α -Melanocyte-stimulating-hormone precursors in the pig pituitary

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1. The occurrence of intermediates from the processing of ACTH-(1–39) [adrenocorticotrophic hormone-(1–39)] to α -melanocyte-stimulating hormone was investigated in normal pig pituitaries by the use of sensitive and specific radioimmunoassays for ACTH-(1–13), ACTH-(1–14), ACTH-(1–13)-NH₂ and ACTH-(1–39). Fractionation by reverse-phase h.p.l.c. revealed ACTH(1–17) and their acetylated analogues. 2. The intermediate lobe contained *NO*-diacetyl-ACTH-(1–13)-NH₂, *N*-acetyl-ACTH-(1–13)-NH₂ and ACTH-(1–13)-NH₂. In addition, the corresponding ACTH-(1–14) peptides (the glycine-extended precursor of the amidated peptides) were detected in lower amounts in both the intermediate lobe and the anterior lobe. ACTH-(1–17), ACTH-(1–13) and their acetylated analogues could not be detected in the anterior lobe or the intermediate lobe. 3. The results suggest that an endopeptidase initially cleaves ACTH-(1–39) at the Lys-16–Arg-17 bond. ACTH-(1–16) is then processed by a pituitary carboxypeptidase to ACTH-(1–14) and ACTH-(17–39) by the aminopeptidase to ACTH-(18–39).

INTRODUCTION

One of the most important and ubiquitous modifications of hormonal peptides is the C-terminal amidation (for review see Mains *et al.*, 1983). The amidation signal in the precursor is the sequence Xaa-Gly-Lys/Arg-Lys/Arg-Yaa. The initial cleavage is performed by an endopeptidase requiring the dibasic amino acids N-terminal to the cleavage point (Fletcher *et al.*, 1980; Loh & Gainer, 1982; Chang *et al.*, 1982; Chang & Loh, 1983). The two basic amino acids are then removed by a carboxypeptidase, yielding a peptide with C-terminal glycine. Finally, glyoxylate is removed to produce the C-terminal amidation (Bradbury *et al.*, 1982; Eipper *et al.*, 1983a).

The ACTH precursor pro-opiomelanocortin contains three amidation sites at least in humans (Takahashi *et al.*, 1981): (1) in the ACTH sequence, (2) immediately N-terminal to the ACTH sequence (the hinge peptide; Seidah *et al.*, 1981) and (3) in the N-terminal fragment of the precursor, which contains the γ MSH sequence (Nakanishi *et al.*, 1979; Takahashi *et al.*, 1981). The amidated products are α MSH, amidated hinge peptide and γ_1 MSH respectively. The ACTH sequence is phylogenetically highly conserved, whereas the γ MSH sequence is less conserved and in some species completely deleted (Kawauchi, 1983). The amidation signal for the hinge peptide is present in man (Seidah *et al.*, 1981; Takahashi *et al.*, 1981), rat (Drouin & Goodman, 1980) and mouse (Notake *et al.*, 1983), but neither in cow (Nakanishi *et al.*, 1979) nor in pig (Boileau *et al.*, 1982).

In a normal anterior pituitary no amidation of pro-opiomelanocortin-related peptides takes place, although generation of α MSH can be induced *in vitro* (Eipper *et al.*, 1983a). In species with a conserved intermediate lobe almost all ACTH molecules are processed to α MSH. The first step in generation of α MSH from ACTH-(1–39) is proposed to be the proteolytic cleavage by an endopeptidase to produce ACTH-(1–17)

(Scheme 1), followed by the action of a carboxypeptidase B-like activity that is present in pituitaries (Hook & Loh, 1984). Finally, ACTH-(1–14) is converted into ACTH-(1–13)-NH₂ by an oxidative mechanism involving peptidyl-glycine α -amidating mono-oxygenase in the presence of ascorbic acid, Cu²⁺ and O₂ (Bradbury *et al.*, 1982; Eipper *et al.*, 1983a). However, the putative intermediates ACTH-(1–17), ACTH-(1–16), ACTH-(1–15) and ACTH-(1–14) have never been detected *in vivo*, and only ACTH-(1–14) has been detected *in vitro*. The purpose of the present work was to examine whether ACTH-(1–17) and ACTH-(1–14) are present in extracts of normal pig pituitaries.

