INTRACELLULAR TRANSPORT OF SECRETORY PROTEINS IN THE PANCREATIC EXOCRINE CELL

IV. Metabolic Requirements

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ABSTRACT

Since in the pancreatic exocrine cell synthesis and intracellular transport of secretory proteins can be uncoupled (1), it is possible to examine separately the metabolic requirements of the latter process. 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 5×10^{-4} M cycloheximide and inhibitors of glycolysis, respiration, or oxidative phosphorylation. In each case, the effect on transport was assessed by measuring the amount of labeled secretory proteins found in zymogen granule fractions isolated from the corresponding slices. This assay is actually a measure of the efficiency of transport of secretory proteins from the cisternae of the rough endoplasmic reticulum (RER) to the condensing vacuoles of the Golgi complex which are recovered in the zymogen granule fraction (16). The results indicate that transport is insensitive to glycolytic inhibitors (fluoride, iodoacetate) but is blocked by respiratory inhibitors (N_2, cyanide, Antimycin A) and by inhibitors of oxidative phosphorylation (dinitrophenol, oligomycin). Except for Antimycin A, the effect is reversible. Parallel radioautographic studies and cell fractionation procedures applied to microsomal subfractions have indicated that the energy-dependent step is located between the transitional elements of the RER and the small, smooth-surfaced vesicles at the periphery of the Golgi complex. Radiorespirometric data indicate that the substrates oxidized to support transport are endogenous long-chain fatty acids.

INTRODUCTION

In the preceding paper (1), we have demonstrated that, in the pancreatic exocrine cell of the guinea pig, the intracellular transport of secretory proteins from the RER[1] cisternae to the condensing vacuoles of the Golgi complex can be uncoupled from protein synthesis. In the uncoupled state, it is possible to investigate whether this type of transport is dependent on metabolic energy and if so, to determine precisely the source of energy used and the site at which it is required.

[1] Abbreviations: IA, iodoacetate; DNP, 2,4-dinitrophenol; ATP, adenosine triphosphate; DOC, sodium deoxycholate; RER, rough-surfaced endoplasmic reticulum. (3 + 37) min indicates a 3-min pulse labeling followed by a 37-min incubation in chase medium; +37 min indicates a 37-min post-pulse incubation in chase medium. Proximal and distal refer to the attached ribosome–acinar lumen pathway of movement of secretory proteins in the exocrine cell.

METHODS

General

Procedures for the preparation of guinea pig pancreatic slices, and for their in vitro incubation, pulse labeling, and cell fractionation are given in reference 1.

Assay for Transport Efficiency

The assay for measuring transport efficiency has been described in reference 1; it determines the specific radioactivity of the proteins of zymogen granule fractions isolated from treated pancreatic slices after a standard 3 min pulse labeling with leucine-^{3}H followed by 37 min incubation in chase medium, and gives the results as per cent of the corresponding value for control (untreated) slices. The assay covers transport from the RER cisternae to the condensing vacuoles of the Golgi complex, i.e., the compartment normally reached by secretory proteins within $(3 + 37)^1$ min. Hereafter, for reasons of convenience, "intracellular transport" will be used to designate movement over this segment of the over-all pathway followed by secretory proteins from attached polysomes to acinar lumina.

Cycloheximide (5×10^{-4} M) was routinely introduced in the chase medium so as to dissociate transport from protein synthesis and to inhibit the latter strongly (~97%) and uniformly in all specimens. Most of the agents that affect transport also inhibit protein synthesis to a varied extent, dependent on agent and dose; hence, without cycloheximide, transport would be studied against a highly variable background of protein synthesis which may affect the findings. Similar results, however, have been obtained in the absence of cycloheximide (data not shown).

The conditions and agents tested were applied to the assays immediately after pulse labeling and were present throughout chase incubation. Further details are given in the legends of figures and tables.

Oxygen Consumption and $^{14}CO_2$ Production

The oxygen consumption of pancreatic slices was measured as in reference 1, and their respiratory quotients were determined by the method outlined in Umbreit et al. (2, see also 3).

$^{14}CO_2$ production from palmitate-1-^{14}C was measured as follows. An aliquot of a complex of labeled palmitate and bovine serum albumin (20%, 2X recrystallized) prepared according to Stein and Stein (4) was added to a Warburg flask containing two pancreatic slices (~100 mg wet wt) in 2.5 ml of

TABLE I

Effect of Temperature and Glycolytic Inhibitors on Intracellular Transport

Post-pulse incubation conditions	Gas phase	Additions	Reincubation conditions	Relative specific activity	Specific activity
		M/liter		%	*dpm/mg protein*
37 min, 37°	O_2	—	—	100.0	80,000
37 min, 27°	O_2	—	—	26.3	
37 min, 17°	O_2	—	—	7.5	
37 min, 4°	O_2	—	—	1.1	
37 min, 4°	O_2	—	37 min, 37°	57.0	
37 min, 37°	O_2	—	—	100.0	45,000
37 min, 37°	O_2	F^- 10^{-3}	—	106.0	
37 min, 37°	O_2	F^- 10^{-2}	—	99.0	
37 min, 37°	O_2	IA 10^{-4}	—	132.0	
37 min, 37°	O_2	IA 10^{-3}	—	53.0	

Sets of slices, pulse-labeled for 3 min at 37° with 5 μc/ml L-leucine-4,5-^{3}H (45 c/mmole), were transferred to chase medium containing 2 mM L-leucine-^{1}H, 5×10^{-4} M cycloheximide, and the indicated additions. They were incubated post-pulse for the time (usually +37 min), at the temperature, and with the gas phase specified. For testing reversibility of transport inhibition, some sets were reincubated under the indicated conditions. At the end of incubation or reincubation, each set of slices was homogenized and fractionated to give zymogen granule fractions as described (1). The specific radioactivity of the proteins in each fraction was determined and is expressed as per cent of the corresponding value for control slices (relative specific activity).

Krebs-Ringer phosphate medium (final concentrations: 19 μM palmitate (54 mc/mmole), 0.8% albumin). The assay was incubated for 2 hr during which time the oxygen consumed was measured manometrically and the $^{14}CO_2$ produced was trapped quantitatively in the center well of the flask with 0.1 ml of 20% KOH wetting a piece of fluted Whatman #1 filter paper 1.7 × 3.5 cm. After incubation, the filter paper was counted directly in Bray's fluid (5) with a Nuclear Chicago Mark I liquid scintillation spectrometer. The efficiency of $^{14}CO_2$ trapping was tested with $^{14}CO_2$ liberated from known amounts of $Na_2\ ^{14}CO_3$ by acid tipped into the flask from the side arm (6). Trapping was quantitative up to the equivalent of 500 μl CO_2 which corresponds to ~5× the CO_2 amount usually produced over 2 hr by a pair of pancreatic slices. Over-all counting efficiency for $^{14}CO_2$ was 46%; counting rates have been corrected to disintegrations/min (dpm). Since at the end of the incubation no further $^{14}CO_2$ was liberated from the medium by acid added from the side arm, we assumed that $^{14}CO_2$ trapping was instantaneous and complete. Hence, acid was not routinely added.

