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Studies on Peptides. XVI.[1,2] Regeneration of Lysine from N^{ε}-Formyllysine by Aqueous Hydrazine or Hydroxylamine and Their Application to the Synthesis of α-Melanocyte-stimulating Hormone

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Treatment of N^{ε}–formyllysine with dilute aqueous hydrazine acetate or hydroxylamine hydrochloride in aqueous pyridine regenerated lysine in satisfactory yield. These methods were adopted to convert N^{α}–acetylseryltyrosylserylmethionylglutamylhistidylphenylalanylarginyltryptophylglycyl–N^{ε}–formyllysylprolylvaline amide to α–melanocyte–stimulating hormone (MSH). The products prepared by two alternate methods behaved quite identically with natural α–MSH on paper and thin–layer chromatographies and their infrared spectra were both identical with that of natural α–MSH. Their *in vitro* MSH activities were $2.3—5.4 \times 10^{12}$ and $2.4—4.1 \times 10^{12}$ U/g respectively.

N^{ε}–Formyllysine has been used by Hofmann, *et al.*[4–12] for the syntheses of peptides related to adrenocorticotropic hormone. The formyl group can survive under various conditions required for the syntheses of complex peptides. It is stable under the condition of alkaline saponification and resists catalytic hydrogenation in the presence of acetic acid or formic acid. Besides, it is worthwhile to note that most N^{ε}–formyllysin–peptides known are soluble in water. This unique property permits one to purify various N^{ε}–formyllysine–peptides by column ion–exchange chromatography.

The removal of the N^{α}–formyl group of amino acids or peptides can be readily achieved with dilute hydrochloric acid[13] in methanol or in aqueous dioxane. Recently Losse, *et al.*[14,15] reported that the formyl group can be removed by catalytic hydrogenation in the presence of concentrated hydrochloric acid or by oxidation with 15% hydrogen peroxide. These conditions could be applied only to N^{ε}–formyllysine–peptides that do not contain acid–labile

1) The amino acids, peptides, and their derivatives (except glycine) mentioned in this communication are of the L–configuration. Their abbreviated designations are those recommended by IUPAC–IUB commission on Biochemistry Nomenclature in July, 1965 and July, 1966; *Biochemistry*, **5**, 2485 (1966), **6**, 362 (1967), *Biochim. Biophys. Acta*, **133**, 1 (1967).
2) Part XV: *Chem. Pharm. Bull.* (Tokyo), **15**, 1922 (1967).
3) Location: *Sakyo–ku, Kyoto.*
4) K. Hofmann and H. Yajima, "Polyamino acids, Polypeptides and Proteins," Ed. by M.A. Stahmann, The University of Wisconsin Press, Madison, Wis. U.S.A., p. 21, 1962.
5) K. Hofmann, H. Yajima, and E.T. Schwartz, *J. Am. Chem. Soc.*, **82**, 3732 (1960).
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9) K. Hofmann, N. Yanaihara, S. Lande, and H. Yajima, *J. Am. Chem. Soc.*, **84**, 4470 (1962).
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12) K. Hofmann and H. Yajima, *Recent Prog. Hormone Res.*, **18**, 41 (1962).
13) J.C. Sheehan and D.D.H. Yang, *J. Am. Chem. Soc.*, **80**, 1154 (1958).
14) G. Losse and D. Nadolski, *J. Prak. Chem.*, **24**, 118 (1964).
15) G. Losse and W. Zönnchen, *Angew. Chem.*, **72**, 385 (1960); *Ann. Chem.*, **636**, 140 (1960).

peptide bonds or oxidation-sensitive amino acid residues. The use of acetyl chloride[16-18] was also proposed for this purpose, but general application of this reagent seems to be limited, because of its ability to acylate other functional groups in the peptide. The application of N^{ε}-formyllysine in the peptide synthesis suffers considerable limitation at the final stage of the synthesis; removal of the formyl group. Therefore, inspite of the many advantageous properties that it possesses, the formyl protecting group on the ε-amino group of lysine has limitation in its application in the peptide synthesis.

In 1960, Hofmann and one of the present author (H.Y.)[5] synthesized the acetyltridecapeptide amide corresponding to the entire amino acid sequence of α-melanocyte-stimulating hormone (MSH, I) except that the glutamic acid residue at position 5 was relplaced by glutamine and the ε-amino function of the lysine residue at 11 was blocked by the formyl group. This compound exhibited the *in vitro* MSH activity of $0.6—2.2\times10^{10}$ U/g, nearly equivalent to that reported in natural α-MSH (2.0×10^{10} MSH U/g).[19] However, selective removal of the formyl group from this acetyl peptide amide in acidic condition was unsuccessful, since the N^{α}-acetyl group of the amino terminal serine residue was split off and partial hydrolysis of the γ-amide bond of the glutamine residue had also taken place.[6] Oxidative cleavage of the formyl group was unsuitable, since there is a methionine residue which is sensitive to oxidation in this peptide hormone. It seems, therefore, that in order to prepare α-MSH from its formyl derivative, another suitable deformylation condition is required. Miyamoto, *et al.*,[20] and Lefrancier and Bricas[17] showed that the N^{α}-formyl group of amino acids could be removed by hydrazine hydrate. We[21] have applied this method and succeeded in removing the formyl group by 10% hydrazine at 37°, from histidylphenylalanylarignyltryptophylglycylserylprolylprolyl-N^{ε}-formyllysylaspartic acid, a partially protected decapeptide of the C-terminal protion of β-MSH. Under this condition, the ariginine residue which is sensitive to basic condition survived. We have also looked into milder conditions required for the removal of the formyl group from the ε-amino group of lysine.

It was found that regeneration of lysine from N^{ε}-formyllysine could be accomplished by treatment with aqeuous hydrazine acetate or hydroxylamine hydrochloride in aqueous pyridine. The pH of the solutions were both controlled at approximately 6. These two conditions were successfully adopted for the synthesis of α-MSH.

