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Studies on Pituitary Polypeptide Hormones

1. THE STRUCTURE OF β -MELANOCYTE-STIMULATING HORMONE FROM PIG PITUITARY GLANDS

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The presence of a melanocyte-stimulating substance in the pars intermedia of the pituitary has been recognized for well over 30 years as a result of the pioneer investigations of Atwell (1919), Hogben & Winton (1922), Smith & Smith (1923), Zondek & Krohn (1932) and others. Subsequently, numerous attempts were made to isolate the melanocyte-stimulating principle in pure form, and these efforts were finally brought to a successful conclusion when highly purified melanocyte-stimulating substances were isolated from pig pituitary glands by Lerner & Lee (1955), Porath, Roos, Landgrebe & Mitchell (1955), Benfey & Purvis (1955) and by Geschwind, Li & Barnafi (1956).

The melanocyte-stimulating hormone isolated by Lerner & Lee (1955) was found to be a highly basic polypeptide (isoelectric point 10.5–11.0), and Porath *et al.* (1955) and Geschwind *et al.* (1956) reported isoelectric points of 5.2 and 5.8 respectively for the melanocyte-stimulating polypeptides which they had isolated. This apparent discrepancy was resolved when Lee & Lerner (1956) showed that both forms of the hormone were present in the pig pituitary; it was therefore proposed that the basic polypeptide which they had originally isolated be called α -melanocyte-stimulating hormone and that the acidic form of the polypeptide hormone isolated by the other three groups of investigators be called β -melanocyte-stimulating hormone.

In this paper experiments which have led to the

elucidation of the complete structure of β -melanocyte-stimulating hormone are described. The amino acid composition of β -melanocyte-stimulating hormone (Roos, 1956) was consistent with an octadecapeptide structure containing the following amino acid residues (abbreviations for the amino acid residues are in accordance with those of Brand & Edsall, 1947): Arg₁, Asp₂, Glu₂, Gly₂, His₁, Lys₂, Met₁, Phe₁, Pro₃, Ser₁, Try₁, Tyr₁. The amino acid sequence of β -melanocyte-stimulating hormone has been determined by application of stepwise methods of degradation to the intact octadecapeptide and to peptide fragments derived from it by selective degradation with trypsin and chymotrypsin. The complete sequence was deduced in this manner, and was subsequently confirmed by partial acid-hydrolysis studies (cf. Sanger & Tuppy, 1951*a, b*).

MATERIALS

The sample of β -melanocyte-stimulating hormone (β -MSH), used in this study, was prepared by one of us (P. Roos) at the Institute of Biochemistry, University of Uppsala, Sweden, according to the procedure described by Porath *et al.* (1955). Crystalline trypsin and chymotrypsin were obtained from the Novo Terapeutisk Laboratorium, Copenhagen, and a highly purified preparation of crystalline carboxypeptidase from The Armour Laboratories, Chicago, U.S.A.

METHODS

N- and C-terminal group and sequence analysis

'Stepwise' degradation from the amino end (*Expt. 1*). The sequence of amino acids from the *N*-terminal position was investigated by means of the phenylthiohydantoin method

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of Edman (1950), as described by Fraenkel-Conrat, Harris & Levy (1955). An aqueous solution (50 μ l.) of β -MSH (2.5 mg.) was applied to a strip of Whatman no. 1 paper (6.0 cm. $\times$ 1.5 cm.). After drying, a 10% solution of phenyl isothiocyanate in peroxide-free dioxan (50 μ l.) was applied to the paper strip, which was then suspended in an atmosphere of aqueous pyridine (1:1, v/v) for 3 hr. at 40°. The paper strip was kept in an evacuated desiccator over conc. H_2SO_4 to remove excess of pyridine, and was then washed by gentle shaking in a stoppered tube with successive portions of benzene (at least three washings with 10 ml. of A.R.-grade reagent), followed by a 1:1 (v/v) mixture of ethanol-ether (peroxide-free; three to five washings), until the reading for extinction coefficient at 268 $m\mu$ of the last wash solution (10 ml. of ethanol-ether, 1 hr. at room temperature) was less than 0.02.

The paper strip containing the phenylthiocarbamyl (PTC) derivative of β -MSH was freed from solvent and placed horizontally in a Petri dish at the bottom of a desiccator, between two beakers containing 50–100 ml. quantities of acetic acid and constant-boiling HCl respectively. The desiccator was evacuated (20–50 mm. Hg) and left at room temperature. The paper strip was withdrawn after 12 hr. and excess of acid was removed in a desiccator over KOH pellets.

The phenylthiohydantoin (PTH) derivative of the *N*-terminal amino acid was extracted into ethanol-ether (10 ml.; 1:1, v/v) by gentle shaking at room temperature for 30 min.; the extinction coefficient of the solution was measured over the range 240–280 $m\mu$ in the Beckmann model DU spectrophotometer, and the approximate yield of PTH calculated from the reading at the maximum (268–270 $m\mu$).

After extraction of the PTH derivative of the *N*-terminal amino acid, the paper strip was resubmitted to the whole cycle of operations described above. In all, β -MSH was submitted to ten successive applications of the degradative procedure.

The PTH released in each successive step was identified directly by chromatography on paper. Sample portions (0.05–0.1 μ mole) were applied to Whatman no. 1 paper (which had been pretreated with 0.5% soluble starch solution) and chromatographed, in the heptane-pyridine (70:30, v/v) and heptane-butanol-formic acid (40:40:20, by vol.) solvent systems, as described by Sjöquist (1953). The PTH compounds were identified by comparing their R_F values with those of control PTH compounds. The heptane-pyridine system, which was found to be the most generally useful solvent system for the PTH compounds under investigation, was made up with technical-grade laboratory reagents and prior equilibration of the paper was unnecessary. Paper sheets were placed directly into a trough containing the solvent and the chromatogram was developed (by descending chromatography in well-sealed all-glass tanks) for 4–6 hr. at constant temp. (25°); in some cases resolution of the PTH compounds was improved by running the chromatograms for periods of up to 24 hr., the solvent being allowed to drip off the end of the paper.

