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Purification and characterization of a γ-melanotropin precursor from frozen human pituitary glands

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A new melanocyte-stimulating peptide has been isolated from acid extracts of frozen human pituitary glands by salt/ethanol fractionation, Sephadex G-75 gel filtration and DEAE- and CM-cellulose ion-exchange chromatography. The peptide is glycosylated, has an *N*-terminal tryptophan residue and an apparent mol.wt. of 16000 as estimated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Its amino acid analysis closely resembles residues Trp^{-105} to Gln^{-29} predicted for the common precursor protein of bovine corticotropin and β-lipotropin by Nakanishi, Inoue, Kita, Nakamura, Chang, Cohen & Numa [(1979) *Nature* (*London*) **278**, 423–427]. This fragment is expected to have melanotropin activity due to the tetrapeptide -His-Phe-Arg-Trp- (residues −51 to −48) of the predicted sequence of the common precursor. It was found to have a molar potency of 1×10^{-5} relative to α-melanotropin in the frog skin bioassay. These characteristics are consistent with the isolated melanotropin peptide being a non-corticotropin, non-lipotropin peptide of the human common precursor protein of corticotropin and lipotropin. The peptide neither potentiates the adrenal weight-maintenance activity of corticotropin-(1–24)-tetracosapeptide when administered to hypophysectomized rats, nor stimulates release of non-esterified fatty acids from isolated rat epididymal cells. A second *N*-terminal-tryptophan glycopeptide was also isolated, which had an amino-acid composition similar to that predicted for the bovine common precursor protein, residues Trp^{-105} to Gly^{-35}.

The amino-acid sequence of the common precursor protein of corticotropin and β-lipotropin has been inferred from DNA sequencing of complementary DNA synthesized with a precursor mRNA template purified from neurointermediate lobes of bovine pituitaries (Nakanishi *et al.*, 1979). The sequence corroborates the model of the common precursor developed by studies on the biosynthesis of corticotropin and β-lipotropin in a mouse anterior-pituitary tumour-cell line (AtT-20/D-16v) and in cultured rat pars-anterior and pars-intermedia cells (Mains & Eipper, 1978, 1979). The model places corticotropin, which can be glycosylated or unglycosylated, in the middle of the precursor protein, separated at its *C*-terminal from β-lipotropin and at its *N*-terminal from a glycopeptide of at least 80 amino-acid residues by dibasic amino-acid sequences (e.g. Lys-Arg). The DNA sequence also predicts that a third melanocyte-stimulating peptide (melanotropin) is present in the protein sequence *N*-terminal to corticotropin in the precursor, apart from and identical with the His-Phe-Arg-Trp tetrapeptide found in corticotropin and lipotropin. This third melanocyte-stimulating peptide was named γ-melanotropin by Nakanishi *et al.* (1979).

A similar, if not identical, precursor molecule is synthesized by the melanotroph of the pars intermedia and the corticotroph of the pars anterior. The cells differ in their processing of the precursor; in the melanotroph it is converted into α-melanotropin, corticotropin-like intermediate-lobe peptide and the endorphins, whereas in the corticotroph corticotropin and lipotropin are final products (Lowry *et al.*, 1977). In corticotropin/lipotropin-containing cells of both lobes of the pituitary, the non-corticotropin, non-lipotropin *N*-terminal fragment of the precursor seems to be stored, at least in part, as an 80-amino-acid glycopeptide (Mains & Eipper,

Abbreviation used: SDS, sodium dodecyl sulphate.

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1978). Recent studies on the incorporation of radioactive amino acids into the common precursor by cultured rat pars-intermedia cells showed cleavage of the nascent molecule to produce an *N*-terminal-tryptophan corticotropin/lipotropin-precursor protein (Gossard *et al.*, 1980). Processing of this protein in the pars-intermedia cell should, according to the above model, give the endorphins, corticotropin-like intermediate-lobe peptide, α-melanotropin and an *N*-terminal-tryptophan glycopeptide. This explains the intense fluorescence after formaldehyde condensation observed in the cytoplasm of pars-intermedia cells, which are almost all melanotrophs (Moriarty, 1969), and in scattered cells of the pars anterior of the rat and other species, and found to be due to *N*-terminal-tryptophan polypeptides (Hakanson *et al.*, 1972).

