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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 Laboratory, 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

on paper in pyridine-acetate buffers at pH 3.5 and 6.5 and by chromatography on paper in butanol-acetic acid-water (4:1:5, by vol.) as described in detail in the preceding paper (Harris & Roos, 1959). The presence of tryptophan, histidine and arginine residues in chromatographically pure peptide fragments was revealed by the use of the appropriate specific reagents (see Block, Durrum & Zweig, 1955), and the complete amino acid composition of each peptide was determined by paper-chromatographic analysis of the respective acid hydrolysates with butanol-acetic acid-water (4:1:5, by vol.) or the two-dimensional system of Levy & Chung (1953) or both. *N*- and *C*-terminal groups were determined by the modified Edman and carboxypeptidase methods respectively as described by Fraenkel-Conrat, Harris & Levy (1955).

Biological assay of enzymic digests of β -melanocyte-stimulating hormone. Tryptic and chymotryptic digests of β -MSH, prepared as described previously (Harris & Roos, 1959), and the pepsin and subtilisin digests described above, were assayed for melanocyte-stimulating activity according to the method of Landgrebe & Waring (1944). Assays were carried out *in vivo* in *Xenopus laevis* and *Rana pipiens* frogs. We are grateful to Professor F. W. Landgrebe and Dr G. M. Mitchell of the Welsh National School of Medicine, and to Dr H. B. F. Dixon, University of Cambridge, for their co-operation in carrying out the assay determinations.

RESULTS

Action of pepsin. No reaction was detected when a solution of β -MSH (0.7% in 0.01N-HCl) was digested with pepsin (enzyme/substrate ratio, 1:100, by wt.) at 25° for 8 hr. When the concentration of enzyme was increased ten times and the reaction mixture was incubated at 37°, a slow reaction was shown to occur, and after 24 hr. no unchanged β -MSH was detected when a sample



Fig. 1. Peptides formed by the action of pepsin on β -MSH, separated by ionophoresis in pyridine-acetate buffer, pH 6.5 (pyridine-acetic acid-water; 10:0.4:90, by vol.); 35–40 v/cm.; 3 hr.

was examined by ionophoresis at pH 6.5 (Fig. 1). As shown, two major components, P₂ and P₅, and three minor components, P₁, P₄ and P₆, were produced by hydrolysis of β -MSH with pepsin.

When chromatographed in butanol-acetic acid-water (4:1:5, by vol.) peptide P₂ was resolved into two components, P2a and P2b, the latter being present in relatively small amount; peptides P₁, P₅ and P₆, on the other hand, chromatographed as pure substances. The chromatographically pure peptides, P₁, P2a, P2b, P₅ and P₆, were analysed for constituent amino acids and for *N*- and *C*-terminal groups, and the results are given in Table 1. Peptide P₄ was present in such small amounts that its amino acid composition could not be determined.

Action of subtilisin. The rate of uptake of alkali when β -MSH (0.25% soln., 2.4 μ moles) was digested with subtilisin (enzyme/substrate ratio, 1:100, by wt.) at pH 8.5 and 30°, is shown in Fig. 2. The reaction was very rapid in the initial stages, and in the first experiment digestion was terminated after 20 min. when approx. 2 μ moles of alkali had been consumed/ μ mole of substrate; on the assumption that 80–100% of the liberated amino groups were being titrated at pH 8.5 (cf. Harris & Roos, 1959), the amount of alkali consumed corresponds to the hydrolysis of approximately two peptide bonds/mole of β -MSH.

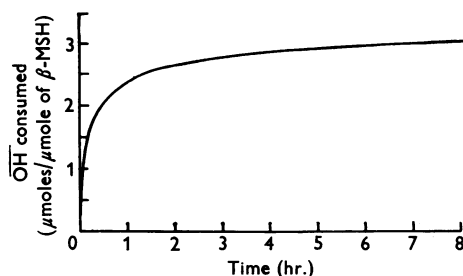


Fig. 2. Rate of uptake of alkali during the reaction of β -MSH (0.25% soln.) with subtilisin (enzyme-substrate ratio, 1:100, by wt.) at pH 8.5 and 30°.

Table 1. Structure of peptide fragments produced by peptic hydrolysis of β -melanocyte-stimulating hormone

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

Peptide (Fig. 1)	Try	His	Arg	<i>N</i> -terminal* residue	<i>C</i> -terminal† residue	Amino acid composition and deduced structure
P ₁	–	–	–	Asp	Glu	Asp.Glu.Gly.Pro.Tyr.Lys.Met.Glu
P2a	–	+	–	Asp	His	Asp.Glu.Gly.Pro.Lys.Met.Glu.His
P2b	–	+	–	Asp	Phe	Asp.Glu.Gly.Pro.Tyr.Lys.Met.Glu.His.Phe
P ₅	+	–	+	Phe	Asp	Phe.Arg.Try.Gly.Ser.Pro.Pro.Lys.Asp
P ₆	+	–	+	.	Asp	Arg.Try.Gly.Ser.Pro.Pro.Lys.Asp

* Identified as their phenylthiohydantoin derivatives.

