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Isolation and Characterization of Corticotropin- and Melanotropin-Related Peptides from the Neurointermediary Lobe of the Rat Pituitary by Reversed-Phase Liquid Chromatography†

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ABSTRACT: A novel procedure utilizing reversed-phase high-performance liquid chromatography for the extraction and purification of peptides from biological tissues has been applied to the isolation of corticotropin-like intermediary lobe peptide (CLIP) and α-melanocyte-stimulating hormone (α-MSH) from the neurointermediary lobe of the rat pituitary. The isolation and characterization of two major forms of CLIP and two major forms of α-MSH are described. The isolated peptides have been identified by using enzymatic digestions and peptide mapping. The main form of CLIP is a peptide which has been modified by phosphorylation of the serine residue at position 31. This is the first peptide of endocrine origin reported to be modified in such a manner. A non-phosphorylated form of CLIP was also present at lower concentrations. The main form of α-MSH was found to be *N*,*O*-diacetyl-α-MSH, with the more familiar mono-*N*-acetyl-α-MSH present to a much smaller extent. Thus, in the rat neurointermediary lobe, the two main corticotropin-related peptides present are mostly in modified forms which are the result of posttranslational modifications. It is only by the use of methodology such as that described in this paper that small alterations in peptide structure may be identified.

It has been known for many years that the intermediary lobe of the pituitary contains large quantities of various peptide hormones. α-Melanotropin (α-MSH)[1] has been isolated from the rat intermediary lobe (Harris, 1956), and more recently corticotropin-like intermediary lobe peptide (CLIP;

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[1] Abbreviations used: RP-HPLC, reversed-phase high-performance liquid chromatography; F_3CCOOH, trifluoroacetic acid; HFBA, heptafluorobutyric acid; ODS-silica, octadecylsilylsilica; α-MSH, α-melanocyte-stimulating hormone; ACTH, adrenocorticotropic hormone; CLIP, corticotropin-like intermediary-lobe peptide; β-LPH, β-lipotropic hormone; RIA, radioimmunoassay; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

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$ACTH_{18-39}$)[2] (Scott et al., 1974) has also been found in this tissue. The relationship of these two peptides to corticotropin (ACTH) has been well recognizd (Lowry & Scott, 1975). α-MSH consists of the sequence $ACTH_{1-13}$ with the amino-terminal serine N-acetylated and the carboxyl-terminal valine in the amide form, whereas CLIP consists of $ACTH_{18-39}$ in all species in which it has been found (Scott et al., 1976). It has been proposed (Scott et al., 1973) that the anterior pituitary secretes ACTH intact, whereas in the intermediary lobe the ACTH is converted to α-MSH and CLIP, which are the main corticotropin-related peptides secreted from this gland. Recently, much more extensive knowledge has been obtained about the biosynthesis of ACTH. It has been shown that ACTH and β-lipotropin (β-LPH) are biosynthesized from a common precursor (Mains et al., 1977) and that this precursor molecule may be processed in different ways to produce different products (Eipper & Mains, 1980). Thus, in the anterior lobe the precursor is processed mainly to ACTH and β-LPH, whereas in the intermediary lobe α-MSH, CLIP, and β-endorphin are the end products of biosynthesis.

Most of the data in these biosynthetic studies have been obtained by using pulse–chase labeling techniques, coupled with sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide gel electrophoresis (Mains & Eipper, 1976) or paper electrophoresis (Roberts & Herbert, 1977), to identify the precursors, biosynthetic intermediates, and end products. However, these studies have not been successful in the complete characterization of the end products, especially with regard to possible posttranslational modifications of the resulting peptides. In light of the new data which have been accumulated on the biosynthesis of ACTH-related peptides, it seemed appropriate to reinvestigate the molecular nature of peptides related to α-MSH and CLIP in the intermediary lobe of the rat by using techniques which will allow positive identification of all the peptides present.

Experimental Procedures

Materials. Bovine luteinizing hormone, follicle-stimulating hormone, thyroid-stimulating hormone, growth hormone, and prolactin were all obtained from the National Pituitary Agency, Bethesda, MD. Synthetic mammalian α-MSH (*N*-acetyl-$ACTH_{1-13}$ amide) was obtained from Dr. W. Rittel, Ciba-Geigy Ltd., Basel, Switzerland, and human $ACTH_{17-39}$ was obtained from Dr. P. J. Lowry, St. Bartholemews Hospital, London, United Kingdom. All other peptides and reagents were obtained as previously described (Bennett et al., 1981a).

Extraction and Isolation of Peptides from the Neurointermediary Lobe of Rat Pituitary. Peptides were extracted from 190 rat neurointermediary lobes and were isolated by reversed-phase high-performance liquid chromatography (RP-HPLC), using the methods described earlier (Bennett et al., 1981a), which combine a reversed-phase extraction procedure (Bennett et al., 1978) with RP-HPLC (Bennett et al., 1980a,b). Briefly, tissue was homogenized in a strongly acidic medium, and the peptides were extracted by using ODS-silica cartridges (C_{18} Sep-Paks, Waters Associates) and were eluted with 3 mL of 80% acetonitrile containing 0.1% trifluoroacetic acid (F_3CCOOH). This eluate was diluted to 18 mL with 0.1% F_3CCOOH and was loaded directly onto a C_{18} μBondapak RP-HPLC column (Waters Associates). The column was then eluted at 1.5 mL/min with a linear gradient over 1 h, from 20% to 40% acetonitrile containing 0.1% F_3C-COOH throughout, and then was purged for 10 min with 80% acetonitrile containing 0.1% F_3CCOOH. A total of 70 fractions of 1.5 mL were collected, and aliquots were dried in vacuo and assayed by radioimmunoassay (RIA) for ACTH and α-MSH as described below. Fractions containing immunoreactive ACTH and α-MSH were further purified by diluting 1:1 with 0.13% heptafluorobutyric acid (HFBA) and loading back onto the same RP-HPLC column, which had been equilibrated with 12% acetonitrile containing 0.13% HFBA. The column was eluted at 1.5 mL/min with a linear gradient over 1 h from 20% to 45.6% acetonitrile containing 0.13% HFBA throughout. The amounts of ACTH and α-MSH in aliquots of the fractions were measured by RIA and the peaks of interest were dried in vacuo and subjected to various analytical procedures.

Radioimmunoassays. (*a*) *ACTH.* Antibodies to $ACTH_{1-39}$ were raised in female New Zealand white rabbits. Each animal was immunized with 0.5 mg of porcine $ACTH_{1-39}$ in complete Freund's adjuvant (Gibco) at 6–8-week intervals. Blood was collected at 10–14-day intervals from the central ear vein. Substantial titers of ACTH antibodies were obtained in three of the six animals used. One of these antisera, designated R123378, was used for the work described here.

