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Biochemistry

Biosynthesis of precursor corticotropin/endorphin-, corticotropin-, α-melanotropin-, β-lipotropin-, and β-endorphin-like material by cultured neonatal rat hypothalamic neurons

(immunoadsorbents/NaDodSO$_4$ gel electrophoresis/tryptic mapping/neonatal culture)

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ABSTRACT **Enzymatically dispersed hypothalamic cells derived from 15-day-old female rats were maintained in tissue culture for 4 days and then incubated for 2 hr in the presence of [^{35}S]methionine. After such incubation, cell extracts contained multiple forms of ^{35}S-labeled products that were specifically bound by immobilized affinity-purified antisera to corticotropin and β-endorphin. Sequential use of these immobilized antisera revealed two molecular species (apparent M_r of 33,000 and 36,500) that contained both corticotropin and β-endorphin antigenic determinants within the same molecule. Substances containing only one of these determinants were also present and co-eluted with corticotropin, α-melanotropin, β-lipotropin, or β-endorphin upon Sephadex G-50 gel filtration. Extracts of similarly labeled rat anterior and intermediate pituitary lobe cells contained two forms of the common precursor molecule corresponding to the same molecular weights estimated for the hypothalamic material and similar to it with respect to other physicochemical parameters. These data suggest that rat hypothalamus synthesizes corticotropin-related and β-endorphin-related products via sequential cleavage of a larger common precursor molecule in a manner similar to the processing pathway demonstrated for the intermediate lobe of the pituitary.**

Both anterior and intermediate lobe pituitary corticotrophic cells synthesize a similar (possibly identical) glycoprotein molecule(s) containing within it the complete sequences of corticotropin (ACTH) and β-lipoprotein (β-LPH) (see Fig. 1). In the anterior lobe, ACTH and β-LPH are the major secretory products, whereas in the intermediate lobe ACTH and β-LPH are present as transient intermediates, which are cleaved to α-melanotropin (α-MSH) [and presumably ACTH-(18–39)] and to β-endorphin and γ-lipotropin (γ-LPH), respectively (2–13). Immunoreactive and bioreactive ACTH, immunoreactive β-LPH, β-endorphin, and α-MSH have been detected in brain extracts of both intact and hypophysectomized animals. These peptides have been localized within the same neuronal cell bodies in the hypothalamic arcuate nucleus and periarcuate regions as well as in extensive fiber pathways within hypothalamic and extrahypothalamic regions in several species, including humans (see ref. 1 for review). We have recently reported that cultured bovine hypothalamic cells incorporate radiolabeled amino acids into material with the immunological (dual antigenic determinants of ACTH and β-endorphin within the same molecule) and physicochemical characteristics of bovine pituitary common precursor material, providing direct biochemical evidence of brain synthesis of ACTH-related peptides (14, 15).

The present study extends these findings in rats by use of cultures of neonatal hypothalamic neurons exhibiting extensive outgrowth of processes. We demonstrate radiolabeled amino acid incorporation into two forms of a common precursor-like molecule and show that these molecules are physicochemically similar to the rat pituitary precursor molecules with respect to apparent molecular weights, immunoprecipitation studies, binding to concanavalin A, and partial tryptic mapping. Additional radiolabeled immunoreactive species similar in size to β-LPH, β-endorphin, ACTH, and α-MSH were also detected.

MATERIALS AND METHODS

Primary Cell Culture of Neonatal Hypothalamic Tissue. Fifteen-day-old female Sprague–Dawley rats were used to prepare hypothalamic cell suspensions for primary culture. Briefly, freshly excised hypothalami from 20 rats were subjected to enzymatic and mechanical dispersion.§ Tissue was incubated in Dulbecco's modified Eagle's medium containing 0.1% trypsin (Difco) at 37°C for 20 min, followed by trituration with pasteur pipettes of decreasing bore size. The resulting cell suspension was centrifuged (22°C, 1000 × *g*, 5 min) and resuspended in 50 ml of modified Eagle's medium containing 10% (vol/vol) fetal calf serum. Cells were plated in 35-mm plastic tissue-culture dishes at 1 hypothalamic equivalent per dish [dishes had been coated for 1 hr with a sterile solution of 5 μg of poly(L-lysine) per ml, M_r 15,000, and incubated for 4 days in a 5% CO_2/H_2O-saturated atmosphere at 37°C]. The medium was changed on the third day of incubation. At day 4 of culture, neurons exhibited extensive outgrowth of processes. Little or no network formation was seen, as judged by phase-contrast microscopy and silver staining.

