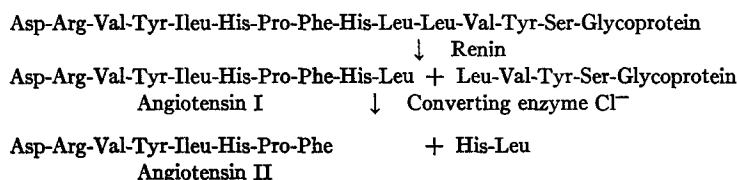# KINETICS OF THE REACTION OF RENIN WITH NINE SYNTHETIC PEPTIDE SUBSTRATES

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The renin-angiotensin system may have a central role in the maintenance of normal blood pressure and in elevating pressures in human essential hypertension. The system is activated by the liberation of the enzyme renin from the kidney into the blood-stream where it hydrolyzes a specific protein substrate, liberating a decapeptide, angiotensin I. A plasma enzyme acts upon this compound releasing a dipeptide, his-leu, and producing angiotensin II, the highly vasoactive octapeptide effector substance of the pressor system (1). These reactions are illustrated in the following equations:

Asp-Arg-Val-Tyr-Ileu-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser-Glycoprotein
$\downarrow$ Renin
Asp-Arg-Val-Tyr-Ileu-His-Pro-Phe-His-Leu + Leu-Val-Tyr-Ser-Glycoprotein
Angiotensin I $\qquad\downarrow$ Converting enzyme Cl⁻

Asp-Arg-Val-Tyr-Ileu-His-Pro-Phe $\qquad$ + His-Leu
Angiotensin II

Renin substrate has been purified (2) from hog plasma, and has been found to exist in five major forms: A, B_1, B_2, C_1, and C_2. All are glycoproteins with molecular weights of about 57,000, and have similar amino acid compositions. All appear to yield the same angiotensin I upon treatment with renin. Differences in the content of sialic acid, neutral hexose, and glucosamine may account for the different chemical properties of the substrates.

Renin substrate, which was prepared from horse plasma, has been treated with trypsin to form a polypeptide fragment which yields angiotensin I upon further treatment with renin. This fragment, a tetradecapeptide, has been purified (3), its structure determined, and it has been synthesized (4).

The kinetics of the reaction of renin with the several forms of substrate have been studied (5). The Michaelis constants (K_m) for A, C_1, and C_2 are nearly the same. The constants for substrates B_1 and B_2 are slightly smaller. The Michaelis constants of substrate A and the tetradecapeptide substrate were identical, and it would appear that the latter is a completely adequate substrate for renin.

It is important to discover the essential structural requirements for a renin substrate. This would permit synthesis of a suitable substrate for the chemical or radio-

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chemical assay of renin. More importantly, it would provide a rational basis for the design of a specific renin inhibitor. It is possible that such an inhibitor might lead to the development of a worthwhile drug for the treatment of human essential hypertension. At the very least, its experimental use would permit a determination to be made of the importance and function of the renin-angiotensin system.

Some of the structural requirements for a renin substrate have been determined and are reported in this paper. In order to accomplish this task, seven peptides, containing from 7 to 12 amino acid residues which represent various portions of the tetradecapeptide molecule, have been synthesized. The product of the reaction of renin with these compounds is biologically inactive. For this reason, it was necessary to devise a highly sensitive chemical method for the determination of the rate of hydrolysis. This method has been applied to the reaction of renin with the seven synthetic peptides, as well as with the tetradecapeptide substrate, under conditions permitting a full description of their kinetic behavior.

Synthesis of Peptide Substrates

All amino acids used were of the l form. All evaporations were conducted under reduced pressure. All peptides and derivatives were examined by descending chromatography on Whatman No. 1 or No. 4 paper. The solvent consisted of n-butyl alcohol–acetic acid–water (4:1:5) except when otherwise indicated. Compounds with free amino groups were first visualized with ninhydrin before staining the imidazole groups with bromphenol blue by the procedure of Durrum (6). The systems used for the countercurrent distributions consisted of (I) secondary butyl alcohol–0.01 M Na_2CO_3–0.01 M $NaHCO_3$ (2:1:1), pH 10.5; and (II) secondary butyl alcohol–0.01 N HCl (1:1), pH 2.4. The distribution pattern was determined by optical density measurements at 279 mμ. Melting points were determined with a Fisher-Johns block and are uncorrected. Amino acid ratios were obtained by automatic ion exchange chromatography of hydrolysates prepared by heating the peptides in 6 N HCl at 110–115°C in evacuated tubes for 24–72 hr. Analytical samples were dried at 110°C under vacuum whenever the melting point permitted; otherwise they were dried at room temperature over magnesium perchlorate or phosphorus pentoxide. Ultimate analyses were performed in a rapid combustion, ultramicro Technicon CHN analyzer employing an integrating catharometer (7).

Z-Pro-Phe-NHNH$_2$.[1]—Z-pro-phe-OMe, 10 g (24.4 mM), mp 73–74°C, was dissolved in 34 ml of ethanol containing 3.8 ml of hydrazine hydrate. The mixture was allowed to stand at room temperature for 65 hr. The resulting crystals were recovered by filtration and were washed with ether. They were dissolved in 100 ml of 0.5 N HCl and the solution was extracted twice with ether. The hydrazide (NHNH$_2$) was precipitated by the addition of 125 ml of 1 M NaHCO$_3$ to the acid solution. The solid was filtered off, suspended in water, and refiltered. Recrystallization from ethanol–water gave 3.4 g, mp 147–148°C, $[\alpha]_D^{25} = -69.9°$ (c 1.01 in methanol).

Analysis: $C_{22}H_{28}N_4O_4$; calculated: C, 64.38; H, 6.39; N, 13.65.
found: C, 64.50; H, 6.45; N, 13.62.

Z-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser-OMe.—Z-pro-phe-NHNH$_2$, 10.26 g (25 mM), was

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[1] The following unusual abbreviations are used in this paper: Z, carbobenzoxy ; OMe, methyl ester; OEt, ethyl ester; Ac, acetate.

dissolved in 330 ml of cold 1 N HCl and 250 ml of water was added. 25 ml of cold 1 M NaNO₂ was added and the resulting azide extracted into 500 ml of ethyl acetate which was then washed with water, 0.5 M NaHCO₃, and again with water. After drying with Na₂SO₄, the solution was concentrated to 50 ml, and 100 ml of dimethylformamide was added.

15.83 g (19.4 mM) of his-leu-leu-val-tyr-ser-OMe·2HCl were dissolved in 600 ml of methanol and 5.6 ml (40.6 mM) of triethylamine were added. The solvents were evaporated, and the residue was suspended in 500 ml of chloroform in order to dissolve the triethylamine hydrochloride. The suspension was filtered and the solid was washed with chloroform and ether. The hexapeptide ester was then dissolved in 500 ml of dimethylformamide and added to the solution of the azide. After standing overnight at 6°C, the reaction mixture was evaporated to 100 ml. 10 volumes of cold 0.5 M NaHCO₃ were added. The resulting precipitate was collected by filtration and redissolved in 1500 ml of the acid solvent system (II). A four stage countercurrent distribution was performed. The upper phases of 0 and 1 were combined and extracted with cold 0.5 M NaHCO₃, then with cold water. Concentration of the solution, followed by chilling to 6°C, caused crystallization. The solid was collected and washed with ether. The product (11.49 g) was recrystallized from 1500 ml of ethanol. After filtration, washing with ether, and drying over P₂O₅, it weighed 7.43 g, mp 226–228°C, $[\alpha]^{29.5}$ = −61° (c 0.5 in 0.05 N HCl containing 50% methanol). Additional product was obtained by concentrating the filtrate; 2.83 g, mp 218–219°C. The amino acid molar ratios were pro, 1.00; phe, 0.92; his, 0.92; leu, 2.00; val, 0.96; tyr, 0.70; ser, 0.92.

Analysis: $C_{58}H_{78}N_{10}O_{13}$; calculated: C, 62.01; H, 7.00; N, 12.45.
found: C, 62.08; H, 7.15; N, 12.45.

Pro-Phe-His-Leu-Leu-Val-Tyr-Ser-OMe·2HCl.—A solution of the protected octapeptide, 8.25 g (7.3 mM), in 250 ml of methanol containing 8.8 ml of 2.5 N HCl and 760 mg of palladium was treated with hydrogen at atmospheric pressure for 150 min. 175 ml of methanol and 150 ml of water were added to redissolve the solids which precipitated during the reduction. The palladium was removed by filtration, and the solution concentrated until a thick gel formed. This was redissolved in methanol and reprecipitated by the addition of five volumes of ether. One-half of this material was purified by countercurrent distribution for 182 transfers in the acid distribution system (II). The major peak was located at this time, and all other tubes were rinsed and refilled. After 794 more transfers (with recycling), the peak tubes (K = 0.41) were removed and the contents were pooled. The solvents were evaporated to 50 ml. After diluting with 100 ml of ethanol and adding 500 ml of ether, the peptide ester precipitated. The solid was redissolved in 200 ml of methanol and reprecipitated with 500 ml of ether. The product was dried over P₂O₅ to give 2.10 g, mp 235–236°C; $[\alpha]_D^{29}$ = −44° (c 0.5 in 0.05 N HCl containing 50% methanol), R_f 0.77; molar ratios: pro, 1.01; phe, 1.01; his, 1.02; leu, 2.00; val, 1.02; tyr, 0.90; ser, 0.95.

Analysis: $C_{50}H_{74}N_{10}O_{11}Cl_2·H_2O$; calculated: C, 55.60; H, 7.11; N, 12.97.
found: C, 55.92; H, 7.32; N, 12.60.

