Processing of the Precursor to Adrenocorticotrophic Hormone and β-Lipotropin in Monolayer Cultures of Mouse Anterior Pituitary†

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ABSTRACT: The synthesis of adrenocorticotrophic hormone (ACTH), β-lipotropin (β-LPH), and β-endorphin has been studied in primary cultures from mouse anterior pituitary by pulse–chase techniques of protein labeling, followed by immunoprecipitation with ACTH- or β-LPH-specific antisera and sodium dodecyl sulfate ($NaDodSO_4$) gel electrophoresis. The forms of the hormones were identified by immunoprecipitation with specific antibodies, by determination of their apparent molecular weights, and by mapping their tryptic peptides. After incubation of cultures with [^{35}S]methionine for 15 min, about two-thirds of the immunoprecipitable label was present as a 29 000 molecular weight form of pro-ACTH-endorphin (29K pro-ACTH-endorphin) and the remainder as a 32 000 molecular weight form. When a chase was performed for 30–120 min, there was a decrease in label in 29K pro-ACTH-endorphin accompanied by an increase in label in 32K pro-ACTH-endorphin and followed closely by the appearance of label in two forms of ACTH with apparent molecular weights of 27 000–28 000 and 23 000. A β-lipotropin-like molecule became labeled with a time course somewhat slower than that of 27 000–28 000 and 23 000 molecular weight forms of ACTH. Label appeared in 14 000 and 4500 molecular weight forms of ACTH after a 30-min chase period. Very little label appeared in β-endorphin-like material even after a 16-h chase period in contrast to β-lipotropin which contained over 95% of the labeled immunoprecipitable material after this time. When cultures were incubated with [^{3}H]-glucosamine and [^{35}S]methionine, all of the ^{35}S-containing forms of ACTH except 4500 molecular weight ACTH became labeled with tritiated sugar. These studies show that proteolytic processing of pro-ACTH-endorphin in primary cell cultures from mouse anterior pituitary is similar to that in mouse pituitary tumor cells (AtT-20/D_{16v} cells) except that it has a slower time course.

Adrenocorticotropin and β-lipotropin are synthesized as part of the same precursor protein in AtT-20/D_{16v} tumor cells (Roberts & Herbert, 1977a,b; Mains et al., 1977) and in pituitary glands from a number of different animals (Nakanishi et al., 1977a; Roberts et al., 1978; Eipper & Mains, 1978; Crine et al., 1978; Loh, 1979; Loh & Gainer, 1979). Although similar forms of the precursor are present in anterior and intermediate lobes of the pituitary, these forms are processed to different end products in the two lobes (Roberts et al., 1978; Eipper & Mains, 1978). The major end products in the anterior lobe are glycosylated and unglycosylated α(1–39)-ACTH[1] (13K and 4.5K ACTH, respectively), β-LPH, and the amino-terminal portion of the precursor (Roberts et al., 1978; Eipper & Mains, 1978). In the intermediate lobe, ACTH is processed further to α-melanocyte-stimulating hormone (α-MSH) and corticotropin-like intermediate lobe peptide (CLIP); β-LPH is converted to β-endorphin and the fate of the amino-terminal portion is uncertain (Scott et al., 1973, 1976; Eipper & Mains, 1978). Regulation of the release of hormones is also different in the two lobes of the pituitary. Release of ACTH and β-LPH from the anterior lobe is stimulated by corticotropin releasing factor(s) (CRF) (Guillemin & Rosenberg, 1955) which is made in the hypothalamus and delivered to the pituitary via a portal circulatory system and inhibited by glucocorticoids (Nakanishi et al., 1977b; Bergland & Page, 1979; Przewlocki et al., 1979; Vale et al., 1978; Guillemin et al., 1977; Krieger et al., 1978; Yasuda et al., 1978; Roberts et al., 1979). Release of intermediate lobe hormones is not regulated by corticotropin releasing factors and glucocorticoids but rather by direct neural input from the brain (Scott & Baker, 1975).

Detailed investigations of the processing of pro-ACTH-endorphin have been made by using AtT-20/D_{16v} tumor cells as a model system, because these cells mimic the corticotrophic functions of the anterior pituitary (Allen et al., 1978; Herbert et al., 1978) in that they produce large quantities of ACTH and β-LPH but not other anterior pituitary hormones (Herbert et al., 1978) and the production of these hormones is regulated by glucocorticoids and CRF. The first form of the precursor synthesized in AtT-20 cells is a 29 000 molecular weight glycoprotein which can be further glycosylated to a 32 000 molecular weight form (Roberts & Herbert, 1977a; Roberts et al., 1978). In the diagram of the precursor (see Chart I), the amino acid chain is represented by the long block, with the different peptide regions named and delineated (by arrows) at sites which have dibasic residues susceptible to trypsin-like cleavage. The 29K glycosylation site is represented as a solid circle. The other glycosylation site, yielding the 32K precursor, is indicated by the dashed circle. Then, by a series of proteolytic cleavages and glycosylation steps these forms are processed to their constituent hormones as shown in Scheme I.

In this study we have asked how normal pituitary cells from mouse process pro-ACTH-endorphin and if the processing events in the AtT-20 tumor cell line accurately reflect the processing events in anterior pituitary cells. We have used monolayer cultures of anterior pituitary cells for these studies

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[1] Abbreviations used: ACTH, adrenocorticotrophic hormone; β-LPH, β-lipotropin; $NaDodSO_4$, sodium dodecyl sulfate; α-MSH, α-melanocyte-stimulating hormone; CLIP, corticotropin-like intermediate lobe peptide; CRF, corticotropin-releasing factor(s); Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; IAA, iodoacetamide; Tris, tris(hydroxymethyl)aminomethane; YADH, yeast alcohol dehydrogenase; [^{35}S]Met, [^{35}S]methionine; Cl_3AcOH, trichloroacetic acid; TPCK, L-(tosylamido-2-phenyl)ethyl chloromethyl ketone; EDTA, ethylenediaminetetraacetic acid.

