Separation of Dissociated Thyroid Follicular and Parafollicular Cells: Association of Serotonin Binding Protein with Parafollicular Cells

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ABSTRACT Parafollicular cells (PC) of the sheep thyroid gland are neural crest derivatives that synthesize and release the biogenic amine serotonin (5-HT) as well as the hormone calcitonin. The thyroid also contains a highly specific serotonin-binding protein (SBP). Separation of dissociated thyroid cells was done to study the cellular localization of SBP and to develop a means of isolating PC for study. Various methods were used to obtain an enriched and purified population of PC. Minced thyroid glands were enzymatically dissociated and the cells were layered on a Ficoll linear density gradient. Fractions obtained from the gradient were examined for cell number, viability, 5-HT concentration, SBP activity, and morphology by electron microscopy. One of the fractions was found to be enriched in PC. High levels of 5-HT and SBP were also found in this fraction, whereas these levels were low where the majority of cells were found. This PC-rich fraction, however, contained numerous follicular cells (FC); therefore, additional approaches to cell separation were used. FC can be stimulated in vitro with thyroid-stimulating hormone (TSH) to become intensely phagocytic. When stimulated cells were incubated in the presence of silica microspheres, the FC engulfed the microspheres, which were toxic to them. PC did not become phagocytic and were unharmed by the microspheres. Suspended cells, after incubation with microspheres, were centrifuged on a discontinuous gradient, and a PC-rich fraction was obtained. Silica, however, interfered with analysis of SBP. Another method to take advantage of the phagocytic potential of FC was therefore used. TSH-stimulated cell suspensions were passed through a column of Sepharose to which thyroglobulin had been coupled. Stimulated FC apparently adhered to the beads and were retained by the columns. Fractions eluting from the columns were greatly enriched with PC. These fractions contained high levels of 5-HT and SBP, and considerably reduced FC contamination was found by quantitative electron microscopy. It is concluded that SBP is localized to PC in the sheep thyroid. The idea that these cells resemble serotonergic neurons in their mechanisms of 5-HT storage is supported.

The thyroid glands of all mammalian species that have been examined have been found to contain the biogenic amine, serotonin (5-HT; 25). The concentration of 5-HT in these glands is particularly high in the rat, sheep, horse, goat, bat, and callithricid primate (8, 20, 22, 25, 31); however, the site of 5-HT storage differs among these species. In the rat, 5-HT is found in mast cells (21). In the sheep, horse, goat, bat, and callithricid primate, 5-HT is not contained in thyroid mast cells but, instead, the amine is concentrated in the parafollicular cells of the glands (8, 11). There is evidence that 5-HT is stored within the same secretory granules that also contain calcitonin (3, 15, 22). In the bat this evidence is radioautographic (22), whereas in sheep the evidence is derived from studies involving subcellular fractionation and partial purification of parafollic-
ular cell granules (3) as well as electron microscopic cytochemistry (15).

In all animals injection of 5-hydroxytryptophan (5-HTP), the immediate precursor of 5-HT, fills parafollicular cells with 5-HT (11-13, 26, 27) and [3H]5-HTP labels parafollicular cell granules with [3H]-5-HT. The radioactive [3H]-5-HT is discharged by exocytosis along with calcitonin when parafollicular cells are exposed to elevated concentrations of Ca²⁺, their natural secretagogue (10). The finding that parafollicular cells synthesize, store, and release 5-HT has made these cells interesting to neurobiologists as well as endocrinologists. The cells are of neural crest origin (18) and have been called paraneurons (9, 17). As such they are potentially very valuable as a model system for examining the cellular biology of 5-HT storage resembling that in neurons. The idea that thyroid parafollicular cells might be analogous to serotonergic neurons has received some support. Serotonergic neurons, both in the brain (35, 36) and in the gut (16), contain a specific 5-HT-binding protein (SBP). The sheep thyroid gland also contains such a protein (16); the properties of sheep thyroid SBP are similar to those of SBP derived from central or peripheral serotonergic neurons (5) but are different from those of 5-HT-binding proteins of rat mast cells (32) or platelets (33). Although the evidence is indirect, it seems likely that the SBP of the sheep thyroid is stored in parafollicular cells, because the activity of the protein shows a regional distribution in the gland that correlates with the concentrations of both 5-HT and parafollicular cells (4).

