CELL MEMBRANE RESORPTION IN THE RAT EXOCRINE PANCREAS CELL AFTER IN VIVO STIMULATION OF THE SECRETION, AS STUDIED BY IN VITRO INCUBATION WITH EXTRACELLULAR SPACE MARKERS

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ABSTRACT

Pancreatic secretion in the rat was stimulated in vivo by pilocarpine injection causing 90% of the storage granules to be discharged within 2 h. Incubation in vitro with [14C]sorbitol indicated that maximal ingestion of this extracellular space marker occurred 3 h after secretagogue injection. Morphological cell membrane measurements on cells with stimulated secretion revealed a simultaneous decrease in amount of membrane bordering the microvilli at the cell apex, lamellar processes, and infoldings present at the latero-basal face of these cells. In 3-h stimulated cells, having the average zymogen granule content characteristic for that phase of secretion, ferritin treatment in vitro showed that the infoldings and related fragmentation vesicles had ingested ferritin and could consequently be considered as being transport vehicles for redundant cell membrane. During stimulated secretion numerous vesicles and vacuoles appeared in the apical cytoplasm. Part of these structures were postulated to be related to the Golgi complex and were discussed in relation to secretory protein transport. Another part of these structures was assumed to have an endocytic nature, although they never contained ferritin.

INTRODUCTION

The exportable proteins of a variety of exocrine and endocrine gland cells have been described as being packed in membrane-bounded granules. In merocrine (eccrine) gland cells, such as the exocrine pancreas cell, the granule membranes fuse during secretion with the cell membrane and become part of it. Meanwhile the secretory proteins are extruded through a gap in the membrane at the place of fusion. It is evident that the gland cell must have a means by which it can remove the extra membrane contributed to the cell membrane during secretion, because no conspicuous volume increase takes place during cell life.

Only a few relevant studies have been published that concern cell membrane reduction during the secretory cycle of endocrine and exocrine cells. In the chromaffin cells of the adrenal medulla, secretion has been found to be merocrine by fractionation experiments with dopamine β-hydroxylase as a marker for the granule membranes (1). At the site of exocytosis, vesicles occur that seemed to be pinched off from the cell membrane and to return into the cytoplasm (2). In the parotid gland massive secretion of enzyme is followed by an enlargement of the acinar lumina. The subsequent reduction of these lumina is accompanied by the appearance of numerous smooth-surfaced vesicles in the cell apices (3).

In all of these investigations it was suggested on
morphological grounds that cell membrane reduction is accompanied by endocytosis. However, these results cannot be interpreted unambiguously, since no evidence was presented to indicate the direction of vesicle movement, that is, it remains questionable whether the vesicles are endocytotic or exocytotic.

We endeavored to prove the endocytotic nature of the membrane reduction process by studying the uptake of extracellular space markers. We have worked with pancreas cells in which secretion was stimulated in vivo by pilocarpine. The phase in the secretory cycle during which vesicular membrane withdrawal is optimal could be detected by a subsequent in vitro incubation of tissues slices in the presence of sorbitol, which cannot penetrate the membrane passively. It appeared that 3 h after the onset of the stimulated secretion the tissue slices had taken in a maximal amount of sorbitol that could not be washed out. Incubation of 3-h stimulated slices with ferritin (which is an electron microscope detectable extracellular space marker) revealed that at least one way of cell membrane resorption in the pancreas cell is achieved by infolding and subsequent fragmentation of the lateral and basal cell membrane.

MATERIALS AND METHODS

Stimulation of the Secretion

Male albino Wistar rats (obtained from Centraal Proefdierenbedrijf TNO, Zeist), weighing approximately 350 g each, were fasted for 24 h, but given free access to water.

The secretagogue was pilocarpine nitrate (Brocacef, Utrecht), dissolved in physiological saline (100 mg per ml). This solution was introduced by tail vein injection at a concentration of about 20% (wt/vol). The animals were killed by decapitation 1, 2, 3, and 4 h after injection.

Experiments with [14C]Sorbitol

Five rats at each time interval after the application of the drug and five unstimulated controls were used in these experiments. The pancreas was freed from adherent fat and connective tissue, and slices of about 0.5 X 0.5 X 2 mm were cut with a tissue sectioner (Ivan Sorvall, Inc., Newtown, Conn.). Approximately 200 mg of slices were preincubated for 10 min in 25-ml flasks, containing 2 ml of Krebs-Henseleit bicarbonate buffer at pH 7.4 with addition as described before (12). Next, the slices were transferred to another 2 ml of the same medium to which was added 1 µCi of [14C]D-sorbitol (7 mCi/mmol, The Radiochemical Centre, Amersham, Buckinghamshire, England). After 30 min of incubation the medium was poured off and the pancreas slices were washed intensively for 10 min at 0°C in several changes of incubation medium containing a 1,000-fold excess of unlabeled D-sorbitol. The pancreas was homogenized with a Potter homogenizer (Potter Instrument Co., Inc., Melville, N. Y.). The radioactivity in homogenate samples was counted in a dioxane based scintillation mixture with a liquid scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.). The amount of DNA per sample was determined according to Burton (4).

