Properties of Renin Substrate in Rabbit Plasma with a Note on its Assay

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1. Rabbit plasma enzymes that degrade angiotensin I are inhibited completely by the combination of 2,3-dimercaptopropan-1-ol (10mm), EDTA (10mm) and chlorhexidine gluconate (0.005%, w/v). These compounds do not modify the reaction of renin with renin substrate and are termed the selective inhibitors. 2. The renin substrate concentration of plasma can be measured as angiotensin I content by incubating plasma plus the selective inhibitors with renin for a time sufficient to allow complete utilization of renin substrate. 3. This reaction obeys first-order kinetics to substrate concentrations of at least 1000 ng. of angiotensin I content/ml. In general, the renin substrate concentrations of normal rabbit plasmas are less than 1000 ng. of angiotensin I content/ml. Thus the time required for the complete release of angiotensin I from normal plasma is inversely related to renin activity and is independent of renin substrate concentration. 4. A method for the assay of renin substrate, taking these reaction kinetics into account, is presented.

Renin substrate, an α_2 -globulin of plasma, is hydrolysed by renin to release the vasoactive decapeptide, angiotensin I (Lentz, Skeggs, Woods, Kahn & Shumway, 1956; Skeggs, Lentz, Kahn, Shumway & Woods, 1956; Peart, 1956; Elliott & Peart, 1957; Skeggs, Lentz, Hochstrasser & Kahn, 1963). Though renin substrate has been purified and its structure partially characterized, there is not a generally accepted method for its assay. Pure renin substrate has been measured in terms of the maximum amount of angiotensin I released when incubated with a high concentration of highly purified renin. Angiotensin I, in turn, has been measured by its biological activity. However, the assay of renin substrate in plasma or crude extracts has not been accomplished by this approach. In consequence, very little is known about physiological or pathological variations of plasma renin substrate concentrations.

Difficulties in measuring renin substrate in plasma, like those of assaying plasma renin activity (Ryan, McKenzie & Lee, 1968), are due largely to plasma enzymes that modify the biological activity of angiotensin I.

In the preceding paper (Ryan *et al.* 1968) it was shown that EDTA, BAL† and chlorhexidine gluconate prevent the actions of converting enzyme

and angiotensinase enzymes of rabbit plasma, and that SBTI prevents the release of kinins. These reagents do not modify the reaction of renin with its substrate and are called the selective inhibitors. Thus renin substrate concentration can be measured after incubating rabbit plasma plus the selective inhibitors with renin for a time sufficient to release the total angiotensin content.

MATERIALS AND METHODS

Prevention of bacterial contamination and glass adsorption of angiotensin. Methods recommended by Cook & Lee (1965) and Lee, Cook & McKenzie (1966) were used to avoid bacterial contamination. All glassware was treated with silicone (Siliclad; Clay-Adams, New York, N.Y., U.S.A.) to prevent glass adsorption of angiotensin (Ryan *et al.* 1968).

Reagents. The following reagents were used: 0.1 M-sodium phosphate buffer, pH 6.0; EDTA-phosphate solution, 40 mM-EDTA (disodium salt) in 0.1 M-sodium phosphate buffer, pH 6.0; SBTI-phosphate solution, 1 mg. of SBTI (3× crystallized; Sigma Chemical Co., St Louis, Mo., U.S.A.)/ml. of 0.1 M-sodium phosphate buffer, pH 6.0; BAL (Dimercaprol; Boots Pure Drug Co. Ltd., Nottingham), 0.4 M solution in arachis oil. All solutions in 0.1 M-sodium phosphate buffer, pH 6.0, also contained 0.01% (w/v) chlorhexidine gluconate [1,6-di-(4-chlorophenyl)diguanido]-hexane gluconate, Hibitane; Imperial Chemical Industries Ltd., Pharmaceutical Division, Wilmslow, Cheshire].

Preparation of renin substrate. At 48 hr. after bilateral nephrectomy, adult rabbits were anaesthetized with intravenous thiopental (20–30 mg./kg.) and then given intravenous heparin (1000 units/kg.). Animals were exsanguinated through a cannula in the common carotid artery and the blood was collected in silicone-coated

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‡ Abbreviations: BAL, 2,3-dimercaptopropan-1-ol; SBTI, soya-bean trypsin inhibitor.

centrifuge bottles. After centrifugation at 1690g at 4° for 30 min., plasma was transferred to dialysis sacs and dialysed against 10l. of water for 24 hr. and then against 10l. of 3mM-EDTA for 24 hr. Both dialysis solutions contained chlorhexidine gluconate (0.01%, w/v). The clear faint-brown supernatant collected after centrifugation of the dialysed plasma was used as renin substrate. If not used immediately, this solution was passed through a Millipore bacteriological filter into sterile silicone-coated glass ampoules. Ampoules were flame-sealed. Stored at 4°, renin substrate was stable for at least 4 months. These preparations contained renin substrate in concentrations of 1000–3000 ng. of angiotensin content/ml.

