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PURIFICATION OF HUMAN RENIN AND INHIBITION OF ITS ACTIVITY BY PEPSTATIN*

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Abstract—Several proteases from human kidney tissue were partially purified by ammonium sulfate precipitation followed by Sephadex G-75 gel filtration chromatography. The incubations of column fractions with ^{14}C-tetradecapeptide renin substrate indicated four major renin activity peaks between the molecular weights of 25,000 and 80,000. The highest specific activity was found in Fraction B (mol. wt 39,500). Significant renin-like activity was also found in Fraction A (mol. wt 58,000) and Fraction C (mol. wt 33,500). None of the fractions contained angiotensinase activity. Disc-gel electrophoresis indicated that Fractions B and C were heterogeneous. The conversion of ^{14}C-tetradecapeptide renin substrate to angiotensin I by major fractions was differentially inhibited by low concentrations of pepstatin. Both the rate of substrate conversion and the sensitivity to pepstatin were influenced by pH. Generally, the proteases were more sensitive to pepstatin at physiological pH. Regardless of pH, all of the kidney proteases were completely inhibited by 10^{-3} M pepstatin. The conversion of human substrate by human plasma proteases was found to be even more sensitive to pepstatin. The concentration of 10^{-5} M pepstatin essentially eliminated plasma renin activity. These results indicate that pepstatin can serve as an efficient inhibitor of both human kidney and plasma renin proteases and as such will be useful in the study of the kinetics of these systems in both research and clinical situations.

MUCH OF THE current research interest in renin (EC 3.4.5.15) is due to its role as the primary initiator of the renin–angiotensin–aldosterone system. This feedback cycle which controls electrolyte homeostasis and blood pressure has been the subject of recent reviews,[1,2] Expectedly, many pathological conditions are associated with changes of plasma renin activity.[3]

Renin has generally been considered simply as an endopeptidase which is elaborated from the kidney into the circulatory system where it reacts with plasma precursor α_2-globulin by cleaving a leucyl–leucyl bond, thus producing angiotensin I, a decapeptide. But as early as 1953, there has been evidence for at least three distinct configurations of a hog renin–protein complex,[4] and human renin or renin-like activity was subsequently ascribed to proteins having molecular weights of 39,000,[5] 42,300[6] and 70,000.[7] Recently it has been clearly shown that there is more than one form of human,[5,8] mouse[9] and rat and pig[10] renin. From accumulating evidence it is apparent that there are many distinct proteinases which can act upon renin substrates to produce angiotensin I; thus, the subject regarding renin as well as renin inhibitors becomes increasingly complex.

Although there are several recent studies regarding various types of inhibitors of animal renin-substrate reactions,[11–14] there is a lack of information regarding inhibitors of human renin. Only naturally occurring phospholipids,[15,16] heparin[17] and

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series A prostaglandins[18] have been demonstrated to be effective inhibitors of human renin activity. Utilizing crude preparations of hog renin, it has been shown that pepstatin, a pentapeptide, is a very potent inhibitor of hog renin activity *in vitro* and produces a hypotensive response in experimental hypertension *in vivo*.[19–21] Pepstatin is an acid protease inhibitor which was isolated from culture filtrates of various species of actinomycetes by H. Umezawa, S. Umezawa *et al.*[22,23] It is a modified pentapeptide and its primary structure has been determined.[24] Pepstatin inhibits proteinase activity attributed to pepsin,[22] gastricsin,[25] cathepsin D from pig liver,[26] and human cathepsin D.[27,28] Physiological studies on five species of common laboratory animals have revealed that pepstatin has a very low toxicity.[22]

The purpose of this study was to determine the effects of pepstatin on various forms of purified human renin by utilizing a synthetic radiolabeled tetradecapeptide (TDP) renin substrate. The effects of pepstatin on human plasma renin activity were also studied.

MATERIALS AND METHODS

*Human Renin Standard.** Throughout the experiments, renin activity was compared with a human renin preparation, Coded 68/356, where one ampule contained 0·1 Goldblatt Unit [GU].[29–31] Protein determination[32] of two ampules indicated that each contained approximately 0·70 mg protein. A dilution was made such that 10 μl phosphate-saline buffer contained 0·001 GU/7·0 μg protein.

