CHANGES IN FINE STRUCTURE AND ACID PHOSPHATASE LOCALIZATION IN RAT THYROID CELLS FOLLOWING THYROTROPIN ADMINISTRATION

BRUCE K. WETZEL, Ph.D., SAMUEL S. SPICER, M.D., and SEYMOUR H. WOLLMAN, Ph.D.

From the National Institute of Arthritis and Metabolic Diseases, and the National Cancer Institute, National Institutes of Health, Public Health Service, United States Department of Health, Education, and Welfare, Bethesda, Maryland

ABSTRACT

Shortly after the administration of 1/40 unit thyrotropin to rats, 24 hours *post*-hypophysectomy, the following sequence of changes has been observed within thyroid follicular epithelial cells: (1) the appearance of apical cell surface activity consisting of pseudopods projecting into the follicular lumen; (2) apparent phagocytic engulfment of colloid droplets lacking indications of acid phosphatase activity; (3) close association and probable fusion of newly formed colloid droplets and dense granules, the latter cytochemically positive for acid phosphatase activity; (4) the appearance of presumptive acid phosphatase activity within colloid droplets; and, (5) further colloid droplet changes, *viz.*, basipetal migration and decrease in size, accompanied by an increase in density and in demonstrable acid phosphatase activity. These changes appeared to represent the resorption and degradation of follicular colloid. Comparable results were obtained using intact and more heavily stimulated animals. Colloid biosynthesis was tentatively visualized in these cells as a separate mechanism involving small vesicles prominent in the Golgi region and beneath the apical plasma membrane of some, but not all, thyroid follicular cells in each specimen.

INTRODUCTION

The injection of thyroid-stimulating hormone (TSH) of pituitary origin results in the rapid appearance of relatively large ($\sim$1 μ) droplets in the apical cytoplasm of rat thyroid follicular epithelial cells (De Robertis, 1942, Nadler *et al.*, 1962, Wollman *et al.*, 1964). Initially, the droplets are found in pseudopod cell processes which project into the follicular lumen; these droplets subsequently appear to pass into the body of the cell. The droplets stain like follicular colloid with the periodic acid–Schiff reaction (PAS) and other methods (De Robertis, 1942, Gersh, 1950).

The incidence of these droplets correlates with apparent resorptive activity (Grant, 1931). For example, after strong TSH stimulation, droplets are most numerous while luminal colloid is disappearing; whereas droplets disappear upon depletion of luminal colloid (Wollman *et al.*, 1964). Several lines of autoradiographic evidence further suggest that the material in the droplets is derived from the lumen of the follicle (Wollman and Spicer, 1961, 1963; Nadler *et al.*, 1962). On the other hand, the participation of these "colloid droplets" in biosynthetic processes is not indicated

by autoradiographic studies on the incorporation of tritiated leucine into thyroid colloid proteins (Nadler *et al.*, 1964) although other cell organelles, such as the endoplasmic reticulum, Golgi zones, and small cytoplasmic vesicles have been implicated in this anabolic role.

Histochemical evidence (Wollman *et al.*, 1964) has suggested that colloid may be degraded at least partially in the colloid droplets. These recent studies have localized non-specific esterase and acid phosphatase to a population of small cytoplasmic granules in the thyroid glands of hypophysectomized, unstimulated animals. Initially appearing colloid droplets, observed following TSH administration, were found to be devoid of demonstrable enzyme, but shortly thereafter the droplets acquired esterase and acid phosphatase activities. The appearance of hydrolytic enzymes within colloid droplets preceded the disappearance of these organelles from the thyroid cell as the response to TSH waned. These findings support the suggestion that colloid droplets represent a mechanism for the resorption and degradation of follicular colloid, and may be the site of thyroxine release.

The present report substantiates and extends many of these previous findings by exploring changes in ultrastructure and in non-specific acid phosphatase localization as manifestations of thyroid follicular cell responses to thyrotropin stimulation after hypophysectomy. The effects of TSH have been evaluated primarily in hypophysectomized animals, providing a base line against which the sequence of changes following hormonal stimulation may be clearly distinguished.

A preliminary account of this work has already been presented (Wetzel *et al.*, 1963). Other authors have recently reported similar results concerning several aspects of this problem (Novikoff and Vorbrodt, 1963).

MATERIALS AND METHODS

The animals used for these experiments were 150- to 200-gram male Fischer rats, 2 to 3 months of age, maintained on Purina Laboratory Chow. Hypophysectomy was performed by the paratracheal route on animals anesthetized with pentobarbital. A rare occurrence of traumatic inflammation in the thyroid gland was noted. TSH with a specific activity of 1.3 international units/mg, prepared from transplantable mouse pituitary tumors, was generously provided by Dr. R. W. Bates and Dr. P. G. Condliffe. The TSH was diluted in 0.2 ml of 0.1 per cent bovine serum albumin solution and injected into the external jugular vein of animals under light ether anesthesia.

Thyroid glands were obtained immediately *post mortem* from etherized, exsanguinated animals by flooding the gland with fixative upon exposure and prior to its removal. When hypophysectomy was performed, the operation was invariably carried out 24 hours previous to necropsy or TSH administration.

Thyroid lobes were obtained from 8 unstimulated, hypophysectomized animals. Hypophysectomized rats treated with TSH were sacrificed at various intervals after stimulation. Following $\frac{1}{40}$ unit TSH, specimens were obtained from 1 animal at $2\frac{1}{2}$ minutes, 3 at 5 minutes, 4 at 10 minutes, 5 at 15 minutes, 3 at 30 minutes, 2 at 60 minutes, 4 at 75 minutes, and from 1 animal each at 85 minutes, 90 minutes, 2 hours, 3 hours, and 4 hours. Two hypophysectomized animals were sacrificed 75 minutes following stimulation with $\frac{1}{4}$ unit TSH. Thyroid glands were also examined from 6 intact, uninjected animals, and from 8 intact animals stimulated with either $\frac{1}{40}$ or $\frac{1}{4}$ unit TSH, from 5 minutes to 3 hours prior to necropsy. Each gland was divided into several pieces which were processed by various techniques in order that the results of different methods could be compared in tissue from the same gland.

For morphological examination in the electron microscope, small blocks of tissue were fixed for 90 minutes in 1 per cent osmium tetroxide buffered according to Millonig (1961 *a*), or for 90 minutes in 6.25 per cent glutaraldehyde in pH 7.4, 0.1 M cacodylate buffer at 4°C (Sabatini *et al.*, 1963), followed by 45 minutes in Millonig's fluid. Tissues were dehydrated and embedded in Epon 812 (Luft, 1961), or in Maraglas (Freeman and Spurlock, 1962) according to the schedule suggested by Spurlock *et al.*, (1963). Other fixatives explored for the anatomical studies included chrome osmium with postformalinization (Dalton and Ziegel, 1960), Palade's acetate-veronal buffered osmium tetroxide (1952), and Caulfield's modification thereof (1957). Sections cut on a Porter-Blum MT-1 microtome with fractured glass knives were collected from the surface of 40 per cent acetone onto etched 65-mesh copper grids covered with a supporting carbonized collodion film. Various staining procedures were used including Karnovsky's (1961) "A" and "B" solutions, Millonig's (1961 *b*) and Reynolds' (1963) lead stains, uranyl acetate staining (Watson, 1958), and several combinations of uranyl and lead methods. Specimens were examined and micrographed in either an RCA EMU-2A or an RCA EMU-3G electron microscope.

For fine structural localization of acid phosphatase, tissues were fixed at 4°C for 18 hours in either 10 per cent formalin containing 2 per cent calcium acetate, or sucrose phosphate formalin (Holt and

Hicks, 1961), or for 2 hours in the glutaraldehyde solution prior to processing according to methods described by Holt and Hicks (1961) and Miller (1962). After fixation, tissues were rinsed 3 times for 5 minutes each in 7.5 per cent sucrose at 4°C, frozen quickly in this solution on a microtome hammer with dry ice, and sectioned at 40 μ in the cryostat. These sections received three 5-minute rinses in 0.33 M (11.5 per cent) sucrose-0.1 M cacodylate buffer at 4°C, during which time they were sandwiched between two layers of lens paper tied over the end of a 4 inch segment of 10 mm glass tubing. The sections were transported in this device through subsequent processing steps until they were removed for final embedding. Sections were incubated at 37°C in Gomori's (1952) medium for acid phosphatase or in the Barka and Anderson (1962) modification, employing a mixture of 25 per cent α- and 75 per cent β-glycerophosphate (Sigma) as substrate. Enzyme incubation times from 5 to 50 minutes were employed, but optimal results were generally obtained in the range of 15 to 40 minutes. Some specimens received a subsequent 0.5- to 3-minute rinse in 2 per cent acetic acid, immediately preceded and followed by a 5-minute rinse in pH 5.0 acetate buffer containing 5 per cent sucrose and 10 per cent formalin, all carried out at 4°C. The acetic acid step was frequently omitted from this postincubation rinse procedure to reveal all reaction product resulting from a given set of incubation conditions. Following 45 minutes postfixation in osmium tetroxide, the processing of cytochemical preparations resembled that employed for the morphological studies.

Experimental conditions were varied in an effort to validate the cytochemical procedure. Variations in the concentration of lead nitrate, substrate, pH, and the ionic strength of the enzyme incubation medium were compared with Gomori's original acid phosphatase incubation medium and with the Barka and Anderson modification thereof. Conditions were routinely adjusted to provide, on the one hand, specimens showing relatively heavy reaction product deposition and illustrating the lack of activity in certain areas, and, on the other hand, specimens with relatively light reaction product deposition and high specificity. Enzyme inactivation was attempted in some specimens by exposure to 100°C for 10 minutes in 4 per cent formalin prior to incubation, or by the inclusion of 10 per cent formalin or 0.01 M sodium fluoride in the enzyme incubation medium. Omission of the glycerophosphate substrate from the incubation medium was also included as a control measure.

RESULTS

Thyroid glands of rats 24 hours following hypophysectomy resembled morphologically and cytochemically the glands of intact animals in most respects. However, certain structures (apical pseudopods and colloid droplets) characteristic of stimulated thyroids, and to a lesser extent of normal thyroids as well, were conspicuously and consistently absent following hypophysectomy. By eliminating these configurations, the use of hypophysectomized animals permitted recognition of the sequence of cellular changes induced by thyrotropin stimulation. Accordingly, most of the results described below were obtained using rats 24 hours after hypophysectomy treated with $\frac{1}{40}$ unit TSH at various intervals prior to necropsy. Results from these animals were compared with those obtained from intact and heavily stimulated animals.

Most of the thyroid follicular cell organelles dealt with in this study have been noted and described previously by other workers (Ekholm and Sjöstrand, 1957, Herman, 1960, Wissig, 1960, 1963). Particular attention will be devoted to the luminal surface of the cells, the population of cytoplasmic dense granules, and the colloid droplets which appeared to be involved in the early cellular response to TSH.

Hypophysectomized, Unstimulated Animals

Twenty-four hours following hypophysectomy, rat thyroid follicular cells appeared as a moderately low cuboidal epithelium with an even apical margin interrupted by a modest number of microvillous projections measuring roughly 0.1 μ in diameter and 0.5 μ in length (Figs. 1 and 2). Within each cell profile, 0 to 50 (usually ~35) relatively small and adielectronic (electron-scattering), single membrane-limited cytoplasmic inclusions—designated herein as "dense granules"—were usually encountered in a perinuclear distribution (Figs. 1 to 3). These organelles have been noted and described in part by other authors (*e.g.*, Wissig, 1960, 1963), on occasion employing different terminology (Young and Leblond, 1963). In hypophysectomized animals, there was an apparent tendency for these organelles to occur in basally located clusters (Fig. 2), although apical granules occurred regularly. The relatively high density of these granules varied somewhat. Individual granules were often inhomogeneous in density and structure, occasionally including membrane elements and, rarely, recognizable cell organelles within their matrix (Fig. 3). Minute (~70 A) particles were usually discernible within the dense granules (Figs. 3 and 4). These minute particles have been examined more extensively by

Wissig (1963), but have not received detailed study herein. Dense granules of this general description ranged widely in size from ~0.03 to 0.6 μ and varied in shape from spherical or ovoid to the rod-like form of some of the smaller granules. Despite the non-uniformity among these dense granules, they have been considered collectively, through most of this report, on the basis of their apparent functional similarities.

Cytochemically, heavy deposition of reaction product indicative of intense non-specific acid phosphatase activity was present in most of the morphologically defined dense granules; but some showed little or no reactivity with the techniques employed (Figs. 2, 5). Presumptive enzyme activity was generally restricted at this stage to the dense granules. Peripheral lamellar elements of occasional Golgi complexes constituted the only other structures showing such activity (Fig. 6), but these accounted for a relatively small proportion of the observed reactive sites.

