STUDIES ON DISPERSED PANCREATIC EXOCRINE CELLS

II. Functional Characteristics of Separated Cells

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ABSTRACT

The functional characteristics of separated guinea pig pancreatic exocrine cells have been examined following dissociation of the gland by a procedure described in the previous paper (*J. Cell Biol.* 1974. **63**:1037). The ability of isolated cells to incorporate labeled amino acids into secretory proteins was assessed biochemically and by quantitative electron microscope autoradiography. Incorporation remained linear for up to 4-h incubation at levels equivalent to those of pancreatic slices; over 95% of the exocrine cells in the population were viable, and all appeared to be equally active in incorporating amino acids. The capacity of separated cells to transport, concentrate, and store exportable proteins was monitored by electron microscope autoradiography on populations pulse labeled with [^{3}H]leucine and chase incubated for 4 h. The same overall pathway previously mapped in pancreatic slices was followed by secretory proteins in separated cells although in quantitative studies a defect was noted in the rate of conversion of condensing vacuoles to zymogen granules. Secretogogue responsiveness was assessed by monitoring discharge of labeled secretory proteins or of amylase in response to carbamylcholine and caerulein to the medium. While the separated cells released secretory proteins linearly for up to 4 h in response to both secretogogues, the net release was ~50% less than previously noted for pancreatic slices and required a ten times higher concentration of stimulant. The defect may represent alteration in receptors due to the protease used for dissociation. Our data indicate, however, that separated exocrine cells retain their ability to process secretory proteins stepwise and vectorially which is consistent with preservation of structural polarity.

In the preceding paper we have described a procedure for dissociation of the guinea pig pancreas into a population consisting primarily of isolated exocrine cells (1). The method includes digestion with an enzyme mixture containing pure collagenase, chymotrypsin, and hyaluronidase, an interposed chelation of divalent cations by EDTA, and repeated pipeting. It is simple to perform, reproducible, and results in cell yields of from 50 to 60% based on DNA content of the final preparation.

Fine structural studies indicated that these isolated exocrine cells retain the main characteristics of their counterparts *in situ* both with regard to polarized distribution of cell organelles and to regional differentiation of the bounding plasmalemma.

In addition to the use of morphologic criteria,

we have also used functional parameters to assess the adequacy of the dissociation procedure. In this paper we report on the ability of isolated exocrine cells to synthesize, transport, and concentrate exportable proteins into zymogen granules and to release the stored products in response to secretogogues. These studies were conducted under conditions simulating those previously used to assess exocrine cell function in pancreatic slices (2–5). In this way the functional characteristics of the isolated cells can be related to data previously obtained on cells *in situ*.

MATERIALS AND METHODS

Materials

L-[4,5-^{3}H]leucine, 50–60 Ci/mmol, L-[U-^{14}C]leucine, 270–320 mCi/mmol, and carbamylcholine Cl were from Schwarz/Mann Div., Becton, Dickinson and Co., Orangeburg, N. Y. Synthetic caerulein, 70% pure, was the kind gift of Professor B. Camerino, Farmitalia, Milano. Ilford L-4 emulsion was from Ilford Ltd., Ilford, Essex, England. Soybean trypsin inhibitor (STI)[1] was from Worthington Biochemical Corp., Freehold, N. J. All other chemicals were reagent grade.

Preparation of Cells

Populations of exocrine cells were obtained from the guinea pig pancreas as described in the preceding paper (1). For incubation postdissociation, the cells were resuspended in KRB containing 1% BPA, a complete L-amino acid supplement (including 0.4 mM leucine), 14 mM glucose, 2.5 mM Ca^{++}, and 1.2 mM Mg^{++}. The medium also contained 0.1 mg/ml STI to block tryptic activity, 100 U/ml K penicillin G, and 50 μg/ml streptomycin SO_4. This medium, referred to as complete KRB (KRBC), was equilibrated with 95% O_2/5% CO_2 to a pH of 7.4; this atmosphere was maintained by intermittent gassing with moist 95% O_2/5% CO_2 at 30-min intervals, the flasks being tightly stoppered in between. All glassware was siliconized.

Labeling Procedures for Isolated Cells

PROCEDURE A: Cells obtained from a single pancreas (0.8–0.9 g wet weight) and representing ~1.7 mg DNA (~2 × 10^8 cells) were suspended in 5 ml KRBC in a 25-ml Erlenmeyer flask. To initiate pulse labeling, the cell suspension was preincubated for 3 min at 37°C, after

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[1] *Abbreviations used in this paper:* BPA, bovine plasma albumin; DNA, deoxyribonucleic acid; KRB, Krebs-Ringer bicarbonate solution; KRBC, Krebs-Ringer bicarbonate solution complete; PCA, perchloric acid; STI, soybean trypsin inhibitor; RER, rough-surfaced endoplasmic reticulum.

which [^{3}H]leucine was added at 60 μCi/ml, and incubation continued for 10 min at 37°C. At the end of this time chase incubation was begun by diluting the suspension to 50 ml in a 250-ml Erlenmeyer flask with KRBC containing 4.0 mM [^{1}H]leucine resulting in an isotope dilution of × 100. The chase medium contained in some cases 0.6 μCi/ml [^{14}C]leucine in order to monitor the ability of the cells to synthesize protein, during subsequent incubation.

After a chase period of 1 h at 37°C, aliquots of the cell suspension (still containing [^{14}C]leucine) were transferred to 25-ml Erlenmeyer flasks, some of which contained hormonal additions, and incubation was continued for a further 3 h at 37°C. At hourly intervals, duplicate 1.0-ml samples were removed, placed in 5-ml conical tubes, and the cells separated from medium by centrifugation at 100 g for 2.5 min. Media were removed from the pellet of cells; media and cell pellets were either immediately precipitated with cold 0.5 N perchloric acid (PCA) for radioactivity measurements or were frozen before assaying for amylase activity.

PROCEDURE B: With the above procedure, the chase was not completely effective in that at the end of a 4-h incubation the specific radioactivity of incorporated [^{3}H]leucine had increased by 10–15%. Consequently, in later experiments where a more efficient chase was required, the following improvements were introduced. Cells were pulse labeled as before with [^{3}H]leucine, but at the end of the pulse the cells were pelleted (100 g for 2.5 min) and washed with chase medium by recentrifugation before resuspension. This step removed most of the soluble unincorporated label and provided a low initial background in the medium by removing labeled proteins and amylase released from damaged cells. Separation of cells from medium for sampling was also improved by layering 1.0 ml of cell suspension over 1.0 ml of KRBC-chase containing 4% BPA before centrifugation. Use of this discontinuous gradient effectively washed the cells once before collection in a pellet.

ANALYTICAL PROCEDURES: Cell pellets and media were precipitated and washed with 0.5 N PCA before preparation for liquid scintillation counting (2). ^{3}H and ^{14}C counting rates were corrected for quenching by use of an external standard. DNA was assayed on hot PCA extracts of washed precipitates (6). Amylase activity was measured on fresh or frozen and thawed cell pellets and supernates diluted in amylase assay buffer containing 0.2% Triton X-100 (5). No loss of amylase activity was noted after freezing and thawing.

Autoradiography

For light and electron microscope autoradiography, cell suspensions were pulse labeled and subjected to chase incubation as in procedure A above except that the pulse medium was [^{1}H]leucine-free and contained 200 μCi/ml [^{3}H]leucine (~4 μM), while the chase medium did not contain [^{14}C]leucine but contained [^{1}H]leucine at 4.0 mM

(× 10,000 isotope dilution). At the end of the pulse and at selected chase intervals up to 4 h, aliquots of cells were fixed with 1% OsO_4 in 0.1 M Na cacodylate, pH 7.4, and embedded in Epon as described (1). Autoradiograms for light and electron microscopy were prepared according to published procedures (3, 7) using Ilford L-4 emulsion.

Quantitative analysis of the autoradiographic grain density on individual exocrine cells was performed at the end of the pulse as follows. A series of low-power (× 3,000) electron micrographs was taken of samples at the end of the pulse. The micrographs included all the cells on a single section so as to minimize variations in grain density due to differences in section thickness and emulsion coat. On the enlarged micrographs, the number of autoradiographic grains over each exocrine cell profile was counted and related to the area (in μm^2) of the cell profile. Surface area was calculated from the mean diameter derived from the largest and smallest axes of individual cells. Only cells whose diameters were greater than half the maximum profile diameter observed (~7.5 μm) were counted in order to ensure identity of cell type.

RESULTS

Ability of Separated Cells to Incorporate Amino Acids into Proteins

The protein synthesizing capacity of the isolated cell population was followed (a) by measuring the kinetics of incorporation of [^{14}C]leucine into proteins of the total cell population, (b) by light microscope autoradiography which should enable us to determine the types of cells involved in incorporation of labeled precursors, the fraction of cells active within each class, and the semiquantitative variations of incorporation among cells of a given type, and (c) by electron microscope autoradiography which allows for quantitation of incorporation on a cell-specific basis.

