Solution B	2.5	-	-
Solution C	-	7.5	7.5
Solution D	0.3	0.3	0.3
Solution E	1.0	1.0	1.0
Water	23.2	11.2	8.7
TEMED	0.015	0.015	0.015

[a] The columns represent volumes (ml) of the various reagents required to make 30ml of gel mixture.

allowed to set. The comb was then carefully removed and the sample wells were rinsed with and then filled with reservoir buffer.

Protein samples were mixed with an equal volume of sample buffer and were heated to 100°C in a boiling water bath for 3 min. After the samples had cooled, they were carefully layered into each sample well of the gel. Electrophoresis was carried out at constant voltage (50V) until the tracking dye had travelled the length of the gel. The gel was then fixed and stained for protein as described in Section 2.6(b).

Rod gels were cast in glass tubes (5mm internal diameter). The gels were prepared in an identical manner to the slab gels except that the stacking gel solution was overlayered with stacking gel buffer before polymerisation was complete. After the gel had set the surface of the stacking gel was rinsed with reservoir buffer. Protein samples were prepared as described above and carefully layered onto the surface of each gel. Electrophoresis was carried out at 2mA per gel.

(b) Detection of protein bands in polyacrylamide gels.

Staining with Coomassie Brilliant Blue

Proteins were fixed in polyacrylamide gels by soaking the gels in 12.5% (w/v) trichloroacetic acid for 30 min at room temperature. Each gel was then stained by immersion in a 0.1% (w/v) solution of Coomassie Brilliant Blue in methanol/acetic acid/water (5/1/4 by volume) for 2h at 40°C. Excess stain was removed by soaking the gel in methanol/acetic acid/water (1/1/8 by volume) at 40°C until a clear gel background was obtained.

Silver staining

The silver staining of proteins in polyacrylamide gels was carried out essentially as described by Wray et al. (1981). Silver stain and developer solutions were prepared immediately before use as follows:

Stain solution: 2.8ml of 14.8M-NH_4OH was added to 42ml

of 0.36% (w/v) NaOH. Silver nitrate (1.6g) dissolved in 10ml of water was added dropwise to this mixture and the solution was made to a final volume of 200ml with water.

Developer solution: 2.5ml of 1% (w/v) citric acid and 0.25ml of 38% (w/v) formaldehyde solution were mixed and made to a final volume of 500ml with water.

Proteins were fixed in polyacrylamide gels by immersion of the gels in methanol/acetic acid/water (5/1/4 by volume) for 1h at 37°C. Each gel was then soaked at room temperature in 50% (v/v) methanol for 24h with four changes of methanol. Protein staining was carried out by soaking the gels in stain solution for 20 min with constant gentle agitation. The gels were washed in water for 5min and then transferred to developer solution until the protein bands appeared. The stain development was stopped by immersion of the gels in 10% (v/v) acetic acid. The stained gels were rinsed in water and were stored in 50% (v/v) methanol in the dark.

Detection of radiolabelled proteins

Polyacrylamide slab gels containing proteins labelled with ^{125}I were initially fixed and stained with Coomassie Brilliant Blue as described above. The stained gels were soaked overnight in 10% (v/v) methanol/3% (v/v) glycerol and were then dried down onto Whatman 3MM filter paper using a model 224 gel dryer (Bio-Rod Laboratories, 2200 Wright Avenue, Richmond, California, 94804, U.S.A). The radiolabelled proteins were then detected by indirect autoradiography of the dried gel using Kodak X-Omat AR5 film with an X-ray intensifying screen (Antony Monk (Eng) Ltd., Sutton in Ashfield, U.K.)

2.7 Determination of relative molecular mass (M_r)

The M_r values of active renin and inactive renin were estimated both by gel filtration chromatography and by electrophoresis in the presence of 0.1% (w/v) SDS.

(a) M_r estimation by gel filtration chromatography

Gel filtration chromatography of all samples was carried out at 4°C. A column (1.6 cm x 65 cm) of Sephadex G100 (superfine grade) was packed and equilibrated in 50mM-Tris/HCl (pH 7.4)/20mM-benzamidine/5mM EDTA/0.01% (w/v) lysozyme (elution buffer). Samples (1-5 milliunits) of the purified enzymes dissolved in 1ml of elution buffer containing 3% (w/v) glycerol were applied to the column and run through at a flow rate of 2ml/h. Fractions of 1ml were collected and assayed for active or inactive renin as described in section 2.4. ^{14}C-methylated marker proteins (0.5µCi each) were included in each sample as internal standards. Fractions were assayed for ^{14}C by liquid scintillation counting of 0.2ml of each fraction in 8ml of Packard scintillator 299.

M_r values were determined by comparison with standard curves of K_{av}, defined as:

$$\frac{\text{elution volume } (V_e) - \text{void volume } (V_o)}{\text{total volume } (V_t) - \text{void volume } (V_o)}$$

versus log M_r. V_o was determined by measuring the elution volume of a 1ml sample (2mg/ml) of Blue Dextran. The ^{14}C-methylated marker proteins used were ^{14}C-methylated bovine serum albumin, ^{14}C-methylated ovalbumin and ^{14}C-methylated carbonic anhydrase. In preliminary experiments, these labelled proteins were found to co-elute with unlabelled bovine serum albumin, ovalbumin and carbonic anhydrase respectively. Their M_r values are given in Table 2.2.

(b) M_r estimation by electrophoresis in the presence of 0.1% (w/v) SDS.

Polyacrylamide gel electrophoresis of proteins was carried out using the gel system of Laemmli (1970). Unknown M_r values were determined by comparison with standard curves of electrophoretic mobility versus log M_r. For slab gels, electrophoretic mobility was defined as the distance migrated by the protein. In the case of rod gels, electrophoretic mobility was defined as:

$$\frac{\text{distance migrated by protein}}{\text{distance migrated by tracking dye.}}$$

The M_r values of the standard marker proteins are given in Table 2.2.

Table 2.2

Subunit M_r values of standard proteins

Protein	Subunit M_r value	Reference
Bovine serum albumin	66,200	Peters (1975)
Pyruvate kinase	57,000	Steinmetz and Deal (1966)
Ovalbumin	43,000	Castellino and Barker (1968)
Aldolase	40,000	Kawahara and Tanford (1966)
Carbonic anhydrase	29,000	Lambin (1978)
Soybean trypsin inhibitor	20,100	Lambin (1978)
β-Lactoglobulin B	17,500	Righetti and Caravaggio (1978)
Lysozyme	14,300	Lambin (1978)

CHAPTER 3

THE RENIN CONTENT OF AUTOPSY KIDNEYS

3.1 Introduction

In spite of its importance, renin has been purified to homogeneity only within the last decade. One problem encountered in its purification has been the low concentration of the enzyme in the renal cortex (Inagami et al., 1977b; Slater, 1980). In particular, 'normal' human renal cortex, which can only be obtained post mortem, has been reported to contain particularly low concentrations of the enzyme (Inagami et al., 1977b). Published procedures for the purification of human renin, using several kilograms of renal cortex as starting material have yielded only a few hundred micrograms of pure enzyme (Slater et al., 1978; Yokosawa et al., 1978; Yokosawa et al., 1980; Higaki et al., 1983).

The renin content of human kidneys obtained post mortem might be expected to vary considerably, depending both on the physiological status of the individual at the time of death, and on the delay between death and the removal of the kidney at autopsy. In the present study, therefore, preliminary experiments were carried out to determine the quantities of renin present in the renal cortex of individual human kidneys and only tissue containing relatively high concentrations of both active renin and inactive renin was selected for use in subsequent purification studies.

3.2 Measurement of renin content of autopsy kidneys

(a) Preparation of extracts of renal cortex

Eleven human kidneys were obtained post mortem from individuals with no history of renal disease. In each case a 1g sample of renal cortex was removed from the kidney and the remainder of the kidney was stored frozen at -20°C. Each sample of cortex was homogenised in 5ml of 50mM-Tris/HCl

(pH 7.4)/20mM-benzamidine/5mM-EDTA/1mM-phenylmethanesulphonyl fluoride/0.5mM-N-ethyl maleimide/0.01% (w/v) Tween 20 (extraction buffer) with a Polytron PT 20s (5 min, 25,000 rev/min) and the homogenates were centrifuged for 30 min at 100,000g and 4°C. Each pellet was washed with a further 5ml of extraction buffer, centrifuged as described above and the wash was then added to the initial supernatant.

(b) Assay of active renin and of inactive renin in extracts

The concentrations of active renin and of inactive renin present in the extracts of renal cortex were measured using the methods described in Section 2.4. For each extract, six measurements of the active renin concentration and of the total renin concentration (defined as the renin concentration measured after trypsin-activation) were made, and the mean values were determined in each case. The inactive renin concentration was calculated as the difference between these means

3.3 Results and discussion

The concentrations of active renin and of inactive renin in the individual extracts of renal cortex (expressed as milliunits/g cortex) are given in Table 3.1. The concentrations of both forms of the enzyme were found to vary considerably. The range of active renin concentrations was 7 to 3,400 milliunits/g cortex. Inactive renin was present in a number of kidneys, representing up to 25% of the total renin present in the renal cortex.

The concentration of inactive renin was derived as a difference measurement between 'total' and 'active' renin. In cases where active renin formed a large percentage (over 92%) of the total renin concentration, the values obtained for this difference measurement were not statistically significant. The problems associated with the measurement of inactive renin in such situations have been discussed by several authors (Millar et al., 1978; Sealey et al., 1980; Derkx et al., 1983) and in these cases it may be appropriate

to assay inactive renin after it has been chromatographically separated from the active form of the enzyme. However, in the present study, in which inactive renin measurements were required only as a guide to tissue levels of the protein, such a separation was considered unnecessary.

The studies described in Chapters 4 and 5 of this thesis were carried out using renal cortex obtained from kidney no. 1 in Table 3.1 (active renin concentration, 3.4 units/g; inactive renin concentration, 1.1 units/g).

Table 3.1

Concentrations of active renin and of inactive renin in extracts of renal cortex prepared from autopsy kidneys.

	Renin concentration (milliunits/g cortex)		
Kidney	Active[a]	Total[a]	Inactive[b]
1	3400 ± 150	4510 ± 170	1110 *
2	2120 ± 90	2330 ± 110	210 *
3	1210 ± 60	1250 ± 80	40
4	404 ± 18	429 ± 35	25
5	134 ± 9	185 ± 13	51 *
6	102 ± 5	111 ± 5	9
7	62.5 ± 3.1	70.9 ± 5.9	8.4 *
8	32.9 ± 2.1	34.6 ± 6.2	1.7
9	22.5 ± 1.9	26.2 ± 3.1	3.7 *
10	11.7 ± 0.9	12.9 ± 1.6	1.2
11	7.3 ± 0.3	9.2 ± 0.8	1.9 *

[a] Active renin and total renin (renin concentration measured after trypsin-activation) were assayed as described in Section 2.4. Mean ± SD of six measurements.

[b] Inactive renin was calculated as the difference between the mean values obtained for the total renin concentration and the active renin concentration. *, $p < 0.05$ (unpaired t-test).

CHAPTER 4

PURIFICATION OF RENIN FROM HUMAN RENAL CORTEX.

4.1 Introduction

Attempts to purify renin over the last 40 years have largely reflected the state-of-the-art in protein chemistry of given periods. Early studies, using conventional techniques such as salt and solvent fractionation, gel filtration and ion exchange chromatography yielded products which were markedly heterogeneous and which were often unstable due to the presence of contaminating proteinases (Haas et al., 1953; Maier and Morgan 1966; Wauldhausl et al., 1970). In recent years, the application of affinity chromatography to this problem has resulted in the isolation of renin from the renal cortex of various species, including man (Inagami et al., 1980).

One problem encountered in the use of affinity chromatography in renin purification has been the lack of a ligand which is both specific for renin and binds the enzyme with sufficient affinity to sequester it from crude extracts of renal cortex. Attempts to use short peptide analogues of renin substrate as ligands have produced adsorbents with only moderate affinities for renin (Majstoravich et al., 1974; Poulsen et al., 1975). The acylated pentapeptide pepstatin has been used with considerable success in the purification of renin from renal cortex (Murakami et al., 1973; Devaux et al., 1976; Inagami et al., 1980). Unfortunately, however, it lacks specificity, acting as an excellent affinity ligand for another tissue proteinase, cathepsin D. The purification of renin using published protocols, therefore, remains a lengthy procedure, involving several steps of conventional chromatography as well as affinity chromatography (Slater, 1981).

In the present study, a new potent peptide inhibitor of renin, H.77 (D-His-Pro-Phe-His-Leu^{R}Leu-Val-Tyr, where R is a reduced peptide bond, $-CH_2-NH-$;

Szelke et al., 1982), has been used as an affinity ligand to achieve the rapid isolation of a highly purified and stable renin preparation from human renal cortex.

4.2 Purification of renin

All buffers contained 20mM-benzamidine,5mM-EDTA and 0.01%(v/v) Tween 20.

Renal cortex (30g) was subjected to two cycles of freezing and thawing and was then homogenised in 40ml of 50mM-Tris/HCl (pH 7.4)/1mM-phenylmethanesulphonyl fluoride/0.5mM-N-ethylmaleimide with a Polytron PT 20s homogeniser (5min, 25,000 rev/min). The homogenate was centrifuged for 30min at 100,000g and 4°C. The supernatant solution was passed through a glass-microfibre filter (Whatman GF/C) and immediately applied to a column (1.6cm x 5cm) of H.77-Sepharose equilibrated with extraction buffer. The column was washed with 100ml of the same buffer, with 100ml of 0.1M-Tris/HCl (pH 7.4)/1M-NaCl and finally with 100ml of 0.1M-sodium acetate (pH 6.0)/1M-NaCl. Renin was eluted with a pH gradient generated by allowing 75ml of 0.1M-acetic acid to mix with 75ml of 0.1M-sodium acetate, pH 6.0.* The flow rate was 0.2ml/min and 5ml fractions were collected.

The renin-containing fractions were pooled and adjusted to pH 6.8 by adding 2M-Tris. The enzyme was then concentrated by ultrafiltration and stored at -20°C in 50%(v/v) glycerol at a concentration of 100µg/ml.

4.3 Results and discussion

In the above procedure crude extract of human renal cortex containing 102 units of renin activity was applied to the column of H.77-Sepharose. At pH 7.4 the renin activity was bound tightly by the immobilised H.77 (Figure 4.1). In contrast, non-specific acid proteinase activity did not bind and emerged from the column with the bulk protein fraction. After washing the column to remove weakly bound proteins, renin activity was eluted with a pH

* The gradient was linear with respect to the acetic acid.

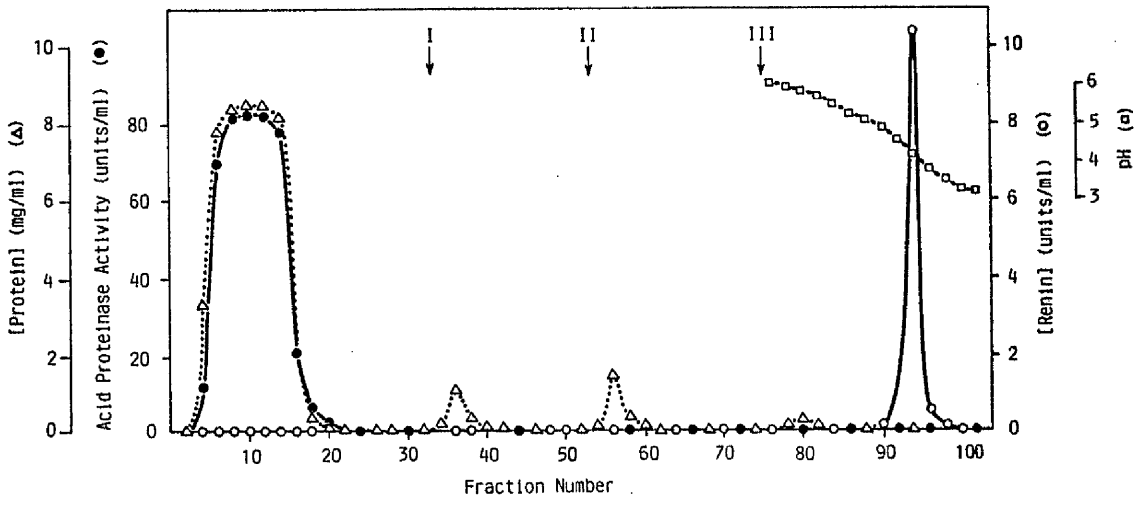

Figure 4.1 **Affinity chromatography of renin on H.77-Sepharose**

Crude extract of human kidney cortex was applied to a column of H.77-Sepharose equilibrated with extraction buffer. The column was washed with 0.1M-Tris/HCl (pH 7.4)/1M-NaCl (arrow I) and then with 0.1M-sodium acetate (pH 6.0)/1M-NaCl (arrow II). Renin was eluted with a pH gradient from pH 6.0 to pH 3.2 in 0.1M-acetate buffer (arrow III).

Table 4.1

Purification of human renal renin

	Protein (mg)	Renin activity (units)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Crude homogenate	645	102	0.158	1	100
Renin eluted from H.77-Sepharose	0.089	77.5	871	5512	76

gradient from pH 6.0 to 3.2. This resulted in a 5500-fold increase in the specific activity of renin from 0.158 units/mg of protein in the crude material to 871 units/mg; 89µg of purified enzyme were obtained, representing a yield of 76% (Table 4.1).

The instability of renin encountered in previous studies has been attributed to the presence of contaminating proteinases and, in particular, to cathepsin D-like activity (Inagami et al., 1980). The purified renin obtained in the present study exhibited no non-specific proteolytic activity towards (^{3}H) acetylhaemoglobin and was found to be stable over a period of several months at pH 6.8 and -20^{o}C.

The specific activity of human renin has been reported to be 400 units/mg by Slater et al. (1978). These authors used the fluorescamine assay of Bohlen et al. (1973) to determine the protein content of the pure enzyme. Values of 830 units/mg (Yokosawa et al., 1978) and 950 units/mg (Yokosawa et al., 1980) have been obtained for specific activity of pure human renin using the method of Lowry et al. (1951) to determine protein concentration. The purified renin obtained in the present study was found to have a similar specific activity (871 units/mg), the dye-binding method of Bradford (1976) being used to measure protein concentration.

