The Quantification of Renin Liberated *in Vitro* from Rat Kidney Slices[1] (34729)

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At present, the exact nature of the stimulus and modifying factors that control renin release is unclear. Proposed mechanisms include a natriastat, barostat, and possible hormonal influences (1). Several laboratories have provided evidence which indicates that the sympathetic nervous system may influence renin release and/or synthesis (2–4). Perhaps the best way to isolate and study each proposed stimulus is *via* an *in vitro* method. However, this requires the ability to measure very low concentrations of renin quantitatively. A modification of the zero-order kinetic assay developed by Schneider *et al.* (5, 6), described below, provides this quantification for dog and rat renin concentrations.

Methods. Thirty mongrel, male dogs, anesthetized with sodium pentobarbital (30 mg/kg), were bilaterally nephrectomized. Fifteen 4-hour, postnephrectomized and fifteen 18-to-24-hr, postnephrectomized animals were bled *via* the femoral artery; the blood was collected in ice-immersed flasks and immediately centrifuged for 20 min at 12,000*g* and 0° in a Sorval RC2-B refrigerated centrifuge. The plasma thus obtained was recentrifuged and then stored frozen at —20° until treated for the extraction of renin substrate by the method of Skeggs *et al.* (7) as modified by Schneider *et al.* (5, 6).

The extraction of substrate consisted of adjusting approximately 2 liters of plasma to pH 2.5 by the addition of 2.5 *N* HCl, followed by 2 hr of continuous stirring at 0°. The plasma was then adjusted to pH 6.0 by the addition of 2.5 *N* NaOH. Ammonium sulfate was added to a concentration of 1.5 *M* and the resultant mixture was stirred for 1 hr at 0°. The mixture was then centrifuged for 15 min at 10,000*g* and 0°. After being washed with 1.5 *M* ammonium sulfate at 0°, the precipitate was discarded and the molarity in the pooled supernatant and wash solution was adjusted to 2.3 *M* by further addition of ammonium sulfate. The mixture was stirred for 20 min at 0° and centrifugation at 10,000*g* and 0° was repeated. The supernatant was discarded and the precipitate was dissolved in a minimum of distilled water, placed into Visking tubing, and dialyzed for 8 hr against cold (7–9°) running tap water. Further dialysis occurred in 4 liters of cold (2–3°) distilled water which was changed four to six times during the next 24 to 36 hr. Clarification of the solution was achieved by centrifugation at 10,000*g* and 0°. The supernatant containing the substrate concentrate thus obtained from each 2-liter aliquot of plasma was pooled for each group of dogs. Since no objectionable endogenous renin concentration, expressed in Goldblatt units per milliliter (GU/ml), was found in the substrate concentrate prepared from the plasma of the 4-hour postnephrectomized dogs, the two preparations were combined to form a final volume of 2.8 liters of concentrate. This volume was thoroughly mixed and divided into 200-ml aliquots and stored frozen at —20° in plastic containers.

Treatment of substrate concentrate for its incubation with renin and tissue medium. To determine the total concentration of renin substrate in angiotensin equivalents (ng/ml) in the partially purified substrate concentrate for the purpose of standardization of the renin assay system and to measure the angiotensinase activity, various concentrations of

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standard dog renin[2] were added to 1-ml portions of substrate concentrate. The samples were then dialyzed against 50 ml of a 0.01 *M* disodium salt of ethylenediaminetetraacetic acid for 6 hr (one change) followed by dialysis for 16 hr against 50 ml of 0.02 *M* phosphate buffer at pH 5.3 (changes at 3, 10, and 13 hr). Saturated NaCl (0.026 ml/ml of sample) and diisopropyl fluorophosphate[3] (0.025 ml/ml of sample) were added to each sample just prior to incubation at 37°. After incubation, the enzymatic reaction was stopped by placing the sample in boiling water for 10 min. The sample volume was raised to 2 ml by the addition of buffer (0.04 *M* phosphate plus 0.11 *M* NaCl at pH 8.3). Finally, the sample was thoroughly mixed, centrifuged, and the supernatant fluid was stored frozen at —20° for the bioassay.

