

# HORMONE-STIMULATED EXOCYTOSIS IN THE TOAD URINARY BLADDER

## Some Possible Implications for Turnover of Surface Membranes

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### INTRODUCTION

It is well established that neurohypophyseal hormones and cyclic 3',5'-adenosine monophosphate (cAMP) increase the permeability of the amphibian bladder to water and enhance the transport of sodium across the mucosal epithelium (16, 17, 27). Recently, we reported that in the toad bladder mucosal epithelium, the altered permeability is paralleled by a marked increase in the endocytic uptake of horseradish peroxidase from the lumen (31-33). One of the possible explanations was that the observed endocytosis might be a secondary effect. The primary effect in the response to hormone and cyclic nucleotide might conceivably be a stimulation of exocytosis, the fusion of membranes surrounding secretion granules with the plasma membrane. This would result in the addition of secretion granule membrane and contents to the apical cell surface. The endocytosis could represent a mechanism by which the cell "retrieves" membrane from the surface and, in so doing, maintains a more or less constant surface area. This hypothesis grew out of observations on gland cells and neurons (1, 2, 21, 22, 24, 25) which indicate such an interrelationship or coupling of endocytosis and exocytosis.

Secretory activity by the bladder mucosal cells has thus far not been widely noted. Choi (6) showed that large granules in the cells share morphological and cytochemical features with cell surface material, and there have been rare

observations (40) of continuity between the granule membrane and the plasma membrane. The present findings indicate that the frequency of such membrane continuities is greatly increased in cells stimulated by oxytocin or the dibutyryl derivative of cAMP as compared to unstimulated controls.

### MATERIALS AND METHODS

The following compounds were used in this study: oxytocin, prepared by Miss P. L. Hoffman at Mt. Sinai School of Medicine by the solid-phase method of peptide synthesis (35). The hormone was found to possess an activity of 500 units/mg when tested in the fowl vasodepressor assay just before use. N<sup>6</sup>-2'-O-dibutyryl adenosine 3',5'-cyclic monophosphate (dibut. cAMP) was purchased from Schwarz Bio Research Inc., (Orangeburg, N.Y.), peroxidase type II from Sigma Chemical Co., (St. Louis, Mo.), and toads (*Bufo marinus* originating from the Dominican Republic) from National Reagents, Inc., Bridgeport, Conn.

### Preparation of Toad Bladders

Toads were kept on wet peat moss. The urinary hemibladders were excised from doubly pithed toads and placed in Ringer's solution of the following composition: NaCl 110 mM, KCl 3 mM, CaCl<sub>2</sub> 1 mM, dextrose 5.5 mM, MgCl<sub>2</sub> 1 mM, NaHCO<sub>3</sub> 3 mM, 1,1,1-Tris (hydroxymethyl)-methylamine hydrochloride (Trizma<sup>®</sup> Sigma Chemical Co.) 10 mM; the pH was adjusted to 8.4 with NaOH. The final

tonicity was found to be 242 mosmols/liter. Hemibladders were mounted as sacs as previously described (33), the mucosal surface facing inward. The mounted hemibladders were filled with 7 ml of full-strength Ringer's fluid and placed in a bath of 25 ml Ringer's fluid per bladder for a 2 hr preincubation.

### Incubation Experiments

Paired hemibladders were used, one serving as an experimental and the other as a control. After preincubation, the Ringer's fluid in the bladders was replaced with fresh 1/5-strength Ringer's fluid. The experimental hemibladder was placed in a fresh bath of full strength Ringer's fluid containing  $10^{-7}$  M oxytocin (seven bladders) or  $5 \times 10^{-8}$  M dibut. cAMP (six bladders). The control hemibladder was transferred to a serosal bath of full-strength Ringer's fluid per se. The serosal bath was replaced at 60-min intervals; the bladders were weighed at 30-min intervals to determine the rate of water loss; at the time of weighing, the mucosal solution was replaced by fresh 1/5-strength Ringer's fluid. Paired experimental and control bladders were fixed after an incubation period of 2-5 hr.

