PROTEIN SYNTHESIS, STORAGE, AND DISCHARGE IN THE PANCREATIC EXOCRINE CELL

An Autoradiographic Study

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ABSTRACT

The synthesis, intracellular transport, storage, and discharge of secretory proteins in and from the pancreatic exocrine cell of the guinea pig were studied by light- and electron microscopical autoradiography using DL-leucine-4,5-H^3 as label. Control experiments were carried out to determine: (*a*) the length of the label pulse in the blood and tissue after intravenous injections of leucine-H^3; (*b*) the amount and nature of label lost during tissue fixation, dehydration, and embedding. The results indicate that leucine-H^3 can be used as a label for newly synthesized secretory proteins and as a tracer for their intracellular movements. The autoradiographic observations show that, at ~5 minutes after injection, the label is localized mostly in cell regions occupied by rough surfaced elements of the endoplasmic reticulum; at ~20 minutes, it appears in elements of the Golgi complex; and after 1 hour, in zymogen granules. The evidence conclusively shows that the zymogen granules are formed in the Golgi region by a progressive concentration of secretory products within large condensing vacuoles. The findings are compatible with an early transfer of label from the rough surfaced endoplasmic reticulum to the Golgi complex, and suggest the existence of two distinct steps in the transit of secretory proteins through the latter. The first is connected with small, smooth surfaced vesicles situated at the periphery of the complex, and the second with centrally located condensing vacuoles.

INTRODUCTION

The purpose of this work was to study, by high resolution autoradiography, the synthesis, intracellular transport, storage, and discharge of a well defined class of proteins: the digestive enzymes produced by the exocrine cells of the guinea pig pancreas. This system has been the object of a number of recent studies involving the biochemical analysis of cell fractions separated by differential centrifugation (1–8). Such studies have provided a sizable amount of data on the secretory cycle but have left several points in doubt and could not provide any information on important steps of the process. This situation is due to the intrinsic limitations of the cell fractionation procedures used so far: incomplete separation, resulting in the contamination of all fractions by structural elements belonging to other fractions, and incomplete fractionation, resulting in the apparent loss of cell structures such as the Golgi complex (9).

Recent advances in high resolution autoradiography (10, 11) have rendered possible the intracellular localization of label with a resolution

of 0.2 to 0.1 μ. It appeared, therefore, that autoradiography would provide a suitable analytical tool to check the conclusions arrived at by cell fractionation studies and, in addition, give information which could not be obtained by other means.

The design of the experiments was simple: radioactive leucine was injected, intravenously, into guinea pigs, whose pancreas was then examined by autoradiography at various times after injection. This examination was performed both in the light- and the electron microscope.

MATERIALS AND METHODS

Male albino guinea pigs, weighing 200 to 250 gm, were fasted 48 hours, then fed 1 hour before the experiment in order to initiate a secretory cycle (3). Under nembutal anesthesia, the animals received by intravenous injection about 1 ml of physiological saline containing from 1 to 5 millicuries of DL-leucine-4,5-H^3 (from New England Nuclear Corp., Boston) with a specific activity of 3570 mc/mM. At times varying from 4 minutes to 15 hours after the injection of the label, a portion of the pancreas was fixed by injecting directly into the tissue 2 per cent OsO_4 in acetate-veronal buffer, pH 7.5 to 7.6. The results were followed under a stereoscopic microscope and those regions which turned brown most rapidly were excised and cut into small blocks whose fixation was continued by immersion in the same fixative at 4° for 2 hours. The blocks were subsequently dehydrated and embedded in methacrylate according to standard methods (12).

The blocks were sectioned for autoradiographic examination in the light- and the electron microscope; the thickness of the sections was $\sim$ 0.4 μ in the first case and $\sim$ 0.06 to 0.1 μ in the second. The methods for autoradiography have been described in detail in previous communications (10, 11). They involve the application to the specimen of a thin uniform layer of Ilford L-4 Nuclear Research emulsion. In the case of electron microscopic preparations the thickness of this layer is reduced to that of a single crystal of silver halide (about 0.1 μ).

Because of the lack of contrast of the autoradiographs in the electron microscope it was essential to stain the sections before examination. This was done, using either uranyl acetate in a 30 per cent ethanol solution (13) or the Karnovsky lead stain (14). As shown by Revel and Hay (15), the latter staining procedure results in a partial removal of the gelatin and in a considerable improvement in image quality. Since, however, this method causes on rare occasions a displacement or a removal of the autoradiographic grains (10), we have used it only for illustration purpose. In all cases where grain counts were made we have used the uranyl stain, which gives less contrast but does not affect the gelatin and is, therefore, safer.

Grain counts were made by taking low power electron micrographs of a number of fields (openings in a 200-mesh screen), each selected on the basis of the uniformity and quality of the section. Since all micrographs were taken at a magnification at which the photographic grains were invisible on the screen, the presence and position of grains did not influence the choice of fields. Although this method of selection is not completely random it gives unbiased grain counts. The reproducibility of such grain counts is good, as demonstrated on this material in our earlier publication (10).

The techniques used in the various control experiments performed in connection with this work will be described, together with the results, in the next section.

RESULTS

A. *Controls*

In order to follow, by autoradiography, the passage of marked macromolecules on or through the various organelles of the cell several conditions must be fulfilled: (1) the period during which the label is available for incorporation must be known; (2) the label used must be specific for the class of macromolecules to be studied; (3) the label once incorporated must be preserved during the fixation of the specimen; (4) it should be ascertained that a rapid turnover of the labeled molecules does not take place. We shall, therefore, begin by a discussion of these various prerequisites.

1. Availability of Leucine-H^3 Following Intravenous Injection

A. IN THE BLOOD: Friedberg and Greenberg (16) reported that various amino acids (glycine, L-alanine, L-glutamic acid, L-histidine, and L-lysine) are removed from the blood to the extent of 80 to 90 per cent within 15 minutes after their intravenous injection in a starved rat. Borsook *et al.* (17) found that the level of radioactivity in the blood, following the intravenous injection of L-leucine-1-C^{14} in mice, falls within 10 minutes to less than 2 per cent of the amount injected. Ul Hassan and Greenberg (18) found significant differences in the metabolism of D-leucine and L-leucine. Because in our experiments we used DL-leucine-4,5-H^3, and because the label is on a different position than in the material used by Borsook *et al.*, we conducted a few experiments to determine the fate of tritiated leucine after intravenous injections.

Two such experiments are illustrated in Fig. 1. The preparation of the animals and the conditions of the injection were as described under Materials and Methods. 200 μc of DL-leucine-4,5-H^3 were used in each experiment. Samples of the blood, about 0.5 ml in volume, were taken at various intervals following injection, from a femoral vein in one case and a mesentery vein in the other. The samples were placed in cold, 1-ml centrifuge tubes containing 0.25 ml of 20 per cent perchloric acid. After centrifugation the radioactivity of the acid-soluble fraction was measured in a Packard Tri-Carb scintillation counter. A correction for quenching was made for each specimen.

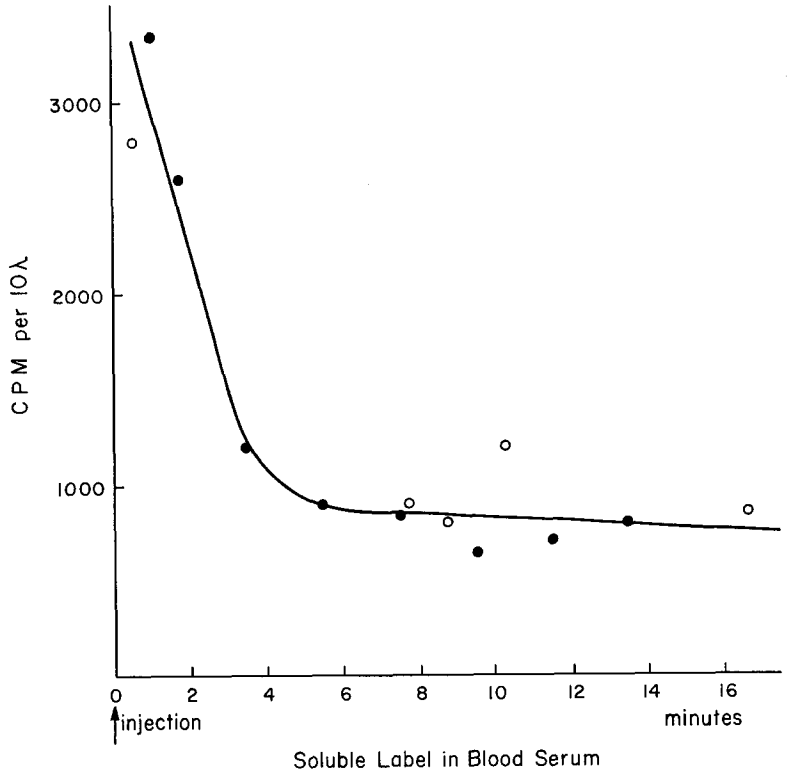


FIGURE 1 Acid-soluble radioactivity in blood serum following the injection, into 700-gm guinea pigs, of 200 μc of DL-leucine-4,5-H^3, shown as number of counts per minute per 10 μl of serum. The counting efficiency was 5.3 per cent. Injection was in the femoral vein. Samples were taken from a mesentery vein (○) in one experiment and a femoral vein (●) in another.

The results show that acid-soluble radioactivity reaches a peak in less than 1 minute after injection and that the measured peak value is within 20 per cent of that expected for a blood volume corresponding to 8.5 per cent of body weight. The radioactivity then decreases very rapidly until it reaches a plateau at about 20 per cent of the peak value at 4 to 5 minutes.