Labeled renin substrate (TDP) and angiotensin II. The 5-L-isoleucine, 3-L-[U-^{14}C]-valine tetradecapeptide[33,34] with a stated sp. act. of 40 mCi/m-mole was obtained from Schwarz BioResearch, Orangeburg, N.Y. High voltage paper electrophoresis and cellulose thin-layer chromatography followed by radioactive strip scanning and quantitation indicated that this material was greater than 98 per cent homogeneous. For the stock solution the substrate was dissolved in 5 ml distilled water (0·51 nmole/10 μl). Angiotensin II (5-L-[U-^{14}C]isoleucine with a sp. act. of 236 mCi/m-mole) was obtained from New England Nuclear, Boston, Mass. For the stock solution the substrate was dissolved in 1 ml distilled water (0·19 nmole/10 μl).

Expression of renin activity. Throughout the experiments, parallel incubations of the experimental renin fractions were made with the W.H.O. calibrated renin. This was done to provide a comparative basis for the expression of renin activity. The following calculations were performed to yield the comparative data: (a) W.H.O. specific renin activity (GU/μg protein) = amount of ^{14}C-angiotensin I formed from ^{14}C-TDP/time; and (b) exptl. specific renin activity (arbitrary units/μg protein) = amount of ^{14}C-angiotensin I formed from ^{14}C-TDP/time. The experimental renin activity (b) was expressed as "specific activity of GU" by comparing the amount of angiotensin I formed from TDP/time/μg renin protein, with the data obtained from comparable incubations of calibrated W.H.O. Standard Renin (a).

Pepstatin. A generous quantity of pepstatin (iso-valeryl-L-valyl-L-valyl-4-amino-3-hydroxy-6-methylheptanoyl-L-alanyl-4-amino-3-hydroxy-6-methylheptanoic acid) was provided by Dr. H. Umezawa, Institute of Microbial Chemistry, Shinagawa-Ku, Tokyo, and Mr. K. Goto, Banyu Pharmaceutical Co., Tokyo.

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Tissue. Human cadaveric kidneys free from obvious disease were obtained at autopsy from individuals who died from traumatic injury. Upon removal from the body they were quick-frozen, and stored (−85°). At the end of the collection period, superficial fat and connective tissue were removed.

Tissue extraction. The following extraction and ammonium sulphate precipitation procedures were slightly modified from previous procedures.[30,35] Five liters of distilled water at 25° were added to the ground kidney tissue. The mixture was stirred for 1 hr at room temperature and then overnight at 5°. The remainder of the steps were carried out at 5°. The particulate material was removed by centrifugation at 5000 *g* for 30 min. The supernatant was stored and the pellet was finely ground in a Waring Blendor with an equal volume of distilled water and stirred overnight. The mixture was centrifuged and the supernatants were pooled, acidified to pH 4·5 with 5 N H_2SO_4 and made 0·8 M with respect to NaCl. The mixture was stirred for 30 min and centrifuged at 5000 *g* for 30 min. The precipitate was discarded and solid $(NH_4)_2SO_4$ was added to the supernatant to make it 2·5 M. The mixture was stirred for 30 min and centrifuged as before. The supernatant was discarded and the precipitate was diluted to 1 M $(NH_4)_2SO_4$, stirred for 20 min and centrifuged at 4000 *g* for 1 hr. The supernatant was dialyzed against three changes of distilled water over 24 hr, removed from the dialysis bags, and centrifuged at 4000 *g* for 20 min to remove insoluble proteins. Disodium EDTA was added to a final concentration of 0·1 M. The mixture was stirred for 5 min and the pH was adjusted to 9·5 with 5 N NaOH. The solution was stirred for 1 hr and crude renin was precipitated by adding $(NH_4)_2SO_4$ to a final concentration of 2·5 M, followed by a constant stirring for 1 hr. The precipitate was collected by centrifugation at 5000 *g* for 30 min and dissolved in 40 ml cold distilled water. The solution was dialyzed against three changes of distilled water over 24 hr. The preparation was again made to 2·5 M with $(NH_4)_2SO_4$, stirred and centrifuged as described. The precipitate was dissolved in a minimal quantity of cold distilled water and dialyzed against five changes of 0·005 M phosphate buffer at pH 6·0. Next, the dialyzed renin solution was stirred with 260 ml CM-Sephadex (Pharmacia) slurry equilibrated with 0·001 M phosphate buffer, pH 5·4. The suspension was stirred for 1 hr at 5° and centrifuged at 5000 *g* for 30 min. The renin preparation was eluted with four 60-ml vol. of 5% (w/v) NaCl in 0·05 M phosphate buffer, pH 5·4, and centrifuged at 5000 *g* for 30 min. The combined supernatants were dialyzed against four changes of distilled water. This purified renin solution was stored (−85°) prior to gel filtration chromatography.

