INTRACELLULAR TRANSPORT OF SECRETORY PROTEINS IN THE PANCREATIC EXOCRINE CELL

III. Dissociation of Intracellular Transport from Protein Synthesis

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ABSTRACT

Experiments have been carried out to determine whether intracellular transport of pancreatic secretory proteins is obligatorily coupled to protein synthesis or whether it is a separable process which can be independently regulated. To this intent, guinea pig pancreatic slices were pulse labeled with leucine-^{3}H for 3 min and incubated post-pulse for 37 min in chase medium containing cycloheximide up to concentrations sufficient to inhibit protein synthesis by 98%. In controls, newly synthesized secretory proteins are transported over this interval to condensing vacuoles of the Golgi complex. Since the latter are recovered in the zymogen granule fraction upon cell fractionation, intracellular transport was assayed by measuring the amount of protein radioactivity found in the zymogen granule fraction after a (3 + 37) min incubation. The results indicated that at maximum inhibition of protein synthesis (5×10^{-4} M cycloheximide), transport proceeded with an efficiency ~80% of control. Parallel radioautographic studies on intact slices confirmed these data and further indicated that all the steps of intracellular transport, including discharge to the acinar lumen, were independent of protein synthesis. We conclude that: (*1*) transport and protein synthesis are separable processes; (*2*) intracellular transport is not the result of a continuous delivery of secretory proteins from attached polysomes to the cisternae of the rough endoplasmic reticulum; and (*3*) transport is not dependent on the synthesis of "specific" nonsecretory proteins within the time limits tested.

INTRODUCTION

Using guinea pig pancreatic slices incubated in vitro, we have previously established that newly synthesized secretory proteins are transported from the cisternae of the rough-surfaced endoplasmic reticulum (RER) to condensing vacuoles of the Golgi complex by means of small, smooth-surfaced vesicles located at the periphery of the complex (1). We have also shown that the condensing vacuoles are subsequently transformed into zymogen granules by progressive filling and concentration of their content (2).

Although the pathway and timetable of the intracellular transport of secretory proteins are well defined, the factors involved in its control are little understood. The experiments reported here were carried out to determine whether intracellu-

lar transport is obligatorily coupled to protein synthesis or whether it is a separable process which can be independently regulated. For this purpose, we have again used guinea pig pancreatic slices incubated in vitro and have examined the effects of cycloheximide, a potent inhibitor of protein synthesis (3), on the intracellular transport of pulse-labeled secretory proteins.

METHODS

General

The animals used were ~500-g male guinea pigs (The Rockefeller University colony), fasted for 24 hr with water given ad libitum. The preparation and incubation of pancreatic slices have been described in detail before (1). Conditions of pulse labeling and chase incubation are described in figure and table legends.

The cell fractionation procedure applied to the slices and the preparation of cell fractions for counting were as previously described (1) except that: following the extraction by centrifugation at 600 *g* (avg) × 10 min of a "nuclear supernate," the resulting pellet was resuspended in 0.3 M sucrose, and the suspension centrifuged at 200 *g* (avg) × 10 min. This supernate and the original 600 *g* supernate were centrifuged at 1000 *g* (avg) × 10 min in a Spinco #40.3 rotor to produce a pair of zymogen granule pellets. After their surfaces were rinsed free of loosely adhering contaminants, the two pellets were resuspended and pooled for chemical and radioactive determinations. All cell fractions were routinely precipitated with 10% trichloroacetic acid (TCA) (final concentration), washed once with cold 5% TCA, hydrolyzed for 20 min at 90° in 5% TCA, washed again with cold 5% TCA, and prepared for liquid scintillation counting without previous lipid extraction, since the latter step removes only an insignificant amount of label. Counting rates have been corrected for quenching by using the external standard of a Nuclear Chicago Mark I liquid scintillation spectrometer. The results are given as disintegrations/minute (dpm).

Procedures for electron microscopy and radioautography were as given in references 1 and 2.

Oxygen Consumption

Oxygen consumption was determined as in Umbreit et al. (4) with the use of standard Warburg manometric flasks and a Bronwill respirometer (Bronwill Scientific Co., Rochester, N.Y.). Incubations were performed with two pancreatic slices (~100 mg wet wt) per flask in 2.5 ml medium of the same composition as before (1) except that the bicarbonate buffer was replaced by Na phosphate buffer, pH 7.4. The gas phase was oxygen. Following 5 min of temperature equilibration, oxygen consumption was measured every 30 min over a 2-hr period. CO_2 was trapped in the center well with 0.1 ml of 20% KOH wetting a piece of fluted Whatman #1 filter paper, 1.7 × 3.5 cm. Under these conditions, the slices incorporate L-leucine-^{3}H into proteins at about the same rate as slices incubated in bicarbonate-buffered medium.