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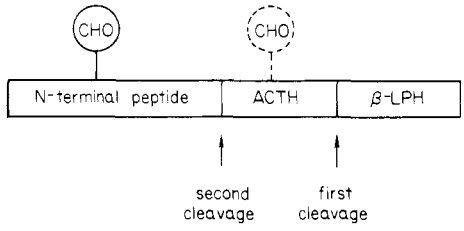
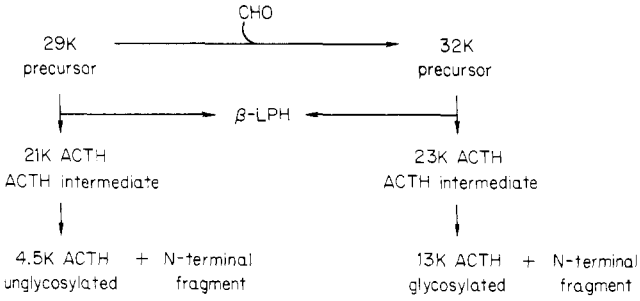
Chart I



Scheme I



because previous work has shown that these cultures retain their responses to physiological regulators such as CRF and glucocorticoids for a number of days in culture (Paquette et al., 1979). Furthermore, radiolabeling techniques that we developed for study of precursor processing in AtT-20 cells are easily adapted to study processing in primary cultures.

In this paper we present a study of the synthesis and processing of the common precursor to ACTH and β-LPH in primary cultures of the mouse anterior pituitary.

Materials and Methods

L-[^{35}S]Methionine (600–1400 Ci/mmol) and D-[6-^{3}H]-glucosamine hydrochloride (10–25 Ci/mmol) were obtained from Amersham. Collagenase, hyaluronidase, neuraminidase, and deoxyribonuclease (DNase) were purchased from Sigma Chemical Co. TPCK-treated trypsin was obtained from Worthington Biochemical Corp.

Primary Culture of Mouse Anterior Pituitary. Anterior lobes were cultured as described previously (Allen et al., 1978) with minor modifications. Briefly, the pituitaries were dissected from adult, male, C57Bl/6J mice; the anterior lobes were separated and minced and the minced pieces incubated 5 min in 0.4 mL of Hepes–BSA–DNase at room temperature. This suspenison was then added to 1 mL of Hepes buffer containing collagenase and hyaluronidase and incubated 40 min at 37 °C. The pieces were washed with Hepes containing neuraminidase and then incubated in 1 mL of the same solution for 10 min at 37 °C. The tissue was drawn through a siliconized, fire-polished Pasteur pipet several times to disperse the cells and then washed with Hepes and finally with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% horse serum. The cells were suspended in serum-supplemented DMEM and equal aliquots were plated in plastic tissue culture miniwells (multiple 16-mm wells) containing serum-supplemented DMEM and incubated at 37 °C in a 5% CO_2–95% air incubator for 4 days to allow attachment of cells.

We found that there was no significant change with regard to the following properties for at least 6 days in culture: (a) level of ACTH and β-endorphin in the cells; (b) stimulation of ACTH release by hypothalamic extract or vasopressin and inhibition of release by glucocorticoids (Paquette, 1977; Allen et al., 1978); (c) distribution of the molecular weight forms of ACTH as compared to the distribution found in the anterior pituitary (Paquette et al., 1979; Allen et al., 1978; Roberts et al., 1978).

Incubation with [^{35}S]Methionine. (A) Pulse Chase. After 4 days in culture, the medium was replaced with 0.3 mL of methionine-free, serum-free DMEM containing 200 μCi of [^{35}S]methionine (final concentration, 1×10^{-3} mM) and incubated for 15 min. The cells were washed 5 min with serum-free DMEM, and then chases were performed for various time periods with 0.5 mL of serum-free DMEM (final methionine concentration, 0.5 mM). The cells were then extracted with one 0.5-mL aliquot of 5 N acetic acid containing 300 μg/mL phenylmethanesulfonyl fluoride ($PhCH_2SO_2F$) and 300 μg/mL iodoacetamide (IAA). The extracts were frozen on dry ice and thawed in a 22 °C water bath 3 times and left at 4 °C overnight. They were then centrifuged at 10000*g* for 10 min at 4 °C, and the supernatants were split into appropriate aliquots for subsequent lyophilization.

(B) Continuous Labeling. After the cells were cultured for 4 days, the medium was replaced with 0.3 mL of methionine-free, serum-free DMEM containing 400 μCi of [^{35}S]-methionine (final concentration, 2.02×10^{-3} mM). The cells were allowed to incubate for appropriate time periods and extracted. The extracts were treated as described in the pulse–chase section and lyophilized.

Incubation with [^{3}H]Glucosamine and [^{35}S]Methionine. After 4 days of culture, the medium was replaced with 0.3 mL of serum-free, low-glucose DMEM (glucose concentration, 5.6 mM) containing 0.5 mCi of [^{3}H]glucosamine (final concentration, 0.044 mM) and 0.2 mCi of [^{35}S]methionine. The cells were allowed to incubate for 8 h and then extracted with 5 N acetic acid with 300 μg/mL of $PhCH_2SO_2F$ and IAA, and the extracts were treated as described previously.

Immunoprecipitation. The double-antibody immunoprecipitation procedure used for purifying ACTH and endorphin proteins in extracts of AtT-20 cells (Roberts et al., 1978) had to be modified for use with anterior pituitary cell extracts, because anterior pituitary cultures contain large amounts of growth hormone, prolactin, and other proteins that contaminate the immunoprecipitate and interfere with detection of labeled ACTH/endorphin proteins on $NaDodSO_4$ gels. AtT-20 cells do not produce growth hormone or prolactin (Yasamura et al., 1966; Herbert et al., 1978). If the proteins which bind nonspecifically are removed from the extract prior to immunoprecipitation of the ACTH/endorphin proteins, the contamination problem is largely overcome. This was accomplished as follows. After dissolving the lyophilized cell extracts in 400 μL of NET buffer [150 mM NaCl, 5 mM EDTA, and 20 mM Tris, titrated with HCl to pH 7.4, followed by addition of Triton-X-100 (0.005 v/v)], nonimmune rabbit immunoglobulin and goat anti-rabbit globulin were added. Then the mixture was incubated for 4–6 h and the precipitate sedimented by centrifugation. The supernatant was removed and immunoprecipitated with ACTH-specific antiserum (Wilma) or β-endorphin-specific antiserum (Bridgette) as described previously (Roberts & Herbert, 1977a; Allen et al., 1978). The preincubation with nonimmune IgG and goat anti-rabbit serum removed almost all of the nonspecifically bound proteins (mostly in the 20–21K region of the gel) without removing ACTH or β-endorphin proteins. The antisera were produced in rabbits by injecting a concentrate of tissue culture medium from AtT-20/D_{16v} tumor cell cultures. Antiserum Wilma was purified on an Affigel column cross-linked to α_p(1–39)ACTH, and antiserum Bridgette was purified on an Affigel column cross-linked to β-LPH (M. A. Phillips, M. Budarf, and E. Herbert, unpublished experiments).

