rial travelled at a faster rate than the corresponding octapeptide amide containing arginine (band B).

A representative experiment was carried out as follows: The crude coupling product (707 mg.) was hydrogenated and the hydrogenation products were subjected to a 560-plate distribution in the solvent system 1-butanol-5% acetic acid containing 0.5% sodium chloride. The desired completely reduced octapeptide amide (band B) was located in tubes 20 to 80, the nitroarginine analog occupying tubes 141 to 200. The contents of these tubes were pooled, the solvents were evaporated *in vacuo*, and the residue was desalted by distribution between 1-butanol and 1% ammonium hydroxide in the manner described. The salt-free residue was dissolved in 5% acetic acid and the filtered solution was lyophilized to give a colorless, fluffy solid; yield 108 mg., $[\alpha]^{25}D$ −30.1° (*c* 0.8 in 10% acetic acid), homogeneous on paper ($R_f^{10}$ 0.67); ninhydrin, Pauly and Ehrlich positive, Sakaguchi negative; completely digestible by LAP, amino acid ratios in digest $his_{1.0}phe_{1.0}arg_{1.0}try_{1.0}gly_{1.0}N^{\epsilon}$-formly$s_{1.0}$-$val_{1.0}$ (arg absent, pro present but not determined); biol. activity $5.0 \times 10^5$ MSH units per g.

PITTSBURGH, PENNA.

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

# Studies on Polypeptides. XVII. The Synthesis of Three Acyltridecapeptide Amides Possessing a High Level of Melanocyte-expanding Activity *in Vitro*[1-3]

BY KLAUS HOFMANN, HARUAKI YAJIMA AND ELEANORE T. SCHWARTZ

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The synthesis of three acyltridecapeptide amides containing of the entire amino acid sequence of the pituitary hormone α-MSH is described. Evidence is presented for the homogeneity and 12-L-configuration of these compounds. All three peptide derivatives were shown to possess a high level of melanocyte-expanding activity *in vitro*. The biological activity of acetylseryltyrosylserylmethionylglutaminylhistidylphenylalanylarginyltryptophylglycyl-$N^{\epsilon}$-formyllysylprolylvaline amide was found to be essentially the same as that reported for natural α-MSH. The relation between structure and biological activity of these α-MSH derivatives is discussed.

Elucidation of the constitution of α-MSH (I) by Harris and Lerner[4] provided the structural foundation for the intrinsic melanocyte-expanding activity of pure corticotropin which was observed by Bell and collaborators.[5] Indeed, the very tridecapeptide sequence of amino acids which constitutes the molecule of α-MSH makes up the N-terminal third of the corticotropin structure. The molecule of α-MSH contains an acetylated serine at the amino end and terminates with a valine amide group in position 13[6]; the amino group of the N-terminal serine in corticotropin is free.

In an attempt to delineate the minimal structural features endowing a peptide with melanocyte-expanding or corticotropic activity we have completed, some time ago,[7,8] the synthesis of two derivatives of α-MSH possessing structures II and III. These blocked tridecapeptide amides which embody within their molecules the entire structure of α-MSH are endowed with *in vitro* melanocyte-expanding activity to a significant degree.

In the present communication we present detailed experimental procedures for the preparation of compounds II and III and record the synthesis of a new derivative of α-MSH (compound IV) exhibiting a level of biological potency approaching that of α-MSH.

In their, by now classical study, on the constitution of the corticotropin molecule, Bell and collaborators[5] established the L-configuration of the constituent amino acids except alanine, aspartic acid, methionine and tryptophan by microbiological procedures. The structure of α-MSH was determined with extremely small amounts of the hormone and information pertaining to the stereochemical nature of its constituent amino acids is not available, but the all-L configuration seems highly probable. Based on the premise that only a molecule possessing the all-L configuration would possess maximal biological activity (an assumption which remains to be verified experimentally) we have employed in our work only those coupling reactions which minimize the chances for racemization. In addition we have purified extensively and have evaluated critically the homogeneity of all subunits prior to their application to the syntheses of the more complex peptides. Such a course of action can be expected to ensure the highest possible degree of purity of the final products.

Studies relating structure to biological activity of complex polypeptides can have fundamental significance only if the compounds to be tested biologically are of a high degree of homogeneity. The biological evaluation of impure samples is likely to provide misleading results.

The general scheme which we employed in the synthesis of the α-MSH derivatives involved the interaction of an acylpentapeptide azide (subunit A) with a partially blocked octapeptide amide (subunit B). Thus, the coupling of the azide of carbobenzoxyseryltyrosylserylmethionylglutamine (subunit A, R = carbobenzoxy, $R^1 = NH_2$) with histidylphenylalanylarginyltryptophylglycyl - $N^{\epsilon}$-tosyllysylprolylvaline amide[9] (subunit B, $R^2$ =

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

(2) The 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) Some of the results reported in this paper were presented at the Atlantic City Meeting of the American Chemical Society in September, 1959.

(4) J. I. Harris and A. B. Lerner, *Nature*, **179**, 1346 (1957).

