INTRACELLULAR TRANSPORT OF SECRETORY PROTEINS IN THE PANCREATIC EXOCRINE CELL

II. Transport to Condensing Vacuoles and Zymogen Granules

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ABSTRACT

In the previous paper we described an in vitro system of guinea pig pancreatic slices whose secretory proteins can be pulse-labeled with radioactive amino acids. From kinetic experiments performed on smooth and rough microsomes isolated by gradient centrifugation from such slices, we obtained direct evidence that secretory proteins are transported from the cisternae of the rough endoplasmic reticulum to condensing vacuoles of the Golgi complex via small vesicles located in the periphery of the complex. Since condensing vacuoles ultimately become zymogen granules, it was of interest to study this phase of the secretory cycle in pulse-labeled slices. To this intent, a zymogen granule fraction was isolated by differential centrifugation from slices at the end of a 3-min pulse with leucine-^{14}C and after varying times of incubation in chase medium. At the end of the pulse, few radioactive proteins were found in this fraction; after +17 min in chaser, its proteins were half maximally labeled; they became maximally labeled between +37 and +57 min. Parallel electron microscopic radioautography of intact cells in slices pulse labeled with leucine-^{3}H showed, however, that zymogen granules become labeled, at the earliest, +57 min post-pulse. We assumed that the discrepancy between the two sets of results was due to the presence of rapidly labeled condensing vacuoles in the zymogen granule fraction. To test this assumption, electron microscopic radioautography was performed on sections of zymogen granule pellets isolated from slices pulse labeled with leucine-^{3}H and subsequently incubated in chaser. The results showed that the early labeling of the zymogen granule fractions was, indeed, due to the presence of highly labeled condensing vacuoles among the components of these fractions.

INTRODUCTION

In the preceding paper (Jamieson and Palade, 1967), we have described the functional and morphological characteristics of an in vitro[1] system of guinea pig pancreatic slices in which the secretory proteins produced by the acinar cell can be conveniently labeled with radioactive amino acids in short, well defined pulses. Because of such labeling, the kinetics of intracellular transport of newly synthesized secretory proteins can be profitably investigated. Rough and smooth microsomal fractions were isolated from the incubated slices by isopycnic centrifugation in a continuous sucrose density gradient. The first fraction consists of healed fragments of the rough endoplasmic reticulum (ER), while the second represents primarily the peripheral elements of the Golgi complex. Kinetic experiments performed on these microsomal subfractions after 3 min of pulse labeling and after various times of incubation in a chase medium indicated that newly synthesized secretory proteins were transported from their site of synthesis in the rough ER to cumuli of smooth-surfaced vesicles at the periphery of the Golgi complex.

[1] In this paper, in vitro will refer to incubated pancreatic slices while in vivo will refer to the gland in situ, i.e., in the animal.

Since in vivo, secretory proteins are transported to, and finally stored in zymogen granules (Keller and Cohen, 1961; Greene, Hirs, and Palade, 1963), experiments were performed on pulse-labeled slices to determine if acinar cells could carry out in vitro the remaining steps of intracellular transport, viz., the transfer of secretory proteins to the condensing vacuoles of the Golgi complex and their storage in zymogen granules prior to discharge. The results of combined cell fractionation and radioautographic studies presented in this paper show that the acinar cells of pancreatic slices can carry out all the operations involved in the secretory process up to and including the discharge of the zymogen granule content into the duct system.

METHODS

Techniques for preparing and pulse-labeling guinea pig pancreatic slices, for isolating cell fractions from homogenized slices, and for assaying these fractions chemically and radiochemically are described in the previous paper (Jamieson and Palade, 1967).

Slices to be used for light and electron microscopic radioautography were pulse labeled in incubation medium containing 200 μc/ml L-leucine-4,5-^{3}H (40 μM) and incubated post-pulse in a chase medium containing L-leucine-^{1}H at a concentration of 2.0 mM (50× excess). Light and electron microscopic radioautography were performed as described by Caro and van Tubergen (1962) on tissue fixed and embedded in Epon as outlined in the preceding paper.

LIGHT MICROSCOPIC RADIOAUTOGRAPHY: 0.5-μ thick Epon sections were mounted on glass slides and coated with Ilford L-4 emulsion. The preparations were exposed for 4–7 days at 4°. After photographic processing, the sections were stained through the emulsion with 1% methylene blue in 1% sodium borate (Richardson, Jarett, and Finke, 1960), a procedure which does not remove or displace the silver grains. The preparations were coated with a clear plastic aerosol spray (Jennings, Farquhar, and Moon, 1959) and were examined and photographed under the oil immersion lens using bright-field illumination. With this technique, a satisfactory image of both tissue structure and radioautographic grains can be obtained.

ELECTRON MICROSCOPIC RADIOAUTOGRAPHY: Thin sections of cells or cell fractions were mounted on grids covered with a carbon-coated formvar film. Ilford L-4 emulsion was applied by the loop method (Caro and van Tubergen, 1962). Exposure times were from 2–4 wk. After photographic processing, the sections were doubly stained with uranyl acetate and lead citrate (Venable and Coggeshall, 1965). This staining schedule does not produce any detectable loss or displacement of silver grains.

MATERIALS: L-leucine-4,5-^{3}H (specific activity 5 c/millimole) was obtained from New England Nuclear Corporation, Boston. Ilford Nuclear Research emulsion L-4 was obtained from Ilford Limited, Ilford, Essex, England.

TABLE I

Chemical Composition of Zymogen Granule Fractions

Source	mg RNA/mg protein	μg phospholipid-P / mg protein
Guinea pig* pancreatic slice	0.01–0.03	1.3–3.3
Guinea pig‡	0.16	—
Dog§	0.005	1.1
Ox‖	0.006	2.2
Mouse¶	0.04	—

* These values are the range from three experiments.
‡ Siekevitz and Palade, 1958 *a* (isolated by discontinuous gradient centrifugation).
§ Hokin, 1955.
‖ Greene et al., 1963.
¶ Van Lancker and Holtzer, 1959.

RESULTS

Intracellular transport in the slice system was investigated in parallel by cell fractionation and radioautography.

Cell Fractionation Studies

ISOLATION AND CHARACTERIZATION OF THE ZYMOGEN GRANULE FRACTION: So that we could follow the transport of secretory proteins into zymogen granules, it was necessary to isolate these granules in a relatively clean cell fraction. The method used is described in the preceding paper (Jamieson and Palade, 1967) and is based, with minor modifications, on that proposed by Hokin (1955) for the isolation of a zymogen granule fraction from the dog pancreas.

CHEMICAL COMPOSITION OF THE ZYMOGEN GRANULE FRACTION: Table I gives the RNA/protein ratio of the zymogen granule fraction isolated by us from guinea pig pancreatic slices and compares it with that of zymogen granule fractions isolated from guinea pig pancreas by discontinuous gradient centrifugation, and from dog, ox, and mouse pancreas by differential centrifugation. RNA was determined in all cases

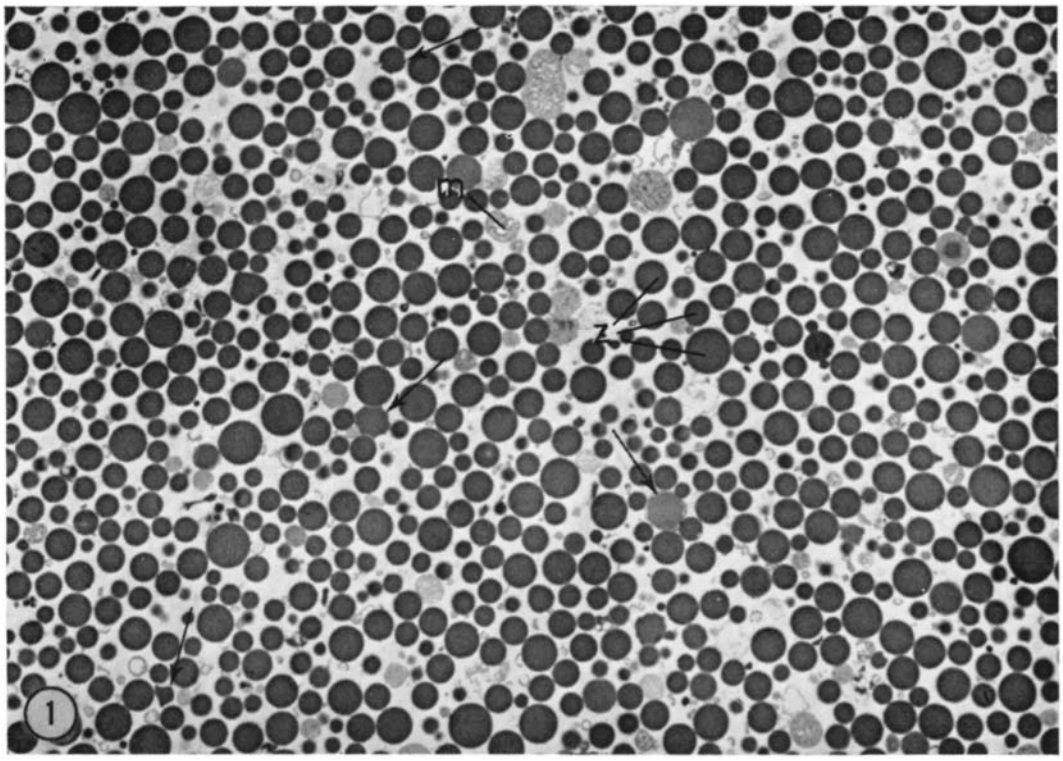


FIGURE 1 Low magnification electron micrograph of the zymogen granule fraction. This field is representative of all levels in the pellet. Zymogen granules (*z*) comprise the majority of structures. A smaller population (~ 5%) of condensing vacuoles (arrows), identified by their scalloped profiles, content of lesser electron opacity, or both features, can be recognized in the fraction. A few mitochondria are seen (*m*). × 3,000.

by the orcinol reaction (Mejbaum, 1939). Insofar as the RNA content reflects residual microsomal contamination, the zymogen granule fraction isolated from pancreatic slices compares favorably to fractions isolated from dog and ox pancreas and is noticeably less contaminated than that isolated by density gradient centrifugation. The phospholipid-phosphorus content of the fractions is given for comparison.

