Glycosylation Events in the Processing and Secretion of Pro-ACTH-endorphin in Mouse Pituitary Tumor Cells†

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ABSTRACT: The glycosylation of pro-ACTH-endorphin and of its cleavage products has been studied in a mouse pituitary tumor cell line (AtT-20/D_{16v}) by pulse labeling with ^{3}H-labeled sugars and amino acids followed by immunoprecipitation and sodium dodecyl sulfate slab gel electrophoresis. The results of this analysis indicate that there are two major intracellular species of pro-ACTH-endorphin (29K and 32K) and two minor species (30K and 34K). All of these species label with [^{3}H]mannose, [^{3}H]glucosamine, and [^{3}H]galactose. In addition, the 30K and 34K species label with [^{3}H]fucose and are the major secreted forms of pro-ACTH-endorphin. Only the 29K and 32K forms are labeled after a short pulse with [^{35}S]methionine. The 30K and 34K forms become labeled during a subsequent chase. Glycosidase digestion of Pronase glycopeptides derived from pro-ACTH-endorphin shows that the 29K and 32K forms contain high mannose type oligosaccharides. The 30K and 34K forms contain complex oligosaccharides with both core and branch sugars. These results suggest that the 29K and 32K forms are converted to 30K and 34K forms by processing of mannose-rich oligosaccharides. Pulse labeling with ^{3}H-labeled sugars shows that pro-ACTH-endorphin is the first species labeled with both core and branch sugars. The label appears later in 21–26K and 12–15K ACTH, suggesting that glycosylation is completed before proteolytic cleavages begin. In support of this model, it is shown that intracellular and secreted forms of ACTH and N-terminal fragments have the same apparent molecular weights on sodium dodecyl sulfate slab gels. Furthermore, glycosidase digestion shows that the intracellular and secreted forms of the latter proteins contain complex oligosaccharides. The N-terminal portion of pro-ACTH-endorphin gives rise during processing to two major fragments (14.5K and 16–17.5K fragments) with very similar tryptic peptide contents. The 14.5K fragment contains a single oligosaccharide while the 16–17.5K fragment contains two.

A mouse pituitary tumor cell line (AtT-20/D_{16v}) has been previously used as a model system for anterior lobe corticotrophs to study the synthesis and processing of pro-ACTH-endorphin (Mains & Eipper, 1976; Mains et al., 1977; Roberts & Herbert, 1977b; Roberts et al., 1978). Processing of this molecule is particularly interesting because (1) it is the precursor to several different hormones and (2) it is present in both anterior and intermediate lobes of the pituitary but is processed to different peptide hormones in each lobe (Roberts et al., 1978). The precursor is processed in AtT-20 cells to glycosylated and unglycosylated forms of α(1–39)-ACTH[1] (12–15K ACTH and 4.5K ACTH, respectively), a β-lipotropin-like molecule (12.5K endorphin), and a β-endorphin-like molecule (3.5K endorphin). In addition, a glycoprotein (14–18K N-terminal fragment) is derived from the N-terminal portion of the precursor (Mains & Eipper, 1978; Roberts et al., 1978; Keutmann et al., 1979).

Processing studies (Mains & Eipper, 1978; Roberts et al., 1978) have shown that the generation of the peptide hormone end products requires at least three proteolytic cleavages which occur in a specific order as diagrammed in Figure 1.

The existence of glycoprotein forms of ACTH generates further complexity in the processing pathway. Heterogeneity is produced by the addition of different numbers of oligosaccharide side chains to pro-ACTH-endorphin. A 29K form has been identified which is a precursor to 4.5K ACTH (via a 21K intermediate), and a 32K form acts as a precursor to 12–15K ACTH (via a 23K intermediate) (Figure 1) (Roberts et al., 1978; Eipper & Mains, 1978).

Recent investigations of the biosynthesis of complex-type oligosaccharides of vesicular stomatitus virus (VSV) G protein have shown that glycosylation of proteins occurs as a highly ordered multistep process generating many intermediates (Kornfeld et al., 1978). The initial step is the attachment of a high mannose type oligosaccharide containing only *N*-acetylglucosamine, mannose, and glucose. Several glucose and mannose residues are subsequently removed to produce the smaller complex-type core followed by the stepwise addition of branch sugars, *N*-acetylglucosamine, galactose, fucose, and sialic acid (Tabas et al., 1978; Hunt et al., 1978; Robbins et al., 1977). Since pro-ACTH-endorphin contains complex-type oligosaccharides (Eipper & Mains, 1977; Roberts et al., 1978), one might expect to find several glycosylation intermediates. In particular, we have looked for molecules which contain high

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[1] Abbreviations used: ACTH, adrenocorticotropin; β-LPH, β-lipotropin; $NaDodSO_4$, sodium dodecyl sulfate; TPCK, tosylphenylalanyl chloromethyl ketone; Bis, *N*,*N*′-methylenebis(acrylamide); Temed, *N*,*N*,*N*′,*N*′-tetramethylenediamine; DMEM, Dulbecco–Vogt minimal essential medium; $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; IAA, iodoacetamide; SAC, *Staphylococcus aureus* Cowan I; BSA, bovine serum albumin; YADH, yeast alcohol dehydrogenase; dansyl, 5-(dimethylamino)naphthalene-1-sulfonyl; DNP, 2,5-dinitrophenol; VSV, vesicular stomatitis virus; Gdn·HCl, guanidine hydrochloride; Tris, tris(hydroxymethyl)aminomethane; GlcNAc, *N*-acetylglucosamine; endoglycosidase H, endo-β-*N*-acetylglucosaminidase H; RIA-ACTH, ACTH activity by radioimmunoassay expressed as nanograms of purified porcine ACTH with equivalent activity. The forms of ACTH, endorphin, and N-terminal fragments are named according to their apparent molecular weights determined by using $NaDodSO_4$ gel electrophoresis (Bio-Phore, 12%; Roberts et al., 1978). The intracellular precursor forms that contain both ACTH and endorphin are 29K and 32K pro-ACTH-endorphin, and the secreted forms are 30K and 34K pro-ACTH-endorphin. Other forms of ACTH or endorphin are designated as follows: 21K, 23K, and 26K ACTH for the intermediate forms of ACTH; 12–15K ACTH and 4.5K ACTH for the glycosylated and unglycosylated forms of α(1–39)-ACTH; 12.5K endorphin for the β-LPH-like molecule; 3.5K endorphin for the β-endorphin-like molecule.

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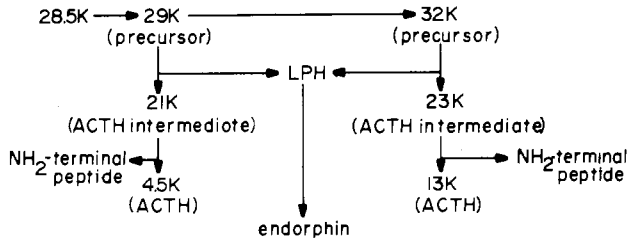

FIGURE 1: Model of processing of pro-ACTH-endorphin.

mannose type oligosaccharides and others which contain smaller complex-type core sugars with different numbers of branch sugars. In this paper, we present evidence that some of these intermediates are produced during the processing of pro-ACTH-endorphin.

Proteolytic cleavage and glycosylation events have been described for precursor proteins in many cell types, but the temporal relationship between these events has not been well characterized. This paper further describes some of the relationships between protein synthesis, glycosylation, cleavage, and secretion of ACTH/endorphin peptides.

Materials and Methods

Materials. D-[6-^{3}H]Glucosamine hydrochloride (20 or 38 Ci/mmol), D-[1-^{3}H]mannose (5 Ci/mmol), D-[1-^{3}H]galactose (22 Ci/mmol), L-[6-^{3}H]fucose (26 Ci/mmol), L-[4-^{3}H]-phenylalanine (26 Ci/mmol), and L-[^{35}S]methionine (500–1000 Ci/mmol) were purchased from Amersham Searle Corp. Other chemicals used were as follows: TPCK-treated trypsin and neuraminidase (Worthington Biochemical Corp.); jack bean α-mannosidase, *Clostridium perfringens* neuraminidase (types IV and IX), ovalbumin (grade VI), fetuin (type III), and bovine thyroglobulin (type I) (Sigma Chemical Co.); endo-β-*N*-acetylglucosaminidase H from *Streptomyces griseus* (Miles Laboratories); electrophoresis-grade acrylamide, Bis, Temed, and ammonium persulfate used for slab gel electrophoresis and Affigel (Bio-Rad); electrophoresis-grade $NaDodSO_4$ (British Drug House).

