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Steps Involved in the Processing of Common Precursor Forms of Adrenocorticotropin and Endorphin in Cultures of Mouse Pituitary Cells†

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ABSTRACT: The initial steps in the processing of the common precursor to adrenocorticotropin (ACTH) and endorphin in mouse pituitary tumor cells (AtT-20) have been investigated. Three forms of the precursor have been resolved by sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide gel electrophoresis with apparent molecular weights of 29 000 (29K ACTH-endorphin), 32 000 (32K ACTH-endorphin) and 34 000 (34K ACTH-endorphin). These forms have a similar peptide backbone, but their carbohydrate content differs. In particular, a tryptic glycopeptide has been observed in 32K ACTH-endorphin which is not present in 29K ACTH-endorphin and has been identified as the tryptic peptide containing the α(22–39) sequence of ACTH. Similar heterogeneity in carbohydrate has been observed in some of the smaller molecular weight forms of ACTH which are resolved by $NaDodSO_4$ gel electrophoresis. Pulse chase and continuous labeling studies using radioactive amino acids and sugars suggest that the 29K ACTH-endorphin is converted to 32K and 34K ACTH-endorphin by the addition of carbohydrate. The glycopeptide and pulse chase studies suggest that 29K ACTH-endorphin is at a branch point in the processing pathways. It can either be converted to 4.5K ACTH by proteolytic processing or to 32K ACTH-endorphin by the further addition of carbohydrate. The 32K ACTH-endorphin can then be converted to 13K ACTH, the glycosylated form of 4.5K ACTH (Eipper, B. A., & Mains, R. E. (1977) *J. Biol. Chem. 252,* 882), by proteolytic processing. A comparison of the distribution of the different molecular weight forms of ACTH and endorphin in mouse pituitary extracts and in the mouse pituitary tumor cells reveals that the pituitary contains all of the forms of ACTH and endorphin seen in the tumor cells, including the three forms of the ACTH-endorphin precursor. However, the molecular weight distribution of the forms in the anterior lobe is very different from that in the intermediate lobe of mouse pituitary.

The pituitary hormone, adrenocorticotropin (ACTH),[1] and the opiate peptide, β-endorphin [β-(61-91)LPH], have recently been shown to be synthesized as part of a much larger precursor molecule in a mouse pituitary tumor cell line (AtT-$20D_{16v}$ line) (Mains et al., 1977; Roberts & Herbert, 1977a,b) and in beef pituitary (Nakanishi, et al., 1977). When mRNA

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[1] Abbreviations used: ACTH, adrenocorticotropin; β-LPH, β-lipotropin; $NaDodSO_4$, sodium dodecyl sulfate; Dulbecco-Vogt MEM, Dulbecco-Vogt minimal essential medium; SAC, *Staphylococcus aureus* Cowan I; YADH, yeast alcohol dehydrogenase; BSA, bovine serum albumin; $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; IAA, iodoacetamide; Tos-$PheCH_2Cl$, tosylphenylalanyl chloromethyl ketone; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; DNP, 2,4-dinitrophenyl; MSH, melanocyte stimulating hormone; Bis, *N,N'*-methylenebis(acrylamide); RIA-ACTH, ACTH activity by radioimmunoassay expressed as ng of purified porcine ACTH with equivalent activity. The forms of ACTH and endorphin are designated by their apparent molecular weights as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The precursor forms that contain both ACTH and endorphin are: 28.5 ACTH-endorphin, the product synthesized in the cell-free system and 29K, 32K, and 34K ACTH-endorphin, the glycosylated forms of the precursor synthesized in the tumor cells. Other forms of ACTH or endorphin are designated: 21K, 23K, and 26K ACTH for the intermediate forms of ACTH; 13K and 4.5K ACTH for the end products of processing of ACTH as seen in tissue culture medium; 12–15K ACTH for the pool of glycosylated ACTH end products seen in intact tumor cells; 11.5K endorphin for the β-LPH-like molecule and 3.5K endorphin for the β-endorphin-like molecule. These molecular weight designations are for identification purposes only, since it is well known that accurate molecular weights of glycoproteins cannot be otained by this method (Segrest & Jackson, 1972).

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from AtT-20 cells is translated in a reticulocyte cell-free protein synthesizing system, a single form of the precursor is synthesized with an apparent molecular weight of 28 500 (Roberts & Herbert, 1977a,b). When AtT-20 cells are labeled with radioactive amino acids, three radioactive forms of the ACTH-endorphin precursor are resolved by $NaDodSO_4$-polyacrylamide gel electrophoresis with apparent molecular weights of 29 000, 32 000, and 34 000 (Roberts & Herbert, 1977a). Smaller polypeptides possessing either the ACTH or the endorphin determinant, but not both, are also resolved by $NaDodSO_4$ gel electrophoresis (Mains et al., 1977). The smaller size classes of ACTH have apparent molecular weights of 20 000–26 000 (20–26K ACTH), 12 000–15 000 (12–15K ACTH), and 4500 (4.5K ACTH) (Mains & Eipper, 1976; Herbert et al., 1978a). Pulse-labeling studies with radioactive amino acids suggest that 13K ACTH (12–15K ACTH) and 4.5K ACTH are both end products of processing pathways in tumor cells and that 23K ACTH (20–26K ACTH) is an intermediate in the pathway (Mains & Eipper, 1976). All of the forms of ACTH are glycosylated except 4.5K ACTH (Eipper et al., 1976; Herbert et al., 1978a). The smaller forms of endorphin resolved by $NaDodSO_4$ gel electrophoresis have molecular weights approximately the size of β-LPH (11 500 daltons, designated 11.5K endorphin) and β-endorphin (3500 daltons, designated 3.5K endorphin).

It has recently been shown that 13K ACTH is a glycosylated form of 4.5K ACTH (Eipper & Mains, 1977). The 13K and 4.5K forms of ACTH are both end products of processing. The discovery of three forms of the precursor suggested that separate processing pathways for 13K and 4.5K ACTH might originate with different forms of the ACTH-endorphin precursor. Thus, in an attempt to understand how the precursor is converted to the different forms of ACTH and endorphin, we have studied the initial steps involved in the biosynthesis and processing of the precursor in the AtT-20-D_{16v} cells.

The AtT-20-D_{16v} cell line is used in these studies as a model system for investigating ACTH and endorphin synthesis and processing in the pituitary. It is important, therefore, to determine if the forms of ACTH and endorphin observed in the tumor cells are also present in the pituitary. ACTH and endorphin are found in both the anterior and intermediate lobes of mouse and rat pituitary. Hence, a study of the distribution of the molecular weight forms of ACTH and endorphin was undertaken in both anterior and intermediate lobes of mouse pituitary in order to answer this question.

Materials and Methods

Incubation of AtT-20 Cells with Radioactive Amino Acids and Sugars. AtT-20-D_{16v} cells were grown in Falcon Microtest Wells with Dulbecco–Vogt minimal essential medium (Dulbecco–Vogt MEM) supplemented with 10% horse serum. For labeling of cultures with radioactive amino acids, Ham's F-10 medium, which has a lower concentration of amino acids, was used instead of Dulbecco–Vogt MEM in order to increase the specific radioactivity of the labeled amino acids. Cells cultured in this medium have a similar growth rate and ACTH content to cells grown in Dulbecco–Vogt MEM (Herbert et al., 1978b). Cells were fed with Ham's F-10 medium containing horse serum 1–2 h prior to labeling and then incubated with radioactive amino acids in 50 μL of the same medium. The same procedure was used for labeling cells with radioactive sugars except that cells were fed before labeling with Dulbecco–Vogt MEM containing 10% horse serum and incubated with radioactive sugars in 50 μL of a low glucose Dulbecco–Vogt MEM (Eipper et al., 1976). Cells were extracted with 5 N acetic acid and 5 mg/mL BSA supplemented with freshly prepared $PhCH_2SO_2F$ and IAA (1 mM each) to inhibit proteolytic activity. The extract was frozen and thawed three times on dry ice to solubilize the cellular protein, incubated at 4 °C overnight, centrifuged to remove insoluble material, and lyophilized. Secreted forms of ACTH were obtained by labeling cells as previously described. Cells were rinsed one time with serum-free medium and then incubated in serum-free medium in order to avoid degradation by the serum of the secreted proteins. The medium was harvested and $PhCH_2SO_2F$ and IAA were added. The medium was centrifuged to remove any contaminating cell debris and immunoprecipitated.

