SUMMARY The phenomenon of plasma renin activation by acid dialysis and preincubation with trypsin was studied in normal human plasma. Activation of plasma renin by exposure to pH 3.3 was shown to require at least one dialysis step and could be inhibited by the presence of Trasylol, indicating the involvement of a protease in acid activation. Amniotic fluid exposed to pH 1.5 to destroy renin and renin substrate was also found to contain an enzyme capable of activating plasma renin. The Michaelis-Menten constant $K_m$ and the molecular weight of activated "renin" were found to be similar to those of normal plasma renin. Inactive renins or renin-like enzymes were partially purified from plasma by affinity chromatography on concanavalin A, precipitation with $(NH_4)_2SO_4$, and isoelectric focusing. Trypsin and acid exposure gave similar results with regard to the activation of this zymogen, suggesting that trypsin and acid dialysis may increase plasma renin activity by the same mechanism. (Hypertension 1: 523-528, 1979)

KEY WORDS • renin • plasma • activation • trypsin • dialysis • characterization

RECENT studies have shown that the renin concentration of plasma can be increased by in vitro techniques such as 1) dialysis of plasma to pH 3.3 followed by dialysis to pH 7.4; 2) preincubation of plasma with proteolytic enzymes such as pepsin or trypsin; and 3) by cold storage. Alterations in the ratio of an inactive renin or renin-like enzyme to the active form have also been shown to occur in vivo following renin stimulation or suppression. In this study, we have explored the molecular mechanism(s) underlying the in vitro phenomenon of plasma renin activation and isolated and partially characterized an inactive renin or renin-like zymogen from normal human plasma.

Methods and Materials

Blood Collection

Blood was collected in Na-EDTA from consenting normotensive subjects, centrifuged at $4^\circ$ and the plasma was either used immediately or snap frozen and stored at $-20^\circ$C to prevent possible cryoactivation.

Measurement of Renin Concentration and Activity

Plasma renin concentrations (PRC) were determined in the presence of angiotensinase inhibitors by measuring the rate of angiotensin I generation by endogenous renin (PRA) and following the addition of several concentrations $(n = 5)$ of standard renin (Mill Hill); if the sample under investigation did not contain sufficient endogeneous renin substrate to maintain a linear rate of angiotensin I generation for 1 hour, plasma obtained from women on oral contraceptive therapy was added. Ammonium sulfate fractions were tested for renin activity by the addition of purified homologus renin substrate from normal human plasma.

Renin concentration was determined from a linear least squares extrapolation of angiotensin I generation as a function of added renin standard. When only renin activity measurements were required, i.e., for the localization of renin following chromatography, samples were incubated as described above, with added renin substrate from plasma of women on oral contraceptive therapy but without the added aliquots of standard renin. All samples were titrated to pH 7.4 with phosphate buffer and incubated...
in the presence of angiotensinase inhibitors.\textsuperscript{10} Generated angiotensin I was measured by radioimmunosassay.\textsuperscript{13}

**Renin Substrate (Angiotensinogen) Quantitation**

Renin substrate was measured by the quantitation of generated angiotensin I following incubation of plasma with homologous renin.\textsuperscript{13}

**Activation of Plasma Renin**

**Acid Dialysis**

The plasma samples were dialyzed to pH 3.3 (0.05 M glycine-HCl) for 24 hours and then dialyzed back to pH 7.4 against 0.05 M NaH\textsubscript{2}PO\textsubscript{4}/Na\textsubscript{2}HPO\textsubscript{4} for 24 hours at 4°C.\textsuperscript{1} A control sample was dialyzed only against the latter buffer at 4°C for 48 hours. Homologous renin substrate (oral contraceptive plasma) was added to all acid-treated-plasma samples to compensate for the irreversible denaturation of human renin substrate, (fig. 1).

