also exist in the solid state and were present in the phosphates.

If future evidence supports the N_7 base pairing scheme for DNA, some interesting deductions follow. If RNA base pairs by hydrogen bonding with DNA in the process of information transfer for subsequent protein synthesis, and if deoxycytosine is in the imino form and cytosine is in the amino form, deoxycytosine–guanine base pairing must be N_7 but deoxyguanine–cytosine must be N_1. Thymine–adenine and deoxyadenine–uracil may theoretically base pair in either form.

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[CONTRIBUTION FROM THE BIOCHEMISTRY DEPARTMENT, UNIVERSITY OF PITTSBURGH SCHOOL OF MEDICINE. PITTSBURGH 13, PENNSYLVANIA]

Studies on Polypeptides. XXIII. Synthesis and Biological Activity of a Hexadecapeptide Corresponding to the N-Terminal Sequence of the Corticotropins[1-4]

BY KLAUS HOFMANN, NOBORU YANAIHARA, SAUL LANDE AND HARUAKI YAJIMA

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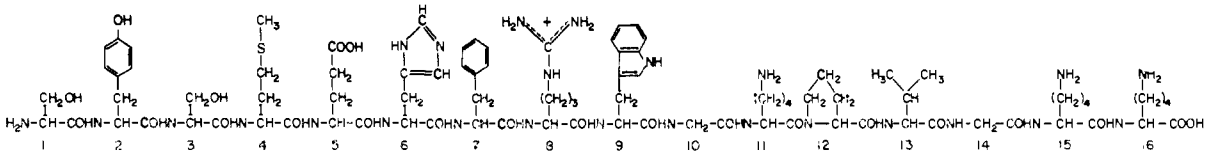
A synthesis is described of the hexadecapeptide seryltyrosylserylmethionylglutamylhistidylphenylalanylarginyltryptophylglycyllysylprolylvalylglycyllysyllysine (14-L) which corresponds to the arrangement of the N-terminal 16 amino acid residues of pig corticotropin. Evidence is presented for the stereochemical homogeneity of this hexadecapeptide. The peptide possessed both *in vitro* melanocyte expanding and adrenocorticotropic activity but the latter activity (<0.1 i.u./mg.) was of the same low order of magnitude as that of a tridecapeptide amide corresponding to the arrangement of the N-terminal 13 amino acid residues in pig corticotropin. It was concluded that the unit of the corticotropin molecule which possesses the full adrenocorticotropic activity of pig corticotropin must be longer than the N-terminal hexadecapeptide but may be shorter than the N-terminal tetracosapeptide.

Definition of the shortest segment of the corticotropin molecule which is endowed with full adrenocorticotropic activity is of considerable significance for the understanding of the physiological function of this hormone. Presumptive evidence[5] has located the adrenocorticotropically active portion of ACTH within the N-terminal 24 amino acid residues, but the smallest fully active sequence remains to be elucidated.

Biological evaluation of homogeneous synthetic peptides of increasing chain-length which correspond to the N-terminal portion of the ACTH molecule appears to provide a rational approach to the solution of this problem.

We have reported[6] that a synthetic tridecapeptide amide which corresponds to the arrangement of the first 13 amino acid residues of the corticotropins possesses low, but reproducible *in vivo* adrenal ascorbic acid depleting and steroidogenic activity in the rat. Thus, the fully active segment must be longer than 13 but may be shorter than 24 amino acid residues.



In the present communication we describe a synthesis of the hexadecapeptide (I) and provide biological data to show that this compound possesses essentially the same low *in vivo* adrenocorticotropic activity as the tridecapeptide amide. We conclude that the smallest fully active segment of ACTH must be longer than 16 but may be shorter than 24 amino acid residues.

The synthesis of (I) is patterned according to the scheme which we developed for the preparation of seryltyrosylserylmethionylglutamylhistidylphenylalanylarginyltryptophylglycyllysylprolylvaline amide.[6]

Two approaches were explored for preparing N^{α}-carbobenzoxy-N^{ϵ}-formyllysylprolylvalylglycyl-

