Competitive Inhibitors of Renin†

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ABSTRACT: Six analogs of an octapeptide sequence, His-Pro-Phe-His-Leu-Leu-Val-Tyr, representing a segment of renin substrate were synthesized. L-Amino acid residues were replaced with D-amino acids as well as with closely related L-amino acids. These were tested as competitive inhibitors of the proteolysis of tetradecapeptide substrate, Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser, by renin. The octapeptide as well as all its analogs were true competitive inhibitors. None of the substitutions resulted in a greater inhibitory constant than that of the model octapeptide, and in several instances, inhibitory constants one order of magnitude smaller were demonstrated. Proteolytic cleavage of these peptides after exposure to renin was also determined by measuring the increment in ninhydrin color as well as by Edman degradation of reaction products. All peptides appeared to be substrates except those in which D-leucine was substituted for L-leucine on either side of the leucylleucine bond which is the cleavage site for renin. The [D-Leu^6]octapeptide had an inhibitory constant of 3 μM and was not cleaved by renin. This peptide also inhibited the reaction between human renin and protein renin substrate in plasma. The availability of such effective competitive inhibitors for renin may be of importance in the understanding of the physiologic role of renin as well as in mapping the specificity of the substrate site.

This investigation was undertaken to begin definition of the substrate requirements for the proteolytic enzyme renin, and in the course of this definition, to plan for the synthesis of competitive inhibitors. Specific competitive inhibitors would be of substantial value in physiologic investigations of the role played by renin in the homeostatic regulation of circulation. The amino acid sequence around the cleavage site of renin substrate was determined by Skeggs and coworkers (1957) and served as the basis of our investigation. Sequence variants of the minimal peptide which serves as a good substrate (Skeggs *et al.*, 1968) were prepared and tested both as substrates and as competitive inhibitors. Substitutions by D-amino acids and L-amino acids of somewhat differing structure were made in the octapeptide. These substitutions were selected because of the prior demonstration that such changes made in substrates for other enzymes resulted in competitive inhibitors: carboxypeptidase A (Stahmann *et al.*, 1946), chymotrypsin (Webb, 1960), and papain (Schechter and Berger, 1967).

Methods

Peptide Synthesis. tert-Butoxycarbonylamino acids were synthesized by the method of Schnable (1967) or purchased from Bachem. Boc-His(Z)·C_6H_6 was a gift from Dr. G. Tregear. Z-D-His(Z)·C_2H_5OH was prepared by the method reported for the L-histidine derivative (Akabori *et al.*, 1958). Dichloromethane was redistilled before use and triethylamine purified by distillation from phenyl isocyanate (Sauer, 1963). Other chemicals were reagent grade and used without further purification. Bio-Beads SX-1, 200–400 mesh, 0.69 mequiv of Cl^-/g, obtained from Bio-Rad or chloromethylated, *uncross-linked* polystyrene insolubilized on Kel-F beads, 200–400 mesh, 0.128 mequiv of Cl^-/g (Tregear, 1972), were used as supports for the solid-phase syntheses. Reactions were conducted in chromatography columns (2.5 × 10 cm), stirred with a turbine agitator attached to an overhead stirrer. Completeness of coupling reactions was tested with ninhydrin (Kaiser *et al.*, 1970). Amino acid analyses were performed on samples of peptidyl-resin dried to constant weight and hydrolyzed for 24 hr at 110° in a mixture of concentrated HCl–acetic acid (1:1) in tubes evacuated to 25 μ. Peptides were hydrolyzed in 6 N HCl under the same conditions but were not dried to constant weight. D-Leucine was determined by the method of Manning and Moore (1968). Derivatization of an acid hydrolysate of the peptide containing D-leucine was accomplished with Leu-*N*-carboxyanhydride. All dipeptides in the mixture were resolved by chromatography on a column (56 × 0.9 cm) of AA-15 resin eluted with pH 5.3, 0.20 M citrate buffer. Standard dipeptides (Bachem) were used to identify the position of elution and to calculate the color yield: L-Leu-L-Leu, 80.5 ml; L-Leu-D-Leu, 85.5 ml; L-Leu-L-Tyr, 90.6 ml.

Enzymatic digestion of peptides was performed on 200-nm samples using the method of Keutmann *et al.* (1970).

Purified peptides were characterized by migration on high-voltage electrophoresis at 67 V cm^{-1} on Whatman No. 3MM paper in pH 3.5 pyridine–acetic acid buffer (E1) relative to leucine. Thin-layer chromatograms were run on 250-μ silica gel GF plates obtained from Analtech and compounds on both electropherograms and chromatograms detected by ultraviolet (uv) absorbance, ninhydrin, or Cl_2–starch–KI reagent in that order. Systems used for development were: T3, butan-1-ol–pyridine–acetic acid–water (30:20:6:24); T4, butan-1-ol–water–acetic acid (100:30:10); or T6, propan-1-ol–30% NH_3 (70:30). Optical rotations were measured in a Perkin-Elmer Model 220 spectropolarimeter equipped with a 1-dm cell thermostated at 20°. The melting point was measured on a Köffler block. Absorbance was measured at 280 and 220 nm with a Cary 15 spectrophotometer.