† Products of reaction with carboxypeptidase.

When a similar reaction was allowed to proceed for 8 hr., 3.0–3.2 μ moles of alkali were consumed/ μ mole of β -MSH, suggesting that 3.0–3.5 peptide bonds had been hydrolysed by the enzyme.

Samples of the two digests, withdrawn after 20 min. and 8 hr. respectively, were submitted to ionophoresis at pH 6.5 and the distribution of ninhydrin-reacting components is shown in Fig. 3. The main products of hydrolysis, peptides S1, S2, S3, S6, S7, S8 and S9, were tested for homogeneity by chromatography in butanol–acetic acid–water (4:1:5, by vol.). Peptides S1, S3, S6, S7 and S9 appeared to be chromatographically pure, whereas S2 and S8 each separated into two components, S2a and S2b and S8a and S8b respectively (Table 2).

Peptides S1, S2a, S2b, S3, S6, S7, S8a, S8b and S9, together with S4 and S5, which were present in relatively minor quantities in the 20 min. digest only, were examined for constituent amino acids by specific colour tests carried out on the intact peptides, and by chromatography of their acid

hydrolysates; in some cases *N*- and *C*-terminal-group analysis was also carried out. The results are summarized in Table 2.

All the components separated by ionophoresis represent single peptides which have been derived from β -MSH by degradation with subtilisin. Peptides S2a and S8a contained methionine sulphoxide and represent oxidized forms of the methionine-containing peptides S2b and S8b respectively. This phenomenon was also encountered among the products of trypsin and chymotrypsin digestion of β -MSH and, as described previously (Harris & Roos, 1959), the methionine-containing peptides can be regenerated from the corresponding sulphoxide derivatives by incubation with 0.2M-thioglycollic acid at 50°. Peptides S2, S4 and S5 represent intermediate peptide fragments which are present only during the early stages of hydrolysis (Fig. 3).

Sites of hydrolysis. From the results presented in Tables 1 and 2 the sites of hydrolysis of β -MSH by pepsin and subtilisin are established as shown in Fig. 4.

Biological assay of enzymic digests. Degradation products of β -MSH, derived from it by degradation with trypsin and chymotrypsin (Harris & Roos, 1959), and with pepsin and subtilisin, as described in the present paper, were assayed for melanocyte-stimulating activity according to the method of Landgrebe & Waring (1944). The peptide fragments listed in Table 3 were present in the various partial enzymic digests which were used for biological assay. In each the enzyme reaction had been terminated by heating the reaction solutions at 100° for 2 min. at pH 3.0. Solutions of undegraded β -MSH, which had been subjected to the same conditions in the absence of enzyme, were used as controls.



Fig. 3. Peptide fragments present in subtilisin hydrolysates of β -MSH, fractionated by ionophoresis in pyridine–acetic acid buffer, pH 6.5 (pyridine–acetic acid–water; 10:0.4:90, by vol.); 35–40V/cm.; 2 hr.; A, 20 min. sample; B, 8 hr. sample.

Table 2. Structure of peptide fragments present in subtilisin digests of β -melanocyte-stimulating hormone

Try, His and Arg were identified as described in Block *et al.* (1955).

Peptide (Fig. 3)	Try	His	Arg	<i>N</i> -terminal* residue	<i>C</i> -terminal† residue	Amino acid composition and deduced structure
S1	–	–	–	Asp	Tyr	Asp. Glu. Gly. Pro. Tyr
S2a	–	–	–	Asp	Met‡	Asp. Glu. Gly. Pro. Tyr. Lys. MetSO
S2b	–	–	–	Asp	Met	Asp. Glu. Gly. Pro. Tyr. Lys. Met
S3	–	+	–	Glu	Phe	Glu. His. Phe
S4	+	Glu. His. Phe. Arg. Try. Gly. Ser. Pro ₂ . Lys. ^A
S5	Lys. Met. Glu. His. Phe
S6	+	–	+	.	Asp	Arg. Try. Gly. Ser. Pro ₂ . Lys. Asp
S7	+	–	+	.	.	Arg. Try
S8a	.	–	–	.	.	Lys. MetSO
S8b	.	.	–	.	.	Lys. Met
S9	–	–	–	Gly	Asp	Gly. Ser. Pro. Pro. Lys. Asp

* Identified as their phenylthiohydantoin derivatives.

