are currently under way in this laboratory.

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Isolation of a Phospholipid Renin Inhibitor from Kidney*

Subha Sen, Robert R. Smeby, and F. Merlin Bumpus

ABSTRACT: The reactivity of renin added to plasma from nephrectomized dogs increases independent of increase in renin substrate concentration. Addition of plasma proteins from normal dogs to plasma from a nephrectomized dog reduces the reactivity of renin. This suggests the presence of a renin inhibitor in normal dog's plasma of renal origin. The inhibitor was isolated from dog's kidney and shown to be a phospholipid similar to bovine phosphatidylserine but differs in fatty acid content and the structure of the amino acid. The phospholipid completely inhibits the reaction of dog renin with dog renin substrate *in vitro* and single, daily intramuscular injections of the compound reduces the blood pressure of chronic renal hypertensive rats.

Renin, an enzyme released from kidney, reacts with an α-2-globulin in plasma (renin substrate) to release a decapeptide, angiotensin I. Converting enzyme, also present in plasma, rapidly splits histidylleucine from the C-terminal end of angiotensin I to yield an octapeptide, angiotensin II. This latter, the only substance of this system with direct biological activity, is the most potent, natural, pressor substance known. Both peptides are rapidly degraded by peptidases named angiotensinases. The renin–angiotensin system, recently reviewed by Peart (1965) and Page and Bumpus (1961) and summarized by the reactions in Figure 1, may play an important role in the regulation of normal blood pressure, salt metabolism, and some types of experimental and clinical hypertension.

The recent intensified interest in measurement of plasma renin concentration (Page *et al.*, 1965) has demonstrated the need to determine if substances

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exist which affect the rate of formation and concentration of angiotensin II in plasma. It was observed that a constant amount of renin added to human plasma samples, treated with EDTA and diisopropylfluorophosphate to eliminate angiotensinase activity, generated varying amounts of angiotensin during 4 hr of incubation (Pickens *et al.*, 1965). Variations in converting enzyme activity were compensated for by measuring the amount of angiotensin formed in rats which respond almost equally to angiotensin I and angiotensin II. In plasma from patients with essential hypertension, neither renin substrate concentration nor the level of endogenous renin could be related to the amount of angiotensin generated by a constant amount of added renin (Bumpus, 1965). Boucher *et al.* (1964) also observed that generation of angiotensin is more rapid in some plasma samples than in others. These observations suggest the presence of renin activators or inhibitors.

Nephrectomized animals respond to renin with a greater blood pressure rise that is longer lasting than in normal animals (Tigerstedt and Bergman, 1898). Plasma from nephrectomized animals generated angiotensin faster than that from normal animals due either to changes in renin substrate or to unknown factors (Brunner, 1962; Hoobler *et al.*, 1964; Bing, 1964). Our report presents evidence that the change of renin reactivity following nephrectomy is due to loss of a renin inhibitor. The isolation of this inhibitor and its partial characterization are described.

Methods and Materials

Assay of Dog Renin and Renin Substrate. Indirect assay was conducted as described previously for human renin (Pickens *et al.*, 1965) except that each sample was extracted with ether. Briefly, the assay involves dialysis of the sample against EDTA solution and then water, followed by incubation for 4 hr at 37° in the presence of DFP. The reaction was stopped by heating the sample in a boiling water bath for 10 min, the precipitated protein was removed, and the sample was evaporated to dryness. The residue from dog plasma samples was then washed twice with ether. Excess ether was removed with a stream of air and the residue was dissolved in 0.9% NaCl solution for assay. Results are expressed in terms of nanograms of angiotensin produced per milliliter of plasma during a 4-hr incubation period. For direct assay, the renin sample was injected into a ganglion-blocked, vagotomized rat and the height of blood pressure reponse was compared with that produced by angiotensin standards. Results were expressed as nanogram equivalents of angiotensin and are averages of assays obtained on at least three different rats.

Renin substrate concentration was measured by incubation as described above with an excess of added dog renin and expressed as units of substrate. A unit of substrate is defined as that amount which will produce 1 ng of angiotensin when incubated with an excess of renin in the absence of salt.

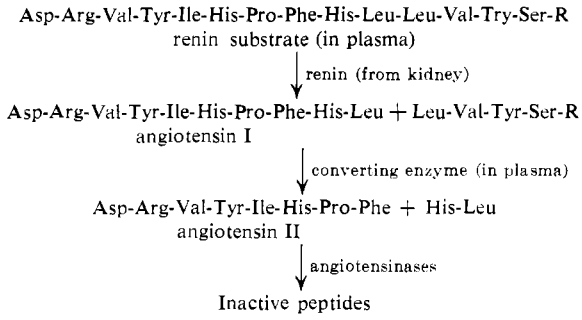


FIGURE 1: Reactions of the renin–angiotensin system.