To determine the nature of the material in this plateau, another animal was injected with 2 mc of leucine-4,5-H^3 and, after 10 minutes, 1 ml of blood was removed and added to 0.5 ml of cold 20 per cent perchloric acid. The ensuing precipitate was sedimented out and the supernate neutralized with potassium bicarbonate and cleared by centrifugation. A 50 μl aliquot of the supernate supplemented with 40 μg of cold carrier leucine was then analyzed by descending chromatography on Whatman No. 1 paper in the following solvent system: 70 per cent secondary butyl alcohol, 10 per cent formic acid, 20 per cent water (19). The position of the cold leucine was identified by the ninhydrin reaction and the distribution of tritium on the chromatogram measured in a scintillation counter. Practically all the radioactivity was found associated with the position of the leucine spot on the chromatogram.

The proportions of the two leucine isomers remaining in the blood 10 minutes after injection were estimated in the following manner: the leucine spot on the chromatogram of a perchloric acid extract was

eluted into 5 ml of M-9 minimal medium for bacteria. When cells of *E. coli* 15 were allowed to grow to saturation in this medium, they were found to have incorporated 30 per cent of the label. If a large excess of tritiated DL-leucine was also added to the medium, the cells incorporated 50 per cent of the label, corresponding to the entire amount of the L isomer added (20).

We conclude, therefore, that, after intravenous injection of the DL-leucine-4,5-H^3, the major fraction of the label is available as a short pulse, of a duration of 3 to 4 minutes. A smaller proportion of the label remains in the blood for at least 15 minutes. It is predominantly in the form of D-leucine although some L-leucine is still present as evidenced by its incorporation by bacteria incapable of utilizing the D isomer.

B. IN THE PANCREAS: The situation within the tissue is different, as shown by the results in Table I. In these experiments[1], the animals were sacrificed 3 to 30 minutes after the injection of 0.2 mc of DL-leucine-4,5-H^3. For all but the 3-minute point, a chaser containing leucine-H^1, 500 times more concentrated than the radioactive leucine, was given to the animal 3 minutes after the first injection. The pancreatic glands were removed, homogenized, and analyzed for total and specific radioactivity of the homogenate and of its acid insoluble fraction. It can be concluded from the results: (*a*) that the chaser

[1] The help of Dr. Philip Siekevitz and Miss M. Ledoux in performing these experiments is gratefully acknowledged.

TABLE I

In Vivo Incorporation of DL-*Leucine-4,5-H^3 into Guinea Pig Pancreas*

The animals received intravenously under light ether anesthesia 0.2 mc of DL-leucine-4,5-H^3; 3 minutes later a chaser diluting the label 500 times was injected (× 100, intravenously; × 400, intraperitoneally) in all animals except the first two (3 min. time point). The pancreatic glands were removed at the times indicated and homogenized in H_2O to give a 1:10 (w:v) homogenate. Total counts and protein amounts were determined in the homogenate and its TCA-insoluble fraction obtained by the Schneider procedure. Protein content was calculated from N determined by nesslerization on Kjeldahl digests. Radioactivity was measured in a Packard Tri-Carb scintillation counter on formic acid–dissolved samples with corrections for quenching for each sample.

Time	Animal weight	Total pancreatic proteins	Total radioactivity CPM × 10^2			Specific radioactivity§ CPM/mg protein		Incorporation
			Homogenate	TCA-insoluble	TCA-soluble‡	Homogenate	TCA-insoluble	
min.	*gm.*	*mg*						*per cent*
3	335	172	1860	451	1409	1420	318	20
	305	128	1310	193	1117	1120	176	
7*	275	96	1294	196	1098	1330	201	19
	275	127	1258	306	952	985	242	
11*	285	117	1561	658	903	1360	575	37
	290	136	1228	365	863	945	280	
15*	270	119	1680	935	745	1370	570	43
	270	111	1243	559	684	1090	492	
30*	245	78	1355	843	512	1460	960	68
	235	111	1530	1078	452	1160	828	

* Chaser at 3 minutes.

‡ By difference.

§ These figures are corrected for differences in weight among animals (average weight 278.5 gm) assuming that after injection the specific activity of the leucine is inversely proportional to the weight of the animal.

TABLE II

In Vivo Incorporation of DL-*Leucine-4,5-H³ into Guinea Pig Pancreas*

The animals received intravenously 0.066 mc/100 gm body weight of DL-leucine-4,5-H³ (specific activity: 3570 mc/mM) under light ether anesthesia. A chaser diluting the label 100 × was injected intravenously at 4½ minutes only in the two animals of the 5½ minute time point. The rest of the procedure was the same as given in Table I except that radioactivity was determined in cold TCA-soluble, hot TCA-soluble, and TCA-insoluble fractions of the homogenate.

Time	Animal weight	Total pancreatic proteins	Total radioactivity CPM × 10²			Specific radioactivity CPM/mg protein		Incorporation
			Cold TCA-soluble	Hot TCA-soluble	TCA-insoluble	Homogenate‡	TCA-insoluble	
min.	*gm*	*mg*						*per cent*
5½*	310	108.5	927	15	211	1063	194	27
	255	95.0	825	18	454	1367	478	
20	290	100.0	846	28	1100	1975	1100	58
	290	106.2	563	27	971	1472	914	
60	300	87.8	455	24	1283	2010	1465	73
	300	99.6	505	36	1421	1967	1425	

* Chaser at 4½ minutes.

‡ Calculated.

stopped the increase of radioactivity in the pancreas; (*b*) that there is in the cell a non-exchangeable pool of acid-soluble, leucine-containing material. It can be estimated roughly that the half-life of a leucine molecule within the pool is of the order of 15 minutes. This fact will determine the length of the pulse of label. As a first approximation, we can say that the specific activity of the leucine used for protein synthesis in the pancreas will fall to 50 per cent of its maximum value within roughly 15 minutes after the injection.

In another series of experiments[1] (Table II), we used conditions and times more similar to those of the autoradiography experiments: a chaser was injected after 4½ minutes for the first time point, and no chaser was used for the other time points. A comparison of the two tables shows: (*a*) that the omission of the chaser does not affect significantly the proportion of incorporated to total label in the pancreas, and (*b*) that the amount of total label in the pancreas reaches ~60 per cent of the 60-minute value in (4½ + 1) minutes and ~90 per cent of that value in 20 minutes. We see, therefore, that the length of the label pulse is increased by the omission of the chaser but that the difference is small. A complicating factor is the fact that we used DL-leucine-H³ since, as in the blood, the incorporation of the D isomer might be inefficient. In experiments of this type it would be preferable to use the L isomer alone.

It might be pointed out that, at the specific activity used in the autoradiographic experiments, 5 mc of leucine-H³ represent approximately 30 per cent of the normal leucine concentration in the blood. The injection of this amount should not disturb unduly the normal flow of leucine through the cells.

TABLE III

Losses of Label During Fixation, Dehydration, and Embedding of Pancreatic Tissue Fixed at Different Times after the Intravenous Injection of DL-*Leucine-4,5-H³*

Time after injection	Chaser at	Label lost in		Total loss
		OsO_4 and buffer	Alcohols and methacrylate	
min.	*min.*	*per cent*	*per cent*	*per cent*
7½	5½	8.3	24.7	33
20	—	2.9	13.2	16.1
20	—	3	16.2	19.2
22	5½	5.8	9	14

2. Specificity of the Label

Siekevitz and Palade (4) have found that DL-leucine-1-C^{14} is efficiently incorporated into the proteins of the pancreas. Thus, after 45 minutes, 2.4 per cent of the total label injected was found in pan-