$^{14}CO_2$ produced from glucose-1-^{14}C and glucose-6-^{14}C by pancreatic slices was trapped by the same procedure.

Materials

L-leucine-4,5-^{3}H (45 or 60 c/mmole), palmitate-1-^{14}C (54 mc/mmole), D-glucose-1-^{14}C (10 mc/mmole), D-glucose-6-^{14}C (7.05 mc/mmole), and $Na_2\ ^{14}CO_3$ (20.2 mc/mmole) were obtained from the New England Nuclear Corporation, Boston. Antimycin A was purchased from Mann Research Laboratories, New York. All other chemicals used were of analytical grade.

RESULTS

Effect of Incubation Conditions and of Metabolic Inhibitors on Intracellular Transport of Secretory Proteins. Cell Fractionation Studies

The experiments described in Tables I and II and Fig. 1 lead to the following findings: Intracellular transport is reversibly inhibited by low

TABLE II

Effect of Nitrogen, Cyanide, and 2,4-Dinitrophenol on Intracellular Transport

Post-pulse incubation conditions	Gas phase	Additions	Reincubation conditions	Relative specific activity	Specific activity
		M/liter		%	*dpm/mg protein*
37 min, 37°	O_2	—	—	100	80,000
37 min, 37°	N_2	—	—	12	
17 min, 37°	N_2	—	37 min, 37° O_2	80	
37 min, 37°	N_2	—	37 min, 37° O_2	70	
37 min, 37°	O_2	—	—	100	90,000
37 min, 37°	O_2	CN^- 5×10^{-5}	—	98	
37 min, 37°	O_2	CN^- 1×10^{-4}	—	90	
37 min, 37°	O_2	CN^- 4×10^{-4}	—	28	
37 min, 37°	O_2	CN^- 5×10^{-4}	—	22	
37 min, 37°	O_2	CN^- 7×10^{-4}	—	10	
37 min, 37°	O_2	CN^- 1×10^{-3}	—	2	
37 min, 37°	O_2	CN^- 5×10^{-4}	37 min, 37° no CN^-	111	
37 min, 37°	O_2	—	—	100	47,000
37 min, 37°	O_2	DNP 1×10^{-5}	—	116	
37 min, 37°	O_2	DNP 1×10^{-4}	—	67	
37 min, 37°	O_2	DNP 5×10^{-4}	—	20	
37 min, 37°	O_2	DNP 1×10^{-3}	—	10	
37 min, 37	O_2	DNP 5×10^{-4}	37 min, 37° no DNP	80	

See Table I for experimental details.

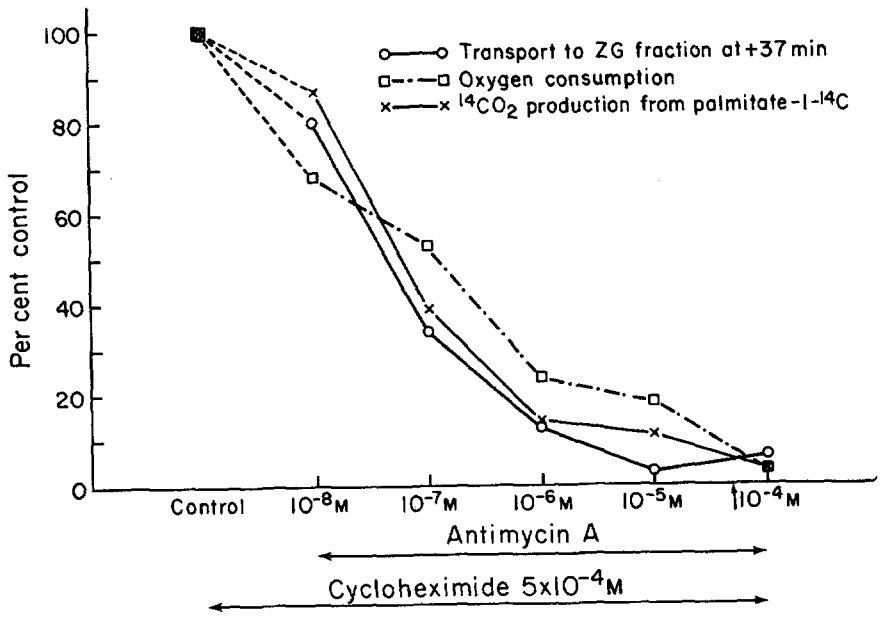

FIGURE 1 Intracellular transport to the zymogen granule fraction, O_2 consumption, and $^{14}CO_2$ production from palmitate-1-^{14}C during transport inhibition with Antimycin A.

Sets of slices (~400 mg wet wt/set), pulse labeled for 3 min with L-leucine-4,5-^3H (5 μc/ml, 45 c/mmole), were transferred to a chase medium containing 2.0 mM L-leucine-^1H, 5×10^{-4} M cycloheximide, and the indicated concentrations of Antimycin A. Antimycin A was added from a 0.02 M stock solution in ethanol. An equivalent amount of ethanol did not affect transport. Chase incubation was for 37 min at 37° with a gas phase of 95% O_2/5% CO_2. At this time, intracellular transport was determined by measuring the specific radioactivity of proteins in zymogen granule fractions isolated from the slices as described in reference 1 and is expressed as the per cent of the value for control slices incubated without Antimycin A. O_2 consumption and $^{14}CO_2$ production from palmitate-^{14}C were determined in separate experiments as described in Methods. Controls—transport: 100% = 51,900 dpm/mg protein; oxygen consumption: 100% = 8.4 μl O_2/mg protein/hr; $^{14}CO_2$ production: 100% = 12,800 dpm/mg protein/hr.

Arrow indicates 5×10^{-5} M Antimycin A.

temperature (Table I); it has a Q_{10} of 3.9 similar to that of many biological processes, e.g., pinocytosis (7), and hence depends on enzymatic processes. Under aerobic conditions, transport is insensitive to glycolytic inhibitors, such as sodium fluoride (F^-) (Table I) at concentrations which effectively block glycolysis (by inhibiting enolase) and glycolysis-dependent processes such as phagocytosis (8). Similar results were obtained with iodoacetate at 10^{-4} M; depression of transport at 10^{-3} M coincided with extensive cytolytic effects.

