AN INVESTIGATION OF THE RENIN ANGIOTENSIN SYSTEM IN RELATION TO BLOOD PRESSURE CONTROL

A thesis submitted by

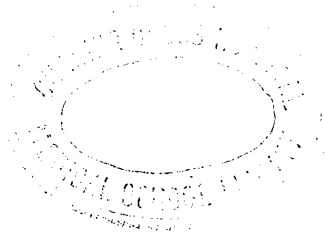
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Abstract

A literature review reveals considerable argument regarding the rôle of the renin-angiotensin system in blood pressure control. It has been claimed that plasma renin activity is not a constant index of angiotensin II under all conditions. The methodologies for estimation of plasma renin activity are controversial. Generation of angiotensin I at acid pH is said to yield artefacts, and increases in plasma renin activity after storage have been reported. Clinical reports of a discrete population of "low renin hypertensives", and the claim that there is diagnostic therapeutic value in renin measurement on a low salt diet, are disputed. The renin angiotensin system is purported to be a defence against volume depletion but unimportant under normal physiological conditions.

In this work assays for plasma renin activity and angiotensin II are developed and assessed. It is shown that generation of angiotensin I at the optimal pH of renin is rectilinear with time, does not yield artefacts, and samples are safely storable at -20°C. In normal and hypertensive subjects plasma renin activity is shown to be a direct, constant, linear function of angiotensin II unaffected by dietary sodium, diuretics or β-blockade. Time-dependent deinhibition of converting enzyme by Captopril is demonstrated for the first time. Plasma renin activity is less responsive to sodium deprivation or converting enzyme blockade in hypertensive subjects. Plasma renin activity on a low salt diet does predict efficacy of therapy although no discrete subpopulation of "low renin hypertensives" was observed. The renin system maintains blood pressure to the same extent in both normal and hypertensive subjects for a given renin level but is unlikely to be pathogenetic in essential hypertension. The rôle of renin as a defence against volume

depletion is supported but considerably extended as the renin angiotensin system also maintains blood pressure under normal physiological conditions.

These results are discussed and a tenable hypothesis for the rôle of the renin-angiotensin system in blood pressure control offered.

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SECTION I

INTRODUCTION

CHAPTER ONE

THE RENIN-ANGIOTENSIN SYSTEM

Discovery and elucidation of the pathway

Towards the end of the nineteenth century, the ideas of Brown-Séquard (1856) on internal secretion of chemical substances into the blood stream came to have a considerable influence on physiological ideas and research.

Tigerstedt and Bergman (1898) decided to investigate the possible endocrine function of the kidney. They found that crude saline extracts of rabbit kidney injected into the anaesthetised rabbit produced a pressor response. The active substance in their extracts caused vasoconstriction and they called it renin. This initial observation was disputed for many years as numerous findings in this field have been. Pearce (1909) and Hartwich and Hessel (1932) were unable to repeat the original work, whereas a pressor effect was demonstrated by Vincent and Sheen (1903), Shaw (1906), and Bingel and Strauss (1909). The use of alcohol at room temperature as extractant could perhaps have accounted for the inconsistent results through enzymic denaturation.

In 1934 Goldblatt, Lynch, Hanzal and Summerville discovered that moderate reduction of the blood flow to the kidneys by means of a silver renal artery clamp produced a form of persistent arterial hypertension that closely resembled the human form of the disease. The constriction of only one renal artery in the dog, with the other kidney untouched (so-called "two kidney hypertension") provided an increase in pressure that usually declined to normal levels within a few weeks. Constriction of both renal arteries or of just one renal artery, the opposite kidney being removed ("one kidney hypertension") provided a form of hypertension which was persistent. This work stimulated several groups of investigators to re-examine the nature of the substances that might cause renal hypertension (Pickering and Prinzmetal, 1938; Hessel, 1938; Helmer and Page, 1939; Swingle *et al.*, 1939).

Goldblatt (1947) further showed that the hypertension which resulted from constriction of the renal artery could not be prevented by complete sympathectomy, destruction of the spinal cord by pithing, or by transplantation of the clamped denerved kidney to the neck. Furthermore it was shown that the hypertension was abolished if the renal veins were tied off, the animal becoming normotensive and eventually succumbing to uremia. The conclusion from these experiments was that this form of hypertension was caused directly or indirectly by a humoural agent released by the clamped kidney. This rekindled interest in Tigerstedt and Bergman's discovery.

In 1940 two groups working independently of each other, Page and Helmer (1940) in America and Braun-Menendez, Fasciolo, Leloir and Munoz (1940) in Argentina,showed that renin itself was not a directly pressor or vasoconstrictive substance. It was found that renin, acting on plasma, produced a new, previously unknown, heat stable, short-acting vasoconstrictor substance. This was the first indication that renin was only the initial activating component in what was eventually revealed as a complex and potent pressor system. Page and Helmer named the new compound angiotonin, Braun-Menendez and his colleagues styled it as hypertensin. It was many years before the compromise name of angiotensin was finally adopted.

There was an immediate interest as to whether or not renin and angiotensin were the mediators of hypertension in human subjects and in experimental hypertension in animals. The first demonstration of the presence of angiotensin in the blood of animals with experimental hypertension was given by Skeggs, Kahn and Shumway in 1951. The blood of a hypertensive dog was subjected to dialysis in an artificial kidney in the belief that if the "hypertensin" were a small molecule it would be dialysed out of the blood. Large amounts of dialysate were collected,

concentrated and assayed for pressor activity in the rat. The positive results provided the first direct evidence that there was a small molecular weight substance with pressor activity in the blood of hypertensive dogs. The first attempt to show the presence of such a substance in human subjects came from the same group the following year (Kahn *et al.*, 1952) with a method (the dialysis approach was not practical in humans) of extraction and concentration of angiotensin from blood. These concentrates were then tested in the rat pressor assay. Whilst the volumes of blood required made the test impractical for clinical use it did provide some evidence of a pressor substance which was later confirmed with the introduction of more sensitive assay techniques. Further reason to believe that renin might be the mediator of hypertension came from the work of Wakerlin and his colleagues (Wakerlin *et al.*, 1953; Kremen and Wakerlin, 1955; Wakerlin, 1958) who demonstrated that the blood pressure of hypertensive dogs could be lowered by injection of kidney extract containing hog renin. Since antibodies to the heterologous hog renin were produced in the dog which cross-reacted with dog renin sufficiently to inactivate it, it seemed reasonable that the blood pressure lowering effect was due to the effective blockade of the renin system. These findings were confirmed in the dog and demonstrated in other species by several independent groups (Helmer, 1958; Haas and Goldblatt, 1959; Frank, 1963; Deodhar *et al.*, 1964; Hill *et al.*, 1970). Taken as a whole these studies provided strong evidence in favour of the importance of renin in the hypertensive mechanism.

In the early 1950's work was begun in several laboratories to try to isolate and purify angiotensin. The amounts that could be obtained from the plasma of hypertensive animals were far too small to permit isolation, therefore the angiotensin was generated *in vitro* by incubation

of crude renin derived from hog kidneys with crude renin substrate derived from horse plasma.

The first pure preparation of angiotensin was isolated in 1954 by Skeggs and his colleagues (Skeggs *et al.*, 1954a). The following year the same group published the results of amino acid analysis showing angiotensin to be a decapeptide containing nine different amino acid residues (Skeggs *et al.*, 1955).

Peart (1956) was the first to isolate and sequence an angiotensin. It was obtained from bovine plasma reacted with rabbit renin. Peart's bovine angiotensin differed from the horse angiotensin of Skeggs in that the former had a valine residue at position 5, whereas the latter had isoleucine. Bumpus, Schwarz and Page (1957), using a homologous incubate of hog renin and hog plamsa, isolated an angiotensin which was identical to horse angiotensin. Human angiotensin was reported by Arakawa, Nakatani, Minohara and Nakamura (1967) to be identical to the species found in the horse and hog.

The discovery that angiotensin existed in two forms was made by Skeggs, March, Kahn and Shumway in 1954(b). For their *in vitro* angiotensin production they prepared renin substrate by ammonium sulphate fractionation of the horse plasma followed by dialysis against distilled water. However, "for reasons which are now obscure" (Skeggs *et al.*, 1976) dialysis against saline was introduced instead. They noted when the substrate prepared in this manner was reacted with renin, the product, although it had the usual pressor activity in the rat, had entirely different chemical properties. This difference was due to the presence in plasma (and crude renin substrate) of a chloride activated enzyme which was named angiotensin converting enzyme and converted the initial product of the renin reaction, the decapeptide angiotensin I, to angiotensin II in the presence of chloride ion. The relationship of

angiotensin II to its immediate substrate angiotensin I was shown by Lentz and his colleagues (Lentz *et al.*, 1956). They found that the converting enzyme removed the carboxyterminal dipeptide (histidyl-leucine) to yield an octapeptide. Shortly after this angiotensin II was synthesised by Rittel, Iselin, Kappeler, Riniker and Schwyzer (1957). Schwarz, Bumpus and Page (1957) reported a similar synthesis later in the same year.

The synthesis of angiotensin II was an important achievement which marked the culmination of work by four groups of investigators led by Schwyzer in Switzerland, Peart in England and Bumpus and Skeggs in America. Availability of the peptides meant that work to try and elucidate their physiological rôle(s) could be undertaken. The relationship between the various components of the renin angiotensin system are shown in Figure 1.1.

Angiotensin II was found to be a very potent vasoconstrictor when it was perfused through isolated rat kidneys in a physiological saline solution, whereas angiotensin I was virtually inactive (Skeggs *et al.*, 1956). Helmer (1957) confirmed these findings showing that aortic strips from the rabbit were unaffected by angiotensin I until exposed to plasma, whereas angiotensin II was immediately effective. Bumpus, Schwarz and Page (1956) showed that angiotensin I had less than 5% of the oxytocic activity of angiotensin II in the isolated rat uterus.

Additional confirmation of the biologically inactive nature of angiotensin I was provided by a converting enzyme inhibitor prepared from the venom of the South American Pit Viper *Bothrops jararaca* (Ferreira *et al.*, 1970). This substance, which is a peptide, specifically inhibits the converting enzyme *in vivo* blocking thereby any pressor action of angiotensin I (Ng and Vane, 1970).

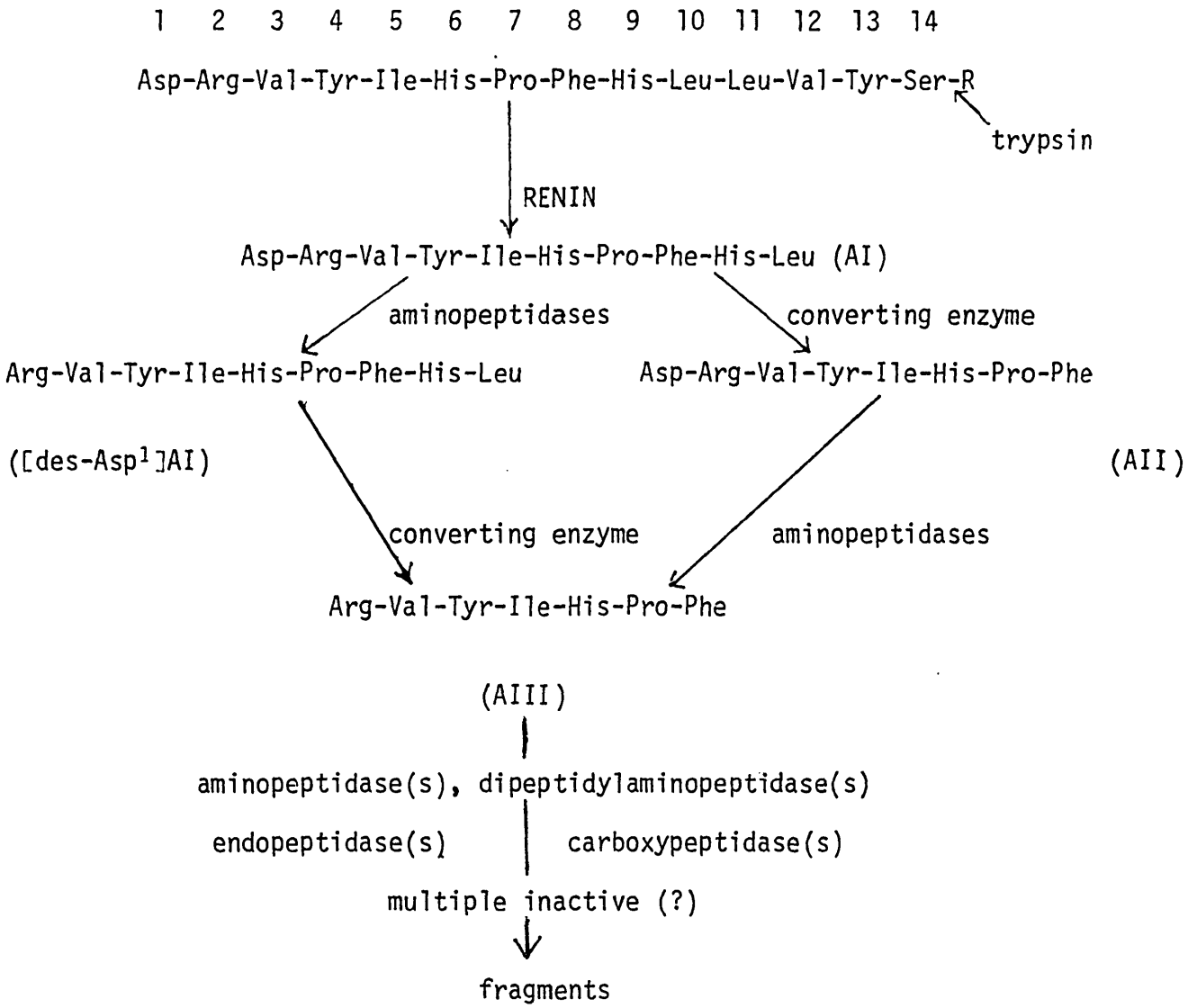


Figure 1:1

The relationship between the components of the renin-angiotensin system.

R = alphaglobulin.

Angiotensin I is not totally void of all physiological activity although it has been shown to be inactive as a pressor molecule. The first studies reporting significant direct effects of angiotensin I were done in the adrenal medulla. In the isolated perfused adrenal, angiotensin I was equal to angiotensin II as a stimulant of adrenal catecholamine release. No evidence of conversion of angiotensin I to angiotensin II was obtained with radioactive tracers, immunoassay of angiotensin I or inhibitors of converting enzyme (Peach, 1971; Peach *et al.*, 1971; Ackerley *et al.*, 1976).

Other functions of angiotensin II have been described. Davis (1963) reported cross circulation studies in the dog from the results of which he inferred that a humoral factor was responsible for the release of aldosterone from the adrenal cortex. It was Laragh, Angers, Kelly and Lieberman (1960) who showed that this humoral factor was angiotensin II, their results being confirmed by Biron, Koiw, Nowaczynski, Brouillet and Genest (1961).

A third form of biologically active angiotensin has also been discovered and described by Blair-West, Coghlan, Denton, Funder, Scoggins and Wright (1971) and Campbell, Brooks and Pettinger (1974); Goodfriend and Peach (1975) and Freeman, Davis and Lohmeier (1975). Des-aspartyl angiotensin II, or angiotensin III, may be generated by the action of the amino peptidase angiotensinase A on angiotensin II. Alternatively, angiotensin III could be produced by the sequential action of angiotensinase A and the converting enzyme on angiotensin I.

Angiotensin III and angiotensin II have been shown to produce similar blood levels of aldosterone in the rat and also be similarly effective in suppressing renin release and renal blood flow in the kidney of the dog (Freeman *et al.*, 1975). However, as a pressor molecule angiotensin III is only 22% as effective as angiotensin II. Whether angiotensin III plays a major part in the renin system *in vivo* is not

certain. Semple and Morton (1975) provided evidence that whilst angiotensin III may exist in blood in concentrations equal to or greater than those of angiotensin II in the rat, the angiotensin III levels in man and in the dog are very low.

Renin substrate

The substrate of the renin reaction, that is the direct precursor of angiotensin I, had always been assumed to be hepatic in origin ever since it was demonstrated in animals that partial hepatectomy caused disappearance of the substrate from plasma (Page *et al.*, 1941). This was confirmed thirty years later by Nasjletti and Masson (1972), who demonstrated the synthesis of renin substrate during perfusion of rat livers for up to 8 hours.

Knowledge of the chemistry of the renin substrate was derived from the work of Green and Bumpus (1954), who managed a partial purification of hog renin substrate, and the work of Plentl, Page and Davis (1943), who showed the substrate present in the alpha-2-globulin fraction of the plasma proteins.

Attempts to purify renin substrate led to the identification of several distinct forms (Skeggs *et al.*, 1963) from an aggregate volume of 3,750 litres of hog plasma. Five major glycoproteins designated A, B_1, B_2, C_1 and C_2 were separated out by DEAE cellulose column chromatography in a descending pH gradient followed by counter-current distribution with polyethylene glycol and lithium sulphate. All five, whilst having different carbohydrate moieties, appeared to have identical amino acid composition and molecular weights approaching 58,000. Reaction rates with homologous renin were identical and the same product was formed in all cases. The reason for the differences observed by Skeggs *et al.* in the content of glucosamine, sialic acid, and hexose is not known. It has been suggested that these differences are, in fact, isolation artefacts

generated by acidification (Eggena *et al.*, 1976). Eggena *et al.* purified a human renin substrate of molecular weight 110,000 by gel filtration and polyacrylamide gel electrophoresis. When electrophoretic mobilities of substrates from eight species were compared, substrates from sheep, cow, pig and rabbit migrated as α-2-globulins. The substrates from man and dog had a more rapid migration than those of the herbivores and should, perhaps, be classified as α-1-globulins (see Gordan and Sachin, 1975; Nasjletti *et al.* (1971). Renin substrate from rat and mouse were shown by Gordan and Sachin to co-migrate with albumin. Substrate has also been found in lymph (Horky *et al.*, 1971), brain (Ganten *et al.*, 1972) and human amniotic fluid (Skinner *et al.*, 1975).

There is dramatic species variation in the susceptibility of plasma substrates to hydrolysis with heterologous renin. For example, human substrate is split only by renin obtained from primates. Although human renin will produce angiotensin I from a variety of substrates, the kinetics of the reaction vary markedly (Braun-Menendez *et al.*, 1946; Gordan and Sachin, 1975; Gould *et al.*, 1966; Schaffenburg *et al.*, 1960). This variation must be borne in mind when interpreting results obtained from heterologous systems.

Plasma renin substrate is synthesised by the liver and is secreted into the circulation and lymph. In isolated perfused livers of rats essentially all the subtrate released into the perfusate was newly synthesised protein (see Nasjletti and Masson, 1971, 1972). This synthesis was shown to be greater in nephrectomised rats than in controls. Skinner *et al.* (1975) have proposed that amniotic fluid substrate enters the circulation. Ganten's work (Ganten *et al.*, 1972) indicates that the renin substrate present in brain is probably locally synthesised.

A variety of conditions have been reported which affect substrate levels. Increases have been reported following administration of ACTH, glucocorticoids, estrogens, angiotensin II, after nephrectomy, during pregnancy, hemodilution and severe hypoxia (Gould *et al.*, 1966; Helmer and Judson, 1967; Gould and Goodman, 1970; Nasjletti and Masson, 1973; Hasegawa *et al.*, 1973; Reid *et al.*, 1973; Page and Bumpus, 1974; Reid *et al.*, 1974; Weir *et al.*, 1975). Decreases in plasma substrate levels have been reported following hepatectomy, adrenalectomy, and hypophysectomy (Hasegawa *et al.*, 1973; Nasjletti and Masson, 1972; Reid *et al.*, 1973; Page and McCubbin, 1968; Ménard *et al.*, 1974). An intact adrenal-pituitary axis is not required for substrate elevation after nephrectomy however (Carretero and Gross, 1967).

The mechanisms responsible for the control of substrate levels are not clearly understood but must involve regulation of hepatic synthesis (Bing, 1972; Nasjletti and Masson, 1971, 1972, 1973). The amount of substrate present in humans is sufficient to generate about 700 $ng.ml^{-1}$ of plasma of angiotensin I. The concentration in humans may vary from 0,8 µM in normals to 6 µM in patients with malignant hypertension (Gould and Green, 1971). The amino-terminal tetradecapeptide of the substrate protein was obtained by treatment with trypsin and the peptide sequence was determined by Skeggs *et al.* (1957). This peptide has full substrate activity. Potent competitive inhibitors of angiotensin I production have been synthesised using part of the tetradecapeptide molecule as a model for the structure (Oparil and Haber, 1974).

Inhibition of renin has also been demonstrated by 1.4 µM prostaglandins A_1 and A_2 (PGA_1 and PGA_2). At low substrate concentrations PGE_2 also inhibited formation of angiotensin I. Based upon the concentration of PG required to inhibit renin, it seems unlikely to be of physiological relevance (Eggena *et al.*, 1975; Kotchen and Miller, 1974).

Renin

Renin-like activity has been identified in many organs since the early discoveries in the kidney. A renin-like enzyme has been reported in uterus and placenta (Anderson *et al.*, 1968; Capelli *et al.*, 1968; Carretero *et al.*, 1971; Chesley, 1974; Ferris *et al.*, 1967), in brain (Ganten *et al.*, 1971a, 1971b, 1971c; Minnich *et al.*, 1972). Reports of renin-like activity have also beenattributed to brain stem, hypothalamus, pineal gland and hypophysis (Fischer-Ferraro *et al.*, 1971; Haulica *et al.*, 1975), to the adrenal glands (Ryan, 1967), large arteries and veins (Dengler, 1956; Ganten *et al.*, 1970; Gould *et al.*, 1964) and the submaxillary gland of the white mouse (Cohen *et al.*, 1972; Inagami *et al.*, 1974; Werle *et al.*, 1957). No definitive proof of identity exists between the various renin-like enzymes and that from the kidney and their physiological rôle is unknown.

In the kidney, renin is synthesised and stored by specialised cells in the afferent arteriole and specialised epithelial cells situated between the two glomerular arterioles at the renal glomerulus. The cells in the afferent arteriole, the juxtaglomerular (JG) cells, are thought to be derived from smooth muscle cells and contain numerous membrane bound granules where renin is stored and presumably synthesised. The specialised granular epithelial cells situated in the angle between the afferent and efferent arterioles and the distal tubule make up the macula densa. The granular cells of the macula densa, together with those in the wall of the afferent arteriole, are collectively termed the JG apparatus (Davis, 1973; Davis and Freeman, 1976; Oparil and Haber, 1974).

Regulation of renin release by the JG apparatus is believed to be influenced by at least three mechanisms. The first depends upon the afferent arteriole acting as a stretch receptor or baroreceptor, such that increased intravascular pressure in the afferent arteriole inhibits

renin release, and conversely low pressure induces release of the enzyme (Oparil and Haber, 1974). The second mechanism is founded upon the modulation of renal renin by changes in the amount of sodium at the macula densa (Davis, 1973). The third control for renin release is neurogenic and via the rich sympathetic innervation of the JG cells (Schrier, 1974; Vander, 1965; Wogermark *et al.*, 1968). The interrelationships between these control mechanisms are not clear (see Davis and Freeman, 1976; Zanchetti *et al.*, 1976).

The molecular weight of hog renin would appear to be 40,000 (Levine *et al.*, 1970; Murakami and Inagami, 1975; Rubin, 1972). A molecular weight of 42,000 has been reported for human renal renin by Waldhausl *et al.* (1970). The Michaelis constant for plasma substrate is of the order of 1×10^{-6}M. The half-life of renin in the circulation is from 30 to 90 minutes, inactivation primarily involving the liver (Johnson *et al.*, 1971; Schneider *et al.*, 1970), with a minor excretory role for the kidneys (Heacox *et al.*, 1967; Peters-Haefeli, 1971).

Acid activation of renin after extraction of rabbit and hog kidney (and of enzyme in amniotic fluid) has led to the suggestion that renin may be stored in an inactive form and on release be activated by a process similar to *in vitro* activation by acidic pH (Boyd, 1972, 1974; Leckie, 1973; Lumbers, 1971; Rubin, 1972; Skinner *et al.*, 1975).

Activation of renin from hog kidney (Boyd, 1974) or rabbit kidney (Leckie, 1973) is accompanied by a reduction in molecular weight. As a result of their findings both Boyd (1974) and Leckie and McConnell (1975) have concluded that the low-activity high molecular weight moiety is a combination of renin with an inhibitory carrier protein. However, activation of renin <u>without</u> loss of molecular weight has been demonstrated by Day and his colleagues (Day and Luetscher, 1974; Day *et al.*, 1976).

The term "prorenin" was coined by Sealey and Laragh (1975), who observed an increase in plasma renin activity in samples stored at -20°C. Osmond *et al.* (1973) had observed a similar phenomenon at 4°C.

Day *et al.* (1976) reported a molecular weight of 63,000 for "big" renin. Prior to activation this molecule had lower activity than "normal" renin and was not responsive to physiological changes which stimulate or suppress normal renin activity.

Further work is required to prove that renin is released from an inactive storage form ("prorenin") and that the active enzyme is formed by limited proteolysis, although such a hypothesis would be in keeping with certain other proteases (e.g. pepsinogen). Explanations for enzyme activation are legion and include restricted proteolysis, lipase activation, or alternatively the inactivation of a renin-inhibitor (Kotchen *et al.*, 1975; Sen *et al.*, 1968). This latter possibility may involve the high-molecular-weight fragment of renin substrate being formed simultaneously with angiotensin I (Lucas *et al.*, 1975).

Angiotensin I

Historically angiotensin I was an inactive precursor of angiotensin II. This view was based upon the weak activity of the decapeptide in isolated smooth muscle assays relative to angiotensin II (Helmer, 1955). Angiotensin I does display significant activity in isolated myocardial preparations (30-50% the activity of angiotensin II) but pretreatment with inhibitors prepared from *B. jararaca* venom, which block converting enzyme, reduced activity of the angiotensin I to less than 2%, indicating that the activity was due to conversion to lower homologues (Ackerley and Peach, 1975; Britton and DiSalvo, 1973; Gerlings and Gilmore, 1973).

Direct actions of angiotensin I have been reported, however, as mentioned earlier, in isolated perfused adrenals. The response of adrenal chromaffin tissue to angiotensin I is dependent upon extracellular calcium ions, is blocked during depolarisation-phase nicotine blockade and is antagonised by angiotensin antagonists (Peach and Westfall, 1972; Peach and Ober, 1974). An intact decapeptide is required for activity; both the NH_2- and -COOH terminal nonapeptides and substituted analogues cause only weak secretion of adrenal catecholamines (Ackerley *et al.*, 1976; Page and Bumpus, 1974).

A host of studies have been attempted to try and ascertain whether or not a direct action can be attributed to angiotensin I specifically. The potential actions include stimulation of the central nervous system (Finkielman *et al.*, 1972; Buckley (1972), release of adrenal catecholamines (*vide supra*), modulation of peripheral sympathetic activity (Johnson *et al.*, 1974; Kadowitz *et al.*, 1972) and regulation of intra-renal blood flow (Itsokovitz and McGiff, 1974; Vane and McGiff, 1975). At the present time, however, no specific receptor site or unique function may be attributed with certainty to angiotensin I. Sustained action by angiotensin I in the presence of a converting enzyme inhibitor suggests but does not prove direct angiotensin I activity, since some conversion by enzymes other than the converting enzyme may occur and be unimpeded by the converting enzyme blockers.

The structure of angiotensin I contains the peptide sequences necessary for all the biological activities of the renin angiotensin system. However, at physiological concentrations the decapeptide exerts little or no action on many of the potential end organs. End-organ specificity of the renin angiotensin system is a function of peptide chain length which is also a key determinant of receptor affinities and

responses of various effector cells. In high enough concentrations angiotensin I will stimulate all tissues that respond to angiotensin II.

The potential nonapeptides of angiotensin II have not been studied as widely as angiotensin I itself. The NH_2 terminal nonapeptide is a substrate for converting enzyme and is hydrolysed also by amino and endopeptidases. In rabbit aortic strips this peptide had less than 5% of the activity of angiotensin II (Lentz *et al.*, 1956; Oparil, *et al.*, 1971). There is, however, no evidence for the existence of this peptide *in vivo* or with intact tissues *in vitro*.

The carboxy terminal nonapeptide is a product of angiotensin I hydrolysis by amino peptidases. It is a very good substrate for converting enzyme (Chiu *et al.*, 1976; Larner *et al.*, 1976; Tsai *et al.*, 1975). In the presence of converting enzyme inhibitor this peptide exhibits less than 1% of the activity of angiotensin II (Ackerley *et al.*, 1976). The peptide does not appear to stimulate prostaglandin synthesis (Blumberg *et al.*, 1976).

Converting enzyme

Converting enzyme has been determined in a wide variety of tissues from many species. As mentioned earlier, it was discovered by chance because of its requirement for chloride ion (Skeggs and his colleagues, 1954b). With the realisation (Ng and Vane, 1967) that conversion in plasma of angiotensin I to angiotensin II was too slow to account for the action of the decapeptide *in vivo* it was then rapidly established that the lungs contained high levels of converting enzyme activity (Bahkle, 1968; Page and McCubbin, 1968; Ng and Vane, 1968; Cushman and Cheung, 1972). The enzyme in lung appears in plasma membranes, presumably from the vascular endothelial cells (Bahkle, 1968; Ryan and

Smith, 1971; Sanders and Huggins, 1971). Pulmonary vessels have been shown to convert more angiotensin I to angiotensin II than vessels from other areas by Aiken and Vane (1970). A strong case may be argued for localisation of converting enzyme in cell types with free access to the vascular space, in order to take account of the rapid conversion of decapeptide to octapeptide across an organ and the low amount of peptide sequestered by the organ (Ryan *et al.*, 1971). The enzyme has been identified from several species in brain, heart, liver, kidney, uterus and lymph (Horky *et al.*, 1971; Granger *et al.*, 1971; Yang and Neff, 1972; Britton and DiSalvo, 1973; Merrill *et al.*, 1973; Poth *et al.*, 1975).

Purification of converting enzyme has been achieved from the lung of rabbit (Cushman and Cheung, 1972), hog (Dorer *et al.*, 1972), humans (Fitz and Overturf, 1972) and calf (Stevens *et al.*, 1972). Erwin Erdös' group have obtained a converting enzyme from hog kidney (Oshima *et al.*, 1974) and also porcine plasma has been used as a source (Lee *et al.*, 1971). Molecular weights for lung enzyme vary between 150,000 and 206,000 (Erdös, 1975; Lee *et al.*, 1971). The exception to this was human lung enzyme reported by Fitz and Overturf to have a molecular weight of 480,000. However, these workers note that about 25% of the enzyme had a molecular weight of 175,000; this indicates perhaps a propensity to form higher aggregates. If this were so it would agree with the molecular weight of 200,000 found by the Erdös group for human lung enzyme.

The enzymes from plasma and hog kidney are inactivated by incubation at 60-65°C for 30 minutes or by pH below 5.0 or above 8.5 (Orekhovich, 1968; Ueda *et al.*, 1971). The pH optimum of the converting enzyme varies according to the substrate, but has always been found to lie between 6.5 and 8.0, usually greater than 7.0 (Cushman and Cheung, 1971).

It has become clear that converting enzyme is, in fact, a rather non-specific dipeptidylcarboxypeptidase; that is it will remove COOH-terminal dipeptides from a variety of substrates (Igic *et al.*, 1972). Converting enzyme is likely to be the same enzyme as kininase II which inactivates bradykinin (Erdös, 1975). There appear to be some general requirements for catalysis. The carboxy terminal residue must be an L-amino acid with a free COOH group. One small caveat to that statement is that if the C-terminal residue is an acidic amino acid then the substrate is less susceptible to hydrolysis (Orekhovich, 1968). Peptide bonds involving proline are not hydrolysed, hence the failure of converting enzyme to subsequently cleave angiotensin II. By the same token [des-Leu^{10}]-AI is not converted and neither is [Pro^{9}-Phe^{10}]-AI, whereas [Ala^{7}]-AII is rapidly hydrolysed (see Ackerley and Peach, 1975; Schwyzer and Turrian, 1960). The tetradecapeptide substrate has been shown by Skeggs' group (Dorer *et al.*, 1975) to be converted by converting enzyme to angiotensin II by repeated removal of carboxy terminal dipeptides. Of the carboxy terminal homologues of angiotensin I only [des-Asp^{1}]-AI, conversion of which yields "angiotensin III", appears to be of physiological significance (Chiu *et al.*, 1976; Tsai *et al.*, 1975), although the enzyme is capable of hydrolysing molecules as small as tripeptides (Erdös, 1975).

The requirement for chloride is variable according to substrate, the low molecular weight substrates requiring 300 mM NaCl for maximal activation (Cushman and Cheung, 1971), whereas angiotensin I requires only 30 mM. Chloride has been identified as an allosteric inhibitor of the enzyme and lowers the K_m of the enzyme for substrate (Cheung and Cushman, 1973; Cushman and Cheung, 1971; Oshima *et al.*, 1974). Bradykinin and [des-Asp^{1}]-AI are less dependent on chloride for maximal activity (Dorer *et al.*, 1974; Erdös, 1975; Tsai *et al.*, 1975). As K_m

decreases so does dependency on chloride. Other halogens and nitrate are the only other monovalent anions which can substitute for chloride.

Converting enzyme is a metalloenzyme inhibited by chelating agents such as ethylenediaminetetraacetic acid (EDTA), ethylene glycol-bis-(β-aminoethylether)-N,N'-tetraacetic acid (EGTA), 2,3-dimercapto-1-propranol (BAL) and cations such as Ni^{2+}, Zn^{2+}, Cd^{2+}, Hg^{2+}, Pb^{2+} and Cu^{2+} (Cushman and Cheung, 1971; Huggins *et al.* 1970). The effects of sulphydryl groups are still the subject of debate (Page and Bumpus, 1974).

Any compound which is a substrate for the enzyme can also function as a competitive inhibitor; structural analogues of angiotensin I as well as hydrolysis products of angiotensin I have been shown to inhibit converting enzyme from lung and plasma (Fitz *et al.*, 1974; Needleman *et al.*, 1972; Tsai *et al.*, 1975; Yang and Neff, 1972).

A group of peptides isolated from the venom of *Bothrops jaracara* inhibit pulmonary conversion of angiotensin I (Bahkle, 1968; Ferreira, 1965; Marshall, 1976). Blockade of converting enzyme by such inhibitors could prove of great value in helping to elucidate the rôle of the angiotensin system and a great deal of work to synthesise inhibitors which could be of use as hypotensive agents *in vivo* is being undertaken.

Angiotensin II and lower homologues

In addition to angiotensin II, angiotensin III, the carboxy terminal heptapeptide of angiotensin II has also received considerable attention because of its activity in the adrenal cortex. The amino-terminal heptapeptide and all other lower homologues studied to date have no activity. The effects of angiotensin II on the cardiovascular system have been widely studied. The heart rate of an animal is slowed by administration of angiotensin II if the animal has intact baroreceptor reflexes

(Page and Olmstead, 1961). When these reflexes are depressed by barbiturate anaesthesia or vagal tone is inhibited by atropine or vagotomy then angiotensin II induces cardiac acceleration (Nishith *et al.*, 1962; Peach, 1972). The cardioaccelerator actions of angiotensin II are not abolished by bilateral adrenalectomy (Krasney *et al.*, 1966; Peach *et al.*, 1970; Peach and Ford, 1968) which implies that the acceleration is not dependent upon angiotensin II stimulated release of adrenal catecholamines. By contrast Boake and Folts (1971) showed a positive chronotropic response induced by angiotensin II in denervated transplanted hearts. This response was prevented by propranolol, thus suggesting a rôle for catecholamines. Evidence is consistent, however, for the rôle of angiotensin II on autonomic ganglia. Studies have shown an increase in heart rate by action on autonomic ganglia and this rise is not produced if the adrenergic nerves to the sino atrial node are quiescent or if the ganglia that give rise to the postganglionic adrenergic fibres of the sino atrial node are destroyed (Aiken and Reit, 1968; Farr and Grupp, 1971; James, 1965). Therefore angiotensin II directly stimulates autonomic ganglia that send postganglionic fibres to the heart.

Angiotensin II has a positive inotropic action in dog heart-lung preparations at 10^{-10} to 10^{-9} molar concentrations (Freer *et al.*, 1976). Increases in myocardial contractile force in point-stimulated left atria, brought about by angiotensin II, are not abolished by β-adrenergic blockade (Freer *et al.*, 1976), extrinsic cardiac denervation (Dempsey *et al.*, 1971) or reserpine pretreatment (Bonnardeaux and Regoli, 1974). "Angiotensin III" also exerts a positive inotropic action (Bonnardeaux and Regoli, 1974).

In spite of the well-established effects of angiotensin II on cardiac rate and contractility, cardiac output is rarely increased in the intact animal. Indeed in the vast majority of cases it has been reported that angiotensin II decreases cardiac output (Brod *et al.*, 1969; Finnerty, 1962; McCaa *et al.*, 1967; Nolan *et al.*, 1967; Page and Olmstead, 1961; Mueller *et al.*, 1970). The common concensus of opionion is that fall in cardiac output is due to the reflex bradycardia following the rise in peripheral resistance and elevation of blood pressure (Brod *et al.*, 1969; Finnerty, 1962; Nolan *et al.*, 1967).

In all species studied arterial blood pressure rises sharply within 10 to 15 seconds of intravenous angiotensin II administration. The duration of the response is dose-dependent, lasting from 3 to 5 minutes. Peach (1977) reported that in normotensive animals on normal sodium diets no fall in blood pressure was observed with angiotensin converting enzyme inhibitors. However, MacGregor and Dawes (1976) have shown that at least one of these inhibitors, [Sar^1-Ala^8]-AII, "Saralasin", is a potent agonist at low concentrations of angiotensin II, which is not a surprising finding with a competitive inhibitor. The structure-activity relationships of angiotensin II and smooth muscle have been reviewed comprehensively by Regoli *et al.* (1974).

Blood flow responses to angiotensin differ qualitatively and quantitatively in different vascular regions and sequential segments of the same vascular bed (Jährhult, 1971). Angiotensin II reduces mesenteric blood flow by arteriolar constriction. In perfused animal limbs the peptide induces vasoconstriction and decreases blood flow. Cutaneous vasoconstriction occurs after angiotensin II administration, but skeletal muscle blood flow is not affected (Forsyth *et al.*, 1971). Cerebral blood flow was decreased.

Pressor concentrations of angiotensin II cause a net fluid accumulation by tissues indicating an increase in post-capillary resistance (Jährhult, 1971). In addition to this action on capillary permeability which is dependent upon pressure changes across the capillary bed, angiotensin II has been shown to increase permeability through the endothelial cells in large arteries (Robertson and Khairallah, 1972).

Angiotensin exerts an action on a wide variety of smooth muscles (see review by Regoli *et al.*, 1974). The vasoconstriction response to angiotensin has two components; a direct action and an indirect one mediated by the sympathetic nervous system. The evidence for a direct action is compelling; the contractile responses of isolated blood vessels to angiotensin II are not altered by α- or β-adrenergic blockade (Palaic and LeMorvan, 1971; Voth *et al.*, 1971), pretreatment with reserpine (Sakurai and Hashimoto, 1965), blockade with atropine (Blair-West *et al.*, 1968; Voth *et al.*, 1971), ganglionic blockade (Page and Bumpus, 1974), methylsergide inhibition of serotonin receptors (Blair-West *et al.*, 1968) or blockade of histidine receptors with metapyramine (Blair-West *et al.*, 1968). The contractions of vascular smooth muscle induced by other agonists (norepinephrine, histamine, serotonin, K^+) are not attenuated by angiotensin antagonists (Bumpus, 1974; Regoli *et al.*, 1974).

The effects of angiotensin II on the central nervous system fall into three major categories. The pressor response was first noted in the early 1960's when cross-perfusion experiments in dogs indicated that injection of angiotensin II into the circulation of the donor resulted in systemic pressor response in the recipient (Bickerton and Buckley, 1961). The doses used were far higher than the physiological

range but subsequent studies have shown that doses of peptide within the physiological range do induce a centrally mediated pressor response. The second action reported in some species is that of a dipsogenic response (Fitzsimmons, 1972). This effect is abolished by saralasin, although at low angiotensin II doses an agonist effect has been reported (Tang and Falks, 1974).

Angiotensin II has also been reported to have a stimulatory effect on antidiuretic hormone (ADH) release from the pituitary (Malvin, 1971). This action is only seen in application of angiotensin II to pituitary fragments; intravenous administration of angiotensin II does not cause elevation of plasma ADH, implying it is the renin-angiotensin system in the CNS which is the potential pituitary activator.

The interaction between angiotensin II and the sympathetic nervous system has also been extensively studied. Early work established that angiotensin II induced ganglionic stimulation (Kaneko *et al.*, 1961), and that responses to angiotensin II were impaired after acute surgical sympathectomy (Zimmerman, 1962) and also angiotensin II facilitated responses to nerve stimulation and drugs or reflexes that evoked norepinephrine release (Benelli *et al.*, 1964). The precise mechanism of action of angiotensin II on the adrenergic nerve terminal remains to be established, although all studies are consistent with a rôle for angiotensin II as a modulator of peripheral sympathetic activity.

Angiotensin II has been implicated in catecholamine biosynthesis, having been reported to stimulate norepinephrine synthesis by possible interference with product inhibition of tyrosine hydroxylase (Davila and Khairallah, 1971). Administration of angiotensin II, *in vivo* and *in vitro*, stimulates the adrenal medulla to release catecholamines (Feldberg and Lewis, 1964; Peach *et al.*, 1966).

Angiotensin II also has a marked steroidogenic activity in the adrenal cortex. By the late 1950's there was an accumulation of evidence regarding a close relationship between renal renin content and the morphology of the adrenal zona glomerulosa (Hartroft *et al.*, 1959). Cross-circulation studies (Denton *et al.*, 1959; Yankopoulos *et al.*, 1959) and the inhibitory effect of nephrectomy on aldosterone secretion induced by acute haemorrhage (Davis *et al.*, 1961; Mulrow and Ganong, 1961) clearly established a humoral rôle for the kidney in the control of aldosterone. Angiotensin II is believed to stimulate the first step of the aldosterone biosynthetic pathway; the conversion of cholesterol to pregnenolone (Aguilera and Marusic, 1971).

Angiotensinases

An angiotensinase is the generic term for any protease or peptidase that catalyses hydrolysis of any peptide bond in angiotensin II. No enzyme has to date been identified for which angiotensin II serves as specific substrate; rather angiotensin II is eliminated from the circulation by non-specific enzymic hydrolysis. The angiotensinases falling into three categories, aminopeptidases, endopeptidases and carboxypeptidases, have been described in tissue homogenates, plasma and urine (Khairallah, *et al.*, 1963; Regoli *et al.*, 1963; Kakimoto *et al.*, 1973).

However, identification and location of an enzyme which can hydrolyse angiotensin II does not, *per se*, indicate that the enzyme does so *in vivo*.

Angiotensin II has a short half-life *in vivo*, less than 10 minutes being commonly quoted and angiotensin III ([des-Asp^1]-A2) has a half-life of half that of angiotensin II or less (Goodfriend and Peach, 1975).

This chapter has attempted to briefly review our knowledge of the renin angiotensin systems up to the time this present study was begun. It can be seen that the renin angiotensin system, whilst being defined in terms of a biochemical pathway, has by no means a clear and obvious dominant physiological or pathological rôle *in vivo*. The pharmacological and physiological studies reviewed in this chapter indicate some of the many actions attributed to angiotensin II since the early discoveries in the 40's and 50's. The most widely reported of these effects is the pressor effect of angiotensin II, which is the most powerful vasconstrictor known, and it is this action as a possible modulator of arterial blood pressure which has instigated a great deal of research over the years. In the next chapter high blood pressure is considered with particular regard to essential hypertension and the putative rôles examined of the renin angiotensin system in the causation and or maintenance of high blood pressure, the two having been continually linked since Tigerstedt and Bergman's original report.

CHAPTER TWO

BLOOD PRESSURE

Introduction

Primary abnormalities of the nervous system, the heart, the blood vessels, sodium and water balance, corticosteroid and catecholamine secretion, the psyche and the renin-angiotensin system have all been considered important at various times in the pathogenesis of hypertension. Despite extensive researches, however, the mechanism of hypertension is understood in three syndromes only and these are rare; phaeochromocytoma, primary hyperaldosteronism and renin-secreting tumour. In each of these conditions an excess of a pressor substance is released from a neoplastic or hyperplastic gland. In other hypertensive syndromes no excess of any known pressor has been found.

Mechanisms controlling blood pressure are inter-related; normally overactivity of one factor is compensated by underactivity or antagonistic activity of others so that the blood pressure is maintained at its original level. Failure of such compensatory devices, of course, could lead to elevation of blood pressure without any pressor mechanism deviating from its normal range.

High blood pressure as a state of risk

High blood pressure, or hypertension, is not easily defined. There is general agreement that high blood pressure is dangerous and treatable and that the risk decreases when blood pressure is controlled (Pickering, 1968; Freis, 1973; Rose, 1973). The problems really begin when an attempt is made to distinguish normal and abnormal pressure. Hypertension is common, that is not in dispute, but if it were a disease state in the ordinary sense of the words a frequency distribution curve for blood pressure would be expected to show two sub-populations; one normotensive and the other hypertensive. Most surveys

in which this has been attempted show a single uninterrupted curve (see Hawthorne *et al.*, 1974). Epidemiological and insurance company data show a graded relation between blood pressure and the risk of subsequently developing cerebrovascular, coronary and renal disease; this relation extends well into the normal range of blood pressure (Pickering, 1968; Freis, 1973). It is observations of this sort, showing no "threshold" as it were, which caused Pickering (1968) to argue that hypertension is not a qualitative entity in the sense that it is present or absent. It must follow that a dividing line drawn between normal and abnormal blood pressure is arbitrary as it would appear to have no epidemiological basis.

Nevertheless, a dividing line is needed in the management of hypertension. It is therefore important to identify a level of pressure above which treatment is beneficial. There is certainly a good case for treating patients with a diastolic pressure over 110 mm Hg (Rose, 1973) and patients with diastolic pressures as low as 90 mm Hg are being investigated to see if treatment is beneficial (Rose, 1973; Peart, 1973). According to Hawthorne and his colleagues over one-third of the 3061 subjects they screened had diastolic blood pressures greater than 90 mm Hg. The fact that the criteria for defining hypertension are arbitrary does not in any way detract from the genuine need for treatment in cases of high blood pressure. Similarly need to treat high blood pressure does not imply the existence of a distinct or discrete pathological entity.

Classification of hypertension

Table 2.1 gives a fairly general classification of hypertensive patients. Pickering (1968) and Kaplan (1973) give more comprehensive

Table 2.1

Classification of hypertension

(a) Essential hypertension

(b) Secondary hypertension

(i) Renal

Renal vascular disease

Renin-secreting tumour

Glomerulonephritis

Pyelonephritis

Polycystic kidney

Collagen disease

(ii) Adrenal cortex

Cushing's syndrome

Primary hyperaldosteronism

Deoxycorticosterone excess

Congenital adrenal hyperplasia

(iii) Adrenal medulla

Phaeochromocytoma

(iv) Coarctation of the aorta

(v) Hypertensive disease of pregnancy

listings. No cause for increased blood pressure is found after investigation of 70-90% of patients. These are considered to have "essential" hypertension. This diagnosis is made on the grounds of exclusion of known causes and, therefore, does not necessarily imply the existence of a single cause.

Secondary hypertension

Extreme hypertension frequently results from renal tumours that secrete renin (Robertson *et al.*, 1967; Kihara *et al.*, 1968; Bonnin *et al.*, 1972; Conn *et al.*, 1972; Brown *et al.*, 1973; Schambelan *et al.*, 1973; MacCallum *et al.*, 1973) and by some other types of renin-secreting tumour or carcinoma (Marosvari *et al.*, 1972; Ganguly *et al.*, 1973; Genest *et al.*, 1975). Furthermore, phaeochromocytomas which secrete norepinephrine can also cause severe hypertension which can be intermittent - brought on by episodic sympathetic stimulation or continuous because of continuous secretion of the catecholamines by the tumour (Goldenberg *et al.*, 1950; Peart, 1953; Hamilton *et al.*, 1953; Leather *et al.*, 1962; Thomas *et al.*, 1966; Remine *et al.*, 1974; Radtke *et al.*, 1975; Tcherdakoff *et al.*, 1974; Ohman *et al.*, 1974; Louis *et al.*, 1975; Manger and Gifford, 1977).

Primary hyperaldosteronism or Conn's syndrome (Conn, 1955) is associated with an adrenocortical adenoma or with bilateral hyperplastic and/or nodular change in the adrenal cortex. On rare occasions there are no detectable adrenal abnormalities. The pathological lesions have been fully recounted by Symington (1969).

Most features of this condition derive from aldosterone excess and include potassium depletion, sodium retention, hypertension and depression of the renin angiotensin system. The hypertension is relatively mild and the malignant phase is rare but not unknown (Kaplan, 1973;

Brown *et al.*, 1972). Figure 2.1 shows a simplistic representation of the effects of these three primary lesions in raising arterial pressure, showing how and where they override the homeostatic mechanisms.

The most common cause(s) of secondary hypertension is renal vascular disease; where there is basically some problem with the kidney(s), which results directly or indirectly in increased arterial pressure to compensate for renal insufficiency. Reduced glomerular filtration rate or renal mass or various lesions of the renal medulla may lead to such renal hypertension.

Essential hypertension

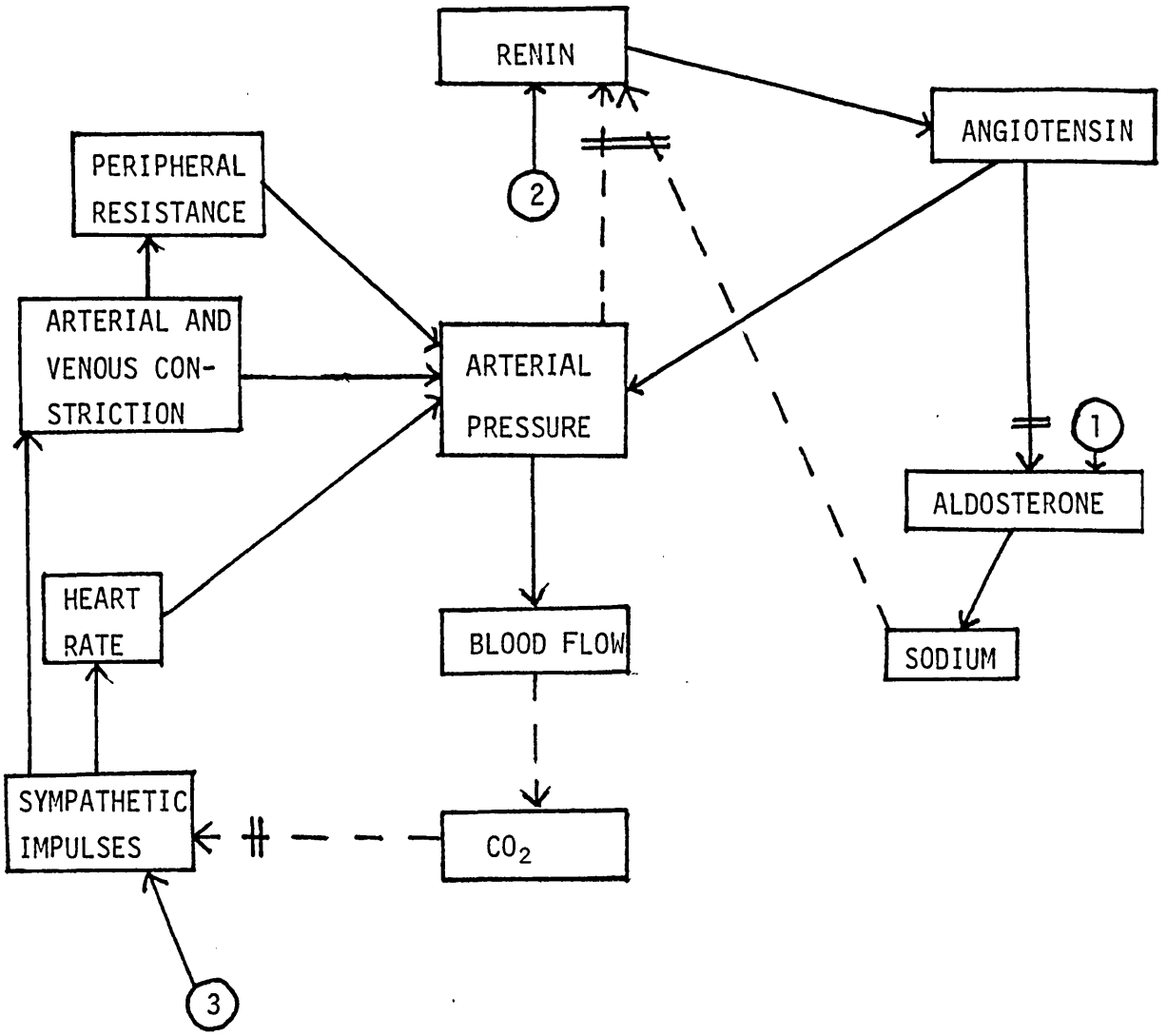
In the vast majority of cases where the blood pressure is elevated no primary lesion or cause is known to exist. Over the years many "hormonal hypotheses" have been offered to account for this persistent primary hypertensive state. Overactivity of the sympathetic nervous system and the renin-angiotensin system and underactivity of prostaglandin synthesis and the kallekrein-kinin system have all, at various times, been considered as important causative agents in essential hypertension.

The sympathetic nervous system

There is some evidence that the nervous system and/or circulating catecholamines are involved in essential and renal hypertension (Dickinson, 1965; Tarazi and Dustan, 1973). Although there is an overlap with the normal range, patients with essential hypertension have been shown, in some studies, to have significantly higher catecholamine levels in blood and urine than normal subjects (Engelman *et al.*, 1970; Esler and Nestel, 1973). Also the plasma concentration of norepinephrine

Figure 2.1

Diagrammatic representation of breakdown of homeostatic mechanisms by autonomous pressor producing lesions.



(1) Conn's syndrome

(2) Renin secreting tumour

(3) Phaeochromocytoma

⟶ Positive effect, i.e. same "direction"

- - - -→ Negative effect, i.e. opposite "direction"

but not epinephrine correlates with blood pressure in essential hypertension and the fall of blood pressure on ganglion blockade is related to the decrease in plasma norepinephrine concentration (Louis *et al.*, 1973). These observations have been interpreted as the increase in plasma norepinephrine reflecting sympathetic nervous overactivity which is in turn responsible for the raised blood pressure. However, whilst stimulation of the sympathetic nervous system has been shown to produce acute rises in arterial pressure, peripheral vasoconstriction (Guyton, 1948), stimulation of the heart (Sugimoto *et al.*, 1966; Cowley and Guyton, 1971) and venous constriction (Guyton *et al.*, 1973), none of these acute neurodynamic effects have been shown to have any long-term effect on blood pressure.

Prostaglandins

Current understanding of the rôle of prostaglandins in relation to blood pressure is rather confused. The immediate effects of infusion of PGA_2 or PGE_2 are renal vasodilatation, increased renal sodium excretion and increased renin secretion (Gross and Bartter, 1973; Patak *et al.*, 1975). Current hypotheses are based upon the idea that long-term formation of prostaglandins will cause a decrease in blood pressure owing to the vasodilatory effects of prostaglandins. However, in the only long-term infusion study reported to date, the result of infusion of PGE_2 for one week into the renal artery or femoral vein indicated a rise in blood pressure in the former and no change in the latter (Hockel and Cowley, 1979).

Kallekrein-kinin system

The kallekrein-kinin system is another potential vasodilator mechanism (Mills and Ward, 1975). Kallekreins are protein enzymes,

circulating in the plasma, found in glandular tissue including the kidney and secreted into urine in approximate proportion to their concentration in the kidney. Kallekrein cleaves a kininogen (two types of which are found among plasma proteins), the major cleavage products of which are kinins, usually either kallidin or bradykinin. Kallidin is usually formed by glandular kallekrein, bradykinin by plasma kallekrein. Kallidin and bradykinin then circulate in the blood for a short time until kininases present in plasma and some tissues (kininase II is angiotensin-converting enzyme) destroy them. These kinins are vasodilators and their rôle in essential hypertension is analogous to that proposed for prostaglandins; that is long-term formation leads to lowering of blood pressure by vasodilation. Once again the evidence is equivocal. Acute falls in blood pressure when 1 ml of a dog's own urine is infused into its circulation have been seen and Croxatto, Albertini, Corthorn and Rosas (1977) have reported moderately depressed levels of renal and urinary kallekreins in various types of hypertension, including essential hypertension in humans, Okamoto spontaneous hypertension in the rat and one-kidney Goldblatt hypertension. However, these correlations and acute studies are not undisputed and by no means prove a rôle of kallekreins in causation or maintenance of blood pressure.

The renin-angiotensin system

Evidence connecting the renin-angiotensin system and blood pressure is plentiful and, often, extremely difficult to interpret. It is extremely likely that the amount of renin and angiotensin II in blood is within the range capable of influencing blood pressure (Chinn and Düsterdieck, 1971; Johnson and Davis, 1973). However, the relationship between plasma renin and angiotensin and blood pressure has been reported

as significantly positive, significantly negative and non-existent (see Davies *et al.*, 1974). Laragh (1974) proposed a sub-classification of essential hypertension into low-renin, normal renin and high-renin as a means of permitting "more specific drug therapy". Thurston and his colleagues, however (Thurston *et al.*, 1978) concluded from their results that such a classification was arbitrary and did not "significantly assist" in predicting blood pressure response to either diuretics or β-blockers combined with diuretics. Also interpretation of renin data requires care as there is considerable controversy over which measurement(s) best reflect(s) the activity of the renin angiotensin system *in vivo* and furthermore there is some argument as to how best to measure these parameters.

CHAPTER THREE

THE RENIN-ANGIOTENSIN SYSTEM AND BLOOD PRESSURE

As indicated briefly in the previous chapter, there is some debate over the rôle of the renin-angiotensin system in blood pressure control and essential hypertension and, furthermore, how best to measure the activity of the renin-angiotensin system *in vitro* to reflect the physiological situation as closely as possible.

Measurement of the activity of the renin-angiotensin system

Three parameters of the system have found recent favour, and account in some form or other for the vast majority of current "renin assays" being performed.

Plasma renin concentration

The plasma concentration of renin can be measured by extracting the enzyme, incubating it with exogenous substrate and measuring the rate of angiotensin I production using either a bioassay (Brown *et al.*, 1964) or a radioimmunoassay (Stockigt *et al.*, 1971).

In order to measure plasma renin concentration *per se* the optimal assay would be one in which other factors in the renin-angiotensin system did not interfere. It would be independent of potential activators or inhibitors, renin substrate and converting enzyme activity. These problems have been discussed by Poulsen (1973). One of the problems is whether or not the amount of substrate present in normal plasma is sufficient to achieve zero-order (i.e. [substrate] independent) kinetics. Therefore exogenous substrate is usually added to the system. However, the purpose of measuring plasma renin concentration in a clinical context is as a function or index of angiotensin I and, therefore, angiotensin II production, hence addition of exogenous substrate may render the results difficult to interpret, in isolation, as measures of the activity of the

renin-angiotensin system *in vivo*. A second and more serious problem involves the methodology of renin extraction used in some cases where acidification (pH of 3) is one of the steps to destroy renin-substrate and angiotensinases. It has been reported that various procedures, acidification among them, activate renin which is inactive at pH 7.5 (Boyd, 1972 and 1974; Skinner *et al.*, 1972; Morris and Lumbers, 1972; Leckie, 1973; Derkx *et al.*, 1976). Obviously such an *in vitro* activation may give rise to discrepancies such that the relationship to angiotensin *in vitro* is different to that *in vivo*, and not "uniformly different".

Plasma-renin activity

Until the introduction of immunoassay techniques for angiotensin most plasma renin methods were based on the bioassay of generated angiotensin. However, angiotensin immunoassay offered a potential increase in both sensitivity and specificity as well as a rather more convenient technique and became a popular alternative. Early approaches used the specific immunoassay of angiotensin II but this was shown to give low results by comparison with previous bioassay levels (Vallotton *et al.*, 1967). It was shown subsequently that materials, such as EDTA, which were added to plasma as anticoagulant and angiotensinase inhibitor, also inhibited the converting enzyme with the result that angiotensin I rather than angiotensin II was the major end product of the reaction (Boyd *et al.*, 1967; Boyd *et al.*, 1969). Fitz, Boyd and Peart (1971) showed that this converting enzyme inhibition was difficult to reverse without simultaneously restoring the unwanted angiotensinase activity. Therefore the reaction was deliberately stopped at the angiotensin I stage by complete inhibition of the converting enzyme and angiotensin I

measured by specific immunoassay as the end product of the reaction (Boyd *et al.*, 1969). This assay was shown to compare much more favourably with bioassays. There are several points, however, which require consideration in regard of PRA estimation. The constancy of the pH during the generation of angiotensin I is very important (Poulsen, 1973). Unbuffered plasma will lose carbon dioxide during incubation with consequent increase in pH. Furthermore some of the commonly used plasma enzyme inhibitors such as EDTA and 8-hydroxy-quinoline may also alter pH, even at low concentrations. The second consideration is at what pH to maintain the system.

There are two schools of thought in this regard. One choice is pH 7.4. as this is physiological pH and should therefore be a closer reflection of the *in vivo* situation. The alternative is to use the optimum pH of the renin reaction, generally agreed to be around pH 5.7. The advantage is a 50% faster rate of reaction which yields greater assay sensitivity at the lower end of the normal range. Oparil, Koerner and Haber (1974) demonstrated significant plasma renin activity in anephric subjects after generation at pH 5.5, but not after generation at pH 7.4, and inferred from the results that generation at optimal pH could be yielding artefacts. So, whilst most workers agree that it is vital to maintain a constant pH there is considerable dispute over which pH to use.

Oparil *et al.* (1974) also showed that the inhibitors of converting enzyme in common use were not 100% efficient over the pH range of generation encountered; that is, an inhibitor system adequate at pH 7.4 was not necessarily adequate at pH 5.7 and *vice versa*.

There is also considerable dispute about the linearity of the production of angiotensin I with respect to time; obviously non-linearity

would severely compromise the use of the assay as an index of angiotensin II. Since there is no one standard procedure for generation of angiotensin I it is therefore essential for each investigator to check the linearity of his system empirically. Addition of exogenous substrate in excess causes more confusion than it relieves since situationsof substrate dependence are possible *in vivo* in cases of liver failure, and it would not therefore yield a true reflection of *in vivo* activity to falsely produce zero-order kinetics.

Finally it is, of course, essential to show that there is a linear relationship between PRA and plasma angiotensin II and whether or not that relationship is identical for normotensives and hypertensives. For example, it has been suggested by Sambhi and his colleagues (Sambhi *et al.*, 1976) that the relationship between angiotensin II and PRA in uncomplicated essential hypertensives during chronic diuretic therapy changes from the pre-treatment relationship. This finding obviously makes clinical interpretation of the assay data extremely difficult.

Measurement of angiotensin II

Direct assay of angiotensin II is only possible after extraction and concentration of angiotensins from plasma. Extraction losses need to be quantified by some kind of radioactive or standard markers (Catt *et al.*, 1967).

Antisera to angiotensin II usually cross-react with the heptapeptide metabolites and sometimes the hexapeptide also. These fragments tend to accumulate in venous blood but the total immunoreactivity does not appear to differ between arterial and venous blood (Cain *et al.*, 1969; Oelkers *et al.*, 1972), although the proportion of angiotensin II to other immunoreactive metabolites may differ (Cain *et al.*, 1969).

Therefore angiotensin II immunoassay is generally regarded as the most direct index of *in vivo* activity of the renin-angiotensin system. However, the volume of blood, complex procedures and sensitivity limitations have combined to make this assay less popular than PRA estimation.

From the above it is clear that plasma renin concentration has no special advantage over plasma renin activity as an index of activity of the renin-angiotensin system and may even yield artefacts as a result of acidification. Plasma angiotensin II would appear to be the most "direct" hormone assay, although the methodology is laborious, the sensitivity limit restrictive and the sample size very high. As a result it is easy to understand the popularity of plasma renin activity as an *in vitro* index of *in vivo* plasma angiotensin status. However, before one can use this PRA assay technique to investigate clinical situations it is vital to assess the assay with regard to the inhibitors, pH of generation, generation of angiotensin I with respect to time and the correlation of PRA with angiotensin II as measured by angiotensin II immunoassay. Obviously before the two assays can be compared they must be individually optimised, and assessed for precision, reproducibility, specificity and sensitivity. Only then can one compare results between PRA and angiotensin II and finally consider their interpretation in a clinical context.

The renin-angiotensin system and blood pressure control

As indicated in Chapter two the underlying mechanism of raised blood pressure is not known for the large majority of cases. Indeed precise biochemical causes are only attributable in three distinct, uncommon syndromes, primary hyperaldosteronism (Conn's syndrome), renin-secreting tumour, and phaeochromocytoma where noradrenaline levels

are elevated and betray hyperactivity of the sympathetic nervous system. It would be of great value if the causative mechanism(s) of essential hypertension were elucidated.

Laragh and his colleagues (Buhler *et al.*, 1972; Laragh, 1974) have suggested that patients with essential hypertension can be divided into three groups: those with high, normal or low renin, on the basis of measurement of PRA in relationship to sodium balance. Low renin hypertensives have been claimed to specifically respond to diuretics and normal renin hypertensive patients to beta-blockers. Laragh has claimed that these patients with normal renin levels are "angiotensin dependent" because of their response to beta-blockers which inhibit renin release. Low renin hypertension is also claimed to be associated with volume expansion which would account for the low renin level and response to diuretics (Jose *et al.*, 1970; Melby *et al.*, 1971; Woods *et al.*, 1969; Spark and Melby, 1971). Others (Padfield *et al.*, 1975; Dunn and Tannen, 1974; Thurston *et al.*, 1978) have been unable to substantiate such claims. However, showing the presence or absence of angiotensin dependence does not indicate a causative rôle *per se* in pathogenesis of hypertension. In order to make any statement about this it is vital to consider the effects of different renin levels, renin blockade and response to drugs of normotensive volunteers and compare and contrast the findings with those in hypertensive patients.

The rôle of the renin-angiotensin system in blood pressure maintenance (as opposed to high blood pressure generation) has been recently postulated as a defensive mechanism which "cuts-in" under conditions of volume depletion or sodium restriction (Haber, 1976). Haber's study with "teprotide", an inhibitor of angiotensin converting enzyme, and studies with saralasin, a competitive inhibitor of angiotensin II (Posternak *et al.*, 1977), have supported this concept.

These studies do not clarify, however, at what stage of volume depletion the system becomes 'active' in blood pressure maintenance, and there is no evidence for a threshold as such. Also, since saralasin and teprotide only inhibit the short-term effects of circulating angiotensin II they would tend to underestimate the rôle of the renin angiotensin system. Furthermore, it has been shown (MacGregor and Dawes, 1976) that saralasin is a potent agonist and only inhibits at high angiotensin levels (Hollenberg *et al.*, 1976; Anderson *et al.*, 1977; Case *et al.*, 1976). Coupled with the fact that in these studies the subjects were invariably rested for 10 minutes prior to infusion of saralasin or teprotide it is possible that these results do underestimate the rôle of the renin angiotensin system.

Further investigation of the rôle of the renin-angiotensin system in normotensive volunteers and patients with essential hypertension may shed light upon whether or not the renin-angiotensin system does have a causative rôle in the pathogenesis of essential hypertension and whether or not low renin hypertensives are a distinct group. Furthermore comparative studies with normal volunteers may indicate to what extent blood pressure maintenance is effected by the renin-angiotensin system and whether this rôle differs between normal and hypertensive subjects; that is whether essential hypertension is a quantifiable disease in terms of the function of the renin angiotensin system.

It was therefore decided to develop and assess radioimmunoassays for angiotensin I and angiotensin II and optimise these with respect to precision, sensitivity and reproducibility and to establish quality control pools for these assays. The angiotensin I assay would then be used to quantify plasma renin activity as described earlier. Plasma

renin concentration was not considered owing to the danger of producing artefacts. Validation of the plasma renin activity assay would be in two distinct parts (a) the radioimmunoassay of angiotensin I with consideration of the antibody-antigen reaction, optimal concentrations predicted from the Law of Mass Action and kinetic analysis of the antibody; cross reactivities, parallelism, recovery, precision and sensitivity, and (b) the generation of angiotensin I under controlled conditions *in vitro*. That is, the choice of the most suitable pH ("optimal" or "physiological"), inhibitor systems to prevent converting enzyme and angiotensinase activities at the pH chosen, the linearity of the generation procedure (i.e. zero-order or at least pseudo-zero-order kinetics) and the limitations of such linear kinetics and also the stability of the samples *in vitro*.

Similarly, assessment of the angiotensin II assay would be conducted on both the radioimmunoassay, paying attention to the criteria outlined for angiotensin I radioimmunoassay, and the extraction procedure which needs to be assessed for both percentage recovery and the consistency of the procedure.

Finally the plasma renin activity assay would be assessed as a function of plasma angiotensin II concentration as reflected by the angiotensin II assay procedure, in samples from both normal subjects and patients with essential hypertension to see if a linear relationship exists and also if the relationship differs between normal and hypertensive subjects or differs with beta-blockers, diuretics, or divergence in dietary sodium intake.

Having ascertained to what limits the assays are co-related it is proposed then to use one or both of these to study the rôle of the renin-angiotensin system in blood pressure control in normal and hypertensive subjects under a variety of conditions of therapy and diet to see

if one can postulate a physiological or pathological rôle for the renin-angiotensin system in the context of blood pressure control.

SECTION II

MATERIALS AND METHODS

CHAPTER FOUR

GENERAL REAGENT PREPARATION

This chapter outlines the preparation and evaluation of the reagents commonly used during this work. Radiolabelled ligands and antibodies, however, are discussed separately in subsequent chapters.

Buffers

The buffers and general laboratory solutions used routinely in the course of this work are formulated and listed in detail in Appendix I.

Reagent Sources

All reagents were of 'Analar' grade. Table 4.1 lists the source of the reagents used with the exception of routine laboratory chemicals.

Standard Solutions

Angiotensin I

250 µg of angiotensin I (^{1}Asp ^{5}Ileu) was dissolved in 1 litre of assay buffer. Approximately 500 two ml aliquots were dispensed into glass vials and freeze dried. The vials were then closed in partial vacuum under nitrogen, sealed manually with metal closures and stored at -20°C until required.

To reconstitute an aliquot 2 ml of distilled water were added to the vial and the lyophilised pellet dissolved. Subsequently the solution was transferred to a "Sterilin" vacutainer (capacity ≈ 30 ml) and diluted with assay buffer, the freeze drying vial and stopper being carefully rinsed. For a high standard of 25 ng.ml^{-1} 18 ml of assay buffer were used as wash and diluent. For a high standard of 20 ng.ml^{-1} 23 ml of buffer were used. After reconstitution an aliquot was stored

Table 4.1 Sources of reagents used in the course of this study

	Reagent	Supplier
1.	Angiotensin I (^{1}Asp ^{5}Ileu)	Uniscience, Cambridge, England
2.	Angiotensin II-amide ('Hypertensin')	C.I.B.A., Horsham, Sussex, England
3.	Angiotensin II (^{1}Asp ^{5}Ileu)	Schwarz Bioresearch, N.Y., U.S.A.
4.	Angiotensin II (^{1}Asp ^{5}Val)	National Institute for Biological Standards and Control, London, England
5.	Heptapeptide 2-8 (^{5}Ileu)	Calbiochem, Bishops Stortford, England
6.	Heptapeptide 2-8 (^{5}Val)	C.I.B.A., Horsham, Sussex, England
7.	Hexapeptide 3-8 (^{5}Ileu)	Schwarz Bioresearch, N.Y., U.S.A.
8.	Hexapeptide 3-8 (^{5}Val)	C.I.B.A., Horsham, Sussex, England
9.	Pentapeptide 4-8 (^{5}Ileu)	Schwarz Bioresearch, N.Y., U.S.A.
10.	Renin substrate	Sigma, St. Louis, Mo., U.S.A.
11.	Renin	National Institute for Biological Standards and Control, London, England
12.	Bovine serum albumin	Sigma, St. Louis, Mo., U.S.A.
13.	Dowex AG50W-X2, 50-100 mesh, $H^{\oplus}$ form	Bio-Rad, Richmond, Ca., U.S.A.
14.	Sephadex DEAE A25	Pharmacia, Uppsala, Sweden
15.	Charcoal ("Norit A")	Sigma, St. Louis, Mo., U.S.A.
16.	o-Phenanthroline	Sigma, St. Louis, Mo., U.S.A.
17.	1-Ethyl 3(3 dimethylamino-propyl)carbodiimide HCl	Sigma, St. Louis, Mo., U.S.A.
18.	Phenylmethylsulphonyl-fluoride (PMSF)	Sigma, St. Louis, Mo., U.S.A.

at 4°C whilst it was in use, which did not exceed 6 weeks.

Angiotensin II

100 µg of angiotensin II-amide ($H_2NAsp^1Val^5$) were dissolved in 1 litre of assay buffer. Approximately 500 two ml aliquots were dispensed, frozen, lyophilised, capped and sealed as described in the previous section. Reconstitution with 2 ml of distilled water followed by dilution with 18 ml of assay buffer yielded a working high standard of 10 $ng.ml^{-1}$. The aliquot after reconstitution was stored at 4°C for up to six weeks.

Non-immune Separation Media

Charcoal suspension

Charcoal has been extensively used as a substance to separate 'bound' from 'free' in competitive binding assays. This method was originally proposed for the vitamin B_{12} assay by Miller in 1957 and then used by Herbert, Gottlieb, Lau and Wasserman in 1964. It was Herbert and his colleagues who applied it to insulin radioimmunoassay in 1965. Since that time it has been broadly used for the assay of many steroid and peptide hormones, including angiotensins.

The validity of the use of charcoal depends upon the possibility of obtaining preferential and relatively complete adsorption of the free hormone or drug with little or no adsorption of the bound moiety. When an assay is carried out in a pool of proteins among which the free and bound species are to be found, it is not known to what extent the bound complex will be adsorbed.

Several authors, especially Ekins *et al.* (1968) and more recently Hunter and Ganguli (1970) and Palmieri, Yalow and Berson (1971) have emphasised

that plain charcoal could be used to give satisfactory separation. In contrast to this, Gottlieb and colleagues (1965) proposed the use of a dextran coated charcoal which they claimed worked as a molecular sieve. This belief is widely held, and the majority of assays employing a charcoal separation system use a dextran coated system. However, it has been shown more recently by Binoux and Odell (1973) that dextran does not have a "molecular sieve" property and does not therefore enable a sort of instant dialysis to occur. The role of dextran appears, from their data, to reduce adsorption sites on the charcoal for both bound and free hormone indiscriminately. Adsorption, then, is a simple physicochemical phenomenon based upon the Mass-Action Laws and adsorption of hormone will be an inverse function of protein concentration. Thus dextran would only have a beneficial effect in separation in the absence of other proteins when total indiscriminate adsorption would otherwise ensue. In the assay systems used in this work the protein concentration of the buffer was found to be a sufficient minimum to yield good separation of bound and free moieties.

Selection of Optimal Charcoal Concentration

A series of 21 tubes containing 250 µl of assay buffer were dispensed in parallel with a series containing 300 µl of assay buffer. To all tubes were added 50 µl of labelled angiotensin I solution. Finally to the set of tubes containing 250 µl of buffer 50 µl of a suitable dilution of antiangiotensin I were added. All the tubes were then vortexed and incubated at 4°C for 24 ± 2 hours. After the incubation period 750 µl of various concentrations of vigorously mixing "Norit A" charcoal suspended in the charcoal buffer described in Appendix I were added. The range of concentrations used was from 18.6 mg charcoal per tube down to 0.775 mg per tube.

After all the dispensations and all the tubes had been vortex mixed they were all spun at 1500 g for 20 minutes at 8°C. The supernatants were then decanted and both supernatants (≡ "bound") and pellets (≡ "free") were counted on a Wallac 80000 gamma counter. The results are shown in Figures 4.1 and 4.2. It can be seen that adsorption of bound angiotensin on to the charcoal is a direct function of increase in charcoal concentration. However, above 6.2 mg of charcoal per tube there is no significant decrease in the amount of free angiotensin present in the supernatant as so-called "non-specific binding". It may be seen that at a concentration of 3.1 $mg.tube^{-1}$ there is optimal difference between "bound" and "free" adsorption but the amount of free angiotensin in the supernatants is increasing at this concentration of charcoal. From these results it was decided to employ 6.2 mg per tube as the routine concentration as this yielded an almost maximal differential between bound and free and had, more importantly, minimal non-specific interference.

This concentration was found to be equally useful for the angiotensin II assay.

Ammonium sulphate solutions

In order to effect a separation of free and bound hormone in the automated assays (see Chapter 10) a preincubated double antibody system was routinely employed and the separation effected by automatic *in vacuo* filtration. However, for very small numbers of tubes or non-routine protocols it was necessary to find a suitable means of separation of bound and free phases independent of the automated equipment. Centrifugation did precipitate the bound complex selectively but, owing to the extremely small precipitate size, this tended to yield imprecise

Figure 4:1

Separation of bound from free radioactivity as a function of charcoal concentration. Results from precipitate counting.

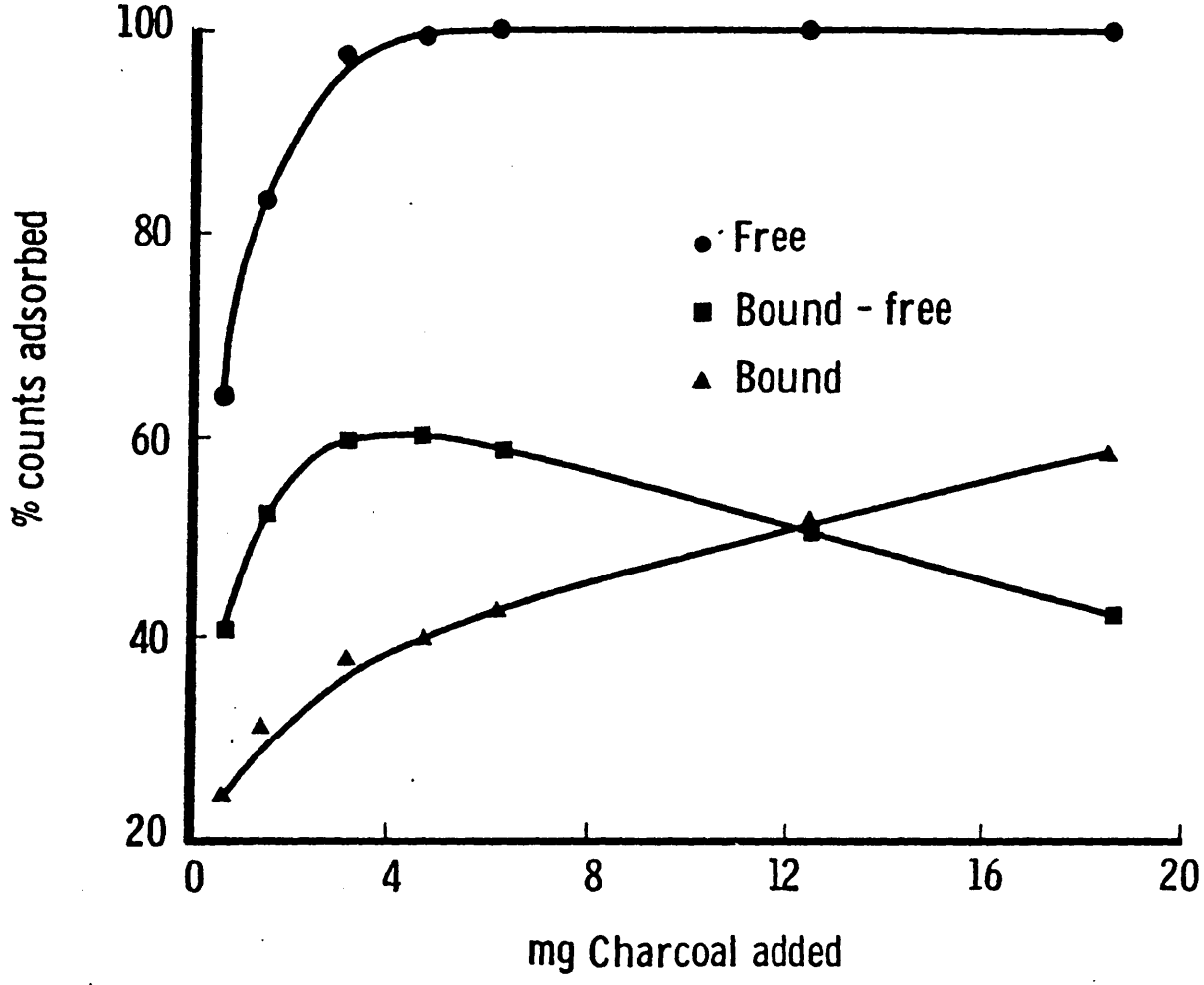
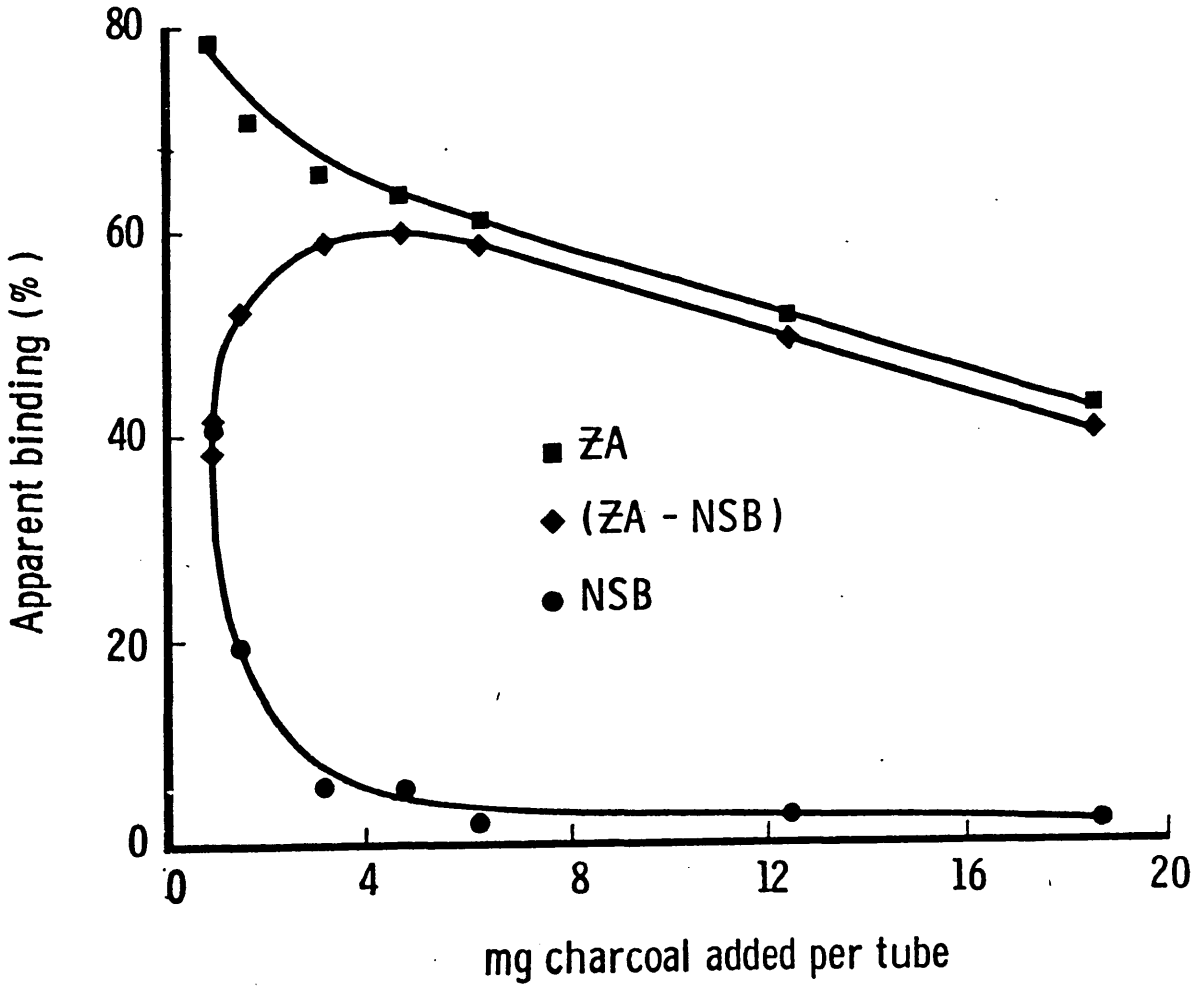


Figure 4:2

Separation of bound from free radioactivity as a function of charcoal concentration. Results from supernatant counting.



results upon decantation since the precipitate was not stable when the tubes were inverted. Initially this problem was avoided by supernatant sampling and counting the "free" aliquot, but this proved unnecessarily time consuming. Therefore an additional separating agent was sought. Charcoal was of no use in this regard since addition of charcoal would precipitate the free hormone, whereas centrifugation would precipitate bound: hence all the counts would appear in the pellet.

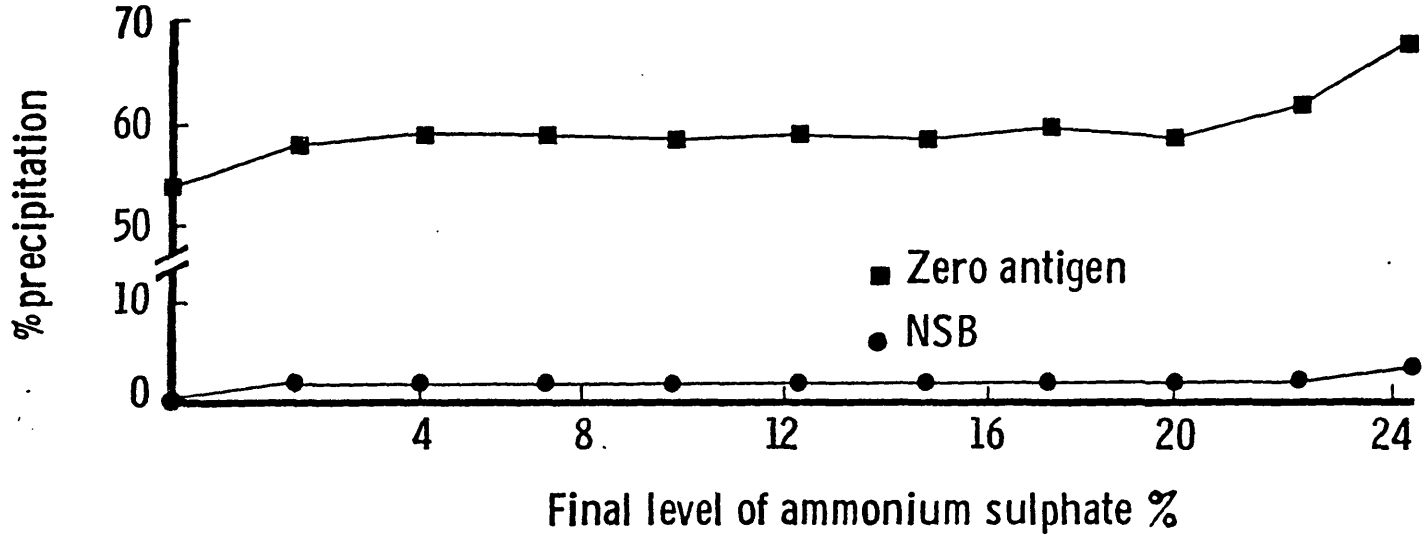
Ammonium sulphate has been used widely to separate bound and free hormone in radioimmunoassays (Chard *et al.*, 1970, 1971). It is particularly useful as it is relatively cheap and, unlike some organic separating agents, does not markedly disturb the equilibrium between bound and free moieties. This is especially true in a double antibody system where, since the disparity between bound and free molecular size is enormous, dilute solutions only are necessary.

Selection of optimal ammonium sulphate concentration

Two series of 33 tubes were set up in parallel, buffer and label being dispensed as in the previous (charcoal estimation) experiment. To one series of tubes 50 µl aliquots of the routine preincubated double antibody solution were added. All the tubes were then mixed, covered with plastic film and incubated at 4°C for 24 hours. after the incubation period 750 µl aliquots of ammonium sulphate solutions of varying strengths were added to all the tubes except three control tubes from each set which were to ascertain zero antigen and NSB levels in the unmodified automated assay. The tubes were vortexed, centrifuged at 1500 g at 8°C for 20 minutes and the tubes were then inverted and allowed to drain for 5 minutes prior to counting. The results are displayed in Figure 4.3. It is apparent from these data that the concentration

Figure 4:3

Separation of bound from free radioactivity as a function of ammonium sulphate.



of ammonium sulphate is not critical up to 20% final (w/v) concentrations. In the absence of ammonium sulphate there is, however, a slight drop in the non-specific binding; from ≈1.3% to 0.5% and a concomitant fall in zero antigen binding (≈57.5% to 54.5%). Whilst neither of these is significant the reproducibility of the estimations was tested using a final concentration of 10% ammonium sulphate as the test concentration and comparing zero antigen and NSB values obtained by this with values obtained using no addition of ammonium sulphate and the addition of 750 µl of assay buffer instead of ammonium sulphate. This second control was to ascertain whether the fall in reproducibility was in fact due to the unstable microprecipitate or was merely due to the smaller discard-supernatant volume. Twenty estimations of each zero antigen and NSB were made and the results are shown in Table 4.2. The experiment indicates that both the volume and ammonium sulphate are important factors in reproducibility, the latter being more significant. Therefore whenever the preincubated assay systems were used not in direct conjunction with the automated filtering equipment 750 µl of 14.67% (w/v) ammonium sulphate (1.11 M) were added to all tubes prior to centrifugation and decantation.

