Extraction, Purification, and Assay of Human Renin Free of Angiotensinase

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ABSTRACT

Three procedures are described for the extraction and purification of renin from 0.05 to 500 g of human kidneys. A uniform yield of renin, free of angiotensinase, resulted from all three procedures.

All tests for renin were carried out in dogs. When more than 10 g of renal tissue was used, renin was determined by the direct method; with smaller amounts, the indirect method, involving the production and the assay of angiotensin, was employed.

Renin substrate free of angiotensinase, suitable for the indirect assay of human renin, was prepared from pooled human serum by a simple procedure.

The angiotensinase-free renin and substrate permitted prolonged incubation for the production of the angiotensin required for the indirect assay. The mean ratio of angiotensin produced (unit per milliliter of serum) to the amount of renin added (unit per milliliter of serum) was 1,482 for an 18-hour period of incubation. The large amount of angiotensin produced permitted the indirect assay of minute amounts of human renin (as little as 0.0005 unit, from 5 mg of renal tissue) in the dog. Unless the angiotensinase present in extracts of renal tissue or serum is first removed, the accurate, indirect assay of the renin is not possible.

ADDITIONAL KEY WORDS renin substrate free of angiotensinase prolonged incubation of renin and substrate angiotensin production sensitive assay renal renin serum renin

After every step involved in any procedure for the extraction and purification of renin, an assay of the solution is desirable for the determination of the yield and degree of purification attained. For the bioassay of the renin there are two main methods—direct and indirect.

The direct method for the bioassay of human renin consists of the determination of the pressor effect of an intravenous injection of the renin-containing solution into an animal. The extract does not have to be free of angiotensinase. If enough renin or angiotensin is available, we believe the ideal animal for the assay is the dog (1) because no anesthesia or time-consuming surgical procedures are required, and because a permanent colony of selected dogs can be maintained, all of which respond uniformly, for years, to the injection of a standard dose of renin or angiotensin. For example, the injection of 1 unit of angiotensin (10 doses in 3 dogs), produced an average increase of blood pressure of 30 mm Hg, with a range of 24 to 33 mm Hg and a standard deviation of ± 3.5 mm Hg.

The potency of an unknown renin or angiotensin solution can therefore be determined directly from the pressor response of the dog. In contrast, the sensitivity of the rat varies considerably (2), both initially in different animals (three- to fourfold), and during the course of the day in the same animal (five- to sixfold). The potency has to be calculated each time by comparison of the response of the test animal to a standard preparation of the pressor agent.

A dog unit of human renin is the quantity...

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which, when injected intravenously into an unanesthetized, trained dog, raises the direct, mean, systemic (femoral) blood pressure 30 mm Hg in about 2 min (1). In the normal human kidney, the average concentration of renin, within a narrow range, is 0.1 dog unit/g of the tissue (see Table 5). For an assay, in duplicate, at least 20 g of human renal tissue is necessary. This method is, therefore, not satisfactory for the determination of the amount of renin that can be extracted from a smaller quantity of renal tissue.

The indirect method for the bioassay of minute quantities of human renin in the dog involves the production of angiotensin by incubating a small quantity of renin with a relatively large amount of a suitable substrate. For the maximum production of the angiotensin, which is necessary for an accurate assay, both renin and substrate must be free of angiotensinase. Since a small quantity of renin can then yield a large amount of angiotensin, the latter can be assayed by the intravenous injection of the angiotensin into unanesthetized dogs.

The dog unit of human angiotensin is the amount which, when injected intravenously into a dog, raises the direct, mean, systemic blood pressure 30 mm Hg in about 1 min. Human angiotensin has not been purified sufficiently to permit an analysis of its amino acid sequence and to express its potency in terms of dog units per microgram of pure, natural, human angiotensin. However, a sample of synthetic beef angiotensin II (valine5-asparaginyl-β-amide, Hypertensin Ciba) was kindly supplied to us by Dr. A. J. Plummer; it is also obtainable as an International Standard from the World Health Organization. Our assay showed that it contains 3.0 dog units of angiotensin per microgram.

Most of the procedures available at present for the extraction and purification of renin from human kidneys are not suitable for the indirect assay of small quantities of renin in the dog. The main drawbacks of these procedures are that a relatively large amount of renal tissue has to be processed (3-5), the extraction and purification require up to 10 days, the average recovery of renin is only about 37% (5) and, most important, angiotensinase is removed only incompletely (3, 6) from the renin solution.