Edman degradation was performed as described previously for small hydrophobic peptides (Niall *et al.*, 1972). Extraction with benzene was omitted and the reaction mixture was evaporated under N_2 before cleavage with trifluoroacetic acid to maximize the yield of Leu>PhNCS. Phenylthiohydantoin derivatives were identified and quantitated using thin-layer and gas–liquid chromatography (Strosberg *et al.*, 1972).

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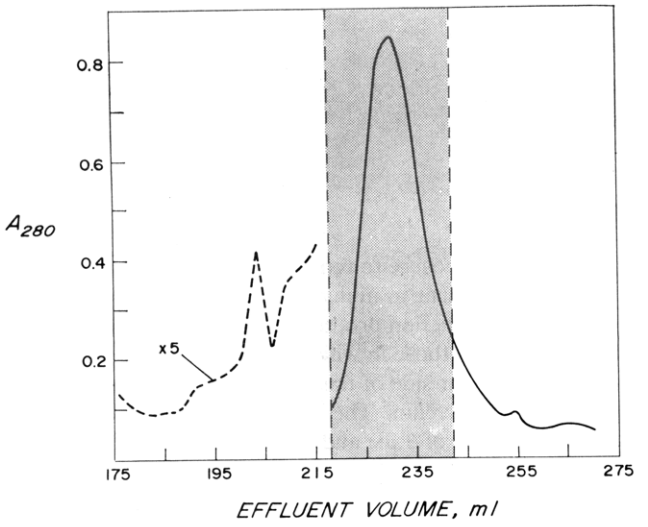

FIGURE 1: Chromatography of crude octapeptide (23 mg) on Bio-Gel P-2 (1.2 × 194 cm) eluted with 0.01 N HCl at 8.7 ml hr^{-1}. Volume, indicated by the shaded area (84%), was lyophilized to yield octapeptide.

TABLE I: Peptide Synthesis.

Step	Reagent	Time Stirred (min)
Prewash	30% Trifluoroacetic acid–CH_2Cl_2	1
Deblock	30% Trifluoroacetic acid–CH_2Cl_2	30
Wash four times	CH_2Cl_2	1
Test with ninhydrin, submit sample to be dried to constant weight for amino acid analysis.		
Prewash	10% Triethylamine–CH_2Cl_2	1
Neutralize	10% Triethylamine–CH_2Cl_2	15
Wash four times	CH_2Cl_2	1
Add	10-fold excess Boc-amino acid as 0.25 M solution in CH_2Cl_2	10
Add	10-fold excess dicyclohexylcarbodiimide as 1.0 M solution in CH_2Cl_2	50
Add	CH_2Cl_2	60
Wash six times	Ethanol, ethanol, CH_2Cl_2, ethanol CH_2Cl_2, CH_2Cl_2	1

His>PhNCS was detected with modified Pauly reagent (Sanger and Tuppy, 1951).

HISTIDYLPROLYLPHENYLALANYLHISTIDYLLEUCYLLEUCYLVALYLTYROSINE (OCTAPEPTIDE) (**1**). Boc-Tyr(Bzl)-resin was prepared using previously reported techniques (Merrifield, 1963). Analysis showed 0.19 mmol of tyrosine/g of resin (27%). Octapeptide was synthesized using the cycle of operations shown in Table I for the addition of each residue. Amino acids were coupled as their N^{α}-*tert*-butoxycarbonyl derivatives. Side-chain protection was: tyrosine, *O*-benzyl; histidine, N^{im}-benzyloxycarbonyl or N^{im}-tosyl. After addition of all residues, 1.82 g of peptidyl-resin was treated with liquid HF in the presence of anisole at 0° for 1 hr (Sakakibara and Shimonishi, 1965). The residue was extracted three times with aliquots of 10% acetic acid solution and the combined extracts were filtered, lyophilized, transferred to a tared vial with additional acetic acid solution, and relyophilized to yield 60.8 μmol of crude octapeptide (24%).

Crude octapeptide (23 mg) was dissolved with heating in 2.0 ml of 0.01 N HCl, and the solution was filtered and then chromatographed on Bio-Gel P-2 (1.2 × 194 cm) eluted with 0.01 N HCl at a flow rate of 8.7 ml/hr. The elution profile is shown in Figure 1. Fractions eluting between 218 and 243 ml were pooled, lyophilized, and relyophilized in a tared flask from 10% acetic acid solution. This fraction was homogeneous by the tests reported below and contained 7.6 μM peptide (84%): acid hydrolysis: His, 1.93; Pro, 1.04; Phe, 1.06; Leu, 1.98; Val, 1.00; Tyr, 0.96; R_F(T3) 0.66; R_F(T4) 0.15; R_F(T6) 0.54; R_{Leu}(E1) 3.42; mp 212–214° dec; $[\alpha]_D^{20°}$ −76.5° (*c* 0.222, 1.00 M acetic acid); $[\alpha]_D^{25°}$ −69.4° (*c* 0.202, 0.03 N HCl containing 75% ethanol); lit. (Skeggs *et al.*, 1968) mp 191–194°; $[\alpha]_D^{24.5°}$ −68.99° (*c* 1.03, 0.03 N HCl containing 75% ethanol).

