Proc. Natl. Acad. Sci. USA
Vol. 78, No. 1, pp. 318–322, January 1981
Cell Biology

Secretory granules of an anterior pituitary cell line, AtT-20, contain only mature forms of corticotropin and β-lipotropin

(organelle purification/deuterium oxide density gradient/prohormone processing/β-endorphin/intracellular transport)

BARRY GUMBINER AND REGIS B. KELLY

Department of Biochemistry and Biophysics, University of California, San Francisco, California 94143

Communicated by George Streisinger, September 22, 1980

ABSTRACT **The pituitary cell line, AtT-20, synthesizes the precursor to corticotropin (adrenocorticotropic hormone; ACTH) and β-endorphin and correctly glycosylates and cleaves it to make the mature forms of the hormones before they are secreted. This cell line was used to study the intracellular transport, packaging, and secretion of these hormones. Secretory granules from the cells were isolated by homogenization and differential centrifugation and isopycnic sedimentation on a ^2H_2O-Ficoll gradient to give a preparation having a specific activity of 90 μg ACTH per mg of protein, which is 30- to 90-fold greater than that of whole cells. The granules have density characteristics and a sedimentation coefficient that are appropriate for spheres of 1000 Å radius. They contain all of the fragments of the initial ACTH/endorphin precursor but almost undetectable amounts of the intact precursor. The fragments constitute about 50% of the protein in the secretory granule fraction and, from density measurements, we estimate that they are present in ≈60,000 copies per vesicle. The cell line secretory granules appear, therefore, to be similar to mature secretory granules in normal differentiated tissues. ACTH first appears in the secretory granule at 30–45 min after synthesis. Cleavage of the precursor to mature ACTH occurs at about the same time in the whole cell. Therefore, proteolysis of the prohormone to ACTH and to β-lipotropin is a metabolic event that can be correlated with the packaging of the hormone into a mature secretory granule. Cleavage of β-lipotropin to β-endorphin occurs later, probably in the secretory granule.**

Morphological studies of highly differentiated secretory tissues such as the pancreas and the parotid gland have delineated the intracellular route of transport of proteins destined for secretion (1). Secretory proteins are synthesized at the rough endoplasmic reticulum, transported to the Golgi apparatus or the condensing vacuole for packaging into mature secretory granules, and then discharged from the cell by exocytosis. The molecular events underlying this process are poorly understood. It is not known how the secretory proteins are segregated and packaged into secretory granules or how specific granule membrane components are acquired. The nature of the recognition event whereby appropriate membranes fuse during transport and secretion remains obscure.

Highly structured tissues such as the pancreas are optimal for morphological studies, but the use of cell lines for the study of biochemical processes is advantageous—e.g., a cell line that retains normal secretory functions could be used to study the molecular events involved in packaging and secretion of proteins. We chose the mouse pituitary cell line AtT-20 as a model secretory system because these cells retain many important biochemical and physiological properties of pituitary corticotrophs. The biosynthetic pathways of the major secretory products—corticotropin (adrenocorticotropic hormone; ACTH), β-lipotropin (β-LPH), and β-endorphin, in these cells have been elucidated. These hormones are first synthesized as a common polypeptide precursor (2–4), and this is then glycosylated and cleaved to the final hormone products (5). In other tissues, the enzymes involved in posttranslational processing of membrane and secretory proteins are located in specific organelles, such as the rough endoplasmic reticulum or the Golgi apparatus (6, 7). If normal transport and processing occurs in AtT-20 cells, it should be possible to use ACTH processing activities to monitor transport between intracellular compartments or as biochemical markers for the corresponding organelles.

The final organelle in the secretory pathway in AtT-20 cells, the secretory granule, has been isolated. We report here a ^2H_2O density gradient procedure that produces a highly enriched population of ACTH-containing secretory granules that has distinct physical properties. The large number of fully processed hormones in the vesicle supports our belief that we have isolated mature secretory granules. We show that proteolytic processing of the ACTH/endorphin precursor is simultaneous with the packaging of the hormones into the mature secretory granule.

MATERIALS AND METHODS

Materials. Ficoll and Hepes were purchased from Sigma. ^2H_2O was obtained from BioRad. Particle Data Laboratories (Elmhurst, IL) supplied the Nonidet P40 (NP40). *Staphylococcus aureus* Cowan I cells were purchased from The Enzyme Center (Boston, MA) (product name IgSORB), and [^{35}S]-methionine was from Amersham.

