A Subclass of Proteins and Sulfated Macromolecules Secreted by AtT-20 (Mouse Pituitary Tumor) Cells Is Sorted with Adrenocorticotropin into Dense Secretory Granules

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ABSTRACT The AtT-20 cell, a mouse pituitary tumor line that secretes adrenocorticotropin and β-endorphin, sorts the proteins it exernalizes into two exocytotic pathways. Cells that are labeled with [^{35}S]methionine or [^{35}S]sulfate can be shown to transport three acidic polypeptides (65,000, 60,000, and 37,000 mol wt) and at least two sulfated macromolecules into storage secretory granules. When the cells are stimulated by the secretagogue 8-bromo-cAMP, these polypeptides are coordinately secreted with mature adrenocorticotropin into the culture medium. In contrast, a completely different set of secreted polypeptides and sulfated macromolecules does not enter a storage form and is transported to the cell surface more rapidly. Their secretion from the cells is constitutive and does not require the presence of secretagogues. These molecules, like a viral membrane glycoprotein described previously (Gumbiner, B., and R. B. Kelly, 1982, *Cell*, 28:51–59) are not found in isolated secretory granules and therefore must reach the cell surface in a different exocytotic vesicle. The segregation of a subclass of secretory macromolecules into the secretory granules, despite the existence of another potential secretory pathway, suggests that these molecules have specific functions related to regulated hormone secretion or storage. Presumably all of the proteins secreted by the regulated secretory granule pathway share some common property that targets them to the secretory granule.

The intracellular route for transport and externalization of secretory and membrane proteins by mammalian cells is now well understood at the morphological level. Proteins destined for export are synthesized at the rough endoplasmic reticulum, transported to the Golgi apparatus, and reach the cell surface by vesicular discharge. The role of signal peptides (reviewed in reference 15), the signal recognition particle (35), and docking proteins (22) in the segregation of extracytoplasmic proteins into the lumen of the rough endoplasmic reticulum (RER)[1] is now known. Of the subsequent sorting steps, by far the best understood is the sequestration of lysosomal enzymes. In some but not all cells, newly synthesized acid hydrolases are specifically tagged with phosphomannosyl groups and separated from other proteins that are in the lumen of the Golgi complex by a specific mannose-phosphate receptor (for review, see reference 31). Steps involved in the sorting and intracellular targeting of secretory and membrane proteins, however, remain unclear.

We have previously shown that the AtT-20 cell line, a mouse pituitary tumor line, externalizes a membrane glycoprotein, a murine leukemia virus glycoprotein gp70, by a "constitutive pathway" that is distinct from the "regulated pathway" for the transport of mature ACTH (11). Although both proteins are synthesized at the RER and are similarly glycosylated in the Golgi apparatus, the discharge of mature adrenocorticotropin (ACTH) is slow and strongly stimulated by 8-bromo-cAMP (8-Br-cAMP), whereas that of gp70 is rapid and not sensitive to 8-Br-cAMP. Furthermore, gp70 does not accumulate in the storage granules that contain mature ACTH. Thus the two proteins are segregated during transport to the cell surface, presumably at a late, or post-Golgi stage. The ability of AtT-20 cells to segregate two glycoproteins into

[1] *Abbreviations used in this paper:* ACTH, adrenocorticotropic hormone; POMC, proopiomelanocortin; gp70, murine leukemia virus membrane glycoprotein; 8-Br-cAMP, 8-bromo-cAMP; RER, rough endoplasmic reticulum; BSA, bovine serum albumin; and TCA, trichloroacetic acid.

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two readily distinguished pathways thus provides an excellent system for the study of the mechanisms involved in sorting membrane and secretory proteins.

In considering the various possible sorting mechanisms it is important to first determine if AtT-20 cells package other secretory products in their secretory granules in addition to the hormones. For example, it is possible that the hormones interact with other secretory components, also destined for the granules, to form protein complexes or macromolecular aggregates that are then segregated. We have asked, therefore, whether AtT-20 cells synthesize and export other secretory proteins in addition to viral proteins and the products of proopiomelanocortin (POMC), such as ACTH and β-endorphin. Because we can experimentally distinguish the two pathways with which AtT-20 cells externalize proteins, we can ask whether any other secretory proteins are specifically segregated along with the mature hormones into the regulated pathway. We found that AtT-20 releases a large number of secretory proteins into the culture medium, many of which seem to be secreted by the constitutive pathway. The cells also segregate, package, and release three acidic proteins and at least two sulfated macromolecules by a pathway indistinguishable from the regulated pathway for the secretion of mature ACTH. These results generalize our previous demonstration of two secretion pathways, one for a viral glycoprotein and one for mature ACTH, to all proteins secreted by AtT-20 cells. Furthermore, the specific segregation of the five secretory proteins with ACTH into the regulated pathway suggests that they may share some common property that targets them to the secretory granule.

MATERIALS AND METHODS

Materials: [^{35}S]Methionine and [^{35}S]Sulfate were obtained from Amersham Corp. (Arlington Heights, IL). Iodoacetamide, phenylmethylsulfonyl fluoride, Ficoll, and 8-Br-cAMP were purchased from Sigma Chemical Co.(St. Louis, MO). Deoxycholic acid was obtained from Aldrich Chemical Co. (Milwaukee, WI) and En'hance from New England Nuclear (Boston, MA). D_2O and reagents for SDS polyacrylamide gels were obtained from Bio-Rad Laboratories (Richmond, CA).

Purified porcine ACTH was a generous gift of Dr. J. Ramachandran. Anti-ACTH antiserum Pelar was produced by immunizing a New Zealand white rabbit with purified porcine ACTH(1–39) conjugated to BSA (25). The antiserum used in immunoprecipitation was affinity purified on an ACTH column according to the method of Mains and Eipper (19). *Staphylococcus aureus* cells (Pansorbin) were obtained from Calbiochem-Behring Corp. (San Diego, CA).

Cell Culture and Labeling with [^{35}S]Methionine and [^{35}S]-Sulfate: AtT-20/D-16v cells were obtained from Dr. J. Roberts and grown as monolayers under 15% CO_2 atmosphere in Dulbecco's modified Eagle's minimal essential (DME) medium supplemented with 10% horse serum. For labeling with [^{35}S]methionine cells were preincubated with methionine-free DME medium for 15 min. [^{35}S]Methionine (1,300 Ci/mmol) in the same medium was then introduced. When labeling was carried out for periods >1 h, the labeling medium also contained 1/20th of the normal amount of methionine. To chase, the labeling medium was removed and cells were rinsed and incubated with complete DME medium without horse serum. To stimulate release from labeled cells, the secretagogue 8-Br-cAMP was added to the chase medium at a concentration of 5 mM. Chase medium from labeled cells was routinely treated with iodoacetamide and phenylmethylsulfonyl fluoride (0.3 mg/ml) prior to storage. Procedures for labeling cells with [^{35}S]sulfate were essentially the same as for [^{35}S]methionine, except that sulfate-free DME medium was used and longer preincubation (1 h) and labeling (3 h) periods with [^{35}S]sulfate (25–40 Ci/mg) were necessary to achieve sufficient labeling.

