Kinetic Constants of the Human Renin and Human Angiotensinogen Reaction

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ABSTRACT

The rate of formation of angiotensin was investigated for the reaction of angiotensinase-free human renin, at various states of purity, with angiotensinogen, in pooled, angiotensinase-free human serum. The velocity constant of this reaction and the Michaelis constant of the renin-substrate complex were determined. The concentration of angiotensinogen and of endogenous renin in human serum was also determined and, on the basis of these results, a procedure was designed for the indirect assay of human renin. This was carried out under a wide range of experimental conditions, for periods of incubation ranging from 10 min to 18 hours, for various concentrations of substrate, and for renin concentrations varying from 0.000025 to 0.20 unit/ml. The absence of angiotensinase made possible the prolonged incubation (18 hours) of a minute quantity of renin with a large amount of the renin substrate. The resultant formation of large amounts of angiotensin permitted its accurate assay in the dog.

ADDITIONAL KEY WORDS assay of minute amounts of human renin velocity constant concentration of endogenous renin in human serum Michaelis constant concentration of angiotensinogen in human serum

The kinetics of the enzymatic reaction of human renin with human serum (homologous angiotensinogen) has been investigated by Helmer and Judson (1) and by Pickens et al. (2). Based upon these two studies, it was assumed that the kinetics was that of a first-order reaction. The same conclusion had been reached previously by Plentl and Page (3) in a study of the reaction of hog renin with hog serum substrate. In contrast, the kinetics was considered of zero order in studies of the reaction of rabbit renin (4) and of dog renin (5) with their homologous substrates. Other studies have been carried out on the reaction of human (6), dog (7) or rabbit (8) renin with ox serum substrate.

In the previous studies, the renin and the substrate employed were not always angiotensinase-free, and, for this reason, the period of incubation was usually brief. It appeared desirable, therefore, to reinvestigate the kinetics of the human renin-human angiotensinogen reaction, when the production of angiotensinase-free human renin and human serum (9) made possible the prolonged (18 hours) interaction of a small quantity of renin with a relatively large amount of serum, and the resultant large amount of angiotensin permitted its accurate assay in the dog. It was considered that the ability to vary within a wide range the time of incubation and the concentration of enzyme and substrate would permit investigation of the nature of the interaction of human renin with its substrate in human serum with a high degree of accuracy. Also this investigation has led to the design of a procedure for the indirect assay of even minute amounts of human renin.

Materials and Methods

Two preparations of angiotensinase-free human renin were employed. Renin preparation I, with a specific activity of 0.60 unit/mg of protein was purified approximately 300-fold in the large-scale,
8-step procedure (10). Renin preparation II with a specific activity of 0.04 unit/mg of protein was obtained by a shorter procedure, in which angiotensinase was removed by treatment with ethylenediaminetetraacetic acid (EDTA) (9). These two preparations of renin, though differing 15-fold in their specific activity, induced the formation of the maximum amount of angiotensin. Furthermore, identical rates of angiotensin formation have been obtained in the present study with three renin preparations which varied in specific activity from 0.028 to 1.5 unit/mg of protein.

The human serum which served as the substrate was pooled from several hundred specimens; after treatment for removal of angiotensinase (9), it was readjusted to pH 6.9 and reconstituted with saline.

Because human serum contains a small quantity of endogenous renin, it was necessary to determine the amount of angiotensin produced during the incubation of the serum alone (in the absence of added renin) for the various experimental periods (5 min to 18 hours).

Table 1 outlines the experimental procedures involved in the production of angiotensin in human serum alone, as well as in the serum with added renin. After the incubation period, 18 hours at 38° C in this specific case, the reaction mixture (10 ml) was cooled to 0°, diluted with cold water to 30 ml and acidified to pH 5.3 with 0.3 ml of N HCl (to facilitate the subsequent coagulation of the protein). The enzymatic reaction of renin was then terminated by raising the temperature to 85° C within 2 min, and after cooling to 0° C, the denatured, insoluble serum and renal proteins were removed by centrifugation. The supernatant solution, containing the angiotensin, was neutralized with 0.14 ml N NaOH, clarified by filtration, and assayed.

