CONDESING VACUOLE CONVERSION AND
ZYMGEN GRANULE DISCHARGE IN PANCREATIC
EXOCRINE CELLS: METABOLIC STUDIES

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ABSTRACT

We have examined, in the pancreatic exocrine cell, the metabolic requirements for the conversion of condensing vacuoles into zymogen granules and for the discharge of the contents of zymogen granules. To study condensing vacuole conversion, we pulse labeled guinea pig pancreatic slices for 4 min with leucine-3H and incubated them in chase medium for 20 min to allow labeled proteins to reach condensing vacuoles. Glycolytic and respiratory inhibitors were then added and incubation continued for 60 min to enable labeled proteins to reach granules in control slices. Electron microscope radioautography of cells or of zymogen granule pellets from treated slices showed that a large proportion of prelabeled condensing vacuoles underwent conversion in the presence of the combined inhibitors. Osmotic fragility studies on zymogen granule suspensions suggest that condensation may result from the aggregation of secretory proteins in an osmotically inactive form. Discharge was studied using an in vitro radioassay based on the finding that prelabeled zymogen granules can be induced to release their labeled contents to the incubation medium by carbamylcholine or pancreozymin. Induced discharge is not affected if protein synthesis is blocked by cycloheximide for up to 2 hr, but is strictly dependent on respiration. The data indicate that transport and discharge do not require the pari passu synthesis of secretory or nonsecretory proteins (e.g. membrane proteins), suggesting that the cell may reutilize its membranes during the secretory process. The energy requirements for zymogen discharge may be related to the fusion-fission of the granule membrane with the apical plasmalemma.

INTRODUCTION

We have previously investigated in the pancreatic exocrine cell the metabolic requirements and the route of intracellular transport of secretory proteins from their site of synthesis on attached polyribosomes (1, 2) to their site of concentration in the condensing vacuoles of the Golgi complex (3-7). We have continued the inquiry and we now report on the conversion of condensing vacuoles into zymogen granules and on the release of secretory proteins from these granules into the acinar lumina.

Hokin and Hokin (8) and Schramm and collaborators, (reviewed in reference 9) have already reported that stored secretory proteins can be discharged in vitro from exocrine gland slices upon appropriate stimulation in a process which is dependent on respiratory energy. However, their results cannot be interpreted unambiguously since they investigated discharge in the presence of ongoing protein synthesis and intracellular transport, operations which also require respiratory energy (7). To gain further insight into this problem, we have investigated discharge in the absence of protein synthesis and under conditions independent...
of the preceding steps in intracellular transport. As before (3), our studies have been conducted on guinea pig pancreatic slices incubated in vitro. A preliminary report of part of this work has been given (10).

METHODS AND MATERIALS

General

Procedures used for preparing and incubating guinea pig pancreatic slices, for pulse labeling their secretory proteins with radioactive amino acids, for fractionating homogenized slices, and for assaying the radioactivity of the proteins in cell fractions have been described before (3, 5). Electron microscope radioautography of thin sections of intact cells and of zymogen granule pellets was performed according to the method of Caro and van Tubergen (11). Radioautograms were micrographed in a Siemens Elmiskop I (Siemens Corp., Iselin, N.J.) at initial magnifications of 3500-5000 for tissue sections, and of 1000 for sections of zymogen granule pellets.

The animals used were male albino guinea pigs, weighing 450-500 g each, obtained from The Rockefeller University colony. Before experimentation they were starved for 24 hr with water given freely.

Radioassay for Zymogen Discharge

To facilitate the study of the metabolic requirements of zymogen discharge we have developed an in vitro radioassay of which the rationale and properties will be described under Results. In practice, ~20 slices (~25 mg wet wt per slice) derived from a single pancreas were placed in a 50 ml flask containing 5 ml of incubation medium in which L-leucine-1H was replaced by 5 sCi/ml of L-leucine-4,5-3H (4.5 Ci/mmole). Following a 10 min preincubation at 4°C, the slices were pulse labeled for 4 min at 37°C, washed with a large excess of chase medium (containing 2.0 mm leucine-1H), transferred in pairs to individual 25-ml flasks containing 5 ml of the same medium, and incubated for a further 80 min at 37°C, which is sufficient for labeled secretory proteins to be transported to their storage site in zymogen granules at the cell apex. At this time secretagogues were added to initiate zymogen granule discharge and the incubation was continued for 30 min to allow labeled secretory proteins to accumulate in the medium. Compounds and incubation conditions to be tested were introduced immediately after the secretagogues. At the end of the assay, the medium was decanted and centrifuged for 20 min at 190,000 g in an International Equipment Co. (Needham Heights, Mass.) A-269 rotor to sediment cell debris. The proteins in the centrifuged medium were precipitated by trichloroacetic acid (TCA)¹ (10% final concentration) after the addition of 2 mg/ml of carrier bovine plasma albumin. After a brief wash with cold 0.9% NaCl, the slices were homogenized in 2.0 ml H₂O, and 0.5 ml samples of the homogenate were treated with 10% TCA. TCA precipitates from medium and tissue were washed three times with cold 5% TCA to remove soluble label; prior extraction with hot TCA and lipid solvents was omitted because it was found unnecessary. The washed precipitates were prepared for liquid scintillation counting as described (3). Except for the assay, all operations were performed at 0°-4°C. Discharge is expressed as percentage of the total radioactivity in proteins (tissue and medium) which appears in the medium.

