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Cell Biology

Suspension culture of separated follicles consisting of differentiated thyroid epithelial cells

(ultrastructure/pseudopods/endocytosis/lysosomes)

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ABSTRACT **We have prepared thyroid follicles in suspension culture to use as a model system *in vitro* for investigation of some properties of the thyroid gland. The follicles were free of endothelial cells, fibroblasts, and other nonepithelial cells. They were prepared by collagenase treatment of minced rat thyroid glands followed by differential filtration of the suspension through nylon meshes. Small clusters of principal thyroid epithelial cells were separated from large fragments and single cells. They were cultured in dilute suspension in Coon's modified F-12 medium in dishes coated with agarose to avoid having the cells attach to the dishes. By culture day 3, most of the clusters formed closed follicles containing a periodic acid-Schiff-positive colloid but without a basal lamina. Follicle walls contained an occasional C cell. The epithelium resembled that in the thyroid of a recently hypophysectomized rat, with normal polarity and organelle complement normal with respect to position and abundance, with basally located lysosomes, no pseudopods, and no colloid droplets. The cells were responsive to thyroid-stimulating hormone (thyrotropin) and to dibutyryl cyclic AMP. Thyroid-stimulating hormone at 10 munits/ml resulted in apical migration of lysosomes and formation of pseudopods and colloid droplets within 30 min; longer exposure resulted in depletion of luminal colloid. The results indicate that the suspended follicles resemble follicles *in vivo* with respect to morphology and responsiveness to thyroid-stimulating hormone in the absence of other cell types.**

There have been many studies of the properties of thyroid cells in monolayer cultures (1–4). These studies were generally of mixed cultures of various cell types. Many characteristic functional properties of the thyroid could be demonstrated in some of these preparations but only for a limited time (5–7). However, the shape of the epithelial cells, their organelle complement, and the relationship of the cells to each other show profound differences from the thyroid *in vivo* and such preparations cannot be considered as adequate thyroid models.

It would be useful to have as a model system *in vitro* a preparation consisting of the principal thyroid epithelial cells alone and organized in follicles. Using methods that have some similarities to those introduced by Mauchamp (8), we have succeeded in preparing a suspension of thyroid epithelial cells free of fibroblasts and endothelial cells and arranged as follicles with lumens. In this paper we describe the method of preparation of these follicles and some of their morphologic properties.

MATERIALS AND METHODS

Dissociation. Thyroids were excised from 5- to 6-week-old Fischer rats killed by asphyxiation with CO_2. The glands were freed from surrounding nonthyroid tissues, minced, and washed in Hanks' calcium- and magnesium-free salt solution. The tissue was then placed in tissue culture dishes, at 37°C for 3 hr, in the complete tissue culture medium (described below) containing 1 mg of collagenase (Worthington, CLS II, 179 units/mg) per ml. At the end of the incubation, the tissue was mechanically disrupted by pipetting. Pieces that passed through a 60-μm mesh but were retained on a 15-μm Nitex mesh (Tetko, New York) were cultured.

Monolayer and Suspension Culture. Clusters were plated and incubated without shaking, either on regular 35-mm tissue culture dishes (Costar) or in suspension on agarose-coated dishes at the same cell density. There were about 1000 clusters, each containing about 30 cells, in 2 ml of Coon's modified F-12 medium (National Institutes of Health Media Unit)—containing 0.5% calf serum (Flow), 50 units of penicillin per ml, and 50 μg of streptomycin per ml—in each dish. Other substrates were tried to prevent attachment of clusters, including bacteriological plastic dishes and Teflon-, silicon-, or gelatin-coated dishes. None of these was satisfactory. Medium without serum was also used where indicated. In this case the Coon's modified F-12 was supplemented with insulin (Sigma) at 10 μg/ml, transferrin (Sigma) at 5 μg/ml, and trace elements as described by Hutchings and Sato (9). Medium was changed every third day by centrifuging the follicle suspension at 200 $\times$ *g* for 5 min and resuspending it in fresh medium.

