THE QUANTITATION AND SIGNIFICANCE OF RENIN IN BIOLOGICAL FLUIDS

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DECLARATION AND ACKNOWLEDGEMENTS

I declare that this thesis is of my own composition and that it is a record of original work conducted in the Department of Human Physiology and Pharmacology, at the University of Adelaide, during the years 1966, 1967, 1968 and 1969.

The experiments described herein have not been submitted for any other degree or diploma.

I would like to express my gratitude to my supervisors, Dr S. L. Skinner and Professor R. F. Whelan for their guidance and interest, and I should also like to thank my colleagues, Dr R. L. Hodge and Dr R. C. Hall, for their interest; Miss F. Katic and Mrs M. Deutrom for the excellent technical assistance they provided; and those colleagues and students who were willing to act as subjects for the investigations described.

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PREFACE

Renin is an enzyme which acts on an α_2-globulin substrate present in plasma to produce the decapeptide angiotensin I. Angiotensin I is converted to the octapeptide, angiotensin II, by the action of a chloride-dependent enzyme present in the blood and the lungs. Angiotensin is a factor in the control of the secretion of aldosterone and is a powerful vasoconstrictor. Therefore, renin is indirectly concerned with circulatory homeostasis.

This thesis describes the experimental techniques and results obtained in studies on the presence of renin in human urine and factors influencing its excretion; the presence of renin in human maternal and foetal tissues; and the contribution of renin and renin substrate levels to the changes in renin activity induced by natriuretic agents, the hormonal variations of a normal menstrual cycle, oral contraceptives and pregnancy.

The role of tachyphylaxis in the constrictor response to angiotensin in normal subjects has been investigated. Studies have also been carried out on the peripheral vascular response to angiotensin in the pregnant woman.

CONTENTS

HISTORICAL SURVEY

In 1836, Richard Bright described the association between hypertension manifested by left ventricular hypertrophy and diseases of the kidney. The recognition of this association and the concept of internal glandular secretion by various organs, including the kidney, as proposed by Brown-Séquard (1889, 1892) and Brown-Séquard and d'Arsonval (1892), led Tigerstedt and Bergmann (1898) to investigate the presence of substances in the kidney affecting the cardiovascular system. In renal tissue they found a non-dialysable, heat-labile substance, soluble in water but not in alcohol, which they called 'renin'. They concluded that the site of action of renin was the peripheral vascular apparatus, more particularly the peripheral ganglia of the vascular nerves.

Tigerstedt and Bergmann's work was the first definitive study of the pressor raising properties of renal extracts to meet with real success. Other workers reported a variety of results. Oliver (1897) failed to demonstrate a constrictor material in renal extracts using isolated frog's mesentery as an assay preparation. Vincent and Sheen (1903) studied the pressor and depressor properties of a number of tissues and concluded that, of all the tissues studied, the kidney was the most likely to produce a pressor response on intravenous injection and that this pressor response was converted to a depressor

response by prior boiling of the extract.

Shaw (1906) and Bingel and Strauss (1909) likewise obtained pressor responses on intravenous injection of renal extracts, but Miller and Miller (1911) and Collip (1928) failed to confirm these findings.

In the light of present day knowledge of the actions and properties of renin it is not surprising that these early workers, using a variety of techniques for extraction and assay, produced such conflicting results.

To study the association between the kidney and hypertension, it is desirable to produce, by experimental manipulation of the kidney, a persistent hypertension which can be directly attributed to the experimental procedure performed. Until 1934, no reliable technique existed which produced persistent renal hypertension. Bilateral nephrectomy was not associated with persistent hypertension (Backman, 1916; Cash, 1926), whereas manipulations of the renal vessels produced varying degrees of hypertension. For example, Katzenstein (1905) observed a transient rise in blood pressure following complete occlusion of the renal vessels; so did Backman (1916) and Cash (1926). Backman and Cash thus showed that, whereas bilateral nephrectomy failed to induce hypertension, hypertension did occur following reduction in functioning renal tissue plus ligation of the renal vessels.

Bridgman and Hirose (1918), however, failed to induce hypertension with partial occlusion of the renal vessels of the dog using aluminium bands to produce constriction of the renal vessels. Techniques applied by other workers met with varying degrees of success (references cited by Braun-Menéndez, Fasciolo, Leloir, Munoz and Taquini, 1946).

In 1934, Goldblatt, Lynch, Hanzal and Summerville described a method for the production of renal hypertension using a screw-clamp which partially occluded the renal vessels. This technique for production of renal hypertension was both dependable and reproducible. The hypertension produced with the Goldblatt clamp was studied in detail by Goldblatt, Lynch, Hanzal and Summerville (1934) and other workers (Goldblatt, 1937; Goldblatt, 1938; Goldblatt, Kahn, and Hanzal, 1939; Blalock and Levy, 1937; Blalock, Levy and Cressman, 1939). It was found to be independent of innervation of the kidney (Blalock and Levy, 1937; Goldblatt, 1937; Page, 1935), although Glenn, Child and Huer (1937), on the basis of transplant studies, suggested that the renal nerves may play a role in the maintenance of renal hypertension. Removal of the clamp on the renal vessel or removal of the clipped kidney led to remission of the hypertension induced by clamping. Actual necrosis of the renal tissue was not necessary for the onset of hypertension (Goldblatt, Lynch, Hanzal and Summerville, 1934), a finding contradictory to the earlier findings

of Cash (1926). Occlusion of the renal vessels, or of the aorta above the renal arteries (Goldblatt, Kahn and Hanzal, 1939) was thus found to be a specific mechanism for the induction of experimental hypertension, and was not reproduced by clamping or ischaemia of vessels to other organs such as the spleen (Goldblatt, Lynch, Hanzal and Summerville, 1934) or intestine (Blalock, Levy and Cressman, 1939).

Partial clamping of one renal artery produced hypertension which was not persistent. Severe and persistent hypertension depended upon the clamping of both renal arteries or unilateral clamping plus contralateral nephrectomy (Goldblatt, 1938).

In 1939, Page described another model for the production of persistent hypertension in the experimental animal due to constriction of the whole renal parenchyma. The constriction was induced by wrapping the kidney in cellophane which caused the formation of a fibrocollagenous hull.

Since these experiments showed a lack of nervous factors in the initiation of renal hypertension, and remission of hypertension occurred following removal of the ischaemic kidney, a humoral mechanism as the predominant factor in the initiation of renal hypertension became a possibility. Interest revived, therefore, in the action of renal extracts on the cardiovascular system, and studies of the changes in the content of pressor material in the kidney induced by renal artery clamping were undertaken.

Prinzmetal and Friedman (1936), and Harrison, Blalock and Mason (1936) found that extracts of clipped kidneys produced a greater constrictor response when injected intravenously into dogs than did extracts of normal kidneys. They also found that preparations of renal tissue from patients with hypertension possessed more powerful pressor actions than did extracts of kidneys from normotensive patients.

The most important point to establish was whether or not the ischaemic kidney released a vasoconstrictor material into the circulation following clamping of the kidney. Transplant studies performed by Houssay and Fasciolo (1937, 1938), Fasciolo, Houssay and Taquini (1938) and Fasciolo (1938a, b, c), in which clipped kidneys transferred from a donor dog to the neck of a recipient induced hypertension in the recipient, suggested that mechanical and nervous factors were not responsible for the initiation of the hypertension and that a humoral mechanism was involved. Moreover, in 1938, Fasciolo, Houssay and Taquini showed that the venous effluent of an ischaemic kidney contained a powerful vasoconstrictor substance which was non-dialysable. Mason and Rozell (1939) were, however, unable to repeat these experiments.

Further evidence for the release of a hypertension-producing substance by the kidney was obtained by Taquini in 1940. Taquini found that following release of a clamp which had completely

arrested the blood supply to the kidney, marked hypertension ensued. Such a finding suggested that a vasoconstrictor material was "washed out" into the circulation after the clamp was released. Furthermore, Page, in 1940, found that the peripheral plasma from renal hypertensive dogs was more powerfully vasoconstrictor than was renal venous plasma, and also more powerfully vasoconstrictor than the peripheral plasma from normal dogs. Since he used the isolated ear artery as an assay preparation, he may have been looking at angiotensin rather than renin itself. This could possibly account for the greater levels of vasoconstrictor material in the peripheral plasma as compared to renal venous plasma.

In 1941, Goldblatt, Kahn and Lewis, in an interesting study of the effect of ureteric clamping on the production of hypertension by renal artery clipping, confirmed the findings of Houssay and Fasciolo that venous effluent from a clipped kidney contained a vasoconstrictor material. Therefore, it appeared that the initiation of renal hypertension was based on a humoral mechanism, the substance involved being renin, since it possessed some of the characteristics of renin first described by Tigerstedt and Bergmann (Prinzmetal and Friedman, 1936; Pickering and Prinzmetal, 1937; Prinzmetal, Lewis and Leo, 1940 a and b).

Considerable enthusiasm was expressed in support of the role of renin in the initiation of not only renal hypertension, but

also of essential hypertension (Goldblatt, 1958), a role experimental observation has yet to justify. The absence of significant or consistent changes in renin levels in essential hypertension (Fasciolo, de Vito, Romero and Cucchi, 1964), or in all cases of renal artery stenosis (Brown, Davies, Lever and Robertson, 1964a), has cast a shadow on these original expectations, and even in the field of experimental hypertension all is not clear.

The findings of Regoli, Hess, Brunner, Peters and Gross (1962), Regoli, Brunner, Peters and Gross (1962), and Gross, Brunner and Ziegler (1965) on the relationship between renin content of the kidneys and renal hypertension and, in particular, the action of the contralateral unclamped kidney in maintaining high renin levels in the clipped kidney, and also the role of neurogenic factors in chronic renal hypertension (McCubbin and Page, 1963; Taquini, 1963), emphasize that the relationship between renin and renal hypertension is complex. Moreover, the occurrence of renal hypertension in rabbits successfully immunized against angiotensin following clipping of the kidney (Hedwall, 1968; Hutchinson and Johnston, 1969) further confuses the picture, especially since antirenin has proved successful in reducing blood pressure in acute and chronic renal hypertensive dogs (Wakerlin, 1958; Haas and Goldblatt, 1963; Deodhar, Haas and Goldblatt, 1964). Perhaps a break through of the effective antibody titre by endogenous angiotensin production results in the failure of remission of renal

hypertension following immunization of experimental animals to angiotensin.

A recent statement by Pickering (1968) comments on the present knowledge of the mechanism of production of experimental renal hypertension: "The mechanism by which renal artery constriction produces hypertension thus remains quite obscure. This is an extraordinary position, considering the relative simplicity of the procedure, its replicability and its reproducibility."

Renin is not the active vasoconstrictor or pressor compound. It is a proteolytic enzyme which acts upon an α_2-globulin substrate to form the decapeptide angiotensin I, which is split by a chloride-dependent enzyme in plasma to the octapeptide angiotensin II, the active pressor agent.

Kohlstaedt, Helmer and Page (1938); Kohlstaedt, Page and Helmer (1940) found that plasma was necessary for the vasoconstrictor action of partially purified renin on isolated, perfused preparations.

In 1940, two groups, Helmer and Page, and Braun-Menéndez, Fasciolo, Leloir and Munoz, published papers describing the formation of a pressor compound from the action of renin on plasma. Helmer and Page described this pressor compound as 'angiotonin', and Braun-Menéndez, Fasciolo, Leloir and Munoz described it as 'hypertensin'. These two terms remained in use until 1958 (Braun-Menéndez and Page, 1958) when the joint name of 'angiotensin' was agreed upon.

Braun-Menéndez, Fasciolo, Leloir and Munoz (1940) concluded that renin was an enzyme which acted on a pseudoglobulin fraction in plasma to produce angiotensin. Plentl, Page and Davis (1943a) similarly concluded that renin was an enzyme. It is therefore apparent that the formation of angiotensin in plasma would be dependent not only upon the concentration of enzyme present, but also upon the concentration of substrate present. In fact, Braun-Menéndez, Fasciolo, Leloir, Munoz and Taquini (1946) showed that variations in the amount of renin substrate in plasma influenced the rate of formation of angiotensin in the presence of a constant excess of renin. They also demonstrated the specificity of the renin-renin substrate reaction on a species basis; for example, pig renin fails to react with human substrate (Fasciolo, Leloir, Munoz and Braun-Menéndez, 1940), a finding characteristic of renins other than those derived from primates (Braun-Menéndez, Fasciolo, Leloir, Munoz and Taquini, 1946). Moreover, renin substrate is relatively specific to the proteolytic action of renin, although under certain conditions of pH it can be split by pepsin to produce pepsitensin, a compound with pharmacological properties similar to angiotensin (Croxatto and Croxatto, 1942; Blair, 1962) and the same amino acid sequence as angiotensin I (de Fernandez, Paladini and Delius, 1965).

Difficulties in the assay of angiotensin produced as a result of the action of renin on substrate were encountered due to the

presence of angiotensinases. Page and Helmer (1940) thought that renin itself destroyed angiotensin. However, Braun-Menéndez, Fasciolo, Leloir and Munoz (1940) showed that the removal of angiotensinases abolished the fall in concentration of angiotensin which was formed on incubation of renin with plasma.

Renin substrate was found to be present in the α_2-globulin fraction of plasma (Plentl, Page and Davis, 1943b) and formed in the liver (Page, McSwain, Knapp and Andrus, 1941; Leloir, Munoz, Taquini, Braun-Menéndez and Fasciolo, 1942).

Skeggs, Kahn, Lentz and Shumway (1957) undertook the isolation and purification of renin substrate, first isolating a polypeptide substrate which would react with renin, and later synthesizing a tetradecapeptide (Skeggs, Lentz, Kahn and Shumway, 1958) which liberated active pressor compounds identical to angiotensin I and angiotensin II when reacted with renin.

Skeggs, Lentz, Hochstrasser and Kahn (1963, 1964) purified hog renin substrate, isolating several forms of substrate - A, B_1, B_2, C_1 and C_2 - of which A, C_1 and C_2 were glycoproteins, differing only in glucosamine and hexose content.

Cook and Lee (1965) found no evidence of multiple peaks of rabbit renin substrate activity following elution from Diethylaminoethyl cellulose column and suggested that the multiple peaks found by Skeggs, Lentz, Hochstrasser and Kahn (1963, 1964) were the result of

partial acid degradation of the renin substrate molecule (Lee, 1969).

Helmer and Griffith (1952) showed that renin substrate levels in plasma could be increased by treating animals with oestrogens. Helmer and Judson (1967), and Skinner, Lumbers and Symonds (1969) showed that renin substrate levels in plasma influenced the rate of formation of angiotensin. Elevations in renin substrate were thought by other workers (Pickens, Bumpus, Lloyd, Smeby and Page, 1965; Haas and Goldblatt, 1967; Ayers, 1967) to be unimportant in determining the rate of formation of angiotensin in plasma since the concentration of substrate normally found in plasma was in relative excess. This is now known not to be so; therefore, elevations in renin substrate can influence plasma renin activity.

Skeggs, Marsh, Kahn and Shumway (1954) found that two forms of angiotensin existed - angiotensin I (the decapeptide) and angiotensin II (the octapeptide) - the conversion of angiotensin I to angiotensin II being dependent upon the presence of a chloride-dependent enzyme in plasma. They extracted and purified the chloride-dependent enzyme from horse plasma and studied its properties (Skeggs, Kahn and Shumway, 1955). More recently, it has been shown by Ng and Vane (1967) that large concentrations of this converting enzyme exist in the lungs.

Determination of the amino acid composition of angiotensin I (Peart, 1955, 1956; Skeggs, Marsh, Kahn and Shumway, 1955) and of

angiotensin II (Lentz, Skeggs, Woods, Kahn and Shumway, 1956) and of the amino acid sequence of angiotensin I (Elliot and Peart, 1956, 1957) led to the successful synthesis of angiotensin, isoleucyl^5 angiotensin being synthetized by Bumpus, Schwarz and Page (1957, 1958) and asparaginyl isoleucyl^5 angiotensin by Schwyzer, Iselin, Kappeler, Riniker, Rittel and Zuber (1957).

Successful synthesis of angiotensin enabled determination of amino acid groupings necessary for biological activity to be made (Page and Bumpus, 1961). In 1962, Smeby, Arakawa, Bumpus and Marsh suggested an α-helical conformation for angiotensin based on optical rotatory dispersal studies, internal hydrogen bonding being necessary for the tertiary structure of the molecule and hence for biological activity. However, the α-helical conformation of angiotensin II in aqueous solutions, as described by Smeby, Arakawa, Bumpus and Marsh, (1962), has not been substantiated by Paiva, Paiva and Scheraga (1963) who suggest a random conformation of the peptide in aqueous solutions.

Renin itself has been subjected to intensive investigation. Early studies on the purification of renin were carried out by Helmer and Page in 1939. Haas, Lamfrom and Goldblatt (1953) purified hog renin and obtained a preparation of high activity, but noted the instability of purified renin at a neutral pH. Renin is a proteolytic enzyme which is remarkably stable over a wide pH range in impure preparations (Haas, Goldblatt and Gipson, 1963). This property makes

it possible to separate renin from renin substrate by acid denaturation of the latter, leaving the renin molecule intact (Skinner, 1967).

Renin specifically attacks the leucyl-leucyl bond of the renin substrate molecule to liberate angiotensin I. However, it does not depend (as do other specific proteolytic enzymes) on the presence of adjacent aromatic or basic amino acids (Skeggs, Kahn, Lentz and Shumway, 1957).

The molecular weight of hog renin (Kemp and Rubin, 1964) and human renin (Warren and Dolinsky, 1966) is in the region of 40,000 to 50,000. Peart, Lloyd, Thatcher, Lever, Payne and Stone (1966) extracted and purified hog renin and on electrophoresis against a specific antiserum, obtained one major and two minor precipitin lines.

Antibodies to renin were first produced by Johnson, Wakerlin and Goldberg (1941) and by Goldblatt, Katz, Lewis, Richardson, Guevara-Rojas and Gollan in 1942, and have been used widely in the investigation of the role of renin in experimental hypertension (Helmer, 1958; Wakerlin, 1958) as a criterion for the identification of renin (Skinner, 1967) and in studies on the location of renin within the kidney (Edelman and Hartroft, 1961). The antigenic and catalytic sites of renin appear to be separate, as shown by the studies of Haas, Goldblatt and Gipson, 1963.

The exact location of renin in the kidney is still not

clear. Tigerstedt and Bergmann, in 1898, concluded that renin was located in the renal cortex and a considerable number of studies have been carried out to determine the 'renin containing cells' of the kidney.

In the renal cortex, renin occurs in association with the glomeruli. The deeper the glomerulus from the outer surface of the kidney, the lower the renin content (Brown, Davies, Lever, Robertson and Tree, 1963). Cook and Pickering (1962) assayed the renin content of the upper and lower halves of individual glomeruli and found that renin was located in the vascular pole of the glomerulus, i.e. in association with the juxtaglomerular apparatus. Edelman and Hartroft (1961) and Hartroft (1963) found fluorescent antirenin localized in the juxtaglomerular cells lining the afferent arteriole, supporting the hypothesis of Goormaghtigh (1945) that the granules of the juxtaglomerular cells contain renin. Indirect evidence for Goormaghtigh's hypothesis was also obtained by Marx and Deane (1963) and Verniory and Potuliege (1964) who showed that a correlation existed between renin content and the degree of granularity of the juxtaglomerular cells.

However, microdissection studies done by Bing and Kazmierczak (1959, 1960, 1962, 1964) and Bing (1964) found that renin was located mainly in those fragments of renal tissue containing tubular remnants, rather than the vascular portions of the

juxtaglomerular apparatus, and furthermore, that renin was present in nephrogenic renal tissue devoid of both granular and non-granular epithelioid cells.

An interesting observation was made by Hess and Pearce (1959) and Marx and Deane (1963). They observed that the glucose-6-phosphate dehydrogenase content of the macula densa parallels the changes in granulation of the juxtaglomerular cells induced by 'renal clip hypertension' (Hess and Pearce, 1959), low sodium diet (Marx and Deane, 1963) and infusions of angiotensin (Marx, Deane, Mowles and Shephard, 1963). Whether renin is formed in the macula densa and stored in the juxtaglomerular cells (Bing and Kazmierczak, 1962) or whether the macula densa acts only as a 'sensing site' which controls the release of renin from the juxtaglomerular cells remains to be determined.

Renin is not exclusively confined to the kidney. Werle, Vogel and Goldel (1957) and Werle, Baumeister and Schmal (1962) described a renin-like enzyme in the submaxillary gland of the white mouse. This enzyme released a vasoactive principle which was similar to angiotensin on incubation with plasma. Turrian (1960) found, however, that it was not subject to control by sodium loading or cortexone administration. Dengler (1956) found a renin-like enzyme in extracts of hog aorta. Gould, Skeggs and Kahn (1964) made a more extensive study of the distribution of pig extra-renal renin,

describing an enzyme of similar biochemical properties to renal renin in a variety of tissues. They found only minute amounts of renin in the placenta and myometrium of the pregnant pig.

Renin-like enzymes occur in the placenta of the rabbit (Gross, Schaetelin, Ziegler and Berger, 1964; Bing and Farrup, 1966), the cat (Stakeman, 1960), the dog (Hodari, Bumpus and Page, 1967) and in human amniotic fluid (Brown, Davies, Doak, Lever, Robertson and Tree, 1964). Furthermore, renin is extractable from a variety of body fluids such as lymph (Lever and Peart, 1962), urine (Brown, Davies, Lever, Lloyd, Robertson and Tree, 1964) and blood (Helmer and Judson, 1963; Lever, Robertson and Tree, 1964; Boucher, Veyrat, de Champlain and Genest, 1964; Pickens, Bumpus, Lloyd, Smeby and Page, 1965; Gould, Skeggs and Kahn, 1966; Haas, Gould and Goldblatt, 1968).

The development of assay techniques sensitive enough to detect renin or angiotensin and to correlate these levels to both the physiological and pathological situations has been of considerable importance.

Assays of renin in blood can be broadly divided into two groups. First, those that measure the so-called 'renin activity' of plasma (e.g. methods described by Boucher, Veyrat, de Champlain and Genest, 1964). Renin activity is determined from the amount of angiotensin formed per unit time as a result of the reaction between

renin and endogenous renin substrate. This is a qualitative assay, since it does not take into account the variations in the levels of endogenous substrate that may occur. The second group of renin assays are those which determine the co-called plasma 'renin concentration' (e.g. methods described by Lever, Robertson and Tree, 1964; Skinner, 1967). The endogenous substrate is removed or denatured and a constant amount of an artificial substrate added to the system prior to incubation. In this system, therefore, the amount of angiotensin formed reflects the levels of enzyme present.

An angiotensin assay was developed by Scornik and Paladini (1961), but suffered from the disadvantage that relatively large volumes of blood had to be taken for extraction of angiotensin. A more recent development has been the radioimmunoassay of angiotensin, using antibodies raised against synthetic angiotensin II (Boyd, Landon and Peart, 1967; Catt, Cain and Coghlan, 1967). This method increases the ease of assaying whilst the adaptation of radioimmuno-assay to determine the amount of end-product formed following *in vitro* incubation of renin with substrate (Boyd, Adamson, Fitz and Peart, 1969) allows for greater ease in determining renin levels in certain experimental situations.

The considerable variations in the methods used for the assay of renin make it difficult to interpret and correlate results obtained from various laboratories (Haas, Gould and Goldblatt, 1968).

The biological role of renin is dependent upon the actions of angiotensin. Angiotensin, weight for weight, is the most powerful pressor substance known. It has both direct and indirect actions in raising blood pressure. The pressor effect of angiotensin was demonstrated in man by Wilkins and Duncan, 1941; Bradley and Parker, 1941; Corcoran, Kohlstaedt and Page, 1941. Direct perfusion of vascular beds has shown that angiotensin is vasoconstrictor in all situations studied, constricting the hand vessels (Wilkins and Duncan, 1941; De Bono, Lee, Mottram, Pickering, Brown, Keen, Peart and Sanderson, 1963; Scroop, Walsh and Whelan, 1965), muscle vessels (De Bono *et al*, 1963; Scroop, Walsh and Whelan, 1965) and the splanchnic vasculature (De Bono *et al*, 1963). Further studies have revealed that it also has a central indirect action, the efferent pathway being the sympathetic nervous system (Bickerton and Buckley, 1961; Laverty, 1963; Scroop, Walsh and Whelan, 1965). Angiotensin does not appear to stimulate the peripheral sympathetic nervous system in man (Whelan, Scroop and Walsh, 1969), but potentiation of the constrictor response to infused noradrenaline by angiotensin has been observed (Scroop and Walsh, 1968). Although animal studies have revealed that angiotensin can release catecholamines from the adrenal medulla of the cat (Feldberg and Lewis, 1964) and the dog (Peach, Cline and Watts, 1966), this has not been satisfactorily demonstrated in man (Vincent, Kashemsant, Cuddy, Fried,

Smulyan and Eich, 1965; Whelan, 1967). Angiotensin also stimulates the contraction of uterine muscle (Gross and Turrian, 1960; Paiva and Paiva, 1960), guinea pig intestine (Gross and Turrian, 1960) and rat colon (Bisset and Lewis, 1962). The latter tissue has been used for continuous assay of changes in angiotensin blood levels (Regoli and Vane, 1964).

There is little convincing evidence for a direct myocardial action of angiotensin. The effects of angiotensin on the myocardium described in the literature can be attributed to one or more of the following: effect of reduction in coronary blood flow; stimulation of sympathetic nerves; or initiation of baroreceptor reflexes as a result of the pressor action of the drug. For example, the negative inotropic effect observed by Downing and Sonnenblick (1963) is probably due to the effect of a reduction in coronary blood flow. Koch-Weser (1964) did, however, describe a positive inotropic effect of angiotensin on kitten myocardial preparations. In the intact animal, angiotensin causes a reflex bradycardia (Middleton and Wiggers, 1944; Johnson and Bruce, 1962; Nishith, Davis and Youmans, 1962; Scroop, Walsh and Whelan, 1965).

In the experimental animal and isolated tissue preparations a reduction in responsiveness to large doses of angiotensin has been observed. This phenomenon is described as "tachyphylaxis". Khairallah, Page, Bumpus and Turker (1966) attributed this loss of

responsiveness to angiotensin to saturation of receptor sites and found that addition of angiotensinases to perfusing fluids reversed tachyphylaxis. Kaplan and Silah (1964) have described a clinical test for detection of high endogenous renin levels in hypertensive patients which is based on a reduction in sensitivity to intravenous angiotensin. A similar reduction in pressor sensitivity has also been observed with pregnant women (Chesley, Talledo, Bohler and Zuspan, 1965) in whom high endogenous renin levels occur.

One of the most important actions of the renin angiotensin system is its role as a factor in the control of aldosterone secretion from the zona glomerulosa of the adrenal cortex. Aldosterone was first isolated by Simpson, Tait, Wettstein, Neher, von Euw and Reichstein in 1953. A humoral mechanism as a factor in the control of aldosterone secretion was implicated by the cross-circulation experiments of Yankopoulas, Davis, Klinman and Peterson (1959) and Denton, Goding and Wright (1959a and b). The work of Davis, Ayers and Carpenter (1961); Davis, Carpenter, Ayers, Holman and Bahn (1961); Mulrow and Ganong (1961); Mulrow, Ganong, Cera and Kuljian (1962); and Ganong and Mulrow (1962) showed that the kidney, and more particularly renin, was involved in the release of aldosterone. It has been further shown that it is angiotensin which regulates aldosterone secretion, not renin (Carpenter, Davis and Ayers, 1961; Biron, Koiw, Nowaczynski, Brouillet and Genest, 1961;

Blair-West, Coghlan, Denton, Goding, Munro, Peterson and Wintour, 1962; Davis, 1962; Ganong, Mulrow, Boryczka and Cera, 1962; Mulrow, Ganong and Boryczka, 1963). The action of angiotensin on the zona glomerulosa is to stimulate the biosynthesis of aldosterone (Kaplan 1964; Bledsloe, Island and Liddle, 1966).

The renin angiotensin system is therefore clearly implicated as a factor in the control of sodium homeostasis through its role as a regulator of the secretion of aldosterone. It is also possible that the renin angiotensin system is involved intrarenally in the control of sodium excretion. Thurau, Schnermann, Nagel, Horster and Wahl (1966) suggested that this system may act as an intrarenal sodium conserving system. This hypothesis remains to be proved. Angiotensin in low doses administered to both man and animals is antidiuretic and antinatriuretic, but when administered to animals in large doses, it is diuretic and natriuretic (Louis and Doyle, 1964). A similar reversal of the antinatriuretic response to angiotensin is seen in human subjects in whom high endogenous levels of angiotensin are present (Laragh, Cannon, Bentzel, Sicinski and Meltzer, 1963) or following infusions of very high doses of angiotensin (Louis and Doyle, 1964). Such a biphasic action of angiotensin on sodium and water excretion is difficult to interpret. Louis and Doyle (1964) postulate that in low doses angiotensin is antinatriuretic by virtue of its vasoconstrictor

action which reduces glomerular filtration rate. They postulate that it also has an inhibitory action on the tubular transport of sodium. With increasing doses of angiotensin, this inhibitory action becomes relatively greater and overrides the action of angiotensin in reducing sodium excretion as a result of the reduction in filtration rate which, moreover, progressively diminishes with increasing dose levels of angiotensin. Angiotensin has been implicated as an inhibitor of the proximal tubular transport of sodium by Leyssac (1965), although this was not substantiated by Thurau, Schnermann, Nagel, Horster and Wahl (1966). Using stop-flow techniques, Vander (1963) demonstrated inhibition by angiotensin of sodium transport in the distal tubule, and more recently an inhibitory action of angiotensin on the ascending limb sodium pump has been described by Healey, Elliott and Pearce (1969).

Since the renin angiotensin system is involved in sodium homeostasis, it is not surprising to find that it is influenced by changes in sodium balance. Sodium depletion and repletion (Veyrat, de Champlain, Boucher and Genest, 1964; Genest, de Champlain, Veyrat, Boucher, Tremblay, Strong, Koiw and Marc-Aurèle, 1964; Brown, Davies, Lever and Robertson, 1966, respectively) are associated with elevations and depressions in plasma renin levels.

Tobian (1964) suggested that renin release is stimulated by changes in afferent arteriolar tone. Many of the factors known to

effect renin release could be mediated by such a baroreceptor mechanism; for example, release of renin by a reduction in renal perfusion pressure (Skinner, McCubbin and Page, 1963, 1964) and sympathetic nerve stimulation (Vander, 1965) could affect afferent arteriolar tone and thus release renin. Sodium depletion by reducing circulating blood volume, and sodium loading by expanding blood volume, would also affect the degree of stretch of the afferent arteriole (Vander and Luciano, 1967a). However, not all experimental findings can be explained on the basis of such a baroreceptor mechanism as has been postulated by Tobian. In particular, the action of diuretics in suppressing the release of renin induced by aortic clamping (Vander and Miller, 1964) and the dissociation of the haemodynamic changes induced by norepinephrine from sodium excretion (Nash, Rostorfer, Bailie, Wathen and Schneider, 1968) are not satisfactorily explained on the basis of a baroreceptor mechanism alone. Moreover, those changes which would stimulate a baroreceptor mechanism would alter either the sodium load or the sodium concentration at the macula densa. This applies also to the potentiating action of the intact sympathetic nervous system on renin release (Vander, 1965; Vander and Luciano, 1967b; Bunag, Page and McCubbin, 1966; Hodge, Lowe and Vane, 1966), although a direct action of the renal nerves on 'renin-secreting' cells cannot be eliminated. The proximity of the macula densa to the juxtaglomerular cells and the

realisation that the sodium load and concentration in the tubular fluid varies with variations in glomerular filtration rate has led to the hypothesis that renin release is controlled by a 'sensing device' at the macula densa. This remains to be proved, as does the mechanism by which renin release is stimulated. It is not osmolality, since both hypertonic mannitol, which increases the distal tubular osmolality, and hypertonic saline, which decreases the distal tubular osmolality (Gottschalk and Mylle, 1959), both suppress renin release (Vander and Miller, 1964). Vander (1967) suggested that it is the sodium load at the macula densa which stimulates renin release. This hypothesis would explain the suppression by diuretics of renin release induced by aortic clamping. Diuretics which inhibit reabsorption of sodium proximal to the macula densa and so increase the load of sodium reaching the macula densa therefore suppress renin release.

Recent studies by Landwehr, Schnermann, Klose and Giebisch (1968) on tubular fluid composition during a reduction of glomerular filtration rate suggest that an inverse relationship exists between sodium concentration of distal tubular fluid and the glomerular filtration rate; that is, if the glomerular filtration rate falls, the sodium concentration of the early distal tubular fluid is high as a result of increased abstraction of water from the loop of Henle (Schnermann, 1968). Such elevations in sodium concentration may then

be postulated to stimulate the release of renin. Thurau, Schnermann, Nagel, Horster and Wahl (1966) have suggested, on the basis of proximal tubular collapse time studies, that when high concentrations of sodium are perfused through the distal tubule, the release of renin which occurs acts via angiotensin to reduce glomerular filtration rate as measured by proximal tubular collapse time. However, this evidence is indirect. A recent hypothesis for autoregulation by renin also based on changes in sodium concentration at the macula densa as a stimulus for renin release has been suggested by Britton (1968). Britton suggests that a high flow rate along the loop of Henle increases macula densa sodium concentration activating a carrier molecule which facilitates transport of renin to the afferent arteriolar cytoplasm, initiating local autoregulation. He postulates that the release of renin into the renal venous effluent may not reflect the cytoplasmic changes in renin content.

It can be seen, therefore, that theories concerning control of renin release from the kidney are controversial and the intrarenal role of the renin angiotensin system remains purely speculative.

SUMMARY

The association between the kidney and hypertension was recognised by Bright in 1836 who suggested that the diseased kidney may produce a circulating pressor substance.

The discovery of such a pressor substance was made by Tigerstedt and Bergmann in 1898. They called this substance renin.

In the early part of this century it was found difficult to isolate a pressor compound from the kidney. A revival of interest in the role of the kidney in experimental hypertension followed the advent of a reproducible experimental model (Goldblatt, Lynch, Hanzal and Summerville, 1934). Subsequently renin was isolated from the kidney and its pressor actions studied. Renin was found to be an enzyme (Braun-Menéndez, Fasciolo, Leloir and Munoz, 1940; Plentl and Page, 1943) which acted on an α_2-globulin in plasma to produce the active pressor compound, angiotensin. Further work revealed that renin splits the leucyl-leucyl bond in the renin substrate molecule, liberating a decapeptide (angiotensin I) which is split by a chloride-dependent enzyme present in plasma to the active octapeptide (angiotensin II). Amino acid analysis and determination of the amino acid sequence of angiotensin led to its synthesis (Bumpus, Schwarz and Page, 1957; Schwyzer, Iselin, Kappeler, Riniker, Rittel and Zuber, 1957).

Angiotensin was found to have both direct and indirect

actions in raising blood pressure and also to be an important factor in the control of aldosterone secretion.

Renin is located in the juxtaglomerular apparatus, possibly in the granulated cells lining the afferent arteriole or in the macula densa portion of the early distal tubule. The release of renin into renal venous blood is stimulated by experimental situations which threaten renal perfusion. Therefore, renin is released under situations of sodium depletion, haemorrhage and constriction of the renal vessels.

Two theories have been proposed to explain the mechanism of renin release. Tobian (1964) has suggested that the granular cells lining the afferent arteriole are stretch receptors, and a reduction in the degree of stretch in the afferent arteriole, such as is induced with small changes in renal perfusion pressure, releases renin. The work of Vander and Miller (1964) led to the conclusion that such a stretch receptor mechanism as proposed above could not account for the inhibition of renin release that occurs when diuretics are administered in the presence of a reduced renal perfusion pressure (induced by aortic clamping). Vander (1967) suggested that the total sodium load delivered to the macula densa region of the distal tubule provides the mechanism controlling renin release. Increases in sodium load would inhibit renin release; decreases in sodium load would stimulate it. Thurau, Schnermann,

Nagel, Horster and Wahl (1966) proposed that an increase in sodium load to the macula densa may be inversely related to sodium concentration, as has been demonstrated when glomerular filtration rate is reduced (Landwehr, Schnermann, Klose and Giebisch, 1968), this phenomenon being due to the increased reabsorption of water in the ascending limb of the loop of Henle at low flow rates.

The presence of renin-like enzymes in tissues other than the kidney has been described. Renin is present in arterial walls, the submaxillary glands of the white mouse and in the foetal membranes and maternal reproductive tissues.

INTRODUCTION

The work described in this thesis is based on the methods of assay of plasma renin activity, plasma renin concentration and renin substrate described by Skinner (1967). Using these methods, it has been possible to study the distribution of renin in tissues and body fluids deficient in renin substrate. This is possible since levels of renin can be detected and quantitated using an artificial substrate prepared from nephrectomized sheep plasma. Since it is also possible to simultaneously estimate 'renin activity' (which, as described previously, is dependent upon both the levels of endogenous renin and renin substrate), plasma renin concentration, and renin substrate levels, studies have been done to determine the relative contribution of both enzyme and substrate to the increased levels of renin activity observed in several physiological situations.

Finally, studies were undertaken to determine the role of tachyphylaxis in the normal constrictor response to angiotensin and to see whether the peripheral vessels contributed to the reduced pressor response seen in pregnant women following the administration of exogenous angiotensin.

The thesis is therefore divided into the following sections:

(1) A description of the methods used for the preparation and

and bio-assay of renin and the methods for measurement of the vascular response to induced drugs.

(2) The quantitation of renin levels in human urine and factors controlling the excretion of renin into human urine.

(3) The role of renin and renin substrate levels in the increases in plasma renin activity observed following administration of the natriuretic agents chlorothiazide and spironolactone.

(4) The presence and quantitation of renin in human maternal and foetal tissues.

(5) The role of renin and renin substrate levels in the changes in plasma renin activity induced by:

(a) the normal menstrual cycle;

(b) administration of oral contraceptive agents;

(c) pregnancy.

(6) The induction of tachyphylaxis to angiotensin in normal subjects.

(7) Peripheral vascular reactivity to angiotensin in normal pregnant women as compared to normal non-pregnant women.

SECTION ONE

METHODS

METHODS

INTRODUCTION:

Since renin is an enzyme, the simplest way to determine the amount of renin present in a sample is to measure the reaction velocity. This is the rate at which angiotensin is formed from renin acting on renin substrate under standard conditions of temperature, pH, substrate concentration, and freedom from end-product inactivation.

Angiotensin is most commonly measured by bio-assay. The blood pressure response of an animal to unknown concentrations of angiotensin is compared with the blood pressure response obtained to known concentrations of angiotensin. Animals used include the rat (Peart, 1955) and the dog (Haas and Goldblatt, 1967).

Angiotensin I is formed from the action of renin on renin substrate (Skeggs, Marsh, Kahn and Shumway, 1954). Therefore, if comparison is to be made using angiotensin II as standard, either converting enzyme must be present in the incubation mixture in excess, or an animal preparation must be used which will convert injected angiotensin I to angiotensin II.

Estimations of renin in plasma can be broadly divided into two groups: those which measure the renin 'activity' of plasma, and those which measure the plasma renin 'concentration'.

Plasma renin activity is defined as the rate of formation

of angiotensin from the action of endogenous renin on endogenous substrate in a system in which angiotensinase activity is inhibited. The amount of angiotensin formed, therefore, depends upon the concentrations of both enzyme and substrate and does not provide an accurate quantitation of renin levels, although it may be a reflection of the *in vivo* ability of plasma to form angiotensin.

Plasma renin concentration refers to the rate of formation of angiotensin formed from the action of renin on a constant (excess) substrate concentration. It permits, therefore, accurate quantitation of the levels of enzyme present, provided there are no essential unaccounted cofactors in the system.

The methods described in this thesis for the assay of renin in plasma, urine, amniotic fluid and tissue extracts are based on the simplified methodology described by Skinner in 1967. Renin levels are determined indirectly by determination of the amount of end-product formed following incubation of renin, either with endogenous substrate, giving an estimation of the so-called 'renin activity', or the incubation of renin with a constant level of an artificial substrate (prepared in this system from nephrectomized sheep plasma), in which case the 'renin concentration' of the sample is determined. To avoid the interfering action of endogenous substrate in determining renin concentration, endogenous substrate is denatured by a low pH treatment which does not adversely affect

renin. End-product inactivation is prevented by low pH treatment and the addition of ethylenediamine-tetra-acetic acid, disodium salt, to the system. The technique, as described by Skinner, is simple and does not involve complicated extraction procedures. The recovery of renin is high.

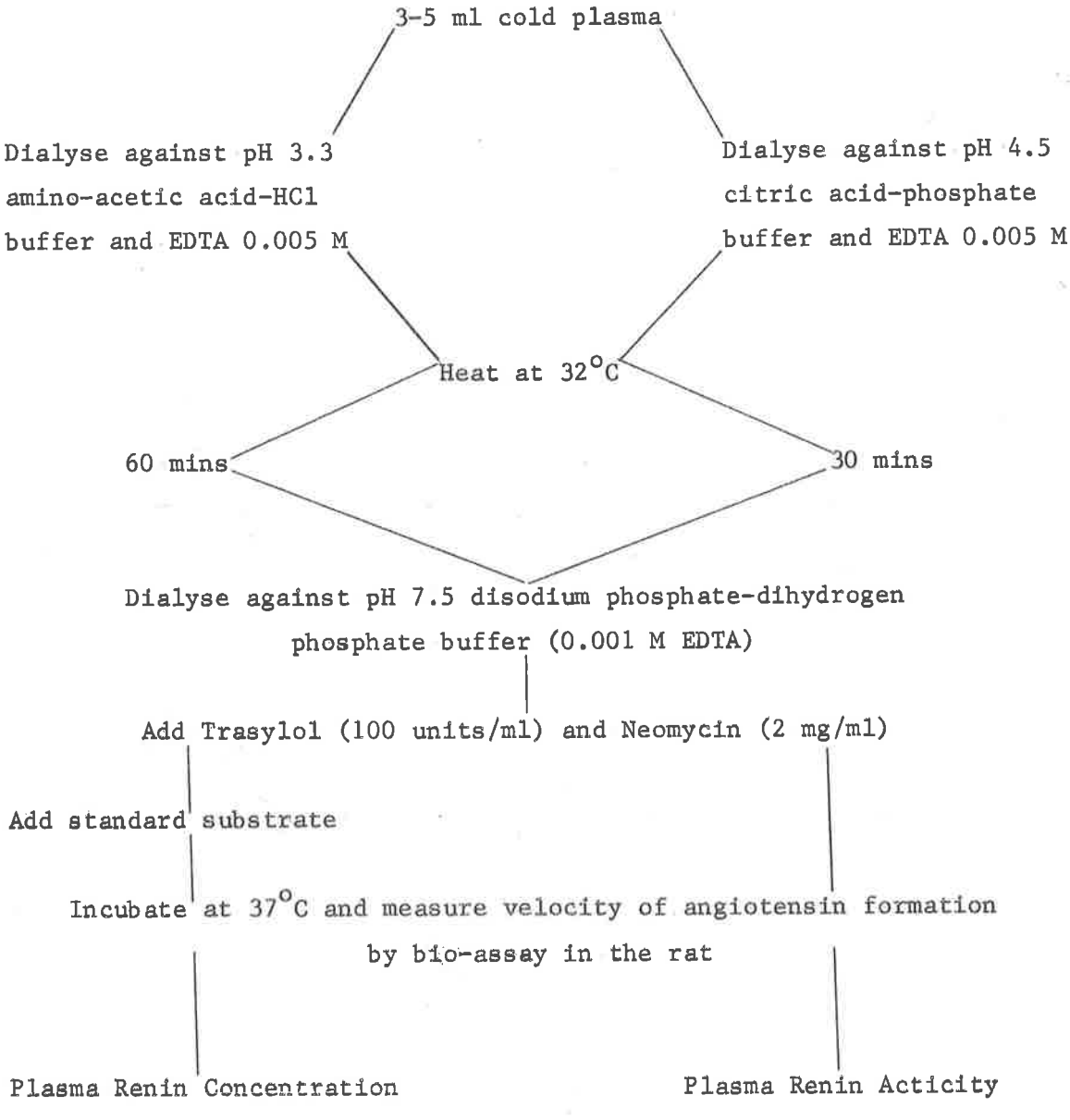
Preparation of plasma for plasma renin concentration and activity estimations:

Heparin (10 or 20 units/ml) was used as anticoagulant and added to samples immediately after withdrawal of blood from an antecubital vein. These concentrations of heparin do not interfere with renin-renin substrate reaction (Sealey, Gerten, Ledingham and Laragh, 1967) in this system (Skinner, unpublished observations). The blood was chilled and spun at 3000 r.p.m. for 30 minutes at 10^{o}C. The plasma obtained was dialysed for 24 hours (Figure 1-1) in size 8/32 Visking cellophane casings against 5 litres of Buffer I or II, with one change, at a temperature of 3^{o}C.

To estimate plasma renin concentration, samples were heated to 32^{o}C after dialysis to pH 3.3 (Buffer I) for one hour. This procedure irreversibly denatures the renin substrate, but renin is unaffected (Skinner, 1967). Following dialysis for 24 hours to pH 7.5 against 5 litres of Buffer III with one change, the samples were decanted, and neomycin sulphate (2 mg/ml), a bacteriostatic agent, and Trasylol (100 units/ml), a kallikrein inhibitor, added.

FIGURE 1-1

Flow sequence for handling plasma for estimation of plasma renin concentration (PRC) and plasma renin activity (PRA).



Restoration of the original volume was accomplished by the addition of cold Buffer III.

Nephrectomized sheep substrate was added to the samples (ratio of two parts of plasma to one part of substrate) and the samples incubated at 37°C, or stored at -20°C.

Plasma for renin activity estimation was dialysed in a similar manner to an initial pH of 4.5 (Buffer II) and heated to 32°C at this pH for 30 minutes. This treatment denatures angiotensinase, but does not denature either renin or renin substrate (Skinner, 1967). Plasma was then dialysed to pH 7.5 against Buffer III and decanted; neomycin sulphate (2 mg/ml) and Trasylol (100 units/ml) were added and the volume corrected by the addition of cold Buffer III. The samples were then incubated at 37°C, or stored at -20°C.

Estimation of renin substrate levels:

Endogenous renin substrate levels were determined by incubation of pH 4.5 treated plasma dialysed to pH 7.5 (i.e. 'activity plasma') with an excess of human renal renin. The concentration of renin added was sufficient to drive the reaction to completion in 10 minutes.

Four parts standard renin were added to one part of plasma and the mixture incubated for 30 minutes at 37°C. For estimation of low substrate concentrations as may be found in amniotic fluid and urine, two parts of renin were added to one part of substrate, giving

a sensitivity limit of 30 ng/ml of angiotensin of the original plasma.

Determination of the renin activity of renin concentration:

Both renin activity and renin concentration were determined by the amount of pressor material (angiotensin) formed following incubation of the samples at 37°C for varying lengths of time.

The pressor material formed was assayed by bio-assay in the ganglion-blocked rat (Peart, 1956).

Bio-assay preparation:

Albino male rats (200 gm) were used. Anaesthesia was induced with sodium pentabarbitone (Nembutal) 60 mg/kg injected intraperitoneally. Ganglionic blockade was effected by pentolinium tartrate (Ansolysen) 2.5 mg/100 gm injected subcutaneously (Peart, 1955). A tracheal cannula was inserted to ensure an adequate airway. A nylon catheter (No. 1, Portex tubing, bore 0.75 mm, external diameter 0.94 mm) was inserted into the jugular vein for injection of standard and unknown solutions (Figure 1-2).

