

# UPTAKE AND FATE OF LUMINALLY ADMINISTERED HORSE-RADISH PEROXIDASE IN RESTING AND ISOPROTERENOL-STIMULATED RAT PAROTID ACINAR CELLS

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## ABSTRACT

The formation and fate of apical endocytic vesicles in resting and isoproterenol-stimulated rat parotid acinar cells were studied using luminally administered horseradish peroxidase (HRP) to mark the vesicles. The tracer was taken up from the lumen by endocytosis in small, smooth-surfaced "c"- or ring-shaped vesicles. About 1 h after HRP administration the vesicles could be found adjacent to the Golgi apparatus. At later times HRP reaction product was localized in multivesicular bodies and lysosomes; in isoproterenol-stimulated cells it was also present in autophagic vacuoles. HRP reaction product was never localized in any structure associated with secretory granule formation. These results suggest that the apical endocytic vesicles play a role in membrane recovery, but that they are degraded and not reutilized directly in secretory granule formation. Additionally, it was found that when isoproterenol was injected before HRP administration, the apical junctional complexes became permeable to the tracer, allowing it to gain access to the lateral and basal intercellular spaces. This permeability may provide an additional route whereby substances in the extracellular fluid could reach the saliva.

**KEY WORDS** electron microscopy · cytochemistry · secretion · endocytic vesicles · lysosomes · junctional complexes

It has been well established for a variety of endocrine and exocrine glands that exportable proteins are packaged into membrane-bounded secretory granules. In merocrine glands, such as the parotid gland and the exocrine pancreas, discharge of these proteins occurs by fusion of the limiting membrane of the secretory granule with the plasma membrane at the luminal surface. Secretory proteins are then exteriorized through openings formed by the breakdown of the fused membranes. Although considerable granule membrane may be added to the plasma membrane,

especially during stimulated secretion, only transient alterations occur in cell size and shape (2, 36, 13). It has been suggested that the excess membrane may be removed in the form of smooth-surfaced vesicles by endocytosis at either the apical (2, 17, 23, 31) or lateral surface (13) of the acinar cells. However, the fate of these endocytic vesicles is unresolved. Biochemical studies of the composition, synthesis, and turnover of cellular membranes in the pancreas (27, 28, 29) and parotid gland (44) suggest that the secretory granule membrane may be reutilized during the formation of new granules. On the other hand, studies on the uptake of exogenous tracers in these glands (13, 23) have indicated that the apical vesicles fuse with lysosomes where the

vesicle membrane is presumably degraded.

The present investigation was undertaken in order to determine whether the apical vesicles in rat parotid acinar cells are endocytic in nature and to study the role that these vesicles may play in membrane recovery. Horseradish peroxidase (HRP) was administered via the main excretory duct to both resting and isoproterenol-stimulated rat parotid glands. Under most experimental conditions, the tracer was confined to the glandular lumen, thus enabling us to study the formation of vesicles from the luminal surface and their fate within the cell.

## MATERIALS AND METHODS

Male and female Sprague-Dawley or Wistar-Furth rats weighing between 200 and 440 g were used. Animals were anesthetized with chloral hydrate (400 mg/kg body weight) administered intraperitoneally. A 1-cm<sup>3</sup> syringe containing a solution of horseradish peroxidase (HRP; type II, Sigma Chemical Co., St. Louis, Mo.), 10 mg/ml in sterile saline, suspended at a height of 8 inches above the animal was connected by polyethylene tubing to a cannula inserted into the main excretory duct of the parotid gland. The HRP solution was allowed to flow by gravity into the gland for varying time intervals (normally 1 h). The maximum hydrostatic pressure was calculated to be 16 mm Hg. Some animals received an intraperitoneal injection of isoproterenol (30 mg/kg body weight; Sigma) either before or after administration of the HRP. The animals either were sacrificed immediately after HRP administration or were allowed to survive for up to 24 h.

The glands were fixed by vascular perfusion with a modified Karnovsky fixative (24) containing 2% glutaraldehyde (Ladd Research Industries, Burlington, Vt.), 2% formaldehyde (Ladd) and 0.025% calcium chloride

in 0.05 or 0.1 M cacodylate buffer, pH 7.4. After perfusion the glands were removed, immersed in fresh fixative, and cut into strips 1 × 5 mm. After 1-h total fixation time, the tissue was rinsed in 0.05 M cacodylate buffer pH 7.4 containing 7% sucrose and stored in the same buffer overnight at 4°C. The tissue was then chopped into 75-μm sections on a Smith-Farquhar TC-2 tissue sectioner and incubated in Fahimi's diaminobenzidine-hydrogen peroxide medium (8) for 45 min at room temperature. Controls included sections from glands that had not received HRP but were incubated in full medium, and sections from glands that had received HRP but were incubated without substrate. In some experiments, after incubation for peroxidase activity, sections were then incubated for acid phosphatase activity in Gomori acid phosphatase medium (14), using CMP as substrate (30). After incubation for acid phosphatase activity, sections were treated with dilute ammonium sulfide in order to convert the lead phosphate to lead sulfide. Sections incubated without substrate served as controls. After incubation the glands were postfixed in 1% osmium tetroxide in 0.05 M cacodylate buffer pH 7.4 containing 7% sucrose, rinsed and stained *en bloc* with 0.5% uranyl acetate in distilled water containing 5% sucrose, dehydrated through a graded series of ethanol and propylene oxide, and embedded in Spurr's resin (37). Thin sections, cut with a diamond knife on a Porter-Blum MT-2B microtome (DuPont Instruments, Sorvall Operations, Newtown, Conn.), were mounted on bare copper grids, stained with Reynolds lead citrate (33), and examined in either a JEM 100-B or JEM 100-C electron microscope.

## RESULTS

### *Resting Acinar Cells*

When HRP was administered retrograde to resting glands, extracellular reaction product was confined to the lumen of the gland (Figs. 1 and

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FIGURES 1-20 All figures are micrographs from rat parotid glands incubated in diaminobenzidine-containing medium.

FIGURES 1-5 Figs. 1-5 are from Sprague-Dawley rats given HRP retrograde for 1 h.