Experiments with Ferritin

Three 3-h stimulated rats and two unstimulated control rats were used in the experiments with ferritin. For the preparation of ferritin the procedure of Amsterdam et al. (3) was essentially followed. The aqueous ferritin solution (Schwarz/Mann, Orangeburg, N. Y.) was centrifuged for 2 h at 200,000 g. The resulting pellet was suspended in 3 ml of Krebs-Henseleit bicarbonate buffer, pH 7.4, and the suspension was dialyzed against two 500-ml changes of the buffer for 24 h at 4°C. The ferritin was used at a concentration of about 20% (wt/vol).

About 25 mg of sliced pancreas was incubated in 2 ml of the ferritin suspension for 30 min under the conditions described for sorbitol. In one experiment, the ferritin incubation time was extended to both 1 and 2 h, respectively. After incubation, one portion of tissue was fixed for light and electron microscopy in 4% glutaraldehyde in Veronal-buffered Henseleit bicarbonate buffer, pH 7.4, and the suspension was dialyzed against two 500-ml changes of the buffer for 24 h at 4°C. The ferritin was used at a concentration of about 20% (wt/vol).

Tissue Processing for Microscopy

Tissue was fixed for light and electron microscopy for 2 h in Veronal-buffered 1% Oso4 at pH 7.4, and en bloc stained for 30 min with 1% aqueous uranyl acetate solution. Dehydration in an upgraded ethanol series and embedding in Epon 812 followed. Zymogen granules were counted under the phase-contrast microscope. To enhance contrast, 1-µm-thick sections were stained in silver methenamine: the sections were floated for 15 min on a 1% aqueous solution of periodic acid in a 2-ml trough at room temperature. After short rinsing with water, the trough was filled with 2 ml of a stock solution of silver methenamine, composed of 18 ml of 3% hexamine,
2 ml of 5% silver nitrate, and 2 ml of 1.9% borax. The sections were incubated in this solution for 10 min at 60°C. Finally, the reaction was stopped with sodium thiosulphate.

Ferritin localization was studied mostly in unstained ultrathin (grey) sections. Some sections were stained, however, with alkaline bismuth nitrate (E. Merck AG, Darmstadt, W. Germany) for 30 min at room temperature according to the method recently proposed by Ainsworth and Karnovsky (5). This staining intensified ferritin electron opacity and enhanced tissue contrast. Electron micrographs were taken with a Siemens Elmiskop I electron microscope.

**Morphological Quantifications**

Three groups of two stimulated animals (killed 1, 2, and 3 h after injection with pilocarpine) and two unstimulated control animals were used to quantify zymogen granule content and cell membrane length. Pancreas slices were prepared and incubated as described for the ferritin experiments, except that ferritin was omitted from the medium.

**Granule Countings**

In order to test the effectiveness of the secretogogue, the numbers of zymogen granules were counted with phase-contrast microscopy in 1-µm silver methenamine stained sections. From each pair of animals 20 cells were selected (10 cells per animal), each cell section showing the nucleus and the luminal cell membrane. It turned out to be impossible to count zymogen granules in 3-h stimulated cells because of the too-small dimensions of the granules present at that time.

**Membrane Measurements**

An attempt was made to quantify changing amounts of cell membrane of the cells during the 3 h of stimulated secretion. The best method is to measure whole cell perimeters in axially sectioned cells bordering the lumen, at each of the three intervals after the onset of stimulated secretion. However, because of the presence of numerous small interdigitations and lamellar processes in the lateral and basal cell membrane (Fig. 17), and microvilli at the luminal cell face, these estimations would have to be done on an ultrastructural level. Obviously, in ultrathin sections, a large variation in apparent cell perimeters can be expected, and hence a very large number of cells would have to be measured. For practical reasons another method was followed.