Pro-Phe-His-Leu-Leu-Val-Tyr-Ser.—The peptide ester, 0.266 g (0.25 mM), was dissolved in 30 ml of water with the aid of 0.4 ml of 1 N HCl. The clear solution was adjusted to pH 7. 3.6 ml of 1 N NaOH were then added. After 5 min, the pH was adjusted to 10.5 and the solution distributed in the countercurrent apparatus using the alkaline distribution system (I). After 200 transfers, the peak tubes (K = 1.44) were removed and pooled, the pH was adjusted to 3, and the volume was concentrated to 40 ml. The pH was adjusted to 7.0 and the resulting precipitate was washed repeatedly with water, with 50% methanol-ether, ether, and finally dried over P₂O₅. The peptide was an amorphous solid, 0.155 g, mp 240–242°C (dec), $[\alpha]_D^{22.5}$ = −48° (c 1 in 0.03 N HCl containing 75% ethanol) and $[\alpha]_D^{32.5}$ = −38.7° (c 0.51 in acetic acid),

R_f 0.66 (*n*-butyl alcohol–1.5 N ammonium hydroxide, 1:1); molar ratios: pro, 1.16; phe, 1.02; his, 1.14; leu, 2.00; val, 0.86; tyr, 0.90; ser, 1.12. Mazur (8) found: mp 228–230°C and $[\alpha]_D^{26}$ = −21° (c 1 in acetic acid).

Analysis: $C_{49}H_{70}N_{10}O_{11} \cdot 2.5H_2O$; calculated: C, 57.68; H, 7.43; N, 13.73.
found: C, 57.65; H, 6.97; N, 13.58.

Z-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser-OMe.—Z-his-NHNH$_2$, 0.606 g (2 mM), in 6 ml of 1 N HCl was chilled to 5°C and 0.55 ml of 4 M NaNO$_2$ was added. After 5 min, 7 ml of ethyl acetate and 2.5 ml of 50% K$_2$CO$_3$ were added and the azide was extracted into the organic phase. A second extraction was performed, the combined extracts were washed three times with water, and were then dried over Na$_2$SO$_4$.

A solution of pro-phe-his-leu-leu-val-tyr-ser-OMe·2HCl, 1.06 g (1.00 mM), in 110 ml of dimethylformamide was chilled to 5°C and 0.3 ml of triethylamine was added. The azide and ester solutions were mixed, stored at 6°C for 16 hr, and then evaporated to 100 ml. The residual solution was added to 400 ml of cold 0.1 M sodium phosphate, pH 7. After the addition of 125 g of NaCl, a precipitate formed. This was collected, washed three times with 50-ml portions of water, and then dissolved in the acid solvent system (II). It was subjected to 200 countercurrent transfers. The major peak was retained, fresh solvents were added, and 1287 additional transfers carried out with recycling. The tubes containing the protected nonapeptide were located (K = 1.15), pooled, and concentrated to 200 ml. The pH was adjusted to 7.5. The resulting precipitate was collected by filtration and dried over P$_2$O$_5$. The compound weighed 0.574 g, mp 232–233°C, $[\alpha]_D^{26}$ = −39.7° (c 1 in ethanol), R_f 0.88; molar ratios: his, 2.12; pro, 0.98; phe, 1.00; leu, 2.00; val, 1.01; tyr, 0.86; ser, 0.90.

Analysis: $C_{64}H_{89}N_{13}O_{14} \cdot H_2O$; calculated: C, 60.11; H, 6.87; N, 14.24.
found: C, 60.26; H, 6.79; N, 14.49.

His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser·OMe·3HCl.—The carbobenzoxy nonapeptide ester, 0.515 g (0.41 mM), in 100 ml of ethanol containing 4 ml of 1 N HCl and 0.4 g of palladium was reduced for 9.5 hr with hydrogen at 50 psi pressure. The product precipitated but redissolved on the addition of 20 ml of water. The palladium was removed and the compound precipitated by the addition of 900 ml of cold ether. It was collected by filtration, washed with ether, and dried over P$_2$O$_5$. The product weighed 0.295 g, mp 231–233°C, $[\alpha]_D^{23}$ = −58.4° (c 1.13 in 0.03 N HCl containing 75% ethanol), R_f 0.40.

Analysis: $C_{56}H_{82}N_{13}O_{12}Cl_3$; calculated: C, 54.40; H, 6.56; N, 14.72.
found: C, 54.07; H, 6.46; N, 14.37.

His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser.—The nonapeptide ester, 0.202 g (0.163 mM), in 35 ml of 0.01 N HCl was mixed with 35 ml of 0.2 N NaOH. After 5 min, the pH was adjusted to 10.5 and an equal volume of secondary butyl alcohol was added. The sample was then subjected to 200 countercurrent transfers in the alkaline system (I), the major peak was retained, and 875 more transfers were carried out with recycling. The tubes containing the major peak (K = 1.46) were removed, pooled, the pH was adjusted to 4, and the solution evaporated to 10 ml. After readjusting the pH to 7 and chilling overnight at 6°C, a precipitate formed which was collected by centrifugation and was washed six times with 5 ml of water, twice with a mixture of alcohol and ether (1:1), and once with ether. After drying over P$_2$O$_5$, the peptide weighed 0.040 g, mp 200°C (dec), $[\alpha]_D^{30.5}$ = −60° (c 0.49 in 0.032 N HCl containing 75% ethanol); molar ratios: his, 1.93; pro, 0.91; phe, 0.95; leu, 2.00; val, 0.92; tyr, 0.79; ser, 0.93.

Analysis: $C_{55} H_{77}N_{13}O_{12} \cdot 2H_2O$; calculated: C, 57.52; H, 7.12; N, 15.86.
found: C, 57.34; H, 6.68; N, 15.62.

Z-Ileu-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser-OMe.—Z-ileu-his-NHNH$_2$, 0.832 g (2.00 mM), in 6 ml of 1 N HCl was cooled to 5°C, 0.5 ml of cold 4 M NaNO$_2$ was added and the mixture was stirred for 2 min. At this time, 2.54 ml of 50% K$_2$CO$_3$ was added and the solution was extracted twice with an equal volume of ethyl acetate. The extracts were combined, washed with water, and dried with Na$_2$SO$_4$.

A solution of pro-phe-his-leu-leu-val-tyr-ser-OMe·2HCl, 1.06 g (1.00 mM), in 110 ml of dimethylformamide was chilled to 5°C and 0.3 ml of triethylamine was added. The azide and ester solutions were mixed, and after standing 40 hr at 6°C, 400 ml of 0.1 M sodium phosphate, pH 7.0, was added and the pH adjusted to 7.0 with 1 N HCl. A flocculent precipitate formed which was collected by filtration and washed with water. It was dissolved in the acid counter-current system (II) at pH 2.4, and 200 countercurrent transfers were carried out. The fractions comprising the major peak were retained in the apparatus while all other tubes were rinsed and refilled with fresh solvents. An additional 1400 transfers were performed with recycling. The tubes containing the main peak ($K = 1.06$) were removed, pooled, and the volume was reduced to 100 ml. The pH was raised to 7.5. The resulting precipitate was recovered by filtration and dried over P$_2$O$_5$. The product weighed 0.327 g, mp 189–190°C, $[\alpha]_D^{26} = -46.0°$ (c 1.0 in ethanol), R_f 0.92; molar ratios: ileu, 0.96; his, 2.20; pro, 1.12; phe, 0.98; leu, 2.00; val, 1.16; tyr, 1.12; ser, 0.80.

Analysis: $C_{70}H_{96}N_{14}O_{15}$; calculated: C, 61.20; H, 7.06; N, 14.28.
found: C, 61.06; H, 7.25; N, 14.30.

Ileu-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser.—The protected decapeptide, 0.25 g (0.182 mM), was reduced in a solution of 50 ml of ethanol containing 2 ml of 1 N HCl and 0.315 g of palladium for 17 hr at 50 psi hydrogen pressure. The catalyst was removed and the filtrate evaporated to remove the ethanol. The residue was diluted to 69 ml with water, the pH was adjusted to 4.5, and 7.8 ml of 1 N NaOH was added. After 10 min, the pH was adjusted to 10.5, and the solution was subjected to 200 countercurrent transfers in the alkaline system (I). The main peak was located, retained in the apparatus, and 1361 additional transfers were made with recycling. The major peak ($K = 2.77$) was identified, the component tubes were pooled, the pH was lowered to 4, and the solution was evaporated to 10 ml. The pH was then brought to 7 and the precipitate which formed was collected by centrifugation, washed with water, methanol–ether (2:7), and ether. After drying over P$_2$O$_5$, the peptide weighed 0.047 g, mp 223–225°C (browning at 218–220°C), $[\alpha]_D^{27.5} = -80.4°$ (c 0.515 in 0.03 N HCl containing 75% ethanol), R_f 0.55.

Analysis: $C_{61}H_{88}N_{14}O_{13} \cdot 4H_2O$; calculated: C, 56.46; H, 7.47; N, 15.11.
found: C, 56.80; H, 7.02; N, 15.08.

Ileu-His-OMe·2HCl.—Z-ileu-his-OMe, 2.67 g (6.4 mM), was dissolved in 85 ml of ethanol containing 20 ml of 1 N HCl. One g of palladium was added, and the mixture was reduced with hydrogen at atmospheric pressure for 90 min. The palladium was removed by filtration, and the solution was dried over Na$_2$SO$_4$. It was evaporated to 50 ml and 500 ml of ether was added to precipitate the peptide ester dihydrochloride. This was recrystallized from alcohol and ether (1:1) and dried over P$_2$O$_5$ and NaOH. Yield: 2.1 g, mp 170–172°C, $[\alpha]_D^{26} = +14.7°$ (c 1.29 in methanol), R_f 0.41.

Analysis: $C_{18}H_{24}N_4O_3Cl_2$; calculated: C, 43.95; H, 6.82; N, 15.77.
found: C, 43.99; H, 6.93; N, 15.09.