D-HISTIDYLPROLYLPHENYLALANYLHISTIDYLLEUCYLLEUCYLVALYLTYROSINE ([D-HIS^1]OCTAPEPTIDE) (**2**). Boc-Tyr(Bzl)-graft copolymer was prepared by refluxing 240 mg of Boc-Tyr(Bzl), 5.00 g of chloromethylated *uncross-linked* graft copolymer, 5.0 ml of ethanol, and 0.081 ml of triethylamine for 24 hr. Analysis showed 0.0397 mmol of tyrosine/g of graft copolymer (31%). The same operations were used to synthesize **2** as **1**. The N-terminal histidyl residue was added as the Z-D-His(Z)·ethanol derivative (Akabori *et al.*, 1958). Treatment of 4.20 g of peptidyl–graft copolymer with liquid HF in the presence of anisole for 1 hr at 0° yielded after extraction, filtration, and lyophilization 27.9 μmol of **2** (17%). Chromatography of 17.1 mg of crude peptide on Bio-Gel P-2 (1.2 × 194 cm) in 0.01 N HCl at a flow rate of 13.8 ml/hr yielded a fraction eluting between 237 and 263 ml which contained 9.9 μmol of homogeneous **2** (92%): acid hydrolysis: His, 2.02; Pro, 1.02; Phe, 0.98; Leu, 2.02; Val, 0.98; Tyr, 0.81; R_F(T3) 0.61; R_F(T4) 0.12; R_F(T6) 0.55; R_{Leu}(E1) 3.25; $[\alpha]_D^{20°}$ +30° (*c* 0.091, 1.00 M acetic acid solution).

HISTIDYLPROLYLPHENYLALANYLLEUCYLLEUCYLVALYLPHENYLALANINE ([PHE^8]OCTAPEPTIDE) (**3**). Boc-Phe was esterified to conventional resin under standard conditions yielding a product which contained 0.14 mmol of phenylalanine/g of resin (20%). The cycle of operations shown in Table I was used to synthesize the peptide. Treatment of 1.35 g of peptidyl-resin with liquid HF in the presence of anisole at 0° for 1 hr yielded, after work-up, 60.2 μmol of **3** (35%). Chromatography of 13.5 mg of crude peptide on Bio-Gel P-2 (1.2 × 194 cm) in 0.01 N HCl gave a fraction eluting between 197 and 225 ml which contained 6.1 μmol of homogeneous **3** (54%): acid hydrolysis: His, 2.04; Pro, 1.01; Phe, 2.06; Leu, 1.94; Val, 0.95; R_F(T3) 0.66; R_F(T4) 0.15; R_F(T6) 0.59; R_{Leu}(E1) 3.25; $[\alpha]_D^{20°}$ −87° (*c* 0.072, 1.00 M acetic acid).

HISTIDYLPROLYLPHENYLALANYLHISTIDYL-D-LEUCYLLEUCYLVALYLTYROSINE ([D-LEU^5]OCTAPEPTIDE) (**4**). Boc-Tyr-(Bzl) was esterified to conventional resin under standard conditions to give a product containing 0.11 mmol of tyrosine/g of resin (16%). Synthesis of **4** was accomplished using the cycle of operations shown in Table I. Treatment of 1.47 g of peptidyl-resin with liquid HF in the presence of anisole at 0° for 1 hr yielded, after work-up, 48.4 μmol of crude **4** (30%). Chromatography of 41.9 mg of crude peptide on Bio-Gel P-2 (2.5 × 194 cm) in 0.01 N HCl yielded a fraction eluting between 719 and 829 ml which contained 8.6 μmol of homogeneous **4** (40%): acid hydrolysis: His, 1.98; Pro, 0.94; Phe, 0.79; Leu, 2.08; Val, 1.01; Tyr, 0.95; R_F(T3) 0.63; R_F(T4)

0.20; R_F(T6) 0.56; R_{Leu}(E1) 3.75; $[\alpha]_D^{20°}$ +21° (*c* 0.047, 1.00 M acetic acid); L-Leu, 1.15; D-Leu, 1.09; L-Tyr, 1.00.