It has been postulated that SBP is a component of the subcellular storage mechanism for 5-HT (5, 37). The protein from neurons is enriched in subcellular fractions containing synaptic vesicles (34), and the binding of 5-HT to SBP is inhibited by reserpine (16, 36), a drug known to interfere with intracellular storage of monoamines (5). Binding of 5-HT to SBP could serve to decrease osmotic pressure within storage vesicles or granules. That exposure of SBP to concentrations of salts found in extracellular fluids antagonizes binding of 5-HT (5) is consistent with this idea. Exocytosis would expose the contents of storage granules to extracellular fluid and thus promote dissociation of an intragranular 5-HT-SBP complex with the consequent liberation of the free amine. Confirmation that SBP is stored in parafollicular cells would strengthen the idea that these cells are useful models for the study of serotonergic mechanisms relevant to neurons.

It would be interesting to study the interrelationships between 5-HT, SBP, and calcitonin, as well as the mechanisms involved in their storage in the thyroid gland; however, an experimental disadvantage of thyroid parafollicular cells is their admixture with follicular cells and their status as a minority of the parenchymal cell population of the thyroid (4). This drawback can be overcome to some extent by using animals, such as sheep or bat, in which parafollicular cells are particularly abundant (4, 23, 24). A major advance would be made in this regard if parafollicular cells could be separated from follicular cells and obtained in an enriched and purified cell preparation. The present study was done in an attempt to develop methods for obtaining such a preparation. In addition, it was hoped that cell separation could be used to provide direct evidence for the localization of SBP in parafollicular cells.

MATERIALS AND METHODS

Fresh lamb thyroid glands (-10 lobes per experiment) were obtained at a nearby slaughterhouse. The glands were placed in chilled Eagle's minimal essential medium with Earle's salts (MEM; Flow Laboratories Inc., Rockville, Md.) containing added glucose (0.15 M), nonessential amino acids (Gibco Diagnostics, Chagrin Falls, Ohio; 0.1 mM), sodium bicarbonate (4.2 mM), glutamine (2.0 mM), and the monoamine oxidase inhibitors, pargyline (10⁻⁶ M) and clorgyline (10⁻⁶ M). These drugs were added to prevent 5-HT metabolism by type A or B monoamine oxidase. This supplemented medium will be referred to as MEM +6. Upon arrival at the laboratory, the glands were trimmed of fat and connective tissue and were minced.

**Dissociation Protocol**

Minced thyroid glands were dissociated by a modification of the method (used for pancreas) described by Amsterdam and Jamieson (2). All dissociation media consisted of MEM +6, equilibrated with 95% O₂ and 5% CO₂ (pH 7.4) at 37°C. 5 g of minced thyroid gland was added to a trypsinizing flask containing 11 ml of MEM +6, bovine serum albumin (BSA; 1%), and either 0.25% trypsin (2 X crystalized; Worthington Biochemical Corp., Freehold, N. J.) or a combination of purified collagenase (325 U/ml, 650 U/ml; Worthington Biochemical Corp.), purified bovine testis hyaluronidase (8,000 U/ml, 360 U/ml; Worthington Biochemical Corp.), purified alpha-chymotrypsin (0.2 mg/ml; Sigma Chemical Co., St. Louis, Mo.), and purified soybean trypsin inhibitor (0.1 mg/ml; 1 mg inhibits 1.45 mg of trypsin; Worthington Biochemical Corp.). EDTA (final concentration, 2.0 mM) was then added to chelate divalent cations. Incubation continued under the same conditions for 10 min more, after which time calcium was replenished by the addition of solid CaCl₂ to yield a final concentration of 2.5 mM. 5 ml of fresh MEM +6 containing fresh enzymes at the above concentrations was also added at this time. After 45 min, the incubation medium was filtered through a 74-µm nylon mesh (Small Parts Inc., Miami, Fla.) to remove any undissociated tissue or cell clumps. Trypsin soybean trypsin inhibitor was added to the trypan-dissociated cells. The weight of the remaining tissue was noted and the cells were counted. Cell viability was estimated by trypan blue exclusion (0.4% trypan blue; 28).

