60 YEARS OF POMC

Purification and biological characterisation of melanotrophins and corticotrophins

Philip Lowry

Emeritus Professor School of Biological Sciences, University of Reading, Reading, UK

Correspondence should be addressed to P Lowry
Email
p.j.lowry@reading.ac.uk

Abstract

The remarkable conservation of the primary structures and anatomical location of dogfish α-melanocyte-stimulating hormone (MSH), corticotrophin-like intermediate lobe peptide (CLIP) and adrenocorticotrophic hormone (ACTH) compared with mammals reinforced the tissue-specific processing hypothesis of ACTH peptides in the pituitary gland. The cloning of dogfish pro-opiomelanocortin (POMC) led to the identification of δ-MSH and simultaneously revealed the high conservation of the γ-MSH sequence during evolution. These studies have also shown that β-MSH is much less conserved during evolution and in some species is not even processed from β-LPH. Human pro-γ-MSH potentiates the corticosteroidogenic activity of ACTH and peptides generated from its N-terminal, in particular big-γ-MSH, appear to have adrenal mitogenic activity. Human big-γ-MSH (from the zona intermedia) may also cause the adrenache. The review finishes with a cautionary note with regard to the misdiagnosis of the ectopic ACTH syndrome in which partial processing of ACTH can result in large concentrations of α-MSH and CLIP, which can interfere in the performance of two-site immunoassays, and the problem of the correct disulphide bridge arrangement in synthetic N-POMC peptides is also discussed.

Key Words
- MSH
- ACTH
- LPH
- adrenal
- POMC

Journal of Molecular Endocrinology
(2016) **56**, T1–T12

Journal of Molecular Endocrinology

Introduction

I hope the reader will forgive me for the personal reflections in this article, but it is probably the last I will write on the fascinating subject of the adrenocorticotrophic hormone (ACTH)/melanocyte-stimulating hormone (MSH) precursor, which we now know as pro-opiomelanocortin (POMC). Who would have predicted that starting a research career on the hormone that caused dogfish skin to darken would lead me on to unravelling the many biological activities present in the most complex multi-peptide hormone precursor involved in many aspects of human physiology and pathophysiology? I was one very lucky scientist. I will describe the characterisation of dogfish MSH and ACTH, the discovery of corticotrophin-like intermediate lobe peptide (CLIP), pro-γ-MSH and big γ-MSH and their involvement in corticosteroidogenesis and adrenal growth, and speculate on their secretion from the zona imtermedia and roles in the development of both the foetal adrenal and prepubertal reticularis zones. I will finish on a note of caution when using a two-site immunometric assay in the diagnosis of the ectopic ACTH syndrome when POMC processing is similar to that in the zona intermedia.

Dogfish melanotrophin

It was 50 years ago when I was first introduced to the peptides derived from pro-opiomelanotropin. For my PhD

http://jme.endocrinology-journals.org
DOI: 10.1530/JME-15-0260

This editorial accompanies a thematic review section on 60 Years of POMC.
The Guest Editors for this section were Adrian Clark and Philip Lowry.

project, I was assigned to work on MSH from the dogfish (*Squalus acanthias*) by James Munro Dodd FRS, the then Professor of Zoology at the University of Leeds. This species of elasmobranch had been carefully chosen to study MSH as it was commercially available, it changed colour and its pituitary was anatomically separated into easily dissected distinct lobes. The comparatively large neurointermediate lobe that contained the MSH bioactivity was easily removed from the many heads of this cartilaginous fish that I needed to isolate enough MSH for characterisation. At that time, only the primary structures of mammalian α-MSH and β-MSH were known, although there was no associated colour change in these species, but MSH bioactivity had been detected in the pituitary glands of a number of lower vertebrates such as frogs, lizards and fish, which were adapted to the background presumably by releasing MSH bioactivity from their respective pituitary glands, which then caused dispersion of melanin in their melanophores.

Dogfish α-MSH

The first MSH I isolated and characterised from extracts of some thousands of dogfish pituitary glands was an α-type MSH (Lowry & Chadwick 1970*a*). Although the elasmobranchs diverged from the main line of vertebrate evolution 300 million years ago (Young 1962), to my surprise, the only difference between the amino acid sequence of this dogfish MSH and that of mammalian α-MSH was a conservative hydrophobic amino acid substitution (methionine for valine) at the C-terminal (Fig. 1). Dogfish α-MSH however lacked the acetyl group at the N-terminal, although it did have the C-terminal amide. Circulating peptides are cleared by the kidneys recognising the free amino group at the N-terminal of the appropriate peptide (Medeiros & Turner 1994), so it would make sense for the dogfish, which would be swimming quickly from dark to light backgrounds, to secrete a melanotrophin with a much shorter half-life due to an unacetylated N-terminal amino group, resulting in more rapid colour change.

Human ACTH

SYSMEHFRWGKPV**G**KK**RRP**V**KVYPN**GA**EDES**A**E**AFPL**E**F

SYSMEHFRWGKPV-**NH_2** **RP**V**KVYPN**GA**EDES**A**E**AFPL**E**F
(αMSH) (CLIP)

Dogfish ACTH

SYSMEHFRWGKPM**G**R**KRRP**I**KVYPN**SF**EDES**V**E**NMGP**E**L

SYSMEHFRWGKPM-**NH_2** **RP**I**KVYPN**SF**EDES**V**E**NMGP**E**L
(αMSH) (CLIP)

Figure 1
A comparison of the primary structures of human and dogfish POMC peptides: ACTH, desacetyl α-MSH and CLIP. Conserved amino acids are emphasized in bold and underlined.

Although the amino acid sequence of mammalian α-MSH was exactly the same as the first 13 residues of ACTH, 50 years ago it was not known whether this was just by chance, whether they were a result of gene duplication, part of the same precursor caused by internal gene duplication, or whether α-MSH was derived from ACTH; peptide processing was poorly understood at that time. The incredible conservation of the α-MSH sequence over 600 million years of evolution suggested that if dogfish ACTH had the dogfish α-MSH amino acid sequence at its N-terminal with the methionine substitution at position 13, there was a distinct possibility that α-MSH was derived from ACTH (Lowry & Chadwick 1970*a*,*b*). This conservation indicated that the receptor(s) involved was also ancient and highly conserved.

The discovery of CLIP

Sandy Scott started his PhD studies to work on fish ACTH at St Andrews University but was seconded to St Bartholomew's Hospital to take advantage of the ACTH antibodies, which were in routine use in radioimmunoassays. During this time, he made some interesting observations regarding the distribution of N- and C-terminal ACTH immunoreactivity in the rat pituitary, and our subsequent collaboration resulted in the discovery of the 18–39 C-terminal fragment of ACTH in the pars intermedia, which we called corticotrophin-like intermediate lobe peptide or CLIP (Scott *et al*. 1973). Thus, there was now a distinct possibility that ACTH was synthesised both in the corticotrophs of the pars distalis and in the cells of the pars intermedia, but that, in the latter, ACTH is further processed to form α-MSH and CLIP (Fig. 1). From research carried out by others described in this issue (Chretien & Mbikay 2016), we now know that this processing in the pars intermedia is by cleavage by prohormone-converting enzyme 2 (PC2) at the Arg^{17}–Arg^{18} peptide bond, followed by removal of the resulting C-terminal basic residues 17–15 by carboxypeptidase E (CPE) and then conversion of the nascent C-terminal Gly^{14} residue to an amide by prohormone-amidating monooxygenase (PAM)

http://jme.endocrinology-journals.org
DOI: 10.1530/JME-15-0260

Published by Bioscientifica Ltd.