Trichloroacetic Acid Precipitation and SDS Gel Electrophoresis: Aliquots of medium from radiolabeled cells were precipitated with equal volume of 10% trichloroacetic acid (TCA) at 0°C. Deoxycholate was added to a final concentration of 0.2 mg/ml as carrier. TCA-insoluble radioactivity was determined by filtering and washing the precipitate on a glass fiber GF/B filter. Alternatively, the TCA precipitate was centrifuged in a Sorvall GLC-4 centrifuge and the pellet extracted with ether prior to application on SDS polyacrylamide gels. For TCA precipitation of materials from D_2O-Ficoll gradients, a higher concentration of deoxycholate (0.5 mg/ml) was used. These latter samples were diluted three fold with 5% TCA before centrifugation.

10–18% exponential gradient SDS polyacrylamide gels were performed according to the method of Laemmli (16). Gels were impregnated with En'hance, dried, and exposed to Kodak XAR-5 film at −80°C. Fluorograms were scanned with an LKB soft laser scanning densitometer for quantitation.

ACTH Radioimmunoassay and Immunoprecipitation: ACTH was quantitated by a sensitive radioimmunoassay as described by Gumbiner and Kelly (10). Immunoprecipitation of ACTH was performed as described previously (11).

Two-dimensional PAGE: Two-dimensional gel electrophoresis was carried out according to the procedure of O'Farrell (24). Isoelectric focusing in the first dimension ranged from pH 3 to 10. The second dimension was a 10–18% exponential polyacrylamide SDS gel.

Isolation and Purification of Secretory Granules: Secretory granules were isolated from AtT-20 cells by the method of Gumbiner and Kelly (10). In brief, cells were homogenized and large debris was removed by centrifugation. The supernatant was centrifuged at 30,000 *g* for 30 min. The resulting crude membrane fraction was then layered over a 40–100% D_2O gradient containing 9.8% Ficoll. To avoid pelleting the granules, a cushion consisting of a discontinuous 9.8%–20% Ficoll gradient in 100% D_2O was employed. Secretory granules were harvested by diluting the peak fractions with homogenization buffer and pelleting at 26,000 rpm in a SW27 rotor (Beckman Instruments, Inc., Palo Alto, CA) for 3 h.

To further purify the granules, we subjected isolated granules from the D_2O-Ficoll gradient to electrophoresis (4). 200 μl of granules in 2.5% Ficoll–250 mM sucrose–10 mM HEPES, pH 7.4, 10 mM KCl and 0.5 mM EGTA was layered on a 4.8ml 5–20% Ficoll gradient in 5 mM HEPES, pH 7.4, 250 mM sucrose (18 × 0.6 cm long). Electrophoresis was carried out at 500 V for 4 h.

RESULTS

Four Polypeptides Are Released with Mature ACTH in Response to 8-Br-cAMP

We noted earlier (10) that purified secretory granule fractions from AtT-20 cells contain at least three major unidentified polypeptides in addition to the proteolytically clipped fragments of POMC. These polypeptides are present in amounts comparable to the hormones and are released in a soluble form when secretory granules are lysed (unpublished observations). If they are contained within the granules then they should be externalized with mature ACTH by the regulated pathway. To determine whether a molecule is externalized by the regulated route or by the constitutive one, we used a pulse-labeling technique that takes advantage of the different kinetic properties of the two pathways. Molecules such as the viral gp70 and precursor ACTH that take the constitutive pathway leave the cell with a half-time of ~40 min, whether or not a secretagogue is present. Molecules like mature peptide hormones that take the regulated pathway enter an intracellular storage compartment from which basal secretion is relatively slow ($t_{½}$, ~3–4 h). Externalization of the stored form is enhanced by the presence of a secretagogue. In this way, we analyzed all the [^{35}S]methionine-labeled proteins secreted by AtT-20 cells. Table I shows an example in which cells were pulse-labeled with [^{35}S]methionine for 20 min and chased for two consecutive 1-h periods. TCA-precipitable radioactivity released into the medium in the absence of stimulation decreased rapidly with increasing chase times. The rapidly secreted products reach the surface with the kinetics of proteins in the constitutive pathway (11). If 8-Br-cAMP was added to the cells after most of the rapid secretion had already occurred, the release of TCA-precipitable radioactivity was stimulated approximately twofold above background.

TABLE I

8-Br-cAMP Stimulates the Release of a Subclass of Secretory Proteins from AtT-20 Cells

	Time of Chase	8-Br-cAMP	Radioactivity in the medium
	min	*mM*	*cpm × 10^{-4}/mg cell protein*
Secretion from [^{35}S]methionine labeled cells	0–60	0	70.0 ± 7.0
	60–120	0	25.5 ± 3.1
	60–120	5	54.9 ± 3.1
Secretion from [^{35}S]sulfate-labeled cells	0–30	0	3.00 ± 0.16
	30–60	0	1.18 ± 0.08
	60–90	0	0.62 ± 0.08
	60–90	5	1.91 ± 0.06

10-cm confluent dishes of AtT-20 cells were pulse-labeled with ^{35}S-methionine (0.2–0.4 mCi/dish) for 20 min or with ^{35}S-sulfate (1 mCi/dish) for 3 hr. The labeled cells were then chased with unlabeled medium for several consecutive periods. TCA-precipitable radioactivity released into the culture medium during the various chase intervals was determined by filtration assays and expressed as cpm per mg total cellular protein. To examine the effect of secretagogues, 5 mM 8-Br-cAMP was added to the cells during the last chase interval. The polypeptide constituents of the rapidly released materials are distinct from those stimulated by 8-Br-cAMP as shown in Figures 1 and 4.

The polypeptide composition of the material released upon stimulation by 8-Br-cAMP differs from the composition of the rapidly released material. Analysis by SDS polyacrylamide gels revealed that four proteins of approximate molecular weights 65,000, 60,000, 37,000, and 15,000 (designated as B65, B60, B37, and B15, respectively) were stimulated along with the previously identified hormone fragments, 4,500-mol-wt ACTH, 13,000-mol-wt ACTH and the N-terminus (Fig. 1, lanes *3* and *4*). The rapidly secreted polypeptides, herein referred to as class A polypeptides (Fig. 1, asterisks), were different from those secreted with the mature ACTH, the class B polypeptides (Fig. 1, arrows). We conclude that AtT-20 cells synthesize four previously unidentified proteins (B65, B60, B37, and B15) that are secreted in a regulated manner similar to the secretion of the mature hormones. These cells also secrete numerous other polypeptides, but with more rapid kinetics and without response to secretagogues.

