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# The Estimation of Renin in Human Plasma

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A method was previously developed in this Laboratory for estimating the enzyme renin in rabbit plasma (Lever, Robertson & Tree, 1963*a*, *b*,*c*, 1964). The present paper describes the adaptation of the method to the measurement of renin in the plasma of man. Preliminary accounts of this work have been published (Brown, Davies, Lever & Robertson, 1963*a*, *b*, 1964*a*).

## METHODS

The technique consists of an estimation of renin concentration by determining the initial velocity of angiotensin formation under standard conditions of incubation with substrate. Angiotensinase-free ox-serum substrate, as prepared for the estimation of rabbit renin (Lever *et al.* 1964), was used throughout the present studies. The technique of incubation and assay, and the tests for contamination with angiotensinase and endogenous substrate, were also as described by Lever *et al.* (1964). The methods of preparation of standard human renin, and of extracting renin from human plasma, however, differed from those used with the rabbit.

*Preparation of standard human renin.* Human kidneys (4·5 kg.) were obtained *post mortem*, and excess of fat and fibrous tissue was removed. The kidneys were then minced and allowed to stand in 10 l. of water at 8° for 36 hr. The mince was filtered through muslin, and 10% (w/v) trichloroacetic acid was added to the filtrate at 8°, with constant stirring, to give pH 2·9. Then NaCl (52 g. to each litre) was added slowly with stirring; the pH was checked after 15 min., and if necessary readjusted to pH 2·9 by adding 10% trichloroacetic acid or N-NaOH. The solution was then filtered at 8° overnight through Whatman no. 50 paper and the filtrate adjusted to pH 5·0 with N-NaOH. Then 2 l. batches of this solution were dialysed in Visking cellophan sacs (28/32 in.) against three 15 l. changes of water at 8° over 48 hr.

The method of renin preparation used to this stage was basically that described by Dexter, Haynes & Bridges (1945). It was found, however, that this renin solution had considerable angiotensinase activity, and therefore further purification was carried out as follows. These later stages were based on results obtained by W. S. Peart, N. N. Mendelsson & N. E. Stone (see Peart, 1959) and W. S. Peart, A. F. Lever, M. W. Lloyd, N. N. Payne, N. E. Stone & A. Taylor (unpublished work) during the purification of pig renin.

The 2 l. batches of renin solution were dialysed in Visking cellophan sacs (28/32 in.) against three 15 l. changes of 5 mM-sodium phosphate buffer, pH 7·0 (0·0240% $NaH_2PO_4$; 0·1074% $Na_2HPO_4,2H_2O$), over 48 hr. at 8°. This solution was then applied at room temperature to a column (65 cm. × 2·5 cm.; dry wt. 100 g.) of lightly-packed DEAE-cellulose equilibrated with the 5 mM-sodium phosphate buffer, pH 7·0. Renin was adsorbed during this application. The column was washed with 3 l. of 0·03 M-sodium phosphate buffer, pH 7·0 (0·140% $NaH_2PO_4$; 0·653% $Na_2HPO_4,12H_2O$), and the eluted protein discarded. Renin was then eluted with 600 ml. of 0·35 M-phosphate–saline buffer, pH 6·0 (0·05 M-$Na_2HPO_4$; 0·3 M-NaCl, adjusted to pH 6·0 with 6 N-HCl), containing neomycin sulphate (0·01%).

The eluate was dialysed against three 10 l. changes of glycine–HCl–saline buffer, pH 3·0 (0·1 M-glycine; 0·9 M-NaCl; 0·018 N-HCl), at room temperature over 24 hr. The precipitate that then formed was removed by filtration

through Whatman no. 1 paper, and the filtrate dialysed against three 10 l. changes of 0·15M-phosphate–saline buffer, pH 5·7 (0·555% $NaH_2PO_4$; 0·179% $Na_2HPO_4$,-$12H_2O$; 0·585% NaCl), at 8° over 36 hr.

*Extraction of renin from plasma.* Blood samples were taken, usually from an arm vein by syringe, into heparin (25 units/ml.). Plasma was separated by centrifugation, and stored at −20°.

It was convenient to extract 40 plasma samples simultaneously. The buffer volumes given in this paper and by Lever *et al.* (1964) are sufficient for dealing with 40 plasma samples (25 ml.) at a time.

