LYSOSOME FUNCTION IN THE REGULATION OF THE SECRETORY PROCESS IN CELLS OF THE ANTERIOR PITUITARY GLAND

ROBERT E. SMITH and MARILYN G. FARQUHAR

From the Department of Pathology, University of California School of Medicine, San Francisco. Dr. Smith's present address is Department of Pathology, Stanford University, and Veterans Administration Hospital, Palo Alto, California

ABSTRACT

The nature and content of lytic bodies and the localization of acid phosphatase (AcPase) activity were investigated in mammotrophic hormone-producing cells (MT) from rat anterior pituitary glands. MT were examined from lactating rats in which secretion of MTH[1] was high and from postlactating rats in which MTH secretion was suppressed by removing the suckling young. MT from lactating animals contained abundant stacks of rough-surfaced ER, a large Golgi complex with many forming secretory granules, and a few lytic bodies, primarily multivesicular bodies and dense bodies. MT from postlactating animals, sacrificed at selected intervals up to 96 hr after separation from their suckling young, showed (*a*) progressive involution of the protein synthetic apparatus with sequestration of ER and ribosomes in autophagic vacuoles, and (*b*) incorporation of secretory granules into multivesicular and dense bodies. The content of mature granules typically was incorporated into dense bodies whereas that of immature granules found its way preferentially into multivesicular bodies. The secretory granules and cytoplasmic constituents segregated within lytic bodies were progressively degraded over a period of 24 to 72 hr to yield a common residual body, the vacuolated dense body. In MT from lactating animals, AcPase reaction product was found in lytic bodies, and in several other sites not usually considered to be lysosomal in nature, i.e., inner Golgi cisterna and associated vesicles, and around most of the immature, and some of the mature secretory granules. In MT from postlactating animals, AcPase was concentrated in lytic bodies; reaction product and incorporated secretory granules were frequently recognizable within the same multivesicular or dense body which could therefore be identified as "autolysosomes" connected with the digestion of endogenous materials. Several possible explanations for the occurrence of AcPase in nonlysosomal sites are discussed. From the findings it is concluded that, in secretory cells, lysosomes function in the regulation of the secretory process by providing a mechanism which takes care of overproduction of secretory products.

INTRODUCTION

Lysosomes are now recognized as a morphologically heterogenous group of cytoplasmic particles which contain acid hydrolases and serve as an intracellular digestive system (1, 2). It has become apparent, primarily as a result of morphological and cytochemical studies with the electron microscope, that the relatively primitive activity of intracellular digestion has been adapted in special-

ized cells of multicellular organisms to subserve a variety of complex functions such as defense (3, 4), absorption (5, 6), differentiation (7, 8), and cell involution (9). Hence, the role of lysosomes in many types of cells has been elucidated; in others, however, especially neurons and secretory cells of exocrine and endocrine glands (2, 10–14), their functions have remained obscure.

We have investigated lysosomal activities in secretory cells by carrying out extensive cytochemical and electron microscope studies on the rat anterior pituitary gland during different phases of secretion (15, 16). In our studies we have paid particular attention to the nature of lytic bodies and the distribution of acid phosphatase (AcPase)[1] activity. In this paper we report our detailed findings on the mammotrophic hormone-producing cell (MT) which we have studied during lactation, when its secretory level is high (17–19), and after removal of suckling young, when hormone secretion is suppressed (19). The findings clearly demonstrate that in cells of the anterior pituitary gland, lysosomes function in the regulation of the secretory process by incorporating and degrading undischarged secretory granules.

MATERIALS AND METHODS

Animals

Thirty-seven primiparous lactating rats, of the Sprague-Dawley strain, were used in these experiments. Animals were housed in individual cages in a room maintained at 24°C and artificially illuminated during the normal daylight hours. They were fed Purina Lab Chow and water ad libitum. Shortly after birth each litter was adjusted to 8 young.

Experiments

Two main experimental groups were established: lactating and postlactating. The lactating group consisted of 12 animals allowed to nurse their young normally and sacrificed on the 3rd to 5th postpartum day. The postlactating group consisted of 25 animals isolated from their litters on the 3rd to 5th postpartum day, and sacrificed at 12, 24, 48, 72, or 96 hr thereafter.

[1] The abbreviations used in this paper are: AcPase, acid phosphatase; ER, endoplasmic reticulum; GP, sodium β-glycerophosphate; IDP, inosine diphosphate; MT, mammotroph or mammotrophic hormone-producing cell; MTH, mammotrophic or lactogenic hormone; ST, somatotroph or growth hormone-producing cell; GT, gonadotroph or gonadotrophic hormone-producing cell; TT, thyrotroph or thyrotrophic hormone-producing cell.

The 3rd to 5th days of lactation were selected for these experiments because the level of MTH secretion is known to be high at this time (19, 20). The total lactation period in the rat is about 28 days.

Techniques for Morphological Studies

FIXATION: Fixation of the pituitary gland was initiated by injecting 5 cc of fixative into the cranial cavity through a fine hypodermic needle inserted into the foramen magnum. In most cases the fixative utilized was 1% OsO_4 in phosphate buffer, pH 7.6 (21); a few glands were fixed in 1.5% glutaraldehyde in 0.067 M cacodylate buffer, pH 7.4 (22) containing 1% sucrose. A few minutes after the injection, the pituitary was exposed, freed from the dura, removed, immersed in a drop of fixative, and cut into pieces of 1 mm^3. These were transferred to a vial containing fresh fixative, and fixed at 4°C for a total of 2 hr in OsO_4, or 19 to 24 hr in glutaraldehyde. The glutaraldehyde stock solution was purified by distillation prior to use, as described below.

DEHYDRATION, EMBEDDING, AND SECTIONING: After fixation most of the OsO_4-fixed tissues were dehydrated in ethanol and embedded in Araldite (23). A few specimens were dehydrated in acetone and stained for 5 to 10 min in 0.5% $KMnO_4$ in absolute acetone (24) prior to embedding. Glutaraldehyde-fixed tissues were transferred directly to 1% OsO_4 in acetate-Veronal buffer (pH 7.4) for 2 hr and then dehydrated in ethanol; some blocks were stained in buffered 0.5% uranyl acetate in Michaelis buffer (pH 5.3) prior to dehydration to enhance membrane contrast (25).

Thin sections were cut with diamond knives on a Porter-Blum MT-2 microtome, mounted on carbon-coated grids, and stained with lead alone, prepared according to Karnovsky (26) or Reynolds (27), or doubly stained with uranyl acetate followed by lead.

Sections of 0.5 to 1 μ were also cut from the Araldite blocks, affixed to glass slides by moderate heating, stained with azure II–methylene blue (28), sprayed with Krylon to retard fading (29), and examined by light microscopy.

Techniques for Cytochemical Studies

FIXATION: Most glands were perfused (30) for 30 to 40 min with chilled 1.5% glutaraldehyde in 0.067 M cacodylate buffer containing 1% sucrose, removed, and immersed in fresh fixative for an additional 2 hr. In a few cases fixation was initiated by injection of glutaraldehyde *in situ* as described for morphological studies; the gland was subsequently removed and cut into strips of 1½ x 1½ x 3 to 5 mm, which were placed in fresh fixative for an additional 4 hr. The most uniform preservation of cell structure and enzyme activity was achieved in perfused specimens, but perfusion was not optimal in all cases.

The fixed whole glands or strips were washed and stored for varying periods (48 hr to 1 week) at 4°C in 0.1 M cacodylate buffer, containing 7% sucrose.

PREPARATION OF NONFROZEN SECTIONS: Nonfrozen sections were cut at 10 or 40 μ from the whole glands or strips by a technique described in detail elsewhere (31, 32). Sections were collected in 0.1 M cacodylate buffer (pH 7.4) containing 7% sucrose and washed overnight (18 to 24 hr) at 4°C to remove traces of fixative. AcPase activity remained unaffected by storage up to 1 week in buffer but was diminished in sections stored for longer periods.

In the initial phases of this work, frozen sections were utilized for incubation, but considerable disruption of fine structure occurred which was attributable to freezing and thawing during sectioning. In order to avoid tissue damage, a new sectioning technique was developed which avoids freezing. Nonfrozen sections were originally prepared with a McIlwain Mechanical Tissue Chopper (32); more recently a new instrument, capable of cutting sections as thin as 10 to 15 μ (31), was utilized.

ACID PHOSPHATASE METHOD: Sections were incubated for 20 min to 1½ hr at pH 5.0 with β-glycerophosphate (Grade II, Sigma Chemical Co., St. Louis, Missouri) as a substrate in media freshly prepared on the day of use. Both the Gomori medium (33) and the modification suggested by Barka and Anderson (34) were utilized. The former required filtering prior to use. Incubations carried out in the latter were generally more satisfactory, for there was less nonspecific lead deposition, particularly in nuclei, and a more intense reaction for a given time period.

After incubation some sections were rinsed briefly in cold 2% acetic acid, and then rinsed twice for 5 min in 0.05 M acetate-Veronal buffer (pH 7.2).

Controls consisted of incubations in which GP or lead were omitted, or 0.01 M sodium fluoride, tartaric acid, or glutaric acid were added to the media. In addition, some sections were incubated in the Barka-Anderson medium at pH 7.0 and others were incubated in similar media at pH 5.0 with IDP as substrate (35).

PREPARATIVE PROCEDURES FOR LIGHT AND ELECTRON MICROSCOPY: For light microscopy, incubated 10 or 40 μ sections were treated with dilute $(NH_4)_2S$ and mounted in glycerogel.

For electron microscopy, 40 μ incubated sections were postfixed at 4°C for 45 min in 1% OsO_4, buffered at pH 7.4 with either phosphate or acetate-Veronal. Some of the sections were treated for 30 to 45 min at room temperature with 0.5% uranyl acetate in acetate-Veronal buffer prior to dehydration. In addition to acting as a membrane stain (25) this technique also proved an effective substitute for the acetic acid rinse, since it removed much of the diffuse, presumably nonspecific lead precipitate (36). When this procedure was utilized, however, the over-all reaction was fainter and the incubation time had to be lengthened to achieve the same intensity of reaction as in untreated sections. All specimens were dehydrated in ethanol and embedded in Araldite. Thin sections were prepared as described above.

Sections 0.5 to 1 μ thick were also routinely cut from the Araldite blocks, affixed to glass slides, and treated for 30 min with 2% $(NH_4)_2S$. After a brief rinse in distilled water, they were dipped for 5 to 10 sec in 1% crystal violet in a solution of 50% acetone in absolute alcohol, air-dried, sprayed with Krylon (29), and examined by phase-contrast microscopy. Such sections were very useful in assessing the results of the histochemical procedure and the general distribution of the reaction product.

Microscopy

Micrographs were taken at magnifications of 2300 to 30,000 on a Siemens Elmiskop I, operating at 80 kv, with a double condenser, and a 50 μ molybdenum aperture in the objective.

Purification of Glutaraldehyde

Glutaraldehyde was obtained as a 25% stock solution from Union Carbide Corporation, New York, and stored at 4°C. Before being used for fixation, it was purified by distillation at atmospheric pressure. Distillate was collected between 100 and 101 °C[2] in 50 cc aliquots. Those samples with a pH less than 3.4 were discarded, whereas those of pH 3.4 or greater were pooled, rebottled, and stored at 4°C. The pH of the distillate remained stable for 3 to 6 months.

The concentration of glutaraldehyde in the pooled samples was estimated from osmolality readings. Under these conditions of collection, the osmolality of the distillate was between 800 and 1200 milliosmols, indicating a glutaraldehyde concentration of 8 to 12%.

The purification procedure was undertaken because of variations in pH and osmolality and in the quality of fixation encountered with different batches of glutaraldehyde. (See also references 37 and 38.) When a distilled stock solution was utilized, greater uniformity of fixation was obtained, particularly in regard to enzyme survival.

OBSERVATIONS

Basis of Identification of Cell Types in the Anterior Pituitary

It is now usually agreed (39–41) that there are at least four morphologically distinct functional cell

[2] According to information provided by Union Carbide Chemical Corporation, the boiling point of a 25% aqueous solution of glutaraldehyde is 101°C.

types in the anterior lobe of the rat pituitary: somatotrophs, mammotrophs, gonadotrophs, and thyrotrophs, so named to denote their association with the production of growth, mammotrophic (lactogenic), gonadotrophic, and thyrotrophic hormones, respectively.[3] With the light microscope, cell types are distinguished by differences in the staining affinities of their secretory granules; with the electron microscope, secretory granule size is the most useful criterion for cell identification. Previous studies from this laboratory (44–47) as well as the work of others (39, 41) have established that, in the rat pituitary, secretory granule size is characteristic for a given cell type; approximate maximal diameters are: ST = 350 mμ; MT = 600–900 mμ; GT = 200 mμ; TT = 150 mμ. In this study we are concerned exclusively with MT.

[3] Recent evidence (39, 42, 43) suggests that there may be an additional morphologically distinct cell type responsible for the production of adrenocorticotrophic hormone (ACTH).

With the light microscope, MT acidophils are distinguished from ST acidophils by their affinity for erythrosin (39). With the electron microscope, MT are identified by their content of large secretory granules which vary in shape from irregularly spherical to polymorphous (39, 46, 47). It is well known that MT hypertrophy during pregnancy and are active during lactation (18, 39, 40).

