Anal. Calcd. for $C_{23}H_{33}O_{18}P$: P, 4.93. Found: P, 4.50.

Proton Magnetic Resonance Spectrum of Glycerophosphoryl Inositol Heptaacetate.—A sample of 20 mg. of the heptaacetate in 0.2 ml. of deuteriochloroform was examined in the 40 mc. Varian V-4300 n.m.r. spectrometer. Two absorptions, separated by 8 cycles, appeared (Fig. 2). Positions of the signals were measured by the side band technique using chloroform as an internal standard.

Acid Hydrolysis Products of Glycerophosphoryl Inositol.—A sample of the P^{32}-labeled diester (R_f = 0.12 in phenol–water) from *Chlorella* hydrolyzed at 100° in 0.1 *N* hydrochloric acid with a half time of approximately twenty minutes. The products were glycerophosphate, 35%, and inositol monophosphate, 65%, as determined by radioactivity measured on one-dimensional paper chromatograms developed in phenol–water solvent.

Acknowledgments.—The authors are indebted to the helpful suggestions of Professors R. E. Glick and R. U. Lemieux.

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

Studies on Polypeptides. XIV. The Synthesis of Peptides Related to the N-Terminus of α-MSH and of the Corticotropins[1,2]

BY KLAUS HOFMANN, THOMAS A. THOMPSON, HARUAKI YAJIMA, ELEANORE T. SCHWARTZ AND (IN PART) HIROYUKI INOUYE

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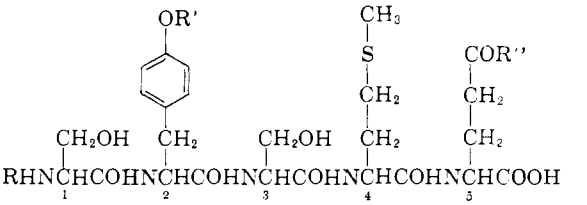
Carbobenzoxyseryltyrosylserylmethionylglutamine was prepared by treating the azide of carbobenzoxyseryltyrosine with the triethylammonium salt of serylmethionylglutamine. Optimum yields were obtained when the solid azide was added to a solution of the tripeptide salt in dimethylformamide. When the coupling was performed in the solvent system ethyl acetate–water, a rearranged product was obtained. A plausible structure for this substance is postulated Decarbobenzoxylation of the carbobenzoxypentapeptide with sodium in liquid ammonia gave seryltyrosylserylmethionylglutamine which was shown by paper chromatography and enzymatic studies to be homogeneous and of the all-L-variety. Acetylation of the pentapeptide with acetic anhydride in sodium bicarbonate solution gave a mixture of N-acetylseryltyrosylserylmethionylglutamine and of N-acetylseryl-O-acetyltyrosylserylmethionylglutamine. The position of the second acetyl group in the latter substance followed from its spectroscopic properties. The crystalline N-acetylpentapeptide was completely digestible by carboxypeptidase. Thus, acetylation was not accompanied by racemization. The preparation of the hydrazides of the carbobenzoxy-, and of the N-acetylpentapeptide is described.

The sequence corresponding to the pentapeptide seryltyrosylserylmethionylglutamic acid (I) represents the N-terminus of the corticotropins,[3-6] and its N-acetyl derivative II occupies positions 1 to 5 in the peptide chain of the melanocyte-expanding hormone α-MSH.[7]

In 1955, we reported the first synthesis of this pentapeptide and (in collaboration with W. F. White) established its identity with a peptic fragment derived from the N-terminus of corticotropin-A.[8,9]

Recently we have prepared the amides of the tridecapeptides carbobenzoxyseryltyrosylserylmethionylglutaminylhistidylphenylalanylarginyltryptophylglycyl-N^{ϵ}-tosyllysylprolylvaline[10] and of acetylseryltyrosylserylmethionylglutaminylhistidylphenylalanylarginyltryptophylglycyl-N^{ϵ}-tosyllysylprolylvaline[11] and have recorded their ability to expand melanocytes *in vitro*. These compounds, which embody within their structures the entire amino acid sequence of α-MSH, were prepared by treating the azides of carbobenzoxyseryltyrosylserylmethionylglutamine (III) and of acetylseryltyrosylserylmethionylglutamine (IV), respectively, with histidylphenylalanylarginyltryptophylglycyl-N^{ϵ}-tosyllysylprolylvaline amide. The azide method was employed to establish the final peptide bond of these tridecapeptide derivatives, since, in our opinion, this procedure is still the method of choice for linking large peptide fragments without risk of racemization.



	R	R'	R''
I	H	H	OH
II	Ac	H	OH
III	Cbzo	H	NH_2
IV	Ac	H	NH_2
V	H	H	NH_2
VI	Ac	Ac	NH_2

Holley and Sondheimer[12] convincingly demonstrated the utility of carbobenzoxyglutamine azide as a reagent for introducing a glutamine moiety into peptides and, using their procedure, we have shown[13] that this azide reacts with simple peptide

(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) All 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) P. H. Bell, THIS JOURNAL, **76**, 5565 (1954).

(4) K. S. Howard, R. G. Shepherd, E. A. Eigner, D. S. Davies and P. H. Bell, *ibid.*, **77**, 3419 (1955).

(5) W. F. White and W. A. Landmann, *ibid.*, **77**, 1711 (1955).

(6) C. H. Li, I. I. Geschwind, R. D. Cole, I. D. Raacke, J. I. Harris and J. S. Dixon, *Nature*, **176**, 687 (1955).

(7) J. I. Harris and A. B. Lerner, *ibid.*, **179**, 1346 (1957).

(8) K. Hofmann and A. Jöhl, THIS JOURNAL, **77**, 2914 (1955).

(9) K. Hofmann, A. Jöhl, A. E. Furlenmeier and H. Kappeler, *ibid.*, **79**, 1636 (1957).

(10) K. Hofmann, M. E. Woolner, H. Yajima, G. Spühler, T. A. Thompson and E. T. Schwartz, *ibid.*, **80**, 6458 (1958).

(11) K. Hofmann, H. Yajima and E. T. Schwartz, *Biochim. et Biophys. Acta*, **36**, 252 (1959).

