

SECRETION AND ENDOCYTOSIS IN INSULIN-STIMULATED RAT ADRENAL MEDULLA CELLS

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ABSTRACT

Insulin was used to deplete the adrenalin stores of rat adrenal medulla cells. Release of secretion was observed to occur by exocytosis. In addition, during the stages of massive release of secretory granules, the insulin-treated preparations showed greatly enhanced endocytic uptake of horseradish peroxidase. The tracer was taken up within vesicles, tubules, multivesicular bodies, and dense bodies. From acid phosphatase studies and from previous work it appears that many of the structures in which peroxidase accumulates are lysosomes or are destined to fuse with lysosomes. Subsequent to the period of intense exocytosis and endocytosis, there is a transient accumulation of lipid droplets in the adrenalin cells. The cells then regranulate, with new granules forming near the Golgi region. These results suggest that under the conditions used, much of the membrane that initially surrounds secretory granules is degraded after release of the granules.

INTRODUCTION

It has been firmly established in a number of secretory systems that exocytosis is the means by which the secretory product is released from the cell (see, e.g., references 2, 10, 22, 46, 54, 67, 69). Exocytosis is the process of fusion of the membrane delimiting a secretory granule with the plasma membrane. This results in addition to the cell surface of new membrane which in some sense must be retrieved if the surface area is to remain constant. Endocytosis, the internalization of vesicles and tubules that bud from the plasma membrane, has recently been suggested by a number of groups as the mechanism by which the cell compensates for the increase in surface (2, 18, 30, 35, 38, 54). But there is no general agreement as to what happens to the internalized pieces of membrane. Are they reutilized as membranes in the packaging of new secretory granules, or are they degraded by the cell either to membrane macromolecules or to smaller molecules? Palade (67) has hypothesized that intact patches of

internalized membranes may be reutilized by the cell in packaging new zymogen granules while Fawcett (24) proposed degradation of these membranes. From biochemical evidence, Meldolesi et al. (55) have recently suggested that retrieved membrane is degraded at most to the point of membrane macromolecules, as total resynthesis of microsomal membranes is not observed during the secretory cycle in guinea pig pancreatic slices. However, Amsterdam et al. (3) report that in rat parotid gland new secretory granules are packaged in membrane whose proteins are newly synthesized from amino acids (see also 80). This might suggest that endocytosed membranes are indeed broken down to small molecules.

Exocytosis has been implicated as the means of secretion in the adrenal medulla on the basis of biochemical evidence in the medulla and in adrenergic nerve terminals (50, 52, 73). Morphological evidence for exocytosis is readily obtained in studies of golden hamster (9, 28, 29) and oc-

casional comparable configurations have been reported in the rat (14). We (34, 35, 37) and others (9, 18, 29) have recently observed endocytosis in the adrenal medulla and have suspected that it serves as a compensatory mechanism for exocytosis. However, further experimental verification is necessary to prove this.

The present report concerns the rat adrenal medulla during and after intensive secretory activity induced by insulin. We have observed exocytosis of secretory material and have studied what we take to be compensatory endocytosis, demonstrable by the use of horseradish peroxidase. Our observations strongly suggest that much of the membrane internalized by endocytosis is degraded in lysosomes. Preliminary reports of our findings have been published (1, 34, 35, 43).

MATERIALS AND METHODS

Male Sprague-Dawley rats weighing approximately 200 g were obtained from Carworth Farms in Rockland County, New York. Rats which were to be administered insulin were not fed for 16–18 h in advance to achieve a greater insulin effect; water was allowed ad libitum. All other rats, including controls for the insulin experiments, were allowed food and water until the time of the experiment. All experiments were done at least two times, with key time points being studied in several independent experiments.

Insulin

Unanesthetized rats were injected subcutaneously with convulsive doses (12–16 IU) of insulin (regular Iletin, Eli Lilly and Co., Indianapolis, Ind.) and sacrificed at intervals from 2 h to 7 days thereafter. To prevent the convulsions that start at 2–3 h after insulin injection, 3 ml of a 5% dextrose solution (32) were administered intraperitoneally starting at 2 h after insulin; this was repeated periodically thereafter at about 1 h intervals until about 10 h after insulin. Animals permitted to survive more than a day after insulin treatment were found to be extremely aggressive and difficult to handle.

Peroxidase

Animals for peroxidase experiments usually received 0.75 ml of a 4% solution of horseradish peroxidase (Sigma Chemical Co., St. Louis, Mo., type II) in isotonic saline injected into a tail vein $\frac{1}{2}$ h before sacrifice. In those experiments in which peroxidase was left in the animals for a longer time (1–3 h), only 0.33–0.50 ml of the 4% solution was

used since some animals tolerated higher doses poorly.

Anesthesia

Peroxidase injection and sacrifice of most animals were preceded by intraperitoneal injection of 3 ml of a 5.5 mg/ml solution (6) of α -chloralose (Sigma Chemical Co.). Animals injected with insulin 24 h or more before sacrifice were anesthetized with ether.

Fixation and Incubation

The animals were sacrificed by perfusion through the aorta by the methods of Holtzman and Dominitz (37). Cold Karnovsky's glutaraldehyde-formaldehyde fixative (48) in 0.1 M cacodylate buffer at pH 7.4 with 0.25% CaCl₂ added or 3% glutaraldehyde (71) in the same buffer were used. Perfusion lasted about 10 min; the adrenal glands were subsequently excised, cut in half to expose the medullae, and immersed in fixative on ice for 90 min. At this time, the medullae were trimmed from the cortices and stored overnight on ice in 0.1 M cacodylate buffer with 7% sucrose at pH 7.4.

The next day, tissues used to demonstrate enzyme activity were cut at a setting of 25 μ m on a Smith-Farquhar tissue chopper. Enzyme activities were demonstrated by the following methods: peroxidase using the 3,3'-diaminobenzidine (DAB) and H₂O₂ method of Karnovsky and coworkers (27, 49), and acid phosphatase using the Gomori method with 5'-cytidylic acid as substrate (60, 65).

For light microscope visualization of acid phosphatase reaction product, sections were immersed briefly in a dilute solution of ammonium sulfide, rinsed in distilled water, and mounted on glass slides in glycerogel. Peroxidase-incubated sections were examined without special visualization.

Incubation Controls

For peroxidase controls, medullae from animals injected only with saline were incubated in full peroxidase medium. Substrate- and peroxide-free media were also used. For acid phosphatase controls, sections were incubated in substrate-free media and in full media to which 0.01 M NaF was added to inhibit enzyme activity. Insulin injection had no detectable effect on tissue behavior in these control procedures.

Preparation for Electron Microscopy

Sections were postfixed in 1% OsO₄ in cacodylate or veronal acetate buffer for 60–90 min. Subsequently, the tissue was rinsed in sucrose, immersed en bloc in a uranyl acetate soak (26) for 30 min at room temperature, rinsed again in sucrose on ice, dehydrated in a

graded series of ethanol and propylene oxide, and embedded in Epon 812 (51).

Tissue for morphological study only was fixed by perfusion of Karnovsky's fluid or glutaraldehyde, then immersed in fixative as small pieces, rinsed with 0.1 M cacodylate buffer with 7% sucrose, post-fixed in cacodylate- or veronal acetate-buffered 1% OsO₄, uranyl soaked, dehydrated in ethanol and propylene oxide, and embedded in Epon.

Microscopy

Thick Epon sections of all material processed for the electron microscope were first examined by phase-contrast microscopy. Thin sections for the electron microscope were cut with Dupont diamond knives (E. I. duPont de Nemours and Co., Inc., Wilmington, Del.) on a Porter Blum MT-2 microtome (Ivan Sorvall, Inc., Norwalk, Conn.) set to give silver-gray to pale gold sections. These were mounted on naked grids and stained with uranyl acetate in 50% ethanol (79) and/or lead citrate (78). Sections were examined with a RCA EMU 3F

microscope and photographs taken at initial magnifications of 2,000–20,000.