(Kumar *et al.* 2016). It should be noted that PC2 does not cleave the trypsin-resistant Arg^{18}–Pro^{19} imido peptide bond in CLIP. The same resistant imido peptide bond affects the processing of substance P from its precursor in a similar way as the sequence around the N-terminal of substance P is –Arg^{-1}–Arg^{1}–Pro^{2}–, and the processing is also between the two arginine residues.

Dogfish ACTH and CLIP

The fact that the rostral lobe in the dogfish pituitary, which contains ACTH bioactivity, is anatomically separate from the neurointermediate lobe made the purification of dogfish ACTH and CLIP relatively easy (Lowry *et al.* 1974). When we found that the amino acid compositions of the purified C-terminal proteolyic fragments from both peptides were identical, complete sequence analysis of the C-terminal region of ACTH was relatively simple. The primary structures of dogfish ACTH and CLIP are shown in Fig. 1, and as expected, dogfish ACTH has the Met^{13} residue substitution. As we had hoped, dogfish CLIP occupied exactly the same position as in mammalian ACTH with an amino acid sequence similar to human ACTH particularly in the acidic residues at positions at 28–30, 33 and 38, which have been assumed to balance or interact with the basic residues that dominate the N-terminal region of the 39mer. Other substitutions also retained the overall hydrophobic/hydrophilic positions in CLIP adding strength to the importance of the whole of the ACTH primary structure.

Dogfish γ-MSH, δ-MSH and β-endorphin

As dogfish POMC cDNA was sequenced (Amemiya *et al.* 1999), it has been much easier to identify the rest of the dogfish melanotrophins and endorphin by their position in the precursor. Figure 2 was originally published by Bennett and coworkers (Bennett *et al.* 1974) and shows the final purification of dogfish α-MSH (peaks B and C) and β-MSH (peak A) from an extract of neurointermediate lobes. The amino acid compositions of the 280 nm absorbing peaks, D and E, were identical, but as there was no associated MSH bioactivity, no further characterisation was carried out because manual sequencing was a laborious technique at that time. It was only after a third cryptic melanotrophin sequence (γ-MSH) was implicated in bovine POMC (Nakanishi *et al.* 1979) that we submitted these dogfish neurointermediate lobe peptides to a fast and simple automated sequenator (MacLean & Lowry 1981). As they had little or no melanotropic activity (compare synthetic bovine γ-MSH), they were initially assumed to be a dogfish γ-type MSH. On examination

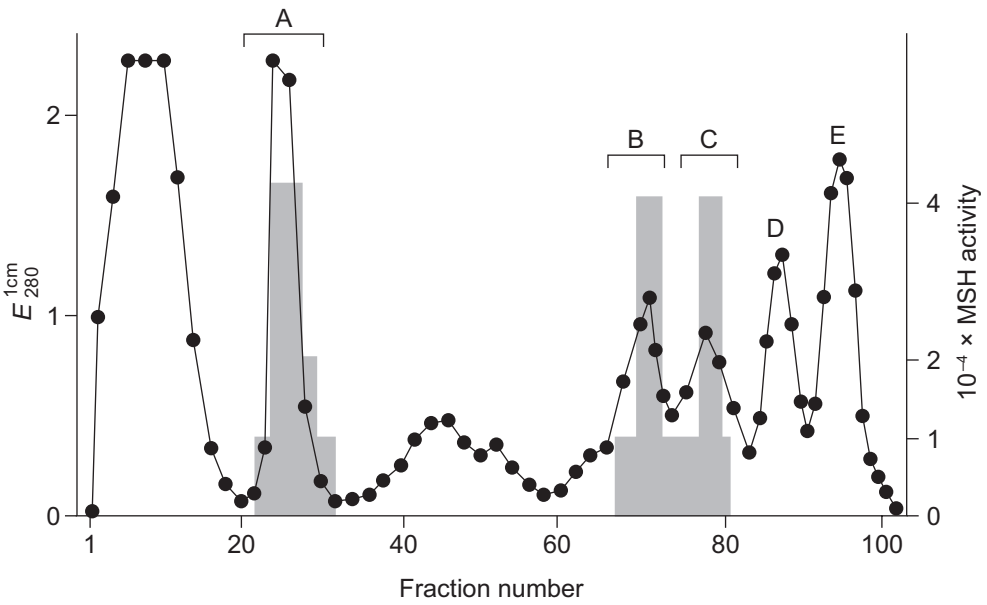

Figure 2
Cation-exchange chromatography of the main MSH-active fractions from a previous gel filtration separation of an extract of 1000 dogfish neurointemediate lobes. The shaded areas represent MSH bioactivity as detected in an *in vitro* frog skin bioassay (Chadwick & Lowry 1970). (A) Dogfish β-MSH, (B) deamidated dogfish α-MSH, (C) dogfish α-MSH, (D) deamidated dogfish δ-MSH and (E) dogfish δ-MSH. This figure was originally published in the *Biochemical Journal* (Bennett *et al.* 1974). Reproduced with permission of The Bioschemical Society.

http://jme.endocrinology-journals.org
DOI: 10.1530/JME-15-0260

Published by Bioscientifica Ltd.

of the complete sequence of dogfish POMC, the third MSH that we had sequenced did not occupy the same position as γ-MSH but occupied a fourth position named δ-MSH by Amemiya and coworkers (Amemiya *et al.* 1999). We thus did not appear to find any evidence for processed dogfish γ-MSH, which from its predicted amino acid sequence would have co-eluted with peaks B and C (Fig. 2). We are now therefore able to conclude that as with mammalian γ-MSH, there is little or no processing upstream of the dogfish γ-MSH sequence (note that no typical upstream double basic processing site is present), giving the naturally occurring dogfish γ-MSH peptide an N-terminal extension of some 50 residues similar to that seen in human γ-MSH (Fig. 3). There are three interesting observations to note: (1) the remarkable conservation evident when comparing the dogfish γ-MSH region with its human equivalent (-YVMGHFRW*NK*F-amide vs -YVMGHFRW*DR*F-amide: Asn/Asp and Lys/Arg are both conserved, single-base substitutions), (2) the positions of the cysteine residues (and consequent disulphide bridges) in the extended N-terminal region and (3) the overall retention of positions of hydrophobic and hydrophilic residues. This lends credibility to the notion that what I now will name 'big γ-MSH' (i.e. N-terminally extended γ-MSH) serves an important physiological function with its own putative receptor; I will return to this later. Big γ-MSH should not be confused with pro-γ-MSH, which appears to be the main processed peptide found in the pars distalis and has the C-terminal extension containing the post-translational N-linked glycosylated moiety. Again, the position of the consensus glycosylation motif and its position C-terminal to the γ-MSH sequence are conserved in dogfish POMC (Fig. 3).

To this day, I do not know why approximately half the dogfish α- and δ-MSHs were also found in the deamidated forms. I can only conclude that it may be an extra control mechanism that the dogfish has evolved to render a peptide susceptible to carboxypeptidase action with an even shorter half-life *in vivo*. In our *in vitro* MSH bioassay, both forms of dogfish α-MSH were equi-active.

