above, the small amounts of contaminating amino acids apparently present in their preparation. A similar problem with contaminants is evident in the data of Porath, *et al.*[7]; moreover, methionine is absent from the qualitative analysis presented by these authors, at the same time that they report that no sulfur-containing amino acids had been found to be present. Also, the analysis of 24- and 48-hr. hydrolysates presented in Table I of this paper gives no evidence in support of the claim of these authors that certain peptide bonds in β-MSH are particularly resistant to acid hydrolysis.[7]

The minimum molecular weight of β-MSH calculated on the basis of the amino acid content is 2177, a value which, as it has been pointed out above, represents the actual molecular weight of the hormone peptide. A molecular weight of 2900 for β-MSH has been determined independently by equilibrium centrifugation.[31]

Another value that may be computed from the amino acid composition is the isoionic point of β-MSH; as pointed out earlier, the calculated value for the isoionic point, *p*H 5.9, is almost identical with the experimental value for the isoelectric point, *p*H 5.8, determined in buffers of 0.1 ionic strength. A value of *p*H 5.2 was reported by Porath and co-workers[7] for the isoelectric point of their MSH preparation in 0.05 M phosphate buffers. Both of these values differ considerably from that reported for the isoelectric point of α-MSH,[5] which is between *p*H 10.5 and *p*H 11.

In view of all the foregoing comparative data, there would appear to be little doubt that the MSH peptide purified by Benfey and Purvis and by Porath, *et al.*, is identical with the β-MSH whose isolation has been described in this paper, just as there can be equally little doubt that α-MSH of Lerner and Lee[5] differs considerably. It is of interest that none of the workers, including ourselves, who have attempted to purify β-MSH have reported any evidence for the existence of α-MSH. Although we have specifically sought it in the course of the present work, we have failed to detect melanocyte-stimulating activity in any peptide as basic as α-MSH. At present, there seems to be no obvious explanation for this discrepancy.

Finally, a few words about the assay methods are in order. All of our earlier assays were performed on hypophysectomized *Rana pipiens* and were quantitated by means of the change in the melanophore index[32] as detected in the web. Subsequently, the assay was made more objective by employing the *in vitro* frog skin method.[12] Excellent agreement has been found between assay results obtained with the two methods. Furthermore, the activities reported above are in good agreement with those reported by other workers for β-MSH. Recently Eakin[33] reported that some of our crude MSH concentrate, containing no more than about 10% β-MSH, stimulated the melanocytes of the albino-hypophysectomized tadpole of *Hyla regilla*, when 8×10^{-6} μg. was injected. Hence, this latter method of assay can detect approximately 10^{-6} μg. of β-MSH.

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(31) A. Ginsburg, P. Appel and H. K. Schachman, *Arch. Biochem. Biophys.*, in press. We are indebted to Miss Pearl Appel and Dr. H. K. Schachman for the molecular weigbt determination. The details of the determination will be reported elsewhere by these investigators.

(32) L. Hogben and D. Slome, *Proc. Roy. Soc.* (*London*), **B120**, 158 (1936).

(33) R. M. Eakin, *J. Exp. Zool.*, **131**, 263 (1956).

BERKELEY, CAL.

[CONTRIBUTION FROM THE HORMONE RESEARCH LABORATORY AND THE DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF CALIFORNIA, BERKELEY]

The Structure of the β-Melanocyte-stimulating Hormone

BY IRVING I. GESCHWIND, CHOH HAO LI AND LIVIO BARNAFI

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By means of chemical and enzymatic degradations, the structure of β-MSH has been shown to be: H-Asp.Glu.Gly.Pro.Tyr.Lys.Met.Glu.His.Phe.Arg.Try.Gly.Ser.Pro.Pro.Lys.Asp-OH (residues numbered 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18). It has been shown previously that the sequence Met.Glu.His.Phe.Arg.Try.Gly also exists in the corticotropins. The implications of these findings have been discussed.