We have described a procedure (7) for the isolation of human renin that resulted in a 600-fold increase of its specific activity and complete removal of angiotensinase and that permitted the assay of the renin by the indirect as well as by the direct method. This procedure was designed, however, for the extraction of a large amount (35 kg) of renal tissue; it requires 8 steps over a period of 10 days and entails a loss of about 40% of the renin. Because it is not practical for the extraction of small quantities of human renal tissue we have developed simplified procedures for the extraction and purification of angiotensinase-free renin, from 100 to 500 g of human kidney, suitable for assay by the direct method in the dog, and from 0.05 to 1.0 g, for assay by the indirect method in the dog. The amount of renin present in 5 mg of normal human renal tissue was found to be adequate for the production of the quantity of angiotensin needed for the indirect assay.

Three simple procedures (A, B, and C) will be described, which can be performed in 3, 2, and 1 day, respectively. Each resulted in an almost complete recovery of renin.

Although the removal of angiotensinase was accomplished previously by various steps, including treatment with trichloroacetic acid (7), in the present procedures it was based upon two different chemical principles: (1) treatment with ethylenediaminetetraacetic acid (EDTA), which was applied to batches of 20 to 2,000 units of human renin, or (2) the fractionation with acid ethanol, which served to remove angiotensinase from preparations containing between 0.005 and 10 units of human renin.

A simple method will also be described for the treatment and fractionation of human serum, to make available a uniform preparation of human renin substrate essentially free of angiotensinase. This served as the substrate for the indirect microassay of human renin, for the production and partial purification...
of human angiotensin, and for a study of the enzyme kinetics of human renin (to be reported).

Methods

PREPARATION OF ANGIOTENSINASE-FREE HUMAN RENIN

The human kidneys used in the three procedures had been stored at −20°C for a day to several months. After being thawed at room temperature, about 2,000 g of these kidneys was ground in a mechanical meat grinder. This batch of ground kidneys served as the starting material for each of the three procedures to be described.

In processing larger amounts of human renal tissue, a motor-driven, 1/3 horsepower meat grinder (Hobart Mfg. Co.), was used. Its capacity was approximately 4 pounds of meat per minute. Smaller amounts of renal tissue were put through a household meat grinder (1/8 horsepower Sunbeam Mix Master). In previous studies on the renin content of hog kidneys, it was found that the yield of renin could not be increased by a more extensive disintegration of the renal tissue, e.g., by grinding for 10 min in a Waring Blendor.

PROCEDURE A

Step 1. The ground renal tissue (300 g) was extracted twice, each time by the addition of 300 ml of water, with stirring for 15 min at room temperature and centrifugation for 15 min at approximately 1,500 × g. The combined supernatant solution (665 ml) containing the renin was diluted with cold water to 1,500 ml, then cooled to 0°C, and all of the following steps were carried out in the cold.

Step 2. The solution was acidified to pH 2.8 by adding drop by drop approximately 9 ml of 5N H₂SO₄ and adjusted to 0.8 M by adding 72 g of solid sodium chloride. After clarification by centrifugation, the supernatant solution (1,450 ml) was stored overnight at 2°C. An appreciable fraction of the angiotensinase present in the crude extract was removed by this procedure.

Step 3. Inactive renal proteins were precipitated and separated by the addition of 204 g of solid ammonium sulfate (2.3 M), stirring for 10 min, and centrifugation. From the supernatant solution (1,500 ml), renin was then precipitated quantitatively by the addition of 305 g of solid (NH₄)₂SO₄ (2.3 M), stirring for 10 min, and centrifugation. The precipitate was then suspended in 12 ml of water and dialyzed for 18 hours at 2°C.

The equipment employed for dialysis in the present study resembles in principle the one constructed almost 40 years ago by Kunitz and Simms (8) for the dialysis of small volumes of protein solutions in collodion sacs.

The dialyzing bags (now seamless tubing, 0.7 to 5 cm in diameter, made of regenerated cellulose by the Viscose process) containing the renal extract were immersed in water, in glass tubes, and a continuous stream of cold (2°C) distilled water flowed through the tube at about 7 ml/min. The dialysis apparatus holds 5 glass tubes each 70 cm long and 7 cm inside diameter, to permit dialysis of renin solutions varying in volume from 1 to 7,000 ml. The glass tubes were rocked mechanically (about 1 cycle in 25 sec). A small glass bead or a glass "marble" inside the dialysis bag passed from one end of the bag to the other and provided a gentle but efficient method of mixing. As an example of the efficiency of dialysis under these conditions, the specific conductivity of a buffered ammonium sulfate solution was measured, and the salt concentration decreased, after 8 and 20 hours of dialysis, to 5% and 0.8% of the original value, respectively.