The cell-free 28.5 ACTH-endorphin precursor was prepared as described previously (Roberts & Herbert, 1977a).

Immunoprecipitations. Immunoprecipitations were performed by either of two different methods. A double antibody immunoprecipitation scheme was used with the ACTH antiserum (Mains & Eipper, 1976; Roberts & Herbert, 1977a). Alternatively, an *S. aureus* Cowan I (SAC) precipitation was used for all immunoprecipitations done with the endorphin antiserum and for some of the immunoprecipitations done with the ACTH antiserum (Kessler, 1975; Martial et al., 1977). In this case, samples were dissolved in 10 mM NaH_2PO_4, 1 mM Na_2EDTA, 1% Triton X-100, pH 7.6, incubated for 10 min at 4 °C with SAC and then centrifuged to remove the SAC. The supernatants were then incubated for 12–16 h at 4 °C with either antiserum Bertha which is specific for the α(11–24) sequence of ACTH (Mains & Eipper, 1976) or with RB-100 antiserum (Guillemin et al., 1977). The specific antibody–antigen complex was precipitated by addition of SAC.

Two approaches were used to demonstrate that all of the ACTH or endorphin proteins were precipitated by the antisera: (1) a second aliquot of antiserum was added to the supernatant remaining after the first immunoprecipitation; no further ACTH or endorphin was precipitated; (2) radioimmunoassay of the supernatants showed that less than 2% of the initial ACTH or endorphin immunoactivity was present after immunoprecipitation.

Specificity of the antisera was demonstrated by showing that excess α_p(1–39)-ACTH inhibited binding of labeled proteins to the ACTH antiserum but not to the endorphin antiserum and that excess β-endorphin inhibited binding of the labeled proteins to the endorphin antiserum but not to the ACTH antiserum (Roberts & Herbert, 1977b).

$NaDodSO_4$ Polyacrylamide Gel Electrophoresis. Bio-Rad 12% acrylamide–$NaDodSO_4$ gels (Tris–acetate, pH 6.5) were used to separate the various molecular weight forms of ACTH or endorphin in the immunoprecipitated cell extracts (Roberts & Herbert, 1977a,b). Molecular weights of ACTH or endorphin proteins were determined relative to YADH using ovalbumin, YADH, carbonic anhydrase, chymotrypsinogen A, ribonuclease A, and cytochrome *c* as molecular weight standards. Gels were eluted in two ways. For tritium or ^{35}S counting of the eluates of the gel slices, elution was done with 300 μL of 5 mM $NaHCO_3$, 0.5 M urea, 0.1% $NaDodSO_4$ at 37 °C on a shaker for 12–16 h. When recovery of the labeled protein was necessary, the slices were incubated with 300 μL of 50 mM Tris, pH 7.6, 0.1% $NaDodSO_4$, 0.1% Triton X-100 at 37 °C on a shaker for 12–16 h. An aliquot of the eluate was counted to locate the radioactivity. Only the peak fractions of each ACTH-endorphin form were used to avoid cross contamination of the different proteins. These fractions were pooled, 100 μg of carrier BSA was added, and the protein was precipitated with Cl_3CCOOH as previously described (Roberts & Herbert, 1977a). Recovery of the protein was generally 80%. These precipitates were then used for tryptic peptide analysis.

Tryptic Peptide Analysis. The Cl_3CCOOH precipitates were dissolved in 100 μL of 0.1 M NH_4HCO_3 (titrated with NH_4OH to pH 8.5), the protein was digested with Tos-PheCH_2Cl-treated trypsin at room temperature (on a shaker) for 16 hours, and the tryptic peptides were analyzed by paper electrophoresis at pH 6.5 as previously described (Roberts & Herbert, 1977a).

Analysis by Radioimmunoassay of Forms of ACTH and Endorphin in the Mouse Pituitaries and AtT-20-D_{16v} Cells. AtT-20-D_{16v} cells were extracted with 5 N acetic acid, 1 mM $PhCH_2SO_2F$ and 1 mM IAA. Pituitaries were removed from adult male mice in Vale's Hepes buffer (pH 7.38) (Vale et al., 1972). Anterior lobes were separated from intermediate–posterior lobes and extracted individually in 500 μL of 5 N acetic acid. Twenty intermediate–posterior lobes were pooled and extracted in 500 μL of 5 N acetic acid. Samples were homogenized 5 min in a siliconized glass homogenizer, heated 2 min at 100 °C, diluted to 2.5 N acetic acid, and centrifuged 20 min at 6000 rpm. [Extraction of pituitaries in cold acetic acid in the presence of proteolytic inhibitors ($PhCH_2SO_2F$ and IAA) did not alter the distribution of the ACTH and endorphin proteins (data not shown)]. Supernatants were removed, frozen, lyophilized, resuspended in 50 μL of gel sample buffer as previously described, and applied to 12% polyacrylamide tube gels (Bio-Rad) or 15% polyacrylamide, 3.5% bis(acrylamide) tube gels as indicated. Samples were preelectrophoresed 35 min at 4 mA/gel and then run for 3 h at 8 mA/gel. Gels were sliced in 1-mm sections and each section was eluted for 12 h at 37 °C with 90 μL per slice of 50 mM Tris, pH 7.6, 0.1% $NaDodSO_4$, 0.1% Triton X-100. Radioimmunoassays were performed as described (Allen et al., 1978).

The antibody used in the ACTH radioimmunoassay is specific for α(4–18)-ACTH and reacts with α-MSH or α(1–13)-NH_2ACTH one-tenth as well on a molar basis as with α(1–39)-ACTH. There is no cross-reactivity with β-MSH (data not shown). The endorphin antibody is specific for β(78–87)-LPH and reacts equally well with β-LPH and β-endorphin on a molar basis (Guillemin et al., 1977). Since purified stocks of ACTH-endorphin and intermediate forms of ACTH are not available, it has not been possible to determine the molar reactivity of these forms with either antibody.

Identification of ^3H-Labeled Sugars. [^3H]Glucosamine. One microwell culture of AtT-20 cells was labeled with [^3H]glucosamine (19 Ci/mmol) for 4 h and extracted as described previously in Materials and Methods. Protein was precipitated with 20% Cl_3CCOOH, washed twice with acetone:ether (1:1), and hydrolyzed in 4 N HCl in an evacuated tube at 100 °C for 6 h (Spiro, 1972). The sample was evaporated to dryness in an evacuated desiccator over solid NaOH, and dissolved in 0.1 N HCl, and 10 μg of unlabeled glucosamine was added. The sample was analyzed by paper electrophoresis at pH 6.5 and by ascending paper chromatography in isopropyl alcohol:HCl:H_2O (68:16.6:18.4) (Brewer et al., 1974). Glucosamine was visualized with ninhydrin. The paper was cut into 8-mm strips, eluted with 300 μL of 0.2 N HCl, and counted. Recovery of ^3H as [^3H]glucosamine was greater than 90%.

[^3H]Mannose. Cells were labeled for 3 h with D-[1-^3H]-mannose (2.1 Ci/mmol) and extracted. Protein was precipitated with Cl_3CCOOH and hydrolyzed in 2 N HCl at 100 °C for 6 h (Spiro, 1977). Ten micrograms of unlabeled mannose was added and the sample was analyzed by repeated ascending chromatography in *n*-butyl acetate:acetic acid:H_2O (3:2:1) (Churms, 1967). Glucosamine, glucose, galactose, and fucose were well separated by this solvent system. Sugars were visualized with an aniline phosphate spray (Dawson et al., 1969). Recovery of ^3H label as [^3H]mannose was 50%.