In another set of experiments, one hemibladder was filled with 1/5-strength Ringer's fluid containing 0.2% peroxidase (33) and placed in a full-strength Ringer's fluid solution containing  $10^{-7}$  M oxytocin. Both mucosal and serosal solutions were replaced after 1 hr, and after a total of 2-hr incubation time the hemibladders were fixed.

### Electron Microscope Preparation

The bladders were fixed in 1% osmium tetroxide in collidine or Veronal acetate buffer (4, 38) or Karnovsky's formaldehyde-glutaraldehyde solution as described previously (33). Peroxidase activity was demonstrated by incubation, in Karnovsky's medium, of bladders which had been fixed, rinsed in buffer, and frozen (29, 30, 33). The aldehyde-fixed bladders were postfixed in osmium tetroxide in phosphate or Veronal acetate buffer (38) on ice, dehydrated in a graded series of ethanol on ice, followed by propylene oxide and embedding in Epon 812 (28).

Thick sections of each block were viewed by phase microscopy to orient the block for cross sectioning through all the tissue layers. Some thick sections of both experimental and control bladders were stained by the periodic acid-Schiff (PAS) method and counterstained with methylene blue for evaluation of possible differences in distribution of polysaccharide-containing granules or amount of extracellular coating material at the surface of the epithelial cells (6).

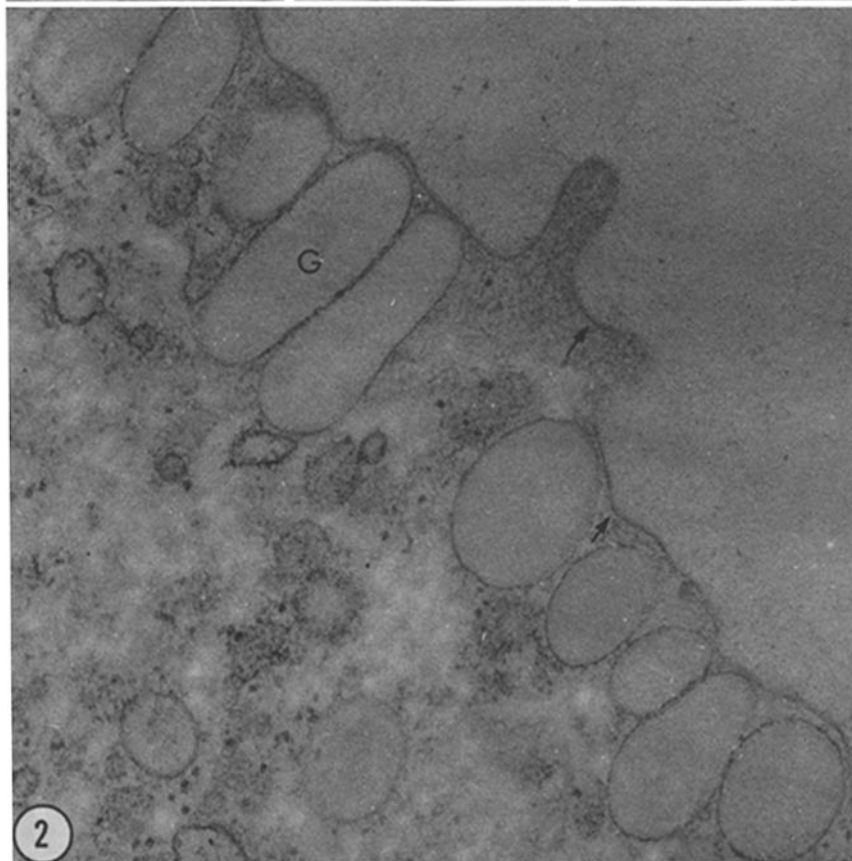
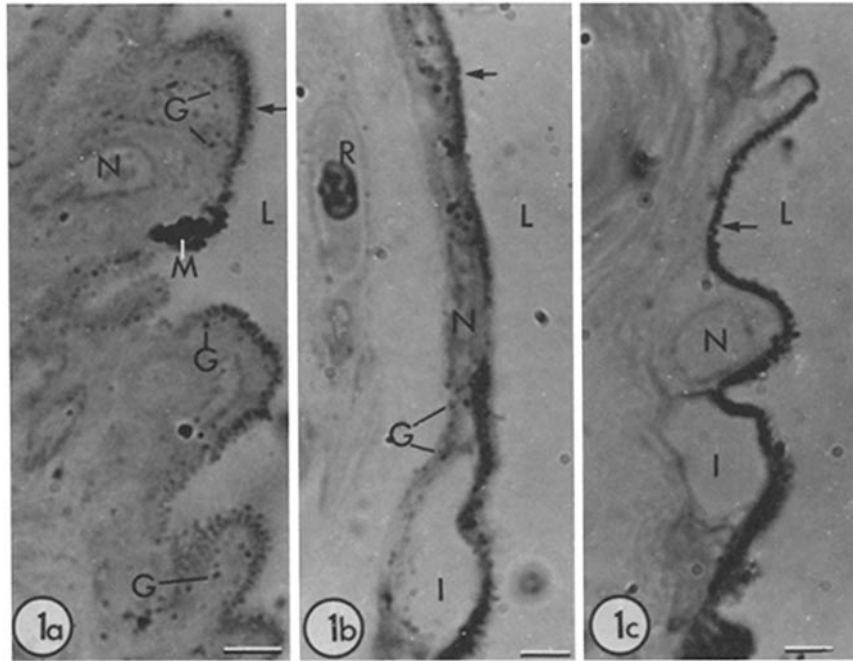
Silver-to-gold sections were cut on a Porter-Blum ultramicrotome (Sorvall) with a diamond knife. Sections stained with uranyl acetate (47) followed by lead citrate (46) were viewed with an RCA EMU-3F electron microscope. To eliminate possible nonobjectivity of the microscopist, "blind" experiments were performed in which unidentified sections from the different experimental conditions were studied for evidence of granule release.