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

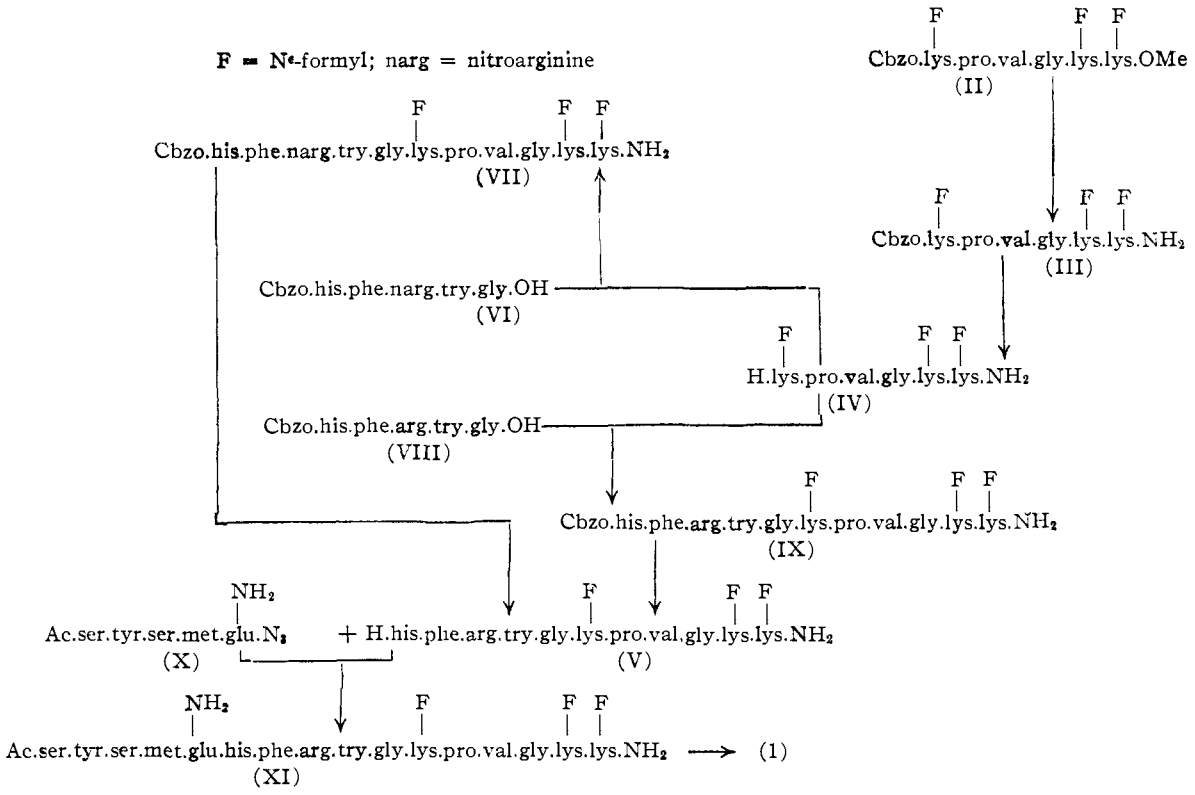
(2) The peptides and peptide derivatives mentioned in this communication (with exception of glycine) are of the L-configuration. In the interest of space conservation the customary L-designation for individual amino acid residues has been omitted.

(3) See *J. Am. Chem. Soc.*, **84**, 1054 (1962), for paper XXII in this series.

(4) A preliminary account of some of the results presented in this paper was communicated to the "First International Symposium on Polyamino Acids," June 19, 1961, "Polyamino Acids, Polypeptides and Proteins" M. Stahmann, editor, University of Wisconsin Press, 1962, p. 2.

(5) 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, *J. Am. Chem. Soc.*, **78**, 5067 (1956).

(6) K. Hofmann and H. Yajima, *ibid.*, **83**, 2289 (1961).



N$^{\epsilon}$ - formyllysyl - N$^{\epsilon}$ - formyllysine amide (III). In the first, the previously described[7] protected hexapeptide methyl ester (II) was converted into the amide in the conventional manner; in the second, the amide of N$^{\epsilon}$-formyllysyl-N$^{\epsilon}$-formyllysine was coupled with N$^{\alpha}$-carbobenzoxy-N$^{\epsilon}$-formyllysylprolylvalylglycine.[8]

N,N'-Carbonyldiimidazole (CDI)[9] proved to be a choice reagent for this purpose since N,N'-dicyclohexylcarbodiimide (DCC)[10] under the conditions used by us (*i.e.*, in dimethylformamide solution) afforded mainly the "acylurea" derivative of N$^{\alpha}$-carbobenzoxy-N$^{\epsilon}$-formyllysylprolylvalylglycine and little of the desired protected hexapeptide amide (III). The melting point, optical rotation and amino acid composition of the products prepared by the two routes were in satisfactory agreement. Hydrogenolysis of (III) over a palladium catalyst followed by conversion of the decarbobenzoxylated material into the hydrochloride of (IV) did not effect the N$^{\epsilon}$-formyl groups.

