Biochem. J. (1970) **118**, 713–718
Printed in Great Britain

Purification and Amino Acid Sequence of Melanocyte-Stimulating Hormone from the Dogfish *Squalus acanthias*

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(*Received* 30 *January* 1970)

A melanocyte-stimulating hormone (MSH) was isolated by gel filtration and ion-exchange chromatography from extracts of the pituitary glands of dogfish. Sequence studies were carried out on the hormone and its enzymically and chemically cleaved fragments. The sequence of the hormone, Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Met, shows that ten of its 11 residues are the same as ten of the 13 residues of mammalian α-MSH. About half of its molecules have the carboxyl group at the *C*-terminus free and about half are amidated; about a fifth have an extra tyrosine residue on the *N*-terminus, thereby making 11 residues the same as in mammalian α-MSH. Unlike the mammalian hormone, however, none of it was found to be *N*-acetylated.

Although the primary structures of MSH† from mammalian sources are well documented (Geschwind, 1966), only preliminary attempts have been made to characterize MSH from lower vertebrates, where the hormone has the obvious function of causing the expansion of melanin within the melanophores of the skin. Burgers (1963) has carried out starch electrophoresis on crude preparations of cod, lizard and frog pituitaries, but no characterization of bands of MSH-active material was made apart from their mobility. Geschwind (1966) has described the chromatography of a preparation of cod pituitaries on CM-cellulose, but apart from the position of the MSH-active fractions relative to the normal positions of a α-MSH and β-MSH on a similar chromatograph no further identification has been reported.

Recently, biologically active peptides showing electrophoretic and chromatographic properties similar to β-MSH have been found in some elasmobranchs, but no chemical characterization was attempted (R. M. Love & B. T. Pickering, unpublished work cited by Pickering & Heller, 1969).

We now report details of the purification and elucidation of the primary structure of the MSH found in the neurointermediate lobe of the pituitary of the dogfish *Squalus acanthias*.

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† Abbreviation: MSH, melanocyte-stimulating hormone.

MATERIALS AND METHODS

CM-cellulose (CMC23) was obtained from Whatman. Sephadex G-25 (fine grade) was purchased from Pharmacia (Uppsala, Sweden) and Bio-Gel P-6 (200–400 mesh) from Bio-Rad Laboratories (Richmond, Calif., U.S.A.). Carboxypeptidase A and carboxypeptidase B (both diisopropyl phosphorofluoridate-inhibited) were obtained from Sigma Chemical Co. (St Louis, Mo., U.S.A.).

Three-times-crystallized trypsin from Worthington Biochemical Corp. (Freehold, N.J., U.S.A.) was treated before use with 1-chloro-4-phenyl-3-toluene-*p*-sulphonamidobutan-2-one (TPCK) by the method of Kostka & Carpenter (1964).

Röhm und Haas (Darmstadt, Germany) supplied the aminopeptidase M.

Neurointermediate lobes were dissected from dogfish heads supplied by A. R. Jenner, Grimsby, Lincs., U.K.

Purification of reagents for use in the Edman degradation. Pyridine was redistilled under reduced pressure and stored under N_2 at −20°C.

Phenyl isothiocyanate was redistilled at 1 mmHg and the fraction that boiled at 55°C was collected.

Trifluoroacetic acid was heated under reflux for 18h over CrO_3 and the fraction that boiled between 71 and 73°C on distillation was collected and stored at room temperature.

Butyl acetate was shaken overnight with aq. 5% (w/v) $KMnO_4$ and then washed with water. It was then dried with anhydrous Na_2SO_4 and redistilled. The fraction that boiled at 78°C was collected and stored at room temperature. Reagents used in other procedures were of the A.R. grade whenever possible. Piperidine was redistilled under reduced pressure before use and stored at 5°C under N_2.

Acetylated dialysis tubing. Na_2CO_3 (5g) and 0.1g of EDTA (free acid) were dissolved in 1 litre of water. After addition of the dialysis tubing (size 8/32; Union Carbide International Co., New York, N.Y., U.S.A.) the mixture was boiled for 10min. The tubing was then washed and boiled in two changes of water. After a final wash the tubing was dried in anhydrous pyridine and left for 16h in 10% (v/v) acetic anhydride in pyridine. This is a modification of the method of Craig & Konigsberg (1961). Acetylation of dialysis tubing has been shown to hinder the passage of relatively small molecules (Craig & Pulley, 1962; Craig & Ansevin, 1963).