MORPHOLOGY OF THE ZYMOGEN GRANULE FRACTION: Thin sections of fixed and embedded pellets of this fraction, cut to include the entire depth of the pellet in the axis of centrifugation, show that it consists mainly of zymogen granules with a few contaminating mitochondria (Fig. 1) and rough microsomes (Fig. 2). The contaminants were evenly scattered from top to bottom throughout the pellet, which did not show any detectable stratification. Zymogen granules isolated from incubated slices were identical to those prepared from fresh pancreatic tissue (Siekevitz and Palade, 1958 *a*; Greene, Hirs, and Palade, 1963) and were characterized by their spherical shape, highly dense, homogeneous, and apparently amorphous content, and a limiting unit membrane which measures ~100 A in thickness, and which can be seen only when sectioned normally.

In addition to zymogen granules, the pellet contains bodies of comparable size but of irregular shape which gives them angular or scalloped profiles (Figs. 1, 2). Like zymogen granules, they are limited by a unit membrane and have an amorphous, homogeneous content of equal or lower density. On account of their morphology, these bodies are identified as condensing vacuoles of the Golgi complex. They represent ~5% of the total particle population of the zymogen granule fraction.

KINETICS OF LABELING OF THE ZYMOGEN GRANULE FRACTION: To determine the ki-

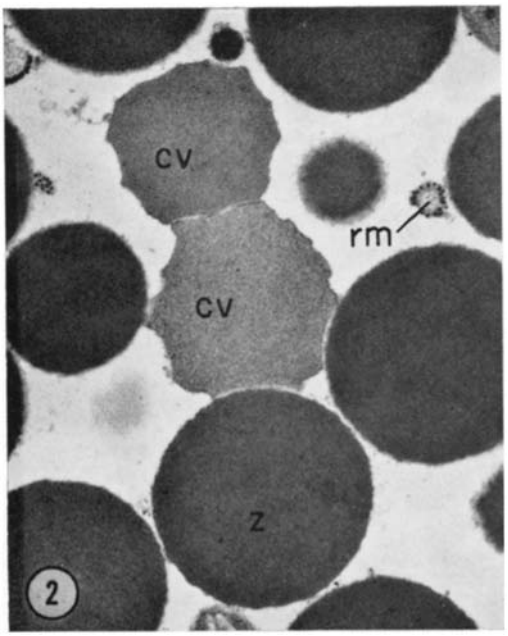


FIGURE 2 Higher magnification field from the zymogen granule fraction. A few contaminating rough microsomes (*rm*) are seen. *cv*, condensing vacuoles; *z*, zymogen granule. × 23,000.

netics of labeling of the zymogen granule fraction, we pulse labeled sets of slices as before (Jamieson and Palade, 1967) with leucine-^{14}C and fractionated them at the end of the pulse and after various times of incubation in chase medium. For these experiments, the fractionation scheme was simplified to the extent that only a zymogen granule fraction and a total microsomal fraction were isolated by differential centrifugation.

1. LABELING OF CELL FRACTIONS DURING 1-HR INCUBATION: The results of a typical short-term experiment are presented in Fig. 3. The specific radioactivity of the proteins of the zymogen granule fraction is negligible initially (i.e., compared to that of microsomal proteins); after +17 min of incubation in chase medium,[2] its proteins are half-maximally labeled and become maximally labeled between +37 and +57 min of incubation. In other cell fractionation experiments not shown, a small but significant amount of labeled protein appeared in the zymogen granule fraction as early as +7 min following the pulse.

[2] The times of incubation in chase medium following 3-min pulse labeling will be referred to hereafter as +17 min of incubation, +37 min of incubation, etc.

The specific radioactivity curve of microsomal proteins declines rapidly after the pulse. The unchanging specific activity of total homogenate proteins indicates an effective chase. The specific activity of the proteins of the post-microsomal supernate remains constant throughout incubation, signifying negligible transfer of labeled proteins from cell particulates to the cytoplasmic matrix, i.e., the cell compartment which is the main contributor to the post-microsomal supernate. After +17 min of incubation, labeled proteins begin to appear in the incubation medium.

2. LABELING OF CELL FRACTIONS DURING 3 HR OF INCUBATION: Fig. 4. shows the kinetics of labeling of proteins in cell fractions isolated from sets of pulse-labeled slices incubated in chase medium for +1, +2, and +3 hr. As before, the specific radioactivity curve of microsomal proteins falls rapidly during the chase. Labeling of proteins of the zymogen granule fraction is essentially complete after +1 hr of incubation, rises slightly during the next hr, and then begins to fall. The specific radioactivity of proteins in the incubation medium shows a slow progressive rise during 3 hr. The specific radio-

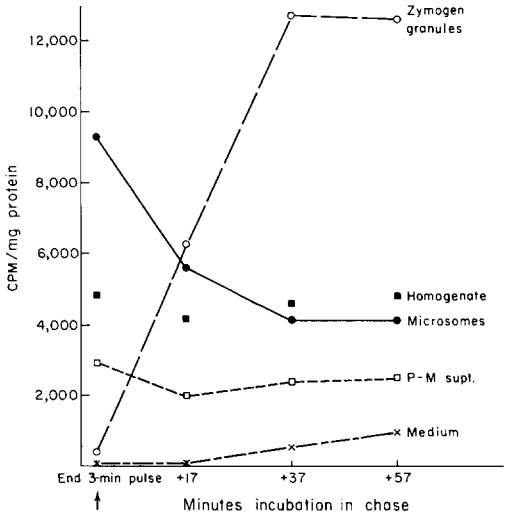


FIGURE 3 Specific radioactivity of proteins in cell fractions isolated from pancreatic slices pulse labeled for 3 min and incubated up to +57 min in chase. Pulse medium: Krebs-Ringer bicarbonate, supplemented with amino acids (minus leucine-^{12}C) and containing 1 μc/ml (4 μM) L-leucine-^{14}C. Chase medium: as above with L-leucine-^{12}C, 0.4 mM. *P-M supt.*, post-microsomal supernate.

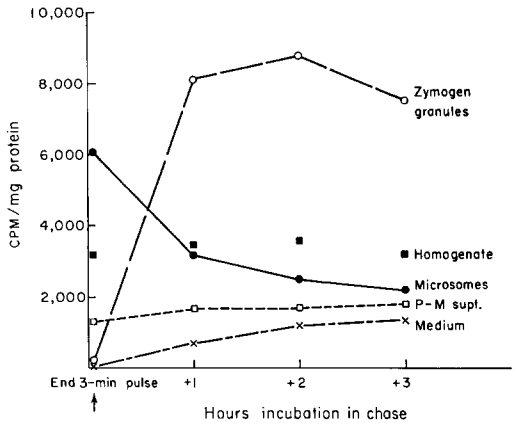


FIGURE 4 Labeling of cell fractions from pulse-labeled slices during 3 hr of incubation. For composition of media, see Fig. 3.

activity of proteins in both the total homogenate and the post-microsomal supernate remains constant throughout incubation.

These cell fractionation data indicate that the zymogen granule fraction begins to accumulate radioactive proteins as early as +7 min after pulse labeling and that it becomes maximally labeled after +37–+57 min of incubation. As was demonstrated in the previous paper (Jamieson and Palade, 1967), secretory proteins are transferred in time from the cisternae of the rough ER to the small vesicles at the periphery of the Golgi complex: the labeled proteins which accumulate in the zymogen granule fraction have come, therefore, from these small vesicles. The kinetics of labeling of zymogen granule fractions in vitro are similar to those of labeling of the same fractions in vivo, as shown by data obtained in the guinea pig (Siekevitz and Palade, 1958 *b*) and the mouse pancreas (Morris and Dickman, 1960). Hansson (1959) found, however, a somewhat slower in vivo labeling of zymogen granule fractions in guinea pig pancreas.