Enzyme Assays

Amylase activity, defined according to Schramm and Danon (13), was measured by Bernfeld’s procedure (12). ATP in tissue extracts was measured spectrophotometrically by determining the amount of nicotinamide adenine dinucleotide phosphate (NADPH) produced by the ATP-hexokinase-glucose-6-phosphate dehydrogenase system (14). The assay contained (in 1.0 ml) 120 µmoles Tris, pH 7.5, 5 µmoles MgCl₂, 2 µmoles glucose, 0.1 µmole NADP⁺, 0.4 unit glucose-6-phosphate dehydrogenase, 1.0 unit hexokinase, and neutralized tissue extract or ATP standards. The tissue extracts were prepared as follows: five pancreatic slices (~300 mg wet wt each) were frozen on dry ice and homogenized in 2.5 ml of 0.5% perchloric acid at 0°C without thawing. The homogenate was centrifuged (2000 g X 15 min) and 2.0 ml samples of the supernatant were brought to pH 7-8 with 3.0 M KHCO₃ at 0°C. The resulting KCIO₄ was removed by centrifugation and the supernatant was stored at −20°C prior to assay.

Materials

All chemicals used were reagent grade and were obtained from the following suppliers: carbamylcholine chloride, ouabain, and antimycin A from Mann Research Labs., N. Y.; cycloheximide from Nutritional Biochemicals Corporation, Cleveland, Ohio; L-leucine-4,5-3H, 45 Ci/mmole from New England Nuclear Corp., Boston, Mass.; NADP⁺.

¹ Abbreviations used: ATP, adenosine triphosphate; DNP, 2,4-dinitrophenol; IA, iodoacetic acid; NADP⁺(H), oxidized and reduced (H) forms of nicotinamide adenine dinucleotide; RER, rough-surfaced endoplasmic reticulum; TCA, trichloroacetic acid.
RESULTS

Conversion of Condensing Vacuoles to Zymogen Granules

GENERAL CONSIDERATIONS: We have shown previously (6) that the conversion of condensing vacuoles to zymogen granules occurs in the presence of cycloheximide, an inhibitor of protein synthesis, indicating that this step in the secretory process, like those immediately preceding it, does not require the continued production of specific, nonexportable proteins. At that time, we suggested that the conversion might be effected by the active extrusion of electrolytes and water from the vacuoles into the cell sap, i.e., by energy-requiring ion pumps similar to those found in the plasma membranes of many cells.

We have now put this assumption to the test by examining the conversion of condensing vacuoles in pancreatic slices whose energy production is restricted by respiratory and glycolytic inhibitors. The results were assessed by electron microscope radioautography. Antimycin A was chosen as the respiratory inhibitor since at a concentration of 0.01 mM it rapidly (in <1.5 min) and severely (>95%) inhibits intracellular transport of secretory proteins from the rough-surfaced endoplasmic reticulum (RER) to condensing vacuoles in parallel with inhibition of O2 consumption by the tissue (7). NaF was used as an inhibitor of glycolysis at a concentration (10 mM) previously found to suppress the aerobic oxidation of glucose.

RADIOAUTOGRAPHIC STUDIES ON CELLS: Pancreatic slices were pulse labeled with leucine-3H for 4 min and subsequently incubated in chase medium for 20 min, i.e., a time sufficient to allow the crest of the wave of labeled secretory proteins to reach condensing vacuoles, yet insufficient to result in heavy labeling of zymogen granules. At this time, antimycin A was added to the medium and incubation continued for a further 20 or 60 min, within which interval a significant proportion of the labeled protein reaches zymogen granules in unblocked slices (5). For controls, pulse-labeled slices were incubated for 20, 40, and 80 min in chase medium without the inhibitor, or for 80 min in chase medium containing antimycin A.

Following the processing of tissue samples for radioautography, a series of low magnification electron micrographs was taken and used to evaluate the distribution of radioautographic grains over structures involved in intracellular transport. The results are given in Table I and illustrated in Figs. 1-3. At the time of addition of antimycin A (20 min chase), 47% of the label was found over condensing vacuoles and only ~7% over zymogen granules. In the absence of the inhibitor, the proportion of label over zymogen granules rose to ~20% during the next 20 min incubation period (40 min control) and reached ~62% in the next 60 min (80 min control). In the presence of the inhibitor, the proportion of label over zymogen granules was ~21% following 20 min of incubation with antimycin A (20 min control + 20 min antimycin A), and rose to ~37% after 60 min of incubation with the drug (20 min control + 60 min antimycin A). Thus the conversion of condensing vacuoles to zymogen granules is not influenced by antimycin A during a 20 min exposure to the inhibitor, although conversion is repressed by ~43% during a further 60 min treatment (20 min control + 60 min antimycin A). Nevertheless, in the absence of respiration, condensing vacuole conversion was considerably less repressed than transport from the RER to condensing vacuoles, which was rapidly (in <1.5 min [7]) and severely (~95%) inhibited by a comparable dose of the drug. Despite the shift in distribution, the percentage of total label over condensing vacuoles plus zymogen granules was the same in slices incubated for 20 or 60 min with antimycin A (~60%) and in slices fixed at the end of the 20 min chase (~54%), indicating that antimycin A completely blocked forward transport from the RER to condensing vacuoles. The efficiency of the block was confirmed by the small percentage of label over condensing vacuoles and zymogen granules in slices incubated for 80 min with antimycin A.