HISTIDYLPROLYLPHENYLALANYLHISTIDYLLEUCYL-D-LEUCYL-VALYLTYROSINE ([D-LEU^6]OCTAPEPTIDE) (**5**). Esterification of Boc-Tyr(Bzl) to graft copolymer under the conditions used in the synthesis of **2** gave a product containing 0.0424 mmol of tyrosine/g of graft copolymer (33%). The cycle of operations shown in Table I was used to synthesize **5**. Boc-D-Leu·H_2O was prepared using the method of Schnable (1967). Treatment of 6.15 g of peptidyl–graft copolymer with liquid HF in the presence of anisole at 0° for 1 hr yielded, after work-up, 75.2 μmol of **5** (31%). Chromatography of 38.6 mg of crude peptide dissolved in 10 ml of 0.01 N HCl on Bio-Gel P-2 (2.5 × 194 cm) eluted with 0.01 N HCl at 33.1 ml/hr yielded 11.9 μmol of homogeneous **5** (48%) eluting between 677 and 767 ml: acid hydrolysis: His, 2.10; Pro, 0.86; Phe, 1.09; Leu, 2.03; Val, 1.00; Tyr, 1.01; R_F(T3) 0.61; R_F(T4) 0.12; R_F(T6) 0.55; R_{Leu}(E1) 3.25; $[\alpha]_D^{20°}$ −26.2° (*c* 0.118, 1.00 M acetic acid); L-Leu, 0.97; D-Leu, 1.03; L-Tyr, 1.00.

HISTIDYLPROLYLPHENYLALANYLHISTIDYLISOLEUCYLLEUCYL-VALYLTYROSINE ([ILE^5]OCTAPEPTIDE) (**6**). Boc-Tyr(Bzl) was esterified to conventional resin under standard conditions to give a product containing 0.090 mmol of tyrosine/g of resin (13%). Synthesis of **6** was accomplished using the cycle of operations shown in Table I. Treatment of 1.43 g of peptidyl-resin with liquid HF containing anisole for 1 hr yielded, after work-up, 125 μmol of crude **6** (88%). Chromatography of 17.6 mg of crude **6** on Bio-Gel P-2 (1.2 × 194 cm) in 0.01 N HCl at 13.9 ml/hr yielded a fraction eluting between 227 and 247 ml. Fractions were pooled, lyophilized, and relyophilized from 6% acetic acid solution to yield 5.5 μmol of homogeneous **6** (55%): acid hydrolysis: His, 2.03; Pro, 1.03; Phe, 1.00; Ile, 0.90; Leu, 1.09; Val, 0.96; Tyr, 0.98; R_F(T3) 0.61; R_F(T4) 0.13; R_F(T6) 0.56; R_{Leu}(E1) 3.50; $[\alpha]_D^{20°}$ −65° (*c* 0.061, 1.00 M acetic acid).

HISTIDYLPROLYLPHENYLALANYLHISTIDYLLEUCYLISOLEUCYL-VALYLTYROSINE ([ILE^6]OCTAPEPTIDE) (**7**). Boc-Tyr(Bzl) was esterified to graft polymer under the conditions used for **2** to give a product containing 0.038 mmol of tyrosine/g of graft copolymer (30%). Synthesis of **7** was accomplished using the cycle of operations given in Table I. Treatment of 7.96 g of peptidyl–graft copolymer with liquid HF in the presence of anisole at 0° for 1 hr yielded, after work-up, 283 μmol of crude **7** (44%). Chromatography of 14.5 mg of this material on Bio-Gel P-2 (1.2 × 194 cm) in 0.01 N HCl at a flow rate of 8.8 ml/hr yielded a fraction eluting between 230 and 247 ml. This was pooled, lyophilized, and relyophilized from 6% acetic acid solution to yield 3.3 μmol of homogeneous **7** (51%). Amino acid analysis of material eluting between 203 and 212 ml indicated the major contaminant of the crude peptide was a failure sequence of the octapeptide lacking phenylalanine: acid hydrolysis: His, 2.00; Pro, 1.07; Phe, 1.00; Leu, 1.04; Ile, 0.76; Val, 0.78; Tyr, 0.86; R_F(T3) 0.63; R_F(T4) 0.13; R_F(T6) 0.56; R_{Leu}(E1) 3.50; $[\alpha]_D^{20°}$ −17° (*c* 0.028, 1.00 M acetic acid).

Determination of Inhibitor Constant (K_i). Human renin (68/356) was obtained from the Division of Biological Standards, London. Tetradecapeptide was purchased (Schwarz/Mann) and the tripeptidyltyrosinol was a kind gift of Dr. T. Kokubu.

In order to determine K_M renin (0.001 Goldblatt unit (GU/ml) was incubated for 1 hr at 37° with incremental concentrations of tetradecapeptide varying between 13 and 108 μM in a volume of 160 μl at pH 5.5 in 0.1 M sodium phosphate containing 0.5% albumin and 0.01% merthiolate (phosphate buffer). The release of angiotensin I was determined at each concentration by radioimmunoassay (Haber *et al.*, 1969), as modified with respect to the separation of free and bound angiotensin (Poulsen, 1969) for high precision. In order to determine the inhibitor constant (K_i) a parallel series of experiments was carried out exactly as described above but in the presence of a single concentration (3–50 μM) of inhibitory peptide. This was dissolved in 0.01 N HCl and concentrations were determined by amino analysis. The peptide solution was diluted to the appropriate concentration with phosphate buffer and the pH adjusted, if necessary, with 1 N NaOH. The cross reaction of the tetradecapeptide with antibody was 1% and with the octapeptides both before and after exposure to renin less than 0.5%. Controls for cross-reactivity were performed with each experiment and appropriate corrections were made when significant.