Z-Tyr-Ileu-His-OMe.—A solution of 3.95 g (12 mM) of Z-tyr-NHNH$_2$ in a mixture of 25 ml of 12 N HCl and 200 ml of water was chilled to 5°C. 12.5 ml of 1 M NaNO$_2$ were added and the resulting azide was extracted into two 50-ml portions of chloroform which were combined, washed with water, with 1% NaHCO$_3$, again with water, and dried with Na$_2$SO$_4$. Triethylamine, 1.75 ml (11.8 mM), was added to the azide solution and immediately followed by 2.1 g (5.9 mM) of ileu-his-OMe·2HCl in 20 ml of dimethylformamide. The clear yellow solution was kept at 6°C overnight. The chloroform was removed by evaporation. The residual dimethylformamide was diluted with 700 ml of water and, after adjusting the pH to 6.5, a flocculent precipitate formed. This was collected by filtration, washed with water, and suspended in 800 ml of 0.01 N HCl. The pH was adjusted to 2, and the suspension was stirred for 1 hr in order to dissolve the tripeptide derivative. This procedure was repeated twice, the extracts were combined, and the pH was adjusted to 6.4. The precipitate that formed was collected by filtration. It was recrystallized from 200 ml of ethanol–water (1:1) to give 1.3 g, mp 196–199°C. An additional 0.55 g, mp 191–193°C was recovered from the mother liquor. Further recrystallization of the crude product from ethyl acetate gave 1.54 g, mp 199–200°C, $[\alpha]_D^{31} = -32.6°$ (c 1.03 in 0.05 N HCl containing 75% ethanol), R_f 0.85.

Analysis: C$_{30}$H$_{37}$N$_5$O$_7$·0.5 H$_2$O; calculated: C, 61.21; H, 6.52; N, 11.90.
found: C, 61.29; H, 6.52; N, 12.18.

Z-Tyr-Ileu-His-NHNH$_2$.—The protected tripeptide, 1.42 g (2.45 mM), after dissolving in a mixture of 27 ml of methanol and 3 ml of hydrazine hydrate, was heated to boiling for 1 min, cooled slowly to room temperature, and finally chilled at 6°C to induce crystallization. The crystals were recovered by filtration, suspended in 300 ml of cold water, refiltered, and washed with cold methanol and ether. The dried material weighed 0.97 g, mp 238–241°C, $[\alpha]_D^{26.5} = -34.4°$ (c 0.32 in 0.01 N HCl containing 90% methanol).

Analysis: C$_{29}$H$_{37}$N$_7$O$_6$; calculated: C, 60.09; H, 6.45; N, 16.92.
found: C, 59.65; H, 6.49; N, 16.57.

Z-Tyr-Ileu-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser-OMe.—Z-tyr-ileu-his-NHNH$_2$, 0.944 g (1.60 mM), in 44 ml of 0.55 N HCl was treated with 1.65 ml of 1 M NaNO$_2$. The pH was raised to 9.3 by the addition of 50% K$_2$CO$_3$, and the azide was extracted into three 40-ml portions of ethyl acetate. The extracts were combined, washed with water, and dried with Na$_2$SO$_4$. The solution (107 ml) contained 0.87 mM, based on its optical absorbance at 275 mμ. To it was added a solution of 1.06 g (1.00 mM) of pro-phe-his-leu-leu-val-tyr-ser-OMe·2HCl in 110 ml of dimethylformamide at 5°C containing 0.3 ml (2.1 mM) of triethylamine. A precipitate formed immediately, but redissolved when a portion of the ethyl acetate was removed by evaporation. The clear solution was chilled to 6°C for 6 hr, and then diluted with 400 ml of 0.1 M sodium phosphate at pH 7.0. The resulting precipitate was collected by filtration and washed with water. The solid was dissolved in the acid solvent system (II) and was subjected to a 200-transfer countercurrent distribution. Two peaks were found: one corresponded to unreacted octapeptide ester and was removed; the other was retained, and an additional 578 transfers were performed with recycling using fresh solvents. The peak ($K = 2.74$) was removed and its component tubes were pooled. After evaporating to 150 ml, the pH was adjusted to 7.5 and crystallization was induced by chilling overnight at 6°C. The recovered and dried product weighed 0.317 g, mp 162–165°C, $[\alpha]_D^{25.5} = -36.1°$ (c 1.02 in ethanol), R_f 0.89.

Analysis: C$_{79}$H$_{105}$N$_{15}$O$_{17}$·2H$_2$O; calculated: C, 60.32; H, 7.00; N, 13.36.
found: C, 60.04; H, 6.59; N, 13.21.

Tyr-Ileu-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser.—The protected undecapeptide, 0.289 g (0.168 mM), was dissolved in a mixture of 35 ml of ethanol and 1.5 ml of 1 N HCl containing

0.2 g of palladium and reduced with hydrogen at 50 psi pressure for 14 hr. The palladium was removed by filtration and washed with water. After removal of the alcohol and adjustment of the volume to 87 ml, the pH was raised to 8 and an additional 10 ml of 1 N NaOH was added. At the end of 5 min, the pH was lowered to 2 and the solution evaporated to 30 ml. The pH was then adjusted to 10.5 and the sample subjected to countercurrent distribution in the alkaline system (I). After 200 transfers, the major peak was located and retained in the apparatus; 1182 more transfers were performed by recycling with fresh solvents. The peak tubes ($K = 3.50$) were pooled, the pH was lowered to 4, and the solution evaporated to 25 ml. The pH was adjusted to 7.3 and the resulting precipitate recovered by filtration, washed with four 9-ml portions of water, once with methanol–ether (2:7), and finally with ether. The peptide, after drying over P_2O_5, weighed 0.071 g, mp indeterminate (above 240°C), $[\alpha]_D^{22.5} = -34.7°$ (c 0.72 in dimethylformamide), R_f 0.75 (n-butyl alcohol–1.5 N ammonium hydroxide, 1:1). Amino acid molar ratios were tyr, 1.70; ileu, 0.96; pro, 0.82; phe, 0.96; leu, 2.00; val, 1.18; ser, 1.00.

Analysis: $C_{70}H_{97}N_{15}O_{15}\cdot3H_2O$; calculated: C, 58.27; H, 7.21; N, 14.57.
found: C, 58.29; H, 7.20; N, 14.13.

Val-Tyr-Ileu-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser.—A solution of 0.107 g (0.066 mM) of val-tyr-ileu-his-pro-phe-his-leu-leu-val-tyr-ser OMe·3HCl (4) in 10 ml of 0.01 N HCl was saponified by the addition of 10 ml of 0.2 N NaOH. After 5 min, the pH was lowered to 10.5 and 10 ml of secondary butyl alcohol were added. The resulting two-phase sample was subjected to countercurrent distribution in the alkaline system (I). After 200 transfers, the major peak was located and all other tubes were emptied and refilled with fresh solvent. At the end of 800 more transfers (with resampling), the tubes containing the peptide peak ($K = 3.83$) were removed, the pH was adjusted to 4, and the volume was reduced to 6 ml by evaporation. The pH was adjusted to 7 and the resulting precipitate was recovered by centrifugation, washed three times with 5 ml of water, once with 5 ml of ethanol–ether (1:1), and twice with 10 ml of ether. The dodecapeptide was dried over P_2O_5 to yield an amorphous powder, 0.036 g, mp 214°C, R_f 0.75.

Analysis: $C_{75}H_{106}N_{16}O_{16}\cdot5H_2O$; calculated: C, 57.09; H, 7.43; N, 14.21.
found: C, 57.26; H, 7.04; N, 13.70.

Z-Leu-Leu-NHNH₂.—10 ml of hydrazine hydrate was added to a boiling solution of Z-leu-leu-OEt (4), 11.6 g (28.6 mM) in 90 ml of methanol. The solution was cooled and, after 48 hr at room temperature, was poured into 1.0 liter of cold water. The resulting oil was extracted into ethyl acetate, which was washed three times with water, and dried with sodium sulfate. The solvent was evaporated to incipient crystallization of the hydrazide. Further cooling followed by filtration provided the dipeptide hydrazide, 9.2 g, mp 152–154°C, $[\alpha]_D^{27} = -47.1°$ (c 1.19 in ethanol).

Analysis: $C_{20}H_{32}N_4O_4\cdot5H_2O$; calculated: C, 61.20; H, 8.23; N, 14.28.
found: C, 60.99; H, 8.13; N, 14.76.

Val-Tyr-OEt-HCl.—Z-val-tyr-OEt (4), 11.05 g (25 mM), was dissolved in 250 ml of ethanol containing 1 g of palladium and 30 ml of 1 N HCl. The mixture was reduced with hydrogen at atmospheric pressure for 3 hr. The palladium was removed and the filtrate was evaporated to dryness. The residue was dissolved in 50 ml of ethanol and precipitated as a gum with 900 ml of ether. The gum crystallized on standing to give 8.53 g, mp 116–119°C, $[\alpha]_D^{28.5} = +28.1°$ (c 1.2 in methanol), R_f 0.78.

Analysis: $C_{16}H_{25}N_2O_4Cl$; calculated: C, 55.73; H, 7.31; N, 8.12.
found: C, 55.63; H, 7.37; N, 8.09.

Z-Leu-Leu-Val-Tyr-OEt.—Z-leu-leu-NHNH$_2$, 3.92 g (10 mM), was dissolved in 200 ml of 0.5 N HCl. The solution was cooled at 3°C and 10 ml of 1 M NaNO$_2$ was added. The azide precipitated and was extracted into two 100-ml portions of ethyl acetate. These were combined and washed with water, 0.5 M NaHCO$_3$, and again with water. The ethyl acetate solution was dried with Na$_2$SO$_4$; since a precipitate appeared during the drying, the ethyl acetate was evaporated and replaced with chloroform which gave a clear solution.

Val-tyr-OEt-HCl, 3.45 g (10 mM), was dissolved in 50 ml of water and chilled to 5°C. The pH was raised to 10 with 50% K$_2$CO$_3$ and the precipitated ester was extracted into two 100-ml portions of ethyl acetate. The combined extracts were washed three times with water and dried with Na$_2$SO$_4$. The dried solution was evaporated to 25 ml and mixed with the chloroform solution of the azide. The mixture was then concentrated to 100 ml and allowed to stand overnight at 6°C. A gelatinous mass of crystals formed; these were filtered off, washed with a small volume of chloroform, and dried over P$_2$O$_5$ to give 3.66 g, mp 200–203°C, raised to 223–224°C by recrystallization from ethyl acetate, $[\alpha]_D^{24.5} = -15.1°$ (c 1.19 in dimethylformamide); molar ratios: leu, 2.11; val, 1.00; tyr, 0.91.