Morphological Studies of Mammotrophs

General Characteristics

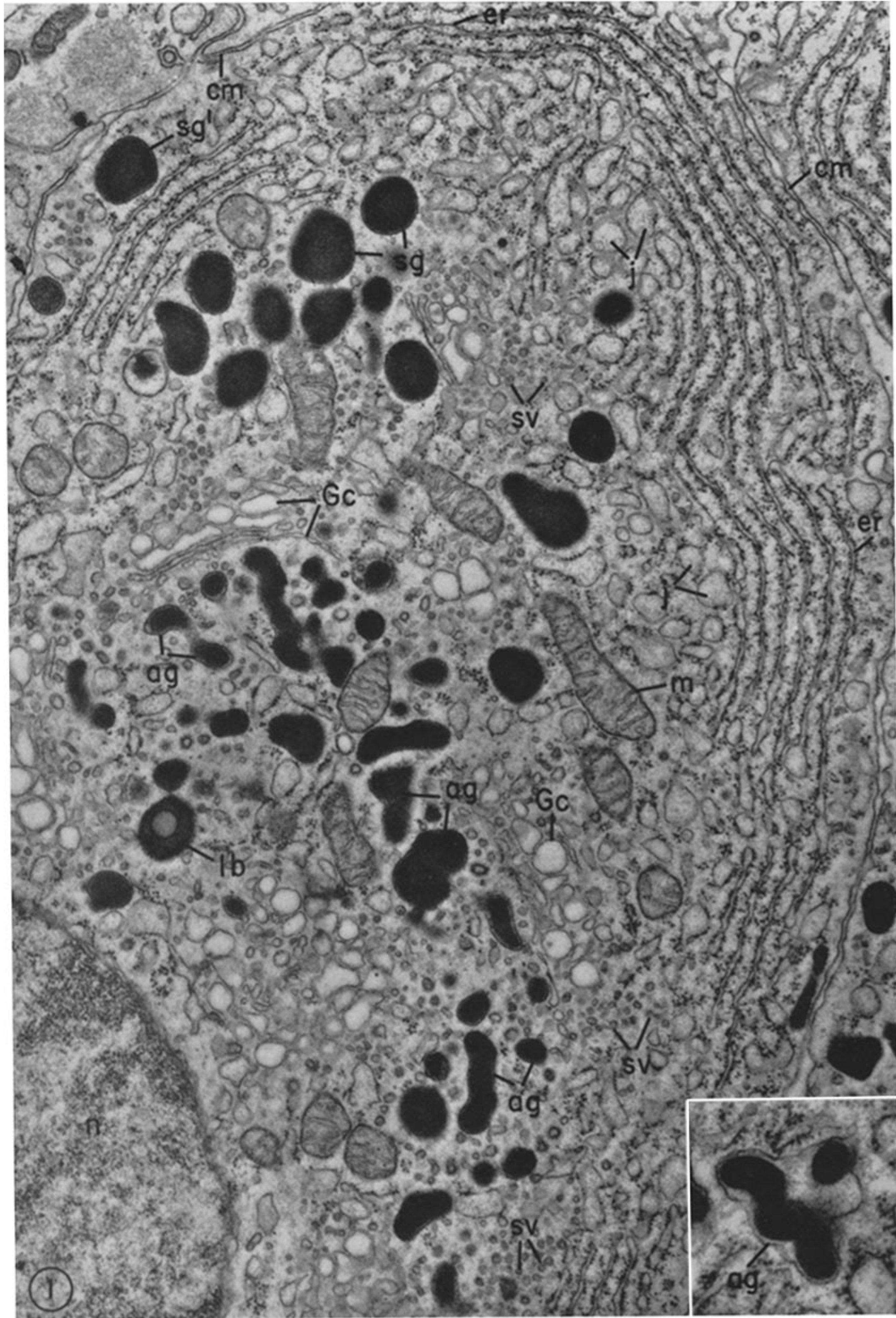
In the pituitary of the normal, nonlactating animal, MT are present in relatively small numbers; their level of secretory activity varies from cell to cell and with the stage of the estrus cycle. During lactation or estrogen treatment, however, when the pituitary is producing increased amounts of MTH, large numbers of MT with cytologic features indicative of active secretion (i.e. enlarged Golgi complex and highly developed rough-

Key to Symbols

ag, aggregating granule
cm, cell membrane
er, endoplasmic reticulum
g, globular residue
Gc, Golgi complex
j, "junction" elements
lb, lytic body
ic, inner Golgi cisterna
li, lipid inclusion
m, mitochondria
me, membranous residue
n, nucleus
oc, outer Golgi cisterna
r, ribosome
s, small immature secretory granule
sg, mature secretory granule
sv, smooth ("intermediary") vesicles
ve, vesicle
va, vacuole

Figure 1 MT cell from the anterior pituitary gland of a rat sacrificed on the 5th day of lactation. These cells are rapidly synthesizing, concentrating, and discharging mammotrophic (lactogenic) hormone; they show abundant peripheral arrays of rough-surfaced ER (*er*) parallel to the cell membrane (*cm*), a large juxtanuclear Golgi complex (*Gc*), many profiles of forming granules (*ag*), and relatively few mature secretory granules (*sg*). Immature secretory granules vary in size and shape and are concentrated in the core of cytoplasm circumscribed by the Golgi cisternae. Mature granules are rounded or ovoid, more uniform in size (diameter, 600 mμ), and are found primarily between the Golgi complex and ER or along the cell membrane (*sg'*). Numerous part rough- and part smooth-"junction" elements (*j*) of the ER (50) are seen between the peripheral ER arrays and the Golgi complex. Clusters of smooth-surfaced vesicles (*sv*) are located in close proximity to the outer cisternae of the latter. Typically, one or several lytic bodies occur in the Golgi region; here a single vacuolated dense body (*lb*) is present. The inset depicts a polymorphous immature granule, which appears to be formed by aggregation of several smaller granules (See also Figs. 2 and 3).

Specimen fixed in 1% OsO_4 in phosphate buffer (pH 7.6) and embedded in Araldite. Section doubly stained with uranyl and lead. Fig. 1 × 24,000; inset, × 40,000.


er
cm
sg'
cm
sg
j
sv
Gc
er
j
ag
m
ag
Gc
lb
sv
ag
n
sv
1
ag

surfaced ER) are present in the anterior lobe (46, 48, 49). In the postlactating animal, after weaning or withdrawal of suckling young, secretion of MTH is suppressed (19), and MT gradually involute to their state in the normal, nonlactating animal.

Lactating Animals

MT from lactating animals (Figs. 1 to 3) are larger than those of the nonlactating. Their most conspicuous features are their peripheral arrays of rough-surfaced ER and large juxtanuclear Golgi complex, which occupies an area of cytoplasm comparable in size to that of the nucleus and contains many profiles of immature or forming secretory granules. Relatively few mature granules are present in the cytoplasm, suggesting that newly synthesized secretory protein is packaged into granules and rapidly discharged, rather than being stored for any period of time.

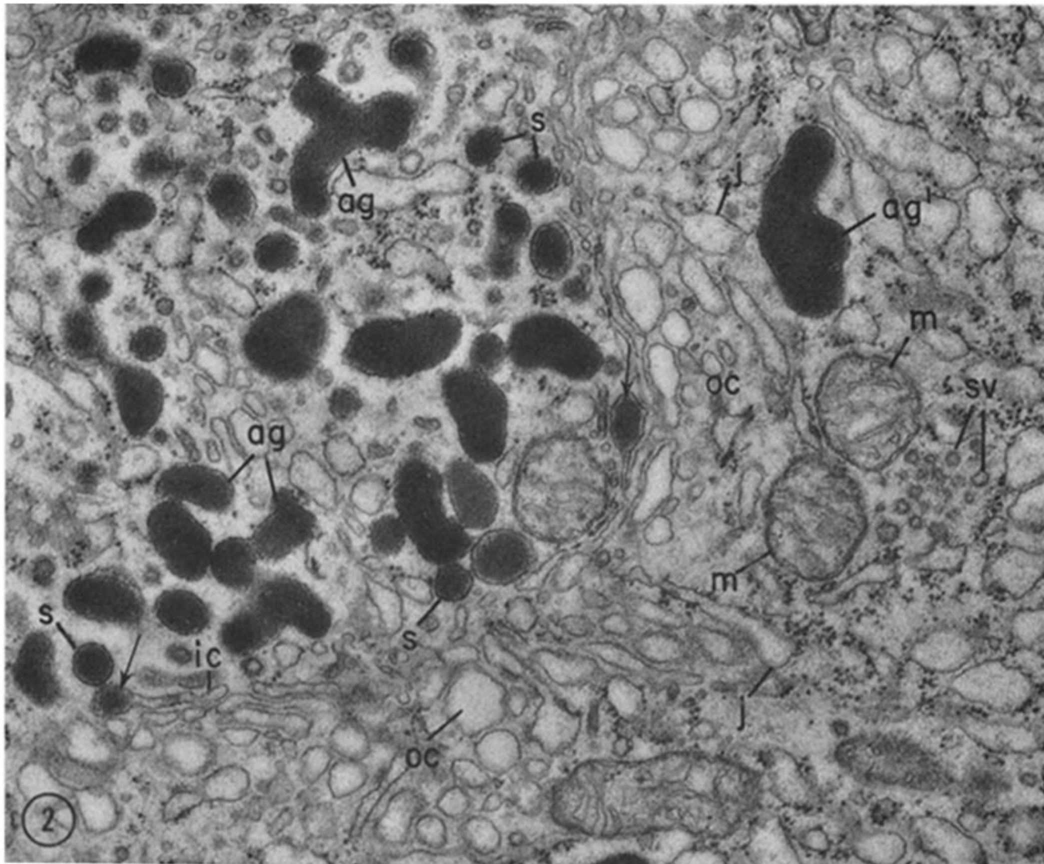
ENDOPLASMIC RETICULUM: The ER is predominantly of the rough-surfaced variety and is concentrated peripherally, where it occurs in groups of 5 to 7 cisternae oriented parallel to the cell membrane (Fig. 1). Between these peripheral stacks and the Golgi complex are a number of irregular profiles, some of which are part rough- and part smooth-surfaced (Figs. 1 and 2), and thus correspond to so called "junction elements" (50). The extensive development and the preferred orientation of the ER is similar to that found in other protein secreting cells, particularly the exocrine cells of the pancreas (50). Whorls of rough-surfaced cisternae constituting *Nebenkern* formations (44, 51) are also occasionally seen.

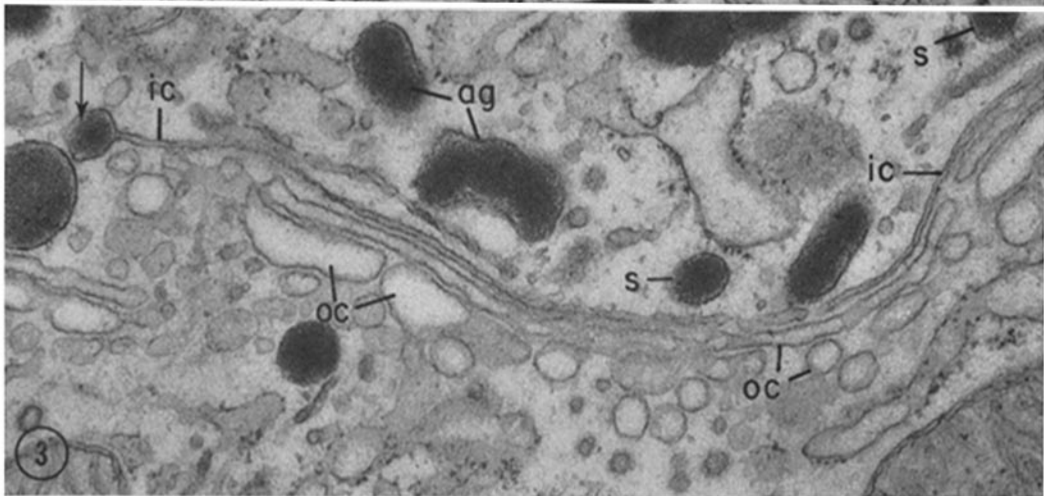
GOLGI COMPLEX: The large Golgi complex is crescentic or S-shaped and consists of stacks of 5 to 8 slightly curved cisternae arranged in concentric layers. Each stack has an inner, or concave, and an outer, or convex surface. The outer cisternae have a content of low density and are dilated or vacuolar, but the cisternae become progressively more flattened and their content more dense toward the concave surface. Clusters of from 20 to as many as 50 smooth-surfaced vesicles are frequently seen along the convex surface of the Golgi complex between the outer cisternae and the ER (Figs. 1 and 2). They resemble similar agglomerations of smooth-surfaced vesicles found in exocrine cells of the pancreas which apparently serve as "shuttle carriers" between the junction elements of the ER and the Golgi complex (52, 53).

Small (100 to 200 mμ) ovoid masses of condensing secretory material frequently occur within the inner, but not within the other Golgi cisternae

FIGURES 2 and 3 Portions of the Golgi complex in actively secreting MT cells from lactating animals. In Fig. 2, a stack of 3 to 5 slightly curved Golgi cisternae occupies the center of the field. To the left, the core of cytoplasm circumscribed by the cisternae is filled with numerous immature granules (*ag*). To the right are seen junction elements (*j*) of the ER, a single cluster of smooth-surfaced vesicles (*sv*), a few mitochondria (*m*), and a single immature secretory granule (*ag'*). The outer cisternae (*oc*) along the convex surface of the Golgi stack are dilated or vacuolar whereas the inner ones (*ic*) along the convex Golgi surface are more flattened. Two small, rounded (100 to 200 mμ) masses (arrows) of condensing secretory material are present within the innermost Golgi cisterna. Other small granules (*s*) are seen along the concave Golgi surface. The polymorphous granules of the Golgi "core" appear to be formed by fusion and aggregation of several of the smaller units which bud from the inner cisterna. Fig. 3, a similar field at higher magnification, shows the dilated, vacuolar outer Golgi cisternae (*oc*), the flatter inner cisternae (*ic*), and the presence of condensing secretory material (arrow) within the latter. From fields such as this the following sequence for formation of MT secretory granules is proposed: (*a*) small spherical granules, 100 to 200 mμ in diameter, are formed by condensation of secretory protein within the inner Golgi cisterna (arrows, Figs. 2 and 3); (*b*) these pinch off and are found along the concave Golgi face (*s*); (*c*) several small granules merge to form larger aggregates (*ag*) of variable shape; (*d*) the polymorphous images subsequently round up to form the ovoid or elliptical mature secretory granules shown in Fig. 1.

Specimen preparation for Fig. 2 is the same as for Fig. 1. Specimen in Fig. 3 fixed in OsO_4 in phosphate buffer (pH 7.6), dehydrated in acetone, and stained in block in $KMnO_4$. Sections doubly stained with uranyl and lead. Fig. 2, $\times$ 36,000; Fig. 3, $\times$ 50,000.


s
ag
j
ag'
m
oc
sv
ag
m
s
ic
s
oc
j
2


ic
ag
s
ic
s
oc
oc
3

(Figs. 2 and 3). In addition, the central core of cytoplasm circumscribed by the Golgi cisternae contains a number of polymorphous secretory granules (Figs. 1 to 3) which consist of aggregates of the smaller 100 to 200 mμ granules. MT secretory granules appear to be produced in several steps: (*a*) small granules are formed by condensation of secretory material within the inner Golgi cisterna; (*b*) these bud from the cisterna and migrate into the Golgi core where (*c*) several (3 to 5) aggregate to form a larger polymorphous image; (*d*) the latter eventually rounds up and condenses to become an ovoid or elliptical mature granule. In the lactating animal, the core of cytoplasm circumscribed by the Golgi cisternae is literally filled with aggregating secretory granules (Figs. 1 and 2). It also contains scattered elements of the rough-surfaced ER and vesicles of varying sizes, some each of the rough-surfaced, smooth-surfaced, and "coated" (54) varieties.

LYSOSOMES AND LYSOSOMAL DERIVATIVES: Structures identified morphologically as lytic bodies could be divided into three main types: dense bodies, multivesicular bodies, and autophagic vacuoles. Several dense bodies and occasional multivesicular bodies are present in the Golgi zone of most MT cells in the lactating animal, but autophagic vacuoles are rarely seen. Dense bodies vary somewhat in size, averaging 450 mμ in diameter. Most of them have a finely particulate content of high density, but a few contain a spherical vacuole of low density and thus correspond to "vacuolated bodies" (6) or "vacuolated dense bodies" (55) described previously in cells of the renal tubule and liver parenchyma, respectively. These terms will be used throughout this paper; however, from its density and uniformity it seems likely that the "vacuole" of the dense body actually represents a droplet of lipid. Multivesicular bodies usually have an irregular outline and contain only a few (2 to 5) vesicles in a matrix of low density. A dense plate or plaque (56, 57) is frequently seen along part of the cytoplasmic surface of its limiting membrane. Occasionally, dense masses, presumed to represent the content of secretory granules, occur within the matrix of the multivesicular body, but this is not as frequent a finding as in the early postlactating animal to be described.