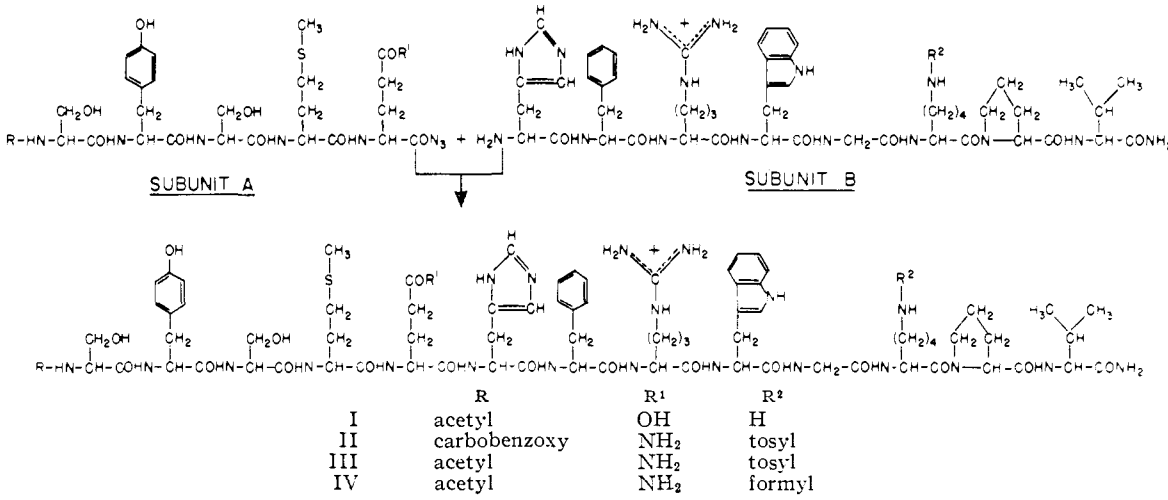
(5) R. G. Shepherd, K. S. Howard, P. H. Bell, A. R. Cacciola, R. G. Child, M. C. Davies, J. P. English, B. M. Finn, J. H. Meisenhelder, A. W. Moyer and J. van der Scheer, THIS JOURNAL, **78**, 5051 (1956).

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

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

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

FINAL STEP IN SYNTHESIS OF α-MSH ANALOGS




| | R | $R^1$ | $R^2$ |
|---|---|---|---|
| I | acetyl | OH | H |
| II | carbobenzoxy | $NH_2$ | tosyl |
| III | acetyl | $NH_2$ | tosyl |
| IV | acetyl | $NH_2$ | formyl |

tosyl) in dimethylformamide containing triethylamine gave a material which contained the carbobenzoxytridecapeptide amide II. The course of the coupling reaction, which was carried out at 0 to +3°, was followed by measuring the decrease in the ninhydrin color intensity of aliquots withdrawn from the reaction mixture at various intervals of time. In order to remove unchanged octapeptide the crude reaction product was subjected to a 200-plate countercurrent distribution in the solvent system 1-butanol–10% acetic acid. The ensuing partially purified material, exhibiting a biological activity of $0.8 \times 10^8$ MSH units per gram,[10] formed a single spot on paper but was still contaminated by an impurity (derived from the azide) which was readily removed by paper electrophoresis. The contaminating substance was clearly distinguishable from the desired acyltridecapeptide amide by its slower rate of migration in an electric field and by its yellow color reaction with the Pauly reagent; the carbobenzoxytridecapeptide amide forms a red color with diazotized *p*-bromoaniline. For purification on a preparative scale the partially purified material was subjected to electrophoresis on a block of cellulose powder in 25% acetic acid and impurities derived from the paper pulp were removed from the desired fraction by a second countercurrent distribution (195 transfers) in the solvent system 1-butanol–10% acetic acid. The highly purified carbobenzoxytridecapeptide amide II was obtained in the form of a colorless fluffy powder by freeze-drying. The final product produced a single ninhydrin negative, Pauly, Sakaguchi, methionine and Ehrlich positive spot on paper ($R_f$ 0.88) and the acid hydrolysate contained the constituent amino acids (with exception of methionine) in the molar ratios expected by theory.[11] The biological activity of the most highly purified material was $1.5 \times 10^8$ MSH units per gram.

The interaction of acetylseryltyrosylserylmethionylglutamine azide (subunit A, R = acetyl, $R^1 = NH_2$) with histidylphenylalanylarginyltryptophylglycyl-$N^\epsilon$-tosyllysylprolylvaline amide[9] (subunit B, $R^2$ = tosyl) afforded a crude product which assayed $5.4 \times 10^8$ MSH units per gram. Distribution in the solvent system 1-butanol–20% acetic acid (185 transfers) removed unchanged octapeptide amide to give a material, still inhomogeneous, possessing a biological activity of $8.8 \times 10^8$ MSH units per gram. Further purification by cellulose-block electrophoresis at *p*H 5.1, and by a second countercurrent distribution (100 transfers) in the solvent system 1-butanol–10% acetic acid followed by freeze-drying from 10% acetic acid gave III in the form of a fluffy colorless powder. The compound formed a single ninhydrin negative, Pauly, Sakaguchi, methionine and Ehrlich positive spot on paper ($R_f$ 0.78), behaved as a single component on paper electrophoresis at various *p*H values, and its acid hydrolysate contained the constituent amino acids in the molar ratios expected by theory. The biological activity of the highly purified acetyltridecapeptide amide III was $2 \times 10^9$ MSH units per gram.