Preparations of Dog Renin Substrate. Normal dog's plasma (400 ml) containing 37,700 units of renin substrate and 30 g of protein, was adjusted to pH 6.5 with 1 N hydrochloric acid and ammonium sulfate was added to a concentration of 1.37 M (83.6 g). The precipitate was removed by gravity filtration and discarded. To the supernatant (390 ml), ammonium sulfate was added to a concentration of 2.0 M (39.4 g). The precipitate was separated by gravity filtration, dissolved in a minimum volume of distilled water, and then dialyzed against distilled water for 24 hr to remove ammonium sulfate. The small amount of protein that precipitated during dialysis was removed by centrifugation and discarded. The final solution (62 ml) contained 86,100 units of renin substrate and 4.6 g of protein. To this solution, ammonium sulfate was added to a concentration of 1.0 M (9.14 g) and the pH was adjusted to 2.5 with 1 N HCl. After 1 hr at room temperature (24°) the pH was adjusted to 4.0 with 2 N NaOH and the precipitate was removed by centrifugation at 7850*g* for 30 min. To the supernatant (98 ml), ammonium sulfate (15.7 g) was added to make the solution 2.0 M and the precipitate was removed by centrifugation. The precipitate was washed once with 2.0 M $(NH_4)_2SO_4$ solution, dissolved in water, and dialyzed against EDTA solution (2.2 g/l.) for 24 hr and then against distilled water for 24 hr. The final solution contained 71,500 units of renin substrate and 1.41 g of protein. Except where noted above, all steps were conducted at 3–4°.

Preparation of Dog Renin. The entire procedure, except where noted, was carried out at 3–4°. Dog renin was prepared using a modification of the procedure of Plentl and Page (1943) followed by ammonium sulfate fractionation. An acetone powder was prepared by mincing 1710 g of dog kidney with 3.8 l. of acetone. The insoluble material was separated by filtration, washed twice with 1600 ml of acetone each time, and air dried overnight (16 hr) at room temperature. The dried acetone powder was mixed with 3 l. of 0.9% sodium chloride solution and the insoluble material was removed by centrifugation at 480*g* for 45 min. The insoluble material was washed with 1 l. of 0.9% sodium chloride and again separated by centrifugation at 480*g*. The combined supernatant fractions were then centrifuged at 70,000*g* for 45 min. The clear

supernatant (3 l.) contained 56 g of protein and renin pressor activity equivalent to 90,000 ng of angiotensin (1.6 ng of activity/mg of protein). To this solution was added 36 g of sodium chloride and the pH was adjusted to 4.0 with glacial acetic acid. The precipitate was removed by gravity filtration and discarded. The filtrate was saturated with sodium chloride at 25° and the precipitate was removed by gravity filtration, dissolved in about 600 ml of water, and dialyzed to remove excess sodium chloride. The solution, after dialysis, had a volume of 650 ml and contained 12.2 g of protein and renin pressor activity equivalent to 59,200 ng of angiotensin (4.8 ng of activity/mg of protein). After dilution to 1870 ml, the solution was brought to pH 7.5 and 269.3 g of ammonium sulfate was added. The precipitate was removed by gravity filtration and discarded, and 295.5 g of ammonium sulfate was added to the filtrate. This precipitate was collected by gravity filtration, dissolved in water, and dialyzed for 24 hr against distilled water to remove ammonium sulfate. The solution (380 ml) after dialysis contained 1.21 g of protein and renin pressor activity equivalent to 70,000 ng of angiotensin. After addition of 188.9 g of ammonium sulfate, the precipitate was removed by gravity filtration, dissolved in water, and dialyzed against distilled water overnight. Following dialysis, the solution had a volume of 300 ml, contained 0.9 g of protein, and had a renin pressor activity equivalent to 92,000 ng of angiotensin (10 ng of activity/mg of protein). To conserve protein, from this stage of purification specific activity was expressed in terms of nanograms of renin activity which gave an absorbance of 1 at 280 mμ. This sample had 98 ng of renin activity/absorbance unit at 280 mμ.

CM-cellulose was washed with 0.001 M phosphate buffer at pH 5.5 and 5 mg of the above protein was added/g of CM-cellulose. A total of 3000 ng of renin activity was used. After 15 min, the CM-cellulose was removed by centrifugation and the supernatant was discarded. The CM-cellulose was washed with 10 ml of 0.001 M phosphate buffer/g of cellulose at pH 5.5 and the wash was discarded. Renin was eluted from CM-cellulose by stirring with 5% sodium chloride in 0.001 M phosphate buffer at pH 5.5 for 15 min (approximately 10 ml of eluting solvent/g of CM-cellulose). The CM-cellulose was washed once with 5% sodium chloride in 0.001 M phosphate buffer. The eluate and wash solutions were combined, concentrated to a small volume under reduced pressure, and dialyzed overnight against distilled water. The yield of renin was 2400 ng of activity (80%) with a specific activity of 308 ng of renin activity/absorbance unit at 280 mμ. This was purified further by chromatography on CM-cellulose.

A column (1.5 × 6.5 cm) was packed by pouring a slurry of CM-cellulose in 0.001 M phosphate buffer into the column and removing the excess solvent. The renin sample from above, containing 2400 ng of activity, was applied to the column. The column was eluted with 5% sodium chloride in 0.001 M phosphate buffer. Fractions of 6 ml were collected and the renin activity was eluted in fractions 65–73. The active fractions were pooled, concentrated to a small volume, and dialyzed overnight against 0.15 M pyrophosphate buffer at pH 7.0. The yield of renin was 1800 ng of renin activity (75%) with a specific activity of 2130 ng of renin activity/absorbance unit at 280 mμ. This enzyme preparation was used for all experiments.