Although little is known of the pathway of corticotropin/lipotropin biosynthesis in the human corticotroph, small amounts of a corticotropin/lipotropin-precursor protein were found in extracts of human pituitaries (Lowry *et al.*, 1976) and human pituitary tumour tissue synthesizes molecular forms of corticotropin similar to those produced by cultured rat pars-anterior and mouse tumour cells (Ishibashi & Yamaji, 1978). Hence, by analogy with the processed rat precursor protein, the human molecule is probably rapidly cleaved in the corticotroph to corticotropin, lipotropin and an *N*-terminal-tryptophan glycopeptide.

During the development of methods to improve the yield of corticotropin and lipotropin extracted from frozen human pituitaries we monitored the chromatographic stages of the purification for *N*-terminal-tryptophan peptides. We report in the present paper the purification of a melanocyte-stimulating peptide (γ-melanotropin; see Nakanishi *et al.*, 1979) with the characteristics expected of the putative *N*-terminal fragment of the human corticotropin/lipotropin-precursor protein.

Materials and methods

Materials

Human pituitaries were collected *post mortem* and stored frozen at −20°C for up to 3 months before extraction.

CM-cellulose (CM-52) and DEAE-cellulose (DE-52) were purchased from Whatman Biochemicals (Maidstone, Kent, U.K.) and fractionated before use by methods similar to those of McMartin & Vinter (1969). Sephadex G-15, G-50 (10–40 μm) and G-75 (10–40 μm) were from Pharmacia (U.K.) Ltd. (Hounslow, London, U.K.).

Reagents for polyacrylamide-gel electrophoresis were of electrophoresis purity grade and purchased from Bio-Rad Laboratories (Richmond, CA, U.S.A.). The standard proteins used to calibrate the gels and L-tryptophyl-L-glycine were from Sigma Chemical Co. (Poole, Dorset, U.K.).

Anthrone and D-(+)-mannose (Gold Label) were from Aldrich Chemical Co. (Gillingham, Dorset, U.K.). Synthetic α-melanotropin and corticotropin-(1–24)-tetracosapeptide (as Synacthen depot) were supplied by Ciba–Geigy (Basle, Switzerland). All other chemicals were of AnalaR grade from BDH Chemicals (Poole, Dorset, U.K.).

Methods

All extraction procedures were carried out at 4°C and centrifugations were carried out in an MSE 18 refrigerated centrifuge (Measuring and Scientific Equipment Ltd., Crawley, Sussex, U.K.) at 10000 **g** for 1 h unless otherwise stated.

Extraction

Frozen human pituitary glands (300; 166 g wet wt.) were homogenized in 0.1 M-HCl (700 ml) and the homogenate was centrifuged. The supernatant was decanted and the residual tissue was extracted with 0.1 M-HCl (300 ml) and centrifuged. The supernatants from each extraction were combined (1200 ml).

Ethanol/salt fractionation

A saturated $(NH_4)_2SO_4$ solution (133 ml) was dripped into the stirred acid extract. The 10%-saturated $(NH_4)_2SO_4$/acid extract was centrifuged and the precipitate was discarded. Ethanol (1200 ml) was added dropwise to the supernatant and the resulting suspension was stirred overnight. The suspension was centrifuged, the precipitate discarded and ethanol (9600 ml) was poured into the supernatant to a final concentration of 87% ethanol. The suspension was centrifuged at 1500 **g** for 2 h and the supernatant was discarded. The precipitate was resuspended in 0.1 M-HCl (1240 ml) and this solution was centrifuged to remove insoluble debris. Solid $(NH_4)_2SO_4$ (542 g) was added to the supernatant and this 80%-saturated $(NH_4)_2SO_4$ solution was centrifuged, the supernatant discarded, and the precipitate resuspended in 0.25 M-formic acid (100 ml). This solution was clarified by centrifugation before gel-filtration chromatography.

