A Radiochemical Renin Assay

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1. A synthetic 3-([¹⁴C]valine)-labelled tetradecapeptide renin substrate was used to measure renin concentration. Renin liberated ¹⁴C-labelled angiotensin I, which was separated from the labelled substrate by paper chromatography. The conversion of substrate into angiotensin I was quantitated by liquid-scintillation counting of radioactivity. 2. The rate of conversion of the substrate into angiotensin I was shown to be linearly related to renin concentration and time under suitable conditions. Angiotensin generation measured in this system agrees well with that measured by bioassay. 3. It is suggested that the use of a pure substrate offers advantages that include the standardization of current units of renin measurement.

There are many different published renin assays (Page & McCubbin, 1968). Most differ widely in the nature and purity of the renin substrate employed. This has led to the expression of assay results in a variety of conflicting units (Haas, Gould & Goldblatt, 1968), none of which have any validity in absolute terms.

Skeggs and co-workers (Skeggs, Kahn, Lentz & Shumway, 1957) isolated a tetradecapeptide from the tryptic digest of hog renin substrate, which was shown to be an adequate renin substrate (Skeggs, Lentz, Kahn & Shumway, 1958; Montague, Riniker & Gross, 1966b). This tetradecapeptide was subsequently synthesized (Skeggs *et al.* 1958). Use of a pure synthetic substrate offers one approach towards standardization of renin assays. It has the added advantage that measurement of angiotensin liberated may be measured by radiochromatographic methods instead of bioassay if a ¹⁴C-labelled substrate is employed. This paper describes the development of a radiochemical renin assay using 3-([¹⁴C]valine)-labelled tetradecapeptide renin substrate.

EXPERIMENTAL AND RESULTS

The 3-([¹⁴C]valine)-tetradecapeptide (¹⁴C-labelled tetradecapeptide) was obtained from Schwartz BioResearch Inc., Orangeburg, N.Y., U.S.A. On paper electrophoresis at 17.5 V/cm for 2 h in 0.6 M-formic acid-2 M-acetic acid (1:1, v/v), pH 1.9, it produced a single peak with R_F 0.53 ($\text{Arg} = 1.00$) and a single peak with R_F 0.34 on descending chromatography with Whatman no. 1 paper in butanol-acetic acid-water (18:2:5, by vol.). Both R_F values were identical with that of Asn¹-Val⁵-tetradecapeptide as synthesized by Riniker (Montague, Riniker, Brunner & Gross, 1966a). The stated specific radioactivity of the

¹⁴C-labelled tetradecapeptide was 40 $\mu\text{Ci}/\mu\text{mol}$ (or 18 $\mu\text{Ci}/\text{mg}$). When bioassayed in the rat by using tetradecapeptide (Ciba Ltd., Basle, Switzerland) as standard and the radioactivity was measured in a liquid-scintillation counter (Nuclear-Chicago) against two internal ¹⁴C standards, the specific radioactivity was found to be 24 $\mu\text{Ci}/\mu\text{mol}$. When the peptide concentration was determined by a micro modification of a quantitative Pauly reaction (Sluyterman, 1960) the specific radioactivity was calculated to be 23 $\mu\text{Ci}/\mu\text{mol}$.

Purified hog renin was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. and the units referred to in this paper are derived from the stated specific activity of that compound (5 units/mg). The incubation buffer used in these experiments was 0.05 M-sodium phosphate-0.9% sodium chloride, pH 7.5, containing 0.01 M-EDTA (sodium salt) and 0.1% neomycin sulphate (Sigma Chemical Co., St Louis, Mo., U.S.A.) (phosphate-saline buffer). Human plasma was treated by the method described by Skinner (1967) for plasma renin concentration by dialysis for 18 h at 4°C against 0.05 M-glycine-0.01 M-hydrochloric acid buffer, pH 3.3, containing 5 mM-EDTA (sodium salt), incubation at 32°C for 1 h followed by dialysis for 18 h at 4°C against 0.1 M-sodium phosphate-0.075 M-sodium chloride buffer, pH 7.5, containing 1 mM-EDTA.

