b. From Nα-Carbobenzoxyhistidylphenylalanylarginyltryptophylglycine (XIII).—The carbobenzoxy derivative (200 mg.) was suspended in 90% v./v. aqueous acetic acid and hydrogenated over a palladium catalyst in the usual manner until the evolution of carbon dioxide ceased. The product was isolated in the manner described under (a) above; yield 162 mg. (82%), $[\alpha]^{28}D$ −12.0° (*c* 1.0 in 1*N* hydrochloric acid); single ninhydrin, Pauly, Ehrlich and Sakaguchi positive spot; R_f^1 0.50, R_f^2 0.74; amino acid ratios in acid hydrolysate $his_{0.97}phe_{1.03}arg_{0.98}gly_{1.05}$ (95%); amino acid ratios in LAP digest $his_{0.97}phe_{1.00}arg_{0.98}try_{1.00}gly_{1.03}$ (90%).

Anal. Calcd. for $C_{36}H_{47}O_8N_{11}\cdot 2H_2O$: C, 54.2; H, 6.4; N, 19.3. Found: C, 53.7; H, 6.6; N, 19.8.

Isolation of DL-Arginine from Racemized Histidylphenylalanylarginyltryptophylglycine.—Crude pentapeptide I (1 g.) [obtained by hydrogenating mother liquor material from preparation of 4-L (IV)[4]] was refluxed for 36 hours with 6*N* hydrochloric acid (110 ml.). The hydrolysate was decolorized with Norit-A, the filtrate evaporated to dryness, the residue freed from excess of hydrochloric acid by repeated evaporation with water and the resultant oil dissolved in water (2 ml.). Flavianic acid (1 g.) was added, the mixture was kept at 4° for 5 hours with occasional shaking. The precipitate was collected by centrifugation, was dissolved in hot 5% ammonium hydroxide and precipitated by addition of 1*N* hydrochloric acid. This process was repeated three times and the final product was washed with ethanol and dried. The flavianate was dissolved in concentrated hydrochloric acid (3 ml.) and the mixture was kept at room temperature for 1 hour and at 0° for 30 minutes and then filtered. The flavianic acid precipitate was washed with several small portions of ice-cold concentrated hydrochloric acid and the combined filtrate and washings were evaporated to dryness. The residue was dissolved in ethanol (2 ml.), the solution was filtered after standing at 4° for 18 hours and the filtrate was decolorized with Norit-A and evaporated. The residue was dissolved in methanol and crystallization of the arginine monohydrochloride effected by addition of aniline. The arginine monohydrochloride was recrystallized from ethanol; m.p. 201–203°, $[\alpha]^{24}D$ ±1° (*c* 2.0 in water); paper chromatographically homogeneous in Partridge and 2-butanol–ammonia systems[15]; R_f's identical with those of arginine. L-Arginine monohydrochloride, m.p. 216–218°, $[\alpha]^{24}D$ +12.6° (*c* 2.0 in water), subjected to the isolation procedure exhibited the properties: m.p. 215–216°, $[\alpha]^{24}D$ +11.2° (*c* 2.0 in water).

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Studies on Polypeptides. XX. Synthesis and Corticotropic Activity of a Peptide Amide Corresponding to the N-Terminal Tridecapeptide Sequence of the Corticotropins[1-4]

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Exposure of carbobenzoxyseryltyrosylserylmethionylglutaminylhistidylphenylalanylarginyltryptophylglycyl-N$^{\epsilon}$-tosyllysylprolylvaline amide to sodium in liquid ammonia resulted in marked destruction of the peptide chain. A systematic study pertaining to stability to 0.5 *N* hydrochloric acid at 100° of certain peptide derivatives corresponding to the α-MSH sequence demonstrated: (a) that the peptide chain of the α-MSH molecule undergoes some hydrolysis under these conditions; (b) that the N-terminal acetyl group and the glutamine amide function are removed from N-acetylseryltyrosylserylmethionylglutamine with formation of seryltyrosylserylmethionylglutamic acid; (c) that histidylphenylalanylarginyltryptophylglycyl-N$^{\epsilon}$-formyllysylprolylvaline amide affords histidylphenylalanylarginyltryptophylglycyllyslylprolylvaline amide; (d) that acetylseryltyrosylserylmethionylglutaminylhistidylphenylalanylarginyltryptophylglycine is converted into stereochemically homogeneous seryltyrosylserylmethionylglutamylhistidylphenylalanylarginyltryptophylglycine; (e) that acetylseryltyrosylserylmethionylglutaminylhistidylphenylalanylarginyltryptophylglycyl-N$^{\epsilon}$-formyllysylprolylvaline amide is converted to seryltyrosylserylmethionylglutamylhistidylphenylalanylarginyltryptophylglycyllysylprolylvaline amide, which was homogeneous stereochemically, exhibited pronounced *in vitro* melanocyte-expanding activity (1.9×10^9 MSH units/g.) and brought about *in vivo* adrenal ascorbic acid depletion and plasma corticosterone elevation in the rat-(activity level <0.1 I.U./mg.).