Chromatography

Gel-filtration and ion-exchange columns were used at room temperature, eluate fractions collected at 4°C and monitored for absorbance at 280 nm with a 1 cm-pathlength optical cell and a Cecil CE292 digital u.v. spectrophotometer (Cecil Instruments, Cambridge, U.K.). Alternate fractions were assayed for *N*-terminal-tryptophan peptides as described below.

Gel filtration. The fractionated extract (100 ml)

was desalted on a column (5 cm internal diam. × 45 cm length) of Sephadex G-15 with 0.25 M-formic acid as eluting solvent at a flow rate of $48\,ml\cdot h^{-1}$. Fifteen-minute fractions (12 ml) were collected and the u.v.-absorbance peak ($K_D = 0.0$–0.21) from the column was pooled (140 ml), loaded on to a column of Sephadex G-75 (5 cm × 87 cm) and eluted with 0.25 M-formic acid at a flow rate of $13.5\,ml\cdot h^{-1}$. Thirty-minute fractions (7 ml) were collected. Fractions containing *N*-terminal-tryptophan peptides ($K_D = 0.49$–0.60) were pooled and the pH was adjusted to 3.0 with 1 M-NH_3 (125 ml) before ion-exchange chromatography.

Ion exchange. CM-cellulose. The *N*-terminal-tryptophan peptide pool from gel filtration was loaded on to a column (1 cm × 90 cm) of fractionated CM-cellulose equilibrated in 0.04 M-ammonium formate (pH 3.0 adjusted with 25 M-formic acid) and eluted with a linear salt gradient from 0.04 M- to 0.5 M-ammonium formate at pH 3.0. The total gradient volume was 800 ml, a flow rate of $10\,ml\cdot h^{-1}$ was used and thirty-minute (5 ml) fractions were collected (see Fig. 1). Fractions 65–73 (pool CM I) and 74–83 (pool CM II), corresponding to *N*-terminal-tryptophan fluorescence peaks, were pooled, mannitol (100 mg) was added to each pool and the solution was freeze-dried.

DEAE-cellulose. Pools CM I and CM II were processed separately on DEAE-cellulose. The freeze-dried pool (CM I or CM II) was reconstituted in 15 ml of 0.01 M-ammonium bicarbonate pH 8.5 and loaded on to a column (2 cm × 50 cm) of fractionated DEAE-cellulose equilibrated in the same buffer. Absorbed peptides were eluted from this column with a linear salt gradient from 0.01 M- to 0.5 M-ammonium bicarbonate. The total gradient volume was 800 ml, a flow rate of $19\,ml\cdot h^{-1}$ was used and fractions (9.5 ml) were collected (Figs. 2*a* and 2*b*).

N-Terminal-tryptophan-peptide peaks from DEAE-cellulose chromatography, the DEAE I peak derived from the CM I peak (Fig. 2*a*) and the DEAE II peak derived from the CM II peak (Fig. 2*b*), were analysed for their amino-acid composition (Table 1), carbohydrate content and by SDS/polyacrylamide-gel electrophoresis.

Peak DEAE I, which was separated into two components on SDS/polyacrylamide-gel electrophoresis, was further purified by gel filtration on a column (1.5 cm × 80 cm) of Sephadex G-50 with 0.25 M-formic acid as the eluting solvent. Fractions

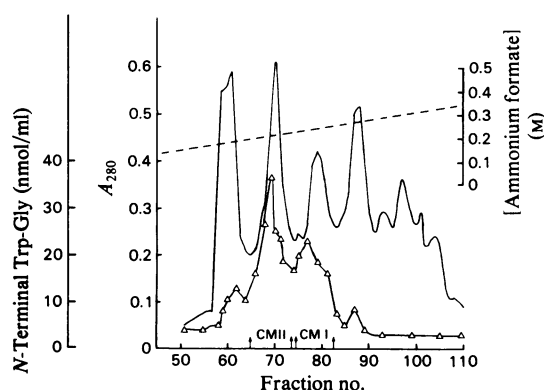

Fig. 1. *Chromatography on CM-cellulose of N-terminal-tryptophan peptides obtained from Sephadex G-75 fractionation of a human pituitary extract*

——, A_{280}; △, distribution of *N*-terminal-tryptophan peptides in fractions obtained from a column (1 cm × 90 cm) of CM-cellulose eluted with a linear gradient (----) of 0.04–0.5 M-ammonium formate (pH 3) as described in the Materials and methods section.