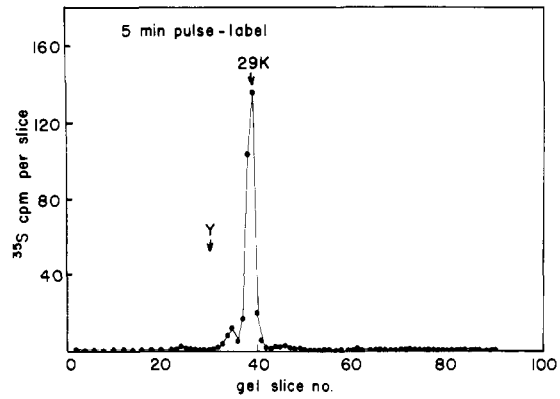

FIGURE 1: Pulse labeling with [^{35}S]methionine; analysis of immunoprecipitated ACTH proteins by $NaDodSO_4$ gel electrophoresis. AtT-20 cell cultures grown in Falcon Microtest wells were incubated with 200 μL of Ham's F-10 medium containing 10% horse serum for 1 h before labeling. One culture well was incubated for 5 min with 100 μCi of L-[^{35}S]-methionine dissolved in 50 μL of Ham's medium plus 10% horse serum and immediately extracted with acetic acid. The extracts were lyophilized, immunoprecipitated with antiserum Bertha, and analyzed by $NaDodSO_4$ gel electrophoresis with 12% Biophore gels. Dansyl-YADH was included as an internal marker for determining relative mobilities of radioactive proteins. Gels were cut into 1-mm slices. Background has been subtracted.

[^3H]Galactose. D-[1-^3H]Galactose-labeled material was prepared and analyzed in the same way as [^3H]mannose-labeled material. Recovery of ^3H label as [^3H]galactose was 75%.

Results

Biosynthetic Relationships of the ACTH-Endorphin Precursors

The primary translation product of ACTH-endorphin mRNA isolated from AtT-20 cells has been identified as a protein with an apparent molecular weight of 28 500 daltons (28.5K) containing one copy each of α(1–39)-ACTH and 3.5K β-endorphin (Roberts & Herbert, 1977a,b). However, when the ACTH-endorphin precursor is isolated from intact cells and analyzed by $NaDodSO_4$ gel electrophoresis, three glycoprotein forms can be identified with apparent molecular weights of approximately 29 000 (29K), 32 000 (32K), and 34 000 (34K) daltons (Roberts & Herbert, 1977a). In order to determine the biosynthetic relationship of these forms of the precursor in AtT-20 cells, a series of labeling experiments with radioactive amino acids was performed.

After a 5-min labeling period, greater than 90% of the radioactivity in ACTH-endorphin is present in the 29K form of the precursor (Figure 1). The remainder of the label is present in the 32K form. After longer labeling periods, radioactivity starts to accumulate in the two larger forms of the precursor (see Figure 2, 10-min label, 0 time chase). In other experiments (data not shown) the 29K pool saturates with label after 30 min, whereas the 32K and 34K pools continue to accumulate label for 60 min.

Pulse-chase experiments were then performed to determine if the 29K form is the precursor of the 32 and 34K forms. The labeling period (pulse) had to be increased to 10 min in these experiments to obtain sufficient radioactivity in the precursors to follow the flow of label accurately in the later chase periods. Although all three forms of the precursor are labeled after this period, more than two-thirds of the radioactivity is still in the

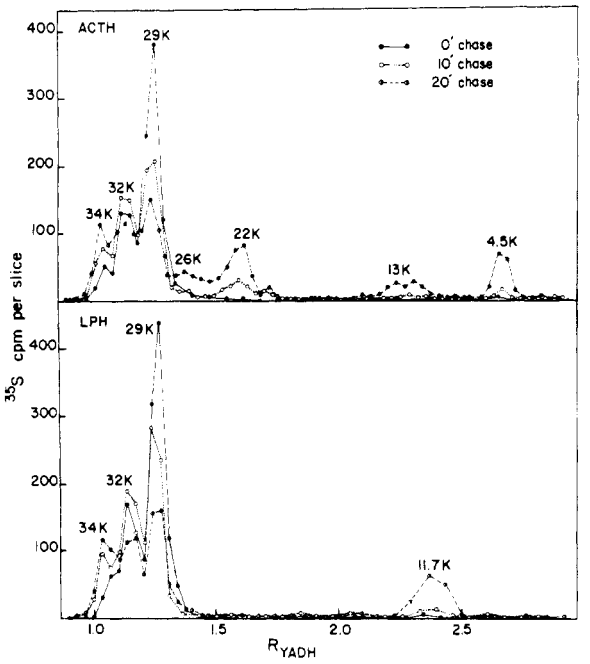

FIGURE 2: Pulse chase with [^{35}S]methionine; analysis of ACTH and endorphin immunoprecipitates. Three microwell cultures of AtT-20 cells containing the same amount of RIA-ACTH were each pulsed for 10 min with 100 μCi of L-[^{35}S]methionine as described in Figure 1. The cells were rinsed quickly with 100 μL of medium and then incubated for 0, 10, or 20 min in 200 μL of unlabeled medium. Cells were extracted as described and aliquots were taken for radioimmunoassay determination of total ACTH and endorphin activity. Cell extracts were lyophilized, immunoprecipitated with antiserum specific for either ACTH or endorphin, and then analyzed by NaDodSO$_4$ gel electrophoresis as described in Figure 1. Gels were cut into 1-mm slices. For analysis of the low molecular weight endorphin-immunoprecipitated material (mobility greater than R_{YADH} = 1.4), every two slices were pooled, eluted together, and counted. Cpm/slice has been plotted against mobility relative to YADH (R_{YADH}) and background subtracted.

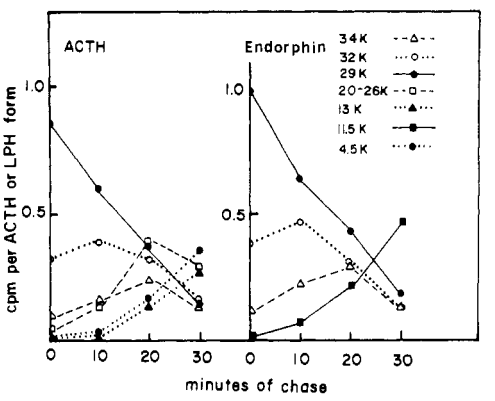

FIGURE 3: Pulse chase with [^{35}S]methionine; summary of NaDodSO$_4$ gel analysis. The quantity of labeled protein present in each form of ACTH or endorphin shown in Figure 2 was determined by summing the cpm in each peak. This was determined for each time period shown in Figure 2 and in addition for a 30-min chase period. The regions of the gel summed for analysis were as follows: 34K ACTH-endorphin, R_{YADH} = 0.95–1.08; 32K ACTH-endorphin, R_{YADH} = 1.09–1.20; 29K ACTH-endorphin, R_{YADH} = 1.21–1.33; 20–26K ACTH, R_{YADH} = 1.34–1.80; 12–15 ACTH, R_{YADH} = 2.10–2.45; 11.5K endorphin, R_{YADH} = 2.13–2.50; and 4.5K ACTH, R_{YADH} = 2.58–2.75. Points lying between two peaks were split in quantity relative to the adjacent peak heights.

TABLE I: Pulse Chase Summary.[a]

form	cpm in forms at 0 time chase	cpm in forms at 10-min chase	cpm gained or lost during 10-min chase	total label gained or lost from all forms
29K ACTH-endorphin	935	630	−305	−305
34K ACTH-endorphin	100	150	+50	
32K ACTH-endorphin	330	390	+60	+220
20–26 ACTH	50	110	+60	
11.7K endorphin	20	70	+50	

[a] Total cpms in each form of ACTH and 11.7K endorphin have been determined from Figure 2 for 0 and 10 min of chase. The forms have been defined as described in legend for Figure 2.