Three routes were investigated for preparing the partially protected undecapeptide amide (V). Coupling by the DCC procedure of (IV) with the protected pentapeptide (VI)[11] gave crude (VII) which was hydrogenated. Homogeneous samples of (V) were obtained from the hydrogenation products by chromatography on carboxymethylcellulose (CMC).[12] Reaction of (IV) with the hydrochloride of (VIII)[11] using the DCC reagent gave crude (IX) which was converted into (V) without prior purification. CMC chromatography was used to obtain homogeneous samples of (V). Comparable yields of highly purified (V) ensued when CDI was employed to bring about the reaction between (IV) and (VIII).

Optical rotation, paper chromatographic behavior, amino acid composition and digestibility by leucine aminopeptidase (LAP) of the products obtained by either of these three routes were in satisfactory agreement. No evidence was obtained for stereochemical inhomogeneity of the partially protected undecapeptide amide (V) which was isolated as the amorphous diacetate tetrahydrate.

Acetylseryltyrosylserylmethionylglutamine azide (X)[13] was reacted with (V) to give crude (XI). Homogeneous samples of the protected hexadecapeptide amide (XI), in yields ranging from 80–90% in a series of experiments, were isolated from the reaction products by CMC chromatography. The compound did not stimulate the rat adrenal cortex *in vivo*[14] but exhibited potent *in vitro* melanocyte stimulating activity at the level of

(7) K. Hofmann, T. Liu, H. Yajima, N. Yanaihara and S. Lande, *J. Am. Chem. Soc.*, **83**, 2294 (1961).

(8) K. Hofmann, E. Stutz, G. Spühler, H. Yajima and E. T. Schwartz, *ibid.*, **82**, 3727 (1960).

(9) (a) H. A. Staab, *Ann. Chem. Liebigs.*, **609**, 75 (1957); (b) G. W. Anderson and R. Paul, *J. Am Chem. Soc.*, **80**, 4423 (1958); (c) R. Paul and G. W. Anderson, *ibid.*, **82**, 4596 (1960).

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

(11) K. Hofmann and S. Lande, *ibid.*, **83**, 2286 (1961).

(12) E. A. Peterson and H. A. Sober, *ibid.*, **78**, 751 (1956).

(13) K. Hofmann, H. Yajima and E. T. Schwartz, *ibid.*, **82**, 3732 (1960).

(14) Ascorbic acid depleting activity was determined in 24 hour hypophysectomized rats according to the method of U. S. Pharmacopeia XV against the USP reference standard. The plasma corticosterone levels were determined 15 minutes following administration, R. Guillemin, G. W. Clayton, J. D. Smith and H. S. Lipscomb, *Endocrinol.*, **63**, 93 (1958). The free steroid was separated chromatographically and assayed by a modification of the method of H. Kalant, *Biochem. J.*, **69**, 93 (1958). We are much indebted to Dr. Joseph D. Fisher of Armour Pharmaceutical Company, Kankakee, Illinois for the ACTH assays.

2.0×10^9 MSH (melanocyte stimulating hormone) units/g.[15]

We have shown[6] that exposure to hot 0.5 *N* hydrochloric acid converted acetylseryltyrosylserylmethionylglutamine into seryltyrosylserylmethionylglutamic acid without causing excessive fragmentation of peptide bonds. Partial hydrolysis with dilute hydrochloric acid served to convert the protected hexadecapeptide amide (XI) into the free hexadecapeptide (I). Apparently homogeneous samples of (I) were isolated from such hydrolysates by CMC chromatography, followed by cellulose-block electrophoresis at pH 6.5 and a second purification on CMC. The final product produced a single Pauly positive spot on paper electrophoresis in pyridinium acetate buffers of pH 3.8, 5.1 and 6.5 and in collidinium acetate buffer of pH 6.9. Paper chromatograms of (I) and (XI) have been reproduced.[4]

Two lines of evidence, one chemical and one enzymatic, served to identify a free carboxyl group as the C-terminus of the hexadecapeptide (I). The lithium borohydride reduction product of the methyl ester of (I)[16] was acid hydrolyzed and the hydrolysate contained 2.35 μmoles of lysine per μmole of peptide. Hydrolysates of the untreated peptide contained 3.03 μmoles of lysine per μmole of peptide.[17]

Carboxypeptidase B,[18] an enzyme which liberates C-terminal lysine and arginine from peptides, removed 1.97 μmoles of lysine per μmole of (I). Since peptides containing C-terminal amide groups are not attacked by this enzyme, it seems evident that the synthetic hexadecapeptide must terminate with a free carboxyl group.

The hexadecapeptide possessed melanocyte expanding activity at a level of 3.7×10^8 units/g. Assays by the *in vivo* rat adrenal ascorbic acid depletion technique of several batches of the synthetic peptide (I) showed a low but reproducible activity of <0.1 i.u./mg. The ability of (I) to bring about the elevation of the plasma corticosterone level agreed closely with the ascorbic acid depleting potency.