In the preceding paper[1] the isolation of the β-melanocyte-stimulating hormone (MSH) from porcine pituitary glands has been described and its characterization presented. The hormone has been found to be an octadecapeptide with the following composition: $Asp_2Glu_2Ser_1Gly_2Pro_3Met_1Phe_1Tyr_1Lys_2His_1Arg_1Try_1$. In view of the well-known specificities of trypsin and chymotrypsin, the presence of only 3 residues of lysine and arginine together and of 3 aromatic residues suggested that digestion with either of these enzymes should result in a small number of cleavage products whose structural sequence should be relatively easy to determine. Furthermore, since 7 amino acids are present as single residues in the hormone, the

(1) I. I. Gescbwind and C. H. Li, THIS JOURNAL, **79**, 615 (1957).

probability of obtaining an unambiguous sequence was enhanced. This paper presents the results of such studies, which have led to the elucidation of the amino acid sequence of the β-MSH molecule.[2a] An identical amino acid sequence for β-MSH has been independently formulated by Harris and Roos.[2b]

Experimental

The N-terminal residue of the β-MSH molecule was determined by the technique of Sanger,[3] and the dinitrophenyl (DNP-) amino acid was quantitatively identified by the 2-dimensional paper chromatographic method of Levy.[4] The C-terminal amino acid was determined by the Akabori procedure,[5,6] reaction with anhydrous hydrazine being carried out for 10 hr. at 105° on 3 mg. of β-MSH. The C-terminal amino acid liberated by this procedure was identified as its DNP derivative.

The sequence of the amino acids at the N-terminus was determined by the paper-strip modification[7] of the phenyl isothiocyanate (PTC) method.[8] Approximately 4 mg. of β-MSH was used for each experiment. The phenylthiohydantoins formed at each step in two complete series of experiments were identified following chromatography on paper in both the heptane–pyridine and in one of the heptane–n-butanol–formic acid systems of Sjöquist.[9] In a third series of experiments the phenylthiohydantoins were hydrolyzed with 5.7 N HCl at 150° for 16 hours[10] to regenerate the parent amino acids, which were identified after chromatography in the system n-butanol–acetic acid–water.[11]

In order to determine the sequence at the C-terminus, kinetic investigations on the action of carboxypeptidase (Worthington) that had been treated with a 50-fold molar excess of diisopropyl fluorophosphate (DFP) were undertaken. In a typical experiment 5 mg. of the hormone was dissolved in 0.9 ml. of 1% $NaHCO_3$, and 0.6 mg. of DFP-treated carboxypeptidase in a volume of 0.1 ml. was added; the solution was then incubated at 38°. At 0, 4, 8 and 20 hr. aliquots were removed and allowed to react with fluorodinitrobenzene (FDNB). The DNP amino acids were extracted from the acidified solution with ether and identified by 2-dimensional chromatography on paper.[4]

For the enzymic digestion, 20 mg. of β-MSH in aqueous solution (2 ml.) were incubated at 38° with 0.5 mg. of trypsin[12] or chymotrypsin (Armour); the pH of the solution was adjusted to approximately 8 with NH_4OH. Tryptic digestion was allowed to proceed for 6–8 hr., whereas chymotryptic digestion was carried out for 18–24 hr. The products of digestion were separated by zone electrophoresis on paper. A sheet of Whatman 3 MM filter paper measuring $17^1/_4 \times 11^1/_2$ inches was prepared, and a line bisecting the longer dimension was drawn across the paper. The entire volume of hydrolysate was then transferred to this line by successive pipettings. In this manner a band about $^1/_4$ of an inch wide was produced. The entire paper was wetted evenly with the buffer (γ-collidine–acetic acid–water, pH 7.0)[13] to within 1 inch of the line and was then placed in a paper electrophoresis apparatus (Durrum type; Spinco Instruments Corp.). The buffer was allowed to rise on each side of the sheet until the two flows merged at the apex. In this manner the original band was compressed into a very narrow line. The run was carried out for 6 hr. at 4° at a potential gradient of 11 volts per cm.; the paper was then dried, and narrow guide-strips were cut from both edges of the paper and sprayed with ninhydrin. The pattern obtained with the tryptic digest is shown in Fig. 1, that with the chymotryptic digest, in Fig. 2.

Appropriate reagents were employed to detect the presence on the paper of arginine,[14] tyrosine,[15] histidine[16] and tryptophan[17] in each of these peptides. In addition, the homogeneity of each peptide was determined by chromatography on paper in the systems n-butanol–acetic acid–water and butanol–acetic acid–pyridine–water.[18] Each band isolated by zone electrophoresis gave rise to only a single spot in paper chromatography. Figure 3 presents a composite pattern of chromatograms (system, n-butanol–acetic acid–water) of the individual peptides derived from chymotryptic digestion.[19]