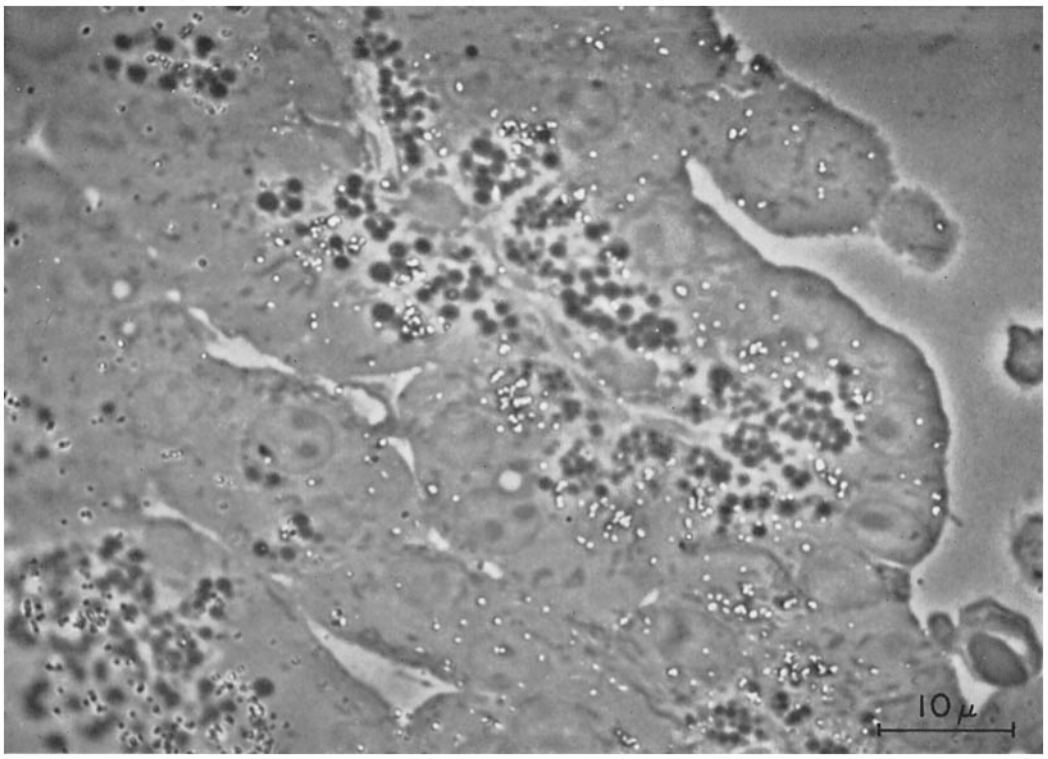
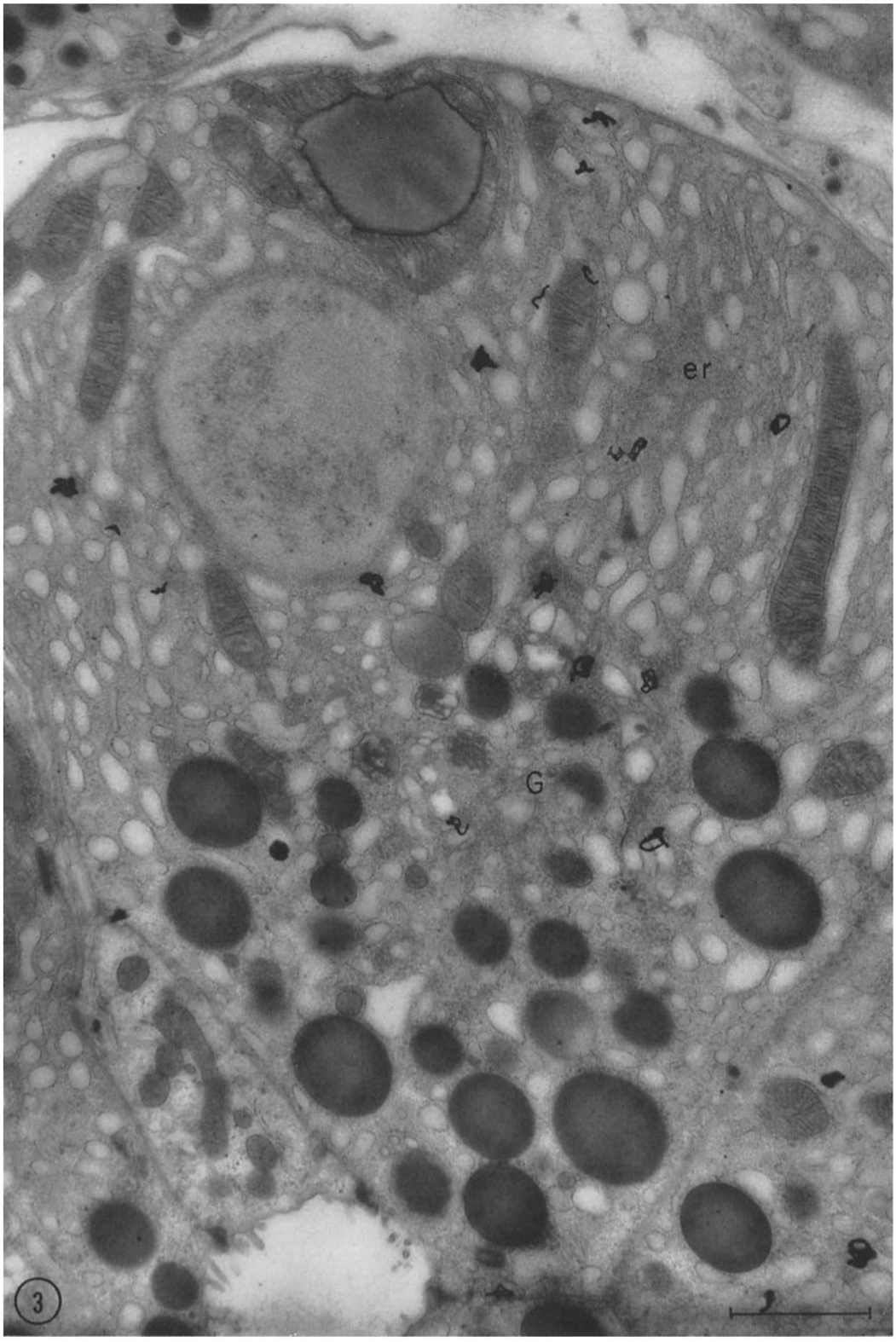


FIGURE 2 Autoradiography of a pancreatic acinus 20 minutes after the injection of DL-leucine-4,5-H^3. The micrograph was taken in phase contrast. The photographic grains, placed a little above the plane of focus, are seen as bright points. Clusters of grains appear in the centrosphere region of many cells, the rest of the cell remaining almost free of label. Although the resolution is fairly good, it is impossible to distinguish clearly the labeled structures. × 1700.

creatic proteins. Schoenheimer and his group (21, 22) have shown that side chains of both D- and L-leucine, labeled with deuterium, are incorporated efficiently into proteins, mostly as leucine. Ul Hassan and Greenberg (18) have reported that almost 90 per cent of the label in the viscera of mice injected with DL-leucine-2-C^{14} is found incorporated in proteins 8 hours after the injection. Borsook *et al.* (17) have found that in the viscera of mice most of the label appears in proteins or as free amino acids at various times after injection of L-leucine-1-C^{14}. It seems, therefore, that, in spite of the known ketogenic activity of leucine, most of it is incorporated into proteins, at least in the pancreas. In this gland, especially after stimulation by a cycle of fasting and feeding, the rate of synthesis of exportable proteins (*i.e.*, digestive enzymes) is considerably higher than that of structural or other non-exportable proteins (6, 8). We expect, therefore, that, in our autoradiographic experiments, the distribution of radioactivity will reflect that of exportable proteins.

At very short times after injection, a difficulty arises from the fact that the OsO_4 used for fixation might cause a non-specific binding of free leucine to various structures. In an experiment in which leucine-C^{14} was added to a pancreatic homogenate, it was found

FIGURE 3 Electron microscopic autoradiography of an exocrine cell 5 minutes after the injection of leucine-4,5-H^3 (a chaser of leucine-H^1 was injected at 4 minutes). Most of the grains are over elements of the rough surfaced reticulum (*er*). A few grains are also found over the small, smooth surfaced vesicles of the Golgi complex (*G*). × 21,000.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that after OsO_4 fixation of this mixture about 4 per cent of the added label was precipitated by cold trichloroacetic acid (TCA) and not extracted by hot TCA, 95 per cent ethanol, ethanol-ether, and acetone, nor removed by the various concentrations of ethanol used in the regular dehydration-embedding procedure. Without OsO_4-fixation the amount of label bound was at least 200 times smaller and undetectable.[1] We assume that this binding of free leucine is responsible for the photographic grains we found over structures normally unlabeled (red blood cells, collagen fibrils) or weakly labeled (nuclei of exocrine cells, centroacinar cells, etc.) when we examined autoradiographs of pancreatic tissue fixed 5 to 6 minutes after the injection of the label. This non-specific labeling disappeared at 15 or 20 minutes after injection, presumably because of the drop in the concentration of free leucine-H^3. At earlier times it could be eliminated completely by injecting, a few minutes after the leucine-H^3, a chaser containing an amount of unlabeled DL-leucine about 100 times greater than that of the labeled leucine used.

It seems clear that this non-specific binding affects only a fraction of leucine molecules and becomes objectionable only when high concentrations of leucine-H^3 are present in the blood. There is good evidence that a large part of the leucine pool in the cell is removed by fixation (see below).

3. Fate of Label During Fixation

To find out whether the incorporated label was preserved during the preparation of the tissue, we measured the losses occurring in the various steps of the fixation and embedding procedures. To this end a fairly large number of small blocks of labeled pancreas was fixed, dehydrated, and embedded in methacrylate. After trimming, the methacrylate was dissolved in acetone, the blocks were completely hydrolyzed in 1:1 concentrated HCl:acetic acid at 100° for 12 hours. Each solution was saved and its radioactivity measured in a scintillation counter. Suitable corrections were made for quenching. The radioactivity remaining in the tissue blocks at the end of the entire preparatory procedure was measured in their acid hydrolysate.

A summary of the results obtained in four experiments is shown in Table III. The major losses occurred during fixation, first wash, and subsequent dehydration steps. A significant but much smaller loss occurred in methacrylate. The losses were somewhat higher for the early time point (7½ minutes).

In one of the 20-minute time points, the various ethanol solutions were pooled and concentrated. Carrier protein (albumin) was added to the concentrate, which was subsequently submitted to chemical fractionation. The radioactivity present had the following characteristics: 30 per cent of it was extractable with chloroform; 60 per cent was soluble in cold TCA; and the remaining 10 per cent was precipitable with cold TCA. We conclude that the largest fraction of the material lost during fixation and embedding consisted of low molecular weight materials, whereas a smaller fraction possibly came from lipids and very little from finished proteins.

