Studies of the renin-angiotensin system in vertebrates.

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SUMMARY

The biochemistry and physiology of the renin-angiotensin system in vertebrates is reviewed. Techniques for measuring some components of this system have been developed. The intention was to develop techniques that could be applied to studies in most if not all vertebrates.

Unlike mammalian renal renin, it was not possible to bioassay renal renin of fish, amphibia, reptiles and birds simply with the rat blood pressure preparation. It was necessary to develop enzyme kinetic techniques based on the hydrolytic action of the enzyme renin on its protein substrate to form the vasopressor peptide angiotensin.

Angiotensin is destroyed in all tissues and body fluids by peptidases (angiotensinases). In developing these techniques it was important to inactivate angiotensinases and this aspect was considered at length. Many enzyme poisons were tested and often their effectiveness was pH-dependent. Diisopropyl phosphite (DP) inhibited

kidney angiotensinases satisfactorily in most of the 40 vertebrate species that were studied. These vertebrates included representatives from the five major classes. DP also inactivated angiotensinase in eel (Anguilla anguilla) plasma and the eel corpuscle of Stannius. In other studies with fish and mammalian plasma, angiotensinases were also effectively inhibited by a combination of phenanthroline and ethylene diamine tetra acetate.

The optimal conditions for the enzymic reaction of renin were considered and it was found that some properties of the fish renin reaction differ from those established in mammals. The eel renin reaction with eel renin-substrate was optimal at 20°C and a lower pH (5.4). Also eel renin was inactivated at a pH above 6.0 .

A simple method for the bulk preparation of ox renin-substrate and its application to the assay of renin is described.

Enzyme kinetic techniques for quantitative estimation of eel renin and eel human plasma renin-substrate were developed. Similarly renin was

detected qualitatively in the kidneys of 39 out of 40 vertebrates.

Renin was most concentrated in the posterior part of the kidney in the eel and sea trout (Salmo trutta) and was detected in eel glomeruli.

The renal renin and plasma renin-substrate concentrations were compared in eels adapted to seawater or freshwater. Renal renin concentration did not differ significantly but plasma renin-substrate was marginally higher ($p =< 0.1$) in freshwater adapted eels. Eel plasma renin-substrate concentrations were similar to human plasma concentrations. In the human, the influence of some clinical conditions, pregnancy and oral contraceptives were assessed.

The content of renin in eel corpuscles of Stannius was less than that in the eel kidney and this may have been due to contamination during their removal from the kidneys. The potent vasopressor substance known to be present in these organs remains unidentified.

A role of renin in the regulation of water and electrolytes in fish is discussed finally.

1. GENERAL INTRODUCTION

1.1. The need to control the osmotic pressure of body fluids; phylogenetic considerations.

The earliest form of life is thought to have been unicellular and to have arisen in the sea. Essential nutrients and oxygen entered the cell by diffusion from the surrounding aquatic medium and waste products were excreted by the same route. Evidence from present day unicelluler organisms suggests that the high intracellular potassium and low intracellular sodium concentrations, vital for normal animal cell metabolism, were maintained against concentration gradients by energy-consuming or active processes. The evolution of multi-cellular organisms, in which each cell was not directly in contact with the external environment, necessitated the elaboration of a circulatory system, at first open and later closed, to distribute nutrients to tissues and to remove metabolites. The electrolyte composition and

osmotic pressure of the circulating extracellular fluid is thought to have been based on the marine environment prevailing at that time. The metabolism of the tissues is adapted to this pseudo-marine environment, the extracellular fluid and is markedly affected by relatively small changes in the concentrations of sodium and potassium ions, osmotic pressure, pH, and many other factors. The character of extracellular fluid must therefore be maintained within narrow limits.

The osmotic characteristics of extracellular fluid may be altered either by changes in electrolyte content or by the gain or loss of water. An obvious means of preventing changes in these factors is to construct an impervious pellicle around the animal, thus inhibiting physical and chemical intercourse with the environment. Unfortunately, certain surfaces must be excluded from this protection in order to allow such processes as gaseous exchange and excretion to continue. It is at these surfaces, such as gills, renal tubules and intestinal mucosa, that the animal becomes vulnerable to salt and water imbalance.

From the time at which the concentration of the extracellular fluid was fixed to the present day, the concentration of sea water has increased. Terrestrial animals have the added problem of water conservation. Thus organisms living in each of the major habitats - marine, terrestrial and freshwater - are presented with different problems of maintaining the concentration and composition of their body fluids.

Marine animals, living in a hyperosmotic environment, tend to gain sodium by diffusion, for example at the gill surfaces, but lose water. This process must be reversed by the active expulsion of sodium ions. In freshwater, conversely, loss of sodium to the hypo-osmotic environment with water influx tends to disturb osmotic equilibrium. In this case, sodium must be actively retained. A number of fish migrate between marine and freshwater conditions and must therefore alternate between these extreme situations. Finally, terrestrial animals excrete large volumes of water containing not only the toxic waste products of metabolism but also variable quantities of electrolytes,

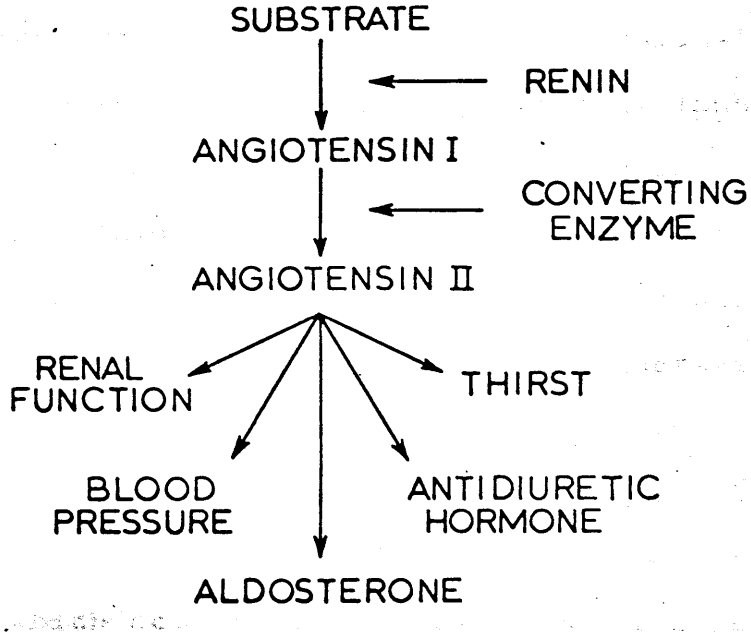
particularly sodium. Like freshwater animals, therefore, they may need to retain sodium.

In vertebrates, a major organ of osmoregulation is the kidney. Renal electrolyte and water excretion is affected by many factors, both neurological and endocrine. Teleologically, however, it seems logical that the kidney itself may have a primary role in controlling excretion. This role may be partly accomplished by the renin - angiotensin - aldosterone system, and it is with this system that the following thesis is concerned.

1.2. The renin - angiotensin - aldosterone system. (Fig 1).

Renin, a renal enzyme is released into the blood stream where it catalyses the hydrolysis of a plasma protein substrate to the decapeptide angiotensin 1. Angiotensin I, with no known direct physiological actions, is further hydrolysed to the octapeptide, angiotensin II, under the control of converting enzymes which are present in plasma and also in many tissues. The properties of these components are discussed in detail in section 2.

Fig.1. The biochemistry and physiology of the renin-angiotensin system.



Angiotensin II maybe an important element in the control of blood pressure and sodium balance. Evidence suggests that angiotensin II may mediate either sodium retention or sodium excretion by a direct effect on the kidney depending upon the prevailing sodium status of the organism (see review by Brown et al, 1971b). Also blood pressure increases markedly in sodium-loaded human subjects infused with angiotensin, thus increasing glomerular filtration and sodium excretion. Infusion in sodium deplete subjects on the other hand has less effect on blood pressure. Angiotensin infusion also leads to an increase in the secretion and plasma concentration of aldosterone (see reviews by Fraser et al, 1969; Brown et al, 1971b), a sodium-retaining corticosteroid which acts on the distal renal tubule. Angiotensin II does not affect aldosterone secretion so markedly in sodium-loaded subjects. Angiotensin II may have further effects on ADH release (Bonjour and Malvin, 1970; Mouw et al, 1971; Tagawa et al, 1971), and thirst (Brown et al, 1969; Fitzsimonds, 1972).

As will be discussed, the precise role of the renin-angiotensin system in lower vertebrates remains unclear, partly through lack of reliable techniques for measurement of the various components. Evidence in man (Gould and Green, 1971; Helmer and Judson, 1967) suggests that the concentration of angiotensin II present in the circulating blood will depend upon the concentrations of both renin and renin-substrate. The following thesis is an account of an attempt to construct and evaluate methods of measuring renal renin and plasma renin-substrate (section 2) and the use of these methods to study the renin-angiotensin system in a variety of species paying particular attention to the migratory eel Anguilla anguilla.

2. METHODS OF STUDYING THE RENIN-ANGIOTENSIN SYSTEM.

2.1. Introduction

In order to study the renin-angiotensin system it is first necessary to measure each of its components in blood or plasma and possible also in tissues. To develop these methods, careful attention must be paid to the physical and chemical properties of the individual components. These are described below.

2.1.1 Properties of the components of the renin-angiotensin system.

2.1.1.1 Renin.

Knowledge of the physical characteristics of renin is rudimentary and studies on it have mainly been confined to mammals. Though it is known to be a protein and enzyme, it has yet to be isolated in crystalline form. Several partial purifications have been reported (Nairn et al, 1960; Haas et al, 1965; Peart et al, 1966a; Lubash & Peart, 1966;

Maier & Morgan, 1966; Skeggs et al, 1967; Newsome, 1969; Rubin, 1972). Properties vary slightly from species to species. For example, the molecular weight of both human and porcine renin is approximately 40,000 (Warren and Dolinsky, 1966; Rubin et al, 1966; Peart et al, 1966a) whereas that from the marsupial Trichosurus vulpecula is heavier at 53,000 (Reid and McDonald, 1969b). Although similar in molecular weight, porcine and human renins have different chromatographic mobilities on starch gel (Peart et al, 1966 b) and there may be several different renins from the same species (Skeggs et al, 1967; Rubin, 1972).

Renin must therefore be recognised by its biochemical properties. It is classed as a peptidohydrolase (E.C.3.4.4.15) which catalyses the hydrolysis of the leucyl-leucine bond in the renin-substrate molecule (Skeggs et al, 1957,1958). Biochemical properties, which are more readily studied in impure preparations have received more detailed attention. The enzyme is

stable indefinitely either at -20° or in a lyophilised state. Dimercaprol and ethylene diamine tetra acetate (EDTA) which chelate various metal ions, do not affect its activity (see Lee, 1969), indicating that it is probably not a metalloenzyme. Further studies (see section 2) tend to corroborate this finding. Sulphydryl reagents such as para-chlorobenzoic acid or salts of cadmium or mercury are without effect on the activity of the enzyme unless used in high concentrations for long periods (see Lee, 1969). This suggests that sulphydryl groups are not components of the active site but may have an important role to play in maintaining the overall tertiary structure of enzyme protein. However, other heavy metal ions such as zinc and copper may cause inactivation (Lee 1969). Renin is also inhibited by sodium deoxycholate, present in bile (Hiwada et al, 1971) and n-heptane (Haas and Goldblatt, 1961). The active centre contains no serine. Small quantities of di-isopropyflouro-phosphate (DFP), which esterifies the free hydroxyl of serine, does not reduce the activity of the enzyme

(Pickens et al, 1965).

It is probably stored in an inactive form since acidification in the region of pH 3.5 of renal tissue (Rubin, 1972; Leckie, personal communication) and amniotic fluid (Lumbers, 1971) increases its activity.

Stability in acid solution varies with species and also with the purity and concentration of the preparation. Canine plasma renin is not inactivated to great extent by pH levels as low as 2.5 during its extraction, even at room temperature for several hours (Brown et al, 1964b). However, partially purified or dilute solutions of renin extracts are less stable (Peart et al, 1966a). At 0^{o} it is less stable in alkaline (pH 10) than in acid (pH 4) solution (Pickering and Prinzmetal, 1938). Further investigations of the effect of pH on renin activity are described in section 2.2.4.

2.1.1.2. Other renin-like enzymes

A number of enzymes other than renin hydrolyse renin-substrate. Among these are the less specific peptidases such as pepsin (Weber et al, 1942; Croxatto & Croxatto, 1942; see also Lee, 1969). One

of the products of the reaction of pepsin with renin-substrate has been characterised as angiotensin I (Franze de Fernandez et al, 1965). However, pepsin is readily distinguishable from renin by its lower pH optimium and also because it is inhibited by poly-l-lysine and is inactivated in neutral or alkaline solution whereas renin is not (Lumbers and Skinner, 1969). These differences have been employed in methods of preparing renin.

Perhaps more interesting is the probable existence of isoenzymes of renin. Renins have been isolated from many tissues including lung, liver, blood vessel walls, spleen, amniotic fluid, submaxillary gland and brain (Gould et al, 1964; Skeggs et al, 1969; Takeda et al, 1969; Symonds et al, 1970; Carretero and Houle, 1970; Lumbers 1971; Johnson et al, 1971; Eskildsen, 1971; Ganten et al, 1971; Carretero et al 1971). Renin isolated from rabbit uterus and placenta is probably identical with the renal enzyme from this animal (Ziegler et al, 1967; Ryan, 1970). However, renin from the dog uterus, though having several

similar properties to renal renin, was able to digest another plasma substrate (Carretero and Houle, 1970). Human pseudorenin (Skeggs et al, 1969) obtained from kidneys and plasma could be separated from human renin by DEAE cellulose chromatography. It was inactive in serum but produced angiotensin from tetradecapeptide and substrate A (see below). The pH optimum for this reaction was lower than renin.

2.1.1.3. Renin-substrate

Renin-substrate is an alpha-2-globulin (Plentl et al, 1943; Cohn et al, 1944,) which is most abundant in plasma but present also in lymph (Friedman et al, 1943; Lever and Peart, 1962; Horky et al, 1971). In common with many other plasma proteins it is probably synthesised in the liver since removal of this organ or hepatic necrosis results in a marked and rapid decrease in plasma renin-substrate concentration (Page et al, 1941; Gould et al, 1966; Ayers, 1967). However, its concentration in the hepatic vein of rats was not higher than peripheral veins (Dauda et al, 1970) nor has it been isolated from

hepatic tissue or indeed from any other tissues (Munoz et al, 1940).

Renin-substrate is stable for several years at -20°. Degradation is accelerated in acid conditions (Dexter et al, 1945; Skinner, 1967). Increasing the ionic concentration of the substrate solution, for example with sodium chloride, prevents damage in acid conditions (Dexter et al, 1945; Lever et al, 1964).

Renin-substrate may not be a single compound but a complex mixture of proteins and/or glycoproteins (Skeggs et al, 1957, 1958, 1964). Gradient elution from diethyl-amino-ethylene cellulose (DEAE) columns with sodium acetate (pH 5.6 down to 4.0) is reported by Skeggs and his co-workers (1964) to separate porcine plasma renin-substrate preparations into at least five fractions, three of which are glycoproteins (A,C, C_2) of similar molecular weight (57,000) and which are hydrolysed with equal efficiency by renin. These and other protein substrates (B,D,E and synthetic tetradecapeptide) are attacked at similar rates by renin. Some doubt has been

cast upon the validity of these results by the inability of later workers to confirm them. Using a different buffer to gradient elute from DEAE cellulose, Cook and Lee (1965) could find no evidence of more than one component, and it is suggested (see Lee, 1969) that some degradation may have occurred in the Skeggs preparation, possibly during the stages of the extraction where low pH levels were used. However Nasjletti et al (1971) using starch-gel electrophoresis found substrate in the albumin and α_2 globulin fractions.

In the past, interest in plasma renin-substrate has been restricted largely to preparing it in a suitable form for use in assays of renin. Frequently, this has meant little more than the addition to substrate-containing plasma of various inhibitors of angiotensinase (see 2.1.1.6. and section 2.2.2.) and converting enzymes (see section 2.1.1.7.). A synthetic tetradecapeptide substrate has also been prepared (Skeggs et al, 1958) but this has the disadvantage that it may decompose to angiotensin spontaneously (Skeggs et al, 1969).

In section 2.2.4. methods of measuring plasma renin-substrate are described and discussed. Preparations for use in the assay of renin are dealt with in section 2.2.3.

2.1.1.4. Renin-renin-substrate specificity.

Renin from a given species may vary in its ability to hydrolyse renin-substrate from other species. This suggests that protein moiety of the substrate molecule from which angiotensin I is detached varies from species to species and that, although renin is capable of releasing angiotensin from relatively small polypeptides (see 2.1.1.3. above), in plasma, the character of this protein moiety influences the rate of hydrolysis. As discussed (in 2.1.1.1.) variations in the structure of renin may also account for part of the variation in reaction rate.

The relationship of the structure and specificity of renin and its substrate to the position of a particular species on the

evolutionary scale is a subject which has received very little attention to date but which would make an interesting and possibly rewarding study. The available information is confused. For example, although it was originally stated that only primate renin was capable of releasing angiotensin from human plasma renin-substrate (Fasciolo et al, 1940), more recent studies (Arakawa et al, 1965) using an improved method of assaying angiotensin demonstrated that rat, porcine and bovine renin also act but with somewhat lower affinity than human renin. Conversely, human renin has a very low affinity for rat substrate. A similar situation exists between the chicken (presumably Gallus domesticus) and toad (Bufo arenarum and B. paracnemis); toad renin releases angiotensin from chicken plasma renin-substrate but the converse does not occur (Nolly and Fasciolo 1971 a). Amphibian renin has a very low affinity for mammalian substrate (Johnston et al 1967b, Nolly and Fasciolo, 1971a), though Capelli et al (1970) could detect renin in Rana

pipiens kidney on incubation with calf (Bos taurus) substrate. Simpson and Blair-West (1971 and 1972) have suggested that marsupial renin-substrate may differ from purified and synthesised substrate (as in figure 2). They demonstrated that whilst antibodies to angiotensin II would react with marsupial angiotensin II, antibodies to angiotensin I would not react with marsupial angiotensin I. This suggested that amino acids 9 and/or 10 may differ in the marsupial renin-substrate.

2.1.1.5. Angiotensin

The term "angiotensin" is used to describe both the decapeptide angiotensin I and the octapeptide angiotensin II. The relationship between these and converting enzyme (see 2.1.1.6.) is described in section 2.1.1.1. and illustrated in figure 1 and 2. Angiotensin II differs from angiotensin I by the lack of a histidyl-leucine dipeptide at its C-terminal end. Two forms of angiotensin are known to occur in nature. Valine 5 angiotensin I and II was first isolated from an equine plasma renin-substrate preparation treated

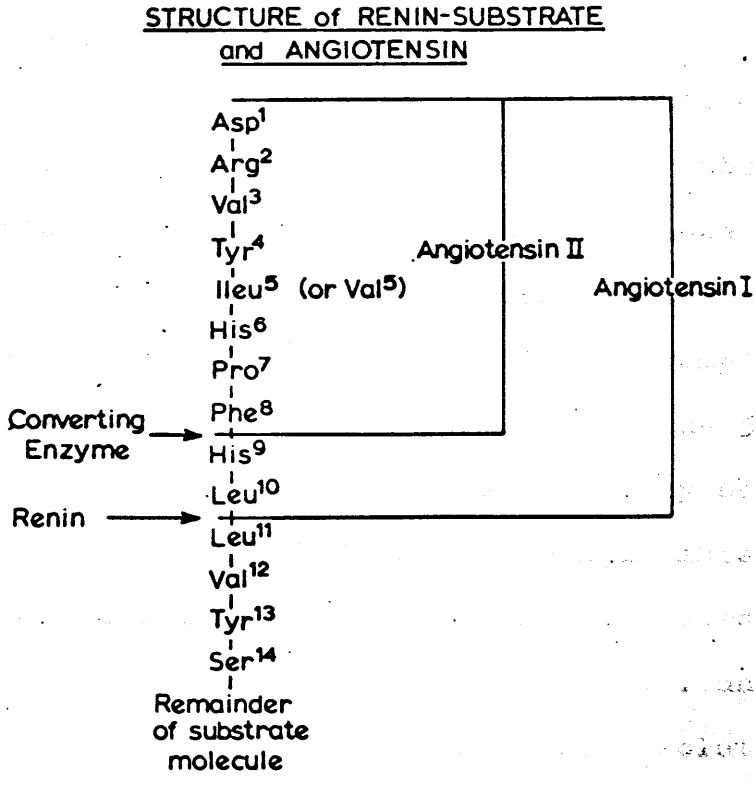


Fig.2. (After Skeggs et al, 1955,'56,'57,'58 and Peart and Elliott, 1957).

with porcine renin by Skeggs et al (1954, 1955, 1956 b and c). It was characterised by Lentz et al (1956a). At the same time, Peart (1955, 1956) and Elliott and Peart (1956, 1957) purified isoleucine 5 angiotensin I from bovine substrate incubated with rabbit renal renin. Since then a number of other forms have been synthesised (see Page and McCubbin, 1968). For example, the pressor effect of asparagine 1 valine 5 angiotensin II is identical to naturally occurring angiotensin II, which is also now available commercially, making it a suitable standard for bioassay procedures (see sections 2.2.3. and 2.2.4.).

Angiotensin is stable in sterile solution at low temperature and also in solid form. It is more susceptible to hydrolysis in alkali than acid (Dexter et al, 1945; Elliott and Peart, 1957). It can also withstand temperatures as high as 100° for periods of several hours.

2.1.1.6. Converting enzyme

Converting enzyme catalyses the removal from the C-terminal end of the angiotensin I molecule, of the dipeptide histidyl-leucine (Lentz

et al 1956). It does not react with the nonapeptide formed from angiotensin I by removing the C- terminal leucine. Whether a specific enzyme for the conversion of angiotensin I to II exists or whether the hydrolysis is catalysed by more general endopeptidases is the subject of some controversy and at present it may be more accurate to speak of "converting enzyme activity".

Such activity has been demonstrated in equine plasma (Skeggs et al, 1956a) and also in homogenates of heart, liver, aorta and ileum (Page and Bumpus, 1968). Lung is particularly rich in converting enzyme activity and may be the source of the plasma enzyme with which it has many similarities (Huggins et al, 1970). The lung in the dog (Ng and Vane, 1967) and also in the rat (Roth et al, 1969) and human (Hollemans et al, 1969) is possibly the major source of activity. There is also evidence for a renal converting enzyme (Franklin et al, 1970; Aitken and Vane, 1972; Leckie et al, 1972) and a renal lymph converting enzyme (Horky et al, 1971).

This renal enzyme also has similar properties to the lung and plasma enzyme though it is possibly non-specific for angiotensin I since it can hydrolyse bradykinin and other small peptides (Yang et al, 1970).

The optimum pH for converting enzyme activity varies from 6.5 to 8, depending on the type of buffer used (Skeggs et al, 1956a) and the enzyme is inactivated at pH 3 or below. It may be a metalloenzyme, or require metal ions possibly calcium, zinc, cobalt or manganese (Dorer et al, 1970; Huggins et al, 1970; Yang et al, 1970; Fitz et al, 1971), for its activity, since both EDTA and cyanide (Skeggs et al, 1956a) dimercaprol (Ryan et al, 1966, quoted in Lee, 1969) dipyridyl (Boucher et al, 1970), phenanthroline (Yang et al, 1970) and hydroxyquinoline (Lehfeldt et al, 1971) cause its inactivation. Monovalent anions such as the halides or nitrate or bicarbonate are also required for its activity (Skeggs et al, 1954; Piquilloud et al, 1970; Huggins et al, 1970; Yang et al, 1970).

The properties of converting enzyme activity vary from tissue to tissue. Chelating agents inhibit that from lung and plasma (Huggins et al, 1970, and see above). DFP inactivates that from artery walls and also from cardiac muscle leaving both plasma, kidney and hepatic enzymes unaffected (see Page and McCubbin, 1968; Yang et al, 1970). There are also species specific differences in converting enzyme (Poulsen and Bing, 1970). No further studies of converting enzyme have been undertaken in this thesis. Further discussion of the properties of converting enzyme are to be found in the work of Oparil et al (1970), Ferreira et al (1970). Piquilloud et al (1970), have developed a synthetic substrate for use in measuring converting enzyme activity. The methods of Huggins and Thampi (1968) and Poulsen and Poulsen (1971) depend on the rate of conversion of angiotensin I by plasma.

2.1.1.7. Angiotensinases.

Peptidases which catalyse the hydrolytic degradation of angiotensin are generally referred

to as angiotensinases. It seems even more probable than with converting enzyme that many so-called angiotensinases are in fact non-specific plasma and intracellular peptidases. The following work will therefore refer to "angiotensinase activity". Nevertheless, an examination of the properties of these peptidases is of considerable importance because methods of measuring both renin and renin-substrate rely on the formation of angiotensin, and angiotensinase activity must therefore be eradicated. In vivo, the length of time that an organism is subject to the effect of circulating angiotensin is also partially governed by angiotensinase. Some peptidases which may not affect angiotensin in vivo, may assume great importance in vitro. For example, plasma pepsinogen is activated to pepsin by acid treatment during extraction of renin and may interfere with substrate-renin incubations.

The properties of 'angiotensinases' are the subject of reviews by Page and McCubbin (1968) and Lee (1969). Further information is listed in Table 1.

TABLE 1. Angiotensinases: Their probable location of action and other properties.

Angiotensin II	Angiotensinase	Location of Angiotensinase	Optimum pH**	Co-Factor	Inhibitor	Non-Inhibitor	Notes	References
Asp_1	Angiotensinase A_1 [1.]	Human serum, [2.] human plasma & RBC [1.3.]	7.4 [1.]	*Ca^{++} [1.2.3.]	EDTA; Ni^{++} [1.2.3.]	/	Asn_1 specific [1.2.]	1. Khairallah & Page 1967.
	Angiotensinase A_2 [1.]	Human serum [1.]	6.8 [1.]	Ca^{++} [1.2]	EDTA [1.]; Ni^{++} [2.]	/	Asp_1 specific [1.2]	2. Nagatsu et al, 1965.
	Aminopeptidase A [2.7]	Human serum [2.] rat kidney [5.]	human plasma [7.]; /	Ca^{++} [5.]	EDTA [5.]	/	Identical with A_2? [2.3.]	3. Khairallah et al, 1963.
Arg_2	Trypsin [8.9.18.]	/	/	/	DFP [13.]; SBTI [14.] Trasylol [15.]	/	No such enzyme demonstrated in human plasma [2],	4. Coleman and Vallee, 1960
Val_3	Pepsin [8.9]	Human urine [10], human plasma [12.]	5.5 [9.]	/	Poly-L-lysine [20.]; pH7+ [10.]	SBTI [14.]	Circulates as pepsinogen [12.]	5. Glenner et al, 1962.
Tyr_4	Chymotrypsin [8.9.18.]	/	6.75 [9.]	/	DFP [13]	/		6. Yang et al, 1968
	Endopeptidase [7.]	Human plasma; rat serum, rat kidney [7.]	/	/	/	/		7. Regoli et al, 1963.
	Angiotensinase B. [1.]	Human plasma [1.]	5.8 [1.]	None [1.]	DFP [1.]; EDTA [1.]	/		8. Riniker & Schwyzer, 1961
Val_5 [18] or Asp_5 [20]	Leucine amino-peptidase [7.8.18.]	Human plasma, rat serum, rat kidney [7.] Intestine and pig kidney [16.]	8 to 9 [17.]	Mg^{++} or Mn^{++} [16.17.]	Cu^{++}, Cd^{++}, Hg^{++} Pb^{++}, EDTA [17.]	DFP & Mercaptides [17.]	Sequential hydrolysis from Asn_1 is more rapid than from Asp_1 leaving a tripeptide [7.8.18.21]	9. Plentl & Page, 1944.
His_6	/	/	/	/	/	/	/	10. Lumbers & Skinner, 1969
Pro_7	Angiotensinase C [6.]	Pig kidney; human urine [6.]	5 to 7 [6.]	/	DFP [6.]	Chelators; Mercaptides [6.]	Only when Pro was amino acid [6.]	11. Riniker et al, 1962, quoted in 6.
	Carboxypeptidase A [8.9.11.18.19]	Pancreas	8.5 [9.]	Zn ++ [4.]	Phenanthroline [4.]	DFP [6.]		12. Mirsky et al, 1948.
Phe_8								13. Schaffer et al, 1957.
								14. Northrop et al, 1948.
								15. Haberland, 1967.
								16. Spackman et al, 1955.
								17. Smith & Spackman, 1955.
								18. Elliott & Peart, 1957
								19. Lentz et al, 1956.
								20. Katchalski et al, 1954.
								21. Carrara et al, 1972.

* Ca^{++} activates angiotensinase A_1 in plasma [1.3.] but not in serum [2.]