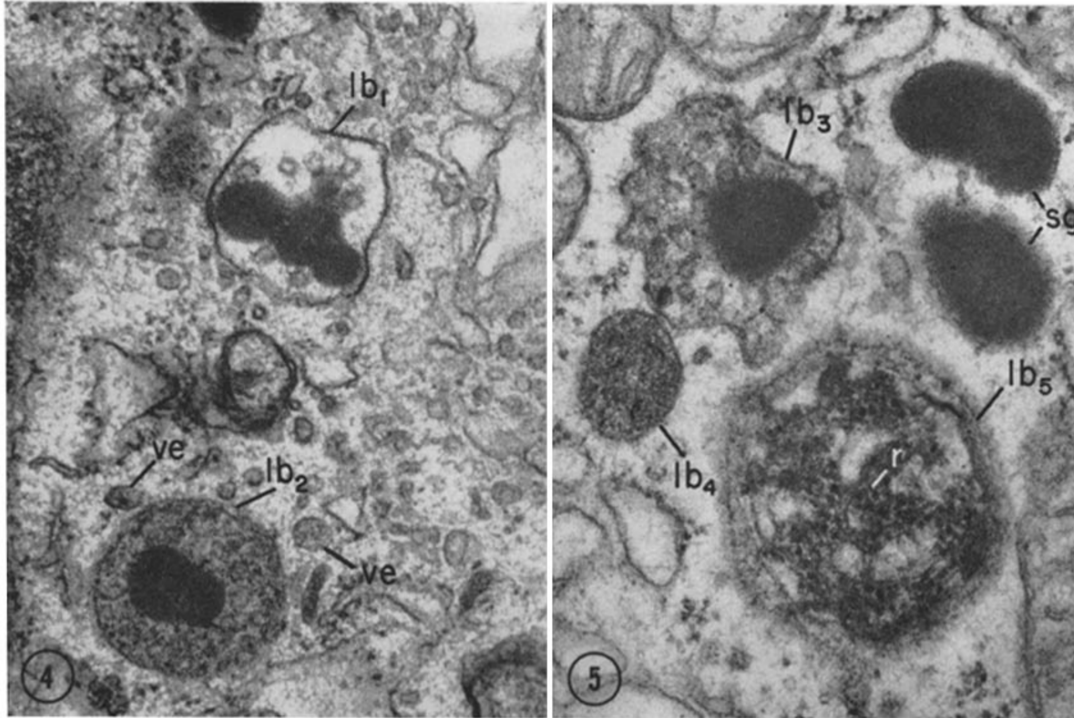
Postlactating Intervals

After removal of the suckling young, there is first a lag of about 12 hr during which secretory granules accumulate, followed by progressive involution of the ER and Golgi complex, a gradual decrease in the numbers of both mature and immature secretion granules, and an increase in the number of lytic bodies. Involutionary events are not entirely synchronous, proceeding at differing rates in individual cells; hence, although the majority of cells show the findings described as typical for each organelle at a given time interval, considerable variation is encountered from cell to cell.

12 hr: No differences are discernible between MT at this interval and those from the lactating animal except for the accumulation of mature secretory granules, primarily at the cell periphery.

24 hr: The rough-surfaced ER is less elaborate in most cells: the peripheral stacks are not so extensive, many having lost their preferential orientation along the cell membrane; large vesicular profiles occur more frequently; and *Nebenkern* formations are not seen. The Golgi complex is reduced in size; the length and average number of the cisternae comprising the stacks are decreased. Small masses of condensing secretory material are still commonly seen within the inner cisterna as are polymorphous (aggregating) granules in the Golgi core.

Increased numbers of lytic bodies are present owing primarily to an increase in the number of multivesicular bodies (Fig. 8) and elements morphologically intermediate between multivesicular and dense bodies (Figs. 10, 12, and 27). All these bodies are concentrated in the Golgi region (Fig. 6) and frequently contain material which, on the basis of its characteristic morphology, can be identified as secretory granules. The secretory material found within multivesicular bodies is commonly multilobulated (Figs. 4 and 9), resembling the content of aggregating granules, whereas that inside dense bodies and intermediate forms is usually rounded or elliptical (Figs. 4, 7, and 13), like mature secretory granules. These findings suggest that the content of aggregating granules finds its way preferentially to multivesicular bodies, whereas that of the mature granules is sequestered into dense bodies. In both cases the incorporated material usually lacks a limiting membrane. Images were encountered occasionally which suggested that incorporation of secretory granules into dense bodies occurs by mergence of the corresponding structures and fusion of their membranes (Fig. 11). No such images were seen in



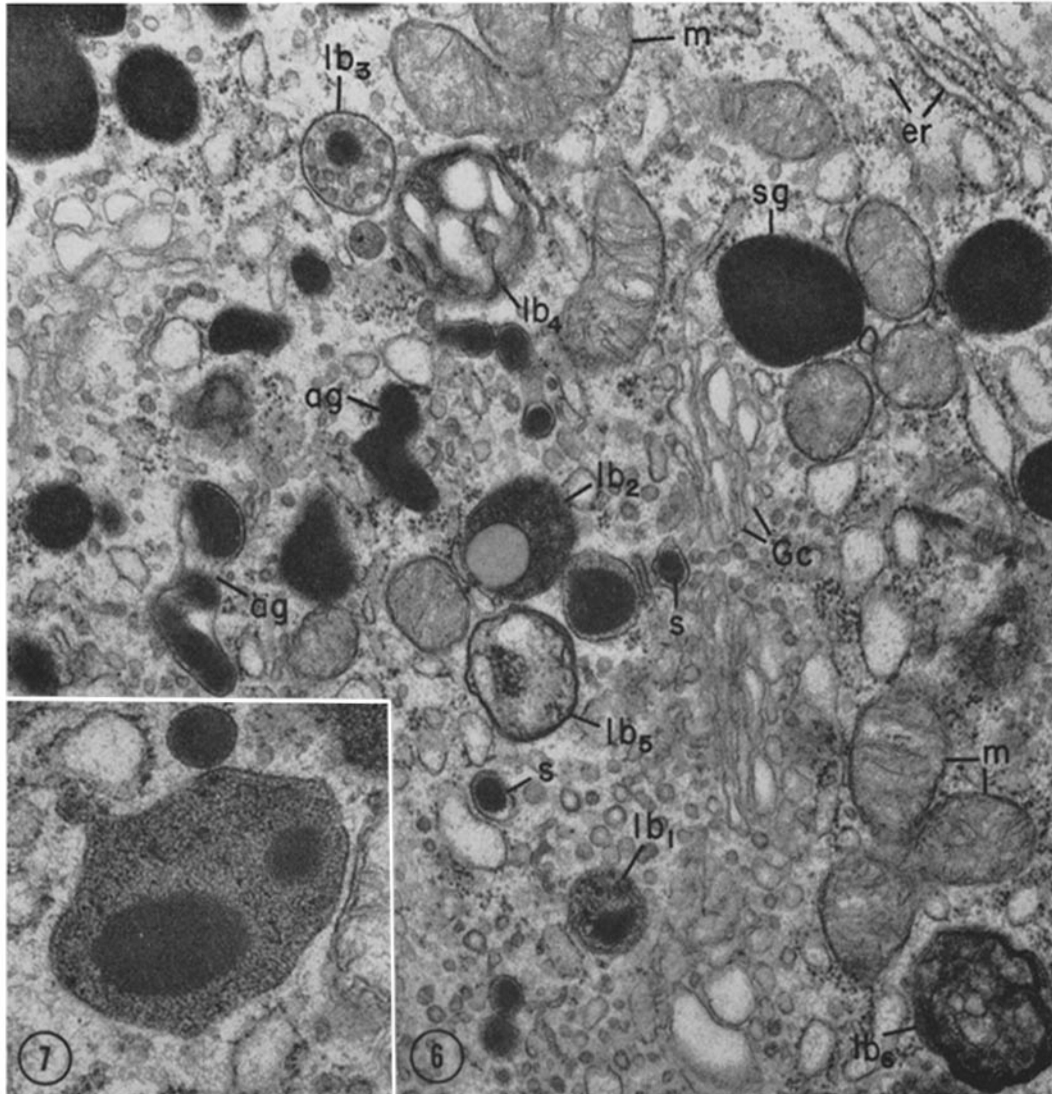
FIGURES 4 and 5 Lytic bodies (lb_1 to lb_5) in MT cells from lactating rats sacrificed 24 hr after separation from their suckling young. Several morphological types are seen: multivesicular bodies (lb_1 and lb_3), one large (lb_2) and one small (lb_4) dense body, and an autophagic vacuole (lb_5). Both multivesicular bodies and one of the dense bodies (lb_2) contain dense material which appears identical with the content of secretory granules. The multivesicular body shown in Fig. 4 has a lighter background matrix than that shown in Fig. 5, and the secretory material it contains is multilobulated, like the content of aggregating secretory granules. The dense body (lb_2) has a moderately dense matrix and, in addition to its content of secretory material, shows faint vesicular residues, suggesting its derivation from a multivesicular body. The autophagic vacuole (lb_5) contains ribosomal aggregates (*r*) and remnants of rough-surfaced ER. Note that several small vesicles (*ve*) with a finely granular content are seen near the dense body in Fig. 4.

Specimens fixed in glutaraldehyde in cacodylate buffer (pH 7.4) and postfixed in OsO_4 in phosphate buffer (pH 7.6). Sections doubly stained with uranyl and lead. Fig. 4, × 56,000; Fig. 5, × 63,000.

the case of multivesicular bodies. It is of interest that, as in the case of granule discharge into the perivascular spaces (47, 58), the dense granule content retains its discrete form for a time after incorporation into the lytic body before undergoing dissolution.

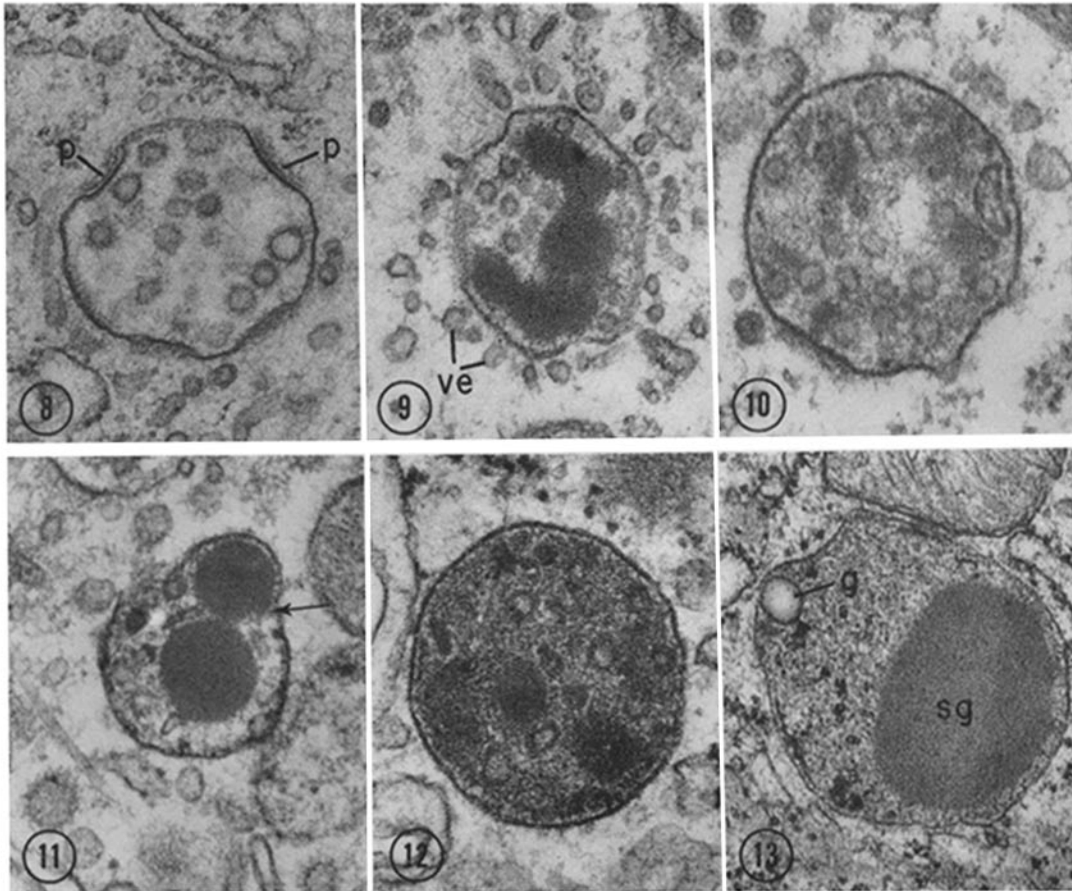
A few autophagic vacuoles are seen at this time interval. Most of these structures are rather small (400 to 500 mμ) and contain ribosomal aggregates and rough-surfaced ER (Fig. 5); secretory granules (Fig. 14) or mitochondria (Fig. 15) are only occasionally included. When secretory granules are present, they are usually membrane-limited (Fig. 14), suggesting that they were included within the autophagic vacuole at the time of its formation, presumably by walling off a bit of cytoplasm by a smooth-surfaced membrane (59).

48 hr: There is a further diminution in the amount and complexity of the rough-surfaced ER and in the size of the Golgi complex; condensing secretory material is rarely seen within the latter, but aggregating granules are still evident (Fig. 6). Among lytic bodies, dense bodies and forms intermediate between dense and multivesicular bodies predominate (Figs. 10 to 12, and 27). The increased number of intermediate forms, the corresponding decrease in the number of typical multivesicular bodies, together with the frequent finding of secre-



FIGURES 6 and 7 Golgi region of an MT cell from a lactating rat which had been separated from its suckling young 48 hr prior to sacrifice. The Golgi cisternae (*Gc*) appear partly collapsed and no condensing secretory material is seen within them, but a number of immature granules are present nearby; several small granules (*s*) and a number of polymorphous aggregating granules (*ag*) are concentrated in the core of cytoplasm circumscribed by the Golgi cisternae. Numerous lytic bodies of several types are also present in the same location: a dense body with heterogeneous content (lb_1), a vacuolated dense body (lb_2), a multivesicular body (lb_3), and three autophagic vacuoles (lb_4–lb_6). The multivesicular body (lb_3) contains a spherical mass of secretory material identical to the content of the small granules. Fig. 7 shows, at higher magnification, another dense body containing two secretory granules which are devoid of limiting membranes.

Specimen preparation as for Fig. 1. Fig. 6, × 35,000; Fig. 7, × 65,000.



FIGURES 8 to 13 Lytic bodies from MT cells of lactating animals sacrificed 24 hr (Figs. 8 and 10) or 48 hr (Figs. 9, and 11 to 13) after removal of suckling young.

