Further Studies on the Determination of Renin in Rat Kidney

Shoichi Harigaya, Ph. D., Hirofumi Sokabe, M.D., and Fuminori Sakai, M.D.

The renin-angiotensinogen reaction in the rat was examined in detail under various conditions of pH and temperature. Angiotensinase activity in the kidney extract and the plasma was also determined at various pH. We found that incubation of acidified kidney extracts with the plasma at 20°C and pH 8.0 for 30 min. gave a more reliable method for the determination of renin in rat kidney.

The determinations of renin are classified into two categories: the direct and indirect method. The former is based on the elevation of blood pressure induced by the intravenous injection of test materials directly into the animal. The pressor activity is due to angiotensin formed in vivo. In the latter, the amount of renin is determined indirectly from the assay of angiotensin produced in vitro as the result of renin-angiotensinogen reaction by incubating test materials with a suitable angiotensinogen source.

We have reported a simple and accurate method for the determination of renin in rat and human kidneys from small specimens, utilizing the renin-angiotensinogen reaction. Further studies in the rat are reported in this paper with special reference to the results on the renin-angiotensinogen reaction and angiotensinase activity under various conditions.

METHODS

The procedures of preparation of renin or angiotensinogen, incubation, determination of angiotensinase activity, and assay of angiotensin were the same as previously reported. Only modifications are given in the following.

A kidney extract prepared by using glass-distilled water, 1 ml./Gm. of tissue, was referred to as the non-treated or original (1/1) concentration. It was diluted by saline to make various concentrations. When the non-treated kidney extract was acidified or alkalized, it was called the treated. After the treatment, pH of the kidney extract was readjusted to a desired value for incubation.

Sodium phosphate buffer (0.15 M NaH₂PO₄ : 0.15 M Na₂HPO₄) containing...
0.002% thimerosal was used. pH of each component of the incubation mixture was
determined by the pH meter prior to mixing in order to assure an accurate pH.

An angiotensin solution of 0.4 µg./ml. was used in the determination of angio-
tensinase activity, and 0.3 ml. of the incubation mixture was injected into the rat
for assay, as in the case of the determination of angiotensin formation by incubating
the kidney extract with the plasma. The bracket method was used throughout for
the bioassay of angiotensin.

The precipitate of heparinized blood obtained by centrifugation (3,000 r.p.m.
for 15 min.) was suspended in 0.9% saline of about the same volume, and centrifuged
again. After washing 3 times as above, the red blood cells were dissolved with
distilled water in a volume of 9 times the cell-precipitate, and referred to as 1/10
concentration. The red cells were thus hemolysed, and we obtained a clear red
solution after removal of ghosts by centrifugation.

**Results**

1. Effect of pH on angiotensinase activity of the plasma and of the kidney

 extract

Fig. 1 shows the pH-activity curves of angiotensinases in the plasma and
the red blood cells. Plasma angiotensinases had optimum pH near 7.0. Those
of red cells also showed a similar curve, although the net activity was far greater.
This meant that the use of hemolysed plasma must be absolutely avoided as
an angiotensinogen source when renin is determined indirectly. Ethylene-

![Graph showing pH-activity curves of angiotensinases](image-url)

**Fig. 1.** The pH-activity curves of angiotensinases in the rat plasma and red
cells. Incubation was carried out at 37°C for 10 min. Angiotensinase activity
was expressed as the amount of angiotensin destroyed in %.
Fig. 2. The pH-activity curves of angiotensinases in the rat kidney extracts.

diaminotetraacetic acid (EDTA) in an amount of $3.3 \times 10^{-3}$M inhibited partly plasma angiotensinase activity at pH 6.5 to 7.5.

Fig. 2 shows the pH-activity curves of angiotensinases in the kidney extracts. The non-treated extract had high angiotensinase activity over the wide range of pH, which would be due to several kinds of peptidases present in the kidney. Alkalization of the extract to pH 9.0 at 0°C for 30 min. resulted in a moderate decrease of the activity especially near pH 5.5. Acidification to pH 3.0 at 0°C for 30 min., on the other hand, strongly inhibited angiotensinases at pH higher than 6.5. EDTA in an amount of $3.3 \times 10^{-3}$M did not affect the angiotensinase activity of acid-treated kidney extract. When the acid- and alkaline-treatment were combined, further inhibition especially between pH 5.5 to 6.5 was recognized.