Isolation of Renin Inhibitor. The acetone extract of 3592 g of kidney, prepared as described above, was evaporated to dryness under reduced pressure. The residue (135 g) was extracted with 1 l. of chloroform–methanol mixture (2:1), filtered, and evaporated to dryness. It was dissolved in 1 l. of chloroform–methanol (2:1) and the solution was washed five times with one-fifth this volume of distilled water at 4° (Folch *et al.*, 1957). The organic phase was then taken to dryness under reduced pressure. The residue (63.7 g) was dissolved in a minimum of petroleum ether (bp 30–60°) and ten volumes of acetone was added (Hanahan *et al.*, 1957). After 24 hr at −24°, the precipitate was removed by centrifugation and washed three times with acetone. This crude lipid (18.5 g) was then fractionated into major phospholipid classes as described by Hanahan *et al.* (1957).

Silicic acid (75 g) (Mallinckrodt, 100 mesh, chromatographic grade), activated by heating overnight at 110°, was suspended in a chloroform–methanol (4:1) system and poured into a column. The flow rate was adjusted to 1.5–2 ml/min. After the silicic acid had reached a constant level, the solvent was allowed to flow until it reached the surface of the silicic acid forming a packed column of 3 × 40 cm. The phospholipid (1.35 g) was dissolved in a small volume of the same chloroform–methanol mixture and pipetted into the column. Silicic acid (1 g) was used for each 0.8 mg of phospholipid phosphorus applied to the column (Hanahan *et al.*, 1957). The column was developed with the following solvents: (1) chloroform–methanol (4:1), volume used 750 ml; (2) chloroform–methanol (3:2), volume used 350 ml; and (3) chloroform–methanol (1:4), volume used 350 ml. The eluate obtained with solvent 1 contained all inhibitor activity and was evaporated to dryness under reduced pressure in a nitrogen atmosphere (yield 412 mg). This fraction contained phosphatidylserine, phosphatidylethanolamine, and possibly neutral lipid in addition to the inhibitor and these were separated by chromatography on another silicic acid column as described by Tinker and Hanahan (1966).

This column, prepared as described before from 69 g of silicic acid, was 3 × 40 cm. The sample (412 mg) was applied to the column in chloroform–methanol (6:1) and it was developed with the following solvent systems: (4) chloroform–methanol (6:1), volume used 550 ml; and (5) ethyl acetate–methanol (3:2), volume used 300 ml. The loading factor was 0.5 mg of phospholipid phosphorus/g of silicic acid. Eluent from solvent system 5 was pooled and evaporated to dryness in a film evaporator under a nitrogen atmosphere (yield 63 mg). If more than 300 ml of eluting solvent 5 was used, phosphatidylserine was

eluted from the column. If phosphatidylserine was present in the sample it was removed using a column similar to that described by Rouser *et al.* (1961). This column was prepared from 25 g of silicic acid with chloroform–methanol (4:1) as described above. The finished column (1 × 20 cm) was washed with 30 ml of chloroform–methanol–ammonia prepared by adding 10 ml of concentrated ammonium hydroxide to 1 l. of chloroform–methanol (4:1). The sample was applied to the column in chloroform–methanol (4:1) and the column was developed with the following solvent systems: (6) chloroform–methanol (4:1), volume used 200 ml; (7) chloroform–methanol (3:2), volume used 150 ml; and (8) methanol, volume used 100 ml. The eluate from solvent system 6 contained the inhibitor and when 75 mg of lipid was applied to the column, 42 mg was recovered in this fraction.

Analytical Procedures. Phosphorus was estimated according to Fiske and Subbarow (1925). Nitrogen was estimated with Nessler's reagent following digestion with H_2SO_4 and H_2O_2 (LaPage, 1957). For glycerol analysis the lipid sample was hydrolyzed in 2 N HCl for 48 hr at 118° (Renkonen, 1962) and then neutralized with NaOH. The solution was taken to dryness and extracted with isopropyl alcohol. Glycerol in the isopropyl alcohol extract was determined fluorometrically as described by Kessler and Lederer (1966).[1]

Thin layer chromatography was conducted with silica gel G plates (activated by heating for 1 hr at 110° before use) using the following solvent systems: chloroform–methanol–water (95:35:6), (65:25:4), (60:35:8), (95:35:4), and (65:30:5); 1-butanol–pyridine–water (60:40:20); and chloroform–methanol–diisobutyl ketone–acetic acid–water (23:10:45:25:4). Color was developed with ninhydrin or by the charring reaction of sulfuric acid. The amino acid chromatograms were run on Whatman No. 1 paper using butanol–acetic acid–water (4:1:5), propanol–water (3:1), and butanol–acetic acid–water–pyridine (30:6:24:20) as the solvent systems. Color was developed with ninhydrin either before or after spraying with periodate reagent (Giri *et al.*, 1952).

Gas–liquid partition chromatography was conducted on the methyl esters of fatty acids obtained after refluxing the lipid with 4% sulfuric acid in methanol. The fatty acid esters were extracted into petroleum ether and dried over anhydrous sodium sulfate. After removal of the petroleum ether under nitrogen, the residue was dissolved in hexane and applied to the column. The esters were separated at 180° on a 0.25-in. diameter, 6-ft long column packed with 13% ethylene glycol succinate on 80–100 mesh Gas-Chrom P[2] using helium at a flow rate of 60 cc/min as carrier gas with an F & M Scientific Corp. Model 810.[3] Standard fatty acid methyl esters were obtained from Applied Science Laboratories.

