Biochem. J. (1974) 139, 593–602
Printed in Great Britain

Adrenocorticotrophic and Melanocyte-Stimulating Peptides in the Human Pituitary

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(*Received* 7 *December* 1973)

The adrenocorticotrophic and melanocyte-stimulating peptides of the human pituitary were investigated by means of radioimmunoassay, bioassay and physicochemical procedures. Substantial amounts of adrenocorticotrophin and a peptide resembling β-lipotrophin were identified in pituitary extracts, but α-melanocyte-stimulating hormone, β-melanocyte-stimulating hormone and corticotrophin-like intermediate lobe peptide, which have been identified in the *pars intermedia* of pituitaries from other vertebrates, were not found. The absence of β-melanocyte-stimulating hormone appears to contradict previous chemical and radioimmunological studies. Our results suggest, however, that it is not a natural pituitary peptide but an artefact formed by enzymic degradation of β-lipotrophin during extraction.

Melanocyte-stimulating and corticotrophic peptides have been identified in the pituitaries of all vertebrate species studied, and several have been isolated and characterized. They belong to two structually related classes, namely those related to adrenocorticotrophin (ACTH) including ACTH, α-melanocyte-stimulating hormone (α-MSH) and 'corticotrophin-like intermediate lobe peptide' ACTH (18–39) peptide (CLIP), and others related to β-melanocyte-stimulating hormone (β-MSH) including β-MSH, β-lipotrophic hormone [β-lipotrophin (β-LPH)] and γ-lipotrophic hormone [γ-lipotrophin (γ-LPH)] (Geschwind, 1966, 1967; Lowry & Chadwick, 1970; Chrétien *et al.*, 1973; Scott *et al.*, 1973). All these peptides [except ACTH (18–39) peptide] contain a heptapeptide core -Met-Glu-His-Phe-Arg-Trp-Gly-, which is necessary for both melanocyte-expanding (Pickering & Li, 1962) and corticosteroidogenic activity (Schwyzer *et al.*, 1971).

Adrenocorticotrophin has been isolated from human pituitaries and its amino acid sequence determined partially, by Lee *et al.* (1961*b*) and completely by Riniker *et al.* (1972) and Bennett *et al.* (1973). Interest in the presence of other related peptides stemmed originally from observations that increased plasma concentrations of melanocyte-stimulating activity in patients with Addison's disease (or with some forms of Cushing's syndrome) could not be accounted for wholly by the co-existent increase in concentrations of ACTH (see review by Harris, 1960). An active melanocyte-stimulating peptide was isolated from a side fraction obtained during the extraction of human pituitaries for growth hormone (Dixon, 1960). Its stucture was determined by Harris (1959) and shown to be similar to β-MSH isolated from other species, except for the presence of an extra four amino acids at the *N*-terminus. The presence of sufficient amounts of β-MSH in human pituitaries to account alone for the bulk of the melanocyte-stimulating activity in the pituitary extracts was shown by radioimmunoassay (Abe *et al.*, 1967*b*). β-MSH was also detected in plasma by radioimmunoassay (Abe *et al.*, 1969) at concentrations that paralleled those of ACTH in a number of physiological and pathological conditions. In contrast, Lee *et al.* (1961*a*) and Lerner *et al.* (1968) have been unable to identify this peptide in human pituitary glands.

α-MSH has never been definitely identified in human pituitary extracts and it has only been detected in minute quantities <1% of the biological MSH activity by radioimmunoassay (Abe *et al.*, 1967*a*). Lee & Lee (1973) have partially characterized a peptide which they called α-MSH, although its amino acid content suggests that it could be an *N*-terminal fragment of ACTH formed by non-specific peptidase activity.

β-Lipotrophin, which has lipolytic activity on rabbit adipocytes, was originally isolated from sheep pituitaries by Li *et al.* (1965) and was shown to be a single-chain polypeptide with amino acids, containing the complete sequence of sheep β-MSH in positions 41–58 of the molecule. Chrétien & Li (1967) later identified another lipolytic peptide, γ-LPH, which had a sequence identical with the 1–58 portion of β-LPH. Both peptides have also been

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isolated from pig and ox pituitaries (see review by Chrétien *et al.*, 1973) and a β-LPH has been identified in human pituitaries (Cséh *et al.*, 1972). It contains 91 amino acids, and evidence obtained from the composition of its tryptic fragments, and its homology with sheep, ox and pig β-lipotrophins strongly suggest that the complete amino acid sequence of human β-MSH is contained within the 37–58 portion of the molecule (Cséh *et al.*, 1972).

The close structural relationship between β-LPH, γ-LPH and β-MSH in the ox, pig and sheep led Chrétien *et al.* (1973) to propose that β-MSH is derived by enzymic cleavage from β-LPH, with the γ-LPH molecule representing an intermediate product of this transformation. The close structural relationship also found between ACTH and α-MSH, in species where these peptides have been studied, has led us to propose that α-MSH is similarly derived from ACTH by a post-translational cleavage mechanism (Scott *et al.*, 1973). This hypothesis is supported by the demonstration of the *C*-terminal fragment of the ACTH molecule [ACTH (18–39) peptide] in the *pars intermedia* of the rat and pig (Scott *et al.*, 1974*a*,*b*).

