1970

A Dialyzable Phospholipid Renin Inhibitor

Donna L. Webber

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A Dialyzable Phospholipid Renin Inhibitor

by

Donna L. Webber

Submitted in partial fulfillment of the requirements for the Senior Scholars Program

Colby College

1970
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ACKNOWLEDGEMENTS

The author wishes to thank the Maine Heart Association for financial aid, the Colby College Departments of Chemistry and Biology for the use of their facilities, the Colby College Senior Scholars' Committee for its book funds, Mr. John A. Cioffi for technical assistance, and Dr. George D. Maier for his help during this investigation.
# TABLE OF CONTENTS

## PART I: LITERATURE SURVEY

<table>
<thead>
<tr>
<th>A. EARLY MECHANISTIC HYPOTHESES</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recognition of Renal Hypertension</td>
<td></td>
</tr>
<tr>
<td>Experimental Induction of Renal Hypertension</td>
<td></td>
</tr>
<tr>
<td>The Juxtaglomerular Apparatus</td>
<td></td>
</tr>
<tr>
<td>B. THE RENIN - ANGIOTENSIN SYSTEM</td>
<td>4</td>
</tr>
<tr>
<td>C. ASSAYS FOR RENIN</td>
<td>15</td>
</tr>
<tr>
<td>D. CADMIUM ION AND ZINC ION EFFECT</td>
<td>15</td>
</tr>
<tr>
<td>E. A RENIN INHIBITOR</td>
<td>18</td>
</tr>
</tbody>
</table>

## PART II: EXPERIMENTAL

| A. MATERIALS AND METHODS                      | 22 |
| Cд\(^{2+}\) and Zn\(^{2+}\) - Indirect Activation of Renin |    |
| Extractions of Dialysands for a Renin Inhibitor |    |
| Discrepancy Between Standard Hypertensin II Batches |    |
| Attempted Isolation of a Dialyzable Phospholipid Renin Inhibitor |    |
| Investigation of Pre-slaughter Steer Diets for High Cд\(^{2+}\) or Zn\(^{2+}\) Content |    |
| B. RESULTS AND DISCUSSION                     | 37 |
| Cд\(^{2+}\) and Zn\(^{2+}\) - Indirect Activation of Renin |    |
| Extractions of Dialysands for a Renin Inhibitor |    |
| Discrepancy Between Standard Hypertensin II Batches |    |
| Attempted Isolation of a Dialyzable Phospholipid Renin Inhibitor |    |
| Investigation of Pre-slaughter Steer Diets for High Cд\(^{2+}\) or Zn\(^{2+}\) Content |    |
| C. SUMMARY                                    | 52 |

## TABLE OF REFERENCES

## ACKNOWLEDGEMENTS
PART I: LITERATURE SURVEY
EARLY MECHANISTIC HYPOTHESES

Recognition of Renal Hypertension

Renal hypertension, high blood pressure originating from a damaged regulatory apparatus in the kidney, is a frequently though not necessarily hereditary disease afflicting about three per cent of the population of the United States. In hypertension, small capillaries under the strain of abnormally and consistently high blood pressure are susceptible to breakage and, if not immediately, death from hemorrhage occurs when a circulating blood clot from the repair of earlier hemorrhage blocks tiny capillaries, often in the brain or heart causing oxygen starvation to that organ or fatal hemorrhage. High blood pressure associated with toxemia of pregnancy is another instance of a secondary effect of unsuspected renal hypertension. At this time, renal hypertension correction, surgically possible in five per cent of all cases, is either severely limited by the scarcity of antihypertensive drugs or is impossible. Clinical data collected early in the nineteenth century by BRIGHT, an English physician, reiterated an hypothesis associating kidney disease with hypertension. This hypothesis dates from 200 B.C. Not until the last years of the nineteenth century, however, was the first successful research done on the relationship between kidney disease and hypertension. In 1896 TIGERSTEDT and BERGMANN reported a saline-extractable "pressor substance" from rabbit kidneys which they named "Renin" (from the Latin ren for kidney).

Experimental Induction of Renal Hypertension

The discovery of renin in the kidney suggested to their contemporary scientists that hypertension could be induced in a test animal by a defect in the normal operation of the kidney. The first successful alteration of the
kidney which produced persistent hypertension was the constriction of renal arterial branches, reducing renal arterial circulation. In 1934, experimenting under his hypothesis that renal ischemia is sufficient to cause hypertension through a mechanism involving a renal secretion, GOLDBLATT achieved persistent hypertension in test animals. GOLDBLATT's technique was to constrict partially a renal artery with a silver clamp, leaving renal arterial branches untouched, thereby creating renal ischemia. Subsequent persistent hypertension developed in the test animal. To reverse this experimental hypertension, the clamp was removed, allowing normal blood flow to be restored and to relieve ischemia. GOLDBLATT observed that permanent hypertension is induced by partial constriction of both arteries.

Juxtaglomerular Apparatus

Granular cells in the juxtaglomerular apparatus (JGA) and in the walls of adrenocortical arterioles were discovered by HUTTER in 1925 and have been the subject of successive postulates. In 1939, and again in 1945, GOORMAGHTIGH ascribed an hormone-secreting relationship of these granular juxtaglomerular (JG) cells to hypertension. In 1941 DUNNIEUE emphasized the adrenocortical location of some of these cells and studied the cells for a possible adrenocortical role. GARRER, et al., in 1959 studied the effects of induced hypotension and drugs on the granular JG cells postulating that the JGA was a target organ for adrenocorticotropic hormone (ACTH). Other laboratories were unable to observe ACTH-induced effects. By 1960 the individual granules in the cytoplasm of the JG cells had been shown to contain all kidney renin. Decreases and increases in JG cell granulation and number with increase and decrease of renal arterial pressure, respectively, had been observed. In the following year TOBIAN

suggested that increased granulation represented activation of the renin in the granules, and inhibition of normal pressure controls, by a decrease in blood pressure (contrast GOLDBLATT\textsuperscript{6}). PAGE\textsuperscript{14} had foreseen the importance of renal arterial pressure twenty years earlier. In later experiments TOBIAN\textsuperscript{15} supported his pressure influence postulate by showing that lowering renal arterial blood pressure deviates the rate of renin secretion in the JGA, that a subsequent increase in the arterial blood pressure decreases the rate of renin secretion furthermore, constriction of the arterioles increases the rate of renin secretion. He was unable to find conclusive evidence that a sharp increase in renal arterial pressure lowers the rate of renin secretion. In these later experiments TOBIAN also observed an inverse relationship between the amount of plasma renin and radical, presumably causal changes in blood volume, indicating the possibility of extra-renal control of renin secretion.

To infer GOLDBLATT'S theory of the involvement in elevated blood volume (and therefore in renin secretion) of ischemia from TOBIAN'S, and not GOLDBLATT'S, blood volume change experiments. An inverse relationship between marked sodium-ion (Na\textsuperscript{+}) depletion (and intake) and plasma renin levels similar to the blood volume and plasma renin level relationship was reported in the same paper. This Na\textsuperscript{+} effect is possibly due to an increase in fluid retention with an increase in body Na\textsuperscript{+} level (and \textit{vice versa}) and thus may be understood in terms of the volume effect. However, the JGA may be capable of directly detecting Na\textsuperscript{+} levels.

Two types of cells are found in the JGA: the macula densa cells and the granular JG cells. TOBIAN postulates that macula densa cells contain renin in a precursor form and that the granules in the cytoplasm of the JG cells secrete the active renin when macula densa cells are tall, and high in endogrmous enzymes other than renin (cf. Leyssac\textsuperscript{16}). This integrated function between the macula densa cells and the granular cells is inferred from data showing \textit{ha} retilular fibers...
separating the two types of JGA cells. Whether a case of hypertension is of renal origin can be determined during surgery by JGA biopsies which show depletion and hypogrammarity of the granular cells with renal hypertension.

LEYSSAC reports the intrarenal coupling of reabsorption of the major fraction of sodium chloride (NaCl) and water to JG filtration to be regulated by an intrarenal mechanism. His experiments indicate that the liberation of renin is signaled by a decrease in tubular pressure due to decrease in NaCl concentration. Angiotensin, a pressor peptide released from the circulating renin substrate by the liberated renin adjusts the tubular reabsorptive capacity, thereby restoring normal tubular pressure (and thus causing restoration of normal JG filtration rate).

LEYSSAC hypothesizes that this "glomerulotubular balance" involves a salt-conservative feedback mechanism in which an unknown signal, possibly the release of a hormone, (aldosterone), is alerted by variations in macula densa cell salt concentration, causing secretion of renin, which, through the glomerulotubular balance mechanism, eventually return salt concentration to normal.

* * * * * * * * * * * * * * *

THE RENIN-ANGIOTENSIN SYSTEM

Angiotensin is a key peptide functioning in the renin-angiotensin mechanism of renal blood pressure regulation, a mechanism which has been revealed and increasingly substantiated over the last thirty years. Roughly summarized, the angiotensin-renin system involves the action on the leucine-leucine
(L-Leu-L-Leu) bond in the renin substrate (a glycoprotein) by the enzyme renin to release the decapeptide, angiotensin I (probably an indirect pressor). A chloride-ion - requiring converting enzyme liberates the octapeptide angiotensin II, a powerful pressor, from angiotensinI. The destructive action of angiotensinases on angiotensin in vitro is probably of minor extent.