** Using angiotensin as substrate except with Leucine aminopeptidase.

EDTA = Ethylene diamine tetra acetate.
DFP = Diisopropyl fluorophosphate.
SBTI = Soya bean trypsin inhibitor.

2.1.1.8. Inactivation of angiotensinase activity.

It is possible to remove angiotensinase activity selectively by fractionating plasma and tissue extracts using acid treatment and salt precipitation (Lever et al, 1964).

Renin and renin-substrate have been studied mainly in renal tissue and plasma. Renal angiotensinase activity is effectively eliminated by small quantities of DFP (Pickens et al, 1965; Yang et al, 1968). The inactivation of plasma angiotensinase activity is more difficult and may require combinations of several compounds. Ryan et al (1968a, b) used a mixture of two metal ion chelators. EDTA, dimercaprol and chlorohexidine gluconate an antibiotic, to inactivate rabbit plasma angiotensinase activity. Inhibitors may also vary in their effectiveness depending on the species from which the plasma is obtained. Further studies of compounds which inhibit angiotensinase activity are presented in section 2.2.2. Control of angiotensin in plasma

and renal extracts from a wide variety of vertebrates proved the main technical problem in the work undertaken for this thesis; consequently it will be considered at length.

2.1.1.9. Renin inhibitors

Three main types of renin inhibitors have been identified.

I. Antirenin.

Antibodies to renin have been raised by injecting partially purified renal extracts into a variety of mammals . The resulting antiserum has been employed as a means of identifying renin in extracts of plasma and to study, by infusing antiserum to inhibit renin, the physiological properties of renin (see Lee, 1969; Page and McCubbin, 1968; Warren and Dolinsky, 1969). Fluorescein-labelled antibodies have been used in attempts to locate intrarenal renin in kidney slices (Edelman and Hartroft, 1961; Hartroft et al, 1964). Unfortunately, as previously stated, pure renin is not so far available, and thus the

conclusions of these studies are severely limited. Impure renal extracts contain a variety of antigenic substances in addition to renin and the use of antisera raised to such extracts may cause changes unrelated to the presence of antirenin.

II. Phospholipid and other naturally-occurring renin inhibitors.

Plasma from normal animals may reduce the rate of angiotensin formation during incubation of plasma samples from nephrectomised animals when renin is added to the samples. This phenomenon has been demonstrated in both the rat (Brunner, 1962) and the dog (Smeby et al, 1967). The responsible inhibitor has been isolated from canine kidneys and identified as a phopholipid (Smeby et al, 1967; Sen et al, 1967) and has subsequently been demonstrated in plasma and red cells of dog and man (Ostrovsky et al, 1967). Treatment of the phospholipid with phospholipase-A produces a lysophospholipid with increased renin-inhibiting capacity. It is probably this lysophospholipid which causes inhibition *in vitro*. The inhibitor is

destroyed by phospholipases B,C and D.

Some information on the properties of renin inhibitors has been obtained indirectly from interference by these compounds with techniques of measuring renin concentration (see section 2.1.2). For example, Boucher et al (1964) and Pickens et al, (1965) found inhibition to occur during incubation of plasma extracts, to a degree which varied with the source of the plasma. Plasma from uraemic human patients suffering from glomerulonephritis also decreases the rate of the renin-catalysed reaction, presumably due to the presence of an inhibitor (Maebashi et al, 1968). Inhibitors may have a degree of species specificity. Schaectelin et al (1968) demonstrated that rat plasma was capable of inactivating rat renin but not hog renin. This inhibitor was inactivated by EDTA and also by acidification.

Heparin competitively inhibits the action of renin but relatively large quantities (5 to 1900 u/ml) are needed (Sealey et al, 1967). The physiological significance of this is

is questionable. It is possible that heparin or some related mucopolysaccharide, located near the site of renin biosynthesis, may bind or combine with renin, thus rendering it immobile and/or inactive. It is known that renin forms complexes with mucopolysaccharides such as chondroitin sulphate and hyaluronic acid (Haas and Goldblatt, 1961). Alternatively, heparin as a polyanion may inhibit renin by precipitating some essential colloidal factor in plasma.

III. Artificial inhibitors of renin activity

The effect of heavy metals and organic substances that poison renin have been considered above in section 2.1.1.1.

2.1.1.10. Factors affecting the rate of the renin-catalysed reaction

Some of the specific factors considered above, will affect the reaction of renin with its substrate. Also in common with other enzyme-catalysed reactions, the action of renin is

affected by conditions of pH, temperature, and substrate concentration.

I. Hydrogen ion concentration

The surface charge of an enzyme protein and also its tertiary structure and therefore its surface conformation, will be affected by the pH of the medium in which it is dissolved. Where, as in the renin reaction, the substrate is also a protein, this may also be altered by changes in pH. Alterations in surface properties of enzyme and/or substrate due to pH fluctuation will alter the efficiency with which the enzyme can react.

Estimations of the optimum pH for renin vary widely from 5.5 to 8 (see Lee, 1969). Since these measurements were made on renin-containing extracts of variable purity, it is probable that the results may have been influenced by the pH sensitivity of contaminants. For example, the rate of the reaction is measured by the rate of formation of angiotensin. Different extracts may have contained angiotensinase activity (see

section 2.1.1.7.) whose pH optimum differed from that of renin. Similarly, the activity of inhibitors of renin may be pH-dependent. The majority of studies indicate that the optimum pH for the renin reaction is approximately 6. Further studies are presented and discussed in sections 2.2.3. and 2.2.4.2.

II. Temperature.

The temperature coefficient (Q_{10}) of an enzyme-catalysed reaction is approximately 2. The initial velocity of the reaction increases with temperature until the enzyme is denatured. Renin is denatured at about 55^{o}. As the temperature approaches this level, although the initial velocity is high, denaturation is marked and progressive and the final yield of angiotensin is low. The best yields of angiotensin are obtained at lower temperature, approximately the physiological temperature of the animal from which the reactants are obtained. This point became of particular importance in work on optimal temperature for the reaction of

eel renin with eel substrate (section 2.2.4.2.).

III. Substrate concentration

The velocity of enzyme-catalysed reactions increases with substrate concentration to a maximum (V_{max}) which is characteristic of the enzyme. The relationship between reaction velocity and substrate concentration is used to calculate the Michaelis-Menten constant (K_m) which is a measure of enzyme-substrate affinity. Published values of K_m for renin vary by over three orders of magnitude (see Page and McCubbin, 1968), probably again because different substrate preparations of different purity from different species were used (see above). Ideally in measuring renin concentration by following the rate of angiotensin formation, substrate must be in excess such that the reaction rate does not slow to any great extent (Section 2.1.2.). The influence of the concentration of renin-substrate in plasma on the physiological effect of renin is also discussed in section 2.1.2.

2.1.2. Methods of measuring concentrations of renin and renin-substrate in biological materials

2.1.2.1. Renin

I. Introduction

Measurement of the renin content of tissues has presented considerable difficulties, as is evident from the long period which elapsed between the discovery of the enzyme (Tigerstedt & Bergmann, 1898) and the development of reliable techniques of measuring its concentration in plasma. In the absence of pure renin, most techniques have used impure standards. Although Goldblatt and his colleagues have made available a partially purified, lyophilised renin preparation for international standardisation, full use has not yet been made of it and comparison of results between research establishments has been difficult. Moreover, although the renin content of tissues such as kidney may be high and readily detectable by biological assay methods, the concentration in plasma, a more suitable medium for study, particularly in *in vivo* experiments, is very low and

cannot be detected by these means. Techniques so far developed can be subdivided into those which attempt to measure renin directly and those which rely on following the rate of formation of the product, angiotensin, of the renin-catalysed reaction. For convenience, these have been called "direct" and "indirect" methods respectively.

II. "Direct" bioassay of renin.

The original qualitative test for renin (Tigerstedt and Bergmann 1898) was adapted to a quantitative method by Pickering and Prinzmetal (1938). This relies on a comparison of the pressor effect of intravenous injections of renin-containing extracts into intact rabbits with the effect of a standard renin preparation. Rats are now more commonly used for the bioassay. The incubation of renin and its substrate occurs in the test animal and the angiotensin released increases blood pressure. It is relatively insensitive and suitable only for extracts containing a large quantity of renin. In common with *in vitro* incubation techniques (see below),

the exogenous renin and endogenous renin-substrate must readily react with each other.

III. "Indirect" enzyme kinetic methods

Renin catalyses the formation of angiotensin I from renin-substrate (see section 1) *in vivo* and *in vitro*. In common with other enzyme-catalysed reactions, the rate of production of angiotensin is directly proportional to the concentration of renin, providing that substrate concentration is not limiting and that conditions such as pH and temperature are standardised (Braun-Menendez et al, 1946; section 2.1.1.10). The angiotensin produced is most commonly measured by means of the rat blood pressure preparation (Skeggs et al, 1953; Peart 1955) or less satisfactorily using strips of artery wall (Helmer, 1955). In these preparations angiotensin I is converted to angiotensin II which raises blood pressure in direct proportion to its mass (section 1). More recently, techniques of measuring concentrations of angiotensin I by radioimmunoassay have been developed (Boyd et al, 1969; Poulsen, 1969; Page et al 1969; Haber et al, 1969; Giese et al, 1970),

which are less time consuming than bioassay and have been successfully applied to renin assay. Finally, Reinharz and Roth (1969) have developed a synthetic substrate (N-carbobenzoxy-pro-phe-his-leu-leu-val-tyr-ser-β naphthalamide) which, in the presence of renin-containing extracts, releases an angiotensin derivative which can be measured fluorimetrically.

Since all these techniques depend on accurate measurement of angiotensin concentration, it is essential that angiotensinase activity should be completely inhibited.

Methods for measuring plasma renin are generally subdivided according to the way in which the renin-substrate is employed. They are described as (i) 'plasma renin activity' and (ii) 'plasma renin concentration' methods respectively.

(i) Plasma renin activity is measured by incubating renin in the sample with its endogenous substrate under standard conditions. Methods of measuring plasma renin activity differ mainly in the steps taken to prevent hydrolysis of the angiotensin after its formation. In the earliest

method (Helmer and Judson, 1963) the plasma sample was merely dialysed at pH 5.5 before incubation so that other factors, including converting enzyme and angiotensinase were modified as little as possible. However, Boucher et al (1964) used the metal ion chelating agent, EDTA thus inactivating converting enzyme and most angiotensinase. Also the angiotensin formed during incubation was adsorbed onto Dowex resin, thus both preventing further destruction and also concentrating the polypeptide to enhance sensitivity in subsequent bioassay. Fasciolo et al (1964) used acidification and Pickens et al (1965) used EDTA and DFP to obtain complete inhibition of angiotensinase activity.

Plasma renin activity methods are rapid and convenient and in some cases possess a high degree of sensitivity. However, they are subject to a number of severe limitations. For example, in no case has the recovery of renin been investigated. It has generally been assumed that the endogenous concentration of renin-substrate in the plasma sample represents an excess and is consequently not rate limiting. There is

evidence that this may not always be so. For example, Skinner et al (1969) demonstrated that as a result of therapy with oral contraceptive steroids, the concentration of renin in the plasma of human female subjects fell slightly although plasma renin activity was markedly increased. These steroid drugs are known to increase the plasma concentration of a number of proteins including renin substrate. The results of Skinner and his colleagues indicate that in the plasma of the untreated subject, renin-substrate concentration is a limiting factor in the renin-catalysed reaction. It may also be a limiting factor in samples from human subjects with liver disease (Ayers, 1967), and nephrotic syndrome (Aida et al, 1965; Medina et al 1972). In vitro studies of the renin-renin-substrate reaction also point in this way (Gould and Green, 1971).

The most recent method of measuring plasma renin activity have attempted to circumvent this problem by adding exogenous renin substrate preparations to the sample before incubation (Boucher et al, 1967). Even this manouvre is

subject to criticism. The majority of measurements have been carried out on human plasma, the renin-substrate content of which has been augmented with bovine plasma renin substrate. No evidence has been presented that the kinetics of the human renin human-renin-substrate reaction and the human renin bovine-renin-substrate reaction are identical. It is at least possible that the velocity curve resulting from this type of incubation will have two components, and that the calculated 'activity' may still largely depend on the initial concentration of endogenous renin-substrate.

(ii) Methods of measuring plasma renin concentration depend on eliminating substances which interfere with the renin-catalysed reaction. These include not only 'angiotensinases' but also endogenous renin-substrate. The plasma extract is then incubated with a known quantity of a standard renin-substrate preparation.

The earliest of these methods, that of Lever et al (1964), removed endogenous renin-substrate by means of DEAE cellulose chromatography, and inactivated angiotensinase activity by

acidification to pH 2.5 or 3 depending on the species from which the sample was obtained. The extract was then incubated at pH 5.7 with renin-substrate prepared from ox blood by acidification and fractional precipitation with salt. Bovine plasma renin substrate preparations were calibrated against a standard renin preparation before use. Samples taken at intervals from the renin renin-substrate incubation were assayed for angiotensin by means of the rat blood pressure preparation.

Subsequently other groups have used slightly different renin-substrate preparations or have inactivated angiotensinase activity with other inhibitors. For example, Cook and Lee (1965) prepared renin-substrate from nephrectomised rabbits, since removal of the kidney increases the plasma concentration of this protein. Skinner (1967) simplified the procedure for extracting renin from plasma, inhibited angiotensinase activity by means of EDTA, and prepared renin-substrate from nephrectomised sheep. Further studies of renin-substrate preparation are presented and discussed in section 2.2.5.

Methods of measuring plasma renin concentration have the advantage that they are not affected by such limiting factors as renin-substrate. With such techniques, renin can be studied in isolation, whereas renin activity assays may only reveal the composite effects of variations in renin, its substrate, and inhibitors or accelerators. However, renin concentration methods are more elaborate and time consuming than the methods of measuring renin activity.

IV. Immunoassay

As previously discussed, (section 2.1.1.9) preparations of renin used to raise antibodies are as yet too impure to raise specific antisera and little progress has been made in measuring renin concentration immunologically. The situation may possibly be remedied in two ways. Investment of time and effort should eventually lead to the isolation of renin in uncontaminated form for use in immunising animals.

V. Juxtaglomerular index

Ruyter (1925 quoted in Page and McCubbin 1968)

and Goormaghtigh (1932, 1940) observed that renin is stored in granules in the renal afferent arteriolar walls. The degree of granulation of renal slices - the juxtaglomerular index or JGI - can be assessed by light microscopy following staining (Bowie, 1935 - 1936) and has been used as a semiquantitative measure of renal renin (Hartroft and Hartroft 1953). The validity of this technique obviously depends on equating the Bowie-stained granules with renin alone. Only circumstantial evidence exists. Granulation is increased in animals subjected to physiological stimuli (see section 4.1.) which should enhance renin biosynthesis (Hartroft and Hartroft, 1953; Pitcock et al, 1959; Tobian, 1960). Flourescein-labelled antirenin (see section 2.1.1.9) is bound to the renin-containing granules (Edelman and Hartroft, 1961; Hartroft et al, 1964). Renin is not distributed uniformly throughout the kidney (see review by Brown et al, 1968) and results may therefore vary with the location from which the sample is taken.

2.1.2.2. Measurement of renin-substrate.

Recent evidence suggests that the concentration of renin-substrate (angiotensinogen) usually present in plasma may have a rate-limiting effect on the production of angiotensin by renin (Skinner et al, 1969; Weir et al, 1970a; Brown et al, 1971a; Gould and Green, 1971). Changes of renin-substrate may, therefore, influence the amount of angiotensin produced by renin. Measurement of the plasma concentration of renin-substrate is of interest for this reason.

Existing techniques are based on the complete conversion of renin-substrate to angiotensin by a high concentration of renin, followed by bioassay of the angiotensin formed. Methods available for man are listed in table 9. Otherwise the laboratory rat has been mainly used in studies on renin-substrate (Helmer and Griffith, 1951, 1952; Mackaness, 1959; Boucher et al, 1967; Carretero and Gross, 1967; Bing and Poulsen, 1969). Data on other mammals and birds, reptiles, amphibia and fish is supplied by Schaffenburg et al (1960)

Romero et al (1970) and Nolly and Fasciolo (1971 a and 1972) and Mizogami et al (1968).

Although the object of this thesis was to study the renin-angiotensin system in vertebrates generally, work was begun on a technique for measuring renin-substrate in the eel. As will be described (2.2.4.1.) it soon became apparent that methods available for measuring renin-substrate in man were generally unsatisfactory.

As can be seen in table 9, values reported for the mean of the normal range for renin-substrate concentration in man vary widely from one laboratory to another. So great is this variation that the upper limit of the normal range for some methods (Ayers, 1967; Imai and Sokabe, 1968) is below the lower limit of the range quoted for others (Maebashi et al, 1965).

As will be discussed (2.2.5.) differences of technique will contribute to this variation: none of the methods listed (table 9) for man reports adequate recovery data, none validates the angiotensin standard (see page 114) and few show

that conversion of substrate to angiotensin is complete at the end of incubation.

With these potential sources of error in mind, one object of the work reported in section 2.2.5.1. of this thesis was to develop and test a method for renin-substrate measurement in man. The method for renin-substrate measurement in the eel (2.2.5.2.) was developed in parallel. Both techniques depend on the inhibition of angiotensinase activity in the specimen of plasma and in the standard renin preparation.

The search for adequate inhibitors of angiotensinase activity was a necessary first step in the development of renin-substrate assay methods for man (2.2.5.1) and the eel (2.2.5.2.) and also in methods for measuring renin in kidney extracts (2.2.3). The plan of this thesis follows these chronological events.

After a preliminary description of the methods used (2.2.1) there follows a summary of the testing of angiotensinase inhibitors (2.2.2). Information gained in these experiments was applied to the

measurement of renal renin (2.2.3) and renin-substrate (2.2.4.). In a final section on methods (2.25.) a technique for mass-production of renin substrate from the nephrectomised ox will be described.

2.2. New methods developed for assay of different components of the renin-angiotensin system in man and other vertebrates

2.2.1 Materials and general methods

2.2.1.1 Materials

I. Metal salts

Metal salts used as inhibitors of angiotensinase activity (section 2.2.2) were of reagent grade and were used without further purification.

II. Other inhibitors

E-Amino caproic acid, L-arginine and poly-L-lysine (molecular weight approximately 175,000) were obtained from Sigma Ltd. This firm also supplied 2,2 bipyridine and 1,10 phenanthroline which were initially dissolved in ethanol (1 g / 80 ml.) and further diluted as required. A 0.4M stock solution of 2.3-dimercaprol ('BAL' BDH Ltd.) in arachis oil was also diluted as required. Hydrogen fluoride (reagent grade) and sodium ethylene diamine tetra acetate ('EDTA', M & B Ltd.) were used as supplied and samples of di-isopropylfluorophosphate (DFP') were obtained from several sources (Sigma Ltd., Koch-Light-Ltd., Kodak Chemical Co. Inc.) to be used in ethanol solution (10% v/v). Diisopropylphosphite was obtained from R. Emmanuel Ltd., Koch-Light-Ltd.,

and the Kodak Chemical Co. Inc., and 'Trasylol' (a polypeptide) from Bayer Ltd. (Germany).

III. Angiotensin

Asn 1 Valine 5 angiotensin II (angiotensin II) and asp 1 isoleucine 5 angiotensin I (angiotensin I) were obtained from Ciba Ltd. and the Schwarz Bio-research Co. respectively. Stock solutions (10 µg/ml) were prepared in buffer (pH 5.7, see below) and further diluted for use as a standard in the rat blood pressure preparation (0.1 µg/ml.) and for use in the assay of human plasma renin substrate (0.1 µM).

A solution of natural angiotensin I was also prepared using eel renal renin and bovine plasma renin-substrate. Eel kidney extract (0.6 ml) and a bovine plasma renin-substrate preparation (48 ml. pH 6.2) were incubated with diisopropyl phosphite (0.36 ml) and buffer (11 ml. pH 6.2) for 15 hours at 37°. The mixture was boiled and centrifuged (2,800 r.p.m., 30 minutes) and the supernatant concentrated at 60° using a rotary evaporator. The residue was dissolved in buffer solution (2 ml. pH 5.7). It contained 3.4 µM angiotensin (as assayed against angiotensin II).

IV. Buffer solution

Phosphate buffers of various levels of pH were

prepared by adjusting the proportions of 0.05M Na_2HPO_4 and 0.05M NaH_2PO_4 (see Gomori 1955). The total ionic strength of these buffers was maintained at 0.15M by adding 0.1M sodium chloride solution. The buffers were sterilised by boiling and sterility was maintained by adding the antibiotic neomycin sulphate (0.2 g/100 ml). Buffers for dialysis procedures were not sterilised but neomycin sulphate was added where stated. Glycine buffers (pH2.5) contained glycine (0.1M), hydrochloric acid (3.6 mM) and sodium chloride (0.5M).

V. Other substances.

Cabbage phospholipase C and phospholipase D from Clostridium perfringens were supplied by Koch-Light-Ltd. and soya bean trypsin inhibitor by Sigma Ltd. Sea water (SG 1.022) was prepared by dissolving a salt mixture ('Meeresalz' Aquatic Hobby Ltd.) in Glasgow tap water.

VI. Equipment

Syringes (Hamilton Co.) of 250 µl and 100 µl capacity were used for general bioassay purposes and for estimation of plasma renin substrate (section 2.2.4) respectively. Incubations of renin extracts and renin-substrate were performed in polyethylene tubes (10 ml,

Nalgene) and aliquots from these were stored in clip-top polythene tubes (5 ml, A.R. Horwell Ltd.).

VII. Animals

Live eels (Anguilla anguilla L.) were obtained principally from Loch Lomond and supplied by Mr. W. Greene of Glasgow. Other sources were the University of Glasgow Research Station, Rowardennan, Stirlingshire; Freshwater Biological Association, Ambleside, Westmoreland; and Messrs. Brammer and Morck Ltd., Billingsgate, London. Prior to use they were kept out-of-doors in a fibre glass tank (6' x 3' and 2.5') through which tap water was running constantly. During experiments one or two eels were kept in 70 litre polythene tanks containing either tapwater or seawater. In each case, the water was circulated by means of an Eheim pump and filtered through a charcoal resin filter. The pH of the water was tested at frequent intervals (sea water - pH 8, tap water - pH7) and the charcoal/resin filter changed when pH fell by one unit. This restored pH to its initial level.

Rats (Wistar) used for the bioassay of renin and angiotensin were supplied by Oxfordshire Laboratory Animal Colonies and were prepared for use as described by Peart (1955). Kidneys from a variety of vertebrates, supplied by the Zoological Society (London), were stored

at - 20°.

2.2.1.2. General Methods

I. Preparation of renin substrate

Bovine renin-substrate for the assay of renal renin was prepared by the method of Lever et al (1964). Its preparation varied slightly with the purpose for which it was intended. It was prepared from plasma from nephrectomised stirks (castrated bulls) for use in the assay of renal renin. The renin-substrate concentration (1.16 μM) was determined from replicate incubations with an excess of human renin. When used to study the inhibition of angiotensinase activity it was diluted to 0.58 μM. In each case, it was stored at - 20°. Further studies of bovine renin substrate preparation are presented in section 2.2.5.

Human renin-substrate was prepared according to Dexter et al (1945) with the following modification. After the final centrifugation, it was dialysed against pH 6.8 phosphate buffer for 24 hours at 4°C and diluted in the same buffer to a renin substrate concentration of 123 nM. At this concentration there was no detectable angiotensinase activity.

II. Extracts of kidney and corpuscles of Stannius

Samples of kidney from several species and cor-

puscles of Stannius from the eel were macerated in phosphate buffer (pH 5.7, 5 ml/g tissue) with a pestle and mortar and acid-washed sand (40-100 mesh, BDH). After standing at room temperature with frequent mixing for one hour, the supernatant was removed by centrifugation and stored at - 20°. It was recentrifuged on each occasion when it was thawed to remove precipitates.

An extract of a large number of eel kidneys was prepared in a similar manner for use in the study of the inhibition of angiotensinase activity (section 2.2.2) and in the development of a method for the assay of eel renal renin (section 2.2.3).

III. Human renal renin. Human renal renin was prepared by the method of Brown et al (1964a). It was diluted in sodium phosphate buffer (pH 5.7) to a concentration of 10 units/ml (one unit of renin, incubated with ox substrate forms 100 ng angiotensin/ml/hr) and stored at - 20°. Under these conditions it is stable for at least six years.

IV. Exsanguination procedures.

Blood for renin and renin substrate assay (section 2.2.4, 2.2.5, 4.2.2) was obtained from eels anaesthetised with MS.222 (2g/l aquarium water, Sandoz Ltd.).

Blood was collected from the ventral aorta into nylon tubes containing lithium heparin (Teklab Ltd.), centrifuged and the plasma stored at - 20°.

Blood for the preparation of bovine plasma renin-substrate (see above and section 2.2.5) was collected from the left carotid artery of an ox previously nephrectomised and treated with heparin (3,500 u/100 lb) prior to anaesthesis. The animals were respired during exsanguination which was kindly performed by Dr. J. Campbell of the Veterinary Department, University of Glasgow. Plasma removed after centrifugation was either processed immediately or stored overnight at 4°.

V. Denaturation of ichthyotoxin

Eel plasma contains a protein which is lethal on intravenous injection into other species (Bertin 1956, Rocca & Ghiretti 1964). This toxin was denatured by immersing incubated plasma extracts in boiling water in a tightly sealed polythene tube for five minutes (see also section 2.2.5). The protein precipitated during this procedure was removed by centrifugation.

VI. Bioassay of angiotensin

Angiotensin in incubation media was assayed by means of the rat blood pressure preparation (Peart 1955).

The limit of the assay is determined by the responsiveness of the rat to angiotensin. The threshold response occurs at approximately 1 ng (0.97 nm)of angiotensin II contained in 0.1 ml of solution.

2.2.2. Inhibition of angiotensinases

2.2.2.1. Introduction

Extracts of biological tissues containing renin and renin-substrate are invariably contaminated with angiotensinases. Since neither renin nor its substrate can be measured in their presence, a necessary first step in developing methods of measuring renin or renin-substrate is the removal or inactivation of these angiotensinases. This section is concerned with the different problems of inhibiting angiotensinase activity in renin-containing extracts of kidneys, plasma and other tissues from a wide variety of vertebrates.

In general, enzyme activity can be inhibited by:-

(i) removing an essential cofactor

(ii) competitive inhibition using compounds resembling the substrate of the enzyme.

(iii) selective protein denaturation.

With these principles in mind, the inhibition of angiotensinase activity has been attempted by displacing essential metal ions, either with heavy metal salts or with metal ion chelating agents. Amino acids and amino acid analogues have been tested as possible competitive inhibitors, and organophosphorus compounds and sulphydryl reagents have also been employed in an attempt to inactivate the active centre of the enzymes.

Finally, the effect of mild protein denaturing techniques has also been investigated. Compounds were tested not only as inhibitors of angiotensinase activity but also for undesirable side-effects such as renin inactivation and toxicity to the rat during the bioassay of angiotensin.

2.2.2.2. Assessment of angiotensinase activity.

I. Hydrolysis of synthetic angiotensin I and II

Angiotensinase activity results in reduced recoveries of angiotensin when incubated with tissue extract. The efficacy of inhibitors of angiotensinase activity was tested by comparing the recovery of angiotensin I or angiotensin II (100 ng/ml incubation medium) from incubated extracts containing inhibitor with that from control incubation lacking inhibitor. In all cases, the same type of angiotensin was used for the incubation and for the bioassay standard.

II. Natural angiotensin

Recoveries of endogenously-formed angiotensin were also studied in control experiments by incubating renal extracts with ox renin-substrate with and without inhibitor. In this case, absence of angiotensinase activity was indicated by a gradual rise in angiotensin concentration during incubation, reaching a maximum

level which was then maintained until the incubation was terminated. Angiotensinase reduced the rate of increase of angiotensin and, when the maximum concentration had been reached, caused a gradual fall in concentration until the incubation was terminated (see Lever et al, 1964).

III. Estimation and expression of recovery

Renal extracts contain little or no renin-substrate and, when incubated alone, angiotensin is not formed. However, in many species, plasma contains variable and appreciable quantities of this substrate. In order to correct angiotensin recoveries for angiotensin released from endogenous plasma substrate by renin, blank samples of plasma were incubated alone. To obtain recovery, the blank value was subtracted from that of the sample with added angiotensin.

2.2.2.3. Results

Recoveries of added angiotensin from tissue extracts incubated in the presence of various inhibitors of angiotensinase activity are shown in Tables 2 to 7.

I. Metal ions

Some of the compounds tested improved angiotensin recovery from incubated plasma solutions, (Table 2)

TABLE 2. Metal ions as inhibitors of angiotensinase.