(12) E. Sondheimer and R. W. Holley, THIS JOURNAL, **76**, 2816 (1954).

(13) K. Hofmann, H. Kappeler, A. E. Furlenmeier, M. E. Woolner, E. T. Schwartz and T. A. Thompson, *ibid.*, **79**, 1641 (1957).

derivatives containing an N-terminal histidine to form the desired peptide bond involving the α-carboxyl group of glutamine and the α-amino group of histidine. Since presently available evidence indicates that peptides obtained by this route are not contaminated significantly by the respective isomers involving the γ-carboxyl group of glutamic acid, we have prepared the N-carbobenzoxy- (III) and N-acetyl-derivative (IV) of seryltyrosylserylmethionylglutamine (V), and have converted them into their hydrazides. These hydrazides, on treatment with nitrous acid, formed solid azides which reacted smoothly with the octapeptide amide mentioned above to give the respective acyl tridecapeptide amides.

At the time these studies were initiated, we recognized the difficulties involved in regenerating a free γ-carboxyl group from the glutamine residue present in the final products but assumed that replacement of a free carboxyl by a carboxamide function would not exert too marked an effect upon the physiological activity. This assumption proved to be justified as far as melanocyte-expanding activity *in vitro* is concerned.[10,11,14]

The present communication describes experimental details relating to the preparation and properties of the pentapeptide seryltyrosylserylmethionylglutamine (V) and of a number of its derivatives. The paper chromatographic and enzymatic techniques described in a previous communication[15] provided the criteria for evaluating the homogeneity of the various peptides.

A mixed anhydride of carbobenzoxymethionine was allowed to react with the triethylammonium salt of glutamine to give carbobenzoxymethionylglutamine which was decarbobenzoxylated by treatment with sodium in liquid ammonia. The ensuing free dipeptide in the form of its triethylammonium salt was then coupled with the azide of carbobenzoxyserine[16] and the resulting carbobenzoxyserylmethionylglutamine was transformed into the free tripeptide. For conversion into the carbobenzoxypentapeptide (III) the triethylammonium salt of the tripeptide was treated with the azide of carbobenzoxyseryltyrosine.[9] Variations in experimental conditions were explored in order to achieve optimal yields in this step.

Initially the reaction was carried out by shaking an ether solution of the azide with an aqueous solution of the tripeptide salt. The cation exchanger Dowex-50 was employed to remove unreacted tripeptide from the crude reaction product which was purified by precipitation from ethanol with water. The carbobenzoxypentapeptide was obtained with superior yields, when the solid azide of carbobenzoxyseryltyrosine was added to a solution of the tripeptide triethylammonium salt in dimethylformamide. This method was adopted for routine preparation of the compound. Acid hydrolysates of the carbobenzoxypentapeptide obtained by both procedures exhibited the expected amino acid composition, but the recovery of methionine was low.[17]

Treatment with sodium in liquid ammonia converted the carbobenzoxy derivative into the free pentapeptide V which was obtained in crystalline form. The compound was homogeneous as judged by paper chromatography in two solvent systems, and was completely digestible by leucine aminopeptidase (LAP) with formation of the expected amino acids. Chymotryptic digestion of the pentapeptide gave seryltyrosine and serylmethionylglutamine (matching R_f's with synthetic specimens). The amino acid ratios in an acid hydrolysate of the pentapeptide were those expected by theory.

Attempts to prepare III by shaking of an ethyl acetate solution of carbobenzoxyseryltyrosine azide with an aqueous solution of the triethylammonium salt of serylmethionylglutamine did not result in formation of the expected material but afforded instead a rearranged product. Following purification by countercurrent distribution, this material was obtained in crystalline form. The compound which was ninhydrin negative but Pauly and methionine positive differed from the carbobenzoxypentapeptide III in R_f-value and melting point. Little difference in optical rotation was noted. The rearranged product formed a methyl ester and hydrazide and thus must have contained a free carboxyl group. The behavior on acid hydrolysis demonstrated that the material did not possess a normal pentapeptide structure. The hydrolysate contained serine, methionine and glutamic acid. Tyrosine was absent, but a fast moving, ninhydrin negative, Pauly positive spot which developed an orange color on treatment with dinitrophenylhydrazine (p-hydroxyphenylacetaldehyde) was present on the chromatograms.

Decarbobenzoxylation of the crystalline carbobenzoxy derivative afforded a material homogeneous on paper which exhibited a positive reaction with the ninhydrin, Pauly and methionine reagents. The paper chromatographic evaluation of an LAP digest of this substance revealed the presence of serine. No other free amino acid was seen on the chromatogram. The compound was completely resistant to the action of chymotrypsin.

These experimental findings support the already assigned structure VIII for the rearranged product. A substance possessing this structure should form serine, methionine and glutamic acid on hydrolysis with acid, but tyrosine should not be present among the products of hydrolysis. The absence of a peptide bond between the residues in positions 2 and 3 renders the product resistant toward chymotrypsin and LAP.

The tendency of some acylamino acid azides to react with amino acid or peptide esters to form urea derivatives rather than peptides has been observed previously.[18,19]

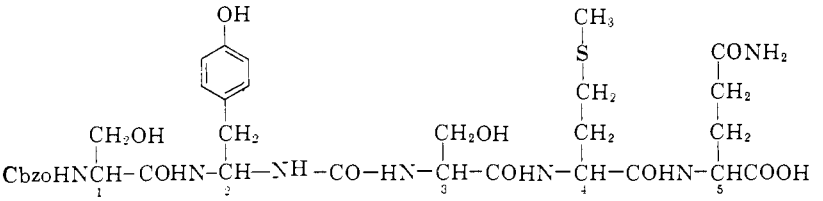
(14) We have completed recently the synthesis of acetylseryltyrosylserylmethionylglutaminylhistidylphenylalanylarginyltryptophylglycyl-N$^{\epsilon}$-formyllysylprolylvaline amide and have found this material to exhibit essentially the same biological activity as natural α-MSH (see paper XVII in this series).

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

(16) J. S. Fruton, *J. Biol. Chem.*, **146**, 463 (1942).