† Products of reaction with carboxypeptidase.

‡ Met released only after incubating the peptide with 0.2M-thioglycollic acid (50°; 12 hr.). No reaction was observed when Met sulphoxide (MetSO) occurred as the *C*-terminal amino acid.

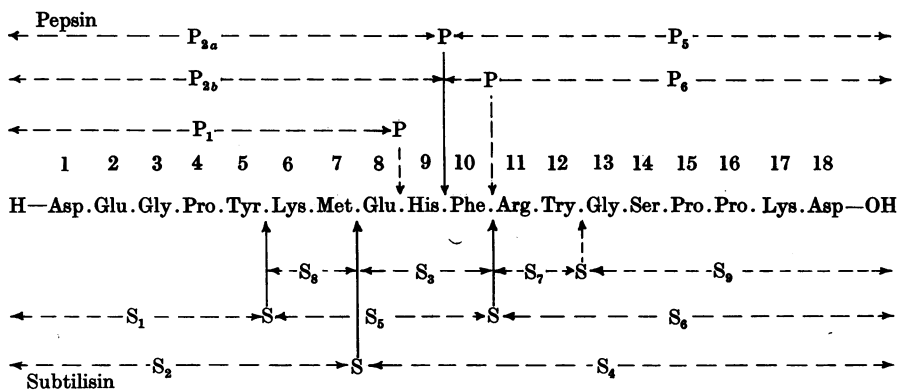


Fig. 4. Hydrolysis of β -MSH by pepsin (P) and subtilisin (S). \uparrow , Major sites of hydrolysis. \uparrow , Minor sites of hydrolysis. (See Tables 1 and 2.)

Table 3. Peptide fragments present in partial enzymic digests of β -melanocyte-stimulating hormone, assayed for melanocyte-stimulating activity

Italicized residues form part of the 'common sequence'.

Enzyme	Peptide fragments identified in partial enzymic digests
Trypsin*	Asp. Glu. Gly. Pro. Tyr. Lys. <i>Met. Glu. His. Phe. Arg</i> (T4) <i>Try. Gly. Ser. Pro. Pro. Lys. Asp</i> (T2)
Chymotrypsin†	Asp. Glu. Gly. Pro. Tyr. Lys. <i>Met. Glu. His. Phe</i> (C4) <i>Arg. Try. Gly. Ser. Pro. Pro. Lys. Asp</i> (C2)
Pepsin‡	Asp. Glu. Gly. Pro. Tyr. Lys. <i>Met. Glu. His</i> (P2a) <i>Phe. Arg. Try. Gly. Ser. Pro. Pro. Lys. Asp</i> (P5)
Subtilisin§	Asp. Glu. Gly. Pro. Tyr. Lys. <i>Met</i> (S2) <i>Glu. His. Phe. Arg. Try. Gly. Ser. Pro. Pro. Lys. Asp</i> (S4)

* Fig. 3, Harris & Roos (1959).

† Fig. 5, Harris & Roos (1959).

‡ Fig. 1, this paper.

§ Fig. 3, this paper.

Since the yields of the various peptide fragments differed considerably, varying between 5 and 10% for C4 and S4 to approx. 80% for P2a and P5 (Table 3), assay determinations were carried out with up to 100 times the amount (in terms of equivalents of β -MSH) of undegraded β -MSH which was required to give a maximum response. Under these conditions no significant melanocyte-stimulating activity was detected in any of the digests studied.

DISCUSSION

Structure and activity of β -melanocyte-stimulating hormone. Some of the peptide fragments listed in Table 3 were present in relatively small amounts; the subtilisin digest, for example, contained only 5–10% of peptide S4, and in this case it is possible that an activity corresponding to 0.1–0.5% of the undegraded hormone might not have been detected despite the large dose which was employed. In addition, assays were in all cases carried out on unfractionated enzymic digests, and the possibility that the activity of a potentially active fragment

may have been inhibited in the presence of other products of digestion has not been eliminated.

Nevertheless, both the *N*-terminal peptide fragment, T1 (positions 1–11 in β -MSH), and the *C*-terminal fragment, S4 (positions 8–18 in β -MSH), which contain five and six respectively of the seven amino acid residues which constitute the heptapeptide structure common to both corticotrophin and β -MSH, appear to be inactive as melanocyte-stimulators. Thus in all the cases which have been studied the selective hydrolysis of any one of the peptide bonds within the common sequence leads to extensive inactivation of the hormone, which suggests that this key heptapeptide structure is an essential constituent of a highly active melanocyte-stimulating polypeptide.