The crude acetyltridecapeptide amide IV (activity $1.5 \times 10^9$ MSH units per gram) which was isolated from the reaction between acetylseryltyrosylserylmethionylglutamine azide (subunit A, R = acetyl, $R^1 = NH_2$) and histidylphenylalanylarginyltryptophylglycyl-$N^\epsilon$-formyllysylprolylvaline amide[12] (subunit B, $R^2$ = formyl) was incubated with thioglycolic acid and was then subjected to a 1250-plate countercurrent distribution in the solvent system 1-butanol–10% acetic acid. Isolation of the material corresponding to the major peak of the distribution pattern followed by freeze-drying from 10% acetic acid solution gave com-

(9) K. Hofmann, T. A. Thompson, M. E. Woolner, G. Spühler, H. Yajima, J. D. Cipera and E. T. Schwartz, THIS JOURNAL, **82**, 3721 (1960).

(10) 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).

(11) 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.

(12) K. Hofmann, E. Stutz, G. Spühler, H. Yajima and E. T. Schwartz, THIS JOURNAL, **82**, 3727 (1960).

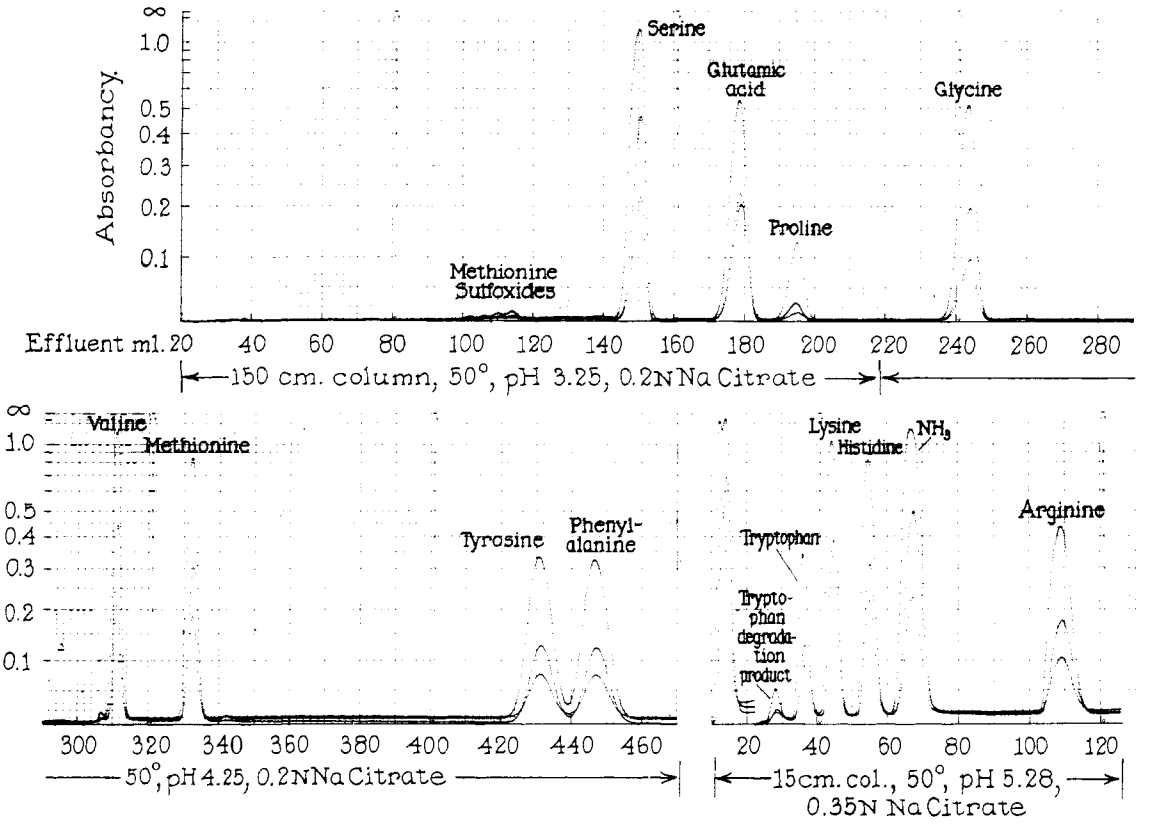



Fig. 1.—Stein–Moore chromatogram of an acid hydrolysate of the acetyltridecapeptide IV.

pound IV in the form of a colorless fluffy powder. The compound proved to be homogeneous on paper and traveled as a single component when subjected to paper electrophoresis at various *p*H values. Analysis by quantitative paper chromatography of an acid hydrolysate of the material showed the presence of the constituent amino acids in the proportions required by theory. As a further check for homogeneity the amino acid spectrum of an acid hydrolysate of the highly purified acetyltridecapeptide amide IV was determined by the Stein and Moore technique.[13] The result is shown on Fig. 1. With the exception of trace impurities (in that region of the chromatogram containing the various stereoisomeric sulfoxides of methionine) the hydrolysate was composed of only the expected amino acids and ammonia, and its quantitative composition was that predicted by theory. The paper chromatographic procedure which we employed to evaluate quantitatively the amino acid composition of peptide hydrolysates[14] is not suitable for the determination of ammonia. However, from the results shown in Fig. 1 it could be readily calculated that two molecules of ammonia (one from the glutamine and one from the C-terminal amide group) were liberated during the acid hydrolysis of the acyltridecapeptide amide IV.

