Separation of Somatotrophs from the Rat Adenohypophysis by Velocity and Density Gradient Centrifugation[1] (36912)

W. C. HYMER,[2] J. KRAICER, S. A. BENCOSME, AND J. S. HASKILL
(Introduced by Nathan Kaufman)

Departments of Physiology and Pathology, Queen's University, Kingston, Ontario, Canada

Cells dissociated from the rat adenohypophysis retain their characteristic morphology at the level of both the light (1) and electron (2, 3) microscope. Recently some progress has been made toward the separation of the different types of cells contained in these suspensions. Ishikawa (3) reported that centrifugation of adenohypophysial cells through discontinuous gradients of dextran A yielded chromophobe and somatotroph-basophil fractions as judged by light and electron microscopy. In a quantitative study, we showed that fractions enriched in chromophobes (60%) or somatotrophs (60%) could be obtained by sedimentation of the cells through a shallow (0.3–2.4%) bovine serum albumin (BSA) gradient maintained at unit gravity (1). Cells separated by this technique incorporated ^{14}C-amino acid into protein at a rate comparable to that of the cells in the intact tissue.

A combination of the equilibrium density gradient centrifugation and unit gravity sedimentation separation methods has proven useful in the isolation of antibody-forming cell precursors (12). In this report we show that cell populations consisting of over 90% somatotrophs can be isolated by sequential velocity and density-gradient centrifugation. The results also show that the separated somatotrophs remain morphologically and functionally intact. Preliminary reports of this method have appeared in abstract form (4).

Materials and Methods. Cell dispersion. For each experiment, cells in minced adenohypophysial tissues obtained from 10 male rats (Sprague-Dawley; 250–300 g) were dissociated by procedures previously described (1). In this method, dispersion was carried out in a 25-ml spinner suspension flask (Belco Glass Company) equipped with a Teflon impeller. A siliconized flask containing 10 ml of a trypsin BSA solution (1/10% trypsin (Difco 1:250) and 1/10% bovine serum albumin BSA (Nutritional Biochemicals, Fraction V)) was prepared in Spinner's Minimum Essential Medium (Grand Island Biological Corporation), buffered with 5.6% sodium bicarbonate to pH 7.3. The solution described above was partially immersed in a 37° water bath. Tissue fragments were kept in constant agitation by the impeller using a water stirrer, approximate impeller speed of 200 rpm. The contents of the flask were gassed with a 95% oxygen–5% CO_2 mixture at 1.5 ft.^{3}/hr. The trypsin solution was not changed during the 2-hr association period. To further aid the dispersion, the pieces were expelled through a Pasteur pipette 20–30 times every one-half hour. Virtually no tissue fragments were seen after the 2-hr period. The final pH of the suspension was 7.3–7.4. Cell yield was 2.0–2.8 $\times$ 10^6 cells from each gland; as before, this recovery accounts for approximately 70–80% of the cells in the tissue (1).

Velocity sedimentation. The unit gravity separation technique for adenohypophysial cells has been described (1). Basically the method involves sedimentation of cells through a shallow (0.3–2.4%) gradient of bovine serum albumin (Nutritional Biochemicals Co., Fraction V) BSA prepared in Medium 199 at pH 7.4. Sedimentation was carried out

[1] Supported in part by grants from the Medical Research Council, National Cancer Institute of Canada, J. P. Bickell Foundation, National Science Foundation, Abbott Laboratories, and PHS research career development award (1-K04-AM-15808;WCH).

[2] Address reprint requests to: Dept. of Biology, Penn State Univ., Univ. Park, PA.