Analysis: C$_{36}$H$_{52}$N$_4$O$_8$; calculated: C, 64.65; H, 7.85; N, 8.38.
found: C, 64.59; H, 7.67; N, 8.62.

Leu-Leu-Val-Tyr-OEt-HCl.—The carbobenzoxy tetrapeptide ester, 2.87 g (4.29 mM), was was suspended in 100 ml of methanol containing 0.5 g of palladium and 5 ml of 1 N HCl. Reduction with hydrogen was carried out at 50 psi pressure for 3.5 hr. After the palladium had been removed, the solution was concentrated to 15 ml, 100 ml of ethanol was added, and the solution was evaporated to a syrup which began to crystallize. When crystallization was complete, the solid was suspended in ether and filtered. After drying over P$_2$O$_5$, the product weighed 2.09 g, mp 253–255°C (dec), $[\alpha]_D^{23.5} = -37°$ (c 1.0 in 0.03 N HCl containing 75% ethanol), R_f 0.89.

Analysis: C$_{28}$H$_{47}$N$_4$O$_6$Cl; calculated: C, 58.88; H, 8.31; N, 9.81.
found: C, 58.59; H, 8.28; N, 9.82.

Z-Pro-Phe-His-OMe.—Z-pro-phe-NHNH$_2$, 3.4 g (8.28 mM), was dissolved in 100 ml of 0.5 N HCl. The solution was cooled to 3°C and 8.5 ml of cold 1 M NaNO$_2$ were added. The resulting azide was extracted into 200 ml of ethyl acetate which was washed with cold water, cold 0.5 M NaHCO$_3$, and again with cold water. The azide solution was dried with Na$_2$SO$_4$ and CaSO$_4$.

His-OMe·2HCl, 2.42 g (10 mM), was suspended in 50 ml of chloroform and 2.76 ml (20 mM) of triethylamine were added. After shaking for 30 min, the chloroform was evaporated and replaced with ethyl acetate. The resulting suspension was filtered to remove amine hydrochloride. A quantitative ninhydrin determination showed the presence of only 3.8 mM of the ester, so a second preparation was made which gave an additional 3.4 mM. The solutions of the azide and the ester were combined, evaporated to 350 ml, and stored at 6°C for 48 hr. The resulting crystals were recovered by filtration and, after drying over P$_2$O$_5$, yielded 2.63 g, mp 150–153°C, $[\alpha]_D^{27} = -51.5°$ (c 1.01 in ethanol) and $[\alpha]_D^{25} = -39°$ (c 1.0 in dimethylformamide). Mazur (8) reports: mp 155–159°C, $[\alpha]_D^{26} = -72°$ (c 1.0 in methanol); DeWald (9) reports: mp 160–162°C, $[\alpha]_D^{23} = -38.2°$ (c 1.0 in dimethylformamide).

Analysis: C$_{29}$H$_{33}$N$_5$O$_6$; calculated: C, 63.62; H, 6.07; N, 12.80.
found: C, 63.72; H, 6.09; N, 13.10.

Pro-Phe-His-OMe·2HCl.—The carbobenzoxy tripeptide ester, 36.7 g (67 mM), was dissolved in 400 ml of methanol containing 12.3 ml of concentrated HCl and 2 g of palladium. Reduction with hydrogen at atmospheric pressure was carried out for 5 hr. The catalyst was

removed and the filtrate was concentrated to 50 ml. Upon addition of this solution to 1800 ml of ether, an oil separated out which crystallized to give 32.11 g upon drying over P_2O_5, mp indeterminate, $[\alpha]_D^{25} = -22.5°$ (c 1.96 in methanol), R_f 0.45.

Analysis: $C_{21}H_{29}N_5O_4Cl_2 \cdot 2H_2O$; calculated: C, 48.27; H, 6.38; N, 13.41.
found: C, 48.29; H, 6.12; N, 13.40.

Z-His-Pro-Phe-His-OMe.—A solution of Z-his-NHNH$_2$, 15.1 g (50 mм), in 160 ml of 1 N HCl was overlaid with 250 ml of ethyl acetate. 52 ml of 1 м NaNO$_2$ were added and the pH raised to 10 with 50% K$_2$CO$_3$. The ethyl acetate layer was separated and the aqueous layer extracted twice more with ethyl acetate. The extracts were combined and dried with Na$_2$SO$_4$.

Pro-phe-his-OMe·2HCl, 22.6 g (46.5 mм), were dissolved in 116 ml of water and two volumes of chloroform were added. The mixture was cooled to 4°C and the pH was adjusted to 9.5 with 40 ml of 50% K$_2$CO$_3$. 42 g of NaCl were added to decrease the solubility of the ester in the aqueous phase. The chloroform layer was separated, and the aqueous phase was extracted twice with an equal volume of chloroform. The chloroform extracts were combined, washed three times with 230 ml of a saturated NaCl solution, and dried with Na$_2$SO$_4$. A quantitative ninhydrin determination showed the presence of 44 mм of the ester.

The azide and the ester solutions were mixed and allowed to stand at 6°C for 48 hr. The solvents were removed by evaporation and the residue was dissolved in 600 ml of chloroform, which was then extracted three times with 500-ml portions of 0.2 м sodium phosphate at pH 9, and three more times at pH 6. The chloroform phase was stirred with cold water and the pH was lowered to 2.0. The organic layer was separated and extracted twice with 250 ml of 0.01 N HCl. Adjustment of the pH of the acid extracts to 9 gave a gummy precipitate which was washed with water and evaporated to dryness. The residue, after drying under vacuum overnight, was dissolved in 30 ml of ethanol and added dropwise to 1 liter of cold ether. The precipitate was collected by filtration, washed with ether, and dried over P$_2$O$_5$ to give 15.92 g, mp 128–130°C, $[\alpha]_D^{25.5} = -57.1°$ (c 0.98 in ethanol).

Analysis: $C_{35}H_{40}N_8O_7$; calculated: C, 61.39; H, 5.90; N, 16.37.
found: C, 61.16; H, 5.79; N, 16.31.

Z-His-Pro-Phe-His-NHNH$_2$.—15.35 g (22.4 mм), of Z-his-pro-phe-his-OMe were dissolved in 144 ml of methanol and 16 ml of hydrazine hydrate were added. The solution was heated to boiling and then allowed to cool and stand at room temperature for 24 hr. The reaction mixture was poured into 1600 ml of water. The resulting oil was extracted with difficulty into two 1600-ml portions of chloroform. The combined chloroform extracts were washed with three 2500-ml portions of a saturated NaCl solution. After drying with Na$_2$SO$_4$, the solvent was evaporated to 300 ml. 700 ml of ethyl acetate were added and the resulting solid was collected. The dried hydrazide weighed 13.45 g, mp 147–149°C. It was recrystallized from methanol–ethyl acetate, from methanol–ether, and was dried over P$_2$O$_5$, 9.44 g, mp 142–144°C.

Analysis: $C_{34}H_{40}N_{10}O_6 \cdot H_2O$; calculated: C, 58.11; H, 6.04; N, 19.94.
found: C, 57.86; H, 5.98; N, 19.47.

Z-His-Pro-Phe-His-Leu-Leu-Val-Tyr-OEt.—The recently described procedure of Mazur and Schlatter (8) was used. 0.343 g (0.50 mм) of Z-his-pro-phe-his-NHNH$_2$ was dissolved in 1.25 ml of dimethylformamide at −40°C and 1 ml of 2 N hydrogen chloride in tetrahydrofuran was added. The mixture was titrated with isoamyl nitrite until a positive starch-iodide test[2]

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[2] One drop of the azide solution removed to a spot plate and acidified with one drop of 0.1 N hydrochloric acid gave a positive test with one drop of 1% starch–0.1 м potassium iodide if excess nitrite were present.

was obtained (0.074 ml). After stirring 30 min at −40°C, 0.37 ml of triethylamine was added, followed by a solution of leu-leu-val-tyr-OEt·HCl, 0.286 g (0.5 mM), in 3 ml of dimethylform-amide at −40°C. The mixture was stirred 30 min longer at −40°C and then stored at −20°C for 5 days. Dilution of the reaction mixture with 10 volumes of water caused a precipitate to form which was recovered by filtration and then washed with water. After drying over P_2O_5, the product weighed 0.51 g, mp 175–177°C (raised to 186–188°C after recrystallization from methanol-ether), $[\alpha]_D^{25} = -77.2°$ (c 1.01 in 0.01 N HCl containing 80% ethanol), R_f 0.86; molar ratios: his, 1.98; pro, 1.04; phe, 1.04; leu, 2.00; val, 1.00; tyr, 2.26.

Analysis: $C_{62}H_{82}N_{12}O_{12}\cdot H_2O$; calculated: C, 61.77; H, 7.04; N, 13.95.
found: C, 61.85; H, 6.85; N, 14.16.

His-Pro-Phe-His-Leu-Leu-Val-Tyr.—The protected octapeptide, 0.264 g (0.20 mM), was dissolved in a mixture of 20 ml of ethanol, 4 ml of water, and 1 ml of 1 N HCl to which 0.2 g of palladium was added. The compound was reduced for 6 hr under hydrogen at 50 psi pres-sure. The catalyst was removed and the alcohol was evaporated from the filtrate. The material was saponified by adjusting the solution to pH 3.3 and then adding 2 ml of 1 N NaOH. After 10 min, the pH was lowered to 7. The precipitate that formed was collected by filtration and washed four times with 20 ml of water, once with 5 ml of ethanol, and three times with 30 ml of ether. The octapeptide, after drying over P_2O_5, weighed 0.162 g, mp 191–194°C (browning at 182°C), $[\alpha]_D^{24.5} = -68.9°$ (c 1.03 in 0.03 N HCl containing 75% ethanol).

Analysis: $C_{52}H_{72}N_{12}O_{10}\cdot 3H_2O$; calculated: C, 57.87; H, 7.30; N, 15.58.
found: C, 57.85; H, 6.64; N, 15.53.

