RADIO-IMMUNOASSAY OF ANGIOTENSIN II

E. G. WILMSHURST, M.B.,B.S., M.R.A.C.P.,

Department of Medicine,

University of Adelaide

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STATEMENT

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University and to the best of my knowledge and belief contains no material previously published or written by another person, except when due reference is made in the text.

E. G. WILMSHURST

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CHAPTER 1

INTRODUCTION

1. RENIN-ANGIOTENSIN SYSTEM

(i) HISTORICAL

The existence of the renin-angiotensin system was first suspected following the work of Tigerstedt and Bergman (1898), who demonstrated a prolonged rise in arterial blood pressure in anaesthetized rabbits following the injection of extracts of rabbit kidney. The renal pressor substance was found in saline extracts of fresh rabbit kidney or of the dry residue obtained after treatment of rabbit kidney with alcohol. The authors coined the name "renin" for the active substance, which was obtained from cortex but not to any appreciable extent from medulla of the kidney. The main properties of the substance were: it was non-dialyzable, stable at 56°C but destroyed by boiling, soluble in water, glycerine and in dilute salt solutions, but insoluble in acetone and in alcohol. The blood pressure rise was not abolished by section of the cervical spinal cord nor by destruction of the spinal cord, indicating that the effect of the pressor agent was independent of the nervous system.

The classic paper of Goldblatt et al (1934) described the production of sustained hypertension in dogs by renal artery constriction with a clip. Constriction of one renal artery led to a slight to moderate rise in blood pressure with a tendency to return towards normal after a period of time, whereas bilateral renal ischaemia commonly produced very high levels of blood pressure.

The authors felt that the hypertension produced by only moderately severe renal artery constriction, with no signs of significant renal functional impairment, resembled benign nephrosclerosis in man, whereas severe constriction from the beginning led to marked elevation of blood pressure, severe disturbance of renal function, and uraemia, the latter picture resembling malignant nephrosclerosis in man. The production of experimental hypertension with a resemblance to clinical varieties of hypertensive disease in man stimulated a large amount of work aimed at defining the role of the kidneys in this situation.

The work of Tigerstedt and Bergman was confirmed in 1938 by Pickering and Prinzmetal and by Landis et al. Pickering and Prinzmetal showed that a prolonged rise in blood pressure may be produced in the anaesthetized rabbit by intravenous injection of extracts prepared from fresh kidney of several species. They confirmed that the active substance, "renin", was present in the cortex but not in the medulla of the kidney and that it had chemical properties suggestive of a protein structure. They showed also that the substance could be assayed biologically by comparison of the blood pressure response produced in unanaesthetized rabbits by the extract with that produced by a standard preparation of renin. Landis et al (1938), working with rabbits, found that "unheated kidney extracts" had a variable effect on blood pressure but that heating to 55-56°C for 20 minutes and filtration produced an extract which consistently elevated blood pressure. The substance responsible for the pressor

activity of renal extracts was destroyed by heating to 65°C, did not pass through a dialysis membrane, and was precipitated with ammonium sulphate.

Goldblatt (1938) and Wilson and Pickering (1938) described the experimental production in animals, by renal artery constriction, of the fibrinoid arteriolar necrosis characteristic of malignant hypertension, in the same distribution as in man except for the sparing of the kidney. The sparing of the kidney prompted the suggestion (Goldblatt, 1938) that the height of the arterial pressure was an important factor in the production of the arteriolar lesions.

In 1939, Page, and Braun-Menéndez et al independently showed that renin had the properties of an enzyme, splitting a plasma constituent into a smaller molecule now known as angiotensin. Page found that injection of purified renin in Ringer's solution did not produce vasoconstriction in experimental animals, but that incubation with "renin-activator" in plasma restored the vasoconstrictor activity. This type of observation led to the concept of renin acting on a plasma substrate to form an active vasoconstrictor substance.

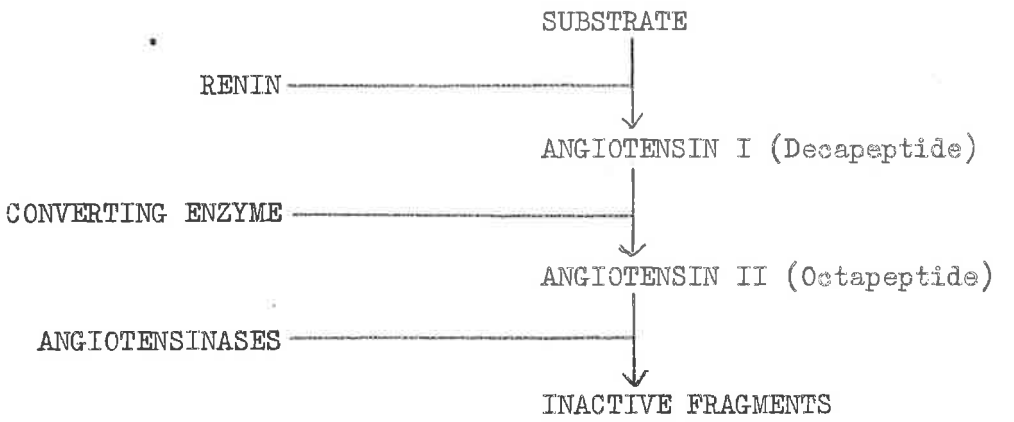
The existence of two forms of angiotensin was demonstrated by Skeggs et al (1954). They showed that the first form, angiotensin I, was formed by the enzymatic action of renin on a plasma substrate and that this was rapidly converted to angiotensin II by the action of a second enzyme present in plasma.

The biochemical definition of the renin-angiotensin system was taken further by Lentz et al (1956). They showed that angiotensin I contained one mole of leucine and one mole of histidine in addition to

the amino acids of angiotensin II, and that conversion of angiotensin I by converting enzyme involved hydrolysis of the phenylalanine-histidine bond to form angiotensin II and histidyl leucine.

The amino acid sequence for horse angiotensin II was described in 1956 by Skeggs et al, and is as follows: asp-arg-val-tyr-iso-his-pro-phe. The amino acid sequence of angiotensin formed by the action of rabbit renin on ox substrate was reported at about the same time (Peart, 1956; Elliott and Peart, 1957); the amino acid sequence was shown to be: asp-arg-val-tyr-val-his-pro-phe-his-leu. It was thought that all the amino acids had the L-configuration and that the N-terminal residue was aspartic acid, not asparagine. The synthesis of angiotensin was achieved shortly afterwards by Bumpus et al (1957) and by Schwyzer et al (1958).

Due to the above work, the concept of the renin-angiotensin system had developed as follows:



(ii) ESTIMATION OF RENIN CONCENTRATION OR ACTIVITY IN BLOOD AND PLASMA

Direct chemical estimation of renin in plasma is not presently feasible and awaits the development and characterization of a pure renin preparation. The currently available methods depend on measurement of the angiotensin generated during the incubation of plasma samples in vitro. The generated angiotensin is measured by bioassay, which is based on either the pressor action or the action in causing contraction of smooth muscle. Most methods do not give a precise measurement of the amount of enzyme present in plasma since there are many factors which affect the rate of enzyme-substrate reaction and thus the amount of angiotensin generated by plasma when incubated in vitro. The lack of a universal renin standard preparation has meant that absolute values for renin activity or concentration obtained by groups using different methods cannot be compared. However, it is still possible to compare patterns of change in response to various stimuli.

The assay techniques differ in that some measure the amount of angiotensin generated after a specific duration of incubation, whereas others employ enzyme kinetic techniques and use either the initial or maximal velocity of angiotensin formation as a measure of the renin content of the plasma samples.

The end product of the in vitro incubation of plasma samples is mainly angiotensin I, owing to inhibition of converting enzyme by substances added to prevent destruction of the generated angiotensin by angiotensinases (Boyd *et al.*, 1967). Renin "concentration" or "activity" estimations using these indirect methods are useful in clinical practice as long as the results are interpreted

Renin Concentration

These assays set out to measure the actual concentration of renin present in plasma samples. After a preliminary purification or partial separation of renin, the plasma is treated to destroy endogenous substrate and to inhibit angiotensinases and the renin is then allowed to react with a standard exogenous substrate preparation so that, as far as possible, the renin concentration is the only variable factor in the reaction mixture (Lever and Robertson, 1964; Cook and Lee, 1965; Brown et al., 1964).

Renin Activity

These methods do not attempt to quantitate renin as such but rather measure the activity of the system (renin and its substrate) and its ability to produce angiotensin. Apart from inhibiting angiotensinases to allow accumulation of angiotensin, and purification of angiotensin in one method (Boucher et al., 1964), no attempt is made to modify the activity of the system. Indeed, this would defeat the purpose of "activity" assays, which should correlate more closely with actual angiotensin (and aldosterone) levels than renin "concentration" methods which measure only one component of the system. Renin "activity" assays have been described by Helmer and Judson (1963), Pickens et al (1965), Boucher et al (1964), Boucher and Genest (1966) and Skinner (1967).

(iii) SUBSTRATE MEASUREMENT

Available methods for substrate measurement rely on the addition of excess renin and assay of the maximum amount of angiotensin generated. For accurate measurement, the added renin should be free

of substrate and angiotensinases and the substrate should not contain renin, angiotensin or angiotensinases. These conditions are not satisfied in any of the available methods, although a method which claims to produce substrate free of renin and angiotensinase has been described (Cook and Lee, 1965) and might resolve this problem.

(iv) ESTIMATION OF ANGIOTENSINASE ACTIVITY

Angiotensinase activity has been universally assessed by examination of the survival of angiotensin II added to plasma. The problems of interference with angiotensinases by the anti-coagulants used and the generation of further angiotensin by the action of renin need to be considered when using this method.

Levels of angiotensinase activity have been reported in a number of hypertensive and other conditions (Hickler et al, 1963). Elevated levels were reported in several hypertensive states, usually those thought to be associated with impairment of renal blood flow, in pregnancy, and in refractory oedema.

(v) BLOOD ANGIOTENSIN ESTIMATION

Scornik and Paladini (1961) described a method whereby angiotensin is extracted from arterial blood collected into 96% ethanol by partition separation with an organic solvent followed by chromatography on an ion exchange resin. The extracted angiotensin was assayed in the nephrectomized, anaesthetized rat by the blood pressure response produced by the samples. Recoveries of approximately 60% of added angiotensin were consistently obtained. The main difficulty with this and other similar methods is that,

because of the low levels of angiotensin in plasma, removal of relatively large volumes of blood is required, with resultant stimulation of the renin-angiotensin system.

A modification of the method of Scornik and Paladini was described by Boucher et al (1964). This method was quicker and simpler, while retaining the same degree of specificity and sensitivity. A mean recovery from blood of 83% of added angiotensin was demonstrated by this method. Arterial blood angiotensin levels were found to be higher than venous levels, which were too low to measure in all but one of the normal subjects studied.

The method of Morris and Robinson (1964) offered a high recovery (mean 85%), the requirement of only 50 ml of arterial blood, and a relatively simple procedure, allowing multiple samples to be assayed in one day. The minimum amount of angiotensin that could be accurately determined in a 50 ml blood sample was 5 ng, corresponding with a concentration of 100 pg/ml of blood. Detectable levels of angiotensin were found in the peripheral arterial blood of patients diagnosed as having either hypertension secondary to renal ischaemia or coarctation of the aorta.

A different approach to the problem of measurement of angiotensin was that of Regoli and Vane (1964, 1966) who measured the circulating angiotensin levels of dogs by allowing blood to drip over various smooth muscle preparations with various sensitivities to different pharmacological agents, the angiotensin concentration being assessed by contraction or relaxation of the rat colon preparation.

This system was sensitive to concentrations of angiotensin II of 0.1-1.0 ng/ml when bathed in Krebs' solution, with somewhat reduced sensitivity when bathed in blood. The advantage of this method is that it permits virtually instantaneous measurement of acute changes in the activity of the renin-angiotensin system, assessing renin activity as well as possible changes in the metabolic clearance of angiotensin. It is, however, less satisfactory under conditions of relatively sustained stimulation of renin activity, when only the initial changes may be measured accurately, and it is very cumbersome.

(vi) SITE OF PRODUCTION OF RENIN

(a) Location in the Kidney

Goormaghtigh (1939) suggested that the histological appearance of the juxtaglomerular cells was indicative of a possible endocrine function. He demonstrated that renal artery constriction in the rabbit led to an increase in the number and size of the granulated cells in the juxtaglomerular apparatus and that an identical picture was seen in the ischaemic kidney of the dog. This led him to postulate that the endocrine activity of the juxtaglomerular cells was related to the production of the hypertensive substance (i.e. renin) present in the ischaemic kidney.

Tobian et al (1959) demonstrated a striking correlation between the amount of juxtaglomerular granulation and the amount of extractable renin in the kidney of the rat under conditions of renovascular and mineralocorticoid induced hypertension.

Pitcock et al (1959) showed the same correlation between juxtaglomerular granulation and renin content in kidneys of normal and sodium-deficient rats, suggesting that the juxtaglomerular cells secrete the pressor substance renin, and that the granules represent either renin or a precursor of renin.

The microdissection studies of Bing and Kozinierczak (1962) demonstrated that the great majority of the renin in the kidney was found in the macula densa of the distal tubule or within the wall of the afferent arteriole. This finding was in agreement with the work of Cook and Pickering (1958), who used a suspension of iron oxide and a magnetic method to separate the various fragments of a "mashed" kidney and found that most of the renin was located in, or very near to, the glomerulus.

The direct fluorescent antibody technique was successfully applied to the problem of localization of the histologic site of renin by Edelman and Hartroft (1961), who found specific staining in the juxtaglomerular cells but not in the glomerular elements or in other structures of the renal cortex.

(b) "Renin" in Extrarenal Tissues

Werle et al (1957) showed that the submaxillary glands of some mice contain high amounts of a renin-like enzyme. Bing and Faarup (1965) demonstrated that there was a correlation between the renin content and the proportion of granulated ducts, that both were higher in males than in females, and that both changed in the same way after castration and under the influence

of various hormones (Trautschold et al., 1966). The content of "renin" in submaxillary glands appeared to be independent of renin content of the kidney and was not altered by sodium and mineralocorticoid administration.

Several groups have shown that the female reproductive organs of some mammals, including humans, contain renin, the concentration of which has been found to be especially high in the rabbit uterus in the last third of pregnancy (Stakemann, 1960; Gross et al., 1964; Bing and Faarup, 1966; Ferris and Mulrow, 1965).

(vii) REGULATION OF ACTIVITY OF THE RENIN-ANGIOTENSIN SYSTEM

(a) Sodium Balance

Luetscher and Axelrad (1954) showed that a low sodium diet in two normal subjects produced a sharp rise in urinary aldosterone excretion and a fall in urinary sodium. These changes were reversed on the resumption of a normal sodium intake. Urinary 17-ketosteroids or 17-hydroxycorticosteroids were not affected and the authors concluded that the effect on aldosterone was independent of pituitary corticotrophin. The finding of increased aldosterone production following sodium restriction in normal man was confirmed by Luetscher and Curtis (1955), Hernando et al (1957), and Bartter et al (1959). A similar response was demonstrated in the sheep by Denton et al (1959) and Blair-West et al (1962).

The role of the renin-angiotensin system in the regulation of adrenal function was clarified by the finding that the inverse

relationship between sodium balance and the secretion of aldosterone was also reflected in the content of renin in the kidney (Gross, 1958; 1959).

Acute sodium depletion or strong diuresis with accompanying reduction in intravascular volume was shown to lead to a prompt increase in plasma renin activity and aldosterone secretion. These changes were diminished after replacement of blood volume by repeated blood transfusions (Gross et al, 1965; Bailie et al, 1966; Brown et al, 1966; Davis et al, 1966; Vander and Luciano, 1967a; Ménard et al, 1967). The replacement of the fluid lost during mercurial-induced diuresis restored the enhanced plasma renin activity to normal (Vander and Luciano, 1967a), whereas rapid infusion of a hypotonic saline solution into the aorta above the renal arteries stimulated renin release (Bailie et al, 1966).

Prolonged dietary sodium restriction was observed to cause an elevation of both plasma renin activity and renal renin and to be accompanied by an increased aldosterone secretion (Davis et al, 1961(b); Blair-West et al, 1963; Brown et al, 1963; Gross et al, 1965; Marieb and Mulrow, 1965), whereas sodium loading had the opposite effect. In response to a high sodium diet given together with sodium-retaining steroids, plasma renin activity fell to undetectable levels and renin disappeared from the kidneys (Gross et al, 1965). Several groups of workers demonstrated that aldosterone secretion was stimulated by

exogenous angiotensin II infusion (Laragh et al, 1960; Genest et al, 1961; Mulrow et al, 1961; Blair-West et al, 1962), and it was shown that the increased aldosterone output in response to various stimuli was markedly reduced after total nephrectomy (Davis et al, 1961; Mulrow and Ganong, 1961(b)). A similar impairment of the response of plasma renin activity to haemorrhage after bilateral nephrectomy was described by McKenzie et al, (1966). It was suggested by these results that the renin-angiotensin system, through the action of angiotensin II, had a specific stimulatory effect on the secretion of aldosterone by the adrenal cortex. Several other factors, namely ACTH, plasma potassium concentration, and plasma sodium concentration, were found to have an effect on the secretion of aldosterone independent of the renin-angiotensin system (Luetscher and Curtis, 1955; Liddle et al, 1956; Laragh and Stoerk, 1957; Moran et al, 1958; Bartter et al, 1959; Denton et al, 1959; Blair-West et al, 1962; Kaplan, 1965).

Recently, evidence has been produced to suggest that there may be at least one further factor (so far unidentified) which has a significant effect on the secretion of aldosterone (Williams et al, 1970; Blair-West et al, 1971).

(b) Fluid Balance

A rapid, significant fall in blood pressure produced by haemorrhage or by drugs was demonstrated to be followed by an increase in plasma renin activity (Davis et al, 1961; Mulrow et al, 1961; 1962; Gross, 1965; McKenzie et al, 1966).

It was not certain whether it was the reduction of intravascular volume or the fall in blood pressure which stimulated the secretion of renin. Brown et al (1966), showed inconsistent effects on plasma renin levels in man following the withdrawal of 400-500 ml of blood, but elevated plasma renin activity and aldosterone secretion rate were demonstrated by Skillman et al (1966) after reduction of blood volume by 15%.

Lowered renal artery perfusion pressure has been shown to stimulate renin release (Skinner et al, 1963). If the artery of one kidney is clamped, the renin content in the clamped kidney increases, whereas it is markedly reduced in the unclamped kidney (Regoli et al, 1962; Gross et al, 1965). These results suggest that a receptor mechanism sensitive to stretch or pressure in the afferent arteriole might account for the acute release of renin associated with lowered renal artery pressure. This situation, however, affects urinary flow and composition, so that it is also possible to postulate a mechanism sensitive to urinary flow and/or composition. Experimental evidence has been obtained suggesting a receptor in the region of the macula densa sensitive to sodium concentration in the tubular fluid (Tobian, 1960; Thurau et al, 1964; 1967). This receptor may assume importance in the release of renin secondary to sodium depletion and other factors changing urinary composition such as diuretics.

Vander and Miller (1964) demonstrated that it was possible to overcome the stimulant effect of lowered renal artery perfusion pressure on renin release by injection of hypertonic saline or diuretics into the renal artery, thus suggesting that the composition of the tubular fluid might be of relatively greater importance than perfusion pressure under these circumstances. Further evidence to support this view was obtained by Bailie et al, (1966), who produced evidence in dogs that renal sodium metabolism plays an important role in the acute regulation of renin secretion independent of renal haemodynamics. However, Newsome and Bartter (1968) showed that the relationship of plasma renin activity to serum sodium concentration could be reversed during overhydration and, therefore, some factor related to body fluid balance, e.g. intravascular volume or renal artery perfusion pressure might be more important than serum sodium concentration in determining plasma renin activity under these conditions, a fact confirmed by the work of Gordon and Pawsey (1971).

(c) Sympathetic Nervous System

A diurnal rhythm of plasma renin activity has been described in man (Gordon et al, 1966(b)), the highest values occurring in the early morning hours and the lowest in the afternoon. Changes from the recumbent to the upright position, whether active or passive, lead to prompt elevation of plasma renin activity (Cohen et al, 1966; Gordon et al, 1966(a); Brown et al, 1966), despite the fact that blood pressure does not fall. Autonomic reflexes, e.g. carotid compression or cold pressor test, in man

have been shown to stimulate renin release and aldosterone secretion or excretion (Gordon et al, 1966(b); 1967); the same effect was seen after the administration of a noradrenaline/adrenaline mixture (Gordon et al, 1966(a); 1967). Patients with severe autonomic insufficiency were found to frequently have a reduction in aldosterone secretion not responsive to indirect stimulation by salt depletion or direct stimulation with corticotrophin or angiotensin II (Slaton and Biglieri, 1967). In one such patient, when the orthostatic reaction was prevented by the infusion of catecholamines, plasma renin activity and urinary aldosterone were elevated (Gordon et al, 1967). Other patients with autonomic insufficiency have been described with apparently completely normal renin-angiotensin-aldosterone and salt-retaining mechanisms (Bliddal and Nielsen, 1970).

Plasma renin activity was found to increase during exercise, an effect which could be prevented with ganglion-blocking drugs (Castenfors et al, 1967). Hodge et al (1966) showed that blocking of the renal nerves may inhibit the release of renin in response to haemorrhage in dogs. Taquini et al (1964) reported decreased renin content in the denervated kidney of the rat compared with the opposite intact kidney. Vander (1965) demonstrated the release of renin from the kidney in dogs in response to electrical stimulation of the renal nerves.

The foregoing evidence suggests that an intact sympathetic innervation is important for the release of renin. However, evidence has been presented (Vander and Luciano, 1967(b)) that the

sympathetic nervous system plays a modifying role but is not essential for the increased renin release produced by natriuretic-induced, acute sodium depletion. It has also been shown (Greene and Vander, 1967) that normal changes in renin secretion in response to sodium deprivation and to assumption of the upright posture occurred in a group of patients with renal homotransplants. A role for the sympathetic nervous system in this situation cannot be completely excluded, however, since increased sensitivity to circulating catecholamines or regrowth of the renal nerves could allow such an action to occur.

(d) Hypoxia

The effect of renal artery constriction on production of renin by the kidney has been shown to be unrelated to hypoxia in the acute situation (Huidobro and Braun-Menendez, 1942; Divry, 1952; Skinner *et al*, 1964). A possible role for chronic hypoxia, however, has been suggested by the demonstration that prolonged hypoxia causes increased juxtaglomerular granulation in rats (Oliver and Brody, 1965) and that plasma and renal renin were increased in rats kept in an environment with reduced oxygen tension (Gould and Goodman, 1970).

(e) Oestrogens

Plasma renin levels have been shown to rise early in pregnancy, returning to normal after delivery (Brown *et al*, 1963). The increase in plasma renin concentration during pregnancy is not as marked as the increase in plasma renin activity; the greater increase in the latter parameter has been shown to be due largely

to the increased levels of renin substrate (Helmer and Judson, 1967). A slight variation in plasma renin during the normal menstrual cycle has been reported (Brown et al, 1964). A significant increase in plasma renin activity values and aldosterone excretion was seen following administration of ethinyl oestradiol to normal subjects, but no apparent change was detectable after the administration of medroxyprogesterone acetate. A substantial percentage of normal subjects given oral contraceptives showed increased plasma renin activity and aldosterone excretion rates during the first and third weeks of administration (Crane and Harris, 1969). These studies confirm the effect of oestrogens on the renin-angiotensin system, acting principally through increased production of renin substrate.

(f) Potassium

In man, high doses of potassium given during the administration of a low-sodium diet, diminished the high plasma renin activity (Veyrat et al, 1966). Simultaneously, the aldosterone secretion rate rose, signifying the direct effect of a high plasma potassium concentration on the adrenal cortex, which is especially pronounced during sodium depletion (Cannon et al, 1966). If, together with the feeding of a sodium-restricted diet, potassium was removed by giving an ion-exchange resin, plasma renin activity increased more markedly than aldosterone secretion. The addition of potassium under these conditions stimulated aldosterone secretion, whereas it lowered renin activity in the plasma (Veyrat et al, 1966). In patients with

renal artery stenosis or malignant hypertension, in whom the plasma renin level was high, potassium administration also led to a marked increase in the aldosterone secretion rate (Cannon et al, 1966).

These observations demonstrate that, under certain experimental conditions, either an additive or an antagonistic effect between plasma renin activity and the plasma potassium concentration on the secretion of aldosterone can be achieved. The effect of the plasma potassium concentration on the aldosterone-producing cells of the adrenal cortex is, therefore, independent of renin release or the plasma renin level.

(g) Feedback Control of Renin Secretion

There is no evidence of a direct feedback mechanism between plasma aldosterone levels or its secretion rate and plasma renin activity. On the other hand, high doses of aldosterone or other sodium-retaining corticoids, as well as sodium administration, reduce renin concentration in the kidneys and plasma renin activity, provided they are given for a sufficiently long period; this suppression of renin production is thought to operate through the increased plasma volume consequent upon these manoeuvres.

Angiotensin has been demonstrated to exert a negative feedback effect on renin release from the kidney which is independent of changes in renal arterial blood pressure or aldosterone secretion (Genest et al, 1965; Vander and Geelhoed, 1965). The specific mechanism by which this effect is exerted and the relative importance of this means of control of renin secretion are at present unknown.

(h) Integration of Control Mechanisms

Considering all the available evidence, it would seem that there are multiple factors affecting the production of renin by the kidney, and that these include at least the renal artery perfusion pressure, tubular sodium concentration, blood volume, plasma potassium concentration, plasma angiotensin concentration, and activity of the sympathetic nervous system. The relative importance of each of these factors probably varies according to the physiological state of the subject at that particular time, with particular reference to state of hydration, sodium balance, blood pressure, and so on.