After ~+20 min of incubation, labeled proteins begin to appear in small amounts in the incubation medium. Although some of them probably represent leakage from damaged cells, especially at the early times, the radioautographic studies reported later in this paper indicate that exocrine cells in vitro are able to discharge labeled proteins in the acinar lumina beginning about 1 hr after pulse-labeling. Junqueira, Hirsch, and Rothschild (1955) and Hansson (1959) have reported that in vivo longer times elapse (~150 min) before labeled proteins appear in the pancreatic juice.

Radioautographic Studies

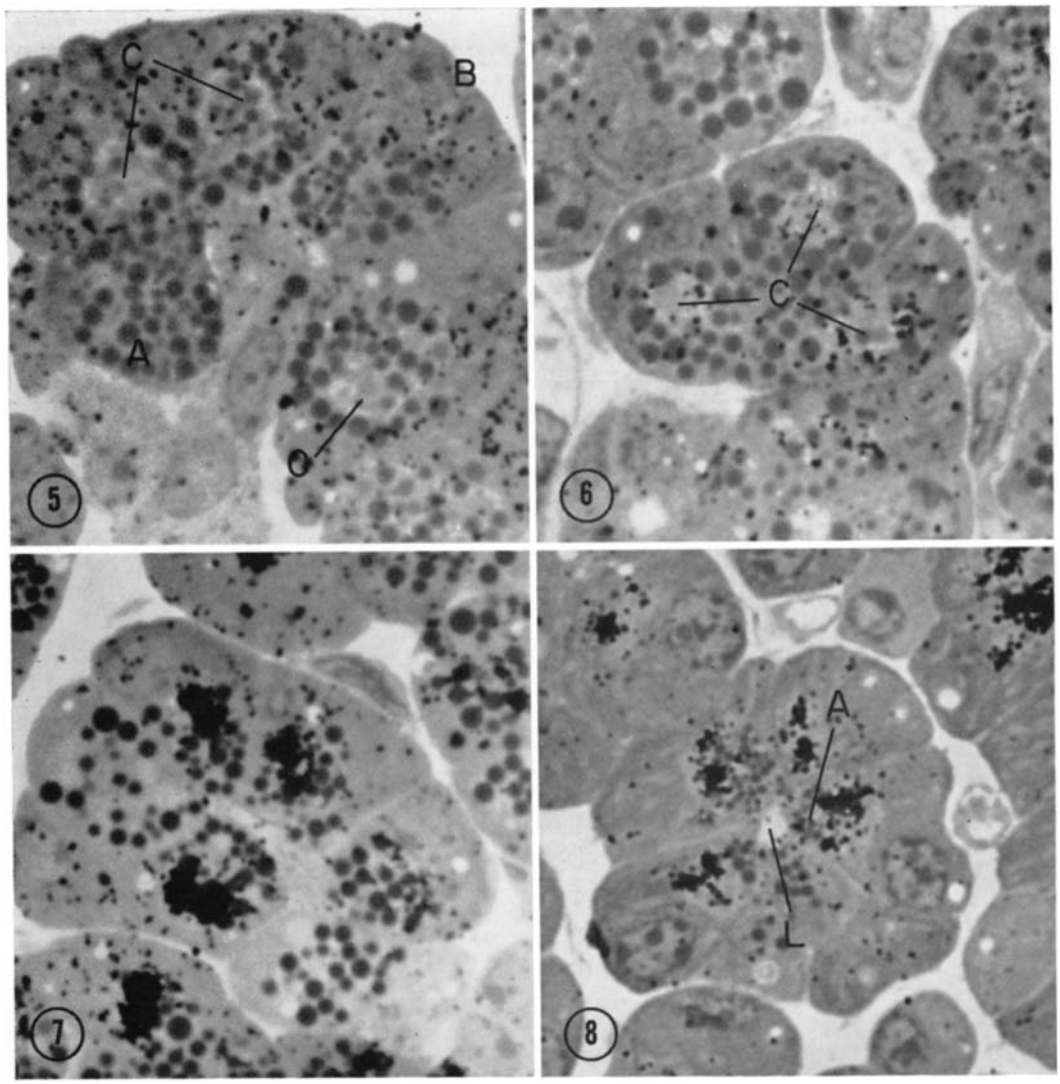
Intracellular transport was also studied by radioautography taking advantage of the fact that the slice system allows heavy labeling of secretory proteins during a short (3-min) pulse with L-leucine-4,5-^{3}H. The conditions are distinctly better than in the whole animal in which neither an equally high dose of labeled amino acids can be administered nor an equally effective chase can be achieved.

The results obtained on slices can be used to complete and correct—if necessary—previous findings on the intact animal. In addition, they represent an independent line of evidence needed for a reliable interpretation of the kinetic data obtained from cell fractionation studies.

INCUBATION CONDITIONS: Slices were pulse labeled for 3 min with L-leucine-^{3}H (200 μc/ml; 40 μM) and incubated post-pulse for further +7, +17, +37, +57, and +117 min in chase medium containing 2.0 mM unlabeled L-leucine (50x excess). Determinations of TCA-soluble and insoluble radioactivity on homogenates of these slices at the end of the pulse and after chase incubation indicated that even with this relatively massive dose of tracer, a true pulse label was obtained.

To ascertain that the incorporated label is retained in the tissue during processing for electron microscopy, we treated specimen blocks from slices at the end of the pulse and at various times during the chase as follows. After fixation, dehydration, and infiltration with Epon, the blocks were hydrolyzed in 1 N NaOH (with heating) and aliquots assayed for protein and radioactivity. It was found that the specific radioactivity of proteins from these blocks was identical to that of proteins from slices labeled under the same conditions but homogenized, precipitated with TCA, and assayed for incorporated radioactivity in the usual way. These results indicate that the tissue-processing schedule extracts negligible protein-associated radioactivity although it effectively washes out soluble, nonincorporated label (cf. Caro and Palade, 1964). We assume, then, that the radioautographic grains mark only label incorporated into protein.

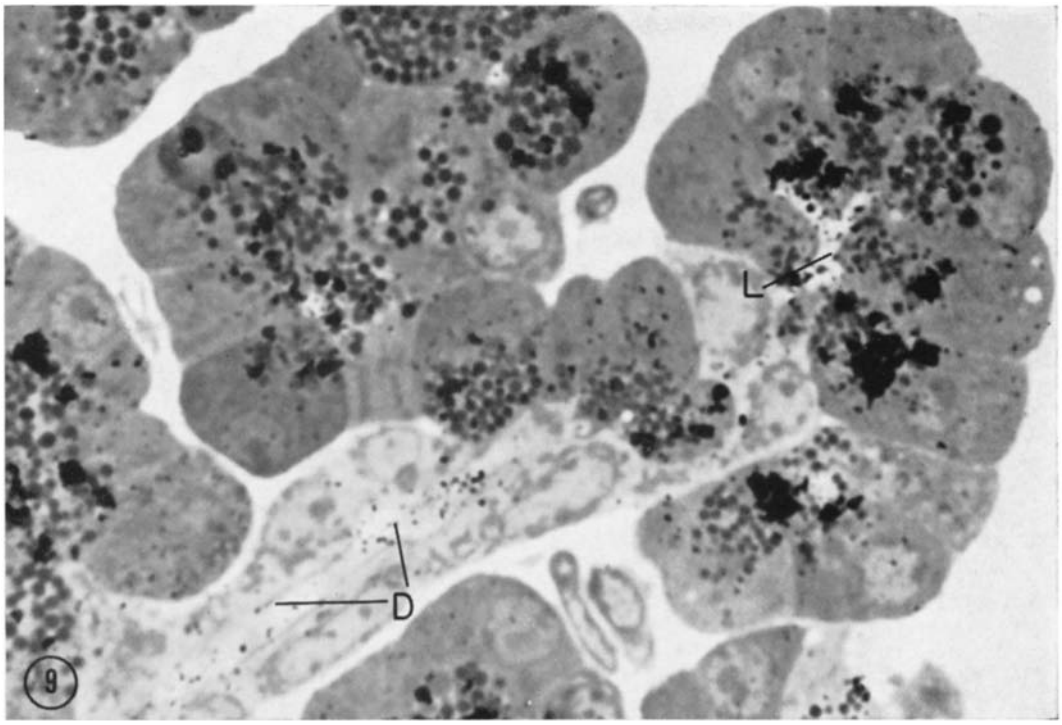
LIGHT MICROSCOPIC RADIOAUTOGRAPHY:



FIGURES 5–9 Light microscopic radioautograph of slices pulse labeled for 3 min with L-leucine-^{3}H (Fig. 5) and incubated in chase for further +17 min (Fig. 6), +37 min (Fig. 7), +57 min (Fig. 8), and +117 min (Fig. 9). *B*, base of cell; *C*, centrosphere region (Golgi complex); *L*, acinar lumen; *D*, collecting duct; *A*, cell apex with zymogen granules. Exposure time 7 days. × 1,750.

Epon sections, 0.5 μ thick, from tissue at the end of the pulse (3-min) and after +7, +17, +37, +57, and +117 min of incubation in chase medium were prepared for radioautography as described under Methods.