Renin was adsorbed from plasma on DEAE-cellulose, eluted, acidified to remove remaining traces of human substrate and angiotensinase, and then freeze-dried. The procedure followed that described for use with rabbit plasma (Lever *et al.* 1964) except for the following details. With human plasma, the eluate from the DEAE-cellulose column was dialysed in Visking cellophan sacs (18/32 in.) against three 10 l. changes of glycine–HCl–saline buffer, pH 3·0 (0·1M-glycine; 0·9M-NaCl; 0·018N-HCl), at room temperature over 24 hr. The dialysed eluate was centrifuged, the supernatant was filtered through Whatman no. 1 paper, and further dialysed, first against one 10 l. batch of 0·15M-phosphate–saline buffer, pH 5·7 (0·555% $NaH_2PO_4$; 0·179% $Na_2HPO_4,12H_2O$; 0·585% NaCl), and then against eight 10 l. changes of water at 8° over 96 hr. This material was then filtered and freeze-dried as described by Lever *et al.* (1964).

*Relation of the initial velocity of angiotensin formation to renin concentration.* The initial velocity of angiotensin formation was determined for a range of dilutions of the standard human renin incubated with the prepared ox-serum substrate. From these data the calibration curve (Fig. 1) was prepared. The incubation times varied from 30 min. for those samples with the greatest renin concentration to 96 hr. for those with the least.

*Angiotensinases.* The following angiotensinases were incubated with various angiotensins at 37° in 0·15M-phosphate–saline buffer, pH 7·5 (0·072% $NaH_2PO_4$; 1·58% $Na_2HPO_4,12H_2O$; 0·585% NaCl); α-chymotrypsin (Worthington Biochemical Corp.); carboxypeptidase A (L. Light and Co. Ltd.); leucine aminopeptidase (prepared from pig kidney by the method of Hill, Spackman, Brown & Smith, 1958) and trypsin (Hopkin and Williams Ltd.).

## RESULTS

*Properties of the standard human renin.* The human renin unit was arbitrarily defined in terms similar to those used for rabbit renin (Lever *et al.* 1964). One unit was taken as the quantity of renin that, when incubated with prepared ox-serum substrate at pH 5·7 and 37°, in a substrate concentration of 30 units/ml. and a total volume of 5 ml., formed 0·1 μg. of angiotensin/ml. of incubation mixture/hr.

The standard renin had a specific activity of 6 units/mg. of protein (the latter being measured by the method of Lowry, Rosebrough, Farr & Randall, 1951). A solution of this renin containing 1·5 units/ml. was free from angiotensinase and substrate by the criteria applied by Lever *et al.* (1964) to rabbit renin.

*Shape of calibration curve.* The relation between renin concentration and initial velocity of angiotensin formation was rectilinear for incubation mixtures with relatively high renin concentrations (Fig. 1). With lower renin concentrations, where incubation for longer than 24 hr. was necessary, the line became curved (Fig. 1) for the following reason. When the prepared ox-serum substrate was incubated along at 37° up to 24 hr., no detectable pressor material was formed (Lever *et al.* 1964). During more prolonged incubation, however, angiotensin-like pressor activity appeared, equivalent to a concentration of 0·015 μg./ml. after 96 hr. incubation. This effect might have been due either to non-enzymic splitting of the substrate, or to traces of ox renin remaining after the preparation of the substrate. The result was to supplement slightly the pressor effect of angiotensin formed by low concentrations of added renin. In the calibration graph published for rabbit renin (Lever *et al.* 1964), no results were included for very low renin concentrations requiring more than 24 hr. incubation for detection. Consequently, the graph was rectilinear. For the measurement of renin in single rabbit glomeruli (Brown, Davies, Lever, Parker & Robertson, 1963*d*), it was necessary to increase the sensitivity of the method by prolonging incubation up to 96 hr. The extended calibration curve for rabbit renin consequently was similar in shape to that shown for human renin in the present work.