Mammalian β-MSH

Fifty years ago, the situation regarding the occurrence of a processed human β-MSH was also somewhat confused. An octadecapeptide that contained the same heptapeptide core MSH sequence (Met–Glu–His–Phe–Arg–Trp–Gly) as α-MSH had been isolated and sequenced from a number of different mammalian pituitary posterior lobes. An equivalent peptide had not been identified in extracts of murine pituitary glands, but a β-MSH-like peptide with a four-amino acid N-terminal extension (Ala–Glu–Lys–Lys) had been isolated from human pituitaries extracted at mild pH (Dixon 1960). In those mammals in which the primary structure of β-lipotrophin (β-LPH) was elucidated, it was revealed that they all contained the sequence of their appropriate β-MSHs (banded by double basic amino acids: Lys–Arg or Arg–Arg), and thus, it was naturally

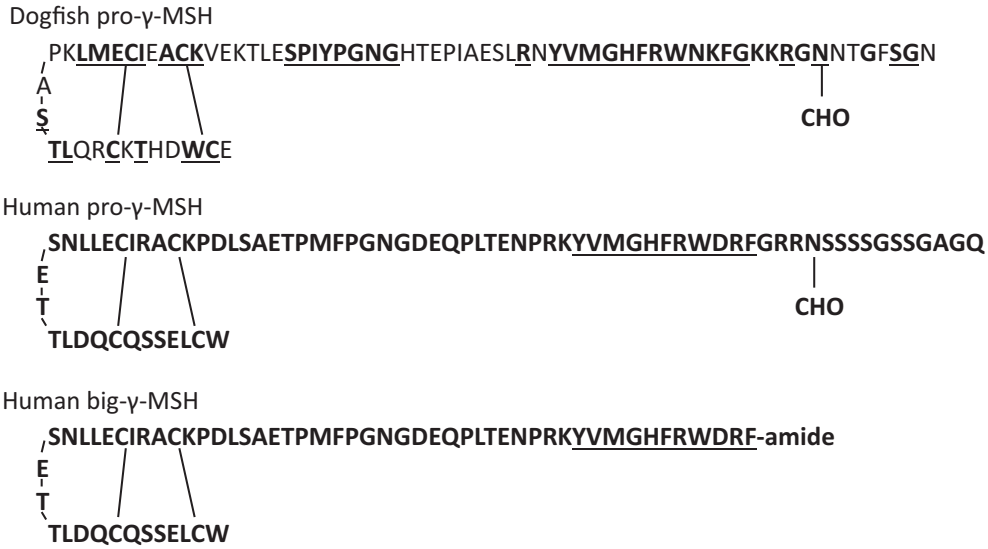

Figure 3
A comparison of the structures of dogfish and human pro-γ-MSHs and the proposed structure of human big γ-MSH. Conserved amino acids in dogfish pro-γ-MSH are emphasized in bold and underlined. CHO denotes an N-linked carbohydrate moiety.

http://jme.endocrinology-journals.org
DOI: 10.1530/JME-15-0260

Published by Bioscientifica Ltd.

assumed that β-LPH could be the precursor of β-MSH by simple proteolytic processing at the double basic residues by trypsin-like activity (Chretien & Mbikay 2016, Smyth 2016). The situation with human β-MSH was atypical, as it appeared not to be cleaved from its β-LPH at the N-terminal Lys–Lys double basic motif but in a non-trypsin-like manner at an Ala–Ala peptide bond. Moreover, when human pituitaries were extracted at strong acid pH to minimise any artificial proteolytic activity, no evidence could be found for a β-MSH-sized peptide: all the β-MSH immunoreactivity eluted on gel filtration in the position of β-LPH (Scott & Lowry 1974). However, if a human pituitary was extracted and incubated at mild pH, β-MSH-like immunoreactive material started to appear in the expected elution position of synthetic human β-MSH. Thus, we concluded that the 22-residue human β-MSH was an extraction artefact being cleaved from β-LPH if mild pH was used during the initial extraction from pituitary glands (Scott & Lowry 1974). Furthermore, when plasma from a patient suffering from Nelson's disease was similarly submitted to gel filtration, all the immunoreactive β-MSH material eluted in the position of β-LPH (Bloomfield *et al*. 1974). This led to the reinterpretation of reports of β-MSH in the corticotrophs of the human pituitary and in the blood of patients with Cushing's and Nelson's diseases, which was in fact β-LPH. The observation could have also been due to the lack of a pars intermedia in the human pituitary gland, but there have never been any reports of β-MSH-like peptide even in extracts of foetal pituitary glands in which there is a distinct zona intermedia; it is more likely to be due to the fact that natural processing by prohormone-converting enzymes rarely takes place after Lys–Lys residues.

Nevertheless, these findings gave rise to the hypothesis that ACTH and LPH were biosynthesised in both the corticotrophs of the pars distalis and the cells of the pars intermedia. ACTH and LPH were released intact from the pars distalis under positive control from the hypothalamus but were processed further in the pars intermedia (ACTH to MSH and CLIP and LPH to β-MSH) and were under negative dopaminergic hypothalamic control (Lowry & Scott 1975).

I have mentioned that murine LPH is not processed to a β-MSH-like peptide nor is the heptapaptide core sequence conserved. The diversity of β-MSH-like sequences is perhaps demonstrated in the two species of dogfish, *Squalus acanthias* (Bennett *et al*. 1974) and *Scyliorhinus canaliculus* (Love & Pickering 1974). They seem to be processed to a 16mer and a 18mer, respectively, and there is little common sequence homology between them, perhaps suggesting that there is no functional pressure on this part of the precursor. However, the opiate pentapeptide sequence (YGGFM) that follows β-MSH in human POMC occupies the same position at the N-terminal of dogfish β-endorphin (Amemiya *et al*. 1999).

The recent proposal by Lee and coworkers that a mutation in the β-MSH region of POMC, which changes the tyrosine 221 residue to a cysteine residue, has led to the proposal that a lack of β-MSH activity is also responsible for obesity (Lee *et al*. 2006). As the natural processing of human LPH to the true β-MSH-18mer has never been observed in the hypothalamus either, there is an alternative explanation to this observation. One unusual feature of normal human POMC is that there is already a single cysteine residue in the joining peptide (Fig. 4), which even after processing leads to the joining peptide is a stable homodimer. This suggests that human POMC with a second cysteine residue in the β-MSH/β-LPH region (Fig. 4) would form into a second inter-chain disulphide bridge, making this mutant POMC a more stable dimer during biosynthesis. There is also the possibility of intra-chain disulphide bridge or aggregation due to tandem multiple inter-chain disulphide bridges. One or more of these scenarios could result in altered processing in the hypothalamus and thus may be responsible for the observed obesity.

Common precursor

I had the temerity to suggest in the review I wrote on finishing my PhD in 1969 that there could be a 'large molecule with all three types of activity' (namely, ACTH, MSH and LPH) (Lowry & Chadwick 1970*b*). Some years later, we did partially purify a potential common precursor from human pituitaries, which had both ACTH and LPH/β-MSH immunoreactivity (Lowry *et al*. 1976*b*, 1977). However, Nakanishi and coworkers put all the speculation to rest 3 years later with the cloning of bovine POMC cDNA (Nakanishi *et al*. 1979).