The substrate concentration was determined by the addition of ample renin (0.1 GU) to convert the substrate molecules to angiotensin during 30 min of incubation. The angiotensin thus formed was a measure of the substrate concentration expressed as nanograms (ng) of angiotensin per milliliter of substrate concentrate.

The angiotensinase activity was estimated by incubating samples of substrate with 0.1 GU of dog renin for 3, 4, 20, and 21 hr. Since all the substrate was converted to angiotensin during the first 3 hr, the loss of angiotensin during the subsequent incubation was taken as a measure of angiotensinase activity. If the samples incubated for 3 hr and the samples incubated to 20 hr contained the same quantity of angiotensin, no measurable active angiotensinase was considered to be present.

The endogenous renin activity of the substrate concentrate was determined by first determining the amount of angiotensin formed on incubation by the addition of known amounts of renin to establish a linear relationship between renin concentration and product at low renin concentrations. The angiotensin formed by incubation of substrate without added renin was due to endogenous renin, the concentration of which could be found by the slope of the relationship established by the incubation of the substrate at two exogenous renin levels.

Measurement of renin in whole rat kidneys, surviving slices, and incubation medium. White male rats, weighing 170 g, were divided into two groups, one of which was placed on a General Biochemicals TD-68441 control diet, and the other placed on a General Biochemicals TD-68503 low sodium diet for 60 days. The control group averaged a 180-g weight gain, while the low sodium group averaged a 90-g weight gain. At the end of this period, the kidneys were removed under ether anesthesia. The right kidney was frozen immediately and held for renin extraction.

The opposite kidney was cooled in isotonic medium on cracked ice. Ten slices, 50 to 70 mg of wet weight, were placed in Warburg vessels containing Robinson's medium (8), one slice to a vessel, and incubated in a gas phase of 100% oxygen at 37°. Incubation time varied from 10 to 120 min. The renin content of the medium was determined and the renin liberation from the slice was expressed in GU/mg/min.

Renin extraction from whole kidneys as well as from incubated slices was done by homogenizing the tissue in a glass and Teflon tissue homogenizer. The renin in the extract was estimated by the zero-order kinetic method and results were expressed in GU/mg of wet weight.

The assay of the renin content of the extract of the right kidney, the slices incubated in the Warburg vessels, and the medium in the incubation flasks was carried out as follows: A sample of 0.25 ml of diluted extract or undiluted medium was added to 0.75 ml of substrate. The samples were then treated precisely as those in the substrate concentration determination; *i.e.*, they were dialyzed against ethylenediaminetetraacetic acid and phosphate buffer; saturated NaCl and diisopropyl fluorophosphate were added just prior to incubation. The samples were incubated at

[2] Standard dog renin was kindly provided by Dr. Erwin Haas, The Mt. Sinai Hospital of Cleveland,, University Circle, Cleveland, Ohio.

[3] A solution (4 g/100 ml) of diisopropyl fluorophosphate (DFP) in isopropyl alcohol supplied by Calbiochem.

37° for an appropriate time to produce an adequate amount of angiotensin to measure on the rat bioassay. The reaction was stopped in boiling water, the volume was raised to 2 ml with buffer, and the sample was centrifuged. The supernate was stored at −20° for the bioassay. The amount of substrate in the 0.75 ml of substrate concentrate used to determine renin concentrations was 1200 ng of angiotensin equivalents, which is well above the limit of substrate required for the application of zero-order kinetics in the estimation of renin in samples of media or extracts.

Rat bioassay for angiotensin. Dibenzyline–Ansolysen-treated postnephrectomized male rats (240 to 300 g) were used for the bioassay as described by Wathen *et al.* (3). Hypertensin (Ciba[4]) was used as the angiotensin standard and all results from the bioassay were expressed in terms of nanograms of angiotensin amide.