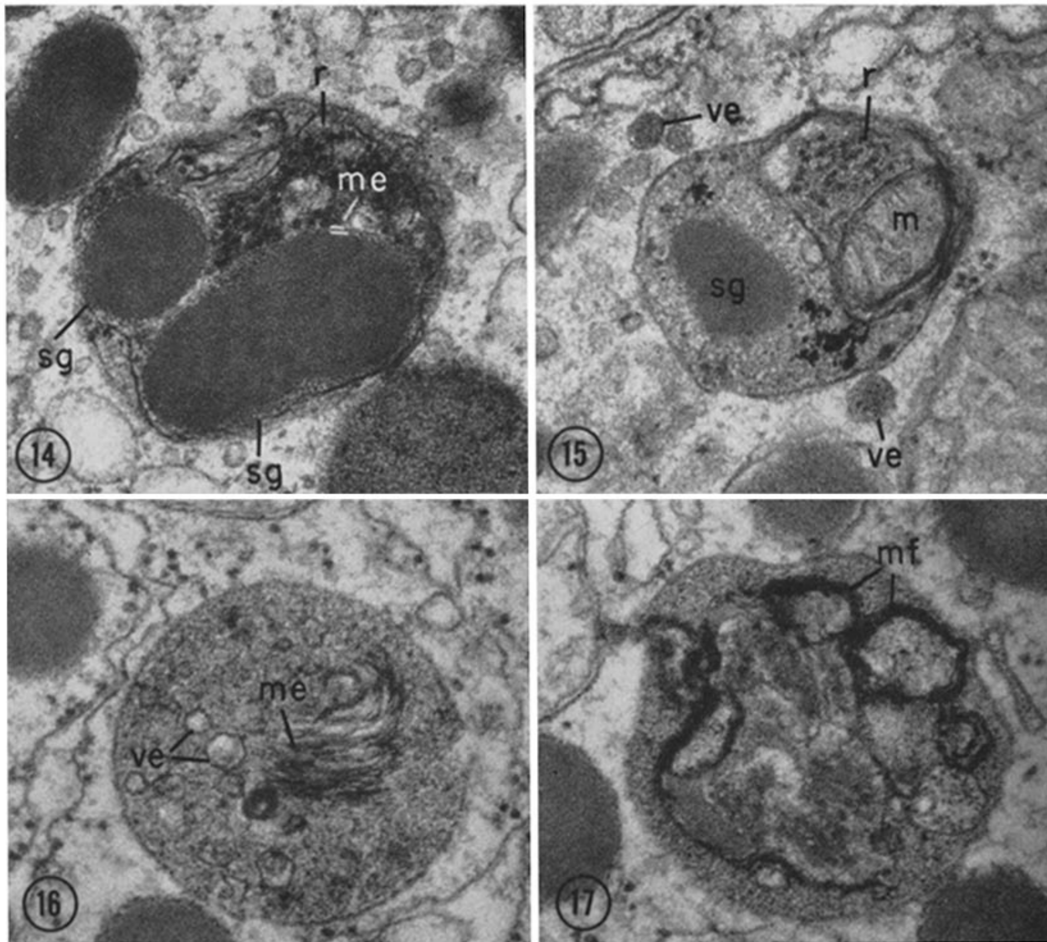
Fig. 8 shows a multivesicular body with its content of small vesicles, light background matrix, irregular contours, and plaques (*p*) of dense material along the outer surface of its limiting membrane. This form is typical of that of "embryonal" multivesicular bodies prior to the incorporation of secretory granules.

Fig. 9 depicts a multivesicular body, similar to that in Fig. 8, which contains a polymorphous mass of secretory material, presumed to represent the content of an aggregating granule. Note that this body is surrounded by a constellation of small vesicles (*ve*).

Figs. 10 to 12 show lytic bodies which are intermediate in appearance between typical multivesicular (Fig. 8) and dense bodies (Fig. 13). All contain vesicular profiles but are more rounded and have a denser matrix than typical multivesicular bodies. These probably represent forms of the latter which have incorporated and partially degraded secretory material and are in the process of being transformed into dense bodies. The body in Fig. 11 appears to have just fused with a secretory granule (arrow).

Fig. 13 shows a typical dense body with a dense, finely particulate matrix, containing a small lipid globule (*g*), dense particles, and an ingested secretory granule which lacks a limiting membrane.

Specimen preparation for Figs. 8 to 10 and 13 is the same as for Fig. 1; that for Figs. 11 and 12 is the same as for Fig. 4. Figs. 8, 12, and 13, × 75,000; Figs. 9 to 11, × 65,000.



Figures 14 to 17 Lytic bodies in MT cells from lactating animals separated for 48 hr (Figs. 14 and 15) and 96 hr (Figs. 16 and 17) from their suckling young.

Fig. 14 shows an autophagic vacuole containing ribosomal aggregates (*r*), membrane remnants derived presumably from rough-surfaced ER (see Fig. 5), and two secretory granules (*sg*). Note that, in contrast to those within dense bodies, illustrated previously, a limiting membrane (*me*) can be recognized around the granules.

Fig. 15 shows a bipolar dense body with a secretory granule (*sg*) at one pole and ribosomes (*r*) and a mitochondrion (*m*) at the other. Such images suggest that dense bodies and autophagic vacuoles can merge. Note the small vesicles (*ve*) with a finely granular content of moderate density located in close proximity to the body.

Figs. 16 and 17 Dense bodies with heterogenous content including vesicular (*ve*) and membranous (*me*) residues, presumably derived from degradation of ingested secretory granules and cytoplasmic membranes. In Fig. 17 some of the residues form membranous whorls or myelin figures (*mf*).

Specimen preparation for Fig. 14 as for Fig. 1; that for Figs. 15 to 17 as for Fig. 4. Fig. 14, × 68,000; Fig. 15, × 76,000; Figs. 16 and 17, × 60,000.

tory granules within multivesicular bodies at this (Figs. 6 and 9) and earlier periods, suggest that multivesicular bodies develop into dense bodies after incorporation and solubilization of secretory granules. At this time, the content of dense bodies is more heterogeneous; in addition to recognizable secretory granules they frequently contain other types of residues, i.e. vesicles, small dense particles (Fig. 13), larger globules (Fig. 13), and spherical vacuoles of low of density (Fig. 6), all of which are presumed to represent residues derived primarily from the digestion of secretory granules.

After this interval one or two autophagic vacuoles are also usually seen per cell profile (Fig. 6). Occasionally, structures with a mixed content–partly that of a dense body and partly that of an autophagic vacuole—are encountered (Fig. 15), indicating that these two types of bodies merge. Rarely, one or more large autophagic vacuoles with a heterogeneous content consisting of several secretory granules and conspicuous membranous residues are seen, but this is not a frequent finding.

72 to 96 hr: The ER and Golgi complex in most cells (Fig. 18) is further reduced in size and complexity. The ER occurs primarily in the form o vesicles or short cisternae, the elongated peripheral arrays having virtually disappeared. The Golgi cisternae are collapsed and only 2 to 3 comprise the stacks; condensing secretory material is not usually seen within the cisternae and aggregating granule forms are less frequent. Lytic bodies are numerous and most of those present have a dense matrix and heterogeneous content consisting of membranous or vacuolar residues (Figs. 16 and 17), and thus correspond to lamellated or vacuolated bodies (6). By far the most common form, however, is the vacuolated body (Figs. 19, 20, and 33), in which the number and size of the corresponding vacuoles varies considerably. Sometimes 2 to 3 small vacuoles are evident. In other instances a single large vacuole is seen to occupy the body almost completely, leaving only a thin peripheral dense rim (Fig. 18), or partially protrudes from it (Fig. 20). Occasionally, also, such a vacuole appears to be located "free" in the cytoplasm (Figs, 21 and 22), and a crescentic dense body is present nearby, suggesting that the lipid vacuole may separate from its dense rim and be released into the cytoplasm. In addition, larger, more irregularly shaped lipid droplets of low density are seen in the cytoplasm (Figs. 18 and 23). These are often located in close proximity to elements of the rough-surfaced ER (Fig. 23).

By 96 hr some cells can be found in most glands in which the ER is more elaborate (i.e. arranged peripherally in short cisternal stacks), and there is evidence of secretory granule condensation within the Golgi complex, suggesting the reinitiation of MTH synthesis in connection with a new estrus cycle.

Acid Phosphatase Localization in Mammotrophs

Sites of reaction were the same in tissue incubated in the regular Gomori or modified (Barka-Anderson) media except as noted.

Lactating Animals

In the lactating mammotroph (Figs. 24 and 25) lead phosphate reaction product was found (*a*) within lytic bodies (i.e. dense bodies and multivesicular bodies), (*b*) within the innermost Golgi cisterna, (*c*) within smooth-surfaced vesicles closely associated with the inner cisterna, (*d*) around the polymorphous aggregating granules of the Golgi region and (*e*) around some of the mature secretion granules. The dense bodies present in the Golgi region typically contained heavy lead phosphate deposits (Fig. 25). In the vacuolated dense bodies reaction product was limited to the dense portion of the content and was not found within the vacuole (Figs. 27 and 33). Relatively few of the multivesicular bodies contained reaction product. In those that were reactive, deposits appeared to be localized in the matrix rather than in the contained vesicles (Fig. 28). Only the innermost Golgi cisterna was consistently reactive, but small, patchy deposits of lead phosphate were occasionally seen in one or two adjacent cisternae of specimens incubated in Barka-Anderson media. In specimens incubated for longer periods (60 to 90 min), the inner Golgi cisterna was completely filled with reaction product. In specimens incubated for shorter periods (30 min) in which the membranes had been well stained by uranyl treatment, the lead phosphate deposits appeared to be distributed throughout the content of the cisternae rather than being associated with their membranes (Fig. 26). It is of interest that AcPase activity is present in the same cisterna in which secretory granules are condensed; frequently, one or several forming granules could be recognized within the heavily reactive cisternae (Figs. 24 and 25). The number of vesicles containing reaction product varied considerably from cell to cell; one or more were commonly seen near the ends of the reactive cisternae (Fig. 26).

Most (Figs. 24 and 25) but not all (Fig. 29) of the aggregating granules of the Golgi region contained considerable reaction product, concentrated in the space between their limiting membrane and dense content. Occasional mature secretion granules were also rimmed with smaller deposits of reaction product (Fig. 24, inset, and Fig. 25), but the deposition was typically patchy, and the number of labeled granules varied greatly from cell to cell.

Postlactating Intervals

In the postlactating animal, the frequency of labeling and the intensity of the aforementioned AcPase-reactive sites varied with the period of elapsed time after removal of suckling young. Initially (up to 12 hr), no definite change in the distribution of reaction product could be recognized. After this lag period, however, there was a progressive decrease in the amount of reaction product found in the inner Golgi cisterna and associated vesicles, and an increase in AcPase activity in lytic bodies.

12 hr: The distribution of AcPase reaction product and the intensity of reactive sites appeared the same as in the lactating animal.

24 hr: By this time the number of cells which showed AcPase reaction product in the inner Golgi cisterna and associated vesicles was definitely decreased, and reaction product was concentrated in lytic bodies. Virtually all the dense bodies were reactive, although some variation in intensity was encountered from one to another (Fig. 29). On the other hand, only about half the multivesicular bodies and intermediate forms contained lead phosphate deposits (Figs. 27 and 28), and the reaction was usually much lighter. Sparsely reactive or unreactive multivesicular bodies could frequently be seen alongside heavily labeled dense bodies (Fig. 27).

Often secretory granules could be recognized within the reactive dense and multivesicular bodies

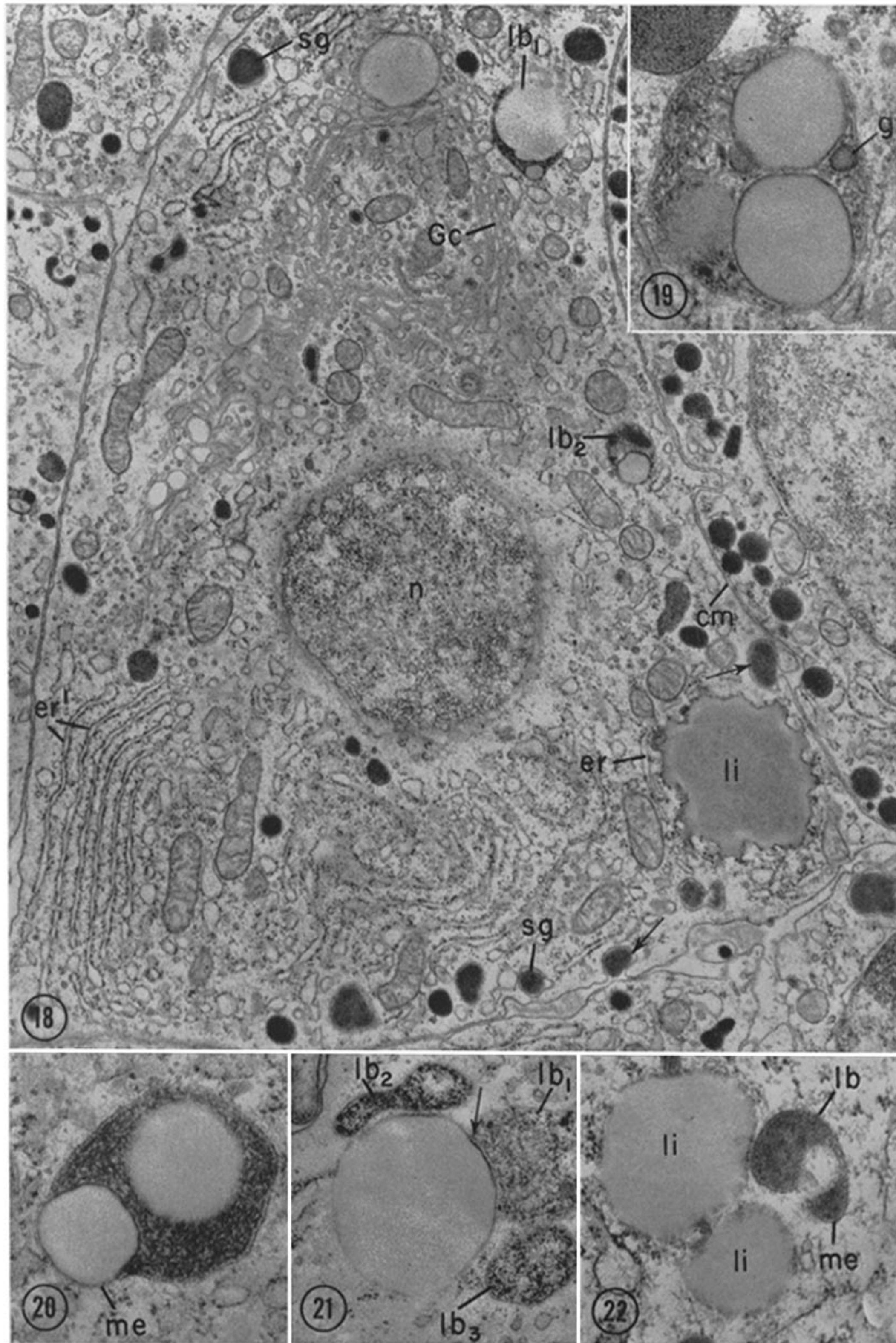
Figures 18 and 19 MT cell from a postlactating (72 hr) animal. The Golgi complex (*Gc*) appears small and inactive: no condensing secretory material is seen within its cisternae and no aggregating granules are associated with it. Only a few mature secretory granules (*sg*) are present, most of which are found along the cell membrane (*cm*). Several are in continuity with the plasma membrane (arrows), indicating that granule discharge is still taking place. In the cytoplasm are several vacuolated dense bodies (lb_1 and lb_2) and a large lipid inclusion (*li*). The latter is closely associated with cisternae of the rough-surfaced ER (*er*). One of the lytic bodies (lb_1) is almost completely occupied by a large lipid vacuole surrounded by only a thin, dense rim. Another vacuolated body containing two such vacuoles and a smaller and denser, globular residue (*g*) is shown in Fig. 19. The presence of a stack of cisternae of the rough-surfaced ER (*er'*) may signal the reinitiation of secretory activity or may be connected with the breakdown of lipid residues (see text).
Specimen preparation as for Fig. 1. Fig. 18, × 15,000; Fig. 19, × 50,000.

Figures 20 to 22 Small fields from MT cells at 96 hr postlactation. These three figures show (from left to right) possible stages in the discharge of lipid residues from dense bodies into the cytoplasm.

Fig. 20 shows a dense body containing two vacuoles, one of which partially protrudes from the body but is surrounded by its membrane (*me*).

Fig. 21 shows a vacuole which does not appear to be surrounded by a membrane, but is in contact with one dense body (lb_1) to the right (arrow) and is located in close proximity to two others (lb_2 and lb_3).

