Measurement of Renin Activity in Human Plasma

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Many methods for measurement of renin activity have been described since Tigerstedt and Bergman first made an extract of kidney and showed that when injected it raised blood pressure. Those methods which rely on the rise of blood pressure following such injections suffer from three main disadvantages. First, the magnitude of response to a given dose may vary and tachyphylaxis can be elicited. Second, relatively large quantities of extracts are required to obtain a measurable response. Third, renin is an enzyme and not a pressor substance as thought at first, and this type of assay does not measure its enzymatic activity as discussed originally by Plentl and Page.2

Methods in which renin is allowed to react with substrate in vitro, and in which the resultant angiotensin is measured, avoid these difficulties.2 Angiotensin does not produce tachyphylaxis except in large doses. The sensitivity of renin measurement can be increased by prolonging the time of incubation. Furthermore, these methods allow control of numerous factors which affect the rate of formation, or destruction, of angiotensin such as pH, temperature, substrate concentration, presence or absence of inhibitors or activators, and angiotensinases. Control of these factors can be achieved by separating renin as much as possible from other plasma constituents, and then adding to it a uniform substrate.2,3 Alternatively, the reaction may proceed under controlled conditions similar to those in whole plasma,4,5 with inhibition of interfering factors or correction for those which cannot be inhibited. It is convenient to make use of both types of methods. We have studied some of the factors concerned in measuring renin activity in human plasma.

Methods

PREPARATION OF RENIN

Renin was prepared from human kidneys by the method of Haas et al.,6 and was kindly assayed by Dr. Harry Goldblatt. This renin contained a small amount of angiotensinase with a pH optimum at 5.5, but at pH 7.5 no angiotensin destruction was observed.

RENIN ASSAY

Approximately 20 ml of heparinized blood were cooled rapidly in a 50 ml polyethylene tube in ice and all steps of the procedure prior to incubation were done at 0 to 4°. Following centrifugation, the plasma was removed, and its volume recorded. Following adjustment to pH 6.5 with 1 N HCl, the plasma was dialyzed, with stirring, against 5 liters of a solution of di-sodium ethylenediamine tetraacetic acid (EDTA, 2.2 g/liter) for 24 hours and then against 5 liters of distilled water for a further 24 hours using Visking 20/100 dialysis tubing. The volume of the dialyzed plasma was measured and the heavy precipitate formed during dialysis removed by centrifugation. A 0.05 ml sample was then removed for substrate assay as described below. To the remaining supernatant was added one drop of a 1:20 solution of di-isopropylfluorophosphate (DFP) in isopropyl alcohol and the solution adjusted to pH 5.5 with HCl or NaOH. A sample was removed for measuring velocity of angiotensin formation. The remainder (usually about 10 ml) was measured, transferred to a polyethylene tube and incubated for four hours at 37°C, 1 ml of saline added, the volume adjusted to 20 ml with distilled water, the pH brought to 5.0 with HCl, and the tube placed in boiling water for 10 min-
UTES. The protein precipitate was removed by centrifugation and the supernate adjusted to pH 7.2 with NaOH, and then to pH 6.8 by addition of 0.1% acetic acid. If the solution was cloudy it was recentrifuged. An aliquot, usually about 15 ml, was evaporated to dryness under reduced pressure and the residue prepared for assay by redissolving in distilled water using 1/20 the original volume.

SUBSTRATE ASSAY
To 0.05 ml of dialyzed plasma was added 1 ml of 0.01 M EDTA in saline, 0.1 ml of a solution of human renin containing approximately 1 Goldblatt unit per ml and 0.85 ml saline. After pH adjustment to 7.5 with NaOH, the mixture was incubated for one hour at 37°C. The pH was then adjusted to 5.0 with HCl, the tube placed in boiling water for 10 minutes, protein precipitate removed by centrifugation and the supernatant assayed. Substrate concentration was expressed as nanograms (ng) angiotensin produced.