When I started working on the adrenal function in the 1970s, there seemed to be some doubt that ACTH was responsible for all aspects of adrenal physiology particularly with respect to adrenal growth (Rao *et al*. 1978) in which immunoneutralisation with an ACTH antiserum did not inhibit compensatory growth following unilateral adrenalectomy in rats. Adrenal growth did however seem to be linked to pituitary corticotroph function as compensatory growth was inhibited by dexamethasone treatment. Once we realised that it was β-LPH that was co-synthesised and co-secreted with ACTH, and we had sufficient purified human LPH, attempts were made to use

http://jme.endocrinology-journals.org
DOI: 10.1530/JME-15-0260

Published by Bioscientifica Ltd.

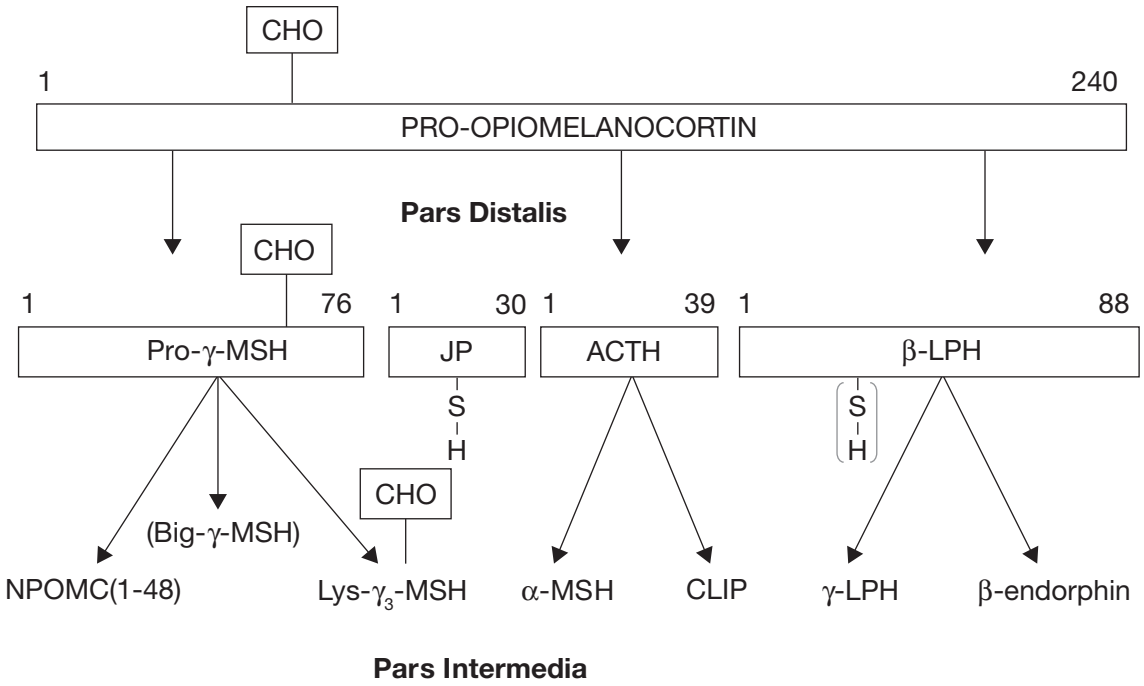

Figure 4
Tissue-specific processing of POMC in the mammalian pituitary gland and ectopic ACTH-producing tumours. The SH groups show the positions of the cysteine residues in the human joining peptide and the Tyr^{221}–Cys mutation in the β-MSH/β-LPH region reported by Lee *et al.* (2006).

it with and without synthetic ACTH to stimulate adrenal growth in hypophysectomised rats, but without any success (P J Lowry and A P Scott, unpublished observations). Nor did β-LPH potentiate the adrenocorticosteroidogenic activity of ACTH *in vitro*.

Isolation and characterisation of human pro-γ-MSH

The cloning of the precursor POMC gene, which confirmed that ACTH and LPH were part of the same precursor, simultaneously provided possible evidence of a large N-terminal more complex glycopeptide, pro-γ-MSH, being co-secreted from the pars distalis corticotroph during stress. The sequence of POMC predicted that pro-γ-MSH had two disulphide bridges and an N-linked polysaccharide moiety downstream of γ-MSH, and that removal of a 26-residue leader sequence would reveal a nascent N-terminal tryptophan on pro-γ-MSH. As tryptophan fluorescence had previously been observed in corticotrophs (Håkanson *et al.* 1975), this property was used to monitor the purification of the putative 'pro-γ-MSH' glycopeptide from human pituitary glands. In the initial extraction, we used strong acid to avoid any proteolytic activity. The gylcopeptide we purified, which we called human pro-γ-MSH, proved to be a 76-residue peptide with an N-terminal tryptophan. It had two disulphide bridges between the cysteine residues at positions 2 and 24, and those at 8 and 20 (Bennett *et al.* 1985), and contained an N-linked polysaccharide attached to the asparagine at position 65 in the region downstream from the γ-MSH sequence (Estivariz *et al.* 1980). As expected, when a homologous immunoassay for human pro-γ-MSH was used to detect this relatively stable glycopeptide in unextracted plasma (Hope *et al.* 1981) in various pathological conditions (Cushing's disease, Nelson's disease, Addison's disease and the ectopic ACTH syndrome), it mirrored the concentrations of ACTH obtained using a laborious extracted assay. Even with the advent of unextracted two-site assays for ACTH (Hodgkinson *et al.* 1984), given the labile nature of the latter compared with pro-γ-MSH, it is surprising to me that the potentially more robust and sensitive immunoassay for pro-γ-MSH has not replaced the ACTH immunoassay for the diagnosis of pathologies of the hypothalamic/pituitary/adrenal (HPA) axis. I will discuss some other shortcomings of the two-site ACTH immunoassay particularly with respect to diagnosing the ectopic ACTH syndrome later.

Initial experiments failed to find any activity with purified human pro-γ-MSH with respect to MSH bio-activity or adrenal growth-promoting properties (Estivariz *et al.* 1980). When added alone to adrenal cells *in vitro*, it

http://jme.endocrinology-journals.org
DOI: 10.1530/JME-15-0260

Published by Bioscientifica Ltd.

did not cause steroidogenesis but did promote RNA synthesis (Al Dujaili *et al*. 1982). It also potentiated the steroidogenic activity of ACTH several-fold in perfused rat adrenal cells *in vitro* (Al Dujaili *et al*. 1981). This potentiation was completely abolished in the presence of actinomycin D, indicating that the increase in RNA synthesis was linked to the potentiating activity of pro-γ-MSH (Al Dujaili *et al*. 1982). This ability to increase ACTH-induced corticosteroidogenesis several-fold is largely ignored in the diagnosis of human adrenal pathology and can be particularly relevant in some cases of the ectopic ACTH syndrome, in which ACTH is processed mainly to desacetyl α-MSH and CLIP, but pro-γ-MSH is still partly intact.