4. Probable Absence of a Rapid Turnover

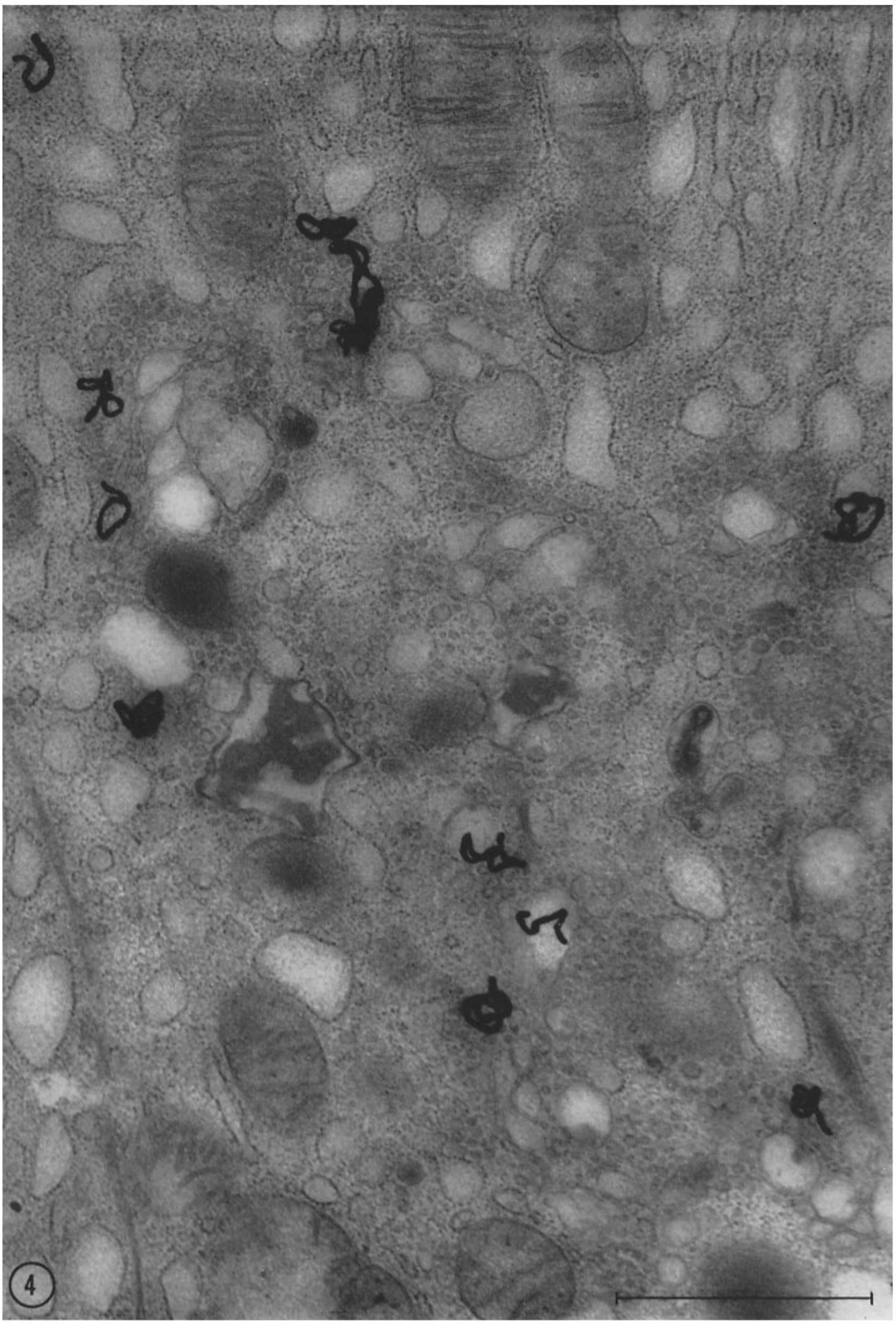
There is no evidence in Tables I and II for a rapid turnover of newly synthesized proteins since in that case the specific radioactivity of the acid-insoluble fraction would be expected to decrease with time. Yet the data do not eliminate completely a fast turnover: they make it, however, dependent on an unlikely condition, namely the preferential re-utilization of breakdown products within the cell.

B. *Morphology*

The fine structure of the pancreatic exocrine cell has often been described. Its general features are fairly constant for all mammalian species so far studied, *i.e.*, the mouse (23–25), rat (26), guinea pig (1), cat (32), and man (27).

The basal region of the cell is occupied by the nucleus, mitochondria, and a large number of preferentially oriented, rough surfaced cisternae of the endoplasmic reticulum. The apical region contains, especially in starved animals, numerous zymogen granules, interspersed with a few rough surfaced elements of the endoplasmic reticulum. In between these two regions, on the apical side of the nucleus, there is a well developed centrosphere or Golgi zone, centered by two centrioles and occupied by numerous smooth surfaced vesicles, cisternae, and vacuoles, *i.e.* the typical components of a Golgi complex.

FIGURE 4 Five minutes after the injection of leucine-4,5-H^3. The Golgi region of an exocrine cell. At this time point, the grains present over this region are found mostly over the clusters of smooth surfaced vesicles located at the periphery of the complex. A few grains mark small vacuoles with a light content situated towards the center of the complex. Vacuoles partially filled with dense material are free of label. × 40,000.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The vesicles measure 40 to 60 mμ in diameter, have a relatively homogeneous content of moderate density and occur in large agglomerations at the periphery of the complex in close juxtaposition to the rough surfaced cisternae of the endoplasmic reticulum. Occasionally such smooth surfaced vesicles are found in continuity with rough surfaced cisternae. Cisternal elements of intermediate appearance—part rough and part smooth—are encountered in the same location (9). Junctional elements between the rough and smooth surfaced parts of the endoplasmic reticulum have been described in many cell types (28–30). In the exocrine pancreatic cell they join rough surfaced cisternae to smooth surfaced elements of the Golgi complex and are usually located along the convex periphery of the centrosphere region, mostly on the basal side (9).

Flattened smooth surfaced cisternae, stacked parallel to one another in small compact piles, are generally present in normally fed animals, but become rarer in our experimental conditions, which are characterized by a high rate of protein synthesis for secretion (3, 4). Under these conditions, the most conspicuous elements of the Golgi complex are its large vacuoles of irregular shape and usually central location. Their content varies in density from very low to as high as that of the zymogen granules. It consists of dense, heterogeneous masses embedded in a light matrix. The varied appearances of these vacuoles can be fitted into a hypothetical series which starts from an "empty" vacuole and leads to the formation of a mature zymogen granule, presumably by a process of progressive accumulation and concentration of secretory proteins. This hypothetical series has been the main argument for the repeatedly advanced, but experimentally unproved postulate that zymogen granules are formed in the Golgi complex (24, 31, 47, 48). For reasons to be given in the course of this article, we propose to give to the large vacuoles of the Golgi complex the general name of *condensing vacuoles*. The smooth surfaced elements of the Golgi complex are frequently grouped in blocks separated from one another by "streamers" of rough surfaced elements and free ribosomes. Finally, a varied number of mature or nearly mature zymogen granules are encountered in the Golgi zone, as well as a few granules of similar size and shape but with a content of low density.

In autoradiographs at the light microscope level, the basal and apical regions of the cell are readily identified, whereas the identification of the centrosphere region is more difficult, often uncertain. Boundaries among the three regions are necessarily arbitrary. In electron microscopical autoradiographs, the identification of every region and of the major components in each region is easily and reliably achieved.

C. *Autoradiography*

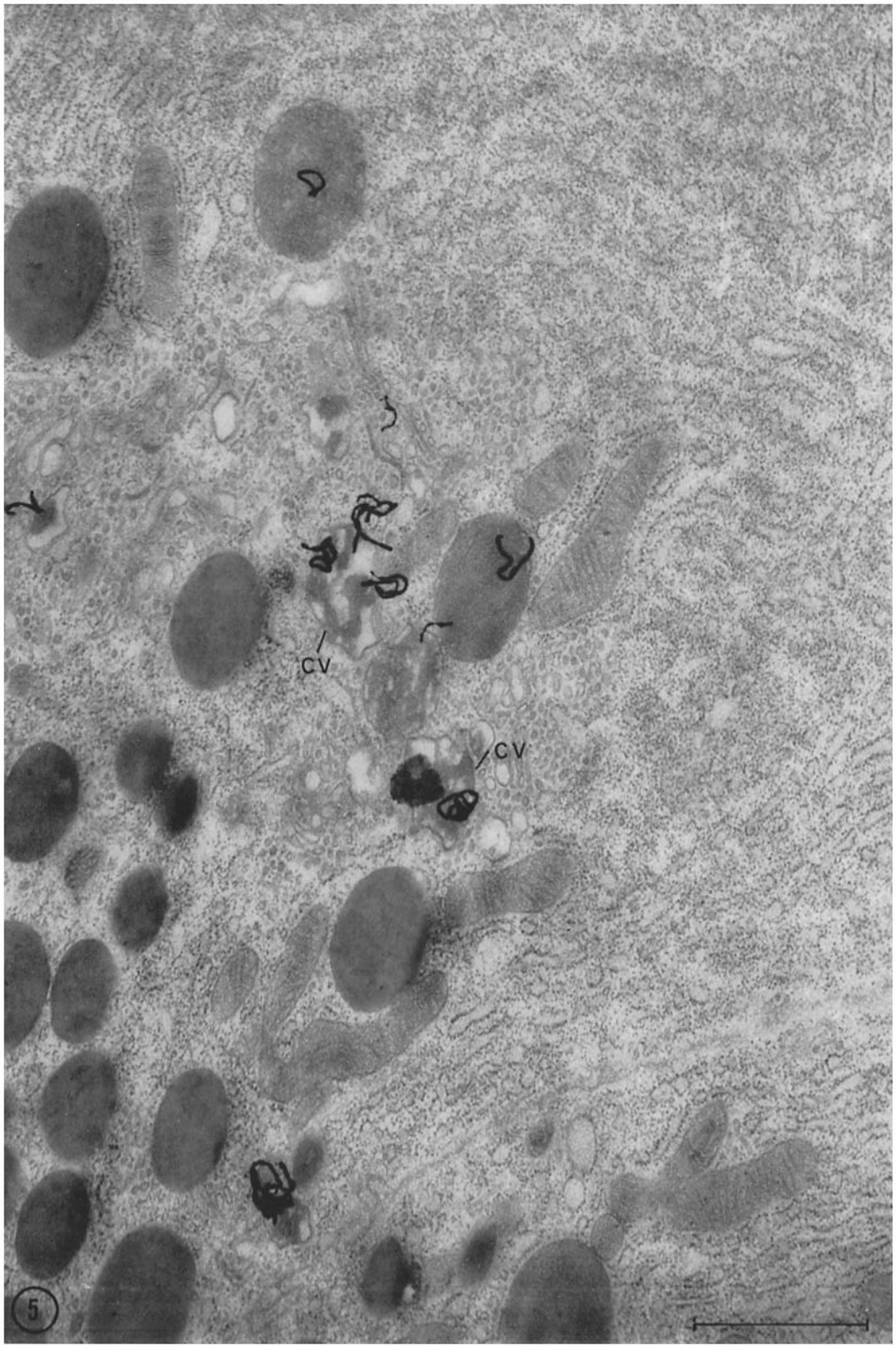
1. Light Microscopic Autoradiography

ACINAR CELLS: A general survey of the time course of the displacement of label within the exocrine cells was carried out at the light microscope level using autoradiographs of thin (~0.4 μ) sections of methacrylate-embedded material examined in the phase contrast microscope. Thirty-two animals were used and specimens of their pancreas collected and examined by autoradiography at 20 time points ranging from 4 minutes to 15 hours after the injection of the label. The time sequence thus established can be divided into four major periods:

(a) *4 to 10 minutes*: Most of the grains are found over the basal part of the cell, to the exclusion of the nucleus. A few grains appear in the centrosphere region and a few others among zymogen granules. Intracisternal granules, when present, are not labeled. No label is found in the acinar lumen.