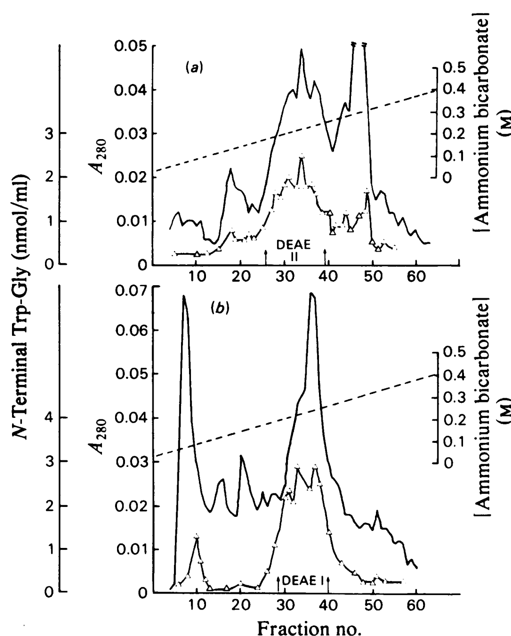

Fig. 2. *Chromatography on DEAE-cellulose of human N-terminal-tryptophan peptides*

The Figure shows chromatography of the *N*-terminal-tryptophan peptide-containing fractions 65–73 (*a*) and 74–83 (*b*) obtained from CM-cellulose after chromatography of a fractionated human pituitary extract and gel filtration on Sephadex G-75. The same column (2 cm × 50 cm) was used and 0.01–0.5 M-ammonium bicarbonate (pH 8.5) linear gradient was developed as described in the Materials and methods section. ——, A_{280}; △, *N*-terminal-tryptophan peptides; ----, salt gradient.

(2 ml) were collected and analysed for carbohydrate content by the anthrone method and for *N*-terminal-tryptophan peptides. Amino-acid analysis and SDS/polyacrylamide-gel electrophoresis were done on the *N*-terminal-tryptophan-peptide peak, DEAE I (G-50), obtained after Sephadex G-50 chromatography.

Peak DEAE II was tested for melanocyte-stimulating, lipolytic and adrenal weight-maintaining activities.

Amino-acid analysis

Freeze-dried peptide samples (approx. 50 μg) (see the Results and discussion section) were hydrolysed with 6 M-HCl (200 μl), containing a crystal of phenol to prevent tyrosine loss, in evacuated Pyrex tubes at 115 °C for 20 h. The hydrosylates were analysed on a Jeol JLC-6AH automatic amino-acid analyser (Japanese Electric and Optical Co., Tokyo, Japan). Serine and threonine values were corrected for destruction on hydrolysis (Scott & Lowry, 1974).

Assay for N-terminal-tryptophan peptides in chromatography fractions

N-Terminal-tryptophan peptides were assayed fluorimetrically after formaldehyde condensation by the method of Hakanson & Sundler (1971). Solutions of L-tryptophyl-L-glycine (1–100 μmol · litre^{-1}, dissolved in 0.05 M-H_2SO_4) were used as standards. Sample or standard (0.2 ml) was mixed with an 18% (v/v) formaldehyde solution (0.1 ml) and 0.05 M-H_2SO_4 (1.5 ml) in a glass tube. This mixture was heated in boiling water for 20 min, 5% (v/v) H_2O_2 (0.1 ml) was added and heating was continued for 20 min. The solutions were chilled and analysed in an Aminco–Bowman spectrophotofluorimeter, with an excitation wavelength of 360 nm and an emission wavelength of 450 nm. Wavelengths are the uncorrected instrumental values. Peptide concentrations are expressed as nmol of tryptophylglycine equivalents per ml of chromatography fraction.