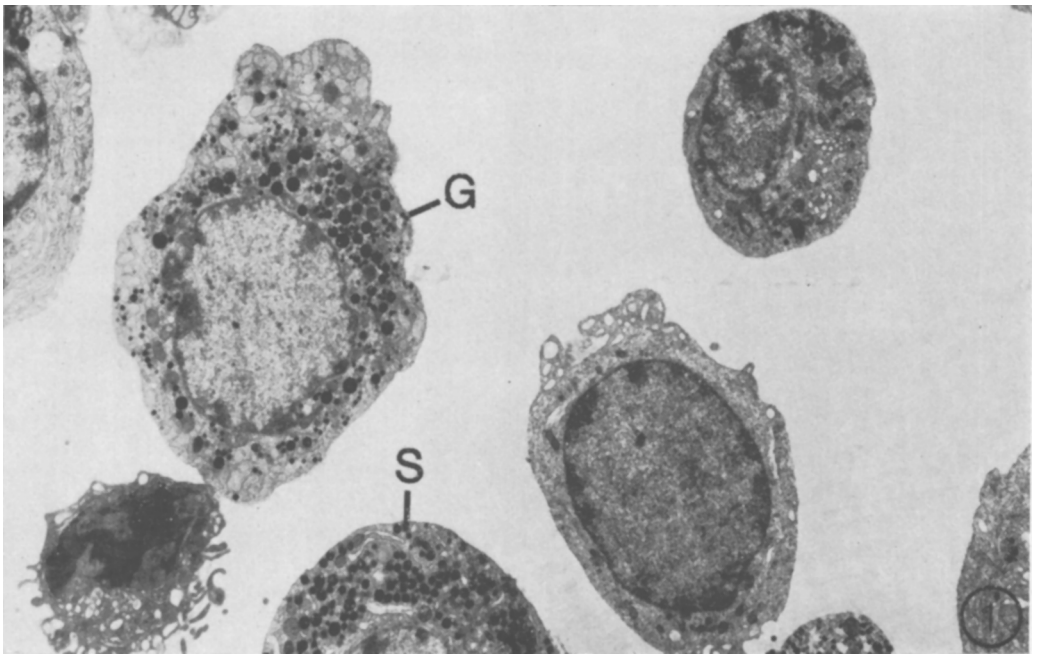

FIG. 1. Electron micrograph of dissociated rat adenohypophysial cells prior to separation by velocity and density gradient centrifugation. Cell types are identified on the basis of secretion granule size. Somatotroph (S), gonadotroph (G). (× 4,000).

for 1.25 hr at 25°. The sedimentation chamber, made of lucite, had an external diameter of 10.8 cm and a height of 11 cm (1). The total capacity of the chamber was 500 ml. Cells were loaded at 1.3 × 10^6/ml and no more than 20 × 10^6 cells were used in any one experiment.

Density gradient centrifugation. Cells recovered from appropriate regions of the velocity sedimentation gradient were pooled, concentrated by centrifugation (700*g*, 15 min), and resuspended in 0.2 ml of pH 7.3 phosphate-buffered saline (PBS) containing 0.1% BSA (Nutritional Biochemicals, Fraction V). These cells were mixed with 6.3 ml of concentrated BSA (~28%, prepared in the isotonic UBSS buffer of Shortman) and were stirred in the mixing chamber. The techniques for preparation and generation of the 12-ml linear BSA gradient (14–28%), centrifugation and subsequent sampling were all carried out exactly as described in Shortman's report (5). Recovery of cells from the gradient was 80–100%. In each experiment, linearity of the gradient was confirmed by refractive index measurements of each cell fraction (~ 0.7 ml). All of the above procedures were carried out at 4°. Cell fractions were diluted (10–15×) with PBS containing 0.1% BSA, centrifuged, and the cell pellets resuspended in either PBS + 0.1% BSA or Medium 199 + 0.1% BSA (both at pH 7.3) prior to microscopic study.

Microscopy. In all experiments, aliquots of cell suspensions at different stages of the separation procedures were prepared for both light and electron microscopic study. Cells were prepared for light microscopy by centrifugation onto microscope slides (Shandon cytocentrifuge); fixation in Bouin-Hollande sublimate, and subsequent staining with Herlant's tetrachrome. These procedures, as well as the criteria used in making the differential cell counts, have been described (1).

For electron microscopy, 0.2 ml of 3% gluteraldehyde prepared in cacodylate buffer (pH 7.4) was added to 0.2 ml of cell suspension. After 15 min the cells were concentrated by centrifugation and processed for electron microscopy according to a fast method (6). To evaluate the quality of the isolated cells, 1 μ sections stained with toluidine blue were

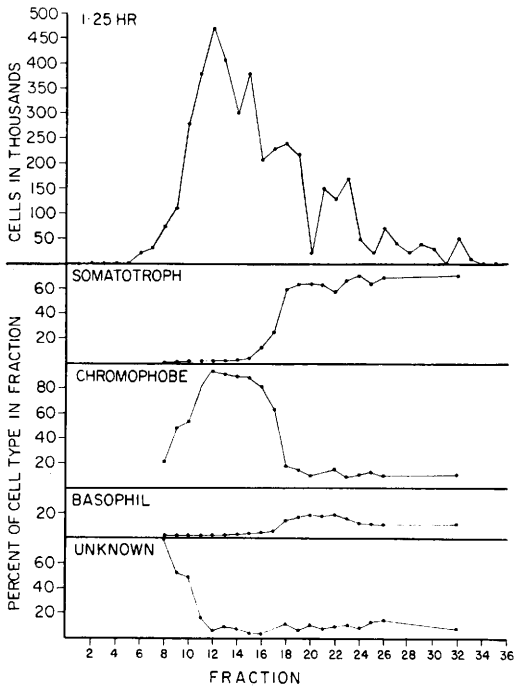

FIG. 2. Top panel: Distribution profile of separated pars distalis cells after 1.25 hr sedimentation at unit gravity. Lower panels: Localization of specific cell types expressed as percentage of cell type in consecutive 10-ml fractions.

prepared from each fraction. Micrographs of cells in thin sections were taken with a Hitachi HU-11C electron microscope at 75 kV.