It should be noted that in slices in which the respiratory block was established after 20 min of chase there was no evidence of backflow from condensing vacuoles to the Golgi peripheral...
TABLE I
Effect of Antimycin A on the Conversion of Condensing Vacuoles into Zymogen Granules

| Subcellular component | 20 min control | 40 min control | 20 min +20 min anti A | 80 min control | 20 min +60 min anti A | 80 min anti A |
|-----------------------|----------------|---------------|------------------------|----------------|------------------------|---------------|
|                       | %              | %             | %                      | %              | %                      | %             |
| Rough ER              | 27.6           | 16.4          | 28.3                   | 13.3           | 29.5                   | 57.6          |
| Golgi peripheral region | 18.7          | 15.4          | 11.9                   | 6.5            | 10.7                   | 32.7          |
| Condensing vacuoles   | 47.0           | 48.6          | 38.8                   | 16.0           | 22.6                   | 5.4           |
| Zymogen granules      | 6.6            | 19.6          | 20.9                   | 62.4           | 37.2                   | 4.3           |

No. of grains counted: 2494, 2996, 2495, 2437, 1216, 1326

Pancreatic slices were pulse labeled for 4 min at 37°C in medium containing 300 µCi/ml leucine-3H (5 µM), washed with a large excess of unlabeled chase medium containing 4 mM leucine-1H, and incubated for a further 20 min in the chase. At this time 0.01 mM antimycin A was added to the test slices and incubation was resumed for control and experimental slices for a further 20 or 60 min. To one set of slices (80 min anti A) the drug was added to the chase medium immediately postpulse and maintained therein for 80 min.

* Grains over nuclei and mitochondria are not included as their contribution to the total cell label is negligible (~ 0.4%).

region or RER elements. The observation indicates that the over-all process is not readily reversible and suggests, in agreement with our previous studies (3, 7), that no continuously patent communications exist between condensing vacuoles and the preceding cell compartments.

radioautographic studies on zymogen granule pellets: Our results indicate that the conversion of condensing vacuoles to zymogen granules proceeds at ~60% of the control rate under conditions in which respiratory-dependent processes such as intracellular transport and protein synthesis are severely (>95%) depressed.

Biochemical determinations, however, showed that after 60 min of exposure to 0.01 mM antimycin A the ATP level of the cell still remained at ~9% of the control value (Table II). This low ATP concentration, presumably maintained by the small but measurable aerobic glycolysis of the exocrine cells (7, 15) could energize condensing vacuole conversion even in the presence of antimycin A, provided the pertinent reaction(s) had a low K_m for ATP.

To test this possibility we investigated the conversion of condensing vacuoles to zymogen granules in the presence of antimycin A (0.01 mM) plus NaF (10 mM). Under these conditions (Table II) the ATP concentration in the tissue fell rapidly with an initial halftime estimated to be ~6 min and reached ~5% of the control value after 60 min. Fluoride alone reduced the ATP content by ~20% but did not interfere with protein transport to condensing vacuoles (7) or with zymogen granule discharge, as shown later.

In experiments in which the effect of antimycin A, or antimycin A plus NaF, was tested, the inhibitors were added to the assays after 20 min of chase incubation and were present throughout the next 60 min. Since we were concerned only with the conversion of condensing vacuoles to zymogen granules, we assessed the results on radioautographs of thin sections of zymogen granule pellets isolated from homogenates of the slices (5). From our previous studies we know that this pellet contains, in addition to zymogen granules, a small population of easily identifiable condensing vacuoles.

The method is as follows. Thin sections of a zymogen granule pellet, cut to include its complete depth in the axis of centrifugation, were mounted on bar grids and used to obtain sequential low magnification micrographs through the pellet. From the enlarged micrographs the number of labeled condensing vacuoles and zymogen gran-
FIGURE 1 Distribution of radioautographic grains over pancreatic exocrine cells at the end of 20 min of chase incubation (30 min control) following a 4 min pulse labeling with leucine-³H. The majority of grains is located over condensing vacuoles (arrows), with some appearing over a zymogen granule (Z). L, acinar lumen. Conditions of labeling are described in the legend to Table I. × 13,000.
FIGURE 2 Pancreatic slice at the end of an 80 min chase incubation (80 min control). At this time the radioautographic grains are mainly located over zymogen granules (Z) near the cell apex, although some are still found over condensing vacuoles (C). Note the thin filaments (arrow) located in a condensing vacuole and in the acinar lumen (L). X 16,000.
FIGURE 3 Pancreatic exocrine cells incubated postpulse in chase medium for 30 min followed by 60 min in the same medium containing 0.01 mM antimycin A. Radioautographic grains mainly mark zymogen granules (Z), although some of them are still associated with condensing vacuoles (C). Arrows outline the periphery of the Golgi region. × 13,500.
TABLE II