The preparation of this compound was repeated four times and no differences in properties and analytical composition could be detected in the final products by our methods of analysis. However, the biological activity of the material derived from different batches varied from $0.6 \times 10^{10}$ to $2.2 \times 10^{10}$ MSH units per gram. The reasons for these fluctuations in biological activity are not clear at the present time.

The results of the analytical evaluation of the synthetic acyltridecapeptide amides cited above warrant the conclusion that these compounds are of a high degree of homogeneity. Since all three compounds were prepared by an azide coupling step from intermediates of established stereochemical purity, we feel justified to assign to them the 12-L-configuration.

That the reaction of acylglutamine azides with peptides containing an N-terminal histidine residue results in normal peptide bond formation has been established by model experiments.[9]

In their description of the isolation of α-MSH from hog pituitaries, Lee and Lerner[15] stressed the marked instability of their most highly purified samples of this hormone. They found, for example, that α-MSH samples which were stored in the dry state at −17° rapidly lost their biological activity.

We have reassayed samples of compounds II and III following storage for one year at 5° in the dry state and have found that the material had lost little of its original biological activity. The reasons for the difference in stability of peptides possessing melanocyte-expanding activity (naturally occurring

(13) S. Moore, D. H. Spackman and W. H. Stein, *Anal. Chem.*, **30**, 1185 (1958). We wish to express our sincere appreciation to Dr. W. H. Stein for these determinations.

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

(15) T. H. Lee and A. B. Lerner, *J. Biol. Chem.*, **221**, 943 (1956).

or synthetic) are not clear at the present time, but it seems likely that oxidation of the methionine residue to a methionine sulfoxide residue may be involved in the inactivation. Since metals are highly effective catalysts for this reaction one may be tempted to attribute differences in stability of various MSH active peptides to variations in their content of trace metal impurities.

It was demonstrated previously[9,16-18] that fragments of the $\alpha$-MSH structure have the ability to stimulate melanocytes *in vitro*. The results presented in this study show that the biological activity (in MSH units per gram) of the acyltridecapeptide amides (II, III and IV) increases from $1.5 \times 10^8$ to $2.0 \times 10^9$ to $2.2 \times 10^{10}$, respectively. Indeed the activity of compound IV is essentially that reported for the natural hormone, *i.e.*, 1 to $2 \times 10^{10}$ MSH units per gram.[15]

Caution is indicated in the interpretation of results relating peptide structure to biological activity particularly when the effects of blocking groups attached to peptides of high biological activity are concerned. The observable biological activity may be that of the intact blocked peptide or may reflect the ability of the target tissue to remove the blocking group with formation of the active parent compound. At the present stage of our understanding it is difficult to decide between these possibilities. The following discussion is based on the assumption that compounds II, III and IV possess intrinsic melanocyte expanding activity which is not the result of their deblocking by the frog tissue. The finding that derivatives of $\alpha$-MSH in which the carboxyl group of the glutamic acid residue is blocked by an amide function and which do not contain a free lysine $\epsilon$-amino group are highly effective melanocyte expanders suggests that the free carboxyl and $\epsilon$-amino groups are not vitally concerned with biological activity.

In a previous study[12] it was shown that histidylphenylalanylarginyltryptophylglycyl - $N^{\epsilon}$ - formyllysylprolylvaline amide exhibits sixteen times the biological activity of histidylphenylalanylarginyltryptophylglycyl-$N^{\epsilon}$-tosyllysylprolylvaline amide. In this pair the more active peptide contained the smaller protecting group attached to the $\epsilon$-amino group of the lysine residue. A comparison of the biological activities of compounds III and IV which differ solely in the nature of the protecting group on the lysine residue again shows the compound containing the smaller blocking group to be the more active one. That this phenomenon is not limited to groups which are attached to the lysine residue follows from a comparison of the biological activities of compounds II and III. These molecules are both $N^{\epsilon}$-tosyllysine derivatives differing by the nature of the N-terminal acyl function (carbobenzoxy in II, acetyl in III). The acetyl derivative exhibits higher activity than does the carbobenzoxylated compound.

We are inclined to interpret these results in terms of the steric interference, by the bulkier group, with the combination of these polypeptides with their receptor sites.

In a recent communication, Guttmann and Boissonnas[19] claim the synthesis of a peptide possessing "the physical, chemical and biological properties of the natural $\alpha$-MSH," but the experimental data supporting these claims are not entirely convincing. For evaluation of the biological activity of their product these workers have employed the *in vivo* Landgrebe and Waring method of assay rather than the more reliable *in vitro* assay of Shizume, *et al.*[10] Since the *in vivo* activity of pure $\alpha$-MSH is unknown, it is difficult to evaluate the true potency of the peptide prepared by these investigators. The final step in the Guttmann–Boissonnas synthesis involves hydrogen bromide deblocking of acetylseryltyrosylserylmethionyl-$\gamma$-benzylglutamylhistidylphenylalanylarginyltryptophylglycyl-$N^{\epsilon}$-carbobenzoxylysyl prolylvaline amide. The blocked acetyltridecapeptide amide was prepared (in 86.6% yield) by a N,N′-dicyclohexylcarbodiimide step and was considered to be analytically and stereochemically pure following a single precipitation from pyridine solution by ether. In our own studies,[9,12] using the N,N′-dicyclohexylcarbodiimide reagent in the preparation of related peptides, we have invariably observed the formation of mixtures and extensive purification was necessary to obtain homogeneous components.