MATERIALS AND METHODS

Materials

Human ACTH-(1–39), ACTH-(1–17), [Phe², Nle⁴]-ACTH-(1–24), ACTH-(1–14), ACTH-(1–13), ACTH-(1–4), ACTH-(5–10), ACTH-(1–10), ACTH-(5–14), ACTH-(18–39), α MSH, *N*-acetyl-ACTH-(1–14), ACTH-(1–13)-NH₂ (desacetyl- α MSH), γ_1 MSH, γ_1 MSH and β MSH were from Peninsula (Belmont, CA, U.S.A.), CRB (Cambridge, U.K.) and Bachem (Bubendorf, Switzerland). The anti-ACTH sera, one of which has already been characterized in some detail (Hummer, 1978), were kindly donated by Dr. L. Hummer (Copenhagen, Denmark). Carrier-free Na¹²⁵I was from New England Nuclear (Dreieich, West Germany) and Seppak cartridges were from Waters Associates (Milford, MA, U.S.A.). Aprotinin, a Trasylol-containing pancreatic extract, was purchased from Novo Industries, Copenhagen, Denmark, and iodogen (1,3,4,6-tetrachloro-3 α ,6 α -diphenylglycoluril) was from Pierce Chemical Co. (Rockford, IL, U.S.A.). All other reagents were the purest available.

Abbreviations used: ACTH, adrenocorticotrophic hormone (corticotropin); α MSH, β MSH and γ MSH, α -, β - and γ -melanocyte-stimulating hormone (melanotropin) respectively.

Scheme 1. Processing sequence of ACTH-(1-39) to α MSH as accepted at the present moment

The degree of acetylation can vary from tissue to tissue and species to species, from non-acetylated peptides through *N*-acetylation to *NO*-diacetylation. The specificities of three of the four antibodies used in this investigation are shown (see the text for details).

Antibody production

ACTH-(1-14), ACTH-(1-13) and ACTH-(1-13)-NH₂ were all coupled to bovine serum albumin by a carbodi-imide. A 1.5 mg portion of peptide was solubilized in 1.5 ml of 100 mM-phosphate buffer, pH 7.4, and *NN*-dimethylformamide was added to 25% (v/v) to solubilize ACTH-(1-14) completely. Then 6 mg of bovine serum albumin in 0.5 ml of phosphate buffer was added, and coupling was initiated by adding 25 mg of 1-(3-dimethylaminopropyl)-3-ethylcarbodi-imide. The reaction time was 18-20 h at room temperature. The mixture was diluted with 6 ml of 150 mM-NaCl, divided into small portions and stored at -20 °C. A 2 ml sample of coupled peptide was mixed with an equal volume of Freund's complete adjuvant. Eight rabbits were each injected subcutaneously with a total of 0.5 ml of the emulsified peptide complex. Successive immunizations were performed at 8-week intervals in Freund's incomplete adjuvant. The rabbits were bled 1 week after the second and subsequent immunizations, and serum was stored at -20 °C.

Iodination of peptides

Iodination of peptides was done by the chloramine-T method (Hunter & Greenwood, 1962). A 5 μ g portion of peptide in 10 μ l of 100 mM-phosphate buffer, pH 7.4, was added to 200-1000 μ Ci of Na¹²⁵I in 10-20 μ l of 100 mM-phosphate buffer. The reaction was started by adding 5 μ g of chloramine-T in 10 μ l of buffer and stopped 30 s later by adding 5 μ g of sodium metabisulphite

in 10 μ l of buffer. Iodination by iodogen was performed in a glass dish sprinkled with 10-20 μ g of iodogen, and to which was added 5 μ g of peptide in 50 μ l of 100 mM-borate buffer, pH 8.5. The reaction was stopped after 5-10 min by dilution with the buffer used for purification on Seppak cartridges. ACTH-(1-13)-NH₂ and ACTH-(1-13) were taken up in 0.1% trifluoroacetic acid and applied on to a Seppak cartridge equilibrated in the same buffer. The peptides were eluted by a linear gradient of methanol in 0.1% trifluoroacetic acid. Iodinated ACTH-(1-14) was taken up in 10 mM-ammonium acetate, pH 4.1, loaded on Seppak and peptides were eluted by a linear gradient of methanol in 10 mM-ammonium acetate, pH 4.1. Fractions (1.5-2 ml) were collected and relevant parts of the radioactive peaks were stored at 4 °C. The iodinated ACTH-(1-39) was adsorbed on 10 mg of Quso G32 in 1 ml of serum. After a wash of the gel by 3 ml of distilled water the iodinated peptide was eluted by 2 ml of 40% (v/v) acetone/1% (v/v) acetic acid and stored in this mixture at 4 °C.

Radioimmunoassays

All radioimmunoassays were performed by the same procedure. Standards or samples (200 μ l) were added to 2000 μ l of appropriate diluted antiserum in radioimmunoassay buffer (20 mM-Veronal buffer, pH 8.6, containing 1% bovine serum albumin and 200 kallikrein-inhibitor units of Aprotinin/ml) in polypropylene tubes. The tubes were kept at 4 °C for 24 h, and 200 μ l of tracer was added (approx. 4000 d.p.m., new tracer). After

another 24 h at 4 °C, 500 μ l of a 5% charcoal suspension was added. The charcoal was coated by stirring in radioimmunoassay buffer for 10 min just before use. After 15 min the tubes were centrifuged at approx. 1500 g for 10 min in a clinical centrifuge to pellet the charcoal. The supernatant and precipitate were separated and their radioactivities counted for 3–5 min. Calculations were done as described elsewhere (Stadil & Rehfeld, 1973).