Agarose-Coated Dishes. Agarose (Sigma), at a concentration of 0.5% in H_2O, was heated for 30 min at 100°C. While still hot, 1 ml of the solution was added to each 35-mm plastic tissue culture dish and allowed to sit for about 1 min. The agarose solution was then aspirated and the dishes were left at room temperature until the remaining thin layer of agarose solidified. Dishes were washed with medium before use.

Thyrotropin and Dibutyryl Cyclic AMP. Bovine thyrotropin (National Pituitary Agency, 3.5 international units/mg) or dibutyryl cyclic AMP (Sigma) was added to some 3-day-old cultures. The thyrotropin was dissolved in 0.1% bovine serum albumin (Calbiochem, A grade) and was present in the medium at a concentration of 10 munits/ml; the bovine serum albumin was present at 10 μg/ml. The dibutyryl cyclic AMP (Sigma) was present at 0.1 mM. Cultures were incubated at 37°C for 30 min and then processed for electron microscopic examination.

Histologic Techniques. Clusters were fixed for 15 min, in suspension, in 2.5% glutaraldehyde (Ladd or Tousimis) in 0.1 M cacodylate buffer at pH 7.3, postfixed in 1% OsO_4 (Fisher) in the same buffer for 20 min, rinsed in H_2O, stained *en bloc* for 1 hr with 1% aqueous uranyl acetate, and dehydrated with a graded series of ethanols. After absolute ethanol, Epon 812 was directly added for embedding. Solutions were changed by centrifugation at 1200 $\times$ *g* for 2 min after each step.

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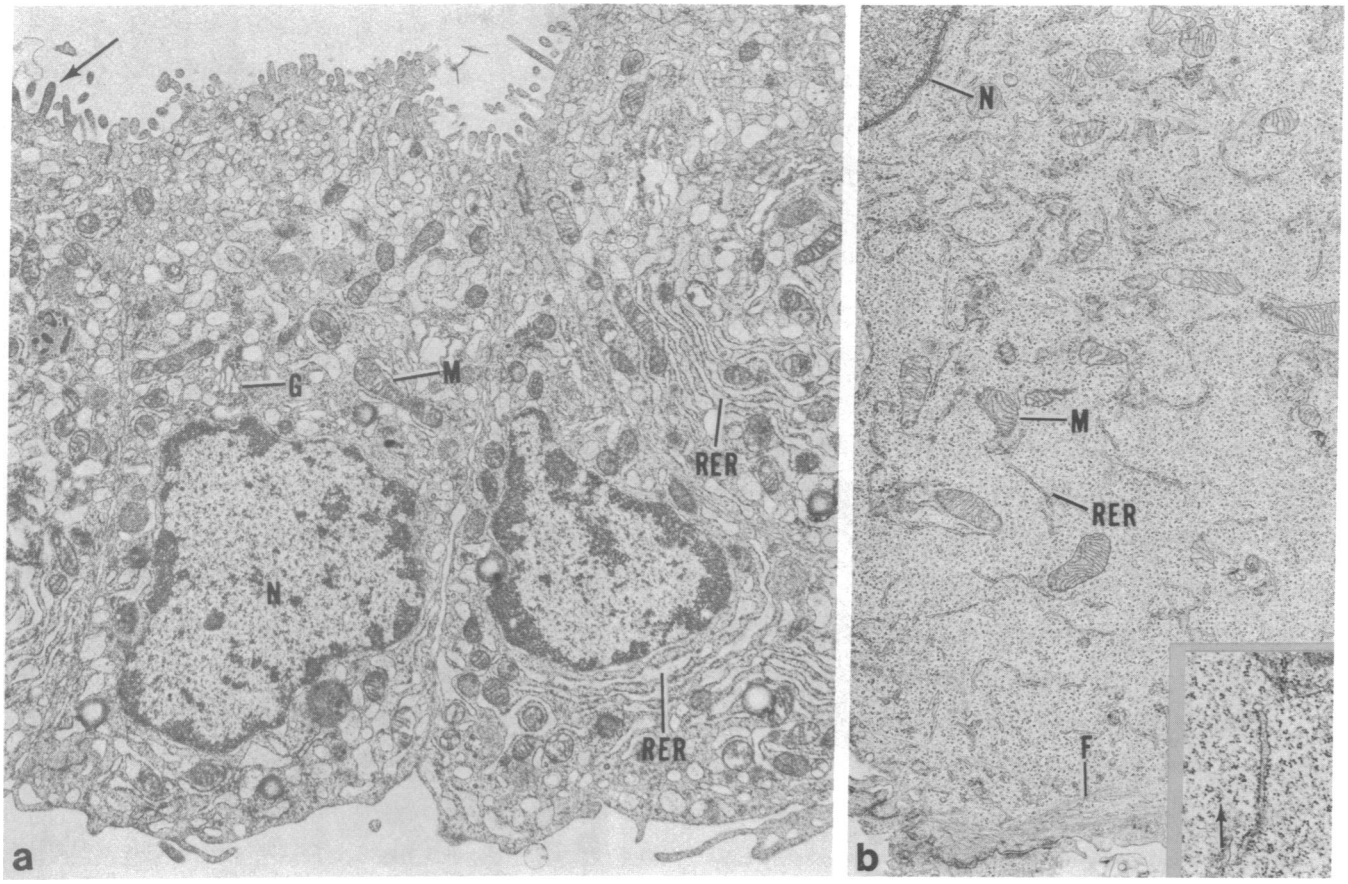