## Experimental[20]

**Carbobenzoxyseryltyrosylserylmethionylglutaminylhistidylphenylalanylarginyltryptophylglycyl - $N^{\epsilon}$ - tosyllysylprolylvaline Amide (II).**—Carbobenzoxyseryltyrosylserylmethionylglutamine hydrazide[21] (458 mg.) was dissolved in 2*N* hydrochloric acid (23 ml.) and water (23 ml.) was added. The solution was kept at 45–50° for 5 minutes and was then cooled in an ice-bath. Insoluble material which had precipitated was removed by centrifugation and a solution of sodium nitrite (42 mg.) in water (4 ml.) was added slowly with ice-cooling. Ice-cold saturated sodium chloride (8 ml.) was added, and the mixture was kept at ice-bath temperature for 10 minutes. The solid azide which had precipitated was collected, washed with small volumes of ice-cold 1*N* hydrochloric acid, saturated sodium chloride and water and was dried for 3 hours at −15° over phosphorus pentoxide and potassium hydroxide pellets; yield 136 mg.

The solid azide (130 mg.) was added to an ice-cold solution of histidylphenylalanylarginyltryptophylglycyl-$N^{\epsilon}$-tosyllysylprolylvaline amide diacetate monohydrate[9] (193 mg.) dissolved in 10 ml. of dimethylformamide containing 0.02 ml. of triethylamine. The mixture was kept at 0 to +3°, and samples (10 λ each) were withdrawn at zero time and at regular intervals thereafter for development with the ninhydrin reagent. After 72 hours the color intensity had dropped to 50% of its original value and no further change was noted after 96 hours. Additional azide (60 mg.) was added at this point and the reaction mixture was kept for an additional

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

(17) R. Schwyzer and C. H. Li, *Nature*, **182**, 1669 (1958).

(18) K. Hofmann, M. E. Woolner, H. Yajima, G. Spühler, T. A. Thompson and E. T. Schwartz, THIS JOURNAL, **80**, 6458 (1959).

(19) St. Guttmann and R. A. Boissonnas, *Helv. Chim. Acta*, **42**, 1257 (1959).

(20) The organic solvents were freshly distilled. Doubly distilled water from which a sizable forerun was removed was used. A Craig-type countercurrent machine of 200 tubes having a capacity of 10 ml. of upper and lower phase each (obtained from H. O. Post, Scientific Instrument Co., Maspeth 78, N. Y.) was employed. Countercurrent distributions were performed at room temperature. Rotations were determined in a Rudolph precision polarimeter model 80 with model 200 photoelectric attachment. The analytical and enzymatic methods used were those described previously.[14] Unless stated otherwise solvents were evaporated *in vacuo* in a flash evaporator at a bath temperature of 40–45°; *Rf* values were determined in the Partridge system, S. M. Partridge, *Biochem. J.*, **42**, 238 (1948).

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

48 hours at 0 to +3°. By that time the ninhydrin color had decreased 90% from its original value.

The solvents were evaporated to dryness under nitrogen and the residue (382 mg.) was subjected to a 200-plate countercurrent distribution in the solvent system 1-butanol–10% acetic acid. One drop of saturated sodium chloride was added to the first 100 tubes to eliminate the formation of emulsions. Spectrophotometric examination at 280 mμ showed the presence of two components, the minor one being located in tubes 109–130, the major one occupying tubes 143–197. The minor component was identified by paper chromatographic comparison with an authentic sample as unchanged octapeptide. The contents of the tubes containing the major component were pooled and the solvents were evaporated. The residue was dissolved in a small volume of 20% acetic acid and the solution was lyophilized to give 248 mg. of a colorless fluffy product, $R_f$ 0.88, biol. activity 0.8 × $10^8$ MSH units per gram, amino acid ratios in acid hydrolysate $ser_{2.8}tyr_{1.4}met_{1.2}glu_{1.4}his_{0.9}phe_{1.1}arg_{1.0}$-$gly_{1.0}N^{\epsilon}$-$toslys_{0.9}val_{1.0}$. Electrophoresis on paper in 25% acetic acid (1200 volts, 1 milliamp. for 4 hours at 5°) revealed the presence of two components traveling toward the cathode, the first (yellow color with the Pauly reagent) was located 4.9 cm. from the starting point, the second (red color with the Pauly reagent) was found 10.9 cm. from the origin. This latter substance proved to be the desired carbobenzoxytridecapeptide amide. For purification on a preparative scale a sample of the partially purified material (100 mg.) was dissolved in 25% acetic acid (1.6 ml.) and this solution was applied to a block (37.7 × 4.7 × 1.0 cm.) prepared from Whatman paper powder with 25% acetic acid. Prior to pouring the block, the cellulose powder was washed with two portions of 1-butanol saturated with 20% acetic acid followed by two portions of 25% acetic acid. The electrophoresis was conducted in a cold room at 0–5° for 18 hours (1200 volts, 8 milliamp.). A contact print obtained from the surface of the block which was stained with the Pauly reagent showed the presence of two components. The zone containing the faster moving material (red color with Pauly reagent) was removed from the block and was eluted with 25% acetic acid. The filtered eluate was lyophilized to give 84 mg. of highly purified material. A second electrophoretic purification using 140 mg. of crude carbobenzoxytridecapeptide amide gave 119 mg. of highly purified material which was combined with the substance obtained from the first run; total yield 203 mg. The electrophoretically purified material was subjected to a 195-plate countercurrent distribution in the solvent system 1-butanol–10% acetic acid. The material located in tubes 137–187 was isolated and lyophilized from 10% acetic acid; yield 145 mg.; single spot ninhydrin negative, Pauly, Sakaguchi, Ehrlich and methionine positive; $R_f$ 0.88, $[\alpha]^{26}D$ −24.6° (*c* 0.7 in glacial acetic acid), amino acid ratios in acid hydrolysate $ser_{2.0}tyr_{1.0}met_{0.7}glu_{1.0}his_{0.8}arg_{1.0}$-$gly_{0.9}N^{\epsilon}$-$toslys_{1.0}val_{0.8}$ (pro and phe present on chromatogram but not determined, try destroyed), biol. activity 1.5 × $10^8$ MSH units per gram. This entire synthesis was performed twice with comparable results.

