# The Brain Renin-Angiotensin System and Extrarenal Renin

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Title of thesis: STUDIES ON THE EXISTENCE OF AN INDEPENDENT BRAIN RENIN-ANGIOTENSIN SYSTEM: A MODEL FOR EXTRARENAL TISSUE RENIN.

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## SUMMARY

An enzyme, capable of catalyzing the reaction with renin substrate to form angiotensin is described in the brain of dogs, rats, sheep and man. This brain tissue iso-renin differs in important characteristics from renal renin. The enzyme persists at control-levels 12 days after nephrectomy. Renin substrate has been found equally in brain tissue of dogs. Angiotensin was extracted from brain of dogs which had been nephrectomized and in which plasma angiotensin was undetectable. This shows that angiotensin can be formed locally in the brain.

An iso-renin of similar characteristics as brain iso-renin is described and partially characterized in aortic tissue and in adrenal glands. The independence of tissue iso-renin from kidney renin is supported by the fact that the two enzymes react differently to the same physio-pathological stimuli.

# STUDIES ON THE EXISTENCE OF AN INDEPENDENT BRAIN RENIN-ANGIOTENSIN SYSTEM: A MODEL FOR EXTRARENAL TISSUE RENIN.

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F O R  U R S U L A

TABLE OF CONTENT

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LIST OF FIGURES

LIST OF TABLES

## LIST OF ABBREVIATIONS

| | |
|---|---|
| AI = | Angiotensin I (decapeptide) |
| AII = | Angiotensin II (octapeptide) |
| ADH = | Antidiuretic hormone |
| BBB = | Blood-brain-barrier |
| CE = | Angiotensin I converting enzyme |
| CM = | Carboxymethyl cellulose |
| CSF = | Cerebrospinal fluid |
| DEAE = | Diethylaminoethyl cellulose |
| DFP = | Diisopropylfluorophosphate |
| DNA = | Deoxyribonucleic acid |
| EDTA = | Ethylenediaminetetraacetic acid |
| n = | Number of experiments |
| PRA = | Plasma renin activity |
| RA = | Renin activity |
| SE = | Standard error of the mean |
| SE-cellulose = | Sulphoethyl cellulose |
| TEAE = | Triethylaminoethyl cellulose |
| TRIS = | 2-amino-2-hydroxymethyl-1-3-propanediol |

# LIST OF ABBREVIATIONS

Amino Acids:

ALA = Alanine

ARG = Arginine

ASP = Asparagine

HIS = Histidine

ILEU = Isoleucine

LEU = Leucine

LYS = Lysine

PHE = Phenylalanine

PRO = Proline

PYRGLU = Pyroglutamyl (pyrrolidone carboxylyl)

SER = Serine

TRP = Tryptophan

TYR = Tyrosine

VAL = Valine

# 1. INTRODUCTION

Thirty-seven years ago Goldblatt and co-workers demonstrated that hypertension could be produced by reducing the blood supply to the kidney by means of a clamp on the renal artery (1). This work was largely confirmed in the following years by several workers (2-10). It was thought, that renin, first described by Tigersted and Bergman in 1898 (11) as a pressor substance of renal origin, was directly responsible for the rise in blood pressure following renal ischemia.

When, however, more specific, accurate and reproducible methods for measurement of "renin activity" and "renin concentration" were developed (12-22), and when it became possible to measure other components of the system such as renin substrate, angiotensin and angiotensin I converting enzyme in different types of renal hypertension and at various times after the onset of hypertension, the apparently simple role of the renin-angiotensin system in renal hypertension became more complicated. More and more exceptions had to be considered.

Early experiments were performed on animals with experimental hypertension of short duration. The finding that plasma renin can be normal or sub-normal in dogs with renal hypertension of 3 months to 4 years duration, resulted in the renin-angiotensin system being discredited to some extent in the pathogenesis of experimental renal hypertension and led to a more critical approach to the problem.

It has become evident that a multitude of factors such as adrenal steroid hormones, catecholamines and electrolytes, among others, are important factors involved in the onset and maintenance of renal hypertension.

New aspects of the renin-angiotensin system have gained attention by the finding of

1) renin activity in various extrarenal tissues such as arteries, adrenal gland, salivary glands, uterus and others (23-64);
2) recently discovered metabolic actions of angiotensin:
    a) On catecholamine synthesis and release (65-69);
    b) on acetylcholine release (70-72);
    c) on release of antidiuretic hormone (73-76);
    d) on thirst regulation (77-87);
    e) on protein synthesis (88-91);
    f) on the membrane-bound sodium pump and on cellular electrolyte regulation (92-98).

The origin of extrarenal renin, and its physiological function have not been clarified as yet. In view of the newly described metabolic actions of angiotensin it appeared a most exciting task to investigate:

1) The characteristics of tissue iso-renin in comparison to renal and plasma renin.
2) Whether extrarenal tissue iso-renin was dependent or independent of the renal renin-angiotensin system.

3) Whether the various metabolic functions of angiotensin could be influenced by the local tissue renin-angiotensin system.
4) The response of tissue iso-renin in a variety of physio-pathological conditions and to various agents.

# 2. REVIEW OF THE LITERATURE

Excellent and recent reviews on the renin-angiotensin system and its role in hypertension are available (99-108). This review of the literature will only deal with aspects that are relevant to extrarenal tissue iso-renin.

## 2.1. *Components of the Renin-Angiotensin-System*

### 2.1. 1. Renin

Renin is a protein. It is heat labile (11), non dialyzable (11, 109, 110), acid and alkali labile (110), insoluble and denatured in organic solvents (109, 110). Resistance to acid and alkali is increased in the cold. Renin can be salted out by ammonium sulfate at pH 2.3 to pH 6 and concentrations of 1.4 to 2.6 M (111).

In spite of many attempts of purification (112-128) renin has not been obtained in pure form. The molecular structure of this enzyme is unknown, and its molecular weight, as estimated on sephadex columns, appears to be between 42,000 and 47,000 (114, 127). There seem to exist several forms of renin in the kidney which are possibly artefacts, due to the chemical treatment during the extraction and purification procedure (127). Purification and subsequent characterization is difficult, because the enzyme is unstable in the purified form.

Since renin is a labile enzyme, the interpretation of described characteristics of the purified enzyme must be done with great caution. Even extraction and purification methods, that are usually considered gentle in protein chemistry may alter the structure and properties of renin. Haas, for example, has described three interconvertible forms of renin: α, β, and γ (119). The α-form was converted to the β-form by a 36 hours dialysis at a pH between 4 and 5. The fact of allowing α-renin to stand at low temperature at pH 3.8 produced the γ-form. Both the β and γ-types could be converted into the α-form by precipitation with 2.5 M ammonium sulfate.

Skeggs et al. (126), following a different approach for purification, described 4 forms of renin I - IV, eluted at characteristic pH values of 5.2, 4.7, 4.5 and 4.25 respectively from a DEAE-cellulose column. When the elution of renins I - IV was followed by ammonium sulfate precipitation at pH 7.5, a chromatographically homogeneous renin I fraction was obtained. Enzymes II - IV thus were converted to type I by this procedure. It is not clear, whether the multiple forms of renin arise from alterations in the structure of the molecule or from the dissociation or transfer of the enzyme from one "carrier" protein to the other. Morgan and Leon (128) have chromatographed unpurified neutral renin extracts on TEAE-cellulose and obtained two different, renin containing fractions.

Peart et al. (124), using DEAE-cellulose, CM-cellulose, SE-cellulose and gel-filtration on Sephadex G 100 described one peak,

containing renin activity. They used, however, acid treatment of pH 2.5 before the chromatography, a procedure which would convert all renin I to renin II according to the findings of Skeggs et al. (126).

### 2.1. 2. Tissue Renin

Dengler was first to describe in 1956 "a renin-like substance" in extrarenal tissue, namely the arterial wall (24). He extracted the enzyme from pig arteries. One year later, Werle et al. (129) described a "blood-pressure increasing principle" in the submaxillary gland of the white mouse. Various groups since then have studied renin in extra-renal tissues. The uterus (32-43), placenta and amniotic fluid (36, 40, 41, 43), submaxillary gland of different species (44-52), the adrenal gland (26), spleen, lung, liver, skeletal muscle and heart (29, 30) have been studied. Though Werle et al. (129) in their early publications already mentioned renin isoenzymes, little work has since been done to characterize their properties and to establish any differences from the kidney enzyme.

The prime interest of most groups was to demonstrate, that the final product of the tissue-renin substrate reaction was angiotensin. The tissue enzymes therefore are frequently referred to as "angio-tensin-forming-enzyme", "renin-activity", or "renin-content". The term "renin-like" substance is equally used.

In this thesis we will employ the term tissue iso-renin as opposed to plasma and kidney renin.

2.1. 2.1. Uterus

Among the extrarenal tissues, most of the attention has been given to the uterus (32-43). Attempts have been made to purify the enzyme from rabbit uterus and it has been shown, that the molecular weight of kidney and uterine renin is approximately the same (ca 40,000) (32). Both enzymes have an optimal activity at pH 6. The enzymes are identical with respect to their mobility on disc-electrophoresis, but the Michaelis constant has been found different (32).

The renin-like enzyme in the uterus of dogs is increased during pregnancy (35, 37, 39). After nephrectomy, the enzyme activity does not drop, but rather increases, an indication, that uterine renin is independent of kidney- and plasma-renin (39). The demonstration of renin synthesis by uterine muscle cells, was made by Symonds in cultures and subcultures of these tissues (41), and by Eskildsen, who transplanted renin synthesizing myometrial tissue into the rabbit eye (130). Uterine renin may be secreted into the blood stream as shown by simultaneous measurement of RA in peripheral and effluent venous blood from the uterus (144, 145). Persisting plasma renin activity in anephric humans may partially be due to uterine renin, since PRA tends to be higher in bilaterally nephrectomized female than male subjects (131). Renin has been found, however, also in anephric male subjects (132). The question of the contribution of extrarenal sources to PRA (131-143) is still controversial and will be discussed later in this thesis. There seems to be a substrate present in the

plasma of dogs which is used by uterine renin and which is not utilized by kidney-renin (34).

## 2.1. 2.2. Salivary Glands

Submaxillary glands of adult mice may contain high concentrations of renin which can exceed the renin content of mouse kidney per unit of weight (44-52, 129). Its concentration is higher in male mice. Undetectable in the first weeks of life, its concentration increases until sexual maturity at approximately 3 months (146). Castration of male mice decreases submaxillary renin activity. It can be increased in castrated male as well as in untreated female mice by testosterone administration (146, 147). Deoxycorticosterone and salt loading on the other hand have no effect on submaxillary gland renin (146).

Takeda et al. (45) measured renin activity in the venous effluent blood and in extracts of the submaxillary gland of the mouse. They reported a decrease following treatment with deoxycorticosterone and 1% sodium chloride in the drinking fluid and by isoproterenol. Sodium restriction did not change submaxillary gland renin.

Following extirpation of the submaxillary gland, PRA and systemic blood pressure decreased in the male but not in the female mouse (45). These findings are contrary to those of Bing et al. (46), who found, that PRA and blood pressure did not change following sialo-adenectomy. More recently, it was shown that secretion of renin from the submaxillary gland of mice into plasma can be achieved by stimulation of

adrenergic α-receptors (148).

Renin activity decreases in the submaxillary gland, when the excretory duct is ligated. It is of interest that the granules in the striated part of ducts of the salivary gland of female rats are morphologically similar to the granules of the juxtaglomerular cells in the kidney and contain high amounts of renin. However, for unknown reasons, the same granules in male rats do not seem to contain any renin (44, 47, 149-151).

Werle classified the submaxillary enzyme as a renin isoenzyme (51). He purified the enzyme on ion-exchange chromatography, sephadex-gel-filtration and column-electrophoresis, and obtained a purified renin preparation with a specific activity of 500 Goldblatt units per mg Protein (51). Rats were immunized with this purified preparation of iso-renin. The induced antibody inactivated in equal amounts renal renin from mouse, pig and dog, but this iso-renin antibody was inactive with human renin. A similar specificity was observed when antibodies were produced against renal renin. Renal renin and submaxillary gland iso-renin from the mouse thus appear to have a similar antigenicity (51).

### 2.1. 2.3. Adrenal Glands

Goormaghtigh and Handovsky described in 1938 the presence of epitheloid cells in the arterioles of the capsule of adrenal glands

similar to the juxtaglomerular cells of the renal afferent arterioles (152).

Granzer showed the presence of an enzyme in adrenal glands (23), capable of consuming a protein of the $\alpha_2$-globulin-fraction in the plasma. Ryan (26) reported a renin-like enzyme in the adrenal gland of the rabbit, which was non dialyzable, stable for several hours at pH 2.6 and $4^{o}C$ and which formed angiotensin I when incubated with renin substrate. The identity of the formed pressor product with angiotensin I has been demonstrated by counter-current distribution. The presence of a renin-like enzyme in adrenal gland has been confirmed by Hayduk et al. (29) and changes in its activity have been reported after sodium restriction and Goldblatt-Hypertension (29). Speculation on the possible role of locally formed angiotensin on aldosterone production and catecholamine synthesis has been entertained, but not substantiated.

### 2.1. 2.4. Arterial Wall

The presence of a renin-like substance in extracts of arteries of pigs was first described by Dengler (24). This substance was similar to renin being precipitable with ammonium-sulfate, thermo-labile, non dialyzable and forming a pressor material when incubated with plasma (24).

These findings have been confirmed by Genest et al. (27), Rosenthal et al. (28), and Hayduk et al. (29, 30). Gould et al. (25) showed that arterial wall (pig) renin could be inhibited by anti-renin. The pH-optimum for angiotensin formation was between 6.7 and 7.5. They demonstrated that most of the renin activity was localized in the adventitia and adjacent media. While Gould et al. (25) concluded that arterial wall and renal renin have identical characteristics, Dengler observed differences in that

a) cocaine did not enhance enzyme activity of renal renin and arterial tissue extract in the same way and that
b) equal amounts of renal renin and arterial tissue extract, as judged by their vasoconstrictor effect in the rabbit ear were not equipressor when injected intravenously into the rat: the renal enzyme being more potent under the latter conditions.

### 2.1. 3. Pseudorenin

In an effort to develop a radiochemical method for renin by using a synthetic $^{14}C$ labeled tetradecapeptide substrate, Skeggs et al. (153) found, that more angiotensin was produced by a plasma sample acting on this artificial substrate than could be accounted for by the renin content in the sample when renin was measured by biological methods. This new enzyme was called pseudorenin. It was shown, that renin and

pseudorenin could be separated on DEAE-cellulose chromatography.

The structural requirements for a substrate of human renin and pseudorenin appeared to be similar, since both renin and pseudorenin hydrolyse the LEU-LEU bond of the tetradecapeptide ASP-ARG-VAL-TYR-ILEU-HIS-PRO-PHE-HIS-LEU-LEU-VAL-TYR-SER and of the nonapeptide HIS-PRO-PHE-HIS-LEU-LEU-VAL-TYR-SER. Both enzymes do not hydrolyse the peptide LEU-LEU-VAL-TYR·OET(oxy-ethylester), HIS-PRO-PHE-HIS-LEU-LEU-VAL and ILEU-5-angiotensin I. This indicates that pseudorenin, like renin, is not a simple leucyl-leucyinase but a specific endopeptidase.

There are marked differences between renin and pseudorenin. The pH optimum of angiotensin formation with pseudorenin and tetradecapeptide renin substrate is 4.5 and with substrate A ph 5, while that of renin with substrate A is 6. Pseudorenin does not form angiotensin with homologous plasma substrate and it does not attack purified substrate A when plasma is added to the incubation. Human pseudorenin does react, however, with purified hog substrate A without the presence of serum,and angiotensin is formed when synthetic tetradecapeptide is added to an incubation of hog serum containing endogenous pseudorenin. The fact that the enzyme is active in a mixture of serum and tetradecapeptide but that there is no angiotensin formation with a mixture of serum and substrate A, indicates that the inhibitory action of serum is directed against the substrate rather than against the enzyme.

Pseudorenin has been purified and partially characterized only from human kidney which was obtained from the morgue. Bacterial

degradation and alteration of proteins and enzymes in these "dead" tissues have to be considered. During the process of purification, no bacteriostatic agent was added. Though all operations have been carried out at 4°C after the first extraction, further bacterial processes may have gone on. Purification has only been done once and has not been repeated under more vigorous conditions in order to see whether the same results were reproducible.

After partial characterization of pseudorenin from human kidney, further distinction of pseudorenin from renin is accomplished on the basis of different pH optima for angiotensin formation, pseudorenin having a maximum activity at pH 4.5 with synthetic tetradecapeptide while renal renin has an optimum at pH 6 with substrate A. It is possible that renal renin forms angiotensin with the tetradecapeptide at a pH of 4.5 as well. In that case renin would interfere in all pseudorenin measurements. This could not be investigated, since none of the renin preparation used, is free of pseudorenin and the pH-curve of renal renin with tetradecapeptide for these reasons is unknown.

Renin has been measured by other authors in all the tissues where pseudorenin has been found. The salivary gland, apparently rich in pseudorenin, contains also considerable amounts of renin. Renin measurements in extrarenal tissues and reported in the literature (see previous chapters) have been done mostly by methods using homologous natural protein substrate prepared from plasma. This excludes interference of pseudorenin according to the work of Skeggs et al. (153). Levine et al. (154) reported recently a procedure for the preparation of hog renin free of pseudorenin.

## 2.1. 4. Renin Substrate (Angiotensinogen)

The enzyme renin acts on a large protein substrate (angiotensinogen) which has thus far only been found in plasma and lymph. There is some evidence, that renin substrate is produced in the liver, since hepatectomy reduces plasma substrate concentrations (155).

On the basis of electrophoretic distribution, renin substrate is located mostly either in the fraction of $\alpha_2$-globulin mobility (dog, pig) or in the albumin-prealbumin fraction (man, rat) (156-159). Green and Bumpus (157) purified the substrate by use of partial acid denaturation followed by ammonium sulfate fractionation.

Skeggs et al. (159, 160) purified substrate from hog plasma using ammonium sulfate fractionation, partial acid denaturation, DEAE-cellulose used both in batches and columns, and counter-current distribution in polyethylene glycol-neutral salt solutions.

Five different substrates were found: A, $B_1$, $B_2$, $C_1$ and $C_2$. These different forms were present also in plasma that had been precipitated only once by ammonium sulfate at pH 6.0, indicating that they were possibly not artefacts produced during purification. The different forms of renin substrate appeared to have similar amino acid composition and a molecular weight of approximately 58,000, as determined by ultracentrifugation. They differed in the carbohydrate portion of the molecule. The different forms of substrate are attacked by renin at approximately the same rate (160).

Renin does not need the whole structure of the substrate molecule, but also acts on fragments of it. Skeggs et al. (161, 162) showed, that a 14 amino acid peptide, which was obtained by degradation of horse renin substrate with trypsin, yielded angiotensin when treated with renin. The structure of this tetradecapeptide was analysed and confirmed by synthesis. Skeggs et al. were then able to show that renin acted on the LEU-LEU bond in the 10-11 position of the renin substrate to form angiotensin I (161). Since in angiotensin I, angiotensin II, the tetradecapeptide and the other various forms of substrate, aspartic acid is the only amino acid that is N-terminal, the decapeptide angiotensin I must represent the first 10 amino acids, counted from the N-terminal of renin substrate.

Levels of renin substrate in the plasma under normal conditions are rather constant. Nephrectomy increases plasma substrate levels in the dog (163, 164) and even more markedly in the rat, in which species it may increase 4-15 fold 24 hrs. after nephrectomy (165). This increase is usually attributed to the removal of renin from the circulation, which implies, that the renin system must be very active normally and substrate turn-over must be high. Adrenalectomy sharply reduces plasma substrate concentrations, which can be restored by treatment with various steroid hormones (166-170). In a recent communication Nasjletti et al. (171) showed, however, that renin substrate and total protein synthesis was increased in the liver after nephrectomy. The substrate concentration in plasma is not necessarily inversely related to the plasma renin concentration.

Pathological conditions, in which high plasma renin activity is found, could be associated with high, normal or low angiotensinogen concentration (172). This demonstrates that the renin substrate relationship in plasma is not a simple one and that various factors in addition to PRA, some of which are still unknown, have to be considered (173, 174).

### 2.1. 5. Angiotensin

Incubation of renin with renin substrate yields a vasoconstrictor substance which is now called angiotensin and which has been first described by Page et al. (175, 176) (angiotonin) and Braun Menendez et al. (177) (hypertensin).

This substance was heat stable, water soluble and dialyzable (175, 176). It was also highly soluble in acetic acid, insoluble in organic solvents, acid stable and alkali labile (177). It was destroyed upon incubation with trypsin. Strong oxidizing agents inactivated angiotensin (175, 176).

Purification and structural analysis were done for angiotensins of human (178), beef (179-182), horse (183, 184), and hog (185, 186). It was identified as a decapeptide (angiotensin I) with identical sequence of amino acids in all species except beef, where isoleucine in position 5 was replaced by valine.

Angiotensin I itself has no vasoconstrictive effect. When injected into the blood, a specific endopeptidase, converting enzyme, removes the HIS-LEU dipeptide from the C-terminal of the decapeptide, thus producing an octapeptide which is called angiotensin II and is the most potent vasoconstrictive compound known.

Angiotensin II was isolated later (187) and its structure determined. The sequence of natural angiotensin has been confirmed by degradation and synthesis by various groups (188-192) and a number of synthetic analogues have been tested for biological activity. Requirements for biological activity are:

1) at least 6 amino acids from the C-terminal;
2) a free C-terminal carboxyl group;
3) a phenyl side group in position 8;
4) proline in position 7;
5) an imidazol group (HIS) in position 6 and
6) a phenolic group in position 4.

The synthetic α-asparagine 1-Valine-5-angiotensin II has been shown to have identical activity to natural angiotensin II and has been used in all laboratories as a standard (Hypertensin$^{R}$ Ciba).

## 2.1. 6. Angiotensin I Converting Enzyme

Lentz et al. (183) found that angiotensin I injected in the blood stream was converted into angiotensin II. The enzyme cleaving the HIS-LEU dipeptide from the decapeptide was called angiotensin I converting enzyme. It was found that angiotensin I was little or not vasoactive on isolated rabbit aortic strip (193, 194) and in the isolated rat kidney perfused with saline. Addition of plasma resulted in marked vasoconstrictive activity (195, 196). These findings indicated the presence in plasma of a converting enzyme capable of forming AII, and indicated also that AI itself was not vasoactive. Other authors however showed, that AI itself could produce contraction of smooth muscle of isolated guinea pig ileum (197, 198), and when perfused in the hind quarters of rat and rabbits.

A possible explanation for these discrepancies was given by Bumpus et al. (201) who found, that some tissues did have converting enzyme activity and that tissue homogenates, which do not convert AI to AII like the uterus, do not respond to AI. The first reports of plasma converting enzyme purification and a method for converting enzyme activity measurement came from Lentz et al. (202) and Skeggs et al. (195, 196). The plasma enzyme can be precipitated with ammonium sulfate. It is stable at pH 4, but rapidly destroyed at pH 3. Maximum activity occurs between 6.5 and 8. Converting enzyme is activated by chloride ions and inhibited by EDTA. DFP has an inhibitory effect only on CE activity in heart and aortic tissue but not in plasma.

Ng and Vane reported the presence of the same converting enzyme in lungs (203). This finding was confirmed by other investigators (204-206). Recent observations, on the release of a factor following perfusion of the lung with AI, however, which constricted the rat colon but had no effect in the rat pressor assay, raised doubts about the validity of early measurements of rapid in vivo conversion of AI during the passage through the lung (207). Furthermore, Biron et al. (208) have found that angiotensin I was only converted at a rate of 30% when injected directly into the pulmonary artery and no conversion at all was found in 2 patients with high pulmonary blood flow due to left to right shunt. Conversion of angiotensin I during the passage through the lung in children seems to be low as well.

The methodological problems for measurement of converting enzyme activity are far from solved. This is clear from the work of Boucher et al. (210), who demonstrated that not one but two different converting enzymes exist. With a specific method, using angiotensin I as the natural substrate and measuring the natural product of the enzyme-substrate-reaction, namely angiotensin II, by its physiological vasoconstrictive activity in the rat pressor assay, Boucher confirmed the chloride activated converting enzyme (209, 210) described by Skeggs et al. (195). This CE (α-converting enzyme according to Boucher) was inhibited by EDTA, dipyridyl and the pentapeptide PYRGLU-LYS-TRP-ALA-PRO, isolated from Bothrops Jararaca venom (211, 212). Using the same substrate, angiotensin I, Boucher described a second different enzyme

in the submaxillary gland of the rat called by Boucher the β-converting enzyme (210). This new CE was a specific dipeptidase, since it removed the C-terminal HIS-LEU of angiotensin I as a dipeptide and not as individual amino acids. The β-CE was shown to be independent of the inhibitors previously described for converting enzyme. It was inhibited, however, by human and rat plasma and Soybean trypsin inhibitor.

The new β-enzyme appears to have very specific substrate requirements. It does not, or to a very slight degree, attack the synthetic tripeptide substrate benzyloxy-carbonyl-PHE-HIS-LEU which was proposed by Piquilloud et al. (213, 214) for measurement of CE activity. The α-converting enzyme on the other hand reacts much faster with this short artificial substrate than with its natural substrate angiotensin I and thus seems to have less specific substrate requirements.

In numerous studies concerning converting enzyme, methods have been employed

a) using a short artificial substrate (212, 213) which is economic and practical but does not necessarily reflect the physiological converting enzyme activity and

b) using spirally cut strip of rabbit aorta, rat uterus or similar preparations which respond to angiotensin II but not to angiotensin I. In these assays, vasoactive substances other than angiotensin II can interfere as demonstrated after angiotensin I perfusion of the lung (207).

Furthermore, in the absence of chloride ion, converting enzyme activity was considered to be absent by definition. This no longer holds true after the description by Boucher et al. (210) of a chloride independent β-converting enzyme.

### 2.1. 7. Antirenin

The first renin-antibodies were obtained by Johnson and Wakelin (215). Haas and his group (216, 217, 218) showed that renin from various species (dog, rat, rabbit, pig, beef, sheep, human) is antigenic in heterologous hosts. Various adjuvants have been studied and can increase antigenicity of the molecule (216-218). The antibody is not fully characterized. It has been shown that antirenin precipitates with the γ-globulins on alcohol fractionation (216). It is stable over a wide range of temperature (-24°C - 55°C) and pH (1.25 - 11.2 at 0°C) and it can be stored in serum without loss of activity at -20°C for many years. Antirenin and renin form a soluble complex. On dissociation at a pH of 11.3, antirenin remains active while the active site of renin seems to be inactivated irreversely (218).

Since no pure renin is available for the production of renin antibodies, the specificity of the reaction must be evaluated carefully. The hog-renin hog-antirenin system is best studied because of its relatively easy availability. Pig renin is highly antigenic in rats and yields less antibodies in human, dog and rabbit (120). Antirenin

is only partly species-specific. For example: Antirenin to hog, rat, rabbit or dog renin, produced in the rat, dog or rabbit can each inactivate hog, rat, rabbit, dog, beef and sheep renins. Inactivation of renin of one species by different heterologous antirenin occurs to variable degrees, however. None of the animal antireninsmentioned above can inactivate human renin, while anti-monkey renin inactivates human renin and anti-human renin inactivates monkey renin. Acetylation of the renin molecule can alter its specificity and acetylated dog renin induces production of antirenin which then is capable of inactivating human renin (216, 219, 220).

### 2.1. 8. Antiangiotensin

A vast literature has appeared during the last years concerned with the production of antibodies against angiotensin I and angiotensin II and their use for radioimmunoassay techniques for measurement of angiotensin (221-233). Usually angiotensin is coupled as a haptene to a large protein in order to produce an antigenic protein-haptene complex. The rabbit is most often used and yields high antibody titers against angiotensin.

Since it is relatively easy to purify the peptide and since, in contrast to renin, pure synthetic angiotensins are available, the production of specific angiotensin antibodies should theoretically be possible. Various substances and especially metabolites of

angiotensin, however, can interfere in the antigen-antibody reaction. There are antibodies available, which are specific for angiotensin I and do not interfere with angiotensin II and vice versa, antibodies which react only with AII and show no inhibition of AI (221). Degradation products of angiotensin such as the 1-9 nonapeptide, 2-8 heptapeptide and the 3-8 hexapeptide, however, frequently interfere with antiangiotensin antibodies and often make high purification of the antigen necessary prior to measurement.

### 2.1. 9. Renin Inhibitors

Page et al. (234) have suggested, that the kidney may be a source of an antihypertensive substance. Since then several authors have confirmed this observation (235-237). Intravenous infusion of renin gives a higher and more sustained pressor response in nephrectomized animals than in normal animals (11) and this increased sensitivity can be reduced by transfusion of normal blood into the nephrectomized animal (234). The early observation of Goldblatt et al. (1) that experimental hypertension became more severe and sustained when the contralateral normal kidney was removed, was also interpreted as being due to the removal of an antihypertensive agent present in the kidney (238-240).

In vitro experiments have shown, that equal amounts of renin, added to plasma samples from different individuals, generated varying

amounts of angiotensin during the incubation (19, 241) and renin added to the plasma of a nephrectomized animal generated more angiotensin than an aliquot of renin incubated with normal plasma (242).

These observations suggested the presence of one or more factors in the plasma which could affect the rate of angiotensin formation.

Sen et al. (242) have isolated a phospholipid, capable of inhibiting renin in vitro and reducing the blood pressure of hypertensive animals in vivo (243, 244).

This substance was present in the blood of human, dog and rat. The isolated phospholipid was not active itself, but was activated by hydrolysis with phospholipase A which yielded the active lysophospholipid (243, 244). These results have not been confirmed (245). Several other groups have extracted vasodepressor substances from the kidney (246-254) and prostaglandins may be important in this respect as shown by Strong et al. (254) and Tobian et al. (246). The physiological importance of inhibitors of the RAS for the regulation of arterial blood pressure and its role in the development of experimental hypertension remain to be investigated.

## 2.1. 10. Angiotensinases

Angiotensin I or angiotensin II, incubated with plasma or tissue homogenates is rapidly inactivated and metabolized by a group of enzymes called "angiotensinases". Some of these enzymes may be specific for angiotensin while others are not. Proteolytic enzymes such as trypsin, pepsin, chymotrypsin and carboxypeptidases hydrolyse angiotensin as well as other peptides and proteins (255).

An aminopeptidase with a relative high degree of specificity has been found in plasma and red cells (256) and has been termed angiotensinase A. It has a pH optimum between 7.5 and 8. It is dependent on $Ca^{++}$ ions and consequently can be inhibited by EDTA. It is precipitated between 1.5 and 2.1 molar ammonium sulfate. A second angiotensinase with different properties from angiotensinase A has been found in plasma and has been called angiotensinase $A_2$. This enzyme is dependent on $Ca^{++}$ ions as is angiotensinase A but has an optimal pH of 6.8 and can be separated from it by Sephadex G 200 chromatography (257, 258).

A third enzyme, which is present in plasma and splits angiotensin II into two fractions of tetrapeptides is called angiotensinase B (257, 259). Its maximal activity occurs at pH 5.8-6; it is inhibited by EDTA but also by DFP.

Acid treatment of plasma at pH 4 destroys completely all angiotensinases (260). Addition of DFP, EDTA, dimercaptopropanol (BAL), 8-hydroxyquinoline, charcoal or Dowex resin 50WX2 ($NH_4^+$) and

epsilon-amino-caproic acid protects angiotensin from destruction. Mercury inhibits plasma angiotensinases but not those present in red blood cells. Tissue angiotensinases are only partly inhibited by EDTA, complete inhibition is achieved by a mixture of EDTA and DFP (13, 260-263).

The quantitation of angiotensinase activity may be performed by using methods, based on the principle of incubating known concentrations of angiotensin with plasma and measuring the degradation of the peptide (264-267). Strong et al. (268) have recently reported a method for the measurement of angiotensinase activity in plasma by radioimmunoassay techniques. With precise and reproducible methods for the measurement of angiotensinases available, it may be possible to get a better idea of the physiological and clinical importance of these enzymes.

## 2.2. *Clearance of Renin and Angiotensin from Plasma*

For many enzymes and hormones it has become common to measure secretion rate, metabolic clearance rate and plasma levels in order to obtain an idea of the activity of the system. Renin secretion rate has been calculated in some studies, but little work has been done on its metabolic clearance rate.

The half-life time of exogenous renin injected in dogs appears to be between 4 and 45 minutes depending on methods and animals used

(269-275). There are, however, two components of disappearance, a fast one and a second which is much slower (269).

Endogenous renin disappears at similar rates as exogenous (270, 272). After nephrectomy, dogs retain exogenous renin for a longer period of time (269). The kidney thus seems to play a role in renin excretion, which is in agreement with the finding that renin activity has been measured in urine by several authors (276, 277), particularly by Hayduk et al. (278).

Braun-Menéndez and his group (100) have reported that disappearance of renin from plasma is delayed in hepatectomized dogs. These findings point toward the liver as a factor controlling the metabolism of renin. Heacox et al. (272) observed no difference in renin activity between the aorta and portal vein, concluding that renin does not disappear in the vascular bed of the splanchnic territory. There was, however, a marked difference between the portal and hepatic vein, PRA levels being lower in the hepatic venous blood, which confirms the role of the liver in the extraction of renin from the blood. These findings have been confirmed by Horky et al. (274) and Tapia et al. (275) who found that part of the renin is excreted into the bile. Most authors agree on the fact that the liver is the predominant organ for renin clearance (269-275), though the inactivation of renin by renin destroying enzymes which are present in the plasma may also be a factor to be considered (279, 280).

The kidney, though involved through renin excretion, seems to play a secondary role in this respect. The effect of other tissues

appears to be negligible. Disappearance of exogenous and endogenous renin is fast. The metabolic clearance rate is around 70 ml plasma/ minute (269). Under any conditions renin disappears completely from the plasma between 20 minutes to a maximum of 2 hours when high renin concentration is used.

Angiotensin must be removed quite fast from the circulation, since the vasopressor response of injected angiotensin in rats and other animals lasts only a few minutes. The short-lived action of angiotensin is explained by two phenomena:

a) Degradation of angiotensin into inactive fragments by a number of enzymes which are collectively called angiotensinases and which have been discussed in a previous section and

b) by binding of angiotensin in various sites of the body, since it had been shown by Bumpus et al. (261) that infused radioactive angiotensin could be recovered in various organs, with the kidneys, adrenal glands and uterus being the main target organs where accumulated radioactivity was detected.