The inhibitor constant was obtained from a Lineweaver–Burk plot. Each point was given a weight inversely proportional to the square of the error to which it was subject (Dowd and Riggs, 1965).

Determination of Proteolytic Cleavage. The release of free α-amino groups was determined by the ninhydrin reaction modified from the method of Skeggs *et al.* (1968). In a typical experiment, peptide (80–150 μM) was incubated with 0.3 GU/ml of hog renin (Nutritional Biochemical Co.) in 110 μl of the phosphate buffer previously described. At varying times, 100 μl of ninhydrin solution (Hamilton, 1966) was added to an aliquot and the mixture was placed in a boiling-water bath for 15 min, 0.5 ml of ethanol–H_2O (1:1) was added to the hot solution and after centrifugation the absorbance was determined at 570 nm. Corrections for α-amino groups initially present were made by subjecting an unincubated sample to the ninhydrin reaction. The extent of cleavage was expressed as leucine equivalents.

When checking for aminopeptidase and carboxypeptidase activity, the incubated mixture of renin and peptide was applied to the amino acid analyzer without prior acid hydrolysis, and the identity and quantity of free amino acids were determined. Endopeptidase activity was also checked by subjecting the incubated mixture of renin and peptide to Edman degradation (see above).

Determination of Inhibitory Effect of the Peptides in Human Plasma. Human blood was drawn in ice-cold tubes containing EDTA as anticoagulant and the plasma was immediately separated at 4°. The plasma was from a normal person receiving an estrogen-containing medication (Orthanoven) for birth control which insured a high renin substrate concentration. Substrate concentration was determined by measuring angiotensin I concentration by radioimmunoassay after incubation of plasma diluted 1:10 in the phosphate albumin buffer (pH 5.5) with 10 μl of human renin solution (0.1 GU/ml) for 1, 2, 3, and 4 hr in order to convert renin substrate completely to angiotensin I. EDTA, BAL (2,3-dimercapto-1-propanol), and 8-hydroxyquinoline were added as angiotensinase inhibitors (Haber *et al.*, 1969).

The determination of the inhibitor constants was performed in a manner analogous with that described above except that a plasma serially diluted in phosphate buffer (pH 5.5) replaced the tetradecapeptide. The same angiotensinase inhibitors were added.

Results

Comparison between Solid Supports Used in Peptide Synthesis. Chloromethylated *uncross-linked* polystyrene insolu-

Action of Renin on Tetradecapeptide Substrate

Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser

renin ↓

Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu + Leu-Val-Try-Ser
(angiotensin 1)

Action of Renin on Octapeptide Substrate

His-Pro-Phe-His-Leu-Leu-Val-Tyr

renin ↓

His-Pro-Phe-His-Leu + Leu-Val-Tyr

FIGURE 2: Cleavage of substrates by renin.

bilized on Kel-F (graft copolymer) has been reported by Tregear (1972) to give approximately the same yields of peptide as conventional resin when used as a support for solid-phase syntheses. The advantages of the graft copolymer are lack of swelling, nonadherence to glass surfaces, and greater ease of analysis by the automated Edman procedure (Niall *et al.*, 1972).

Synthesis of [D-Leu^6]- and [D-Leu^5]octapeptide as well as [Ile^6]- and [Ile^5]octapeptide were carried out in parallel with identical quantities of reagents. In order to have equimolar amounts of peptidyl-polymer in parallel syntheses 4 and 5 as well as 6 and 7, 5.3 times as much graft copolymer was used as conventional resin. Volumes of support in CH_2Cl_2 were approximately equal, however, because the graft copolymer has a greater density and does not swell appreciably. In both instances, the graft copolymer gave higher yields of final product. Yield (calculated as % esterification × % cleavage × % yield from chromatography) was 4.9% *vs.* 1.9% for 4 and 5 and 6.7% *vs.* 6.3% for 6 and 7. Average yield from esterification and cleavage of all syntheses (calculated as % esterification × % cleavage) is 9.6% for the graft copolymer versus 7.9% for conventional resin. The graft copolymer thus warrants further investigation as a replacement for conventional resins in solid phase peptide synthesis.