INCORPORATION OF [^{14}C]LEUCINE INTO PROTEIN: Suspensions of separated cells prepared for incubation by procedure A were exposed to [^{14}C]leucine continuously for 4 h, and in some cases for up to 6 h, and sampled at hourly intervals for label incorporated into acid precipitable protein. The data, shown in Fig. 1, indicate that the cell suspension incorporated [^{14}C]leucine into protein at a reasonably linear rate for up to 4 h; after 5 and 6 h of incubation, the rate declined somewhat (data not shown). Incorporation ranged from 1,700 to 2,000 nmol leucine/mg DNA over 4 h for several different batches of cells obtained with four different batches of pure collagenase.

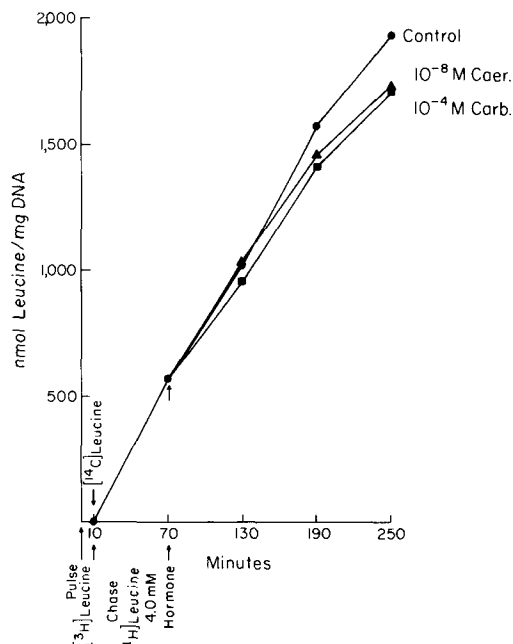During incubation, ~3% of the acid-soluble leucine in the medium was incorporated into

FIGURE 1 Incorporation of [^{14}C]leucine into protein by suspension of isolated cells. Cells were prepared for simultaneous assay of amino acid incorporation and secretogogue responsiveness according to procedure A in Materials and Methods. In this experiment, representative of four identical experiments, the incubation medium consisted of KRBC containing 0.6 $\mu Ci/ml$ [^{14}C]leucine and 4.0 mM unlabeled leucine (final specific activity = 0.15 mCi/mmol). Incubation was at 37°C with continous shaking at 60 cycle/min. At hourly intervals, 1.0-ml samples were removed, cells and medium separated by centrifugation, and incorporation of [^{14}C]leucine into acid precipitable proteins of cells plus medium measured. The data are expressed as nanomoles leucine incorporated per milligram cell DNA. Pulse labeling with [^{3}H]leucine and addition of hormones to two of the flasks at 70 min pertains to the discharge assay on the same set of cells, the results of which are given in Fig. 10.

protein. The DNA content per milliliter of cell suspension decreased by ~10% over 5 h indicating slow cytolysis. Synthesis rates were slightly but consistently diminished when secretogogues were present from the 1st h onwards.

LIGHT MICROSCOPE AUTORADIOGRAPHY: After 10-min pulse labeling with [^{3}H]leucine, light microscope autoradiography indicated that the majority of the silver grains overlay exocrine cells and that, within the exocrine cell population, ~95% were labeled (Fig. 2). Grossly damaged exocrine cells did not incor-

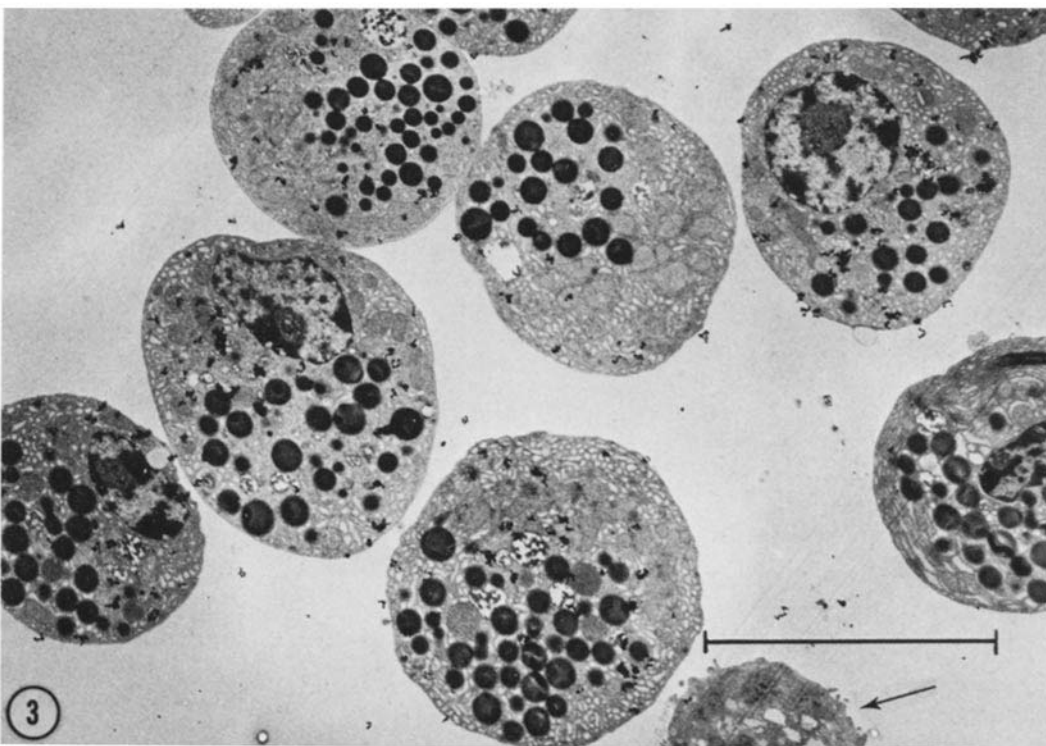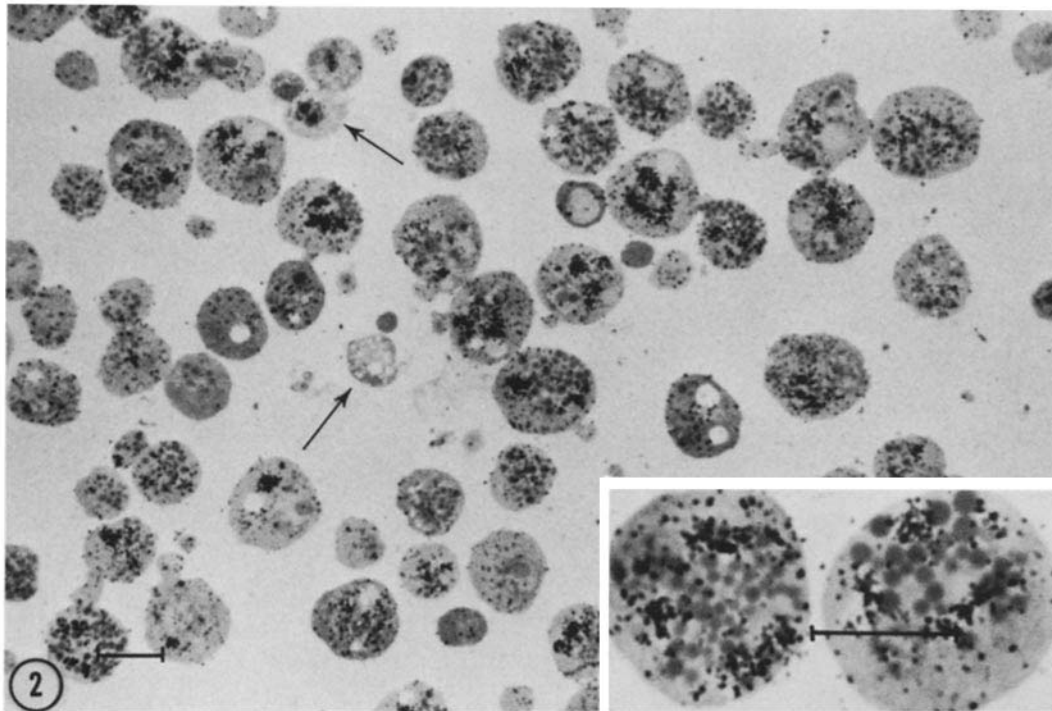FIGURE 2 Light microscope autoradiogram of 0.5-μm section through a pellet of cells pulse labeled for 10 min with [³H]leucine as described under Materials and Methods. The cytoplasm of the majority of the exocrine cells is uniformly covered with silver grains. Unlabeled cells and cell fragments are indicated by arrows. Scale marker = 10 μm. × 860; *inset*, × 2,000.

