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ROLE OF THE GOLGI COMPLEX IN THE INTRACELLULAR TRANSPORT OF SECRETORY PROTEINS

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In the exocrine cell of the guinea pig pancreas, secretory proteins, i.e., digestive enzymes, are synthesized on ribosomes attached to the limiting membrane of the rough endoplasmic reticulum (RER).[1] They are subsequently transported across this membrane,[2] and segregated within the cisternae of the RER. Electron microscopic autoradiography[3] has unequivocally demonstrated that these proteins later appear in the Golgi region, first at its periphery and then in its central condensing vacuoles which, by progressive filling and condensation of their content, eventually become zymogen granules. Small vesicular elements, present at the periphery of the Golgi complex, have tentatively been implicated[3] in the transfer of proteins from RER to condensing vacuoles, but the point could not be proved directly by electron microscopic autoradiography. At present, the resolving power of this technique is not sufficient to show whether labeled secretory proteins are located within these vesicles, or in the cytoplasmic matrix surrounding them.

To define the role of the Golgi complex, especially of its peripheral elements, in the intracellular transfer of secretory proteins, we have reinvestigated the problem by using guinea pig pancreas slices which can be pulse-labeled *in vitro* with radioactive amino acids, and subjected to cell fractionation procedures after labeling.

Materials and Methods.—Preparation of slices: Pancreata removed from starved male guinea pigs were placed in iced incubation medium, and cut in thin slices (~50 mg each) with a Stadie-Riggs blade. Sufficient slices (21–30) for cell fractionation at 3 or 4 successive time points could be prepared from a single pancreas. Hence, a kinetic experiment could be performed on a single pancreas, thus eliminating individual variations among animals.

Incubation medium: The incubation medium was Krebs-Ringer bicarbonate[4] equilibrated with 95% O_2–5% CO_2 and supplemented with amino acids.[5] During pulse labeling, unlabeled L-leucine was replaced by either 1.0 μc/ml (4 μM) L-C^{14}-leucine (for cell fractionation experiments) or 200 μc/ml (40 μM) L-H^3-leucine (for electron microscopic autoradiography). Chase media were 4 mM in unlabeled L-leucine.

Pulse labeling: Three or four sets of slices were placed in 50-ml flasks containing 7 ml of aerated pulse medium, at 4°. Pulse labeling was initiated by incubation for 3 min at 37° with shaking, and was stopped by washing slices with chase medium at 37°. Since temperature equilibration took ~1 min, the actual length of the pulse was 2 min. One set of slices was immediately fractionated; the others were further incubated in chase medium before fractionation.

Cell fractionation: Incubated slices were homogenized in 5 ml of 0.3 *M* sucrose at 4° with a glass-Teflon homogenizer, and the following fractions were successively prepared from the homogenate: (I) nuclei and cell debris, isolated by centrifuging for 6×10^3 *g*-min; (II) zymogen granules, 1×10^4 *g*-min; (III) mitochondria, 1.3×10^5 *g*-min; (IV) microsomes, 6.5×10^6 *g*-min; and (V) postmicrosomal supernate. Rough- and smooth-surfaced microsomes were separated as follows: 0.25 ml of a microsomal resuspension (= 2.5 mg protein) was layered over a linear sucrose density gradient (1.04–2.0 *M* sucrose) and centrifuged to equilibrium ($7^1/_2$ hr) at $123{,}000 \times g$ in a Spinco SW39L rotor. As a result, a narrow band formed at the top of the gradient (smooth microsomes) and a broader one (rough microsomes) about $^2/_3$ of the way down the gradient. Each band was collected with a syringe, diluted to 0.3 *M* sucrose, and pelleted by centrifuging for 1.3×10^7 *g*-min.

Radioactivity measurements: Total homogenates or resuspended cell fractions were precipitated with 10% TCA (final concentration). Acid-soluble radioactivity was measured by liquid scintillation counting of TCA supernatants. Since only 2–3% of TCA-precipitable counts are extractable by lipid solvents and hot TCA, radioactivity in protein was routinely assayed by liquid scintillation counting of samples of washed TCA precipitates dissolved in formic acid. All counts reported were corrected for quenching.