(2) RADIOIMMUNOASSAY TECHNIQUE

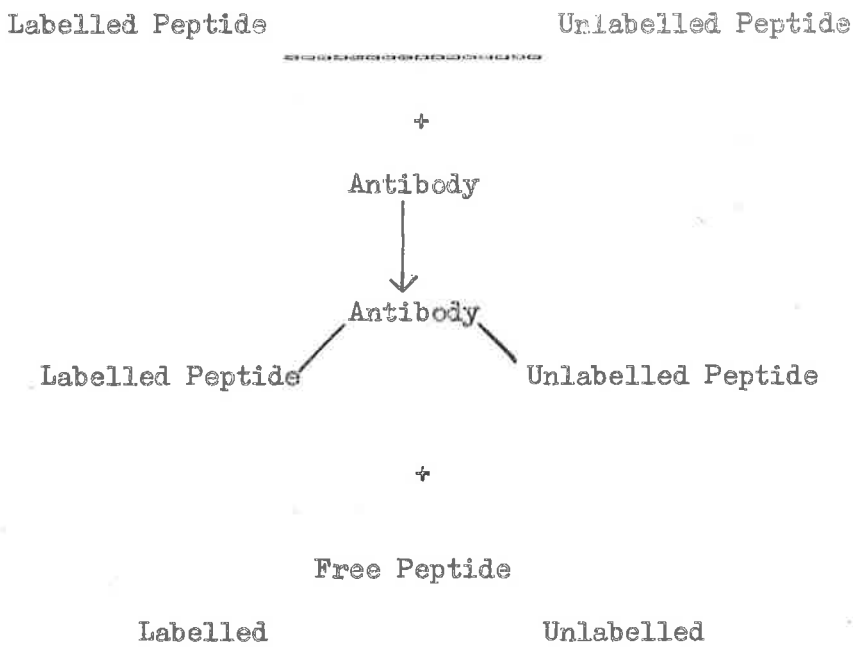
(i) GENERAL

A real understanding of the physiological roles of hormones was hampered for many years by an inability to measure accurately their levels in biological fluids. Bioassays or the indirect method of examination of their effects on target organs were used as a measure of their activity. Most of the available assays were relatively crude bioassays, lacking sensitivity and specificity and often being unable to detect normal concentrations of the hormones concerned. The availability of protein hormones in a purified form and the recognition of their species specificity have led in recent years to the production of specific antisera which could be used to measure the hormones by immunological methods. However, the concentrations of protein hormones in biological fluids are so low that the only two immunological methods which are readily capable of detecting them are the red blood cell haemagglutination inhibition method and the radioimmunoassay procedure.

The red blood cell haemagglutination inhibition method makes use of agglutination of sensitized erythrocytes as the manifestation of an antigen-antibody reaction. It was shown by Boyden (1951) that tannic acid-treated erythrocytes to

which specific proteins are adsorbed are agglutinated by the specific anti-protein antisera and that antigen in small amounts can be detected by the inhibition of the haemagglutination reaction. This effect is due to antigen combining with antibody during pre-incubation so that few free antibody molecules remain available for reaction with the subsequently added sensitized red blood cells. The red blood cell haemagglutination inhibition method has been applied to the measurement of human growth hormone (Read and Stone, 1958; Read and Bryan, 1960) and adrenocorticotrophic hormone (McGarry et al., 1962) in plasma and of human chorionic gonadotrophin (Wide and Gemzell, 1960; 1962) and human luteinizing hormone in urine (Wide and Gemzell, 1962; Wide et al., 1961). It does not, however, provide the sensitivity and precision of the radioimmunoassay technique which has now almost completely replaced it.

The radioimmunoassay method is based on the observations, first reported with insulin-^{131}I (Berson et al., 1956), that, in the absence of precipitation of antigen-antibody complexes, antibodies in low concentration are detectable by their ability to bind ^{131}I-labelled hormone, and furthermore that such binding is competitively inhibited by unlabelled hormone. This competitive binding may be represented as follows:



If a system is set up such that, in the absence of unlabelled hormone, less than 100% of the labelled hormone present is bound to antibody, i.e. antibody binding-sites are saturated, and increasing amounts of unlabelled hormone are added to this system, there will be progressive displacement of labelled hormone from the antibody binding-sites. If a method is available for separation of antibody-bound from free hormone, this displacement can be measured and the results expressed graphically in the form of a standard curve. If unknown samples are incubated under the same conditions with the same amounts of antiserum and labelled hormone but with the unknown sample in place of the standard, the degree of displacement of labelled hormone from the antibody can

be measured and, using this figure, the amount of hormone in the sample can be determined by comparison with the standard curve.

The radioimmunoassay technique was originally described for insulin (Berson and Yalow, 1958; Yalow and Berson, 1959) and has subsequently been applied to a wide variety of protein hormones, e.g. glucagon (Unger et al., 1959; Grodsky et al., 1961), growth hormone (Utiger et al., 1962; Glick et al., 1963; Hunter and Greenwood, 1964), parathyroid hormone (Berson et al., 1963; Sherwood et al., 1966), thyroid stimulating hormone (Utiger et al., 1963; Odell et al., 1963; Utiger, 1965), adrenocorticotrophic hormone (Felber, 1963; Yalow et al., 1964), and many others.

The principal advantages of radioimmunoassay over bioassay procedures are high sensitivity and specificity. Against this it must be realized that the immunologic reactivity may not correlate closely with the biologic activity, as the specific amino acid configurations for each of these properties may reside at different sites on the hormone molecule. If the molecule remains intact it would be expected that the two methods would give identical results, but separation of the two activities could occur, e.g. due to breakdown of the hormone molecule so that biologic activity is lost but immunologic activity is retained, or due to the presence of either biologic or immunologic inhibitors. Under these conditions the results obtained by radioimmunoassay and by bioassay might not be in

close agreement. For this reason, the generally accepted convention is to use the prefix "immunoreactive" before the name of the particular hormone when reporting figures for concentrations when the radioimmunoassay method has been used to derive them; this serves as a reminder that the values obtained by the two different methods are not necessarily interchangeable.

Requirements for Radioimmunoassay

The requirements for a successful radioimmunoassay procedure are as follows:

(a) Pure peptide for use as antigen.

(b) Specific antibody of high titre and affinity.

(c) A method of labelling the peptide to a sufficiently high specific activity without producing significant "damage" to the molecule.

(d) A method of separation of antibody-bound from free peptide.

(a) Purity of Antigen

All antisera are heterogeneous and the hormone antibody produced experimentally usually represents only a small fraction of the total antibody present. Although heterogeneous antibodies are induced when a single protein antigen is used, this does not interfere with the immunoassay. However, contamination of the hormone preparation used for immunization with a protein of potent antigenicity may lead to production of an antiserum containing antibodies

to the contaminant in concentrations much higher than the concentration of hormone antibodies. If the hormone preparation used as a standard in the radioimmunoassay is free of impurities, antibodies to contaminants in the immunizing antigen do not present a problem. A small residual contaminant in the hormone standard may interfere with the assay, but is less troublesome in the radioimmunoassay than in the red blood cell haemagglutination-inhibition method.

(b) Production of Antibodies

The production of antibodies to the larger protein hormones has not presented a major problem and many are sufficiently antigenic to enable the production of antibodies without the use of adjuvants. The smaller peptide hormones, however, are less antigenic and it has usually been found necessary to couple angiotensin to a carrier protein (Deodhar, 1960; Haber *et al.*, 1965) or to adsorb it onto charcoal (Boyd *et al.*, 1967) in order to raise antibodies of sufficient titre and affinity for a radioimmunoassay to be developed.

The sensitivity of the radioimmunoassay technique has been demonstrated to be ultimately dependent on the energy of the antigen-antibody reaction (Berson and Yalow, 1964). Even individual antisera contain heterogeneous antibodies reacting with variable energies, and the potential sensitivity of the assay will depend on the energy of reaction of those antibodies present in high enough concentration to be detectable at the dilution of antiserum employed. This information

can be derived from the slope of the standard curve or the slope of the curve obtained when labelled peptide is incubated with doubling dilutions of antiserum. The titre of the antiserum is not important in the selection of the serum to be used in the radioimmunoassay and the only benefit of a high titre antiserum is that it allows a large number of samples to be assayed with a given amount.

The sensitivity of the assay is determined by the fractional change in bound:free ratio or percentage bound which occurs with the addition of a small amount of standard hormone and this is largest when the initial percentage bound is 50% (Berson and Yalow, 1964). Thus, for high sensitivity it is preferable to use a dilution of antiserum that will bind approximately 50% of the smallest adequate quantity of labelled hormone in the absence of unlabelled hormone.

(c) Labelling of Hormone

Radioactive hormones for use in the radioimmunoassay technique have usually been prepared by iodination of tyrosine residues in the polypeptide with ^{131}I or ^{125}I. The advantage of ^{131}I is that it is relatively easy to obtain the high specific activity usually necessary for sensitivity (to minimize the amount of labelled hormone used). The disadvantage is that, owing to the short half-life of the isotope, it is necessary to prepare freshly labelled hormone at frequent intervals. In the case of ^{125}I-labelled hormone, a single preparation may be used for weeks, the only limitation being the stability of the stored tracer. However, the maximum specific activity that can be achieved is inversely related to the half-life of the isotope and to

achieve equivalent specific activity requires labelling with more atoms of ^{125}I than ^{131}I per molecule of polypeptide. With molecules containing limited amounts of tyrosine, this may not be easily achieved and, even when possible, a high ratio of iodine atoms per molecule could theoretically alter the affinity of the antigen for antibody. This potential disadvantage of ^{125}I as a label is partially offset by the greater efficiency of counting of this isotope compared with ^{131}I.

The iodination is almost universally carried out by the chloramine T method of Greenwood et al (1963) which allows high specific activities to be attained while using relatively small amounts of isotope.

(d) Separation of Bound and Free Hormone

The original method used by Yalow and Berson (1959) for separation of bound and free peptide was chromatoelectrophoresis, and this remains a very satisfactory method when the peptide concerned is adsorbed to paper. The disadvantages of this method are that only small volumes of the reaction mixture can be applied to the paper, with resultant loss of sensitivity, that it is rather tedious and that it is not readily applicable to large numbers of samples. A wide variety of other methods have been used for separation of bound and free hormone, including the following: Coated charcoal (Herbert et al., 1965), talc (Rosselin et al., 1966), Sephadex-coupled antibodies (Wide and Porath, 1966), solid-phase method (Catt et al., 1966), and double-antibody method (Skom and Talmage, 1958). In the choice of method

used, it is usual to select the technique found to provide satisfactory sensitivity and precision for the particular hormone while retaining simplicity and ease of operation.

(ii) ANGIOTENSIN RADIOIMMUNOASSAY

Deodhar (1960) was able to raise antibodies to angiotensin II by coupling the octapeptide to bovine γ-globulin to form an antigen. This antigen was mixed with Freund's adjuvant and injected intraperitoneally in rabbits. The γ-globulin was isolated when the animals were subsequently bled. The presence of antibodies was demonstrated by a precipitin reaction between the rabbit γ-globulin and the angiotensin-bovine gamma globulin complex, by partial inhibition of this precipitin reaction by angiotensin II, and by a precipitin reaction between rabbit gamma globulin from the immunized rabbit and an angiotensin II-protein complex containing a protein other than bovine gamma globulin. The antibody was found to have greater activity in neutralizing the free acid form of angiotensin II than the amide form and reacted to the same extent with synthetic val^5- and natural $isoleu^5$-angiotensin I and this was felt to be in agreement with the studies of Landsteiner (1932) who demonstrated that the amino acid with the free carboxyl group at the carboxyl end of the peptide largely determines the specificity of the antibody. The antibody was used by Deodhar to investigate the effect of an antiogensin inhibitor on the blood pressure of hypertensive animals.

Goodfriend et al (1964) produced antibodies to bradykinin and angiotensin in rabbits by the use of conjugates containing albumin

and the hapten covalently bound. The conjugate was suspended in Freund's complete adjuvant and injected into the toe-pads and leg muscles of rabbits, followed later by intraperitoneal injection of the conjugate in adjuvant. Antibodies were demonstrated by complement-fixation and inhibition techniques. The specificity of the antigen-antibody reaction was indicated by lack of complement fixation with heterologous hapten-albumin conjugates and lack of inhibition by heterologous haptens. The authors considered that the method of conjugating hapten with protein using the carbodiimide reaction had advantages over other conjugating procedures. Thus the reaction occurred under very mild conditions and the method was capable of conjugating a wide range of compounds, and of conjugating haptens directly to proteins without interposing additional groups between the hapten and the carrier. The antibodies produced by this method were used by the authors to study the immunochemistry and biological roles of small polypeptides.

Goodfriend et al (1966) reported immunochemical studies of angiotensin. The antigenic determinants of angio-tensin were investigated by inhibition of complement fixation by analogues of the polypeptide. Among the structural features of angiotensin tested, those which had greatest importance for serologic activity were: conformation as determined by an optical isomer in the middle of the molecule; the presence of the two amino acids at the N-terminus; and the single phenolic hydroxyl. The structural features shown to be important for serologic activity were those previously shown to be

important for biological activity (Bumpus et al., 1961). The antiserum was shown to partially neutralize the biological effects of the free polypeptide on the blood pressure of the rat and also to neutralize the biological activity of angiotensin on the isolated rat uterus.

Haber et al (1965) produced antigenic branch-chain copolymers of angiotensin and poly-L-lysine, using carbodiimide condensation between the carboxyl-terminal end of angiotensin and the ε-amino groups of poly-L-lysine, and coupling the amino-terminal end of angiotensin to poly-L-lysine via m-xylene diisocyanate. The antigens were suspended in Freund's complete adjuvant and injected into rabbits intramuscularly, intraperitoneally and into toe-pads; additional intravenous injection of antigen was given in some cases. Antibodies were detected by alteration of the elution position of labelled angiotensin from a G25 Sephadex column when mixed with immune serum. It was shown that displacement of labelled angiotensin from the antibody could be achieved by the addition of increments of unlabelled angiotensin prior to gel filtration. It was suggested that poly-L-lysine was preferable to other carriers because it was not intrinsically antigenic and the ε-amino groups provided an opportunity for coupling peptides specifically by either their carboxyl- or their amino-terminal ends.

The method was then extended to an assay system for angiotensin (Haber et al., 1965). Gel filtration on a G25 Sephadex column was used to separate bound from free angiotensin after incubation with antibody. The method was shown to be useful for measuring quantitatively the

reaction between antibody and protein antigen and also between antiserum and the small polypeptide antigen.

Valloton et al (1967) reported a radioimmunoassay procedure for angiotensin II and were able to measure plasma levels for the first time. Antibodies were raised in rabbits by injection of copolymers of angiotensin with poly-L-lysine. Val^5-angiotensin II amide ("Hypertensin"-Ciba) was used as a standard and also as isotopically labelled tracer after iodination with ^{125}I. Separation of bound from free angiotensin after incubation with antibody was achieved by gel filtration as before. Binding activity of the antibody was found to be the same for bovine angiotensin II and human angiotensin II but there was diminished affinity for angiotensin I and compounds obtained by enzyme hydrolysis of "Hypertensin". Angiotensin was extracted from plasma by use of an ion-exchange resin prior to measurement in the radioimmunoassay procedure. Further plasma samples were incubated at 37°C for 3 hours prior to extraction of the angiotensin as a measurement of plasma renin activity. Normal values for plasma angiotensin II were derived and results suggested that normal values were to be found in essential hypertension, raised values in malignant hypertension and hepatic cirrhosis, and low values in primary aldosteronism. Plasma renin activity values tended to parallel the plasma angiotensin II levels. The levels reported in this paper were substantially higher than those obtained by later authors and were subsequently adjusted to lower levels when it was realized that adsorption of angiotensin standards to glassware was giving falsely elevated values.

Catt and Coghlan (1967) produced antibodies to angiotensin II in rabbits by immunization with val^5-angiotensin II amide ("Hypertensin"-Ciba) coupled to porcine gamma globulin by carbodiimide condensation. The antibodies reacted equally well with α-aspartyl angiotensin II, and exhibited only slight cross-reaction with angiotensin I. The antibody was used for a radioimmunoassay procedure, ion exchange chromatography being used for separation of bound from free angiotensin after incubation with the antibody. The authors thought that the antigenic portion of the angiotensin molecule which reacted with this particular antibody was close to the C-terminal end of the octapeptide. Since angiotensinases act by removing amino acids from the N-terminal end of the molecule, biologically inactive metabolites of angiotensin II might retain immunological reactivity. The presence of such fragments in circulating blood could lead to disparity between the results of bioassay and radioimmunoassay of angiotensin II unless adequate isolation procedures are performed.

Boyd et al (1967) raised antibodies to angiotensin II by injection into rabbits of val^5-angiotensin II amide ("Hypertensin"-Ciba) adsorbed onto charcoal and mixed with Freund's complete adjuvant. Some injections were given into popliteal lymph nodes and some into the spleen. The antibody was used to set up a radioimmunoassay technique, using dextran-coated charcoal to separate bound and free peptide (Herbert et al., 1965). Venous blood samples were collected into cooled plastic syringes containing EDTA and dimercaprol to inhibit angiotensinases. The angiotensin was extracted from the plasma by

adsorption onto Fuller's earth and subsequently eluted with ammonia in methanol. The assay was sensitive enough to detect 30pg of angiotensin II and was not influenced significantly by angiotensin I.

Catt et al (1967b) reported plasma levels of angiotensin II in normals and in several pathological conditions using the radioimmunoassay as previously described, but using dextran-coated charcoal to separate bound from free peptide in place of the ion-exchange resin. Arterial blood was collected into cold ethanol containing ^{125}I angiotensin II as an internal indicator. Angiotensin was adsorbed onto the cation exchange resin "SE-Sephadex" and, after washing, was eluted with 0.05M triethylamine and dried in a rotary evaporator. Recovery of labelled angiotensin II was 65-70%. The radioimmunoassay procedure was carried out using ^{131}I angiotensin II tracer and isoleu^{5}-angiotensin II standards. The lower limit of sensitivity was 50pg.

Hollemans (1968) raised antibodies in rabbits by injection of copolymers of val^{5}-angiotensin II amide ("Hypertensin") and poly-L-lysine, coupled by carbodiimide condensation. The radioimmunoassay technique reported had a sensitivity of approximately 2.5ng. The antisera tested showed different affinity for val^{5}-angiotensin II amide and isoleu^{5}-angiotensin II, and it was suggested that the use of "Hypertensin" for standards might lead to error in the assay.

Goodfriend et al (1968) described a radioimmunoassay procedure capable of detecting 5-10pg of angiotensin II. Antibodies were raised in rabbits by injection of angiotensin coupled to rabbit serum albumin

by the carbodiimide process. No immunologic differences between val^5- and isoleu^5-angiotensin II were detected. Different antisera had variable affinity for angiotensin and variable specificity as determined with analogues. It was felt by the authors that the technique used to separate antibody-bound and free labelled hormone largely determined the reproducibility and convenience of the assay. A number of techniques were evaluated and the authors finally chose the antibody-coated tube method, primarily on the basis of increased sensitivity.

Gocke et al (1968) described a radioimmunoassay technique capable of measuring angiotensin II levels directly in 0.1 ml of plasma. Antisera were produced in rabbits by injection of val^5-angiotensin II amide ("Hypertensin") conjugated with rabbit serum albumin by the carbodiimide reaction plus Freund's adjuvant. Venous blood was collected into EDTA to inhibit angiotensinases. Separation of bound and free peptide following incubation with antibody was by means of dextran-coated charcoal. Sensitivity of the method was to less than 5pg. The method was extended to measurement of plasma renin activity by incubation of plasma at 37°C in the presence of DFP; it was necessary to use heparinized plasma or to remove the EDTA by dialysis before good correlation between radioimmunoassay and bioassay was obtained - this was thought to be due to inhibition of converting enzyme by the EDTA. There was little cross-reaction with angiotensin I, about 1:70 by weight.

Boyd and Peart (1969) described the development of a radioimmunoassay procedure for angiotensin I and the application of this technique to measurement of plasma renin activitiy. The authors developed this assay because of the problems associated with the application of the angiotensin II radioimmunoassay to measurement of plasma renin activity, namely inhibition of converting enzyme activity by the EDTA and BAL added to inhibit angiotensinases and the difficulty in restoring this activity without also reactivating angiotensinases. Antibodies to angiotensin I were raised in rabbits by repeated injection of angiotensin I adsorbed onto charcoal and emulsified with Freund's complete adjuvant. The lower limit of detection of the assay was 80pg and there was no cross-reaction with val^5-angiotensin II amide. Blood samples were collected into EDTA and BAL. The plasma samples were extracted with Fuller's earth at zero time and after incubation at 37°C for 4 hours. Recoveries for extraction were approximately 80%. The results obtained for plasma renin activity by radioimmunoassay were comparable with those obtained by bioassay, although the radioimmunoassay values tended to be somewhat lower.

Page et al (1969) reported a revised radioimmunoassay procedure for angiotensin II. The previously reported levels from this group (Valloton et al., 1967) had been falsely high because of adsorption of angiotensin standards on siliconized glassware during the drying step. The effect of various anticoagulants was studied and the recovery of added angiotensin from blood samples was best in

the presence of EDTA (recovery 74-88%). The sensitivity of the method was 10pg per sample. Levels of angiotensin II were detected in all patients except anephric subjects. There was 5% cross-reaction with angiotensin I. The radioimmunoassay technique was applied to measurement of plasma renin activity; generation of angiotensin II was greatest in citrated plasma but virtually none was generated in the presence of EDTA. In the plasma renin activity method, plasma samples were incubated in parallel with aliquots to which standardized human renin had been added. It was assumed that the factors affecting renin activity, conversion of angiotensin I to angiotensin II, and destruction of angiotensin II were identical in the aliquots. Endogenous renin concentration was estimated from a comparison of the maximum velocity of angiotensin II generation between the unknown and the sample with standard renin, assuming that substrate was not limiting. Changes in plasma renin activity and plasma angiotensin II were as expected with change in posture and sodium intake, the changes in plasma renin activity being proportionately larger than the corresponding changes in circulating angiotensin. A very low but detectable endogenous renin activity was demonstrated in two out of three anephric subjects, including one male. The source of this renin activity was obscure.

Catt et al (1969) reported plasma angiotensin II levels in normal subjects and in patients with hypertension of varying aetiologies. Arterial blood angiotensin levels were approximately 20% higher than venous levels. Arterial angiotensin II levels in patients with essential hypertension showed a wide scatter, with no

change in mild cases and a tendency towards higher levels in severe cases. Patients with malignant hypertension showed raised levels before treatment but normal levels after treatment. Most of the high angiotensin levels associated with hypertension were encountered in those patients in whom the hypertension was due to underlying renal disease, either renovascular or bilateral diffuse renal disease. Low values of angiotensin II were found in one patient with primary aldosteronism and small quantities were detected in the plasma of two anephric female subjects.

Gocke et al (1969) reported further experience with their radioimmunoassay technique. It was shown that plasma angiotensin II concentration was directly related to plasma renin activity and inversely related to the state of sodium balance. There was some cross-reaction between the antibody and angiotensin I which had 1/70th of the immunoreactivity of the octapeptide, and the antiserum also cross-reacted with hexapeptide and heptapeptide fragments; experiments, however, showed little interference with the assay.

Sundsfjord (1970) reported a further radioimmunoassay for angiotensin II. Antibodies were raised using "Hypertensin"-Ciba coupled to poly-L-lysine as the antigen. Angiotensin was extracted from plasma by ion exchange chromatography prior to assay. The normal range for healthy adults was found to be in agreement with reports by other groups employing extraction procedures prior to assay (Boyd et al., 1967; Catt et al., 1967; Page et al., 1969). The

advantage of this particular method was that maximum binding of antigen to the antibody was achieved within one hour, thus minimizing the risk of non-specific damage to angiotensin which might occur during more prolonged incubation.

CHAPTER 2

ANGIOTENSIN RADIOIMMUNOASSAY METHOD

1. ANTIBODY PRODUCTION
2. IODINATION
3. SEPARATION OF BOUND FROM FREE PEPTIDE
4. ASSAY PROCEDURE
5. SPECIFICITY OF THE ASSAY
6. SENSITIVITY OF THE ASSAY
7. REPRODUCIBILITY OF THE ASSAY
   (a) Within Assay Variation
   (b) Between Assay Variation
8. ASSAY OF ANGIOTENSIN II IN PLASMA SAMPLES
9. MEASUREMENT OF RENIN ACTIVITY, RENIN CONCENTRATION, AND RENIN SUBSTRATE BY BIOASSAY

CHAPTER 2

ANGIOTENSIN RADIOIMMUNOASSAY METHOD

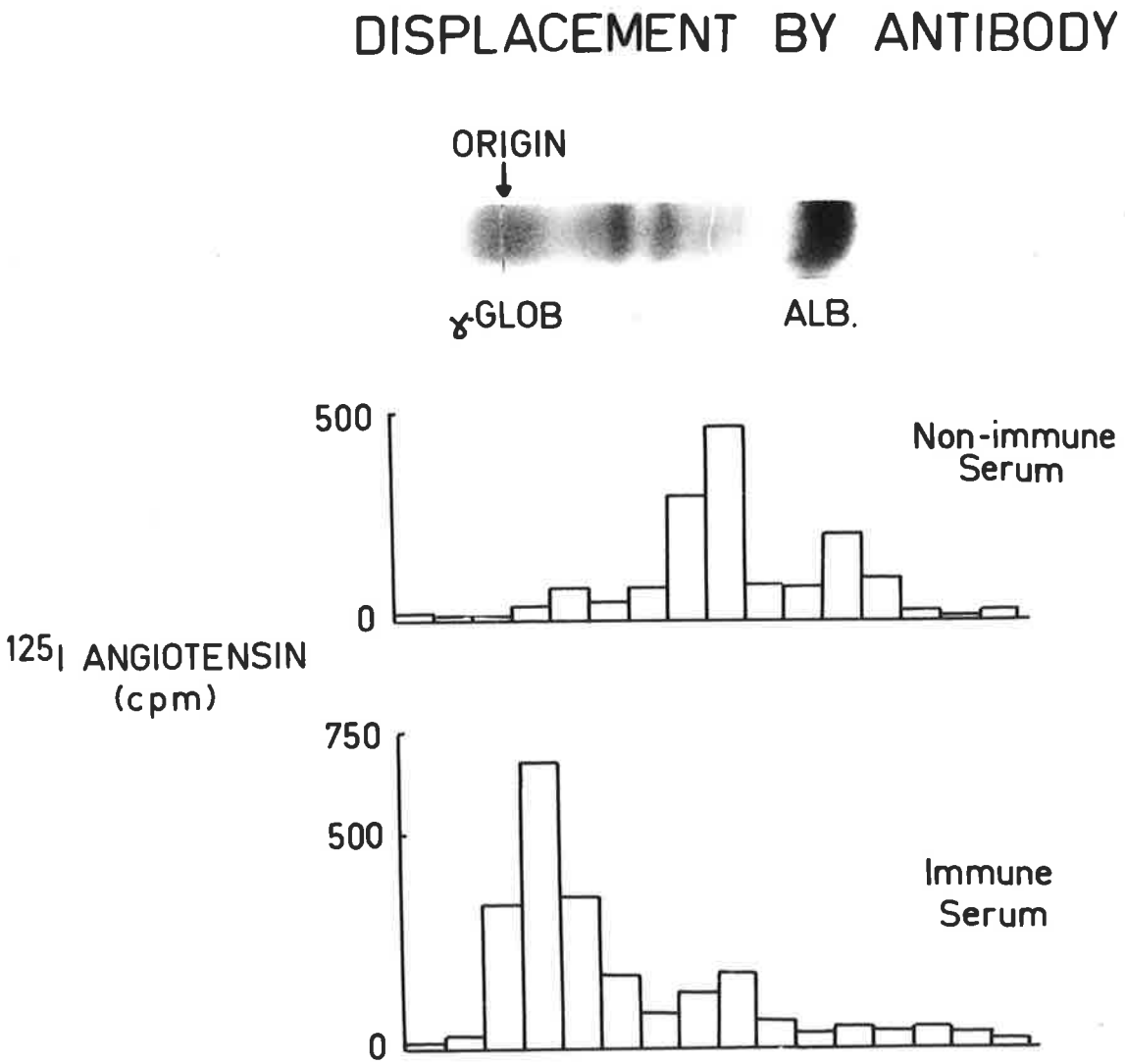
(1) ANTIBODY PRODUCTION

Antibodies were produced in rabbits by injection of val^5-angiotensin II amide ("Hypertensin"-Ciba) adsorbed onto charcoal and emulsified with Freund's complete adjuvant. Six rabbits were used and injections were given intramuscularly or subcutaneously, initially at weekly intervals for four weeks and thereafter at two-weekly intervals. The animals were bled from ear veins each month and the sera were tested for the presence of antibodies by the following methods:

(a) Paper Electrophoresis

In an electrophoretic system using Whatmann 3MM paper and veronal buffer pH 8.6, the majority of the ^{125}I-labelled angiotensin II migrated in the alpha-globulin region, with a further small fraction, presumably non-specifically bound, in the albumin region. When the labelled angiotensin was mixed with control rabbit serum, its electrophoretic mobility did not alter but when it was mixed with serum from successfully immunized rabbits, the majority of the labelled peptide was found in the gamma-globulin region, suggesting that it was bound to antibody (Fig. 1).