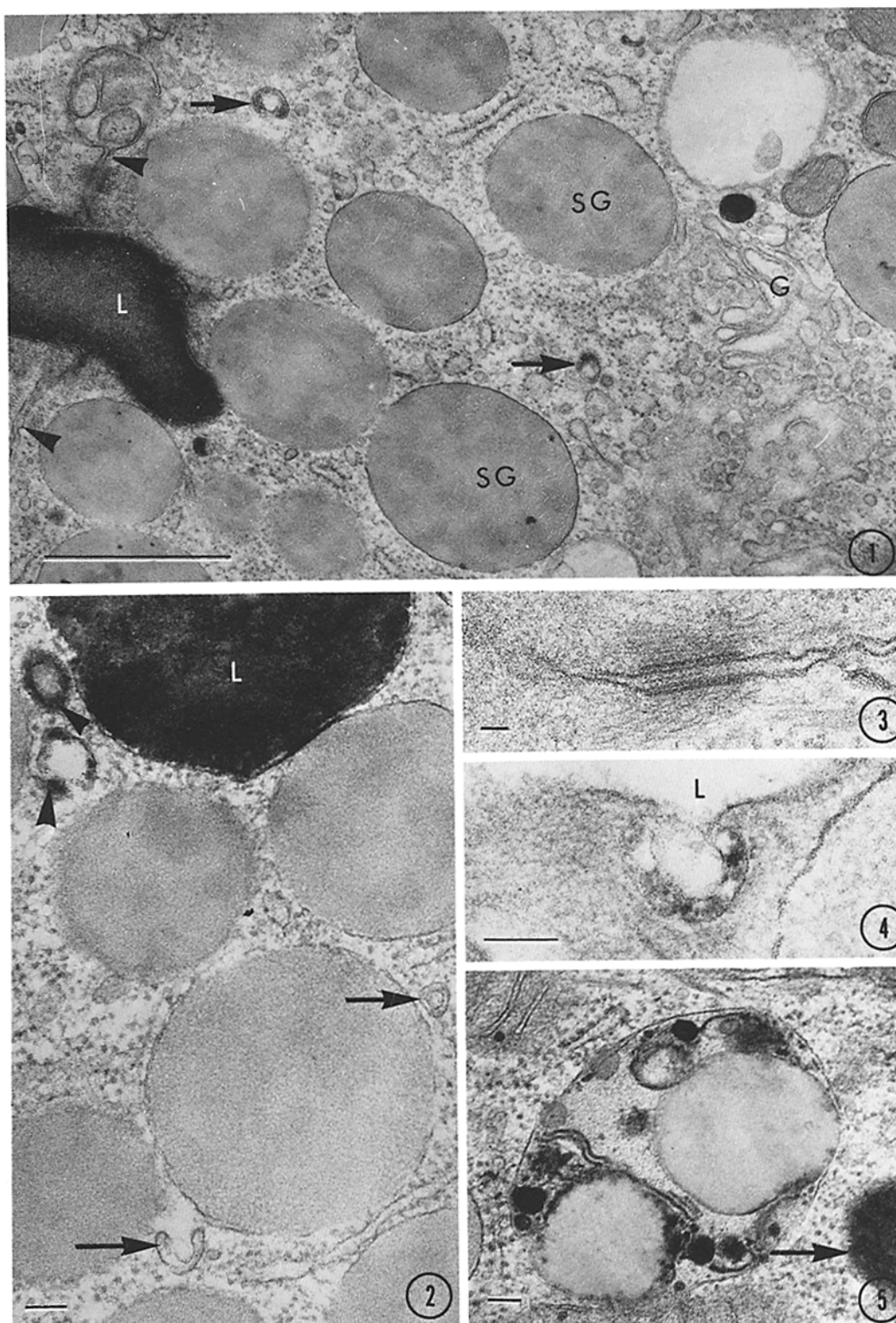
FIGURE 1 HRP reaction product is localized in the lumen (L), in small, ring-shaped vesicles (arrows) and in a dense body adjacent to the Golgi apparatus (G). Secretory granules (SG), Golgi saccules and intercellular spaces (arrowheads) are free of reaction product. Bar, 1 μm. × 29,000.

FIGURE 2 Vesicles containing HRP reaction product (arrowheads) are located adjacent to the lumen (L). Similar vesicles (arrows), free of reaction product, are seen deeper in the cell. Bar, 0.1 μm. × 65,000.

FIGURE 3 No HRP reaction product is present in the junction or in the intercellular space. Bar, 0.1 μm. × 90,000.

FIGURE 4 A vesicle containing reaction product can be seen forming at the luminal (L) surface of the cell. Bar, 0.1 μm. × 115,000.

FIGURE 5 At this time period residual bodies contain no reaction product. Dense inclusions are normal lysosomal constituents (cf. Fig. 9). A portion of a dense body containing reaction product is present (arrow). Bar, 0.1 μm. × 50,000.



2). There was no significant leakage of tracer, and no reaction product was observed in the intercellular junctions (Fig. 3), or in the lateral (Fig. 1) or basal (Fig. 6) intercellular spaces. Intracellularly the reaction product initially was found in vesicles in the apical portion of the cytoplasm. The HRP appeared to be taken up from the lumen in "c"- or ring-shaped vesicles (Figs. 1, 2, and 4) which formed as invaginations of the plasma membrane at the luminal surface (Fig. 4). At early times after HRP administration, these vesicles containing reaction product were frequently seen adjacent to the lumen while similar vesicles which contained no reaction product were located further basally (Fig. 2). By 1 h after administration of HRP, ring-shaped vesicles and small dense bodies containing reaction product were found near the Golgi apparatus (Fig. 1). However, at this time secondary lysosomes (Fig. 5) were free of reaction product. After approx. 5 h, HRP reaction product was localized in the basal region of the acinar cells (Fig. 6). At the later times, the majority of the reaction product was localized in multivesicular bodies (Figs. 6 and 7), secondary lysosomes (Figs. 6 and 9) and small vesicles adjacent to lysosomes (Fig. 9). Reaction product was also present in small "c"- and ring-shaped vesicles located in the basal portion of the cells in association with lysosomes (Fig. 8). At no time was HRP reaction product seen in Golgi saccules or secretory granules.

#### *Isoproterenol-Stimulated*

##### *Acinar Cells*

In the first series of experiments HRP was given for 1 h, then isoproterenol was administered to stimulate secretion. The animals were sacrificed at

varying time intervals after isoproterenol. The secretion produced by isoproterenol washed the extracellular HRP from the glands, and no reaction product was present in either the acinar lumina or the intercellular spaces (Fig. 10). For the first 1-2 h after isoproterenol, reaction product was localized in vesicles and dense bodies in the apical portion of the acinar cells (Fig. 10). Although some of the vesicles and dense bodies were close to the Golgi apparatus, no reaction product was seen in the saccules (Figs. 10 and 11). Some of the dense bodies containing reaction product were also found adjacent to autophagic vacuoles which formed in response to the isoproterenol (36) (Fig. 11). However, by 3 h after HRP administration (Fig. 12), these dense bodies were not observed, but the autophagic vacuoles frequently contained HRP reaction product, presumably obtained by fusion with HRP-containing dense bodies. At later times after isoproterenol, reaction product was localized in lysosomes and autophagic vacuoles in the basal portions of the cells (Figs. 13 and 14). Reaction product was not observed in either Golgi saccules or secretory granules (Figs. 13 and 14).