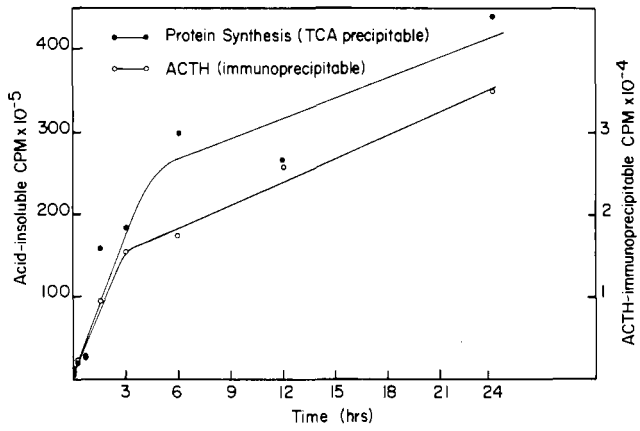


FIGURE 1: Incorporation of [^{35}S]methionine in primary pituitary cultures. Continuous incubations of cells were carried out in serum-free, methionine-free DMEM as described under Materials and Methods by using 400 μCi of L-[^{35}S]methionine/incubation. Cells were extracted with 500 μL of 5 N acetic acid (containing 300 μg/mL $PhCH_2SO_2F$ and IAA). A 4-μL aliquot was pipetted onto a 2.4-cm Whatman No. 1 filter paper disk for use in determining acid-insoluble cpm (●) with a Cl_3AcOH precipitation as described previously. The remainder of the extract was lyophilized and immunoprecipitated with antiserum Wilma, and the ACTH-immunoprecipitable cpm (○) was determined by counting an aliquot of the dissolved immunoprecipitate.

The amount of ACTH or LPH was determined by radioimmunoassay (RIA) as previously described, and excess of antibody over antigen was used for the immunoprecipitation to ensure quantitative precipitation.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. Immunoprecipitates were dissolved in gel buffer and radioactive proteins were analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis with Tris–acetate buffered at pH 6.4 (Bio-Rad Laboratories, 1974). Dansylated yeast alcohol dehydrogenase (YADH) and dansylated myoglobin (Mb) were used as internal standards for all gels. Gels were cut in 1-mm slices and eluted with either 0.5 M urea, 5 mM $NaHCO_3$, 0.1% Triton-X-100, and 0.1% $NaDodSO_4$ for counting directly or 50 mM Tris-HCl, pH 7.6, 0.1% Triton-X-100, and 0.1% $NaDodSO_4$ for tryptic digestion. Pooled gel eluates were made 10% in Cl_3AcOH after addition of bovine serum albumin (BSA) as protein carrier. Precipitates were washed 2 times with acetone–ether (1:1 v/v), dried by evaporation, and dissolved in 0.1 M NH_4HCO_3, pH 8.5, for digestion with TPCK-treated trypsin. Trypsin digestion and paper electrophoresis were accomplished as described previously (Roberts & Herbert, 1977a,b).

Results

Synthesis and Processing of Pro-ACTH-endorphin. To maximize incorporation of label into pro-ACTH-endorphin, we incubated anterior pituitary cultures with high specific activity [^{35}S]Met in Met-free DMEM and in the absence of horse serum (0.036 mM in methionine). Under these conditions, incorporation of [^{35}S]Met into acid-insoluble protein and immunoprecipitable ACTH was linear for at least 3 h (Figure 1). After that time the rate fell off substantially, but incorporation continued for at least 24 h at the reduced rate. It was also found that it took approximately 90–120 min for radioactive forms of ACTH and β-LPH to appear in the culture medium under these conditions. Incorporation of [^{35}S]Met was linear for somewhat longer periods of time in complete media. However, the use of Met-free medium without serum did not substantially alter incorporation of [^{35}S]Met into Cl_3AcOH-insoluble material from cell extracts (8.4×10^6 Cl_3AcOH-insoluble cpm without serum compared to 7.9×10^6 cpm with serum at 2 h and 32×10^6 cpm without serum compared to 41×10^6 cpm with serum at 20 h). Also, the use of Met-free medium without serum did not alter either of the following parameters: (1) incorporation of [^{35}S]Met into immunoprecipitable material (ACTH antiserum) relative to incorporation into Cl_3AcOH-insoluble material for at least 2 h and (2) the distribution of the forms of ACTH for at least 8 h.

Another advantage of using Met as the labeled amino acid in these experiments was that each of the major domains of the precursor molecule [β-LPH, α(1–39)ACTH, and N-terminal fragment] contains a single Met residue. Therefore, it was relatively easy to account for shifts of radioactivity as these regions were cleaved out of the precursor.

Pulse–Chase Experiments. Primary cell cultures were labeled for 15 min with [^{35}S]Met; the label was removed and chases were performed for the time periods shown in Figure 2. Cell extracts were prepared and immunoprecipitated with either ACTH or β-LPH antiserum (Roberts & Herbert, 1977a), with modifications as noted under Materials and Methods. The proteins in the immunoprecipitate were fractionated by $NaDodSO_4$ gel electrophoresis. The results in Figure 2 show that after 15 min of incubation with [^{35}S]Met, 45–65% of the label in the β-LPH immunoprecipitates was present as a 30 000–31 000 molecular weight component with most of the remainder of the label present as a 35 000 molecular weight component. Figure 3 shows similar results for ACTH immunoprecipitates. As chase time was increased, radioactivity decreased in the higher molecular weight forms and increased in the lower molecular weight forms of ACTH (27–28K, 23K, 14K, and 4.5K ACTH) and β-endorphin [12.5K (β-LPH-like) and 3.5K β-endorphin].