FIG. 1. (*a*) Part of a cluster of epithelial cells from a freshly dissociated thyroid gland. The cells are polarized with microvilli (arrow) and Golgi apparatus (G) apical to the nucleus (N). Mitochondria (M) and rough endoplasmic reticulum (RER) are abundant. A basal lamina is absent. (×8000.) (*b*) Part of a principal thyroid epithelial cell after 3 days in monolayer culture (section parallel to the dish). The organelle complement has changed radically from that in *a*. Cisternae of the rough endoplasmic reticulum (RER) are very narrow with a relatively dense matrix. The cytoplasm has many free ribosomes, largely arranged in clusters (polyribosomes). Many mitochondria (M) are present. At the edge of the cell, microfilaments (F) and a tight junction with a neighboring cell are seen. (×6800.) (*Inset*) Detail of the rough endoplasmic reticulum and polyribosomes (arrow). (×16,000.)

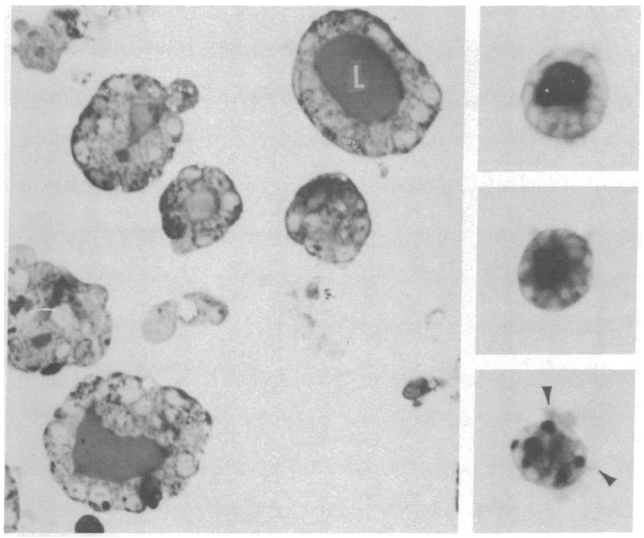

FIG. 2. (*Left*) Thick section of cell clusters, now with closed follicles, after 3 days in suspension culture. The density of lumens (L) varied from follicle to follicle. Lumens are separated from the medium by a layer of cells that may be more than one cell thick in places. (×390.) (*Right*) Whole follicles stained by the periodic acid-Schiff method. The top follicle has a single lumen with a smooth boundary. The bottom follicle has several small lumens (arrowheads). The middle follicle has a lumen with irregular shape suggestive of the recent fusion of tiny lumens with a central larger lumen. (×420.)