Although the human pituitary contains cells that are histologically typical of the *pars intermedia*, it lacks a distinct intermediate lobe such as is present in the rat and pig (Wingstrand, 1966). Evidence discussed elsewhere (Scott *et al.*, 1973) suggests that the formation of α-MSH, ACTH (18–39) peptide and β-MSH from the larger peptides occurs only in the presence of a functional *pars intermedia*. We were therefore interested in re-investigating the presence of these peptides in human pituitaries (which lack this lobe) and for this purpose we used chemical characterization procedures coupled with radioimmunoassay and bioassay of ACTH and MSH.

Materials and Methods

Human pituitaries were collected *post mortem* and kept frozen at −70°C, or immediately acetone-dried.

CM-cellulose (CM-52) and DEAE-cellulose (DE-52) were purchased from Whatman Biochemicals Ltd. (Maidstone, Kent, U.K.), and Bio-Gel P2 (200–400 mesh), P6 (200–400 mesh) and P30 (100–200 mesh) from Bio-Rad Laboratories (Richmond, Calif., U.S.A.). Trimethylamine was obtained from BDH Chemicals Ltd. (Poole, Dorset, U.K.), and was redistilled as described by Bennett *et al.* (1973).

Extraction of peptides

Several extraction procedures, based on those previously published for ACTH-like peptides (Scott *et al.*, 1974*b*) were used. All procedures were performed at 4°C.

(*a*) *Trifluoroacetic acid–acetone.* Twelve frozen pituitaries (6g) were homogenized in 30ml of trifluoroacetic acid (AnalaR grade)–acetone (2:98, v/v). The homogenate was centrifuged at 2500*g* for 15min, the supernatant removed and 3vol. of diethyl ether added with stirring. The precipitate was collected by centrifugation, washed twice with acetone and dried under vacuum.

(*b*) *Acetic acid.* (i) The procedure described by Island *et al.* (1965) was modified as follows. Twenty pituitaries (12g) were homogenized in acetic acid (240ml) and after centrifugation at 2500*g* for 15min, the supernatant was quickly heated in a water bath at 70°C for 25min. The extract was then rotary evaporated, the residue re-extracted with 0.1M-HCl, centrifuged and the supernatant removed. Sufficient acetic acid was added to the supernatant to make the solution 1M with respect to acetic acid. (ii) 12 pituitaries were dried in acetone and the resultant powder (1.35g) was homogenized with 26ml of acetic acid. The homogenate was centrifuged at 2500*g* for 10min and the supernatant removed and freeze-dried.

(*c*) *Acetic acid* (1M). (i) Acetone-dried pituitary powder (200mg) was mixed with 4ml of 1M-acetic acid, boiled immediately, stirred overnight at 4°C, centrifuged at 2500*g* for 10min and the supernatant removed and freeze-dried. (ii) 20 frozen glands (8g) were homogenized briefly in 1M-acetic acid and the mixture was stirred at room temperature for up to 24h. Samples were removed at 0min, 30min, 2h and 16h after the start of the experiment and each was then boiled for 20s and centrifuged, and the supernatants were removed and frozen.

(*d*) *HCl–acetone.* Frozen glands (270; 130g) were homogenized in HCl–acetone (2.5:97.5, v/v) (3ml/g of tissue). After centrifugation at 2500*g* for 15min the supernatant was removed and 3vol. of acetone slowly added with stirring. The precipitate was allowed to settle overnight and collected on a sintered-glass funnel, washed twice with acetone and stored in an evacuated desiccator until required.

Powders obtained from the different extraction procedures were reconstituted in 1M-acetic acid, centrifuged and the supernatants applied to a Bio-Gel P6 column (1.5cm internal diam.×90cm length), which was eluted with 1M-acetic acid at a flow rate of 0.15ml/min and 20min fractions were collected.

Amino acid analysis

Acid hydrolysis and amino acid analysis were carried out as described by Bennett *et al.* (1973). A Beckman 120C analyser was also used for analysing ACTH.

The method of Bennett *et al.* (1972) was used for enzymic peptide hydrolysis.

Assays

(*a*) *Radioimmunoassay*. Immunoreactive ACTH was determined by the method of Rees *et al.* (1971), by using natural human ACTH (Lerner Upton fraction 8B) for standardization and iodination. Two rabbit antisera were used: (i) an antiserum directed towards ACTH (1–24)-peptide (the *N*-terminal sequence) the specificity of which has been published (Orth *et al.*, 1973); (ii) a *C*-terminally directed antiserum, the specificity of which has been described by Ratcliffe *et al.* (1972). The radioimmunoassay for β-MSH used was developed in our laboratory (G. A. Bloomfield, J. J. H. Gilkes, J. G. Ratcliffe, L. H. Rees, P. J. Lowry & A. P. Scott, unpublished results) and uses an antiserum which cross-reacts with peptides related to β-MSH (including synthetic human β-MSH, synthetic bovine β-MSH and ovine β-LPH), and synthetic human β-MSH for standardization and iodination. The method is similar to that described for ACTH (Rees *et al.*, 1971).