**Stimulation of Follicular Cells**

Before the separation of dissociated cells, some experiments included a preincubation period for the activation of follicular cells with thyroid-stimulating hormone (TSH, bovine; Armour Pharmaceutical Co., Phoenix, Ariz.). In one set of experiments, dissociated cells were incubated in MEM +6 that contained TSH (5 mU/ml) and silicamicrospheres (25 inn indiameter, 4.9x 10⁸ spheres/ml; Ludox; E. I. DuPont DeNemours & Co., Wilmington, Del.) for 45 min at 37°C. These cells were then layered on either a linear or a discontinuousgradient. Dissociated cells that were to be passed through chromatographic columns after activation were incubated for 20 min in MEM +6 containing TSH (5 mU/ml, 37°C) or in MEM +6 alone (control).

**Linear Density Gradients**

Linear density gradients of Follicul (polyacrylamide; average molecular weight, 400,000; Pharmacia Fine Chemical Co., Piscataway, N. J.) in MEM +6 were constructed using a two-chambered gradient generator and a peristaltic pump (Buchler Instruments Inc., Fort Lee, N. J.) adjusted to provide a flow rate of 0.5 ml/min. All Follicul solutions were equilibrated with 95% O₂ and 5% CO₂ and were kept at pH 7.4. The 60-ml gradient ranged from 15% Follicul at the sample-gradient interface to 35% Follicul at the bottom of a 100-ml polycarbonate centrifuge tube. The gradients were stored overnight at 4°C. Approximately 3 x 10⁶ cells were suspended in 2% BSA (final volume of 40 ml) and were layered on top of the gradient. The gradients were then centrifuged (2,200 g, 30 min) using a six-place, swing-out rotor (JS-4.2). After centrifugation, successive fractions were collected from the top (29) using a gradient tapping cap (Halpro, Inc., Rockville, Md.). The gradients and fractions were kept on ice throughout this procedure and tubing was siliconized. Fractions were mildly agitated by inversion, and the refractive indices of all gradient fractions were measured to confirm the density of the fractions.

**Discontinuous Density Gradients**

Discontinuous gradients were prepared immediately before their use. 15 ml of 10% Follicol in MEM +6 (refractive index 1.363) was layered over 15 ml of 25% Follicol in MEM +6 (refractive index 1.373) in a 40-ml cellulose nitrate centrifuge tube. Approximately 3 x 10⁶ cells were layered on each gradient (10-ml volume with 2% BSA). The gradients were centrifuged (7,200 g, 30 min; SW 27 rotor; Beckman) and fractions were collected with a Pasteur pipette as shown in Fig. 1.

**Cell Chromatography**

Thyroglobulin (150 mg; Bovine, type I; Sigma Chemical Co.) was coupled to 15 g of cyanogen bromide-activated Sepharose 6MB (200- to 300-µm beads, 0.375 ml) using cross-linking reagent at 0°C. The suspension of cross-linked Sepharose was added to the gradient cell, the cell was washed with 10 ml of 0.1 M Tris, pH 7.4, and the column was eluted with 0.1 M Tris, pH 7.4. Fractions (5 ml) were collected, and 0.1 ml aliquots were assayed for the presence of thyroid-specific protein (thyroglobulin) with the thiocarboxylic acid test (38) and for the presence of thyroxine (39) using the radioactive radioimmunoassay method of Hennet et al. (40) with 125I-labeled thyroxine (New England Nuclear Corp., Boston, Mass.).
washed with 10⁻⁴ M HCl; Pharmacia Fine Chemical Co.) by suspending the thyroglobulin and Sepharose in 75 ml of sodium bicarbonate–buffered saline (0.1 M NaHCO₃ in 0.5 M NaCl, pH 8.5) and rotating end over end for 2 h at room temperature. Unreacted groups were blocked with 1 M ethanolamine (pH 8). The Sepharose was then washed alternately with sodium acetate-buffered saline (0.1 M NaC₃H₅O₇ in 0.5 M NaCl, pH 4) and sodium borate-buffered saline (0.1 M Na₂B₄O₇ in 0.5 M NaCl, pH 8). Columns (0.9 x 10 cm) with an 80-µm nylon mesh filter at the bottom were packed with the activated Sepharose. The flow rate was adjusted to 1 ml/min and the column was equilibrated with MEM 6+ with or without TSH (5 mU/ml). This entire procedure was carried out in a room maintained at 37°C. Approximately 1 x 10⁶ cells in 6 ml were put on each column and 2-ml fractions were collected. Three batches of thyroglobulin coupled to Sepharose were prepared. Chromatographic results were the same with each batch.