Inhibitors of respiration such as N_2 (95% N_2-5% CO_2 in the gas phase), sodium cyanide (CN^-) and Antimycin A (9) effectively and, in the case of the first two, reversibly inhibit transport (Table II). Fig. 1 shows that in the case of Antimycin A transport inhibition is closely paralleled by a decrease in O_2 consumption and $^{14}CO_2$ production from palmitate-1-^{14}C. Intracellular transport is reversibly sensitive to inhibitors of oxidative phosphorylation such as DNP (Table II) which concomitantly increases O_2 consumption, as expected from its uncoupling effect on mitochondria. Oligomycin showed similar but less reproducible and less pronounced effects, presumably on account of its difficulty in penetrating intact cells and of extensive binding to the proteins of the medium (10).

From these data, we conclude that the intracellular transport of secretory proteins is dependent on the continued supply of energy, probably as ATP,[2] produced by mitochondrial respiration

[2] Preliminary data indicate that the concentration of ATP in slices drops progressively during 37 min of

TABLE III

Specific Radioactivity of the Proteins of Zymogen Granule Fractions, Total Microsomal Fractions, and Postmicrosomal Supernate during Transport Inhibition by Antimycin A

Antimycin A	Total microsomes	Zymogen granule fraction	Post-microsomal supernate
(M)	dpm/mg protein	dpm/mg protein	dpm/mg protein
0 (control)	69,920	51,900	28,260
10^{-8}	98,860	41,650	23,500
10^{-7}	84,100	17,760	23,930
10^{-6}	102,000	6,940	16,070
10^{-5}	101,450	1,570	20,350
10^{-4}	100,300	3,100	32,640

For experimental details, see Fig. 1 and Table I.

TABLE IV

$^{14}CO_2$ Evolution from Glucose-^{14}C by Pancreatic Slices

Additions to medium*	$^{14}CO_2$ Evolution
	% Control
D-glucose-6-^{14}C	100‡
" + 10^{-2} M F^-	44
" -D-glucose-^{12}C	1100
D-glucose-1-^{14}C	100§
" + 10^{-2} M F^-	71
" -D-glucose-^{12}C	880

* Medium (2.5 ml) contained 14 mM D-glucose-^{12}C (unless otherwise indicated) plus amino acid supplementation and either 5.3 μc of D-glucose-6-^{14}C or 4.1 μc of D-glucose-1-^{14}C.
‡ 1140 dpm/mg protein/hr.
§ 2120 dpm/mg protein/hr.

rather than glycolysis. Further, because of the nature of the assay we can state that the site of the energy-requiring step in transport is located proximal to the condensing vacuoles, i.e., somewhere between RER cisternae and condensing vacuoles.

For the sake of brevity, complete cell fractionation data are not given for each experiment, but in each case in which transport was slowed down or blocked (see Table III, for an example), a proportionate amount of the nontransported labeled secretory proteins remained in the total microsomal fraction, which consists largely of rough microsomes derived from elements of the RER. Of particular importance, under conditions of complete block, labeled proteins did not appear in the postmicrosomal supernate in amounts significantly greater than in controls. Since this supernate represents mainly the soluble proteins of the cytoplasmic matrix, the finding indicates that in the blocked condition there is no equilibration between the proteins of the RER-Golgi compartments and those of the cell sap.

incubation with 5×10^{-5} M Antimycin A to ~7% of the control value of fresh or incubated slices (~15 mμ moles ATP/mg protein).

Source of Oxidizable Substrate

The fact that intracellular transport was not affected by glycolytic inhibitors while being highly sensitive to inhibitors of respiration, suggested that in the exocrine cell the substrate(s)

TABLE V

Effect of Medium Composition on O_2 Consumption by Pancreatic Slices

Medium	O_2 Consumption
	% Control
Complete*	100‡
+ 10^{-2} M F^-	97
− Glucose	107
− Amino acids	104
− Glucose-amino acids	81

* 14 mM glucose, amino acid supplementation.
‡ 8.4 μl O_2/mg protein/hr.

TABLE VI

$^{14}CO_2$ Evolution from Palmitate-^{14}C by Pancreatic Slices

Medium	O_2 Consumption	$^{14}CO_2$ Evolution
	% Control	% Control
Complete*	100‡	100§
+ 10^{-2} M F^-	99	104
− Glucose	103	119
+ 14 mM sodium acetate	85	57
+ 10 mM L-carnitine	—	178

* Medium (2.5 ml) contained 14 mM glucose, amino acid supplementation, and 19 μM palmitate-1-^{14}C (54.1 mc/mmole).
‡ 8.4 μl O_2/mg protein/hr.
§ 12,800 dpm/mg protein/hr.

