

Phasic Release of Newly Synthesized Secretory Proteins in the Unstimulated Rat Exocrine Pancreas

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Abstract. Pancreatic lobules from fasted rats secrete pulse-labeled proteins in two phases comprising 15 and 85% of basal output, respectively. The first (0–6.5 h) is initially (≤ 0.5 h) unstimulated by secretagogues, probably represents vesicular traffic of Golgi and post-Golgi origin (including condensing vacuoles/immature granules), and notably contains two groups of polypeptides with distinct release rates: (a) zymogens ($t_{1/2} \sim 2.4$ h) and (b) minor nonzymogens plus one unique zymogen ($t_{1/2} \sim 1$ h). The second phase (peak at 9–10 h) is stimuable, probably represents basal granule

exocytosis ($t_{1/2} \sim 50$ h), and contains zymogens exclusively. Newly synthesized proteins released in both phases appear asynchronously, reiterating their asynchronous transport through intracellular compartments. Zymogens in both phases are secreted apically. The sorting of first from second phase zymogen release does not appear to be carrier-mediated, although the sorting of zymogens from other secretory proteins may use this process. Finally, data are presented that suggest that both secretory phases are subject to physiologic regulation.

IT is now known that individual proteins secreted by exocytosis are initially synthesized in the endoplasmic reticulum (ER)¹ and are then transferred to the Golgi complex (Jamieson and Palade, 1967); each polypeptide species has a characteristic ER to Golgi transit time (Lodish et al., 1983; Fries et al., 1984; Rohr and Keim, 1984; Scheele and Tartakoff, 1985). Some proteins passing through the Golgi complex may then be expelled from the cell without storage, via constitutive vesicular secretion (Kelly, 1985). The release of proteins in such a manner cannot be increased by the addition of external stimuli (Gumbiner and Kelly, 1982). In contrast, an alternative intracellular route for newly made secretory proteins in some cells involves their retention in intracellular storage. Numerous studies have emphasized the central role of condensing vacuoles (in concentration of proteins upon exit from the Golgi stacks) as the earliest form of the storage compartment (Palade, 1975). The storage granules, so formed, undergo exocytosis at a low frequency under resting conditions, but exhibit greatly amplified discharge in response to physiological or pharmacological stimulation.

The exocrine pancreas has been considered prototypic in its expression of this regulated secretory granule pathway (Tartakoff and Vassalli, 1978) in which exocytosis of zymogens is exclusively to the apical (luminal) surface. However, recent evidence suggesting that granule and nongranule discharge occur in tandem in cultured pituitary tumor cells (Moore and Kelly, 1985) has inspired us to examine the nor-

mal, highly differentiated exocrine pancreas for evidence of similar tandem pathways, especially in relation to the known secretory polarity of acinar cells.

We present the following novel evidence. (a) Unstimulated pancreatic lobules from starved rats release their pulse-labeled proteins with biphasic kinetics (< 6.5 h and > 7 h). (b) Most of the secreted label in the first phase is in the form of pancreatic zymogens whose discharge is not enhanced by secretagogue addition during the first 30 min of chase (suggesting a nongranule pathway of release). This phase has a half-time of release of only ~ 2.5 h and comprises $\sim 15\%$ of total unstimulated secretion of incorporated label. (c) The second phase of release contains $\sim 85\%$ of all newly synthesized proteins, has a half-time of release of ~ 50 h (that is greatly shortened by secretagogue addition), and represents basal exocytosis from a pool (granules) that shows limited intermixing within the stored population. (d) Both phases of secretion reiterate the asynchronous transport of labeled proteins through intracellular compartments. (e) When discharge kinetics are analyzed on a protein by protein basis, a similar half-time of exit (~ 2.4 h) is observed for all but one of the zymogens, which has a much shorter $t_{1/2}$ (~ 1 h). (f) The latter half-time is matched by at least four minor labeled polypeptides that, in contrast to the zymogens, are not observed in the second phase of release, in isolated granules, or in apical secretion. The timing of their release and insensitivity to secretagogue stimulation suggest a Golgi, rather than granule, origin. (g) Analysis of the release of newly synthesized zymogens into the pancreatic duct in vivo confirms a phasic kinetics and reveals that the zymogen with a $t_{1/2}$ of ~ 1 h is initially a prominent component. (h) Fi-

1. Abbreviations used in this paper: ER, endoplasmic reticulum; LDH, lactate dehydrogenase.

nally, the phasic pattern may be under regulatory control, since pancreatic lobules from different physiologic states (prefasted versus prefed condition) show different profiles of release, altering both the first and second phases.

Materials and Methods

Materials

Materials were obtained as follows: bovine serum albumin (RIA grade), carbachol, and stock chemicals from Sigma Chemical Co., St. Louis, MO; soybean trypsin inhibitor from Cooper-Worthington, Malvern, PA; electrophoresis reagents from Bio-Rad Laboratories, Richmond, CA; [³⁵S]methionine and [³⁵S]cysteine from Amersham Corp., Arlington Heights, IL; and ³H-amino acid mixture from ICN K & K Laboratories, Inc., Plainview, NY.

Tissue Preparations for Pulse-Chase Experiments

Pancreata were dissected from male 150-g Sprague Dawley rats that had been subjected to an overnight (~16 h) fast, except where ad libitum feeding (with standard rodent chow containing 23% protein and 4.5% fat) is specified. Lobules were prepared as described elsewhere (Scheele and Palade, 1975). Acini were prepared with collagenase digestion and mild mechanical shear (Herzog and Farquhar, 1977).

Pulse-Chase Procedure

For labeling in vitro with [³⁵S]methionine or [³⁵S]cysteine, acini or lobules were preincubated (≥3 changes of fresh medium, 37°C, for 30–60 min) in oxygenated Eagle's modified minimum essential medium plus 0.1% bovine serum albumin (RIA grade), 0.01% soybean trypsin inhibitor, and 25 mM buffer (either MOPS or Hepes), pH 7.4. A second preincubation was conducted for 10 min in methionine-free or cysteine-free medium (RPMI 1640, Gibco, Grand Island, NY) with the same additives and buffer. When ³H-amino acid labeling was performed, the second preincubation was replaced by three washes in amino acid-free medium containing the above additives.

After pulse labeling (0.5–10 min, as specified) in Met-free, Cys-free, or amino acid-free media containing 1–1.5 mCi of the appropriate labeled amino acids, the tissue was washed three times in chase medium that contained the original level of nonradioactive amino acid (in ≥100-fold excess of the previous concentration of label). Under these conditions, the pulse-labeling was efficiently terminated, since total acid-insoluble counts remained constant during subsequent chase periods (not shown). Chase collections were made after fixed time intervals (generally 15 or 30 min) with a complete medium change (5 ml) at the end of each time point. Each sample of medium was cleared of any particulates by centrifugation at 100,000 g for 1 h.

Upon removal of the final chase collection, iced 10-mM Na phosphate buffer, pH 8.0, was added to the tissue, and the suspension was sonicated with a probe tip sonicator (Branson Sonic Power Co., Danbury, CT) for ~1 min to achieve complete tissue disruption. Aliquots of medium and the tissue sonicate were divided for acid precipitation and enzyme assays; additional aliquots were mixed with SDS sample buffer and boiled for subsequent gel electrophoresis.