Total radioactivity in the ACTH and LPH immunoprecipitates did not change significantly during the 30- and 60-min chase periods when almost all of the radioactivity was in the precursor forms. For example, there were 1940 cpm in the LPH immunoprecipitate after the initial 15-min pulse in the experiment in Figure 2 and 1920 cpm in the LPH immunoprecipitate after 1 h of chase. Total radioactivity in the LPM immunoprecipitate declined after longer chase periods (to 1200 cpm after 3 h and 800 cpm after 16 h of chase) as radioactivity accumulated in the lower molecular weight forms of ACTH and in β-LPH-like material (Figures 2 and 3). The decline in radioactivity in the LPH immunoprecipitate after 3, 8, and 16 h of chase could be accounted for in part by the increase in label in intracellular forms of ACTH (14K and 4.5K ACTH) and by the secretion of radioactive ACTH and β-LPH into the culture medium. However, since we did not determine the fate of the N-terminal portion of the precursor molecule (which has one Met residue) during processing in these cultures, it was not possible to account for all of the label lost from LPH and ACTH immunoprecipitates during the later chase periods.

We have shown previously that 29 000–31 000 and 32 000–35 000 molecular weight components present in anterior pituitary cells (Figures 2 and 3) comigrate with the 29K and 32K components of pro-ACTH-endorphin from AtT-20 cells on $NaDodSO_4$ slab gels (Herbert et al., 1979a); therefore, in general discussions of molecular weight moieties, we will refer to these forms as 29K and 32K components, respectively, to avoid confusion in terminology.

Summary of Pulse–Chase Experiments. The results of the pulse–chase experiments can be interpreted more readily by plotting the quantity of radioactivity in each molecular weight form of ACTH, β-LPH, or endorphin against time of chase

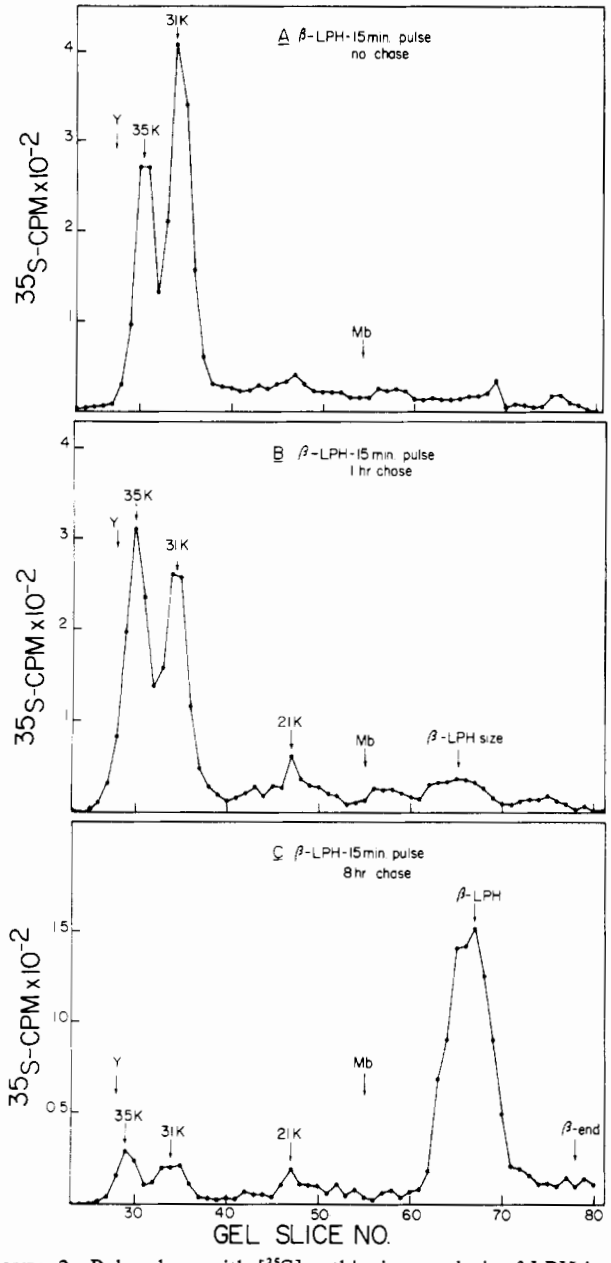


FIGURE 2: Pulse chase with [^{35}S]methionine; analysis of LPH immunoprecipitates by NaDodSO$_4$ gel electrophoresis. Primary cell cultures were grown in 16-mm wells for 4 days with DMEM supplemented with 10% horse serum. The cells were rinsed quickly with medium, pulsed for 15 min with 200 μCi of [^{35}S]methionine in 300 μL of serum-free, methionine-free DMEM, rinsed quickly, and then incubated for 0 (A), 0.5, 1 (B), 3, 8 (C), or 16 h in 500 μL of unlabeled, serum-free DMEM. Cells were extracted as described and aliquots were taken for radioimmunoassay of total LPH/endorphin content. Cell extracts were divided into two aliquots and lyophilized, and one aliquot was immunoprecipitated with antiserum Bridgette for LPH/endorphin and analyzed by NaDodSO$_4$ gel electrophoresis with 12% Biophore gels. Dansyl-YADH (Y) and dansylmyoglobin (Mb) were included as internal markers for determining relative mobilities of radioactive proteins. Gels were cut in 1-mm slices. Background has been subtracted.