H.p.l.c.

Reversed-phase h.p.l.c. was performed at 50 °C on a 4.5 mm $\times$ 250 mm C₁₈-silica column (Techogel 5; 5 μ m particle size, 30 nm pore size). The column was connected to a Mini pump (Milton Roy), and an LKB 11300 Ultragrad gradient-mixer was used for gradient generation. Samples were injected in a 200 μ l loop (Rheodyne), eluted by 1.6 ml/min, and 0.8–1.2 ml fractions were collected. The buffers used were: buffer A, 10 mM-ammonium acetate, pH 4.1; buffer B, 90% (v/v) methanol in 10 mM-ammonium acetate, pH 4.1. Elution and separation of peptides were done by use of several successive gradients (for further details see the legends to the Figures).

Tissue extraction

Pigs (30–35 kg body wt.) were bled and pituitaries removed and processed within 30 min. The anterior lobe, intermediate lobe cells and the posterior lobe were separated by fine needles and soaked in Krebs–Ringer bicarbonate buffer, pH 7.4, supplemented with 10 mM-glucose. The intermediate-lobe cells were diluted in 5 ml of distilled water and boiled for 15 min. The pellet remaining after centrifugation was extracted for 30 min in 5 ml of 0.5 M-acetic acid at room temperature and the supernatant was cleared by centrifugation. The anterior lobe was homogenized for 15 s in a Potter–Elvehjem homogenizer and boiled for 15 min in 5 ml of distilled water. The pellet was extracted and described above for intermediate-lobe cells. In some cases the anterior lobe was cut into two pieces at the most anterior part of the gland before extraction. The extracts were purified on a Seppak cartridge; after the extracts had been loaded on Seppak and a rinse in 0.1% trifluoroacetic acid the peptides were eluted with 80% (v/v) methanol in 0.1% trifluoroacetic acid, evaporated, resuspended in 10 mM-ammonium acetate, pH 4.1, and subjected to h.p.l.c.

Miscellaneous

Peptides were quantified by amino acid analyses on an LKB 4200 amino acid analyser after hydrolysis of the peptides in 6 M-HCl at 100 °C for 20 h.

RESULTS

Iodination of peptides

ACTH-(1–39) was iodinated as indicated above with 200–500 μ Ci of Na¹²⁵I. No further purification of this tracer was needed. ACTH-(1–13), ACTH-(1–14) and ACTH-(1–13)-NH₂ were all iodinated by the chloramine-T method or with the use of iodogen as indicated above. In every instance 1 mCi of Na¹²⁵I was used, and these peptides were purified on Seppak as shown in Fig. 1. All iodinated peptides were stored at 4 °C and were stable for 6–8 weeks. The specific radioactivities of the tracers determined by the method described by Morris (1976) were: ¹²⁵I-ACTH-(1–39), 700 Ci/mmol (1.7 fmol);

Fig. 1. Purification of radioimmunoassay tracers on Seppak

Purification of ¹²⁵I-ACTH-(1–13) (a), ¹²⁵I-desacetyl- α MSH (b) and ¹²⁵I-ACTH-(1–14) (c) on Seppak cartridges is illustrated. In (a) and (b) 0.1% trifluoroacetic acid was used as basal solvent, whereas 10 mM-ammonium acetate, pH 4.1, was used in (c). After application of the iodinated peptide the cartridges were washed in approx. 50 ml of 0.1% trifluoroacetic acid (a and b) or 10 mM-ammonium acetate, pH 4.1 (c). The linear gradients up to 60% methanol were made up of 90 ml (total volume) and developed at 60 ml/h; 1 min fractions were collected. Free [¹²⁵I]iodide was eluted in the wash (not shown). Only the relevant parts of the chromatograms are shown. Arrows indicate fractions routinely used as tracers.

¹²⁵I-ACTH-(1–13)-NH₂, 1900 Ci/mmol (1.4 fmol); ¹²⁵I-ACTH-(1–14), 150 Ci/mmol (10 fmol); ¹²⁵I-ACTH-(1–13), 200 Ci/mmol (7.5 fmol). The amounts of peptide added to each tube in the radioimmunoassays are given in parentheses.

Antisera

Useful antisera were produced as early as 2 months after the first immunization, i.e. after two immunizations. The antisera were diluted 1:100 in 100 mM-phosphate buffer, pH 7.4, kept at –20 °C and finally diluted in radioimmunoassay buffer at the time of use.

Radioimmunoassays

The antisera were diluted to bind approx. 33% of the iodinated peptides when 4000 d.p.m. of new tracer was added. The final antisera dilutions were: HF2, 1:250000

Table 1. Characteristics of antisera used in the radioimmunoassays

Cross-reactivities were determined as described in the text and expressed as amounts of peptide needed to displace tracer to 50% of maximal binding compared with the primary peptide in the assays. K_a values were determined by the method described by Morris (1976).