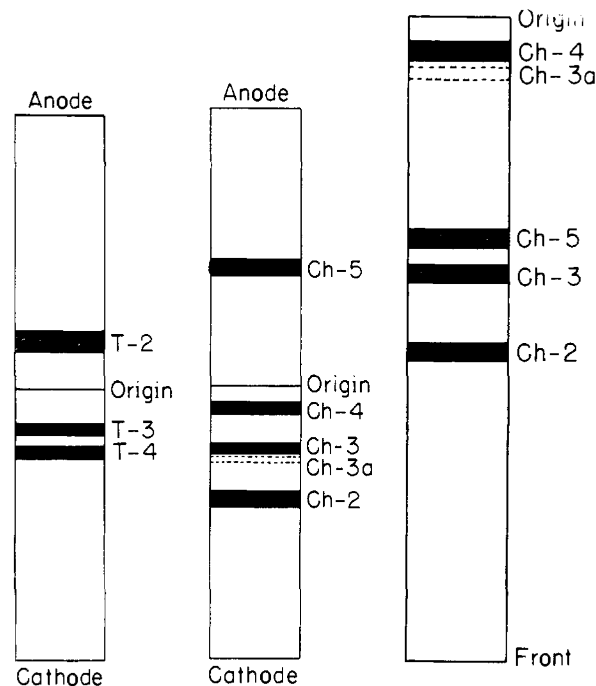


Fig. 1.—(Left) resolution of tryptic digest of β-MSH by zone electrophoresis on paper. System, γ-collidine–acetic acid–water at pH 7.0; 11 volts/cm. for 6 hr.

Fig. 2.—(Center) resolution of chymotryptic digest of β-MSH by zone electrophoresis on paper. Conditions the same as in Fig. 1.

Fig. 3.—(Right) composite pattern of chromatograms of the individual peptides obtained from paper electrophoresis of the chymotryptic digest of β-MSH. System, n-butanol–acetic acid–water.

The areas containing the peptides derived from electrophoresis were located on the paper by means of the guide strip pattern and were excised. To one end of each strip a one-inch length of filter paper was stapled, and the strip was placed, stapled end down, in a large test-tube to which 2 ml. of 1 N NH_4OH had been added; the tube was then stoppered. In this manner all of the peptide was concentrated at the upper edge of the strip by the ascending solvent. The strip was then removed and dried, and 1 inch was cut

(2a) A preliminary account of these findings has been presented (I. I. Geschwind, C. H. Li and L. Barnafi, THIS JOURNAL, **78**, 4494 (1956)).

(2b) J. I. Harris and P. Roos, *Nature*, **178**, 90 (1956).

(3) F. Sanger, *Biochem. J.*, **39**, 507 (1945).

(4) A. L. Levy, *Nature*, **174**, 126 (1954).

(5) S. Akabori, K. Ohno and K. Narita, *Bull. Chem. Soc. Japan*, **25**, 214 (1952).

(6) C. I. Niu and H. Fraenkel-Conrat, THIS JOURNAL, **77**, 5582 (1955).

(7) H. Fraenkel-Conrat, *ibid.*, **76**, 3606 (1954).

(8) P. Edman, *Acta Chem. Scand.*, **4**, 283 (1950).

(9) J. Sjöquist, *ibid.*, **7**, 447 (1953).

(10) A. L. Levy, *Biochim. Biophys. Acta*, **15**, 589 (1954).

(11) S. M. Partridge, *Biochem. J.*, **42**, 238 (1948).

(12) We are indebted to Dr. M. A. Mitz of the Armour Laboratories for a gift of the highly purified trypsin which was employed.

(13) I. M. Lockhart and E. P. Abraham, *Biochem. J.*, **58**, 633 (1954).

(14) J. P. Jepson and I. Smith, *Nature*, **172**, 1100 (1953).

(15) R. Acher and C. Crocker, *Biochim. Biophys. Acta*, **9**, 704 (1952).

(16) F. Sanger and H. Tuppy, *Biochem. J.*, **49**, 463 (1951).

(17) I. Smith, *Nature*, **171**, 43 (1953).

(18) S. G. Waley and J. Watson, *Biochem. J.*, **55**, 328 (1953).

(19) Although comparable over-all patterns were obtained with the unfractionated materials, the bands in the latter instance proved to be markedly crescent-shaped, and several double spots which seemed to be separated by salt fronts (trichloroacetate?) were apparent. Thus, chromatography on paper did not seem to be practicable for separating these peptides on a large scale. In the case of the tryptic peptides, prolonged chomatography was required to resolve all the peptides.

from the upper edge for elution of the peptide.[20] The peptide was then eluted from this small area of paper by allowing 1 N NH_4OH to flow on at the top edge and to drip off the bottom; the eluate was taken to dryness on a steam-bath.

