SYNTHESIS, INTRACELLULAR TRANSPORT, AND DISCHARGE OF SECRETORY PROTEINS IN STIMULATED PANCREATIC EXOCRINE CELLS

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ABSTRACT

Our previous observations on the synthesis and transport of secretory proteins in the pancreatic exocrine cell were made on pancreatic slices from starved guinea pigs and accordingly apply to the resting, unstimulated cell. Normally, however, the gland functions in cycles during which zymogen granules accumulate in the cell and are subsequently discharged from it in response to secretagogues. The present experiments were undertaken to determine if secretory stimuli applied in vitro result in adjustments in the rates of protein synthesis and/or of intracellular transport. To this intent pancreatic slices from starved animals were stimulated in vitro for 3 hr with 0.01 mM carbamylcholine. During the first hour of treatment the acinar lumen profile is markedly enlarged due to insertion of zymogen granule membranes into the apical plasmalemma accompanying exocytosis of the granule content. Between 2 and 3 hr of stimulation the luminal profile reverts to unstimulated dimensions while depletion of the granule population nears completion. The acinar cells in 3-hr stimulated slices are characterized by the virtual complete absence of typical condensing vacuoles and zymogen granules, contain a markedly enlarged Golgi complex consisting of numerous stacked cisternae and electron-opaque vesicles, and possess many small pleomorphic storage granules. Slices in this condition were pulse labeled with leucine-3H and the route and timetable of intracellular transport assessed during chase incubation by cell fractionation, electron microscope radioautography, and a discharge assay covering the entire secretory pathway. The results showed that the rate of protein synthesis, the rate of drainage of the rough-surfaced endoplasmic reticulum (RER) compartment, and the over-all transit time of secretory proteins through the cells was not accelerated by the secretagogue. Secretory stimulation did not lead to a rerouting of secretory proteins through the cell sap. In the resting cell, the secretory product is concentrated in condensing vacuoles and stored as a relatively homogeneous population of spherical zymogen granules. By contrast, in the stimulated cell, secretory proteins are initially concentrated in the flattened saccules of the enlarged Golgi complex and subsequently stored in numerous small storage granules before release. The results suggest that secretory stimuli applied in vitro primarily affect the discharge of secretory proteins and do not, directly or indirectly, influence their rates of synthesis and intracellular transport.

INTRODUCTION

In previous reports we have described the route, timetable, and metabolic requirements of the intracellular transport and discharge of secretory proteins in the guinea pig pancreatic exocrine cell.
(1–4). These studies were performed on an in vitro system of pancreatic slices derived from animals starved for 24 hr before use and represent the situation in the resting, unstimulated gland. Normally, however, the function of the gland appears to be cyclic, at least as far as discharge of secretory products is concerned. It consists of periods of relative starvation during which zymogen granules accumulate in the cell followed by periods of granule discharge initiated by the stimulus of feeding.

The morphologic aspects of secretory granule discharge from the acinar cells of the pancreas (5, 6) and the parotid (7) in response to feeding or administration of secretagogues in vivo have been the subject of several reports. At the same time, numerous biochemical studies have been concerned with the effects of zymogen discharge induction upon rates of synthesis of secretory proteins (8) and upon phospholipid (9) and nucleic acid (10, 11) metabolism in the pancreas. To our knowledge, however, no studies have been concerned specifically with the relationship (if any) between secretagogue action and adjustments in the rate, or changes in the route, of intracellular transport of secretory proteins, although it has been suggested that induction of discharge may result in the transport of secretory proteins through the cell sap (12–14).

The purpose of the present study is to evaluate, by means of radioautographic and cell fractionation procedures, the rate of synthesis and the pathway and timetable of intracellular transport and discharge of secretory proteins in pancreatic exocrine cells previously depleted of their complement of preformed zymogen granules by in vitro incubation with secretagogues.

**MATERIALS AND METHODS**

**General**

Male albino guinea pigs weighing 450–500 g, starved 24 hr with water given freely, were used in all experiments. The preparation of pancreatic slices, the incubation conditions, and the biochemical procedures used have been described before (1, 2). Tissues were fixed for electron microscopy in 1% OsO₄ in 0.1 M K phosphate buffer, pH 7.4, washed with 0.9% NaCl, and stained in block with 0.5% magnesium uranyl acetate in 0.9% NaCl before dehydration and embedding in Epon. Tissue samples to be used for radioautography were not treated with uranyl acetate. For light microscopy, 0.4 μ-

**In Vitro Stimulation of Zymogen Granule Discharge**

Pancreatic slices from starved animals were stimulated in vitro for (usually) 3 hr in a medium containing 0.01 mM carbamylcholine chloride or 10 units/ml pancreozymin (6000 Crick-Harper-Raper units/mg protein) to deplete the cells of preformed zymogen granules. These concentrations of secretagogues produced maximal secretory responses; higher concentrations appeared to be toxic, suppressing discharge, tissue respiration (unpublished observations), and the ability to incorporate amino acids into proteins. In all cases the incubation medium contained, in addition to the usual levels of amino acid supplement, either 0.04 mM or 0.4 mM leucine-H except indicated otherwise.

The secretory response of the slices was assessed by determining morphologically the extent of zymogen granule depletion and by measuring amounts of amylase discharged into the incubation medium. For the latter, the tissue and medium were separated by filtration and the medium centrifuged for 20 min at 190,000 g to remove debris. The tissue was homogenized in H₂O and dilutions of tissue and medium in 0.2% Triton X-100 buffered at pH 6.9 with 0.1 M Na phosphate were assayed for amylase activity. Secretion is expressed as the per cent of amylase in the incubation medium relative to the sum of amylase in tissue plus medium. The amount of amylase (or radioactivity—see Figs. 15 and 16) lost in the 190,000 g debris pellet is negligible.

