decomposed by addition of a few drops of glacial acetic acid, and the solution was evaporated to dryness *in vacuo* at a bath temperature of 40–50°. The residue was washed with a small volume of ether, was dissolved in methanol and the product was precipitated by addition of water; yield 0.70 g. (88%), m.p. 216–217°, $[\alpha]^{24}D$ −26.4° (*c* 0.8 in methanol). A sample for analysis was precipitated twice from methanol with water.

Anal. Calcd. for $C_{34}H_{47}O_{12}N_7S$: C, 52.5; H, 6.1; N, 12.6. Found: C, 52.3; H, 6.3; N, 12.7.

c. Carbobenzoxy Hydrazide.—The above methyl ester (0.60 g.) was dissolved in methanol (240 ml.) at 50° and the solution was cooled at room temperature. Hydrazine hydrate (0.25 ml.) was added and the mixture was kept at room temperature for 24 hours. The clear solution was concentrated to a small volume *in vacuo* at room temperature and the ensuing gelatinous precipitate was collected and dried; yield 0.46 g. (76%), m.p. 201–204°. A sample for analysis was dissolved in a small volume of methanol and precipitated by addition of water.

Anal. Calcd. for $C_{33}H_{47}O_{11}N_9S\cdot 2H_2O$: N, 15.5. Found: N, 15.0.

d. Deblocked Product.—The carbobenzoxy derivative (0.12 g.) was dissolved in sodium-distilled liquid ammonia (approximately 100 ml.) and sodium (approximately 60 mg.) was added in small pieces with stirring until a permanent blue color remained. Dowex-50 (ammonia cycle, 4 g.) was added and the ammonia was allowed to evaporate. The last traces of ammonia were removed by storing the material over sulfuric acid *in vacuo*. The residue was extracted with several small volumes of water and the combined extracts were filtered and lyophilized. The ensuing solid was dissolved in a small volume of water, the *p*H was adjusted to 6 by addition of 10% acetic acid and the peptide was obtained in crystalline form upon the addition of ethanol; yield 73 mg. (74%), m.p. 215–217°, $[\alpha]^{25}D$ −29.6° (*c* 0.45 in 2*N* hydrochloric acid), R_f^A 0.40; ninhydrin, Pauly and methionine positive[28]; LAP liberates serine only. Chymotrypsin did not cleave the compound. A sample for analysis was twice recrystallized from water.

Anal. Calcd. for $C_{25}H_{39}O_{10}N_7S\cdot H_2O$: C, 46.4; H, 6.4; N, 15.1. Found: C, 46.7; H, 6.4; N, 15.4.

PITTSBURGH, PENNA.

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

Studies on Polypeptides. XV. Observations on the Relation between Structure and Melanocyte-expanding Activity of Synthetic Peptides[1-3]

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A number of homogeneous peptides embodying sequences of α-MSH were prepared and were tested for their ability to expand melanocytes *in vitro*. Some of the peptides possessed biological activity. All of the biologically active peptides contained the sequence histidylphenylalanylarginyltryptophylglycine. The significance of the finding that small fragments of a polypeptide hormone possess biological activity is discussed.

The recognition that certain medium sized polypeptides are endowed with hormonal activity, and the availability of synthetic methodology for their production has paved the way to systematic studies on the relation between peptide structure and physiological activity. The thesis that every amino acid residue of a naturally occurring biologically active peptide is essential for activity can now be subjected to rigorous testing. The possibilities for variation are astronomical, but the problem is of key significance if a sound understanding is to be achieved regarding the mode of action of the polypeptide hormones.

The present investigation was initiated in an attempt to delineate the structural requirements for melanocyte-expanding activity and to gain insight into the relation between structure and this physiological effect. A series of peptides and peptide derivatives related to α-MSH was prepared and the ability of these compounds to expand melanocytes *in vitro* was determined.[4] The paper chromatographic and enzymatic techniques described in a previous communication[5] provided the criteria for evaluating the homogeneity of the peptides.

Structural studies of the corticotropins and of the melanocyte-stimulating hormones α- and β-MSH have shown these molecules to contain a common amino acid sequence "core" possessing the structure methionylglutamylhistidylphenylalanylarginyltryptophylglycine. Since all these substances have the ability to stimulate melanocytes, it was suggested[6] that the essential minimum structural requirement for melanocyte-expanding activity may reside in this common sequence.

As a model experiment for a projected synthesis of the octapeptide serylmethionylglutaminylhistidylphenylalanylarginyltryptophylglycine, which embodies within its structure the glutamine analog of the "core" sequence, we investigated the reaction of the solid azide of carbobenzoxymethionylglutamine[7] with the triethylammonium salt of histidylphenylalanylarginine[8] in dimethylformamide. The ensuing carbobenzoxymethionylglut-

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

(2) The amino acid and peptide derivatives mentioned in this communication are of the L-configuration. In the interest of space conservation we have eliminated the customary L-designation for individual amino acid residues.

(3) Preliminary communications describing some of the results reported in this paper have appeared in THIS JOURNAL, **79**, 6087 (1957), and **80**, 6458 (1958).

(4) We wish to express our thanks to Drs. A. B. Lerner and M. R. Wright of the Department of Medicine, Yale University, School of Medicine, for these assays, which were performed according to the method of Shizume, Lerner and Fitzpatrick, *Endocrinol.*, **54**, 553 (1954).

(5) K. Hofmann, M. E. Woolner, G. Spühler and E. T. Schwartz, THIS JOURNAL, **80**, 1486 (1958).

(6) (a) J. I. Harris and P. Roos, *Nature*, **178**, 90 (1956); (b) I. I. Geschwind, C. H. Li and L. Barnafi, THIS JOURNAL, **78**, 4494 (1956); (c) J. I. Harris and A. B. Lerner, *Nature*, **179**, 1346 (1957).