Z-Leu-Leu-Val-OMe.—0.786 g (2.00 mM), of Z-leu-leu-NHNH$_2$ were dissolved in 4 ml of dimethylformamide. 3 ml of 2.67 N hydrogen chloride in tetrahydrofuran were added and the temperature lowered to −40°C. The mixture was titrated with isoamyl nitrite until a positive starch-iodide test was obtained. After stirring 30 min at −40°C, 2.8 ml of triethylamine was added. Val-OMe·HCl, 0.335 g (2.00 mM), in 2 ml of dimethylformamide at −40°C was added to the azide solution. After stirring at −40°C for 30 min, the mixture was stored at −20°C for 3 days. Dilution with 150 ml of water gave a precipitate which was extracted into three 50-ml portions of ether. The combined ether extracts were washed with 50 ml of 1 N HCl and then with three 25-ml portions of water. The ether solution was dried over Na_2SO_4 and evaporated to dryness and desiccated over P_2O_5 to give 0.905 g of the protected tripeptide, mp 114–117°C, $[\alpha]_D^{23} = -49.5°$ (c 1.03 in ethanol).

Analysis: $C_{26}H_{41}N_3O_6$; calculated: C, 63.52; H, 8.42; N, 8.55.
found: C, 63.5; H, 8.39; N, 8.65.

Leu-Leu-Val-OMe·HCl.—A solution of Z-leu-leu-val-OMe, 0.92 g (1.87 mM), in 26.5 ml of methanol and 2.0 ml of 1.0 N HCl containing 0.35 g of palladium was reduced for 5 hr under 50 psi of hydrogen pressure. The palladium was removed and the solution concentrated to 25 ml, and extracted with ether. Evaporation of the aqueous phase to dryness gave 0.577 g, mp 218–220°C, $[\alpha]_D^{24.5} = -71°$ (c 0.99 in methanol).

Analysis: $C_{18}H_{36}N_3O_4$; calculated: C, 54.74; H, 9.21; N, 10.64.
found: C, 54.56; H, 9.39; N, 10.35.

Z-His-Pro-Phe-His-Leu-Leu-Val-OMe.—Z-his-pro-phe-his-NHNH$_2$, 0.535 g (0.78 mM), was dissolved in 2 ml of dimethylformamide and chilled to −40°C. 1.18 ml of 2.67 N hydrogen chloride in tetrahydrofuran was added, followed by 0.118 ml of isoamyl nitrite. After 30 min at −40°C, 0.58 ml of triethylamine and a solution of leu-leu-val-OMe·HCl, 0.308 g (0.78 mM),

in 5 ml of dimethylformamide were added. The mixture was stirred 30 min at −40°C and then stored at −20°C for 48 hr. The addition of 70 ml of water precipitated the crude product which was recovered by filtration and redissolved in 100 ml of 0.02 n HCl. When the pH was adjusted to 6.0, a flocculent precipitate appeared which was collected and washed with water. The dried product weighed 0.392 g, mp 120–123°C, $[\alpha]_D^{24} = -50°$ (c 0.90 in methanol); molar ratios: his, 2.00; pro, 1.06; phe, 0.90; leu, 2.00; val, 1.04.

Analysis: $C_{52}H_{71}N_{11}O_{10} \cdot H_2O$; calculated: C, 60.8; H, 7.17; N, 14.98.
found: C, 60.9; H, 6.98; N, 14.81.

His-Pro-Phe-His-Leu-Leu-Val.—The carbobenzoxy heptapeptide ester, 0.343 g (0.34 mM), in 18 ml of methanol and 2 ml of 1 n HCl containing 0.2 g of palladium was reduced for 7 hr under 50 psi hydrogen pressure. The palladium was removed by filtration and the alcohol by evaporation. The volume of the residual solution was adjusted to 18 ml with water, and the pH to 4.5. The addition of 2 ml of 1 n NaOH caused the separation of a heavy precipitate which largely redissolved during the next 30 min. After filtration, the solution was adjusted to pH 7.0 and evaporated to dryness. The peptide was extracted into 30 ml of alcohol and precipitated with five volumes of ether. The dried heptapeptide weighed 0.209 g, mp 173–176°C, $[\alpha]_D^{26} = -49.7°$ (c 1.45 in methanol), R_f 0.66; molar ratios: his, 2.12; pro, 1.14; phe, 1.02; leu, 2.00; val, 1.04. An analytical sample was recrystallized from ether–0.1 n hydrogen chloride in methanol to give the trihydrochloride, mp 208–211°C.

Analysis: $C_{43}H_{63}N_{11}O_8 \cdot 3HCl$; calculated: C, 53.16; H, 6.86; N, 15.86.
found: C, 53.82; H, 6.79; N, 15.77.

Z-Leu-Val-Tyr-OEt.—To a solution of 6.9 g (20 mM) of val-tyr-OEt·HCl in 100 ml of dimethylformamide containing 3 ml (21.6 mM) of triethylamine was added 7.76 g (20 mM) of Z-leu-OPhNO₂. After the reaction had proceeded 1 hr at room temperature, the mixture was diluted with 1 liter of water. The precipitate was collected and dissolved in ethyl acetate. Extraction with NaHCO₃ solution removed *p*-nitrophenol. The ethyl acetate solution was dried with Na₂SO₄ and evaporated to dryness. The residue was recrystallized from ethanol-water and dried over P₂O₅. Yield: 7.9 g, mp 170°C, $[\alpha]_D^{26} = -24.5°$ (c 0.414 in ethanol).

Analysis: $C_{30}H_{41}N_3O_7$; calculated: C, 64.8; H, 7.45; N, 7.56.
found: C, 65.1; H, 7.65; N, 7.71.

Leu-Val-Tyr-OEt-Ac.—7.53 g (13.6 mM) of Z-leu-val-tyr-OEt was dissolved in 100 ml of ethanol containing 1 ml of glacial acetic acid, and 0.6 g of palladium was added. Reduction was carried out at 50 psi hydrogen pressure for 5 hr. The palladium was removed by filtration, the solution was evaporated to dryness and desiccated over P₂O₅ and NaOH to give 4.5 g of the tripeptide acetate, mp 194–195°C, $[\alpha]_D^{26} = -20.7°$ (c 0.44 in ethanol).

Analysis: $C_{24}H_{39}N_3O_7$; calculated: C, 59.9; H, 8.17; N, 8.72.
found: C, 59.9; H, 8.35; N, 8.75.

Leu-Val-Tyr-Ac.—2.6 g (5.74 mM) of leu-val-tyr-OEt-Ac was suspended with stirring in 0.1 n NaOH for 0.5 hr. Dioxane was then added to complete the solution of the compound and stirring was continued 0.5 hr. The dioxane was removed by evaporation, and the tripeptide dissolved in the alkaline solvent system (I), and subjected to countercurrent distribution. At the end of 200 transfers (K = 0.36), tubes 45 to 66 were pooled and evaporated to dryness. The residue was dissolved in 1 n acetic acid and the pH was adjusted to 3.2 with glacial acetic acid. A second distribution, for the purpose of eliminating NaCl, was run in a secondary butyl alcohol: 0.01 n acetic acid system. After 200 transfers, tubes 35 to 70 (K = 0.25) were pooled

and evaporated. The residue was taken up in ethanol and the tripeptide ester acetate was precipitated with ether and dried over P_2O_5. Yield: 0.77 g, mp 212–214°C (dec), $[\alpha]_D^{24}$ = +34.4° (c 0.44 in glacial acetic acid).

Analysis: $C_{22}H_{35}N_3O_7$; calculated: C, 58.3; H, 7.79; N, 9.26.
found: C, 58.1; H, 8.18; N, 9.51.

Z-Leu-Val-Tyr-Ser-OMe.—The mixed anhydride of Z-leu was prepared by dissolving 2.65 g (10 mM) in 10 ml of tetrahydrofuran followed by the addition of 1.48 ml of triethylamine. This solution was chilled to −5°C and 1.31 ml of isobutyl chloroformate was added. After stirring 10 min at −5°C, the mixed anhydride was combined with a solution of val-tyr-ser-OMe prepared by treating 4.18 g (10 mM) of the ester hydrochloride in 10 ml of dimethylformamide with 1.48 ml of triethylamine at −5°C. The combined solutions were stirred 10 min at 0°C and then heated rapidly to 68°C to complete the condensation.

The reaction mixture was cooled and poured into 200 ml of 5% $NaHCO_3$. The resulting precipitate was recovered by filtration and dissolved in 250 ml of warm ethyl acetate. This solution was extracted successively with 5% $NaHCO_3$, 0.1 N HCl, and water. It was dried with Na_2SO_4 and concentrated to 100 ml. The product crystallized, and was recovered by filtration, and dried. The crude material (mp 183–185°C) was recrystallized from ethyl acetate to give 1.59 g (2.52 mM) of Z-leu-val-tyr-ser-OMe, mp 185–187°C, $[\alpha]_D^{25}$ = −9.6° (c 0.415 in glacial acetic acid).

Analysis: $C_{32}H_{44}N_4O_9$; calculated: C, 61.12; H, 7.06; N, 8.91.
found: C, 60.51; H, 6.59; N, 9.02.

Leu-Val-Tyr-Ser·HCl·H₂O.—To a suspension of 1.58 g (2.51 mM) of Z-leu-val-tyr-ser-OMe in a mixture of 50 ml of methanol containing 3.5 ml of 1 N HCl was added 1 g of palladium. The mixture was treated with hydrogen at 50 psi pressure for 6 hr. The palladium was removed by filtration, 25 ml of water was added, and the methanol was removed by evaporation. The pH was adjusted to 6.2, the volume made to 45 ml, and 7.66 ml of 1 N NaOH were added. After 10 min, the pH was lowered to 6.2 and the solution was evaporated to 25–30 ml where crystallization occurred. The solid was recovered by filtration, washed with water, and dried over P_2O_5. Yield: 1.15 mM; mp 231–233°C.

The product was dissolved in ethanol containing an excess of HCl. The solution was evaporated to dryness. The residue was extracted with ethanol. The solution was clarified by filtration and added to 10 volumes of cold ether. The precipitated peptide hydrochloride was washed with ether and dried over P_2O_5 and NaOH. Yield: 547 mg (1.05 mM), mp 168–170°C.

Analysis: $C_{23}H_{37}N_4O_7Cl·H_2O$; calculated: C, 51.6; H, 7.36; N, 10.47.
found: C, 51.3; H, 7.01; N, 10.38.

