

## STUDIES ON EXPERIMENTAL HYPERTENSION

### XXI. THE PURIFICATION OF RENIN\*

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Partial constriction of the main renal arteries of dogs and other animals is followed by persistent elevation of the blood pressure (1-5). There is good evidence for the view that the liberation into the blood stream of a substance from the kidney initiates the humoral mechanism that determines the elevation of the blood pressure (6). A saline extract of normal kidney capable of producing a pressor effect when injected intravenously into animals was first described by Tigerstedt and Bergman (7). For the active principle in the renal extract they suggested the name renin. Recently (8, 9) it was shown that renin alone is not capable of producing vasoconstriction but must act in the presence of blood plasma or serum. Then it was demonstrated (10, 11) that renin reacts with a globulin in the blood serum to form a heat stable (to boiling) vasoconstrictor and pressor substance, which was named angiotonin (10), and hypertensin (11). It is now recognized that renin is an enzyme and that a globulin in the serum is its substrate (11, 12). To the latter have been given the names, renin activator (10), hypertensin precursor and hypertensinogen (11), preangiotonin and prehypertensin (6).

The elucidation of the humoral mechanism of experimental renal hypertension or of comparable human hypertension will not be complete until the various constituents involved in the mechanism are isolated in pure form. Several methods of preparation and purification of hog renin have been described (13-15). In the main these methods involve fractionation of the kidney extract with neutral salts. Although these methods have resulted in substantial purification of the original extract, yet none of them yielded pure renin.

In the fractionation of a protein solution by neutral salts it is essential for good yield and for purification that the active substance be precipitated within a narrow range of salt concentration so that inert protein may be separated above or below this concentration. In a series of studies on the fractionation of renal extract with ammonium sulfate, by use of the rotating dialysis technique of McMeekin (16), we found that renin was precipitated at all intervals of salt

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concentration between 1.4 and 2.6 molar. The purest fraction was precipitated between 1.7 and 2.0 molar, at pH 6.0, but the yield was low. The purity (units of renin per mg. N) was of the order of that reported by Helmer and Page (13), Swingle *et al.* (14), and Schales (15) (Table II). Similar results were obtained by the use of potassium phosphate as a precipitant. On the basis of these studies it was apparent that sharp separation of renin from the inactive tissue proteins would be impossible by the use of neutral salts alone.

A series of studies on the pH stability of renin showed that at 0°C. renin was stable down to a pH of 2.3. Since the tissue proteins of the kidney are denatured below pH 4.5, it was reasoned that some drastic precipitant, like trichloroacetic acid, added to renal extract, to give a pH intermediate between these values, should denature and precipitate the bulk of the tissue proteins, without inactivating the renin. Subsequent fractionation of the renin remaining in the supernatant solution, with neutral salts and solvents, should then be more effective than when used on the original kidney extract. Based upon this concept, the following procedure for the purification of renin was developed.

#### *Method of Purification*

*Step 1. Extraction.*—Hog kidneys were used exclusively in these studies. All kidneys were frozen within an hour of slaughter of the animals. Immediately before use, the decapsulated kidneys were thawed, and pelvis, peripelvic fat, and part of the medulla were cut off and discarded. The remainder (entire cortex and part of medulla) was ground through a power-driven meat grinder with plate holes 1.5 mm. in diameter. To 3.5 kilos of cold ground kidney substance 5.3 liters of distilled water at 0.5°C. was added, and while the mixture was being vigorously stirred by a heavy duty electric stirrer, 5 per cent NaOH was added to bring it to pH 7.8. The pH adjustment took about 10 minutes and was carried out at room temperature. The pH measurement was made with the Beckman pH electrometer fitted with the shielded external electrodes which permitted immersion of the electrodes directly into the kidney brei. The crock containing the mixture was then placed in a water bath maintained at 0.5°C. and slow stirring was maintained for 18 hours. The extract was then carefully strained through six layers of surgical gauze of 20 × 12 mesh. The tissue residue, resuspended in 2.7 liters of distilled water at 0.5°C., was stirred vigorously, immediately strained through gauze and the two filtrates were then combined. This extract contained both depressor substance and hypertensinase in addition to renin. Because of the initial depressor effect it was not possible to make an accurate determination of the amount of renin.