Adrenal growth activity of N-POMC peptides

Once antisera to pro-γ-MSH were available in the laboratory, we were able to show that rats treated with this antiserum showed a decrease in thymidine incorporation in their adrenal glands, suggesting that pro-γ-MSH may be a mitogenic precursor (Estivariz *et al*. 1980). This was confirmed when either trypsinised pro-γ-MSH or purified human N-terminal POMC (N-POMC) fragments were used *in vivo* or *in vitro* (Estivariz *et al*. 1982). One mitogenic N-terminal pro-γ-MSH fragment that we isolated from human pituitaries, being relatively small, was easily characterised as N-POMC(1–28) from its amino acid composition and N- and C-terminal analyses (McLean *et al*. 1981). Given that this fragment was isolated in significant amounts from a side fraction of large-scale growth hormone purification (using mild pH throughout) and that there was no evidence for large amounts of intact pro-γ-MSH, it was concluded that it had been generated from the latter at the Leu^{28}/Ser^{29} peptide bond by proteolysis during isolation. Additionally, there was no N-terminal tryptophan-fluorescent material eluting on gel filtration in the expected position of an 18mer when strong acid pH was used in the initial extraction. This suggested that N-POMC(1–28) was an extraction artefact (compare human β-MSH). The larger but much less abundant N-POMC peptide that was present in the same growth hormone side fraction was thus more difficult to purify to homogeneity and accurately characterise from its amino acid composition (our original estimate was ~59 residues). More importantly, it did not generate any amino acids on digestion with carboxypeptidase-Y (McLean *et al*. 1981). Given that CPY will remove all C-terminal amino acids (even the imino acid, proline) if a free carboxyl group is present on the substrate peptide, the simple explanation of this would be that the peptide was amidated at its C-terminal, thus blocking the action of CPY. The peptide that fits this property would be human 'big γ-MSH' (i.e. N-terminally extended γ-MSH with phenylalanyl^{61}-amide at its C-terminal), and the peptide that we isolated was by far the most potent as an adrenal mitogen (Estivariz *et al*. 1982). Further evidence for the importance of the γ-MSH sequence being part of the mitogenic POMC peptide is supported by the fact an antiserum raised against a synthetic γ-MSH peptide when injected into rats was the most potent in inhibiting compensatory adrenal growth following unilateral adrenalectomy (Lowry *et al*. 1983). The presence of a C-terminal amide on big γ-MSH would suggest the action of the enzyme PAM, implicating pars intermedia-like processing (see Fig. 4). As the amounts of big γ-MSH isolated were similar to those expected for desacetyl α-MSH found in the human pituitary gland, and the latter (and probably PAM) has been found to be mainly associated with zona intermedia tissue in the human pituitary, it is likely that big γ-MSH is a product of the same cells/tissue.

The human pituitary zona intermedia, the adrenal foetal zone and the adrenarche zona reticularis

In most mammals, the pars intermedia is separated anatomically from the pars distalis by a cleft and usually forms a homogeneous band of cells on the face of the neural lobe; the combination of the two is referred to as the posterior lobe. In humans, higher apes, manatees and dugongs, whales and dolphins, elephants and birds, there is no cleft and no evidence for a functional pars intermedia of the pituitary gland. In the foetal human pituitary, there is an anatomical location of a band of intermedia-like cells referred to as the zona intermedia, and there is also evidence in foetal extracts for significant concentrations of α-MSH-like and CLIP-like immunoreactive peptides (Silman *et al*. 1976). In a comprehensive immunocytochemical study in adult human pituitary glands, using a specific α-MSH antibody, immunoreactive α-MSH cells were found in a total of 97 out of 100 pituitary glands, but only 10 of the 97 showed a marked concentration of α-MSH cells in the area commonly seen in the pars anterior than in the zona intermedia, and in 41 cases, α-MSH cells were completely absent from the zona intermedia (Coates *et al*. 1986).

Secretion of all immunoreactive POMC peptides from rat pars intermedia cells is under common dopaminergic inhibitory control (Jackson & Lowry 1983). The use of

http://jme.endocrinology-journals.org
DOI: 10.1530/JME-15-0260

Published by Bioscientifica Ltd.

a dopaminergic antagonist provided indirect evidence for active zona intermedia-like cells in prepubertal children, as it resulted in a significant increase in plasma α-MSH-like concentrations but not in post-pubertal controls. The elution position on HPLC suggested that the immunoreactive material was mainly desacetyl α-MSH (Facchinetti *et al*. 1995). Desacetyl α-MSH does stimulate adrenal steroidogenesis, albeit at high concentrations, but its secretion would be accompanied by significant concentrations of other potential biologically active intermedia-like processed POMC peptides, e.g. big γ-MSH.

Thus, it may not just be a coincidence that human adrenal development in the foetus and in the prepubertal child occurs exactly at the times when the zona intermedia is active. More specifically, one or more intermedia-like processed POMC peptides could be involved in controlling both the development of the adrenal foetal zone and the development of the prepubertal adrenal zona reticularis; the prime hormonal candidate is big γ-MSH.

In the pituitary glands of mammals with a distinct pars intermedia, the separate tonic inhibition by dopamine of lactotrophs in the pars distalis is anatomically feasible, but as there is no cleft separating the zona intermedia cells in the human pituitary and some appear to migrate into the pars distalis (Coates *et al*. 1986), differential release of big γ-MSH and prolactin would be difficult to control. Thus, the appearance of breast buds in both the neonate and prepubertal child may simply be due to the lower hypothalamic dopamine concentrations needed at these times to increase the release of big γ-MSH from active zona intermedia cells/tissue to stimulate the development of the adrenal foetal and the prepubertal reticularis zones simultaneously allowing the release of significant amounts of prolactin.

Most studies that have been carried out with the melanocortin receptors have been with relatively small synthetic γ-MSH peptides, yet the high conservation of the structures of the dogfish and human big γ-MSHs (and the potent adrenal mitogenic activity of the latter) suggests that there has been evolutionary pressure from a putative receptor for big γ-MSH.

Immunoassay of blood samples from Cushing's disease and the ectopic ACTH syndrome

Radioimmunoassay

Originally, single-site competitive radioimmunoassays were used to detect ACTH in the plasma of patients suffering from Cushing's disease, and antibodies raised against the 1–24 region of ACTH usually gave the best indication of biological activity. Blood samples generally needed a prior extraction technique as the high concentration of proteins in plasma interferes with the binding of radiolabelled ACTH with the limited amount of antibody used in the classical competition immunoassay format. Using ACTH antibodies directed towards the C-terminal region, the measurement of ACTH in blood samples taken from patients with pituitary-dependent Cushing's disease usually gave similar results but with samples from cases with the ectopic ACTH syndrome, much higher readings were often recorded. This was because some ectopic tumours, with regard to processing, behave more like a pars intermedia, resulting in the N-terminal 1–24 antibody (while not reacting with smaller α-MSH-like peptides) still giving a good indication of ACTH biological activity but C-terminal antibodies giving a much higher reading as, generally, they would not distinguish between ACTH and CLIP. In the first ectopic ACTH tumour, I studied (Ratcliffe *et al*. 1973), >95% of the C-terminal ACTH immunoreactivity eluted in the position of CLIP (Fig. 5). After further purification, amino acid and N- and C-terminal analyses were consistent with ACTH(18–39). Only a small amount of bioactive material eluted in the position of ACTH(1–39). MSH bioactive peaks eluted in the positions of LPH and α-MSH. In the second ectopic ACTH-secreting tumour I studied, almost all the ACTH was intact (Lowry *et al*. 1976*a*). Thus, it would appear that ectopic tumours can behave as a pars distalis corticotroph in processing POMC to ACTH, or like a pars intermedia, processing POMC to mainly desacetyl α-MSH and CLIP (Fig. 4). In some ectopic tumours, there thus may be large concentrations of potentiating and mitogenic N-POMC peptides with very little corticosteroidogenic activity (small or negligible amounts of ACTH but large amounts of desacetyl α-MSH). The accompanying large amount of mitogenic N-POMC peptides would give rise to gross bilateral adrenal hyperplasia and Cushing's syndrome with relatively normal or even low ACTH concentrations. This confusion would not be the case if an N-POMC immunoassay was used in diagnosis.