Enzacryl thiolactone linked antibodies

It has been reported by Kardana *et al.* (1979) that linking enzacryl thiolactone, a cross-linked acrylamide polymer, to primary antisera produced, in certain cases, more sensitive radioimmunoassays than did conventional double antibody precipitation. It was decided to investigate this technique with regard to angiotensin II antisera where greater sensitivity could result in direct plasma assay without the need for an extraction process.

Table 4.2

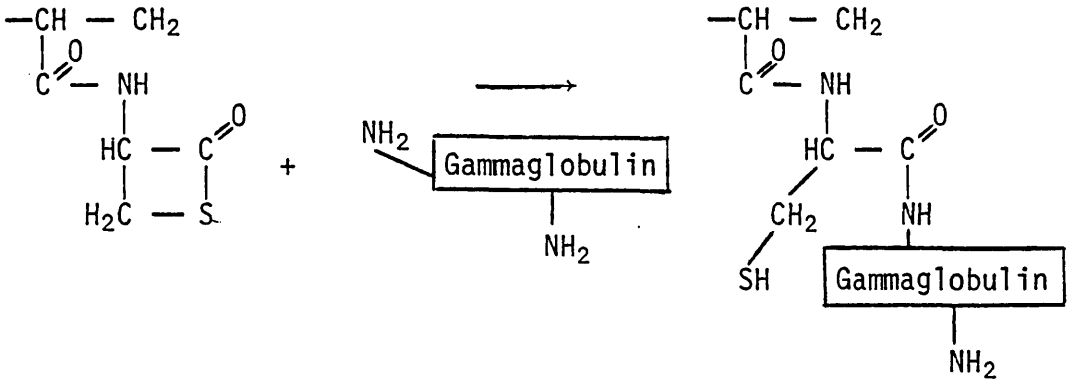
Separation of bound from free moieties by centrifugation in the presence and absence of ammonium sulphate

Separation conditions	No addition		750 µl buffer added		750 µl of 14.7% (w/v) ammonium sulphate added*	
Tube type	NSB	Zero antigen	NSB	Zero antigen	NSB	Zero antigen
Number of estimations	20	20	20	20	20	20
Mean % precipitation	0.51	54.1	0.89	54.8	1.38	57.8
Standard deviation	0.24	4.96	0.26	2.1	0.33	1.63
% Coefficient of variation	46.1	9.2	29.2	3.8	23.9	2.8

*Equivalent to final concentration of 10% w/v

The essence of the method is to isolate IgG from the antiserum and to couple this to cross-linked acrylamide particles. IgG was isolated with Protein A Sepharose CL-4B (Pharmacia Fine Chemicals, Middlesex, England) which selectively binds the Fc fragment (Hjelm *et al.*, 1972; Goudswaard *et al.*, 1978). The IgG fraction was then bound to acrylamide polymer containing a reactive thiolactone which binds to the amino groups of lysine and the hydroxyl groups of serine and tyrosine residues. (See Figure 4.4).

Figure 4.4 Enzacryl polythiolactone coupling to gammaglobulins



The covalent coupling proceeds without the need for any preliminary activation of the beta-thiolactone group.

Materials and Methods

The buffers required are formulated in detail in Appendix I.

An anti-angiotensin II sera which had been previously characterised with respect to affinity, sensitivity and numbers of binding sites was chosen as the test substance.

Isolation of IgG fraction

The Protein A Sepharose (1,5 g) was swollen in 0,05 molar phosphate buffer, pH 7.3 and used to pack a 0.9 x 15.0 cm column. Immediately

prior to use more buffer was washed through the column to establish a base line extinction. The 5 ml sample of undiluted rabbit antiangiotensin II serum was then loaded on to the column and unbound substances eluted with the phosphate buffer until the base line extinction at 280 nm was regained. The bound IgG fraction was then desorbed using 0.1 molar glycine/HCl buffer at pH 2.8 containing 0.5 molar sodium chloride. One ml fractions were collected in tubes containing 0.5 ml of 0.2 molar TRIS to neutralise the glycine buffer. The desorbed protein peak was pooled and dialysed overnight against running tap water and then for three more days against 0.9% (w/v) saline. The volume was then reduced to 10 ml by ultrafiltration using an Amicon PM10. The whole procedure was performed in a cold room at 4°C (see Figure 4.5).

Conjugation

Two ml of the IgG solution (that is, the equivalent of 1 ml of "neat" antiserum assuming no losses) were incubated overnight at 4°C with 20 mg of enzacryl polythiolactone. The pH was adjusted to 10 with molar sodium hydroxide. After 24 hours incubation the complex was centrifuged at 1500 g for 30 minutes at 4°C and the supernatant removed.

The precipitate was washed twice, firstly with 5 ml of acetate buffer (0.1 M, pH 6.0) and the with 2 ml of 0.05 molar acetate buffer, pH 6.0, containing 0.5 molar sodium chloride. After each wash the solution was centrifuged for 15 minutes and the supernatant removed. Finally the precipitate was resuspended in 10 ml of 0.04 molar phosphate buffered saline containing 0.1% bovine serum albumin. Thus this final solution should be equivalent to a $^{1}/_{10}$ dilution of neat antiserum assuming no loss of material.

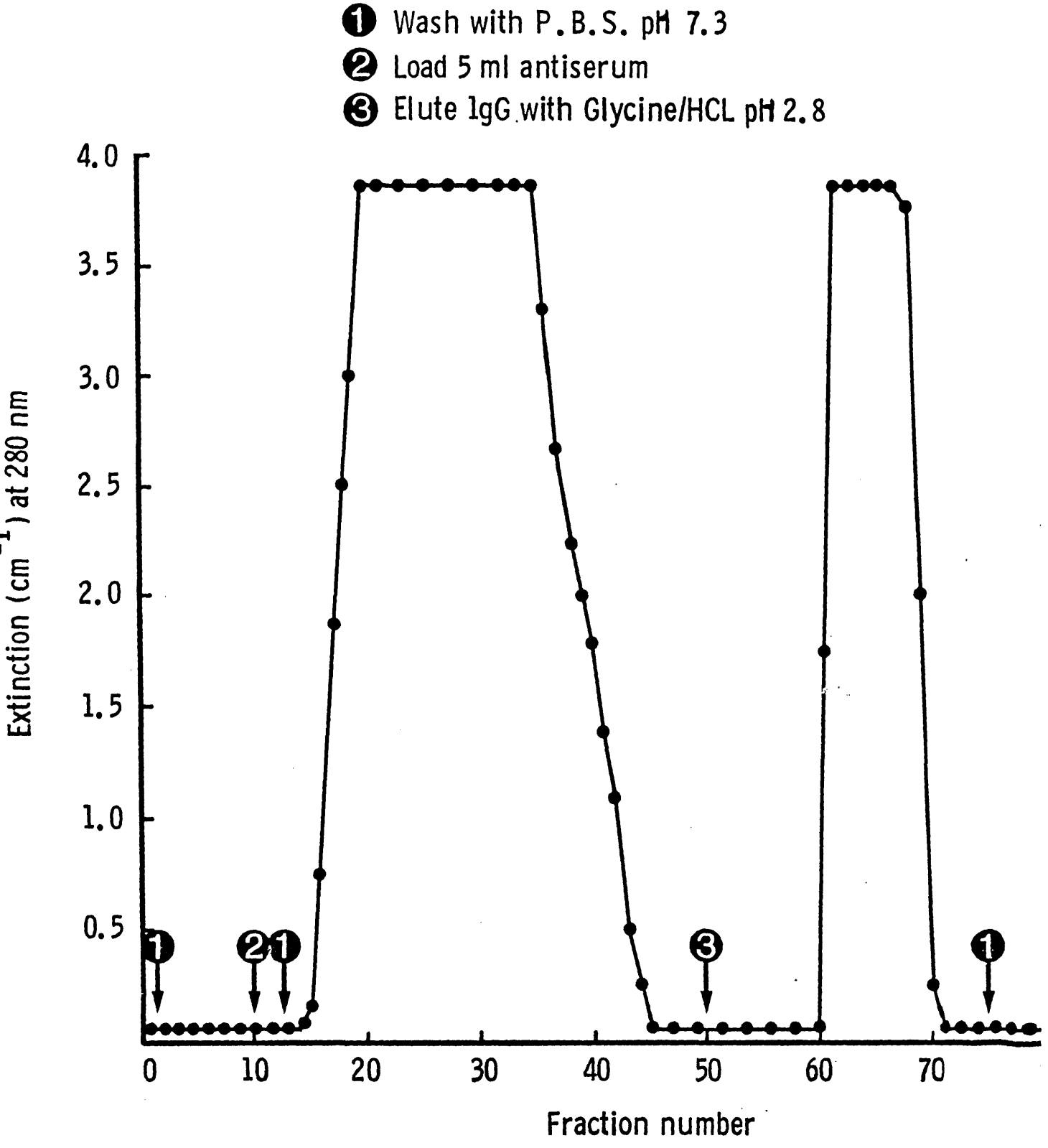


Figure 4:5

Isolation of IgG fraction from whole antiserum by Protein A Sepharose

Results

Dose response curves performed with the antibody-enzacryl conjugated were compared with curves using equivalent concentrations of normal double-antibody complex. No advantage in terms of lowering of detection limit was observed at a variety of antibody-enzyacryl dilutions. Changes in K, the affinity constant, and q, the number of binding sites, when the relative concentration of double antibody and antibody enzacryl were normalised did not differ greatly, implying no substantial loss of, or change in, the antibody population during the preparative procedures.

Discussion

As there appeared to be no particular advantage to this technique for the antibody concerned further study was not done. Kardana and his colleagues (1979) do note that where primary antibody concentrations in the two systems were comparable (as it was in two of their examples) no significant improvement was obtained but only when the primary antibody concentration was lower in the enzacryl system. Thus it is possible that the apparent improvement wrought by enzacryl coupling is in fact due to loss of antibodies at some stage during isolation and coupling yielding a more sensitive dose response curve. This phenomenon would only be observable in assays not previously brought to their limit of detectability by conventional antiserum titration curves.

CHAPTER FIVE

PREPARATION AND EVALUATION OF RADIOLABELLED LIGANDS

The Iodination of Angiotensin II and Purification of Label

Iodination procedure

The labelling of angiotensin II was carried out using the procedure of Greenwood *et al.* (1963). In this method carrier free Na ^{125}I (IMS 30, Radiochemical Centre, Amersham) is oxidised to cationic iodine using chloramine T. The cationic iodine then substitutes for hydrogen in positions 2 and 3 on the tyrosine residue in the peptide. To prevent oxidative damage to the peptide, excess chloramine T is reduced by sodium metabisulphite which also reduces any remaining cationic iodine to iodide. Finally potassium iodide is added to the reaction mixture to solubilse any iodine remaining as I_2 to triiodite.

$I_2 + I^- \rightarrow I_3^-$ Eq. 5.1

Protocol

To a small reaction vessel containing a micromagnetic stirrer the following reagents were sequentially added:-

(1)	0.05 molar phosphate buffer, pH 7.5	30 µl
(2)	Na ^{125}I (IMS 30) in dilute NaOH (2 mCi)	20 µl
(3)	5 µg angiotensin II in phosphate buffer	25 µl
(4)	100 µg chloramine T	25 µl
(5)	240 µg sodium metabisulphite	100 µl
(6)	6 mg potassium iodide	600 µl
		800 µl

Reagents 4, 5 and 6 were added at 20 second intervals. At the end of the reaction 10 µl of the mixture was removed and added to a tube containing 1 ml of 0.05 molar Tris, pH 7.5, buffer. The remainder of the reaction

mixture was loaded on to the DEAE-Sephadex A-25 ion exchange column. The reaction vessel was washed with a further 200 µl of KI which was then added to the column.

Purification of labelled angiotensin

The labelled angiotensin was purified by allowing the reaction mixture to pass through a column (20 x 1 cm) of DEAE Sephadex A-25 (4 g) which had been pre-equilibrated for 48 hours at pH 7.4 with four 250 ml changes of Tris HCl buffer.

At pH 7.5 angiotensin II has a small negative charge (being approximately 1 pH unit below its isoelectric point) and is only slightly retained on an anion exchange column at this pH. However, free I^{125} and metabisulphite, and damaged peptide fragments are completely retained by the column. Furthermore, the iodination procedure alters the pK of tyrosine so that iodinated angiotensin is retained more than the unreacted starting material (Neilsen *et al.*, 1971) and as a result it is possible to obtain pure iodinated angiotensin. In order to effect a good separation it was essential to allow the reaction mixture to run into the column before applying a head of elution buffer. One ml fractions were collected in plastic tubes containing 250 µl of 0.4% BSA to prevent adsorption. The flow rate was set at approximately 1 ml/2 minutes. Up to 40 fractions were collected, and counted on a Panax gamma counter.

Assessment of damage by electrophoresis

Electrophoresis of the reaction mixture

Electrophoresis of the reaction mixture was performed in order to assess the proportion of the I^{125} existing as labelled angiotensin,

labelled fragments and free ^{125}I.

Methods

The 10 µl sample of reaction mixture in 1 ml of Tris was diluted 1:10 by addition of 100 µl of this solution to a further 900 µl of Tris. 100 µl of this were applied as a narrow band 5 cm from one end of a strip of Whatman MMChr paper (approximately 25 x 5 cm) using a syringe and a fine needle. The origin was marked in pencil. After the sample had dried the paper was positioned in an electrophoresis tank with the origin at the cathodic end. The sample was run in 0.07 M barbitone buffer pH 8.6 at 250 volts for 55 minutes. The paper was then removed and allowed to dry. When dry the paper was cut into 1 cm strips numbering 1-25 from the cathodic end and counted on the Panax.

Check of label purity

To confirm that the column had separated contaminants such as peptide fragments and free iodide, a sample of the peak fraction of the DEAE eluate was checked by electrophoresis in a similar manner to that described previously for the reaction mixture electrophoresis.

0.1 ml of a 1/100 dilution of the peak fraction was applied to a 5 cm wide strip of Whatman MMChr electrophoresis paper and the paper was run under identical conditions to those previously described. After being allowed to dry the strip was cut into 1 cm sections and counted. Also a 50 µl aliquot of the peak fraction, suitably diluted to yield approximately 10,000 counts/minute/50 µl (approximately 10 pg of antigen), was incubated overnight at 37°C with antibody at ten times the usual assay concentration. Total immunoreactivity in the presence of excess antibody was >95%.

Separation of label from starting material

There are several methods to check that iodinated angiotensin does separate from unreacted angiotensin. Firstly, there is the original method of Neilsen using macroquantities of unlabelled angiotensin in order to do spectrophotometry on the DEAE eluate fractions and demonstrate discrete peaks. Morton *et al.* (1976) used the DEAE fractions for injection into a rat pressor bioassay to demonstrate separate peaks of activity.

In the present study neither of the above techniques was practicable; the first because of its expense in materials and, the second, because such an assay was not available to this author. Radioimmunoassay was therefore performed on DEAE eluate fractions, at dilution of $^{1}/10$ and $^{1}/100$, of a reaction mixture where non-radioactive sodium iodide had been used as the "label". Two discrete peaks of peptide were observed, the second of which superimposed upon the predicted position of the peak of counts observed on the DEAE A-25 profile in the routine labelling with sodium ^{125}Iodide.

Subsequently, to save time, radioimmunoassay of diluted A-25 column fractions from the routine labelling using I^{125} was carried out. It is essential in such experiments to count the bound and free fractions owing to the gross variation in counts added to each tube. These experiments gave results virtually identical to those achieved by superimposition of the "mock labelling" described above.

Self displacement analysis to determine specific activity

Specific activity may be determined quite accurately by incubation of a series of increasing label concentrations (as unknowns) in the assay system. Routinely an arbitrary dilution of the peak fraction from

the DEAE A-25 column estimated to yield about 10,000 counts/minute was used for the standard curve as this was equivalent to a concentration of about 10 pg per tube of angiotensin. In addition to the standard curve which contained label, standards and antisera, non-specific bindings (no antisera) and zero antigens (no standard) were set up. Also total counts were determined. Also a series of increasing label concentrations (up to 8-fold normal) were incubated with the same amount of antibody.

Classically, interpolation of the concentration of angiotensin from the standard curve is done by superimposition of the percentage binding of the label series at the appropriate points on the standard curve. The mean displacement over several incremental doses being then taken as the concentration of angiotensin label present due to that increment, and from this the specific activity may be calculated. Specific activites were found to be of the order of 750 μCi/μg when the above labelling procedure was followed. This method of data analysis has been called into question by Morris (1976) who proposed that the interpolated doses should be plotted as a fraction of counts added. The intercept on y at x = 0 is the concentration of label per tube. It was claimed that this obviated an error due to competition between labelled and unlabelled antigen. As self-displacement analysis is a widely used technique it was considered essential to check Morris' contention both theoretically and in practice. The validation of the technique is explained fully in the last section of this chapter.

The results of a typical radiolabelling are shown in Figures 5.1-5.4 and Table 5.1 to 5.4.

Figure 5:1

DEAE fractionation of typical label reaction mixture.

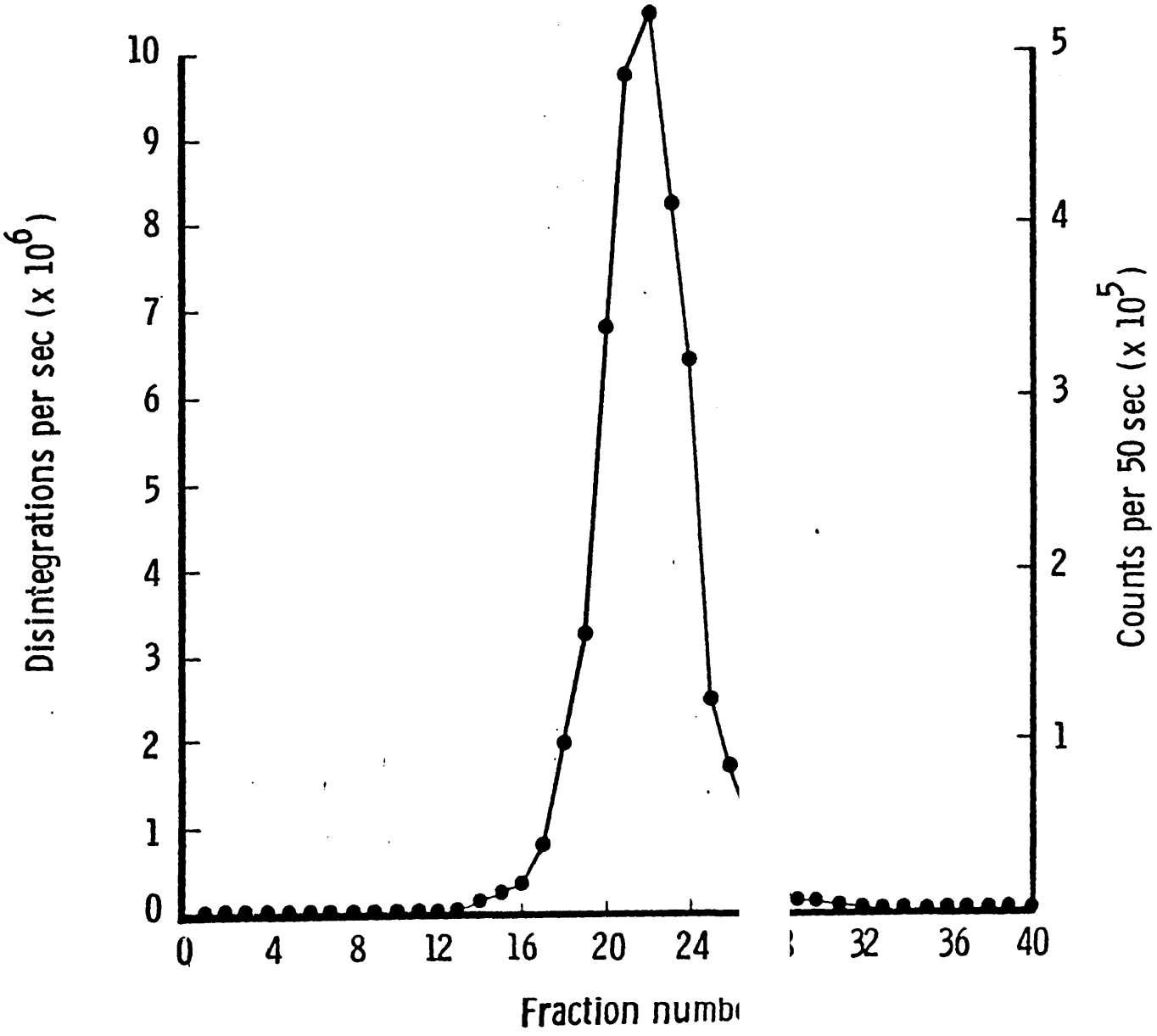


Table 5.1 DEAE Fractionation of reaction mixture

Fraction (ml)	Counts 50"
1	52
2	43
3	40
4	58
5	42
6	63
7	49
8	56
9	53
10	73
11	54
12	897
13	4,421
14	7,638
15	11,961
16	16,061
17	39,302
18	96,942
19	162,496
20	340,034
21	486,053
22	523,486
23	413,413
24	320,619
25	123,633
26	85,418
27	40,443
28	14,311
29	5,344
30	3,813
31	2,842
32	961
33	618
34	33
35	62
36	38
37	44
38	27
39	31
40	24

Counting efficiency 0,1%. Σ 40 = 2,701,998 counts. Counts added = 3,468,302 = t ($\equiv$ 1,875 µCi). Recovery $\frac{\Sigma}{t} \times 100 = 77,9\%$.

Figure 5:2

Electrophoresis of reaction mixture and peak fraction

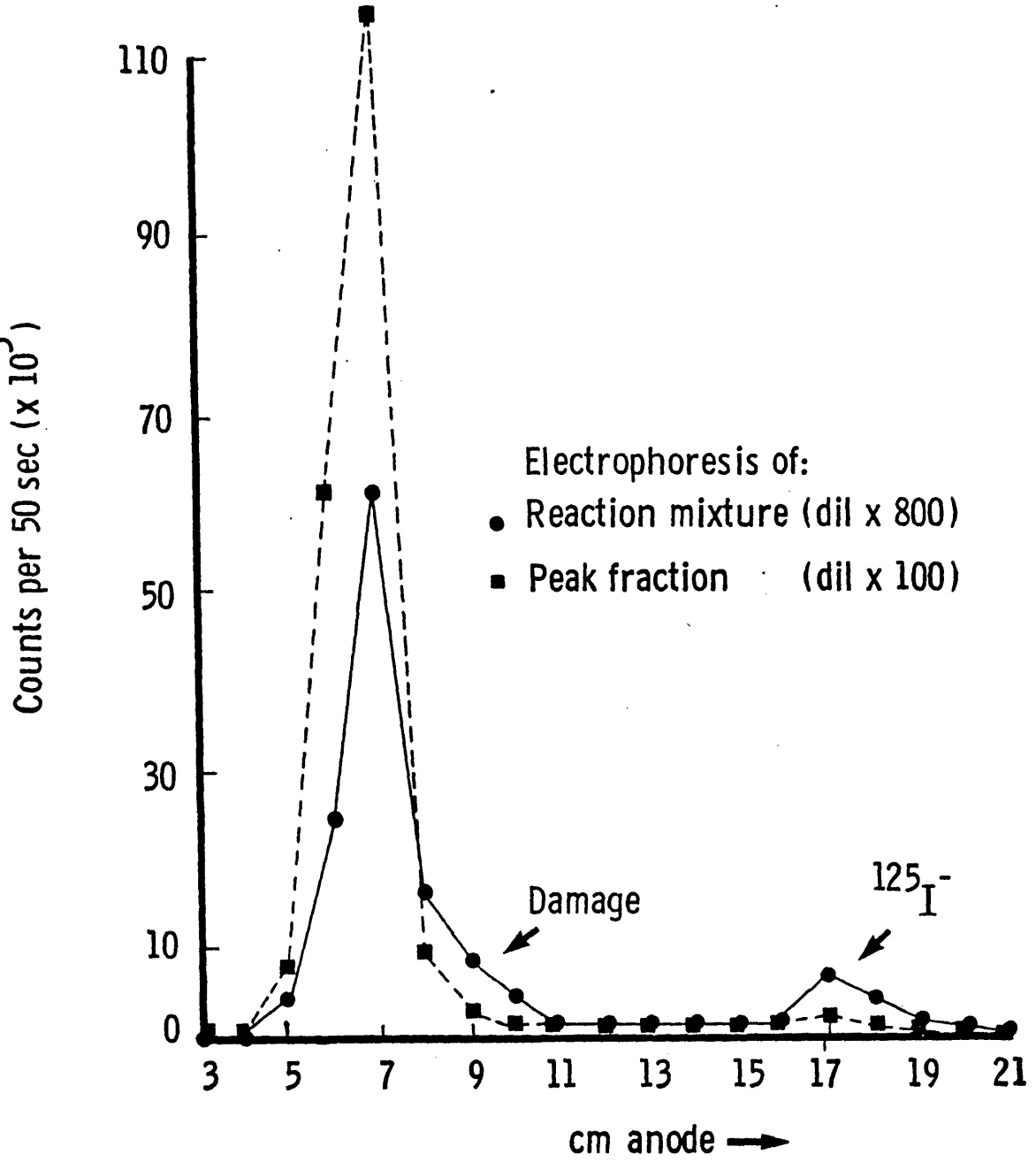


Table 5,2 Electrophoresis of reaction mixture and peak fraction from DEAE A-25 column

	CM. No	Reaction mixture 100 µl of 1/800 50" CT	Reaction mixture 100 µl of 1/100 50" CT
	1	60	67
	2	77	91
	3	90	77
	4	203	171
	5	5,217	8,953
	6	24,353	61,891
	7	61,712	114,502
	8	16,273	10,363
	9	8,773	2,230
	10	4,503	791
	11	1,020	670
	12	805	397
	13	683	338
	14	660	345
	15	994	792
	16	1,886	1,273
	17	7,442	2,586
	18	4,267	975
	19	1,336	88
	20	504	53
	21	52	38
	22	41	54
	23	32	46
	24	27	43
	25	34	40
Total	Σ_1^{25}	141,044	206,874
Angiotensin II	Σ_5^{9}	116,328 (82.5%)	197,939 (95.7%)
Fragments	Σ_{10}^{14}	7,671 (5.4%)	2,541 (1.2%)
Iodide	Σ_{15}^{20}	16,429 (11.7%)	5,767 (2.8%)
Counts added		178,048	263,114
% Recovery		79.2%	78.6%

Figure 5:3

Distribution of angiotensin in DEAE fractions following labelling.

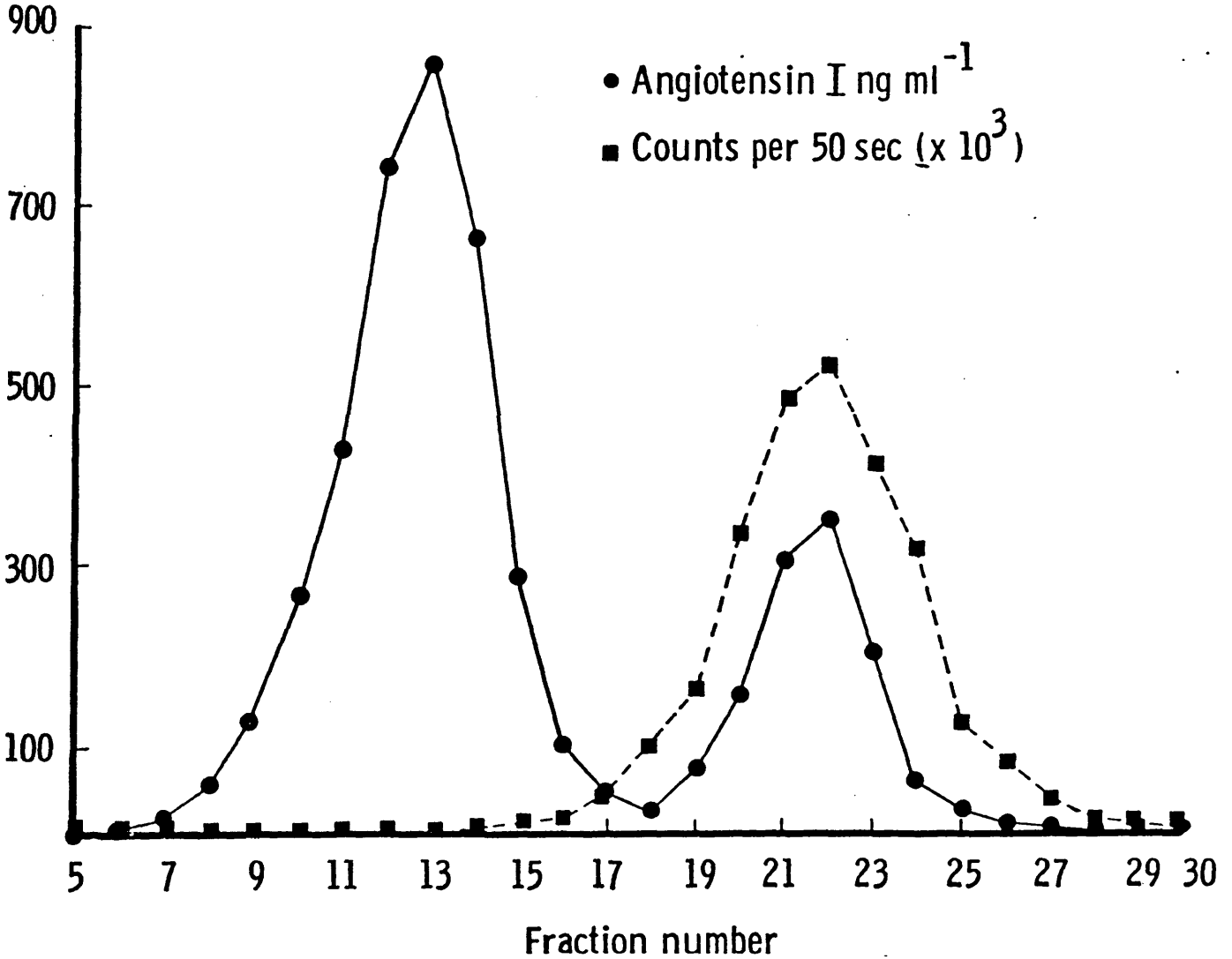


Table 5.3 Angiotensin concentrations in DEAE fractions following labelling

Fraction no.	Angiotensin ng/ml^{-1}
1	<0.1
2	<0.1
3	<0.1
4	<0.1
5	0.7
6	4.8
7	10
8	46
9	120
10	260
11	430
12	740
13	860
14	660
15	290
16	100
17	48
18	22
19	70
20	150
21	310
22	350
23	200
24	60
25	22
26	10
27	<6.4
28	<0.1
29	<0.1
30	<0.1
31	<0.1
32	<0.1
33	<0.1
34	<0.1
35	<0.1

$\Sigma_{1}^{35} \simeq \Sigma_{5}^{27}$ = 4969.9 ng = 95.3% recovery

Figure 5:4

Fractionation of labelling reaction mixture by Sephadex G-25

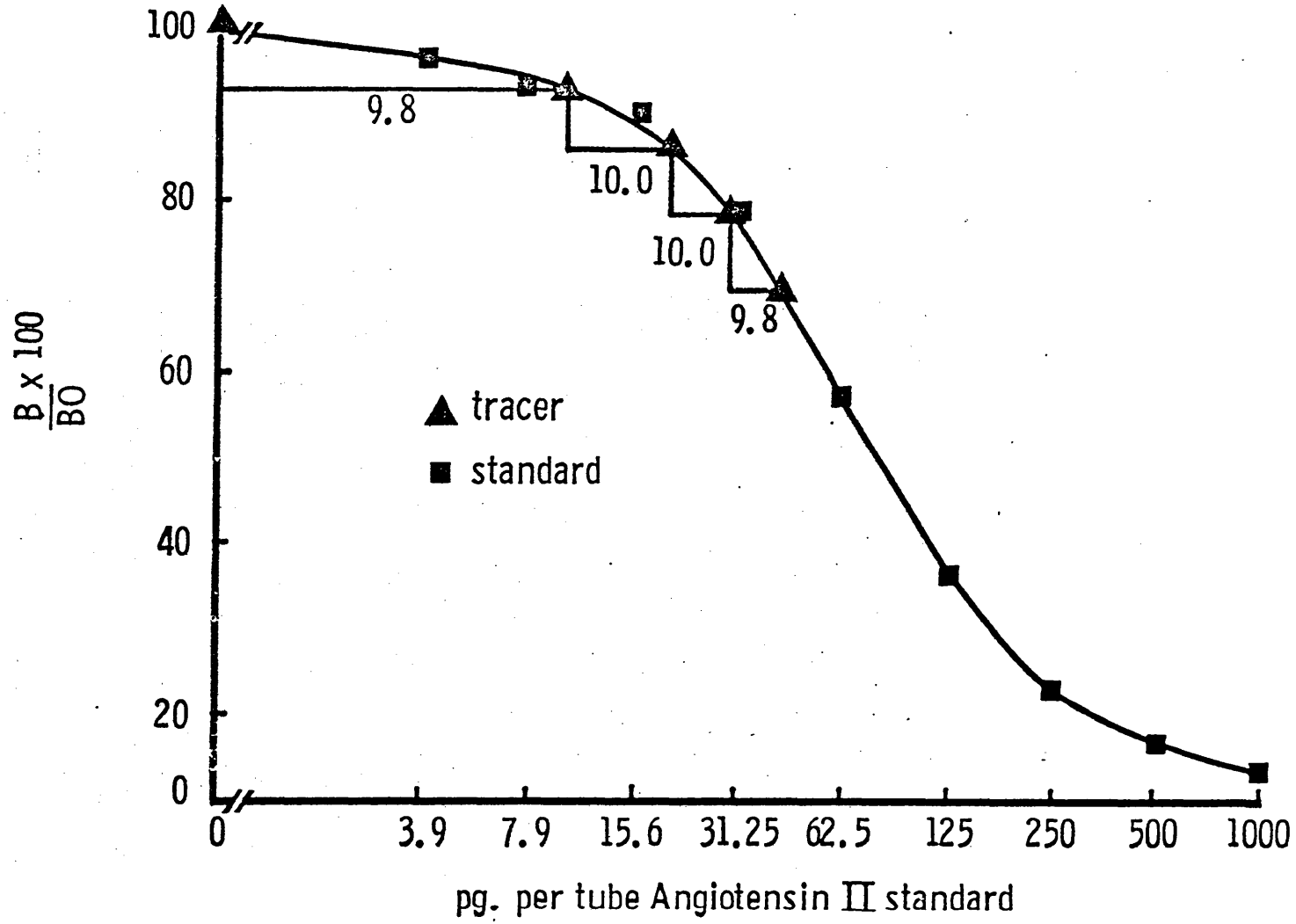


Table 5.4 Evaluation of the specific activity of labelled angiotensin I I by self-displacement analysis

The following protocol was routinely employed:

	Volume in µl			
	Non-specific binding	Zero antigen	Standards	Label assay
Buffer	300	250	150	200- 50
Standard	0	0	100	0
Label	50	50	50	100-250
Antibody	0	50	50	50
Incubation time/temp	24 h/4°C	24 h/4°C	24 h/4°C	24 h/4°C
$(NH_4)_2SO_4$	750	750	750	750

A typical set of results are shown in Figure 5.4.

The Iodination and Purification of Angiotensin I

Iodination procedure

An essentially identical protocol to that used for angiotensin II radiolabelling was employed. Sodium ^{125}iodide is oxidised to cationic iodine which substitutes as the tyrosine residue in the peptide. The oxidising agent, chloramine T, is neutralised rapidly (after 20 seconds) with sodium metabisulphite which also reduces any cationic or free-radical iodine to iodide.

$$I^+ + 2e^- \longrightarrow I^- \quad \text{Eq. 5.2}$$

$$I^\cdot + e^- \longrightarrow I^- \quad \text{Eq. 5.3}$$

Finally potassium iodide is added to solubilise any molecular iodine.

$$K^+ I^- + I_2 \longrightarrow K^+ I_3^- \quad \text{Eq. 5.4}$$

Protocol

(1)	0.05 M Phosphate buffer, pH 7.5	30 µl
(2)	NaI (IMS 30) in dilute NaOH (2 mCi)	20 µl
(3)	5 µg angiotensin I in phosphate buffer	25 µl
(4)	100 µg chloramine T	25 µl
(5)	240 µg metabisulphite	100 µl
(6)	6 mg potassium iodide	600 µl
		800 µl

Reagents 4, 5 and 6 were added at 20 second intervals. A 10 µl sample was taken at this stage for subsequent electrophoretic evaluation. The remainder (790 µl) of the reaction was then loaded on to a Sephadex A-25 anion exchange column followed by a further 200 µl rinsing volume of potassium iodide.

Purification of label

The reaction mixture was purified by being passed through a Sephadex A-25 anion exchange column which had been previously equilibrated with 0.1 molar acetic acid sodium acetate buffer, pH 5.5, for 48 hours at 4°C. The labelled hormone was eluted with 0.1 M sodium acetate acetic acid buffer, pH 5.5, and 1 ml fractions collected in tubes containing 0.25 ml of 0.4% BSA solution to prevent adsorption on to the tube walls. Forty fractions were collected over approximately 2 hours and counted on a Panax gamma-counter.

Assessment of damage by electrophoresis

Both the reaction mixture and the peak fraction of the DEAE eluate were assessed by electrophoresis in a manner analogous to that described for angiotensin II. Incorporation of iodide into angiotensin was estimated by drying the electrophoresis strip, cutting 1 cm sections and counting them in a Wallac gamma-counter. Routinely, about 75-80% incorporation of iodide into angiotensin was seen. Damaged peptide accounted for 5-7% and free iodide about 10-15%. Analysis of the peak fraction by electrophoresis showed that this was usually about 95% pure labelled angiotensin, with minimal damage (1%) and free iodide (2%).

Separation of label from starting material

The separation of label from starting material was demonstrated by radioimmunoassay of the fractions. After column fractionation each fraction was diluted $^{1}/10$ and $^{1}/100$ and assayed for angiotensin concentration. Two peaks were observed, the slower running peak eluting at the radioactive peak fraction numbers.

Self-displacement analysis and determination of specific activity

This procedure has been explained previously for angiotensin II labelling and is further justified at the end of this chapter. Self displacement data indicated that specific activities between 600 and 1000 mCi/mg were customary.

Rapid separation of angiotensin I label

The method of labelling was essentially similar to the above except in that a barbitone buffer, pH 8.6, was employed in the reaction vessel. Considerable time was, however, saved in the separation of peptide from iodide. This was complete within an hour using a Sephadex G-25 column (30 cm x 1 cm). This method, which has been previously reported (Roulston *et al.*, 1979), was finally abandoned as it was found that specific activities were consistently lower (450-550 µCi/µg) due, presumably to the presence of unlabelled starting material in the peptide peak (see Figure 5.5).

Validation of the self-displacement technique for estimation of specific radioactivity of radioimmunoassay tracers

The method of self-displacement for estimation of specific activity of radiolabelled tracers in radioimmunoassays is widely used. The principle is simple: increasing amounts of labelled antigen are incubated with a constant amount of antibody under similar conditions to those employed routinely in the assay (Gocke *et al.*, 1969); the mass of tracer present in the tubes is then determined by interpolating from a standard curve the percentage bound for each label increment; the amount of non-radioactive ligand displaced by each increment is equated to the amount of radioactive ligand in the tubes.

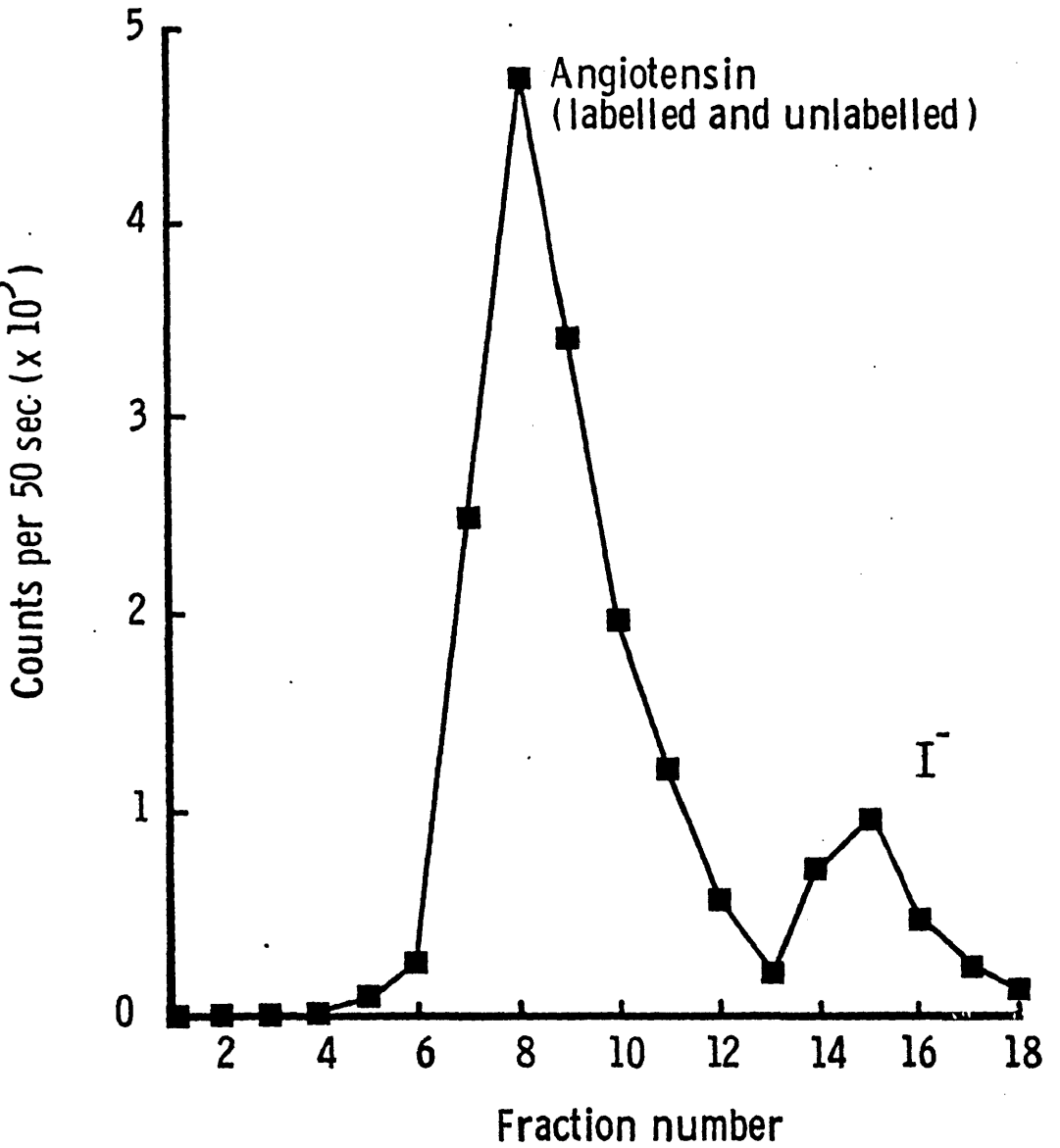


Figure 5:5

Fractionation of labelling reaction mixture by Sephadex G-25

It has recently been claimed, however (Morris, 1976), that this analysis is unsatisfactory as it fails to allow for the fact that an unknown mass of labelled antigen is also present and competing for binding sites on the antibody in each solution containing antigen standard. Further to this, a graphical analysis of the data is claimed by the same author to obviate this error.

As the self-displacement technique enjoys widespread application and popularity, the possibility that the analysis may be invalid was disturbing. It was therefore decided to scrutinise both methods of analysis of self-displacement data, theoretically and practically, in order to ascertain whether or not there is any basis for the hyopthesis of a flaw in the classical methodology and if so, whether descrepant results occur in either the theoretically ideal situation or in practical experimentation. It would be expected that specific activities, as reflected reciprocally by the calculated weight of tracer in the tubes, would be higher when calculated by the classical method if this proposed flaw in the logic exists.

Materials and methods

For repeated practical experiments the angiotensin I radioimmunoassay was used (Roulston and MacGregor, 1978). For the self-displacement assay incremental concentrations up to seven times that used in the standard curve were employed. In addition, data from angiotensin II analysis and other antigens were investigated. In order to test the theoretical basis of the situation, certain assumptions about the nature of the reactants were made in order to facilitate quantitative study. Firstly, it was assumed that labelled and unlabelled ligand were immunologically identical with respect to antibody affinity; secondly, non-specific

binding was assumed to be negligible and separation of bound and free fractions was assumed to be 100%. The reaction was also assumed to be in equilibrium and, therefore, to follow standard kinetics as reviewed comprehensively elsewhere (Ekins *et al.*, 1968; Yalow and Berson, 1968; Roulston and Adam, 1978).

The following values were therefore assumed: (a) a tracer concentration of 20 pg per tube and 1000 counts per minute per picogram; (b) standards of 4, 8, 16, 32, 64 and 128 picograms per tube; and (c) antibody present in such concentration that it bound up to 10 pg of antigen. From these data a statistical curve and self-displacement assay were constructed, and our starting weight of label (20 pg per tube) was calculated by both methods to see if there was any difference in the results predicted in this idea case. Furthermore, by construction of the standard curve and self-displacement curve for the practical assay, we further ascertained whether or not theory and practice were consistent and whether or not there was a discrepancy in the methodology. Table 5.5 shows the construction of the theoretical standard curve based on the axioms outlined. The self-displacement assay, constructed similarly, is also shown. Superimpositions of these data reveals (Figure 5.6) that each increment of label causes a 20 pg displacement in percentage binding on the standard curve indicating that the weight of label used in the standard curve was 20 pg. Similarly, by plotting interpolated doses against counts per minute added, the intercept on y at x = 0 is -20 pg (Figure 5.7). Thus for an ideal situation the two methods give identical results, and no flaw in the original methodology, or its theoretical basis, is apparent.

Table 5.6 shows the results of one of the practical experiments with angiotensin I and the self-displacement results obtained.

Table 5.5 Theoretically calculated antigen bindings for a dose-response curve and self-displacement analysis

Mass of unlabelled antigen added (pg)	Mass of tracer added (pg)	Mass of unlabelled antigen bound (pg)	Mass of tracer bound (pg)	Percentage of tracer bound
0	20	0	10	50
4	20	1.67	8.33	41.65
8	20	2.86	7.14	35.7
16	20	4.44	5.56	27.8
32	20	6.15	3.85	19.25
64	20	7.62	2.38	11.9
96	20	8.27	1.73	8.65
128	20	8.65	1.35	6.75
0	20	0	10	50
0	40	0	10	25
0	60	0	10	16.67
0	80	0	10	12.50
0	100	0	10	10.00
0	120	0	10	8.33

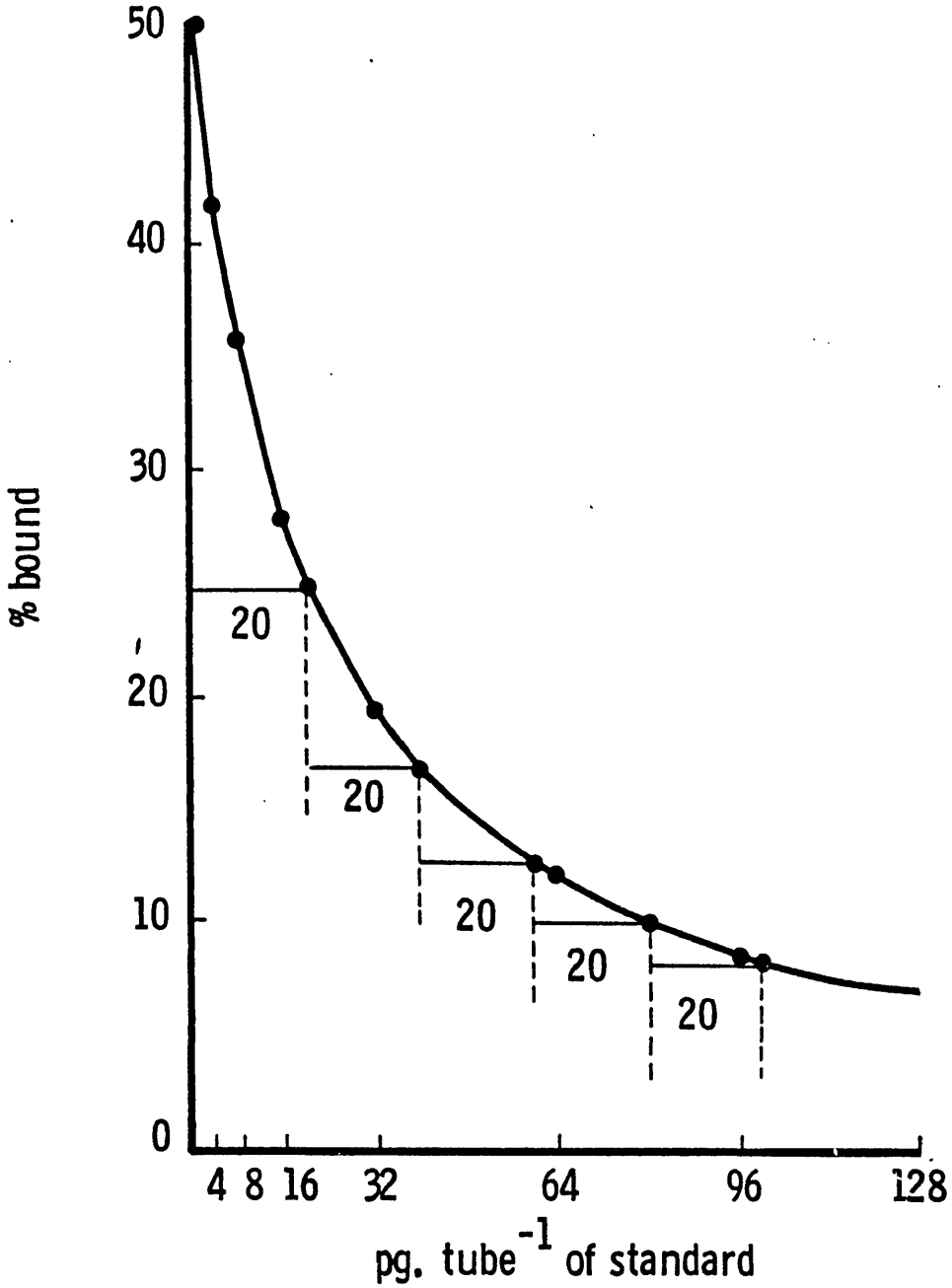


Figure 5:6

Theoretically calculated standard curve and self-displacement

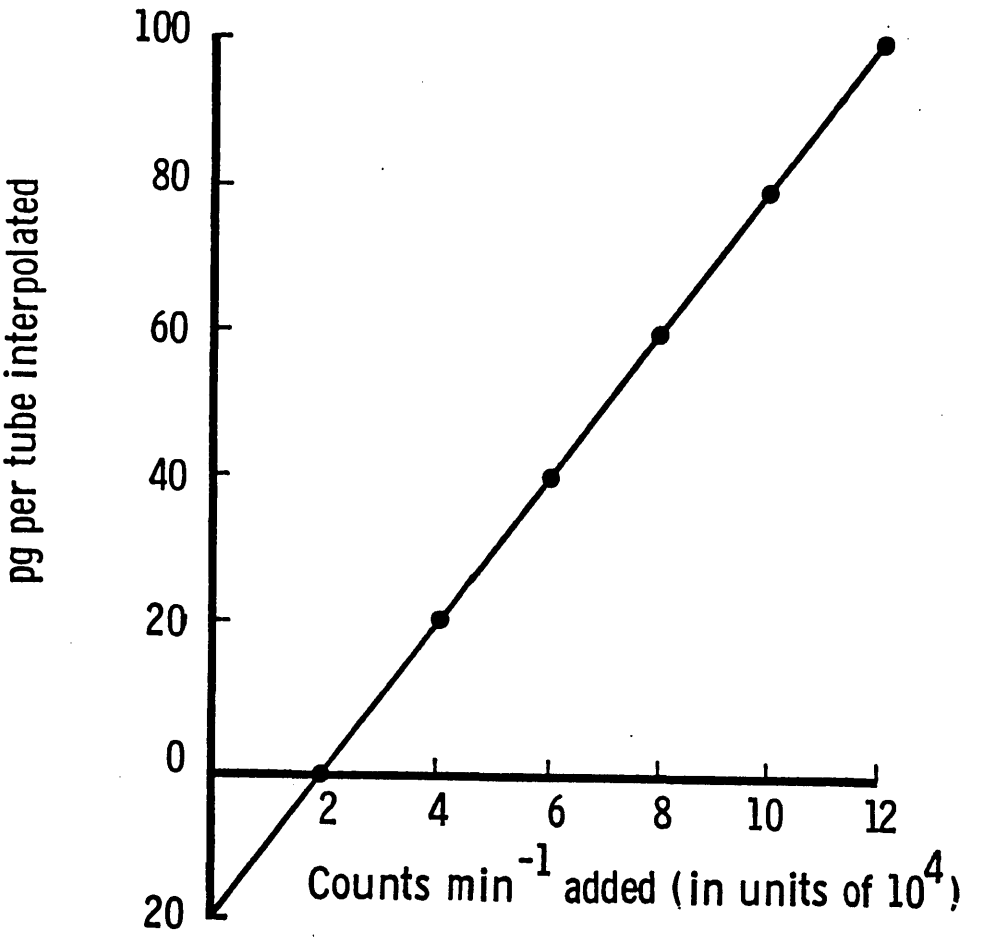


Figure 5:7

Plot of interpolated dose as a function of counts added for the theoretical self-displacement analysis.

Table 5.6 Experimental results for antigen bindings for a dose-response curve and self-displacement analysis

Relative tracer concentration	Unlabelled antigen added (pg)	Percentage of tracer bound
1	0	50.0
1	10	44.5
1	20	39.6
1	40	32.15
1	80	24.9
1	160	16.0
1	320	8.65
1	640	5.10
2	0	33.4
3	0	27.0
4	0	22.1
5	0	17.5
6	0	14.6
7	0	12.20

Superimposition of these upon the standard curve obtained shows that the mean value for the weight of label in the tubes is 38.7 pg (Figure 5.8). Figure 5.9 shows that the graphical determination yields the same value. These results concur very well with the theoretical results obtained above.

Table 5.7 shows the results obtained for further batches of angiotensin I and for a variety of other antigens: angiotensin II, human chorionic gonadotrophin, gastrin, casein, carcinoembryonic antigen, alphafetoprotein, and a putative tumour-associated ovarian antigen. These results provide further corroboration, as the mean percentage bias was less than 0.1%. The differences obtained between the two methods were not significant when assessed using Wilcoxon's signed rank test ($n = 19, \bar{x} = 0.0615, t = 18, P > 0.1$).

Discussion

It is apparent from the theoretical results obtained (Table 5.5, Figures 5.6 and 5.7) that in an ideal situation there is no flaw in the classical self-displacement procedure and that the graphical plot is merely an alternative, perhaps more elegant, way of displaying the result. Also, from the practical results obtained in this laboratory, no difference between the methods could be found (Figures 5.8 and 5.9; Tables 5.6 and 5.7) and therefore one is led to conclude that the hypothesis of an error in the classical analysis is a false one since no discrepancy in results is obtained in theoretical or practical studies.

Furthermore, detailed study of the theoretical premises of the reactions shows that there is no basis for the suggested error; the competition for binding sites between molecules of standard and tracer is immunologically identical with the competition between molecules of

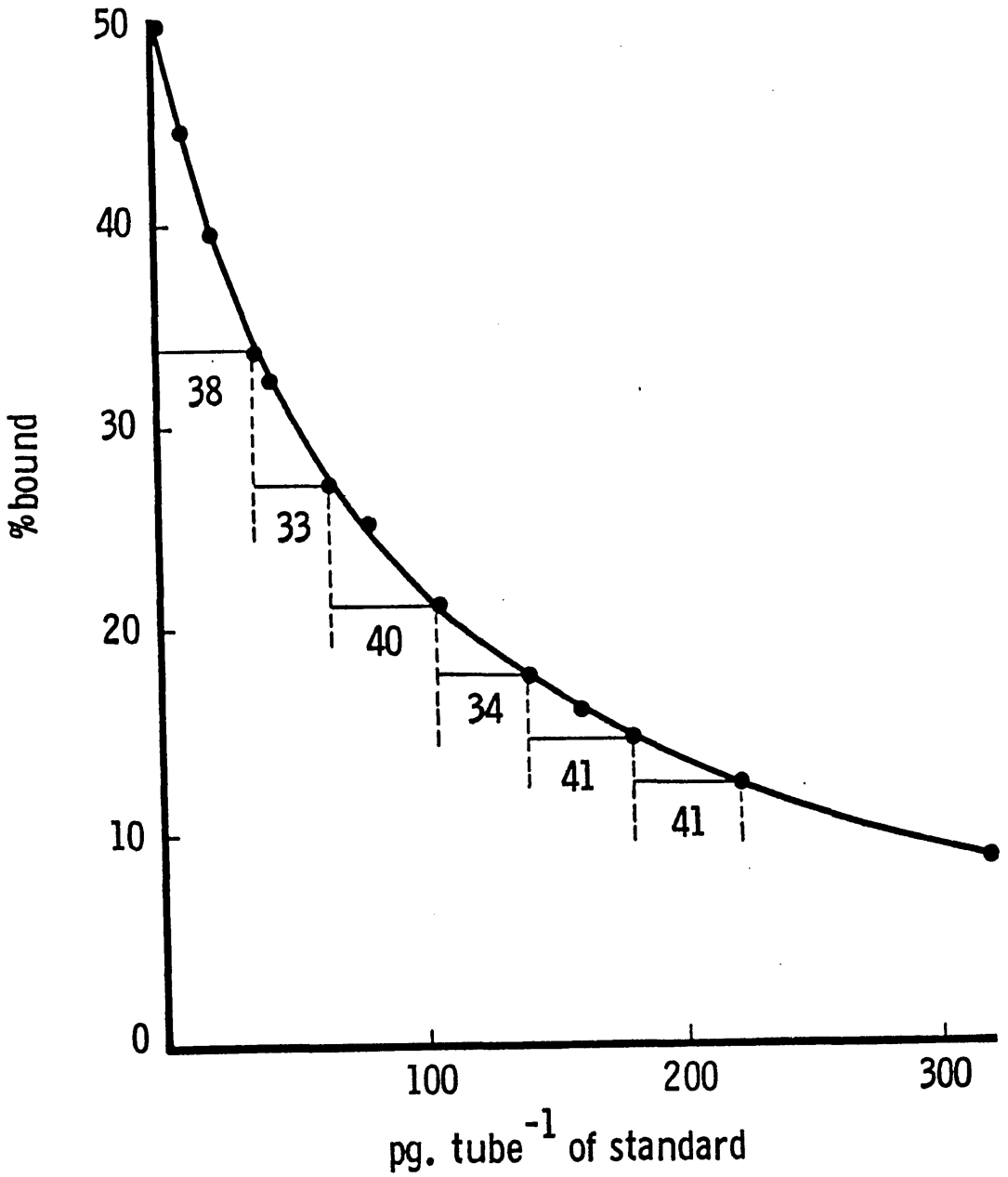


Figure 5:8

Practical dose response curve with self-displacement analysis

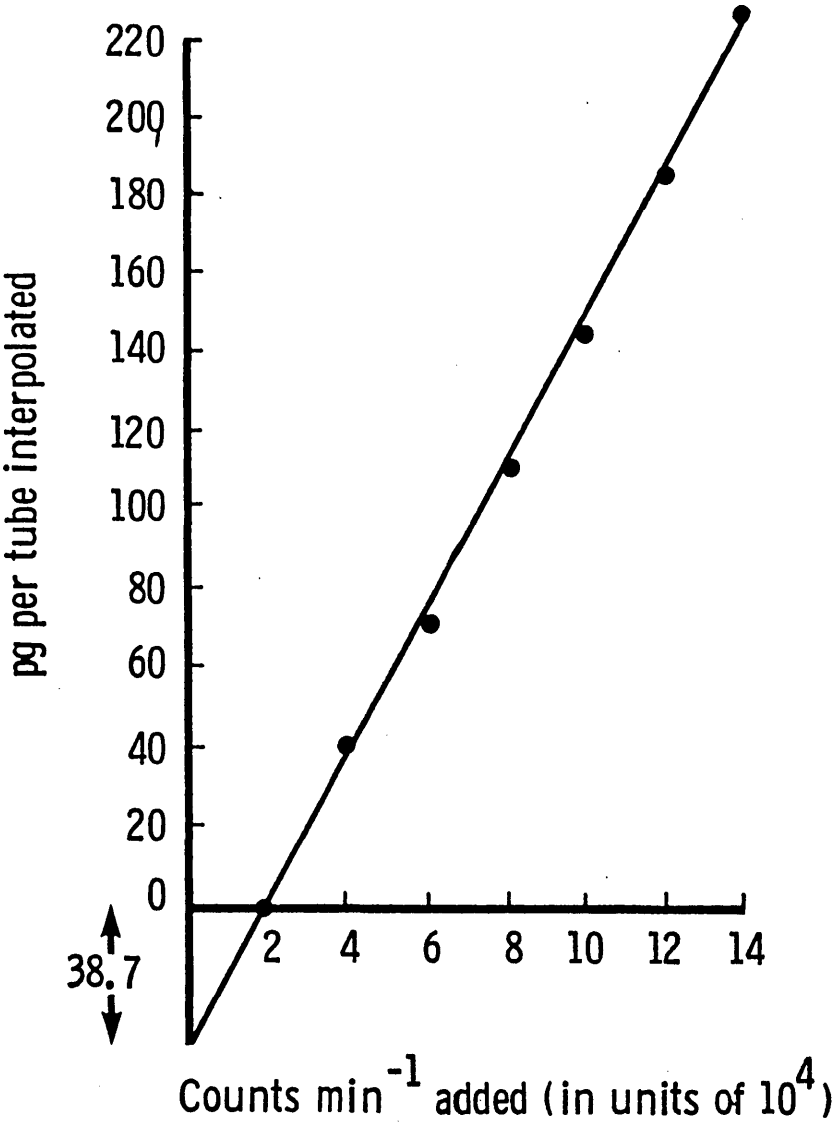


Figure 5:9

Plot of interpolated dose as a function of counts added for the practical self-displacement analysis.

Table 5.7 Comparison of values obtained using classical and modified self-displacement data analyses on a variety of antigens

Antigen	Weight of label per tube (pg)		% Bias $\frac{(A-B)}{A} \times 100$
	Classical calculation A	Modified calculation B	
Angiotensin I	30.5	30.0	+1.67
	8.8	9.0	-2.27
	30.0	30.0	0
	30.0	30.0	0
	40.0	36.0	+10.0
	38.7	38.7	0
	73.5	77.5	-5.4
	110	110	0
	28	28	0
Angiotensin II	4.8	4.4	+8.3
HCG	135	140	-3.7
	100	102.5	-2.5
	97.5	100	-2.56
	160	160	0
Gastrin	7.75	7.15	+7.74
Casein	3,625	3,750	-3.44
CEA	456	456	0
AFP	225	240	-6.67
Ovarian antigen	3,625	3,625	0
			Mean difference = + 0.0615%

tracer alone. Also, as displacement is relative, then absolute total antigen concentration is irrelevant to the analysis. It is, however, worthy of note that any differences in affinity for antibody exhibited between labelled and unlabelled antigen will be equally reflected in terms of the value for weight of label obtained by either of the above methods. Since the result is derived from displacement of unlabelled antigen from a standard curve, then, if the tracer has lower affinity for the antibody than the standard, the result for weight per tube obtained will be proportionately lower, hence the apparent specific activity will be higher irrespective of the method of calculation used.

Finally, it should be stressed that degree of saturation of antibody is assumed constant in the situations considered, otherwise a fully quantitative treatment is not possible for self-displacement analysis theoretically or practically.

CHAPTER SIX

PREPARATION AND EVALUATION OF ANTIBODIES

Preparation and evaluation of antibodies

Of prime importance for radioimmunoassay development is a high affinity, specific antibody for the substance being measured; and several low weight hormones have been inadequately studied because of their poor immunogenicity. The immune response is a complex phenomenon involving many steps between the introduction of an antigen and the production of specific antibodies (Benacerraf, 1968; Franklin, 1968; Sela, 1969; Edelman, 1970; Schechter *et al.*, 1970; Odell *et al.*, 1971; Odell *et al.*, 1972). Although many peptide hormones (for example growth hormone, luteinising hormone, follicle stimulating hormone, thyroid stimulating hormone) appear to possess the necessary antigenic qualities to render them immunogenic, peptides with a molecular weight below 4,000 generally have been found to be poor immunogens when used uncoupled. Although molecular weight is not the only determinant for inherent antigenicity, there does appear to be a generally inverse relationship between molecular size and the ability of a substance to evoke antibody production (Borek *et al.*, 1967; Gill *et al.*, 1967; Sela, 1969; Odell *et al.*, 1971 and 1972). Exceptions are found but are uncommon (Gilliland and Prout, 1965; Roth *et al.*, 1966; Eisentraut *et al.*, 1968; Hansky and Cain, 1969).

Therefore it was decided to conjugate angiotensin to a hapten-carrier molecule, bovine serum albumin, using ethyl carbodiimide (Goodfriend *et al.*, 1964) as a coupling agent. Carbodiimides promote coupling by activation of carboxyl groups to form a C-N bond, unlike glutaraldehyde which forms a bridge between amino groups. For practical purposes the total number of free carboxyl groups approximates to the total sum of aspartic and glutamic acid residues. Likewise the total number of free amino groups approximates to the sum of lysine and

arginine residues. Albumin contains a total of 118 aspartic and glutamic acid residues compared with 73 lysine and arginine residues per mole (Tristram and Smith, 1963). Therefore assuming equal availability of the $-COO^-$ groups as the $-NH_3^-$ groups carbodiimide is the logical choice of coupling agent for albumin. Unfortunately no antibody response at all was observed when this immunogen (using angiotensin I in this case) plus an adjuvant was used in three NZW rabbits. Skowsky and Fisher (1972), however, extending the work of Chopra *et al.* (Chopra *et al.*, 1971), found that bovine thyroglobulin used in conjunction with carbodiimide yielded highly effective immunogens. Thyroglobulin possesses about 1100 aspartic and glutamic acid residues per mole as opposed to 500 arginine and lysine residues Ui *et al.*, 1961; Spiro, 1970). Therefore carbodiimide should yield effective coupling to peptides. The molar ratios of the reactants were chosen based on Skowsky and Fisher's findings using lysine vasopressin and parathyroid hormone as antigen. They found that a ratio of LVP:PTH-Tg-CDI of 118:1:200 gave optimal final incorporation of hormone into carrier (see Table 6.1).

Preparation of immunogen

To 10 mg of angiotensin I (8 of angiotensin II) dissolved in 1.5 ml of deionized water, pH 7.4, was added 42 mg of bovine thyroglobulin. To this solution was added 2.5 mg of 1-ethyl 3 (3-dimethyl aminopropyl) carbodiimide HCl dissolved in 0.5 ml of deionized water. The initial molar ratios approximated to those recommended for optimal incorporation by Skowsky and Fisher (1972). After thorough mixing the reaction was allowed to stand at room temperature for 18 hours at which time an equal volume of one molar hydroxylamine was added and mixed thoroughly. The

Table 6.1 The weights and molar ratios of the reactants used to prepare immunogens

	Antigen used		Hapten carrier	Coupling agent
	AI	AII	B.Tg.	C.D.I.
Molecular weight	1297	1047	6.5×10^5	191.7
Milligrams used	10.0	8.0	42	2.5
µMoles used	7.71	7.64	0.065	13.0
Molar ratio of reactants	118.6	117.6	1	200

mixture was then incubated for a further 5 hours at room temperature. The hydroxylamine was added to reverse a possible tyrosine rearrangement in the presence of carbodiimide. Carraway and Koshland (1968) showed that carbodiimides may alter the aromatic ring of the tyrosine residue, to form either O-arylisourea or N-arylurea. Since the tyrosine in position 4 in angiotensins may be in the locus of the antigenic determinant such rearrangement could lead to antibodies being produced which failed to recognise unmodified angiotensins.

The immunogen was stored in aliquots at -20° until required for use.

Immunisation procedure

The immunogen was given as an emulsion, mixed 50/50 (v/v) with Freund's complete adjuvant (Freund, 1951). This adjuvant is a mixture of water microdroplets, paraffin oil, Arcel A (a detergent or emulsifying agent) and heat killed desiccated mycobacteria. The detergent contains both hydrophilic and hydrophobic groups and binds both the oil and aqueous phases of the immunogen, forming a stable emulsion. The adjuvant is thought to act by delaying the absorption and rapid degradation of the immunogen by circulating proteolytic enzymes. Hence smaller quantities of immunogen may be used. It also enhances phagocytosis of the immunogen by macrophages, an essential step for antibody production.

To an aliquot (300 µl) of immunogen was added 300 µl of adjuvant and the two were mixed extremely thoroughly, until a stable emulsion formed. This emulsion was then injected intradermally into about 40 sites on the back of a New Zealand White rabbit [female, weighing approximately 2.2 kg (5 lb)]. Booster injections were

administered similarly after 28 days and 10 ml blood samples from the central ear artery were taken at weekly intervals. This blood was allowed to clot then spun at 2,000 g for 20 minutes, and the serum stored in small aliquots at -20°C until tested. When an antiserum of suitable quality was obtained the animal was anaesthetised with pentobarbitone and exsanguinated via a carotid catheter. The serum obtained was stored in aliquots at -20°C.

Seven rabbits were immunised as described; three with angiotensin I, four with angiotensin II.

Incorporation of hormone into carrier

Final molar incorporation of angiotensin I on to thyroglobulin was estimated by adding a trace of labelled angiotensin to the conjugation mixture. The reaction mixture was then applied to a 30 x 1 cm Sephadex G-100 column and 1 ml fractions were diluted with 0.1 M ammonium bicarbonate and collected and counted. Estimation of the incorporation of angiotensin was calculated by integration of the peaks of the elution profile (see Figure 6.1). The area under the curve at the void volume being an index of the amount of angiotensin-thyroglobulin, whilst the area under the total volume peak was an index of free I^{125}-angiotensin and polymers thereof. Therefore it may be seen that $\frac{\Sigma Vv}{\Sigma Vv + \Sigma Vt} \equiv$ the fraction of angiotensin added to the reaction mixture which is condensed by the carbodiimide to thyroglobulin. Approximately half the counts appeared in the void volume peak indicating a final molar ratio of 60:1 of angiotensin I with respect to thyroglobulin. For the purposes of these experiments it was, of necessity, assumed that iodinated angiotensin reacted identically to unlabelled angiotensin in the carbodiimide condensation. Similar experiments using angiotensin II as hapten indicated

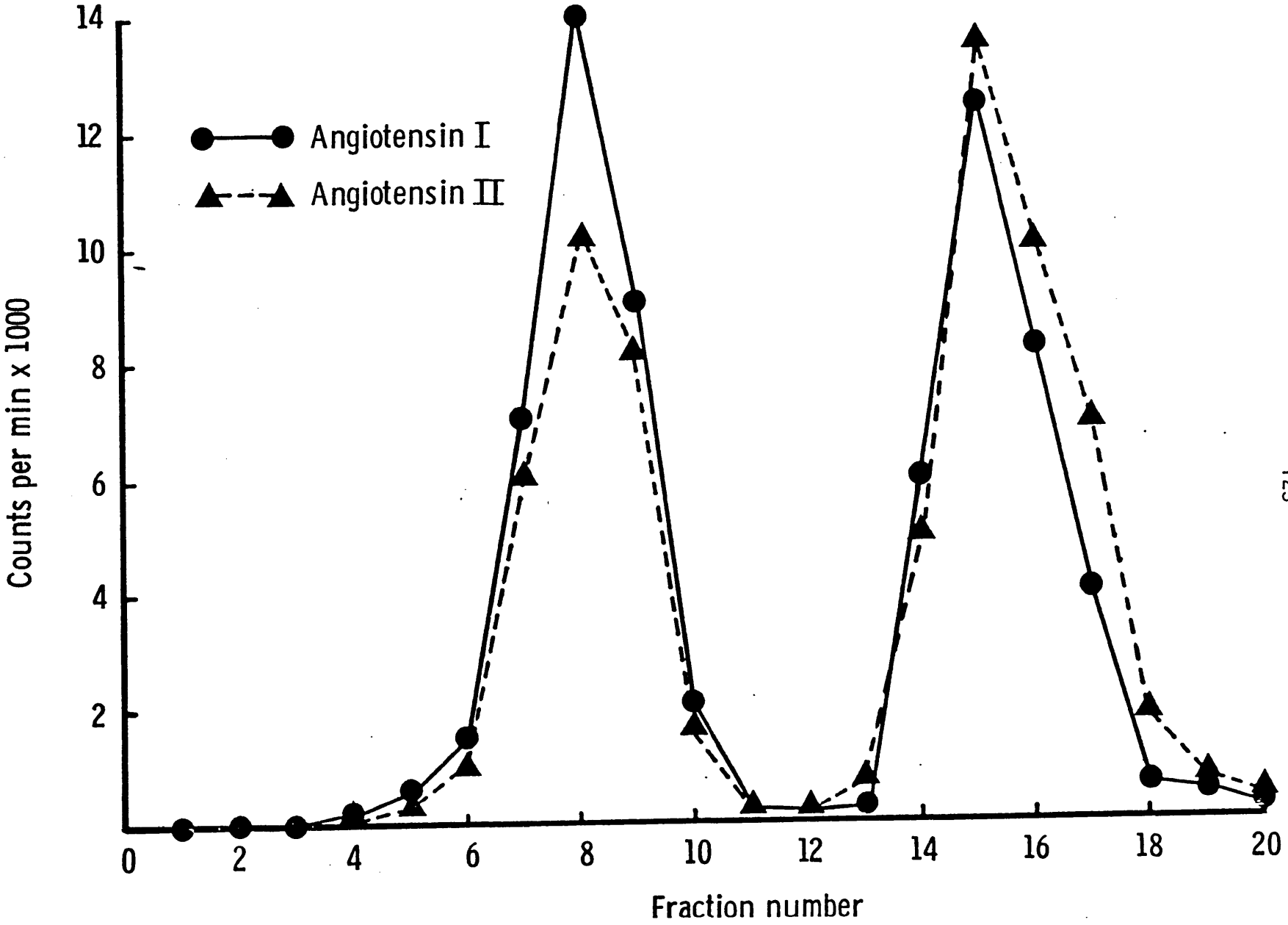


Figure 6:1, Incorporation of hormone into carrier; estimation from Sephadex G-100 column *chromatography data*,

that the final incorporation ratio was of the order of 45:1 of angiotensin II with respect to thyroglobulin.

Assessment of primary antibodies

The choice of an antibody depends upon three main criteria. Firstly, it must have a suitably high titre. The titre may be defined as that dilution of the antibody which will bind 50% of the labelled antigen in the absence of competing ligand. In order to be able to standardise an assay a sufficient quantity of antiserum must be available to enable its consistent use over a long period of time, hence the importance of a high titre.

Secondly, the antiserum must have a high specificity for the antigen to be measured. Cross reactivity with other physiological molecules or drugs may totally prevent an antiserum being of use.

Thirdly, there is the avidity of the antibody for its antigen. In biological terms the avidity of an antibody would manifest itself by its ability to destroy the biological action of the antigen. In antiserum titration (or dilution) curves the ability is indirectly related to the slope of the curve.

Titration curves

Antibody titration curves were usually done with doubling dilutions, 1/100 upwards, of the serum concerned diluted in 1% normal rabbit serum in buffer. The initial 1/100 dilution was done in buffer only, to keep the total serum concentration constant in all dilutions. A constant amount of labelled angiotensin (≃10 pg) was incubated with the serial dilutions of the antibody for 24 hours at 4°C. The bound and free fractions were separated by the addition of charcoal and subsequent

centrifugation and the percentage bound plotted as a function of antiserum dilution. Any antisera yielding a practicable return of counts at a reasonable dilution (50% recovery at 1/5000) were assessed further for cross reactivity and avidity.

Cross reactivity studies

Antiangiotensin I

Assays to produce standard curves were incubated using angiotensin I (^{5}Ileu), angiotensin II (^{5}Val), angiotensin II (^{5}Ileu) and human renin substrate. No inhibition of binding of I^{125} angiotensin I was observed with any antigen other than angiotensin I.

Antiangiotensin II

Since the antibodies were raised against H_2N-Asp^{1}-Val^{5} angiotensin II it was essential to check cross reactivity with human (^{1}Asp-^{5}Ileu) angiotensin II. Also, since angiotensin II is only one component in a series of enzyme reactions involving renin substrate, angiotensin I and the metabolic breakdown products of angiotensin II these other compounds were assessed for cross reactivity. The inhibition of the reaction between the antiserum and ^{125}I-(H_2N-^{1}Asp^{5}Val)-angiotensin II by these substances was assessed in a manner essentially identical to that for angiotensin I. Increasing amounts of the antigen analogue under study were incubated with a constant dilution of the antiserum and a constant amount of the radiolabelled angiotensin II. The percentage cross reactivity of analogue with respect to angiotensin II was defined as the quantity of analogue required to displace 50% of binding of the I^{125} angiotensin II to the antiserum divided into the quantity of angiotensin II standard required to achieve

the same displacement of label, multiplied by 100. Table 6.2 summarises the results obtained with the antibody subsequently chosen as the one for R.I.A. development.

Estimation of antibody avidity

A working value for the affinity constant K was simply achieved using the Scatchard and Odell graphical analyses. Both methods depend, in this context, upon the mass-action law and assume the reaction of antigen with antibody to be an equilibrium. Such an approximation does not however consider allosteric factors and interpretation of the data may be difficult unless further proof of the validity of the approximation can be obtained.

Principle of Scatchard plot

Assuming a starting concentration of antigen p_o and of antibody (expressed as number of binding sites) q_o. At equilibrium application of the Law of Mass Action gives the following equation:-

$$(p_o - p) + (q_o - q) \rightleftharpoons pq \qquad \text{Equation 6.1}$$

where $(p_o - p)$ and $(q_o - q)$ are the concentrations of antigen and antibody free and pq the concentration of the complex.

The affinity (or equilibrium) constant is defined as

$$K = \frac{pq}{(p_o - p)\,(q_o - q)} \qquad \text{Equation 6.2}$$

Therefore rearranging,

$$Kq_o - Kq = \frac{pq}{(P_o - p)} = \frac{B}{F} \qquad \text{Equation 6.3}$$

Table 6.2 Cross reactivity of antiangiotensin II

	Component or analogue	% Cross-reaction (molar basis)
("Hypertensin")	Asp^1NH_2-Val^5-angiotensin II	100
	Asp^1-Val^5-angiotensin II	100
	Asp^1-$Ileu^5$-angiotensin II	100
	Asp^1-$Ileu^5$-angiotensin I	8.5
	Human renin substrate	0
	$Ileu^5$-heptapeptide	100
	$Ileu^5$-hexapeptide	100
	$Ileu^5$-pentapeptide	100
	Val^5-heptapeptide	85
	Val^5-hexapeptide	87

Let B/F ratio = R Equation 6.4

Then $R = Kq_0 - Kq$ Equation 6.5

since at equilibrium p = q = pq

$R = Kq_0 - Kp$ Equation 6.6

Therefore a plot of R vs p (bound/free ratio versus concentration of antigen bound) should yield a straight line of slope -K and intercept Kq_0 on the y axis and q_0 on the x axis.

In order to obtain an accurate result it is essential to know the total weight of antigen present, that is, standard + label, hence a self-displacement analysis is a vital pre-requisite.

Odell plot

This is an alternative way of presenting the data. It is of particular value if the Scatchard is non-linear and the slope variable. The Odell plot is a saturation curve and from the asymptote a mean value for K may be obtained.

The concentration of antigen bound is plotted as a fraction of antigen added. To ensure total saturation an extra "standard" containing 10-fold concentration of the usual highest standard should be incubated. This "infinite antigen" assessment is a true measure of non-specific binding also, as it represents that binding of label which is irreversible, with respect to antigen competition.

From the curve one may interpolate the concentration of antigen required to 50% saturate the antibody. Hence the concentration of free antigen may be ascertained by simple subtraction of antigen bound from antigen added at that point. The reciprocal of free antigen concentration at 50% saturation is equal to K. This is apparent since

$$K = \frac{pq}{(p_o - p)(q_o - q)} \qquad \text{Equation 6.2}$$

At 50% antibody saturation, $q_o = 2q$ *ipso facto* Equation 6.7

$$\therefore \quad K = \frac{pq}{(p_o - p)\, q} \qquad \text{Equation 6.8}$$

since at equilibrium $p = q = pq$

then $pq/q = 1$

$$K = \frac{1}{(p_o - p)} = \frac{1}{(\text{free})} \qquad \text{Equation 6.9}$$

Having obtained values for K and q it is possible to use these to teleologically validate the assumption made initially about the equilibrium kinetics.

$$\text{If,} \quad K = \frac{pq}{(p_o - p)(q_o - q)} \qquad \text{Equation 6.2}$$

$$\text{then} \quad K = \frac{R}{(q_o - q)} \qquad \text{Equation 6.10}$$

$$\text{since} \quad R = \frac{pq}{p_o - p} = \frac{(\text{bound})}{(\text{free})} \qquad \text{Equation 6.3}$$

by definition

$$\text{Thus} \quad Kq_o - Kq = R \qquad \text{Equation 6.5}$$

The unknown in this equation is q - the concentration of antibody binding sites bound to antigen. However, this is the same as p, the concentration of antigen bound. Now

$$p = p_o \times \frac{R}{R + 1} \qquad \text{Equation 6.11}$$

That is, the concentration of antigen bound equals the concentration added multiplied by the fraction bound.

Therefore

$$Kq_o - \frac{Kp_oR}{R + 1} = R \qquad \text{Equation 6.12}$$

multiplying by $(R + 1)$

$$Kq_o (R + 1) - Kp_oR = R(R + 1) \qquad \text{Equation 6.13}$$

rearranging and opening parentheses

$$RKq_o + Kq_o - RKp_o = R^2 + R \qquad \text{Equation 6.14}$$

$$\text{i.e. } R^2 + R (1 + Kp_o - Kq_o) - Kq_o = 0 \qquad \text{Equation 6.15}$$

Hence from this quadratic equation one may predict the bound to free ratio and thereby the percentage bound at any point on the standard curve if K, q_o and p_o (label + standard) are known. It the approximation of K and q_o are good this indicates that the application of Mass Action Kinetics is valid and this is shown by the superimposability of the theoretical curve upon the experimental results.

Methodology

A routine assay to yield a standard curve was incubated in parallel to an assay to produce a self-displacement analysis as described earlier. In addition an "infinite antigen" standard was included for the purpose of assessing 100% saturation. After incubation of the tubes for 24 hours at 4°C the bound and free phases were separated by addition of charcoal, centrifugation at 1500 g and decantation of the supernatant. Both bound and free fractions were subsequently counted on a Wallac 80000 gamma counter.

The results (Figures 6.2 and 6.3, Tables6.3 and 6.4) are for one antiangiotensin I serum and one antiangiotensin II serum. The results obtained on these two antisera indicate that in each case there appears to be only one class of binding site for the respective antigen. This can be seen from the good linearity of the Scatchard data. The results also show that the theoretical dose response curves are good approximations of the actual experimental results, indicating that the values of K and q_0 and p_0* (label) evaluated were good approximations and that the Ekins quadratic equation, derived from the Law of Mass Action, is a good approximation to the *in vitro* kinetic situation. The slight deviations from parity observed in the predicted 'R' values at high concentrations of angiotensin II are possibly due to the fact that the last three points are at >100% saturating concentrations of antisera and with this equation, as with any quadratic, a true asymptote is never reached with finite concentrations of antigen. However, over the working range of the dose response curve the kinetic model holds very well.

The selected antisera were stored at -20°C in 2 ml sealed aliquots at 10 x the prospective working concentration.

Secondary antisera

The double antibody technique for separating free from bound ligand became widely used in the radioimmunoassay field following early work by Utiger *et al.* (1962), Morgan and Lazarow (1962) and Hales and Randle (1963).

The principle of the double antibody technique is very simple. The initial stage of the assay is similar to other protocols in that labelled and unlabelled ligand are allowed to compete for a limiting number of binding sites on the primary antibody. At equilibrium the

Figure 6:2

Scatchard plots for an antiangiotensin I and an antiangiotensin II

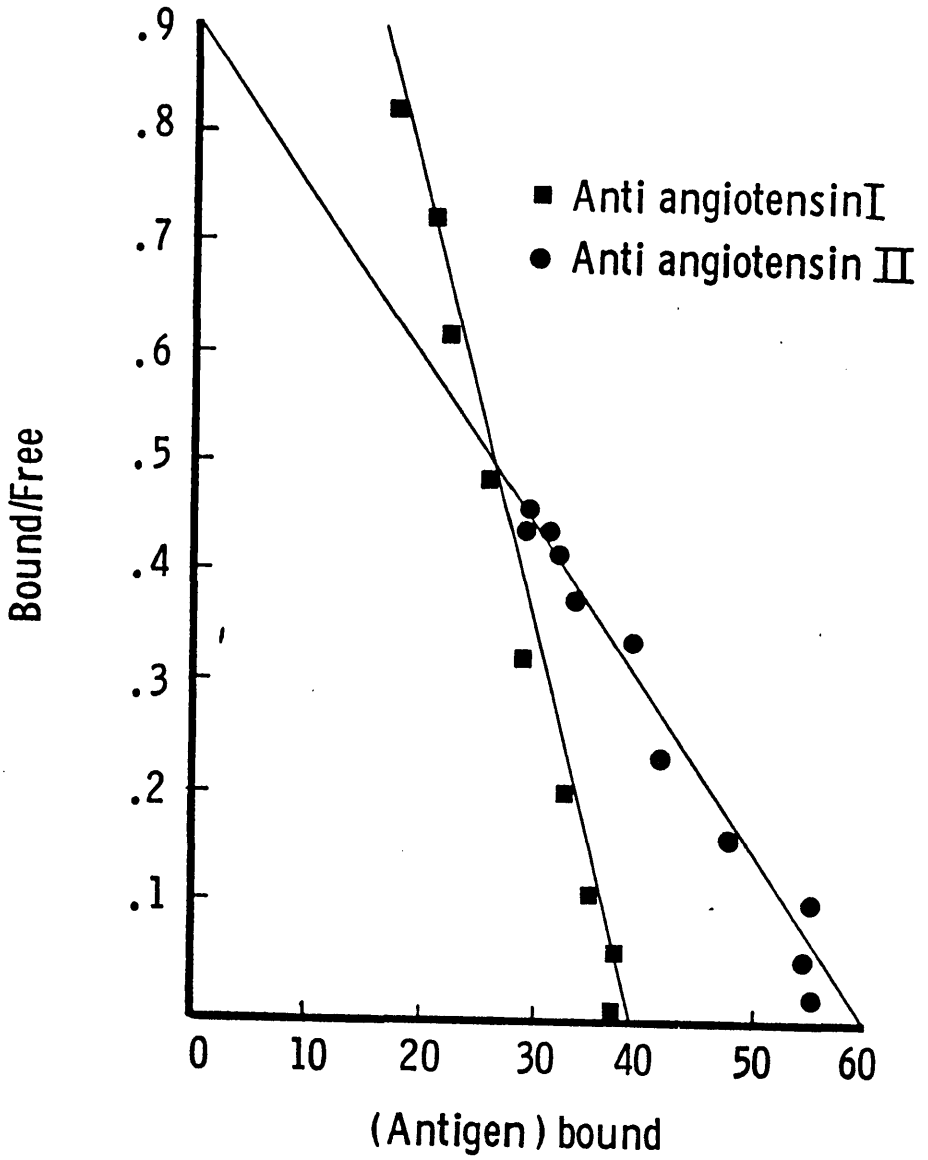
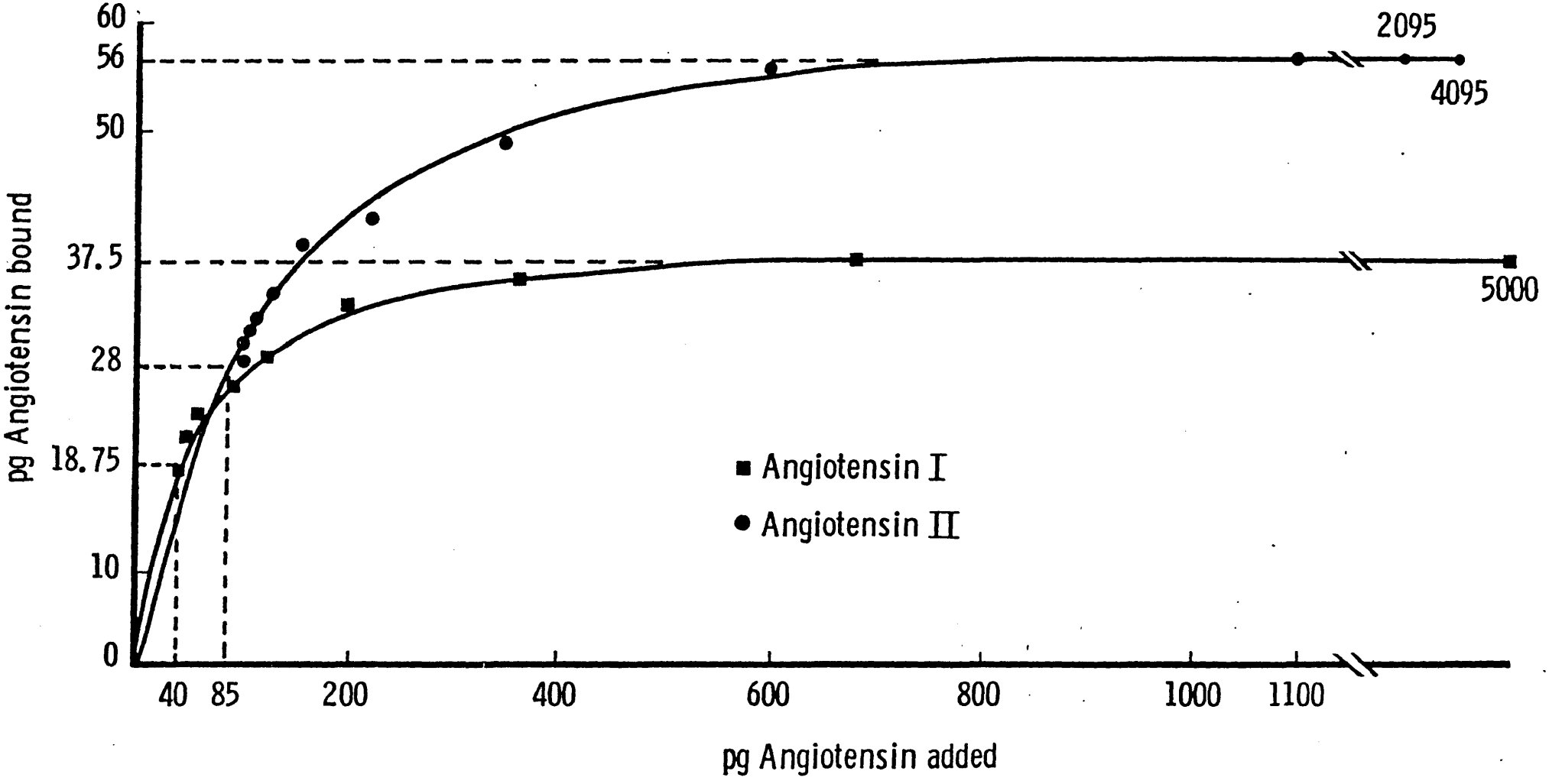


Figure 6:3

Odell saturation analysis for an antiangiotensin I and an antiangiotensin II;



Tabel 6.3 Results of experiment to determine K and q_0 for angiotensin I by Scatchard and Odell plot including results predicted for dose response curve by Ekins quadratic equation.