Columns. Pronase glycopeptides were analyzed on Bio-Gel P-4 (200–400 mesh) columns (0.8 × 100 cm) equilibrated with 50 mM NH_4OCOCH_3, pH 6.0, and 0.02% NaN_3. The flow rate was 4 mL/h, and 600-μL fractions were collected. V_0 was marked with 1 mg of BSA and V_t with 100 μg of mannose. The column was calibrated with ovalbumin, fetuin, and bovine thyroglobulin Pronase glycopeptides, with raffinose and stachyose oligosaccharides, and with bacitracin. Standards (except bacitracin and BSA) were located by the phenol–H_2SO_4 assay (Ashwell, 1966). For the determination of the molecular weights of N-terminal fragments, a Sepharose 6B column (1 × 70 cm) equilibrated with 4 M Gdn·HCl and 0.02% BSA was used. Fractions of 800 μL were collected. Protein standards and samples to be analyzed were dissolved in 6 M Gdn·HCl and 5% 2-mercaptoethanol and heated at 70 °C for 1 h. V_0 was determined with blue dextran and V_t with 2-mercaptoethanol. The column was calibrated with BSA, carbonic anhydrase, and cytochrome *c* which were located by A_{280}.

Incubation of AtT-20 Cells with Radioactive Amino Acids and Sugars. AtT-20 cells were grown in Falcon microtest wells in DMEM plus 10% horse serum and incubated with radioactive sugars and amino acids as previously described (Roberts et al., 1978; Mains & Eipper, 1976) with the following modifications. In order to incorporate sufficient [^{35}S]-methionine during a short labeling period (5 min) for detection of proteins by autoradiography, cells were equilibrated for 10–30 min with methionine-deficient medium before radioactive methionine was added in order to deplete the intracellular methionine pools. Incorporation of ^{35}S into Cl_3CCOOH-precipitable products was linear for 30–60 min after 10 min of equilibration in this medium. For longer labeling periods, complete DMEM without serum was used, and incorporation into Cl_3CCOOH-precipitable products was linear for at least 10 h after a 10-min equilibration period. For short pulse times (1 h or less), deficient DMEM does not appear to alter the processing of pro-ACTH-endorphin or secretion of hormone (as shown by $NaDodSO_4$ gels of immunoprecipitated cell extracts and culture medium).

In order to increase the specific activity of ACTH labeled with ^{3}H-labeled sugars, medium without glucose and horse serum and supplemented with nonessential amino acids and pyruvate (Eipper et al., 1976) was used. Cells were equilibrated in this medium for 30–60 min before labeling to deplete intracellular sugar pools. This procedure increased the incorporation of radioactive sugar precursors into ACTH 5–20-fold without altering the incorporation of [^{35}S]methionine. Incorporation of [^{3}H]mannose, [^{3}H]galactose, and [^{3}H]fucose into Cl_3CCOOH-precipitable products was linear for more than 4 h after an initial lag of 10 min. Incorporation of [^{3}H]glucosamine was linear after 30 min and remained linear for at least 4 h. Use of this medium did not alter the processing or secretion of ACTH.

The metabolic conversion of radioactive sugar precursors during a 2-h incubation period in glucose-free medium was determined by identifying the labeled sugars present in immunoprecipitable ACTH proteins (Roberts et al., 1978). The proportion of ^{3}H cpm recovered from the ACTH immunoprecipitate as the sugar originally added to the incubation was as follows: mannose, 73%; galactose, 68%; glucosamine, 95%.

Cell extracts were prepared for gel electrophoresis as described previously (Mains & Eipper, 1976; Roberts et al., 1978) except that BSA (the carrier protein) was omitted. The omission of BSA decreased nonspecific precipitation of proteins during immunoprecipitation but did not alter either the recovery of ACTH (as determined by RIA) or the distribution of the forms of ACTH. For oligosaccharide characterization, cells were extracted with 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, and 0.5% Triton X-100 containing protease inhibitors ($PhCh_2SO_2F$ and IAA, 1 mM each) to avoid hydrolysis of acid-sensitive sialic acids. Nuclei were removed by centrifugation, and the supernatants were stored at −20 °C. The recovery of the glycosylated forms of ACTH appears to be identical for both methods of extraction. However, there is a loss of unglycosylated ACTH in the neutral extraction method. This loss does not affect the interpretation of the data in these experiments.

Immunoprecipitations. Purified antiserum Wilma and antiserum Henrietta were used for all ACTH immunoprecipitations, and purified antiserum Bridgette was used for the endorphin immunoprecipitations. Antisera Wilma, Henrietta, Violet, and Bridgette were prepared in New Zealand white rabbits by subcutaneous injections of Freund's adjuvant mixed with a concentrate (Island et al., 1965) of AtT-20 culture medium. The primary injection was with 20 μg of immunoreactive equivalent of αp(1–39) (by RIA) followed by monthly booster injections of 10–30 μg each. ACTH antibodies were purified by affinity chromatography by using porcine α(1–39) covalently linked to Affigel, and endorphin antibodies were purified by using human β-lipotropin linked to Affigel (Mains & Eipper, 1976). ACTH- and endorphin-containing proteins were immunoprecipitated by using the SAC procedure (Kessler, 1975; Roberts et al., 1978). The specificity of the

immunoprecipitation was established by competition experiments with an excess of various unlabeled peptides as reported by Roberts & Herbert (1977a). The protein bands on the gels were identified by tryptic peptide mapping (Roberts et al., 1978).

For the isolation of N-terminal fragments, ACTH-containing proteins were first removed by immunoprecipitation. Crude serum containing N-terminal antibodies (antiserum Violet, Bridgette, or Bertha) was added to the supernatant; the sample was incubated for 12–16 h at 4 °C, and the antibody–antigen complexes were precipitated by addition of SAC. The specificity of these antisera was determined by tryptic peptide mapping of the immunoprecipitated proteins and is described under Results.

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis. Analyses of proteins on Bio-Rad 12% acrylamide gels were performed as previously described (Roberts & Herbert, 1977a,b; Roberts et al., 1978). Discontinuous $NaDodSO_4$ slab gel electrophoresis was according to Laemmli (1970), with the modifications of O'Farrell (1975). Ovalbumin, YADH, carbonic anhydrase, ribonuclease A, and cytochrome *c* were included in each slab gel as standards for the determination of molecular weights and were located by Coomassie blue staining. Fluorography of ^3H-labeled proteins was according to Bonner & Laskey (1974).

Tryptic Peptide Analysis. Radioactive proteins separated on $NaDodSO_4$ tube gels were eluted, precipitated, and digested with trypsin for analysis by paper electrophoresis at pH 6.5 (Roberts et al., 1978). Radioactive proteins separated on $NaDodSO_4$ slab gels were located by autoradiography and cut out of the gel. The proteins were eluted with 600 μL of 0.1 M NH_4HCO_3, carrier BSA was added, and the protein was precipitated by addition of Cl_3CCOOH to a final concentration of 15%. Tryptic peptide analysis proceeded according to Roberts et al. (1978).

Tryptic peptides were recovered from paper by eluting 8-mm slices of the paper for 12 h at room temperature with 500 μL of 0.1 M NH_4HCO_3, pH 8.5. Radioactive fractions were pooled and lyophilized.

Preparation of Pronase Glycopeptides. ^3H-Sugar-labeled immunoprecipitates were fractionated by using $NaDodSO_4$ tube gels. Proteins were eluted from 1-mm slices of the gels at room temperature with 50 mM Tris, pH 7.5, 0.1% $NaDodSO_4$, and 0.1% Triton X-100. Carrier BSA (1 mg) was added to appropriate pooled eluates, and the protein was precipitated by addition of 7–8 volumes of acetone at −20 °C. The precipitate was collected by centrifugation and washed once with acetone (−20 °C). The protein was dissolved in 100 mM Tris-HCl, pH 8.0, and 10 mM $CaCl_2$ and digested with 10 μg of Pronase at 37 °C. Toluene was added to prevent bacterial growth. Aliquots of 10 μg of Pronase were added every 24 h for a total digestion time of 4 days. Insoluble material was removed by centrifugation, and the supernatant was lyophilized.