The M_r value of the purified enzyme was determined both by gel filtration and by SDS-polyacrylamide gel electrophoresis. Standard proteins of known M_r were used to calibrate a column of Sephadex G100 (superfine grade) and a standard curve of K_{av} versus log M_r was constructed (Figure 4.2). Gel filtration of the purified renin gave a single peak of renin activity corresponding to an apparent M_r value of 40,000. This value compares well with values obtained in previous studies (Table 4.2). Examination of the enzyme by electrophoresis in the presence of 0.1% (w/v) SDS (Figure 4.3) showed that it was not homogeneous, but consisted of two distinct polypeptides. The M_r

values of these proteins, estimated by comparison with a standard curve of electrophoretic mobility versus log M_r (Figure 4.4) were 40,000 and 17,000 respectively. The larger protein probably corresponds to the renin of M_r 40,000 isolated previously (Table 4.2). The nature of the 17,000 M_r protein is unclear at present, although it may represent a fragment of renin formed by limited proteolysis. Galen et al. (1979) have reported the presence of fragments of renin with apparent M_r values of 25,000 and 20,000 in a renin preparation (specific activity 860 units/mg) isolated from a juxtaglomerular cell tumour of renal cortex.

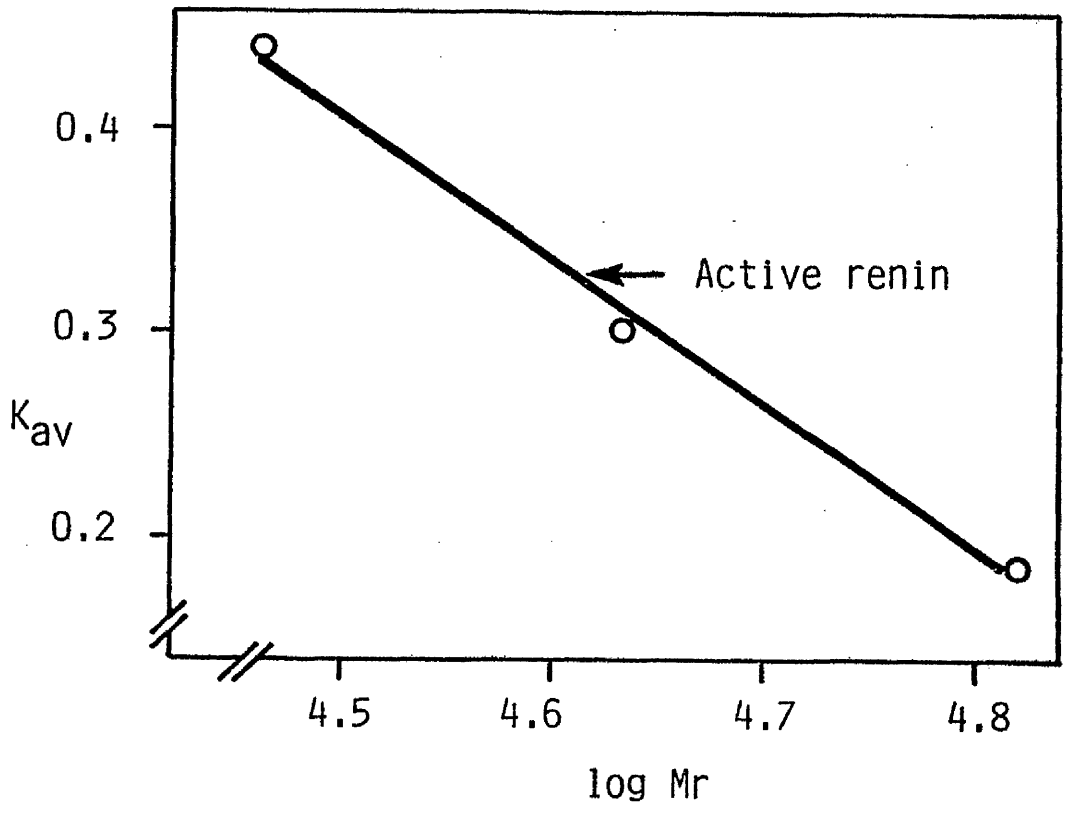

Figure 4.2 **Standard curve of K_{av} versus log M_r for gel filtration of active renin**

Gel filtration of active renin on Sephadex G100 (superfine grade) was carried out as described in Section 2.7(a) using ^{14}C-methylated marker proteins as internal standards. The standard proteins used were bovine serum albumin (66,200), ovalbumin (43,000) and carbonic anhydrase (29,000). M_r values were taken from the sources listed in Table 2.2.

Table 4.2

The reported M_r values of purified active renin

Reference	M_r value	Method of measurement
This study	40,000	Gel filtration
This study	40,000	SDS/polyacrylamide gel electrophoresis
Slater et al., (1978)	40,000	SDS/polyacrylamide gel electrophoresis
Yokosawa et al., (1980)	42,000	Gel filtration
Yokosawa et al., (1980)	41,000	Sedimentation equilibrium
Yokosawa et al., (1980)	40,000	SDS/polyacrylamide gel electrophoresis
Higaki et al., (1983)	40,000	Gel filtration
Higaki et al., (1983)	40,000	SDS/polyacrylamide gel electrophoresis

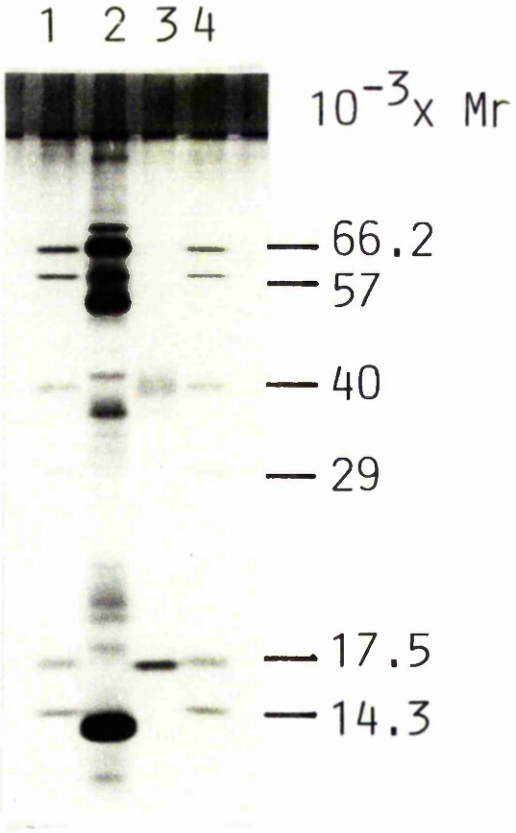

Figure 4.3. SDS/polyacrylamide gel electrophoresis of purified renin

Discontinuous electrophoresis was carried out in a 1mm-thick polyacrylamide slab gel (stacking gel, 3% polyacrylamide; resolving gel, 10% polyacrylamide). Protein bands were detected using the silver staining method of Wray et al. (1981). Track 2, 4µg of crude kidney cortex extract; track 3,100ng of purified renin; tracks 1 and 4, standard M_r marker proteins: 50ng each of bovine serum albumin (66,200), pyruvate kinase (57,000), aldolase (40,000), carbonic anhydrase (29,000), β-lactoglobulin B (17,500) and lysozyme (14,300). Subunit M_r values were taken from the sources listed in Table 2.2.

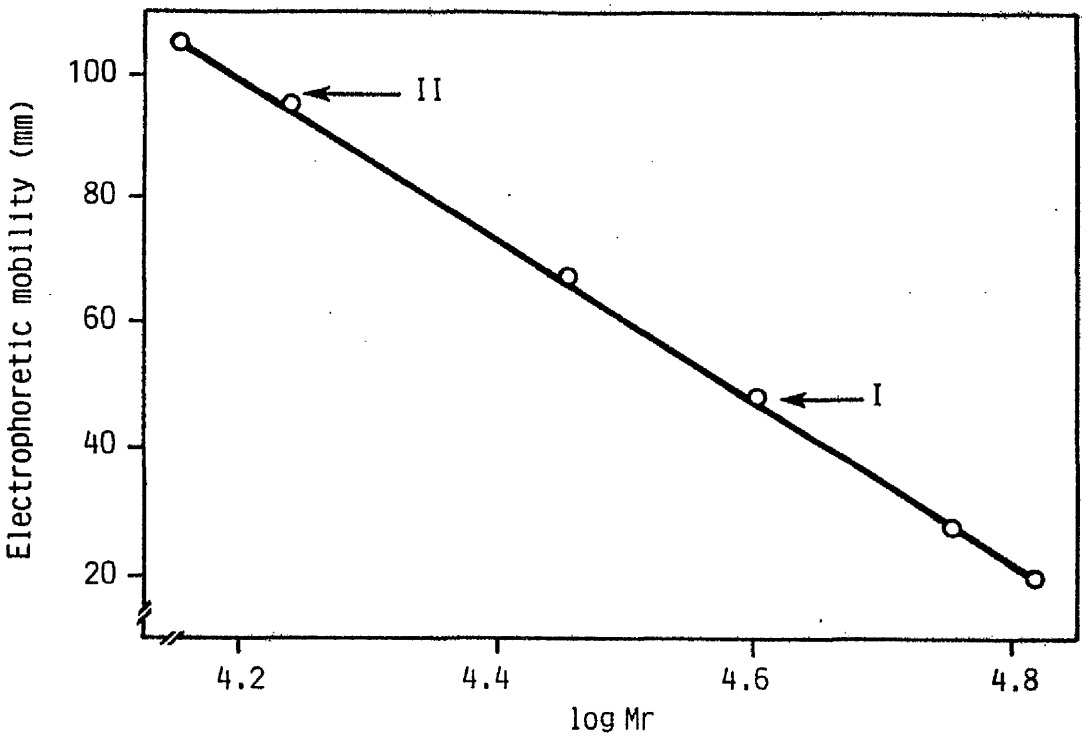

Figure 4.4. **Standard curve of electrophoretic mobility versus log M_r for SDS/polyacrylamide gel electrophoresis of active renin.**

Discontinuous electrophoresis in the presence of 0.1% (w/v) SDS was carried out in a 1mm-thick polyacrylamide slab gel (stacking gel, 3% polyacrylamide; resolving gel, 10% polyacrylamide). The standard proteins used were bovine serum albumin (66,200), pyruvate kinase (57,000), aldolase (40,000), carbonic anhydrase (29,000), β-lactoglobulin B (17,500) and lysozyme (14,300). Subunit M_r values were taken from the sources listed in Table 2.2. The mobilities of the two proteins present in the purified human renin preparation are indicated by arrows I (M_r 40,000 protein) and II (M_r 17,000 protein) respectively.

CHAPTER 5

PURIFICATION OF INACTIVE RENIN FROM HUMAN RENAL CORTEX.

5.1 Introduction

Although inactive renin has been purified to homogeneity from pig renal cortex (Inagami et al., 1982c; Takii and Inagami, 1982), it has not yet been obtained in pure form from human kidney. Partial purifications of human renal inactive renin have been achieved using dye ligand chromatography, hydrophobic interaction chromatography and chromatography on pepstatin-Sepharose to achieve the complete separation of the active and inactive forms of renin (Chang et al., 1981; Atlas et al., 1981; Atlas et al., 1982b; Hsueh et al., 1983). A partial purification of human renal inactive renin has also been obtained using a monoclonal immunoadsorbent, F15-Sepharose (Pau et al., 1981). In the present study, inactive renin has been successfully isolated from human renal cortex using a combination of chromatography on H.77-Sepharose (to remove active renin), ammonium sulphate fractionation, hydrophobic interaction chromatography on octyl-Sepharose and immunoaffinity chromatography on F15-Sepharose.

5.2 Purification of inactive renin

In order to minimise the effects of tissue proteinases on inactive renin during its purification, all manipulations of the enzyme were carried out at 4°C and the proteinase inhibitors benzamidine and EDTA were included in all buffers at concentrations of 20mM and 5mM respectively.

(a) Chromatography of crude extract of kidney cortex on H.77-Sepharose

The preparation of crude extract of renal cortex and its chromatography on H.77-Sepharose have already been described in Chapter 4. The protein-containing fractions which did not bind to the H.77-Sepharose were pooled and inactive renin was further purified from this material as described below.

(b) Ammonium sulphate fractionation

The solution obtained in the above procedure (90ml) was made 1.0M with respect to ammonium sulphate by adding 12.8g of crushed ammonium sulphate over a period of 5 min with constant stirring. After 30 min, precipitated protein was removed by centrifugation for 10 min at 30,000g and 4°C. The supernatant solution (95ml) was adjusted to 2.6M with respect to ammonium sulphate by adding a further 24.8g of crushed ammonium sulphate over a period of 15 min. After the ammonium sulphate had dissolved, the solution was stirred for 60 min and the resultant precipitate was collected by centrifugation for 15 min at 30,000g and 4°C. The precipitated protein was resuspended in 10ml of 20mM-sodium phosphate (pH 7.0)/1.0M-ammonium sulphate and the suspension was dialysed overnight (16h) against 2 litres of the same buffer with one change of dialysis buffer after 3h.

(c) Chromatography on octyl-Sepharose

The protein solution obtained after ammonium sulphate fractionation (22ml) was applied to a column (1.6cm x 50cm) of octyl-Sepharose equilibrated with 20mM-sodium phosphate (pH 7.0)/1.0M-ammonium sulphate. The column was washed with 400ml of the same buffer and then with 400ml of 20mM-sodium phosphate, pH 7.0. Inactive renin was eluted using 20mM-sodium phosphate (pH 7.0)/50% (v/v) ethanediol/0.1% (v/v) Tween 20. The flow rate was 0.5ml/min and 10ml fractions were collected.

The fractions containing inactive renin were pooled, dialysed against 2 litres of 50mM-Tris/HCl for 5h with changes of dialysis buffer every 30 min and then concentrated to a final volume of 5ml by ultrafiltration.

(d) Chromatography on H.77-Sepharose/F15-Sepharose

The protein solution obtained above was passed through a column (1cm x 2cm) of H.77-Sepharose equilibrated with 50mM-Tris/HCl, pH 7.4 which was linked in tandem to a column (1cm x 2cm) of F15-Sepharose equilibrated with

the same buffer. The columns were separated and the F15-Sepharose was washed with 5ml of 50mM-Tris/HCl, pH 7.4 and then with 5ml of 100mM-Tris/HCl, pH 7.4. Inactive renin was eluted with 2ml of 100mM-sodium acetate, pH 4.5. The flow rate throughout this procedure was 0.1ml/min.

The eluate from the F15-Sepharose was immediately concentrated by ultrafiltration to a volume of about 100µl and then adjusted to pH 7.4 by dialysis for 3h against 500ml of 100mM-Tris/HCl, pH 7.4. The purified inactive renin was stored at -20°C in 50% (v/v) glycerol at a concentration of 10µg/ml.

5.3 Results and discussion

The affinity chromatography of crude extract of renal cortex on H.77-Sepharose described in Chapter 4 was also used as the first step in the purification of inactive renin. As noted previously (Chapter 4), at pH 7.4, renin activity was bound tightly by the immobilised H.77. In contrast, inactive renin did not bind and emerged from the column with the bulk protein fraction (Figure 5.1). This material contained less than 0.1% of the renin activity present in the initial extract of renal cortex (Table 5.1).

Ammonium sulphate fractionation of the breakthrough fractions from the H77-Sepharose resulted in a 4-fold increase in the specific activity of inactive renin and a further 35-fold purification was achieved by hydrophobic interaction chromatography on octyl-Sepharose (Figure 5.2). The inactive renin eluted from the octyl-Sepharose (18.3 units) still contained a small quantity of active renin (4.7 milliunits). This was removed by passing the inactive renin through a small column of H.77-Sepharose prior to the final step of purification which involved affinity chromatography using a monoclonal immunoadsorbent, F15-Sepharose (Pau et al., 1981). Inactive renin was bound by the immobilished monoclonal antibody at pH 7.4 and was eluted by a change in pH to 4.5. This step resulted in a further 105-fold purification of the enzyme.

The purification procedure described above produced an overall 14,750-

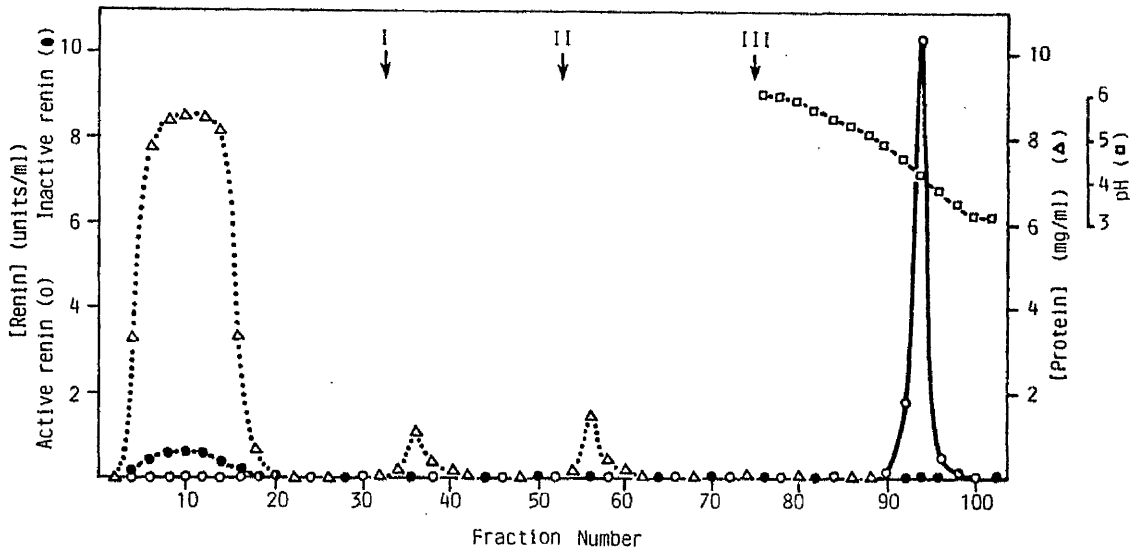

Figure 5.1 **Chromatography of crude extract of kidney cortex on H.77-Sepharose**

Crude extract of human kidney cortex was applied to a column of H.77-Sepharose equilibrated with extraction buffer. The column was washed with 0.1M-Tris/HCl (pH 7.4)/1M-NaCl (arrow I) and then with 0.1M-sodium acetate (pH 6.0)/1M-NaCl (arrow II). Renin was eluted with a pH gradient from pH 6.0 to pH 3.2 in 0.1M-acetate buffer (arrow III).