Antigen added	% Bound	$\frac{B}{F}(R)$	Antigen bound	Predicted value R	Predicted value % Bound
40	45.11	0.822	18.04	0.820	45.05
50	42.06	0.726	21.03	0.698	41.10
60	38.07	0.615	22.84	0.610	37.90
80	32.77	0.487	26.22	0.481	32.50
120	24.18	0.319	29.02	0.330	24.80
200	16.77	0.201	33.54	0.198	16.52
360	9.87	0.110	35.53	0.109	9.80
680	5.50	0.058	37.40	0.057	5.38
5,000	0.75	0.0076	37.50	0.0077	0.76

Table 6.4 Results of experiment to determine K and q_0 for an angiotensin II antibody by Scatchard and Odell plot including results predicted for dose response curve by Ekins' quadratic equation

Antigen added	% Bound	$\frac{B(R)}{F}$	Antigen bound	Predicted value R	Predicted value % Bound
4095	1.57	0.015	56.10	0.0146	1.44
2095	2.68	0.029	56.10	0.0285	2.77
1095	5.10	0.054	55.85	0.0544	5.16
595	9.30	0.103	55.34	0.0994	9.04
345	14.06	0.164	48.51	0.167	14.35
220	18.92	0.233	41.62	0.252	20.11
157.5	25.00	0.333	39.38	0.332	24.92
126.25	27.40	0.377	34.59	0.391	28.13
110.63	29.50	0.418	32.63	0.429	30.01
102.81	30.40	0.437	31.25	0.450	31.02
98.91	30.44	0.437	30.10	0.461	31.54
95.00	31.30	0.456	29.74	0.472	32.08

molecules of ligand bound to primary antibody are rendered insoluble by further incubation with an immunoglobulin directed against the first antibody. If conditions are correct the second antibody aggregates (usually requiring the presence of carrier IgG) to form an insoluble complex separable from the free ligand by centrifugation or filtration.

An alternative approach is the technique of pre-precipitation as described by Hales and Randle (1963) which involves pre-precipitation of the first antibody by the second. This pre-precipitated first and second antibody complex is then added to the assay as a complete binding reagent and then removed, either by filtration or centrifugation after it has been allowed to react with the ligand. This technique has the advantage, in theory, that the first and second antibody complex can be formed under controlled conditions where artefacts resulting from the presence of serum or similar substances can be removed. Furthermore, time is saved in that the second incubation period between ligand-first antibody and second antibody is not required. It has been suggested by Ratcliffe (1974), however, that the technique may produce less sensitive assays than post-precipitation methods, possibly by reduction of the first antibody's effective avidity when pre-complexed.

Protocol for immunisation

Immunogen: The immunogen used was rabbit gamma-globulin (RGG). The hosts (sheep) were immunised with a 50 mg intramuscular injection followed by a second injection of 30 mg 14 days later. The sera were harvested three weeks after the second injection.

Testing of second antibody: The prime requirement of the second antibody was to precipitate the primary antibody. Therefore total specificity is not essential, avidity and titre being far more vital

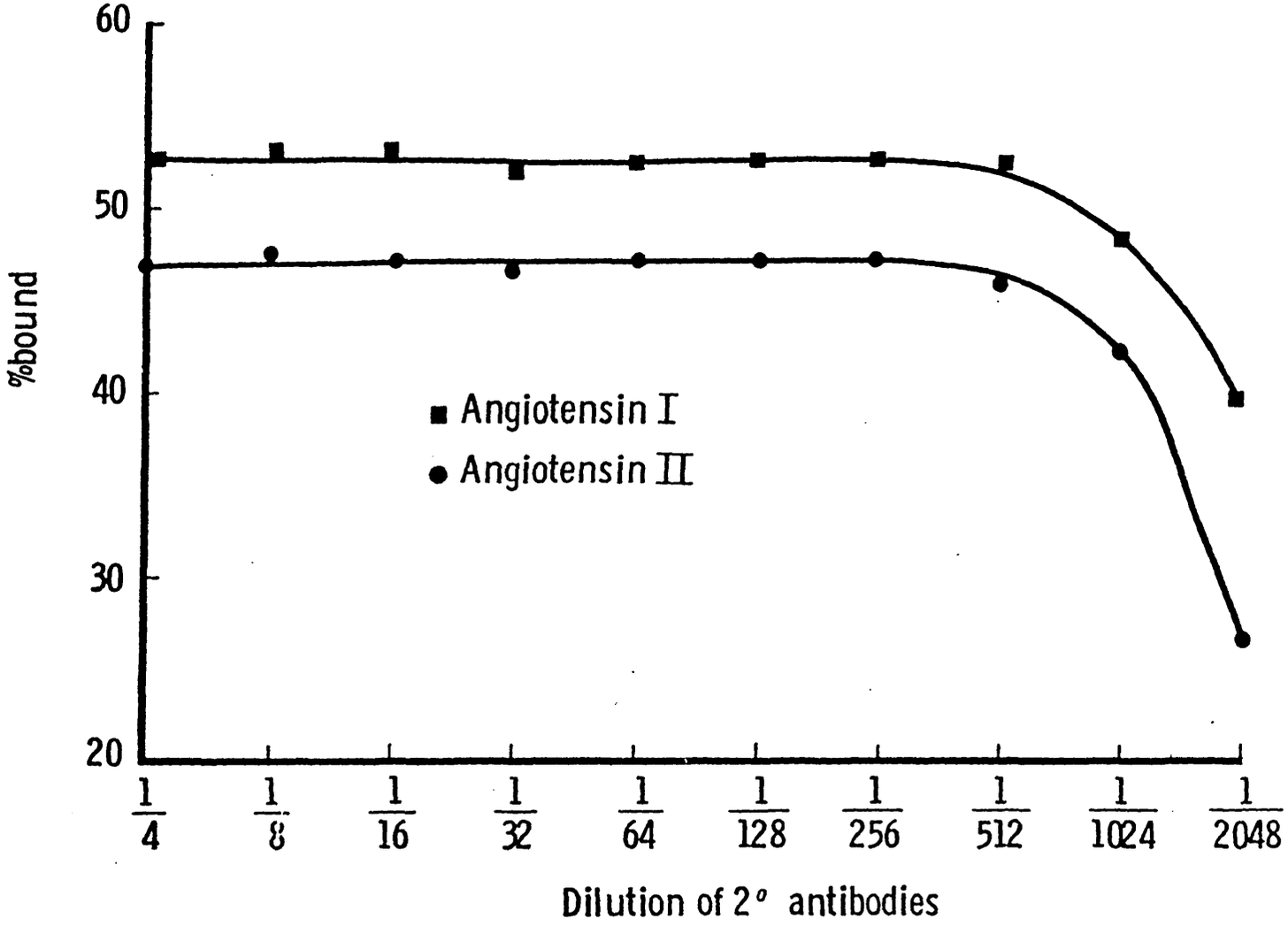
in this case. To tubes containing constant amounts of buffer (250 µl) labelled angiotensin (10 pg in 50 µl), and antiangiotensin (50 µl), was added, after a 24 hour incubation period, 50 µl of serial dilutions of the second antibody. The tubes were then reincubated at 4°C for 48 hours. The second antibody was diluted over a range of 1/4 to 1/2048. The dilutions were performed in 1/4 normal (i.e. non-immune) rabbit serum in an attempt to normalise the total IgG concentration which can have a significant effect on the final titre achieved if varied markedly (Sönksen, 1976).

The dilution chosen for routine work was the maximum dilution achievable which still yielded maximal separation of bound and free fragments as estimated by counting the precipitates post centrifugation at 1500 g for 15 minutes (Figure 6.4).

In order to try to eliminate the second incubation time (48 hours) from the assay procedure, a pre-incubated binding complex was made as follows. 200 ml each of the primary and secondary antisera each at 10 times the determined assay optimal concentration were incubated together whilst being gently mixed at 4°C for 72 hours. After this period they were stored in 2 ml aliquots at -20°C. To test and compare the pre-incubated complex with the post-precipitation technique employed in the second antibody assessment two standard curves were set up to the following protocol for each antigen.

Figure 6:4

Secondary antibody dilution curves.



	Post precipitation	Pre-precipitation
	µl	µl
Buffer	250	250
Label	50	50
Standard	50	50
1° Antibody	25	50 *binding complex
24 Hours at 4°C		24 Hours at 4°C
2° Antibody	25	
48 Hours at 4°C		
Centrifuge		Centrifuge
Decant		Decant
Count precipitate		Count precipitate

*Aliquot diluted 10-fold prior to use.

As may be seen from the results overleaf, there was no appreciable difference in sensitivity of the two curves for either A1 or A2. As a result the pre-precipitated complex was used in all double antibody assays (see Figure 6,5).

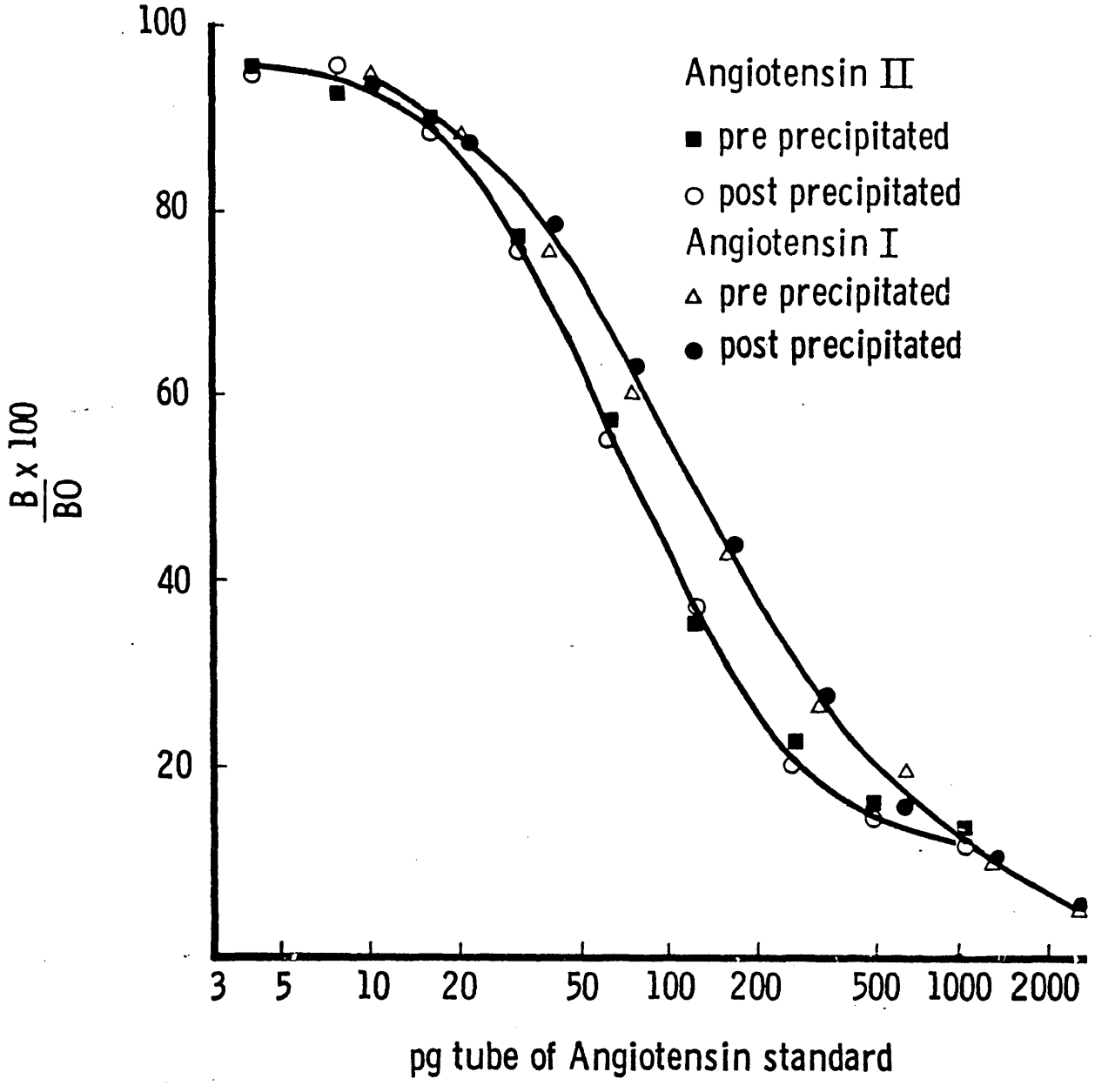


Figure 6:5

A comparison of preprecipitated and postprecipitated double antibody separations for angiotensin I and angiotensin II,

CHAPTER SEVEN

MEASUREMENT OF PLASMA RENIN ACTIVITY

Measurement of Plasma Renin Activity

Theoretical considerations

Having established the closeness of fit of the quadratic equation (Ekins and Newman, 1970 and 1971) to the assay systems under study it is possible to use this to derive a set of conditions that should, in theory, give optimal sensitivity and minimal inherent error.

The initial attempt to define an optimally sensitive system was proposed by Yalow and Berson (1968) who defined the system which yielded the steepest dose response curve as the most sensitive. These conditions were achieved by a dilute concentration (0.5/K) of antibody and a vanishingly small concentration of tracer. Hence from the Ekins' quadratic it may be seen that in the absence of antigen standard a bound to free ratio R of 0.5 would result, since $p = p^* = 0$.

then $R^2 + R(1-Kq) - Kq = 0$ Equation 7.1

$\therefore \quad R^2 + R - \frac{RK(0.5)}{K} - \frac{0.5K}{K} = 0$ Equation 7.2

$\therefore \quad R^2 + \frac{R}{2} - \frac{1}{2} = 0$ Equation 7.3

$\therefore \quad R = 0.5$ (i.e. 33% bound)

These conditions are not, however, ideal in practice. Firstly it can be seen that the use of very small amounts of radiolabelled tracer leads to a great deal of imprecision in counting. Lengthening of counting times cannot, of course, overcome this since the "signal to noise" ratio will remain constant. Secondly it is apparent that there is a flaw in logic which dictates that the steepest slope is synonymous with the most sensitive curve. This is simply illustrated as follows:

if one reduces the concentration of antibody this will effectively reduce the slope of a dose response curve where $\frac{\text{bound}}{\text{free}}$ is plotted against concentration of antigen. However, should the assayist plot $\frac{\text{free}}{\text{bound}}$ as the response metameter the slope will increase. Therefore the definition by Berson and Yalow is seen to be arbitrary, since it is based upon fallacious principles. Therefore in attempting to optimise an assay one should consider other factors than the slope of the curve. Such factors include the precision of measurement of the response metameter and the intrinsic experimental error.

It is however possible to derive optimal concentrations from the theoretical relationship between the response metameter and the concentration of antigen (p) and antibody (q).

It has been shown that the approximations of the Law of Mass Action (see previous chapter) are valid for the case in question. That is $R^2 + R(1 + Kp - Kq) - Kq = 0$ Equation 7.4

where $R = \frac{B}{F}$, or if $R = \frac{F}{B}$ then

$$R^2 + R(1 - \frac{p}{q} - \frac{1}{Kq}) - \frac{1}{Kq} = 0 \qquad \text{Equation 7.5}$$

The slope of the response curve at any point is derived by differentiation.

$$\frac{dR}{dp} = \frac{1}{qR + 1/KR} \qquad \text{Equation 7.6}$$

If we consider a total error at any point on the response curve, ε, for a case when both bound and free fractions are counted and the total count is optimally divided to minimise counting error then it can be shown that

$$\frac{\Delta(R)}{R} = \sqrt{(1 + \sqrt{R})^2 \times \frac{R + 1}{p^*SVTR} + \varepsilon^2} \qquad \text{Equation 7.7}$$

where S = specific activity of label p*

V = volume of incubation mixture.

Combining the last two equations, then, yields:

$$\Delta p = (qR_o + \frac{1}{R_oK}) \cdot \sqrt{\frac{R_o + 1}{R_o p^* SVT} \cdot (1 + \sqrt{R_o})^2 + \varepsilon^2} \qquad \text{Equation 7.8}$$

where Δp is the detection limit of the assay. Hence ideally one chooses values of p* and q which yield a minimum for Δp. To do this without resorting to computer optimisation techniques requires one to assume that ε is a minimum when $R_o = 1$. There is strong experimental evidence to support this supposition (Ekins and Newman, 1971). If we make this assumption we may then partially differentiate the previous equation with respect to p* and q to derive the following expressions

$$\varepsilon^2 STV(\hat{q} - \frac{1}{K})^2 + 2(\hat{q} - \frac{1}{K}) - \frac{4}{K} = 0 \qquad \text{Equation 7.9}$$

and $$\varepsilon^2 STV(\frac{\hat{p}^*}{2})^2 + \hat{p} - \frac{4}{K} = 0 \qquad \text{Equation 7.10}$$

whilst $$\Delta p_{min} + (\hat{q} + \frac{1}{K}) \sqrt{\frac{8}{\hat{p}^* STV} + \varepsilon^2} \qquad \text{Equation 7.11}$$

where $\hat{p}^*$ and $\hat{q}$ are the optimal concentrations. Solving the partial differentials for $\hat{p}^*$ and $\hat{q}$ yields $\hat{p}^* = 4/K$ and $\hat{q} = 3/K$. Using the above expression as a guideline it was therefore possible to dilute the label and antisera to the above concentrations using the information obtained from self-displacement analysis and Scatchard data. The optimal value predicted for $\hat{p}^*$ was 4,2 pg per tube and for $\hat{q}$ 3,2 pg per tube. In practice however values of 10 pg $tube^{-1}$ and 7,5 pg $tube^{-1}$ for $\hat{p}^*$ and $\hat{q}$ were chosen since the range of the curve was such that sensitivity was

not noticeably altered by further dilution whereas the counting times were lengthened considerably.

Assay protocol

The assay was routinely performed in 12 x 75 mm polystyrene tubes (RT 30; available from Slaughter & Co.). Up to 200 tubes were numbered sequentially prior to commencement of the assay.

To the first five tubes was added 300 µl of assay dilution buffer. To tubes 6-10 was added 250 µl of buffer and to all subsequent tubes was added 200 µl of buffer. These dispensations were performed by a hand-held Gilson Pipetman.

Standards

A vial of freeze dried angiotensin I standard was reconstituted in 2 ml of distilled water and diluted further with assay dilution buffer to yield a suitable high standard. Routinely 18 ml of buffer were added to yield a final concentration of 25 $ng.ml^{-1}$. Eight serial doubling dilutions were then made from a 2 ml aliquot of this solution by serial transfer to tubes containing, initially, 1 ml of buffer. The tubes were thoroughly vortex mixed in between addition of standard and subsequent sampling. As a result nine working standards were obtained with values from 25.0 $ng.ml^{-1}$ down to 0.098 $ng.ml^{-1}$. To tubes 11-13 inclusive 50 µl aliquots of the 0.098 $ng.ml^{-1}$ standard were added. 50 µl aliquots of the next standard were added to tubes 14-16 inclusive and this serial dispensation continued until, finally, 50 µl aliquots of the 25.0 $ng.ml^{-1}$ standard were added to tubes 26-28.

Sample aliquots of 50 µl were dispensed by the same means (Gilson adjustable "Pipetman") as the standards. For each patient sample the

"ungenerated" sample aliquots were dispensed and these immediately followed by their respective "generated" counterparts. Care must be exercised in this procedure since the angiotensinase inhibitors tend to cause the formation of precipitates which if allowed to enter the pipette tip cause inaccuracies in the volume taken and transferred. To avoid this the tubes were not mixed post-thawing and any excessively flocculent suspensions were centrifuged for 5 minutes at 1000 x g.

Quality controls

The so-called "renin controls" were treated in every way as a patient plasma sample, the generated and ungenerated halves being dispensed into adjacent pairs or triplets of tubes at various points throughout the sequence. The "angiotensin controls" which do not pass through a generation step were dispensed as single triplets of 50 µl in a manner analogous to sample pipetting in most other radioimmunoassays.

Radiolabelled tracer

After all the standards, samples and quality controls had been dispensed 50 µl of labelled angiotensin I was added to all tubes with an automatic Hamilton syringe (Pat. 3161323). 50 µl aliquots were also dispensed into three other tubes which were capped and set aside until the counting stage. These were to provide an estimate of "total counts". Although not directly required for result analysis or data interpretation the totals do provide a check on the total recovery of label as fraction or percentage bound, and a marked drop in recovery may indicate either an incorrect dilution of antibody or standard or radiolysis of the tracer solution. Routinely the label was diluted to yield a concentration of

10 pg/50 µl for the reasons outlined earlier. The dilution required of each label to achieve this was calculated from self-displacement analyses.

Primary antisera

An aliquot of anti-angiotensin I serum was thawed and diluted by the addition of 18 ml of assay buffer. After being thoroughly mixed, 50 µl aliquots (≡ to 7.5 picograms of binding sites - as estimated from Scatchard data) were added by an automatic Hamilton syringe to all tubes with the exception of the first five.

All the tubes were then thoroughly vortex mixed, covered with plastic film and incubated at 4°C for 24 ± 2 hours.

Separation of bound and free fractions

Post incubation, 750 µl of vigorously mixing Norit A charcoal suspension were added to all tubes in batches of 64 (which equalled the capacity of the centrifuge). The charcoal was added with a Gilson pipetman. After charcoal had been added to 64 tubes they were vortex mixed and centrifuged at 1500 g for 20 minutes at 8°C. Addition of charcoal to the next 64 tubes was so timed to coincide with the end of the centrifugation time of the first run. After removing the first set from the centrifuge and loading the second the supernatants of the first set were decanted into flat bottomed 3 ml tubes which were numbered identically to the assay tubes. These were then capped and placed in a Wallac 80000 gamma counter. After all the tubes had been centrifuged, the supernatant decanted and placed in the counter, the total count tubes were placed in the counter and the tubes set to count for 300 seconds each. The precipitates were also counted for the same length of time.

Analysis of the results

The analysis of results was facilitated by the use of a programmable calculator, the Texas SR 56. The specifically written programme converts the counts bound in each standard to a percentage of the zero antigen binding and then computes a log.logistic transformation which was found in practice to linearise the dose-response curves. From the log-logit plot the slope and intercept were obtained and fed back into the calculator into prescribed data stores. Subsequently sample counts were transferred and interpolated from the line directly without the need for any further reference by the operator to the dose-response curve. A full listing and explanation of the programme and result analysis is given in Appendix II.

Sample Preparation

Sample collection and storage

Five ml of blood were taken from the vein of the arm of patients and volunteers. The blood was then immediately transferred to tubes containing 12.5 mg of dipotassium ethylenediaminetetraacetic acid (K_2EDTA) and mixed thoroughly. The tubes were then rapidly transferred to a centrifuge in a cold room and centrifuged for 15 minutes at 2000 x g at 4°C. The plasma was then pipetted off and transferred to two further tubes containing 12.5 mg K_2EDTA which were capped, suitably labelled, and deep frozen at -20°C until required.

In vitro generation of angiotensin I

The plasma samples were thawed and 1 ml aliquots each dispensed into serially numbered polystyrene tubes. To each tube was then added 1 ml of citric acid-potassium citrate buffer (0.1 molar, pH 5.7). 40 µl

of 0.46 molar 8-hydroxyquinoline were then added followed by 100 µl of a saturated ethanolic solution of phenylmethylsulphonylfluoride (PMSF). After thoroughly mixing each sample half of each sample was transferred into another correspondingly labelled tube. All tubes were then capped and one set immediately frozen at -20°C and the other incubated at 37°C for 3 hours. At the end of this incubation period these samples were frozen at -20°C and stored in the deep freeze until required for radioimmunoassay. Thus since angiotensin I breakdown by converting enzyme is inhibited by hydroxyquinoline, PMSF and EDTA it is possible by assay of both halves of the sample to determine the amount of angiotensin I present before and after incubation at 37°C and by subtraction arrive at a rate of generation of angiotensin I under these standardised conditions. This rate of generation of angiotensin I is commonly referred to as plasma renin activity.

Preparation and storage of quality control pools

Blood was taken from the vein of the arm of twenty normal volunteers on their normal diet and was immediately transferred to tubes containing dipotassium EDTA, a final concentration of 2.5 mg EDTA per ml of plasma being achieved. The tubes were then centrifuged at 2000 x g at 4°C for 15 minutes (as previously described). The plasmas were then pipetted and pooled together in the cold room and 1.2 ml aliquots dispensed into tubes containing EDTA which were capped and stored at -20°C until required. This procedure yielded a pool of normal value quality controls.

A second pool was similarly obtained from volunteers who were on the fifth day of a low sodium intake diet (10 mmol for five days). This gave a pool of high P.R.A. controls.

A low P.R.A. quality control pool was obtained by pooling plasmas from volunteers previously screened and found to have a low P.R.A.

All renin quality control pools were stored at -20°C and an aliquot, once thawed, was used immediately and any remainder discarded.

Further quality control pools were obtained as described above, but the pooled plasmas were mixed thoroughly with 40 µl per ml of plasma of 8-hydroxyquinoline and 100 µl per ml of plasma of PMSF and the whole then incubated at 37°C for 3 hours to generate angiotensin I. The pools were then divided into 0.2 ml aliquots, frozen and stored at -20°C. The "angiotensin control" pools were used as a check on the radio-immunoassay as distinct from the generation and assay procedure *in toto*.

CHAPTER EIGHT

MEASUREMENT OF PLASMA ANGIOTENSIN II CONCENTRATION

Measurement of Plasma Angiotensin II Concentration

The assay was performed in 12 x 75 mm round bottomed polystyrene tubes (RT30, Slaughter & Co.). Up to 200 tubes were numbered sequentially prior to commencement of the assay. To the first five tubes 300 µl of assay dilution buffer were added. To tubes 6-10 250 µl of assay buffer were added and to all other tubes 150 µl of buffer were added. A Gilson hand-held "Pipetman" was used to perform these dispensations.

Standard

A vial of freeze-dried angiotensin II standard was reconstituted in 2 ml of distilled water and diluted further with dilution buffer to yield a suitable high standard, usually 10 $ng.ml^{-1}$. Eight serial doubling dilutions were then made from a 2 ml aliquot of this 10 $ng.ml^{-1}$ solution by serial transfer of 1 ml to tubes initially containing 1 ml of buffer. The tubes were thoroughly vortex mixed in between addition of standard and subsequent sampling. As a result of these dilutions nine working standards were obtained with values from 10 $ng.ml^{-1}$ down to 39 $pg.ml^{-1}$. To tubes 11-13 inclusive 100 µl of the most dilute standard were added. 100 µl aliquots of the next standard were carefully pipetted into tubes 14-16 inclusive and this procedure was continued until finally into tubes 26-28 100 µl of the 10 $ng.ml^{-1}$ standard were dispensed.

Samples

100 µl aliquots of sample were dispensed in triplicate in a manner analogous to the dispensing of standard.

Quality controls

Quality control samples were dispensed in triplicate at various points throughout the sequence of samples and were subsequently treated in the same way as patients' samples.

Radiolabelled tracer

After all samples have been dispensed 50 µl of labelled angiotensin II was added to all tubes by means of an automatic Hamilton syringe (Pat. 3161323). 50 µl aliquots were also dispensed into three additional tubes and these were capped and set aside until the counting stage. These were to provide an estimate of total counts added. As explained in Chapter 7 these "totals" are not directly essential for result analysis or data interpolation, but they do provide a check on the total recovery of label as fraction or percentage bound,and a marked drop in recovery may indicate an incorrect dilution or radiolysis of the tracer.

The label was usually diluted to yield a concentration of 11.4 pg per 50 µl which was calculated from the equilibrium constant ($\hat{p}^* = 4/K$) as explained in the previous chapter to be the concentration for optimal assay sensitivity with this particular antibody.

Primary antiserum

An aliquot of anti-angiotensin II serum was thawed, diluted with 18 ml of assay buffer and thoroughly mixed. 50 µl aliquots ($\equiv$8.5 pg.tube^{-1} of binding sites) were then dispensed into all tubes with the exception of those designated to be for estimation of non-specific binding.

All tubes were then vortex mixed thoroughly, covered with plastic film and incubated in a refrigerator at 4°C for 24 ± 2 hours.

Separation of bound and free fractions

After the incubation the tubes were removed from the refrigerator in batches of 64 and to each was added 750 µl of a vigorously stirring Norit A charcoal suspension. A Gilson "Pipetman" was used for the dispensings. After the charcoal had been added the tubes were vortex mixed and then centrifuged at 1500 g for 20 minutes at 8°C in an MSE Mistral 4L centrifuge. Subsequent batches of 64 tubes were treated at such time as virtually immediate transfer to the centrifuge could be effected. After centrifugation the supernatants from the tubes were decanted into correspondingly labelled flat bottomed 3 ml capacity polystyrene tubes which were then capped and loaded into a Wallac 80000 gamma counter for counting. The precipitates were also loaded and counted. The customary count time was 300 seconds.

Analysis of results

Result analysis was facilitated by the use of a specifically designed programme fed into a Texas SR 56 hand-held calculator. The programme converts the counts bound in each standard to a percentage of the zero antigen (Bo) counts and then in an identical manner to the PRA programme computes a log.logistic transformation to linearise the dose-response curve. The slope and intercept of the log.logit plot were fed back into the programme into prescribed data stores. Subsequently sample counts and the corresponding plasma volumes extracted were fed into the programme and angiotensin II concentrations in $pg.ml^{-1}$ of plasma were computed and displayed. A full explanation is given in Appendix III.

Sample preparation

Blood collection, separation and storage

20 ml of blood were taken from the arm vein into a plastic syringe containing 1 ml of EDTA/orthophenanthroline inhibitor which prevents angiotensin II hydrolysis or synthesis. The blood was then rapidly mixed and transferred to a plastic tube and centrifuged at 4°C for 20 minutes at 2000 x g. The plasma was then carefully pipetted off and stored at -20°C to await extraction.

Extraction of angiotensin II from plasma

The volumes of each plasma sample were measured by drawing them into glass 10 ml pipettes prior to transfer to glass test tubes containing 400 mg Dowex-H^+ resin (which was measured out by a calibrated scoop made in the laboratory workshop). The tube was then capped and the plasma/Dowex mixed on a rotary mixer for 1 hour. After the mixing period the plasma supernates were aspirated and the Dowex residues containing the extracted angiotensins were washed with two volumes of 5 ml of freshly boiled distilled water followed by two volumes of 5 ml of methyl alcohol. After aspiration of the final wash volume 2 ml of a 90% ammonia:10% methanol solution was added to elute the angiotensins from the Dowex. The tubes were tightly capped and taped (to resist pressure due to NH_3 evaporation) and mixed on a rotary mixer for 30 minutes. after mixing the tubes were centrifuged (after releasing the pressure) and then the supernates carefully transferred to Luckhams LP3 polystyrene tubes, each containing 50 µl of 1% (w/v) bovine serum albumin solution to prevent adsorption. The extracts were then reduced to dryness under a directed stream of air whilst standing in a water bath at 37°C. The elution process from the Dowex was then repeated, the second eluate being added

to, and reduced with, the first. The dried extracts were capped and then stored at -20°C until required for assay, when they were brought to room temperature and redissolved in 0.4 ml of Tris buffer, pH 7.5.

Quality controls

Radioimmunoassay controls

Three quality control pools of "Hypertensin" [Angiotensin II (Val^5)-amide] in assay buffer at concentrations 100, 250 and 500 $pg.ml^{-1}$ were made up in buffer and stored in 0.5 ml aliquots in capped polystyrene tubes at -20°C until required for assay. Each aliquot after being thawed and assayed was discarded.

Extraction procedure controls

Owing to the large volumes required it was not feasible to prepare and store batches of plasma in a manner analogous to that for the renin quality controls. A "partial recovery control" was manufactured *in situ* by the subdivision of large plasma samples (≈10 ml) into two equal samples. To one of thesewas added 0.05 ml of assay buffer containing 250 pg of $^1Asp^5Ileu$ angiotensin II. (These 0.05 ml aliquots were obtained from a frozen stock of 0.1 ml aliquots of a 5 $ng.ml^{-1}$ angiotensin II solution.) After the extraction procedure the two samples were assayed and the difference between the two values related to the difference predicted. The percentage recovery was taken as $\frac{\text{pg.tube value in a - pg.tube value in b}}{\text{pg.tube}^{-1}\text{ value of added angiotensin}} \times 100$. For example; a 10 ml sample is subdivided to yield two 5 ml samples. To one (designated 'a') was added 250 pg of angiotensin. After extraction, dissolution in 0.4 ml Tris and assay therefore, if we assume the angiotensin concentrations indigenous to 'a' and 'b' to be x pg, we find that the concentration

of angiotensin in a = $\frac{250 + x}{4}$ pg/tube and the concentration of angiotensin in b = x/4 pg/tube. However, if only 90% of angiotensins were extracted the concentration in 'a' will be $(x + \frac{250}{4}) \times \frac{9}{10}$ and the concentration in 'b' will be $\frac{x}{4} \times \frac{9}{10}$ and the difference will be $\frac{9x + 2250}{40} - \frac{9x}{40}$

$= \frac{2250}{40} = 56.25$ pg/tube.

Thus an actual result of 56.25 would reflect 90% recovery of angiotensin from the plasma ($\frac{56.25}{62.5} \times 100 = 90$). This control was not totally satisfactory, however, as it took no account of the plasma separation stage from whole blood. Ideally one would add the angiotensin to the blood prior to centrifugation, but this was not feasible on a routine basis. A recovery experiment based on this was performed (see Chapter 12) and it can be seen from that experiment that no great losses of angiotensin were incurred during centrifugation and therefore the protocol outlined above, adding angiotensin directly to the plasma, was continued.

CHAPTER NINE

MEASUREMENT OF PLASMA ANGIOTENSIN CONVERTING ENZYME

Measurement of Plasma Angiotensin Converting Enzyme

Principles of procedure

The radioassay for angiotensin converting enzyme in serum or plasma (anticoagulated with heparin not EDTA) is based upon the following sequence of events. The enzyme acts on ^{3}H-Hip-gly-gly (Ventrex Corp., Portland, Maine) to produce ^{3}H-hippurate plus the dipeptide glycylglycine. The pH optimum of the reaction is 8.0. After incubation under controlled conditions the reaction is stopped by acidification and ^{3}H-hippurate separated from substrate by partitioning the solution with ethyl acetate. The partition coefficient of ^{3}H-hippurate and substrate are such that 91% of ^{3}H-hippurate and 7% of substrate enter the organic phase under these conditions. Thus one can quantify angiotensin converting enzyme activity in terms of radioactivity extractable by ethyl acetate, this being a direct function of the rate of substrate hydrolysis.

Methodology

Collection and storage of specimens

5 ml of blood from the vein of the arm were transferred to a plastic tube containing heparin. The blood was centrifuged and the plasma pipetted and transferred to another tube. The plasma was stored at -20°C unless it was to be assayed the same day, in which case it was refrigerated.

Assay protocol

12 x 75 mm glass tubes were used throughout the procedure owing to the destructive effects of ethyl acetate on plastic.

Dilution

To a 50 µl aliquot of each sample or control was added 200 µl of

0.05 M N-2-hydroxyethyl piperazine N'-2-ethane sulphonic acid (Hepes) buffer, pH 8.0, containing 0.01% (w/v) of sodium azide. The samples and controls were then vortex mixed thoroughly.

Assay

Three 12 x 75 mm glass tubes were labelled for each sample, blank and control to be assayed. To blank tubes 50 µl of Hepes buffer was added. To control tubes 50 µl of diluted reference controls were added and to patient sample tubes 50 µl of the relevant diluted patient sample were added. The assay reaction was then begun by the addition of 50 µl of ^{3}H-substrate (16 mMolar) to all tubes which were then covered and incubated at 37°C for 60 minutes. 50 µl doses of ^{3}H-substrate were also dispensed into three scintillation vials to provide a measure of the total radioactivity added.

The incubation was terminated by the rapid addition of 1.0 ml of 0.1 molar hydrochloric acid to all tubes (except the total count vials). Subsequently 1.0 ml of 'Analar' ethyl acetate was added to all tubes. The tubes were then vigorously vortexed and centrifuged at 1000 g for 10 minutes, after which 300 µl of the upper (organic) layers were carefully transferred to scintillation vials. 10 ml of detergent containing scintillant (the detergent is to enable total miscibility with organic and aqueous samples) were then added to all the vials including the estimations of total radioactivity. The vials were then capped and counted in a liquid scintillation counter for 4 minutes each.

Calculation of results

The results were calculated by application of the following formula:-

$$\text{units} = \frac{800}{(\text{total counts}) \times (60 \text{ min}) \times (0.01 \text{ ml})} \times (\text{CPM sample} - \text{CMP blank}) \times 3.33$$

One unit is defined as the generation of one nanomole of ^{3}H-hippuric acid per minute per ml of serum or plasma sample.

800 nm/tube	is the final reaction concentration of substrate.
(Total counts)	is the mean counts per minute added to all tubes, as estimated by the total count estimates.
60 minutes	was the total reaction time.
0.01 ml	was the unknown specimen volume per test (50 µl of 50 µl diluted 1 in 5).
CPM sample	Observed mean CPM for each sample.
CPM blank	Observed mean CPM for each blank.
3.33	is the factor required to convert 300 µl organic (ethyl acetate) aliquot to total organic volume (1 ml).

Quality control samples

Two types of control were used. Firstly, normal human heparinised plasmas, from two volunteers whose ACE activities were at different points in the normal range, were stored as 200 µl aliquots at -20°C until required for assay.

Furthermore a commercial reference preparation was purchased from Ventrex Corporation (Portland, Maine). This control is presented in Hepes buffer (pH 8.0) and contains normal guinea pig angiotensin converting enzyme diluted in normal human serum. Batches were stored up to three months at 4°C.

CHAPTER TEN

AUTOMATED ASSAY SYSTEMS

Automated Assay Systems

Both the plasma renin activity assay and the angiotensin II assay described are time consuming and require many pipetting and tube manipulation steps which in turn limit the size of assay which can be attempted. This chapter describes an automated radioimmunoassay machine under microprocessor control and the adaptation and application of the above assays in an attempt to semi-automate the procedures. A further advantage of this system is that separation of bound and free fractions is achieved by automatic *in vacuo* filtration of a double antibody-antigen complex from free antigen which adds immunospecificity to the separation step.

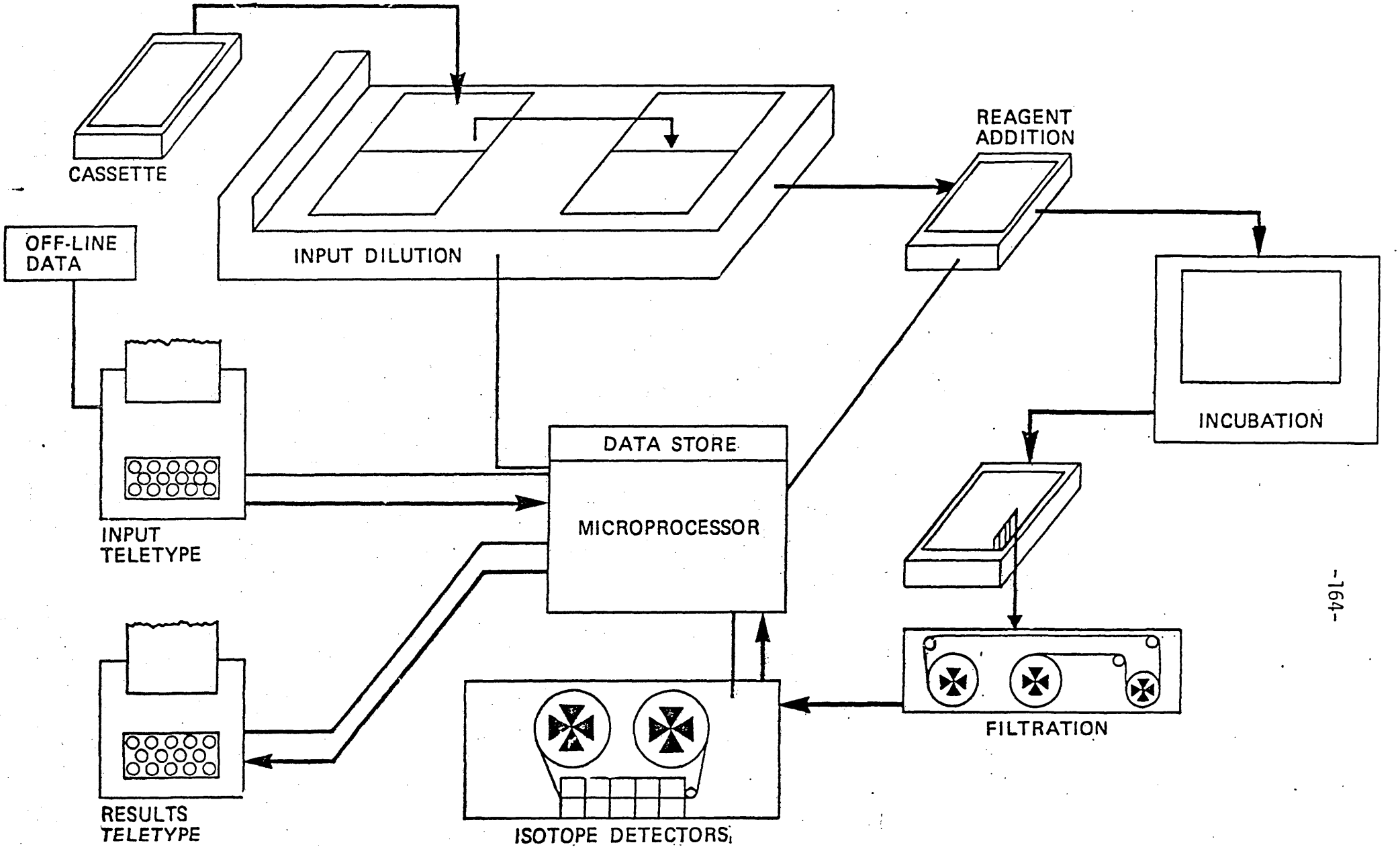
General methodology

Sample input and dilution

The assay was carried out on an automated microprocessor controlled machine, the Kemtek 3000 (see Figure 10.1). Into the "feed" cassette were placed two tubes containing 0.04 molar phosphate buffered saline, pH 7.4, containing 8 millimolar Na_2EDTA and 1 $mg.litre^{-1}$ of bovine serum albumin (Fraction V). These tubes serve as the sources for subsequent dispensations into reaction tubes for estimation of non-specific binding and zero antigen binding. Immediately behind the second tube of buffer was placed a tube containing approximately 2.0 ml of the highest concentration of standard required and this was in turn followed by four empty tubes which serve as diluent tubes. The reaction cassette was filled with sufficient tubes for the entire assay (up to 420 tubes in 28 racks of 15) and the assay may then be started. When 'start' is pressed the first rack of the feed cassette aligns itself adjacent to the probe which transfers 100 µl of buffer into the first ten reaction tubes (NSB estimations). To the next ten tubes 100 µl of buffer from feed tube 2 are dispensed to

Figure 10:1

Diagrammatic representation of the Kemtek automated assay system.



yield ten zero antigens. The feed rack then moves on to the standard tube and the probe begins a process of transfer and dilution of standards, automatically washing and wiping the probe between operations. The operator, prior to commencement of the assay, types in the number of standards (up to nine) and the number of replicates (usually three) required. Hence to assay tubes 21-23 are added 100 µl of undiluted standard to the next three 50 µl of standard and 50 µl of buffer and so on until in tubes 33-36 (standard 5) 6.25 µl of standard were added plus 93.75 µl of buffer. The final four standards were diluted prior to transfer by means of the four empty tubes placed adjacent to the assay standard feed tube. Whilst this dilution and transfer of standards is proceeding the samples are loaded into the feed cassette for subsequent sampling and such instructions as to the dilution at which they are to be assayed are typed in on the "input teletype". After all the samples are transferred to corresponding triplets of assay tubes the reaction cassette is transferred to the "reagent addition" module.

Reagent addition

This module comprised three pumps which, through aligned probes, delivered prescribed volumes of reagents to selected tubes designated (by push buttons) as NSB, ZA, standard, diluted samples, and undiluted samples. The microprocessor had the information that tubes 1-10 were NSB and 11-20 were zero antigens and from the operator instructions it was known how many standard tubes there were (in this case 9 x 3 = 27) and which samples were diluted and which were not. Therefore should selective addition of background sera be necessary to buffer based tubes i.e. NSB, ZA, standard and diluted samples, this could be done automatically by one of the pumps. The other two pumps were used to dispense label (to all tubes) and antibody (to all tubes except NSB). Mixing of

the reaction volume was achieved *in situ* by virtue of the injection velocity which created a transient vortex. After reagent addition the cassette was covered with plastic film and incubated at 4°C for 48 hours or for 24 hours at 37°C.

Separation of bound from free ligand

Separation of bound from free ligand was achieved off-line to the microprocessor by an automatic filtration system. The contents of the tubes in the reaction cassette were removed in sequential batches of five, by five fishtail hypodermic needles attached to pumps, and deposited upon discrete glass fibre discs immobilised in a 35 mm plastic tape fed automatically from a spool. The discs were pre-soaked automatically in an assay buffer containing 0.5% Tween (v/v). The detergent properties of the Tween prevented non-specific adsorption of label to the filter discs. As the reaction volumes,and a subsequent wash volume of buffer, were deposited upon the discs they were held *in vacuo* to facilitate rapid complete filtration.

As the spool moved on to the next batch of five the discs that had just been used passed over a system of spools in a drying chamber and, upon emergence, adhesive tape from a counter-rotating spool was automatically applied to the surface of the plastic tape bearing the discs, thereby preventing any cross-contamination from disc to disc, if the spool was to be rewound prior to counting.

Estimation of radioactivity

To count the gamma emission the filter tape was passed through a counting chamber containing five paired heads located above and below the tape at the same distance apart as the filter discs. The positioning of

the discs precisely in between each pair of counting heads is achieved by a sensor which locates discretely positioned holes at the edge of the plastic tape.

Each disc was counted under each pair of heads and the results were summed to prevent any discrepancy that could be caused by differing counter efficiencies of the various heads. The counting was usually commenced (i.e. if the module was not already in use by another operator) immediately post-filtration; that is, it was not necessary to wait for an entire assay to be filtered before the discs were begun to be counted. If the counting proceeded much quicker than filtration, the exact speeds being dependent upon the amount of workload the other modules had at the time, a switch was tripped by the tautening of the filter tape between the drying chamber and counters and counting and tape feed halted. Once the tape slackened the switch was released and counting restarted without operator intervention.

Analysis of results

A choice of two routine analyses were available. Either a log. logistic transform or a four parameter cubic fit ($ax^3 + bx^2 + cx + d = 0$) were employed at the discretion of the operator. All tube counts were printed out on the results teletype even if they were to be subsequently rejected for the purposes of analysis and/or interpolation.

The microprocessor programme, in addition to interpolating all samples from the curve fit programme used, also gave curve parameters and group standard deviation. The error about each point on the standard curve was also charted, as was the spread and mean of each sample estimation. A semilog plot of the dose-response curve with the ideal curve (that is predicted from the equation superimposed upon the same axes) was also

displayed. This was of great value in overviewing the system at a glance on an interassay basis.

The automated assay of plasma renin activity

The samples were generated manually as described previously in Chapter 7. After thawing the supernatants were transferred to tubes in the feed cassette, each generated sample being immediately preceded by its "blank" counterpart. Separation of the supernatants from the precipitate was, unfortunately, essential to ensure reproducible pipetting without blocking the syringes. The standard was, routinely, 25 $ng.ml^{-1}$ stored in the freeze dried state at -20°C. Reconstitution to 25 $ng.ml^{-1}$ was achieved by the addition of 2 ml of distilled water.

The radiolabelled tracer was identical in form and concentration with that used in the manual assay.

The antibody used was the pre-incubated double antibody complex, the preparation of which was described in Chapter 6. The 2 ml frozen aliquot was thawed and diluted with assay buffer to 20 ml which yielded the working concentration required.

Routinely all samples were programmed in at a dilution to two. In effect this means that the final plasma concentration in the reaction tube is less than 12% (v/v). This is derived as follows:-

$$\text{Generation step dilution } \frac{1000}{2000 + 100 + 50} = 46.5\%$$

50 µl of this in 200 µl reaction volume = 46.5/4 = 11.6%. That is only 23.2 µl of plasma.

The advantage of this is that no background sera was needed as no "serum effect" was observed (see Chapter 11).

The automated assay for plasma angiotensin II

The assay was performed in an analogous manner to that for P.R.A. The reconstituted extracts were assayed at dilution 2 since to do undiluted samples twice as great a dilution when reconstituting would be required to yield sufficient sample. Since this assay has no serum or plasma component once again no 'background' serum need be added. The label used was the same as for the manual assay and the antibody was the pre-incubated double antibody described in Chapter 6. The lyophilised standards were reconstituted to 2 ml with distilled water to yield a working high standard of 10 $ng.ml^{-1}$. The cut-off of the assay was, by virtue of the effective halving of sample size, twice that of the manual assay.

SECTION III

RESULTS AND DISCUSSION

CHAPTER ELEVEN

ASSESSMENT OF THE PLASMA RENIN ACTIVITY RADIOIMMUNOASSAYS

Assessment of the Plasma Renin Activity Assays

Plasma renin activity is measured by the radioimmunoassay of angiotensin I generated *in vitro* in buffered plasma samples under controlled conditions. Therefore in order to assess the system as a whole the assay procedure was examined in two distinct halves; the generation procedure and the radioimmunoassays.

The generation procedure involved buffering the plasma at a suitable pH for a suitable time at 37°C with an appropriate inhibitor to prevent angiotensin I degradation. Thus it was necessary to consider potency of inhibitors at various pH values, the pH of generation to be used and whether or not deviation from physiological pH could yield artefacts. Furthermore the linearity of generation of angiotensin I with respect to time was considered and whether or not the physico-chemical heterogeneity of the samples caused a partitioning of plasma renin activity, which could lead to error in sampling. The radio-immunoassays were assessed in terms of precision, sensitivity, specificity and reproducibility. Finally the manual and automated assays were compared directly using plasma samples in duplicate assay.

Generation Procedures

Choice of inhibitors

The effectiveness of the inhibitors commonly used to prevent angiotensin degradation *in vitro* has been shown to be pH dependent. Oparil *et al.* (1974) showed a difference in the pH profile of renin using two different sets of inhibitors, EDTA-hydroxyquinoline-dimercaprol and EDTA-neomycin-diisopropylfluorophosphatidate. They observed a marked decrease in apparent PRA with DFP-neomycin EDTA at pH values above 6.0 as compared with EDTA-hydroxyquinoline-dimercaprol. This difference is likely to be due to the curves reflecting a titration of relative inhibitor effectiveness as well as PRA; that is, the apparent profile was really the difference between a true pH profile and the degree of inefficiency of the inhibitor. Phenylmethylsulfonylfluoride (PMSF) in ethanolic solution which has been used in renin assays (Goodfriend, 1973) is reported to have a similar mode of action to DFP and has the advantage of being far less volatile than DFP, being therefore far safer to handle. Kodish and Katz (1974), however, reported that this also yielded incomplete inhibition at physiological pH unless used in conjunction with other inhibitors. It was decided, therefore, to investigate various combinations of inhibitors in plasma samples generated at pH 5.7 and 7.4. Owing to the extreme toxicity of diisopropylfluorophosphatidate (DFP) in comparison to PMSF the former was not considered in these studies.

Plasma pool samples were generated for 3 hours buffered either at pH 7.4 or pH 5.7 in the presence of one of the following mixtures of inhibitors in recommended concentrations (Haber *et al.*, 1969; Kodish and Katz, 1974).

1. EDTA, hydroxyquinoline (OHQ) dimercaprol (BAL) and PMSF.
2. EDTA-OHQ
3. EDTA-PMSF
4. EDTA-BAL
5. EDTA-OHQ-PMSF
6. EDTA-BAL-OHQ
7. EDTA-PMSF-BAL

At the end of the generation period the samples were frozen until required for assay. All the generation processes for a particular pH were done at the same time to obviate artefacts which could arise from slight day-to-day temperature variations. Ten samples were tested for each inhibitor mixture, at each pH.

The results are summarised in Table 11.1. The mean plasma renin activity ± ISD for each group is expressed as a percentage of the group mean PRA in the presence of all inhibitors for that particular pH. Thus the relative efficiencies may be seen. It should be noted that these percentages are relative and although the findings agree with observations of other workers in the field they do not, of themselves, prove total inhibition or otherwise of the "100% effective" mixtures.

From the results it is apparent that at pH 7.4 >90% of the maximum inhibition of angiotensin I degradation is achieved by any combination of inhibitors. At pH 5.7, however, EDTA-hydroxyquinoline was not so effective as the others. The EDTA-OHQ-PMSF mixture gave the most complete inhibition being indistinguishable from the complete mixture (+BAL) and this was subsequently used in routine assay work.

Table 11.1

Relative efficiencies of inhibitor mixtures in prevention of angiotensin I degradation *in vitro* at pH 7.4 and pH 5.7.

Inhibitor combination	% Efficiency ± ISD pH	
	7.4	5.7
EDTA-OHQ-BAL-PMSF	100±4.6	100±5.1
EDTA-OHQ	96±3.9	83±8.1
EDTA-PMSF	91±4.8	101±3.7
EDTA-BAL	98±6.2	94±2.1
EDTA-OHQ-PMSF	101±3.9	103±4.9
EDTA-BAL-OHQ	100±4.2	93±5.0
EDTA-PMSF-BAL	95±6.1	98±3.3

Check of absolute efficiency of inhibitors

This was monitored by assessing the survival of iodine labelled angiontensin I in plasma.

To plasma samples containing EDTA-OHQ-PMSF at pH 5.7, which appeared to be the optimal inhibition system and most active pH, was added a small concentration of labelled angiotensin I which had been recently purified as described in Chapter 5. An aliquot of this sample was then transferred and spotted on to a sheet of Whatman chromatographic paper and then subjected to electrophoresis in the manner described in Chapter 5. After 3 hours generation at 37°C a further aliquot of the sample was subjected to electrophoresis. As a control, a plasma sample containing added ^{125}Iodinated angiotensin inhibited by EDTA-OHQ alone at pH 5.7 was also tested before and after generation.

After electrophoresis the strips were dried, cut into 1 cm strips and counted on a Wallac 80000 gamma counter to obtain an electrophoretic profile of I^{125} distribution.

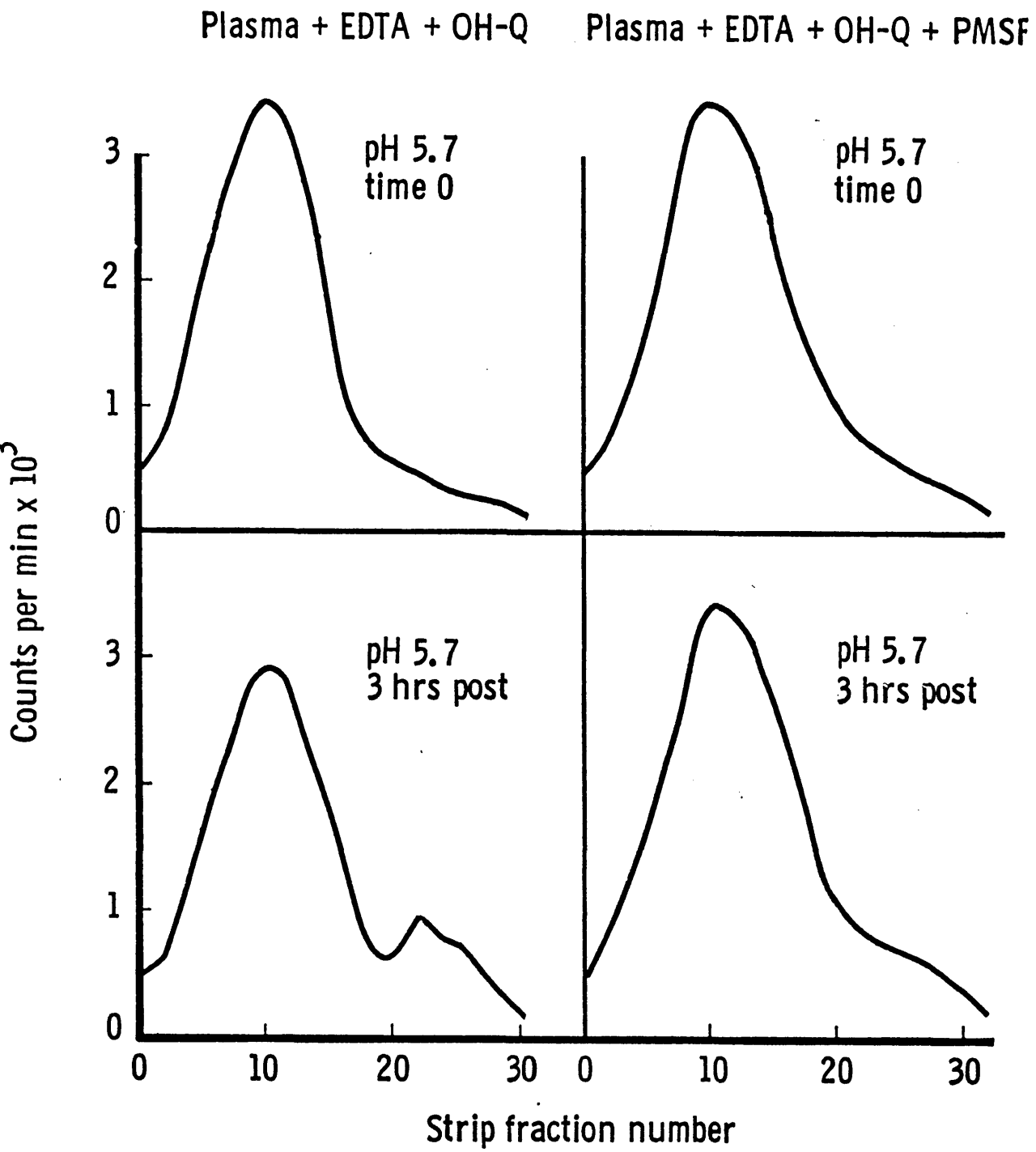
In Figure 11.1 the profiles show the difference between the two systems after generation. It can be seen that there is a definite secondary peak in the EDTA-OHQ system which is not apparent in the EDTA-OHQ-PMSF system after generation or in either prior to generation.

This is indicative of the protection afforded by PMSF in addition to EDTA and OHQ under the conditions employed. These results, together with those from the previous section, show therefore that effectively 100% preservation is afforded by this system, whereas only 80-90% is afforded by EDTA and OHQ alone.

These results verify the findings of Oparil *et al.* (1974) with regard to the unsuitability of OHQ-EDTA at pH 5.5 as they observed a similar phenomenon even with added BAL.

Figure 11:1

Distribution of radioiodine following electrophoresis of plasma containing ^{125}I-angiotensin I under different conditions.



Recovery of added angiotensin I

In order to check that the immunity to degradation was in fact conferred by the inhibitor system and not by virtue of the chloramine T facilitated radioiodination of angiotensin I a small recovery study was performed.

To aliquots of a normal control plasma, measured amounts of angiotensin I standards were added immediately prior to generation for 3 hours buffered at pH 5.7 in the presence of EDTA-OHQ-PMSF. As a control, as in the previous experiment, samples were set up in parallel where PMSF was omitted.

After generation the samples were frozen until required for radioimmunoassay when the concentration of angiotensin I per tube was calculated.

The results are shown in Figure 11.2 and the regression analysis summarised in Table 11.2. It can be seen that with PMSF a constant slope of one is obtained implying a uniform protection of 100% of angiotensin I. In the absence of PMSF a constant slope of 0.86 implies the degree of angiotensinase activity to be an approximately constant 14% irrespective of the starting concentration of angiotensin. This would indicate that to increase OHQ concentration would not make up for the lack of PMSF as the relative degree of protection would not be increased.

Choice of pH

The *in vitro* rate of angiotensin I generation is pH dependent (Lee, 1965). It has been observed that the pH of EDTA collected plasma will vary from physiological levels (7.4) to greater than pH 8.5, according to the length of time the plasma has been stored (Bagni *et al.*, 1972; Osmond *et al.*, 1974). Even when stored frozen at -20°C pH variance (up to 9.0) has been observed (Sealey and Laragh, 1973).

Figure 11:2

Recovery of added angiotensin I from plamsa generated in the presence of PMSF as estimated by radioimmunoassay.

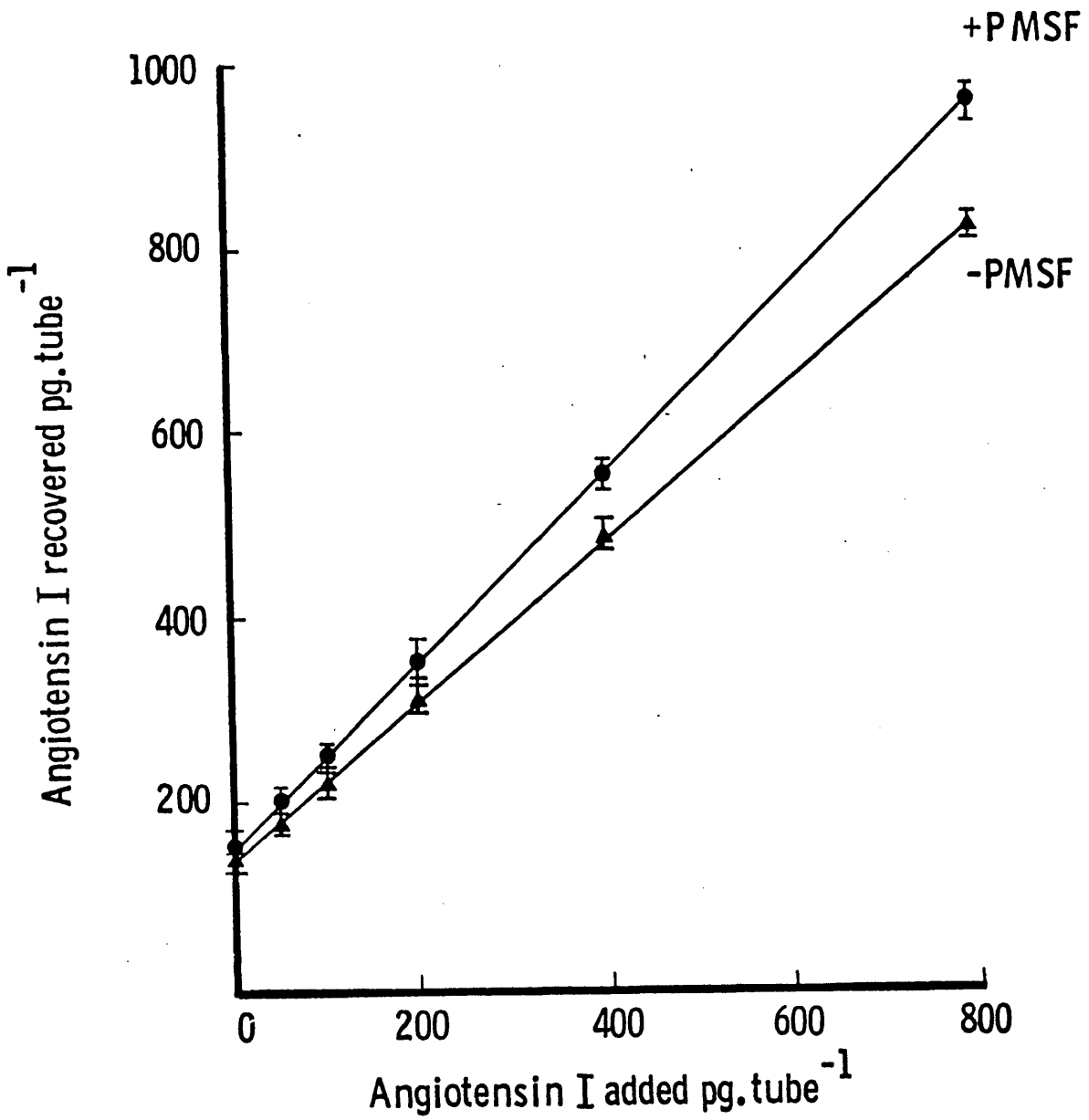


Table 11.2

Recovery of angiotensin I from plasma with and without PMSF. Linear regression data.

Angiotensin I concentration (± ISD) $pg.tube^{-1}$				
Concentration added	Concentration recovered -PMSF		Concentration recovered +PMSF	
0	134	(± 8)	152	(± 8)
50	180	(± 7)	206	(± 8)
100	228	(± 8)	251	(± 10)
200	314	(± 10)	361	(± 14)
400	490	(± 10)	555	(± 9)
800	826	(± 12)	960	(± 11)
n	6		6	
r	0.999		0.999	
m	0.86		1.01	
b	139.0		153.8	

Angiotensin I recovered = angiotensin I added x m + b

Osmond has observed the pH of unbuffered EDTA collected plasma to rise above 8 within the first hour of a 3 hour generation, even when the plasma was initially adjusted to pH 6.0 with acid. Catt (Catt and Douglas, 1974) has reported a rise of 1 pH unit in unbuffered plasma incubated at 37°C.

The need to buffer the plasma in order to obtain a controlled assay system is, therefore, apparent and all workers are now agreed upon this. There is, however, considerable controversy as to the most suitable pH to employ.

Two alternative systems have generally found some favour. In the first of these the plasma is buffered at pH 7.4; in the second it is buffered at pH 5.7 which is the pH optimum of the renin reaction.

The major advantages of generation at pH 5.7 are two fold. Firstly as this is the optimal pH for generation of angiotensin I a greater amount of angiotensin I will be produced per unit time, thus increasing the sensitivity of the assay for low PRA samples. Furthermore, since the chosen pH is on the plateau of the pH profile of renin slight variations in pH *in vitro* do not substantially alter angiotensin I generation rate.

Oparil *et al.* (1974), however, reported significant plasma angiotensin I concentrations when plasmas from seven anephric patients were incubated at pH 5.7 but not after incubation at pH 7.4. From this they concluded that "non-renin related generation of angiotensin I could make a major contribution to total renin activity". An alternative explanation of their data, however, could be that extra renal renin, which has been widely reported (Peach, 1977), may be responsible for some renin activity in these cases and that only at optimal pH is the assay system sensitive enough to measure it. Further scrutiny of the results shows

that the levels of renin activity are in fact very low. The mean plasma renin activity in $ng.ml^{-1}.hr^{-1}$ for the seven patients studied was 0.089 which would hardly appear to be a "major contribution".

Furthermore, the protocol as used by Oparil *et al.* (1974) is open to some criticism. Although the "physiological pH" samples were buffered at pH 7.4, the acid pH (5.5) samples were not buffered, being brought to pH 5.5 by the addition of 0.5 molar HCl (pH 0.3), which could well lead to transient pH in the plasma of well below pH 5.5, and subsequent instability in the pH throughout their 18 hour generation. Thus it is quite possible that some or all of the renin activity picked up in the samples supposedly at pH 5.5 from anephrics could be due to acid activation of inactive renin (Lumbers, 1971) and therefore be an artefact of the procedure.

It was therefore decided to study the PRA of samples from anephric patients and normal volunteers under a variety of conditions.

Four pools of samples were collected. Group one was collected from anephric patients who had previously been determined to have undetectable PRA on the routine assay system. Group two was from anephric patients found to have detectable (0.1 to 0.5 $ng.ml^{-1}.hr^{-1}$) PRA values. Group three was collected from normal volunteers with normal (0.5 to 2.5 $ng.ml^{-1}.hr^{-1}$) PRA and group four from patients with apparently normal renal function but undetectable PRA owing to treatment with β-blockers. The four groups of samples were pooled separately and aliquots generated under three sets of conditions for 18 hours in the presence of EDTA hydroxyquinoline and PMSF to inhibit angiotensin I breakdown.

The three sets of conditions were;generation at pH 7.4 buffered with phosphate, pH 5.7 buffered with citrate and pH 5.5 following adjustment of pH with 0.5 M hydrochloric acid. "Blank" aliquots of all

samples were frozen at the commencement of the generation period and the generated samples were frozen after generation until required for radioimmunoassay.

The data are summarised in Table 11.3. From these figures it would appear that there is no fundamental difference between anephric and normal subjects with regard to the effect of pH on angiotensin generation rate *in vitro*. That is, angiotensin I was generated by renin or a renin-like substance in samples under all sets of conditions or none at all. In the groups yielding detectable PRA values the PRA was a function of pH as expected (Lee, 1965). These results are contrary to those of Oparil *et al.* (1974) and are not consistent with the hypothesis of a fundamental difference in the modes of angiotensin I production with change in pH. It is possibly more likely that their results fell at the sensitivity limit of the assay and therefore the lower levels of angiotensin I generated at pH 7.4 were not detectable for that reason, although one could reasonably have expected from their data, as presented, that the levels would have been within the limits of sensitivity at pH 7.4 in at least four cases.

The results obtained from unbuffered acidic plasmas yielded consistently lower results than their buffered counterparts, both in samples from anephric and normal subjects. Two possible reasons may be offered for this. Firstly measurement of the sample pH post generation showed that in the unbuffered group there was a distinct and reproducible shift from the original pH of 5.5 in all samples that had undergone generation at 37°C. The mean pH was 5.2 ± 0.11 as opposed to 5.5 ± 0.12 in the corresponding "blanks". In the buffered samples no significant changes in the pH were detected with time. This lowering of pH is difficult to explain but, of itself, helps explain the lower

Table 11.3

Effect of pH on plasma renin activity in samples from normal and anephric subjects

pH of generation \ Sample group	Mean plasma renin activity ($ng.ml^{-1}.hr^{-1}$) (n = 6) ± ISD for Groups 2 and 3			
	1	2	3	4
7.4	<0.1	0.27 ± 0.06	0.82 ± 0.05	<0.1
5.7	<0.1	0.35 ± 0.04	1.56 ± 0.12	<0.1
5.5*	<0.1	0.27 ± 0.08	0.98 ± 0.19	<0.1

*Some change observed post generation; see text.

Key to sample groups:

1 From anephric subjects with previously undetectable PRA (<0.1 $ng.ml^{-1}.hr^{-1}$)

2 From anephric subjects with previously detectable PRA (0.1-0.5 $ng.ml^{-1}.hr^{-1}$)

3 From normal subjects with previously detectable PRA (0.5-2.5 $ng.ml^{-1}.hr^{-1}$)

4 From normal subjects with previously undetectable PRA (<0.1 $ng.ml^{-1}.hr^{-1}$)

PRA values as the renin reaction is now 0.5 pH units away from its optimum whereas in the blanks it is only 0.2 pH units away. These two facts added together would yield a falsely high "blank" for the correspondingly generated aliquot and this was apparent when studying the results. The "blank" values were of the same order as those in the buffered pH 5.7 blanks, whereas the generated aliquots yielded values intermediate between the two buffered sets of conditions. Together the net PRA was of the order of those seen at physiological pH. The change of pH from 5.5 to 5.2 whilst not apparently drastic is in fact almost a doubling of hydrogen ion concentration (pH 5.5 ≡ 3.16×10^{-6} M; pH 5.2 ≡ 6.3×10^{-6}) and would therefore be expected to exert a considerable effect.

Secondly, the adjustment of plasma pH to 5.5 by addition of 0.5 molar hydrochloric acid must be regarded as most unsatisfactory as local pH changes before mixing would be extremely drastic and could easily perturb the system. Such perturbations may be reflected by the higher standard deviations in this group of samples and the excessive precipitation seen; more than that routinely caused by the inhibitors. Table 11.4 shows a synopsis of the blank values found in the various sample groups under the differing conditions.

The results indicate that there is no underlying difference in the mode of angiotensin I production at pH 5.7 or pH 7.4 when the systems are buffered and the plasmas not subjected to strong acid additions. Furthermore, they show that to generate angiotensin I in unbuffered acidic plasma is liable to intrinsic error and such results which have been reported elsewhere must be interpreted with caution.

Table 11.4

Effect of pH on "blank" values in samples from normal and anephric subjects.

pH of generation \ Sample group	Mean blank values (ng tube^{-1}) ± ISD for Groups 2 and 3			
	1	2	3	4
7.4	<0.03	0.14 ± 0.39	0.68 ± 0.17	<0.03
5.7	<0.03	0.20 ± 0.21	0.96 ± 0.39	<0.03
5.5	<0.03	0.22 ± 0.09	0.93 ± 0.12	<0.03

pH Profile of renin

Having established a system of inhibition which is apparently totally effective at pH 5.7 and 7.4 and that the optimal pH is the best to employ, a pH profile of renin was constructed to establish the optimal pH of renin in the system under study. This optimum, it is generally agreed, lies between 5.5 and 6.5 (Peach, 1977; Freedlender and Goodfriend, 1979).

Five pooled plasma samples were buffered at each appropriate pH (exact buffer formulations are given in Appendix I) in the presence of EDTA, OHQ and PMSF. Half of each sample was incubated at 37°C for 3 hours whilst the other half was frozen at -20°C. After the generation period the samples were frozen at -20°C until required for radioimmunoassay. The pH of the samples were all checked after the generation period.

No samples were found to have undergone any significant drift from the pH to which they had been adjusted initially.

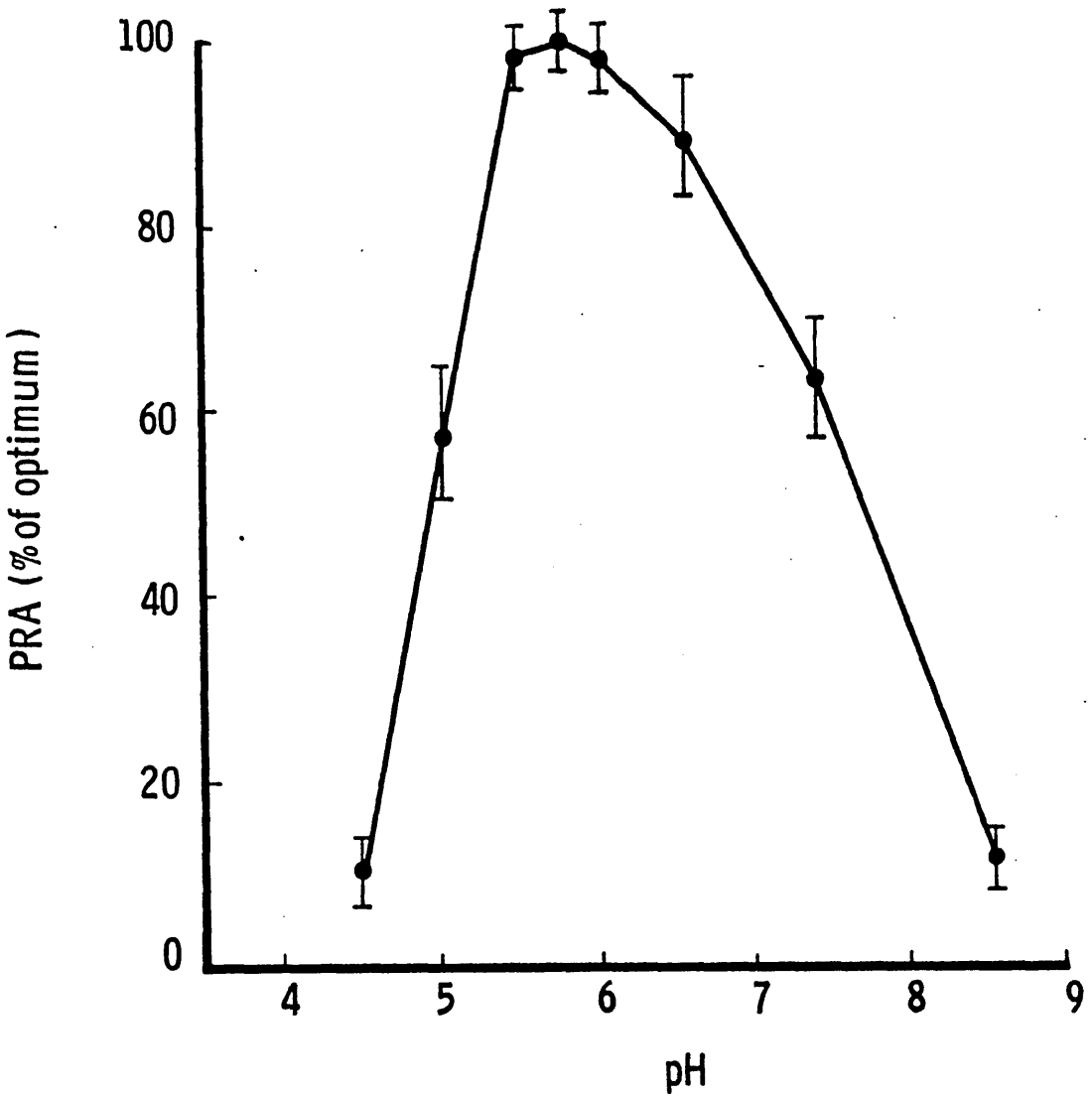
The samples generated at pH 5.7 yielded the highest PRA value and in Figure 11.3 the other results are expressed (mean ± ISD) as percentages of this value.

These results appear to corroborate the findings of other workers, the apparent pH optimum being between 5.5 and 6.0. The highest standard deviation of any group was the group at pH 7.4 and while statistics on a group of five samples are not perhaps terribly reliable indicators it is of interest that this pH is on one of the steepest parts of the curve where slight changes in pH could yield major responses in PRA. Also the lower values of PRA at pH 7.4 could lead to sensitivity problems. Thus pH 5.7 would appear to be the most suitable for use in *in vitro* angiotensin I generation.

It is also noteworthy that outside the range pH 5.7 to pH 7.4 the efficiencies of the inhibitors were not independently tested. This could,

Figure 11:3

pH profile of the renin reaction.



of course, lead to artificially low PRA values at these extreme pHs and if, for any reason, one wished to work at very low or very high pH an independent study of the type described for pH 5.7 and pH 7.4 should be conducted.

The relationship between PRA values at pH 5.7 and pH 7.4

Although the earlier study using normal controls and anephrics' plasma appeared to indicate no fundamental difference in the mode of angiotensin production with pH change and the pH profile similarly indicated an approximately 50% increase in PRA on adjustment to pH 5.7 from 7.4 these results are based on four samples with similar PRA values. It is quite conceivable, therefore, that the apparent 50% increase indicated by both the pH profile and groups 2 and 3 of the pH study is not applicable to the whole range of PRA's which one may encounter. If the relationship is non-linear then it must cast grave doubts on the validity of the assay sensitisation by acidification. Also, it is apparent that there must, kinetically speaking, come a point where substrate and/or product concentration would become rate limiting and this would be expected to affect the acidified plasmas first. Therefore it is necessary to know if a linear relationship exists, and if it does between what limits it holds good.

76 plasma samples from normal subjects and unselected hypertensive patients were generated at pH 5.7 and pH 7.4 for 3 hours in the presence of EDTA, OHQ and PMSF. All samples were then frozen until required for assay.

Samples were always assayed with their corresponding pH controls in the same assay to prevent any interassay artefacts interfering with the interpretation of the data.

The results are shown in Figure 11.4 where PRA values at pH 5.7 are plotted as a function of the corresponding PRA values at pH 7.4. Table 11.5 shows the results of the linear regression analysis.

The results show a good correlation over the range encompassed by this particular population of samples (up to about 7 $ng.ml^{-1}.hr^{-1}$). This would indicate that the acidification does not alter anything fundamental in the generation process only increasing its rate. As may have been predicted from the earlier experiments using plasma from anephric patients no change in the slope was seen at low PRA values.

The relationship between PRA values at pH 5.7 and pH 7.4 in high PRA samples

In order to assess if the linear relationship found in the previous experiment holds good at higher levels up to the limits of the assays the previous experiment was repeated using a different sample population encompassing the range 10 to 50 $ng.ml^{-1}.hr^{-1}$).

The duplicate samples, which had been retained at -20°C throughout, of 24 samples which had been previously assayed for PRA and found to be high (between 10 and 50 $ng.ml^{-1}.hr^{-1}$) were generated in duplicate; at pH 7.4 and at pH 5.7 for 3 hours. They were then frozen until they were required for assay. All samples were evaluated in the same assay.

The results shown in Figure 11.5 and Table 11.6 indicate the continuation of the linear relationship up to the normal assay limits. The population of samples used are not part of the same normal distribution as the previous set and therefore cannot be used in the same linear regression analysis as it would engender false correlations. However, it is possible to compare the two regression lines by the variance ratio test. No significant difference in slopes (F^{1}_{96} 0.444, $p>0.05$) or

Figure 11:4

PRA after generation at pH 5.7 plotted as a function of PRA after generation at pH 7.4.

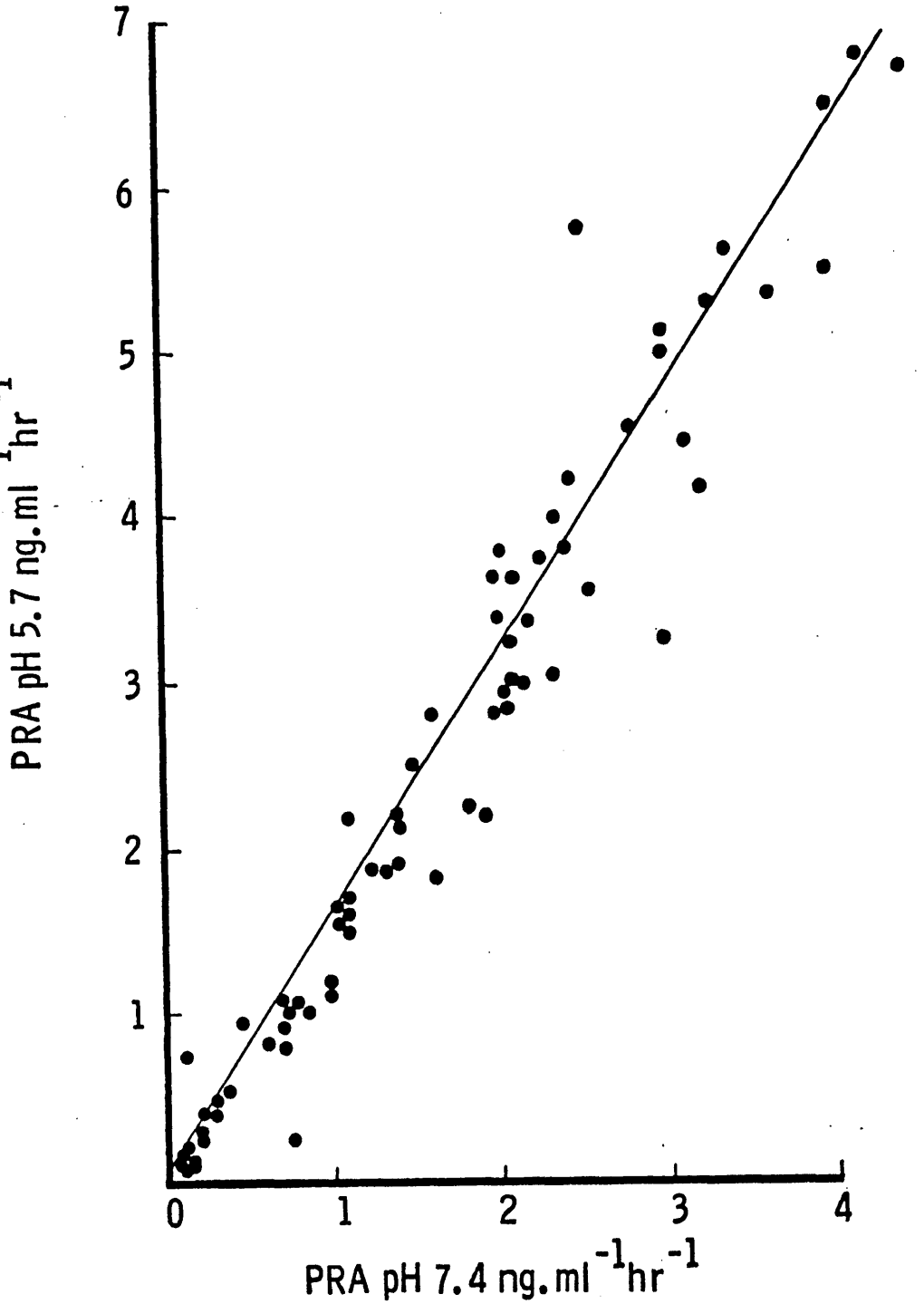


Table 11.5

Correlation of PRA at pH 5.7 with PRA at pH 7.4

No. of samples	76
Mean of y array	2.410
Mean of x array	1.578
Standard deviation of y array (N-1)	1.866
Standard deviation of x array (N-1)	1.181
Standard deviation of y array (N)	1.854
Standard deviation of x array (N)	1.173
Variance of y array (N)	3.438
Variance of x array (N)	1.376
Variance of y array (N-1)	3.484
Variance of x array (N-1)	1.394
Y Intercept	-0.020
Slope after y intercept	1.540
Correlation coefficient	0.974
x Intercept	0.013

Figure 11:5

PRA after generation at pH 5.7 plotted as a function of PRA after generation at pH 7.4 encompassing a wider range of values.