Thick sections were stained with toluidine blue for light microscopy; thin sections were stained with uranyl acetate and lead citrate and examined in a Philips 201C electron microscope.

Periodic Acid-Schiff Staining. After 3 days of suspension culture, follicles were plated on glass coverslips; 3–4 hr later, when clusters had attached, they were fixed in Bouin's fluid and stained by the periodic acid-Schiff method.

Cell Counting. Dissociated tissue was plated on regular tissue culture dishes. Eighteen hours later, cells were fixed and stained. The number of clusters per dish and of cells per cluster were determined by light microscopy. In addition, follicles from suspension culture were dissociated by mild tryptic digestion and the cells were counted in a Coulter Counter.

RESULTS

Dissociation. Thyroid glands were well dissociated by collagenase digestion. The suspensions used consisted of epithelial cells in clusters averaging 30 cells, each cluster being a broken follicle or a portion thereof. Usually, no intact follicles were present. The cells of the clusters maintained the polarization and the organelle complement (Fig. 1*a*) characteristic of the principal thyroid epithelial cell. Some endothelial cells were also present, either in clusters by themselves or attached to the clusters of follicular cells.

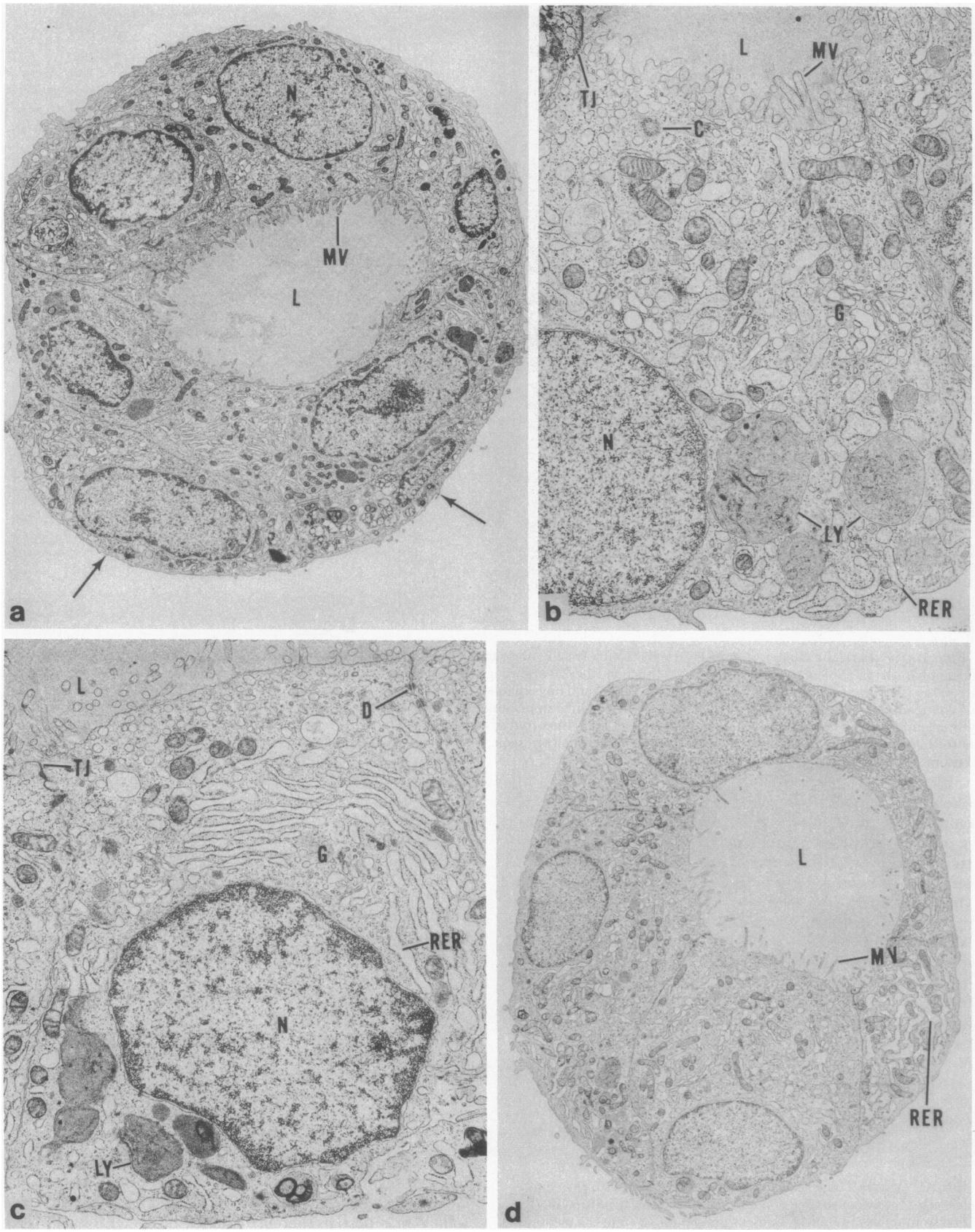

FIG. 3. (*a*) Section of a follicle formed from a cluster of epithelial cells in suspension culture for 3 days. The follicle resembles one *in vivo* in that the cells are polarized with microvilli (MV) extending into a lumen (L) with an electron-dense matrix. Nuclei (N) tend to be basal. There are two cells (arrows) that may be making a double layer. (×3500.) (*b*) Higher magnification of a cell from a follicle similar to the one in *a*. This cell is polarized as *in vivo*. Tight junctions (TJ), microvilli (MV), and centriole (C) are apical, the Golgi apparatus (G) is near the apical end of the nucleus, and the nucleus (N) is basal. The rough endoplasmic reticulum (RER) is somewhat more apical than *in vivo*. Lysosome-related

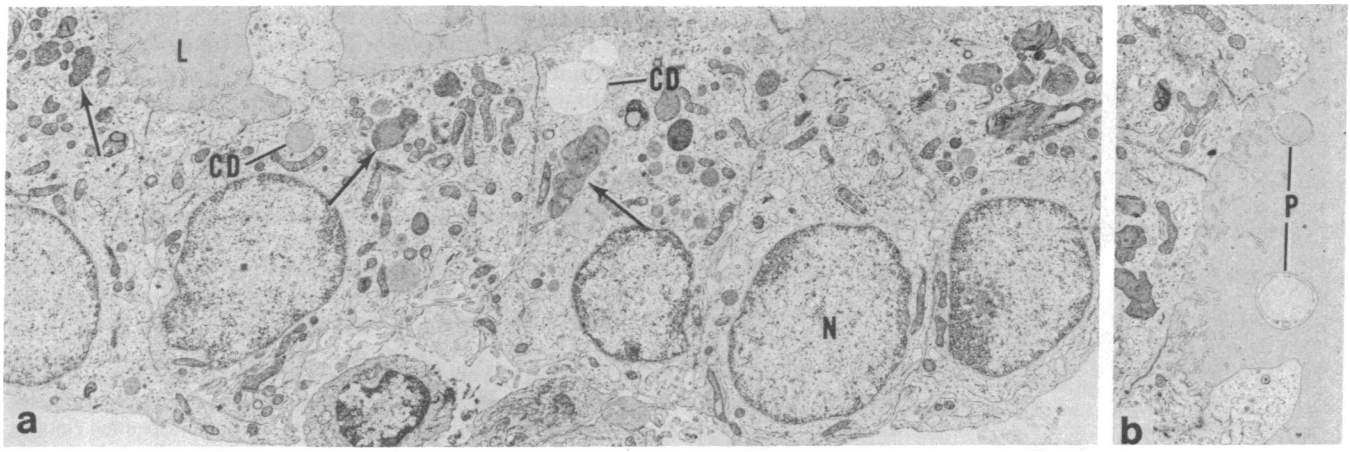

FIG. 4. Parts of follicles stimulated with thyrotropin (10 munits/ml) for 30 min. (*a*) Lysosome-related structures (arrows) have migrated toward the apex and colloid droplets (CD) have been formed. (×4700.) (*b*) Sections of newly formed pseudopods (P) containing colloid droplets are seen in the lumen. (×4700.)