**Assessment of Intracellular Transport**

**Radioautographic Studies:** After 3 hr stimulation of discharge in vitro in the medium described above (containing 0.04 mM L-leucine-¹⁴C), the slices were washed with a large volume of cold leucine-free medium, preincubated for 10 min at 0°C in secretagogue-free medium containing 200 μCi/ml of L-leucine-4,5-³H (58 Ci/m mole), and pulse labeled in the same medium for 3 min at 37°C. The slices were then washed with an excess of warm chase medium containing 4.0 mM leucine-¹⁴C and reincubated for a further 7, 17, 37, or 57 min in chase media.
medium containing the original concentration of secretogogue. At each time point the tissues were prepared for radioautography as previously described (16).

**Cell fractionation studies:** Intracellular transport was assessed in control and secretogogue-treated slices by determining the rate of loss of pulse-labeled secretory proteins from rough microsomal fractions or by measuring the appearance of labeled proteins in the zymogen granule fraction. As will be seen, the composition of the latter fraction is expected to change with time in the case of stimulated slices, and hence it is not strictly comparable to the corresponding fractions isolated from control slices. The content of soluble labeled proteins and of amylase in the postmicrosomal supernate was also determined. The cell fractionation procedures employed have been described in detail before (1, 2), except for the procedure used to obtain rough microsomes. For this purpose, total microsomal fractions were pelleted by differential centrifugation (190,000 g_{av} for 30 min) in an International Equipment Co. A 269 rotor and resuspended by hand in 1.30 M sucrose. Samples of the resuspension were sandwiched in a discontinuous sucrose gradient formed in an I.E.C. SB 283 centrifuge tube as follows: 1.5 ml cushion of 2.0 M sucrose; 3.5 ml 1.35 M sucrose; 1.5 ml microsomal resuspension; 2.0 ml 1.20 M sucrose and 0.3 M sucrose to volume. After centrifugation at 160,000 g_{av} for 10 hr, the band formed above the 2.0 M sucrose layer was collected. By previously described morphologic and chemical criteria this band consisted of highly purified rough microsomes.

**Assays:** The following assays were used: protein, Lowry et al. (17); DNA, the procedure of Burton (18) on hot perchloric acid (PCA);* extracts of the tissue (0.5 N PCA for 20 min at 70°C); amylase, Bernfeld (19) with amylase units as previously defined (20). All cell particulates and extracts for amylase assay were treated with 0.2% Triton X-100 buffered at pH 6.9 with 0.1 M Na phosphate to allow full access of the substrate to the enzyme.

**Materials:** All chemicals were reagent grade. L-leucine-4,5-3H, 44-58 Ci/mMole were obtained from New England Nuclear Corp., Boston, Mass., or from Schwarz Bio Research Inc., Orangeburg, N.Y. Carbamylcholine chloride and atropine sulfate were from Mann Research Labs. Inc., New York. Pancreozymin (CCK), 6000 Crick-Harper-Raper units/mg protein, was the kind gift of Professors Erik Jorpes and Viktor Mutt, of the Karolinska Institute, Stockholm, Sweden.

*This gradient was developed in our laboratory by Mr. A. M. Tartakoff.

Abbreviations used: dpm, disintegrations per minute; PCA, perchloric acid; RER, rough-surfaced endoplasmic reticulum; TCA, trichloroacetic acid.

**RESULTS**

**Morphology of In Vitro Stimulation**

Since the morphologic aspects of zymogen granule discharge induced in pancreatic slices in vitro are similar to those observed in vivo both for the pancreas (5, 6) and the parotid (7) only a brief description of the in vitro process will be given here. In all cases it refers to slices stimulated with carbamylcholine; similar but less consistent results were obtained with pancreozymin.

At the light microscope level, zymogen granule discharge is expressed after 30 min stimulation (Fig. 1) by the formation of numerous pale-staining diverticula and apically located vacuoles which can be shown to be continuous with the acinar lumen and which are the result of the fusion of the granule membrane with the apical plasmalemma. As granule discharge proceeds (Fig. 2), these diverticula coalesce and extend deeply into the cell (1 hr), resulting in a marked increase of the luminal surface. After 2 hr of stimulation the acinar lumen, though still somewhat enlarged in occasional acini, is in general considerably smaller in dimensions than at 1 hr (Fig. 3). The over-all population of granules in the tissue is significantly decreased at this time but the process is not synchronous in all cells. After 3 hr of stimulation (Fig. 4) degranulation is almost complete with only a few residual granules still persisting adjacent to the apical plasmalemma. By now the cells are noticeably shorter in height and generally have more rounded profiles (with truncated apices) than the cells from unstimulated slices (Fig. 5).

Of particular note is the finding that, in the face of continuing stimulation, the circumference of the acinar lumen at 3 hr is no longer enlarged; it is in fact similar in dimensions to that in the unstimulated tissue, although as will be noted later, the cells continue to synthesize and discharge secretory proteins at near maximal rates.

For the purposes of the present study, the electron microscopic appearance of the exocrine cells after 3 hr of stimulation is relevant especially as it pertains to the elements of the Golgi complex. Whereas in the unstimulated cell the Golgi complex consists primarily of numerous small, smooth-surfaced vesicles adjacent to the transitional elements of the rough-surfaced endoplasmic reticulum (RER), a few peripherally disposed stacks of flattened cisternae, and a number of centrally located condensed vacuoles (e.g., Fig. 7 in refer-
ence 1), in the 3 hr-stimulated cell (Figs. 6 and 7), the elements of the Golgi complex are markedly increased in number and occupy most of the apical third of the cell previously populated by zymogen granules. Particularly striking is the increase in the number of stacked cisternae, up to ~10 such stacks often being seen in favorable sections cut through the Golgi region (Fig. 11). These stacks are arranged in a roughly crescentic fashion around the periphery of the Golgi region and in general are oriented such that their concave surfaces face centrally. Up to ~ four to six cisternae comprise a stack, and in most cases the innermost one or two cisternae on the concave face are filled with electron-opaque material (Figs. 6, 11, and 12). In addition to cisternal elements, the complex contains an abundance of small vesicles of both the smooth and coated type (Figs. 6 and 7). The smooth vesicles are fairly uniform in diameter (~45 nm) and are primarily located in a zone between the RER transitional elements and the Golgi stacks, while the coated vesicles are found mainly adjacent to the inner or concave aspect of the stacks or more centrally located in the Golgi region. The coated vesicles are occasionally in continuity with the innermost cisternae of the Golgi stacks, possess a rather wide size range, and frequently contain electron-opaque material. Centrally located in the Golgi complex and frequently adjacent to the concave surface of the Golgi cisternae are variable but usually large numbers of spherical or irregularly shaped storage granules (Figs. 6, 11, 12, and 13). These storage granules range widely in size and possess a content of variable electron opacity approaching, in some instances, that seen in the filled Golgi cisternae and mature zymogen granules. Frequently their limiting membrane is of the coated variety, suggesting that they may represent one extreme of the size gradation of the coated vesicles noted above. This is in contrast to the usual size-discontinuity between the small vesicles of the Golgi periphery and condensing vacuoles seen in exocrine cells from nonstimulated slices. Small storage granules also populate the apical cytoplasm between the Golgi region and the acinar lumen and often lie in close proximity to the apical plasmalemma (Figs. 12 and 14). Condensing vacuoles of the type usually seen in the Golgi region of nonstimulated exocrine cells are absent. Autophagic vacuoles containing remnants of cell debris, intracisternal granules, and small secretory granules were often seen in the Golgi region of stimulated cells (Figs. 11 and 13); increased numbers of autophagic vacuoles have also been noted in pancreatic exocrine cells following in vivo stimulation (6, 22, 23).