*Step 2. Purification with Trichloroacetic Acid.*—To the extract prepared by step 1, while it was being vigorously stirred by means of a heavy duty electric stirrer, 10 per cent trichloroacetic acid was added slowly by means of a dropping funnel to bring it to pH 2.80 ± 0.05. This pH value was found to be critical. During this procedure, which took about 20 minutes, the temperature was not permitted to rise above 10°C. This material was kept overnight in the water bath, at 0.5°C., and then filtered

through E and D No. 613, 50 cm. filter paper, on four 26 cm. funnels, in a cold room maintained between 0° and 3°C. The clear yellowish green filtrate containing the renin was placed in 2½ inch Visking tubes, the surface covered by a layer of toluene 1 cm. thick, and dialyzed for 3 days against running cold tap water. Adequate dialysis was indicated by the disappearance of most of the pigment and a return of the pH to neutrality. Prolongation of dialysis for more than 3 days is undesirable. The bags containing the extract were then suspended in front of a rapidly revolving electric fan, and in 24 hours the material was concentrated by pervaporation from between 5500 and 6000 cc. to 1000 cc. The toluene was then drawn off and the slightly pigmented concentrate containing the renin was filtered by gravity through Whatman filter paper No. 5.

The material at this stage contained 0.70 mg. N per cc. (average) and 4.0 dog units<sup>1</sup> per mg. N<sup>2</sup> (Table I).

*Note:* In step 2, several precautions are necessary if adequate yield and purity of the renin are to be attained. (1) It is important to strain carefully the tissue residue from the extract before adding the trichloroacetic acid. If the acid is added directly to the kidney brei a phospholipid is extracted from the tissue which interferes with subsequent steps in the purification. (2) If the trichloroacetic acid is added to bring the mixture below pH 2.8, partial to complete inactivation of the renin results, depending upon the pH. (3) If the temperature of the trichloroacetic acid filtrate is permitted to rise above 15°C., partial inactivation of renin results. As a consequence, the yield and purity of the renin in this and subsequent steps would be lower than the figures given in Table I.

*Step 3. Fractionation with Acetone.*—The dialyzed concentrate (1000 cc.) prepared by step 2 was adjusted to pH 6.0 with N/1 HCl, cooled to 0.5°C., and 500 cc. cold acetone (0.5°C.) was added slowly, with constant stirring. The concentration of acetone at this stage was 33 per cent, by volume. After 15 to 30 minutes the mixture was filtered through Whatman filter paper No. 5, in the cold room at 0°C., and to the filtrate an additional 500 cc. cold (0.5°C.) acetone was added. This raised the concentration of acetone to 50 per cent, by volume. After standing for 24 hours at 0.5°C. the material was either centrifuged in a brine-cooled centrifuge maintained at 1°C. or filtered in the cold room (1°C.) through 3 gm. celite on a 10 cm. Buchner funnel. The precipitate was washed with 50 per cent cold acetone, and then dissolved in 125 cc. of distilled water previously cooled to 0.5°C. The aqueous solution was then dialyzed for 24 hours against 6 liters of cold distilled water and centrifuged. The clear yellow supernatant fluid was found to contain renin free of any depressor substance or hypertensinase (angiotonase), and highly satisfactory for most physiological studies. This solution contained between 1.2 and 1.5 mg. N per cc. and 15 dog units of renin per mg. N (Table I).

<sup>1</sup> In this study a unit of renin always means a dog unit, as determined in a previous study (17). See footnote, Table I.

<sup>2</sup> Determinations for total nitrogen were made by the Koch-McMeekin micro Kjeldahl method (18) adapted to the spectrophotometer.