Antiserum	$10^{11} \times K_a \text{ (M}^{-1}\text{)}$	Reactivity of test peptides (%)				
		ACTH-(1-39)	ACTH-(1-14)	α MSH	ACTH-(1-13)	ACTH-(1-17)
HF2	3.7	100	0	0	0	100
1835	0.78	1.5	100	2.1	1.6	50
1842	5.5	0	< 0.1	100	0	0
1850	1.5	0	0	0	100	< 0.5

Fig. 2. H.p.l.c. of synthetic ACTH-related peptides

A mixture of 5 μ g of each of ACTH-(1-17), ACTH-(1-14), ACTH-(1-13), α MSH, desacetyl- α MSH and *N*-acetyl-ACTH-(1-13) was subjected to h.p.l.c. The buffers consisted of 10 mM-ammonium acetate, pH 4.1 (buffer A), and 90% methanol in 10 mM-ammonium acetate, pH 4.1 (buffer B). The successive gradients used were: 5% buffer B (0-3 min), 5-30% buffer B (3-10.5 min), 30-46% buffer B (10.5-21.9 min) and 46-52% buffer B (21.9-60 min); 30 s fractions (0.8 ml) were collected and assayed by radioimmunoassay (upper part of the Figure). Peaks are identified as follows: peak I, ACTH-(1-17) (Δ); peak II, desacetyl- α MSH; peak III, ACTH-(1-14) ($\bullet$); peak IV, ACTH-(1-13) ($\blacktriangle$); peak V, α MSH ($\circ$); peak VI, *N*-Acetyl-ACTH-(1-13). *N*-Acetyl-ACTH-(1-14) was

[ACTH-(1-39); 1835, 1:120000 [ACTH-(1-14)]; 1842, 1:35000 [ACTH-(1-13)-NH₂]; 1850 1:20000, [ACTH-(1-13)]. Antiserum HF2 detected ACTH-(1-17) equally well (Table 1), but did not recognize ACTH-(1-13), ACTH-(1-14), ACTH-(1-4), ACTH-(5-10), ACTH-(1-10), ACTH-(18-39) or α MSH. The antibodies are therefore specific for residues 14-17 in ACTH (Scheme 1). It is influenced by the tertiary structure of the peptides, because the [Phe²,Nle⁴]ACTH-(1-24) analogue only reacts with an ED₅₀ of 50%. The detection limit of this radioimmunoassay was 0.2 fmol.

Antiserum 1835 was intended to be directed towards the C-terminus of ACTH-(1-14). However, it also recognized ACTH-(1-17) and ACTH-(1-39), with decreasing affinity (Table 1), and to a lesser extent ACTH-(1-13) and ACTH-(1-13)-NH₂. It did not recognize ACTH-(1-4), ACTH-(5-10), ACTH-(1-10) or the glycine-extended analogue γ_2 MSH. This antiserum is obviously directed towards the ACTH-(10-13) sequence. ACTH-(1-14) has the highest affinity for the antiserum, but the reactivity is influenced by tertiary structures (Table 1). The detection limit is 1 fmol for ACTH-(1-14).

Antiserum 1850, raised by the use of ACTH-(1-13) as immunogen, reacted with ACTH-(1-13), but not ACTH-(1-14), ACTH-(1-13)-NH₂, ACTH-(1-17) or ACTH-(1-39) (Table 1), nor did it react with ACTH-(1-4), ACTH-(5-10) or ACTH-(1-10). This antiserum was therefore C-terminal-directed. The detection limit for this radioimmunoassay is 0.2 fmol.

ACTH-(1-13)-NH₂ was the only peptide reacting with antiserum 1842. This antiserum did not recognize any other of the other listed peptides, including γ_1 MSH (Table 1). This antiserum is end-directed to the amide group and the detection limit is 0.2 fmol.

The precisions are better than 10% for all the assays.

H.p.l.c.

Fig. 2 shows the elution of ACTH-(1-17), ACTH-(1-13), ACTH-(1-13)-NH₂, ACTH-(1-14), *N*-acetyl-ACTH-(1-13) and *N*-acetyl-ACTH-(1-13)-NH₂ (α MSH) from a C₁₈-silica h.p.l.c. column. All the peptides were eluted within 30 min. ACTH-(1-39) was not eluted at the end of the last gradient at 60 min (corresponding to 52% buffer B) but was eluted from the column by a short pulse of 100% buffer B. If elution time

eluted between peaks V and VI (not shown). Only the most important part of the chromatogram is shown. ACTH was eluted at 60 min with 100% buffer B (see Fig. 3).

Table 2. α MSH-related peptides in pig anterior pituitary

Anterior lobes of the pituitary were dissected to separate the most anterior part of the gland from the posterior part and contaminating intermediate-lobe cells after the lateral wings had been removed. The pituitaries were extracted as described in the text. Results are given as means, with ranges in parentheses, for seven determinations. 'Total' of α MSH-related peptides was calculated as the sum of ACTH, ACTH-(1-14) and α MSH.