FIGURE 3 Low-magnification electron microscope autoradiogram typical of the series used for grain density determinations shown in Fig. 4. Arrow indicates an unlabeled vascular or duct endothelial cell. Same experiment as in Fig. 2. Scale marker = 10 μm. × 3,900.

porate label. While many grains were located over the cytoplasmic zone corresponding to the rough endoplasmic reticulum (RER), some local concentrations of grains were seen which corresponded to the Golgi zone, a distribution expected in view of the length of the pulse. Other cell types in the population (endocrine cells, duct cells, vascular endothelia), incorporated little label.

QUANTITATIVE ELECTRON MICROSCOPE AUTORADIOGRAPHY: Incorporation of label on a cell-specific basis was performed as outlined under Materials and Methods. Electron microscope autoradiograms were prepared from the same cell pellet used for the light microscope study above, i.e., cells immediately after a 10-min pulse with [³H]leucine which resulted in an incorporation level of ~2 × 10⁸ dpm/mg DNA or 1-2 dpm/cell. The grain density over profiles of 110 exocrine cells is shown in the histogram in Fig. 4. Part of a typical field used for grain counting is shown in Fig. 3.

The results indicate that ~70% of the cells fell within a fairly narrow grain density distribution ranging from 0.3 to 0.6 grains/μm² of surface profile and that the overall density distribution was symmetric about the mean. From the data we assume that the entire population of exocrine cells in the gland was reasonably synchronized and uniform in its ability to synthesize proteins.

Route and Kinetics of Intracellular Transport of Secretory Proteins in Separated Exocrine Cells

In order to determine the route and timetable of intracellular transport of exportable proteins in isolated cells, kinetic autoradiographic experiments were performed on cell suspensions which had been pulse labeled with [³H]leucine for 10 min and incubated for up to 4 h in chase medium. A pulse period of 10 min was chosen in order to ensure that sufficient label was incorporated so that autoradiograms could be processed within a reasonable time, i.e. after ~2 wk exposure. This pulse period was the same as that used for labeling experiments in which the results were analyzed biochemically. The pulse period was longer than that previously used for pancreatic slices in vitro (3), although as will be seen, the grain distribution after a 10 min pulse was similar to that found in slices after a 3-min pulse followed by a 7-min chase
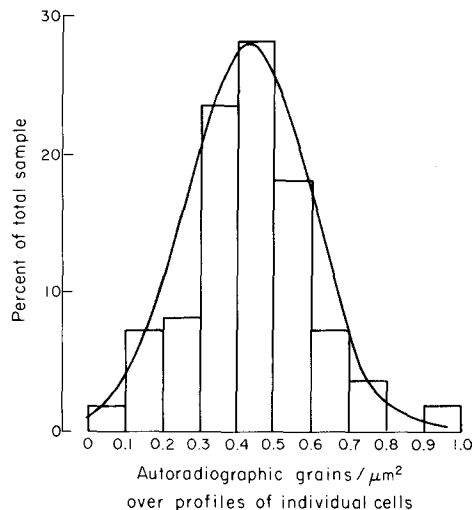

FIGURE 4 Histogram showing autoradiographic grain density distribution over profiles of 110 exocrine cells included in a single thin section. Superimposed is a normal curve derived from the mean (0.43 grains/μm²) of the individual values and its standard deviation (± 0.17).

period. Samples were processed for autoradiography after 30 min, 1 h, and 2 h of chase which included intervals previously used in studies on pancreatic slices (3). Samples were also taken after 4 h of chase which corresponded to the maximum incubation period used for both incorporation and discharge assays in this study.

Grains were scored in relation to regions containing: the cisternae of the rough endoplasmic reticulum and small vesicles and cisternae of the Golgi periphery, two arbitrary classes of condensing vacuoles termed loose and tight based on morphologic criteria (1), and typical zymogen granules. Grains overlying mitochondria and nuclei were also recorded, as were those associated with structures resembling autophagic vacuoles by virtue of their heterogeneous content of membrane pieces and residues of cell organelles. The quantitative grain distribution is presented in Table I. For selected times, the grains were related to the relative cytoplasmic volume of the RER cisternae and zymogen granules using morphometric data presented in the preceding paper. These calculations give an approximate index of protein concentration occurring during transport. Representative electron micrographs of the time points are illustrated in Figs. 5-8.

10-MIN PULSE: At the end of the pulse,

TABLE I
Distribution of Autoradiographic Grains over Cell Components

| | % Autoradiographic grains | | | | |
| | | Chase incubation | | | |
Subcellular component	Pulse 10 min	+ 30 min	+ 60 min	+ 120 min	+ 240 min
Rough endoplasmic reticulum	46.1	14.5	16.7	18.7	16.8
Golgi peripheral region	40.1	19.2	12.0	9.4	11.5
Loosely packed condensing vacuoles	6.3	49.0	29.9	5.4	5.1
Tightly packed condensing vacuoles	0.8	5.6	25.7	28.1	24.3
(Total condensing vacuoles)	(7.1)	(54.6)	(55.6)	(33.5)	(29.4)
Zymogen granules	2.7	8.7	12.3	32.7	38.6
Lysosomes	0.1	0.2	0.2	0.7	0.9
Mitochondria	1.9	1.2	1.9	2.4	1.7
Nucleus	2.0	1.7	1.2	2.0	1.2
No. of grains counted	1711	2022	2802	2492	2241
No. of cells counted	20	27	39	42	34

Autoradiographic background was low ($<$ 0.1%) or undetectable and has not been subtracted from grain counts. Differences in grain distribution over mitochondria, nuclei, and lysosomes are likely not significant but are included to show, for lysosomes, a suggestive upward trend with time.

~46% of the autoradiographic grains were associated with the RER, while a large proportion (~40%) was already associated with the Golgi complex, especially its clusters of peripherally located small, smooth-surfaced vesicles and flattened cisternae (Fig. 5). Accumulation of label over Golgi elements at this time was expected in view of the length of the pulse. Some label (~6%) had already reached loose condensing vacuoles; the remainder was equally distributed over zymogen granules, mitochondria, and nuclei.

If one assumes that the grains over the RER and Golgi periphery at the end of the 10-min pulse initially originated in the RER and were randomly distributed over its cisternal spaces, and then relates these (as percent of total grains) to the fractional cytoplasmic volume occupied by the RER cisternal space, the relative concentration of labeled proteins in this compartment is estimated to be 86%/17.7% = 4.9.

The comparable figure for exocrine cells in pancreatic slices is 86.3%/19.4% = 4.4 based on previous autoradiographic (3) and morphometric data on intact tissue (1).

30-MIN CHASE: During this interval labeled proteins drained from both the RER and Golgi periphery primarily to loosen condensing vacuoles which now possessed ~49% of the total autoradiographic grains (Fig. 6). Discrimination between loose and tight condensing vacuoles is somewhat subjective, although generally a satisfactory distinction can be made.

At this time a significant proportion of tight condensing vacuoles (~6%) and of zymogen granules (~9%) were also labeled. The proportion of grains over other structures remained essentially unchanged.

60-MIN CHASE: By now a large proportion (~26%) of the label had accumulated over tight condensing vacuoles (Fig. 7) which appear to have been derived from loose condensing vacuoles, since the latter contained only ~30% of the label. Condensing vacuoles of both types contained a maximum amount of label at 60 min (sum = 55.6%). At this time ~12% of the label was found over zymogen granules.

Between 30- and 60-min chase, label continued to drain from the Golgi peripheral region, although drainage from the RER had, by 60 min, apparently reached a maximum with ~17% of the label remaining associated with this cell region.