Preparation of a standard renin solution. Renin was prepared by a modification of the method of Skeggs, Lentz & Kahn (1967). Kidneys were removed from adult rabbits under pentobarbitone anaesthesia (30 mg./kg.) or immediately after killing the animal. The kidneys were frozen in an ethanol–solid CO₂ slurry. After partial thawing, the kidneys were minced in a meat grinder. Then 2 ml. of water was added/g. wet wt. of tissue, and this suspension was stirred for 1 hr. at 4°. This was filtered through several layers of sterile gauze. The filtrate was diluted with 6 vol. of water and extracted with 0.1 vol. of toluene. The aqueous phase was collected after centrifugation and its protein determined by the biuret method. Moist DEAE-cellulose (Whatman grade 50; free base form) was added to give 15 g./g. of protein. This suspension was stirred intermittently at 4° for up to 18 hr., then filtered on a Buchner funnel under vacuum. The filtrate was discarded and the DEAE-cellulose pad was resuspended in the original volume of water. The pH was adjusted to 4.6 with acetic acid. After being stirred for 5–10 min., the DEAE-cellulose was again collected by filtration and resuspended in the original volume of water. Then NaCl was added to 0.1 M and the pH was adjusted to 4.6 with acetic acid. This suspension was stirred for 1 hr. at 4° and then filtered. The filtrate contained the renin activity.

After the addition of chlorhexidine gluconate to 0.01% (w/v), the renin solution was free of angiotensinase activity when incubated at 37° or 42° at pH 6.0 for 2 hr. or at pH 7.0 for much longer times. Samples concentrated 20–40-fold by ultrafiltration or by precipitation with (NH₄)₂SO₄ (400 g./l.) contained measurable angiotensinase activity. However, in the following studies, the renin solution was used without concentration and was functionally free of angiotensinase activity.

Assay of the standard renin solution. As shown in this and the preceding paper (Ryan *et al.* 1968), the release of angiotensin from renin substrate, in concentrations of 1000 ng. of angiotensin content/ml., obeys first-order-reaction kinetics. Thus the activity of a given renin preparation can be expressed in terms of the time (*t*₁) required for that renin preparation to release half the angiotensin content of substrate. Renin activity can be measured as the percentage of angiotensin released per unit time by following the method recommended by Ryan *et al.* (1968). The standard renin solution used throughout the studies described below had *t*₁ 3.6 min. at pH 6 at 42°. The activity of this solution was 1155 renin units/ml.

Assay of angiotensin. Angiotensin was assayed as described previously (Peart *et al.* 1966; Ryan *et al.* 1968).

[5-Isoleucine]-angiotensin I (a kind gift from Dr L. T.

Skeggs, jun., Cleveland Veterans Hospital, Cleveland, Ohio, U.S.A.) and [1-asparagine-5-valine]-angiotensin II (Hypertensin; Ciba, Basle, Switzerland) were used as standard reference compounds.

Assay of angiotensinase activity. Samples of renin or renin substrate were incubated at 42° with [1-asparagine-5-valine]-angiotensin II (100 or 200 ng./ml.) in 0.05 M-sodium phosphate buffer, pH 6.0. Angiotensinase activity was measured in terms of the rate of disappearance of angiotensin activity.

Chromatography. Sephadex G-200 was equilibrated with 0.1 M-NaCl in 0.1 M-tris-HCl buffer, pH 8.0. The packed column was 2.7 cm. × 50 cm. A 5 ml. plasma sample was applied and then eluted with the tris-HCl buffer. The flow rate was adjusted to 20 ml./hr.; 4 ml. fractions were collected. Elution was monitored by determining *E*₂₈₀.