Infrared spectra were obtained using a Beckman IR-8 spectrophotometer with samples prepared in KBr pellets or as films prepared on a salt plate by evaporation of solvent. Ultraviolet spectra were obtained using a Beckman DU spectrophotometer.

Phosphatidylserine was obtained from Dr. D. J. Hanahan, and from Applied Science Laboratories. Other phospholipid samples were obtained from General Biochemicals, Inc.

Phospholipid samples were hydrogenated using 50 mg of platinum oxide with 5 mg of lipid dissolved in 18 ml of ethanol and 2 ml of ether at 50 psi for 44 hr in a Parr hydrogenation apparatus.

Isolation of Amino Acid. The phospholipid (12 mg) was hydrolyzed with 5 N HCl in a sealed tube at 118° for 12 hr. The fatty acids were extracted from the hydrolysate with petroleum ether and then the aqueous phase was taken to dryness. The residue was dissolved in water and evaporated to dryness. This process was repeated four more times to remove HCl. The residue was dissolved in water and applied to a 0.8 × 9 cm column of Dowex 50X-12 (100–200 mesh) in the chloride form. The column was washed with 30 ml of water to remove glycerol. The water wash was passed through a column of Amberlite IR-45, free base form (0.9 × 1.5 cm), to remove phosphoric acid. The eluate was taken to dryness and glycerol was identified by mass spectral analysis. The column was then eluted with 30 ml of 2 N ammonium hydroxide. The eluate was evaporated to dryness under reduced pressure to yield 2.9 mg of amino acid.

Preparation of Hypertensive Rats. A silver clip was applied to the left renal artery of Sprague–Dawley rats weighing about 180 g (as described by Wilson and Byrom (1941)) under ether anesthesia. After operation they were permitted food and water *ad libitum.* Blood pressure was measured with a tail cuff using the apparatus of the E & M Instrument Co., Inc., Houston, Texas.

Assay of Renin Inhibitor. The assay system consisted of 4 ml of dog renin substrate containing 10 mg of protein, 0.25 ml of dog renin containing renin activity equivalent to 0.5 ng of angiotensin, 1 ml of ethylene glycol solution containing inhibitor or ethylene glycol alone for control incubations, and 1 drop of DFP in isopropyl alcohol (1:20 dilution). The final solution was usually clear or faintly hazy. The mixture was incubated for 4 hr at 37°; the reaction was stopped by heating in a boiling water bath for 10 min, and the precipitated protein was removed by centrifugation. The angiotensin formed was determined by pressor assay in the pentolinium-treated vagotomized rat (Pickens *et al.*, 1965).

Preparation of Nephrectomized Dogs. Dogs were bilaterally nephrectomized through flank incisions

[1] The glycerol analysis was performed by Dr. C. E. Willis and Mr. S. A. Tytko of the Department of Clinical Pathology, Cleveland Clinic Foundation.

[2] Obtained from Applied Science Laboratories, Inc., State College, Pa.

[3] The gas chromatographic studies were conducted by Dr. Antanas Butkus of our laboratories.

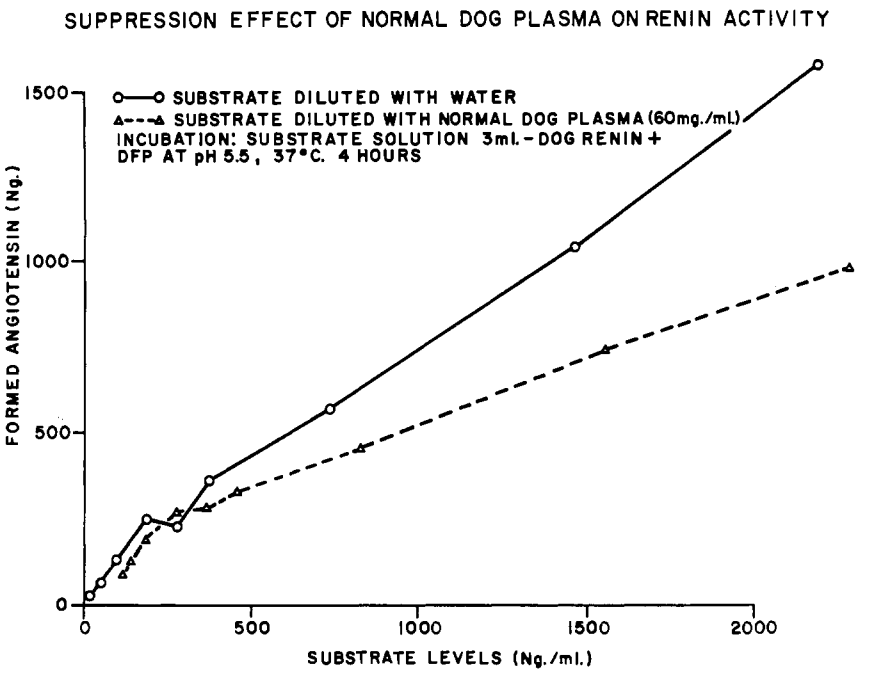


FIGURE 2: Suppression of renin activity by addition of normal dog plasma to purified substrate.

TABLE I: Renin Reaction and Substrate Levels in Plasma from Nephrectomized Dogs.