Fig. 22 includes two lipid droplets (li) and a crescent-shaped dense body (*lb*) which may represent the vacuoles and peripheral dense rim, respectively, of a vacuolated dense body. A unit membrane (*me*) is present around the dense body, but none is visible around either lipid droplet.
Specimen preparation for Figs. 20 and 21 as for Fig. 1; that for Fig. 22 is the same as for Fig. 3. Fig. 20, × 50,000; Figs. 21 and 22, × 40,000.


sg
lb1
g
Gc
19
lb2
n
cm
er'
er
li
sg
18
lb2
lb1
lb
li
li
me
20
me
21
lb3
22

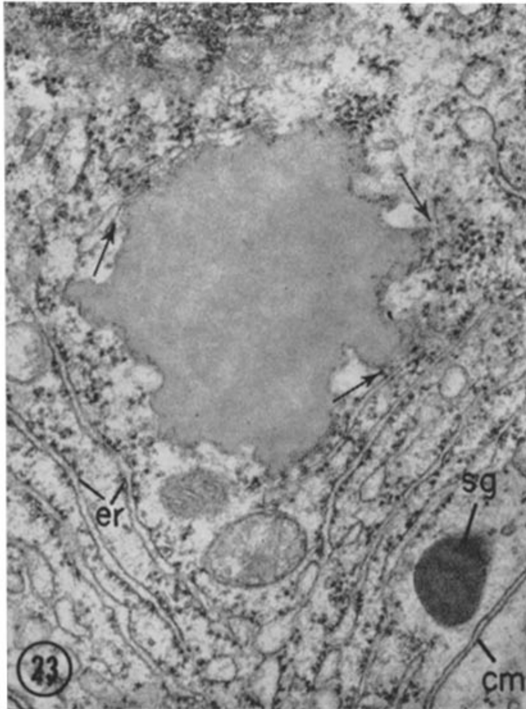


FIGURE 23 Lipid inclusion in an MT cell at 72 hr postlactation. Stacks of rough-surfaced ER (*er*) surround the inclusion and a few cisternae closely approach it in a number of places (arrows). Specimen preparation as for Fig. 1. × 50,000.

(Figs. 28 to 32). The reaction around the immature and aggregating granules of the Golgi region and the mature granules was the same as encountered in the lactating animal.

48 hr: The amount and frequency of lead phosphate deposits in the Golgi complex were further decreased. The Golgi cisternae of most cells were unreactive with only small patches of lead phosphate being seen within the inner cisternae of a few cells. Virtually every cell contained multiple AcPase-positive lytic bodies (Fig. 29). As in the case of previous time intervals, virtually all dense bodies were reactive. The majority of the forms intermediate between multivesicular and dense bodies were reactive at this stage, suggesting that, as multivesicular bodies are transformed into dense bodies, their AcPase reaction becomes more regular and intense.

72 to 96 hr: Reaction product was concentrated virtually exclusively in lytic bodies most of which are of the vacuolated type at this stage (Figs. 32 and 33). Lead phosphate was deposited primarily within the dense part of the content, and was not usually seen within the vacuolar (Figs. 27 and 33) and membranous (Fig. 31) residues or the contained secretory granules (Figs. 29 to 32). Sometimes a circular, empty-appearing area formed part of the content of the lytic body (Fig. 32). These areas probably correspond to lipid droplets which had been partially or completely extracted during the preparative procedures. By 96 hr, the amount of AcPase reaction product found in individual cells was the lowest of any postlactation period, with only the dense peripheral rim of the vacuolated dense bodies reactive.

Control Experiments

No activity was seen when substrate was omitted from the incubation medium or when the reaction was run at pH 7.0 with GP as substrate or at pH 5.0 with IDP as substrate. Activity in all sites mentioned was completely inhibited by 0.01 M NaF or tartaric acid. With 0.01 M glutaric acid in the medium, the reaction in Golgi components and around secretory granules was virtually abolished, but that in lytic bodies was only partially inhibited. Since glutaric acid is a common contaminant of glutaraldehyde solutions, its presence in differing amounts could provide an explanation for the variation encountered in preservation of enzyme activity with different batches of stock (undistilled) glutaraldehyde. (See Materials and Methods.)

DISCUSSION

Main Findings

The main result of our study is the demonstration that in cells of the anterior pituitary gland lysosomes incorporate and degrade secretory granules. It has been traditionally assumed that the hormones produced by anterior pituitary cells are stored in the cytoplasm as granules for a variable period of time and are ultimately released into the circulation as needed. Our results demonstrate that an alternative pathway—an intracellular disposal mechanism—exists for the turnover of secretory protein. Considerable secretory material appears to follow the lysosomal pathway when secretion is suppressed, for the number of lytic bodies (i.e. multivesicular bodies and dense bodies) present and the frequency of finding secretory granules within them were increased in the MTH-producing cells of lactating animals

separated for more than 12 hr from their suckling young, a situation well known to inhibit production and discharge of MTH. Similar findings have also been obtained in other cell types when their secretory activity had been suppressed experimentally, e.g., ST acidophils of thyroidectomized (16) or adrenalectomized (60) animals. Since lytic bodies occur in smaller, though substantial numbers in all the generally accepted cell types (ST, GT, TT, and MT) in the anterior lobe of normal animals, and since granules can be occasionally found within them, we assume that secretory products are more or less continually funneled into the lysosomal system, presumably according to fluctuations in secretory activity. Hence we conclude that in anterior pituitary cells lysosomes operate at the catabolic end of protein turnover and constitute a regulatory mechanism to take care of overproduction of secretory products. This particular regulatory mechanism is unique in that it operates very late in the secretory process on secretory products that are already concentrated into granules.

The Secretory Process in MT Cells

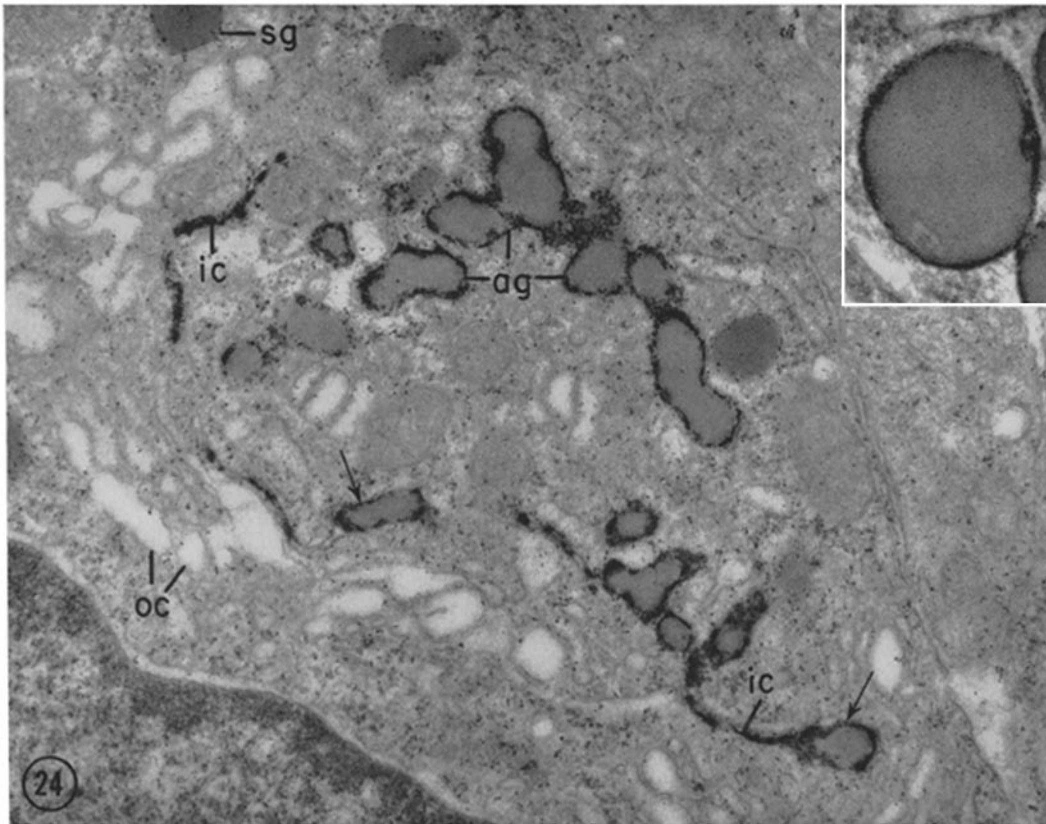
SYNTHETIC PATHWAY: Current concepts of the events that occur during the secretory process in protein secreting cells are based largely on the combined biochemical, morphologic, cell fractionation, and radioautographic studies carried out by Palade and coworkers (50, 52, 53) on the exocrine cells of the pancreas. According to the scheme suggested by their results, secretory proteins are synthesized on ribosomes, transferred into the cavities of the rough-surfaced ER, ferried (via intermediary vesicles) to the smooth-surfaced vacuoles of the Golgi region where they are condensed into zymogen granules, and finally discharged following coalescence of the granule's limiting membrane with the apical cell membrane.

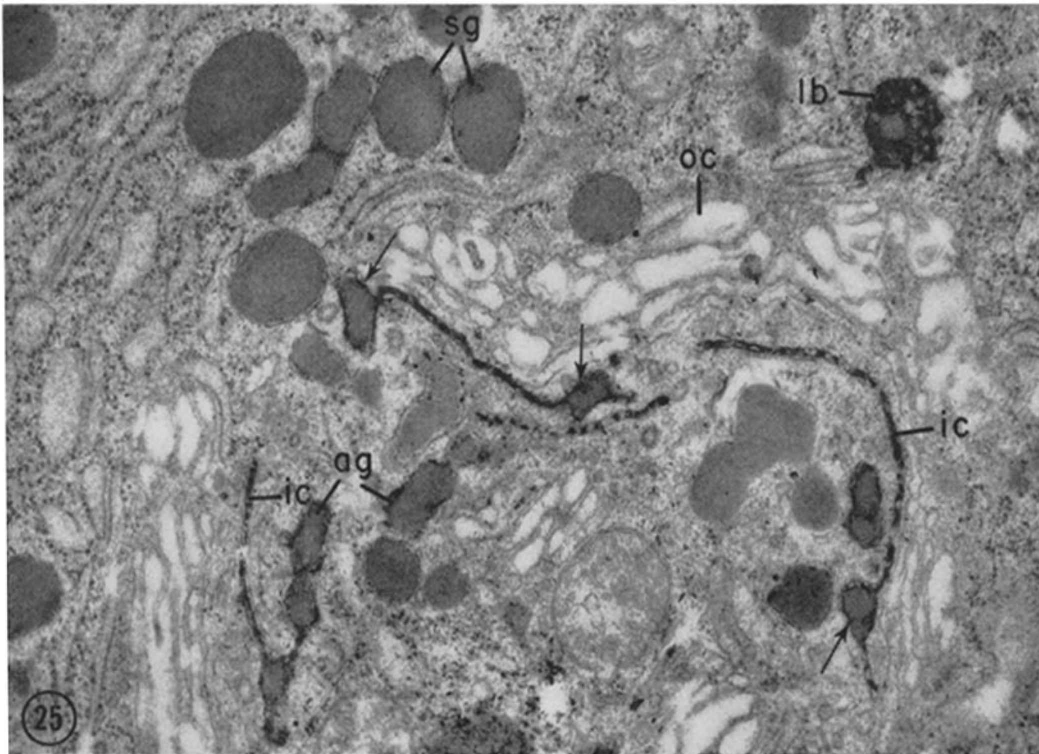
The same general pathway of intracellular passage is probably followed by secreted protein in MT cells. Although no direct evidence is available on the ribosomal and ER-associated events, morphologic parameters suggest that they are similar. The Golgi origin of the granules and their discharge into perivascular spaces by membrane fusion can be directly visualized and were previously described (47, 58). The present findings indicate that the condensation step is somewhat modified; it begins with condensation of small packets of secretory material within Golgi cisternae (rather than in condensing vacuoles), and the mature secretory granule is formed by aggregation of several of these small Golgi-derived packets (cf. Fig. 34). A basically similar aggregation process seems to occur during formation of azurophil granules in rabbit polymorphonuclear leukocytes (61).

DEGRADATIVE PATHWAY: The present findings indicate that when the secretory activity of MT is suppressed, many of the stored mature secretory granules, instead of coalescing with the cell membrane, fuse with dense bodies and become part of their content (Fig. 35). A slightly different pathway appears to be followed by the immature or aggregating granules in the Golgi region: these are segregated into multivesicular bodies (Fig. 35), but their exact mode of incorporation is unknown. However, the fate of the incorporated granules is similar, i.e., degradation within 24 to 72 hr to lipid residues, and multivesicular bodies eventually become dense bodies.

Since AcPase tests clearly indicate that both multivesicular and dense bodies belong to the lysosomal system, it can be assumed that lysosomes represent the protein degrading apparatus in MT cells. This alternative degradative pathway is depicted schematically in Fig. 34.

SECRETORY PATHWAYS IN MT CELLS FROM LACTATING AND POSTLACTATING ANIMALS: During normal lactation large quantities of MTH are continually synthesized and discharged. This high secretory level is reflected structurally in the abundant ribosomes, extensively developed rough-surfaced ER and Golgi complex, numerous immature (aggregating) granules, and frequent images of granules fusing with the cell membrane (48). If, however, the animals are separated from their suckling young, hormone discharge is suppressed, and there occurs initially a lag period of 10 to 12 hr during which synthesis continues and the MTH content of the gland rises (19). These events are reflected structurally in the lack of modification of the protein synthetic apparatus (ER and Golgi complex), the persistence of granule condensation and aggregation images, and the accumulation of mature granules. After 12 hr, synthesis of the hormone is suppressed and glandular hormone content diminishes (19, 20). The morphologic picture agrees with these physiologic events: there is a rapid involution of organelles associated with protein synthesis, and fewer granule condensation, aggregation, and discharge


sg
ic
ag
oc
ic
24


sg
lb
oc
ic
ag
ic
25

images are seen. At the same time, there is an augmentation of the protein degradative apparatus: lytic bodies increase in number and secretory granules are frequently found within them.

That the protein synthetic apparatus (cytoplasmic basophilia) increases in MT during lactation and involutes after weaning has long been known from light microscope studies (62, 63). As already indicated, the existence of a protein degradative apparatus has not been appreciated heretofore, and the suggested relationship between lysosomes and secretory protein turnover in MT represents a new concept.

