IMMUNOCYTOCHEMICAL LOCALIZATION OF AMYLASE AND CHYMOTRYPSINOGEN IN THE EXOCRINE PANCREATIC CELL WITH SPECIAL ATTENTION TO THE GOLGI COMPLEX

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ABSTRACT

Affinity-purified, monospecific rabbit antibodies against rat pancreatic α-amylase and bovine pancreatic α-chymotrypsinogen were used for immunoferritin observations of ultrathin frozen sections of mildly fixed exocrine pancreatic tissue from secretion-stimulated (pilocarpine) rats and from overnight-fasted rats and guinea pigs. The labeling patterns for both antibodies were qualitatively alike: Labeling occurred in (*a*) the cisternae of the rough endoplasmic reticulum (RER) including the perinuclear cisterna, in (*b*) the peripheral area between the RER and cis-Golgi face, and (*c*) all Golgi cisternae, condensing vacuoles, and secretory granules. Labeling of cytoplasmic matrix was negligible. Structures that appeared to correspond to rigid lamellae were unlabeled. Differences in labeling intensities indicated that concentration of the zymogens starts at the boundary of the RER and cis-side of the Golgi complex. These data support the view that the Golgi cisternae are involved in protein processing in both stimulated and unstimulated cells and that Golgi cisternae and condensing vacuoles constitute a functional unit.

KEY WORDS secretory proteins . Golgi complex . exocrine pancreas . immunoferritin cytochemistry . cryoultramicrotomy

The intracellular route that secretory proteins take during their migration from the membrane-bound polyribosomes towards the secretory granules has not yet been mapped in detail. A main problem is what role the Golgi complex plays in protein processing. The Golgi complex in the exocrine pancreatic cell, as in other secretory cells, is composed of a stack of cisternae with peripheral elements consisting of vesicles and tubules at its cis- (immature, proximal, convex, outer) side, and condensing vacuoles and small membranous structures at its trans- (mature, distal, concave, inner) side. Electron microscope autoradiographical and cell fractionation results made plausible that newly formed proteins move via the peripheral elements (11, 26) across the stack of Golgi cisternae (12, 15, 25, 26) towards the condensing vacuoles. On the other hand, Novikoff and co-workers (20, 21) concluded from cytochemical work on marker enzymes of trans-Golgi cisternae and lysosomes that concentration and packaging of secretory proteins are performed by a distinct cell structure, the so-called GERL, that consists of rigid lamellae with dilated portions, the condensing vacuoles. GERL is located at some distance from the trans-side of the Golgi complex. These authors further postulate

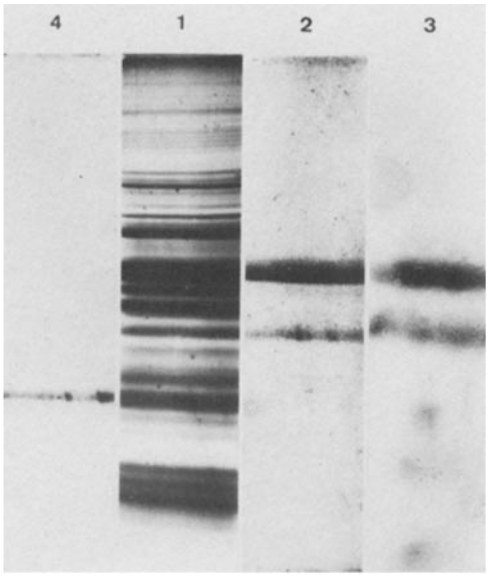


FIGURE 1 Specificity test of the antibodies used. (*1*) SDS electropherogram of rat pancreas homogenate. Coomassie Brilliant Blue staining. (*2*) 30-μm-thick section of the same gel. Indirect immunoperoxidase staining against amylase. Two DAB-positive bands can be seen. (*3*) Starch digestion in agarose gel by a corresponding section, but anti-amylase bound in a first step to the section was allowed to react with fresh amylase from pancreas homogenate in a second step. The digestion pattern coincides with the DAB pattern in *2*. (*4*) As *2*, but stained against chymotrypsinogen. The gel section shows one DAB-positive band.

that secretory proteins may pass directly from the rough endoplasmic reticulum (RER) into the rigid lamellae and to the condensing vacuoles ". . .without passing through either the Golgi apparatus or some 'lock-gate' mechanism in the Golgi zone" (20).