	Content (nmol/g wet wt.)			$\frac{\alpha\text{MSH}}{\text{Total}} \times 100 (\%)$
	ACTH	α MSH	ACTH-(1-14)	
Anterior part	304 (75-640)	0.22 (0.13-0.34)	10.01 (5.00-24.93)	0.10 (0.05-0.29)
Posterior part	141 (97-142)	2.85 (0.55-11.90)	5.63 (4.17-7.96)	2.44 (0.70-7.40)

were shortened to 30 min the peptides could still be identified by u.v. absorption, but they were difficult to separate. *N*-Acetyl-ACTH-(1-14) was eluted between α MSH and *N*-acetyl-ACTH-(1-13) (result not shown).

Analysis of pituitaries

More than 95% of the peptides extracted from the intermediate-lobe cells were extracted by boiling in water. The subsequent extraction by acetic acid was therefore omitted for these cells. The anterior lobe was extracted by acetic acid after extraction by homogenization and boiling in water. The acetic acid extract contained most of the pro-opiomelanocortin and intermediates in processing of pro-opiomelanocortin to ACTH (results not shown). No further ACTH-related peptides were extracted by 5 M-acetic acid.

To establish whether α MSH or acetylated analogues were produced in the anterior lobe, the glands were cut sagittally to obtain the most anterior part of the glands free of contamination of intermediate-lobe cells. Table 2 summarizes the peptide content of the anterior and posterior parts of the anterior pituitaries. The anterior part of the dissected gland varies from 6.6% to 31.4% (w/w) of the whole gland. The α MSH content is significantly lower in the anterior part of the gland than in the posterior part. Less than 0.1% of ACTH-related

peptides are processed to α -MSH, and it is therefore concluded that α MSH is not normally produced by the anterior lobe and that α MSH detected in the anterior pituitary is derived from intermediate-lobe cells.

The peptide content of the pituitary is summarized in Table 3. If it is assumed that all the ACTH in the intermediate-lobe cells is processed to α MSH and intermediates (i.e. all the ACTH in the intermediate-lobe cell extracts is derived from the anterior lobe), the ACTH-(1-14) content of the two lobes can be calculated as follows:

$$\begin{aligned} \text{Measured ACTH-(1-14) in anterior lobe } (M_{AL}) \\ = \text{real ACTH-(1-14) content in anterior lobe } (X) \\ + \text{contamination from intermediate lobe } (IL_C) \end{aligned}$$

and

$$\begin{aligned} \text{Measured ACTH-(1-14) in intermediate lobe } (M_{IL}) \\ = \text{real ACTH-(1-14) content in intermediate lobe } (Y) \\ + \text{contamination from anterior lobe } (AL_C) \end{aligned}$$

By use of the results from Table 2 and the assumption that all ACTH in the intermediate lobe is derived from the anterior lobe IL_C and AL_C can be calculated:

$$IL_C = \frac{Y}{\alpha\text{MSH}_{IL}} \times \alpha\text{MSH}_{AL} = Y \times R_\alpha$$

Table 3. Peptide content in normal pig pituitaries

Results are given as means, with ranges in parentheses, for six determinations. 'Total peptide' is calculated as all α MSH in whole glands plus corrected ACTH-(1-14) in intermediate-lobe cell extract or all ACTH-(1-39) in whole glands plus corrected ACTH-(1-14) in anterior-lobe extracts, as appropriate.

Extract	Content (nmol of peptide/g wet wt.) of anterior lobe				$\frac{\text{Corrected ACTH-(1-14)}}{\text{Total peptide}} \times 100 (\%)$
	ACTH-(1-39)	α MSH	ACTH-(1-14)	Corrected ACTH-(1-14)	
Anterior lobe					
Water	26.1 (4.3-42.7)	3.8 (0.6-8.0)	6.0 (2.5-9.2)	6.1 (2.6-11.0)	12.9 (10.4-19.2)
Acetic acid	5.6 (0.9-17.5)	0.4 (0.01-1.6)	1.1 (0.1-4.0)		
Intermediate lobe					
Water	2.7 (0.3-4.5)	17.7 (2.6-36.5)	4.1 (1.0-10.5)	4.2 (0.4-11.5)	14.9 (3.7-21.4)

and

$$AL_C = \frac{X}{ACTH_{AL}} \times ACTH_{IL} = X \times R_{ACTH}$$

where αMSH_{AL} and αMSH_{IL} represent measured αMSH in anterior and intermediate lobes respectively, $ACTH_{AL}$ and $ACTH_{IL}$ represent measured ACTH in anterior and intermediate lobes respectively, and R_α and R_{ACTH} represent $\alpha MSH_{AL}/\alpha MSH_{IL}$ and $ACTH_{AL}/ACTH_{IL}$ respectively. By substitution, rearranging and inclusion of the peptides contaminating the other lobe, the ACTH-(1-14) content can be calculated in the two lobes:

Anterior-lobe ACTH-(1-14)

$$= \frac{M_{AL} - M_{IL} \times R_\alpha}{1 - R_\alpha \times R_{ACTH}} (1 + R_{ACTH})$$

Intermediate-lobe ACTH-(1-14)

$$= \frac{M_{IL} - M_{AL} \times R_{ACTH}}{1 - R_\alpha \times R_{ACTH}} (1 + R_\alpha)$$

These calculated values are listed as 'corrected' values in Table 3.