Table 5.1

Purification of human renal inactive renin

Step	Protein (mg)	Active renin (units)	Inactive renin (units)	Inactive renin Specific activity (units/mg)	Inactive renin Purification (fold)	Inactive renin Yield (%)
Crude homogenate	645	102	31	0.048	1	100
H.77-Sepharose	614	0.098	30.5	0.049	1	98
Ammonium sulphate fractionation	133	0.082	24.9	0.187	4	80
Octyl-Sepharose	2.7	0.0047	18.3	6.78	141	59
H.77-Sepharose/F15-Sepharose	0.012[a]	0[b]	8.5	708	14750	27

[a] - Based on single protein determination

[b] - No generation of angiotensin I after 5h incubation of 5ng of inactive renin with ox angiotensinogen in standard renin assay (Chapter 2, Section 2.4(a))

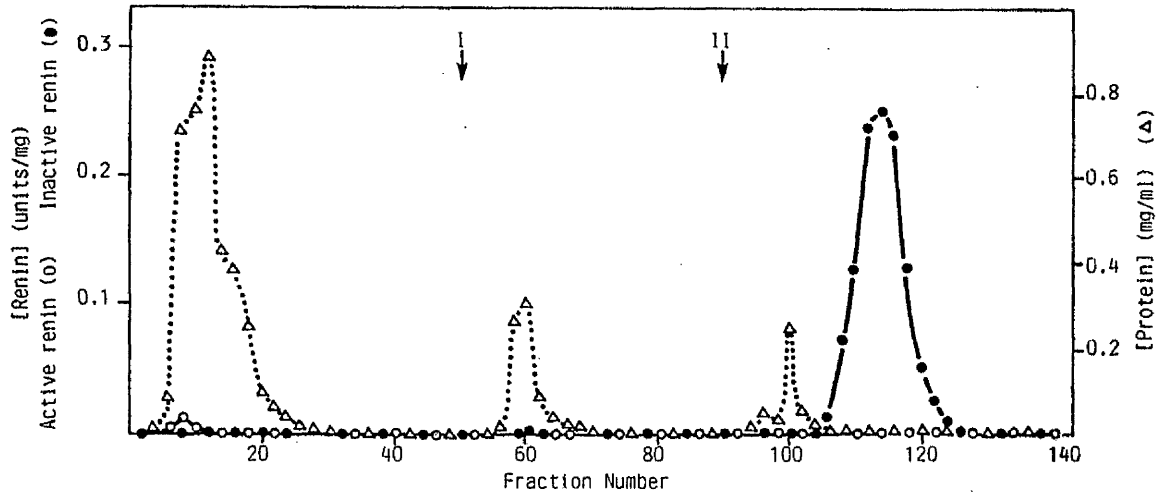

Figure 5.2 **Hydrophobic interaction chromatography of inactive renin on octyl-Sepharose**

Partially purified inactive renin in 20mM-sodium phosphate (pH 7.0)/1.0M-ammonium sulphate was applied to a column of octyl-Sepharose equilibrated with the same buffer. The column washed with 20mM-sodium phosphate, pH 7.0 (arrow I). Inactive renin was eluted with 20mM-sodium phosphate (pH 7.0)/50% (v/v) ethanediol/0.1% (v/v) Tween 20 (arrow II).

fold increase in the specific activity of inactive renin from 0.048 units/mg in the crude tissue extract to 708 units/mg; 12µg of purified protein were obtained, representing a yield of 27%. The purified inactive renin exhibited no activity towards angiotensinogen and was stable over a period of several months at pH 7.4 and -20°C.

Throughout the purification of inactive renin, its concentration was defined as the difference between the renin concentrations measured before and after trypsin activation. The activation of inactive renin was carried out as described in Chapter 2 (Section 2.4(b)) by incubating samples at pH 7.4 with 600 BAEE units of trypsin for 5h at 4°C in the presence of 20mM-benzamidine and 1% (w/v) bovine serum albumin. This method was also used to determine the specific activity of the purified inactive renin. In order to check that the activation procedure resulted in the maximum activation of inactive renin without the concomitant destruction of the enzyme, the effect of varying the trypsin concentration and the time of incubation was investigated (Figure 5.3). Incubation of the purified inactive renin with 600 BAEE units of trypsin at 4°C resulted in an increase in renin activity which reached a maximum value after 3h. The renin activity remained constant during a further 4h of incubation with trypsin indicating that little or no destruction of renin occurred under these conditions. Incubation with 120 BAEE units of trypsin was less effective in activating inactive renin, maximal activation being achieved only after 5h. With 3,000 BAEE units of trypsin, the increase in renin activity occurred rapidly, but was followed by a decrease in activity.

Examination of the purified inactive renin by electrophoresis in the presence of 0.1% (w/v) SDS (Figure 5.4) showed that it consisted of a single polypeptide chain. The M_r value of the protein estimated by comparison with a standard curve of electrophoretic mobility versus log M_r (Figure 5.5) was 48,000. The size of the purified enzyme was also estimated by gel filtration

on Sephadex G100 (superfine grade). Comparison of the elution volume of the inactive renin with a standard curve of K_{av} versus log M_r (Figure 5.6) yielded an M_r value of 51,000. This value is similar to values previously obtained by gel filtration of partially purified preparations of human renal inactive renin (Table 5.2).

The purified inactive renin obtained in the present study is similar in size to the inactive renin which has been isolated from pig renal cortex (Inagami et al., 1982c; Takii and Inagami, 1982). The latter, like human inactive renin, consists of a single polypeptide chain of M_r 50,000 and is completely inactive against angiotensinogen. Trypsin treatment of pig inactive renin has been reported to result in the complete activation of the enzyme and a reduction in its size close to that of the endogenous active enzyme. The specific activity of purified human inactive renin, based on the renin activity generated by trypsin activation, was 708 units/mg of inactive renin. This is close to the value of 871 units/mg obtained for the purified active renin (Chapter 4). However, in contrast to pig inactive renin, trypsin activation of human inactive renin produced only a slight reduction in the size of the enzyme. The M_r of trypsin-activated inactive renin determined by gel filtration on Sephadex G100 (superfine grade) was 47,000 (Figure 5.6) compared with the value of 40,000 for endogenous active human renal renin (Chapter 4). This difference in the behaviour of the pig and human inactive renins presumably reflects a difference in their primary structures.

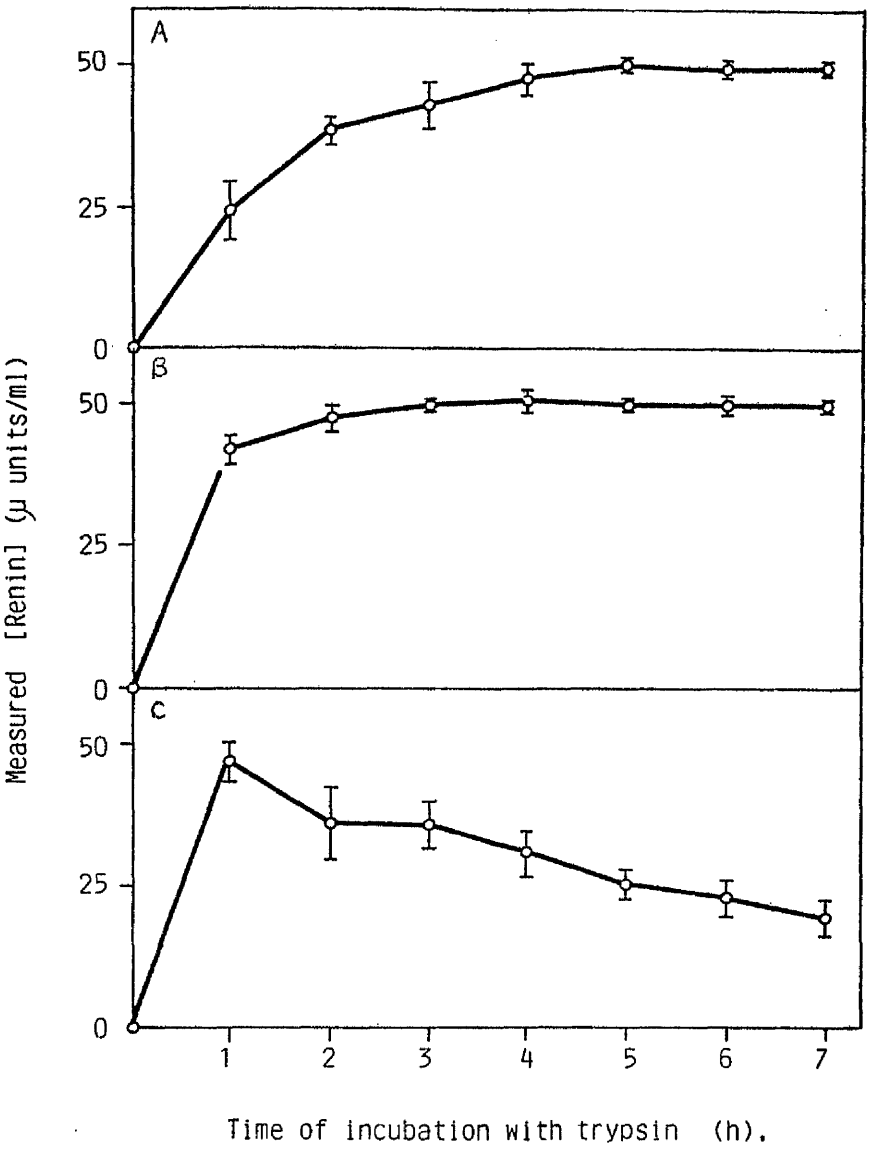

Figure 5.3 **Effect of trypsin concentration on the activation of inactive renin**

Samples of purified inactive renin (70pg/ml) in 50mM-Tris/HCl (pH 7.4)/20mM-benzamidine/5mM-EDTA/1% (w/v) bovine serum albumin were activated using A: 120 BAEE units, B: 600 BAEE units, or C: 3,000 BAEE units of trypsin. The samples (500μl) were activated by adding the trypsin in 50μl of 1mM-HCl and incubating the mixtures at 4°C for varying periods of up to 7h. In each case, the reaction was stopped by adding 50μl of SBTI solution (A: 0.4mg/ml, B: 2mg/ml, C: 10mg/ml) in 50mM-Tris/HCl, pH 7.4 and the renin concentration was then determined as described in Section 2.4(a)

Mean ± S.D. of three determinations.

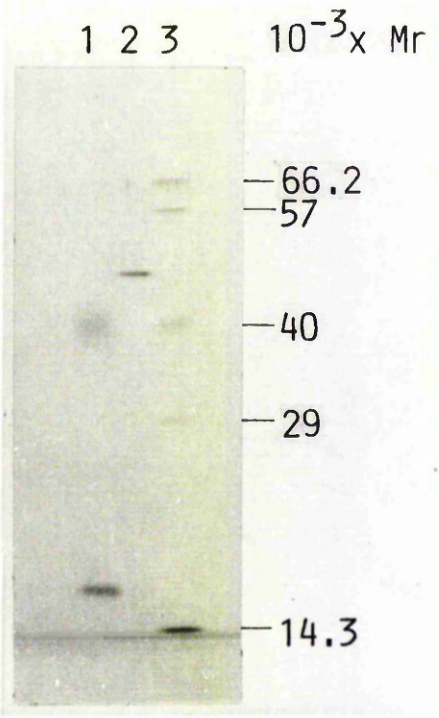

Figure 5.4 **SDS/polyacrylamide gel electrophoresis of purified human renal inactive renin**

Discontinuous electrophoresis was carried out on a 1mm-thick polyacrylamide slab gel (stacking gel, 3% polyacrylamide; resolving gel, 10% polyacrylamide). Protein bands were detected using the silver staining method of Wray et al. (1981). Track 1, 100ng of purified renin; track 2, 50ng of purified inactive renin; track 3, standard M_r marker proteins: 50ng each of bovine serum albumuin (66,200), pyruvate kinase (57,000), aldolase (40,000), carbonic anhydrase (29,000) and lysozyme (14,300). Subunit M_r values were taken from the sources listed in Table 2.2.

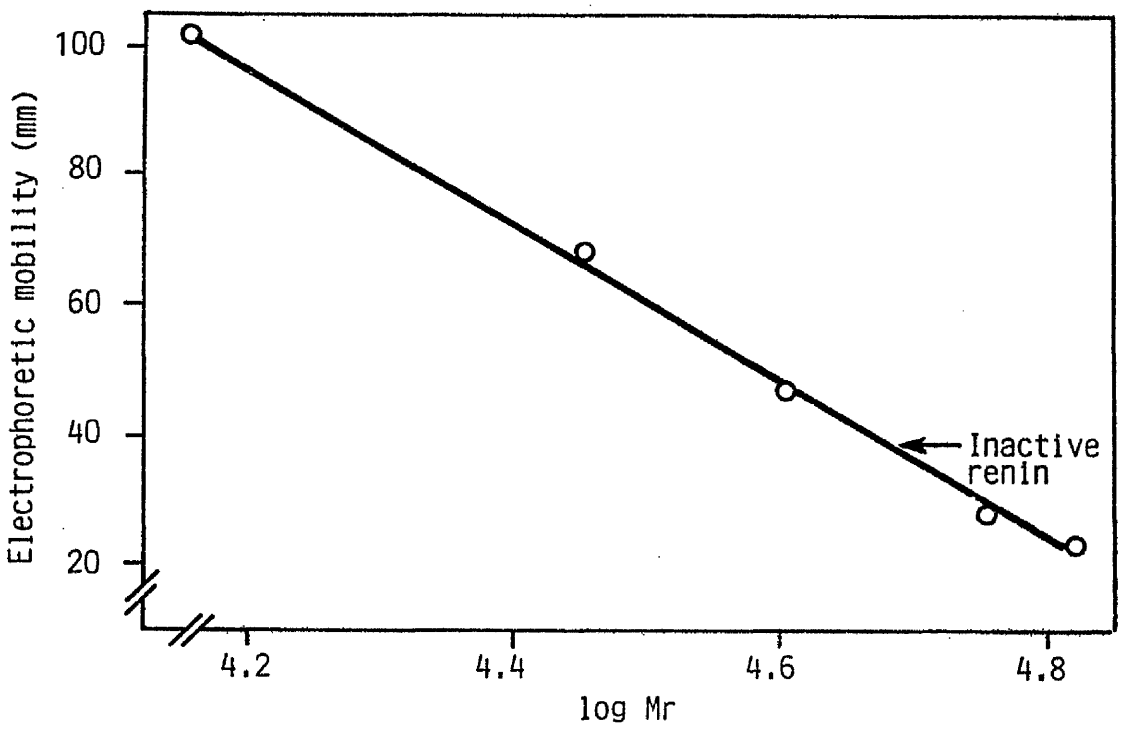

Figure 5.5 **Standard curve of electrophoretic mobility versus log M_r for SDS/polyacrylamide gel electrophoresis of inactive renin**

Discontinuous electrophoresis in the presence of 0.1% (w/v) SDS was carried out in a 1mm-thick polyacrylamide slab gel (stacking gel, 3% polyacrylamide; resolving gel, 10% polyacrylamide). The standard proteins used were bovine serum albumin (66,200), pyruvate kinase (57,000), aldolase (40,000), carbonic anhydrase (29,000) and lysozyme (14,300). Subunit M_r values were taken from the sources listed in Table 2.2.

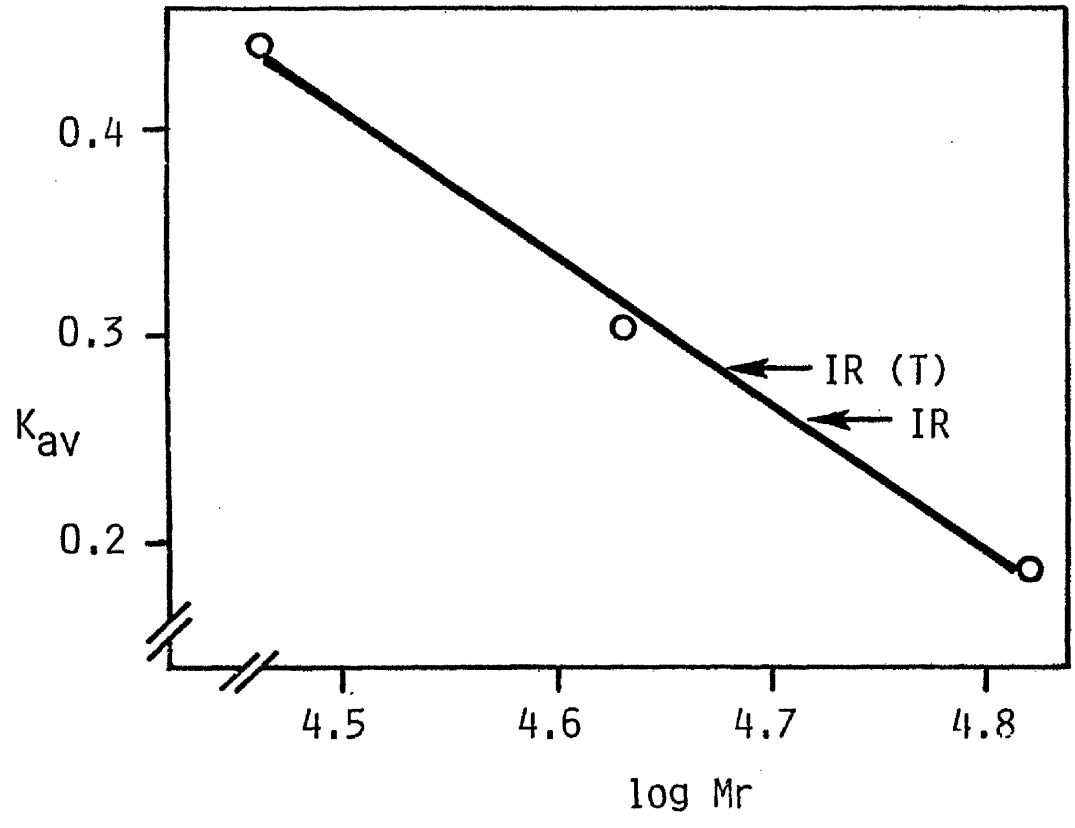

Figure 5.6 **Standard curve of K_{av} versus log M_r for gel filtration of inactive renin and trypsin-activated inactive renin**

Gel filtration of inactive renin and of trypsin-activated inactive renin was carried out as described in Section 2.7(a) using ^{14}C-methylated marker proteins as internal standards. The standard proteins used were bovine serum albumin (66,200), ovalbumin (43,000) and carbonic anhydrase (29,000). M_r values were taken from the sources listed in Table 2.2. The K_{av} values obtained for inactive renin and trypsin activated inactive renin are indicated by the arrows labelled IR and IR(T) respectively.