Salt	Molarity	Kidney extracts (8 mg/ml) Dog		Man		Plasma (20%) Ox		Man	
		R	P	R	P	R	P	R	P
Cupric sulphate	1×10^{-3}	40	+	<10	+	80	-	85	+
Potash Alum.	1×10^{-3}	85	+++	<10	+++	50	+++	50	+++
Zinc sulphate	1×10^{-3}	45	+++	<10	+++	50	+++	30	-
Manganese chloride	1×10^{-3}	20	+	<10	+	50	+++	30	++
Magnesium chloride	1×10^{-3}	20	-	<10	+	45	-	30	-
Barium chloride	1×10^{-3}	30	-	<10	+	50	+++	30	-
Mercuric chloride	1×10^{-3}	50	+	<10	++	90	-	55	-
Plumbous nitrate	1×10^{-3}	70	++	<10	++	50	+++	60	+
Nickel chloride	1×10^{-3}	30	+	<10	+	60	-	70	-
Cadmium sulphate	1×10^{-4}	20	+	<10	+	50	-	70	-
Cobalt nitrate	1×10^{-4}	<20	+	<10	+	30	-	40	-
Control	-	25	-	<10	-	40	-	25	-

% angiotensin II recoveries (R) were estimated after 3/4 hour incubation at 37°C. The precipitates (P, +++ = Large, ++ = Medium, + = small quantity) were assessed at 15 hours. Each result represents the mean of duplicate assays.

although with salts of zinc, magnesium, manganese and barium, the improvement was minimal. Copper sulphate and nickel chloride had the most marked effect in both human and bovine plasma but mercuric chloride, although less effective in human plasma, gave 90% recoveries of added angiotensin from bovine plasma. Unfortunately, copper sulphate caused a small amount of protein precipitation during incubation of plasma. This was also true of alum and zinc sulphate, the chlorides of manganese and barium, and lead nitrate, the extent of precipitation differed between human and bovine plasma. Although the enzyme properties of the precipitates were not tested, they may have contained denatured renin. None of the compounds tested improved angiotensin recoveries from human kidney extracts. Since their effectiveness on plasma angiotensinases was slight and often in combination with protein precipitation which might have included renin, no further studies were made.

II. Chelating agents

Phenanthroline, bipyridine, ethylene diamine tetra acetate (EDTA-di-Na), British anti-lewisite (BAL) and mixtures of EDTA and BAL and of EDTA and phenanthroline were tested with renal and plasma extracts. The results are shown in tables 3 and 4.

TABLE 3. Chelating agents as inhibitors of angiotensinase.

Agent	Molarity mM	pH	Kidney extracts: Dog Q	Dog R	Dog C	Man Q	Man R	Man C	Eel Q	Eel R	Eel C	Plasma: Ox D	Ox R	Ox C	Man D	Man R	Man C
Phenanthroline	0.63	5.7	10	40	<10							25	20	<20	25	75	<20
"	1.56	5.7										25	90	<20	25	80	<20
"	1.56	7.4				0.5	90	<10	7	90	<10						
Bipyridine*	2	5.7	10	50	<10				7	10	<10						
" *	6	5.7	10	100	<10				7	10	<10						
"	2	7.4							7	70	<10						
"	6	7.4							7	100	<10						
EDTA	16.8	5.7				0.5	<10	<10	7	<10	<10						
"	16.8	7.0				0.5	40	<10	7	<10	<10						
"	33.6	5.7										25	70	<20	25	<20	<20
"	33.6	7.0										25	20	<20	25	20	<20
EDTA/ Dialysis*	2.7	5.7										80	100				
dimercaprol	16	5.7													25	40	10

Incubations marked * were for 48 hours, all others were for 16 - 22 hours.

Q = mg. kidney/ml; D = % plasma dilution; R = % angiotensin II recovered; C = % angiotensin II recovered from control sample without the chelating agent

Table 4. Combinations of chelating agents as inhibitors of angiotensinase

Agents mM		pH	Ox Plasma			Human Plasma		
			D	R	C	D	R	C
EDTA +	dimercaprol							
1.68	1	5.7	25	80	<20	25	50	<20
"	4	"	"	60	"	"	55	"
"	8	"	"	55	"	"	65	"
13.4	1	"	"	100	"	"	20	"
"	4	"	"	80	"	"	30	"
"	8	"	"	80	"	"	40	"
67.3	1	"	"	100*	"	"	*	"
"	4	"	"	100*	"	"	*	"
"	8	"	"	100*	"	"	*	"
EDTA + phenanthroline (as AIS, see 2.2.4.1)		See figures 5, 6 and 7						

The angiotensin II recoveries (R = sample, C = control) were assayed after 22 hr. Some assays (*) were extremely difficult because the EDTA at 67.3 mM caused a sharp depression of the rats blood pressure. D = % of plasma in incubation mixture.

Although phenanthroline improved angiotensin recoveries from incubations of both plasma and renal tissue, at concentrations in excess of 1.56 mM it reduced blood pressure markedly when injected intravenously in the rat.

Bipyridine, even at concentrations as high as 6 mM, did not have this effect. It inhibited renal angiotensinase activity slightly more effectively than phenanthroline although this varied with species (see below) Bipyridine used at 6 mM caused protein precipitation from incubated human and bovine plasma, and this was enhanced by frozen storage.

EDTA did not affect the rat blood pressure in the range of concentration used in these experiments (13 mM) but above this it had a hypotensive effect, occasionally. Alone, however, human plasma angiotensinase activity was more effectively inhibited by phenanthroline or bipyridine, and angiotensinase activity in eel kidney extracts was not affected by EDTA at all. EDTA (33.6 mM) was most effective when used in the plasma of the ox where 70% of angiotensin added remained after 24 hours of incubation. If, instead of adding EDTA to the incubation mixture, the undiluted plasma was first dialysed against buffer containing EDTA (2.7 mM, pH 5.7) before dilution and incubation, recovery of angiotensin

was improved. BAL (16 mM) improved angiotensin recoveries in human plasma from the control value of 10% to a level of 40% but further improvement could not be obtained. Moreover, protein precipitation became a serious problem at concentrations in excess of 8 mM.

Phenanthroline inhibited canine renal angiotensinases at pH 5.7 and also ox and human plasma angiotensinases (Table 3). The effects of bipyridine (Table 3) were similar.

The effect of pH on angiotensinase inhibition was studied with EDTA, bipyridine and phenanthroline. Only the two latter substances were effective on eel angiotensinases and then only above pH 7 (fig.3). Because eel renin proved difficult to assay at this pH, the use of these chelating agents was abandoned. Also human angiotensinases were inactivated by phenanthroline and, to a lesser extent, EDTA, in the neutral range (fig.4).

III. Mixtures of chelating agents

It seemed possible that compounds achieving only partial inhibition of angiotensinase activity in a given tissue extract might, when used in combinations of two or more compounds, cause a more complete inhibition. No such synergistic effect was apparent using various mixtures of BAL and EDTA in plasma

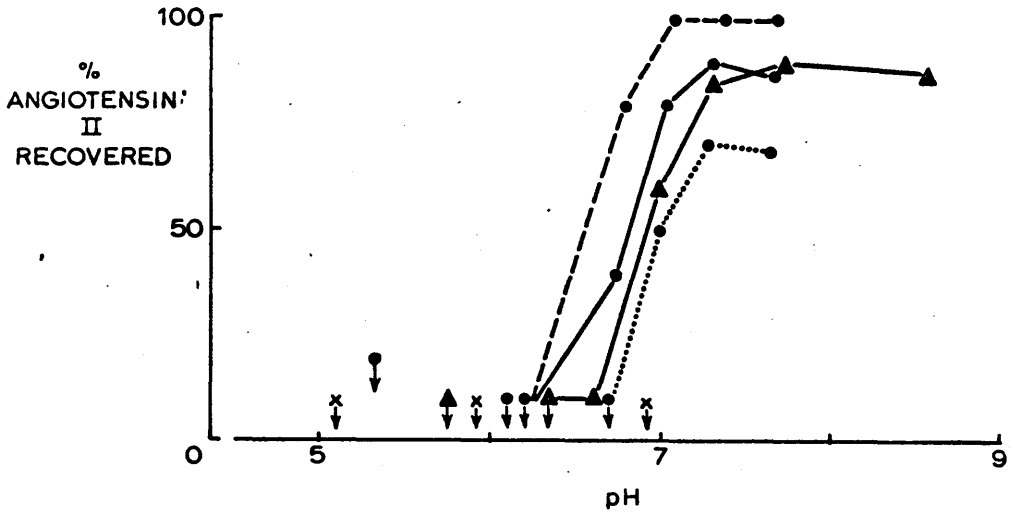


Fig.3. Effect of pH on the recovery of angiotensin II incubated at 37°C. for 16 hours with eel kidney extract (7.2 mg/ml) and with phenanthroline (▲, 1.56mM), EDTA (X, 16.7mM) and bipyridine (●--● at 6 mM, ●——● at 4 mM and ●...● at 2mM). Each point represents the mean of duplicate assays.

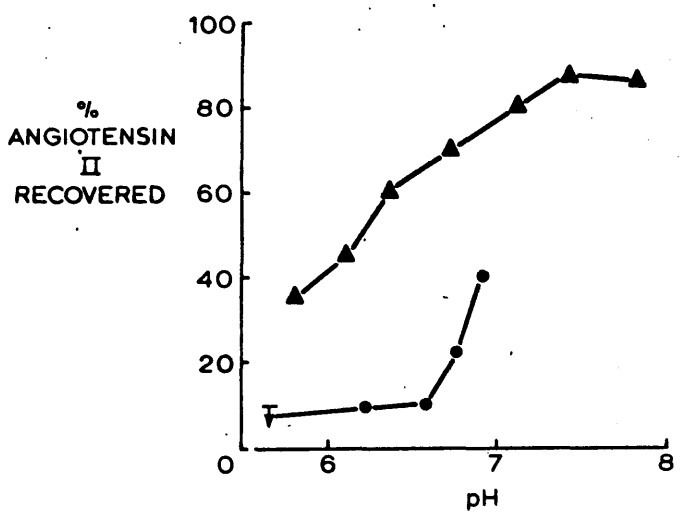


Fig.4. Effect of pH on the recovery of angiotensin II incubated at 37°C. for 16 hours with human kidney extract (0.5 mg/ml) and with phenanthroline (▲, 1.56 mM) or EDTA (●, 16.7 mM). Each point represents the mean of duplicate assays.

(see Table 4), nor were the undesirable effects of the individual components ameliorated by using them together.

The most successful combination to inhibit human plasma angiotensinase activity was EDTA with phenanthroline (Figs. 5, 6 and 7). Also assessing angiotensinase activity by the recovery of endogenously-released angiotensin (see above), inhibition was obtained at pH 6.8 (Fig 6), but marked hydrolysis of angiotensin occurred in more acid conditions. This combination of angiotensin inhibitors was used extensively in studies of human plasma renin-substrate (see section 2.2.4.1.).

The effect of EDTA/phenanthroline varied between species as can be seen from figure 7. It consistently gave a high recovery of angiotensin I over a wide range of pH from bovine, canine and rat plasma extracts. Indeed, recoveries frequently in excess of 100%. Evidence is presented (section 2.2.4.1) that converting enzyme activity in the rat blood pressure preparation is impaired by repeated doses of EDTA/phenanthroline mixture and that a given dose of angiotensin I may therefore give a smaller blood pressure response than an equimolar quantity of angiotensin II. A possible explanation of this recovery in excess of 100% may

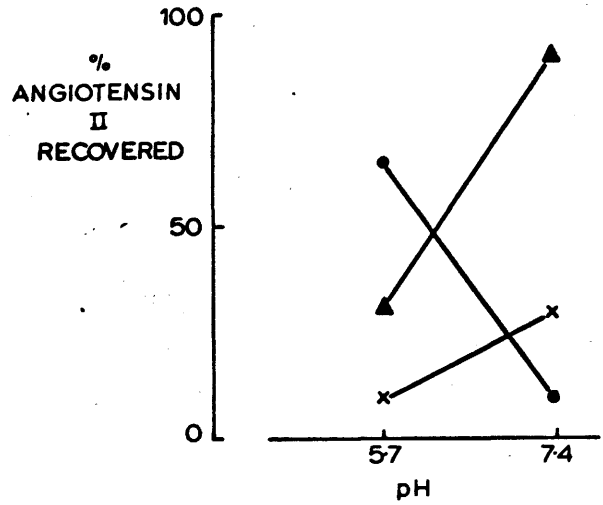


Fig.5. Effect of pH on the inactivation of human plasma angiotensinase by EDTA and phenanthroline. Each point represents the mean of duplicate assays of angiotensin II recovered from 20% diluted plasma after 24 hours at 37°C. X = EDTA (13.4mM); ● = phenanthroline (1.25mM); ▲ = EDTA (13.4mM) + phenanthroline (1.25mM).

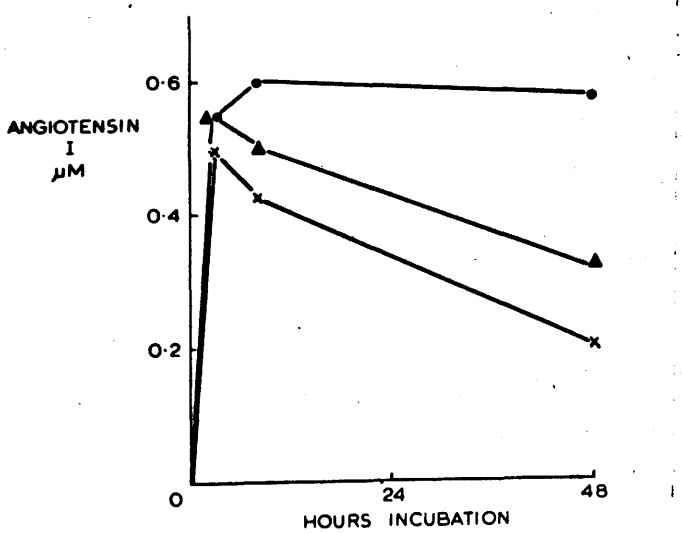


Fig.6. Effect of pH on the reaction of human renin with human plasma using EDTA and phenanthroline as angiotensinase inhibitors. Each point represents the mean of duplicate assays. The pH of the incubation mixtures were 5.9 (X), 6.3 (▲) and 6.8 (●).

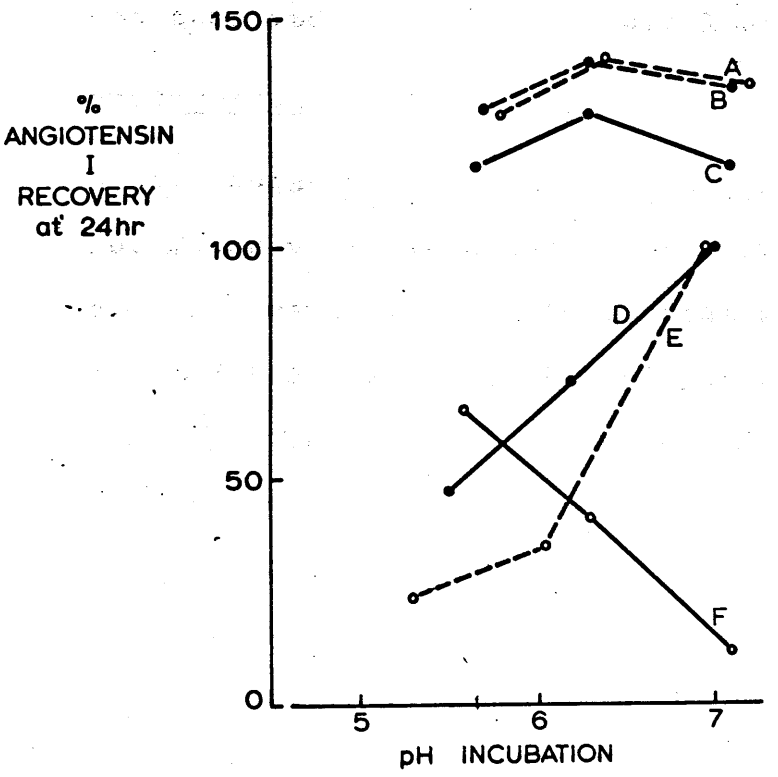


Fig.7. Effect of pH on plasma (diluted to 20%) angiotensinase inhibition in several vertebrates by EDTA (13.4mM) and phenanthroline (1.25mM) at 37°C. A = Ox, B = Dog, C = Rat, D = Eel, E = Cod, F = Rabbit.

therefore have been incomplete inactivation of converting enzyme activity *in vitro*. In the EDTA: phenanthroline treated rat, a mixture of angiotensin I and II may have given an abnormally high blood pressure response when compared to an angiotensin I standard.

IV. Acid denaturation.

It has been suggested that angiotensinases from the kidney of lower vertebrate can be inactivated by means of acid treatment for example by dialysis against glysine-HCl buffer at pH 2.5 (Capelli, Wesson and Aponte, 1970). To test this a number of renal extracts were dialysed in this way and were adjusted to pH 5.7 before incubation. The results summarised in Table 5. It is apparent that while acidification improved the recoveries of angiotensin from the tissues of higher mammals, it was largely without effect in those from fish and amphibia. Moreover, it invariably caused a voluminous protein precipitate.

V. Organophosphorus compounds.

Organophosphorus compounds combine with the primary alcohol group of serine. When this amino acid occurs as part of the active centre of an enzyme, the activity of the enzyme can be inhibited by organophosphorus compounds. Unfortunately, because of their

Table 5. Inactivation of kidney angiotensinase by acidification to pH 2.5.

Species	Extract mg/ml	% Angiotensin I recovered after 24 hr.
Anguilla anguilla	0.8	17.5
(eel)	8.0	10
Salmo trutta	0.8	20
(sea trout)	8.0	10
Rana temporaria	0.8	30
(frog)	8.0	15
?	0.8	70
(Porters Giant tortoise)	8.0	50
Rhea americana	0.8	90
(rhea)	8.0	50
Phoca vitulina	0.8	50
(common seal)	8.0	20
Canis familiaris	0.8	50
(dog)	8.0	10
Rattus norvegious	0.8	10
(rat)	8.0	renin response
Homo sapiens	0.8	87.5
(man)	8.0	22.5
Angiotensin control	-	90

potent inhibition of nerve function, organophosphorus compounds must be handled with extreme caution.

(i) Di-isopropyl fluorophosphate (DFP)

There was a marked variation in the physical appearance of different batches of DFP and also in their ability to inhibit angiotensinase activity. Clear, homogenous or monophasic preparations were without effect on angiotensinase activity but were lethal to the rat. However, some preparations contained an oily, globular component. These preparations inactivated angiotensinase activity in canine and human renal extracts at a concentration of 9.6 mM and from eel kidneys at 14.3 mM over the pH range 3-6.3 (Table 6). The heterogenous appearance of this latter type of preparation and also its reduced toxicity in the rat (see below) suggested that some form of chemical degradation had occurred and that enzyme inhibition was attributable to a derivative rather than to DFP itself.

(ii) Products of hydrolysis of DFP

The products of hydrolysis of DFP are hydrogen fluoride and di-isoprophyl phosphate. The effects of each of these on angiotensinase activity from various sources was tested.

TABLE 6. Organophosphorus compounds as inhibitors of angiotensinase

Agent	Molarity mM	pH	Kidney						Corpuscle of Stannius			Plasma					
			Dog			Eel			Eel			Ox			Man		
			Q	R	C	Q	R	C	Q	R	C	D	R	C	D	R	C
DFP	4.4	+5.7										25	<20	<20	25	10	<10
	11	+5.7										25	<20	<20	25	15	<10
	22 **	+5.7										25	65	<20	25	80	<10
	0.48 *	+5.7	8	33	<20												
	2.4 *	+5.7	8	64	<20												
	4.8 *	+5.7	8	84	<20												
	9.6 *	+5.7	8	100	<20												
	4.8	3.5				5.6	75	20									
	4.8	5.7				5.6	20	<20									
	4.8	6.9				5.6	40	<20									
	4.8	7.6				5.6	30	<20									
	7.25	3.3				5.6	80	<20									
	7.25	4.9				5.6	50	<20									
	7.25	6.8				5.6	60	<20									
	7.25	7.6				5.6	50	<20									
	14.3	3.0, 3.6 4.4, 5.4 and 6.2				5.6	100	<20									
	14.3	6.7				5.6	90	<20									
	14.3	7.2				5.6	80	<20									
DP	36	3.3				1	50	<20									
	36	4.4				1	40	<20									
	36	5.4				1	50	<20									
	36	6.5				1	15	<20									
	36	7.0				1	10	<20									
	36	5.4							0.8	97	35						
	36	5.4							10	50	<20						

See Figure 8 and Table 12 for DP inhibition of eel plasma - and vertebrate kidney angiotensinase respectively. The recoveries of angiotensin II from eel kidney extracts were after 48 hours, otherwise at 20 - 30 hours. ** = Heavy precipitate; * = Dog kidney extract was incubated with ox renin-substrate. The recoveries represent the % angiotensin remaining after all of the substrate had been hydrolysed to angiotensin at 1 hour; + = Experiments carried out with pH 5.7 buffer. The true pH which was not measured, would be lower than this since DFP was subsequently found to be acidic. Q = mg kidney/ml; D = % plasma dilution, R = % angiotensin II recovered, C = % angiotensin II recovered from sample without DFP or DP respectively.

(a) Hydrogen fluoride.

Hydrogen fluoride was without effect on the angiotensinase activity in canine, human and eel renal extracts.

(b) Di-isopropyl phosphite.

Di-isopropyl phosphate is not available commercially and use was therefore made of the phosphite (DP). At a concentration of 36 mM it completely inhibited the hydrolysis of angiotensin by extracts of eel kidney and eel corpuscle of Stannius providing that not more than the equivalent of 1 mg and 4 mg of tissue respectively was used per ml of incubation mixture (Table 6).
As is apparent from fig.8, the efficacy of DP in eel plasma varied considerably with the pH of the incubation medium, but at pH below 5.5 inhibition of angiotensinase activity was satisfactory at 18 hours, the normal period of incubation (see section 2.2.4.2.).

Rats used for assay purposes survived repeated injections of 0.1 ml of 36 mM DP over a period up to 8 hours without exhibiting any adverse effects. The rat blood pressure preparation has therefore been used with DP at a concentration of 36 mM. Higher concentrations appear to be lethal.

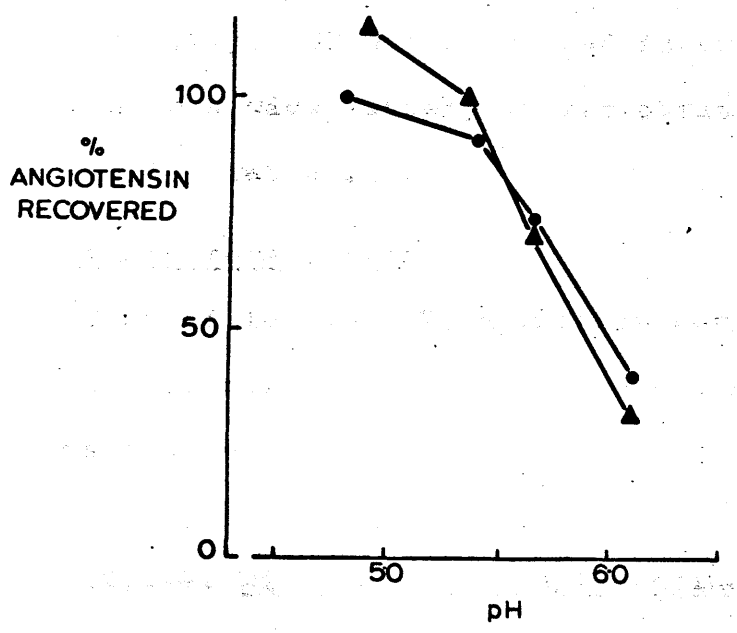


Fig.8. Effect of pH on the recovery of angiotensin I (●) and II (▲) after 24 hours, from eel plasma (20%) with DP as angiotensinase inhibitor. Each point represents the mean of duplicate assays.

For example, ten doses, each of 0.1 ml of 120 mM DP administered to a rat at intervals over a period of one hour resulted in its death. As will be described (2.2.3), DP has been employed with success in the measurement of the renin concentrations in eel renin extracts, and also eel plasma renin-substrate (section 2.2.4.2.). DP was also used in studies of kidney renin in a wide variety of vertebrates (section 4.2.1., Table 12).

VI. Other inhibitors tested

As indicated in Table 7, a further series of potential angiotensinase inhibitors were tested and found to be ineffective.

2.2.2.4. Discussion of angiotensinase inhibition.

The major conclusions of this study were firstly that different species and tissues contain different angiotensinases which are not inhibited by a single antagonist and secondly, that the effectiveness of a given inhibitor varies markedly with pH.

I. Renal extracts

DP was of the most general use in the inhibition of angiotensinase activity in renal extracts. The efficacy of DP varied with pH, being most effective

TABLE 7. SUBSTANCES THAT FAILED TO INHIBIT ANGIOTENSINASE

Agent	Concentration	pH	Kidney extracts: Dog Q	Dog R	Dog C	Man Q	Man R	Man C	Eel Q	Eel R	Eel C	Plasma: Ox D	Ox R	Ox C	Man D	Man R	Man C
Amino Acids																	
EACA	3 & 9 mM	5.7	10	<20	<20	10	<20	<20				25	<20	<20	25	<20	<20
L-arginine	3 & 9 mM	5.7	10	<20	<20	10	<20	<20				25	<20	<20	25	<20	<20
SBTI*	0.06% W.V	5.7	10	<20	<20	10	<20	<20				25	<20	<20	25	<20	<20
Trasylol*	125 i.u./ ml.	5.7	10	<20	<20	10	<20	<20				25	<20	<20	25	<20	<20
Flourides																	
Sodium fluoride	1, 2.5, 6.25 mM	5.7										25	<20	<20	25	<20	<20
Hydrogen fluoride	13.5 mM	5.7							0.7 0.2	<20	<20						
MSF	.025, .125, 1.25 % W.V	5.7							0.5	<20	<20						

Recoveries of angiotensin II from samples (R) and controls without agent (C) were assayed after 2 hours. Q = mg. Kidney/ml; D = % plasma dilution, EACA = ε amino caproic acid; SBTI = soya bean trypsin inhibitor; MSF = methane sulphonyl fluoride.

* See Haberland (1967).

for eel renin extracts at around pH3. In many circumatances, it was less effective than DFP but, since DFP is extremely toxic to the rat and man, DP was adopted as renal angiotensinase inhibitor at pH below 6 for further studies (sections 2.2.3., and 4.2.). DP failed to protect angiotensin in incubations of human and canine tissue and in these cases phenanthroline and bipyridine respectively were more effective. These compounds have previously been employed to prevent hydrolysis of bradykinin by plasma peptidases (Erdos, Renfrew, Sloane and Wohler, 1963) but not, as far as the author is aware, for the inhibition of angiotensinase activity.

II. Plasma angiotensinase

Angiotensinase activity in plasma also contained more than one component. Plasma from a majority of species contains an EDTA-sensitive fraction. Inhibition of the remaining activity required the addition of a second inhibitor. Although this varied in different species, phenanthroline mixtures were used in a new technique for measurement of plasma renin substrate in man (2.2.4.1 & 3.2.2). To the authors knowledge this combination has not been used previously. Ryan et al (1968) used BAL and EDTA with rabbit plasma but studies described above show a lack of effect of

this combination in human and bovine plasma. Hydrogen ion concentration again influenced the action of the EDTA-phenanthroline combination, particularly in cod, eel and human plasma. DP inhibited angiotensinase hydrolysis of angiotensin in eel plasma incubations at pH levels below 6. DP was therefore used in the measurement of renin-substrate in the eel (2.2.4.1.)

2.2.3. Measurement of Renal Renin

As discussed in section 2.1., concentration of renin in extracts of kidney tissues may be sufficiently high for a direct bioassay. The feasibility of applying this to a wide variety of vertebrate species was briefly studied. For reasons discussed below, this approach was not satisfactory for the assay of eel renal renin and effort was therefore concentrated on developing an enzyme kinetic technique (see also section 2.1.2.1. III). Factors affecting the reaction of eel renin with its substrate were studied in some detail and the results are described and discussed below.

2.2.3.1. Direct bioassay of renal renin

I. Pressor responses to untreated extracts

Renal extracts from 39 vertebrate species (see Table 12) were prepared as described in section 2.2.1. and tested on at least two occasions each by intravenous injection into the rat. Where indicated, blood pressure responses were typical of these described for mammalian renin, and examples are illustrated in figures 9a and 9b. However, in amphibia, fish, reptiles and to a lesser extent, birds, the response was of unusually short duration (Figs. 10,11,12).