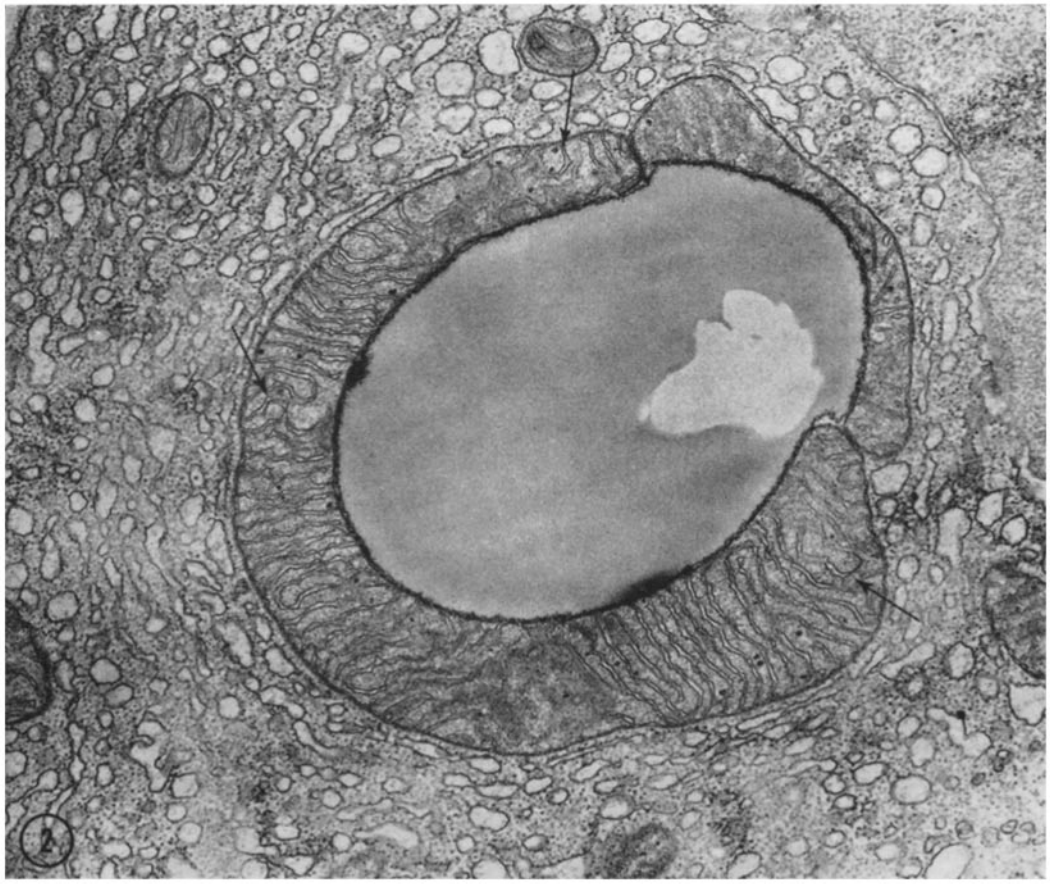

FIGURE 2 Portion of a pancreatic exocrine cell from a starved guinea pig showing lipid droplet surrounded by mitochondria. Note the reticulated appearance of a number of the mitochondrial cristae (arrows). × 17,000.

fed into the Krebs cycle is (are) not derived through the Embden-Meyerhoff metabolic pathway. For a check of this assumption, it was necessary to demonstrate first that F^- entered the cells and indeed suppressed glycolysis while not affecting transport. Table IV shows that this prerequisite was satisfied: the production of $^{14}CO_2$ from ^{14}C-6 or ^{14}C-1–labeled glucose was partially inhibited by F^- at a concentration which does not depress transport (see Table I). As will be shown later, however, glucose oxidation is quantitatively of minor importance to the over-all energy production of the exocrine cell. Complementary experiments showed (Table V) that the omission of glucose and the presence of F^- in the medium did not influence significantly the oxygen consumption of the tissue. The omission of amino acids gave similar results.

By exclusion, we assumed that endogenous fatty acids, known to be present in large amounts as triglycerides in the guinea pig pancreas (11), were the immediate source of oxidizable substrates. Hence, we examined the capacity of pancreatic slices to oxidize trace amounts of palmitate-1-^{14}C to $^{14}CO_2$. The results (Table VI) indicated that the tissue actively metabolized the tracer: although the absolute amount oxidized varied among experiments, as much as 3.3% of the palmitate-^{14}C could be accounted for as $^{14}CO_2$ over 2 hr of incubation.[3] Its oxidation was uninfluenced

[3] The amount of exogenously added palmitate-^{14}C oxidized can be calculated to account for ~10% of the observed O_2 consumption; this estimate is probably low since it does not take into account dilution of the isotope by the endogenous and presumably large pool of fatty acids in the tissue.

by F^- and, in addition, was stimulated only slightly by the omission of glucose though, as seen in Table IV, glucose enters the cells and has a profound istope dilution effect on the oxidation of ^{14}C-labeled glucose. Since glucose has little effect on palmitate oxidation, it is reasonable to assume that the amount of substrate (presumably acetyl coenzyme A) entering the Krebs cycle is derived, to a large extent, from fatty acids, and only to a minor extent from glucose. In support of this assumption is the finding that acetate added to the incubation medium significantly decreased the output of $^{14}CO_2$ from labeled palmitate, most likely by expanding the intracellular pool of acetyl coenzyme A. Further, the addition of carnitine to the medium accelerated the oxidation of labeled palmitate to $^{14}CO_2$, as expected from its reported effect on the rate of oxidation of fatty acids in other mammalian slice systems (12) and from its involvement in fatty acid transport into mitochondria (13).

Finally, the respiratory quotient of pancreatic slices was found to be 0.72, indicating that endogenous fatty acids can account for up to 95% of the oxidizable substrates of the tissue. These findings are in keeping with the frequent occurrence of lipid droplets surrounded by mitochondria, especially in the exocrine cells of starved guinea pigs (14) (Fig. 2) and are consistent with the observed rarity or absence of glycogen deposits in this tissue (15).

Localization of the Energy-Requiring Step of Intracellular Transport

ELECTRON MICROSCOPIC RADIOAUTOGRAPHIC STUDIES: The cell fractionation data reported indicate that the energy-requiring step of intracellular transport is located proximal to condensing vacuoles. A more precise localization in relation to structures known (16, 17) to be involved in this operation (RER cisternae, transitional RER elements, smooth surfaced vesicles at the periphery of the Golgi complex) can be obtained by electron microscopic radioautography of slices pulse labeled with leucine-^3H for 3 min and incubated post-pulse in chase medium containing 5×10^{-4} M cycloheximide and Antimycin A at a concentration (5×10^{-5} M) sufficient to inhibit maximally intracellular transport. For unambiguous interpretation of the results, such an experiment requires that Antimycin A become

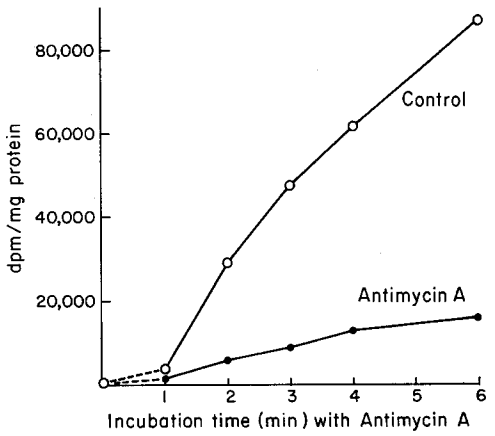

FIGURE 3 Kinetics of Antimycin A activity. Sets of pancreatic slices (~100 mg wet wt/set) were preincubated for 10 min at 4° in medium containing 5 μc/ml of L-leucine-^3H, 45 c/mmole. At 0 time, 5×10^{-5} M Antimycin A was added and the slices were transferred to 37° and incubated for the times indicated. Incorporation is measured in the total protein of the slices. The delay in the onset of incorporation of label during the 1st min is due to the time necessary for the flasks and their content to reach 37° (~½ min) (16).

effective in a fraction of the time (~7 min) normally needed for transport to the peripheral elements of the Golgi complex (16). Preliminary experiments, based on the finding that Antimycin A is a potent inhibitor of protein synthesis in pancreatic slices presumably by virtue of its limiting effect on energy supply, showed that protein synthesis is inhibited within ~1 min after the addition of the drug to the slices (Fig. 3). This time is probably an overestimate, since Antimycin A must first enter the cells and terminate respiration before affecting protein synthesis. It follows that respiration is blocked promptly enough to permit the study of the effect of the block on the first transport steps detectable by radioautography.