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FIGURE 1 Light micrographs of PAS-methylene blue-stained,  $1 \mu$  Epon sections of bladders incubated in vitro for 4 hr. Epithelial cell nuclei are seen at *N*, and the bladder lumen at *L*. The bar represents  $5 \mu$ . Fig. 1 *a*, In a control hemibladder, incubated in Ringer's fluid, stained granules (*G*) can be seen throughout the cytoplasm and adjacent to the apical surface at the bladder lumen (*L*). The cell surface bordering on the lumen (arrow) also stains. The large, stained globules at *M* are characteristic of mucus-secreting cells found in small numbers in the epithelium.  $\times 1170$ . Fig. 1 *b*, A portion of an experimental hemibladder, incubated in Ringer's fluid containing oxytocin. In this region the epithelial cells contain granules (*G*) in the cytoplasm; the cell surface (arrow) near the lumen (*L*) appears to be somewhat more densely stained than in control preparations. The enlargement of the intercellular space (*I*) in Figs. 1 *b* and 1 *c* is typical of epithelial cells in bladders incubated in the presence of hormone (10).  $\times 1150$ . Fig. 1 *c*, A different region of the same experimental hemibladder shown in Fig. 1 *b*. The cytoplasm of the epithelial cells is almost completely degranulated; the apical surfaces (arrow) of the cells are densely stained.  $\times 1150$ .

All electron micrographs are from bladders fixed in osmium tetroxide, except Fig. 6 which is from a bladder fixed in Karnovsky's aldehyde mixture and postfixed in osmium tetroxide. The bar represents  $0.5 \mu$ .

FIGURE 2 Membrane-bounded granules (*G*) grouped below the apical surface of an epithelial cell in a 2 hr control hemibladder. Only the inner dense line of the "unit" plasma membrane is readily visible (arrows); the outer (luminal) line is very difficult to see (compare with Fig. 4).  $\times 61,600$ .