The synthetic scheme is illustrated in Chart 1. This differs from those reported by Guttmann, *et al.*[22] and Schwyzer, *et al.*[23] in the choice of synthetic subunits and of the protecting groups. The former adopted benzyloxycarbonyl and the latter *t*-butoxycarbonyl or phthalyl group for protection of the ε-amino group of the lysine residue at position 11. The partially protected C-terminal octapeptide amide, histidylphenylalanylarginyltryptophylglycyl-N^{ε}-formyllysylprolylvaline amide (II) was prepared according to Hofmann, *et al.*[7] by hydrogenation of the product resulted from the coupling reaction of N^{α}-benzyloxycarbonylhistidylphenylalanyl-N^{G}-nitroarginyltryptophylglycine and N^{ε}-formyllysylprolylvaline amide by dicyclohexylcarbodiimide (DCC).[24] This coupling reaction was also carried out by DCC plus N-hydroxysuccinimide[25] or by N-ethyl-5-phenylisoxazolium-3'-sulfonate[26] in dimethyl-

16) S.G. Waley and J. Watson, *Chem. Ind.* (London), 107 (1953); *Biochem. J.*, **57**, 529 (1954).
17) P. Lefrancier and E. Bricas, *Bull. Soc. Chim. France*, 3668 (1965).
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21) H. Yajima, Y. Okada, Y. Kinomura, and E. Seto, *Chem. Pharm. Bull.* (Tokyo), **15**, 270 (1967).
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23) R. Schwyzer, A. Costopanagiotis, and P. Sieber, *Helv. Chim. Acta*, **46**, 870 (1963).
24) J.C. Sheehan and G.P. Hess, *J. Am. Chem. Soc.*, **77**, 1067 (1955).
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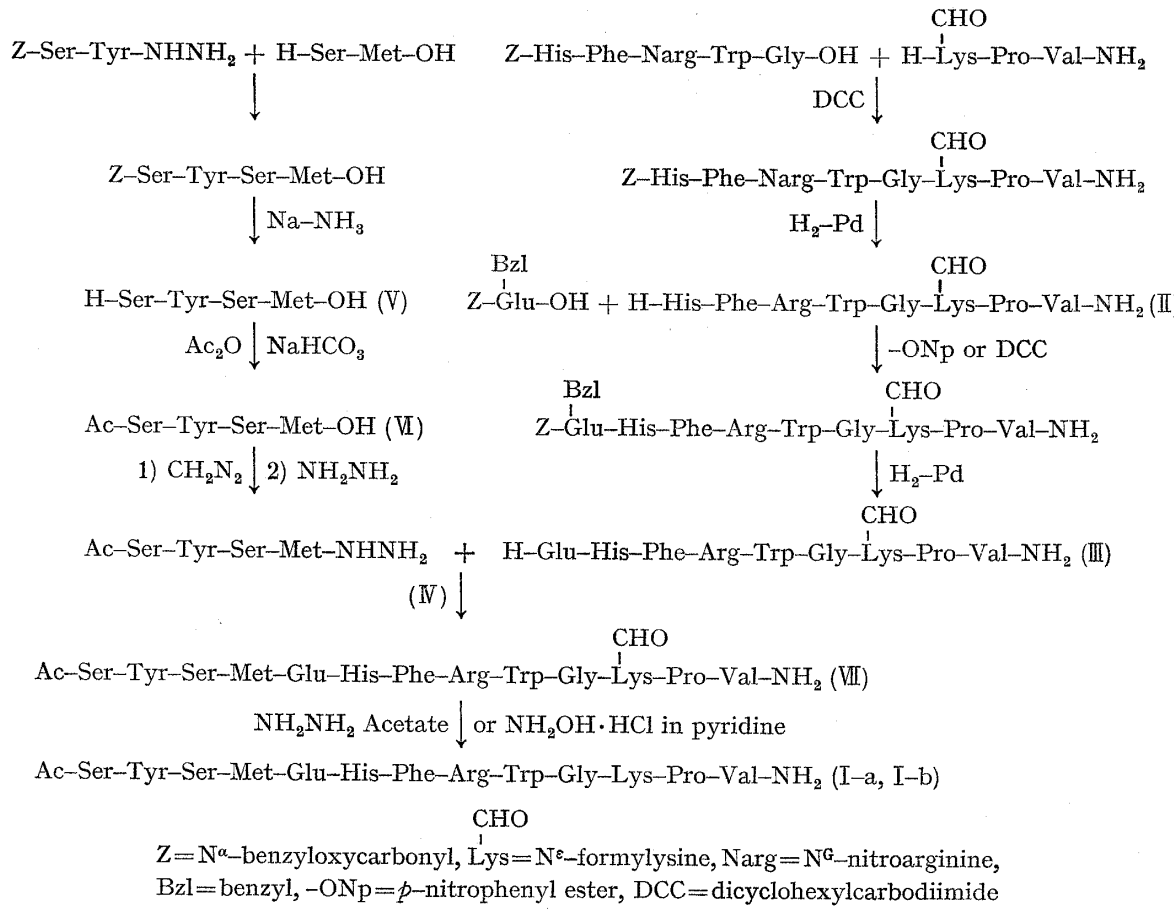

Z=N$^{\alpha}$–benzyloxycarbonyl, Lys(CHO)=N$^{\varepsilon}$–formylysine, Narg=N^{G}–nitroarginine,
Bzl=benzyl, -ONp=*p*–nitrophenyl ester, DCC=dicyclohexylcarbodiimide

Chart 1. Synthetic Route to α-Melanocyte-stimulating Hormone

formamide, but particular advantage was not observed in these cases. The latter gave a minor side reaction product. The partially protected octapeptide amide (II) was purified by column chromatography on carboxymethylcellulose (CM–cellulose). For elution, ammonium acetate buffers gave much better resolution of the desired compound from the contaminant, histidylphenylalanylarignyltryptophylglycine, than pyridine acetate buffers.

This peptide amide (II) was then coupled with N$^{\alpha}$–benzyloxycarbonyl–γ–benzylglutamate[27] either by the DCC or *p*–nitrophenyl ester method.[28] Each product was hydrogenated to give identical glutamylhistidylphenylalanylarginyltryptophylglycyl–N$^{\varepsilon}$–formyllysylprolylvaline amide (III), which was again purified by column chromatography on CM–cellulose using ammonium acetate buffers. Among other various coupling procedures, the mixed anhydride[29-31] of N$^{\alpha}$–benzyloxycarbonyl–γ–benzylglutamate gave some amount of an undesirable contaminant; presumably an ethoxycarbonyl compound.[32-34]

The N–terminal synthetic subunit, N$^{\alpha}$–acetylseryltyrosylserylmethionine hydrazide (IV), was prepared by the different method from that of Schwyzer, *et al.*[23] through the intermediate, seryltyrosylserylmethionine (V) as illustrated in Chart 1. This free tetrapeptide (V) was