Relatively large (to 5 μ) single membrane-limited cytoplasmic droplets—to be described in detail as a later manifestation of the response to TSH—were conspicuously absent from thyroid epithelial cells of untreated, hypophysectomized rats.

Extensive Golgi complexes occurred in several loci within the apical and lateral regions of each follicular epithelial cell, and were recognized by their characteristic, closely applied, lamellar sacs (Fig. 1). A variable number of characteristic Golgi microvesicles measuring ~0.05 μ in diameter invariably surrounded the lamellae (Figs. 1, 18). In some cells, larger ovoid vesicles of relatively constant size (~0.1 μ) and flocculent con-

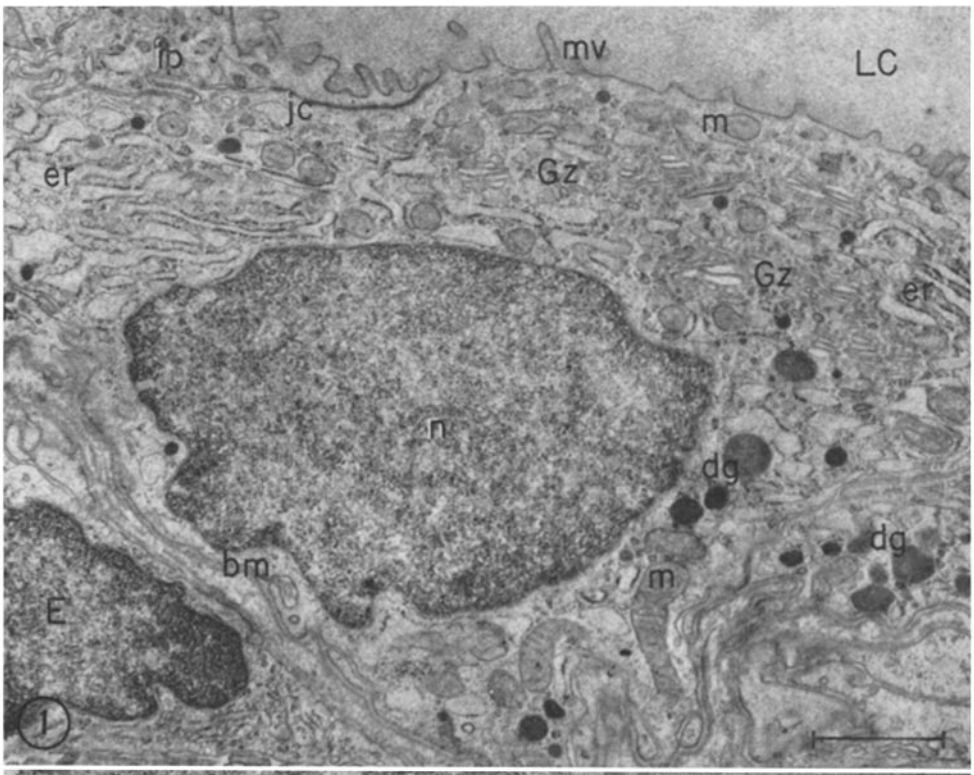
---

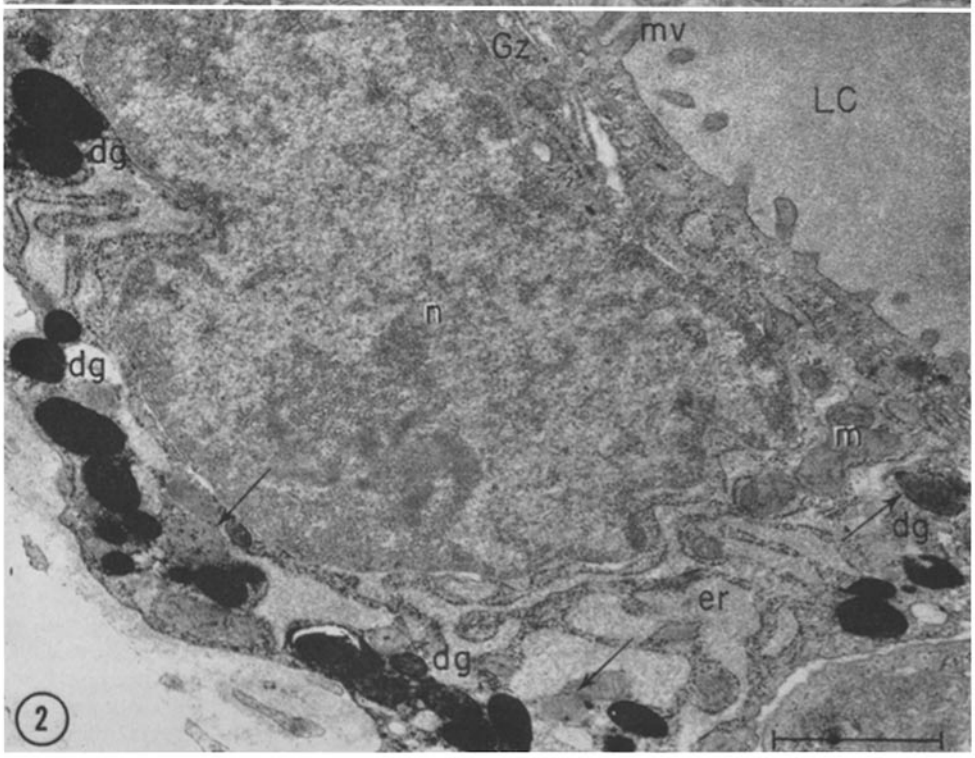
Unless specifically stated, the illustrations represent material processed as follows: (1) Tissue was obtained from animals receiving $\frac{1}{40}$ unit TSH intravenously 24 hours after hypophysectomy; (2) specimens for morphological studies were fixed in Millonig's osmium tetroxide; (3) specimens for cytochemical studies received glutaraldehyde-osmium tetroxide fixation following the methods of Miller (1962) and Sabatini *et al.* (1963); (4) incubation for acid phosphatase was carried out in the Barka and Anderson medium (1962); (5) specimens were embedded in Maraglas (Spurlock *et al.* 1963); and (6) thin sections were stained with Karnovsky's "A" lead stain (1961). The scale marker in each figure represents approximately 1 μ. The following abbreviations have been used:

bm, basement membrane
c, centriole
cd, colloid droplet
dg, dense granule
E, endothelial cell
er, endoplasmic reticulum
F, follicular epithelial cell
Gz, Golgi zone
ip, interdigitating process
jc, junctional complex
LC, luminal colloid
m, mitochondrion
μv, microvesicle
mv, microvilli
mf, "myelin figure"
n, nucleus
P, parafollicular cell
r, free ribosomes
v, vesicle

FIGURE 1 Thyroid follicular epithelial cell from an hypophysectomized, unstimulated animal. Irregular microvilli (*mv*) project into the homogeneous luminal colloid (*LC*), and junctional complexes (*jc*) conjoin closely applied lateral cell membranes. Note the interdigitating processes (*ip*) along the lateral cell surface, and the subjacent basement membrane (*bm*). Extensive Golgi zones (*Gz*) occupy much of the apical cell cytoplasm and ergastoplasmic cisternae (*er*) range throughout the cell. Numerous dense granules (*dg*) of varying size are usually located about the nucleus (*n*) while mitochondria (*m*) are more evenly distributed. Portions of two endothelial cells (*E*) underlie the epithelium. Epon 812 embedding. × 17,500.

FIGURE 2 Acid phosphatase localization in an hypophysectomized unstimulated animal. Note the heavy deposition of black reaction product in most dense granules (*dg*), and its absence from the remainder of the cell and the luminal colloid (*LC*). The predominant basal distribution of the dense granules is dramatically illustrated. Partially reactive granules are indicated (arrows). Compare with Fig. 1 for cytological details. × 22,000.


lb
mv
LC
jc
m
er
Gz
Gz
er
n
dg
dg
bm
m
E
1


Gz
mv
LC
dg
n
dg
m
dg
er
dg
2

tents were numerous in the Golgi zone. These larger vesicles were usually clustered beneath the apical plasma membrane of the same cells (Figs. 7, 18), and some suggestion of the fusion of such vesicles with the cell surface was noted. These vesicles were quite inconspicuous or absent in some follicular epithelial cells and numerous in others; these variations were often evident between adjacent cells and more marked from follicle to follicle. Dilated sacs or cisternae were occasionally seen in close apposition to the Golgi lamellae—a configuration suggestive of transfer of contained material (Fig. 18). Relatively small and pleomorphic dense granules, generally showing strong acid phosphatase activity, were often found in particularly close association with Golgi elements (Figs. 6, 28) as well as distributed throughout the cells.

Ribosome-studded cisternal elements of the endoplasmic reticulum were prominent in most thyroid follicular epithelial cells (Figs. 1, 2, 18) and ranged in array from roughly parallel lamellae to vesiculate profiles dispersed throughout the cells. The volume of these cisternae appeared to vary widely.

Early Changes Following TSH Administration

Specimens removed 2½ minutes following the intravenous injection of thyrotropin showed essentially no changes from the morphology observed in unstimulated animals. Alterations became evident, however, in tissue removed after 5 minutes, and were consistently demonstrated after longer intervals.

The most striking feature of this early response

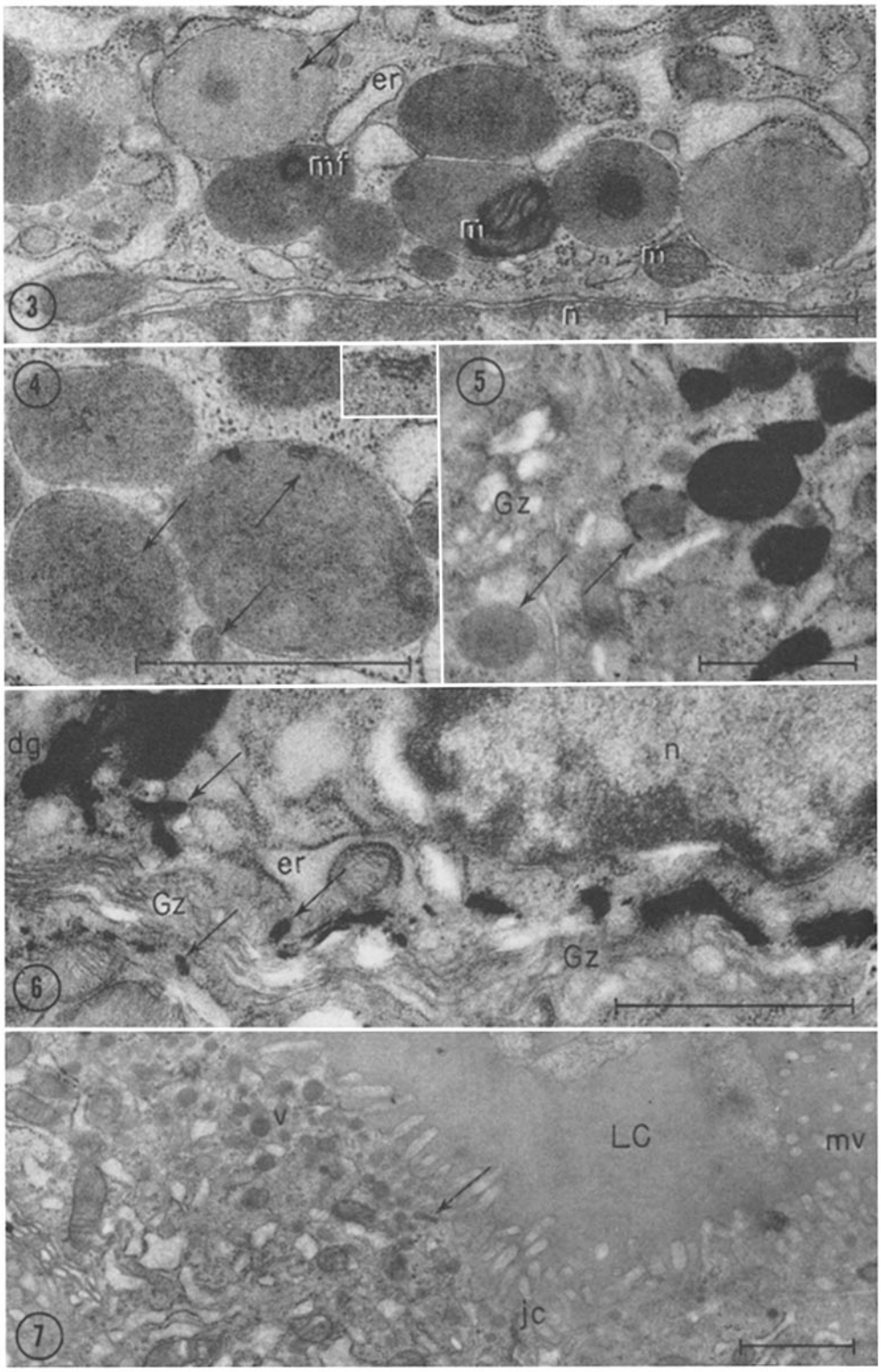
FIGURE 3 A typical perinuclear cluster of dense granules illustrating their morphological heterogeneity. A wide range in size and density is evident among these organelles, and their varied contents include mitochondrial profiles (*m*), a "myelin figure" (*mf*), and a cluster of minute dense particles (arrow). Cisternae of the endoplasmic reticulum (*er*) and occasional groups of free ribosomes (*r*) are seen. The single limiting membranes of the dense granules are generally evident. Removed 5 minutes after TSH injection. Glutaraldehyde–osmium tetroxide fixation. Reynolds' lead stain. × 28,000.

