Improving Assay Methods for Renin "Concentration" and "Activity" in Human Plasma

METHODS USING SELECTIVE DENATURATION OF RENIN SUBSTRATE

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ABSTRACT
Simplified methods are described for the measurement of human plasma renin "concentration" (PRC) and "activity" (PRA) based on the denaturation of renin substrate in which separation and concentration steps are avoided and recovery of renin is complete. In the PRA method effective inhibition of angiotensinase is achieved by warming plasma at pH 4.5 with EDTA followed by dialysis to pH 7.5. Neither renin nor renin substrate is affected by this treatment. In the PRC method, renin substrate is selectively denatured by warming at pH 3.3. After dialysis to pH 7.5 and addition of a standard substrate prepared from nephrectomized sheep, incubation results in a linear increase of pressor material which is assayed without extraction on rat blood pressure against synthetic angiotensin. Specificity is established by nephrectomy and immunological studies. The linear relationship between plasma renin concentration and reaction rate contrasted with the nonlinearity observed with renal renin. The systems are suitable for routine diagnostic use.

ADDITIONAL KEY WORDS
renal pressor system antirenin enzyme assay angiotensin rat blood pressure

Methods
The dual assay sequence is shown in Figure 1. Blood is chilled on withdrawal and centrifuged without delay at 3°C with 20 units heparin/ml. The plasma is dialyzed in size 8/32 Visking casings to either pH 3.3 or 4.5 over 24 hours at 3°C.
against buffers A or B (to be stated in next section) containing disodium EDTA. In the PRC procedure, after dialysis to pH 3.3 using buffer A, the temperature is raised to 32°C for 60 min. Under these conditions endogenous substrate is totally and irreversibly denatured and angiotensinase inactivated and yet renin remains unaffected (see Results). In the PRA methods, pH 4.5 buffer B is used and subsequent heating at 32°C for 30 min at this pH destroys angiotensinase but leaves substrate unaffected. After dialysis to pH 7.5 at 3°C over 24 hours, plasma is centrifuged and a kallikrein inhibitor (Trasylol,\(^1\) FBA 100 units/ml) and neomycin sulfate (2 mg/ml) are added. Deliberate volume reduction of approximately 10% is produced through dialysis by dialyzing against increasing molarity. This volume is partly replaced by Trasylol and neomycin and the original volume is then restored with cold pH 7.5 buffer C. In the PRC method 1 volume of uniform substrate (see below) is added to 2 volumes of the pH 3.3-treated sample and 3 ml of this mixture at pH 7.5 is incubated at 37°C. In the PRA method 3 ml of undiluted pH 4.5-treated plasma is incubated under the same conditions. The incubation mixture is sampled at three appropriate times and without further extraction, assayed for pressor activity against aspartryl\(^1\)-valyl\(^1\), angiotensin II-\(\beta\)-amide (Hypertensin, Ciba) using the initial velocity bracket technique.

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\(^1\)Trasylol was kindly donated by F.B.A. Pharmaceuticals.

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3 - 5 ML COLD PLASMA

DIALYZE AGAINST EDTA (0.005M) OVERNIGHT

\(\text{pH 3.3} \quad \text{pH 4.5}\)

HEAT AT 32°C

FOR 60 MIN FOR 30 MIN

DIALYZE TO pH 7.5 AGAINST EDTA (0.001M) OVERNIGHT.

ADD TRASYLOL 100 UNITS/ML AND NEOMYCIN 2 MG/ML

ADD STANDARD SUBSTRATE

INCUBATE AT 37°C AND MEASURE VELOCITY OF ANGIOTENSIN FORMATION BY BIOASSAY IN THE RAT

PRC IN ARBITRARY UNITS FROM A CURVE RELATING RENIN CONCENTRATION TO VELOCITY.

PRA

FIGURE 1

Flow sequence for dual measurement of plasma renin concentration (PRC) and plasma renin activity (PRA).

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(7, 8) on the ganglion blocked rat (9). At least 2 rats were used in the construction of the velocity curve.

Injections were kept at 0.1 ml or less with a 0.05-ml wash of 0.9% saline. The effect of this volume of saline or unincubated dialyzed plasma was checked on all rats used and found to give pressor responses less than 1 ng of angiotensin. Smaller pressor responses than this were indistinguishable from zero.

