STUDIES ON THE RENIN ANGIOTENSIN SYSTEM IN HUMAN AND DOG WITH SPECIAL REFERENCE TO PRIMARY ALDOSTERONISM AND HEART FAILURE.

Presented by Dr. Pierre Granger, for Ph.D. degree.
Dept. of Investigative Medicine. Chairman: Dr. J.S.L. Browne.

Summary.

The effects of upright posture and sodium restriction on Plasma Renin Activity (PRA) were studied in 60 patients with essential hypertension. 23% had a suppressed PRA. Two of these patients were not cured by adrenalectomy. The incidence of adrenocortical adenomas, nodules and hyperplasia in 2425 autopsied patients further supported the rare occurrence (7%) of primary aldosteronism.

A reproducible and sensitive method for the determination of renin activity in small aliquots of dog's plasma or kidney, using exogenous species specific substrate free of renin and Dowex 50W-X2 (NH_4^+) resin, was established. This technique showed recoveries of 68%.

It was extended and applied to the following studies:

1) Effects of nephrectomy and various medications on substrate concentration.
2) Normal PRA and renal renin (RR).
3) PRA and RR of puppies. Effects of peritoneal dialysis on these parameters.
4) Effects of changes in atrial pressure on renal vein PRA.
5) PRA in dogs with heart failure due to tricuspidectomy.

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by Pierre Granger M.D.

presented to the Department of Investigative Medicine.
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work done in the Clinical Research Institute of Montreal.
Director: Dr. J. Genest.

I dedicate this work to my wife.

Her steady support, particularly in the face of many evening hours, I spent in the laboratory has been a source of gratification. Even though this work has impaired my contribution as a father and a husband, she has succeeded in maintaining the joy of daily living for myself and our children.

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I - INTRODUCTION

Eight years ago, the group of Genest (1-4) demonstrated that intravenous infusions of angiotensin consistently increased aldosterone excretion in normal subjects. This finding, soon confirmed by other groups (5-9) together with the development of sensitive and specific methods for measurement of renin activity (10), led to a better understanding of two main groups of diseases associated with hyperaldosteronism.

Two striking and contrasting syndromes, each with increased aldosterone secretion, were seen at the opposite ends of the spectrum of plasma renin activity. These conditions were referred to as primary and secondary aldosteronism. Primary aldosteronism is a disorder characterized by an autonomous aldosterone-secreting adenoma and suppression of plasma renin activity. The demonstration of suppressed renin activity added to an increased aldosteronuria in primary aldosteronism was extended to essential hypertension by Conn (11) and led him to suggest that an important proportion (20%) of patients with essential hypertension were, in fact, suffering from "normokalemic" primary aldosteronism. In secondary aldosteronism, strong evidence suggested an important role of the renin-angiotensin system in the control of aldosterone secretion and, by extension, of sodium balance and edema formation.

In order to investigate this relationship of aldosteronism to renin, we have studied the renin-angiotensin system in essential hypertension and in experimental cardiac failure. The variations of peripheral plasma renin activity under

standard basal conditions and after stimulation by posture and sodium restriction were evaluated in a case of primary aldosteronism and in 60 patients with essential hypertension. A similar study was also done in an unusual case of "primary aldosteronism". To assess the incidence of adrenal adenomas in the hypertensive population, we have reviewed the autopsy material provided by the department of pathology of the Hotel-Dieu Hospital of Montreal between the year 1955 and 1965.

A sensitive, specific and reproducible microtechnique for the determination of renin activity in rat plasma was recently developed by Boucher and coworkers (12). This method allows serial determinations of renin activity on minute amounts of plasma and is of great value in assessing the role of the renin-angiotensin system. The adaptation of this method to dog has enabled us to study the effect of raising the right and left atrium venous pressure on renal venous blood renin activity, in an attempt to understand the mechanism of high plasma renin activity and edema in cardiac failure. The relationship between right auricular pressure and peripheral plasma renin activity has also been investigated in dogs with cardiac failure produced by the removal of the tricuspid valve.

Since it was possible to measure renin activity on small aliquots of plasma in dogs, we have studied, as a side project, the plasma renin activity, renal renin content and juxtaglomerular index in newborn dogs.

II - REVIEW OF THE LITERATURE

A - COMPONENTS OF THE RENIN-ANGIOTENSIN SYSTEM

1) Renin

In 1898, Tigerstedt and Bergman (13) ascribed the term "Renin" to the pressor substance obtained from rabbits' kidney extracts. Renin remained dormant for 36 years until Goldblatt demonstrated experimental renal hypertension by clamping the renal artery (14). An increase of interest in the relationship of renin to hypertension resulted from this discovery and this led to a better understanding of the chemistry and enzymology of the components of the system. This was achieved mainly by the combined work of Page and Helmer, and Braun Menendez.

Renin was identified as a protein. It was found to be heat labile (13, 15-18), non dialyzable (13, 15-17), acid and alkali labile (16) and insoluble in organic solvents (13, 15-17). It was salted out with ammonium sulfate at pH 6 at concentration between 1.4 and 2.6 M (18). The resistance to acid and alkali was found to be increased in the cold (18). The molecular weight of hog renin has been estimated on Sephadex column to be between 42,000 and 47,000 (19). The molecular structure of renin is still unknown. Many attempts to purify renin were done and the purification achieved by Haas and coworkers (20) is worth mentioning as the first valid purification method. The final product was estimated to be 65% pure by electrophoresis. This method has not been

widely used because it is difficults and tedious. Purity was however increased approximately 56,000-fold by this method. Peart and coworkers (21) achieved a 44,000-fold purification of hog renin. On starch gel electrophoresis, their final product migrated with the pre-albumins. Their purification product was found to be unstable even in the cold. Four forms of renin were obtained by chromatography of purified hog kidney extracts on diethylaminoethyl (DEAE) cellulose by Skeggs and coworkers (22). The rate of reaction of each form with renin-substrate was similar.

Freezing and thawing several times improved the extractability of renin from kidneys and renin can be extracted with water (23).

2) Angiotensin

The vasoconstrictor substance obtained from incubation of plasma with renin "in vitro" was shown by Page and Helmer (24) to be heat stable, water and alcohol soluble and dialyzable. At the same time, Braun Menendez and coworkers (25) showed the presence of a potent pressor substance in acetone extracts of plasma of animals bearing an ischemic kidney. Angiotensin was also found to be highly soluble in acetic acid, insoluble in organic solvents, acid stable and alkali labile (24, 26). Strong oxidizing agents were found to destroy angiotensin rapidly (24).

Purification of angiotensin was achieved and its structure elucidated in horse (27, 28), hog (29, 30), beef (31, 32) and human (33, 34). This product was a decapeptide (angiotensin I) and the sequence of amino acids was identical in

horse, hog and human. Beef angiotensin differed only in that valine replaced isoleucine in position 5.

Since the ability of angiotensin I to induce vasoconstriction in a perfused rabbit's ear was restored by plasma or plasma fraction, Page and Helmer (35) concluded that an angiotensin activator was present in the blood. Skeggs and his group (36) confirmed this hypothesis by the isolation and purification of angiotensin I, an inactive decapeptide, and angiotensin II, an active octapeptide. They also established the rapid conversion of the decapeptide into the octapeptide after removal of the last two amino acids by a chloride ion dependent enzyme contained in the plasma (36, 37). They called it converting enzyme. Studies on the effect of angiotensin on rabbit aortic strips further demonstrated the existence of the two forms of the peptide (38). When injected intravenously, angiotensin I and II are equally pressor, since angiotensin I is converted to angiotensin II by the presence of converting enzyme in plasma (38-40).

Naturally-occurring angiotensins and many of their analogues were synthesized. An extensive review of the synthesis of angiotensin and its analogues was published recently (41). One of these analogues α-asparagine-1-valine-5-angiotensin II, shown to have identical activity to the natural angiotensin, is used by many investigators as a standard.

3) Angiotensinases

When a crude substrate preparation was incubated with renin, the amounts of angiotensin found at the end of incuba-

tion decreased inversely with the time of incubation (24, 42). This phenomenon was due to the destruction of angiotensin by proteolytic enzymes called angiotensinases. The optimal pH of angiotensinases found in plasma, red blood cells and tissues was from 7 to 8 (26, 43-46), whereas the optimal pH of those found in the kidney was 4 (46). The tissue angiotensinases are only partially inhibited by EDTA (44).

An aminopeptidase with a high degree of specificity was found in the plasma and red cells (47). This enzyme, called angiotensinase A, had an optimal pH of 7.5 to 8.0. It was completely inhibited by the addition of ethylene diaminotetracetic acid (EDTA) and reactivated by the addition of calcium ions. A second plasma aminopeptidase was partially separated from angiotensinase A by chromatography on Sephadex G-200 (48, 49). This enzyme required calcium ions and had an optimal pH of 6.8. It was suggested to call this enzyme, angiotensinase A_2 (49). A third enzyme found in plasma had a chymotryptic-like activity (49, 50) and cleaved angiotensin into tetrapeptides. This enzyme called angiotensinase B was inhibited by disopropylfluorophosphate (DFP) and EDTA and had an optimal pH of 5.8-6.

Using various electrophoretic systems, the maximum peak of angiotensinase activity was located in the α-1-globulin fraction in normal plasma (51, 52). In plasma with increased angiotensinase activity, some was detected in the ß2-globulin and albumin fractions (52). This further supported the existence of various enzymes in the plasma.

Angiotensin was found to be inactivated "in vitro" by trypsin (25, 53, 54), pepsin (26, 53, 55), amine oxydase and tyrosinase (56).

The action of the angiotensinases can be completely or partially overcome by acid treatment at pH 4.0 (26), use of charcoal (57), DFP (58), EDTA (44, 10), and Dowex 50W-X2 resin (NH_4+) (10). Epsilon amino caproic acid was found to be a powerful inhibitor of plasma angiotensinase whereas mercury (in the form of meralluride) inhibited the angiotensinase activity of the plasma to a smaller extent. The reverse was true for red blood cell angiotensinases (45). So far, no specific inhibitor of angiotensinase has been found.

4) Renin Substrate (Angiotensinogen)

Renin substrate was shown by electrophoresis to be composed of α_2-globulin (59). It was shown to be produced by the liver (60-62). A most extensive study of the purification of hog renin substrate was done by Skeggs and coworkers (63, 64). The plasma was first treated by a modification of the ammonium sulfate fractionation and partial acid denaturation of Green and Bumpus (65). The product was then adsorbed and eluted after chromatography on a column of DEAE cellulose at pH values characteristic for each type. A molecular weight of about 57,000 was found by ultracentrifugation. The rate of reaction of the different forms of substrate with hog renin appeared to be similar (63, 64).

Crude renin substrate treated with trypsin produced a tetradecapeptide which yielded angiotensin I upon subsequent

incubation with renin (66, 67). The first 10 amino acids were identical, and in the same sequence, as those found in angiotensin I. The tetrapeptide was attached to the C-terminal leucine of angiotensin I. The structure of the tetradecapeptide was confirmed by synthesis (67-69), and thus established the fact that renin acted at a leucyl-leucyl bond of the molecule to liberate angiotensin I.

The substrate concentration in plasma is not necessarily in inverse relation with the renin concentration. Pathological conditions, in which a high plasma renin activity is found, could be associated with high, normal or low angiotensinogen concentration. In pregnancy, the substrate level is increased in humans (43, 70-72). The administration of anovulatory medications to humans (73) and of diethylstilbestrol to rats (74) has been shown to increase substrate concentration. In malignant hypertension and in renovascular hypertension cases, Aida and coworkers (75) find normal levels of renin substrate. During the terminal phase of malignant hypertension, the substrate is found to be increased (71, 43). In contrast, angiotensinogen concentration in dog is not significantly altered by narrowing the renal arteries (76, 77). A reduction of the renin-substrate is reported in cases of cirrhosis of the liver associated with edema (71).

Substrate level is altered by nephrectomy and adrenalectomy, two experimental procedures which have respectively a markedly suppressive and stimulating effect over renin concentration in plasma. Substrate concentration, in these

conditions, is known to be inversely related to the level of plasma renin. Nephrectomized rats increase their substrate level in the first eight hours after nephrectomy and no further increase is detected after 8 hours (78). Nephrectomy increases by 4- to 15-fold the substrate concentration in the rat (79-81). This increase is known to be much less important in dogs (82). In dogs and rats, adrenalectomy reduces considerably the substrate concentration (83, 84, 74, 78). Normal levels of substrate concentration are restored by the administration of DCA to adrenalectomized animals (84). These facts suggest that the decreased substrate concentration could be due to the exhausting effect on substrate of a high circulating renin in the adrenalectomized animals. Similarly, low sodium intake reduces substrate whereas sodium load and DCA overdosage increase it in rats (78).

5) Renin-Inhibitor

The response to intravenous injection of renin is enhanced after bilateral nephrectomy (13), and it is overcome by large transfusions of blood from intact animals (35). This enhancement of pressor response to renin starts 2 hours after bilateral nephrectomy and reaches a plateau within 4 to 8 hours (85, 86). This increased responsiveness was attributed to elevated substrate concentration (80, 81). But, the response of blood pressure to exogenous renin was found significantly increased 2 hours after nephrectomy, whereas substrate concentration augmented only slightly (85). Regoli and coworkers (79) found that plasma from nephrectomized rats

incubated with a small amount of renin generated more angiotensin than normal rat plasma. By dilution experiments, they observed that this difference was not solely due to the increase in substrate concentration following nephrectomy. They concluded that plasma from nephrectomized rats contained a factor accelerating the angiotensin formation or conversely the plasma from normal rats had an inhibiting factor. The presence of a renin inhibitor was also suggested by Boucher et al (10) since the addition of a constant amount of renin to different plasma samples was found to generate varying amounts of angiotensin. This finding was confirmed by Pickens and coworkers (70) and Bumpus (87). Furthermore, the addition of a relatively small amount of dog renin to dog plasma, obtained at different time intervals after nephrectomy, showed that the possibility of generating angiotensin was still increasing even after substrate concentration reached a maximal value (88). These observations led Sen, Smeby and Bumpus (88, 89) to the isolation from dog and hog kidneys of a naturally-occurring phospholipid that inhibited the formation of angiotensin by renin in vitro. This compound was found to reduce the blood pressure of renal hypertensive rats (88, 89). This lipid was termed pre-inhibitor since the active principle was not the phospholipid itself, but rather a lysophospholipid formed by hydrolysis of the phospholipid with phospholipase A (88). Schaechtelin and coworkers (90) observed that renin rat plasma, when incubated at 37° C, at a pH

of 7.4, lost 70% of its renin activity within 2 hours. They suggested the presence of a rat renin inactivating system. The incubation system used by these authors was, however, incompletely protected from angiotensinases and their findings require further confirmations.

B - METABOLISM AND FATE OF RENIN AND ANGIOTENSIN

Only few and incomplete data on the metabolism of renin are available and most of them deal with the effect of the injection of renin into intact and nephrectomized animals. In dogs, 50% of the exogenous renin injected is found after 10 minutes (91) and it disappeared almost completely within 30 to 60 minutes (91, 92). Endogenous renin disappears at a similar rate in rats, but injection of large doses of renin remains in circulation up to 2 hours (93). Nephrectomized and uremic dogs retain exogenous renin for a longer period (92). In the intact animals, a part of the injected renin is recovered from the urine (92). It is known also that measurable amounts of "renin-like" substances are detected in the urine of normal subjects (94). These findings point towards the importance of the kidney as a factor controlling the metabolism of renin. Braun Menendez and his group (83) have reported that disappearance of large doses of exogenous renin is delayed in hepatectomized dogs, whereas lower doses are not affected. Heacox, Harvey and Vander (95) have measured plasma renin activity in an artery and hepatic, portal and renal vein of anesthetized dogs during infusion of renin or

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stimulation of endogenous renin secretion induced by intravenous injection of chlormerodrin. They have observed no difference in the levels of plasma renin activity between the artery and portal vein, but a significant difference between the arterial and hepatic venous renin during control periods and after stimulation of renin secretion or infusion of renin. They have concluded that the liver is the major site of renin inactivation.

Angiotensin is inactivated "in vitro" by various tissues and plasma due to the presence of angiotensinases (43-46). The importance of these enzymes has already been discussed in a previous section. Their presence cannot explain the short pressor response to angiotensin compared to renin in the whole animal (96). In normal rats, infusion of a large dose of tritiated angiotensin is taken up immediately by the adrenals and secondarily by the kidney and the uterus (97, 44). In nephrectomized rats, the distribution is more random, except that a greater concentration is found in the liver. The high concentration of the labeled peptide in the kidney and adrenals suggests that they may represent the target organs or that they are involved in the metabolism of angiotensin (44). During constant infusion of angiotensin at pressor doses, the amounts of angiotensin recovered in venous blood are considerably less than those found in arterial blood (10). An important fraction of the angiotensin entering hind limb (98, 96), kidney, head, part of the body below the

diaphragm (96) and liver (96, 98, 99) is cleared, whereas lung (98, 96) retains only a small fraction. The lung has been suggested, recently, as an important site where conversion of angiotensin I to II takes place (100).

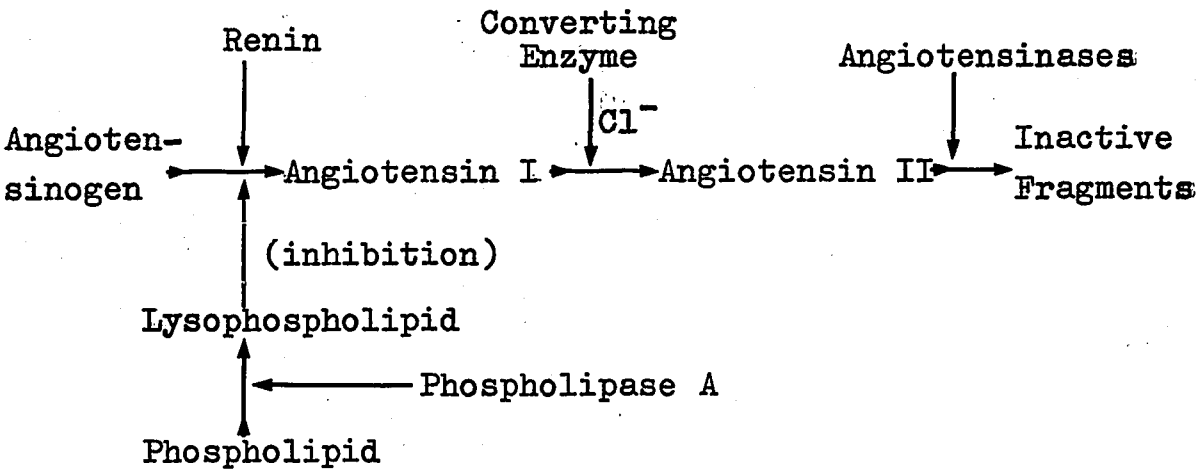
C - SCHEMA OF THE ENZYMATIC REACTION

The enzyme renin acts upon a leucyl-leucyl bond of a substrate, called angiotensinogen or renin substrate and produced by the liver, to form angiotensin I, a decapeptide.

The decapeptide, without any known biological activity, is rapidly transformed to a powerful vasoconstrictive octapeptide, angiotensin II, by a chloride ion dependent enzyme contained in the plasma, and known as converting enzyme. This enzyme removes two amino acids from the C-terminal of the decapeptide. Angiotensin II, one of the most potent vasopressors, is the effector hormone of the system. It is inactivated by various peptidases, known collectively as angiotensinases, to yield inactive compounds. It seems that rapid removal of angiotensin from the blood stream occurs mostly in the tissue to which it is carried and there is no evidence that plasma angiotensinases play an important role in the "in vivo" destruction of angiotensin.

As the research evolves, the classical representation of the enzymatic system is becoming complex. The presence of accelerator or inhibitor of the reaction has been suspected by many investigators. The existence of a renin-inhibitor was demonstrated by the group of Smeby. This inhibitor would

be a lysophospholipid formed by the action of phospholipase A on a phospholipid.



It appears thus that the angiotensin formation in under the influence of many factors and the importance of each factor on the formation of angiotensin is still unknown.

D. - SOURCE OF RENIN

Despite the description of renin-like substances in different organs (101-103), the juxtaglomerular apparatus seems to be the main source of renin. The exact location is still debated but the juxtaglomerular apparatus is implicated as the site of formation of renin on the basis of the results obtained with immunofluorescent techniques, microdissection assays of renin from different fragments of renal tissue, comparison between renin content of the kidney and juxtaglomerular granulations, and cell culture.

1) Structure of the juxtaglomerular apparatus

The juxtaglomerular apparatus is located on the hilar region of the glomerulus. It is made of the granular epithelioid cells contained in the wall of the afferent arteriole,

the macula densa of the distal tubule and the lacis agranular cells found between the macula densa and the glomerulus.

a) Granular juxtaglomerular cells

The juxtaglomerular cells were originally described by Ruyter (104) in mice and by Oberling (105) in human. The juxtaglomerular cells are recognized by the presence of stainable granules and by their location in the media of the afferent arteriole in close proximity to the glomerulus.

Goormaghtigh (106) noted the presence of granules in these cells and emphasized their possible secretory nature. Moreover, on the basis of light microscopic studies on rabbit ischemic kidney, he suggested a secretory cycle. The cytologic characteristics were found to be compatible with such a secretory function in electron microscopy, by Oberling and Hatt (107).

b) Macula densa

This structure is formed by the specialized area of the distal tubule in close contact with lacis cells and juxtaglomerular cells. Macula densa was first described by Peter (108) and Zimmerman (109). It was characterized by the tall columnar epithelial cells and the nuclei lying close together. The Golgi apparatus of these cells are located on the basal side of the nuclei rather than apical as in the other tubular cells (110, 107, 111). An incomplete basement membrane between juxtaglomerular and macula densa cells was observed (112, 107, 113) thus suggesting a functional relationship between

these two types of cells. The evidence pointing toward a functional relationship between the two types of cells will be discussed further in the control of renin secretion.

c) Lacis cells

These cells were thought by Goormaghtigh (114) to be neural elements. The term Polkissen or "polar cushion" was used by Zimmerman (109) to represent the complex formed by the juxtaglomerular cells and the lacis cells. The lacis cells were originally described as agranular cells and distinguished by their clear cytoplasm, their small size and round shape (114, 109). Electron microscopic studies suggested a morphological relationship of these cells with mesangial and granular epitheloid cells (107). Oberling and Hatt (107) postulated that a transformation into granular epitheloid cells occurred.

2) Localization of Renin in the Kidney

The role of the juxtaglomerular cells in the eleboration of renin was suggested by Goormaghtigh (106) on a purely morphologic basis. In early studies, renin was detected in the renal cortex whereas it was absent in the medulla (13, 16). Cook and Pickering (115) introduced magnetic iron oxide into the renal artery and removed selectively ground kidney fragments with an electromagnet. The glomerular fraction (magnetic) contained nearly all the renin whereas the tubular fraction was almost free of it. Further studies revealed that the glomeruli with attached fragments of the juxtaglomerular complex contained more renin that those without. Also, when separating

the glomerulus in two parts, these studies showed that renin was in the half containing the vascular pole. Moreover, by selective destruction or isolation of the glomerular tufts, renin was found located outside the glomeruli (116, 117). Bing and Kazimerczack (118) were able to separate, by dissection, the juxtaglomerular cells from the macula densa. Renin was mainly present in the macula densa and part of the distal convoluted tubules. Brown and coworkers (119, 120) measured renin in single glomeruli and showed that the glomeruli of the outer part of the cortex contained more renin than those of the inner part. They confirmed the results of Bing and Wiberg (116) who measured renin on successive layers of the kidney.

From an histological point of view, a close correlation between the granularity of the juxtaglomerular cells and the renin content of the kidney constituted an indirect evidence implicating the juxtaglomerular apparatus as the source of renin. Marshall and Wakerlin (121) were the first to find a good correlation between renal renin content and juxtaglomerular cell granulations and they noted similarities in the solubility characteristics between canine renin and juxtaglomerular cell granules. Juxtaglomerular index and renin content of the kidney were found to be increased in ischemic kidney (122-124) during sodium deficient diet (125), after adrenalectomy (126-128), and in dogs having a high titer of renin antiobodies (129). Conversely, in the kidney contralateral to the clamped one, or after the administration of salt load alone or combined to DCA (122-124), or after the injection of

renin (130), these two parameters were found to be decreased.

This type of evidence may be considered as indirect or circumstantial, but it is supported by the fluorescent anti-renin antibodies and tissue culture techniques. Studies with fluorescent antibodies permitted a more precise localization of renin. The first attempt to apply this technique to renin was done by Nairn and coworkers (131). Using the "sandwich" technique, hog kidney sections were treated with rabbit anti-hog renin and then with fluorescent goat anti-rabbit globulin. They found specific staining almost exclusively in the glomeruli but not in the juxtaglomerular area. The use of very crude and impure renin extract in the preparation of their antibodies invalidated their results (132). Edelman and Hartroft (133), using a modified technique and a purified renin preparation, observed a specific fixation of the antibodies by the granules of the juxtaglomerular cells and not by the glomerular tuft nor the macula densa. Warren, Johnson and Hoobler (134) also found specific staining only in the granulated juxtaglomerular and macula densa cells.

The ability of cells from human renal cortex to release renin was observed in tissue culture of human ischemic kidneys by Robertson and coworkers (135). When renin was found in the culture medium supernatant, abundant granulated cells, cultured from the diseased kidney, were present but they were unable to confirm this correlation in normal kidneys. Moreover, fluorescent labeled anti-renin was found to be localized in the cytoplasmic granules of the cultured cells.

Before closing this section, we have to stress that in certain conditions, juxtaglomerular cell granularity, renin content of the kidney and plasma renin activity are not always altered in the same manner. Especially in acute experiments, renin release is not always in parallel with the renin content of the kidney or juxtaglomerular cell granularity. For example, acute sodium depletion induced in rats by peritoneal dialysis, hemorrhage or thirst, increases the pressor activity of the plasma without affecting the renin content of the kidney (86, 136). Fisher (137) does not find any significant change in the granulation of the juxtaglomerular cells after peritoneal dialysis whereas Ménard and coworkers (138) find a marked increase in plasma renin activity despite an unchanged juxtaglomerular cell granularity. Rosenthal and coworkers (139) observe a 3-fold increase of plasma renin activity, contrasting with an unchanged juxtaglomerular index in rats injected with fursemide.

E - METHODS OF MEASUREMENT OF RENIN

Braun Menendez and his group (83) pioneered the field of renin methodology. They measured first the pressor response to intravenous injection of crude plasma in anesthetized animals and later the vasoconstriction of vessels of the isolated rabbit ear. They devised another method where the renin was measured by the decrease in substrate. Leloir et al (140) set up an indirect method where renin was estimated by the formation of angiotensin in standardized conditions of incubation. Following this, many research workers developed new methods and many of them modified known methods.

It is impossible to cover all these methods and we shall mention only the original and most recently described methods, since the older ones were neither sensitive nor reproducible.

The direct methods consist of the injection of partially purified renin into the test animal. The concentration of renin is assessed by the rise in blood pressure induced by the "in vivo" angiotensin formation. The most specific direct method was described by the group of Haas and Goldblatt (141). The extract is injected into unanesthetized trained dogs in which the femoral blood pressure is recorded. These authors defined one unit (Goldblatt unit) (141) as the quantity of renin required to produce a rise of 30 mm of Hg of the direct, mean, systemic blood pressure about two minutes after the injection. Because of the difficulty in maintaining a colony of selected trained dogs, this method was used only in a few laboratories. Moreover, since renin concentration is low in plasma, a large amount of blood is needed to produce a detectable effect in the test animal.

Indirect methods consist generally of the incubation of renin with some form of substrate, angiotensin being formed and assayed as such. Homologous or heterologous substrate may be separately prepared and added to the incubation mixture, or the renin may be incubated with the substrate present in the sample. This approach has enhanced sensitivity considerably. The deficiency of the older indirect methods was the failure to inhibit adequately the angiotensinase activity.

The first simple and semi-quantitative method for the determination of circulating renin was evolved by Helmer and Judson (43). Heparinized plasma was dialysed against cold water for 18-20 hours. The dialysate was centrifuged and the supernatant made isotonic by the addition of sodium chloride. Samples of 10 ml were then incubated at 37° C for one hour at pH 5.5. The reaction was stopped by boiling. The angiotensin formed was assayed on spirally cut strips of rabbit aorta or by the blood pressure assay in a 2-day nephrectomized pithed cat when the plasma had a high renin content.

The first quantitative and reproducible method for measurement of plasma renin activity was set up by Boucher and coworkers (10). They demonstrated that Dowex 50W-X2 (NH_4^+) resin did not interfere with the enzymatic reaction when added to the incubation mixture for absorption of the angiotensin formed during the incubation and complete protection from degradation by the angiotensinases. In Boucher's procedure blood was rapidly cooled (0-5° C) to prevent the action of renin on the substrate, collected on EDTA and then centrifuged at 0-5° C. Ten ml of plasma were adjusted to pH 5.5 at 0-5° C and then incubated in presence of 4 ml of moist Dowex 50W-X2 (NH_4^+) resin at 37° C for 3 hours. When enough plasma was available, a 2-hour incubation was carried out. The angiotensin formed was absorbed on the resin. After the incubation period, the incubation mixture was transferred to a glass column, and the angiotensin eluted. The eluate was then concentrated and the dry residue was diluted in 20% ethanol and

assayed on a nephrectomized rat. This simple method was shown to be highly sensitive and reproducible, and showed recoveries of 80-85% of added angiotensin.

Two methods described by Warzynski, Demirjian and Hoobler (142) and by Yoshinaga and coworkers (143) used an incubation mixture essentially identical to the one of Helmer and Judson (43). They improved the method by extracting the angiotensin formed with butanol (142, 143), petroleum ether (143), then the aqueous extract was fractionated on a column of Dowex resin 50W (H^+ form) and the angiotensin eluted with sodium hydroxide (143). Warzynski et al (142) claimed that the butanol extraction excluded atypical pressor substances.

Fasciolo et al (144) improved their first procedure by acidifying the plasma (pH 3.8 at 25° C for 30 minutes), a procedure shown by Leloir and coworkers (140) to inactivate the angiotensinases. The plasma pH was then adjusted to 5.1. One aliquot was not incubated and served as a control. The other aliquot was incubated for 2 hours at 37° C. In both samples, angiotensin was purified by the method of Scornick and Paladini (145) and pressor activity was measured using the arterial blood pressure of anesthetized rats.

A most elaborate improvement in Helmer's method was done by the group of Lever and Brown (146, 147). This method involved the preparation of a semi-purified extract of the enzyme from the blood, a procedure which was time-consuming and required a large amount of heparinized plasma (25 ml). One ml of the renin preparation was added to 4 ml of an ox

substrate preparation which provided an excess of substrate and the mixture was incubated at 37° C for periods of 30 minutes up to 96 hours according to the concentration of renin in the sample to be assayed. The pressor activity formed was then measured in a ganglion-blocked rat. This method was described originally in the rabbit (146) and was applied to human (147).

A method for determination of renin activity in the rabbit described by Lee, Cook and Robertson (148) was essentially similar to that of Lever et al (146). Minor differences were encountered in the purification process of plasma obtained from 4 to 6 ml of blood, and in the use of a purified rabbit renin substrate (149). The renin extract was incubated with the substrate for various time intervals up to 200 hours. The reaction was stopped by freezing and the pressor activity was measured in ganglion-blocked rats.

A simple method, both sensitive and reproducible, was set up by Pickens and coworkers (70). Plasma from 20 ml of heparinized blood was dialysed in the cold for 24 hours against EDTA and then against distilled water for another 24 hours. Following centrifugation in the cold, one drop of a 1:20 solution of DFP in isopropyl alcohol was added and the solution adjusted to pH 5.5. The mixture (10 ml) was then incubated for 4 hours at 37° C. The reaction was stopped by boiling. The supernatant was evaporated and then dissolved in distilled water and assayed in pentolinium treated rats.

Recently, Gould, Skeggs and Kahn (71) established a method in which serum was treated to remove angiotensinases

and incubated with an exogenous substrate. Serum was dialysed overnight at 5° C and pH 7.0 against EDTA. A 0.65 ml aliquot of dialysed serum was incubated with a partially purified hog substrate in presence of phosphate buffer and phenyl-mercuric acetate (used as a bacteriostatic agent) at pH 7.5 and 38° C for 16 hours. The incubation mixture was then heated in a boiling water bath and the supernatant was measured by a direct rat pressor assay.

In all the methods presented above, except for that of Gould et al (71), a large amount of blood is required and they cannot be used for studies involving small animals, or for repeated determinations in short intervals. A method permitting the measurement of renin activity in 0.1 ml of rat plasma was described, recently, by Boucher and coworkers (12). This technique is based on the principle that renin contained in 0.1 ml of plasma, when incubated over a 12-hour period with an excess of homologous substrate, will form detectable amounts of angiotensin. The latter is protected from proteolytic degradation by the addition of Dowex resin 50W-X2 (NH_4^+) to the incubation and is measured by the rat pressor assay. A rat renin-free substrate, prepared according to the procedure of Haas and coworkers (150) from nephrectomized rats, is employed. The procedure is highly reproducible, sensitive and specific.

It is admitted by most workers that the current indirect methods do not measure the renin "concentration" in plasma but rather the "effective renin activity" which is a summation of known and unknown factors involved with the enzymatic reaction.

The angiotensinase is one of the factors that has been efficiently controlled in the recent methods, by the use of EDTA, DFP or Dowex resin. In those methods where the action of angiotensinase was prevented or inhibited, the sensitivity and recovery were greatly improved. This fact is illustrated by the low recovery of added angiotensin with the method of Yoshinaga et al (143) (30 to 35%) contrasting with those found by Boucher et al (10) (84%), and Pickens et al (70) (97%).

In every method, the bio-assay of angiotensin is time-consuming. A physico-chemical method would improve and advantageously replace the bio-assay of angiotensin. Radioimmunoassay shows great promise. Three groups of workers succeeded recently in producing anti-angiotensin and further measuring the circulating angiotensin (151-153). Angiotensin alone is too small a peptide to be antigenic, but when the peptide is coupled to a protein or polymer carrier molecule or to a microparticle of carbon, the induction of antibodies to angiotensin II takes place (152-154). Antibodies produced in this way have been shown to cross react with native angiotensin II (152-156) whereas angiotensin I displays much less affinity for the antibody (151-153). In these methods, anti-angiotensin is allowed to react with labeled angiotensin. The addition of unlabeled angiotensin then displaces the labeled one from the complex and a quantitative relationship could be demonstrated between the amount of unlabeled angiotensin added and labeled angiotensin released from the complex. Valotton et al (151) extended their assay of angiotensin II to the

measurement of plasma renin activity. The plasma was incubated according to Boucher's method (10). The values obtained in normal subjects as well as in various clinical situations were much lower than expected. In the indirect methods (10), the concentration of converting enzyme is not controlled, and most probably the angiotensin formed is a mixture of both angiotensin I and II. This mixture is not affecting the final value of plasma renin activity in the conventional methods since, as far as the biological assays are concerned, the excess of converting enzyme in test animal (38-40) will convert angiotensin I to II. The application of the radioimmunoassay to renin assay should be possible but one must ensure that angiotensin II is the final product of incubation.

A preliminary report of a direct renin antiserum assay in dog was published by Keutel and coworkers (157). The unavailability of pure renin makes such a method hazardous. Moreover, the fluorescent labeled γ-fraction of the antiserum used by Keutel et al (157) reacted with antigen located in the tubules near the glomerulus but not in the juxtaglomerular apparatus.

F - CONTROL OF RENIN SECRETION

With the paramount importance of the renin-angiotensin system in different physiological and pathological states and because of its major role in the control of aldosterone secretion, the identification of the factors controlling production and release of renin into the circulation is pertinent.

1) Baroreceptor Theory

The early experiments of Goldblatt et al (14) with renal artery clamping introduced as a logical hypothesis that ischemia was the main stimulus for renin release. This was contradicted by Huidobro and Braun Menendez (158). In dogs breathing 6-8% O_2 or 2-5% CO_2 and in dogs treated with cyanide, no increase in renal pressor activity was detected. This lack of effect of ischemia on renin release was later confirmed by Skinner and coworkers (159).

Kohlstaedt and Page (160) first claimed that a decrease in pulse pressure was the stimulus for renin release in perfused dog kidneys. Tobian and coworkers (161-163) gave strong evidence of the role of the mean renal arterial pressure in renin release and postulated that the juxtaglomerular apparatus by its situation in the wall of afferent arteriole would act as a baroreceptor or a stretch-receptor. In their experiment, they observed that a high perfusion pressure reduced significantly the granulation of the juxtaglomerular cells in the isolated kidney. Skinner, McCubbin and Page (159, 164, 165) produced support for the stretch-receptor hypothesis. They (159) reported that a reduction of pulse pressure without any change in renal mean arterial pressure and blood flow, did not release renin whereas a slight reduction (10 mmHg) in mean renal arterial pressure was adequate to release renin. They showed that renin release was not stimulated by virtual abolition of pulse pressure achieved by vagotomy and aortic constriction. Also the administration of a ganglion-blocking agent and stimulation of the peripheral end of a severed vagus in order to increase

the pulse pressure, did not prevent the increase in renin release when the mean renal pressure was decreased. Tobian (166) claimed that on the basis of the stretch-receptor theory, he could,explain the conditions characterized by an increase or a decrease of renin secretion, but still some experimental findings remained unexplained. In uni-nephrectomized rats, no increase in renin release and renal renin content is found after clamping the artery of the remaining kidney (86, 167-169). Also the lack of increase in the juxtaglomerular index after clamping the aorta above both renal arteries (170) is not explained by the stretch-receptor theory.

In the light of the baroreceptor theory, the role of the plasma volume in the regulation of renin release has been discussed. With the demonstration that plasma renin activity or circulating angiotensin is suppressed in primary aldosteronism (11, 171-174, 147), the control of renin release by the plasma volume became challenging, since in primary aldosteronism the plasma volume is known to be expanded (175, 176). In the same line of evidence, it has been demonstrated in man, that stimulation of plasma renin activity induced by ethacrynic acid could be prevented by plasma volume expansion (177) and in rats, the expansion of plasma volume prevents the rise in plasma renin activity encountered during peritoneal dialysis with 5% dextrose (138).

The increase of plasma renin encountered in pregnancy (178-181, 144) is difficult to explain since plasma volume

is increased (182, 183). Similarly, in congestive heart failure, the plasma volume is normal or increased and could not explain the high values for arterial angiotensin or plasma renin activity reported in that disease (144, 184-187, 179, 180). Experimental evidence against the role of plasma volume in controlling renin release has also been produced. Recently, it was demonstrated that in volume expanded dogs (60 minutes perfusion of 0.85% NaCl at 0.5 ml/kg in the suprarenal aorta), it was possible to stimulate the renin release by changing the perfusion of physiological saline to a 0.42% solution of sodium chloride (188). In this experiment, the plasma volume was not measured although they reported the volume expansion to be sufficient to suppress the release of ADH. Vander and Miller (189) also demonstrated that the effect of diuretics on renal venous plasma pressor activity of a clamped kidney was independent of changes in plasma volume.

2) Macula Densa Theory

We have already discussed the inadequacy of the stretch-receptor theory in explaining renin release. Moreover, changes in perfusion pressure have an effect on sodium excretion unrelated to the variations in glomerular filtration rate (190-193). The tubular sodium concentration is decreased in isolated kidney preparations perfused at low pressure, as demonstrated by stop-flow techniques (194). The anatomy of the juxtaglomerular apparatus soon led Goormaghtigh (114, 195, 196) to propose that the macula densa fluid was a factor controlling the rate of glomerular filtration via the

juxtaglomerular cells.

The findings suggesting the macula densa as the structure responsible for renin secretion are numerous. An intimate anatomical relationship between the macula densa and juxtaglomerular cells has been demonstrated by light and electron microscopy (107, 111, 113, 197). Moreover, an incomplete basement membrane between juxtaglomerular and macula densa cells was observed (112, 107, 113). A distinct separation between both types of cells was difficult to find in some places, and may have been absent in that infoldings of the membrane of both cell types often appeared continuous. McManus (110, 198) described an unusual location of the Golgi apparatus in macula densa cells in that it was basal rather than luminal. This peculiar location led him to suggest that substances could be transferred from a macula densa cell to a juxtaglomerular cell. In adrenalectomized rats, the presence of dense cytoplasmic bodies in the macula densa cells has been described in association with an increased juxtaglomerular cell granulation (199).

Freidberg (200), studying the distribution of the juxtaglomerular granules and the macula densa in the renal cortex of the mouse, found an inverse relationship between the length of Henle's loops and the number of juxtaglomerular cell granules. These granules were more numerous in the outer cortex than in the juxtamedullary zone. In a subsequent publication, Friedberg (201) found, in studying the distribution of juxtaglomerular cell granules in mice at

different ages, that the adult pattern of distribution of juxtaglomerular cell granules is reached at about the third week of extra-uterine life, a time which correlates with the maturation time of the long Henle loops in rats (202), and the onset of maximal urinary concentrating ability in humans (203). Bing and Kazimierczark (204) found renin in the outer cortex of newborn hog kidneys despite the absence of juxtaglomerular cell granules in that area.