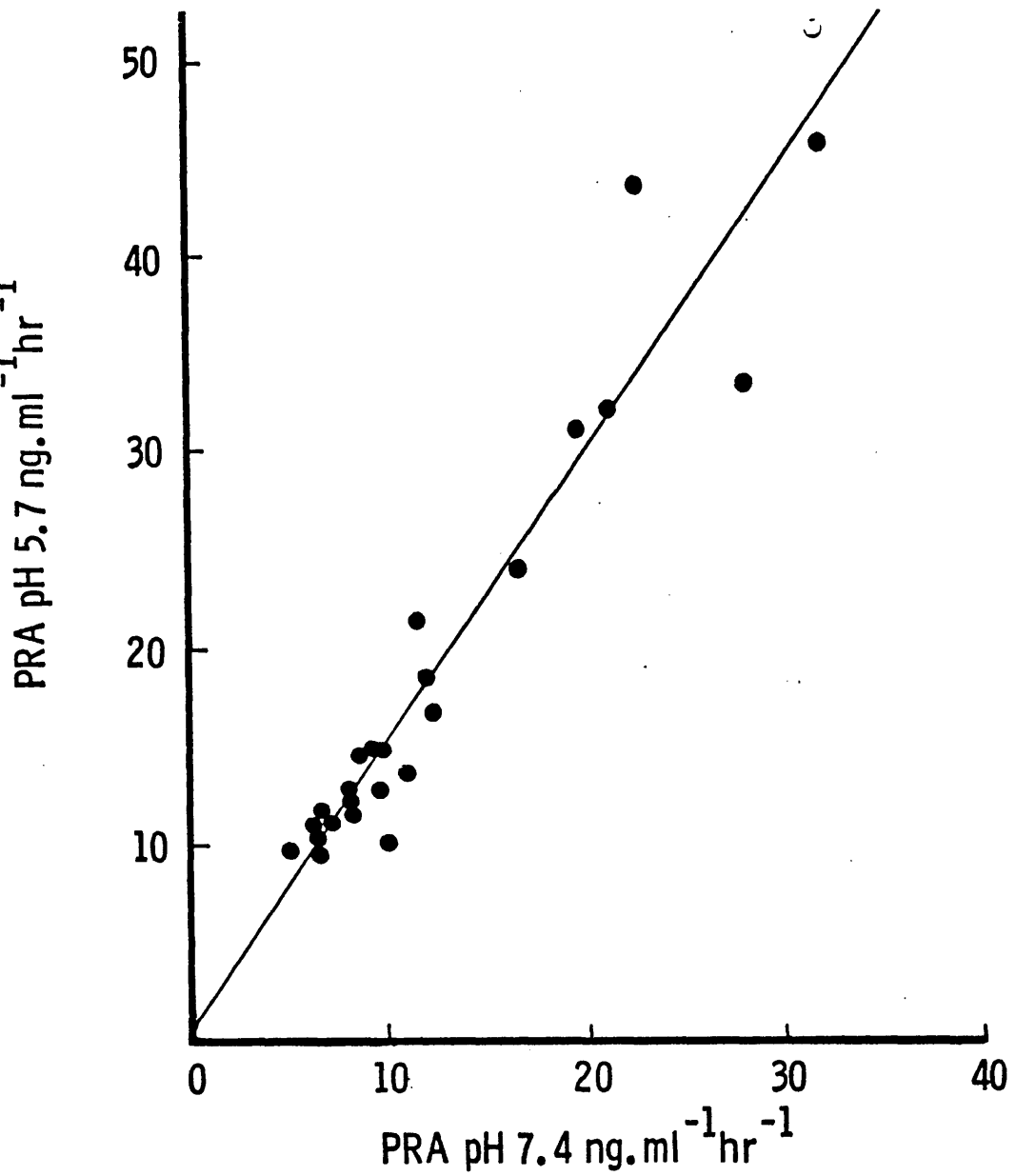


Table 11.6

Linear regression data; PRA at pH 5.7 with PRA at pH 7.4

No. of samples	24
Mean of y array	18.838
Mean of x array	12.517
Standard deviation of y array (N-1)	10.746
Standard deviation of x array (N-1)	7.171
Standard deviation of y array (N)	10.519
Standard deviation of x array (N)	7.020
Variance of y array (N)	110.657
Variance of x array (N)	49.285
Variance of y array (N-1)	115.469
Variance of x array (N-1)	51.428
Y Intercept	0.937
Slope after y intercept	1.430
Correlation coefficeint	0.954
x Intercept	-0.655

elevation (F^{1}_{96} 1.2317, $p>0.05$) was found when this analysis was used.

The results indicate that the linear relationship found in this experiment between the higher PRA values is not significantly different from that obtained in the normal range. It is therefore likely that over the range considered (up to ≈ 50 $ng.ml^{-1}hr^{-1}$) substrate depletion *in vitro* does not appear to affect the PRA and that the use of PRA as an index of plasma angiotensin concentration is as valid in these high samples as in the ones encompassing the normal range. The effects of substrate depletion would be expected to be seen in a decrease in slope of the regression line (Figure 11.5) at higher values as substrate became limiting in the acidified plasmas first.

Rectilinearity of angiotensin I generation with respect to time

One method of adjusting assay sensitivity should it be necessary would be to alter the time of generation at 37°C. To do this one would have to be certain that the generation process was linear within the limits of any proposed variations in time. Samples yielding high PRA values were included in the study in addition to normal value samples in order to see if substrate concentration became apparently rate limiting and if it did so at what level of angiotensin I concentration. The previous pH study indicated, indirectly, rectilinearity up to a PRA of about 50, i.e. 150 $ng.ml^{-1}$ of plasma. Other workers, however, have shown deviations from linearity at lower levels (Bangham *et al.*, 1975). Obviously variations in endogenous substrate concentration, loss of substrate owing to angiotensinase inhibitors and varying dilution conditions would all affect the situation and make it virtually impossible to use the results of other workers obtained in non-identical systems.

Three normal range and three high PRA value samples were incubated at pH 5.7 in seven separate aliquots. These were frozen at timed intervals from 0.5 to 6 hours. All samples were then radioimmunoassayed; the high ones at two dilutions (5x) to ensure that they fell on the standard curve.

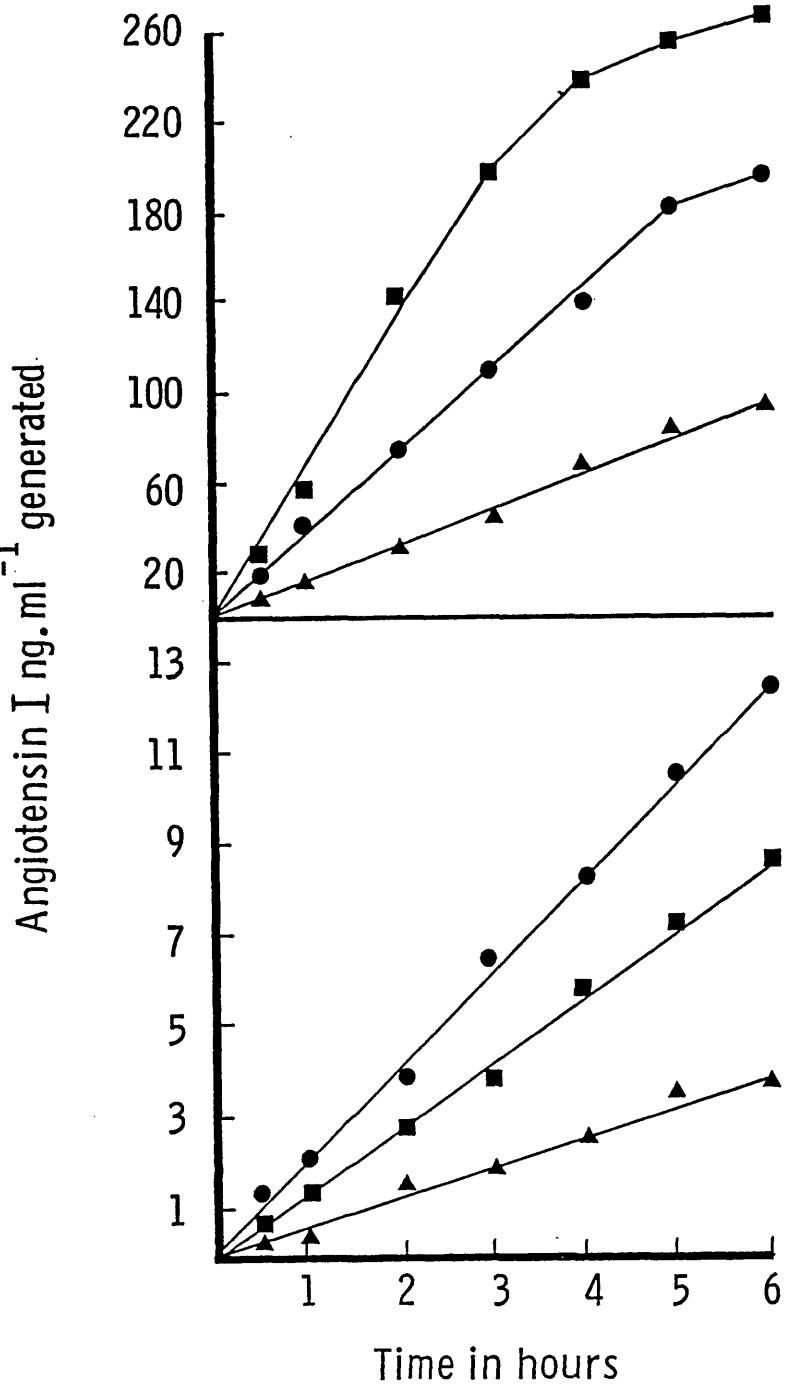
The concentrations of angiotensin I/ml plasma were plotted as functions of time (Figure 11.6).

Rectilinearity was observed up to 6 hours in four of the six samples. In the two highest PRA samples, however, a decrease in rate was observed. This occurred in one case at values >180 ng.ml (60.0 ng/ml/hr), the other case at values >200 ng.ml (66.7 ng/ml/hr).

These results indicate that in PRAs beyond the limits of the normal assay standard curve the rate may not be maximal for 3 hours. Therefore if a result of >66.7 ng/ml/hr is obtained after a 3 hour generation a repeat sample using say a 1 hour generation should be employed. Variation in substrate concentration *in vitro* obviously make this figure (66.7) rather vague as the result is from only one sample. Although in the next highest sample (PRA 46.7 $ng.ml^{-1}.hr^{-1}$) linearity up to 3 hours is observed. Coupled with the linear regression analysis of the previous pH study it may be inferred that PRA values of less than 50 ng/ml/hr obtained in this system are reflections of a rectilinear rate of reaction. Indirect corroborative evidence is discussed in the next section of this chapter. Addition of excess exogenous substrate was not considered appropriate to counteract this loss of generation rate since it has been seen (Reid *et al.*, 1973; Waldhausl *et al.*, 1973; Page and Bumpus, 1974) that the order of magnitude of [substrate] is such that it could affect the rate of reaction, although these results here indicate that this does not happen in this system except in extreme circumstances. However, if one postulates that

Figure 11:6

Angiotensin I generation as a function of time.



[substrate] could be critical to rate *in vitro* it is equally feasible that it could be limiting *in vivo*. Therefore addition of exogenous substrate should be avoided, if at all possible, as it could be misleading. In this system it is clearly possible to obtain linear kinetics for 3 hours over almost the whole range of PRA values (less time for extremely high values).

Addition of exogenous substrate would be a perfectly acceptable step if one really meant "plasma renin activity" in true enzymatic terms. However, one does not, generally. What one means is angiotensin generation rate and if this is possibly dependent *in vivo* or *in vitro* on [substrate] it ought to be so determined, as PRA is used in a clinical context. Addition of excess exogenous substrate, whilst yielding a correct V_{max} and K_m for renin would not of necessity yield a <u>true</u> reflection of *in vivo* angiotensin generation rate. If we consider an extreme example where no endogenous substrate exists, then PRA ≡ angiotensin II concentration = 0. This is the sort of situation which could arise in patients with liver failure, cirrhosis, etc. However, if exogenous substrate is added to the system a false positive result based only upon the enzyme's "potential activity" is obtained which is not clinically meaningful. If, on the other hand, one argues that [substrate] *in vitro* is not a true reflection of substrate concentration *in vivo* one may support the idea of exogenous addition, although one is not to know that this yields a truer physiological situation of itself. However, there is no evidence to suggest, in the very high samples used in this experiment for example, that *in vitro* plasma substrate concentration is not of the same order as *in vivo*. Further, there is no proof that limiting concentration of substrate may not modify the rate of production of angiotensin *in vivo*. It must be remembered that in general PRA is measured as an index of angiotensin concentration *in vivo* and as such

renin should be subject to as many of the potential constraints as would apply therein.

Corroborative evidence for the linearity of PRA with respect to ungenerated angiotensin levels is described in the following experiment where PRA was compared to the "blank value" of angiotensin I for 2752 samples.

Plasma renin activity as a function of angiotensin I concentration

The previous sets of results indicate linear relationships up to PRA values of about 60 $ng.ml^{-1}.hr^{-1}$ with respect to time and pH change. Another, albeit teleological, approach was used to corroborate these findings when PRA was compared with the angiotensin I concentration present in the plasma samples prior to generation. 2752 samples from normal volunteers and unselected hypertensive patients were included in the study.

The results of the comparison are displayed in the bar graphs (Figures 11.7 and 11.8).

Figure 11.7 shows the relationship between mean PRA grouped according to initial angiotensin I concentration (≡ "blank value"). The initial angiotensin I concentrations are plotted as 0.4 $ng.ml^{-1}$ increments and a PRA range of 0 to 11, that is the upper limit of normal subjects on the 5th day of a 10 mmol sodium diet, is included. It can be seen that the relationship is linear over the entire range ($n = 2538$; $r = 0.69$, $p < 0.001$).

Figure 11.8 is similarly constructed, encompassing a much wider range of values, including some of the data from Figure 11.7. It can be seen that at very high levels of PRA (>60 $ng.ml^{-1}.hr^{-1}$) the relationship ceases to be linear although the overall correlation remains significant ($n = 654$; $r = 0.54$; $p < 0.001$).

Figure 11:7

The relationship between PRA and initial angiotensin I concentration.

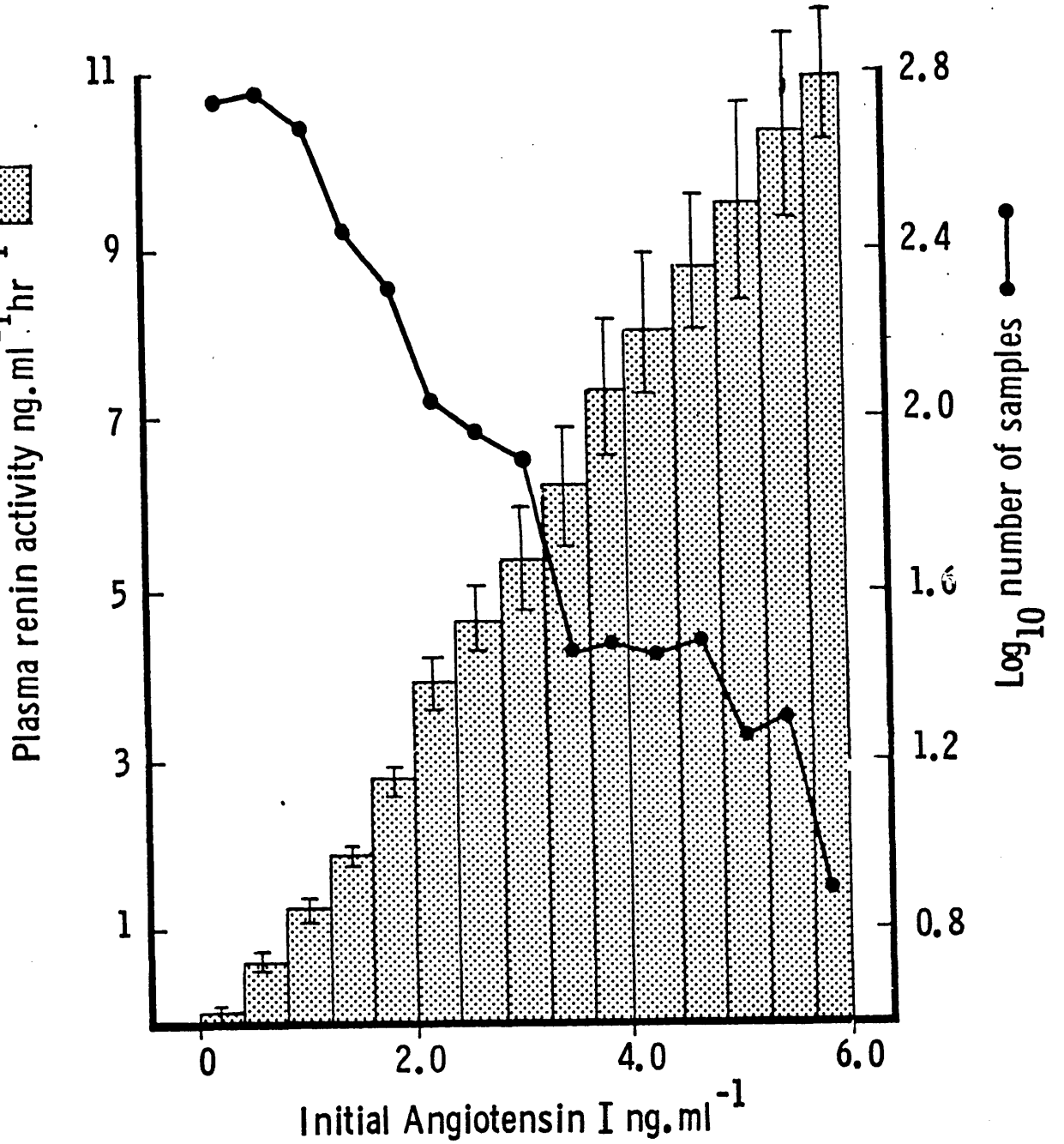
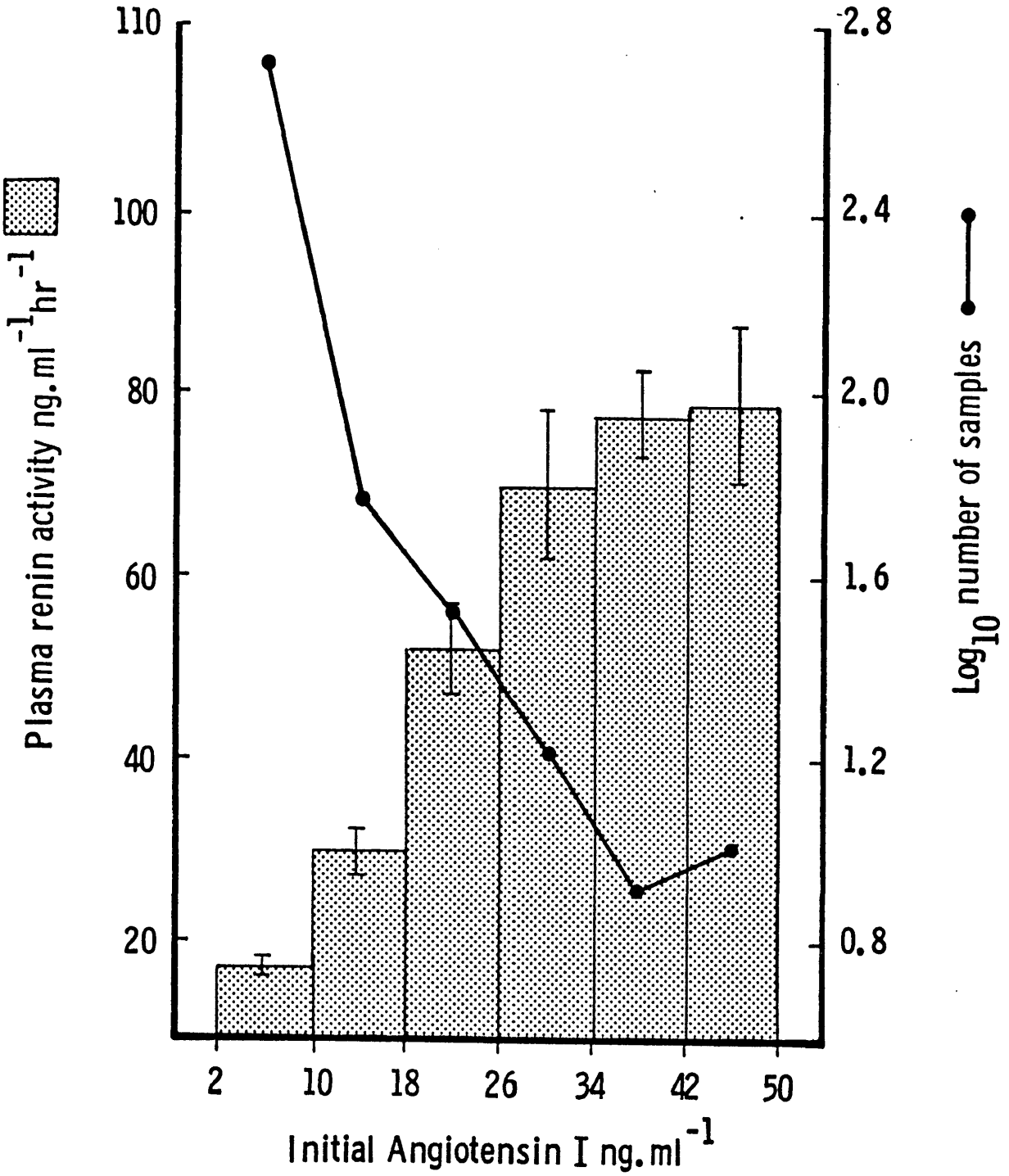


Figure 11:8

The relationship between PRA and initial angiotensin concentration encompassing a wide range of values.



These results provide further evidence that the PRA assay provides a linear index of angiotensin concentration up to very high levels. The loss of linearity above 60 $ng.ml^{-1}.hr^{-1}$ indicates that the two samples studied with respect to generation time in the previous experiment are typical of the population studied in this experiment.

An investigation of sample homogeneity with respect to PRA

Addition of inhibitor solutions, OHQ and PMSF, to the buffered plasmas results in some precipitation. Since this effects a separation of the plasma into effectively two components it was essential to check the distribution of PRA within the samples.

Fourteen plasma samples were generated in the usual way. For the radioimmunoassay, the samples were thawed, mixed thoroughly and samples transferred to the assay tubes. The remainders were then centrifuged and supernatant samples were taken from these and assayed. The amounts of angiotensin I per tube per sample were interpolated from the standard curve in the usual way and a paired t-test and variance ratio analysis performed on the results.

There was no mean difference between the two sets of data, i.e. $t = 0$. The standard deviations were also very similar (101.96 and 104.09) and the variance ratio was not significant ($F_{13}^{13} = 1.0208$). This indicates that the comparison of the two sets of data by a t-test was valid and the lack of difference between the means found indicates that the two sampling techniques yield virtually identical results (see Table 11.7).

Table 11.7

Study of sample homogeneity with respect to PRA

No. of pairs	14
Students t	0
Mean difference	0
Standard deviation of mean difference	12.3923
Degrees of freedom	13.0000
Mean X_1	185.7142
Standard deviation of X_1	101.9588
Mean X_2	185.7142
Standard deviation of X_2	104.0868
Variance ratio F_{13}^{13}	1.0208

Plasma Renin Activity Radioimmunoassays

Having established a degree of confidence in the angiotensin I generation techniques it was necessary to check further the performance of the angiotensin I radioimmunoassays, both manual and automated.

Precision

Using Ekins' definition (Ekins and Newman, 1970) that the precision of measurement of a quantity x, designated Δx, is related to both the precision of the estimate of the response metameter y, designated Δy, and the slope of the dose response curve (dy/dx) at the corresponding point we can express precision as

$$\frac{\Delta y_x}{dy/dx} = \Delta x$$ See Figure 11.9

Table 11.8 lists the precision at each standard point for each assay. Each estimate represents the mean (± ISD) of ten consecutive assays.

Sensitivity

Sensitivity may be defined as the precision of measurement of x when x = 0 (see Figure 11.10).

That is, $\Delta x_0 = \Delta p = \frac{\Delta y_0}{dy/dx}$

The sensitivities of the assays are given at the foot of Table 11.8 and can be seen to be of the order of 2.5 pg per tube in both cases.

Specificity

Cross reactivity

No cross reactivity of the antibody with angiotensin II was observed (See Chapter 6 - "Preparation and Evaluation of Antibodies"). In the

Figure 11:9

Diagram explaining the definition of precision

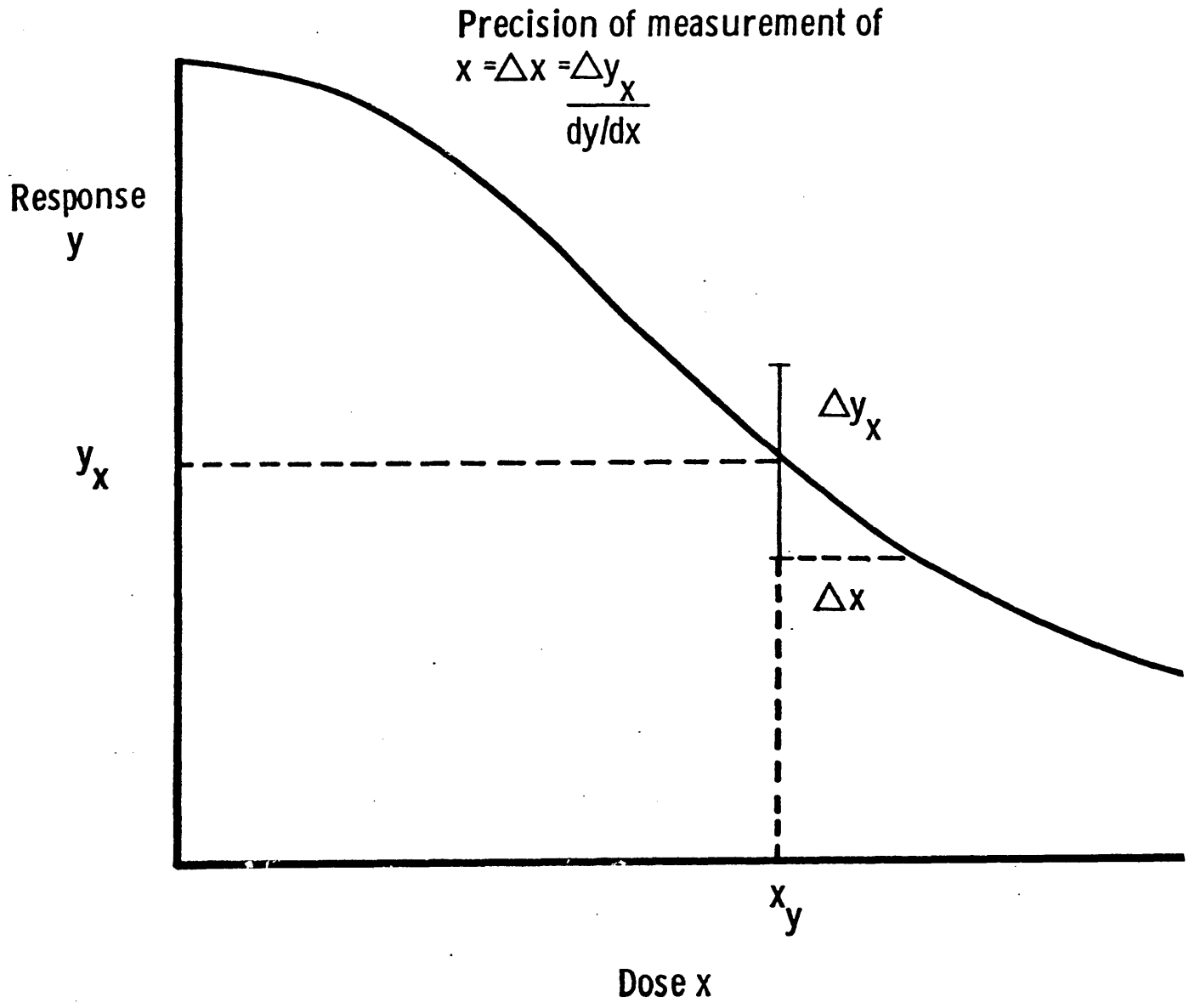


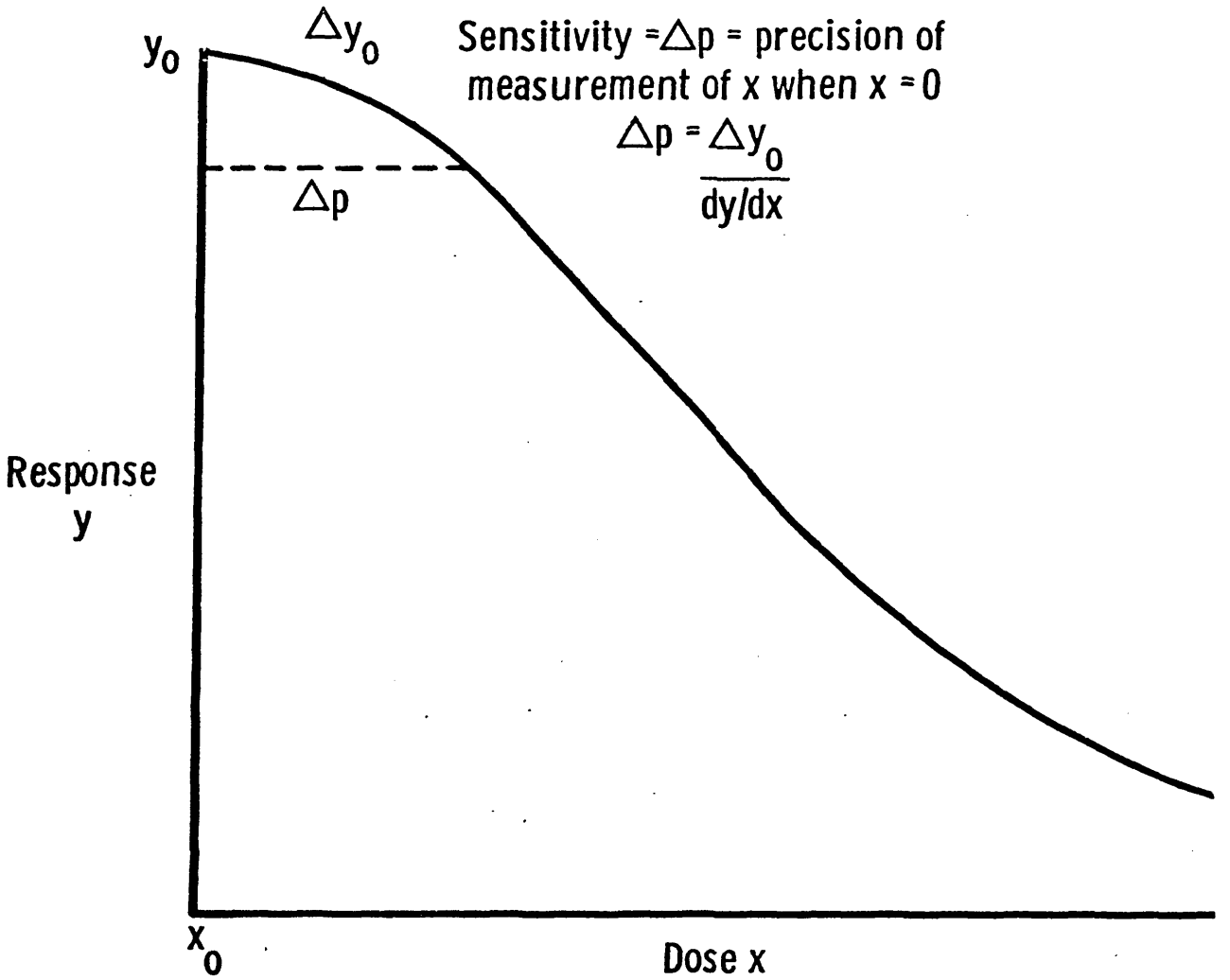
Table 11.8

Precision of angiotensin I radioimmunoassays

Standard concentration pg/tube	Precision pg/tube (± ISD)	
	Manual assay	Automated assay
1250	33.60 (10.84)	35.10 (11.41)
625	20.61 (6.39)	21.40 (8.41)
312.5	13.81 (3.61)	14.64 (3.41)
156.25	13.97 (2.24)	12.14 (2.91)
78.125	4.61 (1.91)	7.99 (1.63)
39.06	4.24 (1.44)	4.33 (1.31)
19.53	4.50 (1.81)	4.51 (1.61)
9.77	2.39 (1.01)	2.36 (1.06)
4.88	2.41 (1.01)	2.25 (1.21)
0	2.38 (1.11)	2.51 (0.77)

Figure 11:10

Diagram explaining the definition of sensitivity.



automated assay non-specific binding as estimated by non-addition of the antibody binding complex prior to filtration was usually between 0,5 and 0,8% of the total count added. In the manual assay the figure was of the order of 1,5% of total. These results show that no appreciable free ligand was retained by the filter discs or left unadsorbed by charcoal.

Recovery

Recovery was estimated by the addition of known amounts of angiotensin I to anephric plasma previously determined as having undetectable angiotensin I concentration (see Table 11,9).

Parallelism

In both the manual and the automated assay the plasma concentration with respect to the total reaction volume was small. In both cases 50 µl aliquots of the generated sample/blank which were 46,5% plasma (v/v) were used. In the manual assay the reaction volume was 350 µl whereas in the automated assay it was 200 µl. Thus the effective plasma concentrations were 6,64% and 11,63% in the reaction mixtures of the manual and automated assays respectively.

In the light of this it would have been surprising to encounter non-specific effects due to serum or plasma interfering with the equilibrium reaction at such dilutions. Nonetheless some studies were done to check for such interference. The assays were checked in two ways. Firstly standard curves were obtained in experiments where no "background plasma" was present and the results compared to curves where 50 µl of anephric plasma (previously determined to have undetectable angiotensin I levels) were added in place of 50 µl of buffer in the automated assay. This gave a plasma concentration of 25% and as can be seen from Figure 11,11 no change in curve shape or binding characteristics could be observed.

Table 11.9

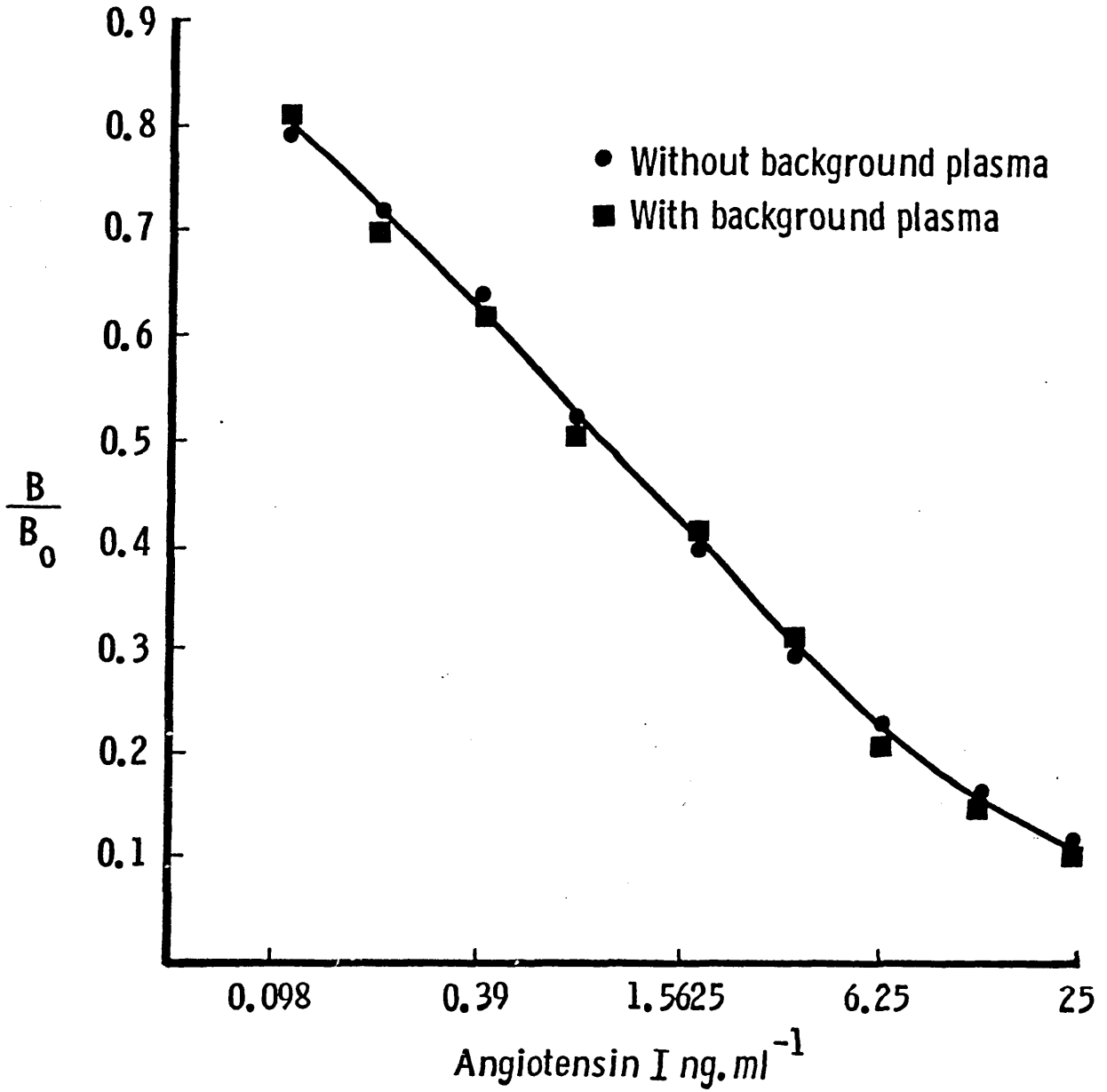
Recovery study.

Values of angiotensin I concentration expressed as a percentage of the total concentration in control samples

Total added concentration $ng.ml^{-1}$	Manual assay		Automated assay	
	Observed concentration $ng.ml^{-1}$	% Recovery $\frac{obs}{exp} \times 100$	Observed concentration $ng.ml^{-1}$	% Recovery $\frac{obs}{exp} \times 100$
0.2	0.22	110.0	0.19	95.0
0.5	0.47	94.0	0.51	102.0
1.0	1.00	100.0	1.01	101.0
2.0	2.12	106.0	2.10	105.0
3.0	3.00	100.0	3.15	105.0
4.0	4.10	102.5	4.08	102.0
5.0	4.95	99.0	5.07	101.4
6.0	6.03	100.5	6.07	101.2
7.0	7.22	103.1	7.36	105.1
8.0	8.13	101.6	8.22	102.8
7.5	7.46	99.5	7.27	96.9
6.5	6.56	100.9	6.45	99.2
5.5	5.41	98.4	5.63	102.4
4.5	4.51	100.2	4.56	101.3
3.5	3.38	96.6	3.51	100.3
2.5	2.57	102.8	2.55	102.0
1.5	1.42	94.7	1.48	98.7
0.8	0.80	100.0	0.82	102.5
0.4	0.38	95.0	0.41	102.5
n = 19	$\bar{x}$	100.3	$\bar{x}$	101.4%
	S.D.	3.85	S.D.	2.59
	C.V.	3.84	C.V.	2.56%

Figure 11:11

Dose response curves in the presence and absence of "background" plasma.



In the manual assay 87,5 µl of anephric plasma were substituted for buffer to obtain a comparable final plasma concentration. Once again no change in the standard curve could be seen.

However, the above experiments do not, of themselves, discount the possibility of the curves being displaced by differential concentrations of plasma components. To test this a parallelism study was performed in which angiotensin containing plasma samples were serially diluted and compared to the standard curves. The dose response curves were linearised by the log-logistic transformation. Linear regression analysis of the results from each samples and the standard curve was then performed to see if all or nearly all of the variance was explained by regression. As correlation coefficients were always >-0,98 this showed that almost all the variance was indeed explained by regression. The significance of differences between the slope of the standard curve and that of each of the samples were then assessed using the variance ratio as described by Snedecor and Cochran (1967). These variance ratios indicated that there was no significant difference in slope of any of the sample curves with respect to those of the standard curves in the automated assay. A set of data using five plasma samples in the automated assay is shown in Table 11.10 and Figure 11.12. The results from the manual assay are shown in Table 11.11 and Figure 11.13. From these results it can be seen that in the manual assay one plasma sample ("G4") does show a statistically significant difference in slope from the standard curve. However, this is due to the extremely high correlation coefficient of that sample ($r = -0,9999$). It will be appreciated that for an ideal correlation ($r = \pm 1$) any difference in slope is <u>statistically</u> significant. It is obvious from the results, however, that such a difference, in this case, is of no practical significance.

Table 11.10

Parallelism study - Automated assay.

Results of linear regression analysis on the standard curve and five serially diluted plasma samples. The variance ratio of each sample with respect to the standard curve is also listed.

Sample name	Linear regression analysis			Variance ratio of samples with respect to the standard curve	
	Correlation coefficient	Slope	Intercept	F^1_{11}	p
Standard curve	-0,9997	-1,4793	1,3991	-	-
U3	-0.9945	-1.5040	0.5556	0.1750	>0.05
U2	-0.9964	-1.5052	0.3732	0.1819	>0.05
U1	-0.9996	-1.4842	0.9845	0.0333	>0.05
G2	-0.9991	-1.5294	1.4208	4.0526	>0.05
G1	-0.9978	-1.5590	1.5893	3.6842	>0.05

Figure 11:12

Parallelism study for the automated PRA assay.

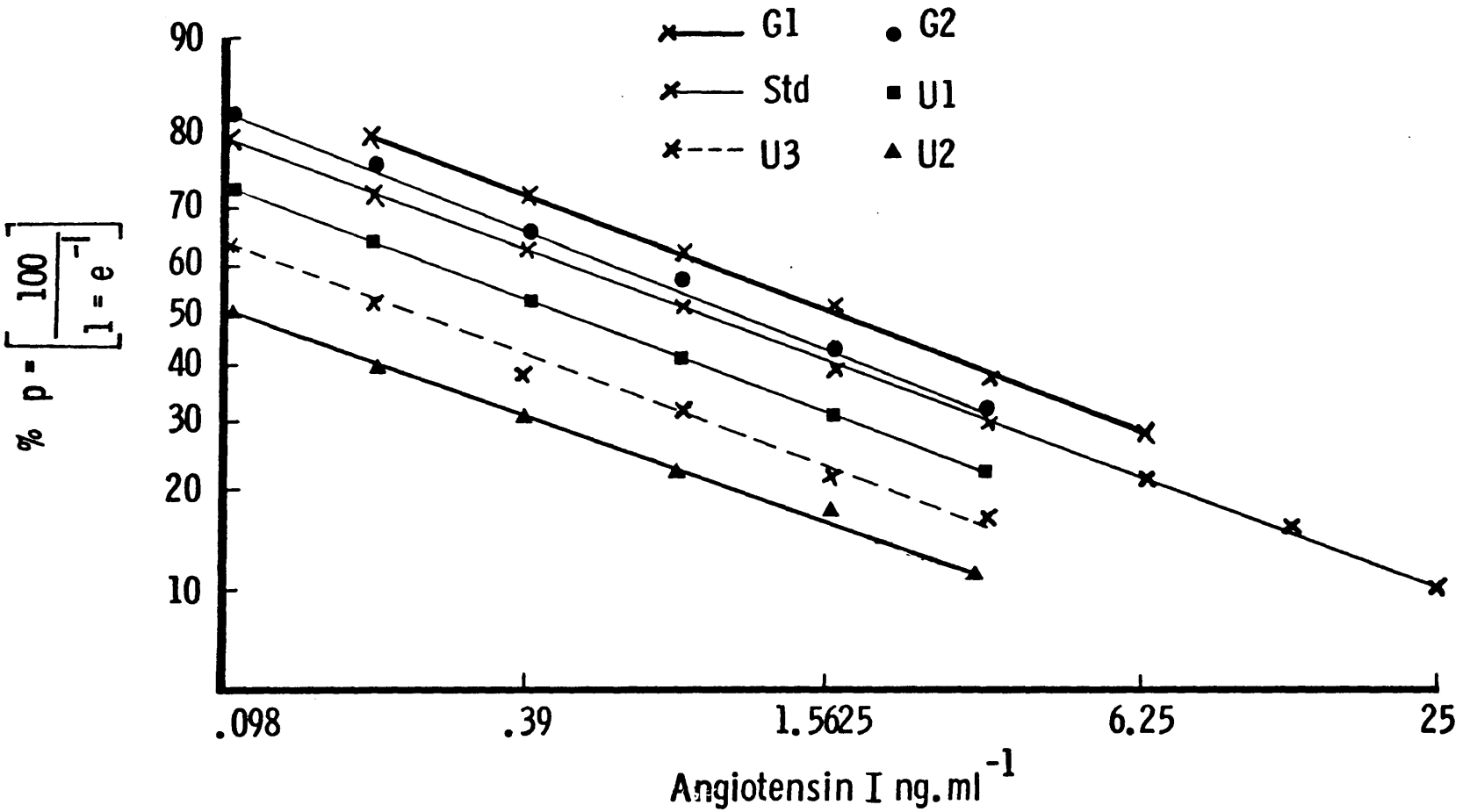


Table 11.11

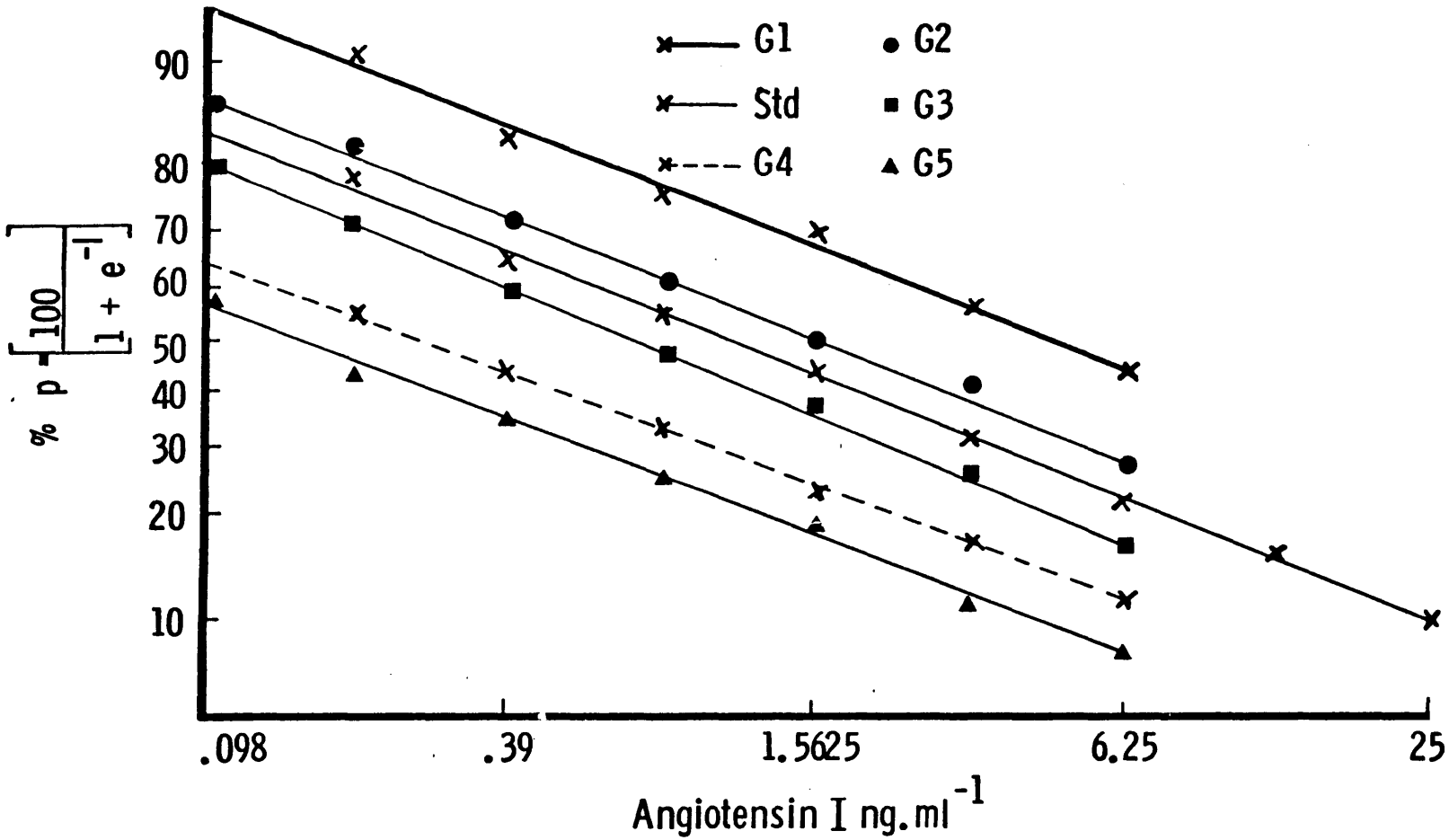
Parallelism study - Manual assay

Results of linear regression analysis on the standard curve and five serially diluted plasma samples. The variance ratio of each sample with respect to the standard curve is also listed.

Sample name	Linear regression analysis			Variance ratio of samples with respect to the standard curve	
	Correlation coefficient	Slope	Intercept	F^1_{12}	p
Standard curve	-0.9992	-1.6015	4.8398	-	-
G1	-0.9977	-1.5670	5.7427	0.5128	>0.05
G2	-0.9974	-1.5881	5.1079	0.0697	>0.05
G3	-0.9991	-1.6206	4.5932	0.2307	>0.05
G4	-0.9999	-1.5064	3.6484	9.1764	<0.025
G5	-0.9978	-1.5274	3.3187	2.4736	>0.05

Figure 11:13

Parallelism study for the manual PRA assay.



Direct comparison of the manual and automated PRA assays

Sixty-five plasma samples were assayed in triplicate by both assay methodologies. Subsequent linear regression analysis of the results showed (see Figure 11.14) a very high correlation coefficient, a slope (m) approaching unity and an intercept (b) tending to zero. ($n = 65$, $r = 0.996$, $m = 1.03$, $b = 0.001$).

Reproducibility

The reproducibility of the assays was assessed by the analysis of the data from the quality control pools on an intra- and inter-assay basis. Tables 11.12 and 11.13 summarise the data. It can be seen from these tables that the two assays yield highly similar coefficients of variation for the various pools both intra- and inter-assay. Whilst, predictably, the intra-assay coefficients of variation are approximately half those of the inter-assay values for the corresponding pool there does not appear to be any significant increase in coefficient of variation between an angiotensin control of a certain value and its corresponding renin control. This is apparent from both intra-assay and inter-assay results and would indicate that the generation procedure to which the renin controls were subjected was consistent and reproducible.

Sealey and Laragh (1975) observed an increase in PRA from normal subjects and from patients with hypertension after storage of samples at -20°C. They reported a mean increase upon re-assay of 26% after two months and 55% after one year. Such results would indicate that long-term storage of plasma quality control pools ("renin" controls) and patients' samples would give invalid results. However, from the results reported in this chapter it may be seen that no swingeing changes take place in renin controls upon long-term storage at -20°C.

Figure 11:14

Comparison of the manual and automated assays for PRA.

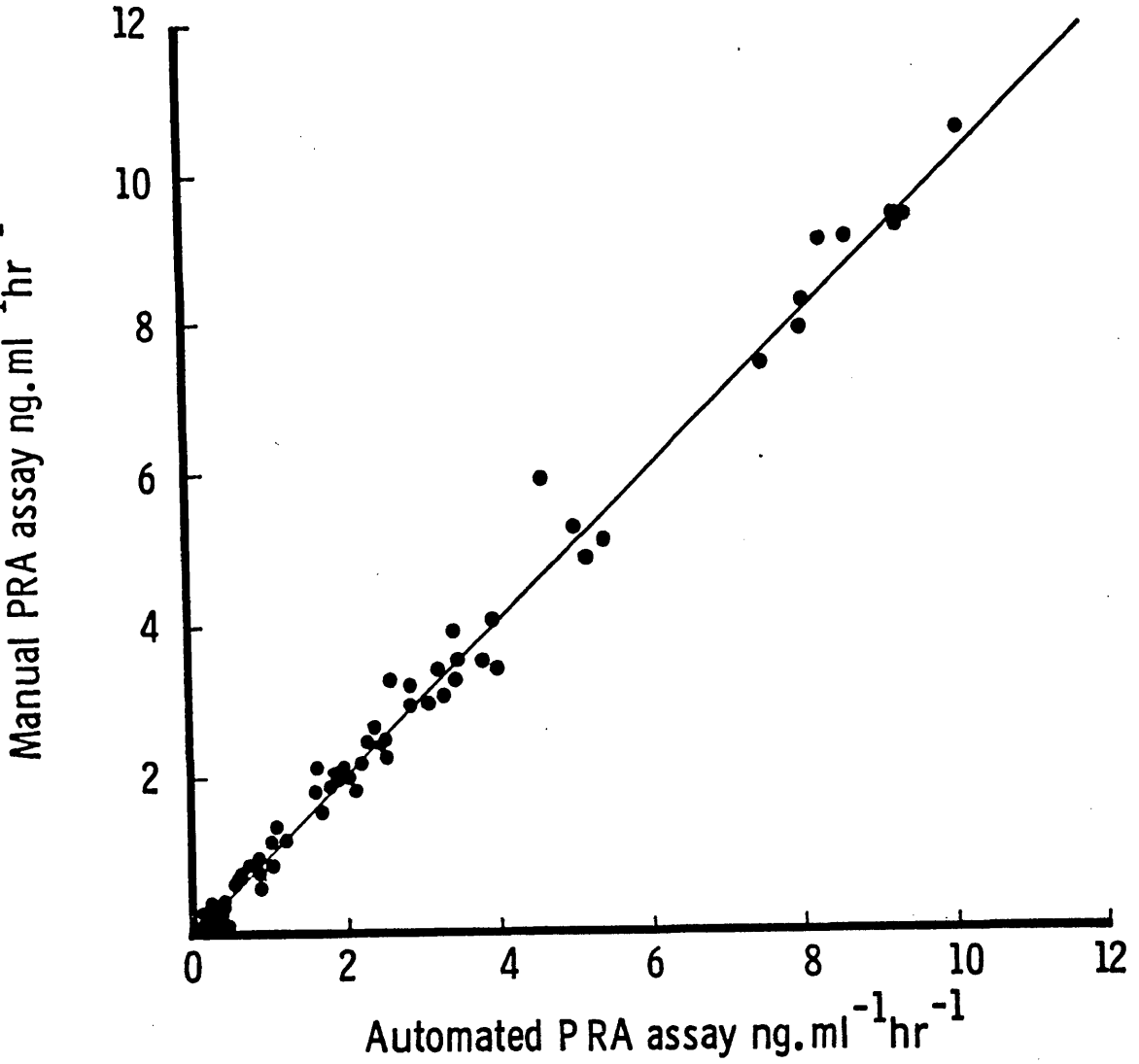


Table 11.12

Intra-assay coefficients of variation of the quality control samples in the PRA assays.

	Renin Controls					
	Manual assay			Automated assay		
Pool	Low	Medium	High	Low	Medium	High
No.	6	6	6	6	5	5
Mean	0.94	1.93	5.91	1.00	2.13	5.69
S.D.	0.058	0.060	0.31	0.058	0.07	0.32
C.V.	6.1	3.1	5.2	5.8	3.3	5.7
	Angiotensin Controls					
Pool	Low	Medium	High	Low	Medium	High
No.	6	6	6	6	6	6
Mean	1.17	2.16	7.65	1.21	2.24	7.77
S.D.	0.078	0.095	0.39	0.068	0.087	0.40
C.V.	6.7	4.4	5.1	5.6	3.9	5.1

Table 11.13

Inter-assay coefficients of variation of the quality control samples in the PRA assays

	Renin Controls					
	Manual assay			Automated assay		
Pool	Low	Medium	High	Low	Medium	High
No.	20	40	36	18	19	19
Mean	0.96	2.00	5.86	1.03	1.92	5.64
S.D.	0.08	0.18	0.72	0.09	0.11	0.58
C.V.	8.33	9.00	12.2	8.7	5.6	10.2
	Angiotensin Controls					
Pool	Low	Medium	High	Low	Medium	High
No.	22	37	35	18	18	18
Mean	1.21	1.94	7.39	1.12	2.10	7.56
S.D.	0.096	0.20	0.95	0.096	0.21	1.01
C.V.	7.9	10.3	12.8	8.6	10.1	13.4

Figure 11.15 shows graphically the PRA value of the medium and high renin and angiotensin quality control pools as a function of time when estimated on the manual assay. These particular pools were chosen as the time course they span was longer than the other pools assessed.

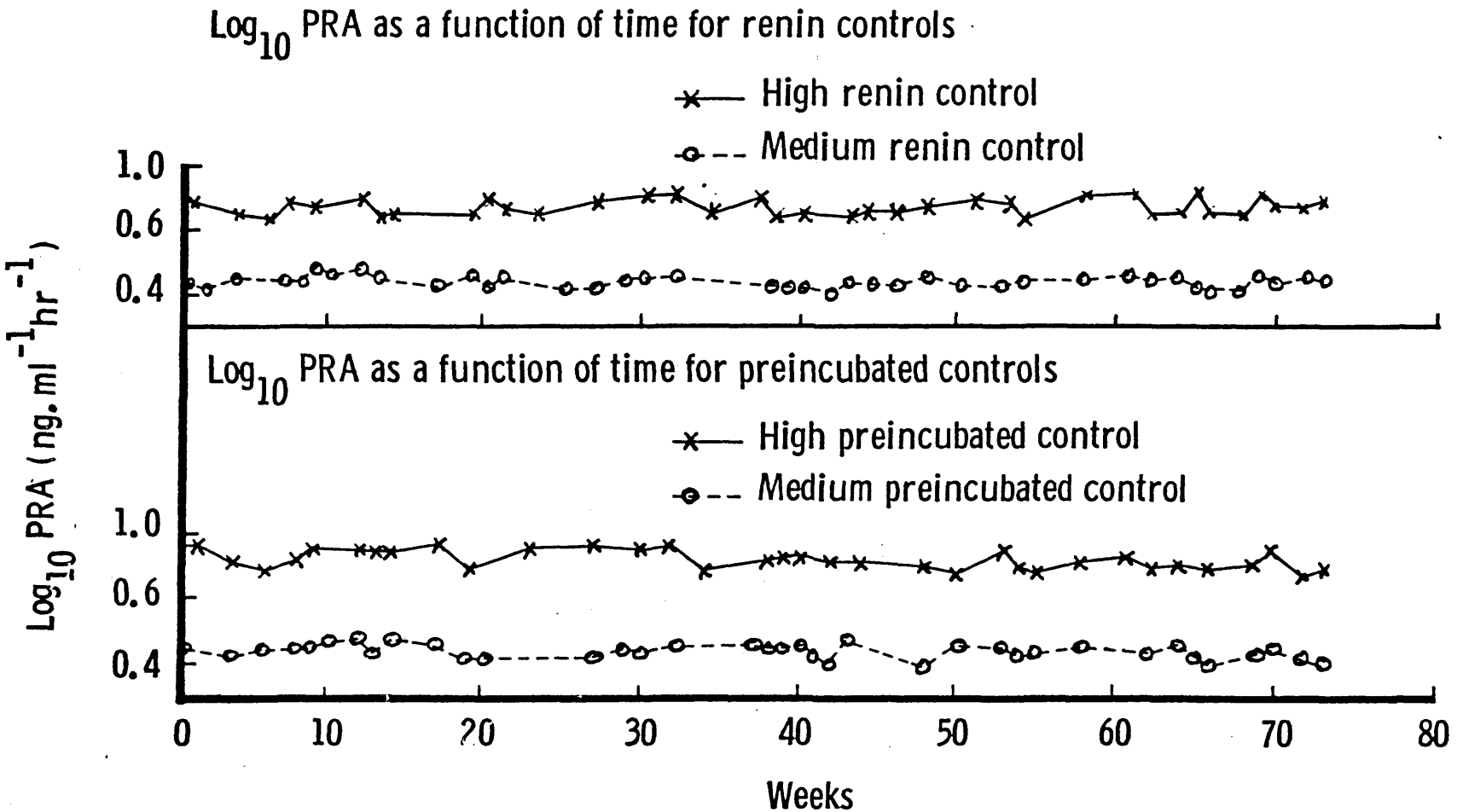
These results show that PRA does not change in samples stored in individual aliquots at -20°C for up to 15 months. This contrasts with the published work of Sealey and Laragh (1975). A major difference between the two studies is that in their experiments the majority of samples appear to have been re-assayed. This would involve plasma samples in serial freezing and thawing, whereas in this study individual aliquots were assayed once only. Osmond *et al.* (1973) found a substantial increase in PRA following storage at 4°C for three days. There is, therefore, always a possibility that an inefficient deep freeze, or temporary deep freeze failure, may cause an increase in PRA. Lijnen *et al.* (1977) who measured total plasma renin concentration biologically by the method of Skinner (1967) and by radioimmunoassay (Lijnen *et al.*, 1976) found no increase in total plasma renin concentration by either method after storage of samples at -20°C for up to one year which indirectly supports the results reported here.

A comparison of the manual and automated radioimmunoassay techniques with respect to assay of PRA

From the data in this chapter it may be seen that in terms of precision, reproducibility, recovery and sensitivity the two assays are virtually identical. This is not, perhaps, any real surprise as the primary reagents (tracer, standard, antiserum) for both assays were from identical sources. Thus the comparison of the techniques is, largely, an academic one and related to such aspects as sample throughput, cost per tube, operator hours per assay, etc., a detailed evaluation of which lies

Figure 11:15

Variation in control values as functions of time.



largely beyond the scope of this thesis. Certainly if PRA evaluation is to become a routine patient or sub-population screening exercise on a large scale as advocated by some authors (Laragh *et al.*, 1979) especially perhaps in the light of angiotensin blocking antihypertensive drugs then an automatic system which caters for large sample numbers is attractive. On the other hand, for small numbers of samples the saving of operator time is minimal and the potential flexibility of a manual system may be more attractive and, of course, involve considerably less capital outlay.

The major point, however, with regard to this study, is that both assays appear to yield directly comparable results over the range under consideration.

CHAPTER TWELVE

ASSESSMENT OF THE ANGIOTENSIN II ASSAYS

Assessment of the angiotensin II assays

The extraction procedure for removing and concentrating angiotensins from plasma was common to both radioimmunoassays in a manner analogous to the angiotensin I generation process being common to both PRA assays. The detailed methodologies have been described in Chapter Eight.

Extraction of angiotensins from plasma

Adsorption on to Dowex resin

As outlined in Chapter Eight the initial removal of angiotensins from plasma was effected by means of mixing the plasma with 400 mg of Dowex-H® resin. The precise weight of Dowex used was not critical as may be seen from the results below where 75 ml of pooled patient plasma was extracted in 15 lots of 5 ml. To five tubes were added 350 mg of Dowex, to a further five were added 400 mg of Dowex and to the final five were added 450 mg of Dowex. After the extraction process all tubes were washed with water and methanol as described and the angiotensins then eluted in two lots of 2 ml of methanolic ammonia as explained in Chapter Eight. They were then dried down and finally reconstituted and assayed for angiotensin II concentration.

The results are shown in Table 12.1. It may be seen from the data that variation of 12.5% in the Dowex weight used did not materially affect relative recovery of angiotensin II in a pool which had an angiotensin II concentration in the upper half of the normal range. (The standard error of the differences being greater than difference between means). In order to save considerable amounts of "weighing time" a plastic tube containing 400 mg of Dowex was carefully scored at the meniscus of the resin and cut. It was then stuck to a wooden spatula (tongue depressor)

Table 12.1

Relative recovery of angiotensin II with respect to varying Dowex resin concentration

	Angiotensin II concentration, $pg.ml^{-1}$		
Wt of Dowex resin / Sample no.	350 mg	400 mg	450 mg
1	36.5	34.4	33.8
2	31.5	32.0	31.4
3	34.0	32.0	30.6
4	41.0	34.4	32.9
5	30.6	31.0	30.7
Mean	32.72	32.76	31.88
S.D.	2.49	1.55	1.41
C.V. (%)	7.61	4.73	4.42

to form a scoop to measure out 400 mg lots of Dowex. The results of 20 aliquots measured by this method and subsequently weighed are given in Table 12.2. From these results it can be seen that the mean was approximately 400 mg and the coefficient of variation was approximately 3.6%. These results indicate that the 'scoop' is precise enough for use in the extraction process. In Table 12.3 is laid out the actual value limits on the samples at various levels of confidence. This does, of course, pre-suppose a normal distribution of values about the mean. It can be seen from Table 12.3 that the distribution appears to be normal, although sample numbers are somewhat limited. It can be seen that one was 98% certain of the sample falling within the upper and lower limit found to be adequate in the previous experiment. Furthermore, it is possible that greater variations in the Dowex-$H^{\oplus}$ concentration could be tolerated so it would appear "safe" to employ the scoop rather than the individual weighing technique.

After a few months of satisfactory use the scoop began to show signs of wear and became loose from its spatula. At this point a new one was made in brass for the author by a member of the Medical Research Engineering Workshop. This was tested for reproducibility and accuracy and found to be equally acceptable.

Recovery of added angiotensin II from plasma

Recovery of angiotensin II was estimated by the addition of 0.05 ml of a 5 $ng.ml^{-1}$ solution of angiotensin II to half of a normal plasma sample previously equally divided into two prior to extraction. As explained in Chapter Eight the difference between the final angiotensin II concentrations in the "spiked" sample and the untreated sample yielded a direct measure of the recovery of angiotensin II.

Table 12.2

Successive sampling of Dowex-H®

Sample no.	Weight (mg)
1	388.9
2	417.2
3	412.5
4	391.0
5	386.5
6	401.1
7	421.3
8	386.9
9	388.6
10	421.4
11	404.3
12	381.1
13	381.6
14	408.4
15	393.3
16	377.2
17	403.8
18	419.9
19	389.7
20	411.1
Mean	399.3
S.D.	14.57
C.V.	3.65%

Table 12.3

Limiting weights of Dowex at varying confidence limits, and the actual and predicted number of samples in each class for a normally distributed population.

Extent of variation from mean (399.3 mg)	Weight range (mg)	Confidence limit	Normal sample distribution (n = 20)	
			Predicted	Observed
1 S.D.	384.72-413.87	67%	13	13
2 S.D.	370.16-428.44	95%	6	7
3 S.D.	355.59-443.01	98%	1	0

Table 12.4 lists the recovery of angiotensin II from eight plasma samples extracted simultaneously with and without added angiotensin II. In addition the results of samples used as extraction controls in 10 successive assays are given to show a batch-to-batch or "inter-extraction" variation. The results show firstly a high degree of consistency and no differences between the intra- and inter-extraction data indicating a high degree of reproducibility in the process. In the light of these results it was felt permissible to use a conversion factor of 89% in assays when no extraction control had been inserted.

Recovery of angiotensin II from whole blood

As a check that the separation of plasma was reproducible and did not lead to possibly discrepant results owing to varying degrees of loss of angiotensin a recovery experiment similar in principle to the previous one was performed.

Twenty ml samples of venous blood were taken into syringes containing 1 ml of phenanthroline inhibitor (the formulation is given in Appendix I). The samples were dispensed into 10 ml EDTA polystyrene tubes. To each sample 50 µl of I^{125}-angiotensin II was added. The tubes were then centrifuged at 4°C and the plasma carefully pipetted and transferred to another tube. All the tubes were then counted in a Wallac 80000 gamma counter for 1 minute. From the counts it was possible to assess the distribution of the angiotensin II and also the reproducibility with which this distribution was obtained. Table 12.5 gives the results obtained on 10 such blood samples. It may be seen that there was a reproducible partitioning of the radioactive angiotensin of 86:14, plasma:PCV. One point of note, however, was that the plasma

Table 12.4

Recovery of angiotensin II from plasma

(a) Within batch

	Untreated sample (pg)	Treated sample (pg)	% Recovery
1	28.3	85.9	92.2
2	49.2	97.0	76.5
3	11.4	69.0	89.0
4	18.6	67.2	77.8
5	10.9	71.0	96.2
6	39.0	96.0	91.2
7	28.9	72.3	69.4
8	58.3	110.9	84.2

n	8
$\bar{x}$	84.56
S.D.	9.24
C.V.	10.93

(b) Between batch

	Untreated sample (pg)	Treated sample (pg)	% Recovery
1	17.1	71.6	87.2
2	6.3	51.4	72.2
3	24.2	79.2	88.0
4	9.6	61.4	82.9
5	31.4	90.8	95.0
6	6.2	59.0	84.5
7	19.1	71.4	83.7
8	28.1	86.1	92.8
9	31.0	95.3	102.9
10	18.0	80.3	99.7

n	10
$\bar{x}$	88.9
S.D.	9.01
C.V.	10.13

Table 12.5

Distribution of ^{125}I-angiotensin II after centrifugation of whole blood and plasma separation

Sample no.	Counts added	Counts in PCV	%	Counts in plasma (volume)	%
1	15502	2293	14.79	13070	84.31
2	14385	1867	12.98	12481	86.76
3	14868	1911	12.86	12833	86.31
4	14811	1941	13.11	12819	86.55
5	15634	1996	12.77	13369	85.51
6	14310	1888	13.19	12402	86.67
7	14803	1907	12.88	12763	86.22
8	15991	2347	14.68	13467	84.22
9	14617	2001	13.69	12333	84.37
10	14719	1961	13.32	12686	86.19
		$\bar{x}$	13.43		85.71
		S.D.	0.74		1.03
		C.V.	5.5%		1.2%

volume to packed cell volume (PCV) approximated to unity in all the samples used. Whether or not a higher percentage recovery would be obtained in the plasma of low PCV such as may be the case in renal failure is uncertain. Interpretation of these data is, empirically, simple. The partition appears to be reproducible and of no cause for concern in terms of source of error in the assay system. However, whether the labelled angiotensin which is in the cell fraction represents a biologically active fraction or just a non-specifically adsorbed percentage of "waste" is not known.

It is not usual to equate plasma (or serum) hormone (or drug) levels with their corresponding whole blood equivalents. It is not perhaps necessary to consider it in the case of direct plasma or serum assays in which one defines the criteria and medium of measurement of the analyte without gross chemical perturbation. In the PRA assay one generates the analyte from plasma. For angiotensin II (and also bradykinin), however, the analyte is removed totally from its native medium and therefore it is perhaps useful to know at which stages and to what extent losses may occur.

Efficiency of inhibitor

The use of an inhibitor mixture of phenanthroline, EDTA and neomycin was intended to perform a dual function. The inhibitor should prevent both angiotensin II formation and its further metabolism so that the subsequent extraction and radioimmunoassay results in a value which reflects the angiotensin II concentration in circulating blood. The first function of the inhibitor, that is, inhibition of synthesis of angiotensin II, was directly assessed by measurement of angiotensin converting enzyme activity levels (by the technique described in Chapter Nine and assessed in Chapter Thirteen) in the samples taken for

angiotensin extraction and radioimmunoassay.

No samples taken into phenanthroline as described had measurable ACE activity even after overnight incubation at room temperature, indicating that the inhibitor effectively blocked angiotensin II synthesis. Angiotensin II degradation could not be directly monitored enzymically as it is not a mono-specific pathway. However, having established that synthesis is blocked it was possible to look at the stability of added angiotensin II in inhibited plasma with respect to time and interpret any decrement in concentration as being due to catabolic enzyme action.

100 ml of blood was collected from a normal volunteer and the plasma was pipetted, pooled and then divided into ten 5 ml aliquots. To eight of these aliquots 50 µl of a 5 $ng.ml^{-1}$ angiotensin II solution ($^{1}Asp^{5}Ileu$) was added. Four of the eight and one of the untreated two were then immediately frozen at -20°C. The other five (4 + 1) samples were left for 2 hours at room temperature and then frozen and stored with the others at -20°C. All ten samples were extracted as a batch, dried down, reconstituted in buffer and radioimmunoassayed.

The results in $pg.ml^{-1}$ of plasma of angiotensin II for the ten samples are shown in Table 12.6. In general terms they may be seen to corroborate the recovery study (Table 12.4). Both groups ('0' and '2') yield between 82 and 84 per cent of the added angiotensin with respect to their untreated sample. The last column shows the angiotensin concentration at 2 hours expressed as a percentage of that assayed from a sample frozen immediately post plasma separation. The mean relative recovery can be seen to approximate to 100%.

The mean percentage recovery after 2 hours is lower than that at time zero. However, as may be seen below this is not significant.

Table 12.6

Stability of angiotensin II *in vitro* in the presence of phenanthroline

Hours at room temp.		0	0	2	2	Relative recovery: $\frac{\text{conc. 2}}{\text{conc. 0}} \times 100$
Samples	AII (pg) added	pg.ml^{-1} AII	% Recovery	pg.ml^{-1} AII	% Recovery	
1 and 6	0	27.0	-	29.2	-	108.1
2 and 7	250	79.0	83.2	73.8	71.4	93.4
3 and 8	250	88.3	98.1	81.4	83.5	92.2
4 and 9	250	78.2	81.9	90.1	97.4	115.2
5 and 10	250	72.4	72.6	77.7	77.6	107.3
n			4		4	5
$\bar{x}$			84.0		82.4	103.2
(σ) S.D.			10.6		11.2	10.0
C.V.			12.6		13.6	9.7
(σ^2) Variance			112.4		125.4	100.0

Difference between sample means $(x_1 - x_2) = (84 - 82.4) = 1.6$. Also variance of the difference $= \frac{\sigma_1^2}{n1} + \frac{\sigma_2^2}{n2} = \frac{112.36}{4} + \frac{125.44}{4} = 59.45$. The standard error of the difference is the square root of the variance of the difference $= \sqrt{59.45} = 7.71$. Thus we can see that the observed difference between means is not significant being about one-fifth the value of its standard error.

Whilst it would always be unwise to place great emphasis on tests of significance performed on only eight samples, such a large degree of difference between the observed means and the standard error of the difference is highly indicative. From these results it may be concluded that the EDTA-phenanthroline mixture prevents signficant angiogensin II catabolism for at least 2 hours; more than sufficient time to thaw the samples and adsorb the angiotensin. Furthermore, as ACE activity was undetectable over this time period, and longer, it would appear to be an effective inhibitor of synthesis as well as breakdown of angiotensin II.

Assessment of the radioimmunoassays for angiotensin II

The radioimmunoassay of angiotensin II has posed problems for a number of years. The major problem has been that in order to measure plasma concentrations of 5 $pg.ml^{-1}$ or below an antiserum of extremely high affinity would be required. The majority of antibodies in current usage appear to have K values of the order of 10^{10} liters per mole, which is not sufficient. Attempts to counteract this insensitivity by increase in sample size have, however, been offset by the introduction of "plasma effects" due to interfering substances in plasma being present at significant levels to affect the true equilibrium. Thus the solution has been, as outlined earlier, to extract and concentrate angiotensins from plasma prior to assay. Whilst this is a laborious and tedious procedure it does have some advantages. The most obvious advantage and the prime purpose of the exercise is to concentrate angiotensin II up to a point where it can be measured by conventional radioimmunoassay. Secondary to this is the advantage that, with the exception of other similar peptides, all other plasma components are effectively removed and upon reconstitution of the dried extract one has, in effect, a homogeneous buffer-based system which is, in principle, identical to the standards with which it is to be compared. However, since Dowex is not specific for angiotensin II it was felt necessary to check by means of recovery and parallelism studies that no other small interfering peptides had been so far concentrated by the extraction as to pose problems. Precision and sensitivity of the assays are dealt with as for the PRA assay, although it must be realised that sensitivity refers to the limits of the standard curve, whereas the final arbiter of the ability to measure samples can rest with the plasma volume initially obtained, the volume of reconstitution and volume of sample assayed.

Precision of the radioimmunoassays

The working definition used in this work was that of Ekins and Newman (1970) in which the variation in dose interpolated (Δx) was set as a function of the slope of the dose response curve $\frac{dy}{dx}$ at that point divided into the variation in the response metameter, Δy.

Ten consecutive manual assays, and ten consecutive automated assays, were used in the estimation of precision and senitivity in the same way as outlined in the previous chapter with respect to angiotensin I in the PRA assays. It should be noted that the values shown in Table 12.7 refer to 100 µl aliquots of standard in both cases. Whilst it may be seen that the values for precision from manual assay to automated assay are similar, it must be remembered that only half the sample size was possible (i.e. 50 µl) with respect to samples in the automated assay which effectively doubled the working cut-off of the system.

Sensitivity

In view of the smaller sample size in the automated system, the sensitivities of the assays, whilst similar as judged from the previous experiment (see foot of Table 12.7), were in fact disparate by a factor of two in favour of the manual system assuming all else (plasma volume and reconstitution volume) to be identical. Since the physiological range of angiotensin II made it necessary to measure very low plasma concentrations (5 $pg.ml^{-1}$ and below) this effectively meant that assuming 90% recovery from 5 ml of plasma one had 22.5 pg reconstitued in 400 µl of buffer. In the manual assay this yielded 5.625 pg which was estimable, whereas in the automated assay this yielded 2.81 pg per tube which was at the limit of detectability. As a result of this rather severe limitation, imposed by the computer programme of the Kemtek 3000 not the design of the machine as such, automated angiotensin II radioimmunoassay was not

Table 12.7

Precision of angiotensin II assays

Standard concentration pg.tube^{-1}	Precision pg.tube^{-1} (± ISD) Manual n = 10 assay	Automated n = 10 assay
1000	28,30 (7,61)	31,65 (6,14)
500	18,11 (4,88)	17,02 (3,90)
250	14,34 (3,91)	12,69 (4,12)
125	11,77 (2,26)	12,19 (2,99)
62,5	6,86 (1,58)	7,21 (1,83)
31,25	5,40 (1,38)	4,90 (1,41)
15,625	3,96 (1,27)	3,62 (1,33)
7,8125	3,66 (1,14)	3,64 (1,29)
3,90625	2,77 (1,11)	2,69 (1,61)

routinely employed or studied to the same extent as either the manual angiotensin II assay or the automated PRA assay.

Specificity

Cross reactivity

As may be seen from the assessment of the anti-angiotensin II serum in Chapter Six, there was considerable and varied cross reaction with a variety of other peptides within the metabolic pathway. However, these figures appear to be typical for other antibodies in common usage (Morton *et al.*, 1976). Therefore such results for angiotensin II as are interpolated from radioimmunoassays must be regarded as an index of angiotensin II concentration rather than a true *in vivo* concentration. The Scatchard data indicated the presence of only one class of binding site with respect to angiotensin II, therefore there did not appear to be any point in attempting further fractionation of the serum to get differentiated activities. It seemed from these data that the binding of angiotensin and analogues were likely to be to this one class of site with differing affinities, as it would be unlikely that analogues and degradation products were bound to a separate class of sites which had no appreciable binding of angiotensin II itself.

Recovery of angiotensin II

The recovery of angiotensin II added to plasma indicates the degree and consistency of the recovery over the whole (extraction plus radioimmunoassay) procedure. It was to be expected that such losses and inaccuracies as were found would be largely due to the extraction process, since by the radioimmunoassay stage the angiotensins were in buffer and should therefore be homogeneous with the standards. However, to check

for the recovery of added angiotensin II from buffer a series of "samples" were made by dissolution of angiotensin II (^{1}Asp^{5}Ileu) in buffer to yield a set of known value artificial samples. These were then assayed as unknowns and the percentage recovery calculated.

Twenty polystyrene tubes were numbered sequentially. To tubes 1 to 19 950 µl to 50 µl (by 50 µl decrements) of buffer were added. To all tubes the requisite volume of a 1 ng.ml^{-1} solution of angiotensin II was added to yield a final volume of 1 ml (that is 50 µl to tube number 1 up to 1000 µl to tube number 20). The tubes were vortex mixed and 100 µl aliquots assayed in triplicate in the manual radioimmunoassay. It can be seen (Table 12.8) that recovery, estimated by the mean of the triplicate value interpolated divided by the concentration per tube calculated to be present and multiplied by 100, was effectively complete and reproducible over the range (5-100 pg.tube^{-1}) covered.

Parallelism

The parallelism of doubly diluted samples with respect to the standard curve was assessed. The main reason for doing this was not, as in the PRA assay, to check against plasma interference as the assay was, as explained previously, totally buffer-based. The major thing to check was the comparability, immunologically, of the standard angiotensin II (^{5}Val)-amide with human angiotensin II (^{5}Ileu). Indications from the previous experiment were that this comparability was of a high order.

Three extracted plasma samples from subjects on low sodium diets (10 mmol for 5 days) were reconstituted in 800 µl of buffer. These were then doubly diluted (400 µl plus 400 µl of buffer) four times to each to yield five aliquots for assay. As well as the above, a standard of human angiotensin II (^{5}Ileu) of concentration 10 ng/ml was doubly diluted eight-fold to yield nine comparable standards to the "hypertensin"

Table 12.8

Recovery of angiotensin II in the manual radioimmunoassay

	Concentration in pg.tube^{-1}		
	Angiotensin added	Angiotensin recovered	% Recovered
1	5	5.2	104.0
2	10	9.6	96.0
3	15	14.0	93.3
4	20	22.0	110.0
5	25	24.4	97.6
6	30	31.2	104.0
7	35	34.8	99.4
8	40	40.0	100.0
9	45	45.2	100.4
10	50	40.6	81.2
11	55	55.7	101.3
12	60	62.6	104.3
13	65	68.6	105.5
14	70	69.9	99.9
15	75	78.8	105.1
16	80	73.4	91.8
17	85	84.4	99.3
18	90	88.6	104.2
19	95	101.0	106.3
20	100	103.4	103.4
		n	20
		$\bar{x}$	100.4
		S.D.	6.38
		C.V.	6.35%

(Val^5-amide) standards. After the "ex-plasma" samples and the human angiotensin II (^{5}Ileu) standards had been assayed as samples on the manual assay they were plotted after a log-logit transformation to linearise the curves. Linear regression analysis was performed on each curve individually to establish that most of the variance was, in fact, explained by regression and then variance ratio tests were done with one standard against the other and each "plasma" sample against both standards.

The results shown in Figure 12.1 and Table 12.9 indicate that the use of the analogue substrate angiotensin II-amide (^{5}Val) does not lead to loss of parallelism with the samples and that the two standards dilute parallel with respect to each other.

Comparability of manual and automated radioimmunoassays

In order to assess the direct comparability of the two assays reconstituted extracts were assayed in parallel in both systems. The samples were reconstituted in 400 µl of buffer thus, in order to do two assays on the extract, duplicate, as opposed to triplicate, estimations were done in both of the assays.

Twenty-five extracts were reconstituted each in 400 µl of buffer and assayed on the 'Kemtek' in duplicate. The remaining volumes of sample were then used in a manual assay set up in parallel. It was necessary to do the automated assay first to ensure that the tip of the sampling probe was immersed in the solution prior to drawing the required aliquot. Although the theoretically necessary volume required for dilution-2 estimations was only 100 µl for a duplicate and 150 µl for a triplicate it was necessary to have a greater volume than this present to ensure no air was taken up. It was considered, briefly, that it may have been possible to reconstitute extracts of 200 µl (instead of the

Figure 12:1

Parallelism study for the manual angiotensin iI assay.

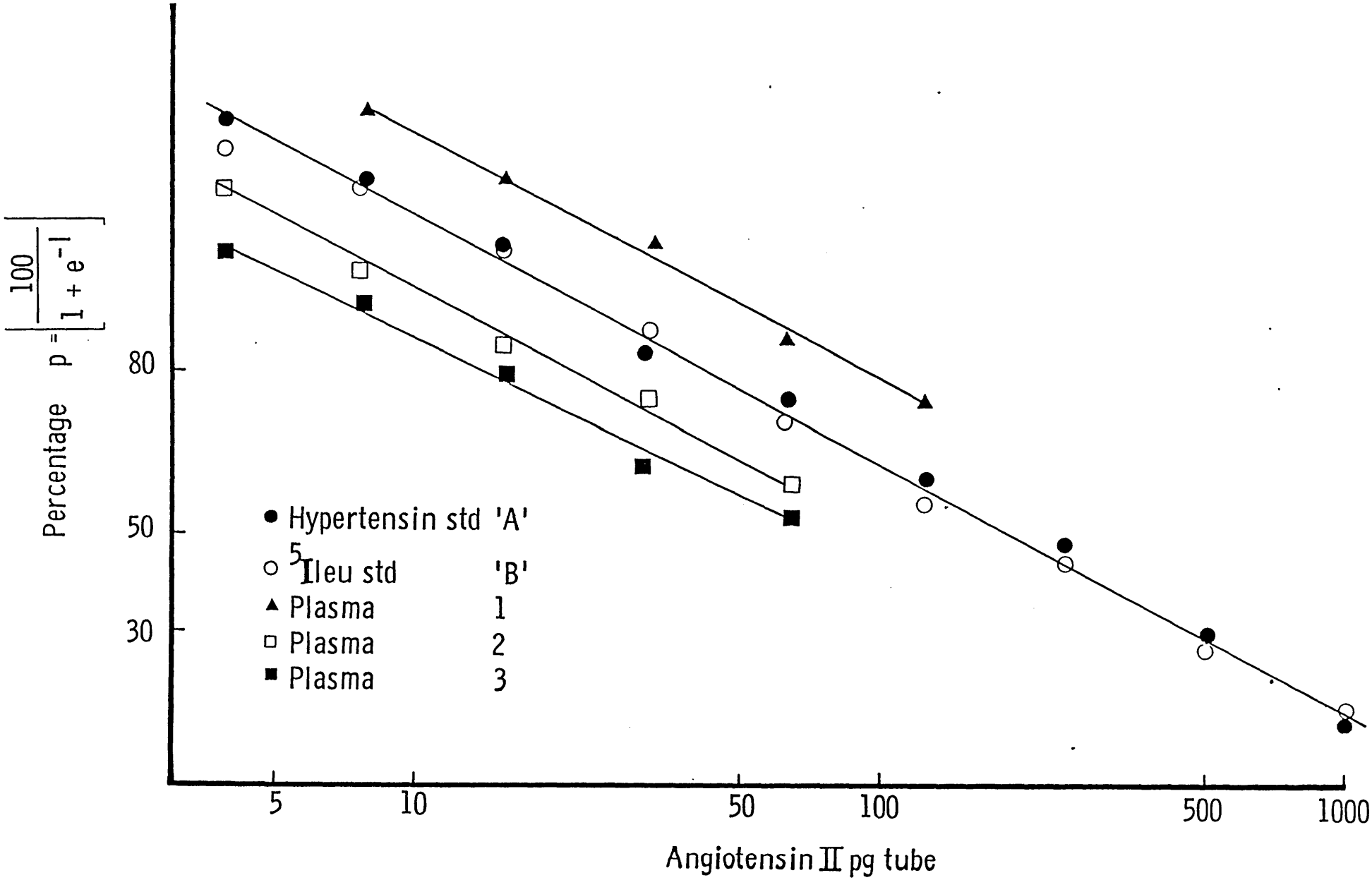


Table 12.9

Results of regression analyses and variance ratios

(a) Regression analyses

	Standard A	Standard B	Plasma 1	Plasma 2	Plasma 3
n	9	9	5	5	5
r	-0.9988	-0.9978	-0.9980	-0.9978	-0.9964
m	-2.1444	-2.1029	-2.1328	-2.0352	-1.9327
b	4.9066	5.7592	5.5845	4.1215	3.5915

(b) Variance ratios with respect to the slope of the lines

Lines compared	F Ratio	Significance
Standard A vs Standard B	$F^1_{14} = 0.3801$	p >0.05
Standard A vs Plasma 1	$F^1_{10} = 0.0128$	p >0.05
Standard A vs Plasma 2	$F^1_{10} = 1.1784$	p >0.05
Standard A vs Plasma 3	$F^1_{10} = 3.9885$	p >0.05
Standard B vs Plasma 1	$F^1_{10} = 0.0487$	p >0.05
Standard B vs Plasma 2	$F^1_{10} = 0.2845$	p >0.05
Standard B vs Plasma 3	$F^1_{10} = 1.6969$	p >0.05

customary 400) to overcome the problem of using a double diluted sample but 200 µl yielded a meniscus base only 3 mm from the centre of the bottom of the tube which left no real margin for error in the probe height. The results of the parallel assays are shown in Figure 12.2. The results of the regression analysis are given in Table 12.10. From this table it can be seen that the results have been analysed in two different ways. Column A indicates the results when all 25 pairs of values were included. Column B indicates the results when one pair was excluded. The pair excluded in the second analysis yielded values of 3.6 $pg.ml^{-1}$ from the manual assay and an undetectable level in the automated assay. In order to include the pair an arbitrary value of 0.01 was assigned to the undetectable result since zero may not be entered into the calculator regression analysis programme. (A reciprocal of the variable is taken during the data processing and "0^{-1}" = ∞). In order to ensure that such an arbitrary designation did not materially affect the analysis the whole was repeated without that particular pair of points. It may be seen that virtually identical correlations and slopes were obtained. The intercept from analysis 'A' was significantly less than 0, whereas it was not in 'B'. This was obviously due to the weighting of the extra pair of values (3.6 - 0.01). In real terms, of course, the fact that y was undetectable meant only that in this assay it was less than 3.9 pg.tube (≡ 3.44 pg.ml of plasma at 89% efficiency of an extraction volume of 5.1 ml). Therefore assignation of a precise value over 100-fold lower than the assay cut-off would, of necessity, tend to alter the weighting of the regression at the low levels and hence significantly shift the intercept to a negative value. The regression line in Figure 12.2 shows all 25 points and an ideal line m = 1.0, b = 0.0, these values for m and b being virtually indistinguishable from the results when n = 24.

Figure 12:2

Comparison of the manual and automated angiotensin II assays.