FIGURE 4 Several dense granules at higher magnification, illustrating the presence of minute (~70 A) particles (*e.g.*, at arrows, also see inset) dispersed throughout their matrices. The smallest of these dense granule profiles contains such tiny particles. Membrane fragments are seen in the largest profile. Removed 5 minutes after TSH injection. Fixation and staining as in Fig. 3. × 40,000; inset, × 90,000.

FIGURE 5 Acid phosphatase reactivity of dense granules. Most of these organelles show heavy reaction product deposition, but reactivity is sparse in one dense granule and absent from the profile of another (arrows). Removed 30 minutes after TSH injection. Uranyl acetate–Reynolds' lead stain. × 23,000.

FIGURE 6 Acid phosphatase localization in an extensive Golgi zone (*Gz*) and in several dense granules (*dg*). Most Golgi lamellae are free of reaction product deposition, but several outer lamellae are strongly reactive. Note the small, pleomorphic, granule-like profiles in association with the Golgi zone (arrows). The nucleus (*n*) and ergastoplasm (*er*) are free of reaction product deposition. This figure illustrates an unusually reactive Golgi zone observed in tissue removed from a normal animal sacrificed 2 hours after the injection of ¼ unit TSH. Similar but less extensive configurations have been observed under most experimental conditions examined, including tissue obtained from hypophysectomized, unstimulated rats. × 35,000.

FIGURE 7 Apical portions of two follicular cells. Numerous ~0.1 μ apical vesicles (*v*) are seen in the cell on the left, but very few are evident in the cell to the right of the junctional complex (*jc*). Elongate profiles (arrow) are occasionally found among these vesicles. Removed 10 minutes after TSH injection. Comparable distributions of apical vesicles were also encountered in tissue from hypophysectomized, unstimulated animals. Glutaraldehyde–osmium tetroxide fixation. Uranyl acetate–Reynolds' lead stain. × 17,000.


er
mf
m
m
n
3
4
5
Gz
dg
n
er
Gz
Gz
6
v
LC
mv
jc
7

was the appearance of surface activity along the apical margin of the cells. Large pseudopods were found projecting into the follicular lumen (Figs. 8 to 14). Pseudopod processes seemed to arise randomly along the luminal-epithelial interface independent of follicular cell limits. With the exception of constantly occurring clusters of free ribonucleoprotein particles, the cytoplasm of these pseudopods was remarkably free of cell organelles (Figs. 8 to 14).

The most rudimentary pseudopods observed consisted of low (0.5 to 1 μ high), broad papillae lacking microvilli and larger projections, and typically free of cytoplasmic organelles (Fig. 8). Some pseudopods retained a relatively simple outline, but were more massive, penetrating as far as 10 μ into the lumen with fleshy profiles as broad as 4 μ (Fig. 9). However, at 10 minutes most pseudopods were quite irregular in outline, possessing long, narrow processes which extended into the surrounding luminal colloid from a stalk of variable diameter (Figs. 10 to 14). The frequency with which elongate profiles were seen branching from the pseudopods, and the relative absence of small, circular, or ovoid profiles above the level of the microvilli, suggested that large portions of most of these protrusions were thin and membranous rather than filamentous in character. Three-dimensional reconstructions based on studies of multiple sections extending at least 4 μ in depth through particular pseudopods have substantiated this impression and provided additional morphological details. For example, broad, horizontal sheets of cytoplasm paralleling the luminal surface just above the level of the microvilli were repeatedly found atop short pseudopod stalks (Fig. 10). Long apical and lateral extensions from the pseudopods were generally found to constitute membranous ridges (Figs. 8, 11 to 14).

Pseudopod projections were often bifid in section, and multiple sections in several instances indicated a flask-like configuration entirely enveloping a globular portion of colloid except for a small apical pore in the pseudopod wall (Figs. 13, 14). No configurations commensurate with the size of most subsequently appearing cytoplasmic colloid droplets have been seen to involve simple invaginations of the apical cell margin.

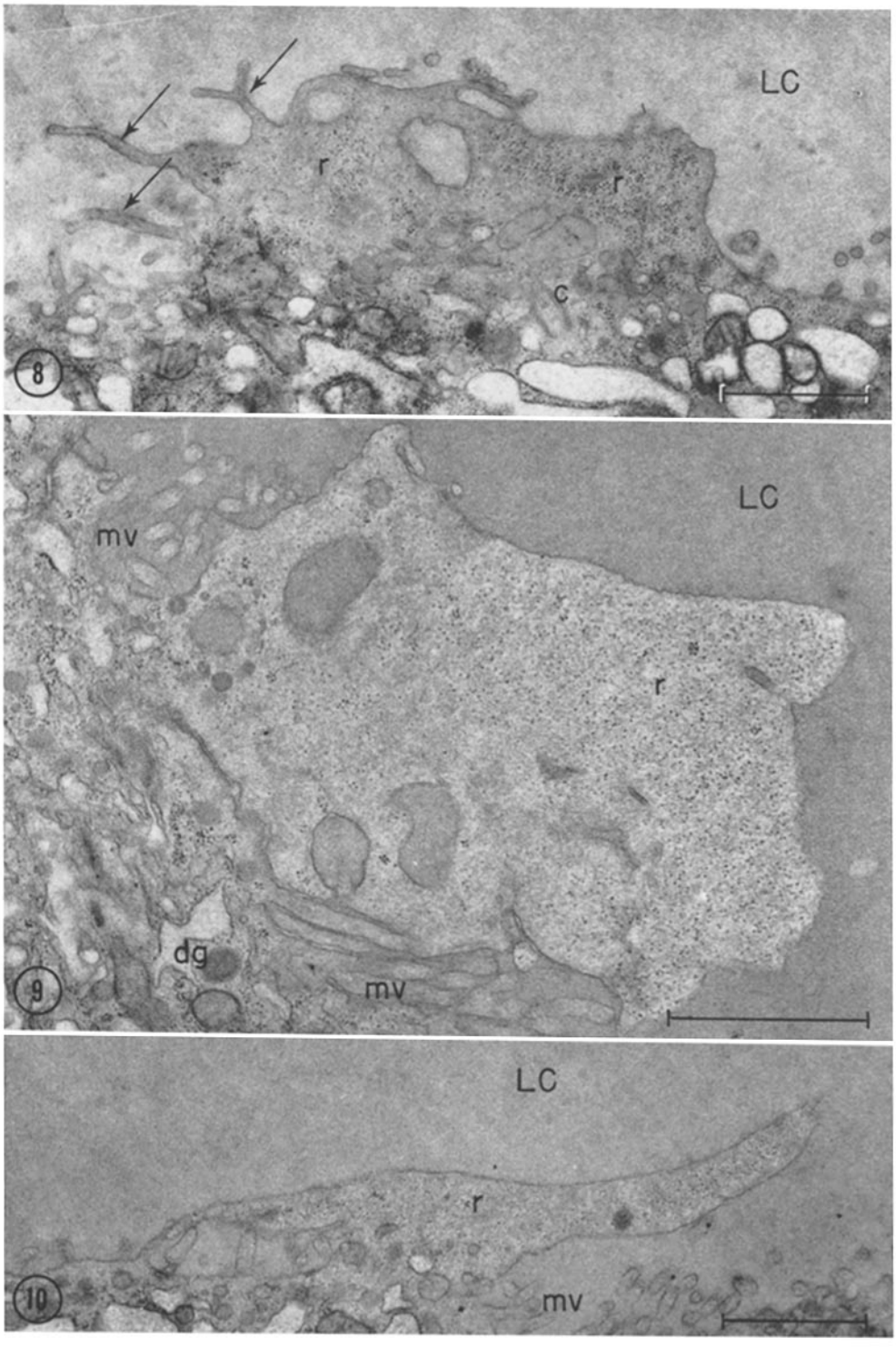
Pseudopods were commonly observed at the latero-apical margins of epithelial cells, in some instances comprising cytoplasmic extensions from two adjacent cells with their apposed lateral cell membranes extending into the pseudopod proper (Figs. 15, 16). These membranes delimiting adjacent cells apparently persisted through colloid engulfment, and overlapping layers of cytoplasm from neighboring cells were occasionally found surrounding colloid droplet profiles (Fig. 16 and probably Figs. 17 and 18). Despite these curious instances of close cooperation (or competition) between adjacent cells at cell junctions, there appeared to be no predilection for pseudopod formation at these sites.

The generally spherical, single membrane-limited droplets which appeared within the pseudopods (Figs. 15 to 19) shortly after the initiation of cell surface activity were variable in size ($\sim$0.3 to 5 μ), although most of them were quite large ($\sim$1.0 μ). The contents of these early inclusions were usually equivalent in density and tex-

FIGURE 8 A low pseudopod characteristically lacking cytoplasmic organelles other than free ribosomes (*r*). Several projections (appearing as ridges in multiple sections) extend from the surface of the pseudopod (arrows). A centriole (*c*) appears in longitudinal section at the base of the pseudopod. Removed 10 minutes after TSH injection. Glutaraldehyde–osmium tetroxide fixation. Reynolds' lead stain. × 21,000.

FIGURE 9 A large pseudopod of relatively simple outline, again lacking most cytoplasmic organelles. Note the absence of microvilli (*mv*) from the pseudopod surface, although they are quite numerous along the adjacent cell margin. Removed 10 minutes after TSH injection. Fixation as in Fig. 8. Uranyl acetate–Reynolds' lead stain. × 29,000.

FIGURE 10 Profile of a low, flat pseudopod attached to the follicular cell by a short stalk. This particular pseudopod consists of a thin, horizontal, discoid structure with no microvilli arising from its surfaces. Removed 10 minutes after TSH injection. Fixation and staining as in Fig. 9. × 21,000.


LC
r
r
c
8
LC
mv
r
dg
mv
9
LC
r
mv
10

ture to the luminal follicular colloid, but in rare instances were less dielectronic. The term "colloid droplet" will be used to describe these organelles, based on the time and site of appearance of these inclusions following TSH administration (De Robertis, 1942), and the correlation of these findings with morphological and histochemical results at the light microscope level (Gersh, 1950, Wollman *et al.*, 1964).

Five minutes after TSH injection, pseudopods were frequently encountered, and the rare colloid droplets occurring at this stage were found within pseudopods. Ten minutes after injection, droplets were more abundant and single pseudopod profiles often contained several of these organelles, although very few were found in the apical cytoplasm of the cells. Fifteen minutes following TSH administration, colloid droplets were commonly encountered in the body of the cell, as well as in pseudopod processes.

Many profiles in the pseudopods appearing as adjacent, independent colloid droplets were found to be confluent when additional sections were examined. Configurations indicative of incomplete cytoplasmic septa between adjacent colloid droplets were observed regularly. Microvilli were not seen within structures identified as colloid droplets, but were encountered with small, round profiles at the bases of occasional pseudopods. Accounting for these latter observations, multiple sections disclosed the presence of lateral concavities in the pseudopod stalks which appeared in section as profiles of luminal colloid and underlying apical cell margin.