Incubation of Cells with L-[^{35}S]Methionine. Four-day cell cultures were washed with neutral saline and modified Eagle's medium containing 0.1% bovine serum albumin, 0.1 mM bacitracin, and 5 μM L-methionine. L-[^{35}S]Methionine (specific activity 581 Ci/mmol, 100 μCi/ml; 1 Ci = 3.7 × 10^{10} becquerels) was added and the cells were incubated for 120 min.

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Abbreviations: ACTH, corticotropin (adrenocorticotropic hormone); β-LPH, β-lipotropin; γ-LPH, γ-lipotropin; α-MSH, α-melanotropin.

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§ The brain was removed from the skull, leaving the hypophysis under the dura. In order to exclude possible contamination by pars tuberalis, we further transected the remaining pituitary stalk above the level of the median eminence with iridectomy scissors under a dissecting microscope (35×) and excluded this area. Conventional H and E staining of cultured material gave no evidence of endocrine-like cells; electron microscopic examination of cultured cells did not reveal the presence of cells with ultrastructural characteristics of those of pituitary origin.

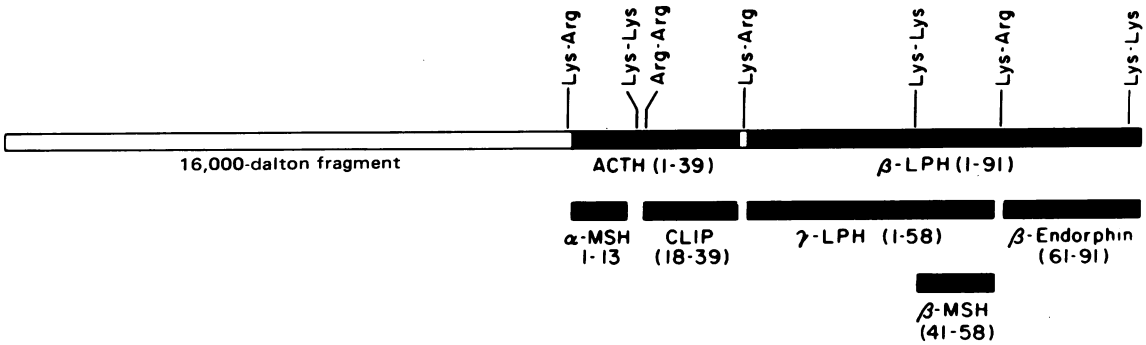

FIG. 1. Schematic representation of the bovine precursor molecule indicating an NH_2-terminal fragment, followed by ACTH-(1–39), which is followed by the β-lipotropin-(1–91) sequence. ACTH-(1–39) contains within it the peptide backbone of α-MSH and CLIP (corticotropin-like intermediate lobe peptide). β-Lipotropin contains within it the sequences of γ-LPH, β-MSH, and β-endorphin. Also indicated are the location of Lys-Arg or Lys-Lys groups that can be cleaved by proteolytic processing to yield the component peptides (ref. 1, as modified from ref. 2).

At the end of this period, medium was removed, made 0.2 M in HCl, and frozen at −70°C. Cells were scraped from the dishes, homogenized in 5 M CH_3COOH containing 0.1% albumin and 1 mM each of phenylmethylsulfonyl fluoride and iodoacetamide (20 hypothalamic equivalents/ml), and centrifuged at 1200 × *g*. The clear supernatant was diluted with an equal volume of H_2O, frozen at −70°C, and lyophilized.

Preparation and Incubation of Dispersed Anterior and Intermediate Lobe Cells with [^{35}S]Methionine. Separate suspensions of anterior and intermediate lobe cells derived from twelve 30-day-old female Sprague–Dawley rats were prepared as described (16). These cells were incubated with [^{35}S]methionine like the hypothalamic cells. This experiment was performed in order to obtain authentic radiolabeled rat ACTH- and β-endorphin-related products for comparison with any ACTH- or β-endorphin-like material detected in the hypothalamic cell preparation. In other experiments (not shown) we established precursor–product relationships for multiple molecular forms of ACTH and β-endorphin in both pituitary lobes.