*Sensitivity limit.* The renin extracted from human peripheral plasma usually gave an initial reaction

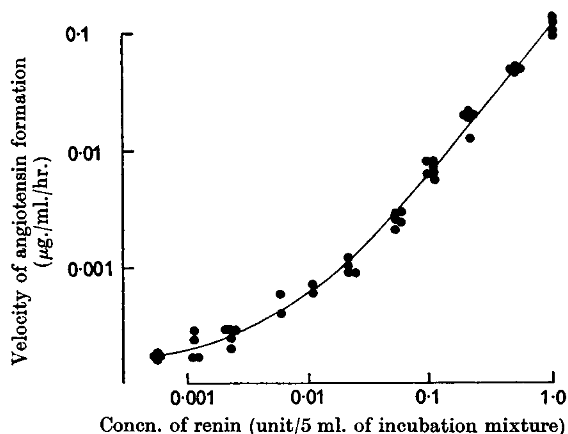


Fig. 1. Calibration curve showing relationship between the initial velocity of angiotensin formation and the concentration of renin in the incubation mixture. The lowest group of four points was obtained with no added renin. Human renin was incubated with ox-serum substrate. Experimental details are given in the text.

velocity that was measurable after less than 24 hr. incubation (Fig. 2). The relevant part of the calibration graph was therefore straight, and the renin concentrations were well within the sensitivity range of the method. For the measurement of lower concentrations of renin, more prolonged incubation was necessary, requiring use of the curved part of the calibration graph. A sensitivity limit was eventually reached when it was no longer possible to distinguish the velocity of angiotensin formation due to added renin from the rate at which pressor material appeared in substrate incubated alone. The standard deviation of 41 measurements (Fig. 1) of the velocity of angiotensin formation when known concentrations of renin were incubated with substrate was 8 %. The sensitivity limit of the incubation technique was therefore taken as the concentration of added renin giving a velocity two standard deviations (16 % velocity) above the rate of formation of pressor material when substrate was incubated alone. This limit represented 0·001 unit of renin/5 ml. of incubation mixture. By taking into account replicate variation between plasma extracts, the effective sensitivity limit with a starting plasma volume of 25 ml. was approx. 0·2 unit/l. of plasma.

*Reaction velocity.* Fig. 2 shows incubation progress graphs obtained with extracts from 25 ml. plasma samples from a normal female, and from subjects with two conditions associated with increased plasma renin concentrations. This Figure emphasizes the wide variation in sampling times necessary for accurate plotting of the reaction velocity.

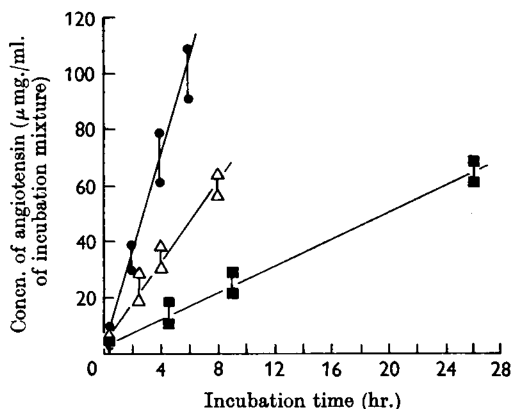


Fig. 2. Typical incubation progress curves of the reaction of human-plasma extract with prepared ox-serum substrate. Extracts of comparable plasma volumes from a normal female (■), a salt-depleted subject (△), and a patient with hepatic cirrhosis and ascites (●) were used. Experimental details are given in the text. Assay bracket limits are shown.

It can also be seen that the velocity line drawn for the sodium-depleted subject passes through the lower part of the early assay brackets. This effect has been seen in a number of incubation progress graphs, obtained with plasma extracts of normal and pathological subjects, and with added renin. Thus, although within the limitations of the assay method used the reaction velocity is rectilinear so long as the substrate concentration remains above 20 units/ml. (Lever *et al.* 1964), there is a possibility that the reaction velocity may initially be slightly faster. Several factors could contribute to this phenomenon. The effective range of 'excess' of substrate concentration (Lever *et al.* 1964) is below that giving theoretical maximum velocity as determined by extrapolation of the $1/v$ axis of a reciprocal plot (Fig. 3). The initial substrate concentration used experimentally might therefore lead to progressive slowing of the reaction as substrate is consumed. Any such retardation is slight while the substrate concentration remains above 20 units/ml., but becomes marked below this value (Lever *et al.* 1964). An alternative explanation derives from the work of Skeggs, Lentz, Hochstrasser & Kahn (1963, 1964), who prepared five chemically distinct substrates from pig blood, and obtained evidence suggesting that these may give slightly different reaction velocities. If a given batch contained several such substrates, the reaction velocity might well deviate from rectilinear before slowing from substrate exhaustion. Since the proportion of the different substrates could also vary between batches, the need for careful recalibration when changing batches is emphasized.

Throughout the present work we have taken straight lines drawn as shown (Fig. 2) as a measure of the initial velocity.