Analytic procedures: Proteins were measured by the method of Lowry *et al.*[6] and RNA by the orcinol reaction on TCA hydrolysates.[7]

Microscopy: Whole slices and cell fractions were fixed for 2–4 hr at 0° in 1–2% OsO_4 buffered at pH 7.4 with 0.1 *M* K phosphate, dehydrated, and embedded in Epon 812. Embedded pellets were oriented so that sections would include the entire depth of the pellet. Thin sections were doubly stained[8] and examined in a Siemens-Elmiskop I. Electron microscopic autoradiography was performed on thin sections of Epon-embedded material according to the method of Caro and van Tubergen.[9]

Results.—Characteristics of the slice system: In contrast to the situation *in vivo*, where pulses of less than 15 min cannot be obtained,[3] well-defined, short (2 min) pulse labeling can be easily achieved with pancreatic slices incubated *in vitro*. Further incubation in an excess of unlabeled leucine rapidly and efficiently reduces soluble counts in slices and stops further incorporation of label into TCA precipitables. Once incorporated into protein, label is stable: the specific activity of total homogenate proteins remains constant for at least 1 hr incubation (see Fig. 4). Minimal fine structural disorganization occurs in the slices during this time.

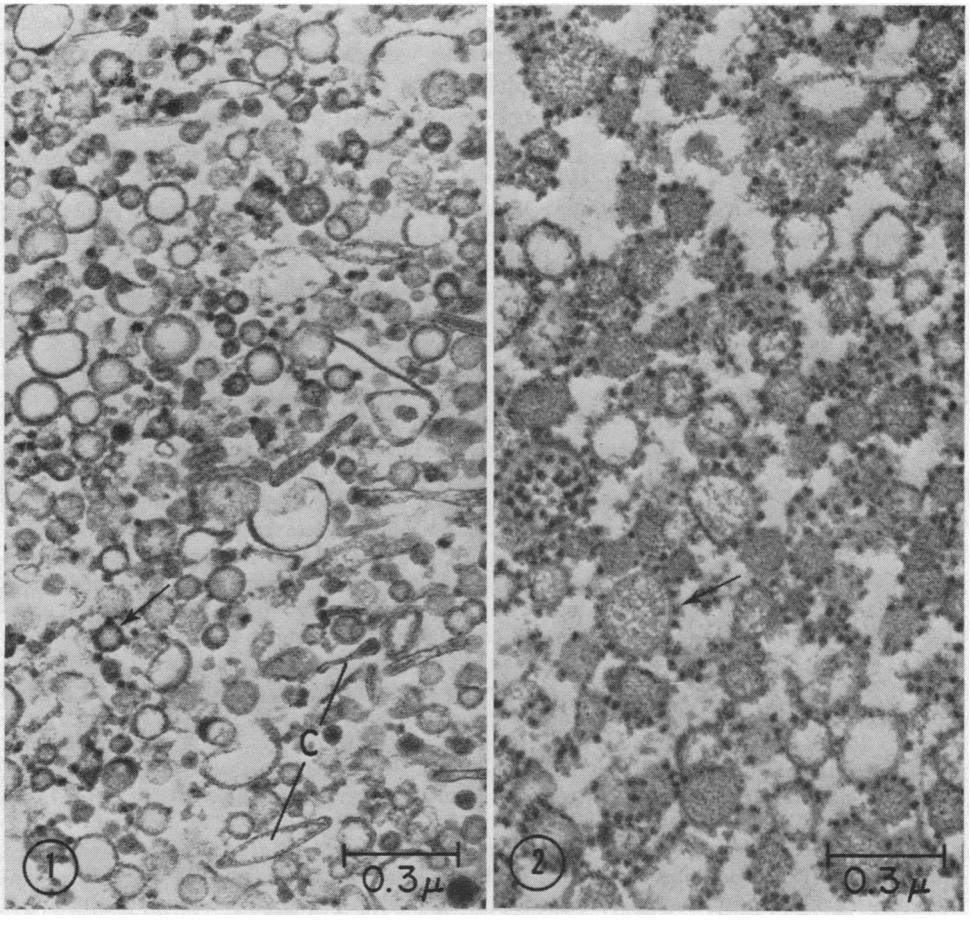

FIG. 1.—Smooth-surfaced microsomes. Rough-surfaced contaminant indicated by arrow. *c*, Golgi cisternae. ×50,000.