Preparation and Use of Affinity-Purified Sepharose-Immobilized β-Endorphin and ACTH Antisera. Immobilized antisera (referred to as immunoadsorbents) were prepared and used for characterization of radiolabeled material as described (14, 15, 17).

Immunoprecipitation with Antiserum to Mouse 16,000-Dalton Fragment. This antiserum is specific for the NH_2-terminal non-ACTH, non-endorphin fragment of the mouse precursor ACTH/endorphin molecule. B. A. Eipper and R. E. Mains kindly provided this preparation and have demonstrated that it also crossreacts with the corresponding NH_2-terminal fragment of rat pituitary precursor ACTH/β-endorphin (5). Immunoprecipitation was performed as described (18).

$NaDodSO_4$/Polyacrylamide Gel Electrophoresis. [^{35}S]-Methionine-labeled immunoreactive ACTH/endorphin material was resolved in a vertical slab-gel apparatus (Bio-Rad model 220), with a 10–20% acrylamide gradient gel, in the buffer system of Laemmli (19). All comparisons of pituitary- and hypothalamus-derived material were made within the same run on different lanes of the same gel along with low molecular weight calibration proteins from Pharmacia. Molecular weight estimates by $NaDodSO_4$ gel electrophoresis are given for comparative purposes only because most studies on the biosynthetic pathway of ACTH-related peptides have used this technique. The anomalous behavior of glycoproteins and basic proteins in this system is well known (20), and such estimates must be viewed with caution.

Sephadex Gel Filtration. Gel filtration on columns packed with Sephadex G-50 or G-200 superfine beads was performed as described (8, 14). Molecular weight estimates were obtained by comparison of elution positions (k_{av}) of the ^{35}S-labeled tissue products with the elution positions of low molecular weight calibration proteins from Pharmacia.

Concanavalin A Binding Studies. These were performed with concanavalin A-Sepharose microcolumns as described (14).

Tryptic Digestion and High-Voltage Paper Electrophoresis of Tryptic Fragments. This was performed as described (6, 15) with the exception that in the present study the tryptic fragments were not purified by immunoprecipitation prior to electrophoresis.

Detection and Characterization of Immunoreactive ACTH and β-Endorphin. In order to detect and separate ^{35}S-labeled material containing the dual antigenic determinants of ACTH and β-endorphin (immunoreactive ACTH/endorphin) from material containing only one of these determinants, we used the immunoadsorbents sequentially, as described in Fig. 2. Immunoreactive ACTH/endorphin obtained by this procedure was subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis, Sephadex G-200 gel filtration under denaturing conditions, concanavalin A-Sepharose microcolumns, immunoprecipitation with antiserum to the 16,000-dalton fragment, and partial tryptic mapping. The sizes of the monodeterminant immunoreactive ACTH and β-endorphin species were determined on Sephadex G-50 columns.

RESULTS

Detection of ^{35}S-Labeled Immunoreactive ACTH/Endorphin. After incubation of all three cell types, ^{35}S-labeled material containing the dual antigenic determinants of ACTH and β-endorphin was detected and separated from ^{35}S-labeled material containing only the ACTH or the β-endorphin determinant by sequential use of the immunoadsorbents (Fig. 2).

$NaDodSO_4$/Polyacrylamide Gel Electrophoresis of ^{35}S-Labeled Immunoreactive ACTH/Endorphin. Fig. 3 depicts the similarity of the electrophoretic profiles of immunoreactive ACTH/endorphin synthesized by the hypothalamic and the anterior and intermediate cell preparations, as obtained by sequential elution from the immunoadsorbents. Material from these sources were resolved into two predominant species with apparent M_r of 33,000 ± 1000 and 36,500 ± 600. These values represent the average ± SD from three gel runs.

