Proc. Natl. Acad. Sci. USA
Vol. 74, No. 10, pp. 4276–4280, October 1977
Biochemistry

In vitro biosynthesis of β-endorphin, γ-lipotropin, and β-lipotropin by the pars intermedia of beef pituitary glands

(endogenous opiates/peptide hormones/prohormones/Edman degradation)

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Communicated by Choh Hao Li, July 25, 1977

ABSTRACT **Labeled amino acids were incorporated into proteins during incubations of isolated cells from the pars intermedia of beef pituitary glands. After 3 hr of incubation with [^{35}S]methionine and [^{3}H]lysine, approximately equivalent amounts of labeled γ-lipotropin and β-endorphins were isolated. They were found to be major synthesis products of the pars intermedia. In contrast, very little labeled β-lipotropin was recovered. Another major synthesis product of the pars intermedia was also purified. Its partial amino acid sequence and molecular weight were determined and it was concluded that this peptide cannot be identified as any known pituitary hormone or protein fragment.**

β-Endorphin is a 31-amino-acid polypeptide that has been isolated from the pituitary gland of various animal species, including human (1–4). Its sequence corresponds to amino acid residues 61–91 of β-lipotropin. The fragment corresponding to the first five residues at the NH_2-terminus of β-endorphin (residues 61–65 of β-lipotropin) is a very strong agonist of morphine known as Met-enkephalin (5). It has been observed that, while β-lipotropin has no morphine-like activity even at high doses (6, 7), β-endorphin is at least as potent as Met-enkephalin in the morphine bioassays and opiate receptor binding assays (1, 3, 6, 7).

The pituitary gland is also known to contain another lipotropic hormone, γ-lipotropin, which is represented by amino acid residues 1–58 of the β-lipotropin molecule (8). γ-Lipotropin contains as its COOH-terminus the complete structure of β-melanotropin, an 18-amino-acid peptide that is 100 times more potent than either β- or γ-lipotropin in lipolysis and melanophore stimulation (9).

Very little is known about the physiological effect of pituitary lipotropic hormones, but β-lipotropin now appears to be a unique molecule containing the structure of two biologically active peptides: β-melanotropin and β-endorphin. This observation led Chrétien *et al.* (9) to propose that β-lipotropin could be the precursor for these two peptides.

The prohormone hypothesis proposed for β-lipotropin is further supported by the analogy between its sequence and the sequence of proinsulin at their respective cleavage sites, which in both cases contain two basic amino acids, a feature likely to be recognized by a trypsin-like enzyme (10).

Chrétien and his collaborators (11–13), using *in vitro* labeling experiments, have demonstrated the biosynthesis of both β- and γ-lipotropin in bovine pituitary slices. More recently, the biosynthesis of β-endorphin has also been proved in whole pituitary slices incubated *in vitro* (14). However, immunohistochemical studies have shown that β-lipotropin is most concentrated in cells of the pars intermedia of the pituitary (15). Similarly, it has also been shown that the major portion of β-endorphin present in the pituitary is also found in the pars intermedia (16, 17). These observations led us to reinvestigate the study of the biosynthesis of β-lipotropin, γ-lipotropin, and β-endorphin in the pars intermedia. This report shows that isolated cells of the bovine pars intermedia can indeed synthesize these three peptides together with another unidentified peptide.