27) E. Klieger, E. Schröder, and H. Gibian, *Ann. Chem.*, **640**, 157 (1961).
28) M. Bodanszky and V. du Vigneaud, *J. Am. Chem. Soc.*, **81**, 5688 (1959).
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32) Th. Wieland, B. Heinke, K. Vogeler, and H. Morimoto, *Ann. Chem.*, **655**, 189 (1962).
33) D. Gillessen, E. Schnabel, and J. Meienhofer, *Ann. Chem.*, **667**, 164 (1963).
34) F.E. King, J.W.C.–Lewis, D.A.A. Kidd, and G.R. Smith, *J. Chem. Soc.*, 1039 (1954).

prepared by coupling of N^{α}–benzyloxycarbonylseryltyrosine azide[35] with serylmethionine[36] followed by treatment of the product with sodium in liquid ammonia. The peptide V was converted to its acetyl derivative, N^{α}–acetylseryltyrosylserylmethionine (VI) and then to (IV) according to the procedure described by Hofmann, *et al.*[35] in the preparation of N^{α}–acetylseryltyrosylserylmethionylglutamine hydrazide. Our synthetic hydrazide (IV) exhibited different mp from that reported.[23] In order to examine the optical purity of our synthetic peptides, N^{α}–acetylseryltyrosylserylmethionine (VI) was digested by carboxypeptidase A.[37] It was found that the hydrolysate contained equimoles of methionine, tyrosine and serine. Next, this N^{α}–acetyltetrapeptide (VI) was treated with acylase.[38] The starting material was fully converted to a ninhydrin positive substance which exhibited the identical *Rf* value on paper chromatography with that of the synthetic tetrapeptide, seryltyrosylserylmethionine (V). These results seem to demonstrate unequivocally that our synthetic subunit of the N–terminal portion of α–MSH possesses the well established optical purity. The discrepancy of the mp between our compound and that reported by Schwyzer, *et al.* is unknown.

The hydrazide (IV) was converted to its azide, which was then allowed to react with the partially protected nonapeptide amide obtained above. The resulting N^{α}–acetyl, partially protected tridecapeptide amide, N^{α}–acetylseryltyrosylserylmethionylglutamylhistidylphenylalanylarginyltryptophylglycyl–N^{ε}–formyllysylprolylvaline amide (VII) was purified by column chromatography on CM–cellulose. The homogeneity of this product was assessed by paper and thin–layer chromatographies and by amino acid analysis.

The [11–N^{ε}–formyllysine]–α–MSH derivative (VII) thus obtained was heated with 100 fold excess of aqueous hydrazine in a boiling water bath for 3 hr. Prior to heating, the pH of the solution was adjusted to 6 with acetic acid. Thioglycolic acid was used to prevent the possible oxidation of the methionine residue during this treatment. The resulting product (I–a) was purified by CM–cellulose using 0.025 M pyridine acetate buffer as an eluant. Chromatographic pattern indicated that very small amount of the starting material remained unchanged. Next, VII in aqueous pyridine was incubated with hydroxylamine hydrochloride at 50° for 48 hr. The product (I–b) was purified on CM–cellulose as stated above. Approximately 50% yield was obtained by both methods. Homogeneity of both products was demonstrated by paper and thin–layer chromatography. Their acid hydrolysates contained the constituent amino acids in the ratios predicted by theory. Destruction of tryptophan and partial decomposition of serine and tyrosine during acid hydrolysis is known.[39] Excellent recovery of methionine and arginine was also confirmed in both cases. The starting material (VII) and the deformylated products (I–a and b) were distinguishable by paper and thin–layer chromatographies and their mobilities on electrophoresis. In order to further identify the deformylated products, the peptides (I–a) and (I–b) were allowed to react with 1–fluoro–2,4–dinitrobenzene according to Sanger[40] respectively. The resulting products were hydrolyzed with 6 N hydrochloric acid and the hydrolysates were analyzed by an automatic amino acid analyzer. No lysine peak was detected in both cases, indicating that the formyl group on the ε–amino group of the lysine residue in the peptide had been removed by hydrazine or hydroxylamine. Smyth[41] demonstrated that the N^{α}–acetyl group of its oxytocin derivative was stable toward the action of hydroxylamine. When N^{α}–acetylseryltyrosylserylmethionine

35) K. Hofmann, T.A. Thompson, H. Yajima, E.T. Schwartz, and H. Inouye, *J. Am. Chem. Soc.*, **82**, 3715 (1960).
36) K. Hofmann, A. Jöhl, A.E. Furlenmeier, and H. Kappeler, *J. Am. Chem. Soc.*, **79**, 1636 (1957).
37) Sigma Chem. Co. Lot. 94B–1520.
38) Crude acylase was prepared from Takagiastase according to Y. Nadai, *J. Biochem.* (Tokyo), **45**, 687 (1958).
39) C.H.W. Hirs, W.H. Stein, and S. Moore, *J. Biol. Chem.*, **211**, 941 (1954).
40) F. Sanger, *Biochem. J.*, **39**, 507 (1945).
41) D.G. Smyth, *J. Biol. Chem.*, **242**, 1592 (1967).

(V) was exposed to hydrazine acetate or hydroxylamine hydrochloride under the identical conditions as stated above, the acetyl group remained uncleaved from the peptide. Therefore, it seems reasonable to assume that the N^{α}-acetyl group of VII was not cleaved by the above treatments.

The synthetic peptide amide (I-a and b) prepared by the two methods were compared with natural α-MSH of porcine origin supplied by Dr. S. Lande of Yale University. No difference among these three compounds were observed on paper and thin-layer chromatography. Their electrophoretic mobilities in pyridine acetate buffers at two different pH values were also identical. The IR spectra of the three compounds were identically superimposed as shown in Fig. 1. From these results, it can be concluded that our synthetic compounds are identical with natural α-MSH. The rotation figure of our synthetic were compounds also very close to that of the synthetic α-MSH of Schwyzer, *et al.*[23]

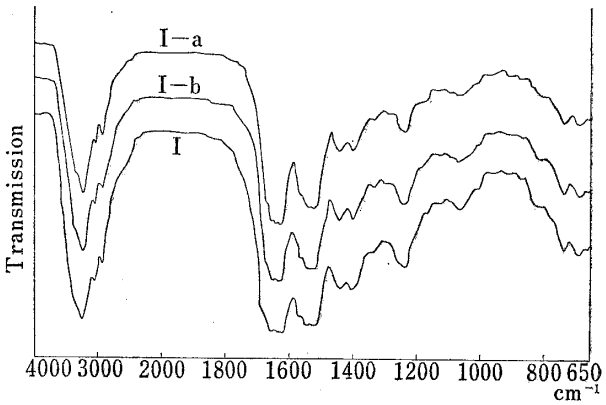

Fig. 1. Infrared Spectra of Natural and Synthetic α-Melanocyte-stimulating Hormone (MSH) in Solid State (KBr)

I, natural α-MSH. I-a, synthetic α-MSH (Prepared by NH_2NH_2 treatment). Ib, synthetic α-MSH (Prepared by NH_2OH treatment). In order to avoid overlapping the three lines in the chart, scale of transmission was omitted.