There are no enzyme cytochemical methods available to localize pancreatic enzymes themselves *in situ*. Immunocytochemistry seems therefore to be the most promising approach for pancreatic secretory proteins. The few of such studies that have appeared so far (14, 22), however, lacked detailed structural information on the Golgi complex and associated substructures.

Recently, a technique was developed which greatly improves the definition of the ultrastructure in ultrathin cryosections (30), and preliminary observations showed that it indeed allows a good identification of the Golgi components (6, 29, 32). In this study, we used this technique in combination with immunoferritin labeling (31) to study the precise localization of two zymogens in unstimulated and secretion-stimulated pancreatic cells.

MATERIALS AND METHODS

Antibody Fractionations

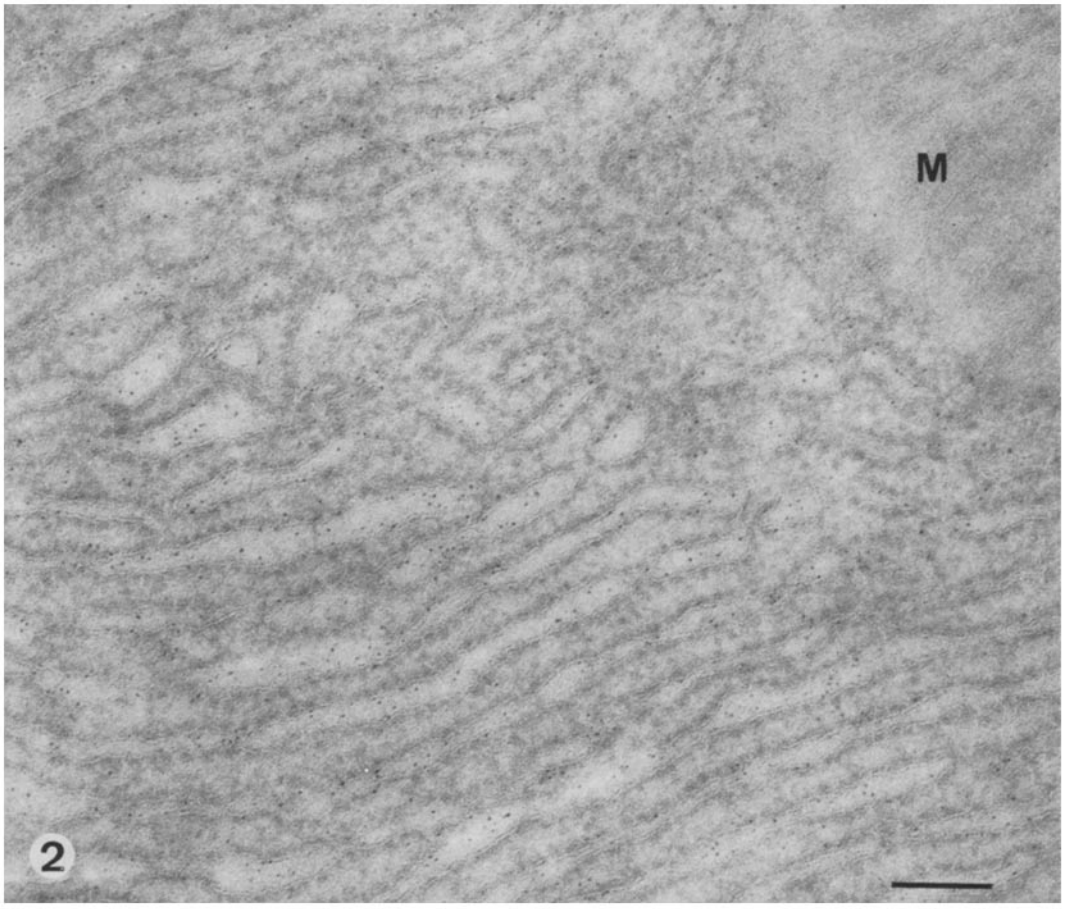
α-Amylase was kindly donated by Dr. W. Linssen (Center for Electron Microscopy, Utrecht, The Netherlands), who purified the enzyme from rat pancreas by glycogen precipitation (17, 18). Bovine pancreatic α-chymotrypsinogen A (6 × crystallized, chromatographically pure) was purchased from Sigma Chemical Co. (St. Louis, Mo.). Rabbit antisera to both zymogens were prepared by repeated subcutaneous immunization of New Zealand white rabbits. Antibody fractions were purified by affinity chromatography on zymogen-loaded Ultrogel AcA22 (LKB Produkter, Stockholm, Sweden), activated with glutaraldehyde (27). The gels were incubated with the antisera at room temperature for 2 h. After thorough washing with phosphate-buffered saline (PBS), the antibodies were eluted with 0.2 M HCl adjusted to pH 2.2 with glycine. The acid eluate was neutralized immediately with 1 M Na_2HPO_4.