Results. Schneider *et al.* (5) have shown that the standard equation for zero-order kinetic reactions accurately describes the reaction of dog renin with homologous substrate when the substrate concentration is adequate. In addition, validation of the assay requires that all angiotensinase activity must be inhibited. These two parameters were determined by incubating 1 ml of substrate with an excess (0.1 GU/ml) of standard dog renin for 3, 4, 20, and 21 hr at 37°. As shown in Table I, the substrate concentration was approximately 1500 ng/ml and all angiotensinase activity was inhibited. The importance of DFP for inhibition of angiotensinase in attaining zero-order kinetic conditions cannot be overemphasized.

The standard equation for zero-order kinetics,

$$P = K_0[R]t,$$

TABLE I. Determination of Substrate Concentration and Angiotensinase Activity.

Incubation time (hr)	3	4	20	21
Angiotensin (ng)	1461	1472	1472	1458

[4] Kindly supplied by Dr. Albert J. Plummer, Ciba Pharmaceutical Co., Summit, New Jersey.

predicts that if P, angiotensin produced, is plotted against $[R]t$, the product of renin concentration and time, then a straight line with a y-intercept of zero and a slope of K_0 should be obtained. In standardizing the assay, both time (30 to 750 min) and renin concentration (2×10^{-4} to 5×10^{-3} GU/ml) were varied. The relationship was determined for dog renin acting on dog substrate and rat renin acting on dog substrate. Figure 1 shows that indeed zero-order reactions for both renins were obtained. The K_0 varied when different renins were used. The K_0 for rat renin was significantly different ($p < .001$) from that found for dog renin. The K_0 for a specific renin is constant, however, for a particular batch of substrate.

Because the substrate extraction procedure does not completely remove all endogenous renin, it is necessary to measure the actual renin level of the concentrate in order to

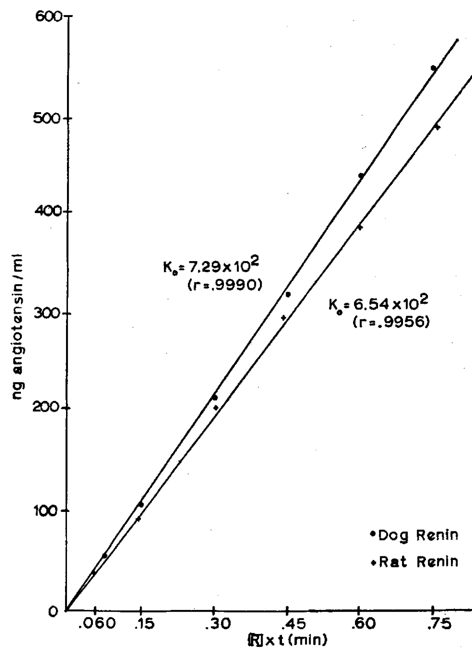

FIG. 1. Dog and rat renin on dog substrate: Rat renin incubated with dog substrate with a K_0 of 6.54×10^2 ng/GU/min. Dog renin incubated with dog substrate with K_0 of 7.29×10^2 ng/GU/min. Each point is determined by at least 10 assays. r is the correlation coefficient. The K_0's show a significant difference ($p < .001$). $K_0 =$ (angiotensin II produced)/[(renin) × Incubation time].

accurately determine the renin concentration in an unknown sample. On incubating substrate concentrate for 300 and 1200 min, the endogenous renin level, using the K_0 determined with dog renin, was calculated to be approximately 1.35×10^{-5} GU/ml. Assuming a large standard deviation of 12% for the rat bioassay, it was calculated that the difference between endogenous renin concentration and the sum of endogenous and exogenous renin concentration need be only 2×10^{-6} GU/ml for significance at the 95% level.

The practical limits of the assay system are the following: (a) The concentration of angiotensin (P) formed during incubation can vary from 15 to 500 ng/ml. (b) Incubation times can vary from 30 to 750 min.[5] (c) Renin concentrations can vary from 0.00003 to 0.03 GU/ml. Thus, the method has a 1000-fold range in assayable renin concentration.