Lysosomes in Secretory Cells

Previous morphologic and cytochemical studies of lysosomes have emphasized their role in disposing of (*a*) exogenous protein taken up by pinocytosis or phagocytosis and segregated into digestive vacuoles (1, 3–6, 13, 14, 64), or (*b*) areas of cytoplasm sequestered into autophagic vacuoles (1, 64, 65). The present study represents one of the first in which the segregation and degradation of endogenous secretory products could be clearly followed. In this work we have taken advantage of the nature of MT secretory granules, which retain their characteristic form, size, and density for a time after fusion with lytic bodies. In many other situations secretory material may be too fluid or not dense enough to serve as an adequate tracer. It should be mentioned, however, that Palay (66) described the occurrence of dense masses morphologically identical with neurosecre-

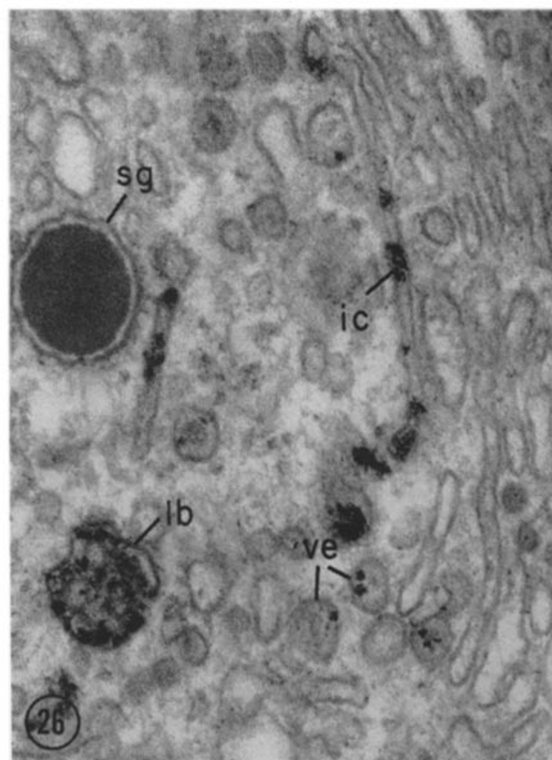


FIGURE 26 Stack of Golgi cisternae from an MT cell at 12 hr postlactation. Deposits of AcPase reaction product are seen within the innermost cisterna (*ic*) along the concave surface of the curved stack, within small vesicles (*ve*) closely associated with the same cisterna, and with a multivesicular body (*lb*). Specimen preparation as for Fig. 25. × 35,000.

FIGURES 24 and 25 Golgi regions of two MT cells from lactating animals; preparations incubated for AcPase. Dense deposits of lead phosphate reaction product fill the inner Golgi cisternae (*ic*), but none are seen in the remaining cisternae. In both figures condensing secretory material (arrows) and AcPase can be identified in the same (innermost) Golgi cisternae. Heavy deposits of reaction product are also seen in a lytic body (*lb*) and around the aggregating small granules (*ag*) in the Golgi cytoplasmic core. Smaller deposits are present around some of the mature secretory granules (*sg*); one such granule showing lead phosphate deposits between the membrane and the dense content is enlarged in the inset to Fig. 24. Note that in Fig. 25 there is considerably less nonspecific reaction product, and the membranes stand out more sharply in the tissue than in Fig. 24, owing to the treatment with uranyl acetate prior to embedding.

Specimen in Fig. 24 fixed in glutaraldehyde in cacodylate buffer (pH 7.4). Nonfrozen section incubated for 30 min in Barka-Anderson medium, postfixed in 1% OsO_4 in phosphate buffer (pH 7.6) and embedded in Araldite. Thin section doubly stained with uranyl and lead. Specimen preparation for Fig. 25 is the same as for Fig. 24, except that postfixation was carried out in 1% OsO_4 in acetate-Veronal buffer (pH 7.4), and the reacted 40 μ section was stained in block in uranyl acetate prior to embedding. Thin section stained in lead alone. × 30,000; inset, × 60,000.

tory material within multivesicular bodies of secretory neurons in the Goldfish hypothalamus. Novikoff and his coworkers (12, 13, 64, 67) have called attention to the close association between lysosomes and the Golgi apparatus in many types of secretory cells. They did not associate lysosomes with degradation of secretory protein but instead focused attention on the AcPase activity around forming secretory granules, which they considered to be lysosomes, and concluded that lysosomal enzymes were involved in condensation of secretory material. This subject will be discussed at greater length in a section to follow. Findings comparable to ours have not yet been reported in other tissues; however, from other work on the pancreatic islets (68–70) and secretory neurons (66, 71), it can be predicted that a comparable role in regulating the secretory process by incorporating and degrading undischarged secretory granules is played by lysosomes in other tissues, particularly endocrine glands.

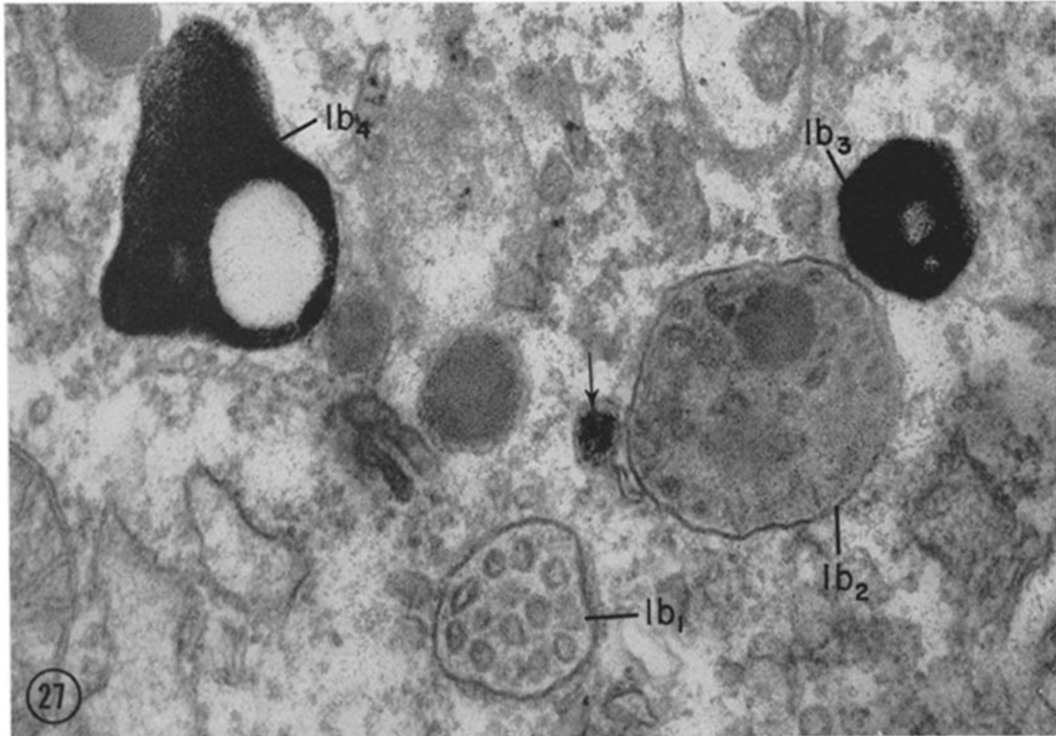
Morphological and Functional Forms of Lysosomes

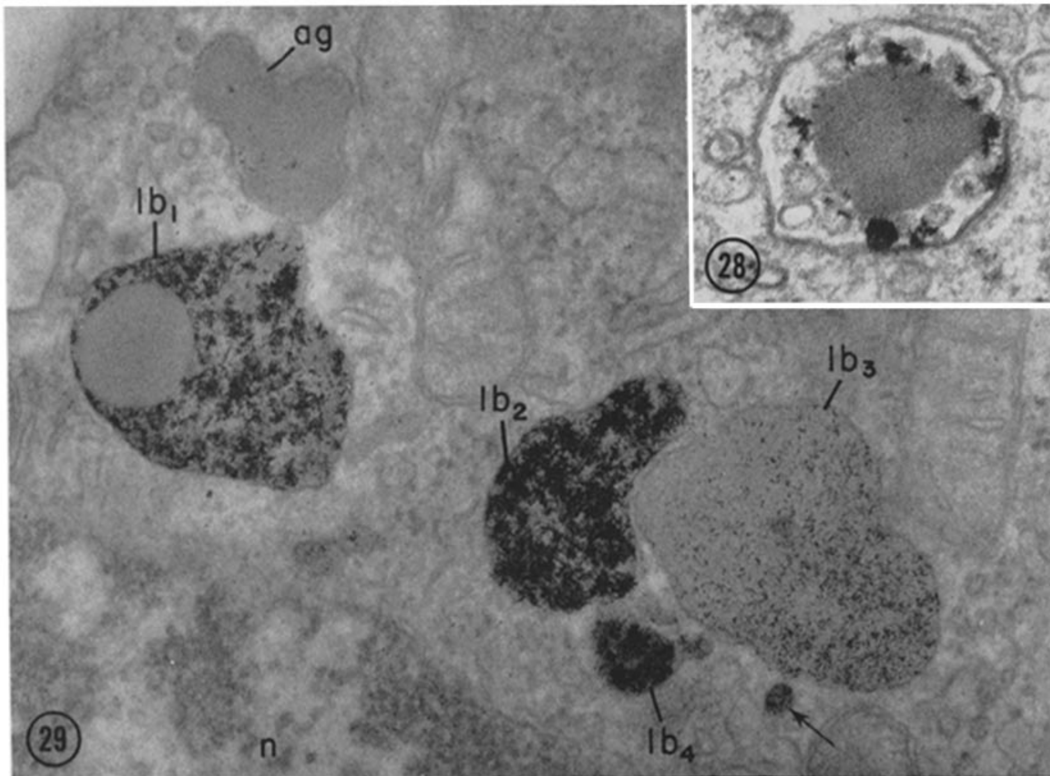
deDuve and Wattiaux (72) have recently proposed a new, more elaborate functional classification for lysosomes. As in the case of previous systematizations (1, 2), they divide lysosomes into two main groups: primary lysosomes, or those which have not yet been involved in digestive events, and secondary lysosomes, which are or have been sites of digestive activity. The main feature of their new classification is the separation of secondary lysosomes into two separate lines—heterophagic and autophagic—based on the origin (exogenous or endogenous) of the material undergoing digestion. Each line is then further subdivided into prelysosomal, lysosomal, and postlysosomal forms. In this scheme the lytic events which we have followed in MT of postlacting animals are autophagic in nature, since the materials undergoing digestion are of endogenous origin; furthermore, the lytic bodies involved correspond to secondary lysosomes or, more precisely, autolysosomes. It should be mentioned, however, that all previously recognized examples of digestion of endogenous material within lysosomes have involved bulk segregation of cytoplasmic components (72), and the term "autophagy" is generally understood to mean envelopment of portions of cytoplasm by a smooth-surfaced membrane to form "autophagic vacuoles." In MT of the postlactating rat two converging, yet distinctive lytic processes take place: (*a*) ER and ribosomes are sequestered and digested within typical autophagic vacuoles, and (*b*) secretory granules are incorporated into and degraded within multivesicular and dense bodies. This last process probably involves membrane coalescence rather than engulfment. It seems reasonable to assume that normally the turnover of secretory protein within lysosomes is the predominant process, and autophagic vacuole formation operates primarily when there is pronounced

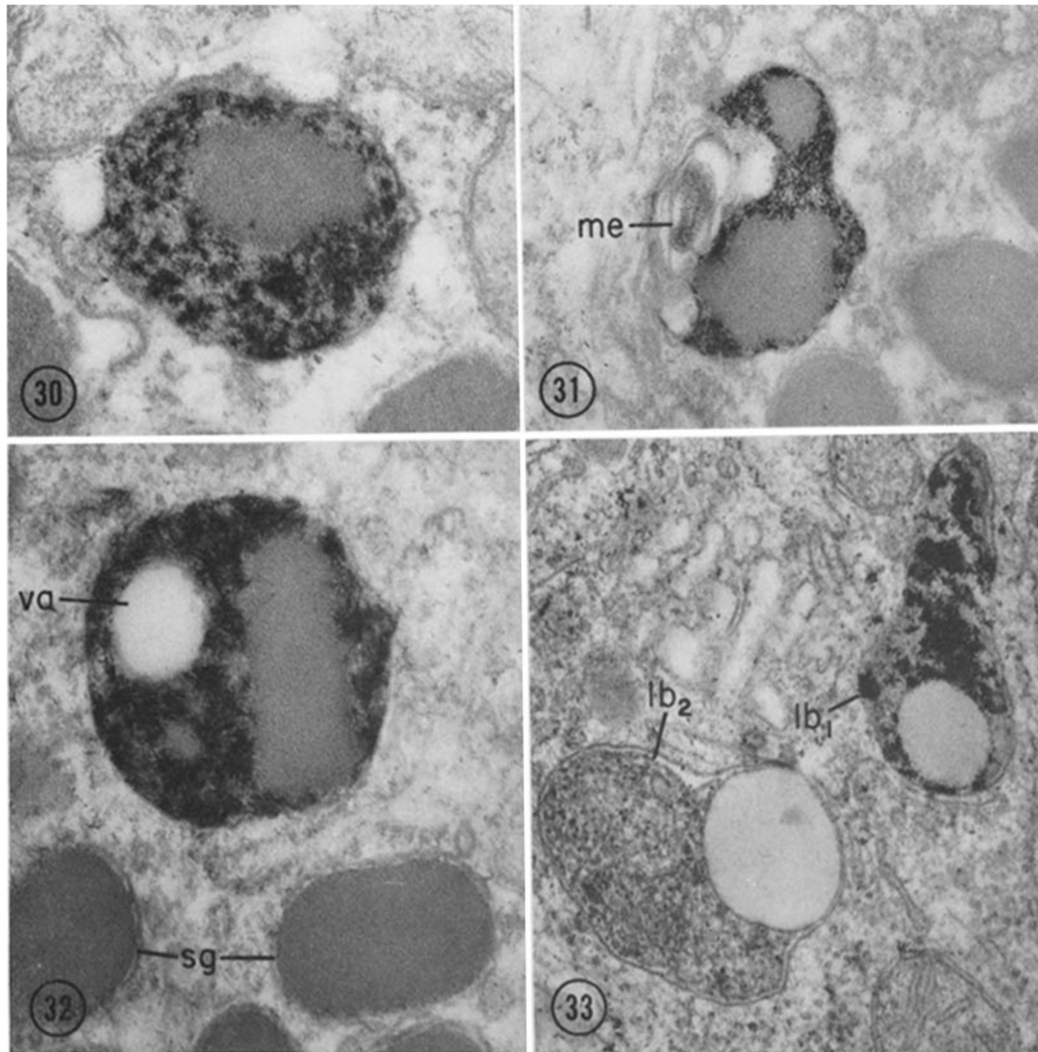
FIGURE 27 Small field from an MT cell at 24 hr postlactation. Specimen incubated for AcPase. Four lytic bodies are shown: a multivesicular body (lb_1) ,a dense body (lb_3), a vacuolated dense body (lb_4), and a form intermediate between a typical multivesicular and a dense body (lb_2). Heavy deposits of AcPase reaction product fill entirely the content of the dense bodies except for that portion of lb_4 occupied by the lipid vacuole. No deposits are seen in the multivesicular body or the intermediate form, but reaction product is present within a vesicle (arrow) adjacent to lb_2. Specimen preparation as for Fig. 24. × 72,000.

FIGURE 28 Another multivesicular body from an MT cell at 24 hr postlactation. In contrast to that shown in Fig. 27, it contains a few deposits of AcPase reaction product and secretory material. Specimen preparation as for Fig. 25, except that incubation was carried out for 1 hr in Barka-Anderson media. X 85,000.

FIGURE 29 MT cell at 48 hr postlactation. AcPase reaction product is present in four dense bodies (lb_1 to lb_4) one of which (lb_1) contains a secretory granule. Lead phosphate crystals are also present in a small vesicle (arrow) located near lb_3. The aggregating granule (*ag*) shown on the upper left is not reactive in this preparation. Specimen preparation as for Fig. 28. X 63,000.


lb4
lb3
lb2
lb1
27


ag
lb1
28
lb3
lb2
lb4
29
n



FIGURES 30 to 33 Lytic bodies in MT cells at 48 hr (Fig. 30) and 72 hr (Figs. 31 to 33) postlactation.

Figs. 30 to 32 show dense bodies containing recognizable secretory granules along with heavy deposits of AcPase reaction product. The body in Fig. 31 contains a membranous (*me*) residue and that in Fig. 32 a vacuolar (*va*) residue.