REACTION VELOCITY OF ANGIOTENSIN FORMATION
To 0.9 ml of dialyzed plasma was added 0.1 ml of a renin solution containing approximately 0.01 Goldblatt units (G.U.) per ml, pH was adjusted to 5.5 with HCl if necessary, and the mixture incubated for four hours at 37°C. Then 1 ml of saline was added, the tube placed in boiling water for 10 minutes and the protein precipitate removed by centrifugation. Results were expressed as ng of angiotensin produced.

ASSAY OF ANGIOTENSIN
Angiotensin formed during incubation was measured by its pressor effect in the rat7,8 given a ganglioplegic agent to increase its sensitivity.9 Animals weighing between 150 g and 200 g were anesthetized by intraperitoneal injection of 100 mg/kg sodium Amytal (isoamylethylbarbituric acid). Atropine (0.65 mg in 1 ml saline) and pentolinium (5 mg in 1 ml 20% polyvinylpyrrolidone) were given by subcutaneous injection. The trachea was intubated and both vagi were cut. The carotid artery was cannulated and connected to a mercury manometer. A fine cannula was placed in one femoral vein for injections.

Standard solutions of angiotensin were made by dilution, at the time of assay, of a stock solution of a crude preparation of natural angiotensin equivalent to the following concentrations of the isoleucyl5-angiotensin II: 33.3 ng/ml, 16.7 ng/ml and 8.3 ng/ml. Injections of 0.1 or 0.2 ml were given and washed through with saline so that the total volume injected was 0.25 ml. Recently the stock solution was prepared from commercially available angiotensin II (Hypertensin, Ciba) by dissolving the contents of a 2.5 mg angiotensin vial in 1 liter of 0.1% bacitracin solution. Using this solution 100 ml were diluted to 758 ml with 0.1% bacitracin to give a stock solution containing approximately 330 ng/ml. This was standardized against a preparation of known activity to determine the concentration of biologically active peptide. This solution is kept as a stock solution and is diluted 1:10 with saline at the time of assay.

RESULTS

ASSAY OF ANGIOTENSIN
Blood pressure rise is not directly proportional to the dose of angiotensin in the rat assay preparation, but to the logarithm of the dose (fig. 1). This linear relationship makes it possible to use rather widely spaced standards of 0.83, 1.67, and 3.33 ng and to interpolate between them to obtain the value of an unknown. The sensitivity of the animals varies considerably, both initially
(three- to fourfold) and during the course of
the day (five- to sixfold). This is illustrated
(fig. 2) by results from several rats, soon after
they were prepared, and several hours later
showing that the linear relationship is main-
tained over a wide range of sensitivity and
that the preparation remains useful for as-
say. The lower limit of sensitivity differs
from animal to animal and figure 3 is a
tracing from a rat in which it was possible to
measure amounts of angiotensin smaller than
1 ng.

It has been found advantageous to wait at
least one hour after preparation of the animal,
because during the first 30 to 60 minutes
sensitivity often increases. Several injections
of standard (usually four to six injections)
are given to insure that sensitivity is approxi-
mately constant; subsequently at least as many
injections are given of the standard as of
unknown. At least three injections were given
for each sample to be assayed.

The accuracy of the assay has been esti-
mated from 10 standard solutions containing
between 10 and 30 ng/ml of angiotensin
measured as unknown samples. The percen-
tage error for these samples varied from 1 to
9% with a standard deviation of ± 5%.

**ANGIOTENSINASE**

Whole plasma destroys angiotensin, and
although it may be possible to measure renin
in the presence of angiotensinases, we have
attempted to inhibit them. Angiotensin de-
struction after four hours incubation could
then not be detected. Since plasma angio-
tensinase A requires calcium ions, this ion
was removed by dialysis against EDTA solution.
However, when calcium-free plasma was in-
cubated with small quantities of angiotensin
for four hours at pH 5.5, loss of angiotensin
still occurred (table 1). In whole plasma,
angiotensinase activity was greatest at approximately pH 7, but that angiotensinase remaining after removal of calcium ions had a pH optimum of approximately 4.5. Di-isopropylfluorophosphate (DFP) in a concentration of 1 part in 2000, reduced the loss of angiotensin so that it was no longer measurable by our method (table 1). Angiotensinase inhibition by DFP was not reversed by dialysis. Its activity was usually less in fresh plasma than in plasma stored in glass bottles for several weeks.