The chromatograms were dried in a current of air at 50–60° and the PTH compounds revealed as bleached spots on an even-purple background by dipping in the developing solution (0.01M- I_2 in 0.5M-KI and 0.5M- $NaNO_3$; 1:1, v/v) (see Fig. 1). In some cases samples of the PTH compounds were hydrolysed with 6N-HCl for 16 hr. at 150° in sealed evacuated tubes, and the amino acids regenerated in this

way were identified by chromatography in butanol-acetic acid-water (4:1:5, by vol.).

Studies of C-terminal sequence (Expt. 2). The sequence of amino acids from the C-terminal end of the molecule was investigated by means of carboxypeptidase (e.g. see Harris, 1955).

β -MSH (1 mg.) and carboxypeptidase (0.05 mg.) were dissolved in 0.1% $NaHCO_3$ (1 ml.) and incubated at pH 8.0–8.5 and 37°. After 4 hr., 0.5 ml. of the reaction solution was transferred to a test tube containing 50–100 mg. of Amberlite IR 120 resin (H^+ form, 20–50 mesh, 8% cross-linking); after 16 hr. the other half of the reaction solution was treated in a similar manner. The tubes were shaken for 1 hr. and the supernatant solutions withdrawn. The resin was then thoroughly washed with water and extracted with aq. 5N- NH_3 soln. (0.5 ml.) for 20 min. The two extracts were taken to dryness *in vacuo* over conc. H_2SO_4 and examined for free amino acid(s) by chromatography on paper in butanol-acetic acid-water (4:1:5, by vol.).

Enzymic cleavage of β -melanocyte-stimulating hormone (Expt. 3)

Use of pH-stat. The enzymic reactions were carried out, in the absence of buffers, by means of a pH-stat (see Jacobsen, Léonis, Linderstrøm-Lang & Ottesen, 1957). In this way the opening of peptide bonds could be followed by continuous titration with alkali (0.05–0.1N-NaOH), and the total alkali uptake recorded by the pH-stat is the sum of the amounts required to titrate all the amino groups liberated during the reaction. The relationship between the amount of alkali consumed and the number of bonds split by the enzyme depends upon the pH at which the reaction is carried out and the average pK of the amino groups which are formed, according to the expression

$$pH = pK + \log \frac{RNH_2}{RNH_3^+}.$$

For example, by comparing the titration curves of ribonuclease, before and after digestion with subtilisin, Richards (1955) estimated that the amino groups which had been released in the reaction had an average pK of 7.4, and that 0.9 mole of H^+ ion had therefore been produced per mole of peptide bond hydrolysed, at pH 8.0.

In the present investigation enzymic reactions were carried out at pH 8.2. The rate of uptake of alkali served merely as a guide for following the course of digestion of β -MSH by trypsin and chymotrypsin, and for this purpose it has been assumed that 80–100% of the amino groups released were titrated at pH 8.2.

Action of trypsin. β -MSH (4.8 mg.) was dissolved in water (2 ml.) at pH 8.2 and 30° in the reaction cell of the pH-stat. Trypsin (10 μ l., 0.5% soln.) was added and the pH was maintained at 8.2 for 30 min. by the automatic addition of 0.05N-NaOH. After 30 min., 0.1N-HCl was added to pH 4.0 and the reaction solution was taken to dryness *in vacuo* over conc. H_2SO_4 .

Action of chymotrypsin. β -MSH (5.0 mg.) was dissolved in water (2 ml.) at pH 8.2 and 30°. Chymotrypsin (10 μ l., 0.5% soln.) was added, and the pH of the reaction solution was maintained at 8.2 for 30 min. by addition of 0.05N-NaOH as described above. Enzymic digestion was terminated by the addition of 0.1N-HCl to pH 4.0 and the solution was then taken to dryness *in vacuo* over conc. H_2SO_4 .

In a second experiment carried out under the same conditions the chymotrypsin reaction was allowed to proceed for 16 hr.

Separation of peptide fragments derived from β -melanocyte-stimulating hormone: ionophoresis on paper

The procedure used was essentially that of Michl (1951) in which ionophoresis was carried out at high-potential gradients in pyridine-acetic acid buffers on filter paper completely immersed in toluene as described by Ryle, Sanger, Smith & Kitai (1955). Samples containing 0.05–0.1 μ mole of each enzymic digest were submitted to ionophoresis on Whatman no. 1 paper at pH 6.5 and at a potential gradient of 30–40 v/cm. for 2–3 hr. When the best conditions for the fractionation of each enzymic hydrolysate had been established, preparative runs were then carried out. Each digest (derived from approx. 2 μ moles of β -MSH) was applied in the form of a narrow band, containing 0.5–1.0 mg. of digest/cm., to Whatman no. 3 paper. In this manner the various peptide components were separated and were located by dipping narrow guide strips cut from both edges of the paper in 0.25% ninhydrin soln. in acetone. The bands were then eluted into a series of small test tubes with 5% acetic acid (0.1–0.2 ml. of solvent was found to be sufficient to effect the complete elution of all the peptide fragments derived from β -MSH), and the peptide solutions were taken to dryness *in vacuo* over KOH pellets and stored at -10° .

Sample portions of each peptide component (0.05–0.1 μ mole) were tested for homogeneity by chromatography on paper in butanol-acetic acid-water (both 4:1:5, and 3:1:1, by vol., systems were used), and were also resubmitted to paper ionophoresis in a pyridine-acetic acid buffer at pH 3.5. If more than one component was revealed by either of these procedures, the entire peptide sample was refractionated and the additional peptide component(s) were isolated as described above.

Characterization of purified peptide fragments: amino acid composition

Sample portions of each of the purified peptide fragments (0.05–0.1 μ mole of peptide) were examined for the presence of tryptophan, histidine and arginine respectively (see Block, Durum & Zweig, 1955), and the complete amino acid composition of each peptide was determined (after hydrolysis with glass-distilled constant-boiling HCl, in sealed evacuated tubes at 105° for 18 hr.) by chromatography on paper in butanol-acetic acid-water (4:1:5, by vol.), and where necessary by the two-dimensional system of Levy & Chung (1953).