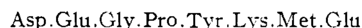
Approximately 0.5 μmole of each peptide was hydrolyzed in 5.7 N HCl for 24 hr. The hydrolysate was then taken for duplicate quantitative amino acid analyses by the method of Levy.[4] A second aliquot (0.5 μmole) was used for N-terminal amino acid analysis by the DNP method. In the case of the peptides derived from chymotryptic digestion, a comparable amount of each peptide was treated for 24 hr. at room temperature with 0.5 mg. of carboxypeptidase in 1% $NaHCO_3$. The solution was then allowed to react with FDNB, and the DNP-amino acids were identified as described above.

Aliquots (2 μmoles) of peptides Ch-3 and Ch-4 (Fig. 2) were also taken for stepwise degradation by a modification[21] of the phenyl isothiocyanate reaction. The procedure was designed so that one complete step could be performed each day. In this manner Ch-3 was degraded for 3 steps and Ch-4 for 4 steps. The phenylthiohydantoins formed were identified after chromatography on paper in the heptane-pyridine system and by regeneration of the parent amino acid. In addition, an aliquot of the aqueous solution that remained at each step after extraction of the phenylthiohydantoins was taken for N-terminal amino acid analysis by the DNP procedure.

Results and Discussion

N- and C-Terminal Amino Acid Analyses and N-Terminal Amino Acid Sequence.—When β-MSH was allowed to react with FDNB, the sole N-terminal amino acid whose presence could be demonstrated was aspartic acid, which was obtained in a yield of 0.6 mole/mole. Since from all the proteins hitherto studied in which aspartic acid occurs in an N-terminal position, its recovery as its DNP derivative has amounted to 0.6–0.7 mole/mole,[22–24] it can be assumed that β-MSH possesses 1 g. mole of N-terminal aspartic acid per 2200 g. These dinitrophenylation experiments permit a determination of the availability of the ϵ-amino groups of the two lysine residues present in the hormone. The optical density of the aqueous solution remaining after extraction of the ether-soluble DNP amino acid was determined at 390 mμ and the amount of ϵ-DNP lysine was determined by calculations based on a millimolar extinction coefficient of 10.2.[25] Without the application of any correction factors for destruction during hydrolysis, 1.68 moles/mole were found; this indicates that both ϵ-amino groups are free to react.[26]

Aspartic acid was also the sole amino acid whose presence could be demonstrated following the first step of the stepwise degradation of β-MSH from its N-terminus by means of the phenylthiohydantoin method. This procedure was applied successfully for 8 successive steps but could be carried no further because the paper support had fallen apart. The octapeptide sequence determined in this manner was



Asp.Glu.Gly.Pro.Tyr.Lys.Met.Glu

At all steps in this procedure the yields in terms of optical density[7] were much greater than the apparent intensity of the appropriate phenylthiohydantoin areas on a chromatogram; thus no values for recovery are presented. The identification of all amino acids in this sequence proved unambiguous. This N-terminal sequence accounts for all the residues of glutamic acid, tyrosine and methionine found in the molecule of MSH, and consequently the presence of any of these in any product of enzymatic hydrolysis would indicate a peptide derived from near the N-terminus.

By means of the hydrazinolysis method, aspartic acid was found also as the C-terminal amino acid; it was obtained in the form of its DNP-derivative in a yield amounting to 13% of theory. Similar poor recoveries of C-terminal aspartic acid by this method have previously been reported.[6] The C-terminal position of this amino acid was confirmed following digestion with DFP-treated carboxypeptidase. It was the sole amino acid that

(20) When the remainder of the strip was sprayed with ninhydrin, little or no coloration was observed, indicating the absence of peptide material.

(21) H. Fraenkel-Conrat and J. I. Harris, THIS JOURNAL, **76**, 6058 (1954).

(22) E. O. P. Thompson, *Biochim. Biophys. Acta*, **10**, 633 (1953).

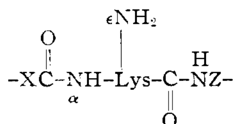
(23) E. O. P. Thompson, *J. Biol. Chem.*, **208**, 565 (1954).

(24) E. W. Davie and H. Neurath, *ibid.*, **212**, 507 (1955).