Step 4. The renin solution (35 ml) obtained after Step 3 was treated further by the addition of 1.4 g of the disodium salt of EDTA to a final concentration of 0.1 M. The solution was then adjusted to pH 9.5, by the addition drop by drop of approximately 1.8 ml of 5N NaOH (7, 9) and stirred slowly for 1 hour. This resulted in practically complete removal of angiotensinase. It represents a modification of the procedure originally employed by Glenner et al. (10) for the inactivation of aminopeptidase A in rat kidney, a procedure which has been adopted recently by Khairallah et al. (11) and by Boucher et al. (12) for the inactivation of angiotensinase in plasma.

Step 5. The renin was precipitated by the
addition of 14 g of solid ammonium sulfate (2.3 M), stirring for 10 min, and centrifugation for 10 min at 20,000 × g. The precipitate was suspended in about 5 ml of water and dialyzed for 18 hours against cold distilled water to remove EDTA and ammonium sulfate. The resultant solution (13 ml) was clarified by centrifugation and assayed by the direct procedure. It contained 2.2 units of renin per ml. The average yield, in 4 experiments, was 0.10 unit of renin per g of renal tissue (range 0.09 to 0.11).

In six individual batches, yielding from 20 to 2,000 units of human renin, the procedure for the removal of angiotensinase caused no loss of the pressor activity of the renin, either as the result of the treatment with EDTA or owing to the alkaline reaction (pH 9.5). The purity of renin was improved almost twofold by steps 4 and 5, resulting in a mean specific activity of 0.064 unit of renin/mg of protein (range 0.02 to 0.15). (See Table 5.)

Renin preparation "A" (pH 6.9) has been stored in the frozen state at −20°C for 14 months and has been thawed and frozen repeatedly without loss of activity. Its enzymatic activity is unimpaired by lyophilization. It has been furnished to the World Health Organization, to be distributed as an international standard of human renin.

Renin, under the conditions of Table 1 (at concentrations of 0.7 to 2.8 units/ml) is precipitated quantitatively by ammonium sulfate, despite the presence of EDTA. At lower concentrations of renin, however (0.02 unit/ml), EDTA interferes with the precipitation of renin by ammonium sulfate. Therefore, the procedure outlined in Table 1 is not suitable for the isolation of human renin on a microscale.

**PROCEDURE B**

Step 1. The experimental details for processing 100 g of human renal tissue are as follows: The frozen-thawed, ground, pooled renal tissue was extracted twice with 100 ml of water, each time by stirring for 15 min at room temperature and then centrifugation. The supernatant solutions were combined, diluted to 500 ml with water, and cooled to 0°C.

Step 2. After the drop by drop addition of about 3 ml of 5N H₂SO₄ (to pH 2.3) and 60 ml of cold (−20°C) 95% ethanol (to 10%), the solution was kept for 1 hour at 0°C without stirring, and clarified by centrifugation. This treatment with acid and ethanol (12) resulted in the removal of angiotensinase without any loss of the renin. The supernatant solution, containing the renin, was adjusted to pH 4.5 with 2.4 ml of 5N KOH and dialyzed overnight against cold distilled water, resulting in a total volume of 740 ml.

Step 3. The fractionation with sodium chloride in step 3 was employed to achieve partial purification. Thus, the addition of about 2 ml of 4N H₂SO₄ (pH 2.8) and of 36 g of solid NaCl (0.8 M), followed by stirring and cen-
trifugation, resulted in the separation of inactive renal proteins.

**Step 4.** The supernatant solution containing renin resulting from Step 3 was purified further. Inactive renal proteins, but not renin, were removed by dissolving 104 g of solid (NH₄)₂SO₄ (1.0 M), stirring for 10 min, and centrifugation. The renin was precipitated from the supernatant solution by the addition of 158 g of solid ammonium sulfate (2.3 M), stirring, and centrifugation. The precipitate was suspended in 5 ml of saline, dialyzed overnight against cold water, and clarified by centrifugation. The resultant solution, 14 ml, contained 0.61 unit of renin per ml, with a specific activity of 0.026 unit/mg. The average yield in 6 experiments was 0.087 unit of renin/g of renal tissue (range 0.078 to 0.093) (see Table 5).