Lipid Stains

A series of light microscope experiments were done in which control and insulin-injected animals were perfused with formol calcium fixative, and medullae were kept immersed in this fixative on ice overnight. The following day, 10–15- μ m frozen sections were cut on a freezing microtome and these sections were stained with either Oil Red O or Sudan Black, by the triethyl phosphate method of Gomori (68). Stained sections were mounted on glass slides in glycerogel for examination by light microscopy.

RESULTS

Phase-Contrast Microscope Observations

Phase-contrast microscopy of Epon sections shows few changes of interest in the cells after

All figures show adrenalin cells of the medulla except as noted. Figs. 3, 4, and 13–17 are from peroxidase-incubated tissue from animals injected with peroxidase. The cells in Figs. 6, 20, and 21 are from animals injected with peroxidase but not incubated for peroxidase activity. The rest of the figures are from animals never exposed to peroxidase. Figs. 1–5 are light micrographs. Bar length is 5 μ m. The remainder of the figures are electron micrographs of aldehyde-fixed, OsO₄-postfixed tissue usually stained en bloc with uranyl acetate and in thin sections with uranyl and lead. Bar length is 0.5 μ m.

FIGURE 1 Phase-contrast micrograph of an Epon thick section of the adrenal medulla of an animal treated with insulin 50 h before fixation. The arrows indicate noradrenalin cells; these contain their characteristic osmiophilic granules. Adrenalin cells are seen at *A* and a blood vessel at *B*. $\times 1,200$.

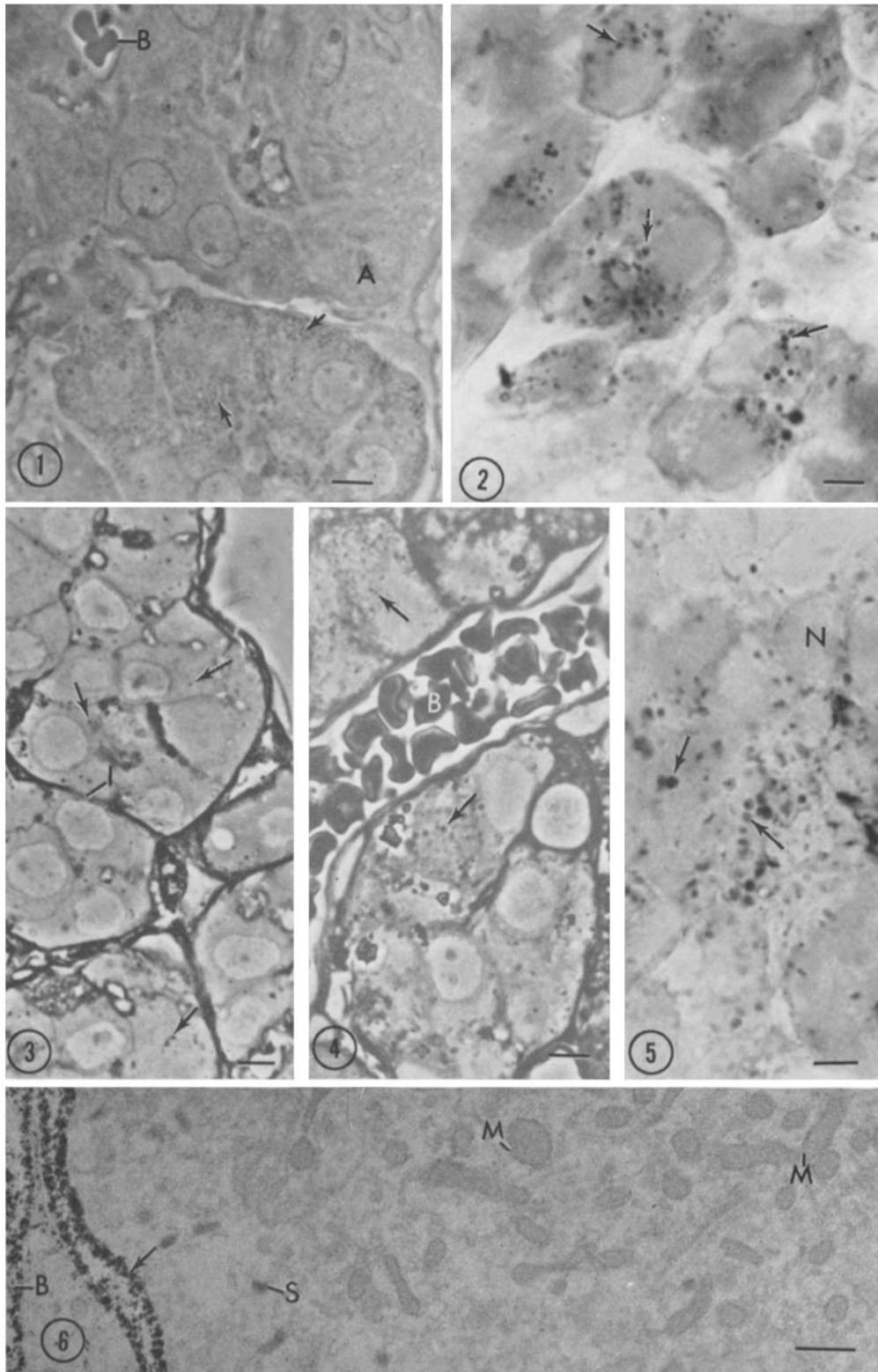
FIGURE 2 Light micrograph of an acid phosphatase-incubated chopper section from an animal treated with insulin 24 h before fixation. Reaction product is seen in numerous intracellular bodies (arrows), the lysosomes. $\times 1,200$.

FIGURE 3 Phase-contrast micrograph of an Epon thick section prepared from a peroxidase-incubated chopper section from an animal not injected with insulin, but given peroxidase 30 min before fixation. Reaction product is seen in small intracellular bodies (arrows) and in intercellular spaces (*I*). $\times 1,200$.

FIGURE 4 Phase-contrast micrograph of a preparation comparable with the one shown in Fig. 3, except that the animal received insulin 4 h before fixation. Reaction product is seen in numerous intracellular bodies (arrows). *B* designates a blood vessel. $\times 1,200$.

FIGURE 5 Light micrograph of an Oil Red O-stained frozen section from an animal treated with insulin 24 h before fixation. *N* indicates nuclei. A few of the many stained lipid globules are indicated by arrows. $\times 1,200$.

FIGURE 6 Electron micrograph of a cell from a preparation injected with insulin 4 h before fixation and with peroxidase $\frac{1}{2}$ h before fixation. 2% lanthanum (11, 70) was included in all fixatives and rinses. As expected for such preparations (cf. Figs. 8, 14), companion portions of adrenal medulla from this animal were shown to contain much intracellular peroxidase. To facilitate detection of lanthanum, the thin section was not stained with heavy metals. Lanthanum is readily visible at the cell surface (arrow), and at the borders of a blood vessel (*B*), but is only rarely seen in structures within the cytoplasm. *M* indicates mitochondria and *S*, some of the few remaining secretory granules. The granules have an intrinsic density but they do not seem to contain lanthanum. $\times 18,000$.



insulin treatment. During the first 24 h the adrenalin cells become somewhat irregular in shape and lipid droplets (see below) accumulate. By 2–3 days after insulin (Fig. 1) the cells again look substantially normal. The most interesting finding in the phase-contrast microscope, confirmed by electron microscopy, is that the noradrenalin cells, identifiable by their content of osmiophilic granules (14), do not undergo appreciable degranulation (Fig. 1).

Light Microscope Cytochemistry

In preparations (“controls”) from peroxidase-injected animals not injected with insulin, peroxidase is demonstrable in the capillaries and intercellular spaces at the earliest time studied, 30 min after injection (Fig. 3). Small peroxidase-containing bodies are also visible in the medulla cells. In animals exposed to peroxidase 4–9 h after insulin, a great number of peroxidase-containing bodies are seen in the medulla cells, and some of the bodies are larger than those seen in the cells of controls (Fig. 4). No peroxidase activity is demonstrable in the medulla cells of saline-injected animals, and no reaction product is seen in DAB- and H₂O₂-free controls.

In peroxidase-injected animals, reaction product is also found in spherical bodies in stellate-shaped cells scattered through the medulla. They are probably phagocytes of the histiocyte type (37).