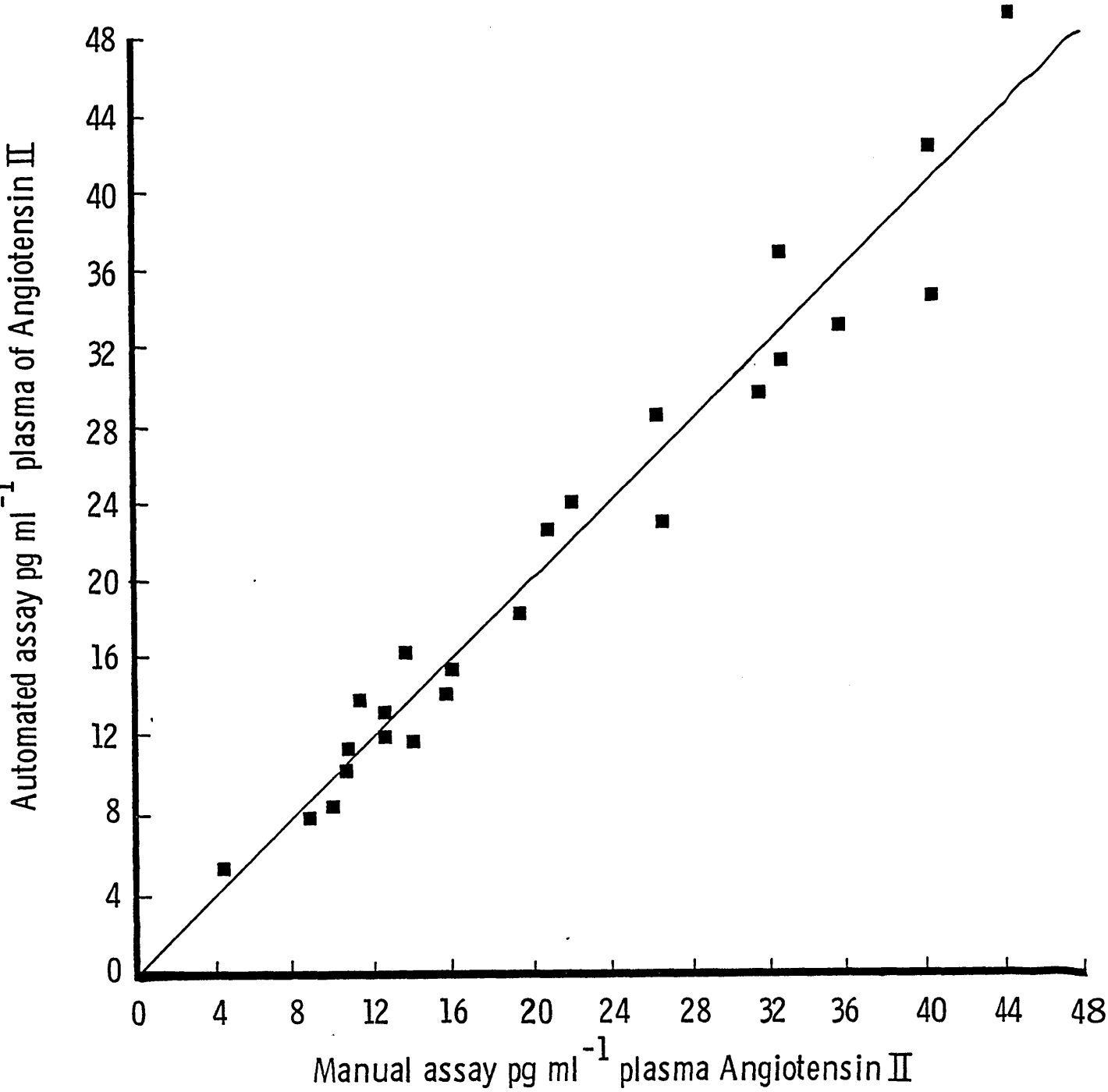


Table 12.10

Regression analysis for comparison of assays.

	A	B
n	25	24
r	0.9797	0.9786
Slope, m	1.007	0.9899
Intercept, b	-0.5067	-0.0044
Σxy	14029.296	14029.26
Σy	508.41	508.40
Σy^2	14022.5001	14022.5000
$\overline{x}$	20.692	21.404
σx^2	12.048	11.7576
$\overline{y}$	20.3364	21.183
Σx	517.3	513.696
Σx^2	14187.81	14174.85
σy^2	12.384	11.893
p	<0.001	<0.001

Reproducibility

The reproducibility of the assays was assessed by monitoring the quality control samples on an intra-assay and inter-assay basis. An estimate of intra-assay reproducibility was obtained by triplicate estimation of ten quality control samples of each group (10, 25 and 50 pg.tube^{-1}) in each assay.

Inter-assay estimation was done by analysing the results of one triplicate estimation of a fresh sample from each group over ten sequential assays. The results are summarised in Table 12.11. It can be seen that the coefficient of variation decreases as the nominal dose increases. This is to be expected as the precision of estimation expected at 50 pg.tube is better than at 10 or 25. This is noticed in both intra- and inter-assay results. Furthermore, this decrease in C.V. is seen in both the manual and automated assays which, with one exception, yield similar variances. The one obviously different result is for the low (10 pg.tube^{-1}) pool on an automated assay. For both intra- and inter-assay results, but especially the latter, there was a much higher degree of variation in the estimation of the low control sample. These values, 8.68 and 15.1%, were significantly higher than their corresponding manual assay values of 5.53 and 9.34% respectively. These discrepant results were due presumably to the dilution sampling of the machine assay which effectively transferred 50 µl of sample not 100 µl. Thus the interpolation from the automated assay would be at about 94% bound on the standard curve compared with say 90% on the manual assay curve. This is a considerable change, especially when one considers 95% to be the sensitivity limit of a dose-response curve in many protein binding assays.

Taken overall the results provide indications that the reproducibility as assessed by quality control data is good for both assays. The one possible doubt is the accuracy of low value sample interpolation

Table 12.11

Reproducibility of angiotensin II assays

(a) Intra-assay results

Nominal value pg.tube^{-1}	No. of estimations	Manual assay			Automated assay		
		Mean	Std.dev.	C.V.%	Mean	Std.dev.	C.V.%
10	10	10.58	0.585	5.53	10.84	0.941	8.68
25	10	24.78	1.407	5.68	26.09	1.342	5.1433
50	10	50.91	2.009	3.95	49.80	2.29	4.596

(b) Inter-assay results

Nominal value pg.tube^{-1}	No. of estimations	Manual assay			Automated assay		
		Mean	Std.dev.	C.V.%	Mean	Std.dev.	C.V.%
10	10	10.26	0.948	9.34	10.81	1.631	15.1
25	10	24.81	2.091	8.43	25.16	2.188	8.70
50	10	51.14	3.036	5.94	50.72	2.916	5.75

from the automated assay, as discussed earlier in this chapter under the heading of sensitivity.

The results from the experiments performed in this chapter indicate that the extraction of angiotensin II from plasma and its radioimmunoassay is a precise reproducible assay system. The results compare well with other described assay systems (Morton *et al.*, 1976). The major limitation on the assays for angiotensin II is the sensitivity limit. Whereas, for example, the PRA assays are capable of measuring values of 10% of those of the bottom of the normal range, neither of these assays for angiotensin II, or indeed any other so far described, can monitor angiotensin II concentrations much below the bottom of the normal range (5 $pg.ml^{-1}$ plasma) using reasonable sample sizes. It is this severe restriction, coupled with the laborious nature of the extraction procedure, which limits the clinical usefulness and prevents application of such assays to routine investigation of blood pressure in most laboratories. It would, therefore, be of great advantage to establish that the PRA assay could be used as a reliable index of plasma angiotensin II concentration as reflected by the assays described in this chapter.

CHAPTER THIRTEEN

ASSESSMENT OF THE RADIOASSAY FOR ANGIOTENSIN CONVERTING ENZYME ACTIVITY

Assessment of the Radioassay for Angiotensin Converting Enzyme

The radioassay used was based upon the method of Ryan *et al.* (1977). The tritiated substrate (p^3-H-benzoyl-glycylglycylglycine) and a guinea pig control serumof nominal value 150 ± 20 units (nmol. min^{-1},ml^{-1}) were kindly donated by Mr. R.N. Piasio of Ventrex Laboratories, Portland, Maine, U.S.A. The principle of the assay as outlined earlier lies in the specific hydrolysis of the substrate to yield a product of ^{3}H-hippurate which partitions selectively in ethyl acetate: 0.1 M HCl to the ratio of 91:9, whereas unhydrolysed substrate partitions 7:93 (Cushman and Cheung, 1971) in the same solvents. Thus it can be appreciated that the stability of the substrate and the selectivity of the hydrolysis are of paramount importance if the assay is to be a specific monitor of ACE activity.

Substrate stability

Substrate stability was assessed in each assay. This was achieved by incubating the substrate in the absence of a plasma sample or control but with only 50 µl of diluent (HEPES) buffer for 1 hour at 37°C prior to the addition of 1 ml of 0.1 M HCl (to stop the reaction), and 1 ml of ethyl acetate (to partition substrate from product). The results of such "enzyme blanks" are shown in Table 13.1 and from these it is apparent that no progressive hydrolysis of substrate occurs over its recommended shelf life of ten weeks, and that the level of substrate invasion of the organic layer appears to be a constant of 4 to 5%.

Specificity of substrate cleavage

Ryan *et al.* (1977) have reported that, upon silica-gel chromatography of the organic phase of samples from normal volunteers in Butan-1-ol/ ethyl acetate/acetic acid/water (1:1:1:1 v/v), all the radioactivity was

Table 13.1

Stability of substrate as assessed by enzyme blanks

	Counts added (300 sec counts)	Counts in 300 µl of ethyl acetate	% of counts in total ethyl acetate	Background count
Week 1	117048.5	1599.2	4.55	26.1
2	118036.2	1648.4	4.66	25.4
3	121114.7	1642.7	4.52	30.2
4	118912.3	1515.3	4.25	28.6
5	119262.0	1699.2	4.75	22.2
6	116864.2	1450.6	4.14	26.1
10	116875.7	1553.0	4.43	29.9

associated with hippurate (R_F = 0,71) and unhydrolysed substrate (R_F = 0,49). No radioactivity was found associated with benzoic acid (R_F = 0,82) or hippurylglycine (R_F = 0.59). These results suggest that other enzymes did not interfere in the reaction. They repeated these experiments with samples from patients with active sarcoidosis and obtained identical results. Thus the alternative routes of hydrolysis do not appear to be manifest *in vitro*.

^{3}H-Benzoyl-glycyl-glycyl-glycine	Substrate, R_F 0.49
① ② ③	Possible points of cleavage
^{3}H-Benzoate + glycyl-glycyl-glycine	Product ① R_F 0.82
^{3}H-Benzoyl-glycine + glycyl-glycine	Product ② R_F 0.71 (by ACE)
^{3}H-Benzoyl-glycyl-glycine + glycine	Product ③ R_F 0.59

However, unfavourable partitioning could account for this rather than specific cleavage at point ② above. If this is so, that is only the product of ACE hydrolysis partitions in ethyl acetate, the assay is just as valid as if it were monospecific.

The specificity of the reaction was further examined by the use of two specific ACE inhibitors, bradykinin-potentiating peptide 9α (also known as SQ 20881 or "teprotide") which was derived from the venom of the South American Pit Viper *Bothrops jararaca* (Ondetti *et al.*, 1971 and for review see Bahkle, 1974) and an orally administrable converting enzyme inhibitor D-3-mercapto-2-methylpropanoyl-L-proline ("Captopril" or SQ 14225) (see Ondetti *et al.*, 1977).

Plasma samples were taken into heparinised tubes and stored at -20°C prior to assay. Samples were taken from patients and volunteers before treatment with ACE inhibitors and whilst undergoing treatment with either of the inhibitors. The results from the patients and volunteers undergoing therapy were expressed as a percentage of their pre-treatment ACE

value. The initial pilot study is summarised in Table 13.2. The results can be seen to be in apparent conflict. Teprotide appeared to totally inhibit ACE activity in the two volunteers studied, but Captopril appeared to have no effect either in one of the volunteers or the two hypertensive patients studied. However, when Captopril was added to these apparently uninhibited samples ACE levels became undetectable. Since these samples had been stored at -20°C for about two months prior to assay it was possible that the inhibition of angiotensin converting enzyme activity by Captopril had diminished with storage. Therefore ACE activity was measured in samples from both normotensive volunteers and hypertensive patients before and during treatment with Captopril. Aliquots of samples taken when the subjects were undergoing Captopril therapy were assayed within 4 hours of sampling and at timed intervals thereafter, each aliquot being stored at -20°C prior to assay. The results (shown in Table 13.3) showed that when the samples were assayed within 4 hours of taking the blood there was no detectable ACE activity irrespective of the dose of Captopril administered (25 mg tds to 150 mg tds). Progressive deinhibition was seen as the time of storage increased. Samples assayed within 24 hours of storage showed detectable ACE activity and activities rose upon more prolonged storage until, after more than 40 days, they approached control levels. These results cannot be explained by non-specific substrate hydrolysis since the pre-Captopril levels do not increase with time. The only tenable explanation is that Captopril is a potent inhibitor of ACE activity, even at low doses, but that the plasma must be assayed within a few hours of sampling as the inhibition is reversible *in vitro*. Despite the obvious problems of assaying samples from subjects receiving Captopril these results do add further proof of the specificity of the assay for ACE.

Table 13.2

Inhibition of ACE activity by Teprotide and Captopril; Pilot study

		nmol/ml/min
Patient MN	Assay 17/1/79	
Date 11.10.78	Pre Captopril	78
11.10.78	2 hours post 25 mg Captopril	78
12.10.78	24 hours post 25 mg tds Captopril	76
18.10.78	Captopril 25 mg tds one week	81
24.10.78	Captopril 50 mg tds one week	81
2.11.78	Captopril 100 mg tds one week	82
9.11.78	Captopril 150 mg tds one week	79
Patient KL	Assay 17/1/79	
24.10.78	Pre Captopril	123
24.10.78	2 hours post 25 mg Captopril	126
30.10.78	Captopril 25 mg tds one week	129
7.11.78	Captopril 50 mg tds one week	131
13.11.78	Captopril 100 mg tds one week	125
20.11.78	Captopril 150 mg tds one week	124
Volunteer PS	Assay 29/3/79	
22. 1.79	Pre Captopril, 8th day of normal sodium diet balance	131
26. 1.79	Captopril 100 mg, 5th day	133
2. 2.79	Off Captopril, 8th day	129
2. 2.79	1 hour post Teprotide 1 mg/kg	8
Volunteer JG	Assay 29/3/79	
2. 2.79	Off treatment	99
2. 2.79	1 hour post Teprotide 1 mg/kg	6.8

Table 13.3

Increase in angiotensin converting enzyme activity with storage time in plasma samples from subjects taking Captopril

Age of sample	Angiotensin converting enzyme activity expressed as a percentage of the pre-Captopril value (± ISD)
<4 hours	0.99 ± 1.39 (n = 5)
18-24 hours	4.62 ± 1.38 (n = 4)
1-20 days	41.0 ± 21.0 (n = 7)
21-40 days	62.6 ± 24.0 (n = 7)
>40 days	98.5 ± 9.4 (n = 23)

Determination of the normal range

The most widely used assay for ACE activity is the fluorimetric assay of Friedland and Silverstein (1976) and it was to this assay that the radioassay was compared. Samples from normal volunteers and patients with active sarcoidosis were measured by the fluorimetric technique by Dr. Roger Bird and his colleagues at the Royal Northern Hospital, Holloway Road, London, U.K. The samples were then assayed by the radioassay method by this author and the results compared by regression analysis. This series of experiments fulfilled two purposes. Firstly it enabled the normal range to be established with direct reference to the range quoted by Bird and colleagues and secondly it enabled us to check that the two assays were 'linear' with respect to each other over their working ranges; since different analogue substrates were used it was, of course, always possible that non-comparable results could be obtained. The results are shown in Figure 13.1. For the 20 samples $r = 0.98$, $m = 0.53$ and $b = -21.5$ ($p = <0.001$). A normal range of 70 to 150 units was taken by interpolation of the upper and lower limits quoted for the fluorimetric assay (16 to 60 units).

Assay reproducibility

The reproducibility of the radioassay was monitored by three quality controls; Two of these were aliquots of normal volunteers' plasmas yielding differing normal range values. The third was a guinea pig serum obtained from the Ventrex Corporation, Portland, Maine, U.S.A. The guinea pig serum had a nominal value of 150 units which placed it at the upper limit of normal for our volunteers. Table 13.4 shows the intra-assay and inter-assay performance with respect to these three controls.

Figure 13:1 Determination of converting enzyme by radioassay and fluorimetric techniques.

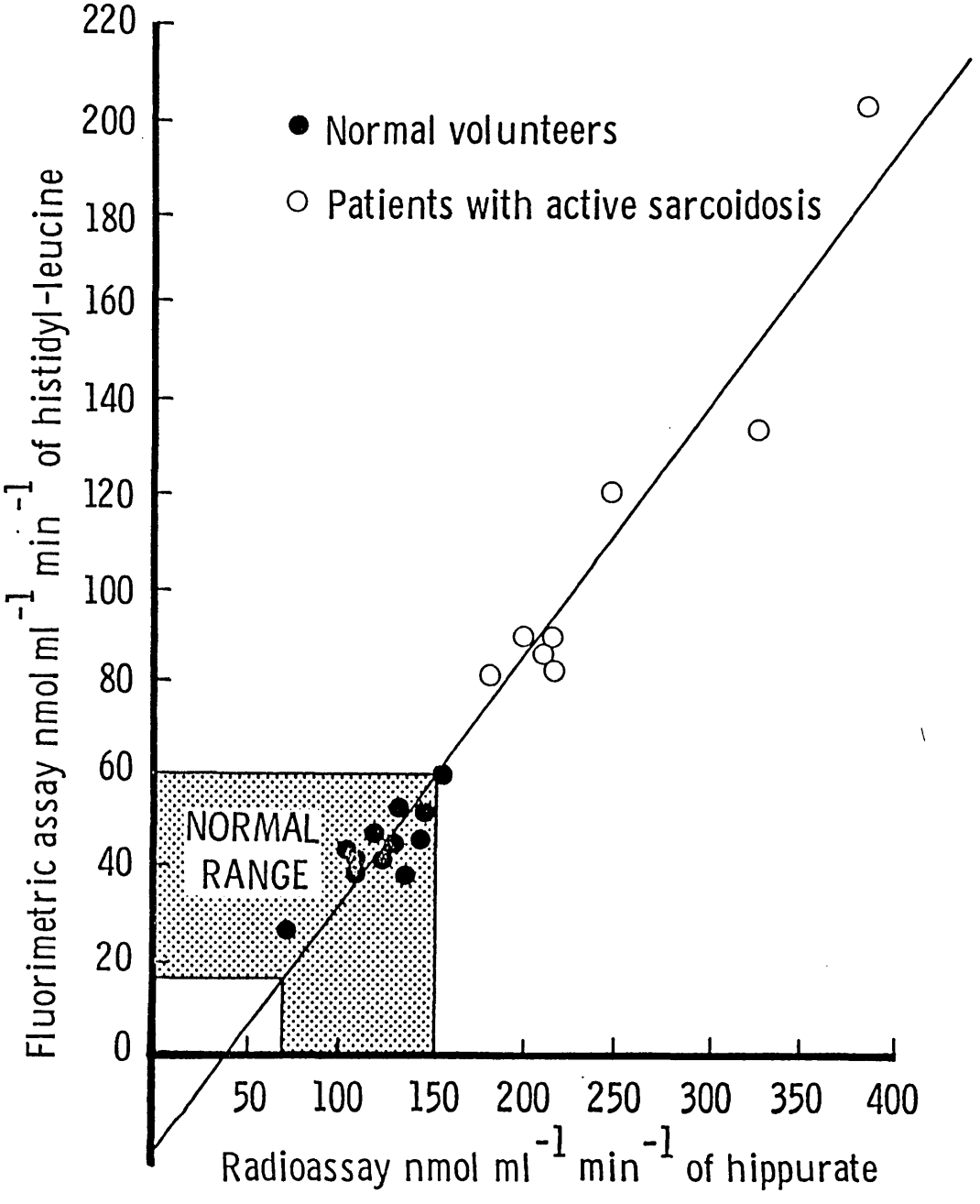


Table 13.4

Reproducibility of the radioassay for ACE activity

(a) Intra-assay

Control	n	Mean ($n.mol.ml^{-1}.min^{-1}$)	Standard deviation	C.V. (%)
Ha	5	63.1	4.1	6.5
Hb	5	108.4	71.6	7.0
Guinea pig	5	159.9	10.2	6.4

(b) Inter-assay

Control	n	Mean ($n.mol.ml^{-1}.min^{-1}$)	Standard deviation	C.V. (%)
Ha	12	65.1	7.7	11.8
Hb	12	110.7	12.4	11.2
Guinea pig	15	162.0	18.7	11.5

These results show that the assay performs reproducibly over the normal range of ACE activity. Further investigation of estimation of elevated ACE activities such as those encountered in active sarcoidosis was not relevant to the use of the assay in regard to blood pressure research. Furthermore the excellent correlation with the fluorimetric technique indicates their validity in ACE monitoring. Also the radioassay was found by Ryan *et al.* (1977) to correlate very well ($r = 0.9$ $p < 0.001$) with the assay of Cushman and Cheung (1971) as modified by Lieberman (1975). The linearity of the correlations between the various analogue substrates provide strong, albeit indirect, evidence that the assays are monospecific for ACE.

Possibly the most important single finding of this study of ACE activity however was with regard to Captopril. Measurement of ACE inhibition by Captopril as a monitor of either patient compliance or of its clinical usefulness and possibly other "second generation" drugs of its type must be carefully assessed. It has been shown here that the drug does not irreversibly inhibit ACE *in vitro* and therefore studies which attempted to interpret ACE levels in patients receiving Captopril must be re-examined critically. Furthermore, any new drug of its class (that is ACE blocker) should be assessed for *in vitro* stability before studies of the drug using ACE assays as a monitor are attempted. If other such drugs are found to be stable in this context or indeed if the decay of inhibition with time can be corrected for, chemically or mathematically, then ACE assay could become a useful tool in investigation of the control of blood pressure and patient compliance to therapy.

CHAPTER FOURTEEN

PLASMA RENIN ACTIVITY AS A FUNCTION OF PLASMA ANGIOTENSIN II CONCENTRATION

As intimated in the conclusion to Chapter Twelve, the plasma angiotensin II assay has a limit of sensitivity at the bottom end of the normal range. It also requires large blood samples (10-20 ml) and a rather laborious extraction process. Plasma renin activity estimation, on the other hand, is able to detect levels well below the normal range and the relative simplicity of the technique means that large numbers of samples may be processed. Furthermore, since a smaller sample size is required than for angiotensin II, repeat sampling, duplicate storage, etc. are feasible for this assay.

Therefore the two assays were compared to see if PRA was a function of plasma angiotensin II as reflected by the angiotensin II assay and, if so, to what extent such correlation existed.

Normal volunteers and patients with high blood pressure who had had no previous treatment, and no underlying cause for their raised blood pressure, were studied. Blood was taken for PRA and angiotensin II from subjects sitting upright between 10 a.m. and 12 noon on their normal diet, the fifth day of high sodium intake (200 mmol + normal diet) and on the fifth day of a low sodium diet (10 mmol). In some of the patients with high blood pressure, measurements were repeated on the sixth week of treatment with either Propranolol (320 mg/day) or Spironolactone (300 mg/day).

The initial correlation data for the whole study are shown in Table 14.1 and Figure 14.1. It can be seen from this that PRA correlates well with angiotensin II over the range studied. However, such correlations are open to criticism as the data does not show, in this presentation, whether or not normotensive volunteers behave in a like manner to hypertensive patients. Table 14.2 shows the normals and patients with high blood pressure correlated separately. It can be seen that there

Table 14.1

Linear regression analysis of PRA as a function of angiotensin II concentration for (a) all subjects studied, and (b) all subjects not on drug therapy.

	a	b
Σx	268.2231	221.5741
Σx^2	409.5975	342.7014
Σxy	71.0902	69.6438
Σy	30.1877	34.8004
Σy	61.8674	46.5154
n	183.0000	148.0000
r	0.8771	0.8552
m	1.6305	1.5980
b	-2.2249	-2.1573
t	24.5771	19.9375
S.E.E.y	0.2684	0.2646
$\bar{x}$	1.4657	1.4971
SD of x	0.3007	0.2732
$\bar{y}$	0.1649	0.2351
SD of y	0.5590	0.5106

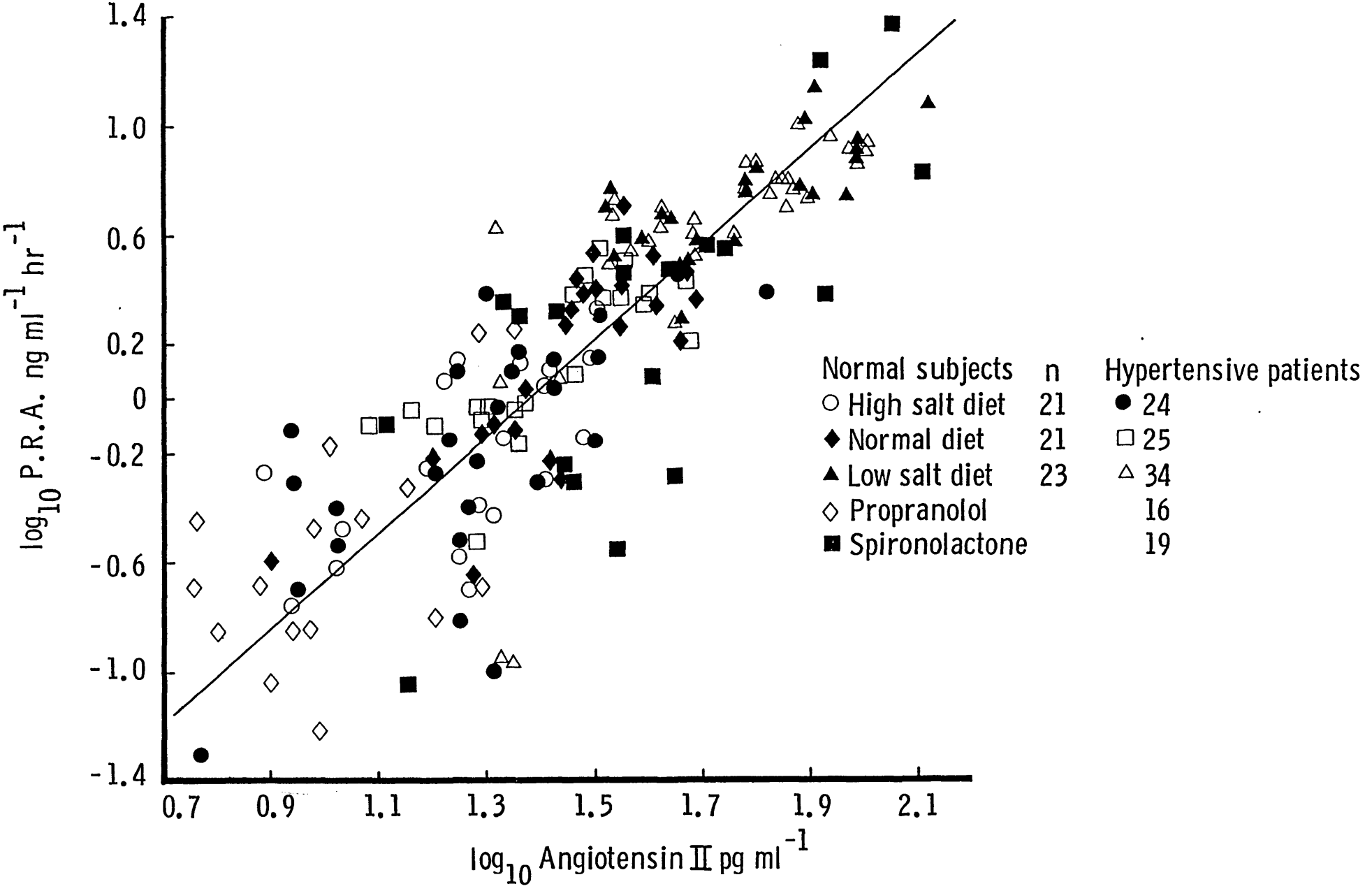


Figure 14:1 Correlation data for PRA as a function of angiotensin II

Table 14.2

Linear regression analysis of PRA as a function of angiotensin II concentration for normal subjects and hypertensive patients, and a comparison of slopes.

	Normal subjects	Hypertensive patients
Σx	98.1113	123.4628
Σx^2	152.9630	189.7384
Σxy	32.5112	37.1326
Σy	16.5573	18.2431
Σy^2	19.6756	26.8398
n	65.0000	83.0000
r	0.8663	0.8479
m	1.5429	1.6421
b	-2.0742	-2.2229
t	13.7692	14.3966
S.E.E.y	0.2454	0.2797
$\bar{x}$	1.5094	1.4875
SD of x	0.2759	0.2724
$\bar{y}$	0.2547	0.2197
SD of y	0.4914	0.5276
Residual F^{81}_{63}	1.2945	p >0.05
Slope F^{1}_{144}	0.3730	p >0.05

is no significant difference in residual variance (which indicated that one was comparing like with like) or slope. However, it will be noted that correlation of all three diets together is not, strictly speaking, valid. This may be understood if one considers that a non-correlated group on a high sodium diet will all have higher values on a normal diet and again on a low sodium diet so that by plotting three totally uncorrelated groups on the same graph one could generate a false positive correlation. It is also not valid strictly as the diets were not obtained by random chance. Therefore it was essential to check the correlation for each salt diet separately. Tables 14.3 and 14.4 show the results for the normal subjects and hypertensive patients respectively. It may be seen that none of the slopes compared differs significantly each from another. However, it could be possible that the different subjects (normotensive or hypertensive) could differ in slope with respect to each other although not with respect to the other sodium intake groups in their blood pressure group. Table 14.5 shows the comparison of the correlation of PRA versus angiotensin II between normals and hypertensive patients on the same sodium diets. Once again no significant difference in slopes could be seen.

A further possible breakdown of the correlation could be envisaged by drug treatment to modify blood pressure if such drugs acted directly on angiotensin converting enzyme. Broadly speaking the two classes of drug most widely used in antihypertensive therapy are beta-blockers which depress the renin angiotensin system and diuretics which stimulate it. Table 14.6 shows the comparison of the slopes of the hypertensive patients on Propranolol with those on Spironolactone. Also these correlations were compared with the correlations obtained in normal subjects and hypertensive patients over similar ranges of values when the subjects were not on drug treatment; that is, when they were on a high

Table 14.3

Linear regression analysis of PRA as a function of angiotensin II concentration for normal subjects on three different dietary sodium intakes, and a comparison of slopes.

	Low salt	Normal diet	High salt
Σx	40.9295	30.4230	26.7588
Σx^2	73.4856	44.7549	34.7225
Σxy	31.0248	6.8013	-5.3149
Σy	17.1165	4.1233	-4.6825
Σy^2	13.6998	2.9982	2.9776
n	23.0000	21.0000	21.0000
r	0.7150	0.6781	0.5925
m	0.8701	1.2160	1.0417
b	-0.8042	-1.5654	-1.5503
t	4.6870	4.0226	3.2065
S.E.E.y	0.1461	0.2413	0.2504
$\bar{x}$	0.7795	1.4487	1.2742
SD of x	0.1718	0.1844	0.1768
$\bar{y}$	0.7441	0.1963	-0.2229
SD of y	0.2090	0.3308	0.3109
Residual	F^{19}_{21} 2.7892 p <0.025	F^{19}_{19} 1.0610 p <0.05	
Slope	F^{1}_{40} 0.9660 p >0.05	F^{1}_{13} 0.1544 p >0.05	
Residual		F^{19}_{21} 2.9596 p <0.01	
Slope		F^{1}_{14} 0.2157 p >0.05	

Table 14.4

Linear regression analysis of PRA as a function of angiotensin II concentration for hypertensive patients on three different dietary sodium intakes and a comparison of slopes.

	Low salt	Normal diet	High salt
Σx	58.1838	35.4805	29.7985
Σx^2	100.9406	50.8964	37.9014
Σxy	38.7398	5.0001	-6.6073
Σy	21.7093	2.9994	-6.4987
Σy^2	16.6117	2.4979	7.7302
n	34.0000	25.0000	24.0000
r	0.8181	0.6906	0.6278
m	1.1586	1.3723	1.6143
b	-1.3442	-1.8277	-2.2750
t	8.0490	4.5804	3.7836
S.E.E.y	0.1659	0.2158	0.3966
$\overline{x}$	1.7112	1.4192	1.2416
SD of x	0.2038	0.1502	0.1981
$\overline{y}$	0.6385	0.1199	-0.2706
SD of y	0.2886	0.2984	0.5095
Residual	F^{23}_{32} 1.7112 p >0.05	F^{22}_{23} 3.3827 p <0.005	
Slope	F^{1}_{55} 0.4821 p >0.05	F^{1}_{45} 0.1882 p >0.05	
Residual	F^{22}_{32} 5.7887 p <0.005		
Slope	F^{1}_{54} 1.3392 p >0.05		

Table 14.5

A comparison of slopes of the correlation of PRA as a function of angiotensin II concentration for normal subjects versus hypertensive patients on three different sodium intakes.

	Low salt	Normal diet	High salt
	Normal vs Hypertensive	Normal vs Hypertensive	Normal vs Hypertensive
Residual	F^{32}_{21} 1,2735 p >0,05	F^{19}_{23} 1,2798 p >0,05	F^{27}_{19} 2,4909 p >0,05
Slope	F^{1}_{53} 1,4115 p >0,05	F^{1}_{42} 0,1354 p >0,05	F^{1}_{41} 1,0193 p >0,05

Table 14.6

Linear regression analysis of PRA as a function of angiotensin II concentration for hypertensive subjects on Propranolol and hypertensive subjects on Spironolactone, with comparison of slopes.

	On Propranolol	On Spironolactone
Σx	16.3741	30.2749
Σx^2	17.2842	49.6119
Σxy	-8.5824	10.0288
Σy	-9.5824	4.9697
Σy^2	7.6886	7.6634
n	16.0000	19.0000
r	0.5376	0.7142
m	1.1988	1.5385
b	-1.7896	-2.1899
t	2.3856	4.2074
S.E.E.y	0.3525	0.4161
$\overline{x}$	1.0233	1.5934
SD of x	0.1874	0.2760
$\overline{y}$	-0.5627	0.2615
SD of y	0.4180	0.5945
Residual	$F^{17}_{14} = 1.3771$	$p > 0.05$
Slope	$F^{1}_{31} = 0.2733$	$p > 0.05$

and low sodium intake (Table 14.7).

The results indicate in all the analyses that the correlation between PRA and angiotensin II (summarised in Table 14.8) was highly significant (r 0.59-0.87 p <0.05-<0.001). The lower correlations in the high salt groups and the group on Propranol were probably due to the angiotensin II assay sensitivity limit causing inaccuracies. Furthermore in all cases comparison of slopes showed no significant difference. In a few cases comparison of the slopes was not a strictly valid procedure statistically speaking as the residual variances were significantly different; but even so subsequent comparison of the slopes showed no significant differences between them.

The importance of these results is two fold. Firstly they indicate that PRA may be used as a function of angiotensin II concentration over a wide range of values. Secondly they show that the correlations do not in essence differ between normotensive volunteers and hypertensive subjects for a given level of PRA and angiotensin whether this is brought about by varying sodium intake or drug treatment. The clinical consequences of this will be examined later.

Table 14.7

Comparison of slopes of the linear regression analyses of PRA as a function of angiotensin II between results from (a) hypertensive patients on Propranolol and normal subjects on a high salt diet, (b) hypertensive patients on Propranolol and hypertensive patients on a high salt diet, (c) hypertensive patients on Spironolactone and normal subjects on a low salt diet, and (d) hypertensive patients on Spironolactone and hypertensive patients on a low salt diet.

(a) Hypertensives on Propranolol vs normals on high salt diet

Residual	F	2.0160	p	>0.05
Slope	F	0.7400	p	>0.05

(b) Hypertensives on Propranolol vs hypertensives on high salt diet

Residual	F	1.2351	p	>0.05
Slope	F	0.3771	p	>0.05

(c) Hypertensives on Spironolactone vs normals on low salt diet

Residual	F	8.2197	p	<0.005
Slope	F	2.0900	p	>0.05

(d) Hypertensives on Spironolactone vs hypertensives on low salt diet

Residual	F	6.4542	p	<0.005
Slope	F	1.2046	p	>0.05

Table 14.8

Summary of linear regression data

Subjects	Diet or treatment	number	Correlation coefficient	
Hypertensive	Low sodium	34	0.8181	p <0.001
Normal	Low sodium	23	0.7150	p <0.001
Hypertensive	Normal diet	25	0.6906	p <0.001
Normal	Normal diet	21	0.6781	p <0.001
Hypertensive	High sodium	24	0.6278	p <0.005
Normal	High sodium	21	0.5925	p <0.005
Hypertensive	Propranolol	16	0.5376	p <0.05
Hypertensive	Spironolactone	19	0.7142	p <0.001
Normal + hypertensive	All diets	148	0.8552	p <0.001
Normal + hypertensive	All diets + Propranol + Spironolactone	183	0.8771	p <0.001
Normal	All diets	65	0.8663	p <0.001
Hypertensive	All diets	83	0.8479	p <0.001

CHAPTER FIFTEEN

THE RELATIONSHIP BETWEEN PRA AND BLOOD PRESSURE IN NORMOTENSIVE VOLUNTEERS AND UNTREATED PATIENTS WITH ESSENTIAL HYPERTENSION

Three groups of subjects were initially studied, grouped on the basis of supine diastolic pressure; normotensive, mild hypertensive and severely hypertensive. Each group was observed and recorded on three different sodium diets as there is some evidence for the involvement of sodium in essential hypertension.

Subjects

Seventy-seven hypertensive subjects and 28 normal subjects, aged between 19 and 65 years, were studied. The patients were untreated with hypotensive drugs and were shown to have essential hypertension by exclusion of known primary causes. They were admitted to the study following three screening visits if their mean supine diastolic pressure by the third visit was between 90 and 130 mm Hg and plasma creatinine was less than 120 $\mu mol.l^{-1}$. The hypertensive patients were subdivided into mildly hypertensive (mean diastolic pressure 90-109 mm Hg) and severely hypertensive (mean diastolic pressure 110-130 mm Hg). The normal subjects had no family history of high blood pressure and all had mean diastolic pressures of less than 85 mm Hg at the third visit. Most of the volunteers were hospital personnel. In the mildly hypertensive group there were 41 subjects, 11 of whom were black. In the group with severe hypertension there were 36 subjects, 15 of whom were black. The severely hypertensive group were significantly older (mean age 50) than the mildly hypertensive group (mean age 44) who were in turn significantly older than the normotensive subjects (mean age 33 years).

Methods

Subjects were studied on their normal diet and on a high sodium intake of approximately 350 mmol/day. This was achieved by supplementation of the normal diet with slow sodium. They were also studied on low (10 mmol/day) sodium diet. The subjects had five days on each diet, 24 hour urine collections being taken during the last two days of each dietary period. The subjects underwent their normal daily routine, were not admitted to hospital but were discouraged from violent physical exercise. All blood pressures were measured on semi-automatic, bias-free ultrasound sphygmomanometers ("Arteriosonde 1217"). Mean values of five readings, taken from subjects on the supine position at 2 minute intervals, were recorded. The blood pressure measurements were made by the same nurse at the same time of day, between 10.00 and 12.00, on the same arm. Following the blood pressure measurement blood samples were taken from the subjects for measurement of plasma electrolytes, plasma renin activity and aldosterone concentration.

Results and Discussion

Table 15.1 summarises the linear regression data for mean arterial pressure as a function of plasma renin activity obtained for the different groups on their different diets. Mean arterial pressure was calculated as diastolic pressure + $\frac{1}{3}$ (systolic pressure - diastolic pressure). There are several points of note in the data in Table 15.1. Firstly, none of the correlations achieve statistical significance implying that PRA is not a determinant of MAP *per se*. The correlation obtained from normal subjects on a low salt diet is the only one which approaches significance. Also it can be seen that severe sodium restriction does seem to lower blood pressure significantly in the hypertensive groups. The mean PRA values also can be seen to be progressively

Table 15.1

Summary of linear regression data for mean arterial blood pressure as a function of plasma renin activity

Subject group	No.	Diet	Correlation coefficient	PRA (± ISD)	MAP (± ISD)	Mean urinary Na^+ mmol
Severe HT	36	LS	0.0106 (p>0.05)	2.00 (1.9)	128.1 (11.4)	21
Severe HT	36	ND	-0.1154 (p>0.05)	0.82 (0.72)	141.5 (9.19)	153
Severe HT	35	HS	0.1170 (p>0.05)	0.50 (0.50)	138.6 (26.1)	245
Mild HT	41	LS	-0.0946 (p>0.05)	3.09 (2.71)	112.9 (10.0)	19
Mild HT	41	ND	-0.1209 (p>0.05)	0.93 (0.68)	121.6 (7.1)	145
Mild HT	41	HS	-0.0279 (p>0.05)	0.49 (0.42)	120.9 (8.2)	293
NT	27	LS	0.3553 (p>0.05)	5.72 (3.28)	89.4 (10.9)	15
NT	28	ND	-0.0933 (p>0.05)	2.14 (1.71)	90.2 (9.9)	167
NT	27	HS	-0.2010 (p>0.05)	0.65 (0.59)	89.6 (10.3)	310

Key to symbols:

MAP Mean arterial pressure $[\text{diastolic} + \frac{1}{3}(\text{systolic-diastolic})]$

PRA Plasma renin activity

HT Hypertensive

NT Normotensive

LS Low sodium diet

ND Normal diet

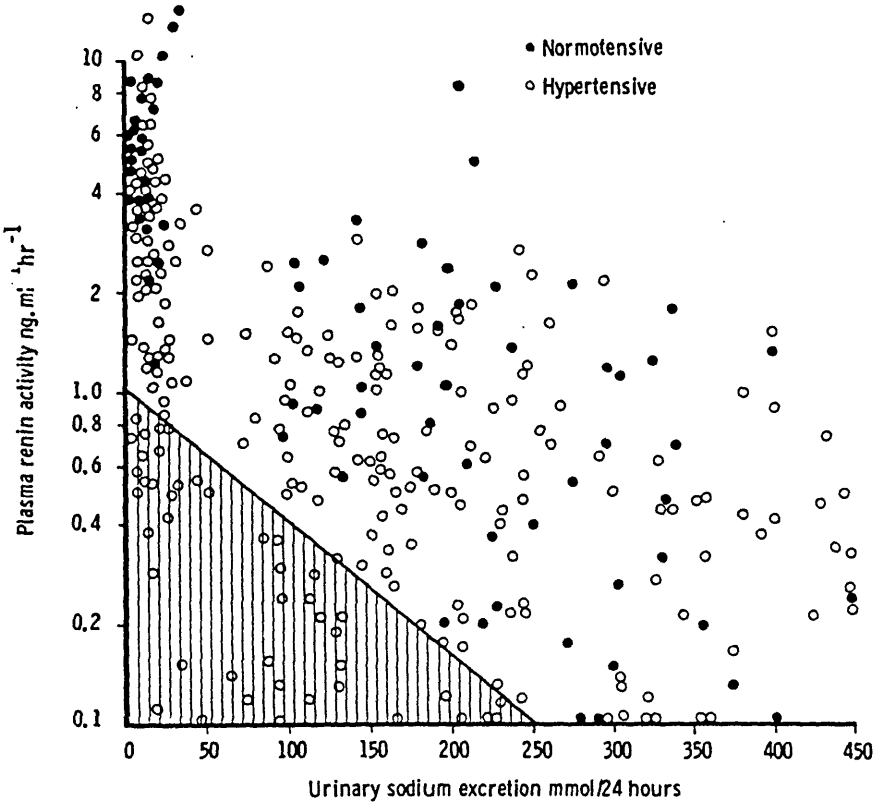
HS High salt diet

lower as the groups mean blood pressure increases; that is, normals less than mild hypertensives who are in turn less than the severe hypertensives for any given diet. It is possible, therefore, to picture a complex functional relationship between PRA and blood pressure which is perhaps affected by sodium concentrations.

It would not be valid to further correlate the data across the different diets as they were not obtained by chance and do not, as a whole, represent a normal distribution. Similarly blood pressure as a function of PRA cannot be evaluated for all subjects on any diet other than the normal diet for the same reason. If all subjects on their normal sodium intake are correlated, for PRA as a function of mean arterial pressure a highly significant inverse correlation is obtained, ($r = -0.44$, $n = 105$, $p < 0.001$). As a control urinary sodium was correlated against mean arterial pressure and found not to be significant ($r = 0.06$, $n = 105$, $p > 0.05$) implying that the correlation was not due to the spread in sodium concnetration found in the subjects on their normal diets. Broadly this appears to indicate that subjects with higher renin levels on a free sodium intake are more likely to be normotensive since 44% of the variability is explicable by regression. Also from Table 15.1 it can be seen that the renin activity of normal subjects responds to changes in sodium intake to a greater degree, in absolute terms, than mild or severe hypertensive patients; interestingly the percentage responses are similar. Thus we may perhaps postulate a situation in which the normal subject has his renin system "set" at a higher basal level than the hypertensive subjects, for a given level of sodium intake, and therefore pro rata responses of PRA to, say, sodium deprivation would yield a greater physiological effect in blood pressure maintenance in these subjects. Figure 15.1 shows the renin profile of the subjects on the three sodium diets. It may be seen that the range of PRA

Figure 15:1

Renin profile with respect to dietary sodium intake.



for hypertensives on a low sodium diet is greater than for normotensives, there being an area of low PRA/low salt (shaded over on Figure 15.1) exclusively populated by hypertensives. These "low renin hypertensives" would be expected to be the non-responding group in such a theory. It should be noted though that the hypertensives do not divide into two groups with respect to PRA when on a low salt diet, merely their range is greater so threshold-type responses are unlikely phenomena to be observed. One could include in this renin response hypothesis an explanation of why the only group in Table 15.1 to come close to any kind of significance is, in fact, the low-salt normals who would respond much more to sodium deprivation than their hypertensive counterparts, and in that respect, when salt and water are depleted it would therefore not be surprising to find that the resultant blood pressure was a direct, if weak, function of the PRA.

The above, then, intimates a certain relationship between blood pressure and plasma renin activity, and also that blood pressure change and change in sodium intake may be a function of the initial blood pressure, and possibly the angiotensin system as estimated by the PRA. The data were therefore re-analysed using the statistical package for the Social Sciences of the University of London Computer. The change in mean arterial pressure (ΔMAP) on going from a high to low sodium diet divided by the change in urinary sodium excretion (ΔNa) was used as an index of blood pressure sensitivity to sodium alteration. Two-tailed Students' t-tests and analyses of regression were carried out to investigate the relationship between this "sodium sensitivity index" and the supine blood pressure on a normal diet and plasma renin activity.

Table 15.2 shows the basal data for the subjects in the analysis. Comparing the three groups, there was no significant difference in 24 hour urinary sodium excretion or pulse rate at the third screening visit. However, it should be noted that the hypertensive patients were older and

Table 15.2

Basal data for the three groups of subjects

	Normal	Mild EH	Severe EH
Systolic BP mm Hg	119 ± 2	161 ± 2	187 ± 3
Diastolic BP mm Hg	75 ± 2	102 ± 1	118 ± 2
Pulse rate/min	75 ± 2	77 ± 2	76 ± 1
Weight Kg	71 ± 2	74 ± 2	75 ± 2
Urinary Na excretion mmol (24 h)	168 ± 10	145 ± 8	153 ± 8
Plasma renin activity ng/ml/h (sitting up)	2.14±0.32	0.93± 0.11	0.81± 0.12
Plasma aldosterone pmol/l (sitting up)	488 ± 51	417 ±51	476 ±41

and heavier and as previously discussed had lower plasma renin activities than normal subjects. There were no significant differences in any parameters studied, other than blood pressure, between the mild and severe hypertensive patients.

In the normal subjects alteration of sodium intake provided large changes in PRA but small changes only in blood pressure, the change in MAP being 0.7 (±1.7) mm Hg between high and low sodium diets . The slope of the arterial pressure-urinary sodium excretion relationship was very low ($\Delta MAP/\Delta Na = 0.004 \pm 0.006$) and this slope did not correlate with the ages of the subjects ($r = 0.17$, $n = 28$). In a subgroup of the normal subjects who were older than 30 years ($n = 12$, mean = 45.2), the change in MAP was 1.7 (±1.05) mm Hg and the slope 0.005 (±0.003), neither value being significantly different from the group as a whole. In the mildly hypertensive group a significant decrease in blood pressure occurred when sodium intake was decreased. This was associated with a decrease in weight and plasma volume. The compensatory rise in PRA was significantly less ($p<0.002$) than in the normotensive subjects. The change in MAP, comparing values on high and low sodium diets, was 8.0 (±1.4) mm Hg, which was significantly greater than in normal subjects ($p<0.002$). The slope of the mean arterial pressure-urinary sodium excretion relationship was 0.03 (±0.006), which was significantly increased by comparison to the normal subjects. The blood pressure response to dietary alteration was similar in white males and females and black females. The four black males with mild hypertension had a significantly greater change in blood pressure.

In the group with severely raised blood pressure, sensitivity to alteration of sodium intake was significantly greater than in the group with mild hypertension despite a lower urinary sodium excretion during the high salt diet. On the low sodium diet, plasma renin activity was significantly less ($p<0.036$) in the group with severe hypertension compared to the mildly hypertensive group. The change in mean arterial

pressure in this group, comparing levels on high and low sodium diets, was 14.5 (±1.4) mm Hg and this was significantly greater ($p<0.002$) than the change which occurred in the mildly hypertensive group. The slope of the mean arterial pressure-urinary sodium excretion relationship was further raised, 0.106±0.023, being significantly greater than the slope found with the mildly hypertensive subjects. This difference between the two groups of hypertensive subjects was also observed in the sub-groups of males and females and blacks and whites, the blacks having the greatest $\Delta MAP/\Delta Na$ slopes (see Table 15.3).

There was a highly significant correlation between the initial blood pressure (measured supine on the third screening visit) - i.e. the severity of the hypertension and the slope of the arterial pressure-sodium relationship ($\Delta MAP \div \Delta Na$) ($r = 0.43$, $n = 105$, $p<0.00001$). No significant correlation between slope and age was found in either normals or hypertensive groups. No correlation existed between slope and plasma volume either. However, there was a significant negative correlation between the slope and charge in plasma renin activity ($r = -0.36$, $n = 98$, $p<0.0002$). Furthermore, multiple regression analysis using the slope ($\Delta MAP \div \Delta Na$) as the dependent variable and correlating it with initial blood pressure and change in plasma renin activity revealed that 46% of the variability in the $\Delta MAP \div \Delta Na$ ratios was attributable to these two variables. Adding in age, weight or change in plasma volume did not improve the multiple correlations.

This analysis demonstrates that the blood pressure to sodium excretion relationships is not only shifted to higher blood pressure levels in essential hypertension but the slope of the relationship is significantly altered in mildly, and further in severely, hypertensive patients as compared with normal subjects. This was not attributable to age but blood pressure and PRA when combined in a multiple regression

Table 15.3

Comparison of blood pressure, renin and electrolytes in the three groups on varying sodium intakes

	Normotensive subjects			Mild hypertension			Severe hypertension		
	High Na	Low Na	Difference	High Na	Low Na	Difference	High Na	Low Na	Difference
Systolic BP mm Hg	119.3±2.8	116.4±2.8	3.1±2.0	160.2±2.4	146.4±2.5	13.8±1.9	189.2±3.1	165.8±3.0	23.5±2.6
Diastolic BP mm Hg	75.4±1.7	75.9±2.1	-0.5±2.0	101.2±1.2	96.2±1.4	5.1±1.4	119.6±1.7	109.3±1.8	10.3±1.5
Pulse rate/min	74.6±2.8	76.4±2.6		78.0±1.5	77.7±1.5		78.6±1.8	79.9±2.0	
24 h urinary Na excretion (mmol)	310±14	15 ±2	294±15	293±15	19±2	274±15	244±20	22±3	222±19
Weight (kg)	70.9±1.9	69.0±1.8	2.38±0.18	74.1±2.4	71.9±2.3	2.14±0.15	75.3±1.9	73.4±1.9	1.83±0.13
Plasma volume (l)	-	-	-	2.89±0.09	2.6±0.08	0.28±0.04	2.78±0.07	2.62±0.07	0.19±0.06
Plasma renin activity ng/ml/h	0.65±0.11	5.71±0.61	-4.79±0.54	0.49±0.07	3.09±0.42	-2.6±0.42	0.5±0.09	2.0±0.32	-1.5±0.3
Plasma aldosterone	257±21	1480±146	-1223±136	271±44	901±44	-630±115	356±32	728±85	-356±91
$\frac{\text{MAP}}{\text{Ur Na excretion}}$		0.004±0.006			0.03±0.006			0.106±0.023	

analysis accounted for almost half the variability (seen in the $\Delta MAP \div \Delta Na$ ratios) and the change in PRA alone was a significant negative correlating factor with the slope. It must, however, be remembered that the slope is not necessarily linear over the range of urinary Na^+ considered and as such must be regarded as an arbitrary index of "sodium sensitivity".

These results tend to support the idea postulated that the renin system is 'set' at a higher level in normotensives and responds more readily to sodium deprivation.

CHAPTER SIXTEEN

THE RESPONSE OF THE RENIN ANGIOTENSIN SYSTEM TO THE LOSS OF SODIUM AND WATER INDUCED BY SPIRONOLACTONE

The results of the previous chapter indicated a possible role for the renin angiotensin system in blood pressure control of normotensive and hypertensive subjects. Broadly, the trend appeared to be that PRA, for a given sodium intake, was decreased as blood pressure increased. Furthermore, in hypertensive patients with low PRA values a much smaller increase in PRA was observed, upon sodium deprivation, than in subjects whose initial renin levels were normal.

Spironolactone has been reported to be a more efficacious hypotensive agent than Propranolol in subjects with a low PRA (Spark and Melby, 1971; Carey *et al.*, 1972). Conversely Propranolol has been reported to be more effective in subjects whose renin levels are normal (Buhler *et al.*, 1973; Karlberg *et al.*, 1976). It was therefore decided to do a drug cross-over trial with Spironolactone and Propranol administered to hypertensive patients whose initial renin levels on a low salt diet covered the whole range encompassed in Figure 15.1 to see if the hypotensive response did vary and if that variation was related to plasma renin activity. Furthermore it was decided to use a competitive inhibitor of angiotensin II, saralasin, in a group of patients on Spironolactone to see if the hypotensive effect, if any, of the inhibitor related to the PRA of the patients on Spironolactone.

Patients and Methods

Twenty-five hitherto untreated patients with essential hypertension from the previous diet study were allocated randomly to one of two treatment groups. In the first they were given 320 mg per day of Propranolol for six weeks and in the second they were given 300 mg per day of Spironolactone for six weeks. At the end of each treatment period therapy was stopped for two weeks and then the alternative drug given.

All patients were seen weekly in the clinical by the same nurse at the same time of day and day of the week. Blood pressure was always measured by the same machine on the same arm. Compliance was checked by tablet counts and by pulse rate slowing on Propranolol and by loss of weight with Spironolactone. PRA and angiotensin II were measured at the end of each treatment period.

Saralasin was infused into 12 patients with essential hypertension who were on 100 mg per day of Spironolactone and had been so for six weeks. After a 1 hour countrol period, blood was taken for PRA estimation. Saralasin was then infused using an incremental infusion of 0.25, 1.25 and 10 $\mu g\ kg^{-1} min^{-1}$, each rate being given for 20 minutes. Blood pressure change with saralasin was calculated from the mean blood pressure in the last 30 minutes before infusion and the mean of the blood pressure in the last 10 minutes of the highest rate of infusion.

Results

The results of the randomised cross-over trial in the 25 patients showed that in those patients with an abnormally low PRA on the low salt diet ($<2\ ng.ml^{-1}\ hr^{-1}$) a significantly greater fall in blood pressure was seen with Spironolactone than in those patients with a normal low salt PRA value. When the drugs were crossed over it was found that Propranolol induced a significantly greater fall in blood pressure in the group with normal low salt renin values than in the low renin group (see Figure 16.1). For each drug the correlation between mean blood pressure fall and the PRA on a low sodium diet was examined. On Spironolactone the blood pressure fall was found to be inversely correlated with the low sodium diet value of PRA (Figure 16.2). The same parameters, when the patients were on Propranolol, yielded a positive

Figure 16:1

Fall in blood pressure with Spironolactone and Propranolol in patients with normal and low PRA values.

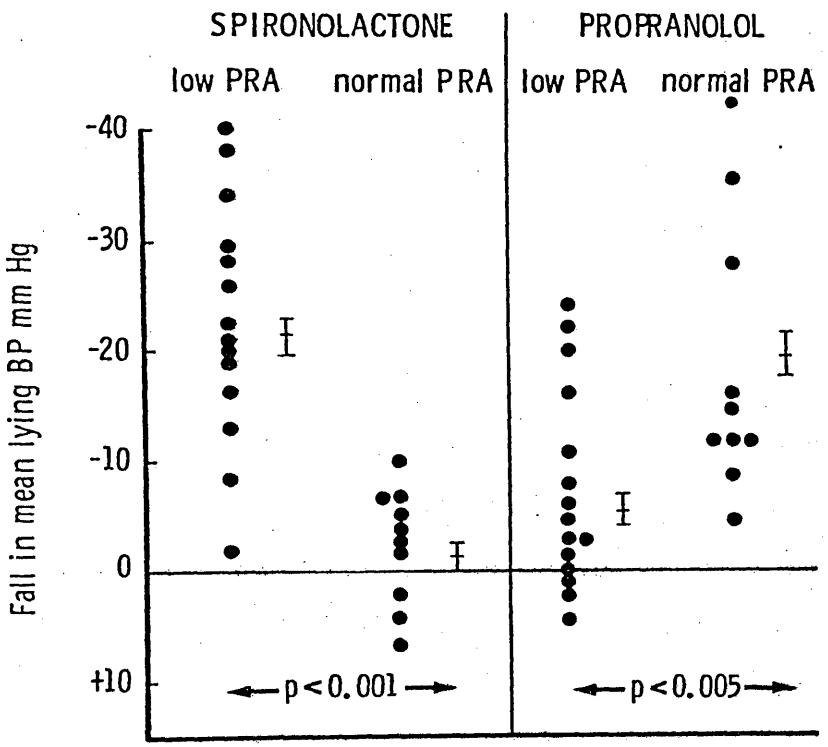
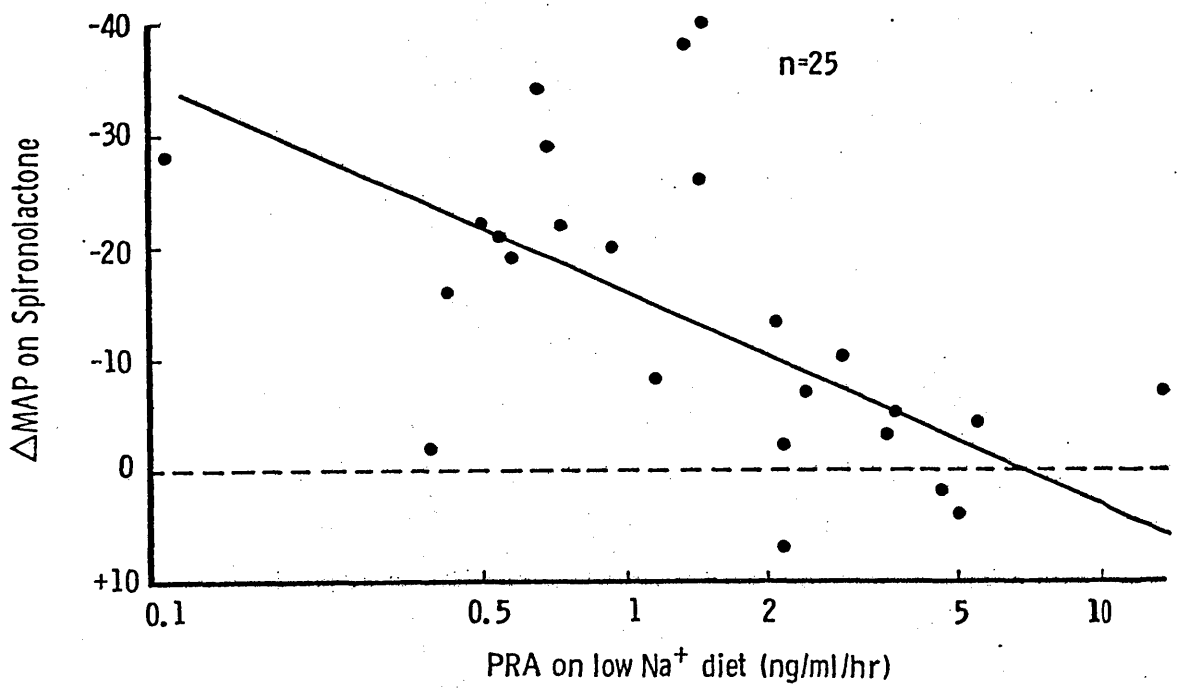


Figure 16:2

Correlation between blood pressure fall and PRA in patients on Spironolactone.



correlation (Figure 16.3). When the difference in blood pressure fall on the two drugs was correlated with low salt diet PRA value a highly significant correlation was observed (Figure 16.4). Analogous correlations using plasma angiotensin II data instead of PRA were also obtained and are summarised in Table 16.1.

On Spironolactone the mean weight loss was 1.82 kg. In 20 patients body composition was remeasured whilst on Spironolactone. Mean fall in exchangeable sodium was 243 ± 46 mmol, mean fall in blood volume was 271 ± 74 ml. Associated with this sodium and water loss PRA rose to a mean value of 4.24 $ng.ml^{-1}\ hr^{-1}$. No significant correlation was found between blood pressure fall with Spironolactone and weight loss or change in body composition. The fall in blood pressure with saralasin infusion was significantly correlated, negatively, with the plasma renin activity before infusion (Figure 16.5).

Discussion

It may be seen from the results of the randomised cross-over trial that Spironolactone was a more effective hypotensive agent in those patients who had a low PRA when on a low salt diet. Propranolol was seen to be more effective in the subjects whose PRA values were normal when salt depleted. The correlations for blood pressure fall against low salt PRA value are in both cases significant, the difference being that for Spironolactone the slope was negative whereas for Propranolol it was positive. Figure 16.4, showing the difference between the two drugs, indicates the cross-over point for predominant hypotensive action of the two drugs.

One interpretation of these results is that in the group of subjects who have a normal PRA on the low salt diet the fall in blood pressure due to Spironolactone induced diuresis is being offset by the

Figure 16:3

Correlation between blood pressure fall and PRA in patients on Propranolol.

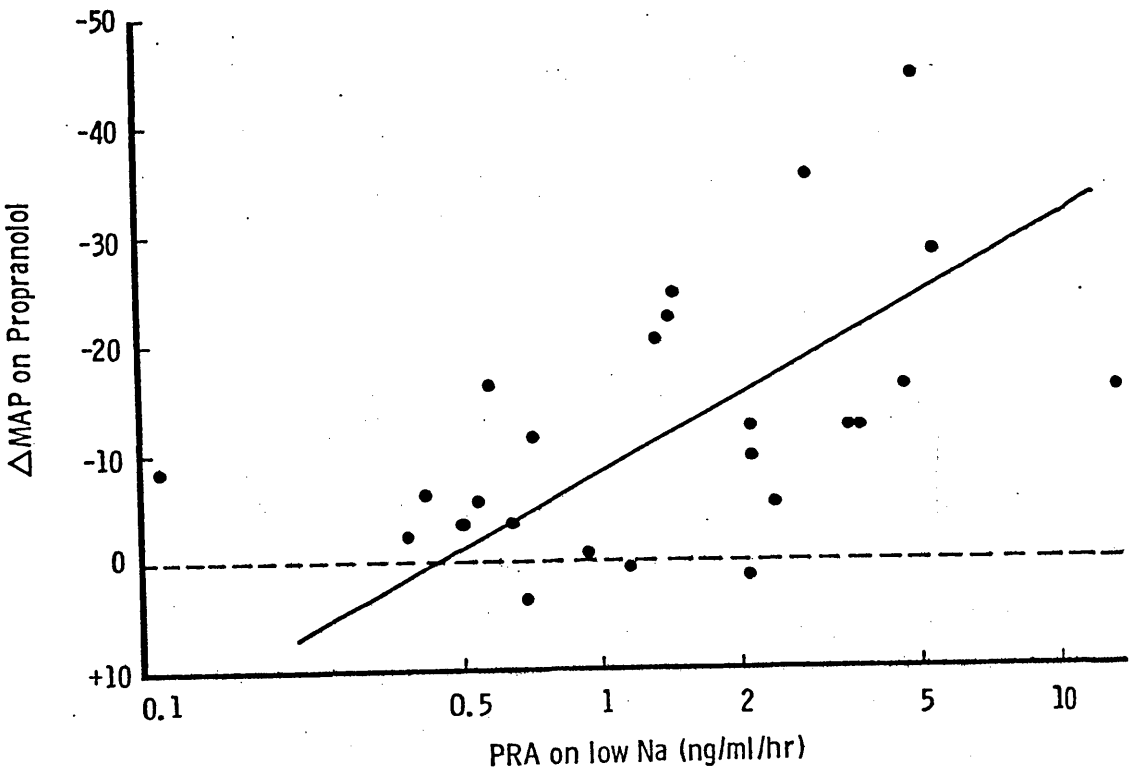


Figure 16:4

Difference in blood pressure fall on Sprionolactone and on Propranolol correlated with PRA.

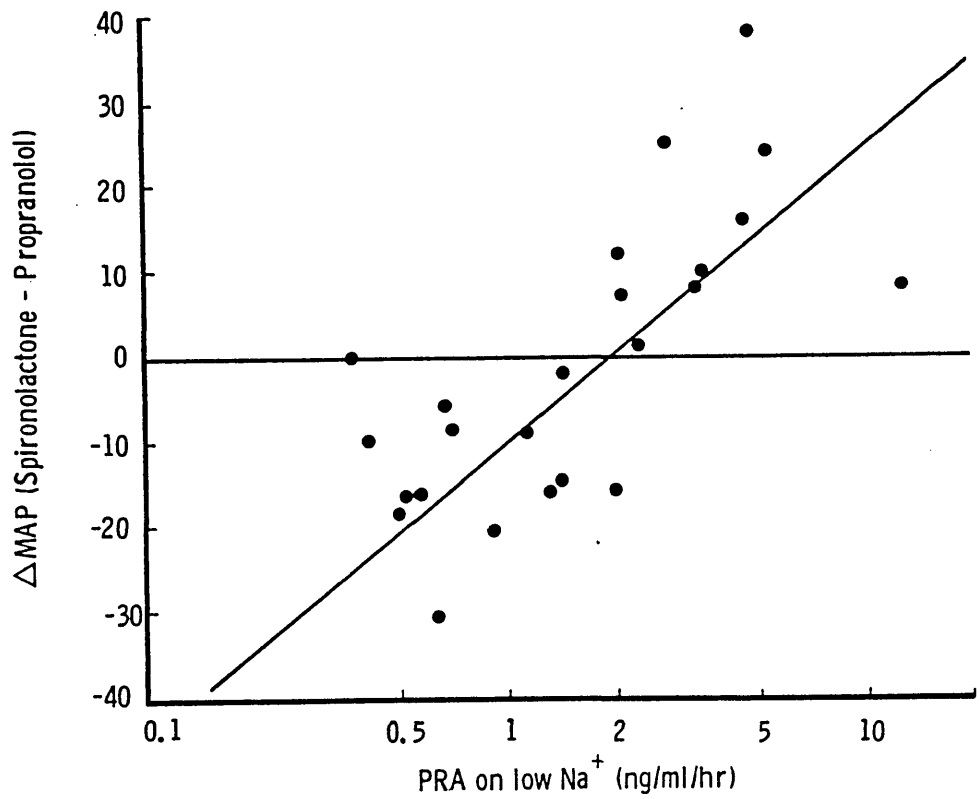


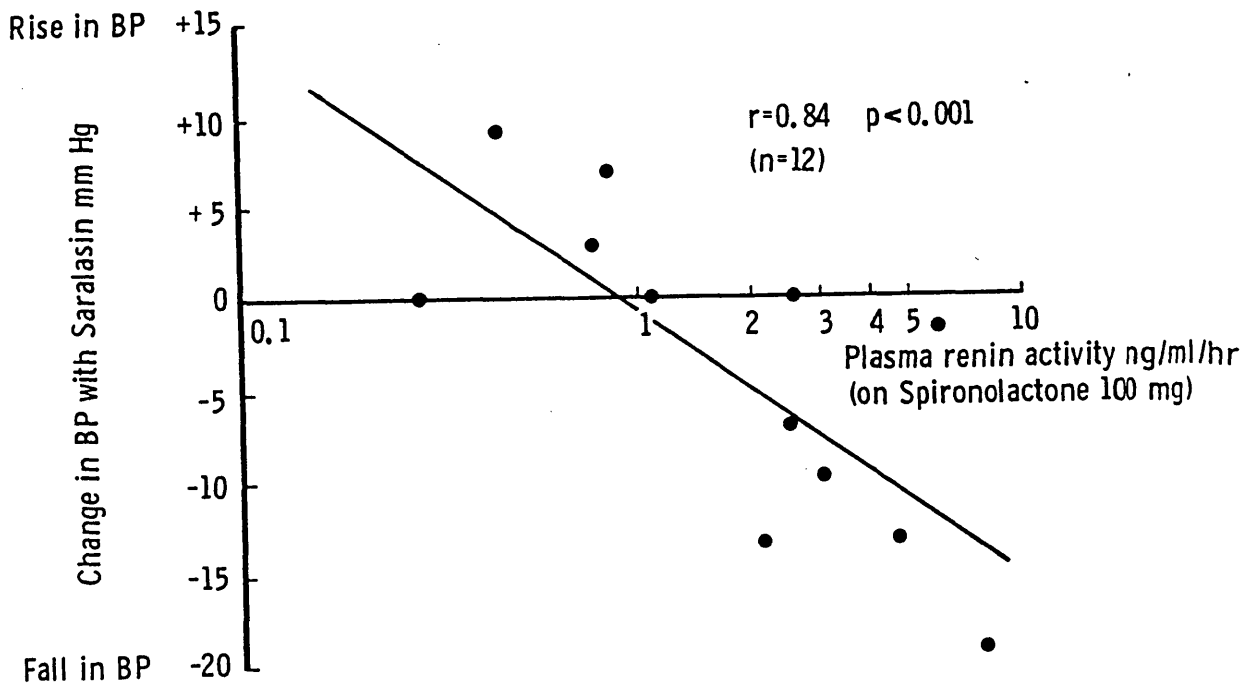
Table 16.1

Synopsis of correlation data between fall in mean supine blood pressure and (a) PRA and (b) angiotensin II values on the low sodium diet.

				n	r	p
(i)	Fall in mean supine blood pressure with Spironolactone	vs	(a) PRA	25	-0.55	<0.005
			(b) AII	25	-0.65	<0.001
(ii)	Fall in mean supine blood pressure with Propranolol	vs	(a) PRA	25	0.50	<0.02
			(b) AII	25	0.47	<0.02
(iii)	Difference in mean supine blood pressure; (Spironolactone-Propranolol)	vs	(a) PRA	25	0.75	<0.001
			(b) AII	25	0.80	<0.001

Figure 16:5

Correlation between blood pressure change and PRA in patients on Spironolactone following saralasin infusion.



response of the renin angiotensin system which increases greatly in such "normal renin" subjects when they are salt and water depleted as we have seen in the sodium-diet study in the previous chapter. The "low renin" group, by this hypothesis, would not be able to respond to the same extent and therefore the blood pressure fall would be greater, hence the negative correlation between blood pressure fall and PRA.

The way in which Propranolol lowers blood pressure is unclear. However, it does lower plasma renin activity and angiotensin II as is shown in Figure 14.1, and this may contribute to its hypotensive effects. If this is the case it would be reasonable to expect it to have a greater effect in the "normal renin" group than in the low renin group and hence yield a positive correlation between blood pressure fall and PRA. Furthermore, this would account for the synergistic hypotensive effect of combined β-blocker and diuretic therapy.

The saralasin study indicated a significant negative correlation between blood pressure change and the plasma renin activity pre-infusion; that is a positive correlation between fall in blood pressure and PRA. As the patients were on Spironolactone their renin angiotensin system was stimulated, in an analogous manner to being on a low salt diet. Therefore in these patients with low PRA a small hypotensive effect was seen and in the "normal renin" group a greater hypotensive response was seen. The highly significant positive correlation found between blood pressure fall and PRA is strong evidence that the renin angiotensin system is helping maintain blood pressure at that time in the patients on Spironolactone. The crossing of the abscissa by the regression line at PRA ≈ 1.0 $ng.ml^{-1}\ hr^{-1}$ illustrates the partial agonist effect of saralasin. That is, under conditions where circulating angiotensin II is low, saralasin, a competitive inhibitor of angiotensin II, has some

angiotensin-like activity and hence will raise blood pressure in the "low renin" group. These results are consistent with, and supportive of, the hypothesis propounded in the previous chapter that with increasing blood pressure there is a decrease in the activity of the renin angiotensin system and a decreased sensitivity of the renin angiotensin system to sodium deprivation. These data would indicate a rôle for the renin angiotensin system in defence against hypotension induced by volume depletion. The results do not, of themselves, provide direct evidence for a rôle in blood pressure maintenance in normal subjects or hypertensive patients on a normal sodium intake. The reason for this is that the major limitation on these experiments is that to discern the correlations it was necessary to use PRA values on a low salt diet or on a diuretic (which has a similar effect in this context) to predict which group of subjects had a renin system responsive to volume depletion and this was not always evident from normal diet values. Therefore, although conclusions may be drawn about the rôle of the response of the renin angiotensin system in blood pressure maintenance to volume depletion, one cannot directly infer the rôle of the renin angiotensin system under normal physiological conditions of free sodium intake. In order to do this, it is necessary to study inhibition of the renin angiotensin system in subjects on their normal diet. Orally administrable inhibitors of the renin angiotensin system have now made such studies possible.

CHAPTER SEVENTEEN

STUDIES INVOLVING *IN VIVO* INHIBITION OF THE RENIN-ANGIOTENSIN SYSTEM

Three specific inhibitors of the renin angiotensin system were used in this study. Two of these, only recently available, are specifically designed orally administrable inhibitors of angiotensin converting enzyme (Kininase II); they are known most widely by their trivial names Captopril and MK 421. Before these agents became available an earlier study was performed using saralasin ([Sar^1-Ala^8]-angiotensin I), a competitive inhibitor of angiotensin II. As this last compound is a peptide oral administration was not possible; also because it is a close analogue of angiotensin it does have some agonist activity particularly evident in the presence of low angiotensin concentrations.

Subjects and Methods

Normotensive volunteers whose diastolic blood pressure was less than 80 mm Hg, over a two month observation period, were included in the studies. Unselected patients with essential hypertension whose diastolic blood pressure was between 100 and 120 mm Hg, over a two month observation period, were also included. The majority of the hypertensive patients had not been previously treated. In those who had had previous therapy this had been stopped at least one month before, if they had been on a β-blocker, and at least three months before if they had been on a diuretic. Patients were excluded if they were taking any other drug, including the oral contraceptive pill, or if they had any evidence of renal failure, heart failure or cerebrovascular disease. It was not possible to age- or sex-match the control subjects with the hypertensive patients.

Blood pressure was measured on an operator-bias-free Arteriosonde 1217 automatic sphygmomanometer, using the mean of five values taken at one or two minute intervals. The blood pressure was always taken by the same nurse, at the same time of day (10 a.m.-12 noon) in the same room

and on the same arm. Blood for plasma renin activity and other tests was taken whilst the subject was sitting upright and had been so for 10 minutes.

The low sodium diet was provided by the metabolic kitchen and contained 10 mmol of sodium. Normal diet varied from study to study; for each study it was either the subjects' usual sodium intakes or a diet containing a fixed 120 mmol sodium for all the subjects. High salt diet was achieved by supplementation of the normal diet with 200 mmol of slow sodium (CIBA).

Mean blood pressure was calculated as the diastolic blood pressure plus one third of the pulse (systolic-diastolic) pressure.

Saralasin was given as an incremental infusion; 0.05 $\mu g.kg^{-1}.min^{-1}$ for 20 minutes and then 1.25 $\mu g.kg^{-1}.min^{-1}$ for 20 minutes. Patients and normals were infused sitting upright with a 1 hour control period before and after infusion. The infusions were carried out on the normal diet or the fifth day of low and high sodium diets. The percentage change in blood pressure was calculated from the change in mean blood pressure in the 20 minutes prior to infusion and the last 10 minutes of the highest rate of infusion.

Captopril was given in graded dosage. In normal volunteers 25 mg was given three times a day on day 1, increasing to 3 x 50 mg on day 2 and 3 x 100 mg on days 3 and 4. One final 100 mg dose was taken on the morning of day 5. In patients with essential hypertension the 25 mg tds dose was continued for one week rising to 50 mg tds for the whole of week 2, 100 mg tds for week 3 and 150 mg tds for week 4. In two patients the diastolic blood pressure fell below 85 mg Hg on the third week of treatment (100 mg tds) therefore their dose was not increased in week 4. However, as the blood pressure was above 85 mm Hg at the end of week 4, they were given 150 mg tds for week 5. For the purposes of data analysis

these "fifth week" results were used rather than the fourth (i.e. second on 100 mg tds) week in these two patients. A matched placebo study was performed in some of the patients with essential hypertension.

MK421 is a very new drug and the manufacturers kindly volunteered to assist in the setting up of a small pilot study in normal volunteers. MK421 in two doses (5 mg or 20 mg) or matched placebos was given in a single dose randomised double blind cross-over trial to assess its hypertensive action and effect on the parameters of the renin-angiotensin system.

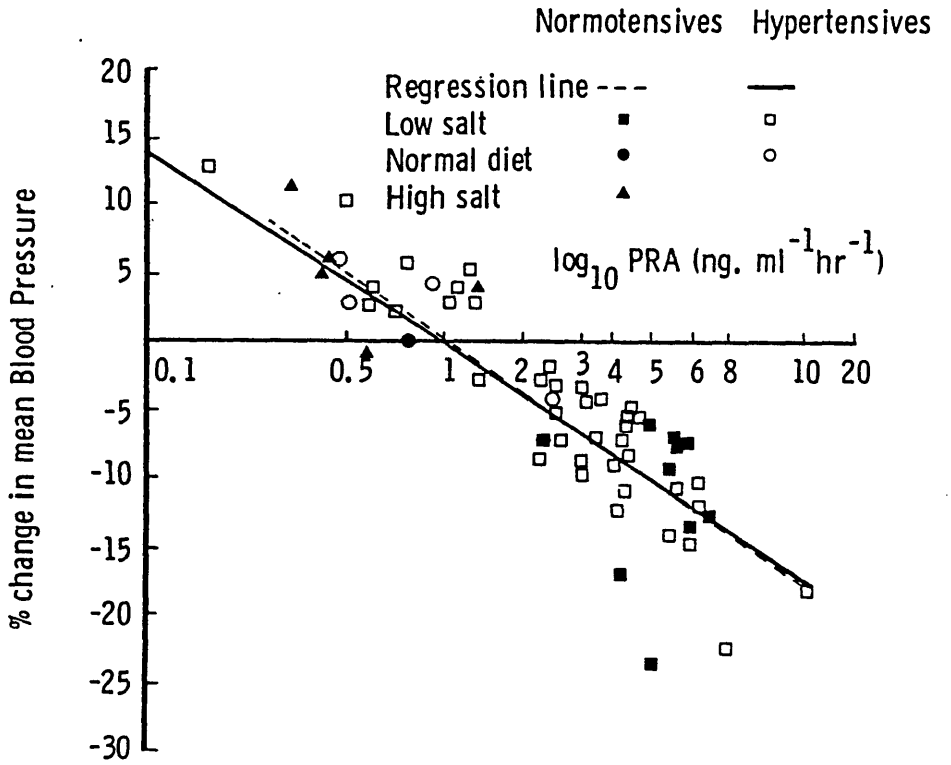
Saralasin study

Sixteen normal volunteers were studied; five on the high sodium diet, one on a normal (unrestricted sodium) diet and ten on a low salt diet. There was a highly significant correlation between the logarithm of the initial PRA and the percentage change in mean blood pressure with saralasin ($n = 16$, $r = -0.85$, $p < 0.001$).

Forty-eight patients with essential hypertension were then studied. Forty-four of these were infused whilst on the low sodium diet in order to make the group mean initial renin levels comparable with the normal volunteers. The four remaining subjects were infused whilst on their normal diets. Once again a highly significant correlation between the logarithm of the initial PRA and the percentage change in mean blood pressure with saralasin was noted ($n = 48$, $r = -0.88$, $p < 0.001$). The two correlations for normotensive and hypertensive subjects are shown in Figure 17.1. There was no significant difference in the slope or elevation of the two regression lines.

Figure 17:1

Correlation between percentage change in blood pressure and log PRA following saralasin infusion in normals and hypertensives.



Captopril study

Eight normotensive healthy male subjects were included in the first study. All were between 21 and 26 years. The subjects were studied on three sodium balances, 10 mmol, 120 mmol and 350 mmol. They were placed on each balance for three weeks with a gap in between each balance in which they ate their normal diets. Potassium intake was a constant 115 mmol in all three diets. Continuous 24 hour urine collections were made whilst the subjects were on the sodium balances. Vigorous excercise was discouraged.

Captopril therapy was begun on day 8 of the balance, measurements being made and blood being taken 2 hours after the first administration, and on days 4 and 5 of treatment.

The results from the eight subjects on a sodium intake of 120 mmol/day showed that a significant fall in blood pressure occurred within 2 hours of the first dose of Captopril and this fall was even greater by the fifth day (see Figure 17.2). The percentage fall in blood pressure with Captopril by day 5 was 16.5%. Figure 17.3 shows the fall in plasma angiotensin II and plasma aldosterone which occurred during Captopril treatment. The mean plasma renin activity rose markedly, from 3.3 $ng.ml^{-1}\ hr^{-1}$ to 41.4 $ng.ml^{-1}\ hr^{-1}$ by the fifth day of treatment. Table 17.1 shows the profile, over the course of the study of renin activities, angiotensin II, aldosterone and blood pressure.

Seven of the subjects were studied on a high sodium (350 mmol per day) diet. Captopril caused a significant fall in blood pressure (see Table 17.2) and plasma angiotensin II and aldosterone fell. PRA rose markedly.

On the low sodium diet (10 mmol per day) five subjects were studied. By the eighth day of the balance PRA, angiotensin II and aldosterone had all risen (see Table 17.3). Captopril caused a fall in blood pressure

Figure 17:2

Blood pressure responses during Captopril therapy in normotensive volunteers on a normal sodium diet.

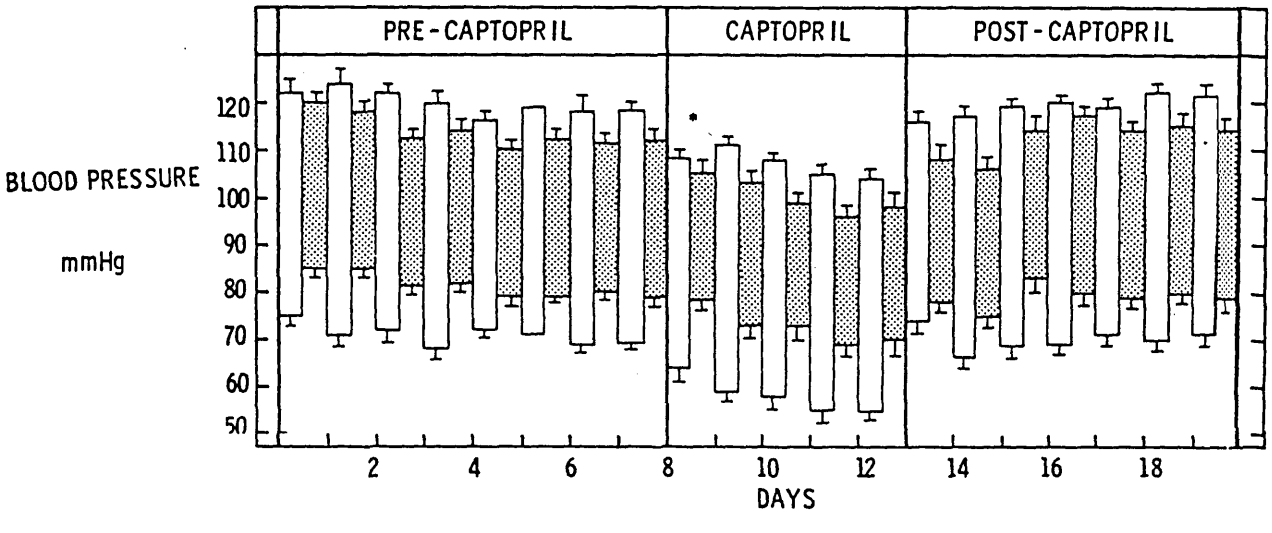


Figure 17:3

Fall in plasma aldosterone and plasma angiotensin II during Captopril treatment in normotensive volunteers on a normal sodium diet.

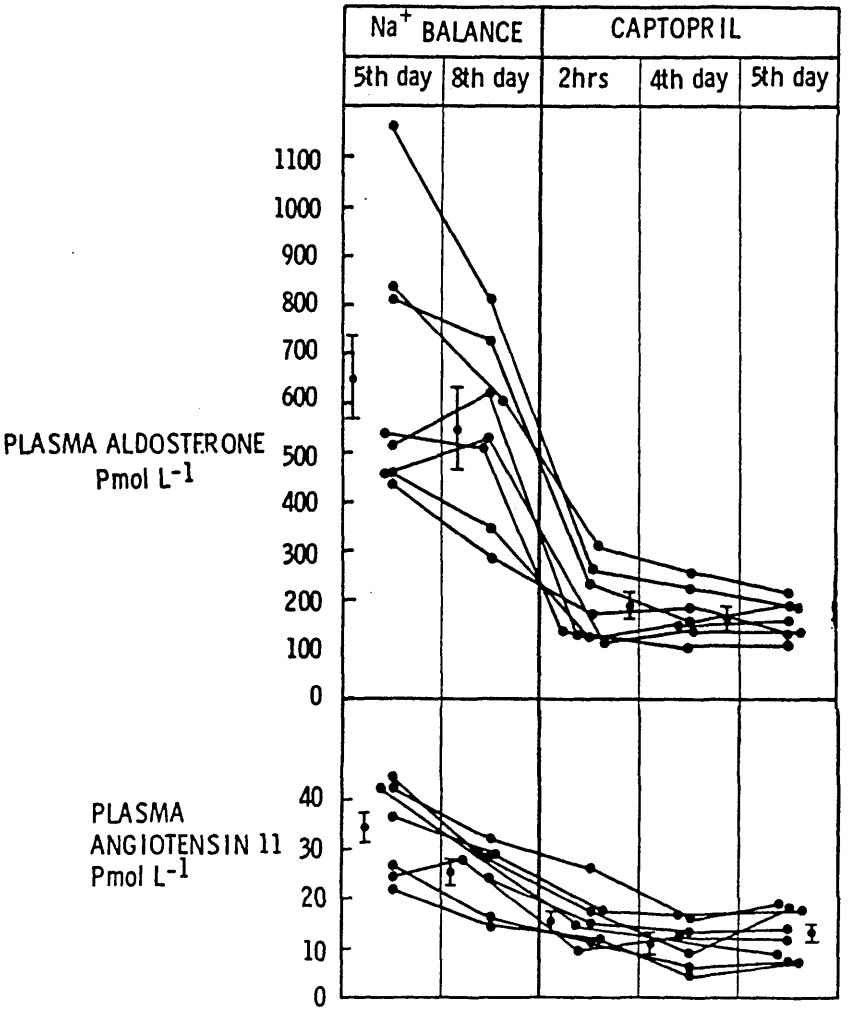


Table 17.1

Supine and standing blood pressure, plasma angiotensin II concentration, plasma renin activity and plasma aldosterone concentration in eight normal subjects on a 120 mmol per day sodium diet, before and during Captopril treatment. Mean values (±SEM) are given.

			Captopril		
	Day 5	Day 8	24h after 25 mg	Day 4 (100mg tds)	Day 5 (100mg tds)
Supine blood pressure (mm Hg)	116/72 (2.2/1.4)	118/69 (1.3/1.3)	103/64*** (1.4/2.8)	105/55*** (2.6/2.1)	104/55*** (2.3/2.6)
Standing blood pressure (mm Hg)	110/79 (2.0/2.3)	112/79 (2.9/2.4)	105/78*** (2.4/2.7)	96/68*** (2.5/3.1)	96/69*** (3.3/3.4)
Angiotensin II ($pg.ml^{-1}$)	34.4 (3.7)	25.3 (2.3)	15.5** (1.9)	11.0*** (1.7)	13.3*** (1.7)
Plasma renin ($ng\ ml^{-1}\ hr^{-1}$)	4.3 (0.8)	3.3 (0.5)	5.7*** (0.7)	45.7*** (9.6)	41.4*** (7.1)
Plasma aldosterone ($pmol.hr^{-1}$)	659 (99)	549 (64)	184*** (26)	164*** (21)	158*** (14)

Significance of differences between values on day 8 of balance before Captopril compared with values on Captopril.

* $p<0.05$

** $p<0.01$

*** $p<0.001$

Table 17.2

Supine and standing blood pressures, plasma angiotensin II, plasma renin activity and plasma aldosterone concentration in seven normal subjects on a 350 mmol per day sodium diet before and during Captopril treatment. Mean values (±SEM) are given.

			Captopril		
	Day 5	Day 8	24h after 25 mg	Day 4 (100mg tds)	Day 5 (100mg tds)
Supine blood pressure (mm Hg)	116/69 (2.7/3.2)	118/72 (3.3/3.1)	115/66** (3.1/2.8)	111/61*** (2.3/2.1)	112/61*** (2.3/3.4)
Standing blood pressure (mm Hg)	112/81 (3.3/3.4)	115/79 (3.3/2.8)	110/76** (4.3/2.0)	103/71*** (3.1/3.9)	103/71*** (3.6/2.2)
Angiotensin II ($pg.ml^{-1}$)	15.4 (2.5)	17.0 (2.6)	11.7 (2.7)	9.6*** (2.4)	7.9*** (3.2)
Plasma renin activity ($ng.ml^{-1}\ hr^{-1}$)	1.9 (0.4)	2.7 (0.4)	6.1* (1.5)	25.6*** (6.6)	27.9*** (5.6)
Plasma aldosterone ($pmol.hr^{-1}$)	201 (44)	318 (53)	112*** (15)	117*** (21)	115*** (16)

Significance of differences between values on day 8 of balance before Captopril compared with values on Captopril.