(7) K. Hofmann, T. A. Thompson, H. Yajima, E. T. Schwartz and H. Inouye, THIS JOURNAL, **82**, 3715 (1960).

(8) K. Hofmann, H. Kapeller, A. E. Furlenmeier, M. E. Woolner, E. T. Schwartz and T. A. Thompson, *ibid.*, **79**, 1041 (1957).

aminylhistidylphenylalanylarginine was purified by precipitation from glacial acetic acid solution with ammonia, and methionylglutaminylhistidylphenylalanylarginine was obtained from the carbobenzoxy derivative by reduction with sodium in liquid ammonia. The analytical evaluation substantiated the assigned structure, demonstrating that normal peptide-bond formation had occurred in the azide coupling step and that no side reactions involving the imidazole nitrogens of the histidine residue had taken place.

The solid azide of carbobenzoxyserylmethionylglutamine[7] reacted with the triethylammonium salt of histidylphenylalanylarginyltryptophylglycine[9] to form carbobenzoxyserylmethionylglutaminylhistidylphenylalanylarginyltryptophylglycine which was purified by countercurrent distribution, and isolated as the monoacetate dihydrate. Decarbobenzoxylation afforded a mixture of substances from which the free octapeptide serylmethionylglutaminylhistidylphenylalanylarginyltryptophylglycine was isolated by chromatography on a column of cellulose powder. Formation of a sharp single spot on paper in two different solvent systems and liberation of the constituent amino acids in the ratios expected by theory on digestion with leucine aminopeptidase (LAP) pointed to the presence of a homogeneous material.

The successful experiments with the azides of the carbobenzoxy di- and tripeptides prompted extension of this approach to the azide of carbobenzoxyseryltyrosylserylmethionylglutamine which was readily obtained in solid form from the previously described hydrazide.[7] This azide reacted smoothly with the triethylammonium salt of histidylphenylalanylarginine[8] to give carbobenzoxyseryltyrosylserylmethionylglutaminylhistidylphenylalanylarginine. The carbobenzoxy group was removed and the free octapeptide purified by cellulose-block electrophoresis followed by countercurrent distribution. The behavior of the highly purified octapeptide toward LAP supported the all-L configuration. Seryltyrosylserylmethionylglutaminylhistidylphenylalanylarginine is the glutamine analog of the N-terminal octapeptide which is liberated from the corticotropins by digestion with trypsin.[10]

The observation that the azide of carbobenzoxyseryltyrosylserylmethionylglutamine reacts normally and with satisfactory yield with the triethylammonium salt of histidylphenylalanylarginine is of considerable practical importance in connection with the development of synthetic routes to more complex polypeptides possessing the amino acid sequences present in α-MSH and the corticotropins, since it allows the introduction of the N-terminal sequence into the rest of the peptide structure in a single step without the risk of racemization.

The partially blocked octapeptide amide histidylphenylalanylarginyltryptophylglycyl-N^{ϵ}-tosyllysylprolylvaline amide was of interest in connection with our studies relating peptide structure to melanocyte-expanding activity, and offered possibilities as an intermediate in the construction of the entire amino acid sequence of α-MSH. The free N-terminal amino group serves as a convenient point for attachment of the rest of the peptide chain, and reduction with sodium in liquid ammonia was expected to remove the tosyl blocking group without causing fission of peptide bonds.

We have prepared this octapeptide amide by the coupling of carbobenzoxyhistidylphenylalanylnitroarginyltryptophylglycine with N^{ϵ}-tosyllysylprolylvaline amide followed by hydrogenolysis. N,N'-Dicyclohexylcarbodiimide[11] was employed in the coupling step. Since three independent groups of investigators[9,12] have shown that this reagent may cause racemization when used to link acyldi- or higher peptides with amino acid or peptide esters we have selected the present scheme of synthesis where a glycine residue contributed the C-terminal carboxyl group. This approach eliminated the possibility for racemization of the C-terminal amino acid residue.

The methyl ester of carbobenzoxyprolylvaline[13] was converted into the amide which was hydrogenated in presence of hydrogen chloride to give prolylvaline amide hydrochloride. This dipeptide amide was coupled with N^{α}-carbobenzoxy-N^{ϵ}-tosyllysine[14] to give N^{α}-carbobenzoxy-N^{ϵ}-tosyllysylprolylvaline amide.

Both the azide and mixed anhydride procedures were employed in this step. The ensuing impure, amorphous material was subjected to exhaustive hydrogenation and the crude N^{ϵ}-tosyllysylprolylvaline amide hydrochloride was purified by extensive countercurrent distribution in two different solvent systems. The final product exhibited a single sharp spot on paper in two solvent systems and was completely digestible with LAP.

Carbobenzoxyhistidylphenylalanylnitroarginyltryptophylglycine benzyl ester[9] was saponified and the resulting crystalline carbobenzoxypentapeptide[15] coupled with N^{ϵ}-tosyllysylprolylvaline amide to give a mixture of compounds which was hydrogenated. The hydrogenation products were separated by countercurrent distribution into histidylphenylalanylarginyltryptophylglycine (slowest moving component), histidylphenylalanylarginyltryptophylglycyl - N^{ϵ} - tosyllysylprolylvaline amide (middle peak) and a faster moving substance which has not yet been identified. The paper chromatographic analysis of the octapeptide amide, isolated from the pooled tubes of the middle peak, revealed the presence of one single component, regardless of whether the papers were sprayed with the ninhydrin, Pauly, Sakaguchi or Ehrlich reagents. Digestion with LAP converted

(9) K. Hofmann, M. E. Woolner, G. Spühler and E. T. Schwartz, THIS JOURNAL, **80**, 1486 (1958).