In acid phosphatase preparations, adrenalin and noradrenalin cells of control animals and animals sacrificed 4–24 h after insulin, contain reaction product in numerous cytoplasmic bodies (Fig. 2). In substrate-free or fluoride-inhibited controls, no reaction product is seen. The number and distribution of acid phosphatase-containing bodies does not change dramatically in the insulin-treated preparations, although sometimes there seem to be a few more large acid phosphatase-containing bodies in insulin-treated cells than in controls.

In Oil Red O- or Sudan Black-stained prepara-

tions, the cortex, as expected, always contains many obvious stained lipid globules, but in medullae from control animals very few stained globules are evident. In cells of medullae from animals sacrificed 24–48 h after insulin, many globules are stained by Sudan Black or Oil Red O (Fig. 5).

Electron Microscope Observations:

Overall Changes

Our attention has been focused on the adrenalin-producing cells which are easily distinguished from noradrenalin-producing cells on the basis of the morphology of the secretory granules after aldehyde and osmium tetroxide fixation (see, e.g., references 8, 9, 14, 19, 37).

During the first 24 h after insulin treatment, heavy degranulation of the adrenalin cells is apparent. At about 24 h, there is an accumulation of lipid bodies and cell borders remain irregular. From 24 h to about 4 days, regranulation occurs until at about 4 days the cells appear substantially normal.

Exocytosis

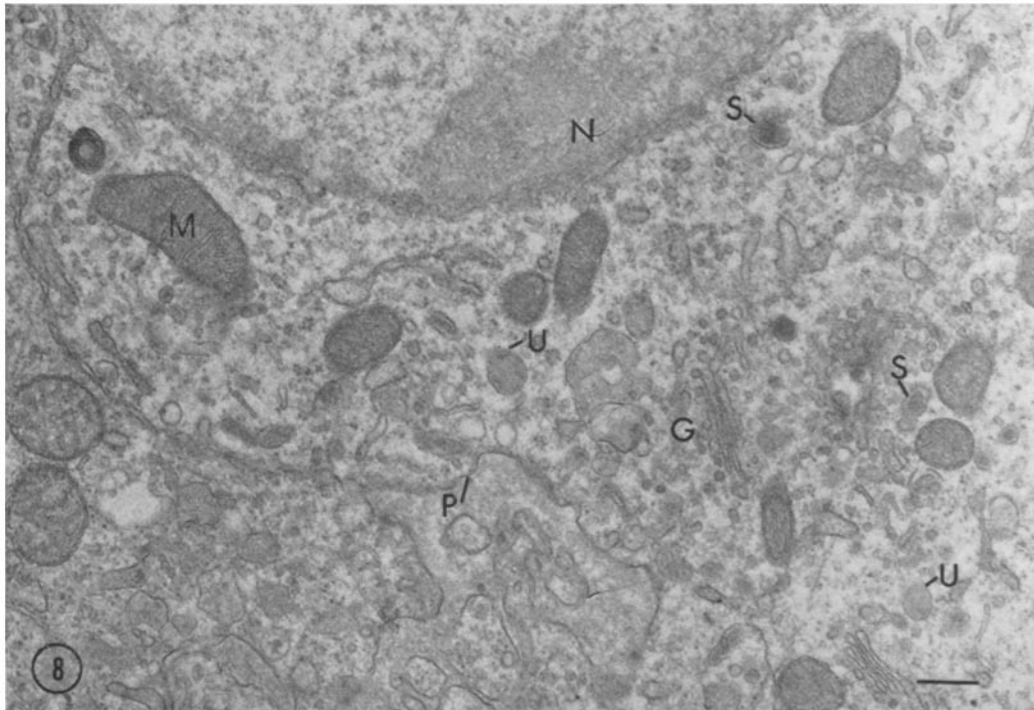
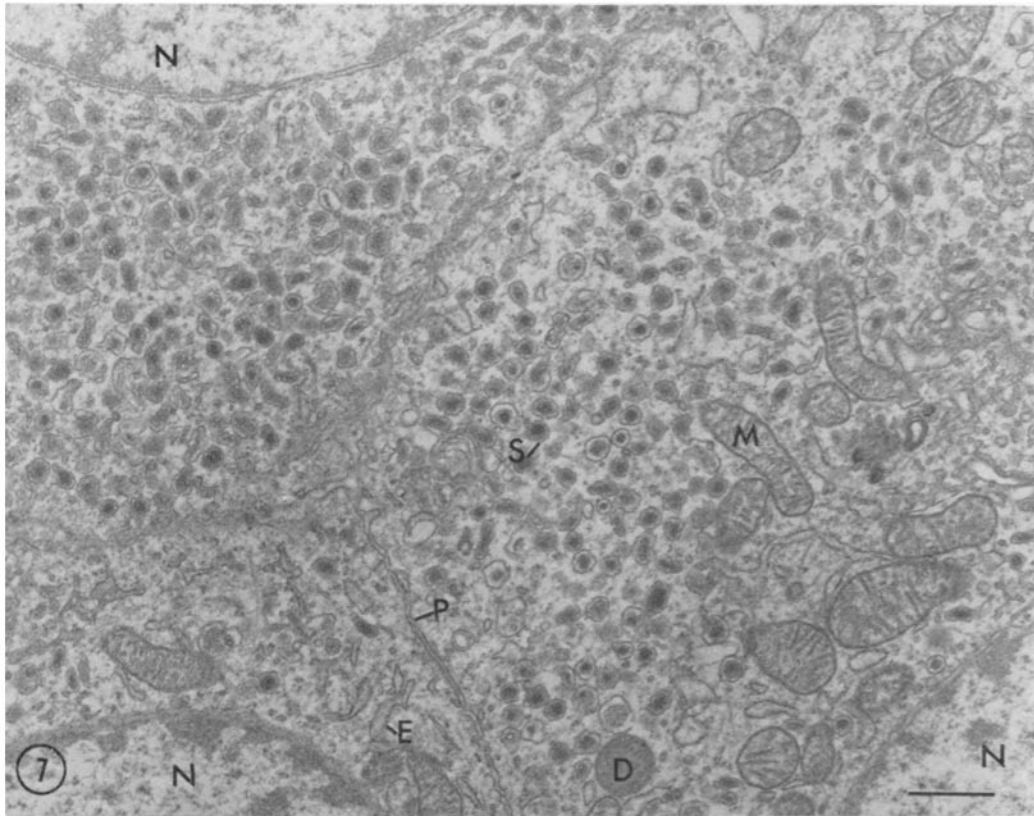
Control cells from medullae of animals not injected with insulin contain large numbers of secretory granules throughout the cytoplasm (Fig. 7) (cf. references 9, 14, 17, 19, 20, 37). The granules are round or oval and most are about 0.1–0.3 μm in diameter. The granules are bounded by a trilaminar membrane and contain a core of opaque, particulate matter.

The most conspicuous change in these cells after 4 or 9 h of insulin treatment is the lack of secretory granules in many of the adrenalin-producing cells (Fig. 8); this is as expected, since Hokfelt (32) has found by biochemical assay that, by 9 h after insulin, only a small percentage of adrenalin remains in the medulla (see also references 8, 14).

Exocytosis of secretory material is readily observed throughout the first few days after insulin.

FIGURE 7 Portions of cells from a control animal. Nuclei are indicated by *N*. Many secretory granules (*S*) are seen in the cytoplasm; *M* indicates mitochondria, *D* a dense body, *P* plasma membrane, and *E*, endoplasmic reticulum. The Golgi apparatus of a similar preparation is seen in Fig. 9. $\times 23,000$.

FIGURE 8 Part of a cell from an animal injected with insulin 4 h before fixation. Only a very few secretory granules remain in the cytoplasm (*S*). *N* indicates the nucleus, *M* mitochondria, and *G* Golgi apparatus. At the cell surface, the plasma membrane shows some foldings or convolutions (*P*). The bodies indicated by *U* are endocytic vacuoles of a type common at this time. $\times 19,000$.



Dense material, similar to the content of secretory granules, is found in the intercellular spaces, and images like the one in Fig. 9 are frequently encountered.