FIGURE 1



Alteration of electrophoretic mobility of ^{125}I-Angiotensin II when mixed with immune serum.

(b) Neutralization of the Pressor Action of Angiotensin II in the Rat Bioassay System

When immune serum at a dilution of 1:100 was mixed with standard solutions of angiotensin II at room temperature for two minutes, there was a significant reduction in the pressor response in the rat bioassay system (Fig.2). The reduction in pressor effect was not seen when standard angiotensin II was mixed with control serum, suggesting a specific neutralization by the antiserum rather than a non-specific effect due to angiotensinase activity. This latter effect became evident with both immune and control sera when higher concentrations were used.

(c) Gel Filtration

The elution position of ^{125}I-angiotensin II from a G25 Sephadex column was determined. Mixing of the labelled peptide with normal rabbit serum did not change the elution position but, when mixed with serum from successfully immunized rabbits, the radioactivity appeared in the void volume, suggesting binding to antibody. Additional evidence that the change in elution position was due to antibody binding was obtained by changing the pH of the void volume fraction to pH 2.0, which will break antigen-antibody linkages, and re-applying to the column, in which case the labelled angiotensin was recovered from its original position (Fig.3).

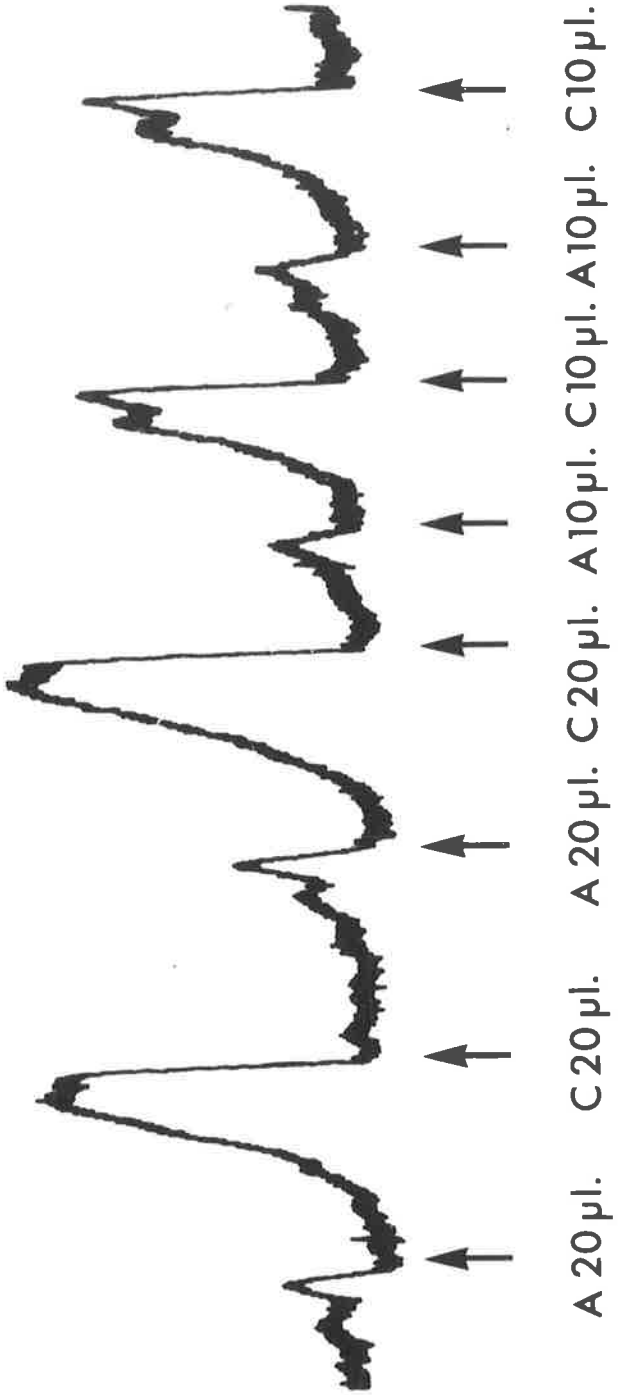
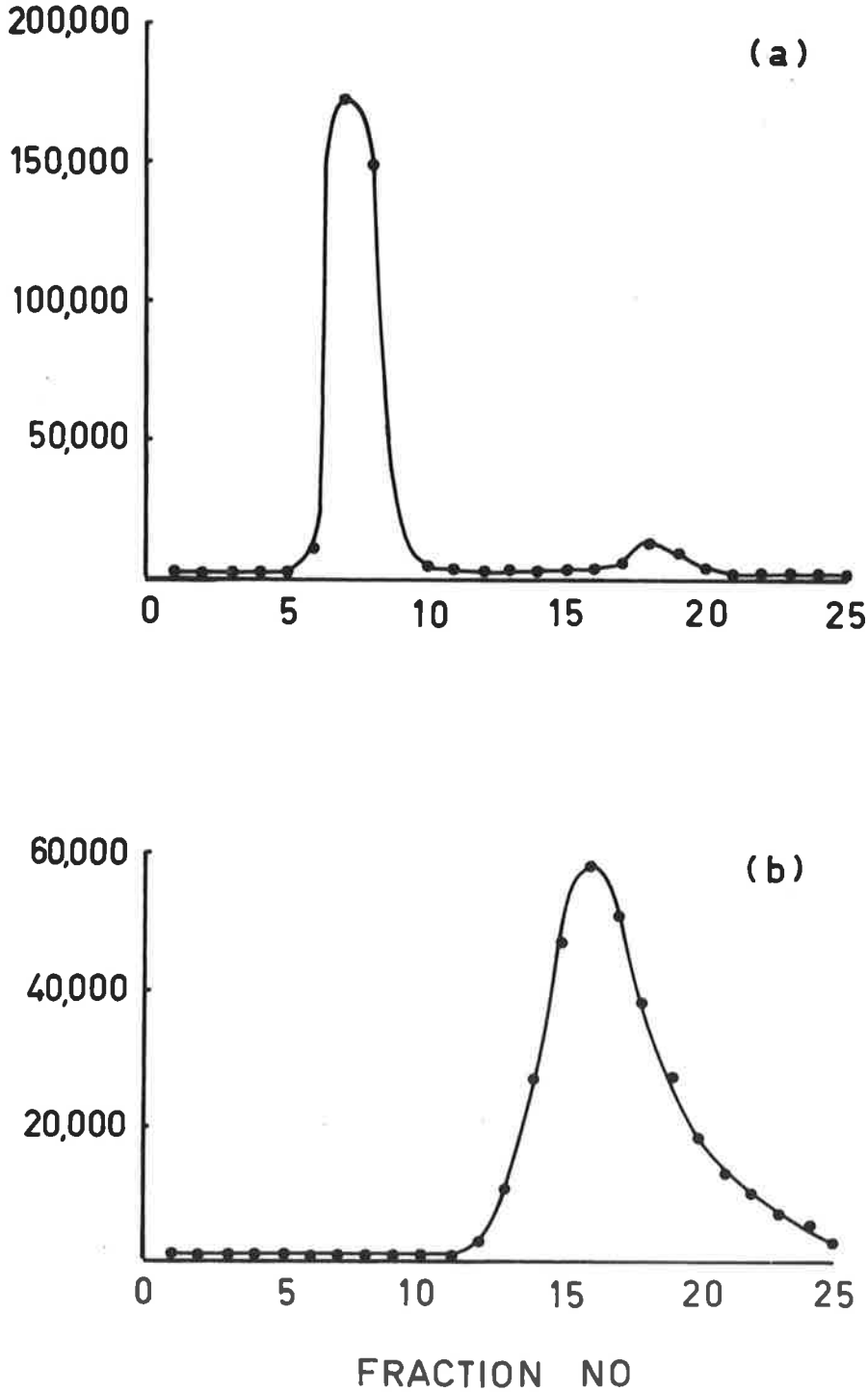


FIGURE 2

Neutralization of pressor action of angiotensin II in rat bioassay system by immune serum.

C = Control serum A = Antiserum

FIGURE 3



Alteration of elution position of ^{125}I-Angiotensin II from a G25 Sephadex column when mixed with antiserum.

(d) Construction of a Standard Curve

Incubation of labelled angiotensin II with increasing dilutions of antiserum resulted in progressively less binding of the peptide. When a suitable dilution of antiserum was chosen, such that approximately 50% of the added ^{125}I-angiotensin II was bound to the antibody, the addition of increasing amounts of unlabelled angiotensin II to the system produced progressive displacement of the labelled peptide from the antibody, enabling a standard curve to be constructed (Fig.4).

Antibodies to angiotensin II were demonstrated in some animals as soon as six weeks after the initial inoculation with antigen, and were detectable in most animals after three months. There was wide variation in the titre of antibodies produced, however, so that in only two rabbits were antibodies of sufficient titre and affinity for application to the radioimmunoassay procedure produced. Alternative routes of injection, e.g. into lymph nodes or lymphatics, the peritoneal cavity and the spleen, were used at various times in an attempt to further raise the level of antibodies, but without any convincing evidence that this made any significant difference. Individual animal variation seemed to be more important in determining the ease of production of antibodies than the injection technique used, but this was not systematically examined.

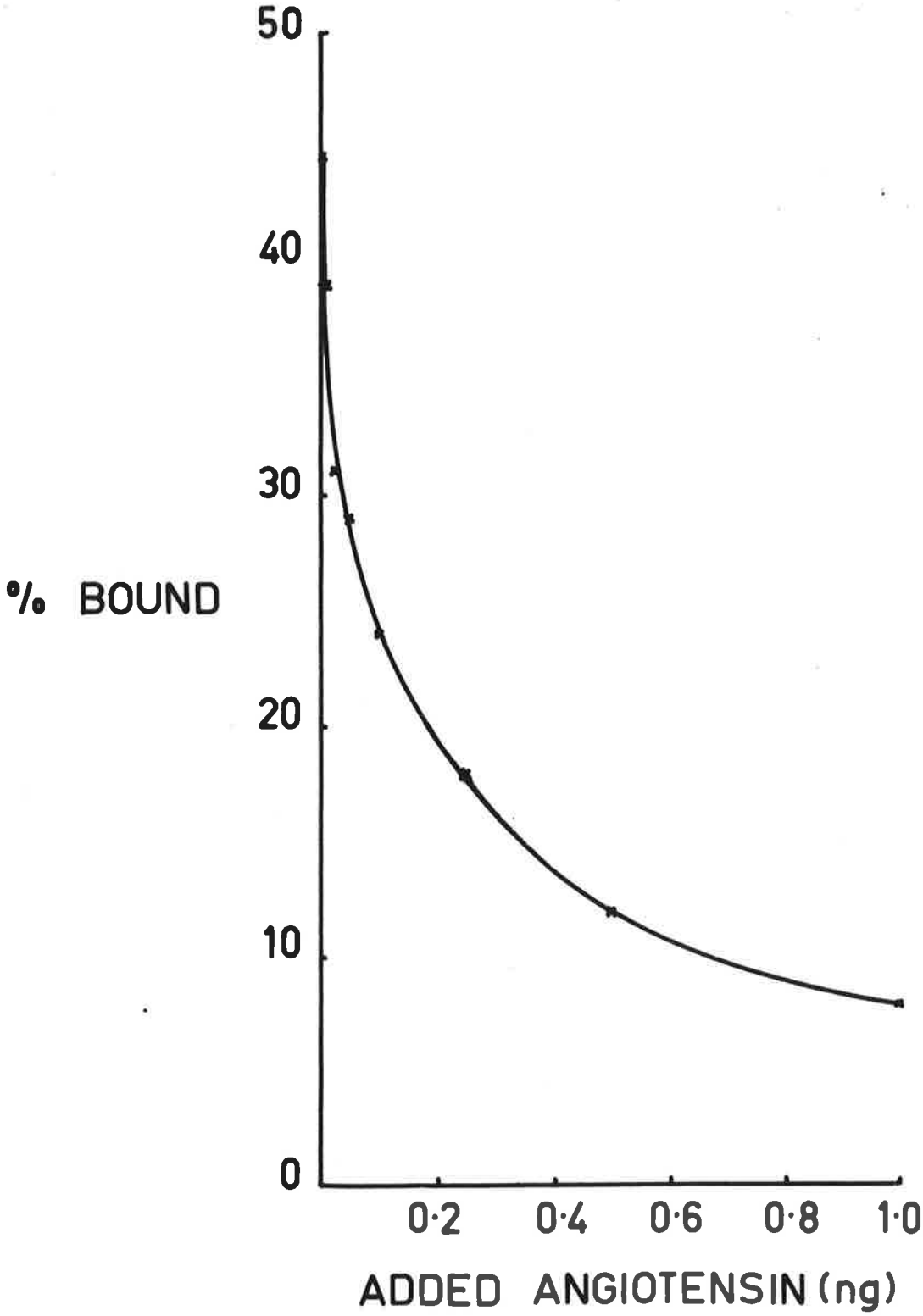


FIGURE 4

Standard curve for radioimmunoassay of angiotensin II.

(2) IODINATION

This was performed by a modification of the chloramine T method of Greenwood et al (1963). In this method, the oxidizing agent chloramine T is used to attach ^{125}I to the tyrosine in the angiotensin II molecule. High activity ^{125}I for protein iodination was obtained from the Radiochemical Centre, Amersham (IMS-3) and was used as soon as it arrived.

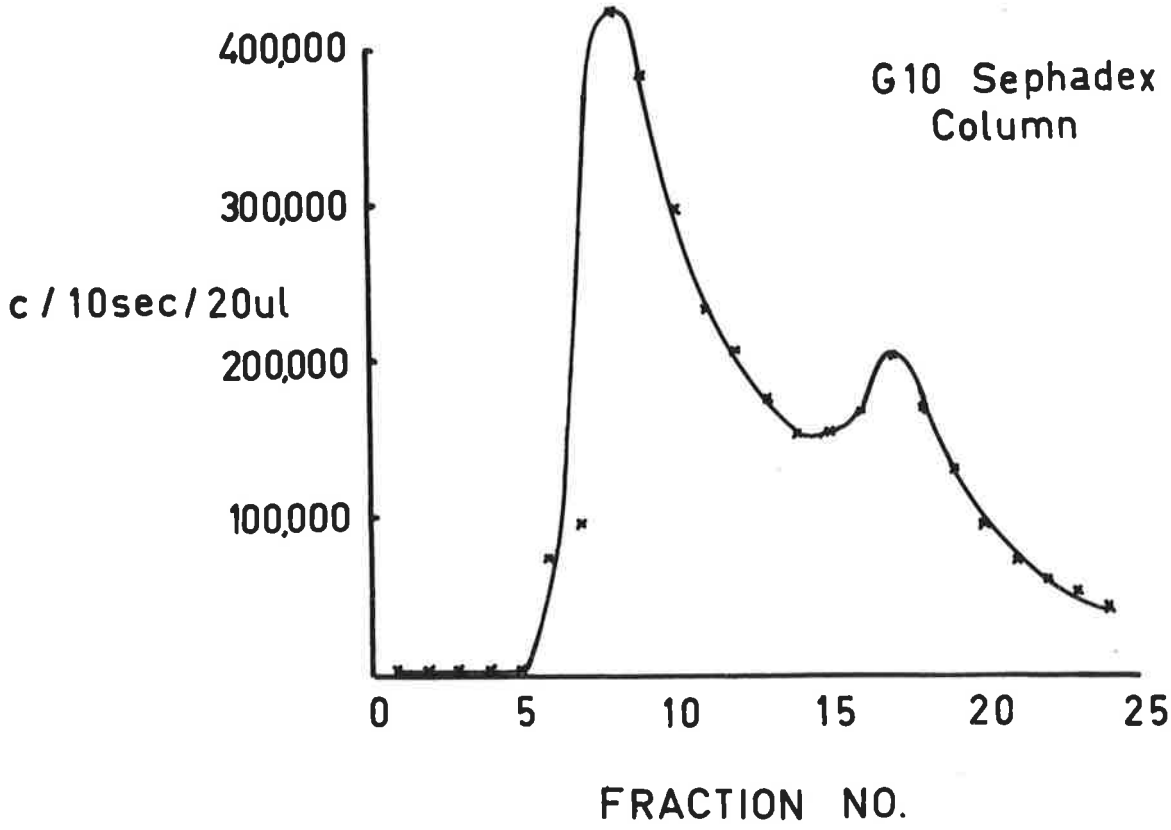
A representative iodination is carried out as follows:
To 2mCi of ^{125}I is added 20µl of 0.5M phosphate buffer pH 7.5 and then 5µgm of val^5-angiotensin II amide ("Hypertensin"-Ciba) or isoleu^5-angiotensin II (Schwartz Bioresearch) in 20µl of 0.05M phosphate buffer pH 7.5, followed by 100µgm of chloramine T in 20µl of 0.05M phosphate buffer. The reaction is allowed to proceed for 10 seconds and then stopped by the addition of 0.1 ml of sodium metabisulphite solution (2.4mgm per ml in 0.05M phosphate buffer). 0.2ml of potassium iodide solution (10mgm per ml) is added to dilute the unreacted ^{125}I and the mixture transferred to a 15 x 1 cm column of G10 Sephadex equilibrated with 0.07M barbitone buffer pH 8.6. An aliquot of the reaction mixture is usually taken at this stage and applied to a paper strip for subsequent electrophoresis and calculation of specific activity of the labelled peptide. The reaction mixture is eluted from the G10 column with 0.07M barbitone buffer pH 8.6 and 15 drop fractions collected; 20µl aliquots of these fractions are counted in a well-type counter (Ecko Scintillation Counter, Type N5504).

The radioactivity is eluted from the column in two peaks, the first representing labelled angiotensin and the second free isotope (Fig.5). Part of the peak fraction from the G10 column is applied to a second column (20 x 1 cm G25 Sephadex) equilibrated and eluted with 0.07M barbitone pH 8.6. 2ml fractions are collected and 20µl aliquots counted. The peak fraction from the G25 column is diluted in buffer containing 1% human serum albumin and stored frozen for subsequent use in the radioimmunoassay procedure.

Specific activity of the labelled angiotensin is determined by electrophoresis of a small aliquot of the iodination mixture on Whatmann 3MM paper in veronal buffer pH 8.6, when two peaks of radioactivity are obtained, corresponding to labelled peptide and free ^{125}I. From the areas beneath the two peaks, an estimate of the fraction of the isotope attached to the angiotensin and thus the average specific activity can be calculated. A similar estimation can be obtained from the areas beneath the two peaks from the G10 column, and this was found to agree with that obtained by electrophoresis. A specific activity of approximately 300µCi per µgm is consistently attained.

The labelled angiotensin is routinely checked for damage after iodination by chromatoelectrophoresis on Whatmann 3MM paper; the radioactivity is found to be confined to a single peak at the origin and the peptide is then considered damage-free. Over the course of several weeks' storage, a small amount of radiation damage occurs and this can be demonstrated on chromatoelectrophoresis as a second small

FIGURE 5



Iodination of Angiotensin II

peak of radioactivity migrating with the protein fraction (Fig.6).

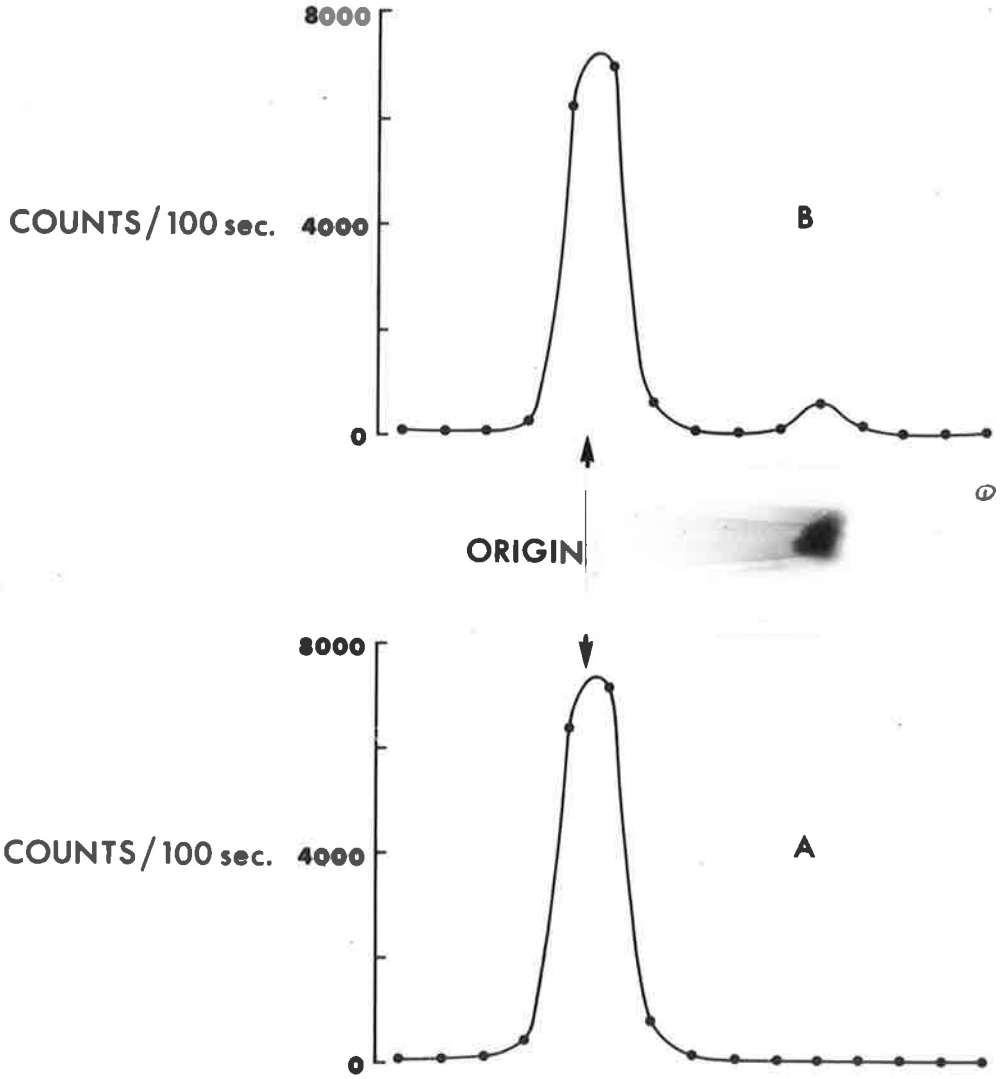
The technique of purification of the labelled peptide by extraction with antibody (Haber et al, 1962) was investigated. This technique uses antiserum to angiotensin II to extract from the iodination mixture the labelled hormone with the greatest affinity for the antibody, followed by acidification to bring about dissociation of the antigen-antibody complexes and subsequent separation of these two fractions by gel filtration. The method was found to yield labelled peptide with slightly increased affinity for the antibody but the increase in sensitivity achieved was only marginal and the purification procedure was not used routinely.

Initially, both val^5-angiotensin II amide and isoleu^5-angiotensin II were iodinated for use in the radioimmunoassay procedure, but it was subsequently found that the latter form was more resistant to non-specific damage than "Hypertensin" and it was, therefore, used routinely in the assay system.

(3) SEPARATION OF BOUND FROM FREE PEPTIDE

This forms an important final step in the radioimmunoassay procedure after incubation of standards or plasma samples with antiserum and labelled peptide. The method used by Yalow and Berson (1959) was chromatoelectrophoresis and this remains a precise and efficient method of separation of bound from free peptide. The chromatoelectrophoretic procedure, however, has the disadvantage that it is not readily applicable to large numbers of samples and, therefore, a wide variety of alternative methods have been used.

FIGURE 6

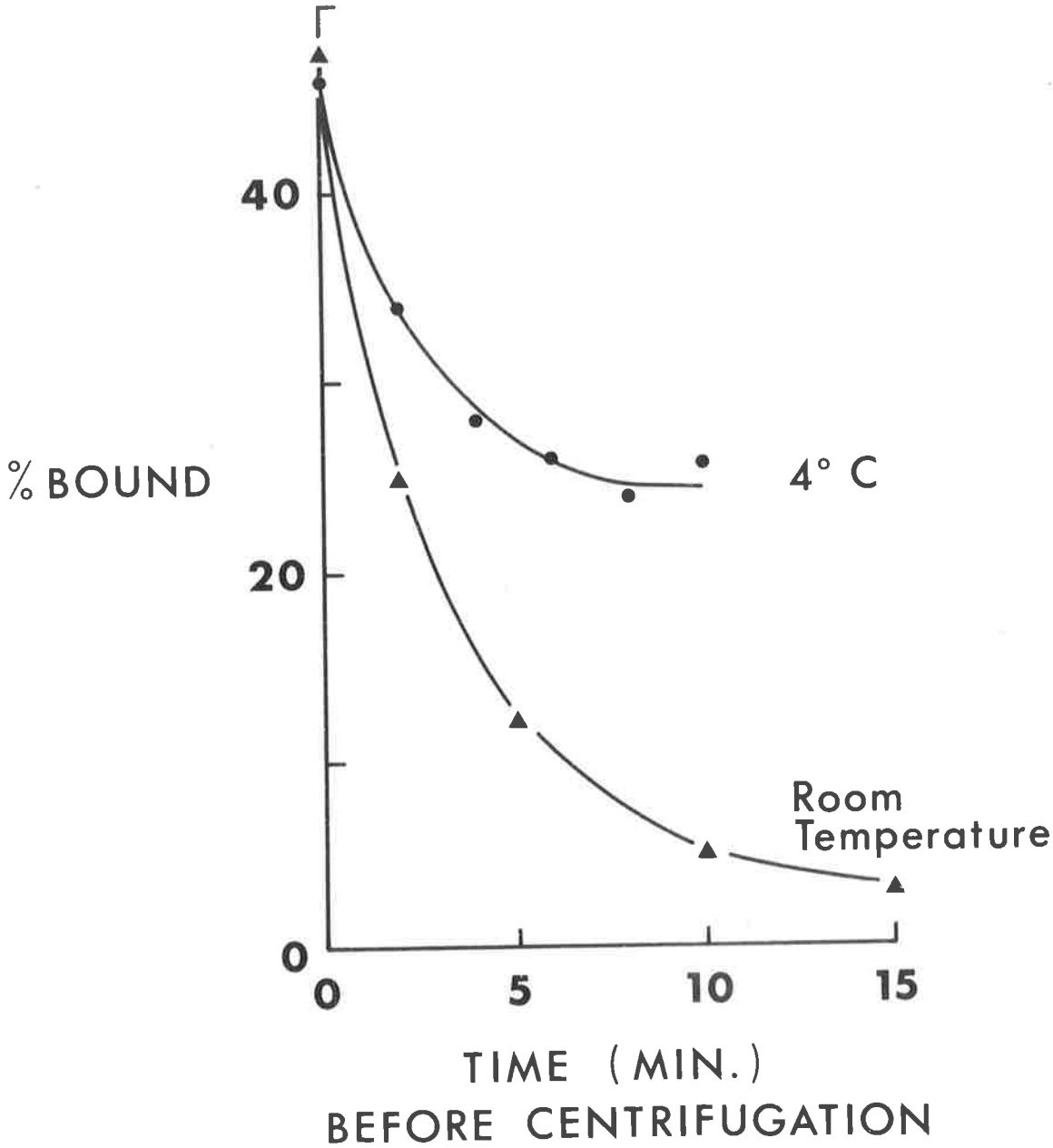


Chromatoelectrophoresis of ^{125}I-Angiotensin II to confirm freedom from damage.