Optical Configuration of Incorporated Amino Acids. The method of Manning and Moore (1968) shows incorporation of one residue of D-leucine into analogs **4** and **5**. Incorporation of the N-terminal histidine as the D form in analog **2** is consistent with data from the enzymatic digestion. All of the expected amino acids are present in the correct ratios except proline and one residue of histidine (His, 1.01; Pro, 0.00; Phe, 0.97; Leu, 2.10; Val, 0.88; Tyr, 1.04). The best explanation for these data is that the D-His–L-Pro bond is not subject to cleavage by the enzymes used (Oparil *et al.*, 1971). Digestion of the octapeptide results in release of two histidines as well as one proline (His, 2.19; Pro, 1.02; Val, 1.00). The latter data are also consistent with the suppression of racemization observed when histidine is incorporated as the Boc-His(Tos) derivative (Windridge and Jorgensen, 1971).

Determination of Inhibitor Constant. The tetradecapeptide is a renin substrate releasing angiotensin I after cleavage between leucine-10 and leucine-11. The octapeptide is also a substrate for renin releasing a penta- and a tripeptide following cleavage of the leucylleucine bond (Figure 2) (Skeggs *et al.*, 1968).

A mixture of the two peptides was expected to compete for the catalytic site of renin. Specific antibody is used to measure angiotensin I release from the tetradecapeptide. Since the antibody does not cross-react significantly with either the octapeptide or its cleavage products, angiotensin I may be measured directly in the reaction mixture.

The cross-reactivity of the tetradecapeptide was measured after incubation of the above-mentioned concentrations without renin for 1 hr at 37° and then adding it to the radioimmunoassay mixture. The apparent angiotensin I values obtained were expressed in percentage of the molar tetradecapeptide concentration added and was 1%.

The cross-reactivity of the various peptides and their cleavage products was determined by incubation with renin but without tetradecapeptide at the relevant concentration for 1 hr at 37°.

The cross-reactivity was expressed as for the tetradecapeptide and never exceeded 0.5%. Each determination for K_i was followed by this series of cross-reactivity experiments and the generated angiotensin I concentrations were subtracted from the small apparent angiotensin I values caused by tetradecapeptide as well as inhibitor peptides and their cleavage products.

The octapeptide should act as a competitive inhibitor for the reaction between renin and tetradecapeptide substrate. The rate of cleavage (v) of the tetradecapeptide will therefore be given by (Dixon and Webb, 1964)

$$v = \frac{V}{1 + \frac{K_m}{[S]}\left(1 + \frac{[I]}{K_i}\right)}$$

V and K_m being maximal velocity and Michaelis constant, respectively, for the reaction between renin and tetradecapeptide. The concentration of the octapeptide is called I. The K_i is the inhibitor constant (or when the octapeptide is a substrate, the Michaelis constant).

The rate of reaction between renin and tetradecapeptide was studied by a Lineweaver–Burk plot ($1/v = \alpha(1/[S] + 1/V)$ with and without the octapeptide. From the intercept with the abscissa ($q_1 = -7.15 \times 10^{-2}\ \mu M^{-1}$) K_m for the reaction between human renin and tetradecapeptide was determined to be 143 μM (SEM = 16 μM, $N = 7$). ($\alpha_1 = 2.44$, $1/V_1 = 1.73 \times 10^{-2}\ \mu M^{-1}$.) In the presence of 40 μM octapeptide, the intercept with the ordinate ($1/V_2 = 1.93 \times 10^{-2}\ \mu M^{-1}$) was unchanged indicating competitive inhibition while the intercept with the abscissa is altered (Dixon and Webb, 1964)

$$-q_2 = \text{intercept} = \frac{1}{K_m(1 + [I]/K_i)}$$

From this the apparent K_i for the normal octapeptide was determined to be 39 μM. ($q_2 = -3.58 \times 10^{-3}\ \mu M^{-1}$, $\alpha_2 = 5.35$.)

A series of octapeptide analogs were then tested for their ability to act as competitive inhibitors (Table II). The first six peptides and the tetrapeptide all act as competitive inhibitors. The phenylalanine-8- and D-leucine-6-substituted peptides were more effective inhibitors than the octapeptide. A Lineweaver–Burk plot demonstrating the activity of the latter as a competitive inhibitor gave the following constants: $q_1 = 5.32 \times 10^{-3}\ \mu M^{-1}$, $\alpha_1 = 3.64$, $1/V_1 = 1.94 \times 10^{-2}\ \mu M^{-1}$; and $q_2 = -2.66 \times 10^{-3}\ \mu M^{-1}$, $\alpha_2 = 8.10$, $1/V_2 = 2.16 \times 10^{-2}\ \mu M^{-1}$, where I was 3 μM and K_i was determined to be 3 μM. The isoleucine-6 analog (Table I) was not sufficiently soluble to allow precise determination of the inhibitor constant. The tetrapeptide described by Kokubu *et al.* (1968) was a far weaker competitive inhibitor than any of the octapeptides tested.

TABLE II: Inhibitors and Substrates of Renin.