ATP Concentration in Pancreatic Slices Exposed to Metabolic Inhibitors

| Incubation conditions          | ATP Amount |
|-------------------------------|------------|
|                               | mmoles/mg protein | %       |
| Control, 60 min               | 14.8       | 100.0   |
| 10 μM F−, 60 min              | 11.8       | 80.0    |
| 0.01 mM antimycin A, 60 min   | 1.3        | 8.8     |
| 10 μM F− + 0.01 mM antimycin A, 10 min | 5.9  | 38.5    |
| " " " 20 min                 | 2.2        | 14.7    |
| " " " 40 min                 | 1.5        | 10.1    |
| " " " 60 min                 | 0.8        | 5.4     |

Details concerning incubation conditions and ATP assay are given under Methods.

ules was determined (5). These structures were considered labeled when more than two silver grains were located on their profiles (e.g., see Figs. 4–7). No attempt was made to score individual silver grains. A similar analysis of the data from intact cells treated with antimycin A gave comparable results. This method of scoring consistently overestimates the proportion of labeled zymogen granules, since condensing vacuoles approaching the end of the filling process are further dehydrated under the conditions of the isolation and appear in micrographs as mature zymogen granules. Condensing vacuoles closer to the beginning of the filling process will likewise undergo dehydration resulting in a content of greater electron opacity than in situ, but retaining their characteristic scalloped or angular profiles presumably due to their high membrane/content ratio.

The results of this experiment (Table III) are expressed as the percentage of labeled zymogen granules relative to the sum of labeled zymogen granules and labeled condensing vacuoles in the pellet. The data show that fluoride alone does not inhibit condensing vacuole conversion nor does it enhance the effect of antimycin A. As might be expected, the proportion of condensing vacuoles recovered in zymogen granules pellets from antimycin A-treated slices is somewhat less than from controls (Table III). This decrease does not alter the interpretation of the data.

SENSITIVITY OF ZYMÖGEN GRANULES TO OSMOTIC SHOCK: According to the above findings, the conversion of condensing vacuoles to zymogen granules is not strictly dependent on a continued supply of metabolic energy. This conclusion is also supported by the observation that zymogen granules do not swell in slices whose energy supply has been severely reduced (e.g., see Fig. 3). Since ion pumps of the type we originally suggested do not appear to be involved, other mechanisms must be considered for condensing vacuole conversion and for the maintenance of the zymogen granule content in a condensed form. For instance, secretory proteins might form os-
motically inactive aggregates upon segregation in condensing vacuoles.

To test this possibility, pancreatic slices were pulse labeled for 4 min with leucine-\(^3\)H and incubated in chase medium for 20 or 80 min in order to allow the bulk of the labeled proteins to be transported to condensing vacuoles or zymogen granules, respectively (e.g., Table I). At the end of the chase periods, the slices were homogenized and total zymogen granule pellets were isolated from the homogenates by differential centrifugation. The pellets were gently resuspended by hand with a Teflon pestle in the solutions indicated in Table IV, and the suspensions were then re-centrifuged to sediment particulate components. We should note that while our granule fractions are practically free of microsomes (5), they contain up to \(~5\%\) contaminant mitochondria (16).

**Table III**

| Incubation conditions | Labeled zymogen granules as per cent of sum labeled (zymogen granules + condensing vacuoles) | No. of labeled structures counted | Per cent of condensing vacuoles in pellet* |
|-----------------------|--------------------------------------------------------------------------------------------|----------------------------------|------------------------------------------|
|                       | 20 min control | 80 min control | 20 min control | 20 min control | 20 min control |
|                       | 20 min control | +60 min F" | +60 min and A | +60 min and A + F" |
|                       | 16.0 | 67.2 | 75.1 | 61.4 | 57.8 |
|                       | 214 | 689 | 225 | 942 | 729 |
|                       | 9.1 | 9.9 | — | 7.4 | 6.6 |

The conditions for pulse labeling and chase incubation are as described under Table I. In order to carry out cell fractionation, the amount of tissue per time point was increased to 500 mg wet wt. Zymogen granule pellets were isolated from the slices by differential centrifugation and processed for radioautography as previously described (5).

* Condensing vacuoles \times 100/condensing vacuoles + zymogen granules.

**Table IV**

| Chase incubation time | Suspending media | % dpm released |
|-----------------------|------------------|----------------|
| 0.3 M sucrose, pH 4.6 | Water, pH 4.7    | 0.2 M NaHCO\(_3\), pH 8.4 | 0.5% Na DOC, pH 7.2 |
| 20 min                 | 50.6 ± 1.4       | 87.2 ± 1.3  | 86.6 |
| 80 min                 | 30.2 ± 1.1       | 87.3 ± 1.2  | 89.8 |

Sets of pancreatic slices were pulse labeled for 4 min with leucine-\(^3\)H and incubated in chase media for 20 or 80 min. Zymogen granule fractions were isolated by differential centrifugation from homogenates prepared from each set of slices. The pellets were resuspended in the media indicated and the suspensions were incubated at 0°C for 5 min. Following centrifugation of the suspensions at 190,000 g for 20 min, the amount of labeled proteins in the supernatant fraction was determined and is expressed as the per cent of total labeled proteins initially present in the suspension (100% = 200,000 dpm for the 20 min set and 320,000 dpm for the 80 min set). Recoveries of radioactivity in the supernatant fraction plus pelletable residue ranged from 87 to 97%. Data shown are derived from four experiments and include the standard error of the means. DOC treatment was performed only once.
Since the latter incorporate negligible amounts of tracer during pulse labeling (5), the proteins they release upon hypotonic shock do not interfere with the assay.