Glycosidase Digestion of Pronase Glycopeptides. Pronase was removed from the glycopeptides by passage through a Bio-Gel P-4 column. Appropriate fractions were pooled and lyophilized, and the glycopeptides were dissolved in 150 μL of buffer and digested at 37 °C in the presence of toluene. Endo-β-*N*-acetylgulcosaminidase H (endoglycosidase H) digestions were done in sodium citrate, pH 5.5, for 2 days by using 5 milliunits of enzyme. α-Mannosidase digestions were done in 50 mM sodium acetate, pH 4.0, and 10 mM $ZnCl_2$ for 5 days with 400 milliunits of enzyme, with a second aliquot of enzyme added after the second day of digestion.

Base Hydrolysis of Glycopeptides. [^3H]Glucosamine-labeled tryptic glycopeptides were prepared as described above. Half of the sample was dissolved in 200 μL of 0.1 N NaOH and 1.0 N $NaBH_4$ and hydrolyzed at 37 °C for 72 h in an evacuated tube. The other half was dissolved in 200 μL of 1.0 N NaOH and 1.0 N $NaBH_4$ and hydrolyzed at 95 °C for 6 h in an evacuated tube (Eipper & Mains, 1977). After digestion, the samples were neutralized on ice with 90% acetic acid, desalted, and analyzed on the Bio-Gel P-4 column. Radioactive fractions were pooled and lyophilized. In order to identify the reduced form of the sugar, the sample was dissolved in 4 N HCl and hydrolyzed in an evacuated tube for 6 h at 100 °C (Spiro, 1972). The HCl was evaporated in an evacuated desiccator over solid NaOH. The sample was dissolved in 20 μL of 2 N HCl and analyzed by descending chromatography for 72 h (Fischer & Nebel, 1955). Aliquots of 10 μg each of glucosamine and $NaBH_4$-reduced glucosamine (glucosaminitol) were cochromatographed as standards and were located with ninhydrin. ^3H-labeled sugars were eluted from 5-mm slices of paper with 400 μL of 0.1 N HCl.

Results

Analysis of [^{35}S]Methionine and ^3H-Sugar-Labeled ACTH and Endorphin by $NaDodSO_4$ Slab Gel Electrophoresis. Figure 2 is a slab gel analysis of [^{35}S]methionine-labeled proteins from cell extracts or culture medium immunoprecipitated with either ACTH or β-endorphin antiserum. The 29–34K proteins are the only proteins which contain both antigenic determinants and are identified as pro-ACTH-endorphin on the basis of their molecular weights and tryptic peptide content (Figure 3A). Tryptic peptide analysis was used to identify the lower molecular weight forms of ACTH, including 20–25K ACTH intermediates (Figure 3B), 13–15K and 4.5K ACTH (Figure 3C), and endorphin (not shown).

Comparative studies by $NaDodSO_4$ gel electrophoresis show that the secreted forms of pro-ACTH-endorphin (30.5K and 34K) are slightly larger than the major intracellular forms of these proteins (29K and 32K). There are also minor intracellular pools of 30.5K and 34K pro-ACTH-endorphin. In contrast, both intracellular and secreted forms of the lower molecular weight cleavage products of pro-ACTH-endorphin are the same size. A plausible explanation of these results is that the 30.5K and 34K pro-ACTH-endorphin molecules arise from the 29K and 32K forms (respectively) by processing of oligosaccharides. Pulse–chase experiments support this hypothesis. As shown in the autoradiogram in Figure 4, label enters 29K and 32K pro-ACTH-endorphin after an 8-min pulse with [^{35}S]methionine and then appears in the 30.5K and 34K proteins after a 30-min chase.

Further support for this possibility was obtained by labeling cells with various ^3H-labeled sugars and isolating the forms of ACTH by immunoprecipitation with ACTH antisera and slab gel electrophoresis as shown in Figure 5. Note first that all of the ^{35}S forms of ACTH that label with methionine except 4.5K ACTH also label with ^3H-labeled sugars, showing that they are all glycoproteins. It can also be observed that [^3H]mannose [a core sugar added to glycoproteins during or just after protein synthesis (Kiely et al., 1976; Bergman & Kuehl, 1977)] labels only the 29K and 32K forms (Figure 5, lane 2). [^3H]Fucose, a terminal branch sugar added later in the processing pathway, labels only the intracellular 30K and 34K forms (Figure 5B, lane 4). [^3H]Glucosamine, both a core and a branch sugar, labels all of these forms (Figure 5A, lane 3). Finally, it can be seen that only the 30K and 34K forms of pro-ACTH-endorphin are found (Figure 5B, lane 1) in culture medium and that they contain all four ^3H-labeled

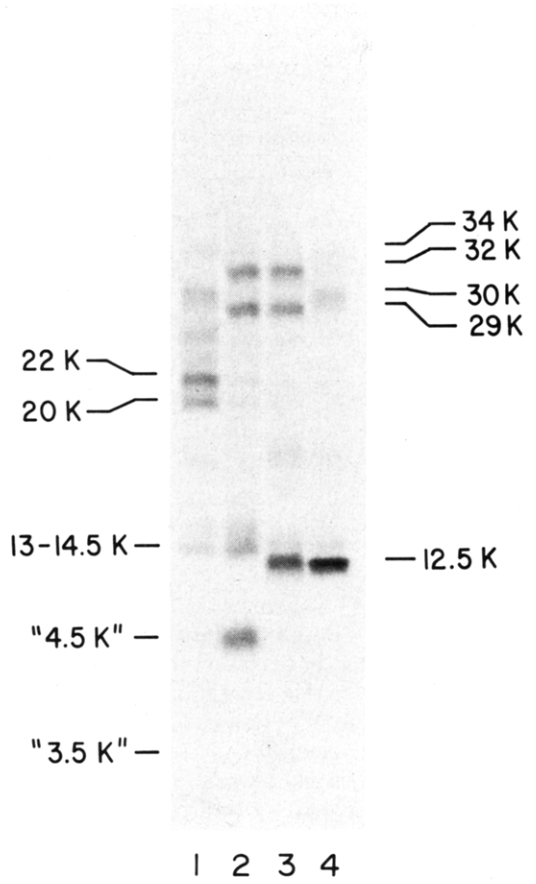

FIGURE 2: $NaDodSO_4$ slab gel analysis of ACTH and endorphin proteins from cell extracts and culture medium. One microtest well culture of AtT-20 cells was incubated with 150 μCi of L-[^{35}S]-methionine dissolved in 50 μL of low-methionine (40 μM) DMEM without horse serum for 2 h. Half of the culture medium and cell extract was immunoprecipiated with antiserum Bridgette (anti-endorphin) and half with antiserum Henrietta (anti-ACTH). The proteins were analyzed by $NaDodSO_4$ slab gel electrophoresis and autoradiography. Lanes 1 and 2 show ACTH proteins in culture medium and cell extract, respectively; lanes 3 and 4 show endorphin proteins from cell extracts and culture medium. See Materials and Methods for molecular weight calculations.