Column chromatography. The protein molecular weight standards and their sources are shown in Table 1. The Sephadex G-75 gel, Blue Dextran 2000, and columns were obtained from Pharmacia Fine Chemicals, Inc., Piscataway, N.J. Pharmacia K26/1000 columns equipped with two flow-adapters were used in gel filtration experiments.

Ascending chromatographic technique was used and buffer [0·05 M phosphate-0·1 M NaCl buffer, pH 6·9 (phosphate–saline buffer)] flow was maintained at 1·2 ml/min with an LKB 12000 pump (LKB Instruments, Inc., Rockville, M.).

The A_{280} of the effluent was monitored continuously (LKB Uvicord II) and 3-ml fractions were collected. Protein concentrations of samples and standards were determined using crystalline human albumin as a standard.[32] The elution volume (V_e) of a substance was determined at the maximum height of the corresponding peak. The

void volume (V_0) was determined before each experiment by passing a 0·2% (w/v) Blue Dextran solution through the column. The bed volume (V_t) was calculated from the internal diameter and the length of the gel bed (2·6 × 91·0 cm). For each standard, the average partition coefficient K_{av} was calculated from the equation $K_{av} = (V_e - V_0)/(V_t - V_0)$.[39] The values were plotted against the logarithm of the corresponding molecular weight; a regression line was constructed by the method of least squares.

TABLE I. PROTEINS USED IN GEL FILTRATION

Protein	Source	Mol. wt.	Log mol. wt.	Reference
Ribonuclease-A	Sigma, type II	13,700	4·13	36
α-Chymotrypsinogen-A	Sigma, type II	25,700	4·39	37
Ovalbumin	Sigma, grade V	44,620	4·64	36
Human serum albumin	CalBiochem, grade A	69,000	4·84	37
Liver alcohol dehydrogenase	Sigma, IX	80,000	4·90	38

The amount of crude renin applied to the column at one time was limited to 20 mg protein so that distinct separations could be obtained. Initially, 120 tubes (360 ml) of column effluent were collected.

Incubation media. The primary incubation media was composed of 0·05 M sodium phosphate–0·1 M sodium chloride, pH 6·9, containing 0·01% (w/v) bacitracin (Sigma Chemical Co., St. Louis, Mo.).

Estimation of renin activity by TLC or electrophoresis. In general, a renin activity incubation was comprised of 10 μl phosphate-saline buffer, 10 μl ^{14}C-TDP (tetradecapeptide), and a 10-μl aliquot from a column fraction tube. Most of the incubations were for 1 hr at 37°. For renin activity assays, a sample from an incubation tube was applied to a cellulose TLC chromatography sheet or electrophoresis paper. Following development, the amount of radioactive angiotensin I was compared to the amount of ^{14}C-TDP and calculated as an expression of renin activity. These results were compared to the results obtained from similar incubations of the standardized human renin preparation. Specific incubation conditions are described in detail in the appropriate figures and tables.

Thin-layer chromatographic (TLC) separation of TDP and angiotensin I. Using a modification of a described paper chromatographic method,[40] the resolution of TDP and angiotensin I was obtained with Eastman cellulose TLC sheets No. 6064 with development in the phosphate-saline, pH 6·9 buffer. The R_f values for TDP, angiotensin I and angiotensin II were 0·1, 0·8 and 0·9 respectively.

Electrophoretic separation of TDP and angiotensin I. The separation of TDP and angiotensin I in the incubation medium was also performed by high-voltage electrophoresis with a model D Electrophorator (Middletown, Wis.).[41,42] This method has an advantage over TLC in that it has the capacity to clearly resolve TDP, angiotensin I, angiotensin II and the dipeptide product histidyl-leucine. Thus, it was used to determine the purity of the TDP substrate as well as the protein standards. In addition, the presence of contaminating converting enzyme, or angiotensinases in the various renin preparations could be determined.