Cells. AtT-20/D-16v cells were grown in monolayers in Eagle's minimal essential medium (Dulbecco's modification) supplemented with 10% horse serum and glutamine. Cells were normally used just before or at confluency. Cells were harvested by incubating at 37°C in 0.02 M sodium phosphate, pH 7.4/0.15 M NaCl/4 mM ethylene glycol bis(β-aminoethyl ether)-N,N,N′N′-tetraacetic acid (EGTA). They were then pelleted at low speed in the cold before use. Approximately 5×10^6 cells were obtained from each 75-cm^2 T flask.

Secretory Granule Purification. Cell pellets harvested from five 75-cm^2 T flasks were suspended in 15 ml of cold homogenization buffer (250 mM sucrose/10 mM Hepes, pH 7.4/2 mM EGTA/1 mM EDTA). They were then homogenized on ice with six strokes of a Kontes Dounce homogenizer (type B pestle).

Large debris and organelles (P_1) were removed by pelleting at $10,000 \times g$ for 5 min in a Sorvall SS34 rotor. The supernatant (S_1) was then centrifuged in the same rotor at $30,000 \times g$ for 35 min to obtain a crude pellet (P_2). This pellet was resuspended in 1–2 ml of homogenization buffer by vigorous repeated pipetting with a 1-ml automatic Pipetman until it appeared as a homogenous milky suspension. The resuspended P_2 was loaded

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: ACTH, corticotropin (adrenocorticotropic hormone); β-LPH, β-lipotropin; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid.

on top of a ^2H_2O-Ficoll density gradient consisting of from bottom to top in a polyallomer centrifuge tube, 1 ml of 20% Ficoll in ^2H_2O; 2 ml each of 17%, 14%, and 11% Ficoll in ^2H_2O; 1 ml of 9% Ficoll in ^2H_2O; and a 29-ml linear gradient of 40–100% ^2H_2O containing Ficoll at a constant 8%, all containing 250 mM sucrose/20 mM KCl/10 mM Hepes, pH 7.4/1 mM EDTA. The ^2H_2O gradient was centrifuged at 26,000 rpm in a Beckman SW27 rotor for 12–15 hr. Fractions (1.7 ml) were collected through a puncture hole in the bottom of the centrifuge tube.

Radiolabeling. Cells were grown in 75-cm^2 T flasks, rinsed once, and incubated for 20 min in 10 ml of modified Eagle's medium lacking methionine and serum. Then, 3 ml of the same medium containing [^{35}S]methionine (≈1000 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) was added to each T flask. After the allotted labeling time, the cells were rinsed with EGTA/phosphate-buffered saline and harvested.

Radioimmunoassay. ACTH radioimmunoassay was performed as described (8). Anti-ACTH antiserum Violet was a gift of E. Herbert. ^{125}I-Labeled ACTH was provided by S. Hane. ACTH for standards (porcine 1-39 ACTH) was a gift of J. Ramachandran. Samples were diluted with 1% NP40 in plastic test tubes and heated to 100°C for 1 min in a temperature block prior to assay.

Immunoprecipitation. Homogenates of whole cells were extracted and lyophilized as described (5). Secretory granules were concentrated by ultracentrifugation after dilution with isoosmotic buffer to reduce the solution density. All samples analyzed by immunoprecipitation were first treated with iodoacetamide and phenylmethanesulfonyl fluoride to inhibit proteolytic activity. Samples were dissolved in precipitation buffer (0.5% NP40/0.4 M NaCl/10 mM Hepes, pH 7.5) and centrifuged for 10 min at 12,000 × *g*. Antiserum was added in excess to ensure complete antigen–antibody reaction. The mixtures were incubated overnight in precipitation buffer. Antigen–antibody complexes were precipitated by using the *Staphylococcus* precipitation procedure (9). The β-endorphin antiserum, the anti-amino-terminal serum Bridget, and the affinity-purified ACTH antiserum used in some experiments were generous gifts of E. Herbert. Additional ACTH antiserum was obtained from J. Ramachandran and purified on an ACTH affinity column as described (10).

Gel Electrophoresis. NaDodSO$_4$/polyacrylamide slab gel electrophoresis was performed in the buffer system originally described by Laemmli (11). Exponential gradient gels from 10–18% acrylamide were used.

Protein was determined by Amido Schwarz staining method (12), modified for Ficoll-containing samples (13), using bovine serum albumin as a standard.