Protein synthesis experiments. Cells (400,000–700,000) were incubated in 20 ml siliconized beakers at 37° for 1 to 3 hr in an atmosphere of 95% O_2:5% CO_2. They were incubated in 1.5 ml of Medium 199 containing 0.1% BSA, 0.02% trypsin inhibitor (Worthington Biochemicals), streptomycin sulfate (7 μg/ml) and a mixture of ^{14}C-amino acids (227 mCi/mmole, New England Nuclear Corp.) at a concentration of 1 μCi/ml. Recoveries of cells after 3 hr incubation were 75–95%. After incubation, the cells were concentrated by centrifugation and tissue proteins precipitated with trichloroacetic acid (TCA, 10% final concentration; 4°, 30 min). Nucleic acids were hydrolyzed (90°, 15 min) and the protein precipitates collected on 0.45 μ Millipore membranes by filtration. Precipitates were washed ten times with 5% TCA, membranes placed in scintillation vials and 1.0 ml of 0.1 *N* NaOH added. Twenty-four hours later 15 ml of Aquasol (New England Nuclear Corp.) was added prior to measurement of radioactivity in a Mark I Nuclear Chicago liquid scintillation counter. Counting efficiency in all samples was 84–87%.

Results. Original cell suspension. In suspensions of dispersed cells the somatotroph is identified by light microscopy as a cell which contains a yellow-stained cytoplasmic granulation (1), and by electron microscopy as a cell which contains cytoplasmic secretion granules 3500–4000 Å in diameter (2). As seen in Fig. 1, the ultrastructural morphology of the somatotroph in the original cell suspension compares favorably to that seen in intact tissue. Other cell types, *viz*, gonadotropes, lactotrophs, *etc.* are also well preserved.

Velocity sedimentation. After 1.25 hr sedimentation in the 0.3–2.4% BSA gradient, cells in the original suspension are distributed as shown in Fig. 2 (top panel). A major cell

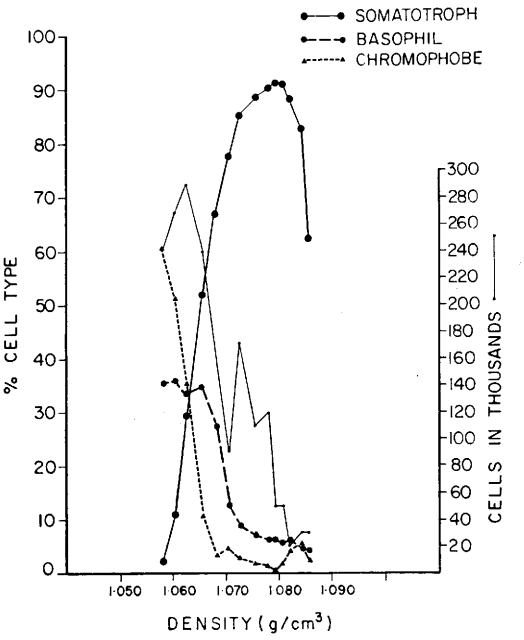

FIG. 3. Distribution and percentage of pars distalis cell types after centrifugation (45 min, 2000*g*) in a linear gradient of 14–28% BSA. These cells were obtained by pooling fractions 18–32 (Fig. 2) from the unit gravity system and concentrating them prior to density gradient centrifugation.