Estimation of renin activity by radioactivity determinations. Two procedures were used to determine the zones of radioactivity on the chromatograms (electrophoretic and TLC). Initially each strip was monitored with a radio-chromatogram scanner model 7201 (Packard Instruments, Downers Grove, Ill.) after which they were cut into 0·5 × 4 cm pieces and put into individual scintillation vials for quantitation by the Packard model 3320 liquid scintillation spectrometer.[42] Alternatively, the strips were quantitated with the chromatogram scanner integrated with the spectrometer of the Packard model 3320.

To determine the radiopurity of the substrate, detect incubation artifacts and possible migration effects due to added inhibitor, incubation tubes which contained only labeled substrate plus incubation solution, or labeled substrate, incubation buffer, plus inhibitor were incubated under control conditions. The incubates were subjected to electrophoresis, or TLC, and assayed for zones of radioactivity.

The ^{14}C in each radioactive zone was calculated as a percentage of the total chromatographed ^{14}C. The amount of product (^{14}C-angiotensin I) formed was calculated from the percentage of ^{14}C in the product and the amount of substrate incubated (assuming a 1:1 mole relationship), and used as the basis for the calculation of enzymic activity.

Estimation of renin activity by rat bioassay. One-hr incubations of radiolabeled TDP and renin eluates from the column were bioassayed by measuring rat blood pressure response according to the bioassay procedures of Pickens *et al.*[43] Appropriate amounts of TDP were injected alone to provide baseline data to account for the small rise in blood pressure due to the TDP.

Composition of column fractions determined by disc-gel electrophoresis. Selected fractions were tested for protein composition by disc-gel electrophoresis. Protein (20–40 μg) was applied to each gel. The over-all approach was that of Davis.[44] Electrophoresis was performed on 7% gels (12 × 0·5 cm) in a Buchler Polyanalyst (Buchler Instrument Div., Fort Lee, N.J.). Sources of chemicals for electrophoresis were acrylamide, Eastman 5521; BIS (*N*, *N'*, methylenebisacrylamide), No. 8383; TEMED (*N*, *N*, *N'*, *N'*, tetramethylethylenediamine), No. 8178 (Eastman Kodak Co., Rochester, N.Y.). Coomassie Brilliant Blue R-250 was obtained from Sigma Chemical Co., St. Louis, Mo. Pepsin and bromphenol blue, used as reference markers, were obtained from Sigma Chemical Co., St. Louis, Mo. The gels were fixed, stained with Coomassie Blue, and destained according to the procedure of Diezel *et al.*[45]

Determination of angiotensinase activity. Column fractions which contained significant renin-like activity were incubated at pH 5·5 and pH 6·9 for 1 hr with ^{14}C-angiotensin II (see ^{14}C-TDP incubations). At the conclusion of the incubation period, aliquots were subjected to electrophoresis and radio-chromatographic scanning.

Estimation of the effect of pepstatin on renin activity in human plasma. Human plasma samples were assayed for renin activity by the Squibb Radioimmunoassay Kit (E. R. Squibb & Sons, Inc., Princeton, N.J., modified from Haber *et al.*[46]). The procedure used with these kits was performed as described in the literature which accompanied them. Aliquots from the samples were taken for control and experimental determination. The control and the experimental aliquots were assayed as described, with the exception that the experimental aliquots were made to 10^{-4} M, 10^{-5} M and 10^{-6} M pepstatin prior to assay.

RESULTS AND DISCUSSION

Renin activity of the ammonium sulfate precipitations. The specific activity of the crude renin preparation obtained from the batch elution of the CM-Sephadex was 0·02 GU/mg protein/hr at pH 6·9. This was determined by incubating ^{14}C-TDP with crude renin and comparing the amount of ^{14}C-angiotensin I formed (renin activity) to the amount formed by a similar incubation of standard W.H.O. renin.

Gel filtration of standard proteins and human renin preparation. The calibration plot of standard proteins used to identify the molecular weights (mol. wt) for the various fractions obtained from column chromatography is shown in Fig. 1. A linear response was obtained for proteins with molecular weights of 70,000 to 13,700 (r = 0·998). At 80,000 mol. wt (tube 60) there was some overlap with the void volume. The majority of the protein from the crude renin preparation was eluted from the column at, or near, the column void volume (Fig. 2); therefore, this procedure provided an efficient method for purifying crude renin preparations.