Peptic digestion of the 39 amino acid residue β-corticotropin molecule affords, among other products, an N-terminal octacosapeptide fragment which is reported to retain the full ascorbic acid depleting activity of the intact hormone. From the results of partial hydrolysis with acid it was inferred that the four C-terminal amino acids may be removed from this fragment with formation of a tetracosapeptide without altering significantly the biological potency.[5]

The smallest fragment of the corticotropin molecule possessing *in vivo* corticotropic activity remains to be established. Indeed, Steelman and Guillemin[6] were able to elicit *in vitro* and *in vivo* corticotropic activity with purified samples of α-MSH, the porcine melanocyte expanding hormone (I) which contains the same N-terminal tridecapeptide sequence as do the corticotropins.[7] The availability for biological studies of the synthetic tridecapeptide amide (VI) which corresponds to the N-terminal third of the amino acid sequence of the corticotropin molecule and which is closely related to α-MSH (I) is of interest in this connection.

(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 and Co. for generous support of this investigation.

(2) The peptides 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.

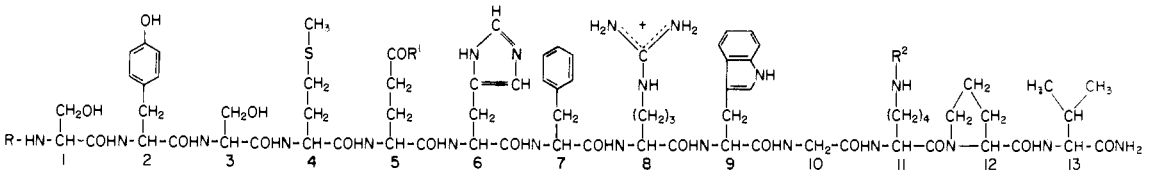
(3) See *J. Am. Chem. Soc.*, **83**, 2286 (1961), for paper XIX in this series.

(4) A preliminary report of some of the results presented in this paper was given at the Brookhaven Conference on Protein Structure and Function on June 8, 1960; K. Hofmann, *Brookhaven Symp. in Biol.*, **13**, 184 (1960).

(5) P. H. Bell, K. S. Howard, R. G. Shepherd, B. M. Finn and J. H. Meisenhelder, *J. Am. Chem. Soc.*, **78**, 5059 (1956).

(6) S. L. Steelman and R. Guillemin, *Proc. Soc. Biol. Med.*, **101**, 600 (1959).

(7) J. I. Harris, *Biochem. J.*, **71**, 451 (1959).



I, R = acetyl; R^1 = OH; R^2 = H
II, R = carbobenzoxy; R^1 = NH_2; R^2 = tosyl
III, R = acetyl; R^1 = NH_2; R^2 = tosyl
IV, R = acetyl; R^1 = NH_2; R^2 = formyl
V, R = H; R^1 = NH_2; R^2 = H
VI, R = H; R^1 = OH; R^2 = H

We have prepared three derivatives of α-MSH (compounds II, III and IV)[8] which embody within their molecules the entire amino acid sequence of this hormone, but which contain protecting groups attached to the N-terminus, the carboxyl group in position 5 and the lysine residue in position 11.

The stability of compounds II and IV has now been explored in a systematic manner under a variety of experimental conditions, and as an outgrowth of these studies a procedure has been developed for converting compound IV into the tridecapeptide amide VI.

Attempts to convert compound II into the tridecapeptide amide V by reduction with sodium in liquid ammonia[9] were most unpromising. The mixture of products resulting from this treatment was subjected to extensive countercurrent distribution and the major, still heterogeneous component was isolated. This material was acid hydrolyzed and the hydrolysate subjected to quantitative amino acid analysis on paper. The chromatogram revealed the presence of the expected amine acids but lysine, proline and valine were present in extremely small proportions. This result pointed to a striking lability of the protected peptide toward sodium in liquid ammonia and eliminated this procedure as a convenient route for the preparation of V.

The availability of N^{ϵ}-formyllysine and its successful application[10] to the synthesis of the blocked acetyltridecapeptide IV provided a route to the free tridecapeptide amide VI.

Of particular importance was the observation that three of the protecting groups, namely, the N-terminal acetyl group, the glutamine amide group and the N^{ϵ}-formyl group attached to the lysine residue, were removable from compound IV with 0.5 *N* hydrochloric acid at 100°. This treatment brought about some hydrolysis of peptide bonds but allowed the isolation of analytically pure VI in satisfactory yields.