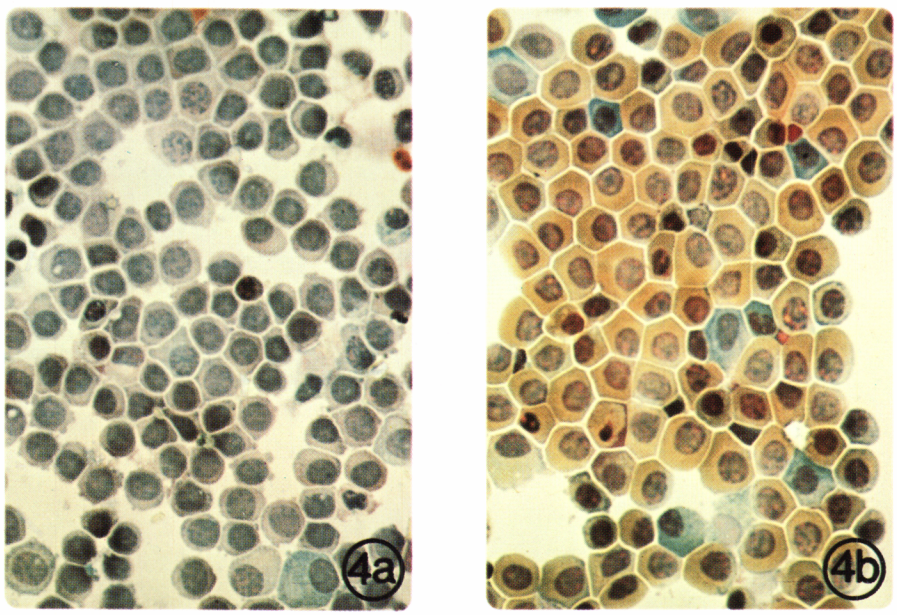

Fig. 4. (a) Photomicrograph of cells recovered from fraction 12 (Fig. 2). Most of these cells are chromophobes. Herlant's stain; × 430. (b) Photomicrograph of somatotrophs obtained after velocity and density gradient centrifugation (recovered from cells encompassed by densities 1.070-1.085) (Fig. 3). The contaminating cells are basophils; × 430.

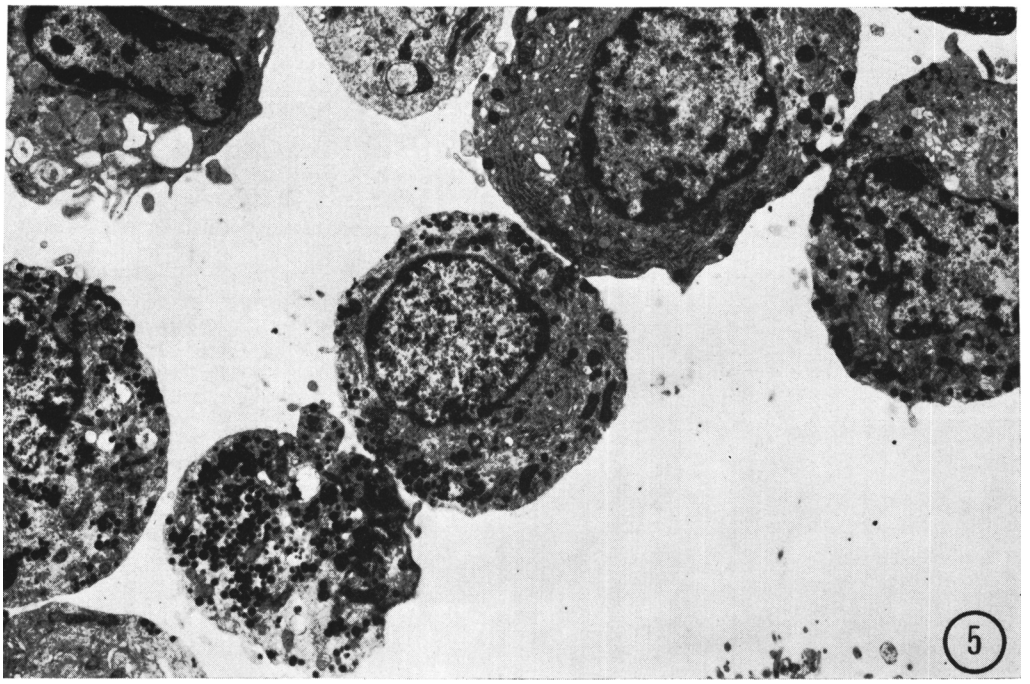

FIG. 5. Electron micrograph of cells recovered from the chromophobe fraction (tubes 11–14, Fig. 2) after unit gravity sedimentation. Most of the cells in this fraction have small cytoplasmic secretion granules (1500 Å–2500 Å). (× 5,400).

peak at fraction 12 (*i.e.,* 120 ml into the gradient), with a progressive decrease in cell numbers to fraction 32 is observed. Few cells are present after fraction 32. In each of 25 experiments the position of these cell peaks was found to be virtually identical to those shown in Fig. 2; however, the number of cells in each peak is variable. A majority of the somatotrophs sediment to an area encompassed by Fractions 18–32 (Fig. 2, lower panel). Sixty to 70% of the cells in this region of the gradient are somatotrophs; chromophobes, basophils and unknowns contaminate this fraction to about an equal extent (~ 10%). Attempts to further purify the somatotrophs by retarding their sedimentation rate with (a) introduction of Ficoll into the gradient or (b) use of shorter sedimentation times have proven unsuccessful.