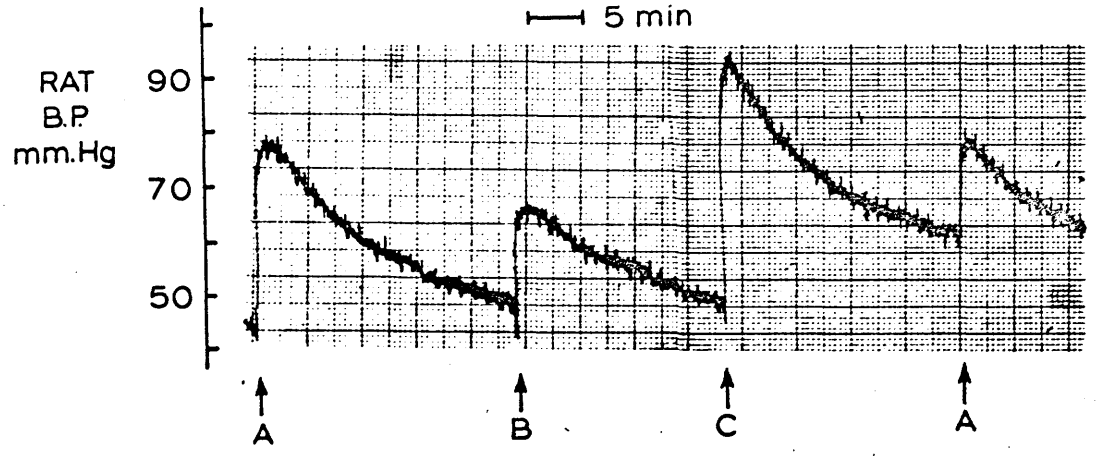


Fig.9a. Blood pressure responses to mammalian renins. The kidney extracts are A = Rat (2 mg), B = Aardvark (20 mg), C = Porpoise (10 mg).

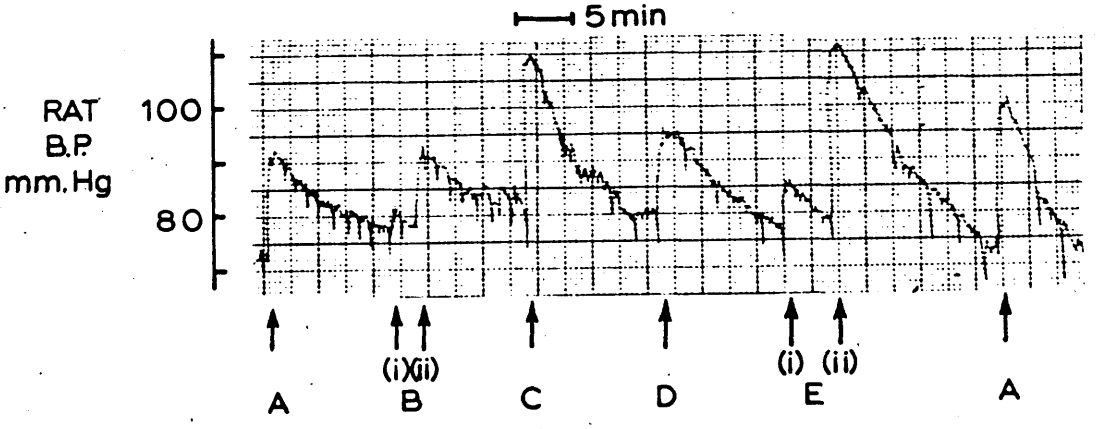


Fig.9b. Blood pressure responses to mammalian renins. The kidney extracts are A = Rat (2 mg), B = Dog ((i) = 2 mg, (ii) = 20 mg), C = Polar Bear (2 mg), D = Crab-eating Mongoose (2 mg), E = Binturong ((i) = 2 mg, (ii) = 20 mg).

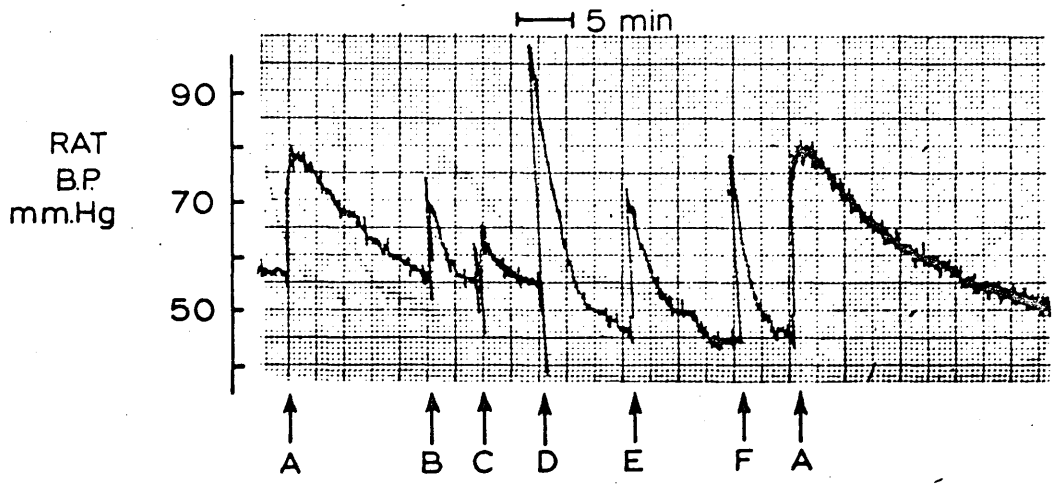


Fig.10. The vasopressive effects of fish and amphibian kidney extracts, compared to rat renin (A = 2 mg kidney), B = Cod (40 mg), C = Sea trout (40 mg), D = Mackerel (20 mg), E = European Eel (40 mg), F = Frog (20 mg).

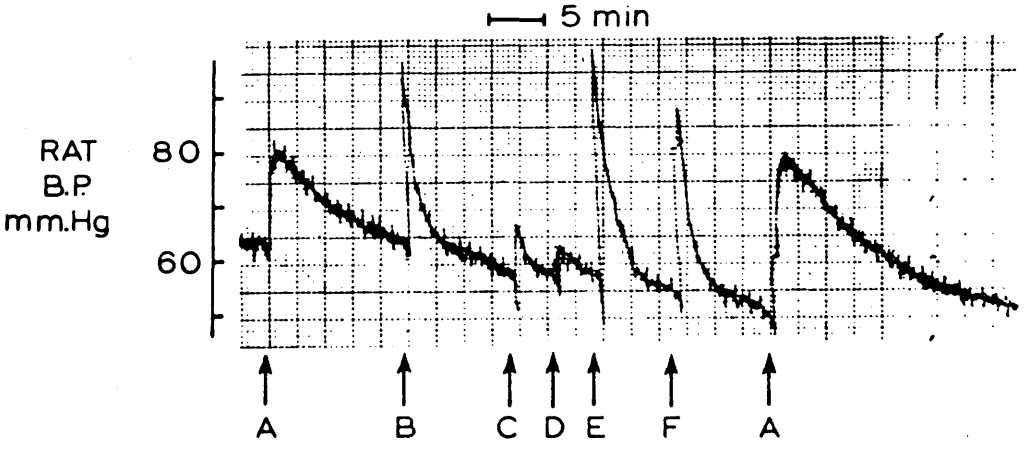


Fig.11. The vasopressive effect of reptile and amphibian kidney extracts, compared to rat renin (A = 2 mg), B = Frog (20 mg), C = Radiated tortoise (40 mg), D = Porters Giant Tortoise (40 mg), E = Russell's Viper (40 mg), F = Blue Spiny Lizard (40 mg)

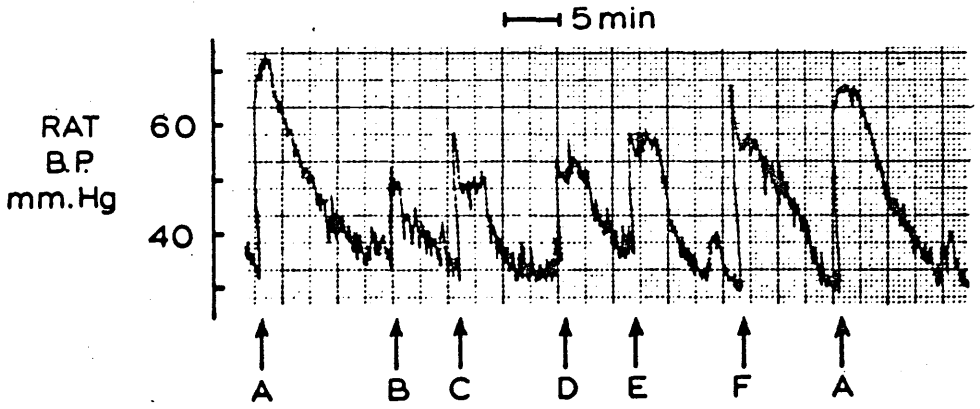


Fig.12. The vasopressive effects of avian kidney extracts, compared to rat renin (A = 2 mg), B = Semi-palmated Goose (40 mg), C = Rhea (20 mg), D = African Crowned Crane (40 mg), E = Humboldts Penguin (40 mg), F = Peafowl (40 mg).

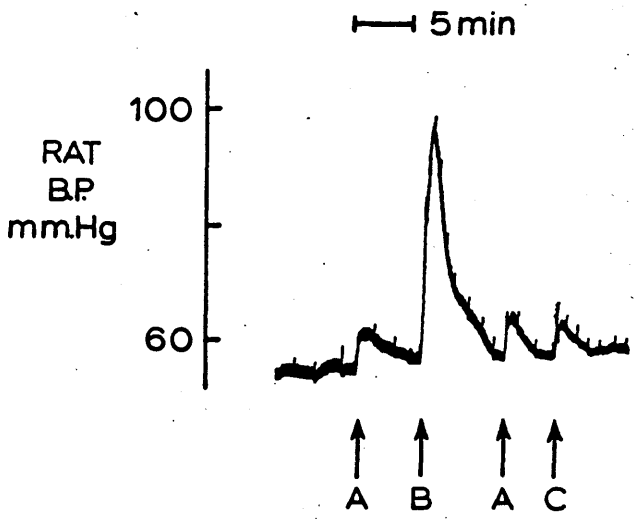


Fig.13. Response of rat blood pressure to i.v. injections of 7 mg cellophane diffused eel kidney extract (A), to 7 mg of same extract, non-diffused (B) and to 0.9 mg of this non-diffused extract (C).

One possible explanation for this was that the extract was contaminated with a short-acting pressor substance in addition to renin. The nature of this contamination was investigated using eel renal extracts.

II. A diffusible pressor substance in extracts of eel kidney

The effect on the rat blood pressure on intravenous injections of eel renal extracts before and after dialysis is shown in figure 13 clearly the short acting pressor substance was diffusible through cellophane membranes (Visking tubing). Incubating the extract at 80^{o} for 30 minutes did not appreciably affect its pressor response (Fig 15). Because, when applying the enzyme kinetic technique (as in 2.2.3.2.) eel renin was shown to be denatured by heat and did not diffuse through cellophane, it is unlikely that the quick-acting response is attributable to renin. The possibility that it was due to angiotensin was considered. Although angiotensin is diffusible and heat-stable it would be unlikely to survive in crude extracts of kidney with high angiotensinase content. Furthermore, comparison of the effects of eel renal extracts and angiotensin II on rat blood pressure (Fig 14) showed a different time-course of response.

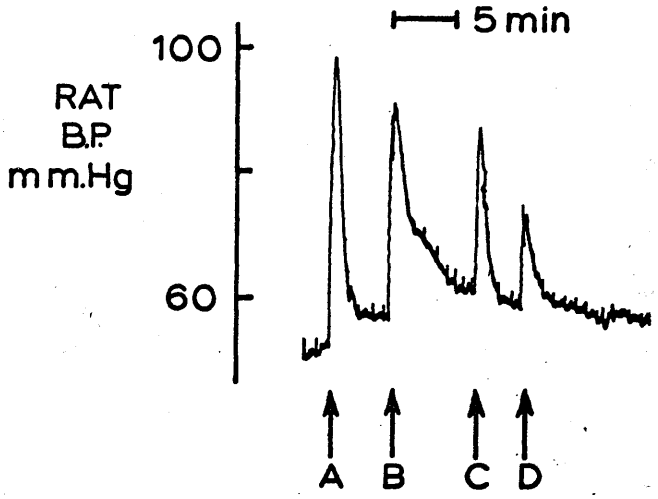


Fig.14. Rat blood pressure response to angiotensin II (A = 8 ng, C = 4 ng) and eel kidney extract (B = 7 mg, D = 3 mg).

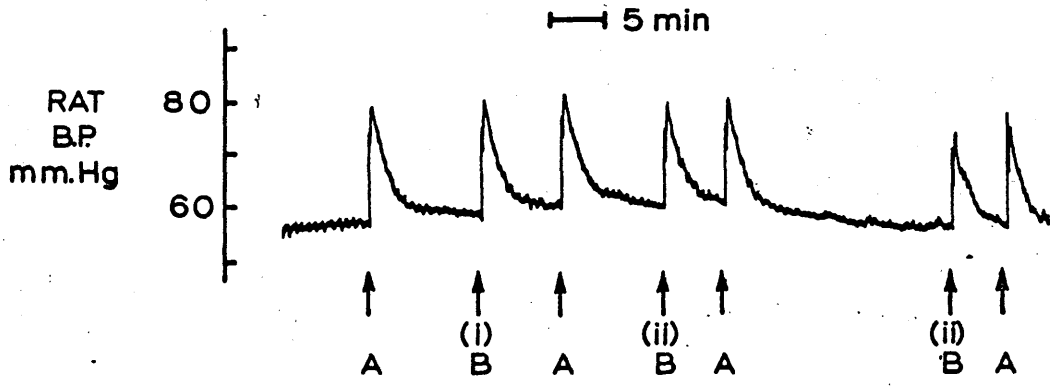


Fig.15. Effect of high temperature on the vasopressive substance in non-diffused eel kidney extracts. A = 4.3 mg of unheated extract, B (I), (II), (III) = 4.3 mg of extract heated at 80°C for 2, 10 and 30 minutes respectively.

Because adrenal tissue is present within the kidney of the eel it seems probable that catecholamines were largely responsible for the pressor effect of the extracts (see discussion 2.2.3.5.). When these are removed by dialysis the amount of renin remaining was too small to be assayable by direct injection in the rat. A more sensitive enzyme kinetic method was developed for this reason.

2.2.3.2. Enzyme kinetic estimation of renal renin in the eel

I. Conditions of incubation

(1) Diisopropyl phosphite ..... 15 µl

(2) pH 6.2 phosphate buffer .....to 450 µl

(3) Ox substrate (see Methods 2.2.1) dialysed to pH 6.2 ..... 2 ml

(4) Eel renin (10% dilution of original extract)..50 µl

The total volume of the mixture was 2.5 ml and its pH lay between 5.6 and 5.7. The mixture was then incubated at 37° for 4 hours. Aliquots were removed at 2 and 4 hours and instantly frozen in a 'dry ice'/acetone mixture before storing at -20° until bioassay.

II. Calibration curve

A calibration curve was made by incubating

different quantities of standard eel kidney renin with a constant amount of substrate in the presence of DP. The relation between the velocity of angiotensin formation and the concentration of standard eel renin is shown in Figure 16. It was also necessary to show that the calibration curve obtained could be used to measure renin in other extracts of eel kidney tissue. This would not have been possible, if such extracts contained inhibitors or accelerators of the renin-substrate reaction in addition to renin. In such an event a serial dilution curve for the extract would not have been parallel with the standard renin preparation and use of the calibration curve to measure renin in the extract would have been invalid.

To test this possibility serial dilution curves were made from 4 extracts of different eel kidneys. As can be seen in Figure 16 these followed the same slope as the calibration curve with standard renin.

III. Expression of results.

Renal renin concentration was derived from the rate of angiotensin formation by reference to the calibration curve (Fig. 16). For the purpose of this study only, one unit of standard eel renin was

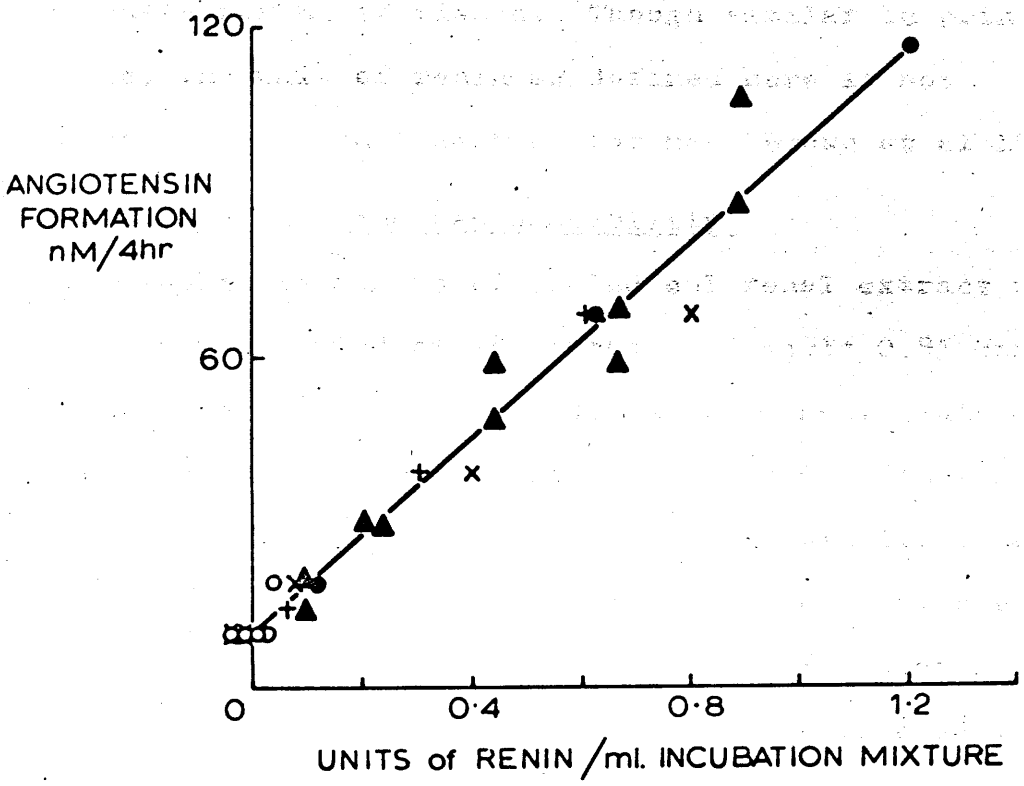


Fig.16. Calibration of eel renin concentration
The quantity of renin is expressed as units/mg of kidney tissue; 1 ml of inbucation mixture contains 0.4 mg. ▲ = standard eel kidney renin pool (see materials section 2.2.1). X, +, ●, O = four individual eel kidney extracts. Ⓧ = Blank incubation mixture (buffer replacing kidney extract).

arbitrarily defined as the amount which produced angiotensin at a rate of 97 nM (100 ng/ml) of incubation mixture per 4 hours of incubation. The concentrations of renin in the extract are expressed as units per mg of tissue. Though similar in principle, the unit of renin as defined here is not identical with that defined for man (Brown et al 1964).

IV. Precision and reproducibility

Replicate assays of pooled eel renal extract were made over a period of 18 months. A result 0.85 units of renin/mg $\pm$ 0.08 SD (n = 9) was obtained. This gives a coefficient of variation of 9.4%. Studies of the recovery of eel renin were not possible because no angiotensinase-free preparations were available. However, there was no significant deterioration in the eel renal preparation over the 18 months of study.

V. Suitability of bioassay standard

Table 8 shows the results of a comparison of angiotensins I and II as standards for bioassay of eel renin-ox substrate incubation product using a pooled incubated material. There was no significant difference ($p = 0.3$ to 0.4) between the results obtained using these two standards. As will be dis-

Table 8. Eel kidney renin method:

validation of bioassay standard

Angiotensin standard	No. of Assays	Sample Mean μM	SD μM	SE μM	Students t test
I (0.97μM)	11	1.769	0.108	0.033	t=0.964 p=0.4 to 0.3
II (0.97 μM	11	1.824	0.156	0.047	

cussed, this was not the case in the method used to measure renin-substrate in human plasma (2.2.4.1).

2.2.3.3. Factors affecting the eel renin-ox substrate reaction

I. Hydrogen ion concentration

The yield of angiotensin after 4 hours of incubation at 37° was studied over a range of pH values. As can be seen from Figure 17 maximum yields were obtained at pH 5.6 which was therefore assumed to be the optimum pH for this reaction under these conditions. When the time course of the reaction over the same range of pH levels was examined (see Fig.18) some evidence of denaturation was revealed at a pH of 6.6 or above. The phenomenon was studied in more detail (see Fig.19) using the following experimental protocol.

Kidney extracts were made as described above but in a range of phosphate buffer solutions (pH 4.6-8.5). They were then dialysed against the appropriate buffer (using 3 changes, each of 5L) of the same pH for 24 hours. The pH was adjusted to 5.6 and the different pH treated extracts incubated as before with substrate at pH 5.6. The results confirm the destruction of eel renal renin at pH

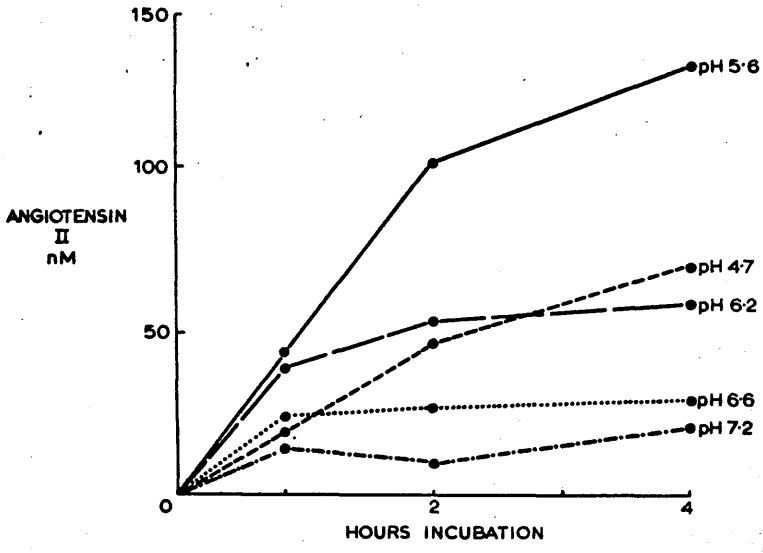


Fig.17. Effect of pH on eel renin-ox substrate reaction. The incubation was carried out at 37°. Each point represents the mean of duplicate assays.

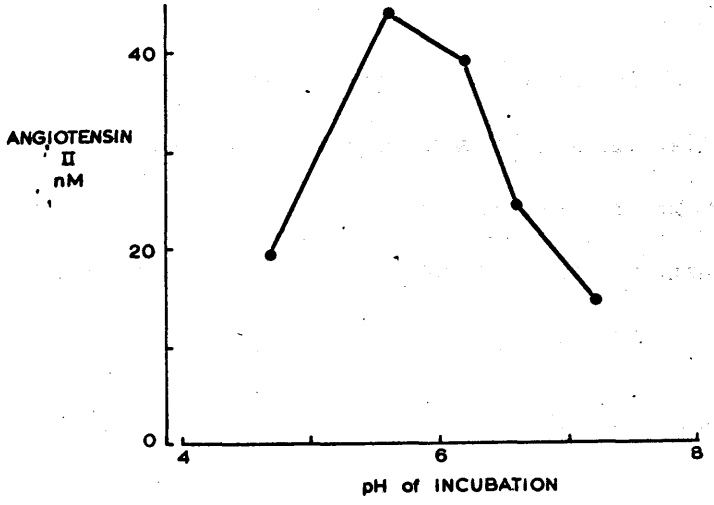


Fig.18. Optimal pH for the reaction of eel renin with ox substrate. This graph has been plotted from the data on fig.17. The angiotensin concentrations at ¾ hour of incubation were used. Each point represents the mean of duplicated assays.

levels in excess of 6.2 (Fig.19).

II. Temperature

Incubations were carried out at a range of temperatures varying from 4°-60°. The results are illustrated in figure 20. Two conclusions may be drawn from these data. Firstly, initial velocity increases with temperature to a maximum at 50°, thereafter decreasing presumably due to thermal denaturation of the enzyme. Secondly, over a 4-hour incubation the highest mean velocity, giving the greatest total yield of angiotensin, occurred at 37° although greater yields (0.58 μM) could be obtained over very long incubation periods (6 days) at the slightly lower temperature of 31°. It must therefore be assumed that some thermal denaturation occurs even at 37°. As will be described in section 2.2.4.2. thermal denaturation of eel renin-substrate may also occur at temperatures less than 37°.

III. Time course of eel renin-ox substrate reaction

Over an extended period of time, the rate of an enzyme-catalysed reaction in vitro under constant conditions of pH, temperature etc., falls progressively with time. This can be due to several factors

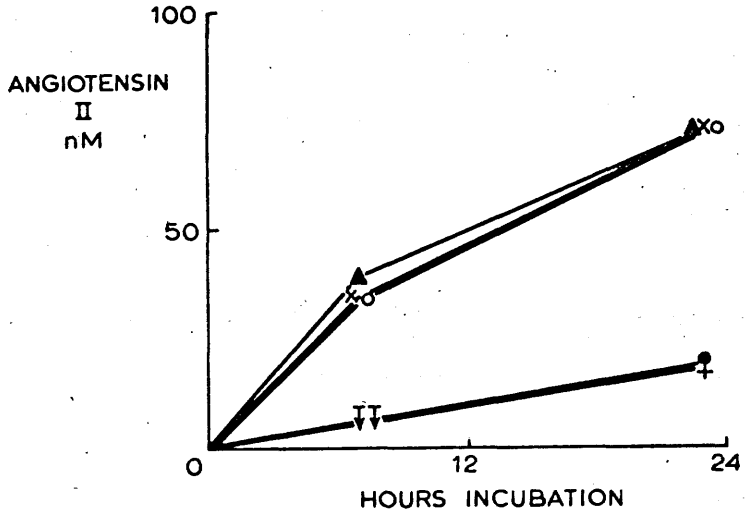


Fig.19. The denaturation of eel renin at pH levels above 6.2. The eel renin has been incubated here with ox substrate at pH 5.7, after the kidney had been minced then diluted and subsequently cellophane-diffused over the following pH range: ▲ at 4.6, X at 5.7, O at 6.2, ● at 6.9, + at 8.5. Each point represents the mean of duplicate assays.

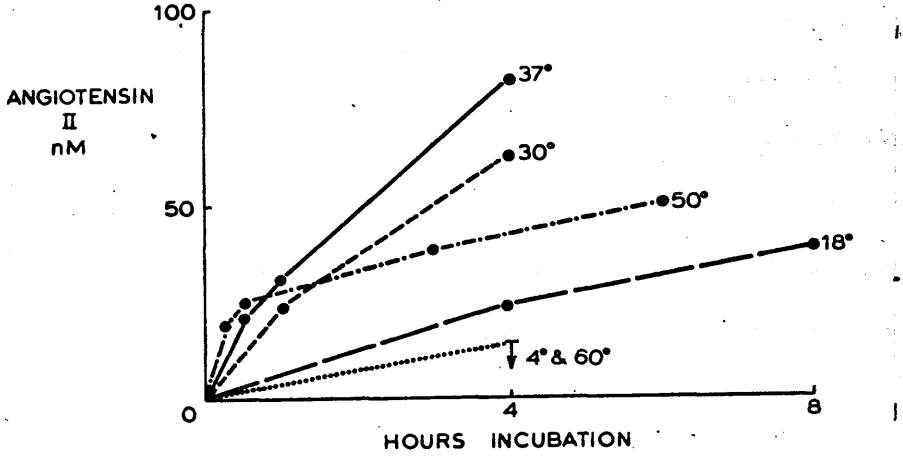


Fig.20. Effect of temperature on the reaction of eel renin with ox substrate. The incubation was carried out at pH 5.7. Each point represents the mean of duplicate assays.

of which the most common are progressive denaturation of the enzyme, approach to and arrival at a state of equilibrium for the reaction, depletion of substrate and release of inhibiting compounds during the reaction. For the purposes of estimating renin concentrations, it is important that the initial velocity is maintained for as long a time as possible and this can be ensured in most cases by using a large excess of substrate.

The time course of the eel renin-ox substrate reaction was therefore compared with that of human renal and human plasma renins with the same substrate. The rate of hydrolysis of ox renin substrate by different quantities of human renal renin is relatively constant (Fig 21) over long periods (Brown et al, 1964). A similar situation exists with human renin extracted from plasma. Figures 22 and 23 however reveal that the hydrolysis of ox substrate by eel renin does not proceed at a constant rate whether incubated at 20, 30 or 37°C. Nevertheless, the rate of angiotensin formation was sufficiently constant to produce an adequate calibration curve. Four other reasons for inconstant velocity of angiotensin formation were considered.