(17) We attribute the low recoveries of methionine from hydrolysates of carbobenzoxy derivatives of peptides containing this amino acid to the interaction of the sulfur atom with benzyl chloride formed during hydrolysis.

(18) J. W. Hinman, E. L. Caron and H. N. Christensen THIS JOURNAL, **72**, 1620 (1950).



VIII

Since α-MSH possesses an acetylated N-terminus, we investigated the N-acetylation of the pentapeptide V. Preliminary experiments were conducted using seryltyrosine as a model compound. The dipeptide employed in these studies was prepared by hydrogenolysis of carbobenzoxyseryltyrosine benzyl ester. This method of preparation is superior to the one described previously.[9]

An excess of acetic anhydride was employed in these experiments in order to ascertain the tendency of the hydroxyl groups to undergo acetylation under the experimental conditions selected.

A ninhydrin and Pauly negative crystalline product was obtained when two equivalents of acetic anhydride were added to an ice-cold solution of the dipeptide in sodium bicarbonate. This substance exhibited a flat absorption band with a maximum at 260 mμ (log ϵ 2.49) and its elemental composition and acetyl content pointed to a diacetylated product. Short exposure to dilute sodium hydroxide converted this compound into a ninhydrin-negative Pauly-positive substance with a sharp absorption maximum at 275 mμ (log ϵ 3.18). This same product was obtained when dilute sodium hydroxide was added to the reaction mixture at the end of the acetylation. The elemental composition and acetyl content of this product agreed with values expected of a monoacetyl derivative. The marked changes in the ultraviolet absorption characteristics which accompany the introduction of the second acetyl group into the seryltyrosine molecule establish the structure of the diacetyl derivative as N-acetylseryl-O-acetyltyrosine. The monoacetyl derivative is N-acetylseryltyrosine.

In a recent communication[20] Guttmann and Boissonnas describe the formation of N-acetylseryltyrosine by exposure of O-acetylseryltyrosine to a *p*H above 7. The latter compound resulted when carbobenzoxyseryltyrosine was exposed to the action of hydrogen bromide in glacial acetic acid solution. A comparison of our product with the material of the Swiss authors is not possible since no experimental details are given in their report.

The behavior of seryltyrosylserylmethionylglutamine (V) toward acetic anhydride (1.3 equivalents) in aqueous sodium bicarbonate was similar to that observed with the model dipeptide. Two ninhydrin-negative products, readily separable by countercurrent distribution, were obtained. One of these crystallized in needles, contained one acetyl group and exhibited a normal tyrosine spectrum. The other, obtained in the form of a gelatinous mass, contained two acetyl groups and its ultraviolet spectrum was markedly different. Treatment with dilute sodium hydroxide converted the gelatinous diacetyl derivative into the crystalline monoacetyl compound. It follows from these findings that the crystalline substance exhibiting the normal tyrosine spectrum is N-acetylseryltyrosylserylmethionylglutamine (IV). The gelatinous material must be N-acetylseryl-O-acetyltyrosylserylmethionylglutamine (VI). Chymotrypsin converted peptide IV into a mixture of N-acetylseryltyrosine and serylmethionylglutamine (matching R_f's with synthetic specimens) whereas carboxypeptidase gave an equimolar mixture of serine, tyrosine and methionine.[21] These results support the all-L-configuration of the acetylpentapeptide IV.

Carbobenzoxyseryltyrosylserylmethionylglutamine (III) and acetylseryltyrosylserylmethionylglutamine (IV) were tested for their ability to expand melanocytes *in vitro*.[22] Both compounds were found to be devoid of biological activity.

Experimental[23]

Carbobenzoxymethionylglutamine.—A mixed anhydride was prepared in the usual manner from carbobenzoxymethionine[9] (5.0 g.) in dry dioxane (20 ml.) with tri-*n*-butylamine (4.2 ml.) and ethyl chloroformate (1.68 ml.). This solution was added slowly with stirring to a chilled solution of glutamine (2.57 g.) and triethylamine (2.44 ml.) in water (20 ml.). The mixture was stirred in an ice-bath for 1 hour and most of the dioxane was removed *in vacuo* at a bath temperature of 50°. The residue was acidified to congo red with 2 *N* hydrochloric acid and was extracted with three 100-ml. portions of ethyl acetate. The ethyl acetate extracts were washed successively with three 50-ml. portions of 2 *N* hydrochloric acid and three 50-ml. portions of water, and were dried over sodium sulfate. Evaporation of the ethyl acetate gave an oily residue which crystallized when methanol was added. The crystalline mass was washed with a small quantity of ice-cold methanol, was dried over phosphorus pentoxide and recrystallized from methanol; yield 3.0 g. (41%), m.p. 159–161°, $[\alpha]^{25}D$ −13.6° (*c* 1.12 in 95% ethanol), R_f^A 0.86,[24] R_f^B phe$^+$,[25] ninhydrin negative, methionine positive.

(19) G. S. Heaton, H. N. Rydon and J. A. Schofield, *J. Chem. Soc.*, 3157 (1956).

(20) St. Guttmann and R. A. Boissonnas, *Helv. Chim. Acta*, **41**, 1852 (1958).

(21) Glutamine was present on the chromatograms but cannot be determined quantitatively by the ninhydrin technique because of pyrrolidonecarboxylic acid formation; N-acetylserine escapes detection by our methods of analysis.

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

(23) 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.

(24) Partridge system; S. M. Partridge, *Biochem. J.*, **42**, 238 (1948).

(25) 2-Butanol–ammonia system; J. F. Roland, Jr., 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.

Anal. Calcd. for $C_{18}H_{23}O_6N_3S$: C, 52.5; H, 6.1; N, 10.2; S, 7.8. Found: C, 52.6; H, 6.1; N, 10.3; S, 7.6.