Monolayer Cultures. When the clusters were plated on regular tissue culture dishes at a density of about 3×10^4 viable cells per 35-mm dish, the cells attached and spread. By 3 days they were flat, especially at the periphery of the clusters. No mitotic figures were seen. Cells were no longer organized into follicles, and not only was their shape changed radically but there was also a gross modification of their organelle complement and the way it was arranged (Fig. 1*b*).

Follicle Reorganization in Suspension Culture. In agarose-coated dishes, closed follicles formed from clusters within 1 day. By 3 days, lumens had enlarged and could be seen by phase-contrast microscopy (data not shown). Most lumens were surrounded by a single layer of cells, but in some places additional cells were evident (Fig. 2). Whole follicles stained by the periodic acid–Schiff method most frequently showed a single lumen, although often there was more than one (Fig. 2 *Right*) and occasionally none was apparent.

Electron Microscopy. Follicles appeared to be composed essentially of epithelial cells. Fibroblasts were never seen in culture. The fate of the fibroblasts is not really known, but it is probable that many became separated by the collagenase treatment and passed through the 15-μm filter. Endothelial cells, initially present, disappeared from cultures within 2 days. Only a few C cells were ever seen in the follicle wall. In follicles maintained in suspension for 3 days, an electron-dense lumen was surrounded by polarized epithelial cells, with microvilli at the apical surface (Fig. 3*a*). At higher magnification tight junctions at the apical end were seen (Fig. 3*b*). Small apical microvesicles with pale content were frequently present. Endoplasmic reticulum was abundant. The Golgi apparatus had an apical location and lysosomes were predominantly basal (Fig. 3*b*). Other dense vesicles, different from the usual lysosome with respect to shape, size, and matrix, were present. They have been shown to be acid phosphatase positive (unpublished observations) and were basally located. A basal lamina was absent (Fig. 3*b*).

Even after 12 days of culture, most cells presented similar ultrastructural features (Fig. 3*c*), although many cell profiles were observed with a reduced amount of rough endoplasmic reticulum (data not shown). The relative abundance of organelles was variable from cell profile to profile.

Follicles also formed in the absence of serum. After 3 days in culture without serum, cell polarization and organelle complement were like those in cells cultured in the presence of added serum although there was usually a lower abundance of rough endoplasmic reticulum (Fig. 3*d*).

Thyrotropin and Dibutyryl Cyclic AMP Stimulation. Follicles cultured in suspension for 3 days responded to acute and to chronic thyrotropin treatment as *in vivo* (10–12). No pseudopods or colloid droplets were seen in unstimulated cells but, within 30 min after stimulation, pseudopods and colloid droplets were observed in many cells (Fig. 4). Also lysosome-related vesicles migrated toward the apex of the cells from the basal area where they were usually located (Fig. 4*a*). Chronic exposure to thyrotropin resulted in depletion of the luminal contents and narrowing of the lumens (Fig. 5). Results with a 30-min exposure to dibutyryl cyclic AMP (not illustrated) were similar to those with thyrotropin as in similar studies done with dog thyroid slices (13).

DISCUSSION

The present results show that it is possible to obtain clusters of principal thyroid epithelial cells arranged as separated thyroid follicles and to maintain them in suspension culture for 12 days. The yield of cells is low, of the order of 2%, but adequate for examination and clarification of many biologic questions.