The results of a typical radioautographic experiment are given in Table VII. During the first 7 min of incubation post-pulse, a substantial amount of labeled protein migrates from its initial localization over elements of the RER to the peripheral region of the Golgi complex in both the control and Antimycin A–treated slices, but thereafter, the distribution of label does not change in the presence of Antimycin A (Fig. 4), and—by comparison with controls—extensive labeling persists over RER elements up to 57

min of incubation in chase medium. Similarly, transport of secretory proteins was blocked at the periphery of the Golgi complex when the slices were incubated post-pulse for 17 min with nitrogen in the gaseous phase though in this case the effect was fully reversible by the reintroduction of oxygen into the flasks (Table VIII and Figs. 6 and 7).

Since Antimycin A was acting for a large part of the first 7 min, the accumulation of label over the peripheral elements of the Golgi complex indicates that transport through the RER cisternae to these elements is energy independent. Furthermore, because the volume of the cell occupied by elements of the Golgi periphery is considerably smaller than that occupied by the RER cisternae, this phase of transport must be accompanied by concentration in this region.

CELL FRACTIONATION STUDIES: At the peripheral region of the Golgi complex are located transitional elements of the RER (part rough-, part smooth-surfaced cisternae) and clusters of small, smooth-surfaced vesicles (16). Since the resolution of the radioautographic technique is insufficient to indicate which of these two structures contains labeled proteins when transport is blocked, we turned again to cell fractionation procedures and carried out experi-

TABLE VII

Distribution of Radioautographic Grains over Cell Components in Slices Incubated Post-Pulse with Antimycin A

Subcellular components	% radioautographic grains				
	Pulse 3 min	Chase incubation* +7 min	+17 min	+37 min	+57 min
Rough endoplasmic reticulum	89.1	50.3 **66.8**	39.6 **62.8**	38.6 **76.8**	37.1 **78.8**
Golgi complex					
Peripheral region‡	5.0	34.9 **24.1**	23.5 **29.4**	19.7 **16.6**	20.4 **15.1**
Condensing vacuoles	1.0	6.2 **3.1**	29.9 **5.5**	35.3 **1.5**	19.9 **1.8**
Zymogen granules	4.4	7.8 **6.1**	6.9 **2.3**	6.4 **5.4**	19.9 **4.6**
Acinar lumen	0.2	0.4 **0.2**	0 **0**	0.5 **0.1**	3.1 **0**
No. of grains counted	992	684 **1620**	359 **968**	823 **884**	405 **1447**

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

* Numbers in lightfaced type refer to slices incubated in chase medium containing L-leucine-^{1}H and cycloheximide; boldfaced numbers beneath refer to slices incubated in the same medium with Antimycin A added.

Grains over nuclei and mitochondria are not included as they represent a small and variable (range 0–4%) contribution to the total cytoplasmic labeling. Grains over zymogen granules at early time points in controls and at all time points in Antimycin A–treated slices are accounted for mainly by labeling of adjacent RER elements (17).

‡ The peripheral region of the Golgi complex comprises, in our terminology, both the transitional elements of the RER and adjacent smooth-surfaced Golgi vesicles.