Using the phase-contrast microscope and 1-µm sections, a selection was made for areas composed of cells having the calculated mean number of zymogen granules characteristic for each particular stimulation time. After trimming, gold-colored sections (approximately 1,000 Å) were cut from these areas, and post-stained with lead acetate. For each pair of animals, 20 electron micrographs of cell membranes were taken at random, with 6 × 9 mm rollfilm and an instrumental magnification of 7,000. Using a photographic enlarger, the negatives were projected on a table at a final magnification of 65,000. With the aid of a curvimeter the following two measurements were performed: (a) the cell membrane with all its lamellar processes, infoldings, and microvilli, provided that continuity with the cell membrane could be seen; (b) the cell outline overlooking all those membrane extensions. The ratio of a and b (called “membrane ratio” in this article) was used as a measure of the “redundancy” of cell membrane at a given time after the application of secretogogue. The membrane ratio only applies to the cell surface area considered and does not of necessity concern the total amount of cell membrane per cell. The ratios were determined separately for the lateral-basal cell membrane and for the luminal membrane between the tight junctions.

**RESULTS**

**Sorbitol Experiments**

This investigation is based on the hypothesis that the contingent removal of cell membrane is accomplished by endocytosis. Since no information is available about the phase in the secretory cycle during which superfluous cell membrane is removed, we marked the extracellular space with labeled sorbitol, which is known to pass biological membranes with great difficulty (6). We then determined the time, after the onset of stimulated secretion, at which a maximal amount of sorbitol stays behind in the tissue slices after an extensive washing. Sorbitol ingestion, which is most likely effected by endocytosis, is in each case expressed as cpm per µg of DNA (called sorbitol index).

As Table I shows, 3 h after secretogogue injection, four of the five pancreases used revealed sorbitol indices higher than those of the respective controls. In addition, at this time the mean index value was the highest. It was concluded that endocytotic processes in pancreas are to be encountered most frequently 3 h after stimulation with secretogogue.

**Morphology of Stimulated Acinar Cells**

The structure of unstimulated pancreas cells is well known (Fig. 1). Structural changes arising during stimulated secretion correspond generally with
Pancreatic slices were incubated for 30 min at 37°C in medium containing [14C]sorbitol. The slices were washed in a medium containing a large excess of unlabeled sorbitol and homogenized in incubation medium.

**TABLE I**

**Ingestion of [14C]Sorbitol by Pancreatic Slices after In Vivo Stimulation of the Secretion Expressed in cpm per µg DNA**

| Experiment number | Time after injection of pilocarpine | Controls | 1 h | 2 h | 3 h | 4 h |
|-------------------|-------------------------------------|----------|-----|-----|-----|-----|
| 1                 |                                     | 13       | 8   | 10  | 9   |
| 2                 |                                     | 13       | 12  | 14  | 9   |
| 3                 |                                     | 15       | 16  | 19  | 15  |
| 4                 |                                     | 8        | 9   | 10  | 11  |
| 5                 |                                     | 6        | 12  | 7   | 7   |
| mean              |                                     | 11.4     | 11.4| 12.0| 9.8 |

* Pancreatic slices were incubated for 30 min at 37°C in medium containing [14C]sorbitol. The slices were washed in a medium containing a large excess of unlabeled sorbitol and homogenized in incubation medium.

**TABLE II**

**Zymogen Granule Counts in Medially-Sectioned Pancreas Cells after In Vivo Stimulation of the Secretion**

| Time after pilocarpine injection | Controls | 1 h | 2 h |
|----------------------------------|----------|-----|-----|
| Number of cells                  | 20       | 20  | 20  |
| Number of acini                  | 14       | 16  | 14  |
| Mean number of zymogen granules per cell | 35.4 | 8.1 | 3.5 |
| Standard deviation               | 8.8      | 7.0 | 2.8 |

A second change resulting from secretion stimulation was a decrease in granule diameter. This was particularly evident in 3-h stimulated cells (Fig. 4), for which precise granule counts consequently could not be made.

During the secretion period concerned, no apparent widening of the acinar lumina occurred. Furthermore, large diverticula of the lumina extending into the cytoplasm, as described for the guinea pig pancreas (7), were not observed.

**Electron Microscopy:** As apparent from the sorbitol experiments, the ultrastructure of pancreas cells 3 h after stimulation should especially reflect endocytosis. Consequently, in the following description attention will mainly be given to the cell membrane and adjacent cytoplasm of 3-h stimulated cells, containing the zymogen granule content, characteristic for this phase of the secretory cycle.

In the apical cytoplasm of these cells vesicles (coated and uncoated), vacuoles and extremely small zymogen granules are present. There is a gradual transition in vesicle content density and texture between the empty smallest vesicles and the electron-opaque, minute zymogen granules (Fig. 14). Frequently, condensing vacuoles were found closely opposed to the luminal membrane (Fig. 15). It appeared that the number of both small empty vacuoles and larger, more dense vacuoles increased with stimulation time.