The difference in composition of leucine aminopeptidase (LAP) digests of the various peptides before and after treatment with acid provided a convenient analytical tool. Both N^{ϵ}-formyllysine and glutamine were incubated separately with the enzyme preparation and were shown by paper chromatography and Stein–Moore analyses[11] to remain unaltered.

The homogeneous product, isolated in the manner outlined in the Experimental section from a partial hydrolysate of histidylphenylalanylarginyltryptophylglycyl - N^{ϵ} - formyllysylprolylvaline amide,[9] was hydrolyzed completely by LAP with formation of the expected mixture of amino acids, but the enzymic digest contained free lysine and not the N^{ϵ}-formyl derivative. N^{ϵ}-Formyllysine is readily distinguishable from free lysine by paper chromatography.[10] The acid-sensitive tryptophan residue was not affected significantly by the acid treatment since the ultraviolet absorption spectrum of the deblocked material was essentially identical to that of the original compound. The resistance of the partially deblocked peptide to the action of carboxypeptidase provided additional evidence for the resistance of the C-terminal amide group to the acid treatment.

The experimental evidence cited justifies the conclusion that the acid treatment had removed the formyl group from the octapeptide amide. It is of interest to note that removal of the formyl protecting group does not alter the melanophoretic activity; both the partially protected[10] and the free octapeptide amide assayed 8.0×10^6 MSH units/g. We have shown previously that formylation of the lysine ε-amino group of an α-MSH analog[3] did not alter the ability to expand melanocytes.

The stability of the N-terminal acetyl group and of the glutamine amide group of N-acetylseryltyrosylserylmethionylglutamine toward hot dilute hydrochloric acid was investigated next.

Samples of the crystalline compound[12] were exposed to the acid treatment and the reaction products were examined by paper chromatography. One major (R_f 0.51) and two minor compounds were present. The major component was separated from these impurities by preparative chromatography on paper, and was crystallized from a mixture of water and ethanol. The compound was ninhydrin positive, was completely digestible by LAP and the digest contained the constituent amino acids in the ratios expected by theory with glutamic acid and not glutamine being present.

These findings and the observation that the deblocked acetylpentapeptide afforded seryltyrosine and serylmethionylglutamic acid on digestion with chymotrypsin established the presence of seryltyrosylserylmethionylglutamic acid. With exception of minor differences in optical rotation the peptide exhibited the properties previously re-

(8) (a) K. Hofmann, M. E. Woolner, H. Yajima, G. Spühler, T. A. Thompson and E. T. Schwartz, *J. Am. Chem. Soc.*, **80**, 6458 (1958); (b) K. Hofmann, H. Yajima and E. T. Schwartz, *Biochim. Biophys. Acta*, **36**, 252 (1959); (c) K. Hofmann, H. Yajima and E. T. Schwartz, *J. Am. Chem. Soc.*, **82**, 3732 (1960).

(9) V. du Vigneaud and O. K. Behrens, *J. Biol. Chem.*, **117**, 27 (1937).

(10) K. Hofmann, E. Stutz, G. Spuhler, H. Yajima and E. T. Schwartz, *J. Am. Chem. Soc.*, **82**, 3727 (1960).

(11) S. Moore, D. H. Spackman and W. H. Stein, *Anal. Chem.*, **30**, 1185 (1958).

(12) K. Hofmann, T. A. Thompson, H. Yajima, E. T. Schwartz and H. Inouye, *J. Am. Chem. Soc.*, **82**, 3715 (1960).

corded for a sample which was prepared by a different route.[13] The results support the conclusion that the acid treatment had resulted in the simultaneous removal of both the glutamine amide group and of the N-terminal acetyl group from the blocked pentapeptide. The somewhat unexpected lability to hot dilute hydrochloric acid of the N-terminal acetyl group may be attributable to a neighboring group effect involving an initial N → O shift of the acetyl moiety.

N - Acetylseryltyrosylserylmethionylglutaminylhistidylphenylalanylarginyltryptophylglycine, prepared by treating the azide of acetylseryltyrosylserylmethionylglutamine[8c] with histidylphenylalanylarginyltryptophylglycine[14] was converted smoothly to seryltyrosylserylmethionylglutamylhistidylphenylalanylarginyltryptophylglycine upon exposure to dilute hydrochloric acid. The paper chromatographically homogeneous free decapeptide was transformed quantitatively into the constituent amino acids by digestion with LAP. The melanocyte-expanding activity of the partially blocked and of the free decapeptide was 3.6×10^6 MSH units/g. and 2.9×10^6 MSH units/g., respectively.[4] The glutamine analog of the free decapeptide commands considerable interest because of its reported *in vitro* corticotropin releasing factor (CRF) activity.[15] The CRF activity of our decapeptide remains to be determined.