These results demonstrate conclusively that an apparently homogeneous peptide which corresponds to the sequence of the N-terminal 16 amino acid residues of the corticotropins possesses adrenocorticotropic activity which is of the same low order of magnitude as that of a peptide corresponding to the N-terminal tridecapeptide sequence. Thus, additional structural elements are necessary to endow a peptide with the full biological potency of ACTH.

Experimental[19]

N^{α}-Carbobenzoxy-N^{ϵ}-formyllysylprolylvalylglycyl-N^{ϵ}-formyllysyl-N^{ϵ}-formyllysine Amide (III). a. By Amination of the Methyl Ester (II).—The methyl ester (II),[7] (250 mg.) was dissolved in methanol (5 ml.), the solution cooled with a Dry Ice–acetone bath and a stream of ammonia was passed through for 10 minutes. The mixture was kept in a refrigerator for one week, the precipitate collected, dissolved in ethanol (5 ml.) and the solution was placed in a refrigerator. The gelatinous mass was washed with ice-cold ethanol and dried *in vacuo;* yield 144 mg. (59%); m.p. 173–177°; $[\alpha]^{26}D$ − 34.7° (c. 1.1 in DMF); ninhydrin negative; amino acid ratios in acid hydrolysate lys 2.99 pro 1.04 val 1.02 gly 0.96 NH_3 1.0 (recovery 97%). *Anal.* Calcd. for $C_{41}H_{64}O_{11}N_{10}$: C, 56.4; H, 7.4; N, 16.0. Found: C, 55.8; H, 7.4; N, 15.8.

b. From N^{α}-Carbobenzoxy-N^{ϵ}-formyllysylprolylvalylglycine and N^{ϵ}-Formyllysyl-N^{ϵ}-formyllysine Amide.—For preparation of N^{ϵ}-formyllysyl-N^{ϵ}-formyllysine amide hydrochloride the N^{α}-carbobenzoxy derivative[8] (2.20 g.) was suspended in methanol (60 ml.) and 10% v./v. of aqueous acetic acid (2.8 ml.) and was hydrogenated over palladium. The ensuing clear solution was separated from the catalyst, evaporated to dryness *in vacuo* and the residue dissolved in methanol (10 ml.). The solution was chilled with a Dry Ice–acetone bath and concentrated hydrochloric acid (0.48 ml.) was added. The hydrochloride, precipitated by addition of ether, was washed with ether and dried *in vacuo;* yield 1.55 g. (89%); R_f^1 0.32.

A solution of the above hydrochloride (760 mg.) in methanol (5 ml.) and triethylamine (0.3 ml.) was evaporated to dryness and the residual oil kept *in vacuo* at room temperature for 30 minutes. This material dissolved in DMF (20 ml.) was added to an ice-cold solution of an acylating agent prepared by adding N,N′-carbonyldiimidazole[9] (340 mg.) to a chilled solution of N^{α}-carbobenzoxy-N^{ϵ}-formyllysylprolylvalylglycine[8] (1.153 g.) in DMF (25 ml.) and stirring the mixture in an ice-bath until evolution of carbon dioxide had ceased. The coupling reaction was allowed to proceed for 4 hr. at room temperature when the solvent was removed *in vacuo*. The residue was dissolved in *n*-butanol saturated with 2% aqueous acetic acid and the solution was washed with six 20 ml. portions of 2% acetic acid, six 20 ml. portions of 3% ammonium hydroxide and seven portions of water saturated with 1-butanol. Emulsions were broken by centrifugation. The solvent was removed *in vacuo*, the residue dissolved in hot ethanol (20 ml.) and the solution placed in a refrigerator for approximately 20 hr. The gelatinous precipitate was collected, washed with ice-cold ethanol and dried; yield 1.10 g. (64%); m.p. 174–179°; $[\alpha]^{28}D$ −35.2° (*c.* 0.94 in DMF); ninhydrin negative; amino acid ratios in acid hydrolysate lys 2.95 pro 1.05 val 1.05 gly 0.94 NH_3 1.02 (recovery 96%); no depression of m.p. when admixed with material prepared according to a. above.

N^{ϵ}-Formyllysylprolylvalylglycyl-N^{ϵ}-formyllysyl-N^{ϵ}-formyllysine Amide Hydrochloride Tetrahydrate (IV).—N^{α} - Carbobenzoxy - N^{ϵ} - formyllysylprolylvalylglycyl-N^{ϵ}-formyllysyl-N^{ϵ}-formyllysine amide (0.890 g.) was hydrogenated over palladium in methanol (50 ml.) containing one equimol of glacial acetic acid. The catalyst was removed by filtration, the filtrate was evaporated to dryness *in vacuo* and the residue was dissolved in ethanol (5 ml.). The product, precipitated by addition of ethyl acetate, was dried *in vacuo;* yield 0.750 g. (87%). For conversion to the hydrochloride the acetate (0.610 g.) was dissolved in water (3 ml.), the solution was cooled in an ice–salt bath

(15) 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 the MSH assays which were performed according to the method of K. Shizume, A. B. Lerner and T. B. Fitzpatrick, *Endocrinol.*, **54**, 553 (1954).