Concanavalin A Affinity Chromatography of ^{35}S-Labeled Immunoreactive ACTH/Endorphin. Aliquots of immunoreactive ACTH/endorphin derived from all three tissue extracts were applied to concanavalin A-Sepharose microcolumns. An average of 43.8% (n = 3) of the hypothalamic, 51.6% (n = 3)

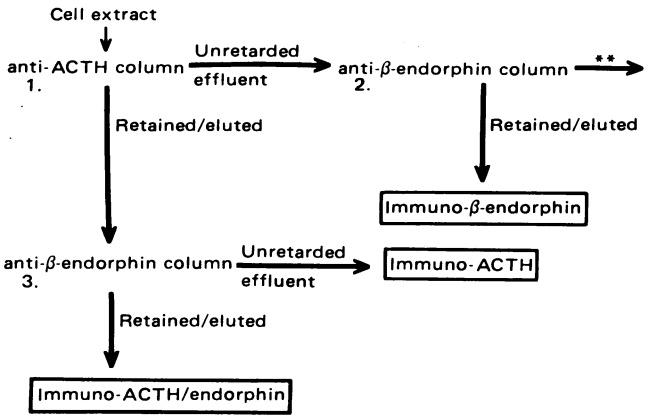

FIG. 2. Sequential use of the immunoadsorbents (anti-ACTH and anti-β-endorphin microcolumns). Each cell extract was first applied to an anti-ACTH column (no. 1). The unretarded effluent was then applied to an anti-β-endorphin column (no. 2); the unretarded effluent from this latter column contained ^{35}S-labeled material unrelated to ACTH and β-endorphin (**), whereas the specifically retained material represented immunoreactive (immuno) β-endorphin. Immuno-ACTH was eluted from the anti-ACTH column (no. 1) and applied to an anti-β-endorphin column (no. 3). The unretarded effluent from this latter column contained immuno-ACTH devoid of the β-endorphin antigenic determinant. The material specifically retained on column 3 represented molecules containing both antigenic determinants within the same molecules (immuno-ACTH/endorphin). All of these ^{35}S-labeled immunoreactive species were further characterized.

of anterior lobe, and 49.2% (n = 3) of intermediate lobe material initially applied was specifically retained and able to be eluted from the columns with buffer containing D-mannose. When buffer containing synthetic ACTH and β-endorphin (10 μg/ml for each) was used instead of mannose buffer, less than 6.2% of the retained ^{35}S radioactivity was eluted in all cases, presumably indicating the specificity of the interaction with this lectin preparation.

Sephadex G-200 Gel Filtration of Immunoreactive ACTH/Endorphin. Because the concanavalin A binding experiments suggested that immunoreactive ACTH/endorphin contained carbohydrate, molecular weight estimates of this material were repeated by gel filtration under denaturing conditions. The profiles (not shown) were again nearly identical for material derived from the three tissues, containing two poorly resolved peaks of apparent M_r of 29,700 and 31,800. These estimates were performed on only one occasion due to the relatively small amount of hypothalamic material available.

Immunoprecipitation of Immunoreactive ACTH/Endorphin with Antiserum to Mouse Pituitary Precursor 16,000-Dalton Fragment. Duplicate 500-cpm aliquots of immunoreactive ACTH/endorphin derived from all three tissues by sequential immunoadsorption were treated with 2 μl of antiserum in the presence of 5 μg each of synthetic ACTH-(1–39) and β-endorphin. In all cases, between 88 and 93% of the ^{35}S activity was precipitated. Highly purified rat precursor ACTH/β-endorphin obtained from whole pituitaries was used for an absorption control because purified 16,000-dalton fragment was not available to us. With this preparation, 81–89% of the total ^{35}S activity remained in the supernatant after immunoprecipitation.

Analysis of ^{35}S-Labeled Tryptic Fragments of Immunoreactive ACTH/Endorphin. Tryptic digests of hypothalamic and pituitary ^{35}S-labeled immunoreactive ACTH/endorphin were subjected to high-voltage paper electrophoresis (Fig. 4). Nearly identical profiles were obtained in all instances. Four