In a second series of experiments the fate of HRP administered after isoproterenol stimulation was investigated. The administration of HRP was begun 1 h after the isoproterenol injection, to allow for the outward flow of saliva. The HRP was administered for 1 h. The animals were sacrificed at varying time intervals after HRP. At all times, little or no reaction product was found in the lumina of the glands. However, in Sprague-Dawley rats HRP reaction product was present in the intercellular spaces at the lateral and basal surfaces of the cells (Fig. 15). Beginning at 1-2 h af-

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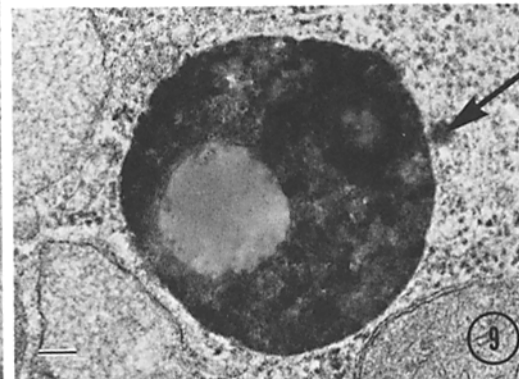
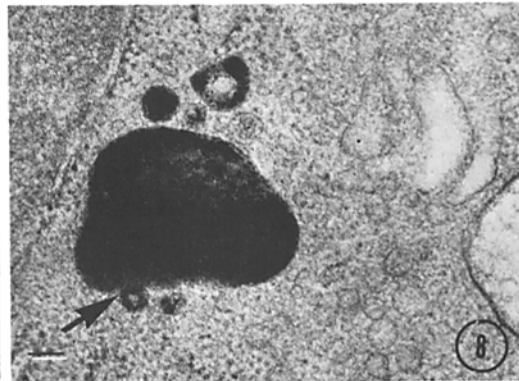
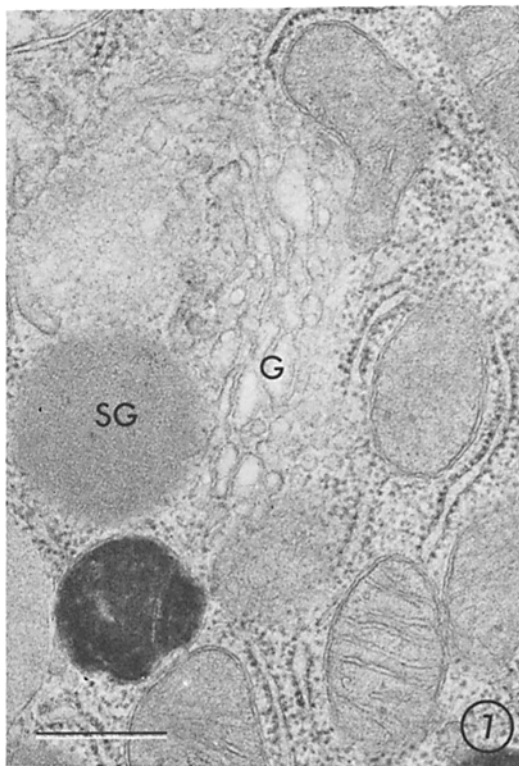
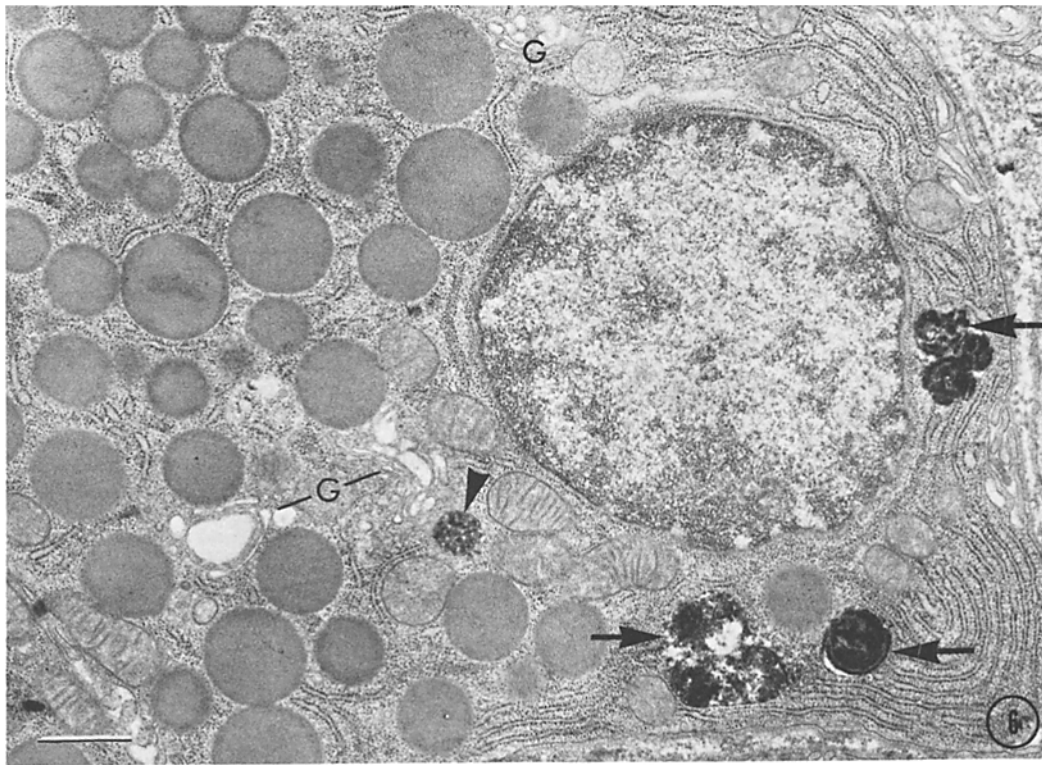
FIGURES 6-9 Figs. 6-9 are from Sprague-Dawley rats given HRP for 1 h and allowed to recover for an additional 4 h.

FIGURE 6 HRP reaction product is now located in a multivesicular body (arrowhead) and lysosomes (arrows) in the basal portion of the cell. Golgi saccules (G), secretory granules, and lateral and basal intercellular spaces are unreactive. Bar, 1  $\mu\text{m}$ .  $\times 12,500$ .

FIGURE 7 HRP reaction product is localized only in a dense body. Golgi saccules (G) and secretory granules (SG) have no activity. Bar, 0.5  $\mu\text{m}$ .  $\times 35,000$ .

FIGURE 8 Several vesicles with reaction product are adjacent to a dense body containing reaction product. One vesicle (arrow) may be fusing with the dense body. Bar, 0.1  $\mu\text{m}$ .  $\times 50,000$ .