The bioassays were conducted according to Shizume, *et al.*[42] at the laboratory of Dr. S. Lande using frog skins from *Rana pipiens* and the results are listed in Table I. The

Table I. The MSH Activities of Synthetic Peptides

Synthetic peptides	*in vitro* MSH activity U/g
H–His. Phe. Arg. Trp. Gly. Lys(CHO). Pro. Val–NH_2	2.0×10^4
H–Glu. His. Phe. Arg. Trp. Gly. Lys(CHO). Pro. Val–NH_2	1.4×10^7
Ac–Ser. Tyr. Ser. Met. Glu. His. Phe. Arg. Trp. Gly. Lys(CHO). Pro. Val–NH_2	2.5×10^9, 2.0×10^{10}
α-MSH prepared by NH_2NH_2 acetate treatment	2.3×10^{12}, 5.4×10^{12}
α-MSH prepared by $NH_2OH\cdot HCl$ treatment	2.4×10^{12}, 4.1×10^{12}

Synthetic α-MSH. lit.[22] 2000 U/mg *in vivo*. lit.[23] $1.2—4\times10^{10}$ MSH U/g
Natural α-MSH. lit[19] 2.0×10^{10} MSH U/g lit.[43] 2.2×10^{12} MSH U/g

synthetic [11-N^{ε}-formyllysine]-α-MSH (VII) exhibited the *in vitro* activity of $0.25—2.0\times10^{10}$ MSH U/g equivalent to that of the structurally related peptide, N^{α}-acetylseryltyrosylseryl-methionylglutaminylhistidylphenylalanylarginyltryptophylglycyl-N^{ε}-formyllysylprolylvaline amide.[5] From this result, it becomes apparent that glutamic acid and glutamine residue at position 5 of these molecules are biologically equivalent as far as this level of MSH activity is concerned.

The two samples of synthetic acetyltridecapeptide amides (I-a and b), equivalent to natural α-MSH, derived from its formyl derivative by hydrazine or hydroxylamine exhibited the *in vitro* activity of $2.3—5.4\times10^{12}$ and $2.4—4.1\times10^{12}$ MSH U/g respectively. These values are nearly equivalent to that of the best α-MSH preparation from natural source[43]

42) K. Shizume, A.B. Lerner, and T.B. Fitzpatrick, *Endocrinology*, **54**, 553 (1954).
43) S. Lande, A.B. Lerner, and G.V. Upton, *J. Biol. Chem.*, **240**, 4259 (1965).

but higher than that of the old standard preparation[19] and those of the synthetic peptides ever recorded in the literatures.[22,23]

When the structures and the activities of N^{α}–acetyl–N^{ε}–formyllysine–tridecapeptide amide (VII) and the synthetic α–MSH (I-a and b) are compared, it can be seen that approximately one hundred fold increase of activity can be attributed to the removal of the formyl group at the ε–amino group of the lysine residue. From this observation, it becomes apparent that the free ε–amino group of the lysine residue is perhaps not essential for MSH activity but is needed to exert the full biological activity. This was not clearly show in the synthetic intermediate peptides which possess relatively low MSH activity. The role of the ε–amino group of lysine in α–MSH may be to serve as a *binding site* between the peptide hormone and the receptor site on the cell. Such indication was recently demonstrated by Stouffer and Watters[44] who treated natural α–MSH with trifluoroacetic acid anhydride. Considerable inactivation occurred and it was suggested that selective trifluoroacetylation of the ε–amino group of the lysine residue in the peptide had taken place.

The experimental results reported herein show that α–MSH can be prepared unequivocally from its N^{ε}–formyl derivative by treatment with hydrazine acetate or hydroxylamine hydrochloride in pyridine. The usefulness of N^{ε}–formyllysine for the synthesis of complex peptides with biological activities has been demonstrated.

Experimental

The general experimental methods are essentially the same as described in the part IV[45] of this series. Amino acid composition of the acid and enzymatic hydrolysates was determined by the method of Moore, Spackmann and Stein.[46] Rf^1 and Rf^2 values refer to the systems of *n*–BuOH, AcOH, H_2O (4:1:5) and *sec*–BuOH, 3% NH_4OH (3:1) on paper chromtography respectively. Rf^2 values are expressed as a multiple of distance traveled by L–Phe under identical conditions. Rf^3 and Rf^4 values refer to the systems of *n*–BuOH, pyridine, AcOH, H_2O (4:1:1:2) and (30:20:6:24) on silica (Kieselgel G, Merck) thin–layer plate respectively. Infrared spectra were determined with Hitachi EPI–S spectrophotometer. NMR spectra were measured at 60 Mc on a Varian associates A–60 spectrometer.

Treatment of N^{ε}-Formyllysine with Hydrazine Acetate——To a solution of N^{ε}–formyllysine (1.50 g) in H_2O (40 ml), 80% hydrazine hydrate (5.1 ml) was added and the pH of the solution was adjusted to 6 with AcOH (5.5 ml). After the solution was heated in a boiling water–bath for 3 hr, the solvent was evaporated and the residue was repeatedly lyophilized. To this residue, 1 N HCl (9 ml) was added and the pH of the solution was adjusted to 7 with pyridine. The solvent was evaporated. Addition of EtOH afforded a crystalline solid, which was recrystallized from H_2O and EtOH; yield 1.27 g (82%), mp 263°, $[M]_D^{29}$ +35.1° (c=2.0, 5 N HCl). (lit.[47] lysine monohydrochloride, mp 263—264°, $[M]_D$ +30.4° in 5 N HCl). Identity of this product with lysine monohydrochloride was confirmed by mixed mp and by comparison of their IR spectra (in KBr). *Anal.* Calcd. for $C_6H_{14}O_2N_2 \cdot HCl$: C, 39.5; H, 8.3; N, 15.3. Found: C, 39.2; H, 8.3; N, 15.2.

