ELECTRON MICROSCOPIC RADIOAUTOGRAPHY OF THIN SECTIONS: THE GOLGI ZONE AS A SITE OF PROTEIN CONCENTRATION IN PANCREATIC ACINAR CELLS

LUCIEN G. CARO, Ph.D.

From The Rockefeller Institute

ABSTRACT

Electron microscopic radioautographs of guinea pig pancreatic exocrine cells were obtained by covering thin sections (∽ 600 A) of OsO_4-fixed, methacrylate-embedded tissue with thin layers of Ilford K-5 nuclear research emulsion. After an exposure of 13 days, at 4°C., the preparations were photographically processed, stained with uranyl acetate, and examined in an electron microscope. The label used was leucine-H^3 injected intravenously 20 minutes before collection of the specimens. Conventional radioautographs of thicker sections (0.4 micron) were also examined in a phase contrast microscope. The advantages obtained from electron microscopic radioautography are: the higher radioautographic resolution (of the order of 0.3 micron) due to the thinness of the emulsion and the specimen, and a high optical resolution permitting a clear identification of the labeled structure. In the guinea pig pancreas this technique demonstrated that, at the time studied, newly synthesized proteins were concentrated in the structures of the Golgi complex and especially in large vacuoles partially filled with a dense material. The vacuoles are probably a precursor to the secretion granules (zymogen granules) in which the label becomes segregated at a later time. These observations demonstrate directly the role of the Golgi complex in the secretion process. They also illustrate the possibilities of this method for radioautography at the intracellular level.

With the rejuvenation of descriptive cytology brought about by electron microscopy, there has arisen a need for high resolution cytochemical methods. Radioautography can, under certain conditions, provide such a method. When the low energy beta emitter tritium (1) is used and when the material under study is reasonably thin, a resolution of the order of 1 micron can be expected (2) with conventional methods, at the light microscope level. Theoretically, much higher resolutions could be obtained by using extremely thin sections as well as extremely thin emulsion layers (3, 4). However, the limitations of the light microscope restrict the improvement that can be achieved by this approach, since fairly thick sections are needed and the finer structural details of the cell are not clearly resolved. The first attempts at direct examination of radioautographs of biological material in the electron microscope were performed by Liquier-Milward on tumor cell nuclei labeled with Co^{60} (5) and by O'Brien and George on sections of yeast cells labeled with Po^{210} (3). These attempts were interesting but mainly exploratory in nature, high resolution being precluded to some extent by the high energy particles emitted in both cases.

Using whole bacteria labeled with tritiated thymidine, van Tubergen (4) was able to demonstrate a considerably higher autographic resolution than was shown in the attempts mentioned (3, 5) and a somewhat better resolution than could be obtained in radioautography at the light microscope level.

In the work presented here we attempted to take advantage of both the high radioautographic resolution obtainable with thin emulsions and thin sections and the high optical resolution given by electron microscopy. The material chosen was the pancreatic exocrine cell of the guinea pig. In the course of investigating, by radioautography, the pathways of protein synthesis and transport in these cells it was found that, 15 to 20 minutes after the injection of a labeled amino acid, concentrations of radioactivity were detected on the apical side of the cell nucleus, and it was suspected that these concentrations were associated with the centrosphere region. The various morphological entities present in this region are not, however, satisfactorily resolved in the phase contrast microscope. It appeared that electron microscopic radioautography might not only provide a clear identification of the Golgi complex, but, furthermore, give us more precise information regarding the association of label with the various structures seen in that complex.

