Proc. Natl. Acad. Sci. USA
Vol. 76, No. 10, pp. 5085–5089, October 1979
Biochemistry

Concomitant synthesis of β-endorphin and α-melanotropin from two forms of pro-opiomelanocortin in the rat pars intermedia

(tunicamycin/β-lipotropin)

P. CRINE, F. GOSSARD, N. G. SEIDAH, L. BLANCHETTE, M. LIS, AND M. CHRÉTIEN

Protein and Pituitary Hormone Laboratory, Clinical Research Institute of Montreal, 110 Pine Avenue West, Montreal H2W 1R7, Canada

Communicated by Elwood V. Jensen, July 30, 1979

ABSTRACT **In the pars intermedia of rat pituitary glands, two forms of a common precursor for corticotropin (ACTH) and β-lipotropin with apparent molecular weights of 34,000 and 36,000 were resolved by sodium dodecyl sulfate/acrylamide gradient slab gel electrophoresis. High-performance liquid chromatographic analysis of [^{35}S]methionine-labeled tryptic fragments of the two forms of the precursor revealed that both contained copies of ACTH-(1–8) and β-lipotropin-(61–69) sequences. When biosynthetic studies were performed in the presence of tunicamycin, the 34,000- and 36,000-dalton forms were replaced by a peptide with an apparent molecular weight of 32,000. It was therefore concluded that the 34,000- and 36,000-dalton forms of the precursor represent two glycoprotein variants of similar polypeptides, differing in the number of asparagine-linked carbohydrate moieties. During pulse–chase incubations with [^{35}S]methionine, the precursor forms were cleaved into two major groups of labeled products: (*i*) β-endorphin and (*ii*) a mixture of ACTH fragments closely related to α-melanotropin. No ACTH-(1–39) was found at the end of a 2-hr chase period, suggesting that ACTH is not a significant hormone product of the rat pars intermedia.**

The pituitary hormones β-lipotropin (β-LPH) and corticotropin (ACTH) belong to a family of peptides derived from a common precursor hormone in At-20 tumors (1–3) and in the rat pars intermedia (4). The complete amino acid sequence for this protein in beef has recently been established (5). Depending on the precise cleavage pattern, this precursor molecule may serve as a source of different groups of peptides with different biological functions. There is now substantial evidence to suggest that the maturation of the precursor hormone in the pars distalis of the pituitary differs from that in the pars intermedia. (6–9). Similarly, it has been proposed that qualitative changes in the expression of the peptide "family tree" may occur during fetal development and could thus be responsible for the alteration in fetal adrenal function that precedes birth (10).

Extensive studies on the biosynthetic pathway of ACTH and β-endorphin have been performed with a mouse pituitary tumor cell line (6). This model has been claimed to provide a fairly accurate picture of the processing events occurring in normal corticotropic cells of the pars distalis (6). However, little is known about the exact mechanism prevailing in the pars intermedia. Previous studies on rat pars intermedia cells have shown that in this tissue β-endorphin is the major opioid peptide resulting from the maturation of a 30,000-dalton precursor containing antigenic determinants for both β-LPH and ACTH (4). In this process β-LPH appears to be an obligatory short-lived intermediate. We now report that the same maturation process of the precursor yields another major end product which has been identified as α-melanotropin (α-MSH).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

MATERIALS AND METHODS

Incorporation of Labeled Amino Acids into Rat Pars Intermedia Cells. Isolated pars intermedia cells from male Sprague–Dawley rats were prepared as described (4). Cells were incubated in Krebs–Ringer buffer containing 25 mM sodium bicarbonate, 2 g of glucose per liter, and 1 g of bovine serum albumin (Sigma, fraction V) per liter. For pulse-labeling studies the medium was supplemented with either [^{3}H]phenylalanine (110 Ci/mmol, 0.5 mCi/ml; 1 Ci = 3.7×10^{10} becquerels), [^{3}H]leucine (120 Ci/mmol; 0.5 mCi/ml), or [^{35}S]methionine (636 Ci/mmol; 12.5 mCi/ml). All the radioactive amino acids were from New England Nuclear. For chase incubations, the incubation medium containing the radioactive amino acid was replaced by buffer supplemented with the corresponding unlabeled amino acid (2 mM final concentration).