The modified zero-order kinetic method for the measurement of rat renin using dog substrate described above was used to estimate renin release (liberation) from surviving rat kidney slices incubated in Warburg vessels. Each of 10 flasks contained one of 10 cross-sectional slices obtained from a single kidney. Figure 2 is a graphic representation of the rate of steady-state liberation of renin from the slices of three left kidneys taken from each of the two groups of rats. The rate of renin liberation into the medium from slices obtained from kidneys of rats on the low sodium diet was seven times faster than from the slices from rats on the control diet. However, the percentage of the total renin content of the slices liberated into the medium in 2 hr of incubation at 37° was the same, 6.2% from the low sodium group and 5.6% from the control. This indicates that the rate of renin release from surviving kidney slices is directly related to the renin content of the slice in the two cases. The steady-state "release" appears to be metabolically dependent and does not represent a renin loss due to failure of metabolism or a simple "washing out" of renin from damaged or failing cells. Slices incubated in a gas phase of nitrogen failed to show a steady-state second hour release, and the total renin loss during 2 hr of incubation was greatly reduced in slices with depressed metabolism.

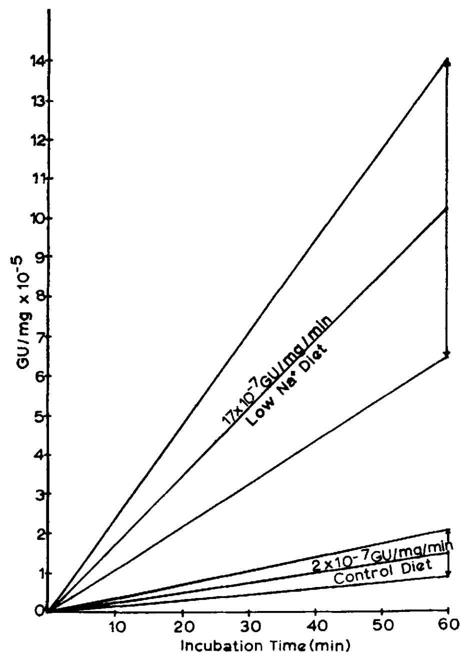

FIG. 2. Rate of renin liberation from surviving rat kidney slices: Rate of renin liberation from the end of the first hour to the end of the second hour of incubation of surviving kidney slices taken from rats on a normal diet and on a low sodium diet. Arrows represent one standard error of the rate of liberation.

Discussion. A thorough *in vitro* study of renin release requires an accurate renin assay. Such an assay is presented in this paper. Outstanding features of this assay include the following: (a) The assay is quantitative. (b) Results are expressed in renin units (GU/ml or GU/mg of tissue) rather than in generated angiotensin. (c) It allows assay of a large number of samples with accuracy.

Preliminary data show that the high renin content of the kidneys found in young rats on a low sodium diet has a marked effect on the amount of renin liberated into the medium when slices of kidney tissue are incubated under conditions that allow a steady-state metabolism, and that the amount liberated is

[5] Although 1500-minute incubation periods were not tried on this substrate preparation, the results from 1500-minute incubations fell on the regression line studies in an earlier substrate preparation.

metabolically dependent and directly related to the renin content of the slice. The metabolic aspects of *in vitro* renin "release" are undergoing active study in this laboratory by the use of the method described herein.

Summary. A bioassay system for the determination of renin liberated *in vitro* from surviving rat kidney slices incubated in Warburg vessels is described. The system is based on zero-order enzyme kinetics obtained by incubating rat renin with concentrated dog renin substrate to obtain a K_0 for the heterologous enzyme–substrate reaction. The method is accurate over a 1000-fold range of renin concentration from 0.03 to 0.00003 GU/ml which encompasses renin concentrations in incubation media and in rat kidney slices. Preliminary results indicate the liberation of renin *in vitro* is metabolically dependent and amounts to about 5% of the renin content of the slice during a 2-hr incubation period. The rate of liberation of renin from slices of rat kidneys obtained from rats on a sodium free diet was several times the rate found for slices taken from controls on a normal diet.

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