At the end of the pulse (Fig. 5), radioautographic grains were generally localized over the basal cytoplasm of the exocrine cell. No grains were seen to overlie either the centrosphere region (Golgi complex) or zymogen granules. The few grains among zymogen granules probably marked rough ER elements scattered in the apical regions of the cell. After +7 min, and especially after +17 min (Fig. 6), however, few grains were seen over the basal cytoplasm; the majority were located centrally in the cell and were often seen to outline the pale-staining centrosphere region. After +37 min, the grains were even more concentrated over the centrosphere region of the cells (Fig. 7) with few grains re-



maining over the basal region. At this time, zymogen granules in the apical region of the cells were generally unlabeled. At +57 min (Fig. 8), the zymogen granules in the cell apex began to accumulate label and after +117 min were highly labeled. At this time, numerous grains were also found over acinar lumina and larger collecting ducts of the gland (Fig. 9).

From these results, it is clear that the initial site of incorporation of label in the pancreatic acinar cell is the basal ergastoplasm. During postpulse incubation in chase medium, the labeled proteins move as a well defined wave through the cells and eventually appear in large amounts in the duct system after 2 hr of incubation.

It should be noted that acini were not uniformly labeled throughout the thickness of the slice. A gradient of labeling from the periphery to the center of the slice was found, though only a few acini, farthest from the surface of the slice, were free of label. Apparently, the thickness of the slice does, to some extent, present a barrier to the penetration of label. Nevertheless, at any time the location of grains over cell structures was the same throughout the depth of the slice, indicating that the penetration barrier merely affected the intensity of the radioautographic response without introducing asynchronous labeling. Thus, the gradient does not alter the intracellular transport schedule.

ELECTRON MICROSCOPIC RADIOAUTOGRAPHY: Since the resolution achieved by light microscopic radioautography does not allow us to determine the relationship of silver grains to the individual elements of the centrosphere and apical regions, electron microscopic radioautography was performed on thin sections cut from the same material used for light microscopic radioautography.

At the end of the pulse (Fig. 10), the radioautographic grains were localized exclusively over elements of the rough ER. No grains were seen over elements of the Golgi complex or zymogen granules.

After +7 min (Fig. 11), although the majority of the label still marked the rough ER, radioautographic grains began to appear over the clusters of small, smooth-surfaced vesicles located at the periphery of the Golgi complex. Little of the label progressed as far as condensing vacuoles at this time.

At +17 min, the situation was intermediate

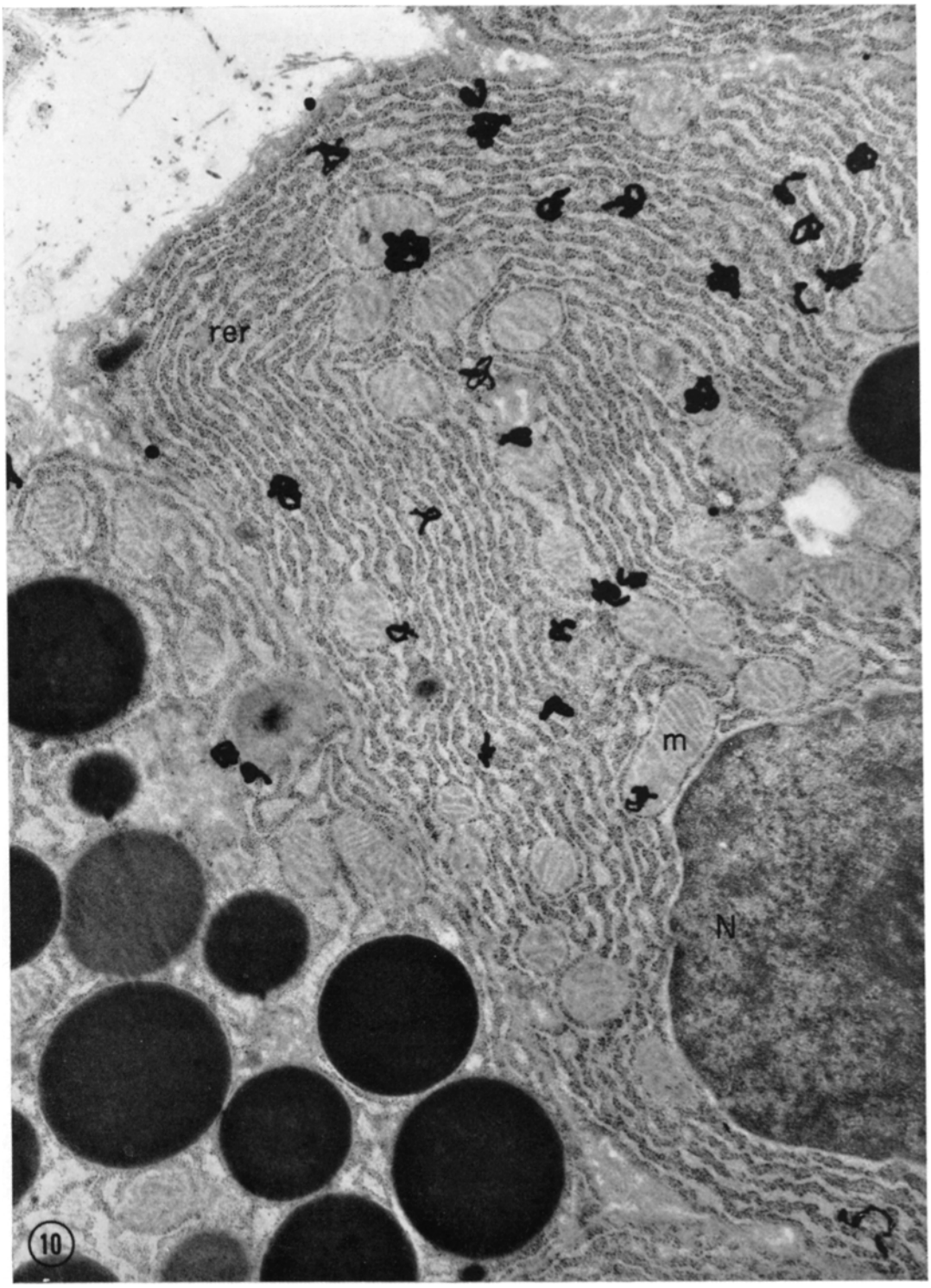


FIGURE 10 Electron microscopic radioautograph of an acinar cell at the end of pulse labeling for 3 min with L-leucine-^{3}H. The radioautographic grains are located almost exclusively over elements of the rough ER (*rer*). A few grains partly overlie mitochondria but, for reasons discussed in the text, most likely label the adjacent rough ER. *m*, mitochondrion; *N*, nucleus. × 17,000.