Acid precipitation was achieved by addition of 1 vol of ice-cold 10% trichloroacetic acid or 1 N perchloric acid plus 1% phosphotungstic acid in the presence of 1.5 mg bovine serum albumin as carrier. Precipitates were collected either by centrifugation (microfuge; Beckman Instruments, Inc., Palo Alto, CA) or by vacuum filtration on glass fiber filters (Whatman, Inc., Clifton, NJ). All precipitates were washed at least eight times with ice-cold 5% trichloroacetic acid.

Cannulation of the Pancreatic Duct in Live Rats

Rats were anesthetized with low dose pentobarbital delivered by intraperitoneal injection. A 2–3-cm longitudinal incision was made in the ventral surface of the abdominal cavity. The pancreatic duct was ligated at the level of entry into the duodenum to induce ductal distension. A nick was made in the duct wall and the duct was cannulated ~3 mm with a piece of polypropylene tubing, which was held in place with a suture. Two ligatures were placed on the common bile duct, which was then transected between the ties so it could make no further contribution to flow collected by the cannula. After a few minutes, an intravenous (inferior vena caval) injection of

2.5 mCi [³⁵S]cysteine in normal saline was administered. In the experiment shown in Fig. 8, protein output was $117 \pm 8 \mu\text{g}/30 \text{ min}$ with a flow rate of $\sim 25 \mu\text{l}/30 \text{ min}$.

Isolation of Pancreatic Zymogen Granules

For this purpose we used the method of DeLisle et al. (1984), with minor modifications. Granules purified by density gradient centrifugation in Percoll were diluted approximately sixfold with 0.3 M sucrose and collected by differential centrifugation at 2,300 g for 60 min.

Analysis of Secretory Proteins by SDS PAGE and Fluorography

Unless otherwise specified, gels were run under reducing conditions (100 mM dithiothreitol) according to the discontinuous system of Laemmli (1970) using 9.5% acrylamide for the resolving gel. Gels were stained with Coomassie Blue, impregnated with Autofluor (National Diagnostics, Inc., Somerville, NJ), dried, and exposed to XAR-5 film (Kodak) at -75°C for fluorography. The fluorographs were compared with the published patterns of rat pancreatic secretory proteins under reducing (Scheele et al., 1980; van Nest et al., 1980) and nonreducing (Iwanij and Jamieson, 1982; Schick et al., 1984; Rausch et al., 1985) conditions. Based on these comparisons, it was possible to make definitive identifications of selected pancreatic zymogens (which, in the discussion of this paper, includes the isozymes of α -amylase [band 2G] and lipase [band 3G], and procarboxypeptidases A and B [bands 4G and 5G]). Presumptive identifications were made of trypsinogen (isozymes 1 and 2, band 7G), chymotrypsinogen (isozyme 1, band 9G) and proelastase (band 6G). Band 10G probably contains both chymotrypsinogen 2 and trypsinogen 3 isozymes. Band 11G contains RNase, but in the 9.5% gels several unidentified dye front proteins are also found in this band.

In some experiments, radioactive bands were excised from the gels, dissolved for 2–5 d in 30% H₂O₂ at 60°C (Iacino et al., 1980), and counted

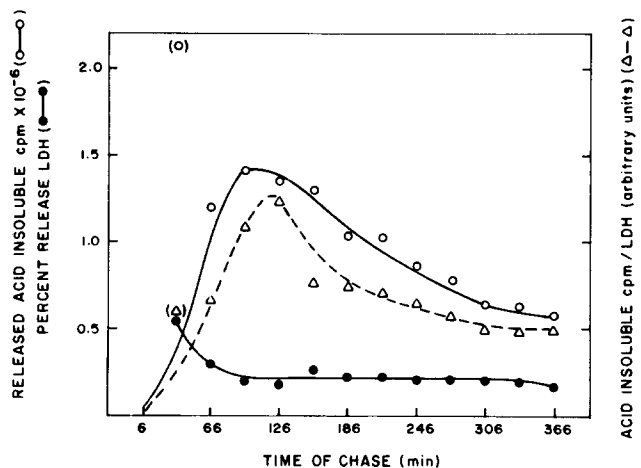


Figure 1. Phasic release of secretory proteins from unstimulated pancreatic lobules. Lobules representing all regions of a pancreas from a fasted rat were pulse-labeled (2 min, [³⁵S]cysteine) and washed as described in Materials and Methods. Chase collections were made every 30 min; LDH was measured within 2 h of sample collection. Acid-insoluble cpm released in the medium were normalized to LDH released during the same period, as shown. Acid precipitation selectively overestimated the labeled protein secreted in the first 30 min² (these values are included in parentheses); the curves are drawn to reflect the position of this time point as judged by quantitative SDS PAGE (not shown).

2. The acid-insoluble radioactivity measured in the medium during the first chase period (6–36 min) was artifactually high due to both greater cell death immediately after the pulse and wash, as measured by release of LDH, and to residual unincorporated amino acid label in the washed precipitates. Nevertheless, we observe significant secretion of labeled protein within 30 min of onset of pulse labeling, in agreement with others (Bieger et al., 1976).

Table I. Ability to Stimulate Discharge of Pulse-labeled Protein and Unlabeled Amylase from Pancreatic Tissue In Vitro

	Pulse-labeled protein chase interval					Amylase
	Pancreatic acini (A)		Lobules (B)			
	15-30 min	30-45 min	30-60 min	60-90 min	90-120 min	
Secretagogue/control	1.1	2.6	3.2	3.8	4.2	>10

Pancreatic acini (A) or lobules (B) were pulse-labeled at time zero (5 min, ^3H -amino acids, [A]; 10 min, [^{35}S]methionine, [B]) and washed as in Materials and Methods. During each chase interval listed, parallel samples were divided such that carbachol (10^{-5} M) was added to one-half. At the end of each interval, medium and tissue were separated and the tissue homogenized; release of acid-precipitable radioactivity and amylase was measured as in Materials and Methods. The release in the presence of secretagogue was divided by that from unstimulated control tissue. A and B are each representative of two experiments.

in Optifluor (Packard Instrument Co., Downers Grove, IL). Equal sized regions of the gels that were nonradioactive were similarly cut and counted for background. The percent distribution of individual labeled zymogens was determined in certain experiments according to Scheele and Tartakoff (1985).

Kinetic Model of Secretory Protein Discharge

We analyzed quantitative data concerning the discharge of individual zymogens in the first phase of release in relation to a three-box kinetic model of first order reactions (ER k_1 Golgi k_2 medium) similar to that used by others (Fries et al., 1984; Moore and Kelly, 1985). Two kinetic outcomes were considered. If $k_1 > k_2$, then in the terminal part of the discharge curve for an individual labeled species (Fig. 6 B), the half-time of release will essentially reflect only the step Golgi k_2 medium. If $k_2 > k_1$ (as occurs when ER exit is very slow), then for extended times (using the same analysis) the half-time will reflect the step ER k_1 Golgi. To encompass both possibilities, the half-time measurements for the appearance of individual labeled species in the medium were made from the descending portions of the release curves. These methods are identical to those employed in the analogous consideration of isotopic decay from one radioactive species to another (Adams, 1973).

Biochemical Assays

Amylase was measured according to Jung (1980) or Bernfeld (1955); Lactate dehydrogenase (LDH) was after Schnaar et al. (1978); protein was measured with fluorescamine according to Udenfriend et al. (1972).