	Before Nephrectomy		24 hr after Nephrectomy		48 hr after Nephrectomy	
Dog	Angiotensin Formed by Added Renin (ng/ml)[a]	Substrate (units/ml)	Angiotensin Formed by Added Renin (ng/ml)[a]	Substrate (units/ml)	Angiotensin Formed by Added Renin (ng/ml)[a]	Substrate (units/ml)
1	0.4	192	2.8	400	5.6	391
2	0.9	179	1.6	256	2.1	250
3	2.2	110	6.7	202	9.4	218
4	2.6	187	5.3	312	8.2	332
5	6.8	90	12.8	250	27.8	229
Av	2.6	152	5.8	284	10.6	284

[a] The values have been corrected by subtracting the amount of angiotensin formed by endogenous renin.

under barbiturate anesthesia. Upon recovery from anesthesia they were permitted water *ad libitum* and were fed the usual diet once a day.

Results

Plasma from dogs was tested for the level of renin, renin substrate, and the amount of angiotensin generated by a constant amount of added renin before and 24 and 48 hr after bilateral nephrectomy. The results show that, as expected, substrate increases during the first 24 hr following bilateral nephrectomy but then remains constant (Collins and Harakal, 1954; Regoli *et al.*, 1961; Bing, 1964; Blaquier, 1965; Cook and Lee, 1965 (Table I)).

In contrast, renin added to dialyzed plasma from dogs nephrectomized for 24 hr generated more angiotensin than before nephrectomy but less than after 48 hr. This change in the amount of angiotensin generated by a constant amount of renin following nephrectomy

was found to be highly significant ($p < 0.001$).[4] This suggested renin inhibitor disappeared in the absence of kidneys.

To test the renin inhibitor hypothesis further plasma from normal dogs was dialyzed against EDTA and distilled water, lyophilized, and added along with dog renin to plasma from nephrectomized dogs (Table II).

TABLE II: Inhibition of Angiotensin Generation by Renin in Nephrectomized Plasma by Normal Plasma Proteins.

Incubation Mixture	Angiotensin Produced (ng/ml)[a]
Nephrectomized plasma + renin	29.0, 14.7
Nephrectomized plasma + renin + 300 mg of plasma protein	17.0, 14.2
Nephrectomized plasma + renin + 600 mg of plasma protein	12.6, 5.4

[a] The data presented are values obtained using plasma from two different nephrectomized dogs and plasma protein preparations from two different normal dogs.

Increasing the amount of dialyzed plasma protein added decreased the yield of angiotensin formed even though renin substrate was concurrently being added. This experiment was repeated by adding the dialyzed plasma protein (60 mg/ml) and dog renin to a purified substrate solution. The yield of angiotensin was again decreased even though renin substrate was increased (Figure 2) by addition of plasma protein.

These experiments indicated that kidney may be the source of a substance that inhibited the rate of reaction of renin with its substrate. The crude lipid fraction obtained from kidney by extraction with acetone was found to be highly active in inhibiting this reaction *in vitro*. This fraction was further fractionated by conventional methods for phospholipids to obtain a phospholipid which possessed the inhibitory activity and was homogeneous in seven solvent systems on thin layer chromatography. The fractionation procedure used and yields from a typical run are outlined in Figure 3. From 0.01 to 0.2 mg of phospholipid was obtained/g of dog kidney in several trials and in one trial 0.4 mg of phospholipid was obtained/g of hog kidney. Before the first silicic acid column the lipid had 4% phosphorous and 1.6% nitrogen while the isolated phospholipid had 3.8% phosphorous, 1.69% nitrogen, and 8.2% glycerol. The ratio of phosphorous:nitrogen:glycerol was 1:1:0.8.