Regranulation

In control cells, rough endoplasmic reticulum is relatively sparse although stacks of parallel cisternae are seen along with individual cisternae (similar to Fig. 12). A well-developed Golgi apparatus is often apparent (Fig. 13).

At 1–2 days after insulin treatment the adrenalin cells start to regranulate. The endoplasmic reticulum is dilated and contains a moderately electron-opaque material (Fig. 11). As in control preparations (see reference 37), new secretory granules arise near the Golgi apparatus (Fig. 10); details of this process are discussed elsewhere (37, 42). By 4–7 days the cells look essentially normal; the endoplasmic reticulum (Fig. 12) is no longer dilated and the granule population has been restored.

Peroxidase Uptake

When cells from animals not injected with insulin are exposed to peroxidase for 30 min, peroxidase is demonstrable in capillaries and intercellular spaces. Within the cells, reaction product is seen in small vesicles, tubules, multivesicular bodies, and dense bodies (Fig. 13). In insulin-depleted preparations studied at 2–9 h after insulin, peroxidase uptake during a 30-min exposure to the tracer is much more extensive.

Reaction product is seen in small vesicles, tubules, irregularly shaped bodies and in a few multivesicular and dense bodies (Fig. 14). While extensive uptake is not seen in every depleted cell at a given time after insulin, the majority of adrenalin cells do contain many bodies with peroxidase. When peroxidase is left in insulin-depleted animals from 1 to 3 h before sacrifice, peroxidase is seen mainly in multivesicular bodies, vacuoles, and dense bodies (Figs. 15, 16). Images suggesting fusions of bodies of the type that sequester peroxidase are quite common during the first day after insulin (e.g., Fig. 18).

That the bodies seen with peroxidase in medulla cells of control and insulin-treated animals are intracellular is strongly suggested by the fact that lanthanum, included in the fixative (35, 38, 70), gains access to very few such bodies, although it does reach the cell surface (Fig. 6).

No obvious differences in morphology are encountered when cells from insulin-injected animals that have not received peroxidase are compared with those that have received peroxidase but have not been incubated for peroxidase activity.

Peroxidase is taken up by cells 1–2 days after insulin treatment, but the intensity of uptake is much more similar to controls than to the earlier stages after insulin. As usual, uptake after 30 min or 3 h of exposure to the tracer is into tubules, vesicles, multivesicular bodies, and dense bodies.

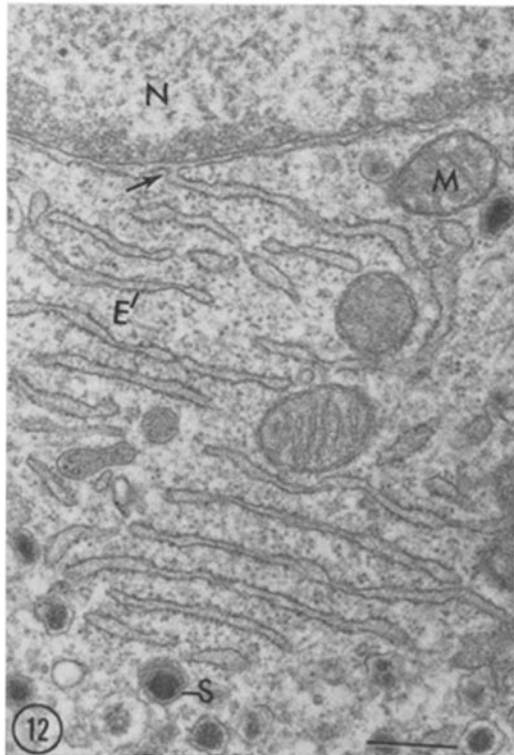
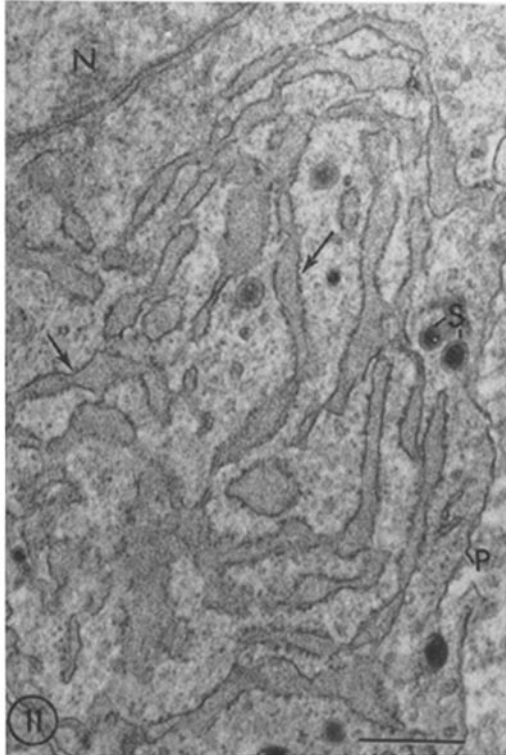
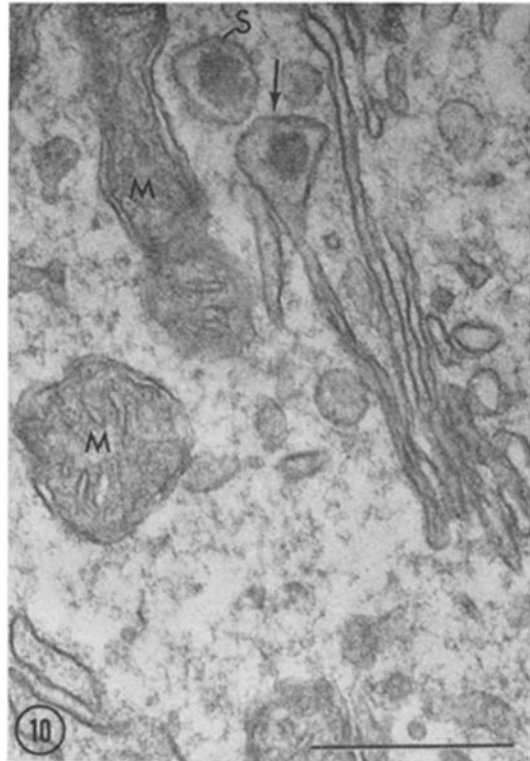
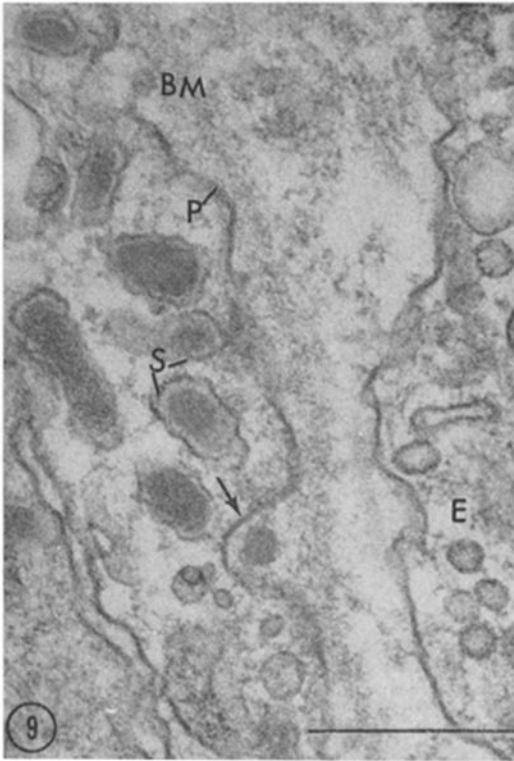
As in our previous work (37) peroxidase-containing vesicles, tubules, and lysosomes often are found near the Golgi apparatus (Figs. 13, 17), but even at the stages of most intensive peroxidase

FIGURE 9 Portion of the cell surface from a preparation 50 h after insulin injection. Secretory granules (*S*) are seen in the cytoplasm near the plasma membrane (*P*). The arrow indicates a secretory granule apparently caught in the process of release from the cell. Basement membrane is present at *BM* and the edge of an endothelial cell at *E*. $\times 57,000$.