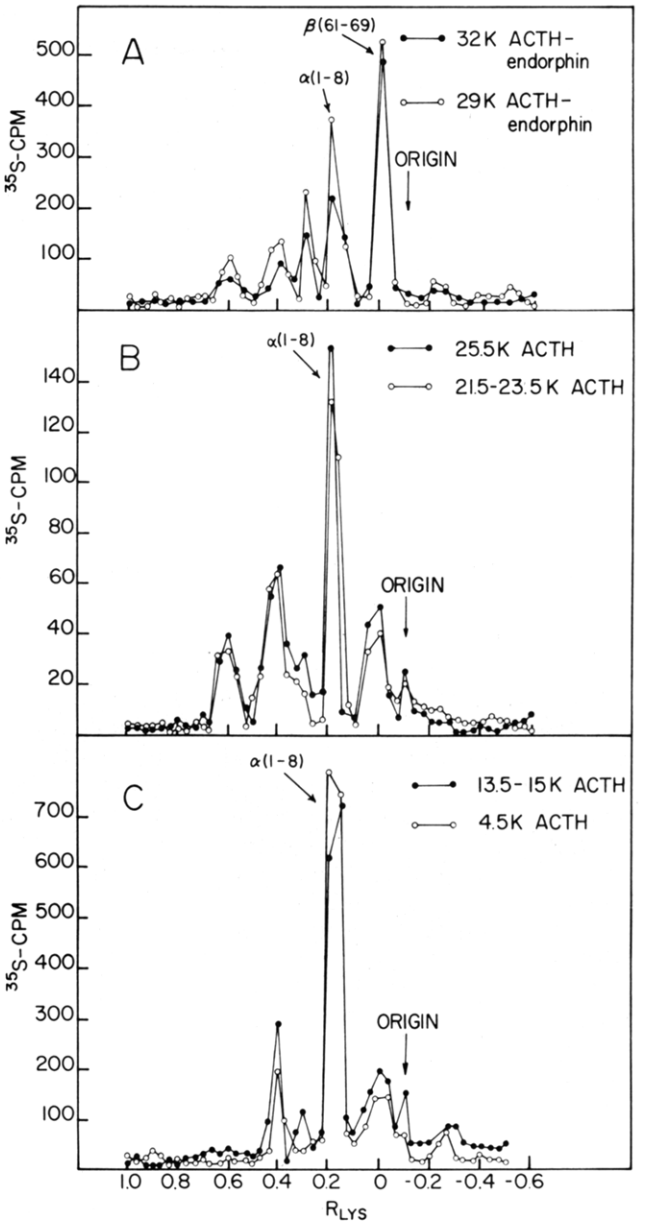

FIGURE 3: Tryptic peptides of [^{35}S]methionine-labeled ACTH proteins. One microtest well culture of AtT-20 cells was incubated with 150 μCi of L-[^{35}S]methionine for 2 h in methionine-deficient medium without horse serum. The ACTH-containing proteins were immunoprecipitated with antiserum Wilma and separated by $NaDodSO_4$ slab gel electrophoresis. [^{35}S]Methionine-labeled proteins were located by autoradiography. The radioactive bands were cut out of the gel, eluted, and digested with trypsin as described under Materials and Methods. Tryptic peptides were analyzed by paper electrophoresis at pH 6.5. Mobility is defined relative to lysine with R_{Lys} of ϵ-DNP of lysine = 0 and R_{Lys} = 1. A background of 15 cpm has been subtracted.

sugars (Figure 5A, lanes 1 and 5; Figure 5B, lanes 2 and 3). The results of this analysis suggest that the 30K and 34K forms are derived from 29K and 32K by the addition of terminal branch sugars. Examination of both intracellular and secreted forms of ^{3}H-sugar-labeled 21–26K and 12–15K ACTH reveals that these proteins label with both core and branch sugars. Therefore, the oligosaccharides of the intracellular forms of the latter proteins appear to be more fully processed than oligosaccharides of 29K and 32K pro-ACTH-endorphin.

Characterization of N-Terminal Fragments. N-Terminal fragments labeled with ^{3}H-labeled sugars or amino acids have been partially purified by immunoprecipitation and $NaDodSO_4$ gel electrophoresis (see Materials and Methods for details). We define an N-terminal fragment as a protein which contains all of the tryptic peptides present in pro-ACTH-endorphin that are not derived from 12.5K endorphin or from 4.5K ACTH. Tube gel electrophoresis resolves two peptides that can be identified as N-terminal fragments by this criterion (not shown). Analysis of N-terminal immunoprecipitates of cell extracts by slab gel electrophoresis resolves these proteins into four bands (Figure 6). Tryptic peptide analysis of each of the [^{3}H]phenylalanine-labeled bands shows that only the 13.5–17.5K class of proteins contains N-terminal peptides. This is illustrated for the 14.5K fragment in Figure 7. It can be seen that only the peptides derived from the N-terminal region of the 29K form of pro-ACTH-endorphin are present in the 14.5K fragment. Peptide maps of the 17.5K, 16K, and 13.5K proteins are identical with the 14.5K map whereas all other protein bands on the gel contain non-N-terminal peptides (not shown).

Analysis of [^{3}H]glucosamine-labeled tryptic peptides (Figure 7) shows that the 16–17.5K forms contain two tryptic glycopeptides whereas the 13.5–14.5K forms contain only one. Both of these tryptic glycopeptides are present in digests of 29–34K pro-ACTH-endorphin and 21–26K ACTH (Roberts et al., 1978). The existence of two N-terminal tryptic glycopeptides could be explained by charge heterogeneity due to different

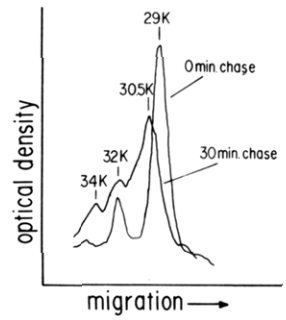

FIGURE 4: Pulse chase with [^{35}S]methionine. Two microwell cultures of AtT-20 cells containing approximately the same amount of ACTH-RIA were each pulsed for 8 min with 50 μCi of [^{35}S]-methionine dissolved in 50 μL of methionine-deficient DMEM. Either the cells were extracted immediately with acetic acid (0-min chase) or the medium was replaced with DMEM containing the standard concentration of methionine for a 30-min chase period followed by acetic acid extraction. The experiments were done at approximately 30 °C in order to slow the processing events sufficiently to allow the transitions in molecular weight to be detected. ACTH was immunoprecipitated by using antiserum Wilma. The proteins were analyzed by NaDodSO$_4$ slab gel electrophoresis, visualized by autoradiography, and quantitated by microdensitometry. Molecular weights were calculated as described under Materials and Methods.

numbers of sialic acid residues present on a single oligosaccharide. However, neuraminidase digestion of the tryptic peptides does not convert the two glycopeptides into one. Accurate molecular weights for glycoproteins cannot be obtained by NaDodSO$_4$ gel electrophoresis (Segrest & Jackson, 1972) but can be estimated by gel filtration in Gdn·HCl (Eipper & Mains, 1978). Molecular weight estimates by this method are 16–17.5K = 10500 and 13.5–14.5K = 8500. The difference in molecular weight is close to the molecular weight of a single N-terminal oligosaccharide (M_r = 2550) (data presented in Table I). Altogether, these data suggest that there are two major species of the N-terminal fragment,

Table I

Pronase glycopeptides	source	mol wt[a]	digestion properties: α-Man	digestion properties: endo-H
29K, 32K pro-ACTH-endorphin	cell	2250–2550	+	+
30K, 34K pro-ACTH-endorphin	cell	2700–3250	–	–
30K, 34K pro-ACTH-endorphin	medium	2900–3100	–	–
21–26K ACTH	cell	2600	–	–
21–26K ACTH	medium	2600	–	–
12–15K ACTH	cell	3250	–	–
12–15K ACTH	medium	3250	–	–
14.5–17.5K N-terminal fragment	cell	2650	–	–
14.5–17.5K N-terminal fragment	medium	2550	–	–

[a] Multiple peaks are reported as a range of molecular weight values. Single peaks are reported as a single value.

13.5–14.5K and 16–17.5K, which contain different numbers of oligosaccharides. Both of these species can be further resolved into at least two bands by gel electrophoresis; however, the structural basis for this separation is not known.

Tryptic peptide maps of N-terminal fragments labeled with [^{3}H]mannose, [^{3}H]galactose, [^{3}H]fucose, and [^{3}H]glucosamine show that both the basic and the neutral glycopeptides contain all of these sugars, suggesting that both of the N-terminal oligosaccharides are complex-type carbohydrates.

Linkage of Oligosaccharides to Protein. Hydrolysis of either the basic or the neutral tryptic glycopeptide prepared either from 29–32K pro-ACTH-endorphin or from 16–18K N-terminal fragments with strong base (1.0 N NaOH hydrolyzes glycosylamine and glycosidic bonds) produced oligosaccharides of 2100 or 1800 molecular weight, respectively,