120-MIN CHASE: By 120 min little further drainage of label from the Golgi periphery had occurred, although the majority of the labeled, loose condensing vacuoles had apparently been converted into tight condensing vacuoles and/or
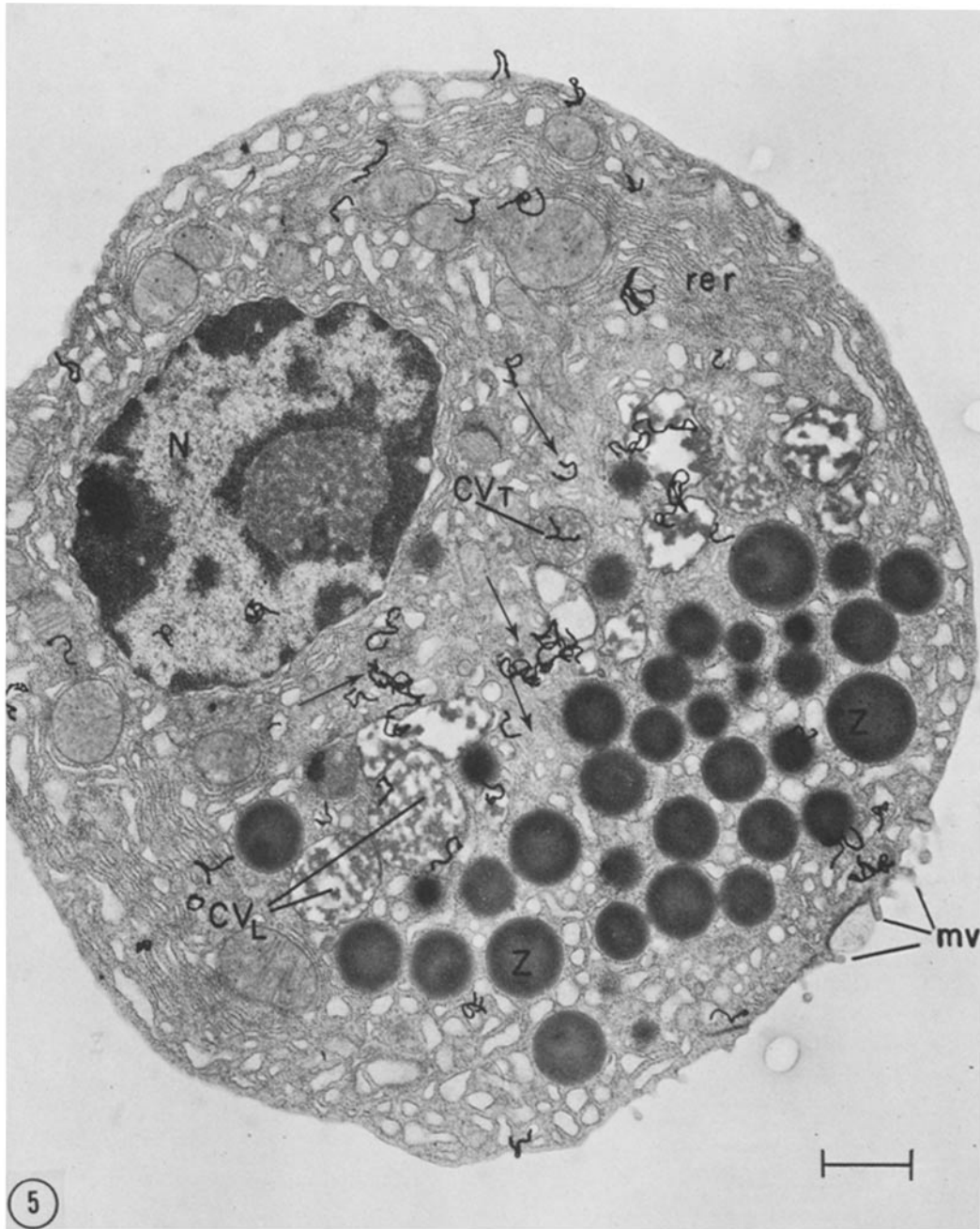
FIGURE 5 Electron microscope autoradiogram of separated exocrine cell at end of 10-min pulse labeling with [³H]leucine. Label is associated with the field of rough endoplasmic reticulum (*rer*), a region of the cell containing elements of the Golgi periphery (arrows), and with some loose condensing vacuoles (*CV*$_L$). *CV*$_T$, tight condensing vacuole; *Z*, zymogen granule; *N*, nucleus; *mv*, microvilli marking the former cell apex. Scale marker = 1 μm. × 12,000.
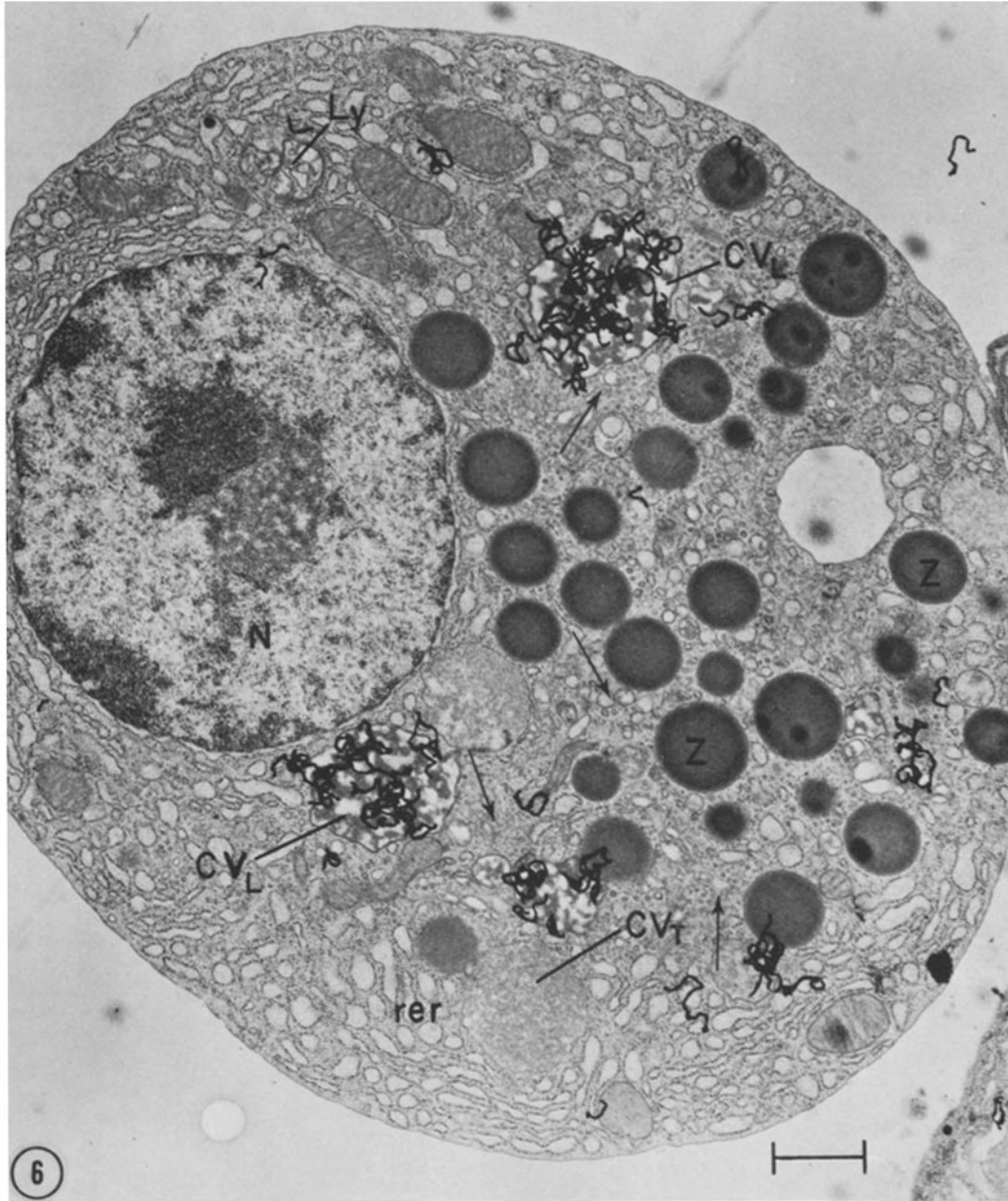
FIGURE 6 Autoradiogram of separated exocrine cell after 30-min chase incubation. Label is predominately located over loose condensing vacuoles (CV_L). Arrows indicate periphery of the complex. CV_T, tight condensing vacuole; Z, zymogen granule; N, nucleus; rer, rough endoplasmic reticulum; Ly, autophagic or lysosome-like body. Scale marker = 1 μm. × 12,000.

zymogen granules which now possessed ~28 and ~33% of the grains respectively over their profiles.

A small but suggestive increase in the proportion of label over lysosome-like structures was seen.

240-MIN CHASE: After 240-min chase (Fig. 8) no further movement of labeled proteins from loose condensing vacuoles was detectable, although tight condensing vacuoles had lost a further 4% of their label; presumably these were converted

to zymogen granules which were now maximally labeled (~39% of the grain distribution). Some labeled zymogen granules appear to have approached the former apical pole of the cell, as is shown Fig. 8.