FIGURE 9 The matrix of the residual body is obscured by HRP reaction product. A small vesicle containing HRP reaction product is adjacent to the residual body (arrow). Bar, 0.1  $\mu\text{m}$ .  $\times 50,000$ .



ter HRP, occasional polymorphonuclear leukocytes (PMN's) were seen near the base of the acinar cells, and by 4–5 h after HRP numerous PMN's were present (Fig. 15). In spite of the presence of the PMN's the intracellular localization of reaction product was essentially the same as that observed in the cells that had received HRP before isoproterenol. Reaction product was localized in lysosomes and in autophagic vacuoles induced by isoproterenol (Fig. 15).

Because of possible alterations in the localization of HRP in the presence of PMN's, these experiments were repeated with Wistar-Furth rats, a strain of rats which does not exhibit mast cell degranulation (6) or vascular leakage (7, 40) after HRP injection. It was first determined that the uptake and fate of HRP in resting cells and in cells stimulated after HRP administration was the same as that seen under identical conditions in Sprague-Dawley rats. HRP was then administered to Wistar-Furth rats after isoproterenol. The localization of reaction product was similar to that seen in the Sprague-Dawley rats, but there was little or no PMN infiltration (Fig. 16). Extensive reaction product was present in the intercellular spaces at the lateral and basal surfaces (Figs. 16 and 17). Reaction product could also be found in the intercellular junctions (Fig. 18), suggesting that the junctions became "leaky" after stimulated secretion. Although less HRP appeared to be taken up by the acinar cells under these conditions, the fate of the HRP-containing vesicles appeared to be similar to that seen in resting cells or in cells that had received HRP before the isoproterenol. At early times, reaction product could be seen in a few ring-shaped vesicles (Fig. 17) in the apical portion of the cells, while at later time intervals reaction product was localized in lysosomes. As

noted for the other experimental conditions, reaction product was not observed in either Golgi saccules or secretory granules.

### Acid Phosphatase

In order to assure that the HRP reaction product was indeed localized in lysosomes, some sections were incubated for peroxidase activity and subsequently incubated for acid phosphatase activity. After incubation for both enzyme activities the reaction products characteristic of each were found localized in secondary lysosomes (Fig. 19) and isoproterenol-induced autophagic vacuoles. The flocculent oxidized DAB typical of HRP activity obscured the lysosomal matrix. Dense aggregates of finely particulate lead sulfide resulting from acid phosphatase activity occurred at the periphery. Both reaction products were present only when sections were incubated in complete media for both enzymes.

### Controls

Although endogenous peroxidase activity has been demonstrated in rat parotid acinar cells (18), this activity is readily inhibited by glutaraldehyde (16). In the present experiment no peroxidase reaction product could be demonstrated in sections from glands that had not received HRP but were fixed and incubated in parallel with experimental glands (Fig. 20). Additionally, no reaction product could be detected in glands that had received HRP but were incubated without  $H_2O_2$ .

Small Golgi-associated vesicles that appeared to be more electron dense than adjacent Golgi saccules were noted in many micrographs (Figs. 1, 7, 11, 14, and 20). Close examination of these vesicles in sections from both control and experi-

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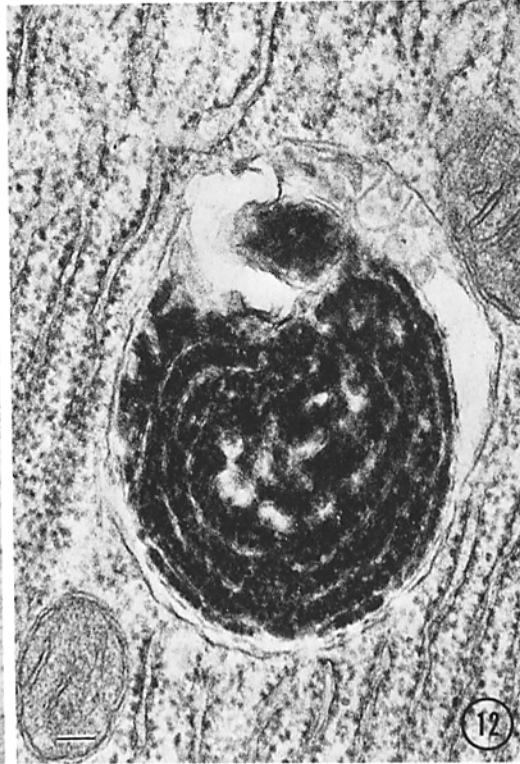
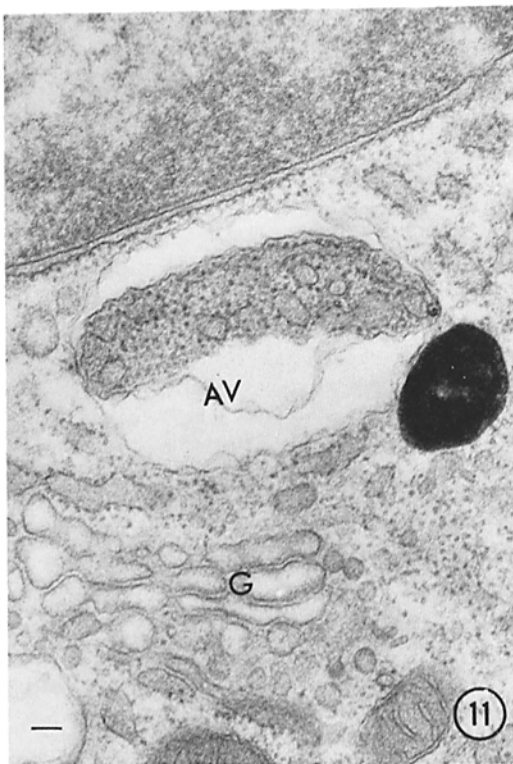
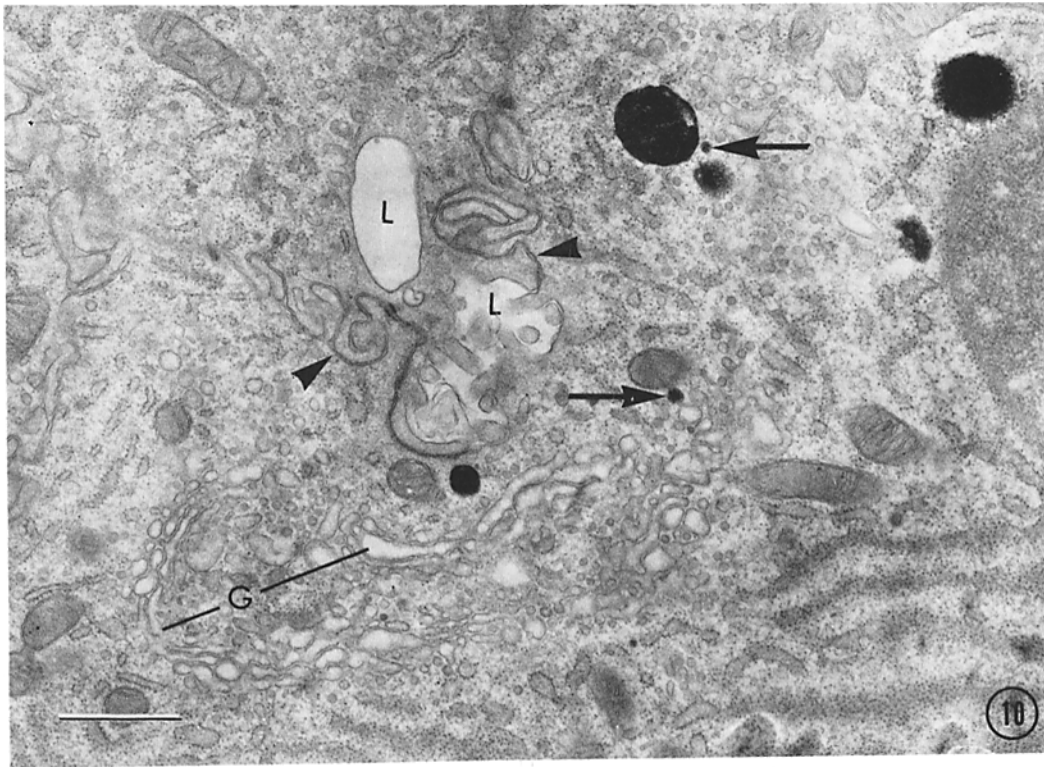
FIGURES 10–14 Figs. 10–14 are from Sprague-Dawley rats given isoproterenol after receiving HRP for 1 h.