2. Effect of pH on renin activity of the kidney extract

Angiotensin forming activity of the acid-treated kidney extract of 1/30 concentration at various pH was determined by incubating with the plasma at 37°C for 10 min. Maximum yield of angiotensin was obtained at pH 6.5 regardless of adding $3.3 \times 10^{-3}$M of EDTA. The result did not mean that the optimum pH of rat renin is 6.5. In the rabbit it has been reported to be 5.7 by Lever, Robertson, and Tree. Angiotensin formation was influenced by the activity of both renin and angiotensinases present in the incubation mixture.

3. Formation of angiotensin at various pH and temperature

The following pH values were chosen to know in detail the effect of temperature, EDTA, and duration of incubation on formation of angiotensin.
(a) At pH 5.5: Plasma angiotensinase activity was low. The angiotensinase activity in the kidney extract was also decreased by alkalization.

(b) At pH 6.5: The maximum yield of angiotensin was obtained, but angiotensinase activity in both the kidney extract and the plasma was also relatively high.

(c) At pH 8.0: Angiotensinase activity of the acid-treated extract and the plasma was relatively low.

(i) Formation of angiotensin at pH 5.5 (Fig. 3)
Incubation of the acid-treated extract of 1/30 concentration with the plasma at 37°C resulted in the maximum formation of angiotensin after
20 min., which gradually decreased afterwards by the influence of angiotensinases. Incubation of the acid- and alkaline-treated extract brought about an increased formation of angiotensin compared to the above conditions. It reached the maximum after 40 min., and then formed a plateau. Addition of EDTA rather decreased the yield of angiotensin. Incubation at 20°C also decreased markedly the activity of renin.

The reason of choosing alkalization at pH 9.0 is apparent in Fig. 4. The acid-treated extract was then alkalized to various pH at 0°C for 30 min. It was diluted to 1/30 and incubated with the plasma at pH 5.5, 37°C for 10 min. The maximum formation of angiotensin was obtained with the extract treated at pH 9.0, which meant that the maximum destruction of angiotensinases was elicited at this pH least affecting the renin activity.

(ii) Formation of angiotensin at pH 6.5 (Fig. 5)

Incubation of the acid-treated extract of 1/30 concentration with the plasma at 37°C resulted in the maximum formation of angiotensin after 10 min., which gradually decreased afterwards by the influence of angiotensinases. Addition of EDTA (3.3×10⁻⁸M) markedly increased angiotensin formation as shown before. It reached the maximum after 20 to 40 min., and then decreased slightly. Incubation at 20°C caused a gradual increase of angiotensin formation with time, but the net amounts were far less than the above conditions. Incubation of the acid- and alkaline-treated extract produced the less amount of angiotensin probably due to the decreased renin activity by the alkalization.

(iii) Formation of angiotensin at pH 8.0 (Fig. 6)

Owing to the decreased activity of angiotensinases at this pH, incubation of the acid-treated extract at 37°C gave an increase in formation of angiotensin
until 20 to 40 min. and the curve reached the maximum. The increasing period was definitely longer than that at pH 5.5 or 6.5. However, angiotensin level in the incubation mixture also gradually decreased afterwards because of remaining angiotensinase activity. Addition of EDTA (3.3×10⁻³M) did not improve the situation. Incubation at 20°C caused a linear increase of angiotensin formation until 80 min. Angiotensinase activity seemed negligible under these conditions, and EDTA did not affect the formation either. Incubation of the acid- and alkaline-treated extract produced smaller amounts of angiotensin.

4. Formation of angiotensin with various concentrations of the kidney extracts

From the above results, the following conditions were selected for further studies:

(a) Incubation of the acid- and alkaline-treated extract at pH 5.5 and 37°C.

(b) Incubation of the acid-treated extract at pH 6.5 and 37°C with 3.3×10⁻³M of EDTA.

(c) Incubation of the acid-treated extract at pH 8.0 and 20°C. Under these conditions, activity of angiotensinases was found to be relatively low and formation of angiotensin was sufficiently high.

Fig. 7 shows angiotensin formation with various concentrations of the kidney extracts under the above conditions. Duration of incubation was 10 min. in (a) and (b), and 30 min. in (c). In lower concentrations angiotensin formation was proportional to the concentration, but in higher concen-
Fig. 7. Formation of angiotensin with various concentrations of the kidney extracts under the various conditions.

concentrations it was gradually decreased owing to the effect of angiotensinases remaining in the incubation mixture. The effect was greatest at pH 5.5, where angiotensinase activity in the kidney extract was preserved relatively high, as shown in Fig. 2. The effect was least at pH 8.0, where angiotensinases in the kidney extract were inhibited markedly.