Incubation of the ¹⁴C-labelled tetradecapeptide with an excess of renin [0.9 Goldblatt unit/ml (Nutritional Biochemicals Corp.)] at 37°C, pH 7.5, for 4 h resulted in a ten-fold increase in pressor activity in a rat bioassay performed by the method of Peart (1955). Further incubation for 25 h gave no further increase in pressor activity. This suggested complete conversion of the substrate into angiotensin I after 4 h incubation, since Montague *et al.* (1966b) had reported a pressor/activity ratio of 10:1 for angiotensin I:tetradecapeptide and we have confirmed this ratio by using unlabelled tetradecapeptide (Ciba Ltd.).

The pressor-active end product of the incubation of ¹⁴C-labelled tetradecapeptide with renin was shown to be

Fig. 1. Paper chromatogram showing the mobility and separation of tetradecapeptide (TDP), angiotensin I (A1), tetrapeptide (TP) and angiotensin II (A2) in 0.05M-sodium phosphate buffer, pH 6.5, on Whatman no. 1 paper, stained with Pauly's reagent (Whitaker, 1967).

Fig. 2. Radioactivity on chromatogram of ^{14}C -labelled tetradecapeptide (▲) alone or (●) incubated with 0.9 Goldblatt unit of renin/ml for 4 h at 37°C . The labelled and unlabelled (Ciba) tetradecapeptides (TDP) remain at the origin, whereas after incubation with renin the radioactive peak coincides with angiotensin I (A1). Solvent system: 0.05M-sodium phosphate buffer, pH 6.5.

Fig. 3. Radioactivity in the angiotensin I spot (as percentage of total radioactivity applied) versus renin concentration for a 4 h incubation of ^{14}C -labelled tetradecapeptide at 37°C in 0.05M-sodium phosphate-0.9% NaCl buffer, pH 7.5, containing 0.01M-EDTA and 1% neomycin. ●, Results of multiple samplings of one incubation experiment; ○, duplicate samples of a separate identical experiment. The conversion of labelled tetradecapeptide into ^{14}C -labelled angiotensin I increases linearly with renin concentration.

similar to angiotensin I biologically, immunologically and chromatographically. It had an identical dose-response curve to angiotensin I in the rat bioassay system. It displaced ^{125}I -labelled angiotensin in a specific immunoassay system for angiotensin I (Johnston, Mendelsohn & Casley, 1969) and had a mobility identical with that of angiotensin I on paper chromatography (Whatman no. 1; butanol-acetic acid-water, 18:2:5, by vol.) and paper electrophoresis (0.6M-formic acid-2M-acetic acid, pH 1.9, on Whatman no. 1 paper).

In preliminary experiments on chromatographic-separation systems with a wide variety of organic-solvent mixtures, difficulty was experienced in obtaining adequate separation of tetradecapeptide and the fragments resulting from its cleavage by renin. It was found, however, that in 0.05M-sodium phosphate buffer, pH 6.5, tetradecapeptide showed a remarkable degree of adsorption to some chromatography papers (Whatman no. 1), whereas angiotensin I, angiotensin II and the tetrapeptide (Leu-Val-Tyr-Ser) migrated with high R_f values. In this system tetradecapeptide remained at the origin and angiotensin I migrated with R_f 0.62, the tetrapeptide (Leu-Val-Tyr-Ser) 0.80 and angiotensin II 0.68 (Fig. 1). Thus the ^{14}C -labelled tetradecapeptide was widely separated from the ^{14}C -labelled angiotensin I (Fig. 2).