In processing less than 100 g of renal tissue, aliquots of 0.2, 0.5, and 1.0 g were ground with sand and the tissue was extracted twice, each time with 5 ml of water. In all of the subsequent stages of purification (Steps 2 to 4, Table 2) the experimental details, as outlined in Procedure B, for 100 g of renal tissue (suitably scaled down), were employed.

**PROCEDURE C**

The procedure outlined in Table 3 represents a modified, abbreviated version of that shown in Table 2. It has been simplified considerably by the omission of several steps, such as the second extraction with water, the first dialysis, the fractionation with 0.8 M NaCl and with 1.0 M (NH₄)₂SO₄ and, finally, the second dialysis. The recovery of renin was improved thereby from 87% to 98% (see Table 5) and the time required for the procedure was reduced from 2 days to 1 day.

As an example, the experimental conditions for the processing of 0.20 g of renal tissue are described here.

**Step 1.** The frozen tissue was thawed and homogenized for 5 min at room temperature with approximately 0.2 g of sand. A 15-ml plastic centrifuge tube with a conical bottom, fitted with a suitably ground conical glass pestle, was used for this purpose. Renin was extracted from the tissue by adding 10 ml of water, and, after 15 min at 25°C, the insoluble residue was separated by centrifugation and discarded. The supernatant solution was cooled to 0°C and all of the following steps were carried out in the cold.

**Step 2.** The solution was acidified to pH 2.2 by the drop by drop addition of 0.33 ml of 0.5N H₂SO₄, adjusted to 10% ethanol with 1.24 ml of cold 95% ethanol, kept for 1 hour at 0°C, without stirring, and clarified by centrifugation. A small amount of renal protein was removed by the addition of 0.30 ml of 0.5N KOH (pH 4.5) and centrifugation.

**Step 3.** Renin was precipitated from the supernatant solution by acidification to pH 2.3 with 0.15 ml of 0.5N H₂SO₄, followed by the addition of 4.25 g of solid ammonium sulfate (2.3 M), stirring for 10 min and centrifugation for 10 min at 20,000 X g. The small amount of precipitate was dissolved in 2.0 ml of water and the resultant renin solution (2.2 ml) was clarified by centrifugation and stored without loss at −20°C. It was assayed by the indirect procedure (see Table 4) and found to contain 0.009 unit of renin/ml; the specific activity was 0.014 unit/mg and the yield 0.102 unit of renin/g of renal tissue.

These same conditions employed for the extraction and purification of renin from 0.2 g of renal tissue were applied also to the processing of 0.05, 0.50, and 1.00 g of tissue, with identical results (see Table 5). The average yield in 4 experiments was 0.098 unit/g of renal tissue (range 0.088 to 0.102).

The amount of renin available from less than 10 g of tissue was too small for assay by the direct method, and the low purity of the

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**TABLE 3**

Outline for Procedure C (Range 0.05 to 1.00 g of Renal Tissue)

<table>
<thead>
<tr>
<th>Step</th>
<th>Procedure</th>
<th>Temperature °C</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Extraction</td>
<td>25</td>
<td>6.4</td>
</tr>
<tr>
<td></td>
<td>Once with 10 ml water</td>
<td>15 min</td>
<td>6.4</td>
</tr>
<tr>
<td>2</td>
<td>Fractionation</td>
<td>0</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>10% ethanol</td>
<td>1 hr</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>10% ethanol</td>
<td>10 min</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>Precipitation</td>
<td>2.3 M (NH₄)₂SO₄</td>
<td>10 min</td>
</tr>
</tbody>
</table>

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renin, as well as residual amounts of ethanol and of ammonium sulfate, precluded direct intravenous injection of the extract. None of these conditions presented any obstacles to the indirect assay of this renin preparation in the dog. The amount of renin obtainable from 0.05 to 1.00 g of renal tissue was adequate for the production of a relatively large amount of angiotensin and for the performance of 6 and 120 individual injections, respectively. The small quantity of renal protein, 0.01 mg/ml, and the serum proteins were removed from the test solutions by the heat-coagulation and centrifugation (Table 4). There was no interference with the test owing to the presence of low concentrations of ethanol, 0.003 to 0.08%, or of ammonium sulfate, 0.0002 to 0.005 M.

