or k_5, with little or no effect on the value of K_S', or K_4. It is unfortunate that the insolubility of calcium sulfate does permit observations of the expected increase in rate arising from an increase in k_5 and a decrease in K_4.

While it is tempting to speculate about the results obtained with lithium, sodium, potassium and tetramethylammonium chlorides and with sodium chloride, bromide, iodide, nitrate and acetate, *cf.*, Table III, it is clear that such speculation must be deferred until comparisons can be made of systems possessing uniform mean ion activities. Such systems and those involving binary mixtures of constant mean ion activity are being investigated.

It is appropriate that some reference be made to the possible nature of the species E'. It is known that α-chymotrypsin may associate to form a dimer at high ionic strengths or a polymer at low ionic strengths.[19-27] In view of the fact that all studies were conducted at *p*H 7.90 ± 0.01 and at enzyme concentrations in the range from 0.035 to 0.15 mg. protein–nitrogen per ml., it is unlikely that a significant amount of dimer was present and we can exclude the possibility that in the transformation $E + M \rightleftarrows E'$, E is the dimer and E' the monomer. The absence of any information relative to the catalytic properties of α-chymotrypsin polymer does not permit us to be as positive about rejecting E as α-chymotrypsin polymer. However, since the experiments with the exception of two points in the case of methyl hippurate–sodium chloride were conducted at values of $M > 0.10$, it does not appear likely that the reaction $E + M \rightleftarrows E'$ can be interpreted as a transformation of polymer to monomer. The two remaining alternatives are that E' is actually EM or that E and E' are different forms of monomeric α-chymotrypsin. The lack of dependence of K_2 upon the nature of the salt suggests that EM is not involved but that in proceeding from an environment of low mean ion activity the conformation of monomeric E is altered to produce new conformational species E' that are more effective catalysts than is E.

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Experimental

The general procedure has been described previously.[5] The two specific substrates were prepared as before.[5,6] All experiments were performed in chemically unbuffered solutions using a *p*H-Stat which poised the reaction systems at *p*H 7.90 ± 0.01 at 25.0°. The enzyme preparation was Armour no. 234. The concentration of enzyme was varied from 0.035 to 0.15 mg. protein–nitrogen per ml., the lower concentrations of enzyme being employed at the higher salt concentration and *vice versa*. This was done to limit possible consequences of dimerization at the higher salt concentrations. Other pertinent details are given in Table I. The upper limits used for calcium chloride and magnesium chloride were determined by the precipitation of enzyme at higher concentrations. Both the calcium and magnesium chlorides were reagent samples stated to contain 99% calcium or magnesium chloride. It was necessary to use mean ion activities in order to interpret the data meaningfully. The values of the activity coefficients of sodium and potassium chloride were interpolated from those given by Stokes and Levien.[28] The activity coefficients of calcium and magnesium chlorides and of sodium sulfate were interpolated from those reported by Stokes.[29] Those of sodium bromide and sodium nitrate were interpolated from the data given by Harned and Owen.[30] The observed initial velocities were corrected for an enzyme blank, evaluated for each salt concentration, and for a blank arising from the hydrolysis of the specific substrate in the absence of enzyme, which also was evaluated for each salt concentration.

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PASADENA, CALIFORNIA

[CONTRIBUTION FROM THE BIOCHEMISTRY DEPARTMENT, UNIVERSITY OF PITTSBURGH, SCHOOL OF MEDICINE]

Studies on Polypeptides. X. The Synthesis of a Pentapeptide Corresponding to an Amino Acid Sequence Present in Corticotropin and in the Melanocyte Stimulating Hormones[1]

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A synthesis of the pentapeptide histidylphenylalanylarginyltryptophylglycine (L,L,L,L) is described. The synthetic product behaved as a single component when analyzed by paper chromatography in two different solvent systems. The behavior of the pentapeptide toward leucine aminopeptidase and trypsin, respectively, was investigated. The former enzyme converted the peptide into an equimolar mixture of the constituent amino acids, and the latter cleaved the arginine-tryptophan bond with the formation of histidylphenylalanylarginine and tryptophylglycine. These results provided conclusive evidence for the stereochemical homogeneity of the synthetic product.

In connection with our studies of synthetic polypeptides corresponding to amino acid sequences which are common to corticotropin and to the

(1) The authors wish to express their appreciation to the U. S. Public Health Service, the National Science Foundation, Armour and Company and Eli Lilly and Company for generous support of this investigation.