EDTA was added to the fluid in the bath during the first dialysis rather than at the time of incubation because it was observed that in high concentration and after repeated doses, it was lethal to rats. If any DFP remained after boiling at the end of the incubation, its concentration was so low, the assay animal was unaffected. Neither EDTA nor DFP, in the concentrations used, affected the velocity of the reaction of renin with substrate. The angiotensinase of kidney renin preparations was partially inhibited by DFP.

RENIN SUBSTRATE
Substrate was assayed by incubation of a small volume of plasma with an excess of renin. For each preparation of renin, the volume of a given solution necessary to provide an excess was determined by experiment as illustrated in figure 4. The amount of renin required for maximum angiotensin yield (fig. 4) was multiplied by 10 and this quantity of renin used for routine substrate determinations. The average substrate concentration of eight samples was 720 ng/ml before and 712 ng/ml after dialysis. Therefore, substrate was not lost during dialysis. Substrate concentration in plasma of 29 normal subjects ranged from 510 ng/ml to 1010 ng/ml, mean 729 ng/ml, in 32 patients with hypertension from 370 to 1320 ng/ml, mean 668 ng/ml, and in 9 pregnant women during the last trimester from 1930 to 3240 ng/ml, with a mean of 2613 ng/ml.

VELOCITY OF ANGIOTENSIN FORMATION AFTER ADDITION OF RENIN
Velocity of angiotensin formation in different samples of plasma, after addition of a constant quantity of renin, varies considerably. This variation is not due to differences in total substrate concentration, nor to renin already present in the plasma. When a constant quantity of renin (approximately 0.005 C.U.) was incubated with a series of dilutions
of two different plasma samples (fig. 5), the velocity at any substrate concentration was approximately three times greater in one plasma than in the other. These observations suggest the presence of an inhibitor in the slower reacting plasma\textsuperscript{11} (or an activator in the faster reacting plasma), or the existence of more than one form of substrate. However, Skeggs et al.\textsuperscript{12} have been unable to demonstrate any difference in the rate of reaction of renin with different hog substrates.

The results of incubation of a constant quantity of renin with plasma from normal subjects, from patients with hypertension, and from women during the last trimester of pregnancy are shown in figure 6. The angiotensin formed by incubation of plasma alone has been subtracted, except in three samples taken during pregnancy (open circles). In the plasma of pregnant women angiotensin was generated faster. The plasma of the majority of hypertensive patients had relatively low rates of angiotensin generation, although in four they were unusually high.

**EVIDENCE THAT THE SUBSTANCES MEASURED ARE RENIN AND ANGIOTENSIN**

The substance measured by our method was nondialyzable and the effect of pH on the rate of the reaction was similar to that of renin with partially purified substrate. The pressor material measured by the rat assay was absent after dialysis and was formed during incubation. Upon injection into the rat, the characteristics of the pressor response were identical to those produced by synthetic angiotensin. The mobility on carboxymethyl-cellulose paper electrophoresis at pH 2.1\textsuperscript{13} was similar to that of angiotensin.

**RECOVERY AND RELIABILITY**

The recovery of added renin or angiotensin and variability have been measured during each step of the method. In nine experiments a quantity of renin varying from 0.0005 to 0.1 G.U. of human renin was added to 10 ml of plasma before, and after, dialysis. After dialysis the recovery varied from 87 to 108\% with a mean of 97\%. There was no greater loss with lower renin concentrations than with higher.

Possible loss of angiotensin during incubation was determined by adding 33 ng of angiotensin to 10 ml of dialyzed plasma and carrying through the complete procedure. The same plasma incubated alone for four hours.
with 33 ng of angiotensin added immediately before boiling served as a blank. In eight experiments recovery ranged from 90 to 108%, mean 99%. In another eight experiments in which 33 ng of angiotensin was added to 10 ml of dialyzed plasma, boiled at pH 5.0, and evaporated to dryness, recovery varied from 84 to 96%, mean 90%.