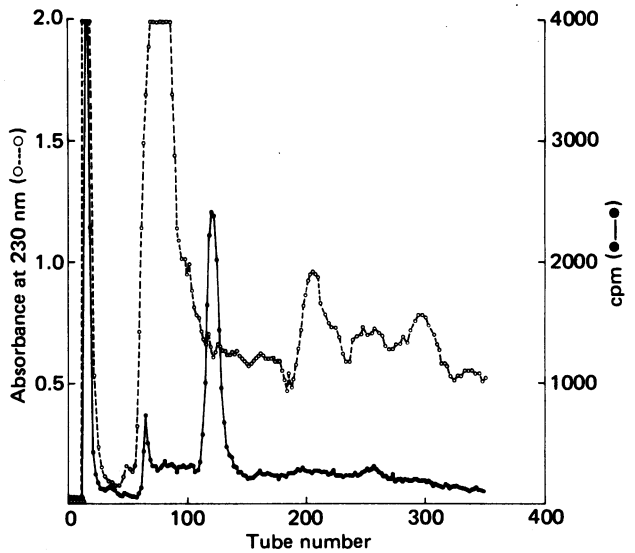

FIG. 1. CM-cellulose chromatography of the labeled proteins extracted from the isolated cells of the beef pituitary pars intermedia incubated *in vitro* for 3 hr with 1 mCi of [^{35}S]methionine and 4.8 mCi of [^{3}H]lysine. The labeled peptides were chromatographed together with 225 mg of sheep pituitary fraction D. The column was eluted with a system of concave gradients (14). Fractions (2 ml) were collected and their absorbances at 230 nm were recorded. A 200-μl aliquot was also taken for radioactivity measurement and the total activities (^{35}S + ^{3}H) were determined.

MATERIALS AND METHODS

Isolation of Intermediate Lobe Cells. Bovine pituitary glands were obtained from a local slaughterhouse. They were removed 15–20 min after the death of the animals and immediately immersed in ice-cold Krebs–Ringer solution containing 0.2% glucose (KRBG). The glands were freed from any remaining pituitary stalk and connective tissue and the posterior parts (pars nervosa plus pars intermedia) were carefully dissected from the anterior lobe and cut into small cubes (about 1 × 1 mm). These fragments were kept in ice-cold KRBG containing 0.1% bovine serum albumin (Fraction V, Sigma). They were further disrupted by several passages through plastic

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Abbreviation: KRBG, Krebs–Ringer buffer with glucose.

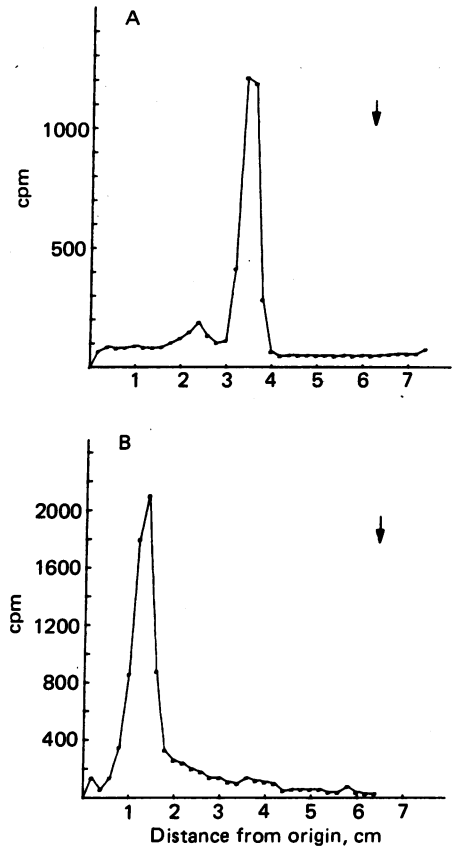

FIG. 2. Polyacrylamide gel electrophoresis at pH 4.5 (*A*) and at pH 8.3 (*B*) of the radioactive material recovered in fractions 110–140 of the CM-cellulose chromatography (Fig. 1). The gel was cut into 2-mm slices and the total activities ($^{35}S + {}^{3}H$) were determined in each slice. The arrow shows the position of the tracking dye at the end of the migration.

