LOCALIZATION OF ESTERASE AND ACID PHOSPHATASE IN GRANULES AND COLLOID DROPLETS IN RAT THYROID EPITHELIUM

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ABSTRACT

Droplets which stain like colloid occur in the cytoplasm of the thyroid follicular epithelium of the rat following stimulation of the gland by thyroid-stimulating hormone (TSH). The occurrence of droplets was remarkably reduced when the lumen became depleted of colloid. Acid phosphatase and esterase were localized in the thyroid droplets and, in addition, in granules largely around the nucleus. Stimulation by TSH resulted in an increase in the number of droplets containing enzyme. Twenty-four hours after hypophysectomy, enzyme-associated granules were localized at the basal end of the cell and droplets were absent. Intravenous injection of TSH resulted in formation of droplets at the apical end of the cell and migration of enzyme-associated granules toward the apical end of the cell. The droplets were first observed approximately 10 minutes after TSH administration and at this time did not appear to contain enzyme. Within 15 minutes many droplets contained enzyme. The granules were largely localized near the nucleus on its apical side 30 minutes after a dose of 25 milliunits of TSH, but were less well localized following one-tenth this dose. These results indicate that the epithelial cell of the thyroid gland contains preformed hydrolytic enzymes associated with granules (lysosomes). When the gland is stimulated by TSH, droplets are formed from colloid derived from the lumen (phagosomes), and hydrolytic enzymes are transferred from granules to the droplets. The droplets may be intracellular organelles for hydrolysis of colloid and liberation of thyroxine prior to the release of thyroxine into the blood.

INTRODUCTION

Droplets of a material which stains like thyroid colloid occur in the cytoplasm of epithelial cells of the thyroid gland (1–11). There are two hypotheses about the origin of these droplets. Some investigators have suggested that the droplets may be freshly synthesized colloid made in the cell prior to secretion into the lumen of the follicle (4, 5, 11). Others have suggested that the droplets may contain colloid derived from the lumen of the follicle (1–3, 8–10). Using the classical methods of histology it did not seem possible to decide whether one or both of these hypotheses are correct.

Recently studies using 2 new techniques have shed new light on this problem (8–10). The first of these techniques is autoradiography. The study makes use of the observation that at short time intervals after injection of radioiodide the luminal colloid of the follicle becomes labeled but not the

epithelial cell (12). After the colloid in the lumen is prelabeled with radioiodine and further formation of protein-bound radioiodine blocked, if droplets are induced by injection of thyroid-stimulating hormone (TSH) they appear to be labeled (8–10). This suggested that the droplets contain material derived from the lumen of the follicle. Further details of this type of investigation will be published in another paper.

The second of these techniques is the histochemical localization of the hydrolytic enzymes, esterase and acid phosphatase. The study makes use of the findings that large molecules can enter a variety of living cells by pinocytosis (13–17), and possibly by other mechanisms. These molecules can enter relatively intact. For example, intravenously administered horseradish peroxidase can be taken into many kinds of cells and become localized in droplets and still retain enzymatic activity (18–20). In the kidney such droplets containing foreign proteins (phagosomes) also have associated hydrolytic activity and contain enzymes such as acid phosphatase (21) and esterase (22) which also occur in lysosomes (23, 24).

We are reporting here some studies of intracellular colloid droplets in the thyroid gland including a study of the extent to which these droplets resemble phagosomes or droplets containing engulfed foreign material found in the kidney and in other tissues.

MATERIALS AND METHODS

Male Fischer rats, 2 to 3 months of age and weighing 150 to 250 gms, were used. They had been fed Purina Laboratory Chow since weaning. Some rats were fed a moderately low iodine stock diet containing 0.25 per cent thiouracil (25).

Animals were ordinarily hypophysectomized through the parapharyngeal approach. However, in one study the rats were hypophysectomized through the ear (26). Completeness of operation was ascertained by examination of the region of the sella turcica with a microscope at a magnification of 8.

The thyroid-stimulating hormone (TSH) used was generously supplied by Dr. Robert W. Bates and Dr. Peter G. Condliffe of the National Institutes of Health. It was prepared from transplantable pituitary tumors in the mouse and had a potency of 1.3 international units per milligram. The hormone preparation was dissolved in an aqueous solution containing 0.1 per cent bovine serum albumin. The TSH in 0.20 ml of solution was injected into the external jugular vein while the rat was lightly anesthetized with ether.