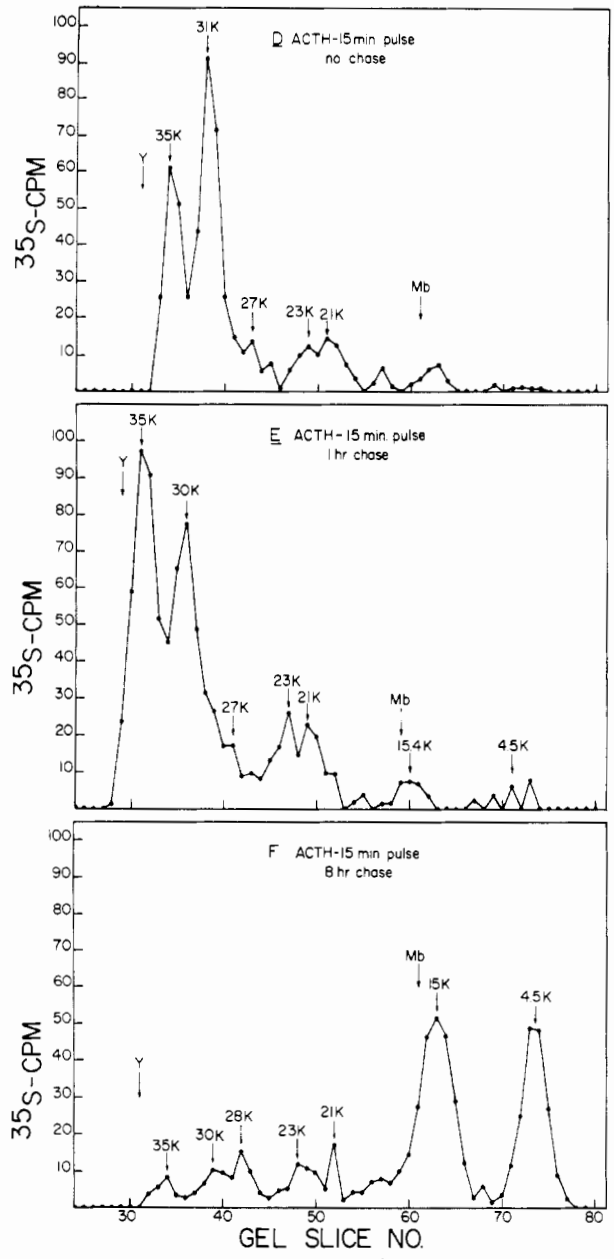


FIGURE 3: Pulse chase with [^{35}S]methionine; analysis of ACTH immunoprecipitates by NaDodSO$_4$ gel electrophoresis. Aliquots from the extracts described in Figure 2 for 0 (D), 0.5, 1 (E), 3, 8 (F), or 16 h of chase were radioimmunoassayed for ACTH content. An aliquot of the cell extract was then lyophilized and immunoprecipitated with antiserum Wilma for ACTH and analyzed by NaDodSO$_4$ gel electrophoresis as described in Figure 2.

as in Figure 4. Clearly demonstrated is the rapid rise and fall of radioactivity in the 29K pool of pro-ACTH-endorphin, followed by the rise and fall of radioactivity in the 32K pool, suggesting that these forms are biosynthetic precursors. The slower accumulation of radioactivity in the 27–28K and 23K forms of ACTH is followed by a decrease to a lower steady level, suggestive of the behavior of processing intermediates. The steady accumulation of radioactivity in the 14K and 4.5K forms of ACTH for the entire 16-h chase period indicates that these forms are end products of processing. It is interesting to note (Figure 4) that radioactivity appears in β-LPH-like protein somewhat later than it does in ACTH intermediates. It is also clearly demonstrated that β-LPH size material is the major component of endorphin-immunoprecipitable protein even after 16 h. The absence of β-endorphin size material is not due to antibody preference for LPH, since antiserum Bridgette has been shown to immunoprecipitate endorphin quantitatively in the presence of β-LPH.

Identification of the Forms of the Hormones. Samples from continuous labeling experiments were immunoprecipitated with the ACTH antiserum and then the β-LPH antiserum. The immunoprecipitates were then fractionated by NaDodSO$_4$ gel electrophoresis and it was found that the first immunoprecipitation had removed all of the radioactive 29K and 32K

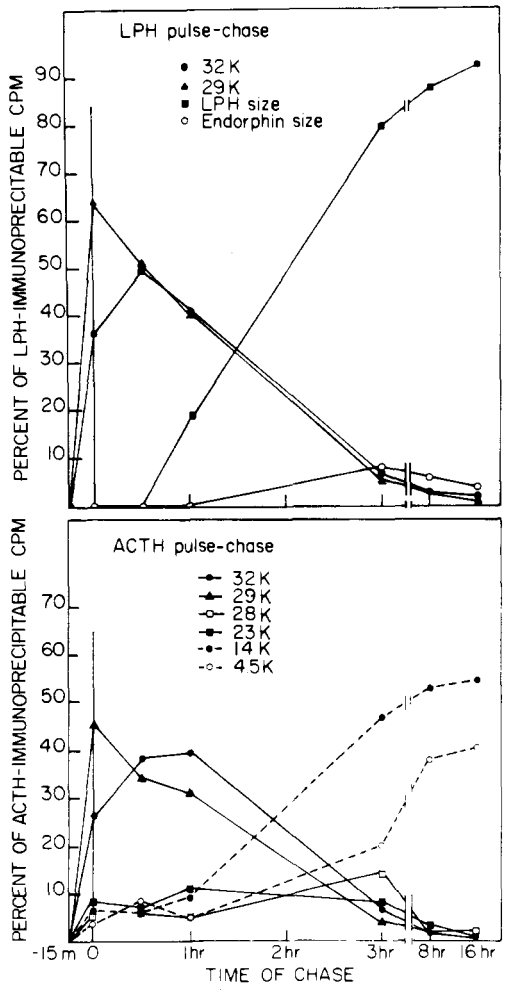


FIGURE 4: Pulse chase with [^{35}S]methionine; summary of $NaDodSO_4$ electrophoresis analysis. The quantity of labeled protein, expressed as a percentage of the total immunoprecipitable cpm, in each form of LPH or ACTH was determined by summing the cpm in each peak and dividing the sum by the number of methionines present in each form [as shown by tryptic mapping in Figure 5 and also Roberts & Herbert (1977a,b)]. This was determined for each time period shown in Figure 2 as well as the 0.5-, 3-, and 16-h time periods. The cpm of points between two peaks was halved for adjacent peaks.

proteins from the sample indicating that the β-LPH and ACTH determinants are present in the same proteins and that antiserum Wilma did not interact with LPH/endorphin determinants.