*Step 4. Fractionation with Ethyl Alcohol.*—The solution obtained by fractionation with acetone (step 3) was diluted with distilled water to make it contain 1.0 mg. N per cc. and adjusted to pH 4.60 with  $n/2$  HCl. 150 cc. of this solution was cooled to  $0^{\circ}\text{C}$ ., and in a cold room maintained at  $0-1^{\circ}\text{C}$ . 125 cc. 50 per cent ethyl alcohol was added by means of a capillary tube. The alcohol was added at the rate of 1 cc. per minute while the mixture was being slowly stirred. After standing for 18 to 24 hours at  $0^{\circ}\text{C}$ ., the material was centrifuged in a brine-cooled centrifuge maintained at  $1^{\circ}\text{C}$ . The precipitate was suspended in 15 cc. 5 per cent ethyl alcohol, allowed to stand 1 hour at  $0^{\circ}\text{C}$ ., diluted to 30 cc. with distilled water at  $0.5^{\circ}\text{C}$ ., and again centrifuged at  $1^{\circ}\text{C}$ . The supernatant fluid was quickly transferred to  $\frac{5}{8}$  inch Visking cellophane tubes and dialyzed for 48 hours against distilled water at  $1^{\circ}\text{C}$ ., with frequent changing of the water. At this stage the faintly pigmented solution was found to contain between 0.25 and 0.30 mg. N per cc. and from 60 to 80 units renin per mg. N (Table I).

*Note:* In step 4 one difficulty may be encountered. An opalescent solution rather than a precipitate may result when the 50 per cent alcohol is added to the renin prepared in step 3. This difficulty is due to two possible contaminants. (1) A phospholipid, tentatively identified as sphingomyelin (see note under step 2), which can be removed from the solution by repeated fractionation with 33 and 50 per cent acetone (step 3). (2) A protein coagulable at 52 to  $53^{\circ}\text{C}$ ., which can be removed by adjustment of the renin solution to pH 6.0 and heating at  $52-53^{\circ}\text{C}$ . for 5 minutes.

*Step 5. Fractionation with Ammonium Sulfate.*—To 28.8 cc. of the solution obtained by fractionation with ethyl alcohol (step 4), 1.2 cc.  $M/1$  sodium acetate buffer, pH 4.20 was added. This was transferred to a  $\frac{3}{4}$  inch Visking cellophane tube and dialyzed, with rotation, against 465 cc., 1.65  $M$ ,  $(\text{NH}_4)_2\text{SO}_4$  containing  $M/25$  sodium acetate, pH 4.20. In 24 hours, at  $0.5^{\circ}\text{C}$ ., the ammonium sulfate concentration inside the bag was 1.55  $M$  and the renin was precipitated. The precipitate was separated by centrifugation in the brine-cooled centrifuge ( $1.0^{\circ}\text{C}$ .), dissolved in 10 cc. of water, dialyzed for 24 hours against distilled water, and centrifuged again. The supernatant fluid containing the renin was dialyzed for 24 hours against 1 per cent NaCl at pH 7.0. This solution contained 130 units of renin per mg. N and was free of pigment (Table I).

#### *Preservation and Sterilization of Renin*

Renin at any particular stage of purification can be preserved by freezing, or by refrigeration in a water bath at  $0.5^{\circ}\text{C}$ ., without the addition of a bactericidal agent. Step 5 renin must be kept in the presence of salt (1 per cent NaCl) at pH 7.0 because, unlike the less pure preparations, it is slowly inactivated when kept dissolved in distilled water. Renin may be sterilized, without loss of potency, by filtration through a Seitz filter, at pH 7.4, and is then best kept in the frozen state.