RESULTS

Secretory Granule Purification. In seven secretory granule purifications, 33- to 88-fold increases in the specific activity of ACTH/protein over whole cells were obtained (Table 1). The average secretory granule specific activity was 74 ± 11 μg ACTH/mg protein (mean ± SD). A variety of homogenization conditions were tried to find the maximum fraction of cellular ACTH that could be recovered in a $10^5 g \times 10$ min pellet, after first removing nuclei and cell debris by low-speed centrifugation. Six strokes with a Dounce homogenizer in the appropriate buffer lysed essentially all of the cells (determined by trypan blue exclusion), released less than 5% of the ACTH to a soluble fraction, and yielded 40% of the cellular ACTH in the high-speed pellet. The differential centrifugation conditions chosen minimized the loss of ACTH during the low-speed centrifugation step but increased the specific activity of the high-speed pellet (P_2). For example, in one purification (see Table 1), 37% of the cellular ACTH was recovered in the P_2, resulting in a 4-fold increase in specific activity.

Table 1. Secretory granule purification summary

Purification step	ACTH Total, μg	ACTH Recovery, %	Specific activity*
Homogenate	41.6 ± 1.3	100	2.7 ± 0.084
P_2	15.5 ± 2.0	37	12 ± 1.6
Peak fractions	2.31 ± 0.26	5.5	89 ± 10

Sample results from a typical purification. P_2 was the 30,000 × *g*, 35-min pellet that was fractionated on the ^2H_2O-Ficoll density gradient. Peak secretory granule fractions were fractions 4 and 5 of the ^2H_2O gradient shown in Fig. 1. Results are expressed as mean ± SD.
* Expressed as μg ACTH/mg protein.

Further purification was achieved by using a Ficoll-containing ^2H_2O density gradient. When centrifuged in a gradient of a membrane-permeable solute, the density of a closed vesicle is determined less by the water it contains and more by the vesicle contents (14). The successful purification with the ^2H_2O gradient presumably results from the permeability of the granules to ^2H_2O and the high density of the granule contents.

The ^2H_2O gradient separated the ACTH-containing structures into two major peaks (Fig. 1). The use of 8% Ficoll throughout the ^2H_2O gradient was critical in obtaining optimal separation between the two peaks. The lighter peak equilibrated within the ^2H_2O gradient and contained a large amount of contaminating protein. The denser peak, containing the secretory granules, equilibrated in the Ficoll gradient at the bottom and included a very small protein peak. Six percent of the cellular ACTH was recovered in the dense peak fractions, which had a very high specific activity (89 μg ACTH/mg protein).

Physical Properties of Secretory Granules. The densities of the secretory granules, determined in isoosmotic Ficoll solutions, were 1.10 g/ml in H_2O and 1.17 g/ml in ^2H_2O. To determine the sedimentation coefficient, the peak fractions from a ^2H_2O/Ficoll gradient were pooled, diluted to one-fifth with isoosmotic ^2H_2O buffer and centrifuged in a swinging bucket rotor at 18,000 × *g* average for varying lengths of time. The amount of ACTH in each pellet was measured by radioimmu-

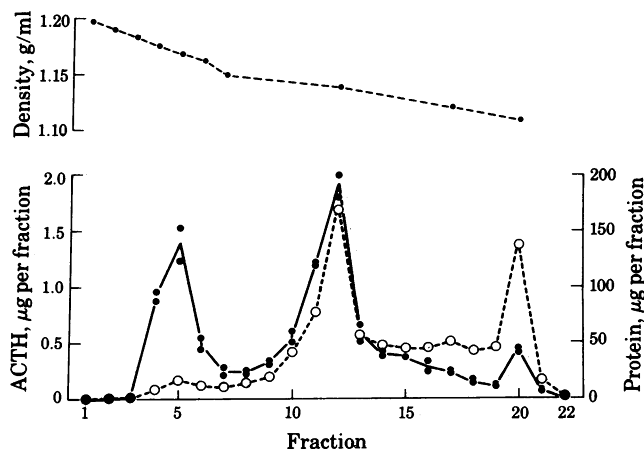

FIG. 1. ^2H_2O density-gradient profile of secretory granule purification (see Table 1). Fractions 4 and 5 are the peak secretory granule fractions. ●—●, μg ACTH per fraction, as determined by radioimmunoassay. ○---○, μg protein per fraction. Each fraction contained ≈1.7 ml.