**SUBSTRATE (ANGIOTENSINOGEN) FOR HUMAN RENIN**

For the maximum production of angiotensin by human renin, human serum or plasma was used as the source of substrate. The angiotensinase naturally present in the human serum or plasma was removed by a procedure which included treatment with EDTA, fractionation with ammonium sulfate, and dialysis.

**Example.** To a batch of 310 ml of human serum, a mixture of 300 specimens, 12 g of solid Na₂EDTA·2 H₂O (0.1 M) was added and the pH adjusted to 8.0 by the drop by drop addition of approximately 4 ml of 5N NaOH. This was followed by slow stirring, for 1 hour, at 26°C. The solution was then cooled to 0°C and adjusted to pH 5.3 by the slow addition of 12 ml of 2.5N HCl. From this solution the substrate was salted out by the addition of 122 g of solid ammonium sulfate (2.3 M) and stirring for 10 min. It was precipitated by centrifugation for 1 hour at 2,000 × g. The precipitate was then suspended in 60 ml of distilled water. After dialysis for 18 hours against cold distilled water, the solution was brought to 280 ml and adjusted to pH 6.9 with approximately 1 ml of 5N NaOH, and to 1.1% saline by the addition of 2.8 g of solid NaCl. For the assay and for the incubation with renin (Table 4), the substrate solution was diluted and restored to the original serum volume by the addition of phosphate buffer and water.

The substrate solution was stored frozen. After 10 months at −20°C, which included repeated thawing and refreezing, it was still

<table>
<thead>
<tr>
<th>TABLE 4</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Procedure for the Indirect Assay of Fractions of a Unit of Human Renin</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Source</th>
<th>Exp. I</th>
<th>Exp. II</th>
<th>Exp. III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate</td>
<td>8.3 ml</td>
<td>As in Exp. I</td>
<td>As in Exp. I</td>
</tr>
<tr>
<td>Water</td>
<td>1.3 ml</td>
<td>Added</td>
<td>Added</td>
</tr>
<tr>
<td>0.5 M phosphate, pH 6.9</td>
<td>0.4 ml</td>
<td>X₁ = unknown</td>
<td>X₁₁ = unknown</td>
</tr>
<tr>
<td>Total volume</td>
<td>10.0 ml</td>
<td>X₁₁ = 0.00050</td>
<td>X₁₁₁ = unknown</td>
</tr>
<tr>
<td>Human renin unit/ml serum</td>
<td>Endogenous</td>
<td>Added</td>
<td>Added</td>
</tr>
<tr>
<td>Angiotensin produced unit/ml serum</td>
<td>Y₁ = 0.23</td>
<td>Y₁₁ = 1.00</td>
<td>Y₁₁₁ = 0.81</td>
</tr>
</tbody>
</table>

Incubation: 18 hours at 38°C.

X₁ = concentration of endogenous renin; X₁₁ = concentration of added, known renin; X₁₁₁ = concentration of added, unknown renin. Y₁ = concentration of angiotensin produced by endogenous renin; Y₁₁ = concentration produced by endogenous plus added, known renin; Y₁₁₁ = concentration of angiotensin produced by endogenous plus added, unknown renin.

Concentration of endogenous renin (units per milliliter serum):

\[
X₁ = \frac{Y₁}{1482} = \text{0.000155}
\]

\[
X₁₁ = \frac{Y₁₁ - Y₁}{1482} = \text{0.00052}
\]

\[
X₁₁₁ = \frac{Y₁₁₁ - Y₁}{1482} = \text{0.00039}
\]
fully active as the substrate for human renin. Lyophilization did not alter the quality of this substrate.

In six of the batches of pooled, treated serum, small residual amounts of EDTA (0.50 to 0.68 mg per ml of serum) were found to be present by chemical analysis. Thus 98.5% of the EDTA originally added to the serum had been removed.

For the analysis of EDTA in the presence of renal proteins, we used a new spectrophotometric procedure (unpublished data). In principle, the light absorption of cadmium-dithizonate was utilized as an indicator, and the assay procedure for EDTA was based on the blocking by EDTA of the formation of the cadmium-dithizonate complex. As little as 5 μg of EDTA may be determined by this method, with a standard error of estimate of ±0.76 μg.

The small but rather uniform amount of residual (protein-bound) EDTA could not be removed or reduced by the precipitation of the serum proteins with ammonium sulfate, by dialysis with continuous stirring against distilled water, or even by an extension of the dialysis from the original 18 hours to 3 days.