(16) J. C. Crawhall and D. T. Elliott, *Biochem. J.*, **61**, 264 (1955).

(17) A band which followed closely the lysine peak was present on Stein–Moore chromatograms of acid hydrolysates derived from the reduction product. This component which is absent in acid hydrolysates of (I) appears to be 2,6-diamino-*n*-hexanol, derived from the C-terminal lysine.

(18) J. E. Folk, *J. Am. Chem. Soc.*, **78**, 3541 (1956).

(19) The organic solvents were freshly distilled. Doubly distilled water from which a sizeable forerun was removed was employed. The melting points are uncorrected. Rotations were determined with a Rudolph Precision Polarimeter Model 80 with a model 200 photoelectric attachment. The amino acid composition of the acid and enzymatic hydrolysates was determined with a Beckman-Spinco Amino Acid Analyzer, Model 120 according to the method of Moore, Spackman and Stein, *Anal. Chem.*, **30**, 1185 (1958). R_f^1 values refer to the Partridge system (S. M. Partridge, *Biochem. J.*, **42**, 238 (1948), R_f^2 values to the system 1-butanol, pyridine, acetic acid, water 30:20:6:24 (S. G. Waley and G. Watson, *ibid.*, **55**, 328 (1953)). The latter are expressed as a multiple of the distance traveled by a histidine marker. The carboxymethylcellulose (Cellex-CM) was obtained from the Bio-Rad Corporation, Richmond, California. Unless stated otherwise, solvents were evaporated at a bath temperature of 40–50° in a rotary evaporator. Absorbancy at 280 mμ served to locate peptides in the various chromatographic fractions.

and 1 *N* hydrochloric acid (0.8 ml.) was added. The solution was then lyophilized; $[\alpha]^{29}D$ −70.5° (*c* 0.56 in 10% acetic acid); single ninhydrin positive spot; R_f^1 0.39; amino acid ratios in LAP digest N^{ϵ}-formlys 3.03 pro 1.05 val 1.00 gly 0.95 (recovery 97%).

Anal. Calcd. for $C_{33}H_{59}O_9N_{10}Cl.4H_2O$: N, 16.5; Cl, 4.2. Found: N, 16.3; Cl, 5.0.

Histidylphenylalanylarginyltryptophylglycyl-N^{ϵ}-formyllysylprolylvalylglycyl - N^{ϵ} - formyllysyl - N^{ϵ} - formyllysine Amide Diacetate Tetrahydrate (V). a. From (IV) plus (VI) *via* (VII) with DCC.— N,N'-Dicyclohexylcarbodiimide (87 mg.) was added to a DMF (dimethylformamide) solution (8.58 ml.) containing (IV) (326 mg.), (VI) (378 mg.) and 10% v./v. triethylamine in DMF (0.58 ml.). After standing at room temperature for 20 hr. DCC (43 mg.) was added followed 8 hr. later by an additional portion (21 mg.) of this reagent. After the last addition the mixture was kept at room temperature for 20 hr. when it was filtered and ether (250 ml.) added to the filtrate. The precipitate collected by centrifugation as washed with ether and dissolved in 250 ml. of *n*-butanol equilibrated with 5% aqueous acetic acid. The butanol solution was washed with three 15 ml. portions of 5% acetic acid and the solvent was removed. The residue (crude VII) was hydrogenated over palladium in 50 ml. of 50% aqueous acetic acid for 10 hr. with two additions of fresh catalyst. The suspension was filtered, the filtrate was diluted with water (150 ml.) and the solution concentrated to a small volume *in vacuo* and then lyophilized. Paper chromatography of the residue (630 mg.) showed the presence of two major components with R_f^1 values of 0.40 and 0.64, respectively. The product was dissolved in 50 ml. of pH 5.75, 0.025 *M* ammonium acetate, and this solution was added to a CMC column (1.5 × 46 cm.) previously equilibrated with the same buffer. The column was then eluted successively with the following ammonium acetate buffers: 0.025 *M*, pH 5.8 (200 ml.); 0.05 *M*, pH 5.9 (200 ml); 0.075 *M*, pH 6.3 (750 ml.); and finally 0.25 *M*, pH 6.9 (350 ml.). Individual fractions (8 ml. each) were collected at a flow rate of 3 to 4 ml. per minute. Fractions 95–136 (0.075 *M* eluates) containing the desired peptide were pooled and the solvent removed first by evaporation, then by lyophilization. Ammonium acetate was removed by repeated lyophilization of the residue to constant weight; yield 264 mg. (42%); $[\alpha]^{29}D$ −43.0° (*c*. 0.35 in 5% acetic acid); R_f^1 0.40; sharp, single ninhydrin, Sakaguchi, Pauly and Ehrlich positive spot; amino acid ratios in acid hydrolysate his 1.00 phe 1.00 arg 1.00 gly 2.01 lys 2.99 pro 1.00 val 1.00 (recovery 89%); amino acid ratios in LAP digest his 0.99 phe 1.00 arg 1.06 try 1.06 gly 1.87 formlys 3.01 pro 1.00 val 0.99 (recovery 85%).