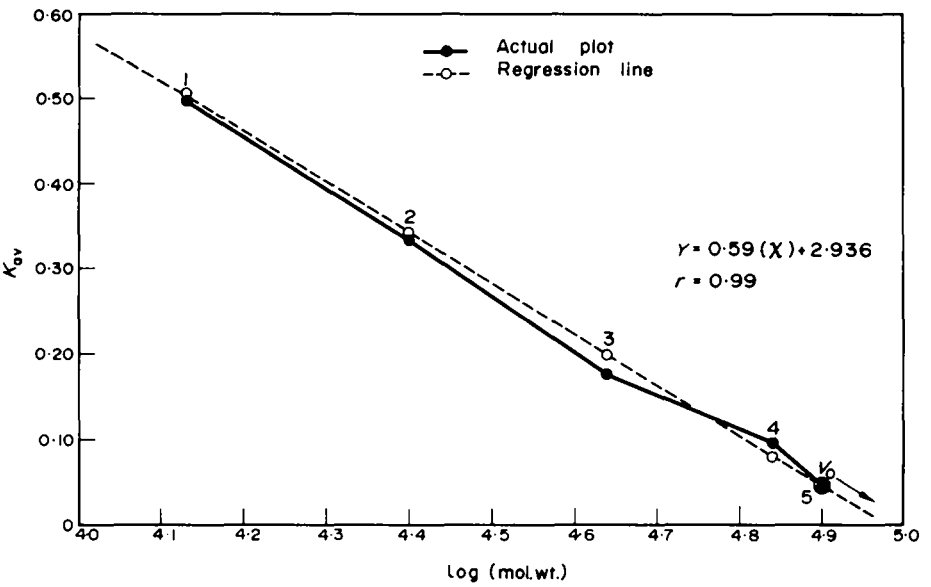

FIG. 1. Calibration plot of the G-75 effluent volumes of protein standards and the calculated least squares regression line. The molecular weight standards are: (1) ribonuclease-A; (2) α-chymotrypsinogen; (3) ovalbumin; (4) human serum albumin and (5) liver alcohol dehydrogenase. The formula is given for the regression line.

Renin activity of various fractions. A survey of renin activity (tubes 55–120) indicated that significant activity was present from mol. wt 80,000 (tube 60) to 25,000 (tube 90). By incubating tube aliquots with ^{14}C-TDP it was found that activity was present in three major fractions (I–III) at pH 6·9 and four activity peaks were present in incubations of pH 5·5 (Fig. 3). The estimated molecular weights which correspond to these peaks are given across the top of Fig. 3. The activity peaks at either pH occurred at approximately the same mol. wt designations with the exception that peak I (pH 5·5) occurred alone at a mol. wt of 73,000.

When the specific activity of the renin activity was calculated it was generally found that regardless of the pH the majority of activity was in three fractions (Fig.

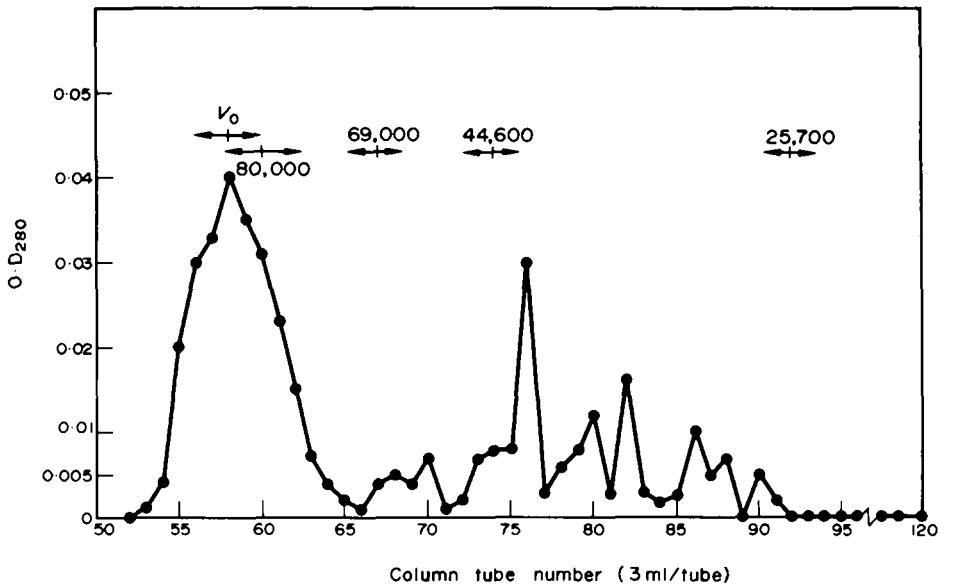

FIG. 2. Elution pattern obtained by chromatography of an ammonium sulfate precipitated preparation of human kidney renin. The bed (2·6 × 91·0 cm) of Sephadex G-75 was packed and eluted with 0·05 M phosphate 0·1 M NaCl buffer, pH 6·9. Elution of molecular weight standards are indicated by the arrows.