Synthetic human $ACTH_{1-39}$ was labeled with ^{125}I (New England Nuclear) by using a modification of the chloramine-T method (Hunter & Greenwood, 1962). The [^{125}I]ACTH was adsorbed onto silica (QUSO-32, Calbiochem) (Rees et al., 1971) and the QUSO-32 eluate was loaded onto an ODS-silica cartridge (C_{18} Sep-Pak, Waters Associates). After being washed with 50 mL of 0.1% F_3CCOOH in water, the [^{125}I]-ACTH was eluted from the cartridge with 2 mL of 80% methanol containing 0.1% F_3CCOOH and was stored at 4 °C. RIA incubations were performed in polystyrene tubes for 2 h at 25 °C in a final volume of 0.8 mL of 0.02 M diethylbarbituric acid (pH 8.6), containing 0.3% bovine serum albumin, 0.4% mercaptoethanol, and 0.18% sodium chloride (referred to as RIA buffer). A total of 5000 cpm of [^{125}I]-$ACTH_{1-39}$ (5–10 pg) was added per incubation tube, and the R123378 antiserum was used at a final dilution of 1:40 000. Synthetic human $ACTH_{1-39}$ was used as the standard. Bound and free fractions of the [^{125}I]ACTH were separated by adsorption onto dextran-coated charcoal (0.25% charcoal and 0.025% dextran in RIA buffer) for 10 min at 25 °C. The range of the assay was from 10 to 4000 pg, and the intraassay variation was 8% and the interassay variation 15%.

(*b*) *α-MSH.* Synthetic mammalian α-MSH was conjugated to bovine serum albumin by the method of Kopp et al. (1977). Rabbits were immunized with the conjugate following the same regime described by ACTH. Three out of the six rabbits gave antiserum, and one of these, designated R830579, was used in these studies. α-[^{125}I]MSH was prepared by the method described above for [^{125}I]ACTH and was purified by the ODS-silica method, without prior adsorption onto QUSO-32. The conditions of the α-MSH RIA were identical with those described for the ACTH RIA, except that synthetic α-MSH was the standard, the incubation time was 3 h, and the antiserum dilution was 1:5120. The working range of the assay was from 25 to 4000 pg, and the intra- and interassay variations were 5% and 12%, respectively.

Peptidase Digestions. All enzymes were purchased from the Sigma Chemical Co. and were the purest grades available. Digestion of peptides with trypsin (diphenylcarbamoyl chloride treated) or α-chymotrypsin [*N*-(*p*-tosyl)-L-lysine chloromethyl ketone treated] was carried out at 37 °C for up to 16 h in 0.2

[2] The numbering system used throughout is the numbering for the sequence of $ACTH_{1-39}$. Thus, α-MSH is numbered as 1–13 and CLIP is numbered as 18–39.

mL of 0.05 M ammonium bicarbonate (pH 8.5) at an enzyme to substrate ratio of 1:100 (w/w) for trypsin or 1:200 (w/w) for chymotrypsin. Pepsin digestion was done in 0.2 mL of 0.01 M HCl at 37 °C for 16 h at an enzyme to substrate ratio of 1:100 (w/w). Carboxyl-terminal digestions were done with carboxypeptidase Y in 0.2 mL of 0.05 M sodium citrate, pH 7.8, at 37 °C at an enzyme to substrate ratio of 1:50 (w/w). Amino-terminal digestion was performed with leucine aminopeptidase in 0.03 M sodium phosphate, pH 7.4, for 16 h at 37 °C at an enzyme to substrate ratio of 1:25 (w/w). "Total" enzymatic digestions were performed with a mixture of carboxypeptidase Y, leucine aminopeptidase, and Pronase P (1:1:2 w/w/w) in 0.2 mL of a buffer consisting of Tris (50 mM), sodium chloride (75 mM), potassium chloride (25 mM), and sodium azide (0.005%), adjusted to pH 7.5 with HCl. Digestions were carried out at 37 °C for 24 h at an enzyme to substrate ratio of 1:25 (w/w) based on the carboxypeptidase Y.

Total acid hydrolysis and amino acid analysis were done as described previously (Bennett et al., 1981a), with the exception that the amino acids produced by exopeptidase digestions and total enzymatic digestions were analyzed on a Jeol 6AH amino acid analyzer with a lithium citrate buffer system (Pico Buffer IV, Pierce Chemical Co.) set up to resolve all of the acidic and neutral amino acids. Polyacrylamide gel electrophoresis was performed as described earlier (Bennett et al., 1981a).

Results

Characterization of ACTH and α-MSH Antisera. The ACTH antiserum R123378 gave parallel and equipotent displacement curves for human $ACTH_{1-39}$, porcine $ACTH_{1-39}$, $ACTH_{1-24}$, human $ACTH_{17-39}$, and human $ACTH_{16-27}$ (results not shown). It gave no measurable (<0.05%) cross-reactivity with α-MSH, β-MSH, $ACTH_{1-18}$, human $ACTH_{20-27}$, and human $ACTH_{22-29}$. There was also no measurable cross-reactivity (<0.01%) with any of the following hormones: bovine luteinizing hormone, bovine growth hormon, bovine prolactin, somatostatin, porcine insulin, human calcitonin, α-, β-, and γ-endorphin, and Met-enkephalin. This antiserum is therefore highly specific and is directed toward the sequence 17–20 of ACTH. The R123378 antiserum can be used in the measurement of ACTH-related molecules, including CLIP. It has been successfully used to date in this laboratory for ACTH-like peptides extracted from human, rat, bovine, and ovine sources and cross-reacts in a parallel manner with all CLIP and $ACTH_{1-39}$ peptides tested.

The α-MSH antiserum R830579 cross-reacted weakly (~0.1%) with human $ACTH_{1-39}$ and $ACTH_{1-24}$ and very weakly (~0.01%) with $ACTH_{1-18}$. It failed to cross-react with any of the other peptide hormones that were tested. In order to characterize the R830579 antiserum more fully, synthetic α-MSH was digested by trypsin and by chymotrypsin, following the procedure described previously (Harris, 1956), and the resulting tryptic and chymotryptic peptides were isolated by RP-HPLC, using the conditions given in Figure 6. The identity of the peptides was confirmed by amino acid analysis and corresponded exactly to the predicted patterns of enzymatic digestion. Only the carboxyl-terminal chymotryptic fragment (α-MSH_{10-13}) and the carboxyl-terminal tryptic fragment (α-MSH_{9-13}) cross-reacted with the α-MSH antibody. None of the other fragments tested (1–8, 1–2, 3–7, and 8–9 of α-MSH) showed any cross-reactivity. Thus the α-MSH antiserum (R830579) is specific for the carboxyl-terminal region of α-MSH, and the presence of the *N*-acetyl group at position 1 does not appear to be an important determinant of the specificity of the antiserum.

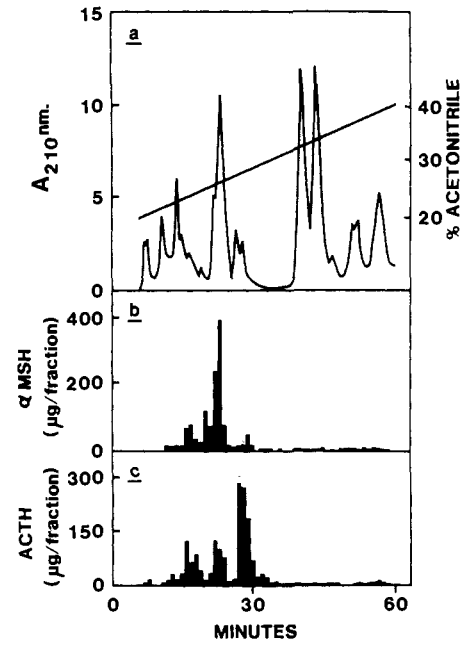

FIGURE 1: RP-HPLC chromatogram of extract obtained from 190 rat neurointermediary lobes. The extract was loaded onto a C_{18} μBondapak column as described under Experimental Procedures, and the peptides were eluted over 1 h with a linear gradient from 20% (v/v) to 40% (v/v) acetonitrile containing 0.1% (v/v) F_3CCOOH throughout, at a flow rate of 1.5 mL/min. The eluate was monitored at 210 nm (a) and 278 nm (not shown). Aliquots from all fractions (1 min) were dried for RIA of α-MSH (b) and ACTH (c). Synthetic α-MSH eluted in fractions 19 and 20, standard CLIP eluted in fractions 27 and 28, and synthetic human $ACTH_{1-39}$ eluted in fractions 30 and 31 under these chromatographic conditions.