It was decided to use a modification of the dextran-coated charcoal method described by Herbert et al (1965) for the radioimmunoassay of insulin. In this method, microfined charcoal is coated with dextran and a standard quantity of this suspension is added to the reaction mixtures after incubation in the assay procedure. The free angiotensin is avidly adsorbed onto the charcoal, but access of the larger antibody-angiotensin complexes is blocked by the coating of dextran. Centrifugation allows easy separation of bound from free peptide. This method is quick, effects almost instantaneous separation of bound and free hormone, and is readily applicable to large numbers of samples. It does, however, require meticulous technique, for dissociation of the antibody-angiotensin complex readily occurs if the incubation mixture is left in contact with the dextran-coated charcoal for more than a few minutes prior to centrifugation. The extent of this dissociation can be seen from Figure 7 which shows the effect of leaving the incubation mixture in contact with the dextran-coated charcoal for increasing periods of time prior to centrifugation. At room temperature, there is rapid dissociation of the antigen-antibody complex so that the binding is not sufficient for the radioimmunoassay procedure within three minutes. The dissociation is less rapid at 4°C but the percentage binding is still significantly reduced by any delay.

The dextran-coated charcoal was prepared by mixing in equal proportions a 10G per 100 ml suspension of neutral pharmaceutical grade charcoal (Merck) in barbital buffer pH 7.4 and an 0.5G per 100 ml solution of dextran, molecular weight 60,000-90,000(Koch-Lite).

FIGURE 7



Effect of prolonged contact with dextran-coated charcoal prior to centrifugation on % binding.

The dextran-coated charcoal was stored at 4°C and did not seem to deteriorate significantly on storage for several weeks. When used in the radioimmunoassay procedure, 1.0 ml of the dextran-coated charcoal suspension was added to each reaction tube following the incubation step and the mixtures were subjected to centrifugation at 3,000 rpm for 30 minutes at 4°C. It was found that, in the absence of antiserum, this amount of dextran-coated charcoal could consistently adsorb 99% of the labelled angiotensin when freshly iodinated, this percentage falling to about 92% over the course of several weeks and presumably indicating some degree of damage to the labelled peptide on storage.

(4) ASSAY PROCEDURE

The assay was carried out in 6 ml polypropylene tubes (Camelec-Adelaide) which did not significantly bind angiotensin when carrier protein was present in the buffer. The buffer used was 0.05M barbitone pH 8.6 containing 1% human serum albumin. The tubes were set up in duplicate containing: diluted antiserum, 0.1ml; ^{125}I-angiotensin II, 0.1ml (approximately 10,000 counts per 100 seconds); standard or plasma sample, 0.1ml; and buffer to a total volume of 1.0ml. The mixtures were incubated at 4°C for 48 hours and then separated with dextran-coated charcoal as described previously. After centrifugation, the supernatants were decanted into 6 ml polypropylene tubes and both precipitates and supernatants were counted in a well-type counter (Ecko Scintillation Counter, Type N5504). Shorter periods of incubation yielded satisfactory results but there tended to be some loss of sensitivity in the lower range and,

therefore, the longer period of incubation was employed routinely for low activity samples.

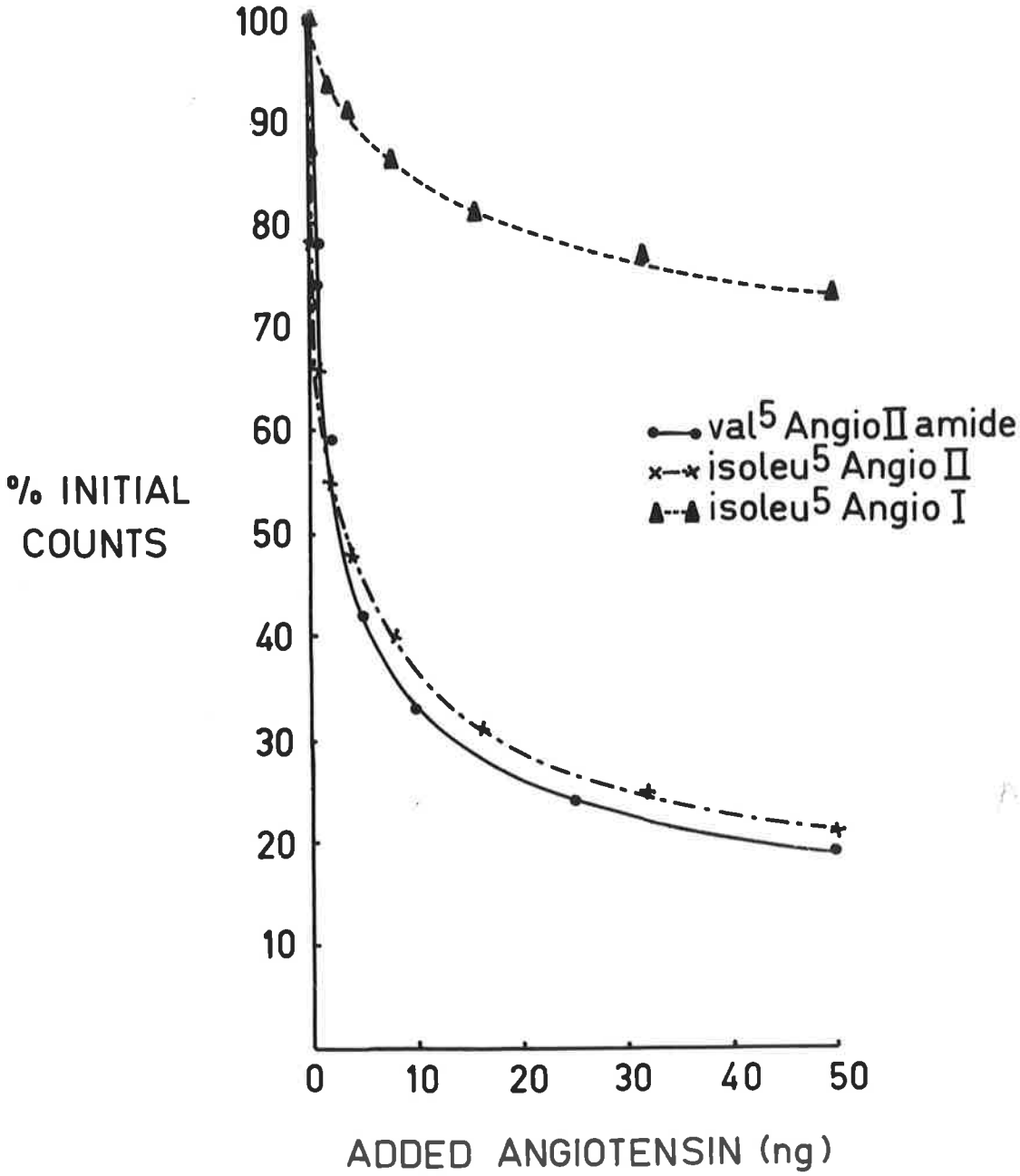
(5) SPECIFICITY OF THE ASSAY

The specificity of the assay for angiotensin II was checked by comparing the displacement of labelled angiotensin II from antibody produced by val^5-angiotensin II amide ("Hypertensin"), isoleu^5-angiotensin II, and isoleu^5-angiotensin I (Fig.8). It can be seen that the displacement produced by the two forms of angiotensin II was virtually identical at all dose levels, indicating identical affinity for the antibody, whereas there was only slight displacement of labelled peptide with the addition of relatively large amounts of isoleu^5-angiotensin I, indicating only slight cross-reaction of the antibody with the decapeptide, of the order of 1-2%.

It has been reported by Cain et al (1969) that biologically inactive fragments derived from the break-down of angiotensin II may retain immunoreactivity and thus give falsely elevated levels of angiotensin. This problem was investigated by incubation of a known quantity of angiotensin II with alpha-chymotrypsin at 37°C until no pressor activity was demonstrable in the rat bioassay system, followed by measurement in the radioimmunoassay. When this was done it was found that displacement of labelled peptide could still be demonstrated, to the extent of 5-10% of that seen prior to chymotrypsin digestion. This persistent immunoreactivity of biologically inactive fragments represents a potential source of error in the radioimmunoassay, although it did not appear to be a significant problem in practice.

FIGURE 8

SPECIFICITY OF ASSAY



Specificity of assay. Comparison of displacement of ^{125}I-labelled angiotensin II by isoleu^{5}-angiotensin II, val^{5}-angiotensin II amide, and isoleu^{5}-angiotensin I.

(6) SENSITIVITY OF THE ASSAY

The lower limit of sensitivity of the assay was 10 picograms of angiotensin II in buffer. In view of the extraction procedure employed prior to assay, with resultant concentration of the angiotensin II from plasma, this enabled plasma concentrations of less than 10 pg/ml to be measured with accuracy.

(7) REPRODUCIBILITY OF THE ASSAY

(a) Within Assay Variation

The within assay variation was determined by the assay of replicate samples within the one run. Representative results are shown in Table 1. It can be seen that for samples in the normal range the coefficient of variation is approximately 5 to 10% whereas in the case of higher activity samples the variation is greater. This difference occurs because the higher values fall on the flatter part of the standard curve where a relatively small alteration in the percentage binding of the labelled peptide will lead to a much greater proportional change in the value of angiotensin II obtained from the graph. For this reason, when maximum accuracy was required, high activity samples were assayed in dilution or with more concentrated antiserum so that the values obtained fell on the region of the standard curve with the steepest slope, thus minimizing the variability in the assay.

TABLE 1

WITHIN ASSAY VARIATION

(pg/ml)

	SAMPLES				
	(a)	(b)	(c)	(d)	(e)
	28	24	35	39	123
	27	23	37	45	100
	27	20	29	38	137
	29	21	32	34	115
	31	24	34	46	99
	25	21	33	41	145
	30	27	36	43	120
	28	23	30	39	119
	29	22	35	47	108
				48	
	27	20	34	39	127
				42	
$\bar{x}$	28.1	22.5	33.5	41.75	119.3
n	10	10	10	12	10
S.D.	1.64	2.06	2.42	4.02	14.06
v	5.84%	9.16%	7.22%	9.63%	11.79%

$\bar{x}$ = Mean v = Coefficient of variation = $\frac{100.S}{\bar{x}}$

n = Number of observations S.D. = Standard deviation

(b) Between Assay Variation

The between assay variation was ascertained by the assay of replicate samples in different assay runs. Representative results are shown in Table 2. It can be seen that the between assay variation is considerably greater than the within assay figure. For this reason, samples from physiological studies or other situations where values were to be compared were all assayed within the one assay run where possible in order to achieve maximum accuracy.

(8) ASSAY OF ANGIOTENSIN II IN PLASMA SAMPLES

For the assay of plasma concentrations of angiotensin II, an extraction procedure was employed to isolate the peptide from plasma prior to its introduction into the radioimmunoassay system; the Fuller's earth method of Boyd et al (1967) was routinely used.

Twenty ml venous blood samples were collected into cooled plastic syringes containing 0.6 ml of 0.2M dimercaprol (Koch-Lite) and 1.0 ml of 0.3M EDTA which were used to inhibit angiotensinases. The samples were transferred to cooled polypropylene tubes and centrifuged at 4°C to separate the plasma. To 10 ml of plasma in 15 ml polypropylene tubes (Camelec) was added 100 mg of acid-washed Fuller's earth, followed by mixing for 10 minutes at 4°C. The supernatants were tipped off and the Fuller's earth washed in turn with distilled water and methanol. The angiotensin was subsequently eluted from the Fuller's earth with 2 ml of 40%, 0.88 S.G. ammonia in methanol (v/v) by mixing at room temperature for 20 minutes. It was found that a second elution

TABLE 2

BETWEEN ASSAY VARIATION

(pg/ml)

	SAMPLES		
	(f)	(g)	(h)
	45	37	50
	42	29	47
	33	24	59
	37	36	39
	44	20	40
	31	28	44
	38	27	49
	43	32	53
	40	21	39
	39	26	48
			50
			41
$\bar{x}$	39.2	28.0	46.6
n	10	10	12
S.D.	4.38	5.44	5.94
v	11.18%	19.43%	12.75%

$\bar{x}$ = Mean V = Coefficient of variation = $\frac{100.S}{\bar{x}}$

n = Number of observations S.D. = Standard deviation

with ammonia in methanol was necessary at this stage to ensure recovery of the majority of the angiotensin II. The eluate was dried in an air stream at room temperature after the addition of 0.1 ml of 1% human serum albumin to prevent drying losses. The dried eluate was subsequently dissolved in human serum albumin buffer prior to assay in the radioimmunoassay system.

Control blanks were set up containing: (a) water, (b) plasma from anephric male subjects, and (c) re-extracted plasma. These blanks were found to produce displacement of labelled peptide of the order of 2 to 3 per cent, corresponding with original concentrations of up to 3 pg/ml. None of the blanks used is completely satisfactory. The water blank invariably gave very low values, suggesting that the error due to the method itself is minimal. The anephric plasma has the disadvantage of being obtained from a subject other than those whose samples are to be assayed. The use of previously extracted plasma as a blank is not satisfactory as the peptides or other substances which may interfere with the assay could be removed completely on the first occasion and their presence would, therefore, not be revealed by the blank.

The extraction procedure was checked in two ways:

(a) Recovery of labelled angiotensin II added to anephric plasma in increasing amounts and then extracted.

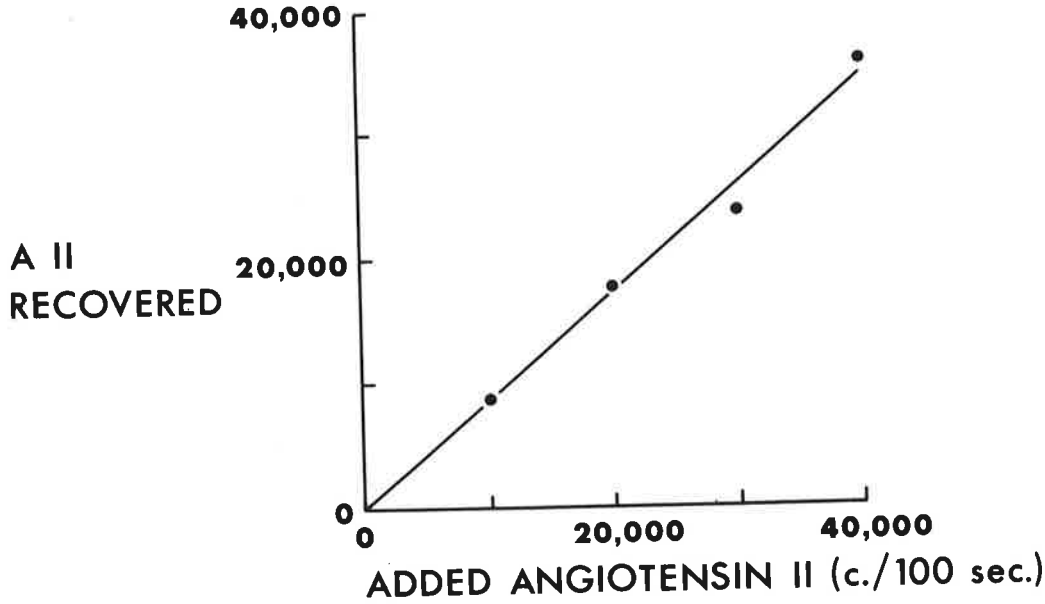
(b) Recovery of unlabelled angiotensin II added to anephric plasma in increasing amounts, extracted and then assayed in the radioimmunoassay system.

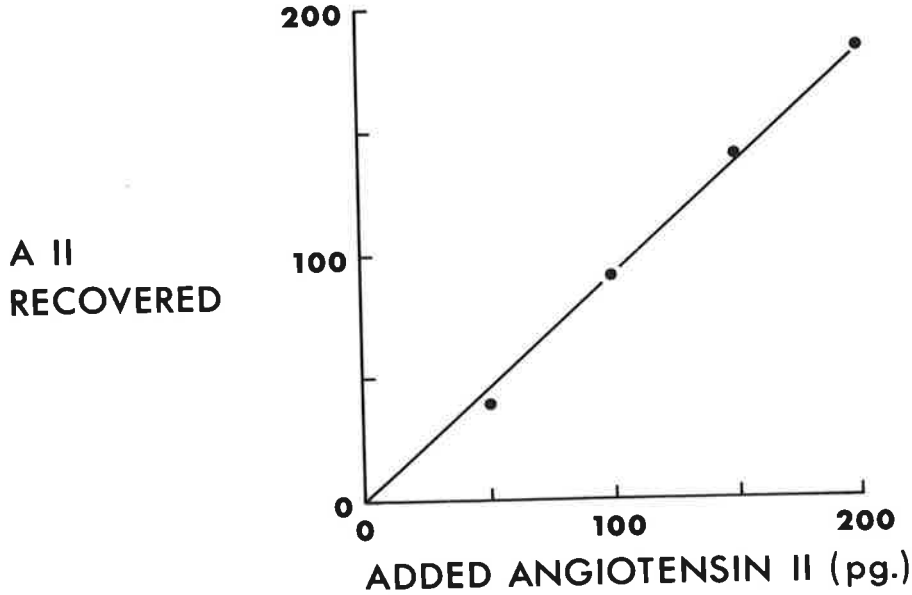
The results of two such experiments are shown in Figure 9. It can be seen that there has been quantitative recovery of both the labelled and unlabelled angiotensin II at all dose levels. The overall recovery for the extraction procedure was approximately 85%, which is of the same order as that reported by Boyd et al (1967).

9. MEASUREMENT OF RENIN ACTIVITY, RENIN CONCENTRATION, AND RENIN SUBSTRATE BY BIOASSAY

Renin activity, renin concentration, and renin substrate were measured by the method of Skinner (1967), except that the bioassay step was performed as described by Gordon et al (1966). Blood samples were collected into bottles containing 0.2 ml of 10% ammonium EDTA to inhibit the action of angiotensinases, the plasma was separated and stored frozen until subsequent processing. In the Skinner procedure, selective denaturation of endogenous substrate and angiotensinases in the case of renin concentration estimation or of the angiotensinases only in the case of renin activity estimation was achieved by dialysis of plasma samples against EDTA-containing buffer at pH 3.3 or 4.5 respectively, followed by heating to 32°C. The samples then underwent dialysis against EDTA-containing buffer at pH 7.5 and tras-ylol and neomycin were added to each sample. In the renin activity method, the prepared plasma samples were then incubated at 37°C for 24 hours. In the renin concentration method, standard substrate, prepared according to the method of Skinner (1967), was added to each sample and incubation was carried out for a period of 6 hours. Renin substrate was determined by the addition of prepared renal renin (Skinner, 1967), sufficient

FIGURE 9





Recovery of labelled and unlabelled angiotensin added to anephric plasma.

to drive the reaction between the renin and endogenous substrate to completion, followed by incubation at 37°C for 40 minutes. The angiotensin generated during each of these procedures was measured in the rat bioassay system.

The rats used for bioassay were pure Lewis strain animals, weighing between 150 and 200 Gm each. This strain of animals had been found to be more sensitive to the pressor action of angiotensin than the other strains tested. Bilateral nephrectomy was performed on each animal several hours prior to being used in the bioassay. Anaesthesia was achieved by intramuscular injection of 50% urethane, calculated on a scale according to body weight, the dose varying between 0.35 and 0.40 ml for the range of weight as above. A tracheostomy was routinely performed. Two fine polyethylene cannulae (PE10*) were introduced into one jugular vein and heparin 0.4 ml (250 units per ml) was administered through these. A larger polyethylene cannula (PE 50*) was inserted into a carotid artery and blood pressure was monitored through a strain gauge attached to a Hitachi recorder model QPD 33 with an input of 1 mV. The animal was given pentolinium (0.2-0.4 ml of an 0.5% solution) and phenoxybenzamine (0.2-0.4 ml of an 0.1% solution) intraperitoneally in order to prevent the effect of the sympathetic nervous system on blood pressure.

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* Intramedic. Clay Adams Division of Becton Dickinson & Co. N.J. 07054.

The concentration of angiotensin in the samples was determined by comparison of the height of the blood pressure increase produced by the samples in rats prepared as above with that produced by injection of a standard solution of val^5-angiotensin II amide ("Hypertensin"-Ciba). Both the standard solution and the sample to be assayed were administered into the jugular vein via microlitre syringes fitted with repeating dispensers*. All samples were assayed at two dose levels and were matched as closely as possible with an injection of standard angiotensin solution at each level. Within assay variation in sensitivity of the bioassay preparation was checked by comparison of the blood pressure responses to standard angiotensin before and after each assay, results being discarded if a variation of greater than 2 mm in deflection was found. Final results were expressed in the form of ng/100 ml/3 hours in the case of renin activity or renin concentration estimations, and ng/ml in the case of renin substrate concentrations. All assays were repeated in at least two rats and samples from physiological or comparative studies were all assayed in the one rat on each occasion wherever possible, in order to minimize the variability of results commonly encountered with the bioassay system.

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* Hamilton Co., Whittier, California.

CHAPTER 3

NORMAL RANGE OF PLASMA ANGIOTENSIN II AND RESPONSE TO PHYSIOLOGICAL STIMULI

1. NORMAL RANGE OF PLASMA ANGIOTENSIN II
2. RESPONSE OF PLASMA ANGIOTENSIN II TO PHYSIOLOGICAL STIMULI
3. CORRELATION BETWEEN PLASMA ANGIOTENSIN II CONCENTRATION AND PLASMA RENIN ACTIVITY

CHAPTER 3

NORMAL RANGE OF PLASMA ANGIOTENSIN II AND RESPONSE TO PHYSIOLOGICAL STIMULI

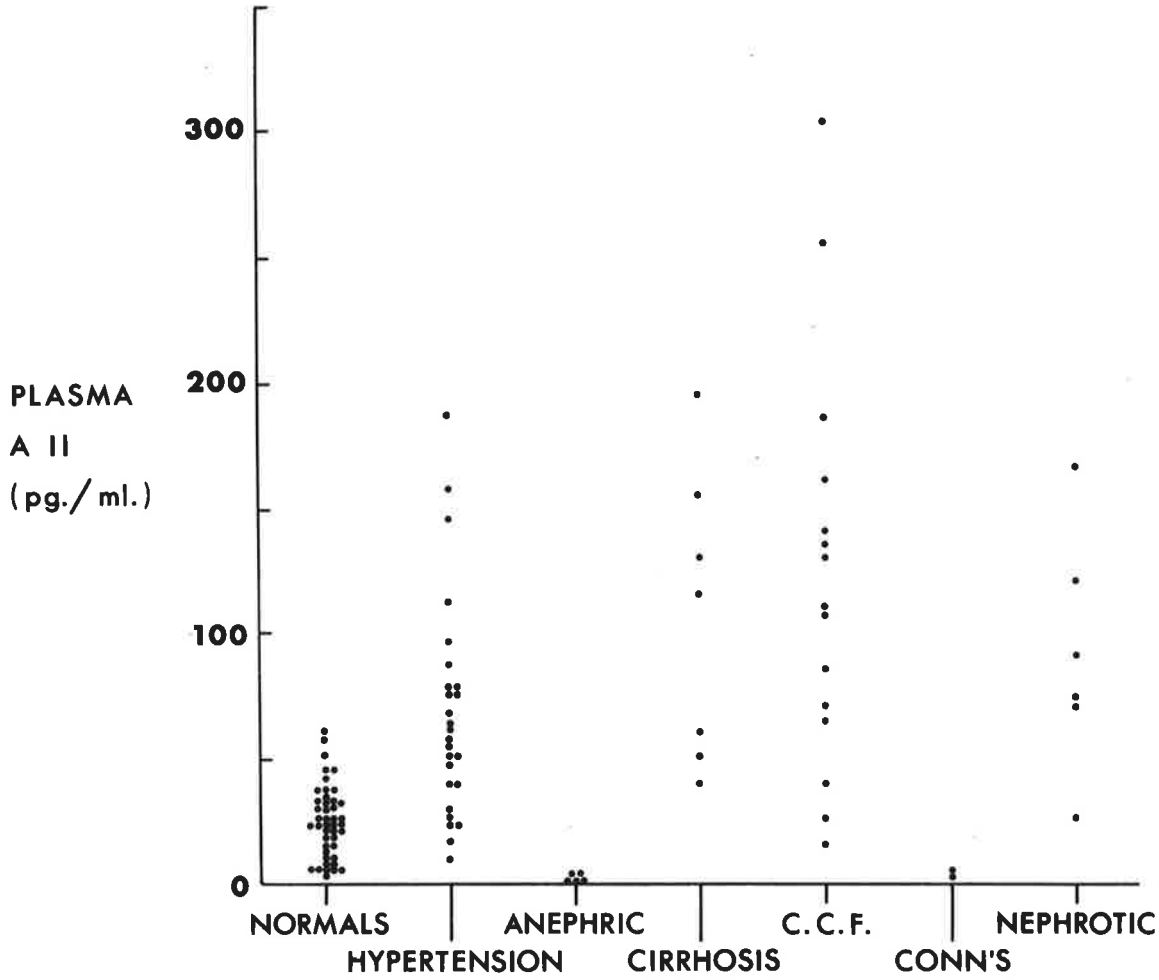
1. NORMAL RANGE OF PLASMA ANGIOTENSIN II

In order to ascertain the normal range of plasma angiotensin II concentration, plasma samples were collected from forty-four normal subjects with no evidence of hypertension, aged between 17 and 45 years. All subjects were on an unrestricted sodium diet and the samples were collected between 8:00 am and 11:00 am in the upright position.