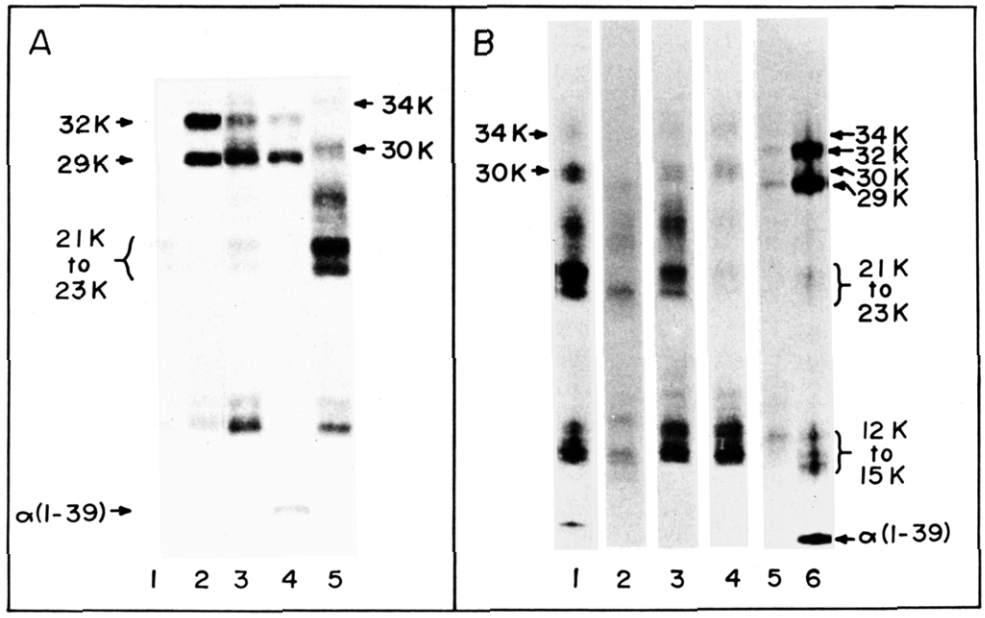

FIGURE 5: NaDodSO$_4$ slab gel analysis of ACTH immunoprecipitates labeled with ^{3}H-labeled sugars. AtT-20 cells were preincubated for 1 h in DMEM without serum and glucose and then incubated for 2 h with the following radioactive precursors dissolved in 50 μL of DMEM without horse serum and glucose: 150 μCi of D-[1-^{3}H]mannose, 150 μCi of D-[6-^{3}H]glucosamine, 100 μCi of L-[4-^{3}H]phenylalanine, 100 μCi of D-[1-^{3}H]galactose, 100 μCi of L-[6-^{3}H]fucose, or 50 μCi of L-[^{35}S]methionine. ACTH proteins were immunoprecipitated from cell extracts by using antiserum Wilma. The proteins were analyzed by NaDodSO$_4$ slab gel electrophoresis and visualized by fluorography. The proteins shown in (A) were analyzed on one slab gel as follows: (lane 1) [^{3}H]mannose culture medium; (lane 2) [^{3}H]mannose cell extract; (lane 3) [^{3}H]glucosamine cell extract; (lane 4) [^{3}H]phenylalanine cell extract; (lane 5) [^{3}H]glucosamine culture medium. The proteins shown in (B) were analyzed on another slab gel as follows: (lane 1) [^{35}S]methionine culture medium; (lane 2) [^{3}H]galactose culture medium; (lane 3) [^{3}H]fucose culture medium; (lane 4) [^{3}H]fucose cell extract; (lane 5) [^{3}H]galactose cell extract; (lane 6) [^{35}S]methionine cell extract. Molecular weights were calculated as described under Materials and Methods.

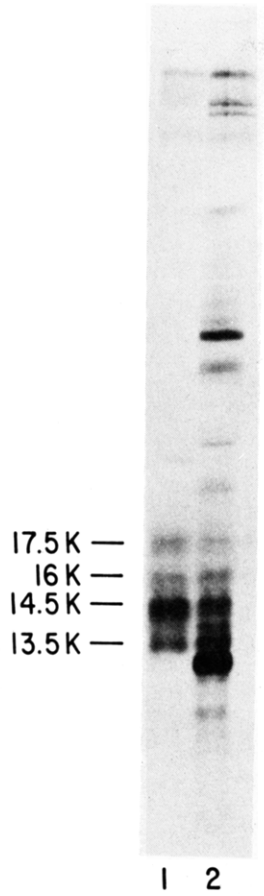

FIGURE 6: $NaDodSO_4$ slab gel analysis of [^{3}H]glucosamine- and [^{3}H]phenylalanine-labeled proteins immunoprecipitated with N-terminal antiserum. Cultures of AtT-20 cells grown in microtest wells were incubated with 150 μCi of D-[6-^{3}H]glucosamine hydrochloride dissolved in 50 μL of DMEM without glucose and horse serum (1) or with 200 μCi of L-[4-^{3}H]phenylalanine (2). ACTH-containing proteins were removed from the cell extracts by immunoprecipitation. The supernatants were immunoprecipitated with antiserum Bertha and analyzed by $NaDodSO_4$ slab gel electrophoresis and fluorography.

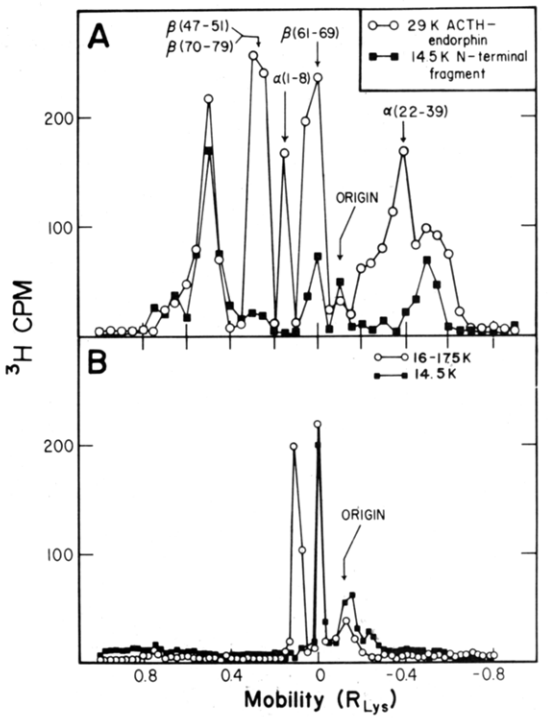

FIGURE 7: Tryptic peptides of [^{3}H]phenylalanine- and [^{3}H]glucosamine-labeled N-terminal fragment. (A) Tryptic peptides were prepared as described under Materials and Methods from [^{3}H]-phenylalanine-labeled 14.5K N-terminal fragment isolated from the gel shown in Figure 6, lane 2. As a reference, 29K pro-ACTH-endorphin tryptic peptides were prepared from an ACTH immunoprecipitate of an extract of cells incubated for 1 h with 100 μCi of L-[4-^{3}H]phenylalanine in 50 μL of Ham's F-10 medium. Peptides were separated by paper electrophoresis at pH 6.5. Mobility is defined relative to the mobility of lysine with R_{Lys} of ϵ-DNP of lysine = 0 and R_{Lys} = 1. Peptides present in α(1–39)-ACTH or in 12.5K endorphin are indicated in the figure (Roberts & Herbert, 1977a,b). (B) Tryptic peptides were prepared as described under Materials and Methods from [^{3}H]glucosamine labeled 14.5K and 16–17.5K N-terminal fragment isolated from the gel shown in Figure 6, lane 1. Peptides were separated by paper electrophoresis at pH 6.5.

as determined by gel filtration (Figure 8A shows result for the basic glycopeptide only). Hydrolysis of these tryptic glycopeptides with mild base (0.1 N NaOH hydrolyzes only glycosidic bonds) generated molecules larger than the oligosaccharides produced with strong base but smaller than the unhydrolyzed glycopeptides (not shown). These molecules are probably formed by hydrolysis of several peptide bonds but not hydrolysis of the oligosaccharide–protein linkage.

Base hydrolysis of the oligosaccharide–protein linkage in the presence of $NaBH_4$ results in the reduction of sugar involved in forming the bond. However, if the linkage is still intact, all of the sugars will exist in the unreduced form. Paper chromatography of an HCl hydrolysate of the products resulting from strong base–$NaBH_4$ treatment of tryptic glycopeptides derived from [^{3}H]glucosamine-labeled 29–32K pro-ACTH-endorphin shows that approximately 30% of the ^{3}H migrates with glucominitol and 60% with glucosamine (Figure 8B). This indicates that there are three *N*-acetylgulcosamine residues in each oligosaccharide and that one of these in involved in the linkage to the protein. In contrast, acid hydrolysis of the glycopeptides treated with mild base results in almost 100% of the ^{3}H migrating with glucosamine (not shown). Hence, the oligosaccharides in both the basic and neutral tryptic glycopeptides are linked via a glycosylamine bond to *N*-acetylglucosamine.

Gel Filtration Analysis and Glycosidase Digestion. Gel filtration analysis of Pronase glycopeptides from either intracellular or secreted forms of 21–26K ACTH, 12–15K ACTH, or 13.5–17.5K N-terminal fragments shows that the sizes of these glycopeptides for a particular form are similar (Table I). This is consistent with the observation that there are no detectable differences in the sugar labeling patterns or in mobilities of intracellular and secreted forms of these proteins on $NaDodSO_4$ gels. The analysis also indicates that the 12–15K ACTH oligosaccharides are larger than the N-terminal fragment oligosaccharides.