Arterial pressure responses were recorded with either a saline-filled glass cannula inserted into the carotid artery and connected to a Condon manometer (Figure 1-3), with an ink-writing pen recording on a Palmer kymograph (speed 8 mm/min), or with a saline-filled nylon catheter (No. 1, Portex tubing) inserted into the carotid artery which was connected through a three-way tap to a Southern Instruments capacitance transducer (A) (Figure 1-4). The output of

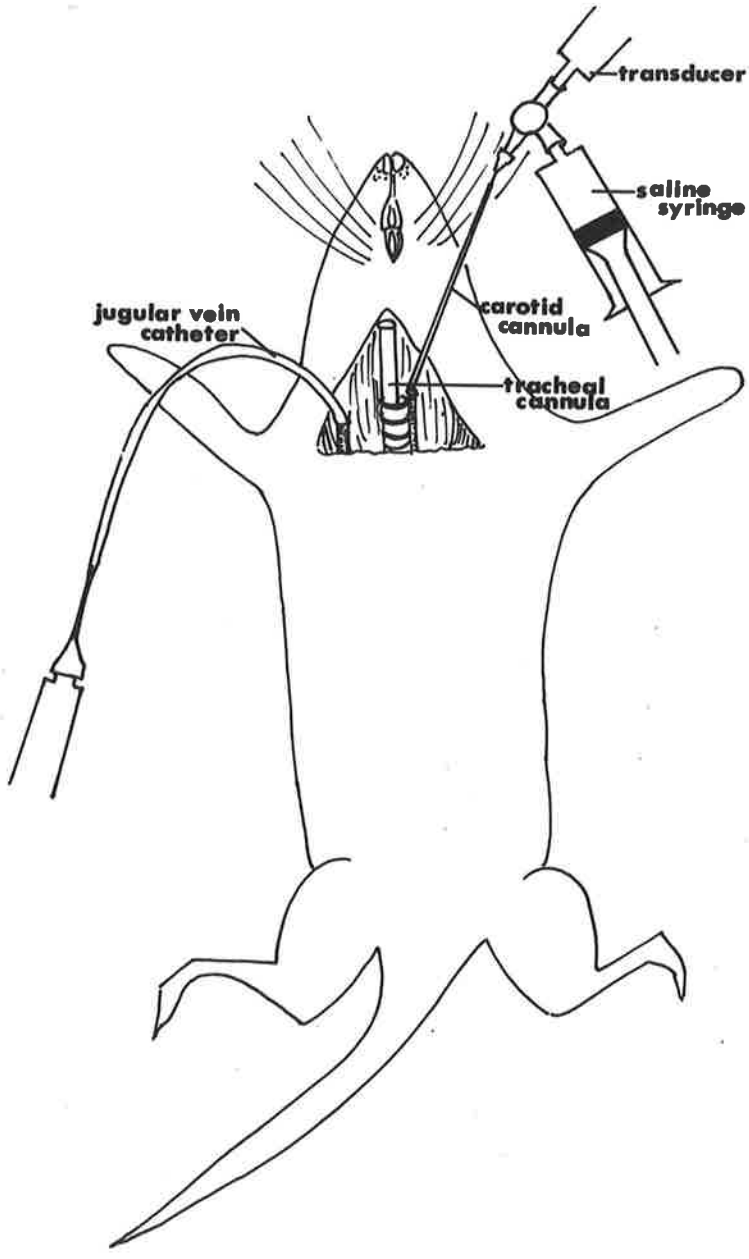


Fig. 1-2 Diagram of a rat prepared for bio-assay. Catheters were inserted into the jugular vein for injection of standard angiotensin and test solutions, and into the carotid artery to record the blood pressure response to intravenous injection of samples.

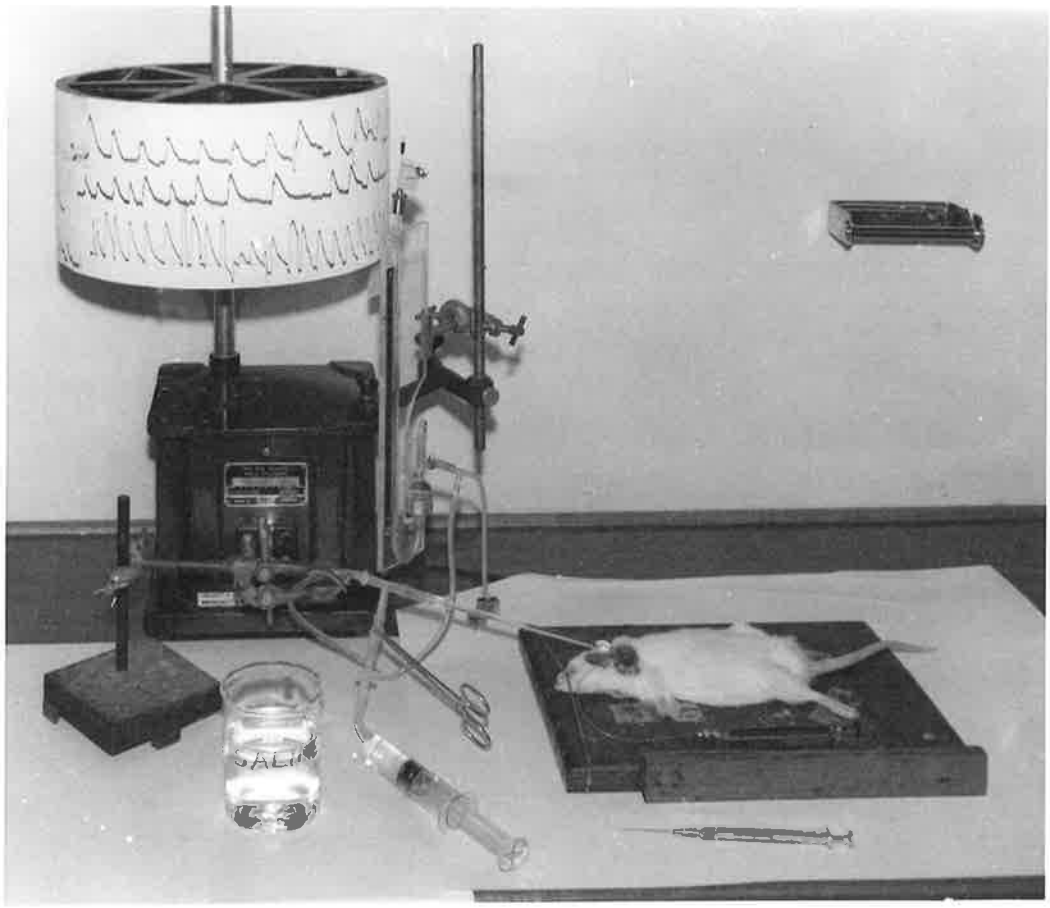


Fig. 1-3 A bio-assay preparation in which the blood pressure responses were measured with a saline-filled glass cannula connected to a Condon manometer. By means of an ink-writing pen, the responses were recorded on a Palmer kymograph.

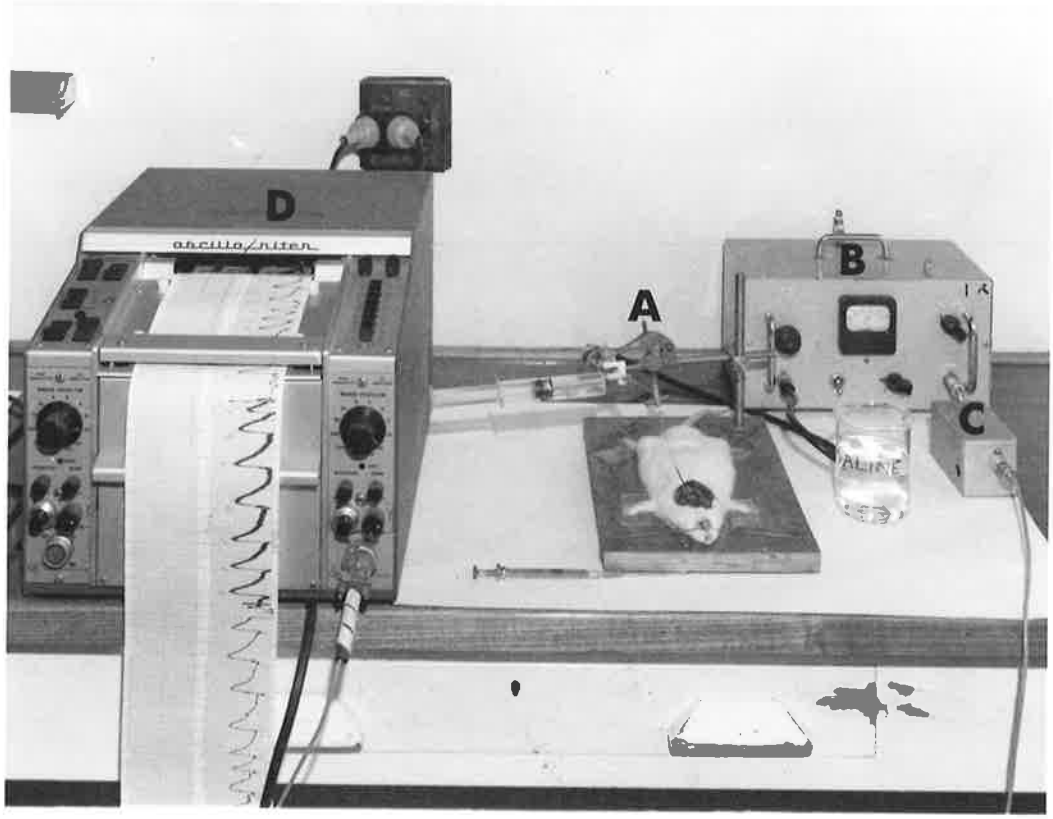


Fig. 1-4 A bio-assay preparation in which the blood pressure responses were recorded by means of a Southern Instruments capacitance transducer (A). The output of the transducer varies the frequency of an oscillator (B) and the frequency change is detected by a discriminator. The output of the discriminator circuit passes through an integrating circuit (C) and is recorded by a Texas recorder (D).

the transducer varies the frequency of an oscillator (B). This frequency change was detected by a discriminator. The output of the discriminator circuit goes through an integrating circuit (C) before being fed into a Texas recorder (D). The integrating circuit damps out the pulse pressure and so gives a mean pressure trace. The recording system was calibrated to record a one mm pen deflection for a one mm Hg rise in pressure. The paper speed was 6 mm/min.

Assay of pressor material:

The pressor content of each sample following incubation was determined by bracket assay, the amount of pressor material in each sample being bracketed between two doses of standard synthetic β-asparaginyl-angiotensin II (Hypertensin, Ciba) (Figure 1-5). All injections were 0.1 ml or less and were followed by a wash in of 0.05 ml of 0.15 M saline.

Expression of results:

The pressor content of each sample after incubation for known periods was determined. The incubation mixture was sampled at three appropriate time intervals during incubation and, without further extraction, each aliquot was assayed for the pressor material generated. At least two rats were used for assay.

Following determination of the pressor content in the three aliquots, a velocity curve of the amount of angiotensin formed to the time of incubation was drawn and the initial velocity was

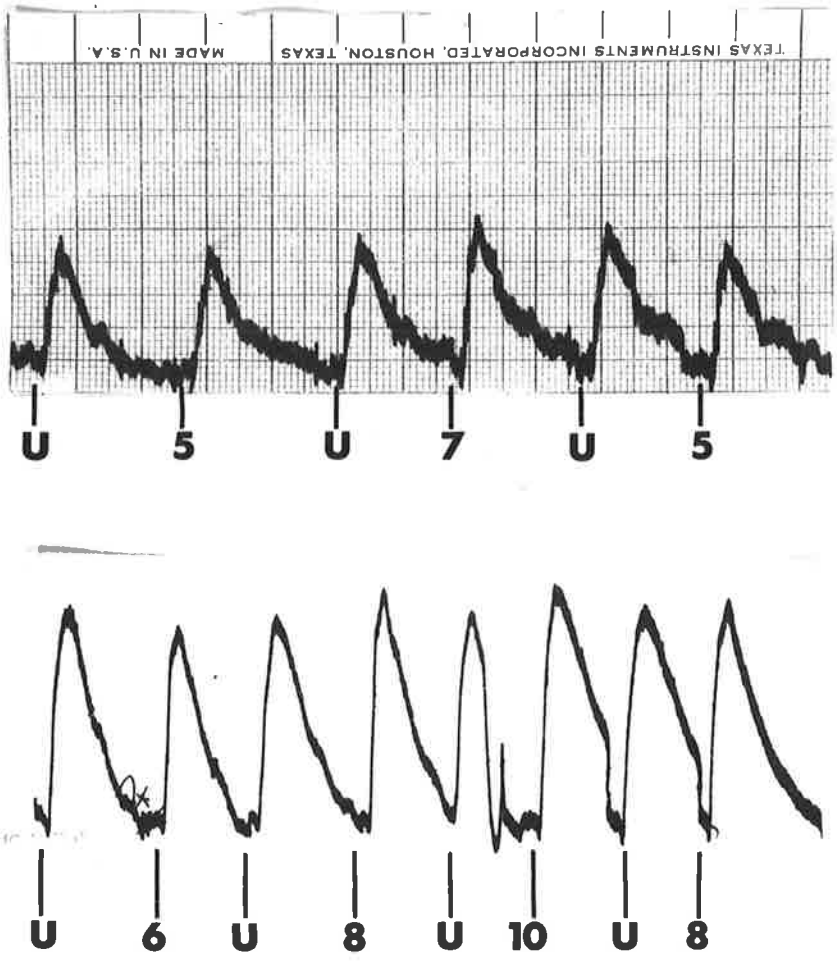


Fig. 1-5 Bracket assays of plasma (U) which contained unknown amounts of angiotensin, using β-asparaginyl angiotensin II as standard. (Numbers refer to nanograms of standard angiotensin injected.) Upper trace: Bracket assay obtained using a capacitance transducer and Texas recorder. Lower trace: Bracket assay obtained using a Condon mercury manometer and a kymograph to record the blood pressure response.

calculated (Figure 1-6) (Lever, Robertson and Tree, 1964; Brown, Davies, Lever, Robertson and Tree, 1964). The results are expressed as follows:-

Plasma renin activity is expressed as the rate of formation of angiotensin in nanograms/ml/hr, at pH 7.5, and 37°C.

Plasma renin concentration is expressed in units/ml, where one unit of renin is defined as the amount of renin which, when incubated at 37°C and pH 7.5, generates angiotensin at the rate of one ng/ml/hr from a concentration of standard sheep substrate of 530 ng/ml.

Since the addition of standard sheep substrate dilutes the renin extract by 1/3, to obtain the original renin concentration the measured velocity is multiplied by 3/2.

Renin substrate is expressed as nanograms of angiotensin/ml, generated following incubation of plasma (pretreated to pH 4.5) with an excess of human renal renin at pH 7.5 and 37°C.

Standard buffers:

Buffer I - Aminoacetic acid - HCl buffer (pH 3.3)

Aminoacetic acid	- 0.05 M
HCl	- 0.01 M
EDTA	- 0.0051 M
NaCl	- 0.0949 M

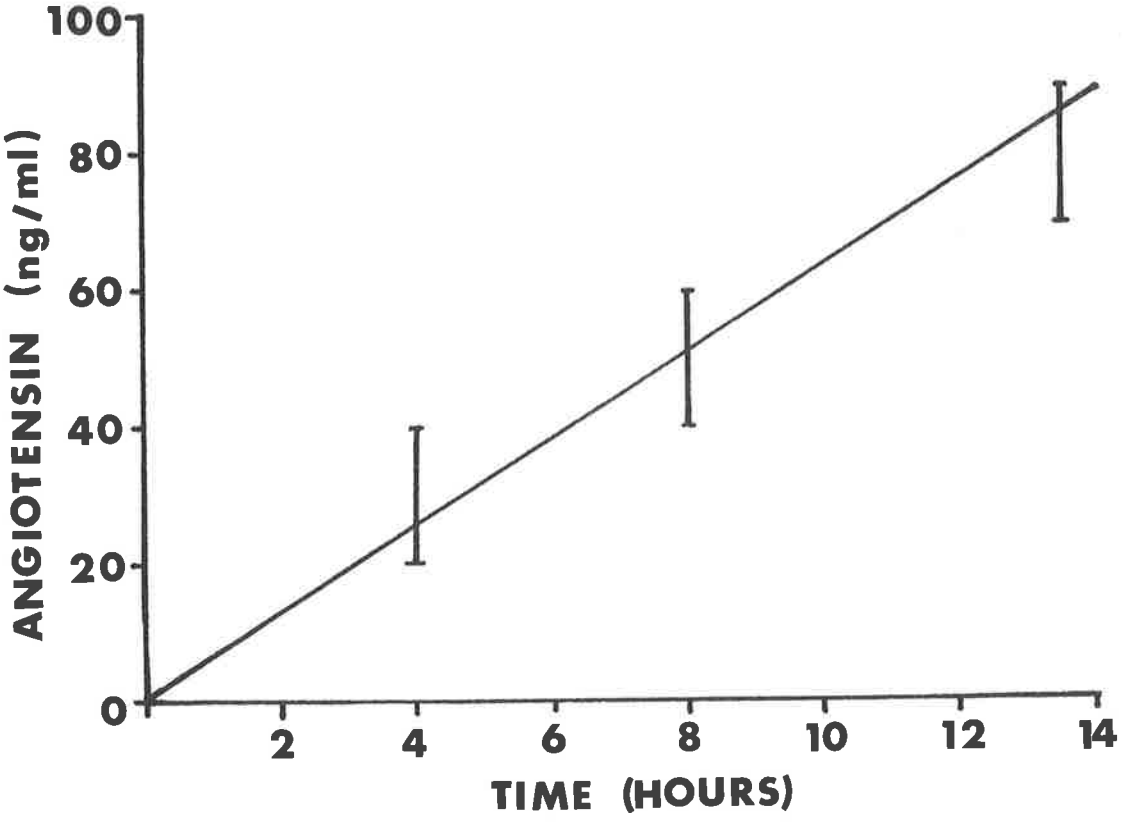


Fig. 1-6 Determination of initial velocity, i.e. the amount of angiotensin formed in ng/ml/hr following bracket assay of the concentration of angiotensin in a sample formed after varying periods of incubation at $37^{o}C$.

Buffer II - Citric acid-phosphate buffer (pH 4.5)

Citric acid	- 0.00278 M
$Na_2HPO_4.12H_2O$	- 0.045 M
EDTA	- 0.0051 M
NaCl	- 0.0821 M

Buffer III - Phosphate-phosphate buffer (pH 7.5)

$NaH_2PO_4.12H_2O$	- 0.0122 M
$Na_2HPO_4.12H_2O$	- 0.0867 M
EDTA	- 0.001 M
NaCl	- 0.075 M

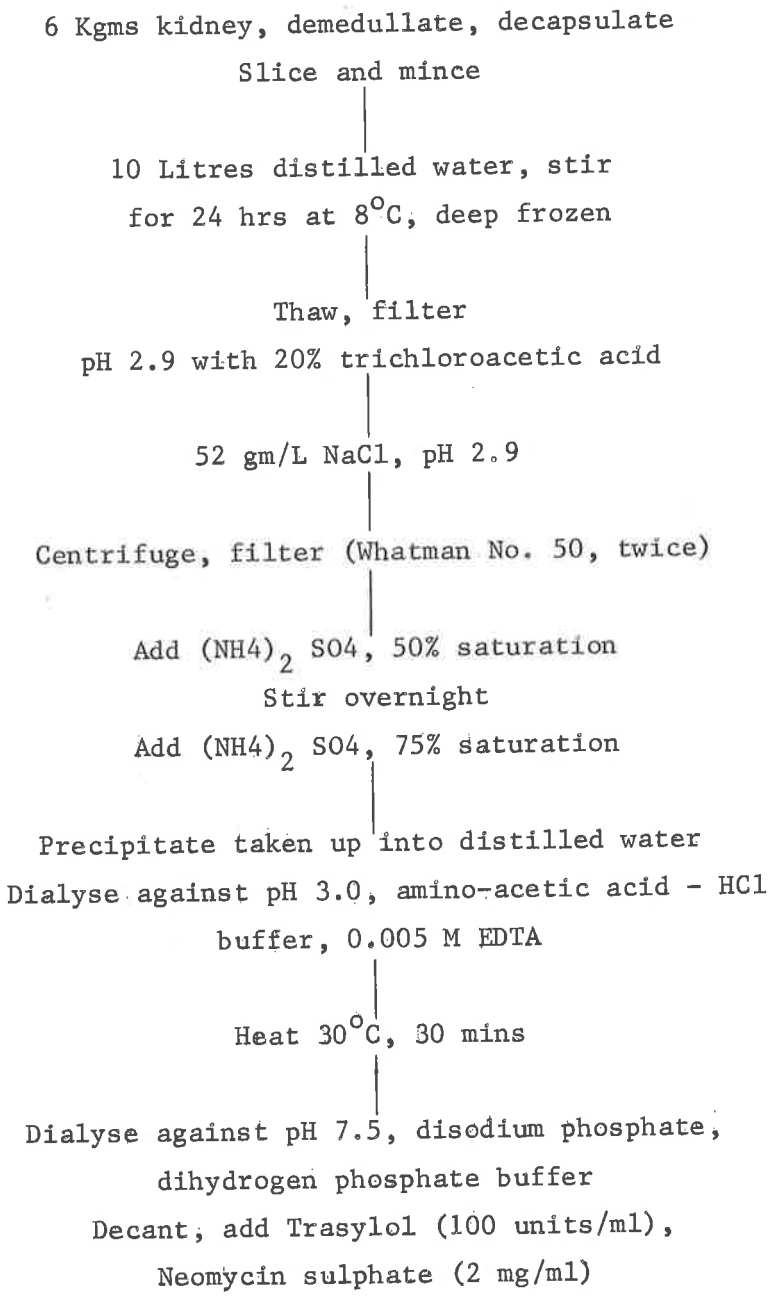
Preparation of human renal renin (Fig. 1-7):

The technique used to extract renin from the kidney is essentially that described by Brown, Davies, Lever, Robertson and Tree (1964). Approximately 6 Kgms of kidneys were demedullated, decapsulated, sliced and minced in a Waring blender. The minced tissue was taken up into 10 litres of distilled water, stirred continuously for 24 hours, at a temperature of 8°C and then deep frozen.

The suspension was thawed, filtered, and the pH of the filtrate adjusted to 2.9 with 20% trichloroacetic acid. The filtrate was stirred continuously during addition of NaCl 52 gm/L, and the pH was readjusted to 2.9 with 20% trichloroacetic acid. The mixture was then stored for 24 hours at 8°C. It was later centrifuged, filtered through Whatman (No. 50) filter paper twice, and sufficient ammonium

FIGURE 1-7

Preparation Human Renal Renin



sulphate added to give a 50% saturated solution. This was stirred overnight, at 8°C, centrifuged and ammonium sulphate added to the supernatant to bring the saturation up to 75%. The precipitate that formed was taken up into distilled water, dialysed in 8/32 Visking cellophane casings against pH 3.0 amino-acetic acid-HCl EDTA buffer (0.005 M EDTA, made 0.16 molar with NaCl) and heated at this pH for 30 minutes. It was then dialysed against pH 7.5 (Buffer III), decanted, and neomycin sulphate (2 mg/ml) and Trasylol (100 units/ml) added.

Renin prepared in this way had a protein concentration of 4 mg/ml, was free of endogenous pressor activity, but exhausted plasma substrate levels during 10 minutes incubation at 37°C. Angiotensinase was present, sufficient to produce 50% destruction of added synthetic β-asparaginyl angiotensin II at the end of 3 hours incubation at 37°C. However, greater than 80% recovery of added angiotensin was obtained at the end of 30 minutes incubation, making the renin preparation adequate for renin substrate determinations.

Preparation of renin substrate:

Renin substrate was prepared according to the method of Skinner (1967). An ewe was heparinized (7500 units intravenously) and exsanguinated 6 days after bilateral nephrectomy.

The plasma was separated by centrifugation at 3000 r.p.m. for 30 minutes and dialysed to a pH of 3.9 against citric acid-

phosphate buffer made 0.16 M with NaCl and containing 0.005 M EDTA. The plasma was then heated for 45 minutes at 32°C, dialysed against pH 7.5 buffer (Buffer III), and stored at -20°C after addition of neomycin sulphate (2 mg/ml) and Trasylol (100 units/ml).

A second batch of renin substrate was prepared in a manner similar to that described above, except that heparin (10 units/ml) was added to the blood following removal from the sheep, and the plasma obtained was dialysed to pH 4.5 (Buffer II) and heated at pH 4.5 for 30 mins prior to dialysis to pH 7.5 (Buffer III).

All renin substrate preparations were angiotensinase free and free of endogenous pressor activity up to and including 100 hours of incubation. Variations in actual concentration of the renin substrate were obtained, but all results expressed in this text are corrected to the rate obtained using a substrate concentration of 530 ng/ml, unless stated otherwise.

Preparation of antirenin:

Antirenin was prepared according to the method used by Skinner (1967). Rabbits were immunized with twice-weekly injections of 1:1 emulsion of Freund's incomplete adjuvant and concentrated renal renin. A third rabbit was immunized with Freund's incomplete adjuvant alone, and 20 ml of blood was taken from the rabbit during an infusion of normal saline to suppress endogenous renin release.

Immunoglobulins were precipitated by the addition of 16%,

and then 14% sodium sulphate, then dialysed against pH 4.1 citric-acid phosphate EDTA buffer (0.001 M EDTA made to 0.16 M with NaCl). The plasma was warmed to 32°C prior to dialysis to pH 7.5 against standard Buffer III, centrifuged and stored at -20°C following the addition of neomycin sulphate.

Preparation of human angiotensin:

Human renal renin (4 ml) was added to angiotensinase-free human substrate (i.e. plasma treated by dialysis to pH 4.5, heated and dialysed to a final pH of 7.5) and the mixture incubated at 37°C for 7 hours. Ultrafiltration yielded a filtrate containing 1 µg/ml of angiotensin, which was stable when incubated alone but rapidly destroyed on incubation with normal plasma at pH 7.5.

Detection of angiotensinase activity:

Human angiotensin and synthetic β-asparaginyl angiotensin II were added to test plasmas, urines and tissue extracts in concentrations of 100-200 ng/ml. Test solutions were considered to be angiotensinase-free if greater than 80% of the added angiotensin was still present after 24 hours incubation when tested against the unincubated control.

Venous occlusion plethysmography:

The technique of venous occlusion plethysmography was used to measure hand and forearm blood flow and to determine the effects of angiotensin and other drugs on the peripheral vessels in man.

Measurement of blood flow to a segment of a limb is determined by enclosing the segment in a rigid container and occluding, intermittently, the venous drainage. During the period of venous occlusion the increase in volume of the segment is proportional to the rate of arterial blood flow (Brodie and Russell, 1905). The upper limb can be partitioned into either the hand (Lewis and Grant, 1925) or the forearm (Freeman, 1935), and the flow in each segment measured. Measurement of forearm flow requires the exclusion of hand blood flow by arterial occlusion at the wrist (Grant and Pearson, 1938).

Water-filled plethysmographs were used in most experiments (Figure 1-8). These are similar to the plethysmograph described by Greenfield (1954) and Greenfield, Whitney and Mowbray (1963). The plethysmograph is sealed by means of an attached rubber sleeve or glove (Lewis and Grant, 1925). It is temperature controlled, the correct water temperature being determined to allow measurement of resting flow (Barcroft and Edholm, 1946).

Intermittent inflation and deflation of the venous collecting cuff is automated by means of a sequence timer (Paton Industries, Adelaide), electrically operated solenoid valves releasing air from reservoirs at constant pressure, the reservoirs being filled from compressed air cylinders using constant pressure valves.

The volume changes were recorded on a Brodie-Starling

Fig. 1-8 A temperature controlled, water-filled plethysmograph showing rubber sleeve and sealing plates used in the measurement of forearm blood flow.

kymograph (Figure 1-9), or by means of a P23 BC Statham transducer connected to a Rikadenki pen writing servo recorder (Figure 1-10).

Calibration of the system was carried out by introduction of known volumes of air into the system, or in experiments where the transducer was used to record blood flow, by the introduction of known volumes of water into the plethysmograph, with the subject in position. The flow was calculated by the method of Barcroft and Swan (1953) (Figure 1-11) and expressed as ml/100 ml tissue/minute.

In a few experiments, a capacitance plethysmograph (Willoughby, 1965; Fewings and Whelan, 1966) was used to measure forearm blood flow (Figure 1-12).

Intra-arterial infusions:

In order to examine the direct effects of drugs on the hand and forearm vessels, infusions of the drug to be studied were given into one arm via intra-arterial infusion into the brachial artery at the elbow. Since the drugs are infused intra-arterially, concentrations of the drug sufficient to produce a local vasoconstriction or a vasodilatation are obtained, whilst dilution or destruction of the drug in the peripheral tissues prevents any systemic effect. Therefore, any central action of the infused drug is avoided as well as any compensatory action of the cardiovascular system to pressor or depressor effects of infused drugs. By using the opposite non-infused hand or forearm as a control, variations in flow due to

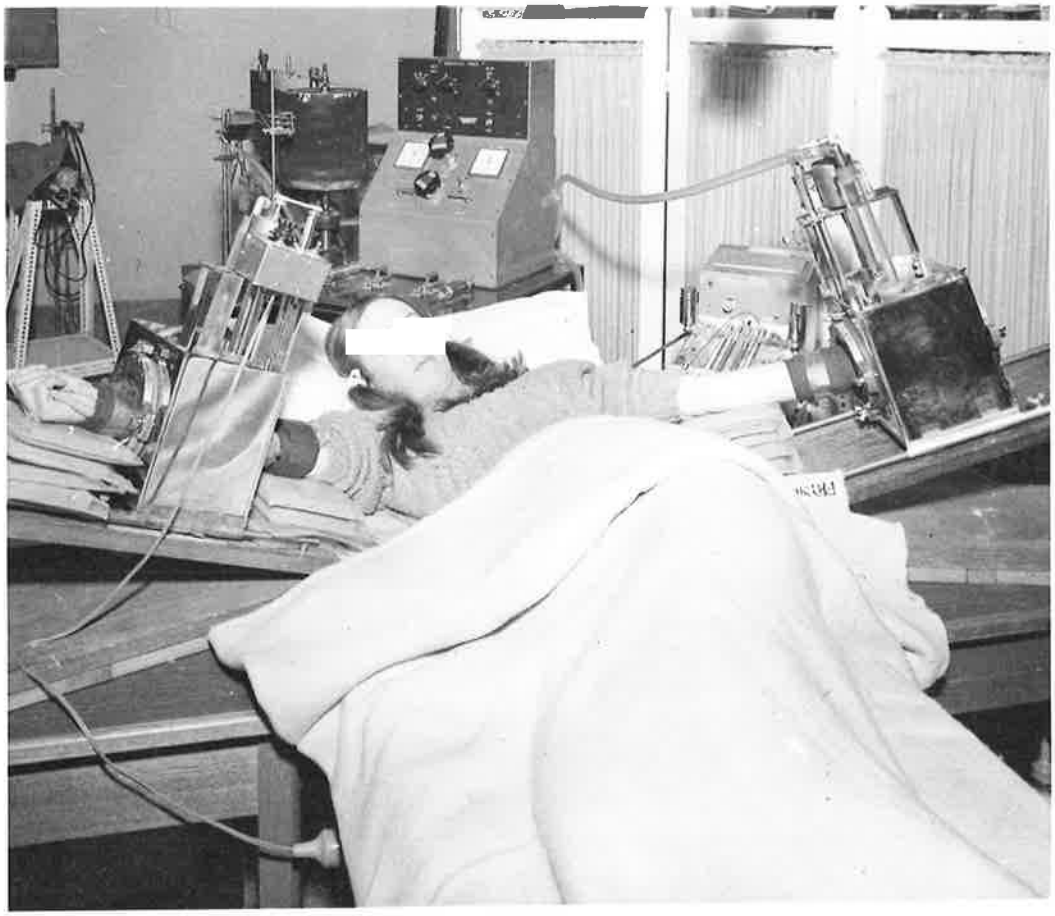


Fig. 1-9 General laboratory set-up showing a subject lying on a couch prepared for measurement of hand and forearm blood flow. In the background is the Brodie-Starling kymograph for recording the blood flows obtained, infusion pump and sequence timer for monitoring venous occlusion cuff inflation and deflation.

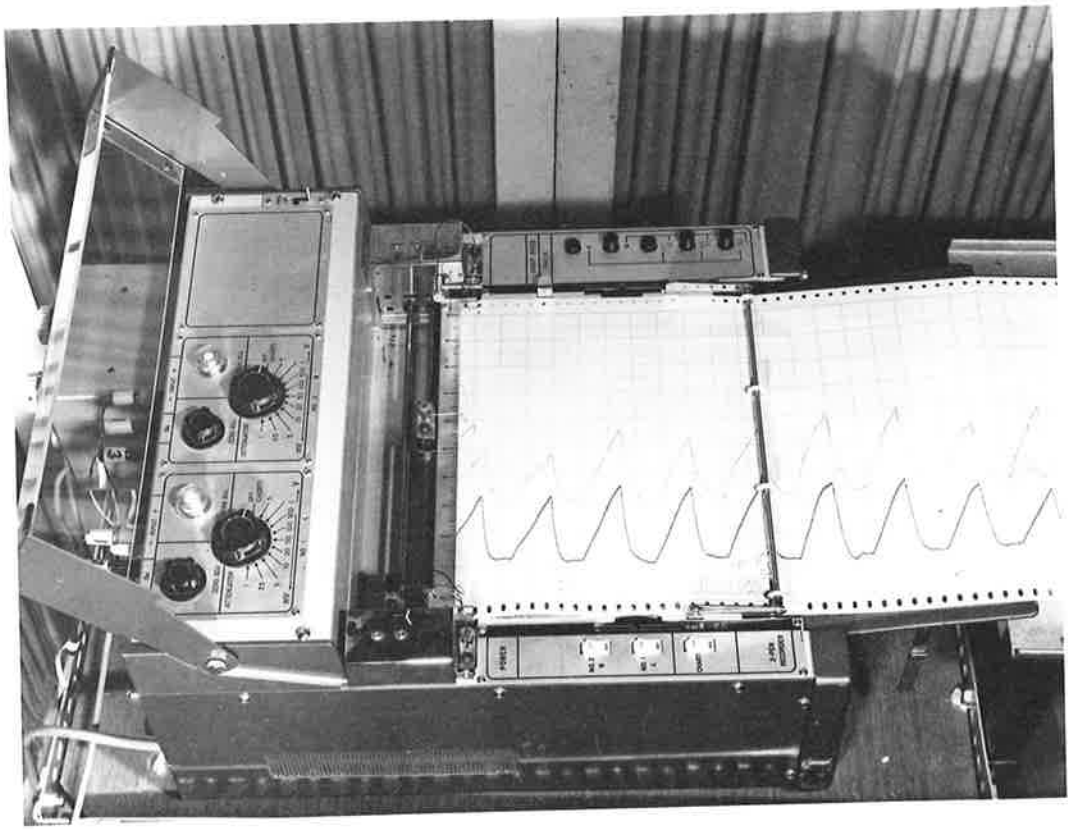


Fig. 1-10 A record of hand blood flow measured by venous occlusion plethysmography using water-filled plethysmographs, and recorded with a P23 BC Statham transducer connected to a Rikadenki multi-pen recorder.

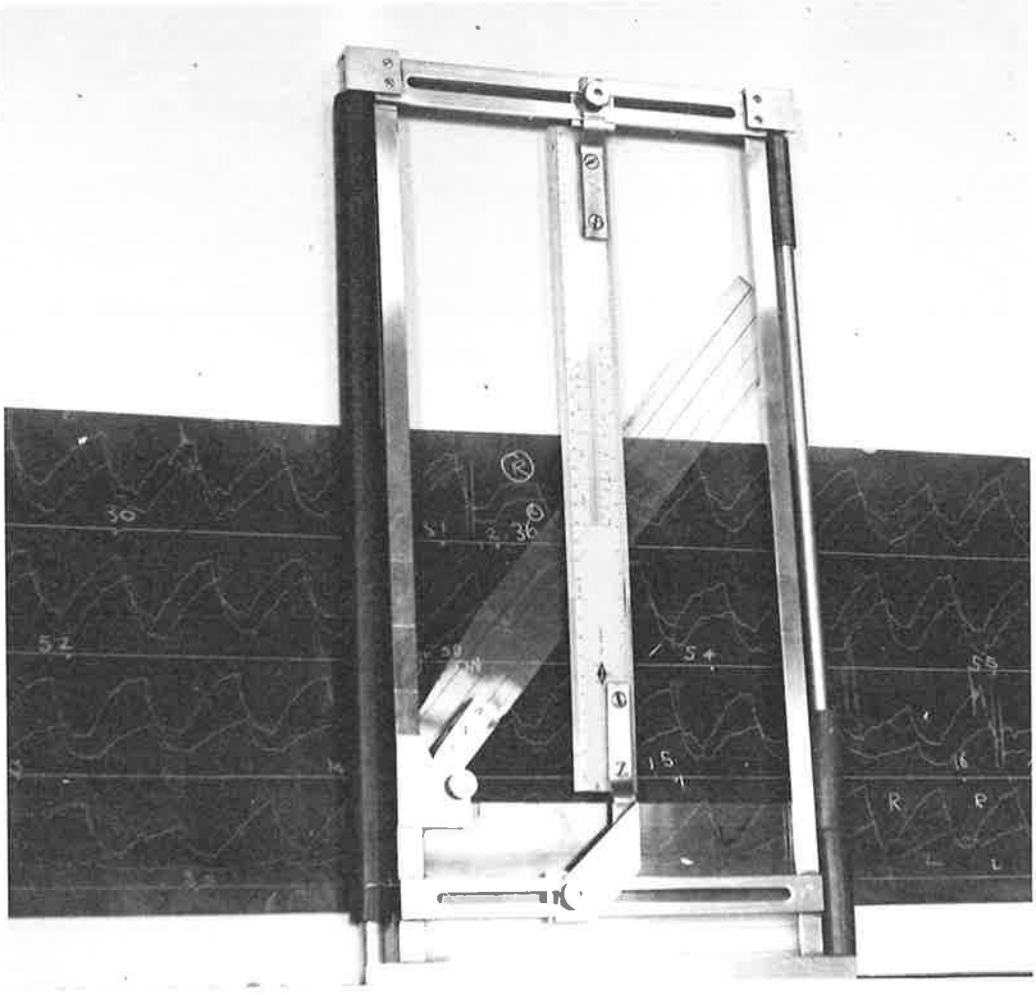


Fig. 1-11 Flow measuring apparatus superimposed on a venous occlusion plethysmographic kymograph tracing.

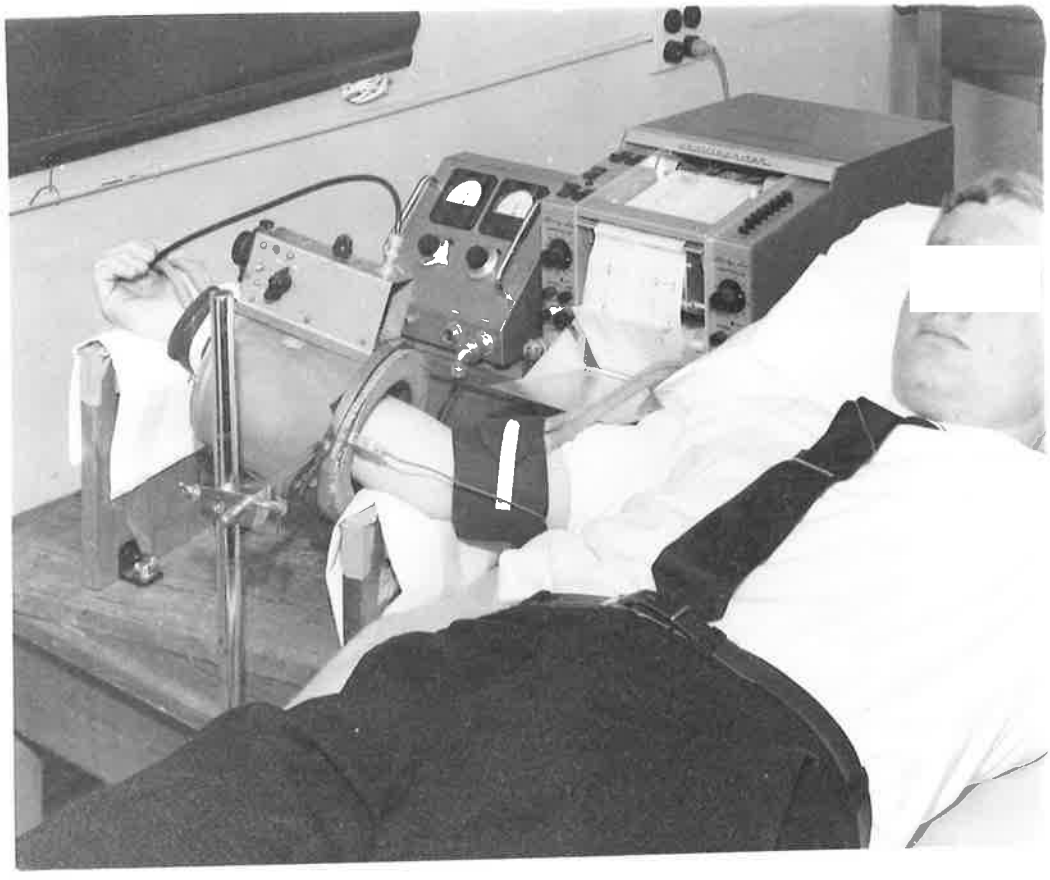


Fig. 1-12 A capacitance plethysmograph used for recording forearm blood flow.

sympathetic activity can be accounted for (Duff, 1952).

Infusions were administered through a 22-gauge needle inserted centripetally into the brachial artery at the elbow under local anaesthesia (2% Lignocaine), the needle being taped into position and remaining *in situ* for the duration of the experiment.

The needles used were short bevel, buttless needles, 3.2 cm long. They were connected by a 30 cm length of polythene tubing (external diameter 0.75 mm, internal diameter 0.5 mm) to a 50 ml syringe driven by a constant infusion pump.

Infusions were given at a rate of 2 ml/min. The total dead space of the system between the artery and the syringe was approximately 0.17 ml, which represented a delay of 5-6 seconds before the drug reached the artery.

During control periods when no drugs were infused, a constant infusion of NaCl (0.15 M) was given. NaCl (0.15 M) was also used as the vehicle for the drugs infused.

Expression of results:

When the dose-response relationship was required for comparisons between the actions of different drugs on subjects, the percentage change in blood flow was determined. This was calculated from the difference between the flow during the two minutes prior to the infusion of the drug and the last two minutes of infusion of the drug, by which time the response to the drug had become stable.

Moreover, an additional correction could be made with regard to flow changes. Since the small doses of the drugs infused to obtain a response did not have any systemic action, the non-infused side was used as a control. Thus, when calculating the percentage change produced by infusion of a drug, allowance for spontaneous variations in flow could be made by assuming that in the absence of the drug the two sides would maintain the same relationship as in the pre-infusion period (Duff, 1952).

SECTION TWO

(A) THE OCCURRENCE AND ASSAY OF RENIN IN NORMAL HUMAN URINE

(B) FACTORS CONTROLLING THE EXCRETION OF RENIN INTO URINE

(A) THE OCCURRENCE AND ASSAY OF RENIN IN NORMAL HUMAN URINE

In 1942, Houssay, Braun-Menéndez and Dexter detected renin-like activity in the urine of dogs following intravenous infusions of large doses of hog renin.

In 1964, Brown, Davies, Lever, Lloyd, Robertson and Tree claimed to have identified a renin-like enzyme in normal human urine. Extrapolation of their results revealed that relatively high concentrations of this renin-like enzyme occurred in normal human urine. This finding raised the possibility of renin being secreted from the macula densa into the tubular fluid, and since angiotensin has been implicated in control of sodium reabsorption by both the proximal (Leyssac, 1965) and the distal tubule (Vander, 1963), secretion of renin into tubular urine could subserve some role in the intra-renal regulation of sodium reabsorption.

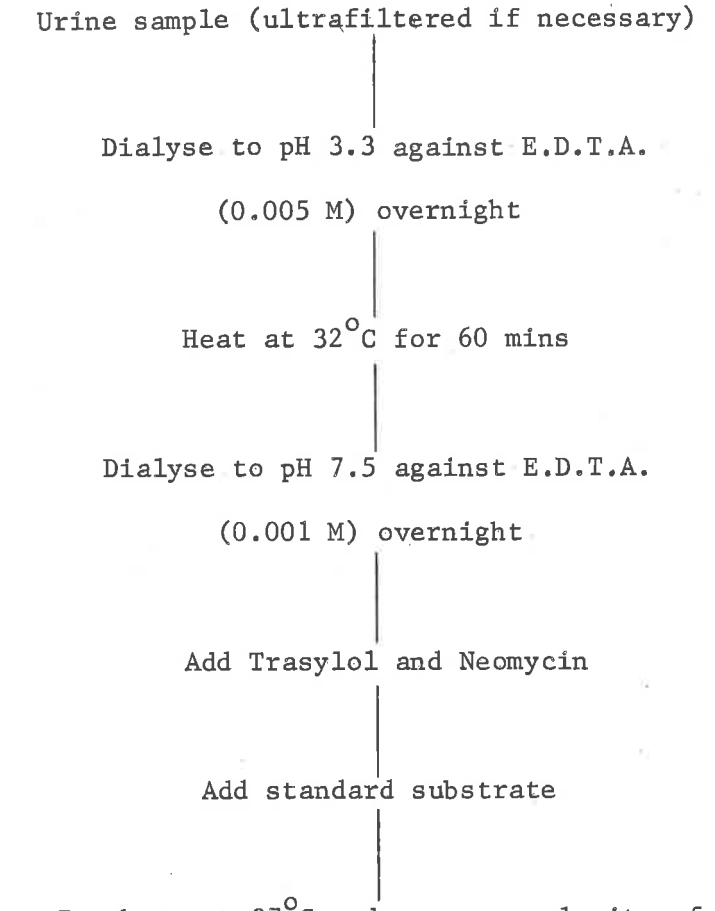
A study was undertaken to identify and quantitate the renin-like enzyme present in human urine.

METHODS:

Renin in urine was quantitatively assayed using the renin concentration method developed for plasma by Skinner in 1967. Figure 2-1 shows the flow sequence for the handling of a urine sample.

Up to 200 ml or more of freshly voided urine were concentrated by ultrafiltration at 750 mm Hg, at a temperature of $3^{o}C$,

FIGURE 2-1


Urine sample (ultrafiltered if necessary)
Dialyse to pH 3.3 against E.D.T.A.
(0.005 M) overnight
Heat at 32°C for 60 mins
Dialyse to pH 7.5 against E.D.T.A.
(0.001 M) overnight
Add Trasylol and Neomycin
Add standard substrate
Incubate at 37°C and measure velocity of
angiotensin formation by bioassay in the rat

in size 8/32 Visking cellophane casings (Figure 2-2). The urine was reduced to a volume of 10 ml or less. Following concentration, it was subjected to the dialysis sequence described for the handling of plasma for renin concentration determination (Figure 1-1, 2-1).

Following dialysis to pH 7.5, the urine samples were decanted, neomycin sulphate (2 mg/ml) and Trasylol (100 units/ml) added, and the volume corrected, if necessary, by addition of standard Buffer III. Samples were stored at -20°C.

Nephrectomized sheep substrate was added to samples in the ratio of one part of substrate to two parts of urine. These were incubated at 37°C. Aliquots of incubation mixture were taken at three time intervals, the duration of which ranged from 15 minutes to 100 hours. These were assayed for pressor activity without further extraction on the ganglion blocked rat, using synthetic β-asparaginyl angiotensin II as standard. The initial velocity of the reaction was determined and the concentration of renin expressed in units/ml (Methods).

Detection of angiotensinase activity in urine, the *preparation of standard sheep substrate*, the estimation of *renin substrate* in urine and preparation of *anti-renin* are described in Section 1 of this thesis.