Anal. Calcd. for $C_{71}H_{107}O_{18}N_{21}.4H_2O$: C, 52.8; H, 7.2; N, 18.2. Found: C, 52.2; H, 7.2; N, 17.9.

b. From (IV) plus (VIII) via (IX) with DCC.—N,N'-Dicyclohexylcarbodiimide (45 mg.) was added to a solution of the dihydrochloride of (VIII)[11] (154 mg.), the hydrochloride of (IV) (90 mg.) in DMF (2 ml.) and 10% v./v. of triethylamine in DMF (0.41 ml.). An additional portion of DCC (25 mg.) was added after 24 hr. at room temperature, and the mixture was kept at room temperature for an additional 24 hr. The suspension was filtered, ether (250 ml.) was added to the filtrate and the precipitate was collected, washed with ether and dried. The residue (crude IX) (247 mg.) contained two major components with R_f^1 values of 0.63 and 0.90, respectively, and a minor component with R_f^1 0.72 (apparently VIII). This material was hydrogenated over palladium in 15% aqueous acetic acid (20 ml.) and the hydrogenated products isolated in the usual manner; yield 209 mg. of a fluffy colorless powder containing two major components with R_f^1 values of 0.40 and 0.64, respectively, and a minor component with R_f^1 0.45. This material was dissolved in 0.05 *M*, pH 6.5 ammonium acetate buffer (30 ml.) and this solution was added to a CMC column (1.5 × 20 cm.) previously equilibrated with the same buffer. The column was then eluted successively with the following pH 6.5 ammonium acetate buffers: 0.05 *M* (250 ml.); 0.075 *M* (250 ml.); 0.087 *M* (500 ml.) and finally 0.25 *M* (250 ml.). Individual fractions (5 ml. each) were collected at a flow rate of 3 to 4 ml. per minute. The acyl urea derivative of (VIII) was present in the 0.25 *M* eluates. The desired material was present in the 0.087 *M* eluates which were pooled and the solvent removed first by evaporation to a small volume then by lyophilization. Ammonium acetate was removed by repeated lyophilization of the residue to constant weight; yield 64 mg. (37%); $[\alpha]^{29}D$ −44.2° (*c* 0.33 in 5% acetic acid); R_f^1 0.40; sharp, single spot ninhydrin, Sakaguchi, Pauly and Ehrlich positive; amino acid ratios in acid hydrolysate his 1.00 phe 1.00 arg 1.00 gly 2.00 lys 2.94 pro 1.01 val 1.00 (recovery 97%); amino acid ratios in LAP digest his 0.83 phe 1.00 arg 0.97 try 1.00 gly 1.90 formlys 3.07 pro 1.00 val 1.00 (recovery 90%).

Anal. Calcd for $C_{71}H_{107}O_{18}N_{21}.4H_2O$: C, 52.8; H, 7.2; N, 18.2. Found: C, 52.2; H, 7.3; N, 18.3.

c. From (IV) plus (VIII) via (IX) with CDI.—N,N'-Carbonyldiimidazole[9] (350 mg.) was added to an ice-cold solution of the dihydrochloride of (VIII)[11] (470 mg.) in DMF (6.0 ml.) and the solution was kept at 5° for 1.5 hr. A solution of the hydrochloride of (IV) (400 mg.) in DMF (2.0 ml.) was then added and the mixture kept at room temperature for 18 hr. The residue remaining following removal of the solvent contained two components with R_f^1 values of 0.72 and 0.63, respectively. This material was hydrogenated over palladium in 95% aqueous methanol containing 5% v./v. of acetic acid (40 ml.) and the desired peptide isolated from the hydrogenated products by chromatography on a CMC column (prepared from 11 g. of the cellulose with 0.025 *M* ammonium acetate buffer). The column was eluted successively with the following pH 6.5 ammonium acetate buffers: 0.025 *M* (500 ml.); 0.05 *M* (500 ml.) and 0.087 *M* (500 ml.). The desired peptide was isolated from the 0.087 *M* eluates essentially in the manner described under (b) above; yield 380 mg. (50%); $[\alpha]^{29}D$ −44.1° (c 0.33 in 5% acetic acid); R_f^1 0.40; sharp, single spot, ninhydrin, Sakaguchi, Pauly and Ehrlich positive; amino acid ratios in acid hydrolysate his 1.00 phe 1.00 arg 0.97 gly 2.00 lys 2.94 pro 1.01 val 1.00 (recovery 95%); amino acid ratios in LAP digest his 1.02 phe 0.98 arg 1.03 try 1.09 gly 1.93 formlys 2.97 pro 0.98 val. 0.98 (recovery 94%).