While the changes noted above were especially evident after 3 hr of stimulation in vitro, condensation of secretory material in Golgi cisternae and coated vesicles and formation of small, irregularly shaped secretory granules was apparent as early as 30 min after the onset of stimulation. Increase in the volume of the Golgi complex was noted after 1 hr of stimulation and reached maximum proportions after 3 hr of stimulation.

The disposition of other elements in the cell was not affected by in vitro stimulation except for an increase in the number of intracisternal granules in the RER cisternae of many of the cells. These granules were originally described by Palade (21).
who observed that they accumulate in guinea pig pancreatic exocrine cells ~2 hr after refeeding starved animals. A similar increase in the number of intracisternal granules was recently observed by Kern and Kern (22) after treatment of guinea pigs with cobalt chloride which appears to act as a potent stimulant for zymogen discharge.

Biochemical Changes Accompanying Discharge

EFFECT OF CARBAMYLCHOLINE ON INCORPORATION OF LEUCINE-^3H INTO PROTEIN: Since the purpose of this study was to evaluate the kinetics and pathway of the transport of secretory proteins in cells depleted of preformed zymogen granules, it was important to evaluate quantitatively the ability of such stimulated cells to incorporate amino acids into proteins. Preliminary experiments indicated that in slices incubated for 3 hr without or with carbamylcholine in media supplemented with all amino acids except leucine (which was supplied in trace amounts) net incorporation began to slow down between 1 and 2 hr of incubation and ceased thereafter, most likely due to exhaustion of the endogenous leucine pool in the slices (Fig. 8, insert; see also reference 1). Consequently, the dependence of protein synthesis on leucine concentration in the medium was investigated in control and carbamylcholine-stimulated slices. The other amino acids in the medium were supplied in the usual concentrations (1). The results are shown in Fig. 8 and are expressed as μmole leucine incorporated per milligram tissue DNA. Because stimulated slices discharge into the medium up to ~45% of the label incorporated during 3 hr of continuous incubation, leucine incorporation was measured in all cases in the combined proteins of tissue and incubation medium. At concentrations ranging from 0.04 mM to 4.0 mM, incorporation of leucine was approximately linear over the 3 hr period although it was consistently slightly less in stimulated slices. The

4 This slight difference in incorporation efficiency tended to disappear when the concentration of carbamylcholine in the medium was progressively lowered to levels producing threshold secretory responses (10^-7 M). No stimulation of incorporation was noted at any of the lower concentrations of carbamylcholine tested.

Figure 6 Low-magnification electron micrograph of the apical third of a pancreatic exocrine cell incubated in vitro for 8 hr with 0.01 mM carbamylcholine. Note the many small, empty smooth-surfaced vesicles at the periphery of the Golgi region (Gv) as well as the numerous profiles of stacked Golgi cisternae (Gc), some of which contain electron-opaque material (Gc'). Arrows denote small storage granules bounded by a coated membrane. Smooth-surfaced vesicles are also found in the cell apex (av). tr, transitional elements of the RER; sg, small secretory granules; L, acinar lumen. × 12,500.
data also show that the net incorporation of leucine-3H was proportional to the absolute concentration of leucine in the medium over the range tested.

Due to the scarcity of highly purified pancreozymin, the effect of this secretagogue on in vitro protein synthesis was investigated only in medium containing 0.4 mM leucine. As seen in Fig. 8, incorporation was depressed significantly after 1 hr of incubation.

No differences between carbamylcholine-stimulated and unstimulated slices were noted in the radioactivity of the tissue trichloroacetic acid (TCA)-soluble fraction which represents in part the soluble unincorporated pool of isotope in the slices. In all cases the TCA-soluble radioactivity remained constant throughout 3 hr of incubation (data not given), having reached plateau levels as early as 5–10 min after the beginning of the incubation (1). About 80% of the acid soluble label in the slices and ~95% in the incubation medium co-chromatographed with authentic L-leucine throughout the 3 hr incubation.

Based on these findings, all preincubations which were to be followed by pulse labeling with leucine-3H were performed in media containing 0.04 mM L-leucine-3H. This concentration enabled the slices to maintain nearly linear rates of incorporation over the 3 hr of the preincubation, yet allowed us to pulse label them with leucine-3H and still obtain a 100-fold increment of leucine-3H (4.0 mM leucine) in the chase medium. Varying the leucine concentration over this range did not affect secretory rates or the morphologic appearance of the slices. All morphologic studies were performed on slices incubated with 0.4 mM leucine.