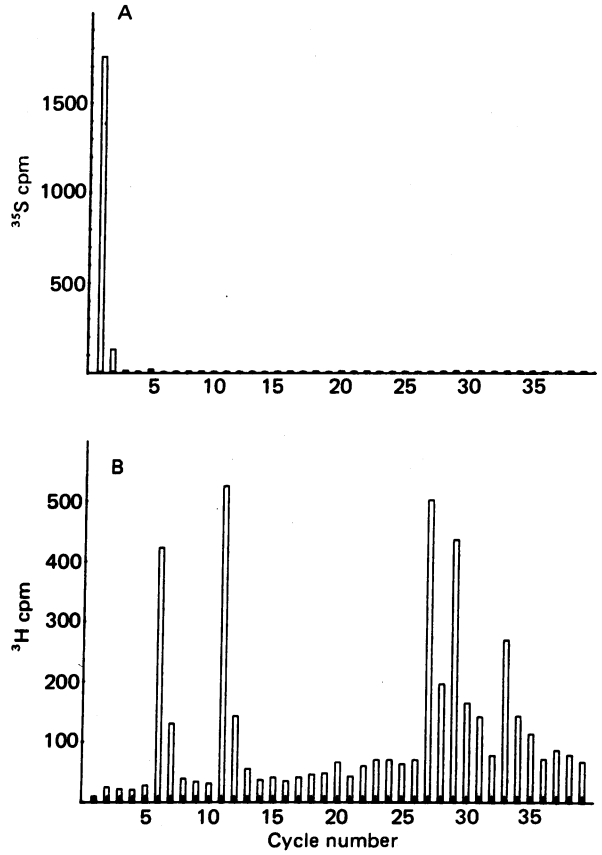

FIG. 3. Sequential Edman degradation of the radioactive peptide recovered in fractions 110–140 of the CM-cellulose chromatography (Fig. 1). The peptide was labeled with [^{35}S]methionine and [^{3}H]lysine. The radioactivity counted in the ^{35}S channel is shown in *A* and the radioactivity due to ^{3}H in *B*. The ^{3}H counts were corrected for ^{35}S spilling over. A total of 5×10^3 ^{35}S cpm and 4.5×10^3 ^{3}H cpm was applied to the sequencer cup.

tubing [1/8 inch (3 mm) inside diameter] attached to a 50-ml plastic disposable syringe and then filtered through a fine nylon mesh. This mechanical treatment separates the cells of the pars intermedia, which is a very loose tissue, from the pars nervosa, which is much more resistant to this gentle mechanical disruption.

Incorporation of Labeled Amino Acids *In Vitro*. The isolated cells from 25 glands were pelleted by centrifugation at low speed in a table-top clinical centrifuge and resuspended in 10 ml of KRBG containing 0.2% bovine serum albumin. The buffer had been thoroughly gassed with O_2/CO_2 (95:5, vol/vol) prior to the incubation and the pH of the gassed solution at 37° was 7.3–7.4.

The cells were preincubated for 1 hr at 37° in an atmosphere of O_2/CO_2 (95:5). The preincubation was performed in a Dubnoff metabolic shaker with a gentle agitation. Approximately 2 hr elapsed between the removal of the glands from the animals and the beginning of the incubation.

At the end of the preincubation, the cells were harvested by centrifugation at low speed and resuspended in 10 ml of fresh KRBG containing 1 mCi of [^{35}S]methionine (New England Nuclear, 420 Ci/mmol), 4.8 mCi of [^{3}H]lysine (New England Nuclear, 80 Ci/mmol), 3 mM dithiothreitol, streptomycin sulfate at 50 μg/ml, penicillin at 19 μg/ml, and lima bean trypsin inhibitor (Worthington) at 180 μg/ml. Incubation was resumed as described above and continued for 3 hr. After the incubation, the cells were pelleted by low-speed centrifugation at 4° and washed with cold KRBG containing 20 mM lysine and 20 mM methionine. The pellet was stored frozen at −20° until it could be further processed.

The frozen pellet was thawed and homogenized in 5 ml of a cold buffer containing 1 mM EDTA (pH 10.35), sheep pituitaries fraction D (18) at 5 mg/ml, and Trasylol (Bayer) at 600 units/ml. Homogenization was performed in a Teflon–glass homogenizer and the homogenate was centrifuged for 10 min at 15,000 × g_{av}. The supernatant was desalted on a column of Sephadex G-25 coarse (1.5 × 60 cm). The fraction excluded from the gel was lyophilized and dissolved in 5 ml of 10 mM NH_4OAc buffer (pH 4.6).

Carboxymethyl-Cellulose Chromatography. The total cell extract dissolved in 10 mM NH_4OAc (pH 4.6) was added to 200 mg of fraction D and chromatographed on a carboxymethyl cellulose (CM-cellulose) column (40 × 1 cm) at 4° as described earlier (14). The protein content of the 2-ml fractions was determined by absorbance at 230 nm and an aliquot of 100 μl was taken for determination of radioactivity by liquid scintillation counting.