FIG. 2.—Rough-surfaced microsomes. Arrow indicates attached ribosomes. ×50,000.

Kinetics of intracellular transfer of secretory proteins: (a) *Small vesicles of the Golgi complex:* To study the transfer of secretory proteins from RER to condensing vacuoles, we isolated smooth microsomes as described. They have an RNA/protein ratio of 0.09 and consist primarily (Fig. 1) of small smooth-surfaced vesicles and flattened cisternae with minimal contamination by rough microsomes. Since in the exocrine pancreatic cell the majority of smooth vesicles and cisternae are located at the periphery of the Golgi complex, we assume that smooth microsomes are derived from this source.[10] The rough microsomes (Fig. 2), with an RNA/protein ratio of 0.3–0.4, are a homogeneous fraction of ribosome-studded vesicles derived from fragmented cisternae of the RER.

To determine the kinetics of labeling of proteins in microsomal fractions, 3 sets of slices were pulse-labeled for 3 min with C^{14}-leucine; from one set, rough and smooth microsomes were immediately isolated, while from the remaining two sets the same fractions were prepared after further incubation in chase medium for 17 and 57 min, respectively. The results are summarized in Figure 3. At 3 min

the specific activity (SA) of proteins of rough microsomes was 2.8× that of the smooth, whereas by 20 min the SA of smooth microsomes was 1.9× that of the rough. By 60 min, the specific activities of both rough and smooth microsomes were decreasing in parallel with that of the total microsomal fraction. Electron microscopic autoradiographs of the smooth microsomal fraction showed that in the 20-min sample 70 per cent of the silver grains appeared over smooth-surfaced vesicles and that there was no preferential association of the autoradiographic grains with contaminant rough-surfaced microsomes. The findings provide direct evidence that the small vesicles at the periphery of the Golgi complex are indeed the structures through which secretory proteins pass in transit from the RER to the condensing vacuoles centrally located in the complex. The SA of the postmicrosomal supernate (which reflects the SA of soluble proteins in the cytoplasmic matrix) remains constant throughout incubation (cf. Fig. 4), indicating negligible equilibration between the labeled proteins of particulate fractions and those of the postmicrosomal supernate (cell sap).

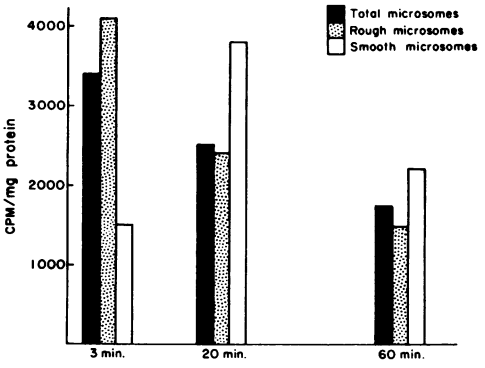

FIG. 3.—Kinetics of labeling of rough and smooth microsomes following pulse labeling (= 3 min) and incubation in chase medium. *Solid bar* is the specific activity of unfractionated microsomes.

(b) *Condensing vacuoles and zymogen granules:* We have also investigated the kinetics of labeling of the zymogen granule fraction, since these granules are the final storage site for digestive enzymes.[11] Electron microscopy of pellets indicated that the zymogen granule fraction consisted mainly of zymogen granules and condensing vacuoles (95:5, respectively), with a small contamination by mitochondria and rough microsomes. As in intact cells, condensing vacuoles are identified in pellets by their irregular shape and by the low density of their content.