As in unstimulated cells, the lateral and basal cell membranes of 3-h stimulated cells showed numerous lamellar processes approximately 0.1 µm thick and 2 µm long (Figs. 5 and 17). The processes mostly ran parallel to the cell membrane, piled in stacks which seemed to originate from multiple plications of the cell membrane. Most of these processes were present on the basal membrane and the lower third of the lateral cell membrane. The lateral processes interdigitate extensively with those of the neighboring acinar cells.

Apart from these lamellar processes, tubular or sheetlike infoldings, enclosing a narrow space, originate from the lateral cell membranes of stimulated cells (Figs. 7-10). Sometimes it can be seen that the infoldings are branched, or have the appearance of fenestrated cisternae. Most cell sections show one or two infoldings, the deeper parts of which appear to fragment into short tubules and vesicles, often forming ringlike configurations (Fig. 10).
Figures 1-4. Light micrographs of pancreatic tissue slices, incubated for 30 min in Krebs-Henseleit bicarbonate buffer. 1-μm thin Epon sections were stained with 1% methylene-azure II blue in sodium borate (15). Fig. 1, Unstimulated control gland. Figs. 2, 3, and 4, pancreas glands stimulated in vivo with pilocarpine for 1, 2, and 3 h, respectively. The number of zymogen granules decreases until 2 h after stimulation. After 3 h the cells seem to accumulate extremely small storage granules around the acinar lumina (L). Golgi complexes (GC) become more prominent in the stimulated tissue, which give the cell apices a foamy appearance. CAC, centro-acinar cell. × 4,200.
Zymogen granule quantification indicated that most secretion granules are discharged during the first 2 h of stimulated secretion, and hence during this time interval a large amount of granule membrane should be added to the cell membrane. In order to measure increases and contingent decreases in amounts of cell membrane, we introduced the concept of the membrane ratio (see Materials and Methods for details). The membrane ratio is a measure of the number and size of microvilli, lamellar processes, and infoldings whose membranes are seen to be continuous with the cell membranes. These ratios were determined for unstimulated and for 1-, 2-, and 3-h stimulated cells, and are listed separately for latero-basal and apical cell membranes in Table III.

At 1 and 2 h after stimulation, the mean latero-basal cell membrane ratios were significantly greater than those of the controls. During the next hour this mean ratio returned to the control value. These results show that an addition of granule membrane to the cell membrane is accompanied by an increase and a subsequent decrease in the number and/or length of cellular membrane infoldings and lamellar processes. As to these structures, the cell regains its normal shape during the third hour of stimulated secretion. In stimulated parotid cells a similar increase of processes and papillar infoldings has been described, without being discussed in direct connection with membrane reduction, however (3).

As is shown in Table III, the same trend can be found in the apical cell membrane. Here, increase and decrease in membrane ratio is caused by changes in membrane length and/or number of microvilli. The differences found are even greater, but, due to the relative scarcity of luminal membrane profiles to be found in only eight of the 20 micrographs used at each post-stimulation time, these results are less certain.

Localization of Ferritin

Ferritin penetrated the tissue as far as the center of the slices, showing a gradient from the periphery to the center.

Ferritin particles could be found throughout the extracellular space of the interstitium, in the intercellular clefs up to the junctional complexes, and in some acinar lumina. At the acinar cell bases most particles were trapped by the basal lamina. In the intercellular clefs ferritin was mainly associated with the lateral cell mem-

### Table III

*Measurement of Cell Membrane Length* in Thin Sections of Pancreas Cells after In Vivo Stimulation of Secretion

|                      | Time after stimulation |                      |                      |
|----------------------|------------------------|----------------------|----------------------|
|                      | Controls 1 h 2 h 3 h    | Controls 1 h 2 h 3 h  |
| Number of           |                         |                      |
| micrographs         | 20 20 20 20            | 8 8 8 8             |
| Mean membrane ratio | 1.27 1.62 1.64 1.24    | 1.75 2.28 2.36 1.76 |
| Standard deviation  | 0.12 0.42 0.39 0.17    | 0.45 0.53 0.63 0.46 |
| P value†            | — 0.01 0.01 >0.05      | — >0.05 0.05 >0.05  |
| Total length of     | 314 471 409 365        | 110 81 158 89       |
| measured membrane   |                         |                      |
| in microns          |                         |                      |