Treatment of N^{ε}-Formyllysine with Hydroxylamine Hydrochloride——To a solution of N^{ε}–formyllysine (1.50 g) in 50% aqueous pyridine (30 ml), hydroxylamine hydrochloride (5.91 g) was added and the solution was stirred at 40° for 20 hr. Paper chromtographic examination of the aliquot revelaed the presence of single spot with Rf^1 0.12. The starting material with Rf^1 0.26 completely disappeared. The solvent was evaporated *in vacuo* and the residue was recrystallized twice from MeOH; yield 1.26 g (81%), mp 264°, $[M]_D^{31}$ +40.3° (c=2.0, 5 N HCl). The IR spectrum of this product (in KBr) was quite identical with that of the authentic sample of lysine monohydrochloride. In the NMR spectrum, the proton signal of CHO (τ 1.98) of the starting material was absent. *Anal.* Calcd. for $C_6H_{14}O_2N_2 \cdot HCl$: C, 39.5; H, 8.3; N, 15.3. Found: C, 39.0; H, 8.6; N, 15.2.

N^{α}-Benzyloxycarbonylseryltyrosylserylmethionine Monohydrate——The entire operation was carried out in a cold room at 4°. N^{α}–Benzyloxycarbonylseryltyrosine hydrazide[35] (8.33 g) was dissolved in 1 N HCl (50 ml) and the solution was cooled in an ice–bath. A solution of $NaNO_2$ (2.07 g) in H_2O (3 ml) was added and the ensuing solid azide was collected by filtration and washed with a saturated soltuion of $NaHCO_3$

44) J.E. Stouffer, and J.A. Watters, Jr., *Biochim. Biophys. Acta*, **104**, 214 (1965).
45) H. Yajima and K. Kubo, *Chem. Pharm. Bull.* (Tokyo), **13**, 759 (1965).
46) S. Moore, D.H. Spackmann, and W.H. Stein, *Anal. Chem.*, **30**, 1185 (1958).
47) J.P. Greenstein and M. Winitz, "Chemistry of the Amino Acids," Vol. III, John Wiley & Sons, Inc., New York, 1961, p. 2097.

and H_2O. This solid azide was added to an ice-cold solution of serylmethionine[36] (4.73 g) and triethylamine (2.8 ml) in 90% dimethylformamide (DMF, 100 ml). After the solution was stirred for 24 hr, an additional azide (prepared from 8.33 g of the hydrazide) was added and the stirring was continued for an additional 24 hr. The solvent was evaporated *in vacuo* and the oily residue was dissolved in 5% NH_4OH, which was washed quickely with AcOEt. The aqeuous phase was acidified with 2 N HCl under ice-cooling and the resulting precipitate was collected. This precipitation procedure was repeated once and the product was recrystallized from MeOH and AcOEt; yield 5.83 g (47%), mp 194—196°, $[\alpha]_D^{20}$ −7.7° (c=1.0, DMF). *Anal.* Calcd. for $C_{28}H_{36}O_{10}H_4S \cdot H_2O$: C, 52.7; H, 6.0; N, 8.8. Found: C, 52.4; H, 5.7; N, 8.7.

Seryltyrosylserylmethionine One and One-half Hydrate (V)——N^{α}-Benzyloxycarbonylseryltyrosylserylmethionine (0.31 g) was dissolved in liquid NH_3 (approximately 50 ml) and Na (approximately 0.10 g) was added in small pieces with stirring until the permanent blue color remained. Dowex 50×8 (ammonium cycle, 3.0 g) was added and the NH_3 was allowed to evaporate at room temperature. A slow stream of N_2 was passed over the residue to remove the last trace of NH_3. The product was extracted with several portions of H_2O and the extracts were combined, filtered and lyophilized. The ensuing powder was dissolved in H_2O. The solution was filtered, the pH of the clear filtrate was adjusted to 6.0 with dilute AcOH and EtOH (about three times of the volume of the solution) was added. The mixture was stored in a refrigerator overnight and the resulting crystalline precipitate was collected and recrystallized from H_2O and EtOH; yield 0.11 g (43%), mp 201—204°, $[\alpha]_D^{25}$ −9.5° (c=1.0, H_2O); Rf^1 0.41, Rf^2 0.70×Phe, single spot positive to ninhydrin, Pauly and methionine tests. *Anal.* Calcd. for $C_{20}H_{30}O_8N_4S \cdot 1.5H_2O$: C, 46.8; H, 6.5; N, 10.9. Found: C, 46.9; H, 6.6; N, 10.9.

N^{α}-Acetylseryltyrosylserylmethionine Monohydrate (VI)——Acetic anhydride (0.3 ml) was added with stirring into an ice-cold solution of seryltyrosylserylmethionine (4.00 g) and $NaHCO_3$ (0.50 g) in H_2O (30 ml). After the mixture was stirred for 3.5 hr, the solvent was removed by lyophilization. The residue dissolved in H_2O (60 ml) was treated with 1 N NaOH (2 ml) for 10 min in an ice-bath. Dowex 50×8 (H^+ form, 6 g) was added to the solution, which was stirred for 10 min. During this period, the pH of the solution reached approximately to 4. The resine was removed by filtration and the filtrate was condensed *in vacuo*. The resulting gel was collected and recrystallized from H_2O; yield 1.05 g(95%), mp 192—194°, $[\alpha]_D^{28}$ −40.0° (c=0.5, 10% AcOH), Rf^1 0.71, Rf^2 0.79×Phe, singel spot positive to Pauly and methionine tests and negative to ninhydrin; amino acid ratios in carboxypeptidase A digestion $Ser_{1.00}$ $Tyr_{0.87}$ $Met_{1.09}$ (average recovery 100%). The peptide (2.6 mg) was treated with acylase according to Nadai.[38] Paper chromatographic examination of the hydrolysate showed the presence of a single spot positive to ninhydrin, Pauly and methionine tests with Rf^1 0.41, identical with that of seryltyrosylserylmethionine. *Anal.* Calcd. for $C_{22}H_{32}O_9N_4S \cdot H_2O$: C, 48.3; H, 6.3; N, 10.5. Found: C, 48.2; H, 6.3; N, 10.2.