Carbohydrate analysis

The anthrone method (Dreywood, 1946) was used to estimate the hexose/methylpentose content of the DEAE I (G-50) and DEAE II peptides. Solutions of D-(+)-mannose (20–2.5 μg · litre^{-1}) were used as standards. Sample or standard (0.5 ml) was frozen in a glass tube and 0.2% (w/v) anthrone in 10 M-H_2SO_4 (1.0 ml) was added. The mixture was heated in a boiling-water bath for 10 min, and allowed to cool. The absorbance of the solution was measured at 585 nm with a Shiymatzu double-beam digital spectrophotometer.

SDS/polyacrylamide-gel electrophoresis

Freeze-dried samples were analysed on SDS/polyacrylamide gels, containing 12% (w/v) acrylamide, prepared and used according to the method of Weber & Osborn (1969). Gels were fixed in 15% (w/v) trichloroacetic acid overnight and protein bands were detected by staining overnight with 0.25% (w/v) Coomassie Blue G250 dissolved in methanol/acetic acid/water (45 : 7 : 48, by vol.) followed by destaining in methanol/acetic acid/water (45 : 7 : 48, by vol.). Glycoproteins were detected after fixing the gels in 15% (w/v) trichloroacetic acid with periodic acid/Schiff reagent (Fairbanks *et al.*, 1971). Sample protein molecular weights were estimated by comparing their mobilities, their R_F values defined by Weber & Osborn (1969), with the mobilities of a set of standard proteins (cytochrome *c*, myoglobin, carbonic anhydrase and yeast alcohol dehydrogenase).

Bioassays

Frog skin melanotropin assay. The melanocyte-stimulating activity of the DEAE II preparation was measured by the reflectometric method of Shizume *et al.* (1954).

Fat-mobilizing assay. The fat-mobilizing activity of the DEAE II preparation was assayed by the method of Smith & Belin (1979).

Adrenal weight-maintenance assay. Hypophysectomized male Sprague–Dawley rats were allowed to recover after pituitary removal for 1 week. Groups of six animals were used to estimate the ability of (I) the DEAE II fraction (10 μg), (II) the DEAE II fraction (10 μg) and Synacthen depot (5 μg), (III) the Synacthen depot (5 μg) or (IV) a control saline (0.9% NaCl) solution to maintain the weight of the adrenals. Each test preparation was administered subcutaneously in iso-osmotic saline (0.5 ml) each day for 6 days. On day 7 the rats were decapitated and the adrenals were removed, defatted, decapsulated and weighed. The significance of differences in weight between the groups was determined by Student's *t* test.

Results and discussion

Extraction and purification

The extraction and salt/ethanol fractionation procedures were not monitored for *N*-terminal-tryptophan peptides. Essentially all the corticotropin and lipotropin extracted from the glands, as determined by radioimmunoassay (results not presented), was recovered after the final desalting stage on Sephadex G-15, however, and a yield of 1.56 μmol of *N*-terminal Trp-Gly equivalents was obtained after Sephadex G-75 chromatography between $K_D = 0.49$ and 0.60. CM-cellulose chromatography resolved this pool into two *N*-terminal-tryptophan peptide components (see Fig. 1), CM I (1.02 μmol) and CM II (0.87 μmol). Freeze drying and DEAE-cellulose chromatography (see Figs. 2*a*

and 2*b*) of CM I and CM II pools resulted in considerable loss of material but produced highly purified products, the *N*-terminal-tryptophan peptides, DEAE I (279 nmol) and DEAE II (221 nmol) eluting as broad peaks that are typical of glycopeptides. A high-cysteine-containing contaminant was removed from DEAE I fraction by gel filtration on Sephadex G-50.

Development of a radioimmunoassay for these peptides will enable optimization of the extraction procedure for their purification and the isolation of related fragments that may not have an *N*-terminal tryptophan residue.