Sequence studies

Phenyl isothiocyanate method. The *N*-terminal sequences in peptide fragments, derived from β -MSH by the above procedures, were investigated by the procedure of Fraenkel-Conrat & Harris (1954). The peptide (0.5–1.0 μ mole) dissolved in water-dioxan (1:1, v/v; 2 ml.) was caused to react with phenyl isothiocyanate (25–50 μ l.) at pH 9.0 and 38° for 2 hr. The reaction was carried out under N_2 in a pH-stat as described by Ottesen & Wollenberger (1953). The reaction solution was extracted, first with benzene, and then with cyclohexane (spectroscopic grade), and the residual aqueous solution was taken to dryness *in vacuo*

over conc. H_2SO_4 . The sodium salt of the PTH peptide was then dissolved in 3*N*-HCl (4 ml., prepared from glass-distilled, constant-boiling HCl), and the extinction coefficient of the solution was measured over the range 235–280 $m\mu$ in a Beckman model DU spectrophotometer. The formation of the PTH derivative of the *N*-terminal amino acid was followed by measuring the increase in extinction coefficient at 265 $m\mu$ as a function of time. When the extinction coefficient at 265 $m\mu$ attained a maximum value (2–4 hr.) the acid solution was extracted with three 5 ml. portions of redistilled ethyl acetate. The combined ethyl acetate extracts were washed with water (two 5 ml. portions), dried over anhydrous Na_2SO_4 and evaporated to dryness *in vacuo*. The yield of PTH was calculated from the total extinction coefficient at 268 $m\mu$, and suitable portions (0.05–0.1 μ mole) were then analysed by chromatography on starch paper.

The 3*N*-acid solution containing the residual peptide was taken to dryness *in vacuo* over KOH pellets; the residual peptide was then redissolved in water-dioxan (1:1, v/v) and the entire degradation procedure was repeated.

Fluorodinitrobenzene method (Sanger, 1945). Peptide T2(3) (Fig. 9a) was caused to react with fluorodinitrobenzene (FDNB) in aqueous solution at pH 8.5. The dinitrophenyl (DNP) peptide was hydrolysed under N_2 in a sealed tube with glass-distilled, constant-boiling HCl (250 μ l.) for 8 hr. at 105° , and both the ether-soluble and aqueous fractions were investigated by chromatography on paper according to Levy (1954).

C-terminal studies. *C*-terminal residues (and, wherever possible, *C*-terminal sequences) in peptide fragments derived from β -MSH by digestion with trypsin and chymotrypsin were investigated by the carboxypeptidase procedure, as described in Expt. 2.

Partial acid hydrolysis. Peptides T1, T2 and C3 (0.5–1.0 μ mole) were dissolved in conc. HCl (A.R. grade; 36%, w/v), and the solutions were incubated at 37° for 1–5 days. The acid hydrolysates were evaporated to dryness *in vacuo* over KOH pellets and the products were isolated and characterized by the methods described above.

RESULTS

N-terminal sequence (Expt. 1). When the 'paper-strip' PTH method was applied to β -MSH the PTH derivative of the *N*-terminal amino acid was obtained in 85–90% yield and was identified as that of aspartic acid. In all, β -MSH was submitted to ten successive applications of the degradation procedure and the *N*-terminal decapeptide sequence was established to be: Asp. Glu. Gly. Pro. Tyr. Lys. Met. Glu. His. Phe(?). The results are illustrated in Fig. 1.

Although the yields of PTH compounds formed at each step decreased progressively, particularly in steps 6–9 (Fig. 1), the intensities of spots produced by chromatography of 0.05–0.1 μ mole samples (i.e. one-twentieth to one-tenth of the total yields based on extinction-coefficient readings at 268 $m\mu$) of the PTH compounds isolated in the first five steps, were comparable with those produced from equivalent quantities of the appropriate

Fig. 1. Stepwise degradation of β -MSH by the phenyl isothiocyanate method. Amino acids identified as their phenylthiohydantoin derivatives by chromatography in heptane-pyridine (70:30, v/v) (1 to 9a) and in heptane-butanolfomic acid (40:20:40, by vol.) (9b). C, control phenylthiohydantoin compounds. *N*-terminal sequence is thus established to be Asp.Glu.Gly.Pro.Tyr.Lys.Met.Glu.His.

control PTH compounds; and although the characteristic 268 $m\mu$ peak was not obtained after step 5, it was still possible to identify the PTH compounds produced in steps 6–10. For example, the histidine PTH spot shown in step 9 (Fig. 1; 9b) was produced from a sample containing only a third of the total yield based on the extinction coefficient at 268 $m\mu$. After the fourth step, small amounts of non-specific PTH compounds began to appear on the chromatograms, but only in step 10 did they become sufficiently serious to interfere with the sequence analysis.

C-terminal group. When β -MSH was digested with carboxypeptidase (Expt. 2) aspartic acid was found to be the only product of reaction; however, even after prolonged periods of digestion with a large excess of enzyme, less than 0.5 μ mole of aspartic acid was produced per μ mole of peptide, but since no other amino acids were produced the result was sufficiently conclusive to establish its identity as the C-terminal amino acid in β -MSH.

Action of trypsin. The rate of uptake of alkali when β -MSH (2 μ moles) was caused to react with trypsin at pH 8.2 and 30° is shown in Fig. 2. There was a very rapid initial reaction, and 1 μ mole of alkali was consumed/ μ mole of β -MSH during the first 2–3 min., which suggested that one of the bonds in the molecule was particularly susceptible to hydrolysis by the enzyme. Thereafter the rate of uptake of alkali decreased rapidly and when approx. 1.8–2.0 μ moles had been consumed/mole of substrate (corresponding to the hydrolysis of two peptide bonds in the molecule) the reaction appeared to be complete.

A sample of the digest (0.1 μ mole) withdrawn after 15 min. was fractionated by ionophoresis at pH 6.5 and 40 v/cm. for 2 hr., and gave the distribution of peptide fragments shown in Fig. 3. After digestion for 30 min., peptide T4 had largely disappeared, and in a separate experiment it was shown to give rise to a mixture of T1 and T3, showing that these two peptides are part of the same sequence. In accordance with the alkali-uptake studies the ionophoresis results showed that three major peptide fragments were produced from β -MSH by the action of trypsin; the two minor components T1a and T3a were formed by the combined action of both trypsin and chymotrypsin, which was present to the extent of 1–2% in the sample of trypsin which was used for the reaction.