**Acetylseryltyrosylserylmethionylglutaminylhistidylphenylalanylarginyltryptophylglycyl-N$^{\epsilon}$-tosyllysylprolylvaline Amide (III).**—Acetylseryltyrosylserylmethionylglutamine hydrazide[21] (150 mg.) was dissolved in 1*N* hydrochloric acid (5 ml.) and a solution of sodium nitrite (15 mg.) in water (2 ml.) was added slowly while stirring under ice-cooling. Solid sodium chloride was added to saturation and the mixture was kept at 0–5° for 10 minutes. The solid azide which had precipitated was collected, washed with a small volume of ice-water and was dried for 3 hours over phosphorus pentoxide and potassium hydroxide pellets at −15° *in vacuo*. The yield of azide varied from 75 to 96 mg. in various experiments. This azide (51 mg.) was added to an ice-cold solution of histidylphenylalanylarginyltryptophylglycyl-N$^{\epsilon}$-tosyllysylprolylvaline amide diacetate monohydrate[9] (100 mg.) in dimethylformamide (5 ml.) containing 0.01 ml. of triethylamine. The mixture was stored at a temperature of 0–3° for 68 hours. An additional quantity of azide (30 mg.) was added and the mixture was stored for another 52 hours at 0–3°. The solvents were evaporated, the residue was triturated with freshly distilled peroxide-free ether, and was dried at room temperature *in vacuo;* yield 180 mg., biol. activity 5.4 × $10^8$ MSH units per gram.

This material was subjected to a 185-plate countercurrent distribution in the solvent system 1-butanol–10% acetic acid Spectrophotometric examination of the upper phases at 280 mμ revealed the presence of two major bands, one located in tubes 9 to 40, the other in tubes 85 to 153. The material corresponding to the slower moving band (55 mg.) was identified as unchanged octapeptide. The faster moving component containing the desired material was isolated from the pooled tubes by evaporation; yield 112 mg., biol. activity 8.8 × $10^8$ MSH units per gram, activity not increased when a 2.11-mg. sample was incubated for 18 hours at 37° with 1 ml. of 10% acetic acid containing 10% v./v. of thioglycolic acid; not homogeneous on paper in Partridge system; amino acid ratios in acid hydrolysate $ser_{4.0}tyr_{2.0}met_{1.4}glu_{2.0}his_{1.0}phe_{1.0}arg_{0.8}gly_{0.9}N^{\epsilon}$ - $toslys_{1.0}val_{1.0}$. For further purification this material was subjected to electrophoresis on a block (37.7 × 4.7 × 0.8 cm.) prepared from Whatman paper powder in 0.1 *M* pyridinium acetate buffer, *p*H 5.1. The paper powder was washed with two portions of 1-butanol equilibrated with 10% acetic acid and with two portions of the pyridinium acetate buffer. The peptide sample (50 mg.) dissolved in buffer (1.6 ml.) was applied to the anode side of the cellulose block, and electrophoresis was conducted at a temperature of 4° for 17 hours (1200 volts, 26 milliamps.). A contact print obtained from the surface of the block revealed the presence of two zones, A and B, which were readily distinguishable by their color reaction with the Pauly reagent. Zone A (yellow color) was located 3.5–6 cm. and zone B (red color) 8–10.8 cm. from the origin. The area of the block which was located between zones A and B was designated as zone C. The portions of the block which corresponded to these various zones were removed, eluted separately with 1-butanol equilibrated with 10% acetic acid, and the filtered eluates were evaporated to dryness. A second electrophoretic separation was performed with 53 mg. of partially purified peptide and corresponding fractions were combined with those derived from the first experiment. The weights and biological activities (in MSH units per gram) of the combined fractions were: material corresponding to zone A (58 mg.), not assayed; material corresponding to zone B (42 mg.), 1.5 × $10^9$; material corresponding to zone C (35 mg.), 0.7 × $10^9$. The material derived from zone B which was contaminated with impurities derived from the paper powder was purified further by a 100-plate countercurrent distribution in the solvent system 1-butanol–10% acetic acid. Evaporation of the contents of tubes 41 to 75 (peak in tube 57) containing the desired highly purified acetyltridecapeptide amide followed by freeze-drying of the residue from 10% acetic acid gave 26 mg. of a colorless fluffy powder; single ninhydrin negative, Pauly, Sakaguchi, Ehrlich and methionine positive spot with $R_f$ 0.78; single spot on paper electrophoresis at *p*H 4.0, 5.1 and 6.0, respectively, in pyridinium acetate buffer (1200 volts for 2.5 hours); $[\alpha]^{26}D$ −40.8° (*c* 0.5 in 10% acetic acid); biol. activity 2.0 × $10^9$ MSH units per gram; distribution coefficient in 1-butanol–10% acetic acid 1.32; amino acid ratios in acid hydrolysate $ser_{1.6}tyr_{1.0}met_{0.8}glu_{1.0}his_{1.0}phe_{1.0}arg_{1.0}gly_{1.0}N^{\epsilon}$-$toslys_{1.1}$-$val_{0.9}$ (pro present but not determined, try destroyed). This entire synthesis was repeated twice with essentially identical results.