**Preparation of Uniform Renin Substrate**

Two ewes were heparinized (7, 500 units intravenously) and exsanguinated 6 days after bilateral nephrectomy. The plasma was immediately separated by centrifugation at 3°C, dialyzed over 36 hours to pH 3.9 against a citric acid-phosphate buffer (10) containing EDTA (0.005 M) which was made 0.16 M with NaCl. It was heated at 32°C for 30 min and then dialyzed back to pH 7.5 against the standard buffer, centrifuged cold and stored at −20°C with neomycin sulfate and was discarded after thawing once.

**Preparation of Standard Renins**

*Human renin* was prepared by the methods of Haas et al. (11), Peart et al. (12) (excluding all chromatographic steps), and Brown et al. (8). The highest specific activity of renin and the lowest angiotensinase activity at pH 7.5 was produced by the method of Brown et al. This renin contained 0.47 mg protein/ml (13) and has been used throughout this study. Because of nonlinearity of the renin reaction using this preparation (see Fig. 6), a value for renin concentration cannot be given. Diluted 2000 times, however, it generated angiotensin at 1 ng/ml per hour. At full concentration the rate would be disproportionately high at greater than 10,000 ng/ml per hour (see Results). Renin was stored in 1-ml aliquots at −20°C with 2 mg/ml neomycin sulfate and was discarded after thawing once.

**Preparation of Antirenin**

Antibody was raised in the rabbit to the standard human renin, concentrated tenfold by ultrafiltration. Two rabbits were injected intramuscularly at multiple sites twice weekly for 5 weeks with 2 ml of a 1:1 emulsion of concentrated renin and Freund’s incomplete adjuvant. A third rabbit (control) was injected with the adjuvant alone. One hour prior to bleeding, an intravenous infusion of normal saline was commenced at 1 ml/min and continued during the collection of 20 ml of heparinized blood (20 units/ml) from each animal. Saluresis was induced in order to suppress endogenous renin production. Double precipitation of immunoglobulins by 16%, and then 14%, sodium sulphate was followed by dialysis to pH 4.1 against citric acid-phosphate buffer containing EDTA (0.005 M) which was made 0.16 M with sodium chloride. The plasma was warmed to 32°C at this pH for 30 min and then dialyzed back to pH 7.5 against the standard buffer, centrifuged cold and stored in 1-ml aliquots with 0.2% neomycin sulphate at −20°C. Samples were discarded after thawing once. Protein concentration of the final extract was 18.8 mg/ml.

**Measurement of Renin Substrate Level**

Renin substrate levels in plasma were estimated at pH 7.5 by the addition standard renal renin to PRA-treated plasmas in a concentration sufficient to drive the reaction to completion during a 10-min incubation at 37°C. Four parts standard renin were added to one part plasma and incubated at pH 7.5 for 30 min at 37°C. Pressor activity of the incubate was then assayed without extraction by the bracket technique (7). A two-to-one ratio of renin to plasma was used for low substrate levels, making an assay sensitivity limit of 30 ng substrate/ml of the original plasma.

**Detection of Angiotensinase**

Human angiotensin and synthetic octapeptide (Hypertensin, Ciba) were added to the test plasma or kidney extract in a volume ratio of 1:9, providing a final concentration of 100 to 200 ng/ml. Test solutions were considered angiotensinase-free if better than 80% of added angiotensin survived at least 24 hours (7) at 37°C assayed against an unincubated control. Correction was made for formation of angiotensin by the endogenous renin during incubation.

**Preparation of Human Angiotensin**

Standard human renin (4 ml) was added to 50 ml of angiotensinase-free human plasma.
(treated by PRA method) at pH 7.5 and incubated for 7 hours at 37°C. The ultrafiltrate, collected in the cold, yielded an angiotensin content of 1 μg/ml. It was stable when incubated alone at 37°C for at least 100 hours, but was rapidly destroyed by the addition of untreated normal plasma at pH 7.5.

**Recovery of Renin**

Recovery of renin through the methods was estimated by recycling plasma samples through the PRC and PRA methods and measuring the fall-off in rate of angiotensin formation after each treatment.