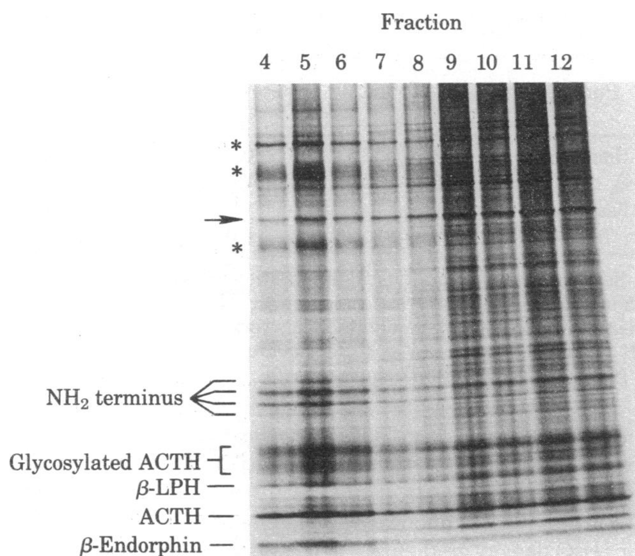

FIG. 2. Protein analysis of ^2H_2O density-gradient purified secretory granules. Four T flasks of cells were labeled with 2.5 mCi (1 Ci = 3.7×10^{10} becquerels) of [^{35}S]methionine for 5 hr, and the secretory granule purification was carried out. Aliquots of the ^2H_2O gradient fractions were precipitated with trichloroacetic acid and examined by NaDodSO$_4$ slab gel electrophoresis. The gel was autoradiographed for 84 hr. Fractions 4, 5, and 6 were the peak secretory granule fractions, as determined by ACTH radioimmunoassay. Asterisks mark unidentified polypeptides that copurify with the secretory granules. Arrow indicates a band with the mobility of actin (M_r 42,000) that does not copurify with the secretory granules.

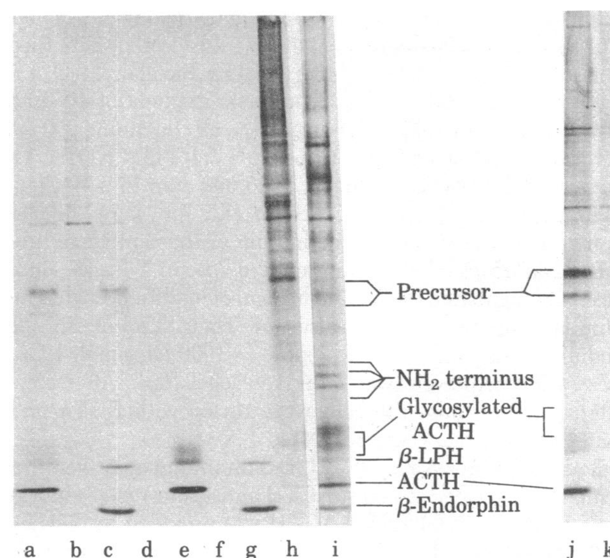

FIG. 3. NaDodSO$_4$ gel electrophoresis of ACTH and endorphin immunoprecipitates of secretory granules. Fractions 4, 5, and 6 from the experiment shown in Fig. 2 were pooled for the sample of labeled secretory granules. Lanes: (i) Total secretory granule sample that was incubated in each precipitation, (e–h) immunoprecipitation of the secretory granules, (a–d) whole cell homogenate, (j and k) a fraction from the light ACTH peak of the ^2H_2O gradient (fractions 10–13, see Fig. 2). a, b, e, f, j, and k were precipitated with an ACTH antiserum in the absence (a, e, and j) or the presence (b, f, and k) of excess (10 μg) unlabeled ACTH to show specific precipitation. c, d, g, and h were precipitated with β-endorphin antiserum, and controls (d and h) contained 10 μg of unlabeled β-endorphin. Gels were analyzed by fluorography. Lane i was exposed one-fifth as long as lanes a–h.

noassay and expressed as a fraction of the total pelletable ACTH. The sedimentation coefficient was calculated for each time point by using a standard equation (15). The average sedimentation rate from five time points was 189 ± 38 S. The secretory granules must be fairly homogeneous in size, as the sedimentation rate did not decrease systematically with increasing centrifugation time (data not shown).