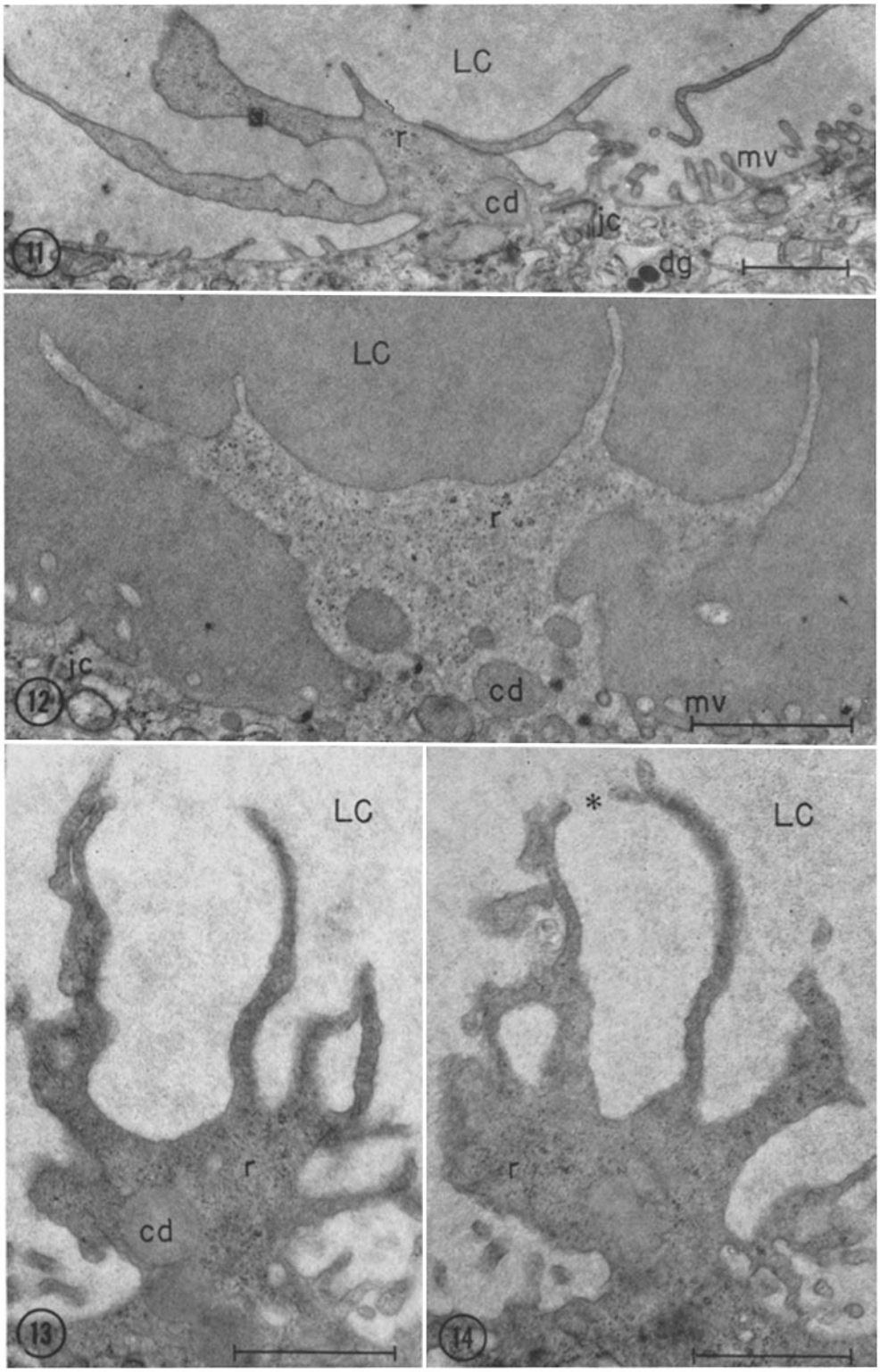
Relatively large droplet profiles located at the level of the luminal surface and surrounded apically by only a thin layer of pseudopod cytoplasm (Figs. 16 and 18) were abundant from 10 to 90 minutes following stimulation. These configurations appeared to represent pseudopod retraction and the transfer of large, newly formed droplets into the apical cell cytoplasm. Smaller droplets often occurred below the level of the luminal surface at the base of seemingly active pseudopods (Figs. 11 to 14).

The electron-scattering properties of both the follicular colloid and follicular cell cytoplasm varied in a given section, occasional cells and infrequent follicles appearing distinctly darker than most. Characteristically, the luminal colloid and the contents of most newly formed colloid droplets were of slightly lower density than the cytoplasm of the surrounding cells (Figs. 8, 11, 13 to 17). However, instances of greater density were noted in the cytoplasm of occasional cells (Figs. 13, 14) and, infrequently, in the contents of particular follicles (Figs. 9, 12). The significance of these observations is obscure; they seemed to bear no apparent relationship to the response to thyrotropin stimulation.

FIGURE 11 Profile of a large, irregular pseudopod process extending into the luminal colloid (*LC*). Microvilli (*mv*) are evident along the remainder of the luminal cell surfaces. A junctional complex (*jc*) conjoins the adjacent cells. Dense granules (*dg*) are seen in the apical cytoplasm. Two small colloid droplets (*cd*) are present at the base of the pseudopod. Removed 15 minutes after TSH injection. × 15,500.

FIGURE 12 A broad pseudopod containing several small colloid droplet profiles at the base of its stalk. Multiple sections of this pseudopod have established the membranous nature of the four thin projections from its surface. Removed 10 minutes after TSH injection. Glutaraldehyde–osmium tetroxide fixation. Uranyl acetate–lead citrate staining. × 23,500.

FIGURES 13 AND 14 These figures represent different sections of the same pseudopod separated by roughly 0.5 μ. The membranous nature of the two thin vertical processes is indicated, and the communication between the follicular lumen and the material between the processes is narrowed in Fig. 14 (*). Repeated sections in this direction reveal a continuity established between the processes, indicating the presence of a pore at the apex of the pseudopod. This configuration suggests a mechanism for the engulfment of luminal colloid by the ultimate closure of such an apical pore. Profiles of three small colloid droplets (*cd*) are evident in the stalk (Fig. 13). Removed 10 minutes after TSH injection. Glutaraldehyde–osmium tetroxide fixation. Reynolds' lead staining. × 23,500.


LC
r
mv
cd
jc
dg
11
LC
r
jc
cd
mv
12
LC
r
cd
13
*
LC
r
14

The disposition of cytoplasmic dense granules during the early stages of response to TSH altered such that these organelles became more abundant apically in the cells (*cf.* Figs. 1, 15). Dense granules were infrequently observed within the cytoplasm of pseudopods. As previously described for the unstimulated gland, most dense granules evidenced intense acid phosphatase activity (Fig. 19). In contrast, newly formed colloid droplets, like luminal colloid, showed no indication of acid phosphatase activity (Fig. 19).

Later Changes Following TSH Administration

Further cytological changes in the thyroid follicular epithelium were observed consistently in glands removed 15 to 30 minutes after the injection of $\frac{1}{40}$ unit TSH into hypophysectomized animals. Some follicles still retained the configurations described for unstimulated glands, while others showed the initially observed changes. Cell surface activity and the apparent formation of large inclusion droplets remained evident. Additional changes were characteristically found in epithelia in which colloid droplets had accumulated, particularly in cells containing more than one colloid droplet profile.

There was an apparent tendency for the basipetal migration of colloid droplets. During this period, the contents of colloid droplets appeared to become more adielectronic and occasionally included material similar to that found in some dense granules (*cf.* Figs. 3, 23). With some exceptions, a gradual transition could be recognized from larger, less dense colloid droplets to more basally located, smaller, denser, and more heterogeneous inclusions (Fig. 27). Of particular interest in this phase of the cellular response was the frequent and intimate association of dense granules with colloid droplets (Figs. 20 to 22, 24 to 26), although membrane configurations indicating granule-droplet fusion were seen only rarely (Fig. 22). Configurations suggesting the possibility of droplet-droplet fusion were occasionally observed within the apices of the cells.

The earliest consistent indication of acid phosphatase activity within colloid droplets appeared in specimens removed approximately 15 minutes after injection of thyrotropin. The amount of reaction product deposition initially found in colloid droplets was slight relative to that evident in adjacent dense granules within the same cell (Figs. 19, 24 to 26).

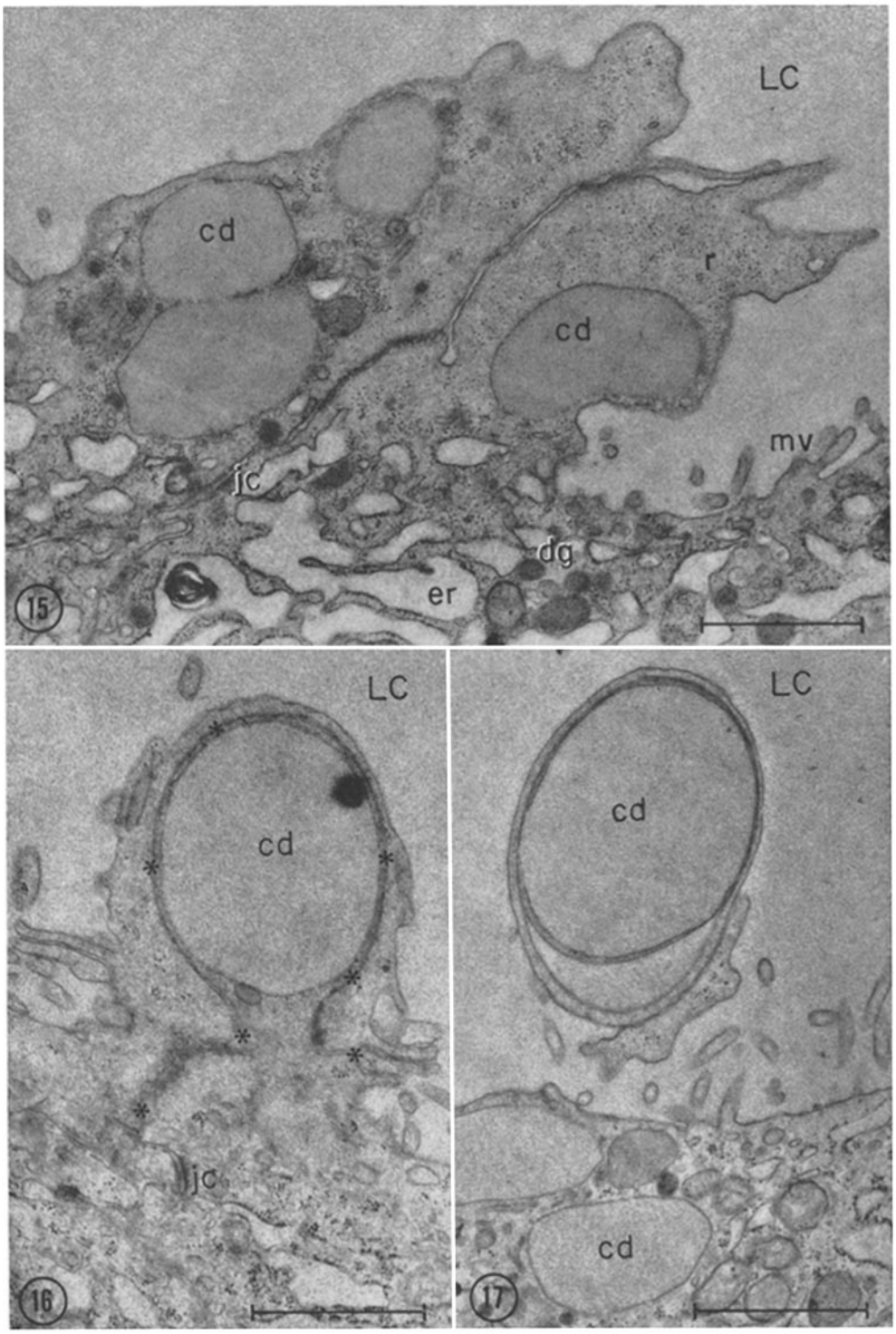
More than half of the colloid droplet profiles usually showed reaction product 30 minutes after TSH injection, and, at this stage, the degree of droplet activity was often variable. Sixty minutes after similar stimulation, most of the droplet profiles encountered evidenced relatively intense enzyme activity. Droplets deeper in the cytoplasm of the cell usually displayed heavier deposition of reaction product than those more superficially located (Fig. 26). The gradation of increasing reaction product deposition in colloid droplets toward the base of the cell corresponded closely with the generally increased density of more basally located colloid droplets (Fig. 27). Acid phosphatase reaction product appeared to be uniformly distributed throughout the droplet.

As late as 1 hour following stimulation with $\frac{1}{40}$

FIGURE 15 A large pseudopod composed of processes from two adjacent cells. The apposed lateral membranes traverse the entire length of the pseudopod. Several colloid droplet profiles (*cd*) are seen in the pseudopod cytoplasm of the cell on the left, and a single profile is evident on the right. Removed 15 minutes after TSH injection. Glutaraldehyde–osmium tetroxide fixation. Reynolds' lead staining. × 23,500.

