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

Peter Arvan* and J. David Castle‡

*Departments of Internal Medicine and ‡Cell Biology, Yale University School of Medicine, New Haven, Connecticut 06510

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 ($t_{1/2}$ ~2.4 h) and (b) minor nonzymogens plus one unique zymogen ($t_{1/2}$ ~1 h). The second phase (peak at 9–10 h) is stimulable, probably represents basal granule exocytosis ($t_{1/2}$ ~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.

I. 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]-l-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 Paraguha, 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 (RPFI 1040, 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 (0.5–30 min, as specified) in Met-free, Cy-f1ee, 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 (Whattman, 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 cava) injection of 2.5 mCi [35S]cysteine in normal saline was administered. In the experiment shown in Fig. 8, protein output was 117 ± 8 μg/30 min with a flow rate of ~25 μl/30 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 Autoradiol (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 min (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).](image-url)
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 $\geq 30$ min after onset of pulse-labeling. In four experiments (two with dispersed 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 (Adamsen, 1973).

**Biochemical Assays**

Amylase was measured according to Jung (1980) or Bernfeld (1955); Lactate dehydrogenase (LDH) was after Schmaar 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). 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

---

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

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

Pancreatic acini (A) or lobules (B) were pulse-labeled at time zero (5 min, $^3$H-amino acids, [A]; 10 min, [35S]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.
The Journal of Cell Biology, Volume 104, 1987

The ratio of secreted label to cell 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 did 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 pulse labeled with [35S]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 reproductible 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 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, 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. [35S]methionine vs. [35S]cysteine), the results were similar.

Figure 3 illustrates that A*-D* (3H-amino acid-labeled) were present in the initial (lane 1) but not later (lane 3) radiolabeled 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, since they are discharged not only from pancreatic lobules, but also 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 ~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 (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 0.2 ± 0.1%/30 min and 0.55 ± 0.1%/30 min, respectively. Amylase output was stimulated ~30-
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 asynronously transported out of the ER (Rohr and Keim, 1984), the compartments that are responsible for both secretory 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. 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 (amyglase [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. 6 A). These bands peaked in the medium slightly before 2 h and for the remainder of their first phase secretion decreased with a t1/2 of ~2.4 h (Fig. 6 B). Amylase and lipase differed in that their release curve was shifted to the right (compatible with slow ER transit, Fig. 6 A). However, the declining portion of their release curves were consistent with a ~4.5- to 5-h time course for the slow-release zymogens.

5. Since condensing vacuole and immature granule labeling are significant within 2 h of chase (Jamieon 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).

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).
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\(_{1/2}\) ≈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 thezymogens 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 inzymogen 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\(_{1/2}\) ≈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*, IG, 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*, IG, and the other zymogens were observed in the first hour of secretion: B* and IG, in contrast to the other zymogens, were barely detectable in the second hour; whereas IG, 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\(_{1/2}\) ≈1 h), but in its second phase it is released like all the otherzymogens (t\(_{1/2}\) ≈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 [35S]methionine 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, [35S]methionine), and washed as described in Materials and Methods. Sequential 1-h chase collections were made, the last in the presence of C6H14H2N (10⁻³ 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 secretion (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 Mr ~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.
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 [35S]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 ± 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 synthesized 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. 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. 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 ~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 transduction 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 ducal 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 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 (t½ ~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 (pro-opiomelanocortin, 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.

The authors gratefully acknowledge the support and encouragement of Dr. Howard Rasmussen, in whose laboratory these experiments were carried out. We thank Dr. L. Arvan and Dr. A. Chang for helpful discussions and Ms. A. Ma for help with the cannulation experiments. We also thank Cindy Davis for help in preparation of this manuscript and Pam Ossorio for help with preparation of the figures.

This work was supported by National Institutes of Health grant GM-26524.

Received for publication 23 July 1986, and in revised form 4 October 1986.

References

Adamson, A. W. 1973. A Textbook of Physical Chemistry. Academic Press, Inc., New York pp. 721-724 and 1046-1048.