FIGURE 10 Part of the Golgi apparatus from a cell 2 days after insulin. The arrow indicates a secretory granule that seems to be forming from an elongate tubule or sac of the Golgi apparatus. Another secretory granule lies nearby (*S*), and mitochondria are seen at *M*. $\times 54,000$.

FIGURE 11 Portion of a cell of a preparation fixed 2 days after insulin injection. The edge of the nucleus is seen at *N*, and the plasma membrane at *P*. The cytoplasm shows many dilated cisternae of rough endoplasmic reticulum containing a moderately electron-opaque material (arrows). A few secretory granules (*S*) are also present. $\times 28,000$.

FIGURE 12 From a preparation fixed 4 days after insulin. The edge of a nucleus is seen at *N*; a mitochondrion is indicated by *M*, and a secretory granule by *S*. The endoplasmic reticulum resembles that seen in controls; the cisternae (*E*) are not dilated but are found in roughly parallel arrays. Connections with the nuclear envelope (arrow) are occasionally observed. $\times 24,000$.



uptake Golgi saccules and secretory granules have not been found to show reaction product.

Lysosomes and Lipid Bodies

As in our previous studies (37), acid phosphatase activity in normal and insulin-injected material is seen in Golgi-associated sacs or tubules, some secretory granules, some of the multivesicular bodies, and especially in many dense bodies (Fig. 21). During the first day after insulin injection, images suggesting transformation of multivesicular bodies and other lysosomes into dense "residual" bodies (16, 22, 33, 60, 61) are especially frequent (Fig. 18). These bodies contain a dense matrix and other material with varying appearance; vesicles, lamellae, patches of dense granular material, and irregular fragments of membrane are frequently encountered. Lipid droplets become quite prominent during the period 1 to 2 days after insulin, and numerous structures intermediate between dense bodies and lipid globules are encountered. For example, sometimes material with the appearance of lipid accumulates as a rim surrounding a dense body (Fig. 19), or as a large globule attached to a dense body. Structures resembling those called "vacuolated dense bodies" by Farquhar (22, 23, 75) are also common (Fig. 20); as in Farquhar's work, these seem to accumulate lipid as relatively small droplets within the dense matrix. As in other conditions of lysosomal breakdown of membranes (e.g., our studies of myelin degeneration, see reference 39), the lipid droplets eventually acquire the featureless appearance shown in Fig. 22. However, as the globules form, portions show a variety of lamellar appearances (Fig. 23) and patches of electron-opaque

material. Lipid droplets are very rare in the control medulla and after 2-3 days after insulin.

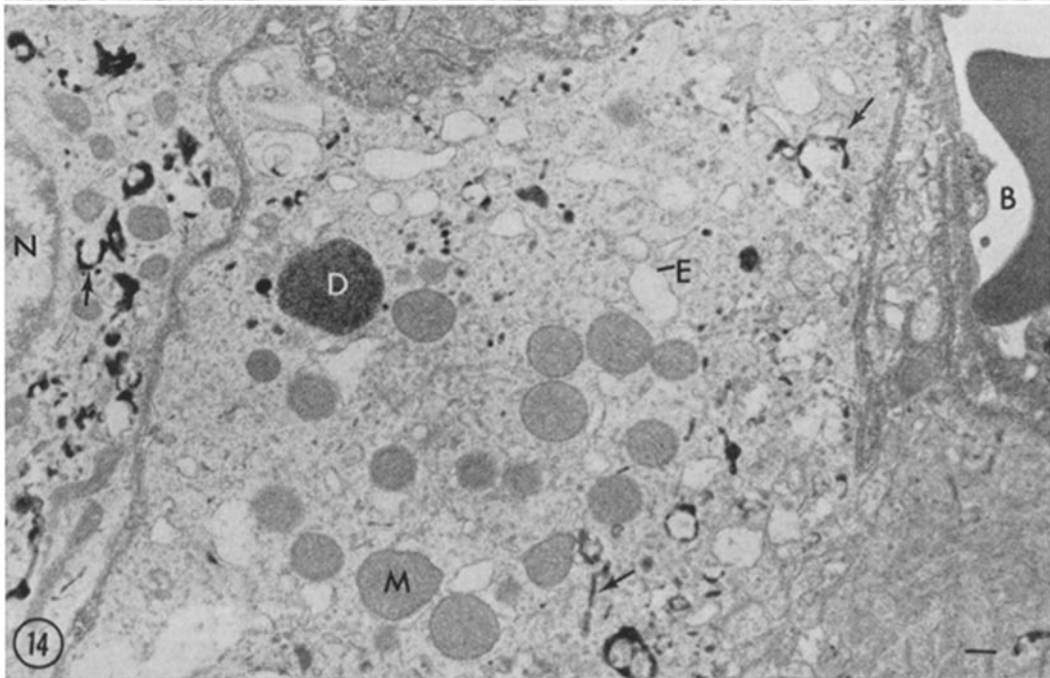
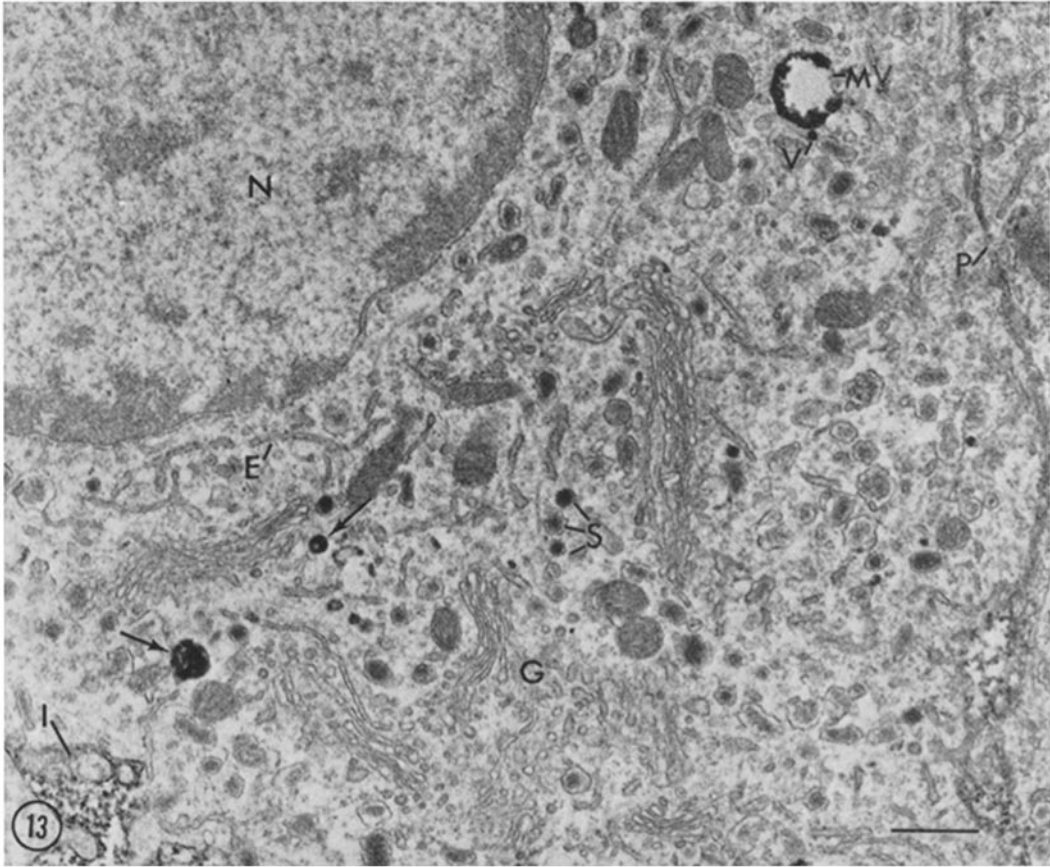
DISCUSSION

The simplest interpretation of our results is as follows: as a result of the presence of insulin, a well-known depleter of the adrenalin stores in the adrenal medulla (see references 8, 14), the adrenalin-producing cells rapidly release most of their secretory granules by exocytosis and, in so doing, add considerable membrane to their surfaces. This membrane is retrieved by endocytosis and much of it accumulates in the lysosomes. The membrane is degraded by the lysosomes, leaving a transient residue of lipid. Meanwhile new secretory granules are formed; their membranes arise from the endoplasmic reticulum-Golgi apparatus systems. These interpretations are in line with a growing body of evidence from study of a variety of cell types.