Serotonin Concentration

Minced thyroid glands (~1 g) were homogenized (30 s, Polytron; Brinkman Instruments Inc., Westbury, N. Y.) in 10 vol of 0.1 N HCl. Dissociated cells were diluted with 1.0 N HCl so that the final concentration was 0.1 N HCl, and the samples were frozen and thawed. Serotonin content was assayed according to the highly sensitive microenzymatic method of Saavedra et al. (30).

Activity of Serotonin-binding Protein

All procedures were carried out at 4°C. Aliquots of dispersed cells were frozen, thawed, and spun (105,400 g, 60 min). The supernate, containing most of the SBP, was partially purified by fractionation with ammonium sulfate (0-60% saturation). Saturated ammonium sulfate (1.5 ml) was added dropwise at 4°C to the supernate (1 ml), which, in turn, was being stirred. The solution was then allowed to equilibrate for 20 min under the same conditions. The precipitate was collected (27,000 g, 15 min) and dissolved in 0.02 M potassium phosphate buffer at pH 7.5. In some experiments, when small numbers of cells were being processed, the pellet remaining after the addition of ammonium sulfate was very small. In this case, ammonium sulfate fractionation was abandoned and the pellet was resuspended in the supernate. The addition of ammonium sulfate was necessary, even if partial purification of protein was not done, to facilitate dissociation of endogenous 5-HT from SBP. The absence of monoamine oxidase inhibitors was also found to improve this dissociation. Pargyline and chloroglyine, therefore, were excluded from the MEM in some of the cell chromatography experiments in order to assay SBP more effectively. Ammonium sulfate was removed by dialysis against 0.02 M potassium phosphate buffer (pH 7.5, 4 h). If neither Ficoll nor BSA were present, protein was measured spectrophotometrically from the ratio of light absorption at 280/260 nm. An aliquot containing 50 µg of protein (or 0.2 ml, if the protein concentration was unavailable) was taken for analysis of SBP. To this aliquot 50 µl of ferrous ammonium sulfate (0.1 mM) and 0.02 M potassium phosphate buffer at pH 7.5 were added to yield a final volume of 0.49 ml. This mixture was incubated at room temperature for 8 min. Trisated 5-HT ([H]5-HT, 0.2 µM, 15.9 Ci/mmol; New England Nuclear, Boston, Mass.) was added and incubation was continued for an additional 15 min. The incubation mixture was then applied to a column of Sephadex G-50 (1 x 10 cm) equilibrated with buffer, as described by Tamir et al. (36) to separate protein-bound [3H]5-HT from the free radioactive amine. The protein-bound [3H]5-HT complex was collected and counted by liquid scintillation. To estimate specific binding, the assay was run in the presence of a 1,000-fold excess of nonradioactive 5-HT. Specific binding was defined as the difference between the [3H]5-HT bound in the absence and presence of nonradioactive 5-HT.

Electron Microscopy Procedures

Intact pieces of thyroid or aliquots of dissociated cells were fixed overnight at 4°C in 4% glutaraldehyde in 0.1 M potassium phosphate buffer at pH 7.5. Dissociated cells were mixed 1:1 with this fixative.

The following morning, the fixative solution containing dissociated cells was centrifuged (5,500 g, 15 min; Beckman J-21B, JA-14 rotor) and the pellet was resuspended in ~0.5 ml of 0.2 M potassium phosphate buffer at pH 7.5. This solution was then transferred to a 400-µl microfuge tube and centrifuged again (10,000 g, 5 min; Beckman microfuge B). The pipette of the tube was then cut off and the pellet was removed with fine forceps.

Intact pieces of fixed tissue were diced into cubes of ~1 mm³ and rinsed with 0.2 M potassium phosphate buffer at pH 7.5. The tissue cubes and cell pellets were postfixed for 2 h at 4°C in 1% osmium in 0.15 M potassium phosphate buffer (pH 7.5), rinsed in isotonic saline, stained en bloc for 1 h at 4°C with uranyl magnesium acetate and rinsed again with isotonic saline. Samples were then rapidly dehydrated in ethanol, cleared in propylene oxide, and embedded in epoxy resin (Epon 812).

Thin sections were stained with uranyl acetate and lead citrate and examined in a Philips EM300 electron microscope.