The amino acid compositions of peptides T1, T1a, T2, T3, T3a and T4 are given in Table 1.

Action of chymotrypsin. The rate of uptake of alkali when β -MSH (2 μ moles) was hydrolysed with chymotrypsin at pH 8.2 and 30° is shown in Fig. 4. Approx. 2 μ moles of alkali were consumed/ μ mole of substrate during the first 30 min. of reaction; subsequently the rate of uptake of alkali became very slow, another 0.7–0.8 μ mole being taken up per mole of substrate during 16 hr. This result suggested that two bonds in β -MSH were hydrolysed rapidly and that this initial reaction was followed by the slower hydrolysis of a third bond in the molecule.

When samples of the reaction mixture, withdrawn after 30 min. and 16 hr. respectively, were submitted to ionophoresis at pH 6.5 and 40 v/cm.

Fig. 2

Fig. 2. Rate of uptake of alkali (0.05N-NaOH) during the reaction of β -MSH (4.8 mg.; 0.24% soln.) with trypsin (0.05 mg.) at pH 8.2 and 30°.

Fig. 3

Fig. 3. Products of tryptic hydrolysis of β -MSH (0.24% soln., enzyme/substrate ratio, 1:100, by wt.; pH 8.2; 30°; 15 min.). Ionophoresis in pyridine-acetate buffer, pH 6.5 (pyridine-acetic acid-water; 10:0.4:90, by vol.); 35-40 v/cm.; 3 hr. (see Table 1.)

Table 1. Amino acid composition of peptides derived from β -melanocyte-stimulating hormone by partial hydrolysis with trypsin (see Fig. 3)

Peptide	Amino acid composition
T1	Asp,* Glu, Gly, Lys,† Pro, Tyr
T1a	Asp,* Glu, Gly, Pro, Tyr†
T2	Asp,† Gly, Lys, Pro ₂ , Ser, Try
T3	Arg, Glu, His, Met, Phe
T3a	Arg, Glu, His, Lys, Met, Phe
T4	Arg, Asp,* Glu ₂ , Gly, His, Lys, Met, Phe, Pro, Tyr

* N-terminal residue. † C-terminal residue.

Fig. 4. Rate of uptake of alkali (0.05N-NaOH) during the reaction of β -MSH (5.0 mg., 0.25% soln.) with chymotrypsin (0.05 mg.) at pH 8.2 and 30°.

the peptide fragments which had been formed were resolved as shown in Fig. 5. In agreement with the predictions made on the basis of the alkali-uptake measurements, three main peptide fragments were found to be present after hydrolysis for 30 min. After 16 hr. peptide C2 had largely disappeared and had given rise to two additional components, C5 and C6. The minor component C4 also disappeared after prolonged digestion, and in a separate experiment it was shown to give rise to additional amounts of peptides C1 and C3. It

Fig. 5. Products of chymotryptic hydrolysis of β -MSH (0.25% soln., enzyme/substrate ratio, 1:100, by wt.; pH 8.2; 30°). Ionophoresis in pyridine-acetic acid buffer, pH 6.5 (pyridine-acetic acid-water; 10:0.4:90, by vol.); 35-40 v/cm.; 3 hr.: A, 30 min. sample; B, 16 hr. sample. (See Table 2.)

Table 2. Amino acid composition of peptides derived from β -melanocyte-stimulating hormone by hydrolysis with chymotrypsin (see Fig. 5)

Peptide	Amino acid composition
C1	Asp,* Glu, Gly, Pro, Tyr†
C2	Arg, Asp,† Gly, Lys, Pro ₂ , Ser, Try
C3	Glu, His, Lys, Met, Phe†
C4	Asp, Glu ₂ , Gly, His, Lys, Met, Phe,† Pro, Tyr
C5	Asp,† Gly, Lys, Pro ₂ , Ser
C6	Arg, Try

* N-terminal residue. † C-terminal residue.

emerges therefore that C5 and C6, and C1 and C3, respectively form contiguous sequences in the β -MSH molecule. The amino acid compositions of peptides C1-C6 are given in Table 2.

N- and C-terminal group analyses. The C-terminal amino acids in peptides T1, T2, C1, C3, C5 and C6 were determined by means of carboxypeptidase and the results obtained are given in Tables 1-3. Like the undegraded β -MSH, T2 and C5 were found to contain C-terminal aspartic acid, showing that the two peptides are derived from the C-terminal portion of the molecule. Similarly, aspartic acid was shown to be N-terminal in C1, T1 and T1a, indicating that these three peptides are derived from the N-terminal portion of β -MSH

Elucidation of amino acid sequence. From the amino acid compositions of the peptide fragments isolated from tryptic and chymotryptic digests, together with the results of *N*- and *C*-terminal group analyses, it was possible to deduce the overall general structure of β -MSH, as shown in Table 3.

The sequence of the *N*-terminal decapeptide in β -MSH, which was determined by stepwise degradation (Fig. 1), agrees in all respects with the partial structure deduced independently from the enzymic studies. From a combination of the two results the sequences in positions 2-4 and 7-9 could be established, and it remained only to determine the sequence of amino acids in positions 13-17 (Table 3).

Sequence studies on peptides C5 and C3. When the phenyl isothiocyanate-degradation procedure was applied to peptide C5, the PTH derivatives of glycine, serine and proline were successively released in the first three steps, and were identified by chromatography in heptane-pyridine. In step 2 the main serine PTH spot was found to be contaminated with glycine PTH (approx. 10 % of the main serine PTH) and similarly the proline PTH in step 3 was found to be contaminated with an 'artifact spot' in the position of serine PTH.