29K form (Figure 2). It was determined that incorporation of [^{35}S]methionine into Cl_3CCOOH-precipitable cpm is linear within 5 to 8 min after addition of the label. Hence, 10-min intervals were used for the chase periods. Thus, the experiment shown in Figure 2 is a 10-min radioactive pulse followed by chase periods of 0, 10, and 20 min with nonradioactive amino acids. Both ACTH and endorphin containing proteins were analyzed by immunoprecipitation and fractionation on NaDodSO$_4$ gels. The gel profiles in Figure 2 are identical in the 29–34K precursor region (as expected if these proteins contain both antigenic determinants) but differ in the molecular weight regions where the proteins contain only one or the other antigenic determinant. The quantity of radioactivity in each form of ACTH or endorphin is summarized in Figure 3 along with data for a 30-min chase period (not shown in Figure 2). The label in the 29K form of the precursor chases immediately, whereas label in the 32K and 34K forms of the precursor continues to accumulate after a 10-min chase period. After the 10-min chase, approximately 40% of the label lost from the 29K form can be accounted for by the gain in label in the 32K and 34K forms. Most of the rest of the label lost from the 29K form can be accounted for by label appearing in lower molecular weight forms of ACTH (Table I). (No radioactive forms of ACTH appear in the culture medium before 60 min of incubation.) Twenty minutes after the start of the chase, the 32K form begins to lose label but the 34K form is still accumulating label. After 30 min, label is being lost from all of the precursor forms. The results in Table I show that there is good agreement between the amount of label lost from the 29K form and the amount of label accumulating in 32K, 34K forms of ACTH-endorphin, 20–26K ACTH, and 11.5K endorphin. These data, together with results of the continuous labeling experiments, suggest that 29K ACTH-endorphin is a precursor to the 32K and 34K forms of ACTH-endorphin. This hypothesis will be elaborated after discussion of the structural properties of the three precursor forms.

Structural Relationships of the Three Precursor ACTH-Endorphin Proteins

Peptide Backbones. All three forms of the precursor contain both the ACTH and endorphin antigenic determinants (Figure 2), suggesting a similarity in their peptide structure. To examine the structural relationships in more detail, these proteins were labeled with a variety of different radioactive amino acids and their tryptic peptides were analyzed by paper electrophoresis. The results of such an analysis with [^{35}S]methionine and [^{3}H]tryptophan labeled material are shown in Figures 4A and 4B. Clearly, the three different precursor forms have similar [^{3}H]tryptophan- and [^{35}S]methionine-labeled tryptic peptides. It also appears that 34K, 32K, and 29K ACTH-endorphin contain similar [^{3}H]tyrosine (Figures 8C and 8D), [^{3}H]phenylalanine (Figures 8A and 8B), [^{3}H]lysine, [^{3}H]-

TABLE II: Glucosamine/Protein Ratios.[a]

form of ACTH or endorphin	^3H cpm	^{35}S cpm	no. of Met residues in ACTH or endorphin forms	^3H cpm/ mol of ACTH or endorphin[b]
34K ACTH-endorphin	107	106	3	3.0
32K ACTH-endorphin	126	115	3	3.3
29K ACTH-endorphin	69	94	3	2.2
26K ACTH	73	23	2	6.3
21K ACTH	60	38	2	3.2

[a] Total ^3H and ^{35}S cpm have been determined from Figure 5 as follows: 34K ACTH-endorphin, slices 34–36; 32K ACTH-endorphin, slices 38–40; 29K ACTH-endorphin, slices 43–44; 26K ACTH, slices 47–49; and 21K ACTH, slices 56–57. [b] These are arbitrary units used to directly compare amounts of glucosamine in each form. The units are adjusted for the total amount of each form present (mol of ACTH or endorphin) by dividing [^{35}S]methionine cpm by the number of methionine residues in each form.

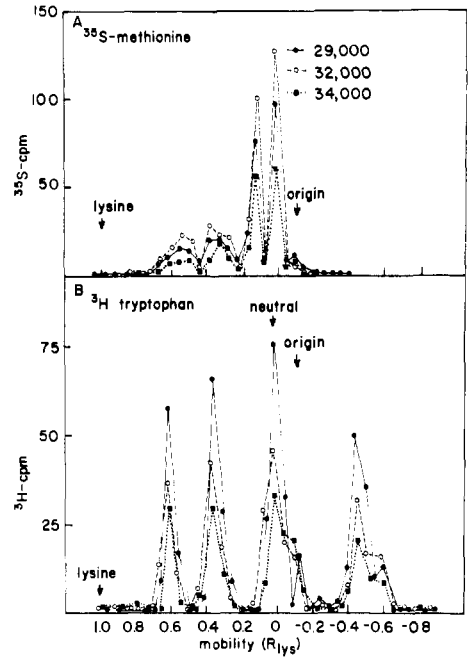

FIGURE 4: Tryptic peptides of [^{35}S]methionine-labeled and [^3H]tryptophan-labeled ACTH-endorphin precursors. One microtest well was incubated with either 200 μCi of L-[^{35}S]methionine (1000 Ci/mmol) or 200 μCi of L-[5(n)-^3H]tryptophan (30 Ci/mmol) in 50 μL of Dulbecco-Vogt MEM, and the ACTH-containing proteins were immunoprecipitated and separated by $NaDodSO_4$ gel electrophoresis as described in Figure 1. Tryptic peptides of the isolated precursors were prepared and analyzed by paper electrophoresis at pH 6.5. Mobility has been defined relative to lysine with R_{Lys} of ϵ-DNP-lysine = 0 and R_{Lys} of lysine = 1.0. Background has been subtracted. Recovery of peptides was 90%.

leucine, [^3H]proline, [^3H]isoleucine, and [^3H]valine (data not shown) labeled tryptic peptides, except that there is a shift in the mobility of one acidic peptide. The implications of this shifted peptide will be discussed later.

Relative Carbohydrate Content of ACTH-Endorphin Precursors. Since the peptide backbones of the 29K, 32K, and 34K forms appear to be similar and since "31K" ACTH-endorphin (the 29–34K pool of molecules) has been shown to contain carbohydrate (Eipper et al., 1976), a possible explanation of the observed differences in molecular weight of the ACTH-endorphin precursors is that they contain different amounts of carbohydrate. A test of this hypothesis is to label cells with both ^3H-labeled sugars and [^{35}S]methionine. Since the number of methionine residues in all the forms of ACTH is known, the ratio of [^3H]/[^{35}S] at steady state labeling can then be used as an estimate of the relative amount of carbohydrate to moles of protein in each form.

The carbohydrate moiety attached to the α(22–39) tryptic peptide of "13K" ACTH (the 12–15K pool of molecule) has been shown to be linked through N-acetylglucosamine to asparagine and is of the complex type, containing glucosamine, mannose, galactose, and fucose (Eipper & Mains, 1977). In addition there is at least one carbohydrate side chain attached to the N terminus of the ACTH-endorphin precursors which also labels with mannose, glucosamine, fucose, and galactose (Phillips & Roberts, unpublished observations). We therefore chose to perform the double-label experiments both with core sugars (glucosamine and mannose) and with two peripheral sugars (fucose and galactose). We postulated that the ACTH-endorphin precursors would label with core sugars, which are thought to be added as a unit through a lipid linked intermediate (Waechter & Lennarz, 1976) during or shortly after synthesis of the protein, and that predominantly the intermediates and end products would label with peripheral sugars, which are generally added later in the smooth endoplasmic reticulum and Golgi (Sharon, 1975; Schachter et al., 1970; Wagner & Cynkin, 1971; Melchers, 1971; Choi et al., 1971).

The ACTH molecules in a cell extract labeled with [^3H]-glucosamine and [^{35}S]methionine were isolated and fractionated (Figure 5). In this 2-h labeling period both the ACTH-endorphin precursors and the 20–26K ACTH intermediates have been labeled to steady state, so the [^3H]/[^{35}S] ratios in Table II should give a reliable estimate of the amounts of glucosamine in these forms. (The 12–15K ACTH molecules continue to incorporate both methionine and glucosamine after 2 h so the ratios for these forms have not been computed.) In addition, when cells were labeled with [^{35}S]methionine and [^3H]mannose, [^3H]/[^{35}S] ratios of 1.0 for 29K and 2.0 for 32K were obtained. (In this experiment an insufficient amount of [^{35}S]methionine and [^3H]mannose was incorporated into 34K for the ratio to be computed.) These comparisons suggest that 29K ACTH-endorphin contains less glucosamine and mannose than 32K ACTH-endorphin and less glucosamine than 34K ACTH-endorphin.