In contrast, the Pronase glycopeptides from the 29K and 32K forms of pro-ACTH-endorphin are smaller (M_r = 2250–2550) than those prepared from either the intracellular or the secreted 30K and 34K molecules (M_r = 2900–3250), reflecting the differences noted earlier in the sugar labeling patterns and mobilities of these forms.

Endoglycosidase H specifically cleaves high mannose sugars between the two *N*-acetylglucosamine residues of the *d-N*-acetylchitobiose group whereas α-mannosidase removes terminal, but not internal, mannose residues. Neither enzyme cleaves complex oligosaccharides. The 29–32K glycopeptides are susceptible to digestion by both enzymes, as reflected by their increased K_D after exposure to these enzymes (panels A and B, Figure 9), while the 34K glycopeptides are not. A similar analysis of Pronase glycopeptides prepared from other forms of ACTH and N-terminal fragment is summarized in Table I. None of the latter glycopeptides are digested by either endoglycosidase H or α-mannosidase, although they are all

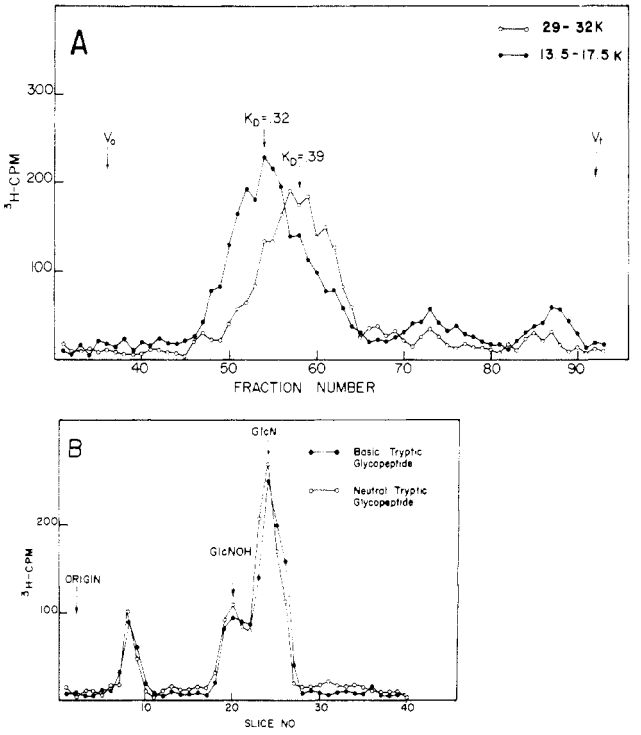

FIGURE 8: Characterization of the oligosaccharide–peptide linkage. (A) Base hydrolysis of [^{3}H]glucosamine-labeled basic tryptic glycopeptides: analysis by gel filtration. Cultures of AtT-20 cells were incubated for 4 h with 100 μCi of [^{3}H]glucosamine dissolved in DMEM without glucose or horse serum. ACTH proteins and N-terminal fragment were isolated for trypsin digestion by immunoprecipitation and $NaDodSO_4$ gel electrophoresis by using tube gels. Tryptic glycopeptides from each of these proteins were prepared, separated, and recovered as described uner Materials and Methods. The samples were then dissolved in 500 μL of 1 N NaOH and 1 N $NaBH_4$, hydrolyzed at 95 °C for 6 h, neutralized with acetic acid, and analyzed by gel filtration on Bio-Gel P-4. BSA marks V_o and mannose marks V_t. (B) Acid hydrolysis of base-hydrolyzed tryptic glycopeptides prepared from 29–32K pro-ACTH-endorphin: analysis by paper chromatography. [^{3}H]Glucosamine-labeled tryptic glycopeptides were hydrolyzed with 1.0 N NaOH and 1.0 N $NaBH_4$, and the resulting oligosaccharides were analyzed by gel filtration on Bio-Gel P-4 (see Materials and Methods). Fractions were pooled, hydrolyzed with HCl, and analyzed by paper chromatography as described under Materials and Methods. The paper was sliced, eluted, and counted. The locations of glucosamine and glucosaminitol (R_{GlcN} = 0.82) are indicated in the figure.

susceptible to digestion by a mixture of neuraminidase, β-galactosidase, and fucosidase (not shown).

Kinetic Analysis of Glycosylation Events by the Double-Label Technique. If branch sugars are added to the protein just before secretion, ^{3}H-sugar-labeled proteins should appear in culture medium before [^{35}S]methionine-labeled proteins. However, if processing of the oligosaccharides is completed during or shortly after protein synthesis is completed, ^{3}H-sugar- and [^{35}S]methionine-labeled proteins should appear almost simultaneously in the culture medium. Figure 10 shows that the time course of the appearance in culture medium of immunoprecipitable ACTH labeled with either ^{3}H-labeled sugars or with [^{35}S]methionine is indistinguishable. There is a 45–60-min lag before ^{3}H- or ^{35}S-labeled ACTH appears in culture medium. Accumulation is linear after that for at least 4 h.

The time course of labeling of the intracellular forms of ACTH with ^{3}H-labeled sugars and [^{35}S]methionine was examined in order to determine which forms are substrates for glycosyltransferases. The 29–34K forms of pro-ACTH-endorphin are the first forms labeled with all of the sugars (Figure 11). Lower molecular weight forms of ACTH become labeled later (not shown), indicating that glycosylation

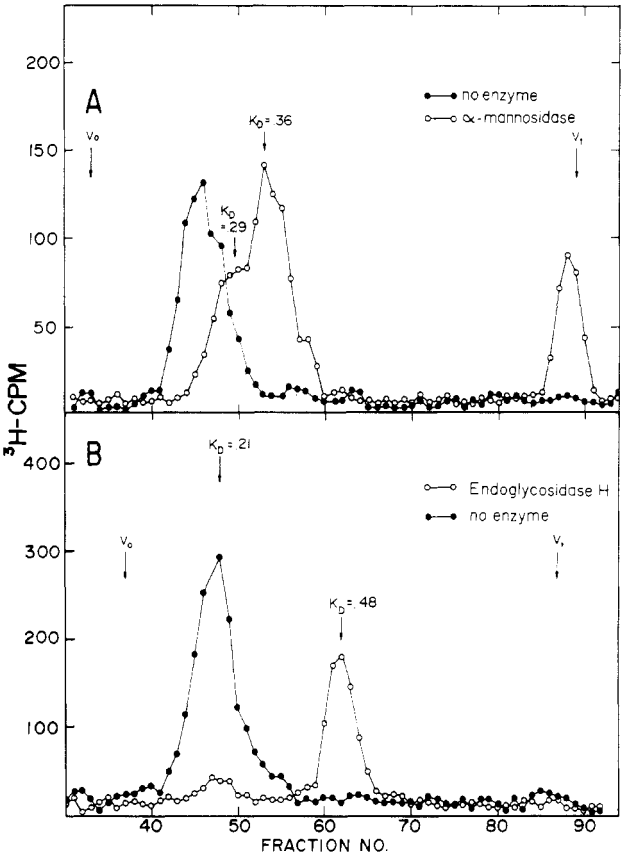

FIGURE 9: Gel filtration analysis of glycosidase-digested Pronase glycopeptides prepared from 29K pro-ACTH-endorphin. Cultures of AtT-20 cells were incubated for 2 h with 200 μCi of [^{3}H]mannose dissolved in 50 μL of DMEM without glucose and serum. Pronase glycopeptides were prepared from 29K pro-ACTH-endorphin and incubated with or without enzyme as described under Materials and Methods. The digestion products were analyzed by gel filtration on Bio-Gel P-4. Panel A shows α-mannosidase digestion products, and panel B shows endoglycosidase H products.