Histologic Procedures

To obtain samples of thyroid tissue, the rats were anesthetized with ether and bled from the heart. The thyroid gland was then removed with the trachea and placed in fixative at 4°C. When tissues were to be stained by the periodic acid–Schiff procedure with hematoxylin counterstain, they were fixed in Bouin's fluid for 18 hours. In preparation for enzyme localization studies, the tissues were fixed overnight in cold 10 per cent formalin containing 2 per cent calcium acetate. Then the fixed glands were dehydrated for ½ hour in each of two changes of 80 per cent alcohol followed by two similar changes of 95 per cent alcohol and subsequently of absolute alcohol. The alcohol dehydration steps and the first of two ½ hour changes in petroleum ether were carried out at 4°C but the second change in cold petroleum ether was left for ½ hour at 24 (±2)°C. The glands were then embedded for 15 minutes in paraffin at 60°C in a vacuum oven. Sections cut at 8 μ thickness from these blocks were stained by a variety of histochemical methods.

The periodic acid–Schiff (PAS) stain (27) was employed to visualize the polysaccharide component of the thyroid luminal colloid and epithelial droplets. A variant of this stain, utilizing a Schiff reagent prepared with toluidine blue (28) rather than basic fuchsin, was employed as a means of staining the polysaccharide of thyroid colloid and droplets blue instead of red. Sections were also stained for 20 minutes with ripened aldehyde fuchsin (29) after a 2 minute oxidation with a solution containing equal parts of 0.5 per cent $KMnO_4$ and 0.5 per cent H_2SO_4 (30). This permanganate-aldehyde fuchsin (PMAF) method has been found to color neutral polysaccharides purple and to stain intracellular bodies in epithelia of aged animals (31).

Consecutive sections were stained for acid phosphatase by the method of Burstone (32–34), employing Naphthol AS-BI phosphate as substrate and fast red violet LB salt as coupling agent in acetate buffer at pH 5.2. Incubation times of 1 to 5 hours were used for acid phosphatase. Additional sections were stained for non-specific esterase using Naphthol AS-D acetate as substrate (33, 34) in conjunction with several diazonium salts in Tris buffer at pH 7.1. Blue BBN salt yielded a blue reaction product, whereas fast red violet LB salt or Garnet GBC afforded red staining (32). Incubation times of 45 minutes for esterase were employed. In some instances, sections were stained by the periodic acid–toluidine blue-Schiff technique after the red esterase or the red acid phosphatase stain; in others, they were stained by the conventional PAS method following

the blue esterase stain as a means of distinguishing the localization of the enzyme and the luminal and epithelial polysaccharide material in the same section.

As a technical variant for normal or stimulated glands from intact rats, fresh frozen 5 to 8 μ cryostat sections fixed in acetone were employed (33) with the modification that sections were first immersed for 10 minutes at room temperature in absolute acetone and then for 2 minutes in 95, 80, and 70 per cent acetone, sequentially. The slides were then incubated in the enzyme substrate media, as were the sections of cold, formalin-fixed, embedded tissue. The localization of the esterase and the acid phosphatase were the same for both methods. However, because of ease of handling, most of the work was done using the formalin-fixed, paraffin-embedded material.

RESULTS

PAS Reaction in Normal Rats and the Response to TSH

The normal thyroid gland contains PAS-positive material (colloid) in the lumen of follicles and also occasionally in intracellular droplets (Fig. 2). These intracellular droplets vary in size from barely visible to the size of the cell nucleus. One hour after injection of TSH almost all follicles had colloid in the lumens and the epithelial cells of almost all follicles had PAS-positive droplets (Fig. 3). There was considerable variation in response from cell to cell and from follicle to follicle. Three and one-half hours after injection of 0.25 units of TSH the lumens of many follicles were depleted of colloid. The epithelial cells of these empty follicles were almost entirely free of colloid droplets. On the other hand, the epithelial cells in those follicles still containing luminal colloid had abundant colloid droplets (Fig. 4). The staining of droplets and luminal colloid using the permanganate-aldehyde Schiff treatment duplicated that using the PAS treatment.

Distribution of Esterase and Acid Phosphatase in the Normal Thyroid Gland and Its Response to TSH

Esterase and acid phosphatase activities found in the epithelial cells of the thyroid gland were localized in droplets and in granules, but no activitiy was visible in the colloid in the lumen of the follicle (Fig. 5). It was generally but not always possible to decide whether a particular small esterase-stained object was a granule or a small droplet. However, the granules were not just non-specific deposits of dye crystals, since at high magnification, as shown below, they were spheroids with smooth surfaces. Three hours after injection of 250 milliunits of TSH the incidence of droplets containing each of these enzymes was greatly increased (Fig. 6).

Tests for identity of the PAS-positive droplets and the enzyme-containing droplets by staining sections sequentially, first for esterase and then PAS, showed that many droplets were positive for both staining procedures (Fig. 7). There was some tendency for droplets toward the apical end of the cells to have a weaker etserase or acid phosphatase reaction than those located more basally. Some of the apically located PAS-positive droplets did not show a visible enzyme reaction, whereas some of the droplets at the basal end of the cell showed an enzyme reaction but were not clearly PAS-positive.