(2) The name of this author was misspelled in communication IX of this series (see ref. 6).

melanocyte stimulating hormones (α- and β-M.S.H.),[3-6] we have now completed a synthesis of the pentapeptide L-histidyl-L-phenylalanyl-L-arginyl-L-tryptophylglycine. The sequence corresponding to this peptide occupies positions 6 to 10 in corticotropin[7-10] and α-M.S.H.[11] and is located at positions 9 to 13 in β-M.S.H.[12,13]

Carbobenzoxy-L-tryptophan[14] was coupled (by the use of N,N'-dicyclohexylcarbodiimide)[15] with methyl glycinate to give methyl carbobenzoxy-L-tryptophylglycinate. Saponification converted the acylated dipeptide ester into carbobenzoxy-L-tryptophylglycine, which had been prepared previously by treating the acid chloride of carbobenzoxy-L-tryptophan with sodium glycinate.[14] Catalytic hydrogenation converted the acylated dipeptide into L-tryptophylglycine. The optical rotation and melting point of our product were in agreement with values given in the older literature.[16] The dipeptide behaved as a single component when analyzed by paper chromatography, exhibiting an R_f value of 0.59 in the Partridge system[17] and traveling somewhat faster than methionine in 2-butanol-ammonia.[18] A similar chromatographic behavior was observed regardless of whether the papers were sprayed with ninhydrin or with *p*-dimethylaminobenzaldehyde (Ehrlich reagent). Leucine aminopeptidase[19] converted the peptide into an equimolar mixture of tryptophan and glycine, demonstrating that no racemization had taken place during the various steps in its synthesis. Unhydrolyzed dipeptide was not seen on the chromatograms.

Treatment with benzyl alcohol and hydrogen chloride converted the dipeptide into its benzyl ester hydrochloride. The free benzyl ester (liberated from the hydrochloride with triethylamine) was then coupled in N,N-dimethylformamide solution with carbobenzoxy-L histidyl L-phenylalanyl-nitro-L-arginine[6]; N,N'-dicyclohexylcarbodiimide served as the condensing agent. The reaction product (carbobenzoxyhistidylphenylalanylnitro arginyltryptophylglycine benzyl ester) was obtained in the form of a pale yellow powder by precipitation from ethanol solution with ether. The yield varied between 80 to 85% in a series of experiments. The analysis of this substance by paper chromatography in two different solvent systems revealed the presence of one major component with an R_f value of 0.89 in the Partridge system. The compound was located below phenylalanine in the 2-butanol–ammonia chromatograms. The material was ninhydrin-negative but was readily identified on the papers by its positive reaction with the Pauly and the Ehrlich reagents; the color spots with both these reagents exhibited identical R_f values. Samples of this material were subjected to hydrogenation over palladium in acetic acid, and the corresponding pentapeptide acetates were isolated in the form of pink powders by freeze-drying. Paper chromatography of these products revealed the presence of one major component which reacted positively with the ninhydrin, Pauly, Ehrlich and Sakaguchi reagents. The material had an R_f value of 0.51 in the Partridge system and migrated at the rate of phenylalanine in 2-butanol–ammonia. In addition to the main product, several minor impurities also were present. Samples of the crude pentapeptide were hydrolyzed with acid, and the hydrolyzates were subjected to quantitative amino acid analyses on paper. Histidine, phenylalanine, arginine and glycine were present in molar ratios of 1:1:1:0.9; tryptophan was absent. The destruction of this amino acid during acid hydrolysis of peptides and proteins is well substantiated. These results indicated that the product contained, as the main component, a pentapeptide possessing the sequence his-phe-arg-try-gly, but they provided no information regarding the stereochemical purity of the substance. In order to gain information on this crucial point, the behavior of the product toward trypsin and leucine aminopeptidase, respectively, was investigated. Trypsin was expected to hydrolyze the arginine–tryptophan bond with formation of histidylphenylalanylarginine and of tryptophylglycine. Both these peptides were available to us, and preliminary experiments demonstrated that they are readily separable by paper chromatography. The analysis of a tryptic digest of the pentapeptide, of approximate enzyme–substrate ratio 1:100, showed the presence of these peptides, but much unhydrolyzed material was also seen on the chromatograms. By the use of a standard curve, prepared with pure histidylphenylalanylarginine acetate,[6] it was possible to evaluate quantitatively the degree of splitting of our peptide. Only 37% of the expected quantity of the tripeptide was found. Increasing the enzyme concentration or extending the incubation time did not alter these results. Obviously, the crude pentapeptide was not homogeneous. Experiments with leucine aminopeptidase led to similar conclusions. The molar ratios of histidine, phenylalanine, arginine, tryptophan and glycine in the enzymatic digests were 1:1:0.4:0.4:0.4, a finding which is in good agreement with the trypsin results. Both techniques demonstrated that the pentapeptide contained only some 40% of the desired all-L isomer[20]