Proline PTH was also the main product of reaction in step 4, although in this case three other relatively minor components were also found to be present after chromatography in heptane-pyridine. Although these results were of a qualitative nature there seemed to be little doubt that the PTH compounds formed were released successively from the

sequence Gly.Ser.Pro.Pro. (positions 12-16) in β -MSH. Aspartic acid was shown to be *C*-terminal by means of carboxypeptidase (Table 3) and the presence of lysine as the penultimate residue could therefore be inferred from the known amino acid composition of C5. The sequence of C5 was thus established to be Gly.Ser.Pro.Pro.Lys.Asp. In a similar manner the PTH derivatives of lysine, methionine and glutamic acid were successively released from peptide C3. Phenylalanine was the main product of reaction when C3 was digested with carboxypeptidase, but after prolonged digestion (enzyme/substrate ratio 1:10, at pH 8.5 and 37° for 16 hr.) some histidine (approx. 20 % of the theoretical yield) was also released by the enzyme, showing that the *C*-terminal sequence in C3 is ...His.Phe. The structure of C3 was thus shown to be Lys.Met.Glu.His.Phe, and this result confirmed the sequence in positions 6-10 in β -MSH (Table 3).

Structure of the β -melanocyte-stimulating hormone

As the result of stepwise degradation studies on the intact peptide, and on peptide fragments derived from it by enzymic hydrolysis, the complete amino acid sequence of β -MSH was established. The results are summarized in Fig. 6.

Confirmation of structure by partial acid hydrolysis studies

In order to confirm the structure proposed on the basis of 'stepwise' degradation methods, peptides T1, T2 and C3 were submitted to partial acid hydrolysis.

Table 3. *Peptides obtained by tryptic and chymotryptic digestion of β -melanocyte-stimulating hormone: a partial structure of the peptide hormone*

Peptide	Deduced partial structure
T1a } C1 }	Asp.(Glu, Gly, Pro).Tyr*
T1	Asp.(Glu, Gly, Pro).Tyr*.Lys*
C3	(Glu, Lys, Met).His*.Phe*
T3a	(Glu, His, Lys, Met, Phe, Arg)
T3	(Glu, His, Met, Phe).Arg
C6	(Arg, Try)*
C2	(Arg, Gly, Lys, Pro ₂ , Ser, Try).Asp*
T2	(Gly, Lys, Pro ₂ , Ser, Try).Asp*
C5	(Gly, Lys, Pro ₂ , Ser).Asp*
	<div style="display: flex; justify-content: space-around; margin-top: 10px;"> CTCTC </div> <div style="display: flex; justify-content: space-around; margin-top: 5px;"> ↓↓↓↓↓ </div>
	<div style="display: flex; justify-content: space-between; align-items: flex-end;"> Asp.(Glu, Gly, Pro).Tyr .Lys.(Glu, Met).His.Phe .Arg.Try.(Gly, Lys, Pro₂, Ser).Asp </div> <div style="display: flex; justify-content: space-between; align-items: flex-end; margin-top: 5px;"> 1 5 6 9 10 11 12 18 </div>

* Amino acids released by carboxypeptidase.

Arrows indicate bonds hydrolysed by trypsin (T) and chymotrypsin (C).

Fig. 6. Amino acid sequence of β -MSH. Arrows indicate points of cleavage by trypsin (T) and chymotrypsin (C), T1-T4, and C1-C6, represent peptide fragments isolated and characterized.

Table 4. *Products of partial acid hydrolysis of peptide T1*

Fractions obtained by ionophoresis at pH 6.5 (T1, *a-i*, Fig. 7) were separately chromatographed in butanol-acetic acid-water (4:1:5, by vol.). *a*, *b*, *c*, *h* and *i* were chromatographically pure; *e* and *g* separated into *e*₁, *e*₂, *e*₃, and *g*₁, *g*₂ respectively; *d* and *f* were not identified.

Partial acid hydrolysate of peptide T1

Band	R_F in butanol-acetic acid-water	Amino acid composition*
T1: <i>a</i>	0.14	Asp
<i>b</i>	0.22	Glu
<i>c</i>	0.15	Glu, Gly
<i>d</i>	—	—
<i>e</i> : { <i>e</i> ₁	0.20	Gly
<i>e</i> : { <i>e</i> ₂	0.36	Pro
<i>e</i> : { <i>e</i> ₃	0.46	Gly, Pro, Tyr
<i>f</i>	—	—
<i>g</i> : { <i>g</i> ₁	0.18	Gly, Pro, Tyr, Lys
<i>g</i> : { <i>g</i> ₂	0.24	Pro, Tyr, Lys
<i>h</i>	0.16	Tyr, Lys
<i>i</i>	0.08	Lys
Sequence:		Asp. Glu. Gly. Pro. Tyr. Lys

* Analysed by chromatography on paper.

Fig. 7. Partial acid hydrolysate of peptide T1 (12N-HCl; 37°; 72 hr.). Ionophoresis in pyridine-acetate buffer, pH 6.5 (pyridine-acetic acid-water; 10:0.4:90, by vol.); 35-40 v/cm.; 2 hr.

Peptide T1. Peptide T1 (0.5-1.0 μ mole) was incubated with 12N-HCl (1.0 ml.) at 37° for 72 hr. and the acid hydrolysate was fractionated by a combination of ionophoresis in pyridine-acetate buffer, pH 6.5 (Fig. 7), followed by chromatography in butanol-acetic acid-water (4:1:5, by vol.). The amino acid composition of each component was determined and the results, summarized in Table 4, showed that all the products of hydrolysis had been derived from the sequence

Asp-Glu-Gly-Pro-Tyr-Lys (positions 1-6 in the β -MSH molecule).

Peptide C3. Peptide C3 (approx. 0.5 μ mole) was hydrolysed with 12N-HCl (0.5 ml.) for 48 hr. at 37°. The hydrolysate was fractionated by ionophoresis at pH 6.5 (Fig. 8), followed by chromatography in butanol-acetic acid, and the amino acid compositions of the fragments isolated in this way showed (Table 5) that they had been derived from the sequence Lys-Met-Glu-His-Phe (positions 6-10 in β -MSH).