(10) (a) W. F. White and W. A. Landmann, *ibid*, **77**, 771 (1955); (b) R. G. Shepherd, S. D. Willson, K. S. Howard, P. H. Bell, D. S. Davies, S. B. Davis, E. A. Eigner and N. E. Shakespeare, *ibid.*, **78**, 5067 (1956).

(11) J. C. Sheehan and G. P. Hess, *ibid.*, **77**, 1067 (1955).

(12) (a) G. W. Anderson and F. M. Callahan, *ibid.*, **80**, 2902 (1958); (b) H. Schwarz and F. M. Bumpus, *ibid.*, **81**, 890 (1959).

(13) R. L. M. Synge, *Biochem. J.*, **42**, 99 (1948).

(14) R. Roeske, F. H. C. Stewart, R. J. Stedman and V. du Vigneaud, THIS JOURNAL, **78**, 5883 (1956).

(15) Samples of the carbobenzoxypentapeptide were subjected to hydrogenation and the optical purity of the ensuing pentapeptide was ascertained by enzymatic digestion. The pentapeptide was completely digestible by LAP demonstrating that the exposure to alkali had not caused racemization.

TABLE I

STRUCTURE AND BIOLOGICAL ACTIVITY OF α-MSH AND RELATED SYNTHETIC PEPTIDES[16]

No.	Peptide	Activity
I	Ac.ser.tyr.ser.met.glu.his.phe.arg.try.gly.lys.pro.val.$CONH_2$	2×10^{10}
II	Cbzo.met.gluta.his.phe.arg.	Inactive
III	Met.gluta.his.phe.arg.	Inactive
IV	Cbzo.ser.tyr.ser.met.gluta.his.phe.arg.	Inactive
V	Ser.tyr.ser.met.gluta.his.phe.arg.	Inactive
VI	His.phe.arg.try.gly.	1.5×10^4
VII	Cbzo.ser.met.gluta.his.phe.arg.try.gly.	0.7×10^6
VIII	Ser.met.gluta.his.phe.arg.try.gly.	0.7×10^6
IX	His.phe.arg.try.gly.toslys.pro.val.$CONH_2$	0.5×10^6

the octapeptide amide into an equimolar mixture of the eight constituent amino acids; undigested starting material was not present on the chromatograms of the digest. Trypsin hydrolyzed the peptide amide with formation of histidylphenylalanylarginine and tryptophylglycyl-N^{ϵ}-tosyllysylprolylvaline amide. The quantitative evaluation of the composition of an acid hydrolysate of the octapeptide amide revealed the presence of seven amino acids; tryptophan was absent.

The octapeptide amide exhibited an absorption maximum at 275 mμ characteristic of tryptophan and the experimentally determined molar extinction coefficient agreed with calculated figures. These results justify the conclusion that the synthetic octapeptide amide is of a high degree of purity and that it does not contain significant proportions of racemized material.

In Table I are presented the structures and biological activities of the peptides whose synthesis is described above. For reference purposes the amino acid sequence and biological activity of α-MSH (I) is also included. In a previous study[7] we have described the synthesis of the pentapeptide seryltyrosylserylmethionylglutamine and of its N-carbobenzoxy- and N-acetyl derivatives and found these compounds to be devoid of melanocyte-expanding activity. We now find that compounds II to V which correspond to sequences 1 to 8 of α-MSH are also inactive. The smallest peptide thus far described which possesses melanocyte-expanding activity is histidylphenylalanylarginyltryptophylglycine (VI).[3,9] Schwyzer and Li[17] prepared this same compound by a route which differed from the one employed by us and reported a biological activity of 3×10^4 MSH units per gram.

Elongation of the peptide chain of compound VI from the amino end gave compounds VII and VIII. These substances which embody within their structure the entire "core" are 47 times more active than peptide VI. This finding supports the concept that the "core" possesses biological activity, but the activity is of a low order of magnitude compared to that of the intact hormones. However, the same degree of activity is also found in peptide IX which corresponds to positions 6 to 13 of the α-MSH molecule and contains only a portion of the "core." It should be noted that all the biologically active peptides which have been described thus far contain the sequence histidylphenylalanylarginyltryptophylglycine. This portion of the structure of the melanocyte-expanding principles seems to play a key role in their physiological function.

The observation that relatively small fragments of a polypeptide hormone exhibit physiolo ical activity is significant. The systematic exploration of this finding may lead to discovery of a special "site" along the polypeptide chain which is connected intimately with those chemical reactions which bring about the observable physiological effects.

(16) Activities are expressed as MSH units per gram; gluta = glutamine; toslys = N^{ϵ}-tosyllysine.

(17) R. Schwyzer and C. H. Li, *Nature*, **182**, 1069 (1958).

Experimental[18]

N^{α}-Carbobenzoxy-N^{ϵ}-tosyllysine.—N^{ϵ}-Tosyllysine, prepared according to Roeske, *et al.*,[14] $[\alpha]^{23}D$ +13.8° (*c* 1.8 in 2*N* hydrochloric acid) (lit.[14] $[\alpha]^{21}D$ +13.6° in 2*N* hydrochloric acid), R_f^A 0.77,[19] R_f^B phe^{-},[20] was carbobenzoxylated to give N^{α}-carbobenzoxy-N^{ϵ}-tosyllysine, $[\alpha]^{27}D$ −14.6° (*c* 1.3 in satd. sodium bicarbonate) (lit.[14] $[\alpha]^{21}D$ −13.3° in 5% sodium bicarbonate). A sample of the carbobenzoxy derivative was hydrogenated in methanol containing 10% v./v. of glacial acetic acid, and N^{ϵ}-tosyllysine was isolated; $[\alpha]^{23}D$ +14.8° (*c* 2.0 in 1 *N* hydrochloric acid). Oxidation with ninhydrin[21] of N^{ϵ}-tosyllysine (three samples) gave, 97.1, 101.0 and 96.7%, respectively, of the quantity of carbon dioxide theoretically expected.