In 1940 two independent research groups, PAGE and HELMER (U.S.)\textsuperscript{18} and BRAUN-MENENDEZ (Argentina)\textsuperscript{19} arrived at the first hint of the renin-angiotensin mechanism by showing that in a reaction between renin and a renin activator (renin substrate) a crystalline pressor peptide (angiotensin) is released. Renin substrate, a protein in the alpha\textsubscript{2}-globulin fraction of plasma, was described three years later by PLENTL, PAGE and DAVIS.\textsuperscript{20} In 1946 BRAUN-MENENDEZ\textsuperscript{21} attributed in vitro destruction of angiotensin to enzymes in plasma, redblood cells, and other human tissues. These enzymes have since been found in most body tissue and are predominantly of one type, given the name "Angiotensinase A", by KEHRA, BUMPUS, PAGE, and SMEBY.\textsuperscript{22} Angiotensinase A is dialyzable against ethylenediaminetetraacetate (EDTA), Ca\textsuperscript{2+} - requiring, optimally active at physiological pH on all forms of angiotensin. Whether Angiotensinase A is significantly active in vivo is uncertain because in vitro experiments for purposes of assay use concentrations of angiotensin much in excess of concentrations existing in vivo. The remaining angiotensinases differ from Angiotensinase A in their inhibition by diisopropylfluorophosphate (DFP). According to studies by BUMPUS\textsuperscript{23} the action by angiotensinases located in the walls of blood vessels may be physiologically significant after the disappearance of pressor response of remaining plasma angiotensin. Circulating angiotensin II may be nonexistent if, as KEHR\textsuperscript{24} note, the renin and converting enzyme are located near (or inside) the walls of blood vessels.
In his early work on the renin-angiotensin mechanism, SKEGs found in circulating-blood assays from malignant hypertensive patients, comparatively high levels of angiotensin (twenty times the normal) and renin, evidence supporting a role of renin in the blood pressure regulation mechanism of human hypertensives. A 1969 report cited the difficulty in measurement of circulating renin activity that removal of blood samples necessary for standard assay creates such a volume depletion that physiological responses occur, thus increasing amounts of circulating renin. This difficulty may explain excessive circulating renin (and angiotensin) levels found by SKEGs. The report included an improved circulating-renin assay and used this assay to demonstrate an increase in circulating-renin activity in rats resulting from volume depletion and sodium depletion, findings which agree with TOBLAN'S results and hypothesis. In 1954 by simple acid denaturation BAAS, LAMFROM, and GOLDBLATT isolated renin, an enzyme they had described as very thermolabile, in a partially purified form. SKEGS, et al, in the same year purified angiotensin I from horse plasma. In the following year GREEN devised a method for partial purification of hog-plasma renin substrate by ammonium sulfate fractionations preceding and following partial acid denaturation. SKEGS, and ELLIOT and PEART determined the amino acid sequence of angiotensin II and by 1956 SKEGS, et al, were able to discover and moderately purify by fractional ammonium sulfate and isoelectric precipitations from horse plasma the Cl- activated converting enzyme. Study of the reaction mediated by this enzyme (conversion of angiotensin I to angiotensin II) and of the available knowledge of other components of the renin-angiotensin system thus revealed the first integrated mechanism explanation of the renin-angiotensin system. Angiotensin II and not angiotensin I was shown to be a powerful pressor. Angiotensin I, the product of the renin substrate hydrolysis by renin, in the rate-determining step of the mechanism, is not directly vasoconstrictive.
Circulating Blood: Pressure normal

Circulating Blood: Volume increase

KIDNEY:
Reabsorption of sodium-ions
Release of renin from juxtaglomerular cells
Renin activity destroyed by renin inhibitors

Release of Angiotensin I
Circulating Blood: Renin substrate (from the liver)

Release of Angiotensin II
Circulating Blood: Converting enzyme

Cl⁻

Angiotensin II activity destroyed by angiotensinases

FIGURE I: BIOCHEMICAL CONDITIONS AND REACTIONS INVOLVED IN THE RENIN-ANGIOTENSIN SYSTEM
Figure I shows the biochemical conditions and reactions of the renin-angiotensin system as accepted in 1967.

SKEGG's clarification of the renin-angiotensin system was a major breakthrough, yet only a starting point for the goal, still not reached, of complete unveiling of the mechanisms of the system. By 1957 in SKEGG's laboratory partial purification of horse-renin substrate had led to a trypsin degradation of horse-renin substrate. The structure of the resultant substrate polypeptide fragment was determined to be that of angiotensin I plus four amino acids. Figure II shows the chemical structure of trypsin-degraded horse plasma renin substrate, relative to the structures of angiotensin I and angiotensin II. According to the structure determination, trypsin, which normally breaks a polypeptide chain only to the (conventional) right of basic amino acids, is alleged to have broken the chain to the right of serine, an hydroxy-amino acid. This curious phenomenon remains a mystery. Reaction with renin converting the renin substrate polypeptide into angiotensin I indicated the sufficiency of the polypeptide as a renin substrate and pointed to a possible chemical assay for renin to replace the SKEGGS rat assay. The polypeptide substrate was synthesized and the renin was shown to break the L-Leu-L-Leu bond of the polypeptide substrate. In an extremely slow in vitro reaction, angiotensin II was broken by renin into two tetrapeptides which inhibited renin in the absence of serum. Serum without angiotensinase activity reduced the inhibitory effect on renin, indicating the probable unimportance of this slow reaction in vivo.

Angiotensin I peptides with isoleucine (Ile) in the fifth amino acid position were isolated from horse and hog plasmas and shown to be identical in corroborating purifications. However bovine-plasma angiotensin I (and II) differs from
Figure II: Chemical structures of trypsin-degraded renin substrate, angiotensin I, and angiotensin II (from horse plasma)
horse- and hog-plasma angiotensin I (and II) by the presence of valine (Val) in the fifth amino acid position. Horse and hog angiotensin II synthesized by SCHWARTZ, BUMPUS and PAGE, and by SCHWYZER in 1957 proved to be partially racemized. Fully active isoleucine-5 angiotensin II was synthesized in 1961 by ARAKAWA and BUMPUS. Bovine angiotensin II (valine-5) was synthesized in 1958 with no racemization difficulties. Figure III shows the chemical structure of trypsin-degraded bovine renin substrate and bovine angiotensin I and angiotensin II. Intra-aortal injections of both forms of the purified angiotensin I produced pressor effects on rabbit blood pressure equivalent to those of purified angiotensin II injections, which is evidence, according to SKEGGS, for fast converting-enzyme action on angiotensin I, the probably biologically inactive form, in the reaction to angiotensin II, the potent pressor. Requirements for biological activity of angiotensin have been studied by BUMPUS and SKEGGS in an effort to understand the mechanism of renin action on its substrate and thereby to develop a synthetic substrate suitable for an automizable chemical assay of renin. SKEGGS has recently synthesized nine such suitable peptide substrates and using a preliminary chemical assay has studied the merits of the relative kinetics of each renin-substrate system. The two substrates suggested for use in chemical assays for renin in SKEGGS' report are shown in Figure IV. Serine-14 has little effect on the Michaelis constant or maximal velocity, however the histidineimidazole group in amino acid position six, proline in position seven, the phenyl side group on phenylalanine in position eight, and a free carboxyl group on the C-terminus are absolute requirements for the renin substrate. In addition the substrate must be at least six amino acids in length.

A clarification of the biochemical relationship between hypertension and
Figure III: Chemical Structures of Trypsin-Degraded Renin Substrate, Angiotensin I, and Angiotensin II (From Bovine Plasma)
FIGURE IV: TWO SYNTHETIC RENIN SUBSTRATES SUGGESTED BY SKEGGS
FOR USE IN AN AUTOMIZABLE CHEMICAL RENIN ASSAY
elevated aldosterone levels was accidentally found by Laragh\textsuperscript{56} and Biron\textsuperscript{57} in 1960-61 during an investigation of plasma renin substrate level regulation. Laragh and Biron both showed angiotensin II to stimulate adrenal aldosterone secretion, but whether the amount of hormone secreted was sufficient to regulate plasma renin substrate at a constant level was not determined.

Renin substrate, in further studies, has been identified as a glycoprotein present in the alpha\textsubscript{2}-globulin, and possibly beta-globulin, fractions of plasma and in circulating lymph. Although renin substrate is thought to originate in the liver, no evidence has been found for the presence of the substrate in this organ.\textsuperscript{59} In 1954 Skeggs\textsuperscript{60} reported purification from hog plasma of five forms of renin substrate (called A, B\textsubscript{1}, B\textsubscript{2}, C\textsubscript{1}, C\textsubscript{2}). Two minor forms D and E were recovered during purification in insufficient amounts to purify satisfactorily. Skeggs modified the classical hog renin substrate purification method of Green and Bumpus\textsuperscript{30} with batch (fractionation) and column diethylaminoethyl cellulose (DEAE-cellulose) chromatography, liquid-liquid partitioning, and countercurrent distribution of renin substrate and serum proteins in aqueous glycol solutions. Ultra centrifugation following this modification of renin substrate purification revealed all five forms of substrate to have approximately the same molecular weight, reported as 57,000 in two reports\textsuperscript{55,62}, and probably reported in error as 58,000 in another.\textsuperscript{63} Amino acid compositions of the five glycoproteins were nearly identical, but carbohydrate portions provided the differences in physical properties. All substrates upon reaction with renin and activated converting enzyme liberated identical angiotensin II molecules. Reaction of these substrates with trypsin yielded the thermostable, dialyzable polypeptide identical to that derived from trypsin degradation of naturally-occurring horse and hog renin substrate. Rates of hydrolysis by renin on all five substrates were close, except in the cases of the two least purified substrates, B\textsubscript{1} and B\textsubscript{2}. 
The enzyme renin has been the most elusive component of the renin-angiotensin system. Although renin is localized in the granular juxtaglomerular cells, as mentioned above, the enzyme is found in arteries and occasionally in other organs. Consequently efforts to purify the enzyme have begun with treatment of the kidney. Ammonium sulfate fractionations, DEAE-chromatography, and triethylaminoethyl (TEAE) chromatography of crude kidney renin preparations have produced only partially purified renin. HASS, LAMFROM, and GOLDBLATT have established the specific activity of pure renin at 780 GOLDBLATT units per mg. One GOLDBLATT unit is the activity of a renin sample equivalent to the activity of 0.45 mg pure angiotensin II. Two groups have recently derived relatively pure renin from kidney preparations by modification of previously used techniques. MAIER and MORIAN used TEAE-cellulose purification (see also MORGAN and LENON), guanidinoethyl (GE-) cellulose, medium Sephadex with four-fold volume reduction, and dialysis against Tris-saline buffer. SHAGS, using a DEAE-cellulose pH gradient, eluted four forms of renin, whose difference may involve only spatial configuration alterations in the enzyme due to carrier protein effect on renin. The spatial configuration and thus the mechanism of the renin-renin substrate reaction have not been elucidated. Renin, due to its thermobility and the large loss of activity encountered in purification, has presented the largest obstacle in renal hypertension research. With the knowledge of renin configuration and mechanism such problems as the renin assay, cadmium-ion (Cd^{2+}) and zinc-ion (Zn^{2+}) (and possibly other divalent cations) effects, and inhibitor studies could be more efficiently attacked.
ASSAYS FOR RENIN