There was considerable variation in the values obtained, ranging from 4pg/ml to 63pg/ml, with a mean value of 25.2 ± 14.5 (S.D.) (Fig.10). This value is comparable with that reported by several other groups (Boyd *et al*, 1967; Catt *et al*, 1967; Page *et al*, 1969; Sundsfjord, 1970) (Table 3).

Plasma angiotensin II concentrations in normal subjects reported by groups assaying plasma samples directly (Goodfriend *et al*, 1968; Gocke *et al*, 1969)(Table 3) have been higher than those reported from the above groups, where an extraction procedure was employed prior to assay. The reason for this discrepancy is not known for certain but it may be that the increase in sensitivity which enables the direct assay of plasma samples has been attained at the price of decreased specificity. Angiotensin levels measured by bioassay have also tended to be higher than those obtained by radioimmunoassay (Genest *et al*, 1964; Morris and Robinson, 1964; Massani *et al*, 1966) (Table 3). The decreased specificity of the bioassay procedures

FIGURE 10



Plasma angiotensin II concentrations in normal subjects and in pathological conditions.

TABLE 3

PLASMA ANGIOTENSIN II CONCENTRATION IN NORMAL SUBJECTS

AUTHOR	RANGE (pg/ml)	MEAN (pg/ml)
Boyd *et al*, 1967	8 - 56	
Catt *et al*, 1967	5 - 47	21 ± 14
Goodfriend *et al*, 1968	<5 - 126	45
Gocke *et al*, 1969	18 - 110	54
Page *et al*, 1969		21 ± 11 (Supine) 25 ± 14 (Upright)
Sundsfjord, 1970	12 - 57	27 ± 14
Present Study	4 - 63	25 ± 14.5
Mulrow, 1964	0 - 60	23 ± 3
Genest *et al*, 1964		70 ± 20
Massani *et al*, 1966		95 ± 12

employed probably accounts for these higher values, although, in one such study, the values obtained agreed closely with the radioimmunoassay values (Mulrow, 1964).

2. RESPONSE OF PLASMA ANGIOTENSIN II TO PHYSIOLOGICAL STIMULI

In order to determine the effects of upright posture and dietary sodium restriction on plasma angiotensin II concentrations, two normal subjects were equilibrated on a diet calculated to contain 10 mEq of sodium per 24 hours. Analysis of a duplicate 24 hour sample of the diet confirmed the low level of sodium intake (9.3 mEq per day). On the day before commencement of the sodium restriction and during seven days of low sodium diet, blood samples were taken for measurement of plasma angiotensin II levels: (a) at 8:00 am following at least 8 hours of overnight strict recumbency, and (b) at 11:00 am after 3 hours sitting, standing or walking. Angiotensin II was extracted from the plasma samples and assayed in the radioimmunoassay system by the usual method. Aliquots of the plasma samples were used for measurement of plasma renin activity by the method of Skinner (1967).

The results of these studies are shown in Figures 11 and 12. It can be seen that the urinary sodium dropped quickly to very low levels, confirming the efficacy of the sodium restriction. The plasma angiotensin II data showed that the values at 11:00 am after 3 hours of upright posture were consistently higher than those at 8:00 am after overnight recumbency. The magnitude of this increase varied considerably, averaging approximately 50%.

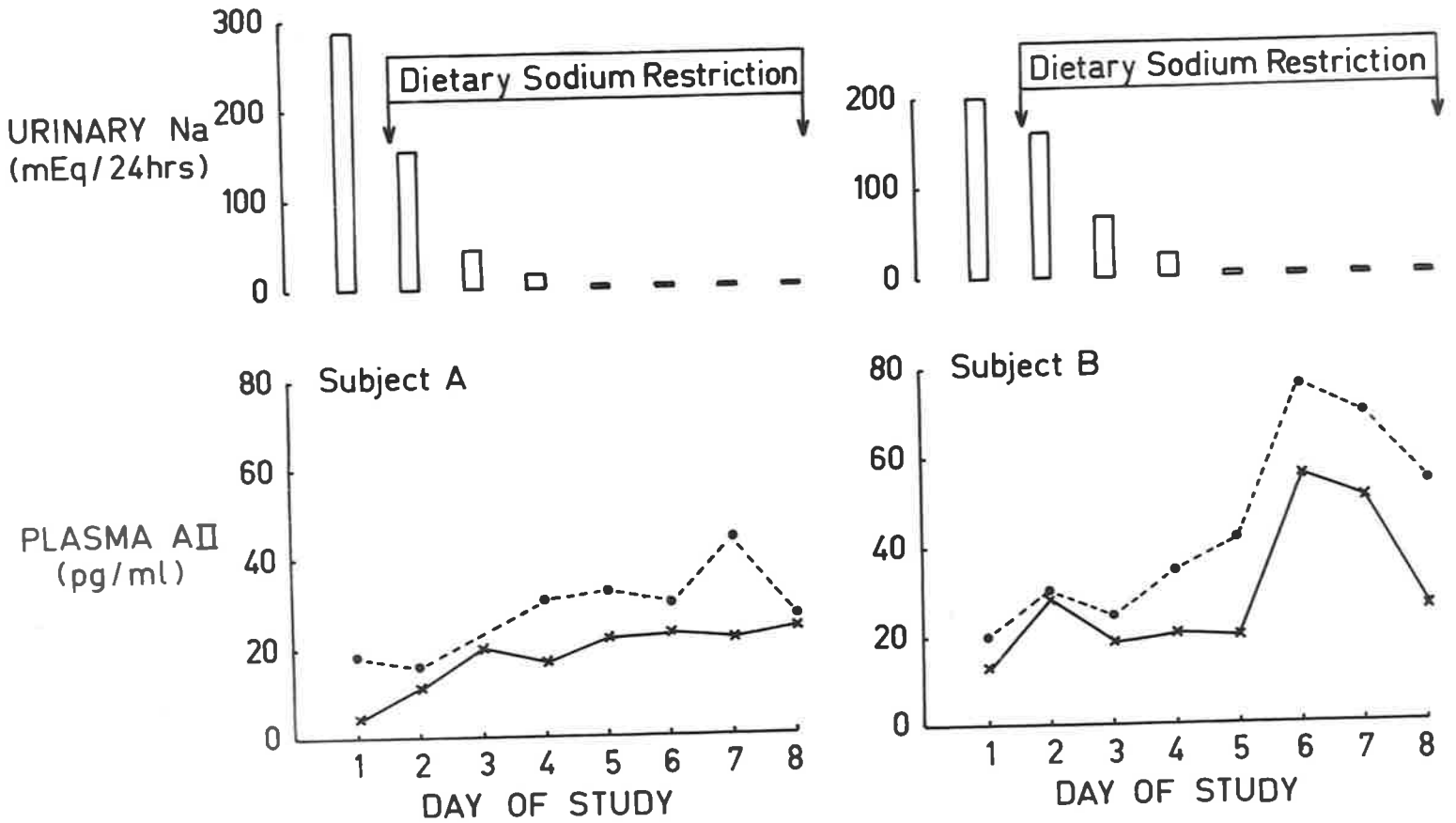
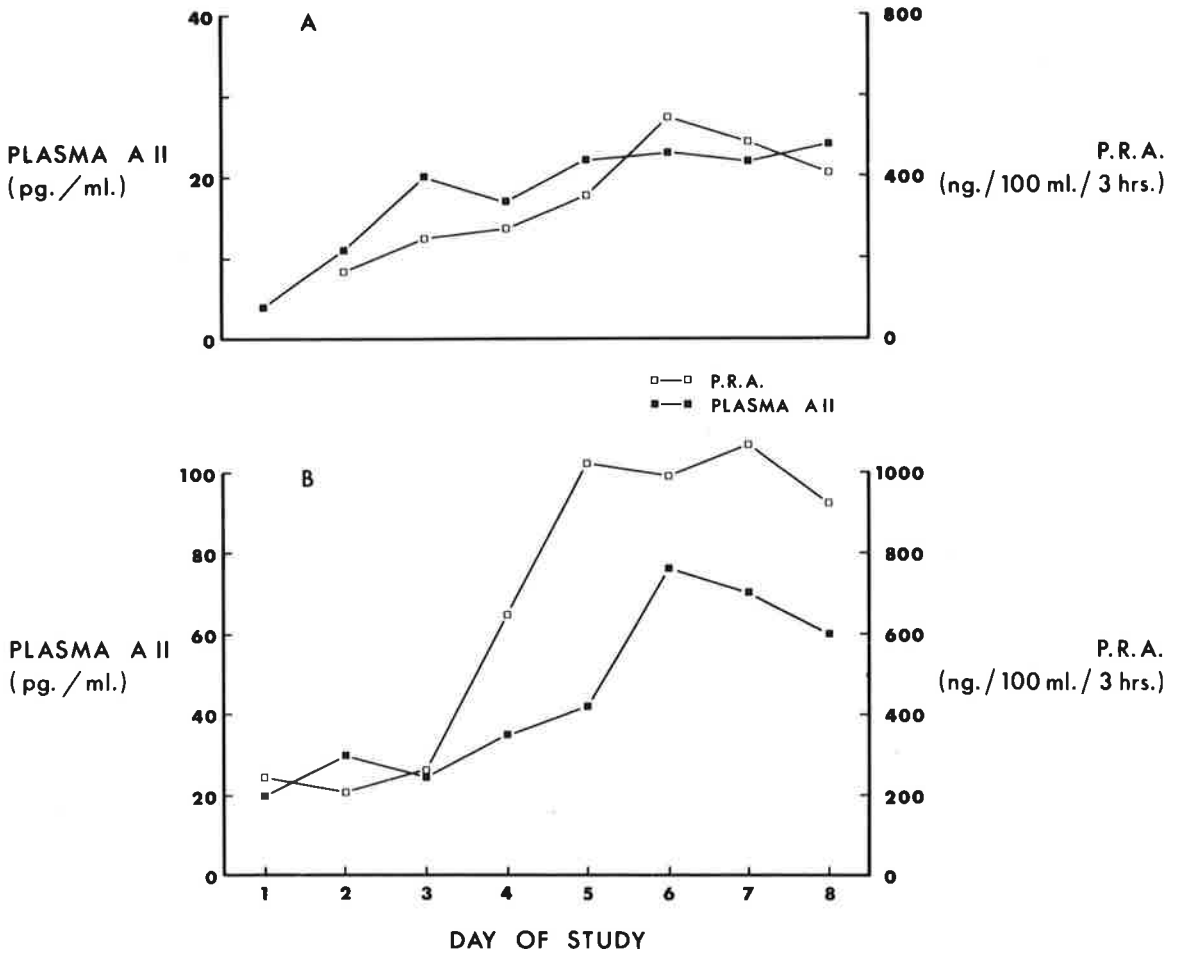


FIGURE 11

Effect of dietary sodium restriction on plasma angiotensin II concentration.

FIGURE 12



Comparison of plasma angiotensin II concentration and plasma renin activity during dietary sodium restriction.

Both the recumbent and the upright plasma angiotensin II levels showed a progressive increase during the period of dietary sodium restriction (Fig.11). The increase was more marked in the case of subject (B), where there was a tendency for the values to fall somewhat during the last two days of the study period, although still remaining elevated above control levels. These changes are of the same order as those reported by Page et al (1969); the increase in angiotensin II concentration was more marked in one subject than the average increase reported by those authors. The period of dietary sodium restriction was, however, longer in the present study.

These changes in plasma angiotensin II concentration in response to posture and dietary sodium restriction are similar to those previously reported for plasma renin activity and aldosterone excretion (Brown et al, 1963; Cohen et al, 1964; Gordon et al, 1966(b)). The consistent increase in the levels at 11:00 am compared with the levels at 8:00 am is presumably due to assumption of the upright posture rather than to the time of day, since levels of plasma renin activity are falling at this time in recumbent subjects (Gordon et al, 1966(b)).

3. CORRELATION BETWEEN PLASMA ANGIOTENSIN II AND PLASMA RENIN ACTIVITY

Plasma renin activity determinations were performed on aliquot samples of plasma from the physiological study, the angiotensin generated in vitro being measured by the rat bioassay system (Skinner, 1967). Comparison of these results with the plasma angiotensin II values showed that similar changes were seen in both

parameters of activity of the renin-angiotensin system. The renin activity responses were proportionally greater in one subject (B), an effect consistent with that reported by Page et al (1969). Figure 12 shows the comparison of these values in the two subjects, in the recumbent position in one instance (A) and in the upright position in the other.

The reason for the proportionally greater rise in plasma renin activity than plasma angiotensin II may be related to the longer half-life of renin in the circulation, to variable tissue extraction of angiotensin, to variations in activity of converting enzyme and angiotensinases in vivo, or may be related to variations in conditions determining the amount of angiotensin generated on incubation of plasma in vitro. Cain et al (1969) recently published evidence that the material measured in venous blood in a radioimmunoassay for angiotensin II consisted of a mixture of angiotensin II and its breakdown products which could vary in composition from sample to sample. In light of these possible variables, it is not surprising that the plasma renin activity and plasma angiotensin II estimations do not correlate more closely.

CHAPTER 4

PLASMA ANGIOTENSIN II LEVELS IN PATHOLOGICAL CONDITIONS

1. CONGESTIVE CARDIAC FAILURE
2. CIRRHOSIS OF THE LIVER
3. NEPHROTIC SYNDROME
4. PRIMARY ALDOSTERONISM
5. HYPERTENSION
6. ANEPHRIC SUBJECTS

CHAPTER 4

PLASMA ANGIOTENSIN II LEVELS IN PATHOLOGICAL CONDITIONS

The angiotensin II radioimmunoassay was applied to the measurement of plasma angiotensin II concentrations in patients with a variety of conditions in which disturbances of the renin-angiotensin system were thought likely to occur, namely, congestive cardiac failure, nephrotic syndrome, hepatic cirrhosis, primary aldosteronism, essential and renovascular hypertension, and in anephric subjects. The results of these studies are shown graphically in Figure 10.

1. CONGESTIVE CARDIAC FAILURE

Plasma renin activity has usually been reported to be elevated in patients with congestive cardiac failure (Brown *et al*, 1964, Veyrat *et al*, 1964; Fasciolo *et al*, 1964). Increased renin secretion has also been demonstrated in dogs with experimentally produced high-output cardiac failure (Davis *et al*, 1964). Congestive cardiac failure could lead to increased production of renin by the kidney through a drop in renal artery perfusion pressure leading to stimulation of baroreceptors at the afferent arteriole level or by an alteration in sodium load or concentration at the macula densa site. Evidence has also been presented (Schneider *et al*, 1969) to suggest that decreased metabolism of renin may contribute substantially to the increased plasma renin of experimental high-output cardiac failure.

The true situation in uncomplicated congestive cardiac failure in man is difficult to ascertain because of the almost invariable treatment of these patients with a low sodium diet and diuretics which will tend to produce an increase in renin levels of its own accord. Treatment of congestive cardiac failure has been reported to produce either an increase in plasma renin levels (Brown et al, 1964) or a fall (Veyrat et al, 1964). It seems likely that the elevated renin levels in congestive heart failure are at least partly due to the therapeutic manoeuvres used in treatment, a proposition supported by the study of Vandongen and Gordon (1970).

Elevated blood angiotensin levels in patients with congestive cardiac failure have been reported by groups employing bioassay methods (Genest et al, 1964; Massani et al, 1966). In the study of Genest et al (1964), the blood angiotensin concentration was reported to usually decrease following total or partial relief of the oedema. Plasma angiotensin II concentration was subsequently reported to be markedly elevated in patients with severe congestive cardiac failure when measured by the radioimmunoassay method (Catt et al, 1969).

In the present study, plasma angiotensin II concentrations in a group of 15 patients with congestive cardiac failure ranged from 16 pg/ml to 303 pg/ml, with a mean value of 122 pg/ml (Fig.10). The majority of these patients were already on treatment with low-sodium diet, digitalis and diuretics when the plasma samples were taken, and it was difficult to decide with certainty whether the

increased angiotensin II levels were largely due to the stimulatory effects of the therapeutic manoeuvres or to the underlying disease process. Some support for the former proposition was afforded by the finding that the plasma angiotensin II concentrations in the three patients from whom plasma samples were taken prior to treatment were 26 pg/ml, 40 pg/ml, and 86 pg/ml, i.e. they were within the normal range or only slightly elevated, and the higher values found in patients who were already on treatment may have been at least partly due to the treatment itself.

2. HEPATIC CIRRHOSIS

Elevated levels of plasma renin have been reported in plasma from patients with cirrhosis of the liver and ascites (Brown et al, 1964; Fasciolo et al, 1964). Renin levels in patients with cirrhosis but no ascites have usually been reported to be normal or only slightly elevated (Brown et al, 1964) but significantly elevated values in this situation have also been reported (Imai and Sokabe, 1968). Blood angiotensin concentrations have been reported to be elevated in patients with cirrhosis and ascites (Massani et al, 1966) and this elevation has been confirmed by more recent radioimmunoassay measurements of plasma angiotensin II (Catt et al, 1969; Gocke et al, 1969; Sundsfjord, 1970). Factors which may contribute to the elevation of plasma renin and angiotensin include low plasma volume, impaired metabolism of renin by the damaged liver, and alterations in sodium balance or sodium handling by the kidney (Schroeder et al, 1970).

Plasma angiotensin II concentrations were measured in seven patients with cirrhosis of the liver (Fig.10). Values ranged from 40 pg/ml to 194 pg/ml, with a mean of 106 pg/ml, confirming the elevated levels reported by others. In this small series, there seemed to be no difference between the levels in the patients with ascites and those without.

3. NEPHROTIC SYNDROME

Plasma renin levels have been reported to be elevated in patients with the nephrotic syndrome and generalized oedema (Veyrat et al, 1964; Imai and Sokabe, 1968). Elevated blood angiotensin levels have also been reported in this situation (Massani et al, 1966; Sundsfjord, 1970).

Six patients with nephrotic syndrome and generalized oedema were found to have plasma angiotensin II concentrations ranging from 26 pg/ml to 166 pg/ml, with a mean of 90 pg/ml. The level of plasma angiotensin II was not significantly correlated with the degree of proteinuria or the severity of the oedema.

4. PRIMARY ALDOSTERONISM

Primary aldosteronism is associated with autonomous hypersecretion of aldosterone from the adrenal cortex from a cortical adenoma or bilateral cortical hyperplasia. The increased aldosterone levels result in hypertension and increased plasma volume, with resultant feedback suppression of renin secretion, so that plasma renin levels are very low in this condition (Conn et al, 1964). Plasma angiotensin II concentration has been shown to be low also in this condition (Catt et al, 1969).

Plasma angiotensin II concentrations were assayed in one patient with primary aldosteronism. The patient was a 15 year old female who presented with severe hypertension, nocturia, hypokalaemia (made worse by a high sodium intake), and suppressed plasma renin activity, not responding to the stimuli of sodium restriction and upright posture. She was ultimately found at operation to have bilateral adrenal cortical hyperplasia. Plasma angiotensin II concentrations of 4 pg/ml in the recumbent position and 5 pg/ml when upright were found after a period of five days on a diet containing only 10 m.Eq. of sodium per 24 hours. Plasma renin activity estimation on aliquots of these blood samples yielded values of 45 ng/100 ml/24 hours and 47 ng/100 ml/24 hours respectively. Both these parameters of activity of the renin-angiotensin system were thus inappropriately low following dietary sodium restriction and it appears that the angiotensin II radioimmunoassay would be a suitable substitute for the renin activity estimation in this clinical situation.

5. HYPERTENSION

Plasma renin levels have been reported to be mostly within the normal range in patients with uncomplicated "essential" hypertension but often elevated in patients with severe or malignant hypertension (Helmer, 1964; Veyrat *et al*, 1964; Fasciolo *et al*, 1964; Brown *et al*, 1964). In the case of patients with hypertension secondary to renal artery stenosis, high plasma renin values have been reported (Helmer, 1964; Veyrat *et al*, 1964; Brown *et al*, 1964), but some values were noted to fall within the normal range. High levels of plasma angiotensin have been reported in patients with renal artery

stenosis (Genest et al, 1964; Massani et al, 1966), but in one study (Mulrow, 1964), normal levels were found in a group of 16 patients with hypertension and renovascular disease, six of whom were cured or improved out of 14 submitted to surgery. Results obtained using the radioimmunoassay procedure for assay of plasma angiotensin II have revealed elevated plasma concentrations in severe and malignant hypertension and in renovascular hypertension (Catt et al, 1969; Gocke et al, 1969). Elevated plasma concentrations of angiotensin II have also been reported in patients with uncomplicated "essential" hypertension (Catt et al, 1969; 1970).

In the present study, plasma angiotensin II concentrations were assayed in a group of patients with hypertension and the results are shown in Figure 10. It can be seen that plasma angiotensin II concentrations vary from values within the normal range to quite high levels. The high percentage of cases with elevated angiotensin II values may be partly explained by the fact that the majority of the cases had been referred for investigation of possible renovascular hypertension. In the group of seven patients in whom renovascular hypertension was unequivocally established, the plasma angiotensin II concentration was universally elevated above the normal range. It was not possible to categorize exactly the aetiology of the hypertension in all of the remainder of the cases, but some of them had evidence of renal parenchymal disease as shown by reduction in renal size or evidence of dysfunction on renal function tests. When the patients with renovascular hypertension and those with suspected renal parenchymal disease were excluded, there remained

a group of 13 patients with "essential" hypertension. In this group of patients, approximately half the plasma angiotensin II levels were within the normal range and the remainder were elevated above normal, so that a mean value of 42 pg/ml was found. This finding is in agreement with the high mean values found in "essential" hypertension by Catt et al (1970). In the case of malignant hypertension, elevated levels of plasma angiotensin II were always found, a finding in keeping with that reported by Catt et al (1969) and Gocke et al (1969).

The role of the renin-angiotensin system in clinical hypertensive disease is not clear. The renin data suggest that the system does not play a major role in mild to moderate "essential" hypertension but that it may contribute significantly to the severity of the disease in accelerated or malignant hypertension, when features of secondary hyperaldosteronism may be superimposed on the underlying hypertensive process. Plasma angiotensin II data, however, have shown elevated mean levels in patients with "essential" hypertension and a highly significant correlation between the plasma angiotensin II concentration and the diastolic blood pressure (Catt et al, 1970). Although the elevated angiotensin II levels need not exert a hypertensive effect (c.f. values in subjects on oral contraceptives (Catt et al, 1970)) and may represent merely a secondary effect of the hypertensive process, a possible contributory role of the renin-angiotensin system to the blood pressure elevation of "essential" hypertension cannot be completely excluded at present.

In the case of hypertension secondary to renal artery stenosis in man or in experimental renovascular hypertension in animals, the renin data suggest that the renin-angiotensin system is of prime importance in the development of the blood pressure increase. Support for this role of the system has been provided by experiments demonstrating a drop in blood pressure in animals with experimental hypertension on infusion of antirenin or antiangiotensin antibodies (Deodhar et al, 1964; Hill et al, 1970; Worcel et al, 1970). However, doubt has been cast on the validity of this concept by the recent demonstration of the production of experimental renovascular hypertension in animals immunized against angiotensin II, together with the lack of effect on the course of established hypertension of active immunization against angiotensin II (MacDonald et al, 1970; Johnston et al, 1970). The failure of antirenin antibodies to affect the blood pressure in animals with hypertension due to renal artery constriction and contralateral nephrectomy (Weiser et al, 1969) adds to this doubt regarding the role of the renin-angiotensin system in this situation.

The suggestion from the latter studies has been that the renin-angiotensin system is not the major factor in the production or maintenance of experimental renal hypertension. The problem raised by this recent evidence indicating a less important role than previously accepted for the renin-angiotensin system has not been completely resolved at the present time.

6. ANEPHRIC SUBJECTS

Bilateral nephrectomy has been shown to cause a marked reduction in plasma renin to very low or undetectable levels (Lever *et al*, 1963). Very low levels of angiotensin II in plasma from human subjects with bilateral nephrectomy have been reported by Catt *et al* (1969).

Plasma angiotensin II concentrations in five anephric patients being maintained on the chronic dialysis program at The Queen Elizabeth Hospital were found to be 0, 0, 3, 0 and 4 pg/ml. Four of the five patients were females and detectable levels of plasma angiotensin II were found in two of this group. The significance of this finding is uncertain. Although a source of a "renin-like" enzyme from the female genital tract is a possibility, this has not been conclusively demonstrated to affect plasma renin levels.

CHAPTER 5

APPLICATION OF ANGIOTENSIN II RADIOIMMUNOASSAY TO MEASUREMENT OF PLASMA RENIN ACTIVITY

1. GENERAL

2. RENAL VENOUS PLASMA RENIN ACTIVITY RATIOS

CHAPTER 5

APPLICATION OF ANGIOTENSIN II RADIOIMMUNOASSAY TO MEASUREMENT OF PLASMA RENIN ACTIVITY

1. GENERAL

One of the original aims in setting up the radioimmunoassay for angiotensin II was for the purpose of replacing the bioassay step in the measurement of plasma renin activity and plasma renin concentration. Until recently, the only method of assessing the activity of the renin-angiotensin system has been by bioassay of the angiotensin generated by plasma when incubated in vitro after inhibition of angiotensinases (Boucher *et al*, 1964; Brown *et al*, 1964; Skinner, 1967). The bioassay techniques depend on the pressor response produced by incubated samples compared with standard angiotensin preparations in animals with pharmacologically blocked sympathetic nervous systems. The bioassay techniques are tedious, lack precision and are not readily applicable to large numbers of samples.

Comparison of the results obtained by bioassay and radioimmunoassay for the angiotensin generated during the Skinner method for plasma renin activity, have shown that the values obtained by the radioimmunoassay method have been consistently lower, usually of the order of 10% of those obtained by bioassay. When samples forming part of a series of physiological studies have been examined by the two techniques, however, the same general trends have been seen, suggesting that there may be some correlation between the two methods.

In order to check whether the substance being measured was actually angiotensin II, several incubated plasma renin activity samples were assayed at doubling dilutions in the radioimmunoassay procedure; it was found that the points obtained coincided closely with the standard curve for angiotensin II obtained under the same conditions, suggesting that the two substances were identical (Fig.13).

A likely explanation for the discrepancy between the two methods is that the end product of the plasma renin activity procedure is a mixture of angiotensin I and angiotensin II, only the latter being measured in the radioimmunoassay but both giving a response in the rat bioassay. It has been shown by Boyd et al (1967) that the presence of edetic acid and 2,3-dimercaprol in incubated plasma samples leads to a higher value for angiotensin by bioassay than by radioimmunoassay and it was suggested that this difference was due to formation of angiotensin I and prevention of conversion to angiotensin II by the 2,3-dimercaprol and edetic acid which inhibited converting enzyme as well as angiotensinases. Subsequent work from this group (Boyd et al, 1968) showed that it was difficult to obtain a complete return of activity of converting enzyme by the addition of various metal ions without also restoring angiotensinase activity. Similar observations were made by Page et al (1969) who demonstrated inhibition of converting enzyme activity by EDTA.

If the fraction of generated angiotensin in plasma samples present as angiotensin II were constant, it would be possible to use the radioimmunoassay procedure to obtain a measure of renin activity.

