The Preparation of Rabbit Renin-Substrate for the Assay of Minute Amounts of Renin

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(Received 9 November 1964)

1. By DEAE-cellulose chromatography and treatment with Cd^{2+} a partially purified renin-substrate has been prepared from rabbit serum. 2. It has the following properties: (a) Angiotensinase activity is undetectable: 0.1 μg . of angiotensin was recovered intact after incubation with the substrate at 37° for 200 hr. (b) It does not contain renin and does not generate vaso-active substances when incubated alone. (c) It is ten times as active as serum in terms of angiotensin produced/mg. of protein. (d) It has not deteriorated after storage for 3 months at 4° at pH 7.5.

Investigation of the function of renin has been handicapped by lack of a sufficiently sensitive method to estimate the minute amount of this enzyme that presumably circulates in the normal animal. The estimation of renin by its direct pressor effect is not sensitive enough. Braun-Menendez, Fasciolo, Leloir, Munoz & Taquini (1946) increased the sensitivity by measuring the rate of formation of angiotensin by incubating renin and its substrate *in vitro*. This procedure was further developed by Lever, Robertson & Tree (1964), who were able to assay renin in normal peripheral blood. Such a method, though theoretically simple, is fraught with practical difficulties. The incubation of blood, or crude extracts of blood, produces vaso-active substances that interfere with the bioassay of angiotensin, and this problem becomes particularly serious when the amounts of renin to be assayed are very small. A further difficulty is that blood contains an enzyme or enzymes ('angiotensinase') that destroy angiotensin, and these must be removed or inactivated if meaningful results are to be obtained.

An ideal substrate preparation for the assay of renin would have the following properties: (1) a high activity in terms of angiotensin produced/mg. of protein present; (2) it should be stable; (3) it should not generate vaso-active substances when incubated alone; (4) it should be free from renin and angiotensinase.

Most methods of angiotensinase removal have used acid-denaturation of the enzymes (Braun-Menendez *et al.* 1946; Lever *et al.* 1964). With acid treatment, we found it difficult to reduce the interfering enzymes to a sufficiently low concentration without at the same time causing a substantial loss of renin-substrate.

We now report a method for preparing rabbit renin-substrate that satisfies the above criteria and is suitable for the assay of minute amounts of renin.

METHODS

Sterile technique. It soon became apparent that angiotensinase activity appeared in the solutions if they became contaminated with micro-organisms. This angiotensinase activity proved very difficult to remove and we decided to exclude micro-organisms as far as possible from the preparative procedures.

Buffers and salt solutions. All salt solutions contained 0.01% of chlorhexidine gluconate B.P. and were bottled in 500 ml. screw-topped bottles that were then autoclaved at 115° for 20 min.

Glassware. All glassware was treated with dimethyldichlorosilane in carbon tetrachloride, well washed, stoppered with cotton-wool plugs and sterilized by dry heat at 170° for 6 hr.

The efficacy of the sterile technique was checked from time to time by inoculation of a sample into culture broth.

PROCEDURE

In several rabbits both kidneys were excised through flank incisions under anaesthesia. Two days later the animals were again anaesthetized and bled from the common carotid artery into 250 ml. centrifuge bottles by means of a cannula and silicone-rubber tube. Special care was taken to avoid bacterial contamination at this stage.

The blood was allowed to clot in an incubator at 37° , the process being accelerated by the addition of a few sterilized glass beads. The serum was withdrawn and centrifuged at 2400 rev./min. (2000g) for 30 min. in 250 ml. centrifuge bottles stoppered with cotton-wool plugs, and then transferred to a sterile weighed 500 ml. blood-bank bottle. The weight of serum was determined by difference and a sample taken for subsequent analysis. 2N-HCl was added to bring

the pH to approx. pH6, with British Drug Houses Ltd. narrow-range indicator paper as external indicator.

Ammonium sulphate precipitation. The bottle of acidified serum was again weighed and analytical-grade ammonium sulphate was added (0.39 g. of salt/g. of serum) and left to stand for at least 1 hr. at 4°. If necessary the preparation can be left overnight at this stage without deterioration.

The precipitate was separated by centrifugation at 20000g for 15 min. at 4°, the centrifuge tubes and caps being sterilized by boiling immediately beforehand and allowed to cool. The supernatant was discarded and the precipitate resuspended in 3.3M-ammonium sulphate (at 4°). The precipitate was again separated by centrifugation at 20000g for 15 min. The supernatant was discarded and the precipitate dissolved in a minimum volume of 0.01M-tris-HCl buffer, pH7.5.

Dialysis. The solution was placed in dialysis tubing that had been sterilized by boiling and dialysed against 0.01M-tris-HCl buffer, pH7.5, until free from sulphate. The precipitate that formed during the dialysis was removed by centrifugation at 20000g for 15 min. and the clear supernatant was applied to a column of DEAE-cellulose.