(b) *10 to 30 minutes*: Only a few grains remain in the basal region, whereas the bulk of the label appears concentrated in the centrosphere zone (Fig. 2). A few granules, similar in appearance to zymogen granules but situated in the centrosphere

FIGURE 5 Twenty minutes after injection. The incorporated label has migrated from the rough surfaced endoplasmic reticulum, now almost completely free of radioactivity, to the Golgi complex. Most of the grains are located over condensing vacuoles (*cv*) at various stages of maturity. The peripheral clusters of small, smooth surfaced vesicles have much less activity at this time point. × 26,000.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region, are heavily labeled. The bulk of the zymogen granules contains little label. The intracisternal granules, the nucleus and the lumen remain mostly unlabeled.

(c) *30 to 60 minutes*: A large fraction of the label remains in the centrosphere region but a few heavily labeled zymogen granules can be found in the apical region. Other structures are weakly or not labeled. Toward the end of this period, the number of grains present over the centrosphere zone becomes comparable to that of grains present over zymogen granules in the apical region.

(d) *60 minutes to 15 hours*: The centrosphere zone loses its label, whereas more zymogen granules become labeled. At all times it is clear that only a fraction of the granules is labeled (that is to say, the distribution of grain counts over the granules does not follow a Poisson distribution (33) although labeled granules can now be found in all parts of the apical region). At 2 to 3 hours most of the label has moved to zymogen granules and some label begins to appear in the lumina of acini and ducts. The situation remains the same until the last time point examined (15 hours), with a gradual decrease in the over-all radioactivity.

EXPERIMENTAL VARIANTS: In a few cases the animals were fed *ad libitum* instead of going through a period of fasting. The sequence of events and its timing remained identical. In other experiments the label was injected intraperitoneally instead of intravenously. In these cases the general picture remained unchanged but the differences between various time points were less pronounced. At the earlier time points (shorter than 2 hours) it was also found that the localizations described were not well defined. This is probably due to a gradual penetration of label into the blood stream and consequently to a lengthening of the period during which the label is available to the cell.

OTHER CELLS: By comparison to the exocrine cells, the other cellular elements of the pancreas show little activity. In particular the islet cells, the centroacinar cells, and the duct cells are found to have only very low levels of incorporation.

2. Electron Microscopic Autoradiography

Using the time sequence established by light microscopic autoradiography for the intracellular transport of secretory proteins, we selected a small number of time points, judged as the most significant, for a more detailed study at the electron microscope level. We shall present here the results obtained for five time points: 5 minutes (4 minutes plus 1 minute chaser), 6 minutes, 20 minutes, 45 minutes, and 4 hours.

A) 5 MINUTES (4 MINUTES PLUS 1 MINUTE CHASER): As illustrated in Fig. 3, a large fraction of the grains appears over cell regions occupied mainly by rough surfaced elements of the endoplasmic reticulum. Qualitatively it seems that a number of these grains are associated with distended cisternae of the reticulum; hence they might be due to decays occurring in the cisternal content.

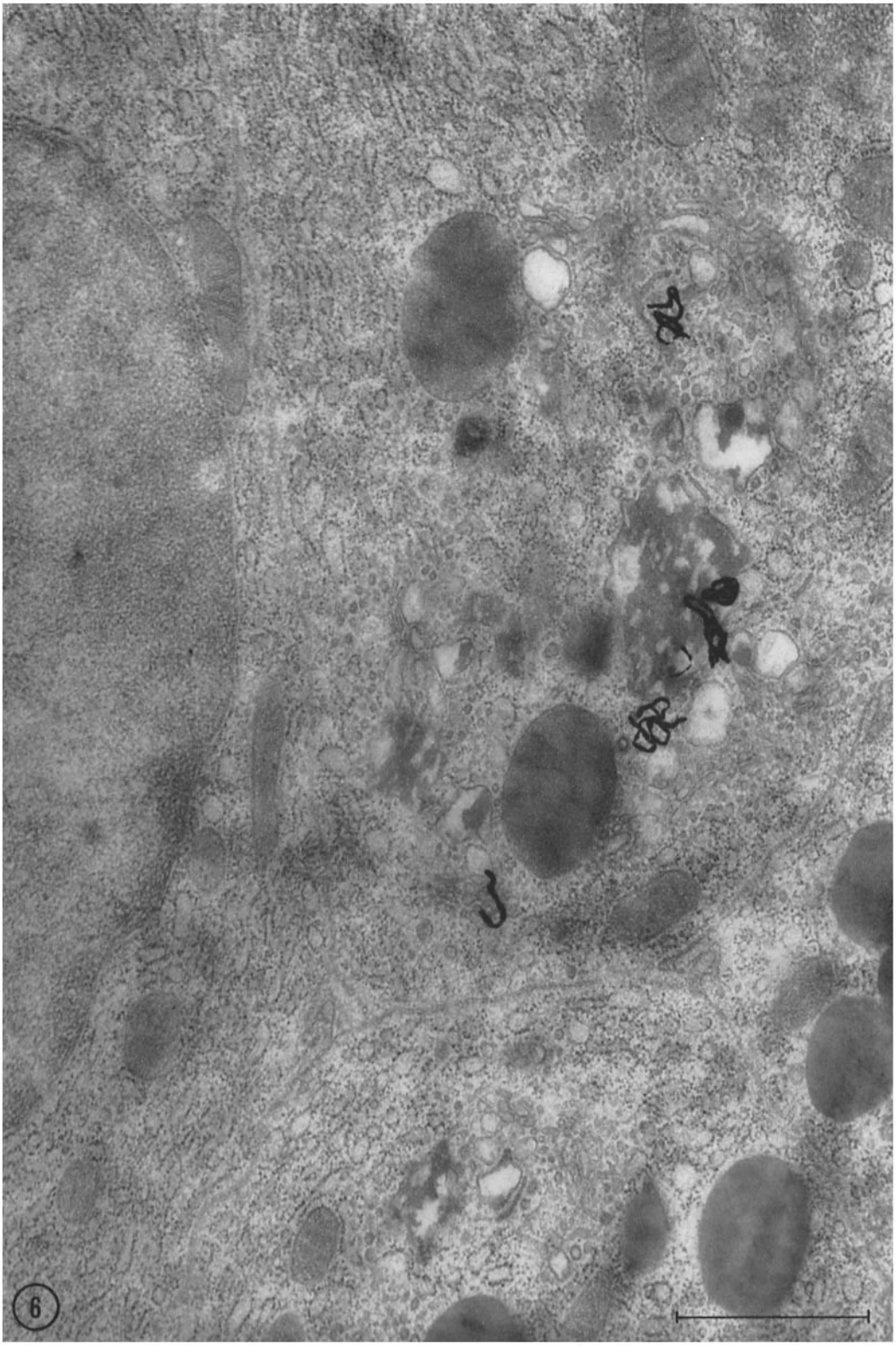
A lesser proportion of the grains is associated with the Golgi complex. As shown in Fig. 4, these grains are located more specifically over the clusters of small, smooth surfaced vesicles at the periphery of the complex. A few grains are found over condensing vacuoles which have a content of low density. Almost no grains are associated with partially filled condensing vacuoles. Other cellular structures are mostly unlabeled.

B) 6 MINUTES: The situation remains similar. Grain counts (see later) show a slight increase in the concentration of grains over the clusters of small, smooth surfaced vesicles at the periphery of the Golgi complex, and over condensing vacuoles with a light content.

C) 20 MINUTES: A drastic and rapid change has occurred in the elapsed 14 minutes (Fig. 5). The rough surfaced elements of the endoplasmic reticulum are now practically free of label, while almost all the grains are over structures of the Golgi complex. Most of these grains are on partially filled condensing vacuoles (Figs. 5 to 8), while only a few appear over other elements of the complex. Other cellular structures are mostly unlabeled.

D) 45 MINUTES: The situation is intermediate

FIGURES 6, 7, 8 Twenty minutes after injection. These micrographs show the high concentration of radioactivity, at this time, over the condensing vacuoles of the Golgi complex. Fig. 6: × 29,000. Fig. 7: × 29,000. Fig. 8: × 26,000.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between the 20-minute and 4-hour time points. The grain counts were too low to include in Table IV, but they were approximately equally divided between condensing vacuoles and zymogen granules.

E) 4 HOURS: Another drastic change in localization has occurred: the label is now almost entirely confined to zymogen granules (Fig. 9), while the elements of the Golgi complex are mostly free of autoradiographic grains. Occasionally it is possible to see the discharge of a labeled granule by a process previously described: fusion of its membrane with the cell membrane at the lumen and ejection of the granule's content (Figs. 10 and 11). Labeled dense material is also found in the lumina of the pancreatic ducts (Fig. 12).

It should be pointed out that in electron microscopical, as in light microscopical autoradiography, intracisternal granules were found unlabeled at all time points examined.

QUANTITATIVE RESULTS: Autoradiographic grains were counted, as described above, on the various preparations. The reproducibility of such counts has been established in an earlier publication (10). The results are shown in Tables IV and V.

