Proc. Natl. Acad. Sci. USA
Vol. 75, No. 10, pp. 4719–4723, October 1978
Biochemistry

Biosynthesis of β-endorphin from β-lipotropin and a larger molecular weight precursor in rat pars intermedia

(endogenous opiates/prohormones/corticotropin/radiolabeled peptide sequencing/pituitary gland)

P. CRINE, C. GIANOULAKIS, N. G. SEIDAH, F. GOSSARD, P. D. PEZALLA, M. LIS, AND M. CHRÉTIEN

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

Communicated by Elwood V. Jensen, July 11, 1978

ABSTRACT **When isolated rat pars intermedia cells were incubated for 10 min with radioactive amino acids, one major labeled protein with a molecular weight of 30,000 ± 1500 was extracted. This protein was shown to contain in its sequence the antigenic determinants for corticotropin and β-melanotropin by immunoprecipitation. When the radioactivity incorporated into this large molecular weight protein during the first 10 min was chased by a further incubation in presence of an excess of unlabeled amino acid, the initial protein was degraded into several smaller peptides including β-endorphin and β-lipotropin. Another 18,000-dalton peptide was also observed and was tentatively identified as a large molecular form of corticotropin. From the kinetics of the maturation of the initial precursor, it is concluded that the initial cleavage of the 30,000-dalton peptide gives rise to β-lipotropin and the 18,000-dalton form of corticotropin. β-Lipotropin is subsequently cleaved to form β-endorphin.**

The intermediate lobe of the pituitary has been shown to contain a number of peptide hormones including corticotropin (ACTH) (1–3), α-melanotropin (α-MSH) (4, 5), corticotropin-like intermediate lobe peptide (CLIP) (5, 6), β-lipotropin (β-LPH) (7–9), β-endorphin (9–11), and β-MSH (4, 12). α-MSH and CLIP are structurally related to ACTH; β-LPH is considered to be the precursor for β-MSH and β-endorphin (13).

Immunocytochemical studies have shown that ACTH and β-LPH immunoreactive peptides occur in all of the parenchymal cells of the pars intermedia (7, 9, 11). Furthermore, when these cells were observed under the electron microscope, staining for ACTH and β-LPH was seen in all the granules and in the structures of the rough endoplasmic reticulum (9, 14). These results agree with the hypothesis of a common precursor for ACTH and β-LPH, which has recently had two good experimental confirmations (15–17).

In both studies, the experimental model was the ACTH-secreting mouse pituitary cell line AtT-20. Mains *et al.* (15) used a double-immunoprecipitation technique to isolate labeled proteins from cells incubated with radioactive amino acids. Roberts and Herbert (16, 17) prepared a cell-free translation product from a poly(A)-containing mRNA fraction obtained from AtT-20 cells. In both cases, the ACTH/β-LPH precursor seems to be a 28,000–31,000 dalton protein that is cleaved into β-LPH, β-endorphin, and several high molecular weight forms of ACTH (15, 18, 19).

The primary purpose of this study was to investigate the ACTH/β-endorphin biosynthetic pattern in the intermediate lobe of rat pituitaries. We report here that, during short incubations with labeled amino acids, rat pituitary intermediate lobe cells synthesize predominantly one 30,000 ± 1500 dalton protein that bears antigenic determinants for both β-MSH and ACTH. Pulse-chase labeling shows that this peptide matures into several products including β-LPH and β-endorphin.

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.

METHODS

Preparation of Rat Pars Intermedia Cells. Sprague–Dawley male rats (Canadian Breeding Laboratory, St-Constant, Quebec) (150–200 g) were killed by decapitation and the pituitary gland was exposed by removing the brain. The posterior lobe (pars nervosa plus pars intermedia) was carefully separated from the anterior lobe with fine needles and immediately immersed in Krebs–Ringer buffer that contained bicarbonate, 0.2% glucose, and 0.1% bovine serum albumin (fraction V, Sigma) and had been thoroughly gassed with 95% O_2/5% CO_2 just before the experiment. This buffer containing the posterior lobes was kept at room temperature. Pooled posterior lobes from 40 animals were washed several times with fresh buffer and were sucked several times through a long plastic tubing (3 mm inside diameter) attached to a 50-ml plastic disposable syringe. This procedure disperses the cells from the pars intermedia which is a very loose tissue (20). The remaining pars nervosa was separated by decantation and the resulting supernatant was then filtered through a fine nylon net (100 mesh) to remove any remaining small fragments of the anterior lobe. The cells were harvested by low-speed centrifugation, resuspended in 1–5 ml of gassed Krebs–Ringer buffer and preincubated for 1 hr at 37° in an atmosphere of 95% O_2/5% CO_2. The preincubation was performed in a Dubnoff metabolic shaker with gentle agitation. Starting from 40 rats, the procedure routinely yielded 10^6 cells. Viability, as determined with the trypan blue exclusion technique, was higher than 90%.