**Units of Measurement**

PRA is expressed as rate of angiotensin formation in nanograms per milliliter per hour. Renin concentration is expressed as units per milliliter, where 1 unit is that amount of renin per milliliter that generates angiotensin at the rate of 1 ng/ml per hour in the presence of a relative excess of the nephrectomized sheep substrate used in this study. The addition of substrate diluted the renin extract by 1/3, and in order to obtain renin concentration in the original plasma, measured velocity was multiplied by 3/2. Since plasma renin concentration was found to be directly proportional to reaction rate over the whole range of renin levels encountered, concentration expressed as units per milliliter of plasma is the same as the corrected velocity in nanograms per milliliter per hour. This value thus provides a figure for the rate of angiotensin formation after removal of native substrate and replacement with standard substrate without volume change. Renal renin is expressed as a dilution of the standard. Renin substrate is expressed as angiotensin released in nanogram per milliliter by a relative excess of angiotensinase-free renal renin.

**Other Enzymes Used**

Pepsin (Sigma, three times crystallized); α-chymotrypsin (Worthington, three times crystallized); and trypsin (Sigma, two times crystallized).

**Results**

**PREPARATION OF PLASMA RENIN, FREE OF RENIN SUBSTRATE AND ANGIOTENSINASE**

In a preliminary experiment, aliquots of pooled normal human plasma were dialyzed to a range of pH from 3.0 to 5.0 against buffers (10) containing EDTA (0.005 M) made 0.16 M with sodium chloride. The aliquots were heated at 32°C for 30 min, dialyzed back to pH 7.5 against the standard buffer and tested for residual substrate, angiotensinase activity, renin content, and nonspecific pressor and depressor effects. Figure 2 illustrates the irreversible denaturation of substrate in this experiment with heating at low pH. The ability of excess renin to release angiotensin was unaffected for treatment down to pH 4.0. Below pH 4.0, substrate concentrations fell sharply and was undetectable below pH 3.5. The minimum substrate level detectable was 30 ng/ml, so that less than 3% survived below pH 3.5.

Digestion of both synthetic and human angiotensin by the treated plasma tested at pH 7.5 at 37°C over 48 hours is shown in Figure 3. Prior treatment of the plasma at pH 3.0 to 3.5 provided complete protection for both angiotensin preparations; pH 3.75 to 4.5 was better than 80% effective and after pH 5.0 treatment 70% survived. Both angiotensins behaved in the same manner.

This study revealed the possibilities of using a pH 4.0 to 4.5 step to inactivate angiotensinase and preserve substrate in a system for
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PRA assay and also of using a pH below 3.5 as a means of additionally inactivating the substrate as a preliminary to PRC measurement. On incubation of the pH 3.75 to 5.0-treated aliquots at pH 7.5 and 37°C, a linear increase in pressor activity of the whole incubate occurred at a rate of 1.5 ng/ml per hour in all samples, while the pH 3.0 to 3.5 samples remained inactive over the 48 hours of incubation. Addition of standard sheep substrate to the pH 3.0 to 3.5 series provoked the generation of pressor activity at the corrected rate of 7.5 ng/ml per hour in all samples. The faster rate using sheep substrate, as shown below, is due to the characteristics of this substrate which make it react five times as fast with human renin as does normal human substrate at the same concentration. Unincubated aliquots did not display pressor activity and depressor responses were not seen.

A closer study was made of substrate denaturation in 15 individual plasmas (11 male, 4 female) by dialyzing each to the pH levels 5.0, 4.5, 4.1 and 3.3, and then returning to pH 7.5 after the treatment described above. The results were identical to those in Figures 2 and 3, except that in 6 plasmas substrate losses of 10 to 70% occurred with pH 4.1 treatment. Under these conditions pH 4.5 was the highest pH level tested which provided freedom from angiotensinase and permitted complete substrate survival.

Incubation of pH 4.5 series for 72 hours at pH 7.5 revealed a linear increase in pressor activity of 1.0 to 2.7 ng/ml per hour. After the addition of sheep substrate to the pH 3.3 series, a linear increase in pressor activity occurred at corrected rates of 5 to 15 ng/ml per hour.

SPECIFICITY OF THE METHODS FOR MEASUREMENT OF RENIN

Antirenin on pressor activity. Total suppression of the pressor activity formed on incubation of 25 normal plasmas passed through both the PRC and PRA methods was induced by antirenin, added in a volume ratio of plasma four to antirenin one. In control experiments, the addition of immunoglobulin from a rabbit injected with Freund's adjuvant alone did not alter pressor activity. This finding indicated that the renin-like material acting in normal plasma after pH 3.3 and 4.5 treatments was immunologically identical to semi-purified human renal renin. Antirenin was free of angiotensinase and renin substrate and did not display pressor activity either incubated alone or with the standard substrate.

