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Studies on Pituitary Polypeptide Hormones

3. THE STRUCTURE OF α -MELANOCYTE-STIMULATING HORMONE FROM PIG PITUITARY GLANDS

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α -Melanocyte-stimulating hormone, which appears to be the major active melanocyte-stimulating substance in the pig pituitary gland (Lerner & Lee, 1955), has been shown to be a highly basic polypeptide (isoelectric point 10.5–11.0), which is readily separable from β -melanocyte-stimulating hormone. The results of amino acid analysis (Lee & Lerner, 1956) suggested that the α -melanocyte-stimulating hormone molecule was composed of at least 37 amino acid residues, and that it contained several amino acids which were not present in β -melanocyte-stimulating hormone (Harris & Roos, 1956, 1959*a*; Geschwind, Li & Barnafi, 1956, 1957*a*).

Of the seventeen different constituent amino acids, alanine, aspartic acid, cystine, threonine and leucine occurred in relatively small amounts; if these five amino acids were considered to be integral constituents of α -melanocyte-stimulating hormone, then it would be necessary to assume that three lysine and two arginine residues were also present, and that consequently from four to six peptide fragments would be formed by hydrolysis of the molecule with trypsin. However, when the α -melanocyte-stimulating hormone preparation was submitted to the action of trypsin, only two major peptide fragments were produced, and these were found to contain all the amino acids present in the original undigested sample, with the exception of the five amino acids listed above; these were subsequently shown to occur exclusively in

relatively minor and non-stoichiometric products of the trypsin reaction.

This unexpected result suggested that the five amino acids, alanine, aspartic acid, cystine, threonine and leucine, were not integral constituents of the α -melanocyte-stimulating hormone molecule, and that they were in fact present in related peptide impurities which had not separated from the hormone under the conditions used for its isolation. When the results of amino acid analysis were re-examined in the light of this possibility, it was found that the following amino acid composition could be deduced for α -melanocyte-stimulating hormone on the basis of whole-number ratios of the other 12 constituent amino acids: Arg₁, Glu₁, Gly₁, His₁, Lys₁, Met₁, Phe₁, Pro₁, Ser₂, Try₁, Tyr₁, Val₁. According to this assumption α -melanocyte-stimulating hormone was likely to be a tridecapeptide, and a close structural similarity to both corticotrophin and β -melanocyte-stimulating hormone could readily be envisaged. It was therefore decided to proceed with structural studies without attempting any further purification of the very limited supply (10–12 mg.) of the hormone preparation which was available to us.

In this paper experiments which have led to the elucidation of the structure of α -melanocyte-stimulating hormone are described, and its chemical and biological relationships to both β -melanocyte-stimulating hormone and corticotrophin are discussed. Preliminary accounts of these findings have already been published elsewhere (Harris & Lerner, 1957; Harris, 1958).

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MATERIALS

The sample of α -melanocyte-stimulating hormone (α -MSH) which was used in the present investigation was prepared by Dr Teh Lee at the Yale University School of Medicine, by a procedure which has already been described (Lee & Lerner, 1956). The crystalline enzymes trypsin and chymotrypsin were kindly donated by the Novo Therapeutisk Laboratorium, Copenhagen, and a highly purified preparation of crystalline carboxypeptidase was obtained through the courtesy of The Armour Laboratories, Chicago, U.S.A.

METHODS

Amino acid composition. The amino acid composition of α -MSH was determined by the procedure of Moore & Stein (1954). We are indebted to Mr M. W. Rees and Miss B. D. Worboys for carrying out this analysis.

N- and C-terminal groups. The N-terminal group in α -MSH was investigated by means of a modification (Fraenkel-Conrat, Harris & Levy, 1955) of the phenyl isothiocyanate method (Edman, 1950). An aqueous solution of α -MSH (1.6 mg.; 0.8–1.0 μ mole) was applied to a strip of Whatman no. 1 filter paper (6 cm. $\times$ 1.5 cm.) and caused to react with phenyl isothiocyanate (20 μ l. of 10% soln. in dioxan) in an atmosphere of aqueous pyridine, at 40° for 3 hr. The paper strip containing the phenylthio-carbamyl derivative of α -MSH was washed with benzene and ethanol-ether (1:1, v/v), and was then placed in an evacuated desiccator containing acetic acid and 6N-HCl for 8 hr. at room temp., in order to form the phenylthio-hydantoin derivative of the N-terminal amino acid. The paper strip was again extracted with ethanol-ether (1:1, v/v) and the extract was submitted to chromatography on paper as described by Harris & Roos (1959a).