Preparation and purification of angiotensin. Two vol. (usually 10 ml.) of dialysed plasma was mixed with 1 vol. of the SBTI-phosphate solution, 1 vol. of the EDTA-phosphate solution, 0.1 vol. of BAL and 0.5 vol. of renin. The mixture was incubated for 80 min. at 42° and then the reaction was stopped by adding 2 vol. of 10% (w/v) trichloroacetic acid. The mixture was centrifuged at 1690g at 4° for 60 min. The supernatant was saved and the precipitate was washed with 2 vol. of 5% (w/v) trichloroacetic acid. The combined supernatants were extracted four times with equal volumes of diethyl ether. The aqueous phase was neutralized to pH 7 with 2 N-NaOH and concentrated in a rotary evaporator to about 1 ml. The resulting yellow viscous fluid was extracted by one of two methods, depending on the countercurrent-distribution system to be used. Most extractions were done with 8 ml. of butan-1-ol previously saturated with 0.05 M-sodium phosphate buffer, pH 7.0. Other extractions used butan-2-ol previously saturated with 15.0% (w/v) NaCl in 0.1 M-sodium phosphate buffer, pH 7.0.

Countercurrent-distribution systems. The first system was modified after that described by Skeggs, Marsh, Kahn & Shumway (1954). Butan-1-ol was 2 vol. with respect to the lower phase, 0.05 M-sodium phosphate buffer, pH 7.0. The second system was described by Skeggs, Kahn & Shumway (1956). Butan-2-ol was 2 vol. with respect to the lower phase, 15.0% NaCl in 0.1 M-sodium phosphate buffer, pH 7.0. Either countercurrent system gave a wide separation of [5-isoleucine]-angiotensin I from [5-isoleucine]- or

Table 1. *Countercurrent partition coefficients (apparent)*

	Butan-1-ol/ 0.05 M-sodium phosphate buffer, pH 7.0	Butan-2-ol/15% NaCl in 0.1 M- sodium phosphate buffer, pH 7.0
[Ile ⁵]-angiotensin I	1.7–2.2	> 6
[Ile ⁵]-angiotensin II	0.17–0.21	< 0.1
[Val ⁵]-angiotensin II	0.11–0.13	< 0.1
[Arg]-vasopressin	0.11–0.13	—*
Adrenaline	< 0.11	—*
Noradrenaline	0.04–0.07	—*

* Not tested.

[5-valine]-angiotensin II (for the first system, see Fig. 1). Apparent partition coefficients of some vasopressor compounds are shown in Table 1.

Selective inactivation of plasma kallikrein, converting enzyme and angiotensinase enzymes. Before the addition of renin, solutions of renin substrate or fresh plasma were treated to contain SBTI (250 $\mu\text{g./ml.}$), EDTA (10 mM), BAL (10 mM), chlorhexidine gluconate (0.005%) and 0.05 M-sodium phosphate buffer, pH 6.0. Thus treated, plasma or plasma extracts are free of kallikrein, converting enzyme and angiotensinase activities, and the reaction of renin with renin substrate is not modified (Ryan *et al.* 1968). These added reagents were designated the selective inhibitors.

Inactivation of angiotensin by trypsin, chymotrypsin and carboxypeptidase A. These studies were done as described in the preceding paper (Ryan *et al.* 1968).

RESULTS AND DISCUSSION

Product of the reaction of renin with plasma of nephrectomized rabbits. When renal renin was incubated with plasma plus the selective inhibitors (SBTI, EDTA, BAL and chlorhexidine gluconate) a substance was formed that, on intravenous injection, caused a sharp transient rise of mean arterial blood pressure. The activity of the substance was not modified by heating in a boiling-water bath for 20 min. at neutral or slightly acid pH. It was soluble in water, 0.15 M-sodium chloride, 5% trichloroacetic acid, 0.5 M-phosphoric acid, 70% ethanol, water-saturated butan-1-ol and water-saturated butan-2-ol. It was not soluble in diethyl ether. Unlike vasopressin, this reaction product was not inactivated when incubated at 37° for 40 min. with 10 mM-thioglycollate. The product lost all activity when incubated at pH 7.8 with trypsin, chymotrypsin or di-isopropyl phosphorofluoridate-treated carboxypeptidase A.

In either the first or second countercurrent-distribution system, the vasopressor product distributed as a single peak like [5-isoleucine]-angiotensin I. [5-Valine]-angiotensin I was not available for comparison. Distribution in the first countercurrent system is shown in Fig. 1 (see Table 1 for apparent partition coefficients of other vasopressor compounds). In the first countercurrent system, the apparent partition coefficient of the reaction product was 1.9.