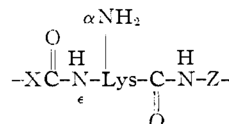
(25) F. Sanger, *Biochem. J.*, **45**, 563 (1949).

(26) It is not possible to decide simply on the basis of these data whether it is the α- or the ϵ-amino groups of lysine that are free. Since it is known that the separation of α-DNP lysine from ϵ-DNP lysine is difficult, assignment of the positions of the free amino groups must depend upon an entirely different consideration, one which, to our knowledge, has not been brought to notice previously.

If we designate a sequence in which lysine is bound through its α-amino group as

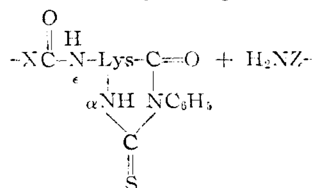


(with X and Z the side chains of the amino acid residues bound to lysine) and if we designate a sequence in which lysine is bound through its ϵ-amino group as



then after reaction with phenyl isothiocyanate of the entire molecule in which such sequences exist, the α- or ϵ-amino groups will occur as phenylthioureido groups, *i.e.*, as $-NHSCHNC_6H_5$.

If such a substituted molecule is then placed in a strongly acid medium, cyclization to the phenylthiohydantoin and rupture of the Lys-Z peptide bond may occur only in the case of the α-phenylthioureido group. The lysine phenylthiohydantoin that is formed, however, would still be bound to X through its ϵ-amino peptide bond, so that the products of reaction may be designated as



When the complete reaction with phenyl isothiocyanate is repeated, two phenylthiohydantoins will be liberated: the expected one next to the original N-terminal amino acid and the phenylthiohydantoin of Z. In the case of β-MSH, the two lysines are present in the sequences ...Tyr. Lys.Met... and ...Pro.Lys.Asp. Consequently, if any ϵ-amino peptide linkage occurred, the phenylthiohydantoins of either methionine or aspartic acid should be found after the second step of the stepwise degradation. In actuality there is found only the phenylthiohydantoin of glutamic acid, arising from the second amino acid residue in β-MSH. These results were confirmed when the aqueous residue remaining after the extraction of the phenylthiohydantoins produced during the first step was dinitrophenylated. After hydrolysis, only DNP-glutamic acid could be found. Therefore, it may be concluded that both lysines are bound in the peptide linkage through their α-amino groups and that it is the ϵ-amino groups that are free to react with FDNB.

could be detected free after as long as 24 hr. of enzymatic digestion, and even after such prolonged periods of digestion, the yields obtained were only about 15% of theory. This suggested that the penultimate amino acid was one which was markedly resistant to the action of carboxypeptidase.

When the carboxypeptidase (3 × recrystallized) was not pre-treated with DFP, phenylalanine also appeared during the reaction with β-MSH. This amino acid was usually present in yields 4 to 6 times greater than those of aspartic acid, except after short periods of digestion (less than 2 hr.). The presence of aspartic acid at both termini of β-MSH accounts for all the aspartic acid found in the molecule.

Digestion with Chymotrypsin.—If chymotrypsin can be considered to possess any sharp specificity, then only four peptides should be found in a chymotryptic digest of β-MSH, since only the peptide bonds adjacent to the one residue each of tyrosine, tryptophan and phenylalanine should be cleaved by this enzyme. As evident in Fig. 2, only 4 main bands are found (band 3a represents a peptide, found only occasionally, which is a product of incomplete digestion and which has been shown by quantitative amino acid analysis to be made up of the amino acids constituting peptide Ch-2 and Ch-4). By the use of the appropriate reagents, tyrosine was located in peptide Ch-5 and tryptophan in Ch-2. Thus, since tyrosine has already been located in the N-terminal sequence, Ch-5 must represent the N-terminal pentapeptide, and, in terms of chymotryptic specificity, tyrosine should be C-terminal in this peptide. Similarly, tryptophan must be C-terminal in Ch-2. In a similar fashion arginine could be located in Ch-2 and histidine in Ch-3. The ninhydrin color given by Ch-4 was that characteristic of a peptide with an N-terminal glycine.

The analyses for the N-terminal amino acids of each of these peptides is presented in the first column of Table I. That these were the sole amino acids found in each peptide served to confirm the homogeneity of each band. Since peptide Ch-5 possessed an N-terminal aspartic acid, its location at the N-terminus of β-MSH was established.