Results

Phasic Character of Unstimulated Secretion of Newly Synthesized Proteins

In initial studies, we examined the release of acid-insoluble radioactivity into the medium, bathing unstimulated pancreatic lobules that had been pulse-labeled with [^{35}S]cysteine and chased at 30-min intervals. Newly synthesized protein was released in a phasic manner over the 6-h time period, with a peak value at 90-120 min of chase (Fig. 1). Approximately 0.5% of the total LDH was released in the first 30-min chase interval (Fig. 1), but this increment decreased to a stable level of $0.21 \pm 0.04\%$ per 30-min chase period. When the phasic radioactive protein output was normalized to the LDH released during the same chase interval, it was evident that the phasic appearance of the curve was unchanged (Fig. 1).² Basal amylase secretion was linear over the course of this experiment, with a mean release of $0.88 \pm 0.29\%/30$ min (not shown in Fig. 1).

Initial Labeled Secretion Occurs by Constitutive Vesicular Discharge

Since constitutive vesicular discharge is both relatively rapid and nonstimulable (Kelly, 1985), after various short chase intervals we examined whether secretagogue addition could

stimulate the discharge of labeled proteins (Table I). Significant carbachol-stimulated discharge of incorporated label could not be observed until times ≥ 30 min after onset of pulse-labeling. In four experiments (two with dispersed

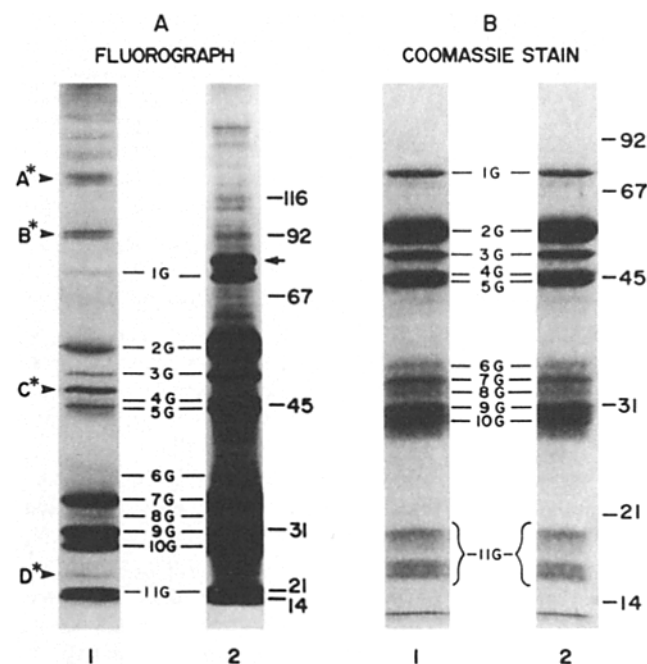


Figure 2. Comparative SDS PAGE patterns of exocrine pancreatic fractions. (A) Lobules from pancreata of two fasted rats were pulse-labeled (7 min, [^{35}S]cysteine) and washed; then a portion of lobules were homogenized as in Materials and Methods. After an 18-min chase, the experiment was terminated and the medium (lane 1) and tissue without chase (lane 2) were prepared for SDS PAGE (9.5% acrylamide) and subsequent fluorography. Samples were loaded by equal unlabeled amylase content. Lane 2 was intentionally overexposed to reveal minor labeled species. Note that a series of labeled bands, A* (M_r 125 kD), B* (M_r 92 kD), C* (M_r ~49 kD), and D* (M_r ~25 kD) is seen in the secretion that represents very minor labeled species in the tissue; in contrast, several more prominent bands (e.g., arrow, lane 2) in the tissue are absent from the secretion. The mobility of band 1G (M_r ~70 kD, lane 2) is slightly retarded (lane 1) by the presence of 1 mg/ml (unlabeled) bovine serum albumin (M_r ~67 kD) in the medium. (B) Coomassie Blue-stained SDS PAGE (14% acrylamide) profiles of isolated zymogen granules (lane 1) and secretagogue-stimulated secretion (lane 2) are shown. The latter sample was discharged from pancreatic acini with 2.5×10^{-5} M carbachol plus 10^{-3} M dibutyryl cAMP for 1 h. Samples were loaded on the basis of equal amylase activity (~ 10 U; Bernfeld, 1955). Note that band 11G (near the dye front in A) can be resolved into a series of low molecular mass bands (M_s 14-19 kD) in this gel.

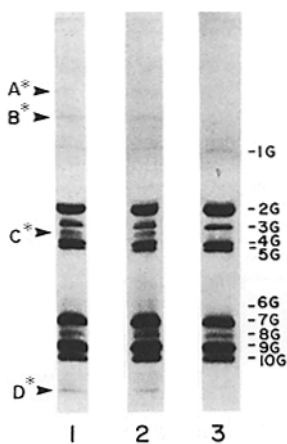


Figure 3. A group of nonzymogen secretory proteins serve as markers for constitutive vesicular discharge. Pancreatic acini were pulse-labeled as in Table I A. Equal numbers of labeled acini were incubated with (lane 2) or without (lanes 1 and 3) 10^{-5} M carbachol for 15-min chase intervals beginning at $t = 15$ min. Lanes 1 and 3 show the labeled output of unstimulated acini at 15–30 min of chase, and 150–165 min of chase, respectively. During each of these time periods, 0.52% of the total unlabeled tissue amylase was released (with a

mean release of $0.51 \pm 0.06\%$ /15 min in the intervening periods). By contrast, the carbachol-stimulated acini (lane 2) released $>11\%$ of total amylase per 15 min. Equal amounts of acid-precipitated radioactivity were loaded in gel lanes. The mobility of band 1G is slightly retarded due to the presence of unlabeled bovine serum albumin. Note that unique bands A*–D* are present at equal levels at early times of chase with or without carbachol, but are not present in the later chase period. Although the intensities of radioactive bands showed differences depending on the original label chosen (^3H -amino acids vs. ^{35}S methionine vs. ^{35}S cysteine), the results were similar.

acini, two with lobules) the ratio of secreted label (secretagogue/control) was only 1.03 ± 0.15 before 30 min, but increased progressively thereafter. After a 2-h chase, secretagogue addition stimulated radiolabeled output by more than an order of magnitude (data not shown), as it did for unlabeled amylase secretion at all chase times (Table I).

We examined the composition of the initial labeled secretion by SDS PAGE with subsequent fluorography. As shown for pancreatic lobules pulsed with ^{35}S cysteine, (Fig. 2 A, lane 1), most of the label was distributed among the group of zymogens 1G–11G, identified by their presence in stained gels of isolated zymogen granules or secretion from stimulated pancreatic acini (Fig. 2 B, lanes 1 and 2, respectively). In addition, a second group of labeled bands (e.g., A*, B*, C*, and D*) were identified (Fig. 2 A) that were not observed in granule or secretory staining patterns.