* $p<0.05$

** $p<0.01$

*** $p<0.001$

Table 17.3

Supine and standing blood pressures, plasma angiotensin II, plasma renin activity and plasma aldosterone concentration in five normal subjects on a 10 mmol per day sodium diet before and during Captopril treatment. Mean values (±SEM) are given.

			Captopril		
	Day 5	Day 8	24h after 25 mg	Day 4 (100mg tds)	Day 5 (100mg tds)
Supine blood pressure (mm Hg)	121/70 (3.4/1.8)	122/69 (0.6/2.5)	110/63*** (4.2/3.0)	100/57*** (4.1/5.3)	100/54*** (6.0/1.1)
Standing blood pressure (mm Hg)	110/78 (3.5/4.0)	109/80 (1.6/4.6)	100/68*** (4.3/6.3)	93/61*** (5.3/4.1)	93/60*** (3.0/5.8)
Angiotensin II ($pg.ml^{-1}$)	59 (11.2)	64.4 (9.3)	39.6 (11.5)	13.2*** (4.7)	11.0*** (2.5)
Plasma renin activity ($ng.ml^{-1}\ hr^{-1}$)	9.7 (2.8)	6.2 (0.9)	18.3** (3.8)	39.5*** (10.3)	44.6*** (3.0)
Plasma aldosterone ($pmol.\ hr^{-1}$)	1235 (260)	1395 (252)	641* (176)	395*** (138)	483*** (173)

Significance of differences between values on day 8 of balance before Captopril compared with values on Captopril.

* $p<0.05$

** $p<0.01$

*** $p<0.001$

which was significantly greater than that obtained on the high sodium diet ($p < 0.005$). The percentage fall in mean blood pressure was 19.6%. During Captopril treatment plasma angiotensin II and aldosterone fell and plasma renin activity rose.

The three diets, taken together, showed that plasma angiotensin II fell on Captopril in all three cases and plasma renin activity levels rose to an order of magnitude above "normal" levels.

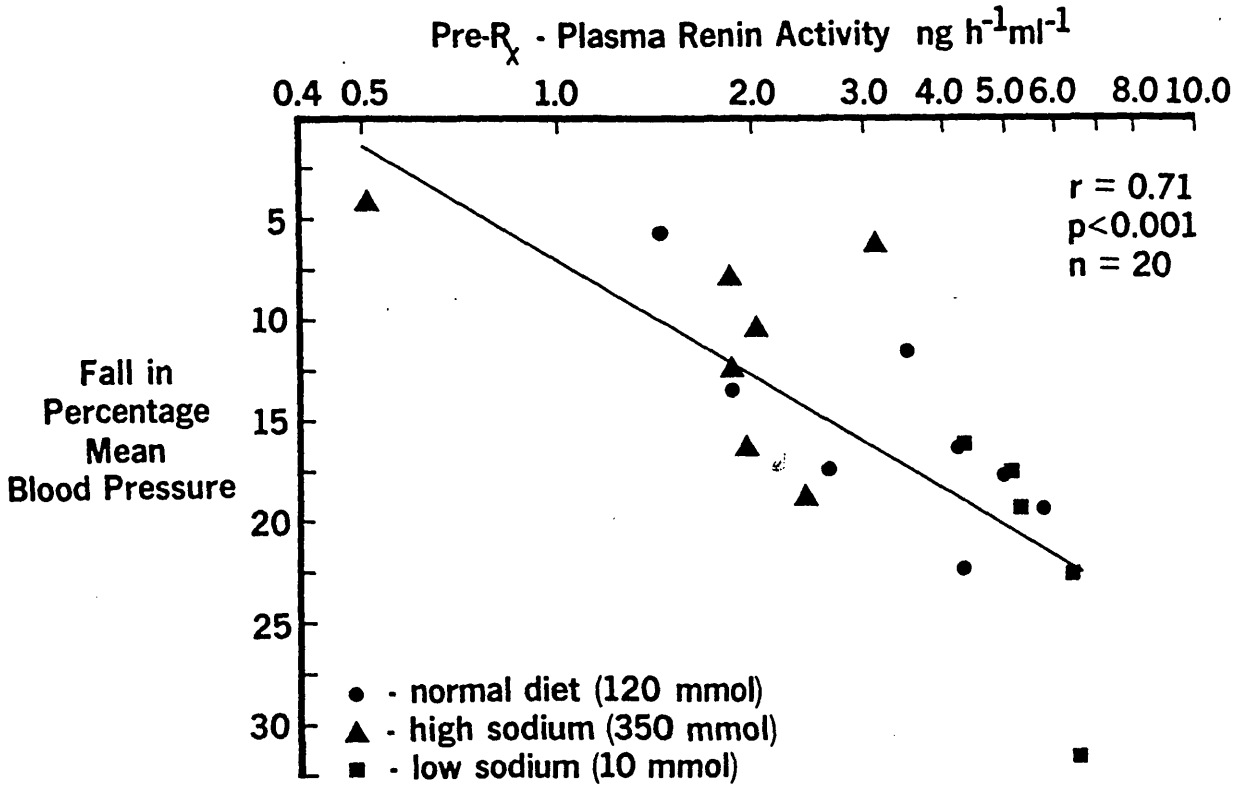
Figure 17.4 shows the correlation between the logarithm of the initial plasma renin activity and the fall in blood pressure ($n = 20$, $r = 0.71$, $p < 0.001$). There was also a significant correlation between the logarithm of the initial angiotensin II concentration and fall in blood pressure ($n = 20$, $r = 0.71$, $p < 0.001$), and a significant correlation between the logarithm of the fall in angiotensin II and the fall in blood pressure ($n = 20$, $r = 0.63$, $p < 0.001$). There was also a significant correlation between initial plasma renin activity, angiotensin II and the fall in angiotensin II against the fall in aldosterone that occurred with Captopril by day 5 ($n = 20$, $r = 0.66$, $r = 0.83$, $r = 0.78$ respectively, $p < 0.001$). These correlations are not strictly justifiable statistically as five of the subjects were studied on the three different diets and the diets were not achieved by chance. Nevertheless they do indicate a close relationship between the fall in blood pressure and the initial levels of PRA and angiotensin II and the fall in angiotensin II in these normotensive subjects.

Eighteen patients with essential hypertension were also studied on Captopril. Four weeks observation without treatment in six of the patients and four weeks on placebo in the other 12 made no significant difference to the mean blood pressures.

Two hours after the first 25 mg dose of Captopril the mean blood pressure fell significantly (144 to 121, $n = 15$, $p < 0.001$). No further

Figure 17:4

Correlation between the logarithm of PRA and fall in blood pressure during Captopril therapy in normotensive volunteers.



significant fall occurred in weeks 1, 2 and 3 but at the highest dose (150 mg tds, week 4) a further significant fall was seen (121 to 114, $n = 15$, $p < 0.01$). Figure 17.5 shows the average blood pressure, supine and standing, for all 18 patients and for the 15 after a single dose. The percentage fall in mean blood pressure at the maximum dose was 14.5%.

Plasma renin activity before active treatment was within the normal range in 13 patients and below normal in five. Plasma renin activities rose 2 hours after the first dose of Captopril and did not change significantly from this value during further treatment in these 15 patients. Table 17.4 shows the mean value for all 18 patients. The fall in blood pressure that occurred with Captopril by the end of the fourth week of treatment was significantly corelated with the logarithm of the pre-treatment of PRA value ($n = 18$, $r = 0.59$, $p < 0.005$). This correlation was in fact significant for fall in systolic, diastolic and mean blood pressures measured supine, sitting or standing 2 hours after the first dose and at subsequent weeks of the trial. The percentage fall in mean blood pressure was also significantly correlated against the logarithm of initial plasma renin activity (see Figure 17.6).

MK421 study

MK421 was given in a single dose randomised double blind cross-over trial using 5 mg, 20 mg and matched placebos to nine normotensive volunteers on a sodium intake of 150 mmol per day. Blood pressure (supine and upright) was measured at -2, -1, 0, +½, +2, +4, +6, and +24 hours and blood for measurement of plasma renin activity, converting enzyme and angiotensin II was taken at 0, +2, +6 and +24 hours after dosing. The subjects were allowed to pursue their normal activities throughout the day.

Figure 17:5

Average blood pressure, supine and standing, during Captopril therapy in patients with essential hypertension.

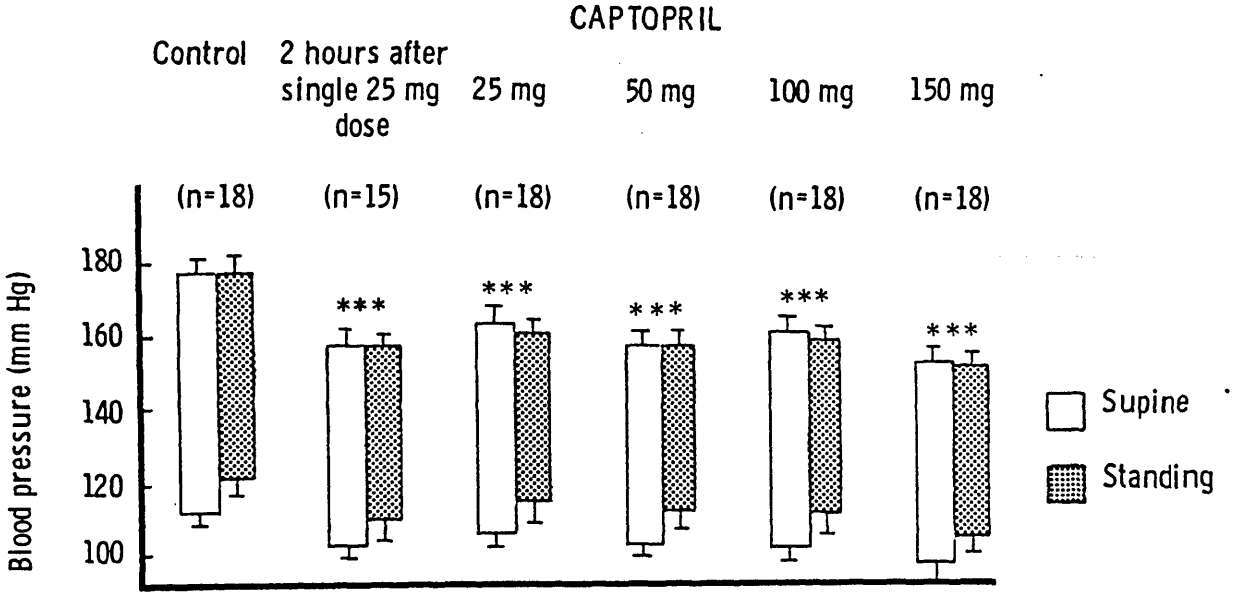


Table 17.4

Mean (±SEM) results of biochemical tests, weight and pulse rate before and during treatment with Captopril at increasing doses

		Captopril treatment			
	Control (n=18)	2 h after single 25 mg dose (n=15)	25 mg† (n=18)	50 mg† (n=18)	100 mg† (n=18)	150 mg† (n=18)
Plasma renin activity ng ml^{-1} hr^{-1}	0.07 (0.12)	1.83 (0.34)**	2.20 (0.36)***	2.76 (0.63)**	2.79 (0.57)**	3.02 (1.36)*
Plasma aldosterone pmol hr^{-1}	303 (31)	201 (34)***	225 (30)**	227 (28)**	198 (13)**	207 (20)**
Plasma potassium mmol hr^{-1}	3.91 (0.07)	3.91 (0.12	4.07 (0.11)	4.19 (0.09)**	4.17 (0.08)**	4.10 (0.077)*
Weight (kg)	76.4 (3.0)	76.3 (3.0)	75.8 (3.0)*	75.5 (3.0)**	75.1 (3.1)***	75.1 (3.0)***
Pulse rate, supine beats/minute	80 (2.5)	79 (2.7)	76 (2.1)	75 (2.4)	78 (3.6)	75 (2.3)

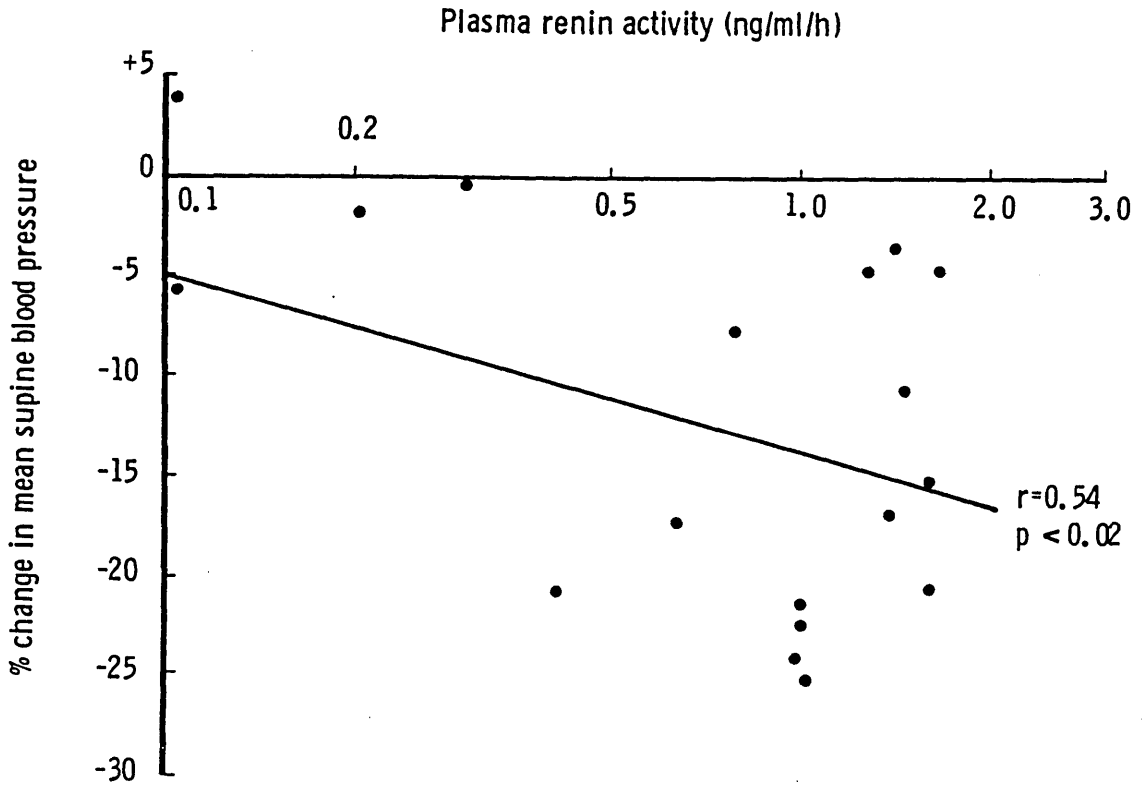
†Dose given thrice daily for one week.

Significance of difference: *p 0.05; **p 0.01; ***p 0.001 compared with control values.

For each plasma renin activity and aldosterone concentration values with increasing doses of captopril were not significantly different from values 2 hours after a single 25 mg dose.

Figure 17:6

The percentage fall in mean blood pressure plotted as a function of logarithm of PRA in hypertensive patients.



Placebo caused no significant change in blood pressure, PRA, angiotensin II or converting enzyme. Either dose of MK421 produced significant falls in blood pressure which were maximal at 4 to 6 hours ($p < 0.001$). Five mg produced a fall of 11.0% in mean blood pressure at 4 hours (10.4% at 6 hours) whilst 20 mg produced a 9.7% fall at 4 hours (9.6% at 6 hours). The blood pressure falls with 5 or 20 mg were not significantly different at any time after dosage. Both doses produced significant rises in PRA and significant falls in angiotensin II and converting enzyme activity. These changes were more pronounced with the higher dose. The results are summarised in Table 17.5.

Discussion

The results of the saralasin infusion demonstrate that the same percentage fall in mean blood pressure for a given PRA was obtained with normal subjects and hypertensive patients. The results indicated also that when plasma renin activity was above 2-3 $ng.ml^{-1}\ hr^{-1}$ then the renin angiotensin system was maintaining blood pressure and doing so to the same extent in normal and hypertensive subjects. Saralasin is a potent agonist however (MacGregor and Dawes, 1976) and when given as a short-term infusion blocks only the immediate effects of circulating angiotensin II. Both of these factors will tend to underestimate the participation of the renin angiotensin system in the control of blood pressure.

The mechanism whereby Captopril lowers blood pressure is not completely resolved. Its only clearly demonstrated rôle is as an inhibitor of angiotensin converting enzyme and this was reflected by the rise in PRA and fall in angiotensin II and aldosterone in the normal

Table 17.5

Results of a single dose randomised double-blind cross-over trial with MK421, an inhibitor of angiotensin converting enzyme. Results expressed as mean (± SEM). n = 9, n = 8*.

Treatment - Placebo

Time in hours	0	+2	+4	+6	+24
PRA	1.68 (0.44)	1.92 (0.42)	-	1.49 (0.16)	1.74 (0.27)
Angiotensin II	*15.8 (2.2)	18.0 (1.9)	-	16.3 (1.4)	16.2 (1.6)
Converting enzyme activity	94.4 (11.1)	100.9 (14.8)	-	98.0 (15.3)	95.9 (12.1)
Mean blood pressure	84.5 (2.6)	-	85.7 (1.54)	87.3 (2.65)	87.0 (2.43)

Treatment - 5 mg MK421

Time in hours	0	+2	+4	+6	+24
PRA	1.76 (0.52)	2.22 (0.59)	-	4.06 (1.15)	1.80 (0.25)
Angiotensin II	14.3 (2.5)	11.3 (2.1)	-	9.9 (3.8)	15.8 (2.0)
Converting enzyme activity	119.0 (18.7)	19.9 (5.3)	-	12.6 (4.5)	30.7 (6.6)
Mean blood pressure	96.9 (2.9)	-	77.3 (2.3)	77.9 (2.5)	82.0 (3.2)

Treatment - 20 mg MK421

Time in hours	0	+2	+4	+6	+24
PRA	2.16 (0.35)	6.89 (1.9)	-	5.22 (1.13)	3.83 (0.53)
Angiotensin II	17.5 (2.2)	8.5 (2.0)	-	8.9 (1.34)	19.6 (2.6)
Converting enzyme activity	105.4 (11.6)	12.6 (4.2)	-	10.4 (3.7)	23.6 (5.1)
Mean blood pressure	86.8 (2.1)	-	78.4 (2.1)	78.5 (2.0)	84.8 (1.50)

subjects studied on high, normal or low dietary sodium intakes. The blood pressure fell significantly with treatment in the normotensive volunteers on all three diets, the fall being greatest on the low salt diet and least on the high salt diet. The results also indicate a close relationship between the fall in blood pressure and the initial plasma renin activity, initial angiotensin II and the fall in angiotensin II. These correlations would suggest that part of the mechanism by which Captopril lowers blood pressure is through reduction of angiotensin II concentrations, and, conversely, that the initial angiotensin II levels were therefore responsible for blood pressure maintenance in the normal subjects on all three diets.

It is possibly surprising that the fall in blood pressure on the low sodium diet was not much higher than on the high salt diet. This is possibly explained by the increased sensitivity of vascular receptors to angiotensin II in salt-loaded normals by comparison with sodium restricted normals (Oelkers *et al.*, 1974). There is also recent evidence to suggest that the absolute number of angiotensin II receptors in resistance vasculature is diminished in the presence of high angiotensin II concentrations (Gunther *et al.*, 1980) which would also tend to auto-regulate the rôle of angiotensin II when the levels increased.

Comparison of the results obtained from the normotensive volunteers with those from patients with essential hypertension reveals that Captopril appears to cause the same percentage fall in mean blood pressure for a given plasma renin activity in both normotensives and in patients with essential hypertension. This suggests that the renin angiotensin system plays no greater rôle in maintaining essential hypertension than in maintaining normotensive blood pressure levels, for a given level of plasma renin activity. These data with Captopril suggest that the renin

angiotensin system is a normal mechanism for blood pressure maintenance in normotensive subjects and in patients with essential hypertension and not simply a defence mechanism in cases of volume depletion. Furthermore, the data indicate that the renin angiotensin system is unlikely to be the cause of the high blood pressure unless there was inappropriately high angiotensin II levels for a given sodium balance or increase in sensitivity to angiotensin II in hypertensive subjects.

It is also of note that the PRA in the normals rose far more dramatically than in the hypertensive patients after Captopril was administered. It has already been postulated (Chapter 15) that the renin angiotensin system could be more 'sensitive' or responsive to sodium deprivation as judged by the greater rise in PRA in normotensives by comparison with hypertensives, an increase which is not significantly related to the age of the groups. However, the mean age difference in the Captopril study (normals 21-26; hypertensives 41-65) must render any inferences open to doubt, although it is perhaps possible that the greater "responsiveness" of the renin angiotensin system in normotensive subjects is a phenomenon inducible by mechanisms other than sodium deprivation. This could imply a common stimulatory mediator or perhaps a coenzyme. Until studies using age- and sex-matched groups are undertaken, however, it would be unwise to dwell upon this possibility.

The results of the single-dose double-blind cross-over trial with MK421 confirm that inhibition of converting enzyme in normotensive man on a normal dietary sodium intake produces a fall in blood pressure similar to that seen with Captopril indicating a rôle for the renin system in blood pressure maintenance under normal physiological conditions.

Williams and his colleages (Williams, 1977; Williams and Hollenberg, 1977) have postulated that it is the vasodilatory action of accumulated bradykinin which causes the fall in blood pressure seen

with Captopril (and, presumably other converting enzyme inhibitors). However, patients do not have symptoms of raised bradykinin levels and it has recently been shown by Matthews, McGrath and Johnston (1979) that plasma bradykinin levels are not elevated by Captopril therapy or by the parenteral inhibitor "Teprotide" (Hulthen and Hokfelt, 1979).

The probability that two specifically designed orally administrable inhibitors of angiotensin converting enzyme, which are chemically dissimilar, should each have a second common hypotensive effect must be regarded as vanishingly small. The overall conclusion must be that the major hypotensive effect of these agents is through converting enzyme inhibition and the significant falls in angiotensin II which this causes.

CHAPTER EIGHTEEN

THE DIFFERING EFFECTS OF SODIUM RESTRICTION, SPIRONOLACTONE, PROPRANOLOL AND CAPTOPRIL IN BLACK AND IN WHITE PATIENTS WITH ESSENTIAL HYPERTENSION

During the course of the work reported in the previous three chapters it was noticed that a higher proportion of black (negro) patients with essential hypertension had low PRA values by comparison to the white (caucasian) hypertensive patients.

From the inhibition studies it has been postulated that the maintenance of blood pressure by renin is, firstly, a direct function of the PRA value; that is the higher the PRA the greater its rôle in blood pressure maintenance. Secondly, it has been suggested that in general the hypertensive subjects tend to have lower renin values. Thirdly, it has been seen that these lower renin subjects have a muted response of the renin angiotensin system to sodium deprivation or angiotensin converting enzyme inhibition.

It was therefore decided to study blacks and whites in order to compare and contrast the effects of dietary sodium perturbation, Propranolol, Spironolactone and Captopril in the two groups. If the rôle of the renin angiotensin system is comparable, in principle, between the two groups, rather as suggested for its rôle in both normotensives and patients with essential hypertension the results should be predictable. Firstly one would expect the more muted response to Captopril, Spironolactone and sodium deprivation to come from the low renin (black) group and this would reflect in the greater hypotensive effects of the low sodium diet and Spironolactone. Conversely Propranolol and Captopril would be expected to be more efficacious hypotensive agents in the normal renin (white) group. Plasma renin activities would be expected to rise with sodium deprivation, Spironolactone and Captopril to a greater extent in the normal renin subjects.

Fifty white (caucasian) and 26 black (negro) patients were studied on a normal, high sodium (normal + 200 mmol slow sodium) and low salt (10 mmol) diets. The blood pressure and plasma renin activities were

measured on all diets. Twenty blacks and 20 whites who were age and sex-matched were then studied in a randomised cross-over trial between Spironolactone (100 mg per day) and Propranolol (320 mg per day). Each period of treatment lasted six weeks.

The effects of Captopril (150 mg tds for six weeks) were also compared in 21 whites and 15 blacks.

The table (18.1) shows the mean results for blood pressure, plasma renin activity and urinary sodium on the three regimes. The PRA in the blacks was less than half the corresponding value in the whites on all three regimes ($p<0.001$). The low sodium diet produced a greater fall in mean blood pressure in the blacks (12.4% as compared with 8.4% in the whites). The increase in PRA on the low sodium diet was greater in the whites than the blacks ($p<0.001$).

The blood pressure lowering effect of Spironolactone was less in the whites (6.6%) than the blacks (13.1%) ($p<0.02$). This was presumably due to the greater increase in PRA in the whites ($p<0.001$) which would offset the diuretic's hypotensive action.

With Propranolol the greater hypotensive effect was seen in the whites and where a mean fall of 14.9% was observed. The blacks had a significantly smaller fall (7.4% $p<0.001$). Similarly the fall in PRA in the whites was significantly greater than the fall in blacks (0.62 and 0.17 ng ml^{-1} hr^{-1} respectively, $p<0.001$).

Captopril was much more effective in lowering blood pressure in the whites as compared to the blacks (17.0% and 7.6% respectively, $p<0.001$). The rise in PRA with Captopril was much greater in the whites (2.54 ng ml^{-1} hr^{-1}) compared to the blacks (0.70 ng ml^{-1} hr^{-1}, $p<0.001$). The correlation between blood pressure fall on Captopril and pre-treatment PRA was highly significant in the two groups together ($n = 36$, $r = 0.69$,

Table 18.1

Mean blood pressure, percentage fall in mean blood pressure, plasma renin activity and urinary sodium levels in black and in white patients with essential hypertension on low, normal and high dietary sodium intakes.

Group	White (n=50)			Black (n=26)		
Dietary sodium	Low	Normal	High	Low	Normal	High
Mean blood pressure	123.7	135.0	135.7	122.3	129.6	138.3
% Fall in mean blood pressure from normal to low salt		8.4%			8.4%	
Plasma renin activity (ng ml^{-1} hr^{-1})	2.97	1.07	0.65	1.31	0.44	0.28
Urinary sodium (mmol)	21	142	281	20	158	280

$p<0.001$). Table 18.2 shows a synopsis of the results obtained. These results are consistent with the idea proposed that the maintenance of blood pressure by the renin angiotensin system is directly related to the level of PRA in the subject concerned. Furthermore the blacks show a much smaller response in elevation of PRA to Captopril and Spironolactone by comparison to the whites, indicating that the higher, pre-treatment, level in the whites is an index of the ability of the system to respond to angiotensin converting enzyme inhibition or sodium deprivation. The response of the white hypertensive subjects, whilst being significantly greater than that in the black hypertensives, was still a lot less than that seen in normotensives, the normotensives pre-treatment PRA also being higher than either of the hypertensive groups.

Table 18.2

Mean blood pressure, fall in blood pressure, plasma renin activity before and during therapy with Propranolol, Spironolactone or Captopril, comparing black and white subjects with essential hypertension.

	Propranolol		Spironolactone		Captopril	
	Whites	Blacks	Whites	Blacks	Whites	Blacks
Mean BP pre-treatment	130	134.6	126	134.6	134.6	140
Mean BP on treatment	110.7	124.6	117.7	117.0	111.7	129.3
% Fall in mean BP	14.9%	7.4%	6.6%	13.1%	17.0%	7.6%
PRA pre-treatment	1.10	0.31	1.05	0.31	1.77	0.66
PRA on treatment	0.48	0.14	3.61	0.86	4.31	1.36
No. of subjects	20	20	20	20	21	15

CHAPTER NINETEEN

GENERAL DISCUSSION

The first part of this work is largely concerned with the development and assessment of assays for the measurement of plasma renin activity and plasma angiotensin II concentration. The radiolabelling techniques were shown to yield tracers of high specific activity with little radiolysis of peptide occurring. The use of ion exchange column chromatography enabled the radiolabelled tracers to be separated successfully not only from iodide but from unlabelled peptide. The "self-displacement" technique for estimation of specific activity is employed in many laboratories and if recent criticism (Morris, 1976) were justified this would have important consequences with regard to tracer assessment. In the present work, however, a detailed evaluation showed the procedure to be valid both theoretically and practically (Roulston, 1979).

Primary antisera were produced in rabbits after injection of a carbodiimide-coupled antigen-thyroglobulin complex. These complexes were made using molar ratios of reactants advocated for other small molecular weight antigens by Skowsky and Fisher (1972) and the hormone incorporations obtained were similar to those found by Skowsky and Fisher. The positive immune response seen in all animals supports the contention that thyroglobulin is a good carrier molecule for small molecular weight antigens, especially as in three animals where an angiotensin-BSA complex was used as immunogen no immune response was seen.

Scatchard (1949) analysis of the antisera indicated the presence of only one class of binding sites for each of the antibodies selected for subsequent use. Estimations of the avidity, as reflected by the equilibrium constants, indicated high affinity. Similar values for the equilibrium constants were obtained when the data were analysed

by the Odell (Odell *et al.*, 1959) saturation technique. The antiangiotensin II exhibited some metabolite cross-reactivity whereas the antiangiotensin I did not cross-react with angiotensin II.

Sealey and Laragh (1975) observed an increase in plasma renin activity in samples from normal subjects and from patients with essential hypertension after the samples had been stored at -20°C. They reported mean increases, upon re-assay, of 26% after two months and 55% after one year. Such results are potentially worrying for the long-term storage of plasma quality control pools and for the assay of samples which have been stored. In the course of this work it was shown that plasma samples stored at -20°C were stable for over 15 months (Roulston and MacGregor, 1978), in direct contrast to the results of Sealey and Laragh. However, in this work all plasma quality controls were stored as separate aliquots and discarded after assay, whereas it would appear that Sealey and Laragh re-assayed the majority serially. It is possible that this serial freezing and thawing accounted for their observed increase in renin-like activity. Osmond *et al.* (1973) noted a rise in plasma renin activity after storage at 4°C for three days, so that deep-freeze failure could account for increased activity. The results reported in this work are supported indirectly by the results of Lijnen *et al.* (1977) who measured total plasma renin concentration biologically (Skinner, 1967) and by radioimmunoassay (Lijnen *et al.*, 1976) and found no increase by either method in plasma samples stored at -20°C for up to one year.

The *in vitro* generation of angiotensin I was found to be reproducibly controlled by the conditions employed. The combination of inhibitors of converting enzyme and angiotensinases was found to be virtually 100% efficient under the conditions of pH and temperature employed over the time course used. The pH profile of renin indicated

that the optimum pH was between 5,5 and 6,0. There has been considerable controversy over which pH is the most suitable for generation of angiotensin I *in vitro*, although almost all workers are agreed on the need to buffer the plasma to keep the pH constant throughout the generation period. Generation of angiotensin I at pH 5,7 has the advantage of increasing the sensitivity of the assay by virtue of generating greater amounts of angiotensin per unit time than physiological pH of 7,4. Also, being the pH optimum, slight fluctuations in pH, up to for example ±0,25 of a unit do not cause swingeing changes in rate. pH 7,4 falls on the steep slope of the renin pH profile and is therefore more subject to change in rate with change in pH, also as less angiotensin I is generated per unit time the sensitivity of the assay is lessened. Oparil *et al*, (1974) reported that in samples from anephric patients generation at pH 5,5 yielded angiotensin I whereas generation at pH 7,4 did not and suggested the possibility that artefacts may arise at pH 5,5 due to acid protease activity. When samples from anephric subjects and subjects with normal renal function but unmeasureable PRA values were generated at acid pH and neutral pH in this laboratory, however, no such threshold of activity was visible. In subjects (anephric or otherwise) with detectable PRA at pH 5,7 detectable PRA was also observed at pH 7,4 although generation proceeded at a slower rate at pH 7,4 as would be expected from the pH profile. The reasons for this difference between the studies are possibly two-fold. Firstly, an arbitrary threshold of PRA could be obtained by virtue of the limit of detectability of their assay which would affect the pH 7,4 samples first. Secondly, the samples used by Oparil *et al*. at pH 5,5 were not buffered but adjusted to pH by 0,5 molar hydrochloric acid. Such a procedure would cause dramatic local pH falls and could generate the acid protease artefacts Oparil *et al*. describe.

PRA values in samples generated at pH 5.7 were shown to correlate well with PRA from identical samples generated at pH 7.4 over a wide range of values. In no case was it possible to demonstrate a fundamental difference in the generation process with change in pH; merely the alteration of rate of production of angiotensin I. One possible situation where PRA at pH 5.7 would not be a linear function of PRA at pH 7.4 would be when the concentration of substrate became limiting during the course of the generation process. Therefore it was essential to check the limits within which generation of angiotensin I at pH 5.7 was rectilinear with respect to time. It was shown by stopping the generation of aliquots of samples at various times that the linear relationship held good up to about 50 $ng.ml^{-1}\ hr^{-1}$. These findings were confirmed for large populations of samples by a new analytical technique in which over 2500 samples were assessed by cluster analysis in which PRA was correlated with the angiotensin I concentration in the samples prior to generation (Roulston *et al.*, 1978).

The addition of PMSF to plasma samples caused some precipitation within the samples. Aliquots of the suspension and the supernatant gave identical results in the manual PRA radioimmunoassay indicating that this physico-chemical heterogeneity did not partition the angiotensin I and enabled supernatant assay to be attempted in the automated assay to prevent the risk of the syringes or pumps becoming blocked as might happen if the whole plasma were used.

The radioimmunoassays for PRA, manual and automated, showed good precision; from about 2.5 pg per tube (49%) to about 34 pg per tube (2.12%) over the range of the dose response curve. The sensitivity of the assays was of the order of 2.5 pg per tube. Specificity was assessed by cross-reactivity studies, recovery studies, parallelism tests and the direct comparability of the two assays. No cross-reactivity with

angiotensin II was observed. Recovery of added angiotensin I was virtually 100% in both assays (coefficients of variation for the manual and automated assays being 3.84% and 2.56% respectively). When samples were doubly diluted and plotted on the same axes as the dose response curve no significant difference in slopes of any sample with respect to that of the standard curve was observed except for one plasma with respect to the manual assay curve. In this case the correlation coefficient of that particular sample was so close to unity that almost any difference in slope would be rendered statistically significant although the two lines exhibited virtual identity.

Addition of anephric "background" plasma to the standard curves did not alter the dose response curve indicating that interfering substances were not present in critical concentrations. Direct comparison of results of samples assayed manually and on the "Kemtek" yielded a very high correlation ($r = 0.99$) with a slope approaching unity and intercept tending to zero. Since the basic reagents were identical in both systems this was not surprising.

The reproducibility of the assays was assessed by the analysis of data from quality control pools. The intra-assay quality control values yielded coefficients of variation of between 4 and 7% for the two assays. Interassay coefficients of variation of between 6% and 13% were observed.

The assay was successfully adapted to an automated system (Roulston *et al.*, 1979). This system has the advantage of a greater sample throughput and is also not operator dependent to such a large extent. For PRA evaluation on a large scale the system is attractive but for small-scale assays the saving in operator time is less significant, but relative waste of reagents (in priming, etc.) is

higher and the supernatant transfer step becomes a major time factor. Furthermore the manual assay system is more flexible and involves considerably less capital outlay.

Extraction of angiotensin II from plasma was achieved by adsorption on to Dowex resin. Routinely, between 80 and 90% extraction of angiotensin II was achieved by this method. These results agree with those quoted by Morton *et al.* (1976). The reproducibility of the technique was assessed both within and between batches and a coefficient of variation of about 10% found in both cases. The plasma samples for angiotensin II immunoassay were taken into a syringe containing a phenanthroline/neomycin/EDTA inhibitor to block both angiotensin-converting enzyme and angiotensinase activity. The efficiency of the inhibitor in prevention of *in situ* angiotensin II formation was checked by assay of samples for converting enzyme activity. No ACE activity was apparent even after incubation of the sample overnight at room temperature. The ability of the inhibitor to prevent angiotensin II degradation was assessed by assay of plasma samples containing a known added concentration of angiotensin II at time zero and after 2 hours. No significant fall in angiotensin II recovery was seen in the samples assayed after 2 hours with respect to those assayed immediately, indicating that the inhibitor mixture effectively prevents angiotensin II hydrolysis for at least 2 hours at room temperature.

The precision of the radioimmunoassays for angiotensin II varied from about 2.8 pg (71.8%) to about 32 pg (3.2%) over the range of the standard curves. The sensitivity of the manual assay was, however, twice as good in practice as that for the automated assay by virtue of the necessity to doubly dilute the sample in the automated system. Since the limit of detectability of the assays is not grossly below the lower

limit of the normal range this doubling of the effective cut-off is a severe restriction.

Specificity of the angiotensin II assays was assessed by cross-reactivity, recovery, parallelism and comparibility of the two assays. The cross-reactivity pattern was similar to that reported by Morton *et al.* (1976) with there being considerable (>80%) cross-reactivity with the metabolites (hepta-, hexa- and pentapeptides). Hence such results as are interpolated from radioimmunoassays for angiotensin II should be regarded as an index of angiotensin II concentration rather than a true *in vivo* concentration.

Recovery, of added angiotensin II to the reconstituting buffer, was virtually total (±6.4%). Since the standards and "samples" were totally buffer-based this was perhaps a predictable result. Parallelism of samples with respect to the "hypertensin" standards and human angiotensin II standard was checked as was the parallelism between the two standards. In all comparisons there was no significant difference in the slopes of the regression lines. Direct comparison of 25 samples measured in both assays yielded a correlation coefficient of ∿0.97 ($p<0.001$), the slope being 1.007 and intercept -0.5067. Removal from the analysis of one pair of values where the automated assay had returned an undetectable result (arbitrarily assigned 0.01) failed to alter the correlation coefficient or slope but brought the intercept virtually to zero.

Reproducibilities, as assessed by analysis of quality control data, indicated intra-assay coefficients of variation of between 4 and 9%. The interassay coefficients of variation were between 6 and 15%. The coefficient of variation of the low value quality control pool in the automated assay was the highest in both intra- and interassay analyses, indicative perhaps of the poorer sensitivity.

Although the assay was adapted to an automated system successfully, the very low normal range (down to 5 $pg.ml^{-1}$ of plasma) meant that the sample dilution necessitated by the machine was a severe limitation on the usefulness of this assay. Unless an antibody of much higher avidity were found or the programme modified to allow the probe to sample differently it would not be possible to routinely use the automated system. This problem with limit of detectability outweighs the advantages which the system offers in terms of higher throughput and less operator time.

Converting enzyme activity was measured using the radioassay technique of Ryan and his colleagues (1977), which employs a tritiated analogue-substrate. The method was checked against that of Friedland and Silverstein (1976) who developed a fluorimetric technique. Samples from normal subjects and patients with active sarcoidosis were kindly supplied, identified only by a code, by Dr. R. Bird (Royal Northern Hospital, London, N.6) after he had assayed them fluorimetrically. The samples were then re-assayed by the author by the radioassay technique. The correlation between the two methods was high ($r = 0.98$, $p<0.001$). The reproducibility of the radioassay was assessed by intra- and inter-assay assessment of quality control pools. Intra-assay coefficients of variation were about 7% and interassay coefficients of variation were about 11.5%.

In preliminary studies with captopril (SQ 14225) it was found that de-inhibition of angiotensin-converting enzyme had apparently occurred whilst the plasma samples were under storage at -20°C. More detailed study showed that this de-inhibition was progressive with time and virtually complete within weeks (Roulston *et al.*, 1980). These findings have recently been confirmed by Dux *et al.* (1981) who used a different

analogue-substrate and demonstrated de-inhibition even after sample storage at -70°C indicating that the results are not a methodological artefact. This finding is clearly important for future studies with converting enzyme inhibitors and also indicates that previous reports regarding inhibition of angiotensin converting enzyme by captopril where the samples were stored prior to assay must be interpreted with great caution, or perhaps the work repeated.

Plasma renin activity was assessed as an *in vitro* index of plasma angiotensin II by correlation with plasma angiotensin II levels. Although PRA has been widely used as a function of angiotensin II the constancy of the relationship has not been comprehensively assessed in differing situations. Some published studies have been criticised for correlations of non-normally distributed data (Gocke *et al.*, 1969). This criticism has also been levelled at workers attempting to correlate plasma renin concentration with plasma angiotensin II levels (see Swales, 1976; Leckie *et al.*, 1976a and 1976b). Furthermore, extrapolation of the correlation curve to include excessively high values and add "weighting" to the regression analysis has also been noted (Swales, 1976). There has been an indication (Sambhi *et al.*, 1976) that the correlation between PRA and angiotensin II is significantly different in patients with essential hypertension who are undergoing chronic diuretic therapy as compared to an untreated group of patients with essential hypertension.

The results from the present work indicate that the relationship between PRA and plasma angiotensin II is linear, highly significant and, more importantly perhaps, constant under all clinical conditions studied. No significant difference was found in the slopes of the regression lines when PRA was correlated with angiotensin II in normotensives and hypertensives on high, normal or low dietary sodium intakes, or with the

data from all diets combined. Also when the normotensive and hypertensive groups were studied separately no change in slope was seen when the salt diets were varied over a wide (350-10 mmol/24 hr) range. Furthermore, when the hypertensive subjects were given a β-blocker (Propranolol) or a diuretic (Spironolactone) the slope of the PRA-angiotensin II relationship was the same on either treatment. When the values on Propranolol were compared with either the normals on a high salt diet or the hypertensives on a high salt diet there was no significant change in slope. Similarly, when the data from the Spironolactone study were compared with either normals or hypertensives on a low salt diet there was no change in the slope of the regression line.

The results of this assessment indicate that the use of PRA as an index of plasma angiotensin II is quite justifiable in studies involving both normotensive and hypertensive subjects on a variety of dietary sodium intakes and when patients are receiving β-blocker or diuretic therapy. The one area of investigation where the relationship would patently be of little use would be when converting enzyme blockers were administered in which case in inverse relationship would be seen.

The second part of this study is concerned with investigation of the rôle of the renin angiotensin system in blood pressure control. The first clinical study examined the relationship between plasma renin activity and blood pressure in normotensive volunteers and patients with mild and severe essential hypertension who were not on any treatment. The subjects were investigated on various dietary sodium intakes.

Correlation of PRA with mean arterial pressure for each group on each diet separately did not yield any significant correlations, implying that PRA was not a useful index of blood pressure within the small ranges considered. In normal subjects on a low salt diet, however, the correlation was positive and approached statistical significance. Also severe dietary sodium restriction did lower blood pressure significantly in the groups with essential hypertension. Furthermore the mean PRA values are progressively lower as the groups' mean blood pressure increases. Indeed correlation of blood pressure of all subjects on a free dietary sodium intake against PRA gave a highly significant inverse correlation ($p<0.001$). From these data it was tentatively postulated that the normotensive subject may have a renin level 'set' higher than the hypertensive subject for a given level of sodium intake and therefore a pro rata response in PRA to sodium deprivation would help offset the fall in blood pressure in normotensive subjects to a greater extent.

'Low renin hypertension' in which a sub group of patients with essential hypertension are said to have an abnormally low renin for a given dietary sodium, is still controversial. Some authors have claimed that this classification is prognostic with regard to vascular complications (Brunner *et al.*, 1972) and therapeutic in regard to selective efficacy of treatment (Buhler *et al.*, 1972). Others dispute this (see Thurston *et al.*, 1978). From the results presented in this study it is apparent that the hypertensive subjects on restricted sodium intake do not divide into two discrete sub groups of 'normal' and 'low' renin. However, what is interesting is that there is a much greater range of PRA on a low salt diet so there is an area on the PRA-sodium profile which is exclusive to hypertensive patients but this does not indicate

of itself the presence of an aetiologically distinct entity. Since there is no clear-cut division one would not perhaps expect to see clear threshold-type responses therefore to dietary or therapeutic change but, rather, a gradual one. The hypertensive patients with the low renins were considered likely to be less responsive to sodium deprivation as judged by the difference in blood pressure fall on sodium restricting normal volunteers and hypertensive patients. In the former the fall in blood pressure is abrogated by rise in PRA, whereas in the latter the response of the renin angiotensin system is muted and blood pressure falls significantly. This perhaps explains why in the normotensive subjects on a low sodium diet the blood pressure was a weak direct function of PRA ($r = +0.36$), almost attaining statistical significance.

Further analysis of the data was attempted using the change in blood pressure on going from a high to low salt diet divided by urinary sodium as an index of blood pressure sensitivity to sodium. The slope of this relationship was found to significantly increase as a function of mean arterial blood pressure, implying perhaps a greater sensitivity to sodium in the more hypertensive patient. There was a significant negative correlation between the slope of the blood pressure-sodium relationship and PRA ($p<0.0002$). Furthermore, multiple regression analysis using the slope of MAP/Na as dependent variable and correlating it with initial blood pressure and PRA revealed that 46% of the variability in the blood pressure sodium ratio was attributable to these two variables; age, weight or plasma volume did not improve the correlations.

These data demonstrated that the blood pressure to sodium excretion relationship is shifted not only to higher blood pressure levels in essential hypertension but the slope of the relationship is altered

progressively as a function of the high blood pressure. This slope was significantly attributable to blood pressure and PRA but not age. These results are consistent with a concept of a renin system which is 'set' at a higher level in normotensives and is more responsive to sodium loss. Findings consistent with those in the present study have been reported by Fujita *et al.* (1980) in hypertensive subjects only and Sullivan *et al.* (1980) in a preliminary report on nineteen subjects.

The response of the renin angiotensin system to the loss of sodium and water induced by Spironolactone was studied in a cross-over trial with Propranolol. The diuretic was seen to be a more effective hypotensive agent in those patients with a low PRA when on a low sodium diet. Propranolol was found to be more efficacious as a hypotensive in the subjects whose PRA values were normal when salt depleted. The correlations between the fall in blood pressure and PRA were significantly negative for subjects on diuretic therapy and significantly positive for subjects on β-blockers. One interpretation of this is that in the normal PRA subjects the fall in blood pressure induced by the diuretic is offset by a rise in the activity of the renin-angiotensin system. Conversely, the depression of PRA by β-blockade would be expected to be more hypotensive in these normal renin subjects. These results indicate that the level of PRA on a low sodium diet would be a useful tool in enabling the clinician to decide whether diuretic or β-blockade would be the most efficacious treatment.

When saralasin was infused into patients on Spironolactone the fall in blood pressure correlated positively with the PRA value. The agonist effect of saralasin is witnessed by the rise in blood pressure in subjects with low PRA values. These data provide evidence that in patients with essential hypertension on Spironolactone the blood

pressure is being maintained to a significant extent by the renin-angiotensin system. These results, however, do not provide direct evidence for a rôle for the renin-angiotensin system in normotensive subjects or in essential hypertensive patients not undergoing diuretic therapy, although the results are consistent with the hypothesis discussed in this chapter.

The use of orally administrable inhibitors of angiotensin-converting enzyme provided direct evidence that the renin-angiotensin system does help maintain blood pressure in normal subjects on a normal dietary sodium intake, and also in patients with essential hypertension. The data indicate that the renin-angiotensin system plays no greater rôle in the maintenance of essential hypertension than in maintaining normal blood pressure levels for a given level of plasma renin activity and is not, therefore, likely to be causative in the pathogensis of essential hypertension.

The final study, a comparison of therapy and sodium diet in black and in white patients with essential hypertension, provided further corroboration for the hypothesis of renin-response and the differing efficacies of classes of hypotensive agents being a function of the "renin status" of the patient. It is important to note that comparison of studies reported from areas where negroes comprise a large percentage of the normal volunteers with studies using caucasians must be undertaken with great caution as these results show a significantly different normal range for blacks and for whites. The blacks as a group had low renin and responded well to diuretic therapy, whereas the whites, with a higher renin level, responded significantly less well to diuretics but significantly better to β-blockade and converting enzyme inhibition.

Whilst it has been demonstrated that PRA is a valid index of plasma angiotensin II as reflected by extraction from plasma and by the radioimmunoassay described, it should be remembered that hormones act on cells, not blood, and the assumption that blood hormone concentration is linearly proportional to the concentration at the level of the target cell and thence proportional to the biological response is by no means proven. Radioreceptor assays may increase our understanding in the near future. Already work on angiotensin II receptors by Gunther and his colleagues (1980) has indicated that a level of autoregulation exists at the hormone-receptor level. Such changes in receptors could cause dramatic changes in biological response with apparently little change in plasma hormone levels or even a change which appears to be paradoxical.

In summary this thesis has demonstrated that the measurement of plasma renin activity by radioimmunoassay of angiotensin I generated at optimal pH (5.7) is a linear function of PRA as measured by assay of angiotensin I generated at pH 7.4. The discrepant results with plasma from anephrics reported elsewhere (Oparil *et al.*, 1974) were not found in this study and an explanation for this has been offered. Furthermore, the storage of plasma samples at -20°C has been shown not to cause artefactual increases in plasma renin activity as suggested by Sealey and Laragh (1975). Possible explanations for their findings have been discussed and the weight of current evidence supports the explanation and the results presented here (Osmond *et al.*, 1973; Lijnen *et al.*, 1979; Roulston and MacGregor, 1978).

The relationship between plasma renin activity and plasma angiotensin II has been shown to be direct and linear in both normotensive and hypertensive subjects. Diuretic or β-blocker therapy or

dietary sodium divergence was shown not to affect the slope of the relationship. Supportive data has been published by Thurston *et al.* (1978). The results of Sambhi and his colleagues indicating a change in the relationship between PRA and angiotensin II on diuretic therapy were not supported by the results of Thurston *et al.* or myself.

PRA has been shown to be an inverse function of blood pressure and also appears less able to respond to sodium deprivation or converting enzyme blockade in hypertensive subjects than in normotensives, indicating the possible existence of a regulatory molecule. The contention of Laragh (1974) and others that plasma renin activity on a low salt diet is a useful predictor of efficacy of therapy by β-blockers or diuretics is supported by this study, although the demonstration of a discrete subpopulation of "low-renin hypertensives" was not seen; rather a greater variation in the PRA of hypertensives on a low salt diet was seen by comparison to normotensives.

The rôle of the renin angiotensin system in relation to sensitivity to change in dietary sodium was shown to be a function of the mean blood pressure, supporting the hypothesis propounded in this thesis; the recent work of Fujita *et al.* (1980) and Sullivan *et al.* (1980) is also consistent with this.

The renin-angiotensin system has been shown to maintain blood pressure to a given extent in both normal and hypertensive subjects for a given level of PRA, but is unlikely to be pathogenetic with regard to essential hypertension.

The concept that the renin-angiotensin system is an important defence mechanism against hypotension induced by sodium or volume depletion (Haber, 1976) is supported by the results in Chapters 15, 16

and 17 but no threshold of onset of activity could be demonstrated. Indeed the renin-angiotensin system was shown to be involved in blood pressure maintenance under normal physiological conditions in both normotensive subjects and in patients with essential hypertension.

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APPENDIX ONE

FORMULATION OF BUFFERS AND GENERAL LABORATORY REAGENTS

Buffers

1. Routine assay reaction/diluent buffer

0.04 Molar phosphate buffered saline, pH 7.4 containing 8 mM disodium ethylenediaminetetraacetic acid (Na_2EDTA), 0.25 mM sodium ethylmercurithiosalicylate (thiomenal) and 1.0 $g.l^{-1}$ of bovine serum albumin (BSA) (Fraction V powder; see Cohn and Strong, 1946).

Compound	Molecular weight	$g.l^{-1}$	Molarity
Sodium chloride	58.44	2.34	0.04
Disodium hydrogen orthophosphate.$12H_2O$	358.15	9.8	0.027
Potassium dihydrogen orthophosphate	136.10	1.4	0.01
Na_2EDTA	372.24	3.0	8×10^{-3}
Thiomenal	404.81	0.1	2.5×10^{-4}
BSA	-	1.0	-

The buffer was stored in plastic Winchester bottles at 4°C when not in immediate use. Fresh solutions were made weekly.

2. Assay rinsing buffer

The buffer used to rinse the probes on the "Kemtek", wash out the reaction tubes and presoak the filter discs at the separation stage was prepared to the same specifications as the above assay reaction/diluent buffer but in addition contained 0.5% (v/v) of Tween 80. This detergent prevented unbound labelled antigen sticking to the filter discs.

3. Angiotensin I generation buffer

0.1 Molar potassium citrate/citric acid buffer, pH 5.7.

Compound	Molecular weight	$g.l^{-1}$	Molarity
Potassium citrate	324.41	27.2	0.084
Citric acid	210.14	3.36	0.016

The buffer was stored at 4°C and replaced monthly.

4. Charcoal buffer

A 7 mM barbital buffer mixture, pH 9.0.

Compound	Molecular weight	$g.l^{-1}$	Molarity
Sodium chloride	58.44	7.6	0.13
Sodium acetate.$3H_2O$	136.03	0.95	7×10^{-3}
Barbitone sodium	206.18	1.44	7×10^{-3}

The pH was adjusted to 9.0 ± 0.2 with 0.2 molar HCl. The buffer was stored at 4°C for up to 14 days.

5. Angiotensin labelling buffer

0.05 Molar phosphate, pH 7.4.

Compound	Molecular weight	$g.l^{-1}$	Molarity
Disodium hydrogen phosphate.$12H_2O$	358	15.56	0.0434
Sodium dihydrogen phosphate.$2H_2O$	156	1.03	0.0066
Sodium azide	65	0.184	0.0028

The buffer was stored at 4°C when not in use.

6. Angiotensin I elution buffer

0.1 Molar acetic acid/acetate buffer, pH 5.5.

Compound	Molecular weight	$g.l^{-1}$	Molarity
Acetic acid	60	1.38*	0.023
Sodium acetate.$3H_2O$	136.09	10.47	0.077

*Equivalent to 1.316 ml of glacial acetic acid.

The buffer was stored at 4°C.

7. Angiotensin II elution buffer

0.04 Molar Tris HCl, pH 7.5.

Compound	Molecular weight	$g.l^{-1}$	Molarity
Bovine serum albumin	-	3	-
Tris	121.14	6.06	0.05
Neomycin	908.7	2.0	2.2×10^{-3}

Adjusted to pH 7.5 with HCl and stored at 4°C when not in use.

8. Electrophoresis buffer

0.07 Molar barbitone, pH 8.6.

Compound	Molecular weight	$g.l^{-1}$	Molarity
Barbitone sodium	206.18	10.3	0.05
Barbitone	184	3.62	0.02

Stored at 4°C.

9. Protein A loading buffer

0.1 Molar phosphate buffered saline, pH 7.3.

Compound	Molecular weight	$g.l^{-1}$	Molarity
Disodium hydrogen phosphate	358.14	35.8	0.1
Sodium dihydrogen phosphate	156.0	15.6	0.1
Sodium chloride	58.44	29.2	0.5

The above stock solution was diluted two-fold with distilled water to yield the working strength solution. The pH was adjusted to 7.3 ± 0.1 with HCl and the buffer stored at 4°C.

10. Protein A elution buffer

0.1 Molar glycine HCl buffered saline, pH 2.8.

Compound	Molecular weight	$g.l^{-1}$	Molarity
Glycine (aminoacetic acid)	75	7.5	0.1
Sodium chloride	58.44	29.2	0.5

The pH was adjusted to 2.8 with concentrated HCl. The buffer was stored at 4°C.

11. Protein A collection buffer

0.2 Molar Tris, pH ≈ 9.5.

Compound	Molecular weight	$g.l^{-1}$	Molarity
Tris	121.14	24.2	0.2

Stored at 4°C. The pH was not adjusted; this solution was used to neutralise eluate fractions.

12. Enzacryl conjugation buffer

Acetate/acetic acid buffer, 0.1 molar pH 6.0.

Compound	Molecular weight	$g.l^{-1}$	Molarity
Sodium acetate	82.04	8.204	0.1

To 910 ml of the above 90 ml of 0.1 molar acetic acid were added. Molar NaOH was used to adjust the pH to 6.0. The buffer was stored at 4°.

13. Acetate salt buffer

An equal volume of 0.5 molar sodium chloride solution was added to the conjugation buffer. The mixture was stored at 4°C.

14. Converting enzyme assay buffer

0.05 Molar "HEPES"*, pH 7.5.

Compound	Molecular weight	$g.l^{-1}$	Molarity
HEPES*	238	11.9	0.05
Thiomersal	404.81	0.2	5×10^{-4}

*HEPES = N-2 hydroxyethylpiperazine,N'-2 ethane sulphonic acid.

15. pH Profile of renin buffers

The activity of renin was assessed at pH 4.5, 5.0, 5.5, 5.7, 6.0, 6.5, 7.4 and 8.6. The following formulations were used:-

(a) pH 4.5. 0.1 Molar acetic acid/acetate.

Compound	Molecular weight	$g.l^{-1}$	Molarity
Sodium acetate	82.04	3.04	0.037
Acetic acid	60	3.78*	0.063

3.78* g ≡ 3.60 ml.

(b) pH 5.0. 0.1 Molar acetic acid/acetate.

Compound	Molecular weight	$g.l^{-1}$	Molarity
Sodium acetate	82.04	5.17	0.063
Acetic acid	60	2.22*	0.037

*2.22 g ≡ 2.12 ml.

(c) pH 5.5. 0.1 Molar acetic acid/acetate; buffer no. 6.

(d) pH 5.7. 0.1 Molar citrate/citric acid; buffer no. 3.

(e) pH 6.0. 0.1 Molar acetic acid/acetate; buffer no. 12.

(f) pH 6.5.

Compound	Molecular weight	$g.l^{-1}$	Molarity
Disodium hydrogen phosphate $12H_2O$	358	13.96	0.039
Sodium dihydrogen phosphate $2H_2O$	156	9.52	0.061

(g) pH 7.4. 0.05 Molar phosphate; buffer no. 5.

(h) pH 8.6. 0.07 Molar barbitone buffer; buffer no. 8.

General Laboratory Reagents

1. Angiotensin extraction medium

A solution comprising 10 ml of methanol and 90 ml of 0.880 ammonia was prepared and used as required in a fume cupboard.

2. Converting enzyme inhibitor solution

Compound	Molecular weight	$g.l^{-1}$	Molarity
Disodium EDTA	372.24	46.4	0.125
Neomycin sulphate	908.7	2.0	2.2×10^{-3}
1.10 Phenanthroline	180.2	5.0	2.77×10^{-2}
Ethanol	46	15.8*	0.343

*15.8 g ≡ 20 ml.

4.64 g of EDTA were dissolved in about 90 ml of distilled water in which 0.2 g of neomycin sulphate had been dissolved. 0.5 g of phenanthroline were dissolved in 1 ml of ethanol and this was added to the neomycin-EDTA solution. The tube in which the phenanthroline had been dissolved was subsequently washed with a further 1 ml of ethanol. The final volume was adjusted to 100 ml with distilled water. The solution was stable for one month at 4°C.

3. Scintillation fluid

Compound	Volume
"Permafluor"	66.25 ml
Toluene	1584 ml
Triton X-100	1100 ml

The solution was stored in a dark bottle to prevent photon absorption and consequent high "background counts".

APPENDIX TWO

PROGRAMME FOR DATA ANALYSIS OF MANUAL PLASMA RENIN ACTIVITY ASSAY RESULTS

The programme as listed (in Figure AI-2) was entered into the calculator. Into memory register 1 (R1) the mean value of the non-specific binding counts (NSB) was entered. Into R2 the mean counts of the zero antigen estimations minus the mean NSB values were stored after dividing the answer by 100 in order to yield a percentage for subsequent $^{B}/_{B0}$ estimations.

The programme contains a "decrement and skip on zero" function which achieved selective branching of the programme after the standards had been processed. This instruction causes the programme to divert to step 85 if a positive integer >1 is present in R0. For iterative sequences this command acts as both a conditional transfer and a counter. Hence to selectively process standards by conditionally branching the programme it was necessary, prior to entering the values, to store in R_0 an integer which equalled the total number of standard tubes; that is (number of standards multiplied by number of replicates) + 1. This value is decremented by 1 prior to branching to step 85 and it may be seen, therefore, that after the last standard had been processed the decrement step for the first sample NDE would result in $R_0 = 0$ and the transfer would then be ignored for the remainder of the programme.

The standard counts were entered singly and the "run stop" button pressed after each one. The programme diverted to step 85 and then calculated for that tube a value of B/B_0 expressed as a percentage.

That is percentage $= \frac{B - NSB}{B_0 - NSB} \times 100.$

After all the standard values had been so calculated a dose response curve was constructed on log.logit graph paper which linearised the dose-response curve over the range employed. From the graph the intercept on the y axis was calculated as a logit value as follows:-

$$\text{logit} = \text{In}\ \frac{p}{100 - p} \quad \text{where } p = \text{percentage, In} = \log_1.$$

For example, if the intercept on y was at 90% $^{B}/_{B_0}$ then the intercept was calculated as $\text{ln}\ ^{90}/10 = 2.1972$. The slope was evaluated in a similar manner by the following formula:

$$\text{Slope (m)} = \frac{\text{In}\ \frac{p_1}{100-p_1} - \text{In}\ \frac{p_2}{100-p_2}}{\log_{10}x_1 - \log_{10}x_2}$$

The values of intercept and slope were stored in registers R4 and R_5 respectively. Also, in R8 the conversion factor to express the results in the usual units of $ng.ml^{-1}.hr^{-1}$ was stored. The value of this factor was 0.67 if the angiotensin concentration was expressed in $ng.ml^{-1}$ or $0.01\dot{3}$ if in $pg.tube^{-1}$.

In order to derive a true value for x from the equation $y = mx + c$ a true value of c, that is y when $x = 0$, should be known. However, on a log.logistic plot extrapolation to $x = 0$ is not possible; hence an arbitrary intercept (c') at say 90% binding was taken. Thus the value of dose interpolated using this intercept will be incorrect. The true value of x, without knowing true c, can be obtained however since true $x = \frac{(y-c)}{m}$, whereas the apparent x yielded from the programme is $\frac{(y-c)}{m} - \frac{(y-c')}{m}$. Therefore the difference to be added to the apparent answer to yield a true x value is $\frac{(y-c')}{m}$; that is, x', the value of x at the arbitrary intercept c'.

Therefore the value of $\log_X$ at $y = c'$ was stored into register 9 (R9).

The sample values were fed in with the "run-stop" (R/S) key being depressed after each entry. After a triplicate blank followed by the

corresponding triplicate generated counterpart had been entered the programme ran through and displayed a value for PRA of that sample in $ng.ml^{-1}.hr^{-1}$.

The calculator stored the sum of the blank sample counts in R6 and the generated counts in R7. Then the contents of R6 were put through the subroutine where they were analysed as follows:-

(i) Divide by 3 to give mean counts.

(ii) Subtract R1 to give net mean counts.

(iii) Divide by R2 to give $\%\ ^{B}/_{B0}$.

(iv) To logit format: $= \text{In.}\ \frac{p}{100-p}$

(v) Interpolate x apparent (= (y-c')/m; i.e. -R4, ÷ R5).

(vi) Add value of $\log_{10}x'$ to give true x.

(vii) Antilog answer.

(viii) Return answer to R6.

The contents of R7 were similarly processed and finally the difference, after unit conversion displayed as $ng.ml^{-1}.hr^{-1}$.

A listing of the programme is given in Table AII-1 and the algorithm in Figure AII-1.

Table AII-1

Listing of the PRA programme

Locus	Command	Locus	Command	Locus	Command
00	*dsz	34	RCL	68	=
01	8	35	7	69	In x
02	5	36	-	70	-
03	STO	37	RCL	71	RCL
04	6	38	6	72	4
05	R/S	39	=	73	=
06	SUM	40	x	74	÷
07	6	41	RCL	75	RCL
08	R/S	42	8	76	5
09	SUM	43	=	77	=
10	6	44	R/S	78	+
11	R/S	45	RST	79	RCL
12	STO	46	÷	80	9
13	7	47	3	81	=
14	R/S	48	=	82	*10^x
15	SUM	49	-	83	=
16	7	50	RCL	84	*rtn
17	R/S	51	1	85	-
18	SUM	52	=	86	RCL
19	7	53	÷	87	1
20	RCL	54	RCL	88	=
21	6	55	2	89	÷
22	*subr	56	=	90	RCL
23	4	57	STO	91	2
24	6	58	3	92	=
25	STO	59	÷	93	R/S
26	6	60	(	94	RST
27	RCL	61	1	95	
28	7	62	0	96	
29	*subr	63	0	97	
30	4	64	-	98	
31	6	65	RCL	99	
32	STO	66	3		
33	7	67	)		

Key to symbols overleaf.

Memory Registers

R_0 = (number of stds x replicates) + 1

R_1 = mean N.S.B.

R_2 = $\frac{ZA - NSB}{100}$

R_3 = % B/B_0

R_4 = Intercept on y (c')

R_5 = Slope

R_6 = Blank sample data

R_7 = Generated sample data

R_8 = Unit converting factor (0.013 or 0.67)

R_9 = $\log_{10}$ of x at $y \equiv c'$

Symbols used

* Indicates that command is a secondary key function

dsz = Decrement and skip on zero

STOn = Store in register n

R/S = Run/stop

SUM = Add to present contents of STO

subr = Subroutine

RCL = Recall store n

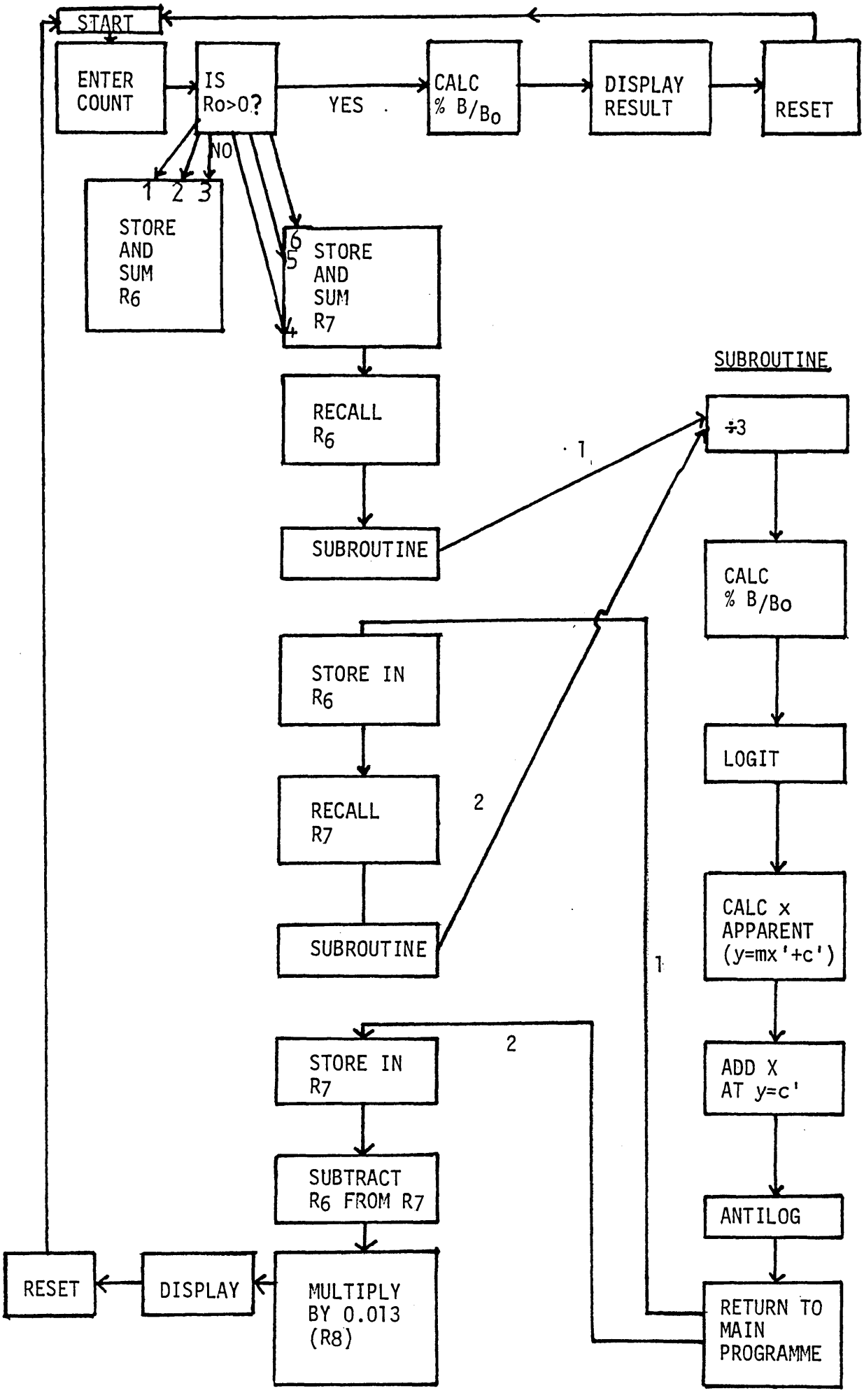
In x = Take $\log_e$

10^x = Take antilog

rtn = Return from subroutine to ruin programme

RST = Reset i.e. go to loc 00

Figure AII-1 Algorithm of PRA programme



APPENDIX THREE

DATA ANALYSIS PROGRAMME FOR THE ANGIOTENSIN II ASSAY RESULTS

As may be seen from the listing and algorithm (Table AII-1 and Figure AII), the programme is substantially analogous to that devised for PRA data analysis. The conditional branch and iterative counting step (dsz) was again used to branch off standard analysis. After getting % B/B_0 for all the standards a log.logit plot was constructed to furnish the slope and apparent intercept c'. These constants were stored in registers 4 and 5 as in the previous programme. The subroutine in this programme calculates the mean counts, converts this through B/B_0 to logit as in the previous programme and finally calculates x apparent as $(y-c')/m$, which is returned and stored in R3. After the value of x' at y=c' had been added to x apparent the whole was antilogged and multiplied by the reconstitution volume in µl of the extract (register 9). This answer was in turn divided by the contents of store 7, the plasma volume of that particular sample, which was fed in and held with the count data entry. Finally the answer was divided by the fractional efficiency of extraction (i.e. 85% recovery = 0.85), which was stored in R6. The final result was displayed as $pg.ml^{-1}$ of plasma.

Example

30% Binding was established by a sample extracted with 85% efficiency from 9 ml of plasma and reconstituted in 400 µl of buffer. The apparent intercept was taken at 90% binding which corresponded with a value for x of 0.01 $ng.ml^{-1}$. The value of y = 20% corresponded with x = 1.0 $ng.ml^{-1}$.

Logit c' $= \ln[90/(100-90)] = 2.197$

Slope m $= \dfrac{\ln[20/(100-20)] - 2.197}{\log 1 - \log .01}$

$= -1.792$

For sample logit y = In 30/70.

$\therefore \log_x$ apparent $= y\text{-}c'/m$

$= [\ln 30/70 - 2.197]/-1.792$

$= 1.699.$

$\therefore$ log true x $= 1.699 + \log .01$ (i.e. x at y = c')

$= -0.301$

i.e. true x $= 0.50$ ng.ml^{-1} extract.

If reconstitution volume was 400 µl then total angiotensin II = 0.5 x 0.4 x 1000 = 200 pg. If extraction efficiency was 85% then true total in 9 ml of plasma = 235.3, i.e. 26.1 pg.ml^{-1}.

Table AIII-1

Listing of data analysis programme for angiotensin II assay

Locus	Command	Locus	Command	Locus	Command
00	*dsz	34	RCL	68	RCL
01	7	35	6	69	5
02	2	36	=	70	=
03	STO	37	R/S	71	*rtn
04	3	38	RST	72	-
05	R/S	39	$\frac{o}{o}$	73	RCL
06	SUM	40	3	74	1
07	3	41	=	75	=
08	R/S	42	-	76	$\frac{o}{o}$
09	SUM	43	RCL	77	RCL
10	3	44	1	72	2
11	R/S	45	=	79	=
12	STO	46	$\frac{o}{o}$	80	R/S
13	7	47	RCL	81	RST
14	RCL	48	2	82	
15	3	49	=	83	
16	*subr	50	STO	84	
17	3	51	3	85	
18	9	52	$\frac{o}{o}$	86	
19	STO	53	(	87	
20	3	54	1	88	
21	+	55	0	89	
22	RCL	56	0	90	
23	8	57	-	91	
24	=	58	RCL	92	
25	*10^x	59	3	93	
26	x	60	)	94	
27	RCL	61	=	95	
28	9	62	In x	96	
29	$\frac{o}{o}$	63	-	97	
30	RCL	64	RCL	98	
31	7	65	4	99	
32	=	66	=		
33	$\frac{o}{o}$	67	$\frac{o}{o}$		

Continued overleaf.

Memory registers

R_0 = (Number of standars x replicates) + 1

R_1 = Mean NSB

R_2 = $\frac{\text{zero antigen - NSB}}{100}$

R_3 = Sample counts

R_4 = Intercept

R_5 = Slope

R_6 = Fraction recovered

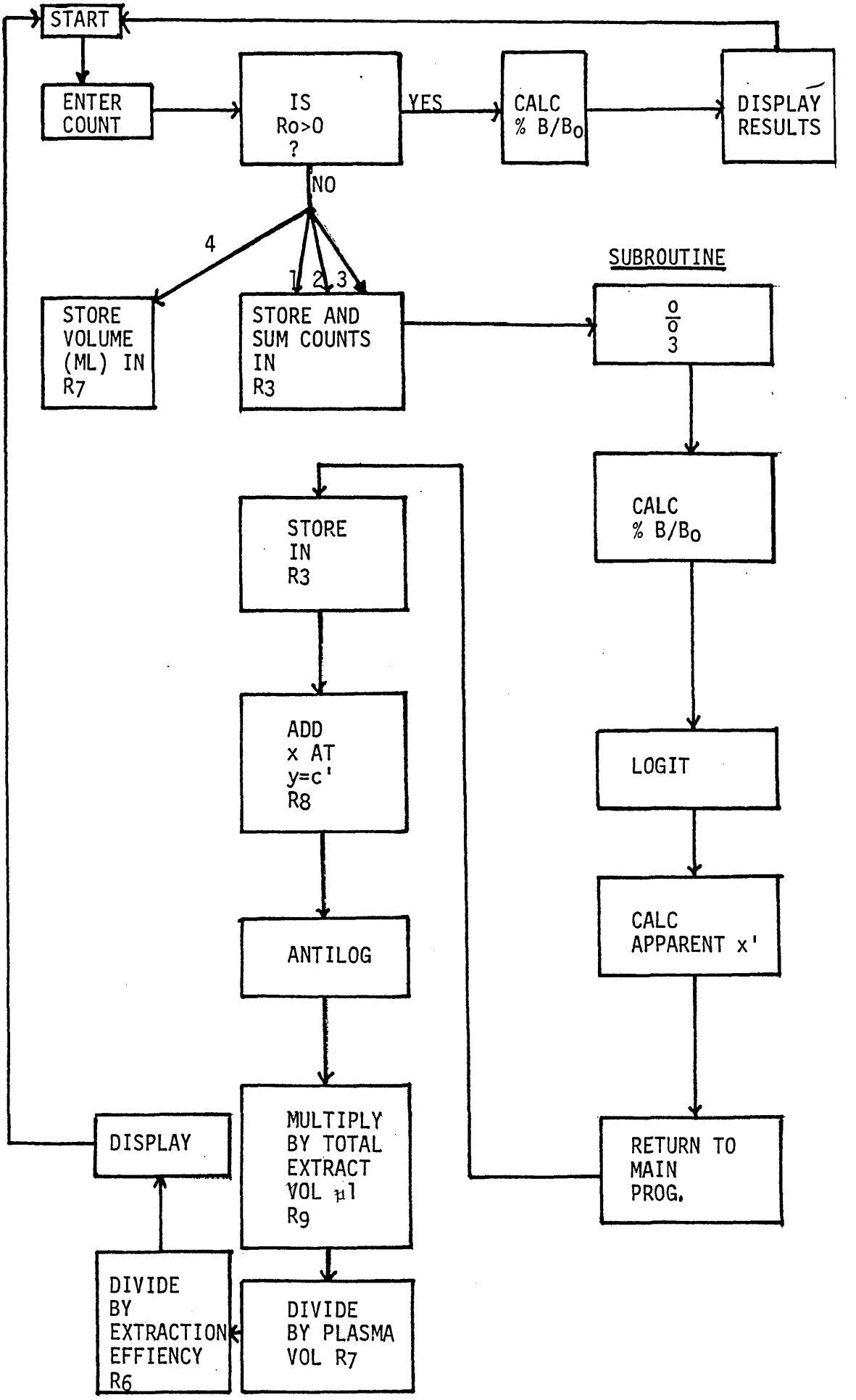
R_7 = Volume of plasma extracted

R_8 = $\log_{10}$ of x at y = c'

R_9 = Reconstitution volume (µl)

For an explanation of the symbols see Table AII-1

Figure AIII-1 Algorithm of the angiotensin II data analysis programme



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1. The anti-hypertensive action of Spironolactone: the importance of the renin angiotensin system to the loss of sodium and water.
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1. A rationale for the development of radioimmunoassays.
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Statement as to the part played by J.E. Roulston in work published conjointly with others:-

Book chapters 1-3

The candidate was responsible with Dr. G.A. MacGregor for the preparation of the manuscripts and was responsible solely for the renin measurements contained therein.

Paper 1 The candidate was responsible for the initial manuscript, mathematical derivations and conclusion in this review article.

Paper 2 The candidate was responsible for the design of the study, biochemical measurements and the preparation of the initial manuscript.

Paper 3 The candidate was responsible for the design of the study, the biochemical measurements, the initial manuscript and was solely responsible for the novel analytical technique employed therein.

Paper 4 The candidate was responsible for the biochemical measurements and assisted in the design of the study and the preparation of the manuscript.

Paper 5 The candidate was responsible for the design of the study, the biochemical aspects of the work and preparation of the initial manuscript.

Paper 6 This was solely the work of the candidate.

Paper 7 The candidate collaborated with Dr. G.A. MacGregor on the design of the study and the preparation of the manuscript and was responsible for the renin estimations reported therein.

Paper 8 The candidate advised with regard to assay assessment and assisted in the preparation of the manuscript.

Paper 9 The candidate was responsible for renin estimations and assisted in the design of the study and the preparation of the manuscript.

Paper 10 The candidate was responsible for renin estimations and assisted in the design of the study and the preparation of the manuscript.

Paper 11 The candidate designed and executed the study and was responsible for the initial manuscript.

Paper 12 The candidate designed the study and was responsible for its practical aspects and the manuscript.

Paper 13 The candidate was responsible for renin estimations and assisted with the design of the study and prepared the initial manuscript.

A rationale for the development of radioimmunoassays

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Abstract
A theoretical study of the principles of radioimmunoassay and allied techniques is presented with a view to their application in practical developmental research. Both equilibrium and sequential saturation methods are discussed and their respective advantages noted. Simple methods for the estimation of the affinity constant by saturation techniques and use of the Scatchard plot are outlined.

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THE technique of competitive binding assay using radiolabelled ligands was first published by Yalow and Berson[1] and Ekins[2]. Since that time the technique has found widespread application to a variety of drugs, hormones and physiomedical molecules of interest to an immense number of workers in the medical-biochemical field.

The popularity of the technique is, possibly, threefold. Firstly, the specificity of the system is exceedingly high, secondly, the sensitivity is very good; estimation of picogram levels being quite attainable and thirdly the technique generally can be reproduced with large numbers of samples with minimal inconvenience. This paper hopes to elucidate the principles of the technique and the options open to the assay user and/or developer to achieve an optimally sensitive assay whether using equilibrium or sequential saturation techniques. Also the postulates presented are equally valid for such modified techniques as immunoradiometric assay, spin immunoassay[5] and enzyme immunoassay[6]. All of these techniques have common fundamental principles; that is the partial saturation of a binding molecule by the ligand to be measured and the competition of this ligand with the "tracer" molecule (often radiolabelled ligand) for these binding sites. Degrees of tracer bound will therefore, be an inverse function of the ligand molecule present in the sample and by graphical interpolation a value for the ligand concentration is obtained.

The majority of assays work using an equilibrium technique. That is to say, the labelled and unlabelled ligands compete simultaneously for the sites available on the binder. The alternative is the so-called sequential saturation analysis, usually used in systems where there is insufficient sensitivity using the equilibrium method[3] or ligand and radioligand have very different affinities[4] for the binder. Sequential saturation lessens or eliminates simultaneous competition; the reaction not being allowed to reach equilibrium.

It is important to differentiate between these two basic approaches as they are influenced, understandably, by different factors.

Equilibrium assays

If we consider "equilibrium" assays first, we can describe the reaction thus:

$$[P + P^*] + [Q] \underset{k_2}{\overset{k_1}{\rightleftharpoons}} [PQ] + [P^*Q]$$

where $[P + P^*]$ = combined molar conc. of ligand (labelled and unlabelled) mixture
$[Q]$ = molar conc. of binding agent
$[PQ] + [P^*Q]$ = combined molar concentration of the complexes between binder and ligand mixture
k_1 = association constant
k_2 = dissociation constant

It is apparent that for the above equation to be true, all of the binding sites on Q must possess identical affinity for P as for P*. For an assay to be sensitive the association velocity must be greater than the dissociation velocity, by a considerable degree since the crux of the assay is dependent on the extent of complex formation at the concentration employed in the incubate. In the above system one varies [P]. Therefore we see that $[P^*Q] \propto 1/[P]$ and this reflects the decrease in the $[P^*]$: $[P]$ ratio as $[P]$ increases. Usually $[P^*Q]$ is measured or $[P^*]$ or both from which a Bound-Free ratio may be obtained. The equilibrium reaction as outlined above has been shown by Ekins[7] to be described by the following quadratic equation:

$$R^2 + R[1 + Kp + Kp^* - Kq] - Kq = 0$$

where R = Bound : Free ratio
K = Affinity Constant (units M^{-1})
p = Conc. of P
p* = Conc. of P*
q = Conc. of Q in terms of numbers of *binding sites.*

The above equation presupposes a univalent binder, chemical identity between P and P* and perfect separation. Thus to optimise an assay p,p* and q expressed in units of K^{-1} (i.e. moles/litre) can be calculated if K is known. Yalow and Berson[8] have also obtained a comparable mathematical expression for the above reaction. Examination of the possible permutations of p* and q shows that there are three distinct possibilities for optimal sensitivity.

Under conditions of minimal tracer concentration

Berson and Yalow defined a set of conditions which yields the steepest dose-response curve, this is the case when p* tends to zero; that is the concentration of label is so low as to be negligible in the reaction. Further to this, they selected an antiserum (binder) dilution which gave 33 per cent binding of the total tracer when unlabelled ligand is absent. The concentration of binder required to achieve these conditions is easily obtained (in units of K^{-1}) from the Ekins equation; which assuming $p = p^* = 0$ simplifies to:

$$R^2 + R(1 - Kq) - Kq = 0$$

$$R = {}^{B}/_{F} = 0.5 \quad \therefore 0.5^2 + 0.5 - 0.5\,Kq - Kq = 0$$

$$\text{i.e. } 0.75 = 1.5\,Kq$$

$$q = 0.5/K$$

The major drawback to assays set up according to these premises is that precision is poor which tends to negate the high sensitivity.

Under conditions defined by tracer concentration equalling 4 K^{-1} and binder concentration of 3 K^{-1}

The second alternative was developed by Ekins and sets the concentration of labelled ligand to four times the reciprocal of the affinity constant i.e. $p^* = 4\,K^{-1}$ or $4/K$.

The dilution of binder is set to $3\,K^{-1}$ or $3/K$.

These values were obtained by mathematical analysis of the equilibrium reaction. This analysis takes into account the precision of experimental determination of ${}^{B}/_{F}$ as well as the sensitivity as defined by slope of dose-response curve. It is apparent that, irrespective of K the bound/free ratio:

$R = 1$ since when $p = 0$

$$R^2 + R(1 + Kp^* - Kq) - Kq = 0$$

$$R^2 + R + RKp^* - RKq - Kq = 0$$

substituting p* and q* in terms of K^{-1} we see:

$$R^2 + R + \frac{4RK}{K} - \frac{3RK}{K} - \frac{3K}{K} = 0$$

That is: $R^2 + 2R - 3 = 0$

i.e. $R = 1$ (or -3)

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Thus a difference may be seen in the optimal requirements when label is present at a significant concentration.

Under conditions of relatively high concentrations

For assays requiring determinations of unknowns of relatively high concentration e.g. Digoxin, it may be envisaged that an exceedingly small sample would be required to measure within the ranges of either of the above methods (concentrations of K^{-1} for example). This would lead to reduced precision, so for these higher concentration molecules a third option is available which is typified by high concentration of binder and an excess of labelled ligand (20-30 per cent say) over binder. (It is apparent if $p^* < q$, sensitivity at low concentrations of p would disappear because q would not be fully saturated.) Another useful application of this method is for systems where the label is of low specific activity and the high concentration used here confers greater precision on the system. An example system would be if p^* was in 30 per cent excess of q and q itself was fifty times the reciprocal of the affinity constant.

Thus $p^* = 1.3q$; $q = 50K^{-1}$ $p = 0$

From Ekins equation we find that R = 2.675, i.e. per cent bound = 72.8 per cent as follows:

$$R^2 + R(1 + Kp^* - Kq) - Kq = 0$$

$p^* = 1.3q \therefore R^2 + R(1 + 1.3Kq - Kq) - Kq = 0$

$Kq = 50 \therefore R^2 + R(1 + (1.3 \times 50) - 50) - 50 = 0$

$$R^2 + R(1 + 65 - 50) - 50 = 0$$

$$R^2 + 16R - 50 = 0$$

Thus $R = 2.675$

per cent bd = 72.8

It is also worthy of note that another fundamental difference between the three systems is the degree of percentage of saturation of binder extant at zero dose of p. In Berson and Yalow's method where p^* tends to zero then per cent saturation of q is, *ipso facto*, zero. Using Ekins method ($p^* = 4K^{-1}$) it is obvious since $p^* = 4K^{-1}$ and $B/T = 1$ then p^* bound = $2K^{-1}$. Since $q = 3K^{-1}$, therefore, 67 per cent saturation is achieved when p = zero. In the high concentration method, however, owing to excess ligand, about 95 per cent of saturation of q is achieved at p = zero. This fact has important consequences. In the methods of Berson and Yalow and Ekins addition of ligand p causes a significant change in saturation (from zero to nearly 100 per cent in the former and from 67 per cent to near 100 per cent in the latter). In the high concentration method, however, there is minimal change. Thus, the dose-response curve constructed using values of p^* and q similar to those described earlier is superimposable upon a curve based on isotope dilution, that is $p/(p^* + p)$. If, however, the concentrations of p^* and q are of the order suggested by Ekins it is seen that the curves are not superimposable; the difference represents the progressive saturation of binder with increasing p. Using Berson and Yalow's method, a direct comparison cannot be made, as p^* tends to zero. Therefore, no finite values for dilution are obtainable as any small finite dose of p would cause infinite dilution of p^*. Thus, in the method of Berson and Yalow the percentage of label bound varies as the reciprocal of the extent by which the dose of p exceeds the saturation level of binding capacity of q at the various dose levels of p. In the "high concentration" method the dose response curve is described by the isotope dilution curve as saturation always tends towards 100 per cent and therefore the binding capacity of q is effectively fixed. For Ekin's method both of the above principles are relevant. It is possibly worth stressing here, that to use a logistic plot for dose response curves, as Berkson[9-15] and Rodbard[16-19] devised in order to linearise a sigmoid dose response curve, is only 100 per cent effective for the "high concentration" method. This is because, for linearity to be ideal the degree of saturation of q should not alter. Hence a logistic-log plot based on isotope dilution will be a straight line.

i.e. As logit y is defined as $\ln \frac{y}{(1-y)}$

$$\text{logit}\left(\frac{B}{Bo}\right) = \ln \frac{\left(\frac{B}{Bo}\right)}{\left(1 - \frac{B}{Bo}\right)}$$

At fixed saturation of q

$$\frac{B}{Bo} = \frac{p^*}{p^* + p}$$

$$\text{Hence logit } \frac{p^*}{p^* + p} = \ln \frac{\frac{p^*}{(p^* + p)}}{1 - \frac{p^*}{(p^* + p)}}$$

Estimation of K

In order to set up assays along the lines intimated above, a knowledge of the affinity constant is required. A reasonably accurate determination of K can be made simply using the Scatchard plot[20] as applied by Berson and Yalow[21] to radioimmunoassays. In this method aliquots of a fixed concentration of binder are saturated with increasing amounts of ligand (cf. a standard dose response curve). The bound to free ratio R is obtained (R = B/T-B) and plotted on y axis versus the concentration of bound ligand on the x axis. This plot not only tells us K, but, gives an indication of the concentration of binding sites. This may be visualised as follows:

Assuming an initial concentration of ligand p_o and an initial concentration of binder q_o. At equilibrium we see that :

$$(p_o - p) + (q_o - q) = pq$$

$$\text{Hence } K = \frac{pq}{(p_o - p)(q_o - q)}$$

$$\text{But } B/F \text{ (i.e. } R) = \frac{pq}{(p_o - p)}$$

$$\therefore K = \frac{R}{(q_o - q)}$$

i.e. $Kq_o - Kq = R$

Since at equilibrium $pq = q = p$. $\therefore Kq_o - Kp = R$

Thus a plot of R versus p will yield a straight line where the slope = $-K$ and the intercept = Kq_o.