Extraction, Isolation, and Characterization of CLIP from the Neurointermediary Lobe of Rat Pituitary. A total of 190 male Sprague-Dawley rats (250–400 g) were sacrificed, and the neurointermediary lobes were removed and extracted within 1 min of death as previously described (Bennett et al., 1981a). The initial RP-HPLC chromatography of the extract (Figure 1) resolved the extracted material into many UV-absorbing peaks and revealed the presence of multiple forms of immunoactive ACTH-like material and multiple forms of immunoactive α-MSH-like material. The main peak of immunoreactive ACTH (fractions 27–30) was rechromatographed in the presence of 0.13% HFBA to yield two major peaks of immunoreactive-ACTH, which were called CLIP 1 and CLIP 2 (Figure 2), both of which corresponded to UV peaks at both 210 and 278 nm. The fractions containing CLIP 1 and CLIP 2 were collected and rechromatographed under the conditions described in Figure 1. Each peak appeared to be homogeneous, and all subsequent data confirmed the purity of these two forms of immunoreactive ACTH-like material.

Acid hydrolysis of CLIP 1 and CLIP 2, followed by amino acid analysis, gave virtually identical results (Table I) for the two peptides, both of which corresponded very closely to the amino acid analysis previously reported for rat CLIP (Scott et al., 1974). Molecular weight determination by $NaDodSO_4$–polyacrylamide gel electrophoresis gave nearly identical apparent molecular weights (5200 for CLIP 1, 5000 for CLIP 2), which corresponded closely to that previously reported for rat CLIP (Main & Eipper, 1980). In another experiment, a mixture of rat CLIP 1 and CLIP 2 could not be resolved by $NaDodSO_4$–polyacrylamide gel electrophoresis. Only a single band with an apparent molecular weight of 5000 was obtained, both by Coomassie Blue staining and by fractionating the gel, eluting the peptides and assaying for ACTH immunoreactivity. Neither CLIP 1 nor CLIP 2 could be bound to Con A–Se-

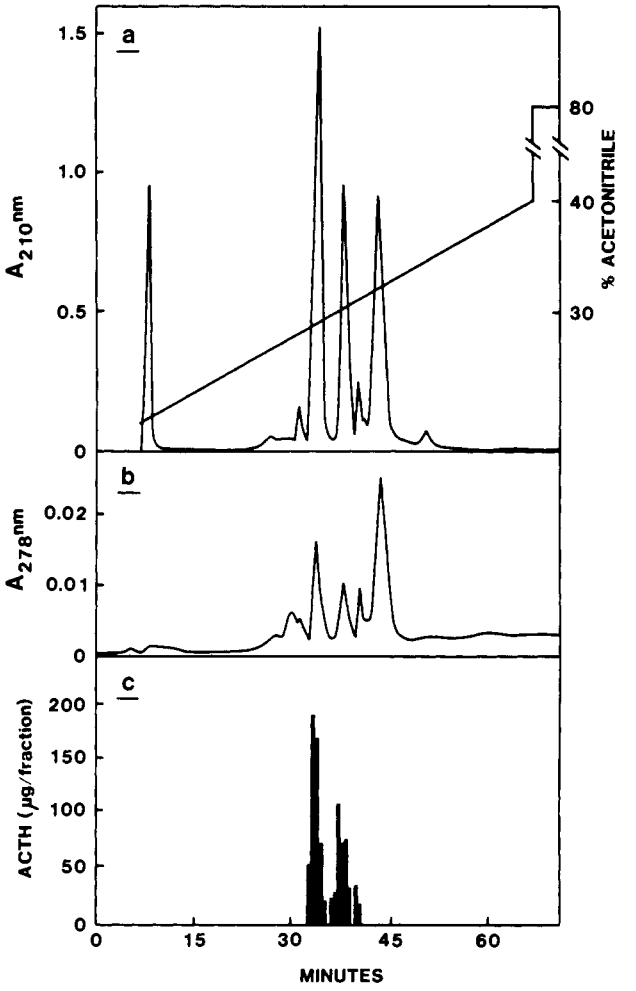

FIGURE 2: Separation of CLIP 1 and CLIP 2. The main immunoreactive ACTH peak in Figure 1c (fractions 27–30) was reloaded into a C_{18} μBondapak column which was then eluted over 1 h at 1.5 mL/min with a linear gradient from 20% (v/v) acetonitrile to 45.6% (v/v) acetonitrile containing 0.13% (v/v) HFBA throughout. The column eluate was monitored at 210 nm (a) and 278 nm (b), and 0.5-min fractions were collected. Aliquots were dried for ACTH (c) and α-MSH radioimmunoassay (not shown). The main peak eluting at 34 min was called CLIP 1, and the second peak eluting at 38 min was called CLIP 2. Both of these peptides eluted much earlier than the two rat $ACTH_{1-39}$ peptides in the HFBA system (Bennett et al., 1981a). A smaller third peak eluting at 40 min was also detected. There was no detectable α-MSH immunoreactivity eluting between 30 and 45 min. The yields for CLIP 1 and CLIP 2 were 580 and 320 μg, respectively.