Peptide T2. Peptide T2 (approx. 1.0 μ mole) was hydrolysed with 12N-HCl at 37° for 48 hr. Under these conditions it was resolved into three major components (Fig. 9a; Table 6). Peptide T2 (3) was subsequently hydrolysed with 12N-HCl for 1 hr. at 100°; the products of hydrolysis were fractionated by ionophoresis at pH 3.5 (Fig. 9b), and their amino acid compositions (Table 7) showed that

Table 5. *Products of partial acid hydrolysis of peptide C3*

Fractions obtained by ionophoresis at pH 6.5 were separately chromatographed in butanol-acetic acid-water (4:1:5, by vol.) and gave the additional components shown.

Partial acid hydrolysate of peptide C3 (Fig. 8)

Band	R_F in butanol-acetic acid-water	Amino acid composition*
C3: a : { a_1 a_2	0.1	Glu, His
	0.28	Glu, His, Phe
b : { b_1 b_2 b_3	0.08	Lys, MetSO, Glu
	0.16	Lys, Met, Glu
	0.5	Phe
c : { c_1 c_2	0.10	Lys, MetSO, Glu, His, Phe
	0.19	Lys, Met, Glu, His, Phe
d : { d_1 d_2	0.05	Lys, MetSO, Glu, His
	0.10	Lys, Met, Glu, His
e : { e_1 e_2	0.09	Lys, MetSO
	0.22	Lys, Met
Sequence:		Lys.Met.Glu.His.Phe

* Analysed by chromatography on paper.

Fig. 8. Partial acid hydrolysate of peptide C3 (12N-HCl; 37°; 48 hr.). Ionophoresis in pyridine-acetate buffer, pH 6.5 (pyridine-acetic acid-water; 10:0.4:90, by vol.); 35-40 v/cm.; 2 hr.

Fig. 9a. Partial acid hydrolysate of peptide T2 (12N-HCl; 37°; 48 hr.). Ionophoresis in pyridine-acetate buffer, pH 3.5 (pyridine-acetic acid-water; 1:10:89, by vol.); 35-40 v/cm.; 3 hr.

Fig. 9b. Partial acid hydrolysate of peptide T2 (3) (12N-HCl; 100°; 1 hr.). Ionophoresis in pyridine-acetate buffer, pH 3.5 (pyridine-acetic acid-water; 1:10:89, by vol.); 35-40 v/cm.; 2 hr.

Table 6. *Products of partial acid hydrolysis of peptide T2 (Fig. 9a)*

Peptide	Amino acid composition
T2: { (1) (2) (3)	Gly *Try, Gly Ser†(Pro ₂ , Lys).Asp‡

* Ehrlich test positive.

† Identified as dinitrophenyl derivative.

‡ Released by carboxypeptidase.

Table 7. *Products of partial acid hydrolysis of peptide T2 (3) (Fig. 9b)*

Peptide	Amino acid composition*
T2(3): a	Asp
b	Ser
c	Ser, Pro, Pro
d	Ser, Pro
e	Lys, Asp
f	Ser, Pro, Pro, Lys, Asp
g	Pro, Pro, Lys
h	Pro, Lys
i	Lys
Sequence:	Ser.Pro.Pro.Lys.Asp

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

they had been derived from the sequence Ser.Pro.Pro.Lys.Asp, which forms the C-terminal peptide in β -MSH (positions 14-18, Fig. 6). From the results given in Table 6 it is clear that T2 (3) arises from T2 by the cleavage of the Gly.Ser bond and that the structure of T2 is Try.Gly.Ser.Pro.Pro.Lys.Asp. Serine was also shown to be the N-terminal group in T2 (3) by the FDNB method.

The results of the partial acid-hydrolysis studies provide additional confirmation for the structure which we have proposed for the β -melanocyte-stimulating hormone (Fig. 6).

DISCUSSION

Elucidation of amino acid sequence. The application of the Edman (1950) and carboxypeptidase methods of stepwise degradation to the intact octadecapeptide, and to peptide fragments derived from it by selective degradation with specific proteolytic enzymes and isolated by high-voltage ionophoresis on paper, have enabled us to deduce

the complete structure of β -MSH. Contrary to the experience of others (for example, Shepherd *et al.* 1956; Elliott & Peart, 1957), the modified procedures for stepwise analysis by the Edman method (Fraenkel-Conrat *et al.* 1955) gave satisfactory results when applied to β -MSH. Successive phenylthiohydantoin derivatives were obtained in good yields and were not extensively contaminated with non-specific phenylthiohydantoin compounds formed by random cleavage of peptide bonds along the chain. The 'paper-strip' method, which had been previously used to determine the *N*-terminal heptapeptide sequence in corticotrophin (Harris & Li, 1954), was used successfully to determine the *N*-terminal decapeptide sequence in β -MSH, and the entire procedure was carried out on as little as 2.5 mg. (1 μ mole) of the peptide hormone.

It is recognized, however, that β -MSH may be a particularly favourable structure for analysis by the Edman (1950) method, since most of the peptide bonds in the molecule are relatively stable to acid. For example, the peptide fragments Gly.Pro.Tyr.Lys (positions 3-6), and Lys.Met.Glu.His.Phe (positions 6-10), are found to remain partly undegraded after incubation in 12*N*-hydrochloric acid at 37° for 72 and 48 hr. respectively, whereas the *C*-terminal fragment Ser.Pro.Pro.Lys.Asp is partly undegraded even after exposure to 12*N*-hydrochloric acid at 100° for 1 hr. In addition, the two bonds which are relatively labile to acid also occur in positions which facilitated the structural analysis. Thus the lability of the Asp.Glu bond in the *N*-terminal dipeptide ensured the formation, in good yield, of the phenylthiohydantoin derivative of the *N*-terminal amino acid, and the specific lability of the Gly.Ser bond (positions 13-14) led to the isolation of the *C*-terminal fragment Ser.Pro.Pro.Lys.Asp (formed in almost quantitative yield from the fragment Try.Gly.Ser.Pro.Pro.Lys.Asp by incubation in 12*N*-hydrochloric acid at 37° for 48 hr.), which could then be degraded by the Edman method. The presence of three basic and three aromatic amino acids, and the fact that seven amino acid residues occur only once in the peptide chain, also facilitated the formation of fragments of suitable size and stability for subsequent characterization by stepwise analysis.