Effect of Thiouracil Feeding on the Thyroid of the Intact Rat

Thiouracil feeding yielded results similar to those found with TSH injection, except that a longer time-interval was required for the effects to appear. With 2 days of feeding there was evidence of colloid resorption. By 4 days most follicles were depleted of colloid. Follicles containing colloid in the lumen had abundant PAS-positive intracellular droplets, whereas those depleted of luminal colloid showed intracellular droplets relatively rarely, and the few droplets present were usually abnormally small. In such follicles a fine PAS-positive "powder" was observed in the cells. With the reaction for esterase (Fig. 8) or for acid phosphatase (Fig. 9), there was strong staining of the droplets in the follicles containing colloid in their lumens and enzyme-containing granules were relatively rare, whereas in follicles depleted of colloid in the lumen enzyme-associated droplets were relatively rare and enzyme-associated granules were abundant.

Effect of Hypophysectomy

It is well known (7) that as early as one day after hypophysectomy intracellular colloid droplets are absent from the thyroid epithelium (Fig. 1). At 6 hours after hypophysectomy through the parapharyngeal approach the incidence of intra-

cellular droplets was much increased above normal. This appeared to be a result of the operative procedure rather than a result of removal of the pituitary gland, since the increase in numbers of droplets also occurred in the sham-operated rats. On the contrary, no droplets were observed 6 hours after hypophysectomy performed through the ear.

Although intracellular colloid droplets were absent one day after hypophysectomy, the epithelial cells still yielded a strong staining reaction for esterase and acid phosphatase. The enzymes were associated with granules localized largely at the basal end of the cell (Figs. 10 and 14). Basally located granules in companion sections stained weakly with the PAS procedure.

Effects of TSH in the Hypophysectomized Rat

To study the properties of the newly formed droplets, we have examined droplets in thyroid epithelium following the injection of TSH into the hypophysectomized rat. The droplets present must have been formed following the TSH injection. Within 10 minutes after intravenous injection of 25 milliunits of TSH into hypophysectomized rats, PAS-positive droplets appeared in the follicular epithelial cells at their apical end. These droplets showed no staining with the acid phosphatase or esterase techniques (Fig. 11). At this time, most of the enzyme-staining granules remained at the basal end of the cells, but a few were more apically located, some close to newly-formed droplets. Within 15 minutes after TSH injection the enzyme-associated granules were no longer localized at the basal end of the cell but were now largely more centrally placed at the sides of the nuclei (Fig. 12). Many intracellular droplets were now stained by the procedures used for detecting esterase and acid phosphatase. By 30 minutes after TSH injection most of the esterase-positive granules were localized in the apical half of the epithelial cells (Fig. 13). At 30 minutes after injection of a smaller dose of TSH (2.5 milliunits) the enzyme-associated granules were less sharply localized, being distributed relatively uniformly in the cells (Fig. 15), and were more similar in their distribution to those in the thyroid of the normal rat (Fig. 7).

Fate of Droplets

Intracellular colloid droplets had a short lifetime. They were not present 6 hours after hypophysectomy through the ear. The time interval after injection of TSH during which abundant droplets were found depended upon the dose of TSH. For example, with a dose of 25 milliunits of TSH the peak droplet response occurred at approximately 1 hour, and by 3 hours the occurrence of intracellular colloid droplets was no greater than in uninjected controls. With 250 milliunits of TSH the peak response was at approximately 3 hours, and droplets were largely gone by 7 hours. At the time the droplets disappeared, enzyme-associated granules were present in abundance.

Localization of Esterase in Droplets

The reaction product from esterase activity was localized partially within the droplet, but also in the wall of the droplet, for the staining of the droplet appeared uniform. If reaction product were localized solely in the droplet surface, the droplet would have appeared stained most darkly at its edge. However, reaction product was localized in the membrane as well as the interior of the drop. For in cases where the droplets were