The coupling of exocytosis and endocytosis has been implicit in "membrane-flow" schemes devised by several authors (e.g., reference 10). Amsterdam's important work on the parotid gland (2, 3), our own studies on nervous tissue (35, 38) and the toad bladder (53, 54), and Douglas's work on neurosecretory material (18) are among recent confirmations of such coupling. Our findings extend the suggestive work of several previous studies (9, 14, 17, 19, 29, 37, 50, 52) and confirm the suspicion that rat medulla, like hamster medulla (9, 28, 29), secretes by exocytosis. Presumably the enhanced rate of secretion in the insulin-treated material is responsible for our ready observation of exocytosis. Or, perhaps, conditions are altered in some subtle way that slows the dispersion of released granules; slowness

FIGURE 13 Portion of a cell from a control animal injected with peroxidase 30 min before fixation and incubated for peroxidase activity. Reaction product is seen in a large body, probably a forming multivesicular body (*MV*); a small vesicle (*V*) nearby also contains peroxidase and presumably was caught shortly before it might have fused with the larger structure. Occasional other bodies in the cytoplasm also contain reaction product (arrows). Some of the secretion granules (*S*) are quite electron opaque, but this is usual with or without peroxidase. The nucleus is seen at *N*, endoplasmic reticulum at *E*, Golgi apparatus at *G*, the cell surface at *P*, and peroxidase in the intercellular space at *I*. $\times 23,000$.

FIGURE 14 From a peroxidase-incubated preparation of an animal injected with insulin 4 h before fixation and with peroxidase 30 min before fixation. *B* indicates the edge of a blood vessel, *N* a nucleus, and *M* mitochondria. Reaction product in the cells is seen in a dense body (*D*) and in many small tubules, vesicles, and irregularly shaped structures (arrows). Dilatation of endoplasmic reticulum (*E*) is often seen at this stage; the significance of this (real or artifact, etc.) is not known. $\times 8,000$.



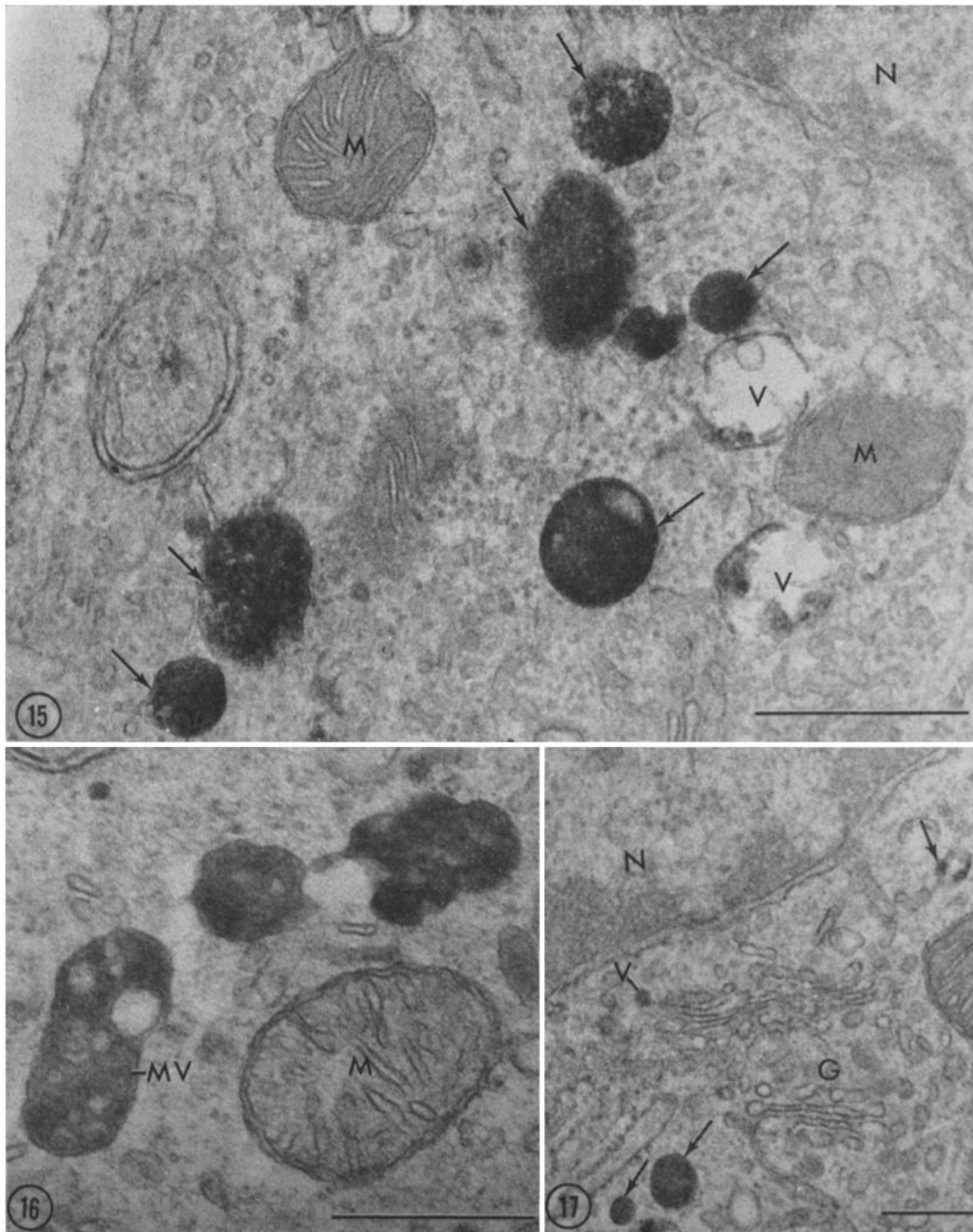


FIGURE 15 From a peroxidase preparation of an adrenalin cell from an animal injected with insulin 6 h before fixation and with peroxidase 3 h before fixation. Some reaction product is seen in two vacuoles (*V*) that probably are early stages in the formation of multivesicular bodies. More intense deposition of reaction product is present in several other bodies (arrows). The larger of these probably are lysosomes (cf. reference 37) and the smaller, endocytic vacuoles of the type shown in Fig. 8. *M* indicates mitochondria. $\times 62,000$.

FIGURE 16 From a peroxidase preparation of an animal injected with insulin 5 h before fixation and with peroxidase 1 h before fixation. Reaction product is seen in a multivesicular body (*MV*). *M* indicates a mitochondrion. $\times 59,000$.

FIGURE 17 Golgi region of an adrenalin cell from a peroxidase preparation of an animal fixed 6 h after insulin and 3 h after peroxidase. Reaction product is seen in a small vesicle (*V*) and larger structures (arrows) near the Golgi apparatus. The Golgi apparatus itself (*G*) shows no reaction product. *N* indicates the edge of the nucleus. $\times 38,000$.