A permanent colony of test dogs was used, without anesthesia, for the assay of human angiotensin and angiotensinogen (the latter being assayed indirectly after its conversion into angiotensin). These dogs were selected because each responded to the intravenous injection (saphenous vein) of a standard dose of angiotensin with an increase of 30 mm Hg of the mean femoral artery blood pressure. This amount of human angiotensin has been designated as 1 unit. The pressor effect resulting within 1 min from the injection of up to 1 unit of human angiotensin is directly proportional to its concentration.

A unit of angiotensinogen is the amount of renin substrate from which there is produced 1 unit of human angiotensin after it is incubated with an excess of human renin in the absence of angiotensinase.

In this paper, angiotensin is determined in dog units but this unit can also be expressed in terms of the quantity of synthetic angiotensin, which produces the same pressor effect (30 mm Hg). The activity of 1 dog unit was found to be identical with that of 0.33 µg of the commercially available synthetic hypertensin (Ciba), the 1-asparaginyl-5-valyl angiotensin octapeptide.

**Results**

**EFFECT OF THE CONCENTRATION OF RENIN ON THE FORMATION OF ANGIOTENSIN**

Table 1 illustrates the production of angiotensin in the serum alone and in a fixed amount of serum with different quantities of renin added.

In the absence of added renin (Table 1, expt. I) a small amount of angiotensin (0.20 unit/ml serum) was formed, as the result of the activity of the endogenous renin. It was necessary, therefore, to subtract 0.20 unit/ml of serum from the amounts of angiotensin formed in experiments II, III and IV in order to determine the enzymatic activity of only the added human renin. The experiments (II-IV) show that the formation of angiotensin increases with the concentration of renin.

The stepwise procedure outlined in Table 1, with the exceptions noted in each individual instance, was applied in essence to the other experiments (Tables 2-5). In these experiments, the concentration of renin was varied between 0.000025 and 1.0 unit/ml, the concentration of the substrate between 0.95 and 3.8 units/ml and the period of incubation from 5 min to 40 hours. Occasionally (Table 2, expts. I and II) for comparative purposes, untreated human serum containing angiotensinase was employed.

**FORMATION OF ANGIOTENSIN DUE TO THE ENDogenous RENIN IN HUMAN SERUM**

Up to four hours at 38° C, the amount of angiotensin formed due to the activity of the endogenous renin in human serum was negligible when assayed in the dog. But in 18 hours of incubation, an appreciable amount of angiotensin was produced which depended, furthermore, on the concentration of the substrate. For example, in the three experiments with 0.95, 1.9 and 3.8 units of angiotensinogen per ml, 0.07, 0.22 and 0.50 unit of angiotensin/ml, respectively, were produced (Table 3).
TABLE 1

Procedure for the Production and Assay of Human Angiotensin

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Expt. I</th>
<th>Expt. II</th>
<th>Expt. III</th>
<th>Expt. IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate</td>
<td>0.5 M phosphate pH 6.9</td>
<td>0.4 ml</td>
<td>As in</td>
<td>As in</td>
</tr>
<tr>
<td>Water</td>
<td>1.3 ml</td>
<td>Expt. I</td>
<td>Expt. I</td>
<td>Expt. I</td>
</tr>
<tr>
<td>Total volume</td>
<td>10.0 ml</td>
<td>As in</td>
<td>As in</td>
<td>As in</td>
</tr>
<tr>
<td>Added human renin</td>
<td>unit/ml serum</td>
<td>0</td>
<td>0.0005</td>
<td>0.0015</td>
</tr>
<tr>
<td>Angiotensin formed</td>
<td>units/ml serum</td>
<td>0.20</td>
<td>0.88*</td>
<td>1.90*</td>
</tr>
</tbody>
</table>

Assay of Angiotensin in the Dog

<table>
<thead>
<tr>
<th>Expt.</th>
<th>X_I</th>
<th>X_H</th>
<th>X_III</th>
<th>X_IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>0.46</td>
<td>0.72</td>
<td>0.83</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>0.29</td>
<td>0.42</td>
<td>0.50</td>
<td>0.72</td>
</tr>
<tr>
<td>III</td>
<td>0.95</td>
<td>0.85</td>
<td>0.95</td>
<td>0.85</td>
</tr>
</tbody>
</table>

Enzyme Kinetics of Human Renin

The reaction of human renin with human substrate was determined under various experimental conditions (Tables 2 and 3) in order to establish whether this reaction, in the physiological range of substrate concentration, follows a first-order or a zero-order course, or whether the Michaelis-Menten law of an enzyme-substrate interaction (11) (equation 1) can be applied. We observed that this reaction, in the integrated form of the Michaelis-Menten equation 2, holds for a wide range of experimental conditions (Table 3). This equation contains a first-order and a zero-order term, and it takes into account the formation and decomposition of a dissociating enzyme-substrate complex, representing the diphasic character of the substrate-dependence curve.