DISCHARGE OF AMYLASE TO THE INCUBATION MEDIUM: In addition to the morphologic assessment of zymogen granule depletion, we measured amylase discharge from the slices to the medium in response to carbamylcholine and pancreozymin. As shown in Fig. 9, during the first 30 min of incubation with or without stimulation, a large amount of amylase appears in the medium most likely as a result of the initial rapid washout of enzyme already present in the duct system of
incubation, while the response to pancreozymin is less well sustained. Between 3 and 5 hr of incubation with the stimulants the rate of discharge decreases noticeably. The maximum net amount of amylase which can be discharged from the slices by carbamylcholine over 5 hr is ~48% of that initially present. We assume that this amount is accounted for mainly by the content of the zymogen granules which are absent or greatly reduced in number after 3 hr of stimulation (e.g., Figs. 4 and 6).

During the first 2 hr of stimulation, the amylase/DNA ratio in the stimulated slices fell from a pre-stimulation value of ~1200 units/mg DNA to ~600 units/mg DNA. Thereafter the ratio remained constant at ~600 units/mg DNA up to 5 hr of stimulation despite the fact that amylase output to the medium was continuing (e.g., Fig. 16).

The enzyme activity appearing in the medium from control slices primarily represents progressive leakage from nonviable cells since this type of amylase discharge is not strongly blocked by conditions which inhibit metabolism in contrast to resting discharge of labeled proteins from pulse-labeled slices (Fig. 17).

Route and Kinetics of Intracellular Transport in Stimulated Exocrine Cells

**Radioautographic Studies:** The route of the intracellular transport of secretory proteins in exocrine cells previously depleted of zymogen granules during 3 hr in vitro stimulation with carbamylcholine was assessed by electron microscope radioautography. For this purpose, the pre-stimulated slices were pulse labeled with leucine-3H for 3 min as described under Methods and then reincubated in chase medium containing 0.01 mM carbamylcholine for a further 7, 17, 37, or 57 min. After processing of the tissue for radioautography, a series of low-magnification electron micrographs of exocrine cells was taken and the percentage distribution of radioautographic grains determined over the following cellular constituents known or presumed to be involved in the transport pathway: (a) cisternae of the RER, (b) the peripheral region of the Golgi complex including its small vesicles and stacked cisternae, and (c) the small storage granules.

The inset gives incorporation data for carrier-free leucine-3H in the medium (~0.09 μm L-leucine) the slices. Thereafter, the response to carbamylcholine is approximately linear up to 3 hr of

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**Figure 8** Time course of incorporation of leucine-3H into total slice protein in the absence and presence of 0.01 mM carbamylcholine or 10 units/ml pancreozymin. The incubation medium was supplemented with a complete set of amino acids and contained the indicated concentrations of L-leucine-3H and 5 μCi/ml of L-cis-3H (58 Ci/mmole). After the indicated times of incubation, the slices and incubation medium were separated. The medium was centrifuged at 190,000 g for 30 min to remove debris and portions of the supernate were precipitated with 10% TCA (final concentration) after adding 1 mg/ml carrier bovine plasma albumin. The slices were homogenized in water and samples of the homogenate were precipitated with 10% TCA. The TCA precipitates of slices and medium were washed three times with cold 5% TCA before determining the incorporated radioactivity. DNA was determined on samples of the homogenates precipitated with 0.5 N PCA. Based on the disintegrations per minute (dpm) found in proteins in the medium plus slices, and knowing the specific radioactivity of leucine-3H in the medium, the molar incorporation of leucine was calculated. The inset gives incorporation data for carrier-free leucine-3H in the medium (~0.09 μm L-leucine).

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*From the shape of the discharge curves this amount can be estimated to represent 8–10% of the total amylase content of the slices before stimulation.*
FIGURE 9 Discharge of amylase to the incubation medium in response to 0.01 mM carbamylcholine or 10 units/ml pancreozymin. Controls consisted of slices incubated without secretagogues at 37°C or at 0°C with or without 0.01 mM carbamylcholine.

TABLE I

Distribution of Radioautographic Grains over Cell Components in Prestimulated Pancreatic Slices Incubated Postpulse with Carbamylcholine

| Subcellular component          | % of radioautographic grains |
|-------------------------------|-----------------------------|
|                               | 3 min (pulse) | +7 min | +17 min | +37 min | +57 min |
| Rough endoplasmic reticulum   | 90.4          | 54.2   | 44.7    | 37.3    | 25.8    |
| (89.1)                        |               | (49.5) | (38.4)  | (24.5)  | (16.2)  |
| Periphery of the Golgi region  | 8.7           | 35.6   | 28.5    | 27.0    | 18.3    |
| Storage granules               | 0.9           | 10.2   | 26.8    | 35.7    | 55.8    |

Sets of pancreatic slices were stimulated for 3 hr before labeling by incubation in a medium containing 0.01 mM carbamylcholine and 0.04 mM L-leucine-1H. They were then washed with leucine-free medium and kept for 10 min at 4°C in a carbamylcholine-free medium containing 300 μCi/ml carrier-free L-leucine-4,5-3H (58 Ci/mmole). For pulse labeling the slices were brought up to 37°C for 3 min; at the end of the pulse one set was fixed and the others were further incubated for the times shown under resumed stimulation in a chase medium containing 4.0 mM L-leucine-1H and 0.01 mM carbamylcholine. Radioautographic grains appearing over mitochondria and nuclei are not included as they represent a variable and small proportion of the total cellular label (~0-4%). For reference, the per cent distribution of grains over the RER in unstimulated slices is shown in parentheses. (Data taken from our previous studies [2]).

The results are given in Table I and illustrated in Figs. 10-14. At the end of the pulse, the large majority (~90%) of the grains marked labeled proteins associated with elements of the RER (Fig. 10). During a subsequent 7 min chase period a large proportion (~36%) of the labeled proteins migrated to the periphery of the Golgi region where it was associated primarily with the small
FIGURE 10 Distribution of radioautographic grains over stimulated exocrine cells at the end of a 3 min pulse-labeling with leucine-$^3$H. The majority of the grains mark elements of the RER. G, elements of the Golgi complex. $\times$ 18,000.
electron-lucent vesicles located adjacent to the RER transitional elements and to a lesser extent with the outermost stacked cisternal elements devoid of electron-opaque material (Fig. 11). After 17 min chase incubation (Fig. 12), the labeled proteins moved more centrally within the complex, where they were associated with the stacked Golgi cisternae containing electron-opaque material and with some small storage granules. By 37 min (Fig. 13) and especially after 57 min of chase (Fig. 14) the label was mainly (up to ~56%) found in association with small storage granules, especially those in close proximity to the apical plasmalemma. For reference, the per cent distribution of radioautographic grains over the RER in unstimulated cells is given in parentheses (data from reference 2).