Electron Microscopic Quantitation

Quantitation was performed to determine the ratio of parafollicular cells to follicular cells in the different fractions collected from cellular chromatography columns. Dissociated follicular and parafollicular cells were not significantly different in diameter. At least 100 cells of each fraction were counted by examining three different grids to obtain a ratio, and this process was repeated up to seven times for each fraction, using different blocks, if available. Other cell types were noted (i.e., red blood cells, lymphocytes, and mast cells); however, cells that appeared damaged (~5%) were not included in the cell counts.

RESULTS

Dissociation of Thyroid Cells

Trypsin was found to be almost as effective in dissociating the cells of the thyroid gland as was the mixture of collagenase, hyaluronidase, alpha-chymotrypsin, and soybean trypsin inhibitor. In both cases, ~50% of the 5 g of tissue was dissociated (determined from the weight of the tissue remaining after filtration). Trypsin yielded a 16-ml suspension that contained ~20 ± 2 (SE) x 10⁶ cells/ml, while the enzyme mixture yielded a suspension that contained 23 ± 2 x 10⁶ cells/ml (p was not significant). Cell viability, as estimated with the trypan blue exclusion test, was >95%. Trypsin, therefore, was used to dissociate thyroid cells in most experiments because it is much less expensive to use than the complex mixture of enzymes, and its action is quickly terminated by the addition of soybean trypsin inhibitor. The 5-HT concentration of the 16-ml trypsin-dissociated cell suspension was 93 ± 16 ng/ml (3.5-6.0 fg/cell), and the SBP activity was 7.9 ± 0.66 x 10⁶ cpm/ml (3.6-4.4 x 10⁵ cpm/cell). The appearance under the electron microscope of thyroid cells dissociated with trypsin is shown in Fig. 2. Erythrocytes, follicular cells (Fig. 2b), and parafollicular cells (Fig. 2a) were numerous in the suspension, whereas mast cells (Fig. 2c), lymphocytes, and polymorphonuclear leukocytes were only occasionally seen. Electron microscope quantitation indicated that the ratio of parafollicular cells to follicular cells in the crude dissociated preparation is 0.51 ± 0.04. This represents a twofold enrichment of parafollicular cells in these preparations as compared with the intact thyroid gland (4).

Linear Density Gradients

Fractions obtained from a Ficoll linear density gradient were found to differ considerably in the various parameters exam-
Electron micrographs of representative types of cells found in the dissociated cell population. (a) Parafollicular cell, containing many secretory granules polarized to one side of the nucleus. (b) Follicular cell, containing numerous profiles of dilated cisternae of rough endoplasmic reticulum. (c) Mast cell with large heterogeneous granules and many filopodia extending from the cell surface. Bars, 1 μm. a, ×10,190; b, ×7,000; c, ×10,260.

A typical example of such a gradient is shown in Fig. 3. Electron microscopic examination revealed that fraction 8, the peak of cells, consisted mainly of erythrocytes, but also contained follicular cells and parafollicular cells. In contrast, fraction 6 (lower density than fraction 8) contained no erythrocytes and was greatly enriched with parafollicular cells (Fig. 4). This fraction also was enriched 23-fold (9.1 × 10^{-2} cpm/cell) over the unfractionated dissociated cells with SBP and fivefold with 5-HT (26 fg/cell). This observation is consistent with the previous suggestion, based on the correspondence between the distribution in the thyroid of SBP, 5-HT, and parafollicular cells, that parafollicular cells contain both 5-HT and SBP (4). 5-HT was also found at the top of the gradient (fraction 2) that contained cellular debris including parafollicular cell granules, and at the bottom of the gradient (fractions 11 and 15) where aggregates of both parafollicular cells and follicular cells were found. SBP was also detected at the bottom of the gradient; however, the determination of the activity of SBP in the top fractions was complicated by the presence of BSA, which bound [3H]5-HT and resulted in high nonspecific binding during the assay of SBP. It is difficult, therefore, to draw conclusions about the activity of SBP in the uppermost gradient fractions that contained cellular debris. It is possible to obtain a fraction that is enriched with parafollicular cells by means of sedimentation of cells through a density gradient. The resulting parafollicular cell fraction, however, is still significantly contaminated with follicular cells; consequently, further attempts were made to improve the separation of these two parenchymal cell types.