*Membrane lengths were determined at a magnification of 63,000, using a curvimeter in two ways: (a) by accurately tracing the membrane contour, and (b) by measuring the general outline of the cell. Membrane ratio = a/b.† The P values were calculated according to the two-sample test of Wilcoxon and refer to the differences between control cells and stimulated cells.
Figures 5-7  Electron micrographs of pancreatic tissue, stimulated in vivo for 3 h and incubated with ferritin in bicarbonate buffer for 30 min. Fig. 5, unstained section. Cell base showing ferritin particles associated with the basal lamina (BL), and with a moderately dense layer adjacent to the outer face of the cell membrane bordering a lamellar process (LP) and an incipient phagocytotic vacuole (V). X 55,000. Fig. 6, unstained section. Free phagocytotic vacuole (V) containing parietal, moderately dense material that is loaded with ferritin. CM, cell membrane. X 55,000. Fig. 7, unstained section. Ferritin is present in the intercellular cleft running diagonally across the picture, and in vesicles (arrows). The linear arrangement of the vesicles in the upper cell appears to reflect their formation by fragmentation of a cell membrane infolding at asterisk (*). Circular profiles of infoldings (If) can be seen in the lower cell. X 55,000.
Figures 8–9  Electron micrographs of pancreatic tissue, stimulated in vivo for 3 h and incubated with ferritin in bicarbonate buffer for 30 min. Fig. 8, section stained with bismuth nitrate. Two acinar cell bases are shown. Ferritin particles can be found in the interstitium between the acini, the basal lamina (BL), the infoldings (If), and in fragmentation vesicles and tubules. The stain has particularly enhanced the contrast of the cell coat present at the outer face of the cell membrane and the membranes of infoldings and vesicles. Some vesicles are unstained (arrows). × 80,000. Fig. 9, Section stained with bismuth nitrate. Ferritin is present in the intercellular cleft between three adjoining cells, and also in infoldings (If), tubules, and vesicles. The membranes of several tubules and vesicles containing ferritin are unstained by bismuth nitrate (arrows). × 80,000.
Figure 10  Electron micrograph of pancreatic tissue, stimulated in vivo for 3 h and incubated with ferritin in bicarbonate buffer for 30 min. Section stained with bismuth nitrate. The basal cell membrane is drawn into an infolding (asterisk) which extends deeply into the cytoplasm. Ferritin particles are present in the interstitium (I), basal lamina (BL), and in the infolding (arrows). To the right, a circular profile of an infolding can be seen. × 70,000.
branes, presumably with the external mucopolysaccharide coating of the cell.

In both stimulated and unstimulated cells the lamellar processes at the cell bases were apparently involved in phagocytosis. As can be seen in Fig. 5, ferritin is ingested by invagination of the cell membrane or by a turning back of lamellar processes to enclose small amounts of extracellular material. In this process the course of the basal lamina remains unaltered. The developing phagocytic vacuoles become isolated from the cell membrane by membrane fusion. The ferritin particles in the vacuoles have a parietal location in a moderately electron-opaque layer, probably representing basal lamina-derived material (Figs. 5 and 6). The free vacuoles had a diameter of approximately 0.5 µm. In cells incubated for 30 min, vacuoles could be found proximal to the nucleus, but most were located within a zone extending to 2 µm from the cell membrane (Fig. 6). This type of vacuole was occasionally found in tissue fixed immediately without incubation.

A remarkable feature of stimulated cells is that ferritin also penetrates the infoldings of the lateral-basal cell membrane which appear to fragment, deeper in the cytoplasm, into short tubules and vesicles (Figs. 7-10). Ferritin particles were found to be present in all of these structures, and ferritin-containing vesicles were encountered up to 3 µm from the cell membrane. These vesicles tend to cluster, and in 30-min incubated cells they have no constant topographical relationship to any particular organelle. In slices from which the extracellular ferritin was almost completely removed by washing, the vesicles and tubules and, to a lesser extent, the infoldings keep their ferritin content. The persistent presence of ferritin in free tubules and vesicles indicates that their bordering membranes originate from the cell membrane. This deduction is supported by bismuth staining patterns. In our hands, bismuth nitrate enhanced the contrast of cell membranes and the membranes of infoldings and fragmentation vesicles more than that of the other membranes (Figs 8-10). The most deeply located vesicles were less heavily stained (Fig. 9).

Ferritin was absent from most acinar lumina. Apparently, ferritin penetrates the acini by way of the interstitium, through the basal lamina into the intercellular cleft, and cannot pass the junctional complex (Fig. 13). In some acini at the margin of the tissue slices, however, ferritin had reached the lumina (Figs. 11 and 12). Most likely, ferritin could enter these lumina because the acini were cut during tissue slicing. It appeared that the ferritin particles were exclusively associated with filamentous material situated in the centers of the lumina. Between this material (which is probably coagulated secretory protein) and the cell membrane lies an electron-transparent, ferritin-free area accurately following the contours of the microvilli, such that particles never appear to adjoin the luminal membrane.