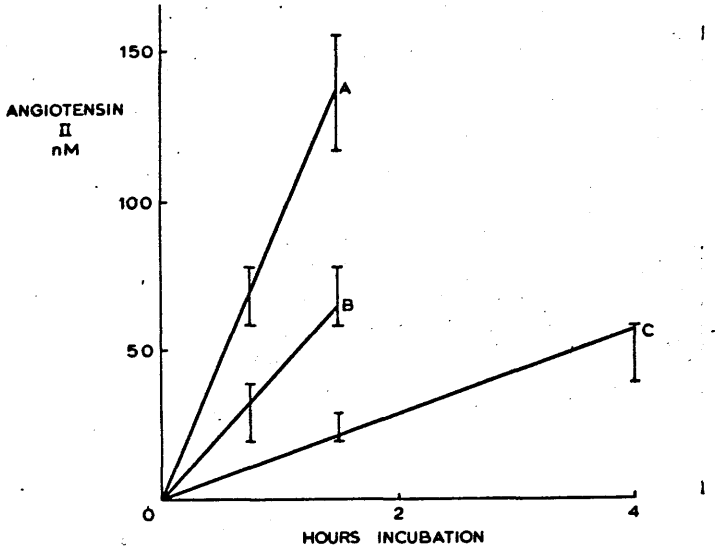


Fig.21. Reaction of three different concentrations of human kidney renin (A = 1 units/ml., B_o=.5 units/ml C =.25 units/ml) with ox substrate at 37°C. Assay brackets are derived using the rat blood pressure preparation.

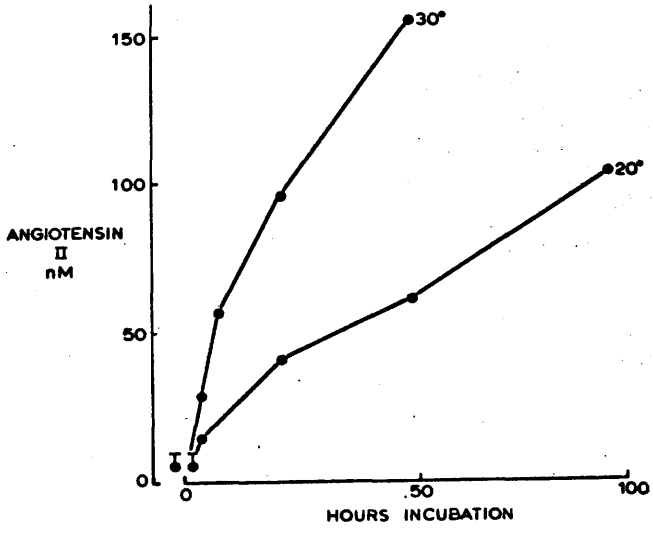


Fig.22. Reaction of eel renin with ox substrate at 20° and 30°C. Each point represents the mean of three assays.

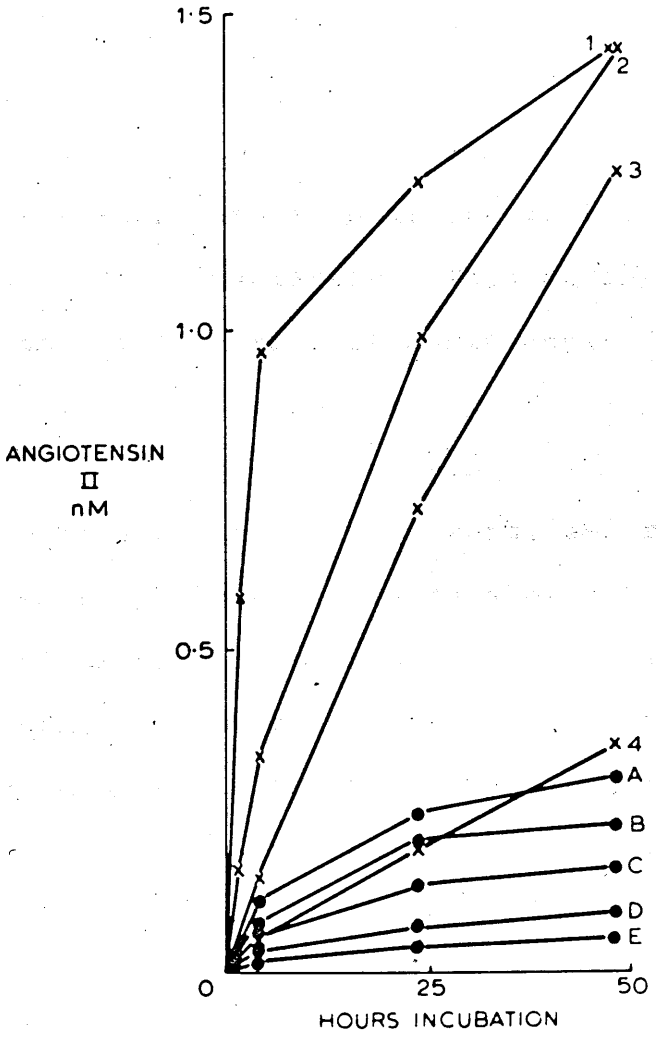


Fig.23. Reaction of human (X) and eel (●) renins with ox substrate. For human renin (Brown et al 1964), 1 = 1 human unit/ml; 2 = 0.2u/ml; 3 = 0.1 u/ml, 4 = 0.02 u/ml. For eel renin, A = 0.92 eel units/ml; B = 0.69 u/ml; C = 0.46 u/ml; D = 0.23 u/ml; E = 0.092 u/ml. Each point represents the mean of duplicate assays.

(i) Effectiveness of DP as an angiotensinase inhibitor

Recoveries of angiotensin II (485 nM) from eel renin-ox substrate incubations in two experiments were 80 and 100% respectively. This strongly suggests that the falling rate of angiotensin formation is not due to angiotensinase activity.

(ii) Presence of pepsin

Unlike human and dog renin, eel renin is inactivated at pH levels in excess of 6.2 (see above). Pepsin shares this characteristic. Because pepsin produces, on incubation with plasma, a peptide indistinguishable chemically and pharmacologically from angiotensin (Croxatto and Croxatto 1942) the presence of pepsin in the eel renal extracts might result not only in a spuriously increased rate of angiotensin formation but also in the subsequent hydrolysis of the octapeptide. These phenomena might account for the failure of the reaction to proceed at a constant rate.

Pepsin, but not renin, is inhibited by poly-L-lysine, (Lumbers and Skinner 1969). The presence of pepsin was, therefore, tested by incubating eel renal extract and ox substrate in the presence of this inhibitor (0.5 mg/ml). The results illustrated in

Figure 24, show that poly-L-lysine did not affect the time course of the reaction. Contamination of the renal extracts by pepsin is not an important factor. Also pepsin would be expected to degrade the angiotensin (see Table 1), but this did not occur.

(iii) Mixed nature of ox substrate preparation

Ox renin substrate preparations may contain several substrates (see Skeggs et al 1964) and these may vary in their affinity for eel renin. Thus, although an apparent excess of total substrate is used, only a small proportion may react with the enzyme and substrate concentration would then be limiting. To test this possibility the following observations were made.

The yield of angiotensin from the ox renin substrate preparation using eel and human renal extracts was compared (Fig. 25). On prolonged incubation, eel renin produced only about one fifth of the angiotensin released by human renal extract. Moreover, when human extract was added to the eel renin incubation, after angiotensin concentration had reached a plateau, angiotensin release recommenced until the final concentration approximated

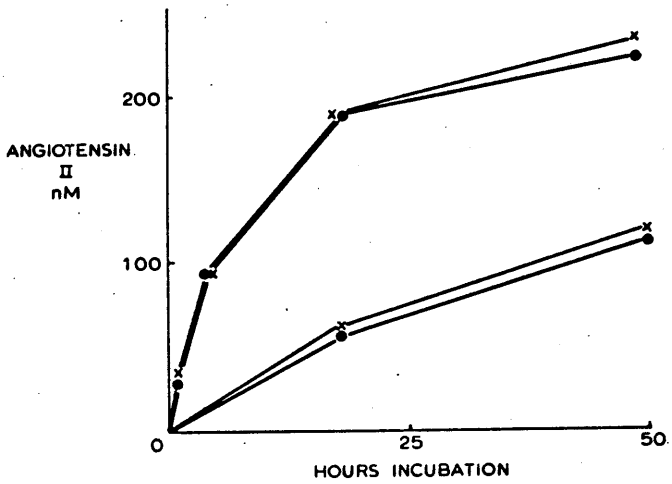


Fig.24. Effect of poly-L-lysine on the eel renin-ox substrate reaction. Incubation at 37°C with (X) and without (●) poly-L-lysine were compared at two concentrations of eel renin (0.92 units/ml for upper and 0.23 units/ml for lower pair of curves.

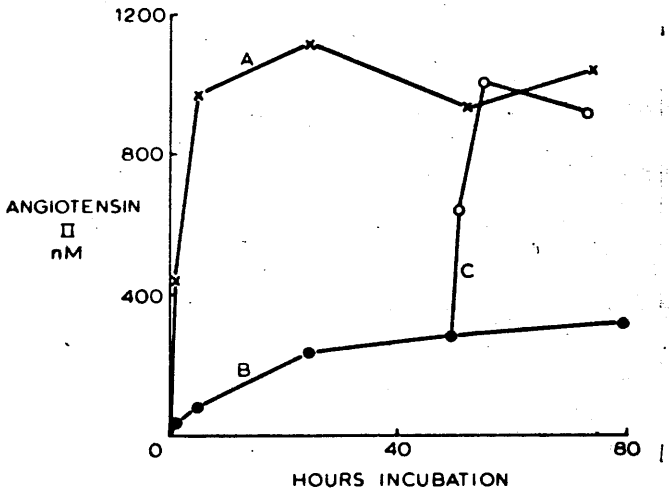


Fig.25. Reaction of high concentrations of eel and human renin with ox substrate. Curve A was produced by a high concentration (1 u/ml) of human renin, curve B by the highest available concentration of eel renin (0.83 u/ml). At point C, when the eel kidney renin - ox substrate reaction was approaching a plateau, human renin (1 u/ml), was added to an aliquot of tube B. Each set of tubes were run in duplicate.

to that obtained with human extract alone. Thus, not all the substrate appears to be available to eel renin-catalysed hydrolysis.

This may not, however, be the explanation for the falling reaction rate. According to Dixon and Webb (1967), an enzyme catalysed reaction should proceed at a constant rate at least until 20% of the substrate is consumed. Since eel renin is capable of releasing 0.58 μM of angiotensin from this substrate, the reaction should proceed at a constant rate until a concentration 20% of this (i.e. 0.116μM) is attained. However this is not the case.

It must therefore be concluded that a heterogenous substrate preparation is not the cause for the progressive fall in the rate of the reaction time.

(iv)Inhibitors

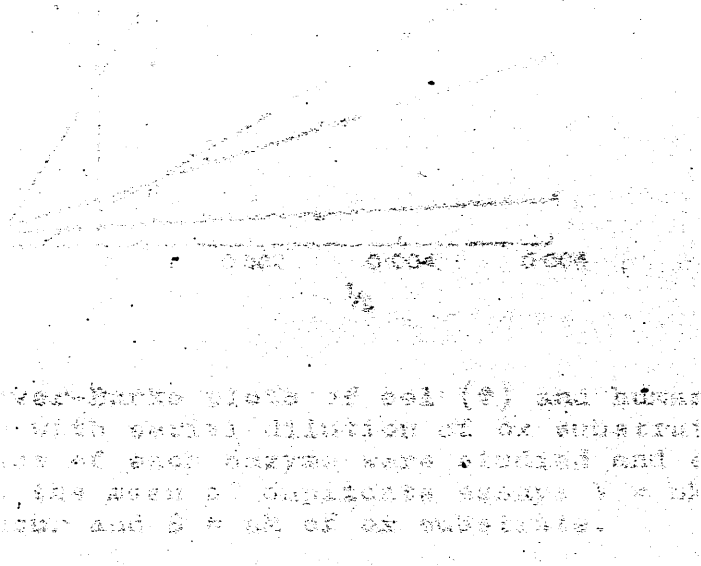
One type of renin inhibitor already studied by other workers is a phospholipid. Phospholipid inhibitors of the type described in section 2.1.1.9 are destroyed by the action of phospholipases. Eel renal extract and ox substrate were incubated in the presence and absence of phospholipases C and D (0.1 mg/ml). The results indicated that neither phospholipase improved the yield of angiotensin.

Phospholipase D had no effect while C reduced the yield still further below the control. The reason for this decrease is not clear.

The inhibitor if any, is therefore probably not a phospholipid. The study was not pursued beyond this point so that other types of inhibitor cannot be excluded.

IV Enzyme kinetics.

As can be seen from Fig. 26, Lineweaver-Burke plots for human and eel kidney renin were linear with serial dilution of ox substrate.



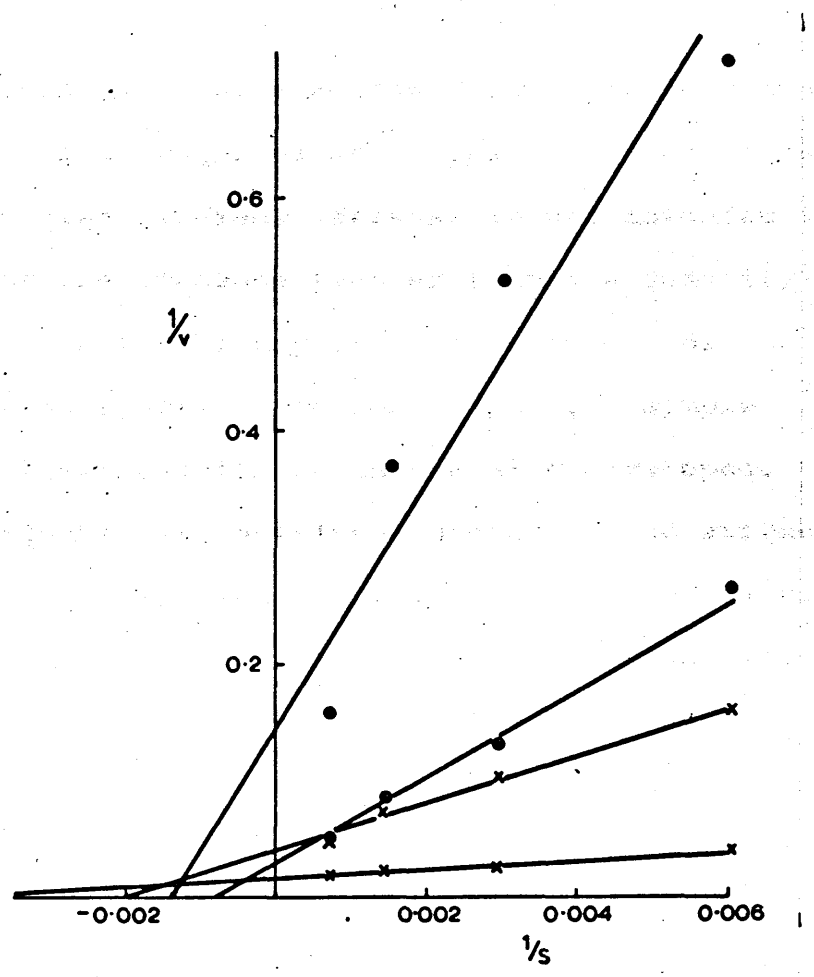


Fig.26. Lineweaver-Burke plots of eel (●) and human kidney (X) renin with serial dilution of ox substrate Two concentrations of each enzyme were studied and each point represents the mean of duplicate assays V = nM angiotensin II/hour and S = nM of ox substrate.

2.2.3.4. A Qualitative test for renin in extracts of kidney tissue.

Development of a method for measuring renin in kidney tissue requires an extensive series of tests for each species studied. Because it was intended to test for the presence (rather than the quantity) of renin in the kidney of a large number of vertebrate species (section 4.2.1.), a simple qualitative test for renin was also developed.

Kidneys were obtained, processed and extracted as described in section 2.2.1.2. The extracts were tested for angiotensinase (see section 2.2.2.), and incubated at 37° with ox substrate and DP as described earlier in this section. Aliquots of the incubation mixture were taken at 4 and 16 hours and angiotensin was determined by bioassay. The results are expressed in Table 12 as the amount of angiotensin formed after 16 hours incubation.

2.2.3.5. Discussion

I. Direct Bioassay

Direct bioassay of eel renal renin by intravenous injection into the rat was found to be of no practical value. Renal extracts were heavily contaminated with another pressor compound which was thermostable and which diffused through cellophane. Since the eel kidney contains chromaffin tissue, this substance is almost certainly catecholamine. That this is so is suggested by the fact that the pressor effect of renal extracts in teleost fish can be obliterated by the α adrenergic blocking agent phenoxybenzamine hydrochloride (Mizogami, Oguri, Sokabe and Nishimura 1968).

When this diffusible component had been removed, the residual pressor response, presumably due to renin, was too small to be measured reliably by direct assay. This could be due either to the lack of renin in the eel kidney or to a lack of sensitivity of the rat to renin from this species. The former possibility is not borne out by subsequent studies (see below) and a likely explanation may be that eel renin does not react with

renin substrate circulating in the plasma of the test rat. These studies indicated the need for more sensitive methods.

II. Enzyme-Kinetic method

The greater sensitivity of the enzyme-kinetic technique has been established in studies of mammalian renin (Lever et al 1964, Brown et al 1964 a and b). The improvement to sensitivity is achieved by the greater quantity of angiotensin which is formed when renin is incubated in vitro with substrate over a prolonged period. As can be seen from the preceding experiments, when eel renal extracts and ox substrate were incubated for long periods, the reaction rate decreased with time. The mechanism of this effect was not discovered. It was not due to thermal denaturation; to reappearance of angiotensinase activity, or to pepsin. An insufficiency of renin substrate was also an unlikely explanation.

For practical purposes as indicated in Fig. 23 the enzyme-kinetic assay was not compromised by the failure to maintain constant velocity of angiotensin formation over a prolonged period.

The amount of renin present in all extracts was sufficient to produce a measurable quantity of angiotensin. It was also shown that dilution of renin-containing extracts produced a decrease of velocity comparable with that resulting from dilution of standard renin (Fig 16). Renin present in the extract could, therefore, be referred to the renin content of the standard.

III. Detection of renin in extracts of kidney tissue

A simple method for detection of renin was tested in a wide variety of vertebrate kidney extracts. The positive results usually found (see Table 12) indicates that renin is present in a particular extract. In these species there should be no difficulty in establishing quantitative methods along the lines described for eel renin (see above).

Negative results (angiotensin content less than 19 nM at 16 hours) should be interpreted with caution. Failure to produce angiotensin could result from absence of renin in kidney tissue, from a failure to extract renin from kidney tissue, from inactivation of extracted renin or from failure of the particular species of renin to react with

ox substrate. For these and other reasons, no conclusion can be drawn as to the amount of renin present in the kidney of a particular species.

2.2.4. Measurement of plasma renin-substrate

The need for a properly validated technique for renin-substrate measurement in man has been mentioned (2.1.2.2) and defects in techniques currently available for man will be discussed (2.2.4.1).

Two methods only (Mizogami et al, 1968; Nolly & Fasciolo, 1971 a & 1972) are available for substrate measurement in fish and these are based on the techniques used in man.

In this part of the thesis two new methods for plasma renin-substrate measurement are described: first a technique for man (2.2.4.1) and then one for the eel (2.2.4.2). The method for man has been submitted for publication (Tree 1972) and the results obtained using the techniques have been reported (Weir et al 1970 a & b; Weir et al 1971; Dusterdieck et al 1970; Love et al 1971; Brown et al 1971; Robertson et al 1971; Medina et al 1972).

2.2.4.1. Measurement of plasma renin-substrate in man

As in other methods for measuring renin-substrate, the present technique is based on the conversion of substrate to angiotensin by an excess quantity of renin, followed by assay of the angiotensin formed.

I. Materials and Methods

The methods of preparation of human kidney renin, human renin-substrate, angiotensin standards and phosphate-saline buffer solutions are to be found in section 2.2.1.

(i) Angiotensinase inhibitor solution (AIS).

This contained 1.56 mM phenanthroline and 16.8 mM disodium EDTA, and was prepared in pH 7.4 buffer (8.6 mM NaH_2PO_4, 41.4 mM Na_2HPO_4, 81.6 mM NaCl) with 2g/l neomycin sulphate. Its total concentration at 0.15 M made it isosmotic to human blood (see ensuing method (ii) on sample collection). This AIS was stored at $+4^{\circ}C$.

(ii) Preparation of the plasma sample

Peripheral venous blood was taken in a plastic syringe and 6.25 ml was transferred immediately to a 25 ml volumetric flask containing 18.75 ml of AIS. Packed cell volume was measured on the remainder using the microhaematocrit technique (Hawksley). Blood and

AIS were throoughly mixed, centrifuged at 1400 g and the supernatant plasma-AIS mixture was removed and stored frozen at -20°C until assay.

(iii) Incubation and assay

2 ml of the thawed plasma-AIS mixture was added to 0.5 ml of human renin solution. The mixture, which had a pH of 6.8 was incubated at 37° for 24 hours. Aliquots of the mixture were taken after 8 and 24 hours incubation and stored frozen until assayed using the anaesthetised rat blood pressure preparation (Peart 1955) and angiotensin I (0.1 μM in AIS) as the standard. The mid-point of each assay bracket was taken as its value and the mean of the 8 and 24-hour aliquots was used to calculate substrate concentration (see later). Where brackets for the two aliquots did not overlap, the sample was reincubated and reassayed and the result accepted only when the brackets overlapped.

Of 1075 samples so far tested, 1059 (98%) were satisfactory on the first incubation and assay and 9 of the 16 failures were acceptable on re-incubation. The remaining 7 samples, less than one per cent of the total, were rejected.

(iv) Calculation and expression of plasma renin-substrate concentration.

Allowing for the dilution of blood with AIS, for the dilution of the plasma-AIS mixture with renin solution and for the molarity of the angiotensin standard, the substrate concentration of the original plasma samples was derived from the expression -

$$A \times \frac{(25 - B)}{(6.25 - B)} \times 12.5\ \mu M$$

Where A = the ratio $\frac{\text{Angiotensin concentration in unknown}}{\text{Angiotensin concentration of standard}}$

and B = the volume of red blood cells in the 6.25 ml blood sample.

Substrate concentration was expressed in molarity rather than as a "concentration" equivalent to the standard angiotensin as in the other methods listed in Table 9. Not only is molarity a more precise means of expressing the concentration of a solute but it enables a comparison to be made of data obtained using different forms of angiotensin as a standard for the bioassay. The assumption

TABLE 9

Author	Mean of normal range μM	Standard used	Plasma or serum
Present Method	1.01 (young men) 0.85 (young women)	Angiotensin I	Plasma
Skinner 1967	1.07	Angiotensin II	Plasma
Maebashi et al 1965	0.87	Probably Angiotensin II	Plasma
Gould and Green 1971	0.76 (young men) 0.70 (young women) 0.92 (older women)	Angiotensin II	Serum
Pickens et al 1965	0.70	Angiotensin II	Plasma
Rossett and Veyrat 1971	0.57 (young men)	Angiotensin II	Plasma
Helmer 1965	0.41	Probably Angiotensin II	Plasma
Ayers 1967	0.33	Probably Angiotensin II	Plasma
Imai and Sokabe 1968	0.29	Probably Angiotensin II	Plasma

Mean values of plasma renin-substrate in normal subjects. For the purpose of comparison with the present method, substrate values have been re-calculated and expressed in terms of molarity.

implicit in all method for measuring renin-substrate is that 1 Mole of substrate is converted by excess renin in the absence of angiotensinase to 1 Mole of angiotensin.

(v) Characterisation of the angiotensin formed during incubation.

The pressor material produced by incubation of renin and substrate in the presence of angiotensinase inhibitor solution was characterised in three ways:

(a) By radioimmunoassay for angiotensin I using an antiserum with a 0.016% cross-reaction against isoleucine 5 angiotensin II (Waite 1972).

(b) By radioimmunoassay for angiotensin II using an antiserum with a 0.6% cross-reaction against isoleucyl-5 angiotensin I (Dusterdieck and McElwee 1971)

(c) By chromatography on a CM-Sephadex (Pharmacia) column capable of separating completely angiotensin I from angiotensin II and with comparable recovery for the two peptides (Leckie et al 1972).

II. Results .

Assay of renin-substrate requires that the substrate is converted completely to angiotensin by

renin, that the angiotensin formed is not subsequently destroyed and that it is measured by comparison with a valid standard.

(i) Nature of the angiotensin formed

Using antisera with negligible cross-reactivity (see materials and methods), radioimmunoassay of the incubation product indicated that most if not all of the pressor material was in the form of angiotensin I (Table 10). Similarly, chromatography of the material on a system capable of separating the two forms of angiotensin showed that a high proportion of the starting material eluted as angiotensin I with little activity detectable in fractions which would contain angiotensin II (Table 10).

(ii) Comparison of angiotensin I and angiotensin II as standards.

Val-5 angiotensin II is often used as a standard for the assay of renin-substrate (Table 9). Because the assayed material - in the present experiments at least - was known to be angiotensin I and not angiotensin II, the two peptides were compared as standards. Six comparisons were made using bioassay.

Table 10.

A	Bioassay of original material, nM	Radioimmunoassay, nM	
		For Angiotensin I	For Angiotensin II
	62	65	1.0
	153	154	2.2
	61	72	5.0
	105	112	2.1
	101	154	5.0
B		CM-Sephadex Chromatography	
		Angiotensin I Recovery (%)	Angiotensin II Recovery (%)
	150	80	<6.7
	185	67	<5.4

A. Comparison of bioassay and radioimmunoassay measurements of 5 samples of incubation product.

B. CM-Sephadex chromatography of two samples of incubation product. Recoveries of synthetic angiotensin I and angiotensin II are in the range 64-93%

(a) Angiotensin I (0.08 μM) against angiotensin II (0.1 μM).

(b) The incubation product (angiotensin I) against angiotensin II (0.1 μM).

(c) Incubation product against angiotensin I (0.08 μM).

(d) Angiotensin I (0.08 μM) against angiotensin I (0.1 μM).

(e) Angiotensin II (0.08 μM) labelled "angiotensin I" (as a means of avoiding observer bias) against angiotensin II (0.1 μM).

(f) Angiotensin II (0.08 μM) against angiotensin II (0.1 μM).

Twenty six assays were made in each experiment. In 13 (indicated as group 1 in Fig. 27), the test rat had been anaesthetised for less than 2 hours and in 13 (indicated as group 2), for longer than this.

The results of the first three comparisons (a,b &c) are shown in Figure 27. In rats which had been anaesthetised for more than 2 hours, angiotensin II produced a falsely low value when used as a standard to assay both angiotensin I (Figure 27A) and the incubation product (Figure 27B). No such discrepancy was apparent when angiotensin I was used as standard

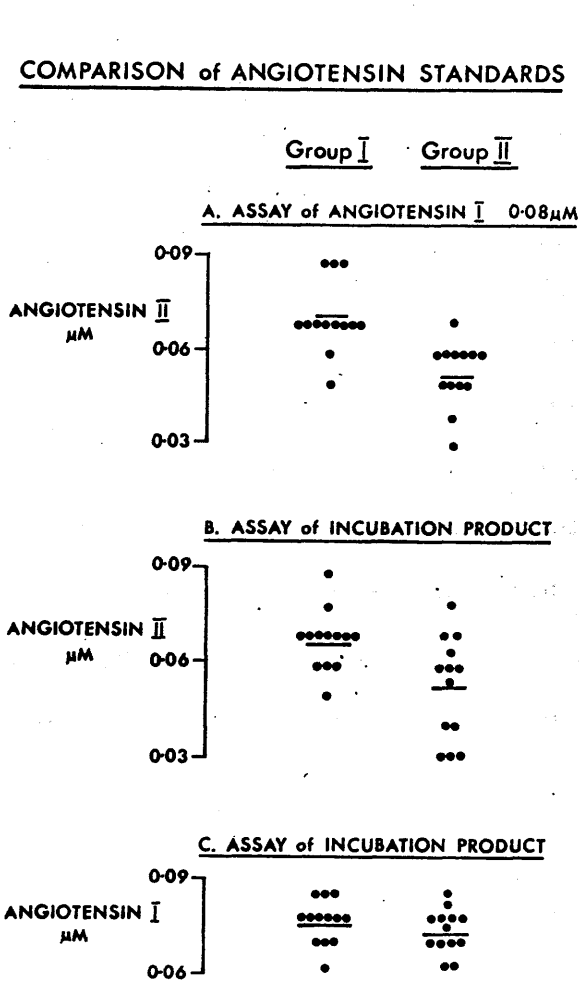


Fig.27. Comparison of assay results in two groups of rats:-
Group I. Those in use for less than 2 hours
Group II. Those in use for more than 2 hours.
See text for details of experiment.

In experiments a and b, the difference between groups I and II were significant (paired $t = 5.48$, $p < 0.001$ and paired $t = 3.47$, $p < 0.01$ respectively). There were no significant differences between groups I and II in experiment c (paired $t = 1.15$, $p > 0.2$). The horizontal bar denotes the mean.