A whole new concept of angiotensin receptors in various organs and cell-organells has emerged (281-284). In our context we will not further discuss these aspects of angiotensin metabolism. The half-life time of angiotensin in the dog, as measured by the multiple organ bath technique, is between 113 and 200 seconds (285). In rat plasma the half life was less than 120 seconds (286), but in man, synthetic angio-

tensin appeared to have a half-life time of 10 to 20 minutes (287). Most of the studies on the metabolism of angiotensin have been done with radioactively labeled octa- or decapeptide, and the conclusions were drawn mostly from data of disappearance of the label from the plasma and location of the tracer at some site in the tissue. We are not aware of any study, in which the tracer, after injection in physiological doses, has been identified as intact angiotensin and it is possible that labeled degradation products of angiotensin (smaller peptides or amino-acids) have been measured. Reported results on the disappearance of angiotensin into tissue may not always reflect the true picture and must be interpreted with caution.

## 2.3. *Interaction of the Components and Critical Evaluation of the Classical Renin-Angiotensin-System.*

Angiotensinogen, the renin substrate, is synthesized mostly in the liver and secreted into the blood stream. It is hydrolysed at its LEU-LEU bond by a specific endopeptidase, renin, which is produced in the juxtaglomerular cells of the kidney and secreted in the blood stream. The enzyme substrate reaction thus takes place in the circulating plasma and yields angiotensin I, which has no known biological activity. This decapeptide is converted to angiotensin II by an enzyme, called converting enzyme. Angiotensin II is believed to be the effector substance of the system and is the most potent vasopressor

compound known. It is degraded by various specific or unspecific enzymes, called angiotensinases.

This simplified outline of the renin-angiotensin-system is summarized in figure 1. Since neither renin nor converting enzyme have been obtained in their pure form, the true kinetics of this system are unknown.

The conversion of AI to AII by the converting enzyme was thought to occur in the plasma and during its passage through the lung (203, 205). The discovery of a second converting enzyme in the submaxillary gland by Boucher (β-converting enzyme) and the presence of an inhibitor of this β-enzyme in the plasma complicated this system (210).

The impossibility up to now to measure the enzymes in their pure form and to identify their regulating inhibitors (or activators), forced many investigators to measure the end products of the enzymatic reaction: angiotensin I and angiotensin II. It was thought that angiotensin I levels in plasma could give an idea of the true renin-activity and that angiotensin II levels could give an idea of the converting enzyme activity. By measuring both angiotensin I and angiotensin II in the blood it was hoped, to get a true picture of the activity of the renin-angiotensin system. Results reported up to date cannot yet be considered fully conclusive, mostly because of methodological reasons:

1) the ordinary way by which blood is withdrawn from patients and experimental animals does not exclude some further

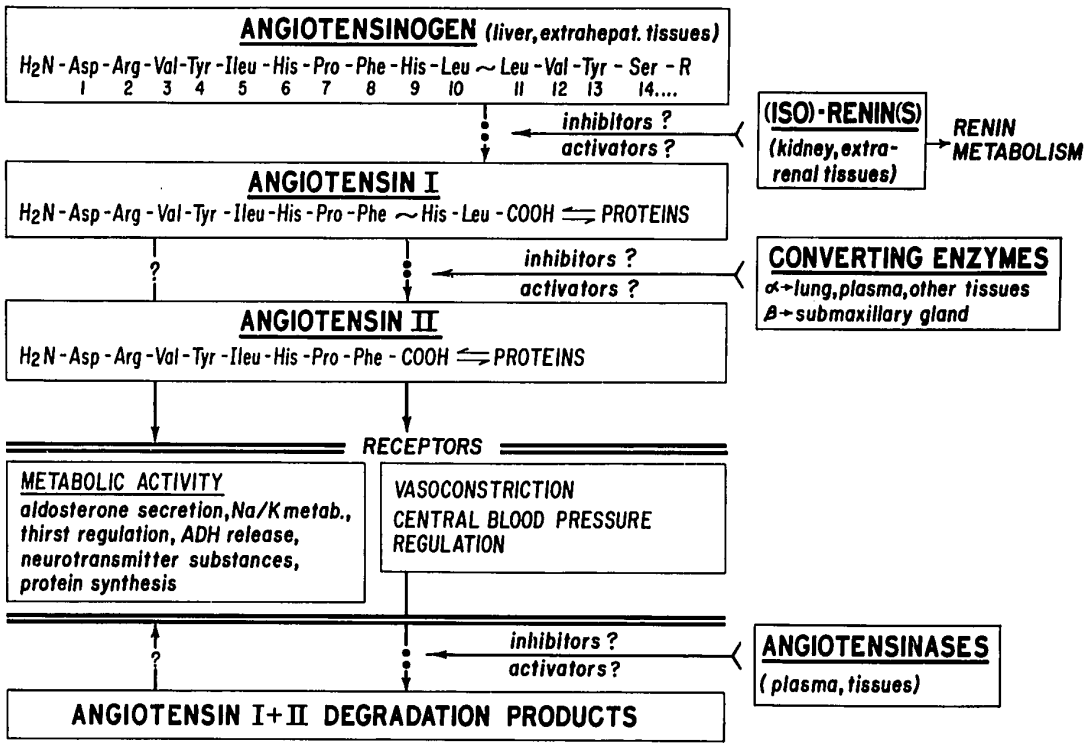


FIGURE 1: *Schematic outline of the Renin-Angiotensin System.*

activity of the enzymes. Renin may continue acting on the substrate and converting enzyme on angiotensin I during the withdrawal and storage time, even if the sample is cooled as quickly as possible. The amounts of angiotensin formed may be small. But with radioimmunoassay techniques, picogram amounts of angiotensin are measured and the slightest activity of the renin-angiotensin system after withdrawal of the blood may easily yield amounts of angiotensin that falsify the pattern of the peptide concentrations in the blood stream when measured by this sensitive assay. A further difficulty in interpreting reported results is the fact that EDTA was considered a converting enzyme inhibitor. Boucher, however, demonstrated that the β-enzyme is not inhibited by this compound (210). Although β-angiotensin I converting enzyme is inhibited by a plasma protein inhibitor, angiotensin I could still be converted under yet unknown conditions. Thus angiotensin II levels, as reported, may, to some extent, be higher than the actually circulating angiotensin II, and, for the same reason, the measured angiotensin I may be lower than the circulating decapeptide.

2) Though good specificity is claimed for the angiotensin antibodies, degradation fragments of angiotensin can interfere in the immunoassay and thus purification of the deca- and octapeptide before measurement is necessary.

It appears evident from the above that methodological problems of measuring the different components of the system must be solved if we want to achieve a better understanding of the renin-angiotensin system.

Some aspects of this complex enzymatic system have not yet been attacked such as,

a) protein-binding of circulating angiotensin, and the biological activity of bound versus free angiotensin,

b) the question whether angiotensin I has biological activity (or activities) has not been solved, since the differentiation of the effects of angiotensin I and II, especially in tissue, has not been possible as yet.

c) The various metabolic actions which have been described for angiotensin in the past three years apart from the stimulating effect on aldosterone must be investigated further and their physio-pathological importance remains to be clarified. Their possible interactions with the role of the renin-angiotensin system in blood pressure volume and sodium regulation has to be determined.

d) The role of tissue renin is far from being clear. Some attempts have been made to relate it to the problem of blood-pressure regulation (submaxillary gland, arterial wall) or to muscle tonicity (uterus). Except for the uterus it is still unclear, whether tissue renin is synthesized in the

tissue itself, whether it represents kidney renin, which is secreted into the blood stream and then adsorbed by the tissue or whether it is a combination of both, adsorbed and synthesized renin.

In all tissues, which are studied so far, renin activity is higher in tissue than in plasma, be it expressed per unit of weight or as specific activity (renin activity per mg protein). Thus, theoretically, angiotensin formation could be even more important in these tissues than in plasma, if renin substrate is available at the tissue level.

It is known that angiotensin influences the handling of sodium by the kidney and other tissues directly or via its influence on steroid secretion (92-98, 103). The action of angiotensin on the release of antidiuretic hormone is well established (73-76). Angiotensin influences catecholamine synthesis, release and re-uptake (65-69) and also affects the metabolism of the cholinergic system (70-72). The effect of angiotensin on whole body water and salt balance via the central nervous system has been described by several authors (73-87). Angiotensin also influences the blood pressure by acting on the central nervous vasomotor system: amounts of the octapeptide, too small to have any effect on the systemic circulation, increase the blood pressure by stimulation of the vasomotor center, situated in the lower medulla (288-296).

It was the purpose of this study to investigate, whether tissue renin is part of a functioning system, that is, whether substrate is available at the tissue level and whether angiotensin can be formed locally in the tissue, independently from the kidney-plasma renin-angiotensin system.

Such an intrinsic, independent tissue renin-angiotensin system, could then influence some of the metabolic actions which were described for angiotensin.

We considered the brain a suitable model for the study of extra-renal renin because

a) some of the newly described and apparently important metabolic actions of angiotensin take place in the brain;
b) the blood-brain-barrier may facilitate the separation of the renal and the brain renin-angiotensin system.

## 2.4. *The Blood-Brain-Barrier*

The composition of the cerebrospinal fluid is characteristically different from plasma or an ultrafiltrate of it, which shows that CSF is not a simple tissue fluid like lymph or pericardial fluid. Its protein concentration is 0.31 mg/ml and the sodium and magnesium content is higher than in plasma while potassium and calcium levels are lower (297, 298, 300, 301). It has been shown that passive diffusion, especially of non lipid soluble solutions, from the blood into the CSF and brain tissue is limited by what is called the blood-brain-barrier (BBB) and extensive studies have been done on the permeability of the BBB for various substances (297-307).

Anatomically, the BBB is a complex system, which has to be divided into several parts:

a) the epithelial layer, which covers the choroid plexus is presumably the basis for the blood-CSF barrier into the ventricular system.

b) Tight junctions (zonular occludentes) that seal the spaces between adjacent epithelial cells have been described.

c) Another part of this barrier system, though probably less effective, may be the ependymal lining of the ventricles which is called the CSF-brain barrier.

The cerebrospinal fluid in the subarachnoid spaces is separated from the brain by the pia and from the dura by the arachnoid. Both ependyma and pia appear to allow relatively free exchange of substances

across these layers, which would account for the effectiveness of drugs when applied directly into these spaces. It is debated, however, whether local variations in selectivity of this barrier do exist by occurrence of tight junctions in parts of these epithelial layers or by variable thickness of glia-cells between the surface and the underlaying nervous tissue.

The failure of trypan blue to stain the brain blue when injected into the blood stream was attributed to the presence of protoplasmatic processes of the astrocytic layer which surround the brain capillaries, and which are the morphological basis of the blood-brain-barrier (300).

It is not clear as yet which part of the vascular system is the most selective barrier, the endothelial cells, the basal membrane of the capillaries consisting of the lamina densa and lamina rara, or the astrocytic layer. There is, however, no doubt that there exists a considerable restraint of the passage from the blood into the brain for substances of even low molecular weight like Hexoses and small peptides (306). It has been demonstrated lately that the permeability for even so small a tracer as $^{24}Na^{+}$ is very low in the human blood-brain-barrier (304).

No studies have been done on the permeability of renin through the blood-brain-barrier. The BBB has been well studied however for another enzyme, horseradish peroxidase, which has been shown not to penetrate into the CSF after injection into the circulating blood. The enzyme is taken up and immobilized by the epithelium of the choroid plexuses (299).

It is generally agreed upon, that if a substance does not pass the blood-cerebrospinal fluid barrier, a similar selectivity and hindrance can be expected from other parts of the barrier system (297-301).

The question whether the permeability of the BBB changes after induction of hypertension is still controversial (302, 303). There is some evidence that proteins may penetrate from the blood into the brain in hypertensive patients which is in agreement with Sonkodi et al. (302) who described that the permeability of the BBB in renal hypertensive rats is increased. There is no correlation between plasma renin activity and the permeability of the BBB (302).

It is not clear, whether angiotensin crosses the blood-brain-barrier from the blood into the brain tissue. Volicer et al. (307) using $^{14}C$ labeled angiotensin concluded from their experiments that angiotensin II penetrates to some extent into the brain. These authors, however, used extremely high doses (0.33 μ Ci labeled angiotensin, which corresponds according to their data to 1400 ng angiotensin per 20 g mouse). Only when even higher doses of labeled angiotensin were injected (2 μ Ci $^{14}C$ angiotensin), could labeled angiotensin be detected in brain tissue by autoradiography. It is questionable, whether these experiments allow any conclusion as to the permeability of the blood-brain-barrier for angiotensin under physiological conditions. Goldstein and Finkielman et al. (personal communication) have found an angiotensin-like substance in CSF of human, which was increased in hypertensive patients in significant corre-

lation with the increase of blood pressure and they conclude, that angiotensin in the CSF originates from the brain tissue. It has been shown for other substances, that cerebral metabolites may be found in the CSF (305).

Polypeptides of comparable size, such as vasopressin, do not pass the blood-brain-barrier (292). Furthermore, the time relationship supports the thesis that the central effect of angiotensin on blood pressure does not come from the CSF-side: infusionsof angiotensin into the vertebral artery have very short-lived after effects while injections into the CSF have long-lasting action (288).

Dickenson et al. (295) support the concept of direct action of blood-borne angiotensin on central vasomotor centers not being mediated via the CSF. Direct vasoconstriction of small brain arteries in certain centers or local deficiency of the BBB are discussed (295) to explain the central action of angiotensin. Joy and Love (291, 292) and Scroop et al. (293, 294) have demonstrated lately that this effect of angiotensin takes place in the area postrema of the medulla. This region, lying in the caudal half of the medulla and protruding into the fourth ventricle, is a known gap in the blood-brain-barrier, which might allow blood-borne angiotensin to reach the brain-tissue directly (288).

Further areas where the brain appears to be devoid of a blood-brain-barrier anatomically are the neurohypophysis, the tuber cinerum, and the pineal body of the intercolumnar tubercle. In these areas, blood-borne angiotensin may influence directly brain function.

# 3. INVESTIGATIVE SECTION

## 3.1. *Methods for Renin and Angiotensin*

Boucher et al. (22) have described a method for measurement of renin activity in the plasma of rats. This method has been adapted by Granger for dogs (104) and is by now internationally used for plasma renin determinations in different species of animals and humans. This method is highly specific and reproducible. Tissue renin activity can be measured by this method essentially the same way as described for plasma. The principles of this method which we used throughout for all determinations of renin activity in plasma, cerebrospinal fluid and tissues are outlined in figure 2.

The renin-containing sample is incubated at 37°C for 12 hours with an excess of semipurified homologous substrate. The lyophilized substrate powder for this purpose is dissolved in 2 ml of a 0.25 M TRIS-buffer solution which is adjusted to pH 5.5 with concentrated phosphoric acid and containing EDTA for inhibition of angiotensinase activity and sodium azide as a bacteriostatic agent. To the mixture of renin, homologous renin substrate and TRIS-buffer, 1 cc of Dowex 50WX2 resin in the ammonium form is added. As described by Boucher et al. (12, 13, 22) this resin has the quality of absorbing angiotensin as soon as it is formed during the incubation. Vigorous agitation is necessary. Bound to the resin, angiotensin is protected against degradation.

**PROCEDURE FOR MEASUREMENT OF RENIN ACTIVITY (DOG)**

1ml PLASMA or CSF or

100mg BRAIN HOMOGENATE (in 3ml ISOTONIC SALINE)

+ DOWEX 50W-X2 ($NH_4^+$)
+ TRIS PHOSPHATE BUFFER (pH 5.5)
+ EXCESS OF DOG SUBSTRATE

INCUBATION FOR 12 Hrs

ELUTION OF ANGIOTENSIN FROM DOWEX RESIN

LYOPHILIZATION

RAT PRESSOR ASSAY

FIGURE 2: *General outline of the procedure for the measurement of plasma and tissue renin activity.*

After incubation, the sample is cooled and the generated angiotensin is purified in the following way: the sample is transferred to a glass column already containing glasswool and 1 cc of moist resin. Proteins and peptides as well as other material are washed out from the column by 15 ml of 0.2 M ammonium acetate at pH 6 and 20 ml of 10% acetic acid followed by 20 ml of distilled water.

Angiotensin is then eluted from the column with 7 ml of 0.2 N ammonium hydroxide into a siliconized recipient. The sample is frozen, concentrated by lyophilization, and determined by the 4 point pressor assay in nephrectomized rats as described by Boucher et al. (22).

All results are expressed as ng angiotensin formed, per 1 hour incubation time at 37°C by 1 ml plasma or CSF or by 1 g wet weight of tissue.

It is clear from the description of the method that the values obtained do not reflect true renin concentration but "effective renin concentration", which is a summation of known and unknown parameters involved in this complex enzymic system. Substrate as the limiting factor is excluded by the addition of excess exogenous angiotensinogen to the sample.

### 3.1. 1. Plasma

Blood was rapidly withdrawn into a syringe containing 2% (V/V) of a 15% EDTA (W/V) solution, the preparation of which has been described (22), and centrifuged as quickly as possible in a refrigerated centrifuge. One ml plasma was pipetted into a plastic test tube, closed with parafilm and kept frozen at -20°C if not processed immediately.

### 3.1. 2. Cerebrospinal Fluid

CSF was obtained in the same way with EDTA by suboccipital puncture of the posterior cisterna. CSF samples which were contaminated with blood were discarded. Processing was identical as for plasma.

### 3.1. 3. Brain Tissue

The animals were killed by an overdose of Nembutal$^{R}$ (Abbott) or by rapid bleeding under anaesthesia. With a motor-driven bone-autopsy saw* the skull-cap was removed and the whole brain taken out. 100 to 200 mg tissue aliquots from various regions were wrapped air tight in parafilm and rapidly frozen. Ten to 15 minutes elapsed, on the average, from the dissection of the skull until freezing of the samples.

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* Lipshaw-Company, No. 450, Ser. D-056, Detroit, Michigan.

During the next 24 hours, the tissue was weighed in the frozen state and transferred into plastic test-tubes. After thawing, it was frozen again. The tissue was then homogenized in an all glass 7 ml Pyrex$^{R}$ homogenizer with approximately 3 ml 0.9% saline solution at 4°C. During the homogenization procedure refrigeration temperatures for the sample were observed. The homogenized tissue was then frozen again at -20°C in a test-tube and later incubated as described above with Dowex 50WX2 ($NH_4^+$) and substrate. The procedure thus included 3 repeated freezings and thawings of the sample, which assured the breaking of the cells and the availability of the intracellular enzyme to the exogenous substrate.

The procedure for measurement of renin activity in other tissues was the same as for brain tissue with minor modifications as described below.

### 3.1. 4. Mesenteric Artery Branches

While brain tissue was taken from the dead animal, arteries from the splanchnic territory were dissected in vivo in the anaesthetized animal from always the same anatomical region (the first complete arterial arcade of the ileum counted from the ileo-coecal end.). The preparation of the tissue took approximately 3-5 minutes. Only the 2nd to 5th order branches of the mesenteric artery were taken. These were freed of connective tissue, the blood was squeezed out of the arterial lumen with a smooth forceps and on Whatman chromatographic paper No. 2.

Approximately 100 mg were weighed for each sample. The incubation for mesenteric artery was prolonged to 24 hours because of the relatively low renin levels in this tissue and the difficulty of obtaining and homogenizing larger amounts of tissue.

### 3.1. 5. Aortic Tissue

The animals were bled by cutting the aorta. A 100 mg piece of thoracic aorta approximately 3 cm proximal to the diaphragm was prepared free of connective tissue leaving the adventitia intact. Incubation time as for all other arteries was 24 hours.

### 3.1. 6. Carotid Artery

The carotid artery was sampled in vivo by a median cut over the trachea, blunt dissection of the common carotid, and ligature at its proximal and distal ends. 100 mg samples were incubated as described above.

### 3.1. 7. Adrenal Gland

100-200 mg aliquots of the whole (cortex and medulla) adrenal gland, carefully dissected free of connective tissue were handled as

described for other tissues. Because of the greater enzyme concentration, they were diluted 1:10 in 0.9 saline solution after homogenization, centrifuged for 10 minutes at 12,000 g and 1 ml of the supernatant was incubated for 12 hours.

## 3.2. *Measurement of Angiotensin in Plasma, CSF and Brain Tissue*

Measurement of angiotensin II in blood raises difficult problems caused essentially by the low concentration of this peptide. Before the advent of radioimmuno-techniques, large quantities of blood had to be collected in order to obtain measurable amounts of angiotensin.

The radioimmunoassay is a highly sensitive method, capable of detecting picogram amounts but there is a general danger for all these techniques: the antibodies are not sufficiently specific and usually not purified enough to exclude interference from related substances.

The antibodies which were used in our experiments have been produced by Wilson et al. (221) with Val-5-angiotensin I and Val-5-angiotensin II in the rabbit. These antibodies are specific for the Octa- and Decapeptide. In amounts up to 1000 pg, angiotensin I did not interfere in the assay of angiotensin II and vice versa. Bradykinin and ARG-Vasopressin did not interfere. There was however interference by the 1-9 nonapeptide, the 2-8 heptapeptide and 3-8 hexapeptide, which are degradation products of angiotensin. This interference was overcome

by purification of the antigen on SE-Sephadex columns, which allows satisfactory separation of angiotensin from the interfering fragments. This procedure has advantages over other methods of measurement of angiotensin because of its higher sensitivity.

Determinations of angiotensin in tissue have been done in parallel by the radioimmunoassay technique and by a more classical method using extraction, purification, concentration, and bioassay in the rat. There was general good agreement of the results obtained by the biological method and radioimmunoassay (221).

Extraction of angiotensin from 20 to 80 g of aliquots of brain tissue was done by homogenization into 5 volumes of ice-cold 80% ethanol in a Virtis homogenizer. By this procedure enzymes are denatured, while smaller peptides are not affected. The homogenate was stirred continuously for 12 hrs. at 4°C. The mixture was then centrifuged at 12,000 g for 10 minutes, the precipitate discarded and 4 ml of Dowex 50WX2 ($NH_4^+$) was added to each 10 ml of supernatant. After stirring for 1 hour in the cold, the mixture was transferred to a column, already containing 1 ml of Dowex 50WX2 ($NH_4^+$) resin.

The column was then washed with 30 ml of 0.2 M ammonium acetate at pH 6 and 30 ml of 10% acetic acid followed by 30 ml distilled water. Angiotensin was eluted from the resin with 14 ml of 0.1 N diethylamine and 14 ml of 0.2 N ammonium hydroxide according to Boucher's method (22) into a siliconized recipient and further processed as described for renin determination. The angiotensin thus extracted from brain tissue was measured in the rat 4 point pressor assay as well as by radio-immunoassay.

### 3.3. *Method for Measurement of Renin Substrate in Plasma and CSF*

The method of Boucher et al. (22) for measurement of renin activity is readily adaptable for the measurement of renin-substrate. The procedure consists of adding to the sample excess amounts of homologous or heterologous renin. The enzyme will then hydrolyse the renin substrate and form angiotensin until all substrate is consumed. The angiotensin formed during the incubation is adsorbed by Dowex 50WX2 ($NH_4^+$) from which it is eluted, purified and assayed as described. Since hog renin* is commercially available in standardized and purified form and since hog renin has a good affinity to dog substrate, it has been used for most of our substrate assays. Two units pig renin are a safe excess of enzyme for the assay of 0.2 ml plasma or 1 ml CSF.

### 3.4. *Method for Measurement of Renin Substrate in Brain Tissue*

Under the experimental conditions used, when tissue homogenates are incubated without excess of homologous or heterologous renin substrate, no angiotensin formation can be observed. This is true for brain tissue as well as for other tissues, where substrate concentrations are suspected to be high, such as in liver. Various possible reasons could explain such negative findings:

a) the substrate may be rapidly destroyed;

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* Nutritional Biochemicals, Cleveland, USA, (NBC).

b) angiotensin may be formed but degraded by active angiotensinases;

c) there may be no substrate pool in the tissue.

It would be unique in the biological field, that an enzyme is found at locations, where its substrate is not available. It is therefore possible that the difficulty of measuring angiotensinogen in tissue is due to methodological problems.

We have tried different approaches for measurement of substrate concentration in brain and other tissues, including differential and density gradient centrifugation. In all our experiments only the procedures which included concentration with ammonium salt yielded positive results, and the brain was the only organ where substrate was detected.

The procedure used for substrate determination in brain tissue was the following: both carotid arteries and one femoral artery of the anesthetized dogs were cannulated with polyethylene tubing. The dogs were then rapidly bled through the femoral artery and ice-cold saline at a pressure slightly above the dogs systolic blood pressure was infused into both carotid arteries. Blood clotting was prevented by 10,000 Units of Heparin given intravenously. The brain was thus rinsed and cooled with 7 liters of ice-cold 0.9% saline and was devoid of blood when removed.

The whole brain was then homogenized in 5 volumes of distilled water and 0.5 volume of a 15% EDTA solution which also contained sodium azide. The homogenate was frozen, thawed and then centrifuged for

20 minutes at 12,000 g. The precipitate being discarded, the supernatant was filtered over glass-wool and brought to pH 5.3 with 2.5 N sulfuric acid. Ammonium sulfate was added very slowly into the solution bringing the concentration to 2.4 molar. This mixture was stirred slowly overnight for 12 hours and was then centrifuged for 10 minutes at 12,000 g. The supernatant contained no substrate and was discarded while the precipitate was dissolved in 0.5 volume of distilled water and dialyzed against water for 24 hours. The resultant solution was clarified by centrifugation and concentrated by lyophylization.

The determination of substrate concentration in this final preparation was performed as described by Boucher et al. for plasma (22), by addition of excess renin, Dowex resin and a TRIS-buffer.

All procedures have been performed at 0-4$^{o}$C, keeping all solutions in an ice bath so that the brain tissue was kept cold right from the beginning through rinsing with cold saline, and enzymatic activity which might destroy the substrate was slowed down. Bacterial degradation was prevented by addition of sodium azide.

## 3.5. *Preparation of Different Renin Substrates*

### 3.5. 1. Dog Plasma "Standard" Substrate

Since the substrate pool in tissue appears to be small and no measurable quantities of angiotensin are formed during the incubation of small quantities of tissue, the determination of renin activity in small volumes of plasma and in tissue by the method of Boucher (22) makes the addition of exogenous substrate necessary. We prepared angiotensinogen from the plasma of 24 hours previously nephrectomized dogs, following the procedure described by Haas et al. (121). The substrate preparation is free of angiotensinase activity and renin. Substrate was always prepared from large pools of plasma (2000-3000 ml) yielding on the average 40 to 50 g of substrate preparation in order to have an identical substrate preparation available for a large number of experiments. Each substrate was tested for "blanks": if incubation of substrate without addition of renin showed the slightest pressor or depressor response in the rat it was discarded. Each substrate preparation was also tested for its angiotensin-forming capacity with given amounts of renin. Substrate concentrations in the zero-order range were always used. The substrate prepared according to the method of Haas et al. (121) and used for the determination of renin activity in blood, CSF and tissue will be referred to as "standard substrate".

### 3.5. 2. Purified Dog Plasma Substrate

Plasma was taken from 24 hrs. previously nephrectomized dogs. It was rapidly cooled and all subsequent precedures were carried out at 0-4°C. The plasma was adjusted to pH 5.3 with 2.5 N $H_2SO_4$ after which ammonium sulfate was added slowly to give a molarity of 1.2. The precipitated proteins were discarded and $(NH_4)_2SO_4$ was added to the supernatant to give a molarity of 2.0. After stirring over night for 12 hrs. this solution was centrifuged at 12,000 g for 10 minutes and the precipitate containing the substrate was dialyzed for 24 hrs. against distilled water.

The dialysate was then further purified on DEAE-cellulose batchwise as recommended by Skeggs et al. (159). Observing the relations of 4 g protein/1 liter water/145 g DEAE-cellulose, the substrate was stirred into the cellulose for 30 minutes after which it was filtered over a vacuum funnel.

The DEAE-cellulose used in this and in the following steps was washed with 0.5 N HCl and twice with 0.5 N NaOH after which it was washed thoroughly with distilled water until the pH was neutral.

After the first filtration water was added to the cellulose, the mixture adjusted to pH 5.6 with 2.5 normal Na $CH_3COO$ and filtered after stirring for thirty minutes.

The DEAE-cellulose was then washed in water again and the pH adjusted near neutral at pH 7.1 with 1 N NaOH. After stirring for 30 minutes the mixture was filtered again. The renin substrate still

being absorbed on the DEAE-cellulose was recovered by stirring it into a 0.04 M phosphate buffer, pH 7. After 1 hour of continuous stirring the substrate was obtained by vacuum filtration. This elution procedure was repeated once. The combined eluates were then dialyzed against distilled water for 24 hours. The dialysate was clarified by centrifugation and then assayed for specific activity in the same way as described for plasma substrate concentration. Protein content was measured by the method of Lowry et al. (308). One mg of protein in this preparation had an angiotensin forming capacity of 50.6 ng.

### 3.5. 3. Heterologous Substrates from Rabbit, Rat, Sheep

All determinations of renin activity in tissue, plasma and CSF of the dog have been done with homologous renin-substrate prepared from plasma of nephrectomized dogs ("standard substrate").

Apart from this "standard preparation", different substrates from

a) dog brain tissue,

b) purified substrate from dog plasma, heterologous plasma substrates from

c) rabbit,

d) rat and

e) sheep as well as

f) synthetic renin substrate have been used for methodological experiments.

The preparation of heterologous substrates from sheep and rabbit was done according to the method of Haas et al. (121). The reason to use the same technique as for the dog's "standard preparation" was, to have approximately the same specific activity for the different substrates. If denaturation of proteins occurred during the purification procedure, it would be similar for the dog, rabbit and sheep substrate, which is important for comparative studies. The specific activity of the standard dog substrate preparation was 6.9 ng angiotensin forming capacity per mg protein versus 6.1 ng per mg protein for the rabbit substrate and 6.3 ng for the sheep substrate. Purified rat substrate was prepared from rat plasma batch wise on DEAE-cellulose in the same way as described above for the purified dog plasma substrate. The specific activity of this preparation was 15.2 ng angiotensin forming capacity per mg protein.

### 3.5. 4. Synthetic Substrate

Besides homologous and heterologous renin substrates of different species and different degrees of purification a synthetic tetradeca-peptide* renin substrate was used for methodological experiments.

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* Commercially available from Schwarz/Mann, Orangeburg, New York. The stock we used throughout was lot. No. W-2120. The amino acid sequence is given with ASP-ARG-VAL-TYR-ILE-HIS-PRO-PHE-HIS-LEU-LEU-VAL-TYR-SER. One mg dry powder contains 0.47 µmoles of this peptide (calculated value).

As mentioned earlier this compound has been first synthesized by Skeggs et al. (161, 162) who showed also, that it represents the first 14 amino acids counted from the N-terminal of the natural angiotensinogen occurring in plasma. 0.01 mg of tetradecapeptide substrate were used per incubation. The angiotensin forming capacity of this amount was above 1000 ng angiotensin, when incubated with excess of renin and it provided zero-order kinetics for RA measurement. The tetradecapeptide itself has a pressor activity when injected into the rat. This "blank" value for 0.01 mg of synthetic substrate corresponded to 40 ng angiotensin and was substracted from the pressor response after sample incubations.

Different mechanisms are possible for the pressor activity of the synthetic substrate:

a) the tetradecapeptide may be hydrolyzed by tissue or plasma renin of the bioassay-rat. Though these test-rats are nephrectomized 24 hrs. previously, the tissue renin does not disappear and the small peptide substrate could penetrate into the tissue and give rise to angiotensin formation.

b) The tetradecapeptide itself may be pressor-active. Our results favor this latter hypothesis, since injection of other renin substrates does not cause a rise in blood pressure and since the "blank" value of the tetradecapeptide is dose-dependent.

## 3.6. *Purification of Tissue Iso-Renins*

While crude tissue homogenates were taken for all determinations of their renin activity, renin was purified from brain tissue, kidney, aortic tissue and adrenal gland for better characterization of this enzyme.

Renin is an instable enzyme in its purified form, and our first goal was not to achieve a high degree of purity but rather to obtain renin preparations from different tissues with a similar specific activity and to minimize partial modification or denaturation of the enzymes.

The method described by Haas et al. (121) for the kidney (Procedure B) was used. Aliquots of tissue were ground in water and stirred for 15 minutes at room temperature. The homogenate was frozen and thawed, then stirred for 15 minutes at room temperature after which it was centrifuged at 12,000 g for 20 minutes. The precipitate was discarded and the supernatant was diluted to 5 volumes relative to the original weight of the tissue. From this step on all operations were carried out at 0 to 5°C.

The solution was brought to pH 2.3 with 4 N $H_2SO_4$, and 0.12 ml 95% precooled ethanol (-20°C) was added per ml of volume. After stirring for 1 hour the mixture was centrifuged at 12,000 g for 10 minutes. The supernatant was brought to pH 4.5 with 5 N KOH and dialyzed for 12 hours against distilled water. The dialysate was diluted with distilled water in 7.4 volumes relative to the original

weight and adjusted to pH 2.8 with 4 N $H_2SO_4$. NaCl was added to obtain a 0.8 M solution. Following stirring for 1 hour, the resulting precipitate was separated by centrifugation and ammonium sulfate was added to the supernatant to give a 1.0 M solution. This mixture was stirred for 10 minutes and centrifuged. Ammonium sulfate was added to the supernatant to obtain a molarity of 2.3. After 1 hour the renin containing precipitate was separated by centrifugation and suspended in 20 to 40 ml of 0.9% saline, which was dialyzed for 24 hours against cold water. The final solution was clarified by centrifugation, separated into different vials and stored at -20°C until use.