The results given in Table IV show that resuspension in 0.3 M sucrose (control) releases 11–17% of the labeled proteins from granule preparations derived from slices incubated in chase medium for either 20 or 80 min. The corresponding figures from granule preparations resuspended in water are ~51% after a 20 min chase, when the label is predominantly in condensing vacuoles, and ~30% after an 80 min chase, when the label is predominantly in zymogen granules. The net release of labeled proteins by osmotic shock is thus ~39% from prelabeled condensing vacuoles and ~14% from prelabeled zymogen granules. It follows that part of the content (or part of the population) of condensing vacuoles is sensitive to osmotic shock, while the majority of the proteins in zymogen granules appear to be present in an osmotically inactive form. In both cases (20 and 80 min chase incubation) the treatment of the zymogen granule suspensions with alkaline medium (pH 8.4) releases the majority of labeled proteins (~87%); the same obtains for sodium deoxycholate at a concentration (0.5%) sufficient to disrupt cellular membranes.

After resuspension in either water or 0.3 M sucrose, the pelletable residue consisted of large numbers of intact zymogen granules bounded by a unit membrane (Fig. 8) and the usual smaller population of condensing vacuoles, as well as partly extracted granules containing a coarse meshwork of electron-opaque material. Since structures of the last type are not present in the original pellets, and since their proportion is about the same after sucrose as after water treatment, they are most likely produced during the mechanical resuspension of the pellets. The pellets

![Figure 8](image)

**Figure 8** Zymogen granules resuspended in water, fixed in suspension with 2% OsO₄ in water (pH ~ 4.5), recovered by centrifugation, and stained in block with 0.5% MgU acetate in isotonic saline. Several intact zymogen granules (Z) bounded by a unit membrane are indicated. A partly extracted granule containing a meshwork of electron-opaque material is shown (Z'). Its unit membrane is marked by opposed arrows. × 48,000.
also contain occasional structures which have the appearance of the "cores" of partly extracted granules freed of their limiting membranes.

**Zymogen Granule Discharge**

**Radioassay for Zymogen Discharge:**
This assay, described under Methods and Materials, is based on our earlier findings (see Table I) that ~65% of the secretory proteins synthesized during a 4 min pulse labeling are transported over an 80 min chase incubation to zymogen granules at the cell apex (see Fig. 2). If at this time secretagogues such as pancreozymin or carbamylcholine are added to the incubation flasks, the slices discharge labeled secretory proteins into the incubation medium via the duct system of the gland (Fig. 9). Fig. 10 shows that the secretory response begins without a lag and is maintained at a linear rate for the duration of the assay. While the discharge of labeled proteins is paralleled by discharge of enzymatic activity (e.g. amylase), the

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2 From separate experiments, not shown here, it was found that the optimum secretory response was obtained with 10 Crick-Harper-Raper Units/ml of pancreozymin or 0.01 mM-0.1 mM carbamylcholine.

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**Figure 9** Radiograph showing labeled secretory proteins in an intralobular duct lumen (DL). Zonulae adherentes (za) in full face view are noted. X 10,000.

**Figure 10** Kinetics of discharge of pulse-labeled proteins. Sets of slices were pulse labeled with leucine-3H for 4 min and incubated in chase medium for 80 min, at which time 0.1 mM carbamylcholine (carbachol) was added. The amount of labeled proteins in the medium was determined after 5, 10, 20, and 30 min exposure to the secretagogue and is expressed as per cent of the sum of protein radioactivity in slices and medium. The lower curve shows the amount of labeled proteins in the medium from control, unstimulated slices. The results are representative of three identical experiments.
radioassay is more sensitive in that the basal, non-stimulated level of radioactivity in the medium is only ~5% of the radioactivity initially present in the tissue (the corresponding figure for amylase is ~20%). It is also selective in that it measures only the discharge of proteins synthesized during the pulse and transported to storage sites during the 80 min chase period; as such it is not affected by the presence in the duct system of proteins synthesized before the pulse.

We are assuming that the labeled proteins which appear in the medium upon the addition of secretagogues derive from the content of zymogen granules. Two lines of evidence support this assumption: (a) after ~40 min of stimulation, the number of zymogen granules harvested from slices by cell fractionation is measurably decreased, and (b) microscopy of the acini during stimulation reveals a progressive depletion of the zymogen granule population: the decrease is already significant after 30 min and becomes particularly striking after ~3 hr (Figs. 11 and 12).

The interpretation of the results given by the assay also depends on the assumption that secretagogues induce discharge without concurrently increasing the rate of intracellular transport of secretory proteins. To check on this point, we determined, as described before (6), the efficiency of transport of pulse labeled proteins from the RER to condensing vacuoles during 37 min of stimulation with pancreozymin or carbamylcholine. The results, given in Table V, indicate that discharge

![Figure 11](image1.jpg)  
**Figure 11** Light micrograph of a portion of a pancreatic slice incubated for 3 hr in chase medium. The apices of the acinar cells are packed with zymogen granules (Z). L, acinar lumen. Osmium-fixed, Epon-embedded tissue; 0.4 μ thick section stained with 1% methylene blue in 1% Na borate. X 1200.