**Trypsin**

Plasma samples or column fractions were adjusted to pH 7.4, 0.1 M NaH\textsubscript{2}PO\textsubscript{4}/Na\textsubscript{2}HPO\textsubscript{4}, and incubated at 37°C for 60 seconds with trypsin (1.0 mg/ml for whole plasma and 0.1 mg/ml for column fractions; Trypsin-Sigma, 10,000–13,000 BAEE units/mg protein, made up fresh as 1.0 mg/ml 0.1 M Na-acetate, 0.02 M CaCl\textsubscript{2}; pH 4.5). The reaction was stopped by the addition of lima bean trypsin inhibitor (0.2 mg/ml of sample; LBTI-Sigma in 0.1 M Na\textsubscript{2}PO\textsubscript{4}/Na\textsubscript{2}HPO\textsubscript{4}; pH 7.4); control samples received aliquots of trypsin vehicle in place of trypsin. At the trypsin concentrations used, an incubation time greater than 60 seconds did not yield greater renin activation, whereas higher concentrations of trypsin led to decreased renin activity.

**Amniotic Fluid**

Human amniotic fluid was dialyzed to pH 1.5 against 31.6 mM HCl, 0.15 M NaCl, 0.07 M KC1 to destroy renin and renin substrate.\textsuperscript{14} Plasma and the acid-treated human amniotic fluid were then dialyzed to pH 4.5 against 27.8 mM citric acid, 45 mM NaH\textsubscript{2}PO\textsubscript{4}, 5 mM EDTA and 82 mM NaCl overnight at 4°C and subsequently incubated (0.1 ml amniotic fluid + 1 ml plasma) at 37°C for 6 hours.\textsuperscript{14} As a control, plasma samples were used to which an aliquot of 0.15 M NaCl was added in place of amniotic fluid. After a second overnight dialysis at 4°C against 0.05 M NaH\textsubscript{2}PO\textsubscript{4}/Na\textsubscript{2}HPO\textsubscript{4} at pH 7.4 the renin concentration of the dialysate was determined.

**Kinetics**

Michaelis-Menten constant \textit{Km} was determined by linear least squares regression analysis of the reciprocals of the quantity of angiotensin I generated as a function of renin substrate concentration.\textsuperscript{18}
Pooled normal human plasma was chromatographed on a Concanavalin A affinity column (Pharmacia Fine Chemicals). The column was equilibrated in 0.1 M Na-acetate, 0.1 M NaCl, 1 mM MnCl₂, and 1 mM MgCl₂ and eluted with 0.05 M α-methyl-D-mannoside in the same buffer.

Isoelectric focusing was performed with an LKB 8121 instrument using a sucrose and pH gradient (4-6) according to the method of Vesterberg and Svensson.¹⁷

Results

Activation of Plasma Renin

Following the activation of plasma from normotensive subjects (n = 9) by acid dialysis, the PRC increased from $1.3 \pm 0.3$ (SE) to $2.6 \pm 0.4 \times 10^{-4}$ IU/ml, ($p < 0.05$) and the PRA increased concomitantly from $4.6 \pm 1.0$ to $10.6 \pm 2.2$ ng angiotensin I/ml/hr ($p < 0.025$). No statistically significant change in the slope of the least squares regression line of angiotensin I generated as a function of added renin concentration was observed upon acid activation ($3.5 \pm 1.8$ vs $4.1 \pm 1.3 \times 10^{-4}$ ng Al/IU). The slope of this regression line also remained unaltered when plasma renin was activated by incubation with trypsin. A comparison of the Michaelis-Menten constant $K_m$ in pooled plasma before and after plasma renin activation indicated no statistically significant change in this parameter.¹⁸