FIGURE 16 Apparent involvement of processes from two adjacent cells in colloid droplet formation. The colloid droplet profile (*cd*) seems to be enclosed within the cytoplasm of the cell on the right, although an overlapping process from the cell on the left persists. The apposed lateral membranes of these cells, sectioned obliquely in part, appear to follow the indicated path (*). Removed 15 minutes after TSH injection. × 25,000.

FIGURE 17 A colloid droplet profile surrounded by two intact layers of cytoplasm. This configuration could arise through the involvement of processes from two adjacent cells, as in Fig. 16. Removed 15 minutes after TSH injection. × 25,000.


LC
cd
r
cd
mv
jc
dg
er
15
LC
cd
jc
16
LC
cd
cd
17

unit TSH, the early manifestations of the response, such as surface activity and apparent colloid droplet formation, persisted in diminished degree, indicating a continued initiation of resorptive processes. Consequently, only the earliest appearance of each particular cell change following stimulation of an hypophysectomized animal afforded a basis for estimating the time intervals involved in the continuous sequence of events constituting the resorptive process.

In addition, many colloid droplets displayed different characteristics at this stage. More frequently, they lay deep in the cell and were smaller in diameter than most initially formed droplets (Fig. 27). These later droplets showed generally increased density (Fig. 27), heavier deposits of acid phosphatase reaction product (Fig. 28), and more heterogeneous contents than droplets observed at earlier times. These later changes in colloid droplets led to difficulty in distinguishing late, altered colloid droplets from larger dense granules such as were observed in unstimulated animals (Fig. 27).

Presumptive secretory elements, such as distended cisternae within the Golgi region and the ~0.1 μ vesicles described above, were encountered somewhat more frequently 1 to 2 hours following the administration of $\frac{1}{40}$ unit TSH. As in unstimulated animals, vesicles of this type were quite numerous beneath the apical cell membrane and in the Golgi zone of some cells and relatively inapparent or absent in other cells. Hormonally dependent variations were not noted or evaluated in other thyroid follicular cell organelles such as the endoplasmic reticulum, microvilli, mitochondria, free ribosomes, lipid droplets, and nuclei.

Additional Experimental Conditions

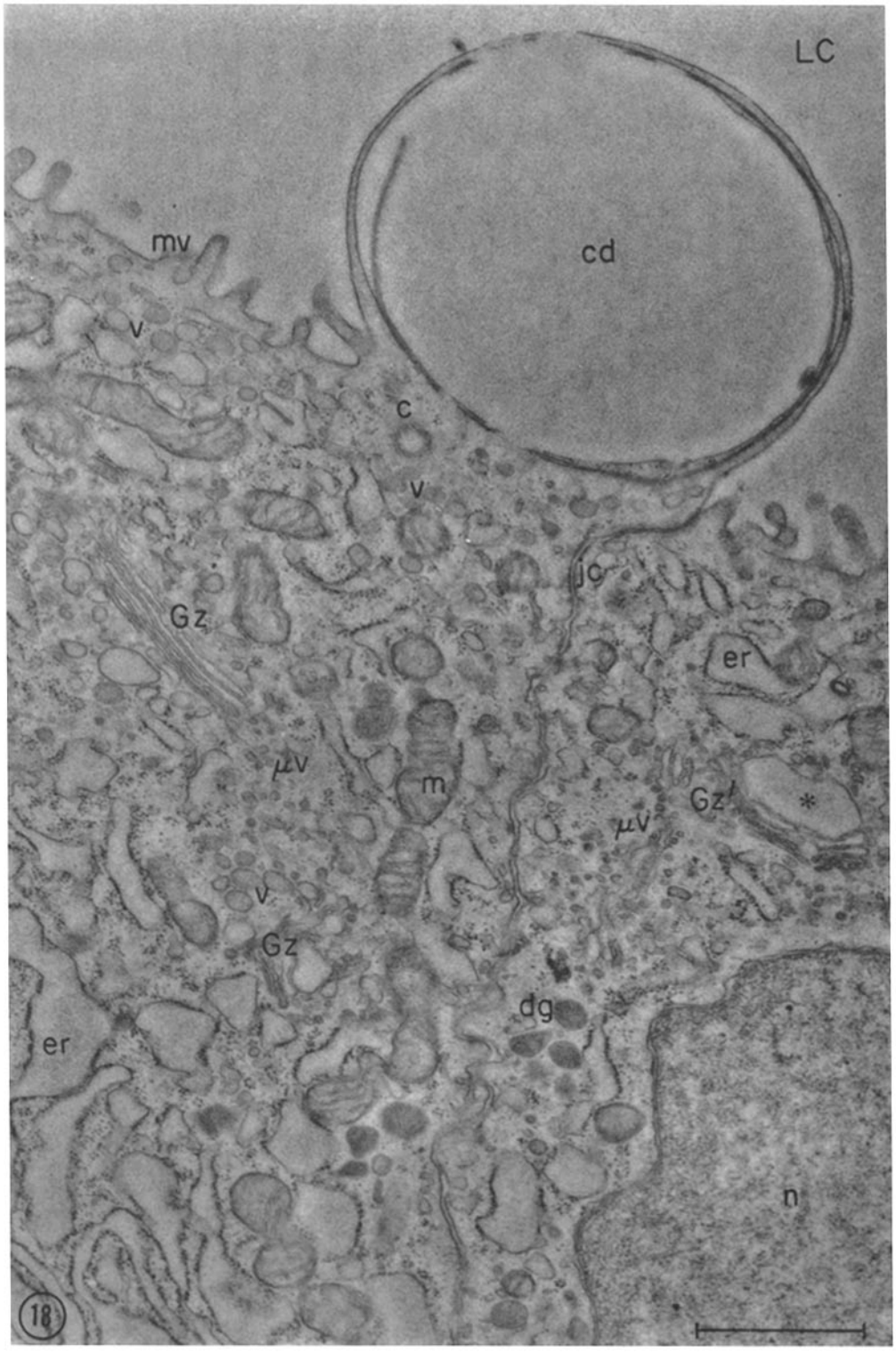
Animals receiving $\frac{1}{4}$ unit of TSH 24 hours following hypophysectomy yielded results substantially similar to those described above, with the same configurations observed as those seen following $\frac{1}{40}$ unit TSH stimulation. The response at this higher dosage level, and at the relatively late times investigated, differed in showing increased and sustained incorporation of colloid droplets from 1 to 2 hours after stimulation, with the resultant accumulation of greater numbers of colloid droplets per cell.

Each of the cytological and cytochemical features described above was verified in normal rat thyroids, in which the entire range of responsive configurations was encountered concomitantly in each specimen. Stimulation of normal animals elicited an intense response which qualitatively resembled that observed in hypophysectomized animals and resulted in the accumulation of large numbers of colloid droplets within the cells.

Parafollicular Light Cells

Ultrastructural features of "parafollicular cells" or "light cells" present in normal rat thyroids have been described by Wissig (1962), Yoshimura *et al.* (1962), and Young and Leblond (1963). Incidental observations obtained during the present studies have essentially confirmed the data of these investigators and have contributed some additional findings. The numerous, small (0.1 to 0.3 μ), membrane-limited cytoplasmic vesicles characteristic of these cells (Fig. 29) appeared to respond more favorably to combined glutaraldehyde–osmium tetroxide fixation than to osmium tetroxide fixation alone. The contents of these

FIGURE 18 Colloid droplet profile (*cd*) enclosed within a thin layer of pseudopod cytoplasm. The overlapping cell processes surrounding the droplet profile may represent the interjection of extensions from an adjacent cell as in Fig. 16. Note dense granules (*dg*), mitochondria (*m*), endoplasmic reticulum (*er*), and centriole (*c*). Vesicles (*v*) of moderate size (~0.1 μ), with contents similar to that of both the ergastoplasm and the luminal colloid, are clustered about the Golgi zones (*Gz*) of the cell on the left. Similar vesicles are present beneath the apical membrane of the same cell. The elements surrounding the Golgi zone (*Gz'*) of the cell on the right consist primarily of microvesicles (*μv*) and a single large cisterna (*) closely apposed to the lamellae. Moderate-sized vesicles are largely absent from this cell. Removed 30 minutes after TSH injection. Uranyl acetate–Reynolds' lead stain. × 25,000.


LC
cd
mv
v
c
v
jc
Gz
er
μv
m
Gz'
*
μv
v
Gz
dg
er
n
18

vesicles in doubly fixed tissue were more homogeneous, of higher density, and more uniform throughout the vesicle population than could be seen without glutaraldehyde prefixation. Variations observed in the abundance of these vesicles and in the ergastoplasm of these cells may reflect developmental or physiological changes in the cells, but no clear indications of hormonal dependency were noted in hypophysectomized or TSH-treated animals. Colloid droplets were not seen in parafollicular cells.

Acid phosphatase localization within the parafollicular cells revealed heavy reaction product deposition in occasional (usually 1 or 2 per cell profile) dense granules, comparable in morphology and cytochemistry to the dense granules of the follicular epithelium (Fig. 29) and morphologically distinct from the smaller vesicles characteristic of these cells. The function of dense granules in parafollicular cells was not evident, although by analogy they may play some role in cellular catabolism. Acid phosphatase reaction product was also observed in the Golgi lamellae of some parafollicular cells and infrequently within typical vesicles in the adjacent cytoplasm—a feature distinguishing these vesicles from the apical vesicles of the follicular cells. The parafollicular cell vesicles, presumably representing a cell secretory product, were generally unreactive following methods for the cytochemical visualization of acid phosphatase. No other organelles within the parafollicular cells showed indication of acid phosphatase activity.

Cytochemical Techniques

As reported by others using the Gomori method for acid phosphatase localization at a fine structural level in other tissues (Goldfischer *et al.*, 1964), reaction product has frequently been encountered within nuclei (in apparent association with nuclear chromatin) and rather randomly scattered throughout the cytoplasm of thyroid epithelial

FIGURE 19 Acid phosphatase localization within dense granules (*dg*) associated with a small, lightly reactive colloid droplet in the body of the cell. Newly formed colloid droplets (*cd*) within a pseudopod projection at the cell surface clearly lack reaction product despite a moderate deposition of "extraneous" reaction product throughout the remainder of the cell. The luminal colloid (*LC*) also remains free of precipitate. Removed 15 minutes after TSH injection. Gomori's acid phosphatase incubation medium. × 17,500.

FIGURE 20 Association of dense granules (*dg*) with a colloid droplet. Note the apparent incorporation of granule contents within the colloid droplet (arrow). Removed 15 minutes after TSH injection. × 22,000.