It was considered possible that the extraction procedures used to purify angiotensin favoured the recovery of angiotensin I over angiotensin II. This was studied by adding various quantities (5–25 $\mu\text{g.}$) of [5-valine]-angiotensin II and [5-isoleucine]-angiotensin I to 10 ml. samples of plasma containing the selective inhibitors. These samples were extracted immediately. The recovery of [5-valine]-angiotensin II was marginally better than recovery of [5-isoleucine]-angiotensin I even when angiotensin II was only 20% (by wt.) the amount of

Fig. 1. Countercurrent distribution of the product (○) of the reaction of renal renin with substrate between butan-1-ol (2 vol.) and 0.05 M-sodium phosphate buffer, pH 7.0 (1 vol.).

angiotensin I added to plasma. However, the recovery of either varied in the range 35–55%.

To confirm that converting enzyme and angiotensinase enzymes were not active, [5-isoleucine]-angiotensin I (2000 ng.) was added to 10 ml. samples of plasma plus the selective inhibitors and then incubated at 37°. Samples (4 ml.) were taken at zero and 21 hr., extracted and applied to the second countercurrent-distribution system. Throughout the incubation there was no loss of angiotensin activity and no measurable conversion into angiotensin II.

From these results it appears that the product of the reaction of rabbit renin with rabbit plasma plus the selective inhibitors is angiotensin I. It could not be either known form of angiotensin II. These results also support the finding (Ryan *et al.* 1968) that the selective inhibitors prevent the actions of converting enzyme and angiotensinase enzymes of sterile rabbit plasma.

Chromatography of rabbit renin substrate. Samples (5 ml.) of plasma of bilaterally nephrectomized rabbits were chromatographed on Sephadex G-200. Results are shown in Fig. 2. Substrate was eluted as a single peak with albumin. By this procedure it appears that, if there is more than one kind of rabbit renin substrate, the various kinds have the same or very similar molecular weights (Andrews, 1965).

Kinetics of the reaction of renin substrate and renin. The following studies were carried out with renin substrate treated with the selective inhibitors as described in the Materials and Methods section. Incubations were run at 42°. The velocity of release of angiotensin by renin was dependent on the renin substrate concentration to concentrations of at least 1000 ng. of angiotensin content/ml. (Fig. 3). Higher substrate concentrations were not studied in detail, though in a single experiment velocity

Fig. 2. Elution of renin substrate in whole plasma (from a bilaterally nephrectomized rabbit) from a column (50 cm. $\times$ 2.7 cm.) of Sephadex G-200. Protein was determined by measuring E_{280} (○); renin substrate was assayed as described in the text (bars). Each fraction contained 4 ml.

Fig. 3. Effect of substrate concentration (ng. of angiotensin content/ml.) on the initial velocity of angiotensin release.

increased with increasing substrate concentration to 1800 ng. of angiotensin content/ml.

With renin substrate concentrations of 1000 ng. of angiotensin content or less/ml., the release of angiotensin followed a characteristic pattern. As shown in Fig. 4, the time required for a single quantity of renin to release half the angiotensin content of three different concentrations of substrate was virtually independent of the initial substrate concentration, $[S]_0$, regardless of whether $[S]_0$ was taken to be the true starting concentration or an instantaneous reference concentration after beginning the incubation. Within the limits of

Fig. 4. Rate of utilization of three concentrations of renin substrate (□, 70 ng./ml.; ●, 110 ng./ml.; ○, 225 ng./ml.) by a single concentration of renin.

substrate concentrations studied, the release of angiotensin obeyed first-order kinetics.

Thus in the general rate equation:

$$-\frac{d[S]}{dt} = k[E]^{\alpha}[S]^{\beta} \quad (1)$$

where t is time, k is a proportionality constant, $[E]$ is renin activity, $[S]$ is substrate concentration, and α and β are order exponents. β being 1, eqn. (1) can, then, be rewritten:

$$\text{Rate} = k[E]^{\alpha}[S] \quad (2)$$

$$\text{and} \quad \log(\text{Rate}) = \alpha \log[E] + \log k[S] \quad (3)$$

If it is assumed that, at low relative conversions of substrate into angiotensin, $[S]$ is unchanged

Fig. 5. Determination of the order of reaction of renin with its substrate (see the text). The slope $[\log(\text{rate})/\log[E]]$ is 0.94. $[E]$ is renin concentration in arbitrary units.