Relative concentration of labeled protein in zymogen granules can be calculated from grain distribution and morphometric data in order to provide an estimate of the maximum extent of concentration of proteins over the entire secretory
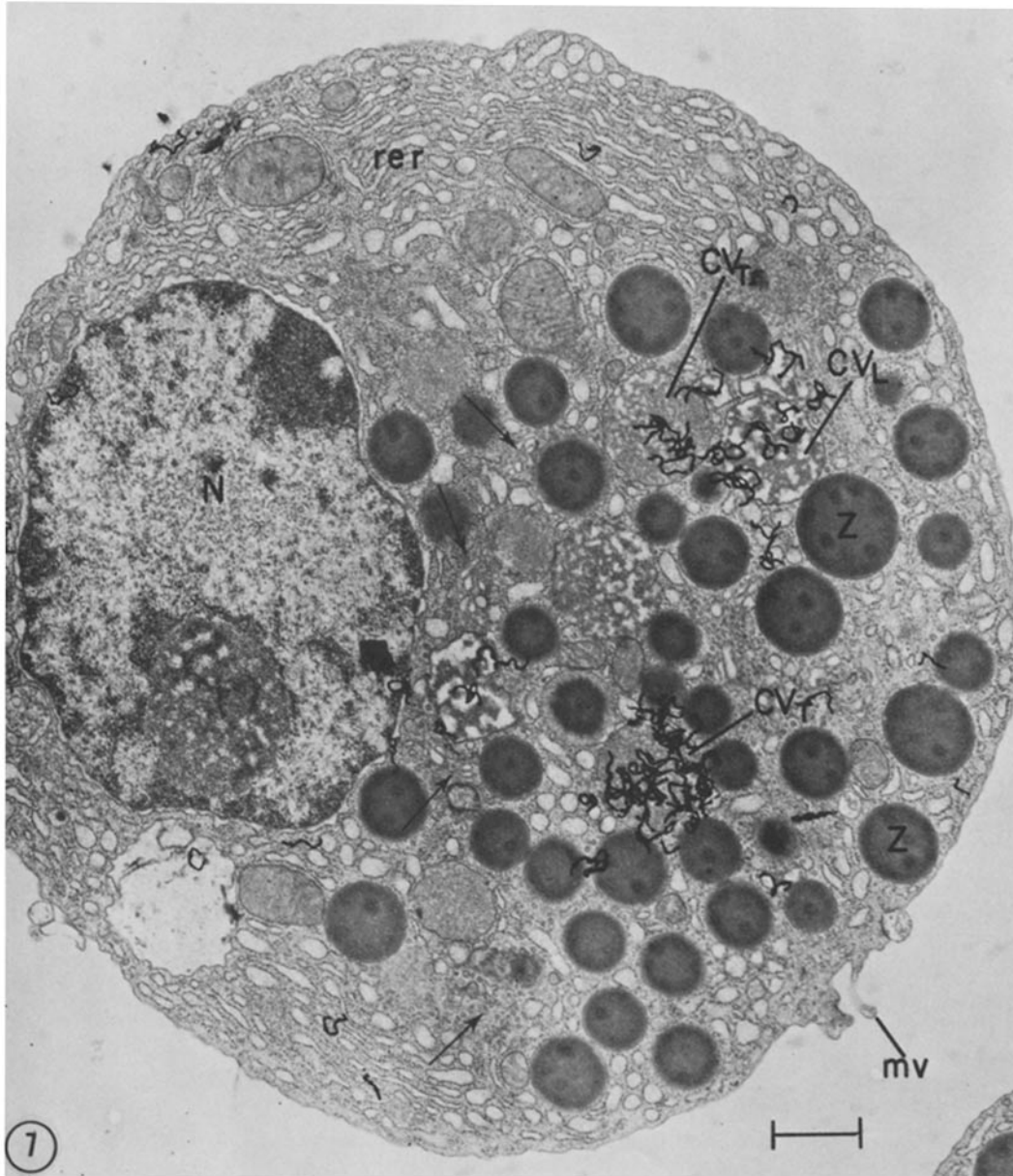

FIGURE 7 Autoradiogram of separated exocrine cell after 60-min chase incubation. Label now appears over tight condensing vacuoles (CV_T) with some remaining in the loose variety (CV_L). Arrows mark periphery of the Golgi complex. N, nucleus; Z, zymogen granules; rer, rough endoplasmic reticulum; mv, microvilli. Scale marker = 1 μm. $\times$ 12,000.
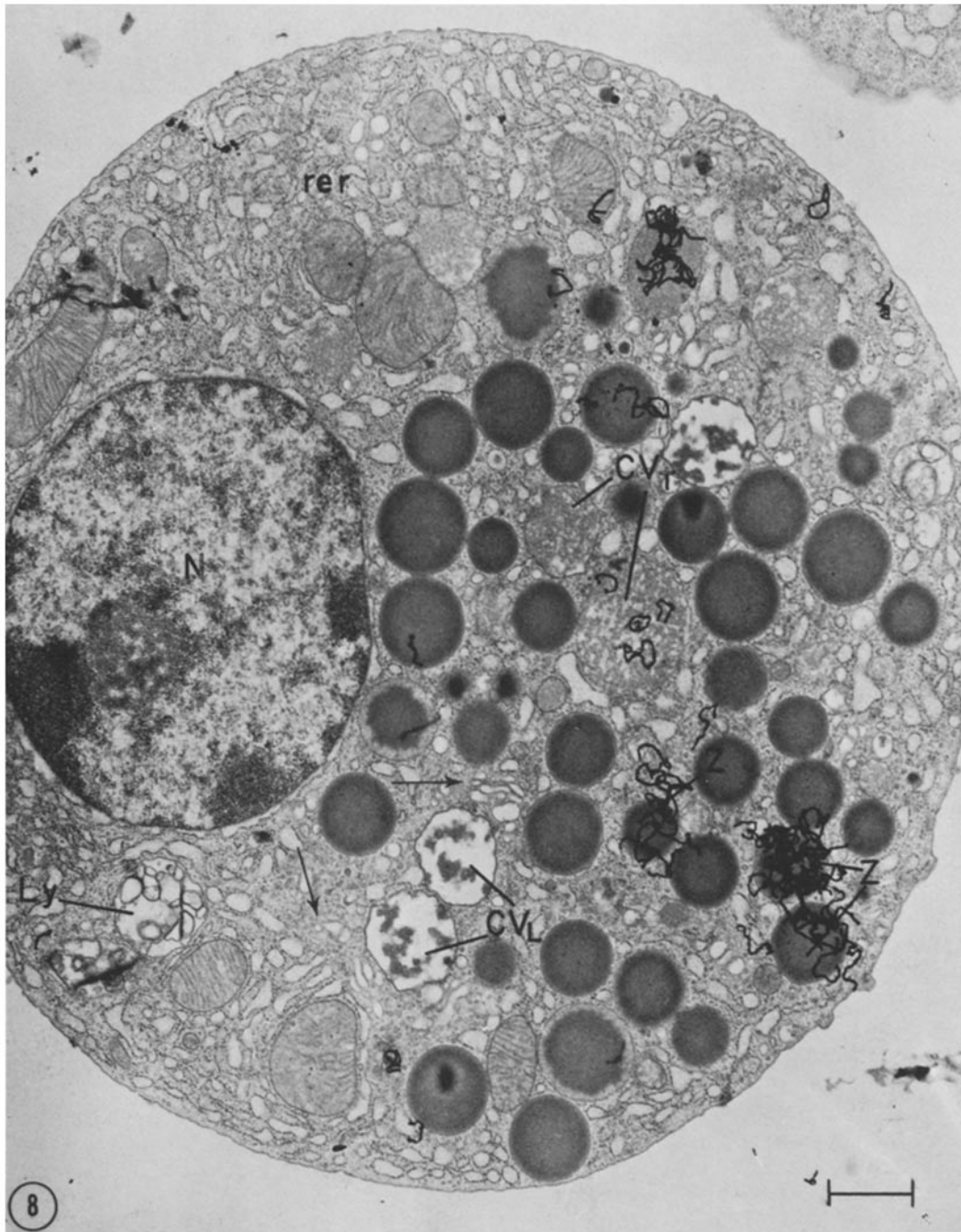
FIGURE 8   Autoradiogram of separated exocrine cell after 4-h chase incubation. Label is now concentrated over zymogen granules (Z) located near to the former apical pole. Some label is seen over tight condensing vacuoles (CV_T), but little is found over loose condensing vacuoles (CV_L). Arrows indicate periphery of the Golgi complex. N, nucleus; rer, rough endoplasmic reticulum; Ly, lysosome-like bodies. Scale marker = 1 μm. × 12,000.

pathway. In this case, however, the compartment cannot be assumed to be randomly labeled as it was for the RER at the end of the pulse, since it is clear from the micrographs that only a certain fraction of the zymogen granules were labeled. Based on counts of the proportion of labeled zymogen granules in the total granule population, we estimated that ~10.4% of granules contained labeled proteins after 240 min of chase. This figure can be used to correct the volume fraction of the total zymogen granule population (Table II in reference 1) downward to correspond to that actually involved in accumulation of labeled proteins. The corrected volume fraction is therefore $0.10 \times 20.2\%$ and the relative concentration of labeled proteins becomes $38.6\%/2.0\% = 19.3$. The comparable figure for relative concentration in zymogen granules in pancreatic slices is ~25 times based on previous autoradiographic (3) and morphometric data (1).