MATERIALS AND METHODS

One male albino guinea pig weighing 250 gm. was starved 48 hours, then fed 1 hour before the experiment in order to initiate a secretory cycle (6). Under Nembutal anesthesia the animal received by intravenous injection 1.2 ml. of physiological saline containing 2 millicuries of DL-leucine-4,5-H^3 (from New England Nuclear Corp.) with a specific activity of 3570 mc./mM. Twenty minutes later a portion of the pancreas was fixed by direct injection into the tissue of 2 per cent OsO_4 in acetate-veronal buffer, pH 7.5. Regions which turned brown rapidly were removed and cut into small blocks, and fixation was continued in the cold, for 2 hours. The blocks were dehydrated and embedded in methacrylate according to standard methods. Fairly thin ($\sim$ 600 A) sections, usually pale silver after xylene vapor treatment (7), were cut on a Porter-Blum microtome and picked up on a specimen screen coated with a Formvar film and a thin carbon layer. Each screen was attached by its edge with a minute drop of rubber cement to a standard microscope slide previously "subbed" with a mixture of 0.1 per cent gelatin and 0.01 per cent chrome-alum.

Under safelight illumination (Kodak Wratten A0 filter) 5 gm. of Ilford K-5 nuclear research emulsion were placed in a Coplin jar with 40 ml. of 0.01 per cent KBr in water at 45°C. After 10 minutes the mixture was stirred with a clean glass rod and was ready to use. A small amount of emulsion was collected by capillarity in the tip of a glass micropipette, and a bubble was formed by blowing gently into the pipette. The bubble, blown to a diameter of about 2 cm., was deposited on the microscope slide in such a way that it covered the specimen screen. The slide was then placed on a horizontal surface until the bubble burst by itself. Of various methods tried (dipping, loops, etc.), the one described here, though far from perfect, was found to yield the most uniform films. The slides were dried under a stream of dry, filtered air, stored in a light-tight container with a small amount of Drierite, and placed in a CO_2 atmosphere at 4°C. (8) for a period of 13 days. They were developed in Kodak D19 for 2 minutes at 20°C., fixed 5 minutes in Kodak acid fixer, and washed briefly in running distilled water.

After drying, the specimen screens were lifted from the glass slides and the sections were stained by a 10 minute immersion in 1 per cent uranyl acetate in 50 per cent ethanol (9) followed by a wash in running distilled water. They were examined and micrographed in a Siemens Elmiskop I.

Thicker sections (0.4 micron) of the same material were also cut and placed directly on a "subbed" slide. The embedding material was removed with amyl acetate and the slide coated by dipping (10) in a solution of 20 gm. of K-5 per 40 ml. of 0.01 per cent KBr, drying in a vertical position, and exposing and developing as described above. Micrographs were taken, using a Zeiss attachment camera, in a Reichert Zetopan phase contrast microscope, with a N.A. 1.25 oil immersion lens. For maximum contrast the slides were covered with a coverslip mounted in water.

In certain preparations a reaction seemed to have occurred with the copper specimen screen, resulting in very high numbers of background photographic grains close to the metal and reduced grain counts in other areas. This effect was minimized by using as thick a layer of Formvar and carbon as was consistent with reasonable image quality. A certain amount of a dense contaminant, probably precipitated silver from the emulsion, was found in most areas of the sections. In most cases it was easily distinguishable from photographic grains (Figs. 3 to 5).

RESULTS

Morphology

The morphology of the pancreatic exocrine cell has been described at the electron microscope level by Sjöstrand and Hanzon (11, 12) for the mouse, Ferreira (13) for the rat, Ekholm and Edlund (14) for man, and Palade and Siekevitz (15) for the guinea pig. In all cases the basal region contains the nucleus and an extensive network of endoplasmic reticulum, while the apical region contains, in the normal animal, numerous zymogen granules and many vesicles of rough-surfaced endoplasmic reticulum. Of more immediate concern to this study is the centrosphere region. It is occupied, in part, by irregular formations of small vesicles, flattened cisternae, and larger vacuoles, all bounded by smooth membranes. Occasional clumps of a material approaching in density the content of the zymogen granules appear in the vacuoles of the centrosphere region. These vacuoles exist in a variety of sizes and shapes (Figs. 5 and 6). Some of them have dimensions similar to those of zymogen granules, but are distinguished by a less homogeneous content (Fig. 5). Vesicles, cisternae, and vacuoles have all been identified by various authors (16, 12, 15) as parts of the Golgi zone. They appear often grouped together in a number of distinct clusters interspaced by mitochondria, elements of the rough-surfaced endoplasmic reticulum, and zymogen granules. These seemingly isolated aggregates might, however, represent sections through various branches of a single continuous structure. In phase contrast the Golgi zone is very poorly defined and its intermingling