Table 5.2

The reported M_r values of renal inactive renin

Reference	M_r value	Method of measurement
This study	51,000	Gel filtration
This study	48,000	SDS/polyacrylamide gel electrophoresis
Atlas et al. (1981)	49,500	Gel filtration
Chang et al. (1981)	51,000	Gel filtration
Inagami et al. (1983)	49,000-50,000	SDS/polyacrylamide gel electrophoresis with immunospecific staining.

CHAPTER 6

THE REVERSIBLE ACTIVATION-INACTIVATION OF INACTIVE RENIN

6.1 Introduction

As discussed in Chapter 1, plasma inactive renin can be activated by dialysis of plasma to a pH of between 3.0 and 3.3 followed by dialysis to neutral pH. The activation of plasma inactive renin which is produced by this manipulation is irreversible and has been shown to depend on the action of endogenous serine proteinases during the dialysis of acidified plasma to neutral pH (Atlas et al., 1978; Leckie, 1978). Recently, Leckie and McGhee (1980) and Hsueh et al., (1981) have shown that the acidification of plasma per se results in the complete activation of inactive renin and that if plasma serine proteinase activity is inhibited, this activation can be reversed by dialysis of the acidified plasma to neutral pH. The reversible activation of inactive renin has also been demonstrated using partially purified inactive renin from human kidney (Chang et al., 1982; Atlas et al., 1982b; Hsueh et al., 1983) or amniotic fluid (Franks et al., 1982), but has not yet been demonstrated using pure inactive renin.

6.2 Reversible acid-activation of pure human renal inactive renin

All buffers contained 20mM-benzamidine 5mM-EDTA and 1% (w/v) bovine serum albumin.

Pure human inactive renin was diluted to a final concentration of 7ng/ml in 100mM-Tris/HCl, pH 7.4 containing ^{14}C-methylated bovine serum albumin (100,000 cpm/ml). 400µl samples of this solution were acidified by dialysis against 1 litre of 100mM-glycine/HCl, pH 3.3 for 24h at 4°C and were subsequently adjusted to neutral pH by dialysis against 1 litre of 100mM-Tris/HCl, pH 7.4 for 24h at 4°C. After each period of dialysis, the samples were assayed for renin activity at neutral pH as described in Section 2.4(a). In

each case, the concentration of ^{14}C-methylated bovine serum albumin was determined by liquid scintillation counting of 50µl of the dialysed solution in 8ml of Packard scintillator 299, and this value was used to correct the renin activity measurement for any changes in sample volume which had occurred during dialysis.

6.3 Results and discussion

Figure 6.1 shows that human renal inactive renin was activated by dialysis to pH 3.3 and that this activation was reversed by dialysis back to neutral pH. If this material was subsequently acidified, then the inactive renin was re-activated indicating that the loss of renin activity during the dialysis of acidified inactive renin to neutral pH was not due to the irreversible denaturation of the enzyme.

The rate of angiotensin I generation by the acid- activated inactive renin was non-linear with time (Figure 6.2) due to the re-inactivation of the enzyme during its incubation with ox renin substrate at neutral pH. When short incubation times were used (10 min or less) to assay renin activity, the maximal activity produced by the acidification of inactive renin (3.93 $\pm$ 0.21 ng AI/h/ng inactive renin) was comparable to that produced by trypsin-activation (4.1 $\pm$ 0.19 ng AI/h/ng inactive renin).

Leckie and McGhee (1980) have postulated that the reversible activation of inactive renin is due to the dissociation and re-association of a renin-inhibitor complex. In contrast, Hseuh et al. (1981) have suggested that acidification induces a partial unfolding of a single chain inactive renin to expose the active site of the enzyme. In support of the latter hypothesis, Atlas et al. (1982a) have reported that the re-inactivation of acidified inactive renin is a first order process, a finding which is consistent with a unimolecular reaction rather than a bimolecular reaction. Unfortunately, however, the inactive renin used in the above study was only partially purified and thus the pseudo-first order reaction

of renin with a large excess of an inhibitor protein could not be excluded as a possible explanation of the experimental data. In the present study, the single chain inactive renin purified from human renal cortex has been shown to be reversibly activated by acidification, indicating that the phenomenon is indeed due to a pH-induced conformational change in a single polypeptide chain rather than to the dissociation of a renin-inhibitor complex.

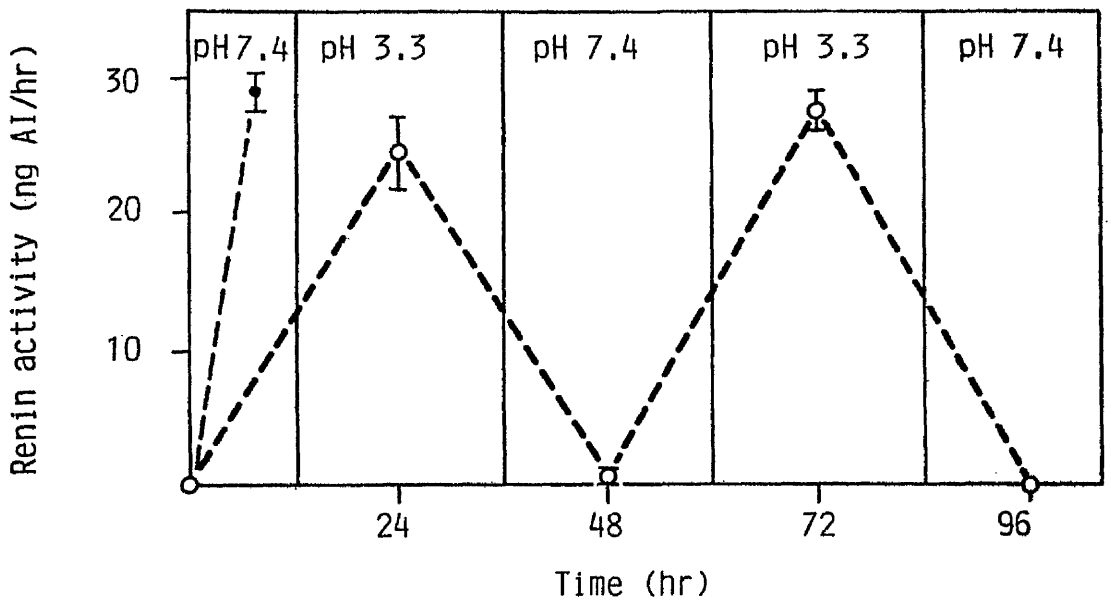

Figure 6.1 **Reversible acid-activation of inactive renin**

Samples of inactive renin diluted to a concentration of 7ng/ml in 100mM-Tris/HCl (pH 7.4)/20mM-benzamidine/5mM-EDTA/1% (w/v) bovine serum albumin were adjusted to pH 3.3 by dialysis for 24h at 4°C against 100mM-glycine/HCl (pH 3.3)/20mM-benzamidine/1% (w/v) bovine serum albumin. The samples were readjusted to pH 7.4 by dialysis for 24h at 4°C against 100mM-Tris/HCl (pH 7.4)/20mM-benzamidine/1% (w/v) bovine serum albumin. After each period of dialysis the samples were assayed for renin activity as described in Section 2.4(a). Each point (O) represents the mean ± SD of five experiments. Samples of inactive renin 7ng/ml) were also trypsin-activated as described in Section 2.4(b); ●, mean ± SD of five experiments.

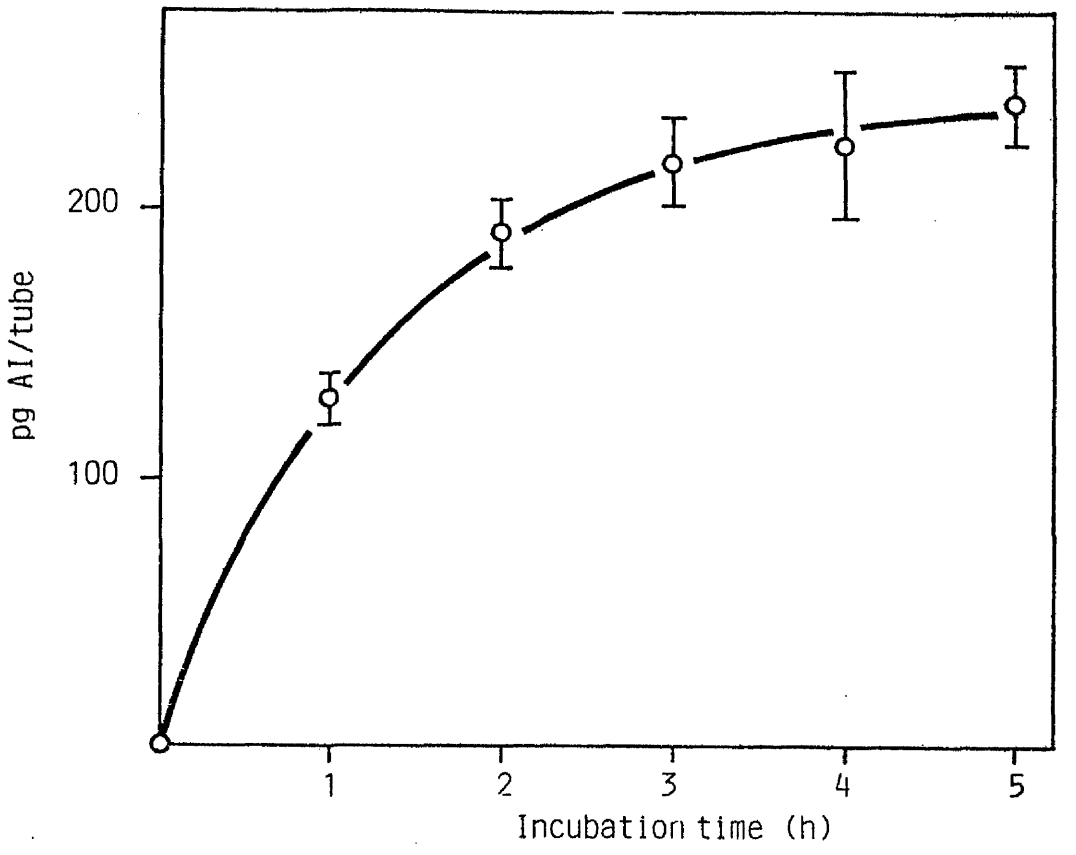

Figure 6.2 **Time course of angiotensin I generation by acid-activated inactive renin**

Samples of acid-activated inactive renin were diluted to a concentration of 70pg/ml in 50mM-Tris/HCl (pH 7.4)/20mM-benzamidine/1% (w/v) bovine serum albumin and immediately assayed for renin activity as described in Section 2.4(a). Incubations were carried out for periods of up to five hours. Mean ± SD of five determinations.

CHAPTER 7

STRUCTURAL COMPARISON OF THE ACTIVE AND INACTIVE FORMS OF RENIN

7.1 Introduction

As discussed in Chapter 1, it has been suggested that inactive renin is a biosynthetic precursor of renin analogous to the well known zymogens pepsinogen and trypsinogen (Sealey et al., 1982a).

The inactive renin purified in the present study consists of a single polypeptide chain and is slightly larger than its active counterpart. The purified protein is completely inactive against ox angiotensinogen, but can be activated by limited proteolysis. Previous studies using partially purified inactive renin have shown that trypsin activated inactive renin and endogenous active renin are similar, both immunologically and with respect to their kinetic properties (Atlas et al., 1982b; Hsueh et al., 1983). These data strongly suggest that inactive renin is indeed a renin zymogen.

In order to obtain direct evidence for a structural relationship between active renin and inactive renin, the two forms of the enzyme have been compared by peptide 'mapping'.

7.2 Peptide 'mapping' of the active and inactive forms of renin

(a) Radiolabelling of proteins

Protein samples were radiolabelled using the method of Bolton and Hunter (1973). In this method the protein is treated with a ^{125}I-labelled acylating reagent, N-succinimidyl-3-(4-hydroxy,5-(^{125}I)iodophenyl)propionate (Bolton-Hunter reagent), which reacts with free amino groups on the protein molecule.

Sample preparation 50µl samples (2µg) of purified active renin, purified inactive renin, and of a mixture of standard proteins (0.4µg each of

pyruvate kinase, ovalbumin, aldolase, carbonic anhydrase and β-lactoglobulin B) were dialysed exhaustively at 4°C against 100mM-triethanolamine/HCl, pH 8.5 and were adjusted to a final volume of 80µl using the same buffer. 20µl of 10% (w/v) SDS was added to each sample. The protein solutions were heated to 100°C in a boiling water bath for 3 min and were then cooled to 4°C.

Radiolabelling of samples 1µg of each protein sample (50µl) was reacted with 300 pmoles of labelled Bolton-Hunter reagent. In each case, the reagent (600µCi) dissolved in 50µl of benzene/0.2% (v/v) dimethylformamide, was pipetted into a small glass vial (5mm x 15mm) and then dried by evaporation of the solvent at room temperature under a flow of nitrogen. The protein sample was added to the dried reagent and the reaction mixture was shaken for 30min at 4°C. The reaction mixture was then transferred to a second glass vial (5mm 15mm) containing 10µmoles of unlabelled Bolton-Hunter reagent (N-succinimidyl-3-(4-hydroxyphenyl)propionate and shaken for a further 30min at 4°C. In order to obviate the subsequent labelling of carrier proteins, unchanged Bolton-Hunter reagent was reacted with 50µl of 100mM-Tris/HCl, pH 8.5 for 10min at 4°C. Finally, 100µl of 100mM-Tris/HCl, pH 8.5 containing 200µg of bovine serum albumin as carrier protein was added to the labelled protein.

Purification of labelled proteins The labelled proteins were separated from the other labelled products of the acylation reaction (acylated Tris and 3-(4-hydroxy,5-(^{125}I) iodophenyl)propionic acid) by precipitation with 1ml of ice-cold 10% (w/v) trichloroacetic acid. In each case, the precipitated protein was allowed to stand for 15min on ice and was then collected by centrifugation at 1000g and 4°C for 5min. Each precipitate was washed once with a further 1ml of ice-cold 10% (w/v) trichloracetic acid and twice with 1ml of ice-cold acetone with the same centrifugation each time.

The labelled proteins were further purified by polyacrylamide gel

electrophoresis in the presence of 0.1% (w/v) SDS. Each protein precipitate was dissolved in 50µl of sample buffer (72.5mM Tris/HCl (pH 6.8)/3% (w/v) SDS/10% (v/v) glycerol/5% (v/v) 2-mercaptoethanol/0.002% (w/v) bromophenol blue and electrophoresis was carried out as described in section 2.6(a) using rod gels (10% resolving gels). After electrophoresis was complete, the gels were frozen using powdered dry ice. The gels were sliced into 1mm segments using a manual gel slicer (Bio-Rad model 190) and the segments were then counted for ^{125}I.

Labelled proteins were eluted from the polyacrylamide gel by crushing the appropriate gel segments with a glass rod and incubating the gel fragments for 24h at 4°C with 300µl of 100mM-ammonium bicarbonate/0.01% (w/v) bovine serum albumin (elution buffer). In each case, the gel slurry was then poured into a 1ml disposable pipette tip containing glass wool and the liquid was separated from the gel fragments by centrifugation for 2min at 500rpm in a benchtop centrifuge. The gel slurry was rinsed with a further 200µl of elution buffer and centrifuged again for 2min at 500rpm. The combined eluates were then frozen and the labelled proteins were stored at -20°C.

(b) Cleveland mapping

One dimensional peptide mapping of labelled proteins isolated from polyacrylamide gels was carried out by the method of Cleveland et al., (1977). A 12.5% SDS/polyacrylamide slab gel was prepared as described in section 2.6(a). Gel 'chips' containing labelled proteins were cut from polyacrylamide gels with a scalpel and soaked for 30 min with occasional shaking in 72.5mM-Tris/HCl (pH 6.8)/0.1% (w/v) SDS. Sample wells were filled with this buffer and each gel 'chip' was pushed to the bottom of a well with a spatula. Spaces around the 'chips' were filled by overlayering with 10µl of the same buffer containing 20% (v/v) glycerol. Finally, 10µl of the same buffer containing 10% (v/v) glycerol and 500ng of V8 proteinase was layered on top of each gel chip and 50µl

samples of a protein mixture containing 5µg each of ovalbumin, aldolase, carbonic anhydrase, soybean trypsin inhibitor and lysozyme in electrophoresis sample buffer were layered into adjacent sample wells. Electrophoresis was carried out at constant current (12mA) until the tracking dye had reached the end of the stacking gel when the current was switched off for 30min to allow digestion of the labelled proteins by the V8 proteinase to take place. The current was then restored and electrophoresis was continued at constant current (25mA) until the tracking dye had travelled the length of the resolving gel. The gel was fixed and stained for protein using Coomassie Brilliant Blue. Radiolabelled protein fragments were detected by indirect autoradiography as described in section 2.6(b).