DISCUSSION

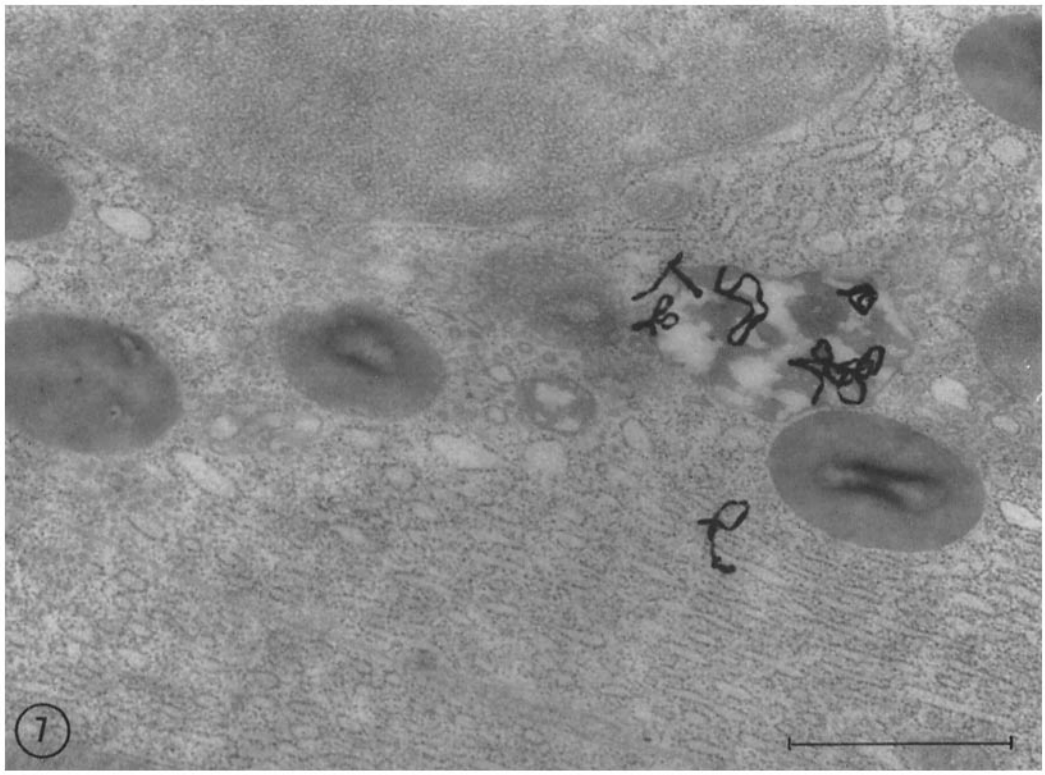
In a comprehensive series of cytochemical studies, Siekevitz and Palade have investigated: (*a*) the distribution of secretory proteins (digestive enzymes) among pancreatic cell fractions (2); (*b*) the variations in the distribution of these enzymes caused by starvation or feeding (3); (*c*) the kinetics of leucine-1-C^{14} incorporation into the mixed proteins (4), as well as into a specific protein, the zymogen α-chymotrypsinogen (6), of these cell fractions. As a result of these studies, they proposed a general hypothesis concerning the synthesis and subsequent intracellular transport and storage of digestive enzymes. According to this hypothesis, the secretory proteins are synthesized on the ribosomes attached to the membrane of the rough surfaced cisternae of the endoplasmic reticulum; they are subsequently transferred into the intracisternal space through which they travel to the Golgi region, where they are concentrated in membrane-bounded vacuoles, which thereby become zymogen granules. These move away from the Golgi zone, and accumulate progressively in the apical region of the cell, awaiting to be released in the glandular lumina following a subsequent food intake.

The beginning and the end of this postulated cycle have been satisfactorily established, for these authors (6) were able to isolate, from the ribosomes attached to the membranes of the endoplasmic reticulum, α-chymotrypsinogen with a specific radioactivity higher than in any other cell fraction, 1 to 3 minutes after the injection of leucine-C^{14}. Forty-five minutes later, the chymotrypsinogen had the highest specific radioactivity in the zymogen granule fraction. The synthesis of exportable proteins on ribosomes is in agreement with current views on protein synthesis (35, 36) and is supported by the works of Korner (37), Takanami (38), and Kirsch *et al.* (39), who showed that ribosomes detached from microsomal membranes can incorporate *in vitro* labeled amino acids into their proteins.

The intracellular storage of digestive enzymes in the form of zymogen granules was postulated more than 80 years ago by Heidenhain (40, 41) and recently confirmed by Keller *et al.* (42) and by Greene *et al.* (43). The latter isolated a reasonably pure zymogen granule fraction from bovine pancreatic homogenates and showed that it contained the same zymogens and enzymes as the bovine pancreatic juice, and that the relative concentration of these various proteins was similar in the two preparations (granules and juice).

The transfer of the newly synthesized proteins from the ribosomes to the intracisternal space was inferred, in the work of Siekevitz and Palade, from the high proteolytic and RNase activities of the microsomal subfraction which contained intracisternal granules, and from a possible precursor→ product relationship between ribosomal and intracisternal radioactive α-chymotrypsinogen. The findings related to the last point, however, are not subject to one interpretation only, since the corresponding microsomal subfractions were most probably contaminated by elements of the Golgi complex. The other stages of the process and, in particular, the passage through the Golgi complex,

FIGURES 7 AND 8 For description see p. 484.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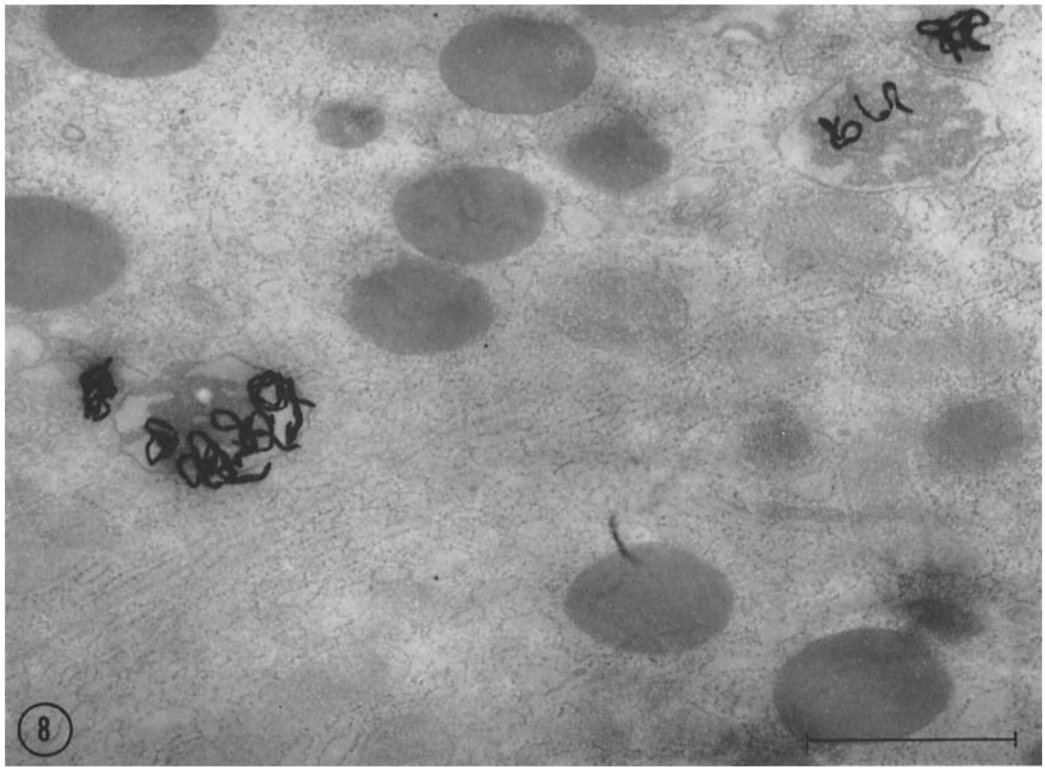
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were assumed on the basis of indirect evidence and as such were entirely speculative in character.