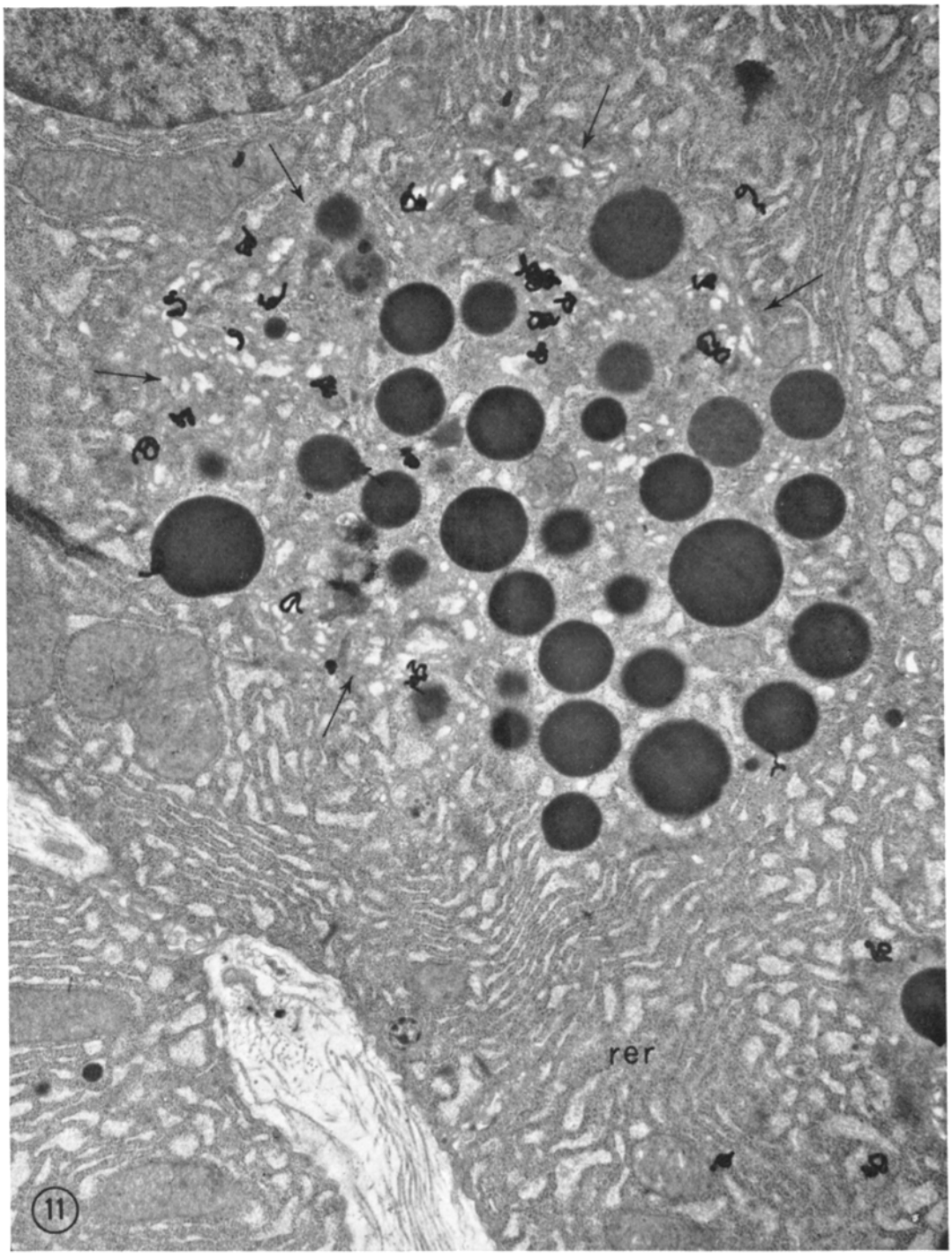


FIGURE 11 Electron microscopic radioautograph of an acinar cell after +7 min of incubation post-pulse. The majority of the label marks the periphery of the Golgi complex (arrows) with little remaining in the rough ER (*rer*). × 17,000.

between that at +7 and +37 min: most of the label had migrated from the basal rough ER and was localized over the periphery of the Golgi complex, while part of it had already reached condensing vacuoles.

After +37 min (Fig. 12), few grains were still found at the periphery of the Golgi complex; most of them now were preferentially and highly concentrated over the condensing vacuoles of the complex. At this time, a small number of grains was already located over zymogen granules.

After +57 min, a larger proportion of the label was found over zymogen granules, especially those adjacent to the Golgi region. Some label remained over the condensing vacuoles though few grains still labeled the periphery of the Golgi complex. By +117 min (which was the longest time point examined by radioautography), the grains heavily labeled zymogen granules grouped near the cell apex and numbers of them were present over fibrous material (presumably discharged secretory protein) in the acinar lumina and collecting ducts of the gland (Fig. 13). The condensing vacuoles of the Golgi complex were now practically devoid of label.

At all times longer than +17 min, a small number of grains persisted over the regions of the cell occupied by rough ER. No significant labeling of centroacinar cells, islet cells, or duct cells was observed at any time. Background label was negligible in all experiments.

Quantitation of radioautographic grain distribution over various components of acinar cells was made at each time point by counting grains on a series of low magnification electron micrographs taken at random (Table II). Analysis of these data gives information about the quantity of labeled material in the compartment represented by the corresponding cell component rather than about the specific radioactivity of its content.[3] Thus, grain counts cannot be compared directly to specific radioactivity data derived from cell fractionation experiments, although similar patterns of labeling occur.

At the end of the pulse, radioautographic grains were associated almost exclusively with the rough ER of the acinar cell; during chase incubation, the numbers of grains over the rough ER progressively decreased. This is consistent with the kinetics of labeling of the rough microsomes which are derived from the rough ER (cf. Jamieson and Palade, 1967). At the end of +117 min, ~20% of the grains were still located over the rough ER. This label probably identifies nonexportable proteins with a long turnover time (see Siekevitz and Palade, 1960; Warshawsky, Leblond, and Droz, 1963).

Concurrently with the decrease in grains over the ER, there was a progressive rise in grains over elements of the Golgi complex. Thus, at +7 min, ~47% of the grains were over this region, with 43% located over the small vesicles at the periphery of the complex. By +17 min, ~58% of the grains were located over the Golgi complex, with the majority still clustered over peripheral elements of the complex. Following +37 min of incubation, the majority of the label (~49%) had migrated into condensing vacuoles. At this time, a small but significant number of zymogen granules was labeled. Labeling of the zymogen granules progressively increased during the next two time points examined (+57 and +117 min) with an over-all decrease over condensing vacuoles. During the same times, a significant percentage of the grains was located in the acinar lumina.

At all time points, the labeling of mitochondria and nuclei was low and generally decreased during the chase.

From radioautography we can conclude that pancreatic acinar cells incubated in vitro are able to perform the entire sequence of operations involved in the secretory cycle and that the timetable of these events is similar to that established in the whole animal by the radioautographic studies of Caro and Palade (1964).

According to our radioautographic data, at +37 min only a small proportion of the label had progressed as far as zymogen granules, the majority being located over condensing vacuoles. From the labeling kinetics of the zymogen granule fraction (cf. Fig. 3), however, protein radioactivity appears in this fraction as early as +17 min and the fraction is maximally labeled between +37 and +57 min. Because zymogen granule fractions are known (this work and Greene et al., 1963) to contain a small population of condensing vacuoles, we assumed that this discrepancy was only apparent and reflected the presence of con-

[3] It should be noted that the method of analyzing radioautographic data reported here (Table II) is valid since the total amount of label remains constant in the tissue during the times of observation (efficient chase incubation).

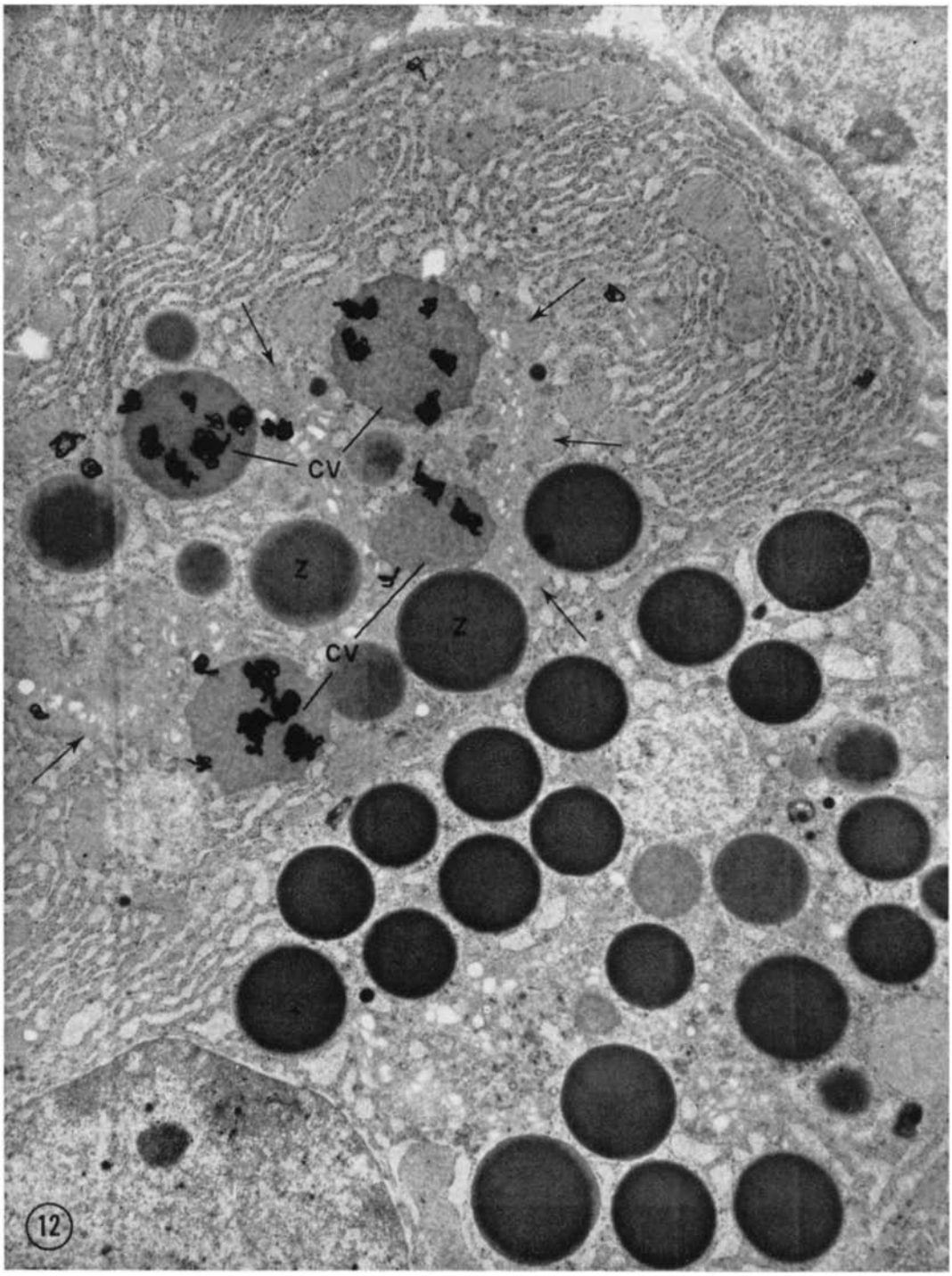


FIGURE 12 Portion of an acinar cell pulse labeled as before and incubated post-pulse for +37 min. Arrows indicate the periphery of the Golgi complex. Radioautographic grains are highly concentrated over condensing vacuoles (*cv*) of the Golgi complex. Zymogen granules (*z*) are unlabeled. × 13,000.