### 3.7. *Ultracentrifugation of Brain and Kidney Tissue*

Ultracentrifugation studies for subcellular localization of renin were carried out in parallel with renal and brain tissue. Techniques used for isolation of subcellular fractions were those of Whittaker (309) and Koenig (310). We followed in detail the following scheme for both tissues:

a) dissection of the caudate nucleus and frontal cortex of the dog brain. One g pieces were weighed rapidly.

b) Immediately after weighing, each aliquot was transferred to ice-cold sucrose solution (0.292 M), minced with scissors, and carefully homogenized in glass vessels with Teflon pestles,

allowing successively 0.635 mm and 0.279 mm clearance. From this point on and during all subsequent procedures, the operations were carried out at 0 to 4°C.

c) Centrifugation for 10 min. at 900 g after adding sufficient ice-cold sucrose solution to make a total of 20 volumes (ml) of sucrose solution per gram of tissue sample.

d) Careful decantation of the supernatant. The pellet (NUC) contains primarily "nuclei and debris".

e) Washing of the NUC pellet. Suspension of the latter in approximately 5 ml of sucrose solution and then centrifugation again at 900 g for 10 minutes.

f) Decanting of the supernatant from NUC pellet. The washed NUC pellet was then frozen at -20°C until measurement of protein content and renin activity.

g) The two supernatants were combined and a solution containing 1% heparin and 10% sucrose was added in amounts to make the supernatant 0.02% (V/W) with respect to heparin. Addition of heparin decreases indiscriminate clumping of particles during centrifugation, but if added to the original homogenate, it releases material from within the nuclei of the cells.

h) Centrifugation of the combined and heparinized supernatant for 20 min. at 11,500 g.

i) Decanting of the supernatant. The resulting pellet (MIT), was frozen at -20°C. This pellet is the primary mitochondrial

fraction containing separated nerve-ending particles ("synaptosomes") as well as mitochondria.

k) Centrifugation of the supernatant at 74500 g for 80 min.

l) Decanting of the supernatant. The resulting pellet (MIC) was frozen again at -20°C until the different parameters were measured. This pellet contains the microsomes as well as some microvesicles, some of which are called "synaptic vesicles". The MIC pellet also contains numerous types of membrane fragments from broken cells.

m) The final supernatant was stored at -20°C.

The primary mitochondrial fraction (MIT) ($P_2$ according to the designation of Whittaker) (309) was submitted to a discontinuous sucrose density gradient centrifugation with densities of 0.29, 0.8, 1.2, and 1.4, resulting in 4 fractions A,B,C, and D, respectively.

In all primary fractions: upernatant, Microsomal, Mitochondrial and Nuclear fractions, renin was measured and proteins were determined according to the method of Lowry (308). The relative specific activity was calculated as % enzyme activity / % protein content. The relative specific activity of the whole primary mitochondrial fraction was taken as 1.00. Thus, relative specific activity greater than 1 unit in the subfractions means relative enrichment in enzyme activity.

## 3.8. *"Disc" Electrophoresis on Polyacrylamide Gel*

The electrophoretic mobility of purified brain tissue renin on 12% polyacrylamide gel was compared with that of purified dog kidney renin and hog kidney renin. The technique used in these experiments was essentially the one described by Davis (311) for plasma proteins with a few modifications.

The electrode-buffer solution at pH 8.3 was made with 6.0 g TRIS and 28.8 g glycine in 1000 ml bidistilled water. In detail the following procedure was observed: 6 stock solutions were prepared and kept at refrigeration temperatures in amber bottles which prevented direct light to reach the reagents.

| | | |
|---|---|---|
| A) | HCl 1N | 48 ml |
| | TRIS | 36.6 g |
| | Tetraethyl methylethylenediamine (TEMED) | 0.23 ml |
| | Water to the volume of 100 ml | |
| | pH of the solution: 8.9 | |
| B) | HCl 1N | 48 ml |
| | TRIS | 5.98 g |
| | Tetraethyl methyethylenediamine | 0.46 ml |
| | Water to the volume of 100 ml | |
| | pH of the solution: 6.7 | |
| | (add HCl 1N, if the pH of the solution is not at 6.7) | |
| C) | Acrylamide | 48 g |
| | Methylene bisacrylamide (BIS) | 0.5 g |
| | Water to the volume of 100 ml | |

| | | |
|---|---|---|
| D) | Acrylamide | 10.0 g |
| | Methylene bisacrylamide | 2.5 g |
| | Water to the volume of 100 ml | |
| E) | Riboflavin | 4 mg |
| | Water to the volume of 100 ml | |
| F) | Sucrose | 40 g |
| | Water to the volume of 100 ml | |

To obtain a gel concentration of 12%, the stock solution C was diluted in the ratios 1:4 = water: solution C.

Ammonium persulfate solution was always prepared freshly by dissolving 140 mg of this compound in 100 ml of water.

The polyacrylamide gel was mixed from the solutions A, C, $H_2O$, and ammonium persulfate in the relations 1:2:1:4.

The gel was allowed to polymerize for 45 minutes in the tubes under a fluorescent lamp; it was covered with a small layer of water. A second layer of gel (stacking gel) was prepared from stock solutions B:D:E:F: in the ratios 1:2:1:4.

The surface of the polymerized first gel was washed with water, dried by capillarity with a soft paper and 0.5 cm of the stacking gel were deposed on the first gel. The second gel was allowed to polymerize for 25 min., being equally covered by a small layer of water. The surface of the stacking gel was washed and dried again as described and the tubes placed in the electrophoresis apparatus. The lyophilized samples were dissolved in a dense sucrose solution (F) so that a layer of the sample could be applied under the electrode buffer right on the

stacking gel. 200 µg to maximal 900 µg total proteins per tube were applied with the sample. Bromphenol blue was added as a marker-dye to the buffer solution. With 5 M Ampere electric current per tube the tracing dye reached the end of the tube (8 cm) after 50 minutes. The electrophoresis was then terminated. The gel was removed from the glass tube, coloured with Amido schwarz (1% in a 7% acetic acid solution) for 1 hour and the non protein bound Amido schwarz removed with a 7% acetic acid solution.

All studies concerning the mobility of the various renin extracts were always done simultaneously, so that the conditions (buffer, solutions, temperature, electric field, etc...) were identical. All samples were done in triplicate.

### 3.9. *Other Methods*

1) Proteins have been measured with the folin phenol reagent after alkaline copper treatment as described by Lowry et al. (308).
2) Plasma sodium and potassium were determined by flame emission spectroscopy (Unicam model SP 900 flame spectrophotometer).
3) Magnesium and Calcium were measured by atomic absorption spectrophotometer (Perkin-Elmer Model 303).
4) Norepinephrine was determined in arterial and brain tissue by a modification (Genest et al. 27) of the trihydroxyindole derivative method of Anton and Sayre (312).

5) Dopamine was measured by the method of Laverty and Taylor (328).

6) Measurement of blood pressure in dogs: the dogs were anaesthetized with sodium thiopenthal (30 mg Pentothal$^{R}$, Abbott, per kg body weight). The femoral artery was punctured and blood pressure recorded on a Grass Polygraph Model 7 PCP B via a Statham transducer No. P 23AC. Mean arterial blood pressure (MABP) was calculated according to the formula

$$\text{MABP} = \text{diastolic BP} + \frac{\text{systolic BP} - \text{diastolic BP}}{3}$$

7) Anaesthesia in dogs for reasons other than measurement of blood pressure was done with sodium pentobarbital (Nembutal$^{R}$, Abbott) at the dose of 30 mg per kg body weight.

8) All experiments reported in this thesis have been done in male mongrel dogs. The dogs were considered adult above 1.5 years of age. The dogs were killed by an overdose of sodium pentobarbital or by rapid bleeding during anaesthesia.

9) Statistics: students unpaired-t-test has been used for calculation of statistical significance whenever possible. In some instances the paired-t-test was utilized.

# 4. RESULTS (METHODOLOGICAL)

## 4.1. *Specificity of the Renin Assay*

Although the specificity of the method of Boucher et al. (22) for measurement of renin activity is widely recognized, verification as to their possible interference with the renin measurement was specially made concerning those substances, which occur in high concentrations in brain tissue, and which may have an effect on the blood pressure when injected directly into the circulation of the rat.

The substances listed in table 1 were added to the complete incubation mixture in concentrations approximating their natural occurrence in brain tissue and ten times this amount. No pressor or depressor substance could be detected in the rat bioassay, if renin was absent from the sample. Catecholamines, Serotonin, Acetylcholine, γ-amino butyric acid (GABA) as well as Histamine thus are not eluted in the angiotensin fraction from the Dowex 50W-X2 ($NH_4^+$) resin column under the conditions used.

A further proof that the product of an enzyme-substrate reaction was indeed measured and not a peptide or other compounds present before the incubation, is given by the first three (1-3) experiments on the specificity of the renin assay:

1) Brain homogenates were incubated for 12 hours at 37°C exactly as described for the measurement of RA. Renin substrate, however, was not added to these incubations. No pressor material was obtained under these conditions.

| Compound | | Bioassay (ng) |
|---|---|---|
| Dopamine · HCl | 20 µg | 0 |
| | 100 µg | 0 |
| Norepinephrine | 6 µg | 0 |
| | 30 µg | 0 |
| Serotonin | 4 µg | 0 |
| | 20 µg | 0 |
| Acetylcholine | 14 µg | 0 |
| | 70 µg | 0 |
| γ-Amino Butyric Acid (GABA) | 120 µg | 0 |
| | 600 µg | 0 |
| Histamine | 2 µg | 0 |
| | 10 µg | 0 |

TABLE 1: *Specificity of the renin assay. The above substances were added to a complete incubation mixture which did not contain renin. After 12 hours incubation, the samples were processed as for RA determination and tested for pressor activity in the rat bioassay.*

2) Identical aliquots of brain tissue homogenates with equal enzyme concentrations were incubated under constant temperature at 37°C with an excess of homologous substrate. The incubation time was increased from 2 to 12 hours. Samples were taken out of the incubation at 2 hour intervals. The product of the enzyme-substrate reaction, angiotensin, increased linearly with the lengthening of the incubation time (figure 3).
3) In a third experiment the incubation time, the bath temperature and substrate concentration for each sample were kept constant. Different amounts of enzyme (crude brain tissue homogenate) were added to the incubation. Angiotensin formation depended linearly on the amount of enzyme present in the incubation (figure 4).

The enzymatic character of the angiotensin forming substance in brain tissue was further proven by the following criteria:

4) whole tissue homogenates were dialyzed against distilled water at 4°C for 12 and 24 hours. Renin concentration before and after dialysis was unchanged. The brain enzyme thus is not dialyzable.
5) The activity of the brain enzyme is pH dependent. Angiotensin-formation reached its maximum at a pH of 5. The incubations have all been done under exactly the same conditions as described for the measurement of tissue renin activity. Using Dowex 50W-X2 ($NH_4^+$) resin and TRIS-buffer adjusted to different pH with concentrated phosphoric acid. Instead of 2 ml as for the usual

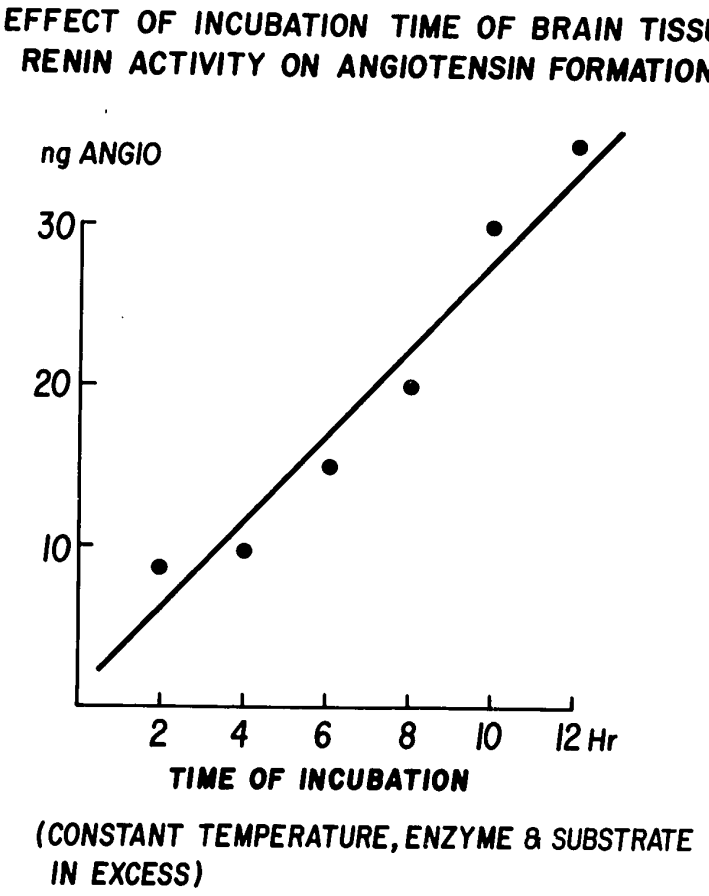


FIGURE 3: *Incubation of equal amounts of brain enzyme for different periods of time under identical conditions. Measurement of angiotensin formation in 2 hour intervals.*

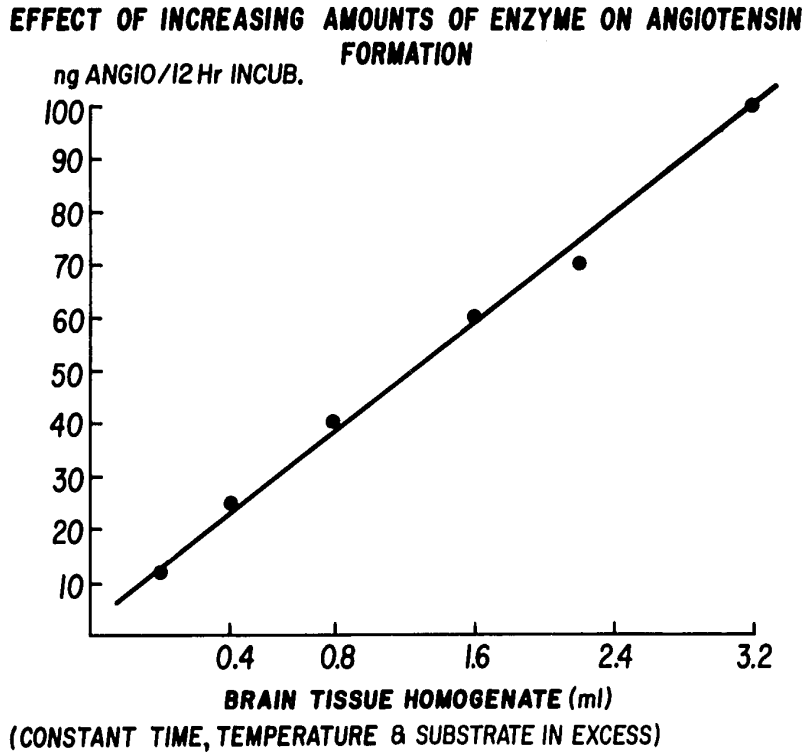


FIGURE 4: *Measurement of angiotensin formation after incubation of different amounts of brain enzyme for 12 hours under identical conditions.*

incubations, 4 ml of buffer were added in order to have a better buffering capacity. pH-values were controlled after the incubation. In addition to the "standard conditions" described above, pH studies were also carried out without Dowex 50W-X2 ($NH_4^+$) resin. In these cases DFP (0.1 ml of a 2% (V/V) solution to 4 ml of the incubation mixture) was added. Plasma of nephrectomized dogs, which contained no detectable renin activity was also added in one series of pH studies. There was no difference between these and the "standard" incubation conditions in the maximal rate of angiotensin formation, which was found to be at pH 5.

6) Brain tissue renin can be precipitated with ammonium sulfate 2.3 molar at pH 2.8.
7) The brain enzyme is stable in acid milieu of pH 2.3 for more than 4 hours.
8) The brain enzyme is denatured and its activity abolished if heated for 10 minutes at temperatures higher than 60°C.
9) Chelating agents like EDTA have no influence on renin activity. The enzyme thus appears to be independent of $Ca^{++}$ and $Mg^{++}$ ions, which are bound by this compound.
10) Soybean-Trypsin inhibitor in concentrations up to 0.01 mg/3 ml and DFP, a frequently used chymotrypsin inhibitor, were added to the renin incubation mixtures. No inhibition of enzyme activity could be observed in these experiments.

11) Brain renin can be inhibited by dog's antirenin to hog renin*. This preparation tested against hog renin had an antirenin titer of 68 units/cc.

The maximal inhibition of brain iso-renin by this antirenin under "standard" incubation conditions (pH 5.5) was 45%. Under these conditions the antibody appears to have higher affinity to kidney renin. Renal renin was inhibited 81%. (Table 2, table 3).

However, brain enzyme could also be totally inhibited by this antibody raised against renal renin when the incubations were done at a less acid pH (table 2). The hydrogen ion concentration of the buffer heavily influences the binding of the antigen to the antibody. In high hydrogen ion concentrations, the equilibrium of Antigen + Antibody⇌Antigen-Antibody complex is shifted to the dissociation of the two components. At pH 2 to 3, full dissociation of the antigen from the antibody is usually achieved. The relative low efficiency of the antirenin in blocking brain renin at pH 5.5 may be explained by this fact. Under these conditions there was a marked difference between brain and kidney renin, the latter being more efficiently blocked.

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* Dr. Erwin Haas, Mount Sinai Hospital Cleveland, kindly supplied this antirenin.

| MODE OF INCUBATION | UNITS ANTIRENIN ADDED TO INCUB. | BIOASSAY ng ANGIOTENSIN | % INHIBITION |
|---|---|---|---|
| 3.5 hrs. | Control | 100 ng | |
| | $2 \times 10^{-2}$ | 75 ng | 25 % |
| 12 hrs. | Control | 32.5 ng | |
| | $2 \times 10^{-2}$ | 25 ng | 23.1% |
| 1 hr. preincub. 6 hrs. incub. | Control | 200 ng | |
| | 1 | 180 ng | 10 % |
| 24 hrs. + 1 ml plasma | Control | 180 ng | |
| | 1 | 120 ng | 33.3% |
| 24 hrs. + 2 ml plasma | Control | 260 ng | |
| | 1 | 200 ng | 23.1% |
| 15 min. preincub. 24 hrs. | Control | 35 ng | |
| | 1 | 27.5 ng | 21.4% |
| 24 hrs. | Control | 25 ng | |
| | 1 | 13.75ng | 45 % |
| 48 hrs. | Control | 60 ng | |
| | 1 | 45 ng | 25.1% |
| pH 7.4 12 hrs. | Control | 90 ng | |
| | $0.25 \times 10^{-2}$ | 30 ng | 66.7% |
| | $0.5 \times 10^{-2}$ | 55 ng | 38.9% |
| | $1 \times 10^{-2}$ | 80 ng | 11.1% |
| | 0.5 | 0 ng | 100 % |
| | 1 | 1.25ng | 98.6% |

TABLE 2: *Inhibition of brain iso-renin (crude brain extract, different quantities) by hog antirenin under various conditions. Incubations were carried out at pH 5.5 if not indicated otherwise. No antirenin was added to the control incubations.*

| MODE OF INCUBATION | UNITS ANTIRENIN ADDED TO INCUB. | BIOASSAY ng ANGIOTENSIN | % INHIBITION |
|---|---|---|---|
| 3.5 hrs. | Control | 160 ng | |
| | $2 \times 10^{-2}$ | 150 ng | 6.25% |
| 12 hrs. | Control | 300 ng | |
| | $2 \times 10^{-2}$ | 200 ng | 33.3 % |
| 12 hrs. | Control | 300 ng | |
| | $1 \times 10^{-2}$ | 250 ng | 16.7 % |
| | $2 \times 10^{-2}$ | 220 ng | 26.7 % |
| | $3 \times 10^{-2}$ | 260 ng | 13.3 % |
| 1 hr. preincub. | Control | 200 ng | |
| 6 hrs. incub. | 1 | 45 ng | 77.5 % |
| 24 hrs. | Control | 400 ng | |
| | 1 | 75 ng | 81.25% |
| 24 hrs. | Control | 400 ng | |
| | 1 | 80 ng | 80 % |

TABLE 3: *Inhibition of kidney renin (crude kidney extract, different quantities) by hog antirenin. All incubations were carried out at pH 5.5. No antirenin was added to the control incubations.*

12) *Enzyme substrate reactions:*

a) *Homologous renin substrates:*

Brain iso-renin generates angiotensin when incubated in the presence of homologous substrate. Equal amounts of brain enzyme (pool of crude brain extract, 0.2 ml) with different substrates were incubated (table 4). The incubations were done under exactly identical conditions (time, temperature, degree of shaking): all measurements shown in table 4 have been done at the same time in the same incubation apparatus. Angiotensin formation with substrate extracted from brain tissue was slightly higher than with the "standard" substrate preparation. The brain iso-renin had a high affinity to purified substrate from dog plasma. (table 4).

It will be shown later in chapter 4.3. that brain iso-renin seems to be a rather species specific enzyme. Angiotensin formation with homologous substrates was higher than with heterologous substrates from sheep, rabbit and rat.

b) *Synthetic renin substrate:*

It has been described that renal renin forms angiotensin with the synthetic tetradecapeptide substrate (153, 161). Kidney renin from dog, appears to have a lower affinity to this synthetic substrate than to homologous "standard" plasma substrate. The brain enzyme, incubated under identical conditions with these two substrates, shows higher angiotensin formation with the tetradecapeptide renin substrate than with homologous substrate (figure 5).

| SUBSTRATE | NO OF INCUBATIONS | MEAN OF ANGIOTENSIN FORMATION | % OF ANGIOTENSIN FORMATION AS COMPARED TO "STANDARD" INCUBATION |
|---|---|---|---|
| "STANDARD" DOG PLASMA SUBSTRATE | 3 | 113 ng | 100 % |
| HOMOLOGOUS BRAIN TISSUE SUBSTRATE | 2 | 132.5ng | 117.3% |
| PURIFIED DOG PLASMA SUBSTRATE | 3 | 300 ng | 265.5% |

TABLE 4: *Angiotensin formation of brain iso-renin with various substrates. Equal amounts of brain homogenate were incubated under identical conditions with excess of different homologous substrates.*

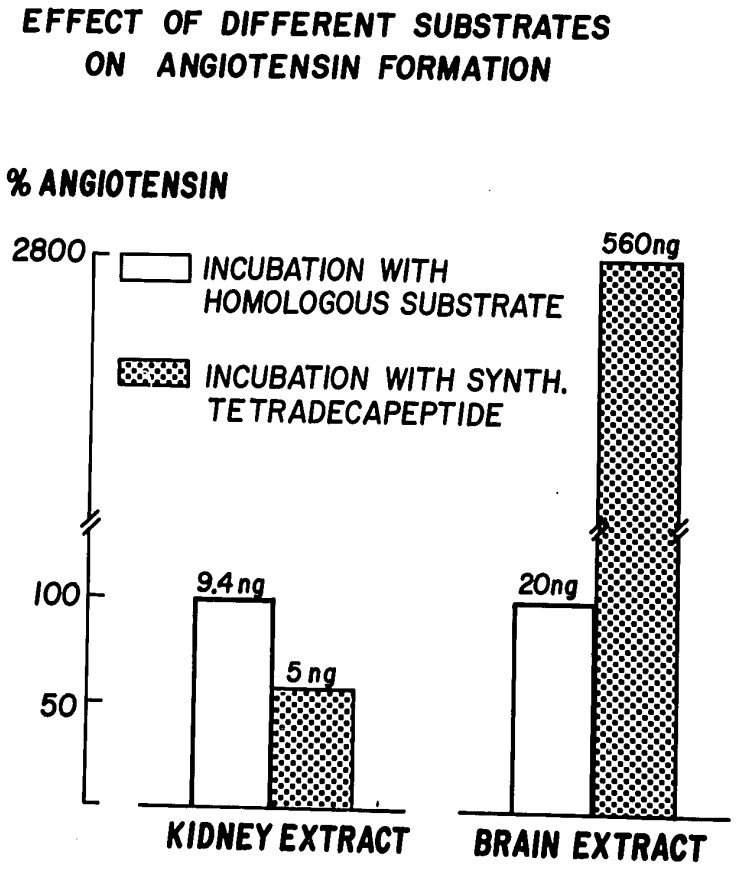


FIGURE 5: *Aliquots of brain and kidney enzyme were incubated under identical conditions with homologous plasma substrate and synthetic tetradecapeptide renin substrate.*

13) *"Disc" electrophoresis of brain iso-renin:*

Partially purified brain iso-renin was applied on a polyacrylamide-gel electrophoretic system following essentially the technique of Davis (311). In an alkaline TRIS-glycine buffer of pH 8.3, brain iso-renin was negatively charged and migrated to the anode as does kidney renin (see also chapter 4.3.).

## 4.2. *Identification of Angiotensin as the Product of the Brain-Enzyme Substrate Reaction*

1) The pressor product, which was generated during the incubation of brain iso-renin with homologous substrate, was absorbed by Dowex 50W-X2 ($NH_4^+$) resin.
2) It was retained by this ion exchange resin in contact with water, 10% acetic acid and ammonium acetate (0.2 M, pH 6), and was eluted with 0.1 N diethylamine and 0.2 N ammonium hydroxide from the resin.
3) The material formed during the incubation, gave a blood pressure rise of short duration when it was injected intravenously into the rat. The pressor curve was identical to that of angiotensin.
4) Heating of the material for 2 hours at 90°C in 0.9% NaCl did not destroy the pressor activity.
5) The pressor effect in the rat bioassay was completely abolished after incubation of the enzyme-substrate product with chymotrypsin in a 2% phosphate buffer, pH 7.5, for 1 hour.

6) A pool of the pressor material was purified on Dowex 50W-X2 ($NH_4^+$) columns. The activity of the angiotensin eluate disappeared completely following dialysis against water for 12 hours.

7) The pressor material could be blocked by angiotensin antibodies (figure 6). 5 µg of the material purified from the incubation mixture when injected into the rat resulted in a blood pressure rise corresponding to 2.5 ng angiotensin II-standard (Hypertensin$^R$,Ciba). The dose response was linear with increasing amounts. The pressor effect of the material was largely suppressed by addition of excess amounts of specific angiotensin I antibodies, which did not cross-react with angiotensin II. Addition of antibodies against angiotensin II further depressed the pressor response in the rat. These experiments indicated that the pressor material, which was formed during the incubation of brain-enzyme with homologous renin substrate consisted mostly of angiotensin I and only a small amount of angiotensin II.

The suppression of the pressor response in the rat bioassay was not due to the injection of the antibodies, since injection of the same amount of the latter did not result in any effect on the blood pressure (figure 6).

The blocking effect of the antibodies on the pressor material was abolished, if the neutral phosphate buffer in which the

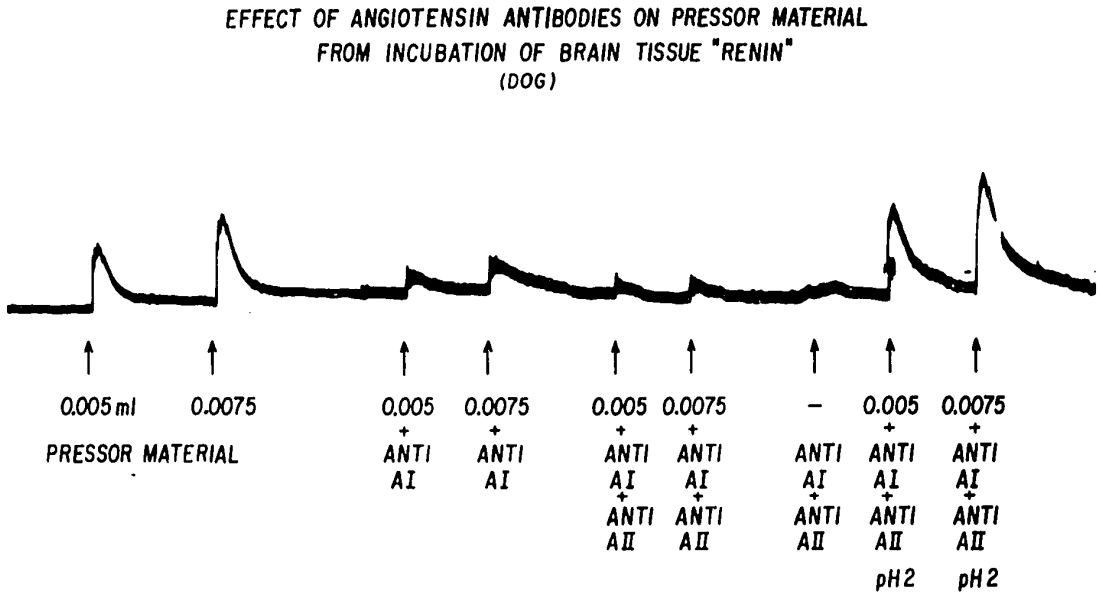


FIGURE 6: *Blood pressure recording in the rat: effect of excess of angiotensin antibodies on pressor material formed during incubation of brain homogenate and homologous dog plasma substrate.*

antibodies were dissolved, was brought to pH 2 to 3 with a few drops of 1 N HCl. At acid pH, the reversible antigen-antibody complex characteristically dissociates and injection of this acidified "pressor material + antibody mixture" showed that the pressor response was fully restored under these conditions (figure 6).

These findings are in agreement with the observations of Boucher et al. (12, 13, 22) who found, that in presence of Dowex 50W-X2 ($NH_4^+$) the angiotensin which was formed during the incubation, was absorbed rapidly by the resin and protected from further degradation. Since the decapeptide is the first product of the renin substrate reaction, it was mostly angiotensin I which was recovered from the Dowex resin. Furthermore, the "α-converting enzyme" was inhibited by EDTA in the incubation mixture. This may partly explain the high angiotensin I/angiotensin II ratio.

The pressor material generated with brain-enzyme and renin substrate was therefore identical to angiotensin with respect to all criteria examined.

## 4.3. *Comparison of Brain Iso-Renin with Kidney Renin*

The experiments described above suggest strongly that the angiotensin formed by the renal and brain enzyme with the same substrate are identical. We found, however, marked differences between the two enzymes themselves:

1) *pH-optimum:*

pH-studies were carried out, under the same conditions as described previously, for kidney and brain iso-renin activity. Under the "standard" incubation conditions and in the presence or absence of resin, kidney and plasma renin have an optimal angiotensin formation between pH 5.5 and 6.5. While maximal angiotensin formation with brain iso-renin occurs at a lower pH of 5.0 (figure 7).

2) *Renin antibodies:*

Brain iso-renin and renal renin activity can be blocked by antibodies against hog kidney renin. Under the incubation conditions used for measurement of tissue renin activity, (pH 5.5) the affinity of these renin antibodies to dog kidney renin was higher than to brain renin. 45% inhibition of the brain enzyme were obtained with these antibodies versus 81% inhibition of renal renin (chapter 4.1., table 2, table 3).

3) *Renin substrates:*

The preparation of these substrates was described in chapter 3.5.. All experiments with different substrates were carried out with purified renins (chapter 3.6.). Each experiment was

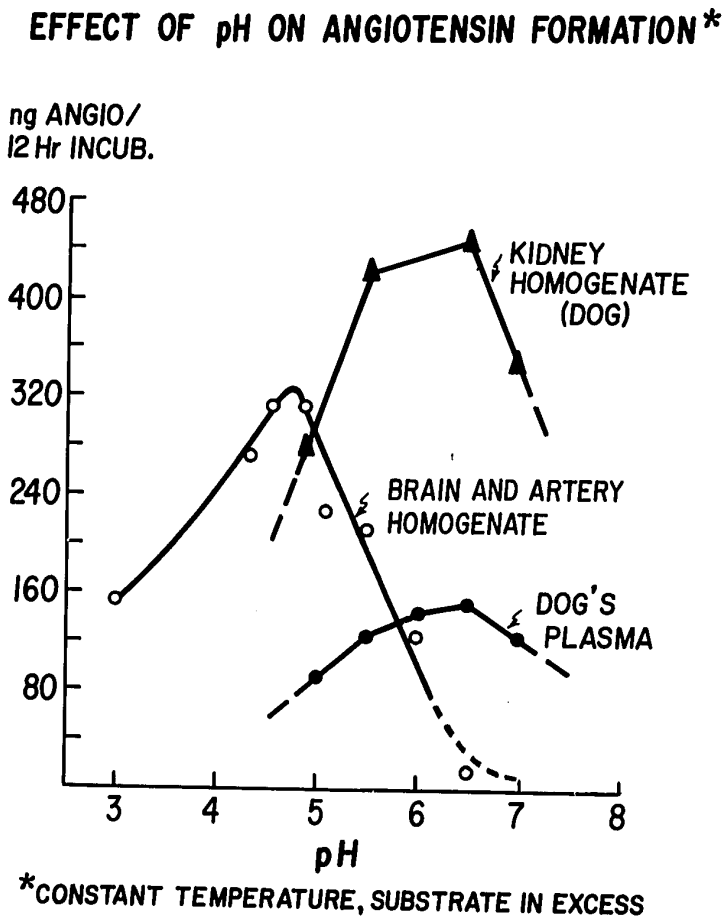


FIGURE 7: *Effect of pH on angiotensin formation. Aliquots of brain-, arterial- and kidney tissue homogenates were incubated with excess of homologous dog plasma substrate; identical incubation conditions were observed.*

carried out (as all the work described in this thesis) in a minimum of 4 assays. Enzyme and substrate concentrations as well as external incubation conditions were kept constant throughout these experiments, unless indicated otherwise.

a) *Homologous substrates*

Brain iso-renin and kidney renin were incubated with "standard" homologous dog substrate. The quantities of enzymes were chosen to yield approximately equal amounts of angiotensin during a twelve-hour incubation period. The angiotensin formation with "standard" substrate for each enzyme was arbitrarily set at 100%. When brain iso-renin and kidney renin were incubated with purified substrate from dog plasma, the angiotensin formation with brain renin increased to 333%, in contrast to 37% with kidney renin. (table 5, figure 8). No explanation can be provided for these results at the moment, although several mechanisms are possible:

a) there may be inhibitors for tissue (brain) enzymes present in the crude "standard" substrate preparation.
b) The more purified substrate is possibly the more "denatured" one and renal renin may have more specific requirements as to the tertiary structure of its substrate than brain renin and thus be less active with the "denatured", purified substrate, while the brain enzyme is more active with it.

| SUBSTRATE USED FOR INCUBATION | ANGIOTENSIN FORMATION WITH | |
|---|---|---|
| | KIDNEY RENIN | BRAIN ISO-RENIN |
| "STANDARD" DOG PLASMA SUBSTRATE | 37.5±2.5 | 22.5±1.4 |
| PURIFIED DOG PLASMA SUBSTRATE | 13.75±3.9 | 75±5 |
| SHEEP SUBSTRATE | 27.5±4.8 | 35.8±1.6 |
| PURIFIED RAT SUBSTRATE | 16.9±1.6 | 9.7±1.1 |
| RABBIT SUBSTRATE | 11.25±1.6 | 16.9±5.7 |
| SYNTHETIC TETRADECA-PEPTIDE RENIN SUBSTRATE | 33.5±7.1 | 576.0±12.5 |

TABLE 5: *Angiotensin formation of purified brain iso-renin and kidney renin with various substrates. Constant amounts of brain and kidney enzyme were incubated under identical conditions with different homologous and heterologous substrates (n=4). Values are given as mean ± standard error of the mean. Angiotensin formation is given in ng per 6 hours incubation at 37°C.*

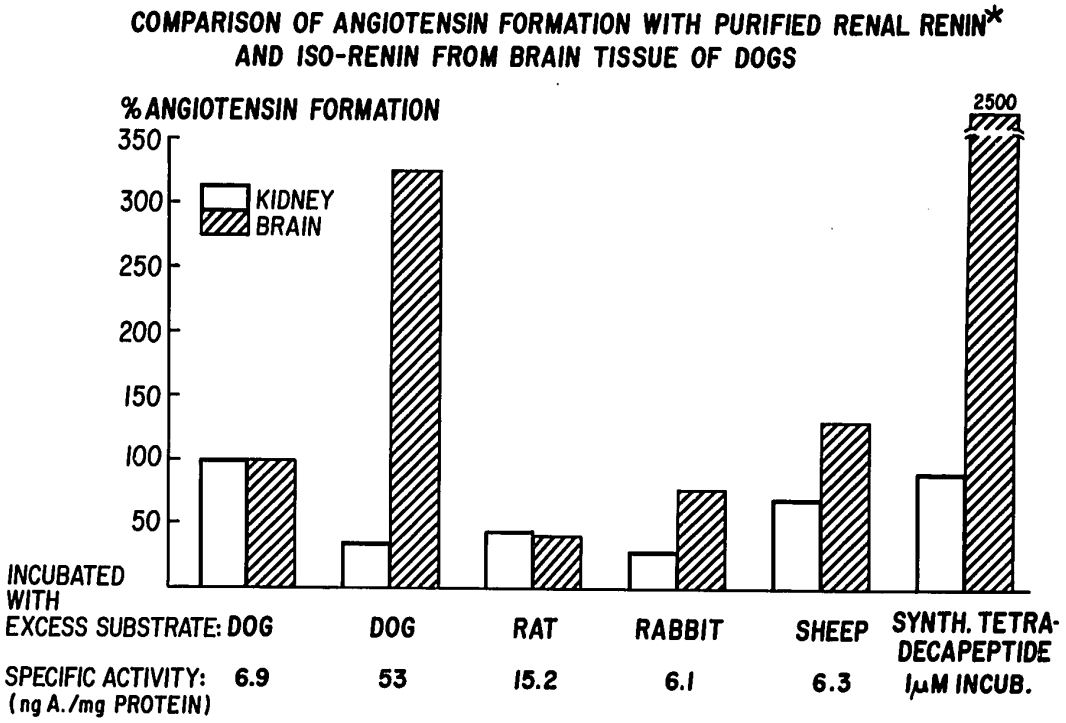


FIGURE 8: *Angiotensin formation of kidney and brain iso-renin with "standard" homologous substrate (=100%) compared to various homologous and heterologous renin substrate preparations.*

b) *Heterologous substrates*

Both brain and kidney renin, incubated with excess of heterologous rat substrate, formed less angiotensin than with homologous dog substrate. The two enzymes reacted similar when incubated with rabbit substrate, forming less angiotensin with this heterologous angiotensinogen than with standard substrate. Equal amounts of enzymes were incubated with excess of sheep substrate. The brain enzyme formed more angiotensin with this heterologous substrate (158%) than it did with dog substrate (100%).

c) *Synthetic substrate*

Equal amounts of kidney and brain renin (as measured by the angiotensin formation with homologous standard substrate) were incubated with synthetic tetradecapeptide substrate (1 μM per incubation). Brain renin showed a much higher affinity than did renal renin which formed only 89% angiotensin as compared to the incubation with standard substrate, while brain renin generated 2500% angiotensin with the synthetic tetradecapeptide substrate (figure 8, table 5).