From these results it is clear that the wave of labeled proteins passes sequentially from the RER cisternae to the peripheral elements of the Golgi complex and from there directly to the small storage granules. According to the data, the rate of drainage of labeled proteins from the RER compartment is either not enhanced or slightly depressed in stimulated cells as compared to resting controls. In contrast to the latter, however, the stacked Golgi cisternae are significantly involved in the transport and concentration of secretory proteins and the innermost (central) cisternae appear to occupy in the transport pathway a position which is equivalent to that of condensing vacuoles in unstimulated cells (2).

As will be shown later (Table II), >80% of the pulse-labeled proteins are associated with sedimentable cell particulates at the end of the pulse and throughout the chase incubation, indicating that the radioautographic response marks mainly proteins in transit through membrane-bounded compartments of the cell, as is known to be the case in cells from unstimulated slices.

**Cell Fractionation Studies:** In parallel with the radioautographic studies, intracellular transport was also assessed by cell fractionation procedures applied to carbamylcholine-prestimulated slices which had been pulse labeled with leucine-3H for 3 min and subsequently incubated for 37 min in chase medium containing 0.01 mM carbamylcholine. Controls consisted of slices treated in the same way except that the secretagogue was omitted from both the preincubation and the chase medium. Transport efficiency was measured either by determining the decrement in the total or specific radioactivity of proteins in the rough microsomal fraction during the 37 min chase period (which gives a measure of the rate of drainage of the RER compartment), or by determining the amount of labeled proteins accumulating during the same interval in the total zymogen granule fraction. Although the complement of typical mature zymogen granules in the prestimulated slices has been largely lost, the small storage granules formed during stimulation are partly recovered (most likely due to the high density of their content) in the pellet obtained by the usual differential centrifugation procedure for the isolation of zymogen granules. Finally, the amount and specific radioactivity of labeled proteins in the postmicrosomal supernatant was measured. These data should give a rough estimate of transport through the cytoplasmic matrix since the postmicrosomal supernate contains (among others) the soluble proteins of the cell sap.

The results of a typical cell fractionation experiment, given in Table II, show that the relative rate of decrease of specific and total protein radioactivity in the rough microsomal fraction during 37 min chase incubation is not changed in the stimulated slices indicating, in agreement with the radioautographic studies, that stimulation does not enhance drainage of the RER compartment. Likewise, the increase in the specific radioactivity of proteins in the zymogen granule fraction is not changed by stimulation. The table also shows that the specific and total protein radioactivity in the postmicrosomal supernatate is comparable for stimulated and control slices, though slightly higher in the former. These data and the finding that the per cent of amylase recovered in the supernate is identical for stimulated and control slices (~15% of the total homogenate) do not support the assumption that the secretagogue enhances transport through the cell sap, i.e., the alternative pathway proposed by several authors (12-14). We should point out that since the supernatant fraction

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6 The specific radioactivity of the labeled proteins in cell fractions has been normalized to the activity of a specific secretory protein, amylase, rather than to total protein. This gives a more reliable estimate of the specific radioactivity of exportable proteins since membrane proteins, ribosomal proteins, etc. would not be expected to be labeled significantly during the short pulse used (24) yet comprise a substantial portion of the total proteins of cell fractions, especially purified rough microsomes.
Distribution of radioautographic grains over a stimulated cell after 7 min chase incubation. Several of the grains overlie small vesicles at the periphery of the Golgi region (Gv) and the outermost empty cisternae of the Golgi stacks (Gc). Saccules of the concave face of the stack contain electron-opaque material (arrows) similar in density to that seen in the small storage granules (sg). Note the multivesicular body which contains dense vesicles (asterisk), ly, presumed lysosome or autophagic vacuole containing cell debris, and possibly intracisternal granules. $\times$ 15,000.
Figure 12  Radioautogram of the apical portion of a stimulated exocrine cell after 17 min chase incubation. At this time the silver grains are heavily concentrated over the parallel cisternae of the Golgi complex (Gc) containing electron-opaque material. The more peripherally located small vesicles (Gv) are less highly labeled at this time. Some label has already become associated with small secretory granules (sg). L, acinar lumen; id, intercalated duct cell. X 15,000.
Figure 13 Radioautogram of a stimulated cell after 37 min chase incubation. Accumulation of silver grains over small secretory granules (sg) is evident. Z, mature zymogen granule; ly, lysosome or presumed autophagic vacuole. × 14,000.
FIGURE 14  Radioautogram of a stimulated cell after 57 min chase incubation. Label is confined mainly to small secretory granules at the cell apex although a few grains persist over secretory granules and other vesicular elements in the center of the Golgi complex. L, acinar lumen. × 18,000.
TABLE II
Effect of Preincubation with Carbamylicholine on Intracellular Transport

| Preincubation | Pulse (L-leucine-\(^{3}H\)) | Conditions (4.0 mM L-leucine-\(^{3}H\)) | Chase incubation | Distribution of radioactivity |
|---------------|------------------------------|----------------------------------------|-----------------|------------------------------|
|               |                              |                                        | Homogenate      | Rough microsomal fraction    | Zymogen granule fraction    | Postmicrosomal supernatant |
|               |                              |                                        | dpm/U amylase   | % dpm                        | dpm/U amylase               | % dpm                        | dpm/U amylase               | % dpm                        |
| 3 hr control  | 3 min 0 min (pulse)          |                                        | 27,000          | 100*                         | 67,800                      | 23.2                         | 618                          | 0.4                          | 12,800                      | 14.7                         |
| 3 “ “         | 3 “ 37 min, control          |                                        | 26,800          | 100                          | 23,700                      | 9.3                          | 16,700                      | 21.8                         | 9,600                       | 15.8                         |
| 3 hr, 0.01 mM carbamylicholine | 3 “ 0 min (pulse)          |                                        | 15,500          | 100‡                         | 33,200                      | 18.4                         | 376                          | 0.3                          | 14,700                      | 16.4                         |
| 3 hr, 0.01 mM carbamylicholine | 3 “ 37 min, 0.01 mM carbamylicholine |                                        | 18,300          | 100                          | 23,800                      | 9.0                          | 12,900                      | 9.6                          | 13,400                      | 17.0                         |