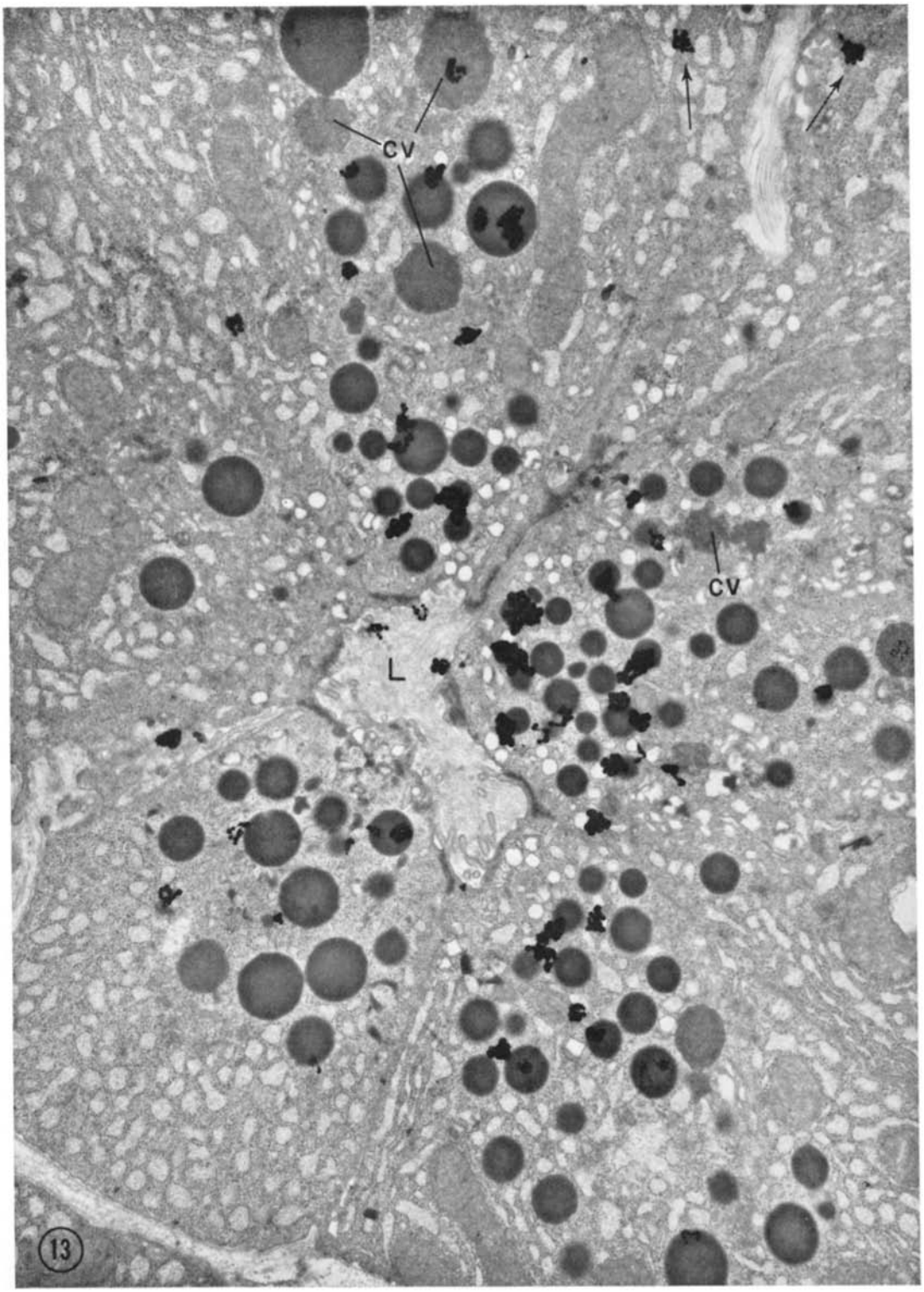


FIGURE 13 Electron microscopic radioautograph of several acinar cells incubated post-pulse for +117 min. Radioautographic grains are located primarily over zymogen granules near the cell apex. Condensing vacuoles (*cv*) are practically devoid of label. Note residual labeling of the rough ER (arrows). Some label marks secretory material in the acinar lumen (*L*). × 13,000.

TABLE II

Distribution of Radioautographic Grains over Cell Components

	% of radioautographic grains					
	3-min (pulse)	Chase incubation				
		+7 min	+17 min	+37 min	+57 min	+117 min
Rough endoplasmic reticulum	**86.3**	43.7	37.6	24.3	16.0	20.0
Golgi complex*						
Peripheral vesicles	2.7	**43.0**	37.5	14.9	11.0	3.6
Condensing vacuoles	1.0	3.8	19.5	**48.5**	35.8	7.5
Zymogen granules	3.0	4.6	3.1	11.3	32.9	**58.6**
Acinar lumen	0	0	0	0	2.9	7.1
Mitochondria	4.0	3.1	1.0	0.9	1.2	1.8
Nuclei	3.0	1.7	1.2	0.2	0	1.4
No. of grains counted	300	1146	587	577	960	1140

The boldfaced numbers indicate maximum accumulation of grains over the corresponding cell component.

* At no time were significant numbers of grains found in association with the flattened, piled cisternae of the complex.

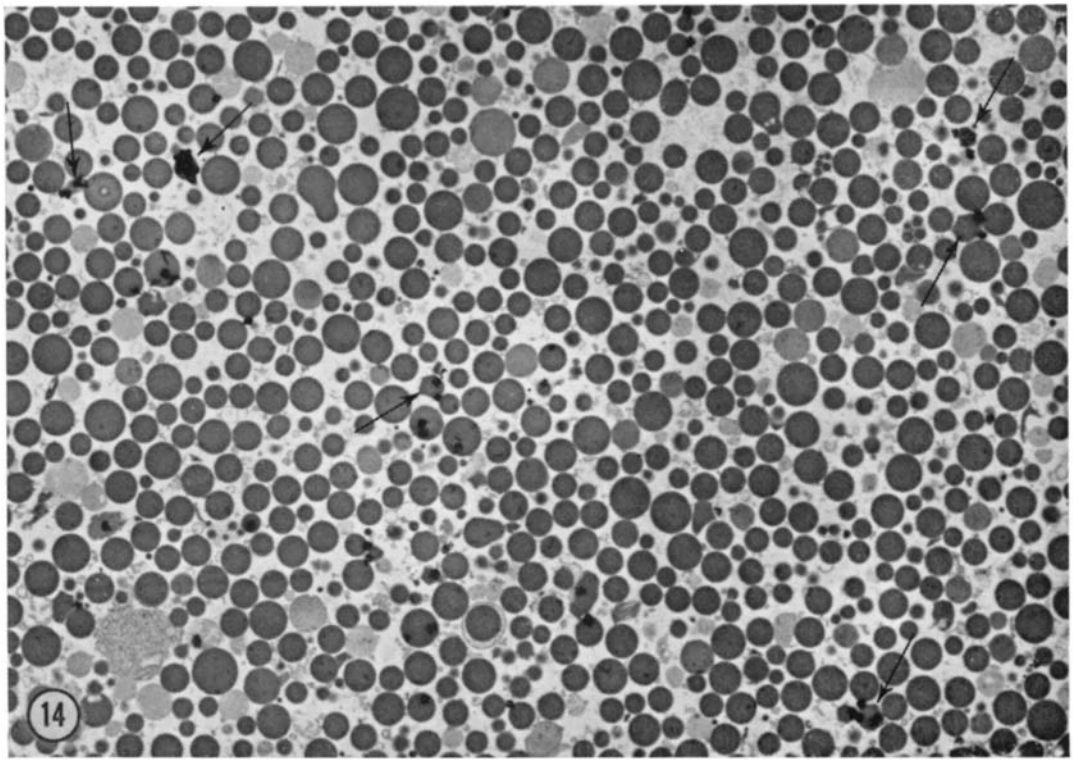


FIGURE 14 Low magnification electron microscopic radioautograph of the zymogen granule pellet derived from slices pulse labeled for 3 min with leucine-^{3}H and incubated +37 min in chase. The arrows indicate highly labeled condensing vacuoles. The majority of the zymogen granules is unlabeled. × 3,000.