Chemical characterization

The amino-acid analyses of the final *N*-terminal-tryptophan-peptide preparations, the DEAE I (G-50) and DEAE II peptides, are compared in Table 1 with the compositions predicted for similar fragments of the corticotropin/lipotropin-precursor protein by Nakanishi *et al.* (1979). The numbering of the amino-acid residues in the precursor fragments is with respect to the *N*-terminal serine residue of the corticotropin part of the precursor molecule (Nakanishi *et al.*, 1979). The isolated peptides have amino-acid compositions similar to peptides in the predicted bovine precursor sequence. The peptide DEAE I (G-50) gave an analysis similar to the bovine precursor protein residues Trp^{-105} to Gly^{-35}. The molecular weight of this peptide, calculated from its amino-acid composition, is 9188, whereas the peptide DEAE II has a calculated molecular weight of 9780 and resembled the bovine precursor protein residues Trp^{-105} to Gln^{-29} in amino-acid composition.

SDS/polyacrylamide-gel electrophoresis analysis of DEAE I (G-50) and DEAE II peaks gave, in both cases, a single band when stained with Coomassie Blue G250, or periodic acid/Schiff reagent. The DEAE I (G-50) and DEAE II peaks are therefore homogeneous glycopeptide preparations; hexose/methylpentose contents of $1.5\,\mu g \cdot nmol^{-1}$ for DEAE I peptide and $1.6\,\mu g \cdot nmol^{-1}$ for DEAE II peptide were determined by the anthrone method. The anthrone measurements were read at 585 nm instead of 620 nm to avoid underestimation of the carbohydrate content due to the high percentage of tryptophan residues in this peptide (Horman & Gollwitzer, 1965).

Apparent molecular weights, by SDS/polyacrylamide-gel electrophoresis, of 16000 and 15000 were estimated for DEAE II and DEAE I (G-50) peptides respectively. SDS/polyacrylamide-gel electrophoresis does not, however, provide reliable molecular-weight determinations of glycoproteins (Weber *et al.*, 1972). Mains & Eipper (1978) termed their *N*-terminal peptide, derived from the rat pars-anterior corticotropin/lipotropin-precursor

Table 1. *Amino-acid analyses of the purified human N-terminal-tryptophan peptides*

Purified peptides (approx. 50 μg) were hydrolysed for 20 h at 115°C with 6 M-HCl (200 μl) and analysed as described in the Materials and methods section. The values for serine and threonine are corrected for destruction during hydrolysis (20%) (Scott & Lowry, 1974). Results are tabulated with the compositions of similar fragments in the predicted sequence of the bovine corticotropin/lipotropin precursor protein (Nakanishi *et al.*, 1979).

	Amino acid composition (residues/mol of peptide)			
Amino acid	DEAE I (G-50)	Bovine corticotropin/lipotropin precursor (Trp^{-105}–Gly^{-35})	DEAE II	Bovine corticotropin/lipotropin precursor (Trp^{-105}–Gln^{-29})
Asx	7.4	8	8.7	8
Thr	3.6	4	4.1	4
Ser	7.1	8	7.6	8
Glx	8.4	8	9.8	9
Pro	5.5	5	5.0	5
Gly	6.0	6	8.0	8
Ala	3.3	3	5.3	5
Cys	3.6	4	3.9	4
Val	1.5	2	3.2	3
Met	2.4	1	2.1	1
Ile	1.1	1	1.3	1
Leu	5.9	6	6.4	6
Tyr	1.3	1	1.1	1
Phe	3.5	3	3.0	3
His	1.3	1	1.8	1
Lys	2.1	2	4.1	2
Trp	1.5*	2	1.6*	2
Arg	4.7	6	5.0	6

* Estimated by absorbance at 280 nm by the method of Roos (1968).

protein, the '16K fragment', because it had an apparent mol.wt. of 16000 when estimated by SDS/polyacrylamide-gel electrophoresis. Re-estimation of the molecular weight of this fragment, however, by gel filtration on Sephadex G-50, G-75 and G-100 and on Bio-Gel A 0.5 M in 6 M-guanidine hydrochloride gave a molecular weight of 11200 ± 500 (Mains & Eipper, 1978). Crine *et al.* (1979) found two non-corticotropin, non-lipotropin glycopeptides derived from the common precursor synthesized by cultured rat pars-intermedia cells. They differed only in polysaccharide content and their molecular weights estimated by SDS/polyacrylamide-slab-gel electrophoresis were 19000 and 17000.