Disc Electrophoresis. Samples from various chromatography fractions during the purification of labeled products were analyzed by disc electrophoresis in polyacrylamide gels at pH 4.5 or pH 8.3 according to Reisfield *et al.* (19) and Davies *et al.* (20). Some gels were stained with amidoschwartz, the others 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 1 ml of 30% (vol/vol)

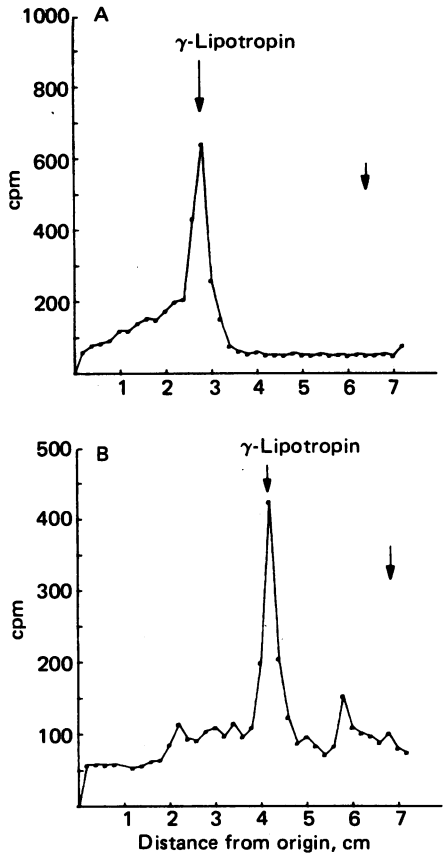

FIG. 4. Polyacrylamide gel electrophoresis at pH 4.5 (*A*) and at pH 8.3 (*B*) of the radioactive material recovered in fractions 59–77 of the CM-cellulose chromatography (Fig. 1). The total radioactivity ($^3H + ^{35}S$) was counted in the 2-mm slices of the gel. The position of purified unlabeled γ-lipotropin run as a reference standard on identical gels is marked on the figures. The position of the tracking dye (right arrow) is also shown.

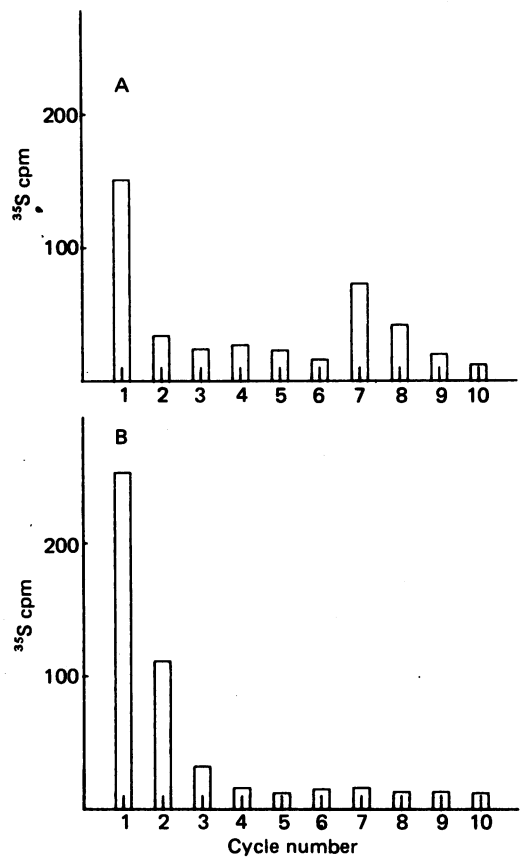

FIG. 5. Radioisotope sequence analysis of the tryptic fragments of γ-lipotropin (fractions 59–77 of the CM-cellulose chromatography shown in Fig. 1). Tryptic digestion was performed for 10 min (*A*) or 24 hr (*B*) at 37° on an aliquot containing 5×10^3 ^{35}S cpm of labeled γ-lipotropin. The enzyme-to-substrate ratio in both cases was 1:50 (wt/wt). The amount of γ-lipotropin (carrier + labeled peptide) used for the incubation was determined by weighing. After the incubation, the digestion was stopped by adding a 10-fold excess of lima bean trypsin inhibitor to the reaction medium. The mixture was lyophilized and mixed with 150 nmol of carrier sperm whale apomyoglobin before sequencing.