From an histochemical point of view, a positive correlation has been established between the granularity of juxtaglomerular cells, renin content of the kidneys and enzymatic activity of the macula densa cells under different experimental conditions (205-209). Such a relation between plasma renin, glucose-6-phosphate dehydrogenase and juxtaglomerular index was not observed, however, by Ortega (210) in adrenalectomized rats if sodium chloride was not added to the diet. In these animals, plasma renin and glucose-6-phosphate dehydrogenase increased moderately despite an almost complete degranulation of the juxtaglomerular apparatus.

The macula densa is ideally located to receive information from the tubular urine and thereby, influence the activity of juxtaglomerular apparatus. The anatomical and histochemical evidence, reported above, suggesting a functional relationship between the macula densa and the juxtaglomerular apparatus led many investigators (195, 196, 107, 111, 189, 208, 211, 212) to propose that renin formation and secretion was under control of the macula densa. The macula densa cells could be sensitive to the osmolality or to the sodium concen-

tration of the tubular fluid passing through. By mathematical analysis of available experimental data from their own studies and from others, Guyton and coworkers (213) concluded that the autoregulation of renal blood flow was most probably controlled by an osmotic feed-back at the site of the macula densa, although they could not rule out the possibility of a sodium feed-back as well. Although renin appears to be located in the juxtaglomerular cell granules, the possibility that renin could be actually synthesized in the macula densa has not been ruled out (see localization of renin in the kidney, page 16).

Strong evidence attesting the role of the macula densa in the control of renin release has recently been reported. Vander and Miller (189) found that osmotic diuretics, acetazolamide and chlorothiazide reversed or prevented the stimulating effect of a suprarenal aortic constriction on renin release. They also demonstrated that other osmotic diuretics such as mannitol, sodium chloride or sodium sulfate were without effect on the base-line level of pressor activity in the renal venous plasma. In their experiments, they found only a positive reciprocal correlation between the renal venous plasma pressor activity and the sodium and water excretion, whereas little or no correlation was found with mean arterial blood pressure, plasma volume, plasma osmolality or sodium concentration, glomerular filtration rate and renal plasma flow. The effect of diuretics or sodium chloride infusion in preventing or reversing the usual renin release

induced by a decresed renal perfusion has been confirmed by White (214), by Vander and Luciano (215), and by Tobian (216). Vander (217) also showed the osmotic diuretics to be effective in reversing the rise in renin release provoked by intravenous infusions of adrenaline or nor-adrenaline and in preventing the same rise induced by renal nerve stimulation. Vander (218) proposed that the signal sensed by the macula densa would be the sodium load and a reciprocal relationship would exist between the sodium load presented at the macula densa and the release of renin.

Vander and Miller (189) reported also that increased ureteral pressure stimulated the pressor activity measured in the renal vein. Vander (218) claimed, however, that the increased intrarenal pressure was without effect on renin release since both, occlusion of the ureter and osmotic diuretics, had an opposite effect on renin release. Leyssac (219), on the other hand, postulated that a decrease in distal intratubular pressure might increase production of renin by a chemical or electrical mechanism at the macula densa. This was supported by the finding that in a clamped kidney the proximal peritubular capillary and intratubular pressures were identical whereas the distal intratubular pressure was decreased as much as fifty per cent.

The strongest evidence implicating the macula densa, although no renin determinations were done, was provided by the group of Thurau (220, 221). They used an original approach to study the autoregulation of renal blood flow. They

first injected by micropuncture, the early proximal convolution of a nephron with lissamine, which permitted them to identify the distal tubule of this same nephron. Although the macula densa of the mammalian kidney is not accessible to micropuncture, the sodium concentration of its tubular fluid was varied by retrograde microinjections of various fluids. The effect of these stimuli at the site of the macula densa was inferred from the quick collapse of the proximal convolution of the same nephron. This collapse was interpreted as a sharp constriction of a preglomerular arteriole reducing the glomerular filtration. They obtained a positive reaction with an injection of 150 mM/L of sodium chloride or sodium bromide solution. The reaction observed with the NaCl solution was present only if the kidneys were rich in renin. The renin depleted kidneys showed no reaction to the injection. When the concentration of the sodium chloride solution was progressively reduced below 150 mM/L, the effect was progressively reduced. The effect was specific in that it did not affect the other convolutions not belonging to the injected tubule. Moreover, the reaction was specific for sodium chloride since choline chloride, sodium sulfate or potassium salts did not cause the collapse of the proximal tubule. Isotonic mannitol was devoid of effect and the osmolality was not considered as the appropriate stimulus. They postulated a feed-back mechanism whereby an increased concentration of sodium at the site of the macula densa induced a local renin release from the juxtaglomerular apparatus generating angiotensin "in situ", and the latter decreasing the glomerular

filtration.

This theory is in complete disagreement with Vander's theory. Admittedly, both approaches are different. Schnermann, and Thurau (220, 221) studied the phenomenon with a single nephron whereas, the whole kidney was used in Vander's work. It is known that renin is mostly located in the outer cortex glomeruli (119, 120), but in clipped kidney, renin content of the deeper glomeruli increases (222). Quantitatively and qualitatively, it is possible that the response of the outer cortex juxtaglomerular apparatus differs from that of the deeper ones. Hence, when working with a single superficial nephron, it becomes difficult to foresee the response of the juxtamedullary nephrons. Moreover, too little is known about the local rate of angiotensin formation to decide which theory is correct.

At the present time, there is some evidence against the role of the macula densa in regulating the granulation of juxtaglomerular cells. In some types of fish (teleosts), the juxtaglomerular apparatus contains granulated cells despite the absence of the distal tubule (223, 224). Atrophy of the macula densa and tubular destruction by sodium tartrate do not affect the granulation pattern of the juxtaglomerular apparatus (225). Similarly, in rats where tubular continuity has been interrupted by chronic pyelonephritis or renal trauma it is still possible to increase the juxtaglomerular cell granularity by adrenalectomy (226).

Before concluding this discussion on the role of the macula densa in controlling renin release, a word must be

said about the juxtaglomerular apparatus as a possible "osmoreceptor" controlling renin release. It has been emphasized that the juxtaglomerular cells could be sensitive to plasma sodium changes. Hartroft (227) reported that despite the administration of vasopressin in order to prevent a decrease in blood volume, the juxtaglomerular cells continue to develop hypergranularity in response to sodium deficiency. In adrenalectomized, uninephrectomized rats, made hypertensive by DCA and cortisone, the juxtaglomerular cell granularity and renal renin content were inversely related to sodium intake (228). In rats made hypertensive by a clip on one renal artery, sodium deficiency increased the granularity of the juxtaglomerular cells of the intact and clipped kidney without affecting the blood pressure (229, 230). An inverse relationship between natremia and plasma renin activity was claimed by Brown et al (231). But definite and convincing evidences against this concept were provided. Meyer et al (177) found a quick increase in plasma renin and hematocrit within 30 minutes following the intravenous injection of ethacrynic acid in normal humans. The natremia was not affected by the medication. When ethacrynic acid was administered concomitantly with a 3.5% solution of polyvinyl-pyrrolidone, the plasma renin activity paralleled the hematocrit values which increased in one patient and decreased in the other two whereas the natremia remained unchanged. Menard, Boucher and Genest (138) found a significant increase in plasma renin activity in rats submitted to thirst or peritoneal dialysis.

These two stimuli respectively increased natremia to 170 mEq of sodium/L and decreased it to 100 mEq of sodium/L. With different diuretics, Vander (189) prevented or reversed renin release following aortic clamping and produced acute changes in plasma sodium which were unrelated to the variations in renal venous pressor activity. Nash et al (188) have demonstrated that the increased renin release evoked by norepinephrine was prevented by increasing both intravascular renal sodium concentration and sodium filtered load. The effect was absent when the intravascular renal sodium concentration was increased without concomittant increase in the sodium load. It must be stressed that under the experimental conditions where the plasma sodium level is altered, similar related changes in tubular fluid composition occur. For this reason, it is difficult to ascribe to the plasma sodium level alone, any effect at the site of the juxtaglomerular apparatus.

3) Hormonal Control of Renin Release

a) Angiotensin

To date, the effect of several physiological substances on the release of renin has been investigated. It seems that angiotensin is one of the most potent inhibitors of renin secretion. Masson and coworkers (232) demonstrated that the injection of hog renin into rats for 4 days resulted in a decrease in secretion of renal pressor material while the renal content remained normal. The administration of angiotensin for a period of two weeks resulted in a decrease of juxtaglomerular cell granularity (233-235).

The most direct approach to this problem was achieved by two groups simultaneously. De Champlain, Genest and coworkers (236, 179, 237) showed in humans that the elevated plasma renin concentrations induced by salt depletion, were reduced by angiotensin infusion given at pressor as well as sub-pressor doses. They also showed that aldosterone and pressor doses of norepinephrine infusions were without effect on plasma renin activity. Thus, they attributed a specific role to angiotensin in this negative feed-back control over renin release. At the same time, Vander and Geelhoed (238, 239) also found the inhibitory effect of angiotensin over renin release in dogs with very low rates of renin secretion and in dogs with an increased renin release induced by ureteral occlusion or aortic clamping during maintenance of constant reduced renal arterial pressure. Geelhoed and Vander (240) also confirmed the lack of acute effects of aldosterone perfusion on plasma renin activity in dogs. Bunag and coworkers (241) confirmed the inhibitory effect of angiotensin on renin release but they showed that large doses of vasopressin, but not oxytocin, also inhibited renin release.

b) Adrenal mineralocorticoids.

Before the description of the inhibitory effect of angiotensin over renin release, the adrenal mineralocorticoids were suggested by Masson and coworkers (242, 243) as possible feed-back substances regulating the activity of the renin-angiotensin system. Using a very crude method of measurement, they showed that the circulating pressor material decreased rapidly after the administration of DCA and salt supplements

while the renal renin content remained unchanged. This effect was observed before the incidence of hypertension. Nevertheless, the administration of cortisone or cortisol also decreased the liberation of pressor material although in a more gradual fashion. The administration of DCA and sodium to rats was reported to decrease the renin content of the kidney (86, 122, 124), and the juxtaglomerular index (122-124). Fukuchi and coworkers (244) reported a slight decrease in the juxtaglomerular index of rats treated with aldosterone for a period of two weeks, whereas Fisher and Tamura (245) reported a more pronounced degranulation of juxtaglomerular cells in rats injected subcutaneously with aldosterone dissolved in oil for three weeks.

Similarly, a low or undetectable level of circulating angiotensin, or plasma renin activity (11, 171-174, 147), as well as a decreased juxtaglomerular index (246-248) were reported in human suffering from primary aldosteronism syndrome. But a suppression of renin release was not found in normal humans during acute aldosterone perfusions (179, 236, 237, 240).

It is difficult to determine to what extent DCA or aldosterone alone are involved in the control of renin release since these hormones do affect the sodium balance and the blood volume. Thus it would appear that mineralocorticoids are practically devoid of any consistent direct effect over renin release.

4) Nervous System

There seems to be doubt that an intrarenal

control mechanism of renin release is operative. However, the innervation of the juxtaglomerelar apparatus has been known for a long time by the histologists (104, 105). Nilsson (249), using a sensitive fluorescent method, characterized the nerve endings surrounding the afferent arterioles as adrenergic. Electron microscopic studies (250, 251) on the juxtaglomerular complex showed the presence of vesiculated processes in the nerve endings located close to the granular juxtaglomerular cells. On the basis of the location and content of these vesicles, the nerve endings were assumed to be adrenergic. These descriptions constitute a more cogent proof supporting the evidence accumulated in favor of the existence of reflex neural control of renin secretion.

A decreased renin release was demonstrated in dogs by Taquini and coworkers (252) following renal denervation. Tobian and collaborators (253) observed fewer granules in the juxtaglomerular apparatus as well as a decreased renin content in the denervated kidney. Vander (217), by direct electrical stimulation of the renal nerves, evoked an increase in renin release. Bunag and coworkers (254) and Hodge and coworkers (255) reported respectively an increase in renin release and in plasma angiotensin during "non-hypotensive" hemorrhage, and this release was prevented by ganglionic blockade or local anesthesia of the renal nerves.

Bozovic and Castenfors (256) demonstrated that the increased plasma renin activity produced by exercise (swimming until exhaustion) or by pain stress (1 ml of 10% NaCl intramuscular) in rats could be prevented by the administration of

pentolinium. To explore further the role of the nervous system in renin release, they (257) studied the effect of bleedings in rats in which the spinal cord was destroyed or transected. They were able to show that the transection of the spinal cord, two hours prior to the bleeding, prevented the expected increase in plasma renin activity. Transection 24 hours prior to bleeding or destruction of the spinal cord was without effect. In rats with cannulation of both carotids, they (258) showed that pentolium pretreatment blocked the expected rise in plasma renin activity found with bleeding whereas hydralazine did not.

The effect of norepinephrine was tested by Scornick and Paladini (259) who showed a significant increase in circulating angiotensin level following intravenous infusion of norepinephrine and an even larger increase when the infusion was coupled with renal arterial blood pressure reduction. Wathen et al (260) demonstrated a rise in renin release when norepinephrine or epinephrine was injected into the renal artery of anesthetized dogs. If norepinephrine was infused into a peripheral vein, no effect over renin release was observed. Intravenous infusions of epinephrine or norepinephrine during maintenance of a constant renal arterial blood pressure by means of a suprarenal aortic constriction was shown by Vander (217) to increase renal venous pressor activity in dogs. De Champlain et al (179, 236, 237) did not find any effect on plasma renin activity in human subjects on low sodium intake infused with pressor doses of norepinephrine. The absence of effects can be ascribed to the facts that their subjects were already stimulated by sodium depletion and that

norepinephrine was infused into a peripheral vein and at pressor doses.

The first evidence to correlate the central nervous system activity with the response of renin secretion has been reported recently by Ueda and coworkers (261). By an electrical stimulation of mesencephalic areas (central gray stratum and adjacent portions), they induced an increased renin release in 6 out of 12 dogs. The renal denervation eliminated almost completely the response. In the experiments where the renin release was increased, the stimulation points were clearly localized in the dorsal portion of the pressor area. In this type of stimulation, the renal blood flow was first decreased and then returned gradually towards control level, whereas the systolic and diastolic blood pressure rose. This suggests that the central nervous system actually plays some role over renin secretion and that there exists a reflex neural control of renin release. We cannot conclude whether this reflex neuronal control is acting directly on juxtaglomerular apparatus or indirectly through hemodynamic or electrolyte changes. Further investigations are needed to clarify the afferent and efferent pathways of these reflexes and to elucidate the effects of stimulation of other areas in the brain stem over renin release. The control of renin release is depending mainly on an intrarenal mechanism. The stretch exerted on the afferent arteriole or the sodium concentration sensed by the macula densa may represent the adequate stimuli to release renin. Most probably, a summation of both factors

is involved. The function of these two structures seems to be modulated by neural and hormonal influences.

G - RELATIONSHIPS OF RENIN TO ALDOSTERONISM

1) In Essential Hypertension and Primary Aldosteronism

a) Primary Aldosteronism

Following the original observation of Conn in 1955 (262-264), primary aldosteronism was observed in many patients and became another curable cause of hypertension. Conn and coworkers (265) in 1964 reviewed 145 cases and stressed the characteristics of this syndrome. Benign hypertension, hypokalemic alkalosis and abnormalities of renal function are the most frequent findings. Malignant hypertension is a rare occurence of the syndrome (266). The symptoms which could be encountered are, by order of importance, weakness, nocturnal polyuria, headache, polydipsia, paresthesias, visual disturbance, intermittent paralysis and tetany. Edema is a rare complaint and has been observed in only 3% of the patients. The physical examination reveals benign hypertension. Tetany is observed in about 9% of the cases and the Trousseau sign is provoked in 17%. The latent or manifest tetany is found mainly in female patients. The blood findings show a hypokalemic alkalosis, hypernatremia, and in many cases increased plasma volume. The absence of hypernatremia in many patients could be explained on the basis of the escape phenomenon to the sodium retaining effect of aldosterone (176, 267-269). The excretion or secretion of aldosterone is increased in most

cases, contrasting with normal values for 17-ketosteroids and 17-hydroxycorticosteroids. The abnormal renal function is reflected mainly by the persistent or intermittent proteinuria of a mild degree, the large volume of urine with low specific gravity unresponsive to water restriction or to vasopressin. Glucose tolerance test is also impaired in 54%, and this impairement has been ascribed to chronic potassium depletion (270). The disease is easily recognized and cured upon removal of a small adrenocortical adenoma.

Bilateral adrenal hyperplasia or occasionally normally-appearing glands are found in a few cases (271, 272). These patients were tentatively termed "congenital aldosteronism" (271) but it is impossible to decide whether the defect resides within or outside the adrenals. Some of these patients present a malignant type of hypertension. Generally, the younger patients of this group benefit from subtotal adrenalectomy whereas the older patients respond poorly to the removal of adrenals.

The diagnostic criteria of primary aldosteronism became, however, inadequate to differentiate Conn's syndrome from aldosteronism secondary to renal artery obstruction (273, 274). The increased aldosterone secretion observed in these patients was found to be due to the large quantity of renin poured into the circulation by the obstructed kidney. The improvement in sensitivity and specificity of methods for determination of renin and angiotensin enabled Genest (172),

Kirkendall (173), Brown (147) and Conn (174) to demonstrate the importance of the suppression of circulating angiotensin or plasma renin activity in the differential diagnosis of primary aldosteronism. Similarly, a decreased juxtaglomerular index (246-248) was observed. This suppression of plasma renin activity is most probably due to the mild but sustained increase in extracellular volume, including intravascular volume (175, 176), just as it is following the daily administration of aldosterone to normal subjects (176). This suppression of renin activity in the plasma may be relieved by spironolactone (275).

Following removal of the tumor, the circulating renin may remain undetectable for a week and then rebounds to supernormal values (174). Hypernatremia has been suggested as responsible for a low circulating renin (231), but it is inconstant in primary aldosteronism, since an escape to sodium-retaining effect of aldosterone is encountered (176). The hypokalemia has also been suggested as the cause of suppressed renin activity, but in man under salt restriction, potassium depletion induced with a potassium ion exchange resin, increases the plasma renin activity (276). Conn (174) emphasized the degree of suppression of plasma renin activity encountered in primary aldosteronism. These patients after 4 hours in upright posture failed to increase their plasma renin activity under normal and restricted sodium intake. In normal subjects, the upright posture was demonstrated to produce a significant

increase in plasma renin activity (174, 277, 278).

b) Essential Hypertension.

Genest and coworkers (279, 280) were first to report a significantly increased aldosterone excretion in essential hypertension. This finding was corroborated by other workers (281-283). In contrast, other investigators (284-287) found a normal aldosterone excretion or secretion in patients with benign essential hypertension. Despite this controversy, the early reports (77, 147, 179) on measurement of plasma renin activity in essential hypertension clearly indicated that, despite a normal mean value, a proportion of these patients had a suppressed renin activity. Observing that the increased aldosterone excretion was accompanied by a suppressed plasma renin activity in some cases of essential hypertension, Conn (11) proposed the hypothesis that 20% of these patients were suffering indeed from primary aldosteronism despite the absence of the usual symptoms and hypokalemic alkalosis. This assumption was also based on two more observations. Hypokalemia could be a late event in primary aldosteronism (265). A prevalence of increased adrenal weight, cortical nodularity and adenomas was found among hypertensive patients examined at autopsy (288, 289).

Special consideration was given to the report of Shamma, Goddard and Sommers (290), since adenomas were found in 20% of 220 autopsied patients and 21% of 78 adrenalectomized patients. This report was probably overestimating the incidence, since they studied selected patients with hypertension.

Furthermore, such a high provalence was not supported by other workers (288, 291). Further support to the hypothesis of "normokalemic" primary aldosteronism was given by the demonstration of the presence of adenomas in the adrenals of six patients with "essential" hypertension in whom plasma renin activity was suppressed and aldosterone excretion or secretion increased, and who were cured by surgery (292, 293). Laragh and his group (287), on the other hand, failed to find suppressed plasma renin and increased aldosterone secretion or excretion in selected patients with essential hypertension.

Conn et al (292) also suggested that the hypertensive patients developing hypokolemia rapidly after thiazide therapy should be the first ones screened for primary aldosteronism. This was not supported by Kaplan (294).

Kuchel and coworkers (295) studied the effect of diazoxide on 29 hypertensive patients. Seventeen responded by an increase in plasma renin activity whereas no change was observed in the other 12 patients. This last group of patients also failed to respond to upright posture and sodium depletion. The serum potassium was significamtly lower in the unresponsive patients. Moreover, 4 of 11 patients, with suppressed renin, had an aldosterone secretion rate consistently elevated; 2 of these patients were explored and adenomas were removed. The renin responsive patients had normal aldosterone secretion rate. They concluded that primary aldosteronism might explain the suppression of plasma renin activity in a

small number of hypertensive patients.

Gunnells and coworkers (296) studied 88 patients from a series of 123 selected patients with essential hypertension. At least one measurement of plasma renin activity after four hours of ambulation under a sodium intake of 180 mEq Na/day was done. Plasma renin was below the normal control values in 11 patients (12%). A careful evaluation of 24 patients among the 88 was undertaken. They measured plasma renin activity in recumbent and upright posture (4 hours) under both normal and low (10 mEq Na/day) sodium intake. Finally, while the patients were still on low sodium diet, 50 mg of hydrochlothiazide were administered orally after the last sample for renin activity and repeated 4 hours later. On the following day, the patients were asked to walk around for 4 hours and then another blood sample was taken for determination of plasma renin activity. Renin was clearly suppressed in 4 of these 24 patients and a marginal suppression was observed in another one. Hypokalemia was present in one and induced by diuretic administration in a second. Aldosterone excretion was significantly increased in the 5 patients with suppressed renin activity.

It seems that the incidence of normokalemic primary aldosteronism has been overestimated. Recent contact with the major research group interested in this field would indicate the incidence of "normokalemic" primary aldosteronism to be around 1 to 8% (297, 297a).

Any enzymatic defect in the adrenals (298) establishing a positive sodium balance will suppress the activity of the renin-angiotensin system. The 2 patients of Sutherland, Ruse and Laidlaw (299) deserve special mention. A father and his son were investigated for benign hypertension and potassium deficiency. The 17-ketosteroids and 17-hydroxysteroids excretion was intermittently elevated. The pregnanediol excretion and aldosterone secretion were increased whereas plasma renin activity was suppressed. These abnormalities disappeared in both patients on dexamethasone 2 mg/day. The condition recurred upon withdrawal of the medication and was relieved by further administration of dexamethasone. The effect of dexamethasone is probably not mediated through ACTH since aldosterone secretion declined with intramuscular administration of ACTH. The response to dexamethasone in these two patients differed from that observed in a normal subject and in a patient with Conn's syndrome. A nodular cortical hyperplasia of the left adrenal was found in the father and the left adrenalectomy failed to improve the hypertension and decreased the aldosterone secretion rate to half the preoperative values.

2) In Congestive Cardiac Failure

Following the isolation and identification of aldosterone in 1953, a series of observations led to the important finding of the control exerted by the kidney on aldosterone production. The first indirect evidence was provided by Deane and Masson (300) who showed that encapsulation of the kidney

or injections of hog renin into rats produced widening of the zona glomerulosa. Genest and coworkers (1-4) first gave direct evidence of the control exerted by renin angiotensin system on aldosterone production. They demonstrated that intravenous infusions of angiotensin consistently increased aldosterone excretion in normal human subjects. This effect appeared to be independent of the pressor effect of angiotensin and was specific since it was not produced by other pressor agents. This fundamental observation was confirmed by other investigators (5-9). Indirect evidence supporting the role of the kidney and the renin angiotensin system on the control of aldosterone secretion was also provided. In hypophysectomized dogs, nephrectomy reduced the aldosterone secretion rate and prevented the substantial increase in aldosterone production following acute blood loss (301). Injection of renin also increased the aldosterone secretion rate (302, 303).

Today, it is accepted that this system is the main regulator of aldosterone secretion and is an important factor in the maintenance of sodium balance. An inverse correlation is observed between the quantity of sodium ingested and the activity of the renin angiotensin system (179, 147, 287, 304), as well as the aldosterone secretion or excretion (179, 287, 304-306).

In normal men (307-309) and dogs (310), angiotensin infusion reduced sodium excretion. In hypertensive patients (307, 308), on the other hand, angiotensin enhanced the sodium excretion despite an increased aldosterone excretion. A similar effect was also observed in cirrhotic patients with ascites (309).

The effect of angiotensin (decreased sodium excretion) in normal men, takes place within minutes. This effect is most probably due to the reduction in renal blood flow and glomerular filtration rate and is not mediated through aldosterone.

Nevertheless, angiotensin exerts a long-term sodium retaining effect mediated through stimulation of aldosterone production. Continuous infusion of angiotensin in normal dogs can maintain a sodium retention only for 1 or 2 days but afterwards the sodium is no longer retained and an escape occurs (311). Such an escape phenomenon is well documented when aldosterone or DCA is administered to normal dogs or humans (267-269, 312). Infusion of angiotensin to dogs with a large arteriovenous fistula, produces virtually complete sodium retention and edema formation (313). This effect of angiotensin is prevented by adrenalectomy when steroid replacement dosage is held constant. The lowering of sodium excretion induced by angiotensin in normal subjects and in patients with adrenal insufficiency (314, 315) persists for a short period after infusion, only in normal subjects (315).

In certain experimental conditions like vena cava constriction (316) and large arterio-venous fistula (313), the administration of mineralocorticoids produces a sodium retention, leading to edema and no escape phenomenon occurs. The major role of aldosterone in the production of edema is suggested by experimental finding. The increase in aldosterone secretion precedes the edema formation during aminonucleoside nephrosis in rats (317). In similar nephrotic rats, aldosterone

secretion is closely associated with the development of edema and adrenalectomy prevents the edema formation (318). Moreover, these adrenalectomized rats, given a normal dosage of aldosterone do not develop edema or ascites whereas they do when they receive large amounts of aldosterone (318). A participation of the renin angiotensin system via aldosterone in the process of edema formation is also suggested by the demonstration of a correlation between the granularity of juxtaglomerular cells, and the amount of ascites in rats rendered nephrotic by the administration of aminonucleosise (319).

Only 50% of the patients with congestive cardiac failure have an increased aldosterone secretion or excretion rate (320-323). The plasma level of aldosterone is normal in heart patients with stationary edema, whereas it is high in those with progressive edema (324, 325). Aldosterone secretion rate was shown by Luetscher and coworkers (326) to be within the normal range in 6 of 7 cardiac patients in whom the circulation was considered "relatively normal" whereas it was high in 4 out of 6 with congestive heart failure. The low aldosterone secretion encountered in some cardiac patients could be explained by the disturbance encountered in aldosterone metabolism. The plasma level of aldosterone as well as its half-life was found to be markedly increased in patients (324, 327) and in dogs (328) with congestive cardiac failure. The hepatic extraction of aldosterone was shown to fall as low as 50% in patients with severe cardiac failure, whereas it was almost complete in patients with only mild failure (326, 327, 329, 330).

There are only a few studies reported on the renin

angiotensin system in patients with heart failure. Merrill, Morrison and Brannon (184) were the first to report an increase vasoconstrictor activity of renal venous blood in 8 of 11 patients with chronic congestive heart failure. De Champlain, Genest and coworkers (179, 180, 185) reported very high levels of arterial angiotensin in 13 of 14 patients with congestive heart failure. Plasma renin activity was also found to be greatly eleveted. After total or partial relief of edema by digitalis, sodium restriction or natriuretic agents, the level of arterial angiotensin and plasma renin activity decreased significantly, contrary to normal and hypertensive subjects. The elevated rate of aldosterone secretion observed in dogs with experimental congestive heart failure secondary to pulmonic stenosis similarly decreased within 90 minutes after digitalization (331). Fasciolo and coworkers (144) reported an increased renin activity in 4 out of 9 cardiac patients. Only untreated patients with marked edema had an elevated renin activity. Brown and coworkers (186) reported in a review two distinct patterns in untreated patients with congestive heart failure. In one group, the plasma renin was low and increased with treatment, whereas in the other group, it was high and decreased with treatment. Johnston, Davis, Robb and Mackenzie (187) measured the plasma renin activity in experimental heart failure. Congestive heart failure was produced in dogs by tricuspid insufficiency combined to pulmonary artery stenosis, and by a large arteriovenous fistula. An increase of the plasma renin activity occured in all 5 dogs

with low-output failure and in 3 of 5 animals with high-output failure. A small arteriovenous fistula, not large enough to produce heart failure, was done in 9 dogs and the plasma renin activity remained unchanged.

From our present knowledge of the factors controlling renin release, the high peripheral renin activity and angiotensin level observed in heart failure and the paradoxical response to salt restriction are not adequately explained. This phenomenon may be of fundamental importance and requires further investigation.

3) In Pregnancy

Pregnancy is a physiological condition where a state of aldosteronism exists (332-335). Renin activity in the plasma of normal pregnant women is increased significantly (144). Genest, De Champlain and coworkers (179, 180) reported a gradual increase of plasma renin activity during the first two trimesters of pregnancy, and they found that renin activity did not increase further, whereas Brown and coworkers (178) found a high level of renin from the beginning of pregnancy. Following delivery, plasma renin activity decreases to normal within 2 to 4 days (336).

Brown and coworkers (337) measured plasma renin in various hypertensive diseases of pregnancy and found an increased level, above the normal pregnancy range, only in the cases associated with hydatidiform mole or rhesus incompatibility. In contrast, Maebashi and coworkers (338) found higher values than normal in pre-eclampsia, but in further

studies these workers showed identical values in pregnant and non pregnant normal subjects. Hodari and coworkers (181) found a significantly higher plasma renin in gravid dogs compared to normal. After experimental production of hypertensive disease of pregnancy, plasma renin activity decreased towards normal values (181).

The placenta acts as an important arterio-venous shunt in the circulation of the mother and could explain a decreased renal perfusion pressure despite the increased plasma volume, and consequently the secretion of renin by the juxtaglomerular apparatus. But renin-like substances were found in the placenta of cats (339) and rabbits (103) as well as in the rabbit uterus (103). This finding was confirmed in placentas from normal women and toxemic patients (340). In both cases the renin-like content was comparable. Identical results were encountered with placentas from normal gestating and toxemic dogs (181). A high concentration of renin was found in the amniotic fluid and it was generally greater than in maternal and umbilical vein plasma (341). The ratio of uterine to femoral vein renin activity is increased in normal gestating and toxemic dogs (181). These findings suggest a fetal, uterine or placental origin of maternal renin. Further evidence favoring this suggestion was provided. Hodari (342) injected renin in the umbilical vein of a fetus within the intact amniotic sac of a normal gestating animal. Following the injection he found a higher renin output in the uterine vein.

In gravid rabbits, Gorden, Ferris and Mulrow (343) showed that DCA and salt load suppressed the renin content of kidney and plasma but not the renin content of uterus. They also demonstrated that the plasma renin activity and the renin content of uterus was not affected by nephrectomy. Ferris and coworkers (344) measured the renin content of gravid rabbit kidneys and uterus. They found a comparable concentration in these two organs.

Although renin has been found in organs other than kidney, it is not clear whether the renin sequestered in these organs is reactive or available for systemic use. If renin is produced and released by extrarenal sites such as uterus, it could tentatively be theorized that smooth muscle cells can produce renin without storing it under the form of granules such as it is encountered in juxtaglomerular cells. These extrarenal sites may also represent only storage sites in an organ having the capacity to concentrate renin. The fetal kidney contribution to uterus, placenta, amniotic fluid and maternal circulating renin, is also not excluded in these investigations.

In mouse, Ruyter (104) described the appearance of juxtaglomerular cells about one week after birth and a good differentiation in mice 2 or 3 weeks old. Friedberg (201) showed that the adult distribution of juxtaglomerular granulations was achieved in mice between the second week and the first month of extra-uterine life. Initially, the granules appeared mainly in the juxtamedullary zone of the kidney.

Similarly, Dauda and Endes (345) were not able to demonstrate the presence of juxtaglomerular granulated cells in rats before the 14th day of extra-uterine life, and they observed a progressive rise in total juxtaglomerular index between the 14th and 60th day of life. Despite the absence of juxtaglomerular cell granules, renin was found in newborn hog kidneys (204). Hodari and coworkers (181, 342) detected a measurable amount of renin in normal newborn dog kidneys. Furthermore, he showed that the renin content of the kidney was much higher in fetuses from gravid dogs with hypertensive disease produced by chronic uterine ischemia than in those from normal gravid dogs. These data are by far too incomplete to assess any role of the renal renin during intra-uterine and early extra-uterine life.

III - INVESTIGATIVE SECTION

CHAPTER I - METHODS

A - CIRCULATING ANGIOTENSIN

Circulating arterial angiotensin was measured by the procedure of Boucher et al (10). Fifty to 75 ml of blood were sampled from the femoral artery and rapidly (4-8 seconds) cooled to 0-5° C. Blood was drawn on EDTA (1 ml of 15% solution of the ammonium salt of EDTA/50 ml of blood). After centrifugation at 0-5° C, 30 ml of plasma were adjusted to pH 6 and the sample transferred to a Dowex 50W-X2 (NH_4^+) resin column equilibrated at pH 6 and maintained to 0-5° C. After washing with 15 ml of ammonium acetate (0.2 N, pH 6), 20 ml of acetic acid (10% v/v) and 20 ml of distilled water, the angiotensin was eluted with 25 ml of 0.1 N diethylamine and 25 ml of 0.2 N ammonium hydroxide into a conical flask already containing concentrated acetic acid. After evaporation and sublimation, the fraction obtained was chromatographed on paper. The ascending chromatographic separation was achieved with a solution of n-butanol-water-acetic acid (45:50:10). The strips were eluted with a mixture of ethanol (95%)-water-hydrochloric acid (1 N) (500:495:5), evaporated to dryness and assayed in nephrectomized rats. The results were expressed in ng per 100 ml of plasma (ng%).

B - PLASMA RENIN ACTIVITY

1) Macrotechnique

Plasma renin activity was measured on 10 ml of plasma

according to Boucher's procedure (10). Blood was sampled from a peripheral vein in the same manner as for determination of circulating angiotensin. After centrifugation at 0-5° C, the plasma was adjusted to pH 5.5 by the slow addition of 1 N hydrochloric acid. After filtration, 10 ml of plasma were incubated for 3 hours at 37° C in presence of 4 ml of moist Dowex 50W-X2 (NH_4^+) resin. The angiotensin produced during the incubation was absorbed on the resin. After incubation, the mixture was transferred to a glass column already containing 1 ml of Dowex resin. The column was washed successively with 15 ml of ammonium acetate (0.2 N, pH 6), 20 ml of acetic acid (10%) and 15 ml of distilled water. Finally, angiotensin was eluted from the column with 15 ml of diethylamine (0.1 N) followed by 15 ml of ammonium hydroxide (0.2 N). The eluate was collected in a conical flask already containing 0.5 ml of concentrated acetic acid. After evaporation and sublimation the dry residue was dissolved in a 20% solution of aqueous ethanol and assayed on nephrectomized rat. The results were expressed in ng of angiotensin formed per liter of plasma per minute of incubation (ng/L/min.).

2) Microtechnique

The method for the determination of renin activity in 1 ml of dog plasma, renal renin content, substrate concentration and capacity of plasma to generate angiotensin was developed by the author and will be reported in detail in chapter III.

C - URINARY ALDOSTERONE

Urinary aldosterone excretion was determined in patients with essential hypertension by the method of Nowaczynski, Koiw and Genest (346). This method involves the purification of aldosterone in three different chromatographic systems. The determination of aldosterone is made with the isonicotinic hydrazide reaction and ultraviolet light absorption at 240 mμ. Results are expressed in μg/24 hours and the normal values range between 2 and 12.

D - JUXTAGLOMERULAR INDEX

Kidney slices about 3 mm in thickness were fixed in Helly's fluid and stained by the Bowie technique as modified by Ortega (210). The juxtaglomerular index was measured semiquantitatively by the method of Hartroft and Hartroft (347).

E - ELECTROLYTES

Sodium and potassium were measured in urine and peritoneal dialysate by flame photometry (Unicam Model SP-900).

CHAPTER II - PLASMA RENIN ACTIVITY AND INCIDENCE OF ADRENOCORTICAL ADENOMAS IN HYPERTENSIVE PATIENTS

In the differential diagnosis of hypertension associated with hyperaldosteronism, the determination of plasma renin activity has become an important diagnostic tool. Conn (11) recently stressed the interest of such determinations to detect "normokalemic" primary aldosteronism among patients with "essential hypertension". In order to appreciate the incidence of this syndrome, we have studied the variations of plasma renin activity in 60 unselected patients with "essential hypertension" under upright posture and by severe sodium restriction, and the incidence of adrenocortical adenoma in hypertensive and normotensive patients examined at autopsy room.

A - STUDY OF THE VARIATIONS OF PLASMA RENIN ACTIVITY IN ESSENTIAL HYPERTENSION AND PRIMARY ALDOSTERONISM SYNDROME

1) Materials and Procedures

The variations of the plasma renin activity were studied in 8 normotensive subjects and 60 patients with essential hypertension in response to upright posture and low sodium intake. The age of the normal subjects ranges between 17 and 32 years, except for one who was 52. Essential hypertension was diagnosed after elimination of known causes and was based upon the following criteria;

- Diastolic blood pressure above 90 mm Hg.
- Absence of retinopathy.
- Renal function within the normal limits.
- Normal intravenous pyelography and renal angiogram.

- Natremia, kalemia and total CO_2 within the normal limits.

Most of the patients were not taking any medication before the investigation. Otherwise, treatment was stopped at least 15 days prior to the investigation. The patients and normal subjects were maintained on a standard diet (135 mEq of sodium and 90 mEq of potassium per day) for 3 days at least prior to the study. On the morning of the fourth day, before arising (between 8 and 9.30 AM) fasting peripheral blood sample was taken for determination of the plasma renin activity. A second sample of blood was taken 4 hours after the patients were ambulant. Thirty of these patients were then given for at least 3 days, a low-sodium diet consisting of 10 mEq of sodium and 90 mEq of potassium per day. At the end of this period, the determination of peripheral plasma renin activity was repeated in similar conditions as those described above (recumbent and after 4 hours in upright posture). One determination was obtained in each patient when he was recumbent and under normal sodium intake. At least another determination was done in each patient under one of the above mentioned conditions, known to stimulate the release of renin. Fifteen determinations of the urinary excretion of aldosterone were obtained in 9 patients after 3 days at least of normal sodium intake (135 mEq sodium and 90 mEq of potassium per day). Values for plasma sodium, plasma potassium and fasting blood glucose were obtained during the hospitalisation in the patients with essential hypertension.

2) Results

In 8 normotensive subjects, the mean plasma renin acti-

vity was 9.8 ng/L/min (± S.D. 12.9) and was increased by upright posture to 31.3 ng/L/min (± S.D. 17.5). After three days of low sodium intake, the mean plasma renin activity was 18 ng/L/min (± S.D. 2.7) in recumbent posture and 48 ng/L/min (± S.D. 18) after 4 hours in upright posture.

Of the 60 patients with essential hypertension, 34 had plasma renin activity responses to upright posture and low sodium diet similar to the group of normotensive subjects. In the other group of 26 patients, plasma renin activity in recumbent position and normal sodium intake was completely or almost completely suppressed. This last group was subdivided into two sub-groups according to the presence or absence of stimulation by upright posture and low sodium intake. This stimulation was partial in 12 patients and very low or absent in the other 14. In a given condition of stimulation (v.g. upright posture and normal sodium intake), the values of each group or sub-group were statistically different from the other, except when comparing the two sub-groups of patients with a partial or complete suppression of plasma renin activity under normal sodium intake and recumbent position (Table I).

A slightly older age with a lower serum potassium and higher blood sugar were observed in the group of patients with essential hypertension in whom plasma renin activity was totally suppressed. But these variations were not statistically significant when compared to the two other groups (Table II).

Urinary aldosterone excretion was measured in four patients who responded to stimulation and in five patients,

TABLE I - Plasma renin activity in response to posture and sodium restriction in 8 normotensive subjects and 60 patients with essential hypertension.

	PLASMA RENIN ACTIVITY (ng/L/min) Na Intake			
	135 mEq/day+		10 mEq/day+	
	Recumbent	Upright	Recumbent	Upright
NORMOTENSIVES	9.8 12.9 (8)	31.3 17.5 (7)	18.0 2.7 (7)	48.0 18.0 (6)
PATIENTS WITH ESSENTIAL HYPERTENSION				
1) STIMULATION	10.9 11.5 (34)	22.0 14.1 (31)	23.8 13.6 (16)	42.4 20.6 (16)
2) SUPPRESSION				
a) partial	2.0 2.7 (12)	7.5 3.7 (6)	6.2 6.0 (8)	16.0 6.4 (7)
b) complete	1.0 1.8 (14)	1.4 2.3 (13)	0.8 1.6 (9)	7.4 6.8 (8)
PRIMARY ALDOSTERONISM (patient R.B.)	0	0	0	6.4

+ : K Intake : 90 mEq/day.

Mean plasma renin activity followed by standard deviation.

Figures in brackets represent the number of determinations.

TABLE II - Age, plasma sodium and potassium, and fasting blood glucose in patients with "essential hypertension.

	Mean age	Mean plasma		Mean fasting Blood Glucose (mg%)
		Na (mEq/L)	K (mEq/L)	
PATIENTS WITH ESSENTIAL HYPERTENSION				
1) STIMULATION	37.7	142.2	4.21	95.0
	11.7	9.7	0.46	12.3
2) SUPPRESSION				
a) partial	39.0	142.9	4.08	94.5
	9.4	2.4	0.31	13.9
b) complete	45.0	143.3	4.05	107.9
	11.6	4.1	0.55	37.1

Mean values with standard deviation.

with suppression of plasma renin activity (Table III). In 2 out of the five patients, unresponsive to sodium restriction and posture, aldosterone excretion was above normal range (Table III, patients R.R. and V.H.).