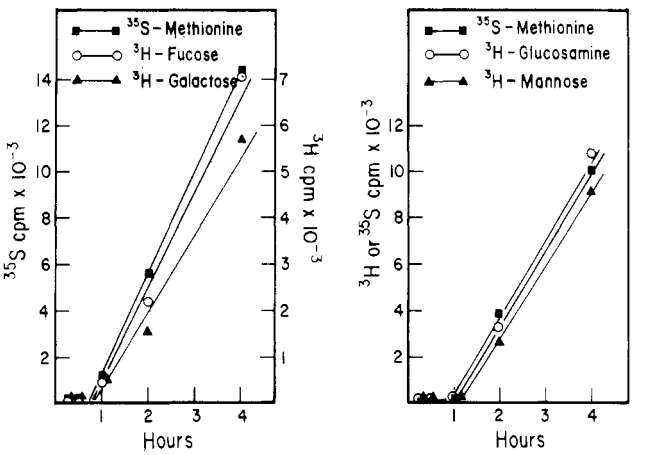

FIGURE 10: Time course of secretion of [^{35}S]methionine- and ^{3}H-sugar-labeled ACTH proteins. One hour before addition of radioactive precursors to cultures of AtT-20 cells, the medium was changed to DMEM without glucose and horse serum. Four sets of five microwells were subsequently incubated with both L-[^{35}S]methionine (20–30 μCi/well) and one of the following sugars (100 μCi/well dissolved in 50 μL in DMEM without glucose and horse serum): D-[6-^{3}H]-glucosamine hydrochloride, D-[1-^{3}H]mannose, D-[1-^{3}H]galactose, or L-[6-^{3}H]fucose. Culture medium was immunoprecipitated with antiserum Henrietta, and the total cpm present in the precipitate are plotted as a function of time of incubation of the cultures.

is completed before proteolytic cleavages occur. This can be verified by plotting the percentage of the total cpm present in each size class of ACTH as a function of time (Figure 12).

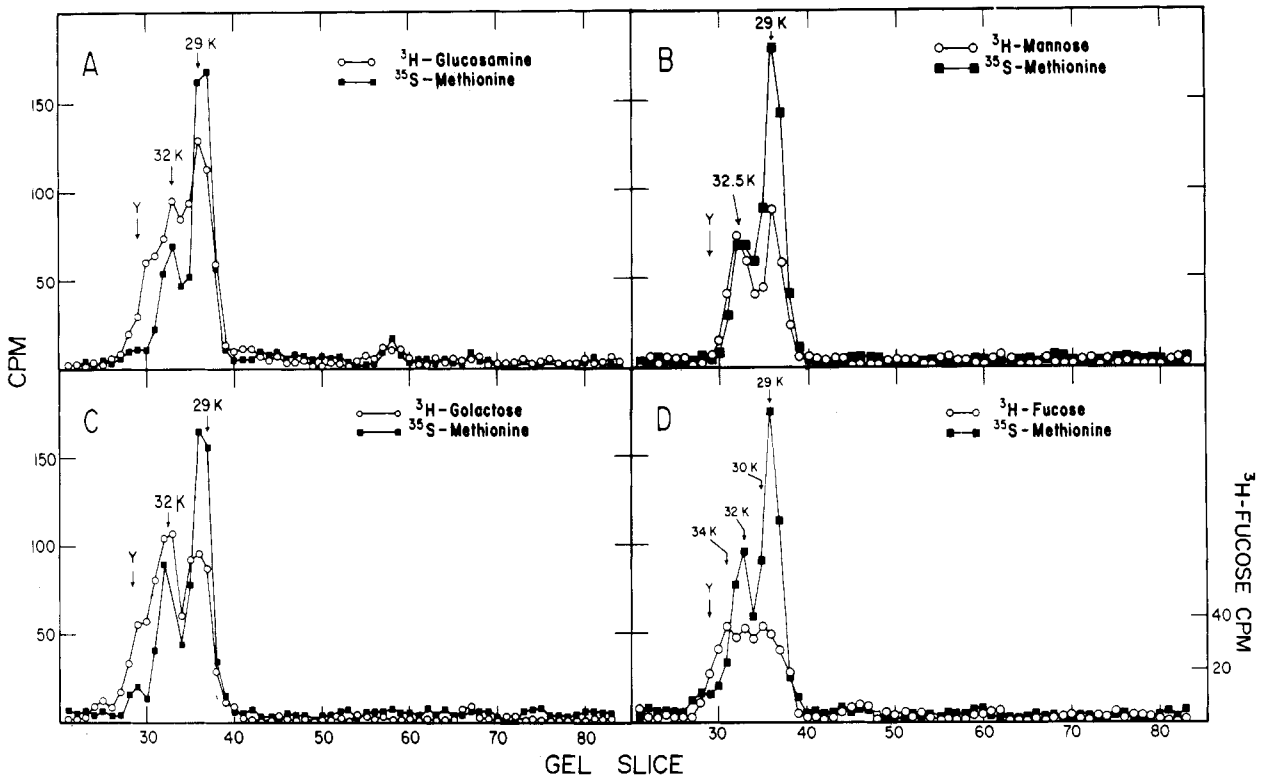

FIGURE 11: Pulse labeling of ACTH proteins with ^{3}H-labeled sugars and [^{35}S]methionine. AtT-20 cells were preincubated for 1 h in DMEM without serum and glucose and then labeled for 20 min with 30 μCi of [^{35}S]methionine and 150 μCi of the following sugars dissolved in 50 μL of the same medium: (A) D-[6-^{3}H]glucosamine hydrochloride; (B) D-[1-^{3}H]mannose; (C) D-[1-^{3}H]galactose; (D) L-[6-^{3}H]fucose. ACTH proteins were immunoprecipitated with antiserum Wilma and analyzed on 12% Biophore gels. The position of dansylated YADH, an internal standard, is indicated. Eluates of gel slices were counted by using a program for quenched ^{3}H/^{35}S counting. ^{3}H cpm were adjusted for ^{35}S spillover, and background was subtracted.

This analysis indicates a precursor–product relationship between the ^{3}H-sugar-labeled forms of ACTH. The 29–34K forms become labeled first; the 21–26K forms become labeled next as the percentage of label in the 29–34K forms decreases. Finally, label enters 12–15K ACTH, and the percentage of label in these forms increases 2–4 h before leveling off. Cells incubated with [^{3}H]mannose show a slightly different time course of labeling. The 29–34K pro-ACTH-endorphin forms are the only ones labeled for the first hour. Both the 21–26K and the 12–15K forms label between 1 and 2 h.

Discussion

In this study, we have attempted to relate glycosylation events to the time course of synthesis and proteolytic cleavage of pro-ACTH-endorphin and hormone secretion. The forms of ACTH or N-terminal fragment labeled with either ^{3}H-labeled sugars or [^{35}S]methionine were separated by immunoprecipitation and gel electrophoresis, and each protein was eluted from the gel for structural analysis. Tryptic peptide analysis was used to classify proteins into families with the same peptide backbone. All of the proteins, except 4.5K ACTH, are glycoproteins. In order to analyze the structure of the oligosaccharides, Pronase glycopeptides were prepared from each protein and characterized by gel filtration and by digestion with specific glycosidases. Caution is required in interpreting some of these results because glycoproteins containing complex oligosaccharides exhibit microheterogeneity and may not be completely separated by gel electrophoresis. Therefore, structural analysis of protein in a single band on a gel may actually represent a composite analysis of several closely related glycoproteins. Despite these limitations, this approach has been useful for the separation of proteins which differ in their oligosaccharides, including the 29K, 30K, 32K, and 34K forms of the precursor and three ACTH intermediates, and to further define some of the steps involved in the glycosylation of pro-ACTH-endorphin.

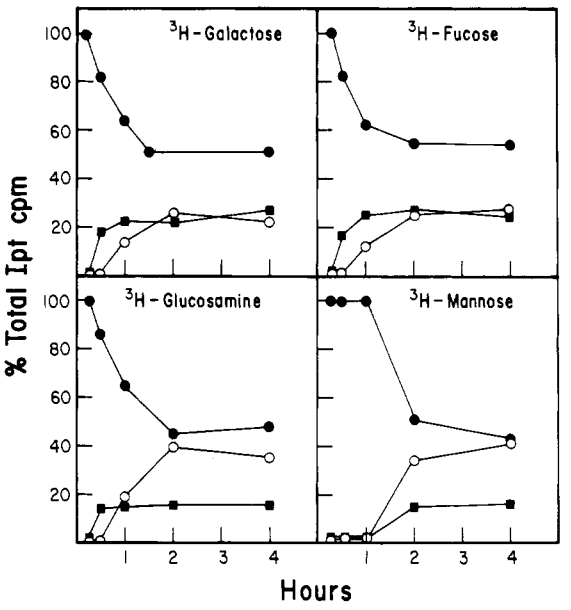

FIGURE 12: Time course of incorporation of ^{3}H-labeled sugars into ACTH proteins. AtT-20 cell cultures were labeled as described in Figure 11. Both culture medium and cell extracts were immunoprecipitated with antiserum Henrietta. The ACTH proteins were separated by $NaDodSO_4$ gel electrophoresis on tube gels (Biophore, 12%). The total amounts of ^{3}H (cell extracts and culture medium) in the 29–34K (●), 21–26K (■), and 12–15K (○) forms were computed and plotted as a percentage of the total immunoprecipitable ^{3}H cpm.