TABLE 2

Experimental Conditions and Results for the Determination of the Michaelis Constant (K_m)

<table>
<thead>
<tr>
<th>Expt.</th>
<th>t (min)</th>
<th>E (unit/ml)</th>
<th>S (unit/ml)</th>
<th>X (unit/ml)</th>
<th>K_m</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>5</td>
<td>0.500</td>
<td>0.6</td>
<td>0.46</td>
<td>0.48</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.2</td>
<td>0.72</td>
<td>0.44</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.8</td>
<td>0.83</td>
<td>0.37</td>
</tr>
<tr>
<td>II</td>
<td>5</td>
<td>0.200</td>
<td>0.6</td>
<td>0.29</td>
<td>0.57</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.2</td>
<td>0.42</td>
<td>0.63</td>
</tr>
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<td></td>
<td></td>
<td>1.8</td>
<td>0.50</td>
<td>0.75</td>
</tr>
<tr>
<td>III</td>
<td>1080</td>
<td>0.0005</td>
<td>0.95</td>
<td>0.45</td>
<td>0.76</td>
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<tr>
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<td></td>
<td></td>
<td>1.9</td>
<td>0.83</td>
<td>0.63</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3.8</td>
<td>0.72</td>
<td>0.45</td>
</tr>
</tbody>
</table>

Average, K_m = 0.56 unit angiotensin/ml serum.

E = Concentration of renin. S = Concentration of renin substrate. X = Concentration of angiotensin formed.

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In this equation: $E =$ concentration of the enzyme (unit renin/ml serum); $X =$ concentration of angiotensin formed in $t$ minutes (unit/ml serum); $S =$ concentration of substrate (angiotensinogen) at time $t = 0$ (unit angiotensin/ml serum); $S - X =$ concentration of substrate at time $t$ (unit angiotensin/ml serum); $K_m =$ Michaelis constant (unit angiotensin/ml serum); $v =$ velocity of formation of angiotensin (unit angiotensin/ml x min); $k_3 =$ velocity constant of the decomposition of the renin-substrate complex into angiotensin and renin (unit angiotensin/unit renin x min).

$$v = \frac{dx}{dt} = \frac{k_n \cdot E \cdot S}{K_m + S}. \quad (1)$$

$$k_3 = \frac{2.3 \cdot K_m \cdot \log \left( \frac{S}{S - X} \right) + X}{E \cdot t}. \quad (2)$$

At a sufficiently high concentration of substrate, when the value of $S$ in equation 1 becomes large, compared to $K_m$, the rate is maximum ($V_{max}$) and independent of the substrate concentration (equation 3).

$$V_{max} = k_3 \cdot E. \quad (3)$$

Dividing equation 1 by equation 3,

$$\frac{v}{V_{max}} = \frac{1}{1 + \frac{K_m}{S}}. \quad (4)$$

DETERMINATION OF $K_m$

Equation 2 was applied to evaluate the Michaelis constant $K_m$ on the basis of the experimental results of Table 2. For example, equation 2 can be written for each of the three different substrate concentrations $S_1, S_2$ and $S_3$ of an experiment where $X_1, X_2$ and $X_3$ are the concentrations of the angiotensin which was produced during the incubation period $t$.

$$k_3 \cdot Et = 2.3 \cdot K_m \cdot \log \left( \frac{S_1}{S_1 - X_1} \right) + X_1,$$

$$k_3 \cdot Et = 2.3 \cdot K_m \cdot \log \left( \frac{S_2}{S_2 - X_2} \right) + X_2,$$

$$k_3 \cdot Et = 2.3 \cdot K_m \cdot \log \left( \frac{S_3}{S_3 - X_3} \right) + X_3.$$  