Incorporation of Labeled Amino Acids *In Vitro*. At the end of the preincubation, the cells were harvested by low-speed centrifugation and resuspended in either 1 ml of prewarmed Krebs–Ringer buffer containing 2 mCi of [^{35}S]methionine (New England Nuclear, 596 Ci/mmol) per ml or 5 ml of the same medium containing 0.5 mCi of [^{3}H]phenylalanine (New England Nuclear, 115 Ci/mmol) per ml. Under these conditions, protein synthesis, as judged by the incorporation of labeled amino acids into trichloroacetic acid-precipitable material was linear for at least 3 hr.

Pulse-Chase Experiments. Pulse-chase labeling experiments were performed with [^{35}S]methionine only. At the end of the labeling period, unlabeled methionine was added to the incubation medium at a final concentration of 2 mM. This resulted in a 1:300 dilution of the radioactive amino acid and proportionally decreased the incorporation of [^{35}S]methionine into trichloroacetic acid–insoluble proteins within the next 10 min. This result agrees with previous reports of the rapid equilibrium of amino acid pools inside the cells with the extracellular amino acids (18, 21).

Protein Extraction. After the incubation, the cell suspension was diluted in 10 vol of cold Krebs–Ringer buffer containing

Abbreviations: ACTH, corticotropin (adrenocorticotropin); MSH, melanotropin; CLIP, corticotropin-like intermediate lobe peptide; LPH, lipotropin; $NaDodSO_4$, sodium dodecyl sulfate.

* Affiliated with the Hôtel-Dieu Hospital of Montreal and the University of Montreal.

10 mM of unlabeled amino acid and immediately centrifuged at low speed. The cell pellet was washed once with cold saline and extracted with 5 M acetic acid/phenylmethylsulfonyl fluoride (0.3 mg/ml)/iodoacetate (0.3 mg/ml)/bovine serum albumin (0.5 mg/ml), containing 5 μmol of the corresponding unlabeled amino acid per ml (21). The cell extract was desalted on a 9.1 ml disposable Sephadex G-25 (medium) disposable column (Pharmacia) previously equilibrated with 1 M acetic acid and coated with 30–50 mg of bovine serum albumin (Sigma fraction V).

CM-Cellulose Chromatography. The fraction excluded from the gel was lyophilized and dissolved in 5 ml of 10 mM NH_4OAc buffer (pH 4.6), and 200 mg of sheep pituitary fraction D (22) was added. The mixture was chromatographed on a CM-cellulose column (40 × 1 cm) at 4° as described (23). The protein content of the 2-ml fractions was determined by absorbance at 280 nm and a 50-μl aliquot was taken for determination of radioactivity by liquid scintillation counting.

Disc Electrophoresis. Samples from various chromatographic fractions during the purification of labeled products or total cell extracts were analyzed by disc electrophoresis in acidic polyacrylamide gels according to Reisfield *et al.* (24).

For the identification of β-LPH and β-endorphin, purified labeled rat β-LPH and β-endorphin obtained from a separate incubation were run on separate gels (25).

Sodium dodecyl sulfate ($NaDodSO_4$) polyacrylamide gels were prepared and used according to Weber and Osborn (26). The molecular weight determination was made by comparing the relative mobility of the unknown molecular species with a set of standard proteins (Pharmacia).

$NaDodSO_4$/urea/polyacrylamide gel electrophoresis according to Swank and Munkres (27) was performed as described (25). Gels containing radioactive proteins were cut into 2-mm slices immediately after the electrophoresis with a Gilson Aliquogel fractionator. Gel fragments were then digested by incubation overnight at 50° in a mixture of 0.2 ml of 60% (vol/vol) perchloric acid and 0.4 ml of 30% (vol/vol) H_2O_2.