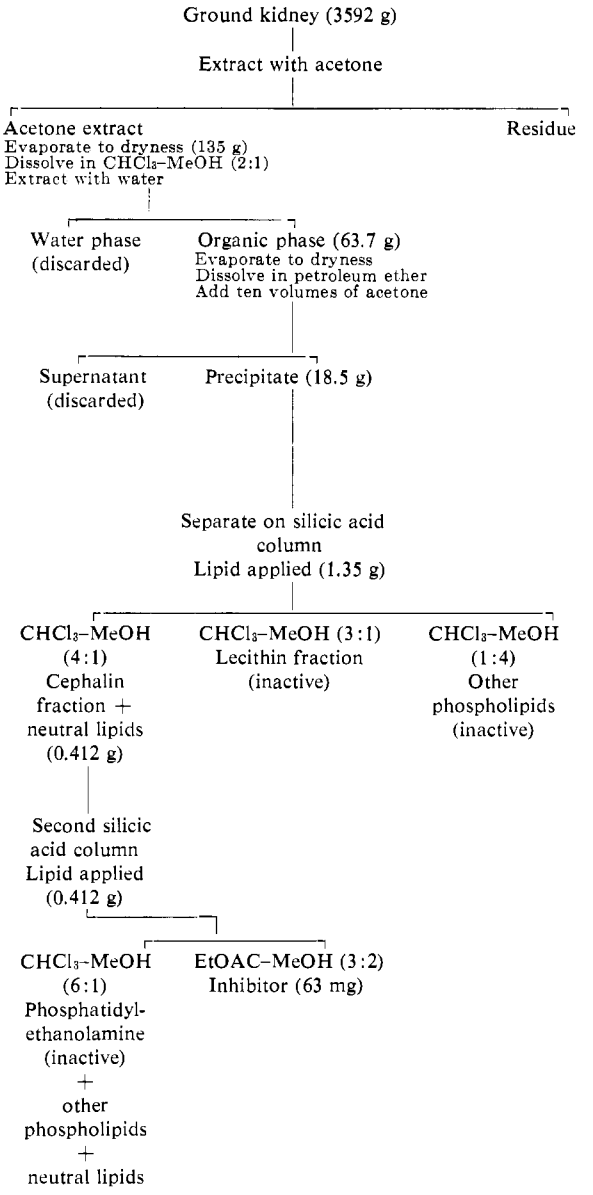


FIGURE 3: Outline of fractionation used to obtain homogeneous phospholipid renin inhibitor.

If kidneys were extracted with chloroform–methanol (2:1), a better yield of crude phospholipids was obtained but it was difficult to separate the inhibitory material from phosphatidylserine. Also, since these solvents destroyed much of the renin activity, acetone is the preferred solvent for extraction of crude lipid.

[4] The statistical analysis of these data was conducted by Professor J. N. Berretoni, Department of Statistics, Western Reserve University, Cleveland, Ohio. His analysis was done in two parts: (1) the additive model, allowing the use of the residual as an error measurement in the analysis of variance, was found to be applicable; and (2) the analysis of variance was then used to test for significance of the data. The change in angiotensin formed by added renin with time was demonstrated to be linear. The amount of angiotensin formed (A) at a given time (T) can be predicted by the equation $A = (A_0 2.11^T/24)$, where A_0 is the amount of angiotensin formed before nephrectomy.

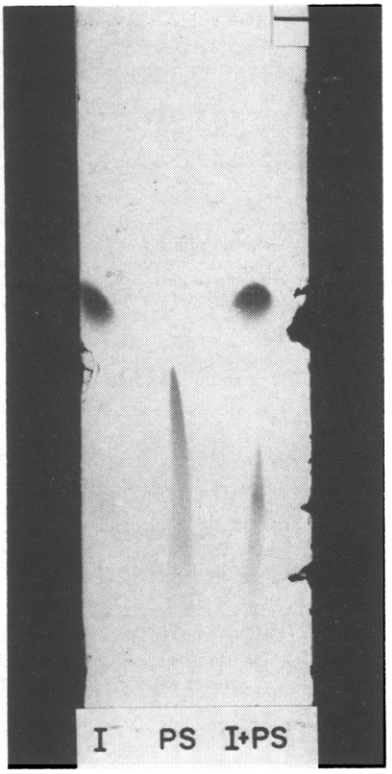


FIGURE 4: Separation of inhibitor (I) from phosphatidylserine (PS) on thin layer chromatography. Color developed with ninhydrin.

The separation of inhibitor from phosphatidylserine was achieved on thin layer chromatography with a solvent system of chloroform–methanol–water (95:35:6) as demonstrated in Figure 4 (D. J. Hanahan, personal communication). The fact that the inhibitor was difficult to separate from phosphatidylserine and that it reacted with ninhydrin suggested that both compounds were similar. On hydrolysis, it yielded an amino acid (R_F 0.39) which reacted with periodate on paper and migrated on paper chromatography differently from serine (R_F 0.15), threonine (R_F 0.32), and homoserine (R_F 0.22). The amino acid could not be extracted from a chloroform solution of the phospholipid with $NaHCO_3$ solution (pH 8). When chromatographed according to Moore *et al.* (1958), the amino acid was eluted from the column as a single peak after tyrosine but before ammonia. The infrared spectrum had a peak at 2.92 μ identified as a hydroxyl group. The ionized amino group should appear about 3.1 μ where a shoulder was seen. These facts suggest this is a β-hydroxy-α-amino acid. The side chain has not been identified. Mild alkaline deacylation, following the procedure of Dawson (1960), yielded a glyceryl-phosphoryl compound (R_F 0.67) which migrated on paper chromatography different from that obtained by deacylation of bovine phosphatidylserine (R_F 0.24). Glycerol was qualitatively identified following hydrolysis by mass spectral data.[5]

Infrared spectra of the inhibitor and phosphatidylserine were very similar (Figure 5). They both exhibited the following peaks of interest which were assigned to the groups shown in parentheses: 3.42 (CH), 3.50 (CH), 3.75 (POH), 5.78 (COOR), 6.1 (COO^-), 6.6 (CONH), 6.9 (CH), 8.1 (P=O), 8.5–8.6 (COR), 9.7 (POC), and 13.9 μ ($(CH_2)_n$). The ultraviolet spectrum showed an absorption maximum at 270 mμ suggesting three conjugated double bonds. The isolated amino acid exhibited no absorption maximum below 220 mμ while the fatty acids dissolved in hexane also exhibited an absorption maximum at 270 mμ.

The fatty acid composition of the inhibitor is shown in Table III. The fatty acid esters were identified by comparing retention times with those of standard esters and by single, symmetrical peaks when unknown and standard esters were chromatographed as a mixture.

TABLE III: Fatty Acid Composition of Isolated Phospholipid.