Materials

All chemicals used were reagent grade. Cycloheximide (Actidione) was obtained from the Nutritional Biochemical Corporation, Cleveland, Ohio. L-Leucine-4,5-^{3}H (60 or 45 c/mmole), was obtained from the New England Nuclear Corporation, Boston.

RESULTS

Effect of Cycloheximide on the Incorporation of L-Leucine-^{3}H into Protein

For determining the effect of cycloheximide on the incorporation of amino acids into protein, sets of slices were incubated for 2 hr in media containing L-leucine-^{3}H and concentrations of cycloheximide from 10^{-6} to 10^{-3} M, and the amount of radioactivity incorporated into total protein was measured. Doses of cycloheximide between 10^{-4} and 10^{-3} M inhibited protein synthesis by 95 and 98%, respectively (Fig. 1), but despite the extent of the inhibition, the tissue remained viable as indicated by the fact that the rate of oxygen consumption remained at ~75% of the control value at maximum inhibition of protein synthesis (Fig. 1). Moreover, cycloheximide inhibition was readily reversible, protein synthesis returning to near control values after a washout period of 30 min[1] even after exposure to 5×10^{-4} M cycloheximide for 60 min (Table I). Finally, and of particular relevance to the interpretation of cell fractionation and radioautographic studies reported below, tissue incubated for 60 min with 5×10^{-4} M cycloheximide showed no structural alterations when compared to control slices (Fig. 2; compare to Fig. 5, in reference 1).

At this same concentration, the effect of the drug is rapid; protein synthesis is more than 90% inhibited in <1.5 min (Table II). A priori, this prompt and efficient inhibition should allow a study of intracellular transport by either cell

[1] A 20-min wash reversed the effect of cycloheximide by ~50%.

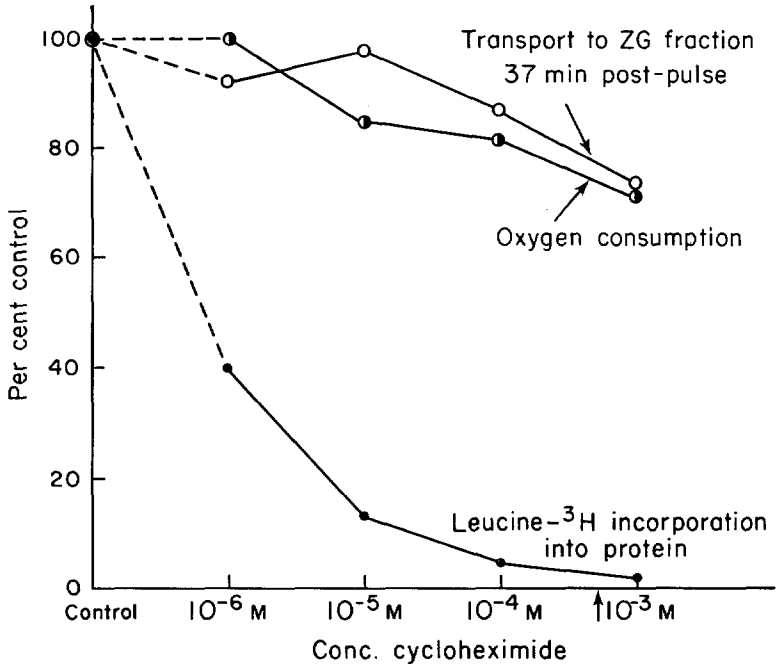

FIGURE 1 Effect of cycloheximide on: (*a*) incorporation of L-leucine-^{3}H into protein, (*b*) intracellular transport of pulse-labeled proteins to the zymogen granule fraction, and (*c*) O_2 consumption by pancreatic slices.

Sets of slices were pulse-labeled for 3 min with L-leucine-4,5-^{3}H (5 μc/ml; 45 c/mmole) and incubated post-pulse for 37 min in medium containing 2.0 mM L-leucine-^{1}H and the indicated concentrations of cycloheximide. Transport was assayed as described under results. The data are averages of five experiments.