Nephrectomy. Plasma collected from 2 patients several weeks after bilateral nephrectomy did not display any pressor activity in the PRA method after 100 hours of incubation. Passed through the PRC method, pressor activity formed at the low rates of 0.6 and 0.9 ng/ml per hour. As with normal plasma, these samples were free of angiotensinase and it was confirmed that substrate was preserved through the PRA method (1500 and 2300 ng/ml) but not the PRC method.

Identity of reaction product. The pressor response of the test animal to the injection of incubated plasma was of similar shape to that induced by synthetic and human angiotensin.
The pressor material was stable to boiling, dialyzable, destroyed by pepsin at pH 5.0 and α-chymotrypsin and trypsin at pH 7.5.

**Pepsitensin.** Formation of pepsitensin (14) was prevented by performing all incubations at pH 7.5. The pH maximum for pepsitensin formation from the standard substrate was found to be pH 5.0 to 6.0 with no formation above pH 7.0 even after 72 hours of incubation.

**Importance of Cofactors**

A similar approach to that of Pickens et al. (2) was used to detect the presence of accelerating or inhibiting cofactors, differing only in the addition of plasma renin, instead of renal renin to the test plasma. Renin (34 units/ml) was added to normal plasmas after treatment by the PRC and PRA methods. After correction for endogenous renin action, the assayed levels were:

- **PRC:** 33.6, 32.1, 32.3, 37.2, 33.9, 33.6, 35.7, 27.7, 28.2, 35.1, 34.6, 31.6, 31.4, 32.7, 35.6, 34.6, 33.2 units/ml. Mean, 33.1 ± 2.5 (coefficient of variation (C), 7.6%).
- **PRA:** 9.0, 6.8, 11.2, 11.5, 10.7, 11.7, 11.7, 7.6, 9.3 ng/ml per hour. Mean, 10.1 ± 1.7 (C, 16.8%).

In neither method were there large variations between plasmas similar to those reported in normal plasma by Pickens et al. (2). Maximum differences in the PRC series were within the expected variation of values derived from double velocity estimations, i.e. total velocity minus endogenous rate (see replicate estimations below). Maximum differences in the PRA series were relatively greater than with PRC treatment and could reflect a small variation in the concentration of a cofactor.

**Recovery of Renin**

Stability of plasma renin through the methods was suggested in a preliminary study in which standard plasma renin was dialyzed to a range of pH from 4.0 down to 2.5 and otherwise treated as in the PRC method. Velocity of angiotensin formation on incubation with the standard substrate was unchanged when plasma renin had been treated down to pH 3.0 and decreased only in samples treated below this level. Thus, after pH 2.75 and 2.5 treatments, only 55% and 22% of the original velocity was obtained.

Confirmation of complete recovery of renin down to pH 3.3 was obtained by recycling plasma samples through the PRC and PRA methods and detecting any fall in velocity of angiotensin formation. Using pooled normal plasma from two separate sources and recycling each once, a small but not significant rise occurred through the PRC method—8.7 to 9.5 and 8.3 to 9.0 units/ml; no change was detected in PRA with recycling. Because loss of renin could not be demonstrated in these studies, variation of replicates rather than recovery of renin became the important source of variability in the assay.

**Replicate Estimations**

Five aliquots of a sample of pooled plasma were passed through both the PRA and PRC methods and tested for reproducibility. Levels obtained were as follows. PRC: 8.7, 8.3, 8.3, 8.3, 8.1 units/ml; mean, 8.3 ± 0.22 (C, 2.6%).

PRA: 0.8, 0.9, 0.8, 0.9, 0.8 ng/ml per hour; mean, 0.84 ± 0.05 (C, 5.9%).

Table 1 shows the agreement between PRC duplicates when estimations were repeated on frozen plasmas after weeks or months over a wide range of renin levels. The mean difference between replicates was 4.1% (sd ± 4.7). The mean difference for PRA was 15% (sd ± 14).