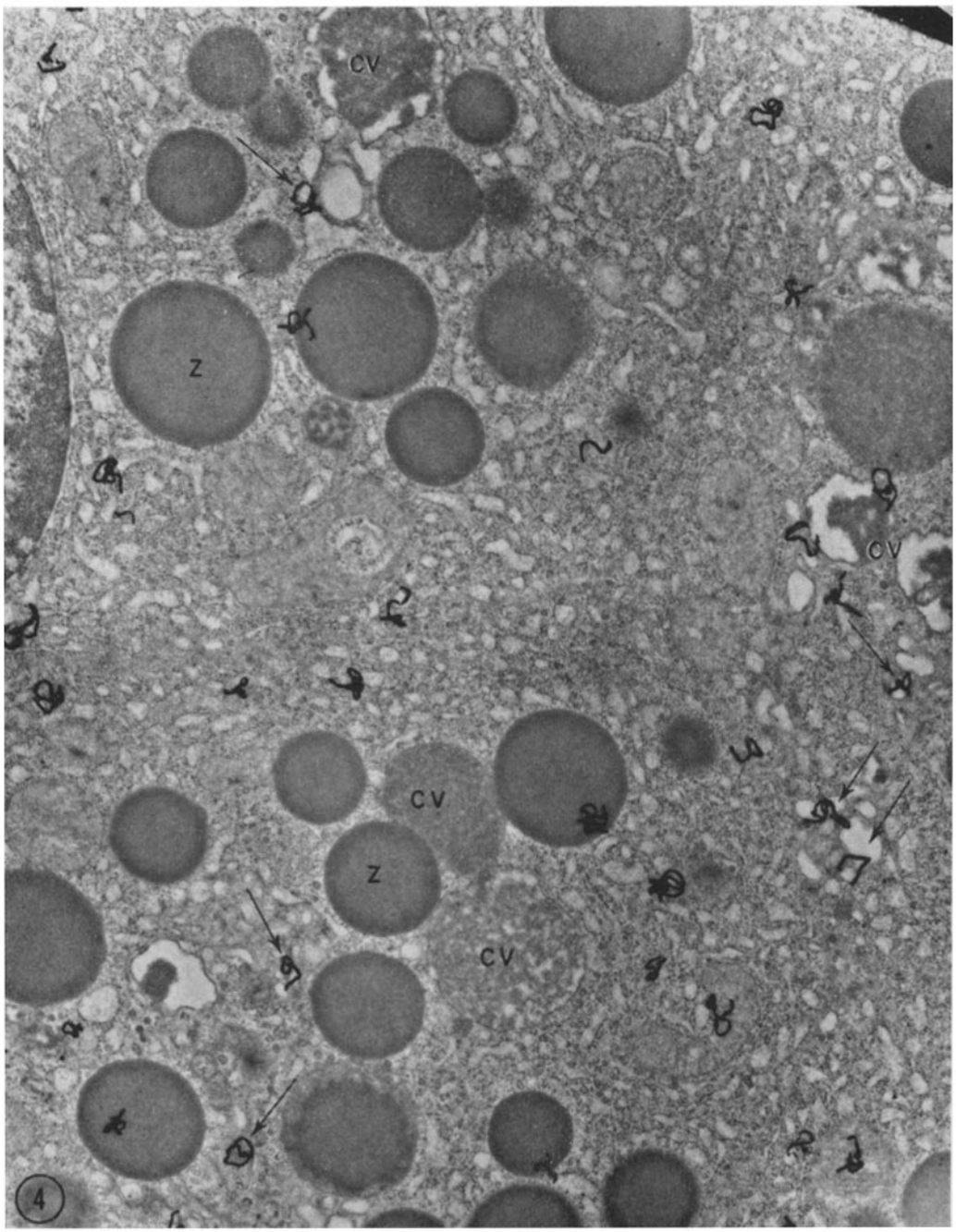

FIGURE 4 Radioautogram of an exocrine cell in a pancreatic slice pulse labeled for 3 min with L-leucine-4,5-^{3}H (200 μc/ml; 60 c/mmole) and incubated post-pulse for 57 min in chase medium containing 4.0 mM L-leucine-^{1}H, 5×10^{-4} M cycloheximide, and 5×10^{-5} M Antimycin A. Radioautographic grains mark elements of the RER and have accumulated over elements of the Golgi periphery (arrows). Condensing vacuoles (*cv*) and zymogen granules (*z*) have much less label than in controls. Note that the general structural organization of the tissue has remained intact under these conditions. (See also Fig. 5.) × 15,000.

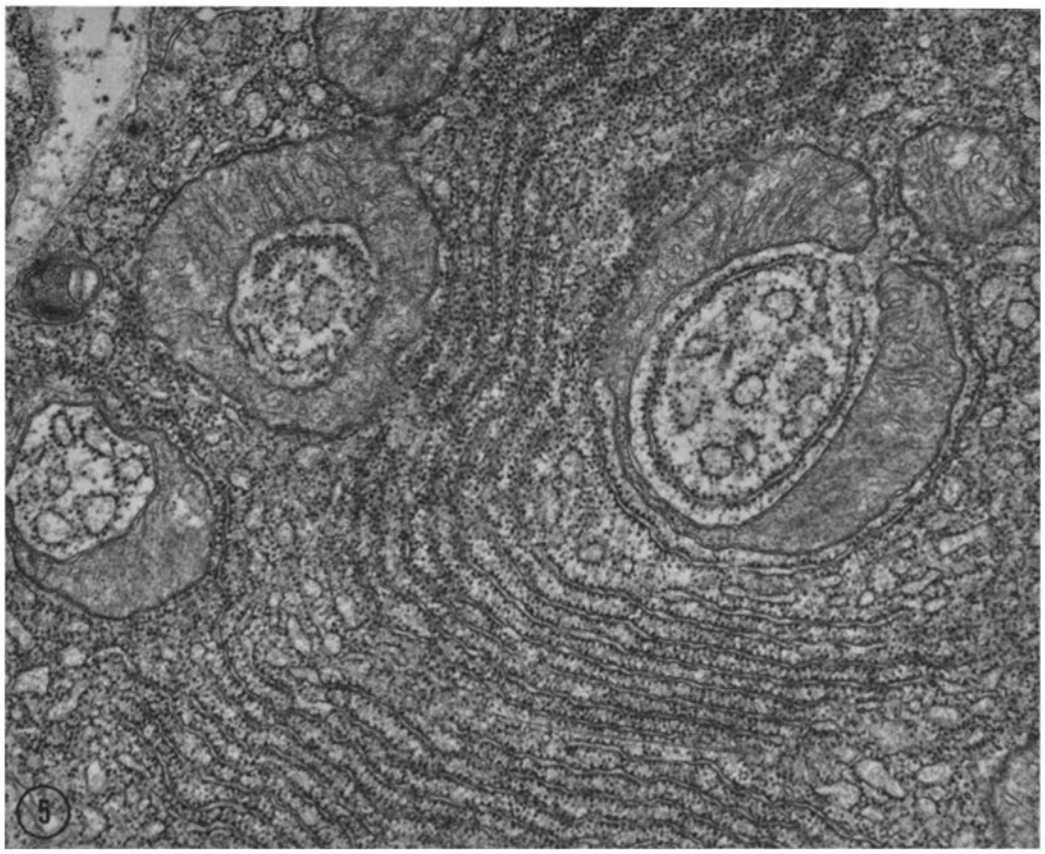

FIGURE 5 Basal portion of an exocrine cell following 40 min of incubation with 10^{-4} M Antimycin A. Note the numerous cup-shaped mitochondria whose concave surface encloses elements of the RER embedded in a pale staining matrix. This configuration was characteristic of Antimycin A–treated tissue and was not observed during block with N_2. × 27,000.

ments (Table IX) in which we compared the total radioactivity of rough and smooth microsomal fractions isolated from slices at the end of a 2.5-min pulse labeling with leucine-^{3}H and after 7 min incubation in a chase medium containing either cycloheximide or cycloheximide and Antimycin A. As previously shown (16), the rough microsomal fraction consists of healed RER fragments, while the smooth microsomes represent the small, smooth-surfaced vesicles of the periphery of the Golgi complex. The results clearly show that in the presence of Antimycin A labeled proteins do not enter the smooth microsomal fraction to any significant extent. Continuation of the chase in the presence of Antimycin A up to +37 min indicated that the block was complete.

These results, together with the radioautographic studies which reveal a concentration of labeled proteins over the Golgi periphery at +7 min, support the conclusion that, in the absence of energy, transport is blocked between the transitional elements of RER and the small vesicles at the periphery of the Golgi complex. Upon cell fractionation, the transitional elements apparently sediment in the rough microsomal fraction.

DISCUSSION

Recent work on the pancreatic exocrine cell has revealed that secretory proteins, upon release from attached ribosomes, are subjected to a number of intracellular transport operations (16, 17). The initial step in the sequence is the vectorial transport of newly synthesized polypeptides to the cisternal space of the RER (18). This step does not require additional energy (19), but depends on chain termination (incomplete pep-

TABLE VIII

Distribution of Radioautographic Grains over Cell Components in Slices Incubated Post-Pulse with N_2

Subcellular components	% radioautographic grains				
		Chase incubation*			
	Pulse 3 min	+17 min O_2	+17 min N_2	+17 min N_2 +17 min O_2	+17 min N_2 +37 min O_2
Rough endoplasmic reticulum	89.1	39.6	74.3	23.2	22.7
Golgi complex					
Peripheral region‡	5.0	23.5	17.0	24.0	16.3
Condensing vacuoles	1.0	29.9	3.7	36.7	22.7
Zymogen granules	4.4	6.9	4.1	15.8	29.7
Acinar lumen	0.2	0	0.7	0.8	8.5
No. of grains counted	992	359	1151	1649	772

* Chase medium contained 4.0 mM L-leucine-^{3}H and 5×10^{-4} M cycloheximide.
See legend of Table VII concerning labeling of mitochondria and nuclei. Label over zymogen granules at the end of the pulse and after +17 min incubation with N_2 is accounted for mainly by labeling of adjacent RER elements (17).
‡ See Table VII.

tides, terminated by puromycin, are also transported [20]) and seems to be controlled primarily by structural restrictions at the ribosome-membrane junction (20, 21).