**Stimulation of Follicular Cells**

In the intact thyroid gland, stimulation with TSH causes follicular cells to phagocytize thyroglobulin-containing colloid. This leads to the formation of membrane-bounded colloid droplets within the follicular cells. These droplets then fuse with lysosomes, ultimately leading to the enzymatic degradation of thyroglobulin to liberate thyroid thyroxine and triiodothyronine (38, 39). When incubated in vitro with TSH, follicular cells were activated to extend pseudopods and to become highly phagocytic (Fig. 5 a). This indicates the relative stability of the dissociated thyroid cells; their responsiveness to TSH is retained. To take advantage of this phagocytic potential, dissociated cells were incubated with TSH and 25-nm silica microspheres. Under these conditions, follicular cells phagocytized the silica microspheres, as shown in Fig. 5 b. The droplets containing silica microspheres also appeared to fuse with lysosomes; however the silica apparently was toxic to...
lysosomes, causing damage to the membranes of these organelles. This resulted in silica microspheres (and presumably hydrolytic enzymes as well) gaining access to the cytosol (Fig. 5c). Follicular cells eventually lysed after phagocytosis of silica. Consequently, when dissociated cells that had been incubated with TSH and silica microspheres were layered on a continuous Ficoll density gradient and centrifuged, a greatly enriched fraction of parafollicular cells was obtained. Few follicular cells were observed in this fraction and those few that were found appeared damaged. Parafollicular cells, however, were unaffected by TSH, did not take up silica, and were healthy in appearance (Fig. 5d).

**Discontinuous Density Gradients**

To maximize the yield of parafollicular cells, discontinuous gradients were used after follicular cell stimulation and incubation with silica microspheres. Experience with the Ficoll linear density gradients indicated that the enriched parafollicular cell fraction occurred at an area of the gradient with a refractive index of 1.370 (a density of 1.10). The discontinuous gradient was designed so that the parafollicular cells would accumulate at the interface between 10 and 25% Ficoll (fraction 4, Fig. 1), whereas erythrocytes would be found at the bottom of this gradient, because they were known to be more dense. Electron microscopic examination of the interface (fraction 4) confirmed the prediction, revealing numerous parafollicular cells (Fig. 6). The top fractions (1, 2, and 3) contained debris from broken cells, whereas the bottom fraction (6) contained many erythrocytes. Unfortunately, attempts to assay 5-HT and SBP proved unsuccessful in preparations containing silica. This was found to be attributable to the affinity of silica microspheres for 5-HT. The technique using silica microspheres is valuable in providing enriched populations of parafollicular cells; however, it is severely limited in that it is difficult to wash out the silica microspheres completely without deleterious effect on the cell yield.

**Cell Chromatography**

The distribution of cells in fractions obtained from a typical thyroglobulin-Sepharose chromatography column through which TSH-stimulated cells were passed is shown in Fig. 7. The greatest number of cells was found in either fraction 4 or 5. To normalize the data to facilitate comparisons between experiments, we designated the fraction containing the peak of cells 0 and numbered consecutively the fractions collected before (negative) and after (positive) this fraction. The 5-HT concentration was highest in the second fraction collected after the peak of cells (22.0 ± 10.4 fg/cell; a fivefold enrichment over the 5-HT concentration of dissociated cells). SBP activity was also found to be maximal in the fractions containing the highest concentration of 5-HT (5.4 ± 0.24 × 10^2 cpm/cell; a 14-fold enrichment over the SBP activity of dissociated cells). Quantitative electron microscope examination of the fractions indicated that the parafollicular cell to follicular cell ratio was 2.02 ± 0.40 in the 5-HT and SBP-rich fraction (Table I and Fig. 8a and b). This represents a 10-fold enrichment in parafollicular cells as compared with the intact thyroid gland (4) and provides a yield of ~4 × 10^6 parafollicular cells per gram of intact thyroid. The other fractions obtained from the column exhibited a 1:1 ratio, which is also an enrichment over the starting material. Relatively fewer follicular cells were evident in the column effluent, probably because the stimulated follicular cells adhered to the Sepharose beads that had been coupled to thyroglobulin. In contrast, in the control experiments that omitted the exposure to TSH, many follicular cells were seen in all fractions of the column effluent (Fig. 8c). The ratio of
parafollicular cells to follicular cells in the absence of TSH stimulation was similar to that of the dissociated cells (Table I).