In other experiments, constant amounts of brain and kidney renin were incubated with different concentrations of the tetradecapeptide renin substrate. Increased angiotensin formation occurred when brain iso-renin was incubated with increasing concentrations of synthetic renin substrate (figure 9).

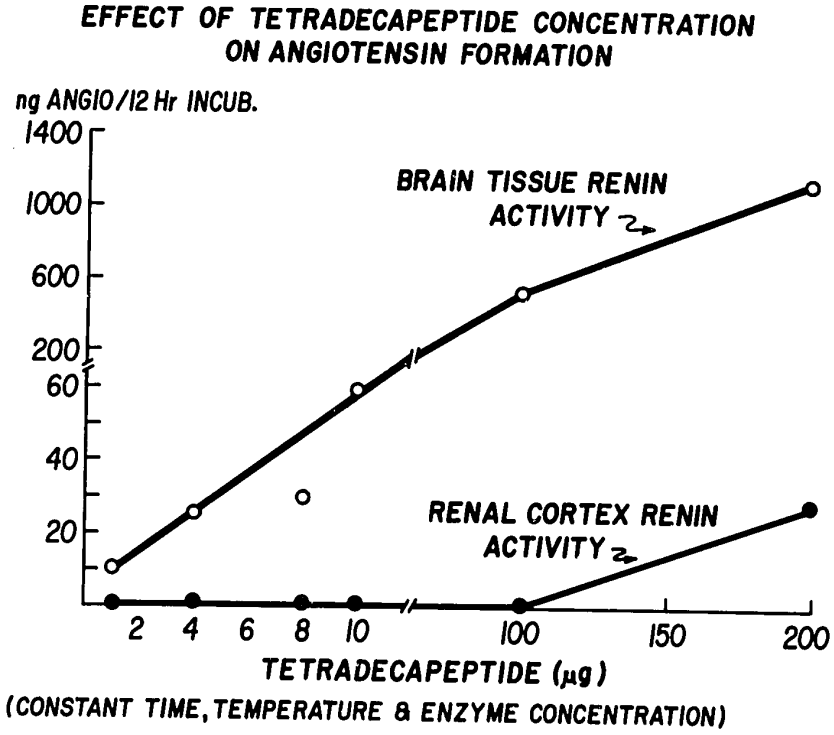


FIGURE 9: *Effect of various synthetic tetradecapeptide substrate concentrations on angiotensin formation. Aliquots of kidney and brain tissue renin (both forming 15 ng angiotensin during 12 hours incubation with homologous dog plasma substrate) were incubated with increasing amounts of synthetic tetradecapeptide.*

Under exactly identical conditions, renal renin showed no angiotensin formation over a large range of substrate concentrations. Only at tetradecapeptide concentrations between 100 to 200 ng/ml could measurable angiotensin formation be detected in a 12 hours incubation period (figure 9).

Brain iso-renin and kidney renin thus are different from each other in their enzyme substrate reactions. Though all experiments were done with purified enzyme preparations, one has to consider the possibility of interference of pseudorenin, especially in incubations with the synthetic substrate on which pseudorenin acts.

No specific assay for pseudorenin is available. At the present time it is therefore impossible to prove the absence or presence of pseudorenin in one of our enzyme preparations. The difference in angiotensin formation between brain and kidney renin cannot be due to the presence of pseudorenin, however, for the following reason: according to Skeggs et al. (153) the kidney contains large amounts of pseudorenin. The brain has not been tested in this respect. If pseudorenin would be present in our purified kidney renin preparation, the effect of kidney pseudorenin on the synthetic substrate should be additive to that of kidney renin and higher angiotensin formation would be expected when these two enzymes are active, than if only one enzyme is responsible for angiotensin formation (as is the case

in the incubations with homologous substrate, on which pseudorenin does not act). Angiotensin formation, however, is lower (89%) with tetradecapeptide than with standard homologous substrate.

Furthermore, the adrenal glands and kidneys, according to Skeggs, contain almost equal amounts of pseudorenin (153). If pseudorenin would be responsible for the high rate of angiotensin formation in our incubations with tetradecapeptide substrate, kidney and adrenal gland extracts should show similar activity. But there is no similarity between the activity of purified enzyme from these two organs with tetradecapeptide substrate. On a weight to weight basis, adrenal gland iso-renin forms angiotensin at a much higher rate than the kidney and thus behaves in the same way as the brain enzyme.

4) *Electrophoretic mobility:*

All renins tested are negatively charged in alkaline buffer and migrated towards the anode. But the different renins migrated at a different speed as shown in figure 10. Pig kidney renin* had a lower electrophoretic mobility than dog kidney renin**. The purified brain and arterial tissue iso-renin preparations migrated between these two "standards".

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* Commercially available from Nutritional Biochemical Corporation, (NBC) Cleveland, USA.

** Kindly supplied by Dr. Haas, Cleveland Ohio.

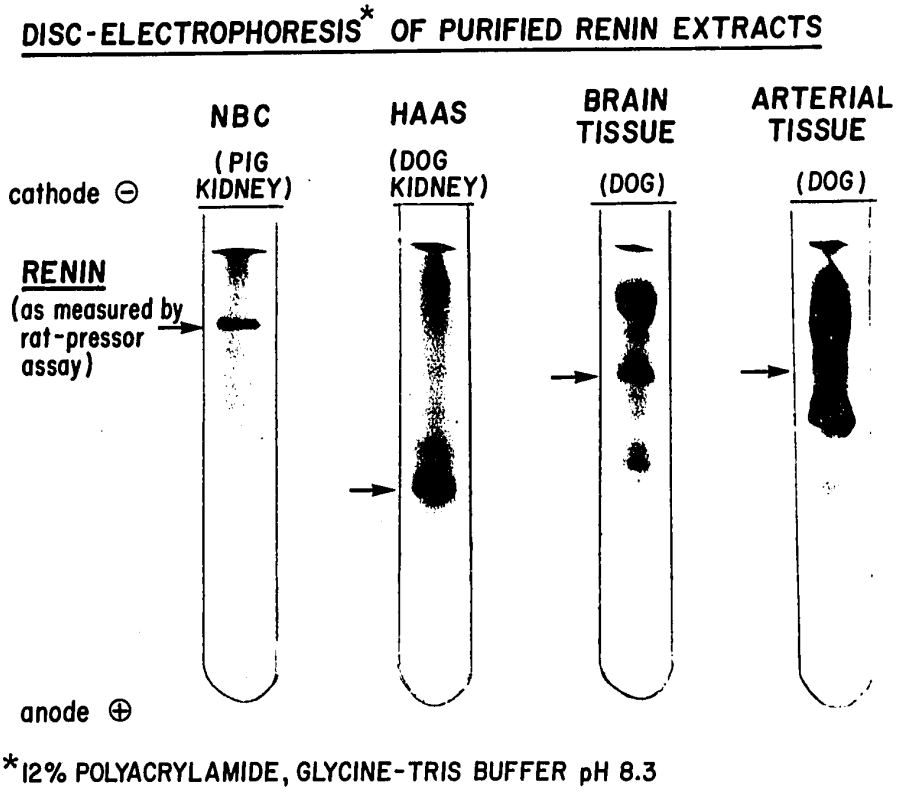



FIGURE 10: *Comparison of the electrophoretic mobility on polyacrylamide gel of kidney renin from pig and dog with brain and arterial iso-renins from dog. The arrows indicate the bands of enzyme activity.*

The enzymatic-activity of these bands in the polyacrylamide gel was measured in the following way: the samples were run in triplicates for each enzyme. One gel was stained with Amido schwarz while the other two remained unstained. The unstained gels were cut in 10 pieces of approximately 0.8 cm each, following the distribution pattern of the stained gel and observing the band distribution. The pieces of gel were transferred into incubation tubes already containing 2 ml of TRIS-buffer (pH 5.5) and kept at 0 to 4°C. The gel was broken into small pieces with a glass pestle and DFP and "standard" substrate were added to the incubation mixture as described. After subtraction of the "blank value" which was found in aliquots of gel containing no protein, the highest activity bands are indicated on figure 10 by the arrows. These findings demonstrated that arterial and brain iso-renin have an electrophoretic mobility different from that of kidney renin under the conditions described.

## 4.4. *The Permeability of the Blood-Brain-Barrier for Renin*

If renin, which is synthesized in the kidney and secreted into the blood stream, would reach the brain tissue, it had to cross the blood-brain-barrier. The choroid plexus is a representative part of this complex barrier system and measurements on both sides of the choroid plexus

a) in the afferent blood and

b) in the CSF, (secreted by the plexus)

are commonly used as indicative for the permeability of a substance through this highly selective barrier system. CSF was obtained by puncture of the posterior cisterna via the suboccipital approach in anaesthetized dogs. At the same time, blood was withdrawn from the cubital vein. Syringes contained 0.2 ml of a 15% EDTA solution for each 10 ml volume. Renin measurements were done in plasma and CSF as described in chapter 3.1.

Parallel measurement of renin activity in plasma and CSF showed that renin activity was undetectable (table 6)

a) in control dogs,

b) in puppies with high PRA,

c) in dogs which had been kept on a virtually sodium free diet for 14 days,

d) in dogs which had been rendered hypertensive by a clip on the renal artery ("Goldblatt Hypertensive Dogs").

e) One dog was unilaterally nephrectomized and 4 weeks later the renin extract of this autologous kidney was re-infused during

| EXPERIMENTAL CONDITIONS | RENIN ACTIVITY | |
|---|---|---|
| | PLASMA | CSF |
| a) Control dogs (n=13) | 0.39±0.2 | 0 |
| b) 2-6 months old puppies (n=8) | 4.38±0.65 | 0 |
| c) Na-free diet for 14 days (n=4) | 12.5 ±3.9 | 0 |
| d) Goldblatt hypertensive dogs (n=3) | 1.81±1.5 | 0 |
| e) Homologous renin infusion (n=1) | 50.00 | 0 |

TABLE 6: *Permeability of the blood-brain-barrier for renin: simultaneous measurement of renin activity in plasma and cerebrospinal fluid under various physiopathological conditions. Values are given as mean ng angiotensin formed / ml / 1 hour incubation ± SE.*

a 30 minutes period. Renin activity was measured at the end of the infusion. While excessively high renin values of 50 ng angiotensin / 1 hour incubation / ml plasma were measured in the blood, no RA could be detected in the CSF. (table 6).

It is evident, from these results, that renin does not readily pass the blood-brain-barrier under various physio-pathological conditions. Our results are in agreement with other reports in the literature, renin being an enzyme of high molecular weight like horseradish peroxydase, and it has been shown that the latter does not pass the blood-brain-barrier (299).

There are various problems concerning the permeability of the blood-brain-barrier for angiotensin, especially if physiological conditions are maintained:

1) it is difficult to measure accurately and specifically small amounts of angiotensin.
2) The degradation of angiotensin into smaller peptides and single aminoacids is rapid. If radioactive angiotensin is used, the exact interpretation of the results is possible only if the label is identified as angiotensin (313-315).

At the present time we have no conclusive results concerning the permeability of the blood-brain-barrier for angiotensin. This problem will be discussed later in the general discussion.

## 4.5. *Ultracentrifugation Studies*

It has been shown in previous chapters that brain iso-renin has some similarities with kidney renin, as it forms angiotensin by hydrolyzing the LEU-LEU bond from several homologous, heterologous and synthetic renin substrates. The renal and brain enzyme, however, are different in various aspects including their pH-optimum for angiotensin formation, their enzyme kinetics with different substrates, their immunological behaviour and electrophoretic mobility. We have equally shown that renin does not pass the blood-brain-barrier. The brain enzyme, therefore, appears to be an isoenzyme of renal renin and is synthesized in situ.

Ultracentrifugation studies of kidney homogenates have been described by several authors (316-318), and it has been demonstrated that renin synthesis does occur in the juxtaglomerular cells. The highest concentration of the enzyme in renal tissue has been found in dense granules of lysosomal character (317).

If a similar pattern of intracellular enzyme distribution could be shown for brain and kidney cells, this could be interpreted as another indication of local renin synthesis in brain cells. If, however, most of the enzyme activity would be bound to external cell membranes, it would favor the hypothesis that brain renin would be concentrated in the brain by absorption of circulating renin.

The ultracentrifugation studies were carried out following the techniques of Whittaker (309) and Koenig et al. (310) as described

previously in chapter 3.7. Kidney and brain homogenates were always done in parallel in order to have identical conditions for the comparison of both tissues.

The distribution of specific renin activity in the primary fractions (Microsomal, Mitochondrial, nuclear fraction and supernatant) showed a similar pattern for all three tissues studied: frontal cortex, caudate nucleus of the brain and renal cortex. Most of the activity in these tissues was found in the primary mitochondrial fraction (table 7, figure 11).

Since the principle of ultracentrifugal separation for these primary fractions is mostly based on particle weight and size (gravity), the primary mitochondrial fractions are especially enriched in mitochondria, but contain also different organelles of the cell which have a similar sedimentation rate. The main constituents of this fraction are mitochondria, myelin, synaptosomes, and other granules of the size of lysosomes.

In order to compare the subcellular distribution of renin in brain and renal tissue, we further separated the constituents of this primary mitochondrial fraction on a discontinuous sucrose density gradient.

The results of the subfractionation of the primary mitochondrial fraction are described in figure 12. The subfraction, coming down in the highest sucrose molarity of 1.4 and which represents, according to Whittaker (309) Koenig et al. (310), dense granules of lysosomal character, contained the highest relative specific activity in the two

| | PRIMARY SUBFRACTIONS | | | |
|---|---|---|---|---|
| FRONTAL CORTEX | MICROSOMAL | MITOCHONDRIAL | NUCLEAR | SUPERNATANT |
| 1. | 4.0 | 9.3 | 2.3 | 6.7 |
| 2. | 0.5 | 1.3 | 0.5 | 0.8 |
| 3. | 1.1 | 11.0 | 5.0 | 5.7 |
| 4. | 0 | 3.5 | 1.4 | 5.0 |
| Mean ± SE | 1.4±0.9 | 6.3±2.3 | 2.3±0.9 | 4.6±1.3 |
| CAUDATE NUCLEUS | | | | |
| 1. | 0 | 4.1 | 0 | 0.5 |
| 2. | 0.1 | 0.8 | 0.7 | 0.1 |
| 3. | 2.2 | 10.6 | 4.2 | 7.7 |
| 4. | 0 | 3.7 | 1.5 | 6.3 |
| Mean ± SE | 0.6±0.5 | 4.8±2.1 | 1.6±0.9 | 3.7±1.9 |
| KIDNEY | | | | |
| 1. | 1.2 | 11.2 | 9.8 | 10.9 |
| 2. | 0.5 | 3.3 | 1.8 | 2.8 |
| 3. | 1.2 | 10.5 | 6.6 | 0 |
| Mean ± SE | 1.0±0.2 | 8.3±2.5 | 6.1±2.3 | 4.6±3.3 |

TABLE 7: *Specific renin activity (ng angiotensin / 1 hour incubation / mg protein) in primary cell fractions which were obtained by ultracentrifugation of brain (frontal cortex, caudate nucleus) and kidney cortex tissue.*

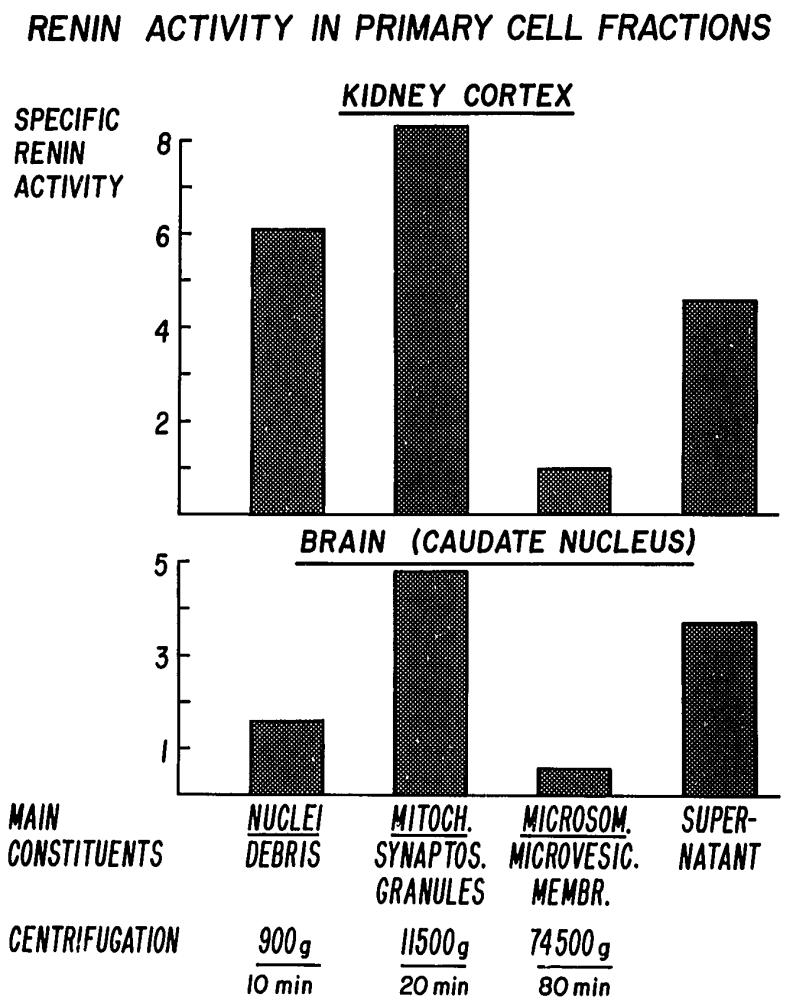



FIGURE 11: *Specific renin activity (ng angiotensin / 1 hour incubation / mg protein) in primary cell fractions which were obtained by ultracentrifugation of brain and kidney tissue.*

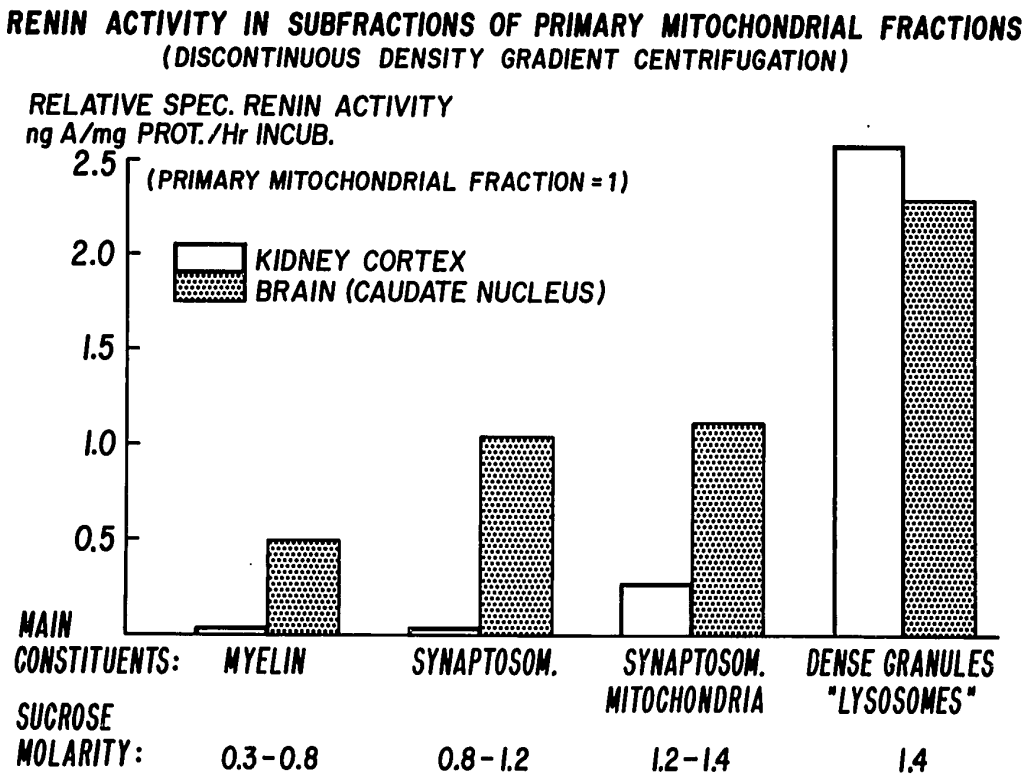



FIGURE 12: *Relative specific renin activity (% renin activity / % protein; primary mitochondrial fraction = 1) in subfractions of the primary mitochondrial fraction of brain and kidney tissue. The subfractions were obtained by discontinuous density gradient centrifugation of the previously isolated primary mitochondrial fraction. Similar results were obtained with frontal cortex tissue.*

brain regions which were studied (frontal cortex, caudate nucleus) as well as in the renal cortex. Similar results have been reported by other authors for the renal cortex (317). The dense granules in the cytoplasma of kidney cells are usually interpreted as renin-storage granules. It has been shown that under various conditions, these granules of the kidney can release renin and it appears that the renin granules of the kidney are actively involved in the regulation of renin secretion (319, 320). Following our criteria the renin containing granules in the brain and in the kidney are similar. It is possible that they fulfil renin-storage function in both organs. The similar pattern of distribution may be interpreted as an argument in favor of local synthesis of renin in the brain.

The question whether the cellular renin distribution was due to unspecific binding of the free enzyme by the different cell organelles, has been tested in two different types of experiments:

a) *Addition of exogenous renin:*

Two aliquots of brain tissue were homogenized in parallel, as described, in a 0.292 M sucrose solution by means of a motor driven teflon pestle. In one of the samples, exogenous hog renin was added to the sucrose solution before homogenization. The samples were then processed under identical conditions and the primary fractions were tested for their renin-content.

The whole-sample renin activity (Microsomal, Mitochondrial, Nuclear fraction and Supernatant) was set at 100%. In the

sample where no renin was added, the supernatant contained only 6.9% of the total sample renin activity which means that over 90% of the tissue renin activity was particle-bound. In the aliquot with added exogenous renin, the increase of renin activity in the supernatant was 2 to 4 times greater than the increase in the primary cell fractions. These results indicated, that the finding of renin to the cellular fractions could not be due solely to unspecific binding of the enzyme on the surface of the cell organelles. If unspecific binding did occur, it was not selective for certain subfractions. Expecially the high concentration of renin in the mitochondrial-subfraction could not be due to unspecific enzyme binding phenomena, since the increase of renin activity due to the added exogenous renin was almost exactly the same in the microsomal fraction and in the mitochondrial fraction (table 8).

**Further** evidence that the high concentration of renin in the mitochondrial fraction was not due to unspecific surface binding but to the intragranular localization of the enzyme is shown by the second experiment:

b) *Osmotic shock:*

After separation of the primary fractions, mitochondrial fractions were submitted to differential density gradient centrifugation as described. However, while one primary mitochondrial fraction was suspended in sucrose as usual, another mitochondrial fraction was suspended in water

| PRIMARY CELL FRACTIONS | RENIN ACTIVITY WITHOUT ADDITION OF EXOGENOUS RENIN "CONTROL" | AFTER ADDITION OF EXOGENOUS HOG RENIN | PERCENT INCREASE IN RENIN ACTIVITY DUE TO ADDED EXOGENOUS HOG RENIN |
|---|---|---|---|
| MICROSOMAL | 2.5 ng (= 2.7%) | 100 ng | 106.9% |
| MITOCHONDRIAL | 75 ng (=82.2%) | 180 ng | 115.0% |
| NUCLEAR | 7.5 ng (= 8.2%) | 60 ng | 57.6% |
| SUPERNATANT | 6.25ng (= 6.9%) | 200 ng | 204.0% |

TABLE 8: *The effect of addition of exogenous hog renin on the distribution pattern of renin activity in primary cell fractions of frontal cortex brain tissue (unspecific binding). Primary cell fractions were obtained by ultracentrifugation. Exogenous pig renin (NBC, Cleveland) was added to the sample prior to homogenization. Renin activity is expressed as ng angiotensin / mg protein / 1 hour incubation.*

(osmotic shock) rather than in isotonic sucrose solution. Analysis of the first sample yielded results similar to those described,with the highest specific activity in the lysosomal subfraction. After osmotic shock with water, the granules were broken, the enzyme activity released by the osmotically sensitive vesicles and higher renin activity was found in the supernatant subfraction (table 9).

These experiments showed that renin can be released from lysosomal vesicles by osmotic shock. RA in the synaptosomal subfraction also decreased following this treatment.

It has been shown (321) that the synaptosomes break away from the neuron during the homogenization and ultracentrifugation procedures. Being thus isolated, they "seal off" the broken end, and show vesicular appearance. The osmotic shock treatment, provoked an opening of these vesicles, when water diffuses into the synaptosomes. Renin escaped under these conditions from the synaptosomes as it did from the dense granules and appeared in the supernatant (table 9).

The fact that renin is present in the isolated synaptosomes would indicate that the brain nerve terminals probably have the capacity of synthesizing angiotensin. The physiological implications of possibly local angiotensin formation in the synaptosomes may be important in view of the effect of angiotensin on neurotransmitter substances (65-72). The presence of renin in brain nerve terminals also indicated that renin activity was not confined to non-neural elements such as glial cells or vascular tissue.

| MAIN CONSTITUENTS OF SUBFRACTIONS OF PRIMARY MITOCHONDRIAL FRACTION | SUCROSE MOLARITY | RELATIVE SPECIFIC RENIN ACTIVITY | |
|---|---|---|---|
| | | FRONTAL CORTEX | FRONTAL CORTEX AFTER OSMOTIC SHOCK |
| A) Myelin ("supernatant") | 0.3 - 0.8 | 0.62 | 1.37 |
| B) Synaptosomes | 0.8 - 1.2 | 1.14 | 0.69 |
| C) Synaptosomes & Mitochondria | 1.2 - 1.4 | 0.62 | 0.66 |
| D) Dense Granules "Lysosomes" | 1.4 | 2.55 | 1.11 |

TABLE 9: *Relative specific activity (% of renin activity / % of protein; primary mitochondrial fraction = 1) in subfractions of the primary mitochondrial of frontal cortex brain tissue, before and after osmotic shock treatment.*

# 5. RESULTS (PHYSIO-PATHOLOGICAL)

## 5.1. *Brain Iso-Renin in Control and Nephrectomized Dogs*

It was shown in previous chapters, that the method proposed by Boucher et al. (22), for the measurement of plasma and kidney renin activity, can be employed for the brain iso-enzyme.

The concentrations of iso-renin in brain tissue were measured in 10 adult dogs kept in individual metabolic cages in rooms with controlled humidity and temperature. They were fed Dr. Ballard's meat, Purina Dog Chow and tap water ad libitum. The dogs were observed at least 4 weeks under these controlled conditions prior to any experiments. The values obtained from these dogs will be referred to as "control" or "normal values" and are given in table 10.

The various brain regions in these dogs contained high renin activity. Areas which consist mostly of white matter, such as the pons and medulla oblongata, contained as much iso-renin as those areas which are rich in neural cells such as frontal cortex and caudate nucleus. For comparison, renin activity in other tissues and in plasma in the same group of dogs is summarized in table 11. Renin activity in plasma was low when compared to tissue renin activity. Brain iso-renin was approximately ten times higher than iso-renin in the mesenteric artery branches, while renin activity in the renal cortex was one thousand time higher than that of brain tissue. The high iso-renin values in extrarenal tissue (as compared to

| BRAIN REGION | RENIN ACTIVITY |
|---|---|
| Frontal Cortex (n=11) | 21.89±1.42 |
| Caudate Nucleus (n=10) | 19.57±2.97 |
| Cerebellum (n=4) | 28.08±5.86 |
| Thalamus (n=5) | 17.12±6.06 |
| Hypothalamus (n=5) | 17.26±2.40 |
| Mid-Brain (n=4) | 18.47±4.31 |
| Medulla Oblongata (n=4) | 16.49±3.47 |
| Pons (n=4) | 12.61±2.24 |

TABLE 10: *Renin activity (ng angiotensin / g wet weight tissue / 1 hour incubation) in different brain regions of "control" adult dogs. No statistical significant difference exists between the brain regions. Values are given as the mean ± standard error of the mean.*

| TISSUE | RENIN ACTIVITY | (VARIANCE) |
|---|---|---|
| Saphenous Artery (n=10) | 2.38 ± 0.33 | (1.11) |
| Mesenteric Artery Branches (n=10) | 2.49 ± 0.30 | (0.91) |
| Thoracic Aorta (n=10) | 3.46 ± 0.48 | (2.16) |
| Carotid Artery (n=10) | 1.68 ± 0.25 | (0.58) |
| Renal Cortex (n=10) | 23,500 ± 4,930 | (24,333) |
| Heart (left ventricle) (n=11) | 33.04 ± 6.51 | (465.04) |
| Lung (n=11) | 17.87 ± 2.18 | (52.27) |
| Liver (n=10) | 140.20 ± 14.84 | (2,413.53) |
| Spleen (n=11) | 77.24 ± 8.60 | (810.98) |
| Skeletal Muscle (n=11) | 4.27 ± 0.67 | (4.97) |
| Adrenal Gland (n=9) | 446.13 ± 94.82 | (4,530.03) |
| Plasma (n=10) | 0.74 ± 0.24 | (0.59) |

TABLE 11: *Renin activity (ng angiotensin / g wet weight tissue (or ml) / 1 hour incubation) in different tissues and in plasma of "control" adult dogs. Values are given as mean and standard error of the mean. The variance is given in brackets.*

plasma) may be due to

a) concentration mechanisms of renin activity out of the plasma into the tissue or

b) local synthesis of renin in extrarenal tissues. The chemical differences between kidney and brain iso-renin described in chapter 4.3, as well as the intracellular localization of iso-renin, and the impermeability of the blood-brain-barrier for renin, favor the second hypothesis of local renin synthesis in extrarenal tissues.

The following experiment was designed to examine the effect of bilateral nephrectomy on brain tissue iso-renin. If brain iso-renin would decrease following nephrectomy, this would speak in favor of the brain enzyme being absorbed from plasma. On the other hand, independence of iso-renin in the brain from nephrectomy and maintenance at high levels would favor the hypothesis of local renin synthesis.