(*b*) *Bioassays*. MSH bioactivity was determined by the method of Chadwick & Lowry (1970), with α-MSH (CIBA–GEIGY) as standard. ACTH bioactivity was measured by the method of Lowry *et al.* (1973) with synthetic human ACTH (CIBA–GEIGY) as standard.

Purification of peptides

The β-MSH-like and ACTH-like peptides in the HCl–acetone extract of human pituitary glands were purified in two batches.

(*a*) *Gel-filtration*. Some of the extract (2.5g) was reconstituted in 20ml of 1M-acetic acid, insoluble material was centrifuged off, and the supernatant was applied to a Bio-Gel P2 column (2.5cm internal diam.×25cm length), the outflow of which ran directly into a Bio-Gel P6 column (2.5cm internal diam.×118cm length). The P2 column effectively desalted the peptide material before it entered the P6 column. Both columns were developed and eluted with 1M-acetic acid at a flow rate of 0.23ml/min and 20min fractions were collected. Peaks of ACTH-like and β-MSH-like activity were pooled separately and freeze-dried.

A peak of large molecular weight containing β-MSH-like immunoreactive material was reconstituted in 1M-acetic acid and resubmitted to gel filtration on a column of Bio-Gel P30 (2.3cm internal diam.×100cm length), which was developed with 1M-acetic acid at a flow rate of 0.12ml/min. Fractions (30min) were collected and the β-MSH-like immunoreactive peak fractions were pooled and freeze-dried.

(*b*) *Ion-exchange chromatography*. (i) Acidic peptides were purified on a DEAE-cellulose column (0.4cm internal diam.×10cm length) at a flow rate of 0.03ml/min with a concave molarity gradient of trimethylamine acetate buffer (pH constant at 5) by using a scintillation vial as the mixing chamber and a 25ml conical flask for the final buffer, the two being connected by a syphon. (ii) Basic peptides were purified on CM-cellulose columns, the dimensions of which varied according to the quantity of material. The 0.4cm internal diam.×10cm length column was developed with a gradient similar to that used for the DEAE-column described above. Larger columns (0.6cm internal diam.×10.5cm length, and 0.6cm internal diam.×25cm length) were developed at a flow rate of 0.1ml/min with a concave gradient formed by a 100ml beaker and a 50ml conical flask connected by a syphon.

Fractions (20min) were collected in all purification steps.

Results

Gel filtration of human pituitary extracts on Bio-Gel P6

(*a*) Fractionation of the trifluoroacetic acid–acetone extract showed that most β-MSH-like immunoreactive material eluted before the large peak of both *N*- and *C*-terminal immunoreactive ACTH (Fig. 1*a*). Melanocyte-stimulating activity was found only in those fractions containing the β-MSH-like and ACTH immunoreactive material.

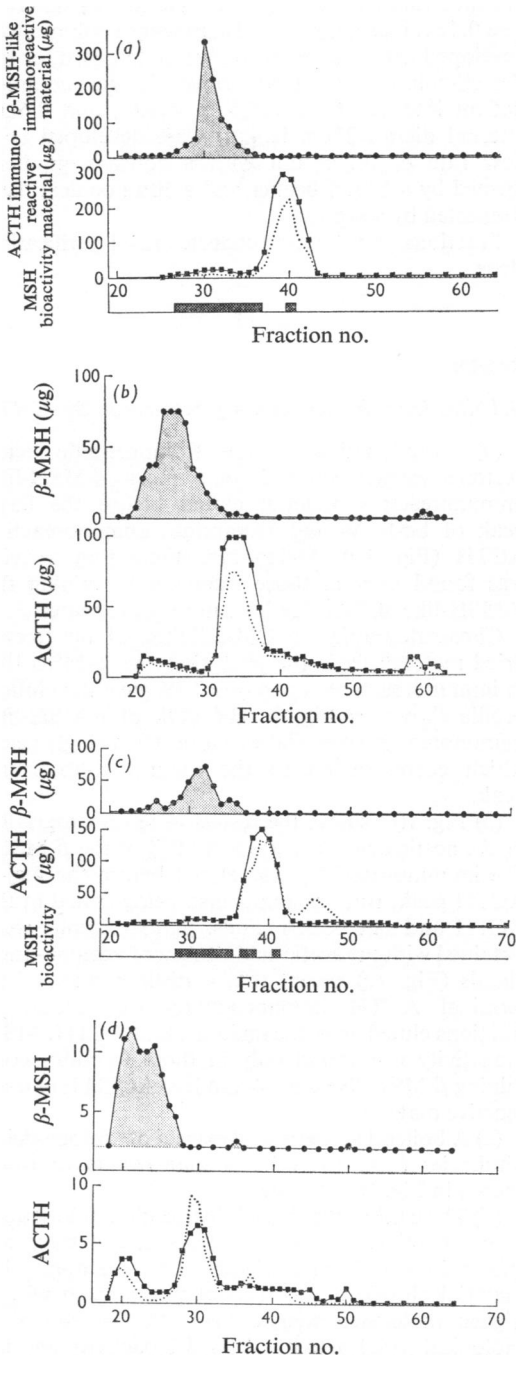
Chromatography on CM-cellulose of the freeze-dried material from the peak that was β-MSH-like in immunoreactivity on Bio-Gel P6 gave the elution profile shown in Fig. 2. The bulk of β-MSH-like immunoreactive material was found in a single peak, which corresponded to the main u.v.-absorbing peak.