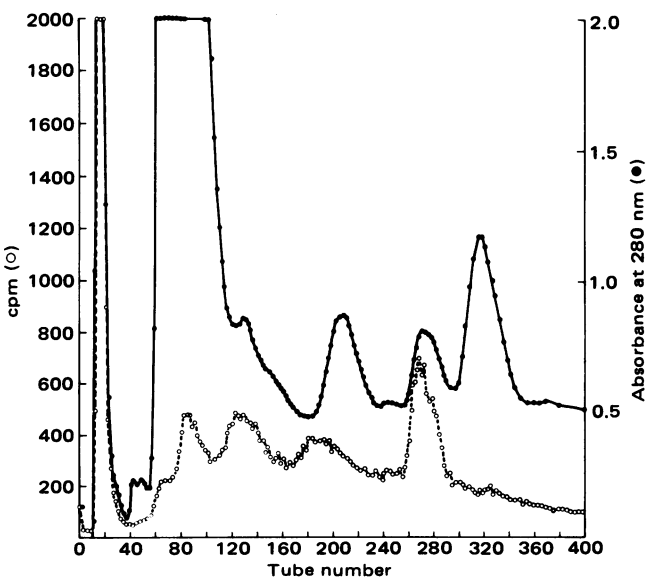

FIG. 1. CM-cellulose chromatography of material obtained after extraction of rat pars intermedia cells incubated *in vitro* for 3 hr with 2 mCi of [^{35}S]methionine. The labeled peptides were chromatographed together with 100 mg of sheep pituitary fraction D. The CM-cellulose column (1 × 40 cm) was eluted starting with 0.01 M NH_4OAc (pH 4.6) for 20 fractions of 2 ml each, followed by a concave gradient made by introducing 0.1 M NH_4OAc (pH 6.7) through a 250-ml mixing flask. This buffer was replaced by 0.2 M NH_4OAc (pH 6.7) at tube 200. Radioactivity was measured in 50-μl aliquots from every second tube.

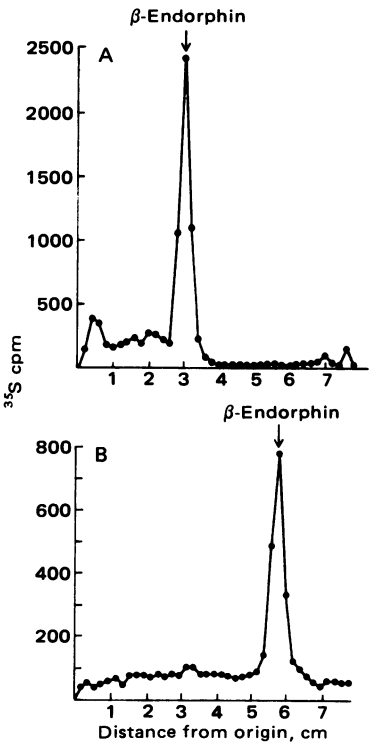

FIG. 2. Polyacrylamide disc gel electrophoresis in $NaDodSO_4$/urea gel (*A*) or in acidic gel (*B*) of the labeled peptides recovered from tubes 258–295 of the CM-cellulose column. The position of standard ovine β-endorphin on separate identical gels is shown.

Immunoprecipitation. ^{3}H-Labeled peptides obtained after a 10-min incubation with [^{3}H]phenylalanine were used for immunoprecipitation studies. An aliquot of the ^{3}H-labeled cell extract that had been previously desalted on Sephadex G-25 was mixed with an excess of either β-MSH antiserum (28) or ACTH antiserum (unpublished results). The amount of antiserum necessary for quantitative immunoprecipitation of the labeled material was previously determined by measuring the radioactivity precipitated by increasing amounts of antiserum. The mixture was incubated for 16 hr at 4° in 0.01 M sodium phosphate/0.15 M NaCl/0.025 M EDTA (P_i/NaCl/EDTA) and 2% Triton X-100 after which an immunoprecipitate was formed by addition of goat antiserum to rabbit immunoglobulins (Calbiochem). The immunoprecipitate was centrifuged at 10,000 × *g* for 4 min through a 1-ml sucrose cushion (1 M sucrose in P_i/NaCl/EDTA/2% Triton X-100), washed several times with P_i/NaCl/EDTA, and dissolved by boiling in 50 μl of a solution containing 12 mg of Tris, 15 mg of dithiothreitol and 10 mg of $NaDodSO_4$ per ml. Immunoprecipitates were analyzed by $NaDodSO_4$ gel electrophoresis. The specificity of immunoprecipitates prepared with ACTH or β-MSH antisera was demonstrated by comparing immunoprecipitates prepared in the presence and absence of excess unlabeled peptide or by using a nonimmune rabbit serum.