Pulse labeling and cell fractionation were performed as before. At 3 min (Fig. 4), the zymogen granule fraction contains negligible radioactivity; its proteins become $^1/_2$ maximally labeled at ~20 min, and maximally labeled between 40 and 60 min incubation in chase medium. The SA curve of the microsomal fraction declines rapidly after the pulse and crosses that of the zymogen granule fraction at ~25 min. Stability of the pulse-labeled proteins of the slices is indicated by the unchanging SA of the total homogenate proteins. After 20 min, labeled proteins begin to appear in small amounts in the incubation medium.

According to these cell fractionation data (cf. also ref. 12), zymogen granules apparently accumulate labeled secretory protein within 20 min. Electron microscopic autoradiography, however, indicates that the content of zymogen granules is not labeled until ~1 hr after the injection of tracer.[3] This large discrepancy could be explained by assuming that the early labeling of the zymogen fraction was due to the presence of highly labeled condensing vacuoles. The assumption was tested by electron microscopic autoradiography of thin sections of zymogen granule pellets isolated from slices pulse-labeled with H^3-leucine and incubated in

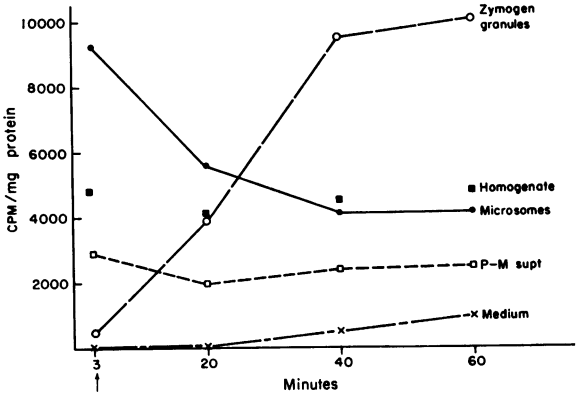

FIG. 4.—Kinetics of labeling of zymogen granule fraction in relation to other cell fractions. Arrow indicates the end of the pulse.

chase medium up to 20 and 40 min. The results clearly showed that the autoradiographic grains were mainly concentrated over condensing vacuoles (Fig. 5). Counts of labeled zymogen granules and condensing vacuoles in the zymogen granule pellet (Table 1) showed that neither component was labeled at the end of the pulse (when the SA of microsomal proteins is $\sim 10^6$ cpm/mg protein), whereas the number of labeled condensing vacuoles doubled between 20 and 40 min. At both 20 and 40 min, the majority of labeled structures were condensing vacuoles, although by 40 min a small but significant number of zymogen granules was labeled. Contaminating mitochondria and rough microsomes were not labeled. These data support our assumption that early labeling of the zymogen granule fraction is accounted for by the presence of labeled condensing vacuoles in the fraction.

(c) *Correlation of cell fractionation data with events occurring in the cell:* Transfer of pulse-labeled secretory proteins through the exocrine cell was also investigated by electron microscopic autoradiography of thin sections prepared from whole slices. The protocol of pulse labeling with H^3-leucine was identical to that used in (*b*) above. At 3 min, the majority of autoradiographic grains were located over the RER. By 20 min, most of the grains were found over the region of the Golgi complex containing small smooth-surfaced vesicles, providing further evidence that the smooth microsomes, which show a marked increase in the SA of their proteins at 20 min, contain the small vesicles of the Golgi complex. By 40 min the majority of grains were heavily concentrated in condensing vacuoles, thus confirming the kinetics of labeling of these structures in the zymogen granule fraction.