(27 C). Nor were significant ($p > 0.1$ by Student's t test) differences found in the three control experiments (d, e & f) in which the individual peptides were tested against a dilution of the same peptide. It is also noticeable in Figure 27 that the scatter of results was greatest when angiotensin II was used as a standard and that the initial value of the incubation product was higher with angiotensin I as a standard than with angiotensin II ($t = 3.0$, $p < 0.01$).

Angiotensin I was adopted as a standard for these reasons.

(iii) Inhibition of angiotensinase

A mixture of phenanthroline and EDTA was chosen for the angiotensinase inhibitor solution because preliminary experiments showed that a combination of the two provided more complete inhibition than either tested alone (Fig. 5). The ability of the AIS solution to inhibit angiotensinase in plasma was demonstrated by 10 assays in which mean recovery of angiotensin I (0.08 μM) incubated for 18 hours with plasma-AIS mixture was 103% ± 3.8 SD. In a further 10 assays in which angiotensin II (0.1 μM) was incubated for 18 hours with plasma, mean

recovery was 105% ± 5.3 SD.

Recovery of angiotensin I (0.1 μM) incubated with human renin (2 units/ml) in AIS for 48 hours was 84 and 67% in two experiments.

Further evidence that angiotensinase was inhibited during the incubation of plasma, renin and AIS was provided by the plateau of angiotensin maintained during prolonged incubation (see sections iv and ix).

(iv.) Completeness of the renin-substrate hydrolysis.

Accurate estimation of substrate depends upon its complete conversion to angiotensin. In the absence of angiotensinase, this would be indicated by the attainment of a plateau of angiotensin concentration during prolonged incubation (Fig 28). For the concentration of renin used (2 units/ml) the plateau was reached before 8 hours incubation whether the initial substrate concentration was high or low (Fig. 28). Aliquots for bioassay were, therefore, routinely taken from the incubating mixture at 8 and 24 hours. The similarity of mean angiotensin values in aliquots taken at these times for replicate estimates from a

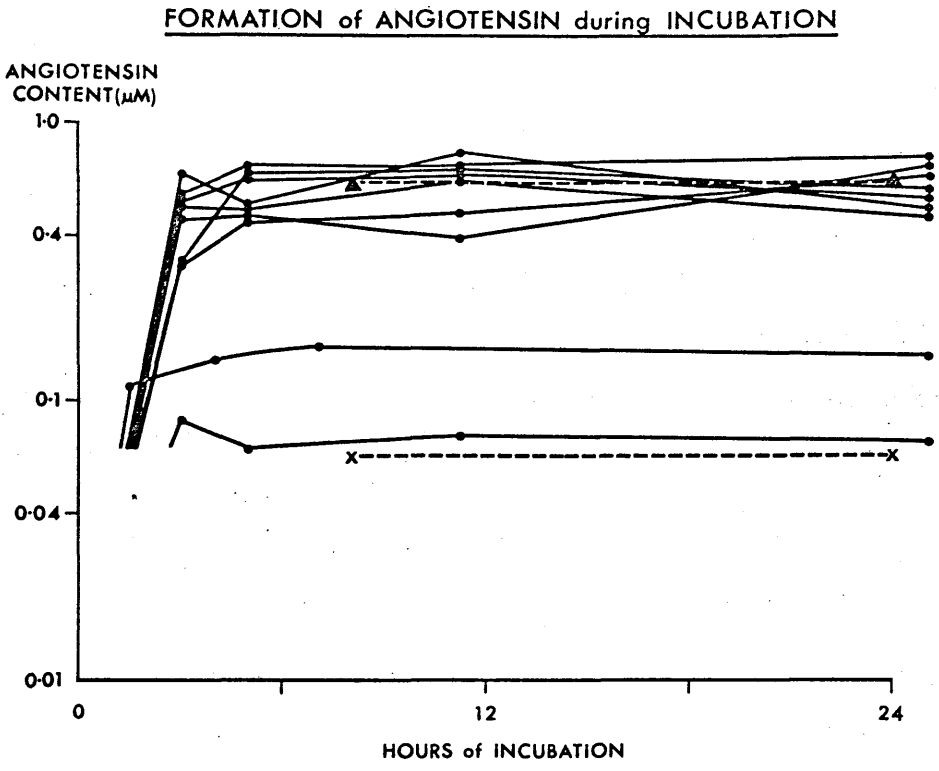


Fig.28. Changes of angiotensin content in nine plasma-AIS mixtures during the course of incubation are shown as solid dots. Triangles represent the mean values at 8 and 24 hours incubation in 43 replicate samples of stock plasma. Crosses represent mean values for 46 replicate estimates from the dilute stock plasma. Further details of this experiment are summarised in Fig.30.

plasma pool (see Fig. 30 and section (x)) also suggested that substrate hydrolysis was complete.

As a further test of complete hydrolysis, an increased concentration of renin (19.5 units/ml) was used for the incubation in 7 experiments. As compared with duplicate incubations using the standard concentration of renin (2 units/ml), the yield of angiotensin was slightly but insignificantly ($p > 0.05$) lower.

(v) Effect of diluting a plasma sample

As a test of the method, 4 plasma samples were assayed in serial dilution. Predicted and measured values agreed well (Fig. 29). Not included in the regression, but falling on the 45° slope was the mean value for 49 estimates of the dilute plasma pool (see section (x)).

These findings also suggest that inhibitors or accelerators of the renin-renin-substrate reaction are not influencing the concentration of angiotensin formed since serial dilution of such agents with substrate would have led to a discrepancy between measured and expected substrate values.

Agreement between these values also indicates that the plateau of angiotensin concentration represents

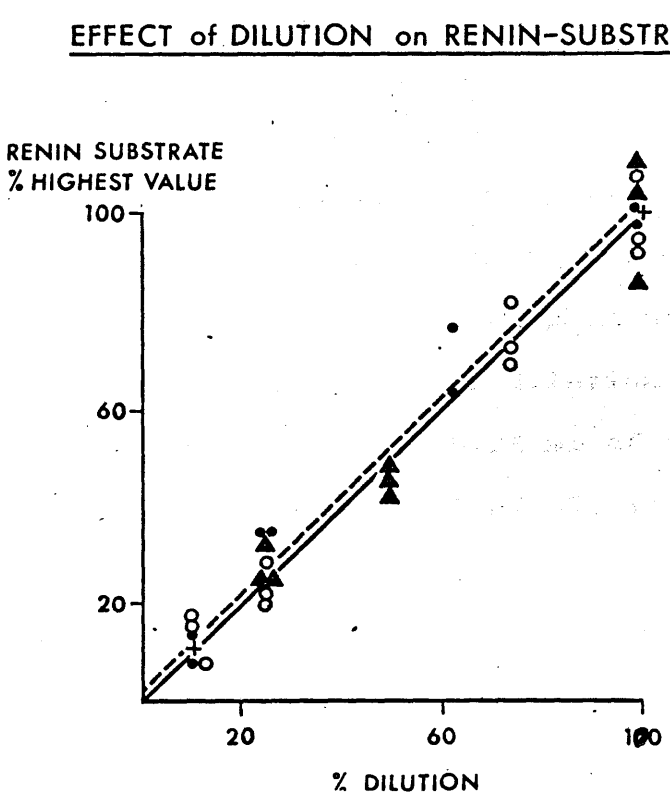


Fig.29. Effect of diluting plasma samples on substrate concentration. Each symbol represents a different sample. Crosses represent replicate estimates of substrate from the undiluted plasma-AIS pool (47 estimates) and from a ten-fold dilution of this pool (49 estimates). The solid line represents a 45° slope, the dotted line the regression for data excluding the two crosses.

complete or near complete hydrolysis of substrate rather than a balanced equilibrium state.

(vi) Recovery of added substrate

In 9 experiments, 6.25 ml of the stock solution of human substrate (0.62 μM) was added to 6.25 ml of blood together with 12.5 ml of inhibitor solution (with concentration of EDTA and phenanthroline increased by 33% to allow for dilution by added substrate). As a control, 6.25 ml of the same blood sample was added to 18.75 ml of AIS in the usual way. The 9 pairs of samples were processed and assayed as described earlier. Mean recovery of added substrate, calculated from the differences between experimental and control values, was 94% ± 32 SD. The large value of standard deviation is attributable partly to the combined variation of the two estimates of substrate used in the calculation of recovery (plasma samples with and without added substrate) and partly to the relatively small quantity of added substrate.

(vii) Effect of angiotensinase inhibitors on the renin-substrate reaction.

Because the angiotensinase inhibitors might interfere with the renin-substrate reaction and thus

with the assay of substrate, aliquots of angiotensinase-free substrate (see methods) were incubated with renin in the presence and in the absence of inhibitor solution. No marked difference was found (0.12,0.13 and 0.13 μM of substrate in samples with AIS and 0,12, 0.12 and 0.11 μM in samples without AIS)

(viii) Effect of delay in transfer of blood to inhibitor.

To test the possibility that substrate loss might occur prior to the addition of inhibitor (by formation of angiotensin and its destruction by peptidases), the effect of delay in the addition of AIS to a blood sample with high renin content (1,040 units/litre) was studied. All of the following results represent the mean of duplicates. When aliquots of the blood samples were kept at +3°C for 5 and 24 hours before the addition of inhibitor, substrate concentration was -5% and +4% different respectively from that in aliquots to which inhibitor had been added without delay. When aliquots were kept at 20°C for 5 and 24 hours the respective differences were -9% and -26%. With AIS added to blood at the outset, but with a delay of 5 or 24 hours at 20°C before centrifugation, substrate values differed

from the original by -8% and -5% respectively.

The practical implication of this study is that whole blood can be left for 5 hours at +20°C and for 24 hours at +3°C before addition of inhibitor without affecting the result materially.

(ix) Effect of storage at -20°C

(a) Plasma-AIS mixtures were prepared from six normal subjects. Each mixture was divided into eight aliquots; one pair of aliquots was assayed directly and the remainder were frozen for varying periods, thawed and assayed. Mean substrate concentration in samples which had not been frozen (0.93 μM ± 0.88 SEM) did not differ significantly ($p > 0.2$ in all instances) from samples thawed at 30 minutes (0.86 μM ± 0.01), 63 days (1.09 μM ± 0.08) and 305 days (1.03 μM ± 0.06).

(b) In another experiment a large pool of plasma was obtained from pregnant women and a plasma-AIS mixture was prepared. A 1/10 dilution of the plasma-AIS mixture was also made. Multiple aliquots of the original and diluted mixtures were stored frozen. Two aliquots of each type were tested with every

batch of substrate samples. The results, illustrated in figure 30, show no tendency for substrate to deteriorate during the 17-month period of storage.

A new batch of renin was introduced at the point indicated in figure 30. No systematic change in substrate value resulted.

(x) Variation of replicate estimates

Forty blood samples were taken in rapid succession from a normal male subject. Assayed in two batches of 20 samples, these had coefficients of variations of 8.4 and 10.7% respectively.

Data from the prolonged storage experiment (Fig. 30) were also assessed in this way. Within-batch replicates had a coefficient of variation of 7.6% and between-batch replicates a value of 15.7%.

(xi) Normal and pathological values of renin-substrate.

Substrate concentration was measured in samples of peripheral venous plasma taken from normal subjects without previous restriction of either diet or physical activity. The values obtained from 19 males aged 23-41 varied from 0.68 to 1.35 μM (mean 1.01 ± 0.21 SD). Values for 30 females (not taking

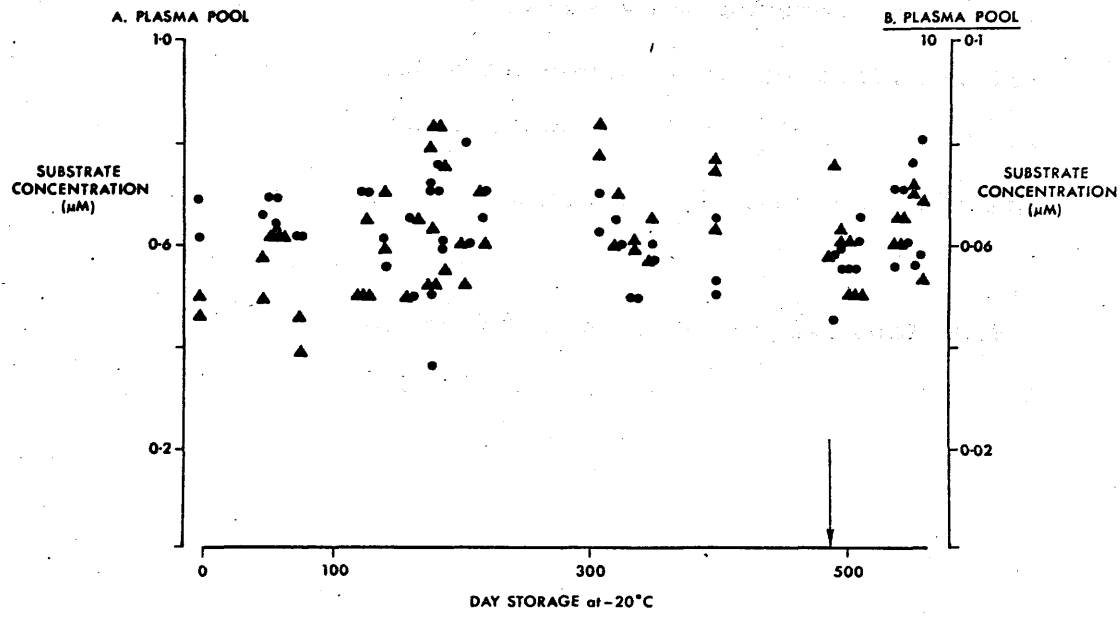


Fig.30. Replicate measurement of renin substrate concentration in samples frozen for different periods. Circles represent values for the undiluted material. Triangles represent a ten-fold dilution of this pool. A new batch of renin was introduced at the point indicated by the vertical arrow.

oral contraceptives and aged 19-34) varied from 0.45 to 1.28 μM (mean 0.85 ± 0.22 SD). Although this difference between the sexes was significant ($t = 2.58$, $p < 0.02$), the females were on average younger than the males and this could have contributed to the differences (see discussion III, below). Taken together, values for males and females had a mean of 0.91 μM (SD 0.23).

Applying the technique to physiological and pathological states, abnormally high values of renin-substrate have been found in normal pregnancy (0.84 - 7.48 μM, mean 3.07, n = 83, Weir et al 1970a) in women taking oral contraceptives (1.53 to 6.13 μM, mean 3.03, n = 12, Weir, Tree, Fraser, Chinn, Davies, Dusterdieck, Robertson, Horne and Mallinson, 1970b), in a patient with advanced renal failure and hypertension (Brown et al 1971a) and in anorexia nervosa (Love, Brown, Fraser, Lever, Robertson, Timbury, Thomson and Tree 1971). Abnormally low values (less than 0.1 and 0.08 μM) have been found in 2 children with an unexplained form of hypokalaemia (Desmit, Cost, Brown, Fraser, Lever and Robertson 1970), in the nephrotic syndrome

(0.36 and 0.43 μM) and in untreated Addison's disease (0.40, 0.36 and 0.16 μM).

III. Discussion

When average values of substrate concentration in normal man are compared, wide variation is apparent amongst the methods currently in use (Table 9). There are several possible explanations for this. Renin-substrate concentration could be falsely low because angiotensin II rather than angiotensin I was used as a standard, because a maximum concentration of angiotensin had not been achieved in the incubation mixture at the time of sampling, because recovery of substrate was incomplete, or because peptidases had destroyed angiotensin formed during the incubation. Tests described in this paper have shown that for the present method at least, none of these factors made an important contribution to the results obtained.

Most of the techniques listed in Table 9 use angiotensin II as standard. The study described in figure 27 suggests that this could lead to inaccuracy if the peptide produced by incubation was angiotensin I. The product of incubation has not

been identified in other techniques, though it is likely to be angiotensin I in those using EDTA as inhibitor (Skeggs et al 1956; Ryan, McKenzie and Lee 1968a; Hollemans, Van Der Meer and Kloosterziel, 1969). The practical implications of this is either that the product of incubation should be identified and the appropriate standard used, or that both standards (angiotensin I and angiotensin II) should be tested and validated.

Previous work has shown that equimolar amounts of angiotensin I and angiotensin II produced comparable effects on blood pressure (Skeggs et al 1956; Brown et al 1964a). The present study may not be in disagreement with this since rats were injected with phenanthroline and EDTA in the present experiments but not in the earlier studies.

Failure to reach a plateau of angiotensin concentration during incubation is a further potential source of falsely low results. Samples for assay could either be taken too early before the plateau had been reached, or, in the presence of angiotensinase, too late during the period when angiotensin concentration was declining (see Lever, Robertson and Tree 1964). Few of the methods listed

in Table 9 routinely establish that a stable plateau of angiotensin concentration has been reached.

Recovery experiments with added substrate are also important. Substrate may be lost during the preparation of samples for assay. Thermal denaturation occurs when some species of substrate are incubated at 37° (2.2.4.2.). Also, inhibitors used in the assay could prevent complete conversion of substrate to angiotensin. None of the other methods listed in Table 1 reports recovery data. Although the recovery is said to be unaffected in the method of Skinner (1967), no details are given of the technique employed to measure recovery or the range of variation.

Angiotensinase was effectively inhibited in the present technique and in most of those listed in Table 1. It is not clear whether this was achieved in the method of Ayers (1967) and that the low value obtained for the normal mean by this method may be attributable to contamination with angiotensinase.

The experiments described earlier suggest that the speed with which blood is added to inhibitor solution is not critical. It may, therefore, be

possible to simplify the method by centrifugation of blood at 4°C immediately after collection with storage of frozen plasma to await assay in angiotensinase-inhibitor solution. Also, the results illustrated in table 10 raise the possibility that the angiotensin I formed by incubation could be measured by radioimmunoassay.

The relatively consistent results for replicates estimated over a 17-month period (Fig 30) indicates that the frozen plasma-AIS mixture is stable and that incubation and assay have not undergone systematic change. It is also useful to have a monitoring system of this type when introducing a new batch of renin (Fig. 30) and to detect major deviation of results in a single batch.

The reservations so far discussed apply to the absolute values obtained by different substrate methods in man. There is less disagreement on the changes of substrate in physiological and pathological states. Where measurements have been made in man, all agree that plasma substrate increases during normal pregnancy (Helmer and Judson 1963, 1967; Skinner et al 1972; Weir et al 1970a) in women taking oral contraceptives (Helmer and Judson

1967; Skinner et al 1969; Weir et al 1970b) and in a proportion of patients with renal hypertension (Helmer 1964; 1965; Gould, Skeggs and Kahn 1966; Brown et al 1971a). Lower than normal values have been observed in patients with hepatic cirrhosis (Gould et al 1966; Ayers 1967) and the nephrotic syndrome (Aida, Maebashi, Yoshinaga, Ichinohe 1965).

2.2.4.2. Plasma renin-substrate measurement in the eel

I. Introduction.

The technical problems likely to be involved in the development of a method for measuring renin-substrate in any species have been considered in the previous section. The problems encountered with the method for the eel were different from those in man: preparation of an angiotensinase-free standard renin proved more difficult, whereas the comparison of angiotensin I and angiotensin II as standard revealed none of the discrepancies apparent in man.

II. Materials and Methods.

The methods of preparation of buffer solutions, angiotensin standards, kidney extracts and also the procedures for eel exsanguination and denaturation of ichthyotoxin are to be found in section 2.2.1.

III. Results.

(i) Inhibition of renal angiotensinase

(a) Preliminary studies with eel kidney extracts

Use of homologous renin is preferable in renin-substrate assay since not all species react with all

species of substrate (Section 2.1.1.4.).

In 4 experiments 0.25 ml of eel plasma was incubated in the presence of 36 mM DP with 1.1 mg of eel kidney tissue/ml in a total volume of 5 ml. at pH 5.5 and a temperature of 20°C. Because a plateau of angiotensin concentration was not reached after 22 hours incubation an increased concentration of eel kidney extract was tested (4.3 mg/ml of incubation mixture). Although the initial velocity of angiotensin formation increased as a result angiotensinase activity was detected. However, DP concentration could not be further increased without affecting the assay rat (page73) An alternative source of fish-kidney renin was therefore sought for the substrate assay.

(b) Preparation and use of sea trout renin in assay of renin-substrate.

Renin was extracted from kidneys of the sea trout as described earlier (page 52). Again, 36 mM DP proved capable of inhibiting angiotensinase (Table 12).

In 7 experiments, when sea trout kidney extract (2.0 mg/ml of incubation mixture) was

incubated with 0.25 ml of eel plasma in the presence of 36 mM DP, a plateau of angiotensin concentration was reached in 8 hours and maintained thereafter for a further 37 hours with only minor decline (Fig. 31).

(ii) Inhibition of plasma angiotensinase

As illustrated in figure 8, adequate inhibition of eel plasma angiotensinase was achieved by DP at 37°C, over the pH range 4.7 to 5.7. Similar results were obtained at the incubation temperature of 20°C at pH 5.4, 100% of angiotensin I and 100% angiotensin II being recovered after 18 hours of incubation.

(iii) Optimum pH .

Accurate measurement of optimum pH for the eel renin - eel renin-substrate reaction was not possible because above a pH of 6, DP (36 mM) did not inhibit the angiotensinase present in the incubation mixture. Data from the experiment illustrated in figure 32 showed that the greatest velocity of angiotensin formation in these circumstances, occurred at approximately 5.4. This pH was chosen for the routine substrate assay.

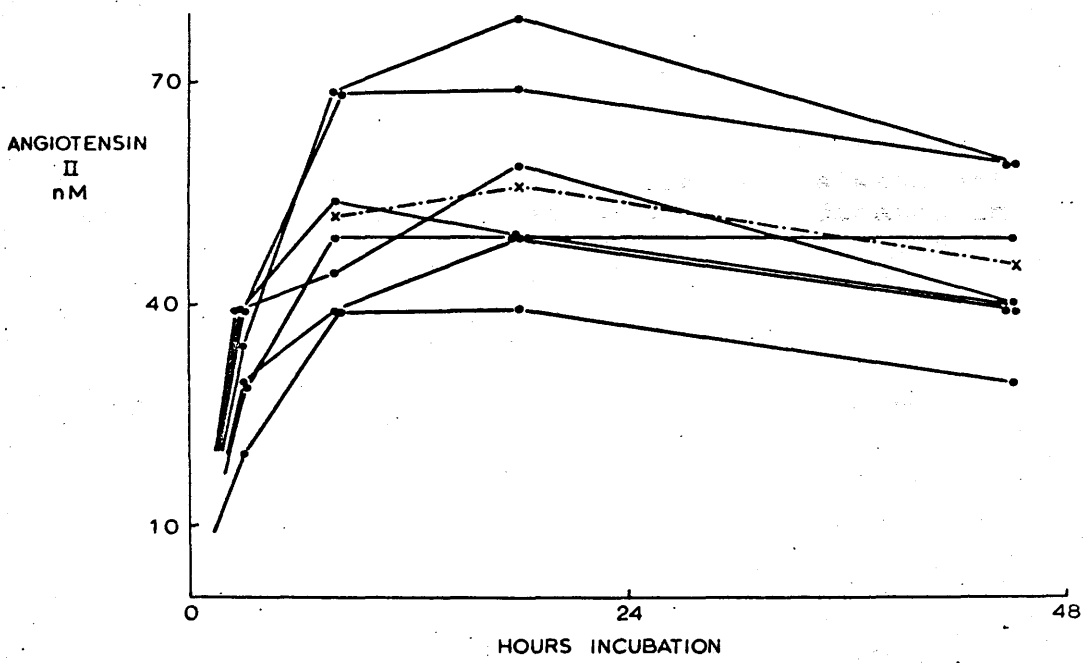


Fig.31. Reaction of sea trout renin with seven different samples of eel plasma. Mean value is shown as X.

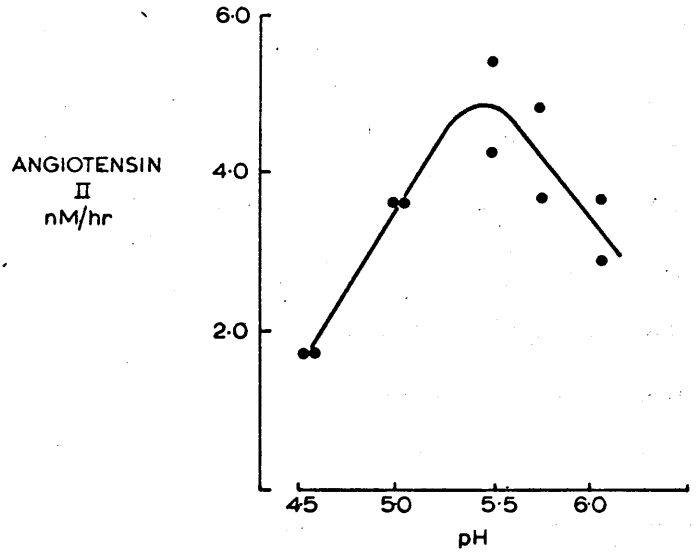


Fig.32. Effect of pH on the reaction between eel renin and eel substrate at 20°C. As indicated in fig. 8 the decline of velocity at pH above 5.5 is likely to be attributable to angiotensinase.

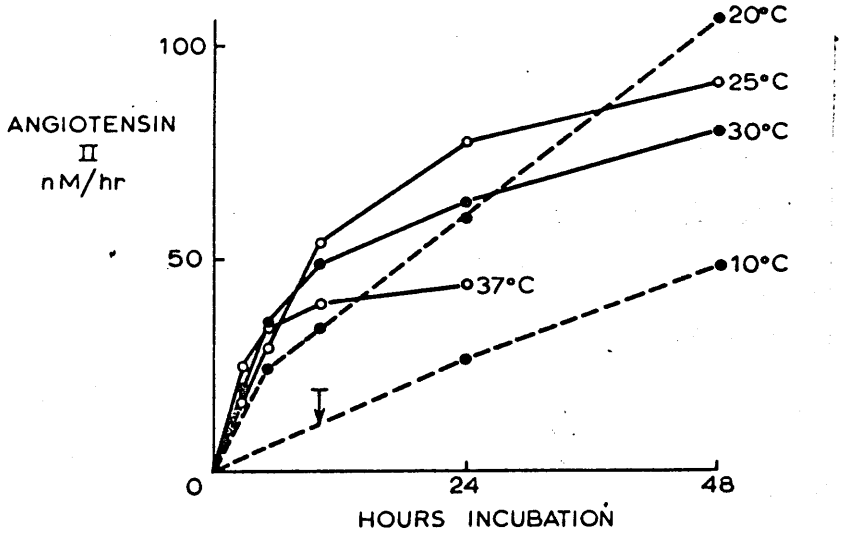


Fig.33. Effect of temperature on the reaction between eel renin and eel substrate at pH 5.4. Each point represents the mean of duplicate assays.

(iv) Optimal temperature

Maximum formation of angiotensin occurred at a temperature of 20°C. Although the initial velocity of angiotensin formation was greater at temperatures higher than this, loss of activity occurred during the process of incubation (Fig 33). Indirect evidence suggests that this loss occurred as a result of thermal denaturation of substrate rather than of the enzyme since the eel kidney enzyme incubated at 37°C with ox substrate formed angiotensin in a linear manner over a prolonged period (Fig 20).

A temperature of 20°C was chosen for the routine assay of the eel plasma renin-substrate.

(v) Technique used to measure plasma renin-substrate concentration in the eel.

The technique was based on observations described in previous sections.

(a) Heparinised samples of eel plasma were obtained and stored as described on page 53.

(b) 0.03 ml of a solution of DP was made up to

4.7 ml with pH 6.2 buffer. 0.05 ml of sea-trout kidney extract (2 mg per ml of incubation mixture) was added.

(c) To this, 0.25 ml of eel plasma was added and the mixture (pH 5.5) was incubated at 20°C. Aliquots were taken routinely at 8 and 18 hours and stored frozen to await bioassay.

(d) Prior to assay the thawed aliquots were placed in boiling water for 5 minutes to inactivate ichthyotoxin. The temperature in the incubating tube rose to 98°C during this process and prolonged incubation in boiling water for 30 minutes did not affect angiotensin concentration as compared with some samples which had been incubated for 5 minutes.

(e) The rat blood pressure preparation was used as before (page 54) for assay of angiotensin formed during incubation (Fig 34) and angiotensin II, 97.2 nM (100 ng/ml) was used as standard.