Sequencing of Labeled Peptides. The purified peptides were subjected to automatic Edman degradations, and the thiazolinones collected in butylchloride were assayed for radioactivity directly in a toluene-base scintillation cocktail [4 g of Omnifluor (New England Nuclear) per liter of toluene].

RESULTS

Identification of Labeled β-Endorphin in Intermediate Lobe Cell Extract. Cells isolated from the pars intermedia of rat pituitaries incorporated radioactive amino acids into proteins during a 3-hr incubation. In one experiment, [^{35}S]methio-

nine-labeled proteins were extracted from the cells and the fraction excluded from the Sephadex G-25 column was added to 200 mg of fraction D and chromatographed on CM-cellulose (Fig. 1). The fraction corresponding to tubes 258–295, previously known to contain carrier sheep β-endorphin (23), contained only one labeled peptide which comigrated with standard β-endorphin both on $NaDodSO_4$/urea/polyacrylamide gel electrophoresis and on polyacrylamide gel at acidic pH (Fig. 2). When this labeled peptide was sequenced, the only radioactivity found corresponded to residue 5 (Fig. 3). In other experiments, [^{3}H]lysine and [^{3}H]leucine were used. A radioactive fraction that comigrated in all systems with the [^{35}S]methionine-labeled β-endorphin was isolated. Microsequencing of this material revealed that [^{3}H]lysine was present at residue 9 and [^{3}H]leucine, at residues 14 and 17 (25). This confirms that our material corresponds to β-endorphin. In the following experiments, this material was used as a marker for the identification of β-endorphin by polyacrylamide gel electrophoresis, either in acidic or in $NaDodSO_4$/phosphate buffer.

Identification of a Large Precursor for ACTH and β-LPH. Intermediate lobe cells were incubated with [^{3}H]phenylalanine (115 Ci/mmol, 0.5 mCi/ml) for 10 min and the proteins extracted from the cells were desalted on Sephadex G-25 and directly analyzed by $NaDodSO_4$ gel electrophoresis. One major peptide with an apparent molecular weight of 30,000 ± 1500 was found (Fig. 4*A*).

A second protein with an apparent molecular weight of 45,000–50,000 was also extracted from the cells under these conditions. This protein represented 10–20% of the radioactivity of the major peak. The major radioactive cell product (30,000 daltons) was immunoprecipitated with either anti β-MSH serum or anti-ACTH serum as shown by $NaDodSO_4$ gel electrophoresis (Fig. 4 *B* and *C*). In each case, immunoprecipitation of labeled material was shown to be specific because the radioactive peaks on the gels were abolished by the addition of an excess of unlabeled β-MSH or ACTH to the sample before the corresponding immunoserum. Moreover, a nonimmune rabbit serum failed to precipitate any radioactivity (data not shown).

Pulse-Chase Studies. In another experiment, intermediate lobe cells were incubated with [^{35}S]methionine (596 Ci/mmol, 2 mCi/ml) in 1 ml of Krebs–Ringer buffer at 37° for 10 min, after which excess unlabeled methionine (final concentration, 2 mM) was added and the incubation was continued. Aliquots (150 μl) of the cell suspension were withdrawn at the end of the 10-min labeling period and then 20, 40, 60, 90, and 120 min later. Fig. 5 shows that, during the chase period with unlabeled methionine, the 30,000-dalton peptide progressively disappeared, giving rise to smaller fragments. After 20 min of chase, two fragments were observed: an 18,000-dalton fragment and a smaller peptide comigrating with β-LPH. After 40 min of chase, a new peptide comigrating with standard β-endorphin appeared. As the incubation with unlabeled methionine proceeded, two fragments increased in importance, the 18,000-dalton peptide and the β-endorphin-sized fragment. Meanwhile, the 30,000-dalton fragment slowly disappeared as its apparent molecular weight slightly increased. The fragment corresponding to β-LPH seemed to have a transient existence and was no longer detected after 90 min. The 40,000–50,000 dalton protein remained constant and represented about 10–20% of the total cpm recovered from the gel throughout the chase incubation.