Acid-denatured renin substrate was prepared by dialysing standard sheep substrate to pH 3.3 against Buffer I and heating for

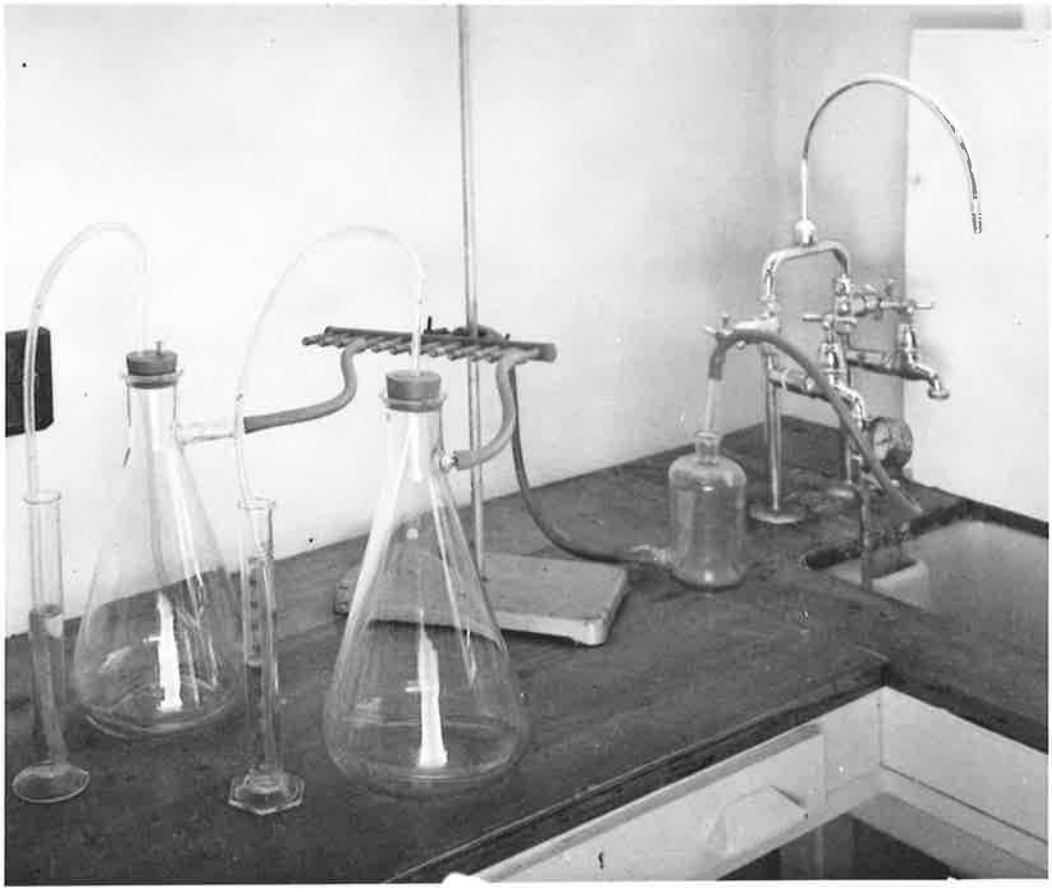


Fig. 2-2 Apparatus used for concentration of urine for renin estimation by ultrafiltration at a negative pressure of 750 mm Hg.

30 minutes at this pH prior to dialysis to the required pH.

Pepsin (Sigma, 3 times crystallized) was stored at 3°C in 0.01 M HCl in a concentration of 1 mg/ml.

Poly-l-lysine hydrobromide (100 mg/ml, Sigma) MW 150,000 was prepared and stored in 0.15 M HCl.

α-*chymotrypsin* (Worthington, 3 times crystallized) was prepared as required in pH 7.5 buffer.

RESULTS:

Occurrence of renin in normal urine:

Prior to the addition of renin substrate to urine, incubation of 26 normal urines, concentrated up to 44 times and passed through the handling sequence (Figure 2-1), failed to generate any pressor or depressor activity over 72 hours incubation at 37°C.

Addition of standard substrate, however, provoked the linear generation of pressor material at rates of 0.5 to 22.4 ng angiotensin/ml of incubate/hour. The rates varied with the extent of initial concentration by ultrafiltration.

Expressed as units of renin relative to the unconcentrated sample, normal urine contained on the average 0.6 ± 0.4 units/ml (mean ± SD) (range 0.1-1.9, n=26). These levels were approximately 1/15 those described for plasma. Only urines containing less than 0.75 units/ml required concentration by ultrafiltration.

Characteristics of the end-product produced by the action of renin in urine on nephrectomized sheep substrate:

The material formed on incubation of dialysed urine with standard sheep substrate gave a pressor response on intravenous injection into the ganglion blocked rat which was similar in shape to that seen with injections of the standard β-asparaginyl angiotensin II.

The pressor material was dialysable, not destroyed on boiling, but totally destroyed by incubation with α-chymotrypsin at pH 7.5 and pepsin at pH 5.7 (Table 1, Appendix). It formed at a linear rate on incubation up to and including 100 hours and during consumption of 120 ng of substrate (Figure 2-3).

The reaction rate increased with a rise in temperature up to and including 50°C (Figure 2-4). Above this temperature no pressor material formed. This was due to inactivation of both the enzyme contained in urine and the standard sheep substrate, since non-heated urine failed to generate pressor material on incubation with standard substrate previously heated to 55°C for one hour, and urine heated to 55°C similarly failed to generate pressor material when incubated with non-heated standard substrate.

Properties of the enzyme present in urine:

Effect of substrate concentration:

Similar curves were obtained for the effect of substrate changes on the reaction rate for renin extracted from plasma and

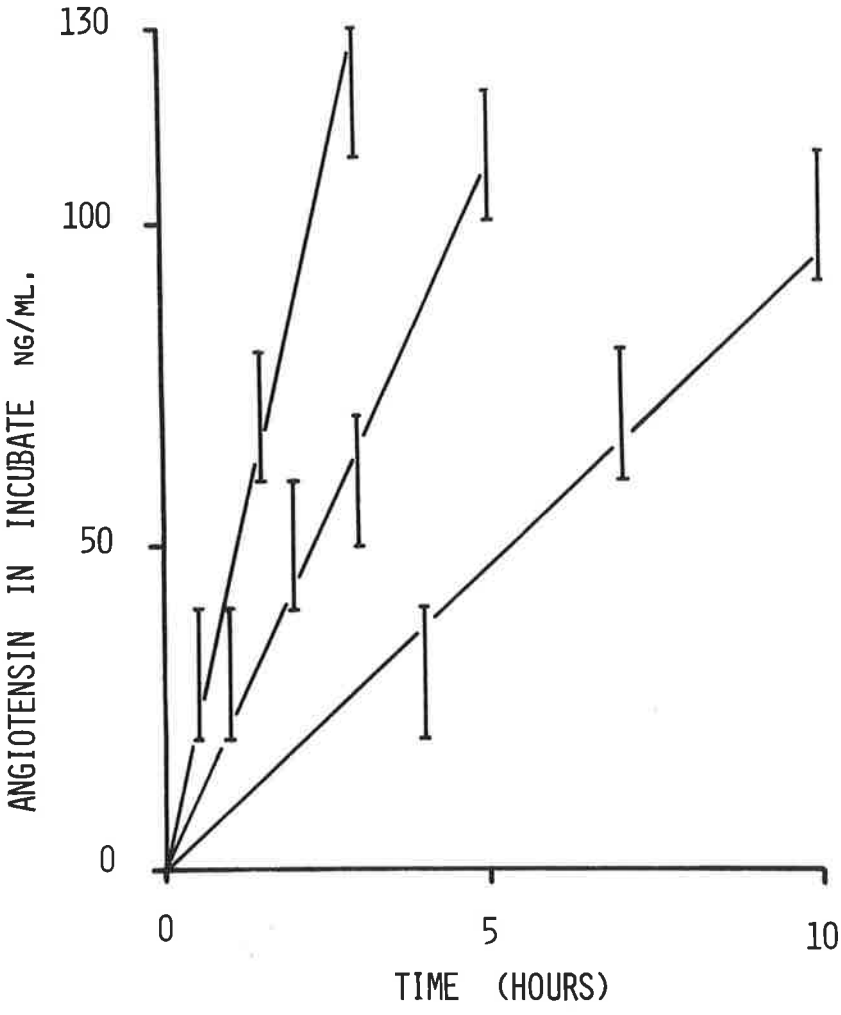


Fig. 2-3 The linear formation of pressor material with time following incubation of urine with standard sheep substrate at 37°C.

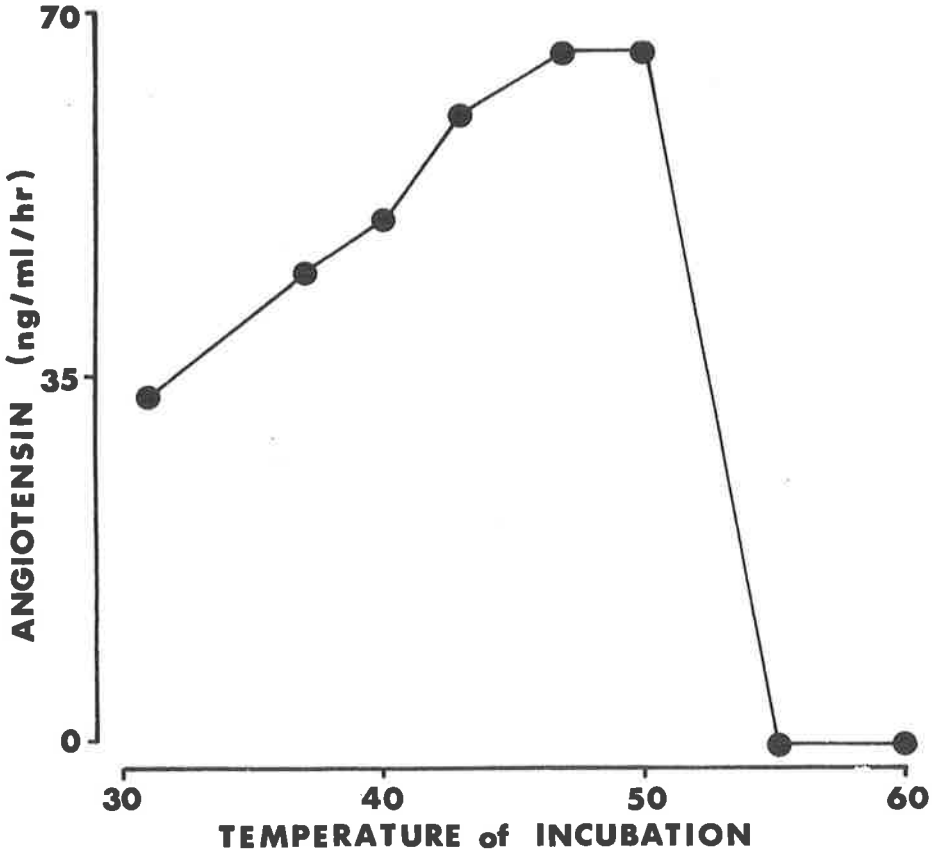


Fig. 2-4 The effect of temperature on the rate of formation of angiotensin by urinary renin acting on standard sheep substrate at pH 7.5 (Appendix Table 2).

urine. The plot for urinary renin is the mean derived from two urine samples containing 9.6 and 4.2 renin units/ml (Figure 2-5). Attempts to determine the Michaelis constant (Km), using either a Woolf or a Lineweaver-Burk plot (Dixon and Webb, 1964), yielded considerable variation. The mean Km of 5 determinations on urine from 4 individuals was 247 ng/ml, the range being 140 to 347 ng/ml (Figure 2-6, Table 3, Appendix). The cause of this spread may be largely accounted for by the error incurred in measuring initial velocity at low substrate concentration. With low concentrations of the substrate, greater than 30% of the substrate must be consumed in order to generate sufficient pressor material for accurate bio-assay.

Effect of enzyme concentration upon the rate of reaction between urinary renin and standard substrate:

The concentration of renin, after serial dilution of enzyme prepared from urine containing both high and low concentrations of enzyme, showed a linear relationship to the velocity of formation of angiotensin (Figure 2-7). Such a direct relationship exists for plasma renin extracted by the same method, but not for renal renin (Skinner, 1967).

Effect of pH on the rate of formation of angiotensin by urinary renin acting on standard substrate:

Urinary renin used to investigate the effect of pH on the reaction between renin and standard substrate was exposed to a pH of

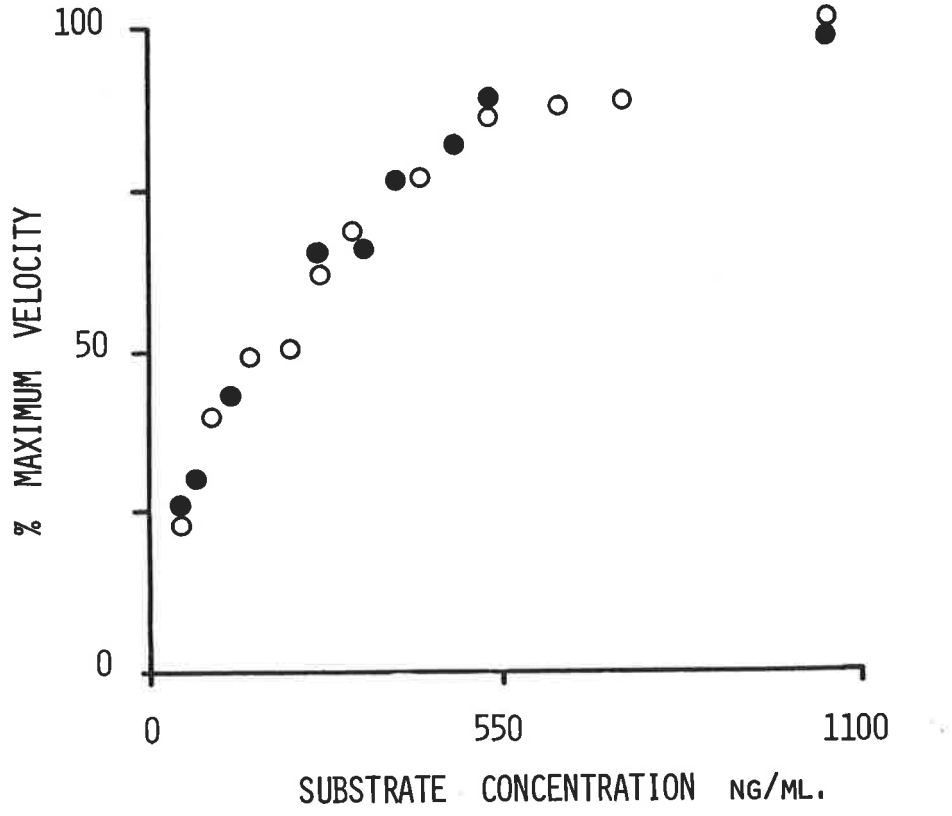


Fig. 2-5 The effect of the concentration of standard sheep substrate on the rate of formation of angiotensin by urinary renin (●) and plasma renin (○), expressed as a percentage of the maximum velocity obtained. The plot for urinary renin is the mean obtained from 2 urines containing 9.6 and 4.2 units/ml.

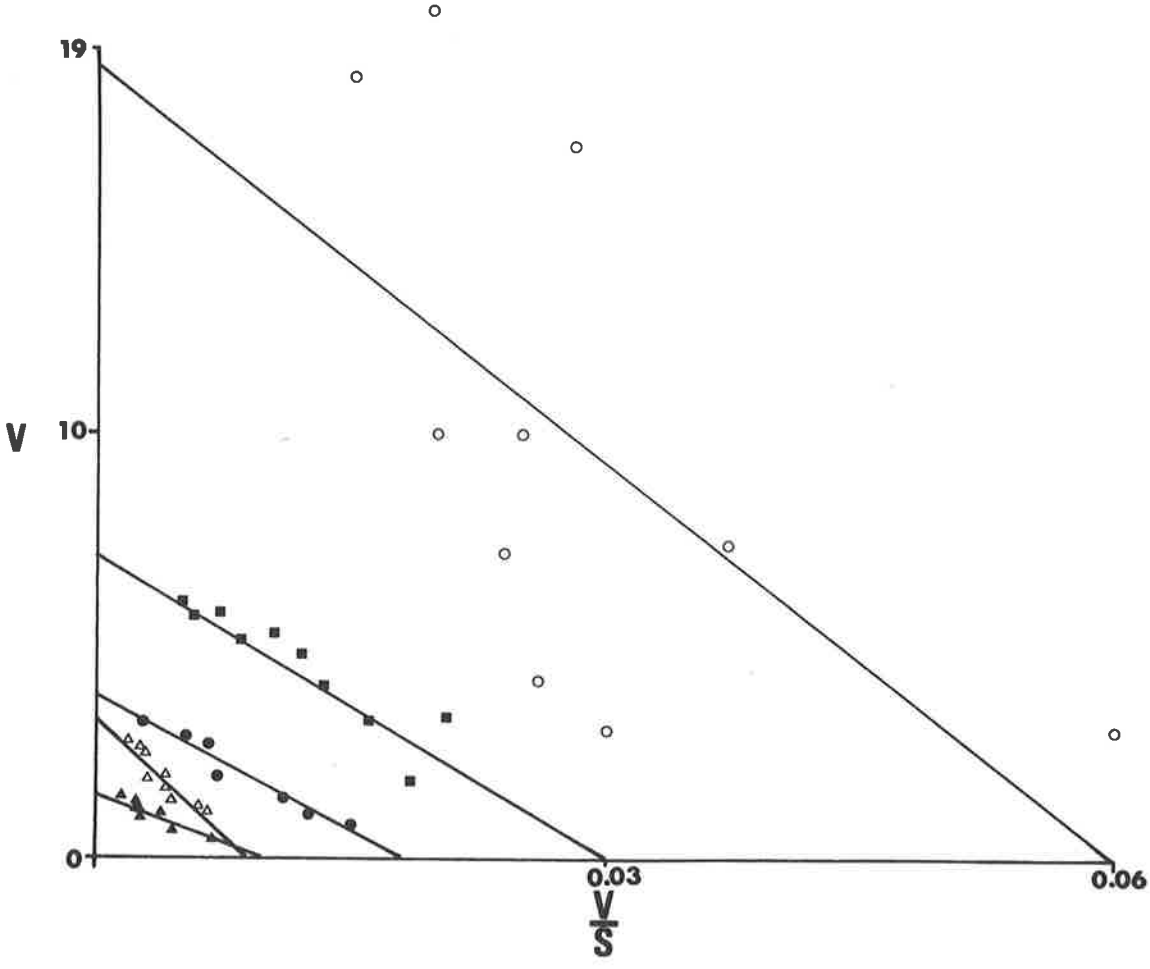


Fig. 2-6 Woolf plots derived from the effect of standard sheep substrate on the rate of formation of angiotensin (V) by 5 urines plotted against the rate of formation of angiotensin divided by substrate concentration. (Appendix Table 3(a), 3(b))

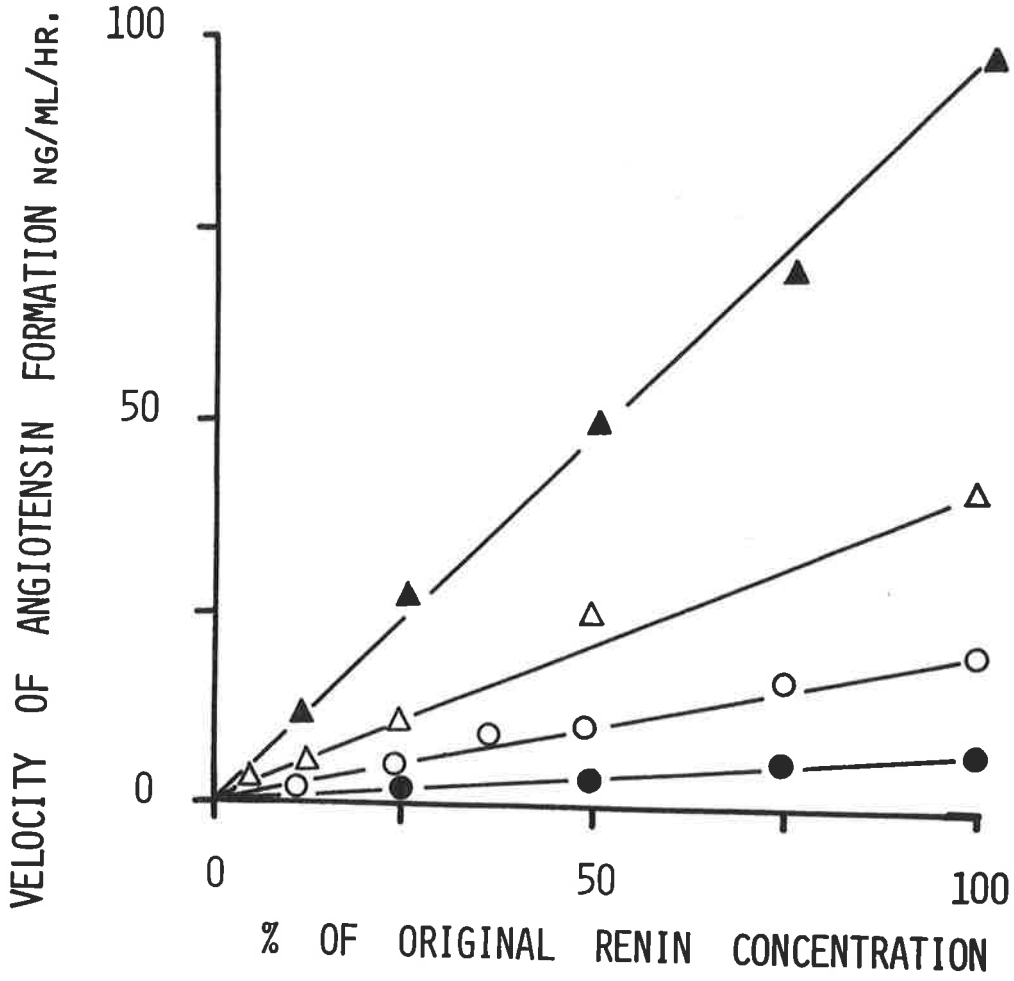


Fig. 2-7 The effect of the concentration of renin on reaction velocity, using 4 separate urine extracts containing 12.0, 30.0, 63.0 and 147 units/ml. Ordinate : uncorrected measured rate. (Appendix Table 4).

9.6, after pH 3.3 treatment, to achieve denaturation of an angiotensinase active below pH 7.0 and to denature pepsin.

The effect of pH on reaction rate was found to be similar to that of plasma renin (Skinner, 1967) (Figure 2-8). The optimum pH of the system was 7.8-8.5, the curve being flat between pH 7.0 and pH 9.0.

The action of antibody to renal renin on the activity of the urinary enzyme:

Incubation of urine, to which one part of antibody to human renal renin had been added to four parts of urine, with standard sheep substrate, failed to produce any pressor material, whereas the control dilutions of urine produced varying amounts of pressor material as did urines incubated with immunoglobulin containing no antibody titre to human renal renin (11 experiments).

Accurate assay of renin in urine necessitates that the following criteria be fulfilled:

(a) That no co-factors should be present in urine in rate-limiting amounts.

(b) That the recovery of renin should be high and consistent.

(c) That the system is free of substances which will inactivate the end-product formed.

(d) That the system is free of the interfering action of

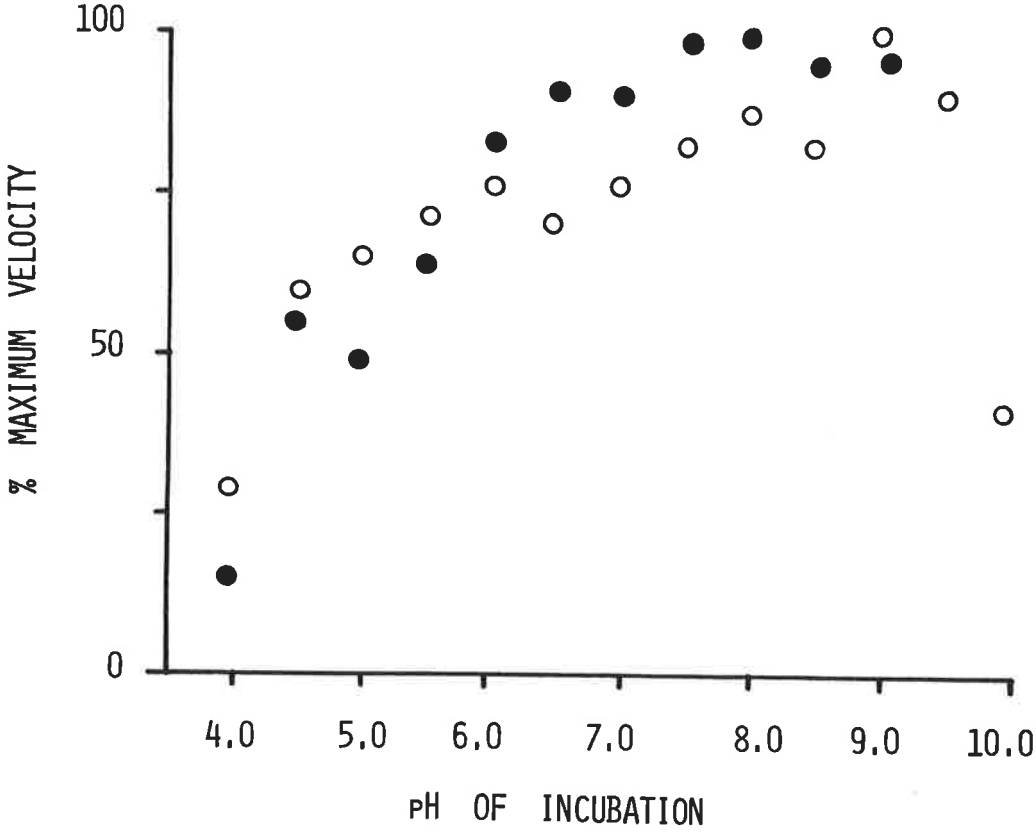


Fig. 2-8 The effect of the pH of incubation on the rate of formation of angiotensin by urinary renin (●) and plasma renin (O) acting on standard sheep substrate. Results are expressed as a per cent of the maximum velocity (obtained at pH 8.0) (Appendix Table 5).

pressor and depressor substances formed by the action of other enzymes during incubation.

(a) Lack of co-factor influence on urinary renin-renin substrate reaction:

The linear relationship between the concentration of urinary renin and the rate of formation of angiotensin made it unlikely that errors in quantitation due to co-factor influences were incurred.

Further evidence of lack of co-factor involvement in the formation of angiotensin from urinary renin and standard sheep substrate was obtained by adding a constant amount of urinary renin extracted from a urine containing a high concentration of renin to 3 urines containing low concentrations of renin. The added enzyme was assayed at 2.6, 2.7 and 3.1 units/ml, while the expected concentration was 3.3 units/ml. These differences were not significant. Furthermore, the addition of renal renin to 3 other urine extracts generated angiotensin at 40, 42 and 44 ng/ml/hr, which was not significantly different from the control sample diluted with standard Buffer III (40 ng/ml/hr).

(b) Recovery of renin and replicate estimations:

Although plasma renin was found to be stable on exposure to the pH treatment used in this study to denature endogenous substrate and angiotensinase (Skinner, 1967), it was necessary to ensure that the urinary enzyme was also stable and, furthermore, that renin was

not lost by ultrafiltration under pressure.

Stability of the enzyme to low pH treatment was established by recycling 4 times a pooled sample of urine through the entire method, excluding ultrafiltration, and estimating the fall in velocity of angiotensin formation after each cycle of the dialysis sequence. Velocity measurements obtained were 2.8, 2.5 and 2.3 ng/ml/hr after the first, third and fourth cycles, respectively.

Possible loss during ultrafiltration was investigated in two ways. Firstly, a sample of pooled urine was concentrated, dialysed and treated as in Figure 2-1, and then diluted to the original volume, re-concentrated and retreated. The velocity of angiotensin formation was the same after each step, being 1.2 ng/ml/hr.

Secondly, 1 ml of stock human renal renin was added to 200 ml of urine and, after saving an aliquot, the remainder was concentrated, and both the concentrate and the ultrafiltrate assayed for renin content.

The ultrafiltrate failed to generate any pressor material on incubation with renin substrate, whilst the concentrate generated angiotensin at the rate of 366 ng/ml/hr. After correction for the 6.2-fold concentration step, the actual rate of 59 ng/ml/hr was not significantly different from the rate of the unconcentrated aliquot (56 ng/ml/hr).

Duplicate estimations (Table 6, Appendix):

(a) Rat assay variation:- Repeat assays of the same samples on the same rat after short intervals of time produced a mean difference in velocities of 4.7% (± 6.0%, n=8).

(b) Repeat assays of the same sample on different rats after storage for weeks or months at -20°C gave a mean difference in velocities of 16% (± 11%, n=13).

(c) Duplicate assay of the same samples, using stored urine and passing it through the method for the second time after intervals of days or weeks, gave an assay variability of 26% (± 19%, n=13).

This rather large assay variability may be a reflection of the extremely low levels of renin found in urine which makes accurate quantitation of these levels more difficult.

(c) Freedom from end-product inactivation:

Angiotensinases present in normal urine:

The following experiments demonstrate that the angiotensinase activity in urine is due to the presence of at least two separate enzymes.

Urine samples to be tested for angiotensinase activity were dialysed against a range of buffers of pH 7.5 to 3.3, both in the presence and absence of EDTA (Figure 2-9). The samples were heated for one hour at their respective pH and then dialysed to a final pH

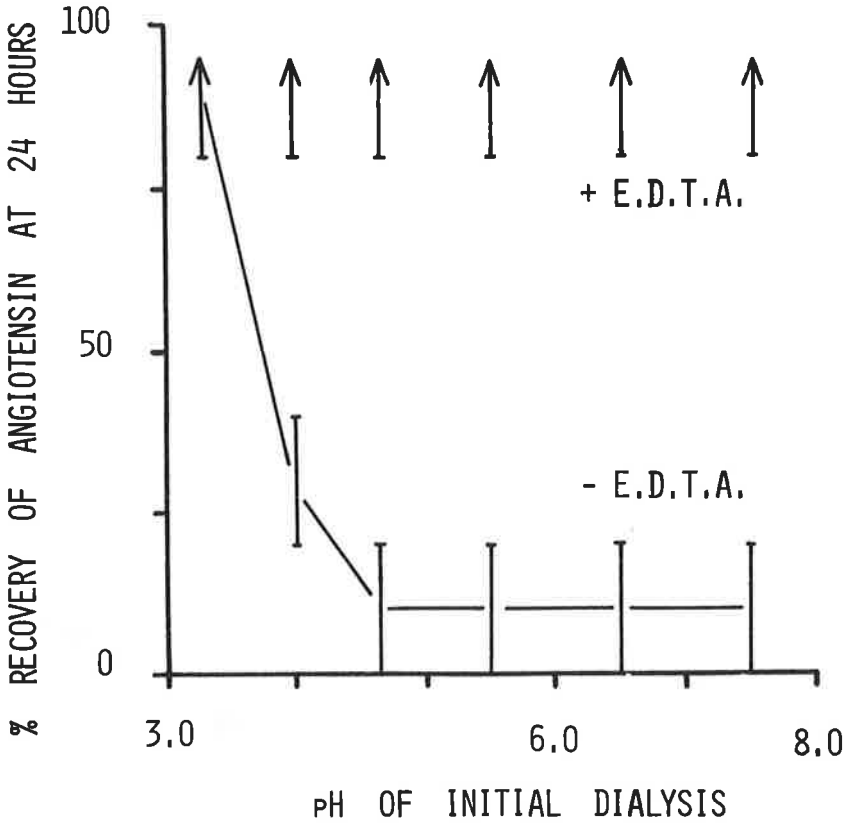


Fig. 2-9 The effect of the pH of initial dialysis and the presence of EDTA on the activity of angiotensinase in urine, tested by incubation of urine with β-asparaginyl angiotensin II at pH 7.5 (Appendix Table 7).

of 7.5. A concentration of β-asparaginyl angiotensin II (250 ng/ml) was added to the samples and an aliquot of the mixture incubated for 24 hours. Comparison with the unincubated control showed that an angiotensinase was present in urine, active at pH 7.5 and resistant to exposure to a pH greater than 3.3. This enzyme was, however, inactivated by a pH of 3.3 or by the addition of 0.001 M EDTA to the system.

Although an initial pH treatment of 3.3 or the addition of EDTA to the system inactivated angiotensinase in samples incubated at pH 7.5 or above, this was not the case with samples incubated at a final pH of less than 7.0. This finding suggested the presence of a second angiotensinase in urine. This second enzyme was found to be denatured, however, by dialysis and heating for one hour at pH 9.6 against 0.16 M glycine-NaOH buffer prior to dialysis to the final pH for incubation with 250 ng/ml of β-asparaginyl angiotensin II (Figure 2-10).

The above findings were obtained with normal dilute urine, but it was anticipated that with ultrafiltration the angiotensinases present would be concentrated in parallel. This was confirmed by the finding that only 20% - 80% survival of β-asparaginyl angiotensin II was obtained in 13 of 20 normal urines concentrated 20-fold and incubated at pH 7.5 following prior dialysis to a pH of 3.3 and treatment with EDTA.

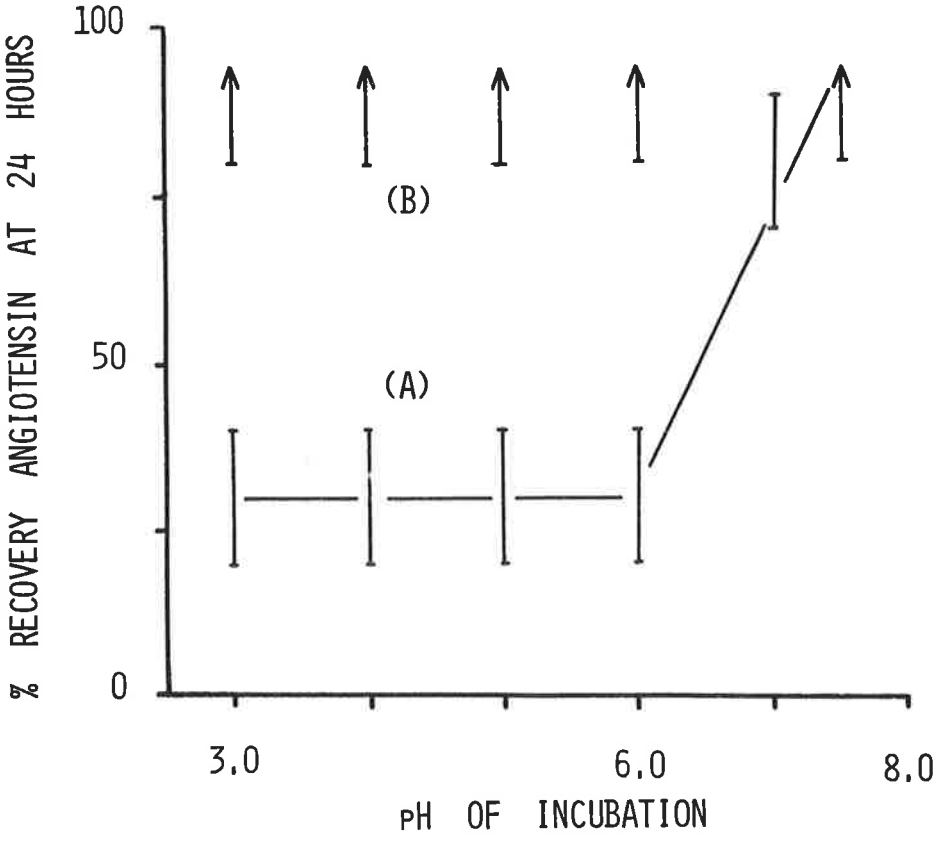


Fig. 2-10 The effect of the pH of incubation on angiotensinase activity in urine incubated with β-asparaginyl angiotensin II. A : Urine incubated with β-asparaginyl angiotensin II without prior dialysis and heating at pH 9.6. B : Urine incubated with β- asparaginyl angiotensin II following dialysis and heating at pH 9.6 (Appendix Table 8).

When, however, the angiotensin which forms as a result of incubation of renal renin on human substrate was used as a substrate to test angiotensinase activity, greater than 80% recovery was recorded after 24 hours of incubation in the 15 urines tested. Further evidence for freedom from end-product inactivation is contained in the finding that the formation of the end-product of the reaction between urinary renin and standard substrate bears a linear relationship to time.

(d) Freedom of the system for the assay of renin in human urine from the formation of depressor and pressor materials by other enzymes:

(1) Presence of kinin-forming enzymes in the system:

Trasylol was used routinely to block the formation of kinins in the present system. It has been shown to be an effective antagonist of urinary kallikrein acting on plasma globulin (Prado, Prado, Brandi and Katchburian, 1963) and does not influence the rate of reaction between renin and renin substrate in a concentration of 100 units/ml, nor does it have any effect on rat blood pressure (Skinner, 1967). Trasylol was added routinely to all samples tested. However, it was also found that no depressor material did form during incubation of 5 concentrated urine samples (to which Trasylol was not added) with 'Trasylol-free' standard substrate.

(2) The presence of enzymes in urine other than renin which are capable of forming pressor material on incubation with

standard substrate:

Since pepsin is known to act on renin substrate to produce a pressor material identical pharmacologically and structurally to angiotensin I (Alonso, Croxatto and Croxatto, 1943; Blair, 1962; de Fernandez, Paladini and Delius, 1965) and, furthermore, is known to occur in normal urine (Bucher, 1947; Mirsky, Block, Osher and Broh-Kahn, 1948), it was of interest to determine whether pepsin in urine was capable of acting on standard substrate under the right pH conditions and, furthermore, whether it was capable of forming pressor substances which would interfere with the accurate assay of renin in urine.

Incubation of urinary renin with standard sheep substrate at pH 7.5 ensured denaturation and destruction of pepsin (Herriott, 1962). However, its importance in systems incubated at a pH of less than 7.0 was emphasized by the following experiments.

(a) Pooled urine was concentrated 50-fold and divided into two parts. One aliquot was dialysed as usual to a pH of 3.3 with EDTA and heating prior to dialysis to a final pH of 7.5. A second aliquot was dialysed to an initial pH of 2.0 and similarly treated. On incubation for 2 hours with standard substrate the results seen in Figure 2-11 (top trace) were obtained. The pH 3.3 treated aliquot diluted 3-fold showed the anticipated pressor activity for this degree of concentration (46 ng/ml/hr), whereas the pH 2.0 treated

urine was inactive. These findings are characteristic of the denaturation properties of renin (Skinner, 1967).

When, however, a further aliquot of the pH 2.0 treated urine was incubated with standard sheep substrate at a pH of 5.6 instead of pH 7.5, pressor material formed at the remarkable rate of greater than 1000 ng/ml/hr. Formation of this pressor material was totally inhibited by dialysis to pH 7.5 prior to dialysis back to a final pH of 5.6 (Figure 2-11, bottom trace).

These findings are characteristic of the denaturation of pepsin (Herriott, 1962), but not renin (Braun-Menéndez, Fasciolo, Leloir, Munoz and Taquini, 1946).

(b) The properties of the acid-stable, alkali-labile enzyme in urine were further investigated by studying its ability to form pressor material from acid-denatured substrate. A further aliquot of the 50-fold pH 2.0 treated concentrate of urine was reacted with both normal and acid-treated substrate at pH 5.6 at 37°C. It was capable of forming pressor material from both substrates at the same fast rate (Figure 2-12).

The same concentrate treated by dialysis to pH 3.3 and then dialysed to a final pH of 7.5 failed to generate pressor material from the acid-denatured substrate, whereas the expected amount of pressor material was generated from normal substrate (Figure 2-12).

These findings are consistent with the known ability of

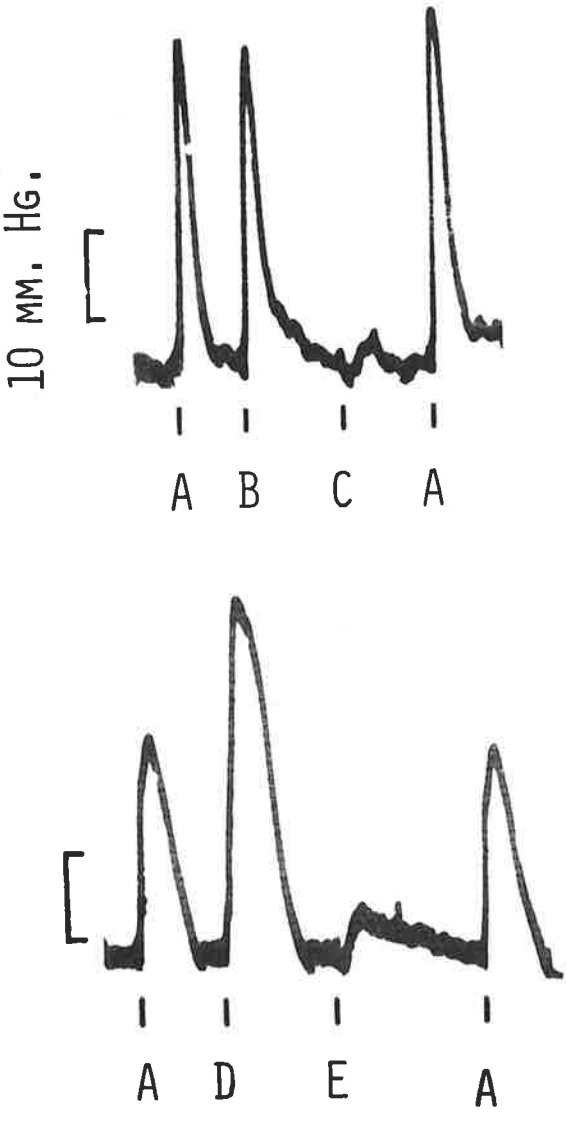


Fig. 2-11 Top trace: Denaturation characteristics of urinary renin at pH 2.0. A : 10 ng angiotensin (0.1 ml). B : 0.1 ml pH 3.3 treated urine after 2 hr incubation with substrate at pH 7.5. C : 0.1 ml pH 2.0 treated urine; otherwise as for B.

Bottom trace: Pepsitensin formation at pH 5.6. A : 10 ng angiotensin (0.1 ml). D : 0.05 ml pH 2.0 treated urine incubated 30 min with substrate at pH 5.6. E : 0.05 ml of same sample after dialysis to pH 7.5 and return to pH 5.6; otherwise as for D.

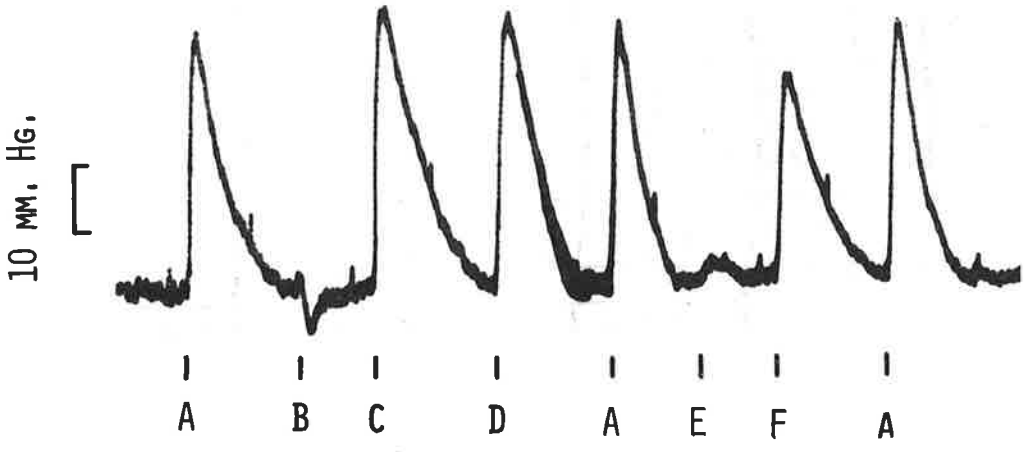


Fig. 2-12 Separation of the effects of urinary renin and pepsin by action on denatured renin substrate.

A : 10 ng angiotensin (0.1 ml)

B : pH 2.0 treated urine (0.04 ml) incubated for 30 min without substrate at pH 5.6.

C : 0.05 ml of B incubated 30 min with standard substrate at pH 5.6.

D : 0.05 ml of B incubated 30 min with denatured substrate at pH 5.6.

E : 0.1 ml of pH 3.3 treated urine incubated 30 min with denatured substrate at pH 7.5.

F : 0.1 ml of pH 3.3 treated urine incubated 30 min with standard substrate at pH 7.5.

pepsin, but not of renin, to form pepsitensin or angiotensin from denatured renin substrate (Braun-Menéndez and Paladini, 1958). We confirmed the ability of pepsin to act on acid-denatured renin substrate by incubating crystalline pepsin with acid-denatured renin substrate at 37°C and pH 5.6. A concentration of 15 µg/ml exhausted the substrate within one hour under these conditions of incubation, whereas a concentration of renal renin which produced substrate exhaustion within 10 minutes when reacted with standard substrate formed an equivalent amount of angiotensin from acid-denatured substrate only after incubations for 7 days or more.

Some characteristics of the action of pepsin on renin substrate:

The reaction between pepsin and renin substrate is not linear with time (Figure 2-13), a curved velocity plot being produced which is probably a reflection of the concomitant destruction of the angiotensin by pepsin as it is produced.

With low concentrations of pepsin added to renin substrate and incubated at pH 5.6, the amount of angiotensin formed is not directly related to the concentration of the enzyme. At concentrations of pepsin of less than 8 µg/ml (Figure 2-14) little pressor material is generated. This may be due to the concomitant destruction of pressor material as it is formed, or may be due to pepsin-inhibitor present in the renin substrate preparation which would tend to combine with pepsin at a pH of greater than 5.4 (Herriott, 1940;

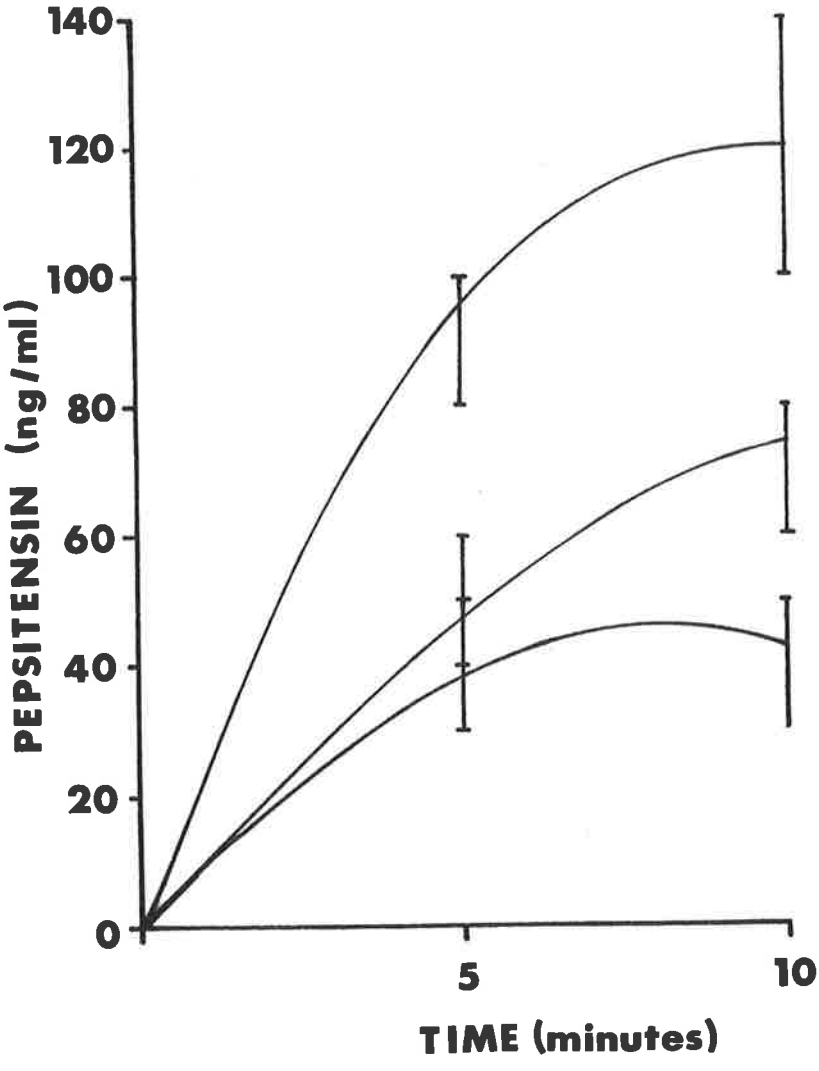


Fig. 2-13 The formation of pressor material with time by pepsin concentrations of 15, 10 and 8 µg/ml acting on standard sheep substrate at pH 5.6 (Appendix Table 9).