Discharge of Exportable Proteins from Isolated Exocrine Cells

The ability of isolated exocrine cells to release exportable proteins to the incubation medium in response to secretogogues was monitored by two methods carried out in parallel on the same cell preparation. (a) The release of amylase, a typical secretory protein, provided a measure of secretogogue responsiveness which was independent of the preceding events in the secretory process in that it measures discharge of preformed exportable proteins temporarily stored within zymogen granules. (b) The appearance in the medium of proteins prelabeled during a 10-min pulse with [³H]-leucine, and allowed to undergo intracellular transport to storage granules during the chase period, served to monitor the release of newly formed secretory proteins. This assay is more dependent on cellular events preceding packaging into storage granules, although it is more sensitive and simpler to perform than measurement of amylase release.

Cells were prepared for chase incubation by either procedure A or B as described under Materials and Methods and indicated in the text. Procedure A was used for the majority of experiments, while procedure B was used where more precise timing of discharge kinetics was required.

BIOCHEMICAL CHARACTERISTICS OF CELL SUSPENSIONS DURING CHASE INCUBA-

TION: After resuspension of the cells in fresh chase medium (procedure B) the acid-soluble radioactivity in the cells represented about 20% of the radioactivity incorporated into proteins during the pulse. During chase incubation, this acid soluble fraction was released exponentially into the medium with a half time of ~8 min. Washout kinetics were not affected by secretogogues. Over 4 h of chase, the total acid insoluble label in control and stimulated cell suspensions (sum of acid insoluble radioactivity in cells plus medium) decreased by 5 and 7%, respectively, to be recovered in the acid soluble fraction of the system. This may be the result of autophagy (? crinophagy) and lysosomal degradation of organelles containing labeled protein, as suggested by the gradual increase in autoradiographic grains over lyso-some-like bodies (Table I) during 4 h of chase incubation. Protease activity was not measurable in the medium over 4-h incubation, indicating effective inhibition of trypsin activation by STI. DNA recovered in cell pellets decreased by ~10% over the same period.

DISCHARGE KINETICS WITH SECRETO-GOGUES ADDED IMMEDIATELY POST-PULSE: In previous studies (5), we have shown that if stimulants are added to pancreatic slices immediately postpulse and are continuously present during the chase, a lag of 20–30 min is seen before labeled proteins begin to appear in the medium. The duration of this lag provides an estimate of the time required for nascent-la-beled proteins to be transported from the RER to a compartment sensitive to secretogogues. Similar experiments were performed on isolated cells with the results given in Fig. 9. Addition of caerulein at optimal doses (10^{-8} M) to cell suspensions immediately postpulse led, after a lag of ~20 min, to the prompt release of labeled proteins to the medium which proceeded at a linear rate for ~2 h. During the remaining 2 h, the discharge rate diminished somewhat. Net discharge above controls after 4 h amounted to ~33% of the pulse-labeled proteins. Over the same period, control-unstimulated cells released ~6% of their labeled proteins. Incubation of cell suspensions with or without added caerulein at 4°C over 4 h suppressed release by ~90%. Similar results (not shown) were obtained at 37°C with N_2 substituted for O_2 in the gas phase. The residual noninhibitable release most likely resulted
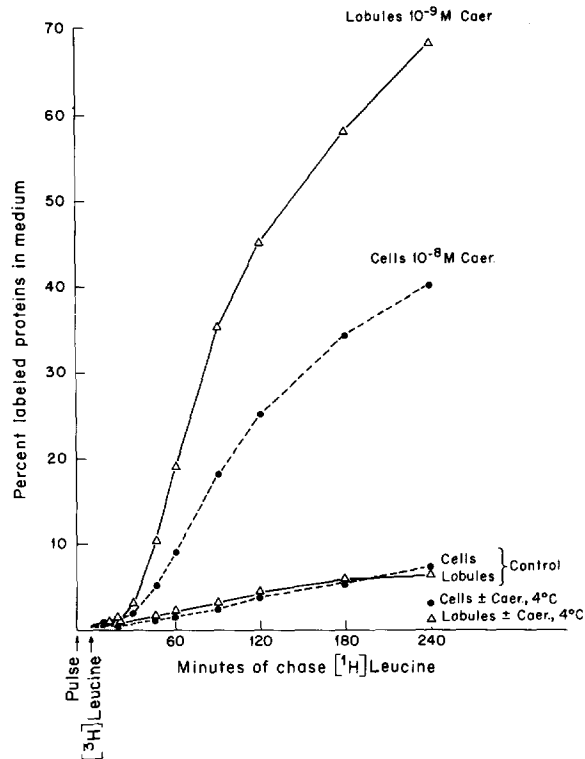
FIGURE 9 Discharge kinetics for labeled proteins from cells prepared according to procedure B. After pulse labeling with [³H]leucine at 60 µCi/ml in KRBC, cells were centrifuged, washed once with chase medium, and resuspended in 50-ml chase containing 4.0 mM [¹H]leucine. Duplicate 20-ml aliquots of the suspension were removed to 50-ml Erlenmeyer flasks and 10^{-8} M caerulein was added to one of them. These flasks were incubated at 37°C. The remainder was apportioned into 25-ml flasks, one of which contained 10^{-8} M caerulein, for incubation at 4°C. All flasks were agitated at 60 cycle/min. At the indicated times, 1-ml samples were removed, and cells and medium separated by centrifugation as described under Materials and Methods. Discharge is expressed as percent of acid-insoluble radioactivity released to the medium (labeled proteins in medium per labeled proteins in medium + cells) × 100. For comparison, discharge kinetics of pancreatic lobules preincubated and pulse labeled under conditions identical to those used for dissociation but with the omission of enzyme and EDTA treatment are shown. Representative of three identical experiments. If preincubation was in media containing normal (2.5 mM) Ca^{++}, the net induced secretion was increased a further 5-10% over that from lobules preincubated in 0.1 mM Ca^{++} containing media shown here. Preincubation in media containing 2.5 mM Ca^{++} corresponds to conditions used in the past for pancreatic slices (2, 5). The concentration of caerulein producing optimal discharge from lobules is 10-fold less than that required for separated cells. Increasing the dose of caerulein applied to lobules to 10^{-8} M suppressed discharge slightly (~ 5%).

from leakage from damaged cells. The difference between noninhibitable release and release from controls incubated at 37°C probably represents resting or spontaneous discharge as noted for pancreatic slices (5).

To determine the effect of the dissociation and incubation media per se on exocrine cell function, pancreatic lobules were preincubated and submitted to pulse labeling and chase incubation under conditions identical to those used for obtaining

isolated cells, except that enzymes and EDTA treatment were omitted. The results, also shown in Fig. 9, indicated that the isolated cells lost ~47% of their secretory responsiveness compared to those of intact tissue. Spontaneous release of labeled proteins from lobules was similar to that from control cell suspensions; the amount of noninhibitable leakage from lobules incubated at 4°C was less than that from isolated cells.

DISCHARGE KINETICS AFTER A 60-

MIN CHASE PERIOD: Release of labeled protein was also examined after a 60-min chase period which allowed pulse-labeled proteins to be transported to storage granules. This type of assay was used to assess the secretogogue responsiveness of most cell preparations during development of the dissociation procedure and for all dose response studies. In these experiments, release of amylase was also monitored.

As shown in Fig. 10, addition of secretogogues after 60-min chase resulted in the prompt release of labeled proteins which began without an apparent lag and which was maintained at a linear rate for the next 3 h leading to a net release above controls of 10–13% of the labeled proteins.

Dose responses of isolated cells to carbamylcholine and caerulein are shown in Fig. 11 and 12. A labeling protocol similar to that used for the studies shown in Fig. 10 was employed; discharge of labeled proteins and amylase after 3 h of stimulation is plotted. Both secretogogues produced sigmoidal log-dose response curves with initial plateau doses being 10^{-4} M and 10^{-9} M for carbamylcholine and caerulein, respectively. Release of amylase and labeled proteins occured in parallel.

The presence of carbamylcholine and caerulein together at optimal doses did not yield secretory responses greater than those seen with the stimulants alone at the same dose (data not shown).

In the experiments reported in this section the rate of release of labeled proteins was approximately half that noted for assays in which hormones were added immediately postpulse (e.g. Fig. 9). The reason for this discrepancy is unclear and is discussed later.