Carbobenzoxymethionylglutamine Methyl Ester.—A solution of carbobenzoxymethionylglutamine (1.7 g.) in absolute methanol was cooled in an ice-bath and diazomethane in ether was added until a faint yellow color persisted. The mixture was kept for 5 minutes, the excess of diazomethane was destroyed by addition of a few drops of glacial acetic acid and the solution was evaporated to dryness *in vacuo*. The methyl ester was recrystallized from a mixture of methanol and petroleum ether (b.p. 30–60°); yield 1.3 g. (74%), m.p. 193–194°, $[\alpha]^{27}D$ −6.6° (*c* 1.15 in methanol).

Anal. Calcd. for $C_{19}H_{27}O_6N_3S$: C, 53.6; H, 6.4; N, 9.9; S, 7.5. Found: C, 53.9, H, 6.4; N, 9.6; S, 7.0.

Carbobenzoxymethionylglutamine Hydrazide.—The above methyl ester (1.0 g.) was dissolved in methanol (100 ml.) at 60° and the solution was cooled in an ice-bath. Hydrazine hydrate (0.59 ml.) was added and the mixture was kept at 2° for 20 hours. The resulting crystalline product was collected, washed with three 50-ml. portions of ice-cold methanol and dried *in vacuo* over phosphorus pentoxide; yield 0.73 g. (73%), m.p. 220–223° dec.

Anal. Calcd. for $C_{18}H_{27}O_5N_5S$: N, 16.5. Found: N, 16.3.

Methionylglutamine.—Carbobenzoxymethionylglutamine (5.5 g.) was dissolved in sodium-distilled ammonia (approximately 300 ml.) and sodium (approximately 1.35 g.) was added in small pieces with stirring until a permanent blue color remained. Dowex-50 (ammonium form, 25 g.) was added and the ammonia was evaporated. The last traces of ammonia were removed by blowing a stream of nitrogen over the residue. The product was extracted with five 30-ml. portions of water, the combined extracts were filtered through a layer of Filter-cel, and the filtrate was lyophilized. The resulting white powder was dissolved in 10% ammonium hydroxide (20 ml.), and the solution was acidified to *p*H 4–5 with glacial acetic acid. Ethanol (approximately 3 times the volume of the solution) was added, and the mixture was placed in a refrigerator to complete the crystallization. The crystals were collected, dissolved in 10% ammonium hydroxide and recrystallized by addition of glacial acetic acid to *p*H 4–5 followed by recrystallization from ethanol. The product was washed with a small volume of ice-water and was dried; yield 3.1 g. (83%), m.p. 232–233°, $[\alpha]^{26}D$ +17.3° (*c* 1.4 in 10% ammonium hydroxide), R_f^A 0.43; R_f^B his$^+$; in a preliminary communication[26] we reported $[\alpha]^{25}D$ +14.1° in 10% ammonium hydroxide and m.p. 220–221°.

Anal. Calcd. for $C_{10}H_{19}O_4N_3S$: C, 43.3; H, 6.9; N, 15.2. Found: C, 43.1; H, 7.3; N, 15.6.

Seryltyrosine.—An ethyl acetate solution (approximately 80 ml.) of carbobenzoxyserine azide[16] (prepared from 2.53 g. of the hydrazide) was added to an ice-cold dioxane solution (approximately 30 ml.) of benzyl tyrosinate (prepared from 3.06 g. of the hydrochloride[27] and 1.39 ml. of triethylamine). The mixture was placed in a refrigerator for 48 hours, the solvents were removed *in vacuo*, the residue was redissolved in ethyl acetate and the solution was washed successively with saturated sodium chloride, 2 *N* hydrochloric acid, saturated sodium bicarbonate and finally with saturated sodium chloride. The solution was dried over sodium sulfate and the solvent was removed *in vacuo*. The ensuing sirup (4.27 g.) was dissolved in a mixture of methanol and glacial acetic acid 6:1 and was hydrogenated in presence of a palladium catalyst. The catalyst was removed by filtration, the filtrate was evaporated to dryness *in vacuo* and the residue was dissolved in 3% ammonium hydroxide. The solution was extracted with ethyl acetate, the aqueous layer was lyophilized and the solid residue dissolved in a small quantity of water. The *p*H was adjusted to 5 by addition of glacial acetic acid, absolute ethanol was added, and the solution was placed in a refrigerator where crystallization soon occurred; yield 1.4 g. (52%), m.p. 256–260°, $[\alpha]^{25}D$ + 43.1° (*c* 0.84 in water) (lit.[9] $[\alpha]D$ + 38.4°), R_f^A 0.42; R_f^B tyr; completely digestible by LAP, amino acid ratios in digest $ser_{1.0}$ $tyr_{1.0}$.

Anal. Calcd. for $C_{12}H_{16}O_5N_2$: C, 53.7; H, 6.0; N, 10.4. Found: C, 53.5; H, 6.2; N, 10.4.

N-Acetylseryl-O-acetyltyrosine.—Acetic anhydride (0.3 ml.) was stirred into an ice-cold solution of seryltyrosine (0.57 g.) in water (15 ml.) and sodium bicarbonate (0.91 g.). The mixture was stirred at ice-bath temperature for 30 minutes and acetic anhydride (0.2 ml.) was added. Stirring was continued for 1 hour and the solution was acidified to *p*H 4 to 5 by addition of acetic acid. The mixture was placed in the first three tubes of a 200 tube countercurrent machine and 295 transfers were effected in the solvent system 1-butanol–10% acetic acid. Spectrophotometric examination of aliquots at 270 mμ located a major band in effluent fractions 149–199. The contents of these tubes were pooled and evaporated to dryness *in vacuo*. The residue was recrystallized from hot water; yield 0.40 g. (52%), m.p. 193–197°, $[\alpha]^{25}D$ −16.4° (*c* 0.90 in water); absorption max. at 260 mμ., log ε 2.49 (in 0.1 *N* hydrochloric acid); ninhydrin and Pauly negative.

Anal. Calcd. for $C_{16}H_{20}O_7N_2$: C, 54.5; H, 5.7; N, 8.0; acetyl, 24.4. Found: C, 54.5; H, 5.9;, N, 7.9; acetyl, 24.8.