*Properties of Purified Renin*

The purest preparation of hog renin prepared by steps 1 to 5, inclusive, has physiological properties similar to less pure renin, although it contains 130 dog units per mg. N. It differs from the initial crude renal extract (step 1) by being free of depressor substance and of hypertensinase (angiotonase). In this study the following properties of this highly purified renin have been demonstrated: (1) it forms a heat stable vasopressor substance, angiotonin (hypertensin), when it is incubated with dog serum or beef serum (prehypertensin), but not with human serum; (2) it produces a slow, prolonged rise of blood pressure, characteristic of renin, when injected intravenously; (3) there is a diminished response to repeated injections of renin in a cat anesthetized with chloralose (tachyphylaxis); (4) there is no reversal of the pressor effect of the renin in a cat previously injected with piperido methyl-3-benzodioxane (933F); (5) there is no potentiation of the pressor effect of the renin by cocaine; (6) the pressor effect is abolished if the renin, dissolved in 1 per cent NaCl at pH 7.4, is heated at 60°C. for 5 minutes; (7) it is non-dialyzable, (8) it is insoluble in half saturated ammonium sulfate, 25 per cent alcohol, and 50 per cent acetone; (9) it gives the biuret reaction; (10) in the Tiselius electrophoresis apparatus, at pH 7.6, it migrates to the anode. These chemical and physical properties suggest that the active substance is a protein, as was surmised by Tigerstedt and Bergman (7). The protein nature of the renin obtained by step 5 precludes application of the usual methods for the establishment of chemical purity, such as the determination of the melting point or boiling point, or elemental analysis. Of the various special methods available for studying the homogeneity of proteins, including (1) Tiselius electrophoresis, (2) ultracentrifuge, and (3) solubility, only Tiselius studies have been made on the purest renin. On this renin two electrophoresis determinations at pH 7.6, phosphate buffer, 0.2 ionic strength, and 4.1 volts per cm., showed a single peak in the schlieren diagram after over 50 minutes of electrolysis. These studies were carried out with the assistance of Dr. Fenn Bernhardt and Dr. Lena Lewis of Cleveland Clinic to whom we owe our thanks. Tiselius studies at other pH values as well as ultracentrifuge and solubility studies are in progress and the results will be reported at a later date.

TABLE I  
*Steps in the Purification of Renin from Hog Kidney*

Step	Yield of renin Dog units per gm. (wet weight) of kidney	Purity of renin Dog units per mg. N
1. pH 7.8 extraction with water.....	*	*
2. Trichloroacetic filtrate.....	0.8	4.0
3. Acetone fractionation.....	0.8	15.0
4. Ethyl alcohol fractionation.....	0.4	60 to 80
5. Ammonium sulfate fractionation.....	0.2	130.0

\* Assays for yield and purity of renin in step 1 extract were not satisfactory, due to the preliminary depressor effect, when the material was injected intravenously. By determining the amount of extract required to raise the blood pressure 30 mm. Hg, after recovery from the initial depressor effect, it was possible to arrive at an approximate yield of 1 to 1.5 unit per gram of kidney tissue (wet weight) and a purity of 0.2 unit renin per mg. N.

A dog unit of renin, as previously defined (17), is the quantity which, injected intravenously, will raise the blood pressure of an unanesthetized dog 30 mm. Hg in 3 minutes. The elevation of blood pressure due to a unit of renin occurs in a characteristic and definite fashion. If the timing is begun from the instant when the intravenous injection (usually 2 cc. of fluid) is completed, there is invariably a lag of 15 or 20 seconds before the rise of blood pressure begins. If there is an instantaneous rise, the presence of some other pressor substance or the effect of excitement should be suspected. If the quantity injected contains a unit of renin, then, by the end of the first 30 seconds, there is usually a rise of 10 mm. Hg. A similar rise occurs during the next half minute, and during each of the remaining two half minute periods there is an additional rise of 5 mm. Hg. By the end of 2 minutes the maximum rise has occurred and it almost invariably persists at this level to the end of the three minute period or longer.

Table I summarizes the results obtained by the 5 steps involved in the procedure for the purification of renin. In column 2 is listed the yield at each step, which is expressed as the number of dog units of renin per gram of original kidney tissue (wet weight). In column 3 the purity at each step is expressed as dog units per milligram total nitrogen.