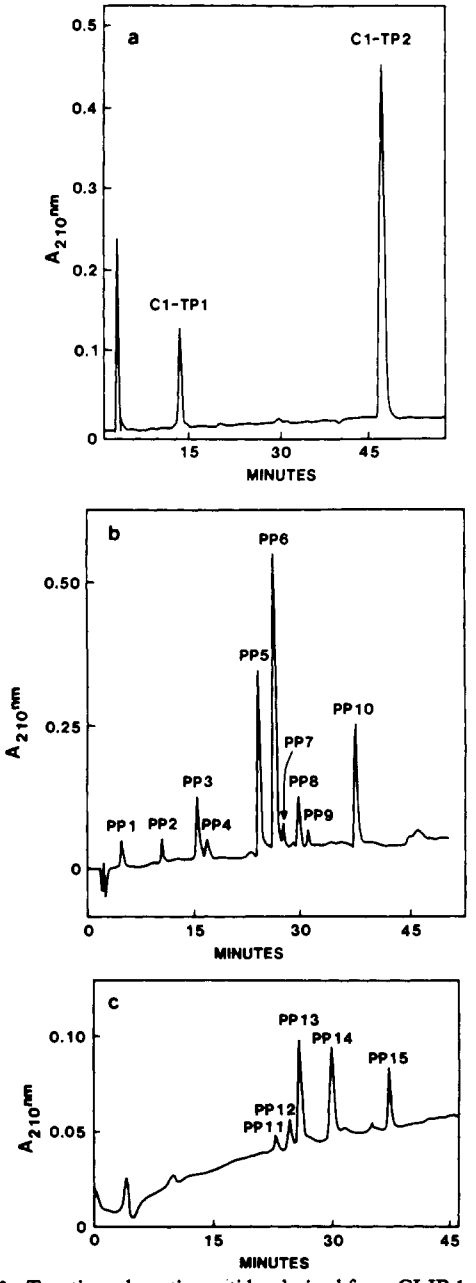

FIGURE 3: Tryptic and peptic peptides derived from CLIP 1 and CLIP 2. CLIP 1 and CLIP 2 were digested with trypsin and the tryptic fragments separated by using a C_{18} μBondapak column which was eluted for 1 h at 1.5 mL/min with a linear gradient of from 1.6% (v/v) acetonitrile to 40% (v/v) acetonitrile containing 0.1% (v/v) F_3CCOOH throughout. (a) The tryptic peptides from CLIP 1 (C_1TP_1 and C_1TP_2) were identified as $ACTH_{18-21}$ and $ACTH_{22-39}$, respectively, by their amino acid analyses (Table I). Corresponding tryptic peptides C_2TP_1 and C_2TP_2 were obtained from CLIP 2 (not shown). C_1TP_1 and C_2TP_1 had identical retention times, but C_1TP_2 eluted 2 min earlier than C_2TP_2. C_1TP_2 and C_2TP_2 were digested with pepsin and the peptic peptides were separated by RP-HPLC using the same conditions as in (a). The peptic peptides derived from C_1TP_2 (b) were called PP_1–PP_{10} inclusive and those derived from C_2TP_2 (c) were called PP_{11}–PP_{15}. Amino acid analyses of these peptides (Table I) has permitted the assignment of tentative sequences (Figures 4 and 5).

pharose columns, suggesting that neither were glycopeptides.

In order to determine the difference between these two forms of rat CLIP, we subjected both peptides to enzymatic digestion. Trypsin cleaved each form of CLIP into two peptides which could be readily separated by RP-HPLC. These two peptides obtained from CLIP 1 were called C_1TP_1 and C_1TP_2 (Figure 3a), and those obtained from CLIP 2 were called C_2TP_1 and C_2TP_2 (data not shown). C_1TP_1 and C_2TP_1 gave identical amino acid analyses (Table I) and had the same retention time by RP-HPLC. They both corresponded to rat $ACTH_{18-21}$. By contrast, C_1TP_2 and C_2TP_2 could be resolved by RP-HPLC, with C_1TP_2 eluting earlier than C_2TP_2 (data not shown). Amino acid analysis of C_1TP_2 (Table I) gave the expected analysis for rat $ACTH_{22-39}$. Amino acid analysis was not performed on C_2TP_2 in order to conserve material for pepsin digestion. Both C_1TP_2 and C_2TP_2 were digested with pepsin, and the resultant peptic peptides were separated by RP-HPLC (Figures 3b,c). The peptic peptides were designated PP_1–PP_{10} for those derived from C_1TP_2 and PP_{11}–PP_{15} for those derived from C_2TP_2. Amino acid analyses were obtained for all of these peptides (Table I). From these analyses, and from the known sequence of CLIP's from other species, it was possible to propose, by homology, a tentative sequence for rat CLIP and to construct peptide maps to account for the observed peptic and tryptic peptides (Figures 4 and 5). For CLIP 1, the three major products of pepsin digestion were PP_6, PP_5, and PP_{10} which corresponded to rat $ACTH_{22-28}$, $ACTH_{29-35}$, and $ACTH_{36-39}$, respectively. For CLIP 2 the major products

Table I: Amino Acid Analyses of Tryptic and Peptic Peptides Obtained from CLIP 1 and CLIP 2

peptide	Asx	Ser[a]	Glx	Pro	Ala	Val	Leu	Tyr	Phe	Lys	Arg	yield (nmol)
CLIP 2	2.2	0.8	4.2	2.8	2.9	3.0	0.8	1.2	1.9	1.0	1.0	
CLIP 1	2.0	0.8	4.1	2.7	2.8	3.0	0.9	1.1	1.8	1.0	1.0	
CLIP 1 tryptic peptides												
C_1TP_1				0.8		1.1				0.9	1.0	
C_1TP_2	2.1	0.6	4.4	2.3	3.0	2.4	0.8	1.1	1.9			
peptic peptides from C_1TP_2												
PP_1	1.0	0.7	1.2		1.0							6.0
PP_2	0.9	0.7	2.0		1.8							3.0
PP_3									1.0			8.0
PP_4			1.1	0.9			1.0					6.5
PP_5	0.7	0.5	2.1		1.9				1.2			12.0
PP_6	1.0		0.9	0.8	0.9	2.2		1.1				18.0
PP_7	0.9			1.1	0	2.0		1.2				2.0
PP_8	1.1		1.3	1.8	1.1	2.6		1.1		1.0	0.9	3.0
PP_9	0.9			2.5		3.4		1.2		1.0	1.0	0.5
PP_{10}			1.0	1.1			1.0		1.0			10.0
CLIP 2 tryptic peptides												
C_2TP_1				1.1		1.0				1.0	1.0	
peptic peptides from C_2TP_2												
PP_{11}			1.0		1.0				1.2			0.3
PP_{12}		0.9	1.1		2.0				1.3			0.4
PP_{13}	2.0	0.5	2.4	1.0	2.1	2.0		1.2	0.6			1.0
PP_{14}	1.7		1.6	1.7	1.0	3.0		1.2		1.1	1.1	0.9
PP_{15}			1.0	1.0			1.0		1.2			1.2

[a] Values for serine were not corrected for breakdown during acid hydrolysis.

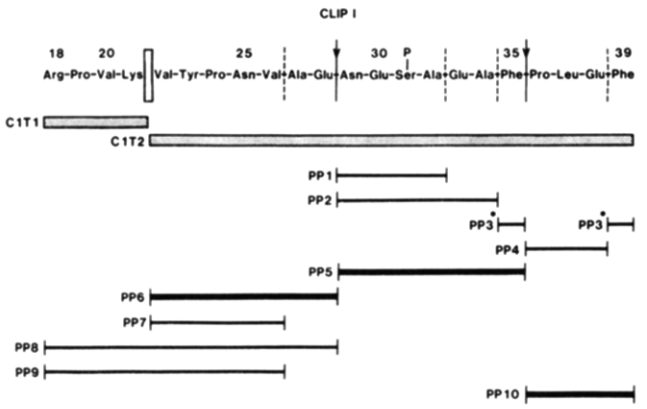

FIGURE 4: Tryptic and peptic peptide map for CLIP 1. From the amino acid compositions (Table I) of the tryptic and peptic peptides (Figure 3a,b) derived from CLIP 1 and from a consideration of the homology found in the 18–39 region of ACTH from a variety of species (Scott et al., 1976), a pattern for the enzymatic digestion of CLIP has been constructed. The tryptic cleavage site is indicated by the stippled bar, the major peptic cleavage sites are indicated by solid lines, and the minor peptic cleavage sites are indicated by dashed lines. PP_5, PP_6, and PP_{10} are designated as major products based on their yield (Table I). PP_3* is the single amino acid phenylalanine which is derived in part from Phe_{35} and in part from Phe_{39}. PP_8 and PP_9 are produced from a small amount of residual CLIP 1 in C_1TP_2 which is present due to incomplete trypsin digestion.