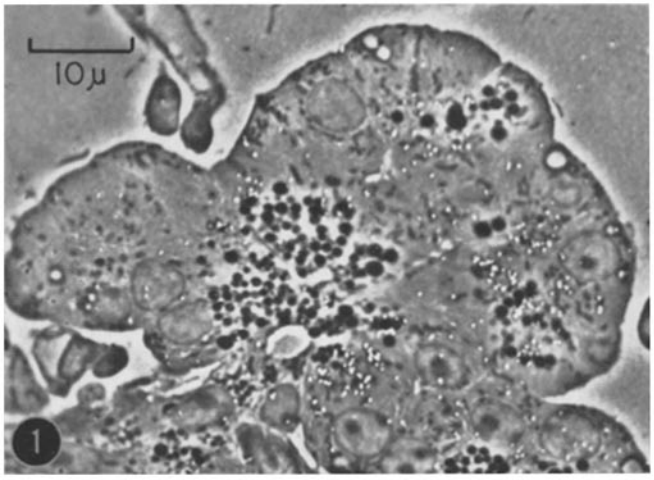


FIGURE 1

Phase contrast photograph of a radioautograph of a guinea pig pancreatic acinus 20 minutes after injection of leucine-H^3. The photographic grains, slightly out of focus, appear as bright points. Very few grains appear in the interacinar spaces, showing low background. This section, 0.4 micron thick, was cut from methacrylate-embedded material. Many cellular details are visible (nuclei, mitochondria, zymogen granules, a few fat globules surrounded by mitochondria, etc.). × 1400.

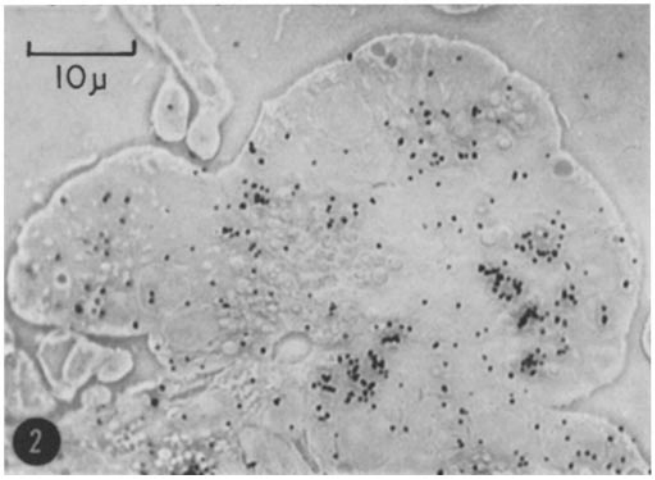


FIGURE 2

Same area in bright field. In several cells clusters of grains are located between the nucleus and the apical aggregates of zymogen granules, presumably the centrosphere region. × 1400.

with other cytoplasmic structures makes its positive identification difficult.

Radioautography

Fig. 1 shows, in phase contrast, a radioautograph of a thin (0.4 micron) section through an acinus of a guinea pig pancreas, fixed 20 minutes after injection of leucine-H^3. Fig. 2 represents the same area in bright field. It will be observed that the photographic background is quite low, so that most grains represent H^3 decays. Examination of the autograph reveals that much of the label is concentrated in preferred regions which, in several cases, appear to be clearly localized between the nucleus and the agglomeration of zymogen granules. An association between these clusters of grains and the centrosphere region might thus be postulated. A small number of zymogen granules are in close contact with photographic grains. Almost invariably such granules are located in or near the assumed centrosphere region. Very few grains appear over the mass of zymogen granules located around the lumen, to the lower left of the center. Some grains are associated with other structures: nuclei, possibly mitochondria, and regions of the cytoplasm other than the centrosphere.