The residual protein-bound amount of EDTA (mean = 0.63 mg/ml serum) was found to be essential for the inactivation of the serum angiotensinase. When the treatment with a high concentration (40 mg/ml) of EDTA was omitted, the subsequent fractionation with ammonium sulfate and dialysis were found to be ineffective for the removal of serum angiotensinase, and even the addition of EDTA, in amounts of 0.6 to 1.8 mg/ml of serum, resulted in only an incomplete blockade of the serum angiotensinase.

**INDIRECT ASSAY OF HUMAN RENIN**

The indirect assay of minute quantities of human renin depends upon the determination of the amount of angiotensin produced by incubation of angiotensinase-free renin with the angiotensinase-free substrate prepared from human serum. In principle, in order to determine the concentration of renin in an unknown preparation, three determinations are required of the amounts of angiotensin which have been produced:

1. by the small amount of endogenous renin present in the substrate preparation,
2. by a known concentration of human renin added to the substrate,
3. by an aliquot of a human renin preparation, of unknown concentration, added to the substrate.

The relatively large amounts of angiotensin produced under these conditions were assayed by the direct method in dogs.

**Method.** In the three experiments of Table 4, buffered substrate (corresponding to 10 ml of the original serum) was incubated for 18 hours at 38°C without added renin (Exp. I), after the addition of 0.0005 unit/ml of human renin (Exp. II), or after the addition of an aliquot of an unknown renin preparation (Exp. III). Each solution was cooled after the incubation to 0°C and diluted to 30 ml with water. It was then acidified to pH 5.3 by the addition of 0.3 ml of 5 HCl and the protein removed by coagulation for 2 min at 85°C, and centrifugation. The supernatant solution containing the angiotensin was neutralized with 0.14 ml of 5 NaOH, filtered, and assayed in the dog.

In order to determine the production of angiotensin by endogenous renin, the substrate preparation obtained from human serum was incubated in the absence of added human renin (Table 4, Exp. I). In 22 experiments, with 13 different batches of substrate, incubation for 18 hours at 38°C yielded an average of 0.23 ± 0.009 S.E. unit of angiotensin (calculated per milliliter of the original serum).

The presence of endogenous renin in the substrate was also ascertained by the addition of antirenin prior to the incubation, which resulted in a complete blockade of the production of the pressor agent (angiotensin).

When human renin in known amount (0.0005 unit/ml), was added and incubated with the substrate for 18 hours at 38°C (Table 4, Exp. II) the angiotensin produced in 10 experiments ranged from 0.9 to 1.0 unit per ml of the incubation mixture. From the aver-
age value (0.97 unit/ml) for total angiotensin which developed as a result of the endogenous and added renin, and the average value (0.23 unit/ml) in the serum alone, it follows that the additional angiotensin produced by the added renin is $0.97 - 0.23 = 0.74$ unit/ml of serum. The ratio of the additional angiotensin produced to the amount of renin added is therefore:

$$\frac{0.97 - 0.23}{0.0005} = \frac{1,480 \text{ units angiotensin}}{\text{unit renin}}.$$ 

In 3 experiments, with slightly smaller or larger amounts of renin (0.0003, 0.0004, and 0.00064 unit/ml), the ratios were similar (1.470, 1.550, and 1.540). This indicates that the production of angiotensin for all practical purposes was linear to the concentration of renin in the range of 0.0003 to 0.00064 unit/ml.

Furthermore, in the 13 experiments it was found that variation of the specific activity of renin, from 0.026 to 1.20 units/mg, and the use of 5 different batches of substrate had no significant effect on the mean ratio: units angiotensin/unit renin $= 1,482$. This mean ratio, $1,482 \pm 24$ se, was therefore adopted as the value with which to calculate the concentration of human renin in an unknown, renin-containing extract, and even to estimate the quantity of endogenous renin in human serum.

The experimental procedure and the method of calculation for the determination of the renin concentration from the amount of angiotensin which had been produced during the incubation of 18 hours at 38°C have been outlined in Table 4. The concentration of renin and of angiotensin are expressed in terms of units per milliliter of the incubation mixture, which is identical to the units per milliliter of the original serum from which the substrate preparation was obtained.

A concentration of endogenous renin ($X_1$) of 0.000155 unit/ml of human serum was found in Exp. I of Table 4. Exp. II of Table 4 served to re-assay by the indirect, micro-method an aliquot (0.00050 unit/ml) of a preparation of human renin which had been assayed previously by the direct procedure, i.e., by the injection of 1.0 unit of renin, in the dog. In this case $X_{II} = 0.00052$ unit/ml serum. The results of the assays by the two procedures therefore agree within 4%.