*Elimination of endogenous substrate and angiotensinase from human-plasma extracts.* The present technique removed angiotensinase and endogenous substrate from 800 consecutive human-plasma extracts, tested by the methods used by Lever *et al.* (1964).

*Recovery of added renin.* At least one plasma sample containing a known quantity of renin was always included in each group of 40. The mean recovery of human renin added to plasma was 37 % (S.D. 6·7 %; 45 experiments). This was close to the value obtained by Lever *et al.* (1964) for renin recovery from rabbit plasma. For convenience, results (Brown, Davies, Lever & Robertson, 1963*a*, *b*, *c*, 1964*a*, *b*; Brown, Davies, Doak, Lever & Robertson, 1963*e*) have been expressed on the basis of an assumed 50 % recovery. Though the starting plasma volume was usually 25 ml., the same proportionate recovery was obtained also with 15, 10 or 5 ml. of plasma, by using columns of the dimensions given by Lever *et al.* (1964). With

50 ml. of plasma, the proportionate renin recovery was slightly less, and with 100 ml. markedly decreased. Recoveries were independent of plasma renin concentration in the range found clinically and experimentally (Brown *et al.* 1964*a*), and were similar in plasma from normal males and from pregnant and non-pregnant females. Recovery was also similar whether the added renin was extracted from human plasma or prepared from kidneys. Portions of 23 plasma samples estimated on different occasions gave results that varied with a standard deviation of 18 % about the mean of the replicates for each sample.

No systematic change in extractable renin was found in plasma on storage for up to 9 months at −20°.

*Effect of prolonged contact between plasma and blood cells* in vitro. Heparin-treated blood was allowed to stand in polyethylene containers at 4° and 20° before separation of the plasma. Compared with samples in which the plasma had been separated immediately, no detectable loss of renin occurred up to 17 hr. Thus, although plasma was always separated as soon as possible, this was not critical.

*Characterization of the enzyme extracted from plasma.* The enzyme extracted from human plasma shared several physicochemical characteristics with renin prepared from human kidneys. Both were similarly heat-labile, and did not diffuse through cellophan. Both were similarly acid-resistant, and both were precipitated from solution at the same pH and molarity. Both were adsorbed on and eluted from DEAE-cellulose in the same ranges of buffer molarity and pH. Direct pharmacological comparison between the concentrated plasma extract and human renin, such as was made between rabbit-plasma extract and rabbit renin (Lever *et al.* 1964), could not be done, since intravenous injections of human renin are usually without effect on rat blood pressure (Shipley & Helmer, 1948).

*Characterization of the reaction.* Incubation of human renin and human-plasma extract with serial dilutions of the prepared substrate at 37° and pH 5·7 gave results (Fig. 3) very similar to those published by Lever *et al.* (1964) for rabbit renin and rabbit-plasma extract. Both human renin and human-plasma extract were active at 37° over a wide pH range (Fig. 4). Though there was a general similarity between the results in the two species, the reaction velocity above pH 6·4 was comparatively faster in man than in the rabbit (Lever *et al.* 1964). Neither angiotensin inactivation nor non-enzymic formation of pressor materials occurred at any of the pH values tested. Though relevant to the identification of the enzyme extracted from plasma, these experiments, performed with only partially purified renin and a hèterologous substrate, cannot establish either the Michaelis constant or the optimum pH range of pure renin.

At pH 5·7 the reaction velocities with the prepared substrate of both human renin and the human-plasma extract were similarly influenced by the incubation temperature (Fig. 5).

*Characterization of the reaction product.* On incubation with ox-serum substrate, both prepared human renin and the plasma extract gave reaction products that shared several characteristics with the synthetic $Asp_{(1)}$-$\beta$-amide $Val_{(5)}$ octapeptide angiotensin. All three were similarly heat-stable, diffusible and soluble in ethanol. A more extensive comparison was made between the two incubation products and pure rabbit renin–ox-serum substrate decapeptide angiotensin (Peart, 1956), synthetic

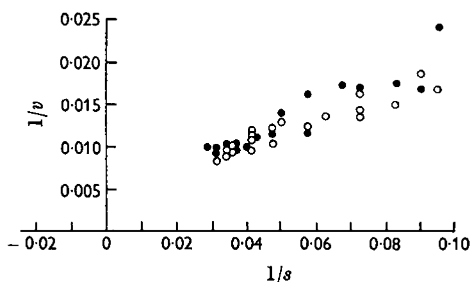


Fig. 3. Effect of substrate concentration on the initial velocity of reaction. Ox-serum substrate was incubated with known human renin (●) and with human-plasma extract (○) at 37° and pH 5·7. Experimental details are given in the text. *s*, Substrate concentration in units/ml.; *v*, % of reaction velocity at 28 substrate units/ml. The results were obtained with four concentrations of human renin and four separate plasma extracts.