Fig. 3 shows an electron microscopic radioautograph of similar material. A number of acinar cells and one centroacinar cell appear on the picture. The photographic grains appear as dense, randomly coiled filaments of silver. The silver halide crystals removed during processing have left a pattern of light, poorly outlined spots in the gelatin layer. Such a pattern is visible over all sections and is especially clear over the dense zymogen granules. In spite of it, most of the structural details present can be clearly recognized. Most of the grains present are seen to be associated with structures of the Golgi complex in three acinar cells. Only two grains are found in the basal cytoplasm. These might be associated with elements of the rough-surfaced endoplasmic reticulum.

Fig. 4 shows an autograph of three acinar cells, cut at the level of the centrosphere region. In this field each grain is seen to be closely associated with vesicles, cisternae, and vacuoles characteristic of the Golgi complex. In some but not all of the cases, the association might be with a vacuole containing dense material, also part of the Golgi complex.

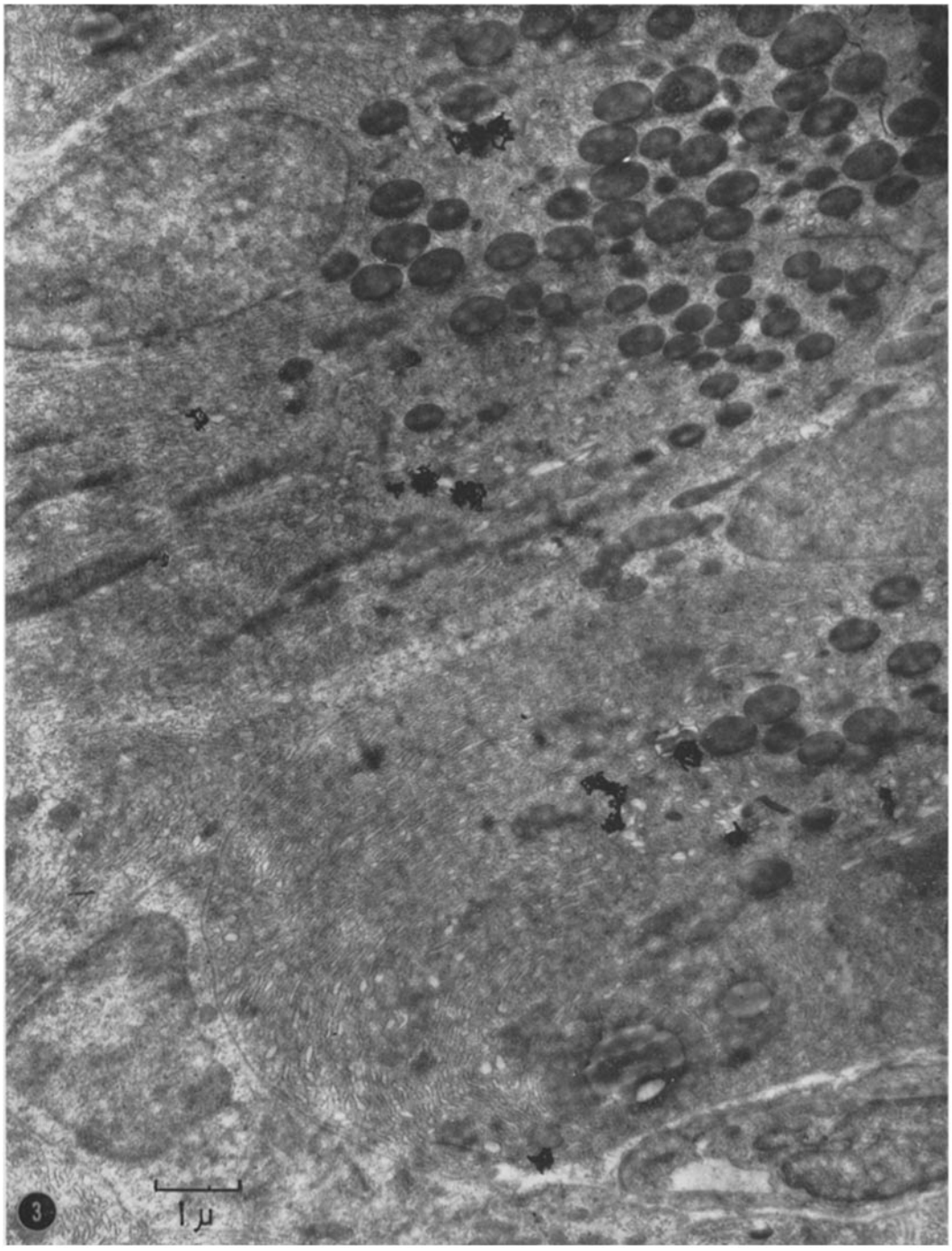
In Fig. 5, three of the grains are associated with almost completely filled vacuoles, while one is associated either with a small vacuole containing a clump of dense material or with a group of vesicles. All these structures are part of the Golgi complex. In Fig. 6, five grains are associated with three partially filled vacuoles included in a small area of the Golgi complex.