(c) Peptide mapping by analysis of V8 proteinase digests using high performance liquid chromatography (HPLC)

HPLC solvents were argon degassed for each experiment. Trifluoroacetic acid (0.1% (w/v)) was passed through a 0.2µm Millipore filter before use.

Samples (200,000cpm) of labelled active renin and labelled inactive renin in 200µl of 100mM-ammonium bicarbonate/0.01% (w/v) bovine serum albumin were exhaustively digested by incubation with 5µl of V8 proteinase solution (1mg/ml in 100mM-ammonium bicarbonate) for 24h at 37°C and then with a further 5µl of the proteinase solution for 6h at 37°C.

Analysis of the protein digests was carried out using a Gilson model 303 computer controlled gradient pumping HPLC system (Scotlab Ltd., Righead Industrial Estate, Bellshill, Scotland) fitted with a µBondapak C_{18} reverse phase column (Waters Associates (Instruments) Ltd., Hartford, U.K.). Each protein digest was lyophilised and resuspended in 25µl of 0.1% (w/v) trifluoroacetic acid. 20µl of this solution was applied to the column using a valve loop injector and the chromatogram was developed over 80min using a 0-40% gradient of

isopropanol in 0.1% (w/v) trifluoroacetic acid at a flow rate of 1ml/min. 300µl fractions were collected and counted for ^{125}I.

7.3 Results and discussion

Peptide mapping techniques involving the detection of resolved peptides using ninhydrin or fluorescamine generally require nanomolar quantities of protein as starting material. In order to compare the active and inactive forms of renin by peptide mapping using the small quantities of material purified in the present study, the proteins were first radiolabelled by reaction with the ^{125}I-labelled acylating reagent, N-succinimidyl-3-(4-hydroxy,5-(^{125}I)iodophenyl)propionate (Bolton-Hunter reagent). This reagent labels both lysine and terminal amino groups.

1µg of each protein preparation was reacted with 300pmoles of labelled acylating reagent (600µCi), and then with an excess of unlabelled reagent in order to ensure complete modification of the proteins. The labelled proteins were separated from other labelled products of the reaction by precipitation with trichloroacetic acid followed by polyacrylamide gel electrophoresis in the presence of 0.1% (w/v) SDS, carried out in rod gels. Examination of these gels by slicing each gel into 1mm segments and counting the segments for ^{125}I (Figure 7.1) showed that both the active and inactive forms of renin had been successfully labelled. As expected the labelled active renin preparation contained two labelled proteins (I and II). The M_r values of these proteins, estimated by comparison with a standard curve of electrophoresis mobility versus log M_r (Figure 7.2), were 41,000 and 17,000 respectively. The labelled inactive renin preparation contained a single labelled polypeptide of M_r 49,000.

The labelled active renin (M_r 42,000, 1.35×10^6cpm) and labelled inactive renin (6.6×10^3cpm) were recovered from the gels by crushing the appropriate gel segments with a glass rod and eluting the proteins into 100mM-ammonium bicarbonate/0.01% (w/v) bovine serum albumin as described in the preceding

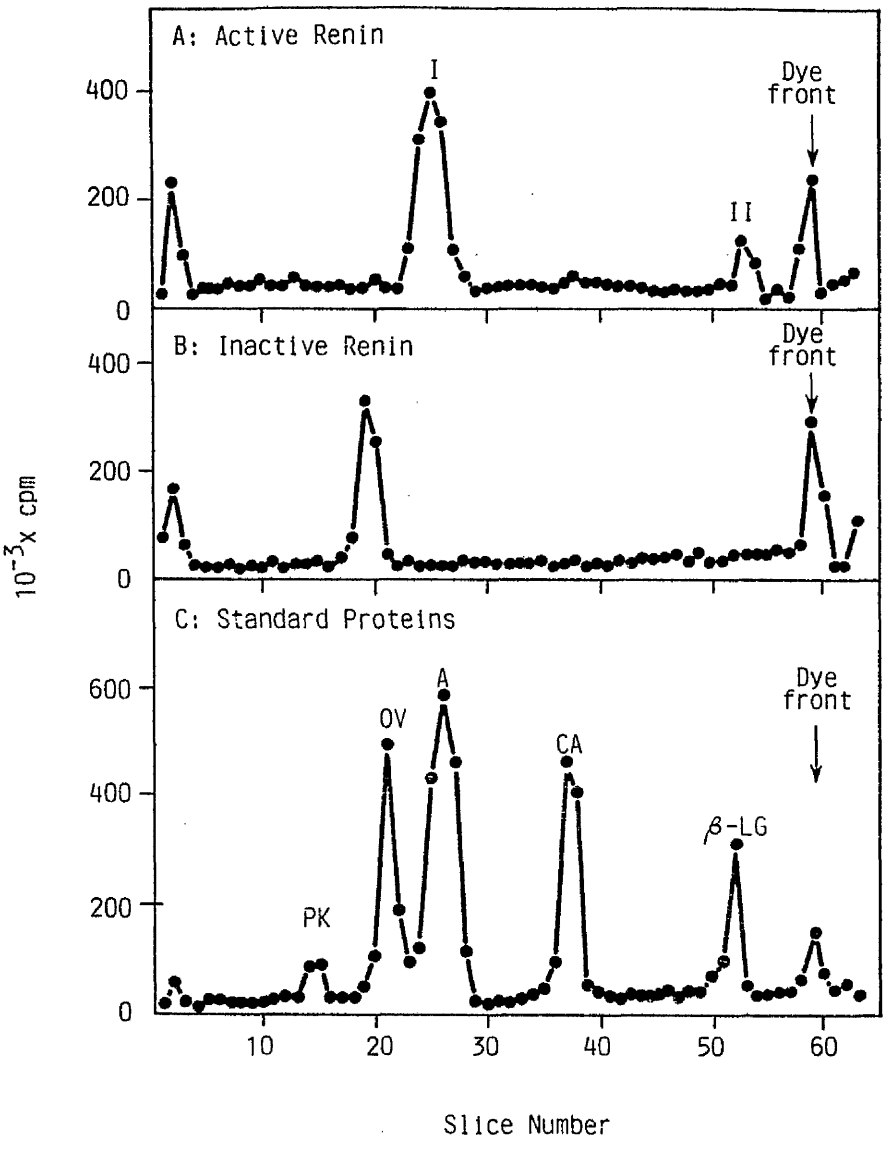

Figure 7.1 **SDS/polyacrylamide gel electrophoresis of ^{125}I-labelled proteins**

Discontinuous electrophoresis was carried out in rod gels (stacking gel, 3% polyacrylamide; resolving gel, 10% polyacrylamide). After electrophoresis was complete, the gels were sliced into 1mm segments and the segments were then counted for ^{125}I. A, labelled active renin preparation; B, labelled inactive renin preparation; C, labelled standard M_r proteins: PK, pyruvate kinase (57,000); OV, ovalbumin (43,000); A, aldolase (40,000); CA, carbonic anhydrase (29,000); β-LG, β-lactoglobulin B (17,500). Subunit M_r values were taken from the sources listed in Table 2.2.

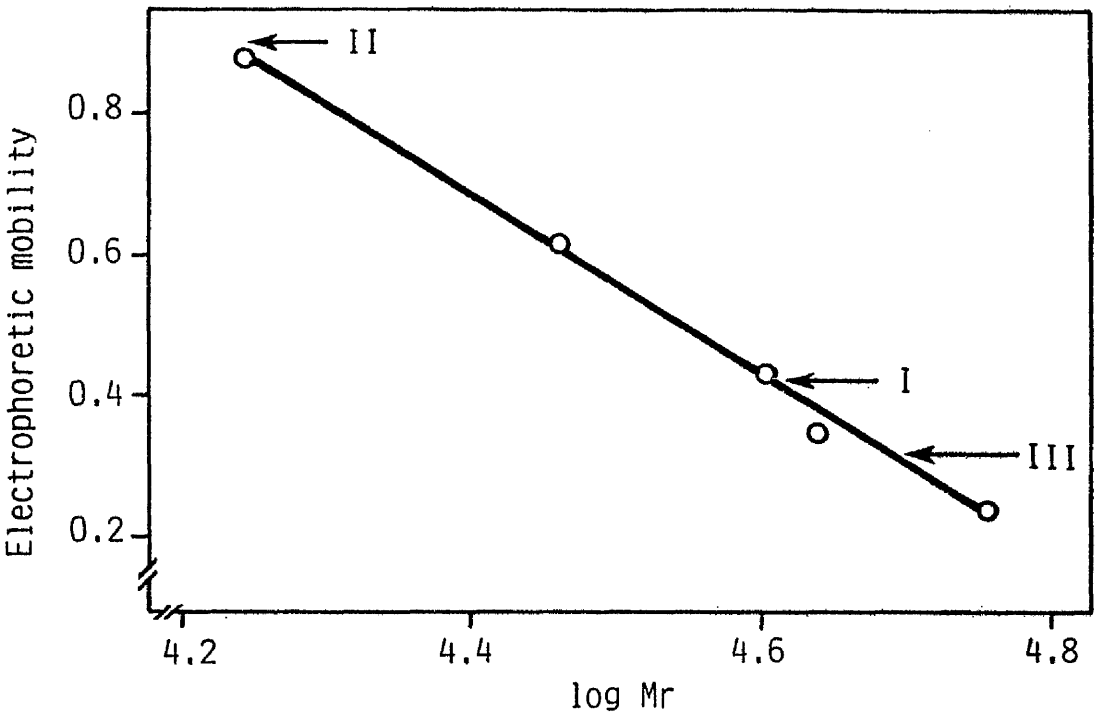

Figure 7.2 **Standard curve of electrophoretic mobility versus log M_r for SDS/polyacrylamide gel electrophoresis of ^{125}I-labelled proteins**

Discontinuous electrophoresis in the presence of 0.1% (w/v) SDS was carried out in rod gels (stacking gel, 3% polyacrylamide; resolving gel, 10% polyacrylamide). The standard proteins used were pyruvate kinase (57,000), ovalbumin (43,000), aldolase (40,000), carbonic anhydrase (29,000) and β-lactoglobulin (17,500). Subunit M_r values were taken from the sources listed in Table 2.2. The mobilities of the two proteins present in the labelled preparation of active renin are indicated by arrows I and II respectively. The mobility of labelled inactive renin is indicated by arrow III.

section (7.2(a)). The recoveries of labelled proteins in this procedure were 8.5×10^5cpm (63%) for the active form of renin and 4.7×10^5cpm (71%) for inactive renin.

In order to check that the labelled proteins had not been fragmented in the above procedure, samples of each eluate were examined by electrophoresis in the presence of 0.1% (w/v) SDS (Figure 7.3). In each case a single polypeptide was observed.

The Bolton-Hunter reagent has been used in numerous studies to label proteins to specific activities in the range 20-200μCi/μg protein (Langone, 1981). Unfortunately, the efficiency of the acylation reaction is dependent on the concentration of protein in the reaction mixture. It is markedly reduced at protein concentrations below 500μg/ml (Bolton and Hunter 1973). In the present study in which the protein concentration in the reaction mixture was only 16μg/ml, the yields of labelled proteins were correspondingly low, being less that 2μCi/μg protein for the labelled active and inactive renins.

In spite of this relatively poor labelling efficiency, the use of the Bolton-Hunter reagent to introduce ^{125}I into proteins has at least two major advantages over direct methods of protein iodination. Firstly, the acylation reaction is extremely mild and avoids the risk of possible protein damage associated with direct iodination methods using chemical oxidants such as a chloramine-T (Hunter and Greenwood, 1972). The risk of oxidative damage to proteins can be reduced by using lactoperoxidase to catalyse protein iodination (Thorell and Johansson, 1971). However, this enzymic method is not appropriate in cases such as the present study where protein iodination is carrried out under denaturing conditions in order to ensure extensive labelling of internal residues. A second advantage of the use of the Bolton-Hunter reagent is that it avoids direct exposure of proteins to labelled iodine. There is some evidence (Hunter, 1971) that proteins may be damaged during direct

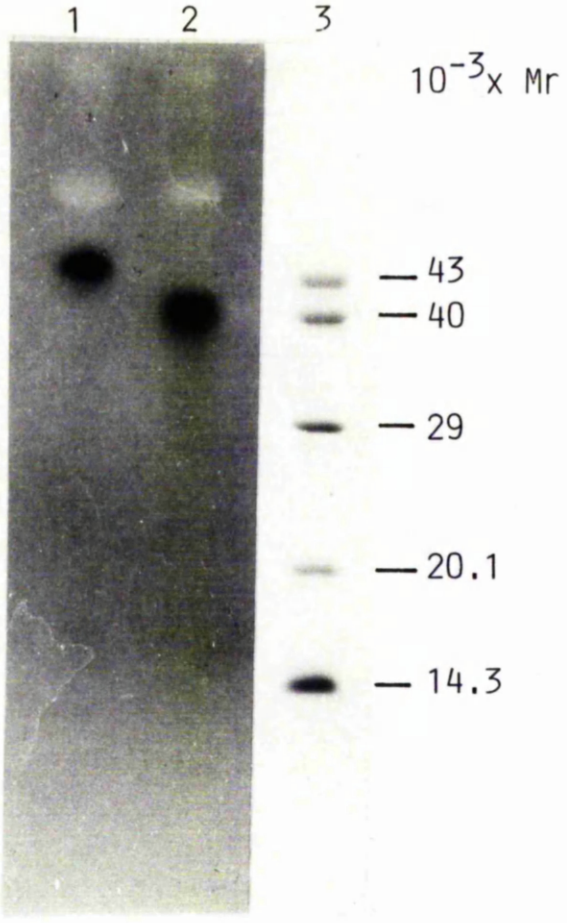

Figure 7.3. SDS/polyacrylamide gel electrophoresis of ^{125}I-labelled active renin and ^{125}I-labelled inactive renin

Discontinuous electrophoresis was carried out in a 1mm-thick polyacrylamide slab gel (stacking gel, 3% polyacrylamide; resolving gel, 12.5% polyacrylamide). The gel was fixed and stained for protein using Coomassie Brilliant Blue. Radiolabelled proteins were detected by indirect autoradiography for 24h as described in Section 2.6(b). Track 1, 5,000cpm of ^{125}I-labelled inactive renin; track 2, 5,000cpm of ^{125}I-labelled active renin; track 3, Coomassie Brilliant Blue stained standard M_r marker proteins: 5µg each of ovalbumin (43,000), aldolase (40,000), carbonic anhydrase (29,000), soybean trypsin inhibitor (20,100) and lysozyme (14,300). Subunit M_r values were taken from the sources listed in Table 2.2.

radioiodination by noxious impurities present in solutions of ^{125}I of high specific radioactivity.

An initial comparison of the active and inactive forms of renin was carried out by limited proteolysis and analysis of the cleavage products by gel electrophoresis in the presence of 0.1% (w/v) SDS (Cleveland mapping). Cleavage of the labelled proteins was carried out using V8 proteinase from S. aureus which cleaves proteins on the C-terminal side of glutamate and aspartate residues (Drapeau, 1976). Although the cleavage patterns produced by the two forms of renin (Figure 7.4) were not identical, four bands (VIII, IX, X and XI) were common to both digests, indicating at least a partial structural homology exists between active renin and inactive renin.

A more extensive comparison of the two forms of renin was carried out by exhaustive digestion of the labelled proteins with V8 proteinase followed by analysis of the digests by reverse phase high performance liquid chromatography (HPLC). Nine labelled peptides were resolved by HPLC 'mapping' of active renin (Figure 7.5). Each of these peptides was also present in the digest of inactive renin indicating that there are extensive regions of common sequence in the active and inactive forms of renin. The digest of inactive renin also contained three labelled peptides (III,VI and XI) which were not present in the active renin digest. These data are clearly consistent with the hypothesis that inactive renin is a renin zymogen.

The activation of a renin zymogen should, by analogy with pepsinogen activation, involve the removal of a short polypeptide from the N-terminal end of the zymogen, thus generating a new N-terminus. Although the Bolton-Hunter reagent labels terminal amino groups in proteins, the peptide maps obtained in the present study (Figure 7.5) do not show the expected extra peptide corresponding to the N-terminal sequence of active renin. Although possible, it is unlikely that this is due to the failure of the HPLC technique to

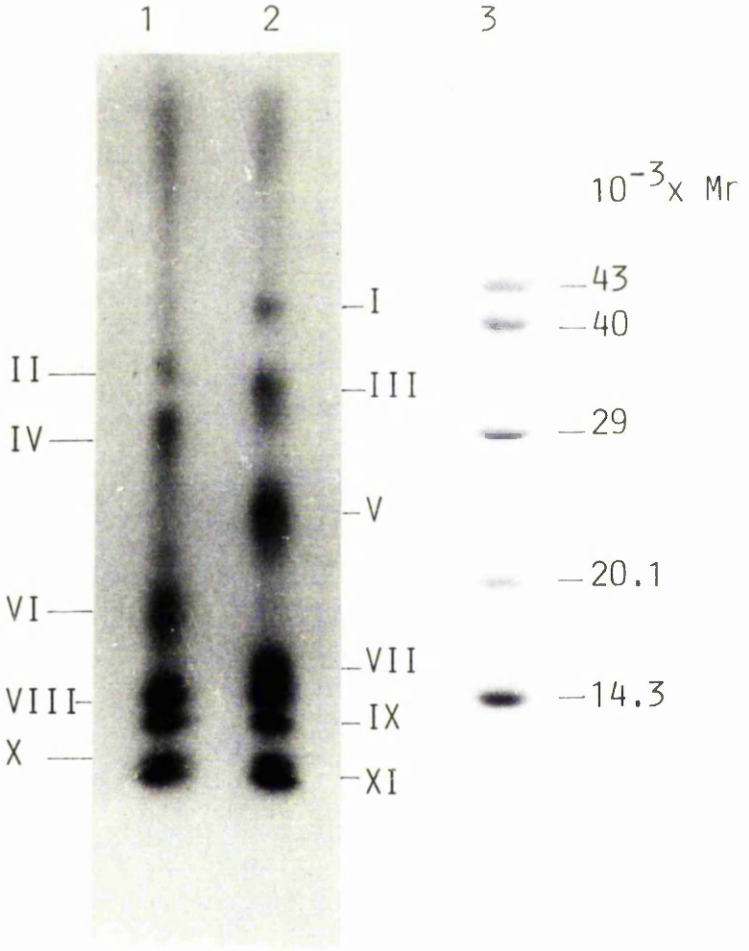

Figure 7.4 Cleveland maps of the active and inactive forms of renin

One dimensional peptide mapping of ^{125}I-labelled active renin and ^{125}I-labelled inactive renin was carried out using the method of Cleveland et al. (1977). Discontinuous electrophoresis in the presence of 0.1% (w/v) SDS was carried out in a 1mm-thick polyacrylamide slab gel (stacking gel, 3% polyacrylamide: resolving gel, 12.5% polyacrylamide). Gel 'chips' containing the labelled proteins were applied to the gel and overlayered with V8 proteinase (500ng) as described in Section 7.2(b). Digestion of the proteins was carried out within the polyacrylamide gel by switching off the current for 30 min when the tracking dye had reached the end of the stacking gel. The gel was fixed and stained for protein using Coomassie Brilliant Blue. Radiolabelled proteins were detected by indirect autoradiography for 7 days as described in Section 2.6(b). Track 1, 5,000cpm of ^{125}I-labelled active renin; track 2, 5,000cpm of ^{125}I-labelled inactive renin; track 3, Coomassie Brilliant Blue stained standard M_r marker proteins; 5µg each of ovalbumin (43,000), aldolase (40,000), carbonic anhydrase (29,000), soybean trypsin inhibitor (20,100) and lysozyme (14,300). Subunit M_r values were taken from the sources listed in Table 2.2.