The colorless solution which resulted when the acetyltridecapeptide amide IV was heated in 0.5 *N* hydrochloric acid at boiling water-bath temperatures for 80 minutes was freed from chloride ions with Amberlite IRA-400 in the acetate cycle. Lyophilization of the Amberlite filtrate gave a material which exhibited a potency of 2.8×10^8 MSH units/g. in two different experiments. Analysis by paper chromatography of this ninhydrin-positive material revealed the presence of one major and of at least four minor components. Chromatography on a column of carboxymethylcellulose (CMC)[16] with ammonium acetate buffers of increasing ionic strength and *p*H serving as the eluting reagents was employed to separate the mixture of hydrolytic products into five components. The tubes containing each of these components were pooled, the solids were recovered by freeze drying and weight and melanocyte-expanding activity were recorded.

The fraction possessing the highest degree of melanophoretic activity was homogeneous when analyzed by paper chromatography, using five different reagents to develop the chromatograms, and migrated as a single component on paper electrophoresis at various *p*H values; the other fractions were heterogeneous.

Acid hydrolysates of the homogeneous material were shown by the Stein-Moore technique[10] to contain the amino acid composition required for a tridecapeptide amide possessing structure VI. The single equivalent of ammonia, which was present in the hydrolysate, must have derived from the C-terminal amide group since the peptide was not altered by carboxypeptidase. The peptide was completely digestible by LAP[17] and the enzymatic digest contained glutamic acid and free lysine. Chymotrypsin liberated seryltyrosine from the peptide.

The experimental findings cited justify the conclusion that the homogeneous product which was isolated from a partial hydrolysate of IV is an acetate salt of the stereochemically pure tridecapeptide amide VI.

Partial hydrolysis with dilute hydrochloric acid has found little application as a deblocking procedure in peptide synthesis, since the protecting groups employed most frequently thus far are resistant to this treatment.

Certain physiological activities of the protected tridecapeptide amide IV and of the deblocked tridecapeptide amide VI are of interest. The tridecapeptide amide VI exhibited *in vitro* melanocyte-expanding activity of 1.9×10^9 MSH units/g. on repeated assays of different samples, and thus retained 10% of the biological activity of the starting material.[18] This tridecapeptide amide which corresponds to the N-terminal third of the corticotropin molecule possesses some tenfold the melanocyte-expanding activity of that hormone.[19]

In connection with attempts to delineate the minimal sequence possessing *in vivo* corticotropic activity, the ability of peptide VI to bring about ascorbic acid depletion and plasma corticosterone elevation in the rat was determined.[20] The highly purified material exhibited consistently an activity of <0.1 I.U./mg. in these tests and the log-dose response curve was linear. The compound was also active when administered by the subcutaneous route.

The protected acetyltridecapeptide amide IV was inactive. It will be recalled that Steelman and Guillemin[6] determined the *in vitro* and *in vivo* corticotropic activity of purified α-MSH isolated from natural sources and found the compound to

(13) (a) K. Hofmann and A. Jöhl, *J. Am. Chem. Soc.*, **77**, 2914 (1955); (b) K. Hofmann, A. Jöhl, A. E. Furlenmeier and H. Kappeler, *ibid.*, **79**, 1636 (1957).

(14) K. Hofmann, M. E. Woolner, G. Spühler and E. T. Schwartz, *ibid.*, **80**, 1486 (1958).

(15) M. P. de Garilhe, C. Gros, J. Porath and E. B. Lindner, *Experientia*, **16**, 414 (1960).

(16) E. A. Peterson and H A. Sober, *J. Am. Chem. Soc.*, **78**, 751 1956).

(17) Initially the LAP digestions of the tridecapeptide were performed in ammonium acetate buffer as described previously.[14] Practically quantitative recoveries of the N-terminal amino acids were attained, but recovery of amino acids located toward the carboxyl end became progressively lower. This behavior of long-chain peptides, which was observed previously with porcine corticotropin (W. F. White, *J. Am. Chem. Soc.*, **77**, 4691 (1955)) was altered markedly when "tris" buffer was employed. A practically quantitative release of all of the constituent amino acids resulted under these conditions.

(18) 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 Shizume, Lerner and Fitzpatrick, *Endocrinol.*, **54**, 553 (1954).

(19) The *in vitro* MSH activity of porcine β-corticotropin is reported as 1.7×10^8 units/g. (C. H. Li, *Lab. Invest.*, **8**, 514 (1959)).

(20) Ascorbic acid depleting activity was determined in 24-hour hypophysectomized rats according to the method of "U. S. Pharmacopeia," Vol. 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**, 349 (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 Co., Kankakee, Ill., for these biological assays.

be active (3.5 I.U./mg. *in vitro;* 0.1 I.U./mg. *in vivo*). The inactivity of our protected tridecapeptide IV which contains the entire amino acid sequence of α-MSH may be a consequence of its differences from that hormone (amide group attached to the γ-carboxyl group in position 5 and a formyl group attached to the ε-amino group of the lysine in position 11). However, the possibility cannot be excluded that the products which were assayed by Steelman and Guillemin may have contained impurities having the ability to bring about ascorbic acid depletion or corticoid production by direct or indirect routes. Such impurities are not likely to be present in our synthetic preparations.