FIGURE 10 1 h after isoproterenol, the acinar cells are free of secretory granules, and the lumen (L) has nearly returned to its resting size. HRP reaction product is localized in vesicles (arrows) and dense bodies in the apical portion of the cell. The lumen (L), Golgi saccules (G), and intercellular spaces (arrowheads) are free of reaction product. Bar, 1  $\mu$ m.  $\times$  17,600.

FIGURE 11 1 h after isoproterenol, a dense body adjacent to an isoproterenol-induced autophagic vacuole (AV) contains reaction product. Golgi saccules (G) are free of reaction product. Bar, 0.1  $\mu$ m.  $\times$  40,500.

FIGURE 12 3 h after isoproterenol, HRP reaction product is localized in an isoproterenol-induced autophagic vacuole. Bar, 0.1  $\mu$ m.  $\times$  50,000.





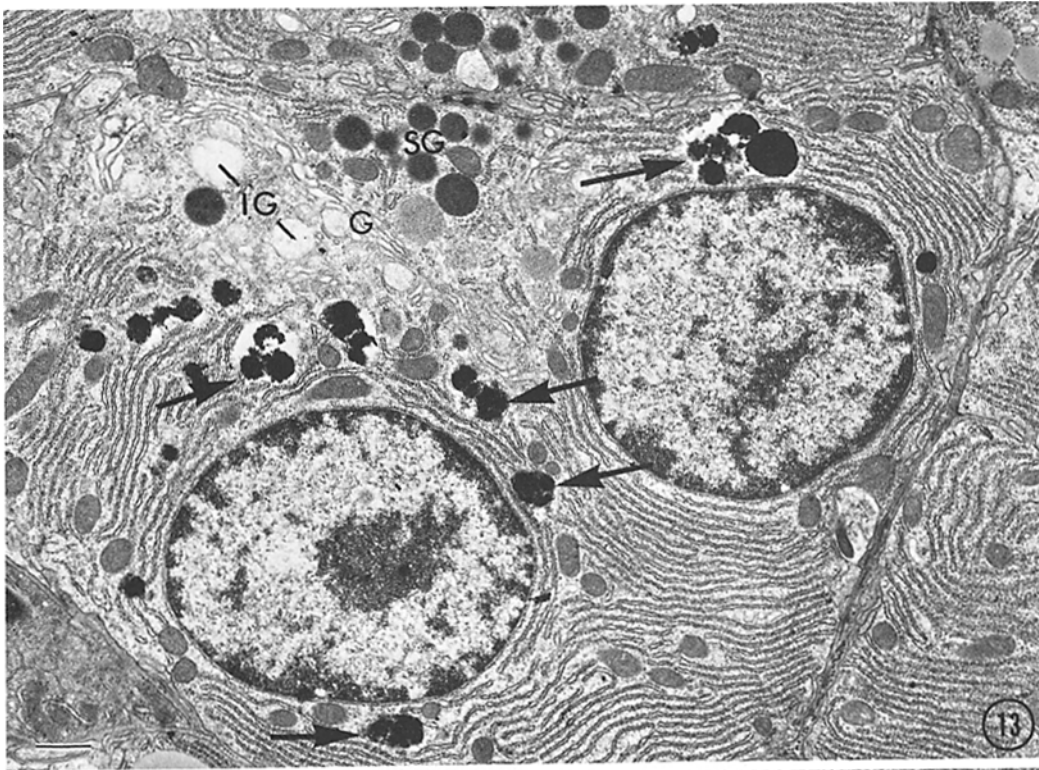


FIGURE 13 7 h after isoproterenol, secretory granules (SG) are beginning to reaccumulate in the apical cytoplasm. HRP reaction product is localized to lysosomes (arrows) in the basal and perinuclear regions of the cell. Golgi saccules (G) and mature (SG) and immature (IG) secretory granules are free of reaction product. Bar, 1  $\mu\text{m}$ .  $\times 7,300$ .