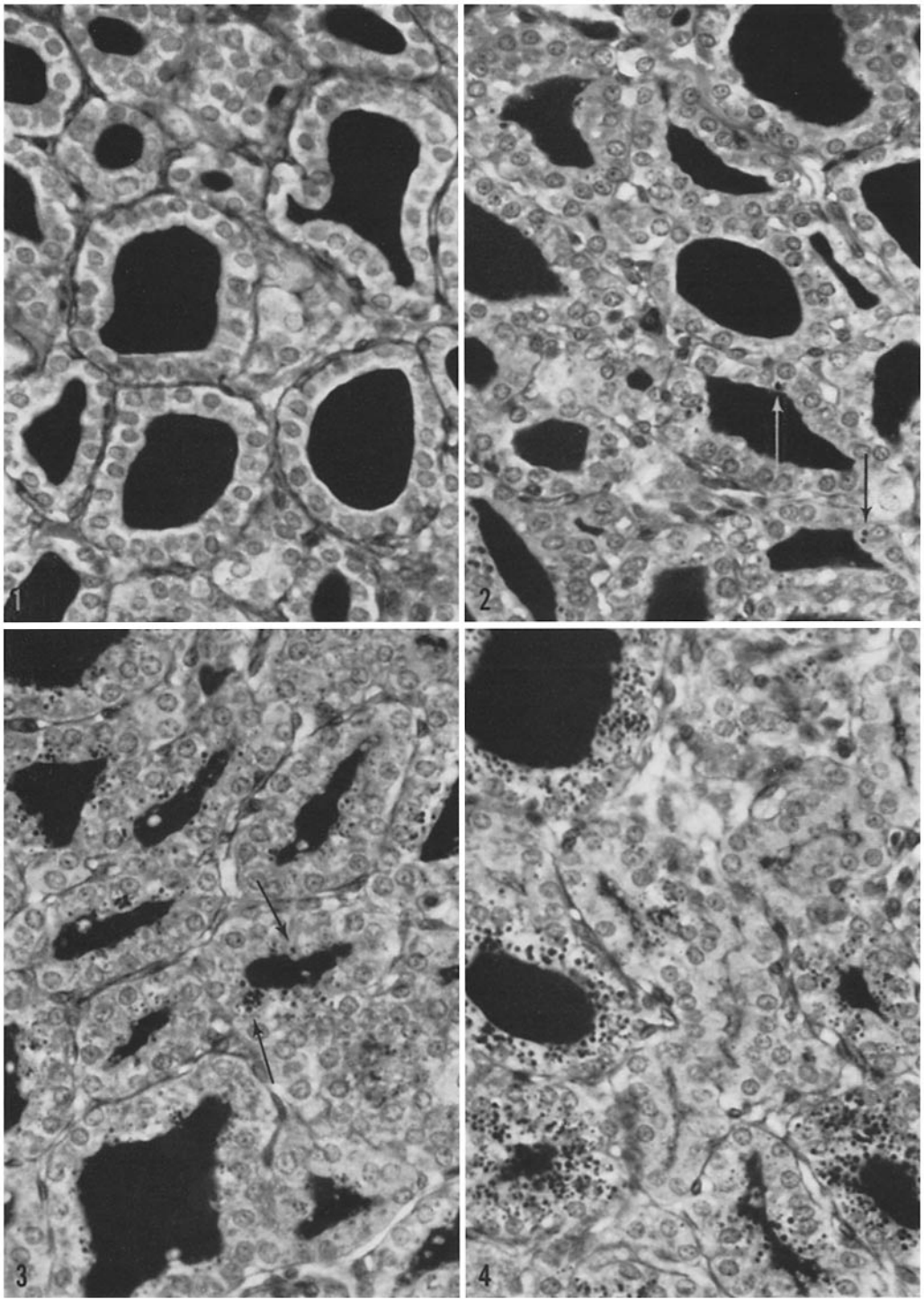
FIGURES 1 to 4 Intracellular colloid droplets in the thyroid gland of the rat. Tissues fixed in cold Bouin's fluid, stained by the periodic acid–Schiff method, and counterstained with hematoxylin. Photographed using green filter. × 500.

Fig. 1, one day after hypophysectomy. Note absence of intracellular droplets.

Fig. 2, normal intact rat. Note occasional intracellular droplets (arrows).

Fig. 3, one hour after intravenous injection of 250 milliunits of TSH. Almost all follicles have intracellular droplets (arrows) in some cells. Note nonuniformity of response.

Fig. 4, 3½ hours after intravenous injection of 250 milliunits of TSH. Follicles with colloid in the lumen have epithelial cells containing many colloid droplets. In follicles in which the luminal colloid is almost exhausted, intracellular colloid droplets are rare.



poorly preserved and had lost their contents, the droplets were PAS-negative in the interior, but reaction product stained the droplet membrane (Fig. 16).

DISCUSSION

Origin of Droplets

The present results are consistent with the hypothesis that in the thyroid gland the intracellular PAS-positive droplets contain colloid derived from the lumen of the follicle and that they should be considered to be phagosomes (24). When droplets first appeared in response to TSH injection, they were localized at the apical end of the cell, in agreement with reports by other investigators (5, 8). They appeared during the phase of colloid resorption from the lumen of the follicle (in agreement with observations *in vivo* by Williams, references 35 and 36), *i.e.* shortly after TSH injection, but then only if there was colloid in the lumen of the follicle. After colloid was depleted from the lumen of the follicle, intracellular PAS-positive droplets were relatively rare.