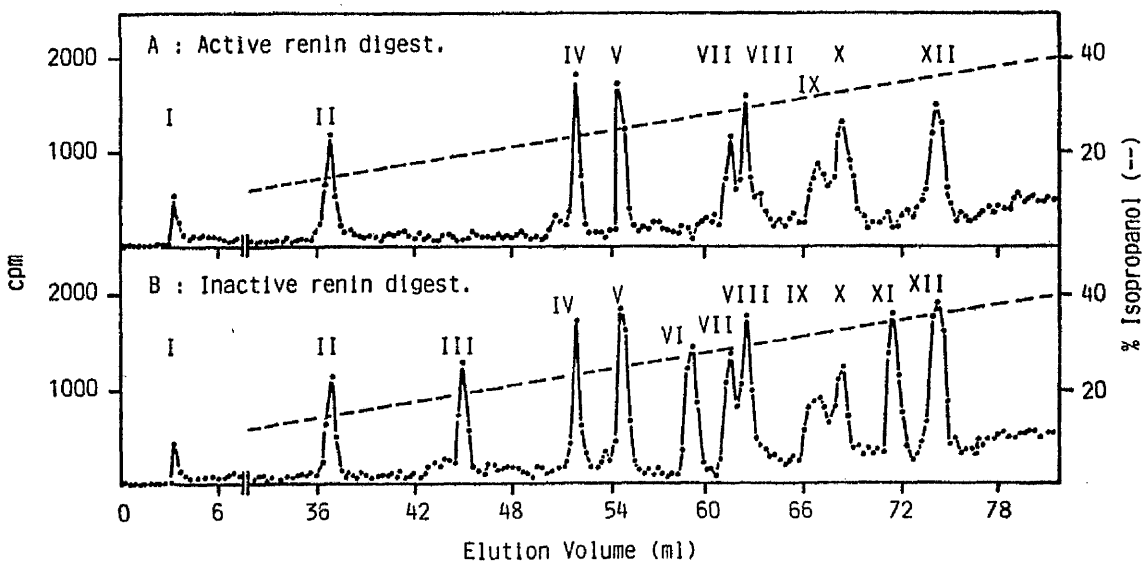

Figure 7.5 **HPLC analysis of V8 proteinase digests of the active and inactive forms of renin**

V8 proteinase digests of ^{125}I-labelled active renin and ^{125}I-labelled inactive renin (circa 170,000cpm of each preparation) were analysed by high performance liquid chromatography. Each digest, dissolved in 25µl of 0.1% (w/v) trifluoroacetic acid, was applied to a µBondapak C_{18} reverse phase column and the chromatogram was developed over 80 min using a 0-40% gradient of isopropanol in 0.1% (w/v) trifluoroacetic acid at a flow rate of 1ml/min. 300µl fractions were collected and counted for ^{125}I.

separate this peptide from the other labelled peptides in the digest of active renin.

One possible explanation for this finding is that the N-terminal sequences of the active and inactive forms of renin are identical. This, however, is unlikely since there is no sequence homology between the N-terminal regions of the active forms and zymogen forms of other aspartic proteinases, such as pepsin or chymosin (Foltmann and Pedersen, 1977), or between the N-terminal regions of the product and precursor forms of mouse submaxillary gland renin (Panthier et al., 1982a). A more attractive explanation for the absence of a unique N-terminal peptide in the 'map' of active renin is that it reflects N-terminal heterogeneity in the active renin preparation used in the present study, the quantities of each N-terminal peptide present in the digest of active renin being insufficient to appear as a distinct peak after analysis by HPLC. Such heterogeneity is not unexpected since the enzyme has been purified from post mortem material and will, therefore, have been exposed, at least briefly, to a variety of tissue proteinases during the period between death and the removal of the kidney at autopsy. Alternatively, N-terminal heterogeneity in active renin could arise if the in vivo activation of inactive renin involved an activating proteinase of broad substrate specificity, or if the activation mechanism involved several sequential cleavage steps. The appearance of active renin (M_r 40,000) as a broad band after electrophoresis in the presence of 0.1% (w/v) SDS, compared to the well defined band produced by inactive renin (Figure 7.6) is consistent with the latter hypothesis. However, since renin is a glycoprotein, this appearance may be due to heterogeneity in the carbohydrate components of renin rather than to heterogeneity in the polypeptide chain. Further experiments will be required to clarify the above points.

The data presented in this chapter indicate that there are extensive regions of common sequence in the active and inactive forms of renin and

therefore suggest that inactive renin is indeed a renin zymogen. This information was obtained from experiments using extremely small amounts of material; the enzyme digests were performed on less than 10pmoles of each protein. Final proof of a precursor-product relationship between inactive renin and active renin will require the total sequence analysis of each protein. This well require much larger amounts of the proteins than are available at the present time.

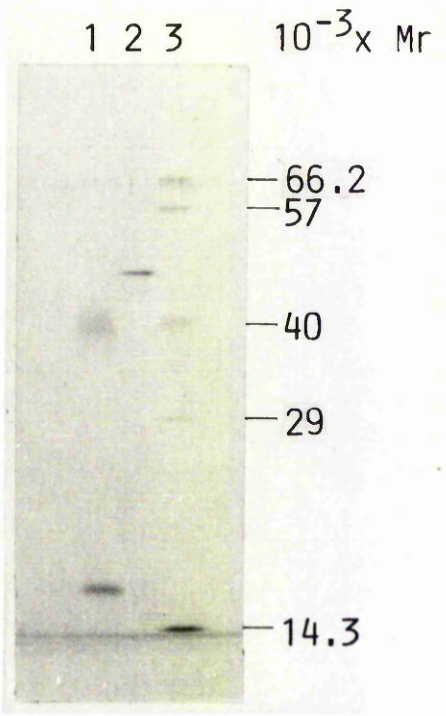

Figure 7.6 **SDS/polyacrylamide gel electrophoresis of purified active renin and purified inactive renin**

Discontinuous electrophoresis was carried out on a 1mm-thick polyacrylamide slab gel (stacking gel, 3% polyacrylamide; resolving gel, 10% polyacrylamide). Protein bands were detected using the silver staining method of Wray et al. (1981). Track 1, 100ng of purified renin; track 2, 50ng of purified inactive renin; track 3, standard M_r marker proteins: 50ng each of bovine serum albumuin (66,200), pyruvate kinase (57,000), aldolase (40,000), carbonic anhydrase (29,000) and lysozyme (14,300). Subunit M_r values were taken from the sources listed in Table 2.2.

CHAPTER 8

GENERAL DISCUSSION

8.1 Introduction

In recent years it has become apparent that many proteins are synthesized as inactive precursors, or zymogens, which are subsequently converted to physiologically active forms by selective enzymatic cleavage. Zymogen activation serves as a rate controlling step in a variety of biological processes, such as blood coagulation, fibrinolysis, complement reaction, differentiation and supramolecular assembly (Neurath and Walsh, 1976). The presence of an inactive form of renin, both in renal cortex and in plasma, which can be activated by limited proteolysis, has therefore prompted some investigators to suggest that this form of renin may be a circulating renin zymogen, and that its activation either within the circulation or at a site distal to its site of synthesis may form the basis of a control mechanism for modulating the activity of the renin-angiotensin system (Schalekamp and Derkx, 1981; Sealey et al., 1982a).

While the existence of a biosynthetic precursor of renin (prorenin) has been demonstrated in vitro, both by the cell free translation of mouse submaxillary gland mRNA (Lund et al., 1982), and by pulse-chase experiments carried out using isolated canine glomeruli (Carlson et al., 1981) and mouse submaxillary gland tissue (Dzau et al., 1982), previous studies have failed to provide conclusive evidence that the inactive form of renin present in renal cortex and in plasma is a precursor of active renin.

The data obtained in the present study show that as expected for a renin zymogen, inactive renin purified from human renal cortex consists of a single polypeptide chain (M_r 48,000) which is slightly larger than its active counterpart (M_r 40,000). Furthermore, peptide 'mapping' of active renin and

inactive renin has shown that extensive regions of common sequence exist in the two forms of the enzyme. Although final proof of a precursor-product relationship between inactive renin and active renin will require the total sequence analysis of each protein, the above results provide the most convincing evidence to date that renal inactive renin is a renin zymogen. Clearly, however, a number of important questions remain unanswered.

1. In man, between 70% and 90% of the total circulating renin is present as an inactive form of the enzyme. Is this material, like renal inactive renin, a renin zymogen and if so, is it released into the circulation from the kidney or from extrarenal sites of renin synthesis?
2. All zymogen activation reactions require the enzyme-catalysed cleavage of a unique peptide bond by 'limited proteolysis'. What enzyme or enzymes are responsible for the activation of inactive renin and where does this process occur in vivo?
3. Does the activation of inactive renin form the basis of a physiological control mechanism for modulating the activity of the renin-angiotensin system?

8.2 Biochemical characteristics of plasma inactive renin

Over the past decade, considerable attention has been paid to the activatable form of renin present in human plasma, partly because plasma is more accessible than renal tissue in clinical situations, and partly because early studies, which demonstrated raised levels of circulating inactive renin in a patient with a Wilms tumour (Day and Leutscher, 1974) and in patients with assorted renal abnormalities (Day and Luetscher, 1975), suggested a functional significance for this material. Unfortunately, the extremely low concentration of inactive renin in plasma has hindered its characterisation. In normal individuals, the concentration of inactive renin in plasma is about 100pg/ml (assuming that, after activation, its specific activity is the same as that of

purified renal renin). Therefore, assuming a recovery of 10%, the isolation of 1mg of plasma inactive renin would require a purification of several hundred millionfold from about 100,000 litres of plasma. Nevertheless, partial purifications of plasma inactive renin have been reported by several workers (Yokosawa et al., 1979; Morimoto et al., 1980; Chang et al., 1981; Atlas et al., 1982b; Inagami et al., 1982b; Hiwada et al., 1983; Hsueh et al., 1983). Of these procedures, the most successful was that reported by Inagami et al., (1982b), who used a combination of hydrophobic interaction chromatography and immunoaffinity chromatography on anti-renin IgG-Sepharose to achieve a 17,500-fold purification of circulating inactive renin from plasma.

Plasma and renal inactive renins behave similarly when applied to Cibacron Blue-agarose, pepstatin-Sepharose, concanavalin A-Sepharose, anion exchange resins and gels containing hydrophobic ligands. Both are completely inactive against renin substrate, but can be activated irreversibly by limited proteolysis and reversibly by acidification. In each case, the activity generated by limited proteolysis is identical to that of active renal renin with respect to pH optimum and reaction kinetics. These properties are summarised in Table 8.1.

One characteristic that distinguishes plasma inactive renin from renal inactive renin is that the former has a greater apparent Mr value by gel filtration. Thus, plasma inactive renin has an Mr value of around 56,000 by gel filtration compared to the Mr value of around 50,000 obtained for renal inactive renin (Atlas et al., 1981). There are several possible explanations for this discrepancy. One is that plasma inactive renin and renal inactive renins differ markedly in their primary structures. However, this is unlikely, given the striking similarities of their other physiological properties and of the catalytic properties of the activated forms (Table 8.1). Moreover, circulating active renin also appears to have a greater Mr value (48,000) by gel filtration

Table 8.1

Properties shared by inactive renins from human renal cortex and plasma [a]

Inactive forms

1. Bound by Cibacron Blue-agarose
2. Not bound by pepstatin - Sepharose
3. Bound by concanavalin A - Sepharose
4. Weak affinity for anion exchange resins
5. Strong affinity for hydrophobic ligands
6. Activated irreversibly by trypsin or pepsin
7. Activated reversibly by acidification

Activated forms produced by limited proteolysis [b]

1. Not bound by Cibacron blue-agarose
2. No activity against haemoglobin
3. K_m with human angiotensinogen (0.8 - 1.3 μM)
4. pH optimum with human angiotensinogen (pH 5.5 - 6.0)
5. Inhibition by pepstatin (K_i = 2.5 - 3.5 μM)
6. Complete inhibition by anti-renin antibodies (titre for 50% inhibition is $4 - 5 \times 10^{-5}$)

[a] data taken from Atlas et al. (1982b)

[b] these properties are also shared by active renal renin.

than active renin extracted from the kidney (Atlas et al., 1981), so that other explanations must be considered. These include the possibilities that:

1. Inactive renin is partially degraded during tissue extraction
2. Renal inactive renin is modified by interaction with a binding protein upon entering the circulation
3. The forms of renin present in kidney and plasma differ in conformation and/or extent of glycosylation, both of which are factors known to affect Mr estimation by gel filtration.

It seems unlikely that proteolytic degradation of inactive renin during its extraction from renal tissue would occur without its simultaneous activation. In addition, the extraction of renins from kidney is generally carried out in the presence of a variety of proteinase inhibitors in order to prevent such degradation taking place. There is more reason to consider the possibility of modification by a binding protein since such a substance does appear to be present in the renal cortex of various animal species. However, Murakami et al. (1980a) have shown that the renin binding protein from hog kidney can bind to inactive renin only after the latter has been activated by trypsin. Attempts to demonstrate the presence of renin binding proteins in mouse plasma using ^{125}I-labelled mouse renal renin as a probe have shown that, while labelled renin will bind to a number of plasma proteins, it does so only if it is first denatured with guanidine hydrochloride and then renatured prior to its incubation with plasma (Poulsen et al., 1979a).

There is precedent for suggesting that a difference in glycosylation might exist between secreted and tissue forms of the same protein since this has been documented for the α-subunit of the pituitary glycoprotein hormones (Kourides et al., 1980). Both renal and plasma inactive renins appear to be glycoproteins, based on their ability to bind concanavalin A (Table 8.1). There are at present, however, no data concerning the composition of the carbohydrate

component of each of these renins.

Finally, it is well known that conformational changes in proteins can lead to significant changes in Stokes radius, and thus in the behaviour of proteins during gel filtration. In this respect it is interesting to note that the Stokes radius of inactive renin is greater in acidified plasma than in normal plasma (Hsueh et al., 1981). Recently, Inagami et al. (1983) have reported the use of electrophoresis in the presence of SDS followed by immunospecific staining of renin to obtain M_r values for renal and plasma inactive renins. Both were found to have M_r values of 49,000-50,000 suggesting that the size discrepancy noted by gel filtration may indeed be due to a difference in the conformation of inactive renin in plasma and kidney. The above value (M_r 49,000-50,000) is similar to that obtained in the present study for purified renal inactive renin (M_r 48,000). It is likely, therefore, that plasma inactive renin is, like renal inactive renin, a renin zymogen.

8.3 Sources of circulating inactive renin

Since the source of circulating active renin is the renal cortex, it is likely that circulating inactive renin is also derived, at least in part, from the kidney. Nevertheless, whereas the concentration of active renin in renal vein samples is 10-20% higher than concurrent arterial values (Millar et al., 1978), similar arteriovenous differences in inactive renin have been difficult to demonstrate. Without question, there are certain patients who seem to secrete relatively large amounts of inactive renin from the kidney (Birkenhager et al., 1978; Derkx et al., 1978b; Millar et al., 1978; Aoi et al., 1979). On the other hand, an apparent negative arteriovenous difference occurs frequently in patients with essential hypertension and in the contralateral kidneys of patients with unilateral renovascular hypertension (Sealey et al., 1980).

If inactive renin were secreted from the kidney then one would expect to find high concentrations of inactive renin in the venous effluent from the

affected kidneys of patients with renovascular hypertension. In this situation, the blood flow through the kidney is reduced and thus inactive renin would be secreted into a diminished volume of blood per unit time. Recently, Hsueh et al. (1983) have examined the levels of plasma inactive renin in a group of eight patients with renovascular hypertension. In all cases, the inactive renin concentration was significantly higher in renal venous blood than in inferior vena caval blood.

Further evidence that inactive renin is released from the kidney includes the presence of inactive renin in the perfusate obtained from transplant kidneys (Atlas et al., 1980) and the fact that the concentration of inactive renin in plasma falls after bilateral nephrectomy (Derkx et al., 1978b). However, the presence of inactive renin in plasma obtained from individuals who have been nephrectomised for several years (Sealey et al., 1977; Weinberger et al., 1977) indicates that there must be an extrarenal source of inactive renin as well.