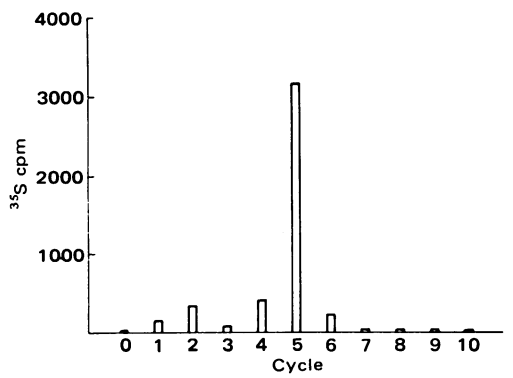

FIG. 3. Partial amino acid sequence analysis of ^{35}S-labeled peptides corresponding to tubes 258–295 of the CM-cellulose column. The total input of radioactivity on the sequenator cup was 10,000 cpm.

When cell extracts from the same pulse-chase experiment were also analyzed by acidic gel electrophoresis (Fig. 6), the major molecular species present after 10 min of [^{35}S]methionine incorporation had an R_F of 0.31. During the chase, it diminished and gave rise to three peptides: one comigrating with standard labeled rat β-LPH, one comigrating with β-endorphin, and one that had a R_F of 0.26. At 20 and 40 min of chase, the peptide comigrating with standard rat β-LPH was clearly apparent as a well-characterized peak on the acidic gels; however, this species disappeared as the chase proceeded and was barely detectable after 2 hr.

DISCUSSION

Previous studies involving labeled amino acid incorporation in whole pituitary slices had proved that β-endorphin was biosynthesized in the pituitary gland (23). However, the elucidation of the detailed biosynthetic pathway required the use of a more specific system in which β-endorphin biosynthesis would represent a high percentage of the total protein synthesis. Immunohistochemical studies have shown that β-LPH and β-endorphin are most concentrated in cells of the pars intermedia of the pituitary (9). These observations led us to reinvestigate the biosynthesis of β-endorphin in isolated pars intermedia cells from beef (29) or rats (25). We have found that, in both species, this tissue can indeed synthesize β-LPH together with its two fragments, γ-LPH and β-endorphin (25, 29).

The results presented in this report prove that, in 10-min pulse experiments, one major labeled protein has a molecular weight of 30,000 ± 1500 and contains antigenic determinants for both ACTH and β-MSH. Another, minor, protein whose amount accounts for 10–20% of the major protein is also synthesized. Its nature is presently unknown.

When the radioactivity incorporated in the cells within the first 10 min of the incubation is chased for 2 hr by an excess of unlabeled amino acid, several fragments are formed from the initial 30,000-dalton peptide and can be separated on $NaDodSo_4$ gel as well as on acidic gel electrophoresis. In both systems, one of them, which is produced in high yield, comigrates with standard rat β-endorphin, isolated from a 3-hr continuous incubation experiment, that has been characterized by electrophoresis and by microsequencing (25). Considering the extremely high resolution of the acidic gels for intermediate lobe peptides, we can identify unambiguously this fragment coming from the precursor as β-endorphin (25).

If the chase incubation is performed for a shorter time (20 min), two major fragments only are formed from the initial 30,000-dalton protein and can be separated by electrophoresis on $NaDodSO_4$ gels. One fragment comigrates with standard rat β-LPH (25) and the other has an apparent molecular weight of 18,000. This peptide has not been fully characterized yet. However, considering the fact that the initial peptide has been shown to contain a common antigenic determinant with ACTH in its structure, we tentatively propose that this peptide could be a high molecular weight form of ACTH. This interpretation