A majority of the chromophobes sediment between Fraction 10 and 16, with some fractions containing over 90% of this cell type. Basophils tend to be concentrated in Fractions 18–24, and are not separable from the somatotrophs. As in a previous study (1) the unknowns (damaged or unclassifiable cells) are confined primarily to the upper regions of the gradient. In other experiments a similar distribution of cell types within the gradient was observed.

Density gradient centrifugation. After the cells in Fraction 18–32 (above) are combined and centrifuged to isodensity in linear gradients of 14–28% BSA, three cell peaks are consistently recovered (Fig. 3). In six experiments the first peak was found at density 1.0601 ± .0007 g/cm^3; the second at 1.0695 ± .0007 g/cm^3; and the third at 1.0761 ± .0007 g/cm^3. Differential counts of the cells banding between 1.0705 and 1.0850 show that 80–92% are somatotrophs, with basophils comprising the major contaminant. Over 90% of the cells recovered at densities 1.078–1.082 are somatotrophs. Approximately 75% of the somatotrophs applied to the gradient have a density greater than 1.070 and can therefore be separated in good purity.

A majority of the basophils, which cosediment with the somatotrophs in the unit gravity system, band at densities

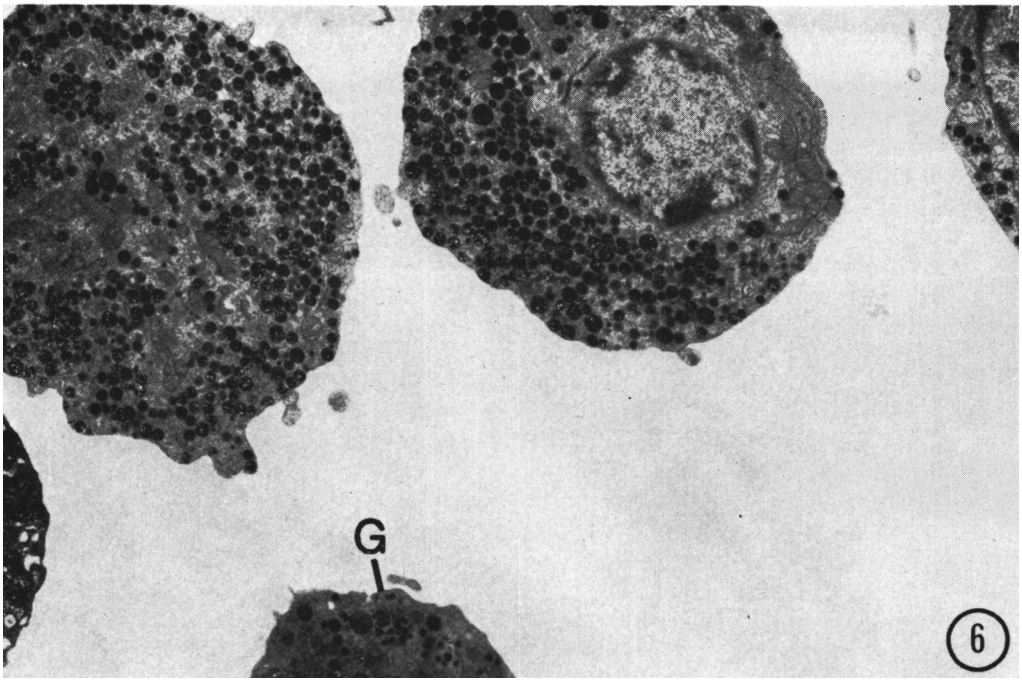

FIG. 6. Electron micrograph of cells recovered from the somatotroph fraction (tubes 18–32, Fig. 2) after unit gravity sedimentation. Four of the five cells are somatotrophs. Gonadotroph (G). (× 5,500).

1.0575–1.0680. Chromophobes appear to be the least dense cell type since they are found at the top of the gradient.

Morphology of separated cells. An example of cells in the chromophobe fraction obtained after separation by velocity sedimentation (Fraction 12, Fig. 2) is shown in Fig. 4a. By light microscopy most of these cells contain no stainable granules and often have minimal quantities of cytoplasm. Study of a chromophobe fraction (tubes 11–14, Fig. 2) by electron microscopy revealed that it consisted of a heterogenous group of cells which contained variable numbers of granules of varying size (Fig. 5). A majority of the cells in this fraction could be identified as gonadotrophs, thyrotrophs and corticotrophs.