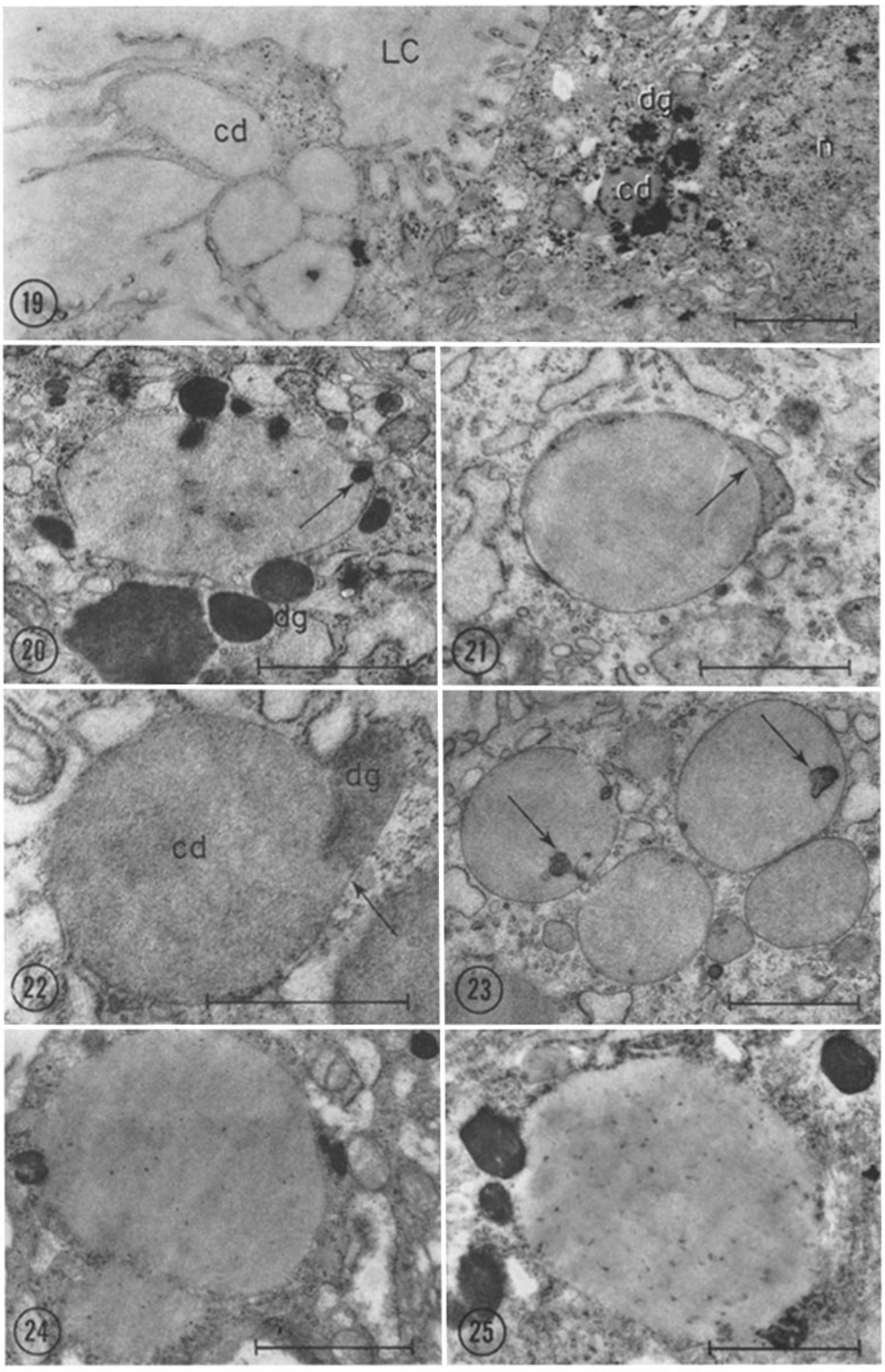
FIGURE 21 Colloid droplet with a dense granule closely applied to its surface. Granule and droplet membranes appear to be separate and intact in this section (arrow). Removed 30 minutes after TSH injection. Uranyl acetate–Reynolds' lead stain. × 22,000.

FIGURE 22 Apparent fusion of dense granules (*dg*) and colloid droplet. Note continuous common membrane surrounding both organelles (arrow). Normal animal sacrificed 3 hours after injection of TSH. Uranyl acetate–Reynolds' lead stain. × 29,500.

FIGURE 23 Membrane fragments (arrows) within colloid droplets similar to those encountered in some dense granules (*cf.* Fig. 3). Removed 30 minutes after TSH injection. Uranyl acetate–Reynolds' lead stain. × 19,000.

FIGURE 24 Acid phosphatase reaction product localized to dense granules closely associated with an essentially unreactive colloid droplet. Removed 30 minutes after TSH injection. Uranyl acetate–Reynolds' lead stain. × 23,500.

FIGURE 25 Acid phosphatase reaction product evident in dense granules closely associated with a lightly reactive colloid droplet. A moderate amount of "extraneous" reaction product is noted in the surrounding cytoplasm in this specimen. Removed 15 minutes after TSH injection. × 22,000.


LC
cd
dg
n
cd
19
dg
20
21
cd
dg
22
23
24
25

cells (Fig. 19). In addition, extensive deposits of reaction product were occasionally found in the cytoplasm surrounding heavily stained inclusions. Although several mechanisms of reaction product deposition and localization may be responsible for these observations—including the activity of appropriate enzymes at these sites—such staining is regarded somewhat arbitrarily in this report as "extraneous" reaction product, as distinguished from reaction product deposition well localized within discrete cytoplasmic loci.

Extraneous staining was encountered with relative frequency in thyroid tissue, in comparison with adjacent portions of lymph node, parathyroid, or loose connective tissue, which routinely showed well localized reaction product (*e.g.*, in macrophage phagosomes; *cf.* Cohn and Wiener, 1963) and remained consistently free of extraneous reaction product. Such extraneous staining was usually abolished by incubating sections in substrate at lower temperatures or for shorter times, or by increasing the length or strength of the postincubation acetic acid rinse. Each of these measures, however, decreased not only extraneous reaction product deposition, but the amount of reaction product found within discrete cytoplasmic organelles. Furthermore, sites such as early colloid droplets, which stained only weakly under optimal conditions for reaction product localization, often lacked reaction product following relatively mild incubation conditions which still produced staining in many dense granules. Increased incubation time or temperature generally appeared to result in heavier reaction product deposition with increasingly less specificity of localization.

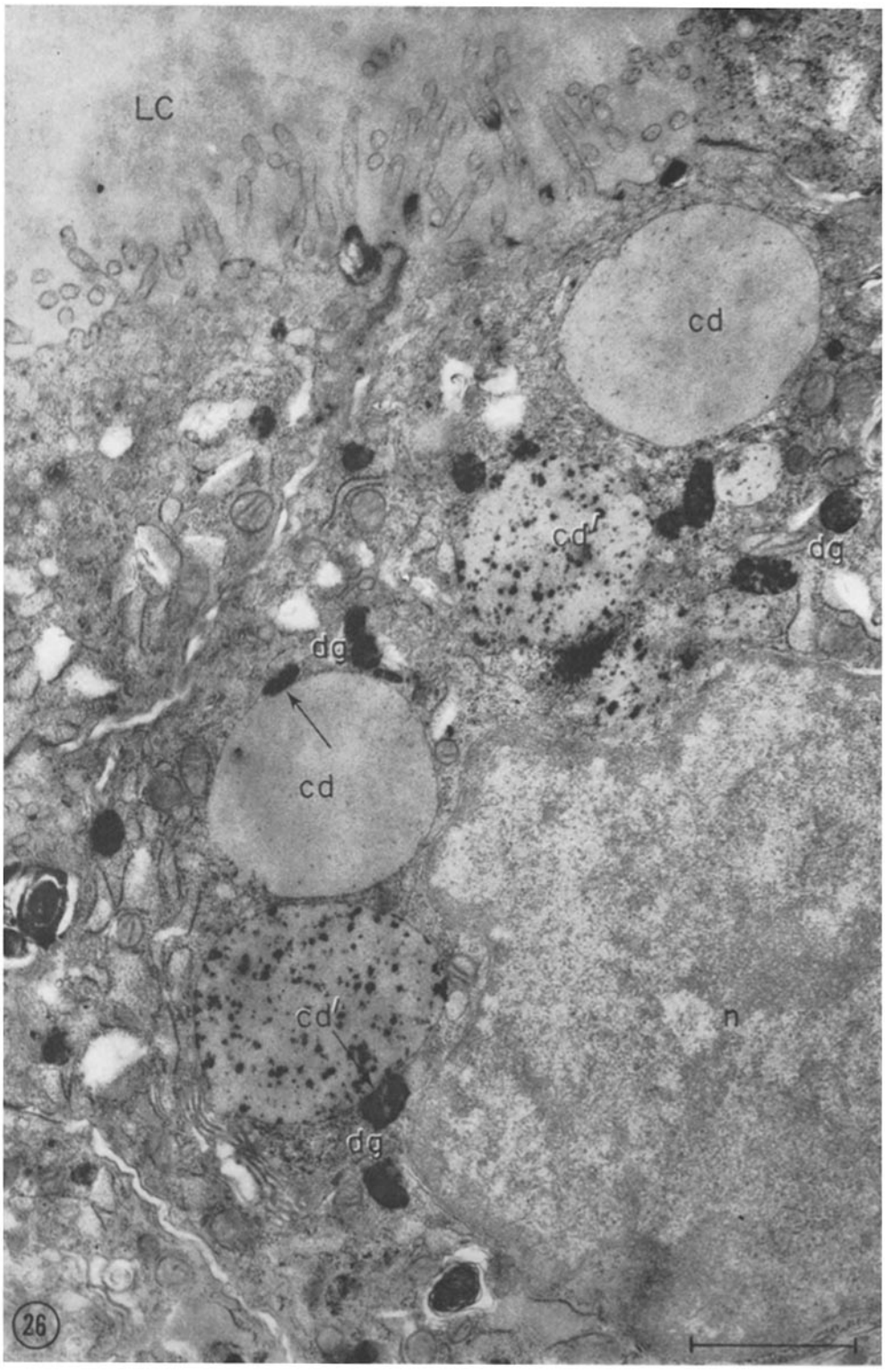
Specimens obtained under representative experimental circumstances were prepared using each of the three preincubation fixation methods described, in order to compare the surviving presumptive enzyme activity. The appearance of such activity within recently formed droplets was most dramatically demonstrated following calcium acetate-formalin prefixation, with heavier reaction product deposition in these and other discrete organelles in tissue free of extraneous reaction product. Unfortunately, the preservation of morphological detail suffered significantly following both formalin prefixation procedures. Glutaraldehyde prefixation unquestionably provided the most intact tissue morphologically, although presumptive enzyme activity was appreciably lower and precise reaction product localization commensurately less sensitive. The results of these three variations in prefixation were in general agreement, however, and no particular cytological locus suffered selective inhibition or enhancement of presumptive enzyme activity.

Each of the enzyme inactivation techniques abolished virtually all of the reaction product deposition within the tissue sections, including extraneous deposits occurring in nuclei and cytoplasm.

Variations in the amount of both localized and extraneous reaction product within a given 40 μ cryostat section were often observed and may be attributable to penetration gradients of either fixative or substrate. By comparison with unstained material, it was determined that subsequent staining of thin sections with lead or uranyl salts did not visibly alter the disposition of the cytochemical reaction product.

On the basis of limited experience, inadequate for selecting an optimal medium, the Barka and Anderson modification was chosen as yielding somewhat more selective reaction product deposition.

FIGURE 26 Acid phosphatase localization in dense granules (*dg*) and, to a variable extent, in colloid droplets within the cell. Of the four large colloid droplets illustrated, two (*cd'*) show moderate reaction product deposition, while the remaining two (*cd*) (perhaps more recently formed) are nearly unreactive. Note the intimate association of several reactive dense granules with the colloid droplets (arrows). Some ice crystal damage is apparent; the section is essentially free of extraneous reaction product. The luminal colloid (*LC*) is unreactive. Colloid droplets exhibiting this extent of basipetal migration and reaction product deposition have not been seen in tissue removed prior to 20 minutes after TSH administration to hypophysectomized animals. On the other hand, these configurations are readily found at later times, and in tissue from intact rats. Unstimulated normal animal. Epon 812 embedding. × 23,500.


LC
cd
cd
dg
dg
cd
cd
n
dg
26

DISCUSSION

Technical Considerations

Several lines of evidence support the assumption that the cytochemical reaction product demonstrated in many dense granules and in certain colloid droplets represents true non-specific acid phosphatase activity in its natural loci. Most importantly, good reaction product localization has been repeatedly achieved in these organelles utilizing methods comparable to those generally used and accepted as yielding enzyme localization (Essner and Novikoff, 1961, Holt and Hicks, 1961, Miller, 1962, Sabatini *et al.*, 1963). Other sites considered to show non-specific acid phosphatase activity, such as tissue macrophage phagosomes (Straus, 1964), have been found to contain well localized reaction product in close proximity to optimally prepared thyroid tissue. The present findings are consistent with results obtained at the light microscope level using both Gomori's (1952) and Burstone's (1962) techniques for the localization of non-specific acid phosphatase (Wollman *et al.*, 1964). Finally, reaction product deposition in these sites was abolished by methods capable of enzyme inactivation.