## RESULTS

The mucosal epithelial cells are lined, at their apical surface, by an extracellular layer of PAS-positive material (Fig. 1). As reported by Peachey and Rasmussen (40) and Choi (6), in unstimulated (control) bladders intracellular granules stainable with the PAS method are scattered in the cytoplasm of the mucosal cells and show some tendency to group near the apical surface. Electron microscopy (Figs. 2 and 3) confirms this distribution; large membrane-delimited granules are found in all regions of the cytoplasm, many of them being near the Golgi apparatus (Fig. 3) or aligned just below the plasma membrane at the bladder lumen (Fig. 2). Peachey and Rasmussen (40) considered the "mitochondria-rich" and "granular" cells to be variants of the same epithelial cell type. We agree with this classification, especially since we encounter in mitochondria-rich cells some granules of the same type seen in the granular cells. In this study we have focused on cells relatively rich in granules.

When bladders are exposed to oxytocin or dibut. cAMP, the distribution and frequency of PAS-staining granules viewed in the light microscope sometimes is markedly changed; fewer granules are present in the cells and the PAS-positive material at the apical surface appears more dense (Fig. 1). However, the light microscope results are not consistent from bladder to bladder, and even within a single preparation there is much variation from cell to cell (compare Fig. 1 *b* and 1 *c*). This variability in appearance probably reflects, in part, alterations in cell geometry, such as the thinning of the epithelium in response to the agonists (10, 14). Electron microscope observations are less ambiguous. Whereas continuities between the plasma membrane and the membrane surrounding the secretion granules are almost never found in unstimulated bladders, after exposure of the bladder to oxytocin (Fig. 4) or dibut. cAMP (Fig. 5) such continuities are regularly observed. Peroxidase uptake is readily demonstrable in bladders incubated with hormone for the same period of time as was used for the observations of exocytosis (Fig. 6).