N^{α}-Carbobenzoxy-N^{ϵ}-tosyllysine Hydrazide.—N^{ϵ}-Tosyllysine ethyl ester hydrochloride[14] (3.51 g.) was dissolved in ice-water (20 ml.) and magnesium oxide (0.44 g.) was added with stirring. Carbobenzoxy chloride (1.75 g.) in ethyl acetate (30 ml.) was added and the mixture was shaken vigorously at 5° for 3 hours. Unreacted carbobenzoxy chloride was destroyed by addition of 1 ml. of pyridine, and the mixture was acidified to congo red with concentrated hydrochloric acid. The ethyl acetate layer was separated, washed and dried in the usual manner and evaporated. The oily residue (4.48 g.) was dissolved in absolute ethanol, hydrazine hydrate (1 ml.) was added and the solution was kept at room temperature for 12 hours. The solid hydrazide was collected and recrystallized from ethanol; yield 2.12 g. (49%), m.p. 136–137°, $[\alpha]^{24}D$ −10.5° (*c* 2.0 in 40% acetic acid).

Anal. Calcd. for $C_{21}H_{28}O_5N_4S$: C, 56.2; H, 6.3; N, 12.5. Found: C, 56.1; H, 6.4; N, 12.7.

Carbobenzoxyprolylvaline Amide.—Carbobenzoxyprolylvaline methyl ester[13] (2.3 g.) was dissolved in methanol

(18) The melting points were determined in capillary tubes and are uncorrected. The organic solvents were freshly distilled. A Craig-type countercurrent machine of 200 tubes having a capacity of 10 ml. of lower and upper phase each (obtained from H. O. Post, Scientific Instrument Co., Maspeth 78, N. Y.) was employed. Rotations were determined in a Rudolph precision polarimeter, model 80 with model 200 photoelectric attachment. The peptides are arranged in the order of increasing complexity and not in the sequence in which they are discussed in the introduction.

(19) Partridge system: S. M. Partridge, *Biochem. J.*, **42**, 238 (1948).

(20) 2-Butanol–ammonia system; J. F. Roland and A. M. Gross, *Anal. Chem.*, **26**, 502 (1954); since the position of the solvent front cannot be determined with this system the R_f's are given in relation to the nearest amino acid on the indicator strip.

(21) B. E. Christensen, E. S. West and K. P. Dimick, *J. Biol. Chem.* **137**, 735 (1941).

saturated with ammonia (75 ml.) and the mixture was kept at room temperature for one week. The solvent was evaporated, the semi-crystalline residue was triturated with ether and the suspension was filtered. The white solid was washed with additional quantities of ether, was recrystallized from a mixture of methanol and water, and was dried; yield 1.05 g. (48%), m.p. 201–203°, $[\alpha]^{25}D$ −80.2° (*c* 1.14 in glacial acetic acid). Treatment with methanolic ammonia of the material recovered by evaporation of the ether washings gave additional quantities of the crystalline amide.

Anal. Calcd. for $C_{18}H_{25}O_4N_3$: C, 62.2; H, 7.3; N, 12.1. Found: C, 62.2; H, 7.3; N, 12.1.

Prolylvaline Amide Hydrochloride.—The carbobenzoxy dipeptide amide (0.52 g.) was hydrogenated in the usual manner over a palladium catalyst in methanol containing one equivalent of hydrogen chloride. The material was recrystallized from a mixture of methanol and ether; yield 0.36 g. (96%), m.p. 266–268°, $[\alpha]^{26}D$ −57.6° (*c* 2.1 in water), R_f^A 0.50, R_f^B N$^\epsilon$-toslys$^+$, amino acid ratios in LAP digest $pro_{1.0}val_{1.0}$.

Anal. Calcd. for $C_{10}H_{20}O_2N_3Cl$: N, 16.8; Cl, 14.2. Found: N, 17.0; Cl, 13.9.

N$^\epsilon$-Tosyllysylprolylvaline Amide Hydrochloride. a. By the Mixed Anhydride Method.—A mixed anhydride was prepared in the usual manner from N$^\alpha$-carbobenzoxy-N$^\epsilon$-tosyllysine (4.72 g.) in freezing dioxane (50 ml.) with tri-*n*-butylamine (2.61 ml.) and ethyl chloroformate (1.04 ml.). This solution was added to an ice-cold dimethylformamide solution (approximately 20 ml.) containing prolylvaline amide hydrochloride (2.72 g.) and triethylamine (1.52 ml.). The mixture was kept at 0° for one hour and at room temperature for one hour and was evaporated to dryness at a bath temperature of 40–45°. The residue was dissolved in ethyl acetate, the extract was washed and dried in the usual manner and evaporated to dryness *in vacuo*. The semi-solid residue was triturated with benzene, the insoluble material was collected, washed with ether and dried; yield 3.2 g. A portion of this material (1.87 g.) was hydrogenated over a palladium catalyst in methanol, containing one equivalent of hydrogen chloride, until the evolution of carbon dioxide had ceased. The catalyst was removed by filtration, the solvent was evaporated and the oily residue dissolved in water. The cloudy solution was clarified by filtration through a layer of Filter-cel and was lyophilized; yield 1.36 g. Examination by paper chromatography revealed the presence of three major ninhydrin-positive components. A portion of the material (0.845 g.) was placed into the first tube of a 25-tube countercurrent machine and 45 transfers in the solvent system 1-butanol-3% ammonium hydroxide containing 1% of sodium chloride were performed. Spectrophotometric examination of aliquots at 263 mμ was employed to locate the material. Effluent fractions 1 to 20, containing the desired compound, were pooled, washed with small portions of water until free of chloride ions (approximately 3 to 4 washings) and the butanol was evaporated. The residue was dissolved in a small quantity of water, the solution was acidified to *p*H 4 with 1 *N* hydrochloric acid and lyophilized; yield 0.724 g. Examination by paper chromatography revealed the presence of two ninhydrin-positive components. The material (0.710 g.) was placed in a 46-tube countercurrent machine and 86 transfers were effected in the solvent system 1-butanol–0.1 *N* hydrochloric acid. Effluent fractions 1 to 16 contained 200 mg. of a material which was discarded; the desired tripeptide amide was located in effluents 17 to 40 which were pooled and evaporated to dryness. The residue was dissolved in a small portion of water and lyophilized to give a white fluffy powder; yield 0.490 g.; a sample for analysis was dried *in vacuo* at 60° for 12 hours; $[\alpha]^{26}D$ −52.5° (*c* 1.39 in water), R_f^A 0.77, single ninhydrin positive spot, R_f^B N$^\epsilon$-toslys, single ninhydrin positive spot, amino acid ratios in LAP digest N$^\epsilon$-$toslys_{1.0}pro_{1.0}val_{1.0}$, amino acid ratios in acid hydrolysate N$^\epsilon$-$toslys_{1.0}pro_{1.0}val_{1.0}$ (trace of lysine present).