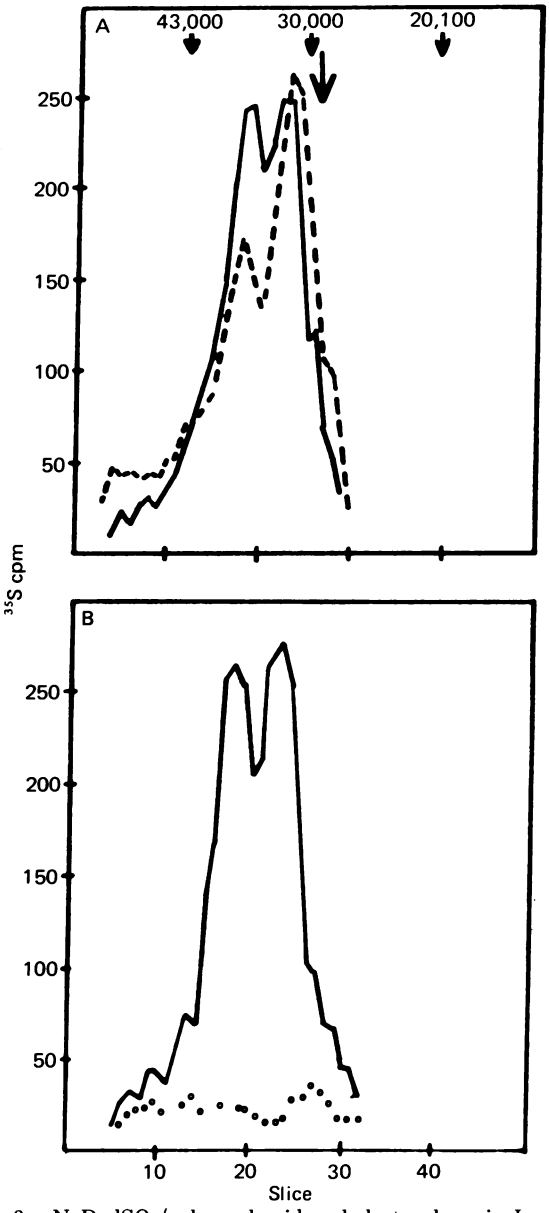

FIG. 3. $NaDodSO_4$/polyacrylamide gel electrophoresis. Immunoreactive ACTH/endorphin derived from anterior (—) and intermediate (---) pituitary lobe (*A*) and hypothalamic (*B*) cells was subjected to electrophoresis on different lanes of the same vertical slab gel. Material from all three tissue sources was resolved into two predominant molecular species of apparent M_r 33,000 ± 1000 and 36,500 ± 600. (*B*) ○, Profile obtained for hypothalamic material when immunoadsorption was performed in the presence of excess ligand [synthetic human ACTH-(1–39) and β-endorphin], indicating the specificity of the binding reaction. Similar adsorption controls were obtained for pituitary-derived material (data not shown). Small arrows indicate the migration positions of the calibration proteins (size shown in daltons); large arrow indicates the position of highly purified mouse pituitary 31,000-dalton precursor ACTH/β-endorphin.

discernable peaks of ^{35}S activity were detected between the origin and migration position of L-lysine. Two well-separated peaks were obtained that migrated with the electrophoretic mobility of synthetic β-LPH-(61–69) and ACTH-(1–8). The profile also contained two poorly resolved peaks [more basic than ACTH-(1–8)], presumably derived from the NH_2-terminal region of immunoreactive ACTH/endorphin. [This assumes that rat γ-LPH does not contain methionine, as has been demonstrated for mouse γ-LPH (21, 22). Previous studies have detected a methionine residue in the rat β-endorphin portion

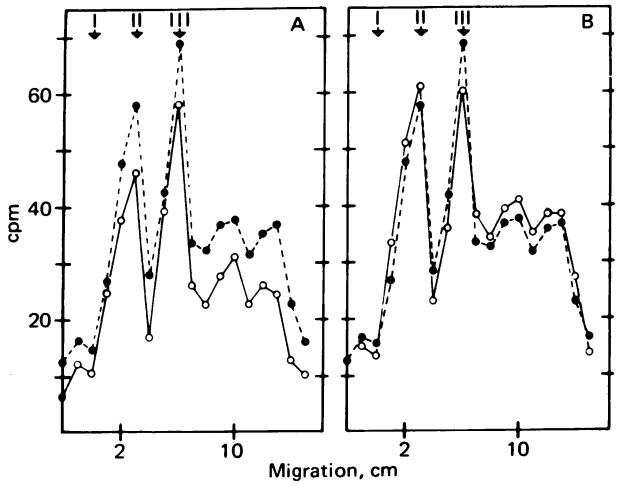

FIG. 4. Tryptic peptides of ^{35}S-labeled immunoreactive ACTH/endorphin. Immunoreactive ACTH/endorphin synthesized by hypothalamic and anterior and intermediate pituitary lobe cells in the presence of [^{35}S]methionine was prepared and material obtained from each cell was separately trypsinized. The ^{35}S-labeled tryptic peptides were analyzed by paper electrophoresis at pH 6.5. (*A*) Similarity of profiles obtained for material synthesized by intermediate lobe and hypothalamic cells. (*B*) Similarity of profiles obtained for the two intermediate lobe forms (33,000 and 36,500 daltons).