The 3-h stimulated cells have numerous ferritin-free vesicles, with either bristle-coated or uncoated membranes, in the cytoplasm adjacent to the luminal membrane. In Figs. 14 and 15 it can be seen that the vesicle diameters vary widely. The classification of apical vesicles is complicated in many cells by having the Golgi system located close to the luminal membrane. In these cases the apical vesicles are mixed with the vesicles of the Golgi system, which are also partly coated, partly uncoated.

In 3-h stimulated cells many fission or fusion patterns were found between vesicles and cell membrane (Fig. 16). The membranes of the pits, either coated or uncoated, show a bismuth nitrate pattern.
Figure 14  Electron micrograph of pancreas tissue stimulated in vivo for 3 h and fixed without previous incubation. Section stained with lead citrate. Several types of vacuoles and vesicles are present in the apical cytoplasm. (1) Condensing vacuoles with scattered flocculent material. Condensing vacuoles can always be distinguished from secretion canaliculi (SC) by not having surrounding terminal web filaments (TW). (2) Smaller vacuoles containing a more homogeneous electron-opaque material, resembling "prozymogen granules." (3) Mature zymogen granules with a homogeneous content. (4) Small, smooth-surfaced vacuoles and vesicles which can be stained with bismuth nitrate (see Fig. 13) and probably are endocytotic. (5) Coated vesicles. (6) Vesicles derived from infoldings. Note the extreme apical position of the Golgi complexes (GC). X 14,000.
FIGURE 15 Higher magnification electron micrograph of the same tissue as in the preceding figure. Section stained with lead citrate. For an explanation of the vacuole and vesicle enumeration, see the legend to Fig. 14. The condensing vacuole (1) seems to be about to fuse with the luminal cell membrane. Note the small diameters and irregular shape of some of the zymogen granules (3). GC, Golgi complexes; L, lumen; TW, terminal web. X 30,000.

FIGURE 16 Electron micrograph of the same tissue as the former two figures. Section stained with bismuth nitrate. At the arrows, pits reminiscent of endo( pinocytosis can be seen. The membranes of the vesicles have the same electron opacity as the luminal membrane. X 52,000.
FIGURE 17. Schematic drawing of a 3-h stimulated exocrine pancreas cell. BL, basal lamina; GC, Golgi complexes; IF, infoldings; JC, junctional complex; LP, lamellar processes; Ly, lysosomes; MV, microvilli; N, nucleus; 1, Golgi vesicles; 2, condensing vacuoles; 3, prozymogen granules; 4, small, mature zymogen granules; 5, apical vesicles and vacuoles reminiscent of endocytosis; 6, coated and uncoated pits; 7, coated vesicle; 8, fragmentation vesicles; 9, phagocytic vacuoles.

staining just as intense as those of the luminal membrane. The pits, however, were also devoid of ferritin particles.

In one experiment, 3-h stimulated tissue was incubated with ferritin during 1 and 2 h, respectively. As is shown in Fig. 13, after 1 h of ferritin incubation ferritin is already present in lysosomes, where it is located between whorls of membrane-like material. After both intervals smooth-surfaced, ferritin-containing vesicles were occasionally encountered in the vicinity of the lysosomes. However, pictures suggestive of fusion of vesicles with lysosomes were never observed. Though it was found that condensing vacuoles frequently bore a coated or uncoated vesicle, it is worthwhile to note that these vesicles never contained ferritin.

DISCUSSION
It has been long established that the exocrine pancreas cell secretes in a merocrine way, and hence must have a regulatory mechanism to compensate for redundant cell membrane after secretory granule discharge. Palade already in 1959 (8) and Hokin (see reference 14) suggested that this regulatory mechanism involves endocytosis. Since that time, it has been proposed for a variety of cell types that secretory granule membrane is withheld by the cells during secretion. In several ultrastructural studies, cell inclusions have been described which suggest an intake of cell membrane by means of endocytosis. However, the scarcity of these structures and an uncertainty about the origin of their content have handicapped an unequivocal interpretation of the membrane vehicle. We partly overcame these difficulties by studying cells with appropriate synchronized secretion, and by using extracellular space markers.

Rat pancreas cells were seen to discharge 80% of their zymogen granules during a 1 h in vivo stimulation with pilocarpine. The relatively large standard deviation of the mean number of storage granules, at this time, implies that the secretion stimulus has affected the cells unequally. Although after 2 and 3 h the cells had a more uniformly empty appearance, it was assumed that the cells did not undergo maximal cell membrane withdrawal simultaneously. In order to study cells in the same phase of the secretory cycle, a selection was made, for electron microscopy, of cells with the average granule content characteristic of that time interval after stimulation.