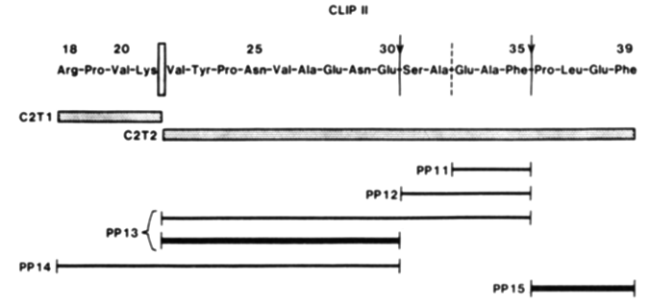

FIGURE 5: Tryptic and peptic peptide map for CLIP 2. The tryptic cleavage site is indicated by the stippled bar, the major peptic cleavage sites are indicated by solid lines, and the minor peptic cleavage sites are indicated by dashed lines. From the amino acid analyses in Table I, PP_{13} is apparently a mixture of $ACTH_{22-35}$ and $ACTH_{22-30}$. PP_{14} is produced from a residual amount of CLIP 2 in C_2TP_2 which is present due to incomplete trypsin digestion. PP_{15} is identical with PP_{10} (Figure 4) and represents the only peptide which was produced from both CLIP 1 and CLIP 2. Note that the main site of pepsin cleavage of C_1TP_2 at residues 28 and 29 (Figure 4) has not been cleaved in C_2TP_2. The bond between positions 30 and 31 (this figure) has apparently been cleaved instead.

of pepsin digestion were PP_{13} and PP_{15}. PP_{13} was a mixture of rat $ACTH_{22-30}$ and $ACTH_{22-35}$, and PP_{15} corresponded to rat $ACTH_{36-39}$. Thus PP_{10} and PP_{15} were identical both in amino acid analysis and elution position and represented pepsin cleavage of C_1TP_2 and C_2TP_2 at the same bond (Phe_{35}–Pro_{36}). However, the other major pepsin cleavage site was clearly different in the two peptides, being at Glu_{28}–Asn_{29} in C_1TP_2 and at Glu_{30}–Ser_{31} in C_2TP_2. Sufficient quantities of PP_5 and PP_6 were obtained to enable the further characterization of these two peptides. The results obtained from carboxypeptidase Y digestion of PP_6 for 24 h supported the presence of asparagine at position 25 and glutamic acid at position 28. Results obtained from leucine aminopeptidase digestion of PP_5 supported the presence of asparagine at position 29 and glutamic acid at positions 30 and 33. Some of the minor peptic peptides, such as PP_8 and PP_9, were clearly derived from pepsin digestion of some residual intact CLIP which had not been completely digested by the trypsin treatment and which was not resolved from C_1TP_2 by the chromatographic conditions used in Figure 3a.

The results of total enzymatic digestion of CLIP 1 and CLIP 2 (Table II) showed only two major differences. These were the low value for serine obtained for CLIP 1 and the unexpected appearance of a novel peak which corresponded exactly to the elution position of *O*-phosphoserine. Both CLIP 1 and CLIP 2 appeared to contain no aspartic acid or glutamine, confirming the results of the exopeptidase digestions of PP_5 and PP_6. The overall pattern of digestion was comparable to that obtained for bovine CLIP, which was used as a standard to optimize the digestion conditions. We conclude that rat CLIP 2 is underivatized rat $ACTH_{18-39}$, with a serine at position 31, and that rat CLIP 1 is rat $ACTH_{18-39}$, with a

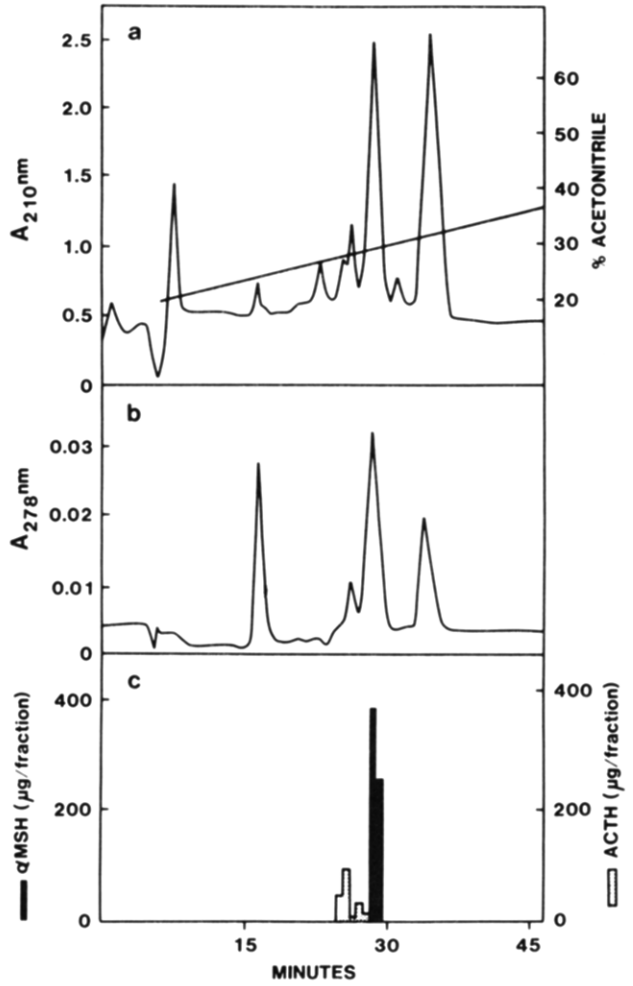

FIGURE 6: Purification of the main immunoreactive α-MSH peak from Figure 1. The main immunoreactive α-MSH peak (fractions 22 and 23 in Figure 1) was rechromatographed on the same RP-HPLC column by using a linear gradient over 1 h at 1.5 mL/min of from 20% (v/v) acetonitrile to 40% (v/v) acetonitrile containing 0.13% HFBA throughout. The column eluate (1-mL fractions) was monitored for UV absorbance at 210 nm (a) and 278 nm (b) and for α-MSH and ACTH immunoreactivity (c). The MSH immunoreactivity eluted as a single peak at 29 min (henceforth designated α-MSH 1) and was free of ACTH immunoreactivity. The yield was 650 μg of MSH 1.

phosphoserine residue at position 31. This is consistent with the finding that CLIP 1 eluted before CLIP 2 in the HFBA system (Figure 2), which tends to separate peptides by the charge differences between them (Bennett et al., 1980b), whereas the two CLIP's are less well resolved in the F_3CCO-OH system which tends to separate peptides more on the basis of differences in hydrophobicity. Furthermore, the different patterns of peptic digestion of CLIP 1 and CLIP 2 can be explained by the prevention of the cleavage of the Glu_{30}–Ser_{31} bond in CLIP 1 by the presence of phosphoserine at position 31. The Glu_{28}–Asn_{29} bond is then cleaved as the most labile alternative site.