For a pair of points the enzyme concentration $E$ and the time of incubation are the same in the two reactions, and by subtraction of the two equations and rearrangement we obtain:

$$K_m = \frac{X_2 - X_1}{2.3 \cdot \log \left( \frac{S_1}{S_1 - X_1} \right) - 2.3 \cdot \log \left( \frac{S_2}{S_2 - X_2} \right)}. \quad (5)$$

$$K_m = \frac{X_3 - X_2}{2.3 \cdot \log \left( \frac{S_2}{S_2 - X_2} \right) - 2.3 \cdot \log \left( \frac{S_3}{S_3 - X_3} \right)}. \quad (5a)$$

$$K_m = \frac{X_3 - X_2}{2.3 \cdot \log \left( \frac{S_1}{S_1 - X_1} \right) - 2.3 \cdot \log \left( \frac{S_3}{S_3 - X_3} \right)}. \quad (5b)$$

Calculated by means of these last three equations, the three experiments of Table 2 resulted in $K_m = 0.56$ unit of angiotensin/ml, on the average.

Thus, according to equation 4, a substrate concentration of $S = K_m = 0.56$ unit/ml is required for the attainment of half-maximal velocity, and this represents a characteristic constant of the human renin-human angiotensinogen reaction.
A fairly uniform value of $K_m$ was obtained under greatly varying conditions of time and renin concentration. Therefore the affinity of renin for the substrate applied equally to the three experiments, in which the concentration of renin varied from 0.0005 to 0.50 unit/ml, and to periods of incubation from 5 min to 18 hours (Table 2).

The value for the Michaelis constant in experiments I and II of Table 2 ($K_m = 0.54$) differs only slightly from that in expt. III ($K_m = 0.61$), although treated human serum served as the substrate in the latter experiment. This indicates that the quality of the substrate was not impaired by the treatment with EDTA and ammonium sulfate, and by the prolonged dialysis which was employed for the removal of the serum angiotensinase.

We evaluated the efficiency of angiotensin formation under physiological conditions of substrate concentration. For example it can be estimated (equation 4) that the formation of angiotensin in the blood probably proceeds at approximately 82% of the maximum possible velocity. This evaluation is based on the numerical value of $K_m = 0.56$ and the concentration of the renin substrate found normally in human serum ($S = 2.5$ units/ml).

$$
\frac{v}{V_{\text{max}}} = \frac{1}{1 + \frac{K_m}{S}} = \frac{1}{1 + \frac{0.56}{2.5}} = 0.82.
$$

**EFFECT OF THE CONCENTRATION OF RENIN AND THE PERIOD OF INCUBATION ON THE RATE OF FORMATION OF ANGIOTENSIN**

In the development of equation 8 we refer to:

- $E_{\text{endog}}$ = concentration of endogenous renin.
- $E_{\text{add}}$ = concentration of added renin.
- $E_{\text{total}} = E_{\text{endog}} + E_{\text{add}}$.
- $X_{\text{endog}}$ = concentration of angiotensin formed during time $t$ due to action of endogenous renin.
- $X_{\text{total}}$ = total concentration of angiotensin formed during time $t$ in the presence of added renin.

In the absence of added renin, equation 2 can be written:

$$
k_3 = \frac{2.3 \cdot K_m \log \left( \frac{S}{S - X_{\text{endog}}} \right)}{E_{\text{endog}} \cdot t} + X_{\text{endog}}.
$$

In the presence of added renin, equation 2 can be written:

$$
k_3 = \frac{2.3 \cdot K_m \log \left( \frac{S}{S - X_{\text{total}}} \right) + X_{\text{total}}}{E_{\text{total}} \cdot t}.
$$

Subtraction of equation 6 from equation 7 and substitution of $(E_{\text{total}} - E_{\text{endog}}) = E_{\text{add}}$, results in:

$$
k_3 = \frac{2.3 \cdot K_m \log \left( \frac{S - X_{\text{endog}}}{S - X_{\text{total}}} \right) + (X_{\text{total}} - X_{\text{endog}})}{E_{\text{add}} \cdot t}.
$$

Under a wide range of experimental conditions, a rather uniform mean value was obtained for the rate constant $k_3$ (Table 3). This constant ($k_3$) was $1.7 \pm 0.045$ se, units angiotensin/unit renin·minute, with a ±12% coefficient of variation. In these 20 experiments, the concentration of renin varied from 0.000025 to 0.20 unit/ml, the concentration of the substrate from 0.95 to 3.80 units/ml, and the period of incubation from 10 min to 18 hours.