In some experiments, whole neurointermediate lobes (pars nervosa and pars intermedia) were incubated directly in the wells of a microtest plate (NUNC Laboratories) containing 50 μl of incubation medium.

Polyacrylamide Gel Electrophoresis of Radiolabeled Proteins. After incubation the isolated cells were collected by centrifugation and washed, and the proteins were extracted in 5 M acetic acid containing bovine serum albumin and protease inhibitors as described (4). The desalted extracts were analyzed by sodium dodecyl sulfate ($NaDodSO_4$) disc gel electrophoresis (4). For slab gel electrophoresis, the 5 M acetic acid cell extract prepared without added bovine serum albumin was lyophylized to dryness and resuspended in 25 μl of 0.0625 M Tris·HCl, pH 6.8/2% $NaDodSO_4$/5% 2-mercaptoethanol (11) containing 2 mmol of the same unlabeled amino acid per liter as the radioactive one used during the incubation. The samples were boiled for 2 min just prior to electrophoresis. In the experiments in which whole neurointermediate lobes were used, the tissue pieces were washed with cold saline at the end of the incubation and immediately boiled for 2 min in 25 μl of the electrophoresis sample buffer containing the appropriate unlabeled amino acid (2 mM) and phenylmethylsulfonyl fluoride (Sigma) (2 mM) to inhibit protease activity.

The radioactive proteins were resolved by slab gel electrophoresis on a 10–30% acrylamide gradient prepared in the discontinuous buffer system of Laemmli (11). [^{35}S]Methionine-labeled proteins were detected by autoradiography of the slab gels. For tritiated peptides fluorography of the gels was performed according to Bonner and Laskey (12) with preexposed Kodak x-ray films (13).

Peptide Mapping of Tryptic Digests. The radioactive protein bands separated by disc or slab gel electrophoresis were

Abbreviations: ACTH, corticotropin (adrenocorticotropin); MSH, melanotropin; LPH, lipotropin; $NaDodSO_4$, sodium dodecyl sulfate; HPLC, high-performance liquid chromatography.

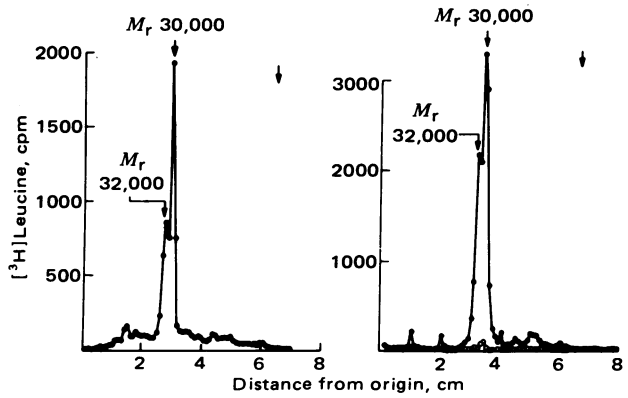

FIG. 1. $NaDodSO_4$ disc gel electrophoresis of [^{3}H]leucine-labeled peptides synthesized by rat intermediate lobe cells during a 20-min incubation. The position of the bromophenol blue tracking dye is marked (arrows). The conditions of electrophoresis are the same as described (4) except for the length of the gels (10 cm) and the thickness of the slices (1 mm). (*Left*) Total extracted peptides obtained directly after desalting on Sephadex G-25. (*Right*) Immunoprecipitates of extracted peptides with ACTH antiserum according to ref. 4. ●, No ACTH added; ○, excess purified ACTH added before immunoprecipitation.

cut out of the gels and washed extensively in 25% isopropanol and then in 10% methanol to remove $NaDodSO_4$. The gel pieces were then dried by lyophilization and 0.5–1 ml of 0.1 M NH_4HCO_3 (pH 8.0), containing 50 μg of bovine serum albumin and 5 μg of diphenylcarbamyl chloride-treated trypsin (Sigma), was added. Tryptic digestion was performed at 37°C for 16 hr. The solution was removed and lyophylized. Recovery of the radioactivity from the gels was always better than 90%. Tryptic fragments eluted from the gels were oxidized by performic acid for 2.5 hr at −5°C as described by Hirs (14).