Anal. Calcd. for $C_{23}H_{38}O_5N_5SCl$: N, 13.2; Cl, 6.7; S, 6.0. Found: N, 13.0; Cl, 6.3; S, 5.9.

b. By the Azide Method.—This entire operation was performed in a cold room at a temperature of 5°. N$^\alpha$-Carbobenzoxy-N$^\epsilon$-tosyllysine hydrazide (1.34 g.) was dissolved in 0.25 *N* hydrochloric acid (70 ml.) and the solution was cooled at 5°. Sodium nitrite (0.21 g.) in water (5 ml.) was added and the resulting precipitate was extracted into ice-cold ether after 2 minutes of standing. The ether solution was washed and dried in the usual manner and was evaporated to dryness at a bath temperature of 5°. The oily azide was dissolved in ice-cold dimethylformamide (5 ml.) and the solution was mixed with a solution of prolylvaline amide hydrochloride (0.75 g.) and triethylamine (0.42 ml.) in dimethylformamide (50 ml.). The mixture was kept at 5° for 24 hours when a second portion of azide (prepared from 0.67 g. of the hydrazide) was added. After standing at 5° for 48 hours the solution was evaporated to dryness at a bath temperature of 45° and the residue was extracted into ethyl acetate. The ethyl acetate solution was washed and dried in the usual manner and the solvent was evaporated. The oily residue was triturated with benzene, the insoluble material was collected and washed with ether; yield 1.42 g. This material was hydrogenated in methanol, containing one equivalent of hydrogen chloride, and the hydrogenation product isolated in the manner described under a above; yield 0.89 g. Examination by paper chromatography revealed the presence of three major ninhydrin-positive components. Purification by countercurrent distribution first in the system 1-butanol–3% ammonium hydroxide containing 1% of sodium chloride (20 tubes, 46 transfers) then in the 1-butanol–0.1 *N* hydrochloric acid system (100 tubes, 250 transfers) gave a material (0.38 g.) which was still contaminated by a ninhydrin-positive impurity; R_f^B N$^\epsilon$-tosyllysine. A third distribution in the 1-butanol–3% ammonium hydroxide system removed this contamination. The final product (0.23 g.) exhibited paper chromatographic properties identical with those of the material prepared according to method a above. The substance was completely digestible by LAP, amino acid ratios in digest N$^\epsilon$-$toslys_{1.0}pro_{1.0}val_{1.0}$; the recovery of N$^\epsilon$-toslys was 90% of theory.

Carbobenzoxyhistidylphenylalanylnitroarginyltryptophylglycine.—Carbobenzoxyhistidylphenylalanylnitroarginyltryptophylglycine benzyl ester[9] (2.18 g.) was dissolved in absolute methanol (45 ml.) by warming slightly. The clear solution was cooled at room temperature and 0.5 *N* sodium hydroxide (9.0 ml.) was added in three portions with shaking. The mixture was kept at room temperature for 1 hour, undissolved material was removed by filtration, and water (10 ml.) was added to the filtrate. The solution was cooled in an ice-bath and acidified to congo red with 2 *N* hydrochloric acid. The mixture was kept at ice-bath temperature for 1 hour, the precipitate was collected, washed with ice-water and dried *in vacuo* at room temperature. The material was dissolved in dimethylformamide (20 ml.) at 90° and water (60 ml.) was added to bring about crystallization. The crystalline compound was collected, washed with a mixture of dimethylformamide and water 1:3, and finally with water, and was dried *in vacuo* at room temperature; yield 1.83 g. (91%), m.p. 241–242°, $[\alpha]^{25}D$ −29.6° (*c* 1.3 in dimethylformamide); R_f^B phe$^+$, single spot, positive with Pauly and Ehrlich reagents, ninhydrin negative.

Anal. Calcd. for $C_{42}H_{48}O_{10}N_{12}.H_2O$: C, 56.1; H, 5.6; N, 18.7. Found: C, 55.8; H, 5.8; N, 18.6.

A sample of the material was converted to histidylphenylalanylarginyltryptophylglycine by hydrogenolysis.[9] This material was completely digestible by LAP; amino acid ratios in digest $his_{1.0}phe_{1.1}arg_{1.0}try_{1.0}gly_{1.0}$.