An expanded model of the processing of pro-ACTH-endorphin is shown in Figure 13. Previous work has demon-

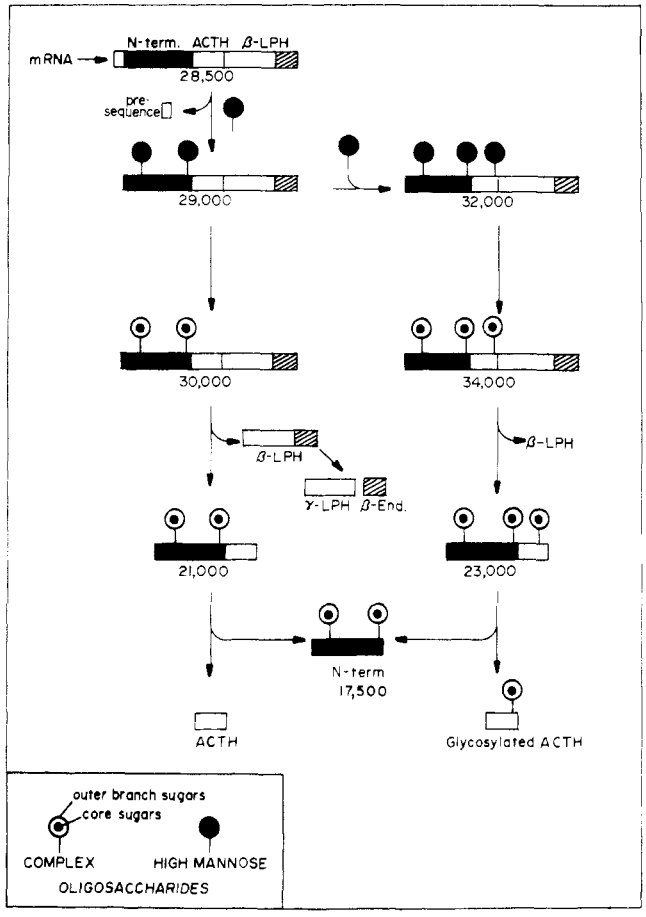

FIGURE 13: Proposed model of processing of pro-ACTH-endorphin. ● indicates a high mannose type sugar, O indicates addition of branch sugars, and □ indicates removal of the signal peptide.

strated that ACTH mRNA from AtT-20 cells directs the synthesis of a protein of 28 500 daltons in a cell-free system (Roberts & Herbert, 1977a). Two cotranslational modifications occur in AtT-20 cells. A signal sequence is removed (Policastro et al., 1981) and one or two oligosaccharides are attached to the nascent polypeptide (as discussed later), resulting in the appearance of a 29 000-dalton glycoprotein (Roberts & Herbert, 1977b; Roberts et al., 1978). An additional oligosaccharide can be attached to the 29K protein to generate 32K pro-ACTH-endorphin (Roberts et al., 1978).

The following results suggest that most of the 29K and 32K proteins contain high-mannose oligosaccharides and that addition of terminal branch sugars to these proteins generates 30K and 34K forms: (1) 29K and 32K pro-ACTH-endorphin are solely intracellular proteins as expected of transient precursor forms while the 30K and 34K forms are found both in cells and in culture medium. (2) 29K and 32K pro-ACTH-endorphin are the first forms labeled during a short pulse with radioactive amino acids. Some of the label can be subsequently chased into 30K and 34K forms. (3) The 29K and 32K proteins label predominantly with core sugars (mannose and glucosamine) while 30K and 34K label with both core and terminal branch sugars (fucose). It should be noted that 29K and 32K forms also label with galactose, an inner branch sugar (Figures 5 and 11). It is not clear why addition of galactose to the precursor forms (29K and 32K proteins) does not cause an observable shift in mobility whereas the addition of fucose (and possibly sialic acid) does cause a shift in mobility (to 30K and 34K proteins). (4) Finally, most of the Pronase glycopeptides prepared from 29K and 32K pro-ACTH-endorphin can be digested with endoglycosidase H and mannosidase, showing that most of these proteins contain oligosaccharides similar to the high mannose type core oligosaccharides described by others (Robbins et al., 1977; Tabas et al., 1978; Hunt et al., 1978). In contrast, Pronase glycopeptides from 30K and 34K forms are not digested by these two enzymes but can be digested by a mixture of exoglycosidases, which remove branch sugars.

Pulse labeling experiments with ^{3}H-labeled sugars were done to determine if glycosylation is completed before or after proteolytic cleavages occur. During a 20-min pulse with ^{3}H-labeled mannose, galactose, glucosamine, or fucose, only pro-ACTH-endorphin is labeled. When the percentage of label in the various size classes of ACTH is plotted as a function of time (Figure 12), it can be seen that 29–34K pro-ACTH-endorphin acts as a biosynthetic precursor, 21–26K ACTH has the properties of a biosynthetic intermediate, and 12–15K ACTH has the properties of an end product. If glycosylation had been completed after proteolytic cleavages, ^{3}H-labeled sugars would have labeled all forms of ACTH at nearly the same time.

Analysis of 21–26K and 12–15K ACTH and of 16–17.5K N-terminal fragment shows that (1) both the intracellular and secreted forms have the same mobilities on $NaDodSO_4$ gels, (2) all forms label with [^{3}H]mannose, -glucosamine, -galactose, and -fucose, and (3) Pronase glycopeptide preparations are not digested with endoglycosidase H or α-mannosidase, but can be digested with exoglycosidases. These data suggest that both the intracellular and secreted forms of these proteins are fully glycosylated and support the model that glycosylation is completed before proteolytic cleavages occur.

Fragments derived from the N-terminal region of the precursor have been isolated from cell extracts by immunoprecipitation and $NaDodSO_4$ gel electrophoresis and identified by tryptic peptide mapping. This analysis reveals that there are two major intracellular species, 14.5K and 16–17.5K N-terminal fragment. Tryptic peptide mapping of ^{3}H-sugar-labeled N-terminal fragment shows that the 14.5K form contains only one tryptic glycopeptide while the 16–17.5K form contains two. Both of these tryptic glycopeptides have complex oligosaccharides attached to the peptide via a glycosylamine linkage to *N*-acetylglucosamine.

The question arises next as to how the 14.5K and 16–17.5K terminal fragments are formed during processing of the precursor. Tryptic peptide analysis of ^{3}H-sugar-labeled pro-ACTH-endorphin reveals that the 29K and 32K forms of this protein contain two N-terminal glycopeptides (Roberts et al., 1978). If we suppose that both glycopeptides are contained within the same precursor molecule as depicted in Figure 13, then cleavage of the precursor molecules would give rise to the 16–17.5K N-terminal fragment. However, this raises a question about the biosynthesis of 14.5K N-terminal fragment which contains only one tryptic glycopeptide. One possibility is that the 14.5K form is generated from the 16–17.5K form by a proteolytic cleavage event which removes a small peptide with one of the oligosaccharides attached. Alternatively, the 29K and 32K forms of pro-ACTH-endorphin could be heterogeneous, consisting of molecules that contain either the basic or the neutral tryptic glycopeptide but not both. Cleavage of precursor molecules containing the neutral tryptic glycopeptide could generate the 14.5K N-terminal fragment. This model, which is not shown in Figure 13, predicts that cleavage of precursors containing the basic tryptic glycopeptide would generate a form of N-terminal fragment containing only the basic glycopeptide. Our failure to detect this molecule could be explained by poor cross-reactivity with our N-terminal

antiserum. This model predicts that the 16–17.5K N-terminal protein would be generated by cleavage of a precursor larger than 29K pro-ACTH-endorphin containing both N-terminal glycopeptides. The latter precursor might be difficult to separate from the 32K forms.

It has been shown by sequencing of pro-ACTH-endorphin cDNA that the bovine precursor contains only one glycosylation site in the N-terminal region of the precursor (Asn-Gly-Ser) (Nakanishi et al., 1979). Since mouse pituitary cells produce a form of the N-terminal fragment which contains two oligosaccharides, either mouse pro-ACTH-endorphin differs in sequence within its N terminus and contains a second glycosylation site or there are two forms of bovine pro-ACTH-endorphin with either one or two glycosylation sites. It is of interest in this connection that the mouse precursor has a glycosylation site in the α(22–39) region of ACTH which is absent in the bovine precursor.

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