Human renin was added to a large volume of human plasma to produce a concentration of approximately 0.0001 G.U. per ml. Ten samples, each of 10 ml, were assayed in pairs daily for five days. The yield of angiotensin varied from 83 to 106 ng, with a mean of 91 ng and the standard error was ±8%. There was no greater similarity between samples assayed on the same day than on others. The loss of added renin and angiotensin throughout the process was approximately 15% and the variability of recovery 85% ± 7%.

OTHER FACTORS

Incubation was conducted at pH 5.5 because it is close to optimum for the reaction of human renin with substrate (fig. 7), and far from the optimum for angiotensinase activity of whole plasma. Other factors that might influence the choice of pH include 1) the activity of the DFP-inhibited angiotensinase is less in more alkaline conditions; 2) the Michaelis constant is lower at pH 7.5 than at pH 5.5 and, therefore, in normal plasma, at pH 7.5 substrate concentrations are less likely to fall below the level necessary for zero order kinetics to apply than at pH 5.5. Incubation for four hours was chosen since at this time normal plasma generated enough angiotensin to assay readily, and the fall in substrate concentration was insufficient to affect significantly the velocity of the reaction. For example, the highest renin activity found in normal peripheral venous plasma generated 50 ng of angiotensin/10 ml plasma at the end of four hours incubation. The substrate concentration was 510 ng/ml, which was reduced by dilution during dialysis to 465 ng/ml. After incubation it fell to 460 ng/ml, a decrease of 1.1%. In samples with abnormally high renin activity, the fall in substrate concentration was greater, and the effect on velocity was increased if the initial substrate concentration was low. The highest renin activity we observed was in plasma of a patient with hepatic cirrhosis and ascites whose plasma generated 585 ng angiotensin/10 ml in four hours. The substrate concentration of this sample was 385 ng/ml, after dialysis 360 ng/ml, and after incubation 302 ng/ml. This reduction of substrate concentration would decrease the velocity by approximately 12% and
the effect on total yield would be approximately one-half of this, because the substrate concentration fell gradually, though not uniformly, throughout the four hours' incubation.

The efficiency of dialysis in removing angiotensin or other pressor substances was tested by adding 1 ml of saline containing 330 ng of angiotensin to 9 ml of plasma. After dialysis for 24 hours, 19 ng of angiotensin (6%) remained, and after 48 hours, 6 ng (2%). In nineteen unincubated plasma samples no pressor activity was measurable, although several samples had abnormally high renin concentrations. Plasma was dialyzed against distilled water rather than saline because of the 20-fold concentration prior to assay.

Precipitation of protein during boiling has been found to be greatest at pH 5.0 and less than 1% remained in solution. Sometimes the centrifuged specimen was turbid after boiling. This turbidity could usually be removed by centrifugation after adjusting to pH 6.8. Maximal protein removal facilitated redissolving the residue after evaporation. Occasionally depressor activity has been observed and this was readily removed by ether extraction of the solid residue before dissolving in distilled water.

**KINETICS OF THE RENIN REACTION**

The kinetics of the renin-renin substrate reaction have been studied previously by Plentl and Page, and we have studied them again under these particular incubation conditions. When renin was incubated with a low concentration of substrate and samples were withdrawn at intervals, the rate of angiotensin formation decreased as the substrate concentration fell, and after three hours, the rate was no longer measurable. The rate of the reaction was proportional to substrate remaining. Under these incubation conditions of low substrate concentrations, the reaction is of first order with respect to substrate.

The integrated form of the first order equation is:

\[ k = \frac{2 - \left( \log_{10} \frac{100 (M - X)}{M} \right)}{t} \times 2.303 \]

where \( k \) = constant depending on the enzyme concentration.

\( M \) = initial substrate concentration.

\( X \) = product formed after time \( t \).

\( t \) = time.

On plotting \( \log_{10} \frac{100 (M - X)}{M} \) against \( t \), a straight line should be obtained if the reaction follows first order kinetics. Within the accuracy of the method the data are consistent with first order kinetics.