**Acetylseryltyrosylserylmethionylglutaminylhistidylphenylalanylarginyltryptophylglycyl - N$^{\epsilon}$ - formyllysylprolylvaline Amide (IV).**—Acetylseryltyrosylserylmethionylglutamine azide (158 mg.) was added to an ice-cold solution of histidylphenylalanylarginyltryptophylglycyl-N$^{\epsilon}$-formyllysylprolylvaline amide[12] (234 mg.) in dimethylformamide (10 ml.) containing 0.028 ml. of triethylamine, and the solution was kept at 0° for 40 hours. Additional portions of freshly prepared azide, 102 mg. after 40 hours and 63 mg. after 80 hours, were added and the solution was kept for 40 more hours following the last addition (total reaction time 120 hours). Freshly distilled thioglycolic acid (2 ml.) was added at this point and the mixture was incubated for 72 hours at 37° under nitrogen. The solvents were removed, the residue was dissolved in 10% acetic acid equilibrated with 1-butanol (50 ml.), and the solution was added to the first five tubes of a 200-plate countercurrent machine. A minor 280 mμ absorbing component, located in tubes 161 to 200, was removed following 400 transfers in the solvent system 1-butanol–10% acetic acid, then 600 additional transfers were carried out. One major band (maximum in tube 121) and two minor bands (maxima in tubes 49 and 81, respectively) were present at this point. Tubes 35 to 57 containing one of the minor components were removed and 250 additional

transfers were performed. Spectrophotometric evaluation of the progress of the separation showed the presence of two clearly separated bands located in tubes 85 to 117 (minor) and tubes 123 to 180 (major), respectively. The tubes containing the slower moving component were evaporated to dryness, the residue was dissolved in a small volume of 10% acetic acid and the solution was lyophilized to give a pale yellow powder: yield 82 mg.; single ninhydrin and methionine negative, Sakaguchi and Ehrlich positive spot, $R_f$ 0.56; biol. activity $1.7 \times 10^8$ MSH units per gram. The chemical nature of this material remains to be elucidated. The tubes containing the faster moving component were pooled and the solvents were removed. The residue was dissolved in a small volume of 10% acetic acid and the solution was lyophilized to give the desired acetyltridecapeptide amide in the form of a colorless fluffy solid; yield 156 mg.; single ninhydrin negative Pauly, Sakaguchi, Ehrlich and methionine positive spot, $R_f$ 0.62; single spot on paper electrophoresis at *p*H 4.0, 5.1 and 6.0, respectively, in pyridinium acetate buffers (1200 volts for 2.5 hours); $[\alpha]^{25}D$ −43.8° (*c* 0.6 in 10% acetic acid); biol. activity of various preparations ranged from $0.6 \times 10^{10}$ to $2.2 \times 10^{10}$ MSH units per gram; distribution coefficient in 1-butanol–10% acetic acid 0.14; amino acid ratios in acid hydrolysate: a, by quant. paper chromatography, $ser_{2.3}tyr_{1.1}met_{1.0}glu_{1.0}his_{1.0}phe_{1.0}arg_{1.0}gly_{1.0}lys_{0.9}val_{0.9}$ (try destroyed, pro present but not determined); b, by the Stein–Moore technique,[13] $ser_{2.0}tyr_{1.0}met_{1.0}glu_{1.0}his_{1.0}phe_{1.0}arg_{1.0}gly_{1.0}lys_{1.0}pro_{1.0}val_{1.0}NH_{3_{2.2}}$ (try largely destroyed).

PITTSBURGH, PENNA.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, DUQUESNE UNIVERSITY]

# The Effects of Optical Configuration of Peptides: Dissociation Constants of the Isomeric Alanylalanines and Leucyltyrosines and Some of their Metal Complexes[1]

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A determination of the dissociation constants of the diastereoisomeric leucyltyrosines and a re-examination of the dissociation constants of several other dipeptides was made. The ability to predict relative acidities of the diastereoisomeric dipeptides on the basis of the folding and unfolding processes which occur as a function of *p*H is presented, and the differences in acidity are postulated to increase with the bulk of the residues attached to the asymmetric centers in the dipeptides. The stability constants of the leucyltyrosine and alanylalanine complexes with $Co^{++}$, $Ni^{++}$ and $Zn^{++}$ were determined and discussed with respect to the effect of optical activity. The possibility of predicting relative rates of hydrolysis of the diastereoisomeric dipeptides as a function of *p*H is presented.