Chain Length: Double Bonds	Retention Time of Std (min)	Retention Time of Sample (min)	% of Total Fatty Acids in Sample
16:0	5.8	5.8	17.7
18:0	10.3	10.4	14.4
18:1	12.2	12.2	12.4
18:2	15.6	15.6	6.1
20:4	37.3	37.8	37.9
Unidentified (5 minor peaks)	—	—	11.5

The high concentration of fatty acid migrating on gas chromatography as arachidonic acid is unusual in this type of phospholipid (Morgan *et al.*, 1963; Gray and MacFarlane, 1961). Reduction of the inhibitor with hydrogen in the presence of platinum oxide or acid hydrolysis completely destroyed the activity. Following reduction the peaks of 18:1, 18:2, and 20:4 fatty acid esters were not observed on gas chromatography and only peaks due to C_{16}, C_{18}, and C_{20} fatty acid esters were found. Pure bovine phosphatidylserine and prostaglandin E_1 and E_1-217 mixtures were obtained following acid hydrolysis or hydrolysis with phospholipase D (Smeby *et al.*, 1967), and the isolated amino acids were all inactive in the assay system.

When a known amount of angiotensin II was added to the incubation system, with or without the presence

[5] The mass spectralanalysis was conducted by Professor F. W. McLafferty, Department of Chemistry, Purdue University, Lafayette, Ind.

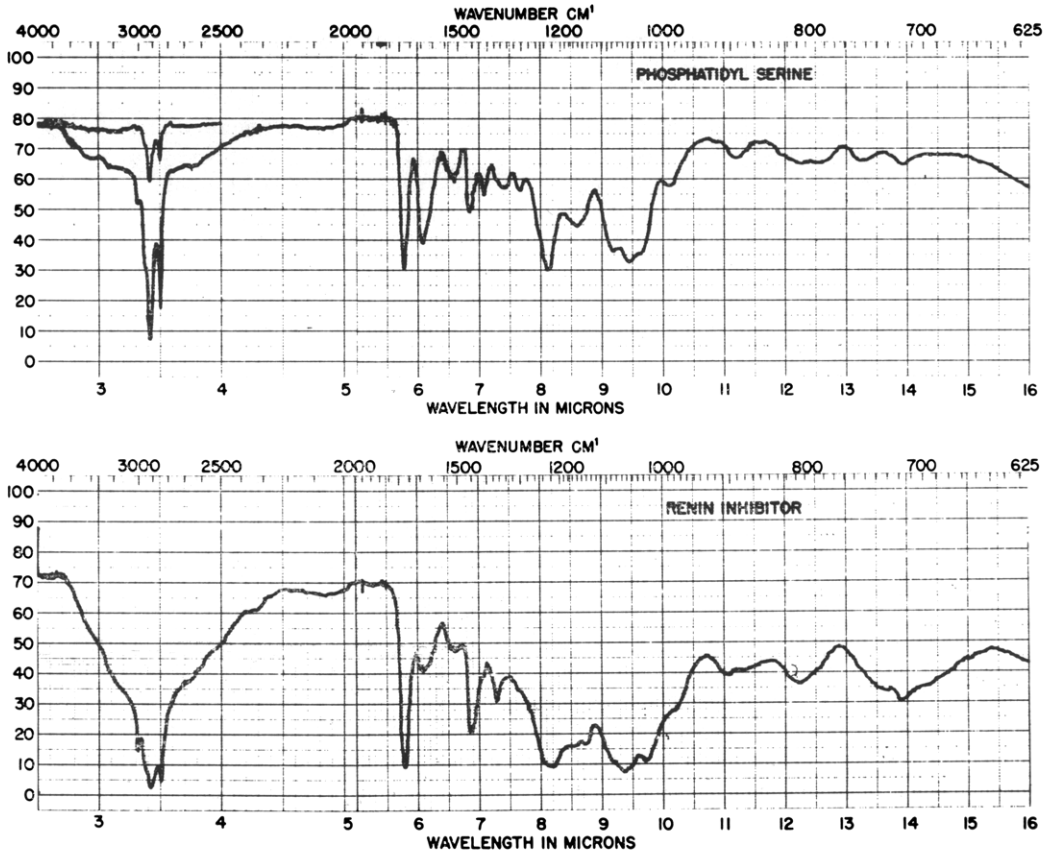


FIGURE 5: Infrared spectra of phosphatidylserine and isolated phospholipid inhibitor run as films on a salt plate.

of inhibitor, the recovery of added peptide was nearly the same. This suggests that the phospholipid inhibits only the reaction of renin with renin substrate and not the pressor response of the rat to angiotensin. In Figure 6, inhibition of renin in the presence of various concentrations of inhibitor is demonstrated. The inhibition increases as the amount of lipid added to the assay system was raised from 0.35 to 3.5 mg.

When renal hypertensive rats were given single, daily intramuscular injections of this phospholipid in a dose of 2.6–6.7 mg/kg the blood pressure fell an average of 42 mm. These data are presented in Table IV. Usually 2–4 days were required to achieve maximum decrease of blood pressure. When the administration of lipid was stopped the pressure gradually rose to the preinjection level. The compound produced no changes in blood pressure when administered to normotensive rats in the same dose range.

Dog renin has been purified about 136 times from a crude extract of acetone-dried dog kidney. This preparation still contained other proteins but was free of lipid inhibitor. This preparation exhibited the same instability as previously reported for hog renin (Haas *et al.*, 1953). The yield of renin and renin substrate in some fractionation steps was greater than 100%. This may be due to removal of inhibitor by this fractionation procedure.