Two lines of evidence argue that the reproducible presence in the medium of both groups of proteins 1G–11G and A*–D* during and after the first 30 min is due to secretion rather than to cell death. First, the newly synthesized pancreatic enzymes (1G–11G) appeared asynchronously in the medium such that the low molecular mass serine protease zymogens (bands 6G–10G) were prominent species earlier than either α -amylase (2G) or lipase (3G), which were initially minor bands (Fig. 2 A). This asynchrony is similar to that observed by others, reflecting asynchronous exit of these proteins from the ER (Rohr and Keim, 1984; Scheele and Tartakoff, 1985). By contrast, intentional cell death (induced experimentally by mechanical forces or detergent treatment) caused the pattern of asynchronous release to be abolished (not shown). Second, bands A*–D* are extremely minor labeled species within the tissue (Fig. 2 A, lane 2) and their disproportionate representation in the initial chase collection (Fig. 2 A, lane 1) suggests that they are secreted selectively.

Fig. 3 illustrates that A*–D* (^3H -amino acid-labeled) were present in the initial (lane 1) but not later (lane 3) ra-

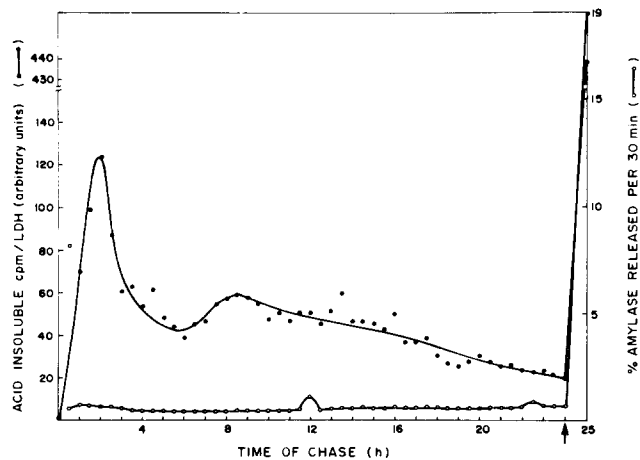


Figure 4. Biphasic release of newly synthesized protein and linear unlabeled amylase discharge from unstimulated pancreatic lobules. Pancreatic lobules from a fasted rat were pulse-labeled with ^{35}S cysteine for 0.5 min at $t = -5$ min, washed, and sequential 30-min chase collections begun (at $t = 0$) for 24 h. Samples were processed and data calculated as in Fig. 1. At $t = 24$ h, carbachol (10^{-5} M) plus dibutyl cAMP (10^{-3} M) were added to the medium (arrow), which resulted in a ~ 30 -fold stimulation of amylase output and a ~ 20 -fold stimulation of label release.

diolabeled secretion of dispersed acini, and the presence of secretagogue (10^{-5} M carbachol, lane 2) did not change the output of these labeled bands (although unlabeled secretory output was stimulated >10 -fold). Most likely these bands originate in acinar cells,³ since they are discharged not only from pancreatic lobules, but also are prominent in the secretion from dispersed pancreatic acini, which are quite poor in duct cell or islet cell contaminants (J. D. Jamieson, personal communication). Therefore, both labeled zymogens and unique labeled proteins (A*–D*) are released (within 30 min of pulse) from nonstimulable compartments. The latter proteins in particular appear to be useful as selective markers for at least one kind of constitutive nongranule discharge.

Basal Granule Exocytosis Occurs as a Second Phase of Release

It seemed unlikely that the decline in labeled protein secretion seen in Fig. 1 represented a decline in the basal exocytosis of labeled storage granules, because the tissue (rather than becoming nonradioactive at 6 h of chase) still retained $\sim 86\%$ of the pulse-incorporated label. To estimate the unstimulated release from this large remaining pool, we examined an extended time course of secretion, during which tissue viability was maintained in vitro by making complete medium changes (with fresh oxygenated medium containing 1 mg/ml bovine serum albumin [RIA grade] and 0.1 mg/ml purified soybean trypsin inhibitor) every 30 min over a 24-h time course. Under these conditions, unlabeled LDH and amylase output showed limited but parallel rises with mean values of release of $0.26 \pm 0.12\%/30$ min and $0.55 \pm 0.11\%/30$ min, respectively.⁴ Amylase output was stimulated ~ 30 -

3. Although we have not formally excluded centroacinar cells as the source of these proteins, their low concentrations and limited protein synthetic capabilities make them unlikely candidates.

4. These values must be considered as estimates, since there is undoubtedly significant new synthesis of cellular amylase and lactate dehydrogenase during the 24-h time period.

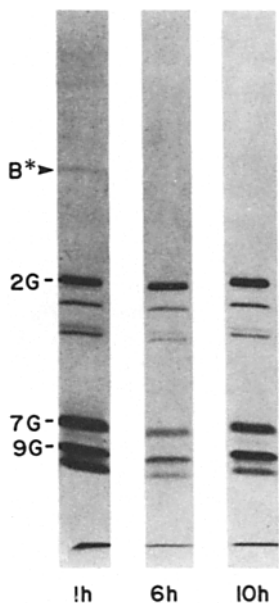


Figure 5. Secretion of newly synthesized proteins at different chase intervals. Lobules from the pancreas of a fasted rat were pulse-labeled as in Fig. 4. Medium samples during selected chase intervals (0.5–1 h, lane 1; 5.5–6 h, lane 2; 9.5–10 h, lane 3) were analyzed by SDS PAGE and fluorography; equal volumes of medium were loaded from each time point. Note that the zymogens are the major secreted labeled proteins at all chase times and that bands 7G and 9G are prominent initially (1 h), less prominent later (6 h), but still later are again prominent (10 h). (The significance of this finding is reviewed in the text and in Table II.)

fold by secretagogue addition at the end of the 24-h period (Fig. 4).

Most striking in these extended experiments was the consistent finding of a second phase of radiolabeled protein release from the pancreatic lobules of fasted rats (Fig. 4). This second phase peaked at 9–10 h of chase⁵ and was lower in amplitude and broader than the first peak; no third phase was detected.

For three reasons, it appears that the second phase represents true (basal) granule exocytosis. First, the large size of the residual tissue pool (after the first phase) and the slow half-time of second phase release ($t_{1/2} \sim 50$ h, data not shown) is compatible with discharge of proteins from the storage compartment (Mains et al., 1986). Second, radioactive protein release clearly was amplifiable by external stimuli during this phase, since a ~ 20 -fold stimulation of label release was observed upon secretagogue addition during the 25th hour of chase (Fig. 4). Finally, related autoradiographic studies indicated that during the first phase of secretion, the labeling of compartments in the secretory pathway was divided among Golgi, condensing vacuoles, immature granules, and a few mature granules (Arvan, P., and A. Chang, manuscript submitted for publication), whereas at chase times during which the second phase of secretion was ongoing, mature granule labeling was predominant (our unpublished observations).

Asynchronous ER Transport is Reiterated in Both Phases of Release

A central finding in these studies is that the pancreatic zymogens are major proteins secreted in both phases of release (Fig. 5). Since individual zymogen species are known to be asynchronously transported out of the ER (Rohr and Keim, 1984), the compartments that are responsible for both secre-

5. Since condensing vacuole and immature granule labeling are significant within 2 h of chase (Jamieson and Palade, 1967; Bieger et al., 1976), intermixing of these structures within the population of preformed zymogen granules must be restricted to some extent; otherwise a monotonic decline without a distinct second peak would have occurred starting at 2 h of chase (see Fig. 4).