In order to determine if the ACTH-endorphin precursors differ in peripheral carbohydrate content, cells were labeled for 2 h with [^{35}S]methionine and [^3H]fucose (Figure 6A) or with [^{35}S]methionine and [^3H]galactose (data not shown). This time period is again sufficient to achieve steady state labeling of the 29–34K and 20–26K molecules, but not the 12–15K molecules. Figure 6A shows that the peaks of [^3H]-fucose and [^{35}S]methionine do not coincide in the 29–35K region of the gel, but do coincide in the regions of the gel where lower molecular weight forms of ACTH appear. This suggests that the predominant forms of the precursor in the cells (29K, 32K, and 34K ACTH-endorphin) do not contain fucose and that the fucose containing proteins in this region of the gel are minor components. Therefore, differences in fucose content of the 34K, 32K, and 29K precursors are not responsible for their differences in molecular weight. In contrast, the peaks of ^3H and ^{35}S do coincide in the 20–26K and 12–15K regions of the gels suggesting that fucose-containing proteins are the major components of these peaks and that differences in fucose content could be responsible for the molecular weight differences of these forms.

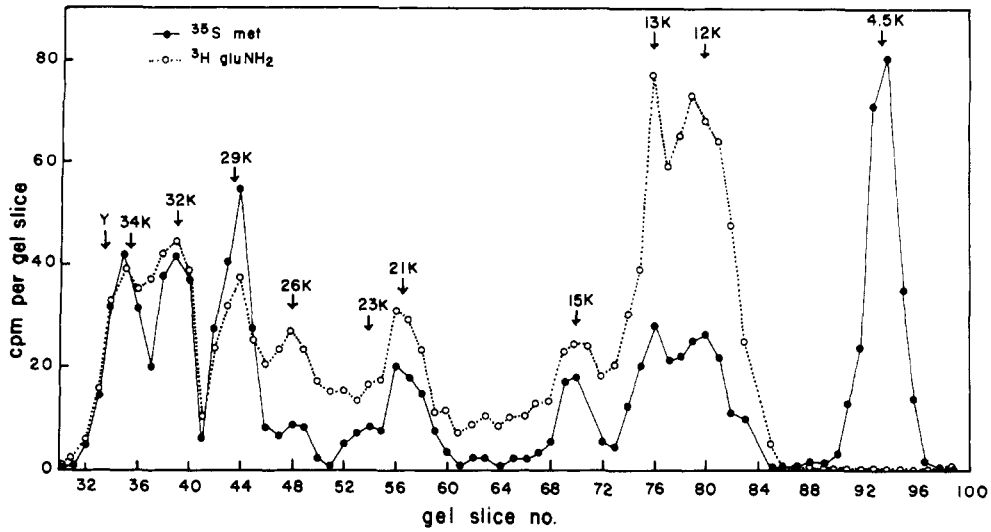

FIGURE 5: [^{35}S]Methionine/[^{3}H]glucosamine-labeled ACTH immunoprecipitates; analysis by NaDodSO$_4$ gel electrophoresis. AtT-20 cells in Falcon Microtest wells were incubated for 2 h with 25 μCi of L-[^{35}S]methionine (1000 Ci/mmol) and 125 μCi of D-[6-^{3}H]glucosamine (19 Ci/mmol) in 50 μL of modified low glucose Dulbecco-Vogt MEM containing 10% horse serum. Cells were extracted, lyophilized, and immunoprecipitated with antiserum Bertha. The specific ACTH-containing proteins were separated by electrophoresis with 12% Biophore gels. Dansyl-YADH was included as an internal standard. Gels were sliced, eluted, and counted using a program for quenched ^{3}H/^{35}S counting (Isocap scintillation counter). ^{3}H cpm are adjusted for ^{35}S spillover and background has been subtracted.

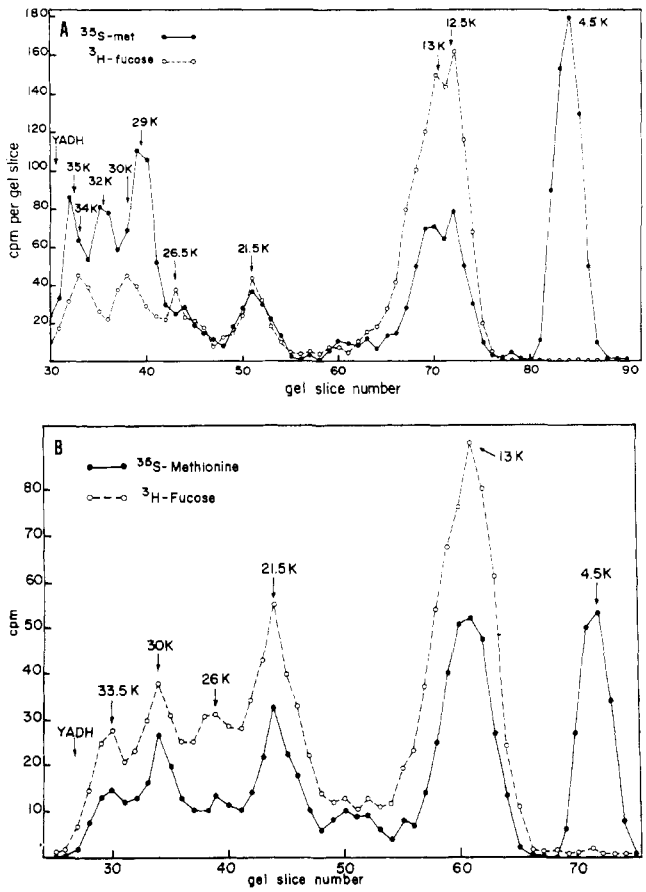

FIGURE 6: [^{35}S]Methionine/[^{3}H]fucose-labeled ACTH immunoprecipitates; analysis by NaDodSO$_4$ gel electrophoresis. (A) Cells were incubated with 25 μCi of L-[^{35}S]methionine (1000 Ci/mmol) and 125 μCi of L-[6-^{3}H]fucose (16.6 Ci/mmol) in 50 μL of low glucose medium for 2 h. The cells were extracted and the ACTH-containing proteins were immunoprecipitated and analyzed by NaDodSO$_4$ gel electrophoresis as described in Figure 5. (B) Cells were incubated with 150 μCi of [^{3}H]fucose (16.6 Ci/mmol) and with 80 μCi of [^{35}S]methionine (1000 Ci/mmol) in 50 μL of low glucose medium for 5 h. The medium was replaced with 100 μL of serum-free low glucose medium and the cells were incubated for 2 h. The ACTH-containing proteins in the medium were immunoprecipitated and analyzed by NaDodSO$_4$ gel electrophoresis.

Since it has been observed that some ACTH-endorphin precursors are secreted into tissue culture medium (Eipper & Mains, 1975), there is a simple explanation for the peaks of [^{3}H]fucose in the 29–35K region of the gel. They might represent a subpopulation of precursor molecules that have escaped proteolytic processing and have passed through the smooth endoplasmic reticulum and Golgi where peripheral sugars have been added, then are stored in secretory vesicles. The major portion of the population of precursor molecules (29K, 32K, and 34K) would be processed proteolytically before reaching the smooth membranes. A test of this hypothesis is to determine if the major species of precursor molecules that are secreted into tissue culture medium contain peripheral sugars. Results in Figure 6B show that in the tissue culture medium the [^{3}H]fucose and [^{35}S]methionine peaks coincide in the precursor region of the gel suggesting that the major species of precursor molecules in culture medium contain peripheral sugars (Figure 6B). In addition the apparent molecular weights of the two major precursor proteins seen in the culture medium, 30K and 33.5K, are the same as those of the fucose labeled precursors seen in cell extracts.