(*b*) Fig. 1(*b*) shows the results of chromatography of the acetic acid extract. Over 98% of the β-MSH-like immunoreactivity was eluted before the major ACTH peak, trace amounts also being eluted in the ACTH and salt peak regions. Similar results were obtained with the acetic acid extract of acetone-dried glands (Fig. 1*c*) except that a distinct peak of *C*-terminal ACTH immunoactivity was found in fractions eluted after the main peak of ACTH. MSH bioactivity was found only in those fractions containing β-MSH-like and *N*-terminal ACTH immunoreactive material.

(*c*) A boiled 1M-acetic acid extract of acetone-dried pituitaries (Fig. 1*d*) gave similar results to those shown in Fig. 1(*a*), (*b*) and (*c*).

(*d*) The elution profile and distribution of immunoactive peptides for the HCl–acetone extract are shown in Fig. 3. The β-MSH-like immunoreactive material eluted in two distinct peaks, each with a higher molecular weight than ACTH and with biological MSH activity. ACTH bioactivity and the

bulk of *N*- and *C*-terminal ACTH immunoactivity were eluted in a single peak. Small amounts of *C*-terminally immunoactive ACTH material were found in a separate peak which was eluted later.



Purification and characterization of MSH and ACTH immunoreactive peptides in HCl–acetone extracts of human pituitaries

(*a*) The first and largest peak of β-MSH-like immunoreactive material (Fig. 3), after refractionation on Bio-Gel P30, was submitted to chromatography on CM-cellulose and gave an elution pattern similar to that shown in Fig. 2. Approx. 5mg of the peptide was isolated. The amino acid analysis of a portion of this material gave the molar ratios shown in Table 1, which are similar to those reported by Cséh *et al.* (1972) for human β-LPH, and indicate that it is the same peptide.

It would appear therefore that the β-MSH-like immunoreactive material isolated from the HCl–acetone extract, and the bulk of β-MSH-like immunoreactive material identified from the other extraction procedures is due to human β-LPH.

(*b*) The second and smaller peak of β-MSH-like immunoreactive material was submitted to chromatography on DEAE-cellulose (Fig. 4). Several immunoactive peaks were resolved and the two major ones (A and B) were studied further. Peak-B material was analysed for its amino acid content. It appeared to be contaminated with a cysteine-containing peptide and was purified by treatment with thioglycollic acid (which may form a mixed disulphide with cysteine residues of the contaminant thus changing its charge and chromatographic properties) and rechromatography on DEAE-cellulose. The amino acid molar ratios (see Table 1) do not correspond to any known human peptide, but because of its acidic charge, β-MSH-like immunoreactivity, and resemblance to sheep γ-LPH and pig γ-LPH, the peptide has been tentatively termed 'γ-LPH' [i.e. β-LPH (1–58)

Fig. 1. *Fractionation of human pituitary extracts on Bio-Gel P6*

Gel-filtration of four separate pituitary extracts on Bio-Gel P6 was as described in the Methods section: (*a*) Trifluoroacetic acid–acetone extract of fresh glands. (*b*) Acetic acid extract of fresh glands. (*c*) Acetic acid extract of acetone-dried glands. (*d*) Boiled 1M-acetic acid extract of acetone-dried glands. The same Bio-Gel P6 column (1.5cm internal diam×90cm length) was used in each fractionation. Amounts of activity are expressed as μg of peptide/fraction. ■, ACTH *N*-terminal immunoactivity; . . . . . , ACTH *C*-terminal immunoactivity; shaded peaks, β-MSH-like immunoactivity. Fractions containing biological melanocyte-stimulating activity in (*a*) and (*c*) equivalent to more than 10μg of α-MSH are represented by dark blocks. The high-molecular-weight ACTH immunoactive peaks found in all runs are not mentioned in the text and are currently being further investigated.