Histidylphenylalanylarginyltryptophylglycyl-N$^\epsilon$-tosyllysylprolylvaline Amide Diacetate Dihydrate.—N$^\epsilon$-Tosyllysylprolylvaline amide hydrochloride (0.72 g.) was dissolved in 13.5 ml. of methanol containing 0.014 ml. of triethylamine per ml. The solvent was removed *in vacuo* at a bath temperature of 30° and the residue was dissolved in 15 ml. of dimethylformamide. This solution was mixed with a solution of carbobenzoxyhistidylphenylalanylnitroarginyltryptophylglycine (1.22 g.) in dimethylformamide (35 ml.), and N,N′-dicyclohexylcarbodiimide (0.280 g.) was added. The solution was kept at room temperature for 20 hours, a few drops of glacial acetic acid were added and the solvent was removed *in vacuo* leaving a semi-solid mass. This material was triturated with a small volume of dimethylformamide, the suspension was chilled in an ice-bath, and the N,N′-dicyclohexylurea was removed by filtration. The crystals were washed with small portions of ice-cold dimethylformamide and the combined filtrate and washings were concentrated to dryness *in vacuo*. Water (100 ml.) was added to the residue, the suspension was cooled in an ice-bath for one hour and the precipitate was collected, washed with water and dried at room temperature over phosphorus pentoxide; yield 1.69 g. Analysis by two dimensional

paper chromatography using the 2-butanol–ammonia[20] followed by the 2-butanol–formic acid[22] systems revealed the presence of three Pauly- and Ehrlich-positive components. A sample of this material (0.74 g.) was hydrogenated in 90% acetic acid (20 ml.) over a palladium catalyst in a stream of hydrogen for 4 hours. Fresh catalyst was added at this point and the hydrogenation was continued for an additional 8 hours. The catalyst was removed by filtration, the filtrate was concentrated to dryness *in vacuo* (bath temperature of 40°), and the pink oily residue was dried for 12 hours *in vacuo* over potassium hydroxide pellets. This material was dissolved in a small volume of water and the solution was lyophilized to give a fluffy pink material; yield 0.63 g. Analysis by paper chromatography revealed the presence of three components A, B, and C exhibiting R_f^A values of 0.52, 0.70 and 0.76, respectively. All these materials reacted positively with the ninhydrin, Pauly and Ehrlich reagents. The A component was identified as his.phe.arg.try.gly, R_f^A 0.51.[9] The B component represents the desired octapeptide; the C component remains to be identified. The crude product (0.64 g.) was placed into the first tube of a 200-tube countercurrent machine and 200 transfers were effected in the solvent system 1-butanol–5% acetic acid containing 0.5% of sodium chloride. Spectrophotometric examination at 280 mμ of aliquots of the upper phases revealed the presence of three bands with peaks located in tubes 26, 120 and 167, respectively. For isolation of the octapeptide the contents of tubes 104 to 137 were pooled and the solvents evaporated *in vacuo* at a bath temperature of 40°. The residue (contaminated with sodium chloride) was triturated with ice-cold absolute ethanol, the suspension was filtered and the filter cake washed with small portions of ice-cold ethanol. The combined filtrate and washings were evaporated to dryness *in vacuo*. The residue was dissolved in 1-butanol (30 ml.) with the addition of 10 ml. of 0.3% ammonium hydroxide, and the butanol layer was extracted with 5 portions of dilute ammonia, then with water until the test for chloride ions was negative. The butanol was evaporated to dryness *in vacuo*, the residue was dissolved in a small volume of 10% acetic acid and the solution was lyophilized over potassium hydroxide pellets. A pink fluffy non-hygroscopic solid was obtained; yield 0.28 g., $[\alpha]^{24}D$ −40.6° (*c* 0.3 in 0.1 *N* hydrochloric acid), R_f^A 0.70; single spot Pauly, Ehrlich, Sakaguchi and ninhydrin positive; R_f^B phe$^+$, amino acid ratios in LAP digest $his_{1.0}phe_{1.0}arg_{1.0}try_{1.0}gly_{1.0}N^{\epsilon}$-$toslys_{1.0}val_{1.0}$. Trypsin converted the peptide into his.phe.arg., R_f^Bmet,$^{+s}$ and try.gly.N$^{\epsilon}$-toslys.pro.val. amide, R_f^B phe$^+$. Amino acid ratios in acid hydrolysate $his_{1.0}phe_{1.0}arg_{1.0}gly_{1.0}N^{\epsilon}$-$toslys_{1.0}val_{1.0}$ (pro present but not determined, try destroyed). A sample for analysis was dried *in vacuo* over phosphorus pentoxide.

Anal. Calcd. for $C_{61}H_{90}O_{16}N_{16}S$: C, 54.9; H, 6.8; N, 16.8; try, 15.3. Found: C, 55.2; H, 7.0; N, 16.2; try, 15.1.[23]

Carbobenzoxymethionylglutaminylhistidylphenylalanylarginine Monoacetate Hydrate.—This entire operation was performed in a cold room. Carbobenzoxymethionylglutamine hydrazide[7] (1.64 g.) was dissolved in water (150 ml.) and 2 *N* hydrochloric acid (80 ml.) with warming, and the clear solution was cooled in an ice-bath. Sodium nitrite (0.24 g.) in water (1 ml.) was added with stirring and the mixture was kept at 2° for 3 hours. The crystalline azide was collected, washed with ice-water and dried over phosphorus pentoxide *in vacuo* at 2°; yield 1.00 g. (60%).

The solid azide was added to an ice-cold solution of histidylphenylalanylarginine[8] (1.16 g.) and triethylamine (0.34 ml.) in dimethylformamide (70 ml.). The mixture was kept at 2° for 10 hours, the solvent was evaporated (bath temperature 45°) and water was added to the residue. The resulting pink granular precipitate was washed repeatedly with water and dried *in vacuo* over phosphorus pentoxide at room temperature. For purification the material was dissolved in glacial acetic acid (4 ml.), water (20 ml.) was added and the turbid solution was clarified by filtration through a layer of Filter-cel. The filtrate was cooled in an ice-bath and the *p*H was adjusted to 6 to 7 by dropwise addition of concentrated ammonium hydroxide. The white precipitate was collected and washed with ice-water. This process was repeated and the final precipitate was freeze-dried; yield 0.8 g. (38%), m.p. 193–195°, $[\alpha]^{25}D$ −15.5° (*c* 1.28 in glacial acetic acid), R_f^A, 0.80, R_f^B phe$^+$; single ninhydrin negative, Pauly, methionine and Sakaguchi positive spot; amino acid ratios in acid hydrolysate $met_{0.9}glu_{1.0}his_{1.0}$-$phe_{1.0}$ $arg_{1.0}$.