A photomicrograph of somatotrophs obtained after velocity and density gradient centrifugation is shown in Fig. 4b. Most of the cells contain yellow cytoplasmic granules. Basophils constitute the main contaminating cell type (~ 9%) in this fraction. Somatotrophs which band between density 1.0605–1.0650 also contain yellow granules, but their staining intensity is considerably less than that of their denser counterparts. The ultrastructural appearance of the cells in the partially purified somatotroph fraction (Fig. 6) (*i.e.*, pooled fractions 18–32 from the unit gravity system) compared favorably with that seen in the original cell suspension. This observation indicates that the somatotrophs were not damaged in the velocity sedimentation procedure. Furthermore, cells in the purified somatotroph fraction obtained by density gradient centrifugation (pooled fractions encompassing densities 1.0697–1.0796) also show good morphological preservation (Fig. 7), although there is at times a tendency for some of the mitochondria of the somatotrophs to be swollen after this step. In accordance with the light and electron microscopic observations, somatotrophs banding between 1.0605–1.0650 g/cm^3 contained fewer granules than those recovered from the higher-density regions. Incubation of the purified somatotrophs (densities 1.070–1.080) for 3 hours in Medium 199 did not appreciably alter the ultrastructure of the somato-

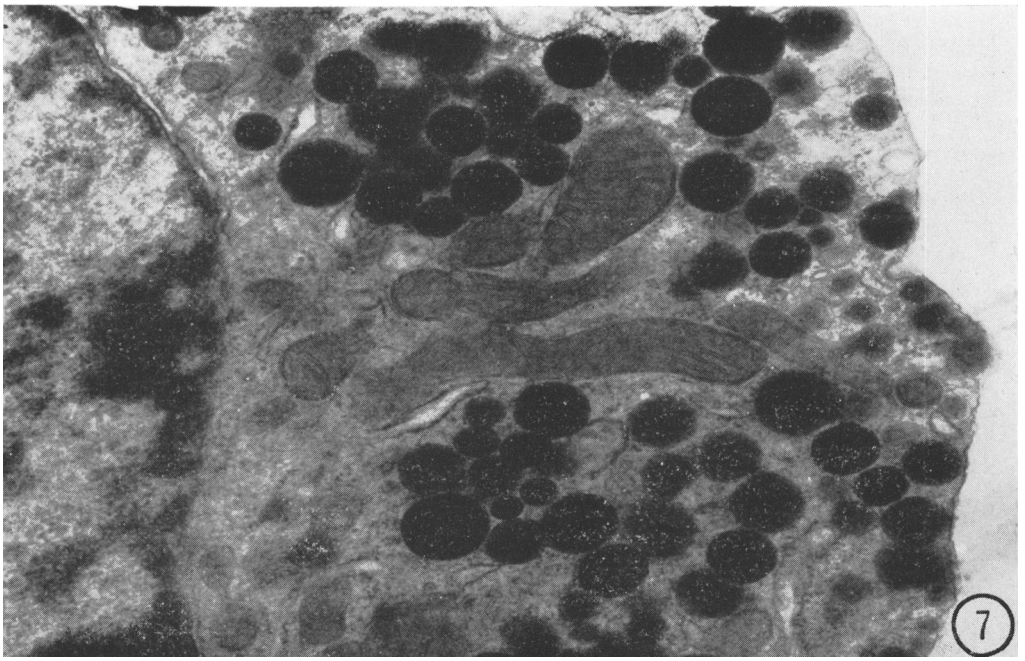

FIG. 7. Purified somatotroph fraction (densities 1.0697–1.0796). Portion of a somatotroph showing excellent preservation of organelles. (× 29,000).

troph (Fig. 8).

It should be pointed out that damaged cells, including some with pyknotic nuclei, are encountered at all stages of the dissociation and separation procedures. We estimate, after examination of cells in 1 μ sections stained with toluidine blue, that a total of 25% of the cells may show some damage after the entire separation procedure. Ten percent are damaged in the original cell suspension, 15% in the fractions obtained from the unit gravity separation step, and 20–25% in fractions obtained from the final density gradient separation procedure.