The effects of cycloheximide on L-leucine-^{3}H incorporation into protein and on O_2 consumption were determined in separate experiments as follows: sets of slices (~100 mg wet wt/set) were incubated for 2 hr in Warburg flasks in 2.5 ml of medium (see Methods) containing 1 μc/ml L-leucine-4,5-^{3}H (70 mc/mmole). At this time, O_2 consumption and the incorporation of leucine-^{3}H into hot-TCA–precipitable proteins of the slices were measured. The data are averages of two experiments.

Arrow indicates 5×10^{-4} M cycloheximide, i.e., the concentration used in subsequent experiments.

fractionation or radioautography, provided transport is not severely affected by the drug.

Effect of Cycloheximide on Intracellular Transport

CELL FRACTIONATION STUDIES: Using pancreatic slices labeled in vitro, we have previously shown by radioautography (2), that after 3 min pulse-labeling with leucine-^{3}H followed by 37 min of incubation in chase medium, ~49% of the incorporated label is transported to the condensing vacuoles of the Golgi complex. We have also shown that these condensing vacuoles are recovered in the zymogen granule fraction isolated from such slices and account for the maximal labeling reached by this fraction after (3 + 37) min[2] in vitro incubation. Radioautographic studies (to be discussed later) demonstrate that labeled proteins are also heavily concentrated in condensing vacuoles in slices pulse-labeled for 3 min and incubated post-pulse for 37 min in a chase medium with a concentration of cycloheximide sufficient to inhibit maximally protein synthesis. We can expect that in this case, as in controls, labeled condensing vacuoles will be recovered upon cell fractionation in the zymogen granule fraction and account for the radioactive protein found in this fraction at the time mentioned.

[2] Notations such as (3 + 37) min refer to 3-min pulse labeling with L-leucine-^{3}H, followed by 37-min chase incubation. +37 min refers to 37-min incubation in chase medium.

TABLE I

Reversibility of Cycloheximide Inhibition

Preincubation			Wash	Incubation		dpm/mg protein*
Time	Leucine-^{3}H	Cyclohex.		Time	Leucine-^{3}H	
	mM	*M*	*min*	*min*	*μc/ml*	
60	0.4	—	30	20	5	45,000
60	0.4	5×10^{-4}	—	20	5	2,700
60	0.4	5×10^{-4}	30	20	5	38,700
20	0.4	—	30	20	5	32,900
20	0.4	5×10^{-4}	30	20	5	30,600

Sets of slices (~100 mg wet wt), preincubated at 37° for the times given in medium containing L-leucine-^{3}H with or without cycloheximide, were washed for 30 min in drug-free medium (except for the set on line 2) and then incubated for 20 min in medium containing L-leucine-^{3}H (5 μc/ml; 12.3 mc/mmole) without cycloheximide.
* Total slice protein.

These findings can be used to devise an assay for intracellular transport in which the end point is represented by the amount of label recovered in the zymogen granule fraction after (3 + 37) min incubation. Using this assay, we have investigated the effect of cycloheximide on intracellular transport as follows:

Sets of slices (10 slices, ~400 mg wet wt of tissue/set) pooled from the pancreata of two animals were pulse labeled for 3 min with leucine-^{3}H, washed with warm isotope-free medium, and transferred to chase medium which contained 2.0 mM leucine-^{1}H, either alone or in the presence of increasing concentrations of cycloheximide (10^{-6}–10^{-3} M). After 37 min of incubation in the chase medium, the slices were recovered and used for preparing zymogen granule fractions in which the amount of TCA-precipitable radioactivity was determined. The results, expressed as per cent of the control values (no cycloheximide) are, in effect, a measure of the efficiency of transport of secretory proteins from the RER to condensing vacuoles.

The averaged data from five such experiments are shown in Fig. 1 in which the amount of label transported in the absence of cycloheximide gave a specific radioactivity of 70,000 dpm/mg protein in the zymogen granule fraction. Between 10^{-6} and 10^{-5} M cycloheximide, transport is unaffected though at the latter dose protein synthesis is only ~15% of control. At 10^{-4} M, and especially at 10^{-3} M, the efficiency of transport is somewhat less than control (~75% at the largest dose used), while protein synthesis is virtually absent (between 4 and 2% of control). It should be noted that transport and oxygen consumption decrease in parallel with increasing concentrations of cycloheximide. Probable relations between the two effects will be discussed in the following paper. In any case, the results show clearly that synthesis and intracellular transport of secretory proteins are not obligatorily coupled processes.