An example of the experimental procedure for the evaluation of an unknown preparation of human renin is shown also in Table 4 (Exp. III). The aliquot of this preparation which had been added per milliliter of serum produced angiotensin ($Y_{III} - Y_I = 0.81 - 0.23 = 0.58$ unit/ml serum) and therefore was found to contain ($X_{III}$) 0.00039 unit of renin. Thus, by this indirect method it was possible to assay human renin, in concentrations of 0.0003 to 0.00064 unit/ml, with an accuracy of approximately ±6%.

There is a critical concentration of the substrate below which the value of the ratio of angiotensin produced to renin used is correspondingly decreased, but above which there is no effect on the ratio. In this study the concentration of angiotensinogen in the batches of pooled sera was such as to assure the maximum ratio of angiotensin to renin.

As an alternative procedure for the indirect

![Figure 1](http://circres.ahajournals.org/)

**FIGURE 1**

Effect of angiotensinase in various preparations of human renin on the production of angiotensin by human renin. The substrate was obtained by fractionation of human serum (free of angiotensinase). Saline plus 0.02M phosphate, pH 6.9, served as the buffer. Graph number 1 refers to human renin after step 1 of procedure A, graph number 2 to step 3, and graph number 3 to step 5.
TABLE 5
Comparison of the Yield of Renin in Preparations A, B, and C

<table>
<thead>
<tr>
<th>Renin preparation</th>
<th>Specific activity (unit/mg protein)</th>
<th>Pooled renal tissue no.</th>
<th>Amount of renal tissue processed (g)</th>
<th>Method of assay of renin</th>
<th>Yield of renin unit/g tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.064</td>
<td>I</td>
<td>500</td>
<td>Direct</td>
<td>0.019</td>
</tr>
<tr>
<td></td>
<td></td>
<td>II</td>
<td>200</td>
<td>Direct</td>
<td>0.090</td>
</tr>
<tr>
<td></td>
<td></td>
<td>III</td>
<td>100</td>
<td>Direct</td>
<td>0.100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IV</td>
<td>300</td>
<td>Direct</td>
<td>0.100</td>
</tr>
<tr>
<td>B</td>
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Average 0.100

Assay of human renin, we graphed the amount of angiotensin produced vs. increasing amounts of a known preparation of human renin with which it was incubated (Fig. 1, graph 3). With such a "calibration curve," the concentration of renin in an unknown preparation could be determined from the amount of angiotensin which it produces in 18 hours under the conditions of Table 4.

COMPARISON OF THE YIELD AND SPECIFIC ACTIVITY OF THE THREE RENIN PREPARATIONS

With a procedure available (Table 4) for the assay of small amounts of human renin, it was possible to determine the concentration of renin in small amounts of renal tissue, as well as in large batches, and for renin preparations of different purity processed by various procedures.

In the 14 experiments, four batches of pooled human renal tissue (I-IV) were processed in amounts ranging from 0.05 to 500 g, and their renin content was determined as indicated, by the direct and the indirect method of assay (Table 5). Preparations A and B were found to be of sufficient purity, free of depressor and anaphylactoid material, to permit their direct assay by the intravenous injection in the dog.

In the 4 experiments (preparation C) using small quantities of renal tissue, the renin content was almost identical (Table 5). This shows that the treatment of human renin at pH 2.2 with 10% ethanol, for the removal of angiotensinase, was tolerated without any loss of activity.

In processing such small amounts of renal tissue (0.05 g), it was noted that renin, even in a very dilute solution (0.004 unit/ml), was precipitated quantitatively with ammonium sulfate and recovered without loss.

A uniform yield of renin, apparently without any loss, was achieved by processing pooled renal tissue (Table 5, batch IV) by any of the three procedures for extraction and purification.

EVALUATION OF INTERFERENCE BY ANGIOTENSINASE

The following three procedures have been employed to investigate the effects of the presence of angiotensinase and to ascertain that this enzyme, or group of enzymes, has been removed from the pooled, treated human serum serving as the substrate, and from the various preparations of human renin. These 4 preparations, A, B, and C (Tables 1–3), and the renin obtained after step 8 of a previous procedure (7), ranged in their specific activity from 0.014 to 1.2 units/mg of protein.