The results of Table 3 indicate that:

- (A) The enzymatic activity of renin was not impaired even in a very dilute solution (Table 3, expt. VII) and during 18 hours of incubation at 38°C.
- (B) There was no loss of the angiotensin that had been produced during this prolonged period of incubation, and angiotensinase apparently had been removed effectively from the renin preparation and from the substrate.
- (C) The velocity of angiotensin formation was constant for various concentrations of the substrate, even when angiotensinogen was lowered to 50%, or was increased to 200% of the normal level of human serum (Table 3, expt. VIII).

The present kinetic study includes the determination of the enzymatic activity of renin at very low concentrations (Table 3, expt. VII), considerably lower than the average concentration (0.00016 unit/ml) found in pooled human serum. The procedure outlined in Table 1 was applied, without any change, in the production of angiotensin;
however for the bioassay of these minute amounts of angiotensin, the anesthetized rat was employed according to the method of Peart (12). (We are indebted to Dr. Anne B. Gould, of the Department of Medicine, for performing these assays in the rat.) For these reactions, the rate constant, determined according to the Michaelis-Menten equation was identical (average $k_0 = 1.7$) with those of the other experiments listed in Table 3 and obtained with much higher concentrations of renin.

The experimental data of Table 3 were utilized again, in the integrated form of the zero-order equation as well as of the first-order equation, to investigate whether the reaction of human renin with homologous substrate, under various experimental conditions, can be expressed by only one of these rate constants. The separate values of $k_{(\text{zero-order})}$, obtained in this manner, were not the same: In experiments I-VII they declined concurrently with an increase of the renin concentration; their value increased in experiment VIII with increased concentration of the substrate.

The value of $k_{(\text{first order})}$ in Table 3, when calculated for various intervals of time and for the three concentrations of substrate investigated, was not constant. For example, the separate values declined from approximately 1.1 to 0.8, when the period of incubation was extended from the initial 10-min period in experiment I to 18 hours in experiments IV-VII. The decrease in the value of the first-order

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### Table 3

**Calculation of Velocity Constants—Effects of Variation of the Time (t), Renin Concentration (E) and Substrate Concentration (S)**

<table>
<thead>
<tr>
<th>Expt.</th>
<th>$t$ (min)</th>
<th>E (unit/ml)</th>
<th>S (unit/ml)</th>
<th>$X_{\text{total}}$ (unit/ml)</th>
<th>$X_{\text{used}}$ (unit/ml)</th>
<th>$k_{(\text{zero-order})}$</th>
<th>$k_{(\text{first-order})}$</th>
<th>$k$</th>
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<tbody>
<tr>
<td>I</td>
<td>10</td>
<td>0.05</td>
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<td>0.7</td>
<td>0</td>
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<td>1.0</td>
<td>1.9</td>
</tr>
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<td></td>
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<td></td>
<td></td>
<td>1.2</td>
<td></td>
<td>1.2</td>
<td>1.1</td>
<td>1.8</td>
</tr>
<tr>
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<td></td>
<td>1.6</td>
<td></td>
<td>0.8</td>
<td>1.1</td>
<td>1.4</td>
</tr>
<tr>
<td>II</td>
<td>60</td>
<td>0.01</td>
<td>2.5</td>
<td>1.0</td>
<td>0</td>
<td>1.7</td>
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<td></td>
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<tr>
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<td>240</td>
<td>0.005</td>
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<td>0.8</td>
<td>1.7</td>
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<tr>
<td>V</td>
<td>1080</td>
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<td>0.0003</td>
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<td>1.3</td>
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<td>1080</td>
<td>0.0005</td>
<td>0.95</td>
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<td>0.07</td>
<td>0.8</td>
<td>1.3</td>
<td>1.6</td>
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<td>0.9</td>
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<td></td>
<td>1.3</td>
<td>0.5</td>
<td>1.6</td>
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</table>

**20 Experiments**

- Mean $1.2, 0.86, 1.7$
- Standard error of estimate $0.052, 0.043, 0.045$
- Coefficient of variation $20\%, 22\%, 12\%$

**Abbreviations**

$k_{(\text{zero-order})} = \left( X_{\text{total}} - X_{\text{used}} \right) \cdot \frac{1}{E_{\text{added}} \cdot t}$

$k_{(\text{first-order})} = 2.3 \log \left( \frac{S - X_{\text{used}}}{S - X_{\text{total}}} \right) \cdot \frac{1}{E_{\text{added}} \cdot t}$

$k_0$, see equation 8 in text. $K_m = 0.56$.  