Peptide (1 2 3 4 5 6 7 8)	K_i (μM)	% of Predicted Cleavage
His-Pro-Phe-His-Leu-Leu-Val-Tyr	39	97
D-His_____	6	30
_____Phe	3	100
_____D-Leu_____	25	0
_____D-Leu_____	3	0
_____Ile_____	7	30
_____Ile_____	>25	20
_____tyrosinol	1020	

Determination of Proteolytic Cleavage. Incubation of the standard octapeptide with renin resulted in an increase in ninhydrin color (Figure 3) with time. The reaction appeared to be complete after 18-hr incubation. The degree of cleavage of the peptide analogs under these conditions is listed in Table II. All the peptides appear to be renin substrates of variable effectiveness except those having a D-leucine on either side of the renin-cleavage site. Thus, the [D-leucine-5]- and [D-leucine-6]octapeptide are competitive inhibitors without being substrates.

The renin preparation was tested for amino- and carboxypeptidase activity by incubation with octapeptide for 18 hr and analyzed for free amino acids on the automatic amino acid analyzer. Aminopeptidase activity was excluded since no N-terminal histidine was detected. However, 6% of the tyrosine and 2% of the leucine were liberated which is compatible with minimal carboxypeptidase activity.

Edman degradation of the unmodified octapeptide yielded both histidine and proline on the first cycle. The result is consistent with the observations of Blombäck *et al.* (1972) who found that when peptides which have an amino-terminal histidine are subjected to Edman degradation both histidine and some of the subsequent amino acid are released. Edman degradation was then performed on 257 μM of octapeptide which had previously been incubated with 3.3 GU of hog renin in a 1-ml volume for 24 hr at 37°. Three residues were now detected after a single Edman cycle, histidine, proline, and leucine. The yield of leucine was 50% as determined by gas–liquid chromatography. Histidine cannot be quantified by gas–liquid chromatography but the sum of proline yields of the first and second cycles was 60%. This suggests that stoichiometric cleavage had occurred at the Leu–Leu bond. Since amino acid analysis had excluded aminopeptidase release of histidine, the results also exclude any other site of cleavage.

Inhibition of the Renin Reaction in Normal Plasma. The K_m for the reaction between added human renin and endogenous renin substrate as present in human plasma was determined to be 0.45 M (SEM = 0.02, $N = 5$): $q_1 = -2.2\ \mu M^{-1}$, $\alpha_1 = 3.38$, $1/V_1 = 7.45\ \mu M^{-1}$. The addition of the normal octapeptide or the D-leucine-5 analog up to concentrations of 63 and 100 μM, respectively, did not result in any inhibition. This indicates that the K_i values are likely to be greater than 150–200 μM if these peptides are capable of acting as inhibitors at all in this medium. Upon addition of the D-leucine-6 analog (30 μM), which had the highest inhibitor constant (3 μM) with respect to synthetic substrate, competitive inhibition could be demonstrated with an apparent K_i of 32 μM ($q_2 = -1.0\ \mu M^{-1}$, $\alpha_2 = 6.8$, $1/V_2 = 6.8\ \mu M^{-1}$).

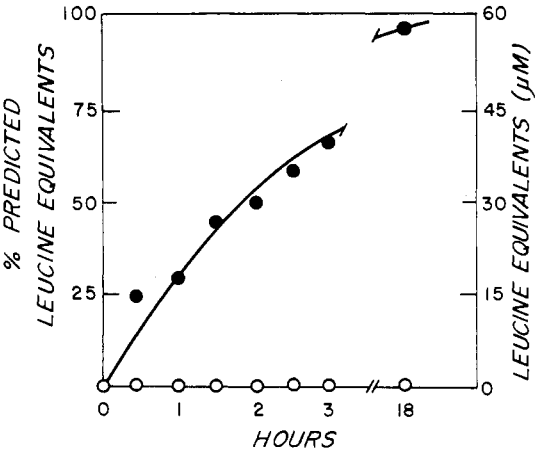

FIGURE 3: The cleavage of the normal octapeptide (60 μM) by renin (0.3 GU/ml) as measured by the increment in ninhydrin color expressed as leucine equivalents (solid circles). Open circles represent the same experiment performed without renin. Temperature was 37°, pH 5.5.

Discussion

The peptide, Pro-Phe-His-Leu-Leu-Val-Tyr-Ser-OMe, which is related to the tetradecapeptide substrate sequence was shown previously to be an inhibitor of the reaction between renin and tetradecapeptide substrate (Skeggs *et al.*, 1964).

We have shown that a closely related octapeptide, His-Pro-Phe-His-Leu-Leu-Val-Tyr, also is a competitive inhibitor for human renin when it acts on the tetradecapeptide. Extensive changes can be made in the sequence of this octapeptide without marked effect on inhibitory activity. A certain degree of sequence specificity must however be preserved since omission of the N-terminal four residues yields a tetrapeptide which has an inhibitor constant three orders of magnitude greater.

All the octapeptide derivatives were cleaved to varying degrees by renin with the exception of the D-leucine-5 and D-leucine-6 analogs. These two peptides are therefore true competitive inhibitors for renin.