Reaction constants (K) calculated by the Helmer’s equation were shown in Fig. 8. It is evident that K values had a linear relationship to the concentration of kidney extract in a wider range under the condition (c). Therefore, incubation of the acid-treated extract at pH 8.0 and 20°C for 30 min. would be a more reliable method for the determination of renin in the kidney of rats. Incubation at pH 6.5 also gave K values having a linear relationship to the con-
centration of kidney extract, but the range was more narrow and the influence of angiotensinases was greater.

**DISCUSSION**

Angiotensinase activity of the kidney extract and the plasma in the rat was investigated under various conditions. Angiotensin formation of the acid-treated and the acid- and alkaline-treated kidney extract was also determined. We found that incubation of the acid-treated extract at pH 8.0 and 20°C gave a more reliable method for the determination of renin in the kidney with minimal influence of angiotensinases.

We have reported a method for the determination of renin in rat and human kidneys by incubating the acid-treated extract at pH 6.5 and 37°C with EDTA. However, the newer conditions at lower temperature would give a more reliable method with the wider range of concentrations of rat kidney extract. In 1944 Sapirstein, Reed, and Southard reported that renin acts even at 0°C. Incubation at the lower temperature was also successful for the determination of renin in fish kidneys.

The so-called indirect method used in the present experiments was based on the renin-angiotensinogen reaction and had several excellent features compared to the direct method. Table I summarizes the characteristics of the direct and indirect methods.

**Table I. The Characteristics of Direct and Indirect Method for the Determination of Renin**

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<th>Direct Method</th>
<th>Indirect Method</th>
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<tr>
<td>Simplicity of the procedures</td>
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<td>×</td>
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<td>Freedom from angiotensinases present</td>
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<td>×</td>
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<td>Tachyphylaxis in the bioassay</td>
<td>×</td>
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<td>Species specificity in the bioassay</td>
<td>×</td>
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<td>Sensitivity difference in the assay animals</td>
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<td>Sensitivity</td>
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The marks (○) and (×) indicate the method which has more and less excellent features, respectively.

The effect of EDTA on angiotensinases was no longer complete. EDTA partially inhibited angiotensinase activity in the plasma at pH 6.5 to 7.5. It did not affect, however, angiotensinases in the acid-treated kidney extract. The incomplete inhibition by EDTA was also shown by Boucher et al., but
Khairallah et al. claimed that it was a specific inhibitor of the angiotensinase in plasma and red cells. The angiotensinase activity in the acid-treated extract was not affected by EDTA. Since the activity decreased markedly at the pH higher than 6.0, the effect of EDTA might be masked. The agent increased angiotensin formation at pH 6.5, but not at pH 5.5 or 8.0, and sometimes rather decreased the formation. Therefore, we have to take into account the inhibition of not only angiotensinases but also renin by EDTA.

**Summary**

1. The renin-angiotensinogen reaction in the rat was examined in detail under various conditions of pH and temperature. Angiotensinase activity in the kidney extract and the plasma was also determined.

2. pH-activity curves of angiotensinases in the plasma, red cells, and kidney extracts were determined. Angiotensinases in the kidney tissue had different pH-characteristics from those of the plasma or red cells.

3. Incubation of the acid-treated extract with the heparinized plasma resulted in the maximum formation of angiotensin at pH 6.5, regardless of the presence of EDTA.

4. At pH 5.5 formation of angiotensin continued to increase until 40 min., and then formed a plateau, by incubating the acid- and alkaline-treated extract of 1/30 concentration at 37°C.

5. At pH 6.5 formation of angiotensin reached the maximum after 20 to 40 min., and then decreased slightly, by incubating the acid-treated extract of 1/30 concentration at 37°C with 3.3 × 10⁻³ M EDTA.

6. At pH 8.0 incubation of the acid-treated extract at 20°C resulted in a linear increase of angiotensin formation until 80 min.

7. Formation of angiotensin with various concentrations of the kidney extracts was determined under the above 3 conditions, where the effect of angiotensinases was relatively low and formation of angiotensin increased with the incubation time for a sufficient length.

8. The reaction constants (K) calculated from the above results had a linear relationship with a wide range of concentrations of kidney extract at pH 8.0.

9. Incubation of the acid-treated kidney extract with the plasma at pH 8.0 and 20°C for 30 min. gave a more reliable method for the determination of renin in rat kidney.
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REFERENCES