Anal. Calcd. for $C_{41}H_{57}O_{11}N_{11}S\cdot 1.5H_2O$: C, 52.5; H, 6.3; N, 16.4; S, 3.4. Found: C, 52.8, H, 6.4; N, 16.2; S, 3.0.

Methionylglutaminylhistidylphenylalanylarginine Monoacetate Pentahydrate.—The carbobenzoxy pentapeptide (0.85 g.) was dissolved in sodium-distilled liquid ammonia (approximately 100 ml.) and sodium (approximately 0.18 g.) was added in small pieces with stirring until a permanent blue color was obtained. Dowex-50 in the ammonium cycle (3.8 g.) was added, the ammonia was evaporated and the residue was kept over sulfuric acid *in vacuo* to remove the last traces of ammonia. The residue was extracted with five 10-ml. portions of water, the combined extracts were filtered through a layer of Filter-cel and the clear filtrate was lyophilized; yield 0.80 g. This material was dissolved in water (5 ml.), the solution was acidified to *p*H 4 by addition of glacial acetic acid and was lyophilized. The ensuing white fluffy solid was dried *in vacuo* over potassium hydroxide pellets at room temperature; yield 0.65 g. (83%), $[\alpha]^{25}D$ −15.6° (*c* 1.4 in water), R_f^A 0.43, R_f^B met$^-$; single methionine, Pauly, Sakaguchi and ninhydrin positive spot; amino acid ratios in LAP digest $met_{1.0}his_{0.9}phe_{1.0}$-$arg_{1.0}$.[24]

Anal. Calcd. for $C_{33}H_{51}O_9N_{11}S\cdot 5H_2O$: C, 45.7; H, 7.1; N, 17.7. Found: C, 45.5; H, 6.8; N, 17.1.

Carbobenzoxyseryltyrosylserylmethionylglutaminylhistidylphenylalanylarginine Tetrahydrate.—Carbobenzoxyseryltyrosylserylmethionylglutamine hydrazide[7] (0.68 g.) was dissolved in a mixture of dimethylformamide (4 ml.) and 2 *N* hydrochloric acid (4 ml.), and water (3 ml.) was added. The solution was cooled in an ice-bath and sodium nitrite (0.061 g.) in water (2 ml.) was added. The mixture was kept at 0° for 10 minutes and ice-cold saturated sodium chloride (10 ml.) was added. The suspension was cooled with an ice–salt-bath to complete precipitation of the azide. The white solid precipitate was collected, washed with ice-cold 1 *N* hydrochloric acid, ice-cold saturated sodium bicarbonate and ice-water and was dried *in vacuo* for 4 hours over phosphorus pentoxide at a temperature of −15°; yield 0.63 g. (93%). The azide explodes when heated in a capillary tube.

The azide (0.63 g.) was added to a solution of histidylphenylalanylarginine[8] (0.42 g.) and triethylamine (0.12 ml.) in ice-cold dimethylformamide (15 ml.), and the mixture was kept at 5° for 3 days. The solvent was evaporated at a bath temperature of 40–45° and the residue was dissolved in 30 ml. of 1-butanol equilibrated with 20% acetic acid. The solution was placed into the first three tubes of a 120-tube countercurrent machine, and 340 transfers were effected in the solvent system 1-butanol–20% acetic acid. Ninhydrin-positive material remaining in tubes 7 to 35 of the machine was identified as unreacted tripeptide. The effluent fractions contained two materials differing by their color reaction with the Pauly reagent. The fastest moving component (yellow color) was located in effluent fractions 5 to 30. This material seems to represent a rearrangement product derived from the azide. The carbobenzoxyoctapeptide (orange color) was located in effluent fractions 121 to 195. These fractions were pooled and evaporated to dryness *in vacuo* at a bath temperature of 40–50°. The residue was dissolved in a small volume of dilute acetic acid and the compound was precipitated by addition of dilute ammonium hydroxide; yield 0.36 g. (35%), m.p. 198–202° dec., $[\alpha]^{26}D$ −22.3° (*c* 0.9 in glacial acetic acid), R_f^A 0.77.

Anal. Calcd. for $C_{54}H_{72}O_{15}N_{14}S\cdot 4H_2O$: C, 51.4; H, 6.4; N, 15.5. Found: C, 51.6; H, 6.2; N, 15.4.

Seryltyrosylserylmethionylglutaminylhistidylphenylalanylarginine.—The carbobenzoxyoctapeptide (0.31 g.) was dissolved in sodium-distilled liquid ammonia (approximately 150 ml.) and sodium (approximately 63 mg.) was added from a capillary tube until a permanent blue color

(22) W. Hausmann, THIS JOURNAL, **74**, 3181 (1952).

(23) T. W. Goodwin and R. A. Morton, *Biochem. J.*, **40**, 628 (1946).