Carbohydrate contributed up to 2000 (17.4%) of the estimated mass of the 16K fragment (Mains & Eipper, 1978). Hexose/methyl pentose residues represent about 15% by weight of the isolated glycopeptides, so, because the total carbohydrate content is probably greater than this, the human peptides appear more glycosylated than the rat 16K fragment. The molecular weights of the DEAE I (G-50) and DEAE II peptides, estimated from amino-acid composition and their measured degree of glycosylation are 11600 and 12200 respectively. We believe that the DEAE II peptide is the human counterpart of the 16K fragment.

Proteolytic cleavage at a pair of basic amino-acid residues in proinsulin, proparathyrin and other secretory polypeptide precursor molecules (Hales, 1978) produces the stored and secreted bioactive form of the peptide. Corticotropin and lipotropin can be cleaved from the predicted bovine precursor protein sequence by one or two of these tryptic-like cleavages. The larger purified peptide, DEAE II, can be produced by an identical cleavage at Lys^{-28}-Arg^{-27} from the precursor in contrast with an atypical cleavage necessary to form the Trp^{-105} to Gly^{-35} peptide, DEAE I (G-50). The DEAE I (G-50) peptide may be produced from the corticotropin/lipotropin precursor in the cell or from the DEAE II peptide by non-specific proteolysis during isolation. Artefactual production of peptides by inappropriate isolation procedures has confused previous studies on lipotropin/corticotropin biosynthesis (Scott & Lowry, 1974) and, although the acid extraction used in this procedure should inactivate known proteinases, it remains necessary to characterize, by use of radioimmunoassay and chemical analysis, the forms present in the gland. Hence, although both peptides were isolated in similar quantities and both should contain by analogy to the bovine precursor, the melanotropic tetrapeptide His-Phe-Arg-Trp, assessment of biological activity was confined to the larger, DEAE II, peptide.

The similar amino-acid composition of the (predicted) bovine and (isolated) human peptides, if due to conservation of amino-acid sequence, may indicate a physiological function for these peptides.

Biological activity

In mammalian physiology, the humoral action of the peptides derived from the corticotropin/lipotropin-precursor protein is well understood only for corticotropin, which is necessary for glucocorticoid production in the cells of the adrenal cortex. Pituitary effects on the adrenal gland, which cannot be explained by the action of corticotropin alone, may be due to some other fragment of the precursor molecule. Removal of the pituitary gland, for example, results in atrophy of the adrenals, which cannot be fully prevented by administration of corticotropin (Segal & Christy, 1968). We tested the DEAE II peptide for its ability to act synergistically with corticotropin and maintain the adrenal weight of hypophysectomized rats. The mean weight ($\pm$ S.D.) of paired adrenals of rats treated with the following dosages were found: (I) Synacthen, 24.2 (± 4.3) mg ($n = 6$); (II) Synacthen plus DEAE II, 19.8 (± 1.8) mg ($n = 5$); (III) DEAE II, 11.6 (± 2.0) mg ($n = 6$); (IV) saline, 11.5 (± 2.8) mg ($n = 6$). Hence the DEAE II peptide failed to potentiate the weight-maintaining action of Synacthen on the adrenals of hypophysectomized rats. In fact a significant decrease ($P < 0.05$) in adrenal weight was observed in rats treated with Synacthen plus DEAE II peptide when compared with the weights of those treated with Synacthen alone.

Both corticotropin and lipotropin stimulate the release of non-esterified fatty acids from isolated adipocytes. However, work by Smith & Belin (1979) on the lipolytic potency of an ovine corticotropin precursor molecule showed it to have a potency greater than that expected from its corticotropin content measured by radioimmunoassay. This could be due to a separate lipolytic activity in the non-corticotropin region of this sheep precursor molecule. The DEAE II peptide, which has the physical and chemical properties expected of a human *N*-terminal corticotropin precursor, was found not to have significant lipolytic activity, however.