Two patients of the sub-group presenting a complete suppression of peripheral plasma renin activity were submitted to an adrenal exploration. The first one (V.H.) was a 55-year-old female known to be hypertensive since 1954. In 1961, she was noted to have hypokalemia, aggravated by the administration of natriuretic agents. She had a normal renal arteriography and urinary aldosterone excretion was 21 and 30 µg/24 hours (Figure 1).

She was admitted for evaluation of the renin-angiotensin system (Figure 2). With a normal sodium intake (135 mEq per day), the peripheral plasma renin activity was 2 ng/L/min in recumbent position and showed no or only minimal response to upright posture (4 ng/L/min). On low sodium diet (10 mEq/day), the plasma renin activity was 0 when recumbent and remained low after 4 hours of ambulation (4 ng/L/min). With a standard diet (135 mEq Na and 90 mEq K/day) she was given 150 mg of spironolactone (Aldactone A) per day during 15 days and 20 mEq potassium per day during 13 days. The plasma renin activity in recumbent position increased progressively to 5 ng/L/min after 6 days of treatment and 7 ng/L/min after 12 days.

At operation, the adrenal glands in situ showed no gross abnormalities. It was then decided to excise the left

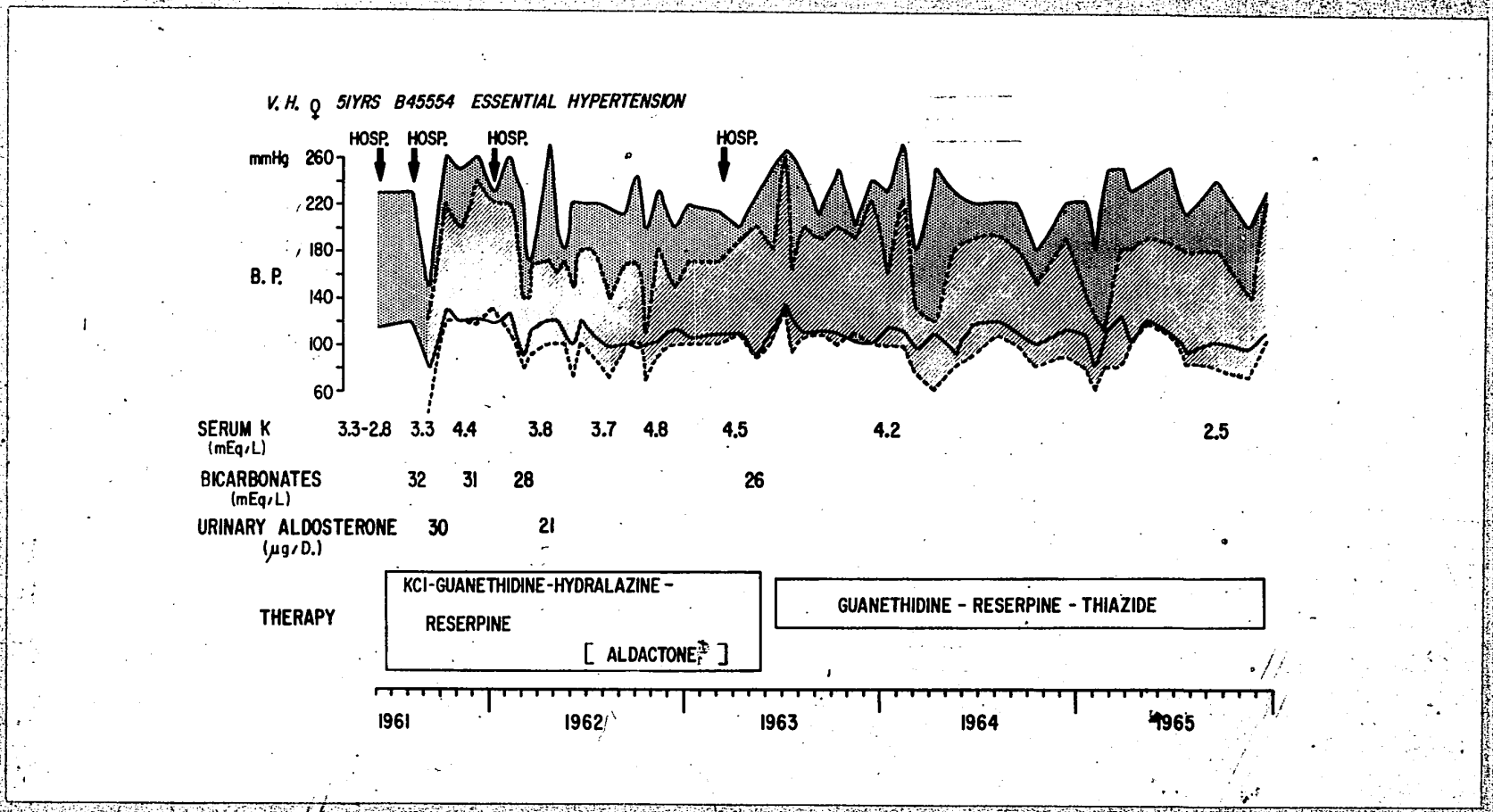


Figure 1 - Follow-up of a 51 year old patient (V.H.) for a period of 4 1/2 years.

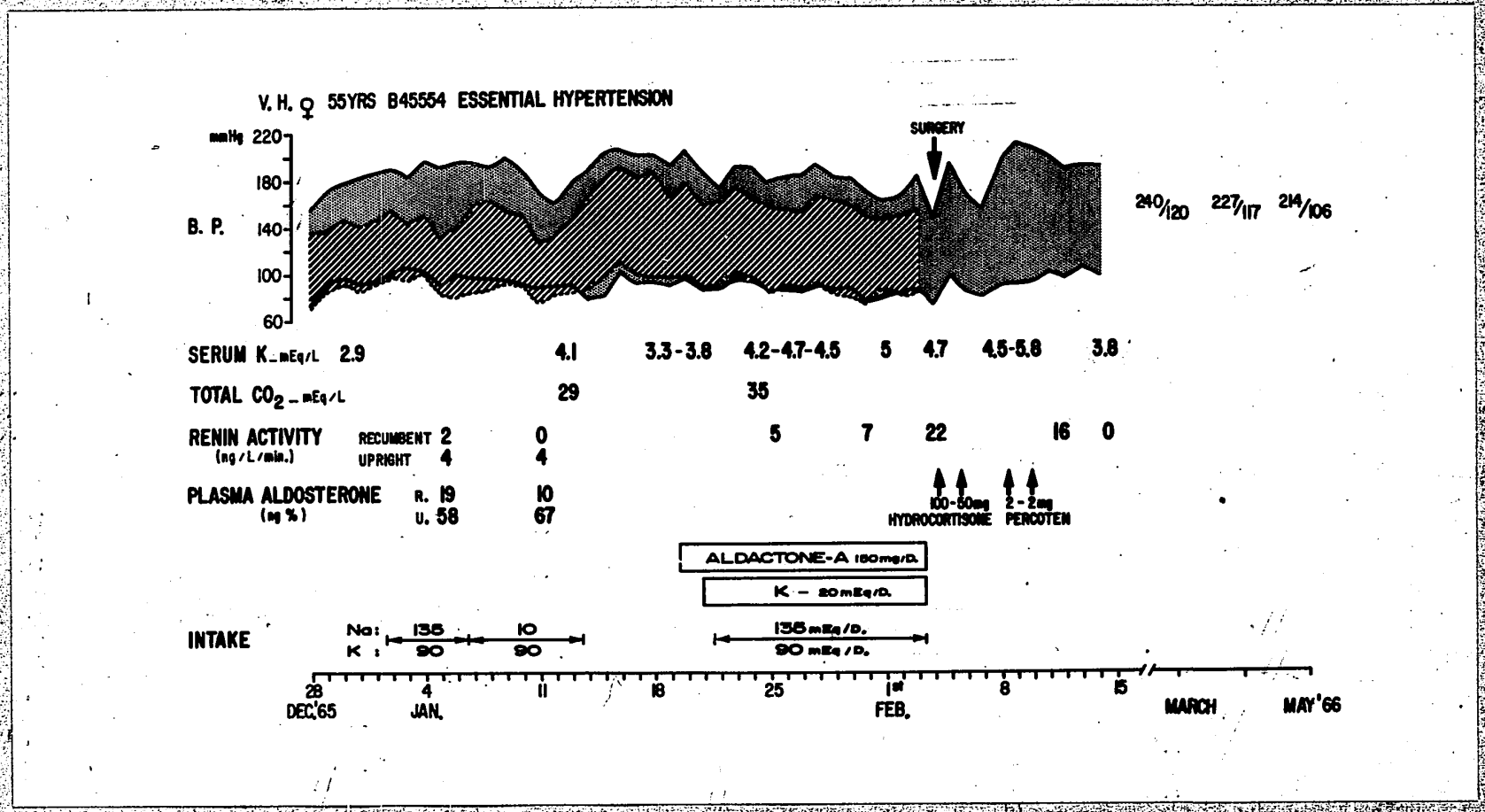


Figure 2.- Effects of posture, sodium restriction and spironolactone on plasma renin activity in a patient (V.H.) with "essential hypertension".

TABLE III - Plasma renin activity and Urinary Aldosterone Excretion (UAE) in patients with "essential hypertension".

Patient	Age	Sex	PLASMA RENIN ACTIVITY (ng/L/min) Na Intake				UAE++ µg/day
			135 mEq/day+		10 mEq/day+		
			Recumbent	Upright	Recumbent	Upright	
L.M.	25	F	15	38	32	29	10 13 11 15
M.R.	28	F	5	12.5	24	-	7
M.S.	50	F	0	-	0	65	8 7.5
S.L.C.	21	F	7.8	46.7	-	-	13
R.R.	25	F	0	2.6	0	10.3	23
C.S.	38	M	0	0	0	11	9 11
R.V.	35	F	0	0	3.5	-	10
R.C.	63	F	0	-	0	20	6
V.H.	55	F	2	4	0	4	30 21

+ : K Intake : 90 mEq/day.

++ : The urinary aldosterone was determined after three days, at least, of normal sodium diet (135 mEq Na and 90 mEq K/day).

adrenal. No tumor was demonstrated on this side but an increased thickness of the cortex as well as several micronodules were seen. Seventy per cent of the right adrenal was also removed (Figure 3). Bilateral kidney biopsies were also taken and showed a marked renal arteriolosclerosis. The Bowie coloration of the juxtaglomerular apparatus showed an absence of granular cells.

The surgical treatment failed to lower the blood pressure and the peripheral plasma renin activity measured in two instances within two years after the operation remained suppressed. These values were obtained without dietary control.

The second patient (L.L.) was a 62-year-old female treated with antihypertensive medication since 1954. Serum potassium was found to be normal. Two determinations of plasma renin activity under normal sodium intake in 1964 showed a suppression. The plasma renin activity was determined twice afterwards on an out-patient basis between 1964 and 1966. Since plasma renin was still suppressed, the patient was admitted for investigation under standard conditions (Figure 4). Plasma renin activity was suppressed and showed only minimal response to upright posture on low sodium diet (10.6 ng/L/min).

On surgical exploration, the adrenal glands were macroscopically normal. The left adrenal was excised and minute sections were done. The right one was also partially removed (60%). Both glands were normal, except for the finding of an extracapsular nodule of 2.5 mm in diameter, close to the

Figure 3 - Cross-section of right and left adrenals from a patient (V.H.) with "essential hypertension".

Figure 3 - Cross-section of right and left adrenals from a patient (V.H.) with "essential hypertension".

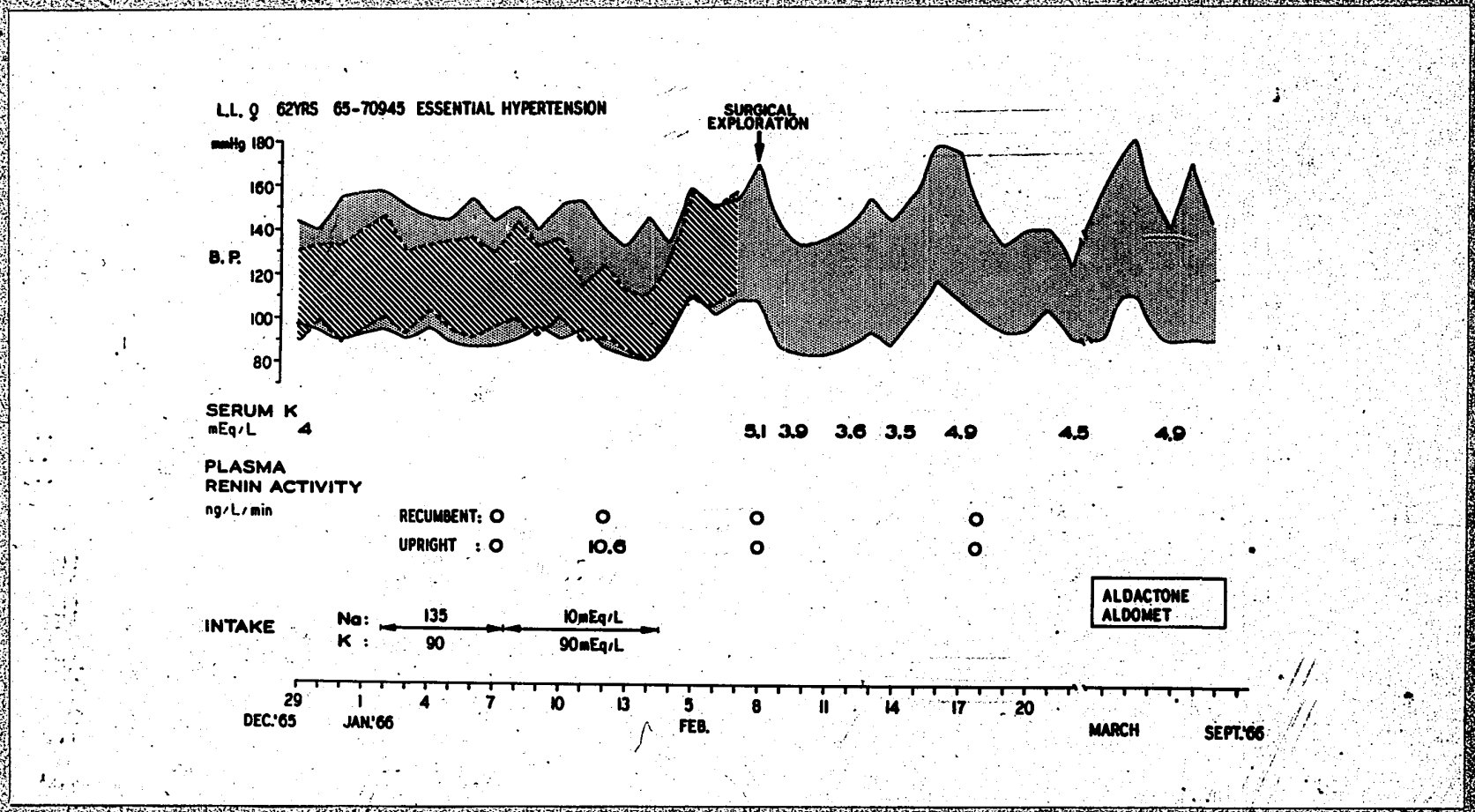


Figure 4 — Effects of posture and sodium restriction on plasma renin activity in a patient (L.L.) with "essential hypertension".

surgical section on the right adrenal (Figure 5). The histological examination revealed that both glands were normal and that the micronodule was encapsulated and contained all the normal three zones encountered in the adrenal cortex. A bilateral renal biopsy showed marked renal arteriolosclerosis and a degranulation of the juxtaglomerular apparatus. The plasma renin activity of the left renal vein prior to adrenalectomy was completely suppressed.

On the ninth post-operative day, while the patient was on a regular diet, the plasma renin activity was also suppressed. No improvement in the blood pressure was encountered and the plasma renin activity measured 4 and 8 months postoperatively remained completely suppressed.

For comparison, the response of a patient with Conn's syndrome to upright posture and sodium restriction are given in Table I. He was a 32-year-old (R.B.) male admitted for hypertension discovered 11 months previously. Headaches, paresthesias, and nocturia were also part of the picture. An hypokalemic alkalosis was found. The glucose tolerance curve was abnormal and the plasma volume was increased. The renal function was within the limits of normal. The renal angiogram was also normal.

Under normal sodium intake (135 mEq Na/day), the plasma renin activity and the arterial circulating angiotensin were completely suppressed in recumbent as well as in upright posture. Under sodium restriction, (10 mEq Na/day), the plasma renin activity remained suppressed in recumbent position and

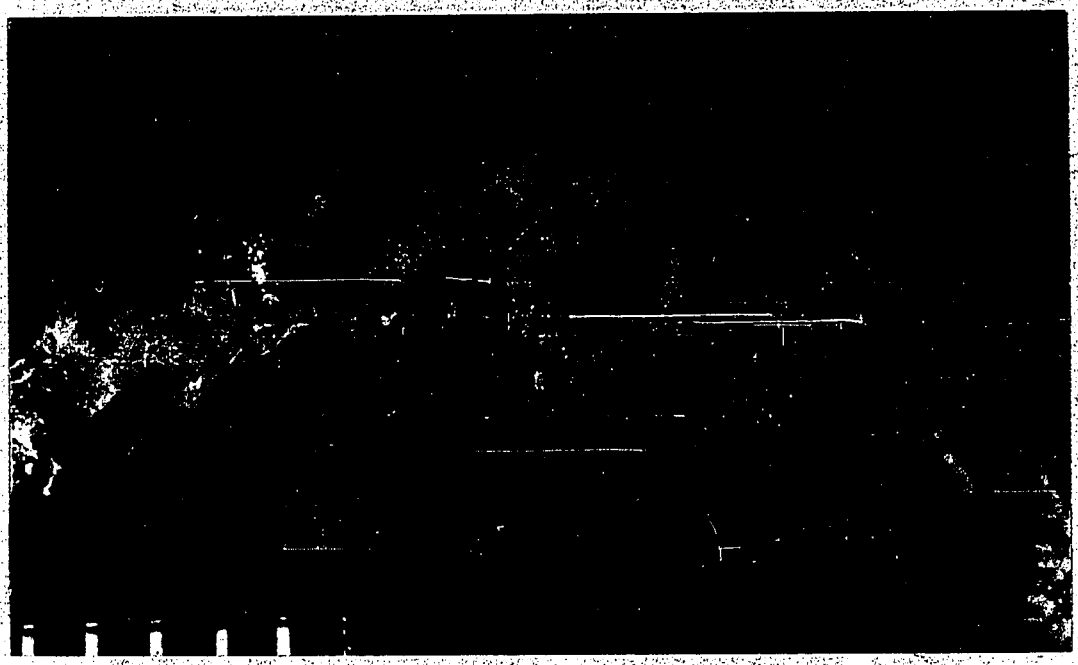


Figure 5 - Cross-section of right adrenal gland from a patient (L.L.) with "essential hypertension".

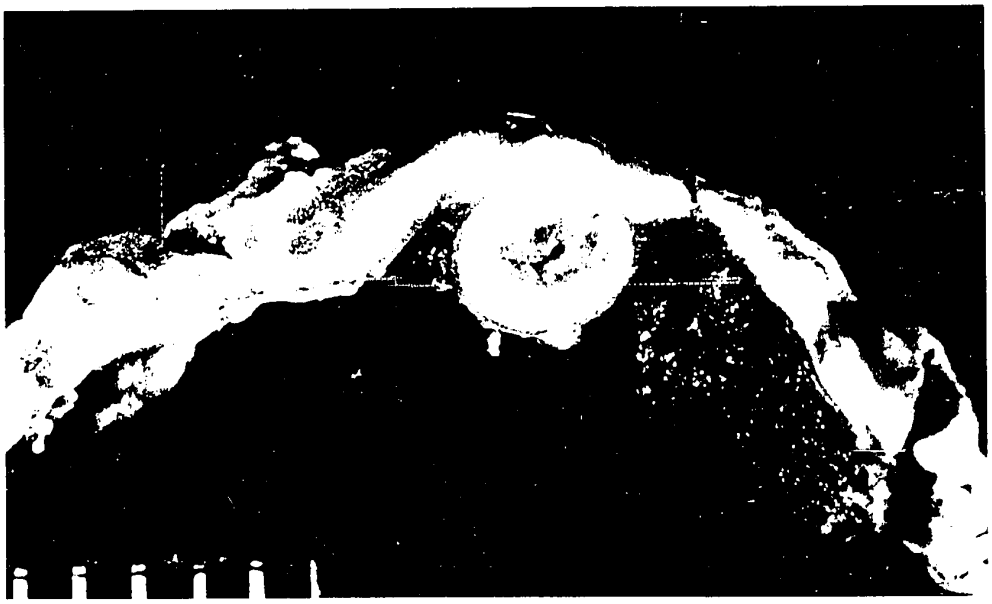
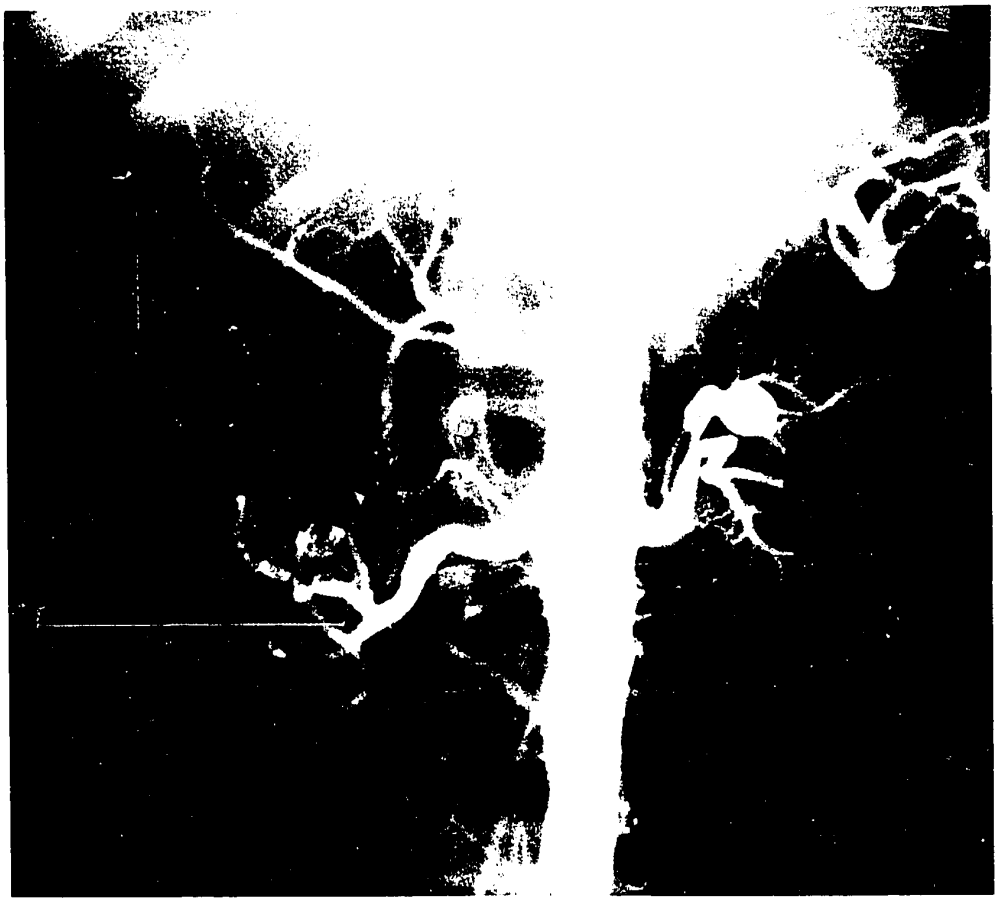


Figure 5 - Cross-section of right adrenal gland from a patient (L.L.) with "essential hypertension".

a detectable level (6.4 ng/L/min) was measured 4 hours after assuming upright posture. Under normal sodium intake, the urinary aldosterone excretion was 23 µg/day. The patient was cured after the removal of the left adrenal gland in which a typical adrenocortical adenoma of 2 cm by 1.5 cm was found. Bilateral renal biopsies were normal and a complete absence of granular cells in juxtaglomerular apparatus was observed.

Beside these studies of the variations of plasma renin activity in essential hypertension, we have investigated an unusual case of a 24-year-old patient (J.C.) suffering from hypertension, hypokalemic alkalosis and nocturia since five months. The administration of natriuretic agents (chlorthalidone) induced tetanic crisis with carpo-metacarpal spasms and a positive Trousseau. Spontaneous tetany occurred twice long after the medication was stopped. In two instances during the hospitalisation, periods of 40 to 50 seconds of hyperventilation resulted in tetany. The renal angiogram showed a tight intra-hilar renal artery stenosis (decreasing the lumen diameter by 75%) with post-stenotic dilatation, located on the superior branch of the left renal artery (Figure 6). Repeated determinations of serum calcium were normal. The renal function was also normal. The urinary aldosterone excretion was 34,26, and 27 µg/24 hours. The plasma volume determined with iodine ^{131} tagged albumin (RISA) was also elevated (44.3 and 51.9 ml/Kg compared to a normal value of 37 ml/Kg for the patient body weight).

Blood from each renal vein showed a complete suppression of the plasma renin activity. The peripheral plasma renin activity, as well as the circulating arterial angiotensin,



[illegible] - [illegible] form of perfect [illegible].

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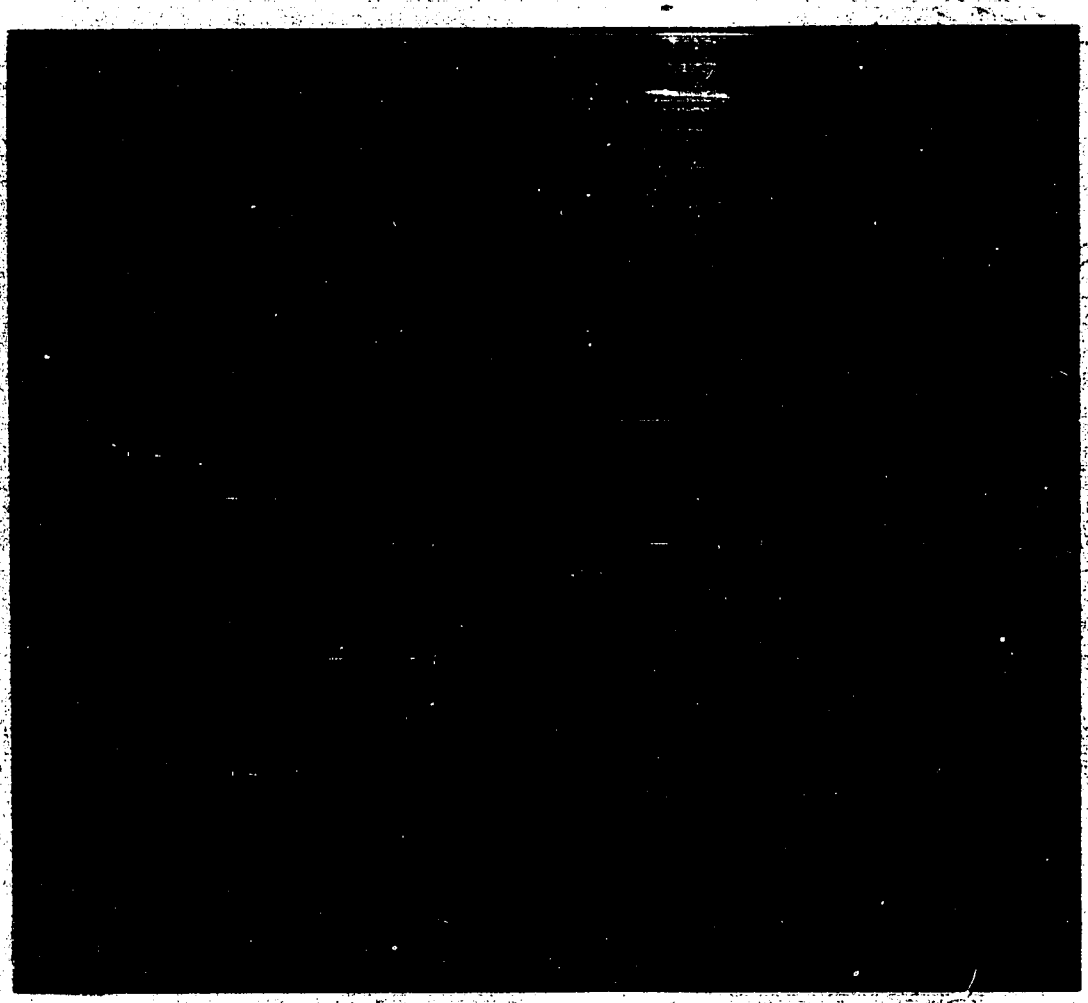

Figure 6 - Renal angiogram of patient J.C.

A tight intra-hilar stenosis of the superior branch of the left renal artery with post-stenotic dilatation was observed. An arterio-venous fistula of about 0.5 cm in diameter was also found in the superior pole of the right kidney.

were suppressed under normal sodium intake (135 mEq Na/day) and were not stimulated by assuming upright posture and by sodium restriction (10 mEq Na/day).

At the laparatomy, a biopsy of both adrenal glands (15%) and of the right kidney were done and the left kidney was removed. The adrenals were macroscopically and microscopically normal. The Bowie coloration of the juxtaglomerular apparatus showed the absence of granular cells. The surgical treatment cured her hypertension and biochemical disturbances. This cure has persisted now for an 18-month follow-up.

B - AUTOPSY STUDY OF ADRENALS IN NORMOTENSIVE SUBJECTS AND IN HYPERTENSIVE PATIENTS

1) Materials

The records of 2425 consecutive autopsies from Hotel-Dieu Hospital of Montreal done between January 1st, 1955, and January 1st, 1965, were studied. These autopsied patients were classified as hypertensive upon one of the following criteria by order of importance:

1. Established diagnosis of hypertension.
2. Pre-mortem systolic pressure reading over 140/90.
3. Pre-mortem systolic blood pressure reading of 160 or more and diastolic blood pressure of less than 90 when the heart weight was over 350 grams for men and over 300 grams for women.
4. When no pre-mortem blood pressure reading was available, or when the patient was admitted in shock, he was considered hypertensive if the heart weight was exceeding 400 grams for men and 350 grams for women. (In less then 7%

of the cases, it was necessary to use heart weight as the determining factor).

All the histological slides of the cases of adrenal cortical adenoma, nodules, hyperplasia, or combinations of these lesions provided by the records were examined. Most of these sections were stained with hematoxilin-Eosin, and at least two slides were obtained from the adrenals of each patient. Special types of hypertension such as Cushing's syndrome and pheochromocytoma were excluded from this series.

After histological examination of the available materials, only the cases presenting one of the following lesions were considered:

- Adrenal adenomas. Adenomas were defined as encapsulated spherical or ovoid homogeneous tumors of the adrenal cortex tissue, over 0.8 cm in diameter, without identifiable zonation composed of cords of cells. Microscopically, some of the cells contained in the adenoma were of a clear type with vacuolated cytoplasm and the architecture resembled that of the zona fasciculata, Scattered throughout the tumor were small groups of giant cells.

- Nodules. The same type of lesions found, located either in the cortex or the capsule, but with a diameter inferior to 0.8 cm were defined as nodules. The "nodules" presenting an architectural orientation resembling the normal adrenal cortex with a clear zonation into a glomerulosa, fasciculata and reticularis zones, were excluded since they can be found in normal humans with a high frequency and their meaning is controversial (289, 290). A clear delineation between adenomas and nodules is missing. We have arbitrarily

chosen 0.8 cm based on Conn's report (265) that in only 4 out of 54 cases of "hypokalemic" primary aldosteronism the adenoma size was ranging from 0.5 to 1 cm.

- Hyperplasia. The glomerulosa was considered hyperplastic when, by simple microscopical observation, there was evidence of increased thickness.

2) Results

a) Incidence of hypertension at autopsy room.

The autopsy records of the Hotel-Dieu Hospital of Montreal (1955-1965) provided 2425 cases of which 55.8% were male. The mean age was 57.1 years (± S.D. 15.1) with extreme ranges from birth to 87 years old. According to the diagnostic criteria of hypertension, 45.5% of the male and 42.7% of the female patients were hypertensive. Both male and female hypertensives represented 44.2% of the whole group and the mean age of the hypertensives was 58.2 years (± S.D. 13.8). The data of autopsy material are illustrated in the following table.

	Autopsied patients	Hypertensive patients	
		number	percentage
Male	1354	617	45.5%
Female	1071	457	42.7%
Total	2425	1074	44.2%

b) "Abnormal" adrenals

Adenomas, nodules or hyperplasia were found in 96 autopsied patients, (mean age : 59.7 ± S.D. 12.8) 75 of whom were hypertensive (Table IV). Of these 75 hypertensive patients, 41 were male and 34 were female (Table V). The incidence of "abnormal" adrenals in the hypertensive population was 7% and they were more frequent among hypertensive females (7.4%) than hypertensive males (6.6%). The prevalence among the normotensive population was much less (1.9%).

c) Adenomas

The most frequent abnormality encountered was the presence of single or multiple adenomas. They were found in 61 patients, 45 of whom were hypertensive. The incidence of these single or multiple adenomas in the hypertensive population was 4.2% compared to 1.1% in the normotensive one. They were also more frequent in the hypertensive females (4.4%) than in hypertensive males (4.2%).

d) Nodules

The findings of nodules in autopsied patients was a rare event compared to adenomas. Single or multiple nodules were observed in 14 hypertensive patients and in only one normotensive. Nine of the hypertensive patients with nodules were female and five male.

e) Mixed hyperplasia

The coexistence of hyperplasia (mixed hyperplasia) with single or multiple adenomas or nodules was observed

in 13 patients out of 76.

f) Hyperplasia

Hyperplastic glands were found in 16 hypertensive patients. Their incidence among the hypertensive population was low (1.4%) and almost identical to that observed for nodules (1.3%).

Tables IV and V give further details respectively about the incidence of "abnormal" adrenals in normotensive and hypertensive patients, and their sex distribution.

C - DISCUSSION

1) Essential Hypertension

The variations of plasma renin activity obtained in unselected patients with essential hypertension showed a complete suppression in 23.3%. This proportion was similar to that reported by Conn (11) for the incidence of "normokalemic" primary aldosteronism. We were inclined to consider the group of patients with a partial suppression as normal, since the percent stimulation was similar to that obtained in normal subjects or stimulated hypertensive patients. This sub-group differed only by low basal level of renin activity in recumbent position under a "normal" salt intake. The study of one case of primary aldosteronism confirmed the suppression (172-174, 147) and the absence of stimulation of plasma renin activity encountered in that disease (174).

Aldosterone excretion was found to be normal in four patients with normal renin activity and elevated in 2 out of 5 patients with essential hypertension and suppressed

TABLE IV - Incidence of "abnormal"[+] adrenals in hypertensive patients vs normotensive patients at autopsy during 10 years (January 1955 to January 1965).

	"Abnormal" Adrenals	Single or multiple		Hyperplasia	
		Adenoma (>0.8cm)	Nodule[+++] (<0.8cm)	mixed[++]	alone
Hypertensive Patients	75	45	14	12	16
Normotensive Patients	21	16	1	1	4
Total	96	61	15	13	20
% of incidence in hypertensive patients	7.0	4.2	1.3	1.1	1.4
% of incidence in normotensive patients	1.9	1.1	0	0	0.3

[+] : "Abnormal" means the presence of adenoma, nodule or hyperplasia.

[++] : Hyperplasia was found concomitantly with single or multiple adenomas or nodules.

[+++] : See definition of nodules.

TABLE V - Sex distribution of "abnormal"+ adrenals found at autopsy.

	"Abnormal" Adrenals	Single or multiple		Hyperplasia	
		Adenoma	Nodule+++	mixed++	alone
MALE					
Normotensive Patients	12	10	0	1	2
Hypertensive Patients	41	25	5	9	11
Total	53	35	5	10	13
FEMALE					
Normotensive Patients	9	6	1	0	2
Hypertensive Patients	34	20	9	3	5
Total	43	26	10	3	7

+ : "Abnormal means the presence of adenomas, nodules or hyperplasia.

++ : Hyperplasia was found concomitantly with single or multiple adenomas or nodules.

+++ : See definition of nodules.

renin activity. In these patients with normal aldosterone excretion, the suppression of plasma renin activity remains difficult to explain.

In an attempt to characterize the different groups of hypertensive patients, we compared the mean values of natremia, kalemia and glycemia. A minimal tendency towards hyperglycemia and hypokalemia was encountered from stimulated to completely suppressed hypertensives. But these values were not statistically different.

In the two patients with essential hypertension and totally suppressed renin activity, the surgical exploration diclosed a micro-nodular adrenal in the first patient (V.H.) and an extracapsular nodule presenting an architectural orientation resembling the normal adrenal cortex in the second one (L.L.). This last nodule was of the type we have excluded from the autopsy study. In both patients, the adrenalectomy failed to improve the hypertension and to increase the level of renin activity in the blood. The persistence of hypertension could be explained by the arteriolosclerotic lesions due to long-standing hypertension.

The suppression of plasma renin activity and arterial angiotensin and an increased aldosteronuria were also observed in a young female patient with severe renal artery branch stenosis. The clinical picture suggested the diagnosis of Conn's syndrome. The adrenals were normal and the 18-month follow-up excluded the possibility of an heterotopic adrenal adenoma. The physiopathology of the renin angio-

tensin system, in our present state of knowledges, cannot explain the pathogenesis of this unusual case.

2) Autopsy Material

In the autopsied patients, the incidence of hypertension was important (44.2%). This prevalence was probably related to the age of these patients. The incidence of hypertension varies with the diagnostic criteria used (288, 348). But using a pressure of 140/90 or over as a demarcation line between normotensive and hypertensive patients, similar incidence was reported (291, 348).

The incidence of "abnormal" adrenals (7%) in our study is inferior to the figure of 20% obtained for adenoma only by Shamma et al (290) in adrenalectomized and autopsied patients. In our study, only 5.5% of the hypertensive patients at autopsy room disclose an adenoma or a nodule. This percentage is close to the evaluation of an incidence of 2 to 8% of "normokalemic" primary aldosteronism, by most of the research groups interested in this matter (297, 297a). The sex distribution is in contrast with the high ratio of female to male patients (2.6 to 1) reported for "hypokalemic" primary aldosteronism (265).

We recognize the disadvantage of such a study mainly for the assessment of a diagnosis of high blood pressure on one pre-mortem reading in many patients, and for the advanced age of the patients. But, these inconveniences further support the conclusion of a low incidence of "normokalemic" primary aldosteronism.

CHAPTER III - DEVELOPMENT OF A MICROTECHNIQUE FOR DETERMINATION OF PLASMA RENIN ACTIVITY IN DOGS, AND APPLICATIONS

A - METHOD FOR ASSAY OF PLASMA RENIN ACTIVITY IN ONE MILLILITER OF DOG PLASMA

It is admitted that presently no method permits the determination of renin "concentration" in plasma or kidney. The currently used methods measure rather an "effective renin activity" which is a summation of known and unknown factors involved with the enzymatic system. Those methods are of great value in assessing the role of the renin-angiotensin system.

Boucher et al (12) have established, recently, a microtechnique for the measurement of plasma renin activity contained in 0.1 ml of rat plasma.

The application of this method to dogs is of great interest and importance. Previous findings of renin measurements in this species were based mostly on the vasopressor activity of crude plasma and yielded conflicting results.

In this chapter, we would like to describe first the study done in order to prepare a substrate for the assay of minute amounts of renin and the effect of nephrectomy and various medications on the substrate concentration in dog plasma. Secondly, we would like to report the work done in order to adapt Boucher's micromethod (12) to dogs.

1) Materials, Reagents and Apparatus

Dowex 50W-X2 resin (100-200 mesh; Baker's analyzed reagent) is first mixed with distilled water. After settling, the resulting suspension of very fine particles is decanted. The procedure is repeated 2 or 3 times until the resin settles rapidly, leaving a clear aqueous suspension. Before use, 500 g. of resin is washed with two liters of 4 N sodium hydroxide. The sodium salt of the resin is then washed with 1 liter of water and 1 liter of 2 N hydrochloric acid and again with 2 liters of water. Then 0.2 N ammonium acetate solution, pH 6, is used to wash the resin cake, until the pH of the eluate reaches 6. The final form obtained is the ammonium salt of the resin, Dowex 50W-X2 (NH_4^+). The resin is stored in 80% ethanol at 0-5° C. It is washed three to five times with distilled water just before use.

Synthetic Valine-5 angiotensin II, aspartic ß-amide (hypertensin, Ciba Company) is used as standard angiotensin. It is prepared by dissolving 0.5 mg of the synthetic angiotensin in 1 liter of a 0.9% sterile solution of sodium chloride. The standard solution is stored at 0-5° C and prepared every two weeks. In order to check the uniformity of each new solution, a blood pressure assay in 18-24 hours nephrectomized rats is performed comparing the old and the new solution.

A 15% (w/v) solution of the ammonium salt of EDTA (Ethylene-Diamine- Tetraacetic Acid) is prepared by the addition of concentrated ammonium hydroxide to EDTA. When the EDTA is

almost dissolved, the pH of the solution is adjusted to 6.5 with 1 N ammonium hydroxide and then the volume of the solution is completed with distilled water.