H_2O_2. Radioactivity was measured in 10 ml of a Triton X-100/toluene (1:2 vol/vol) scintillation cocktail containing 5.6 g of Omnifluor per liter (New England Nuclear). Radioactivity was determined in a Packard liquid scintillation counter (model 3375). For scanning the chromatography columns and the gel fractions, the channel was selected so as to include the entire energy spectra of both ^{35}S and ^3H isotopes.

Sequencing of Labeled Peptides. Automatic Edman degradation on the purified labeled peptides was performed on a Beckman 890 B sequencer. The buffer used was 0.1 M Quadrol (21), and 150 nmol of sperm whale apomyoglobin carrier was added. The thiazolinones collected in butyl chloride were analyzed directly for radioactivity in a toluene-base scintillation mixture (4 g of Omnifluor per liter of toluene). The windows of the counter were set so as to completely exclude ^3H counts from the ^{14}C channel and to minimize the spilling of the ^{35}S counts over the ^3H channel. The ^3H counts were then corrected for the ^{35}S spilling over as previously determined in a control experiment.

RESULTS

Cells isolated from the pars intermedia of beef pituitaries incorporated [^{35}S]methionine and [^3H]lysine into proteins during *in vitro* incubations. The rate of incorporation of radioactive amino acids into proteins was found to be linear for periods up to 3 hr.

Four main fractions were studied in the CM-cellulose chromatography eluate (Fig. 1): (*i*) tubes 59–77, corresponding to the position of carrier γ-lipotropin in the elution pattern; (*ii*) tubes 110–140, corresponding to the major peak of radioactivity; (*iii*) tubes 195–235, where carrier β-lipotropin is found; and (*iv*) tubes 236–270, from which carrier β-endorphin is recovered.

Analysis of the Major Peak of Radioactivity. The fraction corresponding to tubes 110–140 contains a single radioactive band as shown by electrophoresis in acidic as well as basic conditions (Fig. 2). Gel filtration studies on Sephadex G-25 and G-75 also showed that this fraction corresponds to a homogenous material having a molecular weight of around 4000. Its amino acid sequence (Fig. 3) has been partially determined: one methionine residue has been found at its NH_2-terminus, and lysine residues in positions 6, 11, 27, 29, and 33.

Identification of γ-Lipotropin. Tubes 59–77 contained most of the carrier γ-lipotropin from fraction D. This fraction also corresponded to a radioactive peptide that could be identified as γ-lipotropin by electrophoresis on acrylamide gels at pH 4.3 (Fig. 4*A*) and at pH 8.3 (Fig. 4*B*). Further confirmation of its nature has been made by sequencing the fragments obtained after tryptic digestion of the labeled peptide. Fig. 5*B* shows that a 24-hr digestion by trypsin (2% wt/wt) produced a fragment that contains a methionine at its NH_2-terminus. An incubation

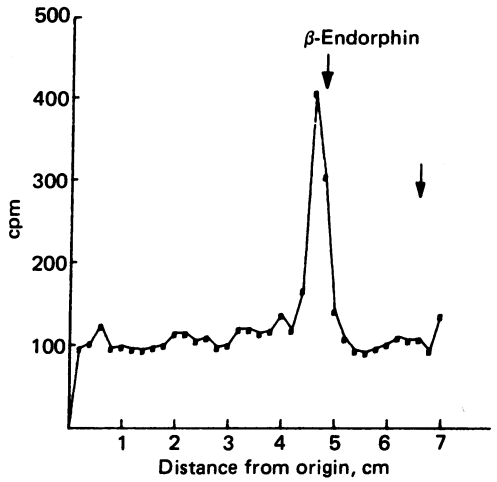

FIG. 6. Gel electrophoresis of purified labeled β-endorphin. The radioactive material recovered in fractions 236–270 of the CM-cellulose chromatography (Fig. 1) was purified on a second CM-cellulose column as mentioned in a previous publication (14). The position of standard β-endorphin is shown on the figure as well as the position of the tracking dye (right arrow).