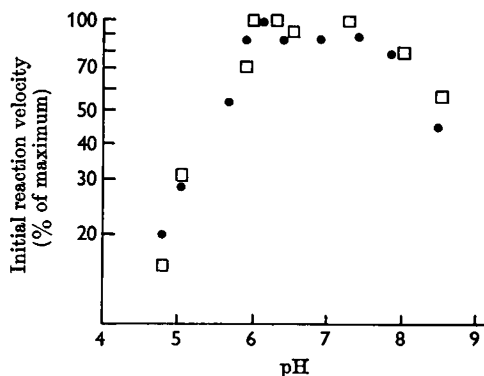


Fig. 4. Effect of pH on the initial velocity of reaction. Ox-serum substrate was incubated at 37° with human renin (□) and human-plasma extract (●). Experimental details are given in the text.

decapeptide angiotensin (80 % of $Asp_{(1)}Val_{(5)}$ decapeptide plus 20 % of $Asp_{(1)}$-β-amide $Val_{(5)}$ decapeptide), a partially purified angiotensin prepared by the action of human renin on human plasma, and the synthetic $Asp_{(1)}$-β-amide $Val_{(5)}$ octapeptide. The pressor activity of all these substances was rapidly destroyed by trypsin, α-chymotrypsin, carboxypeptidase and leucine amino-peptidase.

All six angiotensin solutions showed a constant pharmacological interrelationship when tested on four rats at different doses (Fig. 6). The constancy of the dose–response relationship between the incubation product of human renin, human-plasma extract and synthetic octapeptide was further tested in the course of this work. Each reaction-velocity curve consisted of at least three bracket assays of the incubation product against synthetic octapeptide angiotensin, usually made by a different worker at a different time on a different rat. No anomalous velocity curves were encountered among several hundred made in this way.

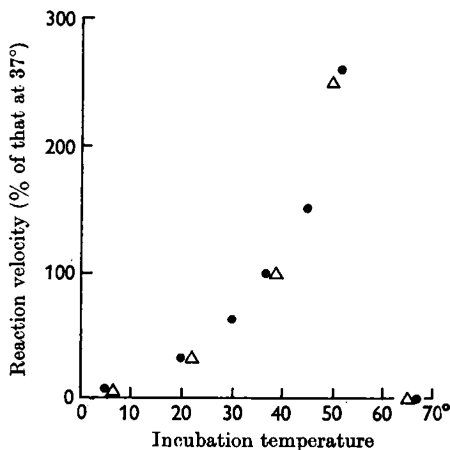


Fig. 5. Effect of incubation temperature on the reaction velocity. Prepared ox-serum substrate was incubated with known human renin (●) and human-plasma extract (△) at pH 5·7. Experimental details are given in the text.

The product of incubation of human-plasma extract with the prepared substrate often gave a pressor response very like that of the synthetic octapeptide angiotensin (Fig. 7). In some rats, however, the effect of the octapeptide was slightly shorter than that of the incubation product (Fig. 8) or the pure decapeptide (Fig. 9). As we have shown, this appearance did not affect quantitative assaying based on the height of the pressor response.

*Renin in the peripheral plasma of normal subjects.* The renin concentrations found in peripheral venous plasma of 68 normal males and 57 normal females aged between 17 and 78 ranged from 2 to 18 units/l. (mean 8·2, S.D. 2·7 units/l.; Brown *et al.* 1964*a*). The females included both post-menopausal subjects and non-pregnant women of reproductive age. Higher renin concentrations occur in normal pregnancy (Brown *et al.* 1963*e*). The sensitivity limit of the method is approx. 10 % of the lowest value found so far in a normal subject.