during the reaction, a plot of $\log(\text{rate})$ against $\log[E]$ should give a straight line of slope α . Six different concentrations of renin were incubated with renin substrate (1100 ng. of angiotensin content/ml.), so that no more than 7% of substrate was used. Results are shown in Fig. 5. Renin units were arbitrary, the lowest being a 12-fold dilution of the highest. The slope of the line in Fig. 5 is 0.94. Thus renin itself obeyed first-order kinetics, and:

$$\text{Rate} = k[E][S] \quad (4)$$

By using eqn. (4), again considering $[S]$ to be essentially unchanged during incubation, a plot of initial rate against $[E]$ gives a straight line of slope $k[S]$ (Fig. 6). With $[S]$ equal to 1100 ng. of angiotensin content/ml. k is 1.99×10^{-3} arbitrary enzyme unit $^{-1}$ hr. $^{-1}$.

The integrated form of eqn. (4):

$$k[E]t = \ln \left(\frac{[S]}{[S-X]} \right) \quad (5)$$

was used to take into account the actual change in substrate concentration, $[S-X]$. The average of the k values calculated for each reaction mixture was 2.11×10^{-3} enzyme unit $^{-1}$ hr. $^{-1}$ and was in good agreement with the k value determined from Fig. 6.

Assay of renin substrate in plasma. From the above kinetic studies it is evident that, so long as the initial renin substrate concentration is 1000 ng. of angiotensin content or less/ml., the time (t_i) for a given renin preparation to release half the angiotensin content is inversely proportional to renin activity and is independent of the actual substrate concentration. In general, renin substrate concentrations of normal rabbits are within the first-order range. Substrate in plasmas of

Fig. 6. Effect of increasing concentrations of renin on the rate of release of angiotensin from a single concentration of substrate (1100 ng. of angiotensin content/ml.).

Fig. 7. Assay of renin substrate. The time required to release the total angiotensin content of renin substrate is independent of the initial renin substrate concentration and inversely proportional to renin activity. $\circ$, Theoretical values, t_i 18 min.; $\bullet$, measured values, with vertical lines indicating limits of bracket assay.

bilaterally nephrectomized rabbits may approach 4000 ng./ml. and should be diluted before assay.

Once t_i for a given renin preparation has been established, that preparation incubated with any normal rabbit plasma under standard conditions for six or more times t_i should cause the release of over 98% of the angiotensin. An example is shown in Fig. 7. Two vol. of plasma was mixed with 1 vol. of the SBTI-phosphate solution, 1 vol. of the EDTA-phosphate solution, 0.1 vol. of BAL and 1 vol. of a standard renin solution. At pH 6 and at 42° t_i of the renin solution was 18 min. Release of angiotensin was essentially complete (more than 98%) after $6 \times t_i$ or 108 min.

This approach to the assay of renin substrate assumes that plasma does not contain substances that modify the reactivity of renin or substrate.

Table 2. *Effect of various concentrations of heparin on the rate of release of angiotensin*

Heparin (units/ml.)	Rate (ng./hr.)	Inhibition (%)
0	500	—
5	500	0
20	500	0
35	333	33
85	125	75
170	75	85

Fig. 8. Effect of heparin on the time required by a given concentration of renin to release the total angiotensin content of renin substrate: ■, renin with heparin (40 units/ml.); ●, renin alone; □, curve for theoretical $t_{1/2}$ 65 min.; ○, curve for theoretical $t_{1/2}$ 9 min.

Pickens, Bumpus, Lloyd, Smeby & Page (1965) have suggested that human plasmas contain variable amounts of an inhibitor or activator of renin. Gould, Skeggs & Kahn (1966), using similar techniques, were unable to find evidence of either. Sealey, Gerten, Ledingham & Laragh (1967) have shown that heparin, added to mixtures of human renin and human renin substrate, decreases the rate of angiotensin formation. Kinetic studies suggested that heparin may be a competitive inhibitor of renin, but the possibility was not ruled out that heparin decreases the reactivity of renin substrate.

Since heparin was the anticoagulant used in this study, its effect on the rabbit renin-rabbit renin

substrate system was determined. Table 2 shows the effect of increasing concentrations of heparin on the rate of release of angiotensin. Fig. 8 shows that heparin (40 units/ml.) increased the apparent $t_{1/2}$ of a standard renin preparation by over sixfold. Lower concentrations (20 units/ml. and less), still in excess of that needed for anticoagulation, had no effect.

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