For two-dimensional peptide mapping the peptides were spotted on a cellulose thin-layer plate together with 10 μg of β-LPH-(61–69) (generous gift from R. Guillemin) and a tryptic digest of 50 μg of ACTH-(1–10) (Ciba–Geigy). These standard peptides had also been oxidized by performic acid. The samples were subjected to electrophoresis in the first dimension for 1.5 hr at 1000 V in formic acid/acetic acid/water (2:8:90) at 4°C. Chromatography in the second dimension was performed in the organic phase of butanol/acetic acid/water (20:50:25).

Small peptides from chase incubations or tryptic fragments of labeled proteins were also extracted from the gel slices as described above and analyzed by high-performance liquid chromatography (HPLC). The recovery of radioactive material from HPLC columns was always better than 80%.

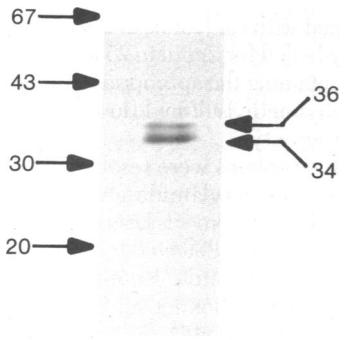

FIG. 2. $NaDodSO_4$ slab gel electrophoretic analysis of [^{3}H]phenylalanine-labeled peptides synthesized by rat intermediate lobe cells during a 20-min incubation. The positions of marker proteins are shown on the left-hand scale in daltons × 10^{-3}: albumin (67); ovalbumin (43); carbonic anhydrase (30). The molecular weight of the two radioactive bands detected by fluorography has been calculated by comparing their mobilities with those of the standard proteins.

RESULTS

Identification and Partial Characterization of Two Precursors for ACTH and β-LPH in the Rat Pars Intermedia. When dispersed rat intermediate lobe cells were incubated for 20 min with [^{3}H]leucine, two major labeled peptides were resolved by disc gel electrophoresis (Fig. 1 *left*). They had apparent molecular weights of 30,000 and 32,000. These two peptides could be specifically immunoprecipitated with antisera against either ACTH (Fig. 1 *right*) or β-MSH (data not shown). When proteins extracted from intermediate lobe cells pulsed for 20 min with [^{3}H]phenylalanine were analyzed by $NaDodSO_4$ slab gel electrophoresis on a 10–30% acrylamide gradient, the labeled peptides were clearly resolved into two major species of 34,000 and 36,000 daltons (Fig. 2).*

Tryptic fragments from a pool of the 30,000- and 32,000-dalton proteins labeled with [^{35}S]methionine and purified by disc gel electrophoresis were analyzed by mapping on a cellulose thin-layer plate. Autoradiography of the plate revealed two major spots (Fig. 3). One comigrated with standard ACTH-(1–8), which is the only methionine-containing tryptic peptide of ACTH. The other spot comigrated with tryptic fragment 61–69 of β-LPH, which contains a methionine residue in position 65 (15). In the rat, the β-MSH sequence does not contain any methionine (15). Therefore, sequence 61–69 is the only tryptic fragment of β-LPH expected to be labeled with [^{35}S]-methionine.

The 34,000- and 36,000-dalton precursor forms, labeled with [^{35}S]methionine, were resolved by slab gel electrophoresis, the

* These molecular weight values are given for identification purposes only. Accurate molecular weights of glycoproteins cannot be obtained by $NaDodSO_4$/polyacrylamide gel electrophoresis. Moreover, the apparent molecular weights computed vary with the electrophoresis procedure used.

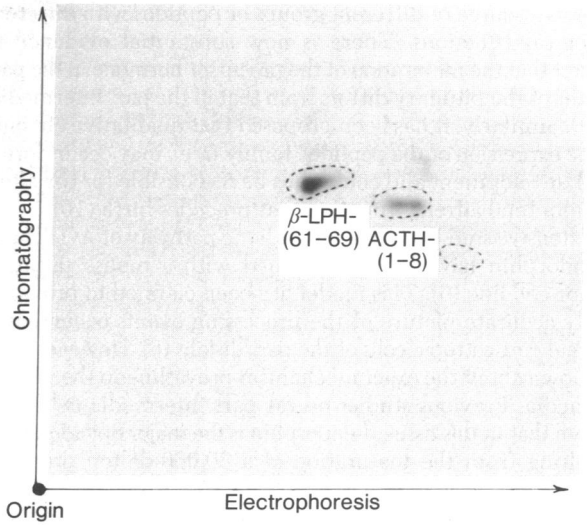

FIG. 3. Two-dimensional tryptic mapping of a pool of 30,000- and 32,000-dalton precursors labeled with methionine that were synthesized during a 20-min incubation and purified by $NaDodSO_4$ disc gel electrophoresis. The radioactive peptides (35,000 cpm) were mixed with 10 μg of β-LPH-(61–69) and 75 μg of a tryptic digest of ACTH-(1–10) and oxidized with performic acid just prior to mapping on the thin-layer cellulose plate. Standard peptides (circled spots) were developed with a cadmium ninhydrin reagent and radioactive peptides were visualized by autoradiography after a 2-week exposure.