Isolation and Characterization of α-MSH from Rat Pituitary Neurointermediary Lobe. The chromatogram in Figure 1 demonstrated the presence of multiple forms of immunoreactive α-MSH in the rat posterior pituitary. The main peak (fractions 22 and 23) had eluted later than standard mammalian synthetic α-MSH which corresponded very closely in elution position to the smaller peak at fractions 20 and 21. The main immunoreactive α-MSH peak (fractions 22 and 23) (α-MSH 1) was further purified by RP-HPLC in the presence of 0.13% HFBA (Figure 6) where it now was resolved from some immunoreactive ACTH, and from several other UV peaks. The smaller immunoreactive α-MSH peak in Figure 1 (fractions 20 and 21) (α-MSH 2) was also further purified by a second RP-HPLC step using a solvent system containing 0.13% HFBA (Figure 7). Both of these forms of α-MSH were now homogeneous in all further RP-HPLC systems. The amino acid analyses of these two peptides (Table III) were

Table II: Amino Acid Analyses of Total Enzymatic Digestions of Rat CLIP 1 and CLIP 2 and Bovine CLIP

	acid hydrolysis of rat CLIP's[a]	rat CLIP 1	rat CLIP 2	bovine CLIP	bovine CLIP theoretical analysis
P-Ser[b]		1.2	0.0	0.0	0
Asp	2.1	0.0	0.0	1.0	1
Thr	0.0	0.0	0.0	0.3	0
Ser	0.8	0.2	1.3	1.2	1
Asn[c]		1.0	1.2	0.5	1
Glu	4.2	2.4	2.8	2.8	3
Gln		0.0	0.0	1.2	1
Gly	0.0	0.0	0.1	1.1	1
Ala	2.9	2.9	3.0	3.0	3
Val	3.9	3.1	2.7	2.1	2
Ile	0.0	0.0	0.0	0.1	0
Leu	0.9	1.1	1.0	1.3	1
Tyr[c]	1.2	0.1	0.1	0.2	1
Phe[c]	1.9	1.3	1.2	1.2	2

[a] The acid hydrolysis of rat CLIP represents the average of the analyses shown for rat CLIP 1 and CLIP 2 in Table I. [b] P-Ser, phosphoserine. [c] For all the CLIP's, recoveries of Asn, Phe, and Tyr were low for the total enzymatic digestions, because the regions Glu_{38}-Phe_{39} and Tyr_{23}-Pro_{24}-Asn_{25} are digested rather poorly by this method.

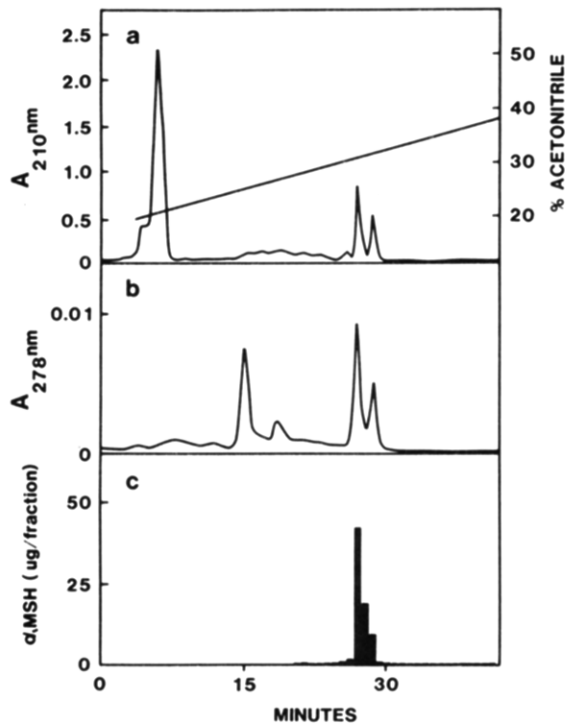

FIGURE 7: Purification of the minor immunoreactive α-MSH peak from Figure 1. The peak of α-MSH immunoreactivity which had the same retention time as synthetic α-MSH in Figure 1 (fractions 20 and 21) was rechromatographed by using the same conditions as described in Figure 6. The eluate was monitored for UV absorbance at 210 nm (a) and 278 nm (b), for α-MSH immunoreactivity (c), and for ACTH immunoreactivity (not shown). The main peak was eluted at 26 min and was nearly completely resolved from a smaller UV-absorbing peak which eluted at 29 min. The amount of immunoreactive α-MSH in this peak (α-MSH 2) was 70 μg.

Table III: Amino Acid Analyses of α-MSH 1 and α-MSH 2

	isolated α-MSH 1	isolated α-MSH 2	synthetic α-MSH	theoretical ratios
Asx	0.0	0.0	0.0	0
Thr	0.0	0.0	0.0	0
Ser[a]	1.8	1.9	1.7	2
Glx	1.2	1.0	1.2	1
Pro	1.1	1.0	1.1	1
Gly	1.5	0.9	1.1	1
Ala	0.0	0.0	0.0	0
Val	0.9	0.9	1.1	1
Met	1.1	1.2	1.0	1
Ile	0.0	0.0	0.0	0
Leu	0.0	0.0	0.0	0
Tyr	0.8	1.0	1.0	1
Phe	0.9	0.9	1.0	1
His	1.1	1.0	1.0	1
Lys	1.0	1.0	1.1	1
Arg	1.1	1.0	1.0	1
Trp[b]	nd	nd	nd	1

[a] Serine values have not been corrected for breakdown during acid hydrolysis. [b] Tryptophan was not determined, but its presence was indicated by the ratio of the UV absorption at 278 nm to that at 210 nm.

nearly identical and corresponded very closely to that obtained for synthetic α-MSH_{1-13}. The smaller peak, α-MSH 2, was indistinguishable from synthetic α-MSH_{1-13} in all chromatographic systems employed, whereas the main peak, α-MSH 1, consistently had a retention time longer than that of synthetic α-MSH. α-MSH 2 was therefore considered to be identical with synthetic α-MSH (*N*-acetylcorticotropin$_{1-13}$ valinamide).

So that α-MSH 1 could be characterized more fully, it was digested with trypsin and the products were compared to those produced from tryptic digests of synthetic α-MSH and synthetic human $ACTH_{1-18}$ amide by RP-HPLC (Figure 8). The carboxyl-terminal tryptic peptides (α-MSH_{9-13}) from synthetic α-MSH and from α-MSH 1 (TP_1) had identical retention times, whereas the amino-terminal tryptic peptides (α-MSH_{1-8}) (TP_2 and TP_3) had clearly different retention times (Figure 8a,b). There was, however, a small peak obtained from the tryptic digestion of α-MSH 1 (TP_2), which corresponded to the elution position of synthetic α-MSH_{1-8}. However, neither this peak nor the main amino-terminal tryptic peptide derived from α-MSH 1 corresponded to the amino-terminal tryptic peptide derived from $ACTH_{1-18}$ amide (i.e., desacetyl-α-MSH_{1-8}) (Figure 8c). The peptides obtained from α-MSH 1 which had been digested with chymotrypsin were compared to those obtained from synthetic α-MSH similarly digested with chymotrypsin (Figure 9). The peptides obtained from both digests were identified by amino acid analysis, enabling the construction of chymotryptic peptide maps of synthetic α-MSH and α-MSH 1 (Figure 10). From these it was clear that the difference between synthetic α-MSH and α-MSH 1 is in the near absence of CP_2 from α-MSH 1 and the appearance of a novel peak (CP_3). Both of these peptides (CP_2 and CP_3) contained only serine and tyrosine and were clearly the first two amino acids in the α-MSH sequence. The difference between the relative amounts of CP_4 and CP_5 in the digests of synthetic α-MSH and α-MSH 1 simply reflects the fact that the Trp_9–Gly_{10} bond is the bond least susceptible to chymotrypsin within the α-MSH sequence. Thus, α-MSH 1 differs from synthetic α-MSH by some modification of the amino-terminal Ser–Tyr.