Inactive renin has been found in the brain and in the pituitary gland (Hirose et al., 1979), and in amniotic fluid (Lumbers, 1971). Renin in human amniotic fluid is almost exclusively inactive and is very similar to circulating inactive renin (Johnston et al., 1979). Available evidence suggests that amniotic fluid inactive renin is synthesized in the chorion (Symonds et al., 1968; Acker et al., 1982). Sustained increases in plasma inactive renin concentration occur throughout pregnancy (Skinner et al., 1975; Sealey et al., 1982b). It is possible that in this situation, plasma inactive renin may be partly derived from the chorion. Further studies will be required to determine whether or not extrarenal sources of inactive renin contribute to circulating inactive renin in normal man.

8.4 Factors involved in the activation of inactive renin

As has been discussed in Chapter 1, renin belongs to the aspartic proteinase group of enzymes. This group also includes the gastric proteinases

pepsin and chymosin, both of which are synthesized as inactive precursors. Under physiological conditions, the zymogens of pepsin and chymosin (pepsinogen and prochymosin) do not require the action of other proteinases to generate active enzymes. In the acid environment of the stomach, pepsinogen undergoes a conformational change which results in an intramolecular cleavage in which a 16 amino acid peptide is removed from the N-terminus of the zymogen. The active species formed (pseudopepsin) then activates other pepsinogen molecules (Kassell and Kay, 1973; Hartsuck et al., 1977). During this conversion, several peptides containing a total of 44 amino acid residues are removed from the amino terminus of pepsinogen. Prochymosin, and cathepsinogen D (the zymogen of the lysosomal aspartic proteinase, cathepsin D) are also activated by an intramolecular mechanism at low pH (Pedersen et al., 1979; Puidzar and Turk, 1981).

Like the zymogens cited above, inactive renin appears to undergo a conformational change at acid pH (Chapter 6). However, although renin activity can be detected if the acidified zymogen is assayed rapidly at neutral pH, this 'activation' process is completely reversible. This finding is not unexpected since the extreme substrate specificity and comparatively high pH optimum exhibited by renin are inconsistent with an intramolecular activation mechanism analogous to that seen with pepsinogen. Clearly, therefore, the activation of inactive renin must, like most other zymogen activation reactions, involve the action of a second proteinase.

The question as to which enzyme or enzymes may be responsible for the activation of inactive renin in vivo has been investigated by examining the effect of proteolytic enzymes on inactive renin in vitro. This type of study has shown that a number of proteinases, including certain enzymes of the coagulation and fibrinolytic systems, as well as a variety of tissue proteinases, are capable of activating inactive renin when added to plasma, amniotic fluid or

Table 8.2

Proteinases reported to be activators of inactive renin *in vitro*

Proteinase	Reference
β-Acrosin	Morris *et al.* (1980)
Arginine esteropeptidase (gamma) subunit of nerve growth factor	Morris *et al.* (1981)
Cathepsins B, D and H	Luetscher *et al.* (1982)
Glandular kallikrein	Derkx *et al.* (1979)
Hageman factor (factor XIIa)	Sealey *et al.* (1979)
Metalloproteinase from *Bitis arientans* venom	Morris and Lawrence (1980)
Pepsin	Morris and Lumbers (1972)
Plasma kallikrein	Sealey *et al.* (1979)
Plasmin	Osmond *et al.* (1978)
Streptokinase	Schalekamp and Derkx (1981)
Trypsin	Morris and Lumbers (1972)
Urokinase	Schalekamp and Derkx (1981)

kidney extracts (Table 8.2). However, there are several points which must be taken into account when interpreting the results of such experiments. Firstly, several studies of this type have used commercial proteinase preparations. Unless the purity of such enzymes is known, it is not certain whether any activation of inactive renin which is observed is due to that particular proteinase, or to a contaminating enzyme. Secondly, studies in which the effects of proteinases are carried out using crude preparations of inactive renin are complicated by the possible involvement of other components in the material under investigation. Plasma, for example, contains a large number of proteinase inhibitors and thus relatively high concentrations of a given proteinase may be required to activate inactive renin in plasma. A further problem with the use of plasma to test putative activators of inactive renin is that the proteinases under investigation may act indirectly by activating an endogenous proteinase zymogen rather than by directly activating inactive renin. Inagami et al. (1982b), for example, have shown that thrombin, urokinase and activated Hageman factor (factor XIIa), which activate inactive renin when added to whole plasma, do not activate partially purified inactive renin.

A second approach has been to investigate the enzymes that activate inactive renin when plasma is dialysed to acid pH and then back to neutral pH (Section 1.3(b)). Two endogenous serine proteinases, activated Hageman factor (factor XIIa) and kallikrein, have been implicated in this process. The activation of inactive renin which is produced by the above procedure does not occur in plasma deficient either in factor XII or prekallikrein. This finding and the results of studies on the activation of inactive renin in fractionated and reconstituted plasmas have led Sealey et al. (1979) to suggest that plasma inactive renin can be activated by a cascade mechanism in which Hageman factor activates inactive renin via the activation of prekallikrein (Figure 8.1).

Both Hageman factor and kallikrein are normally present in plasma as

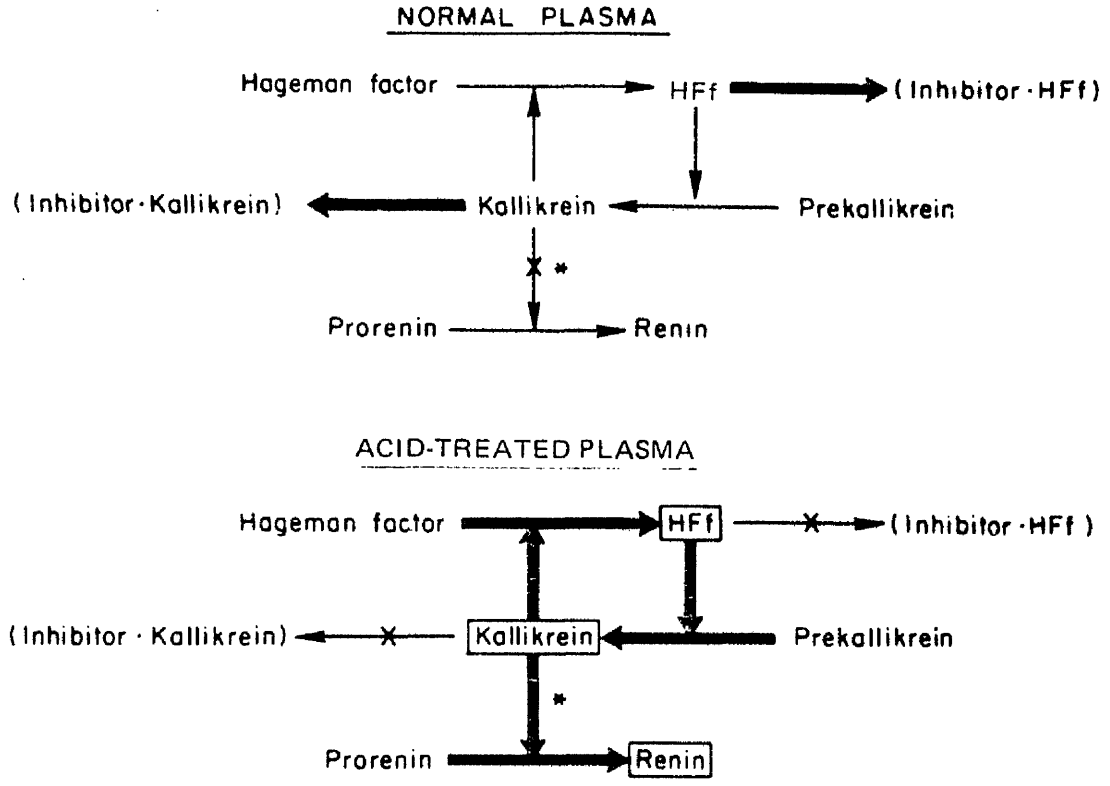

Figure 8.1 **Proposed cascade mechanism for the Hageman factor-dependent activation of inactive renin (prorenin) in acidified plasma as compared to normal plasma[a].**

Heavy arrows indicate the predominant pathway in each case HFf, active Hageman factor fragments. *, Kallikrein may activate prorenin either directly or indirectly. [a]Taken from Sealey et al. (1979).

inactive precursors. Hageman factor is activated on contact with negatively charged surfaces and the activated Hageman factor can then convert prekallikrein to kallikrein. Kallikrein, in turn, accelerates the rate of Hageman factor activation. Thus, the two enzymes and their precursors form a positive feedback loop which would result in the rapid activation of the entire pool of precursors were it not for the fact that the activity of these enzymes is normally maintained at low levels by plasma proteinase inhibitors. Many of these inhibitors, and in particular C_1 inhibitor and α_2-macroglobulin, which inhibit activated Hageman factor and kallikrein respectively, are denatured at low pH (Derkx et al., 1982). Thus, if plasma is acidified and then returned to neutral pH, there is a rapid generation of active kallikrein which can then activate inactive renin.

8.5 Speculations concerning the activation of inactive renin in vivo

While it is clear that a variety of proteinases are capable of activating inactive renin in vitro, little is known about the activation of the renin zymogen in vivo. Inactive renin might merely be a cellular intermediate in renin biosynthesis. If this were the case, then its activation would occur prior to secretion and renin would be released from the cell almost exclusively in the active form. However, as discussed above (Section 8.3), current evidence suggests that significant quantities of inactive renin are released into the circulation. In view of the fact that such a large proportion (up to 90%) of the total circulating renin is present as the inactive form of the enzyme, it is tempting to suggest that this material has a functional significance and that the activation of inactive renin after its release from the cell might form the basis of a control mechanism for modulating the activity of the renin-angiotensin system.

The finding that plasma inactive renin can be activated by glandular (renal) kallikrein in vitro has led to speculation that this enzyme might be

important in the intrarenal activation of inactive renin after its release from the cell. However, some investigators have reported that glandular kallikrein is a relatively poor activator of inactive renin (Hsueh et al., 1981; Inagami et al., 1982b). Furthermore, examination of the distribution of glandular kallikrein and renin in the kidney by the immunohistochemical staining of sections of rat renal cortex has shown that the two enzymes are located in different regions of the nephron, and thus are unlikely to interact in vivo (Orstavvik and Inagami, 1982).

It is often suggested that the coagulation and fibrinolytic cascades are continously active, and that both systems are in a dynamic equilibrium which maintains an intact and patent vascular tree. It is possible, therefore, that one or more of the enzymes belonging to these systems might activate inactive renin in vivo. Certainly, activated Hageman factor (factor XIIa) and plasma kallikrein are potential activators of circulating inactive renin (Sealey et al., 1979). Schalekamp and Derkx (1981) have suggested that plasmin might also be an important activator of the circulating renin zymogen.

The possible participation of plasma kallikrein in the activation of circulating inactive renin is supported by two observations. A positive correlation exists between circulating plasma prekallikrein levels and the proportion of active renin in the circulation (Rumpf et al., 1980). In addition, the proportion of active renin in the circulation is very low in some patients with prekallikrein deficiency (Rumpf et al., 1980; Schalekamp and Derkx, 1981), suggesting that in these individuals the conversion of inactive renin to active renin is abnormal.

Nevertheless, it is unlikely that the conversion of inactive renin to active renin would occur in circulating plasma in which the activities of the coagulation, fibrinolytic and kallikrein-kinin systems are maintained at low levels by endogenous proteinase inhibitors. It is more likely that such conversion would occur only at restricted sites within the vasculature, perhaps on the

surface of blood vessels, or around fibrin clots, where the local generation of active Hageman factor, kallikrein or plasmin can occur.

Current evidence suggests that there is indeed little systemic activation of inactive renin. Experiments in which the levels of circulating active renin are altered by physiological or pharmacological manipulations have, in general, failed to demonstrate reciprocal changes in the levels of circulating inactive renin (Leckie, 1981). For example, dietary sodium depletion and diuretic therapy, both of which stimulate the synthesis and release of active renin, have been shown to cause small, but significant increases in circulating inactive renin levels (Sealey et al., 1980). This data is consistent with the hypothesis that circulating levels of inactive renin are regulated mainly by its synthesis and release from the kidney, or from extrarenal sites of renin synthesis rather than by its conversion to active renin within the circulation.

Clearly, further studies will be required to clarify the physiological role of the renin precursor.

BIBLIOGRAPHY

Acker, G.M., Galen, F.X., Devaux, C., Foote, S., Papernik, E., Pesty, A., Menard, J. and Corvol, P. (1982) J. Clin. Endocrinol. Metab. **55,** 902-909

Aoi, W., Seto, S., Doi, Y., Murayama, M., Tasaki, S., Suzuki, S., Kuramochi, M. and Hashiba, K. (1979) Clin. Sci. **57,** 101s-103s

Atlas, S.A., Laragh, J.H. and Sealey, J.E. (1978) Clin. Sci. Mol. Med. **55,** 135s-138s

Atlas, S.A., Laragh, J.H., Sealey, J.E. and Hesson, T.E. (1980) Clin. Sci. **59,** 29s-33s

Atlas, S.A., Sealey, J.E., Dharmgrongartama, B., Hesson, T.E. and Laragh, J.H. (1981) Hypertension **3,** (Suppl. I) I-30 - I-40

Atlas, S.A., Hesson, T.E., Sealey, J.E. and Laragh, J.H. (1982a) Clin. Sci. **63,** 167s-170s

Atlas, S.A., Sealey, J.E., Hesson, T.E., Kaplan, A.P., Menard, J., Corvol, P. and Laragh, J.H. (1982b) Hypertension **4,** (Suppl II) II-86 - II-95

Bailie, M.D., Derkx, F.M.H. and Schalekamp, M.A.D.H. (1979) Circ. Res. **44,** 32-37

Bing, J., Poulsen, K., Hackenthal, E., Rix, E. and Taugner, R. (1980) J. Histochem. Cytochem. **28,** 874-880

Birkenhager, W.H., De Leeuw, P.W., Falke, H.E. and Van Soest, G.A.W. (1978) Clin. Sci. Mol. Med. **55,** 147s-149s

Blobel, G. and Dobberstein, B. (1975) J. Cell. Biol. **67,** 852-862

Bohlen, P., Stein, S., Dairman, W. and Udenfriend, S. (1973) Arch. Biochem. Biophys. **155,** 213-220

Bolton, A.E. and Hunter, W.M. (1973) Biochem. J. **133,** 529-539

Bouhnik, J., Clauser, E., Strosberg, D., Frenoy, J-P, Menard, J. and Corvol, P. (1981) Biochemistry **20,** 7010-7015

Boyd, G.W. (1977) Lancet **i**, 215-218

Bradford, M.M. (1976) Anal. Biochem. **72**, 248-254

Brown, J.J., Davies, D.L., Doak, P.B., Lever, A.F., Robertson, J.I.S. and Tree, M. (1964) Lancet **ii**, 64-66

Brown, J.J., Leckie, B.J., Lever, A.F., McIntyre, G., Morton, J.J., Semple, P.F. and Robertson, J.I.S. (1983) in Handbook of Hypertension (Robertson, J.I.S. ed.) Vol. 1, pp 278-323, Elsevier, Amsterdam

Carlson, W., Quay, S., Dzau, V., Kreisberg, J., Slater, E. and Haber, E. (1981) in Heterogeneity of Renin and Renin-Substrate (Sambhi, M.P. ed.) pp 33-43, Elsevier, Amsterdam

Castellino, F.J. and Barker, R. (1968) Biochemistry **7**, 2207-2217

Chang, J-J., Kisaragi, M., Okamoto, H. and Inagami, T. (1981) Hypertension **3**, 509-515

Cleveland, D.W., Fischer, S.G., Kirschner, M.W. and Laemmli, U.K. (1977) J. Biol. Chem. **252**, 1102-1106

Cohen, S., Taylor, J.M., Murakami, K., Michelakis, A.M. and Inagami, T. (1972) Biochemistry **11**, 4286-4293

Cook, W.F. and Pickering, G.W. (1962) Biochem. Pharmacol. **9**, 165-171

Cooper, R.M., Murray, G.E. and Osmond, D.H. (1976) Circ. Res. **40**, (Suppl. I), I-171 - I-179

Corvol, P., Devaux, C., Ito, T., Scard, P., Ducloux, J. and Menard, J. (1977) Circ. Res. **41**, 616-622

Corvol, P., Panthier, J-J., Foote, S. and Rougeon, F. (1983) Hypertension **5** (Suppl. I), I-3 - I-9

Day, R.P. and Luetscher, J.A. (1974) J. Clin. Endocrinol. Metab. **38**, 923-926

Day, R.P. and Luetscher, J.A. (1975) J. Clin. Endocrinol. Metab. **40**, 1085-1093

Day, R.P. and Reid, I.A. (1976) Endocrinology **99**, 93-100

De Fernandez, M.T.F., Paladini, A.C. and Delius, A.E. (1965) Biochem. J. **97**,

540-546

Derkx, F.H.M., v.Gool, J.M.G., Wenting, G.J., Verhoeven, R.P., Man in't Veld, A.J. and Schalekamp, M.A.D.H. (1976) Lancet **ii**, 496-498

Derkx, F.H.M., Tan-Tjiong, H.L. and Schalekamp, M.A.D.H. (1978a) Lancet **ii**, 217-219

Derkx, F.H.M., Wenting, G.J., Man in't Veld, A.J., Verhoeven, R.P. and Schalekamp, M.A.D.H. (1978b) Clin. Sci. Mol. Med. **54**, 529-538

Derkx, F.H.M., Tan-Tjiong, H.L., Man in't Veld, A.J., Schalekamp, M.P.A. and Schalekamp, M.A.D.H. (1979) Clin. Sci. Mol. Med. **57**, 351-357

Derkx, F.H.M., Schalekamp, M.P.A. and Schalekamp, M.A.D.H. (1982) Clin. Exp. Hypertension **A4**, 2123-2131

Derkx, F.H.M., Tan-Tjiong, L., Boomsma, F., Man in't Veld., A.J. and Schalekamp, M.A.D.H. (1983) Hypertension **5**, 244-256

Devaux, C., Menard, J., Sicard, P. and Corvol, P. (1976) Eur. J. Biochem. **64**, 621-627

De Vito, E., Cabrera, R.R. and Fascido, J.C. (1970) Am. J. Physiol. **219**, 1042-1045

Drapeau, G.R. (1977) Methods Enzymol. **47**, 189-191

Dzau, V.J., Slater, E.E. and Haber, E. (1979) Biochemistry **18**, 5224-5228

Dzau, V.J., Brenner, A., Emmett, N. and Haber, E. (1980) Clin. Sci. **59**, 45s-47s

Dzau, V.J., Tanaka, A. and Pratt, R.E. (1982) Clin. Exp. Hypertension **A4**, 1973-1985

Foltmann, B. and Pedersen, V.B. (1977) Adv. Exp. Med. Biol. **95**, 3-22

Franks, R.C., Bodola, F., Renthal, R.D. and Hayashi, R.H. (1982) Clin. Sci. **64.** 481-486

Funakawa, S., Funae, Y. and Yamamoto, K. (1978) Biochem. J. **176**, 977-981

Galen, F.X., Devaux, C., Guyenne, T., Menard, J. and Corvol, P. (1979) J.