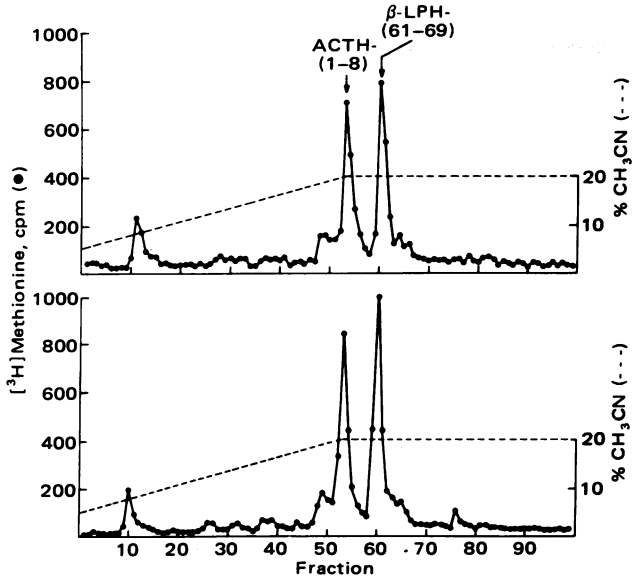

FIG. 4. HPLC elution profile of oxidized tryptic fragments generated from [^{35}S]methionine-labeled precursor forms. The two forms of the precursor [36,000 daltons (*Upper*) and 34,000 daltons (*Lower*)] had been separated by NaDodSO$_4$ slab gel electrophoresis. The radioactive peptides were analyzed together with 10 μg of synthetic β-LPH-(61–69) and 10 μg of synthetic ACTH(1–8). The synthetic peptides had also been oxidized prior to HPLC analysis.

bands were digested with trypsin, and the tryptic fragments were analyzed separately by HPLC. This procedure revealed that tryptic digests of both precursor forms contained the two tryptic fragments ACTH-(1–8) and β-LPH-(61–69) (Fig. 4).

The common precursor for ACTH and β-LPH exists in at least three different glycoprotein forms in AtT-20 cells (16). Analysis of labeled tryptic peptides suggested that the peptide backbone is similar in all forms of the precursor, and the differences in apparent molecular weights are likely to be due to different amounts of carbohydrate.

To test this hypothesis for the precursors from the pars intermedia, we preincubated the cells for 6 hr in a medium containing tunicamycin (5 μg/ml) (generous gift from G. Tamura, University of Tokyo) before adding the radioactive amino acid. Tunicamycin inhibits glycosylation of asparagine residues in glycoproteins (17). Fig. 5 shows that the apparent molecular weight of the most abundant form of the precursor extracted after a 20-min pulse performed on tunicamycin-treated cells was 32,000.

A peptide with the same apparent molecular weight could also be generated by digestion of a pool of the 34,000- and 36,000-dalton forms with endoglycosidase H, an enzyme that hydrolyzes high-mannose carbohydrate side chains from asparagine residues in glycoproteins (data not shown). Peptide mapping of the 32,000-dalton peptide synthesized in the presence of tunicamycin showed that this protein also contains the tryptic segments ACTH-(1–8) and β-LPH-(61–69) (data not shown). The 32,000-dalton peptide is thus likely to represent a form of the precursor free of its asparagine-linked carbohydrate side chains.