Analytical Methods

Reaction rates were determined by continuous measurement of new amino groups as they were formed during the incubation of the enzyme and substrate. Preliminary attempts (10) had been made to accomplish this task using either the ninhydrin reagent or trinitrobenzene sulfonic acid in an alkaline-buffered solution. In the final method now being reported, the ninhydrin method was chosen and especially adapted to the continuous flow method of analysis.

The sample solution was pumped[3] continuously (0.4 ml/min) into the analytical ninhydrin

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[3] The analytical system was composed of AutoAnalyzer equipment manufactured by the Technicon Corporation of Ardsley, New York. The modules employed included a pump II with an air bar, and a 95°C constant temperature bath. The colorimeter was equipped with

reagent (1.2 ml/min) which had been precisely divided into segments by nitrogen gas bubbles (0.24 ml/min). The combined stream was passed through 20 ft of 2 mm i.d. glass coil immersed in a bath at 95°C. Upon emerging, the stream was cooled by passage through a short coil jacketed with cooling tap water and then conducted to the microcolorimeter flow cell. 1 ml/min was aspirated through the flow cell as a solid stream of liquid; the balance was allowed to flow to waste through a 20-ft coil of 2 mm i.d. tubing which had the effect of preventing surging. The entire flow circuit was constructed of glass tubing.

The colorimeter was electrically connected to a recorder employing a four-fold electrical range expansion, and thus the values recorded on the 11-inch chart paper lay between 75 and 100% transmission.

Standardization.—The newly formed amino group produced by the action of renin is, in the case of all of the peptides but one, the N-terminal of the tetrapeptide leu[11]-val[12]-tyr[13]-ser[14]. The single exception is the compound his[6]-pro[7]-phe[8]-his[9]-leu[10]-leu[11]-val[12]-tyr[13] where the product is leu[11]-val[12]-tyr[13].

Both leu-val-tyr-ser and leu-val-tyr were synthesized and suitable standard solutions prepared for analysis.

These solutions were compared with similar standard solutions of L-leucine. The color yields of the two peptides were the same as that of the amino acid within the experimental error of the method. Leucine was thus acceptable as a standard substance and was used throughout the study.

Preparation and Assay of Renin.—The enzyme used was extracted from hog kidneys by the simple acid denaturation method of Haas (11), modified to permit the processing of large amounts of material. The crude extract was fractionated with ammonium sulfate between the limits of 1.0 and 1.75 M at pH 4.0. The preparation was further purified by precipitation as the tungstate. After decomposition of the tungstate complex by calcium, the product was fractionated at pH 4.8 between 33 and 50% acetone and finally dialyzed.

The preparation was assayed by biological methods (5), using a standard renin preparation,[4] and also by the present chemical method. In order to accomplish the latter, an aliquot of the preparation was diluted to an estimated 0.1 Goldblatt unit/ml in a buffer solution consisting of 0.1 M NaCl and 0.05 M sodium phosphate having a pH of 7.5. After warming to 37°C, a sufficient volume of a stock solution of the nonapeptide his[6]-pro[7]-phe[8]-his[9]-leu[10]-leu[11]-val[12]-tyr[13]-ser[14] was added to yield a 20×10^{-6} M substrate concentration. Sampling of the incubation mixture by the analyzer was commenced immediately after mixing and continued for 6–7 min thereafter. The velocity of the reaction (0.24×10^{-6} M/min) was obtained by direct measurement of the resulting recording. Similar velocity measurements were made using appropriate dilutions of the same standard renin solution that was used for the biological assay method. The concentration of enzyme in the preparation was easily calculated from the velocity measurements, and was found to be 45.7 Goldblatt units/ml. The biological assay method yielded a value of 47.6 Goldblatt units/ml. The former value was accepted. The specific activity of the preparation was 12.7 units/mg of protein.

In order to avoid repeated freezing and thawing of the stock renin preparation, 1.0 or 2.0 ml aliquots, sufficient for one day's work, were quick-frozen in 1 ml vials and kept in the freezer until needed.

Preparation and Assay of Stock Substrate Solutions.—Stock solutions of all substrates were

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570 mμ interference filters, and was modified to permit the use of a micro flow cell (1.5 $\times$ 15 mm) with an integral debubblizer. A range expander and recorder were also employed. The recorder was furnished with a slow (10 sec full scale) pen motor in order to increase the available torque.

[4] The standard renin preparation was kindly supplied to us by Dr. Erwin Haas of Mt. Sinai Hospital, Cleveland, Ohio.

prepared by dissolving a carefully weighed amount of the desiccated peptide in 0.01 N HCl. After solution had occurred, the pH was adjusted to between 3.5 and 4.0 with dilute NaOH solution, and the volume adjusted to give a 1.0 mM concentration.

The concentration of hydrolyzable substrate was determined in the stock asp[1]-ileu[5] tetradecapeptide solution by the present chemical method. A very small aliquot of the stock solution, sufficient to yield a substrate concentration of 5×10^{-6} M was added to an ice cold solution of the enzyme containing 0.0915 unit of renin/ml. After initial sampling for 2 min while cold, in order to establish the control value, the mixture was warmed to 37.5°C. The incubation was continued with intermittent sampling for the next 3 hr. At the end of this time, the reaction was very nearly complete. The hydrolyzable substrate was found by this means to be 88% of the theoretical, and the concentration of the stock solution was corrected to this extent.
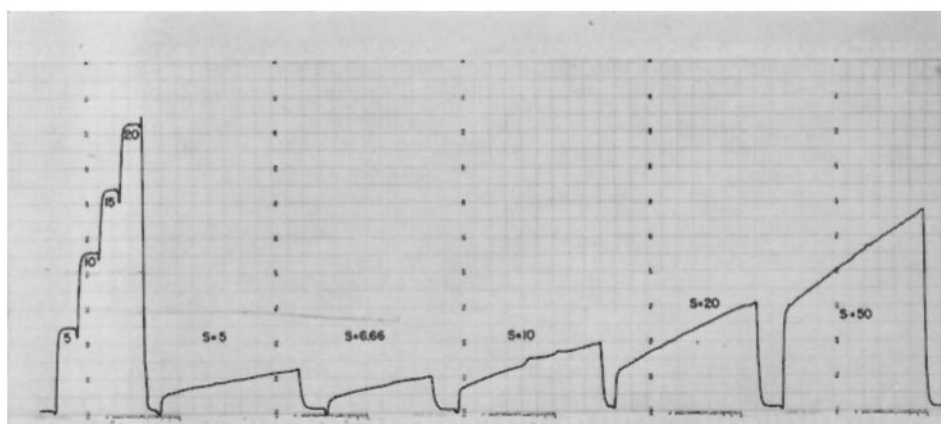

FIG. 1. Measurement of reaction velocities of five different substrate concentrations. Response to 5, 10, 15, and 20×10^{-6} M leucine is shown at left. Recordings from five reaction mixtures containing 5, 6.66, 10, 20, and 50×10^{-6} M of his-pro-phe-his-leu-leu-val-tyr-ser are shown to the right. Renin concentration 0.0915 Goldblatt unit/ml, 1.67 min per division. Temperature, 37°C.

The stock solution of asn[1]-val[5] tetradecapeptide was examined in a similar fashion. In this case, the hydrolyzable substrate was found to be 97% of the theoretical value. The concentration of the substrate stock solution was corrected by this amount.

Experimental Procedure.—The enzyme solution was prepared immediately before use from frozen aliquots of the renin preparation by dilution in a cold buffer consisting of 0.1 M sodium chloride and 0.05 M sodium phosphate having a pH of 7.5. The renin concentrations used in various experiments were 0.0457, 0.0915, or 0.183 unit/ml.

Standard solutions were prepared for each experiment by appropriate dilution of a stock 1.0 mM leucine standard solution in the enzyme solution. Working standard solutions having concentrations from 5 to 20×10^{-6} M, and frequently 50×10^{-6} M, were required and were prepared in intervals of 5×10^{-6} M.

The analyzer was adjusted to 95% transmission, while aspirating the "blank" enzyme solution. Working standard solutions were then sampled for 2-min periods, thus providing the data for the standard curve. Meanwhile, a portion (4–7.4 ml) of the enzyme solution contained

in a siliconized, stoppered test tube was rapidly warmed to 37.5°C. An amount of substrate solution (0.025–0.3 ml) was then added which was adequate to provide the desired concentration (5–50 $\times$ 10^{-6} M). The solution was rapidly mixed and sampling commenced immediately, which was continued until the reaction mixture was exhausted. This usually required from 5–8 min. A recording of a typical experiment is illustrated in Fig. 1.

The peptides his^6-pro^7-phe^8-his^9-leu^{10}-leu^{11}-val^{12}-tyr^{13} and his^6-pro^7-phe^8-his^9-leu^{10}-leu^{11}-val^{12}-tyr^{13}-ser^{14} have low color yields with ninhydrin, and all of their velocity measurements can be made using standard solutions having concentrations between 5 and 20 $\times$ 10^{-6} M.

TABLE I

Kinetic Data Describing Action of Renin on Nine Peptides

Peptide	Renin concentra-tion	$K_m \times 10^6$ M	Average $\times 10^6$ M	$V_{max} \times 10^6$ M/min	$V_{max} \times 10^6$ M/ min per Goldblatt units/ml
	Goldblatt units/ml				
1 2 3 4 5 6 7 8 9 10 11 12 13 14 Asp Arg Val Tyr Ileu His Pro Phe His Leu Leu Val Tyr Ser	0.0457 0.0915	3.66 3.70	3.68	0.48 0.92	10.2
Asn Arg Val Tyr Val His Pro Phe His Leu Leu Val Tyr Ser	0.0457 0.0915	3.18 4.95	4.06	0.37 0.94	9.1
Val Tyr Ileu His Pro Phe His Leu Leu Val Tyr Ser	0.0195 0.1830	30.1 26.0	28.0	0.91 1.45	8.9
Tyr Ileu His Pro Phe His Leu Leu Val Tyr Ser	0.0457 0.0915	23.0 29.2	26.1	0.51 0.78	9.8
Ileu His Pro Phe His Leu Leu Val Tyr Ser	0.0457 0.0915	32.2 29.2	30.7	0.55 0.88	10.9
His Pro Phe His Leu Leu Val Tyr Ser	0.0915 0.1830	52.4 41.3	46.8	1.08 2.13	11.7
His Pro Phe His Leu Leu Val Tyr	0.0915 0.1830	55.2 54.6	54.9	0.74 1.32	7.6
Pro Phe His Leu Leu Val Tyr Ser	0.183 0.366	50.8 56.5	53.6	0.10 0.21	0.55
His Pro Phe His Leu Leu Val	0.183	∞	∞		0

The remaining peptides have very appreciable color yields, and when measuring the reaction velocities at elevated substrate concentrations it was necessary to adjust the baseline using that standard solution as a "blank" which yielded just less optical density than the initial optical density of the reaction mixture. The three standard solutions which are the next most concentrated were then used to produce the standard curve for the experiment.