Extraction of crude MSH. Batches of 1000 neurointermediate lobes (25g) were homogenized in 125ml of ice-cold 1M-acetic acid in a Waring Blendor; 4000 glands were used in all. The insoluble material was centrifuged at 4200*g* at 4°C and re-extracted twice by repeating the above procedure. The combined supernatants were freeze-dried. The dried extracts of 4000 pituitaries were redissolved in 50ml of ice-cold 1M-acetic acid, and solid $(NH_4)_2SO_4$ was added with stirring to saturation. The solution was then left at 4°C for 3h. The precipitate was centrifuged down at 4200*g* and re-extracted and precipitated again by using the above procedure.

The combined supernatants were dialysed in acetylated dialysis tubing against 50 litres of 1M-acetic acid at 4°C for 16h with two changes of acetic acid solution. Any precipitate at this stage was centrifuged off and discarded. The solution was then freeze-dried and stored under N_2 at −20°C.

Purification and separation of peptides. Gel-filtration columns were packed by allowing a slurry of previously swollen gel to settle into the columns already filled with 1M-acetic acid. Flow rates were maintained and regulated by a constant-head device.

After being washed overnight in 1M-ammonium acetate CM-cellulose was packed as a thick slurry and equilibrated with 5mM-ammonium acetate buffer, pH5.8. All column chromatography was performed at 4°C.

The crude MSH preparation was purified by column chromatography on Sephadex G-25, Bio-Gel P-6 and CM-cellulose. 1M-Acetic acid was used as the eluent in both Sephadex and Bio-Gel chromatography. The active fractions were freeze-dried.

A concave gradient of ammonium acetate was employed to develop the CM-cellulose column. Two 150ml mixing chambers were used, containing 5mM-ammonium acetate buffer, pH5.8, and a final chamber containing 100ml of 1M-ammonium acetate buffer, pH7.

Peptides were detected in column effluents by their extinction at 280nm.

Electrophoresis. Electrophoresis was carried out on Whatman no. 1 paper (prewashed with 2M-pyridine) in 1.25M-pyridine–acetate buffer, pH6.5. Peptides were detected by the chloride spray method as modified by Roberts (1966), by spraying either the whole chromatogram in analytical work or a test strip in preparative chromatograms. Ionic charges of peptides were calculated by the method of Offord (1966).

Bioassay. MSH activity of fractions was measured throughout by the method of Chadwick & Lowry (1970).

Amino acid analysis. Peptide samples (usually 20–60nmol) were hydrolysed with a crystal of phenol (to prevent tyrosine loss) in 0.5ml of 6M-HCl in evacuated glass tubes for 24h at 110°C. Analyses were performed on the Technicon amino acid autoanalyser, which was operated by the method of Piez & Morris (1960). Tryptophan was calculated by the method of Edelhoch (1967) before hydrolysis and by amino acid analysis of aminopeptidase digestions. Serine loss on acid hydrolysis was also calculated by this latter method.

Peptide fragmentations. Cyanogen bromide cleavage of methionine was carried out by using the method of Steers, Craven, Anfinsen & Bethune (1965). Treatment with 0.2M-piperidine at 37°C for 2h was employed to convert homoserine lactone into homoserine. Tryptic digestion (usually 100–200nmol; peptide/enzyme ratio, 100:1, w/w) was carried out in 0.2ml of 0.05M-NH_4HCO_3, pH8.5, at 37°C for 4h.

Sequence analysis. For *C*-terminal analysis carboxypeptidase A and/or B were used with a peptide/enzyme ratio 125:1 (w/v) in 0.05M-$NaHCO_3$ at 25°C. Samples were removed at 30min intervals and quenched with 0.1M-HCl. The amino acids released were identified on the analyser. Aminopeptidase M digestions were performed in 0.01M-sodium phosphate buffer, pH7. The peptide/enzyme ratio was 10:1 (w/w) and complete digestion (except for proline residues) was usually effected in 24h at 37°C.

N-Terminal sequence analysis was carried out by using the Edman degradation method of Gray (1967) except that phenyl isothiocyanate was added to the reaction mixture separately instead of it being stored in pyridine. This was used in conjunction with either the 'dansylation' method of Woods & Wang (1967) with the modifications on hydrolysis time suggested by Gros & Labouesse (1969) or, with ambiguous results, the subtractive procedure employing amino acid analysis. When tryptophan was suspected at the *N*-terminal position the Ehrlich reaction was carried out on paper by the method of Smith (1953).