![Figure 12](image2.jpg)  
**Figure 12** Light micrograph of a portion of a pancreatic slice from the same pancreas as in Fig. 11 but incubated for 3 hr with 0.01 mM carbamylcholine. Most of the cells are devoid of zymogen granules. Two unusually "dark" centroacinar cells (ca), cut at the level of their nuclei, appear in the center of the acinus and form one surface of the acinar lumen (L). Mitochondria (m) are visible and a number of cells containing intracisternal granules are indicated (arrows). Same preparation procedures as for Fig. 11. X 1200.
TABLE V
Effect of Secretagogues on Intracellular Transport of Secretory Proteins

Sets of pancreatic slices were pulse labeled with leucine-$^{3}$H for 3 min (6), and then incubated in chase media with the indicated additions for 37 min. At the end of the pulse, and after 37 min chase incubation, the slices were homogenized, fractionated by differential centrifugation, and the radioactivity of proteins in the microsomal and zymogen granule fractions and in the postmicrosomal supernatant determined. Radioactivity data are expressed as % dpm recovered in the fractions relative to the starting homogenate or as specific radioactivity based on amylase measured in the fractions. The data show relative rather than total changes of radioactivity in cell fractions with time since only the postmicrosomal supernatant was completely recovered in our fractionation scheme; recovery of the microsomal and zymogen granule fractions can be estimated to be ~ 30% each.

* Amylase in medium × 100/amylase in medium + homogenate.

| Pulse | Conditions | Amylase in incubation medium* | Microsomal fraction | Zymogen granule fraction | Postmicrosomal supernatant |
|-------|------------|-----------------------------|---------------------|--------------------------|-----------------------------|
| L-leucine-$^{3}$H | 3 min 0 min (pulse) | % dpm/U amylase | % dpm | % dpm | 8500 | 32.4 | 206 | 0.8 | 2730 | 17.5 |
|       | 37 " (control) | 12.0 | 4390 | 14.8 | 5100 | 12.0 | 1550 | 11.7 |
|       | 37 " , 0.1 mm carbamylcholine | 22.2 | 6670 | 19.2 | 4280 | 6.7 | 2400 | 12.0 |
|       | 37 min, 10 U/ml pancreozymin | 20.6 | 6150 | 15.2 | 4100 | 8.6 | 1480 | 12.0 |
|       | 37 min, 3 mm dibutyryl cyclic AMP | 14.5 | 5730 | 14.2 | 4190 | 12.1 | 1900 | 8.4 |

According to our previous work, the total microsomal fraction consists mainly of rough microsomes derived from fragmented and resealed elements of the RER. The decrease of specific and total radioactivity in the total microsomal fraction parallels its rough subfraction during chase incubation (3).

Sets of pancreatic slices were pulse labeled with leucine-$^{3}$H for 3 min (6), and then incubated in chase media with the indicated additions for 37 min. At the end of the pulse, and after 37 min chase incubation, the slices were homogenized, fractionated by differential centrifugation, and the radioactivity of proteins in the microsomal and zymogen granule fractions and in the postmicrosomal supernatant determined. Radioactivity data are expressed as % dpm recovered in the fractions relative to the starting homogenate or as specific radioactivity based on amylase measured in the fractions. The data show relative rather than total changes of radioactivity in cell fractions with time since only the postmicrosomal supernatant was completely recovered in our fractionation scheme; recovery of the microsomal and zymogen granule fractions can be estimated to be ~ 30% each.

* Amylase in medium × 100/amylase in medium + homogenate.

stimulation over this interval accelerates neither the decrease of the specific radioactivity of the proteins of the total microsomal fraction (which for convenience can be taken as a measure of the efficiency of drainage of proteins from the RER cisternae), nor the accumulation of labeled proteins in the zymogen granule fraction (i.e., in the condensing vacuoles recovered in the fraction). Moreover, at the concentration used and over the intervals examined, secretagogues do not alter the rate of incorporation of labeled amino acids into protein (unpublished data). The table also shows that dibutyryl-adenosine 3',5'-cyclic phosphate, an active derivative of adenosine 3',5'-cyclic phosphate, the presumed common mediator of many secretory responses (19), does not affect transport at a concentration sufficient to induce discharge from other exocrine glands (20, 22). In addition, the data show that the discharge induction does not lead to any systematic increase in the total or specific radioactivity of the proteins of the postmicrosomal supernatant which represents primarily the soluble cytoplasmic matrix. This finding does not support the view that, under the influence of secretagogues, secretory proteins are preferentially transported in soluble form along an alternative pathway that passes through the cell sap (21).