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(20) Using the techniques described in this paper, we have also prepared the acetate salts of histidylphenylalanylarginyltryptophan; R_f^{17} = 0.51, R_f^{18} = phe, and of phenylalanylarginyltryptophan; R_f^{17} = 0.66, R_f^{18} = phe$^+$. The former compound exhibited positive

Since the pentapeptide his-phe-arg-try-gly represents an important intermediate for the preparation of certain pituitary hormones, it became of prime importance to have available for further work a method for the preparation of its all-L form. Stereochemically homogeneous samples of the peptide were prepared by the following reproducible procedure. The crude carbobenzoxyhistidylphenylalanylnitroarginyltryptophylglycine benzyl ester was dissolved in absolute ethanol at 50° and the solution was kept at room temperature for one week, when a fraction of the material (19%) had precipitated in crystalline form. The crystals were collected and were recrystallized to constant rotation from absolute ethanol. The crystalline material exhibited a sharp melting point of 183–185° and had a more negative rotation than the starting material. The elementary composition was in good agreement with that expected, and the paper chromatographic evaluation demonstrated the presence of a single component exhibiting a negative ninhydrin but positive Pauly and Ehrlich reactions. Hydrogenation over palladium in acetic acid, followed by freeze-drying, gave a pink powder of the acetate dihydrate of L-histidyl-L-phenylalanyl-L-arginyl-L-tryptophylglycine. This pentapeptide behaved as a single component in both the Partridge and the 2-butanol–ammonia systems regardless of whether the ninhydrin, the Pauly, the Ehrlich or the Sakaguchi test was used for its localization on the papers. On tryptic digestion, this material liberated 95% of the expected quantity of histidylphenylalanylarginine; and leucine aminopeptidase converted the substance into an equimolar mixture of the constituent amino acids. Unhydrolyzed material could not be demonstrated on the chromatograms from either digest. These findings establish beyond doubt the stereochemical homogeneity of this pentapeptide.

The experiments which are presented in this communication demonstrate that the coupling (by means of N,N′-dicyclohexylcarbodiimide) of carbobenzoxy-L-histidyl-L-phenylalanylnitro-L-arginine with the benzyl ester of L-tryptophylglycine affords a carbobenzoxyhistidylphenylalanylnitroarginyltryptophylglycine benzyl ester which is not homogeneous. This follows from the observation that the material resulting from the hydrogenation of the blocked pentapeptide ester was only partially hydrolyzed by two proteolytic enzymes. The results with both enzymes point to racemization of the arginine–tryptophan bond in the crude pentapeptide, but further studies will be necessary to establish this point. One may conclude, however, that caution is indicated in accepting the homogeneity of complex polypeptides which are prepared by the carbodiimide method[21] until their all-L configuration is conclusively demonstrated. Enzymatic techniques, such as the ones described in this communication, are valuable tools for this purpose.

reactions with the ninhydrin, Pauly, Sakaguchi and Ehrlich reagents, the latter, with the ninhydrin, Sakaguchi and Ehrlich reagents only. Enzymatic studies with trypsin and leucine aminopeptidase, respectively, indicated that these products contained only some 40% of the all-L isomers. The preparation of homogeneous samples of these compounds has not yet been accomplished.

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Experimental[22]

Methyl Carbobenzoxy-L-tryptophylglycinate.—Glycine methyl ester hydrochloride (0.413 g.) was dissolved in methanol (5 ml.) and 0.5 *N* sodium methoxide in methanol (5.92 ml.) was added. The methanol was evaporated, dioxane (10 ml.) was added to the residue and the suspension was mixed with a solution of carbobenzoxy-L-tryptophan (1 g.) (m.p. 127–129°)[14] in dioxane (5 ml.). N,N′-Dicyclohexylcarbodiimide (0.618 g.) was added, and the mixture was kept at room temperature for 6 hr. The suspension was filtered, the filtrate was evaporated to dryness *in vacuo* at 40° (bath temperature) and the oily residue was dissolved in ethyl acetate. The solution was washed in the usual manner with 2 *N* hydrochloric acid, water, saturated sodium bicarbonate and water and dried over anhydrous sodium sulfate. Evaporation of the ethyl acetate gave a yellow oil which was triturated with ether. The white solid thus obtained was washed with ether, recrystallized from a mixture of ethanol and water (1:2), and dried; yield 0.71 g. (59%); m.p. 158–159°; $[\alpha]^{27}D$ −11.0° (*c* 2.0, in glacial acetic acid).