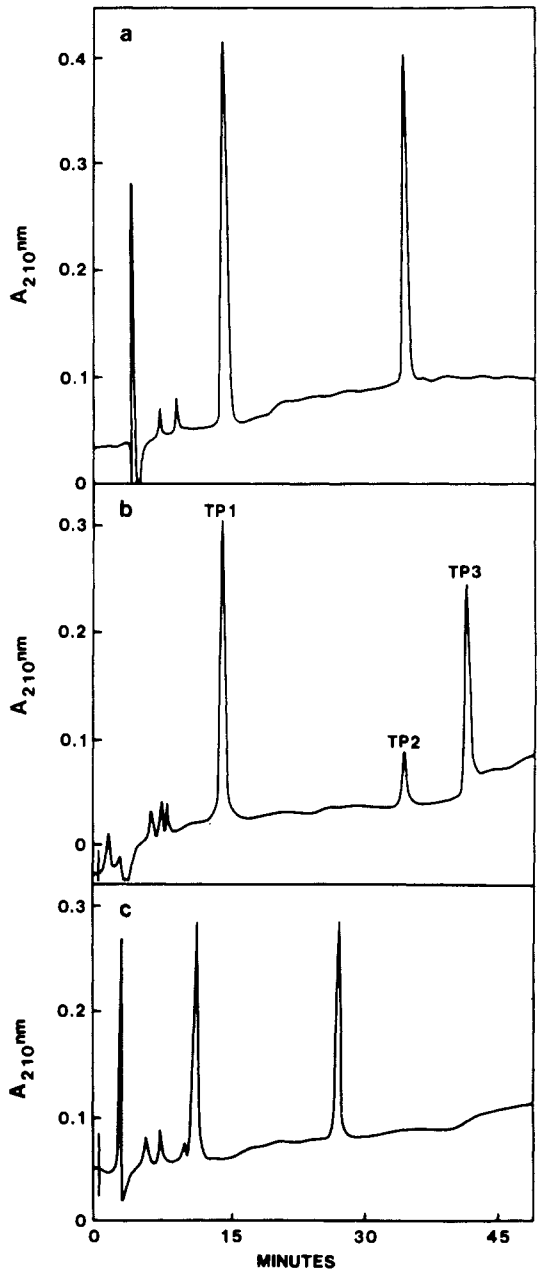

FIGURE 8: Tryptic peptides derived from synthetic α-MSH, rat α-MSH 1, and synthetic $ACTH_{1-18}$ amide. Peptides produced by the digestion of (a) synthetic α-MSH, (b) rat α-MSH 1, and (c) synthetic $ACTH_{1-18}$ amide with trypsin were separated by using a C_{18} μBondapak column which was eluted at 1.5 mL/min for 1 h with a linear gradient of from 12% (v/v) acetonitrile to 26.4% (v/v) acetonitrile containing 0.1% (v/v) F_3CCOOH throughout. The peaks were identified on the basis of their amino acid analyses (not shown). TP_1 corresponded to α-MSH_{9-13}, while both TP_2 and TP_3 corresponded to α-MSH_{1-8}. The peak in (c) eluting at 12 min corresponded to $ACTH_{9-18}$ and that eluting at 27 min corresponded to $ACTH_{1-8}$ (i.e., desacetyl-α-MSH_{1-8}). The two main peaks in (a) had retention times and amino acid analyses identical with those of TP_1 and TP_2.

During the course of this work, the isolation of *N*,*O*-diacetylserine$_1$-α-MSH (Rudman et al., 1979) was reported. This compound could readily be converted to monoacetyl-(*N*-acetyl) α-MSH by treatment with base. When α-MSH 1 was incubated at 37 °C with 0.01 N NaOH for 1 h, it was completely converted to a molecule which was chromatographically indistinguishable from synthetic mono-*N*-acetyl-α-MSH_{1-13} (Table IV). These data strongly suggest that α-MSH 1 is *N*,*O*-diacetylserine$_1$-α-MSH. This is consistent with the finding that α-MSH 1 and α-MSH 2 are resolved in the F_3CCOOH system (Figure 1), which tends to separate

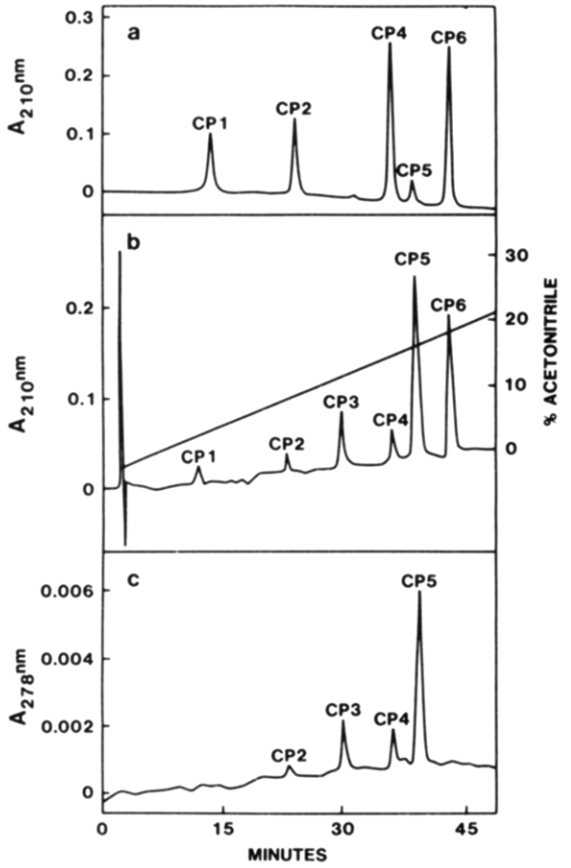

FIGURE 9: Chymotryptic peptides derived from synthetic α-MSH and rat α-MSH 1. Peptides produced from the digestion of synthetic α-MSH and rat α-MSH 1 by chymotrypsin were separated on a C_{18} μBondapak column which was eluted at 1.5 mL/min for 1 h with a linear gradient of from 1.6% (v/v) acetonitrile to 24% (v/v) acetonitrile containing 0.1% (v/v) F_3CCOOH throughout. From the amino acid analyses (not shown), the chymotryptic peptides obtained from synthetic MSH were identified as follows: CP_1, α-MSH_{10-13}; CP_2, α-MSH_{1-2}; CP_4, α-MSH_{8-9}; CP_5, α-MSH_{8-13}; CP_6, α-MSH_{3-7}. All the peptides found in (a) for synthetic α-MSH were also found in (b) for rat α-MSH 1. In addition, a novel peptide, CP_3, was found which corresponded in amino acid analysis to α-MSH_{1-2}. The RP-HPLC of the rat α-MSH 1 digest was also monitored at 278 nm (c) where only CP_2–CP_5 were detected (CP_1 and CP_6 contain no tyrosine or tryptophan).