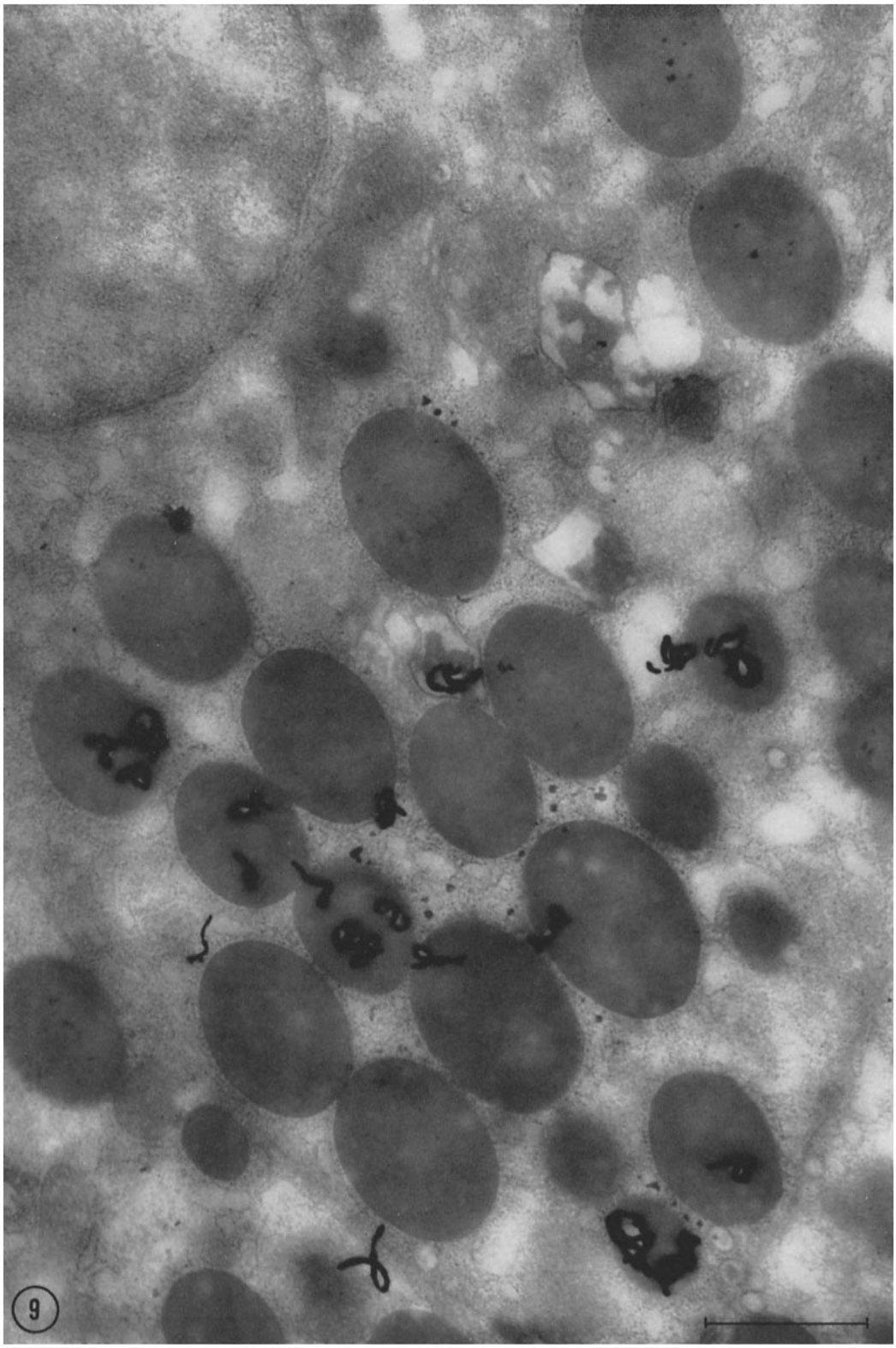
In relation to the many uncertainties involved in this hypothesis, two basic shortcomings of the current methods of cell fractionation must be pointed out. One is that fractionation is never perfect; that is to say, no fraction is exclusively constituted by one cell component, but is contaminated by other components of comparable size or density. Thus in the studies of Siekevitz and Palade, the nuclear and mitochondrial fractions were always heavily labeled but also heavily contaminated with other cellular elements (*e.g.* zymogen granules, microsomes). It was, therefore, impossible to determine without ambiguity which proportion of the label was associated with the main component of the fraction and which with its contaminants. The second shortcoming is incomplete fractionation. In particular, and of most import to this study, the Golgi complex did not appear in any recognizable form among the various fractions. Furthermore, because of the pleomorphic appearance of its components, there is little hope that all the elements of the complex could be isolated in a single fraction. The work of Greene *et al.* (43) reveals that the zymogen granule fraction contains structures similar to the condensing vacuoles of the Golgi complex. Similarly, the "microsome" fractions of Siekevitz and Palade contained some smooth membrane elements, most probably originating also in the Golgi complex. It seems likely, therefore, that the condensing vacuoles are distributed among various fractions mainly according to their density, that is to say, according to their content. Since, as was already shown in our earlier work (45, 46, 9), these vacuoles contain, at certain times after injection, most of the label in the cell and have, therefore, an enormous specific activity, any interpretation based on the results of fractionation studies alone would be erroneous. The present autoradiographic study was, therefore, started in an effort to clarify some of the many problems left unsolved by the studies mentioned.

The results obtained at the light microscope level gave us a general idea of the movement of label through the regions of the cell and were particularly useful in establishing a precise time sequence of events. The results agreed in general with the scheme being tested. Thus we found the label predominantly in the basal part of the cell a few minutes after injection; in the centrosphere region (which, we know, contains the Golgi complex) 15 to 20 minutes later; and in the zymogen granules 1 to 4 hours later. In their general aspects our results also agreed with earlier autoradiographic studies. Hansson (8), for instance, has shown the early presence of incorporated labeled amino acids in the cytoplasm of the cell and their concentration at later times around the acinar lumina. In a recent paper Warshawsky and Leblond (44) obtained similar results and showed in addition that the proximal zymogen region becomes labeled before the apical region. From this finding they postulated an involvement of the Golgi complex in the process of formation of these granules.

In our experiments a higher autoradiographic resolution was already attained at the light microscope level. It was improved in space by the use of thin sections and thin emulsion films (10) and, in time, by the use of pulse labeling obtained by intravenous, rather than intraperitoneal injection of the label. Under these conditions, we were able to demonstrate a well defined third stage, *i.e.*, the concentration of label within the centrosphere region, prior to the labeling of the zymogen granules (45, 46) (Fig. 2). Yet such studies, at the light microscope level, can at best indicate the presence of label within a certain region of the cell; they cannot, because of their low resolution, restrict the localization of the label to a given cell organ. For this further step the electron microscopic studies were indispensable.

The results obtained by electron microscopical autoradiography confirm, at least qualitatively, the early association of the label with the cavities of the rough surfaced cisternae of the endoplasmic reticulum. They suggest that the newly synthesized proteins are rapidly transferred from the rough endoplasmic reticulum to the small smooth vesicles at the periphery of the Golgi complex. An absolute proof of this transfer cannot, however, be given by autoradiography alone. We can use the data on

FIGURE 9 Four hours after injection. The grains are now associated with mature zymogen granules. × 24,000.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the relative distribution of incorporated label (Table IV), combined with those on the relative amount of the total label incorporated into proteins (Table III), to show that some of the label present in the rough endoplasmic reticulum at 5 minutes has left it and has appeared in the Golgi complex at 20 minutes. But we cannot say that all the label follows the same pathway, since some uncertainty is introduced in the data by the length contradicted by none of the known facts. Any other explanation of the autoradiographic results would involve multiple mechanisms for protein synthesis and much more complex pathways. Since we are dealing essentially with a single class of proteins, such multiplicity and complexity are unlikely.

Our results give direct experimental evidence on the role of the Golgi complex in the secretory

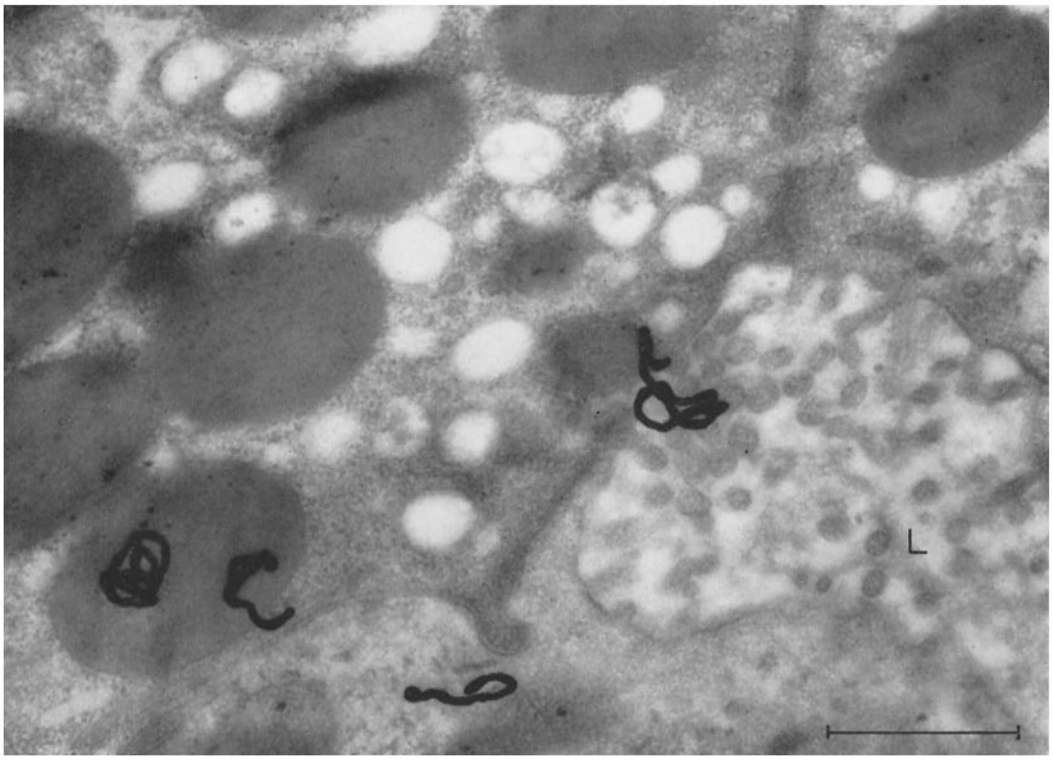

FIGURE 10 Four hours after injection. Labeled granule discharging its contents into the lumen (*L*). × 23,000.

of the intracellular pulse (too long when compared to the speed of the events under study) and by the probable presence of some non-specific binding of free leucine during fixation (as evidenced by the discrepancy between the amounts of label removed by cold TCA and by fixation). The transfer of newly synthesized proteins from the rough surfaced endoplasmic reticulum to the Golgi complex remains, however, a reasonable assumption: it explains satisfactorily our results, as well as those of Siekevitz and Palade (4, 6); fits well currently accepted notions on protein synthesis (35); and is process. They demonstrate convincingly the accumulation of secretory products and their progressive concentration, possibly by water withdrawal, in the large vacuoles centrally located in the Golgi complex, and show that this concentration leads to the formation of zymogen granules. These elements thus deserve the name of condensing vacuoles already introduced under Results. Less well established is the role played by the small vesicles. They might function as shuttle carriers between the part rough and part smooth elements of the endoplasmic reticulum and the

condensing vacuoles. At any rate, it is reasonably clear that they are an intermediate stage, for the location of at least a major part of newly made proteins, between the rough surfaced endoplasmic reticulum and the condensing vacuoles. There is little uncertainty regarding the last stages of the process: the migration of the mature zymogen granules from the Golgi complex to the apical region of the cell; their stability in that region, Golgi complex plays an important role in the economy of membranous systems of the cell.