A 1000-fold excess of bovine serum albumin, poly(L-lysine), the α(1–24) fragment of ACTH, or β-endorphin had negligible effects on the binding of the molecular weight forms of ACTH (32K, 29K, 27–28K, 23K, 14K, and 4.5K) in immunoprecipitations of cell extracts (data not shown).

Levels of ACTH were determined by radioimmunoassay of aliquots from cell extracts, and then the extracts of the 90-min and 6-h continuous labeling incubations were immunoprecipitated with ACTH antiserum Wilma in the presence or absence of a 1000-fold excess of nonradioactive α_p(1–39)ACTH. Analysis by $NaDodSO_4$ gel electrophoresis of the immunoprecipitate of the 6-h labeling experiments (Figure 5) indicated that excess α_p(1–39)ACTH displaced all the major molecular weight forms of ACTH seen on the gels (34K, 29K, 27K, 22K, 15K, and 4.5K). The mobility of the material remaining after competition (at ~21 000 daltons) corresponded closely with that expected for growth hormone. Identical results were obtained for the forms present in the 90-min sample (data not shown).

The radioactive hormones were eluted from the gels and digested with trypsin. The digests were analyzed by paper electrophoresis of pH 6.5. Figure 6A shows that the mobilities of the [^{35}S]Met-labeled tryptic peptides of the 29K protein are almost identical with the [^{35}S]Met-containing tryptic peptides from the 29K form of pro-ACTH-endorphin isolated from AtT-20/D_{16v} tumor cells. The three major peptides present in AtT-20 cells have been identified previously as β(61–91)LPH (the neutral peptide), α(1–8)ACTH peptide, and the split peptide as the N-terminal peptide of the common precursor (Roberts & Herbert, 1977b). The 32K form has the same [^{35}S]Met-tryptic peptides as the 29K form (data not shown). Figure 6B shows that the neutral β(61–69) peptide is missing from the 27–28K form. The peptides derived from the 23K form are similar to those of the 27–28K form. The 14K (Figure 6C) and 4.5K forms of ACTH are missing both the β(61–69)LPH peptide and the N-terminal peptide as one would expect of α(1–39)ACTH (material at the origin was likely undigested protein). Figure 6D shows that the 12.5K (β-LPH/like) form of endorphin has only the [^{35}S]Met-tryptic peptide located in the neutral position. The 3.5K form of endorphin also has only the neutral peptide (data not shown).

Glycosylated Forms of Pro-ACTH-endorphin and ACTH. It has been shown that glycosylation is involved in the processing of pro-ACTH-endorphin in AtT-20 cells. In order to determine if similar glycosylated forms of pro-ACTH-endorphin and ACTH exist in anterior pituitary cells, we incubated cultures of anterior pituitary for 8 h in low glucose DMEM with [^{35}S]Met and [^{3}H]glucosamine. Low glucose medium enhances incorporation of labeled glucosamine into glycoproteins and the double-label approach allows one to identify glycoproteins with more certainty. The cells were extracted and the extracts were immunoprecipitated with ACTH antiserum. The radioactive proteins in the immunoprecipitate were fractionated by $NaDodSO_4$ gel electrophoresis. The results in Figure 7 show that the [^{35}S]Met-labeled peaks that contain ^{3}H label are 35K and 31K pro-ACTH-endorphin, 27K and 23K ACTH intermediates, and 15K ACTH end product. The exact position of the [^{35}S]Met peak for the 23K intermediate is obscured due to interference with 21K material. Since this is probably growth hormone which is unglycosylated, the ^{3}H peak was not affected. The only ^{35}S peak that does not contain significant ^{3}H label is 4.5K ACTH. The negligible amount of ^{3}H label under the 4.5K peak is most likely due to crossover effects of ^{35}S label during counting, although efforts were made to compensate for this. Several other experiments with ^{3}H sugar labels have demonstrated no ^{3}H label associated with the 4.5K peak (data not shown). Therefore, all the forms of pro-ACTH-endorphin and ACTH that are glycosylated in tumor cells (Eipper et al., 1976; Roberts et al., 1978) appear to be glycosylated in mouse anterior pituitary cells. Since the [^{35}S]Met-tryptic maps of 14K and 4.5K components contain only the α(1–8)ACTH peptide, it is likely that the 14K component is a glycosylated form of α(1–39)ACTH and the 4.5K component is α(1–39)ACTH as is the case in AtT-20 tumor cells (Eipper & Mains, 1977). Additionally, the absence of significant ^{3}H label in the 4.5K form (Figure 7) indicates that conversion of [^{3}H]glucosamine to ^{3}H-labeled amino acid in primary cultures was too slow to be detected in these experiments.

Discussion

The kinetics of label accumulation in 29K and 32K forms are very similar to those seen in AtT-20 tumor cells, particularly during early periods. Since it takes more than 90 min for labeled ACTH/endorphin proteins to be released into the