Peptide fragments containing methionine (C3, T3, T3a), which gave single components in ionophoresis at both pH 3.5 and 6.5, were each found to separate into two components when chromatographed in butanol-acetic acid-water (4:1:5, by vol.). The only detectable difference in each was that the component with the lower R_F contained methionine sulphoxide instead of methionine. However, when the material isolated by ionophoresis was treated with 0.2*M*-thioglycolic acid at

50° for 24 hr. before chromatography, only the component with the higher R_F , containing methionine, was obtained. The relatively mild conditions under which the methionine residue in β -MSH is oxidized to the sulphoxide, and subsequently reduced to methionine, suggests that it is in some way implicated in the reactions by which both β -MSH and corticotrophin are inactivated by peroxide and reactivated by thioglycolic acid (Dedman, Farmer & Morris, 1955; Dixon, 1956*a*), although the subsequent results of Dedman, Farmer & Morris (1957), who find that desulphurized corticotrophin is subject to the same reversible oxidation-reduction phenomenon, suggest that other groups may also be involved.

Enzyme specificity. The hydrolysis of β -MSH by trypsin and chymotrypsin occurred, as expected, only at those peptide bonds which conform to the specificity requirements of the two enzymes (cf. Bergmann & Fruton, 1941), although it should be noted that the bond between the penultimate lysine and *C*-terminal aspartic acid residues was not hydrolysed by trypsin under the conditions used (Expt. 3*a*). The Lys.Ala bond in the *C*-terminal Pro.Lys.Ala sequence in the B chain of native insulin is, however, readily hydrolysed by trypsin, and it can only be inferred that with β -MSH the action of the enzyme is inhibited by the additional negative charge on the side-chain carboxyl group of the *C*-terminal aspartic acid residue.

An interesting feature of the β -MSH molecule is that basic and aromatic amino acids occur in adjacent positions. Consequently the resulting Tyr.Lys and His.Phe.Arg.Try segments of the peptide chain are extremely susceptible to hydrolysis by trypsin and chymotrypsin, and by subtilisin (Harris & Roos, 1959). For example, the Arg.Try linkage was found to be completely hydrolysed by trypsin in 2-3 min. (enzyme-substrate ratio 1:100, by wt.), and by terminating the reaction at this stage it was possible to isolate the fragment containing amino acid residues 1-11 (Fig. 6) in high yield. Under similar conditions the Tyr.Lys and Phe.Arg bonds were completely hydrolysed in 30 min. by chymotrypsin; the Try.Gly bond, on the other hand, is hydrolysed at a much slower rate so that peptide C3 (positions 11-18) remains partly undegraded even after 16 hr. It should be noted, however, that after the rapid hydrolysis of the Phe.Arg bond, the Try.Gly bond occurs near to the *N*-terminal arginine residue and consequently its rate of hydrolysis when it is part of peptide C3 is likely to be considerably slower than is its initial rate of hydrolysis in the intact octadecapeptide.

An interesting feature of the trypsin reaction is the formation of the fragment Lys.Met.Glu.His.

Phe.Arg (peptide T3a), owing to the presence of a small amount of chymotrypsin in the trypsin which was used. Studies with chymotrypsin showed that the Phe.Arg bond is hydrolysed more rapidly than the Tyr.Lys bond in the intact octadecapeptide. However, under the conditions of the trypsin reaction (where the chymotrypsin/ β -MSH ratio is only 1:10 000, by wt.) the *N*-terminal fragment Asp.Glu.Gly.Pro.Tyr.Lys.Met.Glu.His.Phe.Arg is formed extremely rapidly by hydrolysis of the Arg.Try bond, so that the Phe.Arg bond, which is now adjacent to the *C*-terminal group, becomes resistant to hydrolysis by chymotrypsin. The Lys.Met bond is hydrolysed less rapidly by trypsin, and it appears that in some of the molecules the adjacent Tyr.Lys bond is split by chymotrypsin before the Lys.Met is split by trypsin. The peptide Lys.Met.Glu.His.Phe.Arg, which is formed during the initial stages of the reaction, thus tends to accumulate, since the Lys.Met and Phe.Arg bonds, which are highly susceptible to the action of trypsin and chymotrypsin respectively in the intact molecule, occur adjacent to the terminal groups in peptide 3a and consequently become resistant to hydrolysis.

The *C*-terminal sequence ...Pro.Lys.Asp forms a particularly unfavourable substrate for carboxypeptidase, and in view of the specificity requirements of the enzyme (cf. Harris, 1955) it is not surprising that release of the *C*-terminal aspartic acid is slow and incomplete. On the other hand, lysine and tyrosine, and phenylalanine and histidine, are successively released from T1 and C3 respectively; in addition carboxypeptidase was also found to hydrolyse the dipeptide Arg.Try (cf. Geschwind, Li & Barnafi, 1957a). The successive release of lysine and tyrosine from the Pro.Tyr.Lys sequence in peptide T1 may be contrasted with the failure of the enzyme to release amino acids directly attached to proline, in tobacco mosaic virus protein (...Pro.Ala.Thr) and in insulin (...Pro.Lys.Ala). Clearly the affinity of the enzyme for *C*-terminal aromatic residues is sufficient to overcome the inhibitory influences of adjacent proline and (with Arg.Try) of basic *N*-terminal residues.

Structural and activity relationships between β -melanocyte-stimulating hormone and corticotrophin

The nature of the relationship between the melanocyte-stimulating and adrenocorticotrophic properties of the pituitary has been the subject of much controversy. At one time it was thought that the two activities were the property of the same hormone molecule (for example, Johnsson & Högborg, 1952; Sulman, 1952), but even before corticotrophin and β -MSH had been isolated in pure form there was considerable evidence to support the view that the main melanocyte-stimulating activity of the pituitary was not associated with corticotrophin (see, for example, Reinhardt, Geschwind, Porath & Li, 1952). Subsequently highly purified preparations of corticotrophin were invariably found to possess a small but nevertheless constant residual melanocyte-stimulating potency, and it is now well established that this is an intrinsic property of corticotrophin and is not due to contamination with MSH (Bell, 1954; Shepherd *et al.* 1956; Dixon, 1956a).