N-Acetylseryltyrosine. a. By Acetylation followed by Alkali Treatment.—Acetic anhydride (0.18 ml.) was stirred into an ice-cold solution of seryltyrosine (0.57 g.) and sodium bicarbonate (0.55 g.) in water (15 ml.). After 25 minutes, additional acetic anhydride (0.065 ml.) was added and the solution stirred for one hour. Sodium hydroxide (0.1 g.) in water (5 ml.) was then added, the mixture was stirred with ice-cooling for 30 minutes, was acidified to *p*H 6 with glacial acetic acid and was lyophilized. The residue was dissolved in 10% acetic acid equilibrated with 1-butanol (30 ml.), the solution was added to the first three tubes of a 200-plate countercurrent apparatus, and 346 transfers in the solvent system 1-butanol–10% acetic acid were carried out. Spectrophotometric examination of aliquots of the upper phases at 275 mμ revealed the presence of a small band in effluent fractions 48 to 108 and a large band in tubes 156–200 and effluents 120–148. The fractions containing the main band were combined and evaporated *in vacuo* to give a solid residue which was recrystallized from water; white prisms, yield 0.45 g. (68%), m.p. 192–194°, $[\alpha]^{22}D$ + 18.3° (*c* 0.87 in water), R_f^A 0.71; R_f^B tyr, ninhydrin negative, Pauly positive; absorption max. at 275 mμ, log ε 3.18 (in 1 *N* hydrochloric acid); liberates tyrosine when treated with carboxypeptidase.

Anal. Calcd. for $C_{14}H_{18}O_6N_2$: C, 54.2; H, 5.8; N, 9.0; acetyl, 13.9. Found: C, 54.3; H, 6.1; N, 9.0; acetyl, 13.6.

b. By Saponification of N-Acetylseryl-O-acetyltyrosine.—A sample of the diacetyl derivative (100 mg.) was dissolved in ice-cold 1 *N* sodium hydroxide (5 ml.) and the mixture was kept at ice-bath temperature for 75 minutes. The solution was acidified to *p*H 4–5 with glacial acetic acid, added to the first tube of a 30 tube countercurrent machine, and 67 transfers were performed in the solvent system 1-butanol–10% acetic acid. The tubes containing material absorbing at 275 mμ were pooled and the solvent evaporated. Treatment of the residue with ether–methanol gave crystals which were recrystallized from hot water; yield 46 mg., m.p. 191–194°, mixed m.p. with material prepared according to a, 191–194°, $[\alpha]^{26}D$ −16.4° (*c* 0.75 in water), R_f^A 0.72; single ninhydrin negative, Pauly positive spot.

Carbobenzoxyserylmethionylglutamine.—An ethereal solution (approximately 130 ml.) of carbobenzoxyserine azide (prepared from 7.30 g. of the hydrazide)[16] was shaken for 48 hours at 1–3° with a solution of methionylglutamine (4.0 g.) in water (130 ml.) and triethylamine (2.01 ml.). The aqueous phase was then separated from the ether layer, was re-extracted with fresh ethyl acetate and was then lyophilized to give a white powder weighing 5.5 g. This material was dissolved in 1-butanol equilibrated with 20% acetic acid, the solution was added to the first five tubes of a 30-tube countercurrent machine and 65 transfers were performed in the system 1-butanol–20% acetic acid. The desired (methionine positive,[28] ninhydrin negative) material was located in the first 31 effluent fractions; these were combined, and evaporated to dryness at a bath temperature of 40–50°. The resulting crystalline material (4.1 g.) was dissolved in 5% ammonium hydroxide, the solution was filtered, and the clear filtrate acidified to congo red with 1 *N* hydrochloric acid and the resulting precipitate was collected.

(26) K. Hofmann, T. A. Thompson and E. T. Schwartz, THIS JOURNAL, **79**, 6087 (1957).

(27) B. F. Erlanger and R. M. Hall, *ibid.*, **76**, 5781 (1954).

(28) G. Toennies and T. T. Kolb, *Anal. Chem.*, **23**, 823 (1951).

The compound was recrystallized from 50% aqueous methanol; fine needles, yield 3.9 g. (54%), m.p. 172–173°, $[\alpha]^{28}D$ −24.8° (*c* 1.4 in absolute ethanol).

Anal. Calcd. for $C_{21}H_{30}O_8N_4S$: C, 50.6; H, 6.1; N, 11.2. Found: C, 50.3; H, 5.9; N, 11.6.

Methionylglutamine was recovered by evaporation of the contents of tubes 2 to 24.

Carbobenzoxyserylmethionylglutamine Hydrazide.—To an ice-cold solution of carbobenzoxyserylmethionylglutamine (3.2 g.) in methanol (300 ml.) was added diazomethane in ether until the yellow color persisted and the mixture was kept for 10 minutes. The excess of diazomethane was destroyed by the addition of a few drops of glacial acetic acid and the solution was evaporated to dryness *in vacuo* at a bath temperature of 50°. The residue was dissolved in methanol (300 ml.), the solution was chilled and hydrazine hydrate (3.0 ml.) was added. The solution was kept at 2° for 12 hours, the resulting crystalline hydrazide was collected, washed with ice-cold methanol and dried; yield 2.9 g. (88%), m.p. 211–212°.

Anal. Calcd. for $C_{21}H_{32}O_7N_6S$: N, 16.4. Found: N, 16.1.

Serylmethionylglutamine.—Carbobenzoxyserylmethionylglutamine (4.05 g.) was dissolved in sodium-dried liquid ammonia (approximately 400 ml.) and sodium (approximately 0.77 g.) was added in small pieces with stirring until a permanent blue color remained. Dowex-50 (ammonium cycle, (20 g.) was added and the ammonia was allowed to evaporate at room temperature. A slow stream of nitrogen was passed over the residue to remove the last traces of ammonia. The product was extracted with several portions of water containing a few drops of 10% ammonia (total volume approximately 150 ml.), and the extracts were combined, filtered and lyophilized. The ensuing white powder was dissolved in a small volume of water containing a few drops of 10% ammonia. The solution was filtered, the *p*H of the clear filtrate was adjusted to 6.0 with dilute acetic acid, and ethanol (approximately three times the volume of the solution) was added. The mixture was placed in a refrigerator overnight and the resulting crystalline precipitate was collected; yield 2.54 g. (82%), m.p. 225° dec.; a sample for analysis was recrystallized by dissolving in hot water and adding ethanol; m.p. 228° dec., $[\alpha]^{27}D$ −17.8°, (*c* 1.0 in 10% ammonia), $[\alpha]^{27}D$ −13.3° (*c* 1.3 in water), R_f^A 0.39, R_f^B his$^+$; amino acid ratios in LAP digest $ser_{1.0}$ $met_{1.0}$gluta[21]; amino acid ratios in acid hydrolysate $ser_{1.0}$ $met_{0.98}glu_{1.0}$.