Upon segregation into the cisternal space, secretory proteins follow its many channels to reach the transitional elements of the ER system at the periphery of the Golgi complex. As our findings show, this step requires neither continued protein synthesis (1), nor metabolic energy. Hence, it apparently depends on diffusion, although some of its aspects, e.g., the concentration of secretory proteins at the periphery of the Golgi complex when transport is blocked, suggest the involvement of additional factors and deserve further investigation.

Our results indicate that the next step in the sequence, i.e., transport from transitional elements to condensing vacuoles, definitely requires energy, presumably ATP generated by oxidative phosphorylation: in the absence of energy, transfer is blocked on the proximal side of the small peripheral vesicles of the Golgi complex. Apparently, the chain of compartments involved in intracellular transport is provided with a lock at this level whose function is energy dependent. At present, it is clear that the energy is used to connect the RER cisternal space with that of the condensing vacuoles, and that the small peripheral vesicles of the Golgi complex participate in the connection. The details of this operation, however, remain obscure: the cell may establish intermittent connections between these two compartments or effect transport between them using the small peripheral vesicles as shuttle carriers (16). Both alternatives imply repeated membrane fission-fusion and this is most probably the energy-requiring event.

Our findings indicate clearly that there are no permanent connections between the compartments mentioned, otherwise labeled secretory proteins would diffuse slowly during the energy block into small vesicles and further on into condensing vacuoles. The lack of permanent connecting channels is suggested by the structural features of the periphery of the Golgi complex, in which clusters of small vesicles are regularly found in the region between transitional elements and condensing vacuoles (16) but where continuous connecting tubules have not been reported. In the past, however, the possibility of artefactual production of isolated vesicles at the expense of connecting tubules during fixation had to be considered in view of the propensity of plasmalemmal infoldings to form vesicles after osmium tetroxide fixation[4] (22).

[4] Small vesicles are also seen in the Golgi periphery of guinea pig pancreas prefixed with aldehydes prior to osmium fixation.

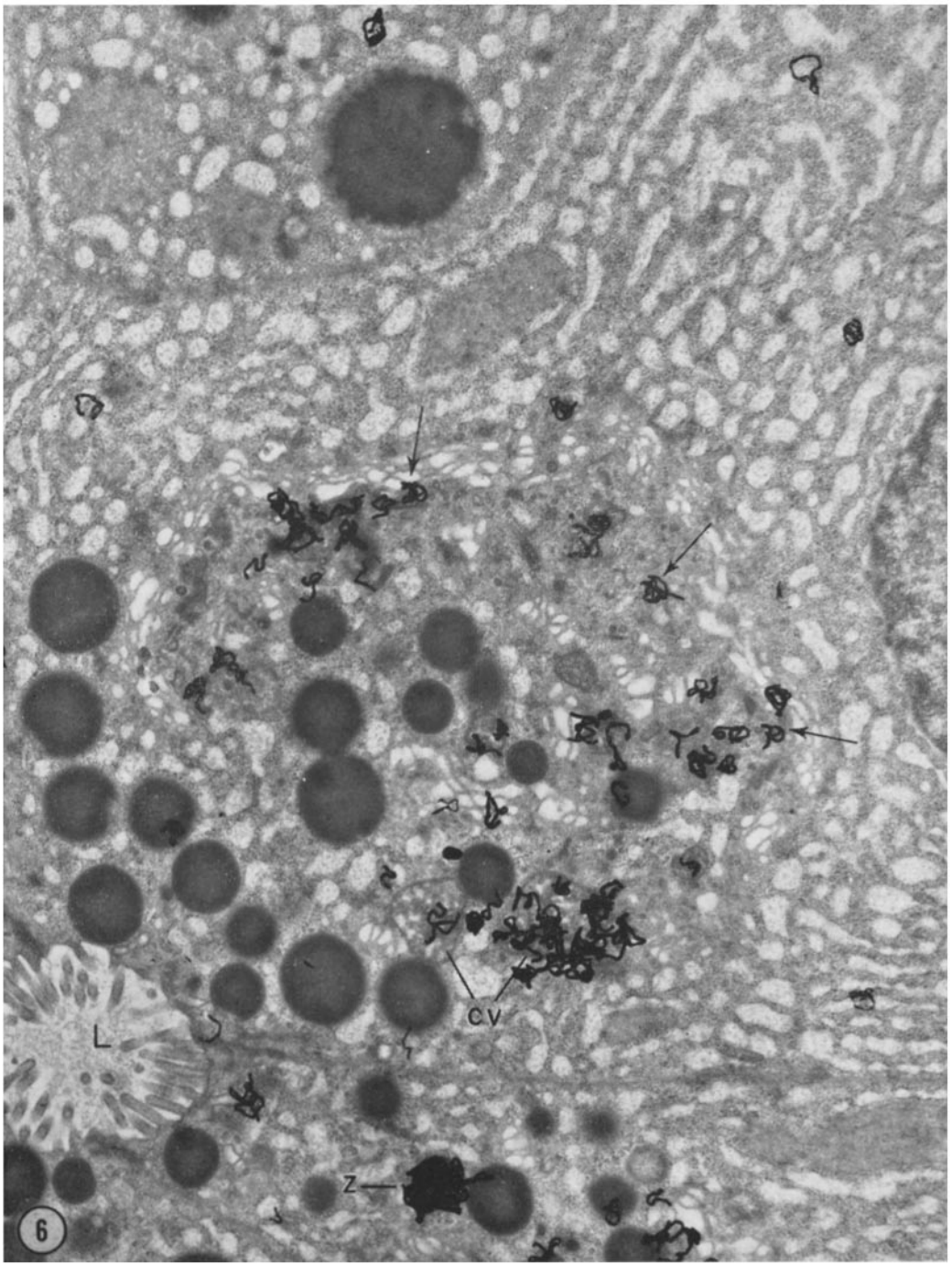

FIGURE 6 Radioautogram of an exocrine cell in a pancreatic slice following reversal of transport inhibition with N_2. Tissue pulse labeled for 3 min with L-leucine-^{3}H (see Fig. 4) and incubated for 17 min post-pulse in chase medium containing 4.0 mM leucine, 5×10^{-4} M cycloheximide, with 95% N_2/5% CO_2 in the gas phase. Transport reversal was obtained by bubbling 95% O_2/5% CO_2 into the flask and reincubating for a further +17 min. Note that the labeled proteins have largely drained from the RER and mark elements of the Golgi periphery (arrows), condensing vacuoles (*cv*), and a zymogen granule (*z*) which is almost obscured by the radioautographic grains. *L*, acinar lumen. × 14,500.