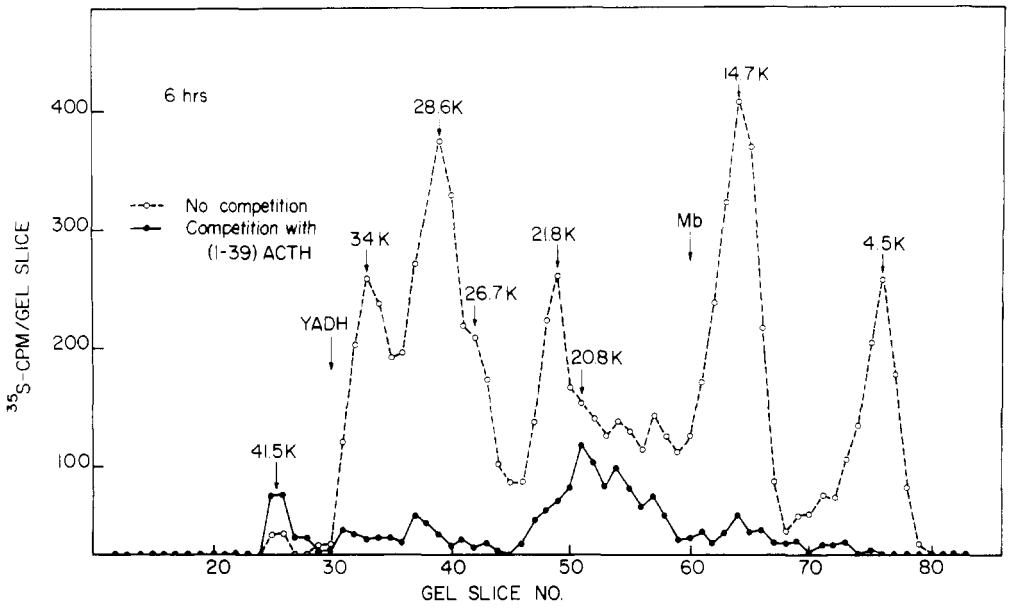


FIGURE 5: Competition of radiolabeled proteins with excess unlabeled ACTH. A continuous incubation of 6 h was carried out in methionine-free medium as described under Materials and Methods with the inclusion of 400 μCi of [^{35}S]methionine. Cells were extracted as described and the extract was split into equal aliquots and lyophilized. The extracts were then immunoprecipitated with antiserum Wilma and no additions (O) or the addition of a 1000-fold excess of porcine α(1-39)ACTH (●) as determined by previous radioimmunoassay.

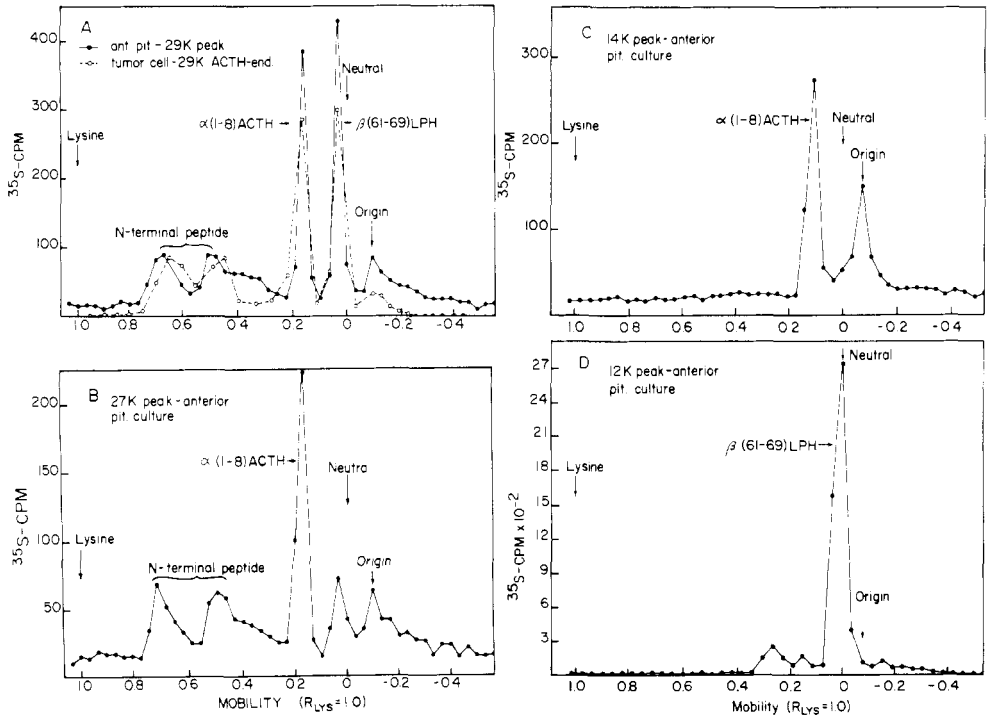


FIGURE 6: Tryptic peptides of the [^{35}S]methionine-labeled ACTH and LPH proteins. A 16-mm well was incubated with 400 μCi of [^{35}S]methionine in 300 μL of serum-free, methionine-free DMEM for either 2 h (to obtain labeled forms of ACTH or LPH greater than 20K) or 8 h (for ACTH or LPH forms less than 20K). The ACTH- or LPH-containing proteins were immunoprecipitated and separated by NaDodSO$_4$ gel electrophoresis as described in Figure 2. Isolated peaks were digested by TPCK-treated trypsin and analyzed by paper electrophoresis at pH 6.5. Mobility has been defined relative to lysine with R_{Lys} of ϵ-dinitrophenyllysine = 0 and R_{Lys} of lysine = 1. Recovery of peptides was always >70%.

medium in the anterior pituitary cultures studied here, loss of labeled hormone from the cells by secretion is not a concern in the early part of the chase period when rapid changes are taking place in the label distribution between the 29K and 32K proteins. The 29K and 32K proteins both have the antigenic determinants of ACTH and β-endorphin as demonstrated in this study (Figure 6) and by sequential immunoprecipitations (results not shown). Additionally, both 29K and 32K protein bands contain three Met-tryptic peptides of pro-ACTH-endorphin that correspond to the Met-tryptic peptides of pro-ACTH-endorphin from AtT-20 cells (Herbert et al., 1979a,b). The latter peptides have been shown to be derived from the ACTH, β-LPH, and N-terminal domains of the precursor molecule (Roberts & Herbert, 1977b). Incubations of the monolayer cultures with [^{3}H]glucosamine have demonstrated that the 29K and 32K proteins are glycosylated as is the case with the 29K and 32K proteins from AtT-20 cells.