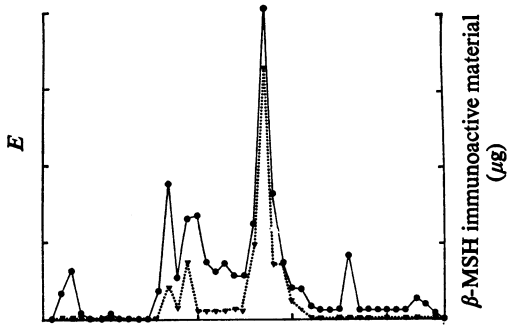


Fig. 2. *Chromatography on CM-cellulose of β-MSH-like immunoactive material obtained from Bio-Gel P6 fractionation of trifluoroacetic acid–acetone extract of human pituitaries*

Absorbance (●) and distribution of β-MSH-like immunoactivity (▼) in fractions obtained from a column of CM-cellulose (0.6cm internal diam.×10cm length) eluted with a concave gradient of 10–300mM-trimethylamine acetate buffer (pH 5) as described in the Materials and Methods section.

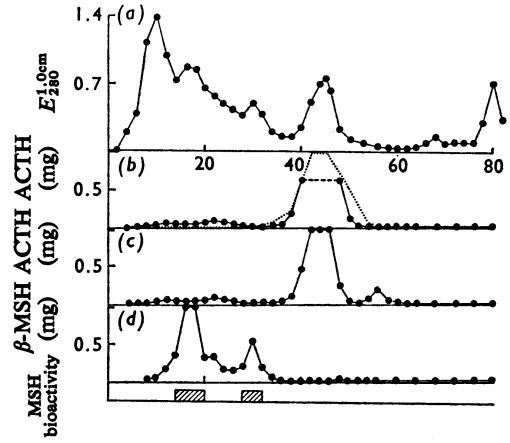


Fig. 3. *Gel filtration of HCl–acetone extract of human pituitaries on Bio-Gel P2 and P6*

E_{280} (*a*); biologically active ACTH (*b*; · · ·). *N*-terminally immunoactive ACTH (*b*; ●); *C*-terminally immunoactive ACTH (*c*) and β-MSH-like immunoactivity (*d*) in fractions obtained by chromatography of an HCl–acetone extract of 135 pituitaries on Bio-Gel P2 and P6 columns connected in series were determined as described in the Materials and Methods section. Amounts are expressed in mg of peptide/fraction. The shaded blocks at the base of the Figure represent those fractions which contain melanocyte-stimulating activity equivalent to more than 100μg of α-MSH. Further purification of the smaller peak of β-MSH-like immunoactive material (fractions 28–33), and the ACTH peak, are shown in Figs. 4 and 5.

peptide]. Peak-A material was purified by chromatography on CM-cellulose and its amino acid composition is shown in Table 1. The molar ratios suggest that this peptide could represent the 35–80 sequence of human β-LPH and we have termed it δ-LPH. MSH bioactivity was associated with all fractions containing β-MSH-like immunoreactivity.

(*c*) Chromatography on CM-cellulose of the Bio-Gel P2/P6-purified ACTH material yielded two peptides with bio- and immuno-activity (Fig. 5). Amino acid analysis after acid hydrolysis (Table 1) showed that they were both homogeneous and had the same amino acid content. It has been reported that under certain conditions both human and pig ACTH undergo deamidation followed by a β shift at the Asn-Gly sequence at positions 25 and 26 (Riniker *et al.*, 1972). The more basic ACTH peptide would appear to be true human ACTH, and the less basic peptide a deamidated form with a β-peptide bond between residues 25 and 26. This was corroborated by the fact that, although asparagine was identified in the enzyme hydrolysis of the more basic ACTH peak, none was identified with the 'deamidated' form, and the yields of amino acids present in the 25–28 region of the molecule were extremely low. Both ACTH species were equipotent with synthetic human ACTH in the bioassay. The total amount of ACTH (amidated form) isolated from 270 human pituitaries was approx. 6mg.

(*d*) Material eluted after the main ACTH peak on Bio-Gel P2/P6, (containing only *C*-terminal immunoactivity) was chromatographed on DEAE-cellulose. Approx. 90% of the activity was distributed in two adjacent peaks. Their amino acid compositions after acid hydrolysis were identical and resembled the 17–39 portion of human ACTH (Table 1). The more acidic peptide probably represents deamidated ACTH (17–39) peptide, as described for ACTH in the previous section. They differ from the *C*-terminally immunoreactive corticotrophin-like intermediate lobe peptides isolated from pig and rat pituitaries and a human carcinoid tumour in possessing an extra arginine residue.