Two-site immunometric assay

As these assays use much higher concentrations of two complementary antibodies (one N-terminally and the other C-terminally directed) than the single antibody used in the competitive immunoassay, there is less

http://jme.endocrinology-journals.org
DOI: 10.1530/JME-15-0260

Published by Bioscientifica Ltd.

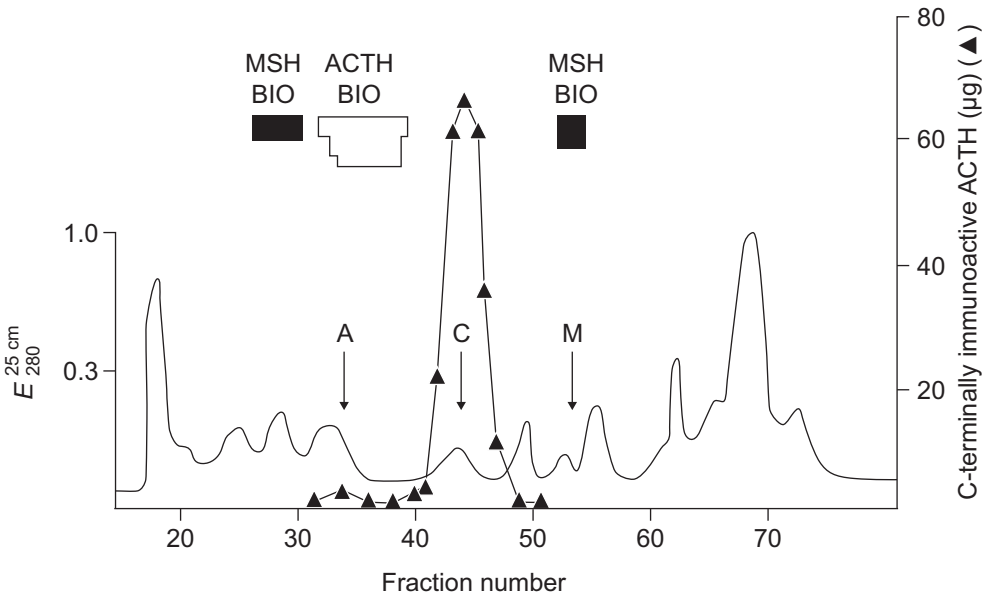

Figure 5
Gel filtration of an ectopic ACTH tumour extract. Shaded blocks represent MSH bioactivity as detected in an *in vitro* frog skin bioassay (Chadwick & Lowry 1970); open blocks represent ACTH bioactivity detected in an *in vitro* rat adrenal cell bioassay (Lowry *et al.* 1973). The triangles show ACTH immunoreactivity measured using a C-terminally directed ACTH antibody (Ratcliffe *et al.* 1973). Arrows and 'A', 'C' and 'M' indicate the expected elution positions of ACTH, CLIP and α-MSH, respectively. Reproduced, with permission, from Ratcliffe JG, Scott AP, Bennett HPJ, Lowry PJ, McMartin C, Strong JA & Wallabaum PR (1973) Production of a corticotrophin-like intermediate lobe peptide and of corticotrophin by a bronchial carcinoid tumour, *Clinical Endocinology*, vol 2, pp51–55. Copyright 1973 John Wiley & Sons.

interference from plasma proteins allowing direct assay of samples and thus avoiding time-consuming prior extraction (Hodgkinson *et al.* 1984). Generally, they give reliable results with samples from patients with pituitary-dependent Cushing's disease as the adult human pituitary gland secretes intact ACTH. Again, it is with plasma samples from patients with the ectopic ACTH syndrome that problems can be encountered.

If the ectopic tumour behaves like a pars distalis and most of the ACTH is the simple 1–39 peptide, then the result of the two-site assay will reflect the amount of ACTH biological activity in the sample. One feature of ACTH which is often ignored is that 20–30% of ACTH present in the human pituitary gland is phosphorylated at the serine residue at position 31 (Bennett *et al.* 1983). This does not affect its biological activity, but if this highly charged negative group interferes with the binding of the C-terminal antibody used in the two-site assay, then a lower reading with phosphorylated ACTH would result. The problem can be more extreme with some ectopic tumours in which the bulk of ACTH(1–39) can be phosphorylated (Massias *et al.* 1994).

The problem of getting erroneous results with the two-site assay becomes even more acute when the ectopic tumour is processing ACTH like a pars intermedia. As the two-site assay signal relies on both antibodies binding to the same contiguous peptide, the high concentrations of ACTH fragments such as CLIP that are present will use up most of the C-terminal antibody, and an inaccurate low reading of any intact ACTH(1–39) present will result. If there is little CPE processing, this would result in high concentrations of steroidogenic ACTH(1–17), which would react with the N-terminal antibody making it unavailable for any binding to intact ACTH(1–39). Processing of ACTH (by CPE and PAM) to desacetyl α-MSH still results in a full agonist at the adrenal gland ACTH receptors. Thus, with ectopic ACTH tumours, significant amounts of adrenal bioactive peptides can be present but with very little signal from the two-site ACTH assay. It is only when the tumour is removed and submitted to immunocytochemical examination that its true endocrine nature can be revealed.

It should also be remembered that when studying rats, a significant amount of its ACTH is glycosylated (Bennett *et al.* 1982). The reason for this is that there is a single base change in the ACTH region of the rat POMC gene, which results in the aspartate residue found at position 29 in human ACTH becoming an asparagine in

http://jme.endocrinology-journals.org
DOI: 10.1530/JME-15-0260

Published by Bioscientifica Ltd.

rat ACTH. This creates the classical N-linked glycosylation motif Asn–Xxx–Ser at residues 29–31. The resulting carbohydrate moiety attached to the side chain of Asn^{29} would not be expected to change the biological activity at the ACTH receptor but would give the peptide a much longer half-life *in vivo*. The bulky carbohydrate group would however interfere with any C-terminally directed antibody giving very low signals in the two-site ACTH immunometric assay. It is not inconceivable that some humans may carry this mutation resulting in glycosylated ACTH with the same assay problems and an adrenocorticosteroidogenic agonist with a much longer half-life in their blood.