Three different solutions of TRIS-phosphate (0.3 M, 0.2 M and 0.15 M) are prepared by dissolving almost completely the required amount of TRIS (2-amino-2-(Hydroxymethyl)-1,3- propanediol) in distilled water. Then 0.04 ml of a 15% solution of the ammonium salt of EDTA and 0.04 ml of sodium azide 1% (w/v) solution are added for every 2 ml (in the case of 0.3 M TRIS solution) or every 3 ml (in the case of 0.2 M and 0.15 M TRIS-solutions) of the final solution. The pH of these TRIS solutions is then adjusted to 5.5 with concentrated phosphoric acid. The volume of the solution is then completed with distilled water. The solution is allowed to settle in the cold (4° C) for 2 to 4 days. The pH is checked and readjusted if necessary to 5.5 with concentrated phosphoric acid. For convenience in the text, henceforth, the solution of TRIS-phosphate buffer with EDTA and sodium azide will be denoted as TP-EN followed by the molarity of the TRIS.

Other materials and instruments used are:

- Silicone (SC 87 drifilm G.E.) in 10% carbon tetrachloride.
- Clear polystyrene plain tubes, disposable and sterile (Falcon 16 x 150 mm, 2040).
- Disposable 5 ml plastic syringes (B.D.).
- Rotary evapo-mix (Buchler Instrument).
- Freeze-dryer (Model FDC2, Thermovac Industries Co.).
- Sorvall superspeed, automatic refrigerated Centrifuge (Model RC2-B).

- Micro-syringes (Starrett Co.).
- Polyethylene tubing, size PE-10 and PE-200 (Intramedic, Clay-Adams Co.).
- Grass Polygraph (Grass Instruments) or Bird kymograph (Phipps and Bird, Inc.).
- Homogenizer (Virtis Model 45).

Plasma from severely bled, diuretic treated or normal dogs, and kidneys from humans and dogs, as well as hog renin (Lyophilized, Nutritional Biochemicals Co.) are used as sources of renin. The kidneys are frozen (at -20° C) and thawed three times and then homogenized in distilled water.

2) Procedure

a) Substrate preparation

The substrate is prepared with plasma of male dogs nephrectomized 24 hours previously after an infusion of angiotensin. This is done by a constant intravenous infusion of valine-5 angiotensin II, aspartic ß-amide (Hypertensin, Ciba, 0.5 mg/liter of 0.9% saline) at a constant rate of 200 ng/kg/min. The infusion is begun half an hour before anesthesia (by a saturated solution of α-chloralose in 0.9% saline) and continued until both kidneys are removed. Twenty-four hours later, a polyethylene catheter (PE-200) is introduced into the femoral artery up to the thoracic aorta and blood is collected on EDTA (1 ml of a 15% solution of the ammonium salt of EDTA/50 ml of blood) at 0-5° C and then centrifuged in the cold (0-5° C).

The method of substrate preparation used up to this stage is basically that described by Haas (150). As an example of the procedure, 19 g. of solid EDTA is added to 500 ml of plasma obtained from a nephrectomized dog and filtered on glass wool. The solution is adjusted to pH 8 by dropwise addition of 5 N sodium hydroxide and stirred slowly at room temperature for one hour. The solution is then cooled at 0-5° C and adjusted to pH 5.3. Then 200 g. of solid ammonium sulfate are slowly added (2.3 M). After centrifugation, the precipitate is suspended in 120 ml of distilled water and centrifuged again. The supernatant is dialyzed against cold distilled water for 18 hours. The formed precipitate is removed by filtration or centrifugation. The solution is freeze-dried and stored at -20° C. About 11 g. of substrate is obtained from such a preparation, and the quantity needed to realize condition of excess substrate is determined by incubating the freeze-dried substrate preparation in increasing amount (50 to 300 mg) in the presence of a constant quantity of dog kidney extracts. At the same time, 150 mg of substrate preparation is incubated without added renin as a control for spontaneous vasopressor substances generation.

b) Incubation mixture for plasma renin activity and renal renin content determination

The amount of freeze-dried substrate necessary to provide an excess of substrate for a minimum formation of 400 ng of angiotensin (usually 150 mg of freeze-dried substrate), is

dissolved in 2 ml of TP-EN 0.3 M or 3 ml of TP-EN 0.2 M.

The plasma to be assayed is collected in a 5 ml disposable syringe cooled at 0-5° C and containing already 0.1 ml of a 15% solution of the ammonium salt of EDTA per 5 ml of blood. The blood is then centrifuged in the cold (0-5° C). Plasma (1 ml) is placed in a disposable Falcon plastic tube and 100 µL of hydrochloric acid (0.2 N) are added slowly while shaking the tube. Then 1 ml[+] of moist Dowex 50W-X2 (NH_4^+) resin and 3 ml of substrate dissolved in TP-EN 0.2 M, pH 5.5, are added. The tube is covered with parafilm paper and incubated. When 2 ml of plasma are incubated, 200 µL of hydrochloric acid (0.2 N) are added to the plasma, and then 2 ml of substrate dissolved in a solution of TP-EN 0.3 M, pH 5.5, and 1 ml of resin are added. In all cases, whether 1 or 2 ml of plasma are incubated, the volume of the liquid incubated is always 4 ml and the final molarity of the TRIS is 0.15 M. The time of incubation is usually 12 hours, although it could be extended to 18 or 24 hours for plasma with low renin activity. The incubation is performed at 37° C with constant vigorous agitation.

For the measurement of renal renin content, the kidney is removed from a dog under light chloralose or pentobarbital anesthesia, by a flank incision. A slice of 0.5 cm width passing through the long axis of the kidney and including

[+] A 5 ml plastic disposable syringe (B.D.) is cut at the 0 mark to give the full opening of the cylinder. This syringe is used to measure the amount of resin.

the medial aspect of the pelvis is obtained, and the medullary tissue is cut out. This slice is placed in a beaker which is stoppered with parafilm paper, and then frozen at -20°C. After freezing, the tissue is left at room temperature for 30 minutes. This sequence (freezing and thawing) is repeated two more times to increase cell rupture and the amount of renin released from the tissue (23). Water (50 ml of distilled water/g. of tissue) is then added to the renal tissue which is homogenized. The solution is centrifuged at 0-5° C and a proper dilution of the supernatant is done (usually 1 g. of original kidney/100 - 300 ml of distilled water). Then 100 µL of this crude extract is added to an excess of dog substrate dissolved in 3 ml of TP-EN 0.15 M pH 5.5, and 1 ml of Dowex 50W-X2 (NH_4^+) resin and incubated for one hour at 37° C, pH 5.5.

When either plasma or kidney extracts are incubated (see Figure 7) a constant vigorous agitation is provided by a Rotary evapo-mix (Buchler Instrument). If incubations have to proceed for 12 hours, they may be started at the end of the afternoon. The enzymatic reaction is stopped by cooling to 0-5° C. This is done by an automatic system in which a timer stops the agitation and heating of the incubation mixture and starts circulation of water at 0-5° C until processing the morning after.

c) Elution of Angiotensin formed

After incubation, the mixture is transferred to a

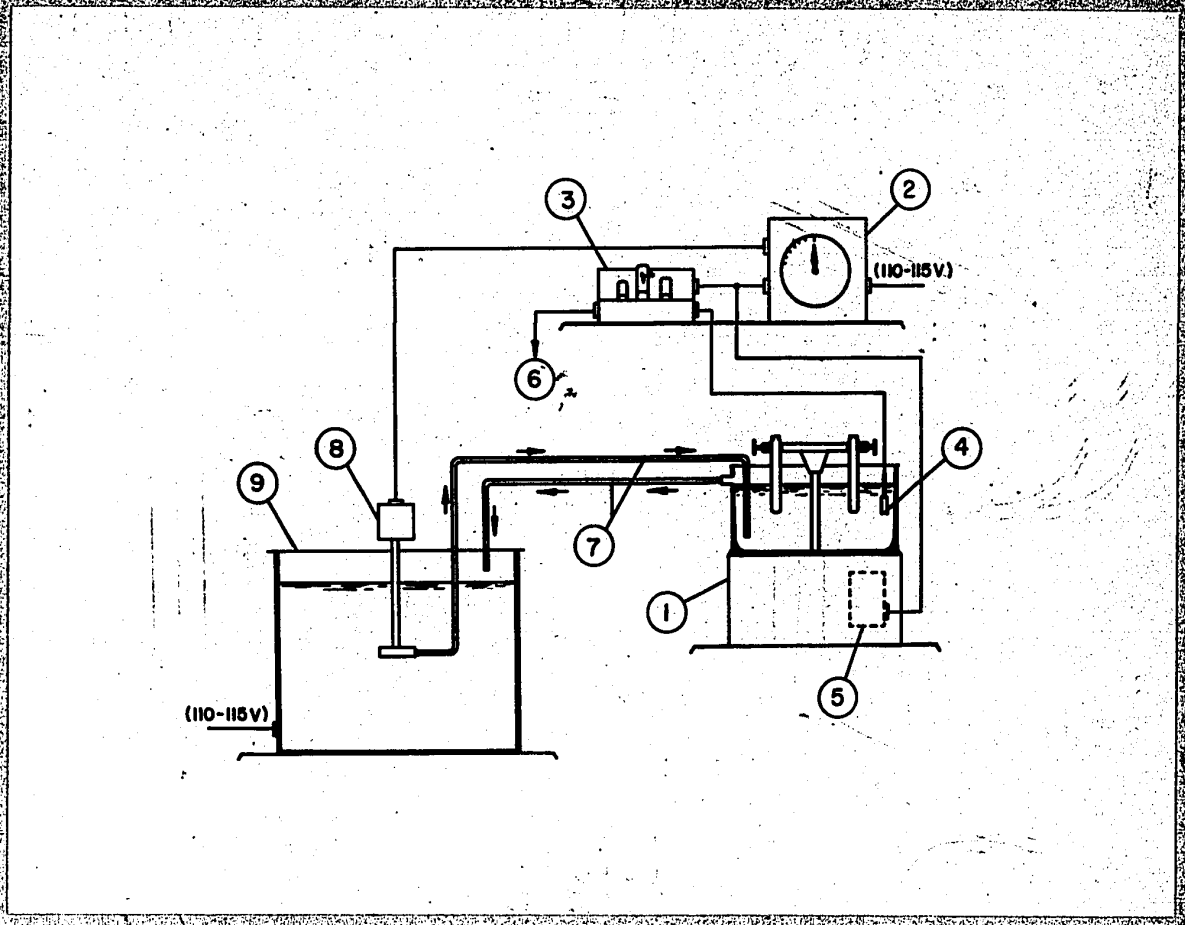

Figure 7 - Schematized set-up for incubations of renin activity determinations (not to scale).

1/ Rotary Evapo-Mix (Buchler Instruments). 2/ Gralab Universal Timer (1/4 min. to 15 hours). 3/ Y.S.P. thermostemp temperature controller, model 63 (circuit on during incubation time). 4/ Temperature probe. 5/ Electric motor connected to outlet receptacle which breaks circuit at 0. 6/ To heating element in bath. 7/ Tygon tubing. 8/ Circulation pump connected to outlet receptacle which makes circuit at 0. 9/ Refrigerated bath (water) 0-5°C. This apparatus was designed by Boucher (12).

glass column (10 cm x 1 cm) containing 1 ml of Dowex 50W-X2 (NH_4^+) resin. The column is washed first with 10 ml of 0.2 N ammonium acetate (pH 6), and then with 15 ml of acetic acid (10% v/v) and 20 ml of distilled water. These eluates are discarded and angiotensin is then eluted with 7 ml of diethylamine (0.1 N), followed by 7 ml of ammonium hydroxide (0.2 N), into a siliconized polypropylene Erlenmeyer flask (50 ml). This eluate is then lyophilized.

The dry residue is dissolved in 1 ml of a solution of sterile saline (0.9%), and is then assayed.

d) Bioassay

Male, albino rats of the Sprague-Dawley strain (100-150 g.) are bilaterally nephrectomized under ether anesthesia 18 hours before use, in order to provide a more stable blood pressure baseline. After pentobarbital sodium anesthesia (3 mg intraperitoneally and 4 mg subcutaneously) a tracheostomy is performed. Both jugular veins are cannulated with PE-10 polyethylene tubing, for injection of unknown and standard angiotensin with micro-syringes. The carotid artery is also cannulated and connected to a mercury manometer, the blood pressure being registered on a kymograph, or to a Statham transducer (P23AC) connected to a Grass Polygraph. When it is needed, subsequent doses of pentobarbital sodium (usually 1.2 - 1.8 mg) are injected subcutaneously.

Only rats responding by at least a 10 mm Hg rise of their blood pressure to the injections of 2.5 ng of standard angiotensin, are used. The volume of injected unknown ranges

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Only rats responding by at least a 10 mm Hg rise of their blood pressure to the injections of 2.5 ng of standard angiotensin, are used. The volume of injected unknown ranges

between 0.08 and 0.001 ml. If the blood pressure is not affected by the injection of 0.08 ml of unknown, the renin activity is reported as zero. When a suitable response to the injection of the unknown is obtained, it is bracketed between known doses of standard angiotensin at two different levels. According to our own experience, the best results are obtained when the assay is performed at doses bracketed between 0.7 and 2 ng of standard angiotensin.

The results obtained are expressed as ng of angiotensin formed per ml of plasma after 12 hours of incubation (ng/ml/12 hours) for plasma renin activity and ng of angiotensin formed per mg of kidney extracts after 1 hour of incubation (ng/mg/1 hour) for the renin content of the kidney.

3) Experimental data concerning the renin-substrate preparation

a) Pentobarbital anesthesia

Preliminary experiments were done to adapt Boucher's micromethod (12) to dogs by stuying the kinetics of the enzymatic reaction. Renin substrate was prepared according to Haas' procedure (150), from plasma of dogs bilaterally nephrectomized through laparotomy under pentobarbital anesthesia. Substrate concentration was studied by incubating the substrate preparation in increasing amounts (50 to 500 mg of freeze-dried substrate) with a constant amount of kidney extracts.

The rate of angiotensin formation was found to be dependent on substrate, prepared from the plasma of dogs nephrectomized 24 hours (4 dogs), 48 hours (2 dogs) and 72 hours

(3 dogs) previously. Two of the three dogs in which the plasma was collected 72 hours post-nephrectomy were salt-loaded (240 mEq Na/day) 3 and 5 days prior to nephrectomy procedure.

Plasma renin activity and/or arterial angiotensin level were measured according to Boucher's method (10) in six of these dogs (Table VI). It was possible to measure detectable renin activity 24 hours post-nephrectomy in 4 out of 5 dogs whereas circulating angiotensin was found in two dogs, 24 hours after nephrectomy and in one, 72 hours after nephrectomy.

Moreover, when performing kinetic studies, five substrate preparations were incubated without added kidney extract as control. Three of these incubations generated vasopressor substances.

Since it was not possible to achieve zero-order kinetics, studies involving time of incubation and possible loss of angiotensin in substrate preparation were done. The time of incubation was reduced to three and six hours in one experiment. The angiotensin was found to be proportional to the substrate concentration (up to 300 mg of substrate), even by reducing the time of incubation to 3 hours.

Angiotensinase-inactivation in the first step of substrate preparation is done by alkalinisation of plasma at pH 8 in presence of EDTA for one hour at room temperature. In order to check if any important losses of substrate were encountered in this part of the procedure, plasma was divided in two equal parts. One of them was processed through the original Haas' procedure (150) and the second part was submitted

TABLE VI - Plasma Renin Activity (PRA) and arterial angiotensin level in nephrectomized dogs.

Dog No.	Time after Nephrectomy (hours)	PRA[+] (ng/L/min)	Arterial[+] Angiotensin (ng %)
1	0	40	
	24	8	100
2	24		12
3	0	10	
	24	3.1	
	48	0	0
4	0	18	
	24	0	
	48	0	
	72	0	
5[++]	0	12.5	
	24	3	
	72	0	6
6[+++]	0	12	
	24	7	
	72	0	0

[+] : PRA and arterial angiotensin level were measured according to Boucher's procedure (10).

[++] : Fed 240 mEq Na/day during 3 days prior to nephrectomy.

[+++]: Fed 240 mEq Na/day during 5 days prior to nephrectomy.

to the same procedure but the angiotensinase-inactivation step was omitted. Such preparations were done in three instances, and kinetic studies comparing both preparations were carried out. In every case, the activity of the substrate prepared with or without angiotensinase-inactivation was comparable.

b) Effects of Enovid (R_X Searle), angiotensin and chloralose anesthesia

Since substrate preparations reported above were not suitable, attempts to increase the substrate level in nephrectomized dogs were undertaken. The plasma of eight consecutive dogs pre-treated with angiotensin before and during the anesthesia procedure for nephrectomy, was processed for substrate preparation. Nephrectomy was performed through laparotomy. The angiotensin pre-treatment was done by a constant intravenous infusion of valine-5 angiotensin II aspartic ß-amide at 200 ng/Kg/min. The infusion was begun half an hour before α-chloralose anesthesia and continued until the second kidney was removed. The total duration of angiotensin infusion was a minimum of 90 minutes. Three other dogs were given Enovid (40 mg of Norethynodrel/day, per os) for periods of 15-18 days. On the last day of treatment, they were nephrectomized after laparotomy under chloralose anesthesia and one of them was infused with angiotensin before anesthesia as described above.

These eleven dogs were bled 48 hours after nephrectomy, and the plasma processed separately according to the proce-

dure of Haas (150). Kinetics of the renin substrate reaction in angiotensin-treated dogs showed that angiotensin formation reached a plateau with 150-200 mg of freeze-dried substrate preparation in 7 out of 8 dogs. Similar results were found with the substrate preparation obtained from the dog treated with angiotensin and Enovid. In contrast, substrate from four control dogs that received the same dose of chloralose and a control infusion of isotonic saline, formed angiotensin in amounts parallel to the substrate concentration (up to 300 mg), when incubated with dog kidney extracts. The results of these four control dogs are presented in figure 8. As with the control dogs, the substrate from the two dogs, given Enovid, also failed to demonstrate zero-order kinetics (Figure 9).

Further evidence of the reproducibility of dog substrate obtained from dogs treated with angiotensin or with angiotensin and Enovid are reported in figure 10 where incubations with a large amount (1 mg) or an excess of kidney extracts (10 mg) were carried out in presence of 150 mg of different substrate preparations.

Substrate obtained from dogs pre-treated with angiotensin as well as from control dogs (chloralose anesthesia) failed to generate vasopressor substances when incubated alone without added renin.

Because of the disadvantages of keeping dogs alive for 48 hours after nephrectomy, and since it is known that maximal substrate concentrations are achieved 24 hours after

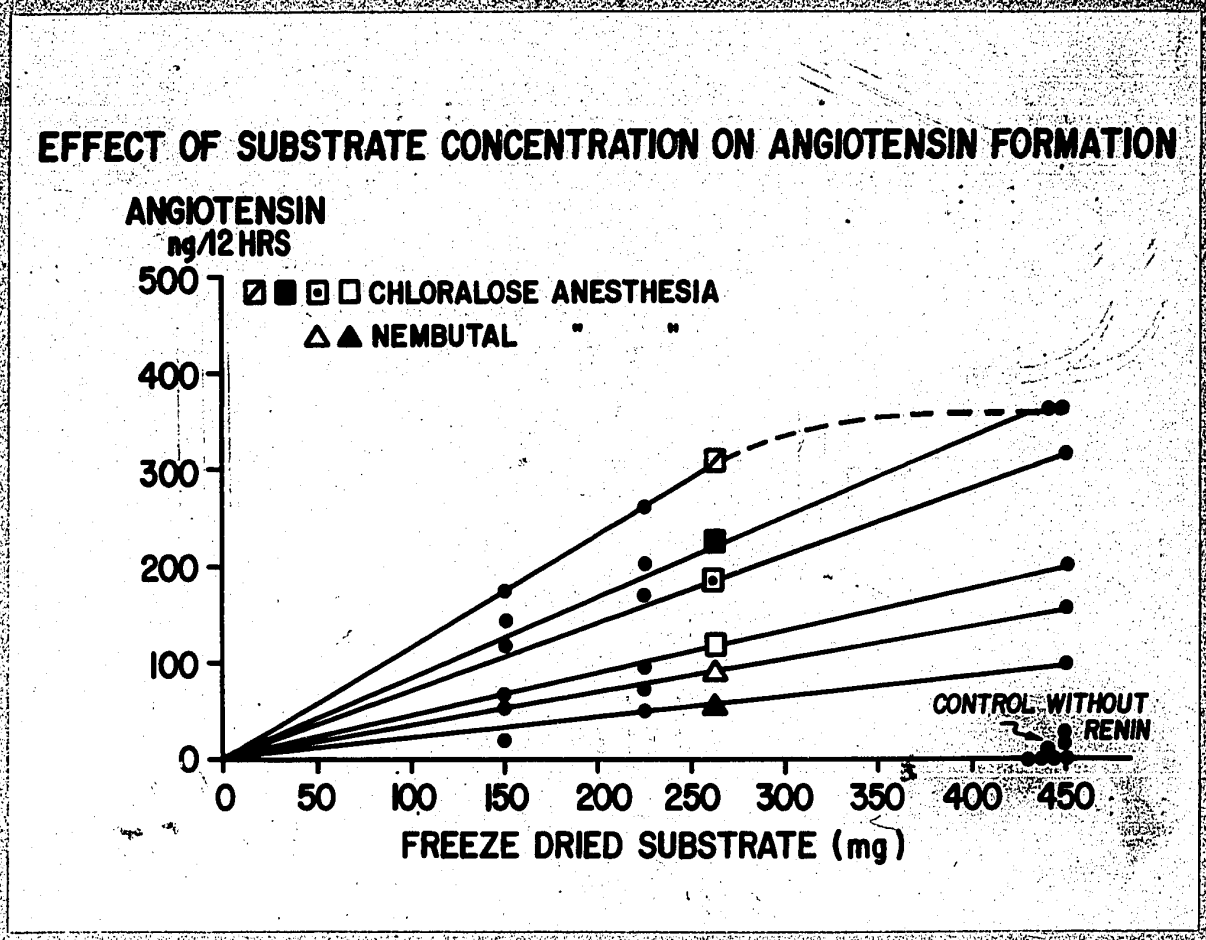

Figure 8

Incubations were carried out at pH 6.5 and 37°C for 12 hours in presence of a constant amount of dog kidney extracts (175 µg).

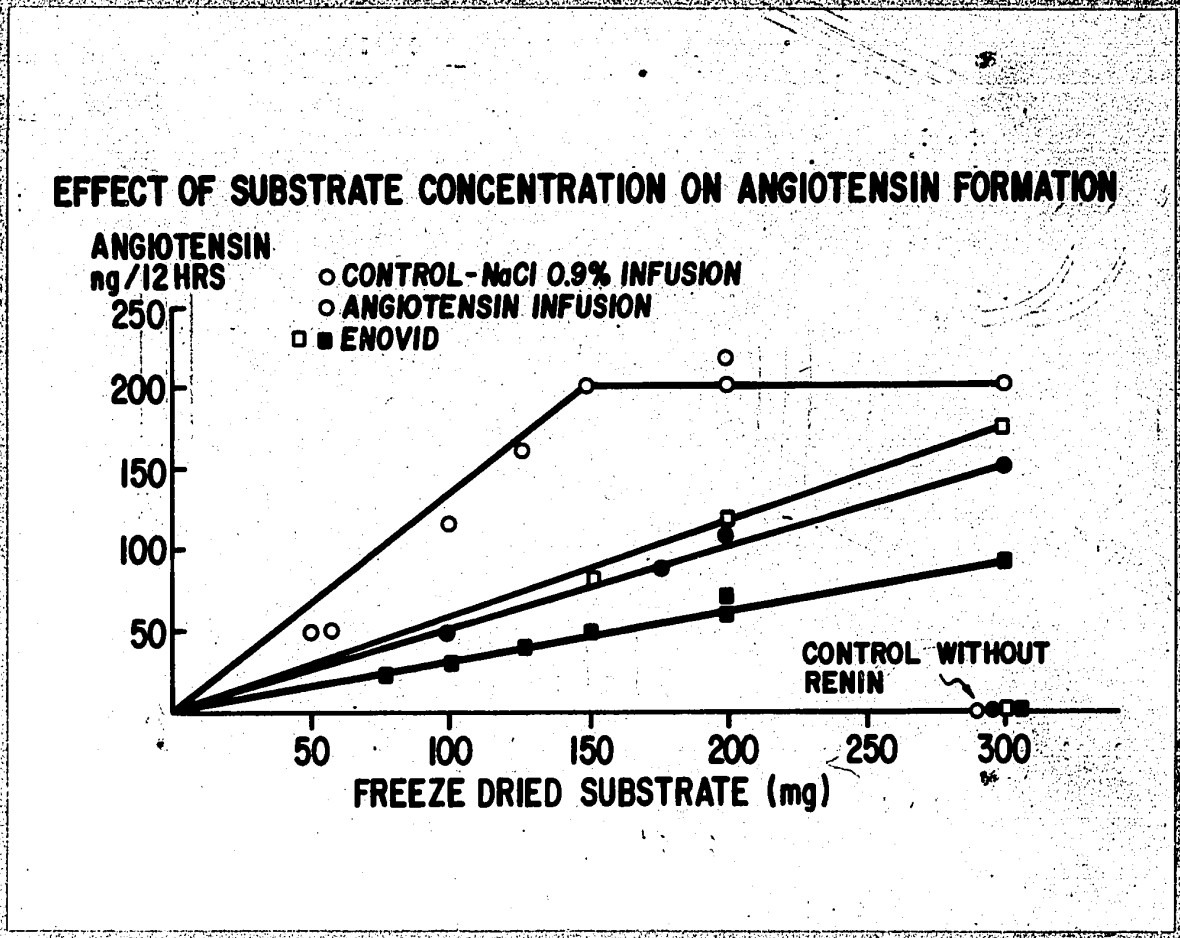

Figure 9

Incubations were carried out at pH 6.5 and 37°C for 12 hours, in presence of a constant amount of dog kidney extracts (150 µg).

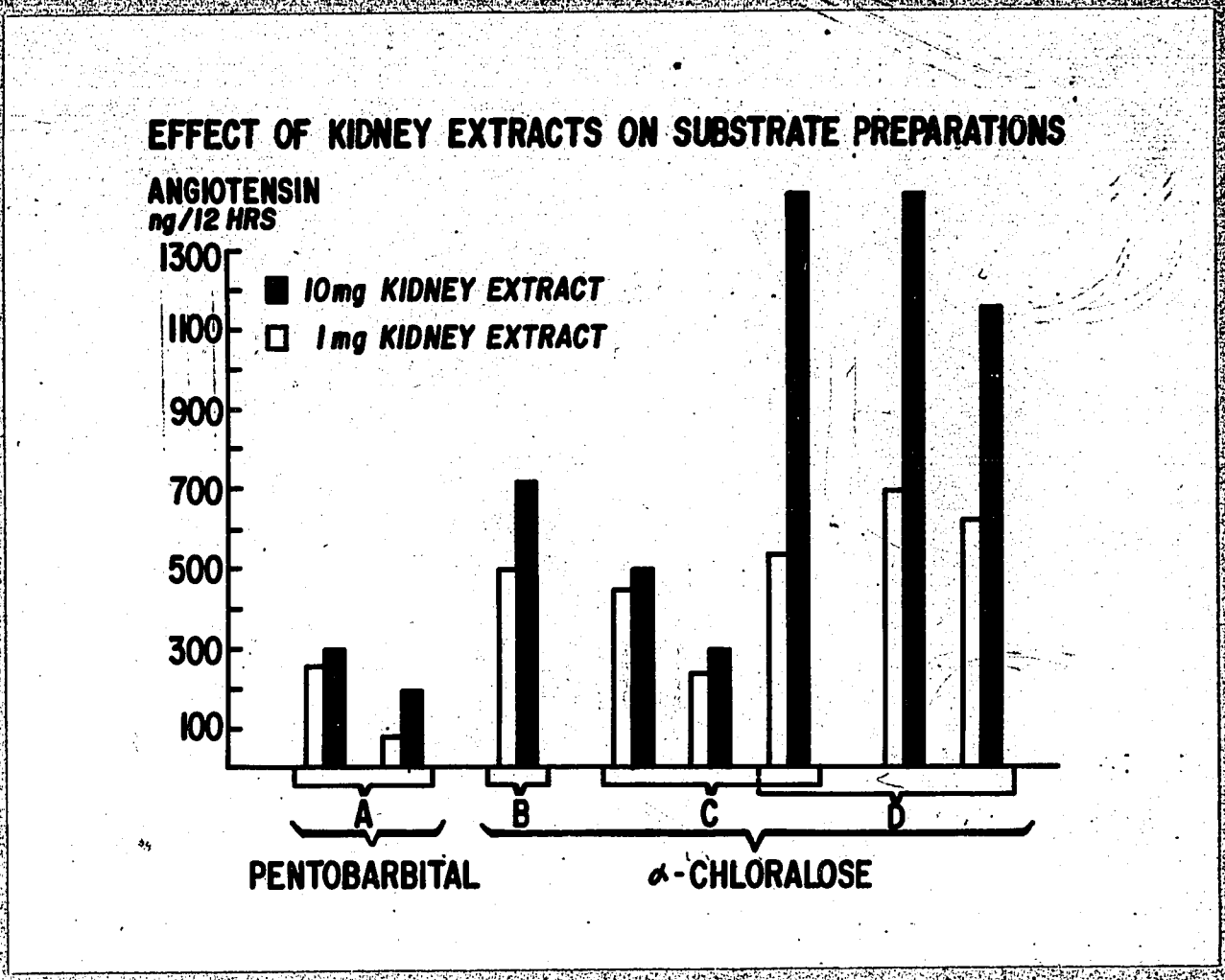

Figure 10

The substrate was prepared from plasma of dogs nephrectomized under pentobarbital (A), or α-chloralose anesthesia (B, C and D) after Enovid (C), or angiotensin (D) pre-treatment. Dog substrate (150 mg) was incubated with dog kidney extracts at pH 6.5 and 37°C for 12 hours.

nephrectomy, we decided to process plasma obtained from 24-hour nephrectomized, angiotensin pretreated dogs. When acquiring further experience with these preparations, it became obvious that they were reproducible and no real differences were encountered when comparing them to the preparations from 48-hour nephrectomized dogs.

The freeze-dried substrate preparation was also found to be stable and reproducible after a long storage in the cold (-20° C). Two substrate preparations were used after one year of storage and they were still suitable for determination of renin activity. One substrate preparation was assayed under controlled conditions on 3 different occasions through 16 days of storage. Table VII shows the reproducibility of the results obtained by incubating this substrate preparation with the same kidney extracts at different times.

c) Effects of various medications on the substrate level and capacity of plasma to generate angiotensin in nephrectomized dogs ("Capacity of angiotensin generation")

Preliminary experiments were done in order to determine the rate of angiotensin formation when incubating hog renin with dog plasma. As shown in figure 11 the substrate contained in 0.1 ml of dog plasma was completely exhausted with one unit of lyophilized hog renin, after 12 hours of incubation at 37° C. Plasma from nephrectomized dogs was used in that experiment to provide a maximal concentration of substrate.

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TABLE VII - Stability of substrate preparation.

Date of experiment	Dog substrate (mg)	Dog kidney extracts (µg)	Angiotensin found (ng)
Day 1	100	125	40
	300	0	0
	300	125	120
Day 11	100	125	50
	300	125	110
Day 16	100	125	50
	300	125	110

The same batch of kidney extracts was used for each experiment. Incubations were carried out at 37°C, pH 6.5 for 12 hours.

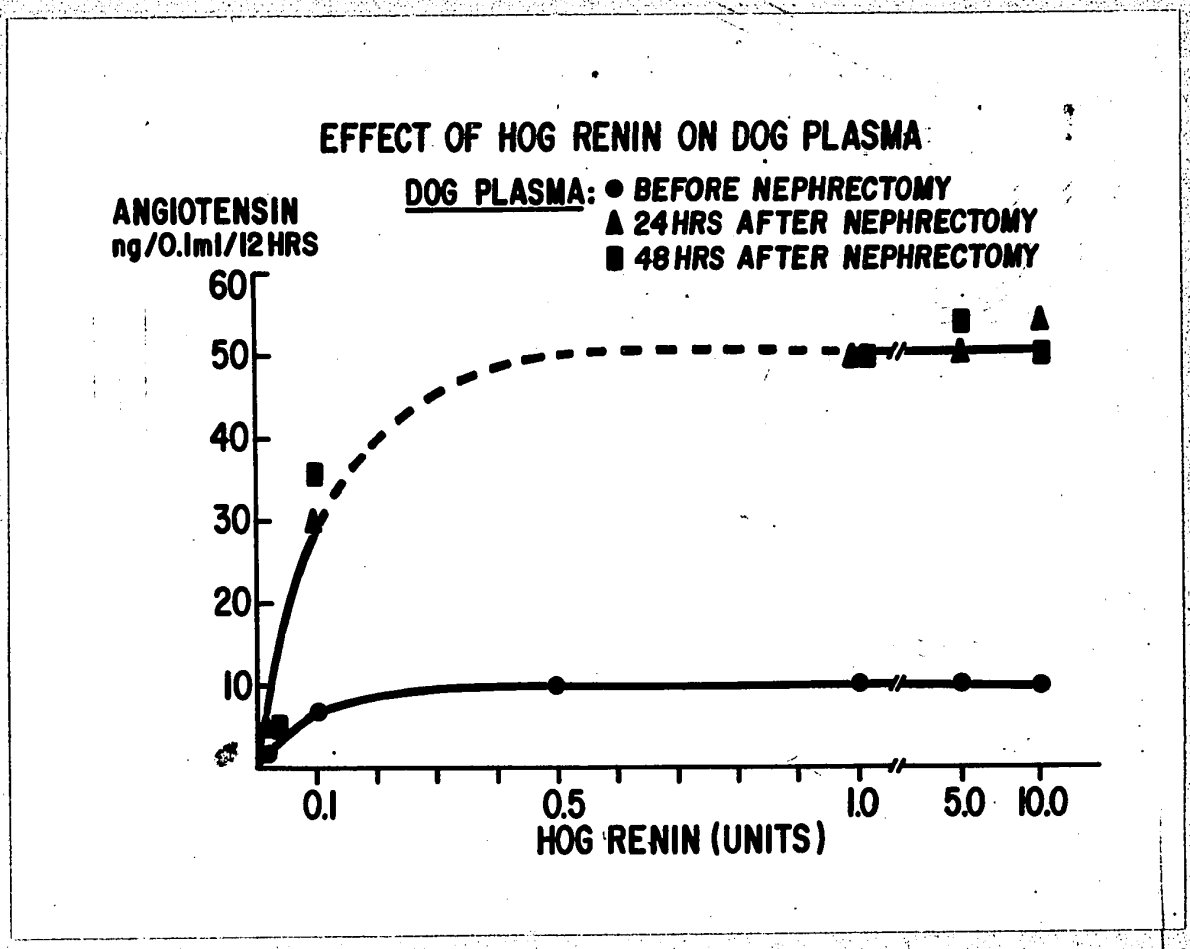

Figure 11

Dog plasma (0.1 ml) was incubated with hog renin for 12 hours at 37°C and pH 6.5.

According to this experiment, 2 units of hog renin were used to measure the substrate contained in 0.1 ml of dog plasma. The results were expressed as ng angiotensin formed per 0.1 ml of plasma for 12 hours (ng/0.1 ml/12 hours).

Since with 0.01 and 0.1 units of hog renin (Figure 11) the angiotensin formation was proportional to renin, 0.02 units were arbitrarily chosen to be incubated with 0.5 ml of plasma in order to evaluate the capacity of plasma to generate angiotensin (or "capacity of angiotensin generation"). The results were expressed as ng of angiotensin formed per 0.1 ml of plasma after 12 hours of incubation (ng/0.1 ml/12 hours).

For the determinations of substrate concentration and "capacity of angiotensin generation", 0.04 ml of a 15% solution of the ammonium salt of EDTA and 0.04 ml of a 1% solution of sodium azide were added to plasma (respectively to 0.1 ml and 0.5 ml) and the volume was brought up to 2 ml with phosphate buffer (0.25 M), pH 6.5. After adding the required amount of hog renin, the mixture was incubated in presence of 1 ml of Dowex resin 50W-X2 (NH_4^+) for 12 hours at 37° C.

Normal values for substrate concentration were determined in 27 dogs. The mean was 15.9 ng/0.1 ml/12 hours ($\pm$ S.D. 6.7 ng) with extreme values of 3 and 37.5 ng.

Substrate concentration was determined before, 24 and 48 hours after nephrectomy performed under pentobarbital (6 dogs) or α-chloralose anesthesia (5 dogs). They were compared to similar determinations done in 13 dogs infused with

angiotensin (200 ng/kg/min) prior to chloralose anesthesia and nephrectomy (Figure 12). In this series of experiments, both kidneys were removed through a flank incision. The increase found in renin substrate was 1.5 to 3.2-fold and it was maximal after 24 hours. The substrate concentration 48 hours after nephrectomy was measured only in 8 of the 13 dogs infused with angiotensin. The increase in renin substrate 24 and 48 hours after nephrectomy was higher in angiotensin-treated dogs. But, the mean substrate concentration 24 hours after nephrectomy in angiotensin-treated dogs was statistically different only from that obtained at a similar interval after nephrectomy in dogs anesthetized with chloralose ($0.002 < p < 0.005$).

Substrate concentration and "capacity of angiotensin generation" were determined simultaneously in 9 dogs before, 24 and 48 hours after nephrectomy. The "capacity of angiotensin generation" as well as substrate concentration did not increase further from 24 to 48 hours after nephrectomy except in one dog (Figure 13). In that dog, both substrate concentration and the "capacity of angiotensin generation" continued to increase from 24-48 hours. In this experiment, the nephrectomy was performed through a flank incision under pentobarbital in 5 dogs and under α-chloralose in 4 dogs, two of which were infused with angiotensin (200 ng/kg/min) prior to anesthesia.

4) Optimal conditions of the procedure

a) Effect of pH on renin activity

In dogs, a maximum yield of angiotensin was obtained

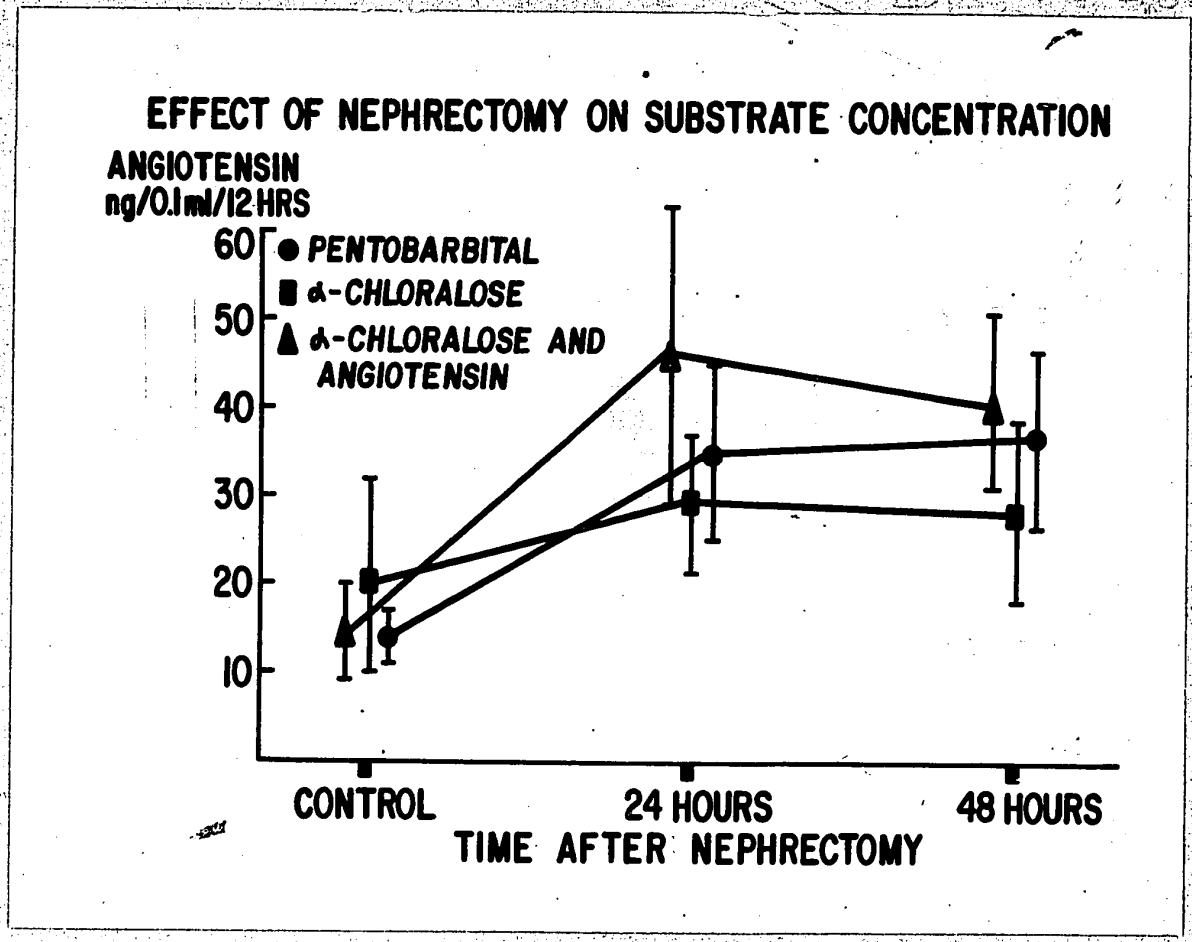

Figure 12

Mean substrate concentration with standard deviation.