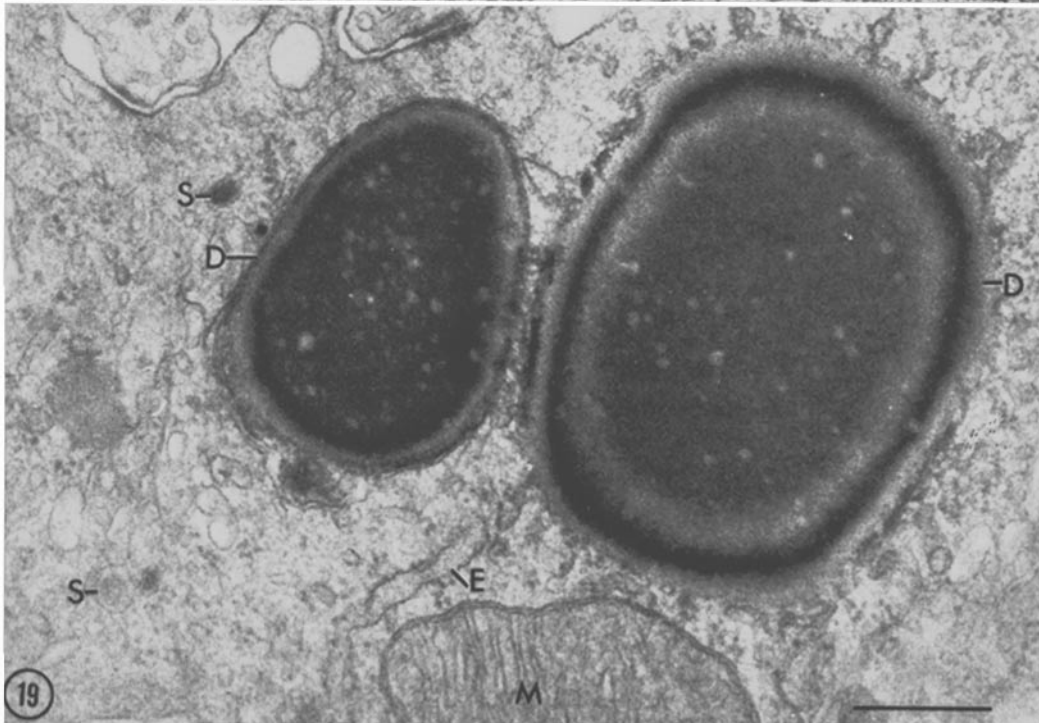
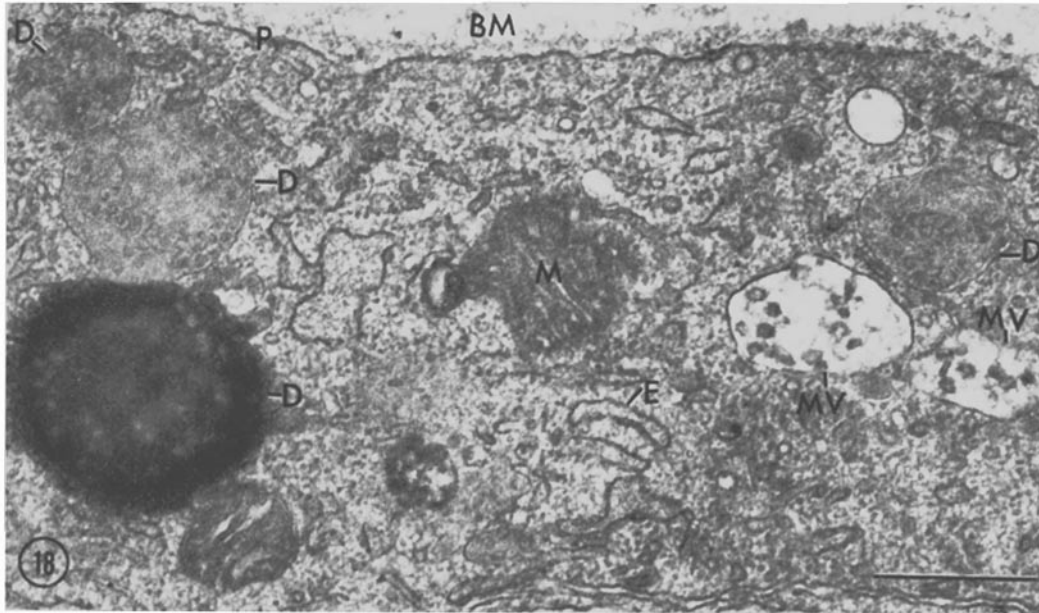


FIGURE 18 Part of a cell from an animal fixed 24 h after insulin. *M* indicates a mitochondrion, *E* a cisterna of endoplasmic reticulum, *P* the plasma membrane, and *BM* basement membrane. *MV* indicates multivesicular bodies, and *D* dense bodies whose content of vesicles suggests that they arise by transformation of multivesicular bodies. The three dense bodies at the left side of the figure are quite closely approximated to one another, as if they might be in the process of fusing. $\times 43,000$.

FIGURE 19 Portion of an adrenalin cell from an animal fixed 24 h after insulin. The two bodies at *D* show central portions similar to the dense bodies in Fig. 18, and peripheral regions of electron opacity and microscope appearance similar to lipid droplets (cf. Figs. 22, 23). *M* indicates a mitochondrion, *S* secretory granules, and *E* a cisterna of endoplasmic reticulum. $\times 36,000$.

in dispersion has been held responsible for the observation of exocytosis in the hamster.

There is nothing particularly unusual about the endocytic phenomena we observe; vesicles, tubules, multivesicular bodies, and so forth have been implicated in endocytosis in a vast variety of tissue (4, 7, 9, 26, 28, 36-38, 41, 44, 53, 76). It is clear that most of the tracer taken up by the control and insulin-treated medulla cells accumulates in bodies that are lysosomes (dense bodies and some multivesicular bodies) or will fuse with lysosomes (vesicles, tubules, vacuoles, some multivesicular bodies). This implies that under our experimental conditions much membrane involved in endocytosis becomes incorporated in the surface membranes of lysosomes.

It should be noted that uptake of peroxidase in sacs or tubules is commonly observed in many cell types by ourselves and others. Pelletier (personal communication), among others, has pointed out that if membrane is directly reutilized after endocytosis, one might expect exogenous tracers eventually to accumulate in Golgi sacs and secretory granules; he thinks that this is the case in pituitary cells stimulated to secrete by various agents (69). Thus far in the medulla we have seen tracer only in the "usual" vesicles, tubules, lysosomes, and so forth, even in tissue exposed to peroxidase for several hours during stages of regranulation. Some peroxidase-containing tubules and vesicles do show up near the Golgi apparatus, but this is frequently the case for lysosomes and related structures (see, e.g., 33, 61, 62), and we have not seen tracer in secretory granules or the sorts of membrane systems from which the granules arise. The situation may be different in nervous tissue (30, 36, 42), other cell types (12, 13), or in the medulla under other conditions; for

example, our experiments cannot rule out a slow recycling of a limited amount of membrane through the Golgi apparatus or very rapid reuse for exocytosis of membrane "retrieved" by endocytosis. And, in cells with contents and secretion granules as variable as in the present material, it would be easy to overlook or fail to recognize tracer-containing secretion granules if such granules were relatively infrequent.