Despite the difficulties encountered in renin purification, assay of renin-content is simpler than assay of angiotensin-content. Use of renin presence in arteries has been made in plasma assays of renin in which a crude preparation of renin from treated plasma is permitted to react with renin substrate and the renin is then assayed indirectly by activity measurement of the reaction resultant in the rat. The rat assay used in the Colby College laboratory, as a direct assay of injected-renin activity, is a modified SKROGS rat assay in which an anaesthetized rat is injected intravenously with a renin preparation and the resultant change in blood pressure is measured with a manometer connected by a cannula to the right carotid artery of the rat. The assay is delicate, lengthy, and accurate only to 5%-15%. A chemical assay of renin was developed in the same laboratory at Colby College by MCDONALD (1968), however as his assay is nearly as arduous as the rat assay, all further work in the laboratory has been performed using the rat assay. SKROGS has partially developed a chemical assay of renin by synthesis of peptides vulnerable as substrates to action by renin however neither his preliminary chemical assay nor his partially-developed assays are suitable for use at this time.

CADMIUM ION AND ZINC ION EFFECT

The roles of Cd$^{2+}$ and Zn$^{2+}$ in renal hypertension were observed by SCHROEDER only recently. Many of his observations are from uncontrolled clinical and autopsy information, however he has conducted three noteworthy laboratory experiments. In an experiment reported in 1962 SCHROEDER observed oral Cd$^{2+}$-induced hypertension in young female rats. In work with both sexes,
females tended to develop hypertension more often than males, however hypertension returned to males later in their lives. SCHROEDER associated shortened life span with hypertension and suggested that the antimetabolite role of cadmium in the kidney was due to an increase in the renal \( \text{Cd}^{2+} - \text{Zn}^{2+} \) ratio. Relative deficiency in renal zinc he says is therefore possibly responsible for essential hypertension. Intravenous and intraperitoneal injections of \( \text{Cd}^{2+} \) also produced hypertension in rats. In a third \( \text{Cd}^{2+} \)-hypertension experiment, a zinc chelate having higher stability as a cadmium chelate (uncommon) was injected into \( \text{Cd}^{2+} \)-induced hypertensive rats. Subsequent lowering of blood pressure was interpreted as a reversal of hypertension. SCHROEDER pointed to the lowering of hypertension-elevated \( \text{Cd}^{2+} - \text{Zn}^{2+} \) ratios as the mechanism of hypertension reversal. Zinc is an essential trace element in mammals while cadmium is probably not essential. In fact, in the mammalian kidney cadmium is a cumulative poison.

Table I shows zinc and cadmium levels and toxicities in man. Both zinc and cadmium rank as very high potentials for pollution and, in view of their toxicity to man, add prospects of death or induced hypertension to pollution. Polluted air contains 0.2-2 \( \text{mg/m}^3 \) zinc which, following approximate calculations, will kill a 150-pound man should he breathe from 35 to 350 meters of it. That such a phenomenon is even conceivable is alarming.

According to SCHROEDER, \( \text{Cd}^{2+} \) is bound to the protein metallothionein in the kidney. KAGI and VALLEE report one molecule of metallothionein from horse kidney to bind nearly irreversibly three zinc atoms and five cadmium atoms. The introduction of excess cadmium to the kidney will not cause metallothionein to lose zinc atoms despite the greater electronegativity of cadmium. Similarly
<table>
<thead>
<tr>
<th>DESCRIPTION</th>
<th>Cd&lt;sup&gt;2+&lt;/sup&gt;</th>
<th>Zn&lt;sup&gt;2+&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Human Tissues:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kidney</td>
<td>130 p.p.m.</td>
<td>210 p.p.m.</td>
</tr>
<tr>
<td>Liver</td>
<td>6.7 p.p.m.</td>
<td>130 p.p.m.</td>
</tr>
<tr>
<td>Others</td>
<td>trace only</td>
<td>13-180 p.p.m.</td>
</tr>
<tr>
<td><strong>Toxicity to Man:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intravenous Injection</td>
<td>highly toxic (1-10 mg/kg body weight is lethal)</td>
<td>moderately toxic (10-100 mg/kg body weight is lethal)</td>
</tr>
<tr>
<td>Oral Ingestion</td>
<td>moderately toxic</td>
<td>slightly toxic (100-1000 mg/kg body weight is lethal)</td>
</tr>
<tr>
<td><strong>Toxicity to Rats:</strong></td>
<td>Toxic Lethal</td>
<td>Toxic Lethal</td>
</tr>
<tr>
<td>Oral Ingestion</td>
<td>0.5 g/da 16 g/da</td>
<td>50 g/da 150 g/da</td>
</tr>
</tbody>
</table>

**TABLE I:** Cd<sup>2+</sup> AND Zn<sup>2+</sup> Levels in Dry Mammalian Tissue<sup>30</sup> and Toxicities in Man<sup>34</sup> and Rats<sup>31</sup>.
introduction of a zinc chelate will not liberate cadmium from metallothionein despite the greater stability of a cadmium chelate than a zinc chelate.

SCHRODER notes that excess cadmium produces hypertension in rats and mice, but he failed to study the effect of excess zinc alone. In 1969 MAIER et al found that both Ca\(^{2+}\) and Zn\(^{2+}\) independently activate a twice-dialyzed renin sample prepared from bovine kidney. Further, when the cations were chelated and removed by a third dialysis, renin activity remained elevated, suggesting cationic-facilitated removal of a dialyzable renin inhibitor rather than direct cationic activation of renin. Dialysis of the crude renin preparation against Tris-saline buffer (Zn\(^{4+}\) and Cd\(^{2+}\)-free) also increases renin activity without removal of contaminants. Figure V shows the effects of the dialyses and cations on renin activity.

A RENIN INHIBITOR

An "antirenin immune serum" which upon injection lowers the blood pressure and increases juxtaglomerular cell granularity and renin content has been studied in the kidneys of dogs treated by GOLDBLATT'S clamped renal artery method since 1940. Injections of hog renin in dogs produces a hog antirenin which deactivates GOLDBLATT-dog renin allowing normal blood pressure to be restored. Injections of animal renin in man fails to produce neutralizing antibodies for human renin. In 1962 ORBISON achieved inhibition of renal hypertension in monkeys by human antirenin and estimated that immunization from renal hypertension in one man would require antirenin from 250,000 monkeys. A partially acetylated human renin found by BASS, GOLDBLATT and GIPSON in 1965 to produce antibodies against human renin is now the only successful attempt to develop an antihypertensive drug, however the scarcity of available human kidneys prevents widespread
use of the drug. Here again enough knowledge of the enzyme renin to produce synthetic renin would constitute a major breakthrough. A successful inhibitor of renin according to SMYKGS\(^62\) would not adsorb on serum proteins, as does the octapeptide PRO-PHE-HIS-LEU-LEU-VAL-TYR-SER in vivo, and would be tightly bound to the active site of the enzyme. Indication that this inhibitor is not an antirenin is given by the experiments of MUIRHEAD, JONES and STIRMAN\(^88\) on the normalizing effects of a renal medulla extract on the elevated blood pressure of test dogs; and of PAGE et al.\(^89\) on similar effects of kidney extracts on human patients as early as 1941. MAIER\(^67\) attributes the dialysis effect of renin activity enhancement (in the absence of removal of impurities) to loss by dialysis of the renin inhibitor. Observations on Cd\(^{2+}\) and Zn\(^{2+}\) effects summarized in Figure V have lead to the present examination of dialysands for an inhibitor whose removal by dialysis is facilitated by Cd\(^{2+}\) (or Zn\(^{2+}\)). Recently SEN, SMYK and BUMPUS\(^90-91\) partially isolated a renin inhibitor from canine kidney. This inhibitor is believed to be a phospholipid similar to bovine phosphatidylserine, however the nature of one of the three side chains is unknown. Inhibition of renin in vivo and in vitro have been demonstrated with this inhibitor.

The method used by SEN, SMYK and BUMPUS to isolate a phospholipid renin inhibitor involves an acetone powder of the kidney, two chloroform-methanol extractions, a lipid precipitation, and column chromatography. If, as suspected by MAIER and co-workers, the removal of an inhibitor by dialysis of a kidney homogenate is facilitated by the presence of added-cadmium ions (or zinc ions) in the homogenate, the isolation of the inhibitor(s) need not be so tedious. We proposed to investigate these assumptions further in an attempt to demonstrate the existence, in steer kidneys, of an inhibitor similar to that reported by SEN, SMYK, and BUMPUS. We first determined to investigate dialysands of dialyses which had enhanced renin activity in earlier work.
Figure V: Effects on Renin Activity of Dialyses and Cation Incubation
PART II: EXPERIMENTAL
MATERIALS AND METHODS:

Preliminary Details

Normal saline is 0.9% NaCl, biological grade, in deionized, then glass distilled, water. Tris-saline unless noted otherwise is 0.05 M Trizma base, 0.9% NaCl, and buffered at pH 7.3 in deionized, distilled water. Tris-2x saline is Tris-saline with a total of 1.8% NaCl.