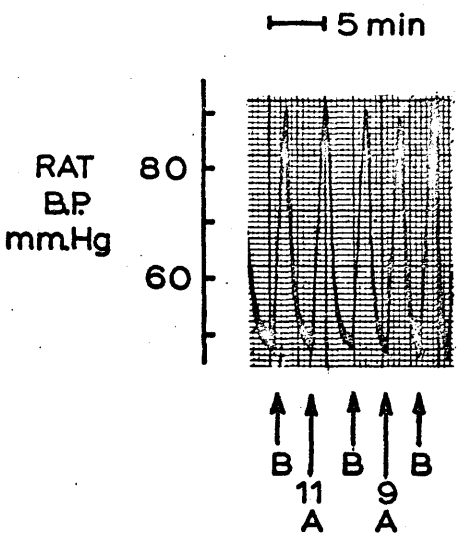


Fig.34. Bioassay of angiotensin from the reaction of sea trout renin upon eel plasma. B = 0.1 ml of incubation product and A = angiotensin II standard (ngs).

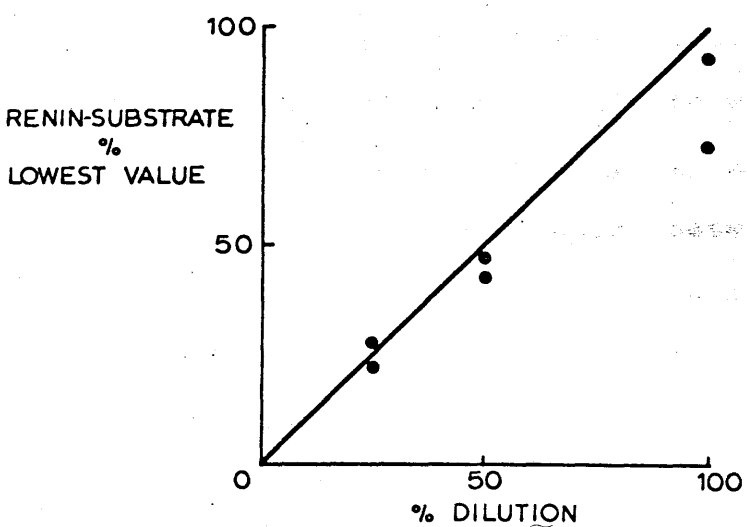


Fig.35. Effect of dilution of an eel plasma substrate sample. The continuous line represents a 45° slope.

(f) Substrate concentration was expressed as in the method for man (page 113).

(vi) Validation of the standards

The product from the incubation of sea trout renin with eel plasma was assayed on nine occasions using both angiotensin I and also angiotensin II as standards. There was no significant difference in concentration between the assays when these two angiotensins were used (Table 11).

(vii) Effect on substrate concentration of diluting plasma

As a test of the method and also of the completeness of substrate hydrolysis, serial dilutions of eel plasma were incubated in duplicate with constant amounts of renin and inhibitor. As in the method for man (Fig. 29), the relation between dilution and measured substrate concentration did not quite follow the expected 45° slope (Fig. 35) because there was some loss of angiotensin at higher plasma concentrations, as the result of angiotensinase activity.

(viii) Results in normal eels

Plasma samples from 18 eels adapted to fresh

Table 11. Eel plasma renin-substrate method: validation of bioassay standard.

Angiotensin standard	No. Assays	Sample Mean μM	SD μM	SE μM	Students t test
I (0.97μM)	9	1.002	0.056	0.019	t=1.597 p=0.1 to 0.2
II (0.97μM)	9	0.946	0.088	0.029	

water or seawater, (see section 4.2.2.) had a mean renin-substrate concentration of 0.98 μM ± 0.29 SD, a result similar to that obtained in man.

(ix) Replicate variation

Six replicate assays of renin-substrate concentration from aliquots of a frozen plasma pool tested at intervals over the course of 7 months had a coefficient variation of 7% with a mean substrate value of 0.95 μM.

2.2.5. Preparation and use of renin-substrate from the nephrectomised ox.

2.2.5.1. Preparation of substrate

Six stirks weighing between 780 and 950 lbs. were subjected to bilateral nephrectomy as described on page 54 . One week after the operation they were anaesthetised with thiopentone sodium, maintained with halothane (Watts and Campbell 1971), and then 35 units of heparin/lb. of body weight was injected intravenously. A glass cannula with a minimum internal diameter of 3.5 mm. was inserted into the left carotid artery and the animals were bled via polyethylene tubing into polyethylene containers. The bleeding lasted for approximately ten minutes and this resulted in the death of the animal.

The plasma was separated by centrifugation at 10^{o} and 1,500 g. for 45 minutes. The 6 to 11 litre batches of plasma obtained from the six animals were dialysed separately at $4^{o}C$ in 36/32 visking membrane against 25 litres of pH 5.7 buffer changed half-daily for 5 days. The composition of this buffer was as follows:-

$Na\ H_2\ PO_4$	-	44 mM
$Na_2H\ PO_4$	-	6.3 mM

NaCl	-	100 mM
Disodium EDTA	-	2.7 mM
Neomycin sulphate	-	0.02 g%

The small quantity of precipitate formed during dialysis was removed by filtration (Whatman 54) and further neomycin was added to make a final concentration of 0.2 g%. The dialysed plasma was frozen and the small precipitate which formed on subsequent thawing was removed by centrifugation.

2.2.5.2. Properties of substrate

The six batches of plasma were tested separately for their properties as a standard substrate preparation.

I. Angiotensinase activity

Was assessed by incubation of 4 ml of substrate with 1 ml of Aspn I. Val 5. —Angiotensin II (97nM) for 48 hours. Recovery of angiotensin, tested in triplicate for each batch, varied from 90 to 110%.

II. Blanks.

Incubated alone, each batch of dialysed plasma formed no detectable angiotensin (less than 9.7 nM) when incubated in triplicate for 48 hours at 37°C. Aliquots of the incubating material were tested at 0, 24 and 48 hours.

III. Substrate concentration

Was assessed as described earlier (2.2.4.1.) by incubating with an excess of human renin. Measured in triplicate, the substrate concentration of the stirks, numbered one to six was 0.98, 1.22, 0.46, 1.51, 1.31 and 0.90 μM, respectively.

IV. Rate of angiotensin formation during incubation with renin.

The individual batches of substrate were incubated with renin (0.005 u/ml) and angiotensin was produced within the assayable range of 20 to 115 nM at a linear velocity over 48 hours.

As a result of these tests dialysed plasma from the first and second stirks (but not the third because of its low concentration of substrate) was bulked (pooled ox substrate I) and subsequently that of stirks 4,5 and 6 (pooled ox substrate II). These pooled substrates had properties similar to those obtained with the individual specimens. They were stored at -20°C in aliquots.

V. The effect of bilateral nephrectomy

The value of bilateral nephrectomy in raising the plasma level of renin-substrate was shown in two stirks (numbers 5 and 6) by serial measurement of

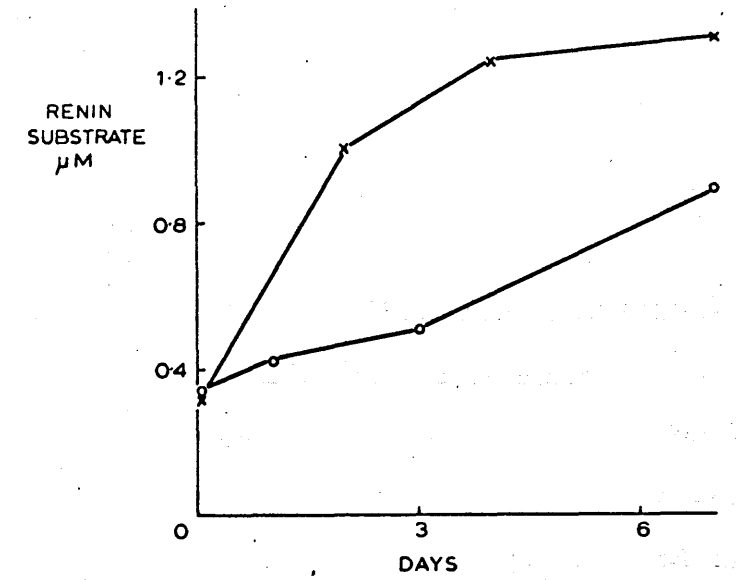


Fig.36. Effect of nephrectomy on renin-substrate concentration in the plasma of two stirks (numbers 5 = X and 6 = O).

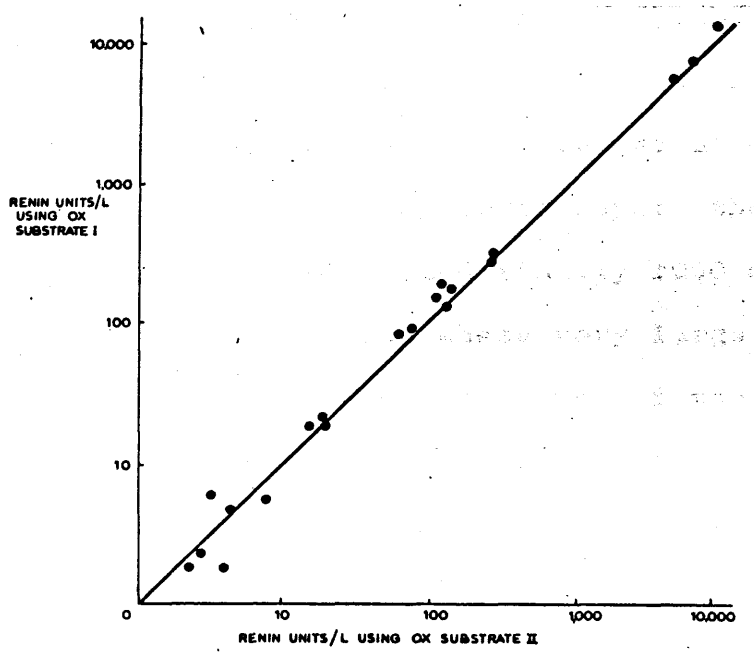


Fig.37. Duplicate assays of human plasma renin (units/L, see Brown et al 1964) using ox substrate prepared by the method of Lever et al (1964) (substrate I) and also substrate prepared as described here (substrate II). The continuous line represents a 45° slope.

substrate at intervals before and after the operation. Renin substrate concentration increased three-fold in one animal and four-fold in the other (Fig. 36).

VI. An unsuccessful attempt to increase substrate concentration by Stilboestrol.

Since the administration of oestrogen has been shown to increase plasma renin substrate concentration in the rat (Helmer and Griffith 1952) and in man (see page 128), it was hoped that an increased yield of renin-substrate could be obtained in the ox using Stilboestrol. In one ox, therefore, Stilboestrol diphosphate was administered intravenously in the following regime:- 2.5 mg. daily for eight days; followed by 10 mg. daily for seven days; then 100 mg daily for thirty nine days and finally 1000 mg daily for fourteen days. Despite these very large doses, which on a weight basis are in excess of the oestrogen dose which has been shown to have an effect in man and the rat, the circulating level of renin substrate remained within the range 0.24 to 0.36 μM throughout.

2.2.5.3. Use of ox renin-substrate

The original intention was to use the new substrate for measurement of small quantities of renin in fish, amphibia, birds and mammals. The new

substrate proved both easier to prepare and superior in quality when compared with the previously used substrate from the un-nephrectomised ox (Lever et al 1964). For these reasons the new substrate was substituted for the old in the routine methods for measuring human renin in the plasma of man (Brown et al 1964 a), the rabbit (Lever et al 1964), and the dog (Brown et al 1964b). The change from the old to the new substrate was monitored by a comparison of duplicate measurements such as those shown in figure 37.

2.3. General discussion of methods

Four new techniques have been described: methods for measuring renal renin in the eel and many other vertebrates (2.2.3 and 4.2.1.), methods for measuring renin-substrate in the plasma of man (2.2.4.1.) and fish (2.2.4.2.) and a technique for producing substrate on a scale sufficient for use in routine assays of renin (2.2.5.).

Inhibition of angiotensinase, a central problem in the development of these techniques, has been described and discussed in a separate section (2.2.2) A large number of possible inhibitors was tested; most were not sufficiently effective, or were lethal to the assay rat. Although a universally effective inhibitor was not found, different inhibitors, or combinations of inhibitors, allowed quantitative recovery of angiotensin in all assays used. These results provide a basis for the development of renin and renin-substrate assays in a whole variety of vertebrates.

Other methodological problems and their relation to earlier work have been discussed in the appropriate sections. These include the need to validate the

the assay standard (2.2.4.1.); this study may have relevance to other assays using angiotensin II as standard and the question is currently under investigation.

3. Studies of the plasma concentration of renin-substrate in man.

Studies of the regulation and function of renin-substrate centre on the following problems. Firstly, it is undecided whether substrate is a rate-limiting factor in the renin-angiotensin system. This problem is approached in two ways, either by determining the Michaelis constant (Km) and the Vmax (see section 2.1.1.10), or by demonstrating in vivo, whether changes in plasma renin-substrate concentration influence the circulating concentrations of angiotensin and, as a consequence, have a physiological role (see Fig.1). If the substrate concentration at Vmax is higher than in normal situations then the importance of understanding the factors that control renin-substrate is greater. Studies of these factors form the second aspect of this research.

The technique developed for the measurement of renin-substrate in man is described in part 2.2.4.1 of this thesis. The method has since been used in several studies of the renin-angiotensin system, most of the assays being performed by the author. Because some of the results have been published a brief survey of the main findings only is given here and reprints of the publications are included as appendices to

the thesis.

3.1. Situations in which the plasma concentration of renin-substrate is increased

Oral contraceptives are known to increase plasma renin substrate concentration. The observation was confirmed and it was further shown that the plasma concentrations of renin and aldosterone did not change (appendices 1 and 2).

Renin-substrate is also known to increase in normal pregnancy. This too was confirmed (appendix 3). It was further shown that the plasma levels of renin and aldosterone did not correlate in individual pregnant women in contrast to other situations (e.g. Brown et al, 1971). Renin substrate data provided a possible explanation for this. Although the plasma level of renin substrate also failed to correlate with aldosterone, the product of renin and renin-substrate did (Fig.38 and appendix 3). This suggested that variation in renin-substrate was influencing the amount of angiotensin formed by renin. Recent enzymekinetic evidence points in the same way (Gould and Green, 1971).

Increased levels of renin-substrate have also been found in a woman with anorexia nervosa (Appendix 4) and a man with severe hypertension and secondary

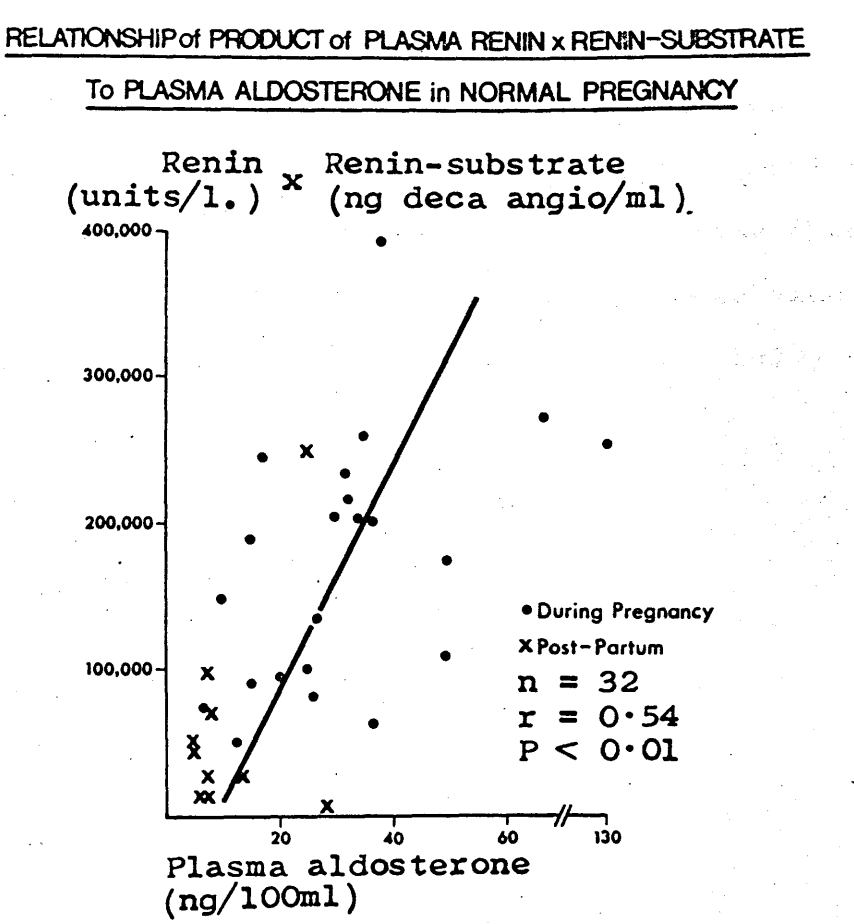


Fig.38. (From Weir, Paintin, Robertson, Tree, Fraser & Young, 1970).

hyperaldosteronism (Appendix 5).

3.2. Situations in which the plasma concentration of renin substrate is decreased.

Decreased values were found in Addison's disease in occasional cases with cardiac failure (Davies et al, to be published), in the nephrotic syndrome (Medina et al 1972) and in two children with an unexplained form of hypokalaemic alkalosis (Desmit et al, 1970).

4.0. Comparative physiology of the renin-angiotensin system.

Biochemical and technical aspects of the renin-angiotensin system have been discussed in parts 1 and 2 of this thesis.

The fourth section consists of a consideration of the literature on the comparative aspects of the renin-angiotensin system (4.1) and a description of several studies on the comparative physiology of renin and angiotensin (4.2.1. - 4.2.6.). The techniques developed specifically for these studies are described in part 2 of the thesis.

4.1. Previous work on the comparative physiology of renin and angiotensin

Understanding of the renin-angiotensin system is based almost entirely on studies in man and laboratory mammals. Because renin seems most closely related to the regulation of sodium balance, and because sodium balance undergoes more extreme physiological changes in lower vertebrates, neglect of these groups is

particularly unfortunate. A comparative study of the evolution of the renin-angiotensin system in vertebrates might also be particularly revealing because the mechanisms controlling salt and water balance have evolved in quite different ways in the various vertebrate classes.

4.1.1. Studies in mammals

Work on renin and angiotensin in man and laboratory animals has been discussed both in part 1 and 2 of this thesis and in reviews by Davies (1964, 1971), Brown et al (1966), Peart (1965), Page and McCubbin (1968), Lee (1969), Coghlan et al (1971) and Ferrario et al, (1972).

Capelli and his colleagues (1970) measured renin and glucose-6-phosphate dehydrogenase in the kidneys of a variety of mammals and whilst renal renin was lower in the desert rat (Dipodomys perblandure) than in the hamster (Mesocricetus auratus) and the white rat (Rattus norvegicus), the G-6-phosphate dehydrogenase staining of the macula densa was more intense. The authors were unable to interpret the observation.

Eichelberger et al (1940) showed that renin-like activity was present in the kidney of the

dolphin (Tursiops truncatus), and, as will be described in section 4.2.1. of this thesis, renin was detected without difficulty in the kidney of the porpoise (Phocaena phocaena). Malvin and Vander (1967) demonstrated that the plasma renin activity in 5 species of Cetacea was similar to that of other laboratory mammals. However, the manner in which the Cetacea regulate salt and water balance is clearly different from terrestrial mammals; a water-load administered through a stomach tube does not induce a diuresis although after a meal, glomerular filtration rate, renal plasma flow and urine flow increased 24-fold in the dolphin (Tursiops gilli) (Malvin and Rayner, 1968). Unfortunately, renin measurements were not made during these experiments.

The response of the marsupial Trichosurus vulpecula to stimulation of the renin-angiotensin system was found to be similar to that of eutherian mammals (Reid and MacDonald 1969 a &b): plasma renin activity was increased by low sodium diet, by mercurial and non-mercurial

diuretics, and by haemorrhage. Sodium chloride loading reduced renin activity. Increased granulation of the juxtaglomerular cells was noted during sodium deficiency. The clearance of injected renin from plasma was found to be similar to that in the dog (Canis familiaris).

In another marsupial, the North American opossum (Didelphis virginiana), injected renin stimulated cortisol, corticosterone and aldosterone whilst sodium depletion caused increased juxtaglomerular granulation. The properties of renin were similar to those in the eutherian mammal, the enzyme being heat-labile non-dialysable, and stable at a pH of 2.6 (Johnston et al, 1967a).

Juxtaglomerular granules have been identified in the embryonic mesonephros of the hog (Sus scropa domesticus) and it was only after degeneration of the mesonephros that the quantity of granules increased in the metanephros (Sutherland and Hartroft, 1968). Bing and Kazimierczak (1964) however, were able to bioassay renin in the subscapular zone of the kidneys of new-born pigs,

where afferent and efferent vessels were absent.

It can be concluded on good grounds, therefore, that renin is widely distributed in mammals and that it bears a relatively consistent relation to sodium metabolism.

4.1.2. Studies in birds

Renin was first demonstrated in birds by Schaffenburg and his colleagues in 1960. Renal extracts from the chicken (Gallus domesticus) produced angiotensin-like material when incubated with homologous plasma. Also in the chicken, Taylor et al (1970) demonstrated aldosterone in adrenal venous blood, as did Phillips and Chester Jones in 1957 after administering ACTH. However, Taylor and colleagues were unable to show a stimulant effect on aldosterone of injection of renin or ACTH yet, after sodium depletion, renal renin and juxtaglomerular index increased. The juxtaglomerular granules were smaller and sparser than in the mammal. A macula densa was also identified. The cells of the avian macula densa have been described (Ogawa and

Sokabe, 1971) as being structurally transitional between those typically seen in mammals and ordinary cells of the distal tubule. McKelvey (1963) also observed granular cells in birds. The adult budgerigar (Sutherland, 1966, referred to in Sutherland and Hartroft, 1968) showed juxtaglomerular granulation, but staining was not present in the afferent glomerular arteriole in either the mesonephros or the metanephros of the embryonic kidney. In the pigeon (Columba oenas), Capelli et al (1970) showed that renal renin reacted with ox substrate to form angiotensin. A structure analogous to the macula densa was recognised although the glomerulus seemed poorly developed. Studying the pigeon and duck (species unstated), Holmes et al (1969) demonstrated an increase in plasma renin activity following haemorrhage and in the pigeon a rise of blood pressure after injection of angiotensin II. Hypophysectomy in the pigeon led to a 4-fold increase of plasma renin activity which was subsequently reduced by ACTH.

4.1.3. Studies in reptiles

Renin has been demonstrated in the kidney tissue of a brackish-water turtle (Malaclemys sp.) (Kaley and Donshik, 1965) a fresh-water turtle (Chrysemys sp.) (Connell and Kaley, 1964; Capelli et al, 1970), and the desert tortoise (Gopherus agassizi) (Capelli et al, 1970). McKelvey (1963) also observed granular cells in reptiles.

Nothstine and colleagues (1971) have demonstrated the presence of the renin-angiotensin system in a fresh-water turtle (Pseudemys sueanniensis) in which kidney extracts were vasopressor and stimulated corticosterone secretion. They could not detect aldosterone in this turtle but could in the crocodile (Caiman sclerops). However, crocodile kidney extract did not influence steroid secretion into postcaval vein plasma; nor was renin identified with certainty.

4.1.4. Studies in Amphibia.

Juxtaglomerular granules were observed by McKelvey (1963) in amphibians. The macula densa has been identified in the juxtaglomerular cells

of the American bull-frog (Rana catesbeiana), and granules were distributed at various sites along the afferent glomerular arteriole (Hartroft, 1966). The juxtaglomerular apparatus in the newt (Triturus cristatus) was composed of cells from the media of the afferent glomerular arteriole which contained granular cells, and cells of the intermediary tubule. However, typical macula densa and lacis cells were absent (Bellocci et al, 1971). The macula densa was also previously noted by Capelli et al (1970) in Rana pipiens.

Capelli and associates (1970) also found that renin was demonstrable in renal extract and decreased to undetectable levels when the frogs were acclimatised to 0.02% sodium chloride solution. In 1968 Ulick and Feinholtz showed that aldosterone secretion in the bull-frog was stimulated by sodium depletion but not by injection of Asn 1 Val 5 angiotensin II. Taylor and Davies (1971) also failed to stimulate aldosterone in the bull-frog with Asn 1, Val 5 angiotensin II and also carp renin even with

quantities that increased the blood pressure. However, the angiotensin-like product from the reaction of carp kidney extract with carp plasma did increase aldosterone and corticosterone secretion and blood pressure in the bull-frog. In the hypophysectomised bull-frog, on the other hand, intravenous infusion of bull-frog renin did increase aldosterone secretion (Johnston et al, 1967b).

Asn 1, Val 5 angiotensin II does affect renal function having an antidiuretic and antinatriuretic effect (Coviello, 1969, 1970) in Bufo paracnemis. Similar results were previously obtained by Henderson and Edwards (1969) with Xenopus laevis when Asn 1, Val 5 angiotensin II was infused in saline. This effect was reversed in animals maintained in distilled water. More recently, Nolly and Fasciolo (1971b) studying Bufo arenarum were unable to demonstrate changes of renal renin as a result of peritoneal dialysis after treatment with the natriuretic diuretic furosemide, or exposure to distilled water or sodium chloride solution (0.11 - 0.35 M).

Nolly and Fasciolo (1971a) also found renal renin concentration to be higher in small toads than large ones. There was twice as much renin in male kidneys as in females, although plasma renin-substrate values did not differ significantly.

4.1.5. Studies in fish

The presence of renin in the kidney of certain fish has been disputed. Some workers have been able to demonstrate the enzyme in all or most of the fish studied (Chester Jones et al, 1966; Mizogami et al, 1968, Capelli et al, 1970; see also section 4.2.1.1.). Others were less certain of its presence particularly in aglomerular teleosts, (Friedman and Kaplan, 1942; Friedman et al, 1942; Kaley et al, 1963; Connell and Kaley, 1964). One possible explanation for this discrepancy is that renin is present in the kidneys of all fish, that there is more in the kidneys of fresh-water fish and that the low levels present in glomerular and aglomerular salt-water fish were undetectable by some of the cruder methods in which angiotensinase inhibition

had not been achieved.

From a morphological standpoint, Capreol and Sutherland (1968) found that there was no macula densa in fish. They (quoting Sutherland, 1966) were also unable to find granular juxtaglomerular cells in the Agnatha (lamprey and hagfish, species unstated). McKelvey (1963) could not identify juxtaglomerular granular cells in elasmobranchs (species unstated). This evidence was supported by Bohle and Walvig (1964) who studied Acanthias vulgaris, and by Capreol and Sutherland (1968) in Raja laevis. Granular cells have been observed in a wide variety of Osteichthyes (Bohle and Walvig, 1964; Bulger and Trump, 1965; Capreol and Sutherland, 1968; Mizogami et al, 1968).

An increase in the amount of renin in kidney tissue following reduction of environmental salinity has been demonstrated in the aglomerular Opsanus beta and in the glomerular kidneys of Alossa sapidissima, Pomelobus pseudoharengus (Capelli et al, 1970) and Anguilla japonica and Tilapia mossambica (Sokabe et al 1968). Also in

Anguilla japonica juxtaglomerular granularity appeared to decrease in the sea water environment. However, in Cymatogaster aggregata juxtaglomerular granularity was increased with increasing salinity (Lagios, 1968). In accord with this, renal renin concentrations were found to be higher in the marine teleosts Tautoga sp. and Paralichthys sp. when compared with the freshwater teleosts Carassius sp. and Ameiurus sp. (Kaley et al, 1963; Kaley and Donshik, 1965).

In a study of Tilapia mossambica, Malvin and Vander (1967) found no differences of plasma renin activity in fish kept in high and low salinity.

Injections of Asn 1, Val 5 angiotensin in the eel (Anguilla anguilla) leads at first to an increase of urine flow and clearances of PAH and inulin, and then to an antidiuresis (Chester Jones et al, 1969). Injected Asn 1, Val 5 angiotensin II and eel renin also raises the blood pressure of the eel (Chester Jones et al, 1966).

A possible role for the corpuscles of Stannius in the regulation of blood pressure and sodium balance is discussed in papers by Chester Jones et al (1966) and Sokabe et al, (1968).

In summary, renin or renin-like enzymes have been extracted from the kidneys of fish, reptiles, amphibians, birds and mammals. A juxtaglomerular apparatus, the probable site of renin storage, has been identified in most of these groups. A clear relation has been demonstrated in mammals between the loss of sodium and water and an increase of renin; by stimulating release of sodium-retaining steroids from the adrenal cortex, the increase of renin would tend to compensate for the sodium loss. A similar mechanism may be at work in birds and amphibians, although the evidence is scanty. In fish the picture is confused. Although renin is certainly present in the kidney of fish it is not clear whether adaptation to a low salt intake is associated with an increase or with a decrease of renal renin. A change from a high to a low-salt environment in fish is associated not only

with a tendency to sodium loss, but also with water influx, a situation dissimilar from sodium and water depletion in the mammal.

4.2. New studies of the comparative aspects of renin and angiotensin

4.2.1. Renin in vertebrate kidney tissue.

Using the methods described on page 106, extracts from a wide variety of vertebrate kidneys were tested for renin both by direct injection and indirectly after incubation with ox substrate. Angiotensinase activity was shown to be controlled by DP in the incubation experiments (table 12). Where a positive result was obtained, the progressive formation of angiotensin during incubation was demonstrated by assay of aliquots taken at different times from the incubation mixture.