Anal. Calcd. for $C_{71}H_{107}O_{18}N_{21}.4H_2O$: C, 52.8; H, 7.2; N, 18.2. Found: C, 53.6; H, 7.4; N, 18.4.

Acetylseryltyrosylserylmethionylglutaminylhistidylphenylalanylarginyltryptophylglycyl - N^{ϵ} - formyllysylprolylvalylglycyl - N^{ϵ} - formyllysyl - N^{ϵ} - formyllysine Amide Monoacetate Octahydrate (XI).—The azide of acetylseryltyrosylserylmethionylglutamine (X) (180 mg.) prepared from 250 mg. of the hydrazide[13] was added to an ice-cold DMF solution (6 ml.) containing (V) (285 mg.) and triethylamine (0.026 ml.). The solution was kept at 4° for 24 hr., then a second portion of azide (171 mg.) was added. Paper chromatographic evaluation of the reaction mixture after additional 24 hr. at 4° revealed the almost complete absence of the amino component. The solvent was removed *in vacuo*, and the crude reaction product was dissolved in 0.005 *M* pH 5.5 ammonium acetate (50 ml.), and this solution was applied to a CMC column (1.5 × 55 cm.) which was then eluted successively with the following ammonium acetate buffers: 0.005 *M*, pH 5.5 (150 ml.); 0.025 *M*, pH 5.7 (350 ml.); 0.0375 *M*, pH 5.8 (150 ml.); 0.05 *M*. pH 5.9 (800 ml.); and finally 0.25 *M*, pH 6.9 (350 ml.). Individual fractions (10 ml. each) were collected at a flow rate of 3 to 4 ml. per minute. The desired product was present in the 0.05 *M* eluates (fractions 78–120); these were pooled, the bulk of the solvent removed *in vacuo* and the residue lyophilized. Ammonium acetate was removed by repeated lyophilization of the residue to constant weight; yield 358 mg. (90%); $[\alpha]^{27}D$ −56.6° (*c*. 0.3 in 10% acetic acid); λ_{max} 280 mμ, Log ε 3.789 (in 10% acetic acid); R_f^1 0.55; sharp, single spot, ninhydrin negative, Pauly, methionine, Sakaguchi and Ehrlich positive; single Pauly positive component on paper electrophoresis in pyridinium acetate buffers at pH 3.8, 5.1 and 6.0, respectively; MSH activity 2.0×10^3 MSH units/g.; amino acid ratios in acid hydrolysate ser 2.08 tyr 1.02 met 0.84 glu 1.00 his 0.98 phe 0.98 arg 0.97 gly 2.00 lys 2.99 pro 0.97 val 1.00 (recovery 95%).

Anal. Calcd. for $C_{96}H_{141}O_{26}N_{27}S\cdot 8H_2O$: C, 50.9; H, 7.0; N, 16.7. Found: C, 50.9; H, 6.7; N, 16.3.

Seryltyrosylserylmethionylglutamylhistidylphenylalanylarginyltryptophylglycyllysylprolylvalylglycyllysyllysine Acetate (I).—A test tube charged with the protected hexadecapeptide amide (XI) (200 mg.) and 0.5 *N* hydrochloric acid containing 0.1% of thioglycolic acid (12 ml.) was immersed in a boiling water-bath for 80 minutes and then immediately cooled at room temperature (gas-phase nitrogen). The