Fourteen mongrel dogs (adult, male) were bilaterally nephrectomized in a one stage operation by lumbar approach, under sterile conditions.

a) Five dogs were killed 24 hours after nephrectomy and blood and brain tissue were sampled in the described way (chapter 3.1.). As shown in figure 13, there was no significant change in brain iso-renin 24 hours after total nephrectomy, while plasma renin activity was undetectable in these dogs.

b) Nine dogs have been kept alive after bilateral nephrectomy for up to 12 days by means of intermittent peritoneal dialysis, using the techniques of Grollmann et al. (322, 323) and

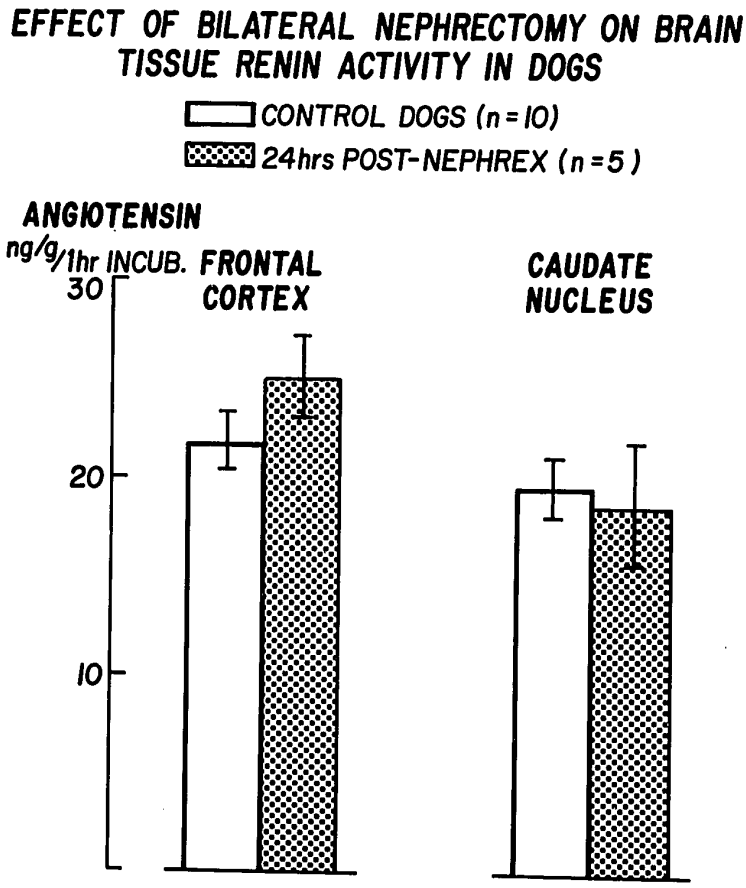
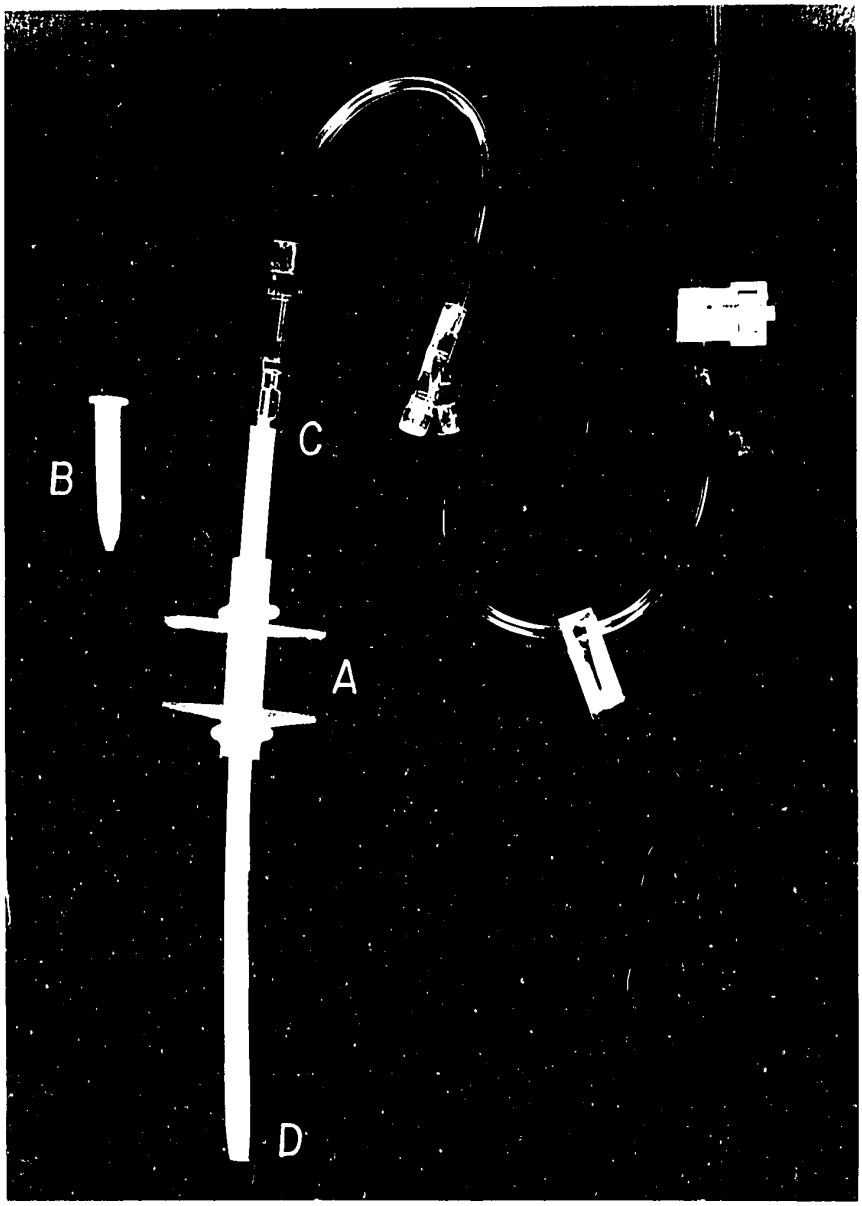



FIGURE 13: *Effect of nephrectomy on brain tissue iso-renin. Values are given as the mean ± standard error of the mean.*


B
C
A
D

Houck (324, 325). Imperinol$^{R}$ (Abbott Laboratories, 2-4 liters) with 1.5% glucose was used for dialysis. The peritoneal lavage was performed under sterile conditions by means of the special device, illustrated in figure 14 and which was implanted into the abdomen at the same time as the nephrectomy was performed.

Peritoneal dialysis could thus be carried out in conscious dogs which were trained to lie quietly on the operating table. The first dialysis was done 12 hours after nephrectomy and meperidine hydrochloride (Demerol$^{R}$, Winthrop) was given intramuscularly for the first 3 dialysing sessions. Vomiting was rather frequent in the dogs one week after nephrectomy and tube feeding with the same diet as described for the "control dogs" was necessary in these cases.

Nine dogs were killed in penthothal anaesthesia and blood and tissue samples were taken 3 to 12 days after nephrectomy. The control of urea and creatinine in nephrectomized dogs by peritoneal dialysis is difficult (322-325). At the day of sacrifice, the 9 dogs of this group were in severe metabolic disorder, which is reflected by a marked increase of urea, creatinine and potassium as shown in table 12:

| | BEFORE NEPHRECTOMY | INCREASE 3-12 DAYS AFTER NEPHRECTOMY |
|---|---|---|
| UREA mg / 100 ml | 35 | 340 |
| CREATININE mg / 100 ml | 1.2 | 10.8 |
| POTASSIUM mEq / liter | 4.8 | 6.4 |

TABLE 12: *Mean increase of urea, creatinine and potassium in bilaterally nephrectomized (NX) dogs, maintained alive by peritoneal dialysis for 3-12 days.*

The concentration of renin found in the brain of these dogs is described in table 13 and figure 15. There was no significant change in brain renin activity 24 hours after nephrectomy. Low values have been found between 3-5 days after nephrectomy but the values increased to the normal level and even above in the dogs which have been nephrectomized 6 to 12 days previously.

The decrease in renin activity in the group of dogs killed 3-5 days after nephrectomy was significant ($p < 0.05$). We have presently no explanation for this result. The potassium retention after nephrectomy may be an important factor, since potassium is known to suppress

| DAYS AFTER NEPHRECTOMY | NO OF DOGS | RENIN ACTIVITY PLASMA | FRONTAL CORTEX | CAUDATE NUCLEUS |
|---|---|---|---|---|
| 1 | 5 | 0 | 25.3 ± 3.9 | 18.4 ± 6.3 |
| 3 | 1 | 0 | 9.4 | 8.4 |
| 4 | 1 | 0 | 9.5 | 8.6 |
| 5 | 1 | 0 | 11.4 | 6.7 |
| 5 | 1 | 0 | 14.8 | 11.8 |
| 5 | 1 | 0 | 22.9 | 7.9 |
| 6 | 1 | 0.2 | 28.2 | 19.2 |
| 11 | 1 | 0 | 5.6 | 10.6 |
| 12 | 1 | 0 | 30.6 | 29.2 |
| 12 | 1 | 0 | 27.8 | 23.1 |
| CONTROL GROUP | 10 | 0.7 ± 0.2 | 21.9 ± 1.4 | 19.6 ± 2.9 |

TABLE 13: *Renin activity (ng angiotensin / g wet weight (or ml) / 1 hour incubation) in plasma and brain tissue of male dogs at various times after nephrectomy. Values are given as mean ± standard error of the mean.*

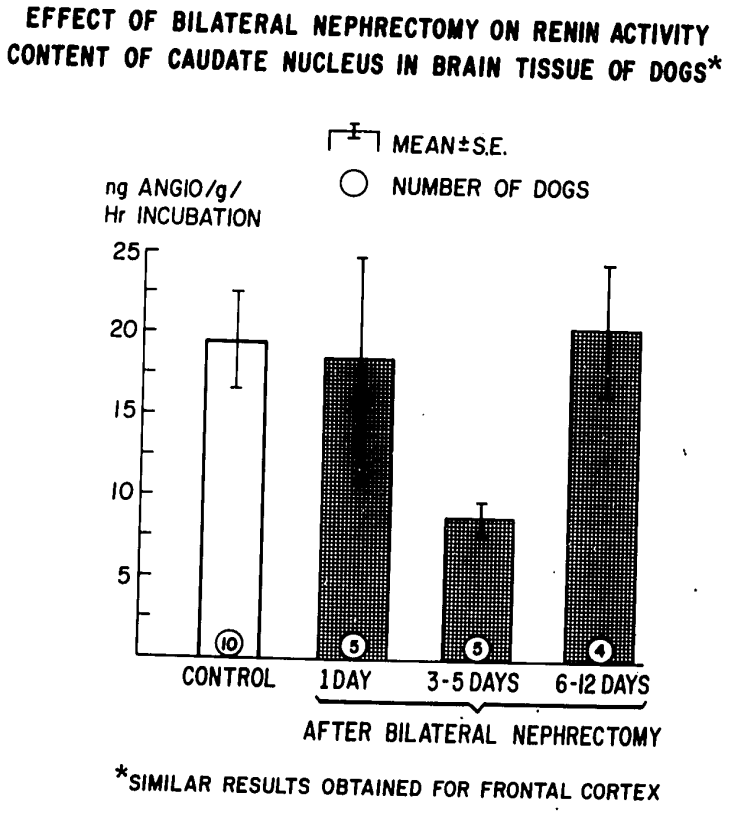



FIGURE 15: *Effect of nephrectomy on brain tissue iso-renin (ng angiotensin / g wet weight tissue / 1 hour incubation). Values are expressed as mean ± standard error of the mean.*

renal renin activity. However, there was no difference in the plasma potassium concentrations between this group of dogs and the dogs kept for a longer period of time where brain renin activity was high.

Values at the same level as in control dogs, 12 days after nephrectomy (table 13), are incompatible with the hypothesis of tissue absorption of plasma renin. If this would be the case, tissue renin in the brain would be expected to decrease gradually after nephrectomy. The high values of brain iso-renin after nephrectomy must be due in our opinion to local renin synthesis. This finding is in agreement with the evidence presented above, that brain renin and renal renin were chemically different and that the brain enzyme like the renal enzyme in the kidney was located intracellularly in similar cell organelles.

## 5.2. *The Presence of Renin Substrate and Angiotensin in Brain Tissue*

The logical question, arising from the finding of an independent iso-renin in brain tissue was, to determine, whether there was a substrate present locally for brain iso-renin and whether local angiotensin-formation could occur in brain tissue itself.

Renin substrate was extracted from brain tissue as described in chapter 3.4.. This extract, incubated with excess renin from dog kidney, with brain iso-renin and with hog renin, yielded significant

amounts of angiotensin as shown in table 14. Because of the difficulty of extracting renin substrate from any tissue and of controlling the losses during the purification procedure, we did not calculate any absolute values of substrate content per gram tissue or other unit. It was thought sufficient at this stage of our studies to know that there is a substrate available in brain tissue with which the brain iso-enzyme as well as renal renins can react to form angiotensin.

Since substrate is present in considerable amounts in the blood, plasma contamination had to be excluded in these experiments. The renin substrate does not pass the blood-brain-barrier, since it was undetectable in the CSF. Renin and renin substrate, having molecular weights of the same order, behaved in the same way in this respect. Plasma substrate contained in the blood vessels of the brain was washed out with large amounts of cold saline as described in chapter 3.4..

If the end product of the enzyme substrate reaction could be demonstrated in brain tissue, this too would be an indirect evidence for the availability of angiotensinogen at this level. We therefore attempted to extract angiotensin from dog brain by the method described in chapter 3.2..

The blood pressure response to the intravenous injection of purified brain extract is shown in figure 16. The pressor material was identified as being mainly angiotensin I, since its effect on the blood pressure was almost completely blocked by anti-angiotensin I.

| BRAIN SUBSTRATE PREPARATION | INCUBATION WITH: | ANGIOTENSIN FORMING CAPACITY OF ALIQUOTS FROM BRAIN-SUBSTRATE |
|---|---|---|
| I | DOG KIDNEY RENIN | 5 ng |
| | HOG KIDNEY RENIN | 20 ng |
| II | DOG KIDNEY RENIN | 8.75 ng |
| | HOG KIDNEY RENIN | 80 ng |
| III | DOG KIDNEY | 25 ng |
| | HOG KIDNEY | 30 ng |
| | BRAIN ISO-RENIN | 125 ng |
| | BRAIN ISO-RENIN | 140 ng |

TABLE 14: *Angiotensin forming capacity of renin substrate extracted from 3 different brains and incubated with different renins.*

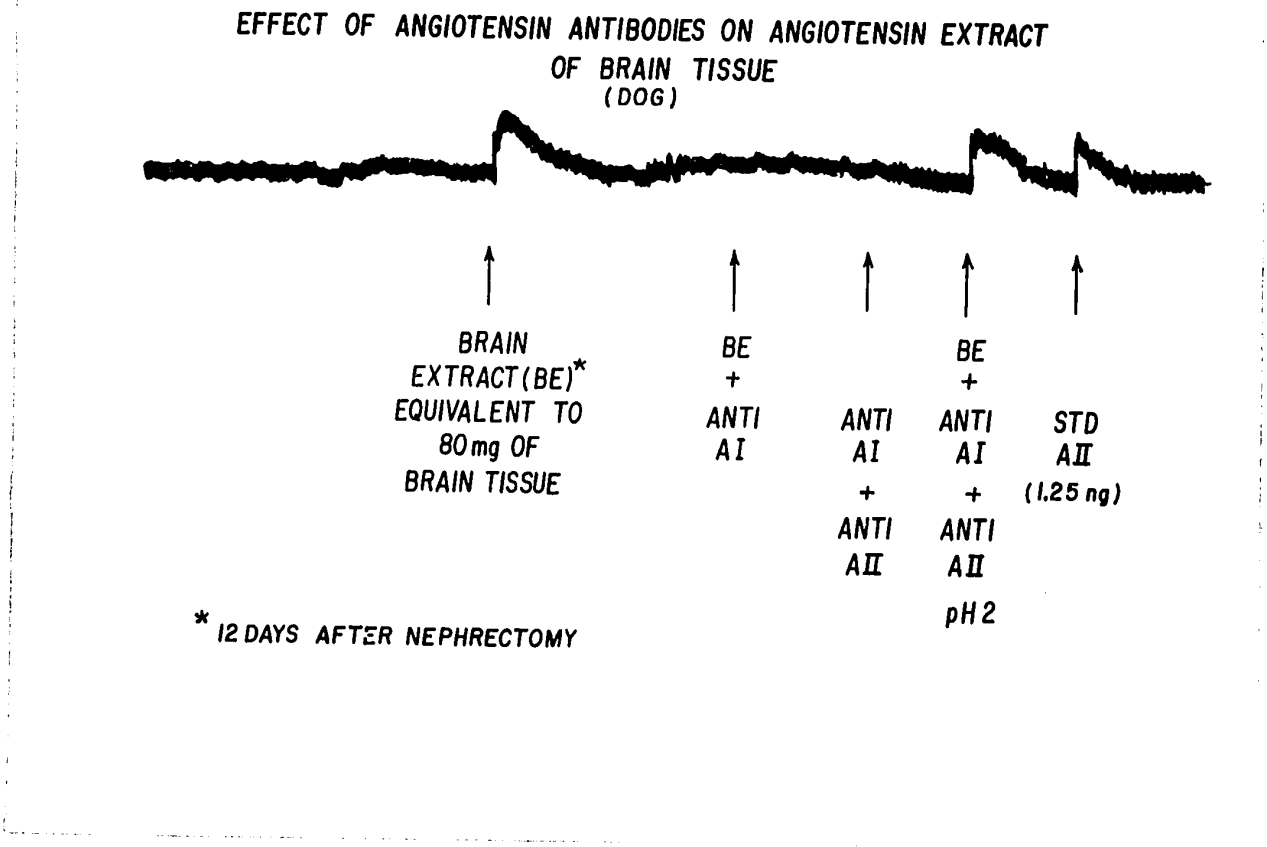



FIGURE 16: *Blood pressure response to the intravenous injection of purified angiotensin extract, obtained from dog brain 12 days after nephrectomy. Effect of angiotensin antibodies on the angiotensin extract.*

Acidification of the antigen-antibody complex to pH 2 with 1 N HCl caused dissociation of the complex and the pressor activity was fully restored.

The recovery of angiotensin added to the extraction procedure was very poor with 10-25%. The calculated value of 2.7 ng angiotensin content per gram of wet brain tissue (uncorrected for losses) has therefore to be taken with caution, and it cannot be concluded from these experiments, which is the ratio of angiotensin I and angiotensin II of the local brain peptides.

The presence of an angiotensin I converting enzyme in brain tissue of rats has been reported (326). These authors, however, used a non-physiological synthetic substrate for the assay which may not reflect true converting enzyme activity. We could not as yet confirm the presence of converting enzyme in brain tissue of dogs. - Large amounts of brain tissue were needed to obtain measurable amounts of angiotensin by the method used and studies on the regional distribution of angiotensin in various brain areas have not been done for this reason.

The question of the passage of angiotensin through the blood-brain-barrier is still unsettled. To exclude the interference of angiotensin from blood, all extractions of brain angiotensin were done in nephrectomized dogs with undetectable angiotensin in plasma as measured by radioimmunoassay. The fact that angiotensin is present in these brains supports our finding that substrate is also available and that a complete, intrinsic and independent renin-angiotensin-system is

functioning in the brain of anephric dogs.

In another experiment we have divided the brain of a "control" dog into two halves of 36.5 and 38.5 g and homogenized each part in 5 volumes (W/V) Ringer solution. While one half was stored at 4°C in the oxygenized Krebs Ringer solution, the other half was incubated in the same medium for 4 hours at 37°C. Angiotensin was then extracted as described from both parts of the brain: the incubated portion of the brain yielded twice the amounts of angiotensin (1.16 ng/gram tissue) as the non-incubated half (0.69 ng angiotensin / gram tissue). These results strongly suggested that renin and renin substrate were present in brain tissue and that angiotensin could be formed locally.

The question arose from these results, as to why there was no pressor material formed, when brain homogenates were incubated without addition of exogenous renin substrate, as described in chapter 4.1. We concluded from those experiments, that no preformed substances interfered in the renin assay and that angiotensin formation occurred only, when renin substrate was added to the incubation. The answer to this apparent contradiction is probably a quantitative one: the endogenous substrate contained in 100 mg brain homogenate might not be sufficient to yield measurable amounts of angiotensin. The incubation of these small quantities of tissue therefore makes the addition of exogenous substrate necessary, while in incubations of large amounts of brain tissue, angiotensin formation as a product of the reaction between endogenous brain substrate and endogenous brain iso-renin could be measured.

The presence of iso-renin and renin substrate in the brain has been recently confirmed through indirect evidence by Fitzsimons et al. (77-83). These workers showed that the injection of angiotensin directly into the hypothalamus caused rats to drink water. The same response could be elicited by the direct injection of renin. Interestingly, the response after injection of renin was delayed and longer lasting than if angiotensin was injected. It was also found, that the synthetic tetradecapeptide substrate, which has a high affinity to brain iso-renin (chapter 4.3), was a potent dipsogen.

## 5.3. *Iso-Renin, Electrolytes and Catecholamines in Brains of Dogs at Different Ages*

Renin activity has been measured in the frontal cortex and caudate nucleus from dogs of different ages, representing different stages of brain development as shown in figure 17.

Four groups of dogs were studied:

1) six 3-day-old puppies were separated from the mother a few minutes before the experiment. The mother's milk was the only nutritional source. The animals were killed, as in the following experiments, under pentothal anaesthesia. Blood was taken from the aorta. The whole brain was removed and iso-renin was measured in different regions.
2) The second group consisted of six 6-week-old equally unweaned puppies like group 1.

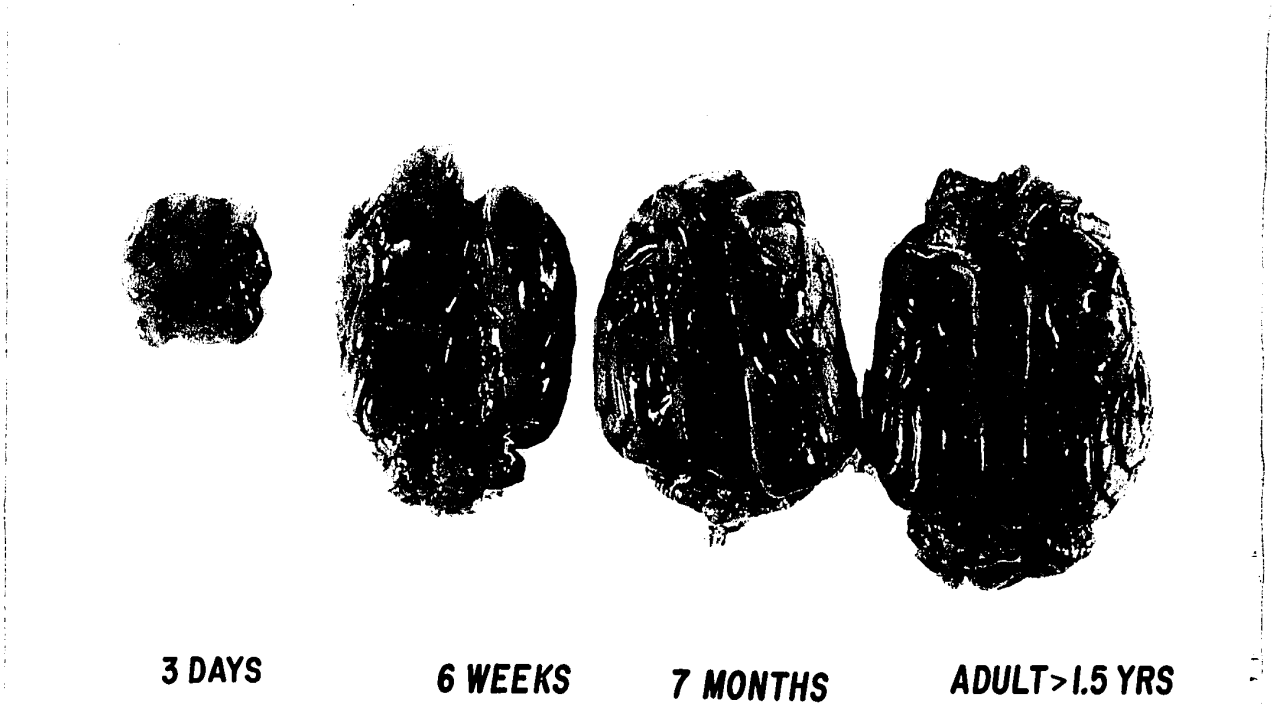



FIGURE 17: *Morphological aspect of dog brains at different ages.*

3) Four dogs were separated from the mother 6 weeks after birth and then kept on a diet consisting of Purina dog chow, Dr. Ballard's Meat and tap water ad libitum until the age of 7 months.
4) A group of ten adult dogs between 1.5 and 4 years of age was kept on the same diet as that described for group 3 at least 4 weeks before the experiment.

Plasma renin activity was significantly higher in puppies than in adult dogs (figure 18 and table 15). In contrast to plasma renin activity, brain iso-renin was lower in 3-day-old dogs and then increased gradually and significantly to control levels observed in adult dogs. Plasma renin activity decreased at the same time. In 7-months-old dogs with a morphologically fully developed brain, iso-renin in the frontal cortex had reached values which were not significantly different from normal values. There existed an inverse relationship between plasma renin and brain iso-renin: with increasing age plasma renin decreased while brain iso-renin increased.

These data confirmed the results reported above, that the kidney and plasma renin-angiotensin system on one hand, and the brain renin-angiotensin system on the other hand are independent from each other. Plasma renin activity has no influence on brain iso-renin. We now can extend these findings to the effect that the renin-angiotensin system in the brain of new-born dogs is apparently not yet fully developed and it increases with morphological and functional brain development, while

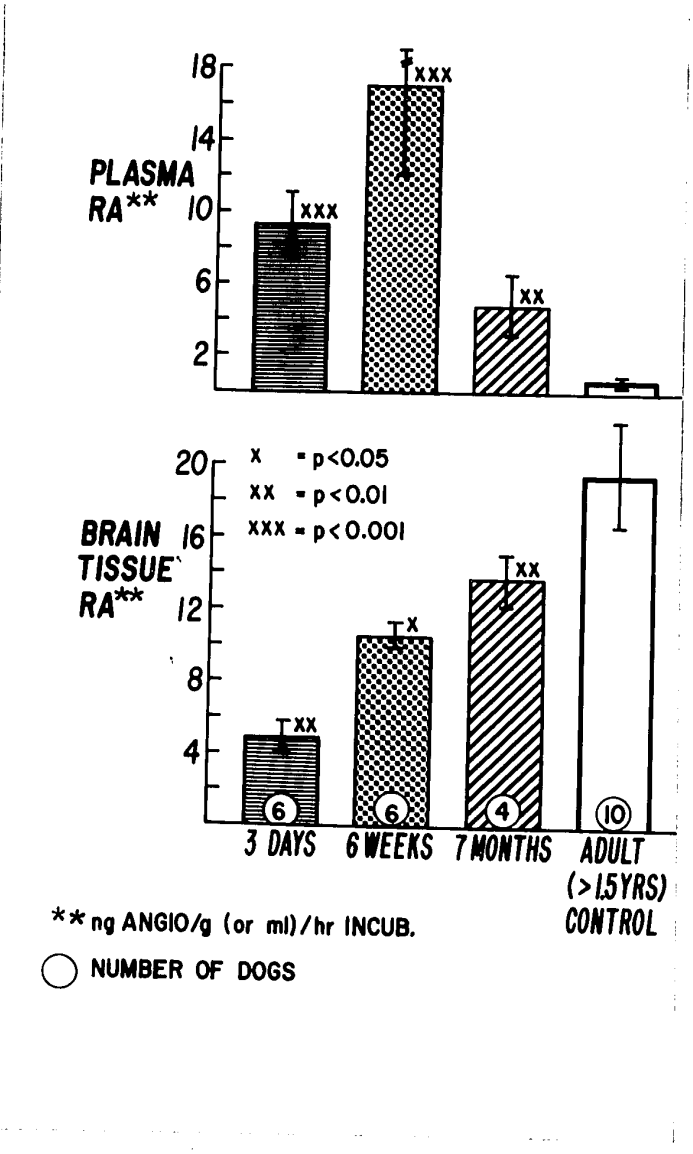



FIGURE 18: *Comparison of brain iso-renin and plasma renin activity (RA) in dogs of different ages.*

| EXPERIMENTAL GROUPS | RENIN ACTIVITY | | |
|---|---|---|---|
| | PLASMA | FRONTAL CORTEX | CAUDATE NUCLEUS |
| 3 days (n=5) | 9.3 ±1.8*** | 6.7 ±0.8*** | 4.8 ±1.1*** |
| 6 weeks (n=6) | 17.0 ±5.3*** | 9.4 ±0.8† | 10.6 ±0.8* |
| 7 months (n=4) | 4.8 ±1.7** | 25.9 ±5.5† | 13.7 ±1.5† |
| Adult (n=11) | 0.7 ±0.6 | 21.9 ±1.4 | 19.6 ±2.9 |

TABLE 15: *Parallel measurement of renin activity (ng angiotensin / g wet weight tissue (or ml) / 1 hour incubation) in brain tissue and in plasma of dogs at different ages.*

*** $p < 0.001$ as compared to adult dogs

** $p < 0.01$ " " " " "

* $p < 0.05$ " " " " "

† no statistically significant difference as compared to adult dogs.

renin synthesis in the kidney of puppies is stimulated. The existence of a stimulated renal renin-angiotensin system in puppies has been reported (327).

*Brain tissue electrolytes in dogs of different ages:*

Tissue electrolytes have been measured in the brain of these dogs by methods described in chapter 3.9. In 3-day-old puppies, brain tissue contained almost 90% water. Similarly sodium, potassium, calcium and magnesium were highest in puppies 3 days of age. Water decreased with age. In 6-week-old dogs it was significantly lower than in 3-day-old puppies and it decreased further in 7-month-old dogs, reaching the lowest values in adult dogs. The tissue electrolytes followed essentially the same pattern: sodium, potassium, magnesium, and calcium were significantly higher in 3-day-old puppies and decreased continuously and significantly with age. (figure 19, figure 20, table 16). There was a highly significant negative correlation between brain tissue water, sodium and potassium on one hand and brain iso-renin on the other hand. The linear regression lines for these parameters are illustrated in figure 21 and figure 22.

Iso-renin activity could reflect angiotensin-formation in brain tissue. The inverse correlation between brain tissue electrolytes and brain iso-renin would then suggest that in the highly hydrated brain tissue of puppies, angiotensin formation is low, while it is high in the tissue of adult dogs, which is "dehydrated" relative to puppies.

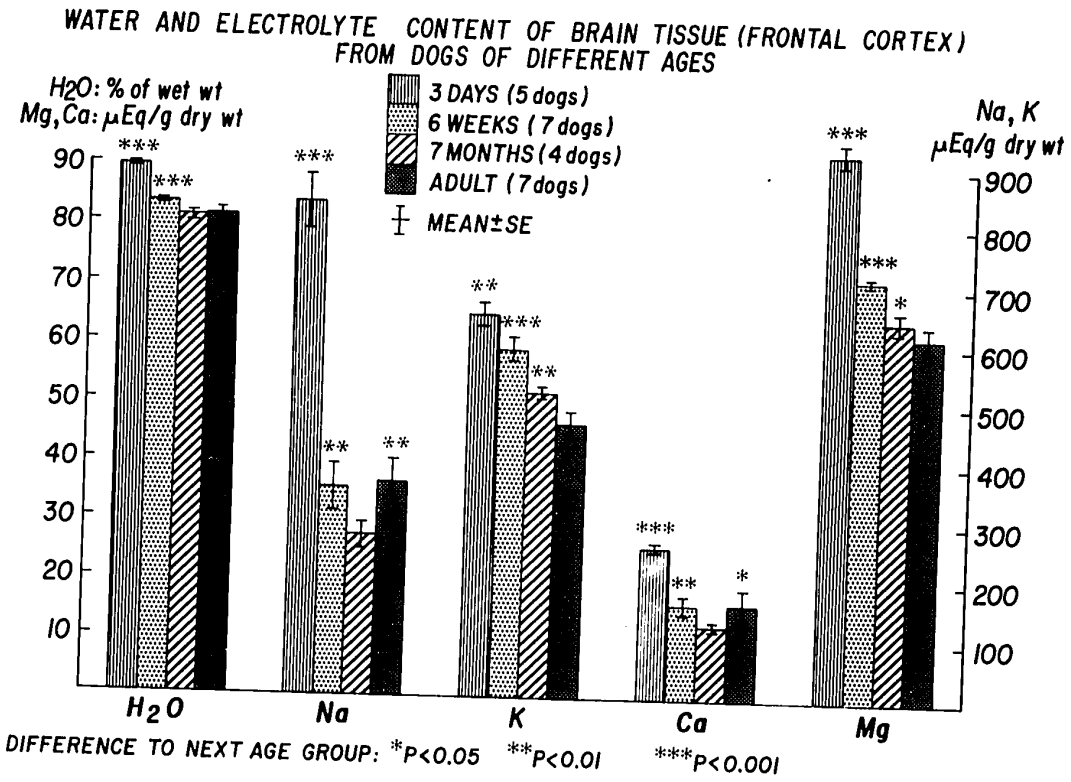



FIGURE 19: *Water and electrolyte content in brain tissue (frontal cortex) of dogs of different ages.*

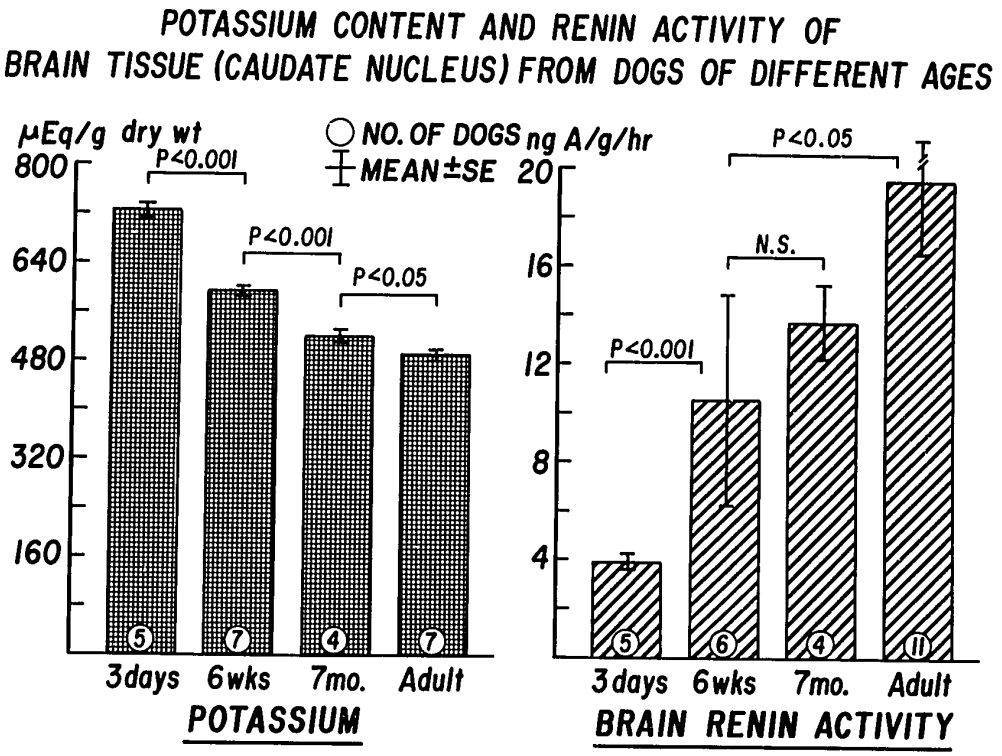



FIGURE 20: *Brain iso-renin and potassium content in brain tissue (caudate nucleus) from dogs of different ages.*

| Age Group | Water | $Na^+$ | $K^+$ | $Ca^{++}$ | $Mg^{++}$ |
|---|---|---|---|---|---|
| 3 days (n=5) | 88.41*** ±0.33 | 635.45*** ±35.97 | 725.75*** ±12.92 | 24.07*** ±1.83 | 89.64*** ±0.66 |
| 6 weeks (n=7) | 82.58*** ±0.11 | 306.86† ±13.03 | 593.73*** ±6.22 | 15.33** ±0.54 | 70.53*** ±0.37 |
| 7 months (n=4) | 81.02† ±0.33 | 274.33† ±11.13 | 520.22* ±10.38 | 12.75† ±0.37 | 64.50† ±0.79 |
| Adult (n=7) | 80.54 ±0.15 | 281.18 ±11.92 | 492.81 ±6.25 | 11.69 ±0.30 | 62.53 ±0.76 |

TABLE 16: *Water content (expressed in % of wet weight) and cation content (expressed in µEq per gram dry weight) in caudate nucleus tissue from dogs of different ages. Values are given as mean ± standard error of the mean.*

*** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$, † = no significant difference, as compared to the next age group.