Before the extracts were subjected to h.p.l.c. the peptides were purified on Seppak columns. More than 99% of the peptides were adsorbed on the column and all were eluted by 80% methanol. Fig. 3 shows the h.p.l.c. elution pattern of a water extract from intermediate-lobe cells purified on Seppak. The main peaks of αMSH -like and ACTH-(1-14)-like material were eluted after the synthetic peptides. When these peptides were treated by 10 mM-NaOH they were eluted as αMSH and *N*-acetyl-ACTH-(1-14) respectively. They were therefore identified as *NO*-diacetyl-ACTH-(1-13)-NH₂ and *NO*-diacetyl-

ACTH-(1-14) respectively (Rudman *et al.*, 1979). Only small amounts of ACTH-(1-13)-NH₂, αMSH , ACTH-(1-14) and *N*-acetyl-ACTH-(1-14) were present. In no instances were ACTH-(1-13) or ACTH-(1-17) or their acetylated analogues detected (see below).

DISCUSSION

The results presented here shows that ACTH-(1-14) and acetylated analogues are present in both the anterior lobe and the intermediate lobe of the pituitary *in vivo*, whereas neither ACTH-(1-17) nor its acetylated analogues were detectable in either lobe. Identification of these peptides and αMSH and ACTH-(1-39) requires sensitive and specific radioimmunoassays. Because cleavage of ACTH-(1-14) to ACTH-(1-13) is a possibility an assay for ACTH-(1-13) was included in this investigation. The amide-directed αMSH assay and C-terminal ACTH-(1-13) assay are both end-directed and hence entirely specific. The ACTH-(1-14) assay is sensitive, but not specific in that it recognizes ACTH-(1-17) and ACTH-(1-39), although with lower potency (Table 1). ACTH-(1-17) is also recognized by the ACTH-(1-39) assay and with equal potency. Exact and correct determinations of ACTH-(1-39), ACTH-(1-17) and ACTH-(1-14) can therefore not be done by the radioimmunoassays alone. None of the assays is influenced by *N*-terminal acetylation or oxidation. The latter is known to occur extensively when chloramine-T is used in the iodination of the peptides (Heward *et al.*, 1979), but assay sensitivity and specificity did not change if iodination was performed with iodogen, which did not oxidize the peptides (M. Fenger, unpublished work).

To solve the problem of cross-reactivity the peptides

Fig. 3. H.p.l.c. of a water extract of intermediate lobe from a pig pituitary

One-fifth the extract was pre-purified on a Seppak cartridge before application to h.p.l.c. (see the text for details). The buffer system was as in Fig. 3, but fractions were collected at 45 s intervals. All fractions were measured with all the four radioimmunoassays. αMSH (○) is read off the left-hand scale, and ACTH-(1-14) (●) and ACTH-(1-39) (△) are read off the right-hand scale. No ACTH-(1-13) was detected.

were separated by h.p.l.c. in a modification of the method of Eipper *et al.* (1983b). The gradients were modified to shorten the elution time and to include ACTH-(1-17) in the chromatogram. ACTH-(1-14), ACTH-(1-17) and ACTH-(1-39) were separated by the combination of gradients used. ACTH-(1-17) was then determined by the ACTH-(1-39) assay, and ACTH-(1-14) can be quantified without interference from ACTH-(1-17). *N*-Acetylated ACTH-(1-17) is not available at present, but from the elution pattern of ACTH-(1-13) and ACTH-(1-14) and their acetylated analogues *N*-acetyl-ACTH-(1-17) is expected to be eluted at approx. 16–17 min, i.e. far ahead of ACTH-(1-14). Therefore all the peptides, including their *N*-acetylated analogues, can be separated by one h.p.l.c. run and quantified by the four radioimmunoassays.

α MSH production can be induced in anterior-lobe cells *in vitro* (Eipper *et al.*, 1983a), but has not been shown to exist *in vivo* in anterior-lobe cells. This is supported by the results presented in Table 2. When the anterior lobe was divided into an anterior and a posterior part the α MSH content in the posterior part was 20–25 times as high as in the anterior part (Table 2). In addition, the size of the anterior part was not correlated to α MSH/total ratio but to the total α MSH content. Although it cannot be definitely proved that α MSH is not produced in the anterior lobe, these data suggest that the α MSH in anterior-lobe extracts is derived from intermediate-lobe cells. α MSH was therefore used as marker of intermediate-lobe cells. The calculations presented in Table 3 are based on the assumption that all ACTH is processed in the intermediate-lobe cells. This assumption represents one of two extremes, and the calculated ACTH-(1-14) contents in the anterior lobe presented in Table 3 are maximum values.