N^{α}-Acetylseryltyrosylserylmethionine Methyl Ester——An ethereal solution of diazomethane was added to an ice-cold solution of N^{α}-acetylseryltyrosylserylmethionine (1.06 g) in MeOH (80 ml) until the yellow color remained and the mixture was kept for 5 min. The excess diazomethane was destroyed by addition of a few drops of glacial AcOH and the solution was evaporated to dryness to give a solid, which was recrystallized from MeOH; yield 0.70 g (70%), mp 161—165° (lit.[23] mp 200—211° decomp.), $[\alpha]_D^{20}$ −45.8° (c=0.7, 10% AcOH); Rf^1 0.80, Rf^2 1.00×Phe. *Anal.* Calcd. for $C_{23}H_{34}O_9N_4S$: C, 50.9; H, 6.3; N, 10.3. Found: C, 50.6; H, 6.6; N, 10.3.

N^{α}-Acetylseryltyrosylserylmethionine Hydrazide Dihydrate (IV)——To a solution of N^{α}-acetylseryltyrosylserylmethionine methyl ester (2.60 g) in MeOH (70 ml), 80% hydrazine hydrate (1.25 ml) was added. The reaction mixture was left stand at room temperature overnight. The gelatinous product was collected by filtration and recrystallized from MeOH; yield 2.50 g (96%), mp 222—225°, (lit.[23] mp 256°), $[\alpha]_D^{29}$ −61.5° (c=0.5, 10% AcOH). Rf^1 0.69, Rf^2 1.21×Phe, ninhydrin negative, single spot positive to Pauly and methionine tests; amino acid ratios in acid hydrolysate $Ser_{2.02}$ $Tyr_{1.00}$ $Met_{0.99}$ (average recovery 90%). *Anal.* Cacld. for $C_{22}H_{34}O_8N_6S \cdot 2H_2O$: C, 45.7; H, 6.6; N, 14.5. Found; C, 45.9; H, 7.0; N, 14.9.

Treatment of N^{α}-Acetylseryltyrosylserylmethionine with Hydrazine Acetate or Hydroxylamine Hydrochloride——N^{α}-Acetylseryltyrosylserylmethionine (10 mg each) was exposed to the action of hydrazine acetate or hydroxylamine hydrochloride in pyridine under similar conditions described in N^{ε}-formyllysine. Each solution was examined by paper chromatography. In both cases, no extra spot besides the original N^{α}-acetyltetrapeptide (Rf^1 0.71) was detected.

Histidylphenylalanylarginyltryptophylglycyl-N^{ε}-formyllysylprolylvaline Amide Diacetate Tetrahydrate (II)——a) By the DCC method: To a solution of N^{ε}-benzyloxycarbonylhistidylphenylalanyl-N^{G}-nitroarginyltryptophylglycine (1.85 g) and N^{ε}-formyllysylprolylvaline amide (prepared from 1.30 g of the hydrochloride[7] and 0.42 ml of triethylamine) in DMF (30 ml), was added DCC (1.24 g). After the mixture was stirred at room temperature overnight, the solvent was evaporated *in vacuo* and the residue was treated with AcOEt. The resulting soild was collected by filtration and washed with AcOEt and H_2O. This product in 60% AcOH (100 ml) was hydrogenated over a Pd catalyst at 40° for 18 hr. After filtration, the filtrate was evaporated. The residue was dried over KOH pellets *in vacuo* and then dissoved in H_2O (400 ml). The solution was applied to a column of CM-cellulose (3×25 cm), which was eluted with the following pH 6.9 ammonium acetate buffers; 0.02 M (1000 ml), 0.05 M (4000 ml) and 0.075 M (100 ml). Individual fractions,

18 ml each, were collected and absorbancy at 280 mμ located the desired compound in the 0.05 M eluates (tubes 67—200), which were pooled. The solvent was evaporated and the concentrate was lyophilized to constant weight, giving a fluffy white powder; yield 1.21 g (49%), $[\alpha]_D^{30}$ −38.0° (c=0.7, 10% AcOH), (lit.[7] $[\alpha]_D^{25}$ −41.2° in 10% AcOH), Rf^1 0.54, single ninhydrin, Pauly, Sakaguchi and Ehrlich positive spot; amino acid ratios in leucine amino peptidase (LAP)[45] digest $His_{1.03}Phe_{1.00}Arg_{0.87}Try_{1.14}Gly_{0.97}$ N^{ε}–formyllys$_{0.93}$ $Pro_{0.97}Val_{1.07}$ (average recovery 87%). *Anal.* Calcd. for $C_{51}H_{72}O_9N_{16}\cdot 2CH_3COOH\cdot 4H_2O$: C, 56.3; H, 6.9; N, 19.1. Found: C, 55.9; H, 7.4; N, 19.1.

b) By the N–hydroxysuccinimide method: N^{α}–Benzyloxycarbonylhistidylphenylalanyl–N^{G}–nitroarginyltryptophylglycine (2.78 g) and N^{ε}–formyllysylprolylvaline amide hydrochloride (1.66 g) were dissolved in DMF (35 ml) and triethylamine (0.52 ml) was added followed by N–hydroxysuccinimide[25] (0.69 g) and DCC (1.24 g). The solution was stirred at room temperature overnight and then the solvent was evaporated. The oily residue turned to the solid by treatment with AcOEt. This solid, after washing with AcOEt and H_2O, was hydrogenated in 60% AcOH (100 ml). Subsequent purification of the desired compound was carried out in essentially the same manner as described in (a); yield 1.55 g (41%), $[\alpha]_D^{29}$ −41.7° (c=0.6, 10% AcOH), Rf^1 0.54.

c) By the Woodward reagent: N–Ethyl–5–phenylisoxazolium–3′–sulfonate[26] (Woodward reagent, 0.25 g) was added to a solution of N^{α}–benzyloxycarbonylhistidylphenylanalnyl–N^{G}–nitroarginyltryptophylglycine (0.93 g) and triethylamine (0.14 ml) in DMF (30 ml). When this reagent went into the solution, N^{ε}–formyllysylprolylvaline amide (prepared from 0.43 g of the hydrochloride and 0.14 ml of triethylamine) in DMF (5 ml) was combined. The solution was stirred at room temperature for 40 hr and then the solvent was evaporated. By addition of H_2O, the residue turned to the solid, which was collected by filtration. Hydrogenation of the product and subsequent purification of the desired compound were carried out in essentially the same manner as described in (a); yield 0.21 g (16%), Rf^1 0.54, $[\alpha]_D^{30}$ −34.0° (c=0.5, 10% AcOH). During the purification procedure, besides histidylphenylalanylarginyltryptophylglycine (0.39 g), another peak (0.15 g) was separated (This acid hydrolysate contained only His, Phe, Arg and Gly).