This plot will only be linear for a homogenous population of antibodies (i.e. binder) and differing slopes over the range of the curve will be representative of a mixed population of binder. Thus, it is possibly wise to check the result by an alternative method if, as is usual in practice, the graph is non-linear and there is doubt as to the line of best fit. Saturation methods are probably the simplest to employ.

Odell[22] has suggested an alternative method for estimation of K based on a saturation method akin in concept to the determination of the Michaelis constant of enzymes. The method utilises progressive saturation of a fixed concentration of aliquots of binder from which a saturation curve is obtained. The concentration of free ligand at 50 per cent saturation of binder = K^{-1}. This is obvious when one plots ligand bound versus ligand added. 50 per cent saturation is then interpolated as total ligand minus bound ligand yielding a molar value of K^{-1} i.e. K expressed in M^{-1}. The validity of this evaluation becomes apparent from the following. Assume a ligand concentration of p_o and a binder concentration of q_o. At equilibrium we see:

$$(p_o - p) + (q_o - q) = pq$$

$$\text{i.e. } K = \frac{pq}{(p_o - p)(q_o - q)}$$

However, at 50 per cent saturation of binder $q_o = 2q$ ex hypothesis.

$$\therefore K = \frac{pq}{(p_o - p)q} \quad (pq = p = q \text{ at equilibrium})$$

Since $pq/q = 1$

$$K = \frac{1}{(p_o - p)}$$

i.e. $K^{-1} = (p_o - p)$ = concentration of free ligand in mol. litre^{-1}.

Either of the above methods is easy to perform in order to obtain a working value of K and for this, values of p^* and q may be calculated to optimise each particular system.

Sequential saturation

In 1963 Hales and Randle[3] modified their insulin assay in order to increase the sensitivity by introducing the sequential saturation technique. This modification of the radioimmunoassay concept

has proved most useful in many cases but it should be appreciated that the above-described equilibrium kinetics are not applicable.

In this method ligand p is premixed with an excess of binder q (as all p binds) prior to addition of p*.

That is: $(p) + (q_0) \rightarrow pq$ (1)

$(p^*) + (q_1) \rightarrow p^*q$ (2)

where $(q_1) = (q_0) - pq$ (3)

Thus competition between p and p* is eliminated. It is immediately apparent that when p = zero then p*q will be the same in both equilibrium and sequential saturation assays, but that in the latter any small addition of p will decrease subsequent (p*q) formation by a greater amount hence a steeper dose-response curve will result and, therefore, if there is no loss of precision then a more sensitive assay will result. If we consider the first equation we see that as q is in excess than pq = p. Whereas in the second equation where p* is in excess to q_1, then p*q will be effectively equal to q_1.

Thus substituting in equation 3:

$$p^*q = q_0 - p$$

Thus, it is clear that the quantity of label which binds to binder (which is the quantity measured) is the difference between the concentration of binder and unlabelled ligand. Hence p*q should be inversely related to p and also linear if high concentrations of reactants are used.

The sequential saturation is obviously dependent on the reverse reaction being negligible.

$$pq \rightarrow p + q_0$$

during the second reaction of p* with q_1. If this is true then our treatment here, considering each reaction as irreversible, is valid. If not, we have a rather nebulous quasi-equilibrium state between binder, labelled ligand and unlabelled ligand. Thus, it is of paramount importance to know association and dissociation rates of ligand and binder under assay conditions prior to applying sequential saturation techniques. It would also follow that conditions which maximise association and minimize dissociation would be optimal.

The time required to reach equilibrium in the 1st step (incubation time, t) is theoretically infinity. However, in practice a finite time is chosen where pq at t is effectively equal to pq at infinity. Formation of pq with respect to time is described by the following equation:

$$\frac{d(pq)}{dt} = k_1(pq) - k_2(pq)$$

where k_1 and k_2 are the rate constants of association and dissociation respectively. Since k_1 and k_2 are difficult to evaluate in practice, t is usually chosen based upon association studies under intended assay conditions. It is also evident from the Law of Mass Action that incubation times are dependent upon reactant concentrations: the higher p and/or q then the shorter the time required to achieve a particular pq concentration. Hence, it may be seen that the tubes with highest p concentration will reach equilibrium faster than those where p is lowest. Thus t must be chosen to be the time for the lowest dose of p to achieve maximal binding. In practice if $p < q$ no large differences in t are seen over the range of the assay. If, however, $p > q$ then t may indeed vary over the assay range. Thus incubation times should be determined in association studies where binder is used in intended assay concentration and where ligand concentration is less than or equal to that concentration. The second incubation time t_2 is the time required to reach sufficient binding between p* and q_1, where p is at its lowest (this may be p = o). Hence this second incubation is determined by the concentrations of p* and qo (as qo determines q_1). This situation is the same as for equilibrium assays.

In equilibrium assays at any point in time and given dose of p the equation:

$$\frac{p^*}{(p^* + p)} = \frac{p^*q}{(p^*q + pq)} \text{ is valid}$$

This does not hold true for sequential saturation analysis. Here the label added at the end of phase 1 will only be diluted by such ligand as is still unbound.

$$\text{i.e. } \frac{p^*}{p^* + p\text{free}}$$

During step 11, as well as new formation of pq and p*q there will be some dissociation of pq formed in step 1 (governed by k_2). Thus if t_2 is extended beyond the time required to equilibrate p* and q_1 in the absence of p, then the total of pq plus p*q will be constant, but, p* will be progressively replacing p on q as dissociation and re-association continues until the p*q/pq ratio equals p*/p — identical to the equilibrium technique. Thus, any enhancement of sensitivity via sequential saturation analysis depends upon termination of step 11 before this full exchange of p* for p can occur. Hence, less p*q is allowed to form in the presence of the same dose of p than in equilibrium assay. Thus t_2 must be rigidly controlled within the assay if inaccuracies are to be minimised. We have stated that t is inversely related to the concentrations of p and q. However, dissociation of pq to p and q by virtue of its 1st order kinetics is concentration independent, that is, as t_2 diminishes so does dissociation of pq. Thus if concentrations of p and q are high to begin with, t_2 may be so short as to render dissociation negligible.

This is shown to great advantage in Rothenburg's[4] folate assay where the label, folic acid, has a higher affinity for binder (milk binding protein) than has unlabelled ligand (methyltetrahydrofolate). Hence as p* has high affinity t_2 can be made very short so there is negligible dissociation of pq, the overall effect being as if p and q bound irreversibly and no dilution via p – p* exchange occurred.

Discussion

Sequential saturation was introduced to increase sensitivity. Such increases are best found when the concentration of p and q are high. When low, the incubation times become long and dissociation velocity a considerable factor, hence a quasi-equilibrium situation develops. Also, the detection limit is only marginally improved (if at all) as the slopes of equilibrium and sequential saturation analyses dose-response curves are identical at low doses of p.

For automated systems where a linear plot is required it is often possible to obtain a linear plot using sequential saturation and high concentration without the necessity to undergo logit transformations. In fact logit-linearation does *not* apply to sequential saturation analyses. Most importantly sequential saturation can exploit differing affinities of p and p* for q as outlined above. Sequential saturation requires two incubation periods, however, thereby doubling the handling and synchronisation. Also, the dose response curves tend to be more limited in range; hence for "wide spectrum" assays an equilibrium approach would be preferable. With low concentrations of reactants poor precision may result using sequential saturation as minor differences in timing and conditions of handling may cause quite significant differences in p*q concentration.

Thus it may be seen, hopefully, that the kinetics of assays are governed by various rules and that application of these may lead to a more sensitive precise assay. While it is rarely possible to design the optimal assay on paper; the above guidelines are intended to help rationalize the research necessary.

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MEASUREMENT OF PLASMA RENIN ACTIVITY BY RADIOIMMUNOASSAY AFTER PROLONGED COLD STORAGE

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Summary

Measurement of plasma renin activities in four separate quality control pools showed no change when measured by radioimmunoassay after being individually stored at —20°C for up to fifteen months, validating their use as long-term quality controls. These results contradict those reported by some authors.

Introduction

An indirect means of assessing circulating angiotensin II levels is the measurement of plasma renin activity (P.R.A.) by radioimmunoassay. A known amount of plasma, which has been previously frozen and stored at —20°C is incubated at 37°C, over a given time, at a controlled pH in the presence of converting enzyme inhibitors, to generate angiotensin I. This rate of formation of angiotensin I is known as plasma renin activity.

Sealey and Laragh [1] in 1975 observed an increase in P.R.A. from normal subjects and from patients with essential hypertension after storage of samples at —20°C. They reported a mean increase upon re-assay of 26% after two months and 55% after one year. Such results are potentially worrying for the long-term storage of plasma quality control pools and for the analysis of samples which may not be assayed immediately.

In view of this we have investigated the effect of long-term storage upon P.R.A. in separate aliquoted samples which were stored for up to fifteen months at —20°C prior to assay.

Materials and methods

(i) Collection, preparation and storage of plasma pools

Blood was taken from 20 normal subjects on their normal sodium intake and

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immediately placed in tubes containing dipotassium ethylenediaminetetraacetic acid (EDTA); a final concentration of 2.5 mg of EDTA per ml of blood being achieved. The tubes were then centrifuged at 2000 × *g* for 15 min at 4°C. The plasma was pipetted, pooled and divided into fractions of one ml and frozen at −20°C. This pool was designated the normal renin control. The procedure was repeated using 20 normal subjects on a low sodium (10 mmol for 5 days) diet. This pool was designated the high renin control.

Two further plasma pools were obtained as above but prior to subdivision into aliquots they were immediately incubated to generate angiotensin I as described below. Following this incubation they were rapidly chilled in a room at −20°C and stored as 0.2 ml aliquots at −20°C. These two pools were designated the normal and high pre-incubated controls.

(ii) Generation of Angiotensin I

The plasma was thawed but the temperature was not allowed to exceed 4°C. It was then mixed with an equal volume of 0.1 molar potassium citrate/citric acid buffer at pH 5.7 followed by 0.46 molar 8-hydroxyquinoline (40 µl per ml of plasma) and 80 µl per ml of plasma of a saturated ethanolic solution of phenylmethylsulfonylfluoride (PMSF). After thorough mixing, half the sample was immediately frozen at −20°C and the other half incubated at 37°C for three hours to generate angiotensin I prior to rapid freezing and storage at −20°C until required for assay.

Radioimmunoassay of samples and subsequent P.R.A. evaluation were performed by methods based upon those of Boyd et al. (1969) [2].

Results

Over the fifteen-month period of this study there was no change either in the normal and high renin controls (Fig. 1) or in the normal and high pre-incubated

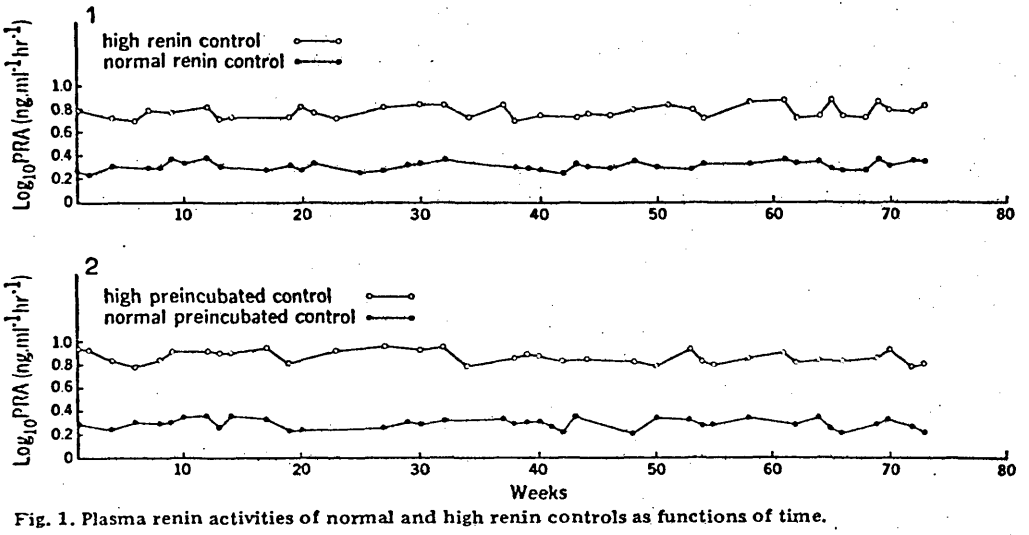


Fig. 1. Plasma renin activities of normal and high renin controls as functions of time.

Fig. 2. Plasma renin activities of normal and high pre-incubated controls as functions of time.

TABLE I

MEAN PLASMA RENIN ACTIVITIES OF THE FOUR QUALITY CONTROL POOLS WITH COEFFICIENTS OF VARIATION

Plasma	No. of samples assayed	Mean P.R.A. (±S.D.) (ng ml^{-1} h^{-1})	Interassay coefficient of variation (%)
Normal renin control	40	2.00 ± 0.18	9.0
High renin control	36	5.86 ± 0.72	12.2
Normal pre-incubated control	37	1.94 ± 0.20	10.6
High pre-incubated control	35	7.39 ± 1.12	12.8

controls (Fig. 2). The mean values and numbers of samples with their coefficients of variation are shown in Table I. The interassay coefficients of variation are not appreciably different for the renin controls and their corresponding pre-incubated controls. This suggests that the method of generation of angiotensin I, in this system, is consistent and reproducible.

Discussion

These results show that P.R.A. does not change in samples stored in individual aliquots at −20°C for up to fifteen months. This contrasts with the published work of Sealey and Laragh who showed a mean increase in P.R.A. of 26% after two months and 55% after one year. A major difference between the two studies is that in their experiments the majority of samples appear to have been re-assayed. This would involve plasma samples in serial freezing and thawing whereas in our study individual aliquots were assayed once only. Serial freezing and thawing may cause an increase in renin-like activity. Osmond et al. (1973) [3] found a substantial increase in P.R.A. following storage at 4°C for three days. There is, therefore, always a possibility that an inefficient deep-freeze or temporary deep-freeze failure may cause an increase in P.R.A. Lijnen et al. (1977) [4], who measured total plasma renin concentration biologically by the method of Skinner (1967) [5] and by radioimmunoassay (Lijnen et al. (1976) [6]) found no increase in total plasma renin concentration by either method after storage of samples at −20°C for up to one year which indirectly supports our results.

The fact that P.R.A. does not change in individually stored samples at −20°C for over one year validates use of such pools, stored in aliquots, as long-term quality controls, and the storage of patients' samples at −20°C for up to one year prior to assay.

Acknowledgements

The authors would like to acknowledge the support of the National Kidney Research Fund, the Medical Research Council and the Mary Smith Scholarship Foundation.

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Measurement of plasma renin activity as a valid estimation of plasma angiotensin status

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SUMMARY Measurement of plasma renin activity is widely used as an indirect assessment of plasma angiotensin II concentration. There has been some controversy over the validity of this assay as an estimate of circulating angiotensin II levels because, during the *in vitro* generation of angiotensin I by renin, over a period of time, substrate concentration may diminish to such an extent that it becomes rate-limiting, giving an artificially low reflection of angiotensin II levels. In this paper the initial angiotensin I concentration, that is the concentration before *in vitro* angiotensin I generation, has been compared with the corresponding plasma renin activity for 2752 individual plasma samples. A linear relationship was found between the initial angiotensin I concentration and the plasma renin activity below 60 ng ml^{-1} h^{-1}. This indicates that, under the conditions of this assay, substrate does not appear to become rate-limiting except at exceedingly high levels of plasma renin activity. These results appear to provide further validation for the use of plasma renin activity measurement as a reflection of the concentration of circulating angiotensin II levels.

Angiotensin I is cleaved by the proteolytic enzyme renin from an α-2-globulin (Peart, 1965; Brown *et al.*, 1966). This decapeptide is subsequently cleaved by a rather nonspecific dipeptidylcarboxypeptidase to yield angiotensin II. Circulating plasma angiotensin II levels may be indirectly assessed by measurement of plasma renin activity (PRA) (Boyd *et al.*, 1969; Salvetti *et al.*, 1972; Fyhrquist *et al.*, 1976). PRA is defined as the rate of production of angiotensin I by a known amount of plasma in a given time under controlled conditions of temperature, pH, and angiotensinase inhibition. This should indirectly reflect plasma angiotensin II concentration since it has been shown that the dipeptidylcarboxypeptidase, responsible for the conversion of angiotensin I to angiotensin II, is present in such concentration, *in vivo*, to be non-rate-limiting (Poulsen and Poulsen, 1971; Ryan *et al.*, 1977). Indeed PRA has been shown to correlate well with plasma angiotensin II concentration in paired samples of normals and hypertensives over a wide range of dietary sodium intake (Gocke *et al.*, 1969). However, doubts about the use of PRA as a quantitative index of plasma angiotensin II concentration have been raised since the rate of the enzymic reaction is dependent upon substrate concentration as well as renin concentration (Brown *et al.*, 1966; Menard and Catt, 1972; Poulsen, 1973).

It has been postulated that the rate of production of angiotensin I *in vitro* will decline as the substrate supply diminishes; and the subsequent PRA will not, therefore, reflect the *in vivo* angiotensin II levels. However, it has been shown that, under the assay conditions normally employed, the formation of angiotensin I *is* linear with respect to time (Rosenthal *et al.*, 1971; Favre and Vallotton, 1973; Krakoff, 1973), indicating that substrate concentration appears to be non-rate-limiting. Another approach is to compare the concentration of angiotensin I present in the plasma sample before *in vitro* generation with its corresponding plasma renin activity value. Such a study using 2752 plasma samples obtained from normal volunteers and unselected hypertensives is described below.

Material and methods

REAGENTS

All reagents were of Analar grade. Angiotensin I ($^{1}Asp^{5}$ Ileu) was obtained from E. R. Squibb and Company and Schwarz Mann Limited. The iodine-125 (^{125}I) derivative of angiotensin I was either purchased from E. R. Squibb or prepared in this laboratory by the method of Hunter *et al.* (1963) using sodium ^{125}I (IMS 30) from the Radiochemical Centre, Amersham. Antisera were raised in rabbits

using a conjugate of angiotensin I condensed with bovine thyroglobulin by the carbodiimide technique of Goodfriend *et al.* (1964). Further supplies were obtained from E. R. Squibb and Company

COLLECTION AND STORAGE OF PLASMA SAMPLES

Five-millilitre blood samples were taken and immediately placed in tubes containing 12·5 mg of dipotassium ethylenediaminetetraacetic acid (EDTA). The blood was then immediately centrifuged at 2000 g for 15 minutes at 4°C in an adjacent cold room. The plasma was transferred carefully to two other tubes and immediately frozen at − 20°C and kept at that temperature until required for assay.

ANGIOTENSIN I GENERATION AND RADIOIMMUNOASSAY

The plasma was thawed but the temperature was not allowed to exceed 4°C; 0·5 ml was then mixed with an equal volume of 0·1 molar potassium citrate/citric acid buffer at pH 5·7 followed by 20 μl of 0·46 molar 8-hydroxyquinoline and 50 μl of a saturated ethanolic solution of phenylmethylsulphonylfluoride (PMSF). After thorough mixing, half the sample was immediately frozen at − 20°C. The other half was incubated at 37°C for three hours to generate angiotensin I before rapid freezing and storage at − 20°C until required for assay.

Radioimmunoassay of samples and subsequent PRA evaluation were performed by methods based upon those of Boyd *et al.* (1969).

Results

Altogether 2752 plasma samples were assayed in duplicate for plasma renin activity. The quality control of the assay has been described previously (Roulston and MacGregor, 1978). Intra-assay coefficients of variation on four independent quality control plasmas were 6·9%, 7·9%, 3·3%, and 5·7%. Inter-assay coefficients of variation for the same pools were 9·0%, 12·2%, 10·6%, and 12·8%.

Figure 1 shows the mean plasma renin activity (± SEM) grouped on the basis of initial angiotensin I concentration with plasma renin activities varying between 0 and 11 ng ml^{-1} h^{-1}, that is, around the upper limit that we have found in normal subjects on the fifth day of a 10 mmol sodium diet. It is apparent that the relationship between PRA value and initial angiotensin I value is linear over the entire range (n = 2538; r = 0·69; P < 0·001).

Figure 2 is similarly constructed, encompassing a much wider range of values, including some of the data from Figure 1. It can be seen from this graph that at very high levels of PRA (> 60 ng ml^{-1} h^{-1}) the relationship is no longer linear although the overall correlation is significant (n = 654; r = 0·54; P < 0·001).

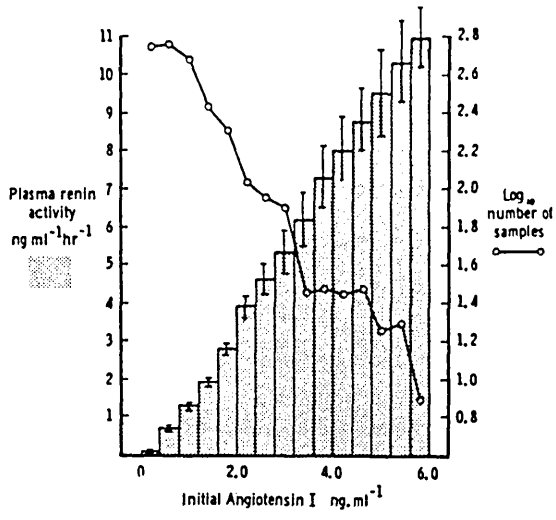


Fig. 1 *Bar graph depicting mean PRA (± SEM) as a function of initial angiotensin I levels rising in increments of 0·4 ng ml^{-1}. Superimposed upon this is the log$_{10}$ of the number of samples in each group.*

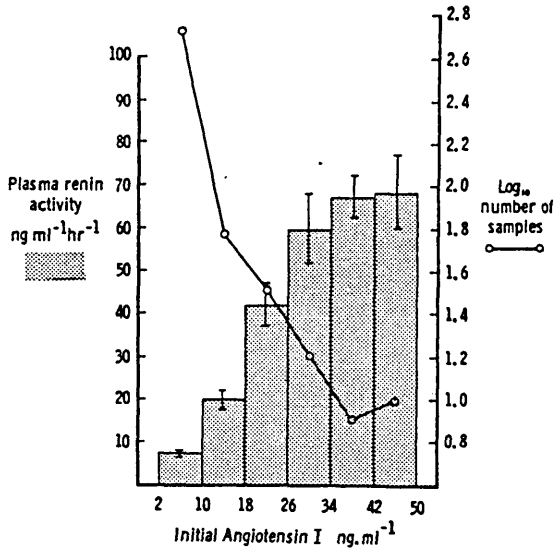


Fig. 2 *Bar graph depicting mean PRA (± SEM) as a function of initial angiotensin I levels rising in increments of 8·0 ng ml^{-1}. Superimposed upon this is the log$_{10}$ of the number of samples in each group.*

Discussion

These results indicate that the PRA value of a plasma sample is linearly related, provided it is below 60 ng ml $^{-1}$ h $^{-1}$, to the initial angiotensin I concentration in the sample. This suggests that, under the conditions of this assay, substrate concentration does not become rate-limiting during angiotensin I generation *in vitro* except at very high levels. This provides further justification for the use of PRA estimation as an index of circulating plasma angiotensin II levels.

The deviation from linearity at PRA values greater than 60 ng ml $^{-1}$ h $^{-1}$ shows that such PRA values may be underestimates owing to the probable rate-limiting effect of substrate concentration and therefore are not true reflections of plasma angiotensin II status. In such cases a more accurate assessment may probably be obtained by incubating the plasma to generate angiotensin I for shorter periods of time. However, such very high values are rare (in this laboratory 18 out of 2752 samples), and these results, although not strictly accurate, are usually of value in diagnosis.

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4

Does the renin–angiotensin system maintain blood pressure in both hypertensive and normotensive subjects? A comparison of propranolol, saralasin and captopril

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Summary

1. Propranolol, saralasin and captopril changed blood pressure in normotensive as well as hypertensive subjects.

2. The percentage change in blood pressure with these three drugs for a given plasma renin activity was similar in normotensive and hypertensive subjects.

3. This suggests that when the renin–angiotensin system is maintaining blood pressure, it maintains the blood pressure to the same extent in percentage terms in normotensive and hypertensive subjects for a given plasma renin activity.

4. Saralasin has marked agonist activity, and probably underestimates the participation of the renin–angiotensin–aldosterone system in the maintenance of blood pressure. The fall in blood pressure that occurred with captopril in normal subjects on their normal sodium intake suggests that the renin–angiotensin–aldosterone system may have an important role in the control of blood pressure in normal subjects on their normal sodium intake. If it does, our results suggest that the renin–angiotensin–aldosterone system plays no greater role in maintaining blood pressure in patients with essential hypertension than normotensive subjects for a given plasma renin activity.

Key words: angiotensin, captopril, essential hypertension, normotensive subjects, propranolol, renin, saralasin.

Introduction

It is not clear how far the renin–angiotensin–aldosterone system maintains blood pressure in normal man on a "normal" sodium intake (Haber, 1976). There is therefore controversy over how important this system is in the maintenance of blood pressure in essential hypertension (Case, Wallace, Keim, Weber, Sealey & Laragh, 1977; Williams, 1977). All drugs that inhibit or block the renin–angiotensin system have other actions, making it difficult to draw conclusions from the use of only one of these agents. In this study, we have compared the effect on blood pressure in normotensive and hypertensive subjects, of three inhibitors of the renin–angiotensin–aldosterone system, propranolol, saralasin and captopril.

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Methods

Unselected patients with essential hypertension, whose diastolic pressure after a 1–2 month period of observation was between 100 and 120 mmHg, and normal male subjects, whose diastolic pressure was less than 80 mmHg after a 1–2 month observation period, were included in the study. All subjects gave their informed consent. The majority of patients had not had previous antihypertensive therapy. In those who had had previous therapy, this had been stopped at least 1 month before if they had been on a β-adrenoreceptor blocker, and at least 3 months before if they had been on a diuretic. The patients were excluded if they were taking any other drug, including the oral contraceptive pill, or had evidence of renal failure, heart failure or cerebrovascular disease. It was not possible to age- or sex-match the controls with the hypertensive subjects. The mean age of the controls was 22 years (range 21–26), all males. Mean age of the hypertensives was 47 years (range 21–63), approximately equal numbers of males and females. All blood pressures were measured by

nurses using semi-automatic ultrasound sphygmomanometers (Arteriosonde 1217) (George, Lewis & Petrie, 1975). The mean value of five readings at 1–2 min intervals, supine, sitting and standing, were taken as the blood pressure in that position. Blood pressure measurements were made at the same time of day in the same room, by the same nurse. All studies were done as outpatients in both the normal and hypertensive subjects. Plasma renin activity was measured by radioimmunoassay (Roulston & MacGregor, 1978). All measurements were made after the subject had been sitting upright for 10 min, between 10.00 and 12.00 hours. The normal range in this laboratory for plasma renin activity in subjects sitting upright between these times on a sodium intake of 100–200 mmol/day is 0·5–2·5 ng h^{-1} ml^{-1}.

For studies with propranolol and saralasin, patients and normal subjects ate either their usual diet or their usual diet with a supplement of 200 mmol of sodium given as 20 slow sodium (CIBA), or a low sodium diet (10 mmol/day). With captopril, the normal subjects were studied on sodium balances. The normal diet was a sodium intake of 125 mmol of sodium/day, high salt diet 350 mmol/day and the low sodium diet was 10 mmol/day. Patients who were studied with captopril ate their usual diet.

Propranolol (160 mg twice a day) was given to normal and hypertensive subjects for 5 days. Blood pressure change was calculated from the blood pressure measured on the day the propranolol was started, and on day 5 of the treatment. In the normal subjects, control observations were made for at least 7 days before the propranolol was started and for 7 days afterwards, so that when they were on a high or low sodium diet, they had been on this diet for at least 7 days before propranolol was started.

Saralasin was infused by using an incremental infusion: 0·05 μg min^{-1} kg^{-1} for 20 min, 0·25 μg min^{-1} kg^{-1} for 20 min and then 1·25 μg min^{-1} kg^{-1} for 20 min. Patients and normals were infused sitting upright with a 1 h control period before and after infusion. Blood pressure was measured every 1–3 min on an Arteriosonde. Saralasin infusion was done either on the normal diet, or on a high salt diet when subjects had been on this diet for 5 days, or the low sodium diet when they had also been on this for 5 days. Percentage change in blood pressure was calculated from the change in mean blood pressure in the 20 min before infusion and the last 10 min at the highest rate of infusion.

Captopril (25 mg thrice daily), was given to patients for 1 week, then increased to 50 mg thrice daily in week 2, 100 mg thrice daily in week 3 and in week 4 the dose was 150 mg thrice daily. If the supine diastolic pressure was less than 85 mmHg, the dose of captopril was not increased, but was kept to the same amount for that week. All patients were studied on their normal diet. It was not possible to treat the normals in the same way. They were given three doses of 25 mg for 1 day, increasing to three doses of 50 mg for day 2 and 100 mg thrice daily for the next 3 days. There was a contr-' , -iod of at least a week before starting captopril in the normals, where they were on a fixed sodium intake of a normal, high or low sodium diet, and a control period of a week after stopping captopril. Blood pressure change in the patients was calculated from the mean supine blood pressure on the day of starting treatment with captopril, and on week 4 of treatment at the maximum dose. Blood pressure change in the normals was calculated from mean supine blood pressure on the day of starting captopril and that of day 5 of treatment.

Percentage change in mean blood pressure was calculated from the change in mean blood pressure calculated as the diastolic plus one-third of the pulse pressure.

Results

Propranolol

Forty-one patients were treated with propranolol on their normal diet only. There was a significant correlation between the logarithm of the initial plasma renin activity before treatment and the percentage change in mean supine blood pressure: $r = 0·62$, $P < 0·01$ (Fig. 1). Eighteen normotensive subjects had propranolol on various diets as shown in the Figure. There was a significant-correlation in the normotensive subjects between the logarithm of the initial plasma renin activity and the percentage change in mean supine blood pressure when the correlation was done for all three diets ($r = 0·63$, $P < 0·01$). The slope and elevation of these two correlation lines for hypertensive and normotensive subjects were not significantly different (Fig. 1).

Saralasin

Forty-four patients were studied on the low sodium diet, four on their normal diet. There was a highly significant correlation in these patients between the logarithm of the plasma renin activity

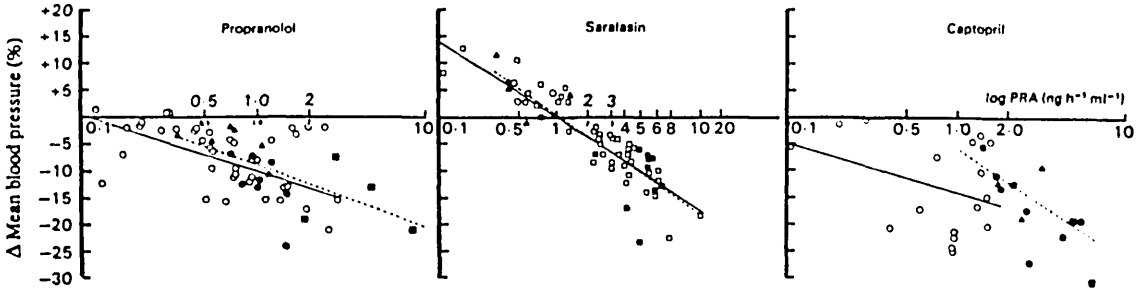


FIG. 1. Percentage change in mean blood pressure sitting upright with propranolol (160 mg bd for 5 days) saralasin (incremental infusion) and captopril (hypertensive 3 × 150 mg/day for 4 weeks, normotensive 3 × 100 mg/day for 5 days) against log initial plasma renin activity (PRA) in normotensive (●, ▲, ■) and hypertensive subjects (○, △, □). ●, ○, Normal diet; ▲, △, high sodium diet; ■, □, low sodium diet. Regression lines for hypertensive (——) and for normotensive (– – – –) subjects are shown.

measured before infusion, and the percentage change in mean sitting blood pressure with saralasin: $r = 0{\cdot}88$, $P < 0{\cdot}001$ (Fig. 1). Seventeen normals were studied, five on a high sodium diet, one on a normal diet, and ten on a low sodium diet. There was a highly significant correlation between the logarithm of the initial plasma renin activity and percentage change in mean sitting blood pressure with saralasin: $r = 0{\cdot}85$, $P < 0{\cdot}001$ (Fig. 1). There was no significant difference in the slope or elevation of the two regression lines (Fig. 1).

Captopril

Captopril was given to 18 hypertensive subjects on their normal diet. There was a significant correlation between the logarithm of the initial plasma renin activity before treatment and the percentage change in mean supine blood pressure on week 4 of treatment: $r = 0{\cdot}54$, $P < 0{\cdot}02$ (Fig. 1). Thirteen normals were studied with captopril given for 5 days, eight on their normal diet, three on a high sodium diet and two on a low sodium diet. There was a significant correlation between the logarithm of the initial plasma renin activity and percentage change in mean supine blood pressure ($r = 0{\cdot}71$, $P < 0{\cdot}01$) with captopril (Fig. 1). There was no significant difference between the regression lines, but the residual variance was such that they are not strictly comparable. This is partly due to the higher plasma renin activity in the normal subjects studied so far (Fig. 1).

Discussion

All three drugs changed the blood pressure in normotensive as well as hypertensive subjects. The percentage change in blood pressure for a given plasma renin activity was very similar in the normotensive and hypertensive subjects for all three drugs. One drawback of this study was that the normals were considerably younger than the patients, but this does not necessarily invalidate a comparison being made between them. Another problem in comparing normotensive with hypertensive subjects is how to compare the blood pressure changes that occur. We have chosen to analyse them in terms of percentage change.

The mechanism whereby propranolol lowers blood pressure is not clear (Gross, 1977). The correlation between blood pressure fall and initial plasma renin activity in both the hypertensive and the normotensive subjects does not necessarily imply that the mechanism of the blood pressure fall is related to, or mediated by, the renin–angiotensin system. However, the results do suggest that, whatever the mechanism, the percentage fall in blood pressure with propranolol is the same in normotensive and hypertensive subjects for a given plasma renin activity. Saralasin, which probably is a more specific inhibitor of the renin–angiotensin system than propranolol, also caused the same percentage change in blood pressure for a given plasma renin activity in the normotensive and hypertensive subjects. The results showed that when plasma renin activity was above 2–3 ng h^{-1} ml^{-1} angiotensin II was maintaining blood pressure to some extent in both normotensive and hypertensive subjects. Saralasin, however, is a potent agonist (MacGregor & Dawes, 1976; Hollenberg, Williams, Taub, Ishikawa, Brown & Adams, 1977; Case, Wallace, Keim, Weber, Drayer, White, Sealey & Laragh, 1976) and, when given as a short-term infusion, blocks only the

immediate effects of circulating angiotensin II. Both of these factors will tend to underestimate the participation of the renin–angiotensin–aldosterone system in the control of blood pressure. Captopril also lowered blood pressure in both the normotensive and hypertensive subjects. The percentage fall in blood pressure was similar in both the normotensive and hypertensive subjects, and was significantly correlated with the initial plasma renin activity. The mechanism of action of captopril is unclear (Gavras, Brunner, Turini, Kershaw, Tifft, Cuttelod, Gavras, Vukovich & McKinstry, 1978; Bravo & Tarazi, 1979). It seems at least in part to be due to the fall in circulating angiotensin II that occurs. Our results would therefore suggest that the renin–angiotensin–aldosterone system is probably maintaining blood pressure to some extent in normotensive subjects on their normal sodium intake, and implies that, at least in percentage terms, the renin–angiotensin–aldosterone system plays no greater role in essential hypertension for a given value of plasma renin activity than in normotensive subjects.

Acknowledgments

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A rapid, automated radioimmunoassay for estimation of plasma renin activity

5

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Abstract

A microprocessor-controlled radioimmunoassay for plasma renin activity (PRA) is described, and its performance assessed and compared with a previously reported manual PRA assay. Owing to the decrease in manual work and the high throughput of this assay system large numbers of samples can be measured. This assay would make it possible to routinely measure PRA in large numbers of patients.

Introduction

THERE is considerable interest shown in the renin angiotensin system and its relationship to blood pressure control (Boyd, 1975; Laragh, 1979). This has led to a series of assays being developed for estimation of the different components of the system (Boyd et al, 1969; Düsterdieck et al, 1971; Tree, 1973; Morton et al, 1976; Fyhrquist et al, 1978; and Millar et al, 1978). The most widely used assay is the estimation by radioimmunoassay of plasma renin activity; that is the measurement of angiotensin I generation rate under controlled conditions of pH, temperature and time.

Nearly all of the assays thus far described are time consuming requiring many pipetting and tube manipulation steps. They also use coated-charcoal which is a rather non-specific separation system. In this paper we describe an automated radioimmunoassay for PRA estimation. The assay is under microprocessor control; it is therefore less time consuming for the biochemist and is capable of a high sample throughput (>300 tubes per hour). The assay employs a highly specific double antibody separation system using an in vacuo filtration technique to minimise non-specific binding effects.

Materials and methods

(i) Assay buffer

The buffer used routinely as diluent in the assay was 0.04 molar phosphate buffered saline containing 8 millimolar di-sodium ethylenediaminetetraacetic acid (E.D.T.A.) 0.2 millimolar sodium ethylmercurithiosalicylate (thiomersal), and 1.0 gram. litre^{-1} of bovine serum albumin.

Requests for reprints should be addressed to J. E. R. at the above address.

(ii) Rinsing buffer

The buffer used to rinse the probes and wash out the reaction tubes at the separation stage was prepared to the same specification as the above assay buffer but contained additionally 0.5% (v/v) of Tween 80. The detergent action of the Tween 80 helps prevent unbound label sticking to the filter discs.

(iii) Angiotensin I

Supplies of angiotensin I were purchased from Serva Feinbiochemica Ltd. of Heidelberg. The amino acid sequence was H_2N-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-OH, the molecular weight being 1297.

The standard, prepared in assay buffer at a strength of 250 ng. ml^{-1}, was stored in 2 ml. freeze-dried aliquots. Reconstitution to 20 mls. yielded a working solution of 25 ng. ml^{-1}.

(iv) Angiotensin II

Angiotensin II was obtained from CIBA Laboratories of Horsham, Sussex. The amino acid sequence was H_2N-Asp-Arg-Val-Tyr-Val-His-Pro-Phe-OH, the molecular weight being 1047. The standard was prepared, stored and reconstituted as described for angiotensin I.

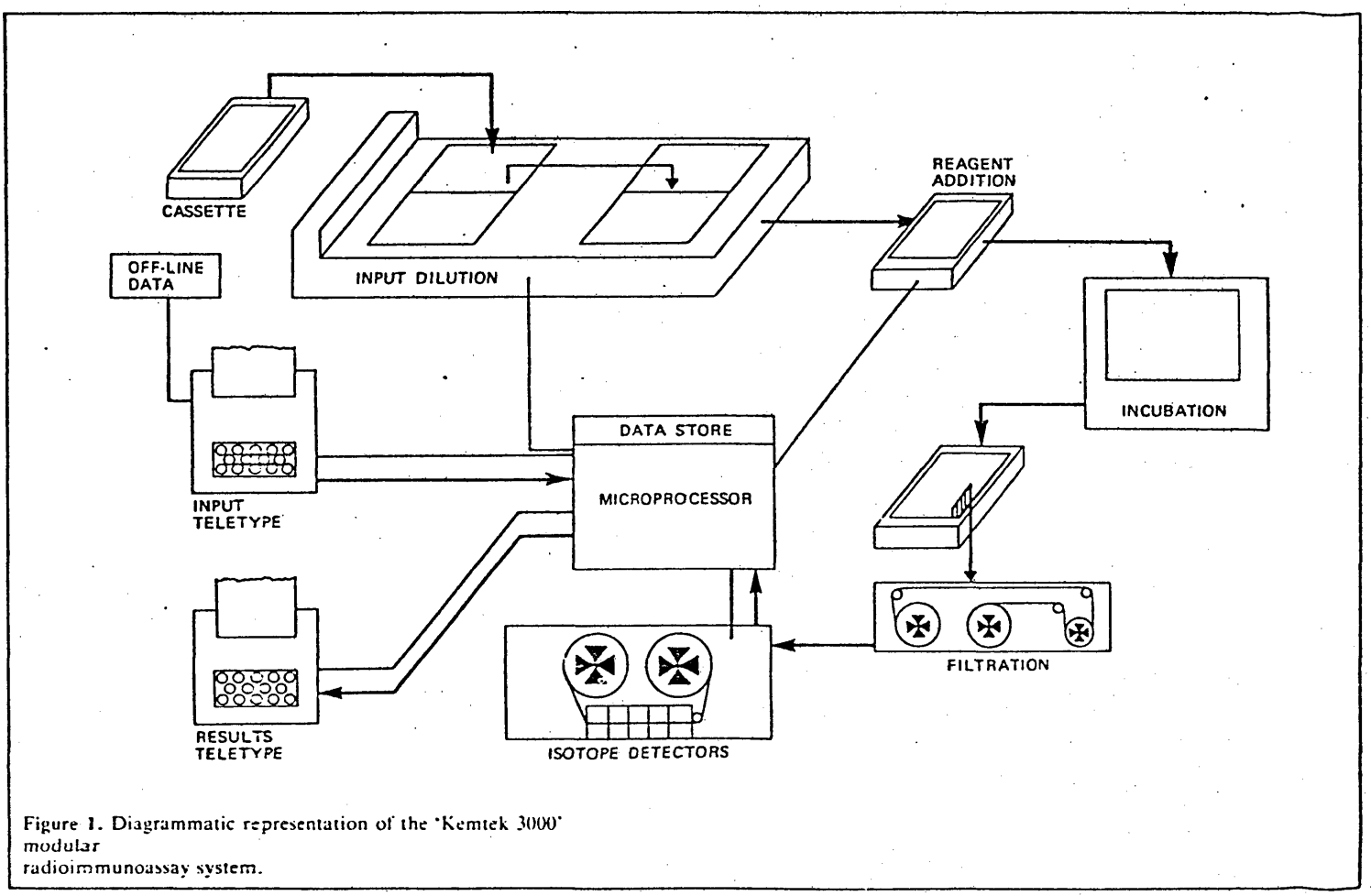


Figure 1. Diagrammatic representation of the 'Kemtek 3000' modular radioimmunoassay system.

v) ^{125}I-Angiotensin 1

Angiotensin I was radiolabelled by the chloramine T method of Greenwood, Hunter and Glover (1963). 5 µg aliquots of antigen in 10 µl of barbitone buffer, pH 8.6, were routinely iodinated with 2 millicuries of Na^{125}I (IMS 30) purchased from the Radiochemical Centre, Amersham. Incorporation of 85 ±10% of the radioactivity into the peptide peak was indicated by the elution profiles obtained from a 30 cm. × 1 cm. Sephadex G25 column. Specific activity as estimated by self-displacement analysis (Gocke et al, 1969; Roulston, 1979) gave values of the order 450-550 µCi/µ g.

(vi) Antiangiotensin I

The primary antiserum was raised in New Zealand white rabbits using the carbodiimide condensation, as described by Goodfriend et al (1964), with thyroglobulin in a molar ratio of 20:1 based on the findings of Skowsky and Fisher (1972). The rabbits were given multiple site injections, subcutaneously, of the antibody-carrier complex in an emulsion with Fruend's complete adjuvant.

The homogeneity of the antibody population, together with values for the equilibrium (affinity) constant and concentration of binding sites was estimated by the Scatchard plot (Scatchard, 1 949) and the Odell saturation analysis (Odell, 1959). The application of these techniques to assessment of radioimmunoassay parameters has been discussed earlier. (Roulston and Adam, 1978).

(vii) Anti-rabbitgammaglobulin (Second antibody)

The secondary antibody was raised in sheep to rabbit gamma globulin (R.G.G.) by direct multiple site subcutaneous injections of purified R.G. G. in emulsion with Fruend's complete adjuvant.

(viii) Preincubated binding complex

Anti-angiotensin I, present in such concentration to yield a bound to free ratio, R, of approximately 1.0 in the absence of unlabelled ligand was mixed with an anti- R.G.G. solution of sufficient strength to yield maximal precipitation at equilibrium.

Continuous mixing of the primary and secondary antibodies at 4°C for 72 hours at ten times working strength was found to be sufficient time for equilibration and 2 ml. aliquots were stored freeze dried. Upon reconstitution to 20 mls. this yielded sufficient antibody for an assay of 400 tubes at a final working dilution of primary and secondary of 1/20,000 and 1/2,000 respectively.

(ix) Plasma samples

The methodology for blood sampling into EDTA tubes and the subsequent cold centrifugation and rapid freezing of the plasma has been described in detail earlier as has the procedure for controlled angiotensin I generation in the presence of citric acid/citrate buffer, pH 5.7, and converting enzyme inhibitors (phenylmethylsulphonylfluoride and 8-hydroxyquinoline) at 37°C. (Roulston et al, 1978).

(x) Radioimmunoassay procedure

(a) Sample input and dilution

The radioimmunoassay is carried out on an automated modular

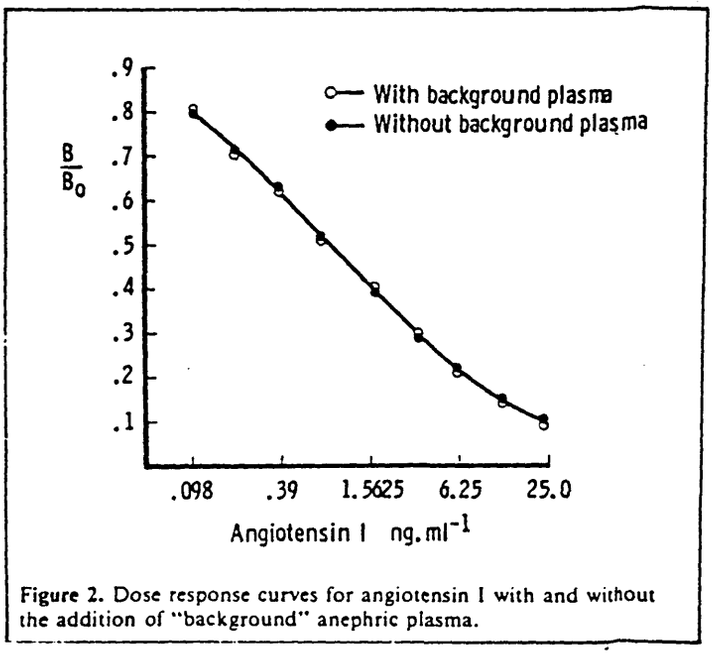


Figure 2. Dose response curves for angiotensin I with and without the addition of "background" anephric plasma.

Table I. Recovery study

Values of angiotensin I concentration expressed as a percentage of the predicted value in control samples.

Predicted concentration ng/ml^{-1}	Observed concentration ng/ml^{-1}	% Recovery $\frac{obs}{exp} \times 100$
0.2	0.19	95.0
0.5	0.51	102.0
1.0	1.01	101.0
2.0	2.10	105.0
3.0	3.15	105.0
4.0	4.08	102.0
5.0	5.07	101.4
6.0	6.07	101.2
7.0	7.36	105.1
8.0	8.22	102.8
7.5	7.27	96.9
6.5	6.45	99.2
5.5	5.63	102.4
4.5	4.56	101.3
3.5	3.51	100.3
2.5	2.55	102.0
1.5	1.48	98.7
0.8	0.82	102.5
0.4	0.41	102.5

n = 19; $\bar{x}$ = 101.4%; S.D. = 2.59; C.V. = 2.56%.

radioimmunoassay machine, the Kemtek 3000 (see Figure 1) which is under microprocessor control (Bagshawe, 1975; Adam et al, In Prep). Into the "feed" cassette are placed two tubes containing assay buffer, for dispensation into the non-specific binding and antigen blank (Bo) tubes, followed by a tube containing approximately 2.0 ml. of angiotensin I standard at a concentration of 25 ng/ml. Adjacent to the standard tube are placed four empty tubes in which the higher doubling dilutions o the standard are performed. The input and dilution module transfers reagents from the "feed" cassette to an adjacent "reaction cassette", containing sufficient tubes for the entire assay, as follows: 100 µl of buffer from feed tube No. 1 to the first ten reaction tubes (non-specific binding estimations), 100 µl of buffer from feed tube No. 2 to reaction tubes 11-20 (Bo estimation), 100 ul of standard from feed tube No. 3, to tubes 21-23, 50 µl of standard +50 µl of buffer to tubes 24-26, 25 + 75 µl to 27-29, 12.5 + 87.5 to 30-32, and finally 6.25 + 93.75 to tube 33-36. The final fou dilutions of standard are performed externally in the four empt tubes located in the feed cassette and are then serially transferred Thus the standard curve encompasses the following values in ng ml^{-1}. 25.0, 12.5, 6.25, 3.125, 1.5625, diluted internally, and 0.78125 0.3906, 0.195 and 0.098 diluted externally, that is dilutions of 1/32 1/64, 1/128 and 1/256.

After all the standards have been diluted and dispensed the fee cassette moves to the samples to be assayed. Instructions how t

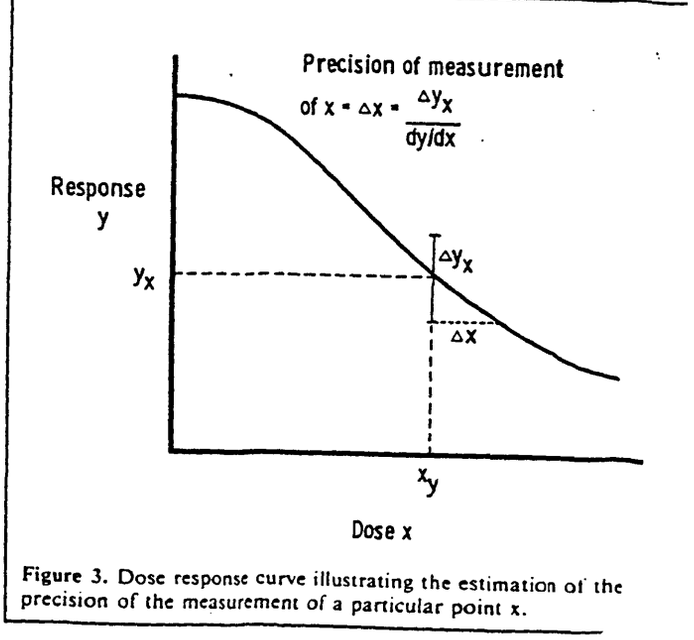


Figure 3. Dose response curve illustrating the estimation of the precision of the measurement of a particular point x.

treat the individual samples, in terms of number and dilution(s) of the determinations required, are conveyed via an input teletype by the operator. Routinely all samples are diluted two-fold; however, if an exceptionally high value is expected it is possible to order either a higher dilution or series of dilutions of that particular sample in order to ensure a value within the confines of the standard curve. One gratuitous advantage of being able to routinely dilute the sample (which is 46.8% plasma at this stage) two-fold is that in the final mixture of 200 µl only 23.4 µl are plasma; i.e. 11.7%. Thus the routine addition of background plasma to obviate "plasma effects" is not necessary (see Figure 2).

After all the samples have been transferred (routinely less than 300 µl of sample will suffice for a triplicate estimation) the reaction cassette is transferred to the reagent addition module.

(b) Reagent addition

This module comprises three pumps which, through aligned probes, deliver prescribed volumes of reagents to selected tubes designated by push buttons as non-specific binding (i.e. the 1st 10), Bo (11-20), standards (21-47), diluted samples (defined by the operator at input) and undiluted samples.

For this assay only two pumps are extensively required; the first is programmed to add 50 µl of label to all tubes and the second to add 50µl of the pre-incubated antibody mixture to all tubes with the exception of the non-specific binding estimations. The third pump, used often to add background plasma to all tubes except undiluted samples, is not required except to add a further 50 µl of assay buffer to tubes 1 - 10 (non-specific binding estimations).

As this module is also on line (there is, if required, an off-line capability) the tubes requiring, in some assays, background plasmas are selected by the module without further instruction or manipulation by the operator.

The label and antibody (or buffer in tubes 1 - 10) are added simultaneously and mixing of the reaction components is achieved by injection velocity which creates a transient vortex. At the end of the reagent addition the cassette is covered with tightly-fitting plastic film and stored at 4°C for 48 hours or overnight at 37°C to achieve equilibrium.

(c) Separation of bound and free ligand

This is achieved off-line to the microprocessor by an automatic filtration system. The contents of the tubes in the reaction cassette are removed in sequential batches of five by fishtail hypodermic needles attached to pumps and deposited upon discrete fibre glass discs, located on a moving 35 mm. plastic tape. The discs, which are pre-soaked in rinsing buffer to prevent adhesion of free label, are held in vacuo as the reaction mixture and subsequent wash volumes of rinsing buffer are deposited upon them.

Following filtration the discs are conveyed over a system of spools in a drying chamber and, upon emergence, adhesive tape from a contra-rotating spool is automatically stuck upon the tape bearing the discs thereby preventing any cross-contamination from disc to disc, if the spool should be rewound after filtration.

Table II. Parallelism study

Results of linear regression analysis on the standard curve and five serially diluted plasma samples. The variance ratio of each sample with respect to the standard curve is also listed. (see text).

Sample name	Linear regression analysis			Variance ratio of samples with respect to the standard curve	
	Correlation coefficient	Slope	Intercept	F ii	p
Standard curve	-0.9997	-1.4793	1.3991	—	—
U3	-0.9945	-1.5040	0.5556	0.1750	> .05
U2	-0.9964	-1.5052	0.3732	0.1819	> .05
U1	-0.9996	-1.4842	0.9845	0.0333	> .05
G2	-0.9991	-1.5294	1.4208	4.0526	> .05
G1	-0.9978	-1.5590	1.5893	3.6842	> .05

(d) Estimation of radioactivity

To count the radioactivity the filter tape is passed through a counting chamber containing five paired heads located above and below the tape at the same distance apart as the filter discs. The positioning of the discs precisely above and below each pair of counting heads is achieved by a sensor which locates discretely positioned holes at the edge of the plastic tape.

Each disc is counted under each counting head and the results summed to prevent any discrepancy that may be caused by differing counter efficiencies of the various heads. Counting can commence immediately post-filtration; that is, it is not necessary to wait for an entire assay to be filtered before beginning to count the discs. In case of the counting proceeding much quicker than filtration a switch, tripped by a tautening of the tape between drying chamber and counters, automatically cuts in and temporarily halts the counting.

(e) Analysis of results

The counts can be analysed in two ways; routinely a cubic fit is employed although a log-logistic analysis is also possible. All the tubes counts are printed out even if subsequently rejected as being outliers for analytical purposes. As well as interpolating all samples from the cubic fit, giving the curve parameters and group standard deviation, the error about each point on the standard curve is also charted as is the spread and mean of each sample estimation. The computer also plots a semi-log dose response curve and superimposes upon this the ideal curve based upon the results obtained. This is of great value in assessment of precision, or, on an interassay basis, monitoring possible reagent degradation.

Results

(i) Precision

Using Ekin's definition (Ekins and Newman, 1970) that the

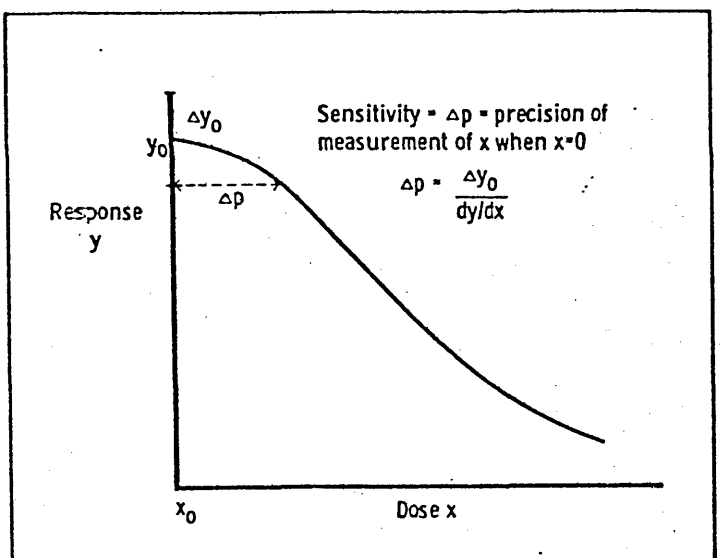


Figure 4. Dose response curve illustrating the estimation of the sensitivity of the curve: that is the precision of the measurement of a point x when x = 0.

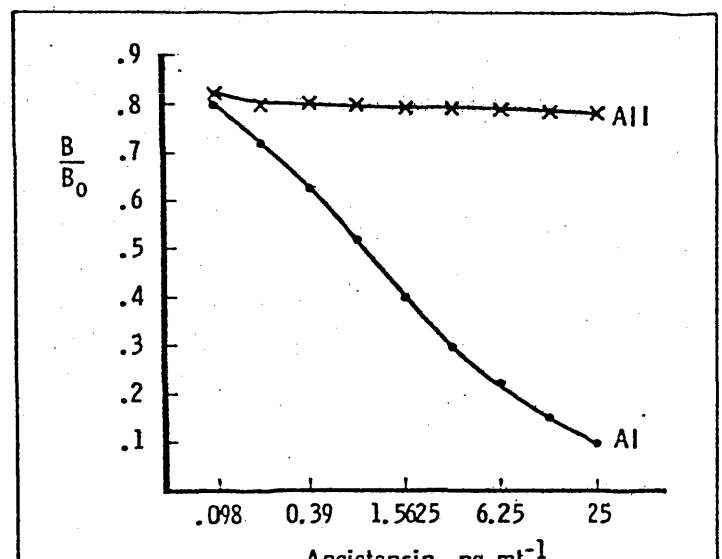


Figure 5. Dose response curves of angiotensin I and angiotensin II using angiotensin I antibody in each case to assess cross-reactivity.

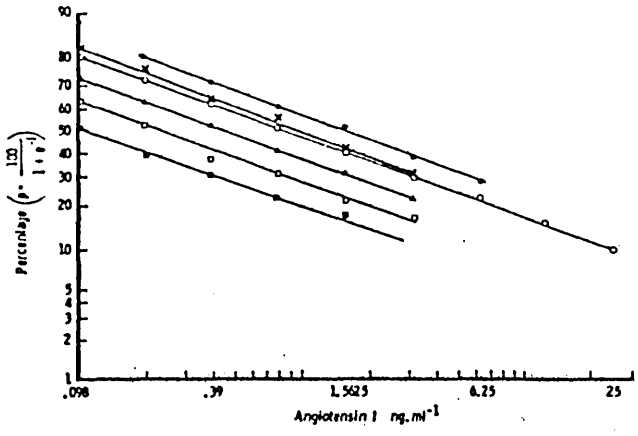


Figure 6. Dose response curves linearised by the log-logit transformation, for the angiotensin standard and five serially diluted plasma samples, showing a high degree of parallelism.

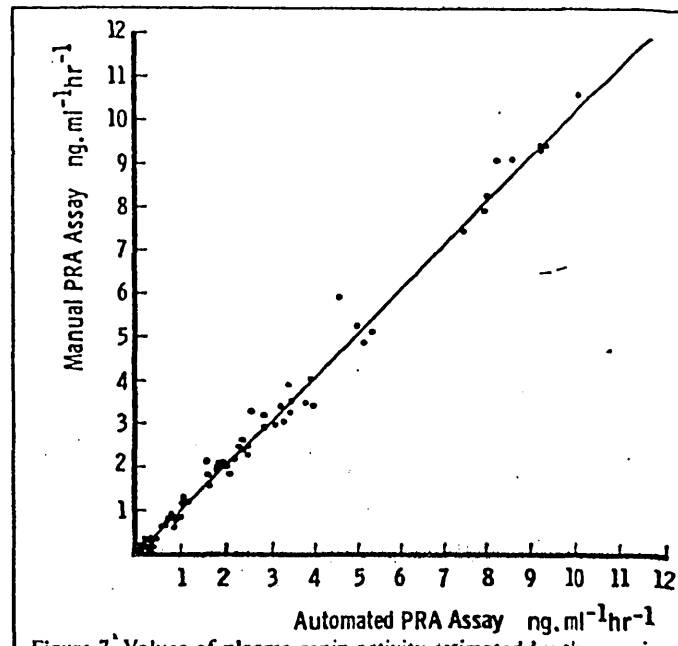


Figure 7. Values of plasma renin activity estimated by the previously published manual RIA technique, plotted as functions of PRA estimated by this automated assay.

precision of measurement of a quantity x, designated Δx, is related to both the precision of the estimate of the response metametery, Δy, and the slope of the response curve (dy/dx) at the corresponding point (see Figure 3), the precision of the assay varies from 20 pg. ml^{-1} in the presence of 0.098 ng.ml^{-1} of unlabelled ligand up to 500 pg.ml^{-1} in the presence of a concentration of 25.0 ng.ml^{-1} of unlabelled ligand.

(ii) Sensitivity

Sensitivity may be defined as the precision of measurement of x when $x = 0$. That is; precision $\Delta p = \Delta x$ when $x = 0$. (See Figure 4). For this assay the sensitivity was usually of the order of 25 pg.ml^{-1}.

(iii) Reproducibility

Intra-assay coefficients of variation on three plasma control pools prepared as previously described (Roulston and MacGregor, 1978) were 5.8%, 3.3% and 5.7%. Interassay coefficients of variation for the same pools were 8.7%, 5.6% and 10.2% respectively.

(iv) Specificity

(a) Cross reactivity

Negligible cross reactivity of the antiserum against angiotensin II was observed (see Figure 5). Apparent binding of ^{125}I-angiotensin I in the absence of antibody was routinely less than 1% of the total radioactivity added.

(b) Recovery

Recovery was estimated by assaying anephric plasma previously determined as having undetectable angiotensin I concentration (i.e. less than 25 pg.ml^{-1}) after the addition of known concentrations of angiotensin I. Mean recovery was 101.4% ± 2.56% (see Table I).

(c) Parallelism

The standard curve was compared to a series of serially diluted plasma samples. The dose-response curves obtained were linearised by the log-logistic transformation (see Figure 6). Linear regression analysis of each sample and the standard curve was performed. All correlation coefficients were >-.993 indicating that almost all the variance is explained by regression. The significance of differences between the slope of the standard curve and that of each of the samples was assessed using the variance ratio as described by Snedecor and Cochran (1967). These variance ratios indicated that there was no significant difference in slope of any of the sample curves with respect to that of the standard curve (see Table II). ($F'_{11} = 0.033$-4.0576; $p > .05$.)

(d) Comparability

The assay has also been assessed directly with respect to a manual radioimmunoassay reported previously (Roulston and MacGregor, 1978). Linear regression analysis of 65 samples assayed in triplicate by both methods showed (see Figure 7) a very high correlation coefficient, a slope (m) approaching unity and a intercept (b), tending to zero. ($n = 65$. $r = 0.996$, $m = 1.03$ $b = .001$).

Discussion

This automated radioimmunoassay for PRA appears to have high precision and accuracy indicating that evaluation of samples over a very wide range of values is possible. The reproducibility, as estimated by the interassay coefficients of variation in quality control pools is of a high order. The assay appears to be highly specific; the antiserum does not cross-react with angiotensin II and apparent binding of I^{125} angiotensin-I in the absence of antibody was consistently less than one per cent.

This assay possesses several advantages over previously described PRA assays. The double antibody separation technique employing in vacuo filtration for phase separation not only yields very low non-specific binding but also means that no tube handling is required as would be necessary with a centrifugation step. The tubes, once positioned in the reaction cassette do not have to be individually handled again throughout the entire assay and a much higher throughput of samples is possible.

The importance of the renin-angiotensin system in the control of blood pressure is not certain. However, with the development of specific drugs that block the renin angiotensin system, particularly the oral converting enzyme inhibitor (Gavras et al, 1978), the routine measurement of PRA in patients with high blood pressure may be indicated and has already been advocated by some authors (Laragh, 1979). A limiting factor in measuring large numbers of PRA samples has been the rather inconvenient assay methodologies. The assay outlined in this paper may overcome this difficulty and enable large numbers of samples to be routinely measured.

Acknowledgements

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Validation of the self-displacement technique for estimation of specific radioactivity of radioimmunoassay tracers

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SUMMARY There has been controversy recently about the validity of the data-analysis of the self-displacement technique for estimation of specific radioactivity. This paper investigates the technique theoretically and provides practical corroboration of the results.

The method of self-displacement for estimation of specific activity of radiolabelled tracers in radioimmunoassays is widely used. The principle is simple: increasing amounts of labelled antigen are incubated with a constant amount of antibody under similar conditions to those employed routinely in the assay (Gocke *et al.*, 1969); the mass of tracer present in the tubes is then determined by interpolating from the standard curve the percentage bound for each label increment; the amount of non-radioactive ligand displaced by each increment is equated to the amount of radioactive ligand in the tubes.

It has recently been claimed, however (Morris, 1976), that this analysis is unsatisfactory as it fails to allow for the fact that an unknown mass of labelled antigen is also present and competing for binding sites on the antibody in each solution containing antigen standard. Further to this a graphical analysis of the data is claimed by the same author to obviate this error.

As the self-displacement technique enjoys widespread application and popularity, the possibility that it may be invalid was disturbing, and it was therefore decided to scrutinise both methods of analysis of self-displacement data theoretically and practically in order to ascertain whether there is any basis for the hypothesis of a flaw in the classical methodology and, if so, whether discrepant results occur in either the theoretical ideal situation or in practical experimentation. It would be expected that specific activities, as reflected reciprocally by the calculated weight of tracer in the tubes, would be higher when calculated by the classical method if this proposed flaw in the logic exists.

Materials and methods

For practical experimentation an angiotensin I radioimmunoassay was used (Roulston and MacGregor, 1978). For the self-displacement assay incremental concentrations up to seven times that used in the standard curve were employed. In order to test the theoretical basis of the situation certain assumptions about the nature of the reactants were made in order to facilitate quantitative study. Firstly, it was assumed that labelled and unlabelled ligand were chemically identical with respect to antibody affinity; secondly, non-specific binding was assumed to be negligible and separation of bound and free fractions was assumed to be 100%. The reaction was also to be in equilibrium and therefore follow standard kinetics as reviewed comprehensively elsewhere (Ekins *et al.*, 1968; Yalow and Berson, 1968; Roulston and Adam, 1978).

The following values were therefore assumed: (*a*) a tracer concentration of 20 pg per tube and, say, 1000 counts per minute per picogram; (*b*) standards of 4, 8, 16, 32, 64, 96, and 128 picograms per tube; and (*c*) antibody present in such concentration that it bound up to 10 pg of antigen. From these data a statistical standard curve and self-displacement assay were constructed, and our starting weight of label (20 pg per tube) was calculated by both methods to see if there was any difference in the results predicted in this ideal case. Furthermore, by construction of the standard curve and self-displacement curve for the practical assay, we further ascertained whether theory and practice were consistent and whether there was a discrepancy in the methodology, as had recently been suggested.

Table 1 *Theoretically calculated antigen bindings for a dose-response curve and self-displacement analysis*

Mass of unlabelled antigen added (pg)	*Mass of tracer added (pg)*	*Mass of unlabelled antigen bound (pg)*	*Mass of tracer bound (pg)*	*Percentage o tracer bound*
0	20	0	10	50
4	20	1·67	8·33	41·65
8	20	2·86	7·14	35·7
16	20	4·44	5·56	27·8
32	20	6·15	3·85	19·25
64	20	7·62	2·38	11·9
96	20	8·27	1·73	8·65
128	20	8·65	1·35	6·75
0	20	0	10	50
0	40	0	10	25
0	60	0	10	16·67
0	80	0	10	12·50
0	100	0	10	10·00
0	120	0	10	8·33

Results

Table 1 shows the construction of the theoretical standard curve based on the axioms outlined. The self-displacement assay, constructed similarly, is also shown. Superimposition of these data reveals (Fig. 1)

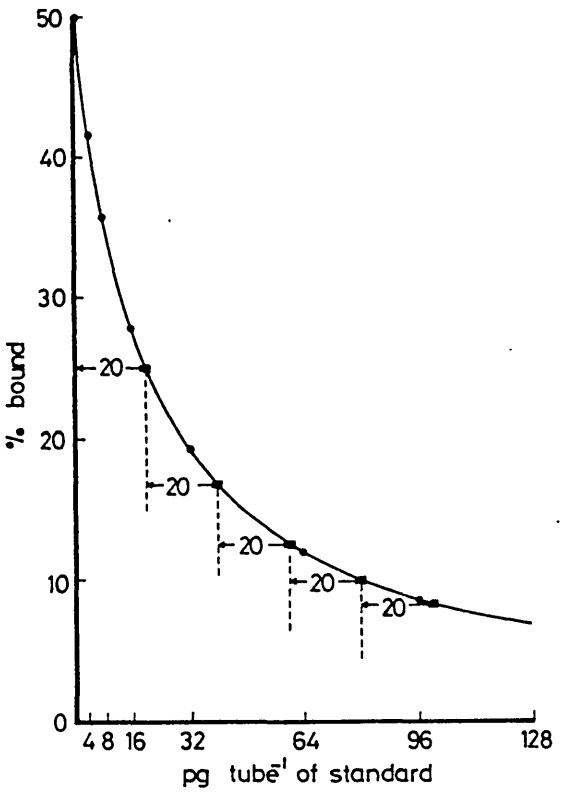


Fig. 1 *Theoretical standard curve showing superimposed self-displacement analysis calculated by the classical technique.*

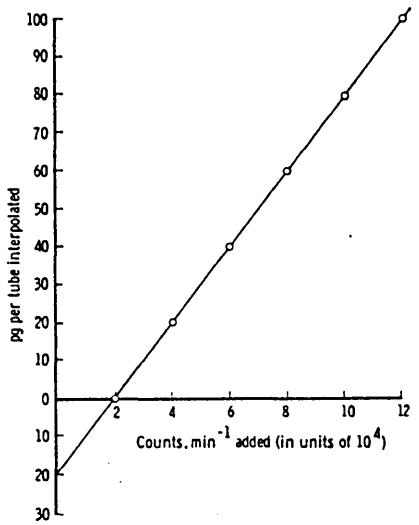


Fig. 2 *Plot of interpolated dose against counts per minute added for the theoretical self-displacement analysis showing the modified calculation technique.*

that each increment of label causes a 20 pg displacement in percentage binding on the standard curve, indicating that the weight of label used in the standard curve was 20 pg. Similarly, by plotting interpolated doses against counts per minute added, the intercept on y at x = 0 is −20 pg (Fig. 2). Thus for an ideal situation the two methods give identical results, and no flaw in the original methodology, or its theoretical basis, is apparent.

Table 2 shows the results of one of the practical experiments with angiotensin I and the self-displacement results obtained. Superimposition of these upon the standard curve obtained shows that the mean value for the weight of label in the tubes is 38·7 pg (Fig. 3). Figure 4 shows that the graphical deter-

Table 2 *Experimental results for antigen bindings for a dose-response curve and self-displacement analysis*

Relative tracer concentration	*Unlabelled antigen added (pg)*	*Percentage of tracer bound*
1	0	50·0
1	10	44·5
1	20	39·6
1	40	32·15
1	80	24·9
1	160	16·0
1	320	8·65
1	640	5·10
2	0	33·4
3	0	27·0
4	0	22·1
5	0	17·5
6	0	14·6
7	0	12·20

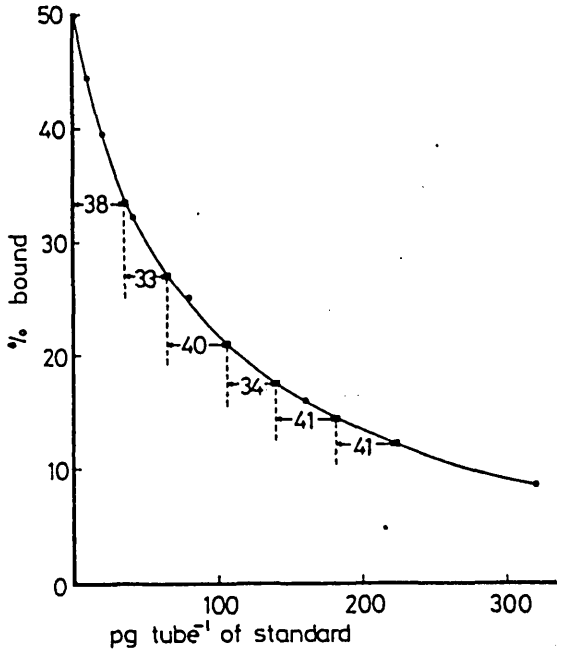


Fig. 3 *Practical standard curve showing superimposed self-displacement analysis calculated by the classical technique.*

mination yields the same value. These results concur very well with the theoretical results obtained above.

Table 3 shows the results obtained for further batches of angiotensin I and for a variety of other antigens: angiotensin II, human chorionic gonadotrophin, gastrin, casein, carcinoembryonic antigen, alphafetoprotein, and a putative tumour-associated ovarian antigen. These results provide further corroboration, as the mean percentage bias was less

Table 3 *Comparison of values obtained using classical and modified self-displacement data analyses on a variety of antigens*

Antigen	*Weight of label per tube (pg)*		*% Bias* $\frac{(A-B)}{A} \times 100$
	Classical calculation A	*Modified calculation B*	
Angiotensin I	30·5	30·0	+ 1·67
	8·8	9·0	− 2·27
	30·0	30·0	0
	30·0	30·0	0
	40·0	36·0	+10·0
	38·7	38·7	0
	73·5	77·5	− 5·4
	110	110	0
	28	28	0
Angiotensin II	4·8	4·4	+ 8·3
HCG	135	140	− 3·7
	100	102·5	− 2·5
	97·5	100	− 2.56
	160	160	0
Gastrin	7·75	7·15	+ 7·74
Casein	3,625	3,750	− 3·44
CEA	456	456	0
AFP	225	240	− 6·67
Ovarian antigen	3,625	3,625	0
			Mean difference = + 0·0615%

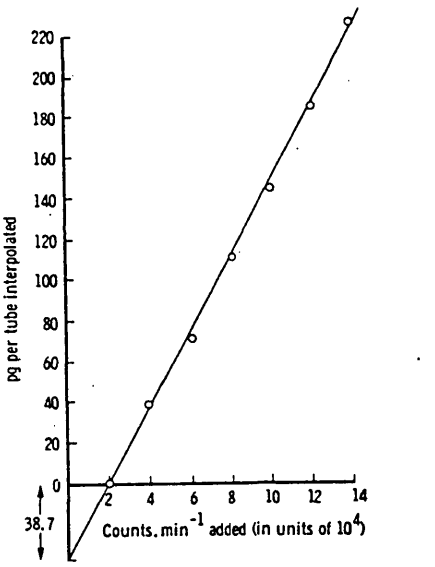


Fig. 4 *Plot of interpolated dose against counts per minute added for the practical self-displacement analysis showing the modified calculation technique.*

than 0·1%. The differences obtained between the two methods were not significant when assessed using Wilcoxon's signed rank test ($n = 19$, $\bar{x} = 0{\cdot}0615$, $t = 18$, $P > 0{\cdot}1$).

Discussion

It is apparent from the theoretical results obtained (Table 1; Figs 1 and 2) that in an ideal situation there is no flaw in the classical self-displacement procedure and that the graphical plot is merely an alternative, perhaps more elegant, way of displaying the result. Also, from the practical results obtained in this laboratory, no difference between the methods could be found (Figs 3 and 4; Tables 2 and 3) and therefore one is led to conclude that the hypothesis of an error in the classical analysis is a false one since no discrepancy in results is obtained in theoretical or practical studies.

Furthermore, detailed study of the theoretical premises of the reactions shows that there is no basis for the suggested error; the competition for binding sites between molecules of standard and tracer is chemically identical with the competition between molecules of tracer alone. Also, as displacement is relative, then absolute total antigen concentration is irrelevant to the analysis. It is, however, worthy of note that any *differences* in affinity for antibody exhibited between labelled and unlabelled antigen will be equally reflected in terms of the value for weight of label obtained by either of the above methods. Since the result is derived from displacement of unlabelled antigen from a standard curve, then, if the tracer has lower affinity than the standard for the antibody, the result for weight per tube obtained will be proportionately lower, hence the apparent specific activity will be higher *irrespective* of the method of calculation used.

Finally, it should be stressed that degree of saturation of antibody is assumed constant in the situations considered, otherwise a fully quantitative treatment is not possible for self-displacement analysis theoretically or practically.

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IS "IDIOPATHIC" ŒDEMA IDIOPATHIC?

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Summary Ten women with "idiopathic" œdema had sodium and water retention and a rapid gain in weight when their accustomed intake of diuretics was suddenly stopped. The magnitude of these changes was directly related to the levels of plasma-renin activity before withdrawal of diuretics. Nine patients became œdematous. Within 10 days of stopping diuretics, plasma-renin activity and urinary aldosterone excretion decreased to normal or below and within 20 days weight-gain and œdema had subsided in seven patients. But the greatest gains in weight, in three patients, were sustained beyond 20 days. Nevertheless, a year later, two of these three patients were free of œdema without the use of diuretics. Intermittent œdema of unknown cause in most, if not all, otherwise healthy women seems to result from their use of diuretics, abetted in some patients by self-imposed fluctuations of sodium and carbohydrate intake, and does not appear to be idiopathic.

Introduction

THERE is a group of women who continuously take diuretics. They are often paramedical workers[1] who are concerned about their weight and appearance.[2] Why they began to take diuretics is rarely clear but some state that they were initially œdematous. All agree that if they stop taking diuretics they become œdematous. This syndrome is often referred to as "idiopathic œdema".

We previously confirmed that two such patients, on stopping diuretics suddenly, had a sudden increase in weight, with the development of œdema, due to acute sodium and water retention.[3] The rise in weight continued for 4–7 days before there was a diuresis and natriuresis. In the next 5–10 days the weight returned to that which it had been before the diuretic was stopped. Both patients remained well thereafter without œdema or the need to take diuretics. In these two patients there was a suggestion that the œdema which followed the cessation of the diuretics was due to the prolonged action of compensatory sodium-retaining mechanisms stimulated by the preceding ingestion of diuretics. We speculated that this compensatory mechanism might involve the renin-angiotensin-aldosterone system.

We present here measurements, in a further 10 patients with so-called idiopathic œdema, of plasma-renin activity and urinary aldosterone before and after stopping the diuretics.

Patients and Methods

Most of the 10 patients gave a history of intense concern

CLINICAL DETAILS

Patient no.	Age	Occupation	Diuretic (mg/day)	Duration on diuretics (years)	First indication for diuretics
1	49	Accountant	Frusemide (40) Spironolactone (100)	9	Oliguria following hysterectomy
2	30	Housekeeper	Frusemide (360)	13	Concern for weight
3	44	Nurse	Frusemide (80) Spironolactone (100)	3	Concern for weight
4	32	Housewife	Chlorthalidone (50) Spironolactone (150)	4	Oedema
5	42	Nurse	Ethacrynic acid (50) Spironolactone (300)	9	Concern for weight
6	44	Secretary	Chlorthalidone (100) Cyclopenthiazide (0·5)	4	Oedema
7	55	Secretary	Frusemide (40)	10	Oedema
8	46	Nurse	Bendrofluazide (10)	3	Worried about oliguria
9	62	Housewife	Frusemide (40)	3	Concern for weight
10	35	Housewife	Frusemide (40)	6	Concern for weight

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about their weight and appearance which usually preceded by many years the start of diuretics. They had been on diuretics from 3–13 years after a diagnosis of idiopathic œdema. The age-range was 30–62 years; 3 of the patients were nurses. It was difficult to ascertain why they had started diuretics. The commonest cause was concern for their weight or œdema (table). The patients had found subsequently that when they stopped diuretics they rapidly gained weight and became œdematous with abdominal discomfort and distension, and they thus had convinced themselves that diuretics were essential to prevent œdema. This conclusion was often shared by their doctors who, on occasions, had tried to stop the diuretics, only to be faced a few days later by a distraught, œdematous patient.

The patients were admitted to the metabolic ward. Their diet was similar to their diet before admission but had a fixed daily intake of sodium and potassium. Control observations were made for at least 5 days before the diuretics were stopped. Observations were then continued for 1–3 weeks depending on the subsequent changes in weight. Throughout the study the patients got out of bed before breakfast, dressed, and remained up and about until evening.

Daily 24 h urine collections were made for the estimation of sodium, potassium, and urinary aldosterone excretion. Urinary aldosterone was measured by radioimmunoassay using a modification of the method described by Pakarinen et al.[4] Normal range in this laboratory for aldosterone excretion is 10–25 nmol/24 h in subjects on a sodium intake of 100–200 mmol/day.

The patients were weighed morning and evening. Venous blood was taken between 10 A.M. and 12 noon every 1 to 3 days with the patients sitting upright, for measurements of plasma-renin activity,[5] urea, creatinine, sodium, and potassium. Normal range in this laboratory for plasma-renin activity in subjects sitting upright between 10 A.M. and 12 noon on a sodium intake of 100–200 mmol/day is 0·5–2·5 ng/ml/h.

Results

Weight (fig. 1)

On stopping diuretics all patients put on weight which reached a maximum at 4–10 days. Pitting œdema appeared in nine of the ten patients. After the weight-gain had reached a plateau, there was a fall in weight to control values within 5 days of stopping the diuretics in two patients (9 and 10). In five patients (4–8) weight returned to normal or near normal within 20 days of stopping diuretics. But in the three remaining patients (1–3) who had the greatest gain in weight (3–5 kg), there was little subsequent loss of weight in the 20 days after stopping the diuretics, and the pitting œdema persisted.

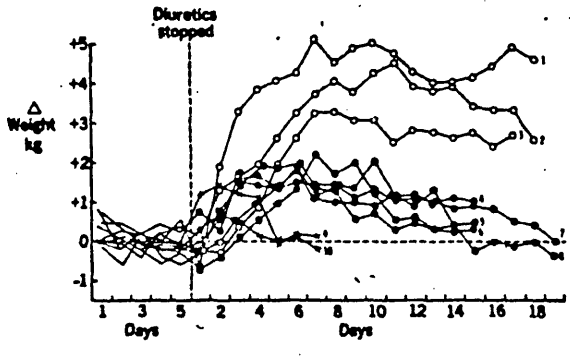


Fig. 1—Changes in weight during control period and after stopping diuretics.

O: patients 1–3 who maintained their gain in weight; ●: patients 4–8 whose weight-gain returned towards normal within 20 days; X: patients 9 and 10 whose weight-gain returned to normal within 5 days.

Plasma-renin Activity (fig. 2A)

Plasma-renin activity while the patients were on diuretics was above normal in 8 of the 10 patients, and was significantly correlated with the maximum gain in weight after stopping diuretics (r=0·87; P<0·001; n=10). Plasma-renin activity before stopping diuretics was also significantly correlated with the amount of sodium retained up to the time of maximum weight-gain (r=0·76; P<0·01; n=10). The three patients (1–3) whose weight-gain was maintained after stopping diuretics had the highest plasma-renin activity while on diur-

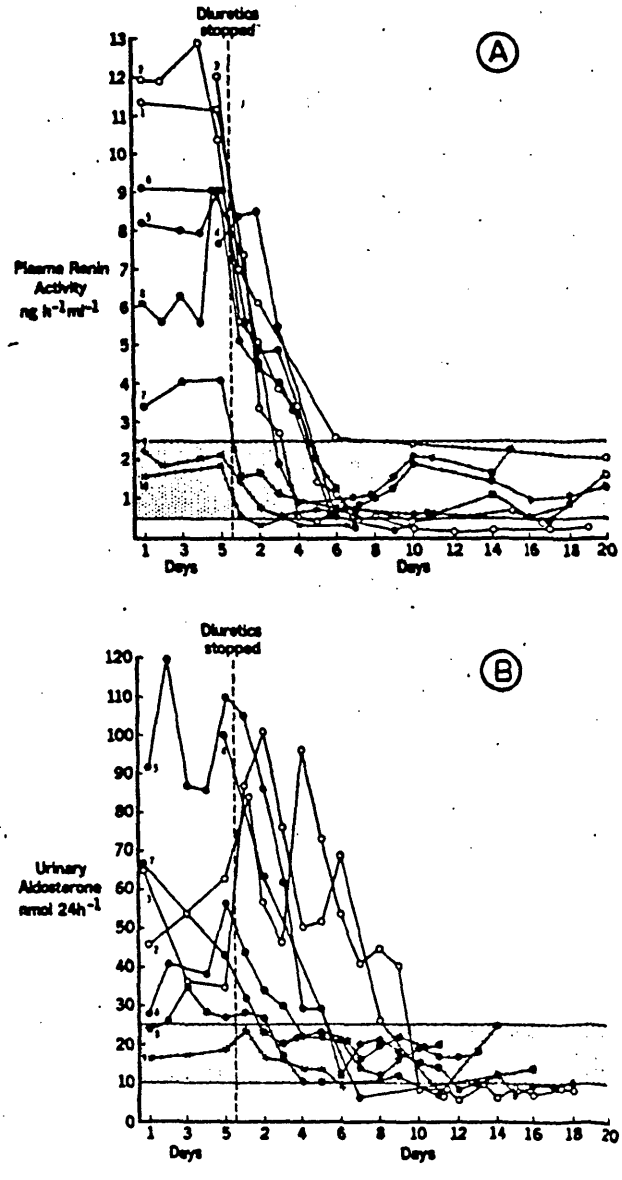


Fig. 2—(A) Plasma-renin activity (patients 1–10), and (B) urinary aldosterone excretion (patients 2–9) before and after stopping diuretics.

Symbols as in fig. 1. Normal range shaded.

etics, range 10·4–13·0 ng/ml/h. The five patients (4–8) whose weight returned to control levels within 20 days had plasma-renin activity above normal but below that of patients 1–3, range 3·4–9·1 ng/ml/h. The plasma-renin activity of the two patients (9 and 10) whose weight returned to control levels within 5 days of stopping diuretics was in the upper range of normal (1·5 and 2·2 ng/ml/h).

Plasma-renin activity fell in all patients to normal or below within 7 days of stopping diuretics. The plasma-renin activity in 2 of 3 patients who had the greatest gain in weight after stopping diuretics, and whose weight-gain and œdema persisted for the 20 days of observation, was in the low range of normal, or below normal, from the 8th to 20th day.

Urinary Aldosterone Excretion (fig. 2B)

Urinary aldosterone excretion while on diuretics was above normal in 6 of the 8 patients in which it was measured but was not significantly correlated with the maximum gain in weight after stopping diuretics (r=0·04) nor with the amount of sodium retained up to the time of maximum weight-gain (r=0·14).

After diuretics were stopped urinary aldosterone excretion fell in all patients (to normal within 6 days in patients 4–9). Urinary aldosterone was measured in only two of the three patients (2 and 3) who had the greatest gain in weight after stopping diuretics and whose weight-gain and œdema persisted for the 20 days of observation. In these two patients urinary aldosterone excretion rose after diuretics were stopped and remained high until the tenth day, when the levels fell to below normal.

Plasma-potassium

Plasma-potassium rose from 3·44±0·24 mmol/l when the patients were on diuretics to 3·98±0·38 ($P<0·01$; n=10) 7–14 days after stopping diuretics.

Patients' Progress

Patients 4–10 have remained well with no subsequent attacks of œdema over the past 1–3 years. None of them have needed diuretics or a sodium-restricted diet. Patients 1–3, whose weight-gain persisted after stopping diuretics, were treated with a low sodium diet (25 mmol/day) which, in all three patients, resulted in a loss of weight and œdema. During the subsequent months sodium intake was gradually increased. Two patients (1 and 3) are now on a normal sodium intake without diuretics. One patient (2) still has occasional attacks of œdema, usually when a period of starvation is followed by a large sodium-containing meal or meals.

Discussion

Our results show that, when these patients with "idiopathic" œdema stopped taking diuretics, sodium and water retention was directly related to the intensity of the stimulation of the renin-angiotensin system by the diuretics. This suggests that the patients' initial weight-gain after stopping the diuretics was due to the high levels of renin and aldosterone which persisted for several days after diuretic withdrawal. Nine of the ten patients became œdematous. Seven patients subsequently lost weight as the plasma-renin activity and urinary aldosterone continued to fall towards normal. Nevertheless, these patients' weights remained above normal for several days after the plasma-renin activity and urinary aldosterone secretion had fallen to normal or below. The three patients whose renin-angiotensin system had been most stimulated by diuretics, and who subsequently had the greatest weight-gain when the diuretics were stopped, maintained this increase in weight and remained œdematous even though the plasma-renin activity and urinary aldosterone at this time was the same, or lower, than those of the other patients who had lost their œdema. This persistence of œdema and weight-gain, despite a fall of plasma-renin activity and urinary aldosterone to normal or below, suggests that sodium continued to be retained in these three patients by some additional mechanism.

The seven patients whose weight returned to normal during the period of observation subsequently continued their usual sodium intake and have not needed, or taken, any diuretics. In the three other patients whose œdema and weight-gain persisted after stopping diuretics, the persistence of the œdema was less obviously related to the previous intake of diuretics. But their subsequent progress suggests that, even in these patients, diuretics may have been responsible not only for the initial development, but also for the subsequent persistence, of the œdema and weight-gain which occurred upon stopping diuretics. These three patients were treated by reducing their sodium intake to a point where there was little or no œdema. During the months that followed it became possible to gradually increase their sodium intake, so that two of them are now comfortable and free of œdema on a normal sodium intake without the need for diuretics. The third patient has not been able to control her sodium and calorie intake (see below) and still becomes œdematous from time to time.