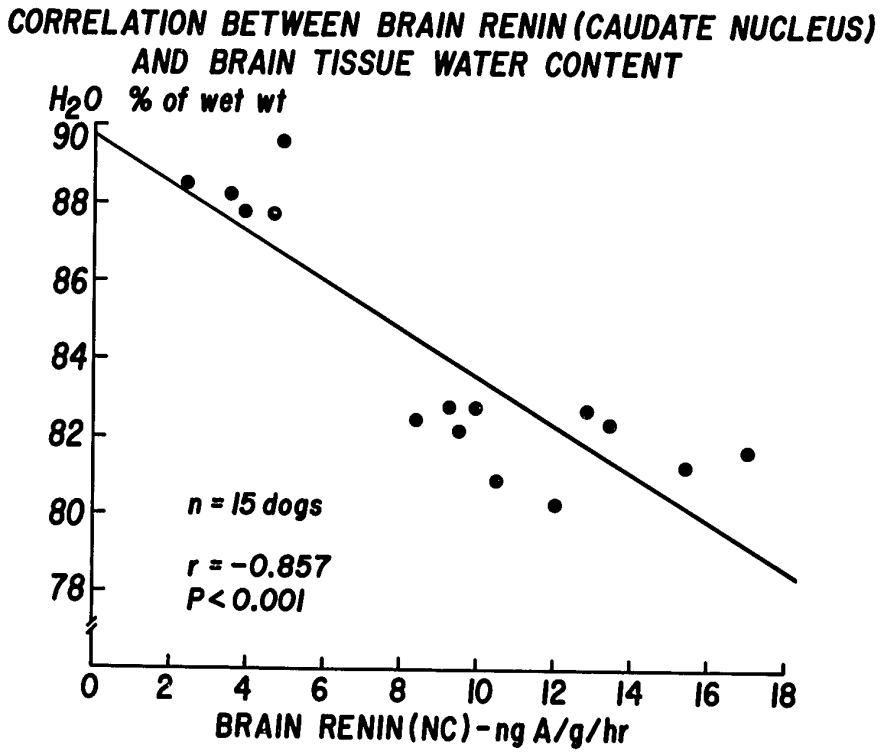



FIGURE 21: *Linear regression line between brain iso-renin (abcisse) and brain tissue water content (ordinate). All age groups are included.*

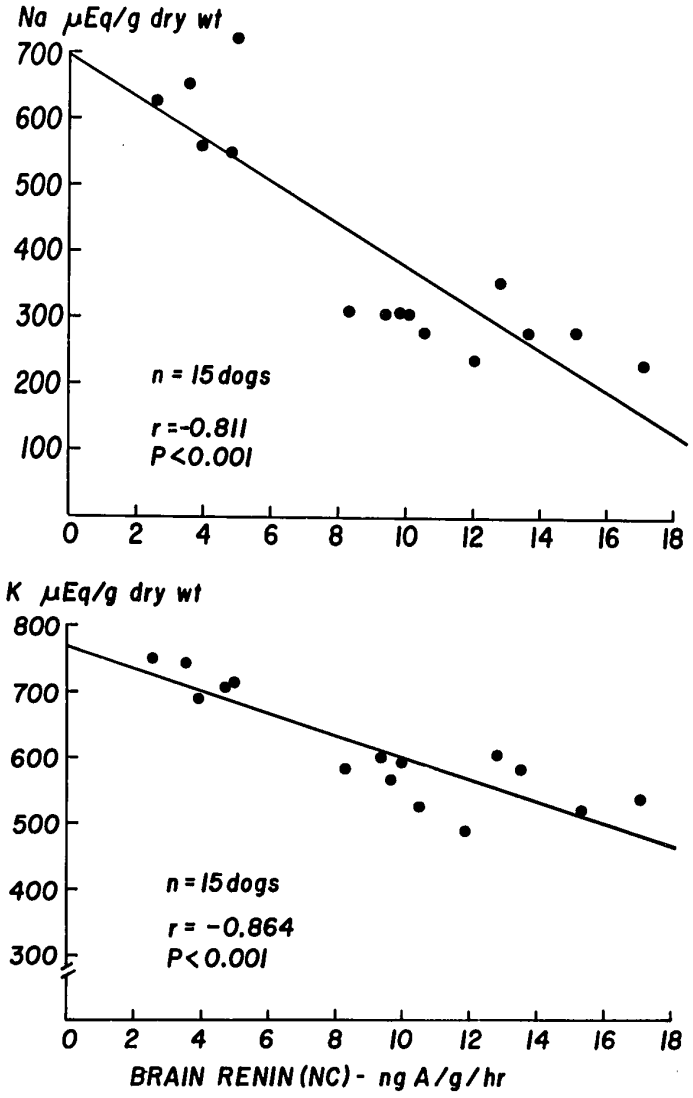



FIGURE 22: *Linear regression lines between brain iso-renin (abcisse) and brain tissue sodium (ordinate, upper graph) and brain tissue potassium (ordinate, lower graph). All age groups are included.*

It would also appear that the brain renin-angiotensin system responds to similar stimuli as the kidney renin-angiotensin system: dehydration, sodium and potassium deficiency.

*Brain tissue catecholamines in dogs of different ages:*

Parallel with iso-renin and electrolytes, norepinephrine and dopamine have been measured in the same groups of dogs at different ages. (Methods for measurement of norepinephrine and dopamine, see chapter 3.9.). The results are given in table 17. There was a significant increase in dopamine content in the caudate nucleus from 6-week-old to adult dogs (> 1.5 years) ($p < 0.005$). The changes in norepinephrine were statistically not significant. An increase in catecholamines in brain tissue with age and brain development has been described previously (329).

The results of a) a parallel increase of brain iso-renin and catecholamines and b) the negative correlation between brain iso-renin and brain tissue water and electrolytes in view of the described effects of angiotensin on neurotransmitter substances (65-72) and on membrane permeability for water and electrolytes (92-98) will be discussed in the general discussion.

| Age | Norepinephrine | | Dopamine |
|---|---|---|---|
| | Frontal Cortex | Caudate Nucleus | Caudate Nucleus |
| 3 days (n=5) | 0.016 ±0.003 | - | - |
| 6 weeks (n=7) | 0.029 ±0.006 | 0.092 ±0.009 | 3.57 ±0.26* |
| Adult (n=7) | 0.040 ±0.009 | 0.112 ±0.023 | 7.74 ±0.98 |

TABLE 17: *Norepinephrine and dopamine content (µg/g wet weight tissue) in brain tissue from dogs of different ages. Values are expressed as mean ± standard error of the mean.*

* $p < 0.005$ vs. adult dogs.

## 5.4. *Iso-Renin in Arterial Tissue*

### 5.4. 1. Characterization

The first extrarenal tissue in which renin had been discovered was the arterial wall of pigs (24). Because of the presumed relationship between the renal renin-angiotensin system and hypertension, it was thought that renin in the arterial wall might be involved in the contraction of blood vessels and thus in the regulation of blood pressure. After the pioneering work of Dengler (24) and Gould et al. (25), research in arterial tissue renin was stimulated by recent work of Genest's group (27-31).

We have now characterized arterial tissue iso-renin from the aorta of the dog using exactly the same methods as for brain iso-renin (chapter 3.5. - 3.8.). The characteristics of arterial tissue renin and brain were found to be similar:

1) incubation of small amounts of arterial tissue iso-renin without addition of exogenous substrate did not yield any pressor substance.
2) The amount of pressor material formed during the incubation was linearly time dependent.
3) The amount of pressor material formed during the incubation was linearly dependent on the quantity of arterial tissue enzyme added to the incubation.
4) The enzyme was not dialyzable.

5) The activity of the arterial tissue enzyme was pH dependent. Maximum rate of angiotensin formation occurred at pH 5 (see figure 7, chapter 4.3.).

6) Arterial tissue enzyme could be precipitated with ammonium sulfate 2.3 molar, pH 2.8.

7) Arterial tissue renin was stable in acid milieu of pH 2.3 for more than 4 hours.

8) The arterial enzyme was denatured and its activity abolished if heated for 10 minutes in boiling water at temperatures higher than 60°C.

9) EDTA and DFP had no influence on the enzyme activity.

10) Enzyme substrate reactions: Purified arterial tissue renin formed less angiotensin with heterologous renin substrates from rat and rabbit as compared to homologous standard plasma substrate. It formed more angiotensin with highly purified dog plasma substrate, with sheep substrate and with synthetic tetradecapeptide renin substrate (figure 23).

11) The mobility of purified arterial tissue iso-renin on "disc" polyacrylamide gel electrophoresis was similar to brain iso-renin but differed from dog and pig renal renin (see figure 10, chapter 4.3.).

The pressor product formed during the incubation of arterial tissue iso-renin with homologous "standard" plasma substrate was identified as angiotensin by the same criteria (chapter 4.2.) described for the characterization of the pressor product formed during the incubation

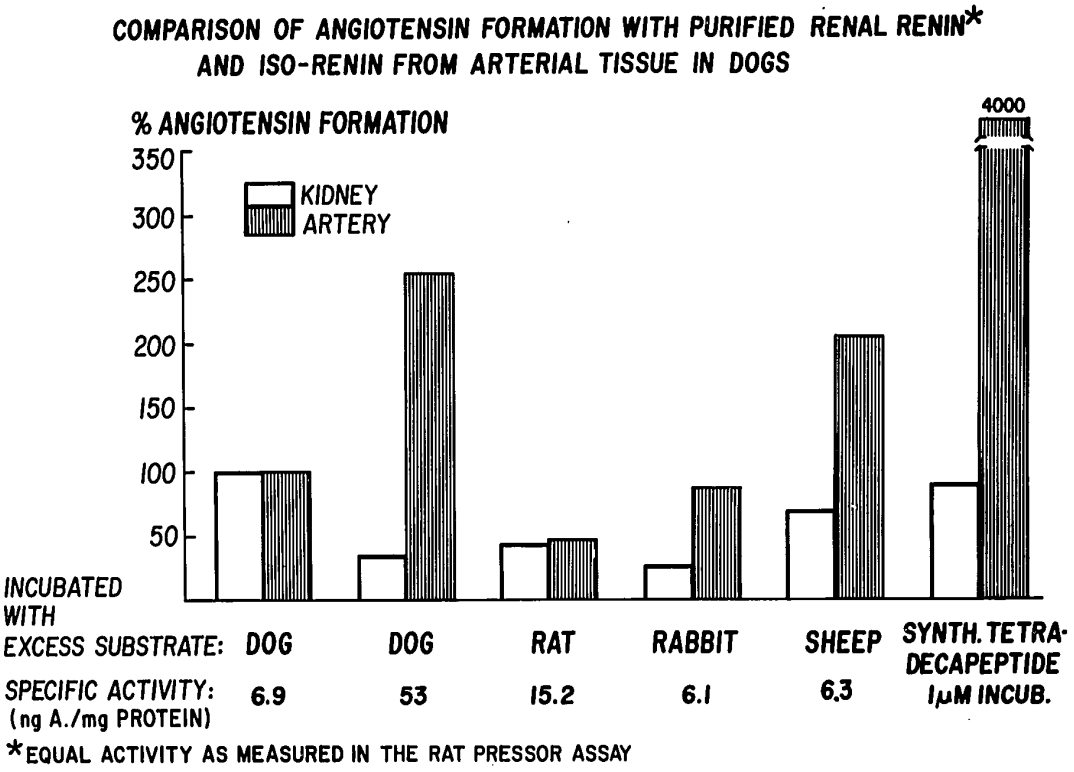



FIGURE 23: *Comparison of angiotensin formation between purified iso-renin from arterial tissue and renal renin. Angiotensin formation with "standard" homologous plasma substrate is arbitrarily set at 100%.*

of brain enzyme and standard dog plasma substrate.

These studies indicated, that the brain and arterial enzymes are similar, but differ in many respects from the kidney enzyme.

### 5.4. 2. Independence of Iso-Renin in Arterial Tissue from Plasma and Kidney Renin

Our interest in arterial tissue iso-renin was stimulated by two observations. As described earlier, exogenous renin substrate is necessary for the measurement of renin activity using the "micro-method" of Boucher et al. (22). For the preparation of this substrate dogs were bilaterally nephrectomized and bled 24 hours later at the time when plasma renin activity was undetectable. The bleeding could be completed in 30 minutes. However, it lasted frequently much longer for reasons other than technical. In some cases bleeding was prolonged for several hours. In these instances it was found, that

a) blood samples at the end of the period contained measurable amounts of renin, while zero values were obtained in the beginning of the bleeding and

b) arterial tissue aliquots contained more renin at the end of the experiment than those obtained at the beginning.

These at first surprising observations were further investigated in the following experiments:

5.4. 2.1. Hemorrhage in Nephrectomized Dogs

Six male dogs were bilaterally nephrectomized by lumbar approach. Renin activity was measured in plasma obtained from the cubital vein before the operation and at 6 hour intervals, after nephrectomy. Twenty-four hours later, the dogs were anaesthetized again and blood samples were taken by means of a polyethylene catheters placed in

a) the aorta just above the diaphragma,

b) the portal vein before the liver and in some cases

c) the inferior vena cava above the diaphragm (figure 24).

The values of plasma renin activity before nephrectomy were all in the normal range (table 18). Six hours after nephrectomy, plasma renin activity decreased to undetectable levels and remained so during the post-nephrectomy period (except dog no. 2, with low but detectable PRA, which will be discussed later). The femoral artery was cannulated with a polyethylene catheter for continuous monitoring of the arterial blood pressure with a Grass model 5 polygraph and a Statham transducer. An acute and severe hemorrhage was then performed in these dogs. A sufficient volume of blood was taken within 10 to 20 minutes to lower the blood pressure to 75/50 mmHg. An intravenous infusion of 5% glucose in saline solution was given and its rate was adjusted according to the blood pressure and condition of the dog after the acute bleeding.

The results of these experiments are given in table 18, table 19, and figure 25. It is evident that plasma renin activity increased following hemorrhage in nephrectomized dogs. Since male dogs were

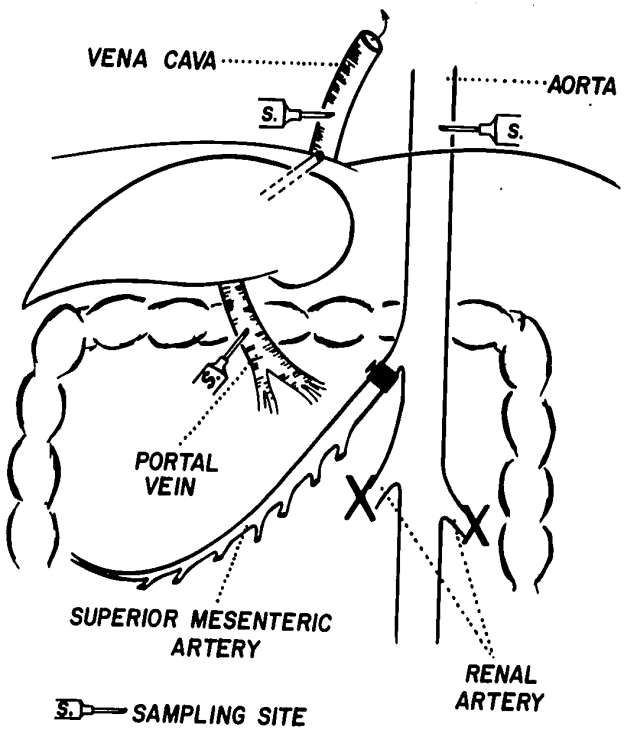



FIGURE 24: *Anatomical illustration of sites where blood was sampled.*

PLASMA RENIN ACTIVITY AFTER BILATERAL NEPHRECTOMY

| DOG NO. | Before Nephrectomy | FIRST DAY 6 | 12 | 18 | 24 hrs. | SECOND DAY Severe Hemorrhage 1 hr. | 2 hrs. | 3 hrs. | 4 hrs. | 5 hrs. | 6 hrs. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 0.12 | 0 | 0 | 0 | 0 | 0.31 | 0.10 | 0.52 | 1.25 | - | - |
| 2 | 0.61 | 0 | 0 | 0 | 0.05 | 0.08 | 0.10 | 0.18 | 0.57 | 1.25 | |
| 3 | 2.04 | 0 | 0 | 0 | 0 | 0 | 0 | 0.01 | 0 | 0.28 | 0.63 |
| 4 | 0.63 | 0 | 0 | 0 | 0 | 0 | 0 | - | - | - | - |
| 5 | 0.52 | 0 | 0 | 0 | 0 | 0 | 0.10 | - | - | - | - |
| 6 | 0.24 | 0 | 0 | 0 | 0 | 0.10 | 0.16 | 0.31 | 0.41 | 0.31 | - |
| MEAN | 0.69 | | | | | 0.08 | 0.08 | 0.26 | 0.56 | 0.61 | 0.63 |

TABLE 18: *Plasma renin activity (ng angiotensin / ml / 1 hour incubation) in male dogs before and after bilateral nephrectomy and following severe hemorrhage for up to 6 hours on the second day after nephrectomy.*

| DOG NO. | PLASMA RENIN ACTIVITY | | | |
|---|---|---|---|---|
| | THORACIC AORTA | | PORTAL VEIN | |
| | Hemorrhage | | Hemorrhage | |
| | before | after | before | after |
| 1 | 0 | 1.25 | - | - |
| 2 | 0.05 | 1.25 | - | - |
| 3 | 0 | 0.63 | 0.10 | 2.7 |
| 4 | 0 | 0 | 0 | 0.52 |
| 5 | 0 | 0.10 | 0 | 2.39 |
| 6 | 0 | 0.31 | 0 | 1.15 |
| Mean ± SE | | 0.59 ±2.3* | | 1.69 ±0.52* |

TABLE 19: *Effect of severe hemorrhage on plasma renin activity (ng angiotensin / ml / 1 hour incubation) in arterial and portal vein blood in nephrectomized dogs. The values "after hemorrhage" represent the sample at the end of each hemorrhage experiment (2 to 6 hours). The difference between the increase in portal vein and aorta was significant ($p < 0.05$).*

*** $p < 0.05$ versus renin activity before hemorrhage.**

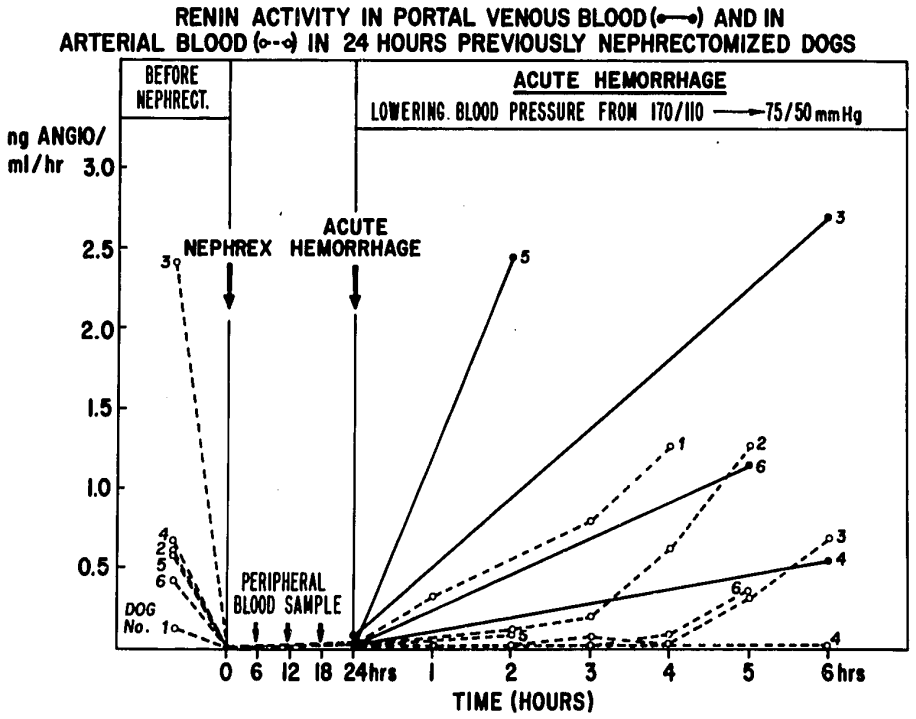



FIGURE 25: *Plasma renin activity in the portal vein and aorta (ng angiotensin / ml / 1 hour incubation) before and after bilateral nephrectomy and following severe hemorrhage for up to 6 hours on the second day after nephrectomy. Each individual dog is represented by a number.*

used (as in all experiments), the uterus was excluded as the source of renin liberation. Plasma renin activity was significantly higher in the portal vein than in the aorta. Since the portal vein drains essentially the splanchnic territory and since plasma renin activity in the arterial blood supply to this region from the aorta was low, these experiments demonstrated that the high renin levels in the portal vein are due to renin liberation from the splanchnic territory. This hypothesis is supported by the fact that iso-renin in the walls of the mesenteric arteries was significantly higher at the end of the bleeding period than in normal control dogs: iso-renin in mesenteric artery branches was at the end of the hemorrhage experiment 8.55 ± 0.76 SE ng angiotensin / g wet weight tissue / hour incubation, versus 2.49 ± 0.3 SE in normal dogs. The difference was significant ($p < 0.05$). These findings of a significant increase in plasma renin activity in nephrectomized dogs following stimulation by ischemia, severe hemorrhage and of a simultaneous increase of mesenteric artery branches iso-renin, support the concept of local iso-renin synthesis in arterial tissue.

One cannot fully exclude the participation of other organs in the liberation of tissue iso-renin into the blood of the portal vein. The spleen, for example, contributes to the blood supply of the portal vein. Our results, however, strongly suggest that the splanchnic territory can contribute to an increase in plasma renin activity after nephrectomy.

An interesting aspect of these experiments is that it took a long time after performance of the hemorrhage before plasma renin activity rose to significant levels. Values within the range found in normal

dogs were only achieved 5-6 hours after such a severe hemorrhage. Renin secretion from the kidney after hemorrhage in contrast occurred rapidly (within minutes). Since the total amounts of kidney renin and the total body extrarenal renin pool, calculated per gram of tissue, are of approximately equal size, it is probably not due to the total amount of renin available which caused the different response to hemorrhage in nephrectomized and in intact dogs, but rather due to different mechanisms of liberation.

Reports on plasma renin activity after nephrectomy are contradictory (131-145). Capelli et al. (131) found significant amounts of renin activity in nephrectomized humans. Other authors did not detect any renin activity in patients which had undergone total nephrectomy (136). Most of the authors favor the hypothesis that the uterus plays an important part in the secretion of renin in female patients (131, 144, 145). Otsuka et al. (143) have done experiments in nephrectomized dogs, similar to ours, and report no significant increase in plasma renin activity after bleeding. They measured plasma renin activity by the micromethod of Boucher et al. (22) and got in fact a very small increase from 0.9 ng angiotensin formation to 1.6 ng angiotensin / ml / 3 hours incubation. These changes were not statistically significant. The increase in plasma renin activity in the same period of 1 hour was not significant either in our hemorrhage experiments. Only the prolongation of the period up to 6 hours after bleeding resulted in a significant increase in plasma renin activity.

It may very well be possible that the severe ischemia over such a long period damages the cells and changes the membrane permeability. Renin from extrarenal sources then "leaks" out of these damaged cells which under more physiological conditions (shorter hemorrhage) may retain the enzyme. Iso-renin thus would be a true tissue enzyme and would be released into the blood-stream only under extreme conditions (6 hours of severe ischemia) provoking severe cell damage and changes in membrane permeability. The uterus is an organ with a physiologically high cell "turnover" and a permanent cell decay which could account preferentially for the leakage of tissue iso-renin into the blood stream from this organ. Contradictory results on PRA in anephric humans (131-145) may partly be due to the conditions of the patients and to the time of dialysis and blood transfusion (transfusion of exogenous renin) as well as to the time of blood sampling for plasma renin activity. In addition, cell-membrane permeability may be altered in severe uremia and acidosis and liberation of tissue iso-renin may occur while well controlled patients show no detectable plasma renin activity, all iso-renin being retained in the tissue. This hypothesis of leakage of tissue iso-renin into the plasma only in cases with cell damage is supported by our hemorrhage experiment and also by the finding of Capelli (131) that plasma renin activity increases with the lengthening of the anephric period. In order to finally conclude on the origin of plasma renin activity in anephric human and animals, its characterization is necessary, however.

The participation of the splanchnic vascular bed in the increase of plasma renin activity in anephric dogs has been further investigated by the following experiments.

### 5.4. 2.2. Hemorrhage in Nephrectomized Dogs with Reduced Splanchnic Vascular Bed

As in the first experiment, 6 male mongrel dogs were nephrectomized and 24 hours later subjected to an acute and severe hemorrhage, lowering the blood pressure to 75/50 mm mercury. In contrast to the experiment above (chapter 5.4. 2.1.) the splanchnic vascular bed was reduced approximately 75% by removing a large part of the small intestine including the supplying blood vessels.

Measurement of plasma renin activity in hourly intervals in the portal vein, vena cava inferior and aorta (see figure 24, chapter 5.4. 2.) revealed that in this experiment plasma renin activity in the portal vein was detectable in some instances but was never higher than in the aorta (table 20). Plasma renin activity, however, was detectable in 3 out of 6 dogs, suggesting that although the splanchnic vessels may play an important role in the liberation of iso-renin from tissue into the plasma, other organs may also liberate tissue iso-renin under the conditions described.

| DOG NO. | SAMPLING SITE | PLASMA RENIN ACTIVITY AFTER BILATERAL NEPHRECTOMY | | | | |
|---|---|---|---|---|---|---|
| | | FIRST DAY | SECOND DAY SEVERE HEMORRHAGE | | | |
| | | | 1 hrs. | 2 hrs. | 3 hrs. | 4 hrs. |
| 1 | Aorta | 0 | 0 | 0 | | |
| | Vena cava | | 0 | 0 | | |
| | Portal vein | | - | 0 | | |
| 2 | Aorta | 0 | 0 | 0 | | |
| | Vena cava | | 0 | 0 | | |
| | Portal vein | | - | 0 | | |
| 3 | Aorta | 0 | 0 | 0 | 0 | |
| | Vena cava | | 0 | 0 | 0 | |
| | Portal vein | | - | - | 0 | |
| 4 | Aorta | 1.25 | 1.25 | 2.50 | 2.50 | |
| | Vena cava | | 2.50 | 3.75 | 0 | |
| | Portal vein | | - | - | 1.25 | |
| 5 | Aorta | 0 | 1.88 | 0 | | |
| | Vena cava | | 2.50 | 0 | | |
| | Portal vein | | - | 1.88 | | |
| 6 | Aorta | 0 | 1.25 | 1.88 | 1.25 | 5.63 |
| | Vena cava | | - | - | - | 3.75 |
| | Portal vein | | - | - | - | 3.13 |

TABLE 20: *Plasma renin activity (ng angiotensin / ml / 1 hour incubation in male dogs after bilateral nephrectomy and following severe hemorrhage on the second day after nephrectomy. The splanchnic vascular bed was reduced in these experiments (75%).*

### 5.4. 2.3. Iso-Renin in Arterial Tissue from Normal and Nephrectomized Dogs.

In chapter 5.1. we have described a group of 9 dogs which were bilaterally nephrectomized and kept alive up to 12 days by peritoneal dialysis. In these dogs, iso-renin has also been measured in arterial tissue from mesenteric artery branches and aorta. Important variations in arterial tissue iso-renin in the individual dogs have been observed (table 21) and cannot be correlated with any of the other parameters measured, serum electrolytes, creatinine and urea.

Our findings described earlier for brain iso-renin hold equally for arterial tissue iso-renin: 3-5 days after nephrectomy tissue iso-renin in the mesenteric artery branches initially drops and then returns to slightly higher levels which are slightly above normal 6-12 days after nephrectomy (figure 26). Similar findings were observed in aortic tissue, except that the iso-renin levels 6-12 days following nephrectomy remained slightly lower than in the control group (figure 27).

Although the differences in iso-renin at various intervals after nephrectomy are not statistically significant, the mere fact, that 12 days after total nephrectomy renin activity was at about the same level as in control dogs, constitutes strong evidence that the tissue enzyme is independent of the kidney and is synthesized locally in the tissue.

One individual mesenteric artery iso-renin value in a dog 12 days after nephrectomy was markedly above control levels (8.00 ng angiotensin / g tissue / 1 hour incubation) while plasma renin activity was zero

| DAYS AFTER NEPHRECTOMY | TISSUE ISO-RENIN: MESENTERIC ARTERY BRANCHES | TISSUE ISO-RENIN: AORTIC TISSUE |
|---|---|---|
| 3 | - | 7.32 |
| 4 | 2.51 | 1.72 |
| 4 | 1.90 | 1.10 |
| 5 | 2.43 | 1.12 |
| 5 | 1.17 | 1.24 |
| 6 | 1.29 | 2.44 |
| 11 | 1.27 | 2.18 |
| 12 | 8.00 | 3.55 |
| 12 | 2.40 | 2.22 |
| Experimental Dogs (Mean±SE) | 2.6 ±0.8 | 2.5 ±0.7 |
| Control Dogs (Mean±SE) | 2.5 ±0.3 | 3.46 ±0.5 |

TABLE 21: *Effect of nephrectomy at various intervals on iso-renin in arterial tissue of dogs. No statistical significant differences between the experimental and control groups.*

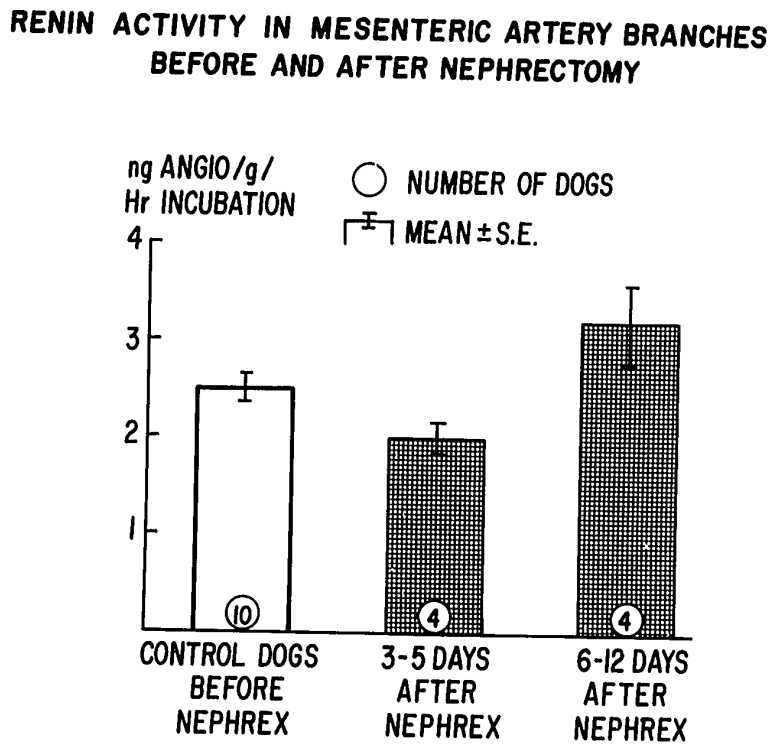



FIGURE 26: *Iso-renin in mesenteric artery branches in control dogs and various intervals after nephrectomy. No statistically significant differences between the control and experimental dogs.*

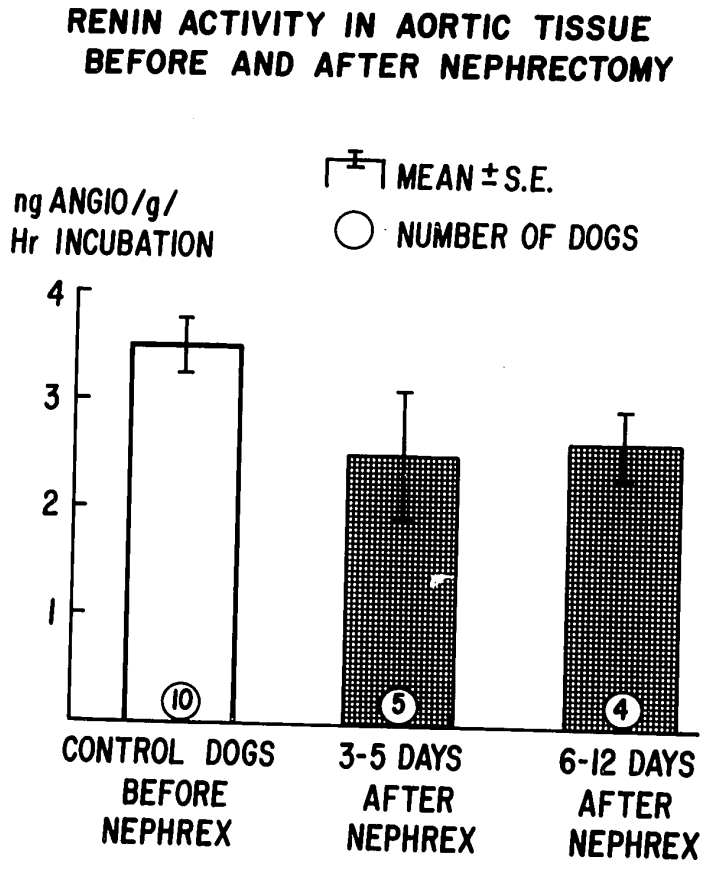



FIGURE 27: *Iso-renin in mesenteric artery branches in control dogs and various intervals after nephrectomy. No statistically significant differences between the control and experimental dogs.*

(table 21). Such high iso-renin in our opinion must be due to local synthesis and cannot be explained by uptake and storage mechanisms.