If it is argued that not all the ACTH-(1-39) in the intermediate lobes is processed and that all the ACTH extracted from intermediate-lobe cells is actually from the intermediate lobe with no contamination from the anterior lobe, the ACTH-(1-14) content can be calculated directly by use of the ACTH-(1-14)/ α MSH ratio in the intermediate lobes. The ACTH-(1-14) contents are then calculated to be 2.1–10.4 nmol/g and 1.4–12.1 nmol/g in the anterior lobe and intermediate lobe respectively. This represents the other extreme, and the calculated ACTH-(1-14) contents in the anterior lobes are minimum values. Still, 9.9–14.1% of ACTH-related peptides from anterior lobes is ACTH-(1-14). The exact content of ACTH-(1-14) can only be calculated if a specific marker of the anterior lobe is used. Thyroid-stimulating hormone (thyrotropin), growth hormone (somatotropin) and prolactin could not be used because these hormones are not extracted efficiently by the procedure adopted here (M. Fenger, unpublished work). However, the results presented here show that ACTH-(1-14) is present *in vivo* in both anterior and intermediate lobes of the pituitary. In the intermediate lobe all the ACTH is assumed to be processed to α MSH, but nevertheless a significant amount of ACTH-(1-14) is present, regardless of which calculation is used. All the pituitaries are extracted within 30 min of removal of the glands from the bled animals. *In vitro* the amidation activity diminishes with a half-life of 18 h (Eipper *et al.*, 1983b; M. Fenger, unpublished work) in the absence of ascorbic acid. Therefore the time taken from bleeding the animals to extraction of pituitaries is too short to account for the low amidation

activity. It is possible that diminished amounts of ascorbic acid *in vivo* could be the explanation of the subtotal processing of ACTH-(1-14) to α MSH in the intermediate lobe. In the anterior lobe ACTH-(1-14) could be a waste product from the production of ACTH-(18-39), which has been shown to stimulate insulin secretion in the pancreas (Beloff-Chain *et al.*, 1983). It is not known if this is the explanation of the occurrence of ACTH-(1-14) in the anterior pituitary. The possibility of a physiological function of ACTH-(1-14) must also be kept in mind, although none as yet has been described.

About 90% of the α MSH and ACTH-(1-14) analogues were eluted as *NO*-diacetylated derivatives (Fig. 3). Small amounts of ACTH-(1-13)-NH₂ and α MSH were identified, but the peaks corresponding to ACTH-(1-14) and *N*-acetyl-ACTH-(1-14) were broad and the latter did not separate from *NO*-diacetyl-ACTH-(1-14). When the charge of the peptides decreases, as is the case for the series of peptides ACTH-(1-17) to ACTH-(1-14), the retention times are increased. *N*-Acetylation and *NO*-diacetylation additionally increase the retention times [compare, e.g., ACTH-(1-13)-NH₂, α MSH and *NO*-diacetyl-ACTH-(1-13)-NH₂ in Fig. 3]. Therefore acetylated ACTH-(1-16) and acetylated ACTH-(1-15) are probably contained in the broadened peaks of the ACTH-(1-14) analogues. These peptides would be detected by the ACTH-(1-14) assay [compare the reactivity to ACTH-(1-17) in the ACTH-(1-14) radioimmunoassay in Table 1]. ACTH-(1-15) and ACTH-(1-16) are therefore probably present, but the confirmation of this requires purification and sequencing of the peptides in fractions 25–32 in Fig. 3. It cannot be completely excluded that *NO*-diacetyl-ACTH-(1-17) is eluted with ACTH-(1-14), but no immunoreactive peak is detected at this position by the ACTH-(1-39) radioimmunoassay.

In summary, ACTH-(1-17) and acetylated analogues could not be detected *in vivo* in pig pituitaries, but ACTH-(1-14) and acetylated analogues are present, and probably also ACTH-(1-15) and ACTH-(1-16) and their analogues. It has previously been assumed that the initial cleavage of ACTH-(1-39) is done by an endopeptidase at the Arg-17-Arg-18 bond, producing ACTH-(1-17) and ACTH-(18-39). This model is based on findings of the final products α MSH/ACTH-(1-14) and ACTH-(18-39) (e.g. Bennett *et al.*, 1982). However, ACTH-(1-17) has never been detected, and its presence could not be established in the present work. The reason for this could be a fast turnover of ACTH-(1-17) to ACTH-(1-16) by the pituitary carboxypeptidase described by Hook & Loh (1984). Another possibility is that the initial cleavage by an endopeptidase is done at the Lys-16-Arg-17 bond, producing ACTH-(1-16) and ACTH-(17-39), which still fulfils the criterion that there should be two basic amino acids *N*-terminal to the cleavage point. The former peptide is then cleaved twice by the carboxypeptidase followed by cleavage of ACTH-(1-14) by peptidyl-glycine α -amidating mono-oxygenase (Eipper *et al.*, 1983a). ACTH-(17-39) is cleaved once by the pituitary aminopeptidase that has been described by Gainer *et al.* (1984). This aminopeptidase specifically cleaves at basic amino acid residues provided that they are not followed *C*-terminally by a proline residue. β -Lipotropin-(60–65) was used as substrate, but the *N*-terminal sequence of ACTH-(17–39) is Arg-Arg-Pro and would therefore be a