RESULTS

Figs. 1, 2 and 3 show the steps involved in the purification of dogfish MSH by column chromatography. The single peak of extinction and MSH activity on Bio-Gel (Fig. 2) was split into two separate active components when submitted to ion-exchange chromatography on CM-cellulose (Fig. 3). The total yield of peptide P_I from 4000 pituitaries was 5.6mg and that of peptide P_{II} was 4.1mg. The specific activities of the two peptides were not significantly different from each other. Peptide P_{II}, however, had only about 3% of the specific activity of mammalian β-MSH (Chadwick & Lowry, 1970).

The results of amino acid analyses are given in Table 1. Both peptides have very similar amino acid compositions. Aminopeptidase M was active in releasing all the amino acids except proline and one methionine residue in both cases. Serine was released as a full residue after aminopeptidase digestion. This also occurred even after exposure to the enzyme for 30min, showing that the *N*-terminus was not acylated. The ionic charges of the two peptides calculated from electrophoresis at

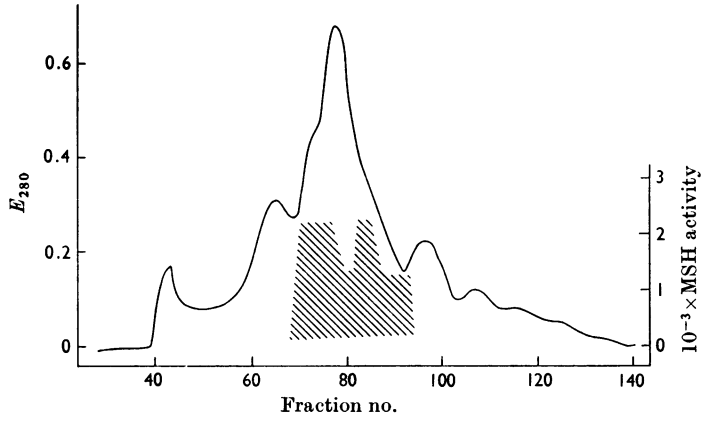

Fig. 1. Gel filtration of an extract of approx. 2000 dogfish neurointermediate lobes on Sephadex G-25. The column (4 cm × 90 cm) was developed at 4°C with 1 M-acetic acid at a flow rate of 36 ml/h; 9 ml fractions were collected and monitored at 280 nm (——); MSH activity is shown by the hatched area.

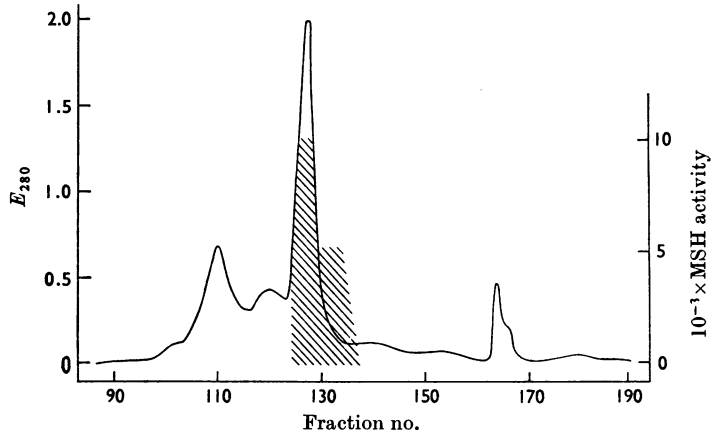

Fig. 2. Fractions 68–93 (Fig. 1) after being freeze-dried were submitted to molecular sieving on Bio-Gel P-6. The column (2.4 cm × 140 cm) was developed with 1 M-acetic acid at 4°C at a flow rate of 7.8 ml/h; 3.9 ml fractions were collected and monitored at 280 nm (——); MSH activity is shown by the hatched area.

pH 6.5, however, differed by one positive charge. Because the terminal amino group was not acylated and glutamate rather than glutamine was released from both peptides by aminopeptidase it was concluded that the *C*-terminal of peptide P_{II} is amidated.

N-Terminal end-group analysis by the 'dansylation' method revealed tyrosine in both peptides, but when the subtractive Edman procedure was used 70% of the serine disappeared with the 0.2 residue of tyrosine at the first cyclization step. The next two amino acids in the sequence were found to be methionine and glutamic acid (Table 2). Digestion of peptide P_I with carboxypeptidase A and carboxypeptidase B released methionine only, which was identified by its DNS derivative. Peptide P_{II} was resistant to attack by these enzymes. Tryptic digestion was carried out on both peptides. The fragments were separated by electrophoresis on Whatman no. 1 paper. After a test strip had been stained the peptides were eluted and portions hydrolysed for analysis. These results are shown in Table 3, together with the electrophoretic information. Peptides TP_IA and $TP_{II}A$ are identical both in composition and ionic charge at pH 6.5. Peptide $TP_{II}A$ was digested with carboxypeptidase B and carboxypeptidase A, which released arginine and then phenylalanine.