Only Secretory Proteins of the Regulated Pathway Are Packaged in Dense Secretory Granules

If class B proteins are secreted by the same regulated pathway as mature peptide hormones, they too should be packaged in the dense ACTH granules that we have isolated. Fig. 2*a* shows the isolation of [^{35}S]methionine-labeled secretory granules on a D_2O-Ficoll density gradient as described by Gumbiner and Kelly (10). SDS gel electrophoresis of fractions across the gradient revealed several polypeptides that co-purified with ACTH and were enriched in secretory granule fractions (reference 10, shown by arrows in Fig. 2*b*, lane *a*). These granule enriched polypeptides are compared with 8-Br-cAMP stimulated secreted components in Fig. 2*b*. Proteins of similar mobility to B65, B60, B37, and B15 were indeed enriched in the secretory granule peak containing the mature hormones. The identity of granule-specific proteins to stimulation-dependent proteins was confirmed by two-dimensional gel electrophoresis (Fig. 3). The pattern of granule-specific proteins (Fig. 3*a*, circles) was identical to that of stimulated, secreted proteins (Fig. 3*c*, circles). The B65, B60, and B37 proteins are acidic and may represent more than three polypeptides as judged by the complex migration pattern of polypeptides in this molecular weight region on two-dimensional gels. (The small hormone fragments were eluted out of the gel during incubation with the buffer of the second dimension and thus were not recovered.) These data show that the composition of proteins secreted by the regulated pathway is almost identical to the composition of proteins contained in purified secretory granules. Note also that none of the rapidly secreted polypeptides (class A) were enriched in the isolated secretory granules. Therefore, like gp70, the majority of these polypeptides appears to be transported to the cell surface in a vesicle different from the mature hormone secretory granules.

A Subclass of Sulfated Macromolecules Is Also Released with ACTH

Secretory vesicles from other cell types are known to contain proteoglycans (for review, see reference 8) in addition to

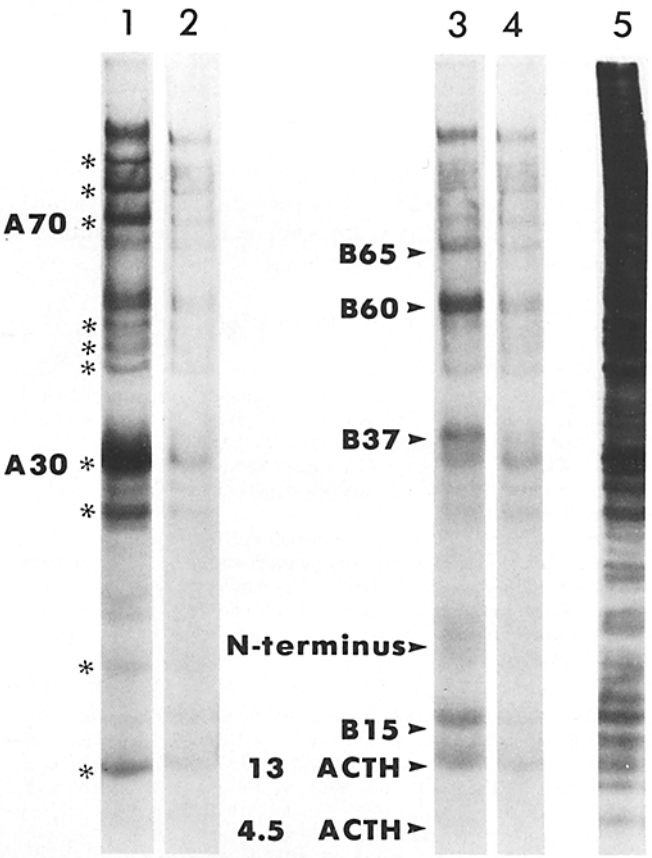

FIGURE 1 SDS polyacrylamide gels of polypeptides secreted from [^{35}S]methionine labeled cells. AtT-20 cells were pulse-labeled with [^{35}S]methionine and chased for an hour with unlabeled medium as described in Table I. After collecting the medium the cells were further chased for an hour in the presence or absence of 8-Br-cAMP. The samples were analyzed on a 10–18% SDS polyacrylamide gel and the fluorogram was exposed for 2 d. (Lanes *1* and *2*) Medium from unstimulated cells during the first and second hour of chase, respectively. (Lanes *3* and *4*) Medium during the second hour of chase in the presence and absence of 8-Br-cAMP, respectively. (Lane *5*) Total labeled cell extract. The polypeptide composition of the stimulation-dependent secretory proteins (arrowheads, class B proteins, see text) is very different from those that are stimulation-independent (asterisks, class A proteins, see text).

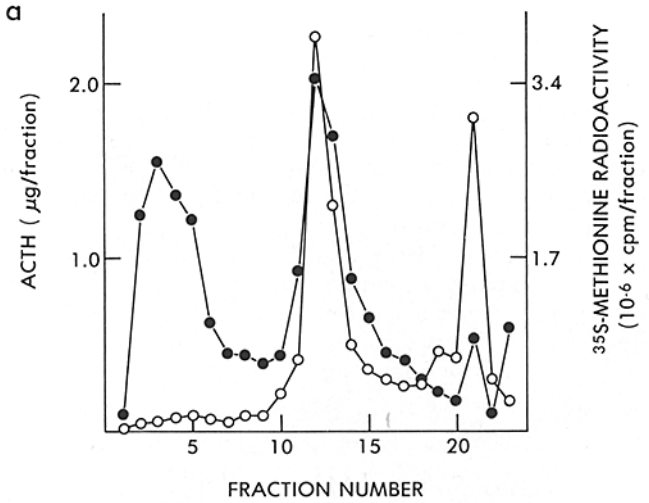

FIGURE 2 Only the stimulation-dependent secretory proteins are enriched in dense ACTH secretory granules. Five 15-cm confluent dishes of AtT-20 cells were labeled with 1.5 mCi of [^{35}S]methionine for 3 h and secretory granules were isolated according to the method of Gumbiner and Kelly (10). (*a*) Purification of crude granule membranes on a D_2O-Ficoll density gradient. Secretory granules enriched in ACTH are recovered in the dense region of the gradient between fractions 2 and 5. ●, amount of ACTH in each fraction determined by radioimmunoassays. O, TCA-precipitable [^{35}S]methionine radioactivity determined by filter assays. Fraction 1 represents the bottom of the gradient. (*b*) Comparison of stimulation-dependent secretory proteins with polypeptides enriched in the ACTH secretory granules. Materials in the second hour chase medium (such as those described in Fig. 1, lanes *3* and *4*) were directly compared to those in the secretory granule fractions (such as fraction 3 in panel *a*) on the same SDS polyacrylamide gel. (Lane *a*) [^{35}S]Methionine-labeled secretory granule peak fraction; arrows indicate the granule-specific proteins that co-purify with ACTH between fractions 2 and 4 as shown previously (10). (Lanes *b* and *c*) Second hour chase medium from [^{35}S]methionine-labeled cells in the absence and presence of 8-Br-cAMP, respectively. Arrows on the right indicate the stimulation-dependent secretory proteins. Note that the two sets of arrows are identical, indicating that only the stimulation-dependent secretory proteins are enriched in ACTH secretory granule.