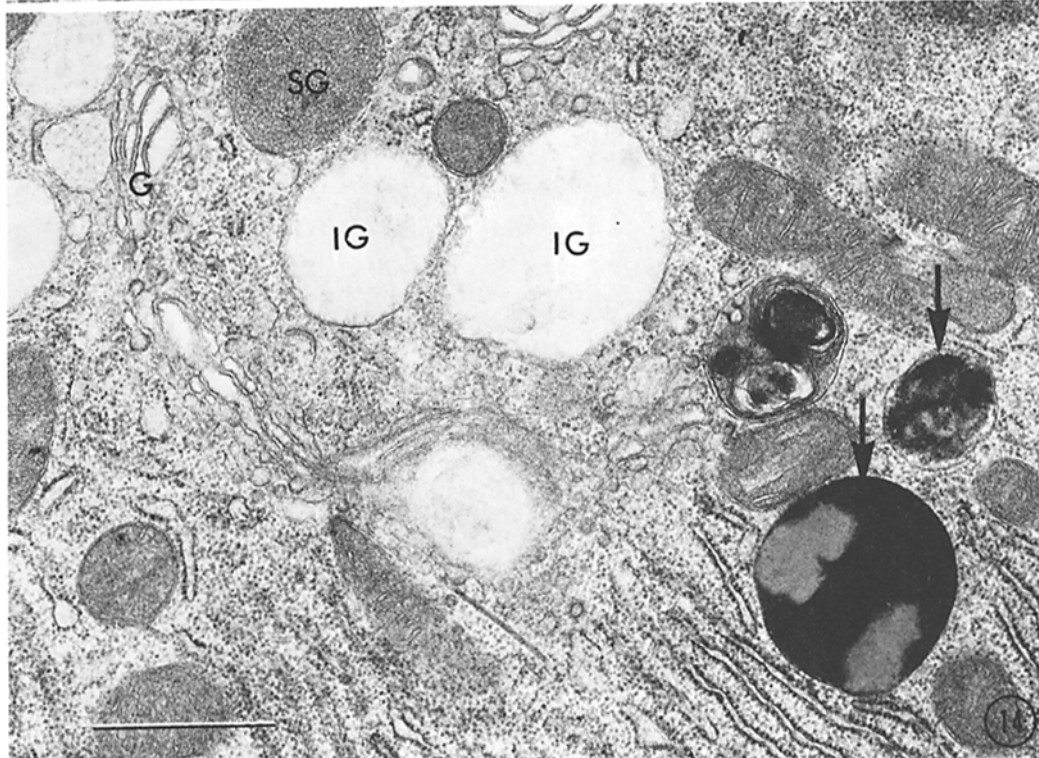


FIGURE 14 7 h after isoproterenol, HRP reaction product is localized to a residual body and lysosomes (arrows). All other organelles including the Golgi saccules (G), immature (IG) and mature (SG) secretory granules are free of reaction product. Bar, 1  $\mu\text{m}$ .  $\times 24,000$ .