TABLE I

N- AND C-TERMINAL AMINO ACIDS AND QUANTITATIVE AMINO ACID ANALYSES OF THE PEPTIDES DERIVED FROM CHYMOTRYPTIC DIGESTION OF β-MSH

Peptid	N-Terminal amino acid	Amino acid analysis	C-Terminal amino acid(s)
Ch-2	Arg	Arg[a]	Try[b]
Ch-3	Lys	Lys,Met,Glu,His,Phe	Phe(His)[c]
Ch-4	Gly[d]	Ser,Pro_2,Lys,Asp,Gly	Asp
Ch-5	Asp	Asp,Glu,Gly,Pro,Tyr	Tyr

[a] Reaction with *p*-dimethylaminobenzaldehyde revealed the presence of tryptophan in the *unhydrolyzed* peptide. [b] Arginine was also found in a quantity equal to that of tryptophan. [c] Phenylalanine was the major amino acid found following digestion with carboxypeptidase; histidine was present in small quantities. [d] DNP-glycine was obtained in a yield of only about 25% that of the N-terminal amino acids of the other peptides.

The amino acid analysis of each of these peptides, presented in Table I, permitted the formulation of an over-all structure for β-MSH, since the second aspartic acid—that occupying the C-terminal position—was located in Ch-4, and the one residue of methionine, whose position had already been established through stepwise degradation of the entire molecule, was found in Ch-3. Aside from the structure of Ch-5, which was known, only that of Ch-2, demonstrated to be the dipeptide Arg.Try., could be determined at this point; this dipeptide has previously been found in chymotryptic digests of the corticotropins.[27-29] To our surprise, this dipeptide was degraded by carboxypeptidase to give tryptophan and arginine. Even though it is now known that this enzyme will attack dipeptides,[27,30] the result was unexpected since the neighboring arginine would have been expected to inhibit effectively any enzymatic action.[31] Carboxypeptidase also attacked Ch-4, releasing only aspartic acid (Table I) as it does in intact MSH. From Ch-5 only tyrosine was released; this is consistent with the finding that the amino acid next in order is proline, which is completely resistant to the action of carboxypeptidase. Only in Ch-3 did the enzyme promote the release of more than a single amino acid, for, in addition to the C-terminal phenylalanine, some histidine was also released, in an amount equal to about 12% of the amount of the phenylalanine.

Digestion with Trypsin.—Simultaneous investigations were carried out to determine the structure of the peptide fragments produced during tryptic hydrolysis. Although in terms of the arginine and lysine content of β-MSH, 4 peptides could be expected, only 3 peptides were in fact found (see Fig. 1).[32] Again, the application of the appropriate specific reagents demonstrated that the tyrosine residue was present in peptide T-2, the histidine and arginine residues in T-4 and the single tryptophan residue in T-3. N-Terminal amino acid analyses, presented in Table II, revealed that peptide T-2 was N-terminal in β-MSH. No N-terminal amino acid was found for peptide T-3 by the DNP method; however, when this peptide was allowed to react with phenyl isothiocyanate and when the phenylthiocarbamyl derivative of the N-terminal amino acid was cyclized, the phenylthiohydantoin of tryptophan was found, establishing this amino acid as N-terminal in T-3. Amino acid analyses of the 3 peptides indicated that T-2 supplied the necessary bridge in our formulation between Ch-5 and Ch-3; T-4, between Ch-3 and

(27) W. F. White and W. A. Landmann, THIS JOURNAL, **76**, 4193 (1954).

(28) P. H. Bell, *ibid.*, **76**, 5565 (1954).

(29) C. H. Li, I. I. Geschwind, R. D. Cole, I. D. Raacke, J. I. Harris and J. S. Dixon, *Nature*, **176**, 687 (1955).

(30) S. Yanari and M. Mitz, *Federation Proc.*, **13**, 326 (1954).

(31) In further experiments it has been found that 1 mg. of carboxypeptidase will completely hydrolyze 1.6 μmoles of either natural arginyl tryptophan or synthetic *l*-arginine-*l*-leucine within 16 hours at room temperature. We are indebted to Dr. F. Carpenter for a gift of the latter peptide.

(32) In the pilot experiments a commercial trypsin preparation was employed and the patterns obtained were far more complex than that shown in Fig. 1. Several of the bands obtained appeared to be identical with bands observed following electrophoresis of the products of chymotryptic digestion.

