A Method for the Measurement of Plasma Renin in the Rat[1] (35261)

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Most of the methods for the measurement of plasma renin cannot be used in the rat because of the large volume of plasma required, hence the need for a micromethod. The one most widely used has been devised by Boucher *et al.* (1). Plasma samples are incubated during 12 hr with an excess of homologous substrate in the presence of Dowex resin which adsorbs and protects angiotensin from angiotensinases. This method is sensitive and specific. However, recovery is not complete, and elution of the resin and concentration of the eluate is time-consuming. Recently, Goodwin *et al.* (2) have applied to the rat the method that Gould *et al.* (3) had used for human plasma. Plasma samples are incubated with hog renin substrate during 16 hr in the presence of angiotensinase inhibitors. Recovery of angiotensin was complete after 16 but not after 24-hr incubation.

The method presently described is also similar to that of Gould *et al.* but, like the Boucher *et al.* (1) procedure, uses homologous substrate. It is simple, accurate, and permits routine determinations of plasma renin in large numbers of samples.

Materials and Methods. Preparation of renin substrate. Blood from 48-hr bilaterally nephrectomized male rats was collected in the presence of disodium ethylenediaminetetraacetate (EDTA). Renin substrate was then extracted and purified by ammonium sulfate fractionation and acid treatment (4).

Following addition of 10 ml of 0.3 *M* EDTA to 100 ml of plasma, the mixture was stirred slowly for 30 min and then adjusted to pH 6 with 5 *N* HCI. Ammonium sulfate was slowly added to a concentration of 0.97 *M*. The solution was stirred for 30 min more and centrifuged. The small precipate was discarded. All the preceding steps were carried out between 0 and 5°. The supernatant was then warmed up to 23°, acidified to pH 2.7, and kept at 23° during 30 min, after which it was cooled and adjusted to pH 4 with 5 *N* NaOH. The heavy precipitate formed was separated by centrifugation; suspended in 0.97 *M* ammonium sulfate solution, pH 4; and centrifuged again. The new precipitate was discarded and both supernatants were pooled. Ammonium sulfate concentration was increased to 1.87 *M*, and after stirring for 30 min, the material was centrifuged. The precipitate, which contained the substrate, was dissolved in a minimum volume of distilled water and adjusted to pH 6. After dialysis against cold distilled water during 24 hr, the substrate was freeze-dried and stored at —20°. Total recovery of the substrate from the original plasma was about 59%. Its specific activity of 70 ng of angiotensin II/mg of proteins represents a 2 to 3 × purification. Substrate activity was measured as previously described (5).

Preparation of substrate for determination of plasma renin was as follows. To process 10 samples, 0.6 g of lyophilized substrate was dissolved in 9 ml of a solution containing 0.15 *M* NaCl, 0.005 *M* EDTA and 0.0001 *M* phenylmercuric acetate, pH 6.5. After addition of 0.09 ml of 1 *M* BAL (2,3-dimercaptopropanol) in arachis oil, adjustment of pH to 6.5 and vigorous shaking to emulsify the oil, the substrate solution was ready to use. The renin substrate concentration was 4600 ng/ml; thus, the amount of substrate contained in 0.9 ml of the substrate solution added to each incubating tube had an activity of about 4200 ng of angiotensin II. When incubated alone for periods up to 24 hr, no

[1] This study was supported by Grant HE-6835 from the National Heart Institute.

pressor or depressor activity could be detected. No angiotensinase activity was observed after incubation of 0.9 ml of substrate and 36 ng of angiotensin II amide during 24 hr.

Preparation of renin. (i) Pooled plasma from normal rats was used as source of plasma renin.

(ii) Purified, angiotensinase-free renin was prepared from rat kidneys according to the procedure C of Haas *et al.* (16) and further purified by chromatography on a G-200 Sephadex column. After elution with 0.15 *M* NaCl, renin was diluted with 0.013 *M* pyrophosphate buffer, pH 5.5, containing 0.15 *M* NaCl and 1 mg/ml of bacitracin to a final concentration of 0.05 Goldblatt units (GU)/ml. Subsequent dilution at the time of use was made with the same solution used to dissolve the lyophilized substrate.