Experimental[21]

Histidylphenylalanylarginyltryptophylglycyllysylprolylvaline Amide Acetate.—Histidylphenylalanylarginyltryptophylglycyl-N$^{\epsilon}$-formyllysylprolylvaline amide diacetate[10] (100 mg.) was dissolved in 0.5 *N* hydrochloric acid (5 ml.) and the mixture was heated in a sealed tube under nitrogen for 60 minutes in a boiling water-bath. The hydrolysate was cooled in an ice-bath, 2 *N* ammonium hydroxide was added until the *p*H reached 7 and the solvent was removed by freeze drying. The residue was dissolved in pyridinium acetate buffer (1.5 ml.) of *p*H 3.8 (0.1 mole of pyridine, 0.5 mole of acetic acid per liter) and this solution was applied to the anode site of a Whatmann cellulose powder block (38.3 × 7.7 × 0.9 cm.) prepared with the same buffer. Electrophoresis was conducted in a cold room at 5° for 20.5 hours (800 volts and 38 milliamp.). A contact print obtained from the surface of the block which was stained with the Pauly reagent showed the presence of a broad zone located between 11 and 20.5 cm. from the origin. This zone was divided into six equal sections (numbered 1 to 6 in decreasing order of mobility) which were removed and separately eluted with pyridinium acetate buffer. The filtered eluates were evaporated to dryness and the solid residues weighed and analyzed by paper chromatography. Fractions 1 (30 mg.), 2 (28 mg.) and 3 (15 mg.) showed the presence of a major component (R_f^1 0.39) which reacted postively with the Pauly, Sakaguchi and Ehrlich reagents. Fractions 4 (19 mg.), 5 (14 mg.) and 6 (8 mg.) were inhomogeneous and contained decreasing proportions of the R_f^1 0.39 material. These fractions were combined and subjected to a second electrophoretic purification. The resulting Pauly positive zone was divided into 5 equal sections from which the peptide material was extracted in the manner described above. From the sections containing the fastest moving component an additional 27 mg. of homogeneous material of R_f^1 0.39 was obtained which was combined with fractions 1 to 3 from the first run. The slower moving material containing mainly the impurities was rejected. The homogeneous material (100 mg.) was subjected to a 200-plate countercurrent distribution in the solvent system, 0.5% trichloroacetic acid–2-butanol. Spectrophotometric examination of the upper phases at 280 mμ revealed the presence of a single band which was located in tubes 75 to 117 (peak in tube 93). The contents of the tubes containing this material were pooled, concentrated to a volume of 20 ml. *in vacuo* and 0.5 *N* hydrochloric acid (3 ml.) was added. The solution was extracted with three 25-ml. portions of benzene which were discarded and Amberlite IRA-400 (acetate cycle) was added to the aqueous phase with stirring until the supernatant solution was free of chloride ions (approximately 7 g. required). The resin was removed by filtration, was washed with four 20-ml. portions of water, and the combined filtrate and washings were concentrated to a small volume *in vacuo* and finally lyophilized to give a colorless fluffy powder, yield 75 mg., $[\alpha]^{24}D$ −37.0° (*c* 0.8 in 10% acetic acid), R_f^1 0.39, R_f^2 Val; λ_{max} 280 mμ, log ε 3.675 (in 10% acetic acid); starting material λ_{max} 280 mμ, log ε 3.700 (in 10% acetic acid); resistant to the action of carboxypeptidase at enzyme substrate ratio 1:50 at *p*H 7.5; amino acid composition of LAP digest (paper chromatography) $his_{0.9}phe_{1.1}arg_{1.1}try_{1.0}gly_{1.0}lys_{0.9}val_{1.0}$ (pro present on chromatogram but not determined); melanocyte-expanding activity 8.0 × 10^6 MSH units/g.