Ellenbogen[4a,b] has recently studied the effects of optical configuration on the dissociation constants of the lysyl and alanyl peptides, and showed that the dissociation constants of these peptides varied by up to 0.26 *pK* unit in changing the amino acid residues on the peptide chain. He determined the dissociation constants of the four isomeric alanylalanines at a constant ionic strength of 0.100, and found that the constants for the LL- were identical to those for the DD-isomer, and those of the LD- were identical to those of the DL-isomer. Since LL is the mirror image of DD and since LD is the mirror image of DL, this is in accordance with theory. However, those of the LL- differed considerably from those of the DL-isomer.

We have extended this work to an examination of the DL- and LL-leucyltyrosines and a re-examination of the isomeric alanylalanines to confirm the effect of optical configuration on the dissociation constants of the peptides and to determine the stability constants of some of their metal complexes.

## Experimental[5]

L-Alanine, $[\alpha]^{25}D$ +14.4° (2 *N* HCl, *c* 1); D-alanine, $[\alpha]^{25}D$ −14.2° (2 *N* HCl, *c* 1) were obtained from the Nutritional Biochemical Corporation. L-Leucyl-L-tyrosine, $[\alpha]^{25}D$ +10.4° ($H_2O$, *c* 1) and D-leucyl-L-tyrosine, $[\alpha]^{25}D$ −15.3° ($H_2O$, *c* 2) were obtained from the Mann Research Laboratories, New York 6, N. Y. *Anal.* Calcd. for $C_{15}H_{22}N_2O_4$ (294.5): C, 61.2; H, 7.5; N, 9.5. Found for LL: C, 61.3; H, 7.4; N, 9.4. Found for DL: C, 61.5; H, 7.4; N, 9.67. All reagents were of C.P. grade.

**Benzyl Esters of L-Alanine and D-Alanine.**—The method of Erlanger and Hall[6] was used for the preparation of the benzyl esters of D-alanine and L-alanine. After recrystallization from methanol–ether, the products were obtained in 40% yield: L-alanine benzyl ester hydrochloride, m.p. 140°, $[\alpha]^{25}D$ −14.7 (0.1 *N* HCl, *c* 1.02); lit. $[\alpha]^{25}D$ −14.3° (*c* 2.11, $H_2O$)[6]; D-alanine benzyl ester hydrochloride, m.p. 140°, $[\alpha]^{25}D$ +14.8° (0.1 *N* HCl, *c* 1).

**Carbobenzoxy-L-alanine Benzyl Ester and Carbobenzoxy-L-alanyl-D-alanine Benzyl Ester.**—Following the method of Boissonnas,[7] the carbobenzoxy-L-alanine[8] was coupled with the benzyl esters of L-alanine and D-alanine. The carbobenzoxy-L-alanyl-L-alanine benzyl ester was recrystallized from methanol–water and melted at 109°[9], $[\alpha]^{25}D$ −1.7° (chloroform, *c* 1). *Anal.* Calcd. for $C_{21}H_{22}N_2O_5$: C, 65.63; H, 6.25; N, 7.29. Found: C, 65.51; H, 6.48; N, 7.54. The carbobenzoxy-L-alanyl-D-alanine benzyl ester, which had not been previously reported, melted at 114°, $[\alpha]^{25}D$ −2.9° (chloroform, *c* 1). *Anal.* Calcd. for $C_{21}H_{22}N_2O_5$: C, 65.63; H, 6.25; N, 7.29. Found: C, 65.60; H, 6.06; N, 7.01.

**L-Alanyl-L-alanine and L-Alanyl-D-alanine.**—The carbobenzoxy-L-alanyl-L-alanine benzyl ester and the carbobenzoxy-L-alanyl-D-alanine benzyl ester were hydrogenated using 10% palladium-on-activated charcoal as the catalyst. The products after recrystallization were obtained in 60%

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(3) Abstracted from a dissertation submitted by N. Solony to the Graduate School of Duquesne University in partial fulfillment of the requirements for the degree of Master of Science, 1959.

(4) (a) E. Ellenbogen, *J. Cell. Comp. Physiol.*, **47**, 151 (1956); (b) THIS JOURNAL, **78**, 369 (1956).

(5) All melting points are uncorrected.

(6) B. F. Erlanger and R. M. Hall, THIS JOURNAL, **76**, 5781 (1954).

(7) R. A. Boissonnas, *Helv. Chim. Acta*, **34**, 874 (1951).

(8) M. E. Carter, R. L. Frank and H. W. Johnson, "Organic Syntheses," Coll. Vol. III, John Wiley and Sons, Inc., New York, N. Y., 1955, p. 167.

(9) B. F. Erlanger and E. Brand, THIS JOURNAL, **73**, 3508 (1951), report the melting point as 138°, essentially the same as both starting reactants and report no rotation. The compound obtained here was insoluble in bicarbonate and dilute acid solutions with which the uncoupled reactants were extracted. The melting point of 109° for the compound obtained was very sharp and on catalytic debenzylation furnished L-alanyl-L-alanine. While the coupled product could conceivably contain some racemized material, its sharp melting point and the purity and ease in obtaining pure LL-dipeptide from it seemed to indicate the coupled product has the structure assigned.