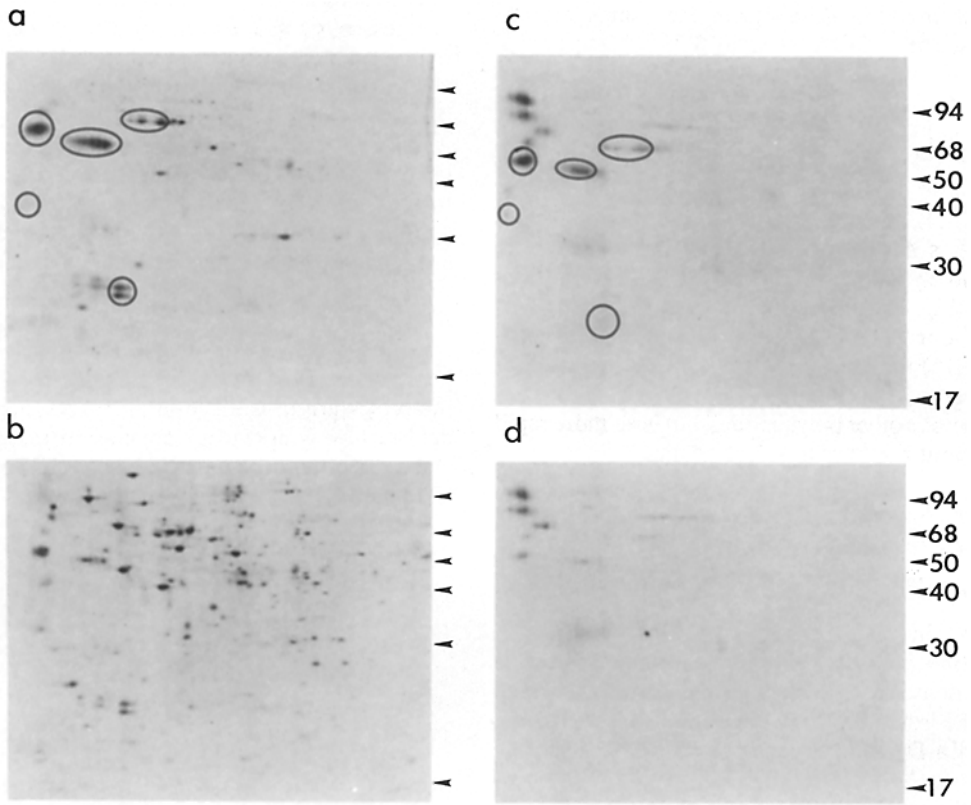

FIGURE 3 Two-dimensional gel electrophoresis of secreted polypeptides and secretory granule polypeptides. (*a* and *b*) [^{35}S]-methionine labeled secretory granule fraction from Fig. 2a (fractions 2–4) and materials from the middle peak (fractions 11–13) of the D_2O-Ficoll gradient were analyzed by two-dimensional gel electrophoresis (24). (Gel *a*) Granule fractions. (Gel *B*) Middle peak. To determine which polypeptides are enriched in the granule fractions approximately equal amounts of total proteins were loaded on each gel and spots which showed higher intensity in the granule fractions (*a*) than in the middle peak (*b*) were circled. (Gels *c* and *d*) Polypeptides released from [^{35}S]methionine pulse-labeled cells (see Fig. 1) were analyzed similarly. (Gel *c*) Second hour chase medium from 8-Br-cAMP-stimulated cells. Gel (*d*) From unstimulated cells. Medium collected from the same number of labeled cells were loaded on each gel and spots which are stimulation-dependent (higher intensity in *c* than in *d*) are circled. The acid electrode of isoelectric focusing is towards the left and the numbers for the molecular weight markers are $\times 10^{-3}$ (phosphorylase, 94; bovine serum albumin, 68; gammaglobulin, 50; alcohol dehydrogenase, 40; carbonic anhydrase, 30; myoglobin, 17). The granule-specific proteins (circles in *a*) isolated from D_2O-Ficoll gradient are identical to the stimulation-dependent proteins (circles in *c*) released into the culture medium.

protein components. Since ACTH is basic, negatively charged molecules such as proteoglycans could play a role in packaging or segregation. Since proteoglycans can be readily labeled in many cases by incorporation of [^{35}S]sulfate, we examined the packaging and secretion of [^{35}S]sulfate labeled macromolecules.

We used a similar protocol to that described in Table I to distinguish sulfated macromolecules released by the regulated pathway from those released by the constitutive one. In Table I and Fig. 4, we show an example in which cells were labeled with [^{35}S]sulfate for 3 h and chased for three consecutive half-hour periods. As shown in Table I, TCA-precipitable radioactivity released into the the medium in the absence of stimulation decreased rapidly with increasing chase times. These products were secreted with the kinetics of the constitutive pathway. When we added 8-Br-cAMP to the medium

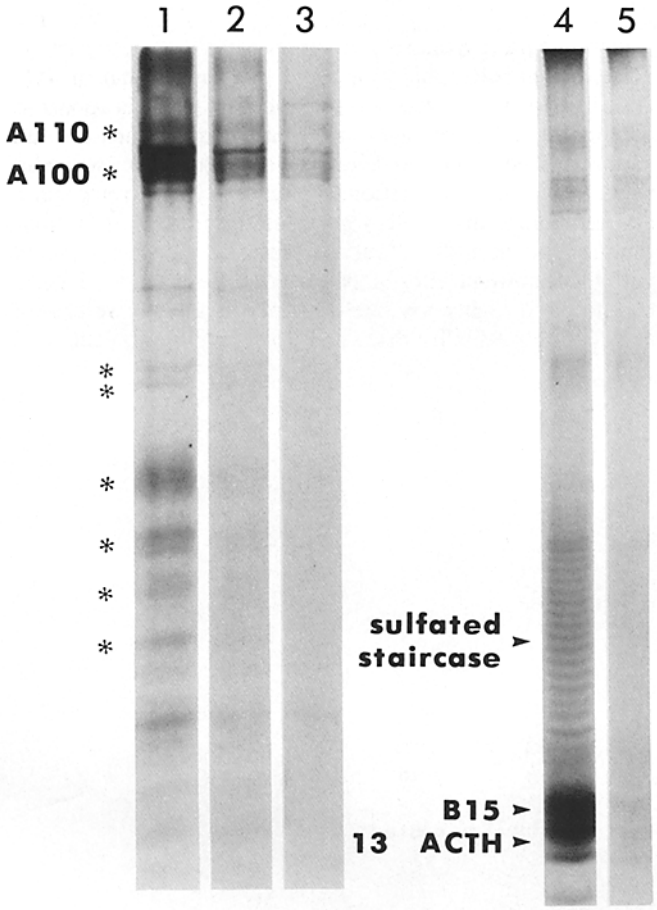

FIGURE 4 Unique sulfated macromolecules are released by AtT-20 cells upon stimulation with 8-Br-cAMP. AtT-20 cells were labeled with [^{35}S]sulfate and chased for three consecutive half-hour periods as described in Table I. 5 mM 8-Br-cAMP was added to the cells during the third chase interval. Chase medium from an equal number of [^{35}S]sulfate labeled cells was TCA precipitated and analyzed on a 10–18% SDS polyacrylamide gel. Fluorograms were exposed for 1 wk for visualization. (Lanes *1*, *2*, and *3*) Medium from unstimulated cells during the chase period, 0–30 min, 30–60 min, and 60–90 min, respectively. Asterisks indicate the class A polypeptides secreted by the constitutive pathway (see text). (Lanes *4* and *5*) Chase medium during the chase interval, 60–90 min, in the presence and absence of 8-Br-cAMP, respectively. The secretagogue stimulates the cells to secrete a number of unique sulfated macromolecules as indicated by arrows (class B polypeptides, see text).

when most of the rapid secretion had already occurred, we observed a two- to threefold stimulation of released TCA-insoluble radioactivity.