Goat anti-rabbit IgG, purified similarly on rabbit IgG-AcA22 gel, was conjugated with ferritin (EM grade, Polysciences, Inc., Warrington, Pa.) after glutaraldehyde activation (13). For immunostaining, we used the antibodies at concentrations of 50–200 μg/ml in PBS.

All micrographs were taken from ultrathin frozen sections of rat or guinea pig pancreas, indirectly immunolabeled with ferritin for the demonstration of amylase or chymotrypsinogen. Unless indicated otherwise, the sections were stained with alkaline and acidic UA, and were embedded in methyl cellulose. Bars, 0.2 μm.

FIGURE 2 Rat; stimulated; amylase. Ferritin is almost exclusively located over the cisternae and membranes of the RER. Comparison with Fig. 3 shows that the UA staining has given the membranes a bilayered appearance and has increased the density of the ribosomes. *M*, mitochondrion. × 66,000.

FIGURE 3 Rat; unstimulated; chymotrypsinogen; unstained. Labeling pattern as in Fig. 2. Membrane delineation and ribosome contrast are less than in Fig. 2. The RER cisternae are somewhat distended during preparation. *M*, mitochondrion. × 40,000.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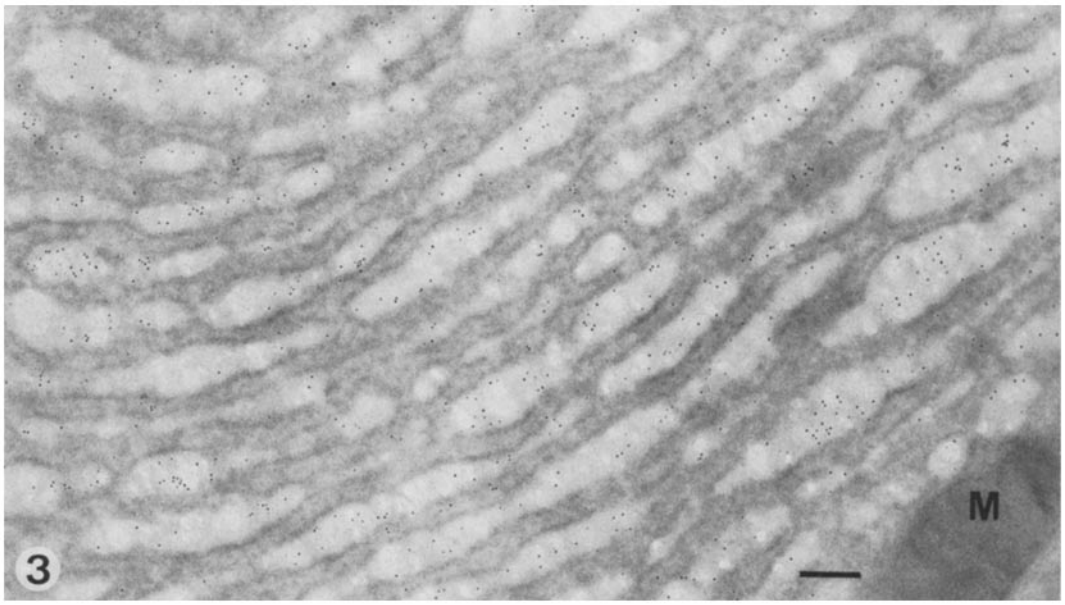
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The affinity-purified rabbit antibody preparations were tested for specificity by the SDS polyacrylamide gel immunoperoxidase (SGIP) method according to Van Raamsdonk et al. (23). SDS gel electrophoresis (16) was carried out with samples of rat pancreas homogenate over a distance of 5 cm of a 4-mm-thick slab gel with a gradient of 9–18% acrylamide. Separate lanes were cut out from the gels and were frozen on the object stage of a CO_2-cooled freezing microtome (type 1205, Jung, Heidelberg, West Germany). A number of 30-μm-thick sections were cut longitudinally from their surface. The rest of the gel (~2 mm thick) was stained with Coomassie Brilliant Blue. Gel sections were incubated for 1–18 h with the purified anti-amylase or anti-chymotrypsinogen immunoglobulin fractions, and subsequently with peroxidase-conjugated goat anti-rabbit IgG (Miles Laboratories, Inc., Elkhart, Ind.) for 1–2 h. The Miles preparation was diluted 1:25 with PBS. The immunoreaction was visualized by the peroxidase-enzyme reaction. This was done by dropping some substrate solution on the gel sections which were spread over glass slides. The substrate solution contained 5 mg/10 ml of 3,3'-diaminobenzidine·4 HCl (DAB) (Serva, Feinbiochemica, Heidelberg, West Germany) in PBS. H_2O_2 was added to a final concentration of 0.01% just before use. The DAB solution without H_2O_2 was stored in 2-ml samples at −20°C. The reaction was stopped after 2–3 min by transferring the sections to distilled water. After a short wash, the sections were dried on the glass slides.