Pulse–Chase Studies. When whole neurointermediate lobes of rat pituitary glands were pulse-incubated with [^{35}S]methionine for 20 min and then chased for 2 hr in a medium in which [^{35}S]methionine had been replaced by 2 mM unlabeled methionine, maturation of the 34,000- and 36,000-dalton proteins into smaller peptides was observed (Fig. 6). These fragments were resolved on NaDodSO$_4$ slab gels into four fractions. Peaks

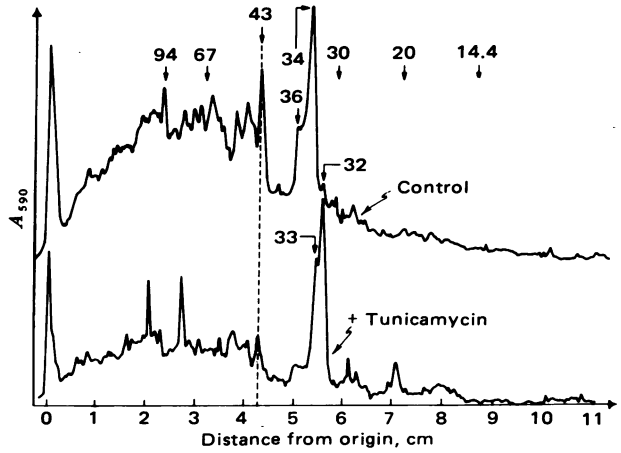

FIG. 5. Densitometric tracings of autoradiograms obtained after NaDodSO$_4$ slab gel analysis of two preparations of ^{35}S-labeled peptides extracted from pars intermedia cells after a 20-min pulse or after a 20-min pulse performed on tunicamycin (5 μg/ml)-treated cells. Numbers are molecular weights × 10^{-3}.

I and II had molecular weights in the range of 16,000–20,000. Peak III comigrated with β-endorphin. The molecular weight of material in peak IV was still lower, but could not be determined by the slab gel electrophoresis method because it migrated with a mobility corresponding to the nonlinear region of the calibration curve. The gel could not be calibrated with molecules smaller than β-endorphin (e.g., β-MSH or α-MSH) because these peptides stain poorly with Coomassie blue and also because they exhibit very broad and poorly defined bands on the slab gels after iodination.

Identification of β-Endorphin. When the [^{35}S]methionine-labeled peptides corresponding to peak III were digested with trypsin and the tryptic fragments were analyzed by HPLC, a major peak comigrating with β-LPH-(61–69) was detected (Fig. 7). Moreover, when this labeled peptide was

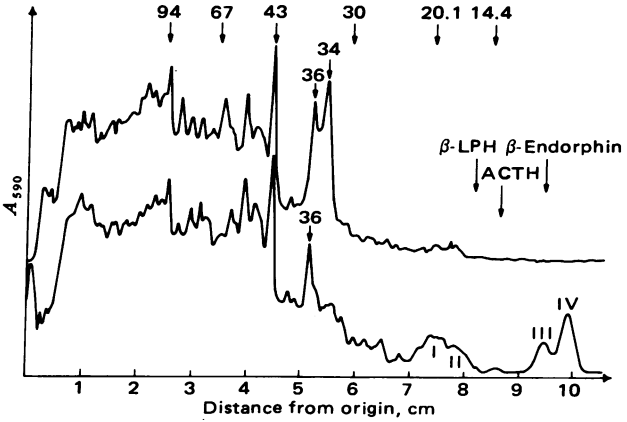

FIG. 6. Densitometric tracings of autoradiograms obtained after NaDodSO$_4$ slab gel electrophoresis of neurointermediate lobe extracts. (Upper curve) Sample was prepared after a 20-min pulse in 50 μl of buffer containing 250 μCi of [^{35}S]methionine. (Lower curve) Sample was pulsed for 20 min in the radioactive medium before a 2-hr chase in the presence of unlabeled methionine (2 mM final concentration). The positions (M_r × 10^{-3}) of the standard proteins (Pharmacia) used as molecular weight markers are indicated as well as the position of several standard ovine hormones (prepared in our laboratory).

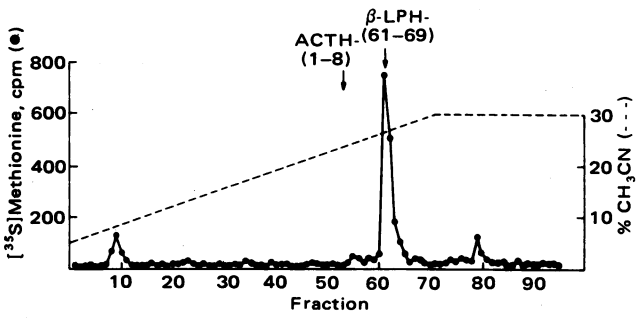

FIG. 7. Peptides migrating under peak III in the electrophoresis depicted in Fig. 6 were digested with trypsin and analyzed by HPLC. Standard synthetic ACTH-(1–8) and β-LPH-(61–69) served as markers.

eluted from the gel without trypsin digestion and the sequence was determined (ref. 15; unpublished results), radioactivity was found in the fifth position only, as expected for the amino acid sequence of β-endorphin (data not shown).