Angiotensin II (Angiotensin II - Ciba, 2.50 mg in 100.0 ml Tris-saline) is used in 0.2-ml injections (0.005 mg per injection) in the rat assay as a standard for units of renin activity. Sodium amytal - Lilly (sterile sodium amobarbital, USP 0.5 g) is divided into 50-mg portions and taken up in 1.25 ml normal saline when needed. This anaesthetic is injected intraperitoneally in the rat in a dose of 0.6 ml plus 0.1 ml per 40 g body weight over 200 g. Atropine sulfate (Burroughs-Wellcome and Co., 0.05 mg in 1-CC ampoules) is injected in doses of 0.5 ml per rat.

Sodium heparin - Lilly (1000 USP/cc) is injected intravenously during the operation preliminary to the rat assay in 0.8-ml doses per rat (800 USP). Young male rats of the SPRAGUE-DAWLEY strain were purchased from Taconic Farms, Germantown, New York. This strain is resistant to peritonitis. Steer kidneys were donated by Alco Packing Company, Winslow, Maine, and were frozen soon after slaughter. An International centrifuge model number HR-1 was used at 15 000 rev./min. A Beckman Zeromatic pH meter was used to make and periodically to check the pH's of buffered solutions. Cadmium and zinc contents of samples were analyzed with a Perkin-Elmer Model 303 Atomic Absorption Spectrophotometer. Dialyses were performed with Thomas Dialyzer Tubing No. 4465-A2, inflated - diameter 5/8 inch.
One worker has questioned the value of the use of rats in renal hypertension research, alleging hypertension in rats to be different from hypertension in humans. The vascular system of rats is particularly sensitive to angiotensin and can be made more so by injection of a "vasoexciter material" from humans. Though adrenalectomy has been unsuccessful in curing renal hypertension in rats and man (contrast dogs) debate has been heard as to whether all of the adrenal gland, which is often mingled with liver tissue in rats, can be removed from the rat. However, evidence that the rat hypertension mechanism is similar to that of humans is found in the formation in both rats and humans of antibodies to various mammalian kidney extracts and the subsequent absence of renin neutralization by these antibodies in both mammals. A modified SKEGGS rat assay contributes one renin sample activity datum per rat. The rat, anesthetized with sodium amytal and atropine sulfate, is prepared for enzyme assay by an operation as follows. The rat is secured to a dissection table, ventral surface upward. One femoral vein is exposed and separated from the accompanying nerve and artery. The left vagus is exposed and cut; the trachea is catheterized; the right vagus is exposed and cut; and the right carotid artery is cleared. The femoral vein is catheterized; sodium heparin is injected and washed into the vein with Tris-saline; and the right carotid artery is cannulated and attached to a simple mercury manometer by which the rat's blood pressure response to femoral injections of Tris-saline, renin samples, and standard Hypertensin II may be measured. A 0.2-ml sample of renin (pH 7.3 in Tris-saline) or renin plus inhibitor, followed by 0.6 ml Tris-saline, is injected. The change in blood pressure from an averaged baseline is recorded and
a subsequent injection in the same rat of 0.2 ml (0.005 mg) standard Hypertensin II plus 0.6 ml Trias-saline is administered. A Trias-saline injection equal to the total volumes (0.8 ml) of the renin and standard Hypertensin II injections precedes the enzyme injection and indicates the volume corrections, if any, to be applied to renin and standard pressor responses. Renin activity (units/ml) of the injected renin sample is calculated as follows:

\[
\text{Activity (units/ml) = \frac{\text{Renin-induced elevation /0.2 ml}}{\text{Hypertensin II-induced elevation}}} \times 5
\]

The blood pressure elevation is calculated from a three-minute baseline (+2 manometer units) of normal blood pressure which precedes injection, and is corrected for volume effect. A maximum range of +2.5 manometer units is permitted between the highest and lowest baseline. Throughout the experiment blood pressure is recorded at 15-second intervals except that the peak for an interval is recorded if the maximum elevation falls within the interval. To insure that the standard does not have pressor potential beyond the maximum blood pressure limit of the rat, an injection of excess Hypertensin II given at the end of the assay must cause blood pressure to exceed the resultant blood pressure peak of the standard Hypertensin II injection.

The time required for a rat assay decreases with experience, however remains excessive considering that only one datum per rat is obtained. That several renin samples be given to the same rat might be proposed as
this would require only one operation, one standard injection, and one excess standard injection, but more than one renin sample injected into the same rat may exhaust the rat's supply of renin substrate. On occasion, other problems of the assay express themselves in unstable baselines and therefore untrue blood pressure elevations. The vagi are regulator nerves which signal the heart muscle to counteract changes in blood pressure. When any strand of a vagus is not severed during the operation, three-minute baselines within two manometer units will not be established and pressor injections will exhibit blood pressure changes which are altered by signals from intact strands of the vagi. When blood or saliva accumulates in the tracheal catheter, irregular baselines predominate. For this reason, the salivary glands should be handled delicately. The most common difficulty encountered in the assay is anaesthesia. The rat's blood pressure will rise from his baseline without provocation when he needs additional anaesthetic. About ten minutes must elapse after the anaesthetic is injected intraperitoneally before a baseline is established. This baseline will be too high with insufficient additional anaesthetic and too low with (as little as 0.05 ml) too much anaesthetic. Because the permitted range of the baselines in the assay is only 2.5 manometer units, the amount of anaesthetic judged by the assayer to be necessary during the assay is critical.

Data from rat assays are averaged and usually yield 15% error or less. If the error is more than 15%, one datum in four (or any multiple thereof) may be eliminated by the following statistical treatment. The
three closest data are averaged and their average deviation, and the absolute deviation of the fourth, from this average are calculated. The fourth datum may be eliminated statistically if the average deviation of the three retained data is more than four times the deviation of the fourth datum.

MATERIALS AND METHODS:

Cd\(^{2+}\) and Zn\(^{2+}\) - Indirect Activation of Renin

Steer kidneys were defrosted, chopped, then frozen and thawed five times to break cell membranes. One-ml Tris-saline, the homogenizing solution, was added per g of wet kidney mince and homogenized at 4\(^{\circ}\) in a Waring blender. A portion of this homogenate (Step I) was stored frozen for eleven months, at which time the homogenate was thawed, an aliquot for assay removed and frozen and in Step II 480 ml homogenate was centrifuged in Step II at 15,000 rev./min. for 2h at 4\(^{\circ}\). Then 250 ml of supernatant and an aliquot for assay were diluted with equal volumes of Tris - 2x saline. The aliquot was frozen and the 500 ml diluted supernatant was dialyzed in Step III for three days at 4\(^{\circ}\) against six changes of 2500 ml each (five volumes) of Tris (0.05 M Trizma base buffered at pH 7.3). The dialyzed sample from Step III was centrifuged and the supernatant was diluted with an equal volume of Tris - 2x saline, and divided into four portions to an 80-ml-portion was added 0.4 ml H\(_2\)O, to a 120-ml-portion was added 1.2-ml 0.1 M CdCl\(_2\) (112.4 p.p.m) and to another 120-ml-portion was added 1.2-ml 0.1 M Zn Cl\(_2\) (65.3 p.p.m.). The cadmium and zinc samples were 0.001 M with respect to their added cation, as higher
Concentrations of zinc caused the sample to precipitate. In Step IV these three portions were incubated for 44 h at 4°C and then centrifuged at 15,000 rev./min. for 2 h at 4°C. A fourth portion was frozen for assay, as was an aliquot from each of the three dialyzed samples from Step IV. In Step V a second dialysis of the renin sample was performed on each of the Step IV supernatants at 4°C against five changes of fifteen volumes Tris-saline; 30 ml of the dialyzed water sample (blank) supernatant was dialyzed against a total of 2250 ml Tris-saline; 30 ml of the corresponding cadmium sample frozen once was dialyzed against a total of 2250 ml Tris-saline; and 60 ml of the corresponding zinc sample was dialyzed against a total of 4500 ml Tris-saline. An aliquot of the dialyzed cadmium sample was assayed immediately, and aliquots of the dialyzed blank and zinc samples were frozen for assay. A third dialysis against Tris-saline-EDTA (0.001 M EDTA, a chelating agent with high stability constants for cadmium and zinc chelates) was done in an effort to remove cadmium and zinc completely from the renin samples. With the exception that EDTA was used and the volumes used in this (final) Step VI dialysis were half those of Step V, the second and third dialyses were identically performed. Samples from each of the final dialyzed renin samples were frozen for renin assay. All supernatants and precipitates except the homogenate precipitate, and all dialyzed samples and dialysands were analyzed for cadmium and zinc content with the atomic absorption spectrophotometer. Calculations from an identical renin-activation procedure were used to correct renin activities for volume changes (Table II) for purposes of comparison with homogenate supernatant activity.

Results of the Cd²⁺ and Zn²⁺ renin activation experiment are shown in
**TABLE II**: VOLUME CHANGES DURING A Cd$^{2+}$- AND Zn$^{2+}$- RENIN ACTIVATION EXPERIMENT
**Kidneys**

**Kidney Homogenate (DB)**
- 0 p.p.m. Cd
- 4.6 p.p.m. Zn

**Homogenate Supernatant (DD)**
- 0 p.p.m. Cd
- 9.1 p.p.m. Zn
- 1X DD volume
- 1.31 units/ml activity

**Dialyzed Supernatant (DG)**
- 0 p.p.m. Cd
- 8.1 p.p.m. Zn
- 1,076 X DD vol
- 2.01 u/ml activity

---

**Legend:**
- P: precipitate
- S: supernatant
- R: renin sample
- D: dialysand
- X: X DD volume
- Cd: cadmium
- Zn: zinc

**Figure VI:** RESULTS OF THE Cd$^{2+}$ AND Zn$^{2+}$ RENIN ACTIVATION EXPERIMENT
MATERIALS AND METHODS: Extraction of Dialysands for the Renin Inhibitor

Approximately 2320 ml dialysand from the cadmium sample dialysis in Step V of the Cd^2+ and Zn^2+ renin activation experiment procedure was thawed and made 0.01 M with regard to EDTA. Then 580 ml of 2:1 chloroform-absolute methanol was added (25% volume); the layers were separated after stirring 1 h.; and the aqueous layer was stored frozen. Partial evaporation of the chloroform layer was achieved at room temperature and atmospheric pressure, however, evaporation to apparent dryness was done at a slightly elevated temperature. Tris - 2x saline (1.5 ml) was used to take up the proposed inhibitor. Two samples were prepared each from 10 ml of the renin-sample dialyzed by Step III of the Cd^2+-Zn^2+ renin-activation experiment, and 9 ml Tris - 2x saline. The blank sample contained one additional ml Tris - 2x saline and the sample presumed to contain the inhibitor ("inhibitor" sample) contained one ml of proposed inhibitor in Tris - 2x saline. The samples were incubated for 22 h at 4^o, and then assayed immediately. The blank sample was expected to be identical in every respect to a sample previously assayed at 2.01 units activity/ml.