Nature and Origin of the Follicles. The follicles generally are largely composed of a single layer of epithelial cells surrounding a periodic acid-Schiff-positive lumen. Such follicles are probably formed by the resealing of a follicle lumen. In those cases in which there was a double layer of cells in the follicle wall, the outer cells were polarized in a direction opposite to the other cells (microvilli toward the medium). Such cells may be remnants of a neighboring follicle *in vivo* whose

structures (LY) are predominantly basal. Note the absence of a basal lamina. (×11,300.) (*c*) Cell from a follicle in suspension culture for 12 days. This cell is similar to that in Fig. 3*b* but is exceptional in having an unusually prominent rough endoplasmic reticulum (RER) located in a relatively apical area. Labels as in *b*. D, desmosome. (×11,300.) (*d*) Follicle formed after putting an open cluster of epithelial cells in suspension culture for 3 days in the absence of serum. It resembles a follicle formed in a medium containing serum in that it has normally polarized cells and a lumen (L) with an electron-dense matrix. The rough endoplasmic reticulum (RER) is very variable, in amount and character, from cell to cell. There is generally less than in follicles maintained in suspension culture in a medium containing serum. (×3700.)

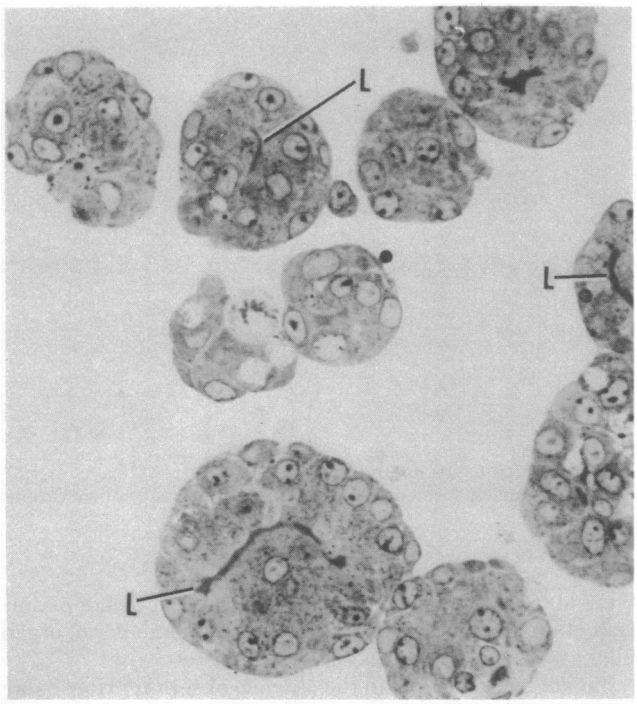

FIG. 5. Sections of cell clusters in suspension culture for 3 days and exposed to thyrotropin for the last 2 days. Note that the lumens (L) have narrowed to form slits, similar to their appearance in chronically stimulated thyroid glands *in vivo*. (×540.)

cells did not detach completely during the dissociation procedure, probably because the two follicles were not separated by a basal lamina (14). There were also clusters containing several tiny lumens. These tiny lumens are probably new lumens formed by the rearrangement of epithelial cells, possibly in a way similar to that observed in embryologic development (15).

The follicles strongly resemble those *in vivo*. The cells have normal polarity and, after several days in culture, an organelle complement like that of the thyroid epithelium of a recently hypophysectomized rat with respect to the basal location of lysosomes (10, 11) and the decreased abundance of rough endoplasmic reticulum (16). The cells respond to thyrotropin with morphologic changes similar to thyroid epithelium *in vivo*: apical migration of lysosomes, formation of pseudopods and colloid droplets (10, 11), and, later, narrowing of the follicular lumen (12). None of these effects requires the presence of nonfollicular cells such as endothelial cells or fibroblasts.

The cells in follicles in suspension culture can therefore be considered a good model for the cells *in vivo*, with respect to both morphology and responsiveness. They are strikingly different from duplicate cells spread in monolayer culture in the same culture medium. One hypothesis to explain the difference is that spreading itself changes the cells (17). Consistent with this is the observation that, if the monolayer culture is seeded at high density, there is less or no accompanying loss of some markers for the differentiated state and there is also much less spreading (18). The extent to which spreading or cell shape affects organelle complement requires further study.

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