Procedure for plasma renin determination. Blood was collected in syringes moistened with a 0.3 *M* EDTA solution and centrifuged in the cold. All incubations were carried out in polycarbonate plastic tubes. Each tube mixture contained 0.1 ml of normal or diluted plasma, 0.9 ml of the renin substrate solution with an activity of 4200 ng, and 1 drop (0.015 ml) of 5% diisopropyl fluorophosphate (DFP) in isopropyl alcohol. Tubes were then incubated at 37° during 16 hr, at the end of which they were cooled in an ice bath, acidified to pH 5 by addition of 1 ml of 0.015 *N* HCl containing 0.15 *M* NaCl and heated in a boiling bath for 4 min. After centrifugation, the supernatant was adjusted to pH 6.8–7.2 with 5 *N* NaOH and assayed. Plasma renin values are expressed as nanograms of angiotensin II produced per 0.1 ml of plasma/16 hr of incubation.

Assay of angiotensin. Female rats weighing 200–300 g were anesthetized with amobarbital, (9 mg/100 g) and treated with atropine (0.40 mg) and pentolinium tartrate (5 mg). Blood pressure was measured with a transducer (Statham P23db) and recorded by a Sanborn recorder. Assays were performed in triplicate and pressor responses were compared with those given by standard doses of valine-5-angiotensin II aspartic β-amide (Hypertensin, Ciba).

Results. Inhibition of angiotensinase activity. Purified substrate, plasma from nephrectomized rats or a combination of both were incubated during 24 hr with 36 ng of angiotensin II in the presence of the angiotensinase inhibitors EDTA, DFP, and BAL. Results from Table I show that BAL was necessary to insure a complete inhibition of the angiotensinases contained in plasma. A similar observation had been made by Ryan *et al.* (7).

TABLE I. Inhibition of Plasma Angiotensinase Activity.

Samples[a]	Angiotensin (ng) recovered after 24-hr incubation	
	BAL	No BAL
EDTA + DFP	40	35
Substrate + EDTA + DFP	36	35
Substrate + plasma + EDTA + DFP	36	16

[a] 36 ng of angiotensin were initially added to each sample.

Characterization of the renin–renin substrate reaction. The effects of pH, time of incubation, enzyme concentration, and substrate concentration on the rate of angiotensin formation were studied to determine the optimal conditions for renin measurements.

a. Effect of pH. Incubation of renin substrate (4200 ng) with normal plasma (0.1 ml) or with purified renin (1.35×10^{-4} GU) at various pH values during 16 hr showed that the optimum pH for both plasma and renal renin was 6.5.

b. Effect of duration of incubation. When 0.1 ml of plasma from normal rats or severely bled rats or renal renin (6.75×10^{-4} GU) was incubated with 4200 ng of substrate at pH 6.5 for various periods of time, a linear relationship was found between rate of angiotensin formation and duration of incubation up to 24 hr. A 16-hr period of incubation was chosen as the most convenient.

c. Effect of enzyme concentration (Fig. 1). Various amounts of rat plasma or of purified rat renin (6.75×10^{-3} GU/ml) were added to 4200 ng of substrate and incubated during 16 hr at pH 6.5. The values obtained indicate that the velocity of angiotensin formation was directly proportional to enzyme concentration.

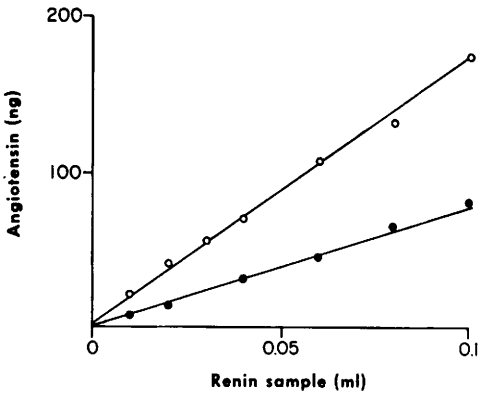

FIG. 1. Relationship between amounts of renin and angiotensin formation: (●) plasma renin; (○) renal renin.

d. Effect of substrate concentration (Fig. 2). Purified renal renin (13.5×10^{-4} GU) and normal rat plasma (0.1 ml) were incubated with various concentrations of rat substrate during 16 hr at pH 6.5. The results show that in all instances the rate of angiotensin formation was initially dependent on substrate concentration until it reached a plateau, indicating that the velocity of the reaction was maximal.

With renal renin, about 3000 ng of substrate were necessary. On the basis of these results, a substrate concentration of at least 3000 ng/ml of incubation has been chosen for routine determination of plasma renin. When high renin levels are expected, diluted plasma is used.