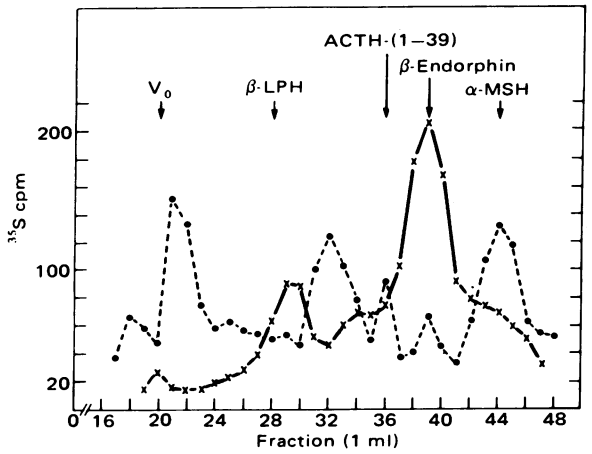

FIG. 5. Gel filtration pattern of monodeterminant ^{35}S-labeled immunoreactive ACTH and β-endorphin material obtained from the immunoadsorbents (see Fig. 2). Multiple peaks are present. The majority of immunoreactive β-endorphin coeluted with β-LPH and β-endorphin marker peptides. At least five peaks of immunoreactive ACTH are evident, two of which coeluted with human ACTH-(1–39) and α-MSH marker peptides. X—X, Reactive only with antibody against β-endorphin; ●--●, reactive only with antibody against ACTH.

of the β-LPH molecule but not in the γ-LPH sequence (7, 23), although these findings appear to disagree with those of Rubinstein *et al.* (24), who detected two methionine residues in the rat β-LPH sequence by amino acid composition analysis.] Fig. 4*A* shows the similarity of the tryptic profiles of intermediate lobe and hypothalamic derived material. A similar pattern (not shown) was also obtained for material derived from the anterior lobe. Because rat immunoreactive ACTH/endorphin exists in two predominant forms (apparent M_r of 36,500 and 33,000), it was of interest to see if any differences could be detected in the tryptic profiles of each of these forms. The species synthesized by intermediate lobe cells were used for this purpose because there was insufficient material available synthesized by hypothalamic or anterior lobe cells. Separate pools of these two species of precursor ACTH/β-endorphin were obtained, each containing less than 9% of the other form, by performing three electrophoretic and gel filtration separations, each time discarding the appropriate regions of the profile. The resolved forms were trypsinized. Fig. 4*B* shows that virtually identical electrophoretic profiles resulted when the same amount of ^{35}S activity was analyzed, indicating the similarity of the peptide backbone in the two precursor forms, at least with respect to the total number of methionine residues and the placement of some of the basic amino acid residues (sites of tryptic cleavages) contained within them.

Gel Filtration of Lower Molecular Weight Immunoreactive ACTH and β-Endorphin Forms Synthesized by Hypothalamic Cells. Hypothalamic ^{35}S-labeled material containing either the ACTH or the β-endorphin antigenic determinant was subjected to Sephadex G-50 gel filtration. Immunoreactive ACTH and β-endorphin were chromatographed on separate columns, and all fractions were immunoprecipitated with the same affinity-purified antisera preparations used to construct the immunoadsorbent to ensure the specificity of the profiles obtained (Fig. 5). The β-endorphin profile contains two prominent peaks of activity that eluted within the regions of the β-LPH and β-endorphin marker peptides. The majority of this activity eluted in the β-endorphin region. The ACTH profile contains at least five peaks of ^{35}S activity. A minor peak eluted in the region of the ACTH marker peptide and a major peak coeluted with synthetic α-MSH. The remaining peaks may represent transient intermediates in the biosynthetic pathway similar to those seen in the pituitary. [The 2-hr labeling paradigm used is insufficient to yield a steady-state labeling profile, underrepresenting the smaller forms (products) and overrepresenting the larger immunoreactive forms (precursor and intermediates) relative to the distribution of molecular species *in situ*]. Similar studies (data not shown) indicate that the major forms seen on Sephadex G-50 patterns derived from anterior lobe cells eluted with ACTH and β-LPH markers whereas those from intermediate lobe cells eluted with α-MSH and β-endorphin markers.