Protein synthesis. Incorporation rates of ^{14}C-amino acids into the TCA precipitable protein fraction of (a) cells in the original suspension, (b) chromophobes (fractions 11–14 from the unit gravity step) and (c) somatotrophs (cells at density 1.070–1.0835) from the density gradient are compared in Table I. The data show that the incorporation rates are essentially linear during the 3-hr incubation period for all cell fractions tested. They also show that the relative specific activity (DPM/cell) of the somatotrophs is about twice that observed in the starting cell suspension; while the chromophobes are much less active.

Growth hormone content of isolated cells. In preliminary studies we have determined, by radioimmunoassay, that the concentration of growth hormone in the initial cell suspension is 30–50 nanograms/1000 cells. The distribution of hormone in the 0.3–2.4% and 14–28% BSA gradients correlates well with the distribution of somatotrophs as judged by light microscopy. In the somatotroph fractions from both gradients, hormone levels range from 100–220 ng growth hormone/1000 cells. Such data not only show that the somatotrophs retain hormone during the dissociation and separation procedures, but on the basis of hormone content are concentrated three to fivefold.

Discussion. In spite of extreme cellular heterogeneity in the mammalian adenohypophysis, a great deal of information has been gathered which enables correlation of specific endocrine function(s) with morphologically distinct cell type(s). Furthermore, it is well documented that secretion of stored hormone from these different cell types is controlled by specific releasing factors. In

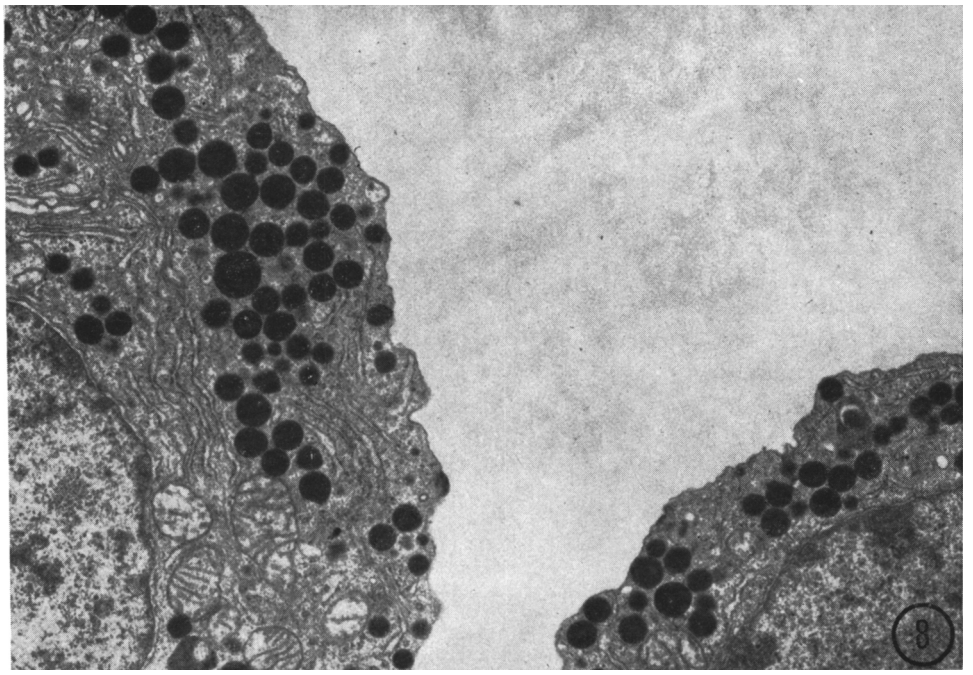

FIG. 8. Electron micrograph of somatotrophs obtained after velocity and density gradient centrifugation. These cells were incubated 3 hr in Medium 199. (× 13,000).

order to study the manner in which one of these hypothalamic factors (*viz*, growth hormone releasing factor) activates intracellular processes leading to secretion of growth hormone, our efforts to date have concentrated on the separation of somatotrophs.

The dissociation technique used in this study provides cells at high yield which, by both light and electron microscopy are virtually indistinguishable from those in intact tissue. In a recent report, Malamed *et al.* (2) showed that the ultrastructural appearance of adenohypophysial cells prepared by the dissociation procedure of Portanova *et al.* (7) also compared favorably with those in the intact tissue. Taken together, the results would indicate that adenohypophysial cells retain good ultrastructural integrity in either Krebs–Ringer bicarbonate (2), PBS or Medium 199.

The cell separation methods employed in this study demonstrate that it is possible to routinely obtain purified populations of somatotrophs at high yield. The first method, *i.e.*, velocity sedimentation at unit gravity, gives separations which are probably based primarily on cell size (8, 9), while those with the second method, *i.e.*, density gradient centrifugation, are based on density differences between the different cell types (5). The results from this study show that the somatotroph is among the most dense cell types found in the adenohypophysis. This appears entirely consistent with the observation that secretory granules isolated from somatotrophs are among the most dense subcellular organelles in the gland (10).