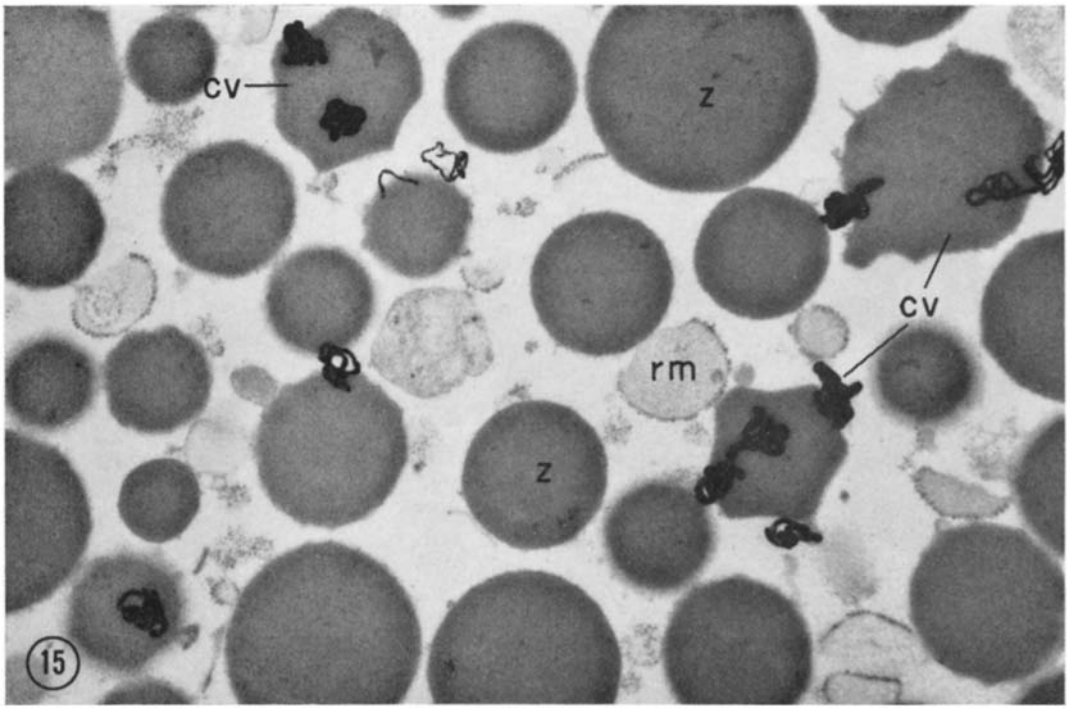


FIGURE 15 Higher magnification view of the same pellet seen in Fig. 14, showing the localization of radioautographic grains over condensing vacuoles (*cv*). *z*, zymogen granules; *rm*, rough microsomes. × 13,000.

densing vacuoles highly labeled at early times after the pulse. Since at present no satisfactory method exists for the separation of condensing vacuoles from zymogen granules, this assumption was tested by performing electron microscopic radioautography on thin sections of zymogen granule pellets derived from slices pulse labeled with leucine-^{3}H for 3 min and incubated for +17 and +37 min in chase medium.

For these studies, the conditions of pulse labeling and incubation in chase medium were identical to those used for tissue radioautography. Thin sections of zymogen granule pellets from each of the three time points were cut through the entire depth of the pellet in the direction of the centrifugal field and mounted on bar-grids which were subsequently processed for electron microscopic radioautography as described under Methods. Grids from the three time points were coated with emulsion at the same time (in order to ensure equal distribution of photographic grains over the specimens), exposed for 3–4 wk, and finally processed photographically. Sequential micrographs were taken in the electron microscope at low magnification (× 750) so as to include the entire depth of the pellet.

At the end of the pulse, no radioautographic grains were found over any component of the zymogen granule pellet. After +17 min, and especially after +37 min of incubation, numerous grains were localized over recognizable condensing vacuoles in the zymogen granule pellets. A typical low magnification field, taken for illustration from the +37-min time point, is shown in Fig. 14. Even at this magnification, radioautographic grains are seen to be preferentially located over condensing vacuoles which are identified in the pellet, as in the cell, by their angular profiles. This is seen to advantage in Fig. 15, which is a higher magnification view of the same pellet.

To quantitate these results, we enlarged photographically the low magnification micrographs to 16 x 20 in. A grid of this size, subdivided into 10- x 10-cm squares, was drawn on a thin plastic sheet and overlaid on the photographic prints. The numbers of labeled condensing vacuoles and

TABLE III

Labeled Condensing Vacuoles and Zymogen Granules in Zymogen Granule Fraction

		Pulse + chase incubation	
	3-min (pulse)	3 + 17 min	3 + 37 min
Condensing vacuoles labeled (%)	0	7.3	14.0
Zymogen granules labeled (%)	0	0.15	0.53
Labeled condensing vacuoles as % total labeled condensing vacuoles + zymogen granules	0	73	65
Condensing vacuoles as % total condensing vacuoles + zymogen granules	4.95	4.06	6.60

zymogen granules were recorded within each 10-cm square (Table III).

No labeled condensing vacuoles or zymogen granules were found in the micrographs from the 3-min zymogen granule pellet. By +17 min, 7.3% of all condensing vacuoles were labeled and at +37 min the number had increased to 14.0%. At this time, the number of radioautographic grains per vacuole was also considerably greater than at +17 min. At +17 min, and especially at +37 min, a small but significant number of zymogen granules was labeled, though at these time points the majority of labeled particles was condensing vacuoles. Contaminating rough microsomes and mitochondria were not labeled at any time.

These data thus support the original assumption that early labeling of the zymogen granule fraction is accounted for by highly labeled condensing vacuoles and satisfactorily explain the apparent discrepancy between cell fractionation data and electron microscopic radioautography.

DISCUSSION

General Premises Used to Interpret the Experimental Data

In interpreting the results of the experiments reported in this and the previous paper, we have assumed that the label identifies newly synthesized secretory proteins in all cell compartments examined, including smooth microsomes. This assumption is justified by the data of Siekevitz and Palade (1960) who showed that the rate of labeling of secretory proteins in the guinea pig pancreas is five to seven times faster than that of proteins retained in the exocrine cell; it is compatible with our radioautographic data which indicate that the majority of the label moves as a single wave from one cell compartment to another; and it is supported by the results of experiments which show that a large and comparable amount of labeled proteins can be extracted by saline-bicarbonate from all fractions examined (rough and smooth microsomes, zymogen granule fraction). This treatment is known to solubilize digestive enzymes and enzyme precursors from zymogen granules (Greene et al., 1963). Incorporation of label into nonexportable protein undoubtedly takes place, but can be considered negligible within the time limits involved in these experiments.

Results of Present Study

SYNTHESIS AND SEGREGATION OF SECRETORY PROTEINS: In the present study, short (2 – 3-min) pulse labeling of secretory proteins coupled with efficient chase incubation has allowed us to demonstrate unequivocally by electron microscopic radioautography and cell fractionation that the single site of incorporation of label into proteins is in the rough ER. The general location of this site has been identified by radioautography, and the structure involved—rough microsomes derived from the rough ER—has been pinpointed by cell fractionation. The existence of a second site of synthesis in the Golgi complex (Sjöstrand, 1962) is excluded since, by the time labeled proteins appear in the complex, the intracellular pool of soluble precursors has effectively been diluted. Further, the label which appears in Golgi elements can be accounted for by label lost from the rough ER (Jamieson and Palade, 1967). The existence of a second site could not be ruled out by previous radioautographic work on intact animals (Caro and Palade, 1964). Thus, the present results put on a factual basis the hypothesis of Siekevitz and Palade (1958 *b*) according to which secretory proteins are synthesized only in the rough ER and subsequently transported to other cell compartments.

Our radioautographic studies indicate that at the end of the pulse only ~3% of the label was located over mitochondria and ~3% over nuclei; during incubation in the chase medium, the per-

centage of grains located over these structures progressively decreased in parallel with the loss of label from the rough ER. It is, therefore, likely that the labeling of mitochondria and nuclei reflects labeling of the adjacent rough ER. Accordingly, the data do not support the assumption of Straub (1958) that mitochondria participate in the synthesis of pancreatic secretory proteins. Similarly, the limited amount of label located over elements of the Golgi complex and zymogen granules at the end of the pulse should be taken as a maximum figure which reflects to a large extent labeling of adjacent rough ER elements.

Our results do not provide further insight into either the actual site of protein synthesis in the rough ER, or the fate of the protein immediately after synthesis. Earlier evidence (Siekevitz and Palade, 1960) had indicated that α-chymotrypsinogen is synthesized on attached ribosomes, and recently Redman, Siekevitz, and Palade (1966) have shown that another secretory protein, α-amylase, is synthesized in the attached ribosomes of pigeon pancreatic microsomes incubated in vitro. In addition, these investigators have demonstrated that newly synthesized amylase is preferentially transported to the content of the microsomal vesicles, which is the in vitro equivalent of the content of the cisternal space. Using liver microsomes, Redman and Sabatini (1966) have also shown that peptides labeled in vitro and discharged by puromycin from attached ribosomes are preferentially transported into the cisternal space, indicating that from the inception of its synthesis, the peptide chain is destined to reach this space. It can be tentatively assumed that these findings (Redman et al., 1966; Redman and Sabatini, 1966) apply to all secretory proteins produced by the exocrine cell.