Secretory Granule Protein Composition. Several polypeptides copurified with the secretory granules. NaDodSO$_4$ gel slab electrophoresis of ^2H_2O gradient fractions after the cells had been labeled with [^{35}S]methionine showed that bands that comigrate with ACTH, β-endorphin, β-LPH, glycosylated ACTH (M_r 13,000), and amino-terminal fragments of the ACTH/endorphin precursor are highly enriched in secretory granule fractions (Fig. 2). Quantitative densitometry of the autoradiograms showed that these polypeptides represent 44%, 47%, and 47% of the labeled protein in fractions 4, 5, and 6, respectively.

Many higher molecular weight polypeptides also copurified with granules and were not present in substantial amounts in nearby fractions. In contrast, a polypeptide that comigrated with actin was present in major amounts in every ^2H_2O gradient fraction. The Coomassie blue staining polypeptide pattern was essentially the same, except that it showed a dominant M_r 30,000 species that was present in every gradient fraction. The M_r 30,000 protein either turns over very slowly or contains few methionine residues.

To confirm that the indicated bands on the gel were indeed ACTH- and endorphin-related peptides, ^2H_2O gradient-purified granules from [^{35}S]methionine labeled cells were subjected to immunoprecipitation with ACTH antiserum and with β-endorphin antiserum (Fig. 3). In each case, specificity was determined by including a control precipitation in the presence of excess unlabeled hormone to displace the labeled hormone. ACTH and a smeared band corresponding to glycosylated ACTH were precipitated from the granules by anti-ACTH antiserum. Bands at the molecular weight positions of β-endorphin and β-LPH were specifically precipitated by an anti-β-endorphin antiserum. Comparison of the immunoprecipitates with the total secretory granule fraction suggested that most, if not all, of the ACTH, endorphin, and β-LPH-size material was immunoprecipitable by the appropriate antiserum. The ACTH/endorphin precursor was only faintly detectable after a very long (≥4 weeks) period of fluorography—by comparison with shorter exposures, it constituted less than 1% of the labeled secretory granule ACTH or endorphin peptides. Precursor forms were readily immunoprecipitated from homogenates of whole cells, and the less-dense peak of ACTH in the ^2H_2O gradient also contained a substantial amount of precursor as well as processed ACTH.

The bands labeled "NH$_2$ terminus" migrated on NaDodSO$_4$ gels in a similar pattern and molecular weight range as the NH$_2$-terminal fragments of the ACTH/endorphin precursor (16). These bands were immunoprecipitated by a crude antiserum (Bridget) that has anti-amino-terminal titers. However, control precipitation to determine specificity was not done because appropriate NH$_2$-terminal fragments were not available.

Comparison of Proteolytic Processing with Packaging into Secretory Granules. The temporal relationship between ACTH/endorphin processing and intracellular transport was examined by labeling AtT-20 cells with [^{35}S]methionine for various lengths of time. After each labeling period, the cells were harvested and secretory granules were prepared. Secretory granule fractions from each preparation were pooled and precipitated with trichloroacetic acid, and the proteins were analyzed by NaDodSO$_4$ gel electrophoresis (Fig. 4*A*). Densitometer scans of the autoradiograms were made, and the peaks were quantitated (Fig. 5*A*). ACTH and β-LPH abruptly accumulated in the isolated granules at 30–45 min of labeling. In contrast, a band having