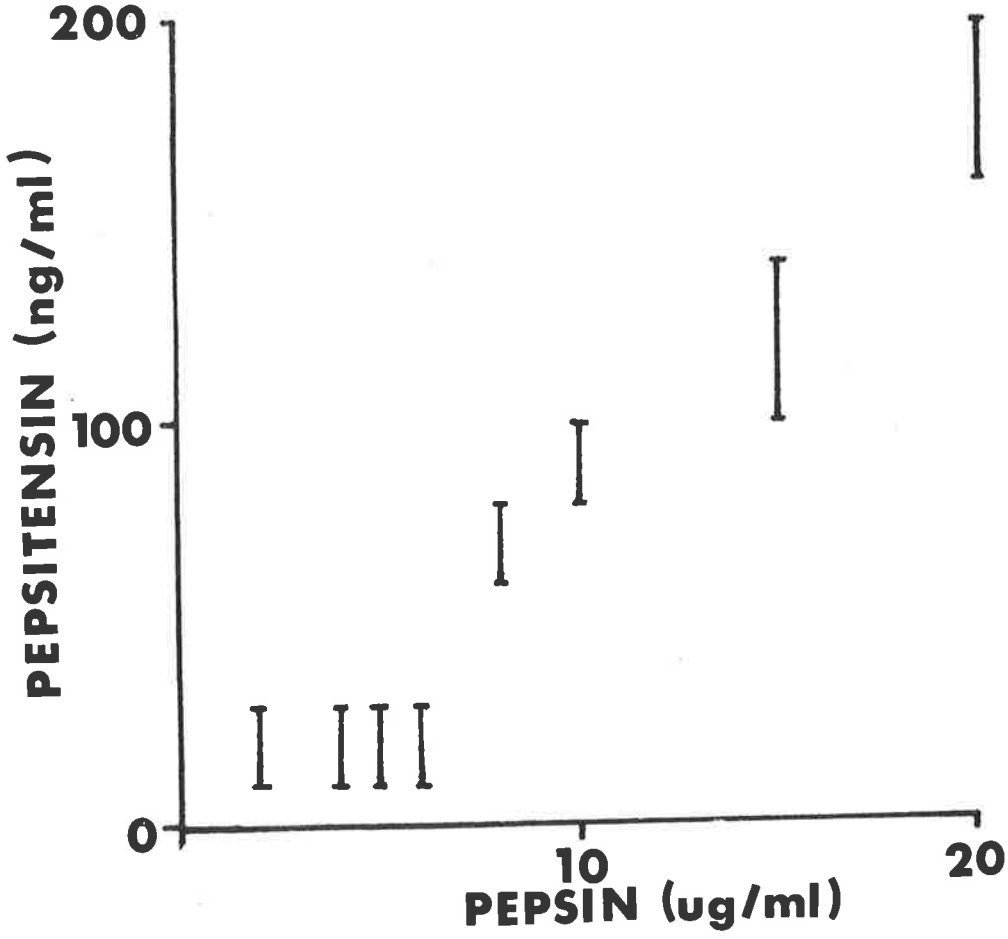


Fig. 2-14 The effect of the concentration of pepsin on the rate of formation of pepsitensin (ng/ml) at pH 5.6 (Appendix Table 10).

Van Vunakis and Herriott, 1956).

(c) Poly-l-lysine has been shown to be an effective antagonist of the proteolytic activity of pepsin (Katchalski, Berger and Neumann, 1954). An almost complete inhibition of the action of pepsin on renin-substrate and the pressor material liberated following incubation of urine with renin substrate at pH of 5.6 was achieved by the addition of poly-l-lysine at a concentration of 0.5 mg/ml to the incubation mixtures. This concentration had no effect on the pressor material formed as a result of incubation of urine with renin substrate at pH 7.5.

It has been suggested that amylase is capable of reacting to produce a pressor material. However, this occurred with impure preparations of amylase and its actual significance is obscure, there being a progressive loss of activity with purification of the enzyme (Walaszek, Bunag and Huggins, 1962). Lack of interference of urinary amylase in the present system is suggested on the basis of immunological data since antibodies to renal renin inhibited the enzyme in urine.

DISCUSSION:

The experiments described demonstrate that there is in urine at least two enzymes capable of reacting with standard nephrectomized sheep substrate to produce pressor substances on intravenous injection into the ganglion-blocked rat. Fortunately,

these two enzymes can be separated on the basis of susceptibility to denaturation by low or high pH; by the optimum pH for incubation; by differences in the rate of reaction of the enzymes on acid-denatured substrate; and by inhibition by specific inhibitor or antibody.

An enzyme which has similar actions to those described for pepsin, in that it acts upon renin substrate to produce a pressor material at a pH of 5.6, is present in urine. This enzyme is resistant to a pH treatment of 2.0, but is denatured by an alkaline pH of 7.5. Like pepsin, it is inhibited by the addition of poly-l-lysine to the incubation mixture.

The second enzyme in urine appears in all respects studied to be identical to plasma and renal renin. This enzyme can be distinguished from urinary pepsin, in that it is denatured by low pH treatment (pH 2.0), although it is stable to a pH of 3.3. On incubation with standard substrate at pH 7.5 a pressor material is generated, the formation of which is linear with time.

Urinary renin is inhibited by antibody raised in the rabbit to human renal renin, but not to rabbit plasma obtained from animals immunized with Freund's incomplete adjuvant alone.

The pressor material formed as a result of incubation of urine with renin substrate at pH 7.5 is similar to angiotensin in that it is dialysable, heat stable, destroyed by the action of

proteolytic enzymes such as chymotrypsin and, furthermore, the pressor response on intravenous injection is identical to that obtained with angiotensin II.

The identity of the end-product obtained from incubation of urinary renin with renin substrate has not been established beyond identification of its peptide nature. It is possible that the peptide formed is angiotensin I which is rapidly converted on intravenous injection into the rat to angiotensin II, since EDTA used in the present system to inhibit angiotensinase would also tend to inhibit the action of any converting enzyme that may be present in the incubation mixture (Skeggs, Kahn and Shumway, 1956).

The enzymatic nature of the reaction between urine and renin substrate at pH 7.5 is apparent from the findings that the production of pressor material is linear with time; that the reaction is temperature dependent; influenced by substrate concentration and also by pH.

Suppression of the reaction by antibody to renal renin and the similarity in the effects of pH and substrate concentration on the rate of reaction between enzyme and substrate suggest a common identity of plasma and urinary enzymes. Although the relationship of angiotensin formation to concentration is linear for both plasma and urinary renin, this is not the case for renal renin acting on standard sheep substrate (Skinner, 1967).

Treatment of urine to a pH of 3.3 was thought initially to be necessary to ensure denaturation of any endogenous substrate that may be present in urine. Although no substrate was found in normal urine, the presence of small amounts of substrate in several urines displaying heavy proteinuria necessitated the routine use of the low pH treatment in these situations.

At least two enzymes are present in normal urine which are capable of inactivating angiotensin as it is formed. Angiotensinase activity was slightly faster against the synthetic β-asparaginyl angiotensin II than it was against the end-product of incubation formed in the present system. This may be accounted for by the finding that the β-aspartyl amide is hydrolysed more rapidly than is the naturally occurring acid (Regoli, Riniker and Brunner, 1963; Nagatsu, Gillespie, Folk and Glenner, 1965).

Inhibition of angiotensinase acting at pH 7.5 was achieved by either pH 3.3 treatment or the addition of EDTA to the system. This treatment failed to inactivate the angiotensinase active at a pH of less than 7.0. However, this angiotensinase is inhibited by prior dialysis and heating at pH 9.6, a finding that suggests that at least part of this activity could be due to the action of pepsin present in urine (Munoz, Braun-Menéndez, Fasciolo and Leloir, 1940; Plentl and Page, 1944).

The linear formation of pressor material on incubation of

urine with substrate further suggests that the present system is free from end-product inactivation, whilst the linear relationship between concentration of the enzyme and formation of the pressor material suggests that there is no co-factor or inhibitor present in this system.

The levels of renin in normal urine are low, being of the order of 0.6 units/ml. This necessitated the use of a concentration step (ultrafiltration) to achieve levels high enough for accurate assay of renin in urine. The low levels found were not consequent upon the loss of renin during treatment of the urine samples, nor were they exaggerated by any loss during ultrafiltration, since recycling of urine samples through the method did not cause any significant fall in the concentration of renin present.

The results presented by Brown, Davies, Lever, Lloyd, Robertson and Tree (1964) suggest that urinary renin concentration is of a similar order to that found in plasma, whilst the concentration of renin in urine as determined by the present assay system is only 1/15 that found in plasma. This discrepancy in results led to an investigation of the properties of the enzyme active at pH 5.6, and it was concluded that this enzyme was very similar to pepsin. This was established by the differential pH treatment of urine described in the text which showed clearly that there are two enzymes in urine capable of producing pressor material on incubation with standard

renin substrate. Since the pressor material formed by the action of pepsin on renin substrate is indistinguishable from angiotensin I (Alonso, Croxatto and Croxatto, 1943; Blair, 1962; de Fernandez, Paladini and Delius, 1965), differentiation of the two enzymes is dependent upon identification of the properties of the enzymes.

It was concluded that some of the 'renin-like' enzyme activity in normal urine incubated at pH 5.6 could be due to the action of urinary pepsin on renin substrate. Since pepsinogen would be present in urine, treatment of urine to a pH of 2.0, which, in the present assay system, results in the denaturation of renin (Skinner, 1967), would convert the urinary pepsinogen to pepsin and subsequent incubation at pH levels of less than 7.0 might produce pepsitensin, especially if the enzyme was concentrated during extraction procedure.

Brown, Davies, Lever, Lloyd, Robertson and Tree (1964) found that the renin-like enzyme in normal human urine resembled renal renin on starch-gel electrophoresis (Peart, Lloyd, Thatcher, Lever, Payne and Stone, 1966), but an enzyme such as pepsin, with an isolectric point of less than 1.0 and a molecular weight of 35,000, might also be expected to migrate with renin in the pre-albumin range.

(B) OBSERVATIONS ON THE ORIGIN OF RENIN IN HUMAN URINE

The development of an assay system for the quantitation of renin in human urine provides a method for studying the renal handling of renin. Although the levels of renin found in urine were considerably less than those described previously, the renal handling of renin is important in view of the suggestion made concerning the action of angiotensin on proximal tubular sodium (Leyssac, 1967) and distal sodium transport (Vander, 1963). Furthermore, in animals, renin appears to induce proteinuria (Pickering and Prinzmetal, 1940; Deodhar, Cuppage and Gableman, 1964; Katz, Sellers and Bonnoris, 1964), and since proteinuria was found to be augmented in conditions associated with elevation in plasma renin levels (Tobian and Nason, 1966), it was of interest to investigate the effects of elevation of plasma renin levels on the excretion of protein in normal subjects. In order to assess the diagnostic potential of urinary renin estimation, urinary renin and plasma renin levels were estimated in a variety of pathological situations.

METHODS:

Experiments were performed on 19 normal males, 8 normal females and 19 hospital patients. Unless otherwise stated, the subjects were requested to pursue their normal pattern of daily activity and no deliberate control was exercised over dietary sodium

or fluid intake.

All blood samples for plasma renin estimation were taken from an antecubital vein with the subject in the sitting position. Samples were taken at 1100 hours or 1600 hours as indicated in the text.

Diurnal variations in urinary renin excretion were determined over 12 hour collection periods between 0900 and 2100 hours. Blood for plasma renin estimation was taken at the times indicated above. Voided urine was stored at $4^{o}C$ during collection periods.

The 24 hour renin clearance was determined from the formula: $$C_{renin} = \frac{U_{renin\ (units/ml)} \times V\ (ml/24\ hours)}{P_{renin\ (units/ml)}}$$

Urinary and plasma renin levels were estimated before and during treatment of 7 normal male subjects with spironolactone, an aldosterone antagonist (Aldactone, Searle), 50 mg twice daily for 12 days which was combined with chlorothiazide (Chlotride, Merck, Sharp and Dohme) 0.5 grams daily during the last 5 days of therapy. In this study, the blood samples were all taken at 1100 hours and the urinary renin output calculated in the 24 hour urine sample collected over the previous 24 hours.

Methods of estimation of parameters measured:

Urinary renin and renin substrate were assayed quantitatively by the methods described previously. Plasma renin concentration was

determined by the method of Skinner (1967). Plasma renin concentration, and urinary renin concentration and output are expressed in the same units.

Sodium was estimated by flame photometry; urinary creatinine, plasma creatinine, and plasma proteins by the Technicon Autoanalyser (Technicon, Ardsley NY, 1965).

Total protein excretion was determined by a modification of the Lowry method, using the Folin-Ciocalteu reagent (Lowry, Rosebrough, Lewis Farr and Randall, 1951). A calibration curve was constructed using bovine serum albumin as standard (Figure 2-15). Standard protein solutions and urine samples were read at 750 mu against a saline blank containing reagents alone.

In order to avoid errors due to reactive groups in amino acids and peptides, urine samples for urinary protein estimation were dialysed for a total of 48 hours in 8/32 Visking cellophane casings against running tap water and then against isotonic saline. This dialysis was performed at atmospheric pressure and loss of protein was therefore avoided (Rowe and Soothill, 1961; King and Boyce, 1963).

Qualitative estimations of protein in urine were made using Albustix (Ames) and the results graded from negative to ++++ by colour matching with the Albustix chart.

Urinary and plasma albumin levels were estimated by the Soothill modification of the Ouchterlony technique (Soothill, 1962).

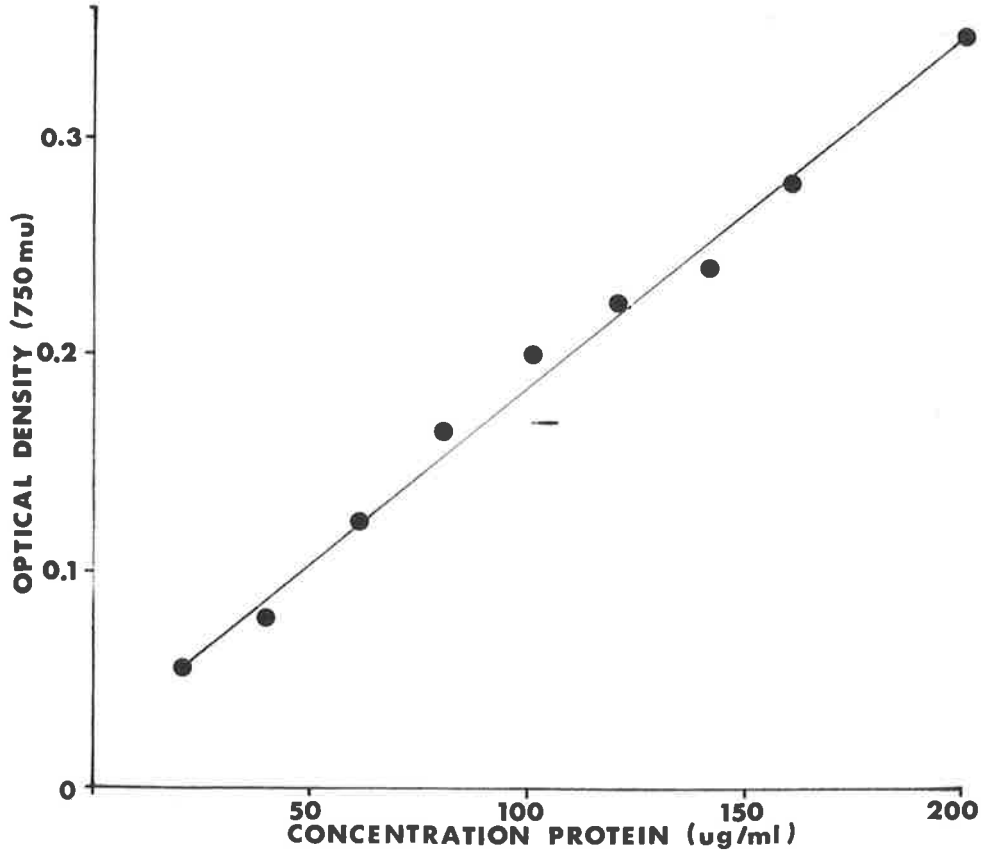


Fig. 2-15 Calibration curve constructed with standard concentrations of protein, using a calorimetric technique. The changes in optical density were read at 750 mμ (Appendix Table 11).

RESULTS:

Normal renin output in urine:

The findings illustrated in Figure 2-16 and Table 2-1 show that the normal renin output estimated in 19 normal males ranged widely from 162 to 7500 units/24 hours (mean 1196). Despite the wide variations in urinary renin output between individuals, the renin output within one individual tended to remain constant (Figure 2-16).

Renin output into urine in 8 normal females ranged from 1013 to 21,730 units/24 hours (mean 6811) (Figure 2-16, Table 2-2). The levels of renin found in the urine of normal females were therefore significantly higher than those found in normal males of comparable age ($0.01>P>0.002$).

No relationship was found between plasma renin concentration and the 24 hour excretion rates of renin into urine (Table 2-1) ($r = -0.17$, n.s.), nor was there any relationship between plasma renin concentration and urinary sodium ($r = 0.33$, n.s.) within the 19 normal males studied. There was no indication that further estimations would have elevated these relationships to a significant level.

Renal clearance of renin:

The renal clearance of renin in 16 normal males ranged from 12 to 893 ml/24 hours (mean 138). The renal clearance of renin in 8 normal females was significantly higher ($0.01>P>0.002$) than that found in males, ranging from 79 to 3149 ml/24 hours (mean 950 ml/

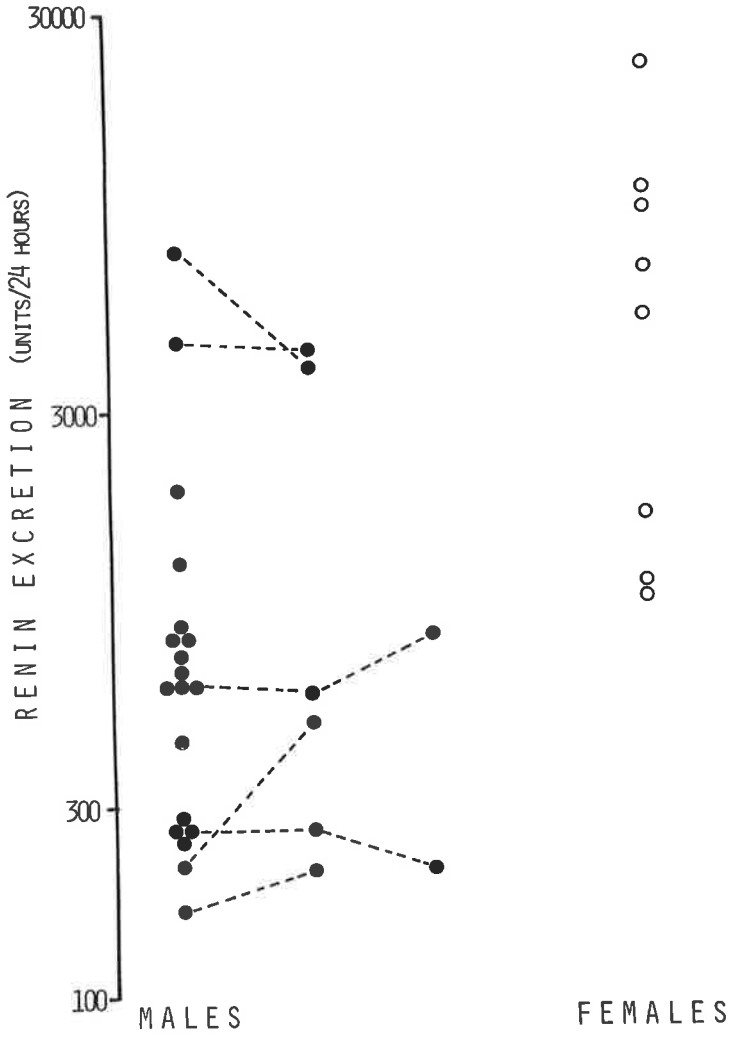


Fig. 2-16 Renin output (units/24 hours) into urine of 19 normal males (●) and 8 normal females (o). Lines join repeat estimations on individuals performed at intervals of weeks or months.

TABLE 2-1

Renin and sodium excretion in normal males

No.	PRC (units/ml)	Renin excretion (units/24 hrs)	Renin clearance (ml/24 hrs)	Sodium excretion (mEq/24 hrs)
1		783		
2		706		
3		614		
4		268		
4a*	9.3	257	27	
5	6.4	596	93	288
5a*	11.5	567	49	
6	6.8	210	31	319
6a*	5.3	490	92	
7	24.0	846	35	347
8	8.3	1,879	226	340
9	9.5	1,234	130	205
10	14.0	162	12	235
11	11.4	273	24	189
12	15.0	675	45	202
13	8.4	271	32	35
14	10.0	815	82	101
15	12.4	287	23	261
16	8.4	7,500	893	
17	10.3	432	42	
18	9.8	4,442	453	
19	13.5	615	46	
Mean	11.2	1,196	138	229

*4a, 5a, and 6a are repeats on subjects 4, 5, and 6, respectively. Repeats were averaged in calculating group means. PRC = plasma renin concentration. Correlation coefficients, renin excretion : PRC (r=-0.17) n.s., renin excretion : sodium excretion (r=0.33) n.s.

TABLE 2-2

Renin excretion in normal females

No.	PRC (units/ml)	Renin excretion (units/24 hrs)	Renin clearance (ml/24 hrs)
1		6,750	
2		1,643	
3	8.8	10,754	1,222
4	6.9	21,730	3,149
5	9.5	1,098	116
6	12.8	1,013	79
7	9.6	9,435	983
8	14.1	2,072	148
Mean	10.3	6,811	950

PRC = plasma renin concentration

24 hours). Assuming a mean normal creatinine clearance of 180 litres/ 24 hours, the renal clearance of renin for males was only 0.077% that of the creatinine clearance, and for females 0.53% that of the clearance of creatinine.

Effect of plasma renin levels on renin excretion:

Elevations in plasma renin levels were induced in 7 normal male subjects by administration of the diuretic spironolactone for 12 days combined with chlorothiazide for the last 5 days. Plasma and urine samples were collected prior to commencement of natriuretic therapy, on the 7th day of therapy with spironolactone and on the 12th day (after 5 days of combined therapy). By the 7th day of therapy plasma renin concentration was elevated to 2.5 times the control levels. This was not associated with any significant change in urinary renin levels (Figure 2-17, Table 2-3). However, by the 12th day of therapy, when the plasma renin levels were elevated to 4.7 times the control ($P<0.001$), there was a mean 1.9-fold elevation in urinary renin level ($P<0.05$) (Figure 2-17, Table 2-3). Consequently, clearance fell to 48.6% of control by the 7th day and to 43% of control levels by the 12th day of therapy (Figure 2-17, Table 2-3).

Creatinine clearance did not alter significantly throughout the course of natriuretic therapy (Table 2-3), whilst total protein clearance fell to 85% of control levels by the 12th day of therapy

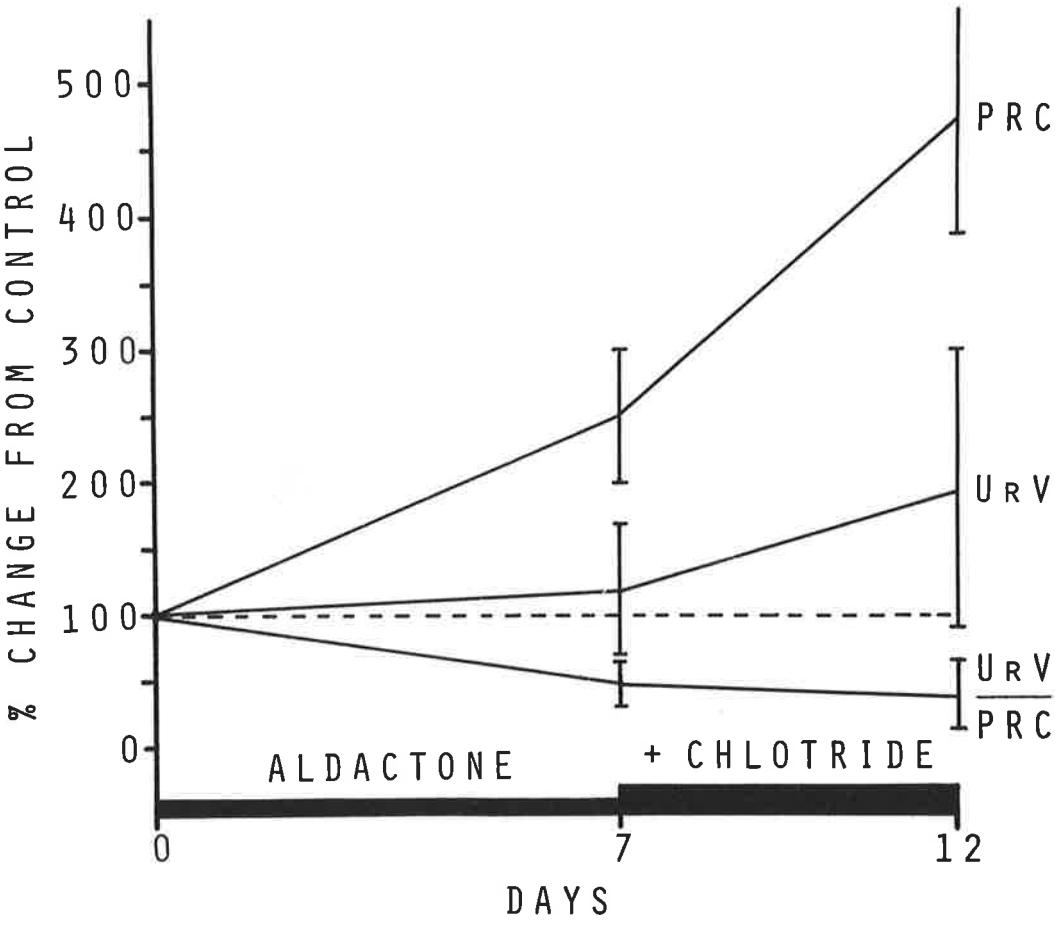


Fig. 2-17 The effect of Aldactone (spironolactone) and Chlotride (chlorothiazide) on plasma renin concentration (PRC), urinary output (URV) and renin clearance ($\frac{URV}{PRC}$) on days 7 and 12 of natriuretic therapy. Results from 7 normal males are expressed as the mean ± SD of control of 100.

TABLE 2-3(a)

Absolute values in plasma renin concentration, and the clearances of renin, creatinine, albumin and protein on days 7 and 12 of natriuretic therapy

		S U B J E C T						
	Day	1	2	3	4	5	6	7
PRC	0	8.4	9.3	10.3	9.8	13.5	5.3	11.5
(units/ml)	7	19.5	23.8	27.5	20.0	23.8	17.3	34.2
	12	31.7	38.4	51.3	55.0	61.3	31.3	46.3
Renin	0	7500	257	432	4442	615	490	567
excretion	7	6983	354	839	3460	613	317	938
(units/24 hrs)	12	5000	962	1062	6000	1628	568	855
Renin	0	893	27	42	453	46	92	49
clearance	7	358	15	31	173	26	18	27
(ml/24 hrs)	12	158	25	21	109	27	18	18
Creatinine	0	193	161	-	153	213	-	163
clearance	7	156	180	-	91	151	-	166
(L/24 hrs)	12	240	174	-	138	220	-	148
Albumin	0				0.095	0.102		
clearance	7				*	0.075		
(ml/24 hrs)	12				0.085	0.069		
Protein	0	18.1	24.6	-	10.6	20.2	-	14.5
clearance	7	14.5	18.5	-	8.9	14.2	-	11.9
(ml/24 hrs)	12	13.3	24.1	-	8.6	15.3	-	14.1

* No detectable albumin

TABLE 2-3(b)

Percentage change from control of 100% in plasma renin concentration, and the clearances of renin, creatinine, albumin and protein on days 7 and 12 of natriuretic therapy

		SUBJECT								
	Day	1	2	3	4	5	6	7	Mean % change	P
PRC	0	-	-	-	-	-	-	-	-	
(units/ml)	7	232	256	267	204	176	326	297	+151	<0.001
	12	377	413	498	561	454	591	403	+371	<0.001
Renin	0	-	-	-	-	-	-	-	-	
excretion	7	93	138	194	78	100	65	165	+ 19	n.s.
(units/24 hrs)	12	67	374	246	135	265	116	151	+ 93	0.05
Renin	0	-	-	-	-	-	-	-	-	
clearance	7	40	56	74	38	57	20	55	-51.4	<0.001
(ml/24 hrs)	12	18	93	50	24	59	20	37	-57.0	<0.002
Creatinine	0	-	-	-	-	-	-	-	-	
clearance	7	81	112	-	59	71	-	102	-15.0	n.s.
(L/24 hrs)	12	124	108	-	90	103	-	91	+ 3.6	n.s.
Albumin	0	-	-	-	-	-	-	-	-	
clearance	7	-	-	-	*	73	-	-	-	
(ml/24 hrs)	12	-	-	-	89	68	-	-	-	
Protein	0	-	-	-	-	-	-	-	-	
clearance	7	80	75	-	84	70	-	82	-21.8	0.001
(ml/24 hrs)	12	73	98	-	81	76	-	97	-15.0	0.05

* No detectable albumin

(Table 2-3). The slight fall in clearance is due to the rise in plasma protein, presumably consequent upon reduced extracellular volume. There was no significant relationship between the changes in renin clearance and glomerular filtration rate (as measured by clearance of creatinine) either between individuals or within the same individuals during sodium depletion, nor was there any significant relationship between the renal clearance of renin and the total protein clearance. This was particularly emphasized in subjects 4 and 5 in whom the control renin clearances differed widely, being 453 and 46 ml/24 hours, respectively, whilst the albumin clearances were similar (0.095 and 0.102 ml/24 hours), as were total protein clearances (10.6 and 20.2 ml/24 hours) (Table 2-3).

The effect of elevations in plasma renin on total protein excretion:

Although natriuretic therapy induced marked elevations in plasma renin concentration, there was no significant rise in total protein excretion (Figure 2-18) or total protein clearance during the course of the therapy.

Diurnal variations in renin excretion:

Renin excretion was measured over the 12 hour periods (0900 to 2100 hours and 2100 to 0900 hours). It was found that there was no significant diurnal variation in renin excretion in the 15 normal male subjects studied (Figure 2-19, left panel). The constancy of renin output within the one individual is also

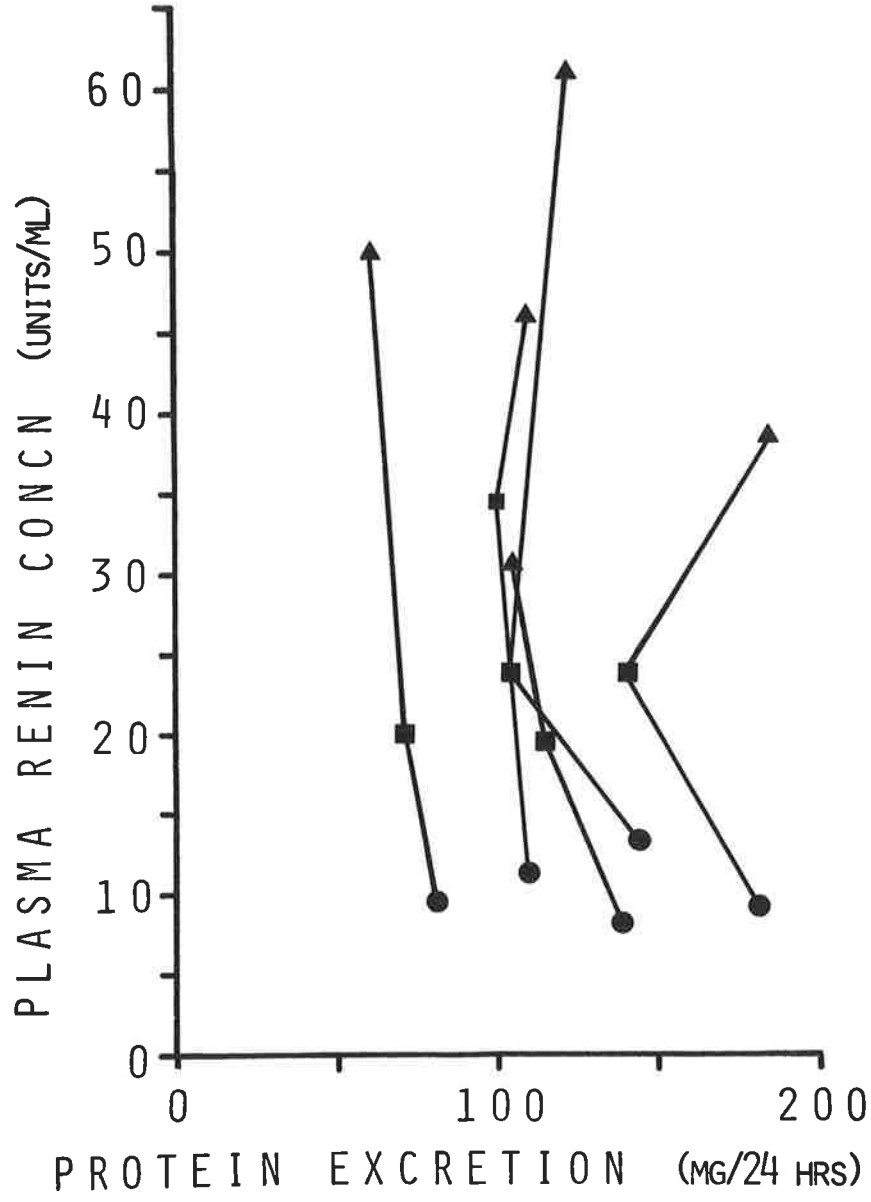


Fig. 2-18 Relationship between plasma renin concentration and protein excretion during treatment with spironolactone. Control values (●). Spironolactone therapy (■). Spironolactone with chlorothiazide (▲). (Appendix Table 12).

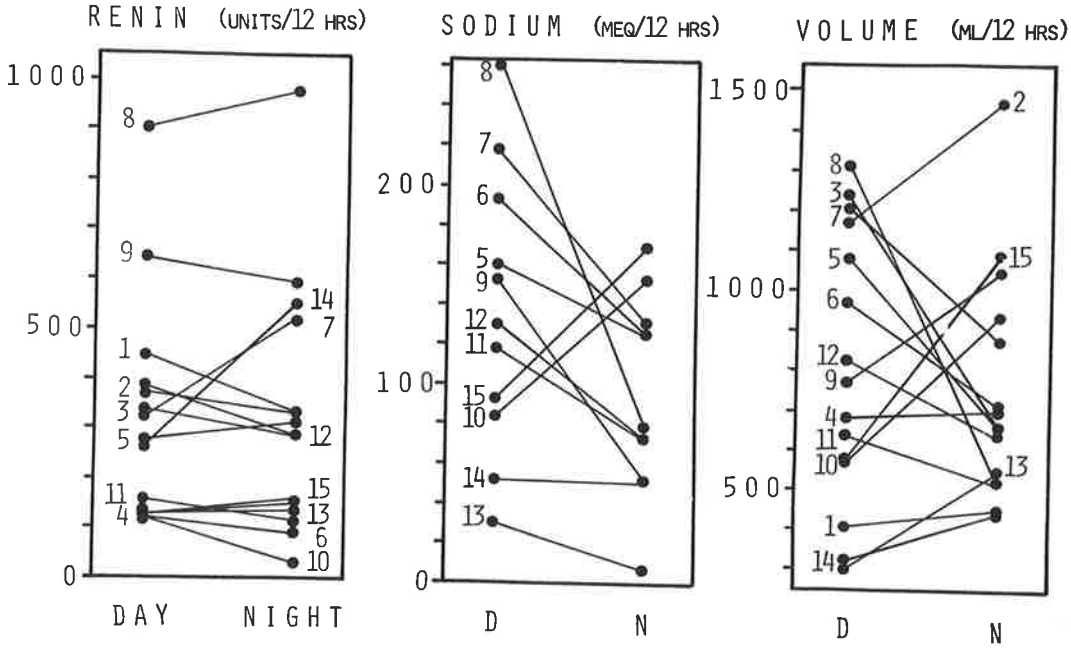


Fig. 2-19 Day (D) and night (N) excretion of renin, sodium and water in 15 normal males. Numbers refer to individual subjects (Appendix Table 13).

apparent from this data. Although there was no diurnal variation in urinary renin output, urinary sodium showed the anticipated (Stanbury and Thomson, 1951) nocturnal decrease in ten out of twelve subjects (Figure 2-19, middle panel, $P<0.05$). Urine volume, however, did not alter significantly (Figure 2-19, right panel). There was considerable variation in both urinary renin output and sodium output between individuals. However, no attempt was made in these experiments to control dietary sodium intake.

No relationship between urinary sodium and urinary renin output was observed in these subjects. In order to detect any occult relationship, the results were expressed as the day/night ratios of renin and sodium excretion within individuals. Again, no significant relationship emerged, nor was there any significant relationship between the day/night ratios of renin output to day/night ratio of volume. When, however, the ratios of day/night renin concentration were correlated with the day/night ratio of volume, a significant negative correlation was found (Figure 2-20), $r = 0.53$, $P<0.05$. This is a finding characteristic of any substance, the excretion of which is independent of urine volume.

Urinary renin levels in disease states:

Table 2-4 shows the data from 17 patients suffering from a variety of pathological conditions. In five of eight severe hypertensives, renin excretion was within normal limits for their sex.

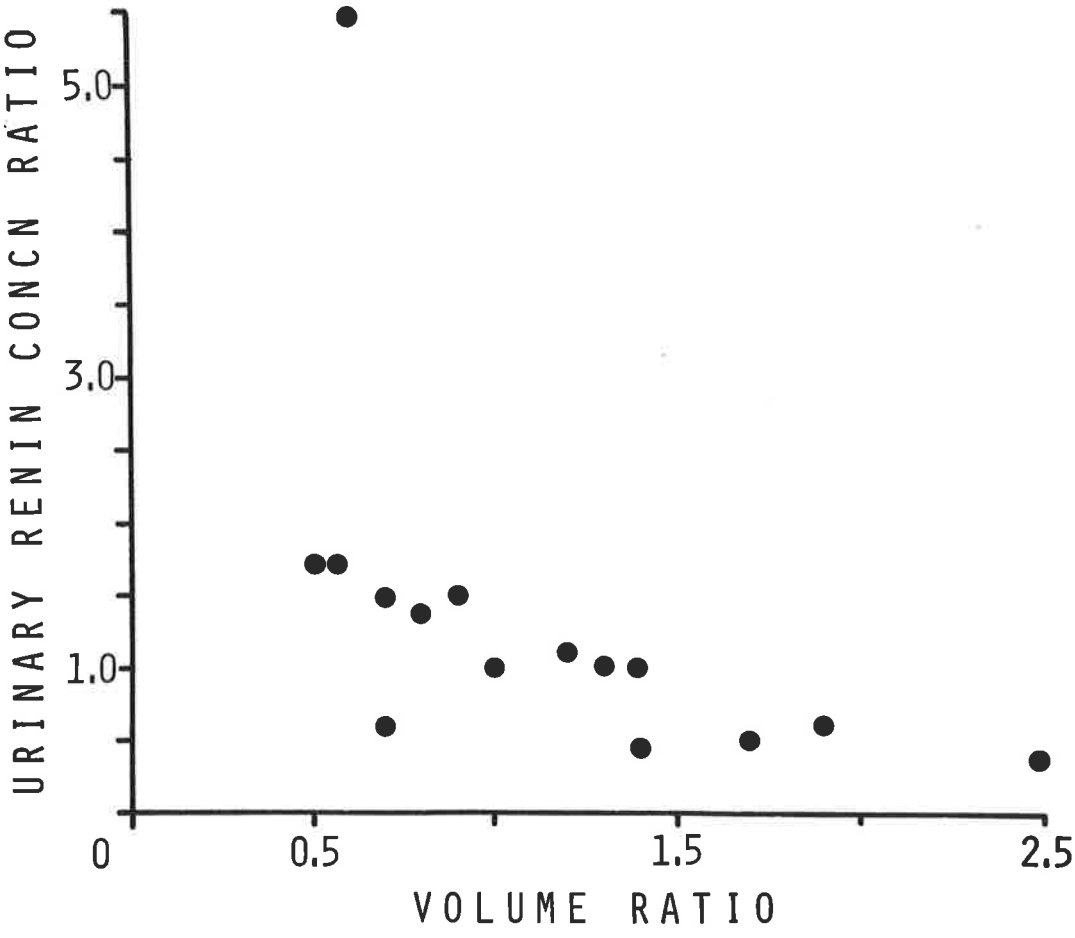


Fig. 2-20 Relationship between the day-night ratio of renin concentration and urinary volume (Appendix Table 13).

TABLE 2-4

Renin excretion, plasma renin concentration, creatinine clearance and renin clearance in patients

No.	Sex	Disease	Renin excretion (units/24 hrs)	PRC (units/ml)	Urinary protein	Creatinine clearance (L/24 hrs)	Renin clearance (ml/24 hrs)
1	M	Essential hypertension	49*	8.4	Negative	84	5.8*
2	M	Essential hypertension	3,300	15.7	+		210
3	M	Essential hypertension	11,874	29.5	+++		403
4	M	Essential hypertension	1,710*	26.8	Negative		64*
5	M	Essential hypertension	135*	28.1	Negative		4.8*
6	F	Malignant hypertension	217	9.6	+++	49	22.6
7	F	Malignant hypertension (renal artery thrombosis)	48,000	384.0	++++	86	125
8	M	Malignant hypertension (polyarteritis nodosa)	55,863	360.0	++++†	39	155
9	M	Cirrhosis with ascites	200	30.0	Negative		6.7
10	M	Acute nephritis	14,000	13.5	Negative		1067
11	M	Acute nephritis	1,251	15.3	++++✓	109	82
12	F	Chronic pyelonephritis	2,687	9.8	++	107	274
13	F	Chronic pyelonephritis	12,750	12.1	++++		1045
14	M	Terminal chronic renal failure	22,150	70.5	++++		314
15	M	Terminal chronic renal failure	20,025	31.5	+++		636
16	M	Nephrotic syndrome	1,230*	11.7	++++	26	105*
17	F	Nephrotic syndrome	6,360	130.0	++++		49

* 12-hour urinary renin output and clearance. † Protein excretion, 1.6 g/24 hours. ✓ Protein excretion, 5 g/24 hours. PRC = plasma renin concentration.

Two patients (numbers 7 and 8) had the highest levels of renin excretion and plasma renin levels yet encountered in this laboratory, yet the clearance of renin was within normal limits. Both of these patients suffered from gross proteinuria. Patient 7 suffered from severe polyarteritis nodosa, whilst patient 8 had a traumatic stenosis of the right renal artery. Right nephrectomy subsequently resulted in a remission of the hypertension in patient 8 and plasma renin levels fell to within the normal range. Since the right kidney was completely anuric and the source of the high renin levels in plasma (because its removal resulted in a fall in plasma renin levels), it is suggested that the high levels of renin in urine resulted from the filtration of renin produced from the ischaemic kidney through the glomeruli of the normal kidney.

Four other patients had elevated urinary renin excretions: numbers 3 (hypertension), 10 (acute nephritis) 14 and 15 (terminal chronic renal failure). Three of these patients also had elevated plasma renin levels, the exception being patient 10.

Although in normal subjects it was found that there was no relationship between urinary renin output and protein excretion, it was thought that, since renin was a protein, its excretion would be increased in patients with heavy proteinuria. This was only the case when the patients also had elevations in plasma renin levels as well as proteinuria. Patients 6, 11, 12, 13 and 16, who had normal plasma

renin levels, also had normal renin excretion and renin clearances despite the presence of heavy proteinuria. Patients 3, 7, 8, 14 and 15 had elevated plasma renin levels, and renin excretion was also elevated above the limits of normal for their sex. Patient 17 was the only exception to this rule. In this patient, heavy proteinuria and elevations in plasma renin levels were not associated with an excess renin output. In summary, therefore, it did not seem that pathological proteinuria was a significant factor in influencing the output of renin into urine.

Renin substrate in urine:

The urine of three patients with heavy proteinuria was examined for renin substrate. In only one of these patients was renin substrate found, the concentration being 0.15 µg/ml, 14% of the levels normally found in plasma (Skinner, 1967).

Renin in ureteric urine:

In two patients, one male and the other female, urine was collected for one hour from catheters passed up the ureters to the renal pelvis. The renin output was 180 units/24 hours and 117 units/24 hours, respectively.

DISCUSSION:

In order to study the renal handling of a substance the following criteria must be fulfilled.

Firstly, quantitative measurement of the substance, both in

plasma and urine, must be possible, and, secondly, it must be established that there is no contribution by the lower urinary tract to urine of the substance being studied. The methods of assay of renin in plasma and urine described in this thesis provide for the quantitation of renin in plasma and urine.

There is no evidence in the literature to suggest that renin is secreted by the cells lining the urinary tract. Furthermore, renin was present in urine collected at the level of the renal pelvis by ureteric catheters in the two subjects. The levels found were lower than those found in normal subjects. However, in these patients urine collections were made for only one hour following anaesthesia for investigation of urinary tract abnormalities. Moreover, the subjects were elderly: hence, a strict comparison with young, active, healthy subjects is probably not justifiable. In conclusion, it seems that the lower urinary tract is not a source of renin in human urine.

Renin output in normal urine showed a wide individual variation for which no obvious reason was apparent. However, the renin excretion in one person tended to be constant. There was no correlation between the renin output and plasma renin levels, or between renin output and glomerular filtration rate, sodium, water, or protein excretion that would account for the wide variations in renin output seen.

It was surprising to find that renin excretion in females was higher than that observed for normal males. There is no apparent reason for this finding. Since the plasma renin levels were of the same order as those observed in normal males, the clearance of renin into urine was also greater in females than in males.

Since renin has been found in the reproductive tissues of man and other animals (Brown, Davies, Doak, Lever, Robertson and Tree, 1964; Gross, Schaechtelin, Ziegler and Berger, 1964; Ferris, Gorden and Mulrow, 1967; Skinner, Lumbers and Symonds, 1968), it is possible that there is a contribution to urinary renin from the reproductive tract of the female of a renin molecule of different size or charge, or different biochemical properties. Although it has been shown that renin from the reproductive tract has similar molecular weight and properties to renal renin in the rabbit (Anderson, Herbert and Mulrow, 1968), the Km for uterine renin is lower, indicating an increased affinity of the enzyme for the substrate. If urinary renin in the female were derived from the reproductive tract, and if it showed a similar reduction in Km as does rabbit renin, then the amount of renin estimated by reaction velocity at a given substrate concentration would appear to be greater.