Ch-2; and T-3, between Ch-2 and Ch-4. It should be pointed out that the yields of all major peptides from chymotryptic and tryptic digestion, calculated on the basis of the actual amino acid analyses corrected for the size of the aliquots taken, were greater than 80% of theory. These yields do not take into account losses in the form of material used for guide strips or due to incomplete elution from the paper.

TABLE II

N-TERMINAL AMINO ACIDS AND QUANTITATIVE AMINO ACID ANALYSES OF THE PEPTIDES DERIVED FROM TRYPTIC DIGESTION OF β-MSH

Peptide	N-Terminal amino acid	Amino acid analysis
T-2	Asp	Asp,Glu,Gly,Pro,Tyr,Lys
T-3	Try[a]	Gly,Ser,Pro_2,Lys,Asp[b]
T-4	Met	Met,Glu,His,Phe,Arg

[a] Detected by means of the phenyl isothiocyanate technique. [b] Reaction with *p*-dimethylaminobenzaldehyde revealed the presence of tryptophan in the unhydrolyzed peptide.

Complete Amino Acid Sequence.—At this point it became possible to formulate the following structure for β-MSH

Asp.Glu.Gly.Pro.Tyr.Lys.Met.Glu.His.Phe.Arg.Try.
Gly(Ser,Pro_2,Lys)Asp

The most striking aspect of this formulation lies in the heptapeptide sequence

...Met.Glu.His.Phe.Arg.Try.Gly...

which occurs identically in all the corticotropins that have been studied hitherto.[28,29,33] In the corticotropins this sequence is followed by

...Lys.Pro.Val...

and at this point in the elucidation of the structure of MSH it seemed possible that the area of identity might be further extended to the Lys.Pro., amino acids which were present in the undetermined sequence of peptide Ch-4. The presence of a Lys.-Pro linkage would also explain the failure of trypsin to split the lysyl bond in this peptide. Subsequently, the structure of this peptide, determined by the stepwise degradation technique with phenyl isothiocyanate,[34] was elucidated as

...Gly.Ser.Pro.Pro.Lys.Asp

Direct identification of the phenylthiohydantoins produced was the chief method relied upon for the determination of this structure, since the other techniques presented certain difficulties. For example, at 2 of the steps when aliquots of the aqueous fractions remaining after extraction of the PTH-derivatives of the amino acids were dinitrophenylated and hydrolyzed to determine the amino acid next in sequence, the expected DNP-proline was almost completely destroyed even with as brief a period of hydrolysis as 4 hr. Furthermore, the PTH of serine was completely destroyed during the acid hydrolysis employed to regenerate the parent amino acid. With this sequence established, the extent of identity between the structures of MSH and the corticotropins is definitely limited to the heptapeptide noted above.

The structures of all peptides derived from the chymotryptic and tryptic hydrolyses of β-MSH are summarized in Table III, and the deduced structure of this hormone is presented.[35] It is of interest that the peptide bond between the penultimate lysine and the C-terminal aspartic acid is not hydrolyzed by trypsin. In the case of the B-chain of insulin, the bond between the penultimate lysine and the C-terminal alanine is apparently hydrolyzed readily,[36] so it must be presumed that it is the second negative charge on the β-carboxyl of aspartic acid that serves to inhibit the approach of the trypsin molecule to the bond that is to be hydrolyzed.

A comparison of the entire structure of MSH and the related area of the corticotropins (Table IV) discloses that the interchange of lysine and serine at two points (positions 6 and 14, Table III) prevents the area of identity, residing in a common heptapeptide core, from extending to 11 amino acids. It would appear extremely probable that the presence of this sequence in the corticotropins explains their intrinsic melanocyte-stimulating activity.[28,37,38] The absence from this sequence of the N-terminal serine that occurs in the corticotropins explains why alteration of this residue by periodate[39] fails to modify the melanocyte-stimulating activity,[40] although for the corticotropic activity the N-terminal serine would appear to be indispensable.

Thus, it is by virtue of an arrangement of a different sequence of amino acids on each side of the heptapeptide core that the molecule becomes one possessing adrenal-stimulating as well as melanocyte-stimulating activity. It is in this common core, too, that the search must be made for the peroxide-sensitive center[37,41] in these two hormones.

Little information has been gained from comparing the structures of the two hormones with respect to the problem of potentiation by alkali of the melanocyte-stimulating activity. We have not detected any activation of β-MSH by alkali, in confirmation of the results of other investigators.[42,43] However, such an activation of the melanocyte-stimulating activity of the porcine

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(34) Confirmation of the structure of Ch-3 was also obtained by this procedure.