Adsorption on and gradient elution from DEAE-cellulose. DEAE-cellulose (Whatman grade 50) was washed with 2M-NaCl and then distilled water until chloride-free. It was then equilibrated with 0.01M-tris-HCl buffer, pH7.5, and autoclaved in 500 ml. blood-bank bottles at 115° for 20 min. Each bottle contained an amount of DEAE-cellulose sufficient to pack a column 1.8 cm. × 30 cm.

Packing the column. This was carried out by filling the column with 0.01M-tris-HCl buffer, pH7.5, inverting the bottle of DEAE-cellulose above it and connecting the two by a short length of wide-bore rubber tubing that had been boiled. The DEAE-cellulose was kept in uniform suspension in the bottle by means of a magnetic stirrer. The outflow from the column was maintained at a constant rate and the air inflow into the bottle was filtered through a sterile cotton-wool plug. In this way micro-organisms were excluded as far as possible.

Gradient elution. The supernatant after dialysis was applied carefully to the DEAE-cellulose column mounted on a Towers fraction collector modified for timed specimens.

A linearly increasing concentration of chloride was used to elute the DEAE-cellulose column. This was achieved by means of a closed system in which a 1 l. separating funnel emptied under gravity into another 1 l. funnel which then dripped into the column. The top funnel contained 1 l. of the high-molarity buffer 0.01M-tris-HCl, pH7.5, containing NaCl (0.38M); the lower one contained 1 l. of the starting buffer, 0.01M-tris-HCl, pH7.5. The rate at which the high-molarity buffer entered the lower reservoir was determined by the rate at which fluid left the column and this was adjusted to 10–20 ml./hr. Air entering the top reservoir was filtered through a sterilized cotton-wool plug. Mixing was achieved in the lower reservoir by means of a magnetic stirrer. In this way a gradually increasing concentration of NaCl was applied to the column (see Fig. 1).

The problem of collecting fractions under aseptic conditions was solved by using capped tubes that were pierced by a hypodermic needle attached to the fraction-collector arm. This arm was lifted by a solenoid when the turntable operated and returned by a spring to pierce the next tube. The effluent from the column was led through silicone-rubber tubing to the needle.

The fraction-collector tubes were capped with aluminium foil and sterilized by dry heat. A lint disk soaked in 0.5% chlorhexidine gluconate B.P. was sealed on top of the aluminium cap with Parafilm.

The renin substrate was eluted as a peak between 0.06M-NaCl and 0.1M-NaCl. The concentration of chloride in any tube is given by the following formula and hence the substrate peak can be located approximately:

$$M_3 = M_1(1 - e^{-x}) + M_2e^{-x}$$

$$\text{where } x = \frac{V_3 - V_0}{V_2}$$

where M_3 is the molarity of effluent at a volume ($V_3 - V_0$), M_1 is the molarity in the top reservoir, M_2 is the molarity in the lower reservoir, V_3 is the volume eluted in the tubes, V_2 is the volume in the mixing chamber, and V_0 is the volume of liquid in column.

Another useful indication of the position of the substrate peak is the presence of a slight pink colour at approx. 0.06M-NaCl.

Fig. 1. Separation of rabbit renin-substrate by gradient elution from DEAE-cellulose. Experimental details are given in the text. ●, Renin-substrate; ■, angiotensinase; □, protein; Δ, chloride.

It is possible to dispense with the gradient elution, washing the column first with 0.01M-tris-HCl buffer, pH 7.5, made 0.03M with respect to NaCl and then eluting the substrate with the same buffer made 0.09M with respect to NaCl. The greater convenience and simplicity more than compensates for the slight decrease in purification.

In the pooled substrate peak it was usual to find a trace of angiotensinase activity. This was removed by adding CdCl_2 to the solution to a final concentration of 1mM- Cd^{2+} and incubating at 37° at pH 7.5 for 2 hr.

A small volume of 0.01M-phosphate buffer, pH 7.5, was added to the solution to a final concentration of approx. 1mM. This reduced the Cd^{2+} concentration to that of the Cd^{2+} at the solubility product of cadmium phosphate.

The precipitate was removed by centrifugation at 20000g. The supernatant was filtered through a Millipore membrane filter (pore size 0.22 μ) mounted in the Swinny hypodermic adapter (V. A. Howe and Co. Ltd., London, W.11) into silicone-treated sterile glass ampoules and stored at 4°.

Renin-substrate prepared in this way has retained its capacity to generate angiotensin after storage for 3 months at 4° at pH 7.5 in sealed silicone-treated glass ampoules, and no vaso-active substances have appeared in the solution over this period of time. The substrate can be concentrated without loss of activity by ultrafiltration or by dialysis against distilled water and freeze-drying.