In view of the relationships which were found to exist between chemical structure and biological activity in the posterior-pituitary hormones oxytocin and vasopressin (du Vigneaud, 1954), there was every reason to expect that possession of melanocyte-stimulating activity by both MSH and corticotrophin would be reflected by a similarity in the arrangement of amino acid residues in their respective molecules. The anticipated structural similarity has now been revealed. As shown in Fig. 10, the heptapeptide Met.Glu.His.Phe.Arg.Try.Gly occurs both in the corticotrophins (Bell, 1954; White & Landmann, 1955; Li *et al.* 1955) (positions 4–10) and in β -MSH (positions 7–13). The tyrosine and proline residues (positions 2 and 12 respectively in corticotrophin) also occur in corresponding positions (5 and 15 respectively) with respect to the common sequence in β -MSH, and but for the transposition of lysine and serine residues between positions 6 and 14 in β -MSH the sequence of amino acids common to both β -MSH and corticotrophin would extend to 11 amino acid

Fig. 10. Amino acid sequence of β -MSH compared with that of the *N*-terminal portion of corticotrophin.

residues. A chemical basis for the intrinsic melanocyte-stimulating activity of corticotrophin has thus almost certainly been revealed.

Corticotrophin is known to be about 1% as active as β -MSH when the melanocyte-stimulating potencies of the two substances are compared by assay *in vivo* in *Rana pipiens* frogs (see, for example, Dixon, 1956*b*). It would appear therefore that corticotrophin does not contain the full complement of structural features which are necessary for full melanocyte-stimulating activity. Alternatively, it could be envisaged that the sequences of the amino acids which in corticotrophin occur on either side of the structure common to both hormones, and which are presumably essential for its adrenal gland-stimulating activity, may at the same time inhibit its potential melanocyte-stimulating potency (see Harris, 1958, 1959; Geschwind *et al.* 1957*a*).

A preliminary communication summarizing the results of this investigation has already been published (Harris & Roos, 1956). The same structure was reported by Geschwind *et al.* (1956, 1957*a*) for the melanocyte-stimulating polypeptide which they had isolated from pig pituitary glands, which showed that it was identical in all respects with the substance isolated originally by Porath *et al.* (1955) and designated β -MSH by Lee & Lerner (1956). Geschwind, Li & Barnafi (1957*b*) have since reported the isolation and structure of a β -type of melanocyte-stimulating hormone from ox pituitary gland; β -MSH (ox) was found to differ from β -MSH (pig) only in position (2) (Fig. 6), where the glutamic acid residue is replaced by serine in the ox hormone. They were, however, unable to confirm the presence, in either pig or ox pituitary glands, of another highly active melanocyte-stimulating substance (α -MSH), which Lee & Lerner (1956) found to be the major melanocyte-stimulating hormone in the pig pituitary gland.

SUMMARY

1. The β -melanocyte-stimulating hormone from pig pituitary gland has been shown to be an octadecapeptide with the amino acid sequence shown at the bottom of the page.

2. The heptapeptide Met.Glu.His.Phe.Arg.Try.Gly is also found in corticotrophin. The tyrosine and proline residues in positions 5 and 15 respectively also occur in corresponding positions with respect to the common sequence in corticotrophin, and but for the transposition of lysine and serine between positions 6 and 14 in β -melanocyte-

stimulating hormone the sequence common to both hormones would extend to 11 amino acids.

3. These common structural features provide a chemical basis for the intrinsic melanocyte-stimulating activity of corticotrophin.

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Studies on Pituitary Polypeptide Hormones

2. THE ACTION OF PEPSIN AND SUBTILISIN ON β -MELANOCYTE-STIMULATING HORMONE

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In the preceding paper (Harris & Roos, 1959) the β -melanocyte-stimulating hormone was shown to be an octadecapeptide containing within its structure a sequence of seven amino acids, Met. Glu. His. Phe. Arg. Try. Gly, which had previously been shown to occur in the corticotrophins. This common structural feature is in all probability an essential constituent of both melanocyte-stimulating and adrenocorticotrophic hormone molecules, and appears to provide a chemical basis for the intrinsic melanocyte-stimulating activity of corticotrophin. Several active degradation products of corticotrophin have been prepared; for example, by limited proteolysis with pepsin (Bell, Howard, Shepherd, Finn & Meisenhelder, 1956), and on the assumption that the complete octadecapeptide structure of β -melanocyte-stimulating hormone may not be essential for its melanocyte-stimulating activity, we have attempted to prepare active fragments by degrading it under carefully controlled conditions with a variety of proteolytic enzymes.

In this paper the degradation of β -melanocyte-stimulating hormone with pepsin and with the bacterial enzyme subtilisin will be discussed.

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MATERIALS

The β -melanocyte-stimulating hormone (β -MSH) was prepared as described previously (Porath, Roos, Landgrebe & Mitchell, 1955). Crystalline pig pepsin was obtained from The Armour Laboratories, Chicago, U.S.A. and crystalline subtilisin, prepared according to Güntelberg & Ottesen (1954), was generously donated by Dr M. Ottesen, Carlsberg Laboratorium, Copenhagen.

METHODS

Action of pepsin. β -MSH (7.0 mg.) was caused to react with pepsin (0.7 mg.) in 0.01N-HCl (1.0 ml.) for 24 hr. at 37°; the enzyme was then inactivated by immersing the solution in a boiling-water bath for 2 min.

Action of subtilisin. (a) β -MSH (5.0 mg.) was dissolved in water (2.0 ml.) and caused to react with subtilisin (0.05 mg.) at pH 8.5 and 30° in the reaction cell of a pH-stat (Jacobsen, Léonis, Linderstrøm-Lang & Ottesen, 1957). A constant pH was maintained by the controlled addition of 0.05N-NaOH and the rate of hydrolysis was followed by recording the uptake of alkali as a function of time. After 20 min. the reaction was terminated by the addition of 0.1N-HCl to pH 3.0.

(b) In a second experiment carried out under the same conditions the subtilisin reaction was allowed to proceed for 8 hr.

Fractionation and characterization of peptide fragments. The peptide fragments derived from β -MSH by hydrolysis with pepsin and subtilisin were fractionated by ionophoresis