A Radiochemical Renin Assay

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(Received 10 August 1970)

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 trypptic digest of hog renin substrate, which was shown to be an adequate renin substrate (Skeggs, Lentz, Kahn & Shumway, 1958; Montague, Riniker & Gross, 1966a). 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 17.5V/cm for 2h in 0.6M-formic acid–2M-acetic acid (1:1, v/v), pH 1.9, it produced a single peak with R f 0.63 (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-Val1-tetradecapeptide as synthesized by Riniker (Montague, Riniker, Brunner & Gross, 1966a). The stated specific radioactivity of the 14C-labelled tetradecapeptide was 40 µCi/µmol (or 18 µCi/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 µCi/µmol. When the peptide concentration was determined by a micro modification of a quantitative Pauly reaction (Slyterman, 1960) the specific radioactivity was calculated to be 23 µCi/µmol.

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 0.05M-sodium phosphate–0.9% sodium chloride, pH 7.5, containing 0.01M-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 18h at 4°C against 0.05M-glycine–0.01M-hydrochloric acid buffer, pH 3.3, containing 5mM-EDTA (sodium salt), incubation at 32°C for 1h followed by dialysis for 18h at 4°C against 0.1M-sodium phosphate–0.075M-sodium chloride buffer, pH 7.5, containing 1mM-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 4h resulted in a tenfold increase in pressor activity in a rat bioassay performed by the method of Peart (1955). Further incubation for 25h gave no further increase in pressor activity. This suggested complete conversion of the substrate into angiotensin I after 4h incubation, since Montague et al. (1966a) 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 4h 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.

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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 RF values. In this system tetradecapeptide remained at the origin and angiotensin I migrated with RF 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 (35nCi, 2.5μg) in 60μl of phosphate–saline buffer, pH 7.5, was incubated at 37°C for 4h with concentrations of hog renin from 0.01 to 0.1 unit/ml. A 10μl sample of the mixture was applied to Whatman no. 1, 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 1h 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 5ml of toluene-2,5-diphenyloxazole-1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene (1 litre: 8.26g:150mg) scintillation mixture. The radioactivities
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 14C-labelled angiotensin I generated (Fig. 3) during a 4h 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 14C-labelled tetradecapeptide was incubated with a constant amount of hog renin (0.05 Goldblatt unit/ml) the amount of 14C-labelled angiotensin I generated was shown to be a linear function of the time of incubation up to 6h (Fig. 5).

Incubation of 14C-labelled tetradecapeptide for up to 72h in phosphate-saline buffer, pH 7.5, or plasma (from 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 4h 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 radioactivity of 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 valine 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 $\mu$mol 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 n-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.

This work was supported by a Grant-in-Aid (G639/522) from the National Heart Foundation of Australia. F.A.M. is a Postgraduate Medical Research Scholar of the National Health and Medical Research Council of Australia. We thank Dr B. Riniker and Dr F. Gross of Research Laboratories, Pharmaceutical Dept., Ciba Ltd., Basle, Switzerland, for generous gifts of tetradecapeptide and angiotensin I and Dr L. T. Skeggs for the tetrapeptide.

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