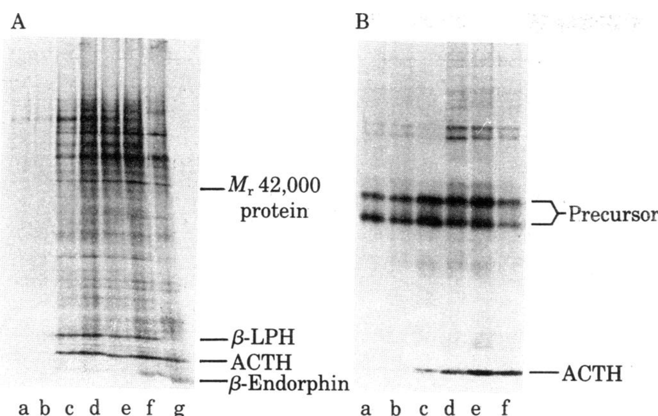

FIG. 4. Correlation of ACTH/endorphin proteolytic processing with packaging into secretory granules. Each of six T flasks of cells was incubated with 800 μCi of [^{35}S]methionine for a specified time. Incorporation of ^{35}S into trichloroacetic acid-precipitable material per milligram of protein was linear with time over the full course of the experiment. The secretory granule preparation was carried out in parallel for all six sets of cells after adding unlabeled cells to serve as carrier. ^2H_2O gradient fractions of density greater than 1.165 g/ml were pooled and analyzed by $NaDodSO_4$ gel electrophoresis and autoradiography (*A*). Aliquots of the homogenates were immunoprecipitated with an ACTH antiserum and analyzed similarly (*B*). Lanes a–f correspond to 15, 30, 45, 60, 90, and 120 min of continuous labeling, respectively. The higher molecular weight polypeptides contaminating the immunoprecipitates bind nonspecifically to SAC, for they could be pelleted in the absence of antiserum. Lane g, secretory granule preparation after 5 hr of labeling (see Fig. 2). The M_r 42,000 protein comigrates with actin.

the mobility of actin (M_r 42,000) labeled linearly with time, a behavior that would be expected of a contaminant adsorbed to the granules. The time course of proteolytic processing of the ACTH/endorphin precursor was determined by immunoprecipitating an aliquot of the corresponding homogenate of each granule preparation (Figs. 4*B* and 5*B*). Mature ACTH (M_r 4500) began to accumulate in the cells at 30–45 min of labeling and accumulated with continued labeling. Thus, within the time resolution of this experiment, proteolytic conversion of the precursor into mature ACTH and β-LPH was concomitant with the packaging of the hormones into secretory granules.

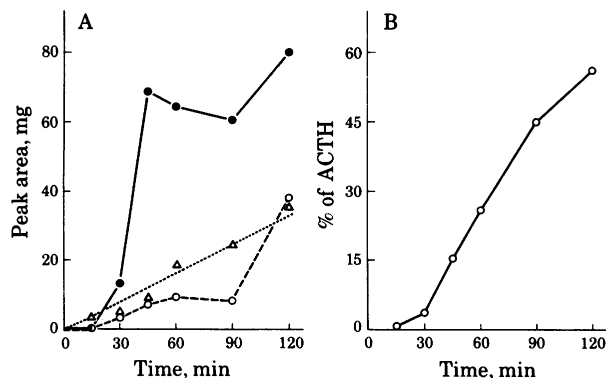

FIG. 5. Kinetics of hormone processing and packaging into secretory granules. Autoradiograms (see Fig. 4) were scanned with a densitometer. Relative peak areas were determined by weighing. (*A*) Kinetics of granule labeling: Radioactivity present as M_r 4500 ACTH (●—●), as β-endorphin (○—○), and in M_r 42,000 protein that comigrates with actin (△···△). (*B*) Time course of the conversion of ACTH/endorphin M_r 30,000 precursor to M_r 4500 ACTH. ACTH (M_r 4500) as percentage of sum of self plus precursor.

A faint band that has the mobility of β-endorphin appeared in granules after 45 min of labeling and increased dramatically at 1.5–2 hr (Figs. 4*A* and 5*A*). Quantitation by densitometry showed that the label in β-LPH decreased by the same amount as the increase in label in β-endorphin. In a separate experiment, cells were labeled for 5 hr with [^{35}S]methionine. In this case, much more label was in β-endorphin than in β-LPH (Fig. 4*A*, lane g). These results suggest that the cleavage of β-endorphin from β-LPH occurs within the secretory granule long after the initial proteolysis of the ACTH/LPH precursor.

DISCUSSION

We have used an ACTH radioimmunoassay to monitor the purification of secretory granules from AtT-20 cells. Because the ACTH detected is presumably contained in other subcellular organelles, such as the rough endoplasmic reticulum and Golgi apparatus, it is not a unique marker for secretory granules. Indeed, more than one peak of ACTH is present in the ^2H_2O density gradient (see Fig. 1). We believe, on the basis of density, sedimentation properties, the large amount of mature forms of hormones contained, and the kinetics of labeling, that the denser component corresponds to secretory granules.