of shorter duration (10 min), performed with the same enzyme-to-substrate ratio, gives the same fragment together with a second fragment, which has methionine in its seventh position (Fig. 5A). No radioactivity was recovered in the first 10 cycles of the sequencing when trypsin was omitted from the incubation medium. These results are in good agreement with the primary structure of γ-lipotropin, which contains lysine at positions 40 and 46, and methionine in position 47.

Purification and Identification of β-Endorphin. The peak corresponding to fractions 236–270 in the first CM-cellulose chromatography (Fig. 1) is known to contain most of the labeled β-endorphin (14).

This fraction was further purified on a second CM-cellulose column with a slower gradient as described earlier (14). The purified material was shown to contain a single radioactive band which comigrated with standard β-endorphin on polyacrylamide gels at pH 4.5 (Fig. 6). When this labeled peptide was sequenced, it was shown that the only ^{35}S radioactivity found corresponded to residue No. 5 (Fig. 7A). Similarly, ^{3}H radioactivity was recovered in fraction 9 of the sequencer, as expected from the structure of β-endorphin (Fig. 7B) (22).

Purification and Identification of β-Lipotropin. The fraction corresponding to tubes 195–235, which contains the carrier β-lipotropin of the fraction D (Fig. 1), was further purified on Sephadex G-75 followed by CM-Sephadex. It contained a labeled peptide coeluting with standard unlabeled β-lipotropin in both cases. The fraction purified on the CM-Sephadex column showed a radioactive band that comigrated with standard β-lipotropin on polyacrylamide gel at acidic pH (Fig. 8). The accompanying band is a contaminant usually found with standard β-lipotropin.

DISCUSSION

The experiments described above show that isolated pars intermedia cells can synthesize β-lipotropin, γ-lipotropin, and β-endorphin.

The possibility that β-endorphin and γ-lipotropin are produced by degradation of β-lipotropin during the isolation process is very unlikely because β-lipotropin is very stable under various pH and temperature conditions (23) and because the

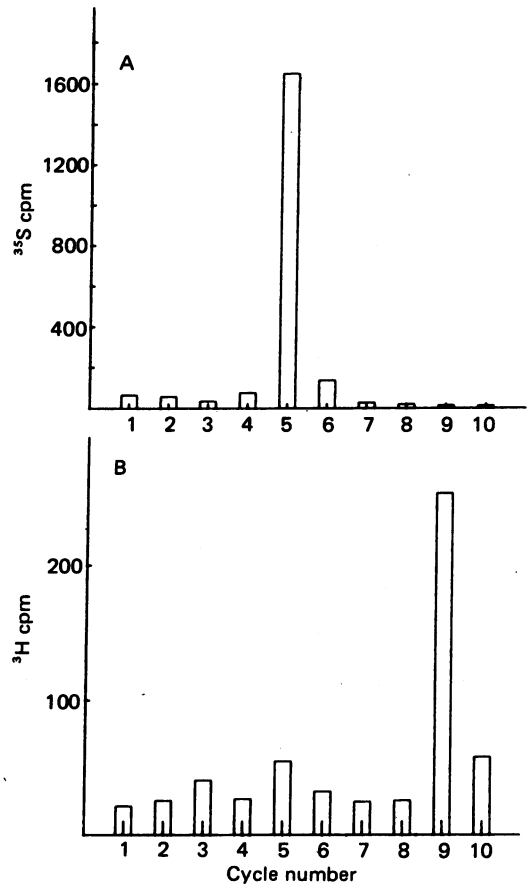

FIG. 7. Radioisotope sequence analysis of purified β-endorphin labeled with [^{35}S]methionine and [^{3}H]lysine. For sequence studies, an aliquot containing 2.5×10^3 ^{35}S cpm and 3×10^3 ^{3}H cpm was used. Radioactivity recovered in the first 10 fractions of the sequence was measured on the ^{35}S channel (A) and ^{3}H channel (B). ^{3}H counts were corrected for the ^{35}S spilling over. ^{35}S radioactivity is recovered in residue 5 and ^{3}H radioactivity in residue 9, as expected from the primary structure of β-endorphin (Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-) (22).

extraction procedure has been carried out at 0° and in presence of an excess carrier sheep pituitary extract (fraction D).