Sets of pancreatic slices (400-500 mg wet wt) were stimulated and labeled as for Table I except that the concentration of L-leucine-4,5-\(^{3}H\) for pulse labeling was reduced to 20 \(\mu\)Ci/ml. At the end of the pulse one set each of control and prestimulated slices was fractionated. The remaining control set was incubated for a further 37 min in chase medium containing 4.0 mM L-leucine-\(^{3}H\), while the remaining prestimulated set was incubated for the same time in the same chase medium containing 0.01 mM carbamylicholine. At the end of the chase, each set of slices was homogenized for cell fractionation. The data are expressed as % TCA-precipitable radioactivity recovered in the cell fractions or as specific radioactivity (dpm per unit amylase). The data are meant to show the relative changes of radioactivity in the cell fractions with time. Only the figures for the postmicrosomal supernatant represent complete recovery. Recovery of rough microsomes from the slices was estimated to be \(\sim 23\%\); recovery of the zymogen granule fractions from unstimulated slices is \(\sim 40\%\) and is indeterminate in the case of stimulated slices.

* 100% = \(11.4 \times 10^6\) dpm.
‡ 100% = \(7.5 \times 10^6\) dpm.
is quantitatively recovered in our cell fractionation procedure, more than 80% of the labeled proteins in transit through the cell must be associated with sedimentable cell particulates.

Using the same experimental approach we found that transport was not accelerated in slices obtained from animals stimulated to secrete in vivo by a combination of refeeding and carbamylcholine injections (data not given).

**Discharge Assay:** The above results suggest that carbamylcholine stimulation does not affect the rate of intracellular transport of secretory proteins. To check on this suggestion by other means, and to assess quantitatively the net output of secretory proteins from prestimulated slices during subsequent reincubations, the following experiments were performed. Sets of slices were preincubated in vitro without (Fig. 15) or with (Fig. 16) 0.01 mM carbamylcholine for 3 hr, after which they were placed in fresh medium lacking secretogogue, and pulse labeled for 4 min with leucine-3H. After a brief wash with chase medium, sets of both prestimulated and nonprestimulated slices were further incubated in 20 ml chase medium containing 0.01 mM carbamylcholine. Secretion controls consisted of nonprestimulated and prestimulated slices incubated for the same times postpulse in chase medium to which 0.1 mM atropine was added to antagonize the effect of residual carbamylcholine. At the indicated times 5-ml portions of the incubation media were removed, and immediately replaced with the same volume of appropriate fresh medium. The portions were centrifuged and assayed for protein radioactivity (see legend to Fig. 8) and amylase. At the end of the experiment the protein radioactivity remaining in the slices was measured and the results expressed as per cent of discharge to the medium of labeled proteins and as units of amylase accumulating in the medium.

Plots of the data during 2 hr of chase are given in Figs. 15 and 16. It can be seen that label begins to appear in the medium after about a 20 min delay from slices stimulated only after the pulse as well as from slices stimulated both before and after the pulse. Thereafter the labeled secretory proteins accumulate in the medium at similar linear rates. This results confirms the cell fractionation and radioautographic data and indicates that both the minimum and average times for transport of labeled proteins over the RER-acinar lumen pathway are unaffected by the secretogogue. Similar lag times were obtained using graded doses of carbamylcholine down to levels (10^-5 M) producing threshold secretory responses.

From the curves for amylase discharge in Figs. 15 and 16 it is also apparent that prestimulated slices continue to discharge enzyme during the 2 additional hr of stimulation, although the net output is only ~one-third that from slices stimulated only postpulse. Since the per cent discharge of radioactive proteins from the slices in the two conditions is about the same, the relative specific radioactivity of proteins in the medium from slices stimulated both before and after the pulse is from 2 to 4.5 times greater than that from slices stimulated only postpulse. This difference can be taken as an indication of the size of the pool of secretory proteins contained in zymogen granules in nonprestimulated slices, i.e., the granule pool at the onset of linear discharge of labeled proteins (45 min postpulse) is ~4.5 times larger than that represented by small storage granules in prestimulated slices.

Given the above considerations on the pool size of secretory proteins in zymogen granules, and keeping in mind that the lag time for discharge of labeled proteins from slices in the two conditions is the same, we can tentatively conclude that discharge of zymogen granules must be random. For otherwise, if discharge of old unlabeled granules were to precede that of newly formed labeled granules, the lag time for the appearance of labeled proteins in the medium would be considerably longer in slices stimulated only postpulse. Although it might be argued that our results could be explained by early discharge of highly labeled condensing vacuoles, we have never observed this to occur in any of our previous radioautographic studies.