In a survey of a small area of the same material the following grain counts were obtained: Golgi zone, 50 grains (30 of them over partially filled vacuoles); zymogen granules, 10 grains (all these granules were in close contact with Golgi complexes); basal cytoplasm, 5 grains; mitochondria, 2 grains. A survey of a larger region from another preparation gave the following figures: Golgi zone, 148 grains (107 of them over partially filled vacuoles); zymogen granules, 22 grains (17 of these on granules which were in close contact with the Golgi complex); basal cytoplasm, 18 grains; mitochondria, 11 grains; nuclei, 9 grains.

Thus, in these preparations, approximately 70 per cent of the total label appeared associated with the Golgi complex, a large proportion, but not all, of it being connected with vacuoles con-

FIGURE 3

Electron micrograph of a radioautograph from the same material. The section was stained with uranyl acetate. The photographic grains appear as dense, twisted filaments. Some of the larger ones probably arose from two silver halide crystals. The non-exposed crystals have been dissolved out in processing and have left a pattern of holes in the gelatin layer especially visible over the zymogen granules. In three different cells, eleven photographic grains appear above structures which are part of the Golgi complex (smooth membrane vesicles, cisternae, and vacuoles). Two isolated grains only seem to be related to rough-surfaced elements of the endoplasmic reticulum or to the cytoplasm between them. × 13,000.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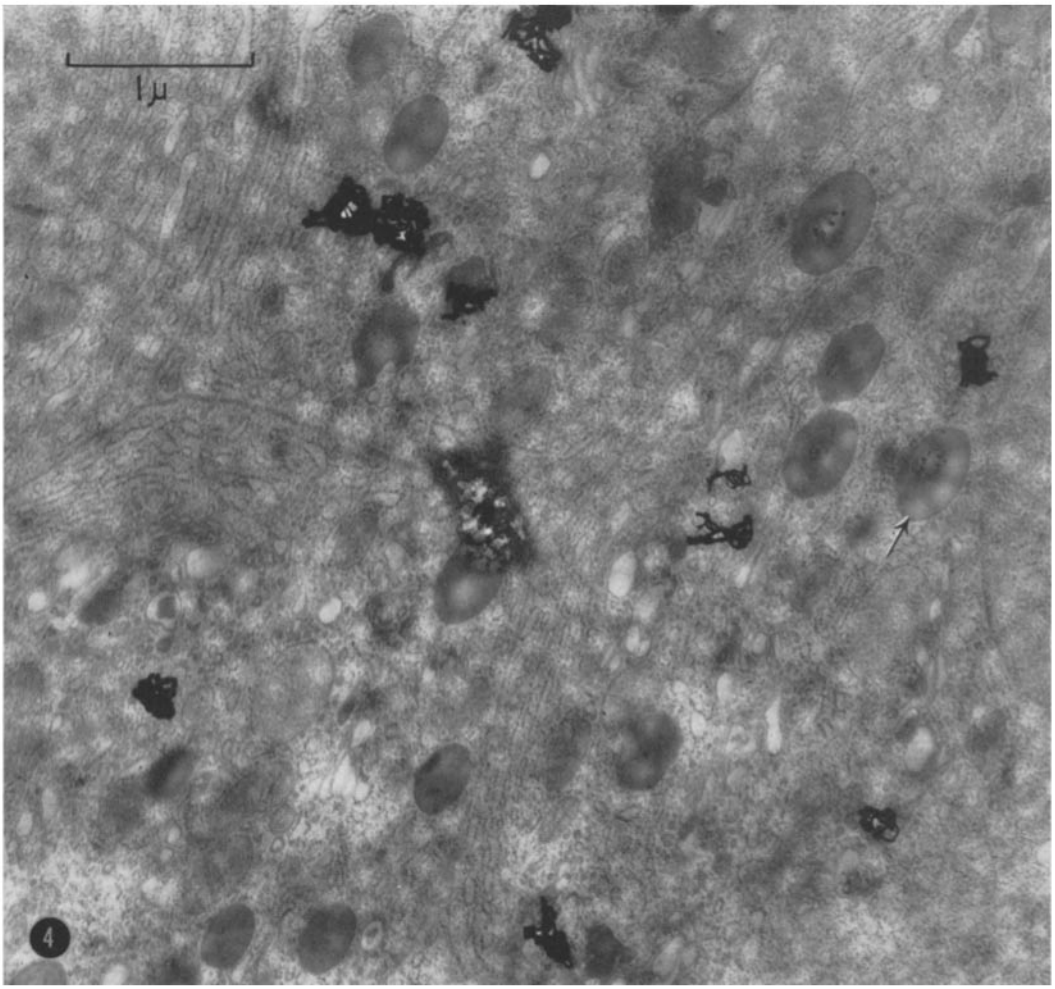