A second dialysand, that from the zinc-sample EDTA - dialysis in Step VI was extracted in the same manner. A total of 1407 ml zinc dialysand was made 0.01 M with respect to EDTA and extracted with 367.5 ml (25% volume) of 2:1 chloroform-absolute methanol. After stirring 1 h, the
aqueous layer was separated from the chloroform layer and frozen. The chloroform layer was evaporated to apparent dryness at room temperature and open to the air, and then was taken up in 3.0 ml Tris-2x saline. The blank prepared was equivalent to a sample previously assayed at 1.3 units activity/ml. This second "inhibitor" sample was prepared to be 3 1/3-times as concentrated as prospective inhibitor as the previous "inhibitor" sample. The two samples from the second extraction were frozen before assay.

A third extraction was performed on the dialysand of the Cd²⁺-incubated sample dialyzed in Step VI against EDTA. In an effort to facilitate drying of the extraction at room temperature and pressure, the extraction was performed with 3:1 acetone-ethanol (25% volume). The layers were separated, the organic layer was evaporated to apparent dryness at room conditions; and samples were prepared with a remin sample previously assayed at 3.02 units/ml. The "inhibitor"-remin sample was prepared, as in the second extraction, to have 3 1/3-times the inhibitor concentration of the "inhibitor"-remin samples of the first extraction.

MATERIALS AND METHODS:
Discrepancy Between Standard Hypertensin II Batches

Standard Hypertensin II prepared from second-batch was assayed in rats and the blood pressure response was compared to the response from a previously-used standard Hypertensin II batch. First-batch standard was freshly defrosted and compared in the same rat with second-batch standards. The second-batch standards were
assayed after having been frozen two days, frozen one day, defrosted one day, defrosted two days, and never frozen. Rises in blood pressure due to volume injections were subtracted from pressor effects.

MATERIALS AND METHODS

An Attempt to Isolate a Dialyzable Phospholipid Renin Inhibitor

Steer kidneys were defrosted at 40, trimmed of excess fat, ground twice in a meat grinder, frozen and thawed five times, and homogenized in Step 1 with 1 ml Tris (0.05 M Trizma base buffered at pH 7.3) per g of wet mince in a Waring blender. In Step 2 1150 ml homogenate was centrifuged at 15 000 rev./min. and 630 ml supernatant was recovered. An aliquot was diluted by one-half with Tris-2x saline and frozen for assay. (Initial assays of this sample indicated excessive activity, and the frozen sample was further diluted with an equal volume of Tris-saline). Then 500 ml homogenate supernatant from Step 2 was made 0.003 M with respect to CdCl2 and was dialyzed for five days against five changes of 2000 ml each (four volumes) of Cd-Tris-saline (0.003 M CdCl2, 0.05M Trizma base, 0.9% saline, buffered at pH 7.3). Each day the dialysand was placed on a magnetic stirrer with 1 l of chloroform and stirred for 24 h. The aqueous layer was removed and stored frozen and the organic layer was placed in a bowl under the hood. Evaporation of all 5 l of the chloroform took nineteen days (approximately) and on the twenty-fifth day Step 3 began. The residue was scraped from the sides and bottom of the vessel with a deionized porcelain spatula
and pulverized to a fine powder. The powder soaked in 30 ml 0.05 M Tris (approx. pH10) for 1 h, and a cation exchanger (4 g, Carboxy-Methyl-Cellulose, capacity 0.72 meq/g, medium mesh, Sigma) with 40 ml Tris was stirred at 4°C for 2½ h. The supernatant from centrifugation (4°C, 12000 rev/min., five min.) was collected and brought to a volume of 50 ml with Tris. The pH was adjusted to 7.38 with concentrated HCl and the sample was filtered by suction through a sintered glass crucible. The resultant "inhibitor" solution was stored at 4°C under a presumed atmosphere of nitrogen, however as the process for establishing a nitrogen atmosphere involved mechanical capping of the flask after submission to nitrogen under pressure, the integrity of the nitrogen atmosphere is questionable. Assuming all inhibitor was extracted from 500 ml homogenate supernatant, the 50 ml "inhibitor" solution contains all the inhibitor which was inside the dialysis sacs prior to Cd-Tris-saline dialysis. The "inhibitor" solution was made 0.9% saline with solid NaCl. In step 4 20 ml of "inhibitor" was incubated for 25 h with 2 ml homogenate supernatant, 2 ml Tris - 2x saline, and 4 ml Tris-saline. The incubated sample, compared in assay with activity of a sample containing 2 ml homogenate supernatant, 2 ml Tris - 2x saline, and 12 ml Tris-saline, represents a 100-fold concentration of "inhibitor" from homogenate supernatant and 800-fold concentration from the sample compared in assay with the incubated sample. A volume correction for renin injections of the renin-inhibitor incubate in the rat assay aquated 0.7 ml inhibitor incubate with 0.2 ml of the homogenate supernatant diluted 1:4 in Tris-saline. The corrected total volume of injections used in the assay of "inhibitor" - renin incubate (only) was 1.3 ml compared to a usual total of 0.8 ml. Activity of the
incubate sample was reported in terms of the eight-fold dilution of the homogenate supernatant sample. Cadmium determinations from spectrophotometric analysis showed the cadmium content of the inhibitor solution to be nearly ten times that in the cadmium-incubated dialyzed sample from Step V of Cd\(^{2+}\) and Zn\(^{2+}\) experiment suggested an investigation of the chelating effects of EDTA on the cadmium left in the renin preparation by an unsuccessful cation exchange treatment in Step 3. As concentrations of 0.01 M EDTA were known to inhibit converting enzyme in vitro, a sample of homogenate supernatant was diluted 1:2 with Tris-saline and compared in assay in Step 5 to an identical sample to which EDTA had been added to a concentration of 0.002 M. Because these EDTA-experiment samples were diluted to the same degree from the homogenate as the renin-inhibitor incubate, their assayed activities are comparable to activity, before expression as a dilution of homogenate supernatant, of the incubate. In Step 6, 75 ml of the sample dialyzed in Step 2 was dialyzed for five days against five changes 1125 ml each (15 volumes) of Tris-saline-EDTA (0.001 M EDTA) and the resultant sample (71 ml) retrieved from inside the dialysis sacs was decanted and diluted four-fold so that it was assayed in Tris-saline. This diluted sample from Step 6 was of the same order of dilution from the homogenate supernatant (four-fold) as the Cd-Tris-saline-dialyzed sample diluted from Step 2. This inhibitor isolation experiment indicated that, when corrected by dilution and other volume change calculations, to expected activities of undiluted samples, the diluted samples showed markedly higher activities than the assayed activities of undiluted preparations. Figure VII shows the results of the renin inhibitor isolation experiment.
Kidneys

STEP 1
Kidney Homogenate (FA)

STEP 2
Homogenate Supernatant (FB) → 1:2 (FC') → 1:2 (FC) → 5.07 units/ml

STEP 2
Dialyzed Supernatant (FD)

(FK) ← 2.33 units/ml

+ Aqueous Dialysand (FE)
+ Organic Dialysand (FF)

STEP 3
"Inhibitor" Solution (FG) → "Inhibitor" - Renin Incubate (FH) → 1.20 units/ml

STEP 4

1:2

1:2 (EDTA)

(FI) ← 4.30 units/ml
(FJ) ← 4.46 units/ml

STEP 6
Tris-saline-EDTA-dialyzed renin sample (FN)

(FI) ← 1:4

5.517 units/ml

FIGURE VII: RESULTS OF THE RENIN INHIBITOR ISOLATION EXPERIMENT

(Ratios indicate order of dilution in Tris-saline or Tris-saline-EDTA)
MATERIALS AND METHODS:

Investigation of Pre-Slaughter Steer Diets for High Cd$^{2+}$ and Zn$^{2+}$ Content

The renin samples from the inhibitor isolation experiment had high activities and were prepared from kidneys of steers slaughtered in January. Previously-assayed renin samples prepared from steers slaughtered in July and in June had less renin activity. Alco Packing Company and the local Agway feed store were consulted as to possible seasonal variations of pre-slaughter steer diets which might introduce varying levels of Cd$^{2+}$ and Zn$^{2+}$ into the steer.
RESULTS AND DISCUSSION:

Cd$^{2+}$ and Zn$^{2+}$—Indirect Activation of Renin

No loss of renin activity from the frozen homogenate was found: the renin activity of the homogenate supernatant was comparable to the activity of an identically-treated sample assayed eleven months earlier. The first dialysis (against Tris in Step III) enhanced renin activity about one-and-a-half times from the homogenate supernatant activity. The loss by dialysis of a renin depressor is implied. A small gain in sample volume from homogenate supernatant volume was experienced with dialysis. Incubation with cations in Step IV further increased renin activity. The blank sample was slightly activated, apparently by scant dilution. This phenomenon is not understood. One-fourth of the added-Cd$^{2+}$ and one-half of the added-Zn$^{2+}$ precipitated during cation incubation. Added-Cd$^{2+}$ activated the renin preparation to a greater extent than did added-Zn$^{2+}$. After completion of dialysis by Tris-saline in Step V, more than 80% of each added cation had been precipitated or dialyzed from the renin samples. Renin activities for all the samples reached their maxima$\pm5.5\%$ after this step, indicating further activation of renin by the removal of the cations. The final dialysis (against Tris-saline-EDTA) removed only about 15% more cation and generally activities did not show further activation. In fact, a slight decrease in activation was displayed by the blank and the Cd$^{2+}$—incubated sample. Some deterioration of the enzyme preparation
is suspected. While the blank and cadmium samples dialyzed in Step VI lost activity, the zinc sample gained 5% activity. The zinc sample is the only final fraction which lost net volume from the homogenate supernatant. A correlation between gain in renin activity and dilution of renin sample is possible.