Due to the four-fold electrical expansion of the recorder scale, the per cent transmission recordings of the standard solutions were very nearly linear with concentration. For this reason, the velocities of the reactions could be determined by direct measurement of the slope of the initial portion of their recordings. All velocity measurements were made during the first 3–5 min of the reaction. Decreasing velocities were often observed after this time, particularly at the lower substrate concentrations.

All reaction velocities were measured in duplicate, and in some cases, triplicate experiments.

The velocity of the reaction of the enzyme with $pro^7-phe^8-his^9-leu^{10}-leu^{11}-val^{12}-tyr^{13}-ser^{14}$ is very slow, and it was necessary in this instance to sample the reaction mixture intermittently over a 100 min period in order to obtain satisfactory measurements.

RESULTS AND DISCUSSION

The kinetic data which describe the action of renin on nine peptides are presented in Table I. These data are derived from the experiments which are graphically illustrated in the Lineweaver-Burk plots of Figs. 2, 3, and 4. The
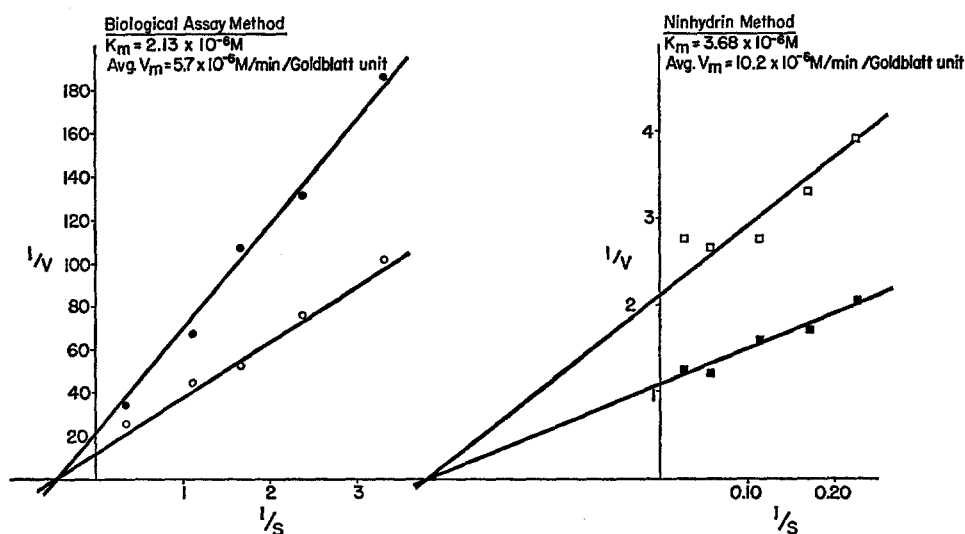

FIG. 2. Lineweaver-Burk plots for the hydrolysis of the asp^1-ileu^5 tetradecapeptide substrate by renin as determined by biological assay and the ninhydrin method. Renin concentration in units per ml: ●, 0.00785; ○, 0.0157; ■, 0.0915; □, 0.0457. Substrate concentration in M × 10^6, and velocity in M × 10^6/min.

constants were obtained by means of the linear equations which best fitted the experimental values as determined by the method of least squares. The mean K_m value obtained with any peptide was used to determine the single intercept on the abscissa which is shown in the graphical presentation.

It was shown by the use of biological assay methods (5) that the tetradecapeptide has a K_z value which is very nearly the same as that of hog substrates A, C_1, and C_2. The maximum velocity (V_{max}) values were also similar. The constants obtained for substrates B_1 and B_2 differed somewhat.

Thus the tetradecapeptide is fully adequate as a substrate when tested in a dilute aqueous solution. When dissolved in plasma, it is susceptible to the
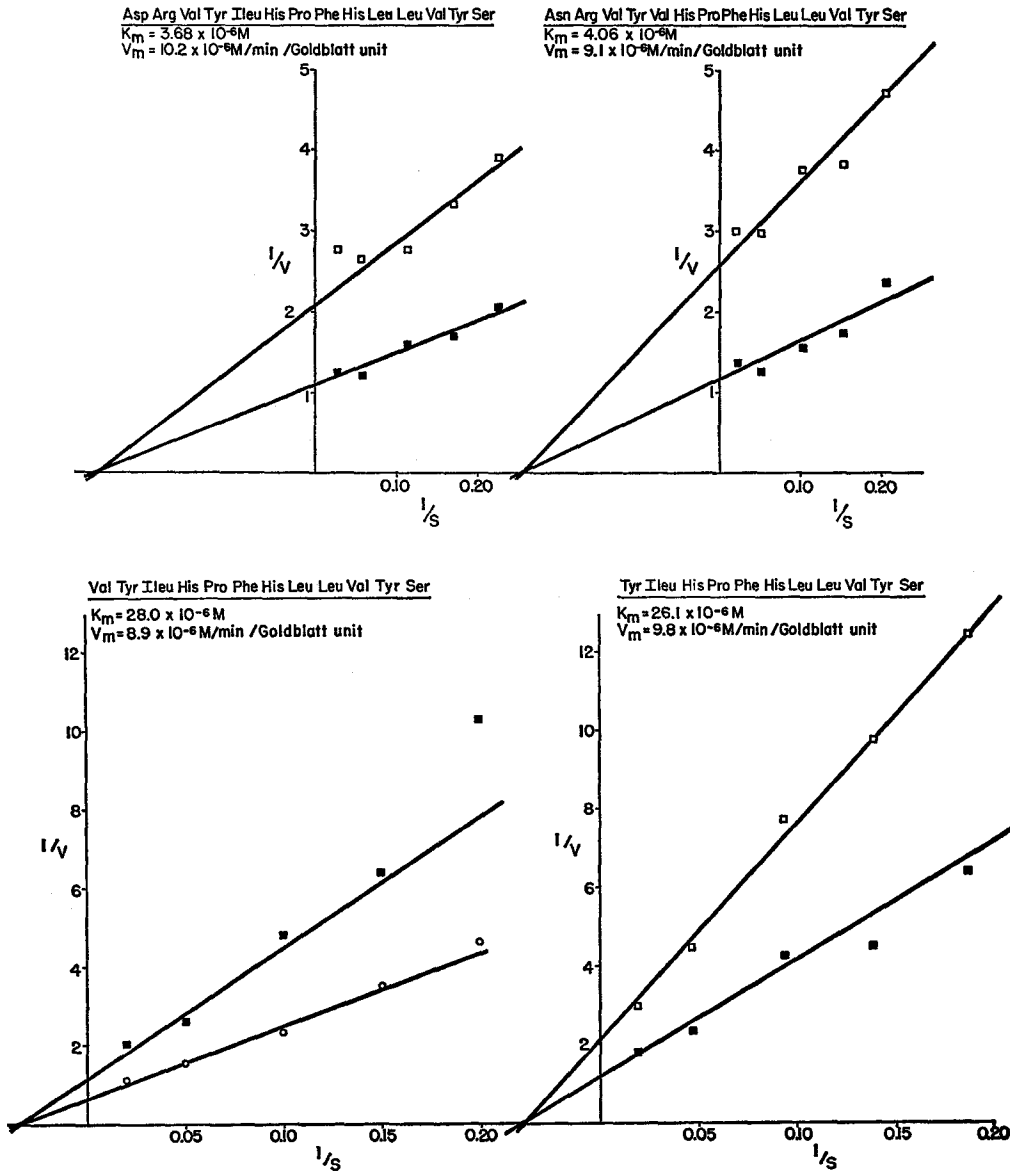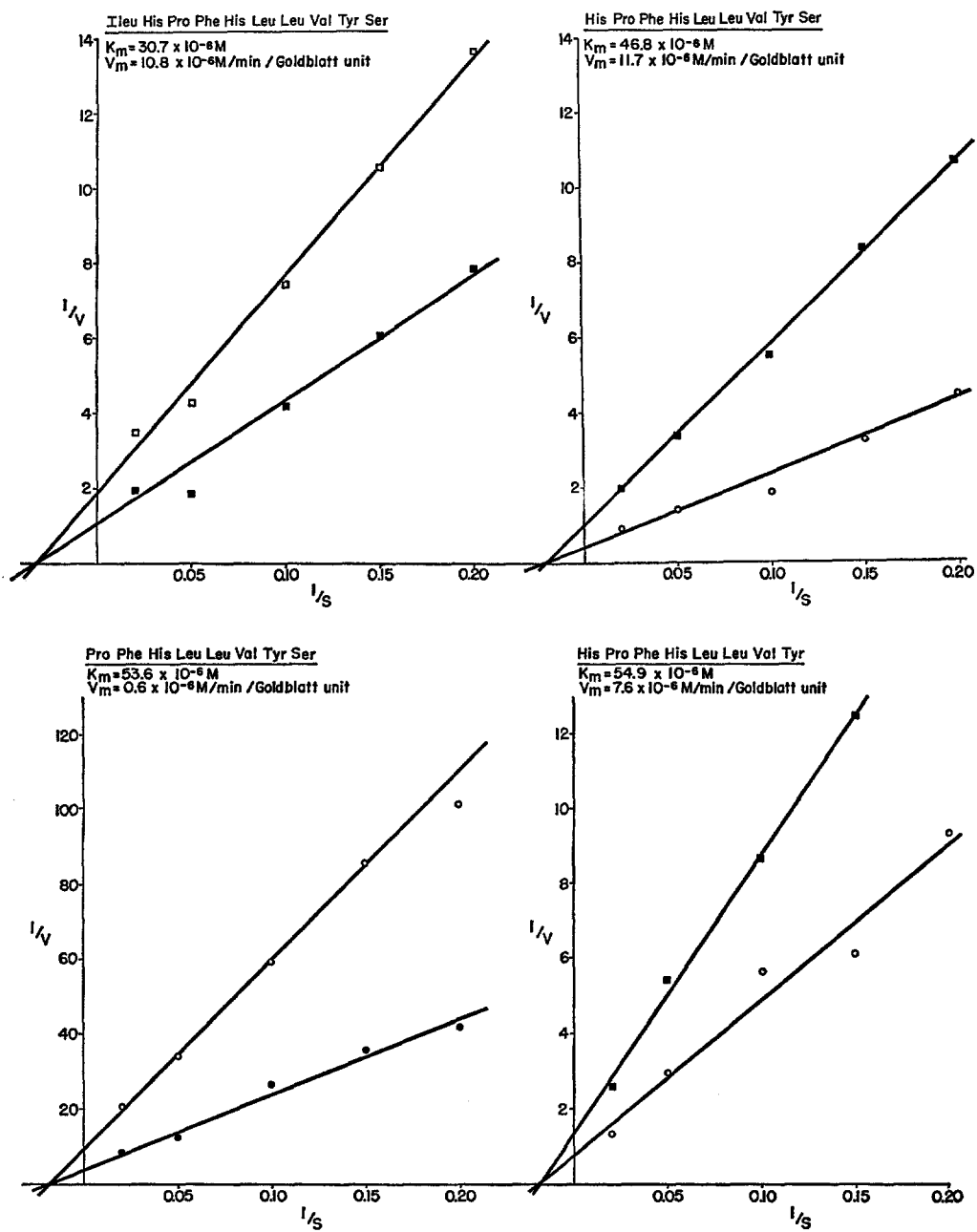
Fig. 3. Lineweaver-Burk plots for the hydrolysis of several synthetic renin substrates. Renin concentration in units per ml: ○, 0.183; ■, 0.0915; □, 0.0457. Substrate concentration in м × 10⁶, and velocity in м × 10⁶/min.