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velocity constant was even more pronounced (from 1.3 to 0.5) in experiment VIII, as a result of the increase of the substrate concentration.

The coefficient of variation calculated for $k_3$ ($\pm 12\%$) was appreciably lower than the corresponding values, $\pm 20\%$ and $\pm 22\%$, respectively, for the zero- and first-order velocity constants.

It appears, therefore, that the renin-angiotensinogen reaction is neither zero-order nor first-order in nature. In contrast, under all of the experimental conditions of Table 3 a fairly uniform value resulted for $k_3$ when the integrated form of the Michaelis-Menten equation 8 was applied to express the kinetics of the renin-catalyzed formation of human angiotensin.

The right side of the Michaelis-Menten equation 8 contains a first-order and a zero-order term. The latter, $(X_{\text{total}} - X_{\text{end}})$ accounted for the major share of the angiotensin production. For example, from the data of the 20 experiments in Table 3 it can be calculated that the zero-order term accounts for an average of 72% of the total angiotensin produced, ranging from 53% at the lower substrate concentration to 84% at the higher level of substrate.

**ASSAY OF ANGIOTENSIN IN HUMAN SERUM**

The experiments outlined in Table 4 were designed, first, to determine the total amount of substrate available in the pooled, treated human serum by the rapid conversion of the substrate into angiotensin by an excess of added human renin. These experiments served, furthermore, to evaluate any possible interference by angiotensinase under a variety of experimental conditions, e.g., (A) an extended range of concentrations of human renin, (B) various periods of incubation, (C) two renin preparations of different purity.

Previously (10), the presence of angiotensinase in the highly purified preparation I of human renin was investigated by the determination of the rate of inactivation of added synthetic angiotensin II-asparaginyl-$\beta$-amide, by incubation with the renin for 10 min at 38°C. Under these conditions, angiotensinase was removed completely from the preparation of human renin resulting from step 8 of the isolation procedure. This has been confirmed in the present investigation by following the formation and recovery of human angiotensin during prolonged periods of incubation of high concentrations of the human renin with the pooled, treated human serum.

In the experiments listed in Table 4, the absence of angiotensinase from the two preparations of human renin and from the pooled, treated human serum was demonstrated by the complete recovery of angiotensin. Any effect of residual amounts of angiotensinase would have been detected by reduction in the amount of angiotensin produced, especially since the concentration of renin in the experiments of Table 4 was so much higher than the renin concentration which was employed in the kinetic study (Table 3).

The data in Table 4 show that: (A) When a relatively high concentration (1.0 unit/ml) of renin preparation I was employed (expt. 1), it induced the complete conversion of the substrate into angiotensin in 10 min at 38°C. When the period of incubation was prolonged to 240 min, the same amount of angiotensin was recovered. (B) 0.03 unit and 1.0 unit of the renin/ml of serum produced the maximum amount of angiotensin during an incubation period of 4 hours (expt. 2). (C) The prolongation of the incubation period to 18 hours, with an excess of renin, still resulted in the maximum yield of angiotensin (expt. 3). (D) When the renin preparations I and II, with specific activities of 0.60 and 0.04, were incubated with human serum, they induced the formation of identical amounts of angiotensin. Thus the two renin preparations served equally well for the production of the maximum amounts of angiotensin necessary for the assay of total serum angiotensinogen. (E) A maximum amount of angiotensin (2.2 units/ml of serum) was found in 4 or 40 hours after its production with 0.006 unit of renin/ml (expt. 7).

Altogether, 40 experiments were performed with 12 different batches of pooled, treated
human serum, to determine the angiotensinogen content. Under the experimental conditions of Table 1, experiment IV, or Table 4, the mean value was found to be 2.35 ± 0.052 SE units angiotensinogen/ml of serum, with a relative standard deviation, (coefficient of variation) \( \frac{SD}{\text{mean}} \times 100 \) of ±14%.

**INDIRECT ASSAY OF HUMAN RENIN**

Since the Michaelis-Menten equation of enzyme-substrate reaction is applicable to human renin and is valid over a wide range of concentrations and for various periods of incubation, it became possible to design an indirect procedure for the assay of even minute amounts of human renin from the amount of angiotensin that it produces, and thus express the concentration of renin in such a preparation directly, in terms of dog units/ml. The comparison of the unknown renin preparation with a standard unit of human renin is therefore no longer required. Any period between 10 min and 18 hours, can be employed for the incubation of renin with its substrate. The indirect procedure of assay was tested over a wide range and it was found to be valid for any concentration of human renin between 0.000025 and 0.20 unit/ml, provided the renin and the substrate were free of angiotensinase.