Beaudoin, A. R., M. Filion, and M. Roberge. 1980. Distinct pools of secretory proteins in the rat pancreas. J. Cell Biol. 101:639-645.

Beaudoin, A. R., M. Filion, and M. Roberge. 1980. Distinct pools of secretory proteins in the rat pancreas. J. Cell Biol. 101:639-645.

Bier, W., J. Seybold, and H. F. Kern. 1976. Studies on intracellular transport of secretory proteins in the rat exocrine pancreas. J. Cell Biol. 70:203-219.

Bieger, W., J. Seybold, and H. F. Kern. 1976. Studies on intracellular transport of secretory proteins in the rat exocrine pancreas. J. Cell Biol. 70:203-219.

Burgess, T. L., C. S. Craik, and R. B. Kelly. 1985. The exocrine protein trypsinogen is targeted into the secretory granules of an endocrine cell line: studies by gene transfer. J. Cell Biol. 101:639-645.

De Lisle, R. C., J. Schulte, T. Tyrakowski, W. Haase, and U. Hopfer. 1984. Isolation of stable pancreatic zymogen granules. Am. J. Physiol. 246:6411-6418.

Elsenhans, A., and B. Kelly. 1985. Four secretory proteins synthesized by hepatocytes are transported from endoplasmic reticulum to Golgi complex at different rates. EMBO (Eur. Mol. Biol. Org.) J. 3:147-152.

Gumbiner, B., and R. B. Kelly. 1982. Two distinct intracellular pathways transport secretory and membrane glycoproteins to the surface of pituitary tumor cells. Cell. 28:51-59.

Havia, J., R. G. J. M. Strous, and C. Poort. 1983. Biosynthesis of the major glycoprotein associated with zymogen-granule membranes in the pancreas. Eur. J. Biochem. 133:449-454.

Herzog, V., and M. G. Farquhar. 1977. Luminal membrane retrieved after exocytosis reaches most Golgi cisternae in secretory cells. Proc. Natl. Acad. Sci. USA. 74:5073-5077.

Iacino, D., G. A. Scheele, and C. Liebow. 1980. Secretory response of the rabbit pancreas to cholecystokinin stimulation. Am. J. Physiol. 239-G247-G254.

Iwanj, V., and J. D. Jamieson. 1982. Biochemical analysis of secretory proteins synthesized by normal rat pancreas and by pancreatic acinar tumor cells. J. Cell Biol. 95:734-741.

Jamieson, J. E., and G. E. Paide. 1967. Intracellular transport of secretory proteins in the pancreatic exocrine cell. II. Transport to condensing vacuoles and zymogen granules. J. Cell Biol. 34:597-615.

Jung, D. H. 1980. Preparation and application of procion yellow starch for amylose assay. Clin. Chim. Acta. 100:7-11.

Kelly, R. B. 1985. Pathways of protein secretion in eukaryotes. Science (Wash. DC). 226:1082-1087.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond.). 227:680-685.

Lodish, H. F., N. Kong, M. Snider, and G. J. A. M. Strous. 1983. Hepatoma secretory proteins migrating from rough endoplasmic reticulum to Golgi at characteristic rates. Nature (Lond.). 304:80-83.

Mains, R. E., E. I. Cullen, V. May, and B. A. Eipper. 1986. The role of secretory granules in peptide biosynthesis. Ann. N.Y. Acad. Sci. In press.

Moore, H.-P. H., and R. B. Kelly. 1987. Secretory protein targeting in a pituitary cell line: differential transport of foreign secretory proteins to distinct secretory pathways. J. Cell Biol. 101:1773-1781.

Moore, H.-P. H., and R. B. Kelly. 1987. Re-routing of a secretory protein by fusion with human growth hormone sequences. Nature (Lond.). 321:443-446.

Moore, H.-P. H., M. D. Waifer, F. Lee, and R. B. Kelly. 1983. Expressing a human proinsulin cDNA in a mouse ACTH-secreting cell. Intracellular storage, proteolytic processing, and secretion on stimulation. Cell. 35:531-538.