The mechanism by which colloid from the lumen gets into the droplets is not known. It is possible that colloid enters the cell by pinocytosis, as observed *in vivo* by Williams (35, 36). The large size of some droplets cannot be used to exclude this mechanism since the osmotic properties of droplets have not been studied. The droplets may swell during fixation so that the diameter of the droplets *in vivo* cannot be estimated from those in histologic sections. It is also possible that the droplets are formed by coalescence of numerous submicroscopic pinocytotic vacuoles. Coalescence of pinocytotic droplets in other tissues has been reported (13, 14, 17, 24).

Consistent with this hypothesis of pinocytosis is the observation of the strong localization of thyroid antibodies at the apical surface of the epithelial cell, suggesting an adsorption of colloid at this site. Bennett (37) has postulated that a preliminary stage in the pinocytosis of proteins is the adsorption of the protein to the cell membrane, and Brandt (38) has observed such an adsorption during pinocytosis in amoebae. The strong fluorescent antibody staining of the colloid at the apical cell surface in the thyroid (39–42) is remarkably similar to that observed during uptake of fluorescent proteins in the ameba.

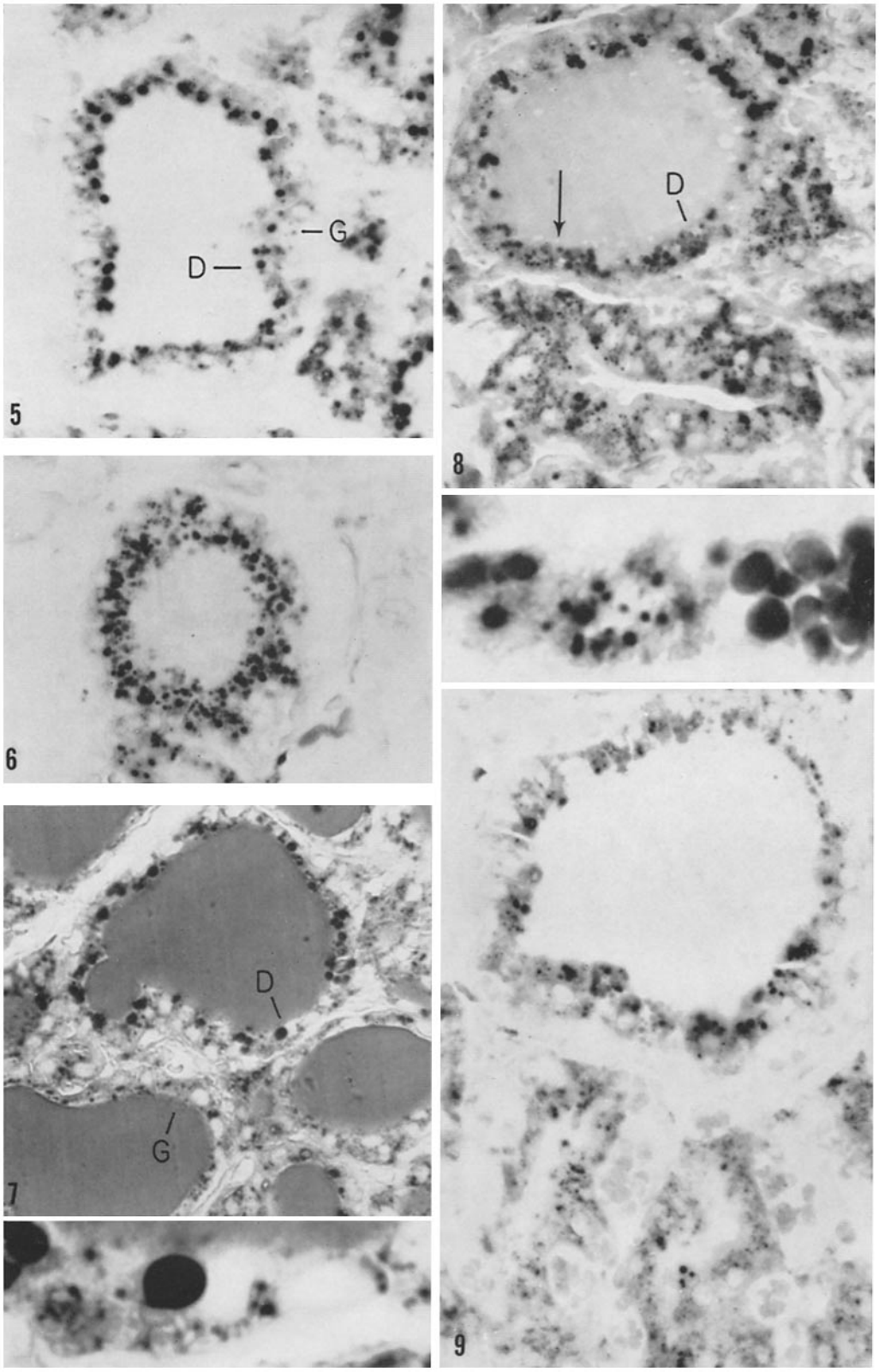
FIGURE 5 Localization of esterase in droplets (*D*) and granules (*G*) in the thyroid epithelium of a normal rat. Note absence of staining in the lumen. In all esterase illustrations, Naphthol AS-D acetate was employed as substrate, and the Blue BBN salt was used as diazonium coupling agent. No nuclear counterstain was used in enzyme preparations. Nuclei appear pale in the illustrations. Orange filter. × 500.