**Levels of PRC, PRA and Renin Substrate**

In midafternoon plasma samples, collected from 45 normal subjects (34 male, 11 female) in the upright position, renin concentration was 9.1 ± 3.5 units/ml of plasma, and activity 1.7 ± 0.5 ng/ml per hour. Lowest levels measured in normal plasmas were 4 units/ml for PRC and 0.7 ng/ml per hour for PRA. Renin substrate in these normal plasmas averaged 1100 ± 300 ng/ml.

In over 200 plasmas from patients with various disease states, PRC values ranged from 0.6 to 600 units/ml, PRA from 0 to 190 ng/ml.
ml per hour and renin substrate from 500 to 6000 ng/ml.

Renin levels have been readily detectable in all normal plasmas so far tested by both methods. The lowest normal velocity is 20 times the velocity threshold (0.2 ng/ml per hour) for the PRC method and 4 times for the PRA method.

Properties of Standard Substrate

Expressed as angiotensin released by excess angiotensinase-free renin, the standard substrate contained 1600 ng/ml with a specific activity of 23 ng/mg total protein (13). Freedom from angiotensinase was demonstrated by better than 90% survival of added angiotensin (Hypertensin and human angiotensin) at pH 7.5 after 100 hours at 37°C. The substrate was stable over the same period and did not display any endogenous pressor activity (less than 10 ng/ml) before and during such prolonged incubations. The rate of reaction of plasma or renal renin with the standard sheep substrate was approximately five times as fast as with normal human substrate at the same substrate concentration. It was stable to freezing and thawing and concentration was unchanged over 12 months stored at -20°C.

Properties of Standard Renal Renin

At full concentration this renin was angiotensinase-free above pH 7.0, but below pH 6.0 synthetic angiotensin (Hypertensin) was completely inactivated during 24 hours at 37°C. This angiotensinase was not selectively abolished by alkaline denaturation and was unaffected by EDTA (0.005 M); 1, 10—phenanthroline (0.0005 M); and Trasylol (200 units/ml). For kinetic studies, renin was diluted at least 20 times and the enzyme mixture was then angiotensinase-free at all pH levels from 4.0 to 9.0. Intravenous injection of standard renin had no effect on rat blood pressure. The addition of standard renin to angiotensinase-free plasma in a ratio of 4:1 at pH 7.5 and 37°C produced maximum yield of angiotensin within 10 min, the level remaining unchanged for at least 24 hours. Deterioration of renin occurred on repeated freezing and thawing but activity was unchanged over 12 months at -20°C.

Standard Plasma Renin

This denatured plasma contained 390 units of renin/ml, was free of substrate and angiotensinase, and displayed no pressor or depressor activity before or during 100 hours incubation. Its ability to form angiotensin on incubation with standard substrate (260 ng/ml per hour) was completely abolished by antirenin. It was stable over 12 months at -20°C.

Characteristics of the Reaction Between Renin and the Standard Substrate

Effect of substrate concentration. The curves in Figure 4 show the effect of substrate concentration on reaction velocity with both standard renal and plasma renin. Standard substrate was diluted with pH 7.5 buffer and reaction velocity was assayed at constant renin concentration. Maximum velocity required substrate concentrations in excess of 1000 ng/ml and as a result of this study, it was decided to add two parts of denatured plasma to one part standard substrate in the PRC method, providing an initial substrate concentration of 530 ng/ml. Incubation was completed when, at most, 120 ng of angiotensin...
had been generated, leaving a residual substrate level of 410 ng/ml. The effect of this drop in concentration on velocity was an 8% slowing from initial to final, and as shown in Figure 5, did not produce discernible nonlinearity using the bracket assay.

**Effect of enzyme concentration.** Figure 6 shows the relation between enzyme concentration and reaction velocity at constant substrate concentration for both renal and plasma renin. Over the same range of reaction rates, extreme nonlinearity was observed for renal renin, but the expected linear relationship was obtained with plasma renin. Nonlinearity with renal renin was confirmed on three separate occasions and could not be accounted for by failure to stop the reaction at high rates, since the reaction with plasma renin was of similar velocity. The highest point on the renal renin plot was obtained with a 40-fold dilution of the standard. Linear relationships were obtained with pH 3.3-treated plasmas from 10 subjects with low, normal and high renin levels, 2 of which are plotted on Figure 6. This finding made invalid any attempt to use renal renin as a standard for construction of a calibration curve from which concentrations of plasma renin could be derived.