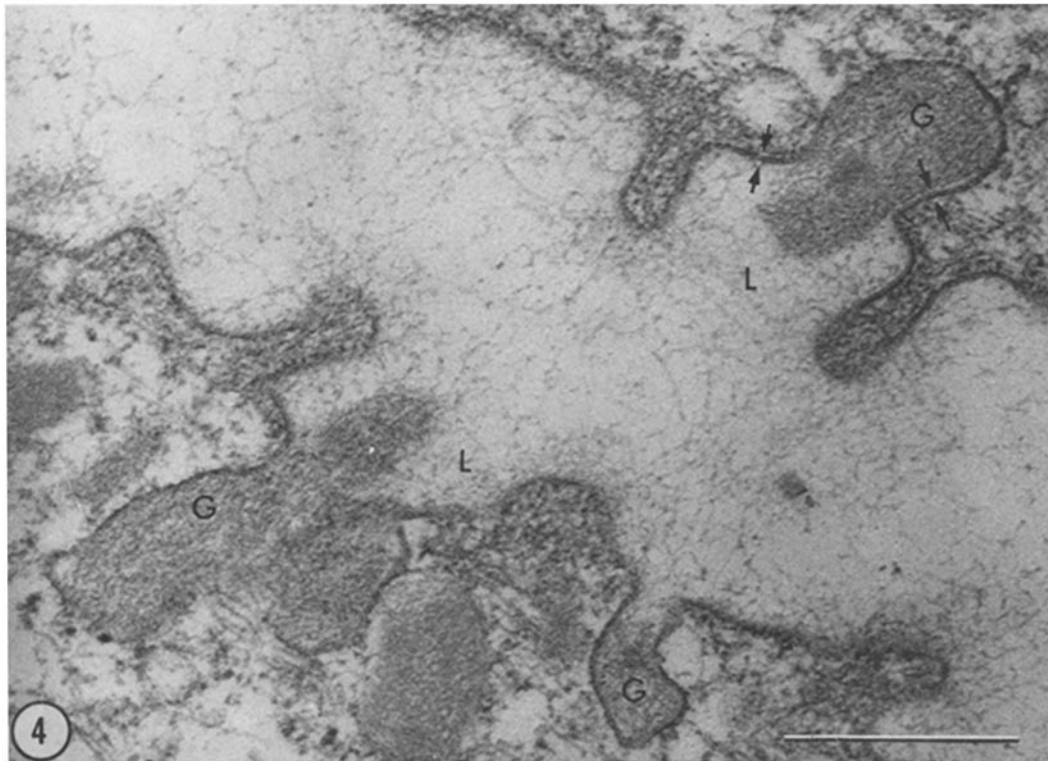
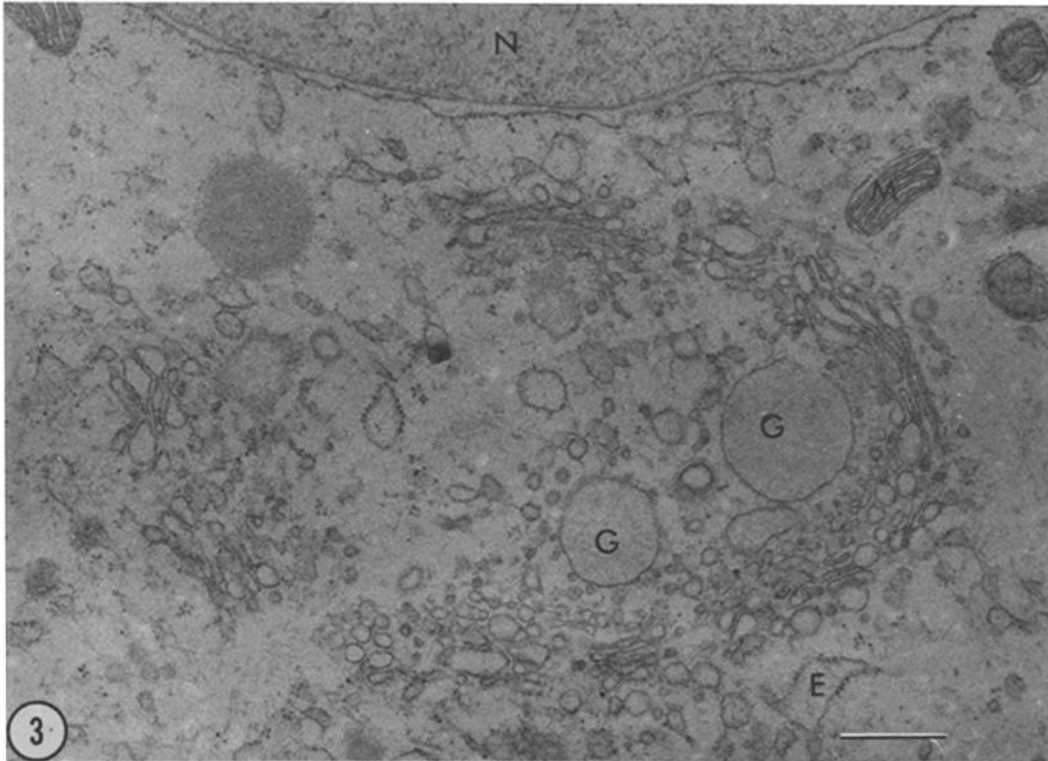
Efforts were made to evaluate possible alterations in details of cell surface morphology that might result from addition of material from the secretion granules. In some preparations the coating found on the extracellular surface of the

apical membrane seemed thickened after oxytocin treatment, and in others the trilaminar structure of the membrane itself (Fig. 4) seemed considerably more distinct than in unstimulated material (7, and Schechter and Schechter cited in reference 5). However, such effects were not always consistent. In the light of the findings of Staehelin et al. (45), which suggest that the apical surface of similar transitional epithelium may be a morphological mosaic, it may not be surprising that the membrane structure differences are not always obvious.