The N-terminal group in α -MSH was also investigated by the fluorodinitrobenzene method (Sanger, 1945), according to the modified procedure described in Fraenkel-Conrat *et al.* (1955).

Reaction with carboxypeptidase. α -MSH (0.8 mg.; approx. 0.5 μ mole) was dissolved in 0.1% NaHCO₃ (0.5 ml.) and caused to react with carboxypeptidase (0.05 mg.) at pH 8.0–8.5 and 30° for 12 hr. The reaction mixture was acidified to pH 4.0, concentrated *in vacuo* over KOH and submitted to chromatography in butanol-acetic acid-water (4:1:5, by vol.).

*Enzymic degradation of
 α -melanocyte-stimulating hormone*

Action of trypsin. α -MSH (2.8 mg.; 0.2% soln.) was digested with trypsin (0.02 mg.) at pH 8.5 and 30°. A constant pH was maintained by means of a pH-stat (Jacobsen, Léonis, Linderstrøm-Lang & Ottesen, 1957) and the course of the reaction was followed by measuring the uptake of alkali as a function of time. After 30 min. the solution was acidified to pH 4.0 and evaporated to dryness *in vacuo* over KOH.

Action of chymotrypsin. α -MSH (4.4 mg.; 0.2% soln.) was digested with chymotrypsin (0.05 mg.) at pH 8.5 and 30°. The reaction was followed in the pH-stat as described above. A sample (1 ml.) was withdrawn after 30 min. and acidified to pH 3.5 to prevent further enzyme action; the remainder was allowed to react for 16 hr. The two samples were then concentrated to dryness *in vacuo* over KOH.

Isolation and characterization of peptide fragments. The peptide fragments derived from α -MSH by reaction with trypsin and chymotrypsin were fractionated, by ionophoresis on paper in pyridine-acetic acid buffers of pH 3.5 and pH 6.5 and by chromatography on paper in butanol-acetic acid-water (4:1:5, by vol.), as described in detail by Harris & Roos (1959a). The presence of tryptophan, histidine and tyrosine residues in chromatographically pure peptide fragments was investigated by means of specific colour tests (Block, Durrum & Zweig, 1955), and the complete amino acid composition of each peptide was determined by chromatography of their acid hydrolysates (6N-HCl; 18 hr.; 100–105°) in butanol-acetic acid-water (4:1:5, by vol.) and phenol-borate buffer, pH 9.3 (Levy & Chung, 1953).

Structural studies on peptide fragments. N-terminal group and sequence studies on peptides isolated from the chymotryptic digest of α -MSH were carried out by a modification (Fraenkel-Conrat *et al.* 1955) of the phenyl isothiocyanate method; the corresponding C-terminal amino acids were investigated by the carboxypeptidase method as described by Harris & Roos (1959a).

Partial acid hydrolysis. Peptide fragments produced by chymotryptic digestion of α -MSH were submitted to partial acid hydrolysis in 12N-HCl at 37° for 3–4 days. The peptide mixtures produced in this manner were separated by ionophoresis and chromatography on paper and characterized as described above.

Identification of the N-acyl substituent. N-acylamino acid and peptide derivatives were revealed on paper chromatograms and ionograms by means of the Pauly & Rydon (Block *et al.* 1955) and *tert.*-butyl hypochlorite (Schwartz & Pallansch, 1958) reagents. The N-terminal peptide (N-acylseryltyrosine, approx. 0.5 μ mole), isolated from a chymotryptic digest of α -MSH, was also hydrazinolysed (Niu & Fraenkel-Conrat, 1955) by heating in a sealed tube with anhydrous hydrazine (0.5 ml.) for 10 hr. at 100°. Excess of hydrazine was removed *in vacuo* over conc. H₂SO₄ and the products of hydrazinolysis were investigated by chromatography on paper in pyridine-aniline-water (9:1:4, by vol.) and in collidine-water (10:2, v/v) (H. Fraenkel-Conrat, personal communication).

RESULTS

Amino acid composition. A portion (2.4 mg.) of the α -MSH preparation was hydrolysed under reflux with 5 ml. of 6N-HCl for 24 hr. and the hydrolysate was analysed for constituent amino acids on a Dowex 50 column by the method of Moore & Stein (1954). Tryptophan was estimated spectrophotometrically (Goodwin & Morton, 1946), and amide nitrogen was determined by the micro-Conway method. The results are summarized in Table 1. From 2.4 mg. of the sample of α -MSH which was taken for hydrolysis, only 1.4 mg. was recovered from the column in terms of amino acids (anhydrous residues) and ammonia. This represents a recovery of approx. 60% in terms of α -MSH peptide and shows that the preparation of α -MSH under study contained up to 40% of non-peptide material (cf. Lee & Lerner, 1956).