Other observations suggest that the carbohydrate composition of the secreted precursor forms is different than that of the precursor forms in cell extracts. The electrophoretic mobility of the α(22–39) containing tryptic glycopeptide shifts from $R_{Lys} = -0.25$ in the 32K precursor form in the cells to -0.40 in the precursor forms in the culture medium, suggesting that the precursor may contain sialic acid in the medium but not in the cells.

Somewhat different results were obtained when cells were labeled with [^{35}S]methionine and [^{3}H]galactose. In this case, the peaks of ^{35}S label do coincide with the peaks of ^{3}H label in the precursor region of the gel. This result is somewhat unexpected since the initial translation products should not contain peripheral sugars. A possible explanation is that galactose is added as one of the first peripheral sugars and that its addition does not significantly shift the molecular weight of the protein. Alternatively, the ACTH-endorphin precursors could contain a second type of core sugar with *N*-acetylgalactosamine linked to serine or threonine which is added during or shortly after synthesis of the protein.

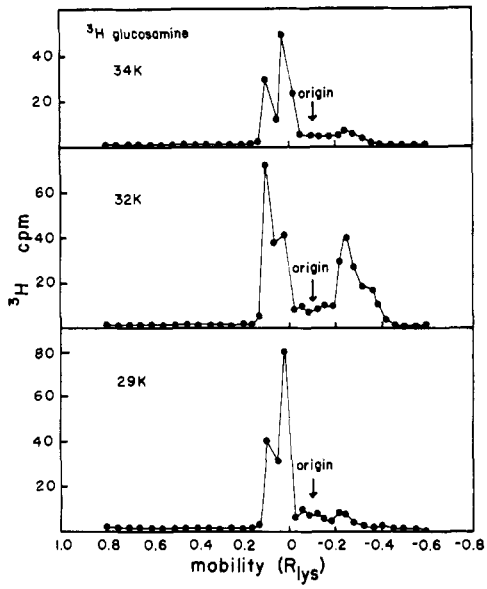

FIGURE 7: Tryptic peptides of [^{3}H]glucosamine-labeled ACTH-endorphin precursors. Cells were incubated with 300 μCi of D-[6-^{3}H]glucosamine (20 Ci/mmol) for 2 h. Tryptic peptide analysis was performed as described in Figure 4. Recovery of peptides was greater than 80%.

[^3H]*Glucosamine-Labeled Tryptic Peptides*

Cells were labeled with [^{3}H]glucosamine and the 29K, 32K, and 34K ACTH-endorphin precursors were isolated and digested with trypsin. Paper electrophoresis of the [^{3}H]glucosamine-labeled tryptic peptides is shown in Figure 7. All three forms of the precursor appear to have in common the basic and neutral glycopeptides (R_{Lys} = 0.1 and R_{Lys} = 0) but only the 32K ACTH-endorphin precursor contains an acidic glycopeptide. The cell-free synthesized 28.5K precursor does not label with [^{3}H]glucosamine or [^{3}H]mannose (Roberts & Herbert, 1977a); therefore its tryptic peptides can be used as unglycosylated markers. In order to identify the acidic glycopeptide, tryptic peptides of [^{3}H]phenylalanine- and [^{3}H]tyrosine-labeled 34K, 32K, and 29K ACTH-endorphin and the unglycosylated cell-free precursor were analyzed by paper electrophoresis (Figure 8). All the tryptic peptides of the 34K, 29K, and 28.5K forms have identical mobilities. The 32K form contains the same tryptic peptides, except that the mobility of one acidic peptide has shifted from R_{Lys} = −0.35 to R_{Lys} = −0.25. This acidic peptide has been identified in the 28.5K precursor as the tryptic peptide containing the α(22–39) sequence of ACTH (Roberts & Herbert, 1977a). The shift in mobility of this peptide has also been observed in analysis of [^{3}H]leucine, [^{3}H]valine, and [^{3}H]proline labeled precursors (data not shown). These amino acids are also known to be contained within the conserved region of the α(22–39)-ACTH peptide. Since the acidic [^{3}H]glucosamine-labeled tryptic peptide derived from the 32K form also has a mobility of R_{Lys} = −0.25, the most plausible explanation is that glycosylation of this peptide alters its mobility. Furthermore, it has been recently shown that 13K ACTH is the glycosylated form of 4.5K ACTH, and that the tryptic glycopeptide obtained by digestion of 13K ACTH is the α(22–39) peptide (Eipper & Mains, 1977). In addition, when the tryptic peptides of [^{3}H]leucine-labeled 13K and 4.5K ACTH are compared, there is a similar shift in mobility due to glycosylation of the α(22–39)-ACTH peptide. The COOH-terminal peptide derived from 4.5K ACTH has a mobility of R_{Lys} = −0.5, and the same peptide derived from 13K ACTH has a mobility of R_{Lys} = −0.4 (data not shown). [The mobilities of these COOH-terminal ACTH peptides are not identical with the mobilities of the peptides containing the α(22–39) sequence derived from the precursor forms since ACTH is not at the COOH terminal of the precursor (Roberts & Herbert, 1977b). Therefore, tryptic peptides derived from the precursor forms contain at least one additional basic residue, either lysine or arginine.] These observations, which show that the α(22–39) region of 32K ACTH-endorphin is glycosylated, suggest that 32K ACTH-endorphin might be a precursor to 13K ACTH, but that the α(22–39) region of 29K ACTH-endorphin is not glycosylated, and it could be a precursor to 4.5K ACTH.

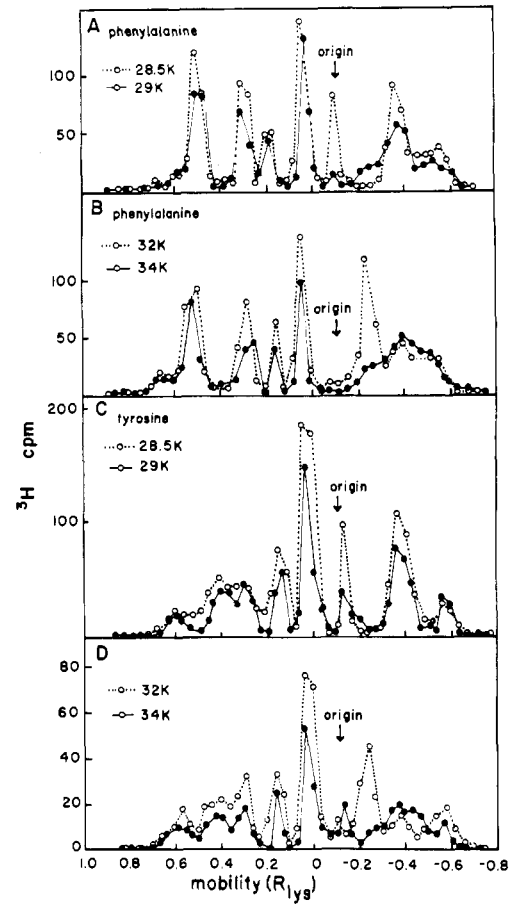

FIGURE 8: Tryptic peptides of [^{3}H]phenylalanine-labeled and [^{3}H]tyrosine-labeled ACTH-endorphin precursors. Cells were incubated with 100 μCi of L-[2,6-^{3}H]phenylalanine (12 Ci/mmol) or 100 μCi of L-[2,3,5,6-^{3}H]tyrosine (80 Ci/mmol) in 50 μL of Ham's F-10 medium containing 10% horse serum for 1 h. Tryptic peptide analysis was performed as described in Figure 4. Recovery of peptides was greater than 70%.

The [^{3}H]glucosamine-labeled tryptic peptides of the 23K and 21K ACTH intermediates have also been investigated (data not shown). The results suggest that 23K ACTH is derived from 32K ACTH-endorphin since it contains an acidic [^{3}H]glucosamine-labeled tryptic peptide and that 21K ACTH is derived from 29K ACTH-endorphin since it does not contain an acidic [^{3}H]glucosamine-labeled tryptic peptide. Both 23K and 21K ACTH contain the basic and neutral tryptic glycopeptides.