Fig. 33 shows two vacuolated dense bodies, one (lb_1) with heavy deposits, and the other (lb_2) with light deposits of reaction product over the dense part of their content. The vacuolar portion is free of such deposits.

Specimen for Fig. 30 fixed in glutaraldehyde in cacodylate buffer (pH 7.4). Nonfrozen sections incubated in Gomori medium for 30 min, postfixed in 1% OsO_4 in acetate-Veronal buffer (pH 7.6), and embedded in Araldite. Thin section doubly stained with uranyl and lead. Specimen preparation for Figs. 31 and 33 as for Fig. 28; that for Fig. 32 is the same as for Fig. 25. Figs. 30 and 32, X 75,000; Figs. 31 and 33, X 60,000.

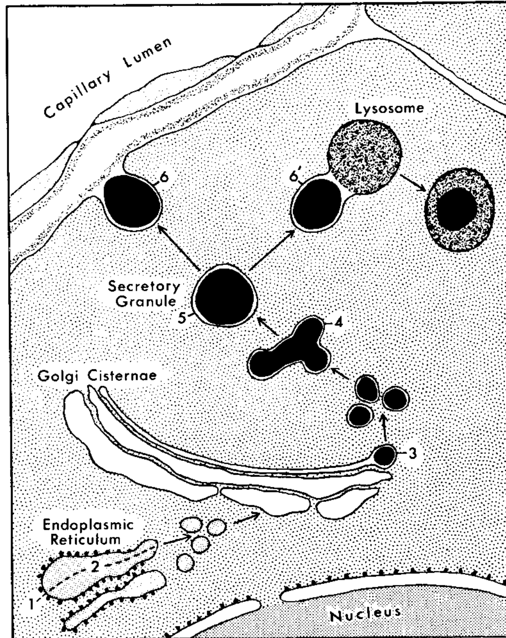


FIGURE 34 Diagrammatic representation of proposed events in the secretory process of MT cells in the anterior pituitary gland. Like zymogen in the exocrine pancreas (*see* reference 50), mammotrophic hormone is probably synthesized on ribosomes (1), segregated and transported by the rough-surfaced ER (2), and concentrated into granules by the Golgi complex. Small granules arising within the inner Golgi cisterna (3) aggregate (4) to comprise the mature secretory granule (5). During active secretion, the latter fuse with the cell membrane (6) and are discharged into the perivascular spaces. When secretory activity is suppressed and the cell must dispose of excess stored hormone, some granules fuse with lysosomes (6′) and are degraded as depicted in Fig. 35.

cellular involution (see below). Our findings demonstrate, therefore, that bulk segregation of cell organelles is not the only facet, or necessarily even the most important facet of cellular autophagy.

A further problem in integrating morphologic and functional lysosome concepts concerns the identification of primary lysosomes. The morphological counterpart of the primary lysosome in MT is not known, but the best candidates at the moment, as in other tissues (64, 67), are the small "Golgi" vesicles which in this case are derived presumably from the inner Golgi cisterna (see Fig. 35). At least AcPase is seen within the inner cisterna and within vesicles closely associated with it. Although it is not known in which direction the vesicles are moving, some of them may transport AcPase and possibly other lysosomal enzymes to lytic bodies. Such a hypothesis is supported by the fact that small AcPase-positive vesicles are frequencly seen in the vicinity of dense bodies and multivesicular bodies.

MULTIVESICULAR BODIES: The present findings clearly indicate that multivesicular bodies can take up and digest proteins and are transformed in the process into dense bodies. This is in accord with previous work on the renal glomerulus (73), nerve cells (57), and cultured cells (74, 75), among others, where segregation of specific proteins by multivesicular bodies has been traced. However, the previous work cited was concerned entirely with segregation of exogenous protein incorporated into the cell by pinocytosis, and the present work represents the first clear evidence for a connection between multivesicular bodies and disposal of endogenous protein. (See, however, the work of Palay (66) whose findings are suggestive of such a relationship.) The available evidence therefore supports the suggestion (76) that multivesicular bodies represent common digestive vacuoles for exogenous and endogenous materials. Since they do not always contain demonstrable AcPase activity, the possibility exists that multivesicular bodies correspond to prelysosomes or, in our case, "autophagosomes" in deDuve and Wattiaux's classification. If so, it follows that the interconnection between heterophagic and autophagic lines can occur at a prelysosomal as well as at a lysosomal level. This problem will probably not be resolved, however, until the mode of formation of multivesicular bodies is understood, and the nature and origin of the contained vesicles is clearly established.[4]

AUTOPHAGIC VACUOLES: Although a few autophagic vacuoles were seen at all postlactation intervals, they seldom contained secretory granules and were typically comprised of rough-surfaced ER and ribosomes. Autophagic vacuole formation therefore appears to serve primarily as a means of disposing of superfluous cellular organelles, and, as in the case of other tissues (65, 77, 78), it occurs more frequently when there is pronounced cellular

[4] Although the mode of formation of multivesicular bodies has not been clearly established, a number of hypotheses have been put forth (see references 72, 76), and a close relationship to the Golgi complex is usually recognized.

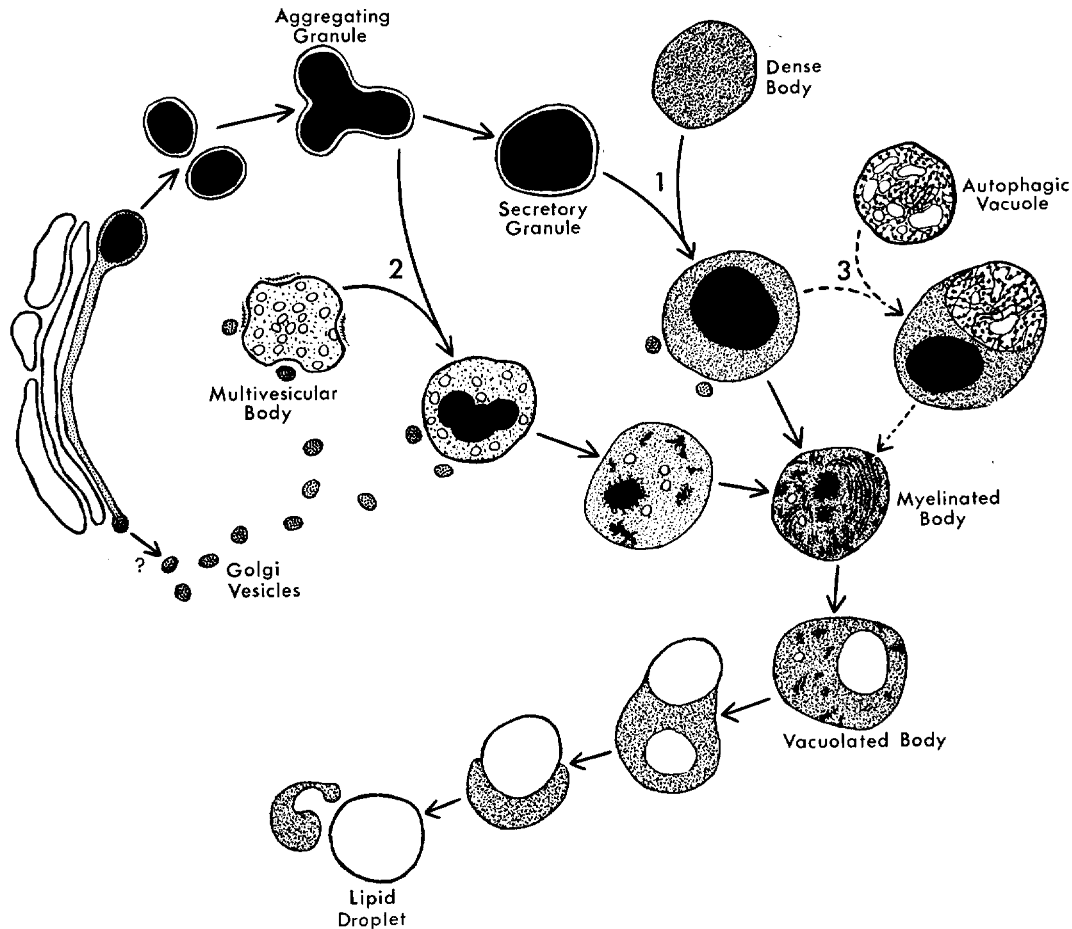


FIGURE 35 Diagram to illustrate morphologic types of lysosomes encountered in MT cells of the anterior pituitary gland and proposed pathways for intracellular digestion. Mature secretory granules are incorporated into dense bodies (arrow 1) and immature or aggregating granules into multivesicular bodies (arrow 2). Rough-surfaced ER and ribosomes are sequestered in autophagic vacuoles which can merge with dense bodies (arrow 3). This last pathway is shown with an interrupted arrow to indicate that autophagic vacuole formation is not obligatory but takes place primarily when there is pronounced cellular involution. In subsequent steps the material entering the lysosomal system through all three pathways is progressively degraded to yield a common residual body, the vacuolated dense body. Below, the "vacuole" of the latter is shown successively protruding from, and eventually separating from the peripheral dense rim, leaving a free lipid droplet and a dense body. The Golgi complex is depicted on the left to indicate that AcPase reaction product and condensing secretory protein occur within the same cisterna, and that AcPase activity may be carried to multivesicular and dense bodies via small "Golgi" vesicles derived from the inner cisterna.

involution, (e.g., ST acidophils after thyroidectomy (16) or adrenalectomy (60)).

DENSE BODIES: In accord with previous work (75), dense bodies with homogeneous content represent the common end stage in the complete digestion of lysosomal residues, regardless of the initial morphologic type of lytic body. Heterogeneity of the dense body's content appears to reflect incomplete digestion of residues, and the nature of the content changes characteristically as digestion proceeds.

Fate of Lysosomal Residues

At early intervals after lactation, secretory granules frequently form part of the content of lytic bodies; at intermediate periods, various

membranous and globular residues are more common; at later stages, one or more pale lipid vacuoles characteristically form part of their content; and finally, at the latest intervals studied (96 hr), small dense bodies with homogenous content predominate. The changes in the nature of the dense body's content reflect the progressive digestion of the incorporated granules along with the ER and ribosomes that enter the system through fusion with autophagic vacuoles. Incorporated protein can undoubtedly be digested readily by the numerous proteases and nucleases present in lysosomes. The frequency of vacuolated dense bodies with lipid droplets after longer postlactation intervals indicates that considerable lipid also accumulates as a by-product of the digestive process. It is not known whether the lipid residues constitute part of the granule content or are derived entirely from the phospholipids of granule and cytoplasmic membranes (41). Disposal of lipid may constitute more of a problem, for lysosomal fractions, except for those from polymorphonuclear leukocytes (72), are known to be poor in lipases and phospholipases (79). While the fate of the lysosomal lipid droplets is not entirely clear, certain images we have seen suggest that they may separate from the dense part of the body as depicted in the diagram of Fig. 35, and aggregate to form larger cytoplasmic lipid droplets. Release of lipid residues into the cytoplasmic matrix may be necessary to bring them into contact with appropriate lipid-splitting enzymes. The frequent association noted between large lipid droplets and rough-surfaced ER suggests involvement of the latter in the metabolism of lipid residues. In this connection it should be mentioned that rat pituitary homogenates have been demonstrated to contain several enzymatic activities capable of hydrolyzing both short and long chain fatty acid esters (80), but nothing is known about their subcellular location.

Acid Phosphatase Reactive Sites

Besides its association with lysosomes, AcPase activity was localized in other sites which are not usually considered to be lytic in nature, and therefore are deserving of special comment. In the lactating animal, AcPase activity was regularly found in the innermost Golgi cisterna, around some of the mature granules, and around most of the immature granules in the Golgi region, as well as in lytic bodies of MT. In MT of the postlactating animal, AcPase activity was restricted largely or exclusively to lysosomes. Our findings thus confirm the association already made by Sobel (10, 11), and Schreiber and his associates (see reference 81) utilizing light microscope histochemistry, between high levels of AcPase and secretion in anterior pituitary cells,[5] and demonstrate the sites of this activity more precisely than could be done by light microscopy. They are also in accord with the findings of Novikoff and his coworkers (12–14), who demonstrated AcPase activity around immature as well as occasional mature secretory granules in a number of tissues (e.g., hepatomas, Paneth cells, pancreatic islets). They do not, however, provide a satisfactory explanation for the occurrence of high levels of AcPase during secretion. It has been suggested that AcPase may be involved in secretory granule condensation (12), or that it may be "carried over" as membrane-bounded sites of activity on the membrane of the secretory granule (71). If AcPase is considered exclusively lysosomal, and if, like secretory protein, it is formed on the rough-surfaced ER and concentrated by the Golgi complex (72), we must conclude from our results that the cell can make and concentrate secretory protein and AcPase not only at the same time, but also in the same Golgi cisterna. The occurrence of AcPase activity around forming and, to a less extent, around mature secretory granules is perplexing since there is no biochemical evidence to indicate that this group of enzymes functions in condensation or transport. It could be explained if, in the process of forming simultaneously secretory granules and primary lysosomes, occasional hybrid granules were made which then end up as lysosomes; however, this does not seem very likely, in view of the fact the majority of the forming granules are heavily labeled with AcPase reaction product. The activity around secretory granules could represent the acid "tail" of another enzyme—a phosphatase of broad specificity—which is involved in transport. With this in mind we ran tests with IDP since we had previously demonstrated (83) IDPase activity around maturing

[5] A similar association between AcPase levels and secretion has been claimed on the basis of biochemical measurements of AcPase activity in pituitary homogenates (10, 81, 82). However, the results of such studies are difficult to interpret in view of the number of cell types present in the anterior pituitary gland, and the fact that the experimental procedures utilized (e.g. thyroidectomy, castration, adrenalectomy) affect more than one cell type.

granules of anterior pituitary cells. Results with this substrate at pH 5.0 were negative, but other phosphates should be tried. Finally, it is possible that a certain amount of AcPase is packaged with each secretory granule in an inactive form which becomes active if the granules are destined for destruction rather than discharge. Activation of AcPase could constitute a mechanism for controlling and triggering the destruction of secretory material, as appears to be the case for yolk platelets of amphibian eggs (72, 84).

This research was supported by grants AM-09090 and 5T1-GM-349, and research career program award 1-K3-GM-25,109, from the United States Public Health Service.

We are grateful for the technical assistance of Mrs. Karin Taylor, Miss Agneta Strömblad, Mrs. Irma Daehne, Miss Cassandra Lista, and Mrs. Jean Sarris. We also wish to thank Mr. Wayne Emery, medical illustrator, for preparation of the diagrams (Figs. 34 and 35).

Received for publication 14 March 1966.