The melanocyte-stimulating activity of the DEAE II peptide preparation is compared with the activity of human corticotropin and α-melanotropin in the frog skin bioassay in Fig. 3. This glycopeptide has an amino-acid composition similar to that predicted for the bovine corticotropin/lipotropin precursor protein sequence Trp^{-105} to Gln^{-29} (see Table 1), and its melanotropin potency, about one-tenth as active as human corticotropin and one hundred-thousandth as active as α-melanotropin, is consistent with a partial amino-acid sequence of His-Phe-Arg-Trp in a large peptide. This tetra-

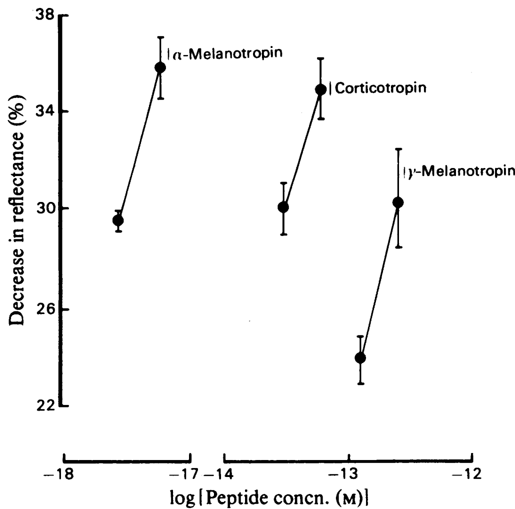

Fig. 3. *Comparison of the melanocyte-stimulating activity of α-melanotropin, the DEAE II peptide and corticotropin*

The percentage decrease in reflectance of frog skin (Shizume *et al.*, 1954) is plotted against, on the logarithmic scale, concentrations of α-melanotropin, DEAE II peptide (γ-melanotropin and corticotropin. The response to corticotropin and DEAE II peptide was parallel to the response to α-melanotropin ($P < 0.001$) in this assay.

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peptide, corresponding to residues 6–9 in corticotropin and α-melanotropin, and residues 50–53 in β-lipotropin, is the smallest peptide fragment of these melanotropin peptides capable of stimulating the melanocyte (Schwyzer, 1977). The bovine corticotropin/lipotropin-precursor protein was predicted to have this core tetrapeptide in its *N*-terminal non-corticotropin, non-lipotropin region at residues −51 to −48, and the peptide containing this sequence, residues −55 to −44, was named γ-melanotropin by Nakanishi *et al.* (1979). γ-Melanotropin has been synthesized by solid-phase methods and a melanotropin potency relative to α-melanotropin of 1.4×10^{-4} in the frog skin bioassay was found (Ling *et al.*, 1979).

Although its melanotropin potency and chemical characteristics suggest the *N*-terminal-tryptophan peptide isolated here may be a human γ-melanotropin precursor, we believe melanocyte stimulation in humans is not likely to be a major physiological action of this peptide (DEAE II peptide). Synthetic analogues of γ-melanotropin failed to stimulate release from cultured rat anterior-pituitary cells of lutropin, follitropin, prolactin, somatotropin or thyrotropin (Ling *et al.*, 1979). Behavioural effects induced in rats by intraventricular administration of melanotropin peptides (de Wied, 1977) and their presence in rat brain (Jacobowitz & O'Donohue, 1978; Watson *et al.*, 1977) and human cerebrospinal fluid (Jeffcoate *et al.*, 1978), however, suggest that γ-melanotropin, along with other fragments of the corticotropin/lipotropin precursor protein, such as α-melanotropin and the endorphins (Burbach *et al.*, 1980), may be part of a co-ordinated system of behavioural control in both man and rat. Purified preparations of these peptides, made available by this isolation procedure, will aid the study of the physiological role of γ-melanotropin and related peptides.

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