The 33- to 88-fold increase in ACTH specific activity over cell homogenate underestimates the actual purification factor achieved, because much of the cellular ACTH may not be in secretory granules. Specific activity measurements suggest that the granule preparation is highly enriched in secretory product. ^2H_2O gradient-purified granules had a specific activity of 89 μg of radioimmunoassayable ACTH per mg protein. The granules also contain the β-LPH and NH_2-terminal fragments of the M_r 30,000 precursor. Because the radioimmunoassay quantifies only the M_r 4500 ACTH fragment, it measures only 15% of the total secretory product. Therefore, the estimated specific activity is 600 μg of secretory product per mg of protein, which means that approximately 60% of the protein in the granule preparation consists of secretory product. Quantitative densitometry scans of $NaDodSO_4$ gel autoradiograms also show that about 50% of the radioactivity of the granule fraction polypeptides is present in the fully processed secretory hormones. The granules must therefore be at least 50% pure (and might be of higher purity if some of the other labeled polypeptides are also granule components).

We have used immunoprecipitations and $NaDodSO_4$ gel electrophoresis to confirm that the granules contain ACTH and endorphin-related peptides. Previous work, including peptide mapping, has shown that this approach is adequate to characterize ACTH and endorphin peptides and their precursors in AtT-20 cells (2–5). All of the hormone products of AtT-20 cells, including β-LPH, β-endorphin, ACTH, and glycosylated ACTH, are present in the purified granules and although the proof is less rigorous, it appears that the granules also contain the NH_2-terminal fragments of the M_r 30,000 precursor. The lack of the ACTH/endorphin precursor in the isolated granules must be reconciled with previous reports of a large proportion of precursor secreted into the medium by AtT-20 cells (5, 17). It is possible that precursor forms preferentially accumulate in the culture medium because they are more resistant to extracellular proteases than the mature hormones or that precursors are released via a pathway that does not involve mature secretory granules. Because cleavage of the precursor is rapid at the time of hormone packaging into granules, the presence of precursor in newly formed granules may be brief. It is unlikely that the lack of precursors in secretory granules is an experimental artifact, as substantial amounts of precursor remain in the less-dense ^2H_2O gradient fractions (see Fig. 3). The lack of precursor

in the isolated secretory granules is consistent with their role as the final intracellular compartment that transports ACTH and the endorphins to the outside of the cell; the role of the less-dense ACTH peak is not yet known.

The isolated vesicles are homogeneous in size as determined by sedimentation analysis and are an appropriate size to be secretory granules. The radius of the granule can be calculated to be 995 Å from the sedimentation coefficient and the vesicle density (14). Secretory granules of radius 1000 Å have been observed in rat pituitary corticotrophs (18) and in AtT-20 cells, (unpublished observations) by electron microscopy.

The number of secretory hormone molecules in each secretory granule can be estimated by using density measurements. The density of a vesicle, ρ_{ves}, can be described by using Eq. 1 (14)

$$\rho_{ves} = (1 - f_s)\rho_c + f_s\rho_s \qquad [1]$$

where ρ_c is the density of vesicle contents, including the limiting membrane and any internal contents; ρ_s is the density of the solvent; and f_s is the fractional volume of the vesicle that is exchangeable water. From the densities of the secretory granule in H_2O and ^2H_2O, we can solve two simultaneous equations to calculate that $\rho_c = 1.30$ g/ml and $f_s = 0.66$. The high density of the vesicle contents presumably reflects the large amount of secretory protein contained within the secretory granule. The granule density in aqueous solution (1.10 g/ml) is consistent with the large proportion of water in the secretory granule. The mass of the granule contents can be calculated to be 1.85 fg by using the measured radius, the fractional volume occupied by contents ($1 - f_s$), and the density of vesicle contents (ρ_c). If we assume that all the membrane of the granule is made up of phospholipids, then we can calculate by using the diameter of the granule, the cross-sectional area, and the molecular weight of the phospholipid (14) that, at most, 30% of the mass of the contents is taken up by lipid. If the remainder is protein and 50% of the protein is derived from ACTH precursors, then there is at least 0.66 fg of ACTH precursor (13,000 molecules) per vesicle. If 50% of the membrane mass were proteins, then there would be 16,000 molecules per vesicle. This would correspond to 64,000 processed hormone fragments per granule. Although the accuracy of this determination is limited by the assumptions made, the number of secretory molecules per vesicle is of the right magnitude expected for a secretory granule. For example, Narcine electric organ synaptic vesicles have 47,000 molecules of acetylcholine (14) and there are 60,000 molecules of vasopressin per posterior pituitary granule (19).