N- and C-terminal groups. No free *N*-terminal group could be detected when the phenyl isothiocyanate and fluorodinitrobenzene methods were applied to α -MSH, and no free amino acids were released when it was caused to react with carboxypeptidase. The absence of end groups suggested the possibility of a *cyclopeptide* structure; alternatively it would be necessary to assume that the α -NH₂ group was not free to react with the end-group reagents, and that the *C*-terminal amino acid did not conform to the specificity requirements of carboxypeptidase (Harris, 1955).

Action of trypsin. When α -MSH (approx. 1 μ mole, based on peptide content) was digested with trypsin at pH 8.5 and 30° in the pH-stat, a very rapid reaction was observed; approx. 1 μ mole of base was taken up during the first 5 min. and the reaction appeared to be complete after 30 min. when 1.2 μ moles of base had been consumed. When the digest was submitted to ionophoresis at pH 6.5, it was resolved into two major components, T₁ and T₂ (Fig. 1); three other components were also present in relatively small amounts, but it was not found possible to characterize them completely. It was established, however, that alanine, aspartic acid, cystine, threonine and leucine occurred exclusively in these three fragments, which were presumably derived by tryptic degradation of peptide impurities present in the α -MSH preparation. On the other hand, the amino acids present in peptides T₁ and T₂ (Table 2) accounted for all the amino acids which were assumed to be present in the tridecapeptide structure proposed for α -MSH (Table 1).

Action of chymotrypsin. When a sample of α -MSH (4.4 mg., containing 2.6 mg., approx. 1.5 μ moles, of α -MSH peptide) was caused to react with chymotrypsin at pH 8.5 and 30°, approx. 2 μ moles of alkali were consumed/ μ mole of substrate during the first 30 min. Thereafter the rate of uptake of alkali became very slow, and after 16 hr., when the reaction appeared to be virtually complete, 2.6–2.8 μ moles of alkali had been consumed/ μ mole of substrate. This result suggested that two peptide bonds in α -MSH were hydrolysed rapidly, and that this initial reaction was followed by a much slower hydrolysis of a third bond in the molecule. Samples of the digest (equivalent to 0.1 μ mole of α -MSH)

were withdrawn after 30 min. and 16 hr. respectively, and by ionophoresis at pH 6.5 the peptide fragments illustrated in Fig. 2 were obtained. Peptide C₁, which did not stain with ninhydrin, gave a positive tyrosine reaction. Peptides C₁, C₃, C₄ and C₅ appeared to be pure by chromatography in butanol-acetic acid-water (4:1:5, by vol.), but C₂ was resolved into two components: C_{2a} and C_{2b}. Samples of the chromatographically pure peptides were analysed for constituent amino acids and for *N*- and *C*-terminal groups, and the results obtained are summarized in Table 3.

Table 1. *Amino acid composition of α -melanocyte-stimulating hormone*

Amino acid	'Anhydrous' residue (g./100 g. of peptide)	Amino acid (mole/1722 g.)*	Deduced amino acid composition of α -MSH†
Aspartic acid	2.04	0.26	—
Threonine	1.08	0.16	—
Serine	9.06	1.50	2
Proline	6.81	1.0	1
Glutamic acid	7.56	0.89	1
Glycine	3.96	0.91	1
Alanine	0.72	0.14	—
Valine	5.68	0.84	1
Methionine	6.86	0.79	1
Leucine	0.80	0.10	—
Tyrosine	8.36	0.80	1
Phenylalanine	9.18	0.96	1
Ammonia	0.97	0.99	1
Lysine	9.79	1.15	1
Histidine	6.56	0.74	1
Arginine	9.18	0.91	1
Tryptophan	11.40	0.96	1

* Based on the molecular weight of the acetyltridecapeptide amide.

† On the assumption that Asp, Thr, Ala and Leu are not integral constituents of pure α -MSH.

Fig. 1. Products of tryptic hydrolysis of α -MSH (0.2% soln.; enzyme-substrate ratio, 1:100, by wt.; pH 8.5; 30°). Ionophoresis in pyridine-acetate buffer, pH 6.5 (pyridine-acetic acid-water; 10:0.4:90, by vol.); 35–40 v/cm.; 90 min. (see Table 2). α -MSH and Lys are shown as 'markers'.