The SGIP tests demonstrated that the antibody fractions were monospecific to rat pancreatic amylase and chymotrypsinogen (Fig. 1). To prove that the two anti-amylase staining bands in the gel sections (Fig. 1, lane 2) resulted from antibodies with an affinity to amylase, sections were immuno-incubated as described for the peroxidase staining, except that fresh (non-SDS-treated) pancreas homogenate was applied instead of the peroxidase goat anti-rabbit IgG. The purpose was to catch amylase from the homogenate on free binding sites of IgG molecules which were already bound to amylase antigen in the gel section. To demonstrate this newly bound amylase in the gel sections, we made use of its enzyme activity. The sections were spread, upside down, over 0.3-mm-thick sheets of 3% agarose gels in 0.2 M phosphate buffer, pH 6.9, containing 2% soluble starch (Merck, Darmstadt, West Germany), and 6 mM NaCl. After incubation for 60–120 min at 37°C in a moistened chamber, the sections were removed and the starch in the agarose sheets was precipitated in a 5:14:1 mixture of water, ethanol, acetic acid. Viewed against a black background, dark lines emerged at places where the starch was digested (Fig. 1, lane 3). The enzyme activity thus demonstrated belonged to the immunobound fresh amylase, as the electrophoresed protein had lost its enzyme activity.

The immunoperoxidase test was also carried out with sections of urea-polyacrylamide gels of rat pancreas. The localization of the zymogens concerned in this type of a gel has been described before (3). Anti-amylase reacted exclusively with the three identified amylase bands and anti-chymotrypsinogen reactivity was seen only over the main (anodic) chymotrypsinogen band. This demonstrated that the test results with the SDS gel sections were not affected by denaturation of the proteins by SDS.