Previous studies on β- and γ-lipotropin have led Chrétien and Gilardeau (23) to propose that β-lipotropin could be considered as the precursor for other pituitary hormones. It had already been shown that β- and γ-lipotropin are synthesized *de novo* in the pituitary gland (11–13). More recently, we have also proved that the pituitary can also synthesize β-endorphin (14). The results presented above show that these three peptides are all synthesized in the pars intermedia of the beef pituitary. Moreover, because the pars intermedia is constituted by a homogenous population of a single type of secretory cells (15), it is most likely that these three peptides are synthesized in the same cells. This conclusion is consistent with the prohormone hypothesis proposed for β-lipotropin.

The amount of β-lipotropin recovered after 3 hr of incubation with radioactive amino acids is very small compared to the amounts of γ-lipotropin and of β-endorphin. This result could be predicted if one considers that β-lipotropin is a transient form readily cleaved into smaller peptides corresponding to the active products.

γ-Lipotropin appears to be very stable in the incubation conditions used during these experiments. Moreover, no labeled β-melanotropic hormone has been detected among the synthesis

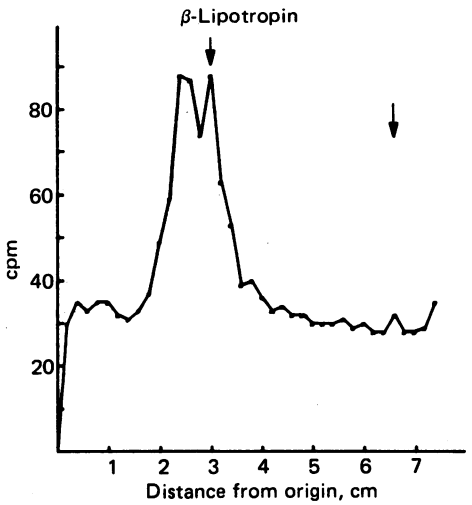

FIG. 8. Gel electrophoresis of purified labeled β-lipotropin. The radioactive material recovered in fractions 195–235 of the CM-cellulose chromatography (Fig. 1) was further purified on Sephadex G-75 and CM-Sephadex. A fraction of this purified material comigrated with standard β-lipotropin.

products. The conversion of β-lipotropin into β-melanotropin thus seems to be a very slow process in the tissue studied. This conclusion agrees with the previous results of Chrétien *et al.* (13).

One important product synthesized by the pars intermedia is a peptide of molecular weight 4000. Its partial sequence provides enough information to conclude that it cannot be identified with any known pituitary peptide or fragment of protein. Although this peptide represents the vast majority of the radioactivity bound to CM-cellulose after 3 hr of incubation, preliminary experiments show that this is no longer the case for shorter incubation periods (10 or 30 min). It is thus conceivable that this peptide represents part of a larger molecule that could be the precursor for corticotropin (ACTH) and β-lipotropin (ref. 24; E. Herbert, personal communication). Alternatively, it is also possible that some other proteins found in the fraction that does not bind to the CM-cellulose column are also synthesized as precursors, which are matured through an enzymatic cleavage step that releases this methionyl peptide.

In any case, the experimental procedure described here seems to be well suited for pulse–chase studies because of the simplicity of the synthesis pattern of the pars intermedia compared to the whole pituitary (14). It is hoped that such experiments will elucidate the manner in which β-lipotropin and related peptides are synthesized in the pituitary.

The authors wish to thank Mrs. O. Théberge for technical assistance and Mrs. D. Marcil for secretarial help. This work was supported by a Program Grant of the Medical Research Council of Canada and Le Ministère de l'Education du Québec. P.C. is a Centennial Fellow of the Medical Research Council.

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