Discussion.—Previous cell fractionation studies of the secretory cycle of the exocrine pancreas have shown that radioactive amino acids are initially incorporated into the secretory proteins of the microsomal fraction, more precisely into the secretory proteins of attached ribosomes[1]; later on, labeled secretory proteins appear in other cell fractions, notably zymogen granules.[1] These studies suggested that secretory proteins were synthesized in the RER and transferred, perhaps through the cisternal spaces,[13] to zymogen granules, but other interpretations were not excluded by the data. Caro and Palade[3] demonstrated by electron microscopic autoradiography that secretory proteins synthesized in the RER pass through the periphery of the Golgi region in transit to the condensing vacuoles of the Golgi complex where they are intensively concentrated, the vacuoles becoming zymogen granules in the process. The periphery of the Golgi region is characterized by the presence of transitional ER elements (part rough and part

TABLE 1

LABELING OF CONDENSING VACUOLES IN ZYMOGEN GRANULE FRACTION

	Pulse and Time in Chase Medium	
	3 min + 17 min	3 min + 37 min
% Condensing vacuoles labeled	7.3%	14.0%
% Zymogen granules labeled	0.15%	0.53%
Labeled condensing vacuoles as % of labeled (zymogen granules + condensing vacuoles)	73%	65%

smooth) and of clusters of smooth-surfaced vesicles. Because of limited autoradiographic resolution, proof that these vesicles function in the transport of secretory proteins from RER to condensing vacuoles could not be obtained. Evidence implicating the general area of the Golgi complex in intracellular transport and zymogen granule formation was also obtained by Warshawsky *et al.*[14] and by van Heyningen,[15] but alternative views of this part of the secretory process have been proposed. For instance, Laird and Barton,[16] Morris and Dickman,[17] and Redman and Hokin[18] have suggested that secretory proteins leave the microsomes and pass, in soluble form, through the cytoplasmic matrix directly into zymogen granules or the glandular lumen, while Sjöstrand[19] assumed that the smooth-surfaced elements of the Golgi complex function in the segregation as well as the synthesis of secretory proteins.

The work reported here has been carried out on an *in vitro* system of guinea pig pancreatic slices which meets two necessary requirements for following the kinetics of rapid transfer of protein from one cell compartment to another: (1) true pulse labeling of secretory proteins and (2) complete cell fractionation, including isolation of, or accounting for, elements of the Golgi complex (small vesicles and condensing vacuoles). In previous cell fractionation studies, these elements were either lost or unidentified.

We have isolated a fraction of smooth microsomes which consists mainly of

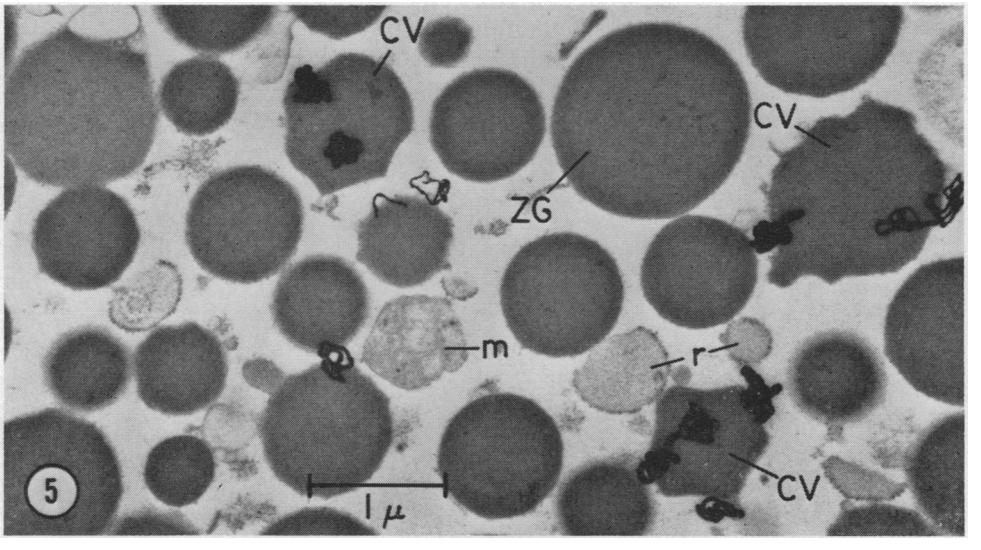

FIG. 5.—Portion of zymogen granule fraction from slices pulse-labeled with H^3-leucine and incubated 40 min in chase. Autoradiographic grains overlie condensing vacuoles (*CV*). *ZG*, zymogen granules; *r*, rough microsomes; *m*, mitochondrion. ×19,000.