From the data presented in Fig. 2, the Michaelis constant (K_m) was calculated according to the method of Lineweaver and Burk (8). By plotting the reciprocal of substrate concentration ($1/S$) against the reciprocal of velocity of angiotensin formation ($1/V$), two straight lines were obtained (Fig. 3). The points where these lines intercept the horizontal axis are equal to the negative reciprocal of the K_m. Within the accuracy of the assay, the values of K_m are similar, thus suggesting that plasma renin and renal renin are identical enzymes. The same K_m was also found for the reaction between plasma renin and two different rat substrate preparations, suggesting that both preparations were identical from the kinetic point of view.

Characterization of the reaction product (Fig. 4). Various plasma samples were incubated with renin substrate, as described for renin determination. After heating and centrifugation the supernatants were pooled to study the nature of the pressor material formed. Antiangiotensin I or antiangiotensin II, in amounts sufficient to neutralize the pressor activity of 200 ng of angiotensin I or II, respectively, were added to 0.5 ml of the pooled samples with an activity equivalent to 15 ng of angiotensin II. After incubation for 15 min at 20°, the samples were bioassayed.

Antiangiotensin I blocked completely the pressor activity of the sample. Pressor activi-

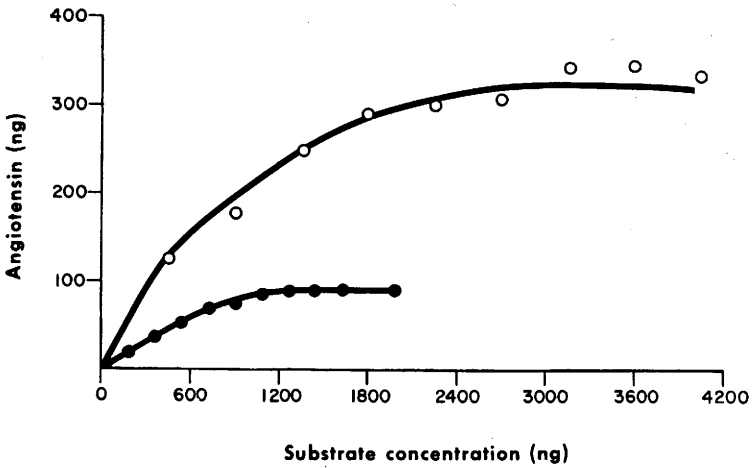

FIG. 2. Effect of substrate concentration on rate of angiotensin formation: (●) plasma renin; (○) renal renin.

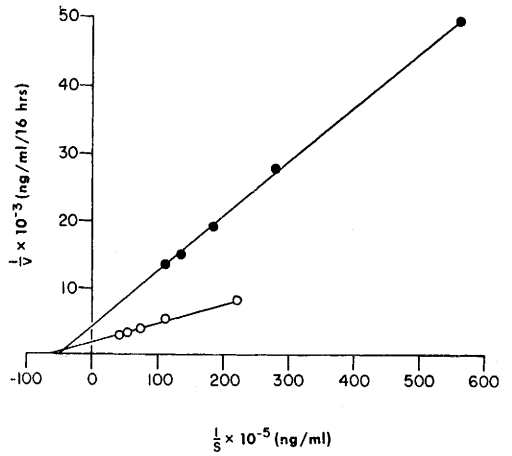

FIG. 3. Lineweaver–Burk plots of the renin–renin substrate reaction: (●) plasma renin; (○) renal renin.

ty reappeared after heating the incubate at 100° for 5 min. Such a treatment very likely denatures the protein moiety of the antigen–antibody complex, hence lets free active angiotensin I. On the other hand, addition of antiangiotensin II did not modify significantly the pressor activity of the incubating mixture. These observations strongly suggest that the pressor substance found in the incubates is angiotensin I.

Recovery of renin and of angiotensin. Angiotensin II (36 ng) or rat renin (1.03×10^{-4} GU) were incubated during 16 hr with 0.1 ml of rat plasma and 4200 ng of rat substrate. In 10 samples, angiotensin recovery averaged 102.7% (range 98–109%); in 9 samples renin recovery averaged 99% (range 85–107%).

Plasma renin in normal rats. a. When blood was obtained by heart puncture under a very light ether anesthesia, plasma renin from 12 rats averaged 47.4 ng (range 22–86 ng).

b. When blood was obtained from the jugular vein following deep anesthesia, plasma renin from 15 rats averaged 96 ng (range 29–149 ng).

c. Following anesthesia with amobarbital, and bleeding from the aorta (5 ml) after clamping both renal pedicles, values averaged 76 ng (range 36–140 ng).