Although not shown here, similar data were obtained with puromycin, a drug which inhibits proteins synthesis (5) by a mechanism different from that of cycloheximide (3). However, at best (10^{-3} M) puromycin inhibits protein synthesis only by 85%, depresses respiration by ~50%, and, as a consequence, allows transport to proceed with an efficiency of about half that of controls.

RADIOAUTOGRAPHIC STUDIES: As an independent check of the cell fractionation data presented above, electron microscopic radioautography was performed on intact cells from slices pulse labeled with leucine-^{3}H and incubated post-pulse either in chase medium containing 4.0 mM leucine-^{1}H or the same medium containing 5×10^{-4} M cycloheximide. At this dose, protein synthesis is inhibited by ~90% within <1.5 min of drug exposure, although both transport and oxygen consumption are less affected than with 10^{-3} M. Table III shows that in the cells of cycloheximide-treated slices, as in those of controls, radioautographic grains appear at (3 + 7) min over the clusters of small vesicles at the periphery of the Golgi complex; at (3 + 17) min over condensing vacuoles where they become markedly concentrated by (3 + 37) min (Fig. 3); and at (3 + 57) min over zymogen granules and—in small but

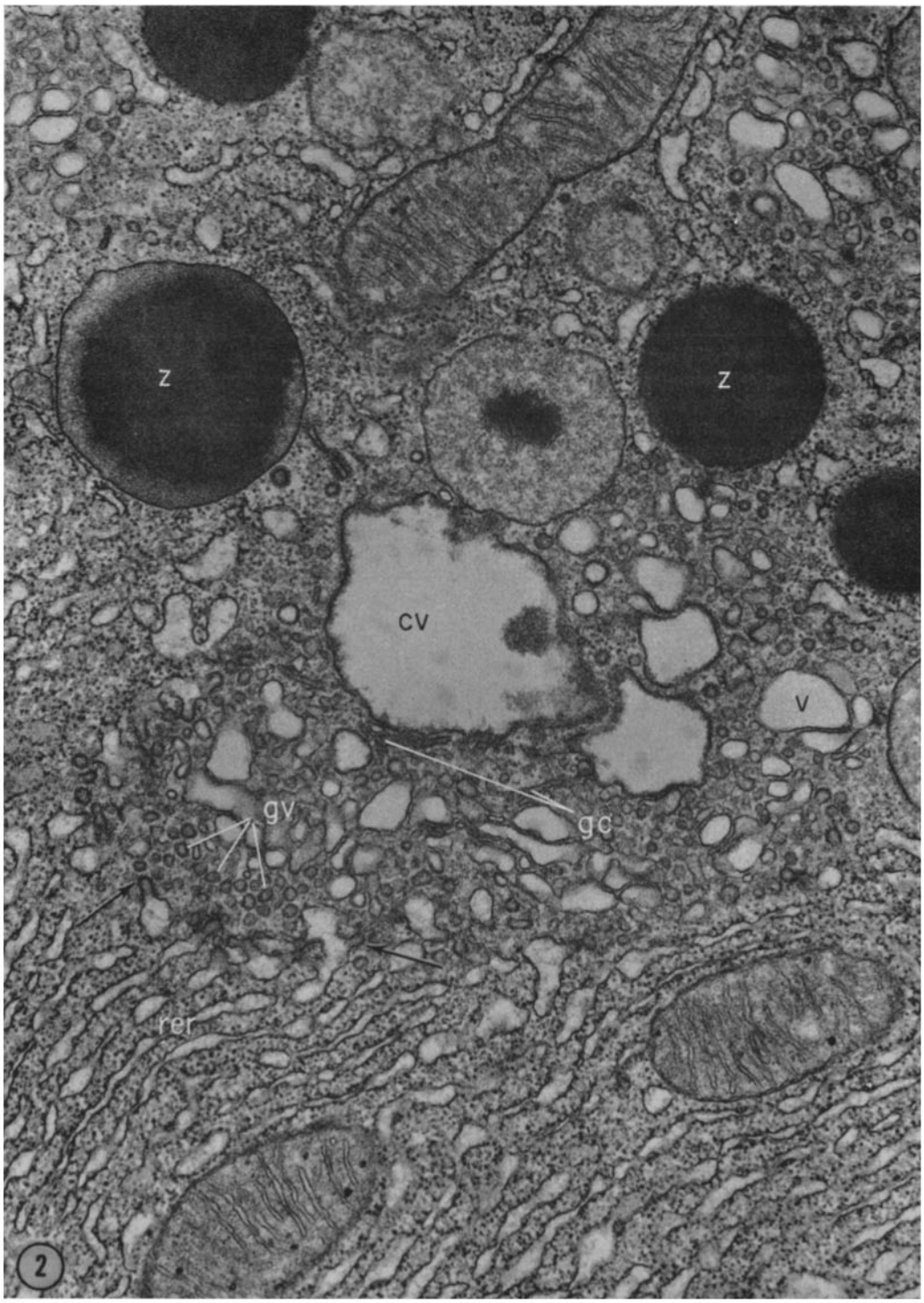

FIGURE 2 Electron micrograph of a pancreatic exocrine cell from a slice incubated for 60 min with 5×10^{-4} M cycloheximide. The section passes through the centrally located Golgi region of the cell. Streamers of RER cisternae (*rer*) approach the periphery of the Golgi complex where they terminate as part rough- part smooth-surfaced transitional elements of the ER (arrows). The Golgi periphery contains numerous smooth-surfaced vesicles (*gv*) and a smaller number of vacuoles (*v*) and cisternae (*gc*). A condensing vacuole with a small amount of content is indicated (*cv*) in the center of the complex. *z*, zymogen granule. × 35,000.

TABLE II

Kinetics of Cycloheximide Inhibition

Incubation time at 37°	dpm/mg protein*	
	Control	Cycloheximide
min		*5×10^{-4} M*
1.5	115	10
4.0	710	20
9.0	1245	35

Sets of slices (~100 mg wet wt) were incubated at 4° for 10 min in medium containing 1 μc/ml of leucine-^{3}H, 2.5 mc/mmole; at this temperature, the label diffuses into the tissue, but is not incorporated into protein (1). Cycloheximide was then added to a concentration of 5×10^{-4} M and the slices were incubated at 37° for the indicated times.
* Total slice protein.