Glutamylhistidylphenylalanylarginyltryptophylglycyl-N^{ε}-formyllysylprolylvaline Amide Acetate (III)——

a) By the *p*–nitrophenyl ester method: Histidylphenylalanylarginyltryptophylglycyl–N^{ε}–formyllysylprolylvaline amide acetate (0.53 g) was dissolved in H_2O (30 ml) and 1 N HCl (1.2 ml) was added. The solution was lyophilized to give a fluffy powder, which was dissolved in DMF (25 ml). To this solution, triethylamine (0.13 ml) and N^{α}–benzyloxycarbonyl–γ–benzylglutamate *p*–nitrophenyl ester[48] (0.48 g) were added consecutively. After the mixture was stirred at room temperature for 20 hr, the solvent was evaporated *in vacuo* and ether was added to the residue to give a solid which was collected by filtration. This solid in a mixture of MeOH (50 ml) and 80% AcOH (5 ml) was hydrogenated over a Pd catalyst for 8 hr. The catalyst was removed by filtration and the filtrate was evaporated. The residue, after drying over KOH pellets, was dissolved in H_2O (300 ml) and the solution was applied to a column of CM–cellulose (3 × 13 cm), which was eluted with H_2O (1000 ml) and then the following pH 6.9 ammonium acetate buffers: 0.01 M (750 ml), 0.02 M (800 ml), 0.03 M (2000 ml) and 0.05 M (600 ml). Individual fractions, 19 ml each, were collected and absorbancy at 280 mμ located the desired material in the 0.03 M eluates (tube 150—222), which were pooled. The bulk of the solvent was removed *in vacuo* and the concentrate was lyophilized to constant weight, giving a colorless fluffy powder; yield 0.47 g (88%), $[\alpha]_D^{22}$ −40.9° (c=0.5, 10% AcOH); Rf^1 0.34, Rf^3 0.55, singlet spot positive to ninhydrin, Pauly, Sakaguchi and Ehrlich tests, a little contamination of ammonium acetate interfered to obtain the reproducible *Rf* value of this peptide; amino acid ratios in acid hydrolysate, $Glu_{1.03}His_{1.06}Phe_{1.00}Arg_{0.98}Gly_{1.03}Lys_{0.95}Pro_{0.86}Val_{0.88}$ (Trp was destroyed, average recovery 84%); amino acid ratios in LAP digest, $Glu_{0.43}His_{0.97}Phe_{1.00}Arg_{0.84}Trp_{0.87}Gly_{1.03}ForLys_{0.93}Pro_{1.03}Val_{1.09}$. (average recovery 94%. Low recovery of glutamic acid is due to the formation of pyroglutamic acid). *Anal.* Calcd. for $C_{56}H_{79}O_{12}N_{17}\cdot CH_3COOH$: C, 56.1; H, 6.7; N, 19.2. Found: C, 56.2; H, 6.5; N, 19.6. (The sample was dried at 100° for 4 hr).

b) By the DCC method: Histidylphenylalanylarginyltryptophylglycyl–N^{ε}–formyllysylprolylvaline amide (0.31 g) was converted to its hydrochloride as described above. This hydrochloride in DMF (15 ml) containing triethylamine (0.07 ml) was allowed to react with N^{α}–benzyloxycarbonyl–γ–benzylglutamate (0.15 g) by DCC (0.16 g). The reaction and subsequent purification procedure were essentially the same as described in (a); yield 0.16 g (53%), $[\alpha]_D^{29}$ −41.2° (c=0.5, 10% AcOH), Rf^1 0.34, Rf^3 0.55, single spot positive to ninhydrin, Pauly, Sakaguchi, and Ehrlich tests; amino acid ratios in acid hydrolysate $Glu_{0.83}His_{0.92}Phe_{1.00}$ $Arg_{1.03}Gly_{0.92}Lys_{0.86}Pro_{0.89}Val_{0.88}$ (average recovery 100%).

c) By the mixed anhydride method: A mixed anhydride, prepared from N^{α}–benzyloxycarbonyl–γ–benzylglutamate (0.23 g) in ice–cold tetrahydrofuran (4 ml) with triethylamine (0.08 ml) and ethyl chloroformate (0.06 ml), was added to a solution of histidylphenylalanylariginyltryptophylglycyl–N^{ε}–formyllysylprolylvaline amide acetate (0.25 g) and triethylamine (0.03 ml) in DMF (8 ml). After the solution was stirred in an ice–bath for 3 hr the solvent was evaporated. The residue was treated with ether and the resulting solid was hydrogenated in a solution of 30% AcOH (28 ml). The product was purified by column

48) G. Losse, H. Jeschkeit, and W. Langenbeck, *Chem. Ber.*, 96, 204 (1963).

chromatography on CM-cellulose as described in (a). An unsymmetical peak was detected in the 0.02 M eluates; yield 0.21 g (85%), Rf^1 0.34, ninhydrin, Pauly, Sakaguchi and Ehrlich positive spot with contamination of a substance of Rf^1 0.44, ninhydrin negative, Pauly, Sakaguchi and Ehrlich positive.

N^{α}-Acetylseryltyrosylserylmethionylglutamylhistidylphenylalanylariginyltryptophylglycyl-N^{ε}-formyllysylprolylvaline Amide Acetate Hexahydrate (VII)——A solution of $NaNO_2$ (0.08 g) in H_2O (2ml) was added to an ice-cold solution of N^{α}-acetylseryltyrosylserylmethionine hydrazide (0.58 g) in 0.5 N HCl (6 ml). The solution was stirred in an ice-bath for 5 min and the pH of the solution was adjusted to 8 with triethylamine. This solution was combined to a solution of glutamylhistidylphenylalanylarginyltryptophylglycyl-N^{ε}-formyllysylprolylvaline amide acetate (0.50 g) in 95% pyridine (14 ml) containing triethylamine (0.06 ml). The solution was stirred for 44 hr and the solvent was removed by lyophilization. The residue was dissolved in H_2O (300 ml) and the solution was applied to a colunm of CM-cellulose (2×12 cm) which was eluted with first H_2O (1300 ml) and then the following pH 6.9 ammonium acetate buffers: 0.01 M (2200 ml), 0.025 M (850 ml) and 0.04 M (1000 ml). Individual fractions, 17 ml each, were collected and absorbancy at 280 mμ located the desired material in the 0.01 M eluates (tube 123—188), which were pooled. The solvent was removed by evaporation and the residue was lyophilized to constant weight, giving a colorless fluffy powder; yield 0.38 g (50%), $[\alpha]_D^{24}$ −46.4° (c=0.3, 30% AcOH), Rf^1 0.59, Rf^2 1.40×Phe, Rf^3 0.66, single spot positive to Pauly, methionine, Sakaguchi and Ehrlich tests; singlet spot on paper electrophoresis (25 v/cm 2.5 hr) at pH 5.0 (mobility −6.3 cm) and 6.1 (mobility −4.3 cm) in 0.1 M pyridine acetate buffers; amino acid ratios in acid hydrolysate $Ser_{1.70}Tyr_{0.95}Met_{0.93}Glu_{1.11}His_{0.93}Phe_{1.00}Arg_{0.87}Gly_{1.04}Lys_{0.89}Pro_{0.98}Val_{0.96}$ (Trp was destroyed, average recovery 100%). *Anal.* Calcd. for $C_{78}H_{109}O_{20}N_{21}S\cdot CH_3COOH\cdot 6H_2O$: C, 51.6; H, 6.9; N, 15.8. Found: C, 51.7; H, 6.8; N, 15.6.