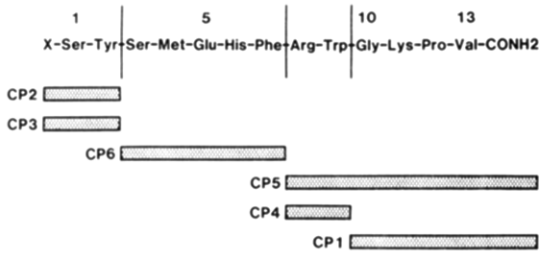

FIGURE 10: Chymotryptic peptide map for authentic α-MSH and rat α-MSH 1. The chymotryptic cleavage sites are marked by a solid line. From the chromatograms in Figure 9, CP_3 is the only novel chymotryptic peptide produced from α-MSH 1 which is not present in synthetic α-MSH. From other data described in the text, and in Table IV, the most likely assignment is that CP_2 represents *N*-acetylseryl$_1$tyrosine$_2$ and that CP_3 represents *N*,*O*-diacetylseryl$_1$tyrosine$_2$.

peptides on the basis of differences in hydrophobicity, and that α-MSH 1 and α-MSH 2 are less well resolved in the HFBA system, which tends to separate peptides more on a basis of charge differences (Bennett et al., 1980b). It is also supported by the finding that the amino terminal tryptic peptides from $ACTH_{1-18}$ amide, synthetic α-MSH, and α-MSH 1 were

Table IV: Conversion of α-MSH 1 to Mono-*N*-acetyl-α-MSH [a]

	retention time (min)
synthetic α-MSH before base treatment	25.8
α-MSH 1 before base treatment	30.8
synthetic α-MSH after base treatment	26.0
α-MSH 1 after base treatment	26.0

[a] Synthetic α-MSH and isolated rat α-MSH 1 were chromatographed by RP-HPLC for 1 h using a linear gradient of from 20% to 32% (v/v) acetonitrile containing 0.1% (v/v) F_3CCOOH throughout, both before and after incubation in 0.01 N NaOH for 1 h at 37 °C.

eluted progressively later from the RP-HPLC column (Figure 8). Thus they represent desacetyl-α-MSH_{1-8}, *N*-acetyl-α-MSH_{1-8} and *N*,*O*-diacetyl-α-MSH_{1-8}, respectively.

Discussion

The work described in this paper demonstrates the versatility and resolving power of our novel RP-HPLC method for the isolation and purification of peptides from biological tissues. The two main forms of immunoreactive ACTH found in the rat neurointermediary pituitary were underivatized CLIP and *O*-phosphoserine$_{31}$-CLIP, with the phosphorylated form predominating by a 2 to 1 ratio. The two main forms of immunoreactive α-MSH found were *N*-acetylserine$_1$-α-MSH and *N*,*O*-diacetylserine$_1$-α-MSH, with the diacetyl form predominating by a 9 to 1 ratio as determined by RIA and amino acid analysis. All four of these peptides were obtained in virtually homogenous forms after only two RP-HPLC steps. Furthermore, RP-HPLC alone has been utilized in the characterization of these peptides, to provide a quick, reliable, and nondestructive method of obtaining peptic, tryptic, and chymotryptic profiles of the isolated peptides. Although rat CLIP has been isolated previously (Scott et al., 1974), it was only identified by amino acid analysis and by ACTH immunoactivity. In this study, we can confirm the amino acid analysis of rat CLIP (Table I) and that it differs in composition from human $ACTH_{18-39}$ by the substitution of a valine for a glycine. From the analyses of the tryptic and peptic peptides obtained from both forms of rat CLIP, a tentative sequence for rat CLIP can be assigned (Figures 4 and 5). The notable features of the sequence are the valine at position 26, asparagine at 25 and 29, and serine at 31.

The sequence Asn-Glu-Ser at 29–31 of rat CLIP is of great importance, since it represents a typical sequence of the Asn-X-Ser or Asn-X-Thr type which are recognition sites for the N-glycosylation of asparagine residues (Jackson & Hirs, 1970). The occurrence of glycosylated CLIP in the rat pituitary has been reported (Mains & Eipper, 1980). The site of attachment of carbohydrate for mouse ACTH has been shown to be an asparagine residue situated between residues 22 and 39 (Eipper & Mains, 1977). The biosynthesis of α-MSH and CLIP from the ACTH–β-LPH precursor has been shown to occur in the neurointermediary lobe of the pituitary, whereas in the anterior lobe, the same precursor is processed only as far as $ACTH_{1-39}$ (Eipper & Mains, 1980). Clearly, there are different mechanisms acting in the two lobes of the pituitary to produce different end products from the same precursor. However it has also been shown that the glycosylated forms of $ACTH_{1-39}$ and CLIP are not the precursors of the nonglycosylated forms (Mains & Eipper, 1979; Roberts et al., 1978). Both the 20–22K ACTH biosynthetic intermediate and the 31K precursor exist in forms which are either glycosylated or nonglycosylated in the ACTH sequence,

presumably at the asparagine 31. The reason why some of these molecules are glycosylated and others are not remains unclear.

The discovery of *O*-phosphoserine$_{31}$-CLIP provides a possible insight into the regulation of glycosylation of CLIP and ACTH. The phosphorylation of serine$_{31}$ may be a post-translational modification which serves to mask the recognition site for the glycosylation of asparagine$_{29}$. Once the serine is phosphorylated, glycosylation of the asparagine either may never occur or may only occur after dephosphorylation. Only phosphorylation of the serine$_{31}$ in the 31K precursor should have any effect on the regulation of the production of glycosylated or nonglycosylated end products. Preliminary experiments on the incorporation of ^{32}P-labeled inorganic phosphate into rat neurointermediary pituitary explants confirmed the presence of phosphorylated and nonphosphorylated CLIP and indicated the presence of the putative phosphorylated precursors, ACTH$_{1-39}$ and the 31K, ACTH-β-LPH precursor (Bennett et al., 1981b).

The identification of the main form of α-MSH as diacetyl-α-MSH confirms the report by Rudman et al. (1979) on the existence of this peptide. In this previous work, the *N*-acetyl-α-MSH and the *N*,*O*-diacetyl-α-MSH were found to be present in approximately equal amounts (Rudman et al., 1979). In contrast, we have found that the diacetyl form of α-MSH predominates over the mono *N*-acetyl form by a ratio of 9 to 1. The combination of the very rapid extraction of fresh tissue with a fast and simple isolation procedure in our study might be one reason for this apparent discrepancy. Whether the diacetyl-α-MSH is a precursor of monoacetyl-α-MSH or vice versa remains to be demonstrated. Since the diacetyl form is the predominant form in the rat neurointermediary lobe, it could represent the main storage form, and the conversion from the diacetyl-α-MSH to monoacetyl-α-MSH could therefore be an important step in the regulation of the secretion of α-MSH.

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