Dialysis vs Titration for Activation of Plasma Renin

In an attempt to obtain renin activation without dialysis against 0.05 M glycine, pH 3.3, the pH was lowered to 3.3 by titration with one molar glycine using a pool of normal human plasma. The plasma was kept at this pH for 18 hours at 4°C before titrating the sample back to pH 7.4 with 0.1 N NaOH. Eighteen hours was chosen since this represents optimum time for acid activation.¹⁹ No activation of plasma renin activity was observed using this titration method as shown in table 1. The above experiment was repeated using only one titration step, i.e., titration of plasma to pH 3.3 followed by dialysis to pH 7.4 or dialysis of plasma to pH 3.3 followed by titration to 7.4; in both cases activation, equivalent to the two-step dialysis method, was attained (table 1). To rule out possible effects of the dialyzing medium as being responsible for renin activation, a plasma sample was titrated to pH 3.3 and then dialyzed to pH 7.4 against a large volume of untreated plasma from the same pool; again activation was observed (table 1).

To determine whether acid dialysis induced the removal of an acid dissociable, dialyzable inhibitor from a renin zymogen, 0.05 M glycine (pH 3.3) which had been previously employed as the dialysis medium to activate plasma renin was concentrated (Diaflo membrane PM 10) and added to activated plasma. The addition of this concentrated fraction to fully activated renin at pH 7.4 did not cause a decline in renin activity ($25.4 \pm 25.4$ ng Al/ml/hr).

Enzymatic Processes in Plasma Renin Activation

To explore the possible involvement of enzymatic processes in acid activation, dialysis to pH 3.3 was performed in the presence of the non-dialyzable protease inhibitor aprotinin (final plasma concentration of Trasylol, 1000 Kallikrein Inactivator units/ml) which was added to plasma before dialysis. Acid activation of plasma renin was inhibited in the presence of this proteolytic enzyme inhibitor. Renin activities of pH 7.4 and pH 3.3 dialysates with and without Trasylol were $21.9 \pm 0.5$ (pH 7.4) and $33.3 \pm 1.2$ (pH 3.3) for control and $20.7 \pm 0.2$ (pH 7.4) and $22.1 \pm 2.2$ (pH 3.3) with Trasylol. No further significant increase of renin activity was evident when samples which had previously been activated by acid dialysis were treated with trypsin. Experiments to investigate whether acid dialysis would cause further activation of trypsin-treated-plasma samples could not be performed since active plasma renin was found to be partially inactivated by prolonged exposure to pH 3.3; when acid-activated plasma was redialyzed at pH 3.3 for 24 hours, a 10 to 50% loss in renin concentration was observed, suggesting that renin activation may be a composite of two processes: partial denaturation of renin by exposure to pH 3.3, combined with activation of a zymogen which makes the dominant contribution to the overall renin activity. Because of this partial instability of active plasma renin to pH 3.3, plasma activated by trypsin generally demonstrated a larger increase in renin activity.

Further evidence in support of an enzymatic process for the activation of plasma renin was obtained from experiments using human amniotic fluid exposed to pH 1.5. The presence of an enzyme in the amniotic fluid (pH 1.5) was observed that was capable of activating plasma renin (12.7 vs 2.7 ng angiotensin I/ml/hr).

<table>
<thead>
<tr>
<th>Step One</th>
<th>Step Two</th>
<th>PRA (ng/Al/ml/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dialysis pH 7.4</td>
<td>dialysis pH 7.4</td>
<td>$9.3 \pm 0.9$ (control)</td>
</tr>
<tr>
<td>titration pH 3.3</td>
<td>titration pH 7.4</td>
<td>$7.2 \pm 1.3$</td>
</tr>
<tr>
<td>dialysis pH 3.3</td>
<td>dialysis pH 7.4</td>
<td>$13.9 \pm 0.7$</td>
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<tr>
<td>dialysis pH 3.3</td>
<td>titration pH 7.4</td>
<td>$14.0 \pm 0.9$</td>
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<tr>
<td>titration pH 3.3</td>
<td>dialysis pH 7.4</td>
<td>$15.5 \pm 1.7$</td>
</tr>
<tr>
<td>titration pH 3.3</td>
<td>dialysis pH 7.4</td>
<td>$14.5 \pm 0.9$</td>
</tr>
</tbody>
</table>

* $\pm$ SE.
†The time the titrated samples were maintained at a given pH value were equivalent to the dialysis period.
‡Dialysis against untreated plasma.
Isolation of “Renin” Zymogen(s)

To investigate whether a “renin” zymogen could be isolated from normal human plasma, several techniques were employed.