Anal. Calcd. for $C_{13}H_{24}O_6N_4S\cdot H_2O$: C, 40.8; H, 6.9; N, 14.6; S, 8.4. Found: C, 40.6; H, 7.2; N, 14.9; S, 7.8.

Carbobenzoxyseryltyrosine Azide.—This entire operation was carried out in a cold room at 5°. Carbobenzoxyseryltyrosine hydrazide (2.62 g.) was dissolved in 2*N* hydrochloric acid (52 ml.) and water (13 ml.) was added. The solution was cooled in an ice-bath and traces of insoluble material were removed by filtration. A solution of sodium nitrite (0.44 g.) in water (5 ml.) was slowly added to the clear filtrate and the oily azide which precipitated soon solidified; it was collected, washed with ice-cold 1 *N* hydrochloric acid, saturated sodium chloride, saturated sodium bicarbonate and water and was dried for 3 hours at −15° over phosphorus pentoxide and potassium hydroxide pellets *in vacuo;* yield 2.13 g. (79%).

Carbobenzoxyseryltyrosylserylmethionylglutamine.— (a) By coupling of carbobenzoxyseryltyrosine azide with the tripeptide triethyl ammonium salt in the system ether-water:

A solution of serylmethionylglutamine (1 g.) in water (30 ml.) and triethylamine (0.38 ml.) was shaken at 5° for 44 hours with an ethereal solution of carbobenzoxyseryltyrosine azide (prepared from 2.28 g. of the hydrazide). The aqueous layer changed into a gelatinous mass during the course of the reaction. The ether layer was removed by decantation, the gelatinous reaction product was brought into solution by the addition of 3% ammonium hydroxide, and the clear solution was extracted with ethyl acetate (emulsions were broken by centrifugation). The aqueous layer was filtered and the clear filtrate was acidified with dilute acetic acid and lyophilized. The residue was redissolved in dilute ammonia and the product precipitated by addition of dilute acetic acid. The precipitate was dissolved in 80% aqueous ethanol (approximately 100 ml.), Dowex-50 in the acetate cycle (5 g.) was added and the mixture was shaken for 1 hour at room temperature. The resin was removed by filtration and the filtrate was evaporated to dryness at a bath temperature of 30–40°. The residue was dissolved in a small volume of ethanol with warming and the acylpentapeptide was precipitated by addition of water; yield 0.71 g. (35%), m.p. 164–170°, $[\alpha]^{30}D$ −16.0° (*c* 1.1 in glacial acetic acid), R_f^A 0.84, ninhydrin negative, Pauly and methionine positive,[28] amino acid comp. of acid hydrolysate $ser_{1.9}tyr_{1.0}met_{0.44}glu_{1.0}$.

Anal. Calcd. for $C_{33}H_{44}O_{12}N_6S\cdot H_2O$: C, 51.7; H, 6.0; N, 11.0. Found: C, 51.1; H, 6.2; N, 10.7.

(b) By adding solid carbobenzoxyseryl tyrosine azide to a solution of the tripeptide triethylammonium salt: Serylmethionylglutamine (1.53 g.) was dissolved in water (6 ml.) and triethylamine (0.56 ml.) and dimethylformamide (45 ml.) were added. The solution was cooled in an ice-bath, carbobenzoxyseryltyrosine azide (1.71 g.) was added, and the mixture was kept at 3° for 3 days. The solvents were removed *in vacuo* at a bath temperature of 30–40°, the residue was dissolved in 5% ammonium hydroxide and the solution was extracted with three 50-ml. portions of ethyl acetate (emulsions were broken by centrifugation). Freeze-drying of the aqueous phase gave a colorless solid which was dissolved in 1-butanol saturated with 20% acetic acid (50 ml.). The solution was placed in the first five tubes of a 30-tube countercurrent machine and sixty transfers in the solvent system 1-butanol–20% acetic acid were performed. The desired (Pauly positive, methionine positive,[28] ninhydrin negative) material was located in effluent fractions 2 to 25. These fractions were pooled and evaporated at a bath temperature of 30–40° to give 2.49 g. of a white powder which was dissolved in 5% ammonium hydroxide at 40°. The solution was filtered through a layer of filter-cel, the clear filtrate was extracted with ethyl acetate and the aqueous phase was acidified to congo red with 1*N* hydrochloric acid. The resulting gelatinous product was collected, washed with water and rapidly dried *in vacuo* at room temperature over phosphorus pentoxide and potassium hydroxide pellets; yield 2.09 g. (68%), m.p. 165–168°; a sample for analysis was purified further by precipitation from ethanol with water; m.p. 167–171°, $[\alpha]^{27}D$ −15.5° (*c* 0.9 in glacial acetic acid), R_f^A 0.85, ninhydrin negative, Pauly and methionine positive, amino acid ratios in acid hydrolysate $ser_{1.8}tyr_{1.0}met_{0.7}glu_{1.0}$.

Anal. Calcd. for $C_{33}H_{44}O_{12}N_6S\cdot H_2O$: C, 51.7; H, 6.0; N, 11.0. Found: C, 51.4; H, 5.9; N, 11.5.

Carbobenzoxyseryltyrosylserylmethionylglutamine Methyl Ester.—The carbobenzoxypentapeptide (1.08 g.) was dissolved in methanol (250 ml.) with slight warming, and the solution was cooled in an ice-bath. Ethereal diazomethane was then added until the yellow color remained and the mixture was kept for 5 minutes. The excess of diazomethane was destroyed by addition of a few drops of glacial acetic acid and the solution was evaporated to dryness at a bath temperature of 50°. The ensuing sirup was washed with ether to give a white solid which was used for preparation of the hydrazide without further purification; yield 1.0 g. (92%), m.p. 174–180° dec. A sample for analysis was precipitated from methanol with water; m.p. 179–184°, $[\alpha]^{26}D$ −29.7° (*c* 1.1 in methanol).