An attempt has been made to determine whether an equation such as that of Michaelis and Menten described the relation between substrate concentration and velocity. The equation is:

\[ v = \frac{V}{1 - \frac{K_m}{s}} \]

where \( v \) = velocity at substrate concentration.

\( V \) = velocity when enzyme is saturated with substrate.

\( s \) = substrate concentration.

\( K_m \) = a constant characteristic of the reaction (the Michaelis constant).

This equation might describe the results observed at low substrate concentration, since when \( s \) is small, \( v \) tends to become directly proportional to it. At high substrate concentration when \( s \) is large, \( v \) tends to become independent of \( s \).

To test the usefulness of this equation in the intermediate range of substrate concentration where \( s \) is equal to \( K_m \), a series of tubes was prepared containing a constant concentration of renin (approximately 0.0035 G.U./ml) and substrate concentration ranging from 42 ng/ml to 220 ng/ml. After incubation for one hour at pH 7.5, the reaction was stopped by boiling, and the angiotensin generated measured (fig. 8). The substrate concentration is the mean of that at the beginning and the end of incubation. The Michaelis constant has been calculated, and the open circles are the values derived from the Michaelis equation. Within the accuracy of the method the experimental values are con-
sistent with those predicted by the equation.

The effect of enzyme concentration on yield of angiotensin has been studied (fig. 9). A plasma sample with high substrate concentration, 1060 ng/ml, was chosen in order that the velocity should be little affected by substrate concentration. The data fall approximately on a straight line (the line does not pass through the origin because this substrate sample itself contained pressor material). Under these conditions velocity is directly proportional to enzyme concentration.

The relation between enzyme concentration and yield of angiotensin was also studied at lower substrate concentrations. Figure 10 presents data plotted according to the method of Lineweaver and Burk from an experiment in which two concentrations of renin, one approximately 10 times greater than the other, were incubated with a series of dilutions of substrate. One series of tubes was incubated for 30 minutes and the other for 5 hours, in order that an easily measurable quantity of angiotensin be formed in both. In this type of plot the intercept on the horizontal axis is equal to the negative reciprocal of the Michaelis constant \( K_m \). Within the accuracy of the assay, the values of \( K_m \) do not differ and, therefore, the substrate concentration giving half maximal velocity is the same for both renin concentrations. Since the readings fall on two straight lines, the ratio of velocities remains constant at any substrate concentration. We conclude

**FIGURE 8**
Comparison of levels of angiotensin formed as determined experimentally with those calculated from the Michaelis-Menten equation.

**FIGURE 9**
Relationship between amount of renin and angiotensin (nanograms) formed in plasma. Numbers on abscissa refer to volumes of a standardized renin sample.

**FIGURE 10**
Lineweaver-Burk plots of the renin-renin substrate reaction at two levels of renin.
that the substrate concentration in normal human plasma provides conditions adequate for renin assay.

**RENIN ACTIVITY IN HUMAN PLASMA**

Peripheral plasma renin activity has been measured in 31 normotensive subjects. The angiotensin formed in 10 ml of plasma after four hours incubation varied from 6 to 50 ng with a mean of 26 ng. The highest plasma renin activity was observed in a patient with cirrhosis and ascites; 585 ng of angiotensin were formed in 10 ml of plasma. Renin activity in renal venous blood obtained by catheterization of the renal veins, or at operation, was usually greater (as much as five times greater) than in arterial blood obtained concurrently, but sometimes there was no difference.

**Discussion**

Over the years, attempts have been made to find the most sensitive biological preparation for measurement of small amounts of angiotensin. The observation of Page and Taylor that ganglioplegics greatly augmented the response, provided a helpful clue and subsequent work has usually employed this principle in rats, cats and dogs, but especially the former because of their size. With various preparations proportionality has been observed between the height of the pressor response and the dose, between the area under the curve and the dose, between the height of the pressor response and the square root of the dose, and between the height of the pressor response and the logarithm of the dose. The latter is valid for our preparation.