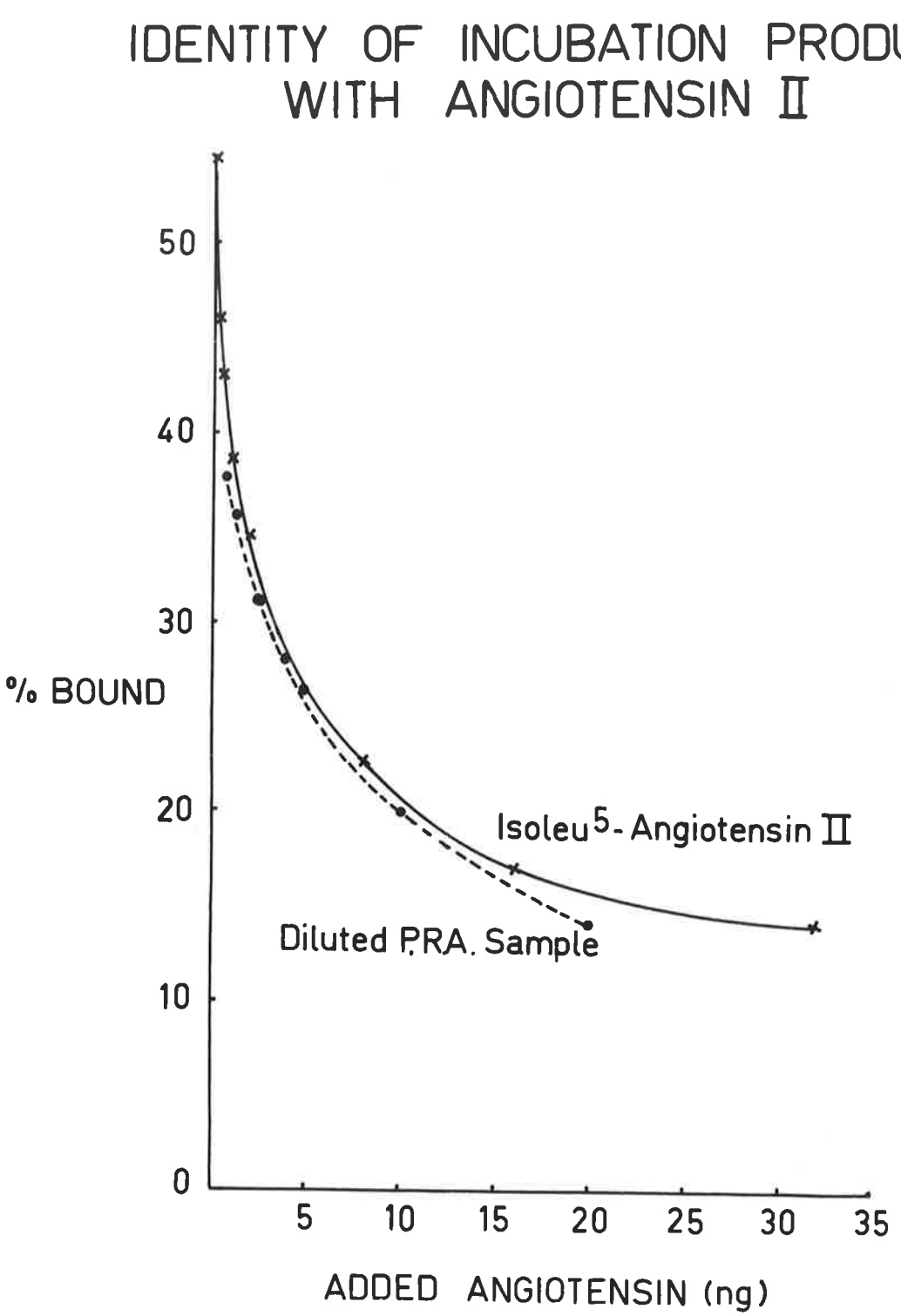


FIGURE 13

Identity of incubation product with Angiotensin II

Experience has shown, however, that the fraction present in this form is variable and the correlation with bioassay results is not close enough to justify the use of the radioimmunoassay for this purpose. Attempts to reverse the inhibition of converting enzyme by the addition of various metal ions has confirmed the experience of others of reactivation of angiotensinases. The alternative approach of producing complete inhibition of converting enzyme activity and measurement of the generated angiotensin in a radioimmunoassay system specific for angiotensin I would seem to be the more satisfactory answer to the problem (Boyd et al, 1969; Haber et al, 1969).

2. RENAL VENOUS PLASMA RENIN ACTIVITY RATIOS

Comparison of plasma renin activity values in both renal veins has been found useful in the diagnosis of renovascular hypertension (Michelakis et al, 1967; Fitz, 1967; Winer et al, 1967). Bilateral renal vein catheterization and simultaneous sampling from both sides in patients with restricted dietary sodium and controlled posture before sampling has been used as a screening test for possible renovascular hypertension. Plasma renin activity has been measured by the method of Skinner (1967) except that the bioassay step has been performed as described by Gordon et al (1966). A significant difference in the values obtained from each side has been found to be a useful test for renovascular hypertension (Pawsey et al, 1970). Since the rat bioassay procedure for measurement of generated angiotensin is relatively imprecise and cumbersome, the possible application of the angiotensin radioimmunoassay to the measurement of generated

angiotensin in these renal venous samples was investigated. Since the procedure involves catheterization of both renal veins and the withdrawing of blood samples simultaneously from the two sides, it seemed likely that the conditions determining the proportion of angiotensin I and angiotensin II generated during an identical period of incubation in vitro would be the same for the two samples, and the problem of varying proportions of angiotensin I and II mentioned previously would be minimized. The ratio of plasma renin activity in the right renal vein to that in the left renal vein should then be very similar for measurement of the generated angiotensin either by radioimmunoassay (specific for the fraction existing as angiotensin II) or by bioassay (measures both angiotensin I and angiotensin II). In order to test this hypothesis, a comparative study was carried out determining the ratios of renal venous renin activity in 27 pairs of samples by both bioassay and radioimmunoassay.

The levels of angiotensin generated in vitro by the Skinner technique and measured both by bioassay and by radioimmunoassay are shown in Table 4. Comparison of the absolute levels determined by the two methods shows a considerable variation in that part of the total generated angiotensin measured by bioassay which is measurable as angiotensin II in the radioimmunoassay, usually of the order of 10% There is however, very good agreement between the ratios (high side : low side) determined using the two methods; the correlation is shown in Fig. 14. From the regression line, if a value of 1.5 is taken as significant for bioassay (Michelakis et al, 1967), the corresponding value is 1.6 for the radioimmunoassay.

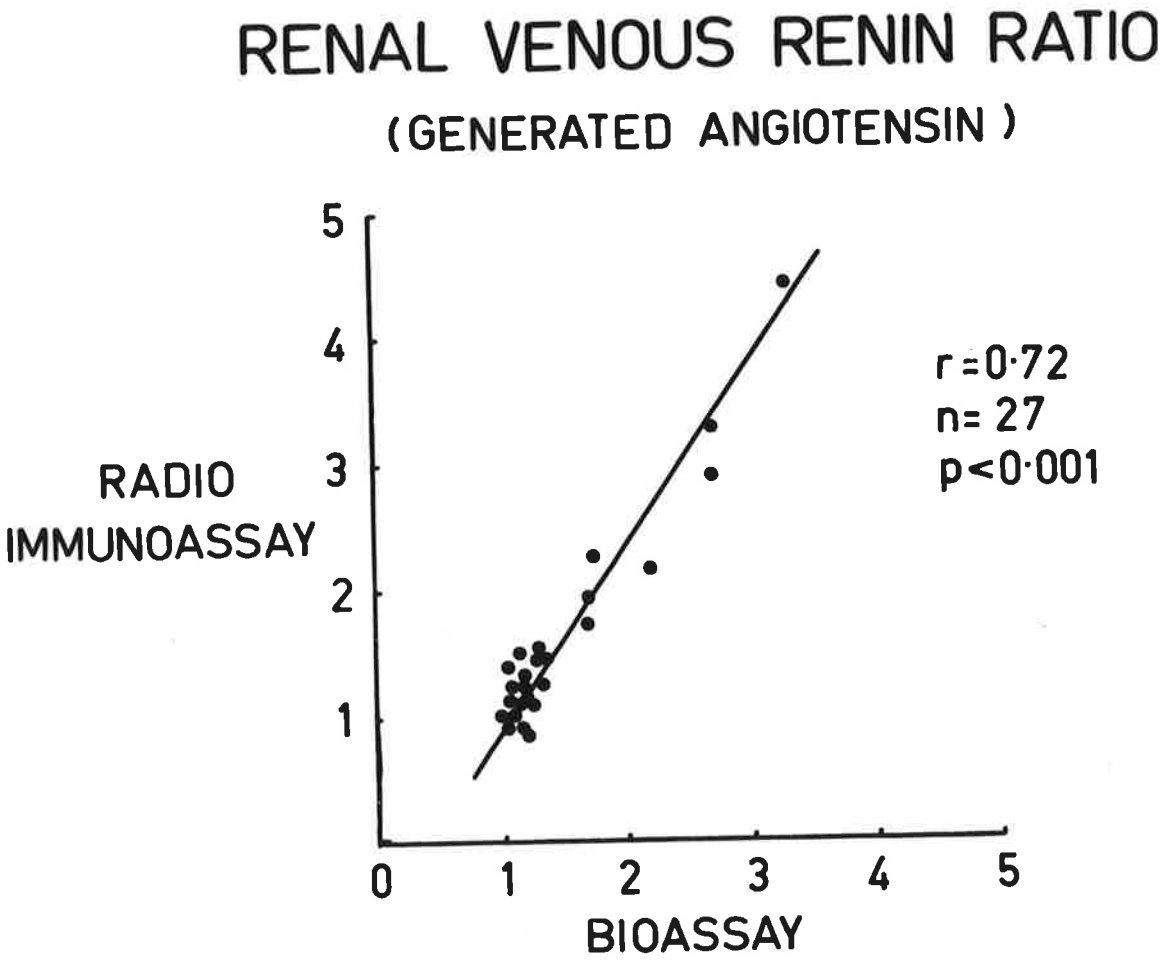
TABLE 4

Comparison of values for renal venous plasma renin activity (PRA) and ratio of high side to low side obtained by radioimmunoassay of generated angiotensin II with those obtained by bioassay in the rat pressor system

	Bioassay (ng/100 ml/3hrs)			Radioimmunoassay (ng/100 ml/3hrs)		
	R.R.V.	L.R.V.	Ratio	R.R.V.	L.R.V.	Ratio
1.	405	900	2.22*	13	27	2.08*
2.	685	563	1.27	48	33	1.45
3.	167	172	1.03	17	17	1.00
4.	1084	1395	1.3	42	60	1.43
5.	1658	1765	1.06	217	200	0.92
6.	1258	2155	1.71*	146	250	1.71*
7.	775	893	1.15	96	96	1.00
8.	1064	1128	1.06	133	188	1.41
9.	978	1119	1.14	127	171	1.34
10.	725	557	1.30	229	175	1.31
11.	253	93	2.72*	67	23	2.91*
12.	921	800	1.15	38	33	1.15
13.	3065	2737	1.12	283	258	1.10
14.	2290	1335	1.72*	138	71	1.94*
15.	7018	2640	2.66*	563	171	3.29*
16.	730	679	1.08	129	104	1.24
17.	784	715	1.10	71	58	1.22
18.	592	458	1.29	109	71	1.54
19.	848	780	1.09	63	67	0.95
20.	589	635	1.08	233	233	1.00
21.	708	779	1.10	292	263	0.90
22.	188	163	1.15	184	150	1.23
23.	90	88	1.02	75	67	1.12
24.	111	93	1.19	92	108	0.85
25.	2319	1341	1.73*	260	116	2.24*
26.	94	111	1.17	92	100	1.09
27.	8318	2503	3.32*	700	159	4.40*

* Significant ratio

FIGURE 14



Renal vein renin PRA ratios; bioassay vs radioimmunoassay.

Using these criteria, 7 out of 27 ratios are significant by both methods of assay. This excellent correlation between results for renal venous renin activity ratios using bioassay and radio-immunoassay suggests that the latter procedure could indeed be applied to this test to replace the more cumbersome bioassay.

CHAPTER 6

RENIN-ANGIOTENSIN SYSTEM IN PREGNANCY

1. INTRODUCTION

   (i) GENERAL

   (ii) TOXAEMIA OF PREGNANCY

   (a) Established Toxaemia
   (b) Renin Activity Prior to Development of Toxaemia

2. PLASMA ANGIOTENSIN II CONCENTRATIONS IN NORMAL PREGNANCY

3. PLASMA ANGIOTENSIN II CONCENTRATIONS IN PRE-ECLAMPTIC TOXAEMIA

CHAPTER 6

RENIN-ANGIOTENSIN SYSTEM IN PREGNANCY

1. INTRODUCTION

(i) General

The excretion of aldosterone is increased throughout pregnancy and returns rapidly to normal following delivery (Venning and Dyrenfurth, 1956; Jones *et al*, 1959; Watanabe *et al*, 1963). The increased secretion occurs as early as the fifteenth week of pregnancy and the actual level reached depends on dietary sodium ingestion in the usual way (Watanabe *et al*, 1963).

The demonstration of a connection between the renin-angiotensin system and aldosterone secretion (Laragh *et al*, 1960; Genest *et al*, 1961; Higgins *et al*, 1962) was soon followed by studies of the renin-angiotensin mechanism during pregnancy. Brown *et al* (1963) showed that the plasma renin concentration was raised throughout pregnancy and returned to normal within two months of parturition. They showed also that, during the late stages of gestation, the plasma renin levels in many of the normal pregnant women were within the normal range, and suggested that this might be due to a decreased demand for sodium at this stage of pregnancy. The increased activity of the renin-angiotensin system during pregnancy was confirmed by Genest *et al* (1965), who showed that there was a progressive and significant rise in plasma renin activity from the fourth to the twenty-first week of pregnancy, followed by stabilization at this high level and then a tendency to decrease slightly after the thirty-first week of pregnancy. Winer (1965) demonstrated a two to eight-fold increase in plasma renin activity in pregnancy, with values being

elevated from the sixth week onwards. The high renin values gradually fell to control levels within a week after delivery.

An explanation of this elevation of plasma renin activity in pregnancy was provided by the demonstration (Helmer and Judson, 1967) that pregnancy was accompanied by a marked increase in renin substrate. The increases in plasma renin activity were, therefore, of much greater magnitude than those in plasma renin concentration, since the amount of angiotensin generated per unit of renin is directly related to the substrate concentration. Confirmation of the probable role of oestrogens in the increased substrate levels during pregnancy was obtained by treating non-pregnant women with oestrogens or oral contraceptive preparations containing oestrogens. This caused renin substrate levels to rise into the range found in pregnancy. The increase in renin substrate during pregnancy was confirmed by Pickens et al (1965), who showed a three- to four-fold increase above normal control values during the last trimester of pregnancy.

Geelhoed and Vander (1968) showed that plasma renin activity was consistently elevated during the third trimester of pregnancy and that the renin levels during labour were significantly higher than the third trimester values for the same women. No significant change occurred during parturition or for the first week post partum, indicating that neither the placenta nor the foetus was the source of the elevated plasma renin. The plasma renin activity had returned to normal by six weeks post partum, a much longer period than that reported by others (Winer, 1965).

Another postulated explanation of the increased activity of the renin-angiotensin system and the increased secretion of aldosterone during pregnancy is that it is related to the high levels of progesterone. It has been shown (Landau et al, 1965) that progesterone has an action antagonistic to that of aldosterone, probably by a mechanism of competitive inhibition at the distal renal tubule, and that administration of progesterone in physiological amounts to normal subjects is accompanied by a natriuresis and followed by increased mineralocorticoid activity. A close correlation between urinary pregnanediol and aldosterone secretion when measured simultaneously in pregnant women has been reported (Jones et al, 1959), whereas there is no correlation between the secretion of these hormones and oestrogens. On the basis of this evidence, it has been suggested that the increased activity of the renin-angiotensin system is necessary to achieve the retention of sodium required for normal homeostasis in the face of the antagonism by progesterone.

(ii) Toxaemia of Pregnancy

Pregnancy toxaemia is a condition characterized by fluid retention, generalized oedema, elevated blood pressure, proteinuria, irritability, headache, and sometimes by the eventual development of convulsions and coma. The prominence of both oedema and hypertension in the symdrome of pregnancy toxaemia suggested a possible role for the renin-angiotensin system in this condition.

(a) Established Toxaemia

Brown et al (1965) found that in none of seventeen patients with pre-eclampsia and hypertension did the plasma

renin concentration exceed the range found in normal pregnancy, and plasma renin activity was not higher in three patients with toxaemia studied by Winer (1965) compared with normal pregnancy. Helmer and Judson (1967) found that renin substrate was not significantly different in pregnant women with raised blood pressure when compared with normal pregnancy, but that the lowest renin concentration and renin activity levels were found in the presence of toxaemia.

However, in toxaemia of pregnancy, another feature, proteinuria, is due to a glomerular lesion which is identifiable on light (Dieckman et al, 1957; Pollack and Nettles, 1960) and on electron (Altchek, 1964; Mautner et al, 1962; Spango et al, 1959) microscopy, and which results in impaired sodium excretion (McCartney et al, 1964). Thus, the more severe the renal lesion the more sodium retention occurs and the greater the degree of suppression of renin (Brown et al, 1965). Interpretation of renin levels in established toxaemia is complicated by this possibly over-riding factor.

(b) Renin Activity Prior to Development of Toxaemia

Gordon et al (1969), in a prospective study, found that mean plasma renin activity in the middle trimester was higher in ten women who subsequently developed toxaemia than in 48 women with subsequent uneventful pregnancy. Following sodium restriction and administration of a diuretic, the plasma renin activity values rose in both groups, but the difference was accentuated. It was

suggested that the elevated renin activity of pregnancy is one of several aetiological factors in the development of toxaemia of pregnancy and that those women with more striking elevation in early pregnancy are more prone to develop toxaemia later in pregnancy.

2. PLASMA ANGIOTENSIN II CONCENTRATIONS IN NORMAL PREGNANCY

The present study was undertaken in order to further clarify the role of the renin-angiotensin system in pregnancy. It was felt that, as all the known effects of the renin-angiotensin system appear to be mediated through angiotensin II, measurement of the latter substance would give the best indication of activity of the system. The study was divided into three parts as follows:

(a) Measurement of plasma angiotensin II concentrations in a group of women representing all stages of normal pregnancy in order to detect any trends which might be evident. These patients were all attending the Antenatal Clinic, The Queen Elizabeth Hospital and all had uncomplicated pregnancy; in particular, they had no evidence of hypertension, albuminuria, or fluid retention.

(b) A prospective study of plasma angiotensin II levels in a small group of patients with normal pregnancy in order to confirm that any trends suggested by the survey outlined in part (a) above were validated by repeated sampling in the same patients.

(c) A preliminary study of plasma angiotensin II levels in a small group of patients with established pre-eclamptic toxaemia.

Plasma samples from subjects in all three groups were collected from ambulant patients, between 8:00 am and 11:00 am. The samples were collected into cooled syringes containing EDTA and BAL, transferred to cooled polypropylene tubes and the plasma separated as soon as practicable. Angiotensin II was extracted from the plasma by the Fuller's earth method and the concentration present assayed in the radioimmunoassay procedure in the usual way.

The results from part (a) of the study are shown in graphic form in Figure 15. Sixty-six patients with normal pregnancy uncomplicated by toxaemia and representing all stages of pregnancy from six weeks to term were included in the study. It can be seen from the figure that 70% of the values for all three trimesters of pregnancy are above the range for normal, non-pregnant women. There is an apparent trend for the plasma angiotensin II values to be somewhat higher during the second trimester than during the first and third, and this is shown in the average values for each trimester (Fig.16). When subjected to single classification analysis of variance, however, this difference in mean values of angiotensin II in the three trimesters proved to be non-significant ($p > 0.05$).

The results of repeated assay of plasma angiotensin II throughout normal pregnancy in the three subjects who completed part (b) of the study are shown in Table 5. Values are elevated in two and high normal in the other, and these high levels are maintained throughout pregnancy. There is some variability but this does not follow a constant pattern.

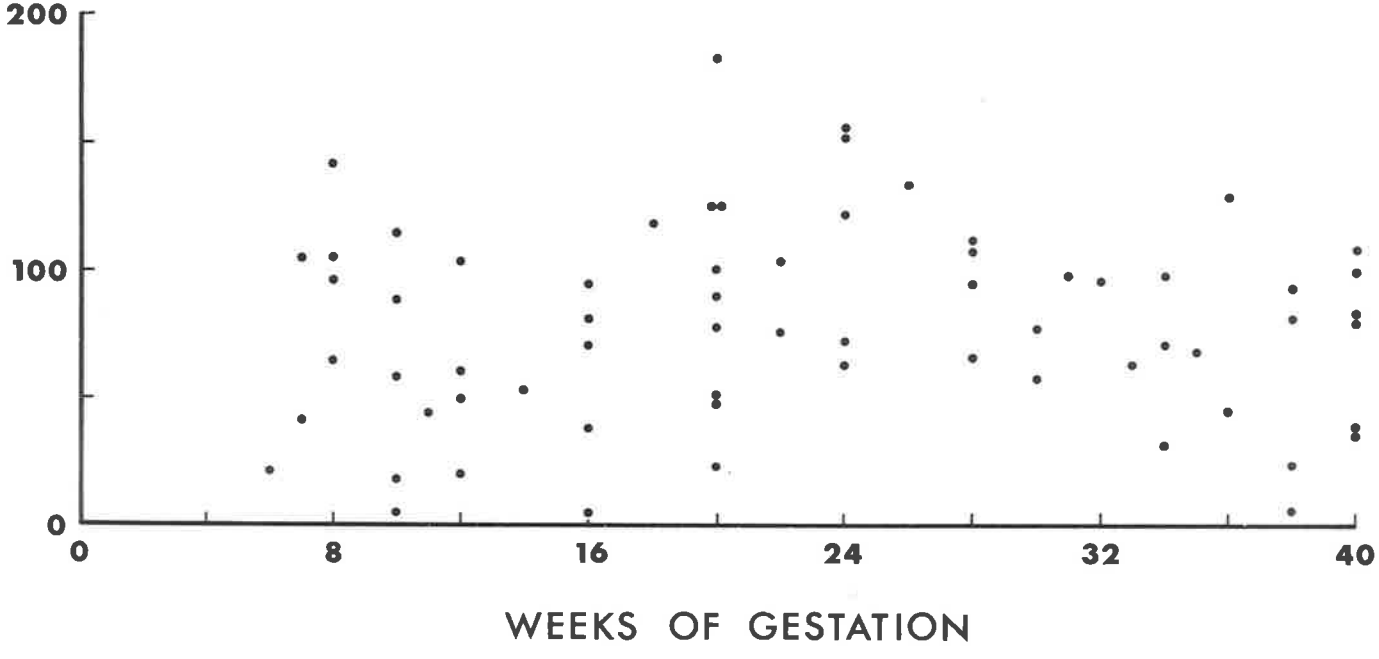


FIGURE 15

Plasma angiotensin II values during normal pregnancy

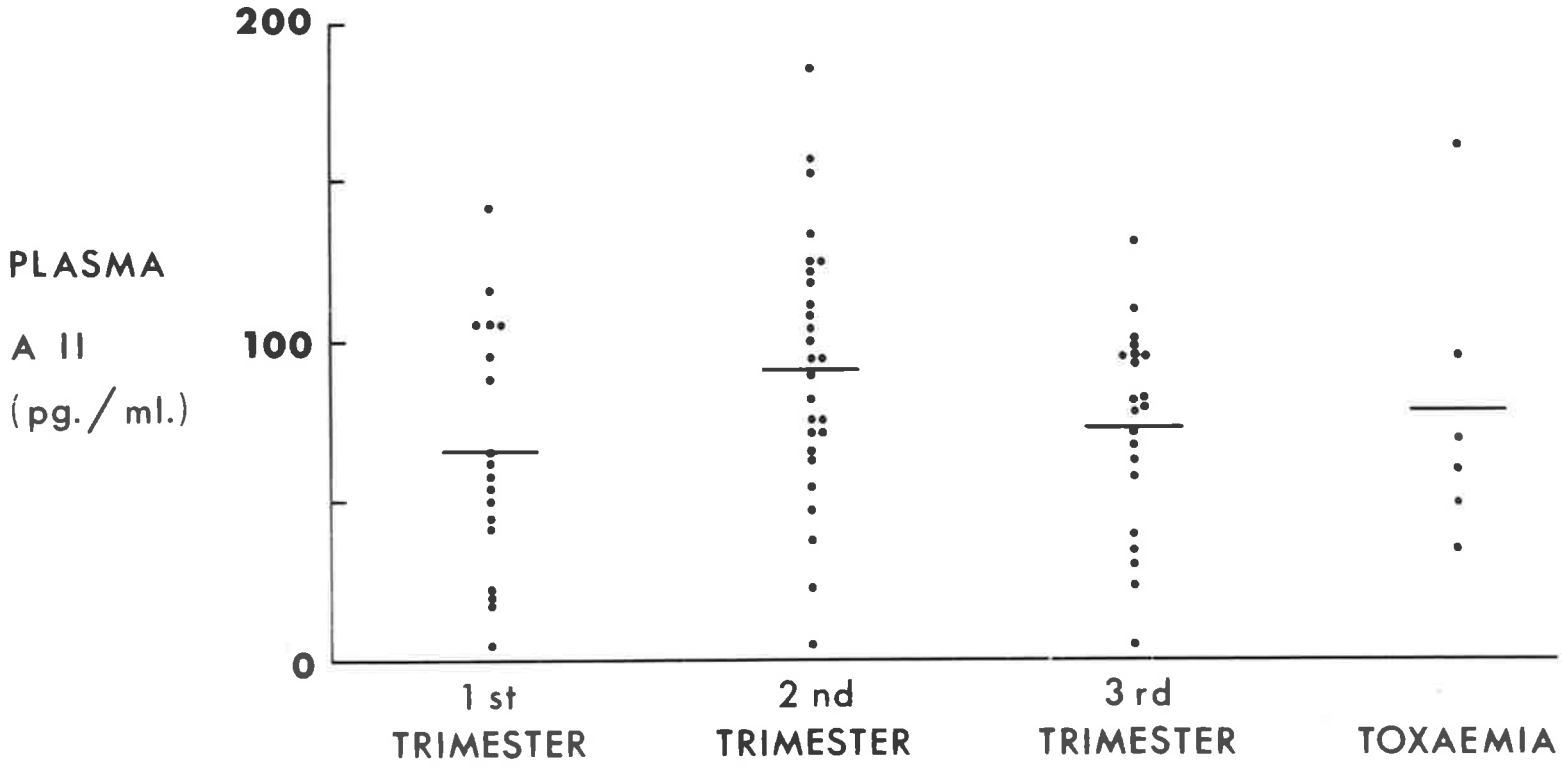


FIGURE 16

Plasma angiotensin II in normal pregnancy vs toxaemia

TABLE 5

Plasma angiotensin II concentrations on repeated sampling throughout pregnancy in three subjects with normal pregnancy

Subject	Weeks of Gestation	Plasma Angiotensin II
A	8	48 pg/ml
	12	42 "
	16	62 "
	24	70 "
	32	41 "
B	8	95 pg/ml
	16	84 "
	24	121 "
	32	100 "
	40	72 "
C	10	65 "
	14	79 "
	18	94 "
	26	70 "
	36	80 "

The results of this study are consistent with the findings of increased activity of the renin-angiotensin system during normal pregnancy in previous studies measuring plasma renin activity, plasma renin concentration, and renin substrate. The finding of increased plasma angiotensin II values throughout pregnancy is consistent with the renin activity data, although the increase in plasma angiotensin II is relatively less than the increase in plasma renin activity reported by some authors (Genest et al, 1965; Winer, 1965). The increase in plasma angiotensin II occurs early in the course of pregnancy and was demonstrable as early as the seventh week in the present study. The increased angiotensin II levels during pregnancy could possibly be due to the increased stimulation of aldosterone production required for sodium homeostasis in the presence of the antagonism of the peripheral effect of aldosterone by the high levels of progesterone (Landau et al, 1965), or could be due to an inappropriate increase secondary to the raised levels of renin substrate and failure to suppress renin secretion.