significant numbers—over acinar lumina.[3] Transport appears to progress less efficiently with time so that at (3 + 57) min the appearance of the label in zymogen granules is delayed by ~20 min. These radioautographic data thus confirm the cell fractionation studies reported above and, in addition, indicate that the cell can complete the intracellular transport of secretory proteins from the RER to zymogen granules and the acinar lumen in the absence of protein synthesis.

Table III also shows that in cycloheximide treated as in control slices, there is a rapid loss of radioautographic grains from over elements of the RER during +7 and +17 min of incubation. Thereafter, the rates of exit of labeled proteins from this cell compartment diverge: at +57 min ~37% of the radioautographic grains remain over the RER in cycloheximide-treated slices as compared to ~16% in the control. It is evident, however, that labeled proteins are lost progressively from this compartment at least during +17 min; hence, we are not simply observing the transport of labeled proteins which have left the RER cisternae during the short time (~1.5 min) taken for the drug to act.

From the known mode of action of the drug (3), it could be predicted that the excess label at +37 and +57 min over the RER is due to incomplete peptides unreleased from ribosomes attached to RER membranes. As a test of this possibility, two sets of slices were pulse labeled with leucine-^{3}H and incubated post-pulse for 37 min in chase medium with and without 5×10^{-4} M cycloheximide. Total microsomal fractions, isolated from the two sets and resuspended in 0.3 M sucrose, were treated with Na deoxycholate (DOC) in 0.5% final concentration so as to solubilize the microsomal membranes. From each treated suspension, a DOC-soluble supernate and a DOC-insoluble pellet consisting mainly of detached ribosomes (6) were separated by centrifugation. The specific radioactivities of proteins in the DOC-soluble fractions were identical in the two cases whereas the specific radioactivity of the hot TCA-insoluble fraction of the pellet was about two times higher in the case of cycloheximide-treated than in that of control slices. This finding supports the assumption that in the radioautographs of treated slices the excess label over the RER elements is mostly due to labeled peptides unreleased from attached ribosomes. The label remaining over this region of the cell in control slices during similar times of incubation may represent nonexportable proteins with a long half-life, as has already been suggested (see 7, 8).