Distribution of Molecular Weight Forms of ACTH and β-Endorphin in Lobes of Mouse Pituitary and in AtT-20-D$_{16v}$ Cells. Extracts of anterior and intermediate-posterior lobes of mouse pituitary and of AtT-20-D$_{16v}$ cells were fractionated by NaDodSO$_4$ gel electrophoresis. The gel profiles of ACTH and endorphin immunoactivity in all the extracts (Figure 9) coincide in the regions of the gels where 29K, 32K, and 34K

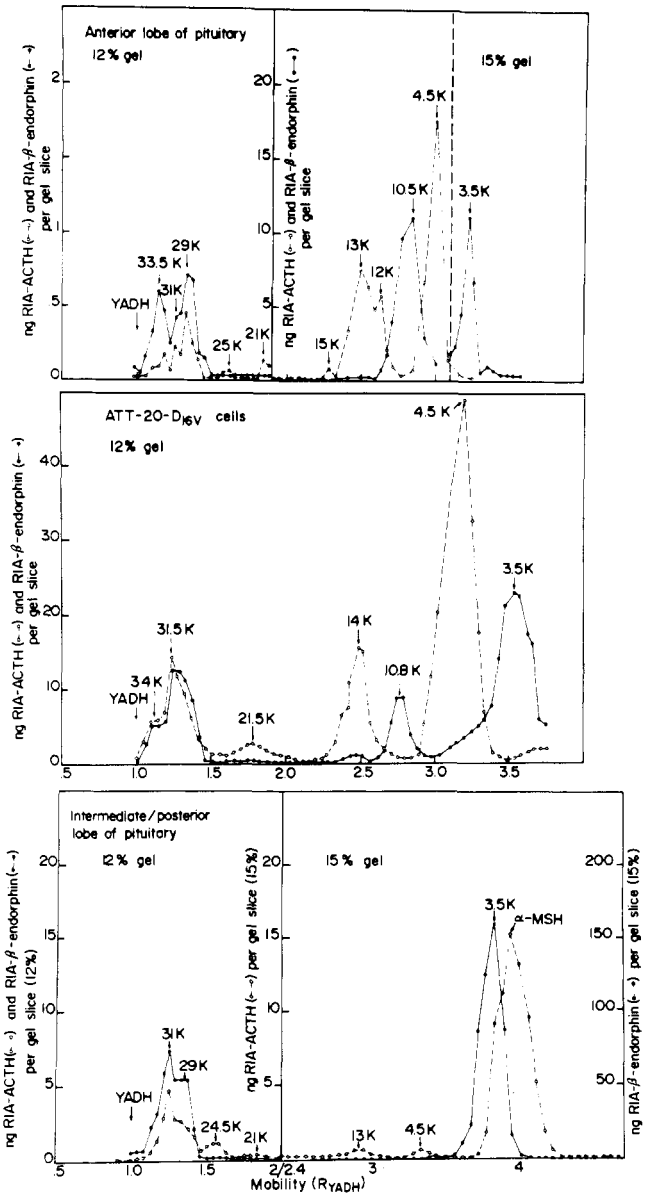

FIGURE 9: Analysis of immunoreactive forms of ACTH and endorphin in the anterior and intermediate-posterior lobes of mouse pituitaries and in AtT-20 cells by radioimmunoassay. Intermediate-posterior lobes of 20 mice were extracted, fractionated on $NaDodSO_4$-polyacrylamide gels, and analyzed by radioimmunoassay as described in Materials and Methods. Fifteen percent polyacrylamide gels were used to obtain resolution of small molecular weight forms. The abscissa represents a continuum of log molecular weight. The difference of R_{YADH} at the juncture of 12% and 15% profiles represents the difference in mobility of small molecules with the two acrylamide concentrations. The same procedure was used in analysis of two anterior pituitary lobes. AtT-20 cells were cultured and extracted as described in Materials and Methods.

proteins run but not in the regions where lower molecular weight proteins run. Hence, only the proteins in the 29–35K region of the gel appear to contain both endorphin and ACTH, whereas lower molecular weight proteins contain either endorphin or ACTH, but not both. These results suggest that the lobes of the pituitary contain the same precursor proteins and the same lower molecular weight forms of ACTH and endorphin as the tumor cells. The same size forms of ACTH and endorphin are observed in gel profiles of immunoprecipitated extracts of tumor cells labeled with amino acids (Figure 2, solid line).

Almost all of the ACTH immunoactivity in the intermediate-posterior lobe (excluding the activity in the α-MSH region) is in the precursor forms of ACTH-endorphin, whereas in the anterior lobe almost all the ACTH immunoactivity (85%) is in the 13K and 4.5K ACTH forms. In the intermediate lobe, ACTH immunoactivity is found in the region of the gel where α-MSH size (1.5–2.0K) material would run. This is in accord with the report by Scott et al. (1974) that α-MSH and an intermediate lobe peptide called "CLIP" [α(18–39)] are present in substantial amounts in the intermediate lobe of the rat pituitary. The molecular weight distribution of ACTH and endorphin is comparable for rats and mice (data not shown).

Figure 9 also shows that endorphin immunoactivity is present in the form of β-LPH size material (11.7K) in the anterior lobe but predominantly in β-endorphin size material (3.5K) in the intermediate-posterior lobe. These results suggest that processing of the ACTH-endorphin precursor is quite different in the two lobes.

Since the same antibodies were used to assay all of the tissue extracts, the differences in distribution of the immunoactive forms of ACTH and endorphin in the two lobes of the pituitary should reflect differences in the amounts of each protein present rather than differences in antigenicity of the forms of the hormones in the two lobes.

Finally, it should be noted that the distribution of immunoactive forms of ACTH and endorphin in the tumor cells is much more like that seen in the anterior lobe than that in the intermediate-posterior lobe.

Discussion

The common precursor to ACTH and endorphin exists in at least three different glycoprotein forms in AtT-20-D_{16v} cells with apparent molecular weights of 29 000, 32 000, and 34 000 (Roberts & Herbert, 1977a). The cell-free product synthesized under the direction of mRNA from AtT-20 cells has an apparent molecular weight of 28 500 and contains all of the lysine, methionine, and phenylalanine labeled tryptic peptides found in the 29K form isolated from the cells (Roberts & Herbert, 1977a,b). Steady labeling and pulse chase experiments show that the first form of the ACTH-endorphin precursor to be labeled in the cells is 29K ACTH-endorphin. The 29K ACTH-endorphin molecule appears to be a precursor of the 32K and 34K ACTH-endorphin.

Analysis of [^{3}H]amino acid labeled tryptic peptides suggests that the peptide backbone is similar in all three precursor forms since there are no tryptic peptides present in one form which are missing from the others. Therefore, the differences in apparent molecular weight of these forms are very likely due to different amounts of carbohydrate.

Analysis of tryptic glycopeptides suggests that the number of carbohydrate side chains in the precursors differs. It was observed that the tryptic peptide containing the α(22–39) sequence of ACTH is glycosylated in the 32K form but not in the 29K or 34K forms. This is interesting because it has recently been shown that 13K ACTH is the glycosylated form of 4.5K ACTH (Eipper & Mains, 1977). The 13K and 4.5K forms of ACTH are the end products of processing in the tumor cells and are the major forms secreted by tumor cells (Allen et al., 1978), and by primary pituitary cell cultures (unpublished observations of Paquette & Herbert). It appears that the decision to produce either 13K or 4.5K occurs as one of the earliest steps in the processing of the precursors. Since the formation of 13K and 4.5K ACTH from ACTH-endorphin precursors involves the generation of an ACTH intermediate (Mains & Eipper, 1976, and Figure 2), it could be predicted that at least two forms of this intermediate should exist. Three forms have been isolated by $NaDodSO_4$ gel electrophoresis,

26K, 23K, and 21K ACTH. ACTH, 23K, contains the glycosylated form of the α(22–39)-ACTH tryptic peptide, and 21K ACTH contains the unglycosylated form of this peptide.