The importance of the correct disulphide bridges in N-POMC peptides

Disulphide bridge arrangement during polypeptide biosynthesis is governed by the 'Anfinsen's dogma', which states that there is enough information in the polypeptide chain to direct the formation between the appropriate cysteine residues. Short synthetic peptides containing the relevant cysteine residues can however form the wrong disulphide bridges. This unfortunately seems to be the case with N-POMC peptides, as early attempts produced short synthetic N-POMC peptides, which were less potent adrenal mitogens than the purified peptide, in particular N-POMC(1–36) which was almost inactive as an adrenal mitogen (Estivariz *et al*. 1988). Rather than forming the natural 2–24 and 8–20 bridge configuration (Fig. 3), the N-POMC(1–36) peptide apparently formed the 2–8 and 20–24 bridges in the so-called calcitonin configuration (Henry Keutmann, personal communication). Early reports of 'calcitonin' immunoreactivity being part of the ACTH precursor in the pars intermedia (Deftos *et al*. 1978) added to this confusion. It should be noted that the 'calcitonin type' was the first disulphide arrangement reported in human N-POMC (Seidah *et al*. 1981) until it was corrected (Bennett *et al*. 1985). The disulphide bridges in fragments of POMC(1–48) were also found to be unstable (H Bennnett, personal communication). Fortunately, Fassnacht and coworkers (Fassnacht *et al*. 2003) before they embarked on their adrenal mitogenic studies *in vitro* with N-POMC(1–28) were aware of the disulphide bridge problem but after two unsuccessful attempts (M Fassnacht, personal communication), embarked on a synthetic strategy to direct the correct alignment in their preparation of N-POMC(1–28). The same peptide preparation, however, seemed to be inactive in rescuing adrenal growth in $POMC^{-}/POMC^{-}$ mice (Coll *et al*. 2006). Although the physiological relevance of the use of herculean doses of both N-POMC(1–28) and ACTH(1–24) could be questioned, the instability of the disulphide bridge arrangement in concentrated solutions in long-term experiments (and the effects this could have on biological activity) would lend caution to the conclusions reached from this study.

Finally, I thank all my collaborators during my PhD at the University of Leeds, during my postdoctoral years at Ciba/Geigy (Horsham, West Sussex, UK), 13 years at St Bartholomew's Hospital and 23 years at the University of Reading for putting up with me.

Declaration of interest
The author declares that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

Funding
This work did not receive any specific grant from any funding agency in the public, commercial or not-for-profit sector.

References

Al Dujaili EAS, Hope J, Estivariz FE, Lowry PJ & Edwards CRW 1981 Circulating human pituitary pro-gamma-melanotrophin enhances the adrenal response to ACTH. *Nature* **291** 156–159. (doi:10.1038/291156a0)

Al Dujaili EAS, Williams BC, Edwards CRW, Salacinski PR & Lowry PJ 1982 Human gamma-MSH precursor potentiates ACTH induced adrenal steroidogenesis by stimulating m-RNA synthesis. *Biochemical Journal* **204** 301–305. (doi:10.1042/bj2040301)

Amemiya Y, Takahashi A, Suzuki N, Sasayama Y & Kawauchi H 1999 A newly characterized melanotropin in proopiomelanocortin in pituitaries of an elasmobranch, *Squalus acanthias*. *General and Comparative Endocrinology* **114** 387–395. (doi:10.1006/gcen.1999.7256)

Bennett HPJ, Lowry PJ, McMartin C & Scott AP 1974 Structural studies of beta-melanocyte stimulating hormone and a novel alpha-melanocyte stimulating hormone from the neurointermediate lobe of the pituitary of the dogfish (*Squalus acanthias*). *Biochemical Journal* **141** 439–444. (doi:10.1042/bj1410427)

Bennett HP, Browne CA & Solomon S 1982 Characterization of eight forms of corticotropin-like intermediary lobe peptide from the rat intermediary pituitary. *Journal of Biological Chemistry* **257** 10096–10102.

Bennett HP, Brubaker PL, Seger MA & Solomon S 1983 Human phosphoserine 31 corticotropin1-39. Isolation and characterization. *Journal of Biological Chemistry* **258** 8108–8102.

Bennett HPJ, Seidah NG, Benjannet S, Solomon S & Chretien M 1985 Reinvestigation of thee disulphide bridge arrangement in human pro-opiomelanocortin *N*-terminal segment (hNT 1-76). *International Journal of Peptide and Protein Reserach* **27** 306–313.

Bloomfield GA, Scott AP, Gilkes JJH, Lowry PJ & Rees LH 1974 Human beta-MSH, a reappraisal. *Nature* **252** 492–493. (doi:10.1038/252492a0)

Chadwick A & Lowry PJ 1970 *In vitro* assay of MSH using skin from the frog (*Hyla arborea*). *General and Comparative Endocrinology* **15** 493–495. (doi:10.1016/0016-6480(70)90124-3)

http://jme.endocrinology-journals.org
DOI: 10.1530/JME-15-0260

Published by Bioscientifica Ltd.

Chretien M & Mbikay M 2016 60 YEARS OF POMC: From the prohormone theory to proopiomelanocortin and to proprotein convertases (PCSK1 to PCSK9). *Journal of Molecular Endocrinology* **56** T49–T62. (doi:10.1530/JME-15-0261)

Coates PJ, Doniach I, Hale AC & Rees LH 1986 The distribution of immunoreactive α-melanocyte-stimulating hormone cells in the adult human pituitary gland. *Journal of Endocrinology* **111** 335–342. (doi:10.1677/joe.0.1110335)

Coll AP, Fassnacht M, Klammer S, Hahner S, Schulte DM, Piper S, Tung YCL, Challis BG, Weinstein J, *et al*. 2006 Peripheral administration of the N-terminal fragment 1–28 to POMC-/- mice reduces food intake and weight but does not affect adrenal growth or corticosterone production. *Journal of Endocrinology* **190** 515–525. (doi:10.1677/joe.1.06749)

Deftos LJ, Burton D, Bone HG, Catherwood BD, Parthemore JG, Moore RY, Minick S & Guillemin R 1978 Immunoreactive calcitonin in the intermediate lobe of the pituitary gland. *Life Sciences* **23** 743–748. (doi:10.1016/0024-3205(78)90075-9)

Dixon HB 1960 Chromatographic isolations of pig and human melanocyte-stimulating hormones. *Biochimica and Biophysica Acta* **37** 38–42. (doi:10.1016/0006-3002(60)90075-5)

Estivariz FE, Hope J, McLean C & Lowry PJ 1980 Purification and characterisation of a gamma-melanotrophin precursor from frozen human pituitary glands. *Biochemical Journal* **191** 125–132. (doi:10.1042/bj1910125)

Estivariz FE, Iturriza F, McLean C, Hope J & Lowry PJ 1982 Stimulation of adrenal mitogenesis by N-terminal pro-opiocortin peptides. *Nature* **297** 419–422. (doi:10.1038/297419a0)

Estivariz FE, Carino M, Lowry PJ & Jackson S 1988 Further evidence that N-terminal pro-opiomelanocortin peptides are involved in adrenal mitogenesis. *Journal of Endocrinology* **116** 201–206. (doi:10.1677/joe.0.1160207)

Facchinetti F, Bernasconi S, Lughetti L, Genazzani AD, Ghizzoni L & Genazzani AR 1995 Changes in dopaminergic control of circulating melanocyte-stimulating hormone-related peptides at puberty. *Pediatric Research* **38** 91–94. (doi:10.1203/00006450-199507000-00016)

Fassnacht M, Hahner S, Hansen IA, Kreutzberger T, Zink M, Adermann K, Jacob F, Troppmair J & Allolio B 2003 N-terminal proopiomelancortin acts as a mitogen in adrenocortical tumour cells and decreases steroidogenesis. *Journal of Clinical Endocrinology and Metabolism* **88** 2171–2179. (doi:10.1210/jc.2002-021318)

Håkanson R, Sundler F, Larsson LI, Ekman R & Sjöberg NO 1975 Peptides with NH2-terminal tryptophan in adrenocorticotrophic hormone and melanocyte-stimulating hormone granules of adenohypophysis. *Journal of Histochemistry and Cytochemistry* **23** 65–74. (doi:10.1177/23.1.163855)