*Plasma renin in abnormal states.* Elevated plasma renin concentrations have been found in salt-deprived normal subjects, in untreated Addison's disease, in hepatic cirrhosis with ascites and

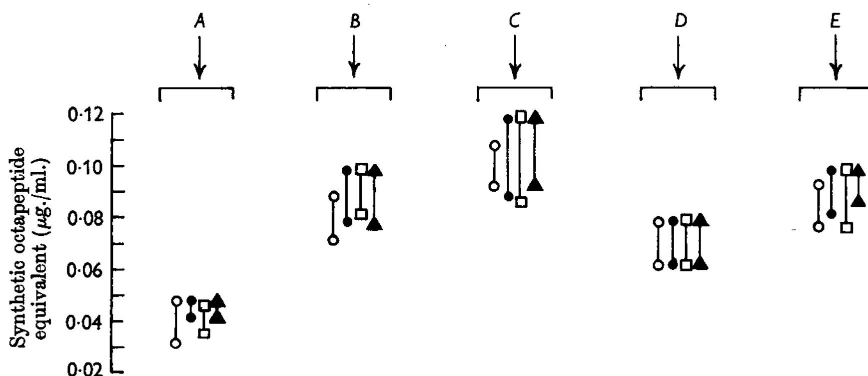


Fig. 6. Assay of five different angiotensin solutions (*A*, synthetic decapeptide; *B*, rabbit renin–ox-serum substrate decapeptide; *C*, human renin–ox-serum substrate angiotensin; *D*, human-plasma extract–ox-serum substrate product; *E*, human renin–human-plasma substrate angiotensin) of unknown concentration against the synthetic $Asp_{(1)}$-β-amide $Val_{(5)}$ octapeptide. Experimental details are given in the text. Quadruplicate assays were carried out by four separate workers (○, ●, □ and ▲), each using a different rat and each giving a different dose of the unknown. Assay bracket limits are shown.

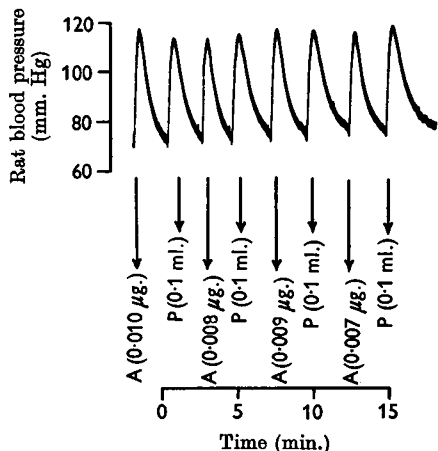


Fig. 7. Typical bracket assay, on the rat-blood-pressure preparation, of the product of incubation of human-plasma extract with ox-serum substrate (P) against synthetic octapeptide angiotensin (A). Experimental details are given in the text. The 0·1 ml. sample of P contains more than 0·008 μg. and less than 0·009 μg. of angiotensin.

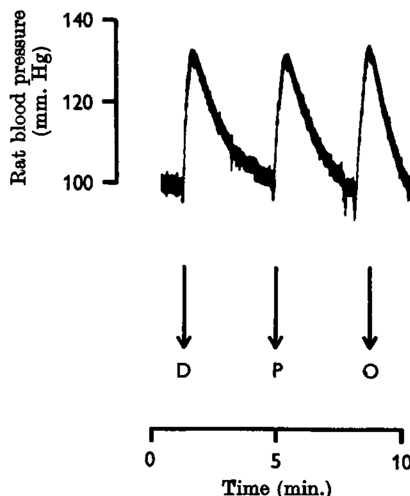


Fig. 9. Comparison, on the same assay rat as in Fig. 8, of similar doses of rabbit renin–ox-serum substrate decapeptide (D), human-plasma extract–ox-serum substrate incubation product (P) and synthetic octapeptide (O).

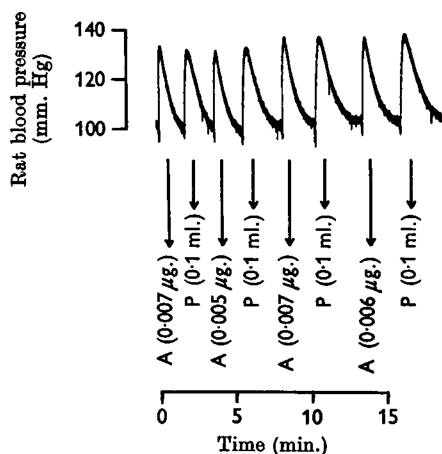


Fig. 8. Bracket assay, on rat-blood-pressure preparation, of the product of incubation of human-plasma extract with ox-serum substrate (P) against synthetic octapeptide angiotensin (A). Experimental details are given in the text. This preparation shows the more prolonged effect sometimes given by the incubation product.