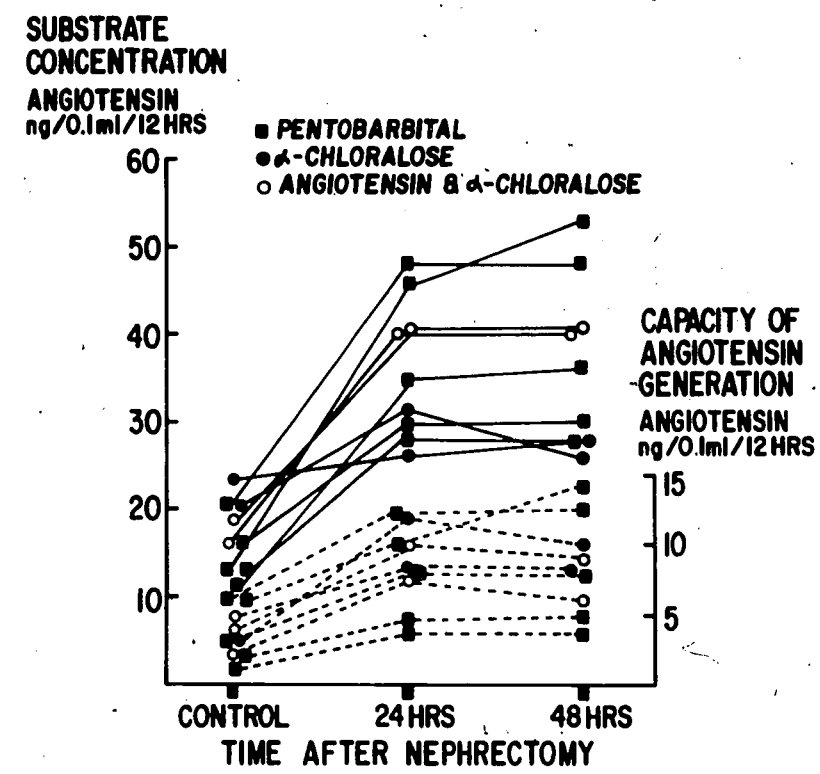

Figure 13

The "capacity of angiotensin generation" followed substrate concentration in every nephrectomized animal studied.

at pH 5.5, 6.0 and 6.5 when kidney extracts (Figure 14) were incubated. Similar results were obtained with dog plasma (Figure 15). An identical curve of pH was found in both cases with high or low renin activity. A maximum yield of angiotensin at pH 5.5 was obtained with human kidney tissue (Figure 14).

b) Effect of substrate concentration on Reaction Velocity

The effect of a constant amount of dog renin (from kidney) on increasing amount of dog substrate was studied extensively (cf. supra). Usually the angiotensin formation reached a plateau with 150 mg of dog substrate, after 12 hours at 37° C. The reaction velocity, when a constant amount of dog plasma was incubated with dog substrate, was also studied. The angiotensin formation reached a plateau when 50, 75 and 100 mg of dog substrate were incubated with three plasmas of different renin content (Figure 16). Usually, a maximal yield of angiotensin up to 400 ng was obtained, in condition of excess substrate, with 100 to 200 mg of freeze-dried dog substrate. A substrate preparation requiring more than 250 mg of freeze-dried dog substrate to provide a zero-order reaction was discarded.

c) Effect of time on the formation of angiotensin

When a constant amount of dog substrate was incubated in presence of a constant amount of dog renin (from plasma or kidney) the angiotensin formation showed a linear relation

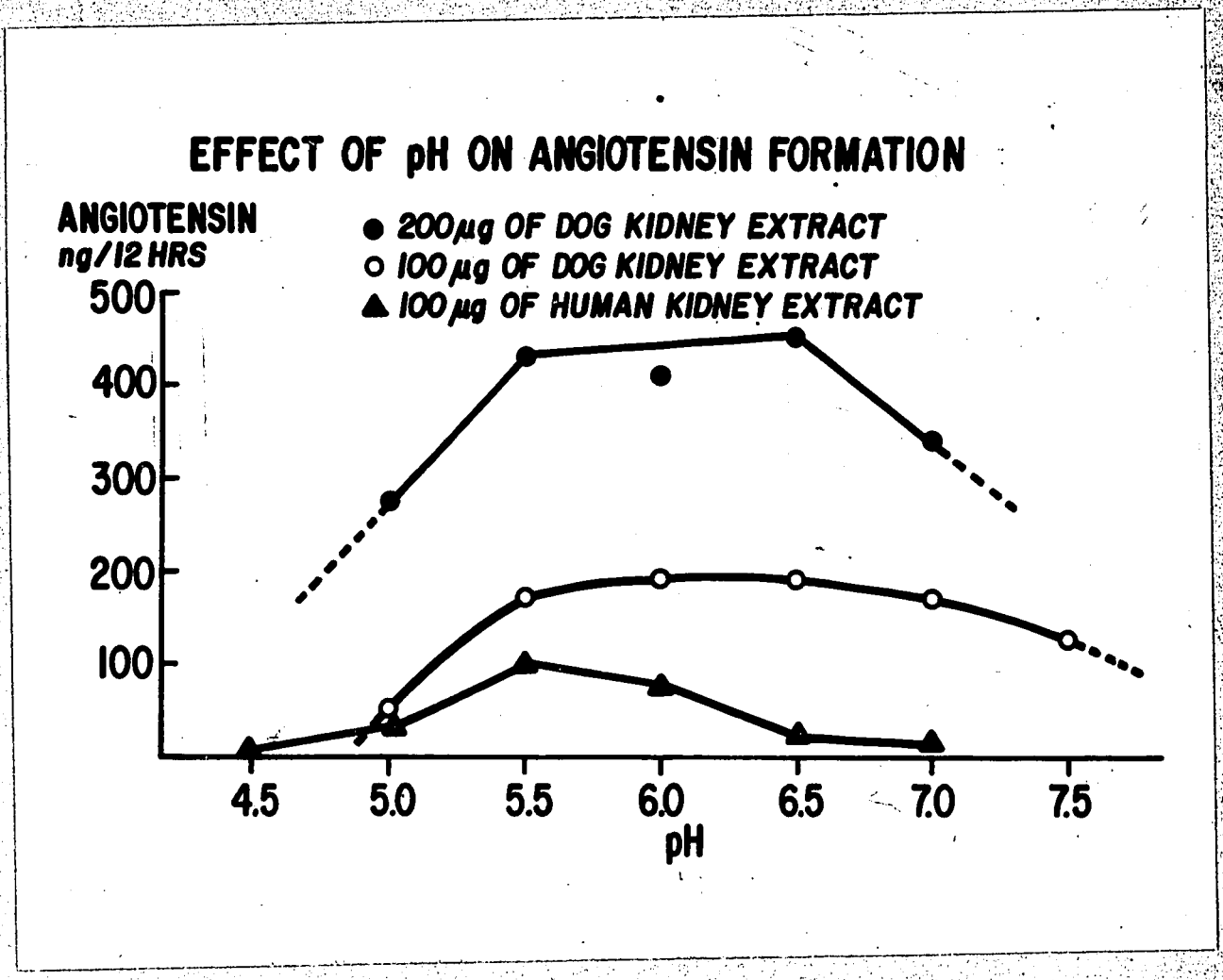

Figure 14

Dog substrate (150 mg) was incubated with dog or human kidney extracts at 37°C for 12 hours.

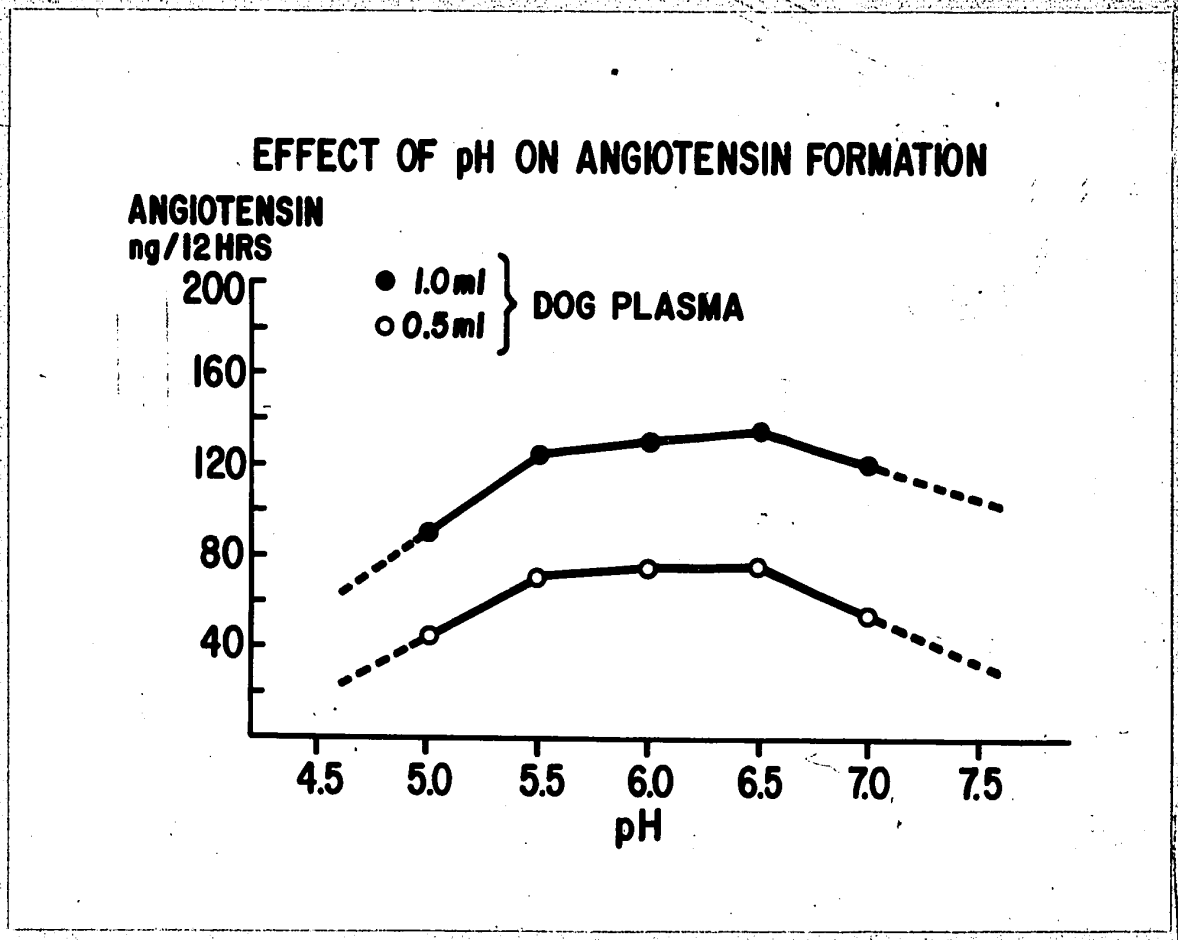

Figure 15

Dog substrate (150 mg) was incubated with dog plasma at 37°C for 12 hours.

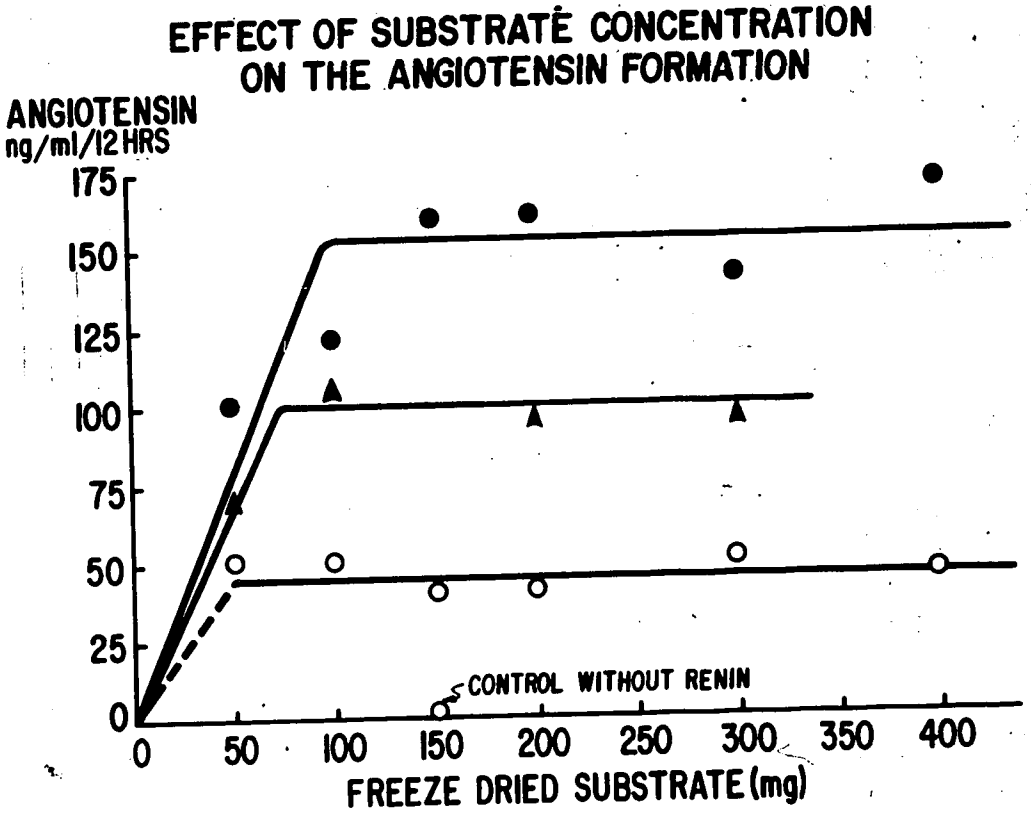

Figure 16

Three different dog plasmas (1 ml) were incubated with dog substrate (150 mg) at pH 6.5 and 37°C for 12 hours.

in function of time, up to 18 hours with large quantities of renin and up to 48 hours with low renin activity (v.g. 10 ng angiotensin/12 hours). The studies were not extended over 48 hours. Figure 17 illustrates a typical incubation progress curve of the reaction of dog renin (plasma) with dog substrate as a function of time. A 12-hour incubation period was therefore chosen for the determination of plasma renin. An 18- or 24-hour incubation period could be performed for plasma with low renin activity.

d) Effect of the concentration of renin on the angiotensin formation

The influence of different amounts of dog kidney extracts on the liberation of angiotensin was studied. The incubations were carried out in presence of a constant amount of dog substrate. The angiotensin formation showed a linear relationship over a large range of dog renin concentration. Figure 18 shows a linear formation of angiotensin up to 500 ng. In two other experiments of this type, conditions of excess substrate were realized with a maximal yield of 600 ng in one instance and 700 ng of angiotensin in the other. These results support our conviction that conditions of excess substrate are realized when less that 400 ng of angiotensin are formed in the incubation mixture.

e) Effects of different incubation media on the formation of angiotensin

When the incubation mixture contained identical portions

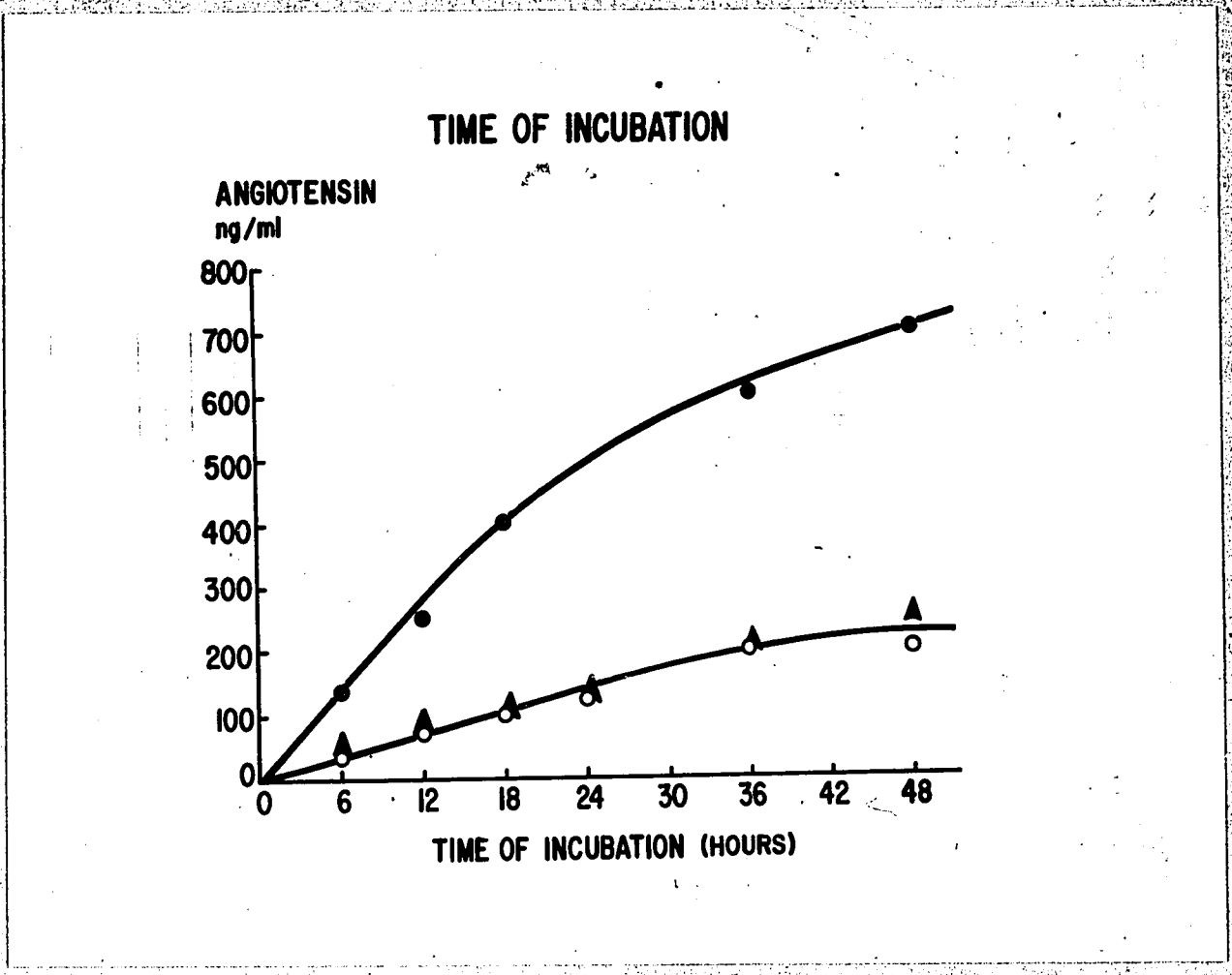

Figure 17 - Effect of time of incubation on angiotensin formation.

Two different dog plasmas (1 ml) were incubated with 100 mg (circles) or 200 mg (triangles) of dog substrate at pH 6.5 and 37°C.

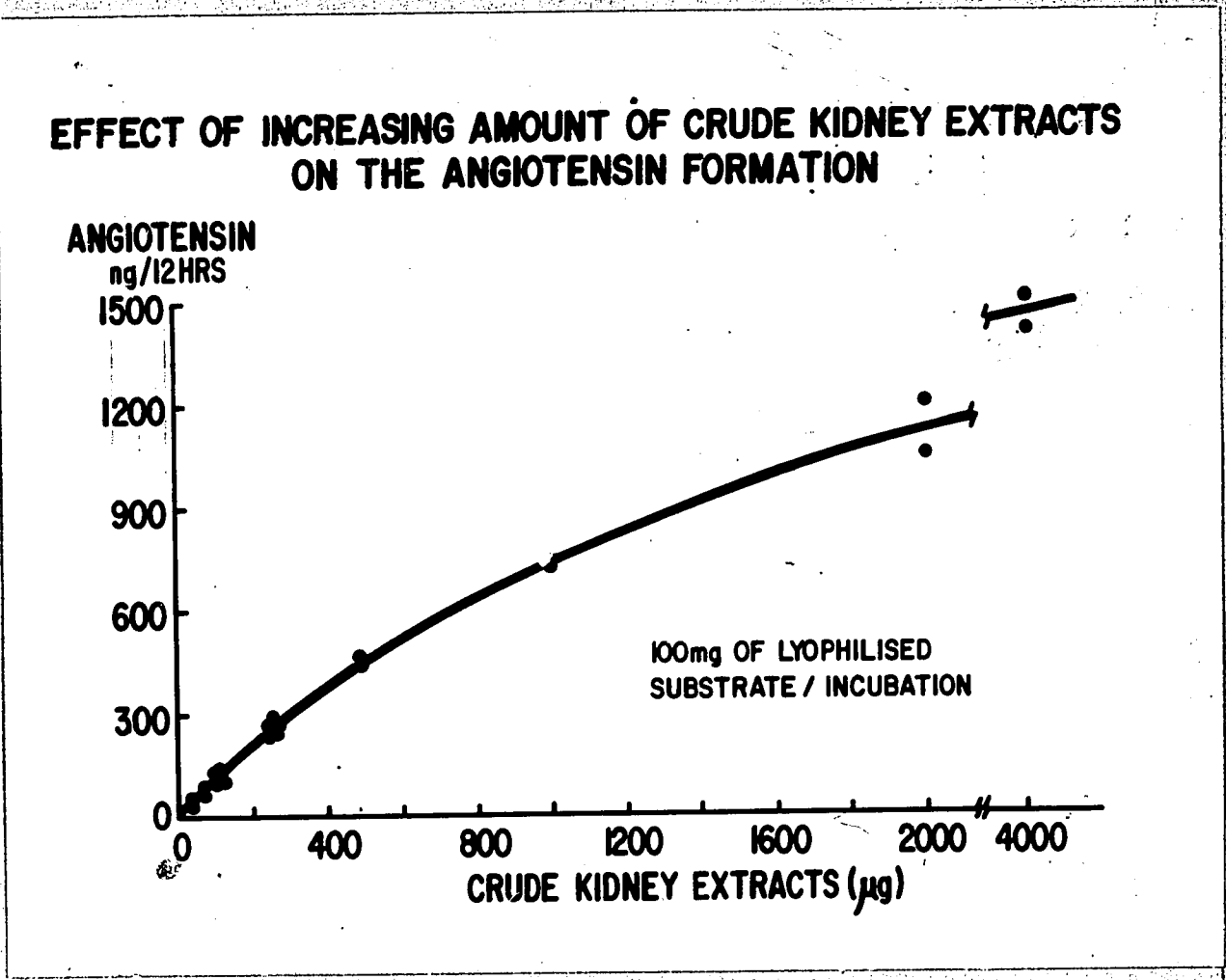

Figure 18

Dog substrate (100 mg) was incubated with dog kidney extracts at pH 5.5 and 37°C for 12 hours.

of renin (dog plasma or dog kidney extracts) and of substrate preparation, a three-fold dilution decreased the angiotensin formation (Table VIII). The addition of sodium chloride to the incubation mixture had no effect on the angiotensin formation, if less than 150 mg was added as shown in table IX and X. The addition of 10 mg of heparin was also without effect on the incubation mixture (Table IX).

In Boucher's original micromethod (12), the substrate was dissolved in water and incubated with 0.1 ml of rat plasma or rat kidney extracts. But in the incubation mixture containing dog substrate dissolved in water and dog plasma or dog kidney extracts, a slight precipitate generally formed after 12 hours. The formation of angiotensin was not affected, however, when that precipitate was found in the incubation mixture. Moreover, to measure detectable amount of angiotensin formed by dog plasma, one or two ml were required. In order to avoid the problem of adjusting the pH on a small aliquot of plasma, it was prefered to use a buffer. Tris-citrate+, Tris-chlorhydrate+ and Tris-phosphate were employed to dissolve the substrate. The molarity of the Tris solutions ranged from 0.05 to 0.5 M.

These buffers did not interfere with the angiotensin formation (Table XI) and the precipitation encountered when dissolving substrate in water was prevented. The Tris-phos-

+ Tris-citrate and Tris-chlorhydrate solutions were prepared in the same way as described for Tris-phosphate in the section of materials, reagents and apparatus.

TABLE VIII – Effect of dilution on angiotensin formation.

No.	Dog substrate (150 mg)	Dog plasma	Tris-phosphate 0.25 M, pH 6.5	Angiotensin found (ng)
1	2 ml	1 ml	0 ml	200
2	2 ml	1 ml	2 ml	200
3	2 ml	1 ml	4 ml	200
4	2 ml	1 ml	6 ml	150

Incubations were carried out at 37°C, pH 6.5 for 12 hours.

TABLE IX – Effect of sodium chloride and heparin on angiotensin formation.

No.	Dog substrate (150 mg)	Dog kidney extracts (µg)	Heparin (mg)	NaCl (mg)	Angiotensin found (ng)
1	3 ml	100	-	-	320
2	3 ml	100	10	-	300
3	3 ml	100	-	430	200
4	3 ml	100	10	430	180

Incubations were carried out at 37°C, pH 6.5 for 12 hours.

TABLE X - Effect of sodium chloride on angiotensin formation.

No.	Dog substrate (150 mg) dissolved in NaCl						Angiotensin found (ng)
	35.7%	15%	5%	1.5%	0.9%	0.5%	
1	3 ml						0
2		3 ml					30
3			3 ml				80
4				3 ml			100
5					3 ml		100
6						3 ml	110

Dog substrate was incubated in presence of a constant amount of dog kidney extracts (125 µg). Incubations were carried out at 37°C and pH 6.5 for 12 hours.

TABLE XI - Effect of sodium chloride, Tris-chlorhydrate (T-Cl), Tris-Citrate (T-Ci) and Tris-phosphate (T-Ph) on angiotensin formation.

No.	Dog substrate (100 mg)				Dog plasma	Dog kidney extracts (µg)	Angiotensin found (ng)
	T-Cl 0.25M	T-Ci 0.25M	T-Ph 0.25M	NaCl 0.9%			
1	3 ml				1 ml	0	130
2	4 ml				0	170	320
3	3 ml+				1 ml	0	12.5
4		3 ml			1 ml	0	120
5		4 ml			0	170	300
6		3 ml+			1 ml	0	12.5
7			3 ml		1 ml	0	120
8			4 ml		0	170	300
9			3 ml+		1 ml	0	20
10				3 ml	1 ml	0	120
11				4 ml	0	170	250
12				3 ml+	1 ml	0	20

+: Substrate was not added to the solution.

Incubations were carried out at 37°C and pH 6.5 for 12 hours.

phate (0.15 M) was chosen.

The addition of chloride ions to the incubation mixture was also shown to have no effect on the angiotensin formation in the system used.

f) Endeavours to eliminate possible interfering substances.

The formation of angiotensin reached a plateau with a pH between 5.5 and 6.5 when incubating dog plasma with dog substrate. pH 6.5 was chosen. Recovery experiments were undertaken and consisted in the addition of a known amount of standard angiotensin into the incubation mixture just before carrying out the incubation. After incubation, the samples were processed and measured in the usual way. The results were expressed as the percentage of angiotensin recovered. The very first recovery experiments were performed at pH 6.5, and the percentage of recovery of added angiotensin varied from a low value of 45% to 60%. For this reason, different attempts to eliminate possible interfering substances were tried.

Moreover, in some samples obtained after incubating plasma at pH 6.5, the oxytocic activity [+](determined on isolated rat uterus) was shown to exceed the values found by rat blood pressure assay whereas a relaxation of the isolated colon[+] was induced by these samples. For determination

[+] We are most grateful to Dr. Hiroshi Kurihara and Mr. Claude Grise who kindly performed rat uterus and colon assays on our samples.

of the oxytocic activity, the time to induce a contraction of the uterus after the injection of unknown was measured and compared to a similar response obtained after standard angiotensin. However, such high values for oxytocic activity were not found when dog kidney extracts were incubated with dog substrate, and the oxytocic activity was comparable to the values found with the rat blood pressure assay or lower (see Table XIII).

In nine experiments, plasma was submitted to salting out with ammonium sulfate before incubation. One ml of plasma was placed in a disposable Falcon plastic tube and a solution of ammonium sulfate was added in order to obtain a 2.4 M solution. A suitable period (30 minutes) was then allowed to obtain precipitation. After centrifugation, the supernatant was discarded and the precipitate was dissolved in the substrate solution. The incubation was carried out after adding one ml of resin. In one of 9 experiments, the values obtained for renin activity after salting out the plasma were comparable to the control values. In 6 of them, the values obtained were lower than the control values. In the last two experiments, when the precipitate was dissolved in the substrate solution, the proteins precipitated and the incubations were not carried out. Moreover, the salting out of plasma was without effect on the oxytocic activity measured after 12 hours of incubation.

Another attempt to remove interfering substances was achieved by treating plasma with resin Dowex 50W-X2 (NH_4^+) before incubation. This was achieved by adding 0.5 ml of

resin to 1.5 ml of plasma. After shaking for 5 minutes at 0-5° C, the solution was centrifuged in the cold. One ml of plasma obtained from the supernatant was added to substrate and resin and a 12-hour incubation was carried out at 37° C. The above mentioned 0.5 ml of resin was immediately transfered to a column for washing and elution. As shown in Table XII, the resin treatment did not affect the angiotensin formation, as evaluated by rat blood pressure assay. The oxytocic activity was also not affected despite the fact that the resin used to treat the plasma had absorbed a large amount of oxytocic substances.

Two enzyme-inhibitors were also used to reduce the oxytocic activity of incubated plasma aliquot. Sodium bichromate was added to the incubation mixture (75 mg) and was found to decrease the angiotensin formation, and less than 40% of the added angiotensin was recovered. Soy-bean trypsin inhibitor was added to the incubation mixture, the amount used varying between 0.01 mg and 0.0001 mg. The soy-bean did not affect the rate of angiotensin formation (Table XIII), nor the measured ocytocic activity (Table XIV). The recovery of added angiotensin was also not improved (Table XIV).

g) Verification of the techniques of washing, elution and freeze-drying of the angiotensin

Verification of the techniques of purification and extraction of the angiotensin formed or added to the incubation mixture was also done. The same amount of angiotensin

TABLE XII - Treatment of plasma with Dowex 50W-X2 (NH_4^+) resin before incubation.

No.	Dog plasma		Resin++	Angiotensin found (ng)	
	intact	treated+		Rat uterus	Rat blood pressure
1	1 ml			750	120
2	1 ml			1600	100
3		1 ml		720	120
4			0.5 ml	200	0
5		1 ml		900	120
6			0.5 ml	600	0
7		1 ml		1250	100
8			0.5 ml	500	0
9		1 ml		2100	100
10			0.5 ml	-	0

+ : 1.5 ml of dog plasma was shaken during 3 minutes in presence of 0.5 ml of Dowex 50W-X2 (NH_4^+) resin at 4°C. After centrifugation in the cold, 1 ml of supernatant (dog plasma) was incubated with substrate and 1 ml of Dowex 50W-X2 (NH_4^+) resin.

++: The resin used to treat the plasma (0.5 ml), was immediately washed and eluted as usual.

Dog substrate (150 mg) was incubated with dog plasma at 37°C and pH 6.5 for 12 hours.

TABLE XIII - Effect of soy-bean trypsin inhibitor on angiotensin formation.

No.	Dog Substrate (150 mg)	Dog kidney extracts (ug)	Soy-bean (0.1 ml)	Angiotensin found (ng)	
				Rat uterus	Rat blood pressure
1	2 ml	140		120	120
2	2 ml	140		110	120
3	2 ml	140	0.01 mg	90	110
4	2 ml	140	0.01 mg	100	100
5	2 ml	140	0.001 mg	-	100
6	2 ml	140	0.001 mg	-	80
7	2 ml	140	0.0001 mg	-	120
8	2 ml	140	0.0001 mg	-	100

Incubations were carried out at 37° C and pH 6.5 for 12 hours.

TABLE XIV - Effect of soy-bean trypsin inhibitor on the recovery of added angiotensin.

No.	Soy-bean (0.1 ml)	Angiotensin added (ng)	Angiotensin found (ng)		Recovery %
			Rat uterus	Rat Blood pressure	
1	0.01 mg	50	-	25	40
2	0.01 mg	50	-	19	
3	0.01 mg	50	-	17.5	
4	0.01 mg	100	-	50	60
5	0.01 mg	100	-	70	
6	0.01 mg	100	-	60	
7	0.01 mg	200	-	100	62.5
8	0.01 mg	200	-	150	
9	0.01 mg	0	2000	0	
10	0.01 mg	0	2000	0	
11	0.01 mg	0	1500	0	

Dog substrate (150 mg) was incubated with dog plasma (1 ml) at 37° C and pH 6.5 for 12 hours.

formed was found after washing the resin column with 15 to 40 ml of acetic acid. The recovery of added angiotensin was not improved when the technique of elution of angiotensin was slightly modified.

Recovery of added angiotensin in unincubated samples (Table XV) was not affected by the addition of EDTA to the samples. Less angiotensin was recovered in two unincubated samples when doubling the usual 15 ml of acetic acid washing (Table XV). Identical samples without added angiotensin were processed through ammonium acetate and acetic acid washing. After washing, the angiotensin was then added directly into the column and eluted (Table XV). The recovery was essentially the same when angiotensin was added before or after washing the column.

A comparison between two different techniques of concentrating the angiotensin fraction obtained after elution was done by submitting samples to freeze-drying and duplicate of these samples to evaporation to dryness and sublimation in siliconized conical flask. The results obtained were essentially the same although the angiotensin measured was slightly higher in the samples submitted to freeze-drying. Lyophilization was preferred to the other technique mainly for practical reasons.

h) Recovery of added angiotensin

It was finally possible to avoid partially the problems encountered with recovery of added angiotensin by carrying out the incubation at pH 5.5. At this pH, the recovery experiments were more stable. The main values in three successive experiments averaged 68%. The oxytocic activity

TABLE XV - Effect of the addition of EDTA and acetic acid washing on the recovery of added angiotensin.

No.	EDTA^{+} (ml)	AA^{++} (ng)	Acetic acid washing	AA^{+++} (ng)	Angiotensin found (ng)	Recovery %
1	0.04	0	15 ml	0	0	
2	0.04	0	15 ml	0	0	
3	-	0	15 ml	0	0	
4	0.04	0	15 ml	100	75	
5	0.04	0	15 ml	100	80	78.3
6	0.04	0	15 ml	100	80	
7	-	0	15 ml	100	85	82.5
8	-	0	15 ml	100	80	
9	-	0	30 ml	100	80	80
10	-	0	30 ml	100	80	
11	0.04	100	15 ml	0	80	80
12	-	100	15 ml	0	80	77.5
13	-	100	15 ml	0	75	
14	-	100	30 ml	0	55	62.5
15	-	100	30 ml	0	70	

$^{+}$: Solution of the ammonium salt of EDTA (15%) was added directly to the substrate preparation.

AA^{++} : Angiotensin was added in the tube containing substrate and plasma.

AA^{+++}: Angiotensin was added directly into the column after washing with ammonium acetate and acetic acid.

Each sample contained dog substrate (100 mg) and heparinized dog plasma (1 ml). The pH of the mixture was 6.5. The samples were not incubated, and the manipulations were performed in the cold room (0-5°C).

measured in 3 plasma samples incubated at pH 5.5 was still as high as the samples incubated at pH 6.5. Nevertheless, if one compares the values obtained by rat blood pressure assay with those obtained by rat uterus assay in table XII, it is obvious that the oxytocic activity bears no relation to the vasoactive response. Recovery experiments performed at pH 5.5 are reported in table XVI.

i) Reproducibility

The results obtained from incubations of identical aliquots of plasma and kidney extracts were found to be reproducible (Table XVII).

5) Results

a) Plasma renin activity

Blood from the forelimb of 43 conscious mongrel dogs fed Purina dog Chow and drinking tap water ad libitum, were taken. After centrifugation, 0.5 ml, 1 ml or 2 ml of plasma were incubated for 12 hours in presence of excess substrate (Table XVIII). One and two ml of plasma from one animal and two aliquots of one ml of plasma from another one were processed separately at the same time (Dog No. 1 and 12, Table XVIII). The results of these duplicate experiments were reproducible. The mean plasma renin activity was 12 ng/ml/12 hours. ($\pm$ S.D. 11.9) with a range from 0 to 40 ng. Fourteen female mongrel dogs were fed by gavage, a diet containing 58.4 to 66.3 mEq Na/day and 40.7 to 42.5 mEq K/day in a volume of 800 ml. The diet was prepared by homogenizing in a "Waring" blender, a commercial meat preparation (Dr.Ballard)

TABLE XVI - Recovery of added angiotensin in incubations carried out at pH 5.5.

No.	Angiotensin added (ng)	Angiotensin found (ng)	Recovery %
	Experiment A		
1	0	30	
2	0	25	
3	0	30	
4	0	20	
5	100	100	
6	100	100	71.3
7	100	90	
8	100	100	
9	200	170	
10	200	160	67
11	200	160	
12	200	150	
	Experiment B		
1	0	12	
2	0	12	
3	0	20	
4	50	40	
5	50	50	67.3
6	50	55	
7	100	75	
8	100	100	70
9	100	80	

Experiment A: Dog substrate (100 mg) was incubated with dog plasma (1 ml).

Experiment B: Dog substrate (150 mg) was incubated with dog plasma (1 ml).

The plasma used in each experiment was sampled from two different normal dogs. Incubations were carried out at 37°C and pH 5.5 for 12 hours.

TABLE XVII - Reproducibility.

No.	Dog plasma	Dog kidney extracts (µg)	Angiotensin found (ng)
1	1 ml		70
2	1 ml		60
3	1 ml		65
4	1 ml		60
5		250	240
6		250	260
7		250	260
8		250	280
9		250	240
10		250	225
11		125	150
12		125	130
13		125	110
14		125	160
15		125	140

Dog substrate (150 mg) was incubated with dog plasma or kidney extracts at 37°C and pH 6.5, for 12 hours.

TABLE XVIII - Plasma renin activity of normal conscious dogs.

Dog no.	Plasma (ml)+	PRA++	Dog no.	Plasma (ml)+	PRA++
1	1	40	21	1	40
1	2	35	22	1	6
2	1	0	23	1	8.7
3	1	25	24	1	5
4	2	5	25	1	25
5	1	25	26	1	15
6	2	0	27	1	12
7	1	15	28	1	15
8	0.5	15	29	1	40
9	0.5	10	30	1	6.2
10	0.5	0	31	1	6.2
11	1	6.2	32	1	11.2
12	1	30	33	1	37.5
12	1	10	34	1	8.7
13	1	37.5	35	1	0
14	1	5	36	1	3.5
15	1	15	37	1	0
16	1	6.2	38	1	3.1
17	1	0	39	1	3
18	1	0	40	1	12.5
19	2	20	41	1	10
20	2	0	42	1	17.5
			43	1	0
Mean ± S.D. = 12 ± 11.9					

+: Volume of plasma incubated 12 hours.

++: Plasma renin activity (ng/ml/12 hours).

and Purina dog chow. The diet was given in two equal portions at 9 and 15 hours for at least 3 days. After sampling peripheral blood for plasma renin activity, the dogs were anesthetized with pentobarbital sodium. Plasma renin activity was repeated 90 minutes later, after infusing Ringer's solution, P.A.H. and creatinine for renal clearance studies.+ The mean renin activity on metabolic balance was 13 ng/ml/12 hours (± S.D. 9.8) with extreme values ranging between 3 and 37 ng. After pentobarbital anesthesia and renal clearance study, the plasma renin activity increased in 10 out of 13 animals whereas a decrease was found in one dog. In 2 dogs, the plasma renin activity remained essentially the same (Table XIX).

The plasma renin activity was measured in three dogs under light pentobarbital anesthesia and in three dogs under light chloralose anesthesia. These animals were still reacting to painful stimuli and plasma renin activity was determined at different time-intervals after the induction of anesthesia. In each case, the last sample was taken when the animals had almost completely recuperated from anesthesia. In all, except one dog under chloralose anesthesia, there was an increase in plasma renin activity (Table XX). This increase was found within

+ We are most grateful to Dr. L. Belleau who permitted us to sample blood for plasma renin activity on his animals kept on metabolic balance.

TABLE XIX - Plasma Renin Activity (PRA) in normal conscious dogs under metabolic balance and the effect of anesthesia.

Dog no.	PRA (ng/ml/12 hours)	
	Conscious	Anesthetized[+]
1	10	3
2	10	15
3	3	-
4	25	50
5	6	200
6	19	19
7	3	30
8	6	30
9	15	35
10	6	60
11	37	60
12	15	30
13	6	100
14	22	45
Mean ± S.D.	13 ± 9.8	46.5 ± 53.1

[+]: Dogs were maintained under perntobarbital anesthesia for 90 minutes before sampling blood for PRA.

TABLE XX - Effect of light intravenous anesthesia on peripheral Plasma Renin Activity (PRA).

Dog no.	Weight (Kg)	Anesthesia		PRA (ng/ml/12 hours)						
		Agent+	Volume (ml)	Time-interval (minutes)						
				0	10	30	45	60	80	120
1	7.7	P	3	37.5	50		30			
2	15.4	P	8	5		18.7		5		5
3	10	P	5.5	0	0	10		6.2		6.2
4	14	C	200	15	6.2			5		0
5	10	C	150	6.2	10	15		25	15	
6	10.9	C	185	0	6.2				15	

+: P: pentobarbital 60 mg/ml.