FIGURE 4

This section cuts three acinar cells at the level of the Golgi zone. Each photographic grain in this autograph appears above regions containing vesicles, cisternae, and vacuoles limited by a smooth membrane and readily identified as components of the Golgi complex. A dense clump of contaminant, probably originating from the emulsion, can be seen in the center. A finer precipitate appears selectively over zymogen granules, usually in the emplacement left by a dissolved crystal (*e.g.*, spot above the one marked by an arrow). Both formations are easily distinguishable from developed grains. The arrow indicates the clear area left by a silver halide crystal removed during photographic processing. × 26,000.

taining a non-homogeneous dense material. Furthermore, those zymogen granules showing some labeling were preferentially located near the Golgi complex. Specific activities were not estimated, but in view of the small relative space occupied by the Golgi zone they should be quite high.

DISCUSSION

Two clear advantages of the method used in this study are apparent. One results from the improved optical resolution provided by the electron microscope. It has thus become possible to identify clearly the Golgi complex as the site of high concentration of label. Furthermore, certain

structures, within that complex, have been shown to possess exceptionally high levels of radioactivity. Such unambiguous identification of structures would have been impossible in phase contrast. The other advantage is the high radioautographic resolution obtained. Even in our general method of radioautographic preparation some effort had been made in that direction. The use of thinner sections than usual, made possible by methacrylate embedding, and of thin layers of emulsion ensured good resolution, at the level of the optical microscope. This, as we have seen, is not always sufficient. Here again the electron microscope becomes the indispensable tool, since sections thinner than 0.1 micron become almost invisible under phase contrast optics.