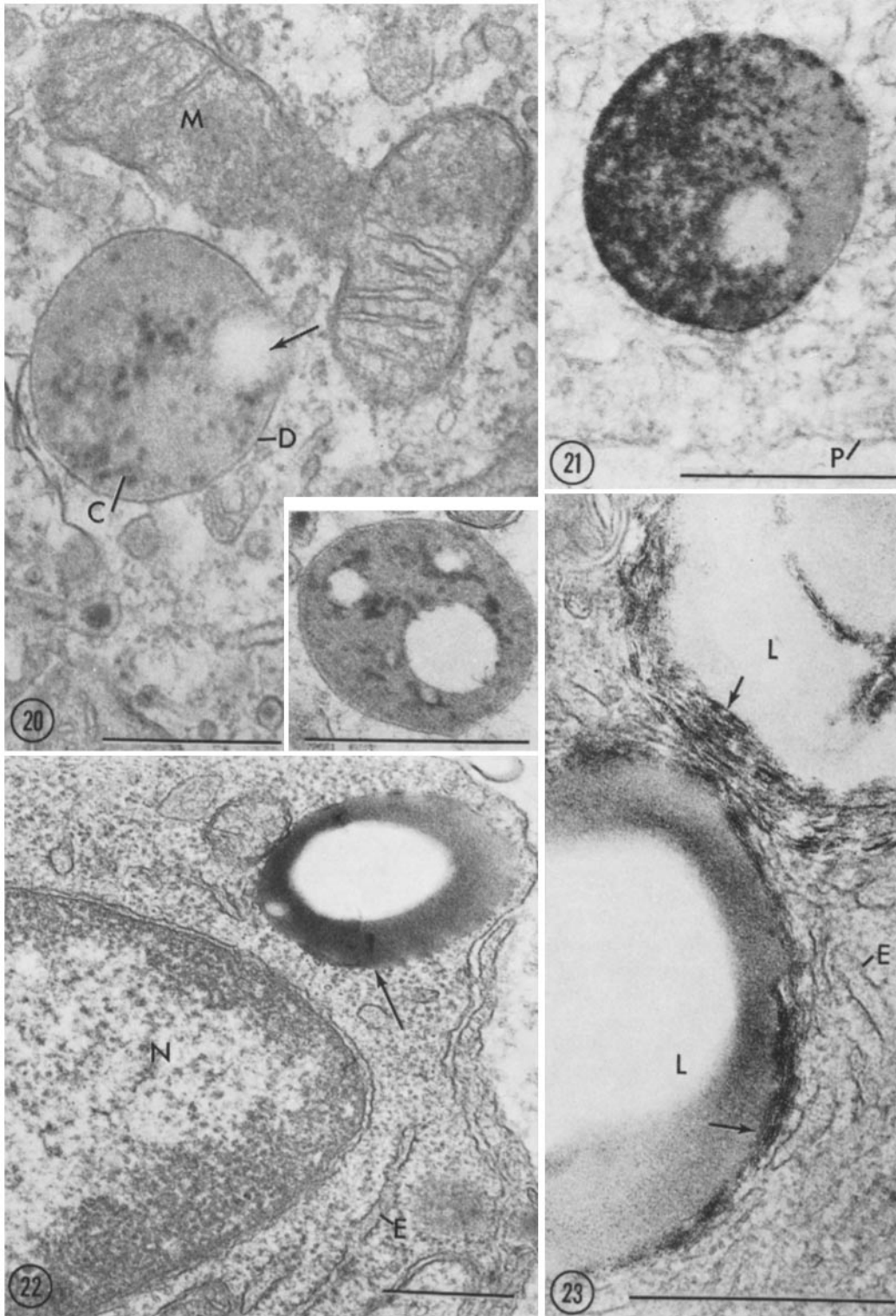
Many of the dense bodies in medulla cells responding to insulin appear to be residual bodies derived from multivesicular bodies. Work in our laboratory (33-35, 37, 38, 42) and elsewhere (4, 26, 31) has made clear that some, perhaps most, vesicles in multivesicular bodies arise by infolding of the surface membrane of the multivesicular body (26, 31, 34, 38, 54); apparently this can occur before or after the body acquires lysosomal hydrolases. There are other possible modes of origins (e.g., references 25, 40) which also involve entry into the interior of the multivesicular body of membranes that were originally outside, and there may be several types of multivesicular bodies (33, 40, 54, 64). But, in any event, it seems highly likely that as new membranes are added to the multivesicular body from vesicles and tubules involved in endocytosis, the body is prevented from expanding enormously by entry of membrane into the interior (cf. references 4, 31, 34, 38, 54). That multivesicular bodies can play roles resembling autophagy is well established (4, 22, 23, 31, 35, 37, 40, 64, 65, 75).

We believe that the lipid that accumulates during the day or two after the height of the endocytic phase largely represents a late stage in the lysosomal digestion (16, 74) of the membrane that is involved in the coupled exocytosis and endocytosis. Farquhar has shown in her studies of the

FIGURES 20 and 21 From preparations given insulin 4 h before fixation and peroxidase 30 min before fixation but not incubated for peroxidase activity. *M* indicates mitochondria and *P* the cell surface. *D*, in Fig. 20, indicates a dense body in which is present some coarse dense material (*C*) and a light region (arrow). A similar body is shown to possess acid phosphatase activity in Fig. 21. The insert in Fig. 20 shows another such body from a control adrenal medulla. Fig. 20: $\times 54,000$; insert: $\times 68,000$; Fig. 21: $\times 68,000$.

FIGURE 22 From a preparation fixed 24 h after insulin injection. The structure at the arrow has the typical electron microscope appearance of lipid droplets. *N* indicates the nucleus, *E* a cisterna of endoplasmic reticulum. $\times 40,000$.

FIGURE 23 From an adrenal cell of an animal 24 h after insulin treatment. The edge of two lipid droplets (*L*) show lamellar arrays (arrows). A cisterna of endoplasmic reticulum is seen at *E*. $\times 81,000$.



pituitary gland (22, 23, 75) that lipid can accumulate in residual bodies of the type we see. The major difference between our study and hers in this regard is that the pituitary residual bodies arise chiefly through autophagy and crinophagy rather than endocytosis. We do not see, in our material, great increase in crinophagy, "conventional" autophagy (or in cell death) that might offer alternative explanations of the lipid accumulation. The lamellae seen at the surfaces of some lipid droplets may reflect the presence (as expected) of polar lipids (see Fig. 23).

Where the lipid goes is not known; presumably it can be reused by the cell. The amounts of lipid in the globules seem roughly comparable to the amounts in the membranes of the original granule population (see reference 42 for suggestive calculations).

Obviously we can not be sure that the membrane that is eventually digested in a lysosome is the same bit of membrane that originally surrounded a secretory granule as opposed to an equivalent region of plasma membrane. However, that it might be is suggested by the observations (9, 29) of coated vesicle formation from surface membranes at the sites of exocytosis in the hamster medulla and by biochemical studies of differences between plasma membranes and secretory granule or vesicle membranes (15, 56-59, 81-84).

Work on a variety of cell types (e.g., references 5, 21, 33, 45-47, 59, 66, 77, 81, 82) including the adrenal medulla (17, 20, 37, 42) has made clear that secretory granules and other membrane-delimited bodies, e.g. lysosomes (33, 35, 61, 65), and melanin granules (63) can be "packaged" in the Golgi region. While the route of packaging and membrane flow probably differ somewhat for different cells and different macromolecules, it seems reasonable to propose (cf. references 42, 59, 81, 82) that membrane destined for the cell surface or for secretory granules can arise in endoplasmic reticulum. However, we do not wish to suggest that there is not any reutilization of membrane, without degradation, in adrenal medulla or in other cell types. It is true that the basic processes we observe in insulin-treated medulla (e.g., sequestration of endocytosed material in lysosomes) are seen in untreated medulla and in many other cell types; and, it seems clear that in insulin-treated medullae a very large proportion of the membrane is degraded rather than reutilized. But particularly in light of the

current uncertainties in biochemical studies, it would be wise to suspend judgment; it might, for example, turn out that lysosomal mechanisms coexist with subtler turnover and reutilization mechanisms and that one or the other is favored under varying circumstances. For example, work on intracellular membranes (see review in reference 72) hints at complex turnover mechanisms; different constituents of a given membrane turnover with quite different rates, which would not be expected for a situation in which lysosomal degradation of large membrane regions is the sole relevant mechanism. Recent data on plasma membranes of endocytosing cells (12, 80), on lysosomal membranes (15), and on secretory granule membranes (3, 55-58, 85) can be interpreted as supporting substantial reutilization (e.g., 12, 55-58, 85) or predominant new synthesis (3, 80) of membranes involved in circulation of the type discussed in the present paper. It may be that different cell types differ and that adequate biochemical evaluation of membrane circulation of the type discussed here will require greater attention to possible reserves of pools of membrane constituents and to variations among different regions of a given plasma membrane and among portions of intracellular membranes (cf. Siekevitz's (72) discussion of membrane "Mosaicism").

Finally, we are aware of the problems in drawing conclusions about dynamic events from static micrographs and in interpreting results on a tissue as sensitive as the adrenal medulla to perturbations. For example, in our early pilot studies we had the strong impression that ether anesthesia of animals not injected with insulin resulted in substantially greater peroxidase uptake than chloralose anesthesia; this is in accord with known effects of these anesthetics on hormone release from the medulla (see reference 14). From our morphological studies we believe that the presence of peroxidase does not significantly affect the cells, which suggests that the presence of the tracer does not alter the circulation of membranes. However, future studies under less abnormal conditions will be useful in further evaluations of the problems under consideration. It should also be borne in mind that insulin effects on the medulla are thought to be largely indirect (14; see also reference 54) so additional work with more directly acting agents is needed.

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