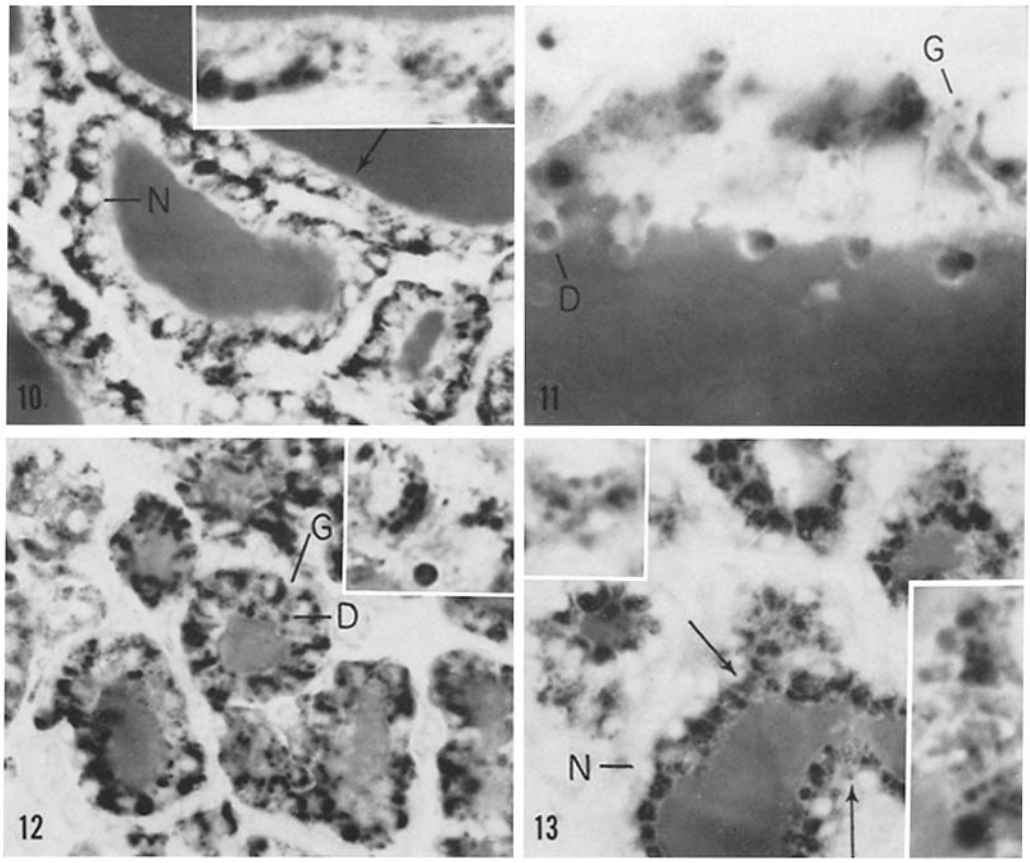
FIGURE 6 Localization of esterase in droplets and granules of a follicle from the thyroid gland of an intact rat 3.5 hours after injection with 0.25 units of TSH. Orange filter. × 500.

FIGURE 7 Localization of esterase activity in the thyroid epithelium of a normal rat. Section counterstained with PAS. Esterase activity is localized in droplets (*D*) and granules (*G*). Many droplets were doubly stained (purple), showing that PAS-positive droplets contained esterase activity. The small area near (*D*) is enlarged in the inset to show granules. Orange filter. × 500; inset × 2000.