Ileu His Pro Phe His Leu Leu Val Tyr Ser

$K_m = 30.7 \times 10^{-6}M$
$V_m = 10.8 \times 10^{-6}M/min$ / Goldblatt unit

His Pro Phe His Leu Leu Val Tyr Ser

$K_m = 46.8 \times 10^{-6}M$
$V_m = 11.7 \times 10^{-6}M/min$ / Goldblatt unit

Pro Phe His Leu Leu Val Tyr Ser

$K_m = 53.6 \times 10^{-6}M$
$V_m = 0.6 \times 10^{-6}M/min$ / Goldblatt unit

His Pro Phe His Leu Leu Val Tyr

$K_m = 54.9 \times 10^{-6}M$
$V_m = 7.6 \times 10^{-6}M/min$ / Goldblatt unit

Fig. 4. Lineweaver-Burk plots for the hydrolysis of several synthetic renin substrates. Renin concentration in units per ml: □, 0.0457; ■, 0.0915; ○, 0.183; ●, 0.366. Substrate concentration in M × 10⁶, and velocity in M × 10⁶/min.

action of the "angiotensinases" while the protein substrates are not. Its activity may also be reduced by binding to plasma protein although this has not been demonstrated.

It was of considerable interest to compare the K_m and V_{max} values of the asp[1]-ileu[5] tetradecapeptide as previously determined by biological assay with those obtained by the present chemical method. In order to reconcile the values obtained by the two methods insofar as possible, the data derived from the biological assay experiment were recalculated, using the substrate concentration which was found by the chemical method. The latter value was 1.5 times the original concentration determined by biological assay.

As recalculated, the K_m obtained by the biological assay method was 2.13 $\times$ 10^{-6} M while the V_{max} was 5.7 $\times$ 10^{-6} M/min per unit. The corresponding values obtained by the chemical method are 3.68 $\times$ 10^{-6} M and 10.2 $\times$ 10^{-6} M/min per unit. The results obtained by the two methods are graphically presented in Fig. 2.

The identical substrate and renin preparations were used in both methods. However, the substrate concentrations in the biological method were in the range of 0.3–3.0 $\times$ 10^{-6} M, while its concentration in the chemical method lay between 5 and 50 $\times$ 10^{-6} M. The renin concentrations were 7.5 times as great in the chemical method and the incubation time much shorter as well. Thus it is possible that the difference in the constants obtained by the two methods is due largely to the difference in concentration of reactants.

The constants obtained for the asn[1]-val[5] tetradecapeptide are essentially the same as those for the asp[1]-ileu[5] compound. The latter compound is the synthetic product (4) having the structure of the material isolated (3) from horse plasma. The former material was very kindly supplied to us by Dr. Franz Gross[5] (12). It contains a val in position 5 which corresponds to the structure of angiotensin I which was isolated from the ox by Elliott and Peart (13). It is of practical significance that the N-terminal asparagine is a fully adequate substitute for aspartic acid. Future synthesis of the tetradecapeptide may be simplified by the use of the asparagine rather than aspartic acid, since asparagine derivatives are more easily prepared.

The removal of asp[1]-arg[2] from the N-terminal of the tetradecapeptide increases the K_m more than eight-fold, from 3.68 to 28.0 $\times$ 10^{-6} M. Although seemingly rather remote from the hydrolyzable leu-leu bond, this highly polar dipeptide group makes a large contribution to the affinity of the substrate for renin.

The K_m values for the next three smaller compounds in the series are nearly the same, and it would appear that val[3] and tyr[4] do not contribute directly to substrate affinity. There is a sudden increase in the K_m value when ileu[5] is removed, and this amino acid may be important. However, in view of its

nonpolar nature, one is tempted to ascribe its value to one of modifying the charge of the adjacent his^6 residue.

Removal of the his^6 residue to yield the compound pro^7-phe^8-his^9-leu^{10}-leu^{11}-val^{12}-tyr^{13}-ser^{14} increases the K_m values very little, if at all. It does have a profound effect upon the V_{max} value, which is approximately 10×10^{-6} M/min per Goldblatt unit/ml in the case of all of the larger compounds. Removal of the his^6 reduces this value to only 0.55×10^{-6}.

It is apparent that the C-terminal serine is not essential. The data suggests that the K_m may be slightly increased, and the V_{max} somewhat reduced by the removal of this residue.

Removal of tyr^{13} from the octapeptide his^6-pro^7-phe^8-his^9-leu^{10}-leu^{11}-val^{12}-tyr^{13} completely eliminates substrate activity. It must be concluded that this aromatic amino acid is essential for the action of renin on its substrate. Conclusions as to the effect of the removal of tyr^{13} upon the K_m cannot be made from these experiments since no hydrolysis occurred and no measurements could be made.

The findings are valuable for the design of a substrate analogue capable of inhibiting renin. It is apparent that such a compound must contain the N-terminal asp-arg structure in order to achieve sufficient affinity for the enzyme. Since tyr^{13} is essential for substrate activity, it seems likely that the basic structure of an analogue would have to contain at least 13 residues.

The development of a chemical assay method for renin utilizing synthetic substrates would permit large numbers of more precise assays to be accomplished. Equally important is the possibility of standardizing the enzyme on the basis of the V_{max} attainable with the substrate that is chosen.

The nonapeptide his^6-pro^7-phe^8-his^9-leu^{10}-leu^{11}-val^{12}-tyr^{13}-ser^{14} or possibly the octapeptide his^6-pro^7-phe^8-his^9-leu^{10}-leu^{11}-val^{12}-tyr^{13} are attractive possible synthetic substrates. Although the K_ms for these compounds are approximately 12 times larger than for the tetradecapeptide, the difficulties of synthesis are very much less. Further, the reaction velocities can be as great as with the tetradecapeptide, providing adequate substrate concentration is provided.

The compound his^6-pro^7-phe^8-his^9-leu^{10}-leu^{11}-val^{12}-tyr^{13}-ser^{14} was used in this paper for the assay of renin. The method is completely practical, providing the purity of the renin is adequate and the ninhydrin "blank" is not unduly large. Improved methods which will allow assay of impure preparations are under development in this laboratory.

SUMMARY

A number of peptides have been synthesized which represent portions of the tetradecapeptide renin substrate molecule, and which contain the hydrolyzable leu-leu bond. An automatic chemical method for determination of the velocity

of the reaction of renin with these compounds was developed. Application of the method at several levels of substrate concentration permitted construction of Lineweaver-Burk plots, and calculation of Michaelis constants (K_m) and maximal velocities (V_{max}). The results show that the maximum affinity of the enzyme (lowest K_m) for substrate is achieved only with the full tetradecapeptide molecule (asp^1-arg^2-val^3-tyr^4-ileu^5-his^6-pro^7-phe^8-his^9-leu^{10}-leu^{11}-val^{12}-tyr^{13}-ser^{14}). Removal of asp^1 and arg^2 from the N-terminal increases the K_m eight-fold. Further, moderate increase in K_m occurs when the next amino acids, val^3, tyr^4, and ileu^5, are removed. The further removal of his^6 results in a marked reduction in the V_{max} . Removal of ser^{14} from the C-terminal of the nonapeptide his^6-pro^7-phe^8-his^9-leu^{10}-leu^{11}-val^{12}-tyr^{13}-ser^{14} does not greatly affect the K_m nor the V_{max} . Further removal of tyr^{13} from this compound results in complete loss of substrate activity. It is suggested that the compounds his^6-pro^7-phe^8-his^9-leu^{10}-leu^{11}-val^{12}-tyr^{13}-ser^{14} or his^6-pro^7-phe^8-his^9-leu^{10}-leu^{11}-val^{12}-tyr^{13} might be used as substrates for the chemical assay and standardization of renin.

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