C: α-chloralose, saturated solution in NaCl 0.9%.

30 minutes following pentobarbital anesthesia. In the dogs where plasma renin activity did not increase under chloralose anesthesia, there was on the contrary a disappearance of plasma renin activity.

b) Renal renin content

The left kidney and then the right one were removed by a flank incision from 4 normal dogs under pentobarbital anesthesia and assayed for renal renin content. Individual values as well as the mean and S.D. are presented in table XXI. The mean renal renin content was found to be essentially the same in both kidneys.

To evaluate the effect of angiotensin on the zonal distribution of renal renin, kidneys were removed by a flank incision under chloralose anesthesia in 8 dogs. In all the animals used, the left kidney was the first one to be removed. In four of them, the left kidney was removed after angiotensin (200 ng/Kg/min) infusion of 60 minute duration and the infusion was begun 30 minutes prior to chloralose anesthesia. Two slices of the left kidney were obtained as described previously (see procedure). After freezing, a section, passing through the middle of the kidney and excluding the medulla, was done on one slice. The lateral part of the organ was called the medial zone. Each pole was also separated from the medullary tissue. The second slice was dissected and the renal renin content was measured separately on the outer cortex, the corticomedullary junction, the medulla and the pelvis. The results of this study are given

TABLE XXI - Renal renin content of normal dogs[+].

	Renal renin content (ng/mg/1 hour)	
Dog no.	Right kidney	Left kidney
1	45	75
2	30	56
3	60	37.5
4	30	22.5
Mean ± S.D.	41.2 ± 14.3	47.7 ± 22.7
	44.5 ± 17.9	

[+]: The kidneys were removed by a flank incision under pentobarbital anesthesia.

in table XXII. In normal dogs, the renin content of the superior pole and the medial zone was comparable, but that of the inferior pole was generally lower. The greatest concentration of renin was observed in the outer cortex, whereas about 75% less was found in the corticomedullary junction. Very low values were found for the medulla, and some activity was detected in the pelvis. After angiotensin infusion, the renin content increased significantly in every zone studied, but the pattern of distribution remained the same.

6) Discussion

a) Substrate

i) Kinetics of renin-angiotensinogen reaction

Dog plasma contains 50% less substrate than that of humans (41). Nephrectomy is known to increase the substrate concentration to a lesser extent in dogs (82) than in rats (79- 81). The variations of substrate concentration encountered in dogs deeply anesthetized with pentobarbital or chloralose, were thought to be explained by the stimulating effect of anesthesia and laparotomy on the release of renin. Subsequently, the level of available substrate in plasma would decrease. This effect of anesthesia was previously shown in our laboratory by Strong and Tremblay (349). In dogs deeply anesthetized with pentobarbital, they frequently observed a 2.5 - to 3 - fold increase in plasma renin activity. Similar elevations were found in anesthetized humans by these same workers. The results obtained in anesthetized dogs (Table XIX and XX) confirm these findings. Moreover, McKenzie

TABLE XXII - Zonal distribution of renal renin content in normal and angiotensin-treated dogs.

Dog no	1	2	3	4	Mean ± S.D.		A-1	A-2	A-3	A-4	Mean ± S.D.	
O.C.	50	40	165	150	101.3	65.4	450	180	200	260	272.5	123.1
C.M.	5	12.5	37.5	41.5	24.1	18.1	37.5	90	120	24.5	68.0	44.8
Med.	-	-	0	10			0	-	5	0		
Pel.	0	18.7	0	-			30	7.5	0	0		
S.P.	25	30	75	60	47.5	24.0	150	100	100	60	102.5	36.9
M.Z.	25	25	50	60	40.0	17.8	300	100	100	100	150.0	100.0
I.P.	20	25	50	40	33.8	13.8	-	60	200	120	126.7	70.2
R.K.	40	20	75	85.8	55.2	30.5	-	-	-	-		

Left kidney

O.C.= outer cortex. C.M.= cortico-medullary junction. Med.= medulla. Pel.= pelvis. S.P.= superior pole. M.Z.= medial zone. I.P.= inferior pole.

R.K.= right kidney.

Dogs no. 1 to 4: Kidneys were removed by flank incision after chloralose anesthesia.

Dogs no. A-1 to A-4: Left kidney was removed by flank incision after angiotensin infusion and chloralose anesthesia.

and coworkers (350) observed that 4 hours after laparotomy, the plasma renin activity was increased on a 3-fold average. This effect on renin release was emphasized by the presence of detectable amounts of renin and angiotensin in the plasma of some nephrectomized dogs (Table VI). This finding may be explained be the presence of renin-like substances in organs other than the kidney (102, 103).

From these observations, it could be tentatively theorized that anesthesia and laparotomy increase renin release before the completion of nephrectomy. A large amount of renin may be taken up and stored in an extrarenal site and explains thereafter that the usual increase in substrate is prevented or diminished. Carretero and Gross (85) observed that infusion of renin (0.4, 2 and 8 units) into nephrectomized rats decreased the substrate concentration whereas in intact animals the substrate was not decreased except when large doses (8 units) were infused.

These possibilities persuaded us to treat the animals before nephrectomy, in order to completely suppress renin release. Salt loading in two dogs, was unsuccessful for producing a suitable substrate preparation. Infusions of angiotensin, known as a potent suppressor of renin release in both humans (179, 180, 236, 237) and dogs (238, 239), allowed us to obtain a substrate preparation sufficiently abundant for providing a zero-order reaction when incubated with dog kidney extracts.

Since progestative medications were reported to increase substrate concentration in humans (73) and rats (74), Enovid (R_x Searle) was administered by gavage in 2 dogs. Two weeks treatment failed to increase substrate concentration as reflected by kinetic studies.

ii) Studies on substrate concentration and "capacity of angiotensin generation"

Substrate concentration, as measured on 0.1 ml of plasma, increased 24 hours after nephrectomy by 3.2-fold in angiotensin-treated dogs and 2.4-fold in dogs anesthetized with pentobarbital. The absence of statistical significance between these two groups was difficult to explain. Two possible explanations may be pointed out. In that series of experiments where the substrate concentration was measured on 0.1 ml of plasma the nephrectomy was performed by a flank incision and we have avoided as much as possible opening the peritoneal cavity. In the first series of experiments, where the substrate concentration was assessed from kinetic studies, the kidneys were removed through laparotomy. Secondly, Boucher et al (12) observed that during summer months, substrate concentration was decreased in nephrectomized rats. This finding could explain the first series of experiments (kinetic studies) performed mostly during summer months, whereas the second series (substrate concentration) was done during springtime. The importance of temperature on renin release was stressed in rats by Rosenthal and coworkers (139). A 3-fold increase in plasma renin activity was found in rats exposed

to high environmental temperature (88° F) for one week. Moreover, these workers produced, by the injection of furosemide, a 6-fold increase in plasma renin activity of these rats compared to a 3-fold one in control rats kept at 72° F.

Simultaneous determinations of substrate concentration and "capacity of angiotensin generation" showed that both parameters increased and reached a maximal value 24 hours after nephrectomy and did not increase further from 24 to 48 hours after nephrectomy. This pattern was observed in angiotensin-treated as well as control (pentobarbital or chloralose anesthesia) nephrectomized dogs. A dissociation between these two parameters was observed in dogs from 24 to 48 hours after nephrectomy by Smeby et al (88). The major source of the differences between our results and theirs resides in our use of hog renin instead of dog renin to measure the "capacity of angiotensin generation".

Finally, it seems that angiotensin infusion prior to anesthesia and nephrectomy, by preventing the liberation of renin due to deep anesthesia in some dogs, permits a maximal increase in substrate concentration after nephrectomy.

b) Procedure

The method described has the advantage of measuring renin on a relatively small amount of dog plasma (1 ml). It is based on the incubation of whole plasma in presence of an excess of homologous substrate and Dowex 50W-X2 (NH_4^+) resin for a long period.

The substrate obtained from one dog nephrectomized after angiotensin infusion permits 50 to 90 determinations of renin activity. The purification of substrate according to Haas et al (150) is easy to perform and the absence of renin in that preparation is obtained by the use of plasma from nephrectomized and angiotensin pre-treated dogs. The substrate concentration necessary to provide a zero-order reaction is determined on each new batch. At the same time, substrate is incubated alone without renin to ensure the absence of spontaneous pressor substances.

When zero-order kinetics apply, the rate of formation of angiotensin is proportional to the amount of renin. These conditions are satisfied in our incubation system when the angiotensin formation is not exceeding 400 ng, a value above the physiological range. When one is interested in studying changes at minimal levels, the time of incubation may be prolonged from 12 to 24 hours or conversely 2 ml of plasma may be incubated.

The presence of "kinin-like" substances was found in the samples when plasma was incubated with substrate. The origin of these substances was unknown, but they were not interfering with the amount of angiotensin measured by rat blood pressure assay.

The optimal pH for the hydrolysis of dog substrate with dog renin is 5.5 to 6.5. The recovery experiments done at pH 6.5 were variable. Such a pH is close to the optimal pH of

angiotensinases in the plasma (26, 43-46), and may explain the variability encountered. A mean recovery of 68% in 3 experiments was found when the incubations were carried out at pH 5.5. On the basis of these results, a pH of 5.5 was chosen for the incubation mixture. Tris-phosphate is used in the medium to provide a better pH control, specially when plasma is incubated.

The pressor product obtained from our incubations increased the blood pressure of nephrectomized rats in the same manner as standard angiotensin and is most probably angiotensin I, since the converting enzyme is inhibited when the incubation is carried out at pH 5.5 in presence of EDTA (351).

The mean normal plasma renin activity in dogs eating Purina dog chow and drinking tap water ad libitum was not different from that obtained in dogs under metabolic balance, despite the smaller standard deviation found in the last group. The wide range of values for plasma renin activity encountered in normal dogs could not then be explained solely by variations in sodium intake. Such a range of plasma renin activity in normal dogs is well known (187).

The zonal distribution of renin was measured in normal kidneys and in kidneys obtained from dogs infused with angiotensin. Renin was mainly located in the outer cortex of kidney of normal dogs. This finding confirmed results obtained by other workers (116, 119, 120). Angiotensin infusion increased significantly the renin content in each part of the kidney, and supported the suggestion that angiotensin might

act by a "feed-back" mechanism (179, 236-239) to suppress renin release.

B - RENIN-ANGIOTENSIN SYSTEM IN NEWBORN DOGS

1) Material and Procedures

Plasma renin activity, renal renin content, substrate concentration and juxtaglomerular index were studied in newborn dogs. Twenty-one normal dogs from four gestating mongrel dogs were studied after 12 hours (11), 48 hours (3), 8 days (1), 12 days (2), 14 days (2) and 15 days (2) of life (Table XXIV and XXV). All the newborn dogs were separated from the mother, only a few minutes before performing the experiment.

A small incision (0.5 cm) was done above the internal third of the clavicle and 2.5 to 4 ml of blood were rapidly withdrawn from the jugular vein. Six out of the 21 newborn dogs were anesthetized and uninephrectomized (left kidney) before sampling blood. The plasma obtained after centrifugation was processed for plasma renin activity. One ml of plasma was incubated for each experiment and a second aliquot of 0.5ml was incubated when the angiotensin formed with one ml of plasma exceeded 400 ng for 12 hours of incubation. Venous blood was also sampled from the forelimb of three mothers, at the time of delivery, and processed for plasma renin activity. The renal renin content and juxtaglomerular index were determined after removing the kidney under light pentobarbital anesthesia. The renal renin content was measured on whole kidney, in 15 newborn and the juxtaglomerular index in 13.

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Eleven of 21 newborn dogs were subsequently used to study the effects of peritoneal dialysis on plasma renin activity (6 dogs 12 hours old, 4 dogs 12-14 days old), renal renin content (4 dogs 12 hours old and 2 dogs 12-14 days old) and the juxtaglomerular index (4 dogs 12 hours old). Each parameter was measured before and 30 minutes after removing the liquid from the abdomen. Peritoneal dialysis experiment was performed after left uninephrectomy in four 12-hour-old and two 14-day-old dogs. Table XXIII gives details about the parameters measured before and after peritoneal dialysis.

The peritoneal dialysis was performed by injecting the animal intraperitoneally with 10 ml of a 5% glucose solution per 100 grams of body weight. The solution was left in the peritoneal cavity for 60 minutes. Then 18 gauge needle was placed in the lower left quadrant and the peritoneal dialysate was allowed to flow freely without applying any pressure to the abdominal wall. Between 80 and 95% of the injected volume was withdrawn. Sodium and potassium were determined in the dialysate and the values were reported in mEq per kg of body weight.

2) Results

The plasma renin activity was found to be very high in newborn dogs 12 and 48 hours old. The mean value was 307 ng/ml/12 hours (S.D. $\pm$ 205) ranging from 50-800 ng. In these animals, the renal renin content was slightly higher (Mean 65.1 ng/mg/1 hour S.D. $\pm$ 24.1) than in normal adult dogs.

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TABLE XXIII - Various parameters before and after peritoneal dialysis.

Age	Dog no.	PRA	RRC	JGI
12 H	36 G $^{+}$		+	+
	36 D $^{+}$	+	+	+
	36 GD^{+}	+	+	+
	36 G2^{+}	+	+	+
	36 D2	+		
	36 S	+		
	41 D	+		
12 D	23 G	+		
	23 D	+		
14 D	42 GD^{+}	+	+	
	42 G2^{+}	+	+	

PRA: Plasma renin activity.

RRC: Renal renin content.

JGI: Juxtaglomerular index.

$^{+}$: The experiment was performed in uninephrectomized dogs.

Despite these high values of renal renin content as compared to adult dogs, the juxtaglomerular index (Mean 0.3, S.D. ± 0.5) was significantly lower than in adult dogs. The substrate concentration as determined in three 48-hour-old puppies was in the range of normal values encountered in the adult dog. The juxtaglomerular cell granules observed in the puppies from mother C were located mostly in the juxtamedullary cortex. In all the animals studied, non granulated cells as well as macular segments were observed mainly in the deeper parts of the cortex.

In the older group (8 to 15 days old), the plasma renin activity was still high with a mean of 285 ng (S.D. ± 186) and the renal renin content with a mean value of 68.6 ng was comparable to the younger group. In the two dogs 15 days old, the juxtaglomerular index was higher than in the first 48 hours of life. Substrate concentration in the two 15-day old puppies was found to be normal in one and slightly above the normal range in the other one. The plasma renin activity, renal renin content, juxtaglomerular index and substrate concentration values in the early-uterine life are reported in Tables XXIV and XXV.

Statistical analysis relating plasma renin activity to renal renin content showed an absence of correlation between both parameters in the first fifteen days of extra-uterine life, with a correlation coefficient, $r = 0.3$.

The plasma renin activity determined at the time of delivery in three out of four mothers was within the normal range.

TABLE XXIV - Plasma Renin Activity (PRA), Renal Renin Content (RRC), Juxtaglomerular Index (JGI) and Substrate Concentration (SC) in newborn dogs (12-48 hours old).

Mother	Newborn dogs							
	Age	No.	Sex	Weight	PRA	RRC	JGI	SC
A	12 H	22G	M	426	333	38	0	
		22D	F	408	266	43	0	
		22GD	F	430	200	50	0	
C		36G	M	540	500+	70	1.5	
		36D	M	530	100+	50	0.5	
		36GD	F	515	400+	70	0	
		36G2	F	570	500+	120	1	
		36D2	F	534	800	-	-	
		36S	F	547	400	-	-	
D		41G	F	184	65	-	-	
		41D	M	232	200	-	-	
B	48 H	15G	M	380	50	85	0	20
		15D	F	338	320	70	0	25
		15GD	M	366	166	55	0	20
Mean					307	65.1	0.3	21.6
± S.D.					205	24.2	0.5	
Normal adult dogs								
Mean					12	44.5	11.6	15.9
± S.D.					11.9	17.9	2.1	6.7

+: PRA was determined immediately after left nephrectomy under pentobarbital anesthesia.

TABLE XXV - Plasma Renin Activity (PRA), Renal Renin Content (RRC), Juxtaglomerular Index (JGI) and Substrate Concentration (SC) in newborn dogs (8-15 days old).

Mother	Newborn dogs							
	Age	No.	Sex	Weight (g)	PRA	RRC	JGI	SC
A	8 D	42G2	M	600	333	113	0	
A	12 D	23G	M	950	560	-	-	
		23D	M	1050	320	-	-	
D	14 D	42GD	M	570	300+	40	-	
		42G2	M	483	400+	50	-	
B	15 D	16G2	M	1125	50	65	3	25
		16D2	M	1200	37	75	3	50
Mean					285	68.6	2	37.5
± S.D.					186	28.2	1.7	
Normal adult dogs								
Mean					12	44.5	11.6	15.9
± S.D.					11.9	17.9	2.1	6.7

+: PRA was determined immediately after left nephrectomy under pentobarbital anesthesia.

In the peritoneal dialysis experiments, a mean of 7.8 mEq (± S.D. 1.37) sodium and 0.3 mEq (± S.D. 0.07) potassium per Kg of body weight were removed with the dialysate. In the 12-hour-old puppies, peritoneal dialysis effected a 1.5-to 6-fold increase in plasma renin activity (Figure 19). A two-fold mean increase was found in that group. The animal presenting the 6-fold increase, started from a relatively low control value of plasma renin activity (100 ng). In the 12 to 14-day-old dogs, the plasma renin activity increased from 2- to 3-fold following peritoneal dialysis (Figure 19).

This increase in plasma renin activity following peritoneal dialysis was consistent in each experiment despite the removal of the left kidney before peritoneal dialysis in 5 of the animals studied. Renal renin content was decreased by dialysis in four 12-hour-old and two 14-day-old puppies (see Figure 20). The left kidney removed in three dogs after peritoneal dialysis showed a renal renin content comparable to the values found in the right kidney after peritoneal dialysis (40, 40 and 70 ng/mg/1 hour). In the 12-hour-old dogs where renal renin content was measured before and after peritoneal dialysis, the juxtaglomerular index was not found to be altered by this experimental procedure as shown in figure 20.

3) Discussion

The role and importance of the fetal kidney in the elaboration of renin is not well defined. The granulated

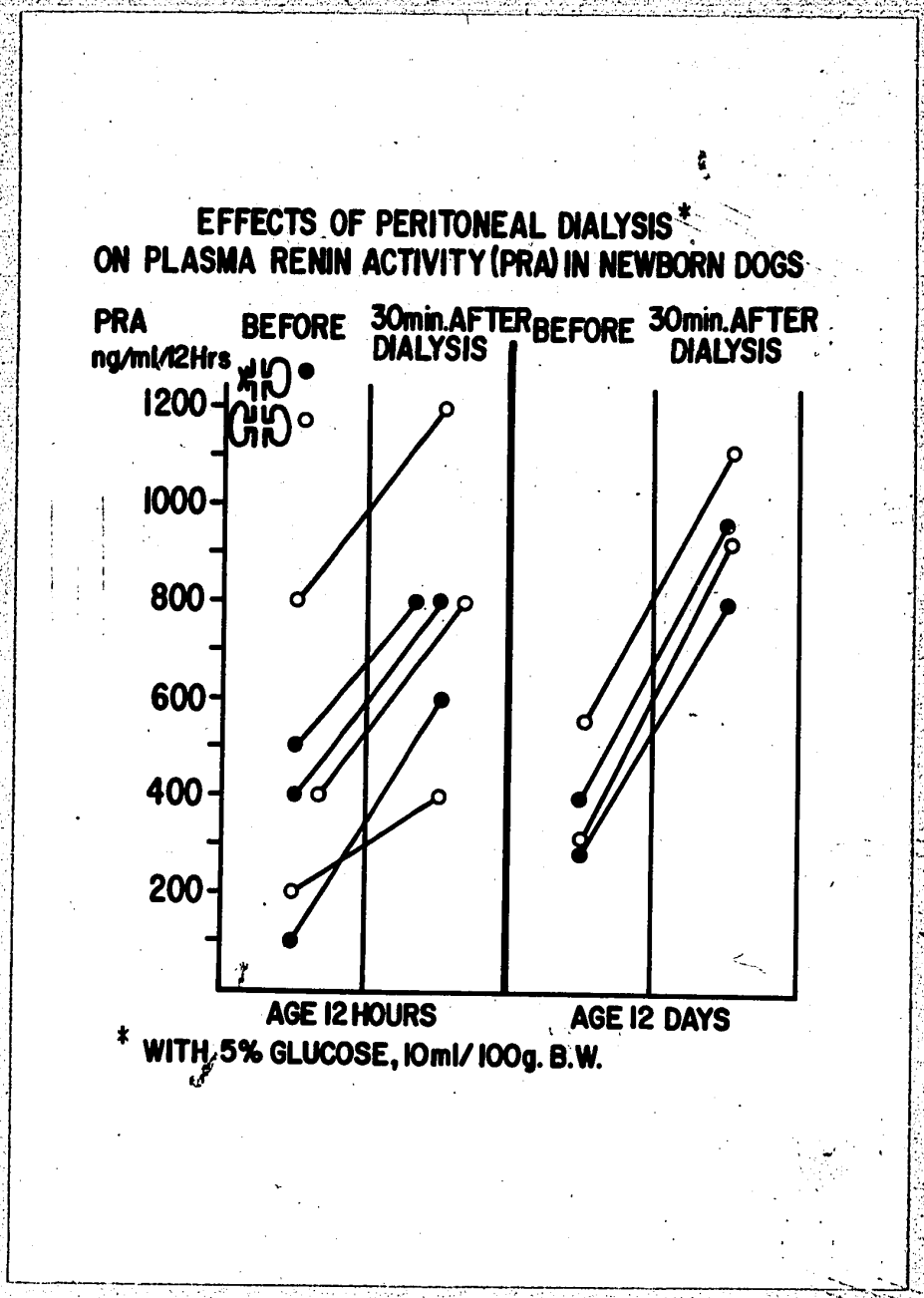


Figure 19

Following peritoneal dialysis, PRA increases from 1.5- to 6-fold in intact and uninephrectomized animals.

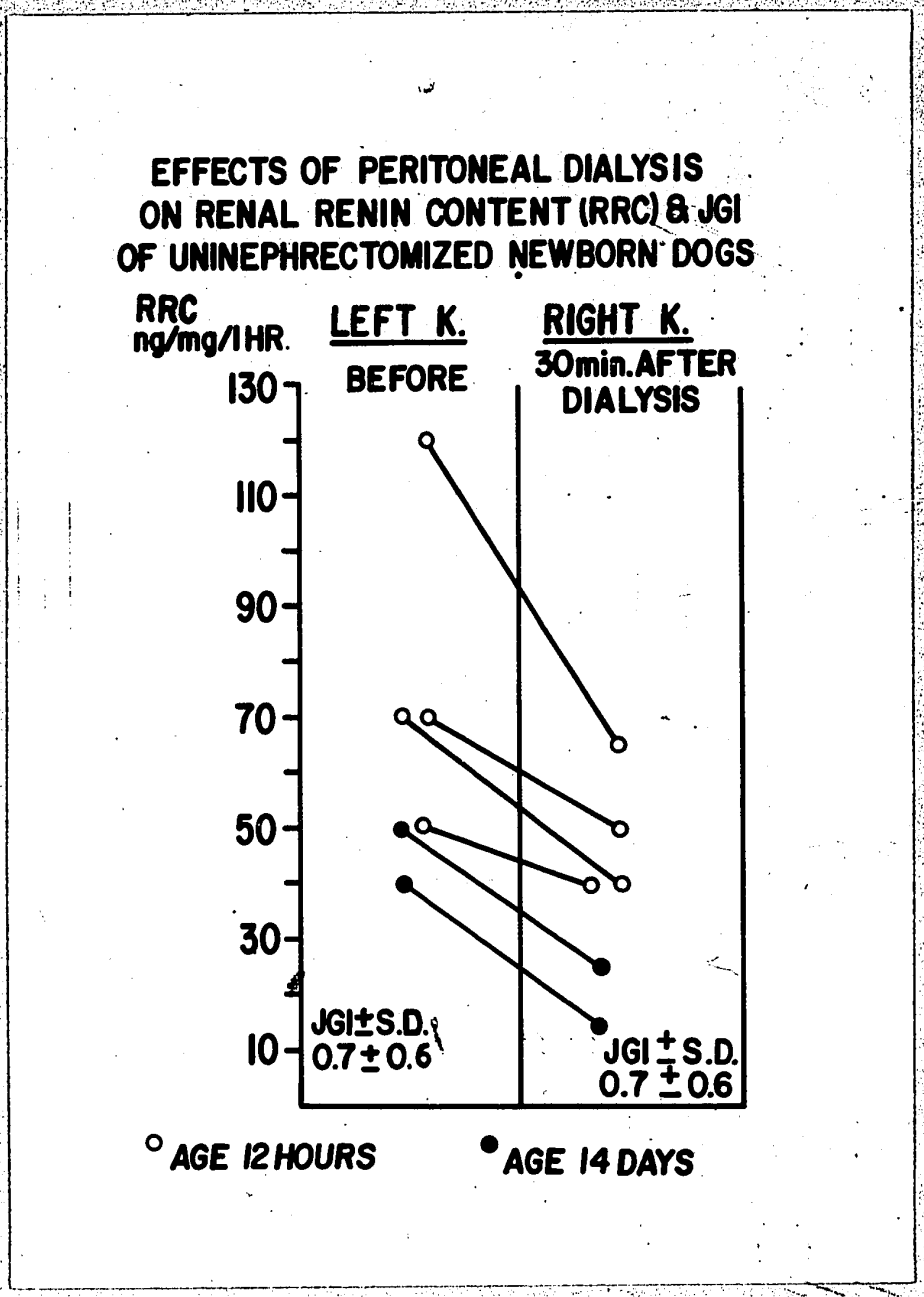


Figure 20

RRC decreased following peritoneal dialysis.

JGI determined in 12 hour old dogs.

juxtaglomerular cells are absent, at least in the first week of extrauterine life, in mice (104, 201) and rats (345). Recently, Hodari and coworker (181, 342) detected a measurable amount of renin in normal dog fetal kidneys.

The main purpose of this investigation was to present a more complete evaluation of the renin-angiotensin system in newborn dogs, during the first fifteen days of life. The plasma renin activity values were much higher than those found in adult dogs, and they were not related to the renal renin content. The renal renin content values were comparable to those found in adult animals, whereas the juxtaglomerular cell granules were either few or absent. The pattern of distribution of juxtaglomerular granulation found in the first two weeks of extrauterine life in mice and rats (104, 201, 345) was also observed in our newborn dogs. The finding of such high values of plasma renin activity raised the question of the origin of the circulating renin in the newborn. Since renin has been found in placenta (103, 181, 339, 340) and uterus (103, 343, 344), and since it can pass the placental barrier (342), it could possibly originate from the mother or the placental-uterine unit. The first possibility seems improbable since the plasma renin activity measured in 3 mothers at the time of delivery was within the normal range. Moreover, the relatively short half life of renin in dog (91, 92) does not support a maternal uterine or placental contribution to the renin activity of newborn dogs after 12 hours of extrauterine life or more.

The renal origin of the circulating renin found in our newborn dogs is suggested by the important amount of renin measured in the kidney. Supportive evidence to confirm this suggestion was brought by the stimulation of renin release by sodium depletion in the peritoneal dialysis experiments. These have been shown to be a potent stimulus for renin release in adult animals (86, 136, 138). The peritoneal dialysis increased plasma renin activity and decreased the renal renin content whereas no concomitant changes were encountered in juxtaglomerular index. The decrease found in renal renin content is suggestive of a storage and release of renin or renin precursors by the kidney despite the absence of juxtaglomerular cell granules.

The existence of such a system cannot be discounted in the adult and could possibly explain the discrepancies encountered between plasma renin activity and the juxtaglomerular index or renal renin content in some experimental conditions (86, 136-139).

CHAPTER IV - STUDY OF ACUTE CHANGES IN RIGHT AND LEFT ATRIAL PRESSURES ON RENAL VEIN PLASMA RENIN ACTIVITY IN DOGS

The suppression of plasma renin activity in primary aldosteronism is most probably explained by the sustained increase in plasma volume. In patients with congestive cardiac failure, the activity of renin angiotensin system is increased despite a normal or increased plasma volume. Although it is postulated that in such patients the "effective circulating volume" is reduced (352), the decrease in plasma renin activity or arterial angiotensin observed in these patients after treatment remained unexplained.

Since one of the earliest and most reliable signs of cardiac failure is increased venous pressure, we have investigated the effects of changes in the right and left auricular pressure on renal venous renin activity.

A - MATERIALS AND PROCEDURES

Mongrel dogs were anesthetized with intravenous pentobarbital sodium (30 mg/kg). After performance of the surgical techniques, isotonic saline was infused (5-10 ml/kg) to replace the blood loss and provide a positive fluid balance. All the experiments were begun 60 minutes after the completion of the infusion. Pentobarbital was then repeated if necessary at least 45 minutes before the beginning of the experiment and was not repeated afterwards except in one dog (Figure 27).

1) Right atrium experiments

In 9 dogs weighing from 10-18.2 kg, a polyethylene catheter (PE-200) and a Foley catheter (Bardex 30 cc) with its lumen tied off were introduced through the right external jugular vein up to the right atrium. The PE-200 catheter was used to measure the venous pressure. A tracheostomy was also performed. The femoral vein and femoral artery were isolated by dissection of the inguinal area. A polyethylene catheter (PE-200) was introduced into the femoral artery and guided in the abdominal aorta just above the level of the renal arteries. Two PE-200 polyethylene catheters were bent in order to fit into the renal veins for blood sampling. They were inserted into the femoral vein and guided by hand, one in the right renal vein and the other in the left one, after laparotomy. Another (PE-200) catheter was also introduced into the femoral vein and guided in the abdominal vena cava just above the level of the renal veins, for registering venous pressure (Figure 21).

Arterial pressure and the different venous pressures were monitored on a Statham Gauge (Model P23AC) and measured with a Grass Polygraph. The calibration of the channels used for venous pressure measurement was done with a water column. The polyethylene catheters (PE-200) were connected with a three-way stopcock to a Grass Polygraph (right atrium, abdominal aorta and inferior vena cava catheters) or to a syringe for sampling (renal vein catheters), and to a bottle of glucose 5%. Glucose was infused at a constant flow rate (0.1 ml/min) in order to avoid clotting. Each catheter was siliconi-

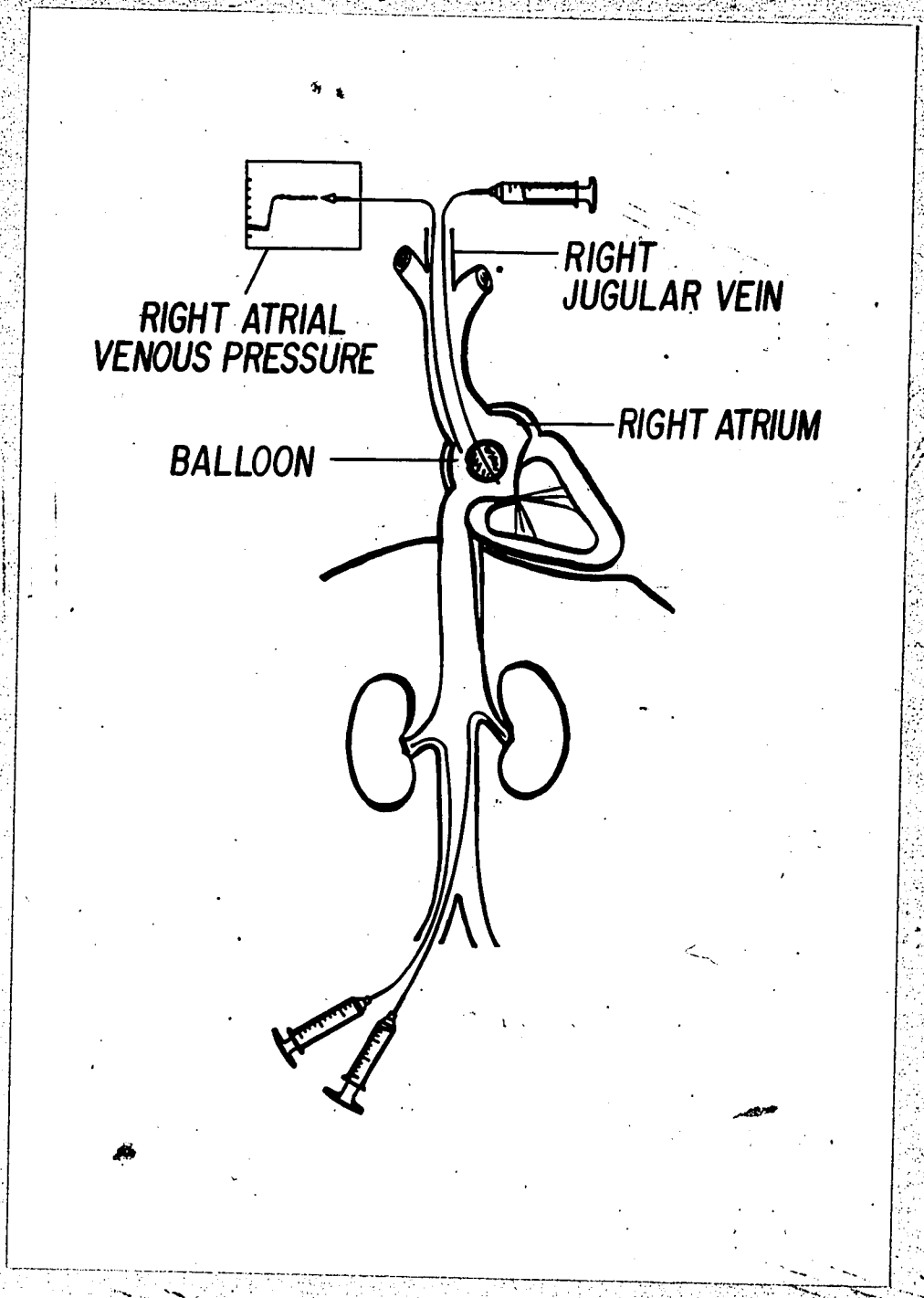


Figure 21 - Experimental preparation for the study of the effects of changes in right atrial pressure on renal venous plasma renin activity.

zed before every experiment.

In five animals, a cutaneous electrode was installed at the apex and an electrocardiogram taken. Venous pressure in the inferior vena cava just above the level of the renal veins was registered in five dogs. The plasma renin activity was measured in the left renal vein of each animal and in the right renal vein of 6 dogs. The arterial blood pressure and the right atrial venous pressure were obtained in every animal studied.

Following a control period, the balloon (Foley Catheter) was inflated by injection of water. The different venous pressures and the arterial pressure were monitored and simultaneous sampling of blood from renal veins were obtained at various intervals during the periods of higher right auricular pressure as well as before inflation and after deflation. A gradual inflation of the balloon without deflation was done only in experiment 1 (Figure 23).

At the end of the experiment, the animal was killed by the injection of a lethal dose of pentobarbital. An autopsy was performed to check the proper position of every catheter and the macroscopic aspect of the kidney. The right atrium was opened after inflating the balloon of the Foley catheter to ensure that it was not obstructing the flow of the inferior or superior vena cava.

2) Left atrium experiments

a) balloon

In five dogs (18.5-24.6 kg), the right external jugular

vein, the femoral vein and artery were dissected. A PE-200 catheter was introduced into the right atrium via the jugular vein for measurement of venous pressure. Another polyethylene catheter (PE-200) was introduced into the femoral artery and pushed in the abdominal aorta just above the emergence of renal arteries. This catheter served to measure arterial blood pressure. A third polyethylene catheter (PE-200) bent in order to fit into the left renal vein, was inserted in the femoral and inferior caval vein and guided by hand into the left renal vein after laparotomy and it was used for renal vein blood samples. The left ureter was also catheterized before closing the abdomen. Urine flow rate (µL/min) and natriuresis (µEq/min,) were determined.

After opening the trachea, an occlusive intratracheal tube was inserted and the lungs were ventilated artificially with a Harvard respiration pump (Model 607D). The rate and stroke of the ventilator were adjusted according to the animal's spontaneous respiration under anesthesia. The respirator circulated air taken from the room. On the outlet, an intermittent hyperinflation valve was intercalated and was used only during the thoracostomy in order to provide maximal lung expansion.

The chest was then opened through the fourth left intercostal space. After retraction of the lung, the pericardium was opened at 1 cm above the left phrenic nerve. The edges of opened pericardium were fixed by silk (3-0) to the thoracic wall. After taking a part of the origin of left atrial

appendage in the tip of a gall duct forceps, a 3-0 silk was sutured in an O-shape around the forceps. An incision of 0.5 cm in the atrial tissue retained by the forceps was done. A Foley catheter with its lumen tied off and a PE-200 polyethylene catheter were inserted into the left atrium. With the help of a second assistant to tie off the O-shape suture, it was possible to open the forceps and introduce the two catheters into the left atrium with minimal blood loss (10-30 ml). The PE-200 catheter was used to register venous pressure. The pericardium was then freed from his suture and the thorax closed.

Right and left atrial pressure, arterial blood pressure, urine flow rate, sodium and potassium in the urine, left renal vein renin activity and heart rate were measured at different intervals before, during and after inflation of the balloon. Venous and arterial pressures were measured with a Grass Polygraph.

b) dilator

We designed a water-tight apparatus which could distend the wall of the atrium with minimal obstruction to the flow. It consisted of a copper tube of 13 cm long covered with tygon tubing and sealed with epoxyresin. The copper tube plus the covering Tygon had an external diameter of 6 mm. Eight stainless steel wires were fixed on the tip of the tube and entered the tube at 3 cm from the extremity. At the other end of the tube, these metallic threads could be manipulated in order to open or close the dilator. The intensity

of the stimulation was defined by the outside diameter in cm ("stretch diameter") of the opened dilator (Figure 22).

The experimental preparation was exactly the same as in the preceding experiment except that right and left atrial pressures were not registered and the Foley catheter in the left atrium was replaced by the dilator. Urine was obtained from the left ureter in 2 dogs. This experiment was performed in four dogs ranging in weight from 21 to 37.7 kg.

B - RESULTS

1) Right atrium experiments

The inflation of the balloon had only transient effects on systolic blood pressure and heart rate. It did not increase significantly the venous pressure in inferior vena cava (Figure 24-26, 30, 31). In the first experiment, the balloon was inflated progressively. A fall in blood pressure (115 to 60 mm Hg) was noted after 20 ml inflation as well as a further fall in blood pressure (40 to 20 mm Hg) and a heart block after 30 ml inflation (Figure 23). Although the degree of stimulation used in the other experiments was not affecting the heart rate and the blood pressure level, the increase in the right atrial venous pressure was not proportional to the inflation of the balloon. This was observed in experiments 1,2,3 and 4 (Figure 23-26). Despite the absence of correlation between the stimulus used and the response observed in venous pressure, the application of the stimulus induced at least a two-fold increase in right atrial venous pressure.