In order to illustrate two examples of the practical application of the kinetic concept developed in the present study, we determined the concentration of endogenous renin in human serum, and we assayed, by the indirect procedure, the concentration of renin in two "unknown" specimens (Table 5). For this purpose, the experimental data of Table 1 were utilized again in Table 5, to demonstrate the method of calculation of the concentration of endogenous renin in pooled, treated human serum serving as the source of substrate (expt. I) and to redetermine, by the indirect method, the renin titer of two preparations of human renin (Table 1, expts. II and III). The concentration of renin in these two specimens had been determined previously, as accurately as possible, by the direct procedure in dogs.

In Table 5, \( E_i \) = concentration of endogenous renin in the serum: experiment I of Table 1. \( E_{II} \) = concentration of added "unknown" renin: experiment II of Table 1.

---

**TABLE 4**

Production of Maximum Amounts of Human Angiotensin (Evaluation of Human Renin and Substrate for the Presence of Residual Angiotensinase)

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Incubation at 38° C</th>
<th>Concentration of human renin (unit/ml)</th>
<th>Angiotensin produced (unit/ml serum)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10 min</td>
<td>1.00</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td>240 min</td>
<td></td>
<td>2.5</td>
</tr>
<tr>
<td>2</td>
<td>4 hours</td>
<td>0.03</td>
<td>2.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.00</td>
<td>2.5</td>
</tr>
<tr>
<td>3</td>
<td>18 hours</td>
<td>0.005</td>
<td>2.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.020</td>
<td>2.7</td>
</tr>
<tr>
<td>4</td>
<td>1 hour</td>
<td>0.06</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.20</td>
<td>2.6</td>
</tr>
<tr>
<td>5</td>
<td>4 hours</td>
<td>0.02</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.03</td>
<td>2.7</td>
</tr>
<tr>
<td>6</td>
<td>18 hours</td>
<td>0.004</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.008</td>
<td>2.5</td>
</tr>
<tr>
<td>7</td>
<td>4 hours</td>
<td>0.005</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>40 hours</td>
<td>0.004</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Average = 2.6</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Average = 2.5</td>
<td>2.5</td>
</tr>
</tbody>
</table>
(0.0005 unit/ml). $E_{III} =$ concentration of added "unknown" renin: experiment III of Table 1 (0.0015 unit/ml). $X_I, X_{II}, X_{III}, X_{IV}$ = concentration of angiotensin produced in experiments I-IV of Table 1 during incubation for 18 hours, at $38^\circ C$ (unit/ml). $S = X_{IV}$ = concentration of substrate at time $t = 0$ (2.50 units/ml); $t = 1080$ (min); $K_m = 0.56$ (unit/ml); $k_3 = 1.7 \frac{\text{units angiotensin}}{\text{unit renin} \cdot \text{minute}}$.

The content of endogenous renin was calculated to be $E_t =$ 0.00013 unit/ml, in this batch of pooled, treated human serum. In 22 similar experiments with 13 different batches of pooled, treated human serum the mean value was $0.00016 \pm 0.0000065$ se unit of endogenous renin/ml of serum with a coefficient of variation of $\pm 19\%$.

That this small amount of angiotensin was due to the catalytic activity of the small amount of endogenous renin in the human serum was shown by the complete inhibition of angiotensin formation when only 0.01 unit/ml of antirenin was added before the incubation.

The indirect assay of the two specimens of added human renin, $E_{II}$ and $E_{III}$, was performed at two levels of concentration of renin (0.0005 and 0.0015 unit/ml). As a result, they induced, in 18 hours, at $38^\circ C$, a depletion of 35% and 76%, respectively, of the substrate originally present in the serum, yet the renin could be determined, under both conditions, with an error of 4 to 10%. This indicates that in the present procedure a wide range of substrate can be utilized with good precision. In contrast, in previous procedures (6-8) the range of utilization of substrate for the assay of renin was limited. Only the initial velocity of angiotensin formation was used in the calculation of the renin concentration, and it was essential to take samples of the incubation mixture sufficiently early.