Table II. Percent Distribution of Pulse-labeled Zymogens Discharged into the Medium at Different Times of Chase

Band	Time of collection		
	0.5–2 h	3.5–5 h	7.0–8.5 h
7G (Trypsinogen)	10 ± 1.3	6.9 ± 0.2	9.6 ± 0.2
9G (Chymotrypsinogen)	15 ± 0.7	12 ± 0.5	17 ± 1.2
2G (Amylase)	27 ± 6.3	39 ± 0.4	31 ± 0.4
3G (Lipase)	4.8 ± 0.4	5.3 ± 0.2	4.6 ± 0.2

Pancreatic lobules were pulse-labeled for 2 min with [³⁵S]cysteine and chased with complete medium changes every 30 min for 8.5 h. Samples of chase media were analyzed by SDS PAGE as in Fig. 5. Individual labeled zymogens were excised from the gel, dissolved, and counted as described in Materials and Methods. The percent distribution of pulse-labeled zymogens for each time interval was calculated according to Scheele and Tartakoff (1985).

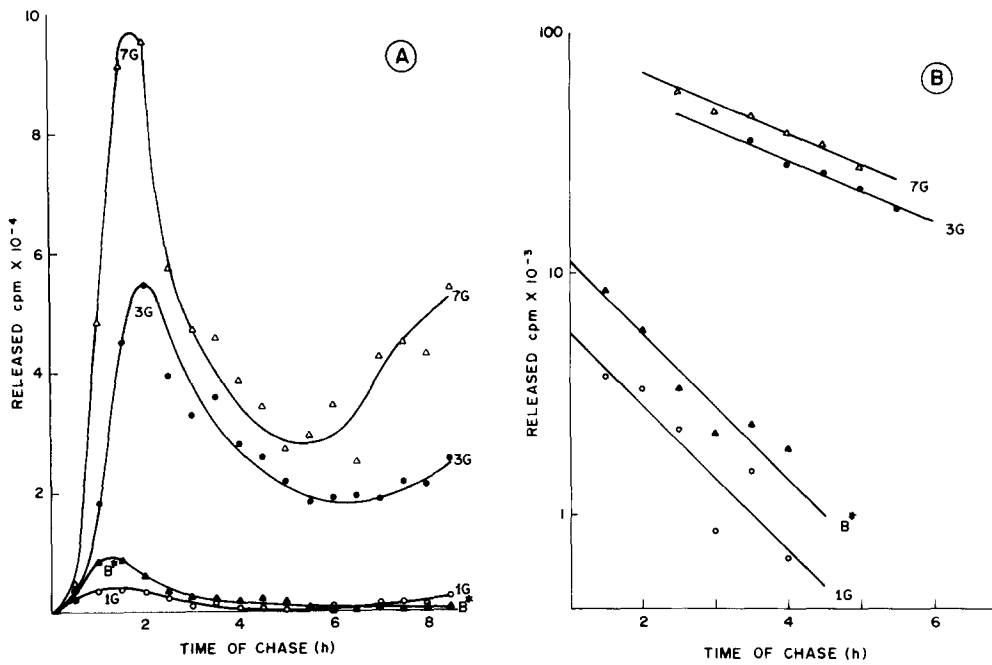
tory phases should initially be rich in those labeled proteins that leave the ER rapidly, but gradually become richer in the labeled proteins that leave the ER slowly.⁶ Therefore, we analyzed three time intervals from the release curve of unstimulated pancreatic lobules (Fig. 4) that represented: (a) the rise of the first phase (0.5–2 h), (b) the fall of the first phase (3.5–5 h), and (c) the rise of the second phase (7–8.5 h). Chase media from each of these intervals were subjected to SDS PAGE under reducing conditions; the radioactivity in each zymogen band was quantitated and the percent distribution for two representative proteins that exhibit rapid ER exit (trypsinogen [7G]; chymotrypsinogen [9G]) were compared with two proteins with slow ER exit (amylase [2G]; lipase [3G]).

The results (Table II) show that for 7G and 9G, their percent distribution was high in the first interval (cf. Table II and Fig. 4), but subsequently fell in the second interval, compatible with the rapidly moving labeled proteins passing through the intracellular compartments responsible for the first secretory phase. However, the percent distribution in these bands rose again in the third interval at the front of the second secretory phase. Radiolabeled amylase (2G) and lipase (3G) exhibited the converse behavior (Table II), exactly as expected for proteins that are slow to exit the ER. Thus, the secretory asynchrony in each phase appears to reiterate the asynchrony in ER exit.

The First Phase of Secretion Contains Different Proteins Discharged with Two Distinct Half-times of Release

To examine in greater detail the kinetics of first-phase secretion for each labeled zymogen as well as bands A*–D*, we quantitated each of the radioactive bands released throughout an 8.5-h time course. The serine proteases (6G–10G) were all secreted from unstimulated tissue with essentially identical kinetics (represented by 7G in Fig. 6A). These bands peaked in the medium slightly before 2 h and for the remainder of their first phase secretion decreased with a $t_{1/2}$ of ~ 2.4 h (Fig. 6B). Amylase and lipase differed in that their release curve was shifted to the right (compatible with slow ER transit, Fig. 6A). However, the declining portion of their

6. The asynchronous transport of zymogens results in a time-dependent shift in the percent distribution of pulse-labeled zymogens secreted from carbachol-stimulated lobules; proteins with rapid ER transit decrease, while proteins with slow ER transit increase with time (Scheele and Tartakoff, 1985).



determine the apparent half-time for discharge (slope of these lines equals half-time). Note that with this analysis, discharge of labeled 7G and 3G occurs with a $t_{1/2}$ of 2.35 h; since these two proteins are known to exit the ER at different rates, their similar discharge rate must reflect a step subsequent to ER exit. In contrast, the measured half-time for B* and 1G is ~ 1 h.

first phase secretion revealed an identical half-time (~ 2.4 h, Fig. 6 B). In fact, using this analysis (see Materials and Methods), bands 2G–11G on average had a half-time for secretion of 2.4 ± 0.35 h before their appearance in the second phase ($t_{1/2} \sim 50$ h) of release. In contrast, the newly described markers of nonstimulated release (A*–D*) peaked earlier in the secretion (≤ 1.5 h, Fig. 6 A), from that point on were discharged with a $t_{1/2}$ of ≤ 1 h (Fig. 6 B), and did not reappear in the second phase. Therefore, despite their differences in ER transit, most of the zymogens were secreted in the first phase by an indistinguishable kinetic process, whereas, on average, the proteins A*–D* exited the cell more rapidly.

A most interesting exception involves 1G, a protein known to be in zymogen granules (Fig. 2 B). 1G was unique in that it showed release kinetics in the first phase similar to that observed with the markers A*–D* (peak release at < 1.5 h, Fig. 6 A; $t_{1/2} \sim 1$ h, Fig. 6 B) yet rose again in the second phase of release. The first phase of 1G secretion is very likely to be apically directed, since it is a major labeled species in the initial ductal secretion collected in vivo (see below).

The contrasting behavior of B*, 1G, and the other zymogens is especially well illustrated in Fig. 7, showing results of an experiment in which pancreatic lobules were briefly pulsed, and subjected to three sequential (1-h) chase incubations, the last in the presence of secretagogues. B*, 1G, and the other zymogens were observed in the first hour of secretion: B* and 1G, in contrast to the other zymogens, were barely detectable in the second hour; whereas 1G, but not B* was detected in increased amounts in the stimulated, third hour, discharge. These data confirm that band 1G has a unique dual character; in its first phase it is released like bands A*–D* ($t_{1/2} \sim 1$ h), but in its second phase it is released like all the other zymogens ($t_{1/2} \sim 50$ h).