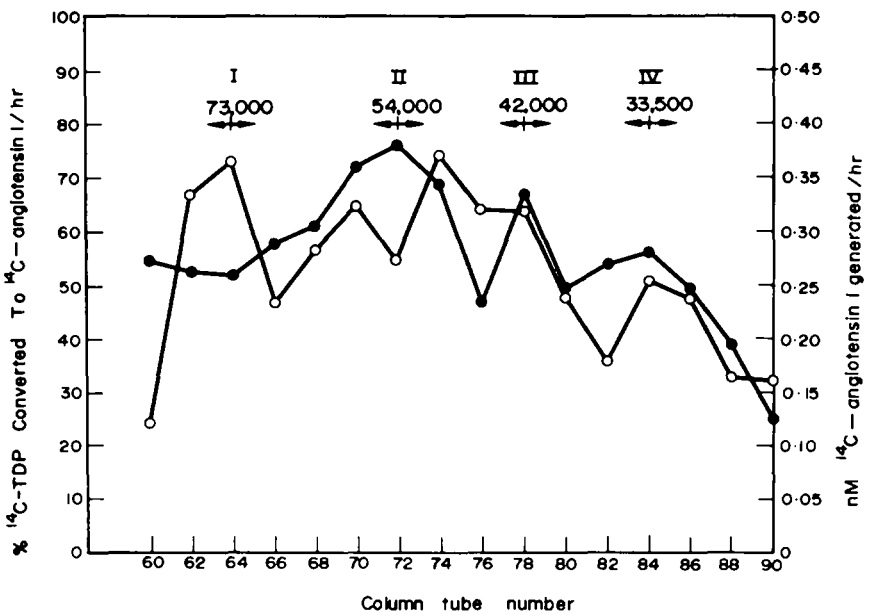

FIG. 3. Renin activity of column fractions plotted as the percentage of ^{14}C-TDP converted to ^{14}C-angiotensin I, and as the amount of ^{14}C-angiotensin I (nmoles) generated. The 1-hr incubations consisted of 10 μl ^{14}C-TDP (0·5 nM) and the 10 μl of a column fraction. The pH was adjusted to either 5·5 or 6·9; ●—●, incubations at pH 6·9; ○—○, incubations at pH 5·5. The Roman numerals refer to major peak designations and the arrows refer to extrapolated molecular weight estimates.

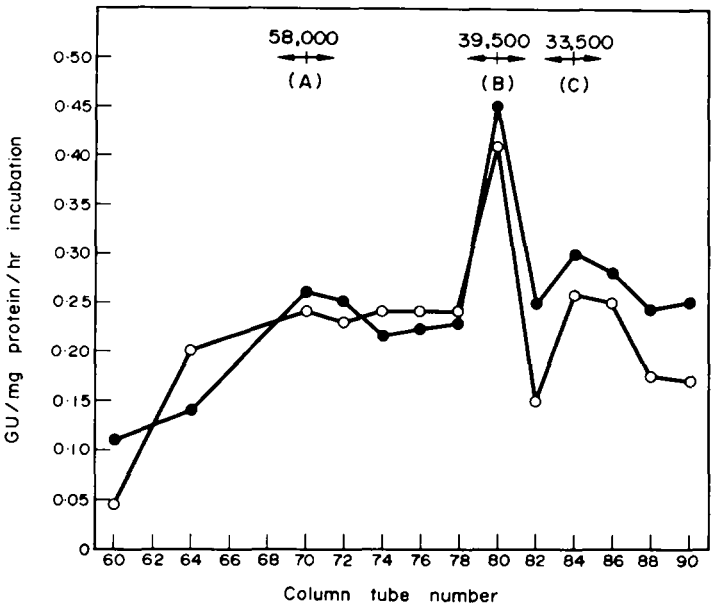

FIG. 4. Specific activity of renin fractions plotted as GU/mg protein/hr of incubation at pH 5·5 and 6·9. These data were calculated relative to the W.H.O. Renin Standard which according to the experimental conditions had a specific activity of 0·140 GU/mg protein/hr. ●——●, incubations at pH 6·0; ○ ·—○, incubations at pH 5·5. The letters refer to major peak designations, and the arrows refer to extrapolated molecular weights.