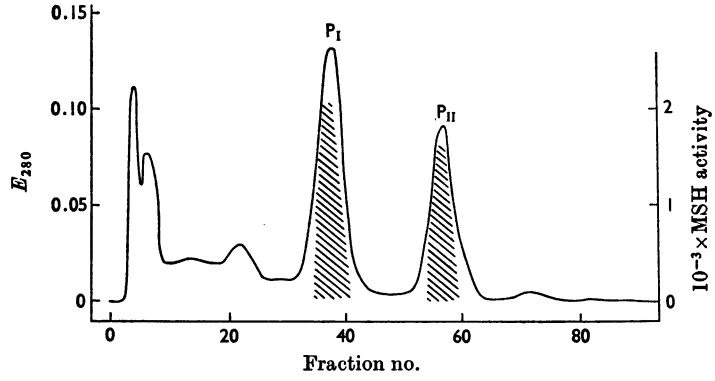

Fig. 3. Ion-exchange chromatography of MSH-active material from approx. 1000 dogfish (after purification on Bio-Gel P-6) on CM-cellulose. The column (0.8 cm × 15 cm) was developed at 4°C with a concave gradient of ammonium acetate buffer from 5 mM (pH 5.8) to 1 M (pH 7) at a flow rate of 5.8 ml/h; hourly fractions were collected and monitored at 280 nm (——); MSH activity is shown by the hatched areas.

Table 1. *Amino acid compositions of acid hydrolysis of MSH-active peptides purified from dogfish pituitaries*

Ionic charges were computed from electrophoretic data as described by Offord (1966).

	Amino acid composition (molar ratios)	
	P_I	P_{II}
Ser*	0.90	0.93
Glu*	1.07	1.10
Pro	0.99	0.93
Gly	1.07	1.20
Met	1.87	1.72
Tyr	0.19	0.22
Phe	0.97	0.98
Lys	1.06	1.10
His	0.97	0.97
Arg	0.96	0.96
Trp*†	0.92	0.95
Ionic charges at pH 6.5	$1\frac{1}{2}^{\oplus}$	$2\frac{1}{2}^{\oplus}$

* Obtained from aminopeptidase digestion.
† Determined spectrophotometrically.

The cyanogen bromide reaction was carried out on peptide P_I and the main fragment was purified by chromatography on a CM-cellulose column packed in a Pasteur pipette. It was eluted by the same gradient that was used in the purification of the hormone and it was located by its u.v. absorption.

The amino acid composition of this fragment is given in Table 3. The *N*-terminal tripeptide sequence of this fragment was determined by the 'dansyl'–Edman procedure and was found to be:

Glu-His-Phe

The sequences of peptides TP_IB and $TP_{II}B$ were also determined by the 'dansyl'–Edman procedure. Tryptophan, however, was identified in the peptide by the Ehrlich stain before degradation. A negative reaction to the Ehrlich stain after degradation was taken as proof that tryptophan had been the *N*-terminal amino acid.

Methionine amide was not identified in peptide $TP_{II}B$, but it was inferred from the charge found on the peptide. The sequences of peptides TP_IB and $TP_{II}B$ were found to be:

TP_IB, Trp-Gly-Lys-Pro-Met
$TP_{II}B$, Trp-Gly-Lys-Pro-Met-(NH_2)

We think that these results can only be explained by the following structures of dogfish MSH, shown in Fig. 4.

DISCUSSION

In the early work in this study pyridine–acetate buffers were used as eluents in some of the column chromatographic, systems. This practice, however, was discontinued for two reasons: (1) the interference encountered when monitoring fractions at 280 nm; (2) the loss of MSH activity [amino acid analyses of samples freeze-dried from pyridine buffers invariably had a low methionine content; this was due to the oxidation of methionine by pyridine impurities to methionine sulphoxide or methionine sulphone even though the reagent had been distilled before use. Lo, Dixon & Li (1961) isolated the methionine

Table 2. *Subtractive Edman degradation of peptide P_{II}*

Peptide P_{II} was subjected to repeated Edman degradation and one-third of the residual peptide was removed for amino acid analysis (expressed as molar ratios). *Italics* indicate where a residue has been depleted.