FIGURE 8 Localization of esterase activity in the thyroid epithelium of a rat fed thiouracil for 4 days. Esterase is localized in droplets and granules. Some esterase-associated droplets occur in the epithelium of follicles from which the colloid was exhausted even though the droplets do not yield a PAS reaction. Note the occurence of a cluster of droplets (*D*) with abnormally small amounts of reaction product. These droplets were PAS-positive and were also clearly stained by the esterase reaction. A small area (*arrow*) is enlarged in the inset to illustrate granules and possibly small droplets. Orange filter. × 500; inset × 2000.

FIGURE 9 Localization of acid phosphatase in the follicular epithelium of the thyroid gland of a rat fed thiouracil for 4 days. Reaction product is localized in granules and droplets. In acid phosphatase illustrations, Naphthol AS-BI phosphate was employed as substrate and the fast red violet LB salt as coupling agent. Green filter. × 500.





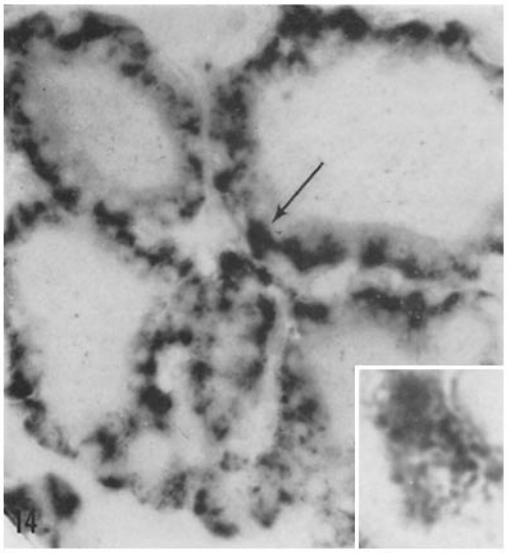
FIGURES 10 to 13 Effect of intravenous injection of 25 milliunits of TSH on the intracellular localization of esterase activity in the thyroid gland of the rat one day after hypophysectomy. Tissues fixed in cold formalin containing 2 per cent calcium acetate. Stained by esterase procedure of Burstone followed by PAS reaction, and photographed using orange filter.

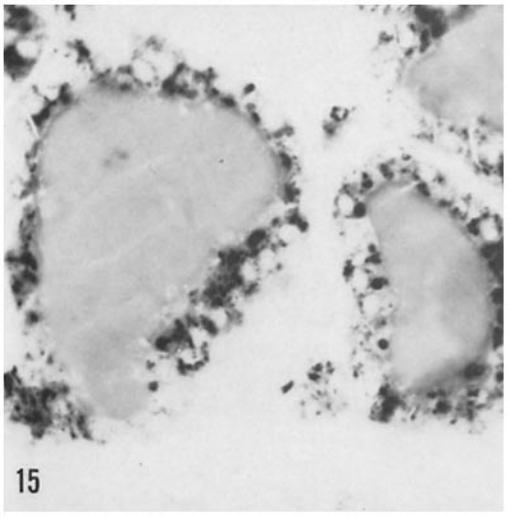
FIGURE 10 Uninjected rat. Note basal localization of reaction product, largely in granules. A small area (arrow) is enlarged in the inset to illustrate granules. (Areas of solid stain appear to be due to fusion of reaction product). No intracellular colloid droplets are present. Nuclei (*N*) are unstained. × 500; inset × 2000.

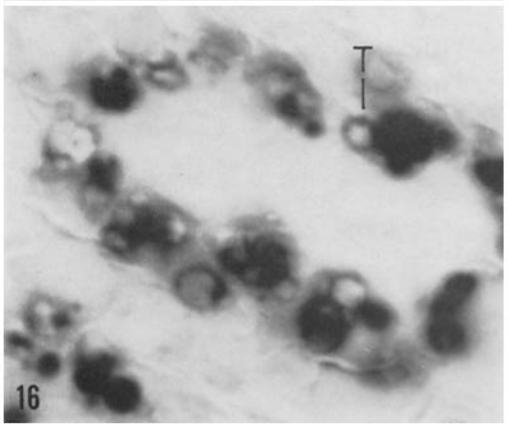
FIGURE 11 Ten minutes after injection of TSH. Reaction product is localized in granules (*G*) still largely at the basal end of the cell. Intracellular colloid droplets (*D*) are present in cytoplasmic projections into the lumen at the apical ends of some cells. These droplets do not contain reaction product. Some granules containing reaction product are at the apical ends of the cells, even in the cytoplasmic projections. × 2000.

FIGURE 12 Fifteen minutes after injection of TSH. Reaction product is localized largely in streaks (*G*) which can usually be resolved into granules (as in inset) located lateral to the nuclei and on the apical and the basal sides of nuclei. Reaction product is also present in droplets (*D*) at the apical end of some of the epithelial cells. × 500; inset × 1250.