DISCUSSION

The present data demonstrate that cells within the hypothalamus synthesize multiple forms of immunoreactive ACTH and β-endorphin. High molecular weight material containing the dual antigenic determinants of ACTH and β-endorphin and determinants recognized by an antiserum directed against the NH_2-terminal non-ACTH, non-β-endorphin sequences of mouse pituitary precursor (5) as well as several smaller molecular species (containing either the ACTH or β-endorphin determinants, but not both) were detected. On the basis of limited characterization (Fig. 5), four of these forms have been tentatively identified as ACTH-(1–39), α-MSH, β-LPH, and β-endorphin. The α-MSH- and β-endorphin-like material appeared to be major products. This pattern of labeling is consistent with (but not proof of) a precursor–product relationship between the high molecular weight material and the smaller forms. Limitations imposed by the small yield of specifically radiolabeled hypothalamic material make it impractical at this time to attempt to elucidate the kinetics of labeling of the multiple forms of ACTH and β-endorphin through the use of pulse–chase experiments.

In support of a precursor function for hypothalamic immunoreactive ACTH/endorphin is the finding that this material is indistinguishable from precursor ACTH/β-endorphin synthesized by rat anterior and intermediate pituitary lobe cells

with respect to the variables of physicochemical characterization used in this study. A precursor function of such material derived from rat pituitary cells has been demonstrated (5, 7, 11). Two predominant size classes of precursor-like material are synthesized by all three tissues. Both these forms are glycoproteins (inferred from the concanavalin A binding experiments) and probably possess a similar peptide backbone [inferred from partial tryptic mapping in this study and shown to be the case for mouse pituitary forms (4, 6)]. The apparent size heterogeneity is probably due to different carbohydrate contents, as demonstrated for the precursor forms synthesized by AtT-20 mouse pituitary tumor cells (4, 6) and for those synthesized by toad pituitary intermediate lobe cells (10).

Although the precursor-like forms appear similar in hypothalamus and anterior and intermediate pituitary lobes, processing of this material appears to differ in the three tissues. In the anterior lobe, β-LPH and ACTH are the major products, whereas in the intermediate lobe β-endorphin, γ-LPH, α-MSH, and ACTH-(18–39) appear to be the end products of processing. Based on our limited observations, processing in hypothalamus more closely resembles that in the intermediate lobe, although differences appear to exist (25).¶

We have previously shown (15) that bovine hypothalamic and anterior pituitary precursor-like material also exists in two predominant forms (apparent M_r by $NaDodSO_4$ gel electrophoresis of 36,000 and 41,500) that are larger than those reported herein for rat forms and previously reported for mouse pituitary forms (M_r = 39,000, 32,000, and 34,000) (4, 6). Thus, although an interspecies heterogeneity appears to exist with respect to apparent molecular weight, within a species both lobes of the pituitary and the hypothalamus synthesize precursor-like material of the same molecular weights. These data suggest that within a species the same mRNA(s) is translated (and, hence, the same gene is transcribed) regardless of the site of synthesis.

We acknowledge the gifts of 16,000-dalton fragment antibody and highly purified mouse 31,000-dalton ACTH/endorphin from B. A. Eipper and R. E. Mains, synthetic β-LPH-(61–69) from S. Udenfriend and S. Stein, and β-endorphin antiserum from N. Ling. We thank P. Crine for making his manuscript available to us before its publication. These studies were supported in part by U.S. Public Health Service Grant NB 02893-15, the Lita Annenberg Hazen Charitable Trust, and by National Science Foundation Grant BNS 78-84506. J. F. McK. is the recipient of a Research Career Development Award (AM 00331) from the National Institutes of Health.

¶ Extracts of hypothalamic tissue maintained in culture contain a significantly lower ratio of immunoreactive α-MSH to NH_2-terminal ACTH than does intermediate lobe tissue similarly incubated.

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