DISCUSSION

Within the time covered by the present experiments, i.e., up to 60 min incubation with cycloheximide, the pancreatic exocrine cell of the guinea pig is able to complete the entire sequence of events involved in the intracellular transport of secretory proteins in the absence of further protein synthesis. Our previous studies have shown that at the end of a 3-min pulse labeling, a large proportion of the labeled proteins has already entered the pool of transportable proteins in the cavities of the RER from which it can be extracted by mild alkaline buffer (1). Under normal conditions, this pool is maintained in a steady state by concomitant synthesis and delivery from attached polysomes and by drainage to the Golgi complex (2). In our experimental conditions, delivery from polysomes practically ceases and the pool is progressively drained to the condensing vacuoles. Apparently, it is large enough at the onset of cycloheximide inhibition to support transport to the next compartment over the interval tested. Each step of the transport sequence is, however, somewhat retarded in relation to its control; this is most likely related to an inhibitory effect of the

[3] In experiments not reported here, the stimulation of discharge of secretory proteins to the incubation medium by carbamylcholine or pancreozymin is unaffected by cycloheximide.

TABLE III

Distribution of Radioautographic Grains in Pancreatic Exocrine Cells Incubated Post-Pulse with Cycloheximide

Subcellular components	% of radioautographic grains				
		Incubation in chase medium*			
	Pulse 3 min	+7 min	+17 min	+37 min	+57 min
Rough endoplasmic reticulum	89.1	45.9	38.4	24.5	16.2
		50.3	**39.6**	**38.6**	**37.1**
Golgi complex					
Peripheral region	5.0	45.2	38.3	15.0	11.1
		34.9	**23.5**	**19.7**	**20.4**
Condensing vacuoles	1.0	4.0	19.9	49.0	36.2
		6.2	**29.9**	**35.3**	**19.9**
Zymogen granules	4.4	4.8	3.2	11.4	33.2
		7.8	**6.9**	**6.4**	**19.9**
Acinar lumen	0.2	0	0	0	2.9
		0.4	**0**	**0.5**	**3.1**
No. of grains counted	992	1146	587	577	960
		684	**359**	**823**	**405**

Sets of pancreatic slices were pulse labeled for 3 min in medium containing 200 μc/ml L-leucine-4,5-^{3}H (60 c/mmole). One set was immediately fixed (3 min pulse) while the others were incubated for the indicated times in chase medium containing 4.0 mM L-leucine-^{1}H with or without 5×10^{-4} M cycloheximide.

* Numbers in lightfaced type refer to slices incubated in control chase medium; boldfaced numbers beneath refer to slices incubated with 5×10^{-4} M cycloheximide. Data are the averages of two experiments.

Grains over nuclei and mitochondria are not included as they represent a small and variable contribution to the labeling of cytoplasmic structures (range 0–4%). For explanation, see reference 2.

drug (~25%) on the respiration of the tissue as will be seen in the next paper. Similar findings have been obtained by Morimoto et al. (9) following the administration of cycloheximide to guinea pigs in vivo.

Our results indicate that transport is not simply the result of the continued synthesis and segregation of secretory proteins into the RER cisternae which, by mass action, moves the secretory proteins to the next cell compartment. Since the maintenance of a steady state concentration gradient within the RER cisternae is not necessary for intracellular transport to proceed, other mechanisms have to be envisaged. They should take into account the fact that the RER compartment is drained centripetally and from one end only (the transitional elements located at the periphery of the Golgi complex) through a lock which continues to operate in the absence of protein synthesis.

Our results also show that the transport of secretory proteins is not dependent on the continued synthesis of other "specific," nonsecretory proteins (e.g., membrane proteins, enzymes involved in catalyzing transport). If such proteins are involved, then their pool is large enough or their life span long enough to allow transport to proceed for at least 60 min. Longer times of incubation in cycloheximide than those so far used may be needed to test the assumption that such proteins are involved.