Hodgkinson SC, Allolio B, Landon J & Lowry PJ 1984 Development of a non-extracted 'two-site' immunoradiometric assay for corticotropin utilising extreme amino- and carboxy-terminally directed antibodies. *Biochemical Journal* **218** 703–711. (doi:10.1042/bj2180703)

Hope J, Ratter SJ, Estivariz FE, McLoughlin L & Lowry PJ 1981 Development of a radioimmunoassay for amino-terminal peptides of pro-opiocortin containing the gamma-MSH region: measurement and characterisation in human plasma. *Clinical Endocrinology* **15** 221–227. (doi:10.1111/j.1365-2265.1981.tb00658.x)

Jackson S & Lowry PJ 1983 Secretion of pro-opiocortin-related peptides from isolated perfused rat pars intermedia cells. *Neuroendocrinology* **37** 248–257. (doi:10.1159/000123553)

Kumar D, Mains RE & Eipper E. 2016 60 YEARS OF POMC: From POMC and αMSH to PAM, molecular oxygen, copper and vitamin C. *Journal of Molecular Endocrinology* **56** T63–T76. (doi:10.1530/JME-15-0266)

Lee YS, Challis BG, Thompson DA, Yeo GS, Keogh JM, Madonna ME, Wraight V, Sims M, Vatin V, Meyre D, *et al*. 2006 A POMC variant implicates beta-melanocyte-stimulating hormone in the control of human energy balance. *Cell Metabolism* **3** 135–140. (doi:10.1016/j.cmet.2006.01.006)

Love RM & Pickering BT 1974 A ß-MSH in the pituitary gland of the spotted dogfish (*Scyliorhinus canicula)*: isolation and structure. *General and Comparative Endocrinology* **24** 398–404. (doi:10.1016/0016-6480(74)90152-X)

Lowry PJ & Chadwick A 1970*a* Purification and amino acid sequence of melanocyte-stimulating hormone from the dogfish (*Squalus acanthias)*. *Biochemical Journal* **118** 713–718. (doi:10.1042/bj1180713)

Lowry PJ & Chadwick A 1970*b* Interrelations of some pituitary hormones. *Nature* **226** 219–222. (doi:10.1038/226219a0)

Lowry PJ & Scott AP 1975 The evolution of ACTH and MSH. *General and Comparative Endocrinology* **26** 16–23. (doi:10.1016/0016-6480(75)90211-7)

Lowry PJ, McMartin C & Peters J 1973 Properties of a simplified bioassay for adrencorticotrophic activity using the steroidogenic response of isolated adrenal cells. *Journal of Endocrinology* **59** 43–44. (doi:10.1677/joe.0.0590043)

Lowry PJ, Bennett HPJ, McMartin C & Scott A P 1974 The isolation and amino acid sequence of an adrenocorticotrophin from the pars distalis of a corticotrophin-like intermediate lobe peptide from the neurointermediate lobe of the pituitary of the dogfish (*Squalus acanthias)*. *Biochemical Journal* **141** 427–437. (doi:10.1042/bj1410427)

Lowry PJ, Rees LH, Tomlin S & Gillies GE 1976*a* Chemical characterisation of ectopic ACTH purified from a malignant thymic carcinoid. *Jouranl of Clinical Endocrinology and Metabolism* **43** 831–835. (doi:10.1210/jcem-43-4-831)

Lowry PJ, Hope J & Silman RE 1976*b* Corticotrophin and lipotrophin. *Proceedings of the Vth Interantional Congress of Endocrinology*, vol 1, pp 71–76. Ed. VHT James. Amsterdam, The Netherlands: Excerpta Medica.

Lowry PJ, Silman RE, Hope J & Scott AP 1977 Structure and biosynthesis of peptides related to corticotrophins and beta-melanotrophins. *Annals of the New York Academy of Sciences* **297** 49–62. (doi:10.1111/j.1749-6632.1977.tb41845.x)

Lowry PJ, Silas L, McLean C, Linton EA & Estivariz FE 1983 Pro-gamma-melanocyte stimulating hormone cleavage in adrenal gland undergoing compensatory growth. *Nature* **306** 70–72. (doi:10.1038/306070a0)

Massias JF, Hardouin S, Vieau D, Lenne F & Bertagna X 1994 Phosphorylated forms of adrenocorticotropin and corticotropin-like intermediary lobe peptide in human tumors. *European Journal of Endocrinology* **131** 341–346. (doi:10.1530/eje.0.1310341)

McLean C & Lowry PJ 1981 Natural occurrence but lack of melanotrophic activity of gamma-MSH in fish. *Nature* **290** 341–343. (doi:10.1038/290341a0)

McLean C, Hope J, Salacinski PR, Estivariz FE & Lowry PJ 1981 Purification and characterisation of amino-terminal pro-opiocortin peptides from human pituitary glands. *Bioscience Reports* **1** 843–849. (doi:10.1007/BF01114817)

Medeiros MS & Turner AJ 1994 Post-secretory processing of regulatory peptides: the pancreatic polypeptide family as a model example. *Biochimie* **76** 283–287. (doi:10.1016/0300-9084(94)90159-7)

Nakanishi S, Inoue A, Kita T, Nakamura M, Chang AC, Cohen SN & Numa S 1979 Nucleotide sequence of cloned cDNA for bovine corticotropin-beta-lipotropin precursor. *Nature* **278** 423–427. (doi:10.1038/278423a0)

http://jme.endocrinology-journals.org
DOI: 10.1530/JME-15-0260

Published by Bioscientifica Ltd.

Rao AJ, Long JA & Ramacahandran J 1978 Effects of antiserum to adrenocorticotropin on adrenal growth and function. *Endocrinology* **102** 371–378. (doi:10.1210/endo-102-2-371)

Ratcliffe JG, Scott AP, Bennett HPJ, Lowry PJ, McMartin C, Strong JA & Wallabaum PR 1973 Production of a corticotrophin-like intermediate lobe peptide and of corticotrophin by a bronchial carcinoid tumour. *Clinical Endocinology* **2** 51–55. (doi:10.1111/j.1365-2265.1973.tb03484.x)

Scott AP & Lowry PJ 1974 Adrenocorticotrophin and melanocyte stimulating peptides in the human pituitary. *Biochemical Journal* **139** 593–602. (doi:10.1042/bj1390593)

Scott AP, Ratcliffe JC, Rees LH, Landon J, Bennett HPJ, Lowry PJ & McMartin C 1973 A new pituitary peptide. *Nature New Biology* **224** 65–67.

Seidah NG, Rochemont J, Hamelin J, Lis M & Chretien M 1981 Primary structure of the major NH^{r}-treminal glycopeptides. *Journal of Biological Chemistry* **256** 7977–7984.

Silman RE, Chard T, Lowry PJ, Smith I & Young IM 1976 Human fetal pituitary peptides and parturition. *Nature* **260** 716–718. (doi:10.1038/260716a0)

Smyth D 2016 60 YEARS OF POMC: Lipotropin and beta-endorphin: a perspective. *Journal of Molecular Endocrinology* **56** T13–T25. (doi:10.1530/JME-16-0022)

Young JZ 1962 Evolution and adaptive radiation of elasmobranchs. In *The Life of Vertebrates*, pp 175–189. Oxford, UK: Clarendon Press.

Received in final form 3 December 2015
Accepted 7 December 2015
Accepted Preprint published online 7 December 2015

http://jme.endocrinology-journals.org
DOI: 10.1530/JME-15-0260

Published by Bioscientifica Ltd.