Palade, G. E. 1975. Intracellular processes of the aspect of protein synthesis. Science (Wash. DC). 189:347-358.

Rausch, U., P. Vasiiloudes, K. Rüdiger, and H. F. Kern. 1985. In vivo stimulation of rat pancreatic acinar cells by infusion of secretin. II. Changes in individual rates of enzyme and isoenzyme biosynthesis. Cell Tissue Res. 242:641-644.

Robberecht, P., M. Cremer, and J. Christophe. 1977. Discharge of newly synthesized proteins in pure juice collected from the human pancreas. Gastroenterology. 72:417-421.

Roberge, M., and A. Beaudoin. 1982. Newly synthesized secretory proteins from pig pancreas are not released from a homogeneous granule compartment. Biochim. Biophys. Acta. 716:331-336.

Rober, G., and V. Keim. 1984. Asynchrony in intracellular transport of newly-synthesized rat pancreatic proteins. Dig. Dis. Sci. 29:965.

Rothman, S. S., and L. D. Issenman. 1979. Secretion of digestive enzyme derived from two parallel intracellular pools. Am. J. Physiol. 226:1082-1087.

Schaller, M., M. M., and M. F. Farquhar. 1981. High resolution analysis of the secretory pathway in mammmotrophs of the rat anterior pituitary. J. Cell Biol. 91:240-246.

Scheele, G. A., and G. E. Palade. 1975. Studies on the guinea pig pancreas. Parallel discharge of exocrine enzyme activities. J. Biol. Chem. 250:2660-2670.

Scheele, G., and A. Tartakoff. 1985. Exit of nonglycosylated secretory proteins from the rough endoplasmic reticulum is asynchronous in the exocrine pancreas. J. Biol. Chem. 260:926-931.

Scheele, G., R. Jacoby, and T. Carne. 1980. Mechanism of compartmentation of secretory proteins: transport of exocrine pancreatic proteins across the microsomal membrane. J. Cell Biol. 87:611-623.

Schick, J., H. Kern, and G. Scheele. 1984. Hormonal stimulation in the exocrine pancreas results in coordinate and anticoordinate regulation of protein synthesis. J. Cell Biol. 99:1569-1574.

Schraar, R. L., P. H. Weigel, M. S. Kuhlenschmidt, Y. C. Lee, and S. Rosenman. 1978. Adhesion of chick hepatocytes to polyacrylamide gels derivatized with N-acetylglucosamine. J. Biol. Chem. 253:7940-7951.

Sharoni, Y., S. Eimerl, and M. Scharmm. 1976. Secretion of old versus new exportable protein in rat parotid slices; control by neurotransmitters. J. Cell Biol. 71:107-122.

Singh, M. 1982. Nonparallel transport of exportable proteins in rat pancreas in vitro. Can. J. Physiol. Pharmacol. 60:597-603.

Tartakoff, A., and P. Vassalli. 1978. Comparative studies of intracellular transport of secretory proteins. J. Cell Biol. 78:694-707.

Uchiyama, Y., and K. Saito. 1982. A morphometric study of 24-hour variations in subcellular structures of the rat pancreatic acinar cell. Cell Tissue Res. 226:609-620.

Vandermeers, A., M. S., P. Bohlen, W. Daiman, W. Leingruber, and M. Weigbe. 1972. Fluorescinc: a reagent for assay of amino acids, peptides, proteins, and primary amines in the picomole range. Science (Wash. DC). 178:871-872.

Vandermeers, A., M. S., and E. Cullen. 1978. Nonparallel transport of exportable proteins in rat pancreas in vitro. Can. J. Physiol. Pharmacol. 60:597-603.

Wassail, R. J., A. K. MacDonald, R. K. Raman, and W. J. Rutter. 1980. Proteins synthesized and secreted at rat pancreatic development. J. Cell Biol. 86:784-794.