Plasma renin in experimental rats. a. Nephrectomized rats. No plasma renin was detected in the plasma of rats which had been nephrectomized 4 or 24 hr previously. Six rats received 10 GU of rat renin subcutaneously at the time of the nephrectomy, and were bled from the aorta 4 hr later. Plasma was diluted 10 times. Values varied from 560 to 900 ng, with an average of 696 ng.

b. Adrenalectomized rats. Six days after adrenalectomy blood was obtained during amobarbital anesthesia from the aorta. Plasma was diluted 10 times. Values ranged from 900 to 1520 ng with a mean of 1020 ng.

c. Stilbestrol-treated rats. After subcutaneous injections of stilbestrol (0.05 mg/day) during 4 days, rats were anesthetized with amobarbital and bled from aorta. Plasma renin ranged from 10 to 22 ng, mean 16 ng.

Discussion. The procedure described in this paper permits the measurement of plasma renin in minute volumes of plasma; it is simple and accurate.

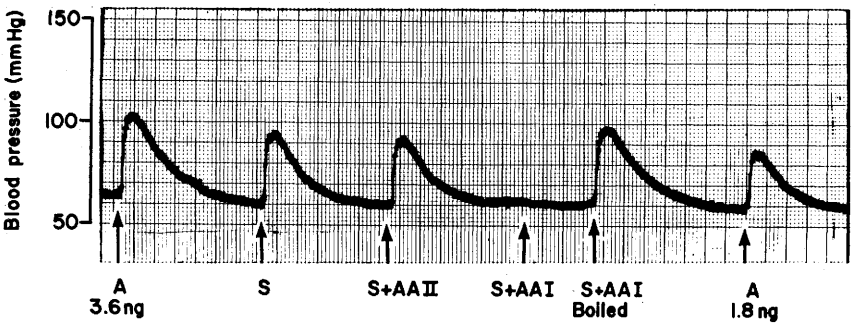

FIG. 4. Effect of antiangiotensin I and II on the pressor activity generated by incubating plasma with substrate. A, angiotensin standard; S, sample; AAI, antiangiotensin I; AA II, antiangiotensin II.

It is based on the determination of the amount of angiotensin formed when an aliquot of plasma is incubated with an excess of substrate, so that the velocity of the enzymatic reaction is proportional to the amount of renin. The sensitivity of the procedure is sufficient to permit the measurement of plasma renin in stilbestrol-treated rats, in which renin levels are consistently lower than normal (14). The degradation of angiotensin by angiotensinases, is completely inhibited by the combination of EDTA (9), DFP (10), and BAL (7), so that complete recovery of angiotensin is achieved.

The present study also shows that unlike other procedures (10, 11), the recovery of renin is complete, thus indicating that renin activators or inhibitors were not detectable in normal plasma under the current conditions, and suggesting that the values obtained may be considered as a better expression of renin concentration, than of renin activity.

The specificity of the method is assessed by the similarities in optimum pH and K_m between renal renin and the enzyme present in plasma, its absence in plasma from nephrectomized animals, and the evidence that the pressor product of the reaction is antigenically identical to angiotensin I. This suggests that only angiotensin I is formed, and that as previously reported, the plasma converting enzyme is inhibited by the association of EDTA and BAL (7, 12). The wide range of plasma renin values in normal rats has previously been reported (1); they may represent differences in response of individual animals to the conditions under which samplings were done. Like others (5, 13, 14) we found increases in plasma renin after adrenalectomy or renin injections, decreases after treatment with stilbestrol, and absence of renin after nephrectomy in the rat. In general, the values are higher than those obtained by using the Boucher procedure. This difference may be attributed to our better recoveries of angiotensin, to the presence of NaCl (15) and/or absence of Dowex resin in the incubation mixtures. This microprocedure differs from others, in that homologous substrate is used instead of hog renin substrate (2), and it does not involve adsorption on and elution of the angiotensin from the Dowex resin (1), thus, saving time and eliminating losses of angiotensin. Its only limitation is the number of samples that can be bioassayed.

Summary. A simple, sensitive, and accurate method for the routine measurement of plasma renin in the rat is described. It is based on the determination of angiotensin I formed when plasma is incubated with an excess of purified homologous substrate. Plasma angiotensinase activity is inhibited by the combination of EDTA, DFP, and BAL, thus permitting complete recovery of angiotensin and renin.

We are indebted to Dr. N. M. Glende for the anti-angiotensins I and II and to Mrs. K. Rice for technical assistance.

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Received Sept. 1, 1970. P.S.E.B.M., 1971, Vol. 136.