The discharge of labeled proteins from nonstimulated slices (Fig. 17) deserves comment. Previously we had concluded that this type of discharge was accounted for by leakage from damaged cells because of its insensitivity to respiratory inhibitors over a 30 min period (23). In the present study, however, in which longer incubation times were employed, ~8% of the pulse-labeled proteins appeared in the medium from nonstimulated slices and up to 75% of this discharge was sensitive to conditions of metabolic inhibition applied to the slices either immediately postpulse (Fig. 17 a) or at a time when the labeled proteins had already reached the storage granules (Fig. 17 b). In view
of the energy requirements for induced discharge, we assume that the majority of the label appearing in the medium from the unstimulated slices may be equivalent to "starvation" secretion known to occur in the resting gland in vivo (41). Upon subtraction of the protein radioactivity from slices incubated postpulse at 0°C, it is evident that the lag time for spontaneous discharge at 37°C is again ~30 min. Our previous radioautographic studies on resting slices which demonstrated labeled proteins in the duct system of the gland at a time when the granule population was heavily labeled (e.g., Fig. 9 in reference 2) are consistent with the above findings. Because atropine is with-

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**Figures 13 and 16** Effect of preincubation in control medium (Fig. 15) or carbamylcholine-containing medium (Fig. 16) on induced discharge of labeled proteins and amylase. Preincubation media were supplemented with a complete set of amino acids including 0.04 mM L-leucine-½H; the pulse medium contained 80 µCi/ml L-leucine-½H (38 Ci/m mole, carrier free), and the chase media were 2.0 mM in L-leucine-½H and contained 0.01 mM carbamylcholine. The values given in the graphs represent the net per cent discharge of protein radioactivity and of amylase in response to carbamylcholine and have been corrected for discharge from secretion controls which were incubated postpulse in chase medium containing 0.1 mM atropine (e.g., Fig. 17). For Figs. 15 and 16, 100% protein radioactivity = 2.8 X 10^6 dpm and 2.8 X 10^6 dpm, respectively. The wet weights of the sets of slices were identical at the beginning of the preincubations. The numbers in parentheses give the relative specific activities of amylase in the medium: per cent dpm discharged per unit amylase discharged. The data in these figures are representative of four identical experiments.

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effect on resting discharge we assume that this type of secretion may be either spontaneous or mediated by endogenous digestive hormones other than cholinergic agents still present in the slices. The small, noninhibitable discharge of labeled proteins from unstimulated slices presumably comes from cells damaged postpulse which rapidly leak their contents to the medium. These findings should be compared to the discharge of amylase from unstimulated slices which, based on its insensitivity to metabolic inhibition, comes primarily from cells damaged during slice preparation (Fig. 9). Such damaged cells apparently do not incorporate amino acids into proteins during the pulse and hence do not interfere with the assay of discharge of labeled proteins.

**DISCUSSION**

**Morphologic Features of Zymogen Granule Discharge**

The general morphologic features of zymogen granule discharge from in vitro-stimulated pancreatic slices are generally similar to those observed after in vivo stimulation of the gland either by refeeding starved animals (5) or by administration of secretagogues (6). In vitro stimulation, however, allows us to apply secretagogues for times and at concentrations in excess of those tolerated by the intact animal and as a consequence, the degree of granule depletion is more complete. Under these conditions the main difference noted was that the elements of the Golgi complex become increased in volume and membrane amount especially at the stage of almost complete degranulation. Increase in volume of the Golgi complex after in vivo stimulation of the canine exocrine pancreas with pancreozymin has been noted in passing by Ribet et al. (23); recently Kern and Kern (22) reported identical morphologic changes in the Golgi complex of pancreatic exocrine cells after in vivo treatment of guinea pigs with cobalt chloride.

Amsterdam et al. (7) have described the morphologic changes which accompany discharge of secretory granules from rat parotid (whose exocrine cells are similar in organization to those of the exocrine pancreas) stimulated in vivo with isoprenaline. These authors showed that secretory
granule discharge is accompanied by massive enlargement of the surface area of the acinar lumina due to insertion of granule membranes into the apical plasmalemma. In their case, granule discharge was virtually complete after \( \approx 50 \) min stimulation, and restitution of the acinar lumens to normal dimensions was completed \( \approx 2 \) hr after cessation of stimulation. In the present study a similar but less marked increase in the surface area of the acinar lumens was seen to accompany zymogen granule discharge. Restoration of the acinar lumens to normal dimensions in in vitro-stimulated pancreatic slices began \( \approx 2 \) hr after the onset of stimulation and was complete at 3 hr even though induced discharge of secretory proteins was continuing. Further, restitution of the luminal profile to normal size continued to completion when the secretory stimulus was abruptly terminated by the addition of atropine to the slices at a time when the acinar lumens was maximally enlarged (\( \approx \frac{1}{3} \) hr).

The mechanism of removal of excess membrane from the acinar lumens after secretory granule discharge is still unknown. As originally proposed by Palade (5) and elaborated on by Amsterdam et al. (7), it may involve internalization of patches of intact membrane from the cell surface, possibly as vesicles, which may move back into the cell to be reutilized in the packing process. This assumption is supported by our morphologic observations which show a marked increase in the number and size of Golgi elements after intense stimulation especially at a time when the acinar lumens has reverted to normal dimensions. No quantitative correlations, however, between amounts of Golgi membranes and changes in dimensions of the acinar lumens were made in this study. Alternatively, as Fawcett has suggested (26), the excess luminal membrane may be degraded, the degradation products being used to resynthesize or reassemble membranes at other sites. Direct proof for one hypothesis or the other requires labeling the apical plasmalemma at the time of maximal luminal dilation as a prerequisite for following its subsequent course during luminal restitution.

Regardless of the mechanism involved, unpublished studies show that the complete cycle of membrane insertion and withdrawal, including the increase in size of the Golgi complex, takes place when discharge is carried out in the presence of cycloheximide at doses sufficient to inhibit protein synthesis by \( > 98\% \). It follows that the excess membrane noted in the Golgi region is most likely not assembled from newly synthesized components; it is either brought back as intact membrane from the cell surface, or is assembled from an intracellular pool of preexisting precursors.

### Protein Synthesis in Stimulated Slices

Our results indicate that stimulation of zymogen discharge in vitro is not accompanied by an increase in the rate of protein synthesis at least over the 3 hr interval examined. This is consistent with the data of Hokin and Hokin (27) and Dickman et al. (28) which showed no change in the rate of synthesis of secretory proteins by pancreatic slices during in vitro incubation with secretagogues. However, initiation of zymogen discharge in vivo, by feeding or secretagogue administration, has generally (but not consistently [29, 30]) been found to result in apparently increased rates of protein synthesis as measured by the ability to incorporate amino acids administered either in vivo (31, 32, 33), or in vitro to pancreatic slices (11, 34). The effects in vivo could be ascribed to an effective increase of the amino acid pool of the gland due to concomitant hyperemia, but differences in in vitro incorporation rates which depend on whether discharge is initiated in vivo or in vitro are unexplained. They may result from a physiologic response initiated by secretagogues only in vivo, yet in rat parotid slices an increase in the rate of synthesis of amylase has been reported upon discharge stimulation in vitro (35). Alternatively, a poststimulation increase in proteinspecific radioactivity may be only apparent due to the substantial reduction in the total protein content of the gland brought about by extensive discharge of preformed zymogen granules. To circumvent this possible source of error, incorporation data should be normalized to a constant denominator, e.g., total cell DNA. The finding that the net incorporation rate of an individual amino acid is dependent of its absolute concentration (36) as well as the presence of a full complement of amino acids in the incubation medium (1) shows again how complex the situation is and...
hence how difficult is the interpretation of incorporation data.