DISCUSSION

According to our functional data, exocrine cells dissociated from the pancreas are able to carry out the main functions of their counterparts in situ, although several differences, discussed later, are apparent. In addition to retaining polarized distribution of organelles involved in the secretory process, the isolated cells also retain functional polarity in that secretory proteins are vectorially transported from their site of synthesis in the former basal pole of the cell to their site of temporary storage in zymogen granules located in the former apical pole. Polarity of function, therefore, is not a necessary consequence of the orienta-

tion of cells with respect to each other or to organization into larger functional units, the acini.

According to our autoradiographic data, all cells in the isolated population appear to be approximately equally active in their ability to incorporate amino acids into proteins. From this, we can assume that within the pancreas in situ regional differences with regard to protein synthesis rates probably do not exist. This is in contrast to the situation in the anterior pituitary where, at any one time, only a certain portion of the population of a given cell type is active in protein synthesis (8). Whether quantitative regional differences exist in the gland with regard to production of different
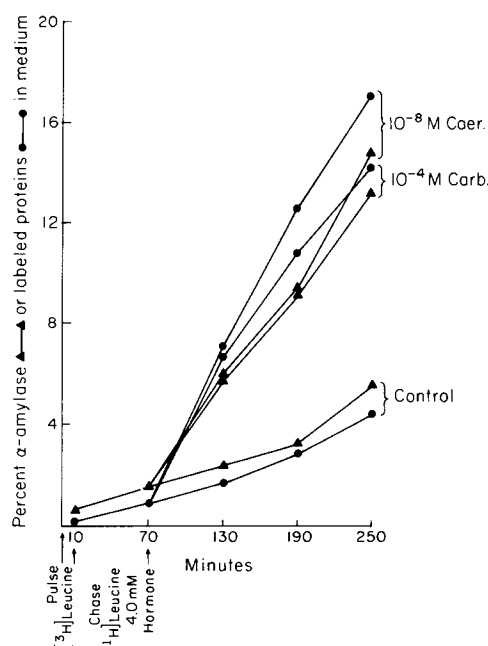
FIGURE 10 Discharge response of cells prepared according to procedure A. Same cell preparation as shown in Fig. 1. After pulse labeling with [³H]leucine for 10 min and chase incubation for 60 min in 4.0 mM [¹H]leucine ([¹⁴C]leucine was also present as indicated in Fig. 1), the cell suspension was apportioned in 3–15-ml samples to 50-ml Erlenmeyer flasks, two of which contained either 10^{-8} M caerulein or 10^{-4} M carbamylcholine. After hourly incubations at 37°C, duplicate 1-ml samples were removed and cells separated from medium by centrifugation. Release of labeled proteins was measured and expressed as in Fig. 9 ([¹⁴C]leucine incorporation was simultaneously monitored by double-label counting of the samples—see Fig. 1); percent release of amylase was determined on the second sample. Representative of four identical experiments.
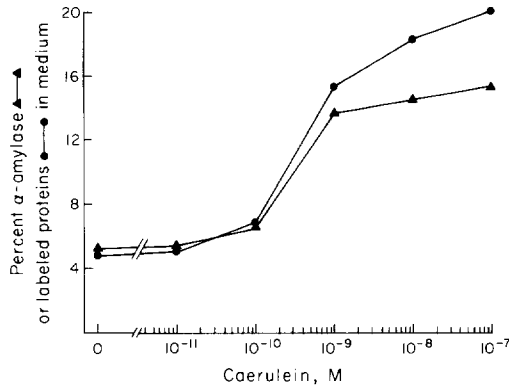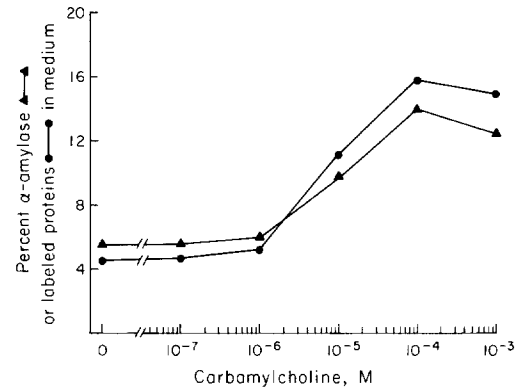
FIGURE 11   FIGURE 12

FIGURES 11 and 12   Dose response curves for release of amylase and labeled proteins from separated cells with caerulein (Fig. 11) and carbamylcholine (Fig. 12). Cells prepared and assayed for discharge according to procedure A and Fig. 10. Release after 3-h stimulation is shown. Representative of three identical experiments.

types of exportable proteins is unknown, although immunocytochemical studies underway on zymogen granule fractions from the bovine pancreas indicate that all granules are alike with regard to their complement of several typical secretory proteins, suggesting that regional differentiation of product packaging both within a given cell and within the gland are unlikely (see also [9]). Along these lines, Tartakoff et al.[2] and Scheele and Palade (10) have shown that the rates of intracellular transport and of release of several defined secretory proteins in the guinea pig pancreas are similar if not identical, although different results have been reported for other species (11).

Using biochemical criteria, the isolated exocrine cell population appears to incorporate amino acids into proteins at a rate somewhat greater than that previously reported for pancreatic slices (5). However, comparison is difficult in that penetration of amino acids into slices is limited by diffusion barriers imposed by the slice thickness as shown autoradiographically (3).

The results of autoradiographic analysis of the intracellular transport of pulse-labeled proteins indicate that the same general pathway is followed in the isolated cells as that previously mapped in exocrine cells *in situ* both in pancreatic slices (3)

[2] Tartakoff, A. M., J. D. Jamieson, G. A. Scheele, and G. E. Palade. Studies on the guinea pig pancreas. Parallel processing and discharge of exportable proteins. Manuscript submitted for publication.

and in the intact gland (12), i.e., sequential and vectorial transport from the RER → Golgi peripheral region → condensing vacuoles → zymogen granules at the cell apex. We should mention, however, that in the isolated cells the stacked cisternal elements of the Golgi complex appear to be involved in the processing of labeled proteins, in contrast to earlier findings in pancreatic slices (3) where labeling of these elements was not apparent. The reason for this difference is not clear, but may be related to the observation that the cisternal elements are somewhat distended in the isolated cells, thus facilitating our ability to recognize them as being associated with labeled proteins.

The timetable of intracellular transport is, however, altered in some respects in the isolated cells when compared to the situation in pancreatic slices. During a 10-min pulse labeling, the rate of transit of the front of labeled proteins from their site of synthesis in the RER to the Golgi peripheral region appears to be the same as that observed in pancreatic slices (3). Similarly, during the first 30-min chase period, labeling of the total condensing vacuole population (loose and tight condensing vacuoles) proceeds at a rate equivalent to that seen in pancreatic slices. However, the next stage in transport—conversion of condensing vacuoles to zymogen granules—is markedly slowed in that by 2-h chase only half as much label appears over zymogen granules as expected from results with pancreatic slices (3). The net result is that transport to the final terminus lags by 1 h. This appears

to be the result of a defect in the final stages of the packaging process which in the isolated cells involves sequential conversion of loose condensing vacuoles to tight condensing vacuoles and finally the transformation of the latter to zymogen granules. We should emphasize that, while the classification of condensing vacuoles as being loose or tight is arbitrary, it has helped us to pinpoint the apparent defect to the level of the conversion of tight condensing vacuoles to zymogen granules. Notwithstanding this, the overall efficiency of drainage of labeled proteins from the RER is somewhat better in the separated exocrine cells compared to that in pancreatic slices (3).

As discussed previously, condensation of secretory proteins may be the consequence of several processes including interaction of secretory proteins with each other to form osmotically less active macromolecular complexes or the interaction of proteins with divalent ions, such Ca^{++} (4). In addition, recent studies by Berg and Young (13) and Tartakoff et al. (14) have indicated that inorganic sulfate is incorporated into a presumed large acidic peptidoglycan in the exocrine pancreas and that the site of incorporation appears to be at the level of the Golgi complex, including its condensing vacuoles (13). It is tempting to speculate that this molecule acts as a matrix or binding protein for pancreatic secretory proteins, many of which have basic isoelectric points (14). The defect in concentration observed in isolated exocrine cells may be due to the inefficient production of this putative matrix glycopeptide due either to substrate deficiencies (sugar, $SO_4=$) in the medium or to defects in their uptake and incorporation. Alternatively, as noted in the previous paper (1), loose condensing vacuoles become distinguishable after exposure of the tissue to the low $[Ca^{++}]$ necessary for efficient dissociation. Perhaps the concentration defect is related to a relative Ca^{++} deficiency which is not readily reversed by incubating cells in media containing normal (2.5 mM) Ca^{++} levels. The deleterious effect of low Ca^{++} levels on the metabolism of isolated hepatic parenchymal cells has been noted recently (15). That Ca^{++} appears to be associated with secretory granule content is indicated by the study of Wallach and Schramm (16) who showed in the rat parotid that Ca^{++} is released to the medium along with amylase.