Table 2. *Amino acid composition of peptide fragments produced by tryptic digestion of α -melanocyte-stimulating hormone (Fig. 1)*

Try, Tyr and His were identified in unhydrolysed peptides as described in Block, Durrum & Zweig (1955).

Peptide	Try	Tyr	His	Amino acid composition*
T ₁	—	+	+	Arg, Glu, His, Met, Phe, Ser, Tyr
T ₂	+	—	—	Gly, Lys, Pro, Val, Try

* Identified by chromatography of acid hydrolysates in butanol-acetic acid-water (4:1:5, by vol.).

C₁, an acidic peptide derivative which did not react with ninhydrin, gave equivalent amounts of serine and tyrosine after hydrolysis with acid; tyrosine was shown to be the C-terminal amino acid by reaction with carboxypeptidase. The residual N-substituted serine was not revealed when a chromatogram of the enzymic digest was developed with ninhydrin, but was located by means of the *tert*-butyl hypochlorite reagent (Schwartz & Pallansch, 1958), and was found to give serine after hydrolysis with acid. In this way C₁ was shown to be an N-acyl derivative of Ser.Tyr, which clearly occupied the N-terminal position in α -MSH.

Peptide C₃, an intermediate product, gave a mixture of peptides C₄ and C₅ by further hydrolysis with chymotrypsin. C₄ was degraded into its constituent amino acids, arginine and tryptophan, by carboxypeptidase and was obviously the dipeptide Arg.Try. C₃ and C₅, like α -MSH itself, were not susceptible to hydrolysis by carboxypeptidase; the fact that they were also unusually basic (to judge from their mobilities during ionophoresis at pH 6.5; cf. Fig. 2), suggested that the CO₂H group of the C-terminal residue in both peptides was not free, and that they were derived from the C-terminal sequence in α -MSH. Glycine and serine were shown to be N-terminal in peptides C₅ and C₂

respectively (C_{2a} differs from C_{2b} only in that it contains methionine sulphoxide, MetSO; cf. Harris & Roos, 1959*a, b*), and the C-terminal sequence in C₂ was shown to be His.Phe by means of carboxypeptidase.

Elucidation of sequence. From the results summarized in Tables 2 and 3 a partial structure for α -MSH could be deduced as shown in Table 4. In this way the proposed tridecapeptide structure was confirmed and all that remained was to determine the sequences of the amino acids in positions 4, 5 and 11–13 in the molecule.

Fig. 2. Products of chymotryptic hydrolysis of α -MSH (0.2% soln.; enzyme-substrate ratio, 1:100, by wt.; pH 8.5; 30°). Ionophoresis in pyridine-acetate buffer, pH 6.5 (pyridine-acetic acid-water; 10:0.4:90, by vol.); 35–40 v/cm.; 60 min.: A, 30 min.; B, 16 hr. (see Table 3). Lys is shown as marker in A.

Table 3. Amino acid composition and partial structure of peptide fragments produced by chymotryptic digestion of α -melanocyte-stimulating hormone (Fig. 2)

Try, Tyr and His were identified in unhydrolysed peptides according to Block *et al.* (1955).

Peptide	R _F †	Try	Tyr	His	N-terminal* residue	C-terminal† residue	Amino acid composition and partial structure
C ₁	0.11	—	+	—	.	Tyr	N-R.Ser.Tyr
C _{2a}	0.12	—	—	+	Ser	Phe (His)	Ser (Glu, MetSO).His.Phe
C _{2b}	0.28	—	—	+	Ser	Phe (His)	Ser (Glu, Met).His.Phe
C ₃	0.10	+	—	—	.	.	(Arg, Gly, Lys, Pro, Try, Val)
C ₄	0.34	.	.	.	.	Arg, Try	(Arg, Try)
C ₅	0.08	.	.	.	Gly	.	Gly.(Lys, Pro, Val)

* Identified as the phenylthiohydantoin.

† Amino acids released by carboxypeptidase.

‡ R_F in butanol-acetic acid-water (4:1:5, by vol.).

Table 4. Peptides obtained by tryptic and chymotryptic digestion of α -melanocyte-stimulating hormone: a partial structure of the peptide hormone

Peptide	Deduced partial structure
C ₁	R- Ser.Tyr*
C ₂	Ser.(Glu, Met).His*.Phe*
T ₁	R- Ser.Tyr.(Glu, His, Met, Ser, Phe).Arg
C ₃	Arg.Try (Gly, Lys, Pro, Val)
T ₂	Try (Gly, Lys, Pro, Val)
C ₄	Arg*.Try*
C ₅	Gly.(Lys, Pro, Val)
	R- Ser.Tyr.Ser.(Met, Glu).His.Phe.Arg.Try.Gly.(Lys, Pro, Val)
	1 2 3 6 7 8 9 10
	C C T C

* Amino acids released by carboxypeptidase. Arrows indicate bonds hydrolysed by trypsin (T) and chymotrypsin (C). R represents unidentified N-acyl group.