Changes in endocytotic activity during the secretory cycle were measured in bulk with sorbitol. This metabolically inactive sugar was preferred to other extracellular space markers because of its relatively small molecular size and consequently rapid penetration into the tissue (6). This permitted a short incubation period. Cells stimulated with pilocarpine in vivo keep their maximal stimulated state in vitro for only 30 min (unpublished results). So, a short incubation time offers a great advantage, apart from avoiding incubation artifacts.

It was found that 3 h after the application of pilocarpine all glands used showed a higher retention of sorbitol after washing than found in the controls. In order to find a satisfactory number of endocytotic profiles for studying ferritin localization, we used 3-h stimulated cells selected with a zymogen granule content typical for that time interval after stimulation.

When these cells were exposed to ferritin in vitro for 30 min, ferritin-containing vacuoles,
infoldings, and vesicles were found in the cytoplasm adjacent to the latero-basal cell membrane. Infoldings, vesicles, and occasionally vacuoles were also encountered in stimulated tissue that had been fixed without a previous incubation. However, the number of vacuoles increased considerably in the incubated slices of both stimulated and unstimulated pancreas. Therefore, they are interpreted as phagocytotic structures induced by the incubation conditions rather than by the secretion stimulus.

On the other hand, infoldings and fragmentation vesicles were extremely scarce in incubated control tissues but were frequently found in the 3-h stimulated cells (selected for their average zymogen granule content) of both incubated and unincubated tissue. The lateral and basal infoldings and vesicles were considered to be the vehicles transporting superfluous cell membrane to the interior of the cell. The following considerations have led to this conclusion. (a) The infoldings and vesicles contain ferritin; (b) They have the same staining affinity for bismuth nitrate as the cell membrane; (c) They are most frequently observed (highest membrane ratio) after 2 h of stimulation when most membrane has been added to the cell membrane (lowest number of zymogen granules per cell)2; (d) The fragmentation of the infoldings (3 h after stimulation), which results in a lower membrane ratio, is accompanied by a maximal sorbitol ingestion by the cells.

It is possible, of course, that sorbitol, as a relatively small and water-soluble molecule, can gain access to the apical cell membrane better than ferritin, so that a direct quantitative comparison of sorbitol and ferritin ingestion is not possible. However, both markers would have to enter the cells by means of endocytosis.

Zymogen granule discharge invariably occurs at the cell membrane at the luminal side of the junctional complexes. Hence, the present findings suggest that the junctional complex allows membrane flow, in spite of its fixed position and its well-known function to separate the lumen from the intercellular cleft. An alternative explanation could be that alterations of lateral membrane ratios are induced by a secretion-associated cell retraction towards the basal lamina (3). In that case the superfluous lateral cell membrane stored in lamellar processes would be internalized before the cell regains its normal stretched shape.

The total amount of cell membrane retracted by means of fragmentation of infoldings could not be quantitatively compared with the amount of granule membrane added to the luminal cell membrane. However, the relative abundance and small size of the fragmentation vesicles suggest a very efficient reduction of cell membrane.

To our knowledge, this type of vesicle formation from infoldings has never before been described for the pancreas, probably because pancreas studies have never before referred to the particular cells selected in this study. However, Fig. 6 in reference 10 shows smooth-surfaced vesicles beneath the basal cell membrane of the guinea pig pancreas cell, which resemble those described above.

In a recent article on stimulated motor end plates it was found that disappearance of synaptic vesicles was accompanied by the formation of complex infoldings of the presynaptic membrane associated with vesicles (11). These structures closely resemble those described in the pancreas cell, and may also be involved in withdrawal of excessive cell membrane.

Determination of membrane ratios at the luminal pole of the cells indicated that at least part of the granule membrane added to the cell membrane was initially stored in microvillous membrane. Presumably, a particular category of vesicles and vacuoles present in the apical cytoplasm also accounts for the membrane reduction during the third hour of stimulated secretion. This assumption is further supported by a similarity in bismuth staining intensity between the cell membrane and the membranes of some apical pits and vesicles. However, the importance of apical membrane withdrawal relative to that of the lateral cell membrane is unknown. A reliable classification of apical pits and vesicles as either endocytic or exocytotic is complicated because ferritin once it had penetrated the acinar lumen, never appeared in these structures. This negative finding can possibly be explained by the persistent separation of the ferritin from the cell membrane between the microvilli.