N^{α}-Acetylseryltyrosylserylmethionylglutamylhistidylphenylalanylarginyltryptophylglycyllysylprolylvaline Amide Diacetate Decahydrate (I)——a) Deformylation by hydrazine acetate: N^{α}-Acetylseryltyrosylserylmethionylglutamylhistidylphenylalanylarginyltryptophylglycyl-N^{ε}-formyllysylprolylvaline amide (53 mg) was dissolved in H_2O (5 ml). To this solution, 80% hydrazine hydrate (0.18 ml) and thioglycolic acid (0.02 ml) were added. The pH of the solution was adjusted to 6 with AcOH and the air of the flask was replaced by nitrogen. After the solution was heated in a boiling water-bath for 3 hr, the solvent was evaporated *in vacuo*. The residue was dissolved in H_2O (250 ml) and the solution was applied to a column of CM-cellulose (1.5×16 cm), which was first eluted with H_2O (750 ml) and then with the following pH 6.9 ammonium acetate buffers: 0.01 M (450 ml), 0.025 M (450 ml) and 0.1 M (300 ml). Individual fractions, 15 ml each, were collected at a flow rate of 4 ml per min. Absorbancy of individual tubes was determined at 280 mμ. The 0.025 M eluates (tube 88—102) containing the desired product were pooled, the solvent was evaporated *in vacuo* and the concentrate was lyophilized to constant weight to give a colorless fluffy powder; yield 32 mg (55%), $[\alpha]_D^{24}$ −55.8° (c=0.5, 10% AcOH), (lit.[23] $[\alpha]_D^{25}$ −58.5° in 10% AcOH), Rf^1 0.42, Rf^2 1.15×Phe, single spot positive to Pauly, Sakaguchi, methionine and Ehrlich tests and positive to ninhydrin on heating; amino acid ratios in acid hydrolysate $Ser_{1.63}Tyr_{0.81}Met_{0.80}Glu_{0.98}His_{1.04}Phe_{1.00}Arg_{1.02}Gly_{1.00}Lys_{1.04}Pro_{1.02}Val_{1.00}$ (Trp was destroyed, average recovery 94%). *Anal.* Calcd. for $C_{77}H_{109}O_{19}N_{21}S\cdot 2CH_3COOH\cdot 10H_2O$: C, 49.5; H, 7.0; N, 15.0. Found: C, 49.0; H, 6.6; N, 14.3.

The peptide (2.19 mg) in 1% aqueous triethylamine (1 ml) was treated with 1-fluoro-2,4-dinitrobenzene (0.01 ml) at room temperature for 3 hr. The solution, after washing with ether, was evaporated. Recovery of the basic amino acids in the acid hydrolysate of this residue were His=0, Arg=44% and Lys=0.

When the starting material (53 mg) was treated with 30 equi-moles of hydrazine acetate in a boiling water-bath for 3 hr, yield of the desired product was 20 mg (35%) and 10 mg of the starting material was recovered.

b) Deformylation by hydroxylamine hydrochloride: To a solution of N^{α}-acetylseryltyrosylserylmethionylglutamylhistidylphenylalanylarginyltryptophylglycyl-N^{ε}-formyllysylprolylvaline amide (53 mg) was dissolved in 50% aqueous pyridine (2 ml). To this solution, hydroxylamine hydrochloride (21 mg) and thioglycolic acid (0.02 ml) were added and the solution was stirred at 50° for 40 hr. The solvent was evaporated *in vacuo* and the product was purified as described in (a); yield 29 mg (48%), $[\alpha]_D^{23}$ −58.3° (c=0.5, 10% AcOH); Rf^1 042, Rf^2 1.15×Phe, identical spot with that of the product obtained in (a); amino acid ratios in acid hydrolysate $Ser_{1.68}Tyr_{0.85}Met_{0.87}Glu_{0.96}His_{1.03}Phe_{1.00}Arg_{0.99}Gly_{0.97}Lys_{1.08}Pro_{1.01}Val_{1.01}$ (Trp was destroyed, average recovery 98%). *Anal.* Calcd. for $C_{77}H_{109}O_{19}N_{21}S\cdot 2CH_3COOH\cdot 10H_2O$: C, 49.5; H, 7.0; N, 15.0. Found: C, 49.5; H, 6.4; N, 14.7.

The peptide (2.10 mg) was treated with 1-fluoro-2,4-dinitrobenzene (0.01 ml) and the product was hydrolyzed by acid as described in (a). Recovery of the basic amino acids were His=0, Arg=48% and Lys=0.

Comparison of the Synthetic Peptides with Natural α-MSH——Synthetic peptides I-a and I-b were compared with natural α-MSH (2.2×10^{12} MSH U/g). They behaved quite identically on paper and thin-layer chromatography; Rf^1 0.42, Rf^2 1.15×Phe, Rf^3 0.49, Rf^4 0.67. Electrophoresis was conducted under the condition of 25 V/cm for 2 hr. Mobilities of these three compounds were identical; at pH 3.0 in 0.1 M AcOH, −0.6 cm, at pH 5.0 and 6.0 in 0.1 M pyridine acetate buffers, −6.7 cm and −10.5 cm respectively. The IR spectra of these compounds were determined in the KBr methods as shown in Fig. 1.

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