TRANSPORT OF SECRETORY PROTEINS THROUGH THE PERIPHERY OF THE GOLGI COMPLEX: The results of kinetic experiments performed on smooth microsomal fractions (Jamieson and Palade, 1966, 1967), isolated from pancreas slices at the end of the pulse and after various times of incubation in the chase medium, provide direct evidence that secretory proteins pass through the small peripheral vesicles of the Golgi complex in transit from their site of synthesis and segregation in the rough ER to their site of concentration in condensing vacuoles. The cell fractionation data also indicate that the specific radioactivity of proteins in the post-microsomal supernate (i.e., of proteins located mainly in the cytoplasmic matrix or cell sap) remains constant at all times examined, indicating negligible equilibration between labeled proteins of the particulate components of the cell and the cytoplasmic matrix.[4] These results do not support the assumption (Laird and Barton, 1958; Redman and Hokin, 1959; Morris and Dickman, 1960) that secretory proteins leave the microsomes and pass, in soluble form, through the cytoplasmic matrix to zymogen granules or the acinar lumen.

Cell fractionation findings are in good agreement with radioautographic data obtained on intact cells labeled either in slices or in the whole animal (Caro and Palade, 1964). The data show that, at the time labeled protein becomes concentrated in the smooth microsomes (between +7 and +17 min), radioautographic grains are concentrated over a region of the Golgi complex containing numerous small, smooth-surfaced vesicles; at this time, no other smooth-surfaced vesicles in the cell (e.g., small clusters of vesicles located among the rough ER cisternae and under the plasmalemma, and small apical vacuoles) or potential sources of smooth vesicles (cell membrane or mitochondria) are labeled. The results of cell fractionation and radioautography thus support and complement each other: radioautography indicates that the smooth microsomal fraction must contain peripheral elements of the Golgi complex while cell fractionation shows that the label is localized to the small vesicles of this region rather than to the surrounding cytoplasmic matrix. The majority of the radioactive protein in the smooth microsomes can be extracted by mild alkaline treatment, indicating that the label is located within the cavity of the small Golgi vesicles and not in their limiting membrane.

From these combined data, it is clear that the small vesicles of the Golgi complex mediate the transport of secretory proteins from the rough ER to condensing vacuoles. This implies transport in bulk or in mass since each vesicle must contain and carry a large number of protein molecules.

[4] From recovery experiments, not shown in this paper, it could be calculated that if the total labeled proteins of the microsomal fraction were transferred completely to the post-microsomal supernate during chase incubation, then the specific radioactivity of proteins in the supernate should increase 2.5- to 3-fold. This did not occur (see Fig. 3).

The transport must also be discontinuous; otherwise, concentration of the solution of secretory proteins at the next step (condensing vacuoles) would not be possible. With these restrictions in mind, we can inquire into cellular mechanisms possibly involved in this operation. It should be recalled that the transitional elements of the rough ER adjacent to the periphery of the Golgi complex are limited by part rough- and part smooth-surfaced membranes. The latter are frequently in contact with smooth-surfaced vesicles or protrude as smooth-surfaced blebs toward the Golgi complex. These blebs are comparable in size to the small, smooth-surfaced vesicles of the region. Likewise, the smooth-surfaced membrane bounding the condensing vacuoles is often in contact with small vesicles or is thrown into small surface blebs. These images suggest repeated fission of, and fusion with, vesicles at both terminals. It can be assumed, then, that secretory proteins are transported from the transitional elements of the rough ER to condensing vacuoles through intermittent tubular connections, or, alternatively, that transport is effected by discrete vesicles that shuttle between the two compartments. Both possibilities are compatible with the data, but the second is more consistent with the fine structural details of the Golgi complex.

The model proposed for the transport of secretory proteins within the periphery of the Golgi complex is reminiscent of the transport of macromolecules in bulk across the vascular endothelium by pinocytic vesicles (Palade and Bruns, 1964; Karnovsky, 1965), though in the case of the pancreatic exocrine cell, transport in bulk operates between two intracellular compartments, the rough ER and the condensing vacuoles.

TRANSPORT OF LABELED PROTEINS TO CONDENSING VACUOLES, ZYMOGEN GRANULES, AND THE ACINAR LUMEN: The radioautographic studies of Caro and Palade (1964) have firmly established that proteins are transported to condensing vacuoles of the Golgi complex where they are intensively concentrated, the condensing vacuoles being converted in the process into zymogen granules. We have confirmed the position of the condensing vacuoles in the secretory cycle by electron microscopic radioautography of pancreatic slices and have demonstrated that the time taken to accumulate labeled proteins within condensing vacuoles is approximately the same in vitro as in vivo. Further, because of the short, well defined label which passes through the cell as a single wave, the arrival and concentration of radioactive proteins in condensing vacuoles are more strikingly demonstrated than was possible in the whole animal.

Earlier cell fractionation (Siekevitz and Palade, 1958 *b*) and radioautographic data (Caro and Palade, 1964) were not in agreement concerning the rate of transport of labeled secretory proteins into zymogen granules. According to the former, the zymogen granule fraction accumulates radioactive protein as early as 10 min after the injection of tracer, whereas radioautography indicated that zymogen granules were not labeled until $\sim$1 hr after injection of tracer. In the present experiments, a similar discrepancy was observed. Since the zymogen granule pellet contains a small population of condensing vacuoles, we assumed that the discrepancy was due to the presence of early and intensive labeling of condensing vacuoles. We tested this possibility by electron microscopic radioautography of sections of the zymogen granule pellet at various times after pulse labeling. The results substantiated the assumption and pointed out the important contribution made by condensing vacuoles to the early labeling of the zymogen granule fraction. Evidently, a correction factor must be applied to previous cell fractionation data which indicated that zymogen granules become labeled shortly after the administration of the tracer (Siekevitz and Palade, 1958 *b*; Hansson, 1959; Morris and Dickman, 1960).

It should be noted here that Schramm and Bdolah (1964) have concluded, on the basis of cell fractionation studies carried out on pulse-labeled rat parotid slices incubated in vitro, that newly synthesized amylase moves from the microsomal fraction sequentially into fractions containing granules of increasing size or density. Since the pancreatic and salivary exocrine cells are similar in organization (cf. Parks, 1961, 1962) it is probable that here, too, early labeling of the granule fractions is due to the presence of labeled condensing vacuoles.

Radioautographic observations on intact cells of slices after 1 hr and especially after 2 hr post-pulse incubation conclusively demonstrate the transformation of condensing vacuoles into zymogen granules and, finally, the discharge of protein into the acinar lumen. The terminal steps in the secretory cycle are events well documented by previous work (Palade, 1959; Caro and Palade, 1964). Hence, we now have a reasonably com-

plete elucidation of the entire intracellular pathway of secretory proteins from their site of synthesis on attached ribosomes to their ultimate discharge into the duct system of the gland. According to the radioautographic data, our interpretations probably apply to the pathway and timetable of transport of all digestive enzymes in the guinea pig pancreas. Exceptions for exportable proteins which represent a small fraction of the total output in the guinea pig or for any protein in other species are improbable but not excluded (cf. Redman and Hokin, 1959; Morris and Dickman, 1960).

We should mention that our radioautographic studies indicate that the piled Golgi cisternae located at the periphery of the complex do not seem to play a direct role in the transport and concentration of labeled secretory proteins. Nevertheless, in the exocrine pancreas of other species (rat, mouse), condensing vacuoles appear to form at the expense of the innermost cisternae of the piles. A comparable situation exists in other secretory cells where the function of the condensing vacuoles is taken over by the piled cisternae of the Golgi complex (Kurosumi, 1963, Bainton and Farquhar, 1966; Smith and Farquhar, 1966; Essner and Novikoff, 1962).

To what extent does the well established sequence of operations involved in the pancreatic secretory cycle apply to secretory processes in other cells? The same sequence seems to operate in glandular cells specialized in the synthesis of proteins for export, provided the secretory product is quantized and stored temporarily in the cell in concentrated form. For example, radioautographic evidence suggests that the model applies to the mammary gland (Wellings and Philp, 1964), and to myelocytes (Fedorko and Hirsch, 1966). In cells in which there is no intracellular accumulation of secretory product or in which the latter accumulates within the ER cisternae (Ross and Benditt, 1965; Revel and Hay, 1963; Rifkind et al., 1962), the extent to which the Golgi complex is involved is not clear.

In conclusion, we now have a satisfactory general understanding of the secretory cycle of the pancreatic exocrine cell based on experimentally established facts from a number of studies. In addition, these studies provide direct or indirect evidence that several types of intracellular transport, some of them new, participate in the cycle. These include: vectorial transport of newly synthesized proteins across the membranes of the rough ER (Redman et al., 1966; Redman and Sabatini, 1966); transport in bulk of materials between cell compartments; concentration of cell products within membrane-bounded structures possibly by intracellular ion pumps; and finally, transport of secretory proteins from zymogen granules to the acinar lumina (Palade, 1959). The forces, molecular events, and control mechanisms operating at each step remain to be elucidated by future work.

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