Specificity of pepsin. Systematic studies of the specificity requirements of the more well-defined and characterized proteolytic enzymes have been undertaken mainly with reference to synthetic substrates of relatively simple structure. The pioneer studies of Bergmann and his associates (for review see Bergmann & Fruton, 1941) gave rise to the general belief that the specificity of an enzyme

was determined by the nature of the side chains, and of other functional groups, present in the amino acid residues which formed the immediate environment of a susceptible peptide bond. More recently longer peptide chains of known amino acid sequence, such as the oxidized A and B chains of insulin (Sanger & Tuppy, 1951; Sanger & Thompson, 1953), and oxidized ribonuclease (Hirs, Stein & Moore, 1956), have become available for study, and although the specificity requirements originally postulated for trypsin have been found to apply to a variety of naturally occurring peptides and proteins, pepsin has been found to possess a much broader and less predictable specificity than was envisaged from the original studies on synthetic substrates (cf. Sanger & Tuppy, 1951; Bailey, Moore & Stein, 1956; Cole, Li, Harris & Pon, 1956). With α -corticotrophin, for example, several bonds, including Phe.Pro, Ala.Phe, Ser.Glu, Ala.Ser, Asp.Glu and Asp.Asp, which occur in the C-terminal ...Asp.Asp.Glu.Ala.Ser.Glu.Ala.Phe.Pro.Leu.Glu.Phe sequence (positions 28-39) in the molecule, were found to be highly susceptible to hydrolysis by pepsin (Cole *et al.* 1956). Prolonged digestion with higher concentrations of pepsin also resulted in the slow hydrolysis of the Glu.His bond (positions 8-9) in the N-terminal portion of the corticotrophin molecule (White & Landmann, 1955; Li *et al.* 1955).

In the present investigation it has been shown that although β -MSH is highly resistant to the action of pepsin, prolonged reaction with a high concentration of enzyme results in the hydrolysis, not as might have been expected, of the Glu.His bond as in corticotrophin, but mainly of the adjacent His.Phe bond in the N-terminal Asp.Glu.Gly.Pro.Tyr.Lys.Met.Glu.His.Phe section of the molecule.

Thus although the Glu.His and His.Phe bonds occur as part of the same common heptapeptide structure in both corticotrophin and β -MSH, the major sites of action of pepsin appear to be different in the two molecules. Unless crystalline pepsin preparations from different sources contain variable proportions of more than one enzyme, it must be concluded that factors other than the nature of the side chains of the amino acids forming a potentially susceptible bond are involved in determining the 'specificity sites' of this enzyme.

Specificity of subtilisin. Although the specificity requirements for the hydrolysis of peptide bonds by the bacterial enzyme subtilisin (Güntelberg & Ottesen, 1954) have not been clearly established, all the available evidence suggests that crystalline preparations of this enzyme exhibit a very broad specificity towards a variety of peptide and protein substrates. Under carefully controlled and well-

defined conditions, subtilisin is capable of degrading native proteins such as ovalbumin (Linderström-Lang & Ottesen, 1947; Ottesen, 1958) and ribonuclease (Kalman, Linderström-Lang, Ottesen & Richards, 1955; Richards, 1955) in a highly selective manner, but in other cases, such as insulin (Haugaard & Haugaard, 1955; Meedom, 1955), the B chain of oxidized insulin (Tuppy, 1953), and glucagon (Sinn, Behrens & Bromer, 1957), extensive and apparently non-specific degradation was found to occur. In the oxidized B chain of insulin, for example, 18 out of 30 bonds were ultimately found to be susceptible to hydrolysis by subtilisin.

The present investigation reveals that a total of four out of the 17 bonds in β -MSH are susceptible to hydrolysis by subtilisin; three bonds, Met.Glu, Tyr.Lys and Phe.Arg, are hydrolysed rapidly and at approximately comparable rates, whereas a fourth bond, Try.Gly, is hydrolysed at a considerably slower rate. The presence of appreciable amounts of the intermediate fragment Asp.Glu.Gly.Pro.Tyr.Lys.Met (peptide S2), and to a lesser extent of Glu.His.Phe.Arg.Try.Gly.Ser.Pro.Pro.Lys.Asp (peptide S4), during the early stages of the reaction indicates that on the average the Met.Glu bond is hydrolysed more rapidly than the others. If one were to consider one particular molecule then once this initial cleavage has occurred, the other susceptible bonds, Tyr.Lys and Phe.Arg, find themselves in an appreciably different environment; thus the proximity of the Tyr.Lys bond to the newly formed C-terminal methionine residue in peptide S2 might be expected to decrease its rate of hydrolysis relative to that of the Phe.Arg bond in peptide S4, and as a result fragment S2 accumulates to a greater extent than S4 during the course of digestion.