## DISCUSSION

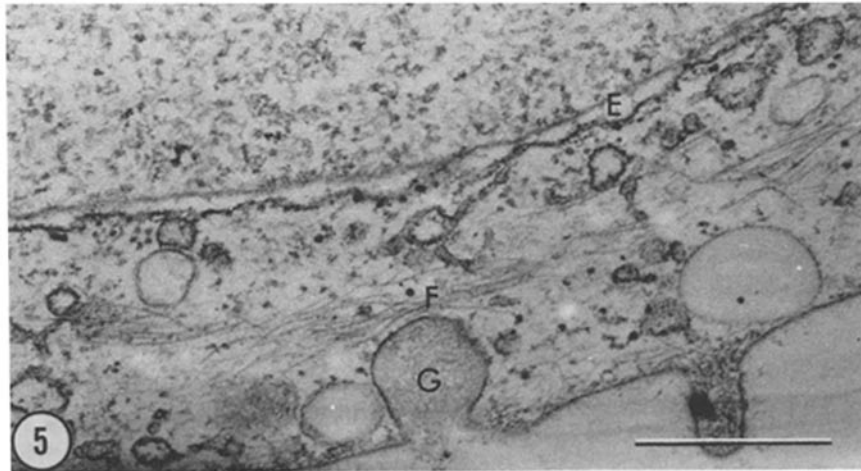
It has been known for some time (12, 39) that many secretory cells release their content by exocytosis. There are indications in various secretory systems that such processes can be stimulated by hormones (12, 39) and perhaps by cAMP (41). Furthermore, it is likely that material packaged into membrane-delimited vacuoles by the Golgi apparatus eventually is released at the cell surface in many cells which would not formally be classified as secretory (18, 36, 42, 48, 49, 52). Apparently, in toad bladder, neurohypophyseal hormone and dibut. cAMP stimulate the fusion of the granule membrane with the apical surface membrane of the mucosal epithelium; concomitantly the content of the granule is released and contributes to the surface coating.

Less well supported is the notion that the secretion granule membrane (or contents) might be responsible for alterations in cell surface physiology. However, Hicks (18) has observed apparent assembly of thickened plasma membrane of rat transitional epithelium in the Golgi apparatus of the superficial cells. Hicks proposes that the membrane is added to the surface by fusion of Golgi-derived vacuoles and notes that the permeability characteristics of the plasma membrane differ markedly from those of the more usual plasma membranes. Thus, it seems reasonable to advance the proposition that the observed hormone- and dibut. cAMP-stimulated changes in the plasma membrane of the mucosal epithelium of the toad bladder could result from the addition to the membrane of new material, derived from the secretion granules. In our case, as in rat transitional epithelium, the Golgi apparatus seems to be the site of formation of the granules. Presumably, the alignment of granules just below the apical plasma membrane

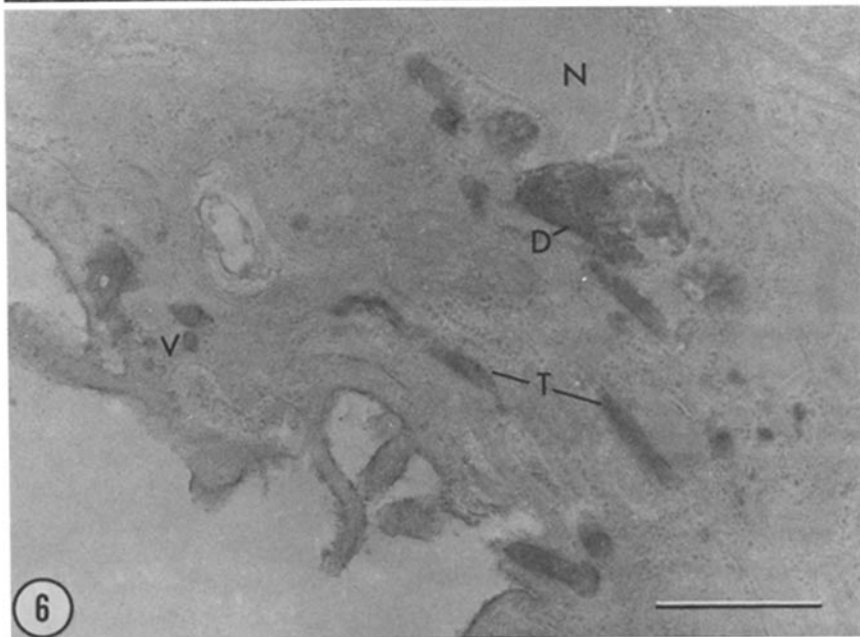


**FIGURE 3** Part of an epithelial cell from a control bladder incubated for 2 hr. Most of the field is occupied by the region of the Golgi apparatus. Membrane-bounded granules (*G*) are found near the concave surface of the Golgi apparatus. The edge of the nucleus is seen at *N*, mitochondria at *M*, and rough endoplasmic reticulum at *E*.  $\times 31,000$ .