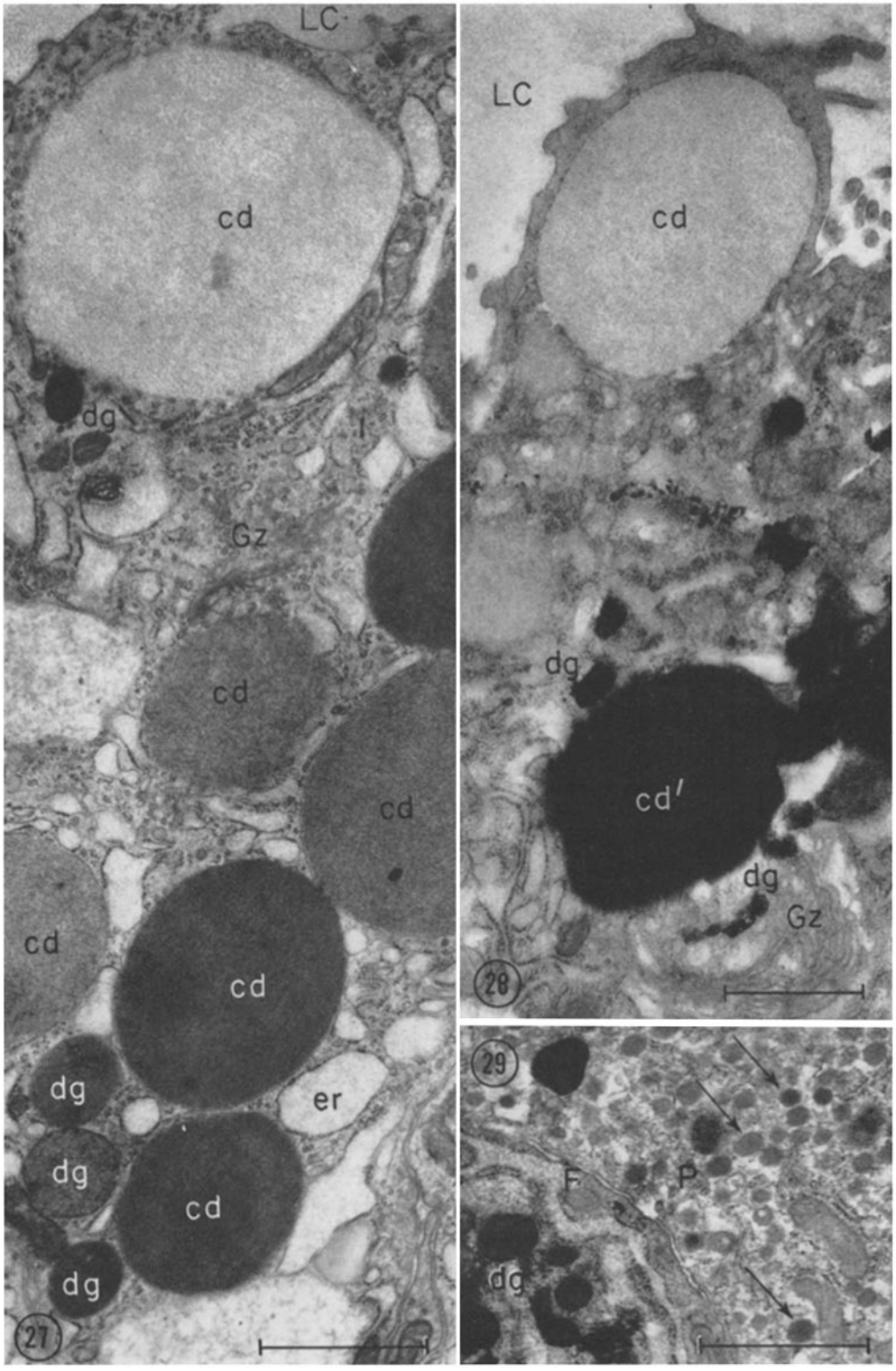
Failure to demonstrate cytochemical reaction product deposition in the luminal follicular colloid, in a few dense granules, or in recently formed colloid droplets (despite convincing reaction product deposition in other sites within the same specimen) suggests the absence of non-specific acid phosphatase from these non-reactive sites. These sites have remained free of reaction product even in heavily incubated specimens showing widespread deposition of "extraneous" lead precipitate. Similar results have been obtained regardless of the type of preincubation fixative used. Concluding the absence of enzyme activity, however, is necessarily inferential and cannot be established with certainty despite efforts to reduce the possibilities of selective enzyme inactivation or loss, and other causes of false negative results.

As a technical matter, the cytochemical results here designated as "extraneous" staining require further consideration. Local concentrations of reaction product in the cytoplasm surrounding heavily stained dense granules and droplets could readily be accounted for by limited diffusion of either active enzyme or free hydrolysate, resulting in displacement of reaction product to the immediate vicinity of its original site within a droplet or granule. Inactivation of localized granule or droplet enzyme would thus also abolish "leakage"

FIGURE 27 Variation in colloid droplet (*cd*) morphology, presumably reflecting the relative age of the droplets. Note the decreased size and increased density generally apparent as the droplets progress basipetally. Note also the similarities between some dense granules (*dg*) and the more basal colloid droplets, in contrast to the apical droplet and the luminal colloid (*LC*). The injection of ¼ unit TSH has produced in this intact animal a characteristically massive response, resulting in the accumulation of large numbers of colloid droplets per cell through continued resorption. The most extensive colloid droplet changes are characteristic of late times (1 hour or more) following TSH injection in hypophysectomized animals. On the other hand, the apical droplet is not noticeably altered in this specimen obtained 3 hours after TSH injection, suggesting its recent origin. × 24,500.

FIGURE 28 Heavy acid phosphatase reaction product deposition within a large colloid droplet (*cd'*) after heavy TSH stimulation of a normal animal. A more recently formed colloid droplet profile (*cd*) within a pseudopod is entirely free of reaction product. Compare with colloid droplets of Fig. 15 showing intermediate reactivity. Dense granules (*dg*), some of which lie in close proximity to a Golgi zone (*Gz*), also contain reaction product. Debris is present on the section midway between the colloid droplets. Obtained 2 hours following the injection of ¼ unit TSH. × 20,500.

FIGURE 29 Acid phosphatase reaction product within the dense granules (*dg*) in a thyroid follicular epithelial cell (*F*) and in a comparable organelle (upper left) within a parafollicular cell (*P*). The characteristic, small (0.1-μ) membrane-limited vesicles of the parafollicular cell (arrows) usually lack acid phosphatase reaction product. Unstimulated hypophysectomized animal. × 23,500.


LC
cd
dg
Gz
cd
cd
cd
cd
cd
dg
er
dg
cd
dg
27
LC
cd
dg
cd'
dg
Gz
28
29
F
P
dg

reaction product deposition, and, indeed, extraneous staining was absent in enzyme-inhibited control preparations. More extensive diffusion of enzyme or of enzyme hydrolysate, followed by adsorption in the offending sites, could possibly explain the nuclear staining observed in some specimens. Again, an explanation dependent upon intact enzyme activity is implied, since all extraneous staining is abolished by procedures of enzyme inactivation.

In considering the possibility of diffusion artifact, it is interesting that satisfactory fine structural preservation is relatively difficult to obtain with thyroid tissues, particularly with regard to membrane continuity. Adjacent portions of other tissues appear to remain free of extraneous reaction product deposition, although these problems persist in thyroid follicular epithelial cells. Such findings suggest that the peculiarity lies in part, at least, with the tissue rather than with the cytochemical methods. Although the possibility cannot be excluded that "extraneous" staining represents the normal occurrence of non-specific acid phosphatase activity in these loci, the naphthol substrates of Burstone (1962) do not reveal activity in nuclei and other sites of presumed false positive staining (Wollman *et al.*, 1964).

Physiological Considerations

This report provides little evidence for influence of hypophysectomy or thyrotropin stimulation on biosynthesis, although changes induced in resorptive mechanisms are readily distinguished. Perhaps the relatively early times investigated following thyrotropin stimulation do not afford a fully developed biosynthetic response. The abundant Golgi elements and rough-surfaced endoplasmic reticulum of these cells—two organelles primarily concerned with the secretion of proteinaceous material (Caro and Palade, 1964)—are quite extensive in thyroid follicular cells. Appreciable Golgi activity is reflected by clusters of 0.1 μ vesicles in this area of some cells in unstimulated animals, and is, perhaps, more frequently encountered in animals receiving thyrotropin.

The disposition of 0.1 μ vesicles within the Golgi zones and beneath the apical plasma membranes of some cells strongly implicates these organelles in the transfer of newly synthesized colloid to the follicular lumen (Nadler *et al.*, 1964). Possible fusion of these organelles with the plasma membrane and the contribution of their contents to the follicular lumen is suggested. The involvement of these organelles in a pinocytotic intake of luminal colloid cannot be entirely excluded, but the evident relationship of the Golgi apparatus to their presence within the cell indicates a secretory phenomenon. The lack of obvious involvement of these organelles in colloid resorptive processes strengthens this position. These vesicles lack demonstrable acid phosphatase activity, further distinguishing them from organelles apparently involved in degradative functions.

A matter of particular interest regarding these presumptive secretory vesicles is the non-uniformity of the vesicle population from cell to cell. Even more variance is found when follicles are compared in regard to the number of such vesicles evident in the Golgi region and underlying the luminal surface. This disparity has been observed in all thyroids examined, irrespective of hormonal conditions, and has failed to corroborate reports emphasizing the uniformity of these cells in regard to their vesicle populations (Nadler *et al.*, 1964).

Consistent with interpretations from earlier light microscope observations (Nadler *et al.*, 1962, Wollman *et al.*, 1964), the present study provides strong evidence for the origin of colloid droplets through engulfment of luminal material by apical cell processes. This engulfment of follicular colloid might well be envisioned as a closure of membranous pseudopod extensions—a mechanism closely akin to phagocytosis.[1] However, the dynamics of pseudopod formation and function must be verified and detailed by other means. Evidence is lacking for any other mechanism for the engulfment of substantial amounts of follicular colloid.

The initial appearance of colloid droplets within pseudopod processes, in conjunction with the continuous sequence of changes leading to the disappearance of these organelles, argues strongly against the apical migration of colloid droplets in a secretory capacity. The extent of pseudopod activity evident under these experimental conditions would appear sufficient to account for the observed formation of colloid droplets, particularly since several droplets may occur in a single pseudopod

[1] The term "phagocytosis" has been chosen, rather than "pinocytosis", on the basis of prevalent usage and connotations in current discussions of cellular ultrastructure, and not from an etymological viewpoint.

within 15 minutes after stimulation. Furthermore, obvious intermediary forms between Golgi-associated vesicles and colloid droplets (Wissig, 1963, Fujita, 1963) have not been evident in these studies. It should be emphasized that large numbers of presumptive secretory vesicles may be encountered in hypophysectomized animals in which colloid droplets are uniformly absent. Certain cells in normal or stimulated animals may also lack colloid droplets and yet contain numerous vesicles. Cells containing colloid droplets may or may not contain such vesicles. Thus, no clear interrelationships have been noted between colloid droplets and presumptive secretory activity in the thyroid follicular epithelial cells of these animals.

The striking physical association observed between dense granules and newly formed colloid droplets represents a prominent and constant feature of cells engaged in colloid resorption. The appearance of acid phosphatase reaction product in colloid droplets closely coincides with their dense granule association. Also, membrane fragments, minute particles, and amorphous dense material comparable to that encountered in dense granule matrices appear within colloid droplets at roughly the same point in the response. A direct transfer of enzyme activity from dense granule to colloid droplet through granule-droplet fusion is considered likely on the basis of the available evidence. Instances of apparent dense granule-colloid droplet fusion were surprisingly rare, possibly because such a process might occur with great rapidity. Analogously, a comparable process occurs within $\frac{1}{10}$ of a second, transferring contents of leukocyte granules to phagocytic vacuoles (Hirsch and Cohn, 1960).

Alternative mechanisms for the appearance of enzyme activity in droplets cannot be eliminated, as for example, transfer across closely apposed intact membranes or the activation of latent enzyme activity within the engulfed colloid. The involvement of other organelles in this process has been tentatively discounted because of their apparent lack of enzyme activity and of association with colloid resorption droplets.

Configurations suggestive of droplet-droplet fusion have been observed in pseudopods and in the apical cell cytoplasm. The converse interpretation must also be considered, in that large droplets may subdivide by means of intrusive cytoplasmic septa. Such configurations might also arise through the inclusion of membranous extensions of the pseudopod surface within newly formed droplets. These configurations apparently do not persist, since spherical or ovoid shapes predominate, particularly in later droplets. Artifactual disruption of cytoplasm intervening between droplets must be considered, although incomplete septa are encountered regularly in relatively well preserved material.

Verification of droplet-droplet fusion is complicated by the identical contents of the organelles involved. Parallel problems arise in a discussion of granule-granule fusion, although such events appear likely.

The fate of colloid droplets is indicated by a progression toward smaller, more adielectronic bodies distributed primarily in the basal regions of the cells. This process apparently can occur in less than 90 minutes, since a complete sequence of droplets can be found routinely in thyroids removed 90 minutes after the stimulation of hypophysectomized animals. The increased density and smaller size of older droplets suggest a process involving dehydration and concentration of droplet contents.