(24) Glutamine was present on the chromatograms but cannot be determined quantitatively by the ninhydrin technique because of pyrrolidonecarboxylic acid formation.

was obtained. Ammonium chloride (75 mg.) was then added and the ammonia was evaporated. A stream of nitrogen was passed over the residue to remove the last traces of ammonia. The solid was dissolved in a small volume of 0.5% acetic acid and the solution was lyophilized. The residue, which contained a mixture of the desired octapeptide and inorganic salts, was dissolved in 0.1 *M* pyridinium acetate buffer of *p*H 5.1 (2 ml.) and the solution was applied to a block (8 × 45 × 1.2 cm.) prepared from Whatman cellulose powder with the same buffer. The material was subjected to electrophoresis in a cold room at 5° for 17 hours at 440 volts and 22 milliamps. Streaking with Pauly reagent located the desired material in an area 2.3 to 9.6 cm. from the point of departure. The cellulose-containing Pauly-positive material was removed and extracted with water. The extracts were filtered and the filtrate was evaporated to dryness. The residue (0.216 g.) which was contaminated with extractives from the cellulose powder was placed in a 200-tube countercurrent machine and 300 transfers were performed in the solvent system 1-butanol–10% acetic acid. Spectroscopic examination of aliquots of the various fractions at 280 mμ revealed the presence of a single band located in tubes 4 to 17 in the machine. The contents of these tubes were pooled and evaporated to dryness *in vacuo*. The residue was redissolved in a small volume of water, the solution was filtered and the filtrate lyophilized; yield 0.150 g., $[\alpha]^{26}D$ −24.9° (*c* 0.9 in 1 *N* hydrochloric acid); R_f^A 0.38, R_f^B tyr; single ninhydrin, Pauly, methionine and Sakaguchi positive spot; completely digestible by LAP, amino acid ratios in digest $ser_{1.9}met_{1.0}tyr_{1.0}his_{1.0}phe_{1.1}arg_{1.0}$.

Carbobenzoxyserylmethionylglutaminylhistidylphenylalanylarginyltryptophylglycine Acetate Dihydrate.—Carbobenzoxyserylmethionylglutamine hydrazide[7] (1.48 g.) was dissolved in 1 *N* hydrochloric acid (90 ml.) and the solution was cooled in an ice-bath. A solution of sodium nitrite (0.19 g.) in water (1 ml.) was added and the mixture was kept at 4° for 10 minutes. The solid azide was collected, washed with ice-cold saturated sodium chloride and ice-water, and was dried *in vacuo* for 12 hours over phosphorus pentoxide at 4°; yield 0.68 g. (43%). The azide was added to a solution of histidylphenylalanylarginyltryptophylglycine[9] (1.04 g.) and triethylamine (0.36 ml.) in dimethylformamide (20 ml.), and the mixture was kept at 4° for 48 hours. The solvent was removed *in vacuo* at a bath temperature of 45° and the resulting oily residue was triturated with water. The solid precipitate was collected by centrifugation, was washed with three 5-ml. portions of water and was dried *in vacuo* at room temperature over phosphorus pentoxide; yield 0.87 g. (56%). This material was dissolved in 1-butanol saturated with 20% acetic acid (40 ml.). The solution was added to the first four tubes of a 25-tube countercurrent machine, and 40 transfers were performed in the solvent system 1-butanol–20% acetic acid. The desired material traveled with the upper phases; colored impurities which remained in the lower phases of the machine were discarded. Effluent fractions 3 to 12 were pooled and evaporated to dryness and the residue was dried *in vacuo* over phosphorus pentoxide at room temperature; yield 0.82 g. (49%), $[\alpha]^{26}D$ −29.4° (*c* 1.0 in ethanol), R_f^A 0.79; single spot ninhydrin negative, Pauly, methionine, Sakaguchi and Ehrlich positive.

Anal. Calcd. for $C_{57}H_{75}O_{15}N_{15}S\cdot 2H_2O$: C, 53.6; H, 6.2; N, 16.4. Found: C, 53.6; H, 6.1; N, 16.2.

Serylmethionylglutaminylhistidylphenylalanylarginyltryptophylglycine.—The carbobenzoxyoctapeptide (0.37 g.) was dissolved in sodium-distilled liquid ammonia (approximately 75 ml.) and sodium (approximately 50 mg.) was added in small pieces with stirring until a permanent blue color remained. Dowex-50 in the ammonium form (3.0 g.) was added, the ammonia was evaporated and the residue was kept *in vacuo* over sulfuric acid to remove the last traces of ammonia. The residue was extracted with five 10-ml. portions of water, the combined extracts were filtered through a layer of Filter-cel and the filtrate was lyophilized; yield 0.37 g. (85%). This material contained four major components, one with R_f^A 0.44 exhibiting a positive reaction with the ninhydrin, Pauly, Sakaguchi and Ehrlich reagents. The crude octapeptide (100 mg.) was dissolved in Partridge solvent (1 ml.) and the solution was applied to a firmly packed cellulose powder column (1.1 × 300 cm.) prepared from Whatman cellulose powder (50 g.) and equilibrated with Partridge solvent. The column was developed with Partridge solvent and 500 fractions (10 drops each) were collected with the aid of an automatic fraction collector. Aliquots from each fraction were spotted on paper and developed with Pauly reagent to locate the various fractions in the chromatogram. Two distinct bands evolved from the column, one collecting in fractions 170 to 330, the other in fractions 420 to 500. The center cut from band one (tubes 211 to 290) contained the homogeneous octapeptide. The contents of these tubes were pooled, the solvent was evaporated at a bath temperature of 40°, and the residue was dried to constant weight over phosphorus pentoxide and potassium hydroxide pellets; yield 57 mg., R_f^A 0.48, R_f^B met^+; single ninhydrin, Pauly, Sakaguchi and Ehrlich positive spot; single spot on paper electrophoresis in pyridinium acetate buffer at *p*H 4.6; completely digestible by LAP, amino acid ratios in digest $ser_{1.1}met_{1.1}his_{0.9}phe_{1.1}arg_{1.1}try_{1.0}gly_{1.0}$.[24] A slight unidentified impurity was present on the two dimensional chromatograms of the digest.

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