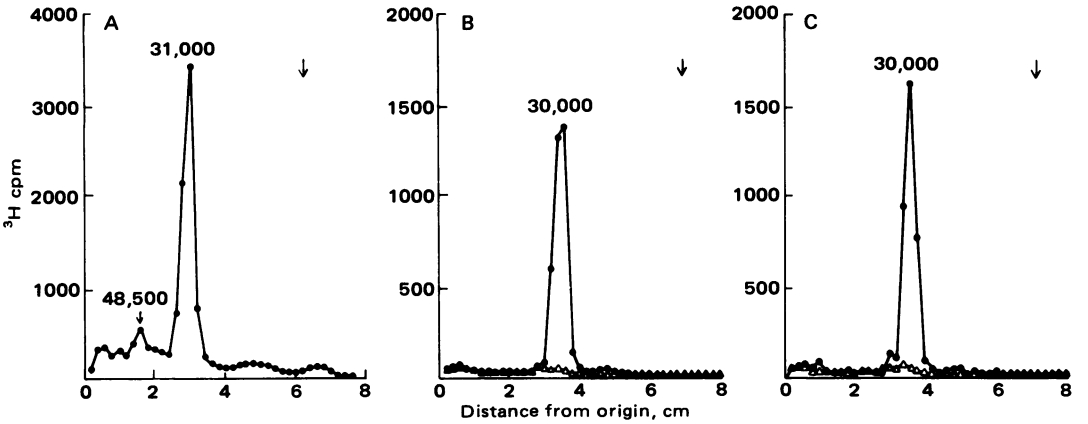

FIG. 4. NaDodSO$_4$ gel electrophoresis of [^{3}H]phenylalanine-labeled peptides synthesized by rat intermediate lobe cells during a 10-min incubation. The position of the bromophenol blue tracking dye is marked (right arrow). The molecular weight of the different species has been calculated by comparing their relative mobilities with those of standard proteins run on a separate gel. (*A*) Total extracted peptides as obtained directly after desalting on Sephadex G-25. (*B*) Immunoprecipitates of extracted peptides with 10 μl of β-MSH antiserum (●). Excess (10 μg) purified β-MSH was added to another aliquot of cell extract before immunoprecipitation with anti-β-MSH (△). (*C*) Immunoprecipitation with 10 μl of ACTH antiserum. No ACTH added (●); excess purified ACTH added before immunoprecipitation (△). In all cases (*A*, *B*, and *C*), an aliquot of the cell extract containing 5000 cpm was used for gel analysis or immunoprecipitation.

is in agreement with the results of Mains and Eipper (19) who have studied the biosynthesis of ACTH and β-endorphin in AtT-20 tumor cells. As soon as β-LPH is cleaved from the precursor, its maturation into β-endorphin is very fast. Even after a 20-min chase, when β-LPH is the predominant form, a small peak of β-endorphin is already present. During this process, γ-LPH is probably formed by the same enzymatic cleavage step. However, rat γ-LPH does not seem to contain any methionine residues (17, 19) and was not detected when intermediate lobe cells were incubated with this labeled amino acid (25).

Even after a 2-hr chase, a fraction of the initial precursor still remained in the cells but its apparent molecular weight had increased from 30,000 to 36,000. This result has also been observed in AtT-20 tumor cell and could be explained by an increased glycosylation of the peptide backbone (17). The extensively glycosylated precursor could represent a stable form that could have its own biological role.

β-Endorphin biosynthesis in the rat intermediate lobe is very similar to the mechanism of ACTH and β-LPH biosynthesis proposed by Mains and Eipper (19) for AtT-20 cells, a tumor cell line derived from the adenohypophysis. However, the