substrate (and maybe the most important substrate) for this aminopeptidase.

The confirmation of this model requires the purification and sequencing of ACTH-(17-39).

Aase Valsted is thanked for skilful technical assistance. Professor J. F. Rehfeld and Professor T. W. Schwartz are appreciated for helpful advices and discussion of this manuscript. Mrs. Werni Lindegaard is thanked for preparing this manuscript and some of the Figures. This study was supported by the Danish Medical Research Council, Research Foundation of Copenhagen, Faroe Islands and Greenland, Haensch Foundation and P. Andersens Foundation.

REFERENCES

- Beloff-Chain, A., Morton, J., Dumnore, S., Taylor, G. W. & Morris, R. (1983) *Nature (London)* **301**, 255-258
- Bennett, H. P. J., Browne, C. A. & Solomon, S. (1982) *J. Biol. Chem.* **257**, 10096-10102
- Boileau, G., Lariviere, N., Hsi, K.-L., Seidah, B. G. & Chrétien, M. (1982) *Biochemistry* **21**, 5341-5346
- Bradbury, A. F., Finnie, M. D. A. & Smyth, D. G. (1982) *Nature (London)* **298**, 686-688
- Chang, T.-L. & Loh, Y. P. (1983) *Endocrinology (Baltimore)* **112**, 1832-1838
- Chang, T.-L., Gainer, H., Russell, J. T. & Loh, Y. P. (1982) *Endocrinology (Baltimore)* **111**, 1607-1614
- Drouin, J. & Goodman, H. M. (1980) *Nature (London)* **288**, 610-613
- Eipper, B. A., Mains, R. E. & Glembostki, C. C. (1983a) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 5144-5148
- Eipper, B. A., Glembostki, C. C. & Mains, R. E. (1983b) *J. Biol. Chem.* **258**, 7292-7298
- Fletcher, D. J., Noe, B. D., Bauer, G. E. & Quigley, J. P. (1980) *Diabetes* **29**, 593-599.
- Gainer, H., Russel, J. T. & Loh, Y. P. (1984) *FEBS Lett.* **175**, 135-139
- Heward, C. B., Yang, Y. C. S., Ormberg, J. F., Hadley, M. E. & Hruby, V. J. (1979) *Hoppe-Seyler's Z. Physiol. Chem.* **360**, 1851-1859.
- Hook, V. Y. H. & Loh, Y. P. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 2776-2780
- Hummer, L. (1978) in *Radioimmunoassay and Related Procedures in Medicine 1977*, vol. 2, pp. 391-403, International Atomic Energy Agency, Vienna
- Hunter, V. M. & Greenwood, F. C. (1962) *Nature (London)* **194**, 495-496
- Kawauchi, H. (1983) *Arch. Biochem. Biophys.* **227**, 343-350
- Loh, Y. P. & Gainer, H. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 108-112
- Mains, R. E., Eipper, B. A., Glembostki, C. C. & Dore, R. M. (1983) *Trends Neuro Sci.* **6**, 229-235
- Morris, B. J. (1976) *Clin. Chim. Acta* **73**, 213-216
- Nakanishi, S., Inoue, A., Kita, T., Nakamura, M., Chang, A. C. Y., Cohen, S. N. & Numa, S. (1979) *Nature (London)* **278**, 423-427
- Notake, M., Tobimatsu, T., Watanabe, Y., Takahashi, H., Mishina, M. & Numa, S. (1983) *FEBS Lett.* **156**, 67-71
- Rudman, D., Chawla, R. K. & Hollins, B. M. (1979) *J. Biol. Chem.* **254**, 10102-10108
- Seidah, N. G., Rochemont, J., Hamelin, J., Benjannet, S. & Chrétien, M. (1981) *Biochem. Biophys. Res. Commun.* **102**, 710-716
- Stadil, F. & Rehfeld, J. F. (1973) *Scand. J. Gastroenterol.* **8**, 101-112
- Takahashi, H., Teranishi, Y., Nakanishi, S. & Numa, S. (1981) *FEBS Lett.* **135**, 97-102

Received 12 August 1985/6 November 1985; accepted 23 December 1985