Anal. Calcd. for $C_{34}H_{46}O_{12}N_6S\cdot H_2O$: C, 52.3; H, 6.2; N, 10.8. Found: C, 52.6; H, 6.2; N, 11.0.

Carbobenzoxyseryltyrosylserylmethionylglutamine Hydrazide.—The above methyl ester (1.0 g.) was dissolved in methanol (50 ml.) and the solution was kept at room temperature for at least 1 hour. The resulting precipitate (trace) was removed by filtration and hydrazine hydrate (0.66 ml.) was added to the filtrate. The solution was kept at room temperature for 24 hours and the ensuing precipitate was collected and dried over sulfuric acid *in vacuo;* yield 0.70 g. (70%), m.p. 194–198°. A sample for analysis was precipitated twice from methanol with water; m.p. 204° dec.

Anal. Calcd. for $C_{33}H_{46}O_{11}N_8S\cdot 1.5H_2O$: C, 50.2; H, 6.3; N, 14.2. Found: C, 50.5; H, 6.0; N, 14.2.

Seryltyrosylserylmethionylglutamine.—The carbobenzoxypentapeptide (4.5 g.) was dissolved in sodium-distilled liquid ammonia (approximately 350 ml.) and sodium (approximately 1.5 g.) was added in small pieces with stirring until a permanent blue color was obtained. Dowex-50 in

the ammonium cycle (20 g.) was added and the ammonia was allowed to evaporate. Nitrogen was passed over the dry residue for 1 hour to remove the last traces of ammonia and the residue was extracted with several portions of water containing a few drops of 10% ammonium hydroxide. The extracts were combined, filtered and lyophilized. The ensuing solid was dissolved in a small volume of water, the *p*H was adjusted to 6 with 10% acetic acid and three volumes of ethanol were added. The solution was stored in a refrigerator for 12 hours and the resulting precipitate was collected. The material was redissolved in water (containing a few drops of 10% ammonium hydroxide) and was precipitated at *p*H 6 by addition of ethanol. The product was then dissolved in a small volume of water with warming, a few drops of acetic acid were added, and the peptide crystallized by addition of ethanol; yield 2.64 g. (70%), m.p. 214–217°, $[\alpha]^{23}D$ −19.4° (*c* 0.9 in 2*N* hydrochloric acid), R_f^A 0.48, R_f^B pro; ninhydrin, Pauly and methionine positive;[28] completely digestible by LAP; amino acid ratios in digest $ser_{2.4}tyr_{1.0}met_{1.1}gluta$[21]; digestion with chymotrypsin gave seryltyrosine and serylmethionylglutamine; amino acid ratios in acid hydrolysate $ser_{2.0}tyr_{1.0}met_{0.6}glu_{0.9}$.

Anal. Calcd. for $C_{25}H_{38}O_{10}N_6S\cdot 1.5H_2O$: C, 46.8; H, 6.4; N, 13.1. Found: C, 46.8; H, 6.3; N, 12.9.

N - Acetylseryltyrosylserylmethionylglutamine. a. By Acetylation of the Pentapeptide without Alkali Treatment: Acetic anhydride (0.53 ml.) was added slowly with stirring to an ice-cold solution of seryltyrosylserylmethionylglutamine (2.57 g.) in water (40 ml.) and sodium bicarbonate (1.34 g.). Following the addition, stirring was continued at ice-bath temperature for one hour. The reaction mixture was added to the first 5 tubes of a 200-plate countercurrent machine and 455 transfers were carried out in the system 1-butanol–10% acetic acid. Inspection of the various fractions revealed crystal formation in tubes 159–187 and precipitation of a gelatinous solid was observed in effluent fractions 182–207. Spectrophotometric examination of aliquots of the lower phases (in the machine) for material absorbing at 275 mμ revealed the presence of a broad band located in tubes 133–199. The contents of these tubes were pooled and evaporated to dryness *in vacuo* at a bath temperature of 30–40° (crystals adhering to the wall of the tubes were dissolved in upper phase solvent and the solutions added to the rest of the material). The ensuing crystalline solid (1.58 g.) was recrystallized from a mixture of water and methanol 3:1; fine needles, yield 1.53 g. (58%), m.p. 215–217°, $[\alpha]^{23}D$ −20.3° (*c* 1.1 in dimethylformamide), $[\alpha]^{25}D$ −39.0° (*c* 0.9 in glacial acetic acid); absorption max. at 274.5 mμ, log ε 3.13 in 0.1 *N* hydrochloric acid; R_f^A 0.60; ninhydrin negative, Pauly and methionine positive[28]; amino acid ratios in carboxypeptidase digest $ser_{1.1}tyr_{1.0}met_{1.1}gluta$[21]; digestion with chymotrypsin gave acetylseryltyrosine and serylmethionylglutamine (R_f^A 0.71 and 0.39, respectively); recovery of tripeptide was 93% of theory.