**FIGURE 4** A part of an epithelial cell from a bladder exposed to oxytocin for 2 hr. The limiting membranes of several granules (*G*) are continuous with the plasma membrane at the apical (*L*) border. Both dense lines of the "unit" membranes are clearly visible (arrows; compare with Fig. 2).  $\times 62,000$ .



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**FIGURE 5** Part of an epithelial cell of a hemibladder exposed to dibut. cAMP for 2 hr. The membrane surrounding a granule (*G*) is continuous with the apical plasma membrane. The nuclear envelope is seen at *E*, and microfilaments are present at *F*.  $\times 52,000$ .

**FIGURE 6** Portion of an epithelial cell of a bladder exposed to peroxidase and incubated to demonstrate peroxidase activity: oxytocin was present in the serosal solution (see reference 33). Peroxidase reaction product is seen in tubules (*T*), vesicles (*V*), and a dense body (*D*). The nucleus is seen at *N*.  $\times 43,800$ .

would facilitate rapid response of the cells to those agents which induce exocytosis.

The possibility that the observed granule fusion, endocytosis, and permeability changes are separate but coordinated responses cannot be ruled out at present. It could be argued, for

example, that the granule contents play some role in protecting or altering the apical cell surface that is only indirectly related to the permeability changes. Alternatively, the fusion of granules with the cell surface might tend to restore "normal" (prestimulation) conditions for example,

by replacing regions of surface membrane removed by pinocytosis.

Finally, mention should be made of the role of lysosomes in endocytosis and exocytosis. A vast variety of tissues (see reference 11 for review) are capable of taking up exogenous substances and adding them, plus the membranes surrounding endocytic vesicles and tubules, to lysosomes. Frequently this occurs by fusion of endocytic vesicles with lysosomes of the multivesicular body type (see e.g., 13, 19, 20, 21, 24). Through the formation of cuplike bodies (20, 21, 23, 24, 26) and other mechanisms by which the internal vesicles may arise (e.g., references 19, 23, 24, 33), membranes originally added to the surface of structures destined to become multivesicular bodies could eventually gain access to the interior of lysosomes and presumably might thereby be exposed to degradation (1). In the present case (33) and in several other situations (1, 18, 20, 21, 25), the lysosomes apparently serve as a terminal organelle in the circulation of membrane among intracellular compartments and the cell surface (cf. discussion in reference 25); multivesicular bodies are prominent among the lysosomal types involved. It is quite possible that this role of lysosomes is a general one applying not only to exocytosis and related processes but to aspects of membrane turnover in the Golgi region (20, 23, 37) and in other parts of the cell as well. The involvement of multivesicular bodies in processes resembling autophagia is well established (19, 21, 23, 24, 44, and Farquhar in reference 11). It has been suggested that some multivesicular bodies may originate more or less directly from tubular invaginations of the cell surface (20, 24) while others may originate near the Golgi apparatus (20, 23, 37, 53).

In the various systems being studied, available data are not yet good enough to establish definitively whether the membrane "retrieved" by endocytosis is the same, in whole or in part, as the membrane that originally surrounded secretion granules or whether some endocytosed membrane is reused without previous degradation. Biochemical information on nerve and gland cells (3, 8, 9, 34, 48-50) can be interpreted as indicating differences in the turnover or composition among secretion granule or synaptic vesicle membranes, plasma membranes, and lysosomal membranes, but some of the similarities (34, 36) may turn out to be as interesting as the differences.

Furthermore, it is difficult to be certain that the restricted region of the cell surface at which secretion or transmitter release occurs is appropriately represented by available preparations of isolated plasma membranes. However, it is probably significant that, in the parotid gland (3), secretion granule membranes appear to be newly synthesized along with the granule contents. In adrenal medulla (1, 15, 21, 24, 51) and a few other tissues (reviewed in reference 43), vesicles that may be involved in endocytosis (1, 15, 21, 24) seem to form from regions of the cell surface at which granule release has just occurred. This suggests that at least part of the secretion granule membrane may have only a limited residence at the cell surface.

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