Stepwise degradation by the phenyl isothiocyanate method. In order to determine the complete structure of α -MSH, peptides C_2 and C_5 were submitted to stepwise analysis from the amino end as described previously (Harris & Roos, 1959a). The phenylthiocarbamyl derivative of C_2 (approx. 1 μ mole) was dissolved in 2 ml. of 3N-HCl at room temperature. A volume (0.2 ml.) of this solution was immediately diluted to 2 ml. with 3N-HCl and its absorption spectrum between 240 and 280 m μ was read at 30 min. intervals. When the extinction coefficient of the solution at 265 m μ attained a maximum value (after 3 hr.) the acid solution was extracted with portions (5 ml.) of ethyl acetate; the ethyl acetate extract gave an absorption spectrum typical of a phenylthiohydantoin (maximum at 268 m μ , ratio 260 m μ /268 m μ , 0.8), corresponding to a total yield of 0.85 μ mole of the phenylthiohydantoin derivative of the N-terminal residue of C_2 , which was identified as that of serine by chromatography in heptane-pyridine (Sjöquist, 1953). In all, peptide C_2 was submitted to three consecutive cycles of the degradation procedure, which resulted in the successive release of the phenylthiohydantoin derivatives of serine, methionine and glutamic acid. The structure of C_2 was thus established to be Ser.Met.Glu.His.Phe.

When the phenyl isothiocyanate reaction was applied to C_5 , the phenylthiocarbamyl derivative of the peptide was found to be extracted into benzene from alkaline (pH 9.0) solution, an observation which again suggested that the terminal carboxyl group was not free.

Partial acid hydrolysis. Peptides C_2 and C_5 (0.5–1.0 μ mole) were each dissolved in 0.5 ml. of 12N-HCl and incubated at 37° for 3 days. The hydrolysate of C_5 was submitted to ionophoresis on paper at pH 6.5 and 35 v/cm. for 90 min., and the distribution of ninhydrin-reacting components is shown in Fig. 3. The amino acid composition of each component is given in Table 5. C_{5b} gave a strong glycine spot, together with weaker spots of proline and valine, when its acid hydrolysate was chromatographed in butanol-acetic acid-water (4:1:5, by vol.), and we suspected that it was probably a mixture of glycine and the diketopiperazine of Pro.Val (Consdén, Gordon, Martin & Synge, 1947). This appeared to be confirmed when the section of the ionogram designated C_{5a} (Fig. 3), and which did not give a colour with ninhydrin, was found to give mainly proline and valine after elution and hydrolysis with acid.

C_{5c} , although less basic than C_5 (to judge from its electrophoretic mobility at pH 6.5), was nevertheless found to have the same amino acid composition, which suggested that it was the free tetrapeptide derived from C_5 by the unmasking of the CO₂H group of its C-terminal residue. A sample of C_5 was

accordingly assayed for amide ammonia by means of the Conway microdiffusion method. In this way the acid hydrolysate of the peptide (an amount equivalent to 8.0 μ g. of α -amino N) was found to contain 2.9 μ g. of ammonia N, as measured by the photometric-ninhydrin method, and since one of the constituent amino acids, proline, does not contribute significantly to the total absorption at 570 m μ , this value for ammonia N is consistent with the presence of one amide group/mole of tetrapeptide. Clearly C_5 is Gly.Lys.Pro.Val.NH₂.

Fig. 3. Products of partial acid hydrolysis of peptide C_5 (Fig. 2; 12N-HCl; 37°; 4 days). Ionophoresis in pyridine-acetate buffer, pH 6.5 (pyridine-acetic acid-water; 10:0.4:90, by vol.); 35–40 v/cm.; 90 min.; (see Table 5). Gly and Lys are shown as 'markers'.

Table 5. Amino acid composition of products of partial acid hydrolysis of peptide C_5 (Fig. 3)

Band	Amino acid composition*
$C_5 a$	(Pro, Val)
b	(Gly) (Pro, Val)
c	(Gly, Lys, Pro, Val)
d	(Gly, Lys, Pro)
e	(Lys, Pro)
f	(Gly, Lys)
g	(Lys)
Structure:	Gly.Lys.Pro.Val.NH ₂

* Hydrolysate contains 1 mole of amide⁺NH₂/mole of C_5 .