Dialyses of cation-incubated renin preparations increased the activities of renin samples approximately three-fold over the activity of the homogenate supernatant. The blank sample activity increased approximately two-fold. (Again, the blank activity-increase phenomenon is not understood). For the final fractions, net volume changes occurring with precipitations and dialyses averaged 1.015-times the homogenate supernatant volume. 97.1% of the added 65.3 p.p.m. Zn$^{2+}$ were recovered from precipitates and dialysands. In this experiment both Cd$^{2+}$ and Zn$^{2+}$ showed indirect activation abilities toward renin; however the overall Cd$^{2+}$-activation of the renin preparation exceeded by 15% that of the overall Zn$^{2+}$-activation. The presence of Zn$^{2+}$ in the kidney homogenate (and the absence of Cd$^{2+}$) concur with expectations that Zn$^{2+}$, and not Cd$^{2+}$, is an essential trace metal in the kidney.$^{70,78}$ Because a small amount of Zn$^{2+}$ is endogenous, the presence of additional Zn$^{2+}$ in the kidney may be expected to have a lesser effect than would additional Cd$^{2+}$, a toxic, cumulative$^{79}$ trace metal. Another possible explanation for the lesser renin activation by Zn$^{2+}$ may lie in the incubation-step precipitation of twice as much (%) of the added Zn$^{2+}$ as the added Cd$^{2+}$. A zinc-chelate having a higher stability
constant for Cd\textsuperscript{2+} than Zn\textsuperscript{2+} will replace Cd\textsuperscript{2+} in a renin-activating role. Perhaps SCHROEDER'S \textsuperscript{77} observation of a "reversal" of Cd\textsuperscript{2+}-induced hypertension by such a zinc-chelate may be more correctly interpreted as a less intense activation of renin by Zn\textsuperscript{2+}. A revised scheme of suspected phenomena of the renin-angiotensin system as elucidated by the Cd\textsuperscript{2+} and Zn\textsuperscript{2+} renin-activation experiment is shown in Figure VIII.

RESULTS AND DISCUSSION:

Extraction of Dialysands for Renin Inhibitor

The cadmium-sample Tris-saline dialysand (from Step V of the Cd\textsuperscript{2+} and Zn\textsuperscript{2+} renin-activation experiment) theoretically should be the dialysand containing the highest concentration of renin inhibitor. The activity of the renin sample dialyzed to produce this dialysand had the highest renin activity in the Cd\textsuperscript{2+} and Zn\textsuperscript{2+} renin-activation experiment, indicating, at this step, the most complete loss of the inhibitor. For this reason the first dialysand extraction was performed on the cadmium-sample Tris-saline dialysand. The "inhibitor"-renin sample and the control-renin sample from the first dialysand extraction were assayed and found to have activities of 1.98 units/ml and 230 units/ml respectively. The latter activity exceeds by 15% the activity shown by previous assay of the sample (\(2.01 \text{ units/ml}\)) A decrease caused by addition of the proposed inhibitor is not conclusively attributable to the presence of a renin inhibitor. If the inhibitor was indeed present, activity would be expected
**INACTIVE: RENIN – RENIN INHIBITOR(S) COMPLEX (in vitro)**

**Zn\(^{2+}\)**
- Inhibitor – Zn\(^{2+}\) Complex
  - + moderately active Renin
    - Little or no Angiotensin I
    - Little or no Angiotensin II

**Cd\(^{2+}\)**
- Inhibitor – Cd\(^{2+}\) Complex
  - + highly active Renin
    - More Angiotensin I
      - More Angiotensin II
    - Much more Angiotensin II
      - Little or no Angiotensin II

**Cd\(^{2+}\)**
- Inhibitor – Cd\(^{2+}\) Complex
  - + highly active Renin
    - Much more Angiotensin I
      - Much more Angiotensin II

**Dialysate**

**Figure VII: Scheme of Suspected Renin-Angiotensin System Phenomena**
to disappear, or certainly to decrease markedly. The decrease in activity shown may reflect any interfering characteristics of the proposed inhibitor solution toward renin activity. The use of heat to complete the evaporation of the chloroform may have altered the structure of a possible phospholipid inhibitor.

The samples of "inhibitor"-renin and control-renin prepared in the second dialysand experiment were incompletely assayed. Preliminary assays reported the inhibitor-renin sample to have an activity in excess of the activity previously assayed for the control-renin sample. The control-renin sample assays reflected a loss of nearly one-half the activity as previously assayed. Because the activity of the Zn$^{2+}$-incubated sample gained (5%) activity during EDTA dialysis, the dialysand of this dialysis was suspected to contain a small amount of renin inhibitor. However, this gain in activity, as previously mentioned, may be due to dilution of the Zn$^{2+}$-incubated sample, and actually no inhibitor may be present in the dialysand extracted in this second experiment.

"Inhibitor"-renin and control-renin samples from the third dialysand extraction experiment were assayed and found to have activities of 2.64 units/ml and 3.30 units/ml, respectively. As in the first dialysand extraction experiment, the "inhibitor"-renin sample showed only a slight decrease in activity. Because the Cd$^{2+}$-incubated sample did not gain activity (and in fact lost
activity) in the Step VI-EDTA dialysis, the "inhibitor"-renin sample was not suspected to illustrate an inhibitor-induced loss of activity in this extraction experiment. The slight decrease in renin activity of the "inhibitor"-renin sample in the first extraction experiment may be interpreted to be independent of inhibitor effect, but the difference of organic solvents used should be noted.

Results of dialysand extraction indicated that a renin inhibitor was not effectively extracted from aqueous solutions. Conclusive evidence for the presence of a renin inhibitor in any of the dialysand extractions was not found.

As reported by SEN, SMEBY, and BUMPUS, the renin inhibitor is suspected to be a phospholipid similar to bovine phosphatidylserine. Phosphoglyceride phospholipids include phosphatidylserines. The general structure and reactions of phosphatidylserines are given in Figure IX with the structure proposed by SEN, SMEBY, and BUMPUS for the renin inhibitor. The structure of the inhibitor is integral for inhibitory activity. Removal of one of the fatty acids from the inhibitor is necessary for conversion from the inactive precursory inhibitor form of the inhibitor to the active inhibitor. Snake venom Phospholipase A performs this beta-cleavage to a lysophosphatide in vitro. Inhibitory activity is destroyed by removal of both fatty acids, of the phosphoamino group, or of the amino acid. Exposure of the inhibitor in the dialysand extraction experiments to water
Phospholipids: general structure

\[ \text{NON POLAR END} \]

\[ \text{Polar End} \]

\[
\begin{align*}
\text{H}_2\text{C} - \text{O} - \text{C} - \text{R}_1 \\
\text{HC} - \text{O} - \text{C} - \text{R}_2 \\
\text{H}_2\text{C} - \text{O} - \text{P} - \text{O} - \text{CH} - \text{CH} - \text{COOH} \\
\text{OH} \quad \text{R}_3 \quad \text{NH}_2
\end{align*}
\]

Phosphatidylserines: general structure and reactions

\[
\begin{align*}
\text{HYDROPHOBIC END} \\
\text{HYDROPHILIC END} \\
\text{(If } R_1 \text{ is } -\text{CH}_2\text{CH}_2\text{R})
\end{align*}
\]

\[
\begin{align*}
\text{Fatty acid } \beta\text{-oxidation to unsaturated or } \beta\text{-OH fatty acid} \\
\text{Lysophosphatidase} \\
\text{Snake venom Phospholipase A}
\end{align*}
\]

\[
\begin{align*}
\text{Phosphatidase B} \\
\text{H}_2\text{O} \\
\text{H}_2\text{C} - \text{O} - \text{C} - \text{R}_1 \\
\text{HC} - \text{OH} \\
\text{H}_2\text{C} - \text{O} - \text{P} - \text{O} - \text{CH} - \text{CH} - \text{COOH} \\
\text{OH} \quad \text{R}_3 \quad \text{NH}_2
\end{align*}
\]

\[
\begin{align*}
\text{H}_2\text{C} - \text{O} - \text{C} - \text{CH}_2 - \text{CH} - \text{R}' \\
\text{HC} - \text{O} - \text{C} - \text{R}_2 \\
\text{H}_2\text{C} - \text{O} - \text{P} - \text{O} - \text{CH} - \text{CH} - \text{COOH} \\
\text{OH} \quad \text{R}_3 \quad \text{NH}_2
\end{align*}
\]

\[
\begin{align*}
\text{H}_2\text{O} \\
\text{Lysophosphatidase} \\
\text{HC} - \text{OH} \\
\text{H}_2\text{C} - \text{O} - \text{P} - \text{O} - \text{CH} - \text{CH} - \text{COOH} \\
\text{OH} \quad \text{R}_3 \quad \text{NH}_2
\end{align*}
\]

\[
\begin{align*}
\text{H}_2\text{C} - \text{O} - \text{C} - \text{CH}_2 - \text{C} - \text{R}_1 \\
\text{HC} - \text{O} - \text{C} - \text{R}_2 \\
\text{H}_2\text{C} - \text{O} - \text{P} - \text{O} - \text{CH} - \text{CH} - \text{COOH} \\
\text{OH} \quad \text{R}_3 \quad \text{NH}_2
\end{align*}
\]

**Figure IX: Phosphatidylserines—General Structure and Reactions**
and air may have oxidized the inhibitor precursor molecule to a form from which activity could not be derived. One end of the phosphatidylserine molecule is hydrophilic; the other is hydrophobic. Extraction from an aqueous dialysand with a solvent, non-aqueous, organic solution is expected to be successful. The solubilities of phosphatidylserines differ and are determined by x-group amino acids and amino acid side chains. The phosphatidylserine amino acid is serine, an hydroxy-amino acid; however, the side chain of the phosphatidylserine-like renin inhibitor is unknown. That the renin inhibitor is soluble in acetone, petroleum ether, 2:1 and 4:1 chloroform, methanol, ethyl acetate, and ethyl ether may be deduced from the SEN, SMEBY, and BUMPUS paper. Phosphatidylserines are usually insoluble in alcohol. Knowledge of the negative charge on the polar group (serine) and the unsaturated fatty acid in the non-polar region of phosphatidylserines may further facilitate understanding of the possible effects of renin inhibitor extraction techniques.