On the other hand, the transient formation of the fragment Lys.Met.Glu.His.Phe (peptide S5, Fig. 4) shows that in a certain proportion of intact molecules the Tyr.Lys bond is split before the Met.Glu bond; the rate of hydrolysis of the Met.Glu bond in these molecules would then be expected to decrease owing to its proximity to the N-terminal lysine residue, so that peptide S5 tends to accumulate during the early stages of the reaction. However, as the reaction proceeds all the intermediate fragments eventually disappear; the only one found to remain after 8 hr. is Arg.Try.Gly.Ser.Pro.Pro.Lys.Asp (peptide S6, Fig. 3), and its survival may in part be due to the influence of the N-terminal arginine residue on the rate of hydrolysis of the neighbouring Try.Gly bond (cf. Harris & Roos, 1959).

The general course of the reaction of subtilisin with β -MSH is reflected in the general shape of the alkali-uptake curve shown in Fig. 2. The rapid

initial reaction, during which approximately three bonds are split per mole of substrate, occurs during the first 90–120 min.; the subsequent part of the hydrolysis curve represents the slower hydrolysis of the Try.Gly bond in peptide S6, and if the reaction had been allowed to go to completion, the β -MSH molecule would have been completely degraded into the five peptide fragments S1, S8, S3, S7 and S9 (Fig. 4).

The action of subtilisin on β -MSH has many features in common with its action on glucagon (Sinn *et al.* 1957), in which the following bonds were found to be split: Glu(NH₂).Gly, Phe.Thr, Ser.Asp, Ser.Lys, Tyr.Leu, Arg.Arg, Glu(NH₂).Asp, Phe.Val, Try.Leu and Met.Asp(NH₂). In almost every case hydrolysis has occurred, either at bonds involving the carboxyl group of aromatic amino acids, methionine and glutamine, or at bonds involving the amino group of both basic and acidic amino acids. The four bonds which are hydrolysed in β -MSH, Met.Glu, Phe.Arg, Tyr.Lys and Try.Gly, fall into one or other of these two categories, and the first three bonds could be considered to combine both of these structural features.

These observations, however, do not appear to apply in all cases, since peptide bonds of an entirely different nature are found to be hydrolysed in insulin (Meedom, 1955) and in the B chain of oxidized insulin (Tuppy, 1953). For example, bonds involving the carboxyl group of leucine residues in glucagon, and of glutamic acid residues in both glucagon and β -MSH, are not attacked to any appreciable extent by subtilisin, whereas in insulin bonds of this type were found to be highly susceptible to the action of the enzyme. Subtilisin does not in fact appear to possess any well-defined 'residue specificity', and for all practical purposes its apparent specificity seems to be largely determined by the overall amino acid sequence and configuration of the substrate.

Thus in any particular substrate a limited number of bonds occurring in certain favourable positions in the structure of the intact molecule are likely to be hydrolysed much more rapidly than others. Once these initial cleavages have occurred, other bonds, which are potentially susceptible to hydrolysis in the intact molecule, become relatively resistant to the action of the enzyme when they occur in proximity to newly formed *N*- or *C*-terminal groups (cf. Harris & Roos, 1959). Hence with subtilisin the sites of initial hydrolysis may play a more important role in determining the subsequent course of hydrolysis than the presence of any predictable combinations of specific amino acid residues. By way of contrast, enzymes such as trypsin, which have a well-defined 'residue specificity', attack peptide bonds at predictable

and reproducible sites, so that the ultimate pattern of hydrolysis is largely independent of the relative initial rates of hydrolysis of the various susceptible bonds.

SUMMARY

1. The action of pepsin and subtilisin on the β -melanocyte-stimulating hormone has been studied. The sites of hydrolysis by the two enzymes have been identified and the peptide fragments produced have been isolated and characterized.

2. Partial enzymic digests of β -melanocyte-stimulating hormone, produced by selective hydrolysis of the octadecapeptide with trypsin, chymotrypsin, pepsin and subtilisin, have been assayed for melanocyte-stimulating activity and found to be relatively inactive. These results suggest that the cleavage of any one of the peptide bonds which occurs within the heptapeptide structure Met.Glu.His.Phe.Arg.Try.Gly, common to both β -melanocyte-stimulating hormone and corticotrophin, leads to extensive inactivation of the hormone.

3. The specificities of pepsin and subtilisin are discussed in the light of their action on β -melanocyte-stimulating hormone and other substrates.

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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

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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).