Identification of α-MSH. When the labeled peptides corresponding to the peak IV region on slab gels were eluted from the gel and analyzed directly on HPLC, three peaks were resolved (Fig. 8). One of these comigrated with standard synthetic α-MSH and therefore probably represents authentic α-MSH. When the peptides eluted from peak IV were digested with trypsin for 2 hr at 37°C, the HPLC elution profile showed a major peak of radioactive material coeluting with the acetylated form of ACTH-(1–8) and a minor peak coeluting with the unacetylated form of ACTH-(1–8) (Fig. 9). These findings indicate that the ^{35}S-labeled peptides migrating under peak IV during slab gel electrophoresis are small molecular weight intermediates related to α-MSH (9).

DISCUSSION

The data presented in this study show that in rat pars intermedia cells the common precursor to ACTH/β-LPH exists in two high molecular weight forms and that maturation of these forms involves concomitant release of β-endorphin and α-MSH. This is an additional reason why we gave the name of pro-opiomelanocortin to this precursor (18).

Analysis of the 34,000- and 36,000-dalton precursor forms observed in this study reveals that each contains within its sequence the antigenic determinants and tryptic cleavage fragments characteristic of both ACTH and β-LPH. The results of

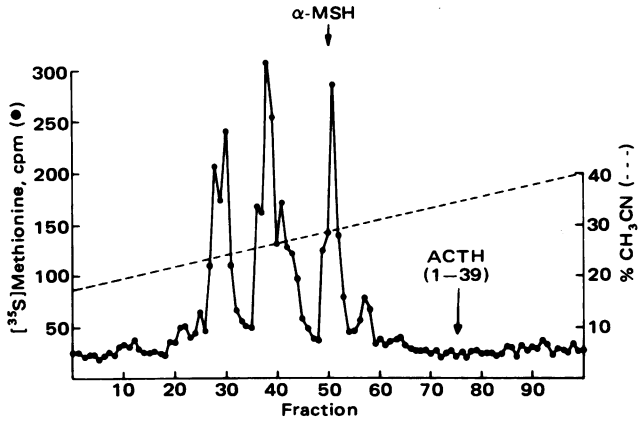

FIG. 8. Peptides migrating under peak IV (Fig. 6) were eluted from the gel and analyzed directly by HPLC. Standard α-MSH (generous gift from C. H. Li) was cochromatographed to serve as a marker.

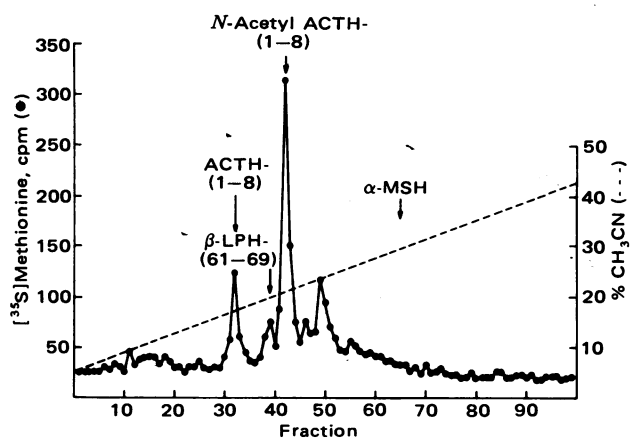

FIG. 9. HPLC analysis of the peptides eluted from peak IV and subsequently digested with trypsin in the presence of 100 μg of synthetic α-MSH. Digestion was for 2 hr at 37°C with an enzyme-to-substrate ratio of 1:100. Identification of *N*-acetyl ACTH-(1–8) was made after amino acid analysis of carrier α-MSH tryptic fragments.

experiments with tunicamycin suggest that these two forms differ mainly in the extent of glycosylation of asparagine residues.