Formation of human 'β-MSH' by prolonged extraction with 1M-acetic acid

We have previously observed that the 1M-acetic acid extraction has a detrimental effect upon the yields of ACTH and ACTH (18–39) peptide from pig pituitaries, probably because of rapid enzymic degradation of these peptides (Scott *et al.*, 1974*b*). Extraction of acetone-dried human pituitaries over a prolonged period in 1M-(6%) acetic acid and

Table 1. *Amino acid analysis of purified β-MSH-like and ACTH-like immunoreactive peptides*

Purified peptides (10–30nmol portions) were hydrolysed for 16h at 115°C with 200μl of 6M-HCl, and analysed as described in the Methods section. The values for serine were corrected for destruction (15% in 16h). Results are tabulated for a comparison with the amino acid compositions of other known peptides. The molar ratios of amino acids of the large-molecular-weight β-MSH immunoreactive peptide are compared with those of human β-LPH, published by Cséh *et al.* (1972). The amino acid compositions of the two smaller β-MSH-like immunoactive peptides isolated from the HCl–acetone extract are compared with those of the 1–58 and 35–80 portions of β-lipotrophin, as calculated from partial sequencing data of Cséh *et al.* (1972). The *C*-terminally immunoactive ACTH-like peptides are compared with the 17–39 fragment of human ACTH. All amino acid ratios for these peptides (determined with a Technicon Analyser) have been rounded off to one decimal place. The values for the two ACTH peptides (determined with a Beckman 120C analyser) have been rounded off to two decimal places. N.d., not determined.

Amino acid	Amino acid composition (mol/mol of peptide)											
	High mol.-wt. β-MSH immunoreactive peptide	Human β-LPH (Cséh *et al.*, 1972)	Acidic β-MSH immunoreactive peptide 'γ-LPH'	Residues 1–58 of LPH(Cséh *et al.*, 1972)	Basic β-MSH immunoreactive peptide 'δ-LPH'	Residues 35–80 of LPH (Cséh *et al.*, 1972)	Acidic C-terminally immunoactive ACTH-like peptides		Human ACTH-(17–39)-peptide	Major ACTH peptide 'not deamidated'	Minor ACTH peptide 'deamidated'	Human ACTH (39 residues)
							1	2				
Asx	10.4	9	7.0	7	3.1	3	2.6	2.7	2	2.07	2.10	2
Thr	3.9	4	1.0	1	2.9	3	—	—	—	—	—	—
Ser	4.3	5	2.1	3	2.8	3	0.9	1.1	1	3.01	2.90	3
Glx	13.1	13	8.5	10	5.8	6	3.9	4.0	4	5.01	5.11	5
Pro	5.8	6	6.4	5	4.0	4	2.9	3.1	3	4.07	4.15	4
Gly	12.0	9	8.0	6	4.3	4	1.1	1.1	1	3.05	3.12	3
Ala	8.8	8	5.8	6	3.0	2	2.7	2.7	3	3.02	3.05	3
Val	2.8	3	1.0	2	2.0	1	2.0	1.6	2	3.00	3.00	3
Met	2.0	2	1.0	1	2.1	2	—	—	—	0.98	0.98	1
Ile	1.7	2	—	—	—	—	—	—	—	—	—	—
Leu	8.1	7	5.0	5	2.0	2	1.0	0.9	1	0.99	1.00	1
Tyr	3.6	3	1.1	1	2.0	2	0.9	0.8	1	2.01	2.01	2
Phe	3.4	3	1.1	1	2.0	3	1.4	1.4	2	2.96	2.98	3
Lys	10.1	10	3.1	4	6.4	6	1.0	1.0	1	4.05	4.16	4
His	2.4	2	2.3	2	1.1	1	—	—	—	0.97	1.00	1
Arg	6.1	5	4.1	4	3.0	3	2.0	1.8	2	3.01	3.09	3
Trp	N.d.	1	N.d.	1	N.d.	1	—	—	—	0.76	0.74	1

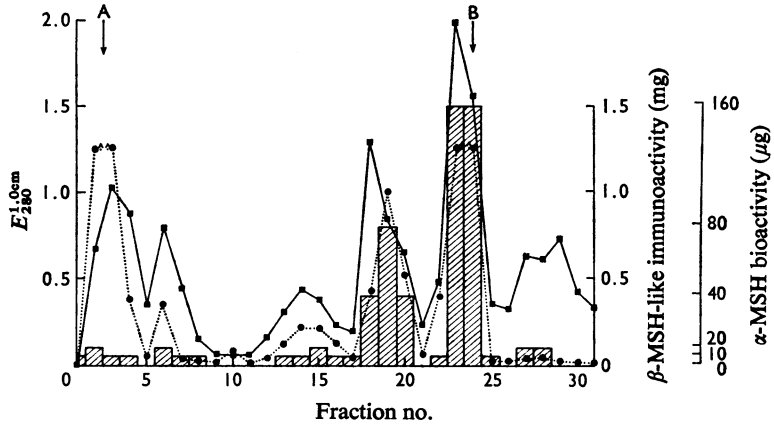


Fig. 4. *Chromatography on DEAE-cellulose of the smaller peak of β-MSH-like immunoreactive material obtained from the Bio-Gel P2/P6 fractionation of the HCl–acetone pituitary extract*

The column (0.4cm internal diam.×10cm length) was developed with a concave gradient of 10–500mM-trimethylamine acetate buffer (pH5) as described in the Methods section. E_{280}; ●, β-MSH-like immunoactivity. Melanocyte-stimulating activity (represented by the hatched blocks) is expressed against an α-MSH standard. Peaks A and B are discussed in the text.