As a result of these alterations, aging colloid droplets come to resemble the larger dense granules encountered in thyroids of unstimulated, hypophysectomized animals. Some thyroid dense granules might indeed represent terminal stages of colloid droplets. The heterogeneous contents of many dense granules may betray the residuum from previous catabolic processes. Further involvement of these same organelles in transferring enzymatic activity to freshly incorporated colloid droplets would constitute an effective enzyme economy measure. An analogous example of the reutilization of catabolic enzymes has recently been reported by Bensch *et al.* (1964) from their studies with tissue culture cells.

The wide structural and dimensional variation encountered among thyroid dense granules has already been emphasized. The failure to demonstrate cytochemical reaction product in some of these organelles further indicates a mixed population. Functional and developmental variations presumably are represented, but discrimination and further classification among dense granules is largely speculative. Of particular interest in this regard are the smaller (0.03 to 0.06 μ) pleomorphic, phosphatase-positive dense granules often encountered in the Golgi region (Figs. 6, 28) and in close approximation to colloid droplets

(Figs. 24, 26) or other dense granules. These small inclusions hypothetically could supply newly synthesized enzymes and associated products as required by organelles such as colloid droplets or other dense granules. Dense granules apparently contribute to other catabolic processes within thyroid follicular epithelial cells, including, for example, autolytic functions (Fig. 3, *cf.* Ashford and Porter, 1962). A rather different circumstance, possibly with very different physiological connotations, may obtain within the parafollicular cell, in which transient acid phosphatase activity appears to be transferred from active Golgi elements to newly formed characteristic vesicles. Analogous situations have previously been described in various secretory sites (Novikoff and Essner, 1962, and Osinchak, 1964).

The lysosome concept, as advanced by de Duve (1959) on the basis of biochemical findings, provides a general hypothesis for the cellular management of catabolic events through the compartmentalization of acid hydrolases within membrane-limited inclusions. The acid phosphatase–containing dense granules and colloid droplets, as well as the reactive Golgi elements described above, clearly compare with the general description advanced for this hypothetical organelle; thus, this report provides further evidence from an additional tissue site to substantiate this general lysosome concept. However, the term "lysosome" appears too general to sufficiently distinguish and characterize the various thyroid follicular cell organelles showing acid phosphatase activity. Beyond a general classification of their enzyme contents, these thyroid cell organelles of apparently diverse origins and interrelationships can be most fruitfully compared on a functional basis with similar organelles in other tissues.

As noted previously, the appearance of enzyme activity within newly engulfed colloid droplets following thyrotropin administration suggests that hydrolases, presumably derived from cytoplasmic dense granules, might function to alter resorbed colloid in some way during thyroxine mobilization (Wollman *et al.*, 1964). This explanation is further indicated by the apparent increase in demonstrable enzyme activity as colloid droplets age and become smaller and more adielectronic. The precise role of acid phosphatase in this process is unresolved; but the additional involvement of esterase (Wollman *et al.*, 1964) perhaps implies relatively general catabolic activity. The participation of non-specific acid phosphatase, accompanied by a battery of other hydrolases including cathepsins, in general catabolic cell activities has been elucidated in several tissue sites including liver (de Duve, 1959) and peritoneal exudate leukocytes (Hirsch and Cohn, 1960). The occurrence of a similar complement of enzymes in thyroid dense granules and in colloid resorption droplets would implement thyroglobulin degradation and the release of thyroxine.

The authors gratefully acknowledge the expert and conscientious technical assistance rendered by Mrs. Esther M. Kendig and Miss Margaretta C. MacLeod in procedures ancillary to electron microscopy, by Mr. Franklin E. Reed in the surgical procedures and animal preparation, and by Mrs. Susan B. Hash and Mrs. Jacqueline G. Henson in effecting the cytochemical procedures.

Received for publication, August 17, 1964.

Note Added in Proof: Since this paper was submitted for publication, three reports have appeared which demonstrate by electron microscope radioautography that intracellular colloid droplets contain iodoprotein: BAUER, W. C., and MEYER, J. S., *Science*, 1964, **145**, 1431; SHELDON, H. H., MCKENZIE, J. M., and VAN NIMWEGEN, D., *J. Cell Biol.*, 1964, **23**, 200; and STEIN, O., and GROSS, J., *Endocrinol.*, 1964, **75**, 787.

REFERENCES

ASHFORD, T. P., and PORTER, K. R., Cytoplasmic components in hepatic cell lysosomes, *J. Cell Biol.*, 1962, **12**, 198.

BARKA, T., and ANDERSON, P. J., Histochemical methods for acid phosphatase using hexazonium pararosanilin as coupler, *J. Histochem. and Cytochem.*, 1962, **10**, 741.

BENSCH, K., GORDON, G., and MILLER, L., The fate of DNA-containing particles phagocytized by mammalian cells, *J. Cell Biol.*, 1964, **21**, 105.

BURSTONE, M. S., Enzyme Histochemistry and Its Application in the Study of Neoplasms, New York, Academic Press, Inc., 1962, 621.

CARO, L. G., and PALADE, G. E., Protein synthesis, storage, and discharge in the pancreatic exocrine cell. An autoradiographic study, *J. Cell Biol.*, 1964, **20**, 473.

CAULFIELD, J. B., Effects of varying the vehicle for OsO_4 in tissue fixation, *J. Biophysic. and Biochem. Cytol.*, 1957, **3**, 827.

COHN, Z. A., and WIENER, E., The particulate hydrolases of macrophages. I. Comparative enzymology, isolation and properties, *J. Exp. Med.*, 1963, **118**, 991.

DALTON, A. J., and ZIEGEL, R. F., A simplified method of staining thin sections of biological material with lead hydroxide for electron microscopy, *J. Biophysic. and Biochem. Cytol.*, 1960, **7**, 409.

DEDUVE, C., Lysosomes, a new group of cytoplasmic particles, *in* Subcellular Particles, (Teru Hayashi, editor), New York, The Ronald Press Co., 1959, 128.

DE ROBERTIS, E., Intracellular colloid in the initial stages of thyroid activation, *Anat. Rec.*, 1942, **84**, 125.

EKHOLM, R., and SJÖSTRAND, F. S., The ultrastructural organization of the mouse thyroid gland, *J. Ultrastruct. Research*, 1957, **1**, 178.

ESSNER, E., and NOVIKOFF, A. B., Localization of acid phosphatase activity in hepatic lysosomes by means of electron microscopy, *J. Biophysic. and Biochem. Cytol.*, 1961, **9**, 773.

FREEMAN, J. A., and SPURLOCK, B. O., A new epoxy embedment for electron microscopy, *J. Cell Biol.*, 1962, **13**, 437.

FUJITA, H., Electron microscopic studies on the thyroid gland of domestic fowl, with special reference to the mode of secretion and the occurrence of a central flagellum in the follicular cell, *Z. Zellforsch. u. mikr. Anat.*, 1963, **60**, 615.

GERSH, I., Glycoproteins in the thyroid gland of rats, *J. Endocrinol.*, 1950, **6**, 282.

GOLDFISCHER, S., ESSNER, E., and NOVIKOFF, A. B., The localization of phosphatase activities at the level of ultrastructure, *J. Histochem. and Cytochem.*, 1964, **12**, 72.

GOMORI, G., Microscopic Histochemistry: Principles and Practice, 1952, Chicago, University of Chicago Press, 273.

GRANT, M. P., The release of follicular colloid from the thyroid of *Amblystoma jeffersonianum* following heteroplastic anterior pituitary implants, *Anat. Rec.*, 1931, **49**, 373.

HERMAN, L., An electron microscope study of the salamander thyroid during hormonal stimulation, *J. Biophysic. and Biochem. Cytol.*, 1960, **7**, 143.

HIRSCH, J. G., and COHN, Z. A., Degranulation of polymorphonuclear leukocytes following phagocytosis of microorganisms, *J. Exp. Med.*, 1960, **112**, 1005.

HOLT, S. J., and HICKS, R. M., The localization of acid phosphatase in rat liver cells as revealed by combined cytochemical staining and electron microscopy, *J. Biophysic. and Biochem. Cytol.*, 1961, **11**, 47.

KARNOVSKY, M. J., Simple methods for "staining with lead" at high pH in electron microscopy, *J. Biophysic. and Biochem. Cytol.*, 1961, **11**, 729.

LUFT, J., Improvements in epoxy embedding methods, *J. Cell Biol.*, 1961, **9**, 409.

MILLER, F., Acid phosphatase localization in renal protein absorption droplets, *in* Fifth International Congress for Electron Microscopy, (Sydney S. Breese, Jr., editor), 1962, New York, Academic Press, Inc., Q-2.

MILLONIG, G., Advantages of a phosphate buffer for OsO_4 solutions in fixation (Abstract), *J. Appl. Physics*, 1961 *a*, **32**, 1637.

MILLONIG, G., A modified procedure for lead staining of thin sections, *J. Biophysic. and Biochem. Cytol.*, 1961 *b*, **11**, 736.

NADLER, N. J., SARKAR, S. K., and LEBLOND, C. P., Origin of intracellular colloid droplets in the rat thyroid, *Endocrinology*, 1962, **71**, 120.

NADLER, N. J., YOUNG, B. A., LEBLOND, C. P., and MITMAKER, B., Elaboration of thyroglobulin in the thyroid follicle, *Endocrinology*, 1964, **74**, 333.

NOVIKOFF, A. B., and ESSNER, E., Pathological changes in cytoplasmic organelles, *Fed. Proc.*, 1962, **21**, 1130.

NOVIKOFF, A. B., and VORBRODT, A., Lysosomes and thyroid function (Abstract), *J. Cell Biol.*, 1963, **19**, 53A.

OSINCHAK, J., Electron microscopic localization of acid phosphatase and thiamine pyrophosphatase activity in hypothalamic neurosecretory cells of the rat, *J. Cell Biol.*, 1964, **21**, 35.

PALADE, G. E., A study of fixation for electron microscopy, *J. Exp. Med.*, 1952, **95**, 285.

REYNOLDS, E. S., The use of lead citrate at high pH as an electron-opaque stain in electron microscopy, *J. Cell Biol.*, 1963, **17**, 208.

SABATINI, D. D., BENSCH, K., and BARRNETT, R. J., Cytochemistry and electron microscopy. The preservation of cellular ultrastructure and enzymatic activity by aldehyde fixation, *J. Cell Biol.*, 1963, **17**, 19.

SPURLOCK, B. O., KATFINE, V. C., and FREEMAN, J. A., Technical modifications in Maraglas embedding, *J. Cell Biol.*, 1963, **17**, 203.

STRAUS, W., Cytochemical observations on the relationship between lysosomes and phagosomes in kidney and liver by combined staining for acid phosphatase and intravenously injected horseradish peroxidase, *J. Cell Biol.*, 1964, **20**, 497.

WATSON, M. L., Staining of tissue sections for electron microscopy with heavy metals, *J. Biophysic. and Biochem. Cytol.*, 1958, **4**, 475.

WETZEL, B. K., SPICER, S. S., and WOLLMAN, S. H., Nonspecific acid-phosphatase activity in rat thyroid glands following TSH stimulation (Abstract), *J. Appl. Phys.*, 1963, **34**, 2525.

WISSIG, S. L., The anatomy of secretion in the follicular cells of the thyroid gland. I. The fine structure of the gland in the normal rat, *J. Biophysic. and Biochem. Cytol.*, 1960, **7**, 419.

WISSIG, S. L., The fine structure of parafollicular (light) cells of the rat thyroid gland, *in* Fifth International Congress for Electron Microscopy, (Sydney S. Breese, Jr., editor), 1962, New York, Academic Press, Inc., WW-1.

WISSIG, S. L., The anatomy of secretion in the follicular cells of the thyroid gland. II. The effect of acute thyrotropic hormone stimulation on the secretory apparatus, *J. Cell Biol.*, 1963, **16**, 93.

WOLLMAN, S. H., and SPICER, S. S., Intracellular colloid droplets in the thyroid gland, *Fed. Proc.*, 1961, **20**, 201.

WOLLMAN, S. H., and SPICER, S. S., Intracellular colloid droplets following thyrotropin injection, *in* Thyrotropin, (S. C. Werner, editor), 1963, Springfield, Illinois, C. C. Thomas, 168.

WOLLMAN, S. H., SPICER, S. S., and BURSTONE, M. S., Localization of esterase and acid phosphatase in granules and colloid droplets in rat thyroid epithelium, *J. Cell Biol.*, 1964, **21**, 191.

YOSHIMURA, F., YONETSU, T., and NAKAMURA, M., Hormonal regulation of parafollicular cell in thyroid gland, *Endocrinol. Jap.*, 1962, **9**, 284.

YOUNG, B. A., and LEBLOND, C. P., The light cell as compared to the follicular cell in the thyroid gland of the rat, *Endocrinology*, 1963, **73**, 669.