REQUIREMENTS FOR PROTEIN SYNTHESIS:
To determine whether discharge is tightly coupled to protein synthesis, we examined the effect of cycloheximide on the secretion assay. The results show that the drug, at a concentration (0.5 mM) sufficient to inhibit protein synthesis by >95%, was without effect on discharge induced by pancreozymin or carbamylcholine (Table VI, Fig. 13), even though present continuously during both the 80 min preincubation and the discharge period. Morphologically, there was no striking difference between cells which had discharged
their zymogen granules in the presence or absence of concurrent protein synthesis (cf. Figs. 12 and 14).

It follows that discharge does not depend on protein synthesis, at least over the time examined (up to a total of ~2.5 hr of exposure to cycloheximide). Since this interval covers a complete secretory wave from segregation into the cisternal space to release into the acinar lumen, the results rule out pari passu synthesis of proteins for secretion products and for their membrane containers. They also indicate that discharge does not depend on the simultaneous synthesis of "specific" non-exportable proteins, such as catalysts, carriers, membrane couplers, etc. If such proteins are needed they must be present within the cell or its membranes in a pool large enough to permit continuous operation over the period examined.

**ENERGY REQUIREMENTS:** Since discharge can be uncoupled from protein synthesis, it becomes possible to examine separately its energy requirements by applying appropriate metabolic inhibitors to pancreatic slices in which protein synthesis is already blocked by cycloheximide. For uniformity, we introduced cycloheximide into our discharge assay at the end of the pulse and maintained it at the same concentration (0.5 mM) throughout all subsequent incubations. We applied the metabolic inhibitors or incubation conditions to be tested at the same time as the secretagogues, i.e. at the end of the 80 min chase incubation, and maintained them in the assay for a standard test period of 30 min. The results of these experiments (Table VI) show that discharge is not affected by glycolytic inhibitors but is strongly inhibited by compounds or conditions which interfere with respiration-dependent ATP production. Since the effect of the inhibitors in blocking discharge is rapid (complete blockage is obtained within 5 min for antimycin A, Fig. 15), and since we have shown that the preceding step in transport, the conversion of condensing vacuoles to zymogen granules, is relatively energy independent, we conclude that under these experimental conditions the discharge process itself is affected. Antimycin A did not suppress the small but significant accumulation of labeled proteins in the medium from control, unstimulated slices (Fig. 15); presumably these proteins represent leakage from damaged cells.

Several other compounds which have been implicated in secretory discharge were also tested in our assay. Dibutyryl-adenosine 3',5'-cyclic phosphate produced a modest secretory response...
in progress indicates that ouabain does not interfere with any of the preceding steps of the secretory process including the conversion of condensing vacuoles to zymogen granules.

**DISCUSSION**

**Conversion of Condensing Vacuoles to Zymogen Granules**

Previous studies on the exocrine pancreatic cell (4, 5) have demonstrated that condensing vacuoles are converted into zymogen granules by the progressive filling and concentration of their content. This conclusion is based mainly upon radioautographic observations which show that condensing vacuoles become progressively more dense and more heavily labeled as secretory proteins accumulate within, until finally they are converted into zymogen granules with a characteristic, highly electron-opaque content. The correlation between changes in electron opacity and degree of
Our results show that the conversion of condensing vacuoles to zymogen granules does not depend to any marked extent on a continued supply of metabolic energy (i.e., ATP or ATP-derived high energy compounds) derived either from glycolysis or respiration. Previously, we had assumed, as already mentioned, that zymogen concentration might be effected by the active extrusion of electrolytes and water from the condensing vacuoles into the cell sap, and we had postulated that this operation might be carried out by endergonic ion pumps located in the condensing vacuole membrane (5). However, this appears not to be the case, since the source of energy needed to drive a pump of this sort has been severely depleted in our experimental conditions without preventing or reversing the conversion step. Further support for this conclusion comes from our morphologic observations, which show that condensing vacuoles and zymogen granules do not swell or lyse in slices depleted of their ATP content by metabolic inhibitors (e.g., see Fig. 3), or treated with 1.0 mM ouabain. It also comes from cell fractionation studies in which we found no evidence for Na$^+$-and K$^+$-ATPase activity in membranes derived from zymogen granule fractions (18). Nucleoside diphosphatase$^5$ (37) and acid phosphatase activities (38) have been detected by histochemistry in the Golgi complex and in forming storage granules of the anterior pituitary and parotid acinar cell, respectively, but the suggestion that these activities play a role in the concentration of secretory products is based primarily upon their location. So far there is no evidence for the involvement of a transport ATPase in the conversion process. Hence, other mechanisms should be considered.

The results of the experiments with isolated zymogen granules suggest that the majority of the proteins in the granule content is relatively stable osmotically, whereas the content of the condensing vacuoles appears to be somewhat more labile, although our results do not rule out the possibility that the condensing vacuole membrane is preferentially more permeable to the content under hypotonic conditions. These findings support the view that secretory proteins, upon segregation in the condensing vacuole, undergo progressive ag-

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$^5$ Determined by isopycnic centrifugation in sucrose gradients.

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$^6$ It has been recently suggested that this activity is due to a partly inactivated polysaccharide synthetase (45), a Golgi enzyme not directly involved in concentration.
aggregation which reduces their osmotic activity in mature zymogen granules. Whether the small proportion of labeled proteins extractable from zymogen granules by water represents a population of sensitive granules or the removal of specific proteins which do not participate in complex formation remains unknown.