Pathway of Intracellular Transport in Stimulated Exocrine Cells

Our studies on stimulated pancreatic slices lead to the general conclusion that newly synthesized secretory proteins are initially segregated within the cisternae of the RER of the acinar cells, transported through their Golgi elements, and finally accumulated in modified secretion granules which upon cell fractionation can be recovered in part in an operationally defined zymogen granule fraction. At the end of the pulse, and during a chase incubation which covered the entire secretory process, the proportion of labeled proteins recovered in the postmicrosomal supernate from both prestimulated and control slices was about the same, i.e., 15-17% of the total labeled proteins of the homogenate. Since the postmicrosomal supernate contains, in addition to other proteins, those of the cell sap, our findings indicate that in stimulated as in control slices, transport of secretory proteins occurs within membrane-enclosed spaces. There is no evidence for their rerouting through the cell sap upon stimulation since, if in operation, such a rerouting should increase to 60-70% the proportion of protein radioactivity recovered in the postmicrosomal supernate and should raise the specific radioactivity of the proteins of this fraction by ~fivefold at some time during chase incubation.

Taken together, the results obtained by radioautography and cell fractionation rule out the long-standing notion that secretory proteins are usually transported through the cell sap and only segregated into membrane-bounded storage granules (i.e., zymogen granules) during periods of relative starvation (12-14). This concept was based primarily on the recovery of a large fraction (up to 85%) of the amylase of pancreatic homogenates in postmicrosomal supernates (12, 13); in our hands, this proportion is generally 15% or less, but it can be increased substantially by prolonging the time of homogenization of the tissue (unpublished observations). A large portion of the secretory proteins found in the supernate fraction is most likely derived from cell particulates mechanically ruptured during homogenization with an indeterminate contribution from soluble enzymes present in the duct system of the gland. Another argument for the concept mentioned above was the finding that chronically stimulated cells continue to discharge actively though apparently depleted of zymogen granules (37).

Although in its general lines intracellular transport in the stimulated pancreatic exocrine cell follows the pathway already established for its resting counterpart, i.e. it involves primarily membrane-bounded compartments, our radioautographic and morphological studies bring forward a number of differences between the stimulated and resting conditions.

First, in stimulated cells the stacked cisternal elements of the Golgi complex, in addition to the peripherally located small vesicles, appear to be actively involved in the processing of secretory proteins. In the resting cell the evidence so far obtained (2) indicates that there is no significant concentration of label over the stacks, while concentration is evident in the condensing vacuoles. This situation may be explained by the fact that the Golgi cisternae are bypassed, the flow of secretory proteins reaching the condensing vacuoles directly via the small vesicles located at the periphery of the Golgi region. Alternatively, the relative concentration of label over the stacks in resting cells may have been too low for detection by radioautography. In any event, in the stimulated cell the concentration of secretory products appears to start more proximally on the pathway since electron-opaque material is commonly seen within the stacked Golgi cisternae, including their terminal dilations, and in small coated vesicles which are located adjacent to the innermost, generally concave faces of the cisternae. These structures appear to take over the role of the condensing vacuoles found in the resting cell since typical condensing vacuoles are not seen in the stimulated cell. In these respects, the route of transport and the sites of concentration found in the stimulated exocrine pancreatic cell of the guinea pig resemble more closely those observed in the exocrine pancreatic cells of other species (38) and in most other cells synthesizing and transporting exportable proteins (reviewed in references 39 and 40).

Second, the structure of the secretory granules formed in the stimulated condition differs markedly from that seen in the resting cell in that they are much smaller in size (range 0.2-0.4 μ) and are notably irregular in profile. Discharge of small storage granules to the acinar lumen by membrane fusion-fission has not yet been observed.
in the stimulated condition but their proximity to the apical plasmalemma, and the fact that they carry labeled secretory proteins which are ultimately secreted into the duct space, strongly suggest that they function in the same manner as mature zymogen granules.

Considering the finding that the amount of smooth membranous elements in the Golgi complex is markedly increased in stimulated cells, and given the observation that the rates of synthesis and discharge of secretory proteins are similar in the stimulated and resting conditions, it follows that the stimulated cell is using more membrane to package its output. The persistence of an enlarged Golgi complex during continued stimulation may reflect an adjustment by the cell to the necessity of concentrating more rapidly its secretory products.

Finally, our study has shown that the rates of synthesis and intracellular transport of secretory proteins are not enhanced by stimulation in vitro. With the reservation that results obtained in vitro may not be entirely applicable to the function of the cell in vivo, our data suggest that the intracellular events concerned with the synthesis and transport of secretory proteins up to the storage site are not directly or indirectly (e.g., by a feedback mechanism) regulated by secretogogues although the details of some operations may be secondarily modified. Under our experimental conditions, only the final discharge step and the preceding storage step are modulated, protein synthesis and transport proceeding at constant rates. Our results thus confirm the long-standing observations of Grossman and his collaborators (37), which indicated that the exocrine pancreas is capable of synthesizing and discharging secretory proteins at near maximal rates during long periods of in vivo stimulation.

We wish to express our thanks to Miss Maryann Dickey for her excellent technical assistance.

This investigation was supported by Public Health Service Research Grant AM-10928 from the National Institute of Arthritis and Metabolic Diseases.

Received for publication 23 September 1970, and in revised form 9 November 1970.

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