The renal clearance of renin in normal males is low, being 0.077% that of the clearance of creatinine. It varied in a manner that was not related to total protein clearance. Although an error

is incurred by assaying only one plasma renin sample during the 24 hour collection, since some diurnal fluctuation in plasma renin levels has been reported (Gordon, Wolfe, Island and Liddle, 1966) and the renin levels appear to be much lower in recumbent subjects (Lumbers, unpublished observations), this would not account for the wide individual differences in clearance seen, since the time of collection of plasma samples was standard in all subjects and differences in plasma levels were compared to variations in renin excretion.

The clearance of renin fell markedly during elevation of the plasma renin level by administration of natriuretic agents. This was not consequent upon a fall in glomerular filtration rate, nor did protein excretion suffer the same degree of suppression. Such a finding indicates that the clearance of renin is not dependent upon filtration of renin alone and suggests that an increased proportion of the renin must be removed from the tubular fluid by reabsorption during sodium depletion.

Another possibility is that there is a constant secretion of renin into urine from tubular cells, possibly from the macula densa, and that this is the only source of renin in urine. Hence, when an elevation of plasma renin is induced, this is not associated with any change in the tubular secretion of renin and therefore the clearance of renin apparently falls. However, although the clearance

of renin falls during sodium depletion, the output of renin into urine rises and, by the twelfth day of therapy, it is 1.9 times control levels, a finding which suggests that renin in urine is derived from filtration and subjected to reabsorption from the tubular fluid. This is further substantiated by finding a high renin output in the urine of one patient with complete anuria of one kidney due to renal artery stenosis. In this patient, since the renin content of the normal kidney can be expected to be low (Regoli, Hess, Brunner, Peters and Gross, 1962) and it is this kidney that is responsible for the formation of urine, the finding of a high renin output in this patient suggests that renin released into the circulation by the anuric, ischaemic kidney is filtered into urine by the normal kidney.

Since renin is a protein, one could postulate that the renal handling of renin would be similar to that of other proteins in urine. The renal handling of proteins appears to vary according to the size of the protein molecule excreted. Hardwicke and Squire (1955) concluded, from a study of the clearance of proteins in proteinuria, that protein reabsorption was non-selective. Their data applies to the reabsorption of proteins of large molecular weight, and recent studies by Harrison and Blainey (1967) suggest that the clearance and excretion of low molecular weight proteins (15,000 to 20,000) is not elevated consistently in heavy proteinuria. Such a

finding indicates that the tubular reabsorption of low molecular weight proteins is different from that of proteins of molecular weight greater than that of albumin.

The lack of association between renin excretion and protein excretion in patients with heavy proteinuria suggests that the tubular reabsorption of a protein of molecular weight of 42,000 (Kemp and Rubin, 1964, and Warren and Dolinsky, 1966) is similar to that of low molecular weight proteins. This finding is substantiated by the data of Blainey and Northam (1967) who showed that the clearance of amylase (molecular weight 45,000) also remains normal in the presence of heavy proteinuria.

That molecular size alone is not the only determinant of the reabsorption of these enzymes is indicated from a comparison of the clearance rates of the enzymes renin, amylase and pepsinogen. The molecular weights of these three enzymes are similar, being 42,300 (renin - Warren and Dolinsky, 1966), 45,000 (amylase - Blainey and Northam, 1967) and 42,600 (pepsinogen - Hirschowitz, 1957), yet the renal clearances are remarkably different, being, respectively, 0.077%, 3% and 30% that of creatinine. Such a wide variation in clearance of enzymes of similar molecular weight is unlikely to be due to filtration differences alone, nor to small differences in molecular weight affecting the rate of tubular reabsorption.

This data suggests, therefore, that a filtration-selective-

reabsorption system for renin is operating in the renal tubules.

Since injections of renin into experimental animals have been shown to induce proteinuria (Pickering and Prinzmetal, 1940; Katz, Sellers and Bonnoris, 1964; and Deodhar, Cuppage and Gableman, 1964) and the findings of Tobian and Nason (1966) are consistent with the association between high levels of renin induced by sodium depletion and proteinuria in the rat, it has been suggested that orthostatic proteinuria in man may be explained on the same basis. However, the present findings do not support this possibility, since no relationship was found between the levels of plasma renin and total protein excretion in normal subjects and, secondly, a rise in plasma renin induced by natriuretic therapy was associated with a fall in protein clearance, no rise in protein excretion being observed. The possibility that high levels of renin may induce proteinuria in man is not excluded in this study, but it seems unlikely that small fluctuations in renin levels due to postural changes are the mechanism responsible for the associated increase in protein excretion seen on assumption of the upright posture (Robinson and Glenn, 1964).

Studies of urinary renin output in disease states revealed that there was no diagnostic advantage to be gained from urinary renin estimations, since elevations in plasma renin were not consistently associated with increases in urinary renin excretion (patients 4, 5 and 9). In patients with very high levels of plasma

renin there was an associated increase in renin excretion. The independence of urinary renin excretion and the presence of heavy proteinuria was clearly demonstrated in these patients. The urinary renin levels only increased in patients with elevated plasma renin levels and not in patients with proteinuria alone. These findings support the conclusions made as to the handling of renin by the kidney.

SUMMARY TO SECTION 2

1. A system for the quantitative assay of renin in human urine has been developed. It is based upon the incubation of urine with nephrectomized sheep substrate at pH 7.5. Angiotensinase activity is abolished by pH 3.3 treatment of urine samples prior to dialysis to pH 7.5 and the presence of EDTA in the incubation mixture. The amount of renin present is determined by an initial velocity technique, the concentration of renin being expressed in units/ml. The interfering action of pepsin is prevented by incubation of enzyme-substrate mixture at pH 7.5.

2. Identification of the urinary enzyme with plasma renin has been established in immunological studies, kinetic studies and the pH dependence of the two enzymes.

3. Experiments have shown that pepsin in urine can form a pressor material indistinguishable from angiotensin on incubation with renin substrate.

4. The presence of at least two separate angiotensinases in urine was established by studying the denaturation characteristics of angiotensinase in urine.

5. Studies on renin output and renin clearance in normal subjects and hospital patients led to the following findings:

(a) Urinary renin output varied widely among normal subjects but within the one subject it remained relatively constant. Renin output was higher in females than in males.

(b) There was no relationship between urinary renin levels and plasma renin levels, glomerular filtration rate, urinary sodium, water or protein excretion. The negative correlation between renin concentration in urine and urine volume indicated an independence between renin excretion and urinary volume.

(c) Although elevations of plasma renin levels induced by natriuretic therapy elevated urinary renin excretion, renin clearance fell, whilst creatinine clearance and total protein clearance remained relatively constant. Excess renin excretion did not occur in proteinuric patients unless plasma renin levels were also elevated. These results and consideration of the clearances of renin, pepsinogen and amylase into urine (enzymes of similar molecular weight) led to the conclusion that renin is filtered and subjected to a selective reabsorption process.

(d) The absence of any significant change in total protein

excretion into urine during elevation of plasma renin levels to five times control levels makes it unlikely that changes in renin levels induced by posture are responsible for the concomitant postural changes in protein excretion.

SECTION THREE

THE EFFECT OF ADMINISTRATION OF THE NATRIURETIC AGENTS, SPIRONOLACTONE AND CHLOROTHIAZIDE, ON PLASMA RENIN ACTIVITY, PLASMA RENIN CONCENTRATION AND RENIN SUBSTRATE LEVELS.

THE EFFECT OF ADMINISTRATION OF THE NATRIURETIC AGENTS, SPIRONOLACTONE AND CHLOROTHIAZIDE, ON PLASMA RENIN ACTIVITY, PLASMA RENIN CONCENTRATION AND RENIN SUBSTRATE LEVELS.

In a previous section of this thesis, experiments are described in which the natriuretic agents, spironolactone and chlorothiazide, were administered to normal man to study the effects of elevation of plasma renin levels on the output of renin into the urine. This study was expanded in order to correlate the actions of such natriuretic agents on the plasma renin activity and plasma renin concentration, and to see whether natriuretic therapy induced a qualitative or quantitative change in renin substrate levels.

A rise in renin activity consequent upon sodium depletion induced either by dietary deprivation or administration of natriuretics is a well described phenomenon (Veyrat, de Champlain, Boucher and Genest, 1964; Conn, Cohen and Rovner, 1964). This elevation in plasma renin activity is consequent upon a rise in plasma renin concentration (Brown, Davies, Lever and Robertson, 1963, 1964). It is not possible, however, to correlate the rise in renin activity with the rise in renin concentration observed by these workers since different techniques were used in the preparation of plasma for estimation of renin activity and concentration.

METHODS:

Five normal male subjects were treated with 0.5 Gm daily of chlorothiazide (Chlotride: Merck, Sharp and Dohme) for 4 days. On the fifth day the dosage of chlorothiazide was increased to 1.0 Gm.

Seven normal subjects were treated as previously described, i.e. they were given spironolactone (Aldactone, Searle) 50 mg twice daily for 12 days. For the last 5 days this was combined with chlorothiazide 0.5 Gm daily. In none of these subjects was any control exercised over dietary sodium intake.

Twenty-four hour urine collections were taken prior to the commencement of therapy, on the 5th day of therapy with chlorothiazide, on the 7th day of spironolactone therapy, and on the 5th day of combined spironolactone and chlorothiazide therapy.

All venous blood samples were taken at 1100 hours following completion of the 24 hour urine collection, the subject being seated. Blood was withdrawn from an antecubital vein and heparin (20 units/ml) was used as anticoagulant.

Plasma renin activity, plasma renin concentration and renin substrate were estimated by the methods described (Skinner, 1967).

Serum sodium, potassium and creatinine were measured by the Technicon Autoanalyser method (Technicon, Ardsley, N.Y., 1965).

To determine whether diuretic therapy caused any qualitative change in renin substrate, a constant amount of renal renin was added

to pooled plasma samples treated as for 'renin activity', i.e. dialysed to an initial pH of 4.5 prior to dialysis to pH 7.5. These plasma samples were obtained from subjects prior to commencement of therapy; on the 7th day of therapy with spironolactone, and on the 5th day of combined therapy with spironolactone and chlorothiazide.

The velocity of formation of angiotensin was determined in the samples following the addition of a constant amount of renal renin, and in control dilutions which were set up in parallel to allow correction for the endogenous velocity of the samples.

To determine whether there was a linear relationship between the concentration of plasma renin treated to pH 3.3 acting on human substrate and the rate of formation of end-product, serial dilutions of pooled pH 3.3 treated plasma were added to pH 4.5 treated plasma. The endogenous velocity of a control dilution of pH 4.5 plasma was again determined to allow for correction of the velocity obtained.

RESULTS:

Treatment with chlorothiazide alone for 5 days produced a rise in plasma renin activity and plasma renin concentration in all subjects (Figure 3-1, Table 3-1). Plasma renin activity rose to 7.1 times control levels ($0.01>P>0.002$), whilst the plasma renin concentration rose to 2.8 times the control levels ($0.01>P>0.002$).

Treatment with spironolactone alone for 7 days produced a 4.5 times control rise in plasma renin activity ($P<0.001$) and a 2.5

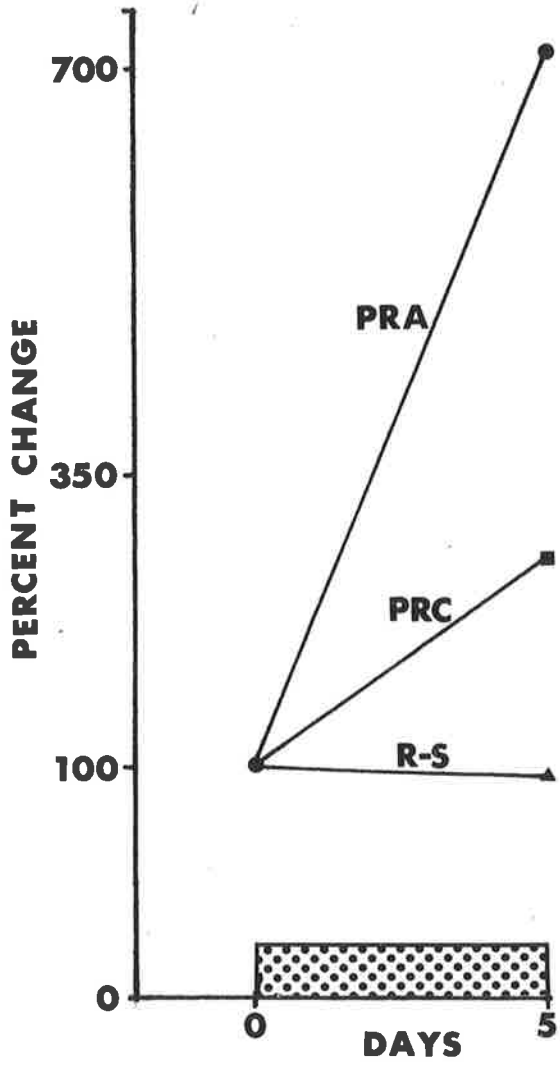


Fig. 3-1 Mean per cent change from control of 100 in plasma renin activity, plasma renin concentration and renin-substrate in 5 normal subjects treated with chlorothiazide.

TABLE 3-1

Absolute values in PRC, PRA and renin substrate before and after chlorothiazide therapy for 5 days.

	PRC		PRA		RENIN SUBSTRATE	
	Normal Range: 9.1±3.5 units/ml		Normal Range: 1.7±0.5 ng/ml/hr		Normal Range: 1.1±0.3 g/ml	
	0	6	0	6	0	6
1	8.3	29.2	0.6	6.0	0.8	0.6
2	20.0	31.3	0.95	4.1	0.6	0.4
3	12.3	37.5	0.8	5.0	1.2	1.2
4	9.9	28.8	0.92	5.4	1.0	1.0
5	11.9	34.2	0.7	6.4	1.2	1.2
Mean % Change		+178		+611		-12
P	0.01>P>0.002		0.01>P>0.002		P>0.1	

times control rise in plasma renin concentration ($P<0.001$) (Figure 3-2, Table 3-2), whilst combined therapy with spironolactone increased the plasma renin activity to 10.2 times control ($P<0.001$) and the plasma renin concentration to 4.7 times the control ($P<0.001$) (Figure 3-2, Table 3-2).

Serum sodium, serum potassium and creatinine clearance remained unchanged during therapy with spironolactone alone and spironolactone plus chlorothiazide (Table 3-2).

Renin substrate levels did not change significantly throughout the course of diuretic therapy.

It was thought that the greater increase in plasma renin activity, as compared with the increase in plasma renin concentration, might be due to a qualitative change in renin substrate induced by diuretic therapy, resulting in a greater affinity of the enzyme for the substrate. This could be due to a change in the substrate molecule or to the addition of a cofactor to plasma. However, when this hypothesis was tested, by adding human renal renin to plasma treated as for estimation of renin activity, the plasmas being pooled control plasmas, pooled plasmas collected after treatment with spironolactone and pooled plasmas collected after treatment with spironolactone and chlorothiazide, angiotensin was generated at rates of 65, 65 and 54 ng/ml/hr, respectively. The slower rate of generation of angiotensin in the plasma sample collected after combined therapy was a

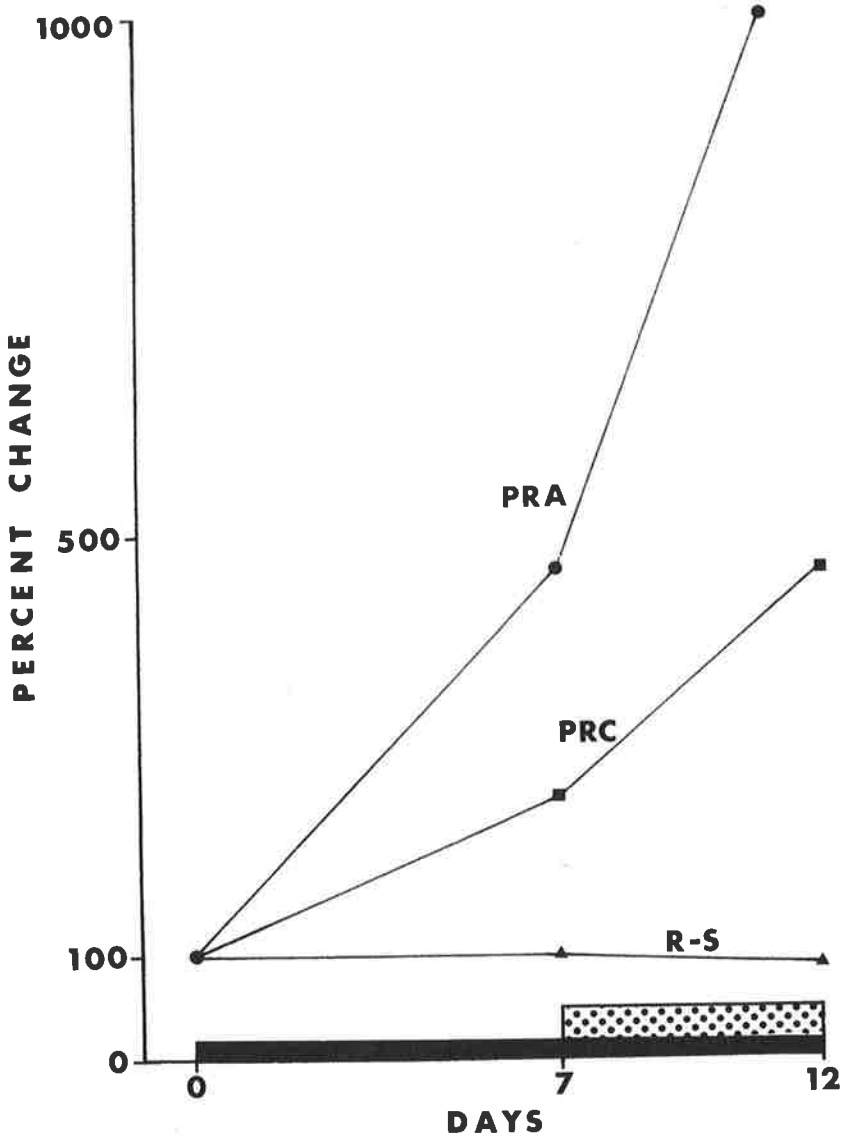


Fig. 3-2 Mean per cent change from control of 100 in plasma renin activity, plasma renin concentration and renin-substrate levels in 7 normal males treated with spironolactone alone for 7 days and spironolactone and chlorothiazide (in combination) for a further 5 days.

TABLE 3-2

Absolute values in plasma renin concentration, plasma renin activity, renin substrate, serum sodium, serum potassium and creatinine clearances before and on the 7th and 12th days of natriuretic therapy.

Subjects	PRC units/ml			PRA ng/ml/hr			Renin substrate µg/ml			Serum Na meq/L			Serum K meq/L			Creatinine clearance ml/min	
	0	7	12	0	7	12	0	7	12	0	7	12	0	7	12	0	7
1	8.4	19.5	31.7	1.4	4.8	10.0	2.0	1.4	1.5	137	136	155	4.3	4.6	4.4	193	156
2	9.3	23.8	38.4	1.4	6.0	15.6	1.4	1.4	1.2	136	135	134	4.6	4.5	4.0	161	180
3	10.3	27.5	51.3	1.0	6.4	15.0	1.2	1.4	1.6	139	136	135	4.3	4.7	4.5	-	-
4	9.8	20.0	55.0	1.3	6.0	14.7	1.9	1.9	1.4	137	141	135	4.1	4.8	4.2	153	91
5	13.5	23.8	61.3	1.4	6.7	14.7	1.6	1.3	1.2	137	140	137	4.3	4.7	4.8	213	151
6	5.3	17.3	31.3	1.4	5.5	12.8	1.8	1.8	1.6	139	135	133	4.3	4.5	4.1	-	-
7	11.5	34.2	46.3	1.3	5.6	9.5	1.3	2.0	1.4	137	136	140	5.2	5.0	4.5	163	166
Mean % Change		+151	+371		+354	+922		+3	-9		0	+1		+6	-1		-15
P		<0.001	<0.001		<0.001	<0.001		n.s.	n.s.		n.s.	n.s.		n.s.	n.s.		n.s.

reflection of the lower concentration of renin substrate in this sample (1.2 µg/ml, as compared with 1.4 µg/ml in the two other plasma samples).

When this experiment was repeated with a lesser concentration of amniotic fluid renin, the rate of formation of angiotensin was 11.1, 11.5 and 9.6 ng/ml/hr, respectively. These findings indicate that there was no increased reactivity of plasma during natriuretic therapy due to increased substrate reactivity or cofactors.

Figure 3-3 shows the linear relationship between the concentration of pH 3.3 treated plasma renin added to pH 4.5 treated plasma and the rate of formation of angiotensin after correction for endogenous velocity has been made.

DISCUSSION:

Both chlorothiazide and spironolactone produced striking increases in plasma renin activity and concentration in the absence of any significant changes in serum sodium, serum potassium and creatinine clearance. Combined therapy with chlorothiazide and spironolactone produced a further rise in plasma renin activity and concentration. This is probably a reflection of the augmentation of the natriuretic action of these drugs when used in combination (Kagawa, 1960), since the drugs have different sites of action on the renal tubule. Chlorothiazide acts on the cortical diluting segment (Earley, Kahn and Orloff, 1961) and spironolactone acts as a

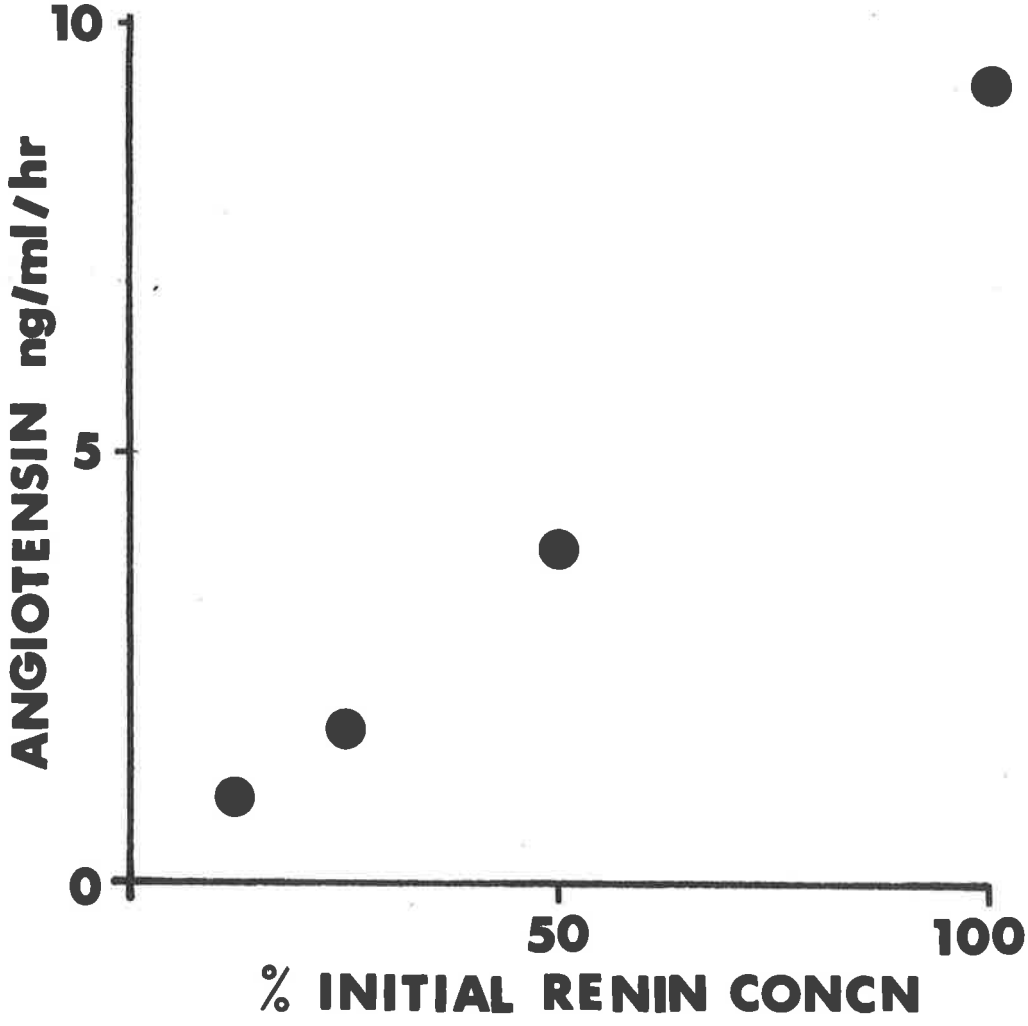


Fig. 3-3 Effect of serial dilution of pH 3.3 treated plasma renin on the rate of formation of angiotensin from normal human substrate.

specific aldosterone antagonist (Vander, Wilde and Malvin, 1960). Both drugs can produce a natriuresis in the absence of significant changes in glomerular filtration rate (Kagawa, 1960; Fuchs and Marlin, 1960).

The elevation of plasma renin activity to above normal levels by the administration of chlorothiazide alone is in contradistinction to the findings of Bourgoignie, Catanzaro and Perry (1968) who found, using comparable doses of hydrochlorothiazide, that an elevation in plasma renin activity in essential hypertensives occurred only when diuretic therapy was combined with restriction of dietary sodium intake to less than 20 meq/day. This may indicate that a hypertensive subject is refractory to moderate stimuli which cause the release of renin in normal subjects.

The explanation for the relatively greater increase in plasma renin activity over plasma renin concentration observed in all subjects studied is not clear. It is not due to any quantitative change in the renin substrate, nor is it due to any qualitative change in renin substrate, resulting in an increased affinity of the enzyme for the substrate.

The linear relationship between the concentration of renin in plasma treated to an initial pH of 3.3 and the rate of formation of end-product from human substrate makes it unlikely that there is any co-factor acting in normal plasma.

However, two further possibilities exist. Firstly, that there is a cofactor present, the release of which is stimulated by natriuretic therapy which is not capable of cross-reacting with either human renal renin or amniotic fluid and which is destroyed by pH 3.3 treatment. Secondly, it is possible that sodium depletion produces some structural or spatial modification to the renin molecule itself, resulting in an increased affinity for substrate. Such a spatial modification of the renin molecule could be easily damaged by exposure to a low pH, such as pH 3.3, whilst being retained during pH 4.5 treatment. This hypothesis would explain the findings observed in the experiments described and would imply that natriuretic therapy induces intracellular changes which are reflected in the production of a modified form of renin.

SUMMARY TO SECTION 3

Spironolactone and chlorothiazide, administered separately or in combination, produce striking elevations in plasma renin activity and plasma renin concentration.

In all subjects studied the rise in plasma renin activity was greater than the concomitant changes in plasma renin concentration. This was not due to an associated increases in renin substrate levels, nor was there any evidence of increased cofactor activity in plasmas from patients receiving natriuretic agents. The formation of pressor material from the action of plasma renin on human substrate bears a

linear relationship to concentration of the enzyme. A hypothesis has been described to explain these findings.

SECTION FOUR

RENIN CONCENTRATION IN HUMAN FOETAL AND MATERNAL TISSUES

RENIN CONCENTRATION IN HUMAN FOETAL AND MATERNAL TISSUES

Renin-like enzymes have been found in a diverse group of tissues, including the submaxillary gland of the white mouse (Werle, Vogel and Goldel, 1957; Turrian, 1960), the arterial wall of the pig (Dengler, 1956; Gould, Skeggs and Kahn, 1964) and the reproductive tissues of various animals.

Renin has been found in the placenta of the cat (Stakeman, 1960) and the dog (Hodari, Bumpus and Smeby, 1967). Gould, Skeggs and Kahn (1964) found very low concentrations of renin-like enzymes in the reproductive tissues of the pig, the concentrations of enzyme present being only 0.6 to 0.4 x 10^{-4} units/gm wet tissue for placenta and myometrium, respectively. The renal concentration of renin extracted by these workers was of the order of 1 unit/gm of wet tissue. When Gross, Schaechtelin, Ziegler and Berger (1964) studied the reproductive tissues of the rabbit, they found high concentrations of renin-like enzymes present, concentrations of the order of 1/10th of those found in the renal cortex.

Very high concentrations of renin have been found in human amniotic fluid (Brown, Davies, Doak, Lever and Robertson, 1964). The levels found were 100 times maternal plasma levels and 20 times the levels found in cord plasma. On the basis of these results, a study was undertaken to quantitate the levels of renin found in the

reproductive tissues of the pregnant female and to locate the source of renin in amniotic fluid.

METHODS:

Tissue extracts:

Tissues were obtained from 6 healthy women undergoing elective caesarean section. In none of these women was there any evidence of preeclamptic toxaemia. Tissues were also obtained from 4 normal women following spontaneous vaginal delivery.

Figure 4-1 is a diagrammatic representation of the sites from which tissues were taken. Chorion laeve was stripped from amnion in regions distant from the placenta. Chorion plate was taken from the foetal surface of the placenta, after dissecting off the amnion and stripping the chorion off the underlying placenta.

Placental tissue itself was dissected free from the maternal surface of the placenta, whilst uterine muscle and decidua were obtained by sharp dissection during caesarean section. Renal cortex was obtained from 4 adults within 24 hours of death, the patients having no evidence of renal disease.

Plasma and amniotic fluid samples:

Amniotic fluid was collected from 6 women at vaginal delivery, caesarean section or during therapeutic abortion. Maternal venous plasmas and venous and arterial cord plasmas were also obtained from these 6 women. Further samples of amniotic fluid were taken serially

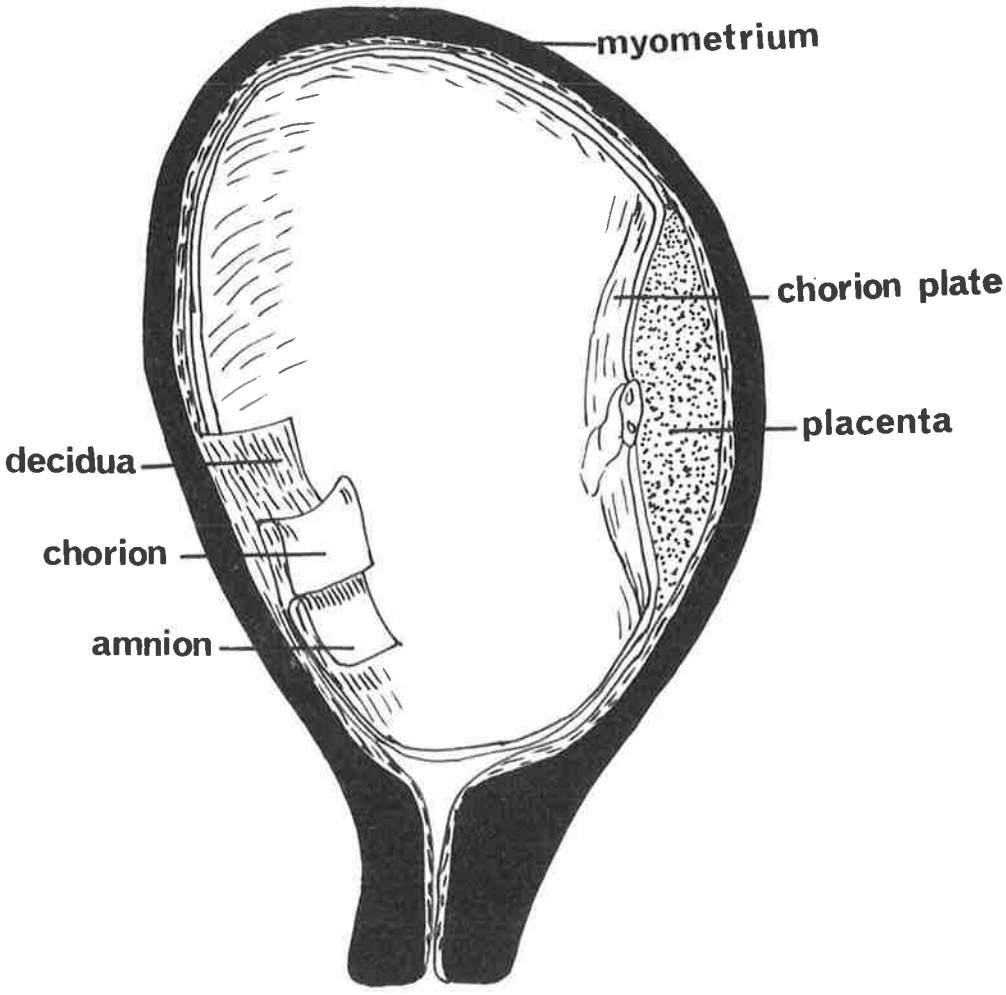


Fig. 4-1 Diagram showing sites of sampling of maternal and foetal tissues for estimation of renin content.

from 5 women following artificial rupture of the membranes, the last samples being taken at the end of the first stage of labour.

Preparation of tissue samples and amniotic fluid for assay of renin:

Amniotic fluid: Amniotic fluid was chilled immediately following collection. Since quantitation of the concentration of renin was desired, the amniotic fluid obtained was treated as for estimation of plasma renin concentration (Methods). It was dialysed to a pH of 3.3, heated for one hour at 32°C at this pH and dialysed to a final pH of 7.5. Following addition of neomycin sulphate (2 mg/ml) and Trasylol (100 units/ml) and correction of the volume, the samples were diluted to enable accurate quantitation of the large amounts of renin present. Standard sheep substrate was added (ratio of two parts of amniotic fluid to one part of sheep substrate) and the mixture incubated at 37°C, with sampling and assay of angiotensin by the methods described previously.

Extraction of renin from tissues:

The tissues obtained were washed 5 times in cold phosphate buffer, at pH 6.8, excess fluid was absorbed into paper, and 0.5 to 1.0 gram of tissue was weighed out and stored at -15°C. Renin was extracted by lysis of the cells in distilled water, followed by freezing and thawing twice, and then homogenization for 30 seconds (Serval Omnimix). The whole tissue homogenate was placed in 8/32 Visking cellophane casings and subjected to the dialysis sequence as

for estimation of plasma renin concentration (Methods). Following the dialysis sequence, the samples were decanted and centrifuged. This step yielded a clear tissue extract to which neomycin sulphate, Trasylol and standard sheep substrate were added in the concentrations described as for the treatment of amniotic fluid. Tissue extracts which contained large amounts of renin were diluted before addition of substrate to allow accurate quantitation of the amounts of renin present to be made.

To determine the effects of concentration of amniotic fluid renin on the rate of formation of pressor material, serial dilutions of amniotic fluid (containing 96 units/ml of renin) were incubated with both standard sheep substrate and human substrate. Correction for an endogenous velocity of 0.5 ng/ml/hr was made when human substrate preparations were used.

The effect of substrate concentration (using substrate from both non-pregnant and pregnant women) on the rate of formation of angiotensin at constant concentration of renin in amniotic fluid was determined. This was compared with the effect of variations in human substrate on the rate of formation of angiotensin at a constant concentration of pH 3.3 treated renin. Control dilutions of human substrate were set up in parallel to correct for the endogenous velocity of human substrate preparations.

RESULTS:

Characteristics of the enzyme in amniotic fluid and tissue extracts:

The amniotic fluid enzyme and the enzyme present in tissue extracts formed angiotensin at a linear rate with relation to time. The formation of pressor material was parallelled by a concomitant consumption of renin substrate. One extract of chorion and one sample of amniotic fluid, each with a high renin content, were incubated with standard substrate until bio-assay indicated substrate exhaustion. Following this, addition of renal renin in excess failed to generate angiotensin from these incubates.

The formation of pressor material bore a linear relationship to the concentration of the enzyme following serial dilution of amniotic fluid when tested against both sheep (Figure 4-2a) and human substrate (Figure 4-2b).

Specificity of the enzyme was indicated from studies using antibody to human renal renin, the enzyme in amniotic fluid being totally inhibited by the addition of one part of antibody to 4 parts of amniotic fluid, whilst the control sample generated 250 ng of angiotensin during a 16 minute incubation.

The Km (Figure 4-3) of amniotic fluid on substrate from pregnant and non-pregnant women was 3.0 and 4.0 µg/ml, respectively. The Km for plasma renin on human substrate was 7.6 µg/ml.

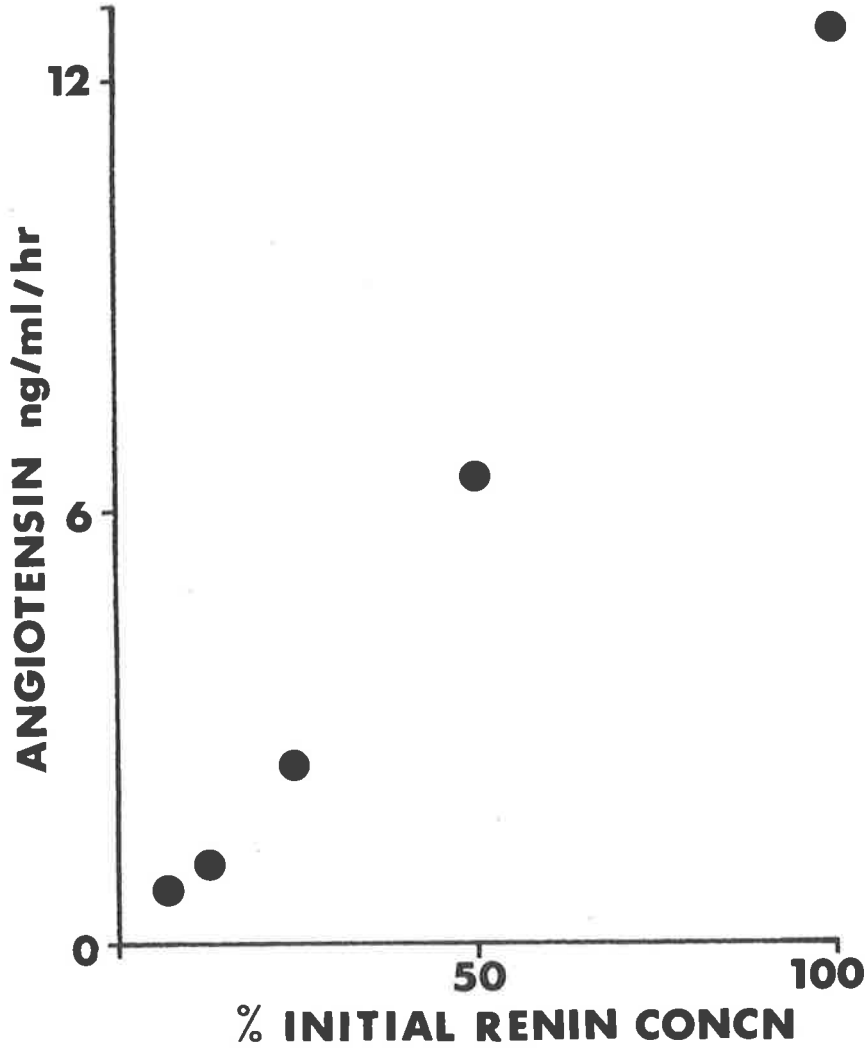


Fig. 4-2(a) Effect of serial dilution of amniotic fluid renin on the rate of formation of angiotensin from standard sheep substrate (Appendix Table 15).

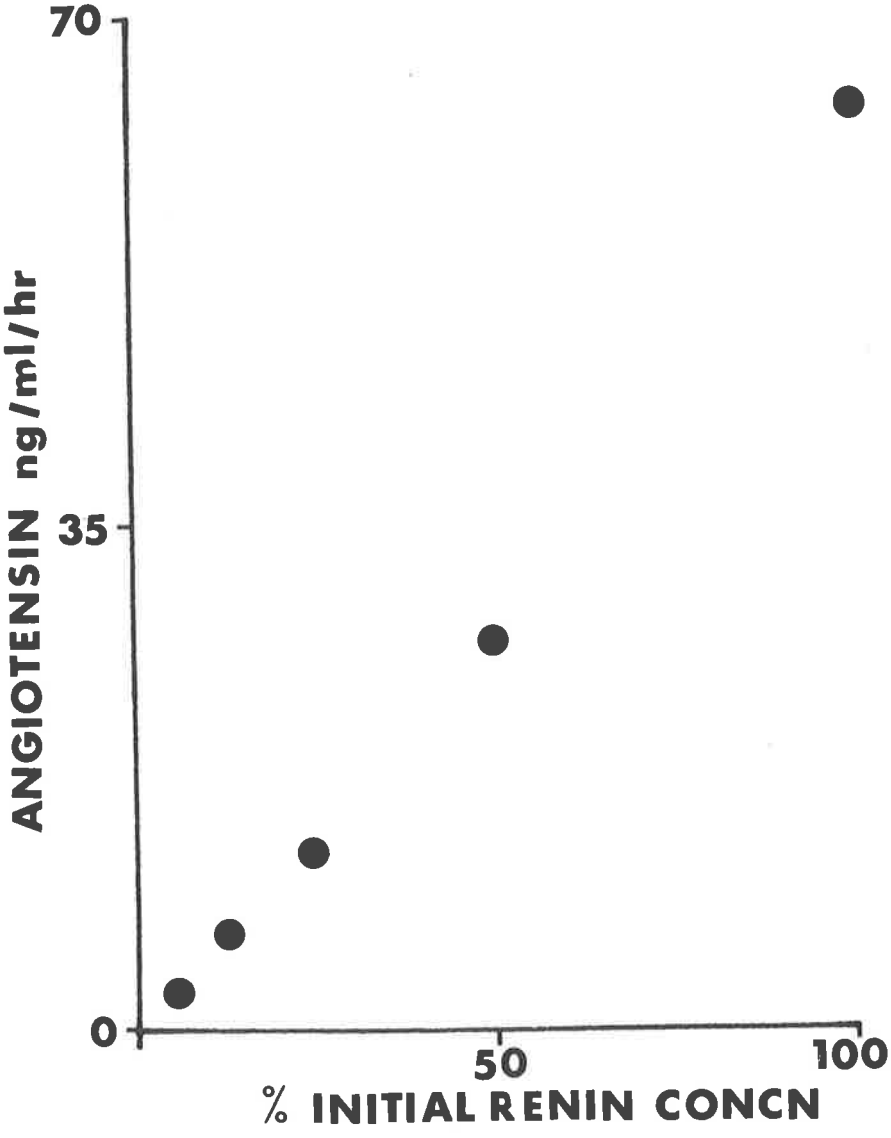


Fig. 4-2(b) Effect of serial dilution of amniotic fluid renin on the rate of formation of angiotensin from human substrate (Appendix Table 15).

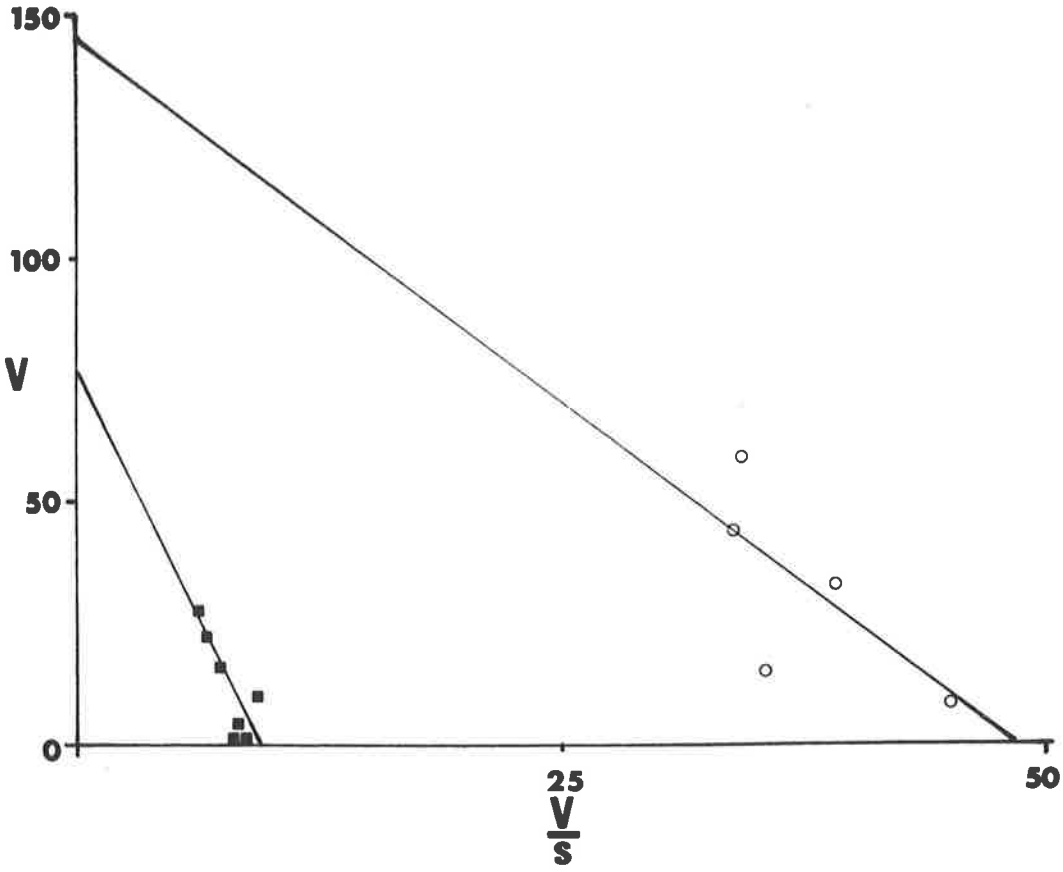


Fig. 4-3 Woolf plots derived from the effect of substrate on the rate of formation of angiotensin (V) plotted against the rate of formation of angiotensin divided by substrate concentration. (■) pH 3.3 treated plasma renin on human substrate. (O) amniotic fluid renin on human substrate. (Appendix Table 16(a), 16(c)).

The presence of substrate in amniotic fluid:

Renin substrate was found in 2 amniotic fluid samples tested, the concentration being of the order of 100 to 150 ng/ml. Curved velocity plots were obtained when these amniotic fluid samples were treated to pH 4.5, prior to dialysis to pH 7.5 and incubated at 37°C without the addition of sheep substrate (Figure 4-4). Since the samples were free of angiotensinase, the non-linearity of these curves is probably associated with consumption of greater than 50% of the substrate. Three other amniotic fluid samples contained no detectable renin substrate.

Angiotensinase activity:

No evidence of angiotensinase activity was present in any of the tissue extracts tested. Angiotensinase activity was tested by incubation of amniotic fluid and tissue extracts with the end-product formed as a result of incubation of human renal renin with human substrate (Methods). Greater than 90% of angiotensin activity remained following incubation of the samples for 12 to 24 hours at 37°C.

Absence of inhibitors in tissue extracts:

One part of amniotic fluid renin was added to nine parts of pooled extracts of myometrium, decidua and placenta. After correction for endogenous renin content of these extracts, the observed renin levels, 57, 60 and 60 units/ml, were not significantly different from

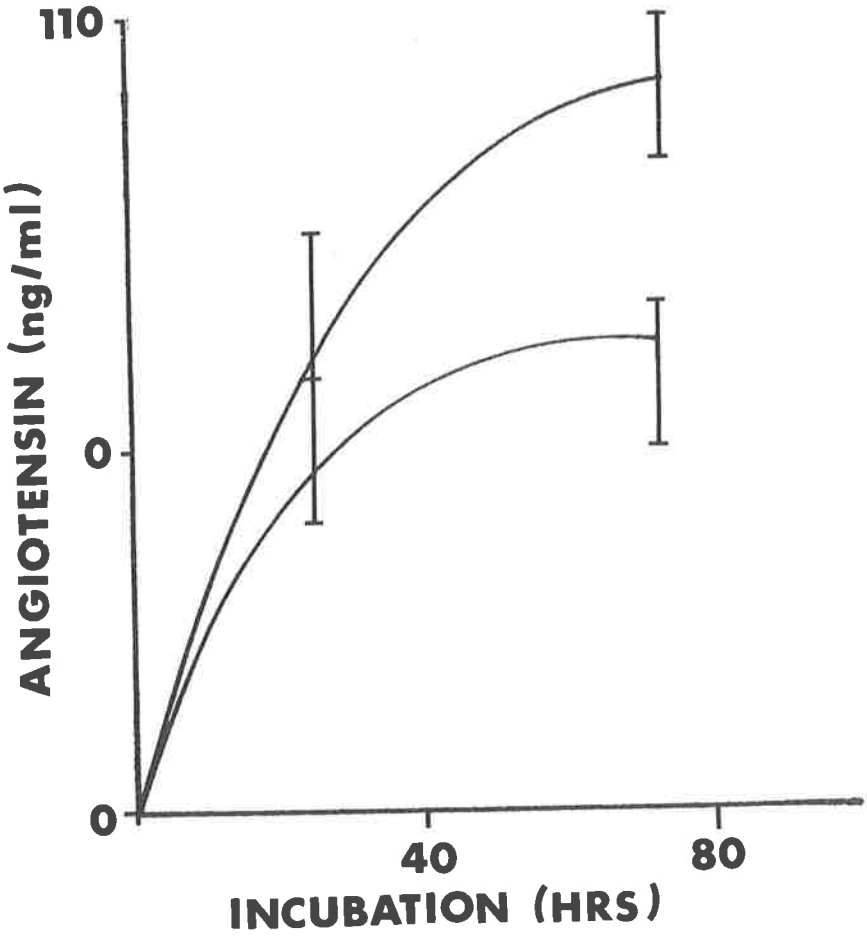


Fig. 4-4 Endogenous plasma renin activity of 2 amniotic fluid samples treated to pH 4.5 prior to incubation at pH 7.5.

the expected rate of 65 ng/ml.