DISCUSSION

Several different methods were tried in an attempt to devise a useful technique that would permit thyroid parafollicular cells to be obtained in an enriched preparation separated from and relatively free of contamination by follicular cells. Such a preparation could be used to confirm the parafollicular cell localization of SBP and would be useful in studying subcellular mechanisms involved in the storage of 5-HT as well as the hormone, calcitonin. Sheep thyroid glands were used as the starting material, in part because the sheep thyroid contains proportionally more parafollicular cells than the thyroid glands of other species, but also because the sheep's thyroid is large and rich in 5-HT. The very fibrous nature of the thyroid was found to be a problem, partially overcome by using the glands of lambs. The 5-HT concentration of lamb thyroid was also relatively higher than that of the thyroids of older sheep.

Trypsin was found to be an adequate agent to use to dissociate thyroid tissue. It was interesting to note that the process of dissociation itself yielded a cell population that was enriched with parafollicular cells as compared with the intact gland. Perhaps this can be explained by the morphological arrangement of the tissue, as parafollicular cells are located most peripherally in the follicles (19) and would be exposed to trypsin before the follicular cells. Parafollicular cells may also be somewhat more stable than follicular cells and survive better. Unfortunately, however, dissociated thyroid parafollicular and follicular cells showed significant overlap in size and density. Isokinetic separation of cells was thus not possible. Sedimentation to equilibrium through a Ficoll linear density gradient did produce some separation of follicular and parafollicular cell populations with consequent enrichment of the parafollicular fraction. SBP and 5-HT were both concentrated in the fraction richest in parafollicular cells. This distribution in the gradients is consistent with the idea that SBP is located in parafollicular cells. The cell populations, however, were not sufficiently separated to enable us to obtain a relatively pure fraction of parafollicular cells. Even the best of the parafollicular fractions contained significant numbers of follicular cells.
FIGURE 6 An electron micrograph of gradient fraction 4 of the Ficoll discontinuous gradient shown in Fig. 1 obtained after incubation with TSH and silica microspheres. Portions of four parafollicular cells (P) are seen in this field. Note the presence of silica microspheres among the debris (arrows). Bar, 1 μm. × 6,300.

The possibility that a subset of follicular cells might contain SBP and sediment with the majority of parafollicular cells cannot be excluded on the basis of these experiments alone. Follicular cell organelles, particularly lysosomes, moreover, would be expected to contaminate any subcellular fractions containing parafollicular cell granules that might be obtained in subsequent studies, even from these enriched cellular preparations. The presence of Ficoll in the gradients was also disadvantageous, because it interfered with the quantitation of protein in the fractions.

Two methods, consequently, were adapted to utilize normal physiological traits of follicular cells to enhance the cells' experimental separation from parafollicular cells. Both depended on the intense phagocytic response of these cells to stimulation by TSH and both were effective. One procedure exposed TSH-stimulated cells to silica microspheres. It was anticipated that the follicular cells might take up the spheres by endocytosis, whereas parafollicular cells, lacking the response to TSH (14) would not. It was also anticipated that the density of follicular cells, laden with silica, would change sufficiently to promote their separation from parafollicular cells by centrifugation through an appropriate density gradient.