Fig. 4. Products of partial acid hydrolysis of peptide C_2 (Fig. 2; 12N-HCl; 37°; 4 days). Ionophoresis in pyridine-acetate buffer, pH 6.5 (pyridine-acetic acid-water; 10:0.4:90, by vol.); 35–40 v/cm.; 2 hr. (see Table 6). Glu, Gly and His are shown as 'markers'.

Table 6. Amino acid composition of products of partial hydrolysis of peptide C_2 (Fig. 4)

Band	Amino acid composition
$C_2 a$	(Glu)
b	(Met, Glu)
c	(Ser, Met, Glu)
d	(Glu, His)
e	(Ser) (Met) (Phe)
f	(His, Phe)
g	(His)
Structure:	Ser.Met.Glu.His.Phe

(Table 5) and forms the *C*-terminal sequence in α -MSH.

When the partial acid hydrolysate of peptide C_2 was submitted to ionophoresis at pH 6.5 and 35 v/cm. for 90 min. it was resolved into the components illustrated in Fig. 4. The amino acid composition of each component is given in Table 6 and the results provided independent confirmation for the sequence Ser.Met.Glu.His.Phe, which had been derived for this peptide from the results of stepwise analysis.

Identification of the N-terminal residue in α -melanocyte-stimulating hormone. For the identification of the acyl substituent on the α -amino group of the *N*-terminal serine residue in α -MSH we were restricted to qualitative paper-chromatographic studies on fragment C_1 (acylseryltyrosine, approx. 500 μ g. of N) derived from 2.6 mg. of α -MSH. When C_1 was caused to react with carboxypeptidase, tyrosine was the only product of reaction to be revealed by ninhydrin; the *N*-substituted serine moiety could, however, be revealed after chlorination (Rydon & Smith, 1952; Schwartz & Pallansch, 1958), and it was provisionally identified as *N*-acetylserine (Harris, 1958) by comparing its R_F with that of a synthetic sample of *N*-acetyl-DL-serine, in butanol-acetic acid-water (4:1:5, by vol.; R_F 0.54), phenol-cresol (1:1, w/w)-borate buffer, pH 9.3 (R_F 0.64), and in *tert*-amyl alcohol-phthalate buffer, pH 6.0 (R_F 0.50).

Additional confirmatory evidence for the presence of *N*-acetylserine was obtained by submitting the *N*-acyldipeptide to hydrazinolysis according to the procedure of Niu & Fraenkel-Conrat (1955). When the hydrazinolysate was chromatographed in pyridine-aniline-water (9:1:4, by vol.) both acetylhydrazide (R_F 0.7) and serine hydrazide (R_F 0.52) were identified; in collidine-water (10:2, v/v) the corresponding R_F values were 0.35 and 0.1 respectively. In both solvent systems samples of synthetic acetylhydrazide and serine hydrazide were chromatographed as controls (cf. Narita, 1958).

The complete structure of α -MSH is thus established as shown in Fig. 5.

DISCUSSION

α -Melanocyte-stimulating hormone has been shown to be an *N*-acetyltridecapeptide amide, with an amino acid sequence identical to that of the *N*-terminal tridecapeptide portion of corticotrophin. The structure of the hormone, deduced from the amino acid sequences of the peptide fragments derived from it by digestion with trypsin and chymotrypsin, confirms the amino acid composition which was deduced for the hormone from the results of amino acid analysis (Table 1). The additional amino acids, aspartic acid, threonine, alanine, cystine and leucine, which also occurred in relatively small amounts in the hormone preparation available to us, were present, as we had suspected, in related peptide impurities which could not be separated from α -MSH under the conditions used for its isolation. It may be recalled that these amino acids were originally believed to be integral constituents of α -MSH; in order to accommodate them it was necessary to assume that the hormone was a polypeptide containing at least 37 amino acid residues (Lee & Lerner, 1956), and this example emphasizes the dangers of taking the results of amino acid analyses at their face value, even when the substance under investigation appears to satisfy the many criteria of purity which were described for α -MSH by Lee & Lerner (1956). Thus when investigating biologically active polypeptides of relatively low molecular weight, such as corticotrophin and the melanocyte-stimulating hormones, which are usually available only in small quantities, more information is likely to be gained by investigating the number and composition of peptide fragments produced by selective enzymic hydrolysis than by attempting to establish purity by a variety of physical and biological criteria which frequently lead to equivocal and misleading conclusions. A knowledge of the chemical structure of a biologically active substance greatly facilitates its eventual isolation in pure form so that meaningful physicochemical and biological studies can

Fig. 5. Structure of α -MSH. Arrows indicate sites of hydrolysis by trypsin (T) and chymotrypsin (C).

then be carried out on a substance of established purity.