These observations do not explain why these patients started to take diuretics. In agreement with other reports[1,2] all our patients were women, many of them paramedical workers. All had an obsessional concern about their weight and appearance. Many of these patients confessed that, in an attempt to control their weight, they varied their intake of food considerably, both before and after they had started to take diuretics: they might starve for several days and then have one or two large meals. Sudden increases in sodium and carbohydrate intake after periods of sodium deprivation and fasting both cause retention of sodium and water. Prolonged low sodium intake stimulates the renin-angiotensin aldosterone system, the effects of which continue for some time after the sodium intake is suddenly raised. The sodium retention which follows a sudden increase in carbohydrate intake is well known[6–9] and may be related to an associated rise in circulating catecholamines.[10] In preliminary experiments in normal women aged 20–26 we have studied the effect of suddenly changing from a low sodium (10 mmol/day), low carbohydrate (80 g/day) diet to a high sodium (350 mmol/day), high carbohydrate (350 g/day) diet. These sudden simultaneous changes in both sodium and carbohydrate intake produced increases in weight of up to 4·2 kg in 36 h (fig. 4) and œdema. As they gained weight these

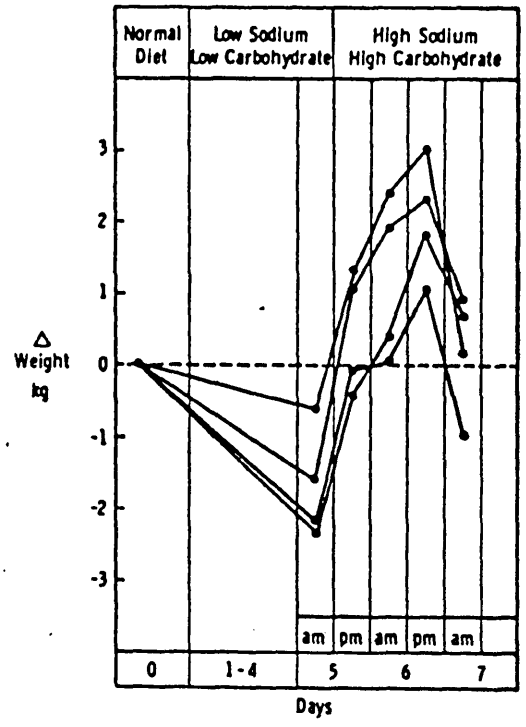


Fig. 3—Changes in weight in 4 normal young women upon changing from their normal diet to a low sodium, low carbohydrate diet, and then to a high sodium, high carbohydrate diet.

normal women complained of the same symptoms as those described by patients with idiopathic œdema, in particular a general feeling of bloatedness and a characteristic tightness across the abdomen and ankles. These observations in normal women suggest that in some patients with "idiopathic" œdema their initial complaints may have been due to fluctuations in sodium and carbohydrate intake caused by their concern for their weight and appearance. Weight-gain, with its attendant discomforts, makes them seek medical advice. The absence of any obvious cause for the œdema, and the patient's persistence, eventually overcomes the doctor's reluctance to prescribe diuretics. Once on diuretics the renin-angiotensin-aldosterone system is persistently stimulated by both the diuretics and the fluctuations in sodium and carbohydrate intake. As a result both the patient and her doctor believe that œdema can only be prevented by the continuous use of diuretics.

Most previous investigators of "idiopathic" œdema have mentioned the patients' tendency to take diuretics. Their reports suggest that their investigations of the cause of the œdema were done within a few days of the diuretics being stopped. It is widely accepted that "idiopathic" œdema results from a reduced blood-volume caused by a capillary leak of albumin. The evidence for the reduced blood-volume is the raised renin and aldosterone levels,[11–17] a tendency to retain sodium and water especially on standing,[17 18] and, after giving a large load of sodium,[19] and the inability of some patients to excrete a water load, particularly on standing.[18] These findings, which are compatible with a state of chronic volume depletion, were probably obtained just after diuretics had been stopped, and were probably due more to the previous prolonged administration of diuretics than to a capillary leak of albumin. Only one attempt[20] has been made to measure the existence of a capillary leak: increases in packed-cell volume on tilting in women with "idiopathic" œdema (10·3%) were compared with the changes in normal women (7·9%). The rate of loss of ^{125}I-albumin from the vascular compartment upon tilting was also studied in six patients but because of difficulties in blood-sampling in the upright position the results were uninterpretable.

There is widespread agreement that, whatever the cause of "idiopathic" œdema, the administration of the diuretics only magnifies the problem.[1 21–23] Our findings, which are consistent with other data,[23] suggest that diuretics not only make the symptoms worse but also perpetuate the œdema. In addition, the condition in some patients may have been initiated and subsequently aggravated by large fluctuations in sodium and carbohydrate intake. It should be noted that "idiopathic" œdema was described[24] only some years after the introduction of oral diuretics.[25] We would suggest that intermittent œdema of unknown cause in most, if not all, otherwise healthy women is due to their use of diuretics, possibly abetted on some occasions by self-imposed fluctuations in sodium and carbohydrate intake—in other words, their œdema is not idiopathic.

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Requests for reprints should be addressed to G. A. M.

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A MICROPROCESSOR-CONTROLLED ASSAY FOR THE ESTIMATION OF HUMAN PLACENTAL LACTOGEN

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A radioimmunoassay for human placental lactogen (HPL) is described using the KEMTEK 3000, which is a modular radioimmunoassay apparatus controlled by a microprocessor. Operation of the KEMTEK 3000 is largely automatic and it requires minimal intervention from the operator. It is capable of 300 reactions per hour so that a large number of estimations can readily be performed. HPL was assayed by a double antibody method on serum samples from pregnant women and patients with trophoblastic tumours.

INTRODUCTION

Since human placental lactogen (HPL) is a sensitive indicator of placental function during pregnancy (Saxena et al., 1968) it is now measured in many laboratories. Its importance as an indicator of the well being of the foetus has been comprehensively reviewed by Chard (1976). This assay, carried out on the fully automated KEMTEK 3000 (Adam et al., in preparation), an updated version of the DARIAS (Bagshawe, 1975), gives ranges of normal and abnormal levels in pregnancy sera in accord with those found by other workers (Genazzani et al., 1971; Letchworth et al., 1971; Teoh et al., 1971; Radiochemical Centre, 1973). Our findings of small amounts of HPL in the serum of some patients with trophoblastic tumours are similar to those of other workers in the field (Yen et al., 1968).

MATERIALS AND METHODS

Antigen

HPL (95%) was purchased from I.C.N. Pharmaceuticals Inc., Life Sciences Group, Cleveland, OH, U.S.A.

Buffer

This was 0.04 M phosphate buffered saline at pH 7.0 containing 0.1% BSA.

Requests for reprints should be addressed to T.A. at the above address.

Antisera

The primary antisera were raised in rabbits using two injections of 1 mg of HPL (plus Freund's complete adjuvant) given a month apart, intradermally at 30—40 sites in each of 6 New Zealand white rabbits. High titres were obtained after 3 weeks.

The second antisera were sheep anti-rabbit γ-globulin. Two injections of 50 mg and 30 mg of purified rabbit globulin were given intramuscularly two weeks apart and the sera were harvested 3 weeks later.

The two antisera were pre-incubated at their optimum concentrations (Hales and Randle, 1963) for 3 days at 4°C. The complexed antisera were stored in freeze-dried aliquots.

Iodination

Iodination was carried out by the Chloramine-T method of Greenwood et al. (1963). Two mCi of carrier-free sodium (^{125}I) iodide (IMS 30) from the Radiochemical Centre, Amersham, Great Britain, were used to label 10 μg of HPL.

Background sera

In the routine assay of pregnancy sera additional protein or serum was not found to be necessary as all the samples were diluted by a factor of 8 with buffer.

The serum of patients with HPL secreting tumours was used undiluted. Hundred μl of a pool of sera from non-pregnant women was therefore added to the non-specific binding (NSB) tubes, the zero antigen tubes and the standard tubes to minimise serum effects.

Brief description of the KEMTEK 3000

The KEMTEK 3000 consists of a control unit which houses the microprocessor and twin floppy discs on which are stored the programme for all assay operations, as well as sample information for up to 60 assays. A teletype is provided for typing in instructions ('input' teletype) and a high-speed, quiet printer for data output.

The dispensing of buffer, standards, and samples into the reaction tubes is performed on the 'input' module of the machine according to standard assay protocols in the microprocessor/disc memory and following specific instructions typed in on the 'input' teletype. The second stage, the addition of the label and the antisera, is performed by the reagent addition module which adds labelled antigen, antibody and pooled serum where appropriate under microprocessor control or alternatively individual reagents can be added with operator intervention.

After incubation the assay is filtered on a module which is not under microprocessor control. The contents of the tubes (treated in batches of 5) are transferred followed by 5 washes to glass fibre discs located at regular discrete sites on flexible plastic tape. After passing through a drying chamber

the discs which retain the antigen—antibody complex are sealed with adhesive tape. After appropriate instructions have been typed in on the 'input' teletype the glass fibre discs are counted in batches of 5 by 5 pairs of photomultipliers. The microprocessor then computes a standard line and reads off the results. The counts, standard line and results are printed on the results teletype. Up to 5 different assays can be processed simultaneously.

The rate of throughput is approximately 300 reactions per hour. In the case of HPL 120 samples can be assayed in triplicate together with standards and control sera in about $1\frac{1}{2}$ h or approximately 180 samples assayed in duplicate in the same time.

Assay procedures

Placental function assay

The complete assay procedure was performed by the KEMTEK 3000. Hundred μl of buffer was dispensed by the machine into each of 10 tubes for the non-specific binding (NSB) estimation followed by 10 tubes for the zero antigen (zeros) assessment. Hundred μl of the highest standard was dispensed into each of 3 tubes from a single supply tube located in the input cassette. The machine then proceeded to make doubling dilutions from the same source of standard to give 8 standards in all. Three 100-μl aliquots of each standard were dispensed sequentially into reaction tubes. The samples were all diluted 8-fold in the routine placental function assay whilst being dispensed into each of 3 tubes in a total volume of 100 μl.

Fifty μl of labelled HPL was added to all tubes and at the same time 50 μl of the double antibody complex was added to all tubes except the NSB tubes. The addition by the dispensing module was timed to proceed at the same rate as the subsequent filtration stage which terminates the reaction. Thus all reaction tubes received the same incubation time.

One hour after addition of the reagents the HPL—antibody complex was separated from the free labelled HPL by vacuum filtration through glass fibre discs, and counted in batches of 5. The standard curve was derived as a cubic fit or as a log-logistic curve (Rodbard et al., 1969; Rodbard and Lewald, 1970) and the HPL samples read off.

Tumour index assay

For this sensitive assay the standards used in the placental function assay were further diluted 10-fold. The samples were used undiluted and therefore it was necessary to add non-pregnant background sera to the NSB, zero antigen and standard line tubes to minimise non-specific effects from plasma proteins (Burr et al., 1969; Van Hall et al., 1971; Vaitukaitis et al., 1972). The antisera complex was diluted 7-fold and the labelled antigen was used at the same concentration as that used in the placental function assay. The incubation time was 3 h at 37° C or overnight at 4° C.

RESULTS

Standard line, zero antigen and non-specific binding

NSB values never exceed 10% of the zero antigen value. The binding of the zero antigen was approximately 35% after one hour's incubation. The coefficient of variation of the 10 zero antigens through 5 consecutive assays varied from 3.2% to 6.4%. The standard line for the placental function assay consisted of 8 points ranging from 2500 ng/ml to 20 ng/ml. For the tumor index assay the range was 250—2 ng/ml. Co-efficients of variation calculated on each point of the standard line for 5 consecutive assays varied from 0.5% to 5.0%.

Label

85—90% incorporation of ^{125}I into HPL was usually obtained. From self displacement studies (Roulston, 1979) specific activities of 90 mCi/mg were commonly found (Figs. 1 and 2). A concentration of 1.4 ng HPL/tube was used in both assays. This corresponded to approximately 1 : 625 of the concentrations obtained from the protein peak at iodination.

Antisera

For the placental function assay the dilution of the primary antiserum was approximately 1/500 and 1/50 for the second antiserum. For the tumour index assay the antisera complex was further diluted 7-fold.

From timed experiments (for the placental function assay) it was found that the antisera gave 35% binding after 1 h incubation. The figure rose to 50% after 3 h. The tumour index assay reaches equilibrium after 3 h at 37°C with the antisera giving ≏33% binding.

In the assay of pregnancy sera equilibrium was not reached and it was

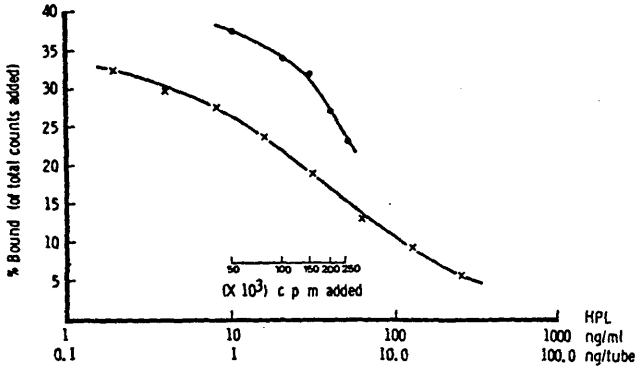


Fig. 1. Dose—response curve for HPL tumour index assay (x———x) and selfdisplacement study (●———●).

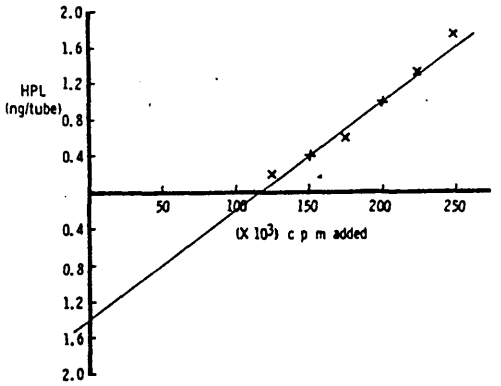


Fig. 2. Self-displacement study to calculate p* (concentration of labelled ligand).

therefore not possible to calculate a true value for the affinity constant (K). However, the tumour index assay reached equilibrium and a Scatchard plot and a double reciprocal plot (Fig. 3) could be made. From these graphs a value of 0.52 $(ng/tube)^{-1}$ was found for K and a value of 1.35 ng/tube was

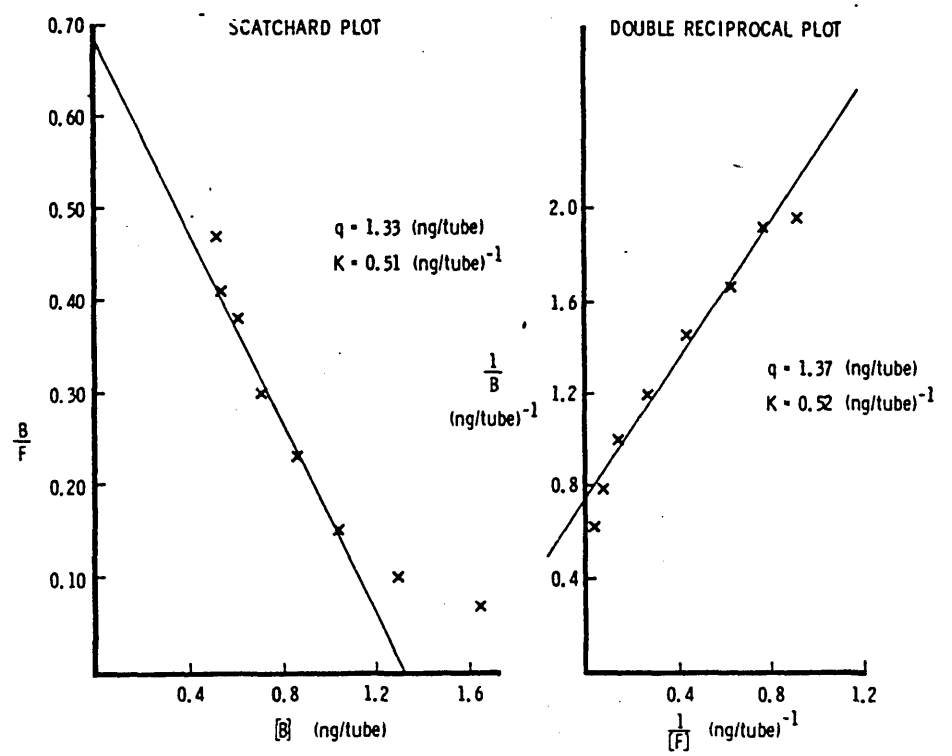


Fig. 3. Scatchard plot and double reciprocal plot. [B] = concentration of bound HPL, [F] = concentration of free HPL, q = concentration of binding sites, K = affinity constant.

obtained for the primary antiserum concentration q (expressed in terms of number of binding sites) when the antiserum dilution was 1 : 3500. Taking the equation of Ekins et al. (1968) in which the equilibrium is described by:

$$R^2 + R\,(1 + Kp + Kp^* - Kq) - Kq = 0$$

where R = bound : free ratio, K = affinity constant, p = concentration of ligand, p* = concentration of labelled ligand, and q = concentration of binding agent (in terms of number of binding sites).

At 33% binding R = B/F = 0.5 when p = 0 i.e. zero antigen conditions, q = 1.35 ng/tube (mean value from Scatchard plot and double reciprocal plot), and p* = 1.4 ng/tube. Then $0.25 + 0.5\,(1 + 1.4\,K - 1.35\,K) - 1.35\,K = 0$, $0.75 = 1.33\,K$, $K = 0.56\ (\text{ng/tube})^{-1}$.

Assuming the molecular weight of HPL to be 21,000 the molar concentration of p* and q are, respectively, 1.33×10^{-9} and 1.29×10^{-9}. The affinity constant K has the value of $0.59 \times 10^{-9}\ M^{-1}$.

Using this value of K it is then possible to calculate the value for R, i.e. B/F for all the points on the standard line. The calculated values for B = R(100)/(1 + R) are shown in Fig. 4 together with the actual values found in the assay. Since the observed values for the dose—response curve effectively superimpose upon those predicted by Ekins' equation it would appear that the experimentally determined values for p* and q are good approximations, and that this assay system follows the equilibrium kinetics described by Ekins and Newman (1970).

Reproducibility

Three samples of different HPL concentrations were each estimated 3 times on 10 consecutive assays over a period of 10 weeks. The intra-assay

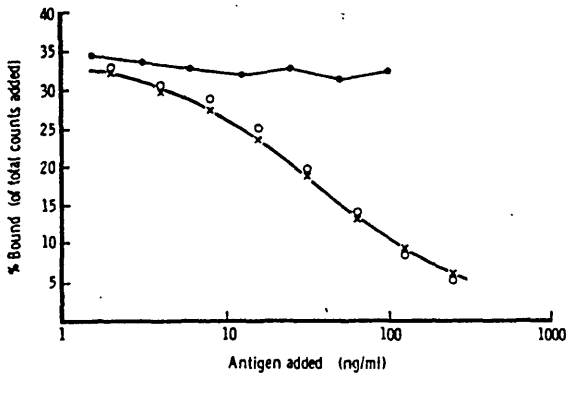


Fig. 4. Dose—response curve for HPL tumour index assay (x———x) found by experimentation. Predicted dose—response curve (o———o) for HPL. HGH dose—response curve (●———●).

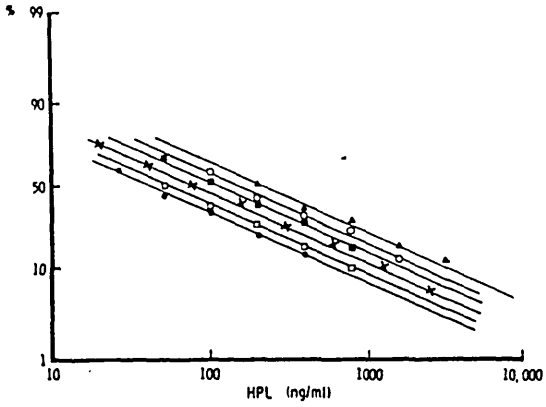


Fig. 5. Log-logistic dose response curve for the pregnancy assay (X———X) and parallelism shown by the doubling dilutions of 5 serum samples.

coefficient of variation was found to be 4.6% and the between assay variation 9.8%.

Parallelism

Five serum samples were diluted X4, X8, X16, X32, X64. The plots (Fig. 5) of the dilutions of the sera against tracer binding parallel the standard curve indicating that the antibody was reacting with HPL alone.

Specificity

No cross-reactivity with growth hormone (HGH) was observed at concentrations of growth hormone up to 100 μg/ml in either the placental function assay or the tumour index assay (Fig. 4).

Recovery

In recovery experiments 2, 4.5, and 7 μg of HPL were added to 3 normal pooled sera, respectively, and analysed. The results are summarised in Table 1.

Comparability in placental function assay

Figure 6 shows the range of HPL concentrations obtained in 300 apparently normal pregnancies, together with means for each week in the last weeks of pregnancy. Figure 7 indicates the mean HPL values obtained in normal pregnancies in our study and in other report studies (Genazzani et al., 1971; Letchworth et al., 1971; Teoh et al., 1971; Radiochemical Centre, 1973).

HPL levels in patients with tumours

The HPL concentration in the serum of 13 patients with untreated inva-

330

TABLE 1

RESULTS OF HPL RECOVERY EXPERIMENTS ON 3 SEPARATE POOLED HUMAN SERA

	Pool 1	Pool 2	Pool 3
HPL added (μg/ml)	2	4.5	7.0
Numbers of estimations	15	17	17
Mean value (μg/ml)	2.02	4.61	6.94
Standard deviation	0.36	0.62	0.82
Coefficient of variation (%)	17.8	13.6	11.9
Mean % recovery	101.0	102.5	99.1
Standard deviation	18.2	13.9	11.8
Coefficient of variation (%)	18.2	13.6	11.9
	Total number of estimations		= 49
	Mean % recovery		= 100.8
	Standard deviation		= 14.5
	Coefficient of variation (%)		= 14.3

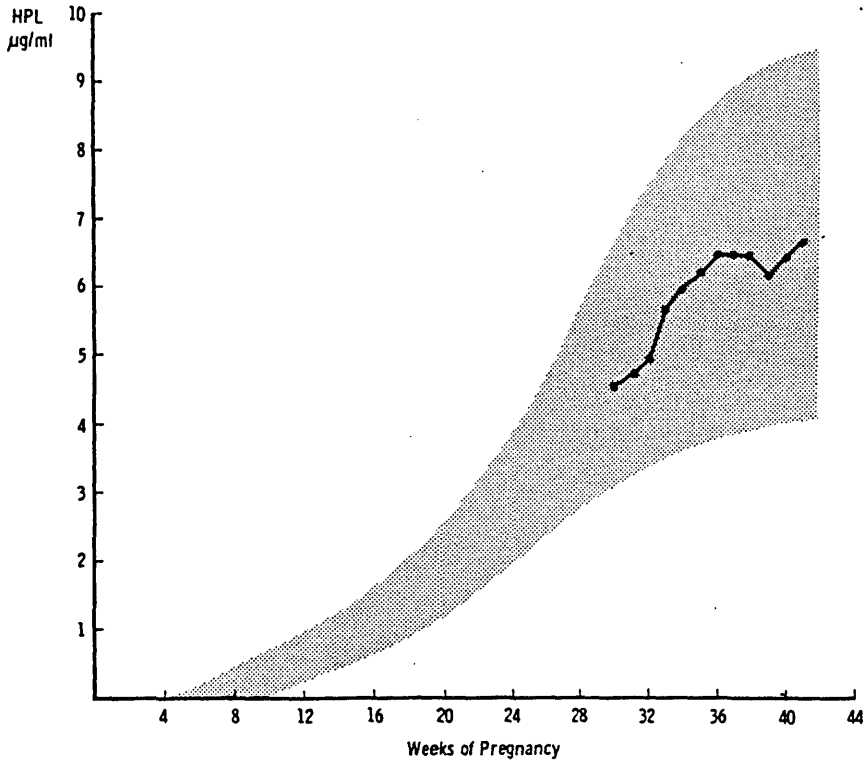


Fig. 6. Spread of HPL levels in 300 apparently normal pregnancies, with the mean values (●——●) during the last 10 weeks.

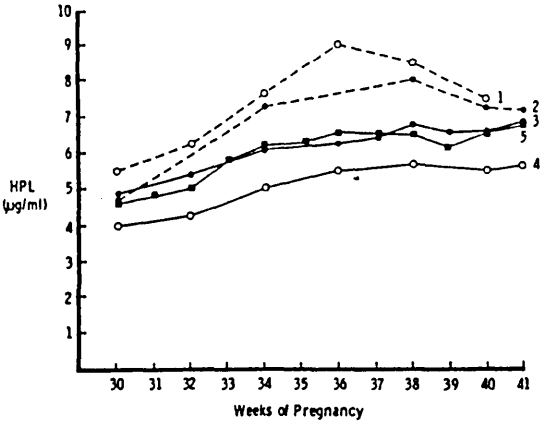


Fig. 7. Comparison of mean HPL levels in normal pregnancies. (1) Teoh et al., 1971; (2) Genazzani et al., 1971; (3) Radiochemical Centre, 1972; (4) Letchworth et al., 1971 and (5) present study.

sive mole or gestational choriocarcinoma were found to range from 277 ng/ml to 4.1 ng/ml. The values started to fall soon after treatment with cytotoxic drugs began. The range of HPL values in 38 patients who had received one course of treatment was 186 ng/ml — <1.9 ng/ml, and in 15 patients who had received 3 or more courses the range was 49 ng/ml — <1.9 ng/ml. HPL was not detected in 4 patients with malignant teratoma.

DISCUSSION

Although the placental function assay is not allowed to reach equilibrium it appears to be adequate for clinical purposes in terms of specificity, reproducibility and recovery and the results correlate well with those of other workers.

HPL results in pregnancy are required quickly and the short incubation time of 1 h is thus obviously an advantage. Since the machine performs all the operations of the assay, the operator has to do little more than type in the instructions and press a few buttons, which facilitates the handling of large numbers of samples. The speed of the KEMTEK 3000 (300 reactions per hour), together with the reproducibility of the assay, further enhances its practicability for this type of assay.

The more sensitive tumour index assay has been shown to follow Ekins and Newman's (1970) equilibrium kinetics. The findings in the choriocarcinoma patients are similar to those of Yen et al. (1968), who found that the rapid disappearance of HPL correlated with the removal of primary disease and that HPL was undetectable despite the presence of active lesions of metastatic disease. Since HPL concentrations fall rapidly after treatment, it is therefore not comparable to human chorionic gonadotrophin as an index of trophoblastic activity (Kardana and Bagshawe, 1975).

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Essential hypertension: effect of an oral inhibitor of angiotensin-converting enzyme

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British Medical Journal, 1979, 2, 1106-1109

Summary and conclusions

Captopril, a specific oral inhibitor of angiotensin-converting enzyme, was given to 18 unselected patients with moderate essential hypertension. Mean blood pressure fell by 14·5% at the maximum dose given, and this fall was significantly correlated with the initial plasma renin activity. The main fall in blood pressure occurred two hours after the first dose of captopril.

These results suggest that captopril effectively lowers blood pressure in patients with essential hypertension and that the renin-angiotensin aldosterone system may maintain blood pressure in essential hypertension. This does not necessarily imply that the renin-angiotensin system is the cause of the high blood pressure.

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Introduction

The importance of the renin-angiotensin aldosterone system in maintaining blood pressure in essential hypertension is disputed.[1,2] Infusions of competitive inhibitors of angiotensin II, such as saralasin, cause no change in blood pressure in most patients with essential hypertension receiving their normal sodium intake.[3] Saralasin, however, is a potent agonist,[4-6] and when given as a short-term infusion blocks only the immediate effects of circulating angiotensin II. Both of these factors will tend to underestimate the role of the renin-angiotensin aldosterone system in maintaining blood pressure. The converting-enzyme inhibitor teprotide, which blocks the enzyme that converts angiotensin I to angiotensin II, has, when given as a single injection, a slight lowering effect on blood pressure in some patients with essential hypertension studied while receiving their normal diet.[3] More recently an oral drug, captopril, that specifically inhibits the angiotensin-converting enzyme has been developed.[7,8] With this drug long-term inhibition of the formation of angiotensin II is now possible.

Preliminary studies with captopril have shown that it is a highly effective antihypertensive agent in patients with renovascular hypertension and in a few patients with essential hypertension who are resistant to other treatment.[9,10]

We report here a study of the effect of captopril on blood pressure and the renin-angiotensin aldosterone system in 18 unselected patients with moderate essential hypertension.

Patients and methods

Eighteen patients (10 men, eight women) with uncomplicated essential hypertension were included in the trial. All had been referred by local general practitioners and were included in the study only if, after an initial observation period of one to two months when they received no treatment, their sitting diastolic pressure was between 100 and 120 mm Hg. The mean age of the patients was 52 years (range 41-65). Patients were excluded if there was evidence of renal failure, ischaemic heart disease, or cerebrovascular disease and if they were taking oral contraceptives or any other drug. Informed consent was obtained from each patient.

PROCEDURE

After the initial assessment period of one to two months all 18 patients were observed weekly for a further four weeks while receiving no treatment. Twelve patients were then treated with a matching placebo three times a day for one month and seen every week. All 18 patients then started to take captopril, 25 mg three times a day. Observations were made two hours after the first 25 mg dose in 15 patients. After one week of captopril the dose was increased to 50 mg thrice daily during the second week, 100 mg thrice daily during the third week, and 150 mg thrice daily during the fourth week. If the supine diastolic pressure was less than 85 mm Hg the dose was not increased but was kept at the same amount as the previous week. Two patients had diastolic pressures below 85 mm Hg during the third week of treatment while taking captopril 100 mg thrice daily; the next week, however, the diastolic pressure was greater than 85 mm Hg, so that the dose was increased to 150 mg thrice daily in the fifth week of treatment. For the analysis of treatment the blood pressure in this fifth week in these two patients was taken as though it was the pressure in the fourth week. In the 16 other patients the diastolic pressure did not fall below 85 mm Hg and the dose of captopril was increased each week.

All patients were seen in the blood pressure clinic in the same room, at the same time of day, on the same day of the week for weekly visits, and by the same nurse. Patients were instructed to take their tablets two to three hours before a visit, and one hour before or two hours after a meal. All blood pressures were measured by nurses using semi-automatic ultrasound sphygnomanometers[11] (Arteriosonde 1217 with recorder). The mean values of five readings made at one- to two-minute intervals with the patient supine, sitting, and standing were taken as the blood pressure in that position. Blood pressure was also measured one minute after a standard period of exercise on a treadmill. Pulse rate was measured on a Cambridge 3048 pulse monitor, with ECG display and recording. Weight was measured at each visit.

Blood samples for plasma renin activity and plasma aldosterone, urea, electrolyte, and creatinine concentrations were taken before active treatment, two hours after the first dose, and at weekly intervals during treatment. Routine haematological and biochemical examinations were done before active treatment and during the second and fourth weeks of treatment. Blood was taken without stasis after the patient had been sitting upright for five minutes. Plasma renin activity was measured by radioimmunoassay.[12] Plasma aldosterone was measured by radioimmunoassay, using a modification of the method described by James *et al.*[13] The normal ranges for plasma renin activity and plasma aldosterone in subjects sitting upright between 10 00 am and noon and with a sodium intake of 100-200 mmol (mEq)/day are 0·5-2·5 ng ml⁻¹ h and 100-600 pmol/l (3·6-21·6 ng/100 ml) respectively. All patients ate their normal diet. Twenty-four-hour urine collections for measurement of sodium excretion were done before active treatment.

Statistical analysis was performed using the University of London computer and North-western University's statistical package for social sciences. Mean blood pressure was calculated as diastolic pressure + (1/3 × pulse pressure). Results are reported as means ± SE of means.

Results

The mean supine blood pressure in the 18 patients before the start of the trial but after the initial assessment period of one to two months was 178 ± 4·5 mm Hg systolic and 113 ± 2·9 mm Hg diastolic. Four weeks' observation in six of the patients and four weeks' treatment with placebo in the 12 others made no significant difference to the mean blood pressures. Mean supine blood pressures for the six patients before and after four weeks of weekly observation were 178 ± 5·2 mm Hg systolic and 115 ± 5·7 mm Hg diastolic (before) and 178 ± 5·3 mm Hg systolic and 114 ± 4·3 mm Hg diastolic (after). Mean supine blood pressure for the 12 other patients before and after four weeks' placebo were 175 ± 5·8 mm Hg systolic and 107 ± 2·7 mm Hg diastolic (before) and 177 ± 6·2 mm Hg systolic and 110 ± 2·5 mm Hg diastolic (after).

Two hours after the first dose of captopril (25 mg) the mean supine blood pressure fell significantly. Blood pressures before and two hours after captopril were 178 ± 5·3 mm Hg systolic and 112 ± 2·6 mm Hg diastolic (before) and 158 ± 5·4 mm Hg systolic and 103 ± 3·2 mm Hg diastolic (after) ($n = 15$, $P < 0.001$). The blood pressure two hours after a single dose of 25 mg of captopril in these 15 patients was not significantly different from the average supine blood pressure at one week, when the dose was 25 mg thrice daily, or during the second and third weeks of treatment at 50 mg and 100 mg thrice daily respectively. During the fourth week of treatment (150 mg thrice daily) the mean supine blood pressure was significantly lower than that two hours after the first dose of captopril, being 150 ± 5·4 mm Hg systolic and 97 ± 3·6 mm Hg diastolic compared with 158 ± 5·1 mm Hg systolic and 103 ± 3·2 mm Hg diastolic ($n = 15$, $P < 0.01$). Figure 1 shows the average blood pressures supine and standing for all 18 patients and for the 15 after the single dose. Changes in sitting and after-exercise blood pressures showed no significant difference from the changes in supine and standing pressures. The percentage fall in mean blood pressure at the maximum dose of captopril as compared with control values was 14·5%.

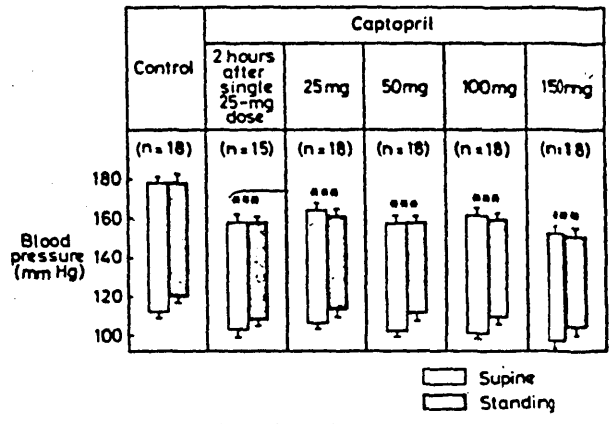


FIG 1—Mean (± SE of mean) systolic and diastolic blood pressures, supine and standing, before and during treatment with increasing doses of captopril (each dose given thrice daily for one week).

Significance of difference: *** $P < 0.001$ compared with control values.

Difference between blood pressures with increasing doses of captopril and values two hours after single 25 mg dose significant only for 150 mg dose ($n = 15$, $P < 0.01$).

Plasma renin activity—Plasma renin activity before active treatment was within the normal range in 13 patients and below the normal range in five. The mean 24-hour urinary sodium excretion in the 18 patients before receiving captopril was 150·6 mmol (mEq) (range 90-198 mmol). Plasma renin activity rose two hours after the first dose of captopril and did not change significantly from this value during further treatment in these 15 patients. The table shows the mean values for all 18 patients. The fall in blood pressure that occurred with captopril at the fourth week of treatment was significantly correlated with the logarithm of the pretreatment plasma renin activity ($r = 0.59$, $P < 0.005$, $n = 18$). This correlation was significant for the falls in supine, upright, sitting, after-exercise, systolic, and diastolic pressures, as well as the mean blood pressure two hours after the first dose and on the second, third, and fourth weeks, but there was no significant correlation for the first week of treatment. The

Mean (± SE of mean) results of biochemical tests, weight, and pulse rate before and during treatment with captopril at increasing doses

	Control (n = 18)	Captopril treatment: 2 hours after single 25-mg dose (n = 15)	25 mg† (n = 18)	50 mg† (n = 18)	100 mg† (n = 18)	150 mg† (n = 18)
Plasma renin activity (ng/ml/h)	0·97 ± 0·12	1·83 ± 0·34**	2·20 ± 0·36***	2·76 ± 0·63**	2·79 ± 0·57**	3·02 ± 1·36*
Plasma aldosterone (pmol/l) ..	303 ± 31	201 ± 34***	225 ± 30**	227 ± 28**	198 ± 13**	207 ± 20**
Plasma potassium (mmol/l) ..	3·91 ± 0·07	3·91 ± 0·12	4·07 ± 0·11	4·19 ± 0·09**	4·17 ± 0·08**	4·10 ± 0·077*
Weight (kg)	76·4 ± 3·0	76·3 ± 3·0	75·8 ± 3·0*	75·5 ± 3·0**	75·1 ± 3·1***	75·1 ± 3·0***
Pulse rate, supine (beats/min) ..	80 ± 2·5	79 ± 2·7	76 ± 2·1	75 ± 2·4	78 ± 3·6	75 ± 2·3*

†Dose given thrice daily for one week.
Significance of difference: *P < 0·05; **P < 0·01; ***P < 0·001 compared with control values.
For plasma renin activity and aldosterone concentration values with increasing doses of captopril were not significantly different from values two hours after a single 25-mg dose.
Conversion: SI to traditional units—Plasma aldosterone: 1 pmol/l ≈ 36 pg/100 ml. Plasma potassium: 1 mmol/l = 1 mEq/l.

percentage fall in blood pressure was also significantly correlated against initial plasma renin activity (fig 2). When patients with abnormally low plasma renin activity were excluded from this analysis the correlation was not significant. There was no significant correlation between the fall in blood pressure that occurred with captopril at each week and the rise in plasma renin activity.

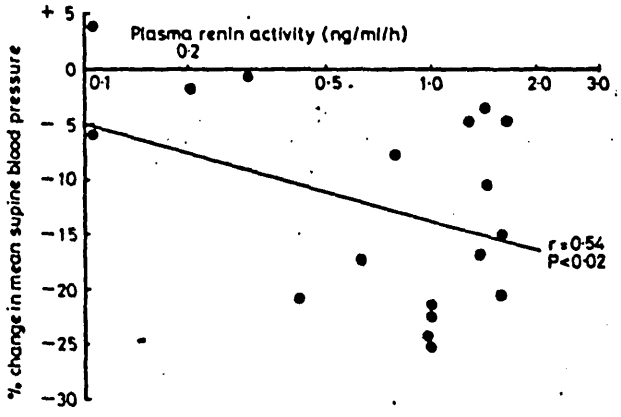


FIG 2—Plasma renin activity before treatment begun plotted against percentage change in mean supine blood pressure at maximum dose of captopril (150 mg thrice daily).

Plasma aldosterone concentration—Two hours after the first dose of captopril the plasma aldosterone concentration fell significantly (table). No further significant change occurred. Before active treatment the concentration showed no significant correlation with the fall in blood pressure that occurred with treatment. There was no significant correlation between the fall in plasma aldosterone at each week and the fall in blood pressure.

Plasma potassium concentration—Plasma potassium concentrations rose significantly during the second week of treatment (table). The mean rise in plasma potassium when the captopril dose was 150 mg thrice daily compared with the control value was 0·19 mmol (mEq)/l ($P < 0·05$). The correlation between this rise and the fall in plasma aldosterone concentration just failed to reach significance ($r = 0·41$, $P < 0·06$).

Weight—The mean weight had fallen significantly by the first week of treatment, and this weight loss increased significantly at the third week; the mean weights in the third and fourth weeks of treatment were not significantly different (table).

Pulse rate—The mean pulse rate showed no significant change except during the fourth week, when it fell significantly (table).

Other blood tests—Plasma sodium concentrations did not change significantly, but a significant fall in plasma bicarbonate concentration had occurred by the first week of treatment, which remained significant to the fourth week. Plasma bicarbonate concentrations before and during the fourth week of captopril (150 mg thrice daily) were 25·1 ± 0·42 and 24·2 ± 0·29 mmol (mEq)/l respectively ($P < 0·02$). Plasma chloride concentrations rose by about 1·5 mmol (mEq)/l, but at no stage was this rise significant. Blood urea, plasma creatinine, and urate concentrations showed no significant changes at each week of treatment. Blood sugar, alanine transferase, bilirubin, alkaline phosphatase, calcium, phosphate, cholesterol, and triglyceride concentrations showed no significant changes at the second and fourth weeks of treatment as compared with control values. The mean haemoglobin concentration showed a significant fall by the fourth week of captopril, from 14·6 ± 0·28 to 13·81 ± 0·29 g/dl ($P < 0·001$). This was associated with a significant decrease in packed cell volume. White cell count did not change significantly. No patient developed or had proteinuria.

Side effects—Captopril was well tolerated, and no patient had to be withdrawn from the trial because of side effects. Two patients complained of headaches on the second, third, and fourth weeks of treatment. One patient had a transient urticarial rash on both hands and arms two weeks after starting captopril. Captopril was continued and the rash disappeared after three days and did not return. One patient lost taste on the fourth week of treatment, necessitating stopping captopril during the fifth week of treatment. Taste was recovered within two weeks of stopping captopril. No other important side effects were recorded, and most patients volunteered spontaneously that they felt well while receiving captopril.

Discussion

Our results show that captopril is an effective antihypertensive drug in unselected patients with moderate essential hypertension. The 14·5% fall in mean blood pressure that occurred is greater than falls we have seen in trials in similar patients using either a beta-blocker or a diuretic alone. The main fall in blood pressure with captopril occurred two hours after the first dose of 25 mg. Blood pressure at the end of the first, second, and third weeks of treatment was not significantly different from this, although a greater fall occurred at the end of the fourth week of treatment, at 150 mg three times a day. This may suggest that increasing the dose of captopril does not greatly increase its blood-pressure lowering effect. In this trial we did not study the duration of action of captopril, but deliberately chose to see patients two to three hours after a given dose. Increasing the dose may well increase the duration of action but not the blood-pressure lowering effect at two to three hours after a single dose, when it might be expected that this effect would be greatest. These results suggest that a randomised trial of different doses o captopril is necessary, as well as an investigation into the duration of action of captopril when given long term.

The mechanism whereby captopril lowers blood pressure is not clear. It is a potent inhibitor of converting enzyme as judged by the rise in plasma renin activity and the fall in plasma aldosterone that occurred. Other workers have shown this[9,10] and have also shown the inhibition of converting enzyme more directly, by measuring converting enzyme[9] and the fall i angiotensin II that occurs.[14,15] The angiotensin-convertin enzyme is also partially responsible for the breakdown o bradykinin. Inhibition of this enzyme could lead to an accumu lation of bradykinin, a potential vasodilator, and this rise ha been claimed to be a mechanism for the fall in blood pressur that occurs.[1,16] Patients do not, however, have symptoms o raised bradykinin concentrations, and more recent work ha shown that there does not appear to be an increase in plasm bradykinin with captopril[17] or with the parenteral converting enzyme inhibitor.[18] Amery *et al* have shown a direct correlatio

between the falls in circulating angiotensin II and blood pressure with captopril.[14] This has also been shown in salt-depleted dogs.[15] The fall in aldosterone that occurs with captopril may also have a blood-pressure lowering effect—firstly, through a postulated but still unproved effect on the sensitivity or tone of the arterioles, and, secondly, through the loss of sodium and water.

Our study shows a significant correlation between the initial plasma renin activity before treatment and the subsequent fall in blood pressure. When patients who had a very low plasma renin activity were excluded, however, the correlation was not significant. This is hardly surprising, as the range of activity was then small. A significant amount of weight was lost, which appeared to increase as the dose of captopril was increased. The weight loss was similar to that seen in similar patients treated with thiazide diuretics: it could be due to sodium and water loss caused by the fall in aldosterone, the fall in angiotensin II, or a rise in another vasodilating substance, perhaps bradykinin in the kidney itself. If this weight loss was due to sodium and water loss, however, it is surprising that the blood pressure did not fall to a greater extent in the second and third weeks of treatment as compared with the two-hour blood pressure after the first dose, when no sodium and water loss could have occurred as there was no change in weight. The fact that the main fall in blood pressure occurred within two hours of the first dose of captopril certainly suggests an immediate action of captopril on blood pressure, and the evidence so far would suggest that it is probably due in part to the immediate fall in angiotensin II that occurs and a decrease in the direct vasoconstrictor effect of angiotensin II.

If the renin-angiotensin aldosterone system is maintaining blood pressure in patients with essential hypertension, either by a direct vasoconstrictor action of angiotensin II or by an effect of aldosterone either directly on arteries or on sodium and water balance, it does not necessarily imply that this is the mechanism of the raised blood pressure. Studies that we have performed in normal subjects receiving a normal diet show that captopril reduces blood pressure to the same extent as it did in these patients with high blood pressure, suggesting that whatever the mechanism of action of captopril it has the same effect on blood pressure in percentage terms in normotensive and hypertensive subjects.[19] If the mechanism of action of captopril is through the renin-angiotensin aldosterone system, this system does not appear to maintain blood pressure to a greater extent in hypertensive than normotensive subjects for a given plasma renin activity.

Captopril in this short-term trial was well tolerated by the patients. Rashes, a reported side effect, were not evident except transiently in one patient. The loss of taste in one patient may be a more important side effect. Out of a total of 47 patients whom we have now treated with captopril, six have developed loss of taste. In four of these six the loss was severe: the captopril had to be stopped and there was appreciable weight loss. The taste recovered within three to four weeks of stopping captopril. The loss of taste with captopril might be related to zinc, but in preliminary studies that we have done in collaboration with the trace-metals unit at the Hospital for Sick Children, Great Ormond Street, we have been unable to show any change in plasma zinc or copper with captopril. The fall in haemoglobin concentration and packed cell volume that occurred with captopril is difficult to interpret in view of the fact that about 450 ml of blood was taken during the trial.

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The renin–angiotensin–aldosterone system in the maintenance of blood pressure, aldosterone secretion and sodium balance in normotensive subjects

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Summary

1. Captopril given for 5 days caused a fall in blood pressure in normotensive subjects. The percentage fall in mean supine pressure was greatest on a low sodium diet (10 mmol/day), 19·6%, least on a high sodium diet (350 mmol/day), 11%, and in between on a normal sodium diet (120 mmol/day), 16·5%.

2. Captopril caused a marked fall in plasma aldosterone in normal subjects on all three sodium intakes.

3. Captopril caused an increase in sodium excretion on the normal (120 mmol/day) and low (10 mmol/day) sodium diet but not the high sodium diet.

4. These results suggest that the renin–angiotensin–aldosterone system is a normal mechanism for maintaining blood pressure and aldosterone secretion in normotensive man. The system may also be involved in the maintenance of sodium balance.

5. These results may lead to a better understanding of the role of the renin–angiotensin–aldosterone system in the maintenance or causation of high blood pressure in essential hypertension.

Key words: aldosterone, angiotensin, captopril, normotensive subjects, renin, sodium.

Introduction

The renin–angiotensin–aldosterone system has been regarded as a defence mechanism to maintain blood pressure in normal man only after volume depletion (e.g. blood loss) or sodium depletion or restriction (Haber, 1976). With the recent development of a specific oral inhibitor of angiotensin converting enzyme (captopril, SQ 14 225) long-term inhibition of the formation of angiotensin II is now possible (Ondetti, Rubin & Cushman, 1977). In this study we have looked at the effect of captopril given for 5 days on blood pressure, aldosterone and sodium balance in normotensive volunteer subjects on three different sodium intakes: low (10 mmol/day), normal (120 mmol/day), high (350 mmol/day). The results suggest that the renin–angiotensin–aldosterone system, like the sympathetic nervous sytem, is not only a defence mechanism but a normal mechanism for maintaining blood pressure. This may help to explain some of the recent controversy over the importance of the renin–angiotensin system in the maintenance or causation of blood pressure in essential hypertension (Case, Wallace, Keim, Weber, Drayer, White, Sealey & Laragh, 1976).

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Methods

Normotensive, healthy, male subjects whose diastolic pressure was less than 80 mmHg after a 2 month observation period were included in the study. All subjects gave their informed consent. Mean age of subjects was 22 years (range 21–26 years). The subjects were studied on three sodium balances, on each balance for 3 weeks, with a gap between each balance period when they ate their normal diet. The low sodium intake was 10 mmol/day, the normal sodium intake was 120 mmol/day and the high sodium intake was 350 mmol/day. The high sodium diet was achieved by supplementing the diet with slow sodium (CIBA). The potassium intake was kept constant at 115

mmol/day for all three diets. All food and drink apart from water was provided by the metabolic ward kitchen. During the 3 weeks of each diet continuous 24 h urine collections were made. The normotensive subjects were allowed to go about their normal activities throughout the study and were not admitted to hospital. They were discouraged from vigorous exercise. Captopril was started on day 8 of the balance, measurements being made 2 h after the first dose of 25 mg. The subjects were allowed to pursue their usual activities during this 2 h period. The dose of captopril was 25 mg three times on day 1, increasing to three doses of 50 mg on day 2 and 100 mg thrice daily for the next 2 days and one dose of 100 mg on the morning of day 5. Daily measurements of blood pressure and 24 h urinary sodium were made. All blood pressures were measured by nurses using semi-automatic ultrasound sphygmomanometers (Arteriosonde 1217) (George, Lewis & Petrie, 1975). The mean value of five readings at 1–2 min intervals, supine, sitting and standing, were taken as the blood pressure in that position. Blood pressure measurements were made at the same time of day between 10.00 and 12.00 hours in the same room, by the same nurse, on the same arm. Pulse rate was measured on a Cambridge 3048 pulse monitor with ECG display and recording. Urinary aldosterone was measured 2–4 days before the start of captopril, during the 5 days of captopril and for 4 days after captopril was stopped. Plasma angiotensin II, plasma renin activity and plasma aldosterone, blood urea, electolytes and plasma creatinine were measured on days 5 and 8 of the balances before captopril, 2 h after the first dose of captopril and on days 4 and 5 during treatment with captopril. All blood was taken without stasis after sitting upright for 5–10 min between 10.00 and 12.00 hours. Plasma renin activity, plasma aldosterone and plasma angiotensin II were measured by radioimmunoassay (Düsterdieck & McElwee 1971; James & Wilson, 1976; Roulston & MacGregor, 1978). Statistical analysis was done using Student's *t*-test (for hormone measurements log values were used) or, for correlations, the method of least squares. Mean blood pressures were calculated as diastolic pressure + ($\frac{1}{3}$ × pulse pressure). Results are reported as the mean ± SEM.

Results

Normal sodium intake (120 mmol/day)

Eight normal subjects were studied on a sodium intake of 120 mmol/day. Captopril caused a significant fall in blood pressure within 2 h of the first dose and this was greater by day 5 (Table 1). The percentage fall in mean supine blood pressure with captopril by day 5 was 16·5%. Blood pressure returned to similar levels to the pre-captopril values in the 7 days after stopping captopril. Plasma angiotensin II and plasma aldosterone fell with captopril and plasma renin activity rose with captopril (Table 1). Mean 24 h urinary sodium excretion 4 days before captopril was 107 ± 4·3 mmol, and during the 5 days of captopril was 135 ± 10·0 mmol. Net loss of sodium during the 5 days of captopril was 142 mmol. All normal subjects lost weight and by day 5 of captopril the mean weight loss was 1·15 kg. Urinary aldosterone excretion fell during captopril. Mean urinary aldosterone in the 4 days before captopril was 50 ± 2·5 nmol/24 h and during the 5 days of captopril was 36·5 ± 3·6 nmol/24 h.

High sodium intake (350 mmol/day)

Seven normal subjects were studied. By day 8 of the high sodium diet in the seven normal subjects, mean 24 h urinary sodium excretion was 334 ± 21 mmol. There was no significant change in weight with the high sodium diet. Plasma angiotensin II, renin activity and aldosterone had fallen (Table 1). Captopril caused a significant fall in blood pressure (Table 1). The fall was less than on the normal sodium diet but the difference was not significant. The percentage fall in mean supine blood pressure was 11·0%. With captopril, plasma angiotensin II and plasma aldosterone fell; plasma renin activity rose (Table 1). There was no detectable change in sodium balance with captopril. Mean urinary sodium excretion in the 4 days before captopril was 324 ± 5·9 mmol and during captopril was 323 ± 7·8 mmol. There was no significant change in mean weight with captopril (71·55–71·40 kg). Mean urinary aldosterone in the 4 days before captopril was 18·7 ± 28 nmol/24 h, and during the 5 days of captopril was 16·3 ± 1·1 nmol/24 h.

Low sodium intake (10 mmol/day)

Five normal subjects were studied. By day 8 of the low sodium diet the five normal subjects had lost 150·6 mmol of sodium and 1·5 ± 0·5 kg. The mean 24 h urinary sodium was 8·6 ± 2·0 mmol. Plasma angiotensin II, renin and aldosterone had risen (Table 1). Blood pressure fell with captopril (Table 1). The fall in blood pressure was significantly greater than the fall on the high sodium diet ($P < 0·005$). The percentage

TABLE 1. *Supine and standing blood pressures, plasma angiotensin II concentration, plasma renin activity and plasma aldosterone concentration on a normal sodium (120 mmol/day), a high sodium (350 mmol/day) and a low sodium (10 mmol/day) intake*

Results are given for days 5 and 8 of each balance, 2 h after the first dose of captopril and for days 4 and 5 of captopril. Mean values ± SEM are shown. Significance of differences between values on day 8 of balance before captopril compared with values on captopril: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

	Day 5	Day 8	Captopril		
			2 h after 25 mg	Day 4 (3 × 100 mg/day)	Day 5 (3 × 100 mg/day)
Normal diet (120 mmol/day, $n = 8$)					
Supine blood pressure (mmHg)	116/72 (±2·2/±1·4)	118/69 (±1·3/±1·3)	108/64*** (±1·4/±2·8)	105/55*** (±2·6/±2·1)	104/55*** (±2·3/±2·6)
Standing blood pressure (mmHg)	110/79 (±2·0/±2·3)	112/79 (±2·9/±2·4)	105/78*** (±2·4/±2·7)	96/68*** (±2·5/±3·1)	96/69*** (±3·3/±3·4)
Angiotensin II (pmol/l)	34·4 (±3·7)	25·3 (±2·3)	15·5** (±1·9)	11·0*** (1·7)	13·3*** (±1·7)
Plasma renin activity (ng h^{-1} ml^{-1})	4·3 (±0·8)	3·3 (±0·5)	5·7*** (±0·7)	45·7*** (±9·6)	41·4*** (±7·1)
Plasma aldosterone (pmol/l)	659 (±99)	549 (±64)	184*** (±26)	164*** (±21)	158*** (±14)
High sodium diet (350 mmol/day, $n = 7$)					
Supine blood pressure (mmHg)	116/69 (±2·7/±3·2)	118/72 (±3·3/±3·1)	115/66** (±3·1/±2·8)	111/61*** (±2·3/±2·1)	112/61*** (±2·3/±3·4)
Standing blood pressure (mmHg)	112/81 (±3·3/±3·4)	115/79 (±3·3/±2·8)	110/76** (±4·3/±2·0)	103/71*** (±3·1/±3·9)	103/71*** (±3·6/±2·2)
Angiotensin II (pmol/l)	15·4 (±2·5)	17·0 (±2·6)	11·7 (±2·7)	9·6*** (±2·4)	7·9*** (±3·2)
Plasma renin activity (ng h^{-1} ml^{-1})	1·9 (±0·4)	2·7 (±0·4)	6·1* (±1·5)	25·6*** (±6·6)	27·9*** (±5·6)
Plasma aldosterone (pmol/l)	201 (±44)	318 (±53)	112*** (±15)	117*** (±21)	115*** (±16)
Low sodium diet (10 mmol/day, $n = 5$)					
Supine blood pressure (mmHg)	121/70 (±3·4/±1·8)	122/69 (±0·6/±2·5)	110/63*** (±4·2/±3·0)	100/57*** (±4·1/±5·3)	100/54*** (±6·0/±1·1)
Standing blood pressure (mmHg)	110/78 (±3·5/±4·0)	109/80 (±1·6/±4·6)	100/68*** (±4·3/±6·3)	93/61*** (±5·3/±4·1)	93/60*** (±3·0/±5·8)
Angiotensin II (pmol/l)	59·0 (±11·2)	64·4 (±9·3)	39·6 (±11·5)	13·2*** (±4·7)	11·0*** (±2·5)
Plasma renin activity (ng h^{-1} ml^{-1})	9·7 (±2·8)	6·2 (±0·9)	18·3** (±3·8)	39·5*** (±10·3)	44·6*** (±3·0)
Plasma aldosterone (pmol/l)	1235 (±260)	1395 (±252)	641* (±176)	395*** (±138)	483*** (±173)

fall in mean supine blood pressure was 19·6%. There was no evidence of postural hypotension on the low sodium diet. With captopril plasma angiotensin II and aldosterone fell, plasma renin activity rose. Mean urinary sodium excretion in the 4 days before captopril was 10·85 ± 0·86 mmol and during the 5 days of captopril was 18·2 ± 2·4 mmol. The net loss of sodium during captopril was 43·6 mmol. There was no significant change in weight with captopril (68·0 ± 2·0–67·6 ± 2·0 kg). Mean urinary aldosterone in the 4 days before captopril was 131 ± 13·0 nmol/24 h and during the 5 days of captopril was 84 ± 13·0 nmol/24 h.

The three diets, taken together, showed that with captopril, plasma angiotensin II fell to approximately the same level irrespective of the diet or starting level of angiotensin II (Table 1). Plasma aldosterone, although showing large falls with captopril, did not fall to the same level on the different diets (Table 1). Considering all 20 normal subjects who were studied with captopril irrespective of diet, there was a significant correlation between initial log(plasma renin activity) and the fall in blood pressure ($r = 0.71$, $P < 0.001$). There was also a significant correlation between the initial log (angiotensin II) and the fall in blood pressure ($r = 0.71$, $P < 0.001$), and a significant correlation between the fall in log(angiotensin II) and the fall in blood pressure ($r = 0.63$, $P < 0.001$). There was also a significant correlation between the initial plasma renin activity, angiotensin II and the fall in angiotensin II against the fall in aldosterone that occurred with captopril by day 5 ($r = 0.66$, $r = 0.82$, $r = 0.78$ respectively, $P < 0.001$). These correlations are not strictly statistically justified as five of the same normal subjects were studied on the three different diets and the diets were not achieved by chance. Nevertheless, they indicate the close relationship between the fall in blood pressure and the initial levels of plasma renin activity, angiotensin II and the fall in plasma angiotensin II.

Discussion

Captopril in normotensive subjects caused a significant fall in blood pressure on a high, normal or low sodium diet. The fall was greatest on the low sodium diet and was least on the high sodium diet. There is controversy about how captopril might lower blood pressure.

Captopril is known to be a potent inhibitor of angiotensin converting enzyme. Inhibition of this enzyme leads to a fall in circulating angiotensin II (Atkinson, Brown, Fraser, Leckie, Lever, Morton & Robertson, 1979; Fagard, Amery, Lijnen & Reybrouck, 1979). As this enzyme is also partly responsible for the breakdown of bradykinin, there could be an accumulation of bradykinin, a potential vasodilator. Recent work has not confirmed an increase in bradykinin with captopril (Mathews, McGrath & Johnston, 1979). Significant correlations have been shown between the fall in angiotensin II and the blood pressure fall with captopril in patients with hypertension (Fagard, *et al.* 1979) and salt-depleted dogs (Morton, Tree, Casals-Stenzel, 1980). Angiotensin II has other actions besides those of direct vasoconstriction of arterioles and these may also contribute to the fall in blood pressure with captopril. The fall in aldosterone that occurs within 2 h of the first dose of captopril may also have a blood pressure-lowering effect.

Our results showing a close relationship in these normal subjects between the fall in blood pressure with captopril and the initial plasma renin activity, the initial plasma angiotensin II and the fall in angiotensin II with captopril suggest that part of the mechanism whereby captopril lowers blood pressure is through the reduction in angiotensin II that occurs. It might seem surprising that captopril did not have a greater effect on blood pressure on the low sodium diet compared with the high sodium diet. This could be explained by the increased sensitivity of vascular receptors to angiotensin II in salt-loaded normal subjects compared with sodium-restricted normal subjects (Oelkers, Brown, Fraser, Lever, Morton & Robertson, 1974). If part of the mechanism whereby captopril lowers blood pressure is through the fall in angiotensin II our results show that the renin–angiotensin system is a normal mechanism for maintaining blood pressure in normotensive subjects rather than just a defence mechanism to maintain blood pressure after volume depletion (Haber, 1976).

The fall in plasma aldosterone that occurred with captopril on all three diets indicates that angiotensin II is an important mechanism in maintaining aldosterone secretion, irrespective of sodium intake in normotensive man. However, the plasma aldosterone achieved during captopril was lowest on the high sodium diet and highest on the low sodium diet, illustrating that other factors besides the renin–angiotensin system are also involved in the changes of aldosterone secretion with different sodium intakes.

Captopril caused an increase in sodium excretion on the normal diet and on the low sodium diet but no change on the high sodium diet. This may be due to the fall in angiotensin II and/or the fall in aldosterone, and these results provide circumstantial evidence that the renin–angiotensin–aldosterone system may be a factor in the control of sodium balance on a low and normal sodium intake but not on a high sodium intake.

The fact that all drugs that block or inhibit the renin–angiotensin–aldosterone system appear to cause the same percentage change in blood pressure for a given plasma renin activity in both normotensive subjects and patients with essential hypertension (MacGregor, Markandu & Roulston, 1979) does suggest that this system plays no greater role in essential hypertension in the maintenance of blood pressure than in normal subjects for a given value of plasma renin activity. Our results with captopril, suggesting that the renin–angiotensin–aldosterone system is a normal mechanism (analogous to the sympathetic nervous system) for maintaining blood pressure in normotensive subjects on a normal sodium intake, necessarily implies that in essential hypertension the renin–angiotensin system is also a normal mechanism for maintaining the blood pressure, but is not the cause of the high blood pressure. The renin–angiotensin system, therefore, can only be the direct cause of high blood pressure if the level of angiotensin II is inappropriately high for the sodium balance or if there is an increase in sensitivity to the various actions of angiotensin II, as compared with normotensive subjects.

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Table 1. Increase in Angiotensin-Converting Enzyme Activity with Storage Time in Plasma Samples from Subjects Taking Captopril.

Age of Sample	No. of Samples	Activity of Angiotensin-Converting Enzyme *
<4 hr	5	0.99±1.39
18–24 hr	4	4.62±1.38
1–20 days	7	41.0±21.0
21–40 days	7	62.6±24.0
>40 days	23	98.5±9.4

*Expressed as a percentage of the values measured before administration of captopril (±1 S.D.).

times a day). Progressively less inhibition was observed as the time of storage increased. Samples assayed after 24 hours of storage all showed detectable angiotensin-converting-enzyme activity; these activity levels rose on prolongation of storage until, after more than 40 days, they approached control levels.

These results show that captopril is a potent inhibitor of angiotensin-converting-enzyme even at low doses but also that the plasma must be assayed within a few hours after the blood sample is obtained. The practical problems in plasma assays performed within a short time of sampling may therefore limit the usefulness of the measurement in subjects taking captopril.

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THE MEASUREMENT OF ANGIOTENSIN-CONVERTING ENZYME IN SUBJECTS RECEIVING CAPTOPRIL

To the Editor: Captopril[1,2] causes long-term inhibition of angiotensin II formation, and studies have shown that it is effective in lowering blood pressure.[3-5] At present there is no direct method to measure plasma levels of captopril, but it is possible to measure the activity of angiotensin-converting enzyme with substrate assay techniques.[6-8] The degree to which the activity of angiotensin-converting enzyme is inhibited by captopril may provide an indirect way to assess the activity of the drug and patient compliance; however, variations in the degree of inhibition have been found.[3]

Therefore, when studying normal volunteers who had taken up to 100 mg of captopril three times a day for five days, we were surprised to find no inhibition of converting-enzyme activity when values after captopril were compared with the respective control values. However, the direct addition of captopril to these plasma samples reduced the enzyme levels to below the limit of detection of the assay. These particular samples had been stored at −20°C for over two months before the assay. We therefore studied the effect of storage on angiotensin-converting-enzyme activity in samples taken from both normotensive volunteers and hypertensive patients before and during treatment with captopril. Aliquots of samples taken during captopril therapy were assayed within four hours of blood extraction and after timed intervals thereafter, with each aliquot stored at −20°C before assay.

The measurements were made independently with either a fluorometric technique[7] or a radioassay method.[8] These two assays have been shown to correlate well over a wide range of values for angiotensin-converting-enzyme activity ($r>0.98$) (Roulston JE, MacGregor GA, Bird R. Unpublished data). The results in Table 1 show that when the samples from subjects who received captopril were assayed within four hours of removal of blood, no angiotensin converting-enzyme activity could be detected in any sample irrespective of the dose of captopril administered (25 to 150 mg three

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Evaluation of a coated tube assay for the estimation of plasma renin activity

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Abstract
An antibody-coated tube radioimmunoassay for the estimation of plasma renin activity (PRA) is described and assessed. From the results obtained it appears to correlate well with a previously published technique. It was reproducible and precise over the normal range of PRA values encountered. However some loss of precision was found at high PRA levels. The advantages of this kit over the majority of manual assays previously described are a reduction in the number of pipetting steps and tube manipulations required. Furthermore the recommended use of icebaths for sample retention is probably not necessary, which would further simplify the assay.

Introduction

The measurement of plasma renin activity (PRA) is the most widely used *in vitro* index of plasma angiotensin II concentration and many assays have been described in the literature since the early reports of Haber *et al* and Boyd *et al* in 1969. However, apart from reports of automated assay procedures (Roulston *et al*, 1979) which require, by their very nature, rather expensive, sophisticated equipment, most of the manual techniques reported involve many pipetting and tube manipulation steps. These considerations have tended to keep sample through-put low in many cases where a simpler procedure would result in an increased sample turnover.

In this paper a commercially available assay kit, the CIS RENCTK*, which may offer a more streamlined PRA assay is described and assessed. This kit makes use of the antibody-coated tube technique which not only reduces the number of pipettin steps but also greatly facilitates the separation of the bound an free phases after the incubation period by removing the need fo centrifugation or filtration.

Owing to the controversy which persists with regard to the mos suitable pH to employ for the generation of angiotensin I *in vitr* (Fleming *et al*, and Oparil *et al*, 1974), the kit offers alternative either a 'physiological' buffer pH 7.4, or a buffer of near optim pH for the renin reaction pH 6.0. The kit was examined using bot generation protocols and the assay was assessed by compariso and correlation with the manual assay technique previousl reported from this laboratory (Roulston and MacGregor, 1978)

*CIS (UK) Ltd, Rex House, 354 Ballards Lane, North Finchley, London N12

Materials and methods

Reagents

Samples

Blood samples were taken from normotensive volunteers and hypertensive patients into tubes containing dipotassium ethylenediaminetetraacetic acid (K_2EDTA). The samples were then centrifuged at 4°C at 1500 g for 20 minutes in a cold room. The plasmas were then carefully pipetted into polystyrene tubes in the cold room, capped and stored at -20°C until required.

The commercial assay kit

(1) ^{125}I-Asp^{1}Ileu^5 angiotensin I was supplied in lyophilised form which upon reconstitution yielded a solution of 14.8 kBq (= 0.4 uCi) per ml. of solution.

(2) Standard Asp^{1}Ileu^5 angiotensin I. Six vials of precalibrated standard of values 0.4, 1.1, 2.2, 3.5, 8.0 and 24 ng ml^{-1} were supplied in lyophilised form each to be reconstituted with 1.0 ml of freshly distilled water.

(3) Antibody coated tubes. 12 × 75 mm polystyrene tubes spray coated with antiangiotensin I serum raised in rabbits to an angiotensin-rabbit serum albumin complex were supplied in sealed packs.

(4) Buffers. A dry sachet of assay reaction buffer was provided which upon solution in 200 mls of water yielded a 0.04 molar phosphate buffer of pH 7.4. Also two angiotensin I generation buffers were provided ready to use. One was a phosphate (+ inert red dye) buffer at pH 6.0 and the other a phosphate (+ inert blue dye) buffer at pH 7.4. A phenylmethylsulphonyl fluoride (PMSF) solution in ethanol was provided in ready to use form.

Angiotensin generation in vitro

Three aliquots of all samples were used to generate angiotensin I under three separate sets of conditions, the routine CXH (Charing Cross Hospital) method (pH 5.7) and the two alternative pH procedures (6.0 and 7.4) provided for in the CIS kit protocol.

CXH procedure

To 500 µl of plasma was added 500 µl of 0.1 molar citric acid/potassium citrate buffer at pH 5.7. To this mixture was added 25 µl of 0.45 molar 8-hydroxyquinoline followed by 50 µl of a saturated ethanolic solution of PMSF. The tubes were capped and mixed thoroughly and then each sample was sub-divided into two aliquots. One half was then immediately frozen at -20°C and the other half was incubated at 37°C for three hours. After the incubation period the incubated samples were frozen at -20°C and so stored until required for assay. No ice baths were used at any juncture.

CIS procedure at physiological pH

To 300 µl of each plasma retained in an icebath was added 10 µl of a saturated ethanolic solution of PMSF followed by 300 µl of ice cold phosphate buffer pH 7.4. The tubes were capped, mixed and subdivided into two aliquots. One half was retained in the icebath at 0° and the other incubated at 37°C for three hours. After the incubation period, both halves of the samples were transferred to a deep-freeze and stored at -20°C until required for assay.

CIS procedure at optimised pH

To 300 µl of each plasma retained in an ice-bath was added 10 µl of a saturated ethanolic solution of PMSF followed by 300 µl of ice cold phosphate buffer pH 6.0. The tubes were capped, mixed and subdivided into two aliquots. One half was retained in the ice bath at 0°C and the other incubated at 37°C for one hour, after which time both the aliquots were transferred to a deep-freeze at -20°C until required for assay.

Radioimmunoassays

All plasma samples, however generated, were assayed in both the CXH and CIS radioimmunoassays. The CXH assay, based on that of Boyd *et al*, has been reported previously (Roulston and MacGregor, 1978).

CIS assay protocol

The assay was performed with all tubes and reagents being retained in ice baths. The following protocol was employed

Tubes / Reagents		Zero antigen	Standards	Sample banks	Sample
Phosphate buffer	ml	0.9	0.8	0.8	0.8
Standard	ml	—	0.1	—	—
Sample	ml	—	—	0.1	0.1
Label	ml	0.1	0.1	0.1	0.1

After the label had been added the tubes were all vortex mixed, covered with tightly fitting plastic film, and incubated in an ice bath in the cold room for 16-24 hours. After incubation the tubes were drained of their contents, washed once with 1 ml of distilled water and then, after draining for five minutes, were counted on a Wallac 80000 gamma-counter.

All samples were assayed by both methods (CXH and CIS) in strict parallel: that is, once a batch of samples had been thawed (45 samples with their respective blanks) they were assayed on the same afternoon in both systems to prevent any discrepancies arising due to refreezing or prolonged storage at 4°C or any other possible time-dependent artefact. Results were calculated in both assays by the optical interpolation of sample values from manually constructed semi-log dose response curves.

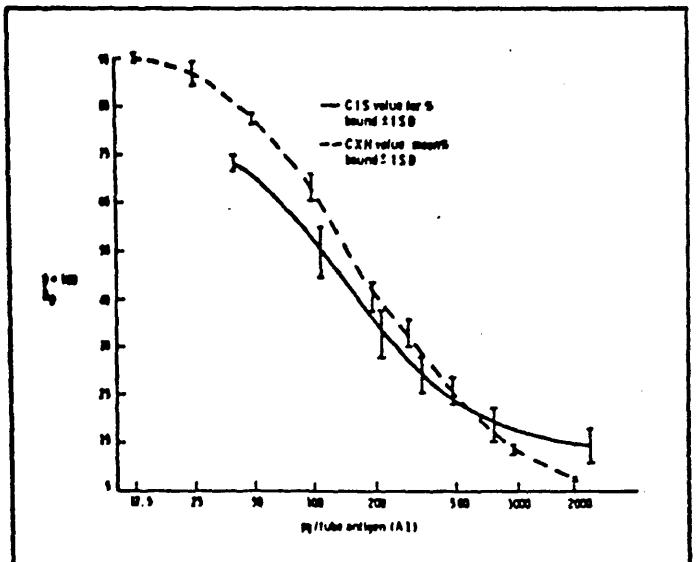


Figure 1. Composite dose response curves plotted on semilog paper showing the superimposability of the individual assays over the whole trial

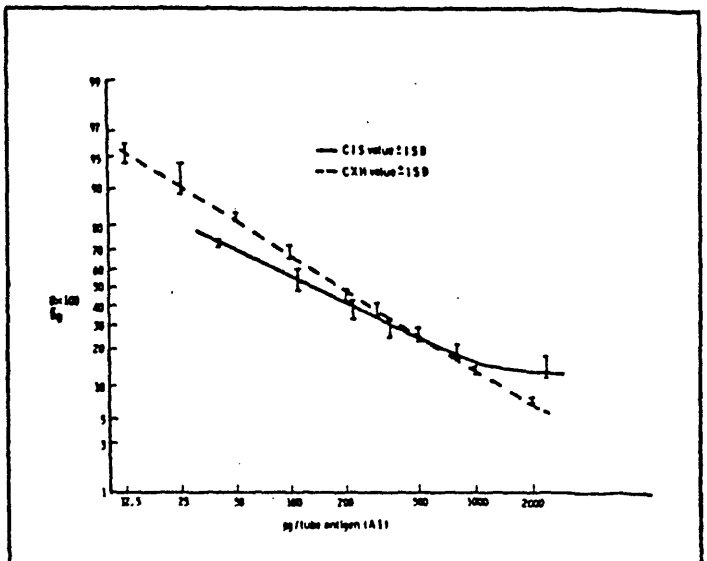


Figure 2. Log logistic transformation of the data used in Figure 1. Note the marked change in slope in the CIS curve at high antigen concentration.

The values of angiotensin I concentration obtained (generated sample — blank) were then transposed into ng. ml^{-1} hr^{-1}, the units in which the final PRA values were expressed. The dose response curves were assessed for precision and reproducibility and the sample data from both assay procedures analysed by linear regression.

Results

Standard curves

Precision

Table 1 shows the precision of the two methods at the various standard points in each of the assays carried out. In the seventh column the mean precision (±1 S.D.), is given at that standard concentration over the whole clinical trial. For the purpose of this table precision, p, was defined as $100-\Delta x$ where $\Delta x = \Delta y/(dy/dx)$, where Δy = the coefficient of variation of the response metameter y, and dy/dx = the modulus of the slope of the dose response curve at that particular point.

Thus is can be seen at Δx is dependent upon two criteria in this definition, the steepness of the slope of the curve at the point x and the variation in the response metameter y at that point. Ideally, as dy/dx increases and Δy tends to zero then Δx will become vanishingly small until finally a precision of 100 units would be recorded. Indeed if $\Delta y = 0$, then $\Delta x = 0$ irrespective of slope. In real terms, values for precision of less than 100 indicate corresponding loss of precision due to significant Δy contributions.

Sensitivity

Sensitivity, or limit of detectability, of an assay may be defined in many ways. If one uses the definition that sensitivity is the precision of estimation of antigen concentration x when $x = 0$ and apply the same rules as for estimation of finite cold antigen concentrations, ie $p_0 = 100-\Delta x_0$, and $\Delta x_0 = \Delta y_0/dy/dx$, this yields values of 3.6 and 3.8 pg $tube^{-1}$ for the CIS and CXH assays respectively. If, however, one uses the more popular, and somewhat more arbitrary definition that sensitivity is the concentration of antigen required to produce a 5% displacement of label binding then this yields values of 6.4 and 12.5 pg $tube^{-1}$ for the CIS and CXH assays respectively.

The marked differences between the results from the two methods are explained by the fact that a low antigen concentration the CIS assay yields a twice-as-steep slope which is 'cancelled out' by a standard deviation about y_0 which is twice the CXH value. Hence by the first method of analysis the sensitivities are approximately equal. The second method arbitrarily fixes Δy_0 at 5, which from these results (Table 2) is too high, and the CIS sensitivity assumes a value half that of the CXH assay.

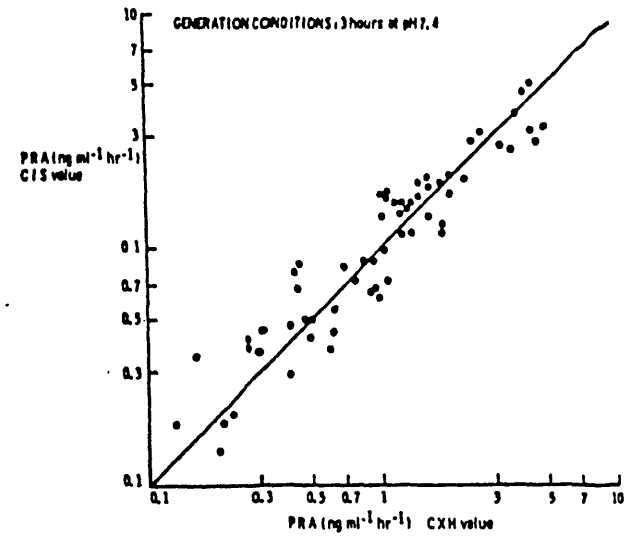


Figure 3. A correlation line for PRA values obtained by the CIS kit as a function of the value in the CXH assay; all samples being generated at pH 7.4 for three hours.

Reproducibility

Figure 1 shows composite dose response curves for the two methodologies where each point is plotted as the mean percentage bound for the whole trial for a particular standard concentration plus or minus one standard deviation. Figure 2 shows the same data after logistic transformation. The kit shows a greater variability especially at high antigen levels. Table 2 summarises the data and gives the value of interassay coefficient of variation of each point over the course of the trial.

Plasma samples

All sample results which fell between the upper and lower standard values in *both* assays were used in the regression analyses. In practice this meant a lower cut off of approximately 0.1 ng ml^{-1} hr^{-1} for both assays. The upper limit was approximately 40 ng ml^{-1} hr^{-1} for the CXH assay but approximately 10 for the CIS kit. Hence regression analyses were performed over a range of 0.1-10. ng ml^{-1} hr^{-1} only.

Effect of generation conditions

Table 3 summarises the results of the samples generated under the three different sets of conditions assayed by the two methods.

Table 1. *Precision study. Assessment of precision of the dose response curves at each standard point both assay procedures over the whole trial. See text for explanation of units.*

Standard pg/tube	Assay number 1	2	3	4	5	Mean value ± 1 SD
CIS RENCTK						
0	99.31	96.76	99.20	99.15	98.49	98.58 ± 1.07
40	98.50	96.03	97.35	97.93	96.64	97.29 ± 0.99
110	92.59	97.29	97.50	95.92	97.07	96.07 ±2.04
220	97.54	99.14	95.68	96.45	98.79	97.52 ± 1.48
350	95.04	98.47	95.81	92.93	94.57	95.56 ± 2.03
800	95.59	98.50	97.33	91.62	97.62	96.13 ±2.73
2400	68.00	75.00	83.90	87.16	74.30	77.70 ± 7.76
Charing Cross Hospital						
0	99.47	99.39	99.57	99.08	99.49	99.40 ± 0.19
12.5	99.40	99.26	99.16	99.27	99.44	99.31 ± 0.11
25	99.12	98.73	98.33	99.93	99.69	99.16 ± 0.66
50	99.77	99.65	99.44	96.49	97.92	98.65 ± 1.42
100	99.00	97.93	97.73	98.45	98.99	98.42 ± 0.59
200	99.27	99.45	99.58	97.55	97.04	98.58 ± 1.19
300	99.13	97.28	96.28	96.91	98.82	97.68 ± 1.24
500	98.60	89.80	97.99	97.70	98.92	96.60 ± 3.80
1000	98.86	95.53	98.50	99.61	99.77	98.45 ± 1.72
2000	99.61	99.99	97.26	98.59	98.57	98.80 ± 1.07

Table 2. *Reproducibility study. A tabulation of the percentage of zero antigen binding for each standard point. The low standard deviations indicate a high degree of reproducibility.*

Assay number	Percentage binding of B/Bo							
pg/tube standard	1	2	3	4	5	$\bar{Y}$	S.D.	C.V.%
CIS assays								
0	100	100	100	100	100	100	2.79	2.79
40	72.9	74.2	70.6	72.0	74.7	72.9	1.66	2.88
110	58.5	59.8	49.0	47.3	57.7	54.5	5.80	10.6
220	45.3	40.0	36.9	31.8	36.25	38.1	4.99	13.1
350	32.0	33.6	29.1	25.4	24.5	28.9	3.98	13.8
800	23.4	19.8	19.6	20.0	13.4	19.2	3.60	18.8
2400	18.4	18.0	15.1	10.6	10.6	14.5	3.81	26.3
CXH assays								
0	100	100	100	100	100	100	1.53	1.53
12.5	95.1	94.3	96.8	94.6	94.1	95.0	1.08	1.14
25	90.3	95.1	93.5	89.4	91.0	91.9	2.36	2.57
50	81.7	84.4	82.8	81.9	82.6	82.7	1.06	1.28
100	66.8	71.2	20.6	69.1	63.6	68.3	3.10	4.54
200	43.7	48.1	48.1	41.1	46.8	45.6	3.07	6.73
300	38.4	40.0	41.5	35.9	34.2	38.0	2.97	7.82
500	25.5	30.6	27.1	22.9	23.5	25.9	3.10	12.0
1000	14.7	13.7	15.00	12.3	12.7	13.7	1.18	8.61
2000	7.91	7.20	7.50	7.50	7.0	7.4	0.35	4.73

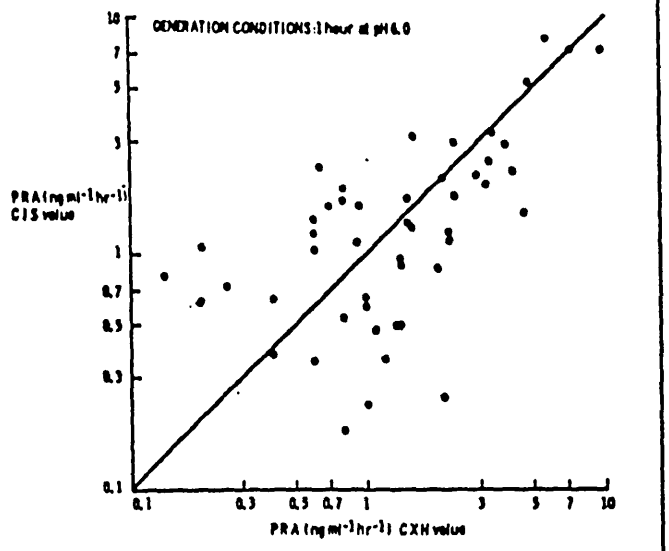


Figure 4. A correlation line for PRA values obtained by the CIS kit as a function of the value in the CXH assay; all samples being generated at pH 6.0 for one hour.

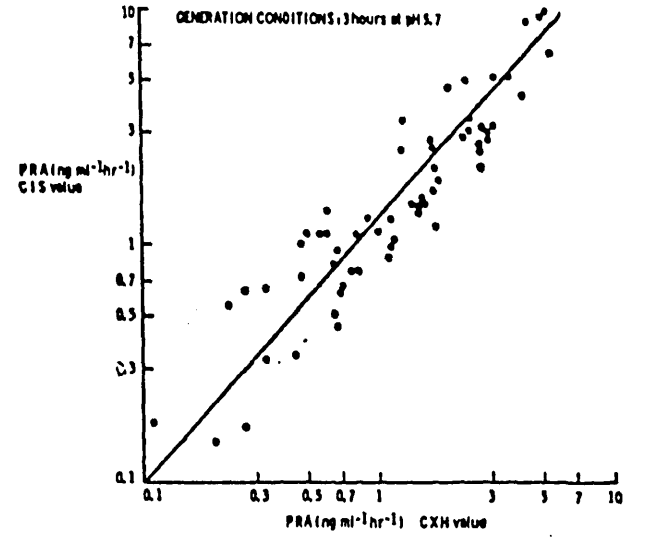


Figure 5. A correlation line for PRA values obtained by the CIS kit as a function of the value in the CXH assay; all samples being generated at pH 5.7 for three hours.

Regression analyses

Figure 3 shows a log-plot of the PRA values of samples generated at pH 7.4 obtained in the CIS assay as functions of the corresponding values obtained with the CXH assay. The results of the samples generated at pH 6.0 and pH 5.7 are illustrated in Figures 4 and 5 respectively. Table 4 summarises the linear regression data. It should be noted that the $\log_{10}$ of the PRA values have been used throughout the linear regression analyses since this transformation results in normally distributed sets of data.

Discussion

The precision studies (Table 1) indicate that both assay methods perform reliably over the normal range of samples encountered. The CXH assay appears from these results to be equally precise at high levels (up to 2000 pg/tube), but the CIS assay shows a marked drop in precision between 800 and 2400 pg/tube. This drop is due almost entirely to a decrease in dy/dx. This flattening of the CIS standard curve is apparent in Figure 1 and Figure 2. With the CIS kit therefore results in high PRA samples may be less precise. Also, as stated previously, the upper cut off is lower than that of the CXH assay (approximately 10 ng ml^{-1} hr^{-1} as opposed to approximately 40 ng ml^{-1} hr^{-1}). The reproducibility of the kit, as judged by the standard curves, is good. Figures 1 and 2 indicate the superimposability of the sequential assays done over the time of the trial. This data is summarised in Table 2.

The mean sample values (Table 3) indicate that, as reported elsewhere in the literature, angiotensin I generation by renin is a function of pH; a pH of 5.7 or 6.0 yielding a greater amount of angiotensin per unit time than a physiological pH of 7.4 (Lee, 1965).

The linear regression data (Figures 3,4 and 5; Table 4) shows the correlations between the two systems. It may be seen that under all types of generation conditions there is no significant deviation from unity in the slope and there is no measurable intercept. This is strong evidence for the direct comparability of the two assays. Furthermore, in two out of three sets of generation conditions highly significant correlation coefficients are observed. Under a third set of conditions (pH 6.0) a correlation coefficient of 0.59 is observed. The reason for this fall could be the shorter (one hour) generation time used at this pH; this gives angiotensin I levels which are on average less than half those obtained from a three hour generation with pH 7.4 buffer

$$\frac{\text{(mean PRA at 6.0)}}{\text{(mean PRA at 7.4)}} \times \frac{1}{3} = \frac{1.75}{3 \times 1.31} = 0.445$$

Thus it is possible that the precision would fall at these lower antigen concentrations. Evidence for this suggestion comes directly from Figure 4 itself. It may be seen that the scatter is much greater at the lower values of PRA than at the higher values. Also since the tubes spend a relatively larger fraction of the total generation time getting from 0° to 37° than in the pH 7.4 methodology there could be considerable interassay variations due to this which would not be apparent in the present study.

The problem of scatter could possibly be obviated or reduced in two ways; firstly by doing a three hour generation as in the CXH methodology and/or not using an icebath. A three hour generation time would effectively treble the total amount of angiotensin generation and this should lead to much greater precision (Figures 3 and 5). Furthermore, avoiding the use of an icebath would mean the interassay variations caused by a variable effective generation time would be reduced since equilibration from 20°C to 37°C would take a much smaller fraction of the total generation time than equilibration from 0°C to 37°C. These results suggest that the use of icebaths for sample retention in the CIS kit may not be necessary. Furthermore, the storage of samples at 0° could possibly give misleading results due to cryoactivation of inactive renin (Osmond *et al*, 1973).

In general terms the kit is easy to use, the particular advantages being the ease of separation of bound and free phases, its sensible (ie visible) sample size (100 µl) and its good rate of counts, approximately 20,000 dpm in the Bo (zero antigen) tubes.

Table 3. *Effect of pH on plasma renin activity. PRA can be seen to be pH dependent. Almost identical results were obtained with both assays.*

Assay method	pH of generation	No. of samples	PRA mean values ng $ml^{-1}hr^{-1}$	Standard deviation
CIS	7.4	67	1.31	1.17
CXH	7.4	59	1.37	1.18
CIS	6.0	64	1.75	1.94
CXH	6.0	58	1.79	1.79
CIS	5.7	65	2.14	2.10
CXH	5.7	66	1.95	1.70

Table 4. *A synopsis of the linear regression analysis performed on the results obtained in the two assay systems under three different sets of generation conditions.*

pH of generation of angiotensin I	7.4	6.0	5.7
Number of pairs, n,	58	49	62
Correlation coefficient, r,	0.935 ($p<.001$)	0.59 ($p<.001$)	0.92 ($P<.001$)
Slope dy/dx	0.88	0.95	0.97
Intercept y at x = 0	0.009	0.016	0.090

Furthermore, as indicated by the standard curves and sensitivity studies, another lower standard (≈20 pg/tube) could be incorporated which would enable measurement of very low PRA levels.

The main disadvantage is the loss of precision at high angiotensin I levels and the inability to measure PRA above approximately 10 ng $ml^{-1}hr^{-1}$ with the sample size recommended.

In addition, as the non-generated half of the sample is frozen with the generated half after the generation period, it could be frozen immediately at time zero. This would remove the possibility of generating artefacts by storage for 1-3 hours at 0°, and simplify the procedure still further.

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