Salt fractionation was employed to separate active and inactive renin. A plasma pool containing 57% active renin and 43% acid activable renin (inactive renin) was fractionated by (NH₄)₂SO₄ precipitation. All renin activity could be precipitated with 2.5 M (NH₄)₂SO₄. The 0–1.5 M precipitate on the other hand contained 40% of the active renin and 84% of the inactive renin. Further subfractionation in 0.5 M (NH₄)₂SO₄ increments indicated that 70% of the inactive renin and 30% of the active renin were located in the 1–1.5 M precipitate whereas 60% of the total active and 16% of the total inactive renin were observed in the 1.5–2.5 M precipitate (fig. 2). To establish that the low concentration of inactive renin in the 1.5–2.5 M precipitate was not due to the absence of a factor responsible for the conversion of the zymogen to the active enzyme (a zymogen convertase), fresh plasma was added to this fraction before activation; no further increase in renin activation was observed.

Pooled plasma was chromatographed on a concanavalin A affinity column to separate active and inactive renin. The unbound plasma fraction contained 69.2% of the inactive and 33.9% of the active renin whereas 66.1% of the active renin and 30.8% of the inactive renin were eluted from the column with 0.05 M α-methyl-D-mannoside. Based on the differential binding of inactive and active renin to Concanavalin A, the presence of a distinct zymogen subspecies with different carbohydrate moieties is suggested.

Fractionation of normal human plasma (n = 5) on Sephadex G-100 in 0.05 M tris-HCl pH 8.4, indicated a single peak of renin activity with an apparent molecular weight between 45 and 55,000 daltons. Following incubation of the individual elution fractions with trypsin, a 3.4-fold increase in renin activity was evident. However, no statistically significant shift in the renin peak was observed suggesting no change in molecular weight (fig. 3). Similar results were obtained with regard to apparent molecular weights when plasma was acid dialyzed or incubated with trypsin prior to chromatography.

On isoelectric focusing active renin of untreated plasma was found to have isoelectric points at pH 5.1 and 5.3 (fig. 4B). Activation of the individual column fractions by trypsin and acid dialysis are shown in figure 4A. In addition to an increase in renin activity at pl 5.1 and 5.3, three additional peaks of renin activity are apparent with pl’s of 4.9, 5.5 and 5.7, respectively. Acid dialysis or trypsin treatment before isoelectric focusing demonstrate the same pattern for renin activity as observed in figure 4B.

Discussion

The phenomenon of activation of plasma renin may be attributable to several mechanisms including: 1) an increase in plasma renin concentration; 2) the destruction of a plasma renin inhibitor; or 3) the generation of an accelerator of the renin reaction. To distinguish among these various possibilities, we performed plasma renin concentration measurements according to the method of Cohen et al. Since this method also allows one to determine whether a change in plasma renin activity is due to a change in enzyme concentration or due to modifiers of the renin reaction. From the rate of angiotensin I production in a plasma as a function of added homologous renin concentration, the PRC is determined by linear least square regression analysis. The slope of this regression line, PRA/PRC, is dependent in addition to the renin concentration on the plasma renin substrate concentration and the presence of modifiers of the renin reaction. Since human renin substrate is irreversibly denatured by ex-
Figure 3. Elution profile of human plasma following chromatography on Sephadex G-100. Continuous line represents optical density, solid line shows renin activity (control), and dotted line shows renin activity following activation of column fractions by trypsin.