RESULTS AND DISCUSSION:

Discrepancy Between Standard Hypertensin II Batches

According to pressor response in bioassay, the first-batch standard Hypertensin II was found to have only two-thirds the activity of the second-batch standard. A renin sample assayed using the first-batch standard Hypertensin II for calculation of renin activity was expected to display 50% higher activity.
than a sample assayed using the second-batch standard. Renin samples assayed, using second-batch standard, in the inhibitor-isolation experiment following this standards experiment, were expected to display two-thirds the renin activity normally shown by similarly-treated and previously-assayed (using first-batch standard) samples. The second-batch standard maintained activity through freezing and defrosting; however, a loss of activity was observed in samples which had been defrosted for more than two days. In the subsequent experiment second-batch standards were stored frozen until use and were not used after having stood defrosted for more than two days.

RESULTS AND DISCUSSION:
An Attempt to Isolate a Dialyzable Phospholipid Renin Inhibitor

The inhibitor-removing action of simultaneous dialysis and cation-incubation on renin in the Cd-Tris-saline dialysis in Step II (see Figure VII) should, theoretically, remove a maximum amount of a renin inhibitor from the dialyzed sample. Extraction of the dialysand with an organic substance, in which a phosphatidylserine-like renin inhibitor is believed to be soluble, should result in the isolation of such an inhibitor. Incubation of a dilution of the kidney homogenate supernatant with an inhibitor-containing aliquot, should depress homogenate supernatant markedly or completely.

Although second-batch Hypertensin II was used to calibrate renin activity in this experiment, the activity of the diluted
homogenate supernatant before dialysis was in excess of, and not diminished (as expected) from, the activity of a similarly-treated and previously-assayed dilution of homogenate supernatant. Further dilution of the diluted homogenate supernatant with an equal volume of Tris-saline (net four-fold dilution) failed to decrease activity significantly (1.6% decrease).

CdCl₂, 0.003 M, in the presence of renin and Tris-saline caused a small amount of precipitate to be formed inside the dialysis sacs during dialysis. The activity of the dialyzed renin sample from Step 2, assayed from a four-fold dilution and corrected to undiluted sample activity, was approximately three-times the activity shown by a preliminary undiluted, dialyzed sample. CdCl₂, 0.001 M, 0.002 M, and 0.003 M, injected with Tris-saline intravenously in the rat caused immediate and marked depression of blood pressure (8-16 units) followed by eventual (about seven minutes) resumption of baseline pressure (contrast Maier, et al. 86). This Cd²⁺ phenomenon suggests that the activity of the diluted, dialyzed sample from Step 2 may have been depressed by the presence of CdCl₂ during assay. Consequently, the activities of diluted and dialyzed samples may have been comparable in the absence of CdCl₂. In Step 5, EDTA, 0.002 M, was found to have no effect on the assayed renin activity of an eight-fold dilution of the homogenate supernatant. The activities of the four-fold dilution and this eight-fold dilution of homogenate supernatant were comparable, ± 15%. However, the eight-fold dilution which had been incubated with "inhibitor" solution showed an activity of only one-third that shown by the control (eight-fold homogenate
supernatant dilution) and the EDTA-incubated, eight-fold dilution samples. Evidence for the presence of a renin inhibitor was again not conclusive, although the activity of the "inhibitor"-renin incubate was depressed to a greater extent in this experiment, when equivalent (all eight-fold) dilutions were compared, than in the previous inhibitor extraction experiments. In this experiment, only chloroform was used to extract an "inhibitor" from the dialysate of Cd-Tris-saline dialysis in Step 2. Previous uses of organic and polar solvent mixtures for extraction of aqueous dialysands may have failed due to the presence of excess aqueous solvents.

The "inhibitor"-renin incubate was found to be high in Cd\(^{2+}\) (approximately 0.002 M) and, according to the Cd\(^{2+}\)-phenomenon investigated above, high Cd\(^{2+}\) content may have been responsible for the renin activity depression observed in the "inhibitor"-renin incubate. Whether a Cd\(^{2+}\)-free "inhibitor" solution will depress the renin activity of an eight-fold dilution of the homogenate supernatant remains to be investigated.

Renin activities assayed in four-fold dilutions of the samples dialyzed in Steps 2 and 6 were compared. Removal of Cd\(^{2+}\) by EDTA-dialysis in Step 6 increased assayed renin activity by more than two-fold. According to this data, the addition of a Cd\(^{2+}\)-rich "inhibitor" solution to a dilution of Cd\(^{2+}\)-free homogenate supernatant may have been responsible for much, if not
all, of the depression of assayed renin activity shown in Step 5.

If the "inhibitor" solution does not contain a renin inhibitor, a possible error in inhibitor extraction lies in exposure to the air of the dried chloroform-extract from the Cd-Tris-saline dialysand. As mentioned above, oxidation of the proposed phosphatidylserine-like inhibitor structure can result in loss of activity. Because the spatial configuration of renin in unknown as yet, inhibitor oxidation possibilities cannot be discussed thoroughly. Another possibility of error lies in the Cd-Tris-saline dialysand. An inhibitor was expected to be in this dialysand because from the previous Cd$^{2+}$ and Zn$^{2+}$ renin activation experiment, Cd$^{2+}$-renin dialysis resulted in continued elevation of renin activity. For this reason, a dialyzable inhibitor-Cd$^{2+}$ complex was believed to exist. If this complex, as in the inhibitor isolation experiment, was dialyzed against Cd$^{2+}$-Tris-saline, the inhibitor-Cd$^{2+}$ complex would have been expected to leave the renin sample in the dialysis sacs and enter the dialysate while Cd$^{2+}$ would have replaced the complex by entering the dialysis sacs. The apparent increase in renin activity shown by the EDTA-dialyzed sample (dilution) over the Cd-Tris-saline dialyzed sample (dilution) may have been due to the depression of rat blood pressure by the presence of Cd$^{2+}$ in the latter sample, and not to a loss of a renin inhibitor. However, if a renin inhibitor were dialyzed from the renin sample
by EDTA-dialysis, extraction of the previous (Cd-Tris-saline) dialysand would have been fruitless. A third reason that the inhibitor failed to be demonstrated in the inhibitor-renin incubate may be that because the initial (homogenate supernatant) activity was abnormally high, the steer kidney used in this preparation contained little or no inhibitor.

Because the activities of diluted and undiluted renin samples were found to be comparable in this experiment, the volume correction calculations used in the Cd\(^{2+}\) and Zn\(^{2+}\) renin activation experiment (and Maier, et al. 86) may have been invalid. Care must be taken to compare samples of equivalent dilution from the homogenate supernatant to avoid this suspected dilution error. The zinc-incubated sample in the Cd\(^{2+}\) and Zn\(^{2+}\) renin activation experiment was the only sample experiencing dilution (0.997 x volume of homogenate supernatant) in the final (EDTA) dialysis. The linear volume correction leading to corrected renin activity of the zinc sample would have led to a slightly higher activity than existed. The zinc sample was also the only EDTA-dialyzed sample reported to have gained activity from the corresponding Tris-saline-dialyzed sample. Invalid volume correction calculations may be responsible. Renin activities of various dilutions of a renin sample are currently being compared in bioassay in an attempt to determine the valid volume corrections.

RESULTS AND DISCUSSION:
Investigation of Pre-Slaughter Steer Diets for High Cd\(^{2+}\) and Zn\(^{2+}\) Contents
Alco Packing Company receives only about eighty steers for slaughter per year. Most of the beef distributed by Alco comes from dairy cattle. Only the steers are raised for beef on a semi-standard program. The steer program recommended by Agway is not used universally and to trace the steer from which the kidney renin preparations were made would not be possible. Therefore, the following information on steer diets can be used only for an indication of dietary-Cd\(^{2+}\) and -Zn\(^{2+}\) effects on the kidneys treated in our laboratory.