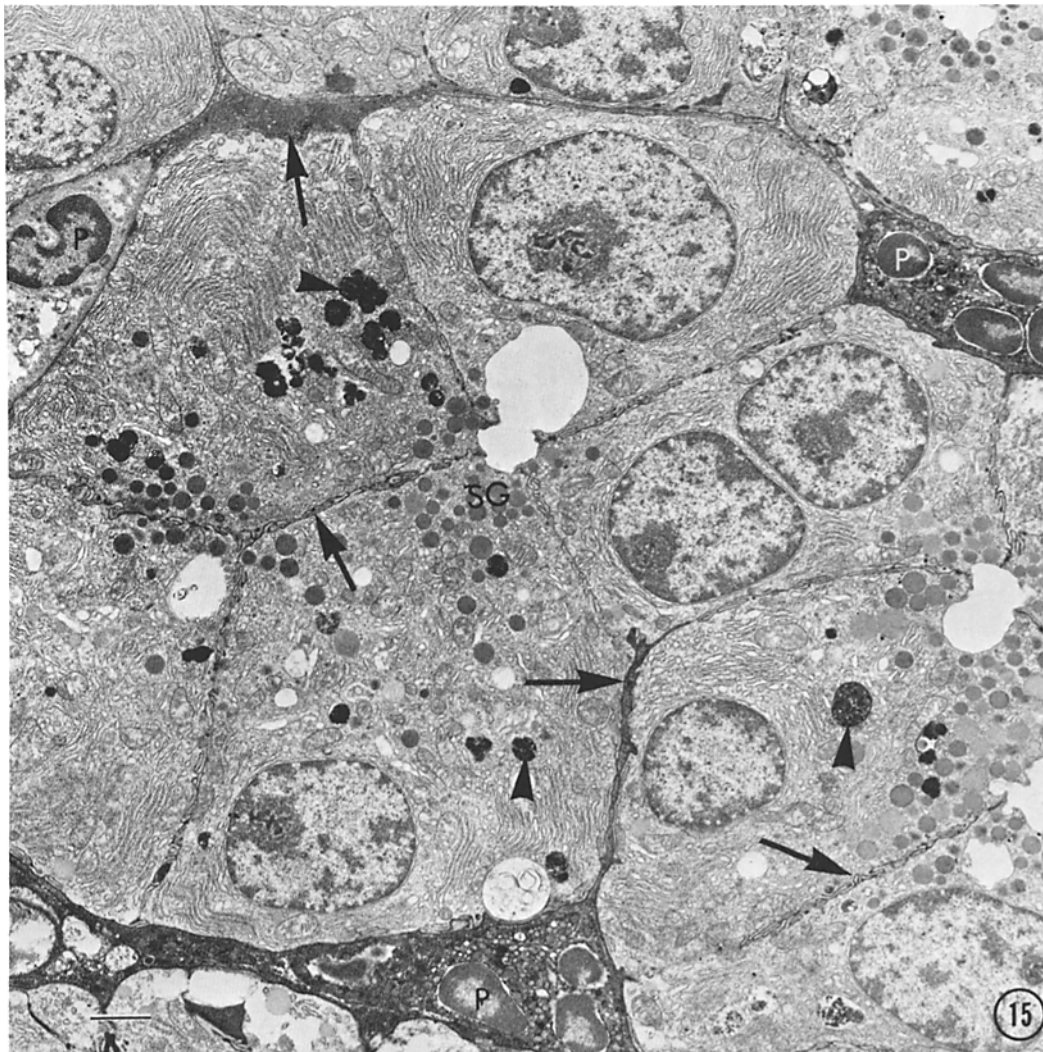


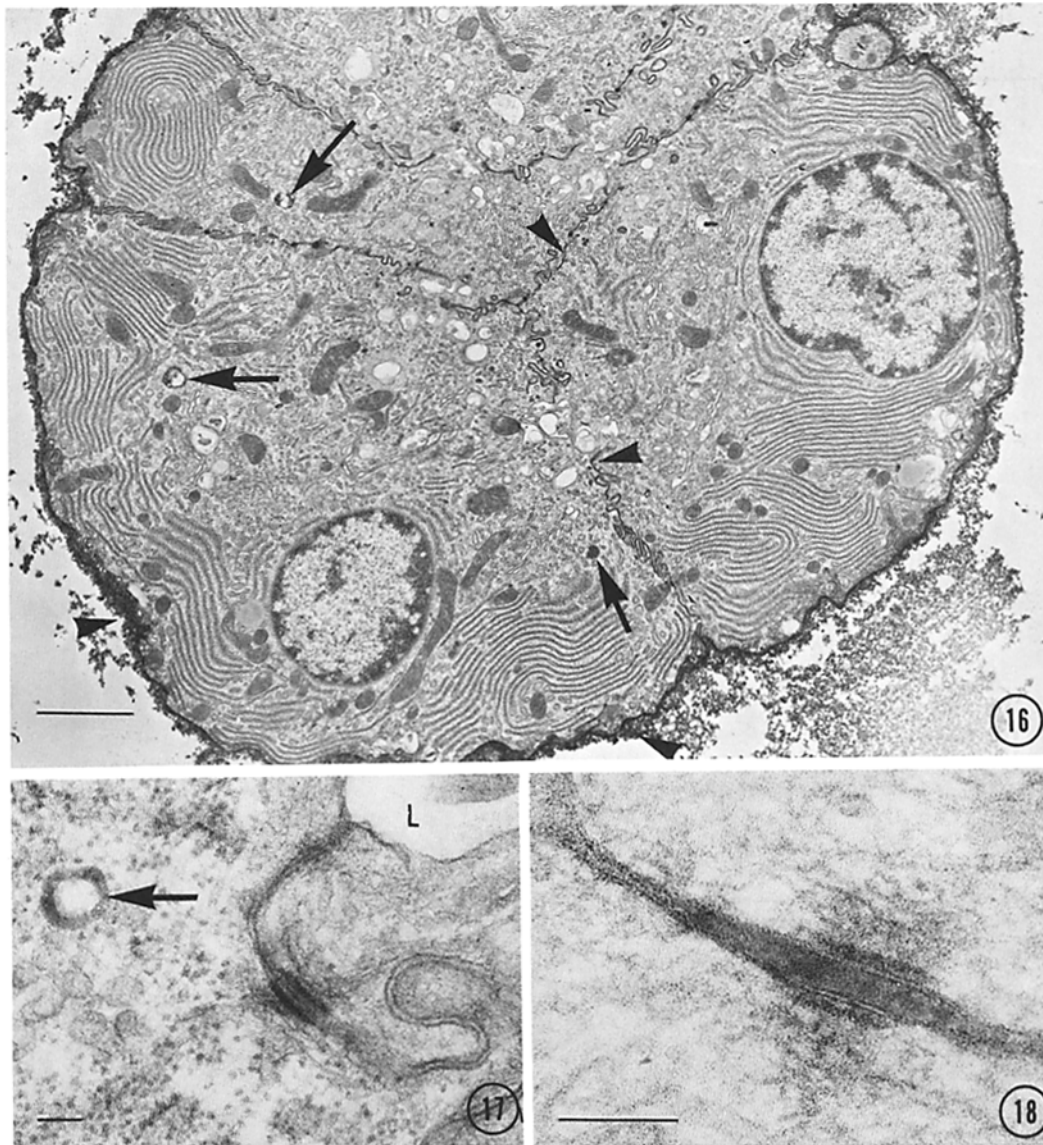
FIGURE 15 From a Sprague-Dawley rat given isoproterenol 1 h before receiving HRP for 1 h. At 6.5 h after isoproterenol, secretory granules (SG) are reaccumulating in the apical cytoplasm. In addition to the intracellular localization of reaction product in lysosomes (arrowheads), reaction product is present in the lateral and basal intercellular spaces (arrows). Portions of three polymorphonuclear leukocytes (P) may also be seen. Bar, 1  $\mu$ m.  $\times 10,000$ .

mental glands revealed that this increased density was due to the presence of a fuzzy coat on the vesicle surface.

#### DISCUSSION

The results of this investigation suggest that in both resting and isoproterenol-stimulated rat parotid acinar cells some membrane is recovered from the luminal surface by endocytosis of small ring- or "c"-shaped vesicles. The use of luminally

administered HRP to mark these vesicles revealed that the vesicles appear to move toward the base of the cell where they fuse with lysosomes and are presumably degraded. At no time was HRP reaction product localized in any organelle associated with secretory granule formation. Additionally, the localization of HRP reaction product in the intercellular junctions, lateral and basal intercellular spaces when isoproterenol preceded HRP suggests that changes in the permeability of the junctional complexes had occurred.



FIGURES 16-18 are from Wistar-Furth rats given isoproterenol 1 h before receiving HRP for 1 h.

FIGURE 16 At 2 h after isoproterenol, the cells are devoid of secretory granules. Intracellularly, reaction product is present in lysosomes (arrows); however, the majority of the HRP reaction product is present in the lateral and basal intercellular spaces (arrowheads). No polymorphonuclear leukocytes are present. Bar, 2  $\mu\text{m}$ .  $\times 6,000$ .

FIGURE 17 Reaction product is localized in a ring-shaped vesicle (arrow) (L, lumen). Bar, 0.1  $\mu\text{m}$ .  $\times 60,000$ .

FIGURE 18 HRP reaction product is present in the junction as well as the lateral intercellular space. Bar, 0.1  $\mu\text{m}$ .  $\times 160,000$ .

Biochemical studies on the pancreas (27, 28, 29) and parotid gland (44, 38) have suggested that the rate of *de novo* membrane synthesis is not adequate to provide sufficient membrane for

new secretory granules, and that the turnover rate is much slower for membrane proteins than for secretory proteins, thus indicating that considerable reutilization of membrane may occur dur-

ing secretory granule formation. The results of the current investigation would suggest that, in

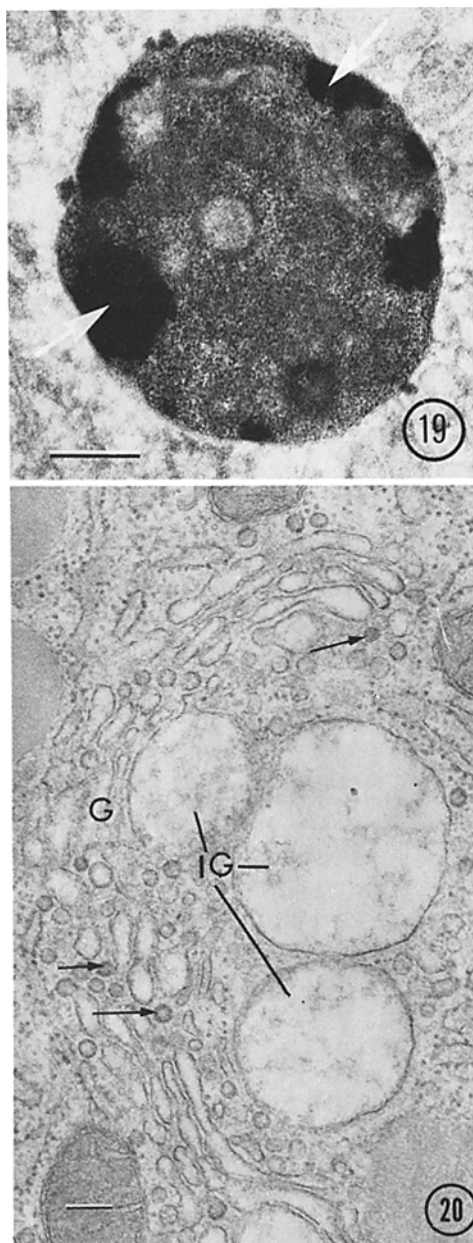


FIGURE 19 From a Sprague-Dawley rat given isoproterenol after receiving HRP for 1 h. The animal was sacrificed 3 h after isoproterenol. After incubation for peroxidase activity, the tissue was incubated for acid phosphatase activity. Both the dense reaction product of acid phosphatase (arrows) and the more flocculent HRP reaction product may be seen in this lysosome. Bar, 0.1  $\mu\text{m}$ .  $\times 125,000$ .

rat parotid acinar cells, at least a portion of the membrane that is recovered from the luminal surface is not reutilized directly but rather is degraded by lysosomes. The components of the degraded membrane might then be available for reuse in the synthesis of new membrane.