^{14}C -labelled tetradecapeptide (35 nCi, 2.5 μg) in 60 μl of phosphate-saline buffer, pH 7.5, was incubated at 37°C for 4 h with concentrations of hog renin from 0.01 to 0.1 unit/ml. A 10 μl sample of the mixture was applied to Whatman no. 1 paper, dried in a stream of air at 90°C and equilibrated overnight in a chromatography tank with 0.05M-sodium phosphate buffer, pH 6.5. Descending chromatography was performed for 1 h at 23°C in the same solvent. During this time the solvent front moved 23 cm. The chromatogram was dried, stained with Pauly's reagent (Whitaker, 1967) and the angiotensin I spot and the origin were separately cut out and placed in counting vials containing 5 ml of toluene-2,5-diphenyloxazole-1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene (1 litre: 8.26 g:150 mg) scintillation mixture. The radioactivities

Fig. 4. Correlation of bioassay and radiochemical assay of the angiotensin I produced from incubation of ^{14}C -labelled tetradecapeptide with hog renin at pH 7.5 and 37°C for 4 h. Points represent the results obtained for incubations at four different renin concentrations.

Fig. 5. Radioactivity in the angiotensin I spot versus time of incubation in phosphate-saline buffer, pH 7.5, at 37°C . The concentration of tetradecapeptide was $36\mu\text{g/ml}$. Hog renin was added to produce a concentration of 0.05 Goldblatt unit/ml ($\blacktriangle$) and shows a linear breakdown with time up to 6 h. No breakdown was observed in buffer without added renin ($\circ$).

of samples were measured in a liquid-scintillation counter (Nuclear-Chicago). The radioactivity (c.p.m.) in the angiotensin I spot was expressed as a percentage of the total radioactivity on the strip.

Increasing renin concentrations up to 0.05 unit/ml in the incubation mixtures produced linear increases in ^{14}C -labelled angiotensin I generated (Fig. 3) during a 4 h incubation. Samples of these incubation mixtures were taken for bioassay in the rat. Fig. 4 shows the close correlation between angiotensin I contents measured by bioassay and by the radiochemical method.

When ^{14}C -labelled tetradecapeptide was incubated with a constant amount of hog renin (0.05 Goldblatt unit/ml) the amount of ^{14}C -labelled angiotensin I generated was shown to be a linear function of the time of incubation up to 6 h (Fig. 5).

Incubation of ^{14}C -labelled tetradecapeptide for up to 72 h in phosphate-saline buffer, pH 7.5, or plasma (from

Fig. 6. Conversion of ^{14}C -labelled tetradecapeptide into ^{14}C -labelled angiotensin I plotted against the renin concentration for a 4 h incubation in phosphate-saline buffer, pH 7.5, at 37°C ($\bullet$) and in a selected plasma treated by Skinner's (1967) method to which exogenous hog renin was added ($\blacktriangle$). The correspondence of results in the plasma and in buffer indicate that no significant accelerator, inhibitor or other tetradecapeptide-cleaving enzyme activity was detected in this plasma during a 4 h incubation.

Fig. 7. Radioactivity in angiotensin I plotted against time in a prolonged incubation of ^{14}C -labelled tetradecapeptide in plasma, treated by Skinner's (1967) method, selected because of its high endogenous renin concentration (24 ng of angiotensin I produced/h per ml by bioassay).

an anephric human), treated by the method of Skinner (1967), showed no appreciable breakdown of the tetradecapeptide to angiotensin I. Exogenous hog renin was then added to samples of the plasma to produce a range of concentrations up to 0.05 unit/ml. Fig. 6 shows the results obtained in this plasma compared with those in buffer. The correspondence of these values indicates that for a 4 h incubation in this plasma, the observed breakdown is due to renin alone and that no inhibitor or accelerator of the reaction is present. Bioassay of samples of these incubations in plasma again revealed good agreement with the results of the radiochemical assay, confirming that the breakdown product was indeed angiotensin.

A sample of human plasma treated by Skinner's (1967) method was selected because of its high plasma renin concentration (24 ng of angiotensin I/h per ml as measured by rat bioassay). Incubation of this plasma with ^{14}C -labelled tetradecapeptide resulted in a linear increase in radioactive angiotensin I with time; however, the incubation time required was more prolonged (Fig. 7).