**Effect of pH on reaction rate.** Reaction velocity was measured over the pH range 4.0 to 10.0. Freedom from angiotensinase and nonspecific pressor activity was confirmed at each level in all reactants. Both standard renal renin and plasma renin displayed identical pH curves with standard substrate (Fig. 7). Rate of reaction increased from pH 5.0 to 9.0 with maximal rate at pH 9.0. A sharp fall-off in rate below pH 5.0 and above pH 9.0 was associated with denaturation of the standard substrate which was stable only inside these limits.

Attempts to study the effect of pH on reaction velocity between plasma renin and human substrate were unsuccessful because of angiotensinase action in the substrate below pH 7.0 and substrate denaturation above pH 8.5. Human substrate for this study was prepared as in the PRA method.

**Heparin, Tranoyl and Neomycin on Reaction Rate**

A comparison in 4 normal subjects of PRA in plasma collected with EDTA (0.005 M) as anticoagulant was not significantly different.
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Enzyme concentration curves. Initial renin concentration = plasma renin (o), 260 units/ml of incubate and renal renin (•), a 40-fold dilution of standard. X = serial dilutions of separate pH 3.3-treated plasmas with renin concentrations of 510 (X-1) and 75 (X-2) units/ml in the original plasma, i.e. 340 and 50 units/ml of incubate. Ordinate = actual measured rate.

FIGURE 6

% OF INITIAL RENIN CONCENTRATION

PLASMA RENIN ACTIVITY (PRA)

If in vitro enzyme activity is to reflect to some extent in vivo activity, the environment of the in vitro reaction should closely simulate physiological conditions. For this reason, activity incubations were performed at pH 7.5 with as little alteration as possible to plasma constituents. Dialysis against EDTA (15) to pH 4.5 followed by warming at 32°C and dialysis back to pH 7.5 was the minimum treatment that would result in effective freedom from angiotensinase and complete survival of endogenous renin substrate. Some incubations were prolonged for periods up to 80 hours in order that sufficient angiotensin accumulated for accurate assay. Such long incubation times were possible because the system was angiotensinase-free and native substrate was stable at 37°C over these periods.

A kallikrein inhibitor (Trasylol) was used in all systems to block the possible formation of bradykinin. Concentration of Trasylol (100 units/ml) was determined from the amount needed to block the formation of depressor material on incubation of plasma at pH 7.5 after acidification to pH 2.0 (16, 17).

Discussion

The method for renin assay described permits the dual measurement of so-called renin "activity" and "concentration" by simplified techniques that display specificity, high recovery and reliability.

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Assay specificity is claimed on the basis of nephrectomy studies and also on the complete suppression of formation of pressor material by antirenin.

Identity of the end product has not been established beyond its peptide nature. It would seem likely that most, if not all, of the peptide would be in the form of the natural octapeptide angiotensin II since prolonged periods of incubation would permit conversion of deca to octapeptide even if converting enzyme was partially inactivated. 0.01 M EDTA has been shown to partially inhibit converting enzyme (18) and it is possible that either the added EDTA or some other step in the present assay could interfere with the action of this enzyme. If the ratio of the two angiotensins was not always the same, an error would be introduced in renin quantitation since angiotensin I has been reported to have slightly less pressor activity than angiotensin II (19). Short of determining the nature of the end-product in every plasma, the presence and importance of this error cannot be established.

Pickens et al. (2) found up to twentyfold variations between normal plasmas in the rate of angiotensin formation in response to added renin. They suggested that this could be due to differences in a reaction cofactor between plasmas. In the present PRA system in normal plasma no such large differences could be demonstrated and the importance of the small variations found must await detailed kinetic studies of this reaction in individual plasmas.

This PRA method offers considerable simplifications over existing methodology and has been found suitable for routine scanning of the hypertensive population. Elevation and suppression of renin activity in clinical situations can be readily detected.

**SUBSTRATE CONCENTRATION IN NORMAL PLASMA**

Adding an excess of renin to plasma treated by the PRA method offered a simple means of determining substrate levels in angiotensinase-free plasma. Extraction of angiotensin from the renin mixture prior to assay was not necessary since neither human renin nor plasma extract themselves had any effect on rat blood pressure.

The mean value of 1100 ng/ml for renin-substrate in normal plasma was slightly higher than that (729 ng/ml) obtained by Pickens et al. (2). Little is known about factors controlling substrate levels under normal conditions although increases in pregnancy (2) and in women taking oral contraceptives are generally recognized. These situations were excluded in the present study.