These findings suggest that the variations in levels found in the tissues tested are not due to the presence of inhibitors.

Concentrations of renin in tissue extracts and amniotic fluid:

Renin content of tissue extracts:

Table 4-1 shows the relative concentrations of renin in tissue extracts obtained from 6 patients undergoing elective caesarean section and from 4 patients following normal vaginal delivery. In any one subject, chorion contained from 1.5 to 12 times as much renin as amnion ($P<0.001$) and 2 to 48 times as much renin as decidua ($P<0.001$).

The concentration of renin in chorion plate which was dissected off the placenta (Patients 7-10, Table 4-1) was less than in peripheral chorion ($P<0.05$) but more than in tissue dissected from the maternal side of the placenta ($P<0.05$).

In order to compare the renin content of tissues extracted from the reproductive tract to the renin content of the renal cortex, 4 cadaver kidneys were assayed for their renin content. The concentration of renin was considerably higher than the levels found in chorion, the actual values being 72,800, 33,600, 78,400 and 44,800 units/gm wet tissue.

Renin levels in amniotic fluid, foetal and maternal plasma.

The renin concentration in amniotic fluid at term ranged

Renin concentration (units/G wet weight) in foetal and maternal tissue collected at caesarean section and vaginal delivery

Caesarean	Amnion	Chorion	Decidua	Myometrium	Chorion Plate	Placenta
1	1400	6050	1270	80	-	80
2	770	9500	1270	32	-	64
3	2020	3180	1700	42	-	42
4	1330	6800	420	42	-	74
5	-	8500	177	37	-	12
6	680	1400	-	-	-	48
Vaginal						
7	-	1270	-	-	212	53
8	-	2120	-	-	740	48
9	-	960	-	-	297	64
10	-	-	-	-	500	21
Mean	1240	4420	967	46.6	437	50.6

from 160 to 2650 units/ml (mean 1058 units) and displayed a 40 to 1 gradient with respect to foetal (mean 26 units) and maternal plasma (mean 27 units) (Table 4-2). There was no consistent arteriovenous difference recorded across the cord. Moreover, foetal and maternal plasma levels were not significantly different. The amniotic fluid renin content of draining liquor did not change consistently over periods of up to 7 hours (Table 4-3).

Comparison of the group data in Tables 4-1 and 4-2 shows that the concentration of renin in chorion is significantly higher (mean 4420 units/gm) than that in amniotic fluid (mean 1058 units/gm) ($P<0.02$), whilst decidua (mean 967 units/gm) and amnion (mean 1240 units/gm) have levels similar to those found in amniotic fluid. These results are summarized in Figure 4-5.

DISCUSSION:

The highest content of renin in the intra-uterine tissues studied was found in chorion laeve. Only in this tissue was the concentration of renin greater than that found in amniotic fluid. This finding would suggest that renin diffused from chorion through the relatively simple membrane barrier constituted by the amnion into amniotic fluid.

Since the gradient between amniotic fluid and maternal plasma is of the order of 40 to 1, there may be some barrier to the outward diffusion into maternal plasma of renin produced by the

TABLE 4-2

Renin concentration (units/ml) in amniotic fluid, maternal and cord plasma

Patient	Delivery	Amniotic fluid	Maternal plasma	Cord Plasma Art.	Cord Plasma Ven.
1	Caesarean	2650	39	26	43
2	Caesarean	160	30	42	42
3	Caesarean	285	21	17	12
4	Vaginal	750	12	-	-
5	Vaginal	-	-	27	26
6	Vaginal	-	-	11	11
7	Vaginal	240	35	-	-
8	Vaginal	2400	-	21	22
9	Vaginal	700	-	-	-
10	Vaginal	1800	-	-	-
11	Vaginal	540	-	-	-
12	Hysterotomy (16 weeks)	560	-	-	-
13	Hysterotomy (14 weeks)	105	-	-	-
Mean*		1058	27	24	26

* In calculating mean of amniotic fluid levels patients 12 and 13 were excluded

TABLE 4-3

Renin concentration (units/ml) in draining liquor

Patient	Hours after A.R.M.	Renin Concentration
7	0	240
	4	385
	5.5	240
8	0	2400
	5	1900
9	0	700
	7	800
10	0	1800
	1.5	1800
11	0	540
	2.25	550

A.R.M. = artificial rupture of membranes

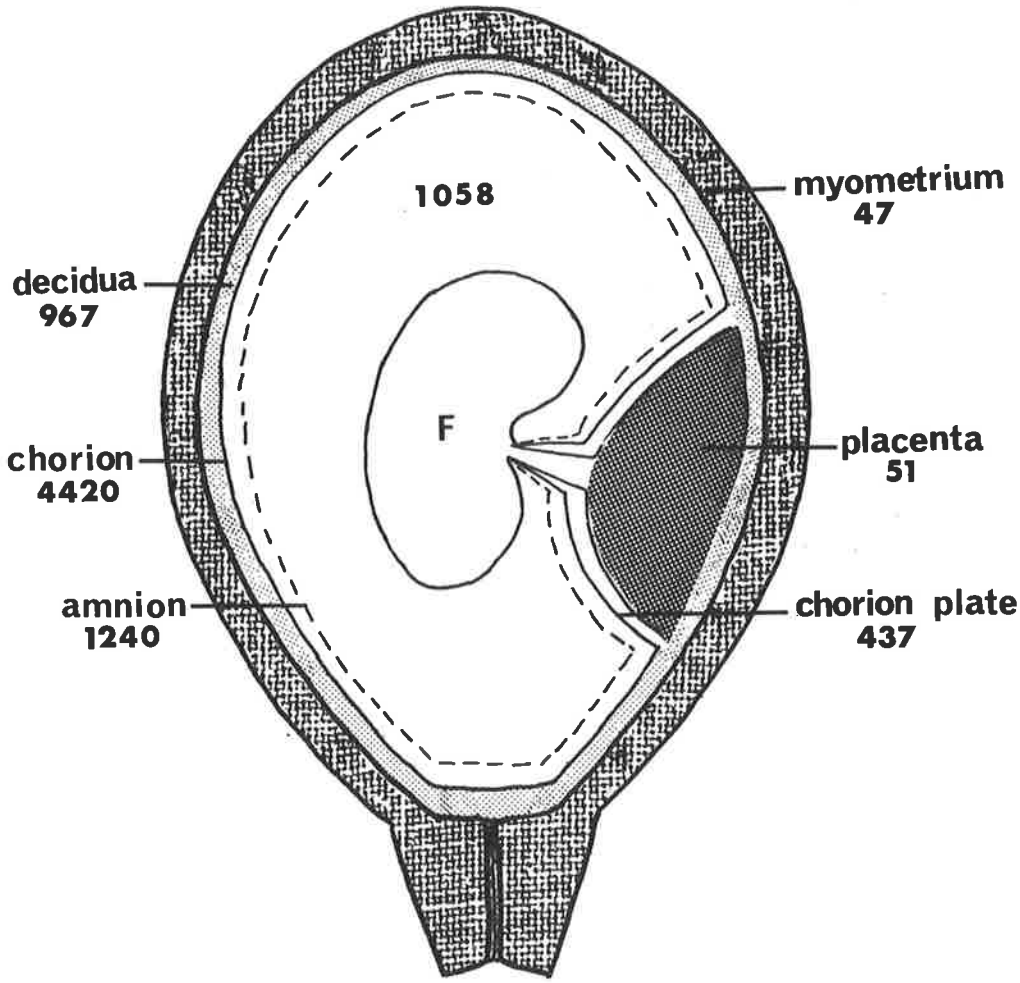


Fig. 4-5 Diagram of the concentrations of renin found in the various maternal and foetal tissues.

chorion. The decidua contains as much renin as is found in amnion and amniotic fluid, whilst the steepest gradient of renin is between the decidua and the myometrium. Therefore, it seems likely that the decidua, which is a thick and relatively avascular layer, constitutes a barrier to the outward diffusion of renin.

These findings suggest that renin in amniotic fluid is derived from the chorion. This implies that the chorion must synthesize and release renin, a concept which has received substantiation from the *in vitro* culture studies of Symonds, Stanley and Skinner (1968). Moreover, since the levels of renin in amniotic fluid do not fall during prolonged drainage of liquor at a time when amniotic fluid is thought to be continuously formed at a slow rate, there is apparently a continuous production of renin and secretion into amniotic fluid.

The presence of high renin levels early in pregnancy makes it unlikely that the foetus is a source of the renin found in amniotic fluid, since the levels obtained are similar to those found at term. Furthermore, foetal plasma renin levels are of the same order as maternal plasma levels, and since renin output into human urine has been found to be low and renin levels during draining of liquor remain constant in amniotic fluid, it is unlikely that the foetus contributes renin in any significant amount to amniotic fluid.

The identity of the enzyme in the reproductive tissues

appears to be similar to that of plasma renin, since it has the same specificity towards antibody inactivation and the generation of pressor material is accompanied by a fall in renin substrate. Since all tissue extracts were treated in the same manner and no inhibitors or non-specific depressor substances were found, it seems likely that a quantitative estimation of the actual content of renin in the different tissues examined has been obtained.

The Km of amniotic fluid acting on human renin substrate is lower than that for plasma renin acting on human substrate. This indicates a slightly higher affinity of amniotic fluid renin for human substrate, a similar finding to that observed with rabbit uterine renin and rabbit renal renin (Anderson, Herbet and Mulrow, 1968).

The intra-uterine distribution of renin with relatively low concentrations in the myometrium and placenta was in contra-distinction to the findings of the distribution of renin in rabbit reproductive tissues. However, this species difference was confirmed by assay of renin content in the tissues of the rabbit. Tissue extracts were taken from a rabbit with 3 foetuses in one cornu and none in the other. Myometrium from each cornu contained a high renin content (400,000 units/gram). Placenta contained relatively little renin (100 units/gram) and amniotic fluid only 30 units/gram. The foetal membranes contained more renin than amniotic fluid (67,000

units/gram), but less than the myometrium. There was no gradient apparent between the amniotic fluid and maternal plasma levels of renin, since the levels in these two fluids were approximately the same.

A preliminary study in the sheep has revealed higher renin levels in maternal plasma than in amniotic fluid.

These findings indicate that there is a considerable difference in the distribution of renin in the intra-uterine tissues and suggest, furthermore, that care must be taken in extrapolating results obtained in experimental animals to the situation in man.

The actual function of renin in the reproductive tract is not clear. It is not known whether it subserves some intra-uterine function. Renin derived from amniotic fluid appears to have the same properties as regards molecular size and charge, although the Michaelis constant appears to be different (Anderson, Herbet and Mulrow, 1968).

It is also not known whether renin produced in the reproductive tissues contributes to the circulating maternal plasma renin levels and hence affects sodium homeostasis in the pregnant woman.

Ferris, Gordon and Mulrow (1967) have shown that the pregnant animal maintains a high plasma renin level after bilateral nephrectomy. However, these results were obtained in the rabbit, and, as already mentioned, there appears to be a species difference in the

distribution of renin in the reproductive tissues. Moreover, in man the source of renin, that is the chorion, is separated from the maternal circulation by the thick and avascular decidua.

Histological examination of the chorion has revealed two layers, a deep trophoblastic layer with some attached decidual cells and a superficial fibroblastic layer, but which of these cell types is the site of synthesis of renin is not yet apparent.

SUMMARY TO SECTION 4

1. High levels of renin were found in the reproductive tissues of the normal pregnant female.

2. The highest levels were found in the chorion laeve. Renin levels in the amnion, decidua and amniotic fluid were similar.

3. The steepest gradient in renin concentration exists between the decidua and the myometrium. These findings suggest that renin is formed in the chorion and diffuses into the amniotic fluid, the outward diffusion of renin into the maternal plasma being prevented by the avascularity of the decidua.

4. The results obtained also indicate that renin is formed and continuously secreted into amniotic fluid.

5. The levels of renin in the chorion are 7.7% of the levels of renin found in the renal cortex.

SECTION FIVE

CHANGES IN PLASMA RENIN ACTIVITY, PLASMA RENIN CONCENTRATION AND RENIN SUBSTRATE LEVELS

(A) DURING THE NORMAL MENSTRUAL CYCLE

(B) DURING ADMINISTRATION OF ORAL CONTRACEPTIVES

(C) DURING PREGNANCY

(A) CHANGES IN PLASMA RENIN ACTIVITY, PLASMA RENIN CONCENTRATION AND RENIN SUBSTRATE LEVELS DURING THE NORMAL MENSTRUAL CYCLE.

Brown, Davies, Lever and Robertson (1964) reported that a rise in plasma renin levels occurred during the second half of the normal menstrual cycle.

Elevations in aldosterone secretion have also been reported during the luteal phase of the menstrual cycle (Reich, 1962).

These findings suggest that there is a physiological elevation in 'sodium-retaining activity' in order to compensate for a tendency to an increase in sodium loss from the body during the late menstrual cycle. The secretion of progesterone during the second half of the menstrual cycle would provide such a sodium losing mechanism, since progesterone can apparently act as a specific aldosterone antagonist, acting in a competitive manner to inhibit the action of aldosterone at a tubular level (Landau and Lugibihl, 1961). Therefore, the elevations in renin levels described by Brown, Davies, Lever and Robertson may be necessary to maintain normal sodium homeostasis during the menstrual cycle.

In order to study the actions of oral contraceptives on the renin angiotensin system, it was first necessary to determine the normal variations in plasma renin activity, plasma renin concentration and renin substrate levels occurring during the course of a menstrual

cycle.

METHODS:

Venous blood samples were taken from 6 normal women at intervals during the course of a normal menstrual cycle. On each occasion the samples were taken from an antecubital vein at 1100 hours, with the subject seated. Heparin (20 units/ml) was used an anti-coagulant. No control was exercised over dietary sodium or fluid intake. The duration of the individual cycles ranged from 24 to 28 days. Samples were taken on three occasions during the menstrual cycle: at the end of the menstrual bleed, at the estimated mid-point of the cycle, and during the luteal phase of the cycle.

To establish whether ovulation had occurred, urinary pregnanediol levels were estimated serially in three normal subjects (Cox, 1963, 1968).

RESULTS:

All six women showed a rise in plasma renin activity ($P<0.01$) in the second half of the menstrual cycle. This was consequent upon a rise in renin concentration in the second half of the cycle ($P<0.001$). Renin substrate levels remained unchanged (Figure 5-1, Table 5-1).

In one subject (subject 6, Table 5-1), a mid-cycle elevation in both renin activity and renin concentration occurred for no apparent reason. However, in the luteal phase, the rise in both renin activity and renin concentration above the levels found in the

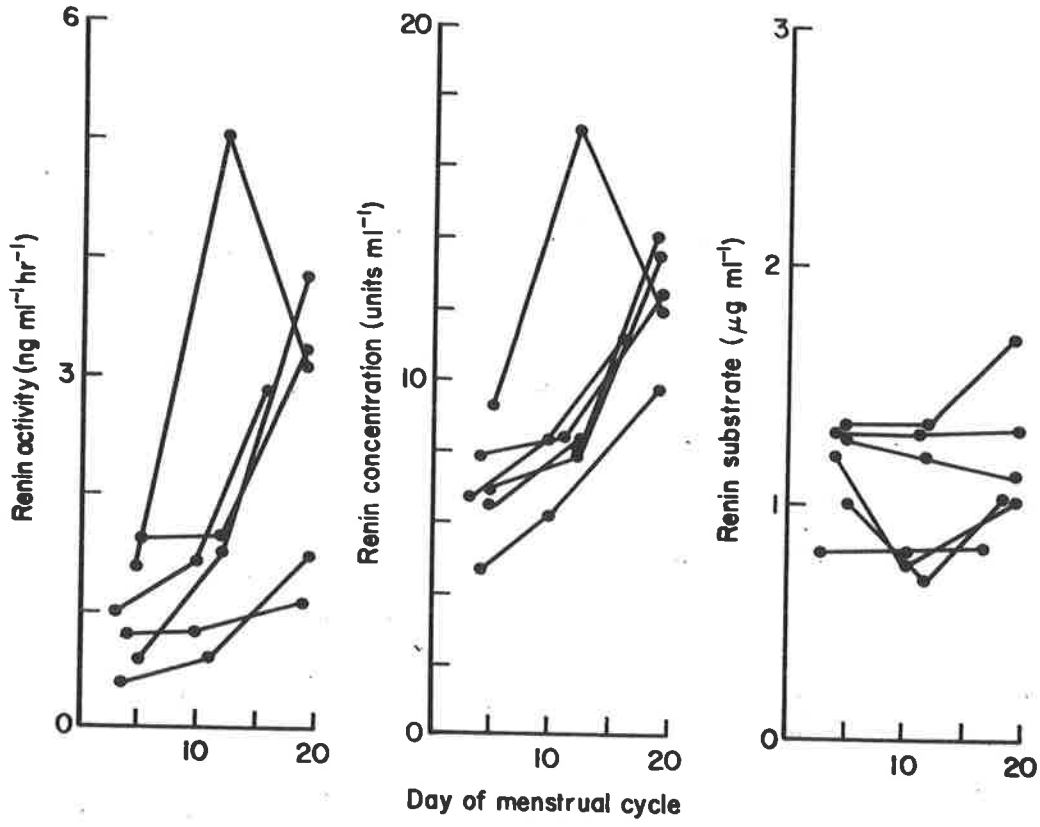


Fig. 5-1 Variations in plasma renin activity, plasma renin concentration and renin-substrate levels during the course of a normal menstrual cycle in 6 women.

TABLE 5-1

Variations in plasma renin activity (PRA), plasma renin concentration (PRC) and renin substrate levels during the course of a normal menstrual cycle.

Subject	Days Menstrual cycle	Activity (ng/ml/hr)	Concentration (units/ml)	Substrate (µg/ml)
1	3	1.0	6.8	0.8
	10	1.4	8.3	0.8
	16	2.9	11.3	0.8
2	4	0.8	4.8	1.2
	10	0.8	6.3	0.7
	19	1.1	9.8	0.9
3	4	0.4	7.9	1.3
	11	0.6	8.3	1.3
	20	1.5	12.5	1.3
4	5	1.6	6.9	1.3
	12	1.6	8.0	1.2
	19	3.2	13.5	1.0
5	5	0.6	6.5	1.0
	12	1.5	8.3	0.7
	19	3.8	14.0	1.0
6	5	1.4	9.3	1.3
	12	5.0	17.0	1.3
	19	3.1	12.1	1.6

first half of the cycle was evident (Figure 5-1).

In the three subjects in whom the excretion of pregnanediol into the urine was measured serially, 1.5, 4.0 and 5.0-fold elevations occurred between days 12 and 19, indicating ovulation.

DISCUSSION:

These findings support the results obtained of Brown, Davies, Lever and Robertson (1964) and show that the elevation in renin activity is due to an elevation in renin concentration, since renin substrate levels are unchanged. As stated previously, this rise in renin activity is associated with elevations in aldosterone secretion, suggesting that a homeostatic mechanism is being invoked to offset the natriuretic action of progesterone which is secreted following release of the ovum and formation of the corpus luteum.

Since renin substrate levels remain unchanged, one would have anticipated that the relative rise in renin activity and renin concentration would be the same. In fact, the renin activity rose to 2.6 times pre-ovulation levels, whilst renin concentration rose to only 1.75 times pre-ovulation levels.

This finding is similar to that described in a previous section in which the relative increases in plasma renin activity and plasma renin concentration were compared following natriuretic therapy.

(B) CHANGES IN PLASMA RENIN ACTIVITY, PLASMA RENIN CONCENTRATION AND RENIN SUBSTRATE LEVELS DURING ADMINISTRATION OF ORAL CONTRACEPTIVES

Since plasma renin activity is influenced by the variations in levels of those hormones responsible for maintaining the normal menstrual cycle, it might be anticipated that oral contraceptives which are a combination oestrogen-progestin therapy would modify this cyclical rhythm in the renin angiotensin system.

It has been reported that both oestrogens and oestrogen-progestin therapy do, in fact, cause a rise in plasma renin activity (Crane, Heitsch, Harris and Johns, 1966; Helmer and Judson, 1967) and that this is associated with a rise in aldosterone secretion (Layne, Meyer, Vaishwanar and Pincus, 1962). Of particular interest, therefore, are the recent reports of hypertension occurring in normotensive women following the administration of oral contraceptive agents (Laragh, Sealey and Ledingham, 1967; Woods, 1967; Clezy, personal communication), although the actual incidence of hypertension in women taking the "pill" is not yet defined (Goodlin and Waechter, 1969).

Since elevation of renin substrate levels have been induced by administration of diethyl stilboestrol to experimental animals and man (Helmer and Griffith, 1952), it is important to determine the

relative contribution made by changes in renin concentration and renin substrate levels to the elevations in plasma renin activity observed in women taking oral contraceptive agents.

METHODS:

Control venous blood samples were taken during the course of a normal menstrual cycle from 8 normal women who had not received oral contraceptives prior to the commencement of this study. Samples were taken from an antecubital vein at 1100 hours, with the subject seated. No deliberate control was exercised over dietary sodium or fluid intake.

Following administration of an oral contraceptive preparation, a second blood sample was collected during the subsequent contraceptive cycle.

Four women received Anovlar (ethinyl oestradial 0.05 mg and norethisterone acetate 4 mg, Schering A. G. Berlin); one received Noracyclin (mestranol 0.15 mg and lynoestrol 5 mg, Ciba, Basle) and three received CTI-441 (Eli-Lilly, Sydney), which is a serial form of therapy, 0.1 mg of mestranol being administered for 7 days followed by an oestrogen-progestin combination, Chlormadinone acetate 1.5 mg and mestranol 0.1 mg for a further 13 days). Heparin (20 units/ml) was added as anticoagulant to samples taken for renin estimation.

The effect of oral contraceptives on the renin angiotensin system in 6 women with hypertension (not of renovascular origin) was

studied. All patients had been taking oral contraceptives for at least 6 months. All patients were receiving both a thiazide diuretic and guanethidine sulphate (Ciba, Basle). Renin levels were studied during contraceptive therapy and within two weeks of stopping therapy.

Enzyme assay and kinetic studies:

Plasma renin activity, plasma renin concentration and renin substrate levels were estimated by the method of Skinner (1967).

Possible kinetic differences between renin substrate from normal women and women taking oral contraceptives were studied by constructing standard substrate curves at constant enzyme concentration. Pooled plasma dialysed to an initial pH of 4.5 was used in this study. This treatment inactivated angiotensinases present.

Plasma renin (pH 3.3 treated) was the source of renin used. Control dilutions were set up in parallel to allow correction for endogenous velocity.

RESULTS:

Table 5-2, Figure 5-2, show the changes induced in the renin angiotensin system by administration of oral contraceptives to 8 normal women. All preparations used had the same qualitative effect.

Despite the normal menstrual cycle rise, which influenced the level of the control samples, a further rise in renin activity was observed in all women studied. However, the time of control

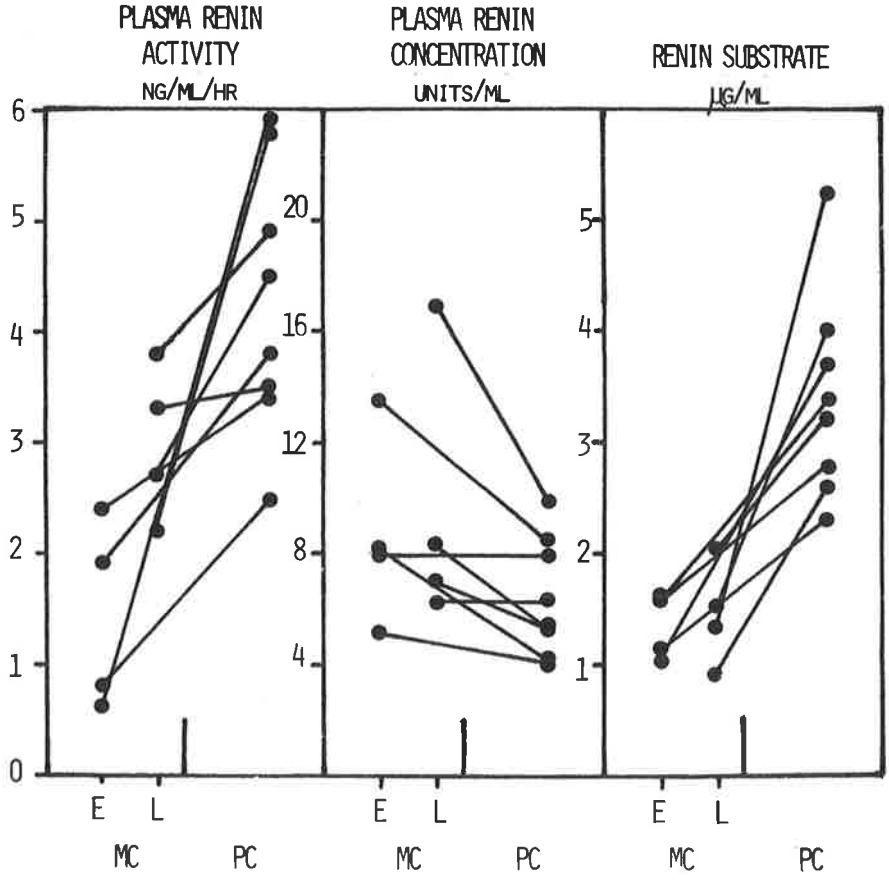


Fig. 5-2 Effect of administration of oral contraceptives on plasma renin activity, plasma renin concentration and renin-substrate levels in 8 normal women.

MC : menstrual cycle

PC : contraceptive cycle

E : control samples taken during the first half of the menstrual cycle

L : control samples taken during the second half of the menstrual cycle.

TABLE 5-2

Changes in plasma renin activity, concentration and substrate induced by oral contraceptives

Subject	Cycle	Day	Activity (ng/ml/hr)	Concentration (units/ml)	Substrate (μg/ml)
1	Menstrual	24	2.7	7.0	1.5
	Anovlar	23	4.5	5.3	4.0
2	Menstrual	21	3.3	8.3	2.0
	Anovlar	19	3.5	5.3	3.2
3	Menstrual	16	2.2	6.3	0.9
	Anovlar	14	5.8	6.3	>2.6
4	Menstrual	4	0.6	8.0	1.6
	Anovlar	14	5.9	8.0	3.4
5	Menstrual	20	3.8	16.5	1.3
	CTI-441	8	4.9	9.8	5.3
6	Menstrual	1	0.8	5.1	1.0
	CTI-441	12	2.4	4.0	3.7
7	Menstrual	9	2.4	8.1	1.6
	CTI-441	21	3.4	4.2	2.8
8	Menstrual	14	1.9	13.5	1.1
	Noracyclin	10	3.8	8.4	2.3

sampling during the previous menstrual cycle did affect the degree rise in renin activity obtained. Thus, in subjects 4, 6, 7 and 8, in whom the control samples were taken in the first half of the normal menstrual cycle when the renin activity is lowest, a mean four-fold elevation in renin activity was obtained following the introduction of contraceptive therapy, whilst the mean elevation in activity in subjects 1, 2, 3 and 5 is 1.7 times control levels. In these subjects, control samples were taken during the second half of the menstrual cycle when the renin activity is at its highest level.

In the normal menstrual cycle the rise in renin activity is associated with a rise in renin concentration. In the contraceptive cycle, on the other hand, the rise in renin activity is associated with a rise in renin substrate levels whilst the renin concentration remains the same or falls. This elevation in renin substrate levels is due to the oestrogenic component of the "pill", since the rise in renin substrate levels were observed in subjects 5 and 6 who, at the time of sampling, were receiving mestranol alone.

Figure 5-3 shows distribution of the levels of renin activity, renin concentration and renin substrate in 8 normotensive women taking oral contraceptive preparations compared to 9 luteal phase normal women and 16 normal males. The differences in the variations in renin activity, renin concentration and renin substrate in luteal phase women and normal males is non-significant, whilst the

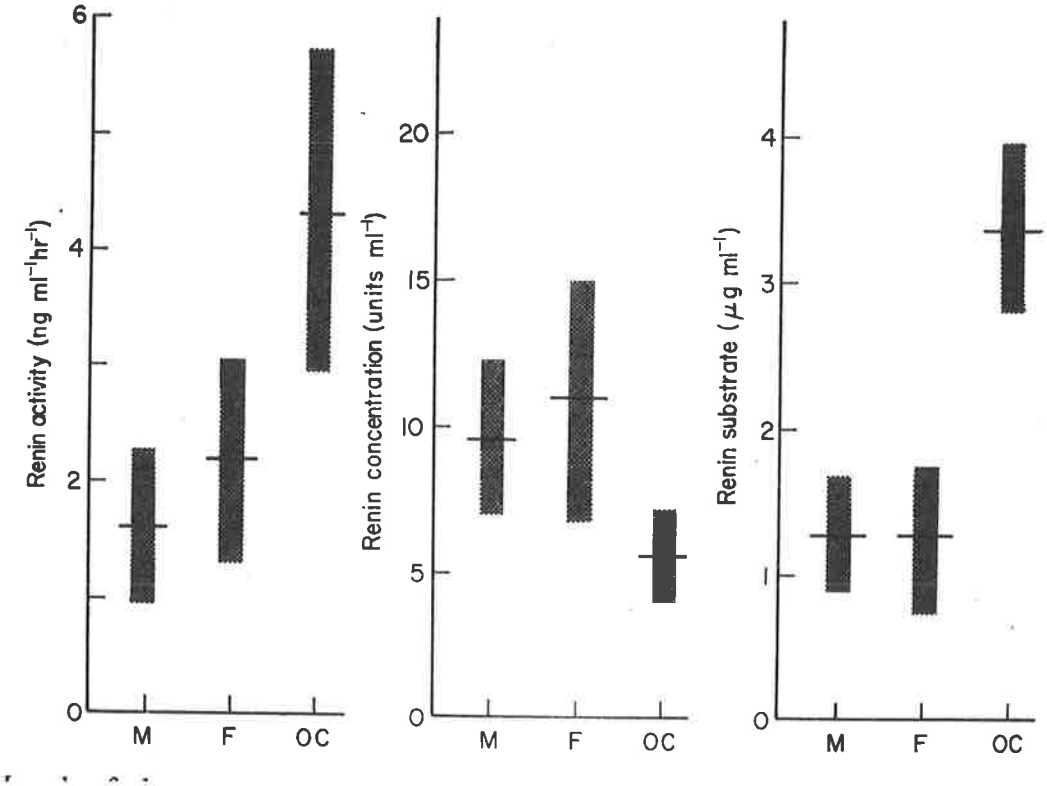


Fig. 5-3 Levels of plasma renin activity, plasma renin concentration and renin substrate in 16 normal males (M), 9 females, luteal phase of menstrual cycle (F), and 8 females taking oral contraceptives (OC). Values are shown as mean ± 1 SD.

elevation in renin activity during oral contraceptive administration is significant ($P<0.002$), as is the elevation in substrate ($P<0.001$) and the suppression of renin concentration levels ($P<0.01$).

Hypertensive women:

The elevations in renin substrate levels observed in hypertensive women taking oral contraceptive preparations were as high, or higher than, those observed in normal women taking oral contraceptive preparations. In patients 1-4 (Table 5-3), the elevation in renin activity was higher than that induced in normal women with contraceptive therapy. Cessation of contraceptive therapy resulted in a fall in renin substrate levels in all women.

The changes in renin activity therefore became a reflection of whether or not cessation of therapy was associated with changes in renin concentration. In patient 1, the concentration of renin did not change markedly and the fall in renin substrate was therefore associated with a fall in renin activity to within normal limits. In patient 2, renin concentration rose to above normal levels, although the renin activity fell to within the normal range, whereas in patients 3 and 4 the relative rise in renin concentration was sufficient to offset the fall in renin substrate, hence the renin activity rose or remained elevated. Patients 5 and 6 were studied whilst on contraceptives only. They showed the previously described changes, i.e. a rise in renin activity, consequent upon a rise in

TABLE 5-3

Plasma renin activity, concentration and substrate in six hypertensive women on and off oral contraceptives

Patient	Oral contraceptives	Activity (ng/ml/hr) (normal range 0.5-3.5)	Concentration (units/ml) (normal range 4-18)	Substrate (μg/ml) (normal range 0.7-2.5)	Serum K (mEq/l) (normal range 4-5.5)
1	On	10	6.3	5.0	4.3
	Off	1.8	8.0	1.5	
2	On	8.5	16.5	>2.2	3.0
	Off	3.3	30.0	1.1	
3	On	7.2	11.7	7.0	1.8
	Off	11.0	23.0	1.8	
4	On	13.0	32.5	3.9	4.3
	Off	13.5	57.0	2.1	
5	On	3.7	3.6	4.5	3.6
6	On	3.1	4.4	6.0	3.7

renin substrate, whilst the renin concentration levels were suppressed.

Enzyme kinetic studies:

In Figure 5-4, the reaction velocity at constant enzyme concentration is plotted against the concentration of substrate using substrate from normal women and women taking oral contraceptive preparations. At equal substrate concentration both substrates yield identical velocities, indicating that no qualitative change has occurred in the substrate molecule as a result of contraceptive therapy, the affinity of the enzyme for the substrate molecule being unchanged. Even at substrate concentrations of 4.5 µg/ml, maximum velocity had not been obtained. Reciprocal plots (Lineaweaver-Burk, Dixon and Webb, 1964) show that the maximum velocity would be obtained at concentrations of substrate of the order of 10 µg/ml. Therefore, in all plasmas studied so far substrate levels are rate-limiting.

DISCUSSION:

Helmer and Judson (1967) have recently found that renin substrate is a rate-limiting factor in the formation of angiotensin by normal plasma. The results described above substantiate these findings.

Other workers have concluded that renin substrate is present in excess (Pickens, Bumpus, Lloyd, Smeby and Page, 1965; Haas and Goldblatt, 1967; Ayers, 1967).

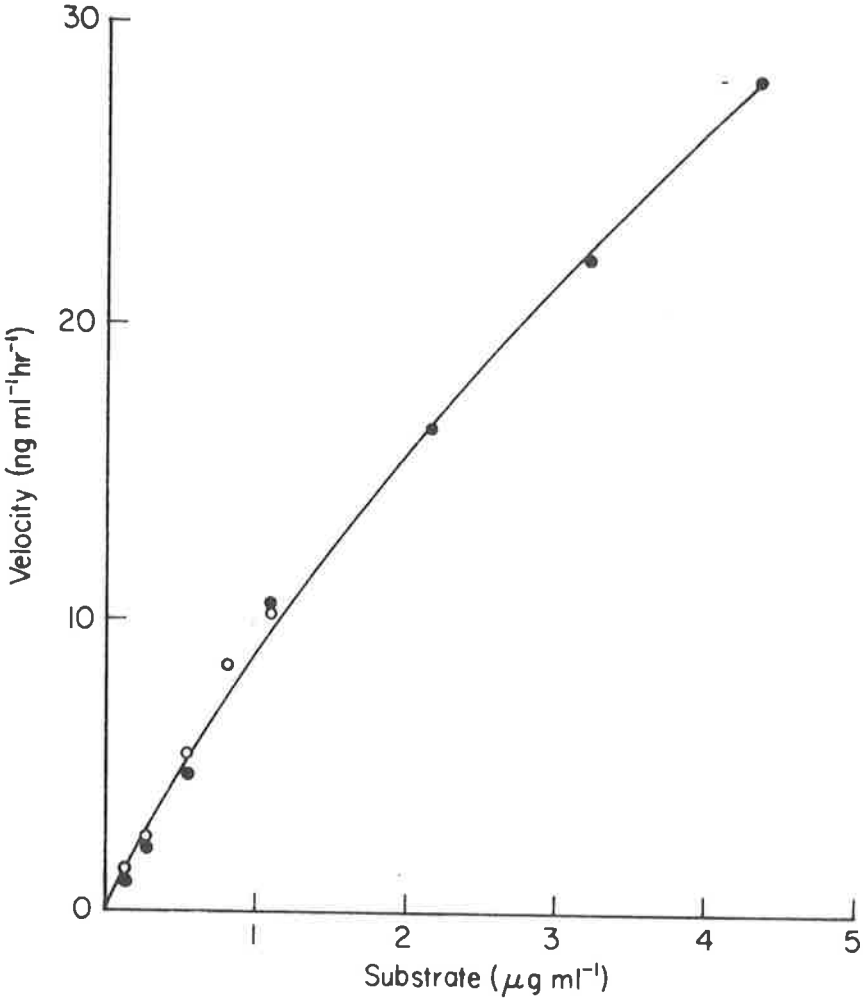


Fig. 5-4 Effect of substrate concentration using human substrate from normal females (O) and substrate from females taking oral contraceptives (●).

There is no apparent reason for this discrepancy in finding. However, it may be dependent upon the different conditions as regards ionic strength and pH to which plasmas are exposed when treated in different laboratories.

The results of the present experiments demonstrate that situations can occur in which the increased formation of angiotensin by plasma is not due to variations in the amount of renin released from the kidney but to increased circulating levels of renin substrate. Such is the action of the oral contraceptive. That the increased 'angiotensin forming ability' is unwanted is suggested by the finding that, associated with the increase in renin substrate levels there is a concomitant fall in renin concentration.

This suppression of renin release could be mediated either directly by the increased levels of circulating angiotensin formed as a result of elevation of substrate levels, or indirectly as a result of the effects on sodium homeostasis due to the increased circulating levels of angiotensin.

In contradistinction to the findings described in this present study, Newton, Sealey, Ledingham and Laragh (1968) found that part of the increase in renin activity could be due to rises in renin concentration as well as elevations in renin substrate. However, due to differences in methodology, it is not possible to explain this discrepancy. Newton, Sealey, Ledingham and Laragh

later suggest that the normalization of endogenous renin activity levels following long-term administration of oral contraceptives is evidence of suppression of the 'true renin concentration'.

Oral contraceptives produce a similar elevation in substrate levels to that seen in pregnancy. In pregnancy, however, the renin concentration is also elevated, whereas with oral contraceptive administration it is suppressed.

In the course of a normal menstrual cycle a rise in plasma renin activity is observed. This is consequent upon a rise in plasma renin concentration. During the pill cycle, however, the rise in plasma renin activity is now consequent upon an elevation in renin substrate levels. As explained previously, this rise in renin substrate is compensated to some extent by a fall in renin concentration. It is therefore interesting to postulate that the aggravation or initiation of hypertension in women taking oral contraceptives is due, in part, to failure of this feed back suppression. There is little evidence at this stage to prove such a hypothesis. However, it is interesting to observe that in two hypertensive women studied the elevations in plasma renin activity were such that they could account for the aggravation of pre-existing hypertension. Cessation of contraceptive therapy resulted in a fall in plasma renin activity to within normal levels. It is suggested that in these women an inability to suppress renin release

adequately in the presence of high levels of renin substrate resulted in undue elevations in plasma renin activity. Although plasma renin activity was further elevated in two other hypertensive subjects, the persistence of an elevated level of plasma renin activity due to a compensatory rise in renin concentration following cessation of contraceptive therapy makes it unlikely that oral contraceptives would aggravate hypertension in these cases.

It is interesting to note that hypertension has been induced by administration of oestrogenic compounds to experimental animals, including the rat (Grollman, Harrison and Williams, 1940; Leatham and Drill, 1960) and the toad (Segura, Lascano and d'Agostino, 1967). The mechanism for the induction of this hypertension is not clear, but the use of experimental animals may help to elucidate the mechanism of hypertension occurring in women taking oral contraceptive preparations.

(C) CHANGES IN PLASMA RENIN ACTIVITY, PLASMA RENIN CONCENTRATION AND RENIN SUBSTRATE LEVELS DURING PREGNANCY

High levels of aldosterone are associated with pregnancy, the secretion of aldosterone being accounted for partly by the increased activity of the renin angiotensin system (Genest, de Champlain, Veyrat, Boucher, Tremblay, Strong, Koiw and Marc-Aurèle, 1965). Genest *et al* described progressive increases in plasma renin activity up to 22 weeks. Since renin activity is influenced by both renin levels and substrate levels, a study of the relative contribution of changes in renin concentration and renin substrate to this elevated maternal plasma renin activity in pregnancy was undertaken. Evidence obtained from the literature suggests that both enzyme and substrate levels are elevated in pregnancy. Brown, Davies, Doak, Lever and Robertson (1963) described increases in plasma renin concentration in pregnancy, the highest levels being obtained in early pregnancy, whilst Helmer and Judson (1967) described a progressive elevation in renin substrate levels during pregnancy.

METHODS:

Venous blood samples were taken from an antecubital vein in 25 normal women at various stages of gestation. All samples were taken at 1100 hours with the subject seated. No control was exercised over dietary sodium or fluid intake. Heparin (20 units/ml)

was used as anticoagulant.

Plasma renin activity, plasma renin concentration and renin substrate levels were estimated by the methods described.

Possible kinetic differences in plasma from pregnant women, compared with plasma from normal subjects, were studied by constructing standard substrate curves of pregnant and normal plasma, using a constant concentration of renin. Amniotic fluid was used as the source of renin, a constant amount being added to serial dilutions of pregnant and normal plasma, and the rate of formation of angiotensin at any particular substrate concentration determined. Control dilutions were set up in parallel to allow correction for endogenous velocity.

The range of plasma renin activity in normal males is 1.7 ± 0.5 ng/ml/hr (mid-afternoon sampling), for plasma renin concentration it is 9.1 units/ml ± 3.5, and for renin substrate it is 1.1 ± 0.3 ng/ml (Skinner, 1967).

RESULTS:

Plasma renin activity:

The plasma renin activity was elevated to above normal, non-pregnant levels in all subjects studied (Figure 5-5, 5-8; Table 5-4). There was no significant positive correlation between plasma renin activity and the duration of gestation.

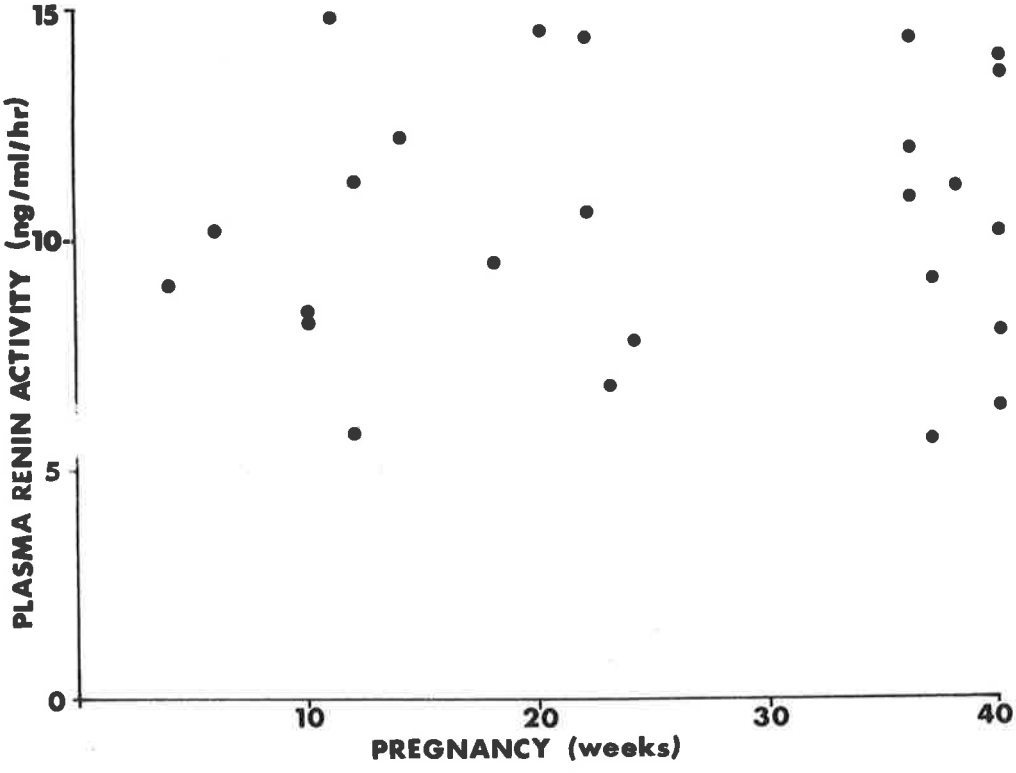


Fig. 5-5 The levels of plasma renin activity in 25 normal pregnant women.

TABLE 5-4

PRA, PRC and renin substrate in normal pregnant women.

Subject	Duration Pregnancy (weeks)	PRC* (units/ml)	PRC† (units/ml) Normal range: 9.1±3.5	PRA (ng/ml/hr) Normal range: 1.7±0.5	Renin substrate (μg/ml) Normal range: 1.1±0.3
1	4	50.0	47.7	9.0	1.3
2	6	82.5	68.8	10.2	3.0
3	10	66	55.0	8.4	3.0
4	10	76.5	63.8	8.3	3.3
5	11	34.5	28.8	14.8	3.0
6	12	136.5	113.8	11.3	4.4
7	12	105	87.5	5.8	1.2
8	14	99	82.5	12.2	6.0
9	18	81	67.5	9.5	5.7
10	20	103.5	86.3	14.5	4.2
11	22	45	37.5	14.4	3.6
12	22	36	30.0	10.6	9.0
13	23	34.5	28.8	6.8	6.0
14	24	33	27.5	7.8	4.2
15	36	25.5	21.3	12.0	5.4
16	36	82.5	68.8	14.4	4.2
17	36	44.1	36.8	11.0	2.7
18	37	43.5	36.3	9.2	7.0
19	37	52.5	43.8	5.7	7.0
20	38	49.5	41.3	11.2	4.8
21	40	33	27.5	8.0	6.0
22	40	36	30.0	10.2	7.0
23	40	25.2	21.0	14.0	6.0
24	40	42.0	35.0	13.7	3.8
25	40	46.5	38.75	6.4	4.2

* Values used in Figures 5-6 and 5-8.

† Corrected for substrate concentration 530 ng/ml.

Changes in plasma renin concentration:

The plasma renin concentration was elevated to above normal, non-pregnant levels in all women studied. The highest values occurred during the first 20 weeks of pregnancy (Figure 5-6, 5-8; Table 5-4), with a peak value in one subject occurring at 12 weeks gestation. The plasma renin concentration had fallen by 22 weeks of pregnancy, although the levels were still above the normal, non-pregnant range. After 22 weeks pregnancy, the levels tended to remain constant.

Renin substrate:

Renin substrate levels were normal in two of the twenty-five women studied, these women being 4 and 12 weeks pregnant. In all other subjects renin substrate levels were above those found in normal, non-pregnant individuals. There was a progressive increase in renin substrate levels during pregnancy ($r = 0.51$, $0.01>P>0.001$) (Figure 5-7, 5-8; Table 5-4).