(35) All the residues of aspartic and glutamic acids in β-MSH possess free carboxylic functions, and hence they are not present as asparagine or glutamine.[1] Furthermore, since one of the residues of aspartic acid is C-terminal, and the other residue of aspartic acid and the 2 residues of glutamic acid could be obtained in the form of their free phenylthiohydantoins in the course of the stepwise degradation of β-MSH with phenyl isothiocyanate, it is evident that neither β-aspartyl nor γ glutamyl linkages can exist in β-MSH.

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TABLE III

STRUCTURE OF THE PEPTIDES OBTAINED FROM THE CHYMOTRYPTIC AND TRYPTIC HYDROLYSIS OF β-MSH

Enzyme	Peptide no.[a]	Structure
Chymotrypsin	Ch-5	Asp.Glu.Gly.Pro.Tyr
Trypsin	T-2	Asp.Glu.Gly.Pro.Tyr.Lys
Chymotrypsin	Ch-3	Lys.Met.Glu.His.Phe
Trypsin	T-4	Met.Glu.His.Phe.Arg
Chymotrypsin	Ch-2	Arg.Try
Trypsin	T-3	Try.Gly.Ser.Pro.Pro.Lys.Asp
Chymotrypsin	Ch-4	Gly.Ser.Pro.Pro.Lys.Asp
Complete amino acid sequence		Asp.Glu.Gly.Pro.Tyr.Lys.Met.Glu.His.Phe.Arg.Try.Gly.Ser.Pro.Pro.Lys.Asp
		1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

[a] See Figs. 1 and 2.

TABLE IV

COMPARISON OF THE STRUCTURE OF β-MSH WITH THE RELATED AREA OF THE CORTICOTROPINS

β-MSH	Asp.Glu.Gly.Pro.Tyr.Lys.Met.Glu.His.Phe.Arg.Try.Gly.Ser.Pro.Pro.Lys.Asp.
Corticotropins	Ser.Tyr.Ser.Met.Glu.His.Phe.Arg.Try.Gly.Lys.Pro.Val.Gly.Lys.

corticotropins is well documented,[28,44] although the magnitude of the activation is open to some question. Inherently the corticotropins possess less than 1% of the melanocyte-stimulating activity of MSH, a fact which may be explained by the nature of the sequences adjacent to the heptapeptide core. Such sequences may be inhibitory, so that treatment of the corticotropins with alkali, by removing some of this inhibitory structure, may consequently potentiate the melanocyte-stimulating activity. In our experience, however, even when potentiation has occurred, the melanocyte-stimulating activity of the corticotropins is less than that found in MSH. This may be a result of the Lys.Ser. interchanges pointed out above. In other words, it may be that the undecapeptide discussed above is necessary for full melanocyte-stimulating activity.

Finally, the finding of a partial structure common to two different hormones is highly reminiscent of the findings in connection with oxytocin and vasopressin.[45] In the case of these latter hormones, two variations occur in a basic nonapeptide structure, and each hormone is endowed with some of the major activities characteristic of the other. Both of these hormones are produced by the hypothalamus and stored in the posterior pituitary. The corticotropins are produced by a group of the cells of the anterior lobe of the pituitary, whereas MSH is produced from an entirely different group of cells, those of the intermediate lobe of the pituitary. This lobe, however, is derived embryologically from the same anlage as the anterior lobe. A full discussion of the biological activity of β-MSH and the biological significance of the structure will be presented elsewhere.

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The Enzymatic Synthesis and Disproportionation of 3-O-α-D-Glucopyranosyl-D-glucose[1,2]

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3-O-α-D-Glucopyranosyl-D-glucose (nigerose) is synthesized during the action of the transferring enzyme of *Aspergillus oryzae* on glucose and maltose. It was characterized by its specific rotation, its paper chromatogram mobility and its crystalline phenylosazone derivative. Evidence from isotope studies indicates that the compound is synthesized by a transfer of the glucosyl moiety of maltose to the three position of cosubstrate glucose molecules. On further treatment of nigerose with the transferring enzyme, it is disproportionated to new glucosyl oligosaccharides.

The α-D-(1→3) glucosidic linkage is a characteristic structural feature of a number of glucosyl polysaccharides and comprises approximately half of the glucosidic bonds in nigeran,[3] a variable frac-

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(2) A preliminary account of a portion of this work has been published in abstract form in *Federation Proc.*, **15**, 325 (1956).

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