Seryltyrosylserylmethionylglutamic Acid.—Acetylseryltyrosylserylmethionylglutamine[12] (100 mg.) was dissolved in 0.5 *N* hydrochloric acid (12 ml.) and the mixture was heated in a sealed tube under nitrogen for 60 minutes in a boiling water-bath. The hydrolysate was cooled in an ice-bath, was adjusted to a *p*H of 7 by addition of 1 *N* sodium hydroxide and the solution was lyophilized. Paper chromatographic analysis showed the presence of a major component (R_f 0.51) and two minor components with R_f^1 values of 0.42 and 0.60, respectively, which reacted positively with the ninhydrin and Pauly reagents. These components were separated on a preparative scale on Whatman No.1 filter paper sheets using the Partridge system.[20] The band containing the major component was eluted with water, the combined eluates were concentrated to a volume of approximately 3 ml. *in vacuo* and dry ethanol (approximately 15 ml.) was added. The mixture was placed in a refrigerator for 12 hours and the crystalline material was collected and recrystallized twice from dilute ethanol; yield 59 mg., m.p. 221–223°, $[\alpha]^{24}D$ −22.7° (*c* 0.7 in 2 *N* HCl), R_f^1 0.51, R_f^2 glu; lit.[13] $[\alpha]^{25}D$ −20.6° in 2*N* HCl, R_f^1 0.51, R_f^2 = glu; composition of LAP digest (paper chromatography) $ser_{2.0}tyr_{0.9}met_{1.0}glu_{1.0}$; glutamine could not be detected on the chromatograms. Chymotryptic digestion gave ser.tyr and ser.met.glu (recovery of dipeptide 99 and 100% of theory, respectively, in two experiments). Peptides were separated by two dimensional paper chromatography first in the 2-butanol–ammonia then in the Partridge system.[20]

Acetylseryltyrosylserylmethionylglutaminylhistidylphenylalanylarginyltryptophylglycine Diacetate Tetrahydrate.—Acetylseryltyrosylserylmethionylglutamine azide[7] (0.136 g.) was added to an ice-cold solution of histidylphenylalanylarginyltryptophylglycine[14] (0.119 g.) in freshly distilled dimethylformamide (6 ml.) containing triethylamine (0.021 ml.) and the mixture was kept at 0° for 48 hours. Freshly prepared azide (0.112 g.) was then added and the mixture was kept at 0° for an additional 48 hours. The mixture was filtered and the filtrate evaporated to dryness *in vacuo* (yield 0.36 g.). Analysis by paper chromatography revealed the presence of three Pauly positive materials with R_f^1 values of 0.52, 0.68 and 0.72, respectively. The R_f^1 0.52 component was ninhydrin positive and its R_f matched that of the pentapeptide component. The R_f^1 0.72 component was methionine positive but failed to produce color with the Ehrlich and Sakaguchi reagents. The ninhydrin negative component (R_f 0.68) reacted positively with the methionine, Ehrlich, Pauly and Sakaguchi reagents and represented the desired blocked decapeptide. The crude preparation was dissolved in 0.001 *M* *p*H 5.5 ammonium acetate (60 ml.) and the solution was added to a (15 × 250 mm.) column prepared from carboxymethylcellulose which was equilibrated with the same buffer. The column then was eluted at room temperature with the following ammonium acetate buffers: 0.001 *M*, *p*H 5.5 (250 ml.); 0.005 *M*, *p*H 5.5 (200 ml.); 0.0075 *M*, *p*H 5.5 (200 ml.); 0.01 *M*, *p*H 5.5 (450 ml.); 0.05 *M*, *p*H 5.9 (180 ml.); and finally 0.1 *M*, *p*H 6.5 (180 ml.). Individual fractions (9 ml. each) were collected in an automatic fraction collector at a flow rate of 3 ml. per minute. Absorbancy at 280 mμ served to locate the peptides in the various chromatographic fractions. The desired blocked peptide was located in tubes 87–147 (0.01 *M* buffer eluates). The contents of these tubes were pooled and lyophilized. Ammonium acetate was removed by repeated lyophilization from dilute acetic acid and the product was dried to constant weight over phosphorus pentoxide *in vacuo;* colorless fluffy powder, yield 118 mg., $[\alpha]^{27}D$ −30.6° (*c* 0.5 in 10% acetic

(21) The organic solvents were freshly distilled. Doubly distilled water from which a sizable forerun was removed was employed. The melting points are uncorrected. Rotations were determined in a Rudolph precision polarimeter model 80 with model 200 photoelectric attachment. Ultraviolet spectra were determined with a Cary 14 recording spectrophotometer. The amino acid composition of the acid and LAP hydrolysates were routinely determined with a Beckman-Spinco amino acid analyzer, model 120, but quantitative paper chromatography was employed in some instances.[14] Solvents were evaporated *in vacuo* in a rotary evaporator at a bath temperature of 40–50°. R_f^1 values refer to the Partridge system (S. M. Partridge, *Biochem. J.*, **42**, 238 (1948)); R_f^2 values refer to the 2-butanol–ammonia system (J. F. Roland and A. M. Gross, *Anal. Chem.*, **26**, 502 (1954)). The carboxymethylcellulose (Cellex-CM) was obtained from the Bio-Rad Corporation, Richmond, Calif.

acid), R_f^1 0.68; single ninhydrin negative, Pauly, methionine Sakaguchi and Ehrlich positive spot; λ_{max} 280 mμ, log ε 3.785 (in 10% acetic acid); amino acid composition of acid hydrolysate $ser_{2.05}tyr_{0.99}met_{1.00}glu_{0.99}his_{1.00}phe_{1.04}arg_{1.00}gly_{1.00}$; melanocyte-expanding activity *in vitro* 3.6 × 10^6 MSH units/g.