Estimation of renin-substrate. This was carried out by incubation with excess of renin at 37° in 0.1M-phosphate buffer, pH 6.0. The tubes were incubated for 20 min. and then boiled to stop the reaction. The angiotensin was assayed on the anaesthetized ganglion-blocked rat (Peart, 1955) with synthetic $\text{Val}_{(5)}$ angiotensin as a standard (Hypertensin; Ciba Ltd.), and expressing the result in terms of the amount of angiotensin produced. Synthetic angiotensin (0.5 mg.) was dissolved in 50 ml. of sterile 0.9% NaCl solution, filtered through a Millipore membrane filter into silicone-treated sterile glass ampoules. These were then sealed and stored at 4°. This solution of angiotensin has remained stable for 9 months.

Estimation of angiotensinase. This term is a generic one for the enzymes that destroy angiotensin. In this work the properties of these enzymes have not been studied in detail. Total angiotensinase activity was estimated by incubating 0.1 μ g. of synthetic $\text{Val}_{(5)}$ angiotensin (Hypertensin; Ciba Ltd.) with a sample of the material to be tested and determining its rate of destruction. At known times the residual angiotensin was assayed in the rat and the initial rate of destruction was calculated. The result was then expressed in terms of μ moles of angiotensin destroyed/min./mg. of protein. In Fig. 1 the angiotensinase activity has been plotted as $10^9 \times$ actual units.

Estimation of renin. In the absence of a universally agreed system for expressing the activity of a renin solution we have followed Pickering & Prinzmetal (1938) and compared the unknown solutions with a renin standard prepared from ethanol-dried rabbit kidney. A 10g. portion of kidney powder was extracted with 50 ml. of 0.9% NaCl solution and the extract filtered through Hyflo Super-Cel. The clear yellow filtrate was diluted to 250 ml. with more 0.9% NaCl and dispensed in 2.5 ml. portions that were stored at -20°. The solution was considered to contain 1 unit/ml. This unit has been used for the work on the location of renin in the kidney published from this Laboratory in the last few years. It cannot be assumed equal to any other renin unit.

As this relatively crude renin solution contained some angiotensinase it could not be used for the prolonged incubations but was used to standardize a more highly purified solution by direct pressor assay on the rat blood pressure. This partially purified renin solution was prepared in the following way.

A 50g. portion of ethanol-dried rabbit-kidney powder was extracted with 250 ml. of distilled water containing 0.01% of chlorhexidine gluconate B.P. The tissue was strained off and the filtrate acidified to pH 3.9 with acetic acid. The precipitate that formed was centrifuged down at 1500g. for 10 min. and the clear supernatant adjusted to pH 6.0 with NaOH. $(\text{NH}_4)_2\text{SO}_4$ was added to give 50% saturation and the solution left at 4° overnight. The precipitate was separated by centrifugation at 20000g for 15 min. and redissolved in a small volume of distilled water. This solution was dialysed against distilled water containing 0.01% of chlorhexidine gluconate. When free of sulphate, acetic acid was added to bring the pH to 3.5 and the solution was left to stand at room temperature (21°) for 45 min. The pH was adjusted to pH 6.0 with NaOH and NaCl was added to give a concentration of 0.9%. After centrifugation at 20000g for 15 min. the supernatant was filtered through a Millipore membrane (pore size 0.22 μ) directly into sterilized silicone-treated glass ampoules that were immediately sealed and stored at 4°. No angiotensinase could be detected in the solution: 0.1 μ g. of angiotensin was recovered intact after incubation with 0.2 ml. of the renin (8 units) for 264 hr. under aseptic conditions in phosphate buffer, pH 6.0, at 37° in a sealed silicone-treated glass tube. This solution has shown no decrease in activity after storage for 12 months at 4°.

When incubated at 37° with an excess of rabbit renin-substrate in 0.1M-phosphate buffer, pH 6.0, 1 unit of renin was found to produce angiotensin at the initial rate of 1800 m μ g./hr.

Protein estimation. Protein was estimated by a colorimetric method (Lowry, Rosebrough, Farr & Randall, 1951).

Chloride estimation. Chloride was estimated by potentiometric titration (Sanderson, 1952).

RESULTS

A typical preparation is illustrated in Scheme 1. The total recovery was 48% of the substrate present in the serum and the overall increase in concentration of substrate relative to that of protein was 11.6. The angiotensinase content was reduced to undetectable levels.

Table 1 shows the changes in activity of the renin substrate and angiotensinase at various stages of the method.