### 5.4. 2.4. Stimulation of Iso-Renin in Arterial Tissue by Clipping of the Superior Mesenteric Artery

Acute hemorrhage has been found to be a potent stimulus for extrarenal renin activity. The following experiment was undertaken to study the effects of relative ischemia in arterial tissue produced by clipping of the superior mesenteric artery and whether changes in tissue iso-renin can be produced independently of plasma renin activity.

Six male mongrel dogs, weighing between 22 and 32 kg were used. A mid-line laparatomy was performed under pentobarbital anaesthesia. The superior mesenteric artery was located and a Goldblatt clamp was placed on it, approximately 1 cm from its origin on the aorta.

The degree of constriction was judged by the pulsations in the peripheral mesenteric artery branches and reported as:

\+ = slightly reduced pulsations

\+ + = pulsations greatly diminished

\+ + + = no pulsations, but vessels still well filled with blood.

Following surgery the dogs were kept on a control diet, consisting of Purina dog chow and Dr. Ballard's meat. Their weight remained steady. Six to 34 days after clamping, the dogs were bilaterally nephrectomized,

receiving an angiotensin infusion of 200 ng/kg/min. two minutes before and during surgery, in order to suppress renin release from the kidneys. Twenty-four hours after nephrectomy the dogs were again anaesthetized and aliquots of aortic tissue and mesenteric artery branches were taken for measurement of iso-renin.

Results are shown in figure 28 and table 22. Iso-renin in the tissue of mesenteric artery branches increased significantly while it decreased in aortic tissue. The induced changes of iso-renin in the two arterial tissues were dissociated and independent of plasma renin activity. Plasma renin activity in the experimental dogs before nephrectomy was not statistically different from control values (figure 28). The individual values after nephrectomy are shown in table 22. Except for dog no. 4 with low but persisting levels of plasma renin activity, it was undetectable in the other dogs 24 hours after nephrectomy. The tissues were sampled after nephrectomy and any possible interference of plasma renin on arterial iso-renin was thus excluded.

There was no correlation between arterial tissue iso-renin and plasma renin. Iso-renin in the mesenteric artery branches was high and plasma renin was virtually undetectable at the time of tissue sampling. There was equally no correlation between arterial tissue iso-renin and plasma renin before nephrectomy. Plasma renin levels at this time were within the normal range.

Plasma renin activity and iso-renin in mesenteric artery branches and aorta have been compared always to values which were obtained from

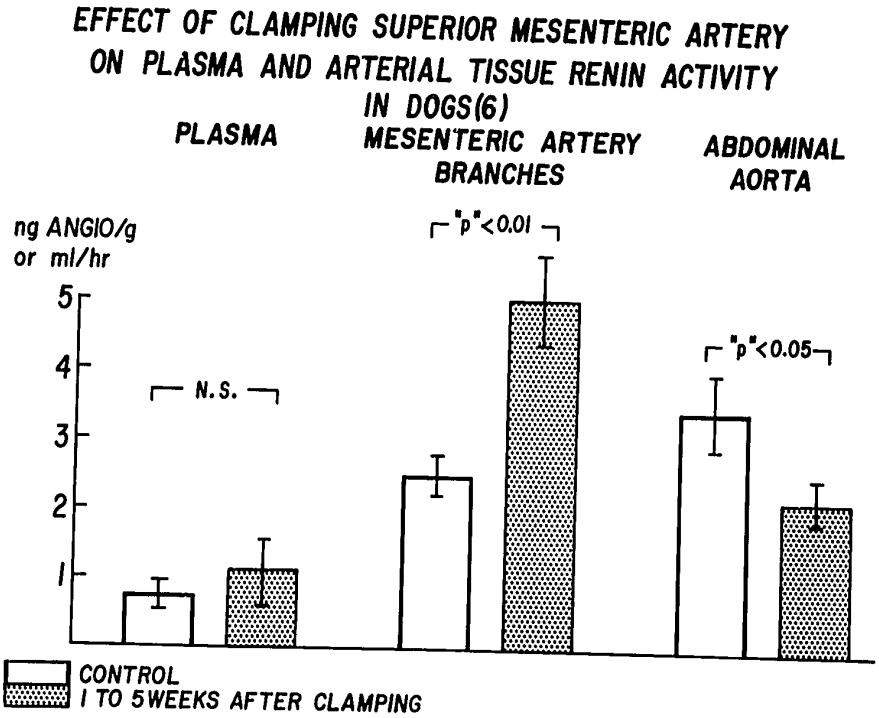



FIGURE 28: *Renin activity in plasma (at the end of the experiment, before bilateral nephrectomy) and in vascular tissue, following clamping of the main trunk of the superior mesenteric artery.*

| DOG NO. | DAYS AFTER CLAMPING | DEGREE OF CLAMPING | RENIN ACTIVITY PLASMA AT DAY OF SACRIFICE | RENIN ACTIVITY MESENTERIC ARTERY BRANCHES | RENIN ACTIVITY AORTIC TISSUE |
|---|---|---|---|---|---|
| 1 | 34 | + + | 0 | 4.13 | 1.92 |
| 2 | 22 | + | 0 | 3.44 | 1.20 |
| 3 | 24 | + + + | 0 | 5.52 | 1.60 |
| 4 | 9 | + + + | 0.12 | 8.01 | 3.02 |
| 5 | 16 | + | 0 | 4.26 | 2.29 |
| 6 | 6 | + + + | 0 | 5.00 | 3.02 |
| MEAN ±SE | 18.5 | | | 5.06 ±0.66** | 2.18 ±0.30* |
| CONTROL DOGS | | | | 2.49 ±0.30 | 3.46 ±0.48 |

TABLE 22: *Results in dogs with a Goldblatt clamp on the superior mesenteric artery.*

** = $p < 0.01$, * = $p < 0.05$ as compared to control dogs.

control dogs (individual metabolic cages, Dr. Ballard's meat, Purina dog chow, tap water ad libitum, constant temperature and humidity). These dogs do not represent a strictly appropriate control group, however, a sham operation and placing of a Goldblatt Clamp on the mesenteric superior artery without reduction of blood flow in the splanchnic territory would have been preferable for the control group.

In these experiments, as described above, it may be possible that the operation itself and the dissection of the main trunk of the mesenteric superior artery free from connective tissue (which includes nervous fibres) may influence tissue iso-renin. We have found a significant decrease of norepinephrine content in the mesenteric artery branches distal from the clamp (figure 29). This may have participated in the changes of tissue iso-renin together with the local ischemia produced by the clamp.

A true sham operation of this kind however has proven to be difficult because the largest Goldblatt Clamp available still causes some degree of constriction of the mesenteric superior artery which can be even increased through uncontrollable "twisting" phenomena in situ after the operation. Nevertheless, these experiments show that stimulation of mesenteric arterial tissue renin is possible independently from plasma renin activity. Local ischemia may have also played an important role in this experiment to stimulate mesenteric artery branches iso-renin. This hypothesis appears supported by the finding of a positive relationship between the degree of constriction of the superior mesenteric artery and the degree of stimulation of iso-renin as shown in figure 30.

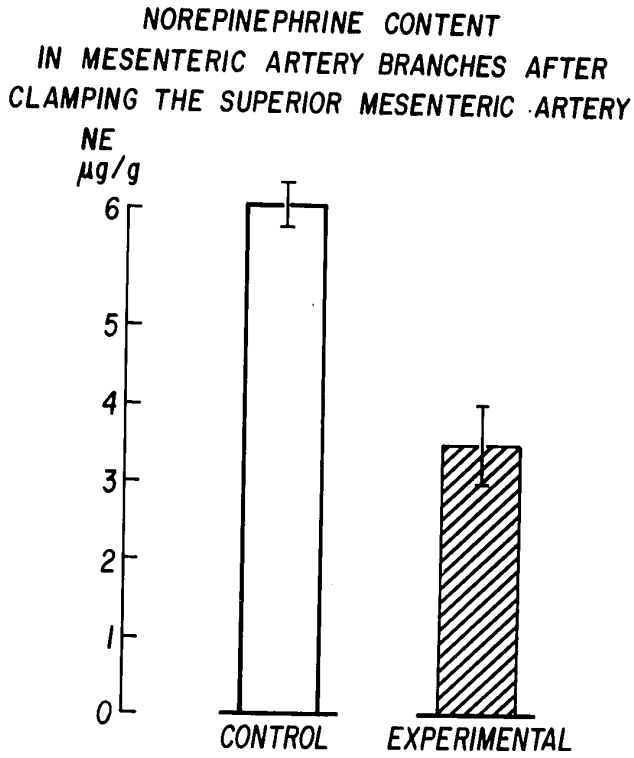



FIGURE 29: *Norepinephrine content (mean ± SE) in mesenteric artery branches (A=5), following clamping of the superior mesenteric artery. The experimental values are significantly ($p < 0.02$) different from those obtained in control dogs.*

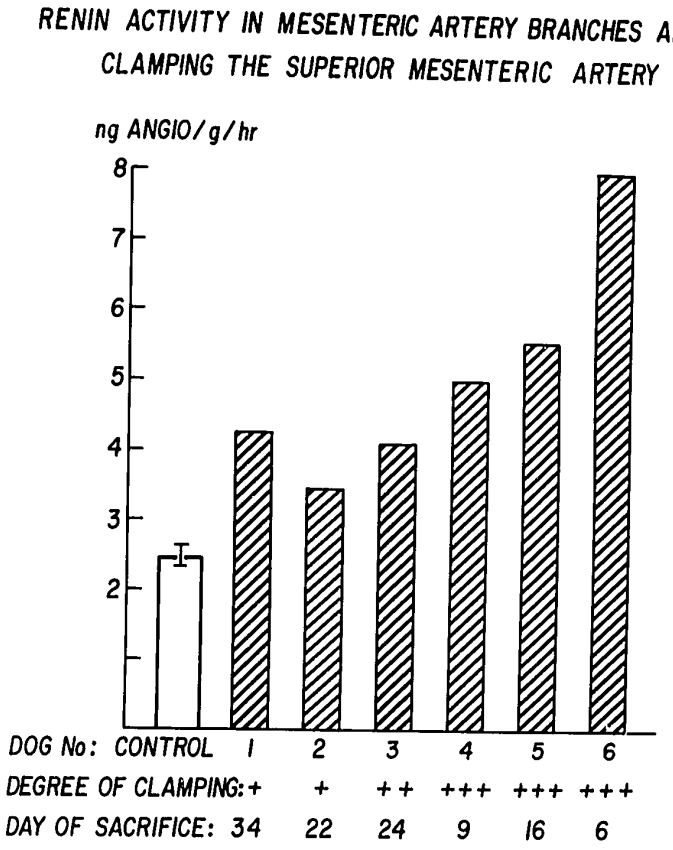



FIGURE 30: *Correlation between the degree of clamping of the superior mesenteric artery and its iso-renin content.*

The blood-flow in the splanchnic vascular bed had not been measured before and after clamping nor at the end of the experiment at the day of sacrifice. But, the efficiency of the clamping can easily be judged in this particular vascular bed where the vessels can be observed directly. Whether local ischemia is the true and only stimulus in this experiment remains to be elucidated. The time relationship may be an important factor also and must be investigated.

Blalock et al. (330) have described that clamping of the mesenteric arteries represents an auxiliary mechanism in the development of renal hypertension. The blood pressure was measured in all dogs before and every three days during the experiment and no significant changes in mean blood pressure were noted after clamping of the mesenteric superior artery.

## 5.5. *Effect of Aldosterone Administration on Plasma Renin Activity, Tissue Iso-Renins and Plasma Electrolytes*

Six male mongrel dogs, weighing between 24.3 and 34.5 kg were kept under "control" conditions (individual metabolic cages, Dr. Ballard's meat, Purina dog chow, tap water ad libitum, constant humidity and temperature) and received 2 mg aldosterone (Ciba) daily for 8 days. The aldosterone was dissolved in 4 ml 0.95 NaCl/mg and was given intramuscularly in two doses of 1 mg.

Plasma renin activity decreased while plasma sodium increased significantly at the end of the experiments. No change in plasma potassium between the control period and the end of the experiment was observed (table 23). Iso-renin was decreased, except in heart muscle, where no significant changes could be observed (table 23, figure 31).

| TISSUE | CONTROL | AFTER ALDOSTERONE TREATMENT |
|---|---|---|
| Mesenteric Artery Branches | 2.49 ±0.3 | 1.16 ±0.22** |
| Carotid Artery | 1.68 ±0.25 | 0.87 ±0.23* |
| Aortic Tissue | 3.46 ±0.48 | 1.43 ±0.10** |
| Brain (Caudate Nucleus) | 19.57 ±2.97 | 10.92 ±1.2** |
| Brain (Frontal Cortex) | 21.89 ±1.42 | 14.28 ±1.3** |
| Heart Muscle | 33.04 ±6.51 | 32.15 ±5.12† |
| Plasma | 0.85 ±0.2 | 0.26 ±0.1* |
| Plasma Sodium | 144.2 ±1.8 | 150.4 ±0.8* |
| Plasma Potassium | 4.5 ±0.1 | 4.5 ±0.1† |

TABLE 23: *Renin activity (ng angiotensin / g tissue (or ml) / 1 hour incubation) and plasma electrolytes (mEq / litre) in dogs (n=6) treated with aldosterone (2 mg / day) for eight days and in untreated controls (n=10). Values are mean ± SE of the mean.*

* = $p < 0.05$, ** = $p < 0.01$, † = no significant difference as compared to control dogs.

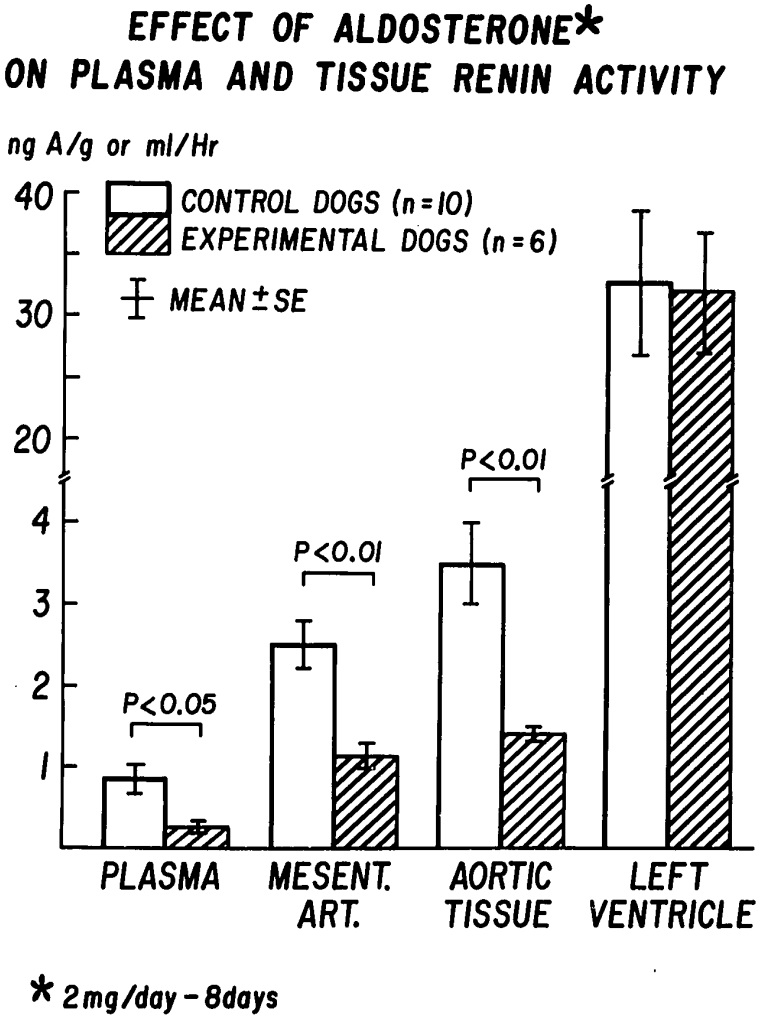



FIGURE 31: *Renin activity in plasma and cardiovascular tissue after aldosterone treatment.*

## 5.6. *Effect of Progesterone Administration on Plasma Renin Activity, Tissue Iso-Renins and Plasma Electrolytes*

The effects of progesterone were studied because of its aldosterone antagonism (331). Six male, mongrel dogs, weighing an average of 23.8 kg received progesterone (Lutocycline$^{R}$, (Ciba), 25 mg per day intramuscularly) over a period of 4 weeks. Results are shown in table 24 and figure 32. There was a slight but significant ($p < 0.05$) increase in body weight at the end of the experiment. Plasma sodium increased and plasma renin activity decreased 4 weeks after progesterone administration. There was no significant change in plasma potassium concentration nor in the arterial blood pressure. Renin substrate concentrations in the plasma increased significantly ($p < 0.05$).

Iso-renin in arterial tissue was decreased significantly in mesenteric artery branches and unchanged in carotid artery and aortic tissue. In contrast, brain tissue iso-renin was stimulated after progesterone treatment, renin activity in the caudate nucleus and in the frontal cortex being significantly higher as compared to control dogs.

It can be concluded from these experiments, that tissue iso-renin is not always stimulated uniformly in one direction: treatment with aldosterone suppressed renin activity in plasma and all other tissues but not in heart muscle. Treatment with progesterone had the opposite effect on brain iso-renin (figure 33) and identical effects on arterial iso-renin.

| TISSUE | CONTROL DOGS | AFTER PROGESTERONE TREATMENT |
|---|---|---|
| Mesenteric Artery Branches | 2.49 ±0.3 | 1.21 ±0.29* |
| Carotid Artery | 1.68 ±0.25 | 0.92 ±0.31† |
| Aortic Tissue | 3.46 ±0.48 | 3.35 ±0.66† |
| Brain (Caudate Nucleus) | 19.57 ±2.97 | 36.9 ±1.9** |
| Brain (Frontal Cortex) | 21.89 ±1.42 | 37.6 ±3.7* |
| Heart Muscle | 33.04 ±6.51 | 11.21 ±2.28*** |
| Plasma Renin Activity | 0.52 ±0.08 | 0.31 ±0.13* |
| Plasma Sodium | 140.7 ±1.2 | 144.9 ±1.6* |
| Plasma Potassium | 4.5 ±0.1 | 4.7 ±1.1† |

TABLE 24: *Renin activity (ng angiotensin / g tissue (or ml) / 1 hour incubation) and plasma electrolytes (mEq / litre) in dogs (n=6) treated with progesterone (25 mg i.m. / day) for four weeks and in untreated controls (n=10). Values are mean ± SE of the mean.*

* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, † = no significant difference as compared to control dogs.

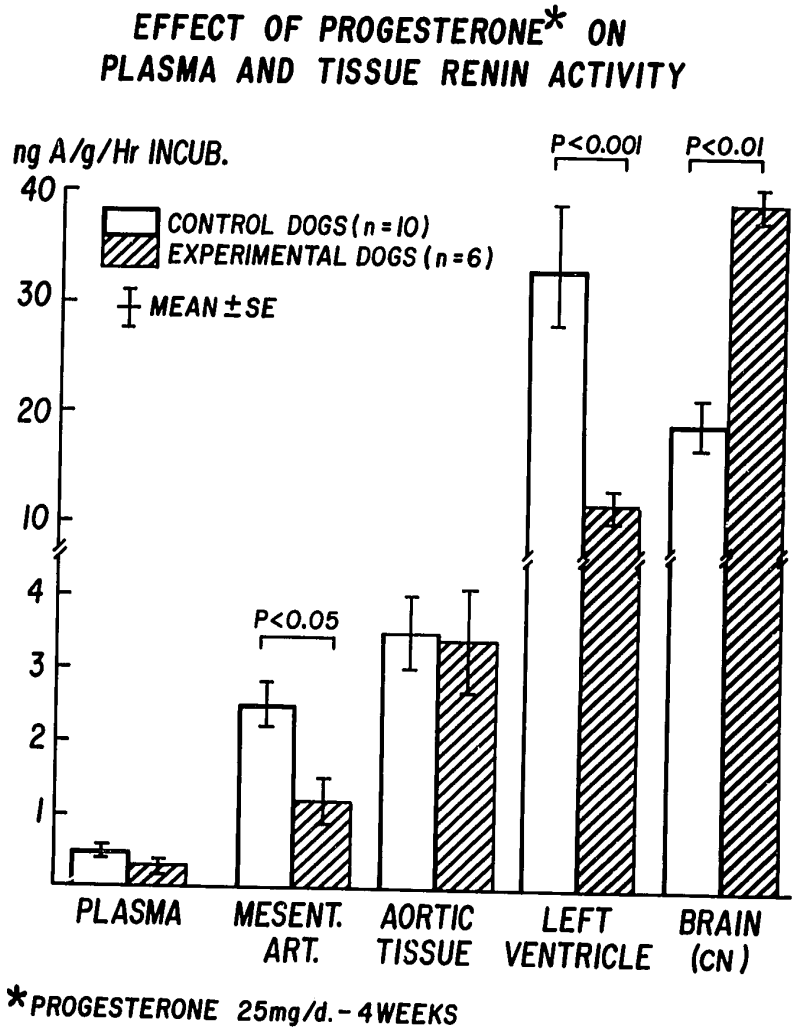



FIGURE 32: *Renin activity in plasma, cardiovascular tissue and brain after progesterone treatment.*

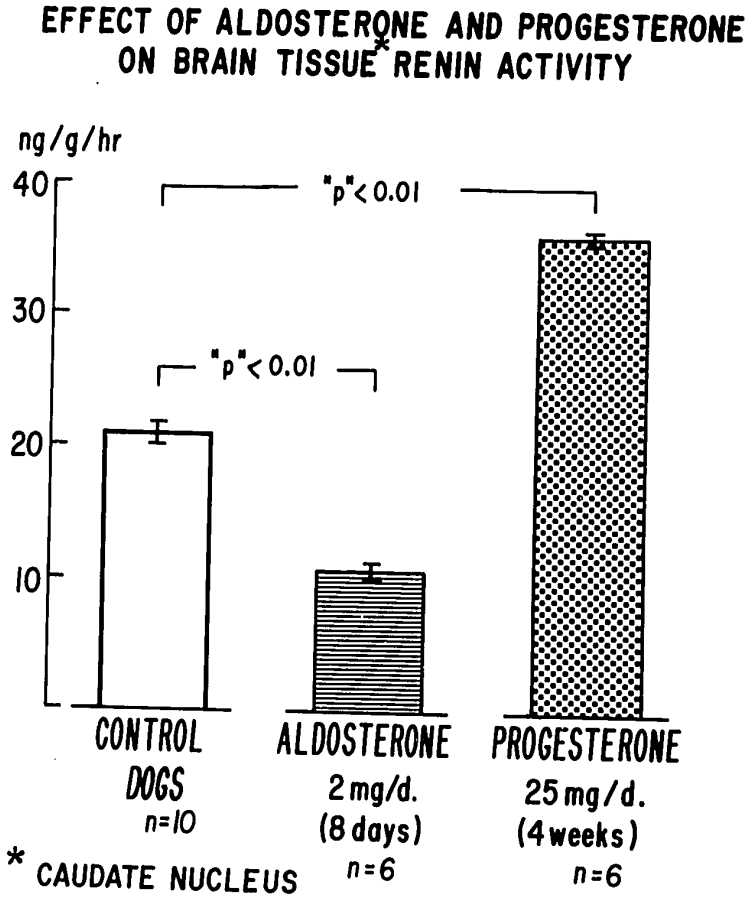



FIGURE 33: *Mean changes in iso-renin of brain tissue (caudate nucleus) after aldosterone and progesterone administration.*

## 5.7. *Effect of Potassium Deficiency on Plasma Renin Activity, Tissue Iso-Renins and Plasma Electrolytes*

Potassium is mainly an intracellular ion. The following experiment was undertaken to see which effects potassium deficiency had on tissue iso-renin. Twelve male mongrel dogs were fed a commercial sodium and potassium deficient diet* which was supplemented for the control group (6 dogs) with sodium chloride and potassium chloride and for the potassium-deficient group with sodium chloride only. The salt supplement was calculated to give a total daily intake of 60 mEq NaCl (= 3.48 g NaCl) and 40 mEq KCl (= 2.96 g KCl) per dog in 300 g of diet. These salt intakes correspond to those reported in the literature for normal intake (332-337).

The weight of the dogs ranged between 15 to 24 kg. The mean weight was equal in both groups and did not change significantly during the experiment. Results are shown in table 25. There was no significant increase in plasma renin activity after 18 days of severe potassium deficiency; renin activity in renal tissue was decreased. Renin activity in the medulla oblongata decreased significantly while renin activity in the thalamic region of the brain increased. The general tendency in all other regions of the brain in which renin has been measured showed increasing renin activity also, though these changes were not always statistically significant. There were no

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* National Biochemicals, Cleveland, (NBC), Cleveland, Ohio.

| TISSUE | CONTROL DOGS | AFTER POTASSIUM DEFICIENCY |
|---|---|---|
| Mesenteric Artery | 3.4 ±0.6 | 3.8 ±0.07† |
| Aorta | 4.16 ±0.59 | 3.5 ±0.67† |
| Adrenal Gland | 408.5 ±50.2 | 428.8 ±66.9† |
| Brain | | |
| Frontal Cortex | 25.53 ±2.9 | 28.39 ±3.6† |
| Caudate Nucleus | 14.9 ±0.74 | 19.81 ±2.1† |
| Thalamus | 15.69 ±1.2 | 22.4 ±2.6* |
| Cerebellum | 26.5 ±2.0 | 35.7 ±6.2† |
| Medulla Oblongata | 26.62 ±4.65 | 12.23 ±145** |
| Pons | 27.1 ±2.2 | 26.2 ±2.8† |
| Kidney | 92,300 ±20.7 | 36,900 ±12.5* |
| Plasma Renin | 0.29 ±0.09 | 0.43 ±0.17† |
| Plasma Potassium | 4.4 ±0.2 | 3.45 ±0.3** |
| Plasma Sodium | 146.93 ±2.6 | 134.15 ±7.9† |

TABLE 25: *Renin activity (ng angiotensin / g tissue (or ml) / 1 hour incubation) and plasma electrolytes (mEq / litre) in dogs (n=6) kept for 18 days on a potassium deficient diet, and in control dogs (n=6). Values are mean ± SE of the mean.*

* = $p < 0.05$, ** = $p < 0.02$, † = no significant change as compared to control group.

statistically significant changes in arterial tissue iso-renin and in adrenal gland iso-renin.

The parallel changes in iso-renin of the medulla oblongata and the kidney following potassium-deficiency are of particular interest in view of the recent findings, that stimulation of the medulla oblongata by electrodes can provoke renin release from the kidney (338, 339). This finding adds support for a direct influence of the central nervous system on renin release from the kidney via the renal nerves, since the stimulating effect could be blocked by renal denervation or by administration of a β-adrenergic blocking agent (propanolol) together with an α-receptor blocking agent (phenoxybenzamine) (338, 339).

Local iso-renin in the medulla oblongata, giving rise to the formation of angiotensin which in turn influences the metabolism of neurotransmitter substances (65-72), could thus influence renal renin secretion. Factors influencing this system and feed-back mechanisms of kidney and plasma renin on brain iso-renin remain to be investigated. The changes in plasma and kidney renin activity on the one hand and the changes in extrarenal tissue iso-renin on the other were not parallel in these potassium-deficiency experiments. While renin activity in some brain regions was stimulated, others showed a decrease or were unchanged. Kidney and different tissues thus did not react uniformly to the same stimulus. This further supports the hypothesis of different and independent renin angiotensin systems.

## 5.8. *Renin Activity in Other Extrarenal Tissues and Renin Activity in Species Other than Dogs.*

We have given priority to studies on the brain renin-angiotensin system, because we considered it a suitable, and from the methodological point of view, an easy model for the study of extrarenal renin-angiotensin systems. Apart from brain and arteries, renin synthesis and local angiotensin formation may be possible also in other tissues. Iso-renin in adrenal glands 12 days after nephrectomy was still higher than in most other organs as shown in figure 34. However, iso-renin in the adrenal gland tissue decreased ($p < 0.001$) from 446 ng angiotensin / g / 1 hour incubation to 110 ng angiotensin / g / 1 hour incubation 3 to 5 days after total nephrectomy and peritoneal dialysis and remains constant at this level even at longer periods after nephrectomy (figure 35).

These results may suggest that both absorption mechanisms and local synthesis do occur in this tissue (especially since there is no barrier to prevent plasma renin from entering the tissue). After nephrectomy the absorbed plasma renin may disappear from this tissue. The persisting levels of iso-renin, following longer periods after nephrectomy, may be due to locally synthesized iso-renin.

Renin from adrenal glands of nephrectomized dogs was purified in the same way as described for brain and arterial iso-renin in chapter 3.6.

Comparison of this enzyme with the tissue iso-renins from brain and aortic tissue revealed that the adrenal gland iso-renin had striking

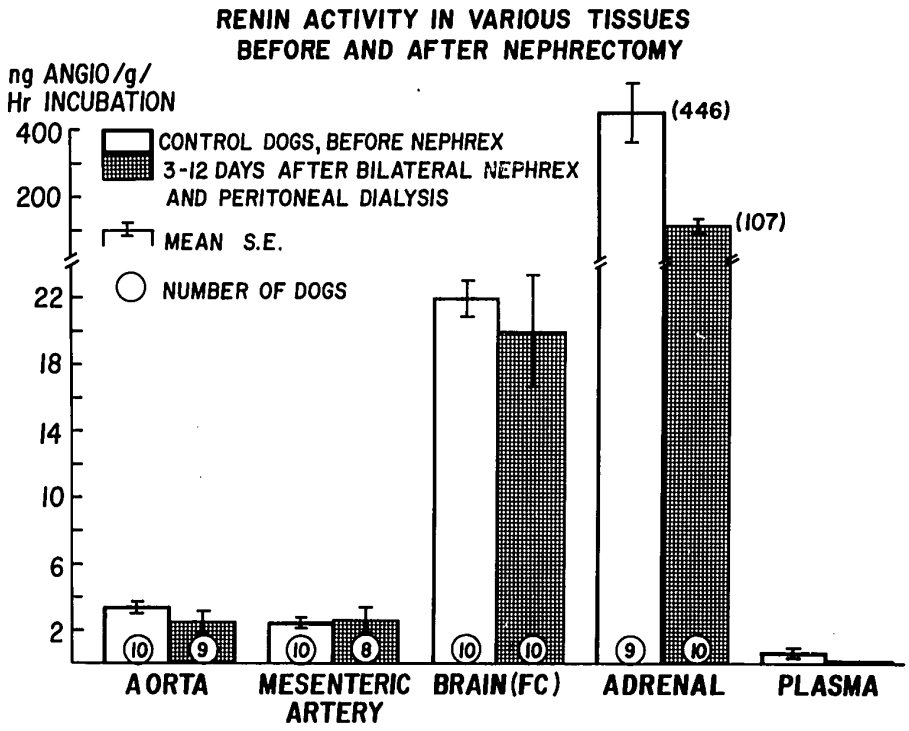


FIGURE 34: *Iso-renin in various tissues and plasma renin activity in dogs before and 3-12 days after nephrectomy. The decrease in adrenal gland and plasma after nephrectomy was significant ($p < 0.001$).*

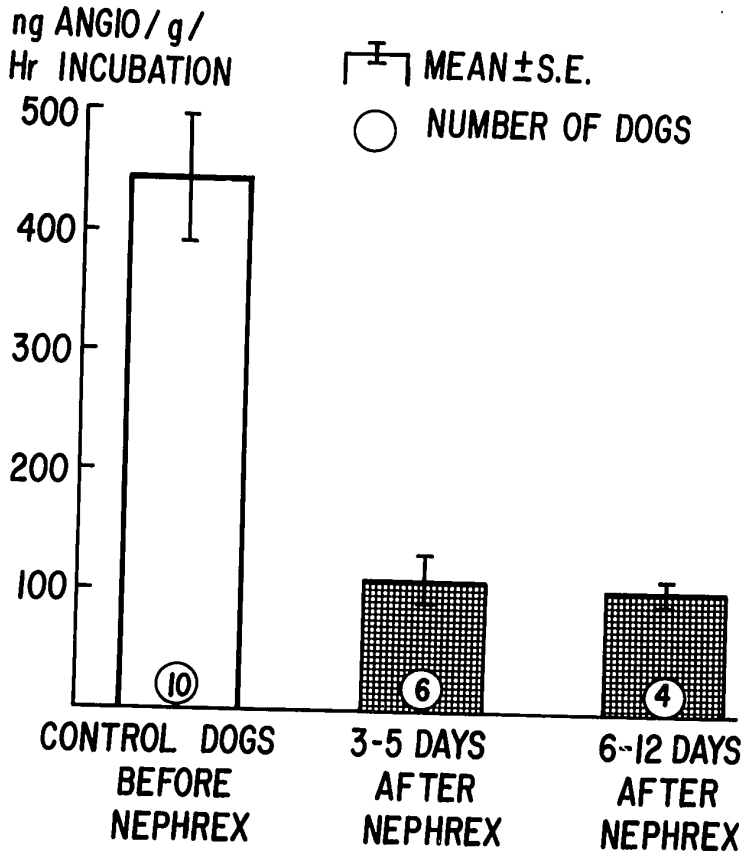


FIGURE 35: *Iso-renin in tissue of the adrenal glands (including medulla and cortex) before and various periods after bilateral nephrectomy. The decrease after nephrectomy was significant ($p < 0.001$).*

similarities with respect to their enzyme substrate kinetics and that adrenal gland iso-renin reacted with homologous, heterologous and synthetic tetradecapeptide substrate in the same way (figure 36, figure 37). Iso-renin was present in both, adrenal cortex and medulla in approximately equal concentrations. The possibility of a participation of local iso-renin and angiotensin in the secretion of aldosterone and catecholamines is of great interest. Further investigation along these lines is strongly indicated. Iso-renin was also measured in lung, liver, spleen and skeletal muscle. (see chapter 5.1., table 11).