Biol. Chem. **254**, 4848-4855

Haas, E., Lamfrom, H. and Goldblatt, H. (1953) Arch. Biochem. Biophys. **42**, 368-386

Hackenthal, E., Hackenthal, R. and Hilgenfeldt, U. (1978) Biochem. Biophys. Acta, **522**, 574-588

Hartsuck, J.A., Marciniszyn, J., Jr., Huang, J.S. and Tang, J. (1977) Adv. Exp. Med. Biol. **95**, 85-102

Higaki, J., Hirose, S., Ogihara, T., Imai, N., Kisaragi, M., Murakami, K. and Kumahara, Y. (1983) Life Sci. **32**, 1591-1598

Hille, M.B., Barrett, A.J., Dingle, J.T. and Fell, H.B. (1970) Exp. Cell. Res. **61**, 470-472

Hirose, S., Naruse, M., Ohtsuki, K. and Inagami, T. (1981) J. Biol. Chem. **256**, 5572-5576

Hiwada, K., Matsumoto, C. and Kokuba, T. (1983) Hypertension, **5**, 191-197

Hsueh, W.A., Luetscher, J.A., Carlson, E., Grislis, G., Elbaum, D. and Chavarri, M. (1978) J. Clin. Endocrinol. Metab. **47**, 792-799

Hsueh, W.A., Carlson, E.J. and Israel-Hagman, M. (1981) Hypertension **3** (Suppl I) I-22 - I-29

Hsueh, W.A., Carlson, E.J. and Dzau, V.J. (1983) J. Clin. Invest. **71**, 506-517

Hunter, W.M. and Greenwood, F.C. (1962) Nature **194**, 495-496

Hunter, W.M. (1971) in Radioimmunoassay Methods: European Workshop (Kirkham, K.E. and Hunter, W.M. eds.) pp 3-23, Churchill-Livingstone, Edinburgh and London

Inagami, T. and Murakami, K. (1977) J. Biol. Chem. **252**, 2978-2983

Inagami, T., Hirose, S., Murakami, K. and Matoba, T. (1977a) J. Biol. Chem. **252**, 7733-7737

Inagami, T., Murakami, K., Misono, K., Workman, R.J., Cohen, S. and Suketa, Y. (1977b) Adv. Exp. Med. Biol. **95**, 225-247

Inagami, T. and Murakami, K. (1980) Biomed. Res. **1**, 456-475

Inagami, T., Murakami, T., Yokosawa., H., Matoba, T., Yokosawa, N., Takii, Y. and Misono, K. (1980) in Enzymatic Release of Vasoactive Peptides (Gross, F. and Vogel, H.G. eds) pp 7-30, Raven Press, New York

Inagami, T., Clemens, D.L., Hirose, S., Okamura, T., Naruse, K., Takii, Y. and Yokosawa, H. (1982a) Clin. Exp. Hypertension **A4**, 607-622

Inagami, T., Okamoto, H., Ohtsuki, H., Shimamoto, K., Chao, J. and Margolius, H.S. (1982b) J. Clin. Endocrinol. Metab. **55**, 619-627

Inagami, T., Takii, Y., Inakagi, T., Clemens, D.L., Chang, J.J., Kisaragi, M. and Okamoto, H. (1982c) Clin. Exp. Hypertension **A4**, 1987-1994

Inagami, T., Chang, J-J., Dykes, C.W., Takii, Y., Kisaragi, M. and Misono, K.S. (1983) Fed. Proc. **42**, 2729-2734

Iwao, H., Minami, T., Ikemoto, F., Takaori, K., Nakamura, N. and Yamamoto, K. (1982) Biochem. Biophys. Res. Commun. **106**, 933-939

James, S.K. and Hall, R.C. (1974) Pflugers Archiv. **347**, 323-328

Jilka, R.L., Familletti, P. and Pestka, S. (1979) Arch. Biochem. Biophys. **192**, 290-295

Johnston, R.L., Poisner, A.M. and Crist, R.D. (1979) Biochem. Pharmacol. **28**, 1791-1799

Kassell, B. and Kay, J. (1973) Science **180**, 1022-1027

Kawahara, K. and Tanford, C. (1966) Biochemistry **5**, 1578-1584

Kawamura, M., Ikemoto, F., Funakawa, S., and Yamamoto, K. (1979) Clin. Sci. **57**, 345-350

Kawamura, M., Ikemoto, F. and Yamamoto, K. (1980) Clin. Sci. **58**, 451-456

Kourides, I.A., Hoffman, B.J. and Landon, M.B. (1980) J. Clin. Endocrinol. Metab. **51**, 1372-1377

Laemmli, U.K. (1970) Nature **227**, 680-685

Lambin, P. (1978) Anal. Biochem. **85**, 114-125

Langone, J.J. (1981) Methods Enzymol. **73,** 112-127

Leckie, B.J., McConnell, A. and Jordan, J. (1977) Adv. Exp. Med. Biol. **95,** 249-269

Leckie, B.J. (1978) Clin. Sci. Mol. Med. **55,** 133s-134s

Leckie, B.J. and McGhee, N.K. (1980) Nature **288,** 702-705

Leckie, B.J. (1981) Clin. Sci. **60,** 119-130

Leckie, B.J. and Semple, P.F. (1983) in Hormones in Blood (3rd edn) (Gray, C.H., and James V.H.T. eds.), vol. 4, pp. 439-488. Academic Press, London

Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. **193,** 265-275

Luetscher, J.A., Bialek, J.W. and Grislis, G. (1982) Clin. Exp. Hypertension **A4,** 2149-2158

Lund, T., Vuust, J., Bravo, R., Boel, E., Orstavik, T.B. and Poulsen, K. (1982) Clin. Exp. Hypertension **A4,** 1965-1972

Lumbers, E.R. (1971) Enzymologia **40,** 329-336

Lush, D.J., Munday, K.A. and Noble, A.R. (1983) J. Physiol. **340,** 57-75

McKown, M.M. and Gregerman, R.I. (1975) Life Sci. **16,** 71-79

Macart, M. and Gerbaut, L. (1982) Clin. Chim. Acta **122,** 93-101

Maier G.D. and Morgan, W.S. (1966) Biochim. Biophys. Acta. **128,** 193-195

Majstoravich, J. Jr., Ontjes, D.A. and Roberts, J.C. (1974) Proc. Soc. Exp. Biol. Med. **146,** 674-679

Matoba, T. Murakami, K. and Inagami, T. (1978) Biochim. Biophys. Acta **526,** 560-571

Meyer, P., Kruh, J., Biron, P., Milliez, R., Chevillard, C., Lorain, M.F. and Pasquier, R. (1965) in Club International sur l'Hypertension Arterielle (Milliez, P. and Tcherdahoff, P. eds) pp 57-74 L'Expansion Scientifique Francaise, Paris

Millar, J.A., Leckie, B.J., Semple, P.F., Morton, J.J., Sonkodi, S. and Robertson, J.I.S. (1978) Circ. Res. **42** (Suppl. I), I-120 - I-127

Millar, J.A., Leckie, B.J., Morton, J.J., Jordan, J. and Tree, M. (1980) Clin. Chim. Acta **101,** 5-15

Misono, K.S. and Inagami, T. (1980) Biochemistry **19,** 2616-2622

Misono, K.S. and Inagami, T. (1982) J. Biol. Chem. **257,** 7536-7540

Misono, K.S., Chang, J-J. and Inagami, T. (1982) Proc. Nat. Acad. Sci. **79,** 4858-4862

Morimoto, K., Matsunaga, M., Hara, A. and Kawai, C. (1980) Hypertension **2,** 680-685

Morris, B.J. and Lumbers, E.R. (1972) Biochim. Biophys. Acta **289,** 385-391

Morris, B.J. and Lawrence, C.H. (1980) Biochim. Biophys. Acta **612,** 137-142

Morris, B.J., de Zwart, R.T. and Brown, C.R. (1980) IRCS Med. Sci. **8,** 721

Morris, B.J. Catanzaro, D.F. and de Zwart, R.T. (1981) Neuroscience Letters **24,** 87-92

Morton, J.J., Semple, P.F., Waite, M.A., Brown, J.J., Lever, A.F. and Robertson, J.I.S. (1976) in Hormones and Human Blood (Antoniaides H.N. ed) pp 607-642, Harvard University Press

Murakami, K., Inagami, T., Michelakis, A.M. and Cohen, S. (1973) Biochem. Biophys. Res. Commun. **54,** 482-487

Murakami, K. and Inagami, T. (1975) Biochem. Biophys. Res. Commun. **62,** 757-763

Murakami, K., Takahashi, S. and Hirose, S. (1980a) Biomed. Res. **1,** 216-222

Murakami, K., Chino, S., Hirose, S. and Higaki, J. (1980b) Biomed. Res. **1,** 476-481

Murakami, K., Takahashi, S., Hirose, S., Takii, Y. and Inagami, T. (1980c) Clin. Sci. **59,** 21s-24s

Murakami, K., Ohsawa, T., Hirose, S., Takada, K. and Sakakibara, S. (1981) Anal. Biochem. **110,** 232-239

Naruse, M. Sussman, C.R., Naruse, K., Jackson, R.V. and Inagami, T. (1983) J.

Clin. Endocrinol. Metab. **57**, 483-487

Neurath, H. and Walsh, K.A. (1976) Proc. Natl. Acad. Sci. U.S.A. **73**, 3825-3832

Nustad, K. and Rubin, I. (1970) Br. J. Pharm. **40**, 326-333

Ogino, K., Matsunaga, M., Saito, N., Kira, J., Takayusu, M. and Ono, K. (1967) Jap. Circ. J. **31**, 1-8

Orstavvik, T.B. and Inagami, T. (1982) J. Histochem. Cytochem. **30**, 385-390

Osmond, D.H., Ross, L.J. and Scaiff, K.D. (1973) Can. J. Physiol. Pharmacol. **51**, 705-708

Osmond, D.H., Lo, E.K., Loh, A.Y., Zingg, E.A. and Hedlin, A.H. (1978) Lancet **ii**, 1375-1376

Panthier, J-J., Foote, S., Chambraud, B., Strosberg, A.D., Corvol, P. and Rougeon, F. (1982a) Nature **298**, 90-92

Panthier, J-J., Holm, I. and Rougeon, F. (1982b) EMBO J. **1**, 1417-1421

Parmentier, M., Inagami, T. and Pochet, R. (1983) Clin. Sci. **65**, 475-477

Pau, B., Simon, D., Galen, F.X., Devaux, C., Soubrier, F., Menard, J. and Corvol, P. (1981) Clin. Sci. **61**, 239s-240s

Peart, W.S., Lloyd, A.M., Thatcher, G.N., Lever, A.F., Payne, N. and Stone, N. (1966) Biochem. J. **99**, 708-716

Pedersen, V.B., Christensen, K.A. and Foltmann, B. (1979) Eur. J. Biochem. **94**, 573-580

Peters, T. (1975) in The Plasma Proteins (Putnam, F.W. ed.) Vol. 1, pp 133-181, Academic Press, New York and London

Poulsen, K., Burton, J. and Haber, E. (1975) Biochim. Biophys. Acta **400**, 258-262

Poulsen, K., Kroll, J., Nielsen, A.H., Jensenius, J. and Malling, C. (1979a) Biochim. Biophys. Acta **577**, 1-10

Poulsen, K., Vuust, J., Lykkegard, S., Nielsen, A.H. and Lund, T. (1979b) FEBS

letters **98**, 135-138

Poulsen, K. Vuust, J. and Lund, T. (1980) Clin. Sci. **59**, 297-299

Puidzar, V. and Turk, V. (1981) FEBS letters **132**, 299-304

Re R., Fallon, J.T., Dzau, V., Quay, S.C. and Haber, E. (1982) Life Sci. **30**, 99-106

Reinharz, A. and Roth, M. (1969) Eur. J. Biochem. **7**, 334-339

Richards, H.K., Lush, D.J., Noble, A.R. and Munday, K.A. (1981) Clin. Sci. **60**, 393-398

Righetti, P.G. and Caravaggio, T. (1976) J. Chromatog. **127**, 1-28

Rumpf, K.W., Becker, K., Kreusch, U., Schmidt, S., Vetter, R. and Scheler, F. (1980) Nature **283**, 482-483

Sagnella, G.A. and Peart, W.S. (1979) Biochem. J. **182**, 301-309

Sagnella, G., Price, R. and Peart, W. (1980) Hypertension **2**, 595-603

Sagnella, G.A. and Peart, W.S. (1981) Clin. Sci. **60**, 639-651

Schalekamp, M.A.D.H. and Derkx, M.H.M. (1981) Clin. Sci. **61**, 15-21

Schwert, G.W. and Takenaka, Y. (1955) Biochim. Biophys. Acta **16**, 570-575

Sealey, J.E., Moon, C., Laragh, J.H. and Alderman, M. (1976) Am. J. Med. **61**, 731-738

Sealey, J.E., White, R.P., Laragh, J.H. and Rubin, A.L. (1977) Circ. Res. **41**, (Suppl. II), II-17 - II-20

Sealey, J.E., Atlas, S.A., Laragh, J.H., Silverberg, M. and Kaplan, A.P. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 5914-5918

Sealey, J.E., Atlas, S.A. and Laragh, J.H. (1980) Endocrine Rev. **1**, 365-391

Sealey, J.E., Atlas, S.A. and Laragh, J.H. (1982a) Clin. Sci. **63**, 133s-145s

Sealey, J.E., Wilson, M., Morganti, A.A., Zervoudakis, I. and Laragh, J.H. (1982b) Clin. Exp. Hypertension **A4**, 2373-2384

Skeggs, L.T., Kahn, J.R., Lentz, K. and Shumway, N.P. (1957) J. Exp. Med. **106**, 439-453

Skeggs, L.J., Lentz, K.E., Kahn, J.R. and Hochstrasser, H. (1967) Circ. Res. **21** (Suppl. II) II-91 - II-100

Skinner, S.L., Lumbers, E.R. and Symonds, E.M. (1972) Clin. Sci. **42,** 479-488

Slater, E.E., Cohn, R.C., Dzau, V.J. and Haber, E. (1978) Clin. Sci. Mol. Med. **55,** 117s-119s

Slater, E.E. (1981) Methods Enzymol. **80,** 427-442

Steinmetz, M.A. and Deal, W.C. Jr. (1966) Biochemistry **5,** 1399-1405

Symonds, E.M., Stanley, M.A. and Skinner, S. (1968) Nature **217, 1152-1153**

Szelke, M., Leckie B.J., Tree, M., Brown, A., Grant, J., Hallett, A., Hughes, M., Jones, D.M. and Lever, A.F. (1982) Hypertension 4 (Suppl II), II-59 - II-69

Takahashi, S., Ohsawa, T., Miura, R. and Miyake, Y. (1983) J. Biochem. **93,** 1583-1594

Takaori, K., Iwao, H., Ikemoto, F. and Yamamoto, K. (1982) Clin. Exp. Hypertension **A4,** 2097-2105

Takii, Y. and Inagami, T. (1982) Biochem. Biophys. Res. Commun. **104,** 133-140

Tewksbury, D.A., Dart, R.A. and Travis, J. (1981) Biochem. Biophys. Res. Commun. **99,** 1311-1315

Thorell, J.I. and Johansson, B.G. (1971) Biochim. Biophys. Acta **251, 363-369**

Thurston, H., Hurst, B.C., Bing, R.F. and Swales, J.D. (1978) Clin. Sci. Mol. Med. **55,** 23s-26s

Tigerstedt, R. and Bergman, P.G. (1898) Skand. Arch. Physiol. **8, 223-271**

Ueno, N., Miyazaki, H., Hirose, S. and Murakami, K. (1981) J. Biol. Chem. **256,** 12023-12027

Vandongen, R., Poessee, M., Strang, K.D. and Birkenhager, W.H. (1977) Clin. Sci. Mol. Med. **53,** 189-191

Wauldhausl, W.K., Lucas, C.P., Conn, J.W., Lutz, J.H. and Cohen, E.L. (1970) Biochim. Biophys. Acta **221,** 536-548

Weinberger, M., Wade, M.B., Aoi, W., Usa, T., Dentino, M., Luft, F. and Grim,

C.E. (1977) Circ. Res. **40** (Suppl. I), 1-4

Wilson, C.M., Erdos, E.G., Dunn, J.F. and Wilson, J.D. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**, 1185-1189

Wilson, C.M. Cherry, M., Taylor, B.A. and Wilson, J.D. (1981) Biochem. Genet. **19**, 509-523

Wilson, C.M. and Taylor, B.A. (1982) J. Biol. Chem. **257**, 217-223

Wray, W., Boulikas, T., Wray, V.P. and Hancock, R. (1981) Anal. Biochem. **118**, 197-203

Yamamoto, K., Ikemoto, F., Kawamura, M. and Takaori, K. (1980) Clin. Sci. **59**, 25s-27s

Yokosawa, H., Inagami, T. and Haas, E. (1978) Biochem. Biophys. Res. Commun. **83**, 306-312

Yokosawa, N., Takahashi, N., Inagami, T. and Page, D.L. (1979) Biochim. Biophys. Acta. **569**, 211-219

Yokosawa, H., Holladay, L.A., Inagami, T., Haas, E. and Murakami, K. (1980) J. Biol. Chem. **255**, 3498-3502