Anal. Calcd. for $C_{22}H_{23}O_5N_3$: C, 64.5; H, 5.7; N, 10.3. Found: C, 64.6; H, 5.7; N, 9.9.

Carbobenzoxy-L-tryptophylglycine.—Methyl carbobenzoxy-L-tryptophylglycinate (0.4 g.) was dissolved in methanol (10 ml.) and *N* sodium hydroxide (1.2 ml.) was added. The mixture was shaken at room temperature for 1 hr., when the bulk of the methanol was removed *in vacuo* (bath temperature, 20°). The resulting solution was extracted with three portions of ethyl acetate, and the aqueous phase was acidified to congo red with 2 *N* hydrochloric acid. The acylated dipeptide was extracted with ethyl acetate, the ethyl acetate extracts were washed with saturated sodium chloride solution, dried over anhydrous sodium sulfate and the solvent evaporated *in vacuo*. The ensuing white foam was dissolved in a small quantity of ethyl acetate and petroleum ether (b.p. 30–60°) was added until the solution became cloudy. On standing, the carbobenzoxy-L-tryptophylglycine separated as a white solid; yield 0.32 g. (82%); m.p. 158–159° (lit.[14] m.p. 156°).

L-Tryptophylglycine.—Carbobenzoxy-L-tryptophylglycine (0.5 g.) was hydrogenated over a palladium catalyst, in 15 ml. of methanol containing 10% of acetic acid (v./v.), until the evolution of carbon dioxide had ceased. The catalyst was removed by filtration, the solvent was evaporated *in vacuo* and absolute ethanol was added to the residue. The resulting solid was recrystallized from a mixture of water and ethanol (1:20); yield 0.26 g. (79%); m.p. 176–178° (lit.[16] m.p. 180°); $[\alpha]^{26}D$ +81.7° (*c* 1.74, in water) (lit.[16] $[\alpha]D$ +78.5° in water); R_f^{17} = 0.59, R_f^{18} = met$^+$; Try:Gly ratio in leucine aminopeptidase digest 1:1.

Anal. Calcd. for $C_{13}H_{15}O_3N_3$: NH_2-N, 5.4. Found: NH_2-N, 5.3.

L-Tryptophylglycine Benzyl Ester Hydrochloride.—L-Tryptophylglycine (3 g.) was suspended in benzyl alcohol (60 ml.), and the solution was saturated with hydrogen chloride while being cooled in an ice-salt bath. Dry benzene (20 ml.) was then added and the water was removed by azeotropic distillation *in vacuo* (bath temperature, 50°). Saturation with hydrogen chloride and removal of the water by azeotropic distillation with benzene was repeated twice, and the solution was subsequently evaporated (bath temperature, 75–80°) at a pressure of 1 mm. The ensuing oil was triturated in a mortar with ether until crystallization occurred. The material was recrystallized twice from absolute ethanol by the addition of ether. Colored impurities were removed by treatment with Norit A; yield 3.2 g. (72%); m.p. 172–174°; $[\alpha]^{25}D$ +27.0° (*c* 1.2, in absolute ethanol).

Anal. Calcd. for $C_{20}H_{22}O_3N_3Cl$: N, 10.8; Cl, 9.2. Found: N, 10.7; Cl, 9.4.

Carbobenzoxyhistidylphenylalanylnitroarginyltryptophylglycine Benzyl Ester.—a. Crude Product.—The preparation of this product was repeated several times with comparable results. A typical experiment was performed as follows: L-Tryptophylglycine benzyl ester hydrochloride (2.52 g.) was dissolved in dry methanol (10 ml.) and tri-

(22) The melting points are uncorrected. Optical rotations were determined with a Rudolph precision polarimeter, model 80, with model 200 photoelectric attachment.