When the reaction velocity at constant renin concentration is plotted against the substrate concentration, the similarity between curves for pregnant plasma and normal plasma is apparent, indicating that there has been no qualitative change in renin substrate levels in pregnancy (Figure 5-9, 5-10).

DISCUSSION:

It has recently been shown that renin substrate levels in

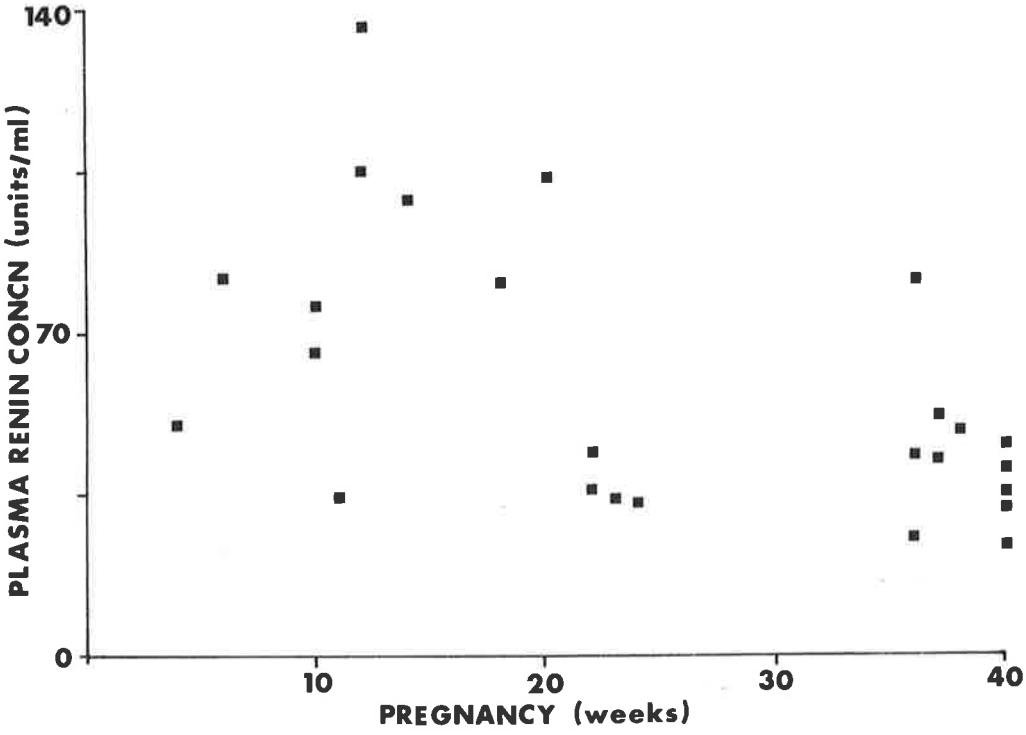


Fig. 5-6 The levels of plasma renin concentration in 25 normal pregnant women.

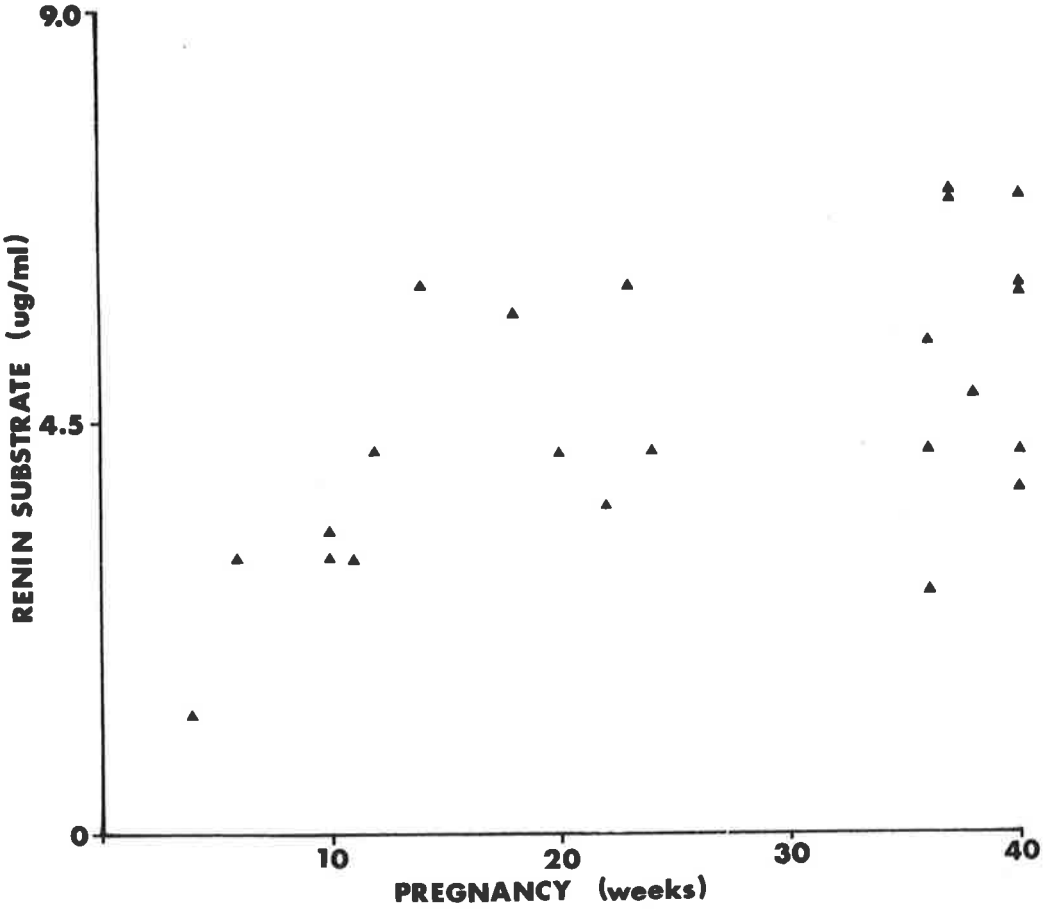


Fig. 5-7 The levels of renin substrate in 25 normal pregnant women.

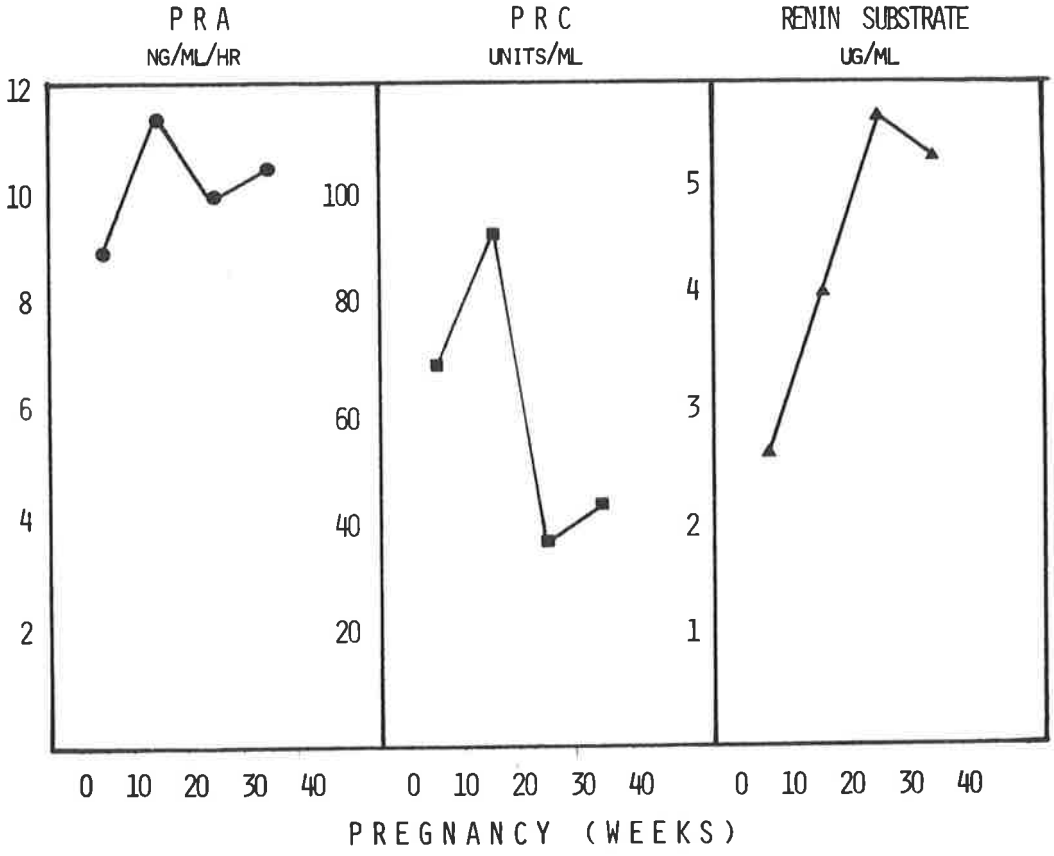


Fig. 5-8 Mean changes in plasma renin activity, plasma renin concentration and renin substrate levels in 25 normal pregnant women. Values represent means of values from women within each 10 week period of pregnancy.

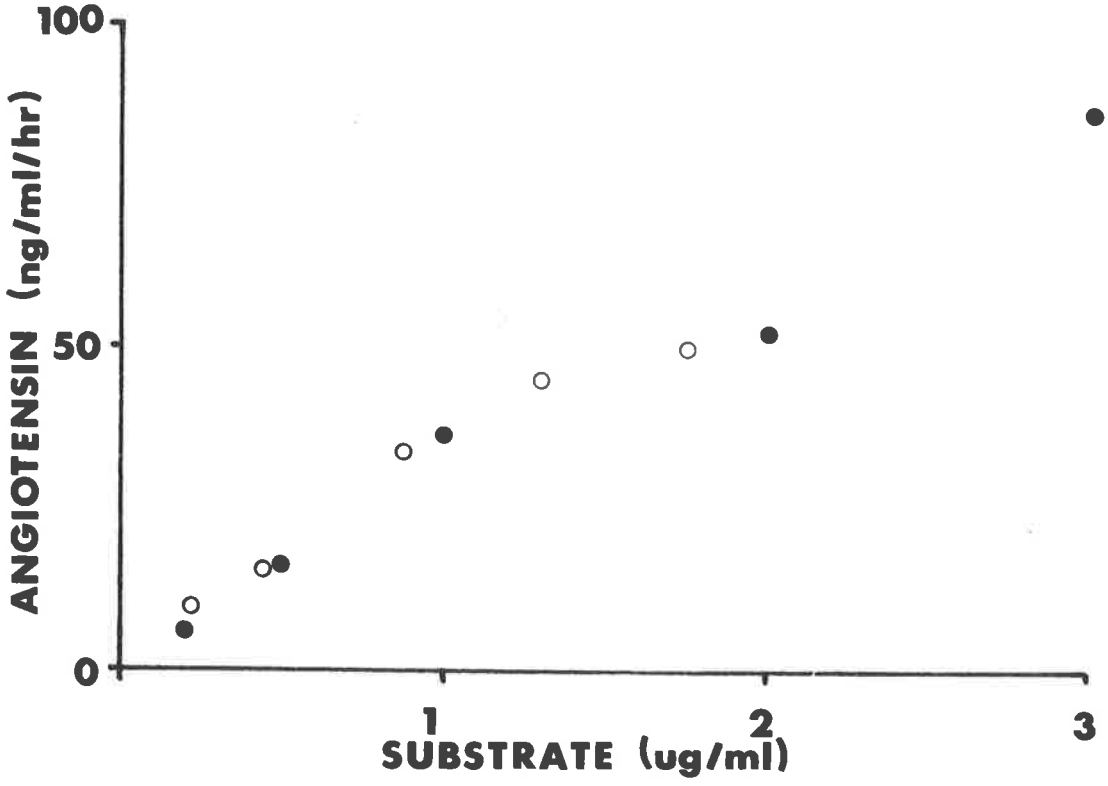


Fig. 5-9 Effect of non-pregnant (O) and pregnant (●) substrate on the rate of formation of angiotensin by amniotic fluid renin.

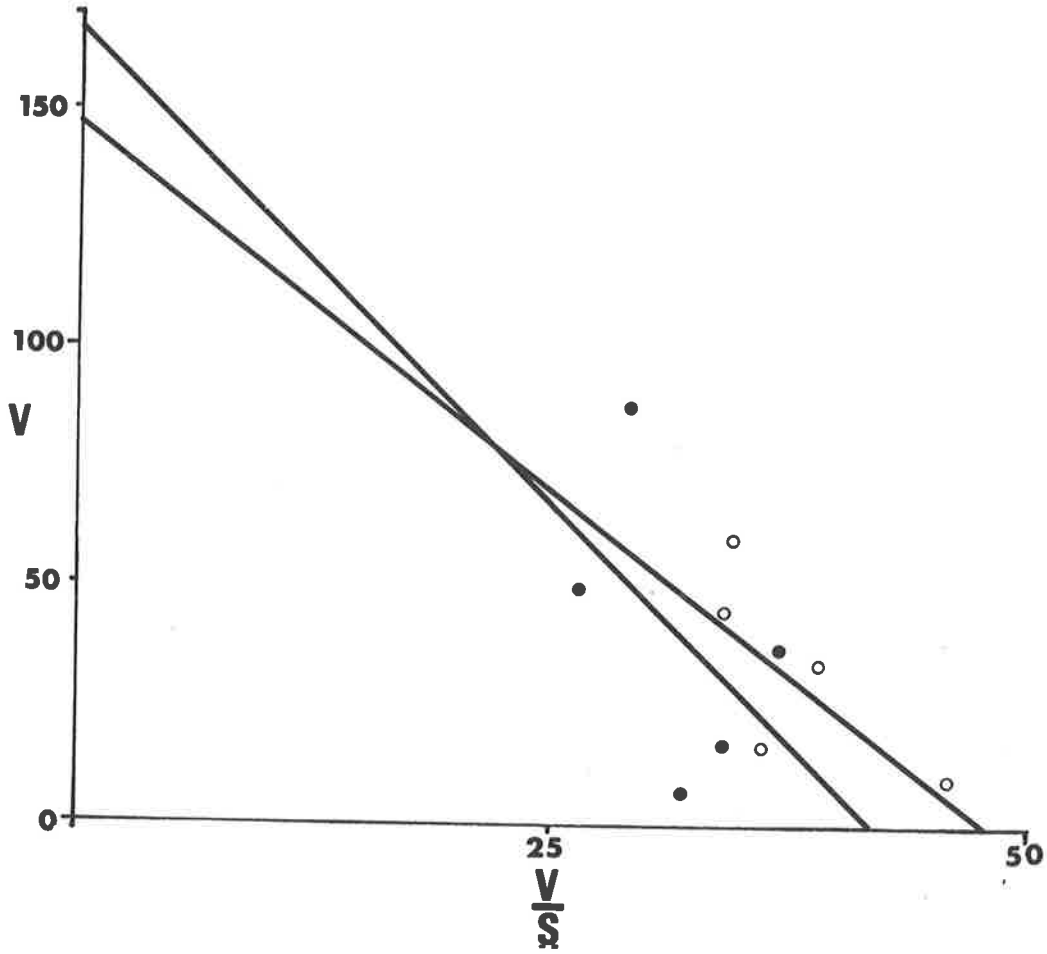


Fig. 5-10 Woolf plot derived from the effect of non-pregnant (○) and pregnant (●) plasma on rate of formation of angiotensin by amniotic fluid renin.

V = angiotensin (ng/ml/hr)

$\frac{V}{S} = \frac{\text{angiotensin (ng/ml/hr)}}{\text{substrate concentration (g/ml)}}$

(Appendix Table 16(a), 16(b))

plasma are present in rate-limiting amounts (Helmer and Judson, 1967; Skinner, Lumbers and Symonds, 1969). Therefore, increases in renin substrate, such as are observed in pregnancy, could increase the renin activity of plasma independent of changes in concentration of the enzyme. The increase in renin activity in pregnant plasma is therefore consequent upon both an increase in substrate and an increase in the circulating levels of renin, there being no evidence of any qualitative change in renin substrate. The contribution made by each to the renin activity varies according to the stage of pregnancy, so that the renin activity of plasma remains relatively constant throughout pregnancy. The release of renin appears to be highest in early pregnancy; later, renin levels tend to fall. Renin substrate levels, on the other hand, show a progressive increase as the duration of pregnancy increases.

The elevation in renin substrate levels is consequent upon the increases in the circulating levels of oestrogens that occur in pregnancy (Siiteri and MacDonald, 1966), since it has been shown that oestrogens stimulate production of renin substrate in man and other animals (Helmer and Griffith, 1952).

A rise in renin concentration in early pregnancy has also been described by Brown, Davies, Doak, Lever and Robertson (1963). The cause of this early rise in renin release is not known. It is possible that increases in progesterone stimulate release of renin

from the kidney, since progesterone has a natriuretic action inhibiting the action of aldosterone on the distal tubule (Landau and Lugibihl, 1961).

Increase in renin concentration, and hence renin activity, may thus be necessary to offset this diuretic action of progesterone. If progesterone is the stimulus for renin release early in pregnancy, since levels of progesterone rise progressively throughout pregnancy, it would be anticipated that renin concentration would also rise progressively. However, it does not: in fact, after 22 weeks the renin levels fall, suggesting feed-back suppression of renin release from the kidney by increased angiotensin levels associated with increasing renin substrate levels.

The hormonal control of sodium metabolism in pregnancy is complex. Firstly, oestrogens have a sodium retaining action (Knowlton, Kenyon, Sandford, Lotwin and Fricker, 1942), and since the maternal levels of oestrogen increase during pregnancy, it might be anticipated that their sodium-retaining effect also increases. Secondly, progesterone appears to play an active role in sodium metabolism during pregnancy. Ehrlich, Laves, Lugibihl and Landau (1962) showed that there is a very close interrelationship between aldosterone and progesterone levels in the pregnant woman. They found that both urinary pregnanediol and urinary aldosterone levels fell concurrently with sodium loading in the pregnant woman and rose

with cessation of the high sodium diet. They also showed that the secretion of progesterone (as measured by urinary pregnanediol) was directly related to the amount of mineralocorticoids given to Addisonian pregnant women. In women receiving no mineralocorticoid supplement, the pregnanediol levels were very low. These results imply that progesterone is therefore actively concerned in the control of sodium homeostasis in the pregnant woman.

As regards the relationship between aldosterone excretion and maternal renin levels in the pregnant woman, this relationship appears to be obscure. Venning, Primrose, Caligaris and Dyrenfurth (1957), Rinsler and Rigby (1957), and Martin and Mills (1956) noted progressive rises in aldosterone secretion during the course of pregnancy. However, we have found that the plasma renin activity, although elevated, does not rise progressively during pregnancy. Genest, de Champlain, Veyrat, Boucher, Tremblay, Strong, Koiw and Marc-Aurèle (1964) noted a progressive rise in renin activity up to the twenty-first week of pregnancy, the levels then stabilizing or dropping slightly. Therefore, the progressive rise in aldosterone excretion contrasts with the high, but comparatively stable, levels of renin activity.

If renin activity is a true reflection of the *in vivo* ability of plasma to form angiotensin, a dissociation therefore exists between the renin angiotensin system and aldosterone secretion,

suggesting that other factors are involved in the control of aldosterone secretion in the pregnant woman. Obviously, more studies are required, in particular application of radioimmunoassay of angiotensin levels in the pregnant woman would be helpful.

It is possible that there is a contribution to maternal plasma renin levels of renin from some extrarenal source, since renin has been found in high concentrations in the human reproductive tract (Skinner, Lumbers and Symonds, 1968). In this regard, it is interesting to note that the variations in maternal plasma renin levels that occur in pregnancy show a similar pattern to the variations that occur in maternal plasma levels of chorionic gonadotrophin (Jones, Delf and Stran, 1944). This is excreted only from the pregnant reproductive tract. It, like renin, is a protein hormone and the tissue of origin is, like renin, believed to be the chorion. Therefore, it is not unlikely that those factors which result in the release of chorionic gonadotrophin into the maternal circulation in early pregnancy also result in the release of renin from the reproductive tract.

SUMMARY TO SECTION 5

1. Plasma renin activity, plasma renin concentration and renin substrate levels have been studied during the course of the normal menstrual cycle following administration of oral contraceptive agents and during normal pregnancy.

2. A rise in plasma renin activity consequent upon an elevation in plasma renin concentration occurs in the second half (luteal phase) of the menstrual cycle. This is probably the result of the natriuretic action of progesterone which is secreted during the luteal phase of the normal menstrual cycle.

3. Administration of oral contraceptives to normal women produced an elevation in plasma renin activity which was greater than the rise in renin activity seen during the luteal phase of a normal menstrual cycle. This elevation in plasma renin activity was due to a concomitant increase in renin substrate levels induced by the oestrogenic component of the pill. Plasma renin concentration levels were suppressed below the levels found prior to administration of the oral contraceptive agent. The changes in renin substrate induced by oral contraceptives are quantitative; no qualitative change in renin substrate was observed. An hypothesis based on failure of feed-back suppression of renin release from the kidney has been advanced to explain the 'hypertensive effect of the pill'.

4. Plasma renin activity levels are elevated in early pregnancy and remain elevated throughout the course of pregnancy. The elevated levels of plasma renin activity in early pregnancy are associated with increased levels of plasma renin concentration. As pregnancy progresses renin substrate levels increase, whilst the renin concentration levels fall, although they remain elevated to

above normal non-pregnant levels. These findings suggest that the elevation in renin substrate as pregnancy progresses contributes to a greater exteht to the increased plasma renin activity and thus invokes a suppression of the initially high renin secretion from the kidney. No explanation for the early marked increases in renin levels is apparent. The control of sodium homeostasis in pregnancy is complex and humoral agents such as progesterone may be actively involved. It is also possible that renin may be released from the reproductive tract into the maternal circulation during pregnancy.

SECTION SIX

THE SENSITIVITY OF THE HAND BLOOD VESSELS TO ANGIOTENSIN AND NORADRENALINE IN PREGNANT AND NON-PREGNANT WOMEN

THE SENSITIVITY OF THE HAND BLOOD VESSELS TO ANGIOTENSIN AND NORADRENALINE IN PREGNANT AND NON-PREGNANT WOMEN

A reduction in the pressor response to intravenous infusions of angiotensin is characteristic of pathological conditions associated with high circulating levels of renin and presumably angiotensin (Kaplan and Silah, 1964). A similar association occurs in pregnancy. The sensitivity to intravenous infusions of angiotensin is reduced in the pregnant woman when the pressor response obtained for a given dose of angiotensin is compared to that obtained in non-pregnant women (Chesley, Talledo, Bohler and Zuspan, 1965).

Elevations in renin levels and renin activity in pregnancy are well described (Genest, de Champlain, Veyrat, Boucher, Tremblay, Strong, Koiw and Marc-Aurèle, 1965; Helmer and Judson, 1967; Lumbers, Skinner and Symonds, unpublished observations). Since the response to angiotensin by the hand vessels was found to be reduced in patients with renovascular hypertension (Scroop and Whelan, 1968), a situation in which there is an associated decrease in the pressor response to angiotensin as well as high renin levels, it was of interest to see whether the decreased pressor responsiveness of the pregnant woman was also associated with a reduction in the sensitivity of the peripheral vessels to the constrictor action of the drug.

METHODS:

Nine pregnant female volunteers were studied. Their ages ranged from 18 to 26 years, all were nulliparous, more than 32 weeks pregnant and had no clinical evidence of toxaemia of pregnancy. Six female volunteers, who were not pregnant and not taking oral contraceptive preparations, were also studied. Their ages extended over the same range as that described for pregnant women.

Hand blood flow was measured by intermittent venous occlusion plethysmography. The laboratory temperature was maintained at 24-25^{o}C. The subjects rested recumbent on a couch for at least 30 minutes prior to infusions of drugs. During this time recording apparatus was applied and a 22-gauge, buttless needle inserted centripetally into the brachial artery under local anaesthesia with 2% xylocaine.

Measurements of flow were made with the subject in the left lateral position to avoid compression of the inferior vena cava by the pregnant uterus.

Hand blood flow was recorded 2-4 times every minute, using water-filled, temperature controlled plethysmographs (Greenfield, 1954; Greenfield, Whitney and Mowbray, 1963). A P23BC Statham transducer connected to a Rikadenki multipen recorder was used to record blood flow. The plethysmograph temperature was maintained at 32-34^{o}C.

NaCl (0.15 M) was infused intra-arterially via a constant infusion pump at a rate of 2 ml/min during control periods and also used as the vehicle for the infused drugs. Drug concentrations were calculated so that the minute doses of the drugs were contained in 2 ml. Angiotensin was infused for 5 minutes and noradrenaline for 4 minutes. The doses of drugs infused were such that the vessels of the infused limb were exposed to high concentrations, whilst the concentration of drug reaching the general circulation was insufficient to have any systemic action. Therefore, the non-infused hand was used as a control, and allowance for spontaneous variation in flow could be made by assuming that in the absence of the infused drug the ratio of flow in the control and infused hands would remain the same (Duff, 1952).

The drugs used were β-asparaginyl angiotensin II (Hypertensin, Ciba) and noradrenaline bitartrate (Levophed, Winthrop). Doses of angiotensin are expressed as weight of the amide and noradrenaline as weight of the base. Ascorbic acid (1:50,000) was added to all noradrenaline solutions.

All non-pregnant subjects received infusions of 100, 200, 400 and 800 ng/min of both noradrenaline and angiotensin. One pregnant subject received 200 and 400 ng/min of angiotensin and noradrenaline, whilst 8 pregnant women received 100, 200, 400 and 800 ng/min of angiotensin and at least two of the following doses

of noradrenaline - 100, 200, 400 or 800 ng/min.

Blood for plasma renin estimation was taken from an antecubital vein at the end of the experiment. Samples were taken from 7 of the 9 pregnant subjects and all non-pregnant subjects. All subjects had been recumbent for 3-4 hours prior to samples being taken. Heparin (20 units/ml) was used as anticoagulant.

Plasma renin activity, plasma renin concentration and renin substrate levels were estimated according to the method of Skinner (1967) as described previously.

RESULTS:

Figure 6-1 shows the individual log-dose response curves to both noradrenaline and angiotensin which were obtained from both pregnant and non-pregnant women (Figure 6-1a and 6-1b, respectively).

The mean per cent fall in hand blood flow, plotted against the log-dose of angiotensin and noradrenaline, respectively, is shown in Figure 6-2.

The per cent fall in hand blood flow obtained in pregnant women was significantly less than that obtained in non-pregnant women for the following doses of angiotensin - 100 ($0.01>P>0.02$), 200 ($P>0.001$) and 400 ng/min ($0.02>P>0.05$) - but not for doses of 800 ng/min. There was no significant difference in the per cent fall in flow obtained with noradrenaline in pregnant, as compared with non-pregnant, women.

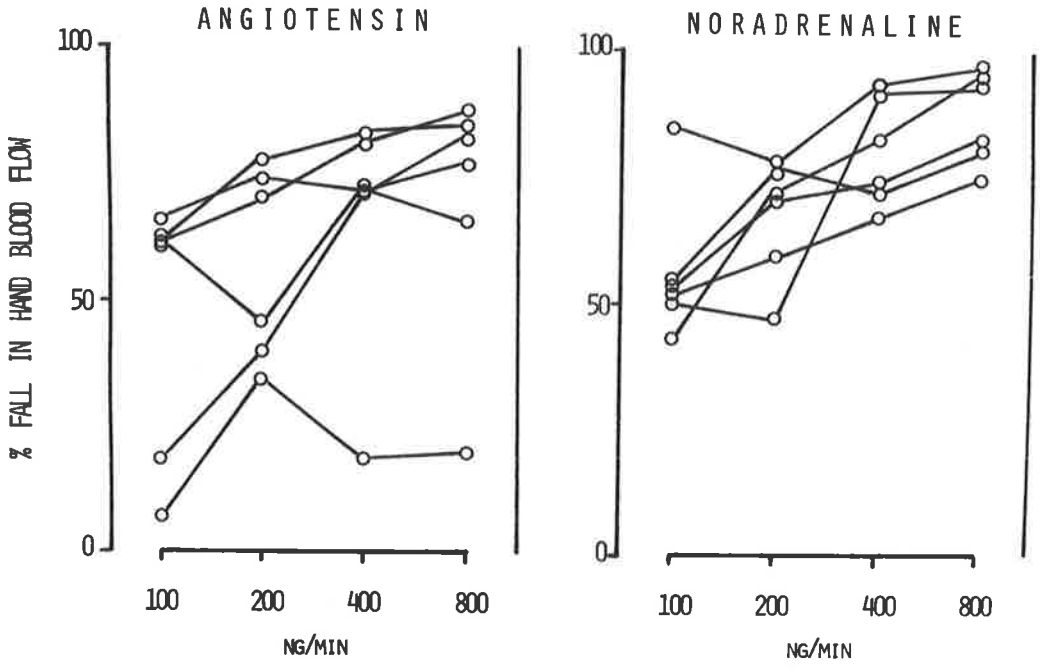


Fig. 6-1(a) Per cent fall in hand blood flow during intra-arterial infusions of angiotensin and noradrenaline in 6 normal non-pregnant women. (Appendix Table 17(a))

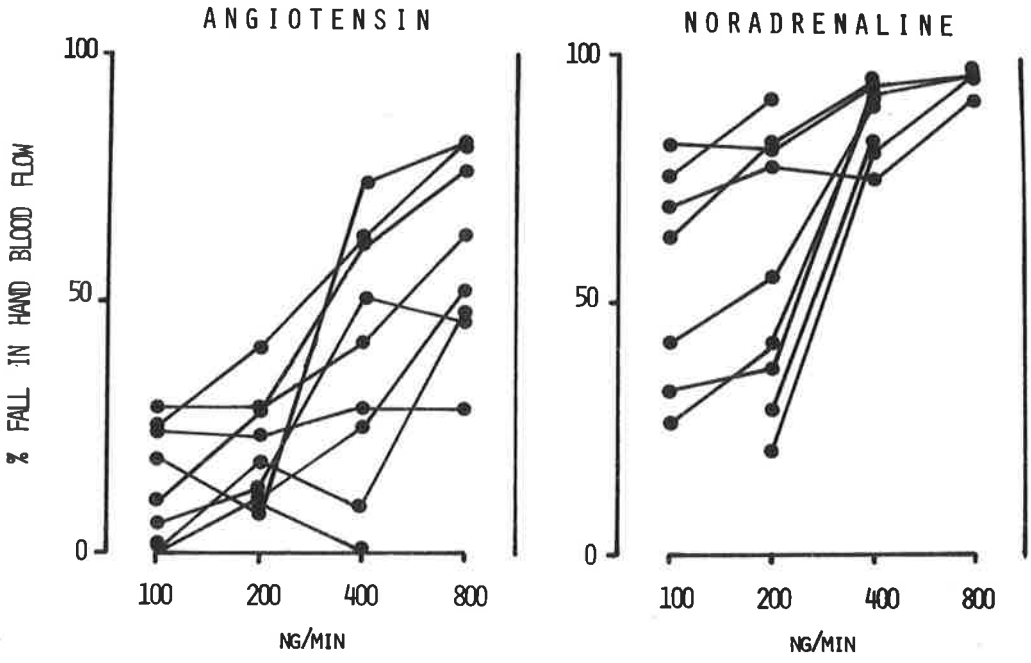


Fig. 6-1(b) Per cent fall in hand blood flow during intra-arterial infusions of doses of angiotensin and noradrenaline in 9 normal pregnant women. (Appendix Table 17(b))

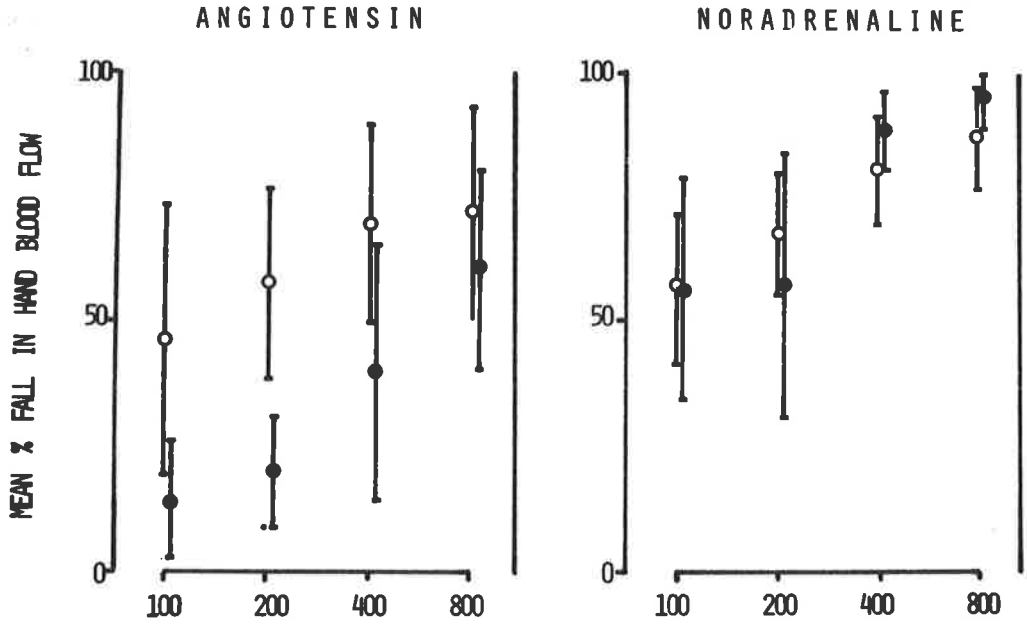


Fig. 6-2 Mean per cent fall (± 1 SD) in hand blood flow obtained on infusion of angiotensin and noradrenaline to non-pregnant (○) and pregnant (●) women.

Plasma renin activity, plasma renin concentration and renin substrate levels were significantly higher in pregnant subjects than in non-pregnant subjects. In one pregnant subject, the renin activity and renin substrate levels were within normal limits. Renin concentration was also suppressed, but a quantitative assay was not completed. No explanation for this finding was apparent (Figure 6-3, Table 6-1).

DISCUSSION:

The reduction in the sensitivity of the hand vessels to intra-arterial infusions of angiotensin indicates that the reduction in pressor sensitivity to the intravenous administration of angiotensin in pregnant women (Chesley, Talledo, Bohler and Zuspan, 1965) is a reflection of decreased responsiveness of the vessels and not the result of a modification of the central action of angiotensin or of baroreceptor mechanisms.

The presence of elevated plasma renin levels in all but one of the subjects studied suggests that reduced vascular sensitivity may be a consequence of the raised levels of circulating angiotensin and is in keeping with the results obtained in patients with renovascular hypertension (Scroop and Whelan, 1968).

The mechanism responsible for the reduction in sensitivity to angiotensin in pregnancy and other situations associated with elevated circulating levels of angiotensin is not clear.

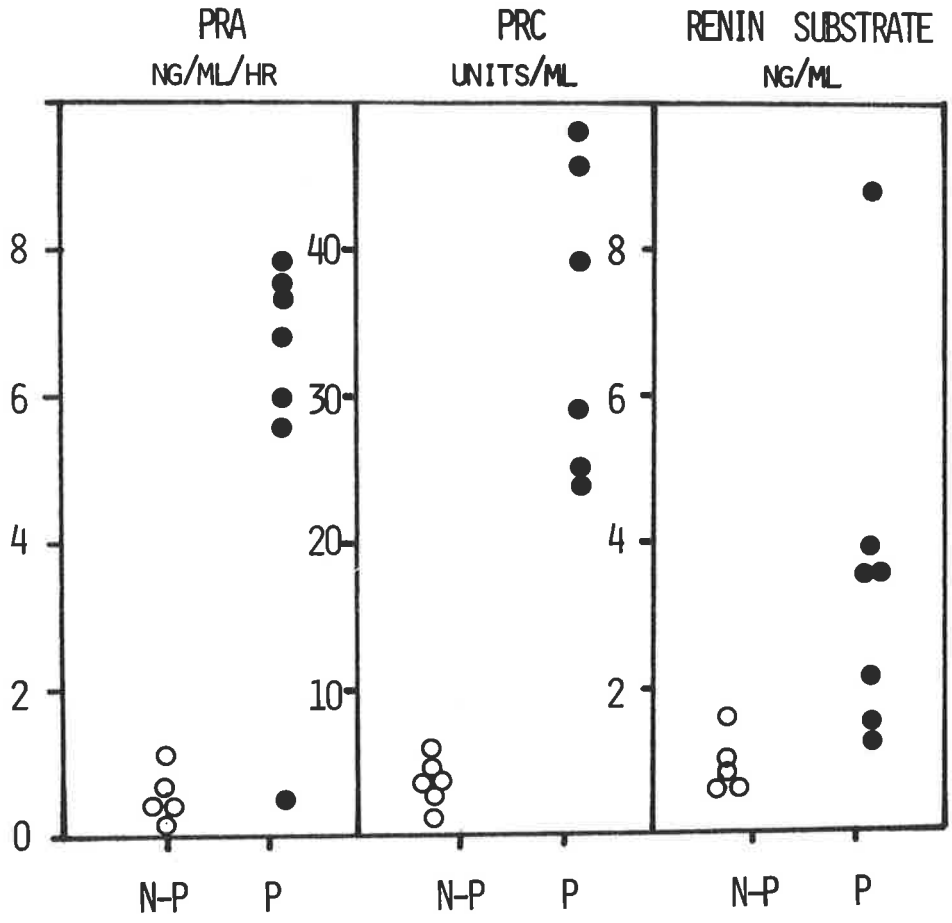


Fig. 6-3 Plasma renin activity, plasma renin concentration and renin substrate levels in non-pregnant (○) and pregnant (●) women.

TABLE 6-1

Plasma renin activity, concentration and renin substrate levels in pregnant and non-pregnant women.

Subjects	Plasma renin activity (ng/ml/hr)	Plasma renin concentration (units/ml)	Renin substrate (µg/ml)
1	0.5	3.5	0.8
2	-	3.5	1.6
3	0.3	2.5	0.6
4	0.2	1.3	0.6
5	0.4	2.8	0.9
6	1.1	5.2	1.1
7	7.8	28.8	8.8
8	6.8	46.0	2.1
9	7.5	38.4	3.9
10	5.6	24.2	3.6
11	0.5	-	1.2
12	7.4	75.2	1.5
15	6.0	48.0	3.6

1-6 Non-pregnant

7-15 Pregnant

The reduction in sensitivity to angiotensin was specific, since the sensitivity to noradrenaline remained unchanged. It is therefore unlikely that non-specific factors, such as dilution of the drug as a result of increased blood flow (mean blood flow: non-pregnant subjects 9.3 ml/100 ml of tissue/min, pregnant subjects 21.3 ml/100 ml tissue/min) or a decrease in vascular tone are responsible for the reduction in sensitivity to angiotensin.

Pregnancy is associated with elevations in the circulating levels of progesterone and oestrogens. Alterations in vascular responsiveness induced by these compounds is probably not responsible for the reduction in sensitivity to angiotensin observed, since such an effect would tend to be non-specific and hence the sensitivity to noradrenaline would be affected in a similar manner. Furthermore, the administration of oestrogens to man and experimental animals does not alter either the pressor response (Hettiaratchi and Pickford, 1968) or the renal response to angiotensin (Chesley and Tepper, 1967). Administration of progesterone does alter both the pressor response to angiotensin in animals (Hettiaratchi and Pickford, 1968) and the renal response in man (Chesley and Tepper, 1967). However, progesterone is an aldosterone antagonist and therefore is natriuretic (Landau and Lugibihl, 1961). This action would probably stimulate release of renin from the kidney and give rise to elevations in renin levels such as are seen in the luteal phase of the menstrual cycle

(Brown, Davies, Lever and Robertson, 1964; Skinner, Lumbers and Symonds, 1969). Therefore, the reduction in pressor and renal responses to angiotensin following the administration of progesterone may be consequent upon the increased production of endogenous angiotensin.

In all situations in which there is a reduction in the sensitivity to angiotensin there is an elevation of plasma renin levels and presumably endogenous angiotensin levels (Kaplan and Silah, 1964; Scroop and Whelan, 1968). Elevations in plasma renin activity were found in all but one of the pregnant women studied. In this subject, there was no apparent reason for the normal renin activity levels obtained as she did not have any clinical evidence of pre-eclamptic toxaemia. The levels of renin activity found in pregnant women were 12 times the levels seen in non-pregnant controls and due to elevations in both renin concentration and renin substrate levels (Table 6-1).

The levels of renin activity and renin concentration in normal non-pregnant subjects were low compared to the levels obtained by Skinner (1967) (Table 6-1). This may be attributed to the fact that Skinner's normal range is for mid-afternoon ambulant subjects, whilst the non-pregnant controls had been recumbent for 3 to 4 hours prior to blood samples being taken. Whether such a postural effect occurs in pregnant subjects is not clear at this stage.

Tachyphylaxis to angiotensin implies a saturation of receptor sites for angiotensin so that no further sites are available for association with the infused drug (Page and McCubbin, 1968). Administration of high doses of angiotensin to the hand or forearm for periods of up to one hour fails to result in a reduction in the degree of constriction obtained (Lumbers and Whelan, to be published). Tachyphylaxis is a complex problem, since it is dependent not only on the doses of the drug infused, but also on the levels of angiotensinases present. Whether or not it is responsible for the reduction in vascular sensitivity to angiotensin in pregnant women cannot be decided with certainty, since it may not be justifiable to extrapolate the results obtained in the normal subject (Lumbers and Whelan, to be published) to pregnant women where endogenous levels of angiotensin have been elevated for months.

Talledo, Rhodes and Livingstone (1968) and Page (1947) have described elevations in circulating angiotensinases in pregnant women. The physiological role of circulating angiotensinases as regulators of the action of angiotensin is uncertain. The half life of angiotensin in the blood of pregnant women is, however, reduced, but is of sufficient duration to make it unlikely that a reduction in angiotensin concentration had occurred in the blood reaching the hand in pregnant women, as compared to non-pregnant women, following the infusion of angiotensin into the brachial artery at the elbow.

Hodge, Ng and Vane (1967) concluded that circulating angiotensinases play a minor role in the destruction of angiotensin II, as compared to the removal of angiotensin across the peripheral vascular bed. It is possible that circulating levels of angiotensinase may reflect tissue angiotensinase activity. Therefore, the increased levels observed in plasma in pregnancy (Talledo, Rhodes and Livingstone, 1967; Page, 1947) may reflect an increase in tissue angiotensinase activity which could be interpreted as a protective mechanism against the constrictor potential of the increased levels of endogenous angiotensin.

Finally, it could be hypothesized that the reduction in sensitivity to angiotensin is the result of reduced sympathetic tone. Ginsburg and Duncan (1967) mention the possibility that reduced sympathetic tone is responsible for the increased hand blood flow seen in pregnant women. Although there is no evidence of augmentation of peripheral sympathetic activity by angiotensin in man (Whelan, Scroop and Walsh, 1969), there is evidence of potentiation of the action of released noradrenaline by angiotensin (Scroop and Walsh, 1968). It is conceivable that a reduction in sympathetic tone could lead to a reduction in the amount of noradrenaline released into the vessel wall which would account, in part, for the reduced sensitivity to angiotensin observed in pregnant women.

SUMMARY TO SECTION 6

The hand vascular sensitivity to angiotensin is reduced in pregnant women as compared to non-pregnant women. This reduction in sensitivity is specific to angiotensin, since it does not occur with noradrenaline.

It suggests that the reduction in sensitivity to the pressor response to angiotensin seen in pregnant women is due to a reduction in peripheral vascular sensitivity.

Non-specific factors such as increased hand blood flow, or changes in vascular responsiveness induced by oestrogens or progesterone are not responsible for this reduction in sensitivity to angiotensin as the sensitivity to noradrenaline would be similarly affected.

Elevations in plasma renin activity, renin concentration and renin substrate levels are associated with the reduction in sensitivity to angiotensin observed.

SECTION SEVEN

TACHYPHYLAXIS TO ANGIOTENSIN IN MAN

TACHYPHYLAXIS TO ANGIOTENSIN IN MAN

In 1964, Kaplan and Silah observed that the blood pressure response to intravenous angiotensin was diminished in patients with renovascular hypertension. Scroop and Whelan (1968) reported a reduction in sensitivity of the hand blood vessels to angiotensin in patients with renovascular hypertension. A diminution in the pressor response to intravenous angiotensin has also been described in pregnant women (Chesley, Talledo, Bohler and Zuspan, 1965) and there is also a reduction in the sensitivity of hand vessels to angiotensin (Lumbers, to be published).

In all these cases, the reduced sensitivity to angiotensin is associated with elevations in plasma renin levels and presumably circulating levels of angiotensin. It has been suggested that the reduced vascular sensitivity to angiotensin in these subjects may be a manifestation of tachyphylaxis of the vessels to angiotensin.

The present study was undertaken to determine whether tachyphylaxis of human vessels could be induced to angiotensin and other drugs.

METHODS:

The subjects were volunteer medical students.

Laboratory temperature was maintained at 23-26^{o}C and the subjects rested recumbent on a couch for at least 30 minutes prior

to infusion of drugs. During this time recording apparatus was applied and a needle inserted into the brachial artery.

Blood flow through the hand and forearm was measured by intermittent venous occlusion plethysmography using water-filled plethysmographs. The temperature of the plethysmograph was maintained at 32°C for hand and 34°C for forearm measurements.

Records of blood flow were obtained 3-4 times every minute.

Blood flow was recorded by a strain gauge transducer (Statham P23Bc) connected to a Rikadenki multi-channel servo recorder. In some of the early experiments forearm blood flow was recorded with capacitance plethysmographs (Willoughby, 1965; Fewings and Whelan, 1966).

Intra-arterial infusions were administered through a 22-gauge buttless, short bevel needle inserted into the brachial artery centripetally, under local anaesthesia with 2% xylocaine. The needle was connected to a constant infusion pump which delivered the infusions at a rate of 2 ml/min.

Saline (0.15 M) was infused during control periods and also used as a vehicle for the drugs infused.

The doses of drugs infused were such that, with the exception of vasopressin, they did not produce any systemic effects. Therefore, the uninfused limb could be used as a control and correction for spontaneous variations in flow made by assuming that

in the absence of the infused drug the ratio of flow between the two limbs would remain the same.

Percentage change in blood flow was calculated as described previously.

Drugs infused:

β-asparaginyl angiotensin II (angiotensin amide), Hypertensin, Ciba, β-aspartyl angiotensin II (angiotensin acid) 33902Ba, Ciba, noradrenaline bitartrate monohydrate (Levophed, Winthrop), acetylcholine chloride (Roche), vasopressin (Pitressin, Park Davis), isoprenaline hydrochloride (Isuprel, Winthrop) and propranolol hydrochloride (Inderal, I.C.I.) were administered.

The doses of noradrenaline are expressed as weights of the base, of angiotensin as weights of the acid or amide, respectively, acetylcholine as weights of the salt, and vasopressin as units/ml.

Drugs were administered either as repeated, short infusions of 1-2 minutes' duration, with a 2-5 minute interval between each infusion, or as prolonged infusions continuing for periods of 30 minutes to one hour.

RESULTS:

Angiotensin:

Repeated infusions:

Angiotensin acid (8 experiments) and angiotensin amide (3 experiments) were given as repeated, short infusions into the

brachial artery, and the hand or forearm blood flow recorded. The intervals between infusions were of 2 to 4 minutes and were sufficient to allow the blood flow to return to near control levels before the next infusion began.

Infusions of 16, 32, 100, 200 or 400 ng/min were infused into the forearm (10 experiments) and doses of 200, 400 or 800 ng/min were infused into the hand. The reduction in response obtained was the same with successive infusions (Figure 7-1).