REFERENCES

1. DE DUVE, C., The lysosome concept, *in* Ciba Foundation Symposium on Lysosomes, (A. V. S. de Reuck and M. P. Cameron, editors), Boston, Little, Brown and Co., 1963, 1.
2. DE DUVE, C., From cytases to lysosomes, *Fed. Proc.*, 1964, **23,** 1045.
3. COHN, Z. A., HIRSCH, J. G., and WIENER, E., The cytoplasmic granules of phagocytic cells and the degradation of bacteria, *in* Ciba Foundation Symposium on Lysosomes, (A. V. S. de Reuck and M. P. Cameron, editors), Boston, Little, Brown and Co., 1963, 126.
4. HIRSCH, J. G., and COHN, Z. A., Digestive and autolytic functions of lysosomes in phagocytic cells, *Fed. Proc.*, 1964, **23,** 1023.
5. STRAUS, W., Occurrence of phagosomes and phago-lysosomes in different segments of the nephron in relation to the reabsorption, transport, digestion, and extrusion of intravenously injected horseradish peroxidase, *J. Cell Biol.*, 1964, **21,** 295.
6. MILLER, F., and PALADE, G. E., Lytic activities in renal protein absorption droplets. An electron microscopical cytochemical study, *J. Cell Biol.*, 1964, **23,** 519.
7. WEBER, R., Behaviour and properties of acid hydrolases in regressing tails of tadpoles during spontaneous and induced metamorphosis *in vitro*, *in* Ciba Foundation Symposium on Lysosomes, (A. V. S. de Reuck and M. P. Cameron, editors), Boston, Little, Brown and Co., 1963, 282.
8. SCHEIB, D., Properties and role of acid hydrolases of the Müllerian ducts during sexual differentiation in the male chick embryo, *in* Ciba Foundation Symposium on Lysosomes, (A. V. S. de Reuck and M. P. Cameron, editors), Boston, Little, Brown and Co., 1963, 264.
9. SLATER, T. F., GREENBAUM, A. L., and WANG, D. Y., Lysosomal changes during liver injury and mammary involution, *in* Ciba Foundation Symposium on Lysosomes, (A. V. S. de Reuck and M. P. Cameron, editors), Boston, Little, Brown and Co., 1963, 311.
10. SOBEL, H. J., The localization of acid phosphatase activity in the rat pituitary and thyroid glands, and its relation to secretory activity, *Endocrinology*, 1961, **68,** 801.
11. SOBEL, H. J., Relationship of three lysosomal enzymes to the Golgi zone and secretory activity in the rat pituitary and thyroid glands, *Anat. Rec.*, 1962, **143,** 389.
12. NOVIKOFF, A. B., Cytochemical staining methods for enzyme activities: their application to the rat parotid gland, *Jewish Memorial Hospital Bulletin*, 1962, **7,** 70.
13. NOVIKOFF, A. B., and ESSNER, E., Pathological changes in cytoplasmic organelles, *Fed. Proc.*, 1962, **21,** 1130.
14. NOVIKOFF, A. B., ESSNER, E., GOLDFISCHER, S., and HEUS, M., Nucleosidephosphatase activities of cytomembranes, *in* The Interpretation of Ultrastructure, (R. J. C. Harris, editor), New York, Academic Press Inc., 1962, 149.
15. SMITH, R. E., Acid phosphatase activity of rat adenohypophysis during secretion, *J. Cell Biol.*, 1963, **19,** No. 2, 66A (abstract).
16. SMITH, R. E., Cytochemical and electron microscopic studies of enzyme fluctuation in the rat adenohypophysis during secretion, Ph.D. Thesis, University of California, San Francisco, 1964.
17. FOLLEY, S. J., The Physiology and Biochemistry of Lactation, London, Oliver and Boyd Ltd., 1956.
18. EVERETT, N. B., and BAKER, B. L., The distribution of cell types in the anterior hypophysis during late pregnancy and lactation, *Endocrinology*, 1945, **37,** 83.
19. GROSVENOR, C. E., and TURNER, C. W., Pituitary lactogenic hormone concentration and milk secretion in lactating rats, *Endocrinology*, 1958, **63,** 535.
20. GROSVENOR, C. E., and TURNER, C. W., Release

and restoration of pituitary lactogen in response to nursing stimuli in lactating rats, *Proc. Soc. Exp. Biol. and Med.*, 1957, **96,** 723.

21. MILLONIG, G., Further observations on a phosphate buffer for osmium solutions in fixation, *in* 5th International Congress for Electron Microscopy, Philadelphia, 1962, (S. S. Breese, Jr., editor), New York, Academic Press Inc., 1962, **2,** P-8.
22. 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.
23. LUFT, J. H., Improvements in epoxy resin embedding methods, *J. Biophysic. and Biochem. Cytol.*, 1961, **9,** 409.
24. PARSONS, D. F., A simple method for obtaining increased contrast in araldite sections by using postfixation staining of tissues with potassium permanganate, *J. Biophysic. and Biochem. Cytol.*, 1961, **11,** 492.
25. FARQUHAR, M. G., and PALADE, G. E., Cell junctions in amphibian skin, *J. Cell Biol.*, 1965, **26,** 263.
26. KARNOVSKY, M. J., Simple methods for "staining with lead" at high pH in electron microscopy, *J. Cell Biol.*, 1961, **11,** 729.
27. 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.
28. RICHARDSON, K. C., JARETT, L., and FINKE, E. H., Embedding in epoxy resins for ultrathin sectioning in electron microscopy, *Stain Technol.*, 1960, **35,** 313.
29. JENNINGS, B. M., FARQUHAR, M. G., and MOON, H. D., Staining methods for osmium-methacrylate sections, *Am. J. Path.*, 1959, **35,** 991.
30. PALAY, S. L., MCGEE-RUSSELL, S. M., GORDON, S., JR., and GRILLO, M. A., Fixation of neural tissues for electron microscopy by perfusion with solutions of osmium tetroxide, *J. Cell Biol.*, 1962, **12,** 385.
31. SMITH, R. E., and FARQUHAR, M. G., Preparation of nonfrozen sections for electron microscope cytochemistry, *Scientific Instruments News* (RCA), 1965, **10,** 13.
32. SMITH, R. E., and FARQUHAR, M. G., Preparation of thick sections for cytochemistry and electron microscopy by a nonfreezing technique, *Nature*, 1963, **200,** 691.
33. GOMORI, G., Microscopic Histochemistry, Principles and Practice, Chicago, University of Chicago Press, 1952.
34. BARKA, T., and ANDERSON, P. J., Histochemical methods for acid phosphatase using hexazonium pararosanilin as coupler, *J. Histochem. and Cytochem.*, 1962, **10,** 741.
35. NOVIKOFF, A. B., and GOLDFISCHER, S. B., Nucleosidediphosphatase activity in the Golgi apparatus and its usefulness for cytological studies, *Proc. Nat. Acad. Sc.*, 1961, **47,** 802.
36. FARQUHAR, M. G., and PALADE, G. E., Adenosine triphosphatase localization in amphibian epidermis, *J. Cell Biol.*, 1966, **30,** 359.
37. POWELL, T. E., 3RD, PHILPOTT, C. W., and MASER, M. D., On the hydrogen ion concentration and osmolality of fixative components, *J. Cell Biol.*, 1964, **23,** No. 2, 110 A (abstract).
38. FAHIMI, H. D., and DROCHMANS, P., Essais de standardisation de la fixation au glutaraldéhyde. I. Purification et détermination de la concentration du glutaraldéhyde, *J. Micr.*, 1965, **4,** 725.
39. HERLANT, M., The cells of the adenohypophysis and their functional significance, *in* International Review of Cytology, (G. H. Bourne and J. F. Danielli, editors), New York, Academic Press Inc., 1964, **17,** 299.
40. PURVES, D., Morphology of the hypophysis related to its function, *in* Sex and Internal Secretions, (W. C. Young, editor), Baltimore, The Williams & Wilkins Co., 1961, 161.
41. MCSHAN, W. H., and HARTLEY, M. W., Production, storage and release of anterior pituitary hormones, *Reviews of Physiology, Biochemistry and Experimental Pharmacology*, 1965, **56,** 264.
42. SIPERSTEIN, E. R., and ALLISON, V. F., Fine structure of the cells responsible for secretion of adrenocorticotrophin in the adrenalectomized rat, *Endocrinology*, 1965, **76,** 70.
43. KUROSUMI, K., and KOBAYASHI, Y., Corticotrophs in the anterior pituitary glands of normal and adrenalectomized rats as revealed by electron microscopy, *Endocrinology*, 1966, **78,** 745.
44. FARQUHAR, M. G., and RINEHART, J. F., Electron microscopic studies of the anterior pituitary gland of castrate rats, *Endocrinology*, 1954, **54,** 516.
45. FARQUHAR, M. G., and RINEHART, J. F., Cytologic alterations in the anterior pituitary gland following thyroidectomy: An electron microscope study, *Endocrinology*, 1954, **55,** 857.
46. HEDINGER, C. E., and FARQUHAR, M. G., Elektronenmikroskopische Untersuchungen von zwei Typen acidophiler Hypophysenvorderlappenzellen bei der Ratte, *Schweiz. Z. Allg. Path. und Bakt.*, 1957, **20,** 766.
47. FARQUHAR, M. G., Origin and fate of secretory granules in cells of the anterior pituitary gland, *Trans. New York Acad. Sc.*, 1961, **23,** 346.
48. PASTEELS, J. L., Recherches morphologiques et expérimentales sur la sécrétion de prolactine, *Arch. Biol. (Liège)*, 1963, **74,** 439.
49. HYMER, W. C., MCSHAN, W. H., and CHRISTIAN-

SEN, R. G., Electron microscopic studies of anterior pituitary glands from lactating and estrogen-treated rats, *Endocrinology*, 1961, **69,** 81.

50. PALADE, G. E., SIEKEVITZ, P., and CARO, L. G., Structure, chemistry and function of the pancreatic exocrine cell, *in* Ciba Foundation Symposium on The Exocrine Pancreas, (A. V. S. de Reuck and M. P. Cameron, editors), London, J. and A. Churchill, Ltd., 1962, 23.
51. HAGUENAU, F., The ergastoplasm: Its history, ultrastructure, and biochemistry, *in* International Review of Cytology, (G. H. Bourne and J. F. Danielli, editors), New York, Academic Press Inc., 1958, **7,** 425.
52. 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.
53. JAMIESON, J. D., and PALADE, G. E., Role of the Golgi complex in the intracellular transport of secretory proteins, *Proc. Nat. Acad. Sc.*, 1966, **55,** 424.
54. ROTH, T. F., and PORTER, K. R., Yolk protein uptake in the occyte of the mosquito *Aedes aegypti* L., *J. Cell Biol.*, 1964, **20,** 313.
55. 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.
56. PAPPAS, G. D., and PURPURA, D. P., Fine structure of dendrites in the superficial neocortical neuropil, *Exp. Neurology*, 1961, **4,** 507.
57. ROSENBLUTH, J., and WISSIG, S. L., The distribution of exogenous ferritin in toad spinal ganglia and the mechanism of its uptake by neurons, *J. Cell Biol.*, 1964, **23,** 307.
58. FARQUHAR, M. G., Fine structure and function in capillaries of the anterior pituitary gland, *Angiology*, 1961, **12,** 270.
59. ASHFORD, T. P., and PORTER, K. R., Cytoplasmic components in hepatic cell lysosomes, *J. Cell Biol.*, 1962, **12,** 198.
60. FARQUHAR, M. G., unpublished observations.
61. BAINTON, D. F., and FARQUHAR, M. G., Origin of granules in polymorphonuclear leukocytes. Two types derived from opposite faces of the Golgi complex in developing granulocytes, *J. Cell Biol.*, 1966, **28,** 277.
62. DESCLIN, L., Détection de substances pentosenucléiques dans les cellules du lobe antérieur de l'hypophyse du rat et du cobaye, *Compt. Rend. Soc. Biol.*, 1940, **133,** 457.
63. DESCLIN, L., Contribution à l'étude de la structure et du functionnement de l'hypophyse pendant le post-partum, *Arch. Biol. (Liège)*, 1945, **56,** 261.
64. NOVIKOFF, A. B., Lysosomes in the physiology and pathology of cells: contributions of staining methods, *in* Ciba Foundation Symposium on Lysosomes, (A. V. S. de Reuck and M. P. Cameron, editors), Boston, Little, Brown and Co., 1963, 36.
65. SWIFT, H., and HRUBAN, Z., Focal degradation as a biological process, *Fed. Proc.*, 1964, **23,** 1026.
66. PALAY, S. L., The fine structure of secretory neurons in the preoptic nucleus of the goldfish (*Carassius auratus*), *Anat. Rec.*, 1960, **138,** 417.
67. NOVIKOFF, A. B., ESSNER, E., and QUINTANA, N., Golgi apparatus and lysosomes, *Fed. Proc.*, 1964, **23,** 1010.
68. HELLERSTRÖM, C., TÄLJEDAL, I., and HELLMAN, B., Quantitative studies on isolated pancreatic islets of mammals, *Acta Endocrinol.*, 1964, **45,** 476.
69. LAZARUS, S. S., VOLK, B. W., and BARDEN, H., Localization of acid phosphatase activity and secretion mechanism in rabbit pancreatic β-cells, *J. Histochem. and Cytochem.*, 1966, **14,** 233.
70. BURTON, P. R., and VENSEL, W. H., Ultrastructural studies of normal and alloxan-treated islet cells of the pancreas of the lizard, *Eumeces fasciatus*, *J. Morphol.*, 1966, **118,** 91.
71. 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.
72. DEDUVE, C., and WATTIAUX, R., Functions of lysosomes, *Ann. Rev. Physiol.*, 1966, **28,** 435.
73. FARQUHAR, M. G., and PALADE, G. E., Functional evidence for the existence of a third cell type in the renal glomerulus, *J. Cell Biol.*, 1962, **13,** 55.
74. ROBBINS, E., MARCUS, P. I., and GONATAS, N. K., Dynamics of acridine orange–cell interaction. II. Dye-induced ultrastructural changes in multivesicular bodies (acridine orange particles), *J. Cell Biol.*, 1964, **21,** 49.
75. GORDON, G. B., MILLER, L. R., and BENSCH, K. G., Studies on the intracellular digestive process in mammalian tissue culture cells, *J. Cell Biol.*, 1965, **25,** No. 2, pt. 2, 41.
76. MERKER, H. J., Über das Vorkommen multivesiculärer Einschlusskörper („Multivesicular bodies") im Vaginalepithel der Ratte, *Z. Zellforsch. u. Mikr. Anat.*, 1965, **68,** 618.
77. ELLIOTT, A. M., and BAK, I. J., The fate of mitochondria during aging in *Tetrahymena pyriformis*, *J. Cell Biol.*, 1964, **20,** 113.
78. BRANDES, D., BUETOW, D. E., BERTINI, F., and MALKOFF, D. B., Role of lysosomes in cellular lytic processes. I. Effect of carbon starvation in

Euglena gracilis, *Exp. and Mol. Path.*, 1964, **3**, 583.

79. TAPPEL, A. L., and DEDUVE, C., Discussion, *in* Ciba Foundation Symposium on Lysosomes, (A. V. S. de Reuck and M. P. Cameron, editors), Boston, Little, Brown and Co., 1963, 111.
80. VANHA-PERTTULA, T. P., and HOPSU, V. K., Esterolytic and proteolytic enzymes of the rat adenohypophysis. I. Studies with homogenate and its fractions, *Histochemie*, 1965, **4**, 372.
81. SCHREIBER, V., Biochemical estimation of elevated acid phosphatase activity in anterior pituitaries of rats and golden hamsters fed methylthiouracil, *Endocrinology*, 1962, **70**, 923.
82. HIROSHIGE, T., NAKATSUGAWA, T., MATSUOKA, Y., and ITOH, S., Acid phosphatase activity of the rat pituitary and hypothalamus in relation to secretory activity, *Jap. J. Physiol.*, 1966, **16**, 94.
83. SMITH, R. E., and FARQUHAR, M. G., Modulation in inosine diphosphatase and thiamine pyrophosphatase activity of rat adenohypophysis during secretion, *J. Cell Biol.*, 1964, **23**, No. 2, 87A (abstract).
84. BRACHET, J., Discussion, *in* Ciba Foundation Symposium on Lysosomes, (A. V. S. de Reuck and M. P. Cameron, editors), Boston, Little, Brown and Co., 1963, 422.