To evaluate the precision of the indirect assay of added specimens of human renin, a series of experiments was performed under the conditions of Table 1, experiment II, and the concentration of renin was calculated according to Table 5. Three preparations of renin were employed, each with a different history of purification (9, 10). The specific

### Table 5

**Method of Calculation of the Renin Concentration from the Concentration of Angiotensin Produced in 18 Hours at 38° C (Experiments I-IV of Table 1)**

<table>
<thead>
<tr>
<th>Step</th>
<th>Formula</th>
<th>Calculation</th>
</tr>
</thead>
<tbody>
<tr>
<td>$E_I$</td>
<td>$E_I = \frac{2.3 \cdot K_m \cdot \log \left( \frac{S-X_{\text{end}}}{S-X_{\text{total}}} \right) + X_{\text{end}}}{k_3 \cdot t}$</td>
<td>Calculated 0.00013 unit of renin/ml serum</td>
</tr>
<tr>
<td>$E_{II}$</td>
<td>$E_{II} = \frac{2.3 \cdot 0.56 \cdot \log \left( \frac{2.50-0.20}{2.50-0.88} \right) + 0.20}{1.7 \cdot 1080}$</td>
<td>Calculated 0.00048 unit of renin/ml serum</td>
</tr>
<tr>
<td>$E_{III}$</td>
<td>$E_{III} = \frac{2.3 \cdot 0.56 \cdot \log \left( \frac{2.50-0.20}{2.50-1.90} \right) + (1.90-0.20)}{1.7 \cdot 1080}$</td>
<td>Calculated 0.00135 unit of renin/ml serum</td>
</tr>
</tbody>
</table>

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activity varied from 0.026 to 1.20 units/mg protein and the renin content of each preparation had been determined accurately by the direct procedure. Each of the five batches of human serum serving as the substrate had been pooled from approximately 300 specimens and treated for the removal of angiotensinase.

In 10 experiments, after the addition of 0.00050 unit of renin/ml, the mean value was found to be 0.00053 ± 0.000011 SE unit of renin/ml, with a coefficient of variation of ± 6.5%.

Discussion

In this study an attempt was made to mimic normal physiological conditions as closely as possible. The incubation of the human renin with its substrate (human serum) was performed in a neutral, buffered solution, containing saline and serum proteins, which included the homologous angiotensinogen. In previous studies, salt and buffer had been omitted (2), which resulted in a lowered velocity of reaction of renin; or the incubation was performed in an acid solution, pH 5.5 to 5.7 (1, 6), presumably, to minimize interference by angiotensinase; or heterologous substrate (ox serum) was employed for the assay of the human renin (6). Although each of these adjustments was of practical importance for the assay of the human renin, the conditions were unphysiological for the study of the enzyme kinetics of the renin.

The validity of assuming a first-order reaction in the assay of renin had been explored previously only over a narrow range of renin concentrations—a 2-fold variation of hog renin (3), a 4-fold variation for human renin (1) and a 10-fold variation for human renin (2). In this study an 8,000-fold variation of the concentration of human renin was employed (Table 3), and the results indicate that the reaction of human renin cannot be expressed by a first-order constant. Furthermore, in order to demonstrate a first-order rate of reaction, the effect of a variation of the incubation period had been previously explored. For example, Plentl and Page (3) recommended a rather narrow time interval: “Not less than 5 minutes and not more than 10 minutes,” Helmer and Judson (1) employed a reaction time of 1 to 2 hours, while Pickens et al. (2) incubated renin with human angiotensinogen for 1 hour. Such single, short periods are not adequate to permit a classification of the order of activity of renin. It is clear from the results of Table 3, that it is necessary to use various periods, ranging from 10 min to 18 hours, to establish the fact that a first-order reaction concept cannot be applied to renin. In contrast, the rate constant $k_3 = 1.7$, according to the Michaelis-Menten equation 8, was found to be equally valid for the quantitative determination of renin for any time interval between 10 min and 18 hours and for any renin concentration between 0.000025 and 0.2 unit/ml.

The pressor activity of 1 unit of angiotensin is identical to that of 0.33 μg of the synthetic, commercially available valine-B-angiotensin II amide. The kinetic constants can be recalculated, therefore, and they can be expressed also as:

$$k_3 = 0.56 \frac{\text{μg angiotensin}}{\text{unit renin \cdot minute}}$$

for the rate constant and as:

$$k_m = 0.185 \frac{\text{μg angiotensin}}{\text{ml serum}}$$

for the Michaelis constant.

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