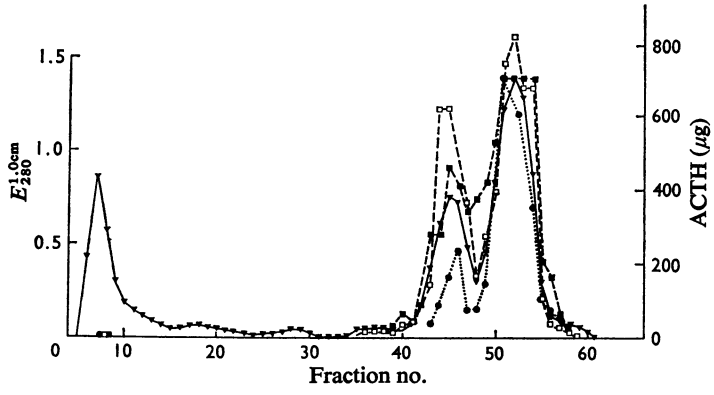


Fig. 5. *Chromatography on CM-cellulose of immunoreactive and biologically active ACTH material already partially purified by gel-filtration on Bio-Gel P2/P6*

The column (0.6cm internal diam.×25cm length) was developed with a concave gradient of 300–500mM-trimethylamine acetate buffer (pH5) as described in the Methods section. E_{280} (▼); *N*-terminal (■) and *C*-terminal (□) ACTH immunoactivity and corticosteroidogenic activity (●) are expressed in μg of active peptide/fraction. The results show the presence of two ACTH peptides in the HCl–acetone extract of human pituitaries. The corticosteroidogenic potency ratios of these peptides were measured against CIBA–GEIGY synthetic human ACTH: major ACTH peptide (fractions 50–55) = 0.96 (fiducial limits $p > 0.05$, 0.7–1.4); minor ACTH peptide (fractions 43–47) = 0.88 (0.7–1.1).

chromatography of samples, removed at various intervals, on Bio-Gel P6 indicated that the large β-MSH-like immunoreactive peptides, β-LPH and γ-LPH, were converted into smaller peptides, one of which had a molecular weight smaller than ACTH (i.e. a similar elution position to 'β-MSH'). These changes were eliminated by prior boiling.

Discussion

We have demonstrated that all the bioactive and immunoreactive β-MSH-like material in acid extracts of human pituitaries is eluted before ACTH on Bio-Gel P6 and is therefore accounted for by peptide(s) with molecular weight(s) greater than 4500. The peptide responsible for the bulk of this activity has

been purified and its amino acid composition shown to closely resemble β-lipotrophin, a peptide with 91 amino acids (Cséh *et al.*, 1972). In contrast, pig pituitary extracts contain in addition to β-LPH-like material, a β-MSH-like immunoactive and biologically active peptide which is eluted on Bio-Gel P6 in the expected position for β-MSH, with a molecular weight of approx. 2000 (A. P. Scott & P. J. Lowry, unpublished results). Pig pituitaries also contain two other peptides in substantial amounts, namely α-MSH and ACTH (18–39) peptide, but we have failed to identify either in human pituitaries.

We propose that the human pituitary does not normally produce β-MSH, α-MSH or ACTH (18–39) peptide, and present the following evidence to support this hypothesis.

(1) Lerner and his colleagues repeatedly failed to isolate β-MSH and α-MSH from human pituitaries (Lerner *et al.*, 1968), using extraction procedures that have produced excellent quality β- and α-MSH from sheep, pig, ox, monkey and horse pituitaries.

(2) The initial isolation of human β-MSH was from a side fraction of a growth-hormone-extraction procedure (Dixon, 1960), and it is possible that its identification may be related to inappropriate extraction techniques. This is supported by the appearance of β-MSH-like immunoreactive material in the expected elution position of β-MSH on Bio-Gel