ethylamine (0.90 ml.) was added. The solution was evaporated to dryness *in vacuo* at a bath temperature of 30°, and the residue was dissolved in N,N-dimethylformamide (20 ml.). To this solution was added carbobenzoxy-L-histidyl-L-phenylalanylnitro-L-arginine[6] (4.14 g.) dissolved in N,N-dimethylformamide (30 ml.) and then N,N'-dicyclohexylcarbodiimide (1.61 g.). The reaction mixture was kept at room temperature for 16 hr., a few drops of glacial acetic acid were then added and the N,N'-dicyclohexylurea was removed by filtration. The filtrate was evaporated to dryness at 1 mm. pressure (bath temperature, 50°) and the remaining yellow sirup was dissolved in ethyl acetate by the addition of saturated sodium bicarbonate. The ethyl acetate solution was washed successively with water, 5% aqueous acetic acid, water, saturated sodium bicarbonate and water and was dried over anhydrous sodium sulfate. The ethyl acetate was removed *in vacuo*, leaving a yellow sirup which was dissolved in absolute ethanol (30 ml.). The product was precipitated by the addition of ether and was dried *in vacuo* at room temperature over phosphorus pentoxide. A pale-yellow powder was obtained; yield 5.4 g. (86%); $[\alpha]^{28}D$ −23.2 (*c* 1, in N,N-dimethylformamide); R_f^{17} = 0.89, R_f^{18} = phe$^+$, ninhydrin reaction negative, Pauly and Ehrlich reactions positive. A sample for analysis was precipitated three times from ethanol with ether and dried *in vacuo* at 80° for 12 hr.

Anal. Calcd. for $C_{49}H_{54}O_{10}N_{12}$: C, 60.5; H, 5.6; N, 17.3. Found: C, 59.6; H, 5.9; N, 17.2.

b. All-L Form.—A sample of the above crude material (4.9 g.) was dissolved at 50° in absolute ethanol (40 ml.), and the mixture was kept at room temperature for one week, when crystallization occurred. With the availability of seed crystals, the crystallization was usually complete after 40 hr. The crystals were collected, washed with ice-cold ethanol and recrystallized to constant rotation from absolute ethanol; yield 0.95 g. (19%); m.p. 183–185°; $[\alpha]^{27}D$ −29.0° (*c* 0.89, in N,N-dimethylformamide); R_f^{17} = 0.89, R_f^{18} = phe$^+$, ninhydrin reaction negative, Pauly and Ehrlich reactions positive. A sample for analysis was twice recrystallized from ethanol and dried at 80° *in vacuo* for 6 hr.

Anal. Calcd. for $C_{49}H_{54}O_{10}N_{12}$: C, 60.5; H, 5.6; N, 17.3. Found: C, 60.1; H, 5.7; N, 17.3.

Histidylphenylalanylarginyltryptophylglycine Monoacetate Dihydrate.—a. Crude Product.—Several batches of crude carbobenzoxypentapeptide benzyl ester were converted to the pentapeptide acetate for enzymatic studies. A typical experiment was performed as follows. A sample of the benzyl ester (500 mg.) was dissolved in 20 ml. of 90% acetic acid (v./v.) and hydrogenated over a palladium catalyst for 8 hr. Fresh catalyst was added after 4 hr. of hydrogenation. The catalyst was removed by filtration, and the filtrate was evaporated to dryness *in vacuo*. The pink residue was dissolved in water (5 ml.) and the solution lyophilized over potassium hydroxide pellets. The ensuing pink powder was employed for analysis of the amino acid composition and for enzymatic studies with trypsin and with leucine aminopeptidase; yield 370 mg. (90%); R_f^{17} = 0.51, R_f^{18} = phe, ninhydrin, Pauly, Sakaguchi and Ehrlich positive. All samples contained several minor impurities. Amino acid ratios in acid hydrolyzate: his, 1; phe, 1; arg, 1; try, 0; gly, 0.9. Trypsin released 37% of the expected quantity of his-phe-arg. Amino acid ratios in the leucine aminopeptidase digest: his, 1; phe, 1; arg, 0.4; try, 0.4; gly, 0.4.

b. All-L Form.—All-L carbobenzoxypentapeptide benzyl ester (500 mg.) was dissolved in 90% acetic acid (v./v.) and hydrogenated over palladium. The product was isolated in the manner described for the racemized form, dissolved in water, the solution decolorized with Norit-A and the solvent removed by lyophilization over potassium hydroxide pellets; yield 385 mg. (94%); $[\alpha]^{27}D$ −10.0° (*c* 0.85, in *N* HCl); R_f^{17} = 0.51 (single spot), R_f^{18} = phe (single spot). Trypsin released 95% of the expected quantity of his-phe-arg. Leucine aminopeptidase released equimolar proportions of his, phe, arg, try and gly. Unhydrolyzed pentapeptide was not seen on the chromatograms.[23] A sample for analysis was dried *in vacuo* at 80° for 8 hr.