3. PLASMA ANGIOTENSIN II CONCENTRATIONS IN PRE-ECLAMPTIC TOXAEMIA

In part (c) of the study outlined previously, plasma samples were obtained from six women with established toxaemia of pregnancy as evidenced by a blood pressure reading of greater than 130/90 mm Hg together with proteinuria and/or fluid retention. The plasma angiotensin II values in this group of patients were not significantly different from those of normal pregnancy (Fig.16).

The finding that plasma angiotensin II is not significantly higher in pregnant women with established toxaemia compared with normal pregnancy is consistent with similar reports regarding renin values (Brown et al, 1965; Winer, 1965; Helmer and Judson, 1967). While this result would seem to indicate that the renin-angiotensin system is not concerned with the development of toxaemia of pregnancy, it is possible that these values are in fact inappropriately high in the presence of increased sodium retention (Davey et al, 1951). Before a role for the renin-angiotensin system in the syndrome of toxaemia can be completely discounted, further studies embodying careful sodium balance data will be required. This is, of course, difficult or impossible to achieve since toxaemia is usually recognized only once it is fully developed. Prospective studies similar to that of Gordon et al (1969) but commencing just prior to the onset of toxaemia would be necessary in order to provide an answer to this problem.

CHAPTER 7

SHEEP STUDIES

1. INTRODUCTION
2. CANNULATION OF RENAL LYMPHATICS
3. PLASMA AND RENAL LYMPH RENIN CONCENTRATION, RENIN ACTIVITY, AND ANGIOTENSIN II CONCENTRATION IN NORMAL SHEEP
4. RENIN AND ANGIOTENSIN II LEVELS IN SHEEP FOLLOWING SODIUM DEPLETION
5. EFFECT OF MERCURIC CHLORIDE INFUSION ON PLASMA AND LYMPH RENIN AND ANGIOTENSIN II
6. EFFECT OF MERCURIC CHLORIDE INFUSION ON RENAL LYMPH FLOW RATE AND RENAL HISTOLOGY
7. EFFECT OF MERCURIC CHLORIDE INFUSION ON URINARY RENIN CONCENTRATION
8. DISCUSSION

CHAPTER 7

SHEEP STUDIES

The studies described in this chapter were carried out in collaboration with Dr. G.H. McIntosh, from the C.S.I.R.O. Division of Nutritional Biochemistry, Kintore Ave., Adelaide, who performed the surgical procedures described.

1. INTRODUCTION

The use of fine plastic cannulae for the collection of lymph in conscious rats was introduced by Bollman et al (1948). These techniques afforded many advantages over the previously used glass cannulae and were adapted to the sheep by Lascelles and Morris (1961), who extended the scope of these preparations to enable lymph to be collected from a wide variety of organs.

Lever and Peart (1962) showed the presence of a pressor material in renal lymph from anaesthetized dogs. The material was not detectable in lymph derived from other organs and was shown by biochemical and pharmacological methods to be indistinguishable from dog renin. Constriction of one renal artery led to an increased concentration of this material in the lymph from the ischaemic kidney.

Higgins et al (1964) demonstrated in thoracic duct lymph of dogs with secondary hyperaldosteronism due to thoracic inferior vena cava constriction the presence of increased pressor and aldosterone-stimulating activities compared with normal dogs. A marked decrease or disappearance of the renin-like activity from thoracic duct lymph

was produced by nephrectomy, indicating a renal origin of the material. The results were thought to indicate increased release of renin from the kidney into renal lymph of dogs with secondary hyperaldosteronism.

The relative importance of this route of renin secretion into the blood stream has been difficult to assess because of the difficulties of quantitating lymph flow and of measuring small arterio-venous differences in the enzyme concentration across the kidney by currently available, relatively insensitive assay methods. Investigation of the intra-renal handling of renin could be expected to help clarify the situation. Rappeli and Peart (1968) investigated the renal excretion of renin infused intravenously in rats. The administration of both mercuric chloride and maleic acid, agents with an action at the renal tubular level, led to a sixfold increase in the amount of renin appearing in the urine. The authors felt that the most likely explanation for this result was a decreased renal tubular reabsorption of renin following glomerular filtration. Lumbers and Skinner (1969) described urinary renin concentrations in human subjects which were approximately 7% of plasma levels. Considerable variation in renal output and clearance of renin was found but levels remained similar over several months in any one subject. Natriuretic administration led to fivefold elevations of plasma renin, twofold increase in renin excretion, and caused renin clearance to fall to 43% of control values. The authors suggested that renin was filtered at the glomerulus and selectively reabsorbed.

On the basis of the above evidence it seems reasonably certain that renin is filtered at the glomerulus and subsequently partially reabsorbed by the renal tubules. However, the origin of the renin present in renal lymph is uncertain. The possible sources of lymph renin are: (a) direct access to lymph from plasma; (b) access to lymph following tubular reabsorption of filtered renin; and (c) secretion of renin into the renal interstitial tissue from the juxtaglomerular region and thence into lymph. The present studies were undertaken in an attempt to clarify the intrarenal handling of renin, to identify the source of the enzyme in lymph, and to determine whether the conditions were such (e.g. renin substrate and converting enzyme available) that angiotensin II could be produced locally in the kidney. This would have important implications in intrarenal regulation of blood flow and sodium reabsorption (Thurau, 1964).

2. CANNULATION OF RENAL LYMPHATICS

An area of skin over the flank of the sheep was clipped of wool and sterilized. An incision was made below the lateral processes of the lumbar vertebrae parallel to the spine and the kidney was approached and mobilized retroperitoneally. The efferent lymphatics were identified and followed towards the renal hilus, where one or two readily accessible lymphatics were prepared for cannulation while the others were tied off. The number of renal lymphatics usually varied from two to six. One or two lymphatics were cannulated with polyethylene tubing (usually 0.55 mm I.D.,

0.90 mm O.D.) at a point about 2 to 3 cm from the hilus of the kidney. The cannulae were tied into place, anchored with stay sutures, and brought out through the psoas major muscle above the line of incision.

3. PLASMA AND RENAL LYMPH RENIN CONCENTRATION, RENIN ACTIVITY, AND ANGIOTENSIN II CONCENTRATION IN NORMAL SHEEP.

Renal lymph was collected from normal sheep after cannulation of renal hilar lymphatics as described above. Lymph and blood samples were collected simultaneously into cooled polypropylene tubes containing EDTA and BAL. Samples were assayed for plasma renin activity, plasma renin concentration, and renin substrate by the method of Skinner (1967), and for angiotensin II by radioimmunoassay. The structure of sheep angiotensin II has been shown to differ from human angiotensin II only at the position of the fifth amino acid, where valine is present instead of the isoleucine of the human form (Cain et al, 1970). The antibody used for the radioimmunoassay was shown to have identical affinity for these two forms of angiotensin II.

Results of the study are shown in Table 6. They may be summarized as follows:

(1) Lymph renin concentration was consistently higher than plasma renin concentration (6-16 times as high, mean 12 times).

(2) Renin activity was significantly higher in renal lymph than in plasma but the difference was less marked than in the case of renin concentration values (4-8 times as high, mean 5 times).

TABLE 6

	Renin Activity (ng/100ml/3hrs)	Renin Concentration (ng/100ml/3hrs)	Renin Substrate (ng/ml)	A II Concentration (pg/ml)	
Jugular Plasma	464	2,745	104		1
Renal Lymph	1,664	42,400	98		
Jugular Plasma	112	470			2
Renal Lymph	575	7,625		25	
Jugular Plasma	157	660			
Renal Lymph	778	7,670		21	
Jugular Plasma	610		186		3
Renal Lymph	2,510		135		
Jugular Plasma	345	1,125	55	9	4
Renal Lymph	2,607	6,940	40	219	
Jugular Plasma	430	870	173	11	5
Renal Lymph	1,940	9,340	164	58	

(3) Renin substrate was slightly lower in renal lymph than in plasma (mean 109 ng/ml vs. mean 130 ng/ml).

(4) Angiotensin II concentration was much higher in renal lymph than in plasma on the two occasions on which they were compared. Plasma levels were comparable with those reported by Cain et al (1970) (14 ± 11 (S.D.) pg/ml).

These results confirm the previous report of higher renin concentration in renal lymph than in plasma (McIntosh and Morris, 1971). The higher renin activity values in renal lymph than in plasma indicate the presence of adequate renin substrate in the lymph compartment, which was confirmed by measurement of substrate. The observation that the difference in lymph:plasma ratio was not as marked as in the case of renin concentration could be explained by the lower concentration of substrate in lymph compared with plasma, a situation which was not unexpected in view of the lower protein concentration in lymph than in plasma (McIntosh and Morris, 1961). The values obtained for renin substrate in jugular venous plasma, however, were lower than those previously reported in normal sheep. This finding is probably explained by the demonstration that the renal renin used in the assay of the renin substrate was not entirely free of angiotensinase activity. The final value was thus the result of generation of angiotensin together with breakdown of a fraction of this generated angiotensin. The substrate figures probably indicate the general trend correctly, but are not accurate in terms of absolute values.

The demonstration of angiotensin II in renal lymph by specific radioimmunoassay suggests the presence of converting enzyme in lymph or interstitial tissue; or, alternatively, the angiotensin II may gain access to the lymph after it has already been formed. This possibility was investigated by infusion of ^{125}I-labelled angiotensin II into the renal artery of a sheep for one hour and collecting urine and renal lymph. The renal lymph, urine, and plasma samples were counted in a well-type counter and the following results were obtained:

	Counts/100 sec/ml
Renal artery plasma (at termination of perfusion)	37,620
Renal vein plasma (at termination of perfusion)	37,030
Renal lymph	44,550
Urine	522,570

It can be seen that there was a small arterio-venous difference across the kidney, that the concentration of counts was higher in renal lymph than in plasma, and that a great deal of radioactivity was present in the urine. In order to ascertain whether the radioactivity represented intact angiotensin II, samples of urine and renal lymph were incubated with an excess of angiotensin II antibody and then subjected to gel filtration on a G25 Sephadex column. The results are shown in Figure 17. In this system, intact angiotensin II is bound to antibody and appears in the void volume, whereas fragments of angiotensin II with less immuno-reactivity are not bound to the

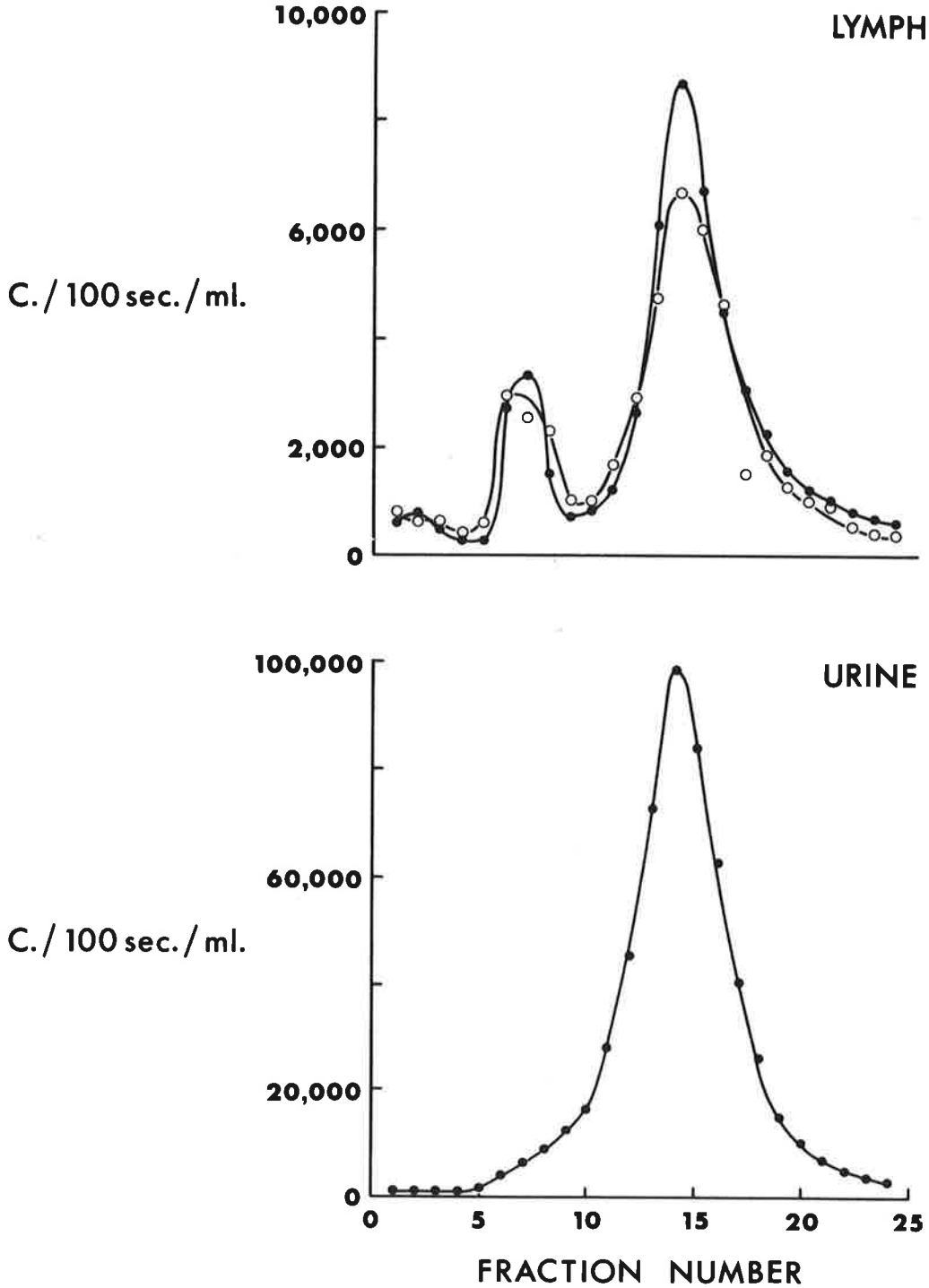


FIGURE 17

Gel filtration of urine and lymph containing labelled peptide after incubation with angiotensin II antibody

same extent. It can be seen that, in the case of renal lymph, a small fraction of the radioactivity appeared in the void volume (approximately 15%) whereas almost none of the radioactivity present in urine demonstrated persistent immunoreactivity. It was concluded from this experiment that the urinary radioactivity represented completely denatured fragments. The renal lymph contained a small fraction of the radioactivity with intact immunoreactivity, but at least part of this could be accounted for by the ability of fragments to retain their reactivity with antibody. It was concluded that the contribution to lymph angiotensin II concentration made by peptide gaining direct access in this manner would be slight.

4. RENIN AND ANGIOTENSIN II LEVELS IN SHEEP FOLLOWING SODIUM DEPLETION

Sodium depletion was induced in three sheep by production of a parotid fistula. Plasma and renal lymph samples were collected and assayed for renin concentration, renin activity, and angiotensin II concentration. The effect of sodium depletion on these parameters is shown in Table 7. It can be seen that there was an increase in both plasma and lymph renin activity, renin concentration, and angiotensin II concentration following sodium depletion but no consistent changes in renin substrate. The changes are as would be expected following the stimulation of renin production by the kidney consequent upon sodium depletion.

TABLE 7

		Renin Activity ng/100ml/3hrs	Renin Concentration ng/100ml/3hrs	Renin Substrate ng/ml	A II Concentration pg/ml
A8-018 (Parotid drainage commenced 8/6/70)					
5/6	Jugular plasma	112	470		
	Renal lymph	575	7,625		25
7/6	Jugular plasma	157	660		
	Renal lymph	778	7,670		21
11/6	Jugular plasma	412	1,210		
	Renal lymph	723	9,290		169
12/6	Jugular plasma	604	1,400		
	Renal lymph	1,215	16,600		
A8-026 (Parotid drainage commenced 2/2/70)					
3/2	Renal lymph	360	3,256	82	
	Jugular plasma	305	1,270	130	
	Renal vein plasma	281	1,170	153	
5/2	Renal lymph	4,480	19,170	27	
	Jugular plasma	2,270	3,640	52	
A8-141 (Parotid drainage commenced 10/1/70)					
8/1	Renal lymph	2,607	6,940	40	219
	Jugular plasma	345	1,125	55	9
	Renal vein plasma	365	1,250	27	
9/1	Renal lymph	3,760			94
	Jugular plasma	387			
	Renal vein plasma	522			
14/1	Renal lymph	4,096			
21/1	Jugular plasma	1,326		46	16
22/1	Renal lymph	3,070		20	86
23/1	Jugular plasma	1,140	6,200	22	30
	Renal lymph	1,704	15,760	21	1,041

5. EFFECT OF MERCURIC CHLORIDE INFUSION ON PLASMA AND LYMPH RENIN AND ANGIOTENSIN II

In an attempt to clarify further the intrarenal handling of renin and to assess the contribution of renin reabsorbed from the renal tubules to the renal lymph renin, mercuric chloride was administered to three sheep in order to assess the effect of a tubular blocking agent on plasma, lymph and urinary renin, and plasma and lymph angiotensin II.

The results from these three sheep are shown in Figures 18, 19 and 20.

A8-521 The dose of mercuric chloride given in this case was too large (1 Gm) and the animal quickly developed toxic effects and died within 24 hours. The results obtained prior to death are shown in Figure 18. It can be seen that during the short period in which observations were made, there was an increase in renin activity, renin concentration, and angiotensin II concentration in both plasma and lymph, the changes being of relatively greater magnitude in renal lymph.

A8-509 This animal survived much longer following administration of a lower dose of mercuric chloride (75 mg of mercuric ion). Following the infusion, all parameters showed a progressive rise, again with relatively greater changes in renal lymph than in plasma, except for the values on the fifth day after infusion when there was a sharp decrease in all three parameters

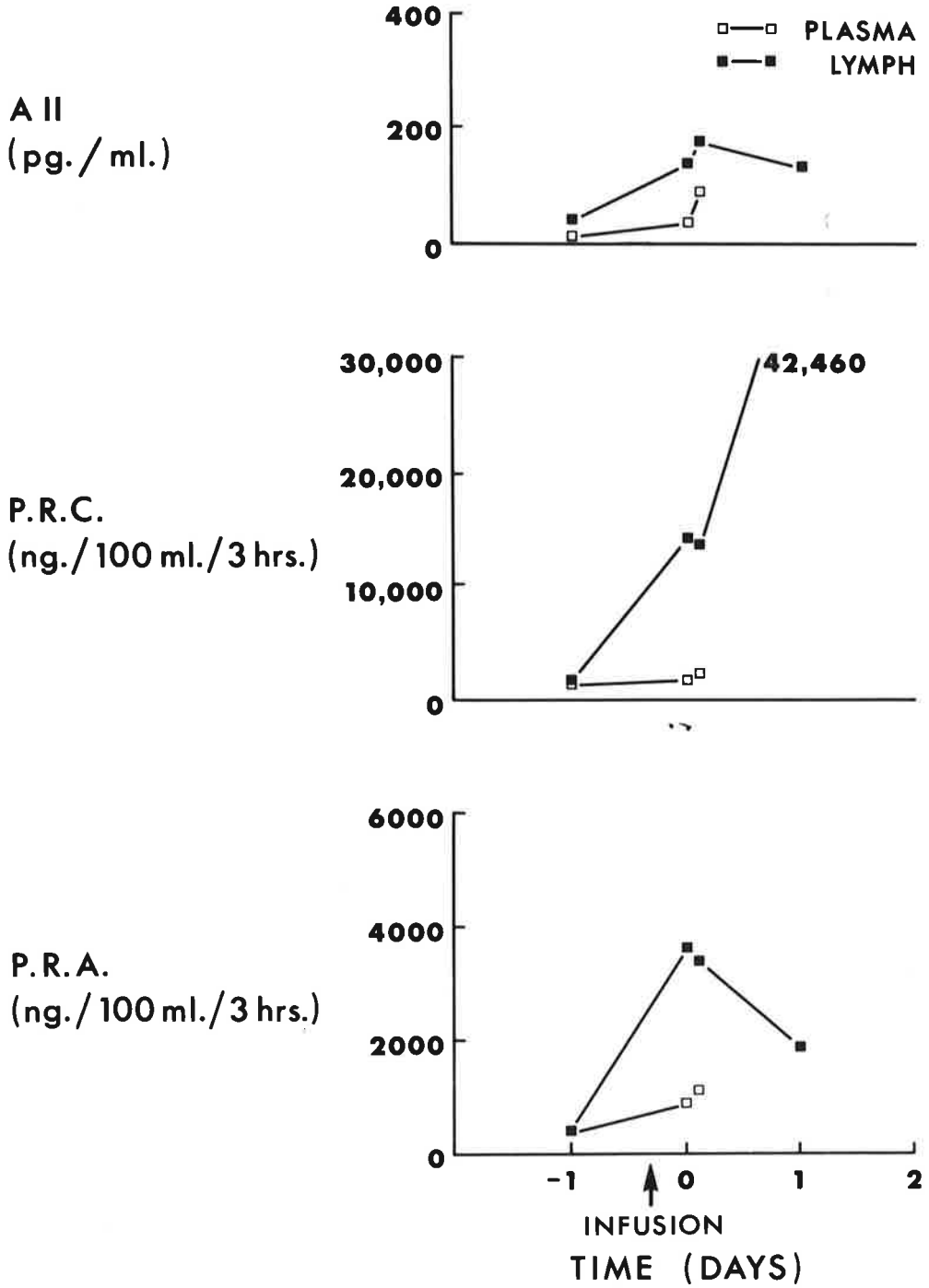


FIGURE 18

Plasma and lymph AII, PRC and PRA following administration of mercuric chloride.

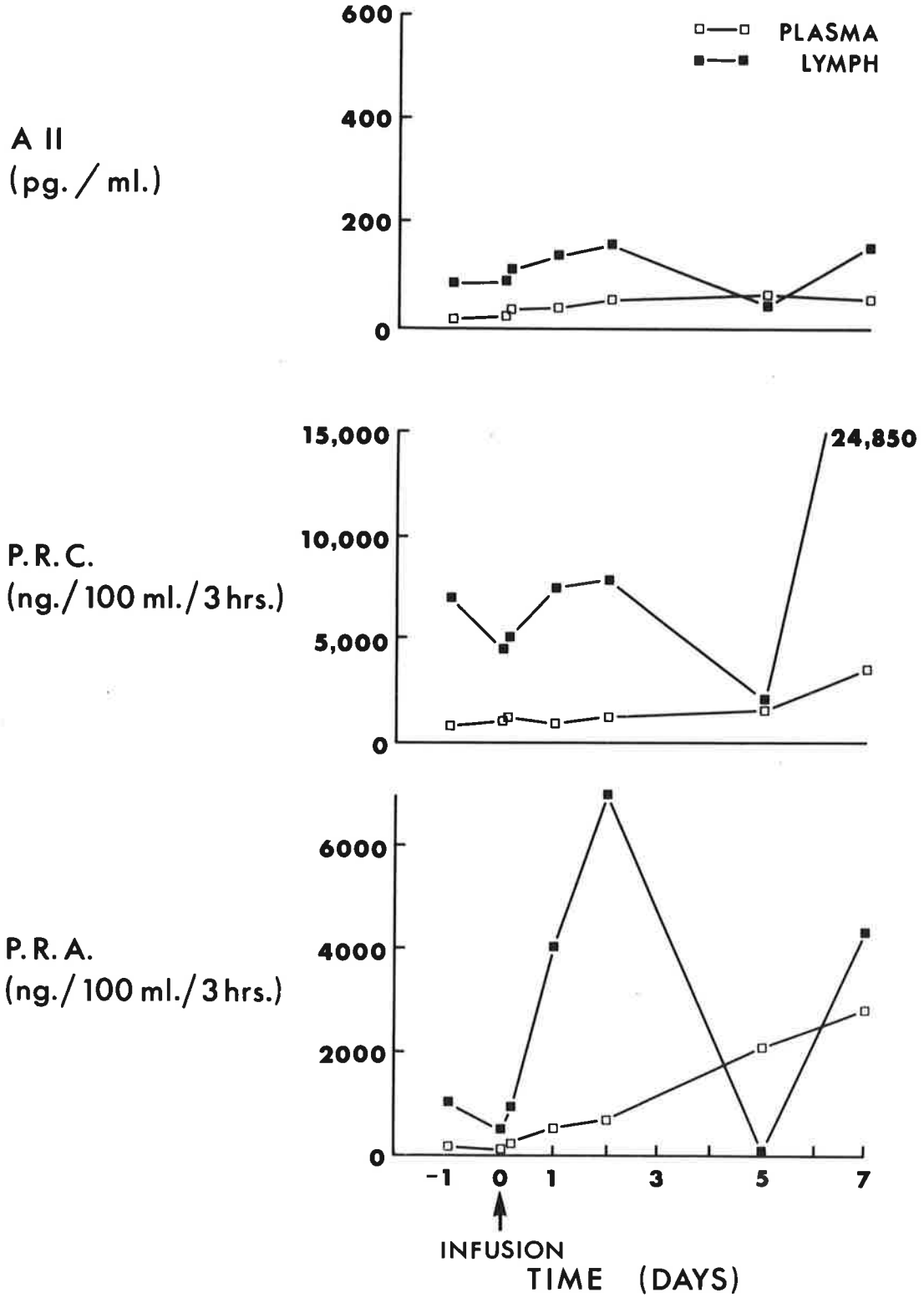


FIGURE 19

Plasma and lymph AII, PRC and PRA following administration of mercuric chloride.