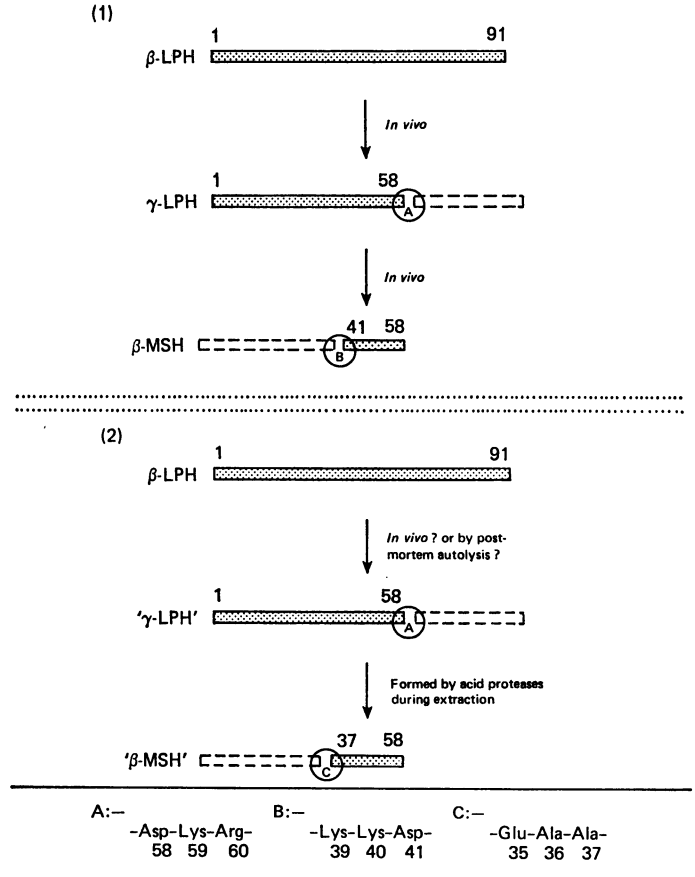


Fig. 6. *Proposed mechanisms of formation of β-MSH molecules from the β-lipotrophins*

The peptides are represented diagrammatically by bars. The first scheme (1) shows the mechanism of formation of pig, ox and sheep β-MSH, as proposed by Chrétien *et al.* (1973). The second mechanism (2) is proposed by us for the formation of 'human β-MSH'. The positions where peptide cleavages occur are circled (A, B, C), and the portions of the amino acid sequences in these regions are shown at the bottom of the figure.

P6 after extraction of pituitaries in dilute acetic acid, that can be eliminated by boiling or the use of stronger acids suggesting that 'β-MSH' may be formed by the action of proteolytic enzymes.

(3) The peptide resembling β-LPH accounts for the bulk of β-MSH-like immunoreactivity in the human pituitary. Small amounts of two other immunoreactive peptides with molecular weights of about 6000 were also identified, one of which resembled residues 1–58 of β-LPH. A similar fragment (i.e. γ-LPH), has been isolated from ox, sheep and pig pituitaries (Chrétien *et al.*, 1973). In these species, the sequence of β-MSH is contained within the 41–58 portions of the corresponding β-LPH and γ-LPH molecules. The mechanism of formation of β-MSH from these two peptides, as proposed by Chrétien & Li (1967), is shown in Fig. 6. This involves two peptide cleavages of β-LPH, with the γ-LPH molecule being an intermediate breakdown product. The pairs of basic amino acids near the cleavage points in the β-LPH molecule implicate a trypsin-like enzyme. It is apparent that human β-MSH can also be formed by fragmentation of the human β-LPH molecule. The fact that the extra amino acid sequence at the *N*-terminus of the human β-MSH molecule lies in positions 37–40 of all known β-LPH and γ-LPH molecules strongly supports the suggestion that human β-MSH is derived from the human γ-LPH molecule by cleavage between residues 36 and 37. The nature of the amino acids at the new site of cleavage, however, (two alanines) indicates that a trypsin-like enzyme cannot be involved, i.e. the mechanism of cleavage differs strikingly from that found in the other species. The formation of many other hormones, including insulin, gastrin and α-MSH from their precursor molecules also appears to involve trypsin-like enyzmes, and it is therefore the unusual nature of the cleavage reaction that leads to the formation of 'human β-MSH', which suggests that it occurs artifactually rather than *in vivo* (Fig. 6).

(4) α-MSH, β-MSH (1–18) and ACTH (18–39) peptide have only been identified in animals with a distinct *pars intermedia* and their absence in the human pituitary is probably associated with the lack of this lobe (Scott *et al.*, 1973). This situation has also been found in another animal without a *pars intermedia*, the finback whale (*Balaenoptera physalus*) (Geschwind, 1966).

(5) Preliminary studies on plasma from two patients with Nelson's syndrome have shown that the circulating β-MSH-like immunoreactive material also resembles β-LPH in molecular weight (G. A. Bloomfield, A. P. Scott, L. H. Rees, J. J. H. Gilkes & P. J. Lowry, unpublished results).

In conclusion, our observations indicate that 'human β-MSH' does not exist in nature, but is an artifactually produced fragment of the β-LPH molecule. This suggests that other published radioimmunoassays for 'human β-MSH' may also be measuring β-LPH, and that it is this peptide that is the β-MSH-like immunoreactive material which has been shown to be present in the same cells that contain corticotrophin in the human pituitary (Phifer *et al.*, 1972). The fact that the 'human β-MSH' molecule is an extraction artefact may also explain why no one has yet discovered its function in human beings.

We acknowledge the help and advice of Dr. J. G. Ratcliffe, Professor J. Landon and Miss Glenys Bloomfield. This work was supported by a grant from the Tenovus Institute. We are grateful to Dr. Lesley Rees for the gift of the ACTH (*N*-terminal) anti-serum, and to Dr. W. Rittell (CIBA–GEIGY) for gifts of synthetic α-MSH, human β-MSH, bovine β-MSH and human ACTH.

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