Anal. Calcd. for $C_{36}H_{51}O_{10}N_{11}$: C, 54.2; H, 6.4; N, 19.3; NH_2-N, 1.8. Found: C, 54.3; H, 6.5; N, 19.5; NH_2-N, 2.1.

Enzymatic Procedures.—Partially purified (through a second ammonium sulfate fractionation) leucine aminopeptidase was prepared from swine kidneys according to the procedure of Spackman, Smith and Brown[19] and was dialyzed against 0.005 *M* "tris" buffer at *p*H 8 for 12 hr. The solution had a C_1 of 7.2 and contained 0.6 mg. of protein per ml. The enzyme solution was stored in the frozen state in a refrigerator and was preserved by the addition of toluene.[24] For activation, an 0.3-ml. aliquot of thawed enzyme solution was incubated for 3.25 hr. at 40° with 0.25 ml. of 0.01 *M* magnesium chloride, 0.05 ml. of 0.1 *N* ammonium acetate buffer (*p*H 8.5) and 0.4 ml. of water. An 0.2-ml. aliquot of this solution was then added to a solution containing the peptide (approximately 10 μM) in water (0.56 ml.), 0.01 *M* magnesium chloride (0.20 ml.) and 0.1 *N* ammonium acetate buffer of *p*H 8.5 (0.04 ml.). One drop of toluene was added and the mixture was incubated for 24 hr. at 40°. The enzyme was then denatured by immersing the digestion mixture in a boiling water-bath for one minute, and the coagulum was removed by centrifugation. The coagulum was washed with a 0.3-ml. portion of water, and the supernatant liquor and washings were combined and evaporated to dryness *in vacuo* at room temperature over phosphorus pentoxide. The dry residue was dissolved in water (0.1 ml.), and aliquots of this solution were used for quantitative paper chromatography. Trypsin (Armour, containing 50% of magnesium sulfate) was employed for the tryptic digestions. The peptide (approximately 10 μM) was dissolved in water (0.85 ml.) and 0.1 *N* ammonium acetate buffer of *p*H 8.5 (0.05 ml.), and 0.1 ml. of a trypsin solution (15 mg. of enzyme in 10 ml. of water) was added. The mixture was incubated at 37° for 4 hr., heated for one minute in a boiling-water bath and evaporated to dryness *in vacuo* at room temperature over phosphorus pentoxide. The residue was dissolved in water (0.1 ml.) and aliquots of this solution were employed for paper chromatography.

Analytical Procedures.—The paper chromatograms were prepared by the descending technique on Whatman No. 1-papers. Amino acids and peptides were located by spraying the papers with a dilute ninhydrin solution.[25] The following qualitative tests were employed to detect the presence of certain amino acids; the Pauly test for histidine,[26] the Sakaguchi test for arginine[26] and the Ehrlich test for tryptophan.[27] Fowden's quantitative photometric ninhydrin technique[28] was employed for amino acid and peptide determinations using the Moore and Stein ninhydrin reagent.[29] Standard amino acid (or peptide) curves were prepared by spotting various concentrations of the desired compound (0.2–0.6 μM) on paper. The chromatograms were developed with 2-butanol–ammonia,[18] the spots were cut from the papers and eluted and their color intensities were determined photometrically with a Klett–Summerson colorimeter using a No. 540 filter. Two-dimensional chromatograms were employed for analysis of more complex amino acid mixtures. These chromatograms were first developed with the 2-butanol–ammonia system[18]; they were then carefully dried and developed in the second dimension with the 2-butanol–formic acid system of Hausmann.[30] Peptides (2–5 mg.) were hydrolyzed with double-distilled (from glass) 6 *N* hydrochloric acid in sealed tubes for 20 hr. at 110°. The hydrolyzates were evaporated to dryness *in vacuo* at room temperature over phosphorus pentoxide and potassium hydroxide pellets, and the residues were dissolved in water. Aliquots of these solutions were used for quantitative amino acid analyses.

(23) A sample of the pentapeptide acetate was kindly analyzed by Dr. W. F. White of the Armour Laboratories with the following results; R_f^{17} = 0.53 (single spot), R_f^{18} = phe$^+$ (single spot). The peptide was completely digestible by leucine aminopeptidase. We wish to thank Dr. White for these determinations.

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PITTSBURGH, PENNSYLVANIA