Our results agree in general with those obtained by cell fractionation studies (4, 6). The timing of the sequence of events has been, of course, modified by the introduction of an intermediate step between microsomes and zymogen granules, *i.e.* the passage through the Golgi complex. In particular, it is clear now that the labeling of the zymogen

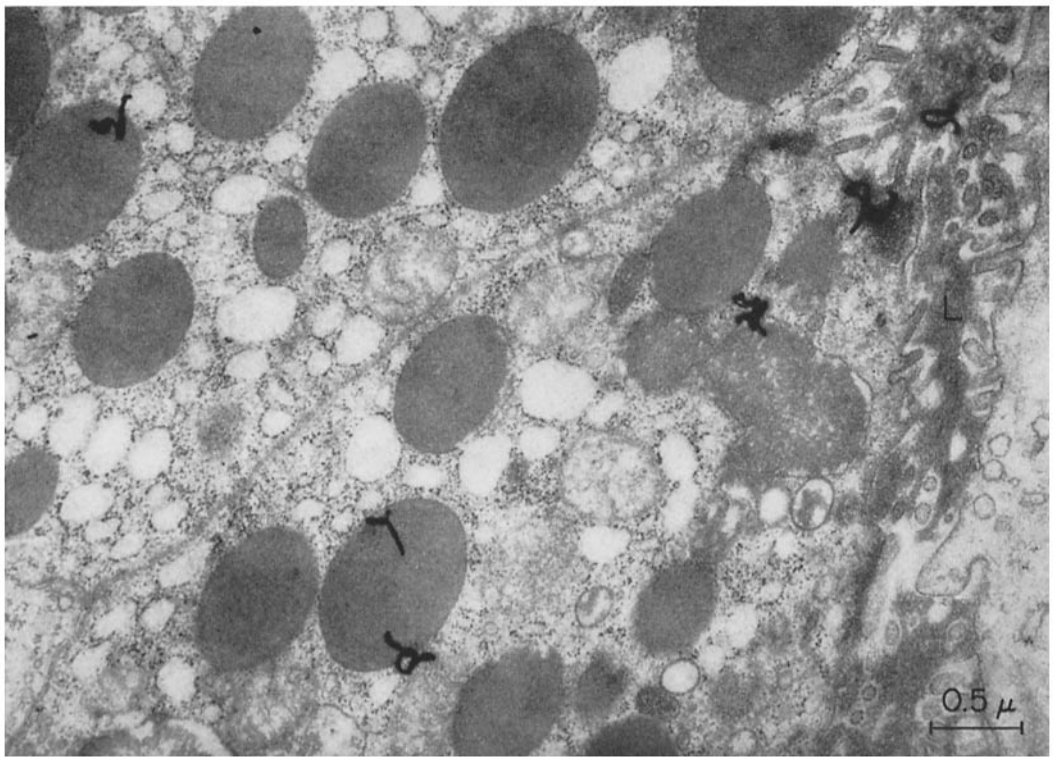

FIGURE 11 Four hours after injection. Apical part of a cell showing various stages in the discharge of labeled material. *L*, acinar lumen. × 23,000.

that is to say, the lack of exchange of label between granules; and the eventual discharge of their content in the acinar lumen. As a consequence of the process described, the membrane of a Golgi vacuole becomes the limiting membrane of a zymogen granule. During the discharge of the granule contents into the acinar lumen, this membrane can be seen to fuse and become continuous with the cell membrane (34). Some possible consequences of these observations have been considered by Palade (34). It seems likely that the granule fraction, at times as early as 20 minutes, was most probably due to the contamination of that fraction by partially filled condensing vacuoles (*cf.* 43). Similarly, it seems, as was already postulated by Siekevitz and Palade (6), that the labeling of nuclear and mitochondrial fractions was spurious and due to contamination since nuclei and mitochondria account only for a small and constant proportion of the label (Table IV).

Our results leave in doubt the functional significance of the intracisternal granules, *i.e.* the

relatively large dense bodies found within the rough surfaced cisternae of the exocrine cell (49).[2] These granules were shown to contain digestive enzyme and zymogens in high concentrations (3) and were assumed to represent an early intermediary step in the secretory process, on the basis of kinetic data on the *in vivo* incorporation of leucine-1-C^{14} into the α-chymotrypsinogen of a series of microsomal subfractions (6). These data were compatible with a precursor → product relationship between the zymogen of a subfraction consisting almost exclusively of ribosomes and that of another ("heavy") subfraction rich in intracisternal granules: the corresponding curves crossed over between 15 and 45 minutes after the injection of the tracer. In our autoradiographic experiments, however, no significant amount of label was found associated with intracisternal granules at any of the time points examined. Moreover, our results show that, at the time of the assumed cross-over, most of the label had already reached the condensing vacuoles of the Golgi region and, therefore, strongly suggest that the kinetic data obtained by cell fractionation reflect the contamination of the "heavy" microsomal subfraction by elements of the Golgi complex.

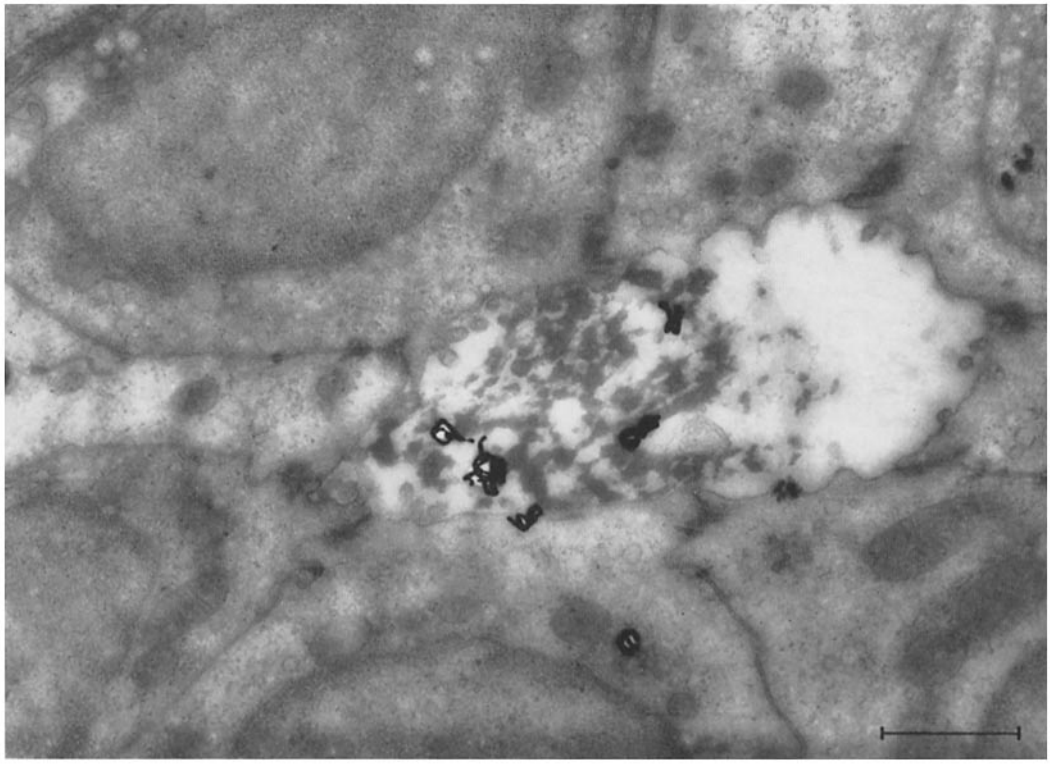

FIGURE 12 Four hours after injection. Labeled secretion material in a pancreatic duct. × 17,000.

More recent studies (9) have pointed out the absence of intracisternal granules from a sizeable proportion of exocrine cells, the difficulty of controlling their formation by variations in feeding schedule, their frequent accumulation in the rough surfaced or intermediate cisternae found at the periphery of the Golgi region, and their inability to reach the elements of the Golgi complex in a morphologically recognizable form. These findings led to the assumption that the intracisternal granules are the result of a block in the intracellular transport of secretory proteins from rough

[2] These granules represent an almost exclusive peculiarity of the pancreatic exocrine cell of the guinea pig; they are absent or only occasionally present in the exocrine cells of other species (9, 49).

TABLE IV

Distribution of Grain Counts over Various Cellular Structures at Different Times after Injection of DL-*Leucine-4,5-H*^{3}

Time after		Ergasto-		Zymogen		Mitochon-	Total grain
Label	Chaser	plasm*	Golgi region	granules	Nucleus	dria	count
min	*min*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	
4	1	67	27	1	2	3	557
6	—	53	39	2	5	1	157
20	—	11	73	10	3	3	518
240	—	11	10	73	4	2	167

* Rough surfaced endoplasmic reticulum and surrounding cytoplasmic matrix.

TABLE V

Distribution of Grain Counts over the Various Components of the Golgi Complex at Two Times after Injection of DL-*Leucine-4,5-H*^{3}

Time after			Condensing vacuoles	
Label	Chaser	Small vesicles	Light content	Dense content
min.	*min.*	*per cent*	*per cent*	*per cent*
4	1	51	42	7
20	—	24	15	61

surfaced cisternae to condensing vacuoles (9). The results here reported are compatible with this view. In any case, they definitely indicate that the intracisternal granules are not an obligatory step in the formation of zymogen granules. Their connections with the secretory process and their exact position on the timetable of the intracellular transport of secretory proteins remain to be clarified by future work.

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