Many smooth muscle preparations such as guinea pig ileum, rabbit intestine, rat uterus, and rabbit aorta have been used for angiotensin assay. The spirally cut rabbit aortic strip is very sensitive, but is difficult to prepare and slow in operation. Pressor assay in the rat is adequately sensitive, and if several readings are obtained from each sample, each compared with injections of standard, it is reasonably accurate.

The relation between substrate concentration and velocity in formation of angiotensin is the subject of contradictory reports. Plentl and Page, when using low substrate levels, obtained results which demonstrated that the reaction of renin with its substrate is of first order. When the substrate level is high, substrate is not rate limiting and the reaction is zero order. Helmer has applied the findings of Plentl and Page generally to the estimation of renin in human plasma when it was incubated in the presence of sodium chloride. Taquini et al. and Hoobler et al. using conditions very similar to those of Helmer, report that in some plasma samples the addition of more substrate makes no measurable difference in reaction rate, suggesting zero order kinetics. We have demonstrated that plasma samples, when incubated in the absence of any salts and under the conditions described here, always formed angiotensin at a velocity completely dependent upon renin concentration and that further addition of substrate did not change significantly the rate of formation of angiotensin.

The observation that plasma substrate concentration was raised during pregnancy confirms that of Helmer. In a small number of patients with cirrhosis of liver, plasma contained less substrate than normal.

It has been pointed out by Plentl and Page and later by Brown et al. that it is necessary to measure reaction velocity. Our method does not permit removal of samples at intervals during incubation but, insofar as velocity falls off, due to consumption of substrate, an approximate correction can be applied either from an empirical curve or from determination of $K_m$. Since in our method renin concentration is lower and substrate concentration is higher than that of Brown et al., the correction is likely to be smaller. In normal plasma it is so small (1%) that it can be ignored. In samples with greater renin activity, especially if the initial substrate concentration is low, the correction is larger. In none of those examined has it been greater than approximately 6%. We have not made the correction, since the corrected figure would have no added clinical significance.
It has been suggested that it is desirable to assay renin under conditions similar to those in whole plasma, without inactivating angiotensinase. Dialysis alone, by decreasing Ca++ concentration, may partially inactivate angiotensinase activity. It may be possible to measure renin in the presence of angiotensinase, but application of the first order reaction equation does not correct for losses due to angiotensinase activity. In the method we have used, plasma angiotensinase is inactivated by EDTA and DFP to the degree that its effect is negligible. These inhibitors provide a useful alternative to destruction by acidification at high salt concentration. It is hoped that DFP may also prove useful by inactivating some renal angiotensinase, remaining in renin prepared from human kidney.

Variance in angiotensin yield with a constant amount of renin added to different samples of normal plasma suggests clearly the presence in plasma of an unknown factor which alters the rate of the renin-renin substrate reaction. Page and Helmer presented evidence that a renin inhibitor is present in blood from dogs made tachyphylactic with renin. Boucher et al. have also observed that angiotensin formation is more rapid in some plasma samples than in others. Hoobler et al. and Brunner have found angiotensin formation more rapid in plasma of nephrectomized rats than in normal.

Our method, as well as others, does not measure the true concentration of renin in plasma, but rather "effective renin activity" as it is affected by other plasma constituents, including renin substrate.

Summary
A method is described for estimating plasma renin activity by using renin substrate present in plasma. This method differs from other indirect renin assay methods by (1) incubation in the absence of ions thus establishing conditions for zero order kinetics for the reaction between endogeneous renin and substrate and (2) the use of angiotensinase inhibitors di-sodium ethylenediamine tetraacetic acid (EDTA) and di-isopropylfluorophosphate (DFP). Recoveries of renin added to plasma in levels similar to those occurring in plasma are 85% ± 7%.

The incubation was done at pH 5.5 which was shown to be the optimum for human renin reacting with human substrate.

By incubating human plasma samples with known quantities of human renin, evidence was obtained suggesting that factors other than enzyme or total substrate concentrations affect the velocity of angiotensin formation. This variability of reaction rate may be explained by the existence of an inhibitor or activator in this system or by a variation in the type of substrate.

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