α -MSH appears to be the first example of a naturally occurring polypeptide [other than cyclic polypeptides such as gramicidin-S (Synge, 1945) and the tyrocidines (Paladini & Craig, 1954; King & Craig, 1956)] which does not possess either a free *N*-terminal or *C*-terminal residue. In so far as it contains a *C*-terminal residue in the amide form, α -MSH resembles the posterior pituitary-gland hormones, oxytocin (du Vigneaud, Ressler & Trippett, 1953) and vasopressin (du Vigneaud, Lawler & Popenoe, 1953); more recently Narita (1958) has isolated an *N*-acetylseryl peptide from tobacco mosaic virus protein which in all probability represents the *N*-terminal peptide of the protein. The occurrence of *N*-acetyl groups in polypeptides and proteins emphasizes the need for vigilance when investigating the number of polypeptides present in enzymic digests, since *N*-acetylpeptide fragments may not be revealed by the methods which are usually employed for their detection. It is also becoming increasingly apparent that absence of end groups cannot in itself be taken as evidence for the existence of *cyclopeptide* chains in protein molecules.

The hydrolysis of α -MSH by trypsin and chymotrypsin, which resulted in the cleavage of the molecule into two and four peptide fragments respectively, occurred in accordance with the expected specificities of the two enzymes. As in β -melanocyte-stimulating hormone (β -MSH), the Arg. Try bond was found to be highly susceptible to hydrolysis by trypsin; the Lys. Pro bond, on the other hand, was not attacked by trypsin, and it may be recalled that similar bonds involving the imino group of proline residues in corticotrophin (e.g. Bell, 1954) were also found to be resistant to hydrolysis by the enzyme. Chymotryptic digestion of α -MSH resulted in the rapid hydrolysis of the Tyr. Ser and Phe. Arg bonds, but, as in β -MSH, the Try. Gly bond in the *C*-terminal peptide fragment

was hydrolysed at a much slower rate. It is of interest that the corresponding Tyr. Ser bond in the *N*-terminal Ser. Tyr. Ser sequence in corticotrophin is hydrolysed very slowly by chymotrypsin, presumably because of its proximity to the free α -amino group present in the *N*-terminal serine residue. When this group is blocked by acetylation, as in α -MSH, the neighbouring Tyr. Ser bond becomes highly susceptible to hydrolysis by chymotrypsin.

Structural and activity relationships between α - and β -melanocyte-stimulating hormones and corticotrophin

It has already been shown (Harris & Roos, 1956, 1959*a, b*; Geschwind *et al.* 1956, 1957*a*) that β -MSH contains a sequence of seven amino acids in common with corticotrophin, and that this common structure is in all probability responsible for the intrinsic melanocyte-stimulating activity of corticotrophin. The present work reveals an even closer structural relationship between α -MSH and corticotrophin.

The amino acid sequences of the two melanocyte-stimulating hormones together with the related *N*-terminal sequence in corticotrophin are shown in Fig. 6. The heptapeptide Met. Glu. His. Phe. Arg. Try. Gly is common to all three molecules and, but for the transposition of lysine and serine residues between positions 6 and 14 in β -MSH, the common sequence would extend to 11 amino acids. All three molecules possess melanocyte-stimulating activity but whereas the activities of α - and β -MSH are of the same order of magnitude (α -MSH appears to be about twice as active as β -MSH when assayed by the *in vitro* method of Shizume, Lerner & Fitzpatrick, 1954; A. B. Lerner, personal communication), corticotrophin is found to be only 0.5–1% as active as β -MSH when the two substances are assayed under comparable conditions (Dixon, 1956; Geschwind *et al.* 1957*a*).

Fig. 6. Amino acid sequences of α - and β -MSH, compared with the related *N*-terminal portion of corticotrophin.

Shepherd *et al.* (1956) have reported that the smallest active degradation product which they were able to isolate from corticotrophin (the *N*-terminal fragment, containing 24 amino acid residues) retained the same ratio of melanocyte-stimulating to adrenocorticotrophic activity as the undegraded hormone (39 amino acid residues), and this observation gave a strong indication that the structure responsible for melanocyte-stimulating activity was associated with that part of the molecule which was also essential for adrenocorticotrophic activity. It has now emerged that the specific sequence of amino acids required to produce a highly active melanocyte-stimulating substance is to be found within the *N*-terminal tridecapeptide fragment of corticotrophin.