In other experiments we have found that centrifugation of cells in the original suspension in linear gradients of 12–28% BSA gives populations of somatotrophs of about the same purity (60–75%) as those obtained by unit gravity sedimentation alone. This shows that neither separation method by itself gives somatotroph fractions as pure as those obtained when both methods are used; *i.e.*, a purity of about 90%.

Results from the current morphological studies demonstrate that a majority of the somatotrophs are well preserved after the 5 hr required to separate them. In a previous report (1), the functionality of the separated

TABLE I. Incorporation of ^{14}C-Amino Acids Into TCA Precipitable Protein of Dispersed and Separated Rat Adenohypophysial Cells.

Cell fraction	Incubation time (min)	DPM/cell × 10^{-3}
Dispersed cells[b]	60	2.63 ± 0.15[a]
	120	5.67 ± 0.42
	180	7.29 ± 0.21
Chromophobes[c]	60	0.93 ± 0.12
	120	1.65 ± 0.11
	180	1.85 ± 0.10
Somatotrophs[d]	60	6.75 ± 0.43
	120	9.56 ± 0.60
	180	14.26 ± 0.54

[a] Standard error of mean.

[b] In all cases, total cells, purified chromophobes or somatotrophs were incubated in Medium 199 containing 0.1% BSA and ^{14}C-amino acid mixture at a concentration of 1 μCi/ml. Cell recoveries after incubation were 70–95%.

[c] Fractions 11–14 from the unit gravity system (Fig. 2).

[d] Cells which banded between 1.070–1.0835 g/cm^3 in the 14–28% BSA gradient (Fig. 3).

cells was indicated by the findings that (a) the cells incorporated ^{14}C-amino acids into protein in linear fashion at a rate comparable to that *in situ* and (b) that the separated cells excluded trypan blue. Results from this study also show that the isolated somatotrophs incorporate ^{14}C-amino acids into protein in linear fashion during a 3-hr incubation period. This rapid incorporation rate agrees well with our previous data obtained on less pure cell populations (1) and is consistent with the radioautographic studies of Kobayashi *et al.* (11).

Finally, the presence of immunoreactive growth hormone in the isolated somatotroph invites study of the mechanisms by which growth hormone releasing factor may act on this cell type. Such studies are currently under investigation.

Summary. Fractions containing over 90% somatotrophs can be separated from suspensions of adenohypophysial cells by the techniques of velocity and density gradient centrifugation. These isolated somatotrophs, as shown by radioimmunoassay, contain growth hormone. Light and electron microsopic studies show that the isolated somatotrophs retain their structural integrity. These somatotrophs incorporate ^{14}C-amino acids into protein in linear fashion during 3-hr incubation.

The expert technical assistance of Allan Morris, Oriette Morris, Margaret Evans and Blake Guggins is acknowledged.

1. Hymer, W. C., Evans, W. H., Kraicer, J., Mastro, A., Davis, J., and Griswold, E., Endocrinology, in press.

2. Malamed, S., Portanova, R., and Sayers, G., Proc. Soc. Exp. Biol. Med. **138**, 920 (1971).

3. Ishikawa, H., Endocrinol. Japan. **5**, 517 (1969).

4. Hymer, W. C., Haskill, J. S., and Kraicer, J., Fed. Proc. **31**, 235 (1972).

5. Shortman, K., Austral. J. Exp. Biol. Med. Sci. **46**, 375 (1968).

6. Bencosome, S. A., and Tsutsumi, V., Lab. Invest. **23**, 447 (1970).

7. Portanova, R., Smith, D. K., and Sayers, G., Proc. Soc. Exp. Biol. Med. **133**, 573 (1970).

8. Peterson, E. A., and Evans, W. H., Nature (London) **214**, 824 (1967).

9. Miller, R. G., and Phillips, R. A., J. Cell Physiol. **73**, 191 (1969).

10. Hymer, W. C., and McShan, W. H., J. Cell Biol. **17**, 67 (1963).

11. Kobayashi, T., Kobayashi, T., Kigawa, T., Mizuno, M., Amenomori, Y., and Watanabe, T., Endocrinol. Japan. **12**, 47 (1965).

12. Haskill, J. S., and Marbrook, J., Cellular Immunol. **3**, 448 (1972).

Received June 7, 1972. P.S.E.B.M., 1972, Vol. 141.