Discussion

The kidney has long been known to possess antihypertensive properties. Page *et al.* (1941) were able to prepare extracts of kidney which lowered blood pressure in renal hypertensive dogs and human beings. Hamilton and Grollman (1958) extracted a substance

FIGURE 6: *In vitro* inhibition of the renin reaction in the presence of varying amounts of inhibitor.

TABLE IV: Blood Pressure Reduction in Hypertensive Rats by Isolated Phospholipid.

Rat	Blood Pressure When Compd Administered (mm)	Min Blood Pressure Achieved (mm)	Max Decrease (mm)	Time Required to Achieve Max Response (days)	Dose (mg/kg)
1	200	150	50	5	5.4
2	190	140	50	5	5.4
3	190	130	60	5	2.9
4	180	140	40	2	2.8
5	180	150	30	3	2.6[a]
6	180	140	40	3	2.6[b]
7	200	170	30	2	6.7
8	180	160	20	2	5.6
9	200	140	60	7	5
10	190	150	40	4	4.8
11	190	145	45	6	5.4
12	200	145	55	4	5 6
13	180	155	25	2	5.4
Av	190	147	42	3.8	—

[a] No response at 1.4 mg/kg. [b] No response at 0.6 mg/kg.

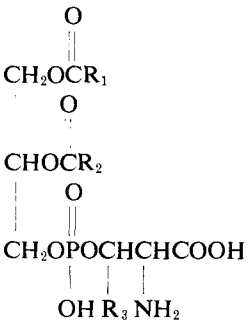
from hog kidneys with aqueous acetone that lowered the blood pressure of hypertensive rats when administered orally. They did not isolate this substance and none of its chemical properties were described. It is not known whether the phospholipid described in this report is the same as the substance described by Hamilton and Grollman (1958), but it is suspected that the two compounds are different, for the substance described by them was insoluble in petroleum ether. Muirhead *et al.* (1966) described a neutral substance obtained from dog renal medulla which lowered the blood pressure of renoprival or renal hypertensive dogs. Again, the chemical properties were not well defined but its solubility in organic solvents and its neutral character suggests it is different from our lipid. Lee *et al.* (1965) isolated a vasodepressor fatty acid, "medullin," from kidney medulla which was similar to prostaglandin E_1. However, prostaglandin E_1 and E_1-217 do not possess renin-inhibitory activity. Further, transplantation of a normal kidney into the circulation of a hypertensive human being (Merrill *et al.*, 1956) or rat (Tobian *et al.*, 1964) results in a reduction in blood pressure.

Page and Helmer (1940) showed that the sensitivity of nephrectomized dogs to renin can be reduced by transfusion with blood from normal dogs and this strongly suggested that an inhibitor was present in normal blood. In cross-circulation experiments between normal and 24-hr nephrectomized rats, the sensitivity of the normal partner increased (Bing and Magill, 1963). It was concluded that a humoral principle was responsible for the increased sensitivity. Our experiments suggest the increased sensitivity is due to reduced concentration of inhibitor. We interpret the transfer of sensitivity by cross-circulation as being a result of dilution of inhibitor present in normal rats.

Blaquier (1965) concluded from kinetic studies that a change in concentration of inhibitor did not occur 24 hr after nephrectomy of rats. It is possible that the change in inhibitor concentration was not great enough following nephrectomy for 24 hr to affect kinetic measurements. Quite impure hog kidney extracts containing renin were used and the rate of reaction could have been modified by inhibitory factor(s) present in the renin itself. The rate of reaction of hog renin with rat substrate may not measure the rate of reaction of rat renin with rat substrate (Arakawa *et al.*, 1965).

The increase in rate of angiotensin production by added renin during the first 24 hr following nephrectomy might be explained by the increase in renin substrate. However, after 24 hr the level of substrate remained constant but the rate of angiotensin production continued to increase. This increase in renin reactivity in plasma from nephrectomized dogs was reversed by addition of normal plasma protein. Following this observation, an *in vitro* assay system was established for this inhibitor and a phospholipid inhibitor was isolated from kidney. The properties of the compound, its infrared spectrum, and analytical data indicate it is nearly identical with phosphatidylserine. On hydrolysis it yielded a β-hydroxy-α-amino acid different from serine or threonine. The most probable structure for

this substance is



where R_1 and R_2 are the hydrocarbon chains of fatty acids. R_3 is an unknown side group of the amino acid. This phospholipid contains an unusually high amount of a fatty acid migrating on gas chromatography as arachidonic acid (Gray and MacFarlane 1961), and biological activity is lost following reduction of this acid. It is not known whether or not this substance is involved in the regulation of normal blood pressure or in the production and maintenance of experimental or clinical hypertension. This compound may not have been detected in other studies of renal phospholipids (Tinker and Hanahan, 1966; Gray and MacFarlane, 1961; Marinetti *et al.*, 1958; Dawson, 1960) because of its difficult separation from phosphatidylserine.

Recently, McCay (1966) reported on microsomal phospholipids that inhibited gulonolactone oxidase. The inhibition of gulonolactone oxidase is reversed by tocopherol or EDTA while these agents are without effect on the inhibition of renin by the phospholipid we have isolated.

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