Newly Synthesized Pancreatic Proteins are Secreted in a Phasic Manner to the Apical Surface In Vivo

To examine the release of newly synthesized pancreatic proteins in vivo, the pancreatic duct of fasted anesthetized rats was cannulated (see Materials and Methods) and the animals were then given an intravenous injection of [35 S]cysteine at time zero. Subsequent half-hour collections of unstimulated secretion were made and each collection was immediately boiled in 2% SDS to minimize proteolysis. The acid insoluble radioactivity in 6 h of collection is shown in Fig. 8 A;

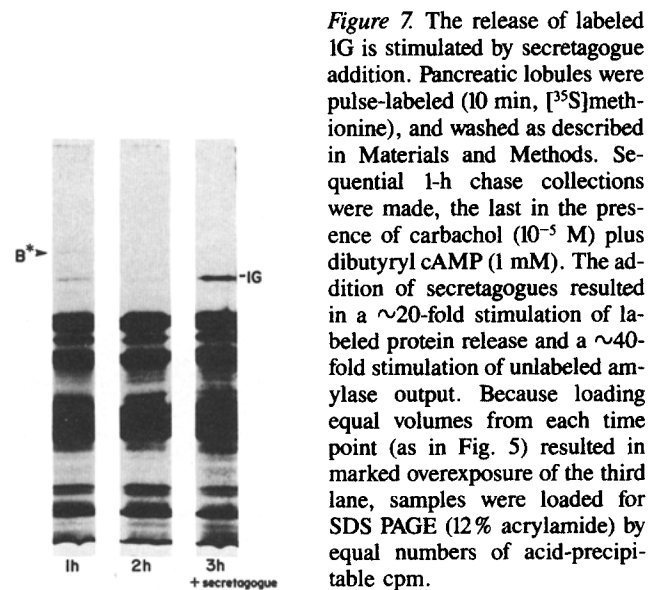


Figure 7. The release of labeled 1G is stimulated by secretagogue addition. Pancreatic lobules were pulse-labeled (10 min, [35 S]methionine), and washed as described in Materials and Methods. Sequential 1-h chase collections were made, the last in the presence of carbachol (10^{-5} M) plus dibutyryl cAMP (1 mM). The addition of secretagogues resulted in a ~ 20 -fold stimulation of labeled protein release and a ~ 40 -fold stimulation of unlabeled amylase output. Because loading equal volumes from each time point (as in Fig. 5) resulted in marked overexposure of the third lane, samples were loaded for SDS PAGE (12% acrylamide) by equal numbers of acid-precipitable cpm.

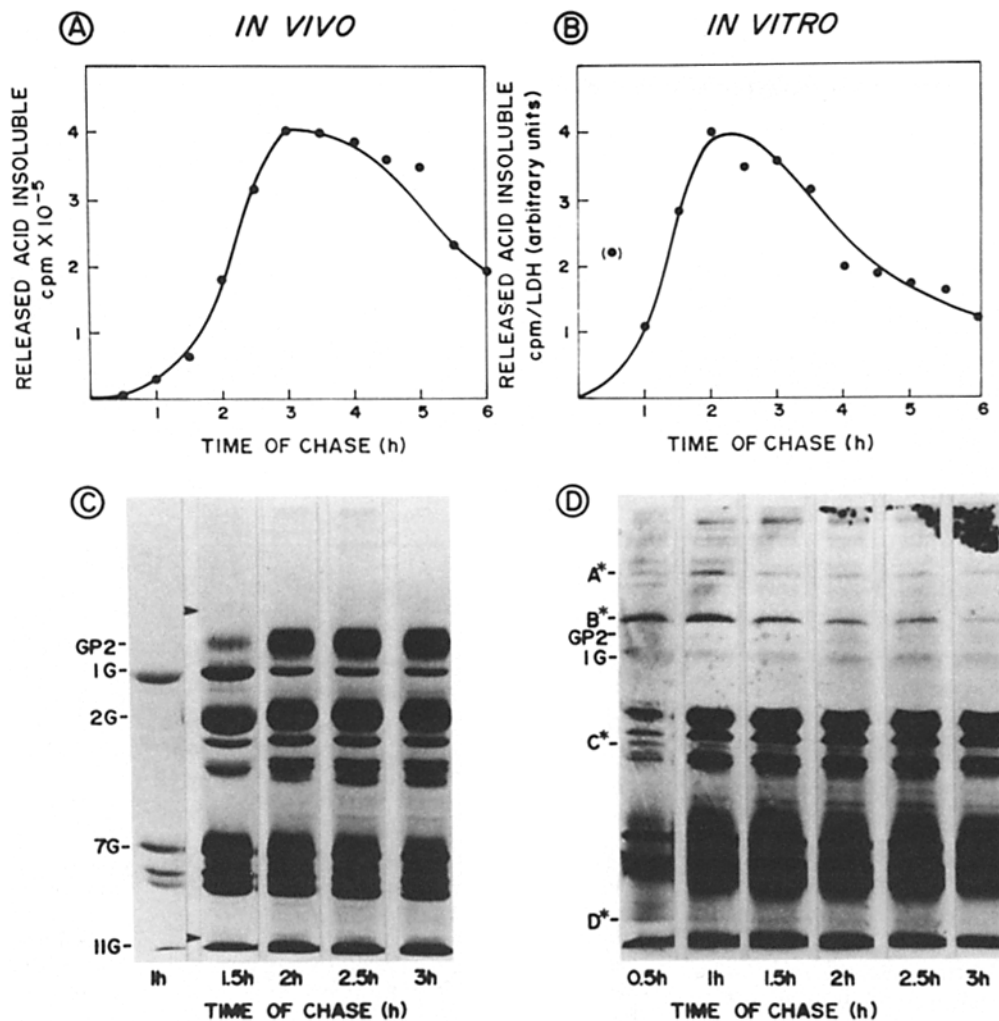


Figure 8. Comparison of labeled apical secretion collected in vivo with lobule secretion in vitro. (A) Cannulation of the pancreatic duct and collection of [³⁵S]cysteine-labeled secretion was performed as described in the text. Acid-precipitable cpm from each time point is shown. (C) SDS PAGE profiles of labeled proteins from cannulated secretion as a function of time after injection. (B) Secretion of [³⁵S]cysteine-labeled proteins from pancreatic lobules. Pulse labeling was performed as in Fig. 4. (D) SDS PAGE profiles of labeled secretion as a function of time. The mobility of band IG is slightly retarded due to the presence of unlabeled bovine serum albumin. Bands A*, B*, C*, and D* decline as the chase progresses. Note that bands A*–D* are far more prominent in D than in C.

for comparison, the release of pulse-labeled proteins from pancreatic lobules over a 6-h time course is shown in Fig. 8 B. The key point of this figure is that the apical secretion from rat pancreas in vivo showed a phasic release similar to that seen in vitro (we were unable to sustain the experiments in vivo long enough to demonstrate a second phase). The peak of the first phase was slightly delayed in vivo, presumably reflecting differences in labeling conditions and exceedingly slow ductal drainage. In the presence of secretin stimulation (to maintain brisk pancreatic flow rates, Robberecht et al., 1977; Beaudoin et al., 1980) the peak of this phase actually may be as early as 80 min (however, we chose to avoid secretin because of its potential effects on granule discharge).⁷