Depending on the age of the steer, a fattening program including extra protein is used in pre-slaughter diets for two to six months. In this program the bulk of the steer diet consists of grain feeds, "55% Beef Mix" and "32% Beef Mix-ND," regardless of the season. (55% Beef Mix contains grains and hormone diethylstilbestrol, and 32% Beef Mix-ND (non-drug) is used for extra protein when the daily limit of hormone has been eaten). Another grain feed, "32% Beef Mix" (containing hormone), may be used in approximately double quantities in place of 55% Beef Mix. The remainder of the steer diet consists of hay (free choice or limited), corn silage (free choice), and high moisture corn (free choice). Beginning in May, pasture grass or legume roughage is available, and a steer slaughtered in June or in July will have had two or three months of pasture-grass contributing to his diet. However, the bulk of his pre-slaughter diet remains a Beef Mix grain.
<table>
<thead>
<tr>
<th>SUBSTANCE</th>
<th>BEEF MIX</th>
<th>CADMIUM CONTENT* (μg/g dry weight)</th>
<th>ZINC CONTENT* (μg/g dry weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat Bran</td>
<td>32% - ND</td>
<td>0.88</td>
<td>99.4-100.4</td>
</tr>
<tr>
<td>Cane molasses</td>
<td>32% - ND</td>
<td>0.83-0.86</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>55%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corn gluten</td>
<td>32% - ND</td>
<td>0.46-0.57</td>
<td>45-52</td>
</tr>
<tr>
<td></td>
<td>55%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alfalfa</td>
<td>55%</td>
<td>-</td>
<td>5.7-29.6</td>
</tr>
<tr>
<td>Dried grains</td>
<td>32% - ND</td>
<td>0.3-0.49</td>
<td>6-51.4</td>
</tr>
<tr>
<td>winter wheat</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>winter rye</td>
<td></td>
<td></td>
<td>35-43.6</td>
</tr>
</tbody>
</table>

**TABLE III:** CADMIUM AND ZINC CONTENT IN PRE-SLAUGHTER STEER DITES

* according to SCHROEDER, et al.,*74
Examination of the declared contents of a typical pasture seed (Forage Mixture Number 1-2) sold by Agway revealed no information about Cd$^{2+}$ or Zn$^{2+}$ contents. The 55% Beef Mix was advertised to contain five substances suspected by Schröeder, et al.\textsuperscript{74} to be high in Cd$^{2+}$ (more than 0.5 mg/g dry weight) or Zn$^{2+}$ (more than 15 mg/g dry weight). The 32% Beef Mix-ND contains one additional substance which is believed to be high in both Cd$^{2+}$ and Zn$^{2+}$. Table III shows this data. As previously stated, these data cannot give conclusive evidence as to dietary effects of seasonally varying Cd$^{2+}$ and Zn$^{2+}$ effects. No such seasonal variation is observable, however, but a high winter Cd$^{2+}$ or Zn$^{2+}$ diet has not been disproved in such special cases as the steer from which the January kidney preparation was made.

By the standards given above for high Cd$^{2+}$ and Zn$^{2+}$ content per g dry weight, the Purina Laboratory Chow, used by our laboratory to feed the assay rats, is high in both Cd$^{2+}$ (0.63 mg/g dry weight) and Zn$^{2+}$ (58.1 mg/g dry weight). However, the quantities eaten per day by a rat are so small that a rat must eat four-to-five-times his body weight in Laboratory Chow to induce hypertension, according to calculations based on Schröeder's\textsuperscript{73} Cd$^{2+}$-induced hypertension experiment in which 0.5 mg/ml drinking water was sufficient to induce hypertension.

**SUMMARY:**

Crude renin preparations from steer kidneys were incubated
with Cd$^{2+}$ and with Zn$^{2+}$. Elevation of renin activity resulted in both preparations. Dialyses of each preparation against Tris-saline, and finally EDTA, removed the cations; however, renin activity remained elevated. An indirect activation of renin by Cd$^{2+}$ and, to a lesser extent, Zn$^{2+}$, was thus demonstrated.

Removal of the cations by Tris-saline and EDTA-dialyses was suspected to involve the loss by dialysis of a renin inhibitor. An isolation technique for a proposed phospholipid renin inhibitor similar to bovine phosphatidylserine, reported by another laboratory, was thought to be unnecessarily arduous due to the probable location of an inhibitor in the Tris-saline- and EDTA-dialysands (the sample outside the dialysis sacs) of Cd$^{2+}$ and Zn$^{2+}$-incubated renin samples.

Extractions of Tris-saline-and EDTA-dialysands of Cd$^{2+}$- and Zn$^{2+}$-incubated renin samples for a phospholipid renin inhibitor were attempted using various mixtures of organic and polar solvents. Renin samples incubated with reconstituted "inhibitor"-ex extractions did not demonstrate marked or total activity depression.

The dialysand of an highly active kidney homogenate supernatant dialyzed against Cd-Tris-saline was extracted with chloroform and evaporated. The proposed "inhibitor" residue was reconstituted, treated, and incubated with a renin sample. Marked
depression of renin activity resulted; however, this may have been due either to a high Cd\textsuperscript{2+}-content or to the presence of an active renin inhibitor.

Dilution of renin samples and the assayed resultant activities were not linear according to initial observations. Data of earlier experiments had been interpreted using a linear dilution-volume relationship and, pending further investigation of a possible non-linear relationship, will be held in abeyance.

Pre-slaughter steer diets were examined for, but failed to show a winter increase in Cd\textsuperscript{2+}- or Zn\textsuperscript{2+} content. Such a phenomenon would have explained the uniquely high activity of the January steer kidney renin preparation in terms of diet-induced renal hypertension. Little or no inhibitor would be expected in such a kidney preparation.

Major areas which have not been investigated are, first, extraction of the EDTA-Tris-saline dialysand for a phospholipid renin inhibitor, and second, incubation of a (Cd\textsuperscript{2+}-free) renin sample with a Cd\textsuperscript{2+}-free "inhibitor" solution extracted in the manner described in the inhibitor isolation experiment reported here.
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2. Ibid., p. 38.
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4. Ibid., p. 17.
5. Ibid., p. 25.


59. Ibid., p. 43.
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82. Ibid., p. 165.
83. Ibid., p. 163.
84. Ibid., p. 115.
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88. Ibid., p. 23.
89. I. H. PAGE et al., in C. C. WINTER, loc. cit., p. 28.


92. E. B. Frolich, University Hospital, Oklahoma City, Oklahoma (personal communication).


A Dialyzable Phospholipid Renin Inhibitor

by

Donna L. Webber

An Abstract
Submitted in partial fulfillment of the requirements for the Senior Scholars Program

Colby College
1970
A Dialyzable Phospholipid Renin Inhibitor
by Donna L. Webber

A phospholipid renin inhibitor has been isolated in another laboratory from canine kidney by a lengthy procedure. In our laboratory, Cd\textsuperscript{2+} and Zn\textsuperscript{2+} have been shown to activate renin samples, and subsequent dialyses of Cd\textsuperscript{2+}- and Zn\textsuperscript{2+}-incubated renin samples have simultaneously removed the cations and increased further the renin activities. The existence of a dialyzable inhibitor-cation complex is suspected. We determined to develop a shorter, simpler method for inhibitor isolation from cation-incubated renin dialysands (the sample outside dialysis sacs). Demonstration of marked or total inhibitor-caused depression of renin activity in proposed-inhibitor - renin incubates would constitute success of an inhibitor isolation procedure.

In the first experiment, indirect Cd\textsuperscript{2+} - and Zn\textsuperscript{2+} - renin activation was demonstrated. Dialysands from Cd\textsuperscript{2+} - and Zn\textsuperscript{2+} - renin sample incubates were reserved for the second experiment.

A literature survey was made to familiarize the author with the historical and contemporary theories and developments in renal hypertension research. A study of phosphatidylserines followed.

In the second experiments, three of the Cd\textsuperscript{2+} - and Zn\textsuperscript{2+} - incubated renin sample dialysands reserved in the first exper-
iment were extracted for a phosphatidylyserine--like renin inhibitor reported to be isolated in another laboratory. The reported isolation was achieved by an extraction from a complete-kidney preparation, however, our extractions were from dialysands of complete-kidney preparations. For this reason we expected to develop a shorter, simpler isolation procedure than the reported method. A conclusively successful inhibitor isolation procedure was not achieved in the second experiments.

In the third experiments, a relationship between activities of renin samples assayed with first-batch standard Hypertensin II and those assayed with second-batch Hypertensin II was established.

In the fourth experiments, a Cd\(^{2+}\)-incubated renin sample was dialyzed against Cd-Tris-saline and the dialysand was extracted with chloroform. We hypothesized that the inhibitor-Cd\(^{2+}\) complex would dialyze into the dialysand, a solution of low inhibitor-Cd\(^{2+}\) complex concentration, and that the Cd\(^{2+}\) from the Cd -Tris-saline would replace the complex inside the dialysis sacs (where the concentration of free Cd\(^{2+}\) would be low because most of the Cd\(^{2+}\) would be involved in the complex). The extraction was thoroughly stirred, separated from the aqueous phase, and evaporated. Upon reconstitution, the proposed "inhibitor" solution was treated with a cation exchanger. The resultant depression of bioassayed activity of an "inhibitor"-renin sample incubate could not be conclusively identified as inhibitor-induced. The cation exchange treatment was incomplete and the lowering effect
on the assay-rat's blood pressure of a concentration of Cd\textsuperscript{2+} similar to that remaining in the "inhibitor"-renin incubate may have been partly or totally responsible for apparent depression of incubate activity. Nonetheless, the homogenate supernatant had abnormally high renin activity and dialyses did not increase renin activity as markedly as in the previous Cd\textsuperscript{2+} and Zn\textsuperscript{2+} renin activation experiment. Possibly the homogenate supernatant contained little or no renin inhibitor(s), and dialysand extraction was therefore unable to isolate any inhibitor. Injections of 0.002 M EDTA were found to have no effect on assayed renin activity (i.e., in vivo), although 0.01 M EDTA is known to inhibit the converting enzyme in vitro. The fourth experiments also indicated a possible non-linear relationship between renin sample dilution and activity, although linear volume corrections had been assumed previously and used for comparison of renin activities.

The fifth experiment was an investigation of, and a failure to find, a possible winter-associated increase in dietary Cd\textsuperscript{2+} or Zn\textsuperscript{2+} in typical pre-slaughter steer feeding programs.

Two suggestions for further work on renin inhibitor isolation stand out after the fourth experiments: first, extraction for a dialyzable, phospholipid inhibitor from the dialysand of the Cd\textsuperscript{2+}-incubated, Cd-Tris-saline-dialyzed renin sample; and second, incubation of a Cd\textsuperscript{2+}-free proposed "inhibitor" solution with a dilution of the (Cd\textsuperscript{2+}-free) kidney homogenate supernatant and assay of this incubate for evidence of inhibitor-induced
renin activity depression.