peripheral vesicles and fragmented cisternae of the Golgi complex. From the kinetics of passage of labeled proteins into this fraction, we conclude that the secretory proteins pass within the small vesicles of the Golgi complex in transit from their site of synthesis and segregation in the RER to condensing vacuoles. Autoradiographic studies[3] had already implicated the periphery of the Golgi region in transfer; our experiments give direct evidence that definite structures present in this region, i.e., the small, smooth-surfaced vesicles, are involved in this operation. Our results also rule out the assumption that newly synthesized proteins move in soluble form through the cell sap (cytoplasmic matrix) on their way to zymogen granules.

In interpreting our data, we have assumed that the label identifies newly synthesized secretory proteins in all compartments examined, including smooth microsomes. The assumption is justified by the data of Siekevitz and Palade[1] who showed that labeling of secretory proteins is 10–20 times higher than that of proteins retained in the exocrine cell. The assumption is also supported by our autoradiographic data which show that most of the label moves as a single wave from one cell compartment to another.

The mechanism whereby the small vesicles of the Golgi complex transfer secretory protein remains unknown. It is clear at least that we are dealing with transport in bulk reminiscent of transport of macromolecules across vascular endothelia (Palade and Bruns[20] and Karnovsky[21]). In the pancreatic cell, however, this transport in bulk takes place between two intracellular compartments (RER and condensing vacuoles).

In the past, cell fractionation data[12] and autoradiographic observations[3] were not in agreement concerning the time secretory proteins need to reach the zymogen granules. Our results explain satisfactorily this discrepancy: the early labeling of zymogen granule fractions is due to the presence in this fraction of highly labeled condensing vacuoles. In earlier cell fractionation studies, the presence of condensing vacuoles in zymogen granule fractions was not recognized. Our results, and those of Caro and Palade,[3] indicate that secretory protein is progressively accumulated within condensing vacuoles where it is concentrated, perhaps by removal of water and electrolytes. This might be accomplished by "pumps" located in the condensing vacuole membrane and operating between two cell compartments (vacuole content and cytoplasmic matrix).

Summary.—We have demonstrated by cell fractionation techniques, performed on guinea pig pancreatic slices, that the small vesicular elements located at the periphery of the Golgi complex transfer newly synthesized secretory proteins from the cisternae of the RER to the condensing vacuoles of the Golgi complex. It follows that nascent proteins, once segregated in the cisternae of the ER, remain within and are transferred through membrane-bounded compartments of the cell until their ultimate discharge from zymogen granules into the glandular lumen.

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*LINCOMYCIN, AN INHIBITOR OF AMINOACYL SRNA BINDING TO RIBOSOMES**

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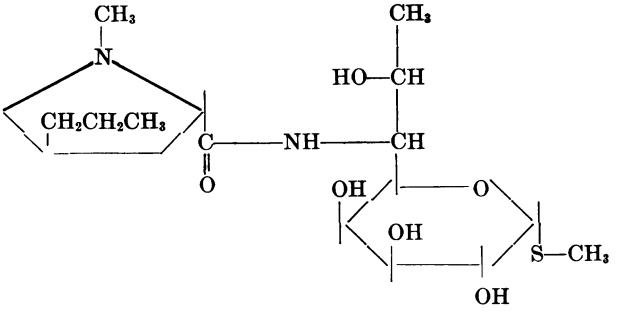
Lincomycin is an antibiotic produced by *Streptomyces lincolnensis* and it has recently been shown to possess the following chemical structure.[1]



Previous studies[2] have shown that lincomycin inhibits protein synthesis in gram-positive bacteria without interfering with DNA and RNA synthesis. This study was initiated to attempt to explain this specificity of lincomycin inhibition and to define the site of action of lincomycin within the over-all protein-synthesizing system.

For this investigation, it was necessary to develop *in vitro* protein-synthesizing systems which utilize combinations of ribosomes, hybrid ribosomes formed by the