solution was diluted with water (12 ml.) and Amberlite IRA-400 in the acetate cycle was added with stirring in small portions until the supernatant was free of chloride ions (approximately 8 g. required). The resin removed by filtration, was washed with four 10 ml. portions of water and the combined filtrate and washings were lyophilized. The ensuing fluffy colorless material (200 mg.) was dissolved in water (40 ml.) and the solution added to a CMC column (1.5 × 23.5 cm.) which was eluted successively with the following ammonium acetate buffers: 0.075 *M*, pH 6.3 (300 ml.); 0.1 *M*, pH 6.5 (300 ml.); 0.15 *M*, pH 6.7 (400 ml.); and 0.175 *M*, pH 6.9 (500 ml.). Individual fractions (10 ml. each) were collected at a flow rate of 3 ml. per minute. The 0.15 *M* eluates (fractions 83–107) were pooled, the bulk of the solvent was removed *in vacuo*, the rest by lyophilization. Ammonium acetate was removed by repeated lyophilization to constant weight; yield 59 mg.; contains one major and one minor component with R_f^2 values of 1.25 and 0.56 × histidine, respectively. This material was subjected to electrophoresis on a block (38.5 × 7.7 × 0.9 cm.) prepared with Whatman paper powder in a pH 6.5 pyridinium acetate buffer (pyridine 10, acetic acid 0.4, water 90 v./v.). The sample (50 mg.) in buffer (0.5 ml.) was applied to the anode side of the block and electrophoresis conducted at 4° for 8.5 hr. (900 volts, 35 milliamps). A contact print from the surface of the block revealed the presence of two ninhydrin positive zones which were located 3.6–6.7 and 10.7–12.5 cm., respectively from the origin. The section containing the slower moving zone was eluted with 1% acetic acid and the solvent was removed. The residue dissolved in water (25 ml.) was applied to a CMC column (1.5 × 7 cm.) which was then eluted with 0.075 *M* ammonium acetate buffers of pH 7.0 (150 ml.) and pH 7.8 (300 ml.). Individual fractions (5 ml. each) were collected at a flow rate of 2.5 ml. per minute. The pH 7.8 eluates were pooled and lyophilized to constant weight; yield 41 mg.; $[\alpha]^{30}D$ −57.1° (c. 0.20 in 5% acetic acid); R_f^2 1.25 × his single ninhydrin, Pauly, Sakaguchi, methionine and Ehrlich positive spot; single Pauly positive component on paper electrophoresis in pyridinium acetate buffers at pH 3.8. 5.1 and 6.5 and in collidinium acetate buffer at pH 7.0; MSH activity 3.7×10^8 MSH units/g.; adrenal ascorbic acid depleting activity <0.1 i.u./mg.; amino acid ratios in acid hydrolysate ser 1.93 tyr 0.97 met 0.93 glu 1.00 his 0.93 phe 1.00 arg 0.97 gly 2.07 lys 3.03 pro 1.10 val 1.03 (recovery 93%); amino acid ratios in LAP digest ser 2.00 tyr 0.94 met 0.88 glu 0.94 his 0.88 phe 1.00 arg 1.00 try 1.00 gly 2.00 lys 3 24 pro 1.00 val 1.06 (recovery 85%).

Determination of C-Terminal Carboxyl Group.—The hexadecapeptide (I) (2.77 mg.) was dissolved in water (0.5 ml.) and methanol (0.5 ml.) was added. The solution was cooled with an ice-bath and diazomethane in ether was added until the yellow color remained for 10 minutes. A drop of glacial acetic acid was added and the solution was evaporated to dryness over KOH and P_2O_5 *in vacuo*. A solution of $LiBH_4$ (1.8 mg.) in tetrahydrofuran (0.8 ml.) was added and the mixture was heated for 5 hr. (bath temperature 75°). The mixture was acidified with 3 *N* hydrochloric acid and evaporated to dryness over KOH and P_2O_5. Amino acid ratios in acid hydrolysate ser 1.62 tyr 0.73 met 0.81 glu 1.00 his 1.00 phe 1.05 arg 1.00 gly 2.05 pro 1.13 val 1.11 lys 2.35. A peak which followed closely the lysine peak was present on the chromatogram.

Enzymatic Procedures.—The LAP digestions were carried out in the manner previously described.[6] Carboxypeptidase B (Worthington Lot 6020) (1 mg.) was suspended in 0.1% sodium bicarbonate (1 ml.) at 4°, and the pH was adjusted to 8.0 by addition of 0.1 *N* sodium hydroxide. A solution of 0.1 *M* diisopropyl phosphofluoridate (DFP) in isopropanol (0.03 ml.) was added and the mixture kept at room remperature for 1 hr. Peptide (I) (2.12 mg.) was dissolved in 0.025 *M*, pH 8.0 "tris" buffer (0.3 ml.) and the DFP treated enzyme solution (0.3 ml.) was added. The mixture was incubated for 24 hr. at 40° when enzymatic action was terminated by addition of acetic acid. The solution was evaporated to dryness over P_2O_5 and KOH and the residue analyzed with an amino acid analyzer. The lysine liberated from 0.36 μmoles of peptide was 0.71 μmole. Trace quantities of other amino acids were also present in the hydrolysate.

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