Our observations are in agreement with other studies on the uptake and fate of exogenous tracers. The uptake of exogenous tracers in endocytic vesicles and the eventual delivery of the tracer to lysosomes have been shown in a wide variety of cell types including parotid acinar cells (23), pancreatic acinar cells (13), adrenal medulla (1, 21), cerebral cortex (42), choroid plexus (3), vas deferens (9), cultured glial and heart cells (45), cultured neurons (41, 20), cultured anterior pituitary cells (43), and macrophages (39). However, studies of HRP uptake in small intestine (5), seminal vesicles (25, 26) and in somatotrophs of the anterior pituitary (32) have shown the presence of reaction product in Golgi saccules and, in some instances, secretory granules. The reasons for these discrepancies in localization of HRP reaction product are unclear. Mata (25) has suggested that the presence of HRP reaction product in Golgi saccules is dependent upon sufficient tracer being taken up by the cells. In the present study, especially at the early time intervals in resting glands, the entire lumen was frequently filled with HRP reaction product, and all of the forming vesicles contained reaction product. In both resting and stimulated cells, enough tracer was taken up to heavily label secondary lysosomes and autophagic vacuoles. Although a few HRP-containing vesicles may have fused with Golgi saccules or secretory granules, delivering undetectable amounts of HRP, the percentage of vesicles involved in such events must be extremely low. It therefore appears that in our experiments the acinar cells received the maximum possible amount of tracer. Prolonged administration of HRP might have provided for additional uptake of tracer, but this could also cause degenerative changes in the acinar cells. More likely, the dis-

FIGURE 20 From a Sprague-Dawley rat which was given isoproterenol 7 h before sacrifice but did not receive HRP. There is no reaction product present in any organelle including Golgi saccules (G) and immature secretory granules (IG). Some small Golgi-associated vesicles (arrows) appear denser than the saccules due to the presence of a membrane coat. Bar, 0.5  $\mu\text{m}$ .  $\times 33,000$ .

crepancies in localization are related to basic metabolic differences between the various cell types studied. It may be that in certain cells endocytosed membrane is recycled and utilized directly in the formation of new secretory granules while in other cells the membrane is first degraded by lysosomes. Recycling of synaptic vesicle membrane has been demonstrated in frog neuromuscular junctions (22, 19). The finding of Pelletier (32), studying the anterior pituitary in which somatotrophs but not mammatrophs have HRP reaction product in Golgi saccules, would indicate that, even in the same tissue, differences in membrane reutilization may exist among the various cell types. The resolution of these differences may have to await further studies similar to those of Pelletier in which the two pathways can be studied under identical experimental conditions.

One major problem in using HRP for this type of study is that HRP is a soluble-phase marker and does not mark the membrane itself (39). Therefore, it is possible that the HRP-containing vesicles fuse with lysosomes only long enough to empty their contents. Once empty, the vesicle ghosts may then enter into secretory granule formation. However, it is hard to envision a situation in which no HRP would be carried with the vesicles.

It is also possible that the presence of HRP in the lumen induced the formation of endocytic vesicles, but previous reports on parotid (2, 17, 36), submaxillary (4), and pancreatic acinar cells (13) have shown the presence of apical vesicles. Additionally, in this study unreactive vesicles with a similar morphology were present in the acinar cells. It therefore seems more likely that the vesicles were not induced by the tracer but rather do represent retrieval of luminal membrane and are only passively marked by HRP.

An unexpected finding of this study was the presence of HRP reaction product in the junctional complexes and in the lateral and basal intercellular spaces when isoproterenol was administered before HRP. It appears that after stimulated secretion the junctional complexes become leaky, at least to low-molecular-weight proteins such as HRP (mol wt, 40,000). In pilocarpine-stimulated rat exocrine pancreas, ferritin (mol wt, 250,000) did not appear to be able to penetrate the junctional complexes (13). It is possible that this increased permeability of junctional complexes is a normal process of secretion and that isoproterenol stimulation merely accen-

tuates this process. Garrett and Parsons (11) have shown that, in rabbits given close arterial injections of HRP, the junctional complexes between submandibular duct cells are permeable to the tracer. When the HRP is given retrograde (12), the tracer appears to pass between not only the duct cells but also the acinar cells. However, in that study the HRP was administered at a relatively high pressure, 60–70 mm of mercury, which may have forced the junctions open. In the present study, in which the HRP was administered at a pressure of approx. 15–16 mm of mercury, no leakage of tracer occurred, except in stimulated glands. Previous work on ion permeability has suggested that certain epithelial tight junctions may be leaky (10), and a more recent study on gallbladder epithelium has shown that approx. 8–15% of the junctions are normally permeable to peroxidase and colloidal lanthanum (15). Additionally, anesthetic ether will also make the junctional complexes of pulmonary epithelium permeable to HRP (34). This evidence suggests that under certain conditions the epithelial junctional complexes may be leaky. If this increased permeability is a normal process of secretion, this could provide a mechanism whereby substances such as immunoglobulins or plasma proteins might reach the saliva directly. An alternative to the HRP passing between the acinar cells would be intracellular transport and exocytosis at the basal or lateral surfaces as has been shown in intestinal epithelium (35) and seminal vesicle cells (25, 26). In the present study, the presence of HRP reaction product in the junctional complexes and the fact that neither the HRP-containing vesicles nor lysosomes were found associated with the lateral or basal membranes suggest that the tracer was not transported through the acinar cells.

The authors would like to thank Mrs. Betty Ho, Mr. Robert Kelly, and Miss Regina Auth for their excellent technical assistance and Mr. Steve Coriell and Mr. Steve Floyd for preparation of the micrographs. We are also indebted to Dr. Murray Robinovitch for demonstrating the technique of parotid duct cannulation.

*Received for publication 6 June 1977, and in revised form 26 September 1977.*

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