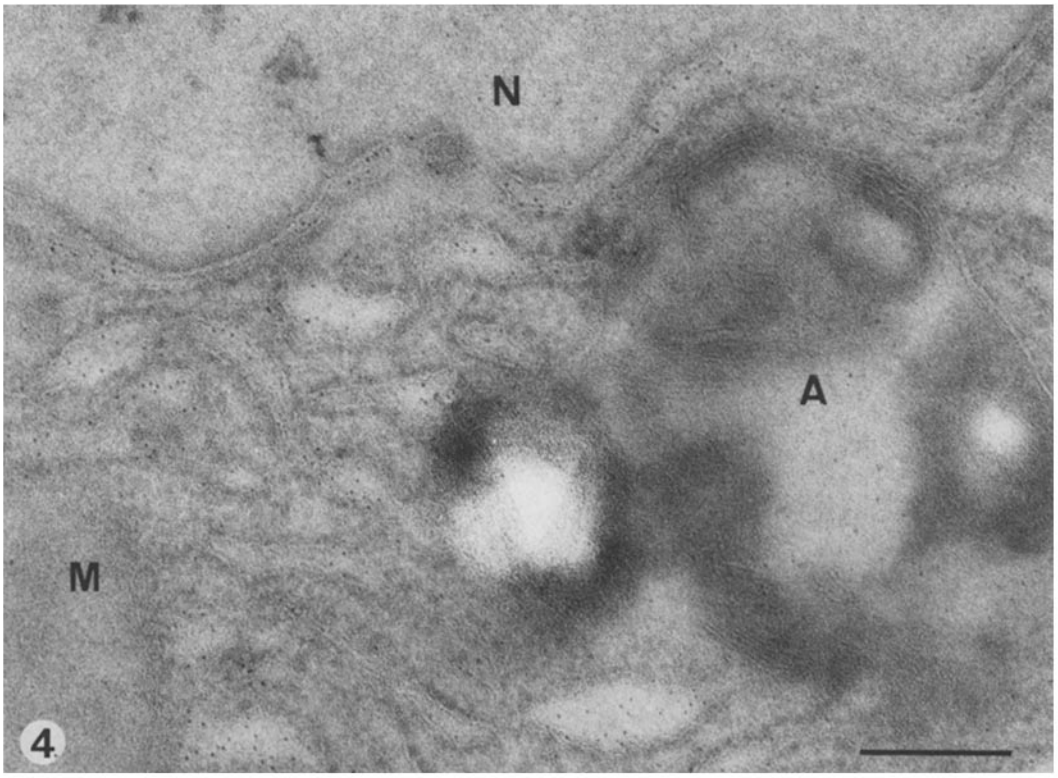
Tissue Processing

Male Wistar albino rats (Centraal Proefdierenbedrijf TNO, Zeist, The Netherlands), weighing ~300 g, were starved for 24 h, but had free access to water. Occasionally, secretion was stimulated by injecting a solution of pilocarpine nitrate (Brocacef, Utrecht, The Netherlands) in physiological saline (100 mg/ml) (3 h before the animal was sacrificed) into a tail vein at a dose of 3 mg/100 g body weight. Unstimulated and 3-h stimulated rats, and overnight-fasted male guinea pigs (same source) were sacrificed by decapitation. Pancreatic fragments with natural edges were fixed for 1 h in a large volume of a mixture containing 2% formaldehyde and 0.2% glutaraldehyde (final concentration) in 0.1 M phosphate buffer, pH 7.4 at 4°C, and were stored for up to 1 wk in 2% formaldehyde in 1 M sucrose. The defined labeling patterns as described in this study were not significantly influenced by protein extraction from the mildly fixed tissue. The use of higher concentrations of glutaraldehyde up to 2% lowered the intensity of labeling, especially that of the RER, but had no effect on the labeling pattern. We therefore routinely used 0.2% glutaraldehyde in the mixture. Small pieces (~1 mm^3), taken from the edges, were used for cryoultramicrotomy. Immunolabeling was performed as described before (30, 31). Briefly, ultrathin sections were cut from 1-M sucrose-infused tissue blocks at −70°C on a Sorvall MT-2B ultramicrotome (DuPont Instruments-Sorvall, DuPont Co., Newtown, Conn.) with a cryoattachment. The sections were picked up on ionized Formvar-carbon-coated copper grids and were indirectly immunolabeled at room temperature with rabbit anti-amylase or rabbit anti-chymotrypsinogen followed by ferritin-labeled goat anti-rabbit IgG. Sections incubated with normal rabbit IgG served as controls. Usually, the sections were then treated for 10 min with 2% alkaline (pH 8), and for 2 min with 0.2% acidic (pH 4) uranyl acetate (UA), to stabilize and contrast the membranes. Finally, the sections were embedded in a film of methyl cellulose (Tylose MH 300, Fluka AG, Buchs, Switzerland). The interference color of Formvar film plus methyl cellulose was silver (see reference 30 for details). The sections were viewed in a Philips EM 301, operating at 60 kV.

RESULTS

Ultrastructure

The ultrastructure of the exocrine pancreatic cell of the rat and guinea pig has been described