4). Fraction B (mol. wt = 39,500) had the highest specific activity while Fraction C (mol. wt = 33,500) and Fraction A (mol. wt = 58,000) had lower and nearly equal specific activities. The highest specific activity occurred at pH 6·9 and was 0·440 GU/mg protein/hr; by comparison the W.H.O. Renin Standard under similar incubation conditions has a specific activity of only 0·140 GU/mg protein/hr. Recent investigation of human kidney renin by Skeggs *et al.*[5] has indicated that there are four forms of renin with a mol. wt of approximately 39,000 and many forms of pseudorenins with molecular weights from 44,000 to 158,000. Although we made no attempt to resolve these forms, our data are in general agreement with those of other workers who have found that the majority of human renin activity occurs at about 40,000 mol. wt.[5,6] Recent data[9] regarding renin purified from rat and pig kidney have indicated renin activity from proteins with molecular weights of 38,000 and 60,000 (pig), and 39,000 and 34,000 (rat). It would appear that these may correspond to Fractions B, A, B and C respectively. Human kidney renin activity occurring with a protein of mol. wt 70,000 has also been reported, which may correspond to the activity found in tube 64 (mol. wt = 73,000).

The methodology used in this study would indicate that three major fractions of renin activity which occur between the mol. wt of 33,000 and 58,000 can be obtained. When the products of the column fraction plus ^{14}C-TDP at pH 6·9 were tested for biological activity by rat bioassay, it was found that Fractions II–IV gave net increased pressure responses of 20, 15 and 13 mm Hg, respectively, while the response to Fraction I was only 3 mm Hg. These responses were predicted by the results presented in Fig. 3.

Proteins of active fractions. Using analytical polyacrylamide disc-gel electrophoresis, fractions obtained from the Sephadex G-75 column chromatography showed different patterns in each of the three fractions tested. The results indicate that Fractions B and C were heterogeneous protein solutions, while Fraction A contained only one band with a relative mobility (R_m) of 3·0. Fraction B contained two bands with R_m of 2·0 and 3·9. Six bands were visible from Fraction C which were represented by the following R_m values: 3·9, 4·3, 5·3, 6·2, 7·0 and 8·2. Since these fractions were separated primarily on the basis of molecular size, it is possible that a particular fraction contains more then one molecular species. These molecular species may be either isozymes or other proteins. Previous countercurrent distribution data have indicated that there are many proteins with renin-like activity which have subtle chemical and physical differences that cannot be resolved by column chromatography techniques.

Angiotensinase activity. When incubations of aliquots from column effluent tubes (tubes 60–90) were performed with ^{14}C-angiotensin II as the substrate, no angiotensinase activity was detected. Previous work[42] demonstrated that the majority of angiotensinase activity in human lung preparations occurred in a molecular weight range of 150,000–200,000. This range was excluded in the V_0 of the present study.

Effects of pepstatin on kidney renin activity of various column fractions. When column fractions (tubes 60–90) were incubated for 1 hr with ^{14}C-TDP at either pH 5·5 or pH 6·9 in 10^{-3} M pepstatin the complete inhibition of renin activity resulted. However, no inhibition was obtained from any tubes at either pH during similar incubations in 10^{-8} M pepstatin. When Fractions I–IV were incubated at pH 6·9 in various molarities of pepstatin ranging from 10^{-8} M to 10^{-3} M (in increments of 10^{-1} M) no inhibition resulted until the minimum concentration of 10^{-6} M pepstatin was used. At 10^{-4} M pepstatin, approximately 30 per cent inhibition was obtained at pH 5·5 (Table 2). The same results were obtained when incubations were made with the W.H.O. renin. Fraction B which had the highest specific activity was 35 and 31 per cent inhibited at pH 5·5 and pH 6·9, respectively, at 10^{-4} M pepstatin. There were, however, large quantitative differences regarding the inhibitory effects of pepstatin on the various renin fractions at pH 6·9. Fraction IV was more than twice as resistant to pepstatin at pH 6·9 as was Fraction II, while Fractions I and III as well as Fraction B were intermediate.