Anal. Calcd. for $C_{65}H_{89}O_{20}N_{17}S\cdot 4H_2O$: C, 50.9; H, 6.4; N, 15.5. Found: C, 51.6; H, 6.5; N, 15.0.

Seryltyrosylserylmethionylglutamylhistidylphenylalanylarginyltryptophylglycine.—The acetyl decapeptide (50 mg.) was dissolved in 0.5 *N* hydrochloric acid (4 ml.) and the solution was heated in a sealed tube in a boiling water-bath for 70 minutes. The solution was cooled, diluted with water (16 ml.) and Amberlite IRA-400 (acetate cycle) was added with stirring until the supernatant was free of chloride ions (amount required approximately 6 g.). The resin was removed by filtration, was washed with four 15-ml. portions of water and the combined filtrate and washings were concentrated to a small volume *in vacuo* and the residue was lyophilized. The ensuing powder was dissolved in 0.001 *M*, *p*H 5.5 ammonium acetate buffer (7 ml.) and the solution was added to a (10 × 200 mm.) column prepared from 7 g. of carboxymethylcellulose and previously equilibrated with the same buffer. The column then was eluted at room temperature with the following ammonium acetate buffers: 0.001 *M*, *p*H 5.5 (120 ml.); 0.005 *M*, *p*H 5.5 (120 ml.); 0.01 *M*, *p*H 5.5 (120 ml.); 0.025 *M*, *p*H 5.7 (240 ml.); 0.05 *M*, *p*H 5.9 (120 ml.); 0.1 *M*, *p*H 6.5 (120 ml.); and finally 0.25 *M*, *p*H 6.9 (120 ml.). Individual fractions (6 ml. each) were collected with the aid of an automatic fraction collector set at a flow rate of 2 ml. per minute. Absorbancy at 280 mμ served to locate the peptides in the various chromatographic fractions. The desired peptide was located in tubes 78–100 (0.025 *M* ammonium acetate eluates). The contents of these tubes was pooled and lyophilized. The residue (30 mg.) on paper chromatography showed the presence of one major component (R_f^1 0.51) and a minor component (R_f^1 0.41). For further purification the material was redissolved in 0.001 *M* ammonium acetate buffer and applied to a CMC column (10 × 130 mm.) which was eluted in the manner described above with the following ammonium acetate buffers: 0.001 *M*, *p*H 5.5 (100 ml.); 0.01 *M*, *p*H 5.5 (120 ml.); 0.017 *M*, *p*H 5.6 (300 ml.). Absorbancy at 280 mμ served to locate the peptides in the various chromatographic fractions. The contents of tubes 56–73 (0.017 *M* ammonium acetate eluates) which contained the desired material were pooled. The peptide was isolated and freed of ammonium acetate by lyophilization in the usual manner; yield 21 mg., $[\alpha]^{27}D$ −29.2° (*c* 0.4 in 5% acetic acid), R_f^1 0.51; single ninhydrin, Pauly, methionine, Sakaguchi and Ehrlich positive spot; R_f^2 pro$^-$; λ_{max} 280 mμ, log ε 3.773 (in 10% acetic acid); amino acid composition of acid hydrolysate $ser_{1.95}tyr_{0.93}met_{1.00}glu_{1.02}his_{1.07}phe_{1.07}arg_{0.91}gly_{1.04}$; amino acid composition of LAP digest (paper chromatography) $ser_{1.9}tyr_{1.0}met_{1.0}glu_{0.9}his_{1.0}phe_{1.0}arg_{1.1}try_{1.0}gly_{1.0}$; melanocyte-expanding activity *in vitro* 2.9 × 10^6 MSH units/g.