Like the [^{35}S]methionine-labeled secretory products, the molecular composition of the material released upon stimulation differs from the composition of rapidly released material. Analysis of the labeled medium on SDS polyacrylamide gels shows that the stimulated material mainly consisted of a ladder of equally spaced bands ranging in molecular weights of about 25,000 to about 35,000 (referred to as the "sulfated staircase"), a major band of ~15,000 mol wt (B15) and a minor band of ~13,000 mol wt (Fig. 4, lanes *4* and *5*). Occasionally we also observe stimulation of labeled material that migrated to the interface of the stack and the running gels. The 15,000-mol-wt protein (B15) might be the same as that observed in the [^{35}S]methionine experiments. We found the minor 13,000-mol-wt protein to be the glycosylated form of mature ACTH (see Fig. 6). Few, if any, of these molecules could be detected in the medium during the first hour of chase in the absence of 8-Br-cAMP. In contrast, a distinct set of molecular species (Fig. 4, lanes *1–3*, asterisks) was rapidly released into the medium during the early chase periods and their secretion was not affected by 8-Br-cAMP (Fig. 4, lanes *4* and *5*). Thus, 8-Br-cAMP stimulates AtT-20 cells to secrete only a subclass of the numerous sulfated secretory products synthesized by AtT-20 cells. We conclude, therefore, that the major sulfated secretory products also fall into two classes which correspond to the regulated and to the constitutive pathways.

Sulfated macromolecules externalized by the same regulated pathway as mature ACTH should be in the same secretory granule. Fig. 5 *a* shows the isolation of [^{35}S]sulfate-labeled secretory granules on a D_2O-Ficoll gradient as described in Fig. 2 *a*. Both the sulfated staircase and the B15 polypeptide were found at high levels in the dense fractions that contain ACTH granules (Fig. 5 *b*). These sulfated macromolecules continue to copurify with ACTH during electrophoresis of intact secretory granules in Ficoll density gradients, following the procedure of Carlson et al. (4) (data not shown). These data further confirm our conclusion that purified secretory granules contain the molecules that are secreted by the regulated pathway.

Newly Identified Proteins Secreted by the Regulated Pathway Are Not Related to ACTH(1–39)

The coordinate secretion of class B polypeptides and ACTH by AtT-20 cells prompted us to examine if any of these proteins are related to ACTH(1–39). Proteins B65, B60, and B37 are not related to ACTH because (*a*) they are higher in molecular weights than the largest ACTH precursor known (30,000 mol wt; see references 20, 29); (*b*) they were not immunoprecipitated by anti-ACTH(1–39) antiserum (10; unpublished data). The two major sulfated class B proteins, B15, and the sulfated staircase are also unrelated to ACTH(1–39) because they were not precipitated by an anti-ACTH(1–39) antiserum (Fig. 6). Note that the 31,000–29,000-mol-wt precursors of ACTH, the 23,000-mol-wt biosynthetic intermediates and the 13,000-mol-wt glycosylated ACTH recovered in the chase medium were sulfated (Fig. 6, lanes *b* and *d*). No sulfation of the unglycosylated 4,500-mol-wt form of mature ACTH was detected.

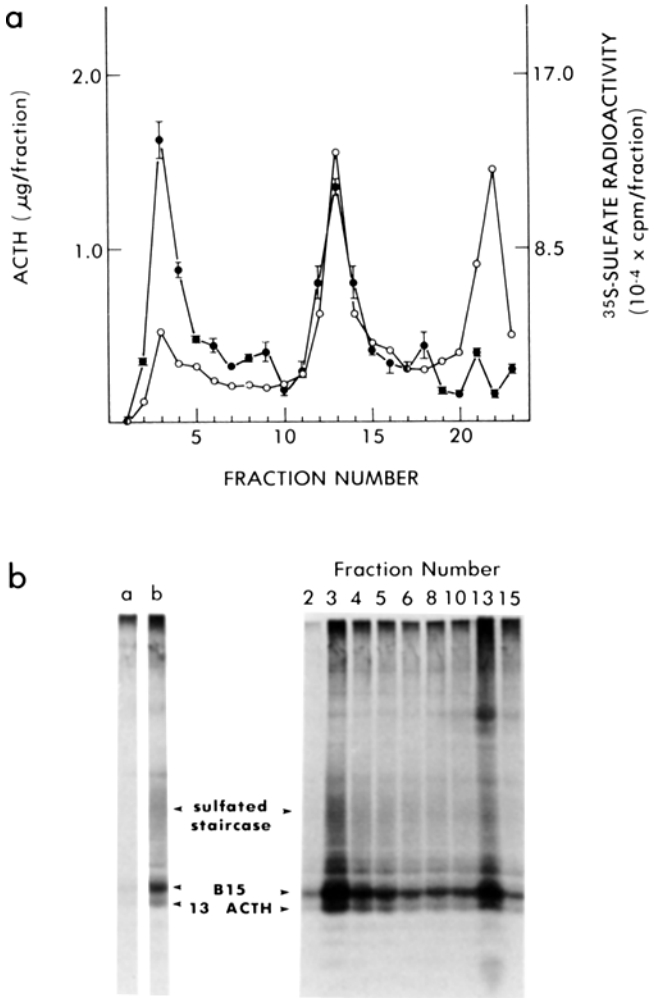

FIGURE 5 The "sulfated staircase" and the sulfated B15 polypeptides are enriched in secretory granule fractions. Five 15-cm confluent dishes of AtT-20 cells were labeled with 5 mCi [^{35}S]sulfate for 4 h and secretory granules were isolated as described in Fig. 2. (*a*) D_2O-Ficoll density gradient purification of secretory granules, showing profiles of ACTH (●) and TCA-insoluble [^{35}S]sulfate radioactivity (○). The dense secretory granules were recovered in the peak between fractions 2 and 4. (*b*) SDS gel electrophoresis of the fractions from *a*. One-half of the labeled materials from each fraction in *a* were TCA precipitated and run on a 10–18% polyacrylamide gel and the fluorogram was exposed for 1 wk to visualize [^{35}S]sulfated labeled bands. The sulfated staircase and the B15 protein are enriched in fraction 3 which has the highest specific activity of ACTH (see *a*). The polypeptide pattern is compared to the stimulation-dependent polypeptides (those described in Fig. 4) on the same gel. (Lane *a*) Second-hour chase medium from [^{35}S]-sulfate labeled cells in the absence of 8-Br-cAMP. (Lane *b*) In the presence of 8-Br-cAMP.

AtT-20 Cell Externalizes Its Secretory Products by Two Distinct Pathways

The kinetics of protein secretion suggest that the secretory products of AtT-20 cells can be categorized into two classes that correspond either to the regulated or to the constitutive pathway. To determine whether this generality holds true, we quantified the secretion characteristics of a few individual proteins from each class and compared them to those characteristic of the ACTH precursor, viral gp70 and mature ACTH (Fig. 7). Radioactivity in each polypeptide from Fig. 1 was quantitated with a densitometer and plotted as a function of chase time. Class A proteins (asterisks in Fig. 1) are characterized by their rapid secretion ($t_{1/2}$, 30–40 min) and their lack of sensitivity to 8-Br-cAMP. Class B components (arrows in Fig. 1) on the other hand, are released slowly and are strongly stimulated by 8-Br-cAMP. Sulfated molecules secreted by these cells also fall into either of the two categories (such as polypeptides A110 and A100, see Fig. 4). The route for secretion of class A proteins thus resembles the constitutive pathway previously described for ACTH precursor and viral gp70 (11), whereas class B proteins seem to use a regulated pathway similar to the secretion of mature ACTH. These results indicate that the two routes previously defined for gp70 and mature ACTH are generalized pathways for the secretion of all proteins by these cells.

DISCUSSION

Previously we had shown that mature ACTH and the viral membrane protein take two different paths to the cell surface. Here we generalize this conclusion by showing that in AtT-20 cells all major secretory proteins of AtT-20 cells appear to use one or the other of the two pathways to reach the cell surface. Three criteria were used to distinguish the two pathways: (*a*) kinetics of secretion, (*b*) sensitivity to secretagogues, and (*c*) storage in secretory granules. By these criteria, four unique proteins and the "sulfated staircase" are co-segregated with the mature hormones, packaged in storage granules and secreted in a highly regulated manner. Although we cannot exclude the possibility that these polypeptides are contained

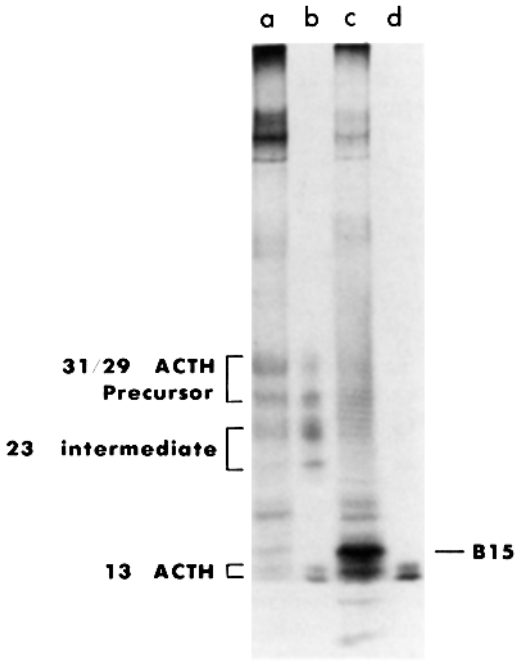

FIGURE 6 Immunoprecipitation of [^{35}S]sulfate-labeled polypeptides released into the culture medium by anti-ACTH(1–39) antisera. Materials secreted by [^{35}S]sulfate-labeled cells as described in Fig. 4 were immunoprecipitated with an anti-ACTH(1–39) antiserum. The total TCA-insoluble materials and the immunoprecipitates were analyzed on a 10–18% SDS polyacrylamide gel and the fluorogram was exposed for 4 d. (Lane *a*) TCA precipitate of medium during 0–30 min of chase in the absence of 8-Br-cAMP. (Lane *b*) Immunoprecipitate of materials in lane *a*). (Lane *c*) TCA precipitate of medium during 60–90 min of chase in the presence of 8-Br-cAMP. (Lane *d*) Immunoprecipitate of materials in lane *c*.

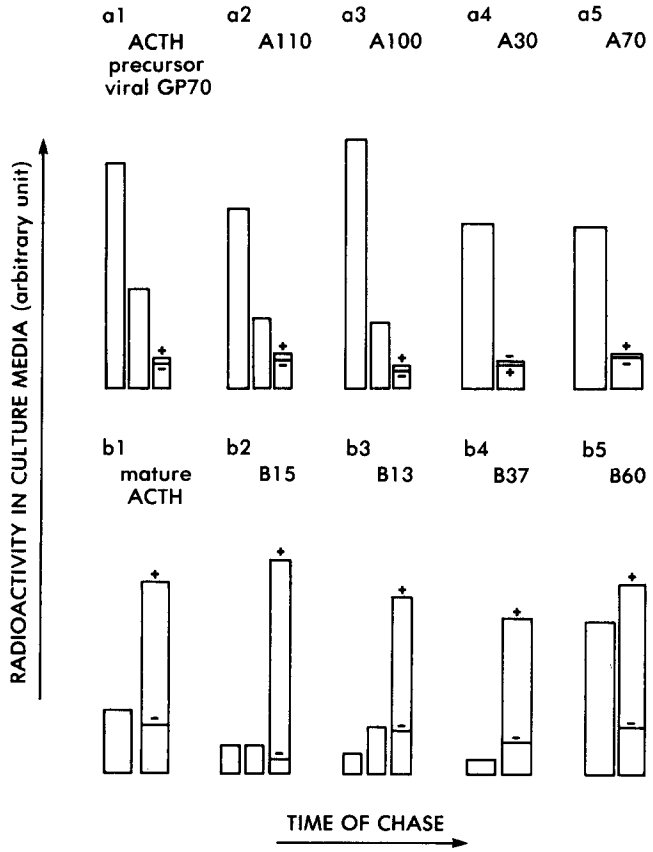

FIGURE 7 Secretion characteristics of the two classes of polypeptides released by AtT-20 cells. The kinetics of secretion and the sensitivity to secretagogues of a number of secretory polypeptides chosen from each class are summarized here. Radioactivity in each polypeptide from Fig. 1 and Fig. 4 was quantitated with a densitometer and plotted as a function of chase time. Chase intervals are 0–30 min, 30–60 min, and 60–90 min for those with three chase points, and 0–60 and 60–120 min for those with two chase points. +, secretion in the presence of 8-Br-cAMP. −, secretion in the absence of 8-Br-cAMP. (*a1–a5*) Class A polypeptides. (*b1–b5*) Class B polypeptides. The size of each polypeptide in $\times 10^{-3}$ molecular weights is indicated.

within a different population of dense secretory granules from that for mature ACTH, we consider it unlikely because so far we have been unable to separate them from ACTH in all purification procedures employed. If they are indeed contained within the same secretory granules as mature ACTH, we estimate that they make up about half of the secretory granule protein content. The proteolytic products of POMC make up most of the other half.

Since there is an alternative route to the cell surface for the five class B macromolecules, their presence in granules suggests a significant function related to hormone secretion. These macromolecules could in principle be unidentified hormones secreted by the pituitary in addition to ACTH. If so, such hormones would come under the same physiological regulation as ACTH. Alternatively, the proteins packaged with mature ACTH could be the proteases needed to cleave and process the hormones from their polypeptide precursors. A thiol protease is thought to cleave the POMC at dibasic amino acids in the rat pituitary intermediate lobe (18). It is unlikely that these proteins are processing enzymes, however, since they are present in granules in amounts nearly equimolar to the hormones. Yet another potential function for intragranular proteins would be to bind to the hormone precursor and regulate the sites of cleavage by intragranular proteases. Not all dibasic amino acids in POMC are sites for proteolytic activity; indeed the polypeptide products of POMC are different in the anterior and intermediate lobes of the pituitary (32). Nonhormone proteins in secretory granules could also cause the formation of storage complexes by binding to the hormones. For example, chromaffin granules that store both adrenalines and the enkephalin hormones (17, 34) contain large amounts of chromogranin A (36) and parathyroid cells release secretory protein I in addition to the parathyroid hormone (5). Recent data show strong homology between chromogranin A and secretory protein I (6). We do not yet know whether any AtT-20 secretory granule proteins are related to these two 70-mol-wt proteins. Such large quantities of nonhormone protein however cannot be essential for the sorting and packaging of hormones in general, since within the limits of measurement, insulin (13), prolactin, and growth hormone granules (30) do not contain large amounts of other proteins.

We found that AtT-20 cells also secrete two classes of sulfated molecules by two different pathways. Most of the sulfated staircase material is released on stimulating the AtT-20 cells. A similar stimulation dependent release of sulfated material has been observed from mast cells (14), adrenal medulla (21), parotid (3), and pancreas (2, 28). Although prolactin granules contain sulfated macromolecules, the release of most of the sulfated material could not be accounted for by exocytosis of granules (9). Our findings with AtT-20 cells suggest that these mammotrophs may release most of their sulfated macromolecules via a constitutive pathway not involving secretory granules.

Some of the sulfation that we observe is presumably due to sulfation of carbohydrate chains since [^{35}S]sulfate labels only the glycosylated 13-mol-wt form of mature ACTH and not the 4.5-mol-wt form. Although protein sulfation occurs in both constitutive and regulated pathways its role in the sorting of proteins between the pathways can not be totally ruled out until the sites of sulfation in these proteins are determined. Hoshima et al. (12) also found sulfation of POMC-derived peptides in the intermediate lobe of the rat pituitary and suggested that sulfation may play a role in the differential processing of POMC in the anterior and intermediate lobes of the pituitary (32). Since the AtT-20 cell is derived from the anterior pituitary and the sulfation pattern of POMC and its products found here is apparently identical to those found in the intermediate lobe, it is unlikely that sulfation controls the differential proteolytic processing in the two lobes.

The peculiar molecular form of the sulfated staircase suggests that it may be a proteoglycan. Recent data (T. L. Burgess, H.-P. Moore, and R. B. Kelly, manuscript in preparation) are consistent with this suggestion. The sulfated material is sensitive to proteolytic enzymes, is not made in the presence of inhibitors of proteoglycan biosynthesis and yields a material with the electrophoretic mobility of glycosaminoglycan after pronase digestion. The characteristic spacings are a property of the oligosaccharide chains and the heterogeneity in mobility reflects a heterogeneity in size. The presence of high molecular weight, sulfated proteoglycans has been reported in many secretory granules including chromaffin granules (36), prolactin granules (7), zymogen granules (28), and synaptic vesicles.[2]

[2] S. S. Carlson and R. B. Kelly. 1983. A unique, highly antigenic proteoglycan-like material in cholinergic synaptic vesicles. *J. Biol. Chem.* In press.

The role of sulfated proteoglycan in secretory granules is not known with certainty. Since there is an alternative route to the surface, the presence of a specific proteoglycan in the granules supports the hypothesis that they serve a function related to regulated secretion. Seveal experiments support the proposal that sulfated macromolecules are involved in packaging of secretory proteins (26). Sulfated glycosaminoglycans can cause the aggregation of zymogen granule contents in vitro (27). The contents of mast cell granules, rich in heparin, stay aggregated even after exocytosis (1). Finally, the contents of prolactin granules, rich in sulfated glycosaminoglycans, remain aggregated after membrane removal (7, 9, 30), whereas the contents of growth hormone–containing granules that lack such sulfated macromolecules, dissociate under identical conditions (30).

Sulfated proteoglycans released from granules upon exocytosis could also perform an extracellular function which requires their secretion by the regulated pathway. For example, specific proteoglycan inserted into the extracellular matrix at sites of neurotransmitter release or hormone secretion could give rise in part to specialized règion of extracellular matrix at the release sites. Preliminary evidence for such a relationship has been found at synaptic junctions in the electric organ (K. M. Buckley and R. B. Kelly, unpublished observations). Such specialized regions could also exist at sites of exocytosis in pituitary (33).

Macromolecules destined for secretion by the constitutive pathway of AtT-20 cells presumably differ in some way from those stored in secretory granules. It seems likely to us that a specific segregation mechanism exists for the regulated pathway, and that the constitutive pathway nonspecifically "leaks" proteins to the surface. This is supported by our finding that most of the newly synthesized ACTH is secreted by the constitutive pathway when its entry into the regulated pathway is inhibited by chloroquine (23). Also, we draw analogy to the targeting of lysosomal enzymes to lysosomes. Lysosomal enzymes lacking the mannose-6-phosphate recognition marker are secreted from the cells (for review, see reference 31). In contrast to the sorting of lysosomal enzymes, the segregation of secretory proteins into secretory granules employs a mechanism which we do not yet understand. In the case of AtT-20 cells two major classes of sorting mechanisms can be proposed. Either all five macromolecules in secretory granules and POMC share a similar recognition signal, or POMC and the five macromolecules interact to form a complex and some member of the complex is recognized by the sorting mechanism. Identifying the nature of the signal is clearly a major challenge.

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REFERENCES

1. Anderson, P., S. A. Slorach, and B. Uvnas. 1973. Sequential exocytosis of storage granules during antigen-induced histamine release from sensitized rat mast cells in vitro. An electron microscopic study. *Acta Physiol. Scand.* 88:359–372.
2. Berg, N. B. 1978. Sulfate metabolism in the exocrine pancreas. II. The production of sulfated macromolecules by the mouse exocrine pancreas. *J. Cell Sci.* 31:199–211.
3. Berg, N. B., and B. D. Austin. 1976. Intracellular transport of sulfated macromolecules in parotid acinar cells. *Cell Tissue Res.* 165:215–225.
4. Carlson, S. S., J. A. Wagner, and R. B. Kelly. 1978. Purification of synaptic vesicles from elasmobranch electric organ and the use of biophysical criteria to demonstrate purity. *Biochemistry.* 17:1188–1199.
5. Cohn, D. V., J. J. Morrissey, J. W. Hamilton, R. E. Shefstall, F. L. Smardo, and L. L. H. Chu. 1981. Isolation and partial characterization of secretory Protein I from bovine parathyroid glands. *Biochemistry.* 20:4135–4140.
6. Cohn, D. V., R. Zangerle, R. Colbrie-Fischer, L. L. H. Chu, J. J. Elting, J. W. Hamilton, and H. Winkler. 1982. Similarity of secretory protein I from parathyroid gland to chromogranin A from adrenal medulla. *Proc. Natl. Acad. Sci. USA.* 79:6056–6059.
7. Giannattasio, G., and A. Zanini. 1976. Presence of sulfated proteoglycans in prolactin secretory granules isolated from the rat pituitary gland. *Biochim. Biophys. Acta* 439:349–357.
8. Giannattasio, G., A. Zanini, and J.Meldolesi. 1979. Complex carbohydrates of secretory organelles. *In* Complex Carbohydrates of Nervous Tissue. R. U. Margolis and R. K. Margolis, editors. Plenum Press, New York and London. 327–346.
9. Giannattasio, G., A. Zanini, P. Rosa, J. Meldolesi, R. K. Margolis, and R. U. Margolis. 1980. Molecular organization of prolactin granules. III. Intracellular transport of sulfated glycosaminoglycans and glycoproteins of the bovine prolactin granule matrix. *J. Cell Biol.* 86:273–279.
10. Gumbiner, B., and R. B. Kelly. 1981. Secretory granules of an anterior pituitary cell line AtT-20, contain only mature forms of corticotropin and β-lipotropin. *Proc. Natl. Acad. Sci. USA.* 78:318–322.
11. Gumbiner, B., and R. B. Kelly. 1982. Two distinct intracellular pathways transport secretory and membrane glycoproteins to the surface of pituitary tumor cells. *Cell.* 28:51–59.
12. Hoshina, H., G. Hortin, and I. Boime. 1982. Rat pro-opiomelanocortin contains sulfate. *Science (Wash DC).* 217:63–64.
13. Howell, S. L. 1974. The molecular organization of the β granule of the islets of Langerhans. *Adv. Cytopharmacol.* 2:319–327.
14. Jaques, L. B. 1975. The mast cells in the light of new knowledge of heparin and sulfated mucopolysaccharides. *Gen. Pharmacol.* 6:235–245.
15. Kreil, G. 1981. Transfer of proteins across membranes. *Annu. Rev. Biochem.* 50:317–349.
16. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.
17. Lewis, R. V., A. S. Stern, J. Rossier, S. Stein, and S. Udenfriend. 1979. Putative enkephalin precursors in bovine adrenal medulla. *Biochem. Biophys. Res. Commun.* 89:822–829.
18. Loh, Y. P., and H. Gainer. 1982. Characterization of proopiocortin-converting activity in purified secretory granules from rat pituitary neurointermediate lobe. *Proc. Natl. Acad. Sci. USA.* 79:108–113.
19. Mains, R E., and B. A. Eipper. 1976. Biosynthesis of adrenocorticotropic hormone in mouse pituitary tumor cells. *J. Biol. Chem.* 251:4115–4120.
20. Mains, R. E., B. A. Eipper, and N. Ling. 1977. Common precursor to corticotropins and endorphins. *Proc. Natl. Acad. Sci. USA.* 74:3014–3018.
21. Margolis, R. K., S. D. Jaanus, and R. U. Margolis. 1973. Stimulation by acetylcholine of sulfated mucopolysaccharide release from the perfused cat adrenal gland. *Mol. Pharmacol.* 9:590–594.
22. Meyer, D. I., E. Krause, and B. Dobberstein. 1982. Secretory protein translocation across membranes—the role of the "docking protein." *Nature (Lond.).* 297:647–650.
23. Moore, H. P., B. Gumbiner, and R. B. Kelly. 1983. Chloroquine directs ACTH from a regulated to a constitutive secretory pathway in AtT-20 cells. *Nature (Lond.).* 302:434–436.
24. O'Farrell, P. H. 1975. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* 250:4007–4021.
25. Orth, D.N. 1979. Adrenocorticotropic hormone. *In* Methods of Hormone Radioimmunoassay. Second edition. B. M. Jaffe and H. R. Behrman, editors. Academic Press, Inc., New York. 245–284.
26. Palade, G. E. 1975. Intracellular aspects of the process of protein synthesis. *Science (Wash. DC).* 189:347–358.
27. Reggio, H. A., and J. C. Dagorn. 1978. Ionic interactions between bovine chymotrypsinogen A and chondroitin sulfate ABC. A possible model for molecular aggregation in zymogn granules. *J. Cell Biol.* 78:951–957.
28. Reggio, H. A., and G. E. Palade. 1978. Sulfated compounds in the zymogen granules of the guinea pig pancreas. *J. Cell Biol.* 77:288–314.
29. Roberts, J. L., and E. Herbert. 1977. Characterization of a common precursor to corticotropin and β-lipotropin: cell free synthesis of the precursor and identification of corticotropin peptides in the molecule. *Proc. Natl. Acad. Sci. USA.* 74:4826–4830.
30. Slaby, F., and M. G. Farquhar. 1980. Characterization of rat somatotroph and mammotroph secretory granules. Presence of sulfated molecules. *Mol. Cell. Endocr.* 18:33–48.
31. Sly, W. S., and H. D. Fischer. 1982. The phosphomannosyl recognition system for intracellular and intercellular transport of lysosomal enzymes. *J. Cell. Biochem.* 18:67–85.
32. Smyth, G. D., and S. Zakarian. 1980. Selective processing of beta-endorphin in regions of porcine pituitary. *Nature (Lond.).* 288:613–615.
33. Vila-Porcile, E., and L. Oliver. 1980. Exocytosis and related membrane events. *In* Synthesis and Release of Adenohypophyseal Hormones. J. Jutisz and K. W. McKerns, editors. Plenum Press, New York. 67–103.
34. Viveros, O. H., E. J. Diliberto, E. Hazum, and K. J. Chang. 1979. Opiate-like materials in the adrenal medulla: evidence for storage and secretion with catecholamines. *Mol. Pharmacol.* 16:1101–1108.
35. Walter, P., and G. Blobel. 1982. Signal recognition particle contains a 7S RNA essential for protein translocation across the endoplasmic reticulum. *Nature (Lond.).* 299:691–698.
36. Winkler, H. 1976. The composition of adrenal chromaffin granules: an assessment of controversial results. *Neuroscience.* 1:65–80.