Anal. Calcd. for $C_{27}H_{40}O_{11}N_6S$: C, 49.4; H, 6.1; N, 12.8; acetyl, 6.6. Found: C, 49.2; H, 6.7; N, 12.6; acetyl, 6.7.

b. By Acetylation Followed by Alkali Treatment.—Acetic anhydride (0.25 ml.) was added slowly with stirring to an ice-cold solution of seryltyrosylserylmethionylglutamine (1.19 g.) in water (20 ml.) and sodium bicarbonate (0.64 g.). Following the addition, stirring was continued with external cooling for 1.5 hours. The solution was lyophilized, the residue was dissolved in ice-cold 1*N* sodium hydroxide (10 ml.) and the mixture was stirred with ice-cooling for 45 minutes. The solution was acidified to congo red with 1*N* hydrochloric acid and was placed in a refrigerator where crystallization soon occurred. The crystals were collected and recrystallized from methanol–water 1:3; yield 1.02 g. (84%), m.p. 215–217°, no depression of m.p. when mixed with a sample prepared according to a above, $[\alpha]^{23}D$ −20.6° (*c* 1.0 in dimethylformamide), R_f^A 0.62, amino acid ratios in carboxypeptidase digest $ser_{1.0}tyr_{1.0}met_{1.0}gluta$.[21]

c. By Saponification of N-Acetylseryl-O-acetyltyrosylserylmethionylglutamine.—The diacetyl derivative (1.19 g., see below) was dissolved in water (20 ml.) and 1*N* sodium hydroxide (9.4 ml.) was added, and the solution was cooled at 0°. The solution was kept at 0° for one hour, glacial acetic acid was added to *p*H 3 and the solution was evaporated to dryness at a bath temperature of 40–50°. The residue was redissolved in a small volume of water. The solution was seeded with the monoacetyl derivative and was kept in a refrigerator over night. The resulting crystals were collected, and recrystallized from methanol–water 1:3; yield 0.86 g. (79%), m.p. 215–217°, no depression of m.p. when admixed with the material prepared according to a and b above, $[\alpha]^{24}D$ − 19.9° (*c* 1.1 in dimethylformamide), R_f^A 0.60, amino acid ratios in carboxypeptidase digest $ser_{1.1}tyr_{1.0}met_{1.0}gluta$.[21]

N-Acetylseryl-O-acetyltyrosylserylmethionylglutamine.—Effluent fractions 170–217 from experiment a were pooled and evaporated to dryness *in vacuo* at a bath temperature of 40–50°. Precipitation of the residue from methanol with water afforded a gelatinous product; yield 0.62 g. (22%), m.p. 187–191°, $[\alpha]^{25}D$ −20.8° (*c* 0.9 in glacial acetic acid); absorption max. at 260 mμ, log ε 2.70 in 0.1 *N* hydrochloric acid; R_f^A 0.68; Pauly and ninhydrin negative, methionine positive.[28]

Anal. Calcd. for $C_{29}H_{42}O_{12}N_6S\cdot H_2O$: C, 48.6; H, 6.2; N, 11.7; acetyl, 12.0. Found: C, 48.2; H, 6.5; N, 11.5; acetyl, 11.8.

N-Acetylseryltyrosylserylmethionylglutamine Methyl Ester.—Ethereal diazomethane was added to an ice-cold solution of N-acetylseryltyrosylserylmethionylglutamine (1.5 g.) in dimethylformamide (20 ml.) and methanol (15 ml.) until a faint yellow color remained. The solution was kept for 5 minutes, the excess of diazomethane was destroyed by addition of a few drops of glacial acetic acid and the solution was evaporated to dryness *in vacuo*. The residue was washed with ether and dried; yield 1.45 g. (92%), m.p. 204–209°. A sample for analysis was precipitated from methanol with water; $[\alpha]^{23}D$ −28.0° (*c* 0.8 in methanol).

Anal. Calcd. for $C_{28}H_{42}O_{11}N_6S\cdot H_2O$: C, 48.8; H, 6.4; N, 12.2. Found: C, 48.4; H, 6.4; N, 12.2.

N-Acetylseryltyrosylserylmethionylglutamine Hydrazide.—N-Acetylseryltyrosylserylmethionylglutamine methyl ester (1.45 g.) was dissolved in methanol (150 ml.), hydrazine hydrate (1.5 ml.) was added and the solution kept at room temperature for 20 hours. The ensuing gelatinous mass was collected and dried over sulfuric acid *in vacuo;* yield 1.35 g. (93%), m.p. 192–196°. A sample for analysis was twice precipitated from methanol with water; m.p. 217–220° dec.

Anal. Calcd. for $C_{27}H_{42}O_{10}N_8S\cdot H_2O$: C, 47.1; H, 6.4; N, 16.3. Found: C, 46.6; H, 6.5; N, 16.0.

Rearranged Product. a. Carbobenzoxy Derivative.—A solution of serylmethionylglutamine (1.3 g.) in water (60 ml.) and triethylamine (0.5 ml.) was shaken at 5° for 48 hours with an ethyl acetate solution of carbobenzoxyseryltyrosine azide (prepared from 2.97 g. of the hydrazide). The ethyl acetate layer was removed, the aqueous phase was re-extracted with three additional portions of ethyl acetate which were discarded. The aqueous phase was lyophilized, the residue dissolved in a small quantity of water, and the product was precipitated in the form of a gelatinous mass by acidification with dilute acetic acid. The material was collected and dried; yield 2.03 g. (75.7%). This material was dissolved in 1-butanol equilibrated with 20% acetic acid (60 ml.) and the solution was placed in the first six tubes of a 15-tube countercurrent machine. Thirty-eight transfers were carried out in the solvent system 1-butanol-20% acetic acid and the contents of effluent fractions 2 to 22 (containing ninhydrin-negative, Pauly and methionine positive material) were combined, and evaporated to give fine needles; yield 0.93 g. (35%), m.p. 195–198°. This material was recrystallized from 1-butanol saturated with 20% acetic acid; m.p. 198–201°, $[\alpha]^{24}D$ +1.85° (*c* 1.0 in dimethylformamide, $[\alpha]^{32}D$ −15.0° (*c* 1.0 in glacial acetic acid), R_f^A 0.77, R_f^B phe, ninhydrin negative, Pauly and methionine positive[28]; amino acids present in acid hydrolysate ser, met and glu, tyr absent; Pauly-positive spot with R_f^A 0.96 present on chromatogram which forms orange color with dinitrophenylhydrazine.

Anal. Calcd. for $C_{33}H_{45}O_{12}N_7S\cdot H_2O$: C, 50.7; H, 6.1; N, 12.5. Found: C, 50.7; H, 6.0; N, 13.5.

b. Carbobenzoxy Methyl Ester.—The rearranged product (0.80 g.) was dissolved in dimethylformamide (40 ml.) and an ethereal solution of diazomethane was added until the yellow color persisted. The excess of diazomethane was

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-

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(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).

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