Some limitations must be noted. The silver halide crystals in the emulsion used are still fairly large. They were found, by measurement in the electron microscope, to have a diameter slightly less than 0.2 micron. When the emulsion was applied as described above, these grains formed a closely packed monolayer over the section. The distribution of these grains is not, however, always uniform, and empty areas as well as areas where a double layer was formed occurred occasionally in the best preparations. This makes precise quantitation impossible at this point. A certain sacrifice in image quality and in resolution has to be accepted. It is caused by the several layers of Formvar, carbon, and gelatin in which the specimen is sandwiched. This is not a serious drawback, since enough contrast can still be achieved to make identification of all important structures possible. In this respect the staining of the section proved to be an essential step. To avoid possible complications, we chose to stain the section, through the emulsion, after processing, but staining with various stains before adding the emulsion layer might be possible. In this case careful controls would have to be done to ensure that grains do not arise as a result of chemical or, in the case of uranium stains, radioactive reactions.

Calculations based on the distribution of silver halide crystals, the thickness of the section, and certain simplifying assumptions regarding the range of the emitted beta particles lead us to expect a radioautographic resolution somewhat better than 0.5 micron and probably of the order of 0.3 micron (average distance of grain from source). This is confirmed by the results of van Tubergen (4). Attempts at measuring the actual resolution obtained with thin-sectioned material are being made and will be reported elsewhere. It seems certain that extremely high resolution could be achieved by using thinner sections and thinner emulsions with a high density of very fine grains. We have tried to show, however, that useful results could be obtained with available emulsions and current electron microscopical techniques.

The biological implications of these results will be discussed in more detail in a later publication. We shall therefore limit the present discussion to a few remarks. Evidence has been presented to show that incorporated leucine-H^3 is concentrated in the Golgi region of pancreatic acinar cells 15 to 20 minutes after injection of the label into the blood stream. In a series of integrated morphological and biochemical studies Siekevitz and Palade (15, 6, 17, 18, 19, 20) have shown that leucine is mostly incorporated into newly synthesized proteins, and that at very early time points it is associated with the microsomal fraction, whereas at later times it appears in the zymogen granule fraction. They postulated that the Golgi zone played a role in the concentration and "packaging" of newly synthesized enzymes into zymogen granules. Several authors (*e.g.* 12, 21, 22) have described the partially filled vacuoles of the Golgi region and attributed to them, on morphological grounds, the role of zymogen precursor (see Palay (22) for a general review of the literature on this subject). We have shown that newly synthesized proteins are accumulated in the Golgi complex and particularly in association with partially filled vacuoles. Taking into consideration other available evidence, this implies that these proteins are funneled through the Golgi complex in transit from their site of synthesis, on the rough elements of endoplasmic reticulum, to their site of storage, the zymogen granules. Our findings support therefore the hypothesis that the Golgi complex plays an important role in the formation of secretory granules.

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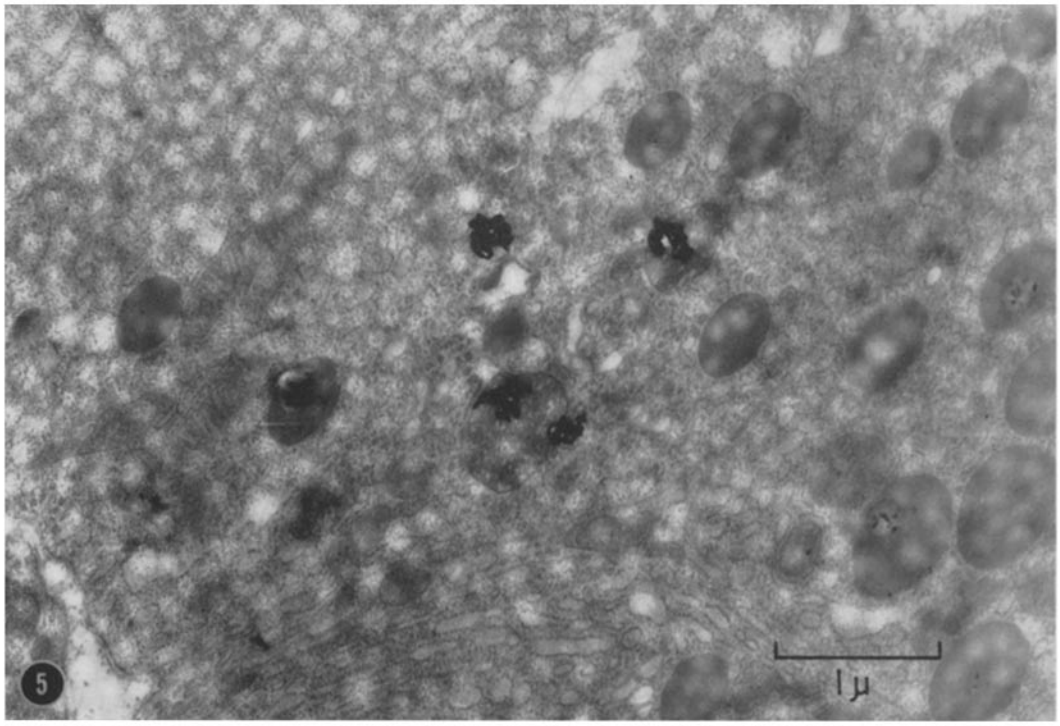
FIGURE 5