Comparison of SDS PAGE profiles confirmed that the pancreatic zymogens were the major secreted proteins in this phase of release, both in vivo (Fig. 8 C) and in vitro (Fig. 8 D). However, several differences in the two patterns were observable. First, in addition to the zymogens, GP-2, a membrane glycoprotein that is apparently released into the secre-

tion (Havinga et al., 1983) was found at a relatively much higher level in the cannulated secretion than it was in vitro. Second, in contrast to the first hour of chase in vitro when bands A*–D* were at their highest levels (together, ~7% of the total labeled protein output) and then declined subsequently; there was no detectable output of these bands in the initially collected labeled apical secretion. Although minor bands in the range of M_r ~100 and ~25 kD were seen in the cannulated secretion at various times of chase (arrowheads, Fig. 8 C), these bands could not be definitely identified as bands B* and D* when run on gels side by side (and there was no evidence of bands A* or C*; data not shown). Further, with the possible exception of the minor band at ~100 kD, there were no minor bands that showed the characteristic phasic decay exemplified by A*–D* in vitro. Although we feel that the data are equivocal on the point of whether bands A*–D* are totally excluded from the apical secretion, it is clear by comparison of Fig. 8 C–D that these bands are scant in their relative proportions to the apically secreted zymogens.

Physiologic Regulation of the Phasic Character of Release

To gain further insight into the possible physiological significance of the two phases of secretion, we compared (Fig.

7. The decline of this phase in vivo (Fig. 8 A) cannot be due to tissue depletion of radioactivity, since this is incompatible with the lengthy turnover time of rat pancreatic digestive enzymes (Vandermeers et al., 1968) and since secretagogue infusion results in a clear stimulation of labeled discharge over the resting level (Roberge and Beaudoin, 1982).

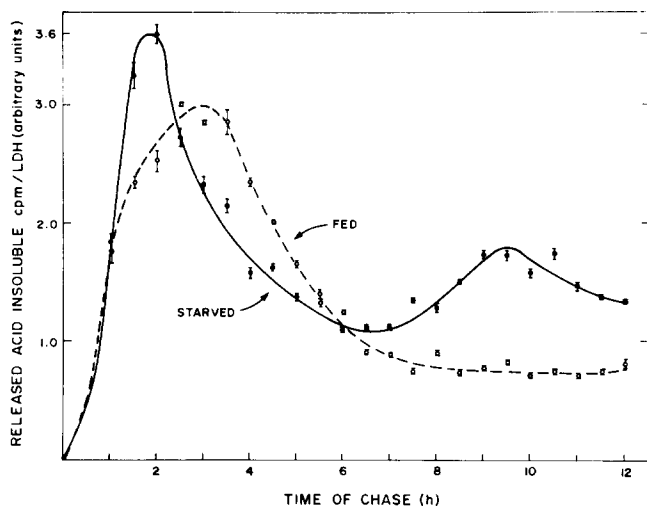


Figure 9. The phasic pattern of labeled secretion in lobules from fasted rats is different from fed rats. The averaged results of five experiments with fasted animals and of two matched experiments (done side by side) with fed animals are shown (two additional unmatched experiments on fed rats have been performed that reveal a pattern similar to that shown here). In each case, lobules were pulsed with [35 S]cysteine (0.5 min), and chase collections were made as in Fig. 4. Note a change in the shape of the first phase and apparent absence of the second phase in the tissue from fed animals. Points represent mean values; the space between error bars represents two standard deviations.

9) the labeled protein output by pancreatic lobules from rats that had been subjected to overnight fast (as in all experiments discussed so far) to that from rats that were permitted to feed ad libitum (on their usual nocturnal cycle). The results over a 12-h incubation (shown from five averaged experiments, including two experiments in which side by side incubations were run with lobules of fed rats for peak height and width comparison) indicated that omission of the fast altered the phasic pattern of labeled protein output: the first phase had a different shape and lasted longer while the second peak, normally occurring at ~ 9 h of chase, was not present (Fig. 9). While the SDS PAGE pattern of first phase proteins from both kinds of animals appeared identical, we did not observe the second reiteration of asynchronous zymogen transport (which marks the onset of the second phase) in times up to 11.5 h of chase from lobules of fed rats (data not shown). So far we have not extended the incubation times of lobules from fed rats to check for a delayed second phase of release; however, we know that at the end of 12 h, these lobules still retained $80 \pm 3\%$ of the original pulse-incorporated radioactivity (a slightly lower value was obtained from lobules of starved rats). Further studies will be required to explore unstimulated secretion from fed animals in detail. Nevertheless, the data obtained so far suggest that the phasic character of unstimulated protein release is under physiologic control.

Discussion

We have sought to assess the quantitative contribution of nongranule secretory pathways to the unstimulated secretion of rat exocrine pancreatic tissue *in vitro*. By employing conditions that enable extended observations of newly synthe-

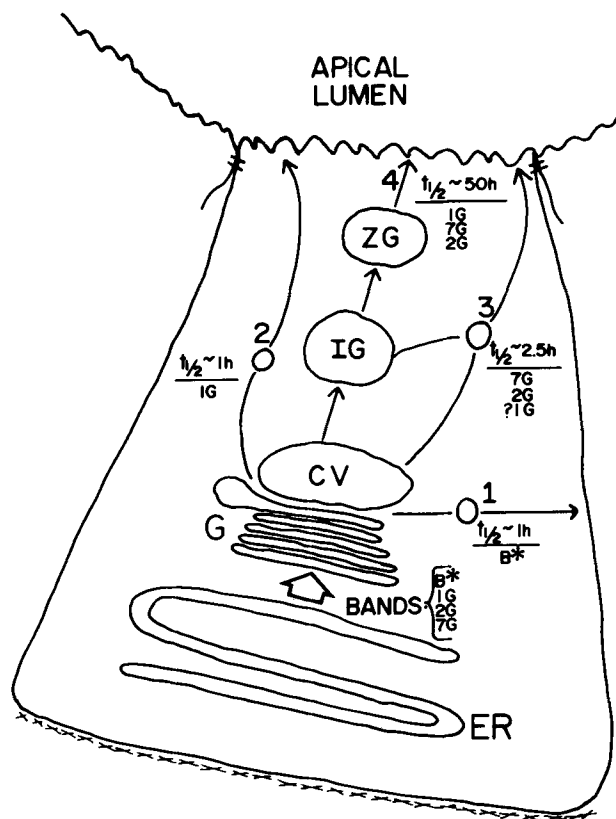


Figure 10. Schematic model of the routes of protein discharge in the pancreatic acinar cell. The model shown is based on kinetic studies of secretion described in the text; four representative protein bands are shown. For simplicity, asynchrony in ER exit is omitted from the model since our data (Fig. 6 B) suggests that the half-time for the discharge step (at least for 7G and 2G) is not controlled by the ER exit rate. The structural correlates shown in this model are compatible with the data presented herein, but have not been established by direct experimentation. G, Golgi complex; CV, condensing vacuole; IG, immature granule; ZG, mature zymogen granule.

sized protein secretion, we have revealed two phases of release (first, constitutive vesicular; second, granule). Both secretory phases reiterate the asynchronous exit of labeled proteins from the ER.⁸ Such reiteration in the second phase indicates that there are restrictions on the intermixing of new and old granules within the storage population. This observation is consistent with previous reports (Singh, 1982; Sharoni et al., 1976), and makes unlikely the possibility that the first secretory phase is due to preferential exocytosis of newly formed granules.