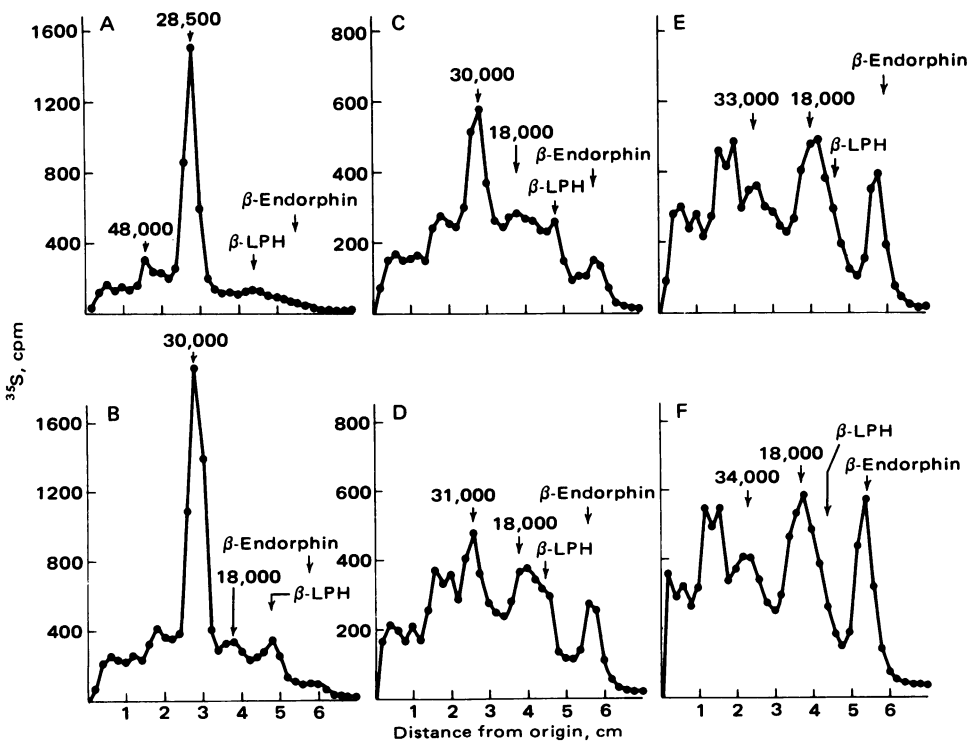

FIG. 5. NaDodSO$_4$ gel electrophoresis of desalted extracts of intermediate lobe cells obtained during a pulse-chase labeling experiment. The cells were incubated with 2 mCi of [^{35}S]methionine per ml for the first 10 min, after which a 300-fold excess of unlabeled methionine was added to the culture medium and the incubation was continued for up to 2 hr. Samples were taken from the incubation medium at various times, extracted, and desalted on Sephadex G-25 before electrophoresis. The positions of standard labeled rat β-LPH and β-endorphin (25) and the molecular weights corresponding to the position of the peaks are marked. (*A*) Pulse, 10 min; no chase; (*B*) chase, 20 min; (*C*) chase, 40 min; (*D*) chase, 60 min; (*E*) chase, 90 min; (*F*) chase, 120 min.

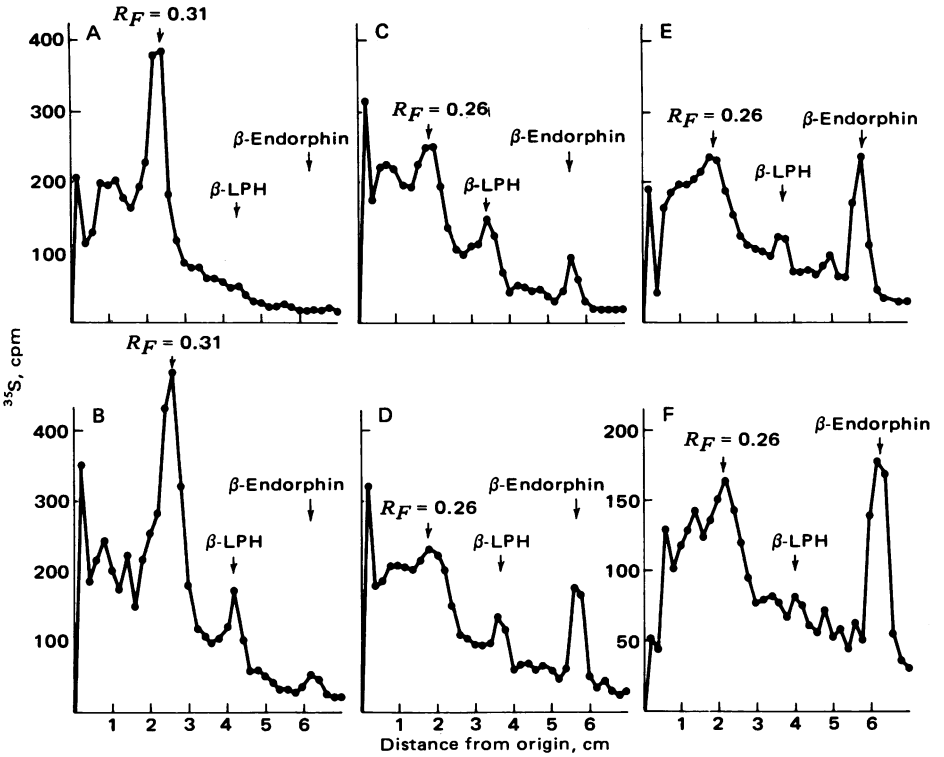

FIG. 6. Acidic gel electrophoresis of desalted extracts of intermediate lobe cells obtained during a pulse-chase labeling experiment. Labeling and chase conditions were as in Fig. 5. The positions of standard labeled rat β-LPH and β-endorphin are marked. (*A–F*) As in Fig. 5.

control of hormone secretion in the intermediate lobe seems to be different from that in the anterior lobe (2, 3). The experiments described in this report conclusively show that the pars intermedia of the pituitary is a highly specialized tissue for the synthesis of the large molecular weight precursor that is transformed into β-endorphin with β-LPH as an intermediate. This constitutes an excellent model for studying the biosynthesis of β-endorphin in normal tissue.