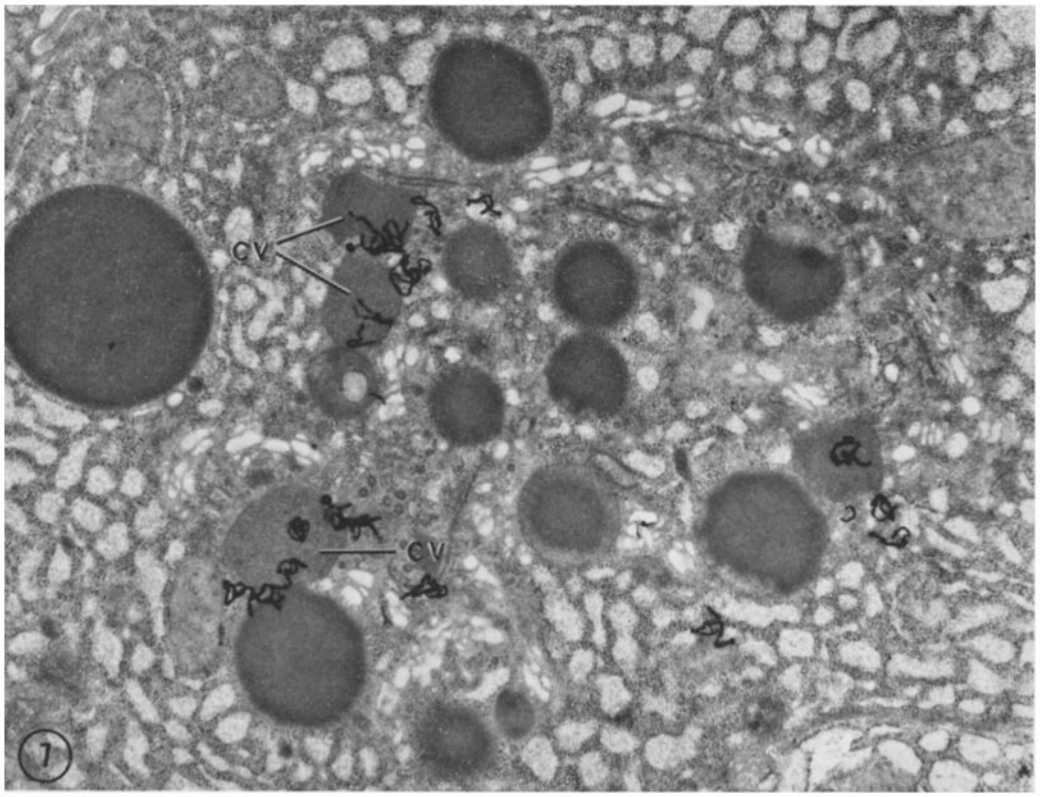

FIGURE 7 Tissue treated in same manner as Fig. 6, showing more clearly the accumulation of labeled proteins in condensing vacuoles (*cv*). × 14,500.

TABLE IX

Labeling of Microsomal Subfractions from Slices Incubated Post-Pulse with Antimycin A

	dpm in gradient load (total microsomes)	dpm recovered in rough and smooth microsomes	% dpm recovered in rough and smooth microsomes	dpm in smooth microsomes as % rough and smooth microsomes
			%	%
3 min (pulse)	101,260	50,430	49.8	17.0
+7 min	66,880	31,660	46.0	43.0
+7 min with Antimycin A	72,790	37,060	50.9	18.8

Three sets of slices, ~500 mg wet wt/set, were pulse labeled for 2.5 min in medium containing 10 μc/ml L-leucine-^{3}H (45 c/mmole). At the end of the pulse, one set was homogenized while the other two were incubated for 7 min in a chase medium containing 2.0 mM L-leucine-^{1}H and either 5×10^{-4} M cycloheximide or 5×10^{-4} M cycloheximide and 5×10^{-5} M Antimycin A. At the end of the chase incubation, these sets were homogenized. From each of the three homogenates a total microsomal fraction was isolated by differential centrifugation and subfractionated into rough and smooth microsomes by isopycnic sedimentation in a linear sucrose density gradient (16). The data compare the total TCA-precipitable radioactivity in these subfractions.

The demonstration of an energy-dependent lock at the periphery of the Golgi complex confirms our previous assumptions (16) and explains why the cell can perform a number of secondary operations related to the transport of secretory proteins, such as the concentration of their originally dilute solution within condensing vacuoles, and the division of the secretory product into quanta. The production of intracisternal granules (23) may also be related to a relative slowdown in the function of this lock.

Under conditions of complete block of intracellular transport, we found no evidence that labeled proteins in the RER cisternae equilibrate at any time with those of the cell sap (final supernate). This finding renders unlikely the existence in this cell of other pathways of intracellular transport, such as transport of secretory proteins in soluble form through the cytoplasmic matrix, and indicates that the segregation of secretory proteins in the RER cisternae is an obligatory (18, 20) and apparently irreversible step.

Following their transport to condensing vacuoles of the Golgi complex, secretory proteins are intensively concentrated within these structures which, in the process, are transformed into zymogen granules (17, 24, 25). Our preliminary findings indicate that membranes derived from a common zymogen granule-condensing vacuole fraction have a nucleoside triphosphatase activity of limited substrate specificity; we are currently studying this activity in more detail to find out whether it is related to the intracellular concentration of secretory products.

Finally, as is well known from the previous studies of Hokin and Hokin on pigeon pancreas (26) and of Schramm on rat parotid (27), the discharge of secretory proteins from the zymogen granules of exocrine cells is dependent on respiratory energy. The morphological basis of this step has been well documented (28) and may also involve repeated membrane fission-fusion.

On the basis of our respiratory studies, it is apparent that fatty acids are the main oxidizable substrates of the exocrine cell. This finding is consistent with the reported high concentrations of tri- and di-glycerides in the guinea pig pancreas (11) and with the characteristic intimate association of mitochondria and lipid droplets frequently found in the pancreatic exocrine cells of starved guinea pigs (14).

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