The presence of renin in the brain of rats and dogs has been described simultaneously by Fischer-Ferraro et al. (53) in Buenos-Aires. We equally found iso-renin in rat brain and have also established its presence in the brain of sheep. Measurements of iso-renin in human brain biopsy material obtained during neurosurgery*, are shown in table 26.

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* I wish to thank Dr. Alain Godon, Hôtel-Dieu Hospital, Montreal, and Dr. Philip L. Gildenberg, Cleveland Clinic, Cleveland, for their kind cooperation.

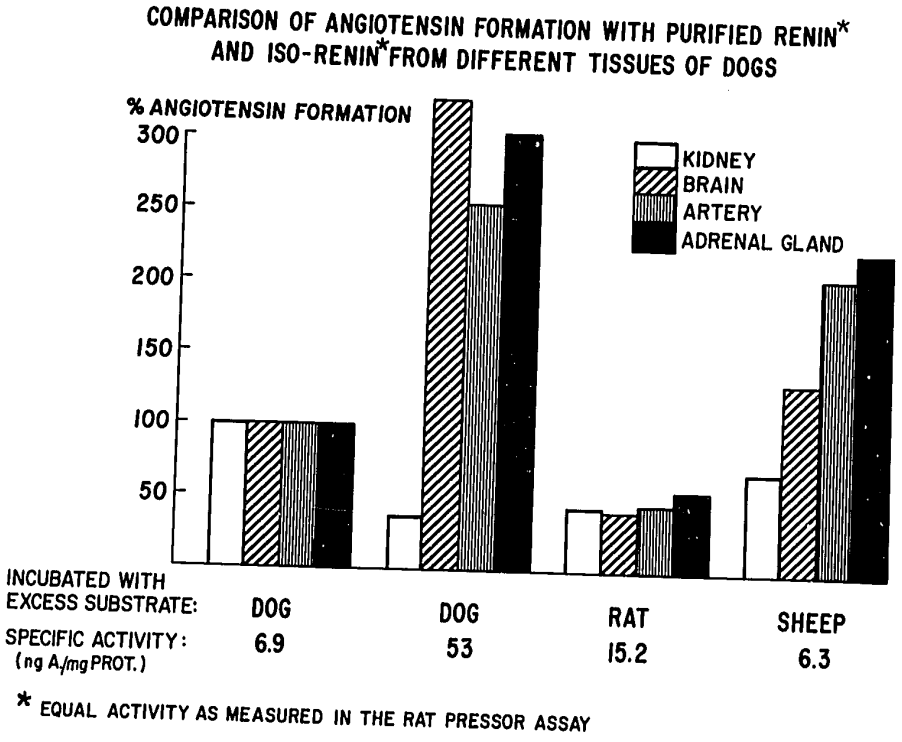


FIGURE 36: *Comparison of angiotensin formation between purified renins from kidney, brain, artery and adrenal gland with various substrates. Angiotensin formation with "standard" homologous plasma substrate was arbitrarily set at 100% (n=4).*

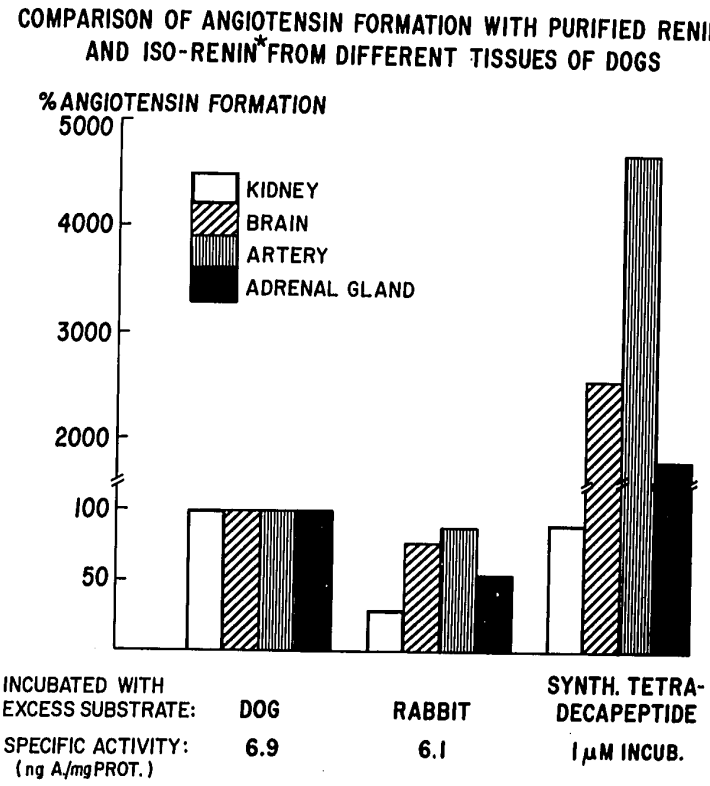


FIGURE 37: *Comparison of angiotensin formation between purified renins from kidney, brain, artery and adrenal gland with various substrates. Angiotensin formation with "standard" homologous plasma substrate was arbitrarily set at 100% (n=4).*

| NO. | ORIGIN OF BRAIN TISSUE | SUBSTRATE | ng ANGIOTENSIN / g / 1 HOUR INCUBATION |
|---|---|---|---|
| 1 | Temporal Lobe | Sheep | 25.7 |
| 2 | Frontal Cortex | Sheep | 7.46 |
| 3 | Cortex | Sheep | 20.42 |
| 4 | Hypophysis (chromophobe adenoma) | Sheep | 57.1 |
| 5 | Glioblastoma | Sheep | 37.88 |
| 6 | Glioblastoma | Sheep | 51.4 |
| 7 | Glioblastoma | a) Sheep<br>b) rat | 26.12<br>3.37 |
| 8 | Glioblastoma | Sheep | 57.87 |
| 9 | Temporal Lobe | Sheep | 44.8 |
| 10 | Meningioma | a) Sheep<br>b) Human<br>c) Synthetic Tetra-decapeptide | 15.25<br>4.80<br>339.76 |

TABLE 26: *Measurement of renin activity in human brain biopsy material.*

# 6. GENERAL DISCUSSION

Renin does not penetrate the blood-brain-barrier as shown in chapter 4.4. This is not surprising, since renin has a molecular weight of approximately 40,000 and it has been shown previously by Brightman (299) that other enzymes of comparable size do not penetrate from the blood into the brain. It could therefore be expected, that plasma renin does not interfere to any significant degree in the measurement of brain tissue iso-renin. Plasma renin, which could be absorbed on the walls of brain arteries and capillaries without penetrating into the brain tissue, does not influence measurably brain iso-renin, since parallel measurements in saline-washed or unwashed brains showed that there was no difference in levels of brain tissue iso-renin.

In our experiments in nephrectomized dogs, the addition of plasma renin activity from circulating blood to brain iso-renin is excluded, since plasma renin activity was undetectable in these dogs. It is most unlikely that the persistence of iso-renin in extra-renal tissues at control levels 12 days after nephrectomy would be due to a slow degradation of renin in these tissues, the degradation of renin in plasma being a matter of minutes (chapter 2.2.).

Furthermore, the initial drop of brain iso-renin 3-5 days after nephrectomy, which was followed by a subsequent rise in brain iso-renin, after a longer postnephrectomy period, strongly suggests local renin synthesis (chapter 5.1.).

The independence of renin synthesis in the brain from the kidney is also supported by the finding that brain iso-renin is low in puppies while renin activity in kidney and plasma is much higher than in normal control dogs. This dissociation of brain iso-renin on one hand and the kidney-plasma renin-angiotensin system on the other hand is fully compatible with the idea that renin synthesis is regulated independently in these two organs.

The physico-chemical characterization and comparison of brain iso-renin with kidney renin gave further support to the independence of the two enzymatic systems: brain iso-renin and kidney renin differ in important properties such as pH-optimum for angiotensin formation, different affinity to various substrates, different affinity to renin antibodies and different migration speed on polyacrylamid gel electrophoresis. Further purification and characterization of purer enzymes is necessary, however, to show whether the enzymes themselves differ or whether tissue factors alter their characteristics in the described way (4.3.).

The purification of renin is a formidable task, and we have contented ourselves in this work with rather crude preparations which had the advantage of being stable over a long period of time, but were "purified" enough to allow some methodological work and preliminary characterization. Being aware of the instability of purer renin preparations and also aware of the fact that apparently "gentle" procedures might entail alterations of the enzyme and change its characteristics, we did not attempt further purification and compared

only preparations, which were obtained through similar procedures.

Ultracentrifugation studies, carried out in parallel with kidney and brain tissue showed a similar distribution pattern of renin activity in both organs. The subcellular distribution does not allow us to be certain, however, about the site of synthesis and the mode of action of the enzyme. The storage of brain iso-renin and kidney renin in cellular granules of identical density, and the absence of unspecific absorption on cell membranes, together with several other criteria (nephrectomy, angiotensin extracted from brain) further support, nevertheless, the concept of local iso-renin synthesis in brain tissue.

These studies revealed, furthermore, that the enzyme is not confined to connective tissue, glia and vasculature in the brain but is present in neural cells and in synaptosomes. We concluded from these experiments that brain iso-renin is synthesized locally in the brain tissue itself.

It is a well accepted fact in molecular biology and genetic science that all cells of an organism contain an identical number of deoxy-ribonucleic acid molecules (DNA) such that the juxtaglomerular cells of the kidney, the tubular cells of the kidney, any cell of the adrenal gland, arterial wall, heart, brain, etc... are identical with respect to their DNA content in a given species. DNA in turn is responsible for the control of protein and enzyme synthesis: if a specific code for a specific enzyme is present in the DNA pattern of one cell of an

organism, this code is present in all cells of this organism. In other words: if an enzyme can be synthesized in one cell, it can, theoretically, be synthesized in all other cells of this organism.

The presence of a specific DNA code in the nucleus of the cell, however, does not mean that this site of the DNA molecule is biologically active. The cells rule over a number of known and probably even more unknown mechanisms which control the activity of DNA. A large number of genes are not active at specific times or never active in certain cells at all, while others are highly activated. This is the genetic base of cell differentiation and specialization.

It follows from the above that if renin can be synthesized in the juxtaglomerular cells of the dog kidney it can, theoretically, be synthesized also in other organs. Interesting in this respect are the findings of Granger et al. (327) that no typical juxtaglomerular cells can be seen microscopically in the kidney of puppies. The activity of the renal renin-angiotensin system in young dogs is nevertheless high as reflected by increased renin activity in the kidney and plasma (chapter 5.3.). It thus appears that fully differentiated juxtaglomerular cells are not necessary for renin synthesis. Renin synthesis in other than the juxtaglomerular cells of the kidney, namely in smooth muscle cells of the renal arterioles, has been found recently in cases of vascular tumors, described histologically as haemangiopericytoma, which were accompanied by high plasma renin activity and hypertension ("primary reninism") (340-343).

The omnipotency of cells towards renin synthesis was supported by the work of Symonds et al. (41), who showed that renin synthesis can occur in uterine muscle cell cultures, which were kept over several generations and by the method of Eskildsen (130), who found renin synthesis in uterine cells, transplanted into the rabbit eye.

Though teleological thinking is risky for the explanation of experimental results, it has proven to be intellectually stimulating: given the high iso-renin content in extrarenal tissues and given the high gradients of renin from low levels in the plasma to high levels in tissue (which, if due to absorption from circulating blood, would require energy consuming active mechanisms) it would be easier to conceive that the cells synthesize the enzyme locally (since this possibility is already built into the cell by its DNA code) rather than develop active uptake mechanisms. Also: even if the release of antidiuretic hormone (73-76) and central control of water uptake (77-87) may partly be controlled by the renal renin system and the circulating angiotensin, it would hardly be conceivable, that the kidney equally controls the synthesis and release of neurotransmitter substances (65-72) and protein synthesis in several organs (88-91). It would be more appropriate to think that the local tissue renin-angiotensin system exerts some of these metabolic activities.

In order to investigate whether the enzymatic system in the brain is active and whether angiotensin can be produced locally, we attempted to extract angiotensin from brain tissue of nephrectomized dogs with

undetectable plasma renin and angiotensin levels. While there is no doubt that the blood-brain-barrier is closed for renin, the latter being undetectable in the cerebrospinal fluid under various physiopathological conditions, this question is still unsettled for angiotensin. Few authors have attacked this problem experimentally. Volicer et al. (307) injected $^{14}C$ labeled angiotensin intravenously in mice and extracted the peptide from brain tissue. Autoradiographic studies, and the larger distribution volume of angiotensin compared to inulin space, led them to the conclusion that angiotensin penetrates the blood-brain-barrier. To the best of our knowledge this is the only reference in favor of the permeability of angiotensin through the blood-brain-barrier. Since the minimal dose of these authors was 1400 ng angiotensin per mouse of 20 g body weight in a single i.v. injection and also, since following administration of their lowest dose, the activity found in the brain "was diminished more than could be explained by the decrease of the dose", it can be assumed that the high doses of angiotensin may have altered the permeability of the blood-brain-barrier in these experiments. Though Volicer et al. (307) have not monitored blood pressure in their experiments, doses of 1000 to 2000 ng angiotensin injected into 20 g mice would rise blood pressure levels to excessively high readings. It has been shown that increase in blood pressure increases the permeability of the blood-brain-barrier (302, 303).

Finkielman and his group in Buenos Aires (personal communication) have found angiotensin-like substances in the cerebrospinal fluid of human. Their results have been obtained by biological assay. The levels of angiotensin in cerebrospinal fluid of normotensive patients was 60 pg/ml, which is above normal plasma angiotensin levels. These workers found increasing angiotensin levels in cerebrospinal fluid with increasing systolic blood pressure. There was no correlation, however, between the angiotensin levels in plasma and cerebrospinal fluid. This led Finkielman et al. to the conclusion that angiotensin in the cerebrospinal fluid originated from brain tissue and not from plasma.

In our experiments angiotensin was extracted from brain tissue of dogs while interference of plasma angiotensin was excluded, these dogs being nephrectomized and plasma angiotensin being undetectable.

We have also shown that renin substrate is present in brain tissue and therefore concluded that angiotensin can be formed locally in the brain. The work of Fitzsimons et al. (81) is fully compatible with our results of an active renin-angiotensin system in the brain. Injection directly into the brain tissue of renin alone and of renin substrate alone, gives rise to the same drinking response as does the injection of angiotensin. The injected renin thus appears to act on the local substrate and the injected substrate is hydrolyzed by the local renin, both reactions giving rise to local angiotensin and eliciting the typical drinking response.

Our concept of local formation of angiotensin in brain tissue is supported also by the work of Fischer-Ferraro et al. (53) who extracted angiotensin from this organ. The possible interrelationship of the brain renin-angiotensin system and kidney-plasma renin-angiotensin system remains to be investigated. The permeability of the blood-brain-barrier for angiotensin is of the utmost importance for this point and changes of the permeability under various pathophysiological conditions remain to be studied.

It is worthwhile mentioning that the brain arteries (pool of basilar artery, middle cerebral artery and anterior cerebral artery) and the choroid plexus, which represent part of the anatomical basis of the blood-brain-barrier also contain high quantities of renin (preliminary unpublished observation). One may be led to the speculation that this local renin would be directly involved in the permeability of the blood-brain-barrier since it has been shown that angiotensin has marked effects on the permeability of ions and water through membranes (92-98) and it also affects the size of intercellular spaces ("door opening effect") as shown first by Constantinides et al. (344) and as confirmed by Robertson et al. (88).

Local deficiency in the blood-brain-barrier is another possibility of feedback mechanisms between the brain and kidney renin-angiotensin system. The area postrema, situated in the caudal medulla and composed of paired mounds of loose vascular tissue, that bulge into the lumen of the fourth ventricle, is such a region, where angiotensin may penetrate freely (288). This region interestingly appears to be

directly involved in hypertension mediated by the central nervous system (288, 290-294). Other areas where the blood-brain-barrier is absent are the tuber cinerum, the pineal body and the neurohypophysis (288), the latter being of particular interest in view of the stimulating effect of angiotensin on the release of antidiuretic hormone (73-76).

It has to be pointed out that all possible interrelations of the kidney and brain renin-angiotensin systems are still in the realm of speculation; no experimental work has yet been done in this subject.

Another interesting aspect of possible interaction between the brain renin-angiotensin system and the kidney renin-angiotensin system is suggested by the finding of Passo et al. (338, 339) who found that adrenergic stimulation of the medulla oblongata stimulates renin secretion in the kidney. Since angiotensin influences the metabolism of neurotransmitter substances (65-72) and since renin is present in this brain region, it may well be that local angiotensin in the medulla oblongata has an influence on the central regulation of renin secretion in the kidney.

The metabolic effect of angiotensin on neurotransmitter substances is of particular interest (65-72). Angiotensin has been shown to enhance the response elicited by post-ganglionic sympathetic nerve stimulation (68). This may be due to an increased norepinephrine release (345-348). Other authors have presented evidence that the increase in norepinephrine may be due to prevention of re-uptake of this neurotransmitter by angiotensin (67, 349).

Hughes and Roth (68) have studied both, uptake and release of norepinephrine and showed recently that the potentiating effect of angiotensin may be explained by a facilitation of norepinephrine release during sympathetic nerve stimulation. These authors attribute this effect to a "modulating" action of angiotensin on membrane transport processes.

It has been reported by Boadle et al. (65) that angiotensin stimulates also the synthesis of norepinephrine in various tissues. Angiotensin can stimulate the output of acetylcholine from the cat cerebral cortex, possibly through changes in the release mechanism as shown by Elie and Panisset (70). Angiotensin has also been shown to reduce the amino barbital sleeping time in rats (350), to produce marked electroencephalographic arousal in cats (351), to stimulate acetylcholine output from peripheral nerve endings (71, 72) and to increase the total acetylcholine brain content in rabbits and mice (352).

With this background of work being done on the effect of angiotensin on neurotransmitter substances, it is exciting and challenging to investigate the role of the brain renin-angiotensin system on the cerebral metabolism of these substances and the vast physiological implications of local angiotensin as related to these functions.

The angiotensin-induced potentiation of electrical nerve stimulation does not appear to be due to an inhibition of metabolizing enzymes, since Hughes et al. (68) reported no observable changes in the metabolic pattern with or without angiotensin. The increased

release of norepinephrine may be due, however, to a modification in the ionic balance in the sympathetic nerve terminals, since angiotensin has been shown to have marked effects on cellular electrolyte and water metabolism (92-98) a hypothesis also favored by Hughes and Roth (68).

Angiotensin in physiological concentrations stimulates bulk transport of sodium and water across the small intestine (96, 97) and across the colon (98) of rats. Barraclough et al. (353) concluded from their experiments that the antidiuretic effect following small infusions of angiotensin in vivo in the rat is due to a direct effect of the peptide on tubular transport mechanisms. Munday et al. (95) have recently presented evidence, that angiotensin increases transtubular sodium and fluid transport in kidney slices by stimulating a potassium independent, ouabain insensitive sodium pump.

The effect of angiotensin to increase intracellular sodium content in arterial tissue has been shown in vivo by Villamil et al. (92), Friedman et al. (354) and Jamieson et al. (355). Friedman et al. (356) have also shown that angiotensin stimulates sodium uptake in "in vitro" incubations of arteries.

Andersson et al. (84-87) have found that the dipsogenic effect of hypertonic sodium chloride infusion into the lateral ventricle of the brain can be potentiated by angiotensin. These authors suggested that angiotensin facilitates the transport of sodium into brain cells

and that intracellular sodium concentration determines the activity of these cells.

In view of the effect of angiotensin on cellular water and electrolyte metabolism as discussed above, it is most tempting to speculate on the physiological implications of local angiotensin in brain tissue. We have presented preliminary evidence in this thesis (chapter 5.3.) that there exists a highly significant inverse relationship between tissue water, sodium and potassium on one hand and brain iso-renin on the other hand, brain iso-renin being low in the highly hydrated tissue of puppies and being significantly higher in adult dogs with "dehydrated" cells (relative to puppies). It would thus appear, in these conditions, that the brain renin-angiotensin system responds to similar stimuli as the kidney renin-angiotensin system: stimulation by dehydration, sodium and potassium deficiency and suppression by hydration and repletion with these ions. We have also found a parallelism between the increase of brain iso-renin with brain development and the increase of norepinephrine and dopamine (chapter 5.3.). A significant increase in catecholamine content in rat brain during post-natal development has been described also by Loizou et al. (329). Fischer-Ferraro et al. (53) have found that brain angiotensin levels in various regions corresponded to their norepinephrine content, and there is evidence that the dipsogenic effect of angiotensin depends on the integrity of the catecholaminergic system (357). These preliminary results and evidence from the literature support our view that the brain tissue renin-angiotensin system

might be important in cellular electrolyte and water metabolism and that some of the metabolic functions of angiotensin might be explained via this basic mechanism.

Extreme caution is necessary, nevertheless, when it comes to define accurately the possible physiological function of extrarenal renin-angiotensin systems in the brain, arterial and adrenal gland tissue. At the present time, we do not possess the methodological tools to assess a complete picture of the functioning, complex enzymatic system in tissue. While renin activity can be measured quantitatively with sufficient accuracy, other parameters like renin substrate(s), converting enzyme(s), differentiation of the effect of angiotensin I and angiotensin II and the affinity of angiotensin receptor sites are either not measured at all or are measured rather qualitatively.

Renin activity in a tissue homogenate may not reflect its activity in vivo. The fact that the adrenal gland contains more renin activity than the brain and the brain more than the arteries does not imply that in vivo angiotensin formation in these tissues is strictly related to that observed under in vitro incubation conditions. Furthermore, it is conceivable that equal amounts of angiotensin may have different effects in different tissues.

Interpretation of the results at this state of knowledge is mainly qualitative. Renin synthesis does occur in extrarenal tissues. Renin substrate is present at the tissue level. Local angiotensin formation takes place in tissue. These statements are true for the brain of dogs and probably also for adrenal gland and other

extrarenal tissues in the dog. We have preliminary evidence that extrarenal renin exists also in brains of man, sheep and rats (chapter 5.8.) and there is evidence in the literature that extrarenal tissues in other species contain also renin (chapter 2.1. 2.).

Our physiological experiments in dogs with aldosterone and progesterone treatment, clamping of the mesenteric artery, severe bleeding and potassium deficiency have shown three important findings:

a) tissue iso-renin can react differently and dissociate from kidney renin in response to the same stimuli, showing the independence from the kidney enzyme.

b) Different tissues react differently to the same stimulus: the tissue renin-angiotensin systems thus are not only independent from the plasma but different tissues may also be regulated independently.

c) Tissue iso-renin content can be altered by changes in tissue electrolyte content.

The possibility of an increased cellular sodium content in arterial tissue in hyperaldosteronism has been formulated in 1956 by Genest et al. (358) and reformulated more recently by Conn et al. (359, 360). Woodbury and Koch (361) found an increase of the ratio of extracellular to intracellular sodium in brain and skeletal muscle after administration of aldosterone to mice. Friedman et al. (362) observed equally an increase in total extracellular sodium. Lim and Webster (363), using intact diaphragms of rats, reported an increase of intracellular sodium under the influence of aldosterone. Llaurado (364)

found a doubling of the ratio of extra- to intracellular sodium, indicating that aldosterone induces an accumulation of sodium into the extracellular space.

Much less work has been done on the effect of progesterone on tissue electrolytes. It may be sufficient to point out in this context that progesterone is considered a peripheral inhibitor of the action of aldosterone, as shown by Laidlaw et al. (331). These two steroids, having profound effects on tissue electrolytes, also alter significantly tissue iso-renin as shown in this thesis (chapter 5.5.-5.6.). Our experiments suggest that the tissue renin-angiotensin system may be involved in the regulation of tissue water and electrolytes. Since the effector agent of this enzymatic system, namely angiotensin, is capable of influencing membrane transport of sodium, potassium and water, the tissue renin-angiotensin system could provide a powerful tool for this fundamental biological mechanism. Angiotensin may not exert this function by its influence on the sodium pump only (95) but also by stimulating the rate of translation of ribonucleic acid, coded for a protein or proteins which are involved in sodium transport, as suggested by Davies et al. (90). The effect of angiotensin on the synthesis of proteins and nucleotides has been demonstrated by Robertson et al. (88), Khairallah et al. (89) and by Trachewsky (91).

The classical kidney renin-angiotensin system is closely linked to hypertensive disease by the triple action of angiotensin: vasoconstriction, aldosterone secretion and its sodium retaining effect. The newly discovered metabolic actions of angiotensin such as stimulation of catecholamine synthesis (65), acetylcholine release (70, 72), release of antidiuretic hormone (73-76) as well as induction of thirst (77-87) were easily integrated in this line of thought.

More recently, central nervous mechanisms in the onset and maintenance of high blood pressure have gained the attention of several research groups.

Bickerton and Buckley (365),using cross-perfusion experiments, were the first to point out the possible role of angiotensin in cardiovascular effects mediated by the central nervous system. Dickinson (295) found that small doses of angiotensin, infused intravenously, had no cardiovascular effect, but the same doses, when infused into the vertebral artery, resulted in a significant rise of blood pressure. He suggested the contraction of small brain arterioles to be the cause of this effect. This suggestion has now been made improbable by recent evidence that the response depends on angiotensin sensitive neural structures in the portions of the brain stem supplied by the vertebral arteries (288-294, 296).

The hypothalamus and midbrain being excluded by the work of Joy and Lowe (290-292), Joy (366) and Gildenberg (367), the lower medulla and more precisely the area postrema, is now considered by most authors (see 288 for review) to be the site, where peripheral

angiotensin acts on the central nervous system to rise the blood pressure.

The blood-brain-barrier is absent in the area postrema, which consists of a loose network of neuroglia, through which runs a plexus of arterioles and capillaries. The histological appearance of this area has been described as a chemoreceptor zone by Ingram (368).

The mechanism by which the blood pressure is raised, is still a matter of discussion and different mechanisms may apply in different species of animals (289, 294, 295). Ferrario et al. (288), Rosendorf et al. (369), Yu and Dickinson (370), Severs et al. (371) and Ueda et al. (372) conclude that the central effects of angiotensin are mediated primarily by increased sympathetic vasomotor discharge followed by increased peripheral resistance and, to a lesser extent, by withdrawal of parasympathetic discharge. We have shown that iso-renin is present in the area postrema itself and that local angiotensin formation is possible in these structures. It appears valid to conclude, that local angiotensin could exert the same functions as peripheral angiotensin.

Future research will show, whether the local angiotensin formation in metabolically active tissue such as adrenal glands, arterial walls and brain tissue is of physiological significance. The theoretical possibilities of an intrinsic independent renin-angiotensin system in these tissues are most exciting and challenging in view of the

metabolic functions which could be influenced by local angiotensin.

Further work, however, is necessary to obtain a more comprehensive picture of the activity of the tissue renin-angiotensin system. The components of this complex enzymatic system have to be characterized more precisely and specific methods for measurement of the various parameters of the renin-angiotensin system in tissue must be established. A simple and reliable method for the routine extraction and measurement of tissue angiotensin is of the utmost importance.

The event of potent and specific inhibitors of some of the components of the renin-angiotensin system, especially renin and angiotensin antibodies, as well as angiotensin analogues, may provide a powerful tool to learn more about the physiological role of local angiotensin by blocking receptor sites or by inhibiting enzyme activity.

Tissue culture techniques would allow a new approach to the problem of the mechanism by which the tissue renin-angiotensin system influences such a variety of metabolic activities. These techniques might also provide an answer to the question, whether angiotensin is able to influence directly enzymatic activity or whether there is a common basic mechanism, such as changes in cellular electrolyte metabolism, which is at the basis of the diversity of functions of angiotensin.

The finding of an intrinsic, independent extrarenal tissue renin-angiotensin system opens new perspectives as to the regulation of blood pressure and this system may, above this function, have fundamental importance in the physiology of cells.

## 7. CLAIMS TO ORIGINALITY

1) An angiotensin forming enzyme is present in brain tissue of dogs, rats, sheep and human.
2) The blood-brain-barrier is impermeable for renin, and renin is undetectable in the cerebrospinal fluid.
3) The enzyme content of brain tissue is not altered by nephrectomy.
4) Brain iso-renin differs markedly in its physio-chemical properties from kidney renin.
5) Brain iso-renin is present in nerve-cells and synaptosomes and is not confined to non-neural elements of the brain.
6) Renin substrate is present in brain tissue of dogs.
7) Local angiotensin formation occurs in brain tissue. The brain of dogs thus possesses an intrinsic renin-angiotensin system, independent of the classical kidney-plasma renin-angiotensin system.
8) Iso-renin from the adrenal gland and arterial wall has similar characteristics as brain iso-renin.
9) The response of tissue iso-renin under various physio-pathological conditions and to various agents is independent of plasma renin and kidney renin. Iso-renins in different tissues respond differently to the same stimulus.
10) A highly significant, negative correlation between tissue sodium, potassium and water on one hand and brain iso-renin on the other hand is described.

Some of the work, which is described in this thesis, has been published in the following journals:

1. Ganten, D., Hayduk, K., Brecht, H.M., Boucher, R., and Genest, J.: Evidence of renin release or production in splanchnic territory. Nature, 226: 551-552, 1970.

2. Ganten, D., Hayduk, K., Brecht, H.M., Boucher, R., and Genest, J.: Stimulation of renin in mesenteric artery in dogs. Proc. of the Canadian Cardiovascular Society, 23rd Meeting, Ottawa, 15-17 Oct. 1970, p. 9.

3. Ganten, D., Brecht, H.M., Hayduk, K., Constantopoulos, G., Boucher, R., and Genest, J.: Contenu des branches de l'artère mésentérique supérieure en rénine, norépinéphrine et cations après clampage du tronc principal. Proc. Association des Médecins de langue française du Canada, 40e Congrès, 2-5 déc. 1970, Montréal, p. 55.

4. Ganten, D., Boucher, R., Hayduk, K., and Genest, J.: Freisetzung von Renin im Splanchnikusbereich. Internationales Klausurgespräch, Titisee. Med. Welt, 21 (N.F.): 1631-1650, 1970.

5. Ganten, D., Minnich, J., Granger, P., Hayduk, K., Barbeau, A., Boucher, R., and Genest, J.: Presence of an Angiotensin forming enzyme in brain tissue of dogs. Clin. Res., 19 (2): 531, 1971.

6. Ganten, D., Minnich, J.L., Granger, P., Hayduk, K., Brecht, H.M., Barbeau, A., Boucher, R., and Genest, J.: Angiotensin-forming enzyme in brain tissue. Science, 173: 64-65, 1971.

7. Ganten, D., Boucher, R., and Genest, J.: Renin activity in brain tissue of puppies and adult dogs. Brain Research, 33: 557-559, 1971.

8. Ganten, D., Marquez-Julio, A., Granger, P., Hayduk, K., Karsunky, K.P., Boucher, R., and Genest, J.: Renin in dog brain. Amer. J. Physiol., 221 (6): 1733-1737, 1971.

9. Ganten, D., Granger, P., Ganten, U., Boucher, R., and Genest, J.: An intrinsic renin-angiotensin system in the brain. In: Hypertension '72, edited by J. Genest and E. Koiw, Springer-Verlag, Heidelberg-Berlin-New York, 1972, p. 423.

10. Ganten, D., Constantopoulos, G., Kusumoto, M., Ganten, U., and Boucher, R.: Renin, norepinephrine and electrolytes in dog brains of different ages. IV International Congress of Endocrinology, Washington 1972, abstract no. 592, p. 235.

The presence of renin and angiotensin in brain tissue has been described simultaneously also by:

1. Goldstein, D.J., Fischer-Ferraro, C., Nahmod, V.E., and Finkielman, S.: Angiotensin I in renal and extra-renal tissues. Medicina, XXX, (1): 81-83, 1970.

2. Fischer-Ferraro, C., Nahmod, V.E., Goldstein, D.J., and Finkielman, S.: Angiotensin and renin in rat and dog brain. J. Exp. Med., 133 (2): 353-361, 1971.

## 8 SUMMARY

An enzyme, capable of catalyzing the reaction with renin substrate to form angiotensin is described in the brain of dogs, rats, sheep and man. This brain tissue iso-renin differs in important characteristics from renal renin. The enzyme persists at control-levels 12 days after nephrectomy. Renin substrate has been found equally in brain tissue of dogs. Angiotensin was extracted from brain of dogs which had been nephrectomized and in which plasma angiotensin was undetectable. This shows that angiotensin can be formed locally in the brain.

An iso-renin of similar characteristics as brain iso-renin is described and partially characterized in aortic tissue and in adrenal glands. The independence of tissue iso-renin from kidney renin is supported by the fact that the two enzymes react differently to the same physio-pathological stimuli.

## 9. RESUME

Une enzyme, capable de catalyser la formation d'angiotensine au contact de l'angiotensinogène, est décrite dans le cerveau chez le chien, le rat, le mouton et l'homme. L'iso-rénine cérébrale est différente de la rénine rénale. La concentration de l'iso-rénine cérébrale n'est pas modifiée par la néphrectomie (12 jours).

L'angiotensinogène a été trouvé également dans le tissu cérébral du chien. L'angiotensine a été extraite de cerveaux de chiens néphrectomisés dont la concentration d'angiotensine plasmatique était indécelable, démontrant que l'angiotensine peut être formée localement dans le tissu.

Une iso-rénine dans le tissu aortique et surrénalien est décrite et partiellement caractérisée. L'indépendance de l'iso-rénine tissulaire, de la rénine rénale et plasmatique est démontrée par le fait que les deux enzymes réagissent de façon différente sur les mêmes stimuli physio-pathologiques.

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