Although exact comparisons cannot be made due to differences of renin standardization and species, the data generally agree with the results obtained by others. Gross *et al.*[19] found 50 per cent inhibition of hog renin–rat substrate with $0{\cdot}58 \times 10^{-4}$ M pepstatin, while other investigators found 50 per cent inhibition of hog renin–bovine substrate with $0{\cdot}32 \times 10^{-6}$ M pepstatin.[20] These data indicate that differences in substrate (hog vs bovine) are reflected in the renin inhibitory effects of pepstatin. Correspondingly, it appears that ^{14}C-TDP and rat renin substrate systems are comparable in that similar concentrations of pepstatin have similar effects upon the renin substrate reaction.

Effects of pepstatin on human plasma renin activity. Results regarding the effects of pepstatin on human plasma renin activity (Table 3) show that a concentration of 10^{-5} M pepstatin essentially eliminated renin activity over a wide range (0·56 to 10 ng angiotensin I/ml plasma/3 hr). It should be noted that plasma renin activity levels greater than 4·0 ng of generated angiotensin I are indicative of high renin states.

TABLE 2. INHIBITION OF HUMAN KIDNEY RENIN ACTIVITY BY PEPSTATIN*

Column fraction	pH 5·5			pH 6·9		
	% ^{14}C-A I† formed (control)	% ^{14}C-A I formed (10^{-4} M pepstatin)	% Inhibition	% ^{14}C-A I formed (control)	% ^{14}C-A I formed (10^{-4} M pepstatin)	% Inhibition
I	47·0	30·0	36·1	33·4	20·1	40·0
II	35·1	23·5	33·0	47·6	20·6	56·7
III	33·1	21·3	35·7	42·0	27·0	36·0
IV	32·9	24·5	25·5	35·1	26·1	25·6
W.H.O. renin	33·1	23·1	30·1	26·4	17·6	33·3

Aliquots (10 μl) from peak activity tubes were incubated for 1 hr in a medium containing: 10 μl phosphate-saline buffer, pH 6·9, 10 μl (0·51 nM) ^{14}C-TDP and 10 μl of various concentrations of pepstatin.

† ^{14}C-A I = ^{14}C-angiotensin I.

TABLE 3. INHIBITION OF HUMAN PLASMA RENIN ACTIVITY BY PEPSTATIN*

	^{14}C-A I (ng formed)			^{14}C-A I (ng formed)			^{14}C-A I (ng formed)		
	Control	10^{-4} M Pepstatin	% Inhib.	Control	10^{-5} M Pepstatin	% Inhib.	Control	10^{-6} M Pepstatin	% Inhib.
N	10			10			7		
$\bar{x}$	5·10	0·08	98·90	4·84	0·07	97·40	3·54	1·98	40·72
Range	0·6- 10·0	0·05	93–100	1·4–8·0	0–0·4	88–100	1·3–7·6	0·5–4·3	16–83
S. D.	3·00	0·16	2·3	2·22	0·10	3·63	1·93	1·23	23·08
S. E.	0·94	0·05	0·7	0·70	0·03	1·14	0·73	0·46	8·74

* Human plasma samples were incubated and assayed as described in the literature which was provided in the Squibb Radioimmunoassay Kits for the measurement of renin activity. Three concentrations of pepstatin were used in the experimental incubations.

according to the methodology and standardization used. The ID_{40} of human plasma renin activity was found to be approximately 10^{-6} M pepstatin. Whether the apparent difference in kidney and plasma sensitivity to pepstatin is due to differences in the renin-like enzymes or to differences in enzyme-substrate concentration remains to be answered. Interestingly, neither the kidney nor the plasma renin systems appear to be as sensitive to pepstatin as some of the other proteases studied. As examples, gastricin and pepsin had an ID_{40} of $4{\cdot}2 \times 10^{-7}$ M and $5{\cdot}4 \times 10^{-9}$ M pepstatin respectively.[25] To the contrary, some proteinases such as trypsin, kallikrein, thrombokinase and chymotrypsin have been found to be completely resistant to concentrations of pepstatin above 1×10^{-4} M.

From studies regarding the effects of pepstatin on various renin–angiotensin systems, it is apparent that pepstatin should be profitably used in delineating some of the ramifications of the renin–angiotensin system. Furthermore, considering the reported low toxicity of pepstatin and its high degree of effectiveness in inhibiting renin, the possible therapeutic value of pepstatin in the treatment of diseases associated with high renin states in man would seem to pose an important question.

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