A comparison of the structures of α - and β -MSH suggests that the *N*-terminal, Asp.Glu.Gly.Pro, and *C*-terminal, Pro.Lys.Asp, sequences in β -MSH are not specifically essential for its activity, since they can be replaced by acetylserine and valine amide respectively in α -MSH; and in a similar manner it could be argued that acetylserine and valine amide do not specifically form part of the structure essential for activity in α -MSH. In this connexion it is perhaps significant that the only difference in structure between β -MSH isolated from pig and ox pituitary glands (i.e. the replacement of Glu by Ser in position 2, Fig. 6; Geschwind, Li & Barnafi, 1957*b*), occurs outside the structure common to both α - and β -MSH. When α -MSH is obtained from other species it will be interesting to see to what extent variations in structure are compatible with biological activity.

All the available evidence points to the conclusion that the structure which is specifically essential for melanocyte-stimulating activity is to be found within the Tyr.Lys.Met.Glu.His.Phe.Arg.Try.Gly.Ser.Pro sequence (positions 5–15) in β -MSH, and the Tyr.Ser.Met.Glu.His.Phe.Arg.Try.Gly.Lys.Pro sequence (positions 2–12) in α -MSH and corticotrophin. Furthermore, the fact that the lysine and serine residues (positions 6 and 14 in β -MSH, positions 3 and 11 in α -MSH) can be interchanged suggests that some variations are possible, even within these two undecapeptide structures.

It is recognized that other conditions, such as the distribution of charged groups and the overall length and configuration of the peptide chain, may have to be satisfied before highly active structures can be produced, but these additional requirements would appear to be of a less specific nature. Any final conclusions must await the synthesis of active polypeptides modelled on the above undecapeptide structures, and in this connexion a synthetic octapeptide Ser.Met.(Glu.NH₂).His.Phe.Arg.

Try.Gly has recently been shown to possess a small but nevertheless significant melanocyte-stimulating activity (Hofmann, Thompson & Schwartz, 1957) when assayed by the *in vitro* method of Shizume *et al.* (1954).

The other outstanding question concerns the melanocyte-stimulating activity of corticotrophin. Despite the fact that it contains the entire tridecapeptide sequence of α -MSH, its observed activity is from 100 to 200 times less than that of the two melanocyte-stimulating hormones. A possible explanation is that although corticotrophin is potentially a highly active melanocyte-stimulating hormone, the additional structural features which are essential for its adrenocorticotrophic properties may at the same time inhibit, or mask, its potential melanocyte-stimulating properties. The length of the peptide chain does not appear to be of major importance since active corticotrophin molecules containing from between 39 and 24 amino acids appear to possess the same low melanocyte-stimulating activities (Shepherd *et al.* 1956). The most obvious difference between corticotrophin and α -MSH is that the *N*-terminal serine residue in the latter occurs as its *N*-acetyl derivative. Can it be that a free CH₂·(OH)·CH·(NH₂)-structure, which has been shown to be essential for adrenocorticotrophic activity (Dixon, 1956), is also an inhibitor of melanocyte-stimulating activity, and does this explain why the α -MSH molecule is substituted in this position?

At present, although there are no clear-cut answers to these questions, there is some experimental evidence to support this view. For example, when the *N*-terminal serine residue in corticotrophin is oxidized with periodate, loss of adrenocorticotrophic activity is accompanied by a significant increase in the melanocyte-stimulating activity of the periodate-modified corticotrophin (Dixon, 1956; H. B. F. Dixon & A. B. Lerner, unpublished results). Similarly, Shepherd *et al.* (1956) have shown that when corticotrophin is subjected to mild alkaline hydrolysis (0.1*N*-sodium hydroxide; 100°; 15 min.), which results, specifically, in the cleavage of peptide bonds in the *N*-terminal Ser.Tyr.Ser segment of the molecule, loss of adrenocorticotrophic activity is again accompanied by a significant increase in melanocyte-stimulating activity. Although a conclusive evaluation of these results must wait the isolation and characterization of the periodate- and alkali-degradation products of corticotrophin, it seems to be established that corticotrophin can be changed into a predominantly melanocyte-stimulating hormone and that the *N*-terminal serine residue plays an important role in determining which of its two biological properties shall predominate.