Prolonged incubation of substrate with renin. To determine the smallest amount of renin that can be assayed, we have incubated in sterile silicone-treated glass tubes for as long as 264 hr. and found that the rate of angiotensin formation remained constant over this period of time provided that the substrate was in excess. Fig. 2 is the blood-pressure record from the assay rat in such an experiment. Injections (*D* and *E*) show that the substrate and renin preparations when incubated alone for 264 hr.

Table 1. *Purification of rabbit renin-substrate*

Experimental details are given in the text.

Material	Angiotensin (μ g./mg. of protein)	$10^9 \times$ Angiotensinase activity (μ moles/mg. of protein)	$10^3 \times$ Substrate/angiotensinase ratio
Serum	0.05	1750	0.286
Material after dialysis	0.084	1480	0.568
Substrate peak from DEAE-cellulose	0.582	2.0	291
After CdCl_2 treatment	0.582	0.56*	1040

* In most substrate preparations there was no angiotensinase activity detectable after 200 hr. incubation with angiotensin.

Fig. 2. Detection of minute amounts of renin by incubation with renin-substrate. The blood-pressure record from the common carotid artery is shown. Injections: A, 0.1 ml. dose (0.1 unit of renin); B, 0.1 ml. dose (0.02 unit of renin); C, 0.2 ml. dose (0.00005 unit of renin) incubated with substrate for 264 hr. at 37°; D, 0.2 ml. dose (substrate preparation incubated alone for the same length of time); E, 0.2 ml. dose (0.00005 unit of renin) incubated alone for 264 hr. at 37°; F, 0.05 ml. dose of synthetic Val₆ angiotensin (5 μ g.); G, 0.05 ml. dose (substrate incubated with 100 μ g. of angiotensin/ml.); H, 0.05 ml. dose (renin incubated with 100 μ g. of angiotensin/ml.); F, repeat; D, repeat; C, repeat; A, repeat.

Fig. 3. Prolonged incubation of renin with substrate. The initial rates of angiotensin formation by 0.0001 unit (○) and 0.00005 unit (●) of renin when incubated with excess of renin-substrate in 0.1 M-phosphate buffer, pH 6.0, at 37° in sealed sterile silicone-treated glass tubes are shown. The horizontal bars represent doses of synthetic angiotensin that are just larger and just smaller than the dose of unknown solution, each bar being defined by a minimum of two responses.

25 μ g. of angiotensin (injections C, and Fig. 3). A similar tube incubated for 145 hr. produced 15 μ g. of angiotensin (Fig. 3). This rate of formation of angiotensin, about 0.1 μ g./hr., was produced by 0.00005 unit of renin and is probably the lowest that can be measured.

At these very low concentrations renin solutions rapidly lose activity but may be stabilized by diluting with buffer containing bovine albumin (1 mg./ml.).

DISCUSSION

Under conditions of substrate excess, when zero-order kinetics apply, the rate of formation of angiotensin is proportional to the amount of renin present. In practice, the limit to the smallest quantity of renin that can be measured is set by the time required to produce an assayable amount of angiotensin. A rate of formation of angiotensin about 0.1 μ g./hr. is probably the lowest rate that can be measured, so that 0.00005 unit of renin is the lower limit of the assay method. This represents a several thousand-fold increase in sensitivity over the direct injection of renin into the test animal.

The key to the assay of such minute amounts of renin is the complete removal of angiotensinase without producing non-specific vaso-active substances. Although chromatography on DEAE-cellulose removes the major part of the angiotensinase activity (Fig. 1 and Table 1), the small amount that remains is sufficient to interfere during

under aseptic conditions in phosphate buffer, pH 6.0, in sealed tubes, produced no vaso-active substances, but when incubated together produced

prolonged incubations. This residual angiotensinase activity we found could be entirely removed by adding cadmium chloride (final concentration 1 mM) and incubating at 37° for 2 hr. Renin substrate is unaffected by such treatment but renin is inactivated by this concentration of Cd^{2+} in chloride solutions. In the presence of phosphate, however, the Cd^{2+} concentration is reduced to a level at which there is no denaturation of renin even on prolonged incubation (W. F. Cook & M. R. Lee, unpublished work).

Removal of endogenous renin was not a problem in this study because the serum used in the preparation was derived from nephrectomized animals. Nephrectomy also increases the concentration of renin substrate in the blood. Normal serum, however, could be used because renin elutes from DEAE-cellulose at a higher chloride concentration than renin substrate, and any trace of renin still

contaminating the substrate would be inactivated by the cadmium chloride treatment.

We are grateful to Professor G. W. Pickering for his advice and encouragement, and to Mr P. R. Williams for technical assistance. Mr Graham Smith derived the equation for calculating the chloride molarity. M. R. L. is the holder of a Beit Memorial Fellowship.

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