These expectations were partially confirmed. Follicular cells were stimulated by TSH even when exposed to that hormone in vitro. Dissociation in the presence of trypsin, therefore, does not destroy all TSH receptors on follicular cells. Parafollicular cells, moreover, seemed not to respond to TSH. The silica microspheres, however, proved to be extremely toxic to follicular cells once these cells had taken up the spheres. It appeared that the internalized silica led to the intracellular lysis of vacuoles containing the microspheres, enabling the spheres to gain access to the cytosol. Fusion of thyroid lysosomes with phagosomes occurs soon after endocytosis of material by fol-
natural product taken up by follicular cells stimulated by TSH however, adsorbs 5-HT and interferes with the biochemical activity of SBP. The silica experiments thus confirmed that parafollicular cells would show any special affinity for this protein, thus being enriched after the passage of the columns. (No attempt was made to determine the concentration of SBP that parallels the distribution of parafollicular cells.) The second procedure to take advantage of the sensitivity of follicular cells to TSH involved cell chromatography. The natural product taken up by follicular cells stimulated by TSH in situ is thyroglobulin (36). This protein was therefore coupled to Sepharose to provide an appropriate target for TSH-stimulated cells. It was anticipated that follicular cells stimulated by TSH might "attempt" to phagocytize thyroglobulin immobilized on the column and thus either become immobilized on the column themselves or at least be impeded in their passage through the columns. (No attempt was made to determine whether follicular cells would phagocytize Sepharose beads that were not coupled to thyroglobulin as well as the coupled beads.) Parafollicular cells, on the other hand, not only are not stimulated to become phagocytic by TSH but these cells, furthermore, never reach the luminal colloid and are not exposed to thyroglobulin in situ (19). It seemed unlikely, therefore, that parafollicular cells would show any special affinity for thyroglobulin and thus be retarded in their passage through the columns. These expectations apparently have been confirmed by our experiments. In control preparations, when dissociated thyroid cells were not exposed to TSH, both follicular and parafollicular cells passed through the columns and little separation of the two cell types occurred. On the other hand, after exposure of the dissociated cells to TSH, relatively fewer follicular cells appeared in the column effluent. Parafollicular cells continued to traverse the columns and consequently a separation of follicular and parafollicular cells did take place. The cellular fractions obtained from the columns after exposure of cells to TSH were enriched with parafollicular cells and had greatly reduced follicular cell contamination. These fractions were also free of potentially interfering substances such as silica or Ficoll and should prove to be extremely valuable for further study of parafollicular cells or the storage of 5-HT and calcitonin. More important, however, when cells were exposed to TSH, cell fractions passing through the columns were enriched both with 5-HT and SBP. As the proportion of parafollicular cells in the fractions eluting from the columns increased, moreover, so too did the concentration of 5-HT and the activity of SBP.

Two techniques, using quite different means of separating thyroid cells, therefore, both show an enrichment of 5-HT and SBP that parallels the distribution of parafollicular cells. The amine and its binding protein sediment with parafollicular cells in Ficoll density gradients and after stimulation with TSH elute with parafollicular cells from columns that retard the passage of follicular cells. These experiments, involving separation of thyroid cells, thus complement previous observations that 5-HT and SBP are most concentrated in those thyroid regions that contain the highest proportion of parafollicular cells (4). 5-HT is known to be contained in, and to be a marker for, parafollicular cells of the sheep thyroid (4, 8). It is concluded that the neural crest-derived parafollicular cells are the site of SBP storage in the thyroid gland. The view that these cells are paraneurons and that they resemble serotonergic neurons in their amine storage mechanism is strengthened.

The authors wish to acknowledge Ms. Laraine Field, Mr. Perry Gershon, and Dr. Thomas Tagliente for their able assistance. They would also like to thank Mrs. Helen Mantulin for her excellent ultrathin sectioning, Dr. Daniel Linkie for the use of his centrifuge, and Dr. Thomas Pretlow for his knowledge and guidance in cell separation techniques.

This project was supported by National Institutes of Health grants NS12969, AM19743, NS12506, and GM07182.

Received for publication 27 June 1980, and in revised form 6 October 1980.

### Table 1

| Material                     | Control                  | TSH-stimulated          | \( P \) |
|------------------------------|--------------------------|-------------------------|--------|
| Intact thyroid gland         | 0.19 ± 0.06*             | —                       | —      |
| Dissociated cells (not incubated) | 0.51 ± 0.04             | —                       | —      |
| Dissociated cells (incubated for 20 min) | 0.66 ± 0.04             | (5) 0.34 ± 0.03         | <0.05  |
| Column fractions             |                          |                         |        |
| -2                           | (7) 0.65 ± 0.04          | (5) 1.09 ± 0.14         | <0.02  |
| -1                           | (2) 0.70 ± 0.02†         | (5) 0.91 ± 0.09         | <0.01  |
| 0                            | (6) 0.78 ± 0.02          | (5) 1.06 ± 0.07         | <0.01  |
| 1                            | (7) 0.86 ± 0.07          | (5) 2.02 ± 0.40         | <0.02  |
| 2                            | (5) 0.68 ± 0.03          | —                       | —      |

* Ratio of cell volumes determined stereologically from electron micrographs (4).
† Range.
§ Pellet too small for quantitation.
FIGURE 8  Electron micrographs of (a) and (b) parafollicular cells observed in fraction 1 from a thyroglobulin-Sepharose column. The dissociated cells had been incubated with TSH before being applied to the column. (c) A corresponding fraction from a control experiment that did not include incubation with TSH. Parafollicular cells (P) are seen; however, note the unstimulated follicular cells (F) that are now numerous in the column effluent. Bars, 1 μm. a, x5,990; b, x3,700; c, x6,780.

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