Test for Angiotensinase in the Four Preparations of Human Renin, by Incubation with Human Angiotensin. A renin concentration of 0.005 unit/ml was selected for the tests, which...
represents 10 times the concentration now employed by us for the indirect assay of human renin (Table 4). The human angiotensin, with a specific activity of 120 units/mg of N, was produced enzymatically and purified 600-fold. When such angiotensin, at a concentration of 3.0 units/ml, was incubated with each of these 4 preparations of renin in saline—0.02 M phosphate buffer pH 6.9, it was recovered without loss of activity after 18 hours at 38°C, indicating that the 4 renin preparations were completely free of angiotensinase.

Test for the Stepwise Removal of Angiotensinase from Renin Preparation A. This is presented as an example of a test for angiotensinase under conditions (Table 4) in which angiotensin is being produced by small amounts of renin during a period of 18 hours at 38°C. The removal of angiotensinase in the course of the purification of human renin was evaluated (Fig. 1) for preparations of human renin which had been obtained after steps 1, 3 and 5 of procedure A, outlined in Table 1.

The interference by angiotensinase is reflected by the experimental results (Fig. 1) in two ways:

For step 1 and step 3 of preparation A renin, not free of angiotensinase, the formation of angiotensin as expressed by the ratio units angiotensin/unit renin was only 100 and 600, respectively, but for step 5 renin, which was free of angiotensinase, the ratio was 1,360.

The effect of the angiotensinase accompanying the renin became apparent also as the result of incubation of the serum with an excess of renin. The recovery of angiotensin amounted to only 0.3 unit/ml serum (12%) (Fig. 1, graph 1) and to 1.0 unit/ml serum (38%), graph 2, when compared with the complete recovery (2.6 unit/ml serum, graph 3) when angiotensinase-free renin preparation A, after step 5, was used.

The Enzymatic Formation of Human Angiotensin and its Recovery in the Course of Prolonged Incubation at 38°C. The new procedure for the indirect assay of human renin in the dog (Table 4) consists of incubation for 18 hours at 38°C, at a concentration of approximately 0.0005 unit of renin per ml of serum. In order to prove the absence of detectable amounts of angiotensinase in the renin the serum, a concentration of the renin 13 times higher was added to the serum and the period of incubation was extended to 40 hours (Fig. 2).

Graph 1, in Figure 2 shows that when 0.0065 unit/ml of human renin was added to pooled, untreated human serum, the angiotensin was inactivated by the serum angiotensinase almost as rapidly as it was being formed during the incubation period; only 0.2 unit/ml of angiotensin was recovered after 1 hour of incubation.

When, however, the same amount of renin was added to the treated substrate obtained from human serum the maximum amount (2.2 units/ml) of angiotensin was produced in about 3 hours (Fig. 2, graph 2), and this value remained fixed without any loss in the course of further incubation, for 6, 9, 18, and 40 hours at 38°C.

Discussion

The removal of angiotensinase from renin-containing extracts previously required a combination of several procedures (7). These included fractionation with NaCl in acid solution, treatment with alkali, followed by inactivation with trichloroacetic acid. In the previous study, two alternative and simpler
procedures were employed for the complete removal of angiotensinase, i.e., either the treatment with acid ethanol or the chemically more specific treatment with EDTA. This is based, presumably, on the chelation of calcium ions, but no attempt has been made to reactivate angiotensinase (by the addition of calcium ions) as has been done for aminopeptidase A of rat kidney (10) or for angiotensinase of plasma (11).

Relatively large amounts (50 g of human renal tissue) were employed in our previous procedure (4) for the extraction and purification of an amount of renin which could be assayed by the direct method. In contrast, one of the procedures presently employed for the extraction, purification and indirect assay requires the processing of less than 0.05 g of renal tissue.

Small amounts of renal tissue also were extracted by Alonso et al. (6). There was, however, no further purification of the crude renal extract, aside from a brief incubation for 30 min at pH 3.8 and 37°C designed for the inactivation of angiotensinase. Reinvestigation in various laboratories, however, has shown that this procedure was inadequate for the destruction of angiotensinase in renal tissue and that it resulted in only partial removal of angiotensinase from the renal extract (14, 15).

The procedures of Helmer and Judson (16), of Boucher et al. (12), of Fasciolo et al. (17), and of Pickens et al. (2) were designed for the assay of renin present in human plasma and not for the extraction and purification of human renin from renal tissue.

References
Extraction, Purification, and Assay of Human Renin Free of Angiotensinase
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