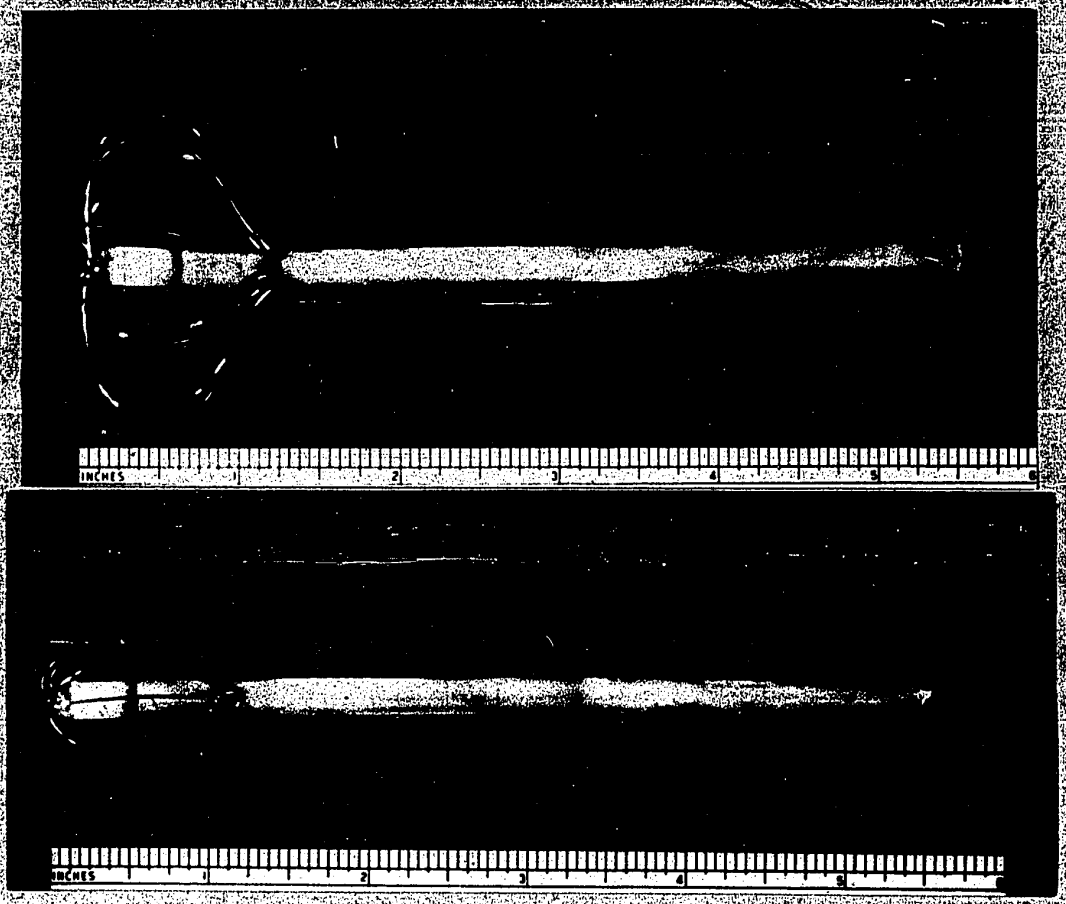
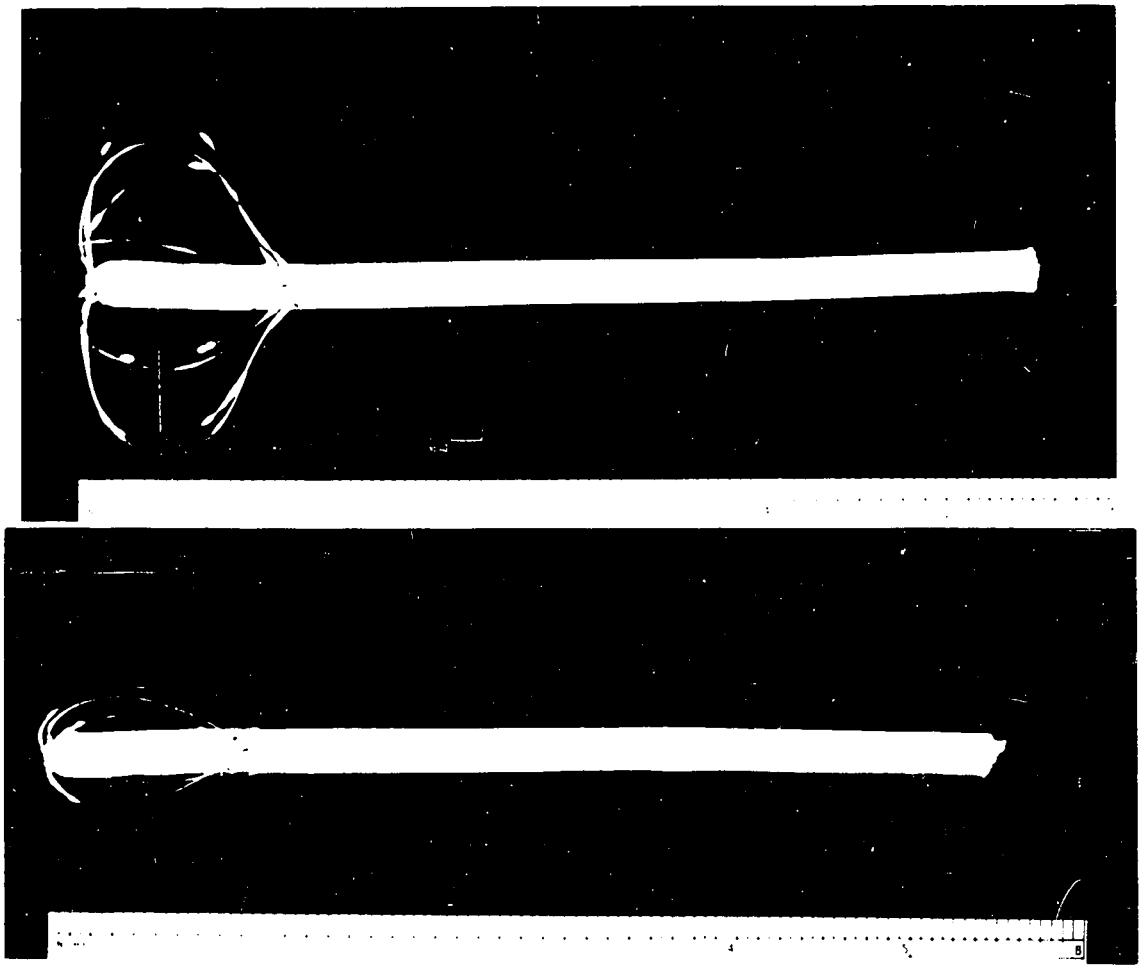


Figure 22 - Apparatus devised for atrial dilatation.

When inserted into the atrium, the dilator may be manipulated by pushing or retracting the wires shown on the right side to the desired "stretch diameter".



In five out of nine dogs (Figure 23-25, 27, 30), renal vein plasma renin activity increased following a rise in the right atrium venous pressure. In these 5 experiments, it was possible to observe a decrease in renal venous renin activity after deflation when right atrial pressure returned to normal. The stimulus appeared to be short-lived specially in experiment 2 and 3 (Figure 24,25). In experiment 8 (Figure 30) an unexplained change in the base line level of renal venous renin activity appeared on the 60th minute. Only a slight increase in renal venous renin activity was observed in experiment 4 (Figure 26) following stimulation, but afterwards this activity paralleled the changes observed in venous pressure. In experiments 6,7 and 9 (Figure 28, 29, 31), the renal vein renin activity was not stimulated and did not parallel the changes in right atrial venous pressure.

2) Left atrium experiments

a) Balloon

The body weight of five animals used in this experiment was superior to that of animals used in the preceding experiment, but the balloon in the left atrium was inflated with 5 ml of water and the increase in left atrial venous pressure was comparable to the right atrium experiments. The left renal vein plasma renin activity during the inflation period was not different from the pre-inflation or post-deflation periods, except in experiment 2 (Figure 33) where two values out of four were higher than the controls. The increase in the right atrium venous pressure after the inflation of the balloon in the left atrium was minimal.

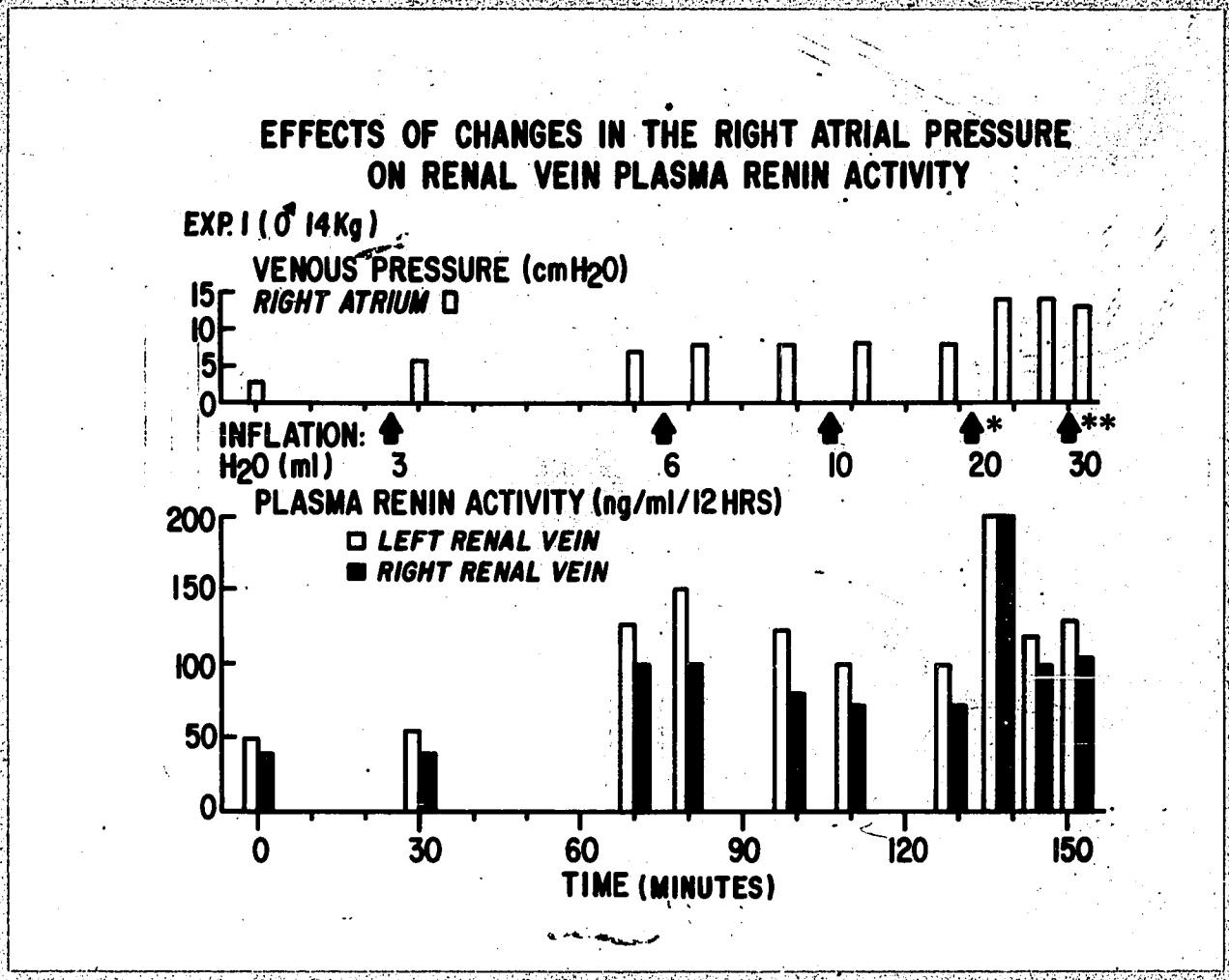


Figure 23

* Systolic blood pressure fell from 115 to 60 mm Hg.

** Systolic blood pressure fell from 40 to 20 mm Hg and a heart block was noted.

Progressive inflation of the balloon significantly increased renal venous renin activity.

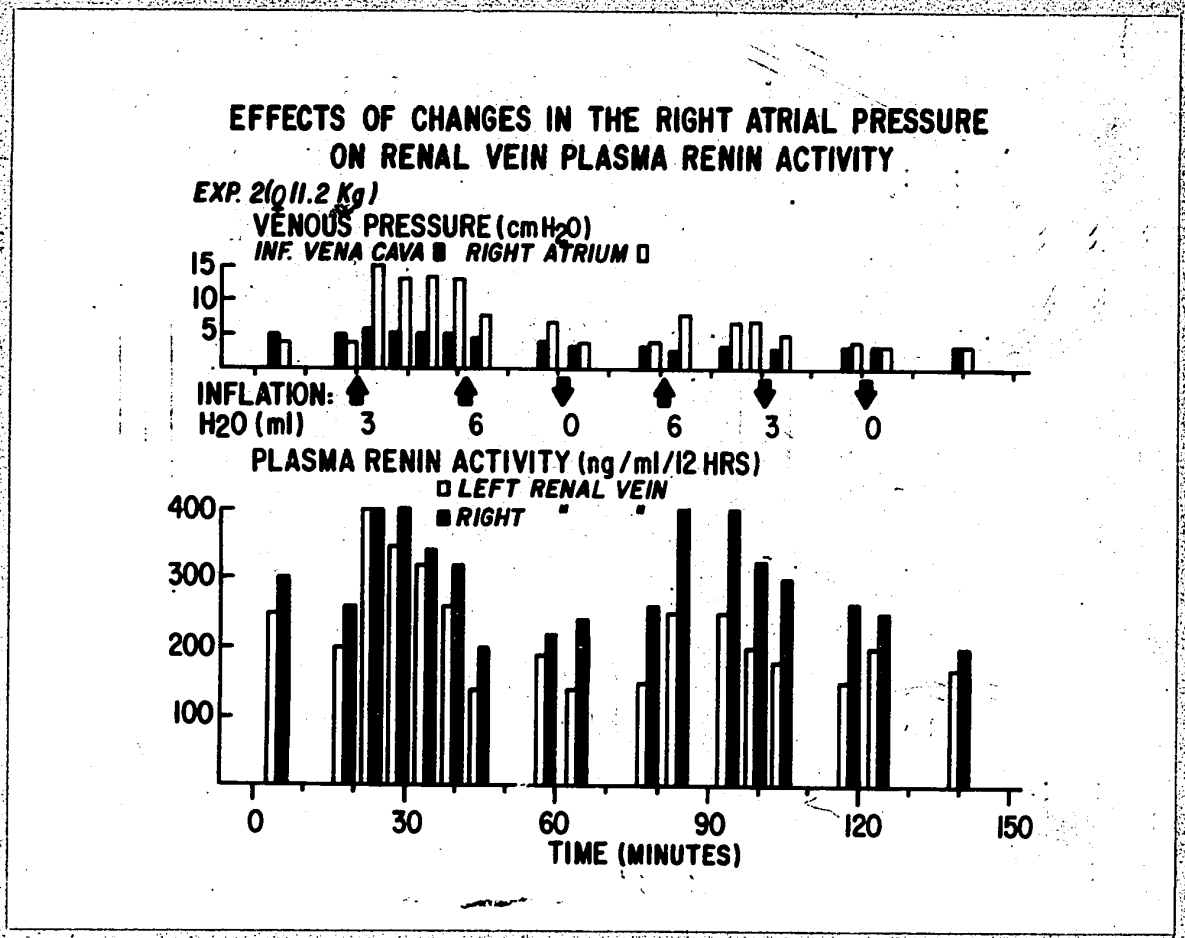


Figure 24

In two successive inflations, the plasma renin activity of both renal veins increased. Right atrial venous pressure was not proportional to the inflation of the balloon. The venous pressure in the inferior vena cava rose slightly but not to the same extent as the right atrial pressure.

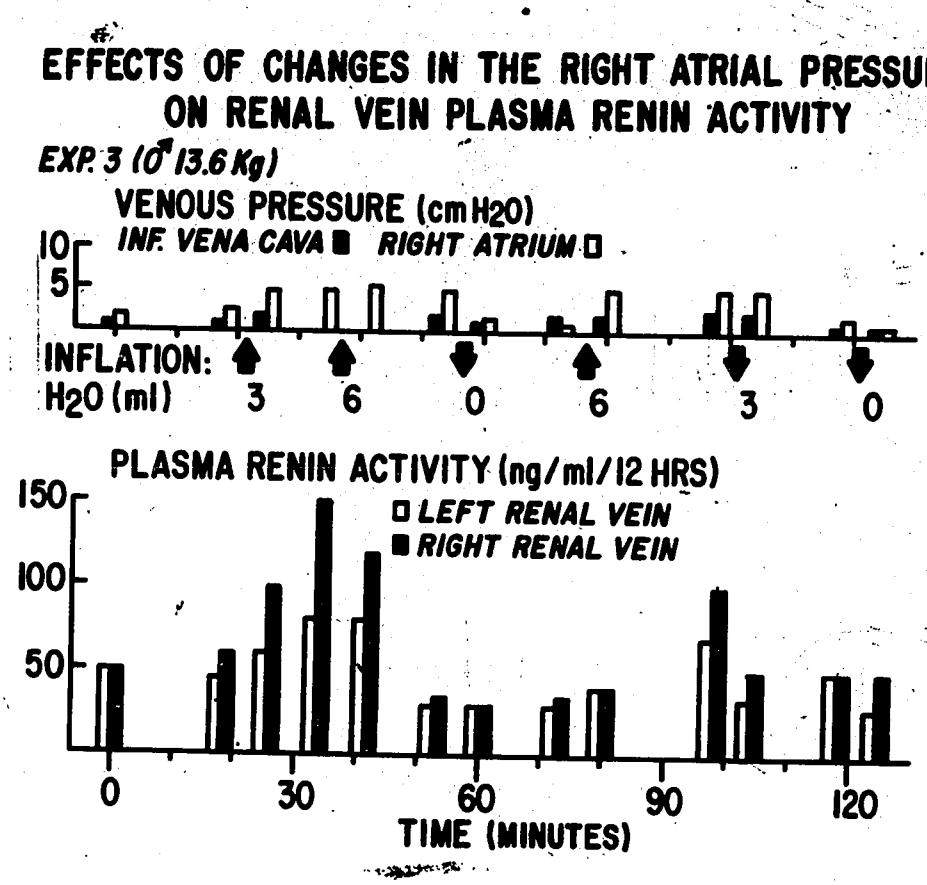


Figure 25

Plasma renin activity in both renal veins increased following inflation of the balloon in the right atrium on two successive trials. The venous pressure in the inferior vena cava remained almost unchanged when increasing the right atrial pressure.

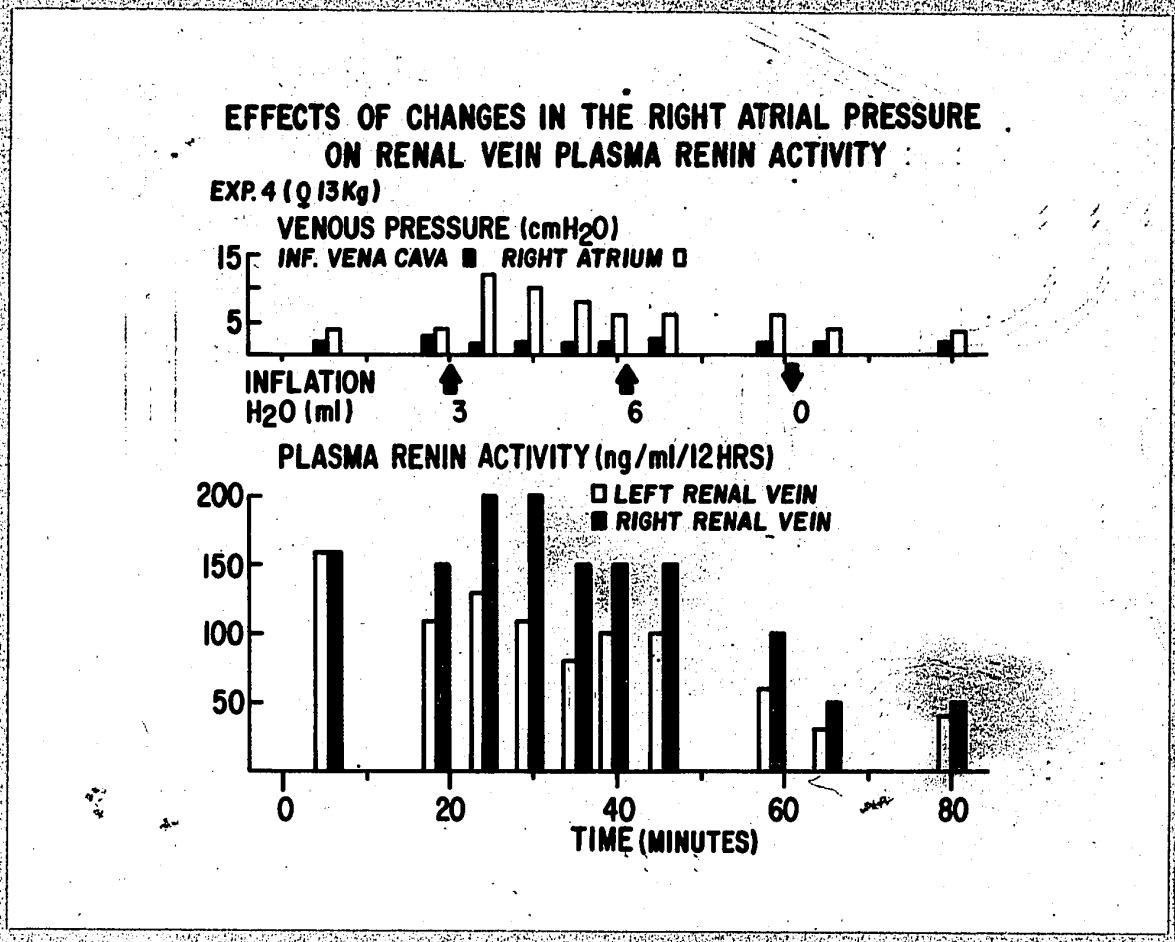


Figure 26

A slight increase in renal venous renin activity occurred following the rise in right atrial venous pressure. Afterwards renin activity paralleled the changes in venous pressure of the right atrium. Inferior vena cava pressure was not affected by the procedure.

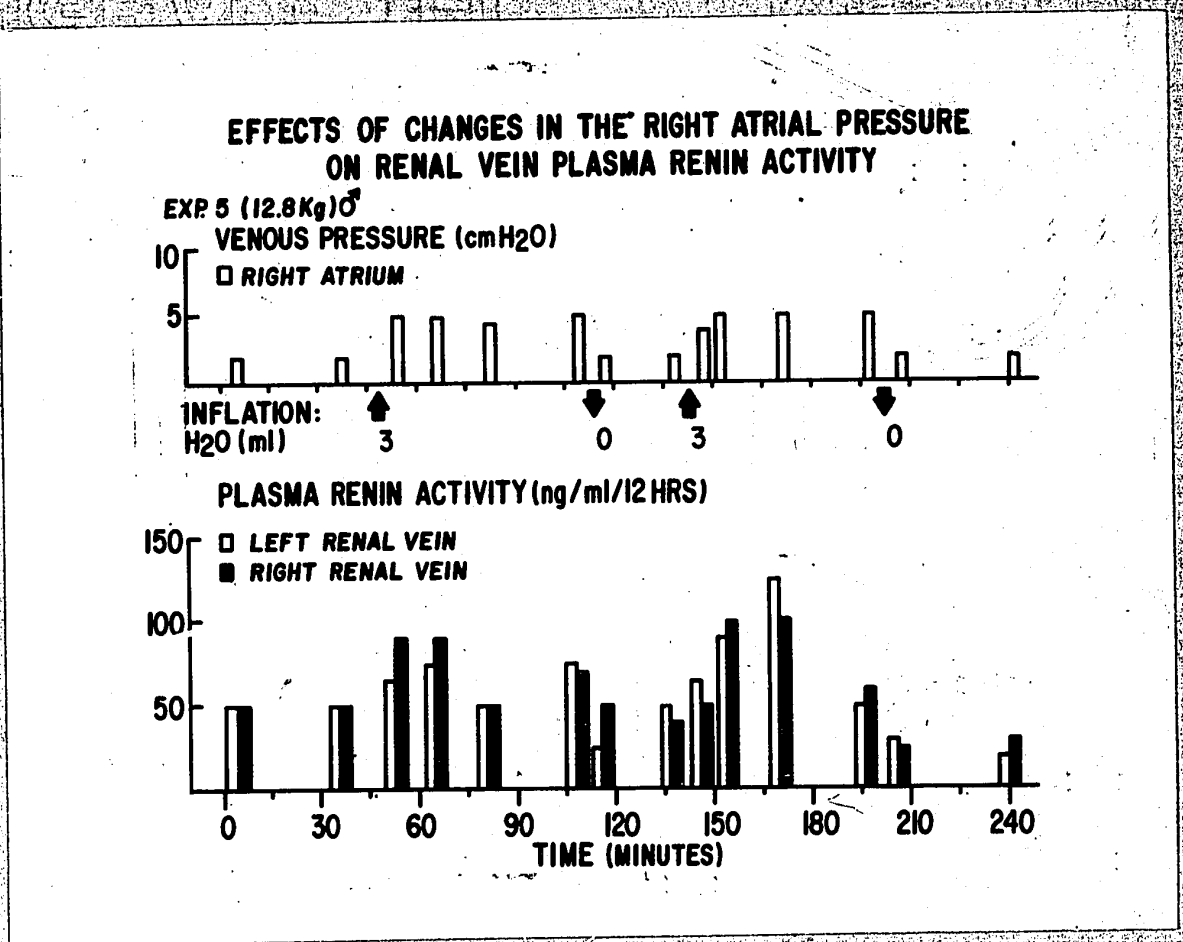


Figure 27

A temporary increase in renal venous renin activity followed each inflation of the balloon in the right atrium. Pentobarbital (30 mg) was administered at the 113th minute.

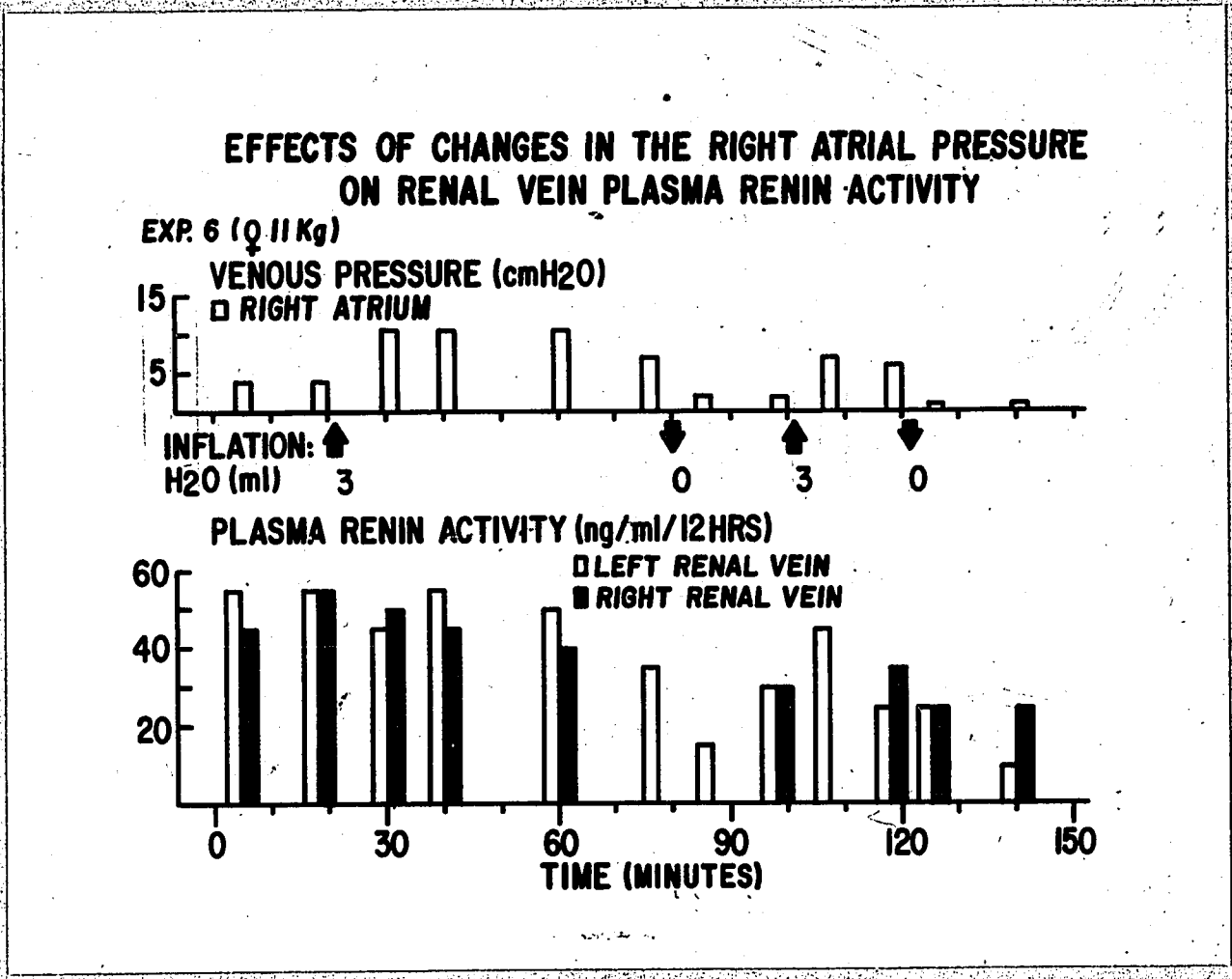


Figure 28

The inflation of the balloon increased the right atrial venous pressure but did not affect plasma renin activity.

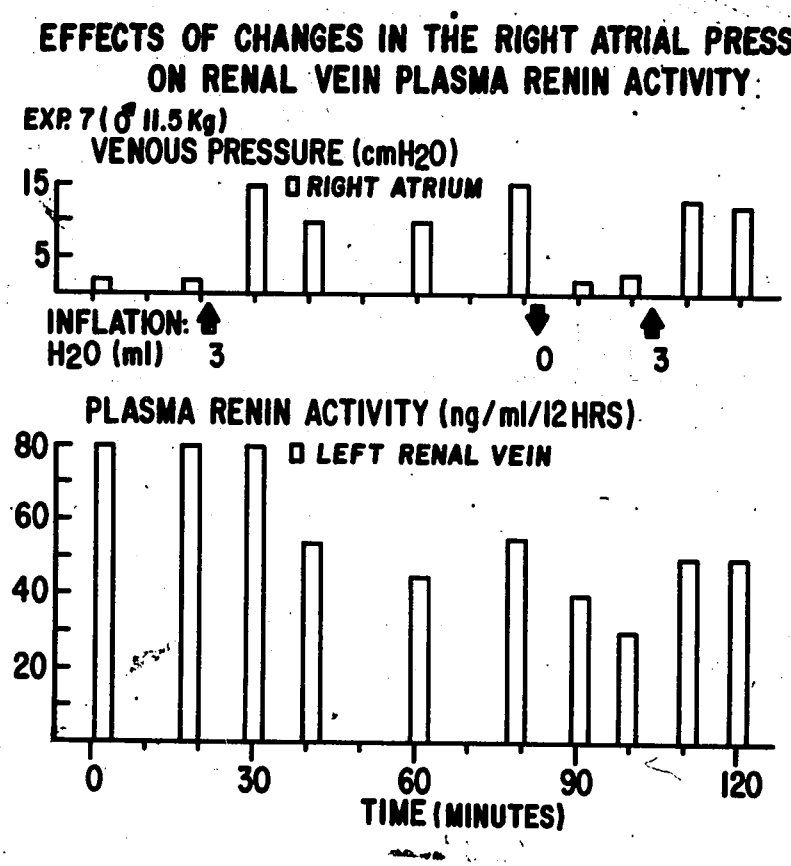


Figure 29

Despite the marked elevation in right atrial venous pressure, left renal vein renin activity was not increased.

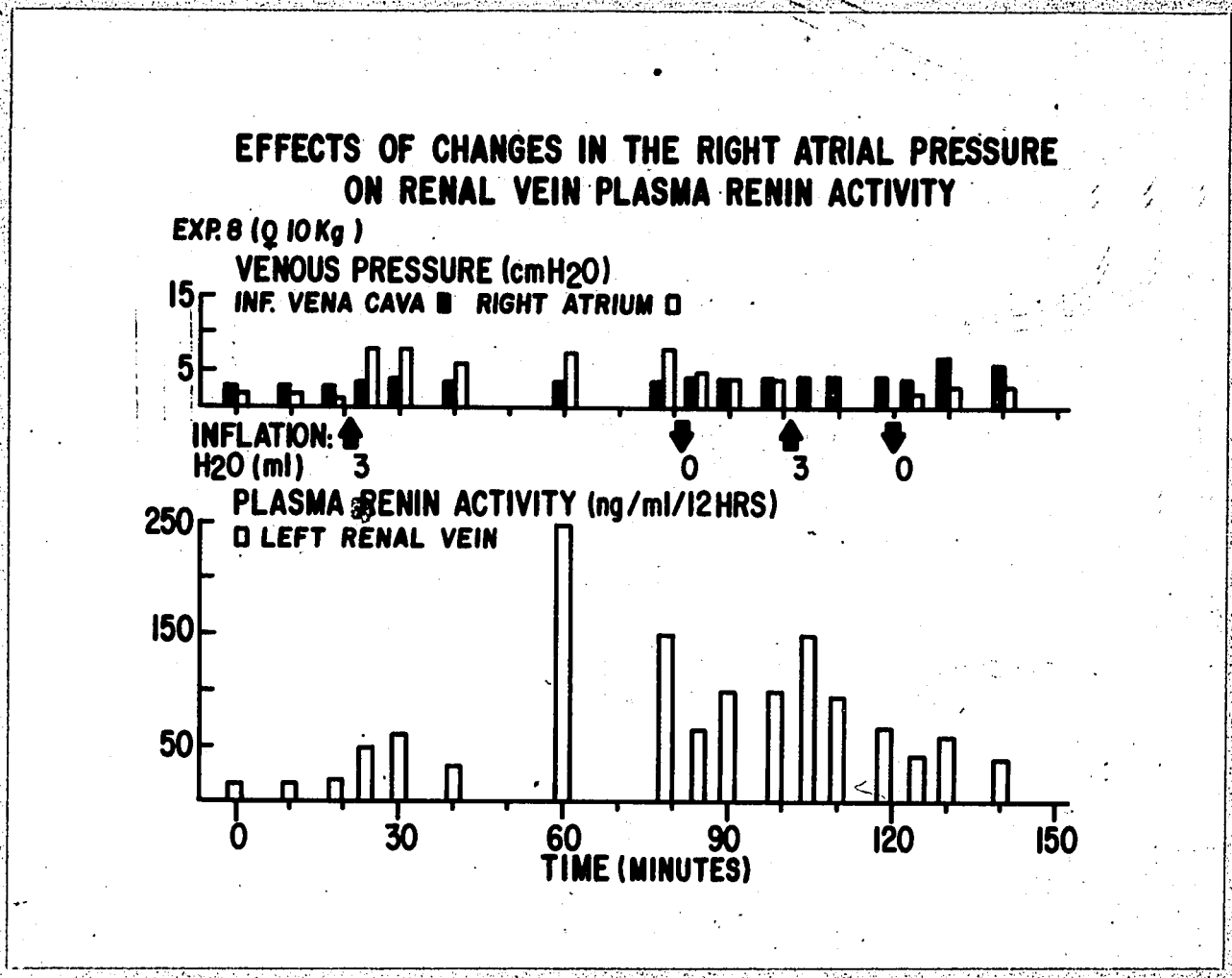


Figure 30

The right atrial pressure and renal venous renin activity rose significantly following the inflation of the balloon. The inferior vena cava pressure was not affected. An unexplained increase in the baseline level of renin activity was observed on the 60th minute of the experiment.

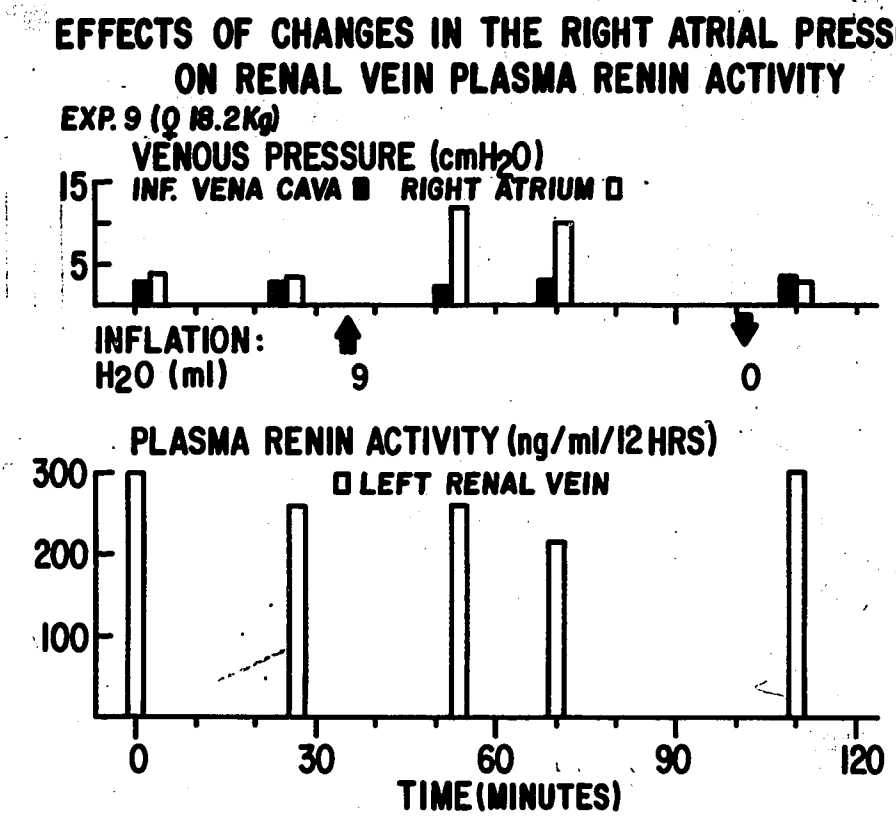


Figure 31

Renal venous renin activity and inferior vena cava pressure were not affected by the inflation of the balloon despite an important increase in right atrial venous pressure.

The urine output measured from the left ureter increased significantly only in the first experiment (Figure 32) passing from 50 to 500 µL/min. after inflation of the balloon. All the 5 individual experiments are reported in figure 32-36.

The stimulation had no effects on the level of arterial blood pressure, although the heart rate increased slightly.

b) dilator

In four dogs, the non-hypotensive dilatation of the left atrium had no effects on the left renal vein plasma renin activity, except in experiment 3 (Figure 39), where the plasma renin activity tended to decrease following the first stimulation. The dilatation of the left atrium was devoid of effects on the level of arterial blood pressure except in experiment 2 (Figure 38). In that experiment, the first dilatation (stretch diameter : 5.5) lowered the blood pressure from 135 to 95 mm Hg and the second one (stretch diameter : 4.5) lowered the blood pressure for a second time from 130 to 70 mm Hg. A decrease in the heart rate was observed during these two dilatations (138 to 120 and 126 to 110). The hypotension and the bradycardia encountered in this animal had most porbably a neural reflex origin, and it disappeared almost immediately after withdrawing the stimulation. In the other animals, the dilatation produced only a slight increase or had no effect on the heart rate.

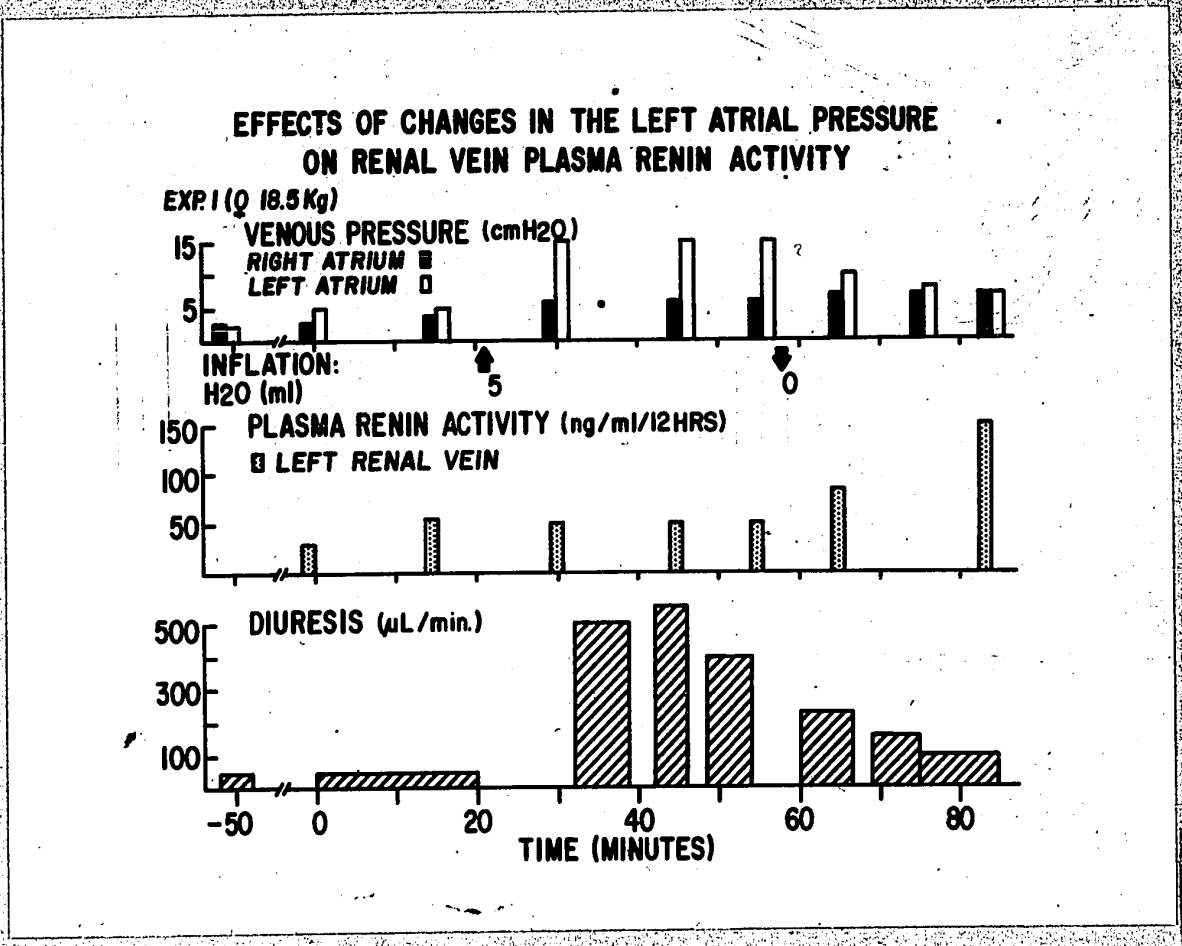


Figure 32

A marked diuresis ensued the inflation of the balloon in the left atrium. Renal venous renin activity and right atrial venous pressure did not change. An important increase in left atrial pressure was found following the inflation of the balloon.