Excepting rabbit kidneys, which contain a large amount of renin the results are expressed as the concentration of angiotensin-like material present in the mixture after 16 hours incubation. Although the original extracts were prepared in an identical manner (200 mg kidney tissue/ml of extract), different dilutions of extract were used in the incubation depending on their renin and angiotensinase content. Generally, the dilution was greatest when the angiotensinase content was high and the

Table 12 Renal renin assay and renal angiotensinase inhibition by DP, in vertebrates.

Species	Direct Assay		Indirect Assay		% Angiotensin II recovered at 62 hr.			
	Extract injected mg.	**Renin response mm.Hg	Extract mg/ml.	nM A II at 16 hr.	Extract mg/ml	% A II	Extract mg/ml.	% A II
PISCES								
Scomber scombrus (Mackerel)	20 & 40	Unlike renin	1.8	19	0.5	100	5.0	50
Gadus morhua (Cod)	" " "	" "	1.8	19	0.67	85	6.7	<10
Salmo trutta (Sea trout)	" " "	" "	1.8	780	0.4	100	4.0	10
Anguilla anguilla (Eel)	" " "	" "	1.3-5.4	33-213	1.0	76	"	<10
AMPHIBIA								
Rana temporaria (Frog)	" " "	" "	0.8	<19	"	60	"	<10
REPTILIA								
Vipera russelli (Russells viper)	" " "	" "	1.8	19	"	100	"	15
? (Porters giant tortoise)	" " "	" "	0.8	19	"	40	"	<10
? (Blue spiny lizard)	" " "	" "	1.8	29	"	80	"	10
Testudo radiata (Radiated tortoise)	" " "	" "	1.8	29	"	90	"	<10
AVES								
Balearica pavonina (African crowned crane)	20	(15)	1.8	39	"	100	"	15
Spheniscus humboldti (Humbolts penguin)	20	(22)	0.8	44	"	40	"	<10
Rhea americana (Rhea)	20	(30)	1.8	19	"	80	"	<10
Pavo cristatus (Peafowl)	20	(20)	0.8	19	"	20	"	<10
? (Semipalmated goose)	20	(11)	1.8	44	"	70	"	<10
MAMMALIA								
MARSUPALIA								
Protemnodon dorsalis (Pretty-face wallaby)	40	18	1.8	107	"	75	"	<10
Macropodus sp. (Wallaby)	40	20	0.8	24	"	40	"	<10
? (Silver grey phalanger)	40	14	1.8	242	"	80	"	20
Thylogale sp. (Short-tailed pademelon)	-	-	-	-	"	50	"	<10
PRIMATA								
Comopithecus hamadryus (Sacred baboon)	40	10	0.8	146	"	<10	"	<10
Cercopithecus aethiops sabaeus (Green monkey)	40	18	1.8	39	"	70	"	<10
Erythrocebus patas (Patas Monkey)	40	Unlike renin	0.8	19	"	<10	"	<10
Homo sapiens (Human)	1 unit	3	0.4	15	0.004	90	0.04	60
EDENTATA								
Myrmecophaga tridactyla (Giant anteater)	20	3	1.8	63	0.4	80	4.0	30
Cyclopes didactylus (Dwarf anteater)	-	-	1.8	680	"	90	"	60
PHOLIDOTA								
Manis sp. (Pangolin)	20	26	0.8	340	"	50	"	<10
LAGOMORPHIA								
Oryctolagus cuniculus (N.Z.-white rabbit)	40	25	0.4	68(1hr)	1.6	100*	-	-
RODENTIA								
Myocaster coypus (Coypu)	40	32	1.8	160	0.4	80	4.0	<10
Rattus norvegicus (Norway rat, Wistar strain)	2	28	0.4	780	"	100*	"	90*

Table 12 (continued

Species	Direct Assay		Indirect Assay		% Angiotensin II recovered at 62 hr.			
	Extract injected mg.	**Renin response mm.Hg	Extract mg/ml	nM A II at 16 hr.	Extract mg/ml	% A II	Extract mg/ml	% A II
CETACEA								
Phocaena phocaena (Porpoise)	10	48	0.4	1070	0.4	100*	4.0	90*
CARNIVORA								
Canis familiaris (Domestic dog)	40	28	0.4	29	0.004	90	0.04	60
Thalarctos maritimus (Polar bear)	2	32	0.4	1450	0.4	100	4.0	70
Herpestes urva (Crab-eating mongoose)	2	23	0.8	680	"	<10	"	<10
Arctictis binturong (Binturong)	10	25	0.8	68	"	20	"	<10
PINNIPEDIA								
Halichoerus grypus (Grey seal)	20	31	0.4	97	"	90*	"	80*
TUBULIDENTATA								
Orycteropus afer (Aardvark)	20	19	1.8	390	"	70	"	<10
ARTIODACTYLA								
Choeropsis liberiensis (Pygmy hippopotamus)	20	35	0.4	242	"	100*	1.6	100*
Camelus sp. (--Camel)	20	20	0.4	68	"	95*	1.6	100*
Lama guanicoe (Guanaco)	20	19	1.8	233	"	70	4.0	<10
Bos taurus (Domestic ox)	20	16	0.8	63	"	40	"	<10
Ovis musimon (Mouflon)	20	20	1.8	291	"	80	"	10

Notes:

1. * = % angiotensin II recovered after 48 hours, not, as in other experiments, 62 hours.
2. ** = The mean response to two or three injections in different rats.
3. Figures in parentheses refer to vasopressor responses that were not entirely like renin (See Fig.12)
4. For Anguilla anguilla, indirect assay data are from Figure 23 and the angiotensin recovery data represents the mean recoveries from six individual kidney extracts, incubated for 20 hours.
5. In control samples without DP, angiotensin was undetectable (<10%) after ½ hour.

period of incubation shortest when renin content was high as in the rabbit (table 12).

The positive findings were as follows:-

4.2.1.1. Pisces

I. The presence of renin in renal extract

A renin-like enzyme was detected in large amounts in kidney tissue of the sea trout and eel and in small amounts in kidney tissue from the cod and mackerel (table 12). As described on page 83 , fish kidney extract also contained a diffusible, heat-stable, pressor material.

II. Location of renin in the kidney of fish

The data quoted above were derived from extracts of entire kidneys. An incidental observation made on one eel and confirmed in a single sea trout, suggests that renin may not be distributed symmetrically in the teleost kidney.

There are variations in the morphology and anatomy of the kidney in the adult teleost (Ogawa, 1961 and 1962; Hickman and Trump, 1969). "Head", anterior and posterior divisions of the kidney were recognised here in the sea trout and

anterior and posterior divisions in the eel (Fig. 39). Sections of kidney tissues were therefore taken from these regions in the sea trout and eel. As can be seen in table 13, most renin was found in the posterior kidney.

III. Association of renin with glomeruli in the eel

In a further preliminary experiment, an eel was anaesthetised with M.S. 222 and, using the method of Nash (1931), 20 ml of Prussian Blue (2% potassium ferrocyanide solution and 2% ferric ammonium citrate in distilled water) was injected into the exposed ventral aorta in a cephalad direction. The kidney was then examined under a dissecting microscope (magnification x 20) and 16 single glomeruli were teased out as described by Brown et al (1965). The glomeruli were stored at -20°. On thawing, glomeruli were incubated singly or in groups of up to 7 at 37° with 2 ml of ox substrate. The angiotensin formed was assayed using angiotensin II as standard. The results are expressed as the amount of angiotensin present after 2½ hours incubation. A

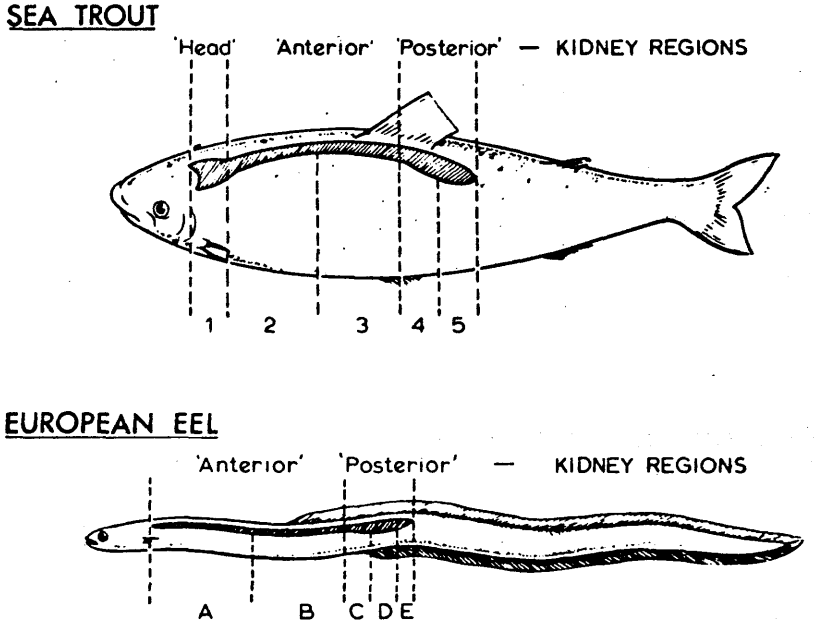


Fig.39. Diagramatic representation of zones of fish kidney extracted for renin (see table 13).

Kidney region		Eel		Sea Trout	
		Section (Fig.39)	Angiotensin II nM /hr.	Section (Fig.39)	Angiotensin II nM/hr.
Head		/	/	1	10
Anterior	A		10	2	39
	B		10	3	290
Posterior	C		144	4	290
	D		258	5	290
	E		258	/	/

Table 13. Renal renin assay of zones of fish kidney.

detectable amount of angiotensin was formed in all instances (Table 14), the rate of angiotensin formation being comparable with that obtained with single glomeruli (Brown et al, 1965).

4.2.1.2. Amphibia

Renin could not be detected by direct or indirect assay in the kidney of Rana temporaria.

4.2.1.3. Reptiles and birds

A small amount of renin-like activity was demonstrable in each of the nine reptiles and birds when renal extracts were incubated with substrate. On direct injection some of the extracts produced a rapidly acting pressor response.

4.2.1.4. Mammals

Renin was demonstrable both by direct and indirect assay in most mammalian species studied. Particularly large amounts were found in the rabbit, polar bear and the porpoise. It may be relevant that the porpoise was pregnant at the time of death; plasma renin concentration is known to be high in human pregnancy (Brown et al, 1963).

TABLE 14.

RENIN CONCENTRATION IN EEL GLOMERULI

No. of glomeruli.	Angiotensin (nM) at 26 hours.
1	10
1	34
1	15
2	19
4	36
7	36

See text for the method of renin assay.

4.2.1.5. Discussion

The positive finding in this study is that a renin-like enzyme has been identified in kidney extracts of a wide variety of vertebrates. Taken with earlier work (page 157 to 170) these observations make it likely that renin is present in all vertebrate classes.

As stressed earlier, (page 36) negative findings using this technique do not indicate that renin is absent in a particular species. Renin could not be demonstrated in kidney tissue of the frog for example. This is most likely to be a technical failure since renin is known to be present in other amphibian kidneys (page 163)

The observation on fish renin described above suggests that at least part of the renin in the kidney of the eel is associated with the glomerulus. Similar observations have been made in mammals (see review by Brown et al, 1968; Cook, 1971), but not to date in fish. The observation is of interest in relation to the possibility that renin may regulate glomerular

filtration and the flow of blood through the glomerulus (see Brown et al, 1972). Large changes of glomerular filtration and renal blood flow occur in fish on changing from a fresh water, to a marine environment (see page 192). As will be discussed renin is suitably placed to mediate or contribute to these effects in the fish. The possible significance of these findings is discussed on page 189.

4.2.2. Renal renin and plasma renin-substrate in eels adapted to sea water and fresh water environments.

The source of eels and the nature of their accommodation during the experiments are discussed on page 51. Initially, all eels had been adapted to fresh water. Nine were further maintained in fresh water and nine - matched individually for weight and length - were adapted to sea water by increasing the salinity of their environment gradually over 4 days to a specific gravity of 1.022. This was achieved by adding an amount of "Meersalz" (630 gm) every 12 hours, which was sufficient to raise

the specific gravity by 0.0028. After the period of adaptation the eels were maintained in sea water for between 9 and 31 days.

Matched pairs of eels were studied at the same time, between 2 and 3 being present in a tank at any one time. The system of matching was necessary because the experiment lasted from July to November (1970) and water temperature varied from 7 to 17°. Also, the hours of daylight were decreasing during the course of the experiment.

At the conclusion of the experiment a sample of blood was taken (see page 53) for renin substrate measurement, the eels were killed and the kidneys were removed, weighed and extracted for renin.

4.2.2.1. Renal renin

There was no significant difference in renal renin content in the eels adapted to fresh water and sea water environments (students' $t = 0.25$, $p = 0.8 - 0.9$). Nor was there a significant difference when data from the

individual pairs were assessed by paired t test ($t = 0.23$, $p = 0.8$ to 0.9, Table 15).

4.2.2.2. Plasma concentration of renin-substrate.

The results illustrated in Table 15 suggest that adaptation to sea water is associated with a decrease of plasma concentration of renin-substrate. In seven of the nine matched pairs substrate concentration was lower in the sea water eel. However, these differences did not achieve statistical significance at the 5% level (students $t = 1.45$, $p = 0.1$ to 0.2; paired t test, $p = 0.05$ to 0.1).

4.2.2.3. Discussion

The experiments described above have shown that adaptation to sea water in the eel is not associated with any significant change of renal renin. It is also likely, but of only marginal statistical significance, that the plasma concentration of renin-substrate decreases in these circumstances.

The relation of these findings to earlier work and their significance are discussed on page 191.

Table 15. Renal renin and plasma renin-substrate concentrations in eels adapted to sea water and freshwater environments.

	Renal Renin					Plasma Renin-Substrate				
	Range u/mg	Mean u/mg	SD u/mg	n	Paired t test	Range µ M	Mean µ M	SD µM	n	Paired t test
Fresh-water eels	0.28-3.01	1.09	0.78	9	p=0.8-0.9 t =0.23	0.68-1.60	1.08	0.33	9	p=0.05-0.1 t = 2.22
Sea-water eels	0.28-1.95	1.17	0.60	9	DF = 8	0.53-1.16	0.89	0.21	9	DF = 8
Fresh- and sea-water eels	0.28-3.01	1.13	0.68	18	/	0.53-1.60	0.98	0.29	18	/

4.2.3. Preliminary study of the pressor material in extracts of the corpuscle of Stannius in the eel.

In a previous study (Chester Jones et al, 1966) it had been shown that saline extracts of the corpuscle of Stannius from the eel, produced a prolonged pressor response when injected intravenously in the rat (Figure 40). The possibility that the response was due to renin was considered, although it was apparent that the response produced by renal renin was briefer in duration. Further studies were begun recently and preliminary results are included here.

4.2.3.1. Effect of corpuscle extract in the nephrectomised rat.

One possible explanation for the slow pressor response to injected corpuscular extract is that it stimulates renin release from the nearby kidney (the corpuscle is closely apposed to the surface of the kidney). To investigate this, a corpuscle extract was tested on 3 nephrectomised rats.

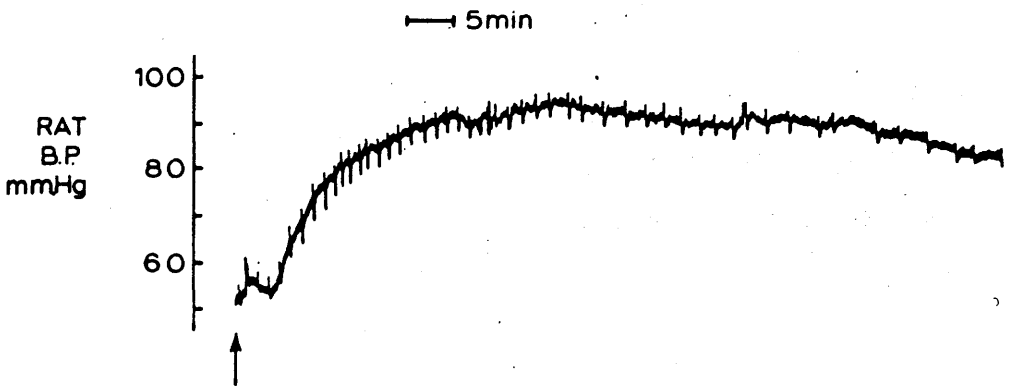


Fig.40. The vasopressive response to 19 mg of extracted corpuscles of Stannius after i.v. injection into the nephric rat at the time indicated by the arrow.

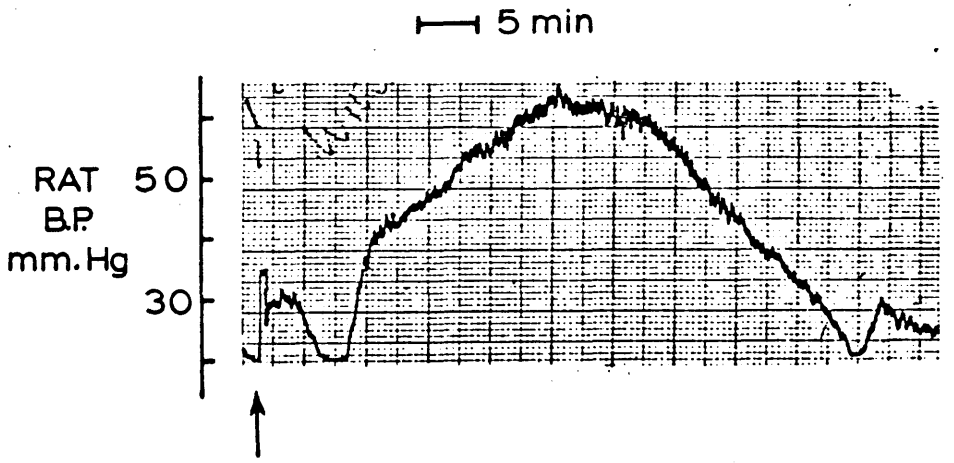


Fig.41. The vasopressive response to 22 mg of extracted corpuscles of Stannius after i.v. injection into the anephric rat, at the time indicated by the arrow.

Pressor responses were obtained on 2 of these occasions; the most marked, illustrated in figure 41, being similar to responses seen in test rats with intact kidneys. It is unlikely, for these reasons, that the corpuscular extract acts by stimulating renin release from the kidney.

4.2.3.2. Comparison of corpuscle extracts and kidney renin.

Dialysed extracts of eel kidney injected into the anaesthetised rat produced little or no pressor response. Incubation of the same extract with ox substrate in the presence of DP as an inhibitor of angiotensinase (page 85), led to rapid production of angiotensin-like pressor material as described earlier (page 85).

Also injected into the rat, a corpuscle extract produced a pressor response on 3 of 3 occasions to 11 or 22 mg extract. The time course and height of these responses when present, was always considerably greater than the pressor effect with renin-containing extracts. Incubation of the same extracts of corpuscle with

ox substrate or eel plasma (Fig 42) and DP as above produced an amount of angiotensin-like material which was difficult to differentiate from the blank containing ox substrate or eel plasma and DP incubated alone.

This experiment suggests that the corpuscle pressor effect is not attributable to an enzyme which reacts in a renin-like manner with ox or eel renin-substrate.

A further possibility tested was that the extract contained an accelerator of the renin-substrate reaction. This was investigated by adding to an eel renin-renin substrate incubation an amount of corpuscle extract which on incubation with substrate had no pressor effect. No augmentation of the renin-substrate reaction occurred when the corpuscle extract was added (Fig 42).

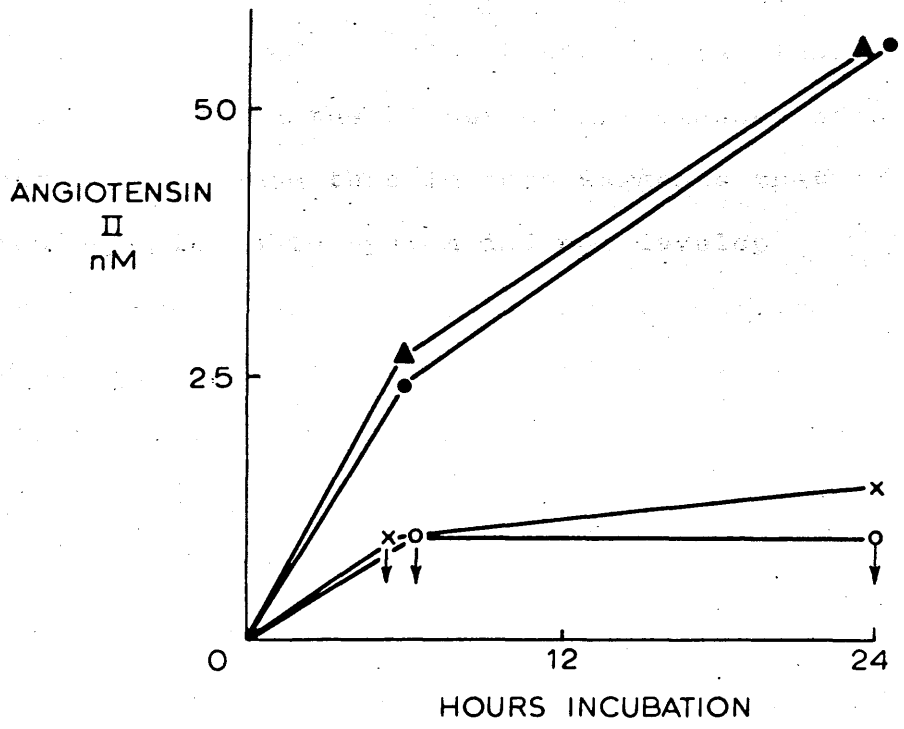


Fig.42. Effect of corpuscle of Stannius extract (4 mg/ml) on the reaction of eel renin (1 mg kidney/ml) with eel substrate (20% v.v. eel plasma). O = eel substrate alone, X = eel substrate + corpuscle extract, ▲ = eel substrate + eel renin, ● = eel substrate + eel renin + corpuscle extract. Each point represents the mean of duplicate assays.

4.3. Discussion of data obtained

4.3.1. The wide distribution of renin amongst the vertebrate classes

Together with earlier studies (pages 157- 170) the results obtained in this thesis suggest that renin is present in the kidney of all classes of vertebrate. Because this in turn suggests that the renin-angiotensin system did not develop during the evolution of mammals,birds, reptiles or amphibia, it is unlikely that the enzyme is specifically involved in a mechanism peculiar to one of these classes. Thus, although renin can increase the osmolality of urine (see Page and McCubbin,1968), it is unlikely that renin evolved primarily in relation to the mechanism responsible for increasing urine concentration above that of plasma, because birds and mammals only have this capacity.

A similarly broad distribution of renin is found amongst warm- and cold-blooded vertebrates regardless of whether they are carnivorous, omnivorous or herbivorous or whether their habitat

is terrestrial or aquatic. It is unlikely therefore that these were important selective factors in the evolution of the renin-angiotensin system.

4.3.2. Role of renin in vertebrates

Opinions on the physiological or pathological role of renin are divided. Because at least six distinct pharmacological effects have been produced by injection of renin or angiotensin, it is sometimes assumed that similar effects occur physiologically or pathologically. Evidence on this point is scanty and almost exclusively obtained from studies in man and laboratory mammals (see Brown et al, 1971b).

For example, injected renin and angiotensin have distinct effects on renal function, leading to marked changes in the volume and sodium content of urine. The mechanism of these effects is not understood, though it is likely that changes in both glomerular filtration and tubular reabsorption are involved. It is also possible that endogenous renin and angiotensin act

locally within the kidney of the mammal (Brown et al, 1970). The present finding of renin in close association with the glomerulus of the fish (4.2.1.1.) is relevant because large changes of glomerular filtration occur in migratory fish on changing from a fresh-water to a sea-water environment (Hickman and Trump, 1969). Although renin is suitably located anatomically to produce these changes by direct vasoconstriction of either afferent or efferent arterioles, no significant changes of renal renin were observed when the eels were acclimatized to sea water. Although the changes of renin substrate observed in the present experiments were significant only at the 10% level, they were in a direction which would have led to a greater amount of angiotensin being formed in the fresh water environment. Taken together the present observations on substrate and the experiments of Sokabe et al (1966, 1968) suggest that a gradual decrease of activity in the renin-angiotensin system occurs during acclimatization to a sea-water environment. For

this to be linked with the decrease of glomerular filtration which occurs at this time the predominant effect of renin and angiotensin in the kidney would have to be that of vasoconstriction of the efferent arteriole. Clearly, further study is needed.

Studies here (Table 12) and elsewhere, on renin in the kidneys of different species of fish show that there was generally more renin extractable from those in fresh water from those in sea water (Capelli et al, 1970; Mizogami et al, 1968). This is likely to mean that there is more renin in the fresh water fish, but for reasons which are discussed on page 16 there are other possible explanations for the difference and a proper calibration of techniques is needed before a valid comparison can be made.

Migrating fish have a lower glomerular filtration rate and a <u>lower</u> rate of sodium excretion from the kidney, when acclimatized to sea water. Thus, the kidney does not help to correct the potential disturbance of sodium balance which confronts the sea water fish, and

the excess sodium is eliminated mainly by way of the gills (see reviews by Conte, 1969 and Hickman and Trump, 1969). If angiotensin does regulate glomerular filtration under these circumstances, its vasoconstrictor effect would have to be exerted on the efferent glomerular arteriole.

4.3.3. Stimuli to renin.

The stimuli to renin release in the mammal have not been identified. Although there is little doubt that reduction of blood volume or loss of sodium can lead to renin release, the way in which this occurs is far from clear. However, with a few exceptions reduction of plasma volume and loss of sodium occur together in the mammal (see review by Brown et al, 1966).

The migrating eel may represent an interesting exception in the fish. On changing from fresh-water to sea-water, the eel is confronted with a reversal of its osmotic environment. In fresh water, there is a tendency to a sodium loss and influx of water, and in the sea, the eel is exposed to sodium influx and water

loss. Since sodium gain would increase total body sodium and water loss decrease plasma volume, a study of the renin in the migrating fish could increase understanding of the stimuli to renin release.

4.3.4. Renin and aldosterone in fish

The ability of renin and angiotensin to stimulate aldosterone secretion in mammals is well-known and it is likely that sodium balance in the mammal is at least partly controlled by a feed-back mechanism involving renin and aldosterone (see Brown et al, 1966). The status of aldosterone in fish is less certain. The subject is reviewed by Chester Jones et al (1969) and Idler (1968) and is briefly re-stated here: Phillips et al (1959) isolated small quantities of aldosterone from the plasma of the Pacific salmon (Oncorhynchus nerka), although Idler, working with blood from the same species at the same stage of maturation was unable to confirm the observation. Similarly, Sandor et al (1966, 1967) were unable to demonstrate the

ability of adrenocortical tissue in the eel (Anguilla anguilla) to synthesise aldosterone, and subsequently Hargreaves and colleagues (1970) similarly failed with Poecilia latipinna and Salmo gairdneri. However, Truscott and Idler (1968) showed that adrenal tissue of the herring, (Clupea harengus harengus) could form aldosterone from corticosterone. Subsequent studies have failed to identify aldosterone in the Agnatha (Weisbart and Idler, 1970), the Selachii (Simpson and Wright, 1970) and the Chondrostei (Idler and Sangalang 1970). As Idler suggests, the technical difficulty in identifying the extremely small amounts of steroid present could explain this apparently conflicting evidence. Alternatively another steroid hormone may regulate sodium in fish. For example Ball and colleagues (1971) have provided evidence to suggest that cortisol under ACTH control may regulate the transfer of gut and possibly gill electrolytes during the migration of Anguilla anguilla from fresh-water to sea-water.

If a function of renin and angiotensin is to stimulate the secretion of aldosterone or other steroids, renin could act as a local tissue hormone. The site of adrenal steroid formation in the fish, the interrenal, is embedded in the wall of the posterior cardinal sinus upstream of the renin-containing posterior kidney. Renin released from the kidney would enter the cardinal sinus and it may be that angiotensin formed in venous blood could stimulate interrenal steroid production without recirculation in arterial blood, the whole system acting via a "portal" circulation. In fact renin may act as a local hormone in amphibians too, since Belocci and colleagues (1971) have found interrenal tissue to be located close to the glomerular arteriole and renal tubule. The possibility that renin and angiotensin acting as a local tissue hormone may stimulate steroid production has been discussed (Brown et al, 1968).

The ability of injected aldosterone to affect sodium transport mechanisms in fish is more certain (see review by Chester Jones et al,

1969). Aldosterone is capable of correcting the defective sodium transport mechanism in the gill of the adrenalectomised eel. It may be, therefore that release of aldosterone (or some other mineralocorticoid) is stimulated by renin in the fresh-water fish.

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