Based on the present findings in unstimulated tissue, we conclude that the classical model of zymogen secretion from storage granules accounts for the behavior of, at most, 85% of pancreatic secretory protein.⁹ The model shown in Fig.

8. Rothman and Isenman (1974) used results of protein radiolabeling experiments to support a hypothesis involving two parallel intracellular pools of secreted zymogens in the rabbit exocrine pancreas. The second phase observed herein is very unlikely (because of its large magnitude) to represent first phase secretion from a cell subpopulation in which intracellular transport is unusually slow. Rather, the present data suggest the existence of parallel intracellular pools; however, these data do not support the interpretation (made by those authors) that the rapid turnover pool of secreted proteins is in the cytosol.

9. We consider this an upper estimate because, although $\sim 85\%$ of the radio-

10 is intended to present a more comprehensive view in which all of the labeled secretory proteins are eventually released as part of basal secretion. However, we emphasize that the paths shown are based primarily on kinetic distinctions; the structural correlates for these paths remain to be rigorously established.

In aggregate, nongranule release mechanisms (comprising paths 1-3, Fig. 10) account for the fate of ~15% of pulse-incorporated label (i.e., the first phase). The intracellular containers that give rise to these paths appear to be devoid of label by 7 h of chase, indicating that the first phase of secretion does not derive, directly or indirectly, from mature zymogen granules.

Since bands A*-D* are not clearly identified in the apical secretion *in vivo*, we have designated pathway 1 as going to the basolateral surface. Another possibility is that these proteins are discharged apically, but are proteolytically or otherwise modified *in vivo* so that they are not detected by our analysis. Although their acinar cell origin seems likely based on their presence in dispersed pancreatic acini and their prominence during early phasic discharge (~20% of total labeled secretion at 30 min), further biochemical and immunological characterization will be needed to confirm their origin and establish their mode of intracellular transport. However, we can already exclude that these polypeptides are precursors or otherwise related to zymogens, since Scheele et al. (1980) have demonstrated by *in vitro* translation experiments that rat pancreatic zymogens are synthesized as polypeptides whose electrophoretic mobilities are exceedingly close to those of the finally secreted products.

Pathways 2 and 3 (Fig. 10) represent the predominant routes of release of pancreatic zymogens in the first phase; both paths lead to the apical surface, based on the results of ductal cannulation experiments *in vivo*. Although these paths almost certainly do not derive from mature granules, we are impressed by the long delay between the peak Golgi labeling time (≤ 30 min) established autoradiographically in the pancreas and other secretory tissues (Jamieson and Palade, 1967; Salpeter and Farquhar, 1981; Arvan, P., and A. Chang, manuscript submitted for publication) and the peak release of labeled zymogens in our studies (~2 h, Fig. 9). A simple interpretation that reconciles these findings is that the first phase of zymogen secretion derives primarily from vesicular traffic exiting condensing vacuoles (peak labeling 40-60 min of chase) and/or immature granules (peak labeling 1-2 h of chase). Since these structures are involved in surface area reduction as well as concentration of content, we hypothesize that constitutive vesicular secretion of the zymogens is in some way coupled to granule maturation. Further, since labeled zymogens within 30-45 min of the pulse are already contained in structures capable of stimulated discharge, we hypothesize that some of these immature structures can give off labeled secretion either in the form of constitutive vesicular discharge (in the absence of stimulation) or by wholesale exocytosis (in the presence of stimulation). Thus, the origins of the constitutive and storage pathways may involve common structures.

The possibility that a fraction of the vesicular discharge occurs before the arrival of label in the condensing vacuole

activity is tissue-associated after the first phase of secretion, a fraction of this label is incorporated into protein other than secretory protein.

compartment may explain the earliest (nonstimulable) release of zymogens (including 1G), implying multiple branch points in secretory routing. Pathway 2 (Fig. 10) was especially constructed to account for the rapid half-time of release of 1G (~1 h) compared with the other zymogens. However, there is no experimental basis to structurally distinguish pathway 2 from pathway 3; we can only conclude at this time that there is a mechanistic difference in the surface delivery of 1G from the other zymogens. We feel justified in treating the other zymogens (2G-11G) as a cohort, because, despite their varying exit rates from the ER (Rohr and Keim, 1984; Scheele and Tartakoff, 1985), they share a common discharge rate ($t_{1/2}$ ~2.4 h) suggesting a mechanistically similar secretory pathway (pathway 3, Fig. 10).

Pathway 4 (Fig. 10) is the classic route of exocytotic discharge from the storage compartment, comprising the second phase of secretion in these studies. By extrapolation of the rising slope of the second phase in Fig. 9 we estimate that the earliest basal granule discharge does not occur in lobules from fasted rats until at least 3.5-4 h postpulse and is not the predominant unstimulated pathway until ~7 h. The assignment of this pathway reflects its quantitative importance (~85% of secretion), its long half-time of release (~50 h), its ability to be stimulated, and autoradiographic evidence identifying mature granules as the major label source during this phase (our unpublished observations).

The current findings provide new insights into secretory sorting operations. Moore and Kelly (1986) have proposed that proteins secreted by constitutive vesicular discharge can be distinguished from granule proteins by differences in polypeptide sorting domains. However, a completely satisfactory proposal has not been made to explain why each of the granule proteins they have examined in detail (proopiomelanocortin, Gumbiner and Kelly, 1982; proinsulin, Moore et al., 1983; trypsinogen, Burgess et al., 1985; human growth hormone, Moore and Kelly, 1985) has been found to be released in part by constitutive vesicular secretion. We too find that granule proteins (pancreatic zymogens) are released by both granule and nongranule mechanisms. Further, the proportions of labeled zymogens (relative to each other) in the first phase of secretion appears to be the same as that in the second phase (Table II and data not shown); i.e., there is no competition evident between the zymogens (which are present in widely different copy number) for a putative carrier.

Therefore, we propose that although sorting domains might be important to facilitate selected protein entry into condensing vacuoles, these domains are not important in distinguishing all the proteins that are divided between constitutive vesicular and granule discharge routes (as exemplified by pathways 3 and 4 in Fig. 10). This proposal is compatible with the idea that there is vesicular traffic to the cell surface from the condensing vacuole and immature granule compartments. Since in the pancreatic acinar cell, the majority of the first phase secretion is accounted for by pathway 3, we are reluctant to correlate the terms regulated and constitutive secretory pathways with carrier-mediated and nonmediated sorting events (Moore and Kelly, 1986), because much of the constitutive vesicular secretion may originate after carrier-mediated sorting steps are completed.

Finally, we are greatly intrigued by the reproducible differences between pancreatic tissue from fasted and fed rats. It

has been shown that during the feeding cycle of rats, the numbers of pancreatic granules, condensing vacuoles, and Golgi elements are regulated (Uchiyama and Saito, 1982). The altered shape of the first phase of secretion in fed animal tissue provides further reason to suspect physiological control of what is currently viewed as an unregulated or constitutive process. We suspect the latter may be an oversimplified view and feel this to be a promising area for future investigation.

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