This work was supported by a Program Grant from the Medical Research Council of Canada and the Jos Rhéaume Foundation. F.G. is a Medical Research Council Fellow; P.C. is a Medical Research Council Centennial Fellow; C.G. is a Killam Foundation Fellow.

1. Shapiro, M., Nicholson, W. E., Orth, D. N., Mitchell, W. M., Island, D. P. & Liddle, G. W. (1972) *Endocrinology* **90**, 249–256.
2. Kraicer, J., Gosbee, J. L. & Bencosme, S. A. (1973) *Neuroendocrinology* **11**, 156–176.
3. Moriarty, C. M. & Moriarty, G. C. (1975) *Endocrinology* **96**, 1419–1425.
4. Baker, B. I. (1973) *J. Endocrinol.* **57**, 393–404.
5. Scott, A. P., Lowry, P. J., Ratcliffe, J. G., Rees, L. H. & Landon, J. (1974) *J. Endocrinol.* **61**, 355–367.
6. Scott, A. P., Ratcliffe, J. G., Rees, L. H., Landon, J., Bennett, H. P. J., Lowry, P. J. & McMartin, C. (1973) *Nature (London) New Biol.* **244**, 65–67.
7. Moon, H. D., Li, C. H. & Jennings, B. M. (1973) *Anat. Res.* **175**, 529–538.
8. LaBella, F., Queen, G., Senyshyn, J., Lis, M. & Chrétien, M. (1977) *Biochem. Biophys. Res. Commun.* **75**, 350–357.
9. Pelletier, G., Leclerc, R., Labrie, F., Côté, J., Chrétien, M. & Lis, M. (1977) *Endocrinology* **100**, 770–776.
10. LaBella, F., Downey, G., Queen, G. & Pinsky, C. (1976) *Can. J. Physiol. Pharmacol.* **54**, 946–948.
11. Bloom, F., Battenberg, E. O., Rossier, J., Ling, N., Leppeluoto, J., Vargo, T. M. & Guillemin, R. (1977) *Life Sci.* **20**, 43–48.
12. Thody, A. J. (1969) *Gen. Comp. Endocrinol.* **13**, 477.
13. Chrétien, M., Seidah, N. G., Benjannet, S., Dragon, N., Routhier, R., Motomatsu, T., Crine, P. & Lis, M. (1977) *Ann. N. Y. Acad. Sci.* **297**, 84–107.
14. Moriarty, G. C. (1973) *J. Histochem. Cytochem.* **21**, 855–894.
15. Mains, R. E., Eipper, B. A. & Ling, N. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 3014–3018.
16. Roberts, J. L. & Herbert, E. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 4826–4830.
17. Roberts, J. L. & Herbert, E. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5300–5304.
18. Mains, R. E. & Eipper, B. A. (1976) *J. Biol. Chem.* **251**, 4115–4120.
19. Mains, R. E. & Eipper, B. A. (1978) *J. Biol. Chem.* **253**, 651–655.
20. Kraicer, J. & Morris, A. R. (1976) *Neuroendocrinology* **20**, 79–96.
21. Richelson, E. & Thompson, E. F. (1973) *Nature (London) New Biol.* **241**, 201–204.
22. Li, C. H., Barnafi, L., Chrétien, M. & Chung, D. (1965) *Nature (London)* **208**, 1093–1094.
23. Crine, P., Benjannet, S., Seidah, N. G., Lis, M. & Chrétien, M. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 1403–1406.
24. Reisfield, R. A., Lewis, U. J. & Williams, E. D. (1962) *Nature (London)* **195**, 281–283.
25. Seidah, N. G., Gianoulakis, C., Crine, P., Lis, M., Benjannet, S., Routhier, R. & Chrétien, M. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 3153–3157.
26 Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412.
27. Swank, R. T. & Munkres, K. D. (1971) *Anal. Biochem.* **39**, 462–477.
28. Pezalla, P. D., Clarke, W. C., Lis, M., Seidah, N. G. & Chrétien, M. (1978) *Gen. Comp. Endocrinol.* **39**, 163–168.
29. Crine, P., Benjannet, S., Seidah, N. G., Lis, M. & Chrétien, M. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 4276–4280.