Prolonged infusions:

In thirteen experiments, each on a different subject (6 with forearm blood flow measurement and 7 with hand blood flow recording), angiotensin amide was infused continuously for 30 to 60 minutes. Doses of 16 and 32 ng/min were infused into the forearm, and 200, 400 and 800 ng/min into the hand. The per cent reduction in flow from the resting level was calculated for each 5 minute period of the infusion and the results of all experiments are shown in Figure 7-2.

During all infusions into the hand of doses less than 800 ng/min, and all but one of these into the forearm, the constrictor response was sustained throughout the infusion period. Similarly, the constrictor response was maintained with prolonged infusions of angiotensin acid (32 ng/min into the forearm and 200 ng/min into the hand). Infusions of 800 ng/min of angiotensin amide into the hand

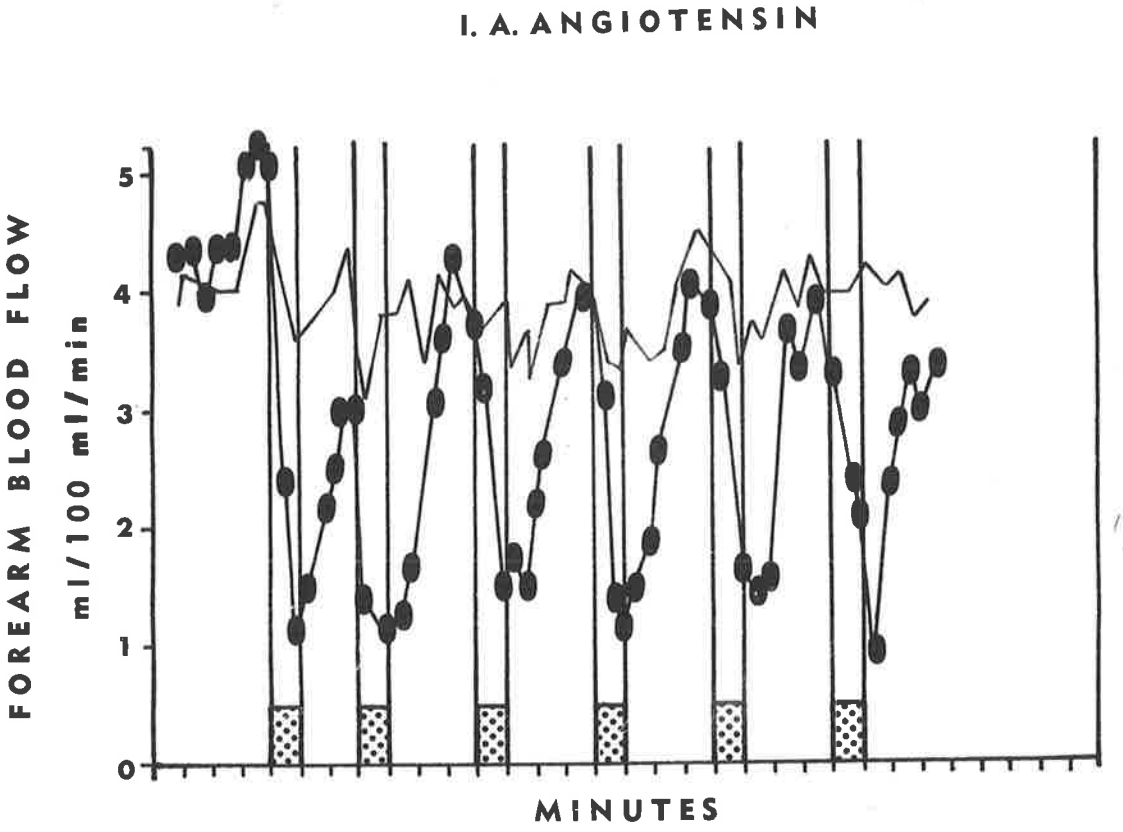


Fig. 7-1 The effect of repeated 1-minute infusions into the brachial artery of angiotensin amide (16 ng/min) on forearm blood flow. (●) infused side.

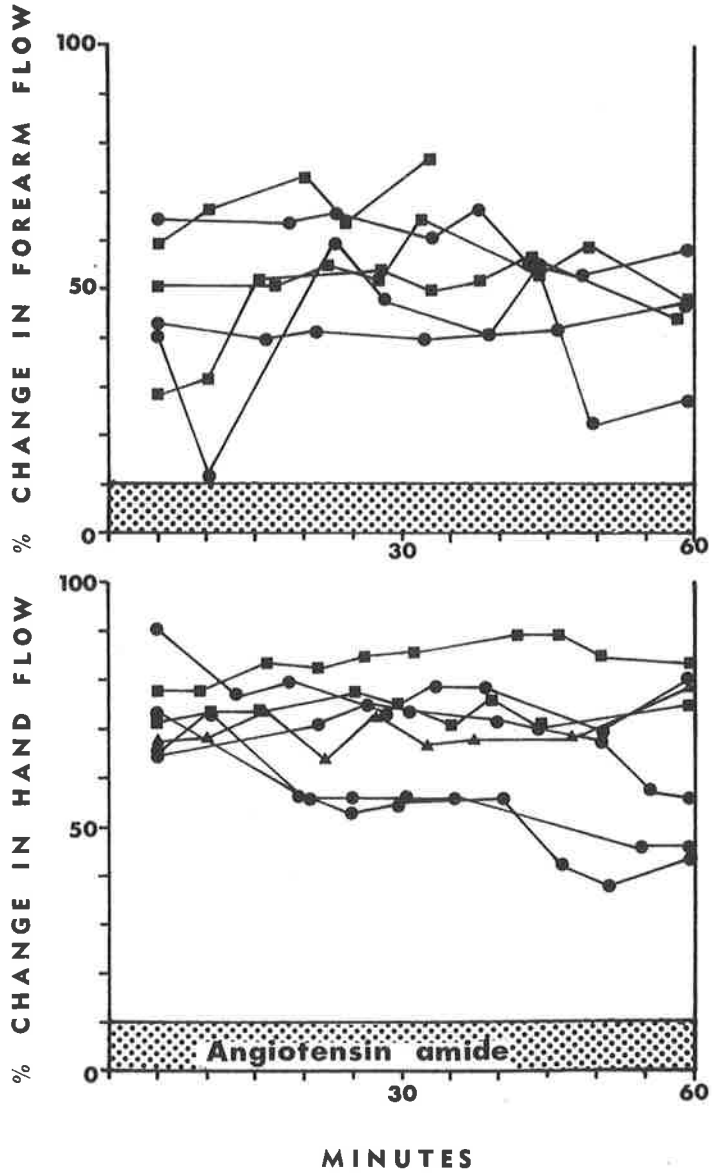


Fig. 7-2 Per cent fall in forearm blood flow (upper frame) and hand blood flow (lower frame) during continuous infusions of angiotensin amide. Doses of 16 ng/min (■) and 32 ng/min (●) were administered and forearm blood flow recorded. Doses of 200 ng/min (▲), 400 ng/min (■) and 800 ng/min (●) were administered and hand blood flow recorded.

resulted in a progressive diminution of the constrictor effect in three out of four subjects.

In view of the reduced constrictor response of the hand vessels to sustained infusions of 800 ng/min of angiotensin, it was decided to administer larger doses of angiotensin into the forearm vessels to see whether a similar reduction in constrictor response could be obtained. The forearm vessels are more sensitive to angiotensin than are the hand vessels (Scroop, Walsh and Whelan, 1965), and doses of the order of 100, 200, 400 and 800 ng/min of angiotensin would be expected to cause complete vasoconstriction. In fact, both flow and arterial pulsation were abolished within one minute of the onset of the infusion, but then the flow began to recover slowly and after 5 minutes had returned to 30-50% of the pre-infusion value. The flow remained at this level for the remainder of the infusion. This pattern of response was observed with doses of 100 to 800 ng/min in 4 infusions of the acid and 3 infusions of the amide of angiotensin (Figure 7-3).

Vasopressin:

Repeated infusions:

Repeated, short infusions of vasopressin were administered to two subjects. In one, hand blood flow was measured and doses were administered which ranged from 0.0025 to 0.1 units/min, infused for 2 to 3 minutes. The hand blood flow did not alter significantly

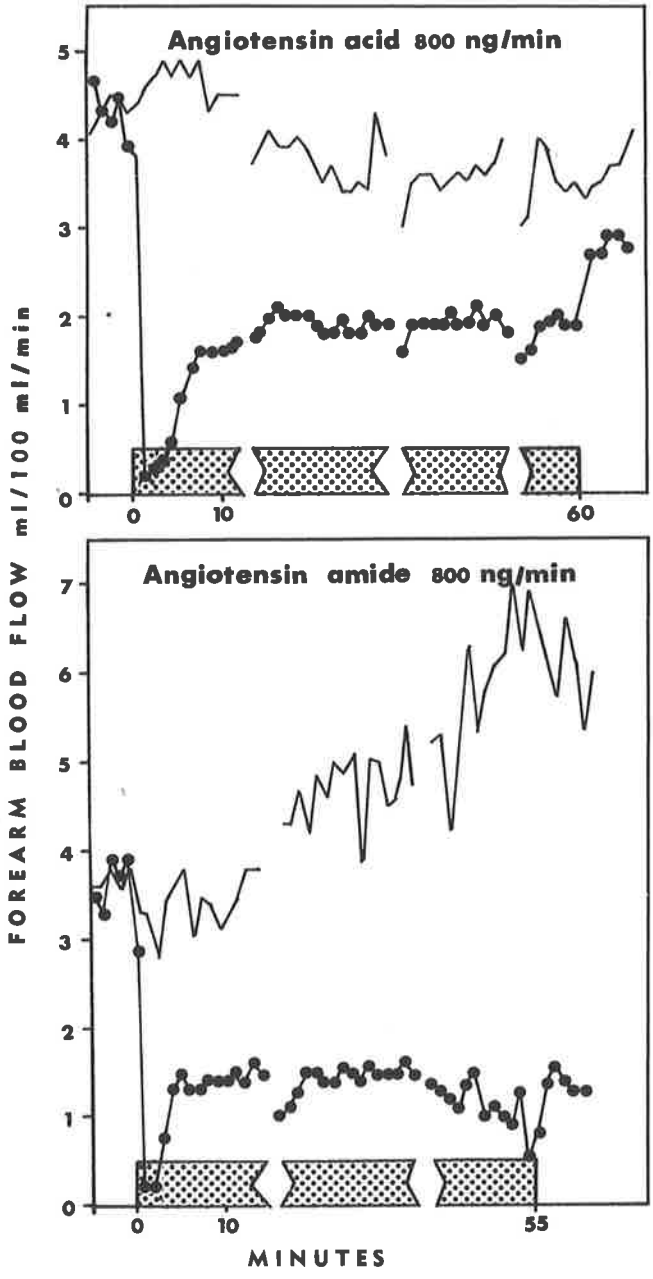


Fig. 7-3 Effect of continuous intra-arterial infusions of angiotensin acid (800 ng/min) (upper frame) and amide (lower frame) on forearm blood flow. (●) infused side.

with any of these doses, although with higher doses pallor and bradycardia was observed, indicating that sufficient was being given for a systemic effect to be produced. Since continuous blood pressure recordings were not made, it was not possible to ascertain whether a significant rise in blood pressure had occurred. Intra-arterial noradrenaline was subsequently infused into this subject and the fall in hand blood flow that occurred indicated that the infused drugs were, in fact, reaching the hand vessels. In one other subject, vasopressin (0.0025 units/min) was infused into the brachial artery and the forearm blood flow recorded. Repeated 2 minute infusions were given, with a period of 2 to 4 minutes between each infusion (Figure 7-4). A progressive reduction in the constrictor response was obtained. When, after the seventh infusion, a period of 12 minutes was allowed to elapse, during which saline only was infused, the response to the same dose of vasopressin was restored (Figure 7-4, lower frame). After a further control period of 17 minutes, 0.0025 units of vasopressin per minute were infused continuously for a period of 10 minutes. The initial constriction lasted for 3 to 4 minutes, after which blood flow rose rapidly, and by the seventh minute of infusion was well above the pre-infusion level (Figure 7-4, lower frame). The flow through the opposite control forearm also showed a rise during the infusion period.

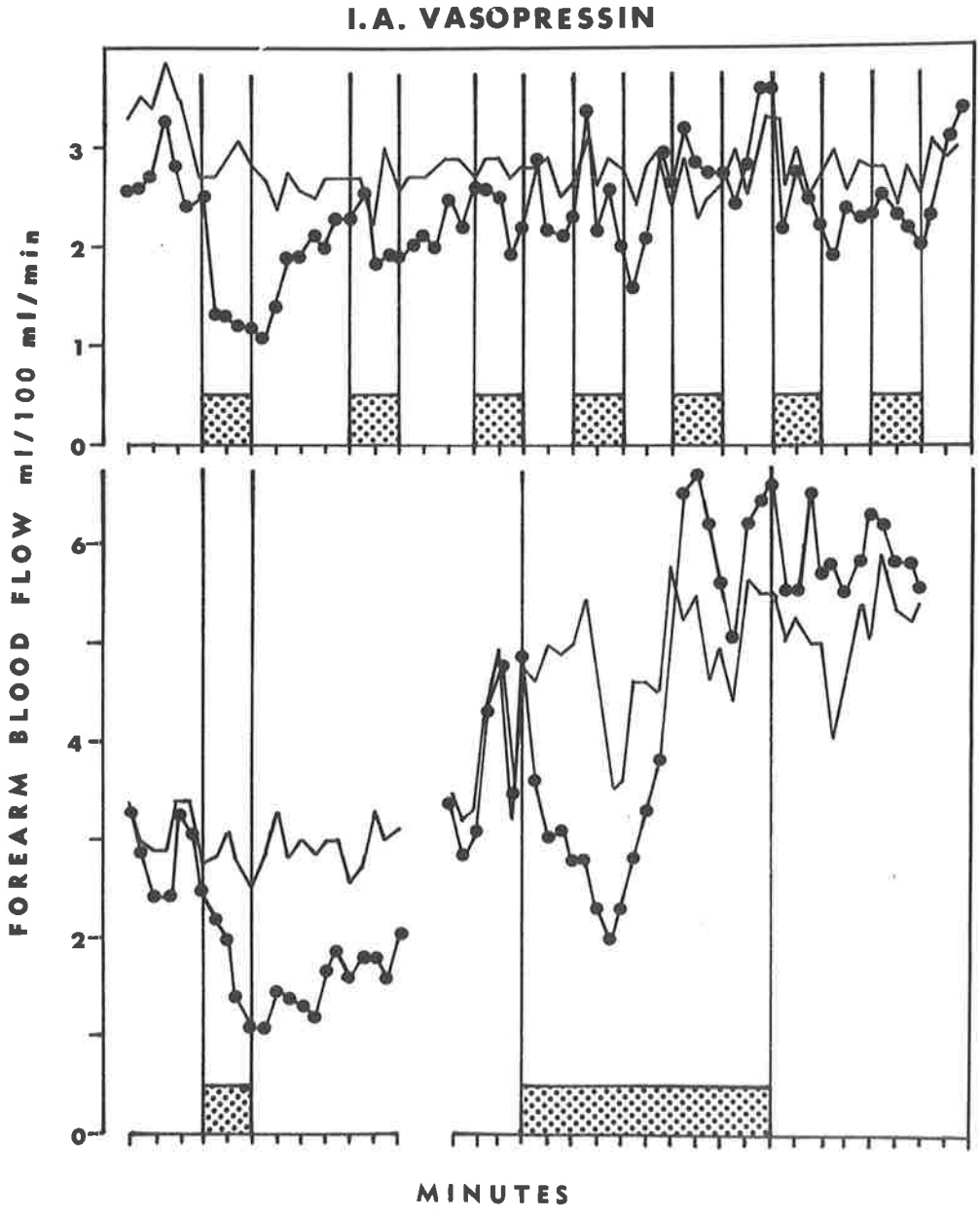


Fig. 7-4 Effect of infusion of 0.0025 units/min of vasopressin into the brachial artery on forearm blood flow. (●) infused side. Upper frame: repeated 2-minute infusions. Lower frame: a 2-minute infusion following a rest period of 12 minutes. After a further period of 17 minutes vasopressin was infused continuously for 10 minutes.

Noradrenaline:

Repeated infusions:

Frequent short infusions of noradrenaline 64 and 400 ng/min, respectively, were administered intra-arterially into two subjects in one of whom the forearm and in the other the hand blood flow were measured (Figure 7-5). No reduction of the constrictor response was seen in either of these subjects.

Prolonged infusions:

An hour-long infusion of 800 ng/min of noradrenaline was given intra-arterially in one subject, and forearm blood flow recorded. The flow fell to a very low level within 1-2 minutes of the onset of the infusion, but over the next 3 to 5 minutes gradually rose to a value of 50% of the pre-infusion levels and remained at this level throughout the remainder of the infusion period (Figure 7-6). Similar results were obtained in three other subjects in whom noradrenaline (800 ng/min) was infused for 20 minutes. In three subjects, 800 ng/min of noradrenaline was infused intra-arterially for 15 minutes. Following this, isoprenaline 0.05 µg/min was infused intra-arterially for 2 minutes. Propranolol hydrochloride 100 µg/min for 8 minutes abolished the dilatation observed with 0.05 µg/min of isoprenaline, but only partially abolished the reduction in constrictor response seen with noradrenaline 800 ng/min in two out of three subjects.

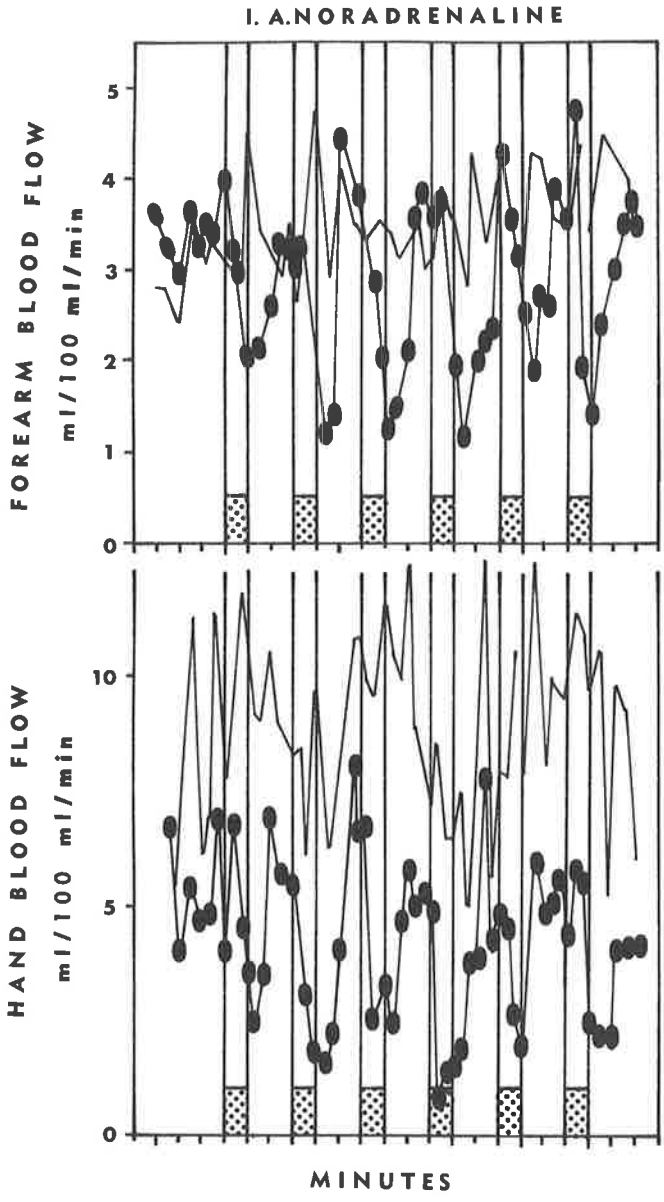


Fig. 7-5 Effect of repeated infusions of 64 ng/min of noradrenaline into the forearm (upper frame) and of 400 ng/min into the hand (lower frame). (●) infused side.

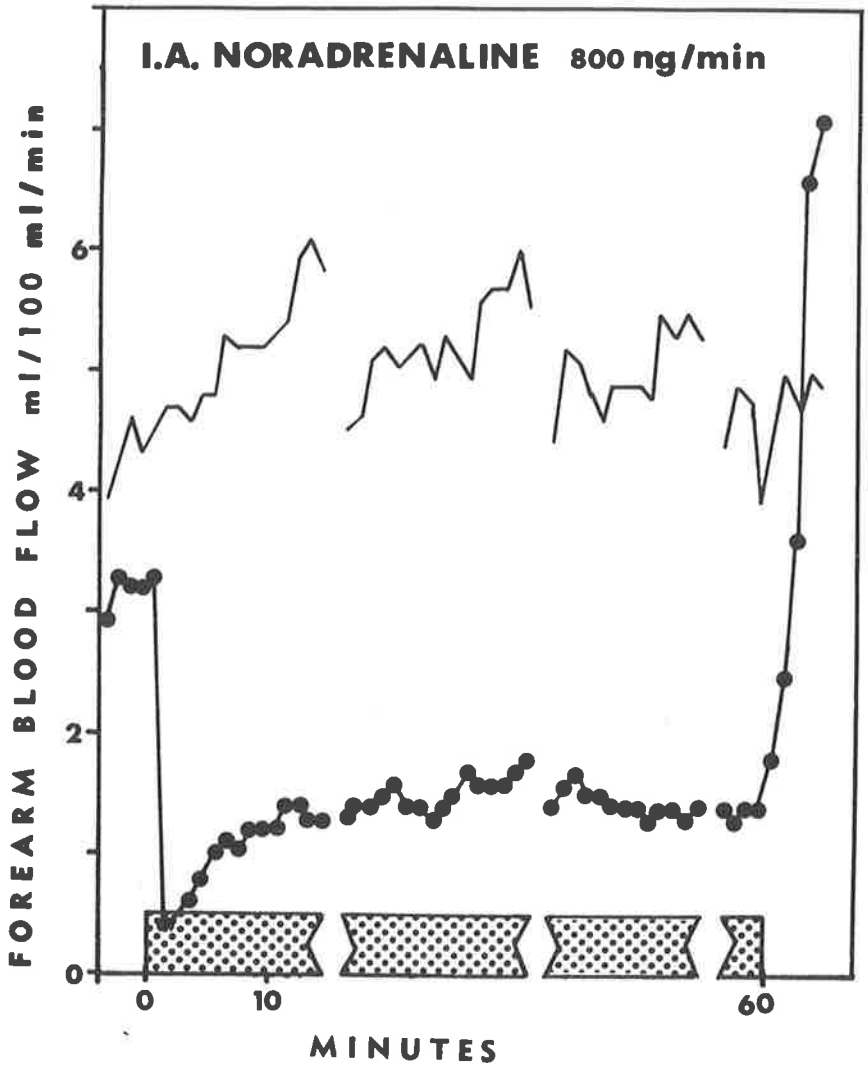


Fig. 7-6 Effect of a prolonged infusion of 800 ng/min of noradrenaline into the forearm. (●) infused side.

Acetylcholine:

Acetylcholine was given intra-arterially into the forearm of one subject in both frequent, short infusions of 4 mg/min and also in a continuous 15-minute infusion of 1 mg/min (Figure 7-7). There was no reduction in the vasodilator response obtained in either case.

DISCUSSION:

Haas and Goldblatt (1964) point out that the term tachyphylaxis was first used by Champy and Gley (1911) to describe the rapidly developing refractory state of an animal against the toxic effects of crude tissue extracts containing foreign proteins by the previous administration of small amounts of the same extract. The phenomenon was thus a manifestation of the immune response, and it was considered by Haas and Goldblatt (1964) not justifiable to apply the term to the refractory state to renin evoked by previous administration of the same substance.

However, the term has gained considerable usage in the pharmacological world, being used to describe the development of a state of refractoriness to a drug by an isolated tissue preparation or by the whole animal.

A more recent definition in accord with the current usage of the term is "the loss of response to a drug following its repeated or continuous administration in large amounts" (Page and McCubbin, 1968). Tachyphylaxis to renin was first described by Tigerstedt and

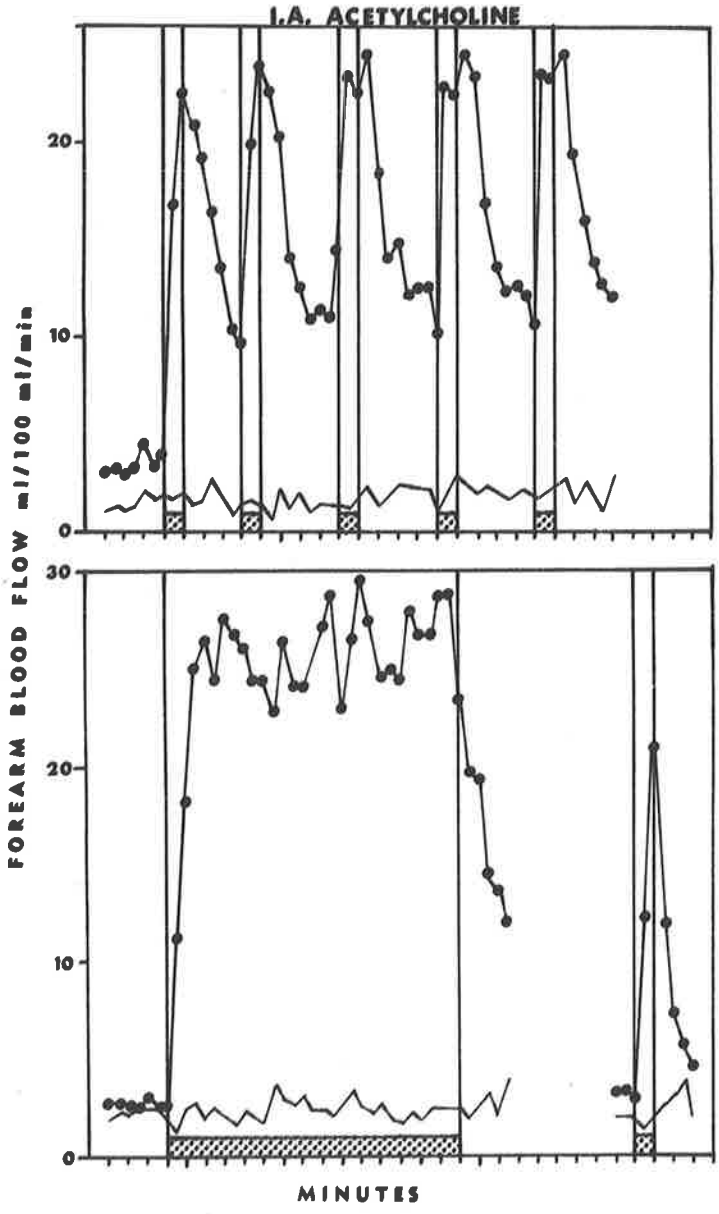


Fig. 7-7 Repeated infusions of acetylcholine (4 mg/min) (upper frame) and a continuous infusion (15 minutes) of 1 mg/min. (●) infused side.

Bergmann in 1898. Following the recognition that renin was an enzyme and acted on a substrate to produce angiotensin I, it was thought that the loss of the pressor action of renin was due to consumption of circulating substrate. However, in 1962, Gross and Bock demonstrated that the development of tachyphylaxis to renin was concomitant with the development of tachyphylaxis to pressor doses of angiotensin. These findings indicated that the refractoriness to renin was due not only to consumption of substrate, but also to refractoriness to the action of the peptide at the receptor.

Tachyphylaxis, therefore, can be explained on the basis of the receptor concept of drug action, as the result of filling all receptor sites so that none remain accessible. If, as Paton (1961) states, the stimulant action of a drug is proportional to the rate of association of the drug with its receptor, attachment and filling of all receptor sites by angiotensin would result in loss of constrictor action of the drug.

Such a basis for the mechanism of production of tachyphylaxis to angiotensin has been postulated by Page and Bumpus (1961) and Khairallah, Page, Bumpus and Turker (1966), and for tachyphylaxis to noradrenaline in the cat by Burn and Rand (1959).

The present experiments were designed to determine whether the development of tachyphylaxis to angiotensin played any part in the normal constrictor response to angiotensin, and to determine

whether the administration of doses of angiotensin comparable with the levels of angiotensin seen in patients with renovascular hypertension were associated with a reduction in constrictor potency on repeated or continuous administration.

The development of a progressive reduction in constrictor response to vasopressin with either repeated or continuous infusions indicated that tachyphylaxis was demonstrable in human forearm vessels with doses of a drug which reduced blood flow by only 50%. However, progressive reductions in the constrictor response to angiotensin similar to that seen with vasopressin or in tissue preparations with angiotensin could not be demonstrated with either repeated or continuous infusions of angiotensin to normal subjects.

The return of forearm blood flow to 30 to 50% of resting levels when very high doses of angiotensin (800 ng/min) were infused into the forearm may represent tachyphylaxis to the drug, but it was by no means a complete phenomenon, and the fact that a similar pattern was seen with an infusion of a large dose of noradrenaline suggests that the recovery of flow may be non-specific and represent a physiological dilator response to ischaemia which opposes the constrictor action of the drug.

The doses of angiotensin which can be safely administered to a normal subject may be insufficient to produce tachyphylaxis to the drug. However, infusions of 32 ng/min into the forearm vessels

would result in an arriving concentration of 1.6 ng/ml, and infusions of 800 ng/min into the forearm and hand would result in arriving concentrations of 40 and 16 ng/ml, respectively. Such levels are in excess of those observed for normal subjects (0.021 ng/ml) and pathological states such as renal artery stenosis (0.218 ng/ml), chronic nephritis (0.251 ng/ml), and malignant hypertension (1.727 ng/ml) (Catt, Cain and Coghlan, 1967). Abolition of the pressor response in animals requires intravenous doses of the order of 20 to 50 µg/Kg by injection and 5 to 15 µg/Kg by continuous infusion (Gross and Bock, 1962; Bock and Gross, 1961; Doyle, Louis and Osborne, 1967). These doses cannot be safely administered to normal subjects and indicate the difficulty of inducing tachyphylaxis in the whole animal. In isolated artery preparations, concentrations of the order of 100 µg/ml in the bathing solutions and injections of 0.25 µg into perfusing solutions are used to induce tachyphylaxis (Bohr and Uchida, 1967; Khairallah, Page, Bumpus and Turker, 1966). Addition of angiotensinase to perfusing solutions of isolated ear artery preparations reverses the tachyphylaxis induced by prior injections of angiotensin.

Factors contained in plasma, such as noradrenaline, albumin and serotonin, also cause apparent reversal of tachyphylaxis (Freckelton and Speden, to be published; Teh, to be published). Therefore, in both the whole animal and the normal subject the

development of tachyphylaxis is inhibited by the above mentioned factors.

The present experiments have shown that tachyphylaxis to angiotensin does not play a role in the response to angiotensin in physiological situations. Whether it plays a role in the reduction in sensitivity of the vessels of patients with renovascular hypertension is less certain. Although the doses administered were in excess of those seen in arterial blood in pathological situations (Catt, Cain and Coghlan, 1967) and venous plasma (Boyd, Landon and Peart, 1967), factors such as duration of exposure of vessels to angiotensin and alteration in plasma factors may play a role in inducing tachyphylaxis. It is unlikely that tachyphylaxis is dependent upon reduction of angiotensinase levels in such pathological conditions, since pregnancy, which is associated with reduced pressor responsiveness, and hand vascular sensitivity to angiotensin is also associated with increased levels of circulating angiotensinase (Page, 1947; Talledo, Rhodes and Livingstone, 1967).

SUMMARY TO SECTION 7

Tachyphylaxis of the hand and forearm vessels to angiotensin could not be demonstrated.

Tachyphylaxis to vasopressin was, however, readily demonstrated.

It is unlikely that in normal subjects tachyphylaxis to angiotensin plays a role in the normal vascular response to angiotensin. If tachyphylaxis plays a role in the reduced vascular responsiveness to angiotensin in patients with renovascular hypertension, it is probably attributable not only to the elevated levels of angiotensin, but, in addition, to other factors such as duration of exposure to angiotensin and alteration of plasma constituents.

STATISTICAL METHODS

1. Calculation of mean, variance and standard deviation:

$$\text{Mean} = \bar{x} = \frac{1}{n}\Sigma x$$

$$\text{Variance} = S^2 = \Sigma x^2 - \frac{1}{n}(\Sigma x)^2$$

$$\text{Standard deviation} = S = \sqrt{S^2}$$

2. Student's t-test was used as a test for significance, using

either $t = \dfrac{x - u}{S\sqrt{n}}$ (n-1 degrees of freedom)

for paired data

or Olivetti Programma 101, Number 136 for unpaired data.

3. Correlation coefficients were calculated using the formula:

$$r = \frac{\Sigma xy - \Sigma x \dfrac{\Sigma y}{n}}{\sqrt{\{\Sigma x^2 - \dfrac{(\Sigma x)^2}{n}\}\{\Sigma y^2 - \dfrac{(\Sigma y)^2}{n}\}}}$$

with n-2 degrees of freedom

$$t = \frac{r\sqrt{n-2}}{\sqrt{1-r^2}}$$

or using the Bravais Pearson coefficient of linear correlation, Olivetti Programma 101, Number 132.

* Reference: Statistical Methods in Biology
Bailey N. T. J. (1959)
The English Universities Press Ltd.

TABLE 1

Urine No:	1	2	3	4
17	30-50	0	50-70	0
20	20-40	0	20-40	0
22	50-70	0	70-90	0
24	80-100	-	100-120	0

1. Amount of pressor material formed following incubation of aliquots of 4 urines incubated at 37°C with standard substrate.
2. Effect of boiling prior to incubation on the formation of pressor material by 3 of 4 urines incubated at 37°C with standard substrate.
3. Concentration of pressor material (ng/ml) following boiling of samples (column 1) for 1 hour.
4. Concentration of pressor material (ng/ml) remaining following incubation of samples (column 3) with α-chymotrypsin at 37°C for 1 hour.

TABLE 2

Effect of temperature on the rate of formation of angiotensin (ng/ml/hr) by urinary renin acting on standard sheep substrate at pH 7.5.

Temperature (°C)	Angiotensin (ng/ml/hr)
31	33.0
37	45.0
40	50.1
43	60.0
47	66.0
50	66.0
55	0
60	0

TABLE 3

Effect of the concentration of standard sheep substrate on the rate of formation of angiotensin (ng/ml/hr) by 5 urines.

TABLE 3(a)

Substrate concentration ng/ml (S)	Angiotensin (ng/ml/hr) (V)		
	Urine 1	Urine 2	Urine 3
1200	18.4	2.7	6.0
1000	19.8	2.6	5.7
800	-	2.5	5.8
600	16.8	1.8	5.1
500	10.0	1.9	5.3
400	10.0	1.6	4.8
300	7.2	1.3	4.0
200	7.4	1.2	3.2
160	4.2	1.1	3.3
100	3.0	-	1.8
50	3.0	-	-
Km	298 ng/ml*	347 ng/ml*	238 ng/ml*

* (1) Km - calculated from Woolf plot V : V/S

(2) Slope (b) = -Km

(3) (b) calculated from Bravais-Pearson coefficient of linear correlation.

$$b = \frac{\Sigma xy - \frac{Tx\ Ty}{n}}{\Sigma x^2 - \frac{(Tx)^2}{n}}$$

$x = V/S, \quad y = V$

TABLE 3(b)

Substrate concentration ng/ml (S)	Angiotensin (ng/ml/hr) (V)	
	Urine 4	Urine 5
1066	3.2	1.4
533	2.8	1.3
480	-	1.1
400	2.7	1.07
266	1.9	0.9
133	1.4	1.0
80	1.0	0.6
53	0.8	0.4
Km	212 ng/ml*	140 ng/ml*

TABLE 4

Effect of the concentration of urinary renin on the rate of formation of angiotensin (ng/ml/hr) using 4 urines.

% initial concentration	Angiotensin ng/ml/hr*			
	Urine A	Urine B	Urine C	Urine D
100	8.0	20.0	42.0	98.0
75	5.8	16.7	-	70.0
50	4.3	10.0	25.5	51.0
37.5	-	8.6	-	-
25	10.3	4.0	11.0	28.0
12.5	-	2.8	6.0	12.0
6.25	-	-	2.6	-

Urine A (12 units/ml)

Urine B (30 units/ml)

Urine C (63 units/ml)

Urine D (147 units/ml)

* measured velocity

TABLE 5

Effect of the pH of incubation on the rate of formation of angiotensin by renin in 3 urines (A, B, C) acting on standard sheep substrate.

pH of incubation	Angiotensin ng/ml/hr			% Maximal velocity			*Mean % maximal velocity	Plasma renin % maximal velocity
	A	B	C	A	B	C		
4.0	0	-	0.8	0	-	28.6	14.5	29
4.5	-	-	1.5	-	-	53.6	54.5	59
5.0	12.0	7.5	1.5	52.2	37.5	53.6	48.6	65
5.5	-	12.5	1.8	-	62.5	64.3	64.4	71
6.0	19.5	-	2.2	84.8	-	78.6	83.0	76
6.5	-	-	2.5	-	-	89.3	90.8	71
7.0	21.0	19.0	2.5	91.3	95.0	89.3	91.2	76
7.5	22.6	20.0	2.7	98.3	100	96.4	99.8	83
8.0	23.0	18.7	2.8	100	93.5	100	100	88
8.5	-	18.0	2.7	-	90.0	96.4	94.7	83
9.0	21.0	17.5	2.8	91.3	87.5	100	96.2	100

* Corrected for a mean % value of 100% at pH 8.0

TABLE 6

Variability of repeat determination of the concentration of renin in urine.

TABLE 6(a)

Duplicate assays of the same samples using the same rats.

Concentration of renin units/ml		Mean of A and B	% Difference (x) of A and B from mean
A	B		
4.63	4.3	4.47	7.38
20.0	20.0	0	0
10.5	10.5	0	0
7.5	7.5	0	0
5.0	4.8	4.9	4.0
9.6	8.7	9.15	9.84
1.3	1.3	0	0
2.35	20.0	2.175	16.09

$$\bar{x} = 4.66$$

$$SD = 6$$

$$S^2 = \Sigma x^2 - \frac{(\Sigma x)^2}{n}$$

TABLE 6(b)

Duplicate estimations of samples following storage of one sample at -20°C and assay some weeks later.

Concentration of renin units/ml		Mean of	% Difference (x) of A and B
A	B	A and B	from mean
3.3	4.63	3.97	33.5
3.3	4.3	3.8	26.3
22.4	20.0	21.2	11.32
10.6	10.5	10.55	0.95
8.8	7.5	8.15	15.95
5.2	5.0	5.1	3.9
5.2	4.8	5.0	8.0
10.8	8.7	9.75	21.5
10.8	9.6	10.2	11.76
1.68	1.25	1.47	29.25
1.68	1.27	1.48	27.7
2.3	2.35	2.325	2.15
2.3	2.0	2.15	13.95

$\bar{x}$ = 15.9, SD = 10.9

TABLE 6(c)

Duplicate estimations of samples, one aliquot of which has been dialysed after a long interval following storage at -20°C.

Concentration of renin units/ml		Mean of	% Difference (x) of A and B
A	B	A and B	from mean
18.0	12.0	15.0	40.0
30.0	30.0	30.0	0
30.0	39.9	34.95	28.31
30.0	29.25	24.63	2.5
30.0	39.9	34.95	28.3
30.0	29.25	29.63	2.5
39.9	29.25	34.58	30.8
97.5	63.0	80.25	43.0
97.5	45.0	71.25	73.68
63.0	45.0	54.0	33.3
2.49	2.145	2.32	15.09
24.0	17.0	20.5	34.14
4.2	4.20	4.2	0
0.26	0.36	0.31	32.3
0.69	0.50	0.595	31.9
0.27	0.33	0.30	20.0

$\bar{x}$ = 26.0, SD = 19.3

TABLE 7

Effect of the pH of initial dialysis and the addition of EDTA (0.005 M) on the destruction of β-asparaginyl-angiotensin II by urine incubated at pH 7.5 for 24 hrs (± 0.001 M EDTA, respectively)

pH of initial dialysis	Angiotensin remaining following incubation	
	+ EDTA	- EDTA
7.5	80-100	0-20
6.6	80-100	0-20
5.6	80-100	0-20
4.7	80-100	0-20
4.0	80-100	20-40
3.3	80-100	80-100

TABLE 8

Destruction of β-asparaginyl-angiotensin II by urine on incubation at a pH of 7.5 or less, with and without prior dialysis and heating to pH 9.6.

	% Angiotensin remaining after 24 hrs incubation	
pH of incubation	Urine not treated to pH 9.6	Urine treated at pH 9.6
7.5	20-40	80-100
7.0	20-40	80-100
6.0	20-40	80-100
5.0	20-40	80-100
4.0	70-90	80-100
3.0	80-100	80-100

TABLE 9

Effect of time on the rate of formation of pepsitensin by pepsin acting on standard sheep substrate at pH 5.6.

Concentration pepsin µg/ml	Concentration of pepsitensin after 5 min incubation	Concentration of pepsitensin after 10 min incubation
15	80-100	100-140
10	40-60	60-80
8	30-50	30-50

TABLE 10

Effect of the concentration of pepsin (µg/ml) on the amount of pepsitensin formed from standard sheep substrate after incubation for 10 min at pH 5.6.

Concentration pepsin µg/ml	Pepsitensin ng/ml
20	160-200
15	100-140
10	80-100
8	60-80
6	10-30
5	10-30
4	10-30
2	10-30

TABLE 11

Calibration curve for standard protein solutions (µg/ml) related to optical density at 750 mµ.

Protein µg/ml	O.D.
20	0.06
40	0.08
60	0.12
80	0.165
100	0.20
120	0.225
140	0.24
160	0.28
200	0.35

TABLE 12

The effect of elevations in plasma renin concentration (PRC) on the urinary excretion of protein (mg/24 hrs).

		Subject				
	Day	1	2	4	5	7
PRC (units/ml)	0	8.4	9.3	9.8	13.5	11.5
	7	19.5	23.8	20.0	23.8	34.2
	12	31.7	38.4	55.0	61.3	46.3
Protein excretion (mg/24 hrs)	0	137.8	182.0	80.7	143.2	110.4
	7	115.9	140.8	72.6	103.96	100.1
	12	103.5	185.6	66.4	122.5	108.9

TABLE 13

Day and night urinary renin concentrations (units/ml), urinary renin outputs (units/12 hrs), volumes (ml/12 hrs) and urinary sodiums (mEq/12 hrs).

No.	Renin conc$^{n.}$ units/ml		Renin output units/12 hrs		Volume urine ml/12 hrs		Na^{+} mEq/12 hrs	
	D	N	D	N	D	N	D	N
1	1.1	0.73	449	334	410	455	-	-
2	0.32	0.23	374	332	1170	1475	-	-
3	0.27	0.43	336	287	1245	660	-	-
4	0.20	0.20	133	135	680	690	-	-
5	0.26	0.51	275	321	1080	631	162	126
6	0.13	0.13	122	88	968	700	193	126
7	0.27	0.6	325	521	1205	869	217	130
8	0.7	1.88	904	975	1310	520	262	78.0
9	0.84	0.57	641	593	763	1040	153	52
10	0.22	0.04	125	37.4	569	936	84.2	151.6
11	0.25	0.22	156	117	630	525	117.3	72.2
12	0.47	0.47	384	291	825	625	130	72.2
13	0.43	0.25	131	140	302	550	29.1	6.2
14	0.8	1.3	262	553	327	440	51.1	50.1
15	0.24	0.14	136	152	575	1075	92.0	169.4

TABLE 14

Effect of serial dilution of plasma renin (pH 3.3 treated) renin on the formation of angiotensin (ng/ml/hr) from human substrate.

% Initial renin concentration	Angiotensin ng/ml/hr*
100	9.3
50	3.9
25	1.8
12.5	1.0

* Corrected for EV = 0.5 ng/ml/hr

TABLE 15

The effect of serial dilution of amniotic fluid on the rate of formation of angiotensin (ng/ml/hr) from standard sheep substrate and human substrate.

% Initial concentration amniotic fluid renin	Angiotensin ng/ml/hr	
	Standard sheep substrate	Human substrate*
100	64.0	12.7
50	27.0	6.5
25	12.4	2.6
12.5	6.7	1.2
6.25	3.0	0.9

* Corrected for endogenous velocity of 0.5 ng/ml/hr

TABLE 16

The effect of substrate concentration on the rate of formation of angiotensin (ng/ml/hr) by amniotic fluid renin acting on plasma from normal and pregnant subjects, and plasma renin acting on plasma from normal and "oral contraceptive treated" subjects.

TABLE 16(a)

Effect of the concentration substrate in normal human plasma on the formation of angiotensin by amniotic fluid renin.

Substrate (S) concentration µg/ml	Angiotensin ng/ml/hr (V)	V/S
1.75	60.3	34.46
1.313	45.0	34.27
0.875	34.4	39.31
0.438	15.7	35.84
0.219	10.0	45.66

Km = 3.0 µg/ml

TABLE 16(b)

Effect of substrate concentration in pregnant plasma on the rate of formation of angiotensin by amniotic fluid renin.

Substrate (S) concentration µg/ml	Angiotensin ng/ml/hr (V)	V/S
3.0	87.6	29.2
2.0	53.0	26.5
1.0	37.0	37.0
0.5	17.0	34.0
0.2	6.4	32.0

Km = 4.0 µg/ml

TABLE 16(c)

Effect of substrate concentration in normal and "oral contraceptive treated" plasma on the formation of angiotensin by pH 3.3 treated plasma renin.

Substrate (S) concentration µg/ml	Angiotensin ng/ml/hr (V)	V/S
4.31	28.3	6.57
3.24	22.2	6.85
2.16	16.5	7.64
1.08	10.5	9.7
0.54	4.7	8.7
0.27	2.3	8.5
0.135	1.2	8.9

Km = 7.6 µg/ml

TABLE 17

Dose response curves obtained with intra-arterial infusions of angiotensin in (a) Non-pregnant and (b) Pregnant women.

TABLE 17(a)

Non-pregnant women

Subject	Per cent constriction							
	Angiotensin (ng/min)				Noradrenaline (ng/min)			
	100	200	400	800	100	200	400	800
1	61.1	70.0	81.7	87.8	51.8	58.9	67.3	74.8
2	65.7	74.3	71.6	65.6	54.4	77.0	72.1	78.8
3	62.1	77.5	83.1	84.8	85.2	77.6	91.4	94.3
4	5.6	34.5	29.4	30.2	42.0	71.0	82.4	95.0
5	62.3	45.8	73.0	77.5	53.3	70.7	73.5	82.9
6	17.7	39.5	72.8	82.4	49.5	47.3	93.4	97.3
Mean	45.8	56.9	68.6	71.4	56.0	67.1	80.0	87.1
SD	±26.7	±19.1	±19.8	±21.6	±14.96	±11.8	±10.8	± 9.6

TABLE 17(b)

Pregnant women

Subject	Per cent constriction							
	Angiotensin (ng/min)				Noradrenaline (ng/min)			
	100	200	400	800	100	200	400	800
7	18.9	8.0	74.0	82.1	74.5	90.8	-	-
8	0	18.6	9.0	47.8	63.3	81.9	93.6	-
9	10.9	29.0	63.4	77.4	33.0	36.9	95.1	-
10	24.9	41.4	62.2	82.5	82.3	81.1	93.6	96.1
11	28.8	29.7	41.7	63.5	68.5	76.6	74.7	91.0
12	0	11.0	24.8	52.4	25.9	41.1	91.6	96.4
13	6.3	13.2	51.6	45.9	41.9	54.8	90.0	-
14	24.0	23.1	29.0	29.1	-	20.2	79.6	95.9
15	-	9.9	0.6	-	-	27.7	82.4	-
Mean	14.2	20.4	39.6	60.1	55.6	56.8	87.6	94.8
SD	±11.5	±11.3	±25.5	±19.5	±21.9	±26.5	± 7.6	± 2.6

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