Seryltyrosylserylmethionylglutamylhistidylphenylalanylarginyltryptophylglycyllysylprolylvaline Amide Acetate.—The blocked acetyltridecapeptide amide IV[8c] (50 mg.) was dissolved in 0.5*N* hydrochloric acid (3 ml.) and the solution was heated in a sealed tube under nitrogen in a boiling water-bath for 80 minutes. Water (15 ml.) was added to the cooled hydrolysate and chloride ions were removed with Amberlite IRA-400 (acetate cycle) which was added to the stirred solution (amount required approximately 4 g.). The resin was removed by filtration, was washed with four 15-ml. portions of water and the combined filtrate and washings were lyophilized. The colorless fluffy residue (49 mg.) on paper chromatography showed the presence of one major component (R_f^1 0.43) and of at least four minor components. The biological activity of various preparations of this crude product was 2.8 × 10^8 MSH units/mg. The product was dissolved in 0.005 *M* ammonium acetate of *p*H 5.5 (10 ml.) and the solution was added to a CMC column (10 × 200 mm.) prepared from 7 g. of CMC and equilibrated with the same buffer. The column was eluted with the following ammonium acetate buffers: 0.005 *M*, *p*H 5.5 (120 ml.); 0.025 *M*, *p*H 5.7 (120 ml.); 0.05 *M*, *p*H 5.9 (120 ml.); 0.075 *M*, *p*H 6.2 (200 ml.); 0.1 *M*, *p*H 6.5 (200 ml.); 0.15 *M*, *p*H 6.7 (100 ml.); and 0.25 *M*, *p*H 6.9 (100 ml.). Individual fractions (6 ml. each) were collected at a flow rate of 2.5 ml. per minute. Absorbancy at 280 mμ served to locate the peptides in the various chromatographic fractions. The contents of appropriate tubes were pooled, the solvent and ammonium acetate removed by freeze drying, and the weights and *in vitro* MSH activity (in MSH units/g.) of the ensuing solids were recorded. Fraction 1 (tubes 3–9), 6 mg., 2.2 × 10^5; fraction 2 (tubes 41–56), 9 mg., 3.1 × 10^8; fraction 3 (tubes 63–100), 17 mg., 7.3 × 10^8; fraction 4 (tubes 119–141), 18 mg., 1.9 × 10^9; fraction 5 (tubes 157–169), 3 mg., 8.3 × 10^8. The paper chromatographic evaluation of these various fractions demonstrated heterogeneity for fractions 1 and 3. Fraction 2 (major component R_f^1 0.52) consisted mainly of the N-terminal decapeptide; amino acid ratios in acid hydrolysate $ser_{1.8}tyr_{0.84}met_{0.70}glu_{1.0}his_{1.0}phe_{1.0}arg_{1.0}gly_{1.0}$. Fraction 4 (0.1 *M* eluates), the desired tridecapeptide amide, proved to be homogeneous using the ninhydrin, Pauly, Sakaguchi, methionine and Ehrlich reagents to localize the material on the chromatograms. The compound traveled as a single band when subjected to paper electrophoresis at *p*H 3.8, 5.1 and 6.0; $[\alpha]^{24}D$ −42.3° (*c* 0.34 in 5% acetic acid), R_f^1 0.43; R_f^2 tyr; λ_{max} 280 mμ, log ε 3.829 (in 10% acetic acid) (starting material log ε 3.810); amino acid ratios in acid hydrolysate $ser_{2.0}tyr_{1.0}met_{1.0}glu_{1.0}his_{1.0}phe_{1.0}arg_{1.0}gly_{1.0}pro_{1.0}val_{1.0}NH_{3(1.0\pm0.2)}$ (try largely destroyed); amino acid composition of LAP digest (paper chromatography) $ser_{1.8}tyr_{1.0}met_{1.0}glu_{0.9}his_{1.0}phe_{1.2}arg_{1.0}try_{1.1}gly_{1.0}lys_{0.9}val_{1.0}$ (pro present but not determined). The compound remained unchanged when subjected to the action of carboxypeptidase at an enzyme-substrate ratio of 1:50 at *p*H 7.5; chymotrypsin digestion gave ser.tyr (recovery of peptide 92% of theory at an enzyme–substrate ratio of 1:25 at *p*H 9). Peptide isolated by two dimensional paper chromatography first in the 2-butanol–ammonia then in the Partridge system.[20]

Enzymatic Procedure.—Partially purified (through second ammonium sulfate fractionation) leucine aminopeptidase was employed (D. H. Spackman, E. L. Smith and D. M. Brown, *J. Biol. Chem.*, **212**, 255 (1955)). For activation, thawed enzyme solution (0.3 ml.) was incubated for 3 hours at 37° with 0.25 ml. of 0.01 *M* magnesium chloride, 0.05 ml. of 0.5 *M* "tris" buffer *p*H 8.5 and 0.4 ml. of water. To an aqueous solution (0.32 ml.) containing the peptide (approximately 1 μ *M*) was added 0.01 *M* magnesium chloride (0.2 ml.) and 0.5 *M* "tris" buffer *p*H 8.5 (0.08 ml.) and the *p*H was adjusted to 8.5 with dilute ammonium hydroxide if necessary. Activated enzyme solution (0.4 ml.) was added and the mixture incubated at 37° for 24 hours. For digestion of complex peptides additional enzyme (0.5 ml.) was added after 4 hours of incubation and incubation was continued for 20 hours. The enzyme was denatured by heating for 2 minutes in a boiling water-bath and the coagulum was removed by centrifugation. The coagulum was washed with one 0.5-ml. portion of water and the combined supernatant and washing were evaporated to dryness *in vacuo* over phosphorus pentoxide. The residue was dissolved in 0.2 *N* sodium citrate buffer *p*H 2.2 and suitable aliquots of this solution were employed for Stein–Moore analyses.[11]

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