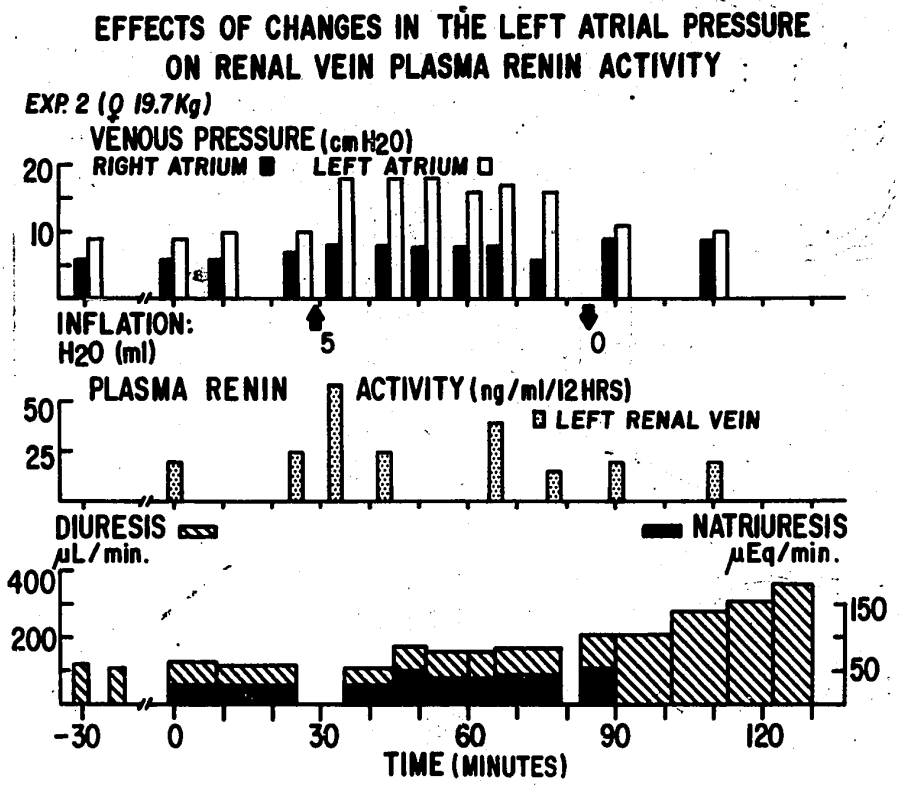


Figure 33

Natriuresis, diuresis and right atrial venous pressure were not affected during the period of higher left atrial venous pressure. Renal venous renin activity was found to be increased in 2 out of 4 determinations during that period.

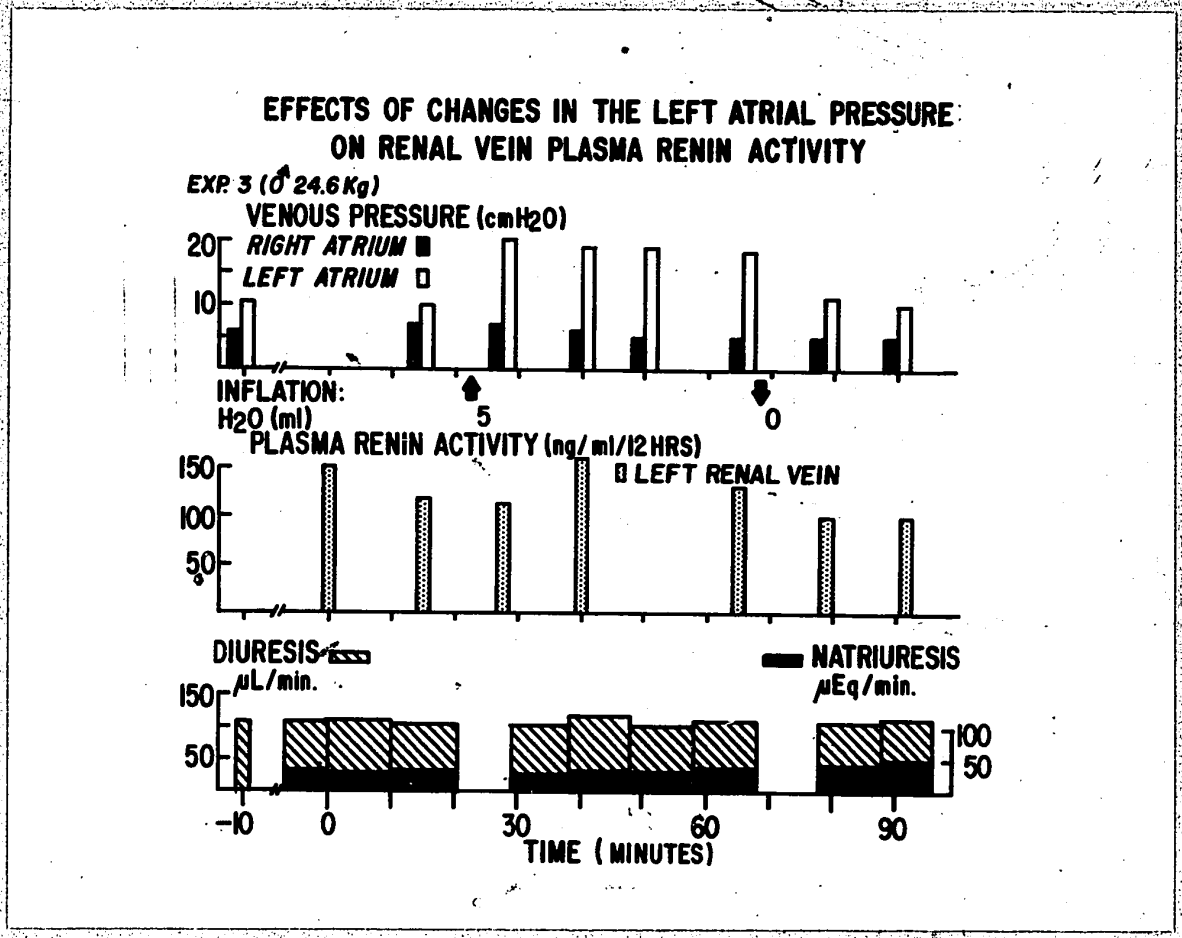


Figure 34

The inflation of the balloon increased left atrial pressure but right atrial pressure, renal venous renin activity, diuresis and natriuresis remained unchanged.

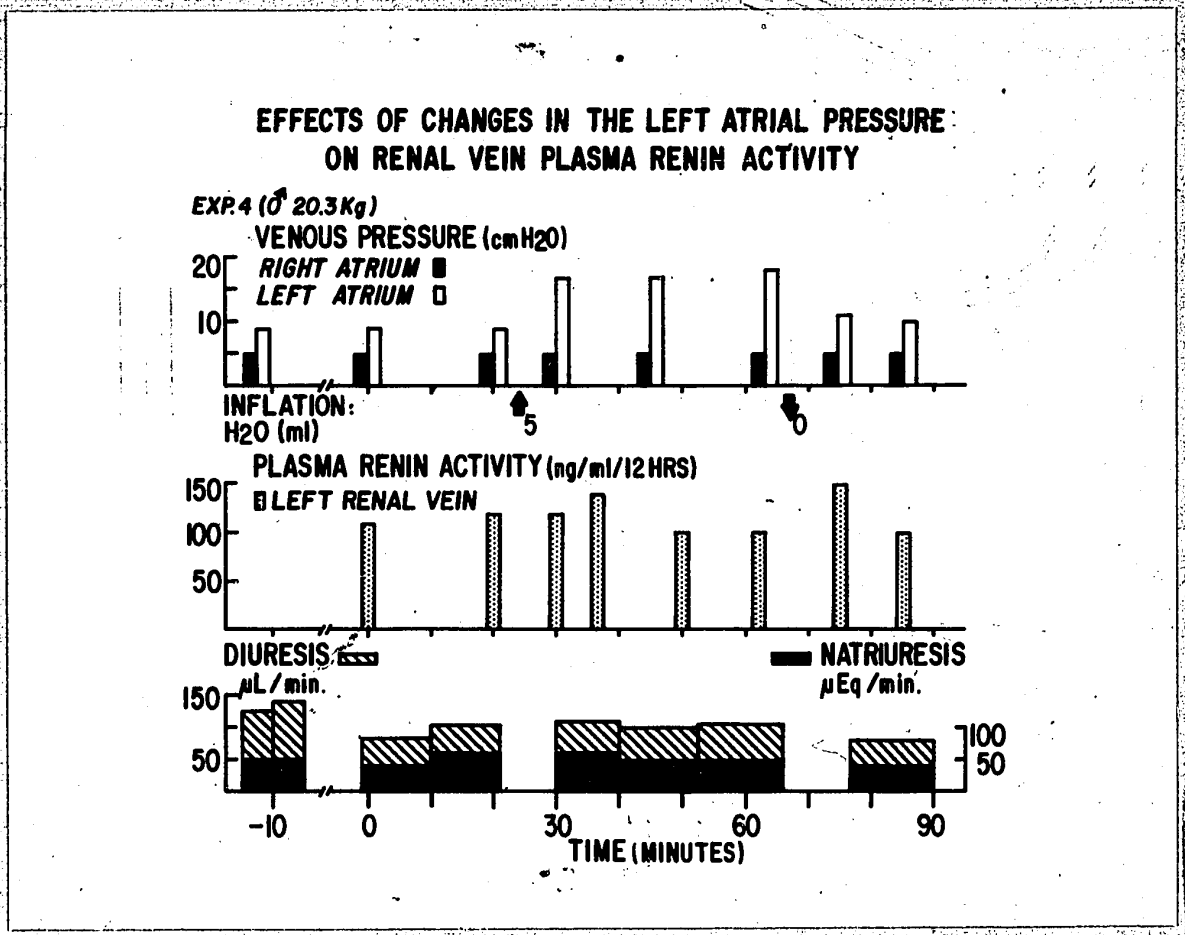


Figure 35

Despite an increase in left atrial pressure following inflation of the balloon, renin activity and the other parameters were not altered.

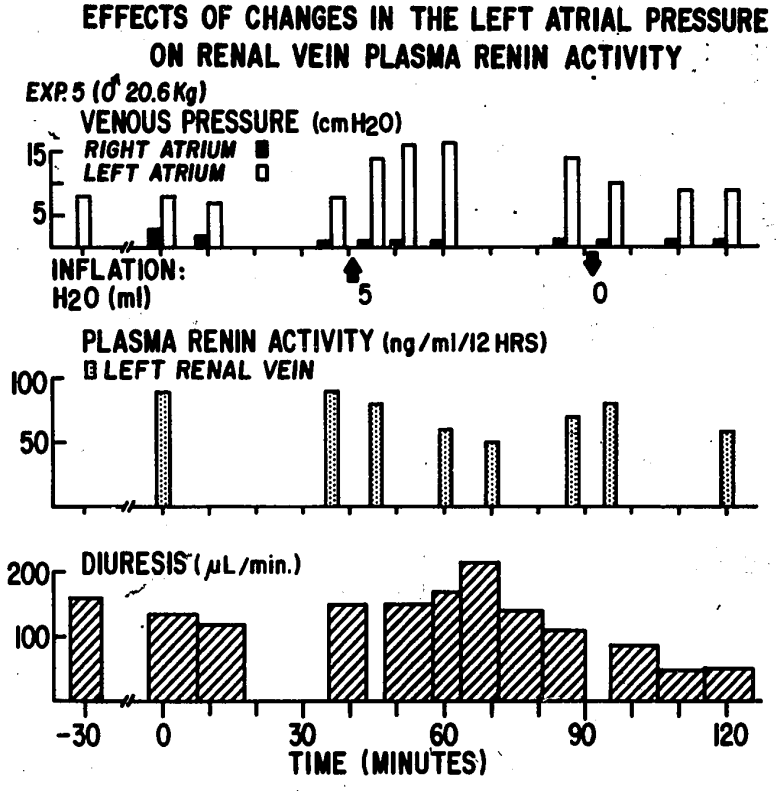


Figure 36

Values for left renal venous renin activty and diuresis were essentially the same in pre- and post-inflation periods. In the post-deflation period, diuresis decreased but renin activity remained unchanged.

In two animals (Figure 39, 40), the diuresis and natriuresis were measured from the left ureter and were not increased by stretching the left atrium.

C - DISCUSSION

By manoeuvres which increased the volume and the pressure of the left atrium, a diuresis and a decrease in the plasma level of antidiuretic hormone was observed respectively by Henry, Gauer and Reeve (353) and by Share (354). The diuresis was not a constant phenomenon in all the dogs studied, following distension of left atrium. In these experiments, a large degree of distension was employed. The balloon in the left atrium was inflated with water to a volume of 1 ml/Kg of body weight. This stimulation increased the left atrial pressure by 15 to 20 cm of water. Such a distension increased the heart rate and the cardiac output measured in two dogs was found to be decreased (353). Nevertheless, the level of arterial blood pressure was not affected.

Smaller degrees of distension of the right and left atrium were employed in our experiments. Thus, sodium excretion was not increased in four out of five dogs following inflation of the balloon in the left atrium.

We assume that the increase in renal venous renin activity observed by distending the right atrium in five out of nine dogs is probably not due to a reduction in renal perfusion pressure. A similar effect, if present, would have probably been observed following the inflation of the balloon

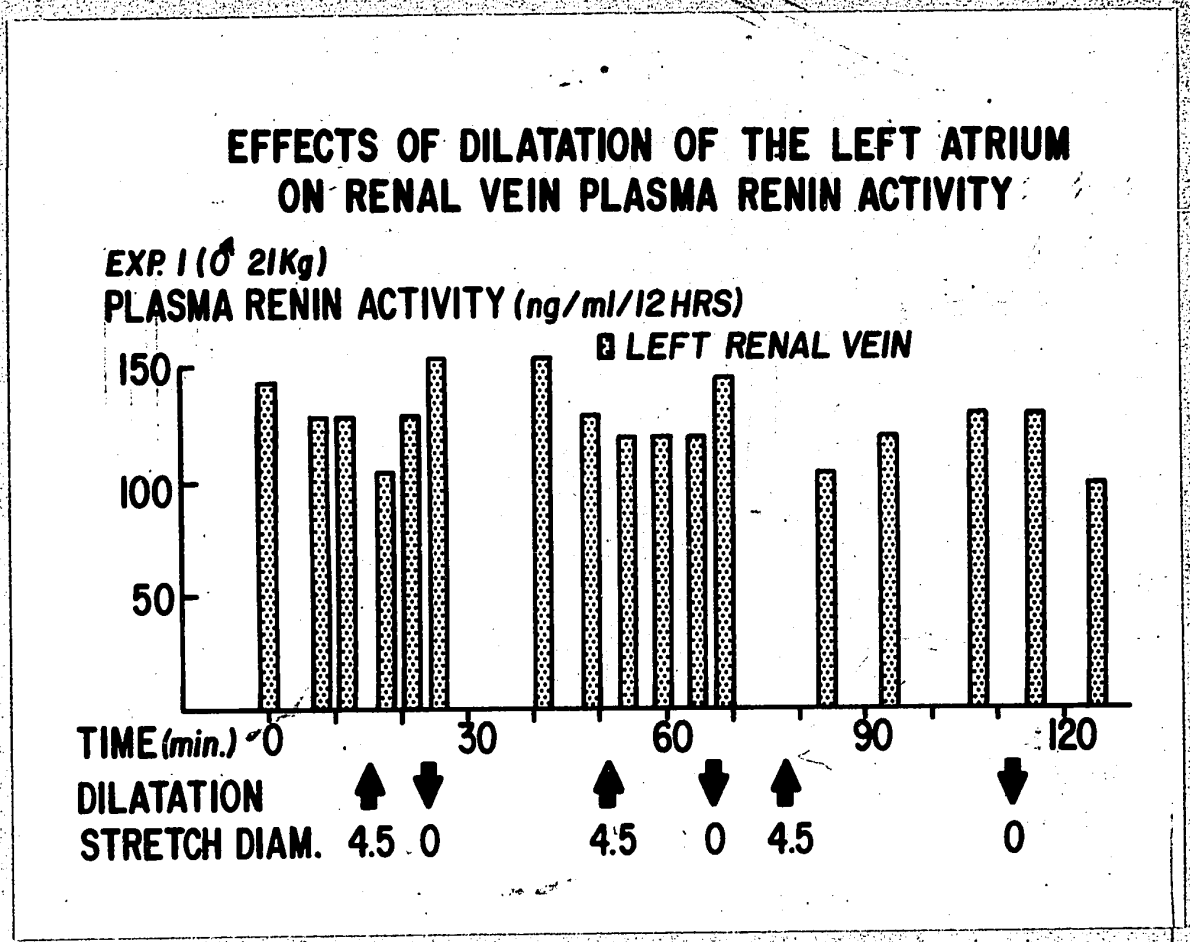


Figure 37

Stretching the left atrium with a dilator had no effect on renal venous renin activity.

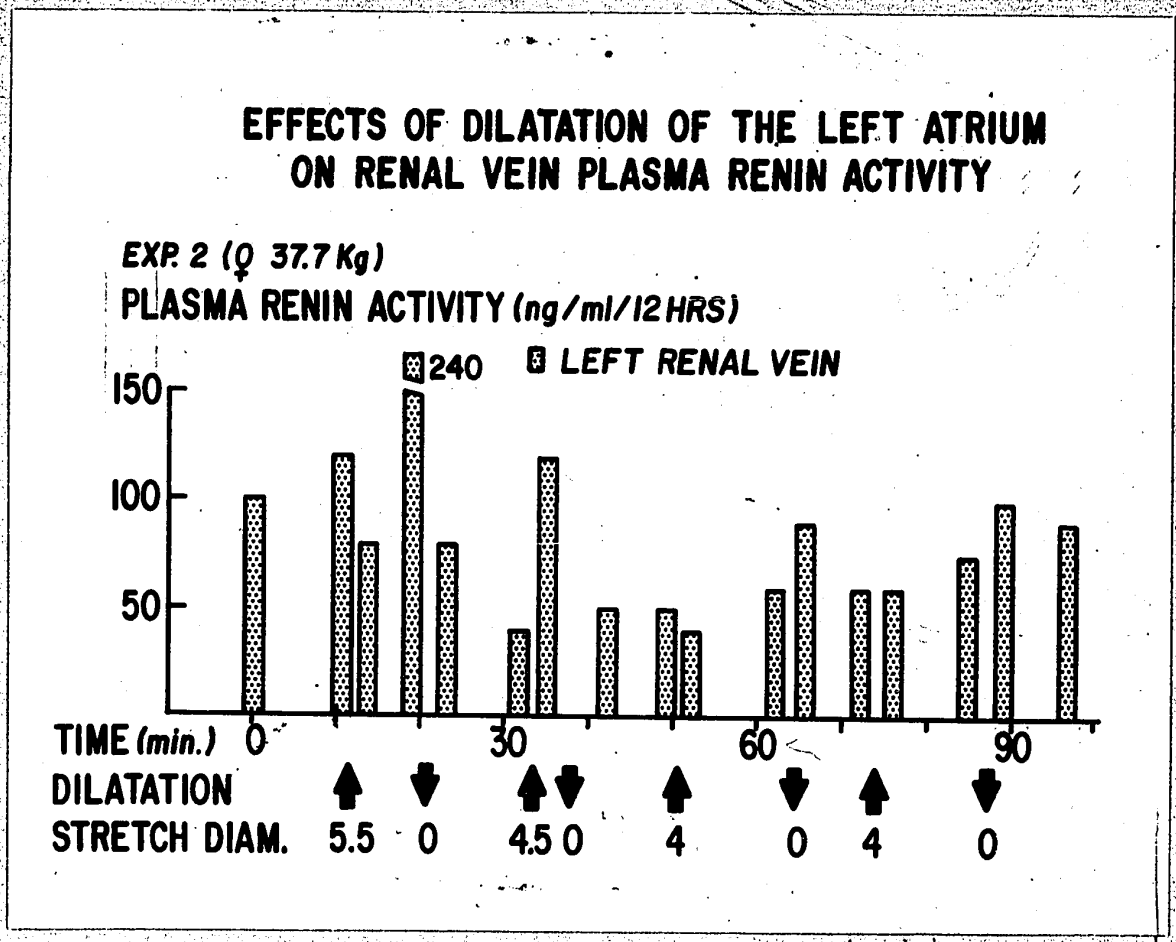


Figure 38

Following the two first dilatations, hypotension and bradycardia occurred. The other stimulations had no effect on systolic blood pressure, heart rate and renal venous renin activity.

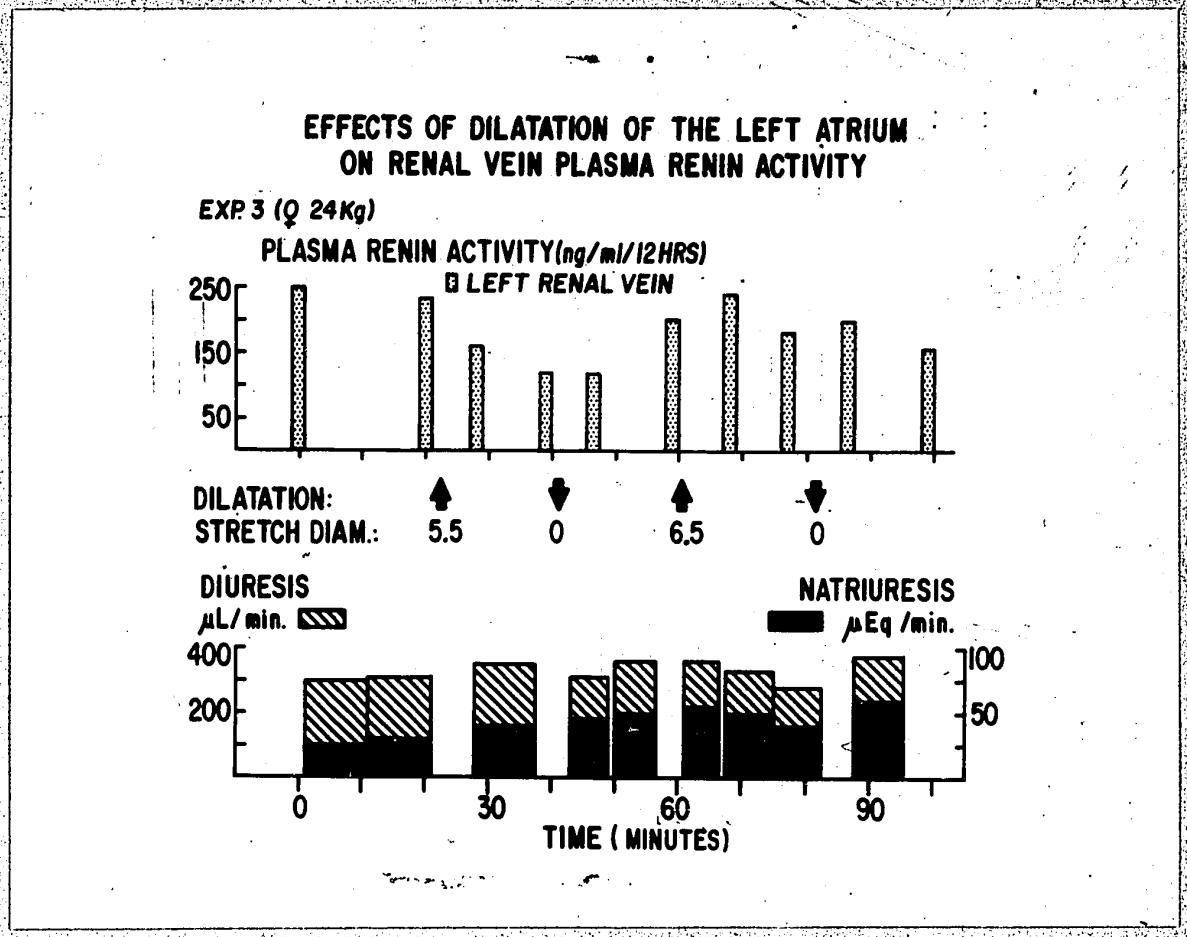


Figure 39

The first dilatation of the left atrium decreased plasma renin activity. Natriuresis and diuresis were not affected by two successive dilatations.

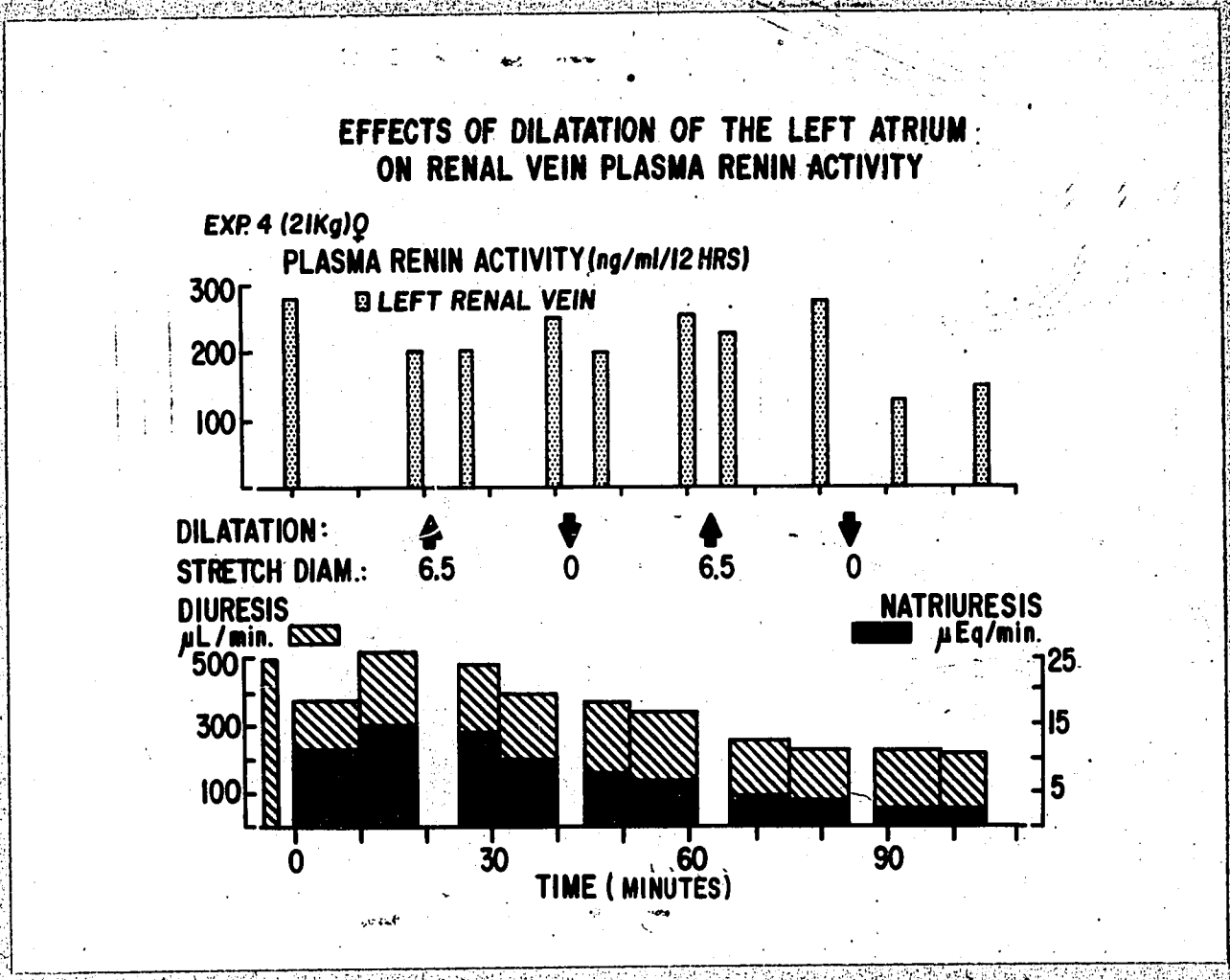


Figure 40

A gradual decrease was observed in diuresis and natriuresis. The values of plasma renin activity were essentially the same except for the last two controls.

in the left atrium. From our results, the response to the stimuli in the right atrium experiments appeared to be short-lived.

The respiratory rate in the right atrium experiments was not controlled but this factor was minimized by performing the experiments on tracheostomized animals. Moreover, the respiratory rate was not altered significantly following the inflation of the balloon in the right atrium.

An increase in renal venous pressure does not seem to be involved in the release of renin. Following the inflation of the balloon in the right atrium, the pressure in the abdominal vena cava (above the level of renal veins) did not increase significantly. Skinner et al (159) showed also that partial renal venous constriction did not alter renin release.

The response of renin release to a change in right atrial venous pressure may be effected through stimulation of receptors located in the right atrium. Evidence has accumulated to indicate a possible reflex neural control of renin release along with a complete intrarenal control mechanism. The effect of atrium distension on renin secretion could be explained by such a neural pathway.

Before drawing any conclusion on the existence of a neural pathway involved with renin release following atrial distension, further studies are required with experimental preparations such as vagotomy and renal denervation.

CHAPTER V - STUDY OF PLASMA RENIN ACTIVITY IN CARDIAC FAILURE IN DOGS

A correlation between the right atrial pressure and the renal venous renin activity has been previously discussed. Extending this observation, we have investigated the effect of tricuspidectomy on peripheral plasma renin activity in dogs with congestive cardiac failure.

A - MATERIALS AND PROCEDURES

The tricuspid valve was completely removed from 9 female mongrel dogs (14.9-20.4 kg). The tricuspidectomy was performed by open heart surgery. The operation was done aseptically under sodium pentobarbital (30 mg/kg) anesthesia. The operated dogs were divided into two groups according to the presence or absence of ascites and edema evaluated by the clinical examination and gain in body weight. All the studies were performed before the operation and between 3 to 17 weeks after valvulectomy. The dogs were maintained under metabolic balance during 3 days at least before the study. They were fed by gavage with a diet containing 58.4 to 66.3 mEq of sodium and 40.7 to 42.5 mEq of potassium in 800 ml. The diet was prepared by homogenizing in a "Waring" blender, a commercial meat preparation (Dr. Ballard) and Purina dog chow. The diet was given in two equal portions at 9 and 15 hours. One of the dogs who became edematous was given hydrochlorothiazide (50 mg/day) during 5 days. This last study was achieved under metabolic balance[+].

[+] These experiments were performed by Dr. Louis Belleau. We are most grateful to him for the blood samples he supplied us.

B - RESULTS

The removal of the tricuspid valve increased the central venous pressure from 3 to 7-fold (Table XXVI). Five out of nine animals presented the symptoms of congestive heart failure. All five dogs had pronounced ascites and edema. The mean central venous pressure (18.4 cm H_2O) was higher in the group with congestive heart failure compared to the group without ascites or peripheral edema (14.6 cm H_2O).

In dogs with increased central venous pressure but without congestive heart failure, the plasma renin activity increased in one and decreased in the other three (Dog No. 1 to 4). The dog No. 4 became edematous and was studied a second time in the 17th week following the valvulectomy. Plasma renin activity was still low. The other 5 dogs (Dog No. 5 to 9, Table XXVI) developed congestive heart failure and the determinations were done within 3 to 8 weeks after the operation. The plasma renin activity increased significantly in dogs No. 5 and 9. A small increase was observed in dog No. 6 and 7, whereas the plasma renin level remained unchanged in dog No 8.

In dog No. 5, hydrochlorothiazide was given during 5 days in order to decrease the central venous pressure. The peripheral plasma renin activity decreased significantly after treatment whereas the central venous pressure passed from 22 to 15.3 cm H_2O.

C) DISCUSSION

In this preliminary experiment, each dog served as its own control. This was necessary because of the restricted num-

TABLE XXVI - Plasma Renin Activity (PRA) in experimental heart failure.

Dog No.	CONTROL (pre-surgery)				TRICUSPIDECTOMY							
					Without CHF				With CHF			
	PRA	CVP	BP	BW	PRA	CVP	BP	BW	PRA	CVP	BP	BW
1	10.0	-	160	17.4	0	9.0	122	-				
2	6.2	2.3	128	19.2	15.6	16.5	136	22.9				
3	19.0	4.5	157	18.7	6.2	14.5	141	22.5				
4	37.5	6.5	160	17.7	2.5	18.5	159	19.8	5	18.5	177	22.4
5	10.0	5.0	148	20.4					120	22.0	132	21.0
									25+	15.3	153	-
6	3.1	3.0	142	14.9					6.2	19.2	132	24.5
7	25.0	2.7	147	16.6					32.5	19.8	150	20.9
8	6.2	3.0	133	19.0					7.0	15.0	160	24.3
9	6.2	3.3	159	19.7					26.0	15.9	151	24.2

CVP: Central Venous Pressure (cm H_2O). BP: Systolic Blood Pressure (mm Hg).
BW: Body Weight (kg). CHF: Congestive Heart Failure.

+: Hydrochlorothiazide (50 mg/day) was administered during 5 days under metabolic balance.

ber of animals used and the variability of normal values for plasma renin activity in dogs. Signs of congestive heart failure occurred following the removal of the tricuspid valve in 6 out of 9 dogs. Plasma renin activity was significantly increased in only 2 of these 6 dogs who developed edema. In 2 other a slight although not significant increase was noted.

In dog number 5, the highest experimental values for central venous pressure and plasma renin activity were found. When given hydrochlorothiazide, both parameters decreased significantly. The level of arterial blood pressure in this animal was also inversely related to the peripheral plasma renin activity. Such a correlation between renin activity and arterial blood pressure was observed in the other dogs with congestive cardiac failure but it was not obvious in the 4 non-edematous dogs studied after removal of the tricuspid valve.

Renin activity was found to be increased in humans suffering from congestive heart failure. In dogs, the lesion underlying the syndrome of congestive heart failure seems to have an importance in the release of renin since Johnston et al (187) found a high renin activity in all the 5 dogs with tricuspid insufficiency combined to pulmonary stenosis whereas only 3 out of 5 dogs with a large arteriovenous fistula presented hypereninemia. Accompanying this underlying mechanism, the release of renin may be influenced by sudden elevation of the right atrial venous pressure. Occasional stimulation of renin release may explain a parallel increase in aldosterone production. This factor combined to the long half-life of aldosterone

in congestive cardiac failure (324, 327, 328) due to hepatic venous congestion and decreased hepatic clearance of aldosterone (326, 327, 329, 330), may explain the occurrence of edema.

GENERAL DISCUSSION

An aldosterone secreting adenoma effects an important degranulation of juxtaglomerular apparatus and thus suppresses totally plasma renin activity. This suppression is most probably due to the sustained expansion of intravascular volume encountered in primary aldosteronism. The suppression and absence of stimulation of plasma renin activity was observed in a case of "hypokalemic" primary aldosteronism.

Establishment of diagnosis of primary aldosteronism is of great importance since most of the patients with this disease are cured or improved upon removal of an adrenal adenoma. Suppression of renin activity in these patients is a most important additional diagnostic criteria. It was suggested in 1964 that primary aldosteronism could be diagnosed despite the absence of kypokalemia by finding suppressed renin activity and high aldosterone secretion or excretion rate in hypertensive patients. On the basis of indirect evidence, it was suggested that 20% of the patients with essential hypertension might really have primary aldosteronism.

A complete suppression was observed in 23% of our patients with essential hypertension. In five patients with suppression of renin activity, the aldosterone excretion was above the normal in only 2 patients. It appears thus that in essential hypertension, suppression of renin activity is not necessarily accompanied by supernormal values for aldosterone excretion and the basis of this suppression remains obscure.

Failure to correct or improve by adrenalectomy 2 patients with essential hypertension and suppressed renin activity, illustrates the limited values of renin assay for screening "normokalemic" primary aldosteronism.

A low incidence of primary aldosteronism among hypertensive population was further supported by the finding of a low incidence (5%) of adenomas and nodules in hypertensive patients at autopsy room.

The renin- angiotensin system was studied in dogs usually by adapting current methods described for man. These methods require a large amount of plasma. If renin is measured on a small amount, the time of incubation may be prolonged. Since substrate concentration is known to be inferior in dog compared to man, it is often risky to assume that zero-order kinetics apply when the substrate contained in plasma is used. We have described a sensitive and reproducible method for determination of plasma renin activity in dogs. This method consists in the incubation of 1 ml of dog plasma with an excess of exogenous dog substrate preparation free of renin.

Hayduk (355) using this technique to study peripheral plasma renin activity in dogs following clamping of one renal artery, showed as preliminary results, an increase in renin activity during the first week after clamping and then a gradual decrease in the following days.

This method has also been extended to measurement of renin content of kidney. Renin can be measured in less than

.10 mg of kidney tissue.

Since this procedure permits serial determinations on a small aliquot of plasma, plasma renin activity has been used to measure renin activity in newborn dogs and to study the effects of changes in right and left atrial pressure on renal venous renin activity.

In newborn dogs, an evaluation of the renin-angiotensin system was provided by measuring simultaneously plasma renin activity, renal renin content and juxtaglomerular index. A complete absence of juxtaglomerular cell granules was observed. The plasma renin activity was high despite a normal renal renin content during the first 15 days of extra uterine life. Stimulation effected by peritoneal dialysis showed an important release of renin. A storage and release of renin by the kidney seemed possible in the absence of juxtaglomerular cell granules, since peritoneal dialysis decreased renal renin content. This observation may suggest that in vascular structures where renin-like substances were found, a similar storage and release in the smooth muscle cells devoid of granules may occur.

Study of the effects of right atrial pressure on renal venous renin activity showed that an acute rise in the right atrial pressure may induce a release of renin. This effect was absent when the left atrial pressure was increased or when the left atrium was dilated. There exists a possibility that this effect on renin release is modulated by a neural

or neurohumoral pathway. In dogs with experimental heart failure, an increase in plasma renin activity was found only in a small proportion of animals rendered edematous. Despite the absence of a correlation between right atrial venous pressure and peripheral plasma renin activity following tricuspidectomy in dogs, a control of renin release by distension of the right atrium is not excluded.

It seems that renin release is influenced mainly by renal perfusion pressure and composition of tubular urine at the macula densa level. These two mechanisms are not exclusive and may influence each other. Both may be altered and thus explain the low or high renin activity found respectively in Conn's syndrome or secondary aldosteronism due to heart failure. We have previously discussed the inadequacy of baroreceptor and macula densa theories to explain renin release in congestive heart failure.

Since increased venous pressure is one of the earliest signs of heart failure, we have sought the presence of atrial receptors sensitive to changes in venous pressure. The response to right atrial distension was sometime of short duration and was present in 5 out of 9 dogs studied. The occurence of edema in heart failure may be explained on the basis of temporary increase in renin-angiotensin-aldosterone due to sudden rise in venous pressure, and the decreased hepatic extraction of aldosterone encountered in that disease.

CONCLUSIONS

1. Measurements of plasma renin activity in patients with essential hypertension showed that a large proportion of this group has a suppressed renin activity and in 23% renin activity cannot be stimulated by upright posture and low sodium intake.

In three out of five patients with complete suppression of renin activity, aldosterone excretion was normal. The basis of the suppression in these patients remains obscure.

Despite its limitation as a screening procedure for "normokalemic" primary aldosteronism, the renin assay may be considered of importance in the differential diagnosis of "hypokalemic" primary aldosteronism.

Circumstantial evidence of the low incidence of "normokalemic" primary aldosteronism among hypertensive patients was provided by an autopsy study of the adrenals in normotensive and hypertensive patients.

2. We have described a sensitive and reproducible method for determination of plasma renin activity on a small aliquot of plasma or extracts of renal tissue.

This method is based on the priciple that a small amount of renin may be detected when incubated with an excess of exogenous renin-free dog substrate, for a long period of incubation.

The recoveries of added angiotensin average 68%.

Normal values were determined in a large series of dogs and showed a great variability.

3. The application of this method to newborn dogs showed a high level of circulating renin and a normal renin content when compared to adult dogs. These newborn dogs were also characterized by the absence of juxtaglomerular cell granules. Renin release was stimulated by peritoneal dialysis and a storage of renin in the kidney despite the absence of granular cells was suggested by this experiment.

4. The effects of changes in right and left atrial pressure on renal venous renin activity were studied. The results suggest that a rise in right atrial pressure produces by ways, undetermined at present, an increase in renal venous renin activity.

In dogs with congestive cardiac failure due to removal of tricuspid valve, plasma renin activity was not consistently increased.

In the light of these two findings, the possible role of the renin-angiotensin-aldosterone system in edema formation has been discussed.

CLAIMS TO ORIGINALITY.

The major contribution of this thesis to the field of renin-angiotensin system, consisted in the establishment of a simple, sensitive and reproducible method for the determination of renin activity on a small amount of dog's plasma (1 ml) or kidney.

In the past, plasma renin activity was measured in dogs by adapting the current methods described for humans. Generally the substrate contained in the plasma sample to be assayed, was used for the subsequent formation of angiotensin. Since the substrate concentration in dog plasma is relatively low, the results obtained with large quantities of renin were not always consistent with a zero order reaction. In our method, an exogenous but species specific substrate preparation was used and zero order kinetics were shown to apply for values of renin activity well above the physiological range.

Determination of plasma renin activity on small aliquots of plasma provided a possibility to study renin-angiotensin system in newborn dogs. Plasma renin activity was measured and compared to juxtaglomerular index and renal renin content in the first 15 days of extrauterine life. This study showed that despite the absence of juxtaglomerular cell granules, the amount of renin contained in the kidney was comparable to that found in adult dogs, whereas the plasma renin activity was much higher. The circulating renin was shown to originate most probably from the kidneys of the newborn dogs as suggested

by peritoneal dialysis experiments. These findings seemed to indicate that the kidneys of newborn puppies may store and release renin in the absence of juxtaglomerular cell granules. This work is the first to give such a complete evaluation of the renin-angiotensin system in early extra-uterine life.

The study of the effects of changes in right or left atrial pressures on renal vein plasma renin activity constituted another original contribution. This investigation showed that an increase in the renal vein plasma renin activity may be produced in half of the experiments when the right atrial pressure is increased. The same stimulation when applied to left atrium, was without effects on plasma renin activity.

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