Since intracellular transport can now be uncoupled from protein synthesis, it becomes possible to determine independently its energy requirements by the use of well known metabolic in-

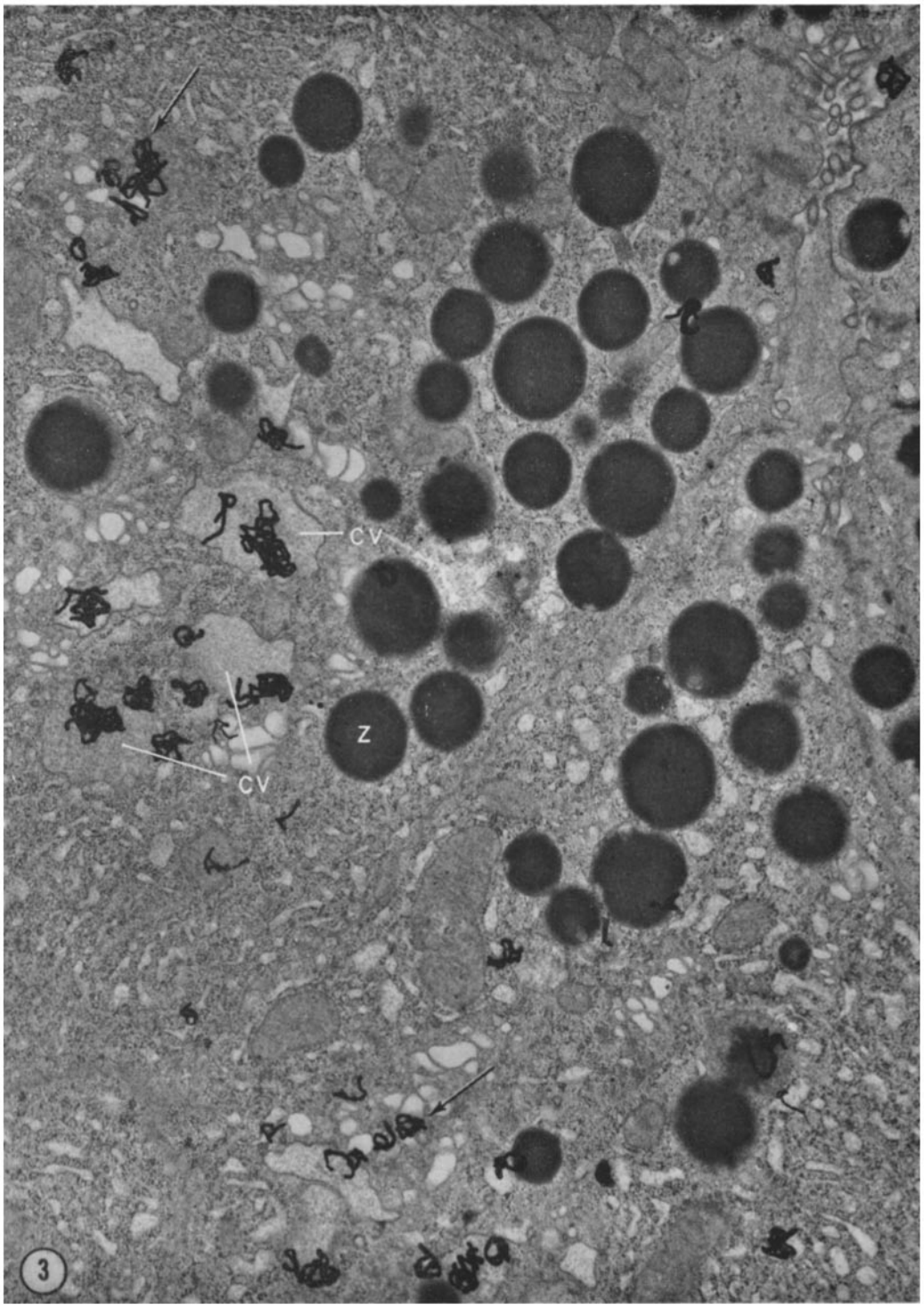

FIGURE 3 Electron microscopic radioautograph of a thin section from a pancreatic slice pulse labeled for 3 min with L-leucine-4,5-^{3}H (200 μc/ml; 60 c/mmole) and incubated post-pulse for 37 min in chase medium containing 4.0 mM L-leucine-^{1}H and 5×10^{-4} M cycloheximide. Radioautographic grains mainly mark condensing vacuoles (*cv*) of the Golgi complex, although some label remains over peripheral elements of the complex (arrows), and over the RER. z, zymogen granule. × 19,000.

hibitors. This aspect is dealt with in the companion paper.

We wish to express our thanks to Miss Maryann Dickey for her excellent technical assistance.

This investigation was supported by United States Public Health Service Research Grant No. AM-10928 from the National Institute of Arthritis and Metabolic Diseases.

Received for publication 31 May 1968, and in revised form 31 July 1968.

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