Despite the condensation defects, it has been possible to arrive at an estimate of the degree of concentration of secretory proteins as they moved from the RER to their final station, the zymogen granules. The figures obtained indicate a relative increase in concentration of ~4 times on going from the RER cisternae (relative concentration intially 4.9 times) to zymogen granules (~20 times final). For comparison the relative net concentration of labeled proteins in zymogen granules of exocrine cells in slices is ~5.5 times computed from previous autoradiographic (3) and morphometric data (1). Apparently, then, the net concentrative capacity in the isolated cells is not drastically affected. While it would also have been of interest to estimate relative concentration of proteins in the intermediate stations, i.e. elements of the Golgi complex including condensing vacuoles, this was not possible in the present study due to the small size and complex morphology of these compartments which necessitates more refined morphometric analysis.

According to our discharge data, the isolated exocrine cells release their store of secretory proteins in response to secretogogues at a rate two to three times less than that of pancreatic slices (5) or lobules (10). Despite this lowered release efficiency, discharge from isolated cells appears to take place by exocytosis of zymogen granule content and the site of exocytosis is restricted to the former apical plasmalemma as indicated by preliminary light and electron microscope observations.

That this lowered discharge efficiency is not simply a reflection of a slowdown in intracellular transport is indicated by the observation that amylase release is similarly affected. Since amylase is released primarily from the cell's store of preformed, exportable proteins in zymogen granules, it should not be adversely affected by the preceding events of intracellular transport, as assays for release of pulse-labeled proteins might be.

The reasons for decreased secretory responsiveness remain to be determined. A likely possibilty is that hormone receptor sites have been altered by the protease treatment used during dissociation since, based on studies with other cell types, hormone receptors appear to be proteins located on the external surface of the limiting plasmalemma (17) and are susceptible to proteolysis (18, 19). Receptor site alteration may consist of a decrease in the number of such sites, a decrease in their binding capacity for hormones, or both.

Decreased binding to available receptors is supported by our data which shows that the effective hormone concentrations required for optimal release are ~10 times greater than those required for pancreatic slices (4, 5) or lobules (10) incubated under similar assay conditions. Finally, since the simultaneous application of optimal concentrations of both caerulein and carbamylcholine to the same cell preparation does not enhance discharge, it appears unlikely that the two receptor types are located on uniquely different exocrine cells in the population. Our data do not rule out, however, the possibility that *in situ* both receptors are located only on some individual cells of an acinus and that excitation is amplified by electronic spread through low-resistance pathways known to couple cells in many epithelia (20, 21).

Discharge efficiency is sensitive to the experimental conditions employed in the assays. In the case of cells which received secretogogues immediately postpulse (e.g. Fig. 9), the total volume of the cell suspensions used and the dilution of cells in the medium were considerably larger than for cells receiving secretogogues after 1 h of chase (e.g. Fig. 10). For the latter experiments, in addition, several variables (up to 12) were tested on each preparation of cells which necessitated smaller individual incubation volumes. Whether depletion of metabolites, accumulation of toxic byproducts, alterations of membrane phospholipids by elevated levels of lipase in the medium (22), or other factors are responsible for the different responses in the two types of assays remains to be determined.

In summary the preparation of isolated pancreatic exocrine cells described here should prove useful and satisfactory for a number of studies not requiring prolonged incubation times. As it stands, however, the system exhibits a number of defects including those related to inefficient concentration of proteins in condensing vacuoles, suboptimal response to secretogogues, and a slow decay with time as evidenced by loss of DNA from the system. For the future it will be imperative to attempt to repair these defects in order to restore the cells of their full functional potential. Certain approaches are already evident, e.g. elevating [Ca^{++}] and providing additional supportive substrates (sugars, SO_4 =) to corect possible defects in the concentrative process. Other approaches should include incubation of cells in more complete tissue culture media containing proteins and other factors (23) which would support exocrine cell growth in vitro

and some means of removal or entrapment of lipase in the medium, since this enzyme has already been shown by us to alter the lipid composition of the plasmalemma (22). Prevention of further damage to and reparation of the cell surface during the more extended incubation periods will be essential for studies relating to the surface properties of isolated exocrine cells including characterization of receptors for peptide secretogogues and lectins and investigations into the dynamics of the plasmalemma during exocytosis.

REFERENCES

1. AMSTERDAM, A., and J. D. JAMIESON. 1974. Studies on dispersed pancreatic exocrine cells. I. Dissociation technique and morphologic characteristics of separated cells. *J. Cell Biol.* **63**:1037-1056.
2. JAMIESON, J. D., and G. E. PALADE. 1967. Intracellular transport of secretory proteins in the pancreatic exocrine cell. I. Role of the peripheral elements of the Golgi complex. *J. Cell Biol.* **34**:577-596.
3. JAMIESON, J. D., and G. E. PALADE. 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.
4. JAMIESON, J. D., and G. E. PALADE. 1971. Condensing vacuole conversion and zymogen granule discharge in pancreatic exocrine cells: metabolic studies. *J. Cell Biol.* **48**:503-522.
5. JAMIESON, J. D., and G. E. PALADE. 1971. Synthesis, intracellular transport, and discharge of secretory proteins in stimulated pancreatic exocrine cells. *J. Cell Biol.* **50**:135-158.
6. BURTON, K. 1956. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochem. J.* **62**:315-323.
7. CARO, L. G., and R. P. VAN TUBERGEN. 1962. High-resolution autoradiography. I. Methods. *J. Cell Biol.* **15**:173-188.
8. HOPKINS, C. R., and M. G. FARQUHAR. 1973. Hormone secretion by cells dissociated from rat anterior pituitaries. *J. Cell Biol.* **59**:276-303.
9. KRAEHENBUHL, J. P., and J. D. JAMIESON. 1972.

Solid phase conjugation of ferritin to Fab-fragments for use in antigen localization on thin sections. *Proc. Natl. Acad. Sci. U. S. A.* **69**:1771–1775.

10. SCHEELE, G. A., and G. E. PALADE. Studies on the guinea pig pancreas. Parallel discharge of exocrine enzyme activities. *J. Biol. Chem.* In press.

11. ROTHMAN, S. S. 1967. "Non-parallel transport" of enzyme protein by the pancreas. *Nature (Lond.).* **213**:460–462.

12. CARO, L. G., and G. E. PALADE. 1964. Protein synthesis, storage, and discharge in the pancreatic exocrine cell. *J. Cell Biol.* **20**:473–495.

13. BERG, N. B., and R. W. YOUNG. 1971. Sulfate metabolism in pancreatic acinar cells. *J. Cell Biol.* **50**:469–483.

14. TARTAKOFF, A. M., L. G. GREENE, and G. E. PALADE. Studies on the guinea pig pancreas. Fractionation and partial characterization of exportable proteins. *J. Biol. Chem.* In press.

15. HOWARD, R. B., J. C. LEE, and L. A. PESCH. 1973. The fine structure, potassium content, and respiratory activity of isolated rat liver parenchymal cells prepared by improved enzymatic techniques. *J. Cell Biol.* **57**:642–658.

16. WALLACH, D., and M. SCHRAMM. 1971. Calcium and the exportable protein in rat parotid gland.

Parallel subcellular distribution and concomitant secretion. *Eur. J. Biochem.* **21**:433–437.

17. CUATRECASAS, P. 1973. Insulin receptor of liver and fat cell membranes *Fed. Proc.* **32**:1838–1846.

18. CUATRECASAS, P. 1972. Properties of the insulin receptor isolated from liver and fat cell membranes. *J. Biol. Chem.* **247**:1980–1991.

19. KONO, T. 1969. Destruction and restoration of the insulin effector system of isolated fat cells. *J. Biol. Chem.* **244**:5777–5784.

20. LOWENSTEIN, W. R., S. J. SOCOLAR, S. HIGASHINO, Y. KANNO, and N. DAVIDSON. 1965. Intracellular communication: renal, urinary bladder, sensory, and salivary gland cells. *Science (Wash. D. C.).* **149**:295–298.

21. LOWENSTEIN, W. R. 1973. Membrane junctions in growth and differentiation. *Fed. Proc.* **32**:60–64.

22. MELDOLESI, J., J. D. JAMIESON, and G. E. PALADE. 1971. Composition of cellular membranes in the pancreas of the guinea pig. II. Lipids. *J. Cell Biol.* **49**:130–149.

23. LEVINE, S., R. PICTET, and W. J. RUTTER. 1973. Differentiation—control of cell proliferation and cytodifferentiation by a factor reacting with the cell surface. *Nat. New Biol.* **246**:49–52.