

# HORMONE SECRETION BY CELLS DISSOCIATED FROM RAT ANTERIOR PITUITARIES

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## ABSTRACT

A new procedure has been developed for dissociating anterior pituitary tissue and producing a viable suspension of single cells. The procedure involves incubation of small tissue blocks in 1 mg/ml trypsin (15 min), followed by incubation in 8  $\mu$ g/ml neuraminidase and 1 mM EDTA (15 min), followed by mechanical dispersion. Cell yields are  $\sim$ 55%, based on recovered DNA. By electron microscopy five types of secretory cells (somatotrophs, mammotrophs, thyrotrophs, gonadotrophs, and corticotrophs) plus endothelial and follicular cells can be identified and are morphologically well preserved up to 20 h after dissociation. Throughout this period, the cells incorporate linearly [ $^3$ H]leucine into protein for up to 4 h at a rate 90% greater than hemipituitaries, and they synthesize, transport intracellularly, and release the two major pituitary secretory products, growth hormone and prolactin. Immediately after dissociation the cells' ability to respond to secretagogues (high  $K^+$  and dibutyryl cyclic AMP) is impaired, but after a 6–12-h culture period, the cells apparently recover and discharge 24% and 52%, respectively, of their content of prelabeled growth hormone over a 3-h period in response to these two secretagogues. This represents a stimulation of 109% and 470% over that released by cells incubated in control medium. The results demonstrate that function and morphologic integrity are preserved in this cell system. Therefore it is suitable for the study of various aspects of pituitary secretion and its control.

## INTRODUCTION

The most commonly used method for studying pituitary hormone secretion *in vitro* employs paired halves or quarters of the gland for comparing experimental with control preparations (1). With this system, only one experimental variant can be studied with each set of hemipituitaries. Additional limitations to the use of large pieces of pituitary tissue become obvious

when attempts are made to study the secretory process at the subcellular level. The variety of cell types present coupled with the fact that even small molecular weight precursors (amino acids) do not uniformly penetrate such large pieces of pituitary tissue generally precludes a detailed analysis of the secretory process in a given cell type. As a result of these problems, our understanding of the

secretory cycle in pituitary cells is patchy and largely deductive, being based on knowledge derived from other more accessible secretory systems. The availability of a system consisting of a suspension of single, dispersed pituitary cells in which cell structure and function is essentially the same as *in situ* would circumvent most of these difficulties. It would (a) overcome individual variations between glands, since the cell population from a number of glands would be randomized; (b) eliminate the diffusion problems encountered in even small tissue fragments; and (c) ultimately provide a suitable starting material for the sub-fractionation of the heterogeneous pituitary cell population into preparations consisting of a single cell type. Several methods for dissociating pituitary tissue have been reported previously (2-7); however, to date, no study has appeared in which the morphology and secretory functions of single cell suspensions have been compared directly with that of undissociated pituitary tissue. In the present work, maintenance of unimpaired morphology and secretory activity have been the primary goals in the development of the dissociation procedure. A method fulfilling these requirements has been devised for preparing a suspension of viable and stable single anterior pituitary cells which is therefore suitable for the study of a number of important aspects of the secretory process.<sup>1</sup>

## MATERIALS

### *Chemicals and Isotopes*

Reagents were obtained from the following sources: Bovine pancreatic trypsin (type III, 2X crystallized) was purchased from Sigma Chemical Co., St. Louis, Mo. Chromatographically pure neuraminidase (*Clostridium perfringens*); crude collagenase (*Clostridium histolyticum*); chromatographically pure soya bean trypsin inhibitor (SBTI)<sup>2</sup>; and deoxyribonuclease I (RNase-free) were purchased from Worthington Biochemical Corp., Freehold, N.J. Dibutyl cyclic AMP (A grade) was purchased from Calbiochem, San Diego, Calif.; bovine serum albumin (fraction V), from Miles Laboratories, Inc., Kankakee, Ill.;

<sup>1</sup> A preliminary report of these findings was published previously (8).

<sup>2</sup> Abbreviations used in this paper: BSA, bovine serum albumin; KRB/BSA, Krebs-Ringer bicarbonate solution containing a complete amino acid supplement, 14 mM glucose, and either 0.3 or 0.5% BSA; PCA, perchloric acid; RER, rough endoplasmic reticulum; SBTI, soybean trypsin inhibitor; STH, growth or somatotrophic hormone.

cycloheximide (Actidione), from Nutritional Biochemicals Corporation, Cleveland, Ohio; streptomycin sulfate and potassium penicillin G, from Eli Lilly and Company, Indianapolis, Ind.; and L-[4,5-<sup>3</sup>H]leucine (specific activity 52-58 Ci mmol), from Schwartz Bio Research Inc., Orangeburg, N.Y. The rat growth hormone (NIAMD-Rat GH-B-1) and rat prolactin (NIAMD-A-Rat prolactin-RP-1) used as standards were provided by the Rat Pituitary Hormone Distribution Program, National Institute of Arthritis and Metabolic Diseases, Bethesda, Md.

### *Animals*

The animals used in this work were 90-120 g Sprague Dawley males (Holtzman Animal Farms, Madison, Wis.). Young males were chosen because the secretion of growth hormone or somatotrophin (STH) was used as an index of the secretory activity of the dissociated cell preparation, and STH secretion is maximal in such animals (9).

### *Incubation Media*

The incubation medium used throughout these studies consisted of a Krebs-Ringer bicarbonate solution (10) containing a complete amino acid supplement (11), 14 mM glucose, and BSA. The media were gassed with 95% O<sub>2</sub> and 5% CO<sub>2</sub> (to pH 7.4), and this atmosphere was maintained either by intermittent gassing at 20-30-min intervals or by continuous gas flow. For long-term incubations, potassium penicillin G (100 U/ml) and streptomycin sulphate (50 µg/ml) were added to the medium to prevent bacterial growth. For tissue blocks, BSA was present at a concentration of 3 mg/ml; for dissociated cells the BSA concentration was increased to 5 mg/ml, and the Ca<sup>++</sup> concentration was reduced to 0.1 mM. Increasing the BSA concentration reduced cell blebbing and cell adhesion to glassware, and decreasing the Ca<sup>++</sup> concentration reduced cell reaggregation. To prepare Ca<sup>++</sup>- and Mg<sup>++</sup>-free media the salts were simply omitted, but in media containing increased K<sup>+</sup>, the Na<sup>+</sup> concentration was reduced to maintain constant osmolality. Pure trypsin, neuraminidase, and SBTI were added as salt-free powders. When other additions were made to the media, the pH was readjusted (with 0.1 N HCl or 0.1 N NaOH) as necessary.

All glassware was siliconized and all media Millipore-filtered through 0.45-µm filters before use. It was not found to be necessary to maintain completely sterile conditions.

## METHODS

### *Incubation Procedures*

The incubation conditions differed from one experiment to another as described below. In incuba-

tions involving dissociated cells, unless indicated otherwise, each sample contained  $\sim 2 \times 10^6$  cells/ml.

**CONTINUOUS INCORPORATION OF [ $^3\text{H}$ ]LEUCINE:** Small tissue blocks or 2-ml aliquots of cell suspension were placed in 25-ml Erlenmeyer flasks and incubated from 0 to 240 min in 5 ml KRB/BSA containing 10  $\mu\text{Ci/ml}$  [ $^3\text{H}$ ]leucine (58 Ci/mmol). At the end of the incubation period, the blocks were rinsed for 10 min at 5°C in KRB/BSA supplemented with 2 mM [ $^1\text{H}$ ]leucine (wash medium) and then homogenized in 1 ml of 1 mM EDTA (containing 0.5 mg/ml BSA) to provide samples for radioactivity and chemical assays.

In the case of cell suspensions, at the end of the incubation period 5 ml wash medium at 5°C was added. The cells were pelleted by centrifugation at 70 *g* for 5 min (in an International Clinical Centrifuge, International Equipment Company, Needham Heights, Mass.) and resuspended in 1 ml of 1 mM EDTA containing 0.5 mg/ml BSA. Samples were taken for radioactivity and chemical assays as for tissue blocks. Some cells were incubated continuously in [ $^3\text{H}$ ]leucine (100–300  $\mu\text{Ci/ml}$ ) for 4 h and prepared for autoradiography as described below for the pulse-chase experiments.

**INCORPORATION OF [ $^3\text{H}$ ]LEUCINE INTO STH:** Tissue blocks and aliquots of cell suspension were incubated in [ $^3\text{H}$ ]leucine for 4 h and washed as described above for the continuous incorporation experiments. The tissue blocks were homogenized in 1 mM EDTA as before, whereas the cells were suspended in 1 mM EDTA and frozen and thawed three times. Microscope examination indicated that this treatment caused 100% cell disruption, and previous work (12) has shown that 85% of the STH is extracted from the tissue by homogenization in 1 mM EDTA. 0.1-ml samples of each of the tissue and cell homogenates were taken for estimation of total radioactivity. The remainder of each homogenate was centrifuged at 4000 *g* for 12 min (to remove cell debris), and 0.8-ml samples of the supernate were taken and their free [ $^3\text{H}$ ]leucine was removed by gel filtration on a 50  $\times$  0.8-cm column of G-25 Sephadex, using 0.01 M ammonium bicarbonate as eluent. The void volume peak, indicated by monitoring the BSA content at  $A_{280}$  nm, was collected and lyophilized. The ensuing powder was dissolved in 0.5 ml 0.2 M Tris-HCl buffer (pH 6.8) and added to an equal volume of 20% glycerol containing 0.1% bromophenol blue. 10- $\mu\text{l}$  samples were taken for assays of total radioactivity and duplicate aliquots submitted to gel electrophoresis for the isolation of STH as described below.

**PULSE-LABELING EXPERIMENTS:** Cell suspensions were incubated 5 min at 37°C in 5 ml prewarmed KRB/BSA containing 0.4  $\mu\text{M}$  [ $^1\text{H}$ ]leucine (instead of the usual 0.4 mM) and [ $^3\text{H}$ ]leucine (500

$\mu\text{Ci/ml}$ ). At the end of the incubation period, 10 ml of prewarmed chase medium (KRB/BSA containing 2 mM [ $^1\text{H}$ ]leucine) were added and the suspension centrifuged for 5 min at 70 *g*. The pellet was then resuspended in 10 ml prewarmed chase medium and incubation continued for up to 240 min at 37°C. Duplicate 0.5-ml aliquots of cell suspension were removed at the beginning and at the end of the 5-min pulse and at 15, 30, 60, 120 and 240 min postpulse. Each sample was further diluted with 5 ml chase medium at 5°C and centrifuged at 70 *g* for 5 min. The cell pellet obtained was resuspended in 0.5 ml chase medium and an equal volume of dilute Karnovsky's fixative (13) added. The cells were then processed for autoradiography as described below.

**RELEASE OF STH:** Ability to release STH was assessed both in recently dissociated cells and cells cultured 15 h. With recently dissociated cells, duplicate 2-ml aliquots of cell suspension were incubated for 360 min in (a) KRB/BSA containing 10  $\mu\text{Ci/ml}$  [ $^3\text{H}$ ]leucine, or (b) the same medium plus 57 mM  $\text{K}^+$ , or (c) the same medium plus 5.0 mM dibutyryl cyclic AMP. Following incubation, the suspension was centrifuged at 70 *g* for 5 min. The cell pellets obtained were washed (by resuspension in KRB/BSA containing 2 mM [ $^1\text{H}$ ]leucine) at 5°C, and again spun at 70 *g* for 5 min. The STH was extracted (by freezing and thawing in 1 mM EDTA) and the homogenate subjected to gel filtration (to remove free [ $^3\text{H}$ ]leucine) and gel electrophoresis (to isolate STH) as in the experiments for incorporation of [ $^3\text{H}$ ]leucine into STH. In addition, a 1.8-ml aliquot of the incubation medium was centrifuged for 12 min at 4000 *g* (to remove debris) and chromatographed on G-25 Sephadex. The void volume peak was collected and prepared for STH separation by gel electrophoresis as described for tissue extracts. As an aid to STH identification on the gel, 200  $\mu\text{l}$  of unlabeled EDTA-pituitary extract (prepared as above) was included in the glycerol-bromophenol blue mixture.

In cells cultured 15 h a different experimental protocol was employed: Here, advantage was taken of the preincubation period to prelabel the secretory product, and before incubation with secretagogues, the cell suspension was washed to reduce the amount of free [ $^3\text{H}$ ]leucine remaining in the medium. The cells were incubated in KRB/BSA for a preliminary period of 5 h and then [ $^3\text{H}$ ]leucine (100  $\mu\text{Ci/ml}$ ) was added for the last 10 h of the 15-h preincubation. The cells were washed three times by repeated centrifugation (at 70 *g* for 9 min) through 4% BSA, resuspended in 12 ml KRB/BSA, and divided into 2-ml aliquots ( $\sim 4 \times 10^6$  cells/ml). Each aliquot was centrifuged at 70 *g* for 5 min, resuspended in 2 ml of control (KRB/BSA) or experimental media containing secretagogues (57 mM  $\text{K}^+$ , or 0.1, 0.5, or 5 mM dibutyryl cyclic AMP), and incubated at 37°C

for 3 h. ~90% of the free [<sup>3</sup>H]leucine in the medium was removed by the washing procedure. Since the washing and subdivision of the cell suspension required at least 60 min to complete, it could be assumed that at the end of this period (i.e., the beginning of the period when STH release was measured), most of the labeled secretory product resided within secretory granules (see Fig. 18). At the end of the incubation period the <sup>3</sup>H-labeled STH was extracted from the cells and the incubation medium as described for recently dissociated cells.