Part of a Golgi zone, surrounded by a rough-surfaced endoplasmic reticulum, zymogen granules, and mitochondria. All grains are over elements of the Golgi complex. Three grains appear over almost completely filled vacuoles. × 23,000.

FIGURE 6

Another similar zone. All five grains visible are associated with partially filled vacuoles of the Golgi region. × 23,000.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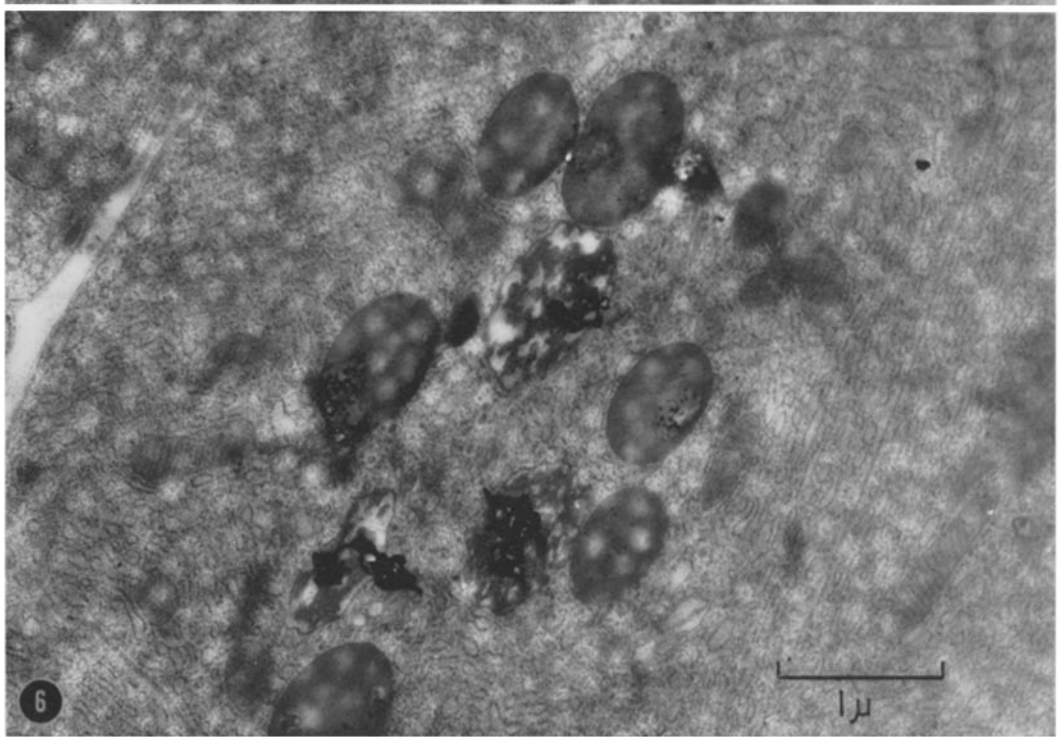
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