**PLASMA RENIN "CONCENTRATION"**

The observation that denaturation of renin-substrate and inactivation of angiotensinase with survival of renin occurred consistently under controlled conditions, led to a simple method of renin estimation in which the effect of substrate variation was eliminated. Such an approach to enzyme assay is only valid if the denatured substrate cannot complex readily with the enzyme and thereby act as an inhibitor. Skeggs and his associates (20) have described the production of a heat stable substrate on denaturation of natural substrate by boiling at pH 2.0. The formation of such a fragment could lead to a variable error in velocity estimations with added substrate.

That no alternative substrate capable of interfering in the reaction forms with the more gentle denaturation described here is shown by failure of denatured plasmas to form angiotensin on incubation prior to the addition of standard substrate and also by failure of renin added in excess to generate angiotensin from these plasmas during an incubation that would exhaust normal substrate. In addition, serial dilution of denatured plasma yielded a direct relationship between plasma enzyme concentration and reaction velocity (Fig. 6) which would not have been observed if the enzyme mixture contained suboptimal amounts of an alternative substrate.

This method of substrate removal offers considerable advantages over the separation procedures of Lever et al. (7) and Lee et al. (21). Renin was found to be stable through the denaturation step, and enzyme recovery resolved itself into volume recovery and bio-
assay reproducibility. When performed under ideal conditions with all plasmas being handled in the one batch, the coefficient of variation for replicates was only 2.6%. Duplicates re-assayed after weeks or months displayed a mean difference of 4.1 ± 4.7%. Replicates were closer for PRC than PRA probably as a result of the faster rates in the PRC system permitting more accurate velocity determination.

The standard substrate from nephrectomized sheep displayed several interesting features. It was prepared very easily by a modification of the PRA method and, besides fulfilling all of the criteria of Lever et al. (7), it displayed the fortunate property of reacting with human renin approximately five times as fast as the isologous substrate. Addition of one part substrate to two parts denatured plasma resulted in such high rates of angiotensin formation that incubations were complete within 15 hours for normal plasma. Assay threshold remained at 0.2 ng/ml per hour and the mean normal level was now elevated fiftyfold above this, enabling suppression below normal to be accurately determined.

A low level of renin was found in two bilaterally nephrectomized patients when assayed by the PRC method whereas no PRA was detected. This is not necessarily a reflection of greater specificity of PRA measurements but rather that the rates are too low to be detected by the latter method. A low but measurable level of renin has also been found in nephrectomized humans by Brown et al. (22) (and personal communication) and could reflect an extrarenal source of renin. Alternative possibilities, however, are the formation of pepsitensin (14) or anephrotensin (23). Pepsitensin formation can be excluded in systems incubated at pH 7.5 (14) and this has been confirmed in the present study, but with incubations at pH 5 to 6 as used in most renin assay methods, pepsitensin formation would be expected. Although the formation of anephrotensin is maximum at about pH 4.0, such formation might occur on prolonged incubations even at high pH levels. This cannot explain the formation of pressor material in the present experiments, however, since the PRA values were zero and furthermore incubation of the pH 3.3-treated plasmas alone without added substrate did not generate pressor activity.

An important difference between renal and plasma renin was revealed by enzyme concentration plots (Fig. 6). The non-linearity obtained with renal renin was similar to that published by Brown et al. (8) in a human renin-ox substrate system. The upper section of their curve which is claimed to be rectilinear (8) actually deviates considerably from the expected 45° slope of a direct relationship on a log-log plot. Non-linearity in the lower section of their plot is considered to be due to renin contamination in the substrate, a problem not encountered with nephrectomized sheep substrate. Reactions similar to these have been found in systems containing suboptimal amounts of accelerating cofactors (24). Because of this finding the use of a calibration curve constructed with renal renin becomes unnecessary and indeed invalid.

It is probable that renin measurements made under the present conditions, where substrate and cofactor variations are eliminated, do reflect actual renin concentration, but confirmation of this claim must await kinetic studies in pure systems. With this qualification it is reasonable to refer to renin levels in such systems in arbitrary units. The unit used here cannot be compared with that of Goldblatt et al. (25) or Brown et al. (8) and this will only be possible when a standard substrate becomes widely available.

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