Our results confirm the earlier studies of Hokin (28) and the more recent observations of Burwen and Rothman (29) which showed that only 20-25% of the protein content of zymogen granule suspensions is extracted by water as long as the pH of the suspending medium is between 4.5 and 6.6.\(^4\) As reported by Hokin (28), and confirmed by Greene et al. (17) and by ourselves (see also reference 5), increasing the pH of the suspending medium to > 7.0 results in the immediate clearing of the suspension with concomitant release of the granule content. Since granule membranes can be recovered quantitatively by centrifugation from the lysed suspension, it is likely that the alkaline extraction affects primarily the content. It may alter the degree of aggregation of secretory proteins within the granules leading to increased osmotic activity, swelling, and eventually to mechanical rupture of the limiting membrane. Preliminary observations using differential interference contrast optics support this contention.

The reactions involved in this putative aggregate formation are, of course, unknown; they may involve ionic or other interactions among secretory proteins or between these proteins and other substances present in the vacuole content, such as divalent cations, binding or matrix substances, etc. Most likely interactions between the granule content and its limiting membrane contribute little to the process since the surface area of the membrane is such that it could accommodate only a small fraction of the content. The occurrence of intracisternal granules in the RER cisternae under certain physiologic conditions (30), the appearance of discrete dense masses in condensing vacuoles and zymogen granules in poorly fixed or damaged cells, and the presence of filamentous or fibrous structures in condensing vacuoles and in the content of acinar lumina (e.g., see Fig. 2) are further indications that secretory products can form large aggregates under certain conditions. Along this line we should mention that at least one secretory protein, bovine procarboxypeptidase A, is actually a molecular aggregate of three discrete proteins, two of which possess enzymatic activity (31), although this observation pertains strictly to bovine procarboxypeptidases.

\(^4\) Under our conditions, the pH of the water or 0.3 M sucrose used to resuspend the granule preparations was \(\sim 4.7-4.8\), i.e., within the range of granule stability.
Complex formation among proteins and small molecules resulting in the loss of solubility of the latter has been established in the storage granules of the adrenal medulla (35, 36), posterior pituitary (45), and splenic nerve endings (46). Finally, crystalline or highly ordered cores have been described in hepatic peroxisomes (33) and in the storage granules of eosinophil granulocytes (32), \( \beta \) cells of the pancreatic islets (34), and acinar cells of the salivary glands (B. Tandler, personal communication); although in these cases the cores may be the result rather than the cause of reduced osmotic pressure.

**Metabolic Requirements of Zymogen Discharge**

Our studies indicate that the discharge of zymogen granules by secretogogues does not require continuous protein synthesis, but strictly depends on respiratory energy. There is therefore a second step in the pathway of intracellular transport which can be described as an energy-requiring lock: the first of these locks (7) connects two intracellular compartments, i.e. the cisternae of the RER and condensing vacuoles, whereas the second lock connects an intracellular compartment represented by the zymogen granule with the acinar lumen. In contrast to the first, the second lock is regulated by secretogogues.

The nature of the energy-requiring step in zymogen granule discharge, as well as the means whereby secretogogues initiate discharge, remains unknown. Morphologic studies (39) have shown that zymogen discharge involves the movement of the granule to the cell apex where its membrane fuses with that of the cell surface and where its content is extruded by exocytosis into the acinar lumen. Presumably part of the energy requirement could be related to membrane fusion-fission and the attendant reorganization or synthesis of membrane components, but the possibility that energy is also necessary for the propulsion of the granule to the cell apex or is needed to support the activity of as yet unknown enzymatic mechanisms involved in discharge must be considered.

Finally, our previous studies (6) and the results reported here clearly show that secretory proteins can be transported, concentrated, stored in zymogen granules, and discharged from the cell into the acinar lumen (i.e., carried through an entire secretory wave of 60-90 min duration) in the absence of further protein synthesis. In particular, the data can be taken to indicate that synthesis of membrane proteins for the containers involved in transport is not required for the processing of the content, at least over the times examined. This implies that the cell most likely reutilizes extensively its intracellular membranes or components thereof during the secretory process. In this regard at least two sites of membrane reutilization or circulation can be postulated in the acinar cell: (a) between the RER and the Golgi region, and (b) between this region and the apical plasmalemma. Our previous studies have shown that secretory proteins are transported from the RER cisternae to condensing vacuoles of the Golgi region within a membrane-enclosed compartment (3), and that functional continuity between the RER and the Golgi region is established through an energy-operated lock (7). Protein transport between these compartments may be effected by the small vesicles which pinch off from the cell surface and move back into the cell for reuse. In any case, some mechanism must exist to remove the excess membrane contributed to the cell surface during zymogen granule discharge and to compensate the Golgi complex for membrane lost if the membrane balance of the cell is to be maintained. Circulation of assembled membrane or disassembled macromolecular membrane components would imply that the intracellular life span of the proteins of the containers should be considerably longer than that of their contents (which is 60-90 min). Turnover rates for the membrane constituents of the pancreatic exocrine cell are not available, but in the case of hepatic parenchymal cells the half-lives of membrane proteins are approximately two orders of magnitude longer (41) than the mean transit time of their contained secretory products.
plasma protein (42), and lipoprotein droplets (43).

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