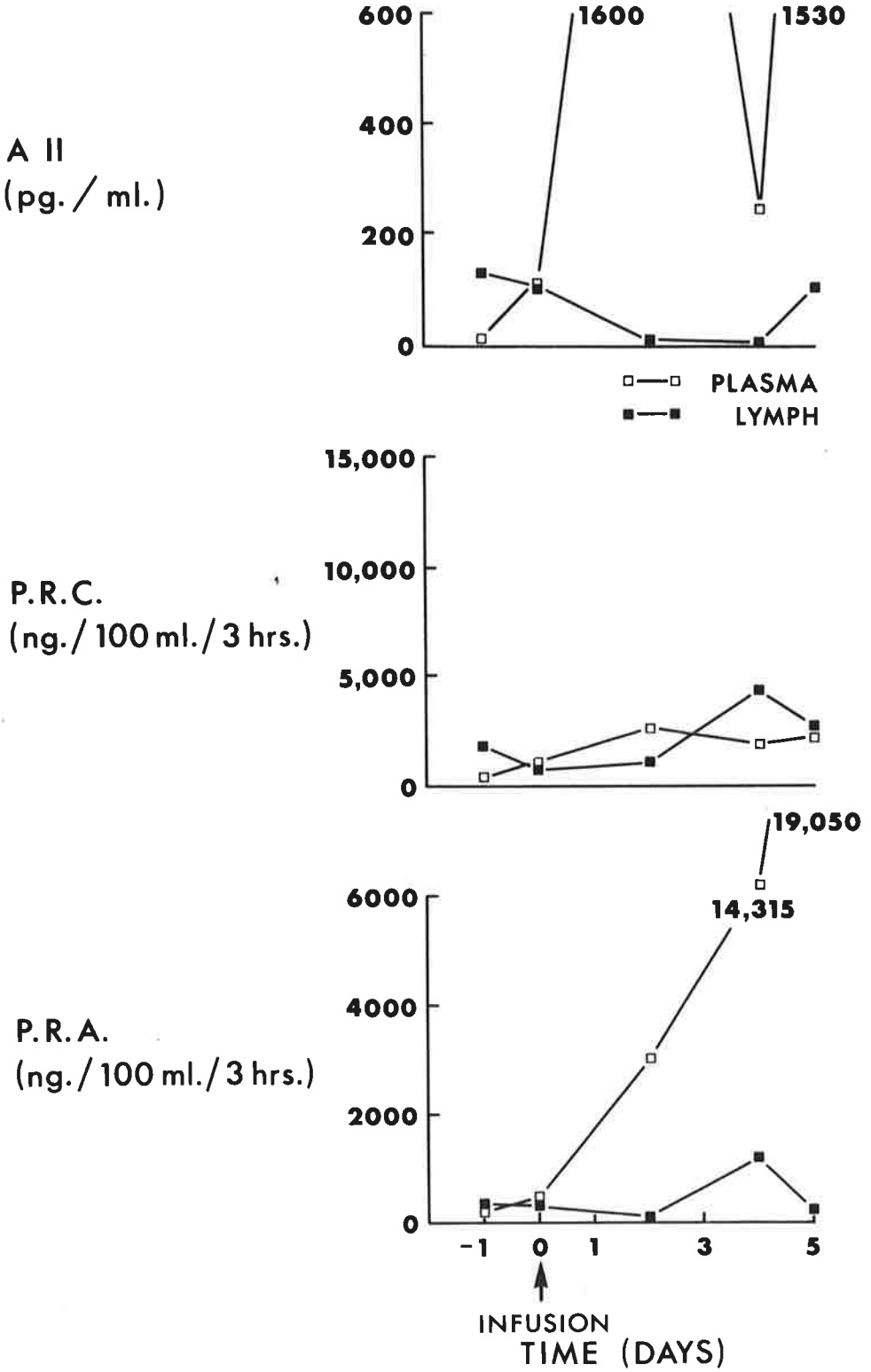


FIGURE 20

Plasma and lymph AII, PRC and PRA following administration of mercuric chloride.

measured in lymph while the plasma values continued to rise or remained stationary.

A8-519 The response seen in this animal given a smaller dose still (63 mg of mercuric ion) was different from that seen in the other two. The plasma values of renin activity, renin concentration, and angiotensin II concentration rose following infusion and remained high. In renal lymph, on the other hand, all three parameters showed an initial decrease lasting one to three days followed by a modest later rise to levels which significantly exceeded the pre-infusion levels only in the case of renin concentration.

All three animals given mercuric chloride suffered severe scouring with marked loss of fluid and electrolytes from the gastro-intestinal tract and developed acute anuria within 24 hours. This fluid and electrolyte loss would be expected itself to act as a stimulus to renin secretion.

6. EFFECT OF MERCURIC CHLORIDE INFUSION ON RENAL LYMPH FLOW RATE AND RENAL HISTOLOGY

Following the infusion of mercuric chloride, there was an initial increase in urinary output in each animal studied. This increase, however, was of short duration and was followed by complete renal shutdown within 24 hours in each instance. The renal lymph flow rates remained at the control levels during this stage of diuresis but rose to significantly elevated values after renal shutdown had ensued. The changes in lymph flow rates are shown in Table 8.

TABLE 8

Day of Study	Lymph Flow Rate (ml/hr)	
	A8-509	A8-519
-1	1.3	1.7
0	Mercuric Chloride Infusion	
1	2.1	
2	25.5	2.0
4		33.0
5	33.0	35.0
7	19.2	

Renal histology was checked in each animal after death. It was found that the mercuric chloride infusion had produced severe disruption of the renal architecture in each case, with particularly severe effects on the renal tubules, which showed complete coagulative necrosis. The interstitial tissue space was markedly increased, an effect consistent with the increased lymph flow rates observed.

7. EFFECT OF MERCURIC CHLORIDE INFUSION ON URINARY RENIN CONCENTRATION

In two of the sheep given mercuric chloride infusion, hourly urine collections were made over the first six hours following commencement of the infusion. The urine samples were concentrated by vacuum dialysis and the concentrated samples were submitted to the Skinner (1967) method for determination of renin concentration, the angiotensin generated from sheep substrate being measured in the rat bioassay system. In one instance (A8-509) the prepared samples were found to consistently give depressor responses in the bioassay so that no values for renin concentration could be obtained. In the other animal (A8-519) values for urinary renin concentration were as follows:

U 1	386 ng/	100 ml/	3 hours	
U 2	798 "	"	"	
U 3	163 "	"	"	
U 4	1191 "	"	"	
U 5	3326 "	"	"	
U 6	514 "	"	"	

The results show that there was an increase in the urinary renin concentration, reaching a peak value in the fifth hour after commencement of the mercuric chloride infusion.

8. DISCUSSION

These studies have confirmed the previous observation (McIntosh and Morris, 1971) that the concentration of renin in renal lymph is higher than in plasma. They have also demonstrated that the renin activity and angiotensin II levels in renal lymph are higher than the corresponding plasma figures. The renin substrate results were inconclusive, but there was a suggestion that the levels were slightly lower in renal lymph than in plasma.

The response of renin concentration, renin activity, and angiotensin II concentration in both plasma and renal lymph to sodium depletion was consistent with increased secretion of renin from the kidney in response to this stimulus, all parameters showing a similar degree of elevation.

The administration of the renal tubular toxin mercuric chloride led to increases in plasma renin and angiotensin II in all cases but changes in renal lymph which were variable and possibly dose-dependent.

The interpretation of these results presents a number of difficulties. The parallel increases in renin activity, renin concentration, and angiotensin II concentration in plasma and lymph would be consistent with an origin of renin from any of the postulated sources and this would apply also to the situation of dietary sodium depletion where all parameters showed similar changes. The labelled angiotensin II infusion experiment suggested that direct access of intact angiotensin II to renal lymph from plasma was unlikely.

A contribution to renal lymph renin from tubular reabsorption could not be completely excluded but this is unlikely to be of any magnitude for the following reasons: (a) blocking of tubular reabsorption of renin with mercuric chloride led to an increase in urinary renin but the lymph renin concentration rose over this period, and (b) increased levels of renin were seen in the renal lymph after complete cessation of renal flow had occurred.

It seems likely that the renin in renal lymph gains access to this compartment through direct secretion into the renal interstitium from the juxtaglomerular region. To explain the dissociation between plasma and lymph values of renin and angiotensin II following mercuric chloride infusion in one animal and on one occasion in another, it is necessary to postulate a differential effect on the secretion of renin into the renal interstitial tissue and the route of secretion into plasma. This dissociation can be partly accounted for by the increased lymph flow seen after the mercuric chloride infusion, flow rates increasing up to 15 times. This is not the whole explanation, however, as similar increases in flow rates were seen in all animals despite the variation in results. The mercuric chloride led to considerable disruption of the renal architecture, with particularly severe effects on the renal tubules, and it is possible that a selective impairment of renin secretion into the renal interstitium could have occurred. An alternative explanation would be that the severe toxic changes could lead to oedema and ischaemia of the kidney; if the renal blood flow were low enough, a greater percentage of the renin secreted could gain access to the lymph.

This would lead to higher lymph values under conditions of greater renal damage and lower values when the damage was less severe. However, the higher values in lymph than in plasma under normal conditions is evidence against this hypothesis.

In view of the demonstration that plasma renin concentration can rise to high levels while the renin concentration in renal lymph is very low, a significant contribution to the plasma renin from this source seems unlikely. Further experiments using smaller doses of mercuric chloride might help to clarify some of these points.

CHAPTER 8

DISCUSSION

The initial response to the demonstration of a potent pressor substance of renal origin and the experimental production of hypertension resembling clinical hypertensive disease in man was optimism that further investigation of the renin-angiotensin system would provide answers to many of the questions regarding the clinical situation. Further investigation, however, revealed that, although the renin-angiotensin system was probably important in the aetiology of renovascular hypertension and in most cases of malignant or accelerated hypertension, its role in the aetiology of essential hypertension was by no means clear. Measurements of activity of the renin-angiotensin system using methods available up to the present have not consistently shown any increased activity in subjects with essential hypertension when compared with normal subjects.

A role for the renin-angiotensin system in the aetiology of essential hypertension cannot be completely discounted, however, for it may be possible that the "normal" levels found in essential hypertension are inappropriately high in the face of increased avidity of sodium retention or some other factor which would normally suppress the activity of the system. In this context, the substantial group (approximately 20%) of patients with essential hypertension and suppressed plasma renin activity are of interest

(Channick et al, 1969), and it has been suggested that some as yet unknown mineralocorticoid might be present in excess in such patients (Woods et al, 1969).

There is also a possibility that plasma levels of renin and angiotensin do not reflect the concentrations present at the receptor sites of importance for the action of these substances, and studies of the tissue binding of angiotensin may provide some of the answers to this problem (Goodfriend and Lin, 1970).

Investigations of the role of the renin-angiotensin system in normal sodium and water homeostasis have shown that angiotensin acts as a trophic hormone for the release of aldosterone from the adrenal cortex, as well as producing vasoconstriction and having a probably minor direct effect on renal tubular function, and in addition has a complex but possibly important effect on the autonomic nervous system. The control of aldosterone secretion depends not only on the angiotensin II concentration in plasma but also on the concentrations of ACTH, potassium, sodium, and probably other as yet unidentified factors (Blair-West et al, 1970). There is thus a complex homeostatic mechanism for sodium and water, involving the above factors as well as the Antidiuretic Hormone mechanism.

Methods of assessing the activity of the renin-angiotensin system up to the present time have not been completely satisfactory. Measurements of aldosterone excretion or aldosterone secretion rates can be made with considerable accuracy using double isotope methods but, as the secretion of aldosterone is affected by other factors, these

measurements do not necessarily reflect the activity of the renin-angiotensin system. Methods previously available for the assay of angiotensin in blood were not satisfactory because of the large volumes of blood required and the lack of sensitivity, which meant that values in normal subjects could often not be measured. Plasma renin activity and renin concentration methods enabled much useful information to be obtained but both are relatively non-specific and both have the disadvantage of a final bioassay step to measure the amount of angiotensin generated, thus introducing the inaccuracies inherent in this type of estimation.

Following the introduction of the radioimmunoassay technique for measurement of peptide hormones (Yalow and Berson, 1956) and the demonstration of antibodies to angiotensin (Deodhar, 1964), the application of this method to the assay of angiotensin in blood seemed a logical development, in the hope of achieving increased sensitivity and specificity. The radioimmunoassay procedure promised these advantages as well as ease and simplicity of handling of large numbers of samples. Application of the radioimmunoassay procedure to the measurement of plasma angiotensin II concentrations has certainly provided increased sensitivity, so that angiotensin II concentrations can be assayed with relative ease in normal subjects. There are, however, potential disadvantages which necessitate careful technique and strict attention to control procedures in order to avoid erroneous results.

The first major problem is that of specificity. Cain et al (1969) demonstrated that antibodies to angiotensin II differ in their ability to react with biologically inactive fragments of angiotensin II, thus raising the possibility of disparity between bioassay and radioimmunoassay results. These authors found that the largest fraction of the angiotensin II measured in venous plasma was in the form of the biologically inactive hexapeptide, but that the levels correlated reasonably well with arterial levels and could be used for routine clinical purposes. The antibody used in the present study has been shown to cross-react with biologically inactive fragments but the extent of the cross-reaction has not been characterized exactly. Although part of the angiotensin II measured in venous plasma during these studies may have been in the form of inactive fragments, it was felt that the assay gave a valid measure of the activity of the renin-angiotensin system. The results obtained using this antibody were comparable with those reported by other groups (Boyd et al, 1967; Catt et al, 1967; Page et al, 1969; Sundsfjord, 1970).

Non-specific effects of plasma extracts on the binding of labelled angiotensin II by antibody present a problem. It is necessary for the samples to be assayed and the standard curve reaction mixtures to have as nearly identical constitution as possible, since non-specific effects on antibody binding might be interpreted as displacement of labelled peptide by angiotensin II present in the samples. When plasma samples are assayed directly, the problem can be partially overcome by making up the standards in anephric plasma so that both

standard curve samples and samples to be assayed contain the same amount of plasma. This solution is not entirely satisfactory as the anephric plasma and the sample to be assayed may differ in constitution other than in the content of angiotensin. The methods reported which assay angiotensin in plasma directly (Gocke et al, 1969; Goodfriend et al, 1969) have yielded values higher than those obtained when a prior extraction procedure is employed, and some of this apparent loss of specificity may be related to non-specific effects of other constituents in plasma.

In the case of extracted plasma samples the situation is again not completely satisfactory. It is possible to use as blanks extracted anephric plasma, water, or to re-extract the plasma samples from which the angiotensin has already been removed by the extraction procedure. None of these is entirely satisfactory, however, for none ensures the same constitution, apart from angiotensin content, of samples and blanks. If non-specific effects on antibody binding are due to small peptides present in extracted plasma, the same peptides may not be present in the re-extracted blank sample. In practice, it was found that some variation in the percentage bound did occur in the case of the various blanks but the magnitude of this variation was such that it corresponded with a concentration of less than 3 pg/ml in the original sample.

It was found that ^{125}I-labelled val^{5}-angiotensin II amide ("Hypertensin"-Ciba) was more susceptible to non-specific damage than labelled isoleu^{5}-angiotensin II. The degree of damage was found to

have a linear relationship with the time of incubation in the radioimmunoassay procedure and produced a substantial error at 24 hours. The labelled isoleu^5-angiotensin II was not affected by prolonged incubation under these conditions and was, therefore, used routinely in the radioimmunoassay method.

Because of the very small concentrations of peptide being assayed, errors due to absorption of the hormone to glassware are very likely to occur and this was the reason for the high values for plasma angiotensin II initially reported by Vallotton et al (1967). In the present assay, in spite of the use of polypropylene equipment exclusively, absorption of hormone to tubes was troublesome unless the buffers used contained carrier protein. It was found that a concentration of human serum albumin of 1% was necessary in order to eliminate this problem.

The method of separation of bound and free hormone after incubation in the assay procedure was found to require careful attention to technique as significant dissociation of the antigen-antibody complexes occurred if there was any delay after the addition of the dextran-coated charcoal prior to centrifugation. This effect was greatly exaggerated at room temperature.

In spite of efforts to maintain constant conditions from one batch of assays to another, occasional batches yielded less satisfactory results for reasons which were not always apparent, and this is presumably an indication that all the factors affecting the reaction are not presently recognized. Although this variation from assay to assay did occur, it was found that when the same antibody and the same

labelled hormone were used, the percentage change in binding of tracer with the addition of standards was relatively constant irrespective of the initial binding figure. When the percentage change differed from that expected, there was usually some technical problem with that particular assay and all samples were reassayed in another batch.

Within the limits outlined above, the radioimmunoassay method afforded a precise, specific measure of the activity of the renin-angiotensin system and the procedure was used to investigate the role of this system in a variety of normal and abnormal situations.

Plasma angiotensin II concentration in normal subjects on unrestricted sodium intake was found to range from 4 pg per ml to 63 pg per ml, with a mean value of 25.2 ± 14.5 (S.D.). These results are comparable with those reported by several other groups (Boyd et al, 1967; Catt et al, 1967; Page et al, 1969; Sundsfjord, 1970). The effect of posture on plasma angiotensin II concentration was found to be similar to that for plasma renin activity, the values in the upright position being consistently higher than those measured following overnight recumbency. The parallel behaviour of plasma angiotensin II and plasma renin activity was also seen following dietary sodium restriction in the physiological studies in normal subjects. Although the values of these two parameters of activity of the renin-angiotensin system tended to parallel each other, the agreement was not perfect. This is not surprising when one considers the different techniques by which these values are obtained; the plasma angiotensin II concentration represents the concentration of

hormone consequent upon the in vivo action of renin and converting enzyme on the one hand and angiotensinases on the other, while the plasma renin activity represents the ability of plasma samples to generate angiotensin in vitro under unphysiological conditions following treatment to inhibit angiotensinases. It seems likely that the plasma angiotensin II concentration would be the better measure of activity of the system since it is more representative of the in vivo situation. However, this must be balanced against the demonstration by Cain et al (1969) that the substance assayed in venous blood may be largely inactive fragments of angiotensin II and, while this may be adequate for purposes of clinical screening of patients, arterial concentrations of angiotensin II should be used for precise physiological studies. It may be that even arterial levels do not represent the concentrations necessary for physiological action and tissue levels may be more critical in this regard (Goodfriend and Lin, 1970). This problem is currently not resolved, but, in the meantime, plasma angiotensin II concentration almost certainly presents a more precise assessment of activity of the renin-angiotensin system than plasma renin activity.

The application of the radioimmunoassay to measurement of plasma angiotensin II concentrations in situations of secondary hyperaldosteronism has confirmed high levels in patients with congestive cardiac failure, nephrotic syndrome, and hepatic cirrhosis with ascites. The role of the renin-angiotensin system in these conditions is difficult to assess, and this is complicated by the fact that the majority of these patients were being treated with diuretics and/or

sodium restriction at the time the samples were taken, so that the elevated angiotensin II levels may have been a secondary effect of the treatment rather than an integral part of the disease process. Support for this view was obtained by the finding of some values within the normal range in patients with untreated congestive cardiac failure.

In one case of primary aldosteronism associated with bilateral adrenal cortical hyperplasia, low levels of plasma angiotensin II, at the lower limit of the normal range, were found, and these correlated well with the plasma renin activity estimations which were also of this order. Both parameters failed to increase in response to the normal stimuli of dietary sodium restriction and upright posture. Plasma angiotensin II levels could be used as an alternative to plasma renin activity measurement in the screening of patients with hypertension for cases of primary aldosteronism.

In the group of patients with hypertension in whom plasma angiotensin II concentrations were assayed, values were found to range from within the normal range to quite high levels. Many of these patients were referred for investigation of possible renovascular hypertension and, in the group in whom the diagnosis was confirmed, the plasma angiotensin II was universally elevated. When this group was separated from the remainder, there were still many patients who had elevated angiotensin II levels. Some of these patients had strong evidence of renal parenchymal disease and this may have been the cause of the elevation (Catt et al, 1969). In the group with uncomplicated

"essential" hypertension, many of the angiotensin II values were within the normal range, but a substantial number were elevated so that the mean value was significantly above normal. A possible role for the renin-angiotensin system in benign essential hypertension has not been completely excluded but the nature of such a role is not evident at present. In the case of malignant or accelerated hypertension, however, the plasma angiotensin II concentration was almost invariably elevated whatever the aetiology of the hypertension, and here it may represent an important contributory factor to the clinical situation.

When the angiotensin II radioimmunoassay procedure was applied to the measurement of angiotensin generated during the in vitro method for plasma renin activity (Skinner, 1967), the values obtained were found to be much less than those obtained by bioassay. When assayed in doubling dilution, the curve obtained was found to coincide with the standard curve, confirming the identity of the substance measured as angiotensin II. The reason for the lower values obtained by radioimmunoassay is the inhibition of converting enzyme by the EDTA added to inhibit angiotensinases. As the immunoassay is specific for angiotensin II, only the hormone present in the form of the octapeptide is detected by this means and the values would be much lower, depending on the degree of inhibition of converting enzyme. If the degree of inhibition were constant from sample to sample, the angiotensin II radioimmunoassay could be used to assess renin activity in this way, even though the absolute values would be much lower. This, however, was not the case when comparisons were made of renin activity values obtained by the two

methods and the correlation was not close enough to enable this use of the radioimmunoassay technique. The only exception to this was found to occur in the case of simultaneously drawn and identically processed samples from the two renal veins, collected to investigate the presence of significant renal artery stenosis. In this case, the ratio of renin activities (high side:low side) was found to correlate closely by the two methods. This presumably comes about because the two samples are collected simultaneously from the two sides and processed identically, so that the conditions determining the fraction of angiotensin present in the form of the octapeptide are relatively constant and the ratio gives a valid indication of differences in renin activity.

Attempts to reverse the inhibition of converting enzyme by the addition of various metal ions were not successful because of simultaneous activation of angiotensinases and this approach to the problem was not practicable. It would seem more desirable to ensure complete inhibition of converting enzyme and to use an angiotensin I radioimmunoassay to measure the generated angiotensin and this approach has been adopted by a number of groups (Boyd et al, 1969; Haber et al, 1969).

A survey of the levels of angiotensin II in the plasma of women during normal pregnancy revealed high mean values throughout, with no significant differences between the various stages of pregnancy. Values were elevated as early as the seventh week of pregnancy and remained high right up to term. This finding is in agreement with the previously reported high plasma renin activity values throughout

pregnancy (Genest et al, 1965; Winer, 1965) and is presumably related to the high levels of renin substrate reported in pregnancy (Helmer and Judson, 1967; Pickens et al, 1965). The increased activity of the renin-angiotensin system and the increased secretion of aldosterone during pregnancy may be related to the antagonistic effect of progesterone on the peripheral action of aldosterone (Landau et al, 1965) so that increased levels of mineralocorticoid are required to ensure adequate sodium homeostasis. Measurement of plasma angiotensin II concentrations in a small group of women with established pre-eclamptic toxaemia yielded values which were not significantly different from those found during normal pregnancy. This finding would suggest that the renin-angiotensin system is not of major importance in this situation.

The sheep studies have confirmed the previous finding of increased concentrations of renin in renal lymph compared with plasma (McIntosh and Morris, 1971). They have also demonstrated increased values for renin activity and angiotensin II concentration and slightly lower values of substrate in lymph compared with plasma. Renin concentration, renin activity, and angiotensin II concentration were all found to increase in parallel with the plasma values following sodium depletion. The origin of the renin and angiotensin II in renal lymph were investigated by infusion of isotopically-labelled angiotensin II into the renal artery, followed by isolation and identification of radioactivity in renal lymph and urine, and by administration of a tubular blocking agent to determine the effect on renal lymph levels of

renin and angiotensin. The evidence obtained from these experiments suggested that the renin in lymph was secreted directly into this compartment from the juxtaglomerular region of the kidney and that any contribution from renin reabsorbed from the renal tubules after glomerular filtration was of minor importance. Similarly, a significant contribution to the angiotensin II in renal lymph from plasma or urinary sources seems unlikely. A disparity between the renin and angiotensin levels in renal lymph and plasma following administration of mercuric chloride to three sheep was thought to represent either a specific effect on the mechanism by which renin gains access to the renal lymph or the consequence of a marked reduction or disorganization of renal blood flow. Either effect could follow the severe derangement of renal architecture produced by this agent.

CHAPTER 9

SUMMARY

The development of a sensitive and specific radioimmunoassay for angiotensin II is described. The radioimmunoassay provided a precise, specific measure of the activity of the renin-angiotensin system in the investigation of the role of this system in a variety of normal and abnormal situations.

Plasma angiotensin II concentrations in normal subjects on an unrestricted sodium intake were found to range from 4 to 63 pg/ml, with a mean value of 25.2 ± 14.5 (S.D.). The values in the upright position were found to be consistently higher than those taken after overnight recumbency. Plasma angiotensin II concentrations were found to increase in response to dietary sodium restriction in normal subjects.

Plasma angiotensin II values were found to be elevated in congestive cardiac failure, nephrotic syndrome, and hepatic cirrhosis, although this increase may have been partly due to the diuretic therapy employed in these conditions. Elevated levels of angiotensin II were invariably found in patients with malignant or accelerated hypertension or hypertension secondary to renovascular disease. In patients with "essential" hypertension, many of the angiotensin II values were found to fall within the normal range but the mean level was significantly elevated, suggesting that a possible role of the renin-angiotensin system in this condition cannot be completely excluded at present.

The application of the angiotensin II radioimmunoassay to measurement of angiotensin generated in vitro during renin activity methods has been prevented by the fact that the hormone generated under these conditions is largely in the form of angiotensin I which reacts to only a minimal extent in the angiotensin II assay. The percentage of the generated hormone in the form of the octapeptide was found to be variable and the only situation where the present assay could be used to measure renin activity in this way was in the case of renal vein renin activity ratios. In this situation, the samples were drawn simultaneously from the same patient and processed identically so that the conditions determining the percentage of the generated angiotensin in the form of the octapeptide were relatively constant and the ratios obtained correlated closely with those obtained by bioassay, suggesting a clinical application of the method for this purpose.

Levels of plasma angiotensin II were found to be elevated throughout normal pregnancy, a finding consistent with the previously reported high values of plasma renin activity. Plasma angiotensin II levels in a small group of patients with established pre-eclamptic toxaemia were not significantly different from those found at a similar stage of normal pregnancy.

Studies in sheep have confirmed the previously reported high concentrations of renin in renal lymph and have, in addition, demonstrated increased renin activity and angiotensin II levels compared with plasma. Renin concentration, renin activity, and angiotensin II levels in lymph increased in parallel with the plasma

values following sodium depletion. Infusion experiments using ^{125}I-labelled angiotensin II suggested that plasma and urinary sources for the angiotensin II in renal lymph were unlikely. Furthermore, experiments using a tubular blocking agent suggested that renin reabsorption from the renal tubules following glomerular filtration did not contribute significantly to the renin in renal lymph. Thus the renin and angiotensin II measured in renal lymph are probably a consequence of direct secretion of renin into the lymph compartment from the juxtaglomerular region of the kidney.

The angiotensin II radioimmunoassay procedure thus represents a precise means of assessing the activity of the renin-angiotensin system in man and sheep and has a number of useful applications, including physiological studies in man and sheep and clinical studies in man.

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