Figure 4. A: Elution profile of human plasma renin activity following isoelectric focusing (pH 4-6) and activation by acid (solid line) or trypsin (dotted line). B: Elution profile of renin activity of untreated human plasma.

Exposure to pH 3.3, renin substrate was added to the plasma following acid activation to equalize substrate concentrations in acid and neutral dialyzed samples. Under these conditions, therefore, changes in the slope are an indication of changes in modifiers of the renin reaction. Since upon activation of plasma by either acid exposure or trypsin, no change in slope was observed, activation of an accelerator or destruction of an inhibitor of the renin reaction do not appear to be the mechanism of renin activation in normal human plasma. The kinetic data obtained before and after activation also seem to rule out an induced structural alteration in the active renin molecule, since no change in the Michaelis-Menten constant (Km) was observed. A lack of change in the Km values upon acid activation have also been reported by Derkx et al. using sheep renin substrate. Further evidence against the hypothesis that acid exposure causes activation by an alteration of native active renin was the observation that repeated exposure of plasma to pH 3.3 caused a partial denaturation, not activation of active plasma renin. This effect of acid treatment is not normally evident since it is masked by the renin activation phenomenon.

We, as well as several other investigators have demonstrated the existence of a "renin" zymogen in plasma. Salt fractionation, affinity chromatography with Concanavalin A, and isoelectric focusing techniques have been employed to partially separate the active enzyme from an inactive zymogen. Whether the active end product of the activation process is renin, a renin isozyme or another enzyme capable of cleaving the leucyl-leucyl bond of renin substrate to generate angiotensin I is at present not clear. The similarity of the Km, and molecular weight observed before and after acid or trypsin activation favor the postulate that the zymogen is prorenin. On the other hand, the absence of a change in the pl of the additional forms of plasma "renin" when plasma was acid treated prior to isoelectric focusing indicate that these additional activated enzymes may not be the normal plasma renin. Although the presence of a plasma prorenin has been suggested, conclusive data demonstrating that the end product of the activation process is normal plasma renin are lacking. Definite proof will require further purification and characterization of the zymogen in order to compare the physical chemical properties of its active end product with those of purified plasma renin.
The observation that activation of plasma renin by acid exposure can be inhibited by the presence of the protease inhibitor Trasylol supports the involvement of an enzymatic process as a mechanism for activation. Supporting evidence for an enzymatic mechanism are: 1) the activation of "renin" by preincubation with proteolytic enzymes such as trypsin; 2) the involvement of a serine protease in cold storage "renin" activation; 3) activation of plasma renin by preincubation with amniotic fluid previously exposed to pH 1.5; 4) the requirement of plasma for activation following purification of inactive "renin"; and 5) the observation that plasma samples lacking factor XII of the clotting system demonstrated reduced cryoactivation. Morris and Lumbers have proposed that activation of renin in human amniotic fluid also occurs via proteolytic enzymes. A postulated mechanism for "renin" activation based on the involvement of an enzymatic process is shown in figure 5. Acid dialysis and trypsin may initiate the generation of the same active end product, i.e., trypsin may activate a serine protease that can alternately be activated by acid exposure and dialysis to remove the dialyzable inhibitor we have described. Although trypsin is known to activate other serine proteases, the possibility that trypsin may act as the convertase in the activation of "renin" zymogen cannot be excluded. The above mechanism could be expanded to account for the multiphasic aspects of acid activation indicated by Atlas et al. by postulating either that the observed renin zymogens have different pH optima with respect to the active convertase or that several convertases are activated at pH 3.3 with different optima for the renin zymogen substrates.

Elucidation of all the parameters of the mechanism of plasma renin activation will have to await the further purification of the individual components. It is evident from this study, however, that the phenomenon of plasma renin activation is not simply an acid-induced conversion of inactive to active renin but that a plasma enzyme system is involved that may have physiological significance in the control of blood pressure by regulation of the plasma "renin" concentration.

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