The behavior of 27–28K forms of ACTH is similar to the 21–26K ACTH intermediates in the AtT-20 cells with respect to (1) kinetics of labeling, (2) incorporation of [^{3}H]glucos-

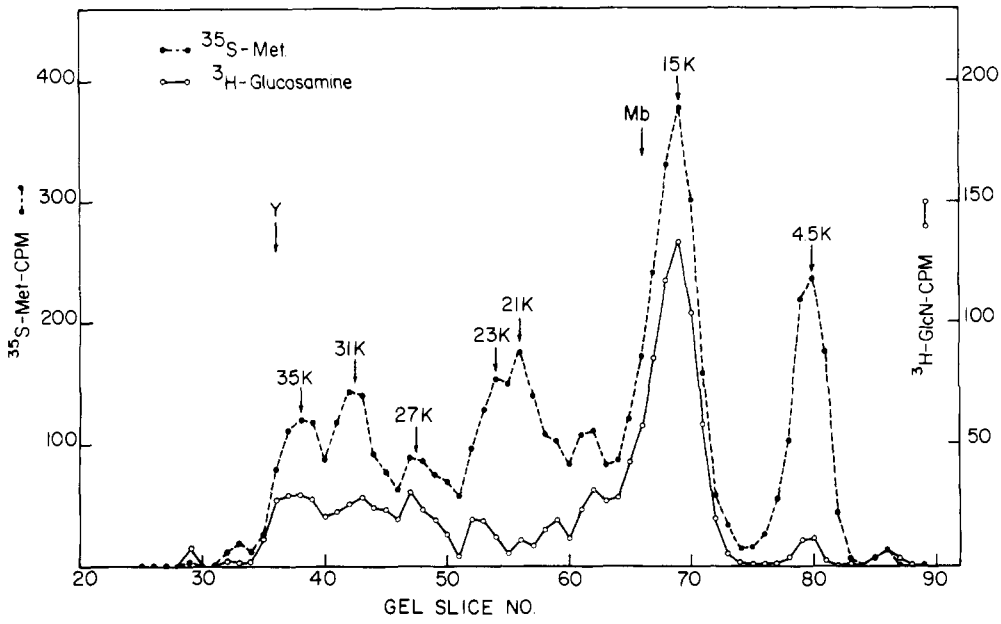


FIGURE 7: [^{35}S]Methionine/[^{3}H]glucosamine labeling of ACTH forms; analysis by NaDodSO$_4$ gel electrophoresis. Primary cultures in 16-mm wells were incubated for 8 h with 300 μL of serum-free, low-glucose DMEM containing 0.5 mCi of D-[6-^{3}H]glucosamine and 0.2 mCi of L-[^{35}S]methionine. The cells were extracted as described and the extract was lyophilized and immunoprecipitated with antiserum Wilma. The specific ACTH-containing proteins were separated by NaDodSO$_4$ gel electrophoresis as described for Figure 2. The gel was cut in 1-mm slices and cpm were eluted and counted by using a program for quenched ^{3}H cpm counting (Isocap scintillation counter). ^{3}H cpm are adjusted for ^{35}S spillover and background is subtracted.

amine, and (3) content of Met-tryptic peptides. These forms contain the Met-tryptic peptides derived from the ACTH and the N-terminal regions of the precursor but not from the β-LPH region.

It is of interest that label appears in β-LPH-like material somewhat later (after 30 min of chase) than in 23K and 27–28K ACTH. This result appears to be incompatible with the scheme proposed for processing of pro-ACTH-endorphin in AtT-20 cells (Roberts et al., 1978) as presented in the introduction. The first proteolytic event depicted in that scheme is a cleavage between the ACTH and β-LPH domains. This cleavage should give rise to ACTH intermediates and β-LPH simultaneously. The most likely explanation of this finding is that the detection of β-LPH is not sensitive enough to demonstrate the precise time that label enters these forms. An alternative explanation is that processing is more complicated than previously thought (Roberts et al., 1978; Herbert et al., 1979b). Studies are now in progress to test these alternatives.

Label first appears in 14K and 4.5K forms of ACTH after a chase of $\sim$1 h and continues to accumulate in these forms for at least 16 h of chase time. This result indicates that 14K and 4.5K are end products of processing in the primary cultures as in the AtT-20 cells. The 14K form is also glycosylated as in the AtT-20 cells. Very little label appears in β-endorphin-like material during the chase period in contrast to β-LPH-like material which contains 95% of immunoprecipitable label after 16 h of chase. Therefore, β-LPH appears to be a major end product of processing in anterior pituitary cells in agreement with a number of other studies (Rubinstein et al., 1977; Liotta et al., 1978).

The turnover time of a protein can be estimated from the time it takes to completely chase label from the protein. For a secretory protein, turnover time is a function of the rates of synthesis, processing, degradation, and secretion of the protein. Figure 4 shows that it takes 3 h to chase label from 29K and 32K pro-ACTH-endorphin and 8 h to chase label from ACTH intermediates in mouse anterior pituitary cultures. The turnover time of the lower molecular weight forms of ACTH and β-LPH cannot be estimated from the results in Figure 4 because the chase time is not long enough. We have, however, carried out continuous labeling studies for several days in culture and have found that it takes about 4–6 days for steady-state labeling of the forms of ACTH to occur (in complete DMEM with 10% horse serum). In other studies we have found that mouse anterior pituitary cells in monolayer culture secrete 10–20% of their stores of 14K and 4.5K ACTH and β-LPH per day in the absence of added "CRF" (basal secretion) without depleting the stores of these hormones. Therefore, the turnover time based on secretion rates is 5 days or longer and is roughly the same as turnover time estimated from the labeling studies.

The turnover of ACTH and endorphin proteins in monolayer cultures of anterior pituitary cells is much slower than the turnover of these hormones in AtT-20/D$_{16v}$ cultures. In AtT-20 cultures the constitutive rate of secretion of ACTH/endorphin proteins is 10 times as high as in anterior pituitary cultures relative to the cellular content of these proteins, and steady-state labeling of the ACTH/endorphin proteins is reached in about one-tenth the time it takes in anterior pituitary culture (8–10 h) in DMEM plus 10% horse serum.

The pulse–chase results, together with the results of tryptic peptide mapping and [^{3}H]glucosamine incubations, suggest that processing of pro-ACTH-endorphin in mouse anterior pituitary cells is very similar to that demonstrated earlier for AtT-20 cells, while the data presented for turnover shows that primary cells process pro-ACTH-endorphin at a slower rate than the AtT-20 cells. Paquette et al. (1979) have shown that culturing of anterior lobe cells by the method used here does not alter the molecular weight distribution of the forms of ACTH in the anterior lobe. Allen et al. (1978) have demonstrated that anterior pituitary cultures and AtT-20 cells have similar responses to partially purified CRF and dexamethasone. Therefore, studies of ACTH/endorphin with primary cultures can complement work done with AtT-20 tumor cells by providing data from a source more closely

resembling the anterior pituitary. Such studies will provide a sound basis for accurate models of anterior pituitary function.

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