Renin cannot be obtained in a pure form. The purified renin preparation used in the cleavage study was shown not to contain aminopeptidase activity but contained minimal carboxypeptidase activity. The failure to cleave the [D-leucine]-octapeptide analogs tends to exclude significant peptidase activity other than renin.

The most potent inhibitor, the D-leucine-6 analog, was active in inhibiting the reaction between human renin and its natural protein substrate present in plasma. The greater apparent inhibitor constant relative to that determined when tetradecapeptide was substrate may have been caused by binding of the peptide to other serum proteins, cleavage of the peptide by the peptidases in plasma not completely inhibited by the mixture of enzyme inhibitors or simply caused by the difference in reaction medium between plasma and buffer.

The [D-leucine-6]octapeptide was shown to be an effective competitive inhibitor at pH 5.5. Relative lack of solubility prevented determination of inhibitory properties at physiologic pH. Preliminary work, however, indicates this problem

can be overcome. A series of inhibitors will then be synthesized which will both permit mapping the active site of renin and provide a powerful tool for demonstrating the physiologic role of the renin–angiotensin system.

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References

Akabori, S., Okawa, K., and Sakajama, F. (1958), *Nature (London) 181*, 772.

Blombäck, B., Hessel, B., Iwanaga, S., Reuterby, J., and Blombäck, M. (1972), *J. Biol. Chem. 247*, 1496.

Dixon, M., and Webb, E. C. (1964), Enzymes, New York, N. Y., Academic.

Dowd, J. E., and Riggs, D. S. (1965), *J. Biol. Chem. 240*, 863.

Haber, E., Koerner, T., Page, L. B., Kliman, B., and Pernode, A. (1969), *J. Clin. Endocrinol. 29*, 1349.

Hamilton, P. B. (1966), *Advan. Chromatogr. 2*, 3.

Kaiser, E., Colescott, R. L., Bossinger, C. D., and Cook, P. I. (1970), *Anal. Biochem. 34*, 595.

Keutmann, H. T., Parsons, J. A., Potts, J. T., Jr., and Schleuter, R. J. (1970), *J. Biol. Chem. 245*, 1491.

Kokubu, T., Ueda, E., Fujimoto, S., Hiwada, K., Kato, A., Akutsu, H., Yamamura, Y., Saito, S., and Mizoguchi, T. (1968), *Nature (London) 217*, 456.

Manning, J. M., and Moore, S. (1968), *J. Biol. Chem. 243*, 5591.

Merrifield, R. B. (1963), *J. Amer. Chem. Soc. 85*, 2149.

Niall, H. D., Tregear, G.W., and Jacobs, J. (1972), *in* Chemistry and Biology of Peptides, Meienhofer, J., Ed., Ann Arbor, Mich., Ann Arbor Science Publishers, p 695.

Oparil, S., Tregear, G. W., Koerner, T. J., Barnes, B. A., and Haber, E. (1971), *Circ. Res. 29*, 682.

Poulsen, K. (1969), *Scand. J. Clin. Lab. Invest. 24*, 285.

Sakakibara, S., and Shimonishi, Y. (1965), *Bull. Chem. Soc. Jap. 38*, 1412.

Sanger, F., and Tuppy, H. (1951), *Biochem. J. 49*, 463.

Sauer, J. C. (1963), Organic Synthesis, Collect. Vol. 4, Wiley, New York, N. Y., p 560.

Schechter, I., and Berger, A. (1967), *Biochem. Biophys. Res. Commun. 27*, 157.

Schnable, E. (1967), *Ann. Chem. 702*, 188.

Skeggs, L. T., Kahn, J. R., Lentz, K. E., and Shumway, N. P. (1957), *J. Exp. Med. 106*, 439.

Skeggs, L. T., Lentz, K. E., Hochstrasser, H., and Kahn, J. R. (1964), *Can. Med. Ass. J. 90*, 185.

Skeggs, L. T., Lentz, K. E., Kahn, J. R., and Hochstrasser, H. (1968), *J. Exp. Med. 128*, 13.

Stahmann, M. A., Fruton, J. S., and Bergmann, M. (1946), *N. Y. Biol. Chem. 164*, 753.

Strosberg, A. D., Fraser, K. J., Margolies, M. N., and Haber, E. (1972), *Biochemistry 11*, 4978.

Tregear, G. W. (1972), *in* Chemistry and Biology of Peptides, Meienhofer, J., Ed., Ann Arbor, Mich., Ann Arbor Science Publishers, p 175.

Webb, E. C. (1960), *in* Steric Aspects of the Chemistry and Biochemistry of Natural Products, Biochemical Society Symposium, No. 19, Grant, J. K., and Klyne, W., Ed., Cambridge, Cambridge University Press, p 90.

Windridge, G. C., and Jorgensen, E. C. (1971), *Intra-Sci. Chem. Rep. 5*, 375.