FIGURE 13 Thirty minutes after injection of TSH. Reaction product is localized at the apical end of the cell especially near the nucleus (*N*). Reaction product can be largely resolved into granules and droplets as in the insets for the areas designated by arrows. × 500; insets, × 2000.







Esterase and Acid Phosphatase Localization

Esterase and acid phosphatase were localized in both granules and droplets. Results with the two enzymes differed only in that localization of the reaction product of esterase was crisper than that of acid phosphatase.

Other investigations of the localization of esterase and acid phosphatase in the thyroid gland have been made (43–47), but the authors did not mention localization of the enzymes in droplets. (However, Sobel's photomicrographs (46) show intracellular localization of enzyme and some structures which may be droplets.) This may be due to the fragility of the droplets. For example, droplets were not preserved when tissues were fixed with 10 per cent formalin containing 1 per cent calcium chloride instead of 2 per cent calcium acetate. Moreover, PAS-positive droplets were not preserved when fresh-frozen sections were made and then fixed in 10 per cent formalin containing 2 per cent calcium acetate, or in acetone or in alcohol (R. Malmgren and S. H. Wollman, unpublished observations).

Dynamics of Enzyme Localization

In the thyroid gland of the hypophysectomized rat, both esterase and acid phosphatase were localized on granules located largely at the basal end of the epithelial cell. These granules are considered to be lysosomes because they contain acid phosphatase and esterase, enzymes charac-

FIGURE 14 Localization of acid phosphatase in the follicular epithelial cells of the thyroid gland of a rat 1 day after hypophysectomy. Reaction product is associated with granules (see inset enlargement of area at arrow) largely at the basal end of the cell. See Fig. 10 for the corresponding localization of esterase activity. Green filter. × 500; inset × 2000.

FIGURE 15 Localization of esterase activity in the thyroid epithelium of a rat (one day after hypophysectomy) 30 minutes after injection of 2.5 milliunits of TSH. Note that droplets and granules are reactive and that the granules are not restricted to the apical end of the cell. Compare with the more apical distribution after 10 times the dose of TSH in Fig. 13. PAS counterstain. Orange filter. × 500.

FIGURE 16 Localization of esterase activity in the droplet membrane. Thyroid gland from intact rat one hour after injection of 250 milliunits of TSH. Note local thickening of membrane (*T*), suggesting fusion of granule and droplet. Orange filter. × 2000.

teristic of these bodies (24). After injection with TSH the enzyme-associated granules migrated toward the apical end of the cell in the direction of newly formed droplets. No esterase or acid phosphatase was detected associated with these newly formed droplets. Enzyme-containing granules and "enzyme-free' droplets were observed in close proximity, as early as 10 minutes after TSH injection. By 15 minutes after TSH injection many droplets contained these enzymes. This suggests that the enzyme is transferred from granule to droplet, or that the granule and its associated enzymes are incorporated into the droplet after its formation. This postulated relationship of granule and droplet is reminiscent of that observed by Rose (17) for the microkinetosphere (corresponding to our granule) and the pinocytotic vacuole or phagosome (corresponding to our droplet) (also see Koehring, reference 48). The fusion of granule and droplet membrane has not been observed in the present study. In certain cases, however, esterase was observed to be localized in the droplet surface. Recent unpublished studies by Wetzel, Spicer, and Wollman using the electron microscope show that the acid phosphatase-associated granule is a somewhat dense body surrounded by a membrane, and that this granule comes in close contact with or fuses with the droplet membrane prior to the appearance of acid phosphatase in the droplet.

The present work, then, suggests that intracellular PAS-positive droplets in the thyroid gland are organelles containing certain hydrolytic enzymes and colloid derived from the lumen of the follicle. They may therefore be organelles which function to hydrolyze thyroglobulin and release thyroxine. De Robertis has suggested that proteolytic enzymes which hydrolyze thyroglobulin are in the lumen. The hydrolytic enzymes reported here were intracellular and were never detected in the lumen. In accordance with this finding is the report that leucine amino peptidase activity is localized in the cell rather than in the lumen of the follicle (49). The endogenous substrates for acid phosphatase and esterase activities are not known. It may be that the esterase activity functions endogenously as a proteolytic enzyme, since many proteolytic enzymes including cathepsin C have esterase activity (50).

It is unknown whether other enzymes associated with lysosomes in liver (23), such as β-glucuronidase, β-galactosidase, β-*N*-acetylglucosaminidase and α-mannosidase, are associated with the granules and with the intracellular colloid droplets (although β-glucuronidase is localized in the epithelial cell, see reference 47). It would be appropriate for some of them to be present, since thyroglobulin is a glycoprotein.

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