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2 The increase of membrane ratios can be explained by (a) a decrease of cell volume and/or by (b) addition of membrane to the cell membrane. Anyhow, it is induced by granule discharge. The subsequent decrease in membrane ratios is mainly accompanied by fragmentation of the infoldings (factor b) and not by an increase of cell volume (factor a) by apparent refilling with secretory granules.
In a previous investigation we found with light microscope autoradiography that, in in vivo stimulated cells, newly synthesized proteins pass to the lumen continuously, apparently without accumulating in storage granules (12). In the guinea pig pancreas cell stimulated for 3 h in vitro, the exportable protein is stored, before secretion, in electron-opaque, irregularly shaped granules (7). This type of "alternative" storage granule was never encountered during the present investigation. By contrast, our finding of condensing vacuole-like structures closely apposed to the apical cell membrane, with the Golgi complex in the vicinity, suggests that part of the vesicle-vacuole population is involved in secretory protein transport as well. Autoradiographic experiments are in progress to test this possibility.

Finally, the ultrastructure of tissue incubated with ferritin for 1 and 2 h was interesting with respect to the fate of the redundant cell membrane. After that time, lysosomes appeared to be studded with ferritin particles probably originating from both vesicles and vacuoles at the latero-basal cell face. Apparently, fusion had occurred between lysosomes and endocytic vesicles. During this process the membranes of both lysosomes and target organelles keep their structural integrity (13). Hence, the presence of ferritin in the lysosomes suggests that at least part of the ingested cell membrane constituents is degraded before its eventual reutilization. No ferritin-containing vesicles were found in the vicinity of condensing vacuoles. This could be in contrast to the data of Hokin (14) which suggest that the empty endocytic vesicles take up secretory protein again, without becoming part of a general membrane pool. Our findings would not conflict with some recent evidence obtained from stimulated parotid tissue (9), indicating that membrane proteins are not derived from cell membrane fragments returning to the membrane store in the Golgi area, but are synthesized de novo concomitantly with the exportable protein.

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REFERENCES

1. VIVEROS, O. H., L. ARQUEROS, and N. KIRSHNER. 1969. Mechanism of secretion from the adrenal medulla. V. Retention of storage vesicle membranes following release of adrenaline. Mol. Pharmacol. 5:542.
2. GRYNSZPAN-WINOGRAD, O. 1971. Morphological aspects of exocytosis in the adrenal medulla. Philos. Trans. R. Soc. Lond. Ser. B. Biol. Sci. 261:291.
3. AMSTERDAM, A., I. OHAD, and M. SCHRAMM. 1969. Dynamic changes in the ultrastructure of the acinar cell of the rat parotid gland during the secretory cycle. J. Cell Biol. 41:753.
4. BURTON, K. 1956. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of DNA. Biochem. J. 62:315.
5. ANSWORTH, S. K., and M. J. KARNOVSKY. 1972. An ultrastructural staining method for enhancing the size and electron opacity of ferritin in thin sections. J. Histochem. Cytchem. 20:225.
6. GOODFORD, P. J., and E. H. LEACH. 1966. The extracellular space of the smooth muscle of the guinea pig Taenia coli. J. Physiol. (Lond.) 186:1.
7. JAMIESON, J. D., and G. E. PALADE. 1971. Synthesis, intracellular transport, and discharge of secretory proteins in stimulated pancreatic exocrine cells. J. Cell Biol. 50:135.
8. PALADE, G. E. 1959. In Subcellular Particles. T. Hayashi, editor. The Ronald Press Company, New York, 64.
9. AMSTERDAM, A., M. SCHRAMM, I. OHAD, Y. SALOMON, and Z. SELINGER. 1971. Concomitant synthesis of membrane protein and exportable protein of the secretory granule in rat parotid gland. J. Cell Biol. 50:187.
10. JAMIESON, J. D., and G. E. PALADE. 1967. Intracellular transport of secretory proteins in the pancreatic exocrine cell. J. Cell Biol. 34:577.
11. KORNELIUSSEN, H. 1972. Ultrastructure of normal and stimulated motor end plates. Z. Zellforsch. Mikrosk. Anat. 130:20.
12. KRAMER, M. F., and C. POORT. 1968. Protein synthesis in the pancreas of the rat after stimulation of secretion. Z. Zellforsch. Mikrosk. Anat. 86:245.
13. LUCY, J. A. 1969. In Lysosomes in Biology and Pathology, 2. J. T. Dingle and H. B. Fell, editors. North-Holland Publishing Co., Amsterdam. 313.
14. HOKIN, L. E. 1971. In The Exocrine Pancreas. L. T. Beck and D. G. Sinclair, editors. J. & A. Churchill Ltd., London. 27.
15. RICHARDSON, K. C., L. JARETT, and E. H. FINKE. 1960. Embedding in epoxy resins for ultrathin sectioning in electron microscopy. Stain Technol. 35:213.