Accuracy of the method was assessed by duplicate chromatograms on 40 different incubation mixtures. Duplicate assays showed a correlation coefficient of 0.992.

DISCUSSION

The 3-([^{14}C]valine)-labelled tetradecapeptide has been shown to be a suitable renin substrate. ^{14}C labelling in the valine³ position of the tetradecapeptide enables measurements of small amounts of it and the liberated angiotensin I to be made. After separation by a simple and rapid chromatographic procedure, a linear relationship between the concentration of added renin or time of incubation and the percentage breakdown of labelled tetradecapeptide in both phosphate-saline buffer, pH 7.5, and a selected treated human plasma had been demonstrated. Further, the labelled breakdown product of the labelled tetradecapeptide has been shown to be identical with angiotensin I on paper chromatography, to be immunologically similar in a specific angiotensin I immunoassay system, and also shows the expected biological activity in the rat.

The radiochemical assay described in this paper has several distinct advantages over existing biological renin assays. It employs a chemically defined pure substrate. The initial substrate concentration and rate of angiotensin formation can be accurately quantitated. The method is rapid and simple, and many measurements can be made concurrently. Further, the substrate concentration and angiotensin liberation can be measured in terms of μmol of peptide. Since the kinetics of the tetradecapeptide-renin reaction have been well studied (Montague *et al.* 1966a; Skeggs, Lentz, Kahn & Hochstrasser, 1968) the reaction rate allows calculation of values of renin concentration independent of arbitrary units.

The range of renin concentration used in these experiments is higher than those found in normal human plasma (Haas *et al.* 1968), but not in kidney. Renin concentrations in the reported physiological

range (0.1–0.5 m-unit/ml) also give a linear liberation of angiotensin I, but longer periods of incubation are needed to liberate enough ^{14}C -labelled angiotensin I to give reliable radioactivity measurements. Long periods of incubation, however, add to the difficulties of assaying plasma renin by this method because of the associated problems of non-specific breakdown of ^{14}C -labelled tetradecapeptide by bacterial contamination, non-specific plasma peptidases or pseudorenin (Skeggs, Lentz, Kahn, Dorer & Levine, 1969). This problem may be overcome by using labelled tetradecapeptide with a higher specific radioactivity or by prior extraction and concentration of the renin from plasma.

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REFERENCES

- Haas, E., Gould, A. B. & Goldblatt, H. (1968). *Lancet*, i, 657.
- Johnston, C. I., Mendelsohn, F. & Casley, D. (1969). *Proc. Aust. Soc. med. Res.* 2, 271.
- Montague, D., Riniker, B., Brunner, H. & Gross, F. (1966a). *Am. J. Physiol.* 210, 591.
- Montague, D., Riniker, V. & Gross, F. (1966b). *Am. J. Physiol.* 210, 595.
- Page, I. H. & McCubbin, J. W. (1968). *Renal Hypertension*, p. 29. Chicago: Year Book Medical Publishers Inc.
- Peart, W. S. (1955). *Biochem. J.* 59, 300.
- Skeggs, L. T., Kahn, J. R., Lentz, K. E. & Shumway, N. P. (1957). *J. exp. Med.* 106, 439.
- Skeggs, L. T., Lentz, K. E., Kahn, J. R., Dorer, F. E. & Levine, M. (1969). *Circulation Res.* 25, 451.
- Skeggs, L. T., Lentz, K. E., Kahn, J. R. & Hochstrasser, H. (1968). *J. exp. Med.* 128, 13.
- Skeggs, L. T., Lentz, K. E., Kahn, J. R. & Shumway, N. P. (1958). *J. exp. Med.* 108, 283.
- Skinner, S. L. (1967). *Circulation Res.* 20, 391.
- Sluyterman, L. A. Ae. (1960). *Biochim. biophys. Acta*, 38, 218.
- Whitaker, J. R. (1967). In *Paper Chromatography and Electrophoresis*, vol. 1, p. 96. Ed. by Zweig, G. & Whitaker, J. R. New York: Academic Press Inc.