in some cases of renal hypertension. Abnormally low plasma renin concentrations have been seen in salt-loaded volunteers and in patients with hyperaldosteronism due apparently to autonomous adrenal-cortical overactivity (Brown *et al.* 1963*a*, *b*, *c*, 1964*a*, *b*). Of the first 500 abnormal plasma samples examined, the lowest renin concentration was 0·6 unit/l., well above the sensitivity limit of approx. 0·2 unit/l. The method has no upper limit of sensitivity, since plasma extracts can be diluted if necessary before incubation. The highest plasma renin concentration found so far is 6300 units/l.

*Effect of* 500 *ml. bleed on plasma renin.* Blood (500 ml.) was removed over 3–5 min. from an arm vein of each of seven healthy young adult males. Further 50 ml. blood samples were taken after 3, 7 and 13 min. in four subjects, and after 20, 40 and 60 min. in the remaining three. The subjects were recumbent throughout. No changes in arterial pressure were found at any stage. No significant changes in plasma renin concentration occurred at these intervals after the haemorrhage.

## DISCUSSION

Several structurally different angiotensins have been prepared from naturally occurring materials. The decapeptide angiotensins formed respectively by the action of pig renin on horse plasma (Skeggs, Marsh, Kahn & Shumway, 1955) and of rabbit renin on ox serum (Peart, 1955, 1956) were shown to be identical in amino acid sequence, apart from isoleucine in position 5 in the pig renin–horse-plasma substrate angiotensin, instead of the valine of the rabbit renin-ox-serum substrate decapeptide (Elliott & Peart, 1956, 1957; Lentz, Skeggs, Woods, Kahn & Shumway, 1956; Skeggs, Lentz, Kahn, Shumway & Woods, 1956*b*). Skeggs, Kahn & Shumway (1956*a*) demonstrated that the pig renin–horse-plasma substrate decapeptide was without vasoconstrictor effect on isolated perfused

rat kidney, and that conversion into a vasoactive octapeptide was caused by an enzyme present in plasma.

The structure of the angiotensin formed by the action of human renin on ox serum is at present unknown. In these studies, therefore, we had either to assay the incubation product against a standard solution of angiotensin prepared from human renin and ox-serum substrate, or to show that the incubation product could be assayed accurately against a more readily available pure angiotensin. Skeggs *et al.* (1956*a*) found that pig renin–horse-plasma substrate decapeptide and octapeptide angiotensins gave identical blood-pressure responses on intravenous injection into the intact rat. However, we have seen that in some animals slightly more prolonged pressor effects are given by both the incubation product and by a pure decapeptide angiotensin than by the synthetic octapeptide (Figs. 8 and 9). Despite this observation, quantitative assays of the incubation product against pure octapeptide, pure decapeptide and standard human renin–ox-serum substrate angiotensins are, as we have shown, consistently valid.

We have therefore used the commercially available synthetic octapeptide as a routine. This means that, although renin concentrations have been expressed in arbitrary units, these are ultimately referable to absolute quantities of a freely available synthetic angiotensin. Comparison between the results of different groups of workers should in this way be facilitated though the possibility that different substrate batches with the same substrate concentration may show different characteristics must again be stressed. A more immediate advantage in the present work has been the ability to avoid reliance solely on a stock solution of standard renin. This has been particularly valuable, since we have found that some solutions of human renin tend to lose activity slowly if repeatedly thawed and sampled.

## SUMMARY

1. A method is described for the extraction and estimation of renin from human plasma.

2. Criteria of identity of the extracted enzyme, the enzyme–substrate reaction and the reaction product are given.

3. Recoveries are consistent, and in the range found with a similar procedure for rabbit plasma.

4. The method is sufficiently sensitive to measure peripheral-venous-plasma renin in both normal physiological circumstances and in conditions associated with depression of plasma renin concentration.

We are grateful to Professor W. S. Peart for continued encouragement and advice, to Professor R. R. Porter for providing the leucine aminopeptidase, and to Mr A. B. Tattersall of Ciba Ltd. for supplies of synthetic angiotensin. Technical assistance was provided by Miss C. Evans, Miss A. Leigh, Mr I. MacNamara, Mr J. Van der Puije and Mr A. Wiseman. A. F. L. and D. L. D. held Wellcome Research Fellowships during this study.

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