### *Processing of Tissues for Microscopy and Autoradiography*

Dissociated cells were examined directly (unfixed and unstained) using Nomarski differential interference-contrast optics. Others were fixed, embedded, and examined by bright field in stained preparations of thick (0.5–1 μm) sections. Fixation was accomplished by adding the cell suspension to an equal volume of dilute Karnovsky fixative (13) (containing 1% formaldehyde and 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4). After 30 min fixation at 25°C, the cell suspension was transferred to 0.4-ml polyethylene tubes and packed by centrifugation at 10,000 *g* in a Microfuge 152 (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) according to a method used previously for collection of polymorphonuclear leukocytes (14). The tips of the tubes containing the pellets were cut out, placed in vials, and postfixed for 1 h at 4°C in 1% OsO<sub>4</sub> in acetate-Veronal buffer (pH 7.4). Cells prepared for morphologic examination were stained in block for 30–60 min with buffered 0.5% uranyl acetate at 25°C (15), dehydrated in graded ethanols, and embedded in Epon (16). Those prepared for autoradiography were dehydrated and embedded directly without prior staining. Tissue blocks were similarly fixed and embedded except that some specimens prepared for autoradiography were fixed for 1¼ h in OsO<sub>4</sub> alone (1% in 0.15 M Sorenson's phosphate buffer, pH 7.6). Sections were stained with lead alone or doubly stained with aqueous uranyl acetate and lead citrate (17) and examined in a Siemens Elmiskop 101.

Autoradiography of thick (0.4 μm) and ultrathin sections was carried out essentially as described by Caro and van Tubergen (18), with Ilford L-4 emulsion (Ilford, Ltd., Ilford, Essex, England), and analyzed by light and electron microscopy, respectively.

### *Radioactivity and Chemical Assays*

**RADIOACTIVITY:** For assay of total protein radioactivity, samples of tissue homogenates or incubation media were precipitated with 1 ml 1.0 N

perchloric acid (PCA) and kept for 60 min at 5°C. The acid insoluble precipitates were then washed (3X) by centrifugation at 4000 *g* for 12 min and resuspension in 0.5 N PCA. The final pellet was resuspended in 0.8 ml 0.5 N PCA and hydrolysed 30 min at 75°C. After cooling, the precipitate was again pelleted (4000 *g* for 12 min) and 0.5 ml of the supernate taken for DNA assay. The drained pellet was finally dissolved in 0.4 ml 95% formic acid, and 0.1-ml aliquots were counted in Bray's fluid (19) with a Nuclear-Chicago Mark 1 liquid scintillation spectrometer (Nuclear-Chicago, Des Plaines, Ill.). Counting rates were corrected for quenching (with an external standard) and for background.

**CHEMICAL:** DNA was assayed according to the method of Burton (20).

### *Cell Yield Estimation*

The DNA content of undissociated pituitary halves was compared with that of the cell suspension derived from the other halves. Hemocytometer counts were used to supplement the results obtained by DNA assay. Counts were also carried out on duplicate 20-μl aliquots of the cell suspensions used in the amino acid incorporation and STH release experiments, in order that the results could be expressed in terms of total cell number. An indication of cell viability was obtained in using the trypan blue dye exclusion method (21).

### *Gel Electrophoresis*

Electrophoresis was carried out on 10-μl samples of tissue extracts or incubation media (in 10% glycerol containing 0.05% bromophenol blue). 10% polyacrylamide gels were used according to the standard Ornstein and Davies procedure described by Maizel (22). Either slab gels (37 V, 14 mA) or cylindrical gels (2 mA/tube) were run for 6 h and the gels stained with 0.5% Coomassie brilliant blue. The electrode buffer was 0.05 M glycine-NaOH at pH 9.5. National Institutes of Health rat hormone standards served to locate the STH and prolactin bands. To measure the distribution of radioactivity, the stained gels were sliced into 1-mm sections and dissolved overnight in 0.5 ml 20% H<sub>2</sub>O<sub>2</sub> at 50°C. Each sample was counted in Bray's fluid and the percentage radioactivity in STH determined by counting the activity in the STH band and comparing it to the total dpm in a duplicate aliquot of the sample loaded on the gel.

## RESULTS

### *Dissociation Procedure*

During the course of this work a number of different dissociation media and procedures were

tried and the most successful in terms of yield of viable single cells is given in detail. The key steps in the procedure are: (a) a short (15-min) incubation in trypsin, followed by (b) a short (15-min) incubation in neuraminidase-EDTA mixture, and finally (c) mechanical dispersion.

Animals are decapitated, their pituitaries removed, the posterior and intermediate lobes lifted away with a small spatula, and each anterior lobe cleanly chopped with a razor blade into about 40–50 rectangular tissue blocks. The blocks obtained from each gland are pooled in 20 ml KRB/BSA at room temperature. Usually 12–24 pituitaries are used per experiment. After their collection is completed, the pooled blocks are transferred to a 15-ml conical centrifuge tube and allowed to settle out. The KRB/BSA is decanted and replaced with 5 ml KRB/BSA containing 1 mg/ml trypsin at 25°C. The blocks and trypsin medium are transferred to a 25-ml Erlenmyer flask and incubated for 15 min at 37°C in a shaker bath (65–70 strokes/min). After the trypsin incubation, 5 ml DNase (2 µg/ml) in KRB/BSA is added to the flask and the contents poured into a conical centrifuge tube. This step is included because following the trypsin treatment the blocks are agglutinated by “DNA-histone material” derived from damaged cells (23). DNase treatment is continued until the tissue blocks fall freely to the bottom of the tube (about 1 min). The supernate is then decanted and replaced with 5 ml prewarmed KRB/BSA containing 1 mg/ml soybean trypsin inhibitor (SBTI) and the tube incubated 5 min at 37°C. The supernate is again decanted, replaced by 5 ml Ca<sup>++</sup>- and Mg<sup>++</sup>-free KRB/BSA containing 2 mM EDTA, and incubated 5 min at 37°C. After decantation, 4 ml of Ca<sup>++</sup>- and Mg<sup>++</sup>-free KRB/BSA containing 1 mM EDTA and 8 µg/ml neuraminidase is added. The blocks and medium are again transferred to a 25-ml Erlenmyer flask and incubated in a shaker bath for 15 min at 37°C. Following this final incubation, the blocks are transferred again to a conical centrifuge tube and washed three times with 20 ml Ca<sup>++</sup>- and Mg<sup>++</sup>-free KRB/BSA by the settling out and decantation procedure described above. These last rinses serve to dilute out the EDTA and neuraminidase. It should be mentioned that, although the above steps involve several rinses in centrifuge tubes and transfers to and from Erlenmyer flasks, the tissue blocks remain intact throughout these procedures so that

cell loss during the transfers is negligible and handling of the tissue is simplified.

The final preparation of the cell suspension is accomplished by mild shearing using a Pasteur pipette with a flame polished tip (~0.5 mm diameter). When the medium becomes milky the remaining blocks are allowed to settle out, and the cell suspension is decanted into a second tube containing KRB with 0.1 mM CaCl<sub>2</sub>, 1.2 mM MgSO<sub>4</sub>, and 5 mg/ml BSA. More Ca<sup>++</sup>- and Mg<sup>++</sup>-free KRB/BSA is then added to the residual blocks and pipetting continued more vigorously. The collection and pipetting is continued until the blocks are completely dispersed. This procedure ensures that newly dispersed cells are transferred to KRB containing divalent cations with minimal delay and subjects them to a minimum of pipetting. If pipetting is carried out in the presence of Ca<sup>++</sup> and Mg<sup>++</sup> the yield of single cells is reduced and the suspension contains very large numbers of cell aggregates; if dispersion is attempted in the presence of EDTA, cell fragility is greatly increased and the proportion of viable cells produced sharply declines.

Before its use and in order to remove debris (capillary-connective tissue elements, subcellular components from broken cells, etc.), the cell suspension is spun through BSA as follows: 5 ml of 4% BSA in KRB is layered below the cell suspension using a hypodermic syringe equipped with a 5-inch needle. The tube is spun for 9 min at 70 g, thus producing a cell pellet from which the BSA carrier medium can be decanted and the cells subsequently resuspended (by mild pipetting) in the media required. For long incubations (12–15 h) the cells are maintained on a shaker bath with slight but continuous agitation (40 strokes/min), and the cell concentration is kept below  $2 \times 10^6$  cells/ml. Under these conditions there is some reaggregation into small clumps of 5–10 cells, but these small aggregates can be readily dispersed by gentle pipetting.

#### *Evaluation of the Dissociation Procedure*

The yield of cells is 50–55% on a DNA basis, and, by dye exclusion, more than 95% of the cells are viable.

Fig. 1 shows the cell yields obtained when the periods of incubation and concentrations of the dissociating agents employed in the procedure are varied. The data shows that the procedure de-

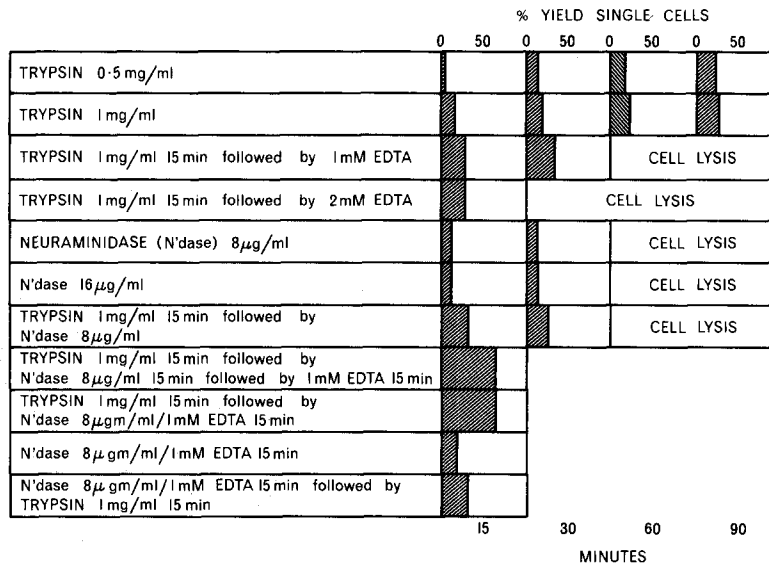


FIGURE 1 Efficiency of the dissociating agents employed in producing a single cell suspension expressed in terms of percentage of the total cell population. Cell counts were made at the end of each 15-min incubation period. Cell lysis indicates complete or gross deterioration of cell morphology as observed in fixed and embedded preparations.

scribed is based on the shortest possible exposure to dissociating agents at the lowest effective concentrations. Lowering the trypsin concentration below 1 mg/ml reduces the cell yield. Prolonged incubation with this enzyme improves the cell yield but is still much less effective than the two step procedure (cell yield ~20% as compared to ~50%). Both EDTA and neuraminidase are cytotoxic and produce cell lysis if used at concentrations higher than those adopted for the final procedure, or if tissues are exposed to them at the suggested concentrations for periods exceeding 30 min. Neither EDTA nor neuraminidase is an effective dissociating agent when used alone, and cell yields are poor (<20%) when they are used together without prior trypsin treatment. Attempts to replace trypsin with crude collagenase resulted in cell yields of <20% (data not shown).

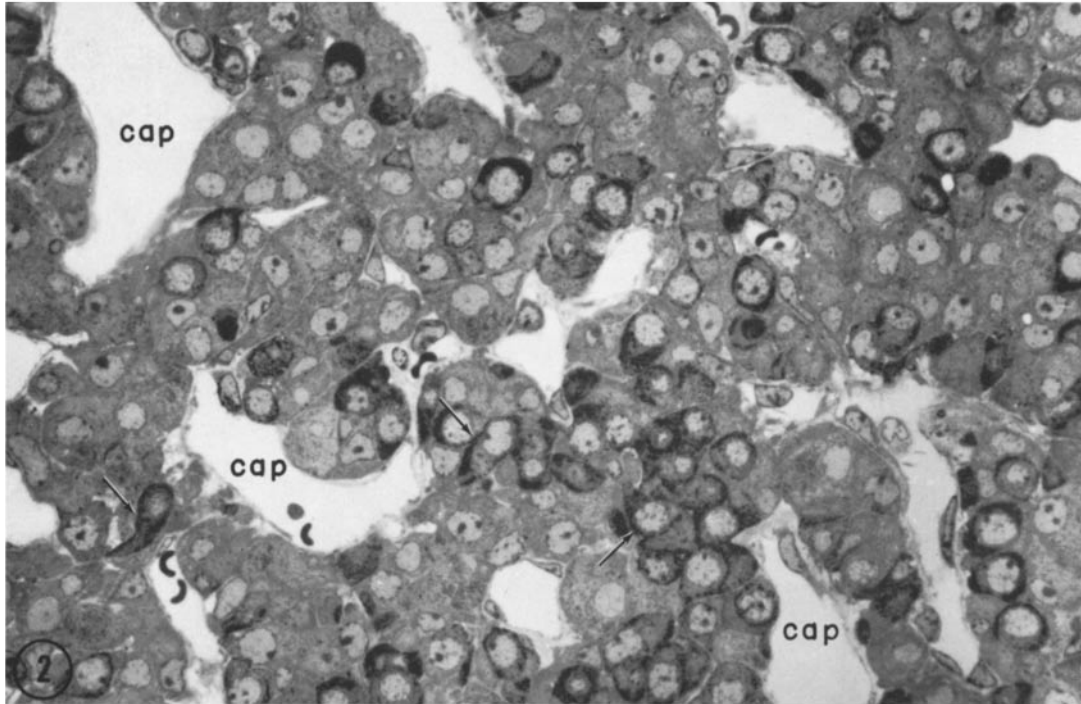
### Morphology of the Dissociated Cells

**LIGHT MICROSCOPY:** Figs. 2-3 are photomicrographs showing the changes in tissue organization and rounding-up of parenchymal cells which occur following successive steps in the dissociation procedure. In small intact tissue blocks before exposure to enzyme treatment (Fig. 2), the cells vary in size and shape and are arranged in groups and cords interlaced with capillaries. After 15-min

incubation in trypsin (Fig. 3 a), the overall appearance of the pituitary cells is similar except that there is a general loosening of the tissue, especially of the capillary networks, which swell and pull away from the cell cords. After incubation in neuraminidase plus EDTA (Fig. 3 b), a general rounding-up of parenchymal cells is evident. The final cell suspension, (Fig. 3 c) obtained immediately after pipetting, contains free single cells. Some cells show small peripheral blebs, but otherwise display good morphological preservation.