Table I shows that the yield as a result of step 2 is, on an average, 0.8 unit of renin per gm. of kidney substance. There is no loss of yield at the end of step 3, but there is a fourfold increase in purity over step 2 renin (15 instead of 4 units per mg. N). Step 4 involves a 50 per cent loss of yield of renin obtained by step 3, but there is a four- to fivefold increase in purity over step 3 renin (60 to 80 instead of 15 units per mg. N). Step 5 involves an additional 50 per cent loss of yield of the renin obtained by step 4 but it gives a twofold increase in purity over step 4 renin (130 instead of 60 to 80 units per mg. N).

TABLE II  
*Comparison of Purity of Renin Prepared by Different Methods*

Author	Helmer and Page (Fraction D)	Swingle <i>et al.</i>	Schales	Katz and Goldblatt			
				Step 2	Step 3	Step 4	Step 5
Purity of renin: (dog units per mg. N)	3.0	10.0	16.0 to 20.8	4.0	15.0	60 to 80	130

In order to make a direct comparison between the results obtained by other investigators and those reported here it was necessary to recalculate their data and express their results in terms of dog units, as defined here (see footnote, Table I).

Recently (17) we described a method for the bioassay of renin (see footnote to Table I). It was shown that a response to an amount of renin that will raise the blood pressure of an unanesthetized dog 30 mm. Hg is independent of the body weight of animals (between 8 and 25 kilos). The unit that has been suggested as a standard (see footnote, Table I) was therefore defined without reference to the amount of renin or of nitrogen per kilo body weight of the test animal. Since the activity of the renin preparations of other investigators has been expressed as the amount of nitrogen in the extract, per kilo body weight, required to raise the blood pressure a certain amount, it was necessary to recalculate their results in order to make a comparison with those reported here.

*Calculations:* Helmer and Page (13) stated that 0.027 mg. N of their fraction D renin, per kilo of body weight, raised the blood pressure of a dog 30 mm. Hg. Since we have found that the pressor effect of a quantity of renin sufficient to raise the blood pressure of dogs weighing from 8 to 25 kilos is independent of body weight, we have assumed an average weight of 12 kilos, for the purpose of these calculations. This is the average weight of the dogs used as test animals in this study. Therefore,  $12 \times 0.027$  mg. N = 0.324 mg. N; that is, a dog of 12 kilos requires an amount of renin containing 0.324 mg. N to give a rise of mean blood pressure of 30 mm. Hg. This means that this product contained 3.0 of our dog units of renin per mg. N.

Swingle, Taylor, Collings, and Hays (14) stated that 0.016 mg. N per kilo was required to raise the blood pressure of a dog 40 mm. Hg. For a 12 kilo dog, therefore, 0.192 mg. N was required to raise the blood pressure 40 mm. Hg. Since we have shown that it requires almost 2 units to raise the blood pressure 40 mm. Hg, this means that an amount of renin containing at least 0.095 mg. N would have been required to raise the blood pressure one unit (30 mm. Hg). Therefore this product contained 10 of our dog units of renin per mg. N.

Schales (15) stated that 0.004 to 0.005 mg. N per kilo body weight raised the blood pressure of a rabbit 30 mm. Hg. He also stated that the response in the rabbit was the same as in the dog. In a dog weighing 12 kilos this would mean that a quantity of renin containing from 0.048 to 0.060 mg. N would be required to raise the blood pressure 30 mm. Hg. Therefore this product contained 16.6 to 20.8 of our dog units of renin per mg. N.

## SUMMARY

1. Extraction of finely ground fresh hog kidney with distilled water adjusted to pH 7.8 with sodium hydroxide, followed by successive treatment, as described, with trichloroacetic acid and acetone, gives renin in good yield of a purity suitable for physiological studies and a good starting material for further purification. It contains 15 dog units per mg. N.

2. Further successive purification of this material with ethyl alcohol and ammonium sulfate gives a preparation containing 130 dog units per mg. N. The purest preparation hitherto reported (15) contained 16.0 to 20.8 units per mg. N. Preliminary Tiselius electrophoresis studies suggest homogeneity, but further studies to establish purity are in progress.

3. The properties of the most purified renin indicate that it is a protein. Its chemical and physiological properties correspond to those of the material in crude renal extract which induces an elevation of blood pressure when it is injected intravenously.

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