As indicated by the results of pulse–chase experiments, the two precursor forms differ in metabolic stability. After a 2-hr chase period, substantial amounts of the larger precursor initially present remained intact, whereas the smaller form had completely disappeared. Interestingly, Loh and Gainer (19) also observed a faster degradation rate for the same precursor synthesized in the presence of tunicamycin. These observations suggest that critically positioned carbohydrate moities may protect the precursor from proteolytic enzymes.

Chase incubations also released peptides with apparent molecular weights of 16,000–20,000, but further analysis did not provide definitive answers on their nature. It is not known whether these peptides represent the end-products of the maturation process or simply transient metabolic intermediates. ACTH-(1–39) production was not observed in these studies and therefore is probably not a significant hormone product in rat pars intermedia. Perhaps it represents a short-lived intermediate in the maturation of the precursor to α-MSH, as proposed by Lowry *et al.* (9).

Two other major end products of the maturation process of the precursor have been characterized in our laboratory (unpublished results). They have apparent molecular weights of 19,000 and 17,000 in the slab gel system and correspond to glycoprotein variants of the NH_2-terminal fragment of the precursor.

We thank D. Marcil for prompt and expert typing, R. Routhier for assistance in the HPLC analysis of tryptic digests, and M. Dennis for critical reading of the manuscript. Tunicamycin was a generous gift from Dr. G. Tamura (University of Tokyo). Synthetic β-LPH-(61–69) was a gift from Dr. R. Guillemin (Salk Institute) and ACTH-(1–8) was prepared for us by Dr. S. St-Pierre (University of Sherbrooke). Synthetic α-MSH was generously provided by Dr. C. H. Li (University of California, San Francisco). This work was supported by grants from the Medical Research Council of Canada (PG2, MA6612), and by "Le Conseil de la Recherche en Sante du Quebec".

1. Mains, R. E., Eipper, B. A. & Ling, N. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 3014–3018.
2. Roberts, J. L. & Herbert, E. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 4826–4830.
3. Roberts, J. L. & Herbert, E. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5300–5304.

4. Crine, P., Gianoulakis, C., Seidah, N. G., Gossard, F., Pezalla, P. D., Lis, M. & Chrétien, M. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 4719–4723.
5. Nakanishi, S., Inoue, A., Kita, T., Nakamura, M., Chang, A. C. Y., Cohen, S. N. & Numa, S. (1979) *Nature (London)* **278,** 423–427.
6. Eipper, B. A. & Mains, R. E. (1978) *J. Supramol. Struct.* **8,** 247–262.
7. Liotta, A. S., Suda, T. & Krieger, D. T. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 2950–2954.
8. Lissitsky, J. C., Morin, O., Dupont, A., Labrie, F., Seidah, N. G., Chrétien, M., Lis, M. & Coy, D. H. (1978) *Life Sci.* **22,** 1715–1722.
9. Lowry, P. J., Silman, R. E. & Hope, J. (1977) *Ann. N.Y. Acad. Sci.* **297,** 49–62.
10. Silman, R. E., Holland, D., Chard, T., Lowry, P. J., Hope, J., Robinson, J. S. & Thorburn, G. D. (1978) *Nature (London)* **276,** 526–528.
11. Laemmli, U. (1970) *Nature (London)* **227,** 680–685.
12. Bonner, W. M. & Laskey, R. A. (1974) *Eur. J. Biochem.* **46,** 83–88.
13. Laskey, R. A. & Mills, A. D. (1975) *Eur. J. Biochem.* **56,** 335–341.
14. Hirs, C. H. W. (1967) *Methods Enzymol.* **11,** 197–199.
15. Seidah, N. G., Gianoulakis, C., Crine, P., Lis, M., Benjannet, S., Routhier, R. & Chrétien, M. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 3153–3157.
16. Roberts, J. L., Phillips, M., Rosa, P. A. & Herbert, E. (1978) *Biochemistry* **17,** 3609–3618.
17. Takatsuki, A., Kohno, K. & Tamura, G. (1975) *Agric. Biol. Chem.* **39,** 2089–2091.
18. Chrétien, M., Benjannet, S., Gossard, F., Gianoulakis, C., Crine, P., Lis, M. & Seidah, N. G. (1979) *Can. J. Biochem.*, in press.
19. Loh, Y. P. & Gainer, H. (1978) *FEBS Lett.* **96,** 269–272.