We have shown that packaging of the secretory product into secretory granules is closely associated in time with proteolytic processing of the ACTH/LPH precursor. Due to the limited time resolution of the experiment, it is not possible to decide whether proteolysis of the ACTH precursor occurs within the secretory granule or just before the packaging. However, a reasonable interpretation is that proteolytic processing is initiated by the transfer of the precursor into a new intracellular compartment—most likely either the Golgi apparatus or the secretory granule itself. The conversion of proinsulin to insulin is initiated in the Golgi apparatus and continues in the secretory granule as it matures (6). Proteolytic processing of posterior pituitary hormones seems to occur within secretory granules as they are being transported along the axon (20). Our data suggest that the final cleavage of β-LPH to β-endorphin occurs within the secretory granule. This is consistent with previous findings that β-endorphin appears in AtT-20 cells with a much slower time course than the initial proteolysis of the ACTH/LPH precursor (17). It is not clear why there should be such a long lag before the β-LPH in the secretory granule is cleaved. This event may be a slower component of an overall cleavage process, perhaps reflecting the presence of the cleavage enzymes within the secretory granule. We have no evidence to support the idea that β-endorphin and ACTH are packaged into separate granules.

The packaging results show the advantage of using posttranslational processing activities for studying intracellular transport and secretion. The secretory granules that we isolated appear to be the final intracellular compartment involved in transporting hormones out of the cell and have many of the important attributes of secretory granules from normal differentiated secretory tissues. The AtT-20 cell line thus retains normal secretory functions and is well suited for studies of molecular events involved in packaging and secretion of proteins.

We would like to thank our colleagues at the University of California, San Francisco, for their advice and Leslie Spector for her work in preparing this manuscript. We are especially grateful to Dr. J. L. Roberts for advice and encouragement and Drs. E. Herbert and J. Ramachandran for gifts of antiserum. This research was supported by National Institutes of Health Grant NS 09878. B.G. was supported by a National Science Foundation predoctoral fellowship and National Institutes of Health Predoctoral Training Grant GM 07449-03.

1. Palade, G. E. (1975) *Science* **189**, 347–358.
2. Mains, R. E., Eipper, B. A. & Ling, N. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 3014–3018.
3. Roberts, J. L. & Herbert, E. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 4826–4830.
4. Roberts, J. L. & Herbert, E. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5300–5304.
5. Roberts, J. L., Pillips, M., Rosa, P. A. & Herbert, E. (1978) *Biochemistry* **17**, 3609–3618.
6. Steiner, D. F. (1976) in *Peptide Hormones*, ed. Parsons, J. A. (University Park Press, Baltimore, MD), pp. 49–61.
7. Molnar, J. (1976) in *The Enzymes of Biological Membranes*, ed. Martinosi, A. (Plenum, New York), Vol. 2, pp. 385–411.
8. Rees, L. H., Cook, D. M., Kendall, J. W., Allen, C. F., Kramer, R. M., Ratcliff, J. G. & Knight, R. A. (1971) *Endocrinology* **89**, 254–261.
9. Kessler, S. W. (1975) *J. Immunol.* **115**, 1617–1624.
10. Mains, R. E. & Eipper, B. A. (1976) *J. Biol. Chem.* **251**, 4115–4120.
11. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
12. Schaffner, W. & Weissmann, C. (1973) *Anal. Biochem.* **56**, 502–514.
13. Carlson, S. S., Wagner, J. A. & Kelly, R. B. (1978) *Biochemistry* **17**, 1188–1199.
14. Wagner, J. A., Carlson, S. S. & Kelly, R. B. (1978) *Biochemistry* **17**, 1199–1206.
15. Cotman, C., Brown, D. H., Harrell, B. W. & Anderson, N. G. (1970) *Arch. Biochem. Biophys.* **136**, 436–447.
16. Keutmann, H. T., Eipper, B. A. & Mains, R. E. (1979) *J. Biol. Chem.* **254**, 9204–9208.
17. Mains, R. E. & Eipper, B. A. (1978) *J. Biol. Chem.* **253**, 651–655.
18. Siperstein, E. R. & Miller, K. J. (1970) *Endocrinology* **86**, 451–486.
19. Cross, B. A., Dyball, R. E. J., Dyer, R. G., Jones, C. W., Lincoln, D. W., Morris, J. F. & Pickering, B. T. (1975) *Recent Prog. Hormone Res.* **31**, 243–286.
20. Brownstein, M. J., Russell, J. T. & Gainer, H. (1980) *Science* **207**, 373–378.