	Tyr	Ser	Met	Glu	Pro	Gly	Phe	Lys	His	Arg
Peptide P_{II}	0.2	0.9	1.8	1.0	0.9	1.2	1.0	1.1	1.0	1.0
Step 1	*0*	*0.3*	1.4	1.2	0.9	1.2	1.0	1.0	0.9	0.9
Step 2	0	*0*	*0.7*	1.1	0.9	1.2	1.0	1.0	1.1	1.0
Step 3	0	0	*0.6*	*0.4*	0.9	1.3	0.8	1.0	1.0	0.9

Table 3. *Amino acid compositions of peptides obtained from enzymic and chemical cleavage of the MSH peptides*

Origin of peptide	Peptide	Ser	Hsr*	Glu	Pro	Gly	Met	Tyr	Phe	Lys	His	Trp	Arg	Ionic charge at pH 6.5
Tryptic digest of peptide P_I	TP_IA	0.7	—	1.2	—	—	0.9	0.2	1.1	—	1.0	—	1.1	$\frac{1}{2}^{\oplus}$
	TP_IB	—	—	—	0.8	1.1	1.0	—	—	1.1	—	1†	—	$1^{\oplus}$
Cyanogen bromide cleavage of peptide P_I	Main fragment	—	0.6	1.1	1.0	1.2	—	—	0.9	1.1	1.0	1‡	1.0	—
Tryptic digest of peptide P_{II}	$TP_{II}A$	0.5	—	1.0	—	—	0.9	0.2	1.0	—	0.9	—	1.2	$\frac{1}{2}^{\oplus}$
	$TP_{II}B$	—	—	—	1.0	1.1	1.0	—	—	1.0	—	1†	—	$2^{\oplus}$

* Homoserine.
† Determined qualitatively on paper by the Ehrlich reaction.
‡ Determined spectrophotometrically.

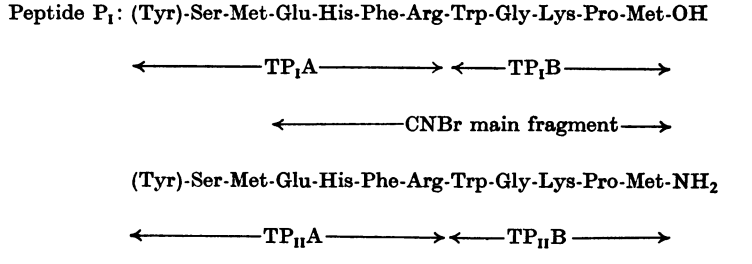

Fig. 4. Amino acid sequences of the two forms of dogfish MSH.

Dogfish MSH: Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-*Met*-(NH_2)
Tyrosyl dogfish MSH: *Tyr*-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-*Met*-(NH_2)
Mammalian α-MSH: *Ac-Ser-Tyr*-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-*Val*-NH_2

Fig. 5. Comparison of dogfish MSH with mammalian α-MSH. *Italic* type indicates residues in which variations are found.

sulphoxide analogue of α-MSH from bovine pituitary glands by using pyridine as one of the solvents in their countercurrent procedures and the analogue had 0.5% of the activity of the unoxidized hormone].

Although serine was present as an *N*-terminal amino acid it was not detected by the DNS chloride procedure. This was probably due to the instability of the DNS derivative of serine to acid hydrolysis. Gros & Labouesse (1969) have also noticed this instability of serine to acid hydrolysis while studying the pure derivative.

The presence of the two forms of dogfish MSH is noteworthy. The deamidated form (P_I) could be an artifact of the extraction procedure, but in view of the mild conditions used this is unlikely. It would simply reflect the occurrence of the deamidated form in the pituitary, as amidation of the *C*-terminal would be the last step in the formation of the hormone.

The greater proportion of the hormone (80%)

was found to have serine as the *N*-terminal residue and for simplicity we will call this hormone dogfish MSH. Some 20% of the hormone, however, had an extra residue (tyrosine) at the *N*-terminal and this can be called tyrosyl dogfish MSH. The occurrence of these two hormones is due either to polymorphism or to microheterogeneity in the dogfish *Squalus acanthias*.

The sequences of these two hormones are compared with that of mammalian α-MSH in Fig. 5. Dogfish MSH is deficient in an acetylated seryltyrosyl sequence, and has methionine substituted at the *C*-terminus (desAc-Ser^{1}-Tyr^{2}-[Met^{13}]-α-MSH). Tyrosyl dogfish MSH, although still having the methionine substitution at the *C*-terminus, is only deficient in an acetylated seryl residue (desAc-Ser^{1}-[Met^{13}]-α-MSH). The remaining ten residues are the same as in α-MSH.

The evolutionary and physiological significance of these differences and our failure to find β-MSH in the dogfish pituitary have been discussed (Lowry & Chadwick, 1970).

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