Double-label experiments using [^{35}S]methionine and ^{3}H-labeled core sugars (glucosamine and mannose) or ^{3}H-labeled peripheral sugars (fucose, galactose, and glucosamine) substantiate the tryptic glycopeptide results. The 32K and 29K forms probably contain very little peripheral carbohydrate; however, 32K clearly contains more mannose and glucosamine than 29K. This difference is most likely due to the addition of another core carbohydrate to the α(22–39) sequence of ACTH. ACTH, 34K, contains relatively more glucosamine than 29K. It may be derived from 29K by addition of peripheral glucosamine or by addition of another carbohydrate side chain.

The data presented here, together with the knowledge that 11.5K endorphin is located at the COOH terminus of the precursor molecule, and that ACTH is adjacent to 11.5K endorphin near the middle of the molecule (Roberts & Herbert, 1977b), suggest the model of processing depicted in Figure 10. The 29K form of ACTH-endorphin is the first form synthesized in the tumor cells. Its peptide backbone is similar to the 28.5K cell-free precursor, but 29K ACTH-endorphin contains at least one carbohydrate side chain attached to the region of the molecule located N terminal to ACTH. It is possible that this carbohydrate side chain is attached to the amino-terminal portion of the growing nascent chain, since the unglycosylated precursor is not observed in vivo. Glycosylation of nascent chains has been observed with ovalbumin (Kiely et al., 1976) and with immunoglobulins (Bergman & Kuehl, 1977).

The 29K precursor can be processed in one of three ways: (1) it can be further glycosylated by addition of another carbohydrate side chain to the α(22–39)ACTH region to yield 32K ACTH-endorphin. The 32K protein can then be proteolytically cleaved to generate 11.5K endorphin and an ACTH intermediate (23K ACTH); (2) 29K ACTH-endorphin can be proteolytically processed to generate 11.5K endorphin and an ACTH intermediate (21K ACTH); (3) 29K ACTH-endorphin can be further glycosylated to generate 34K ACTH-endorphin. The 34K ACTH-endorphin is proteolytically processed to generate 11.5K endorphin and a heavily glycosylated intermediate, perhaps 26K ACTH. The 23K and 21K ACTH intermediates are subsequently cleaved to generate 12–15K ACTH and 4.5K ACTH, respectively, together with a glycoprotein of about 90 amino acids (Roberts et al., unpublished results), the N terminus of the ACTH-endorphin precursor molecule. ACTH, 4.5K, could also be derived from the 34K ACTH-endorphin precursor since 34K, like 29K, contains the unglycosylated α(22–39) sequence of ACTH. Quantitative pulse chase studies corroborate this model of processing since they show that all the label lost from the 29–34K pool of ACTH-endorphin precursors ("31K ACTH/endorphin") can be accounted for in smaller forms of ACTH and endorphin (Mains & Eipper, 1978). Cleavage of the 26K ACTH intermediate may yield a different form of the N-terminal glycopeptide from that generated by cleavage of 21K and 23K ACTH and, in fact, we have recently identified two forms of the N-terminal glycopeptide (Roberts et al., unpublished observations).

Double-label experiments with [^{35}S]methionine and [^{3}H]fucose suggest that peripheral sugars are added to 20–26K and 12–15K ACTH, since the fucose and methionine labeling patterns coincide in this region of the gel (Figure 6A). NaDodSO$_4$ gel analysis of [^{3}H]fucose- and [^{35}S]methionine-labeled ACTH-endorphin precursors suggests that the major

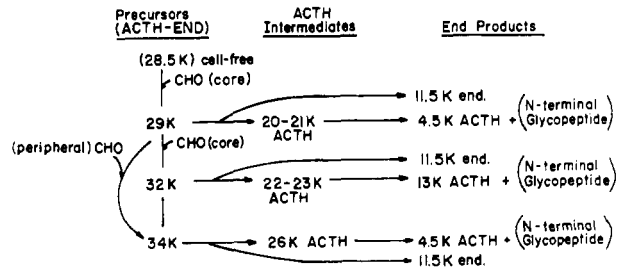

FIGURE 10: Model of processing of ACTH-endorphin precursors.

forms of the precursor present in cell extracts (34K, 32K, and 29K ACTH-endorphin) are proteolytically processed before peripheral sugars are added. However, two precursor forms with slightly different apparent molecular weights (33.5K and 30K ACTH-endorphin) which do contain fucose are present as minor species in cell extracts and as the major species in tissue culture medium (Figures 6A and 6B). It therefore appears that precursor forms which escape proteolytic processing are further glycosylated before they are secreted. NaDodSO$_4$ slab gel electrophoresis of amino acid labeled cell extracts substantiates this hypothesis. At 1 h of labeling three distinct bands of 29 000, 32 000, and 34 000 apparent molecular weight are observed, but, after longer labeling, two additional bands of 30 000 and 33 000 molecular weight appear (Roberts, unpublished observations).

Since the subcellular organelles involved in the processing of secreted proteins are known for many cell types, the subcellular location of some of the steps in the processing of the ACTH-endorphin precursor can be predicted. The synthesis of the ACTH-endorphin precursor in AtT-20 cells is similar to that of other secreted proteins in that it occurs on rough microsomes (Roberts & Herbert, 1977a). Many proteins which are synthesized on rough microsomes have been shown to contain a highly hydrophobic signal sequence at the N terminus of the molecule which is postulated to allow ribosomes synthesizing these proteins to attach to the membranes (Blobel & Dobberstein, 1975; Chan et al., 1976; Habener et al., 1976). It is not yet known whether ACTH contains such a signal sequence, but current studies on the structure of the N terminal of the forms of the ACTH-endorphin precursor synthesized in the cells and in the cell-free system should answer this question. Mannose and *N*-acetylglycosamine are thought to be attached to proteins in a block transfer involving a lipid-linked intermediate which probably takes place in the rough endoplasmic reticulum (Waechter & Lennarz, 1976). The 29K, 32K, and 34K precursor forms label primarily with these two core sugars. Since the first form of ACTH-endorphin to be labeled in vivo (29K ACTH-endorphin) contains carbohydrate, the first carbohydrate side chain may be added directly to growing nascent chains. This is very likely since this side chain is attached close to the N terminus of the ACTH-endorphin precursor (unpublished data) which would make it accessible rather early during synthesis to glycosylating enzymes. Shortly after synthesis another core group consisting of *N*-acetylglucosamine and mannose is attached to some of the 29K precursor molecules to generate the 32K form. It is not yet known whether the glycosylation of 29K ACTH-endorphin to yield 32K ACTH-endorphin is a random event, occurring with only a fraction of the 29K precursors, or a specific event, recognizing a subpopulation of the 29K precursors (i.e., there might be two forms of the precursor differing only in the carbohydrate attachment site). Peripheral sugars are beginning to be added to the different carbohydrate side chains at about the same time as the first proteolytic

cleavage occurs. These glycosyltransferases are generally located in the smooth endoplasmic reticulum and Golgi (Sharon, 1975; Schachter et al., 1970; Wagner & Cynkin, 1971). Additional studies are being performed to determine the precise nature of the carbohydrate side chains in the various forms of ACTH and the intracellular site of addition of the different sugars.

The presence of all of the forms of ACTH and endorphin in both AtT-20 cells and in mouse pituitary provides some assurance that the processing pathways being studied in AtT-20-D_{16v} cells are not tumor cell artifacts (Figure 9). It is interesting to note that the anterior and intermediate lobes of mouse pituitary appear to have the same forms of the ACTH-endorphin (29, 32, and 34K ACTH-endorphin forms) but a very different distribution of lower molecular weight forms of ACTH and endorphin. Furthermore, the end products of processing bearing either ACTH or endorphin determinants appear in equivalent amounts in both lobes. In the intermediate lobe, αMSH size material is present in approximately the same amount as 3.5K endorphin, while in the anterior lobe the amounts of 4.5K plus 13K ACTH approximately equal the amounts of 3.5K plus 11.5K endorphin. This suggests that the processing of the precursors follows a different pattern in the two lobes.

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