**ELECTRON MICROSCOPY:** By electron microscopy it can be seen that all of the cell types observed in the intact tissue are represented; there is no indication of any cell type being preferentially lost. Although not every cell profile present can be identified with certainty (especially those which include only part of a given cell), most can be reliably categorized. At least five secretory cell types, somatotrophs (Fig. 4), mammotrophs (Fig. 6), gonadotrophs (Fig. 7), thyrotrophs (Figs. 5 and 8), and corticotrophs (Fig. 5), can be recognized based on their morphologic features reviewed elsewhere (24, 25).<sup>3</sup> In addition, several

<sup>3</sup> As their names imply these cell types are responsible for the production of growth, prolactin, gonadotrophic, thyrotrophic, and adrenocorticotrophic hormones, respectively.



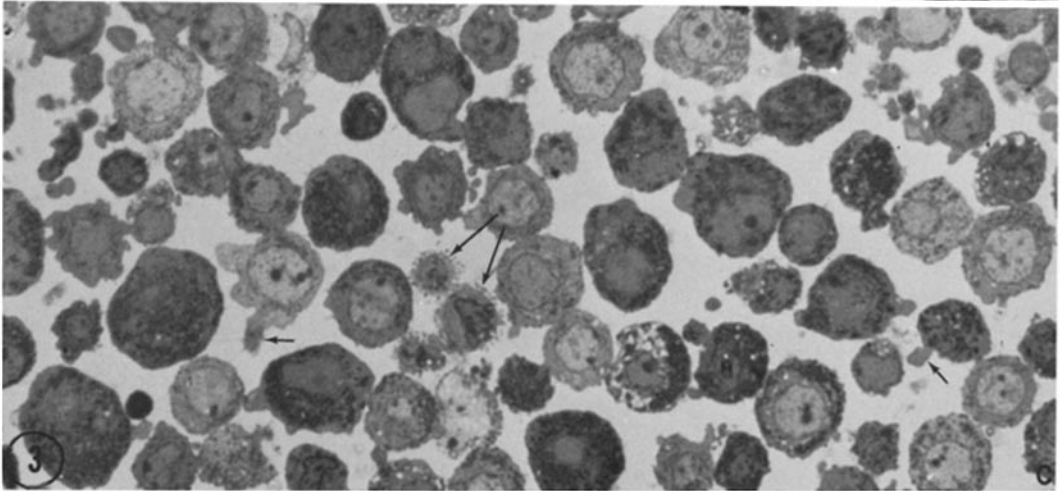
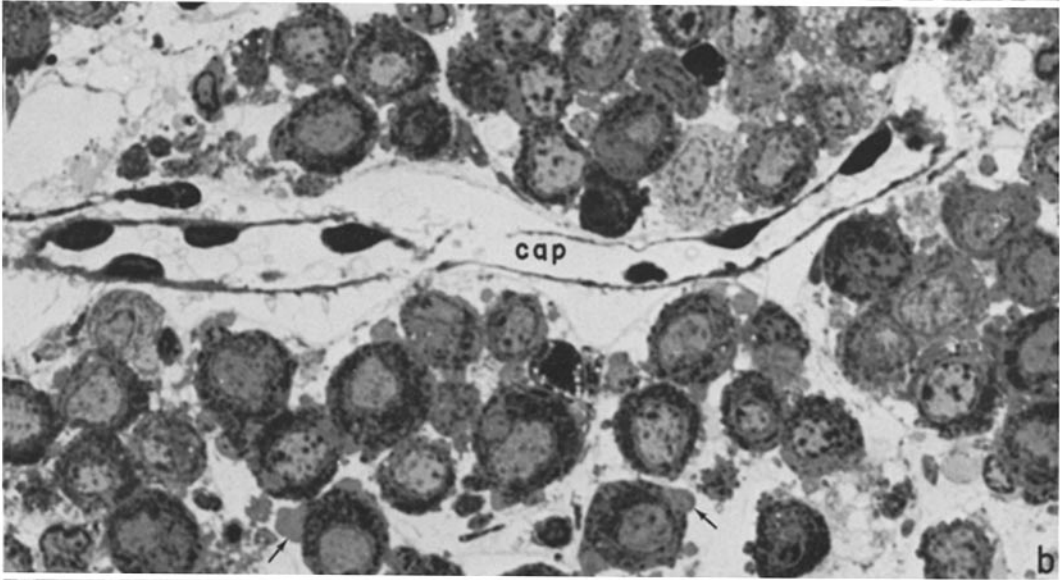
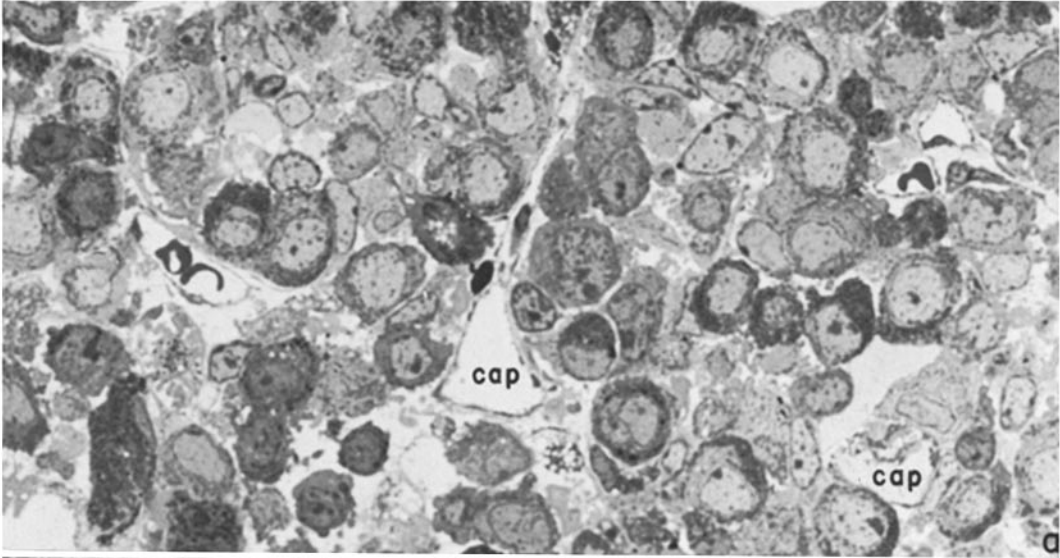
**FIGURE 2** Light micrograph showing a section taken from a small block of intact, undissociated anterior pituitary tissue. The cells vary in size and shape and show complicated interdigitations. Many are angular in contour with the nucleus located at one pole of the cell (arrows). *cap*, capillaries. Epon section (0.5  $\mu\text{m}$ ) of tissue fixed in dilute Karnovsky's fixative, postfixed in 1%  $\text{OsO}_4$  in cacodylate buffer; stained with azure II-methylene blue.  $\times 600$ .

nonsecretory cell types, including follicular cells (Fig. 10), endothelial cells (Fig. 9), and adventitial cells (mononuclear phagocytes), are present. Figs. 4–10 illustrate the fine structural organization of the cells as they appear immediately after dissociation. The cells lose their varied shapes, round-up, and in the process some redistribution

of their intracellular components takes place; most notably the Golgi apparatus assumes a more predominantly juxtannuclear position. With the exception of the Golgi apparatus there is no obvious change in the distribution of the subcellular organelles. It is clear that for many types of studies in which the fine structural organization

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**FIGURE 3** Light micrographs of Epon sections showing progressive loosening of tissue during sequential steps in the dissociation procedure. (a) Central area of a small tissue block fixed after 15-min incubation in 1 mg/ml trypsin. Some removal of intercellular material and rounding-up of cells is evident mainly in the vicinity of capillaries (*cap*). (b) Similar small tissue block after 15-min incubation in trypsin (1 mg/ml) followed by 15-min incubation in neuraminidase (8  $\mu\text{g}/\text{ml}$ ) and EDTA (1 mM). The intercellular spaces are considerably enlarged, and almost all of the cells are rounded-up. Some peripheral cell blebbing can be seen (arrows). (c) Cell suspension produced by gently pipetting the tissue blocks shown in b. Immediately following dissociation, blebbed cells are common (short arrows). However, on being returned to medium containing  $\text{Ca}^{++}$ , blebs are much less frequent. Most of the cells present in the field are secretory elements which contain variable numbers of secretion granules, and which (except for the blebs) have relatively smooth contours. Near the center of the field (long arrows), there is a cluster of small cells with the elaborate ruffled cell margins typical of dissociated nonsecretory elements, i.e., follicular cells and endothelial cells. (See Figs. 9 and 10.) Preparation for microscopy as for Fig. 2. *a*,  $\times 850$ , *b* and *c*,  $\times 950$ .





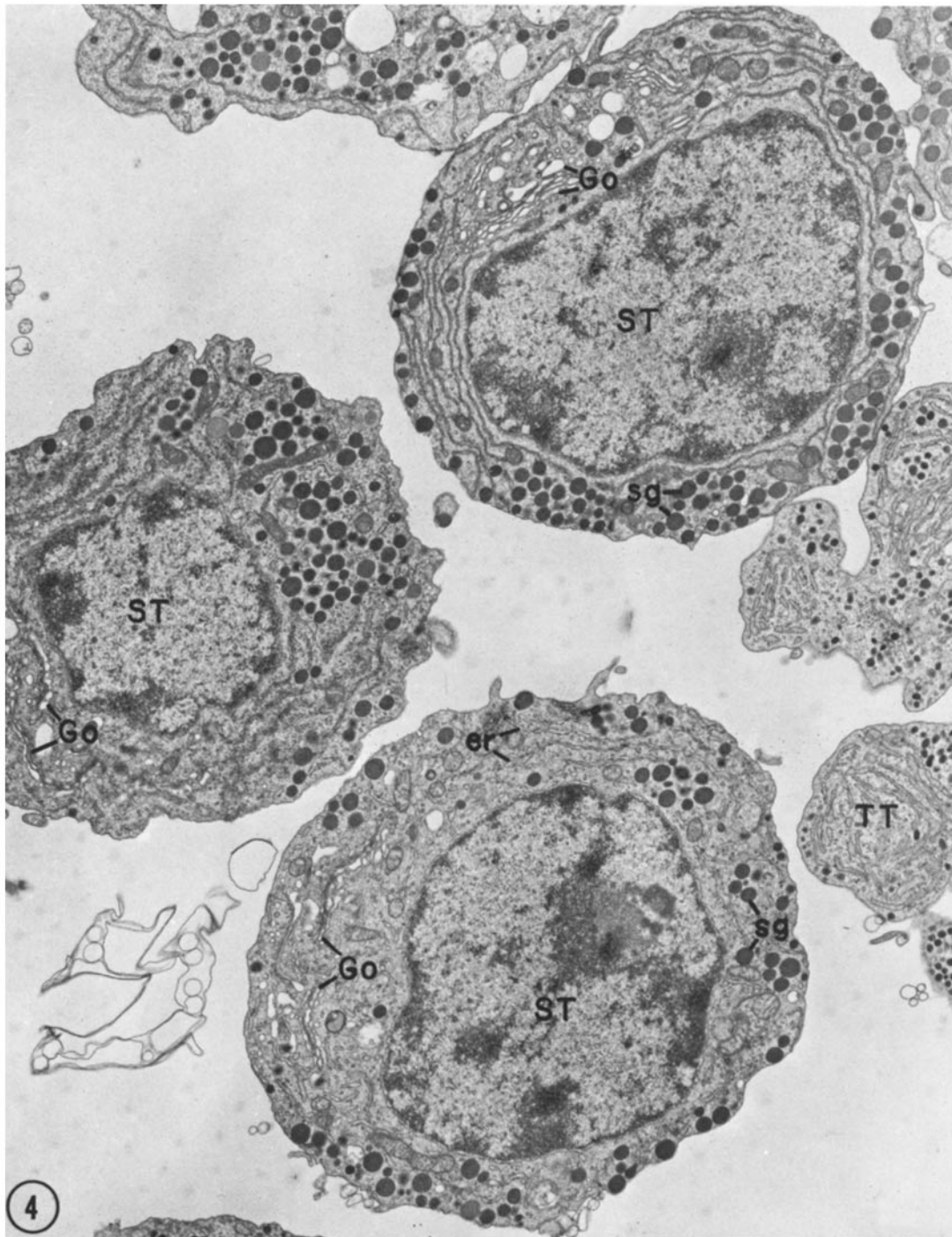
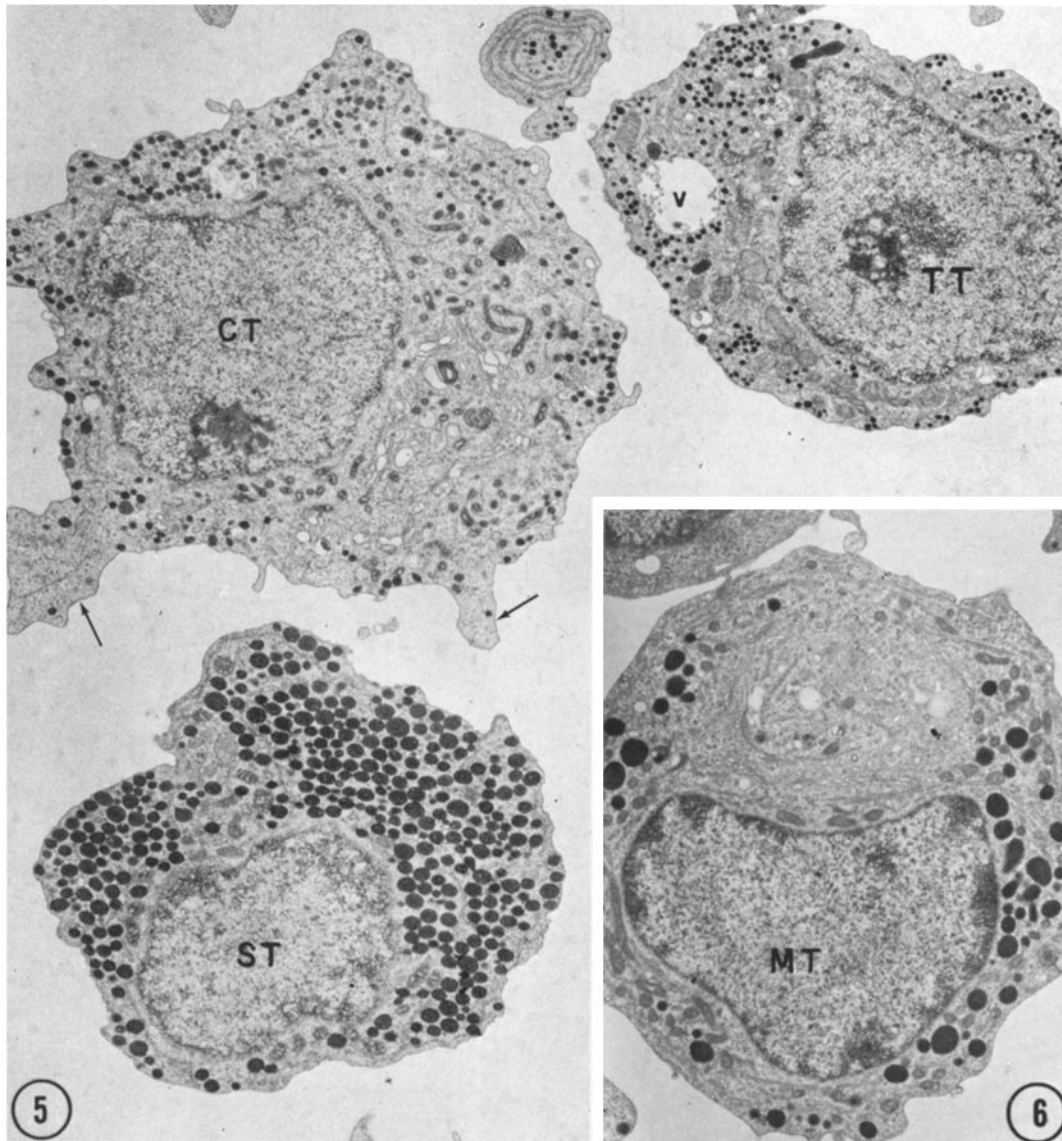


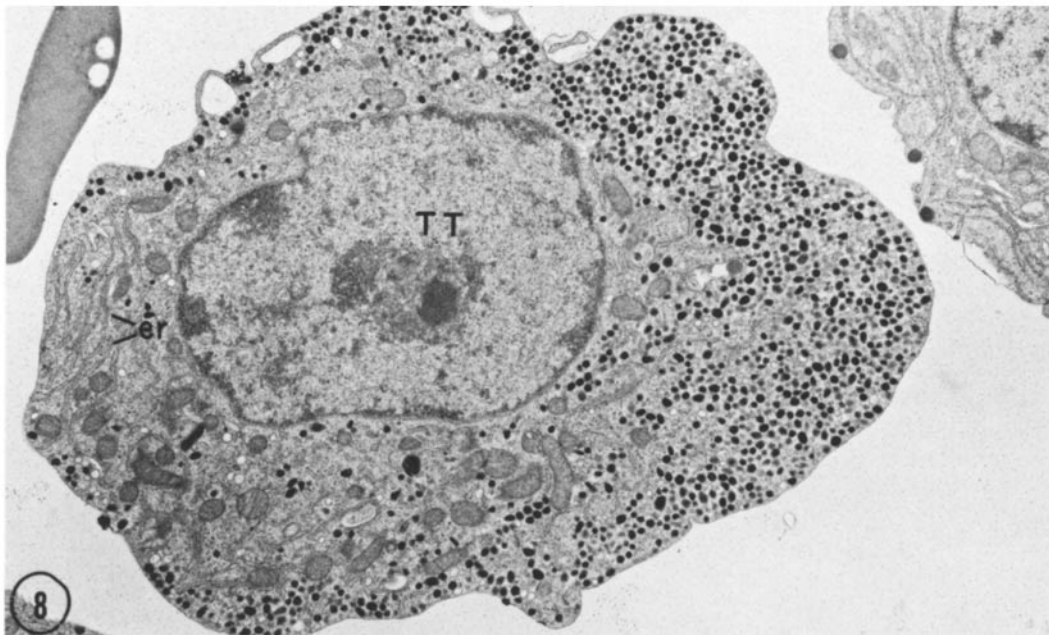
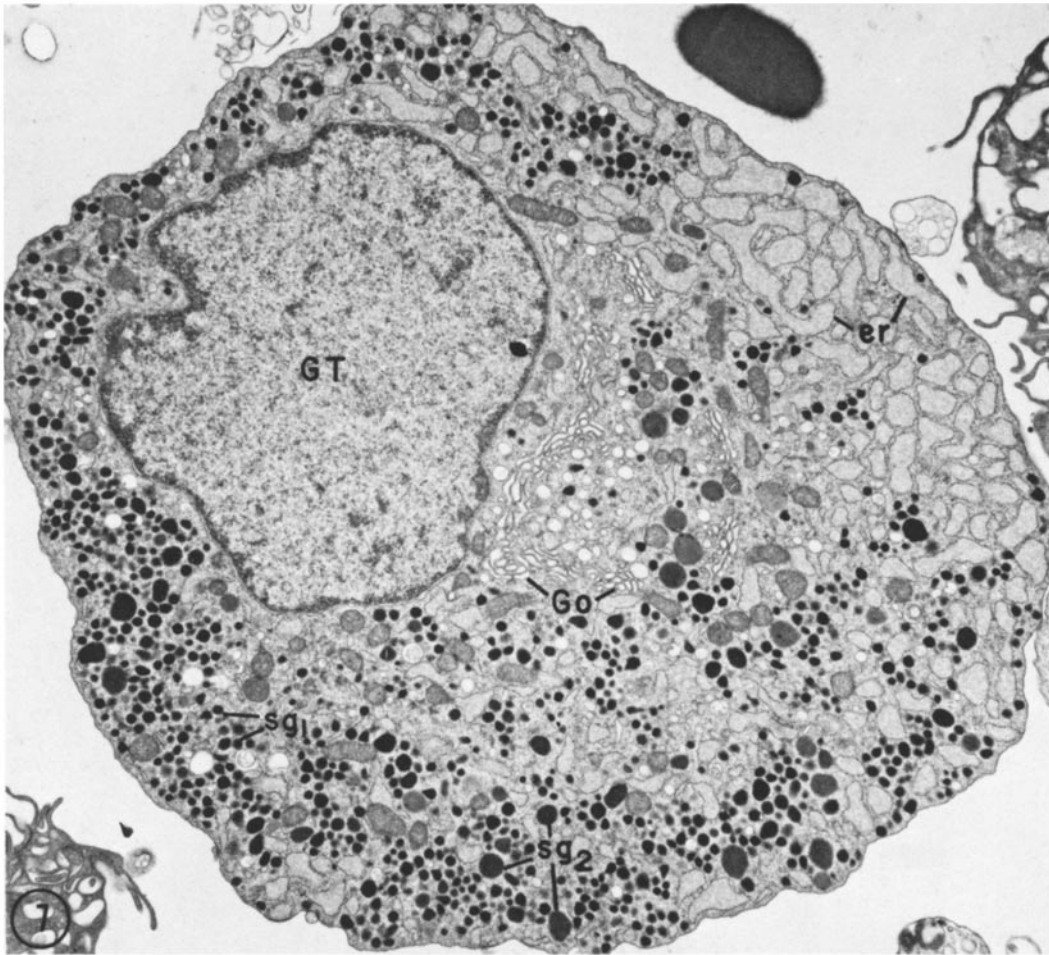
FIGURE 4 Electron micrograph of a group of three somatotrophs or growth hormone-producing cells (ST) from a cell suspension fixed immediately after dissociation. The cells are rounded-up with cytoplasm more or less evenly distributed around the nucleus and the Golgi apparatus (Go) located to one side. The cells can be readily recognized on the basis of their well-preserved fine structural organization — especially their flattened elongated RER cisternae (er) and 350–400 nm spherical secretion granules (sg). A portion of the cytoplasm of a thyrotroph (TT) containing smaller (~100–150 nm) secretion granules is also present to the right. Specimen fixed in dilute Karnovsky fixative, postfixed in OsO<sub>4</sub>, and stained in block with uranyl acetate. Section doubly stained with uranyl acetate and lead. × 10,000.



FIGURES 5 and 6 Low power electron micrographs of several pituitary cell types from a cell suspension fixed immediately after dissociation. The cells can be distinguished by virtue of differences in the size (i.e., maximal diameter) of their secretion granules: mammothrophs (*MT*) = 600–900 nm; somatotrophs (*ST*) = 350–400 nm; corticotrophs (*CT*) = 200–250 nm; thyrotrophs (*TT*) = 100–150 nm. All the cells appear morphologically intact, and their only unusual features are several large blebs (arrows) on the corticotroph and a vacuole (*v*), probably lysosomal in nature, in the thyrotroph. Fig. 5,  $\times 9,000$ , Fig 6,  $\times 7,000$ .

of the whole cell needs to be displayed (e.g., autoradiographic counting) rounded-up single cells have a distinct advantage for sampling purposes over intact pituitary tissues with their complicated shapes and elaborate interdigitation of

cell processes (Fig. 2). In cell suspensions incubated for 15 h or longer in KRB/BSA there is less than a 10% cell loss (indicated by cell counts and by dye exclusion). Electron microscope examination shows that the structural organization of all



of the cell types is for the most part maintained (Figs. 14–19), and there is no indication of preferential loss of any cell type.

### *Evaluation of Secretory Activity*

The ability of the dissociated cells to synthesize, transport, and release secretory products, was analyzed and compared with that of cells in small tissue blocks derived from paired halves of the same pituitaries. Early in these studies it became clear that some aspects of the secretory process were either altered or impaired in recently dissociated cells. Subsequent experiments indicated, however, that if the cells were incubated in KRB/BSA for 6–15 h after dissociation they would recover. Thus, where appropriate, comparative studies have been carried out on recently dissociated cells and cells preincubated for 15 h. The latter will subsequently be referred to as “cultured cells.” To provide data on a specific secretory product, the elaboration of growth hormone (STH) was investigated. STH was chosen because: (a) in young male rats it is known to comprise up to 60% of the total newly synthesized protein released in vitro (26), and (b) a simple procedure is available for the isolation of STH by polyacrylamide gel electrophoresis (27, 28).

**INCORPORATION OF [<sup>3</sup>H]LEUCINE INTO TOTAL PROTEIN:** As shown in Fig. 11, recently dissociated cells, cultured cells, small tissue blocks, and hemipituitaries all linearly incorporate [<sup>3</sup>H]leucine into total protein over a period of 4 h. It is of interest that recently dissociated cells and cultured cells incorporate the labeled precursor at a rate 90% greater than that of tissue blocks, and 140% greater than that of hemipituitaries. Our autoradiographic results presented elsewhere (25)

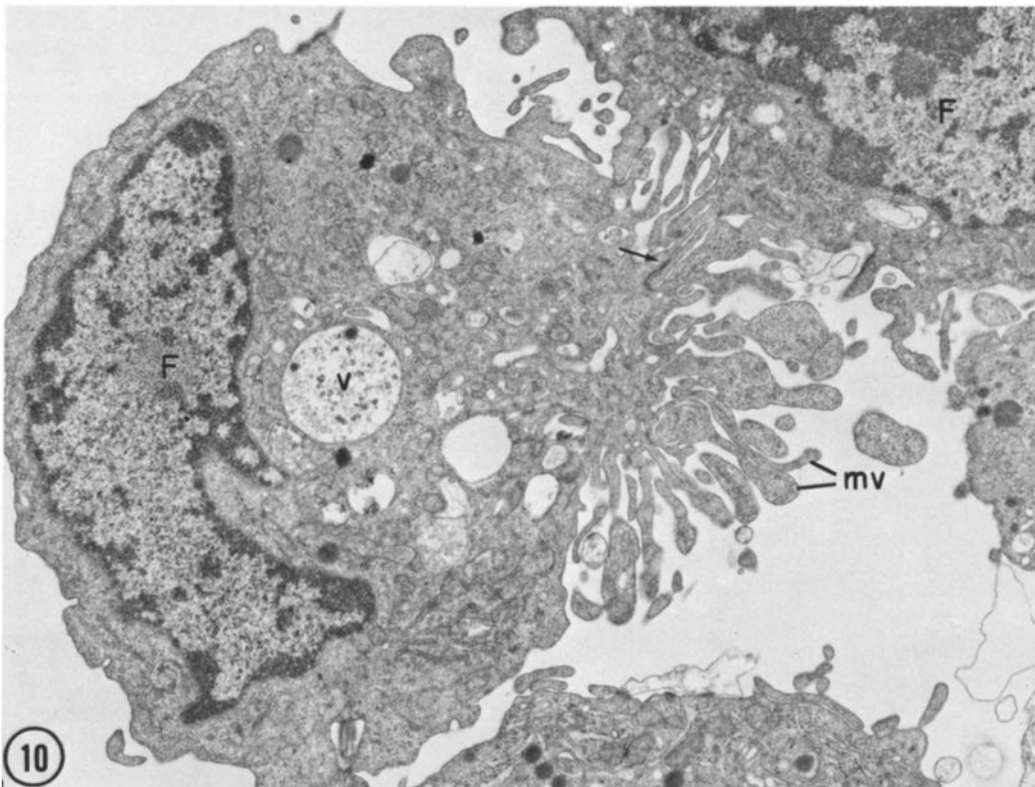
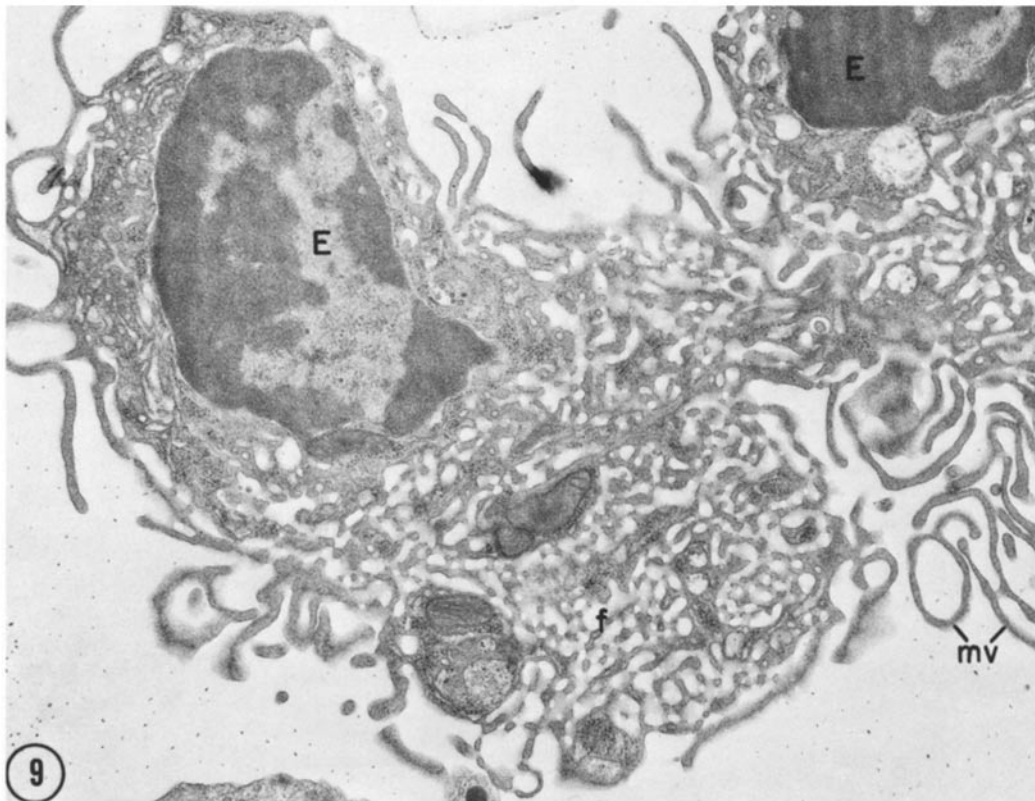
indicate that amino acids do not penetrate to the center of large tissue fragments (as indicated by decreasing grain concentration from periphery to center); hence more uniform accessibility to the tracer may be at least partly responsible for the higher rate of [<sup>3</sup>H]leucine incorporation into a single cell suspension as compared to that found in intact tissue blocks.

**INCORPORATION OF [<sup>3</sup>H]LEUCINE INTO STH:** To determine the ability of suspensions of pituitary cells to incorporate amino acids into a specific anterior pituitary secretory product, we compared the ability of recently dissociated cells, cultured cells, and tissue blocks to incorporate [<sup>3</sup>H]leucine into STH over a 4-h incubation period. Fig. 12 shows representative profiles of radioactivity found in the gels prepared from these specimens. The results obtained show that recently dissociated cells, cultured cells, and tissue blocks all incorporate [<sup>3</sup>H]leucine into STH. In accord with the work of others using hemipituitaries (26), two main peaks of radioactivity representing incorporation into STH and prolactin were found in tissue blocks. The proportion of total label incorporated into STH is about 25%. Recently dissociated cells differ from tissue blocks, in that they incorporate leucine into several major peaks in addition to those represented by these two hormones. As a result, the proportion of the total label incorporated into STH is reduced to 12–13%. However, in cultured cells, these additional peaks of radioactivity are greatly reduced, and the proportion of total label (~25%) incorporated into STH is similar to that found in tissue blocks. We have not examined further the additional labeled components elaborated in recently dissociated cells.

**AUTORADIOGRAPHY:** Having established that suspensions of pituitary cells are capable of incor-

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**FIGURES 7 and 8** A gonadotroph (*GT*) and a thyrotroph (*TT*) from a cell suspension fixed immediately after dissociation. Both cells appear intact and well-preserved. The cytoplasm of the gonadotroph, which is the largest cell in the pituitary, is asymmetrically arranged around the nucleus with the Golgi complex (*Go*) located on the side containing the more abundant amount of cytoplasm. This cell contains two types of secretion granules: one type (*sg*<sub>1</sub>), the more numerous, is relatively small (~200 nm) in size, and the other (*sg*<sub>2</sub>) of which there are fewer, are much larger in diameter (~400 nm). As seen here, the RER (*er*) of gonadotrophs is typically dilated and has a content of moderate density. The overall size of the thyrotroph is much smaller, the RER is more flattened, and the secretion granules are of a single type and smaller (~100–150 nm) than those of the gonadotroph. Note that after aldehyde fixation the shape of both types of gonadotroph granules and the thyrotroph granules is variable but usually oval in comparison to the regularly spherical somatotroph granules. × 9,600.



porating [ $^3\text{H}$ ]leucine into STH, we pulse-labeled the preparation and carried out electron microscope autoradiography. In this way it was possible to assess the cells' ability to transport and concentrate secretory products, and to determine the intracellular route taken by the newly synthesized protein over a 2-h period. For these experiments we used cultured cells, since our incorporation data (see above) indicated that the amount of [ $^3\text{H}$ ]leucine incorporated into STH per unit time in cultured cells is about twice that found in recently dissociated cells.

Immediately after a 5-min pulse with 500  $\mu\text{Ci/ml}$  [ $^3\text{H}$ ]leucine or after the various chase intervals, exposed grains are present over  $\sim 50\%$  of the cells in the preparation as seen by light microscopy (cf. Fig. 13). By electron microscopy only about half of the somatotrophs are heavily labeled (Fig. 14). A few of the unlabeled cells appear vacuolated, but the morphology of most is not obviously different from that of labeled cells. In order to rule out the possibility that this heterogeneity of uptake could be related to either (a) death of cells after prolonged incubation or (b) a transient asynchrony of the incorporation process, we incubated both recently dissociated and cultured cells for longer periods (up to 4 h) in [ $^3\text{H}$ ]leucine. A similar heterogeneity of uptake (Fig. 13) and uneven distribution of label among somatotrophs was observed in all preparations. We also compared uptake by somatotrophs in cell suspensions to that by somatotrophs in tissue blocks. Interpretation of

grain distribution in the latter is complicated by the fact, already mentioned, that cells in tissue blocks do not have equal access to the labeled precursor, and there is a gradient of decreasing grain concentration from periphery to center. Nevertheless, a similar heterogeneity in amino acid uptake can be discerned among somatotrophs at the same level in undissociated pituitary blocks. It can be concluded that somatotrophs of young adult male rats normally vary in their ability to incorporate labeled precursors.

At the end of the 5-min [ $^3\text{H}$ ]leucine pulse, the majority of the autoradiographic grains are diffusely distributed over the nucleus and the cytoplasm of somatotrophs (Fig. 15). The cytoplasmic grains can be related primarily to the rough endoplasmic reticulum (RER). The level and distribution of the nuclear labeling does not change appreciably with time, but the pattern of cytoplasmic labeling does: At 15–30 min post pulse, there is a striking concentration of cytoplasmic grains over the Golgi apparatus (Figs. 14 and 16). At 60 min post pulse the proportion of grains over the Golgi is reduced, and an increased number of grains can be related to secretory granules. At 120 min post pulse the Golgi area is almost free of label, and the majority of the grains are related to secretory granules (Fig. 17). The distribution of grains gives an indication of the intracellular route followed by the pulse-labeled secretory product, and thus shows that somatotrophs in cell suspensions are capable of transporting newly synthesized

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FIGURES 9 and 10 These two figures illustrate the principal nonsecretory elements present in the anterior pituitary—endothelial cells (*E*) and follicular cells (*F*). Whereas secretory cells are rounded and have relatively smooth contours after dissociation, these cell types have highly irregular and elaborate contours with numerous microvillous processes (*mv*).

The *endothelium* of pituitary capillaries is of the fenestrated variety (78). *In situ*, cell organelles are concentrated in the perinuclear cytoplasm from which a peripheral layer extends to form a thin capillary lining layer, containing  $\sim 50$  nm fenestrae bridged by thin diaphragms. Fig. 9 shows that after dissociation, the thin peripheral layer retracts forming a large entangled, convoluted mass in which the characteristic fenestral organization (*f*) can still be recognized, but the material comprising the diaphragms appears to have been removed. The presence of fenestrae after enzyme treatment and dissociation with resultant detachment from the basement membrane, demonstrates the relative rigidity of these structures.

The *follicular cells* are unique pituitary constituents which *in situ* are arranged in groups forming the lining of small follicles or ductules and are attached to one another along their luminal surfaces by typical apical junctional complexes (cf reference 24). Fig. 10 shows that after dissociation, follicular cells can be easily distinguished from endothelial cells by their lack of fenestrae, peripheral convolutions which are typically broader and usually (but not always) confined to one pole of the cell—i.e., that side which *in situ* faces the follicular lumen. As seen here both follicular cells and endothelial cells frequently occur in groups in cell suspensions still attached to one another by remnants of occluding zonules (arrow). *v*, vacuole. Fig. 9,  $\times 20,000$ ; Fig. 10,  $\times 16,000$ .

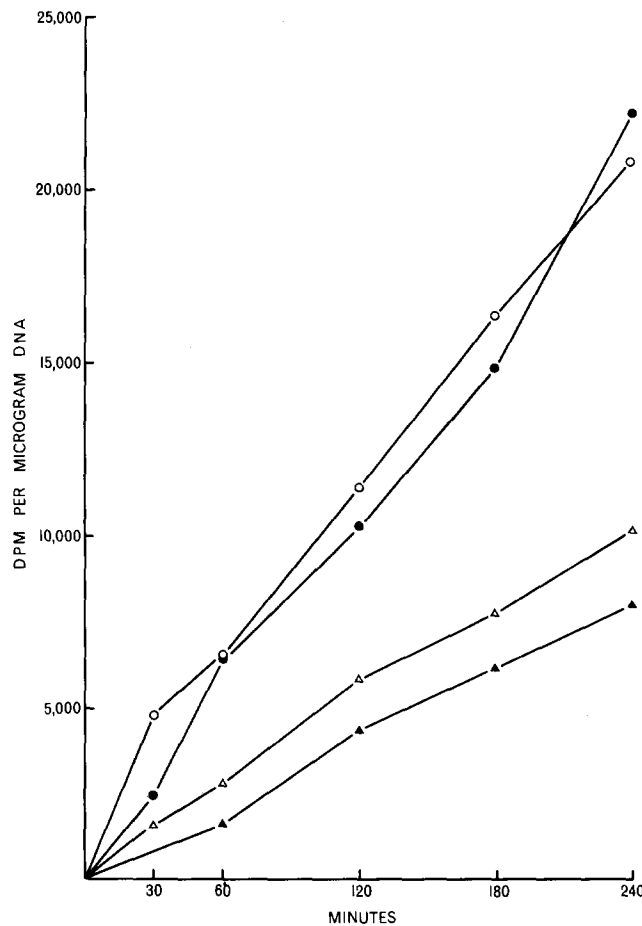


FIGURE 11 Time course of incorporation of [ $^3\text{H}$ ]leucine into total protein in acutely dissociated cells ○—○, cells dissociated and cultured 15 h, ●—●, small tissue blocks △—△; and hemipituitaries ▲—▲. The incubation medium contained 10  $\mu\text{Ci/ml}$  [ $^3\text{H}$ ]leucine (52 Ci/mmol).

secretory product from the RER to the Golgi apparatus where it is presumably packaged into secretory granules.

Labeling of the nucleus is a consistent finding in the somatotroph at all time points, but its precise metabolic significance is not clear. However, in general the relative numbers of grains found over the nucleus parallels that over the cytoplasm, so that significant nuclear label is not seen in the absence of cytoplasmic label and vice versa. Moreover, if protein synthesis is inhibited by preincubating dissociated cells for 5 min before the pulse and during the pulse with  $5 \times 10^{-4}$  M cycloheximide (29), nuclear labeling is abolished. Furthermore, nuclear label is seen in undissociated cells in tissue blocks; it is also present in blocks or cell suspensions regardless of whether  $\text{OsO}_4$  or

aldehyde primary fixation is employed. It is thus concluded that the nuclear label represents newly synthesized protein.

#### *Release of Secretory Product*

The data shown in Table I, indicate that recently dissociated cells, incubated in the presence of [ $^3\text{H}$ ]leucine release spontaneously 12.5% of their total labeled STH into the medium during a 6-h incubation period. Addition of 57 mM  $\text{K}^+$  or 5 mM dibutyryl cyclic AMP, both of which are potent secretagogues for STH release (26, 28, 30), does not increase significantly the amount of labeled STH released. In cells which had been cultured for 15 h and prelabeled, however, significant increases in the amounts of labeled STH re-

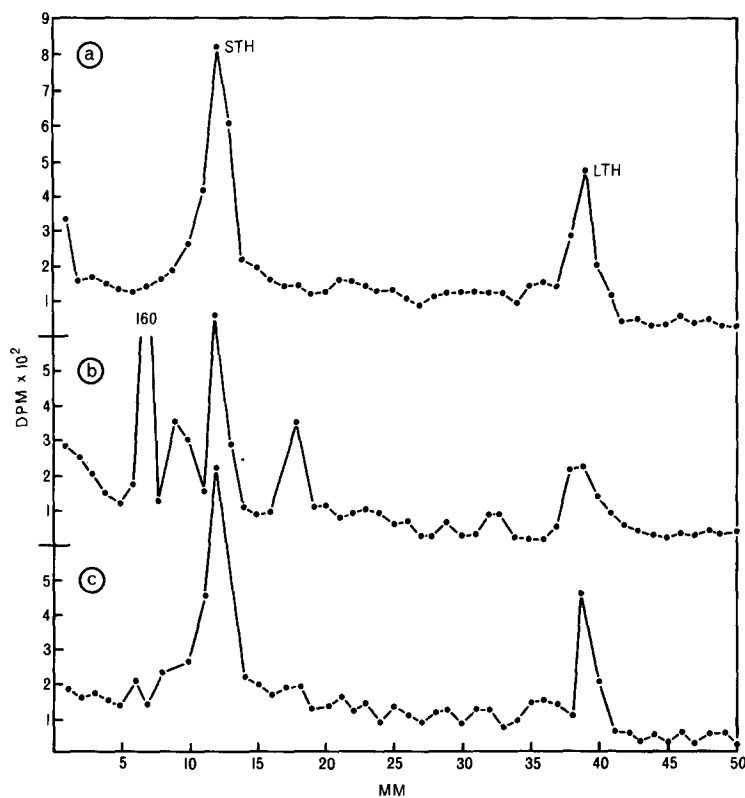


FIGURE 12 Polyacrylamide gel electrophoresis of proteins containing incorporated [ $^3\text{H}$ ]leucine derived from EDTA extracts of (a) tissue blocks, (b) recently dissociated cells, and (c) cells cultured 15 h. 4-h incubations in media containing  $10\ \mu\text{Ci/ml}$  [ $^3\text{H}$ ]leucine were followed by extraction in  $1\ \text{mM}$  EDTA, removal of free [ $^3\text{H}$ ]leucine on G25 Sephadex and electrophoresis on 10% slab gels at pH 9.5. Duplicate aliquots of the load samples contained  $\sim 50,000$  dpm. Growth hormone (STH) and prolactin (LTH) were identified using NIAMD rat hormone standards. Top of gel to the left. Starting gel 1 cm long. 0 mm on axis indicates beginning of separation gel.

leased were observed in the presence of both high  $\text{K}^+$  and dibutyryl cyclic AMP during a 3-h period. The data shown in Table II indicate that the amount of STH released by controls represents 11% of the total labeled STH synthesized during the incubation period. No difference was observed in the amount released by cells incubated in medium containing  $0.1\ \text{mM}$  dibutyryl cyclic AMP, whereas those incubated with  $57\ \text{mM}$   $\text{K}^+$  or  $0.5\ \text{mM}$  dibutyryl cyclic AMP released 24% and 22%, respectively, of the total labeled STH synthesized. The 4% of the labeled STH released at  $5^\circ\text{C}$  probably represents uncontrolled leakage from damaged cells. The most pronounced stimulation occurred in cells incubated with  $5\ \text{mM}$  dibutyryl cyclic AMP, since 52% of the total labeled STH was released during the 3-h period. This represents a stimulation of  $>400\%$  of the basal release at  $37^\circ\text{C}$ . In hemipituitaries incubated in vitro cyclic

AMP is known to stimulate both STH synthesis and its release (26). We have not evaluated the effect of cyclic AMP on biosynthesis of STH in dissociated somatotrophs quantitatively, but their morphology suggests a high rate of production as well as release of secretory product, since the number of secretory granules per cell is reduced and the Golgi apparatus is enlarged (Fig. 19). Similar findings have been reported by Pelletier et al. (31) for somatotrophs in hemipituitaries incubated with cyclic AMP in vitro.

#### DISCUSSION

The dissociation procedure for anterior pituitary tissue developed during the present study is rapid and, in terms of cell yield, it is more efficient in our hands than those previously available. It has the advantage that only specific agents of high purity are employed so that reproducibility is ensured,



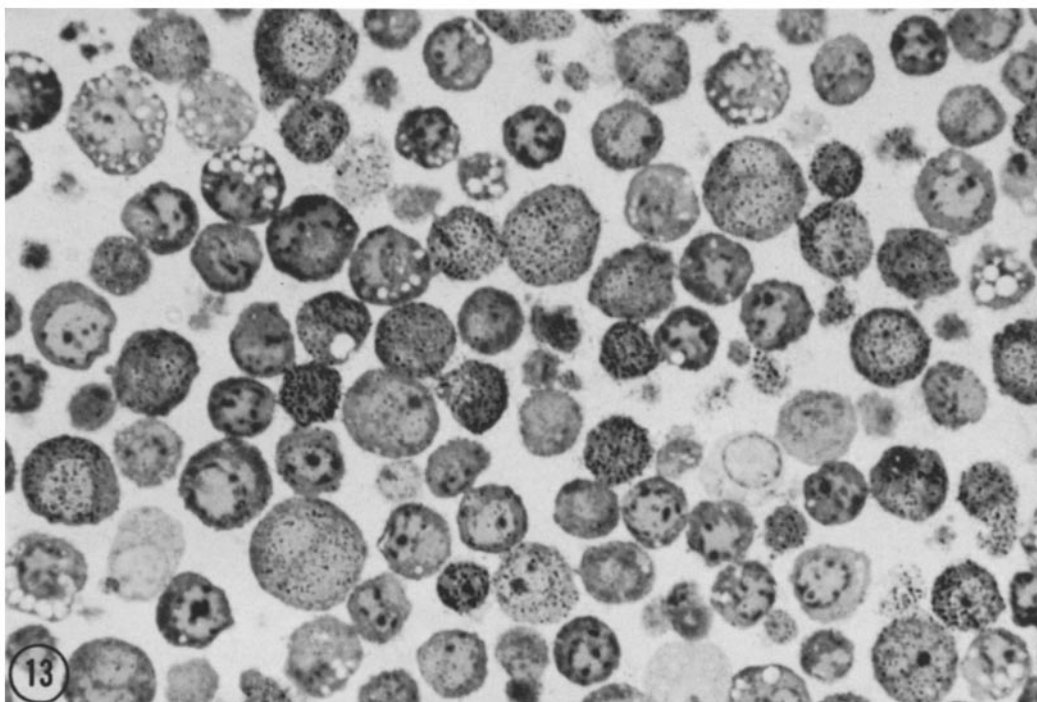


FIGURE 13 Light micrograph showing distribution of autoradiographic grains over a suspension of pituitary cells incubated continuously for 4 h with [ $^3\text{H}$ ]leucine (100  $\mu\text{Ci/ml}$ ). Approximately 50% of the cells show moderate to heavy concentrations of autoradiographic grains, whereas the remainder are unlabeled or sparsely labeled. A small percentage of the unlabeled cells are vacuolated, but most do not appear different from labeled cells.  $\times 600$ .

and the specific effects of these agents (e.g., on the cell surface) are known. Most importantly, it provides a suspension of single cells which are morphologically and functionally intact, and are therefore suitable for combined morphological and biochemical studies on pituitary cell types.<sup>4</sup>

#### *Cell Dissociation Procedures*

A number of successful attempts have been made recently to produce viable single cell suspensions from a variety of tissues (e.g., liver [32, 33], thyroid [34], adrenal [5, 35, 36], corpus luteum [37], and exocrine pancreas [38]), including the anterior pituitary (2-6, 39-41). Most of these procedures have involved the use of collagenase, hyaluronidase, trypsin, pronase, EDTA,

and calcium-free media, alone or in various combinations. To our knowledge, neuraminidase has not been used previously as a dissociating agent.

Our finding that an initial protease treatment is essential for cell dissociation is in agreement with the recent study of Amsterdam and Jamieson (38). Crude proteolytic enzyme extracts or proteases other than trypsin (e.g., Viokase [6], pronase [40]) have been used for pituitary cell dispersion, but none has the dual advantage of being well characterized and of having available a selective inhibitor (such as SBTI) which is effective under physiological conditions. A number of reports have emphasized the importance of specifically inhibiting protease activity, since these enzymes, which are known to adsorb to the cell surface and survive conventional washing procedures (42-44), can cause injury to the cell surface and prevent reappearance of the cell coat material removed by the enzyme action even at very low concentrations (42, 44). However, when protease treatment is

<sup>4</sup> Recent unpublished work by one of us (CRH) has shown that cells cultured 15 h are also capable of responding to hypothalamic releasing factors (LRF).

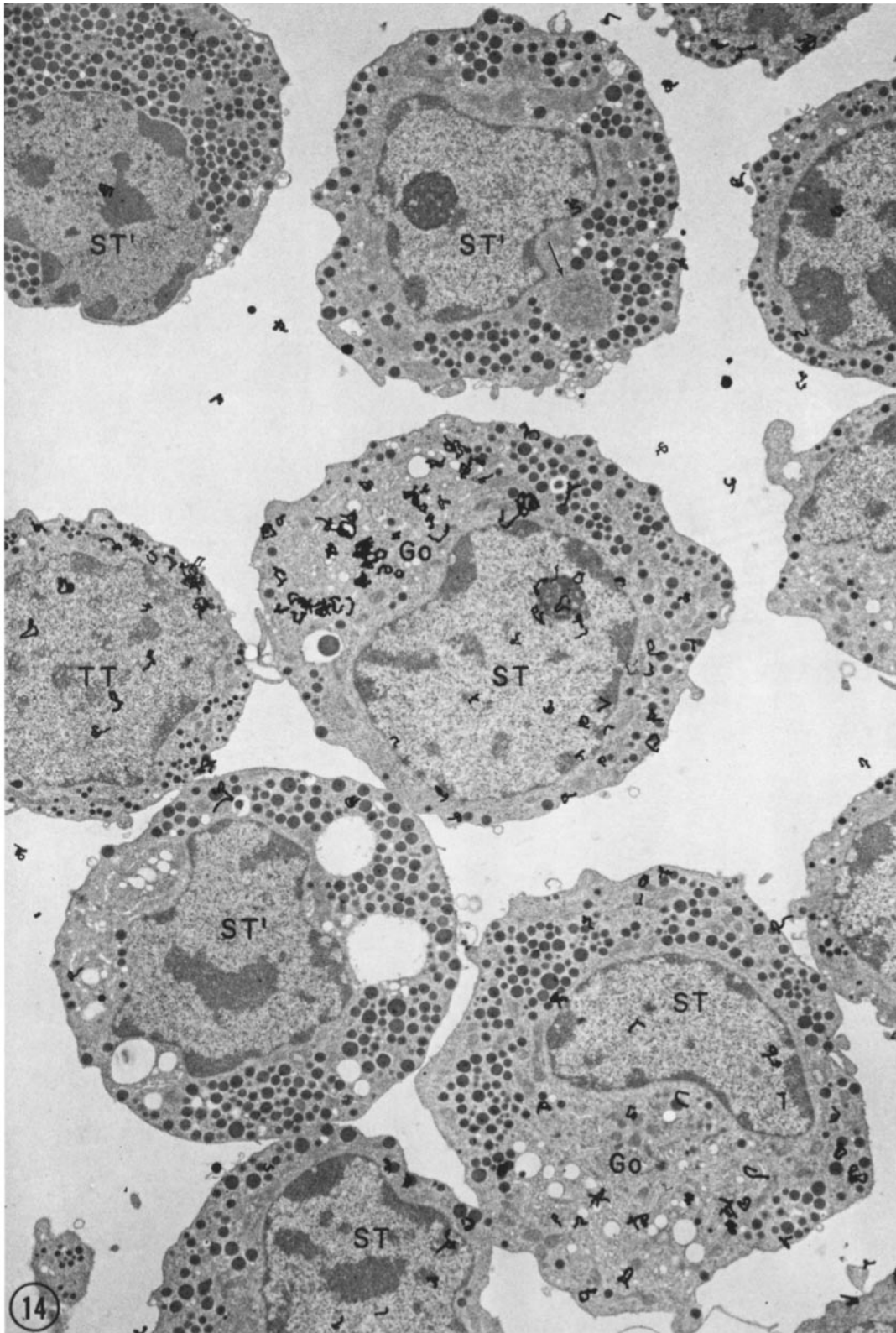


FIGURE 14 Electron microscopic autoradiogram showing a group of somatotrophs cultured for 15 h and given a 5-min pulse with [ $^3\text{H}$ ]leucine, followed by a 15-min chase incubation. Three of the somatotrophs (*ST*) show a heavy concentration of grains, primarily over the Golgi (*Go*) region, whereas three others (*ST'*) show very few grains. Except for one cell on the lower left which is vacuolated, the unlabeled cells do not show any obvious differences from labeled cells.  $\times 7,200$ .

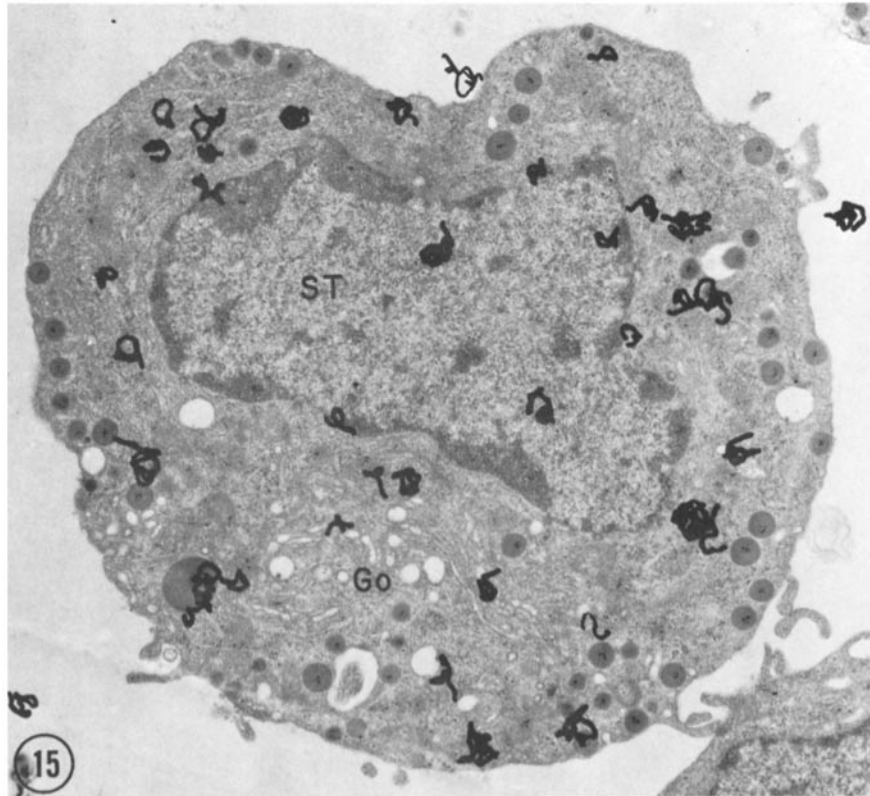


FIGURE 15 Autoradiogram of a somatotroph (ST), cultured for 15 h after dissociation and given a 5-min leucine pulse, followed by a 1-min chase incubation. The radioautographic grains are rather evenly distributed over nucleus and cytoplasm with the majority being associated with the rough ER. Go, Golgi region.  $\times 13,000$ .

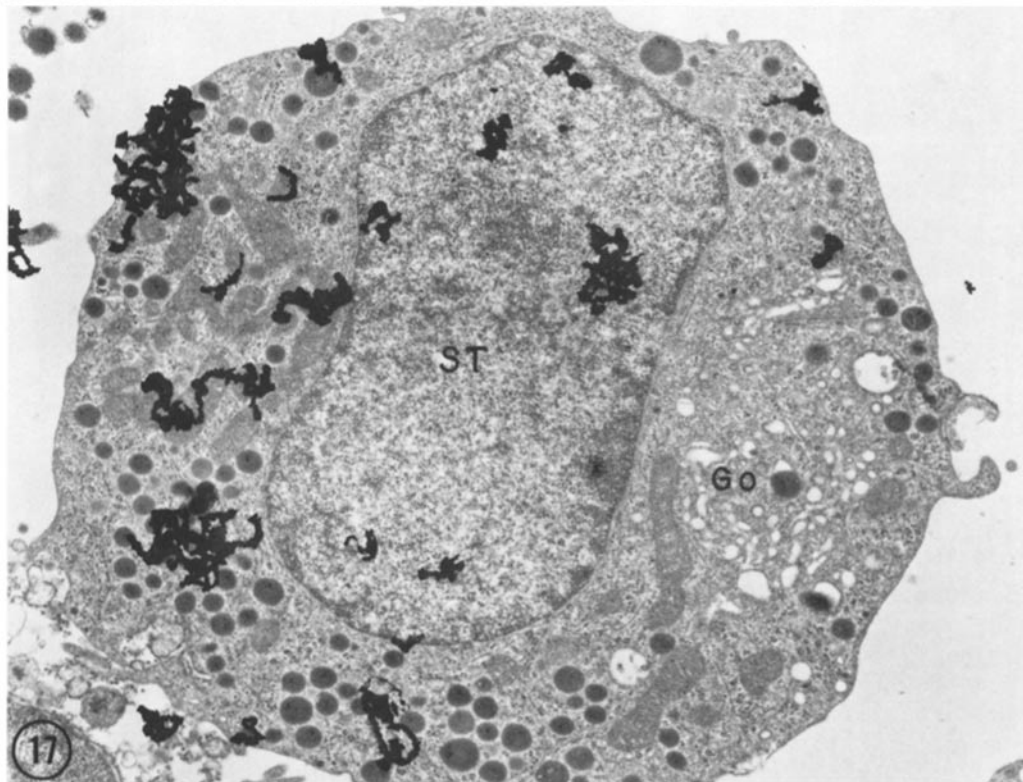
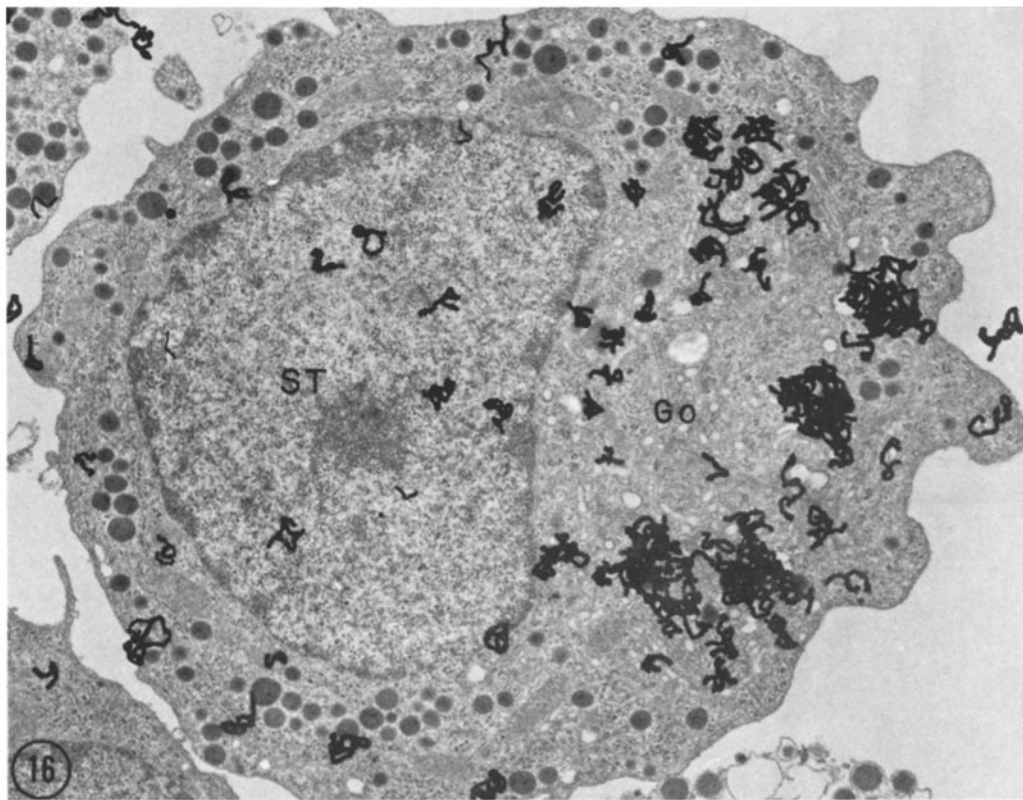
brief and efficiently terminated with appropriate inhibitors, removal of cell coat material is reduced and resynthesis and replacement of these cell surface components occurs within hours (45-47).

The probable efficacy of neuraminidase as a dissociating agent was suggested by indications that sialic acid groups are involved in cell adhesion (48, 49), and reports that the specific enzymic removal of these groups decreases cell aggregation (50, 51). The rationale behind the procedure we have designed is based upon the knowledge that anterior pituitary cells have few or at least limited areas devoted to intercellular junctions of any kind<sup>5</sup> and a limited connective tissue stroma, so

<sup>5</sup> In comparison to the situation encountered in the liver, exocrine pancreas, thyroid, intestine, and other lumen-lining epithelia, the total area of cell surfaces devoted to junctions is much more limited in anterior pituitary cells. Only shallow gap junctions and occasional desmosomes connect secretory ele-

ments to one another (or secretory to nonsecretory elements). The only other junctions encountered are those present between nonsecretory elements (follicular cells and endothelial cells).

that cell adhesion must depend primarily on properties of the cell coat, or more precisely, on the surface glycoproteins. Accordingly, cell dissociation should be promoted by removal of surface glycopeptides and most, if not all, of the sialic acid groups. Quantitative results on other cell types (52-54) suggest that removal of the latter can be accomplished either by prolonged tryptic digestion or brief, sequential trypsin-neuraminidase treatment. The results we obtained on pituitary cells indicated that although neuraminidase treatment alone is not effective for dissociation, a brief sequential incubation in trypsin and neuraminidase enhanced cell yield by 20% (over that obtained with trypsin alone) and allowed the trypsin incubation to be reduced from 90 to 15 min. At the same



FIGURES 16 and 17 Autoradiograms of two somatotrophs prepared and pulsed as in Fig. 15, and fixed after a 15-min (Fig. 16) or 120-min (Fig. 17) chase incubation. Note that after a 15-min chase most of the autoradiographic grains are concentrated over the Golgi region (*Go*) whereas after a 120-min chase most are concentrated over the secretion granules, and the Golgi region is free of grains. At both chase intervals, and indeed at all intervals studied, there is considerable nuclear label which consistently accounts for 15-20% of the total grains. Fig. 16,  $\times 12,000$ ; Fig. 17,  $\times 14,000$ .

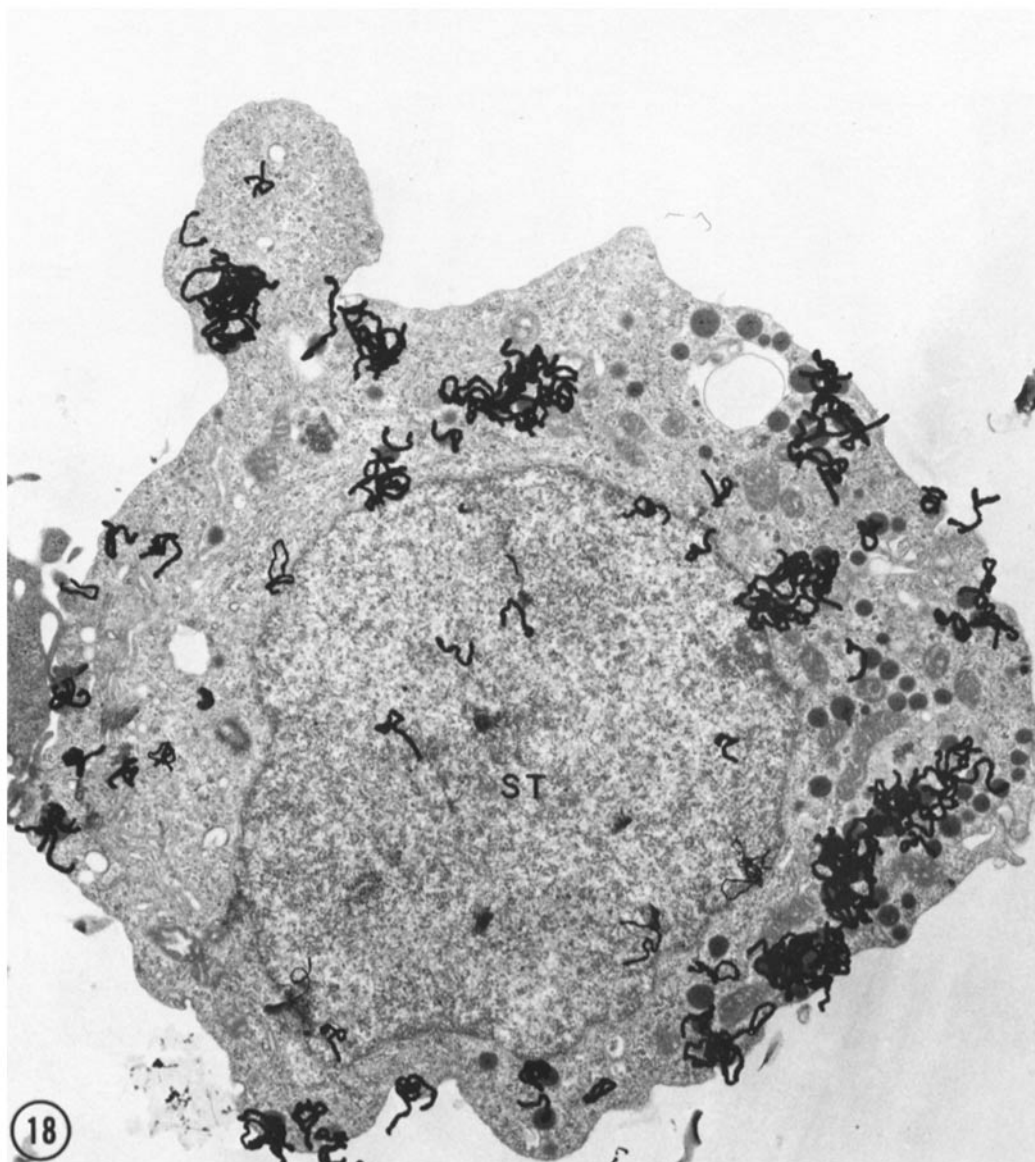


FIGURE 18 Autoradiogram of a cell incubated overnight in the presence of [ $^3\text{H}$ ]leucine ( $10\ \mu\text{Ci/ml}$ ) to show distribution of grains over the "prelabeled" cells which served as starting material for studies on growth hormone discharge (Table II). Heavy concentrations of grains occur over secretion granules.  $\times 12,000$ .

time preservation of the fine structure was similar to that obtained with trypsin alone or even somewhat better.

It should be noted, however, that neuraminidase proved to be cytotoxic for pituitary cells at high concentrations ( $16\ \mu\text{g/ml}$ ) or after prolonged incubation. Furthermore, even at low concentrations and short intervals of exposure this enzyme is

known to affect a number of important reactions at the cell surface (55-59), including the damage of specific receptor sites in fat cells (54, 60). However, it has been reported that cultured cells (60) and mouse tumor cells (61) treated with neuraminidase rapidly (i.e., within 6-12 h) replace the sialic acid removed. It is further known that L-glutamine, an essential precursor in the syn-

TABLE I  
Effect of High  $K^+$  and Dibutyryl cAMP on Release of [ $^3H$ ]STH from Acutely Dissociated Cells

	Cell content*	Medium content*	% total [ $^3H$ ]STH released
KRB Control	142,547 $\pm$ 7,127	20,363 $\pm$ 509	12.5
57 mM $K^+$	145,378 $\pm$ 10,176	21,723 $\pm$ 1,303	13
5.0 mM Db cAMP	142,213 $\pm$ 15,643	27,088 $\pm$ 1,083	16

Cell suspensions were incubated 6 h with 10  $\mu$ Ci/ml [ $^3H$ ]leucine, and STH was isolated using gel electrophoresis as described in Materials and Methods. Data derived from five experiments. Values are mean  $\pm$  SE.

\* dpm  $^3H$ -labeled STH/ $10^6$  cells

TABLE II  
Effect of High  $K^+$  and Dibutyryl cAMP on Release of [ $^3H$ ]STH from Cells Cultured 15 h

	Cell content*	Medium content*	% total [ $^3H$ ]STH released
KRB Control	148,970 $\pm$ 7,448	17,871 $\pm$ 893	11
KRB at 5°C	186,933 $\pm$ 17,863	7,788 $\pm$ 389	4
57 mM $K^+$	138,520 $\pm$ 16,622	33,245 $\pm$ 4,986	24
0.1 mM Db cAMP	164,520 $\pm$ 18,040	20,500 $\pm$ 1,845	11
0.5 mM Db cAMP	140,383 $\pm$ 7,019	39,595 $\pm$ 2,373	22
5.0 mM Db cAMP	93,951 $\pm$ 4,697	101,780 $\pm$ 13,702	52

Cell suspension preincubated for 15 h, and for the last 10 h in the presence of 100  $\mu$ Ci/ml [ $^3H$ ]leucine. The cells were then washed, incubated for 3 h as shown above and the STH isolated by gel electrophoresis as described in Materials and Methods. Data derived from three experiments. Values are means  $\pm$  SE.

\* dpm  $^3H$ -labeled STH/ $10^6$  cells

thesis of complex carbohydrates (62), is required for this process to take place. The failure of dispersed pituitary cells to respond to secretagogues immediately after enzyme treatment and their recovery or partial response after an overnight incubation could be explained on the basis of damage to the cell surface, followed by the regeneration of the required surface components during the succeeding 15-h incubation period.

Like neuraminidase, EDTA treatment for long periods or at supraoptimal concentrations is cytotoxic. In our experience, exposure for more than 45 min to EDTA or calcium-free media causes swelling and eventual lysis of dispersed cells. Since reduction in the divalent cation concentration has been found to be essential for obtaining suspensions of single cells, we have included limited exposure to EDTA at the lowest effective

concentration. In previous in vitro studies on pituitary tissue, the short-term effects of exposure to divalent cation-free media have been shown to be rapidly reversible when the tissue is returned to medium containing calcium and magnesium (63), and our observations confirm this finding.

#### Secretory Functions in Dissociated Pituitary Cells

According to our biochemical results, dispersed pituitary cells, like their undispersed counterparts, (a) linearly incorporate amino acids into proteins, (b) actively incorporate amino acids into two secretory proteins, STH and prolactin, and (c) release newly synthesized, labeled secretory products into the incubation medium. Beyond this, our autoradiographic results indicate they are capable

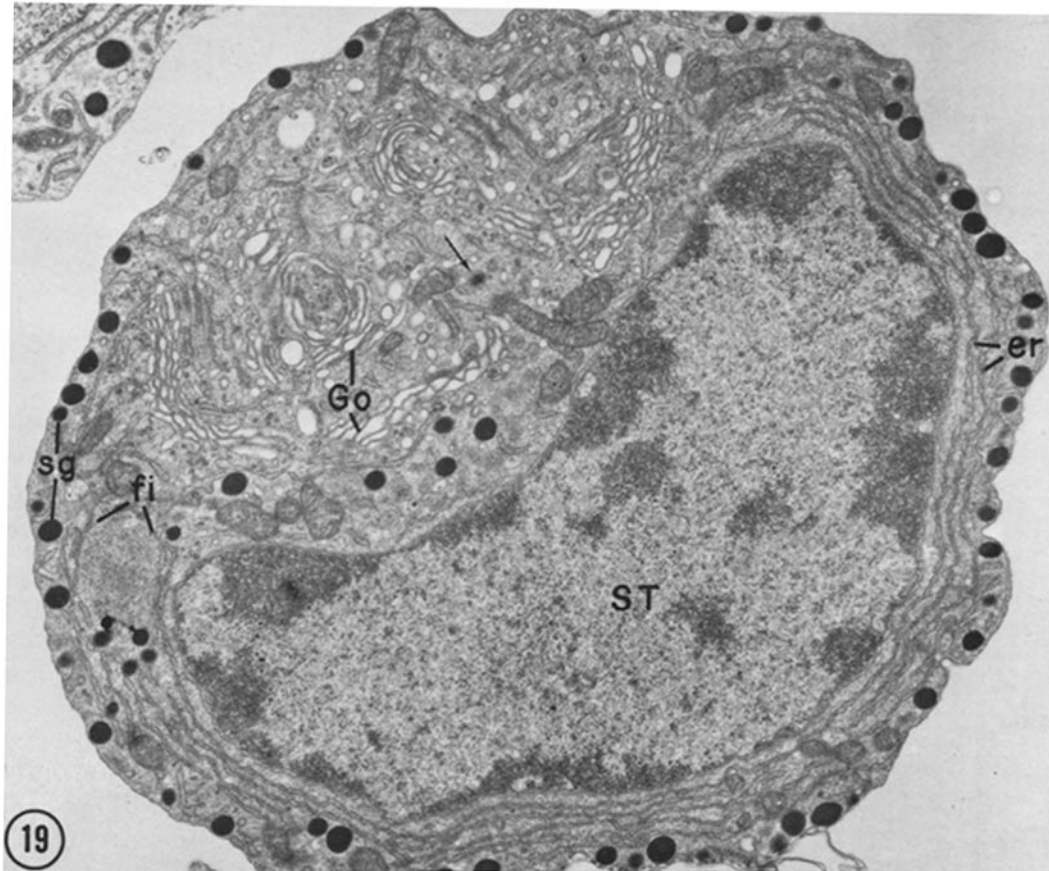


FIGURE 19 Somatotroph from a preparation of cells preincubated overnight in KRB/BSA, followed by 3-h incubation in KRB/BSA plus 5 mM dibutyryl cyclic AMP. The secretion granules (*sg*) are largely depleted with only a few remaining located mainly lined up along the cell membrane. The RER (*er*) is arranged in parallel rows to one side of the nucleus. The Golgi apparatus (*Go*) is quite large and consists of elaborate stacks of dilated cisternae which appear to push the nucleus to one side. Note, however, that although the Golgi apparatus appears enlarged, there is no evidence of secretory material condensing within it, and with one exception (arrow) no small immature granules are seen in the cytoplasm. The absence of such images suggests that concentration may be impaired or omitted in these stimulated cells which are known to be producing and releasing increased amounts of growth hormone. A spherical collection of finely fibrillar material (*fi*) surrounded by rough ER is present at one pole of the nucleus.  $\times 13,000$ .

of transporting newly synthesized, labeled secretory protein from RER  $\rightarrow$  Golgi  $\rightarrow$  granules. We found, however, that there are some differences in the levels at which these operations are carried out by dissociated cells vs. tissue blocks and by acutely dissociated cells vs. cells cultured 15 h. There are two aberrations in acutely dissociated cells which render them undesirable for many studies. One is the fact that the proportion of newly synthesized protein represented by secretory product is reduced, and the other is the fact that their ability to

respond to secretagogues (high  $K^+$ , cyclic AMP) by increased release of STH is impaired. The simplest interpretation of these results is that a period of recovery is necessary for regeneration or repair of cell surface components which were adversely affected by the dissociation procedure, and that the additional proteins synthesized by acutely dissociated cells are related to these repair processes.

The series of secretory operations which are carried out by dispersed cells which have been

allowed to recover for 6–15 h are very similar to those of intact tissue blocks, except that the rate of amino acid incorporation into dispersed pituitary cells (whether recently dissociated or cultured 15 h) is greater than that of tissue blocks. Similar increased rates of amino acid incorporation have been obtained in single cell suspensions of other tissues (33, 38). The general conclusion in all these studies, including ours, has been that the increased incorporation rates reflect increased accessibility of cells to precursors. However, a recent report by Schreiber and Schreiber (64) raises the possibility, which must be considered, that the increased uptake may be due to the fact that the dissociation procedure temporarily destroys the active transport system for amino acids.

High  $K^+$  and cyclic AMP are known to stimulate the release of STH (28, 65) as well as other anterior pituitary hormones (1, 31) from pituitary tissue incubated *in vitro*. Our data clearly demonstrate that high (57 mM)  $K^+$  and dibutyryl cyclic AMP likewise significantly increase the rate of secretory product released from dissociated somatotrophs, provided the cells have been cultured for 15 h. Furthermore, the fine structural organization (i.e., abundant RER and enlarged Golgi apparatus) of somatotrophs stimulated for 3 h by incubation with 5 mM dibutyryl cyclic AMP is similar to that recently described by Pelletier et al. (31) in somatotrophs of hemipituitaries incubated in monobutyryl cyclic AMP for similar periods.

The intracellular pathway followed by newly synthesized secretory product in dissociated somatotrophs is essentially the same as that described in other protein secreting cells, especially those of the exocrine pancreas (66, 67), parotid (68), pituitary pars intermedia (69), beta cells of the pancreatic islets (70), and prolactin cells (mammotrophs) of the anterior pituitary (71, 72). The only information available on the timetable of events in the secretory process of somatotrophs from intact tissue comes from a brief paper by Racadot et al. (73) who carried out an *in vivo* autoradiographic study, and from a recent abstract by Howell (74) who used combined cell fractionation and autoradiography to investigate growth hormone secretion by pituitary tissue incubated *in vitro*. In general the present results on dispersed somatotrophs are similar to those obtained previously in undissociated tissue and the more detailed data available on the other secretory systems cited above. In all cases there is relatively rapid (5–15 min) transport of secretory product to the Golgi

apparatus followed by less rapid (20–60 min) transport to secretory granules. Thus the time interval needed for traversal of the intracellular secretory pathway indicated by autoradiography of dissociated somatotrophs is in agreement with estimates of 2 h derived by following the appearance of labeled newly synthesized STH in the medium (26) in which hemipituitaries are incubated.

#### *Previous Work on Dispersed Pituitary Cells*

Several other workers have recognized the desirability of having available a suspension of dispersed pituitary cells for studies on pituitary secretion and have attempted to produce such a system. Ishikawa (39) and Portanova et al. (2) used prolonged incubation in trypsin for pituitary dissociation. These latter workers established that such trypsin-dissociated cells retain their ability to discharge ACTH after treatment with hypothalamic extracts. Thus they were the first to investigate the ability of acutely dissociated cells to respond to secretagogues. Later with Malamed (75), they established that such cells maintain their structural integrity when viewed with the electron microscope. Ishikawa (39) and Hymer et al. (3, 41, 76) used such trypsin-dissociated cell suspensions as starting material for attempts to separate pituitary cell types. Bala and co-workers (4) and Kudo et al. (7) used collagenase (according to Rodbell's (77) method for adipose tissue); and Vale et al. (6), a combination of collagenase, hyaluronidase, and pancreatic enzymes, for pituitary dissociation. Finally Leavitt et al. (40) utilized pronase as a dissociating agent and studied estrogen uptake and binding to the resultant dispersed pituitary cells. All these efforts, except those of Hymer et al. (41), were hampered by low cell yields and variable or limited responses to secretory stimuli. Vale et al. (6), like us, found that recently dissociated cells responded poorly to a number of stimuli and that a longer period of culture (>72 h) (during which the cells became firmly attached to the culture dish) was necessary to obtain a response to various secretagogues, including hypothalamic releasing factors.

It is very difficult to compare our results directly with those obtained by others, since in most cases the data presented (e.g., yield and monitoring procedures) are incomplete, and none of the previous papers has provided such a thorough combined structural and functional analysis of the resultant cell suspension or has attempted to compare the



suspensions obtained to undissociated pituitary tissue. What we can say is that the procedure given here represents the most successful in our hands of the methods presently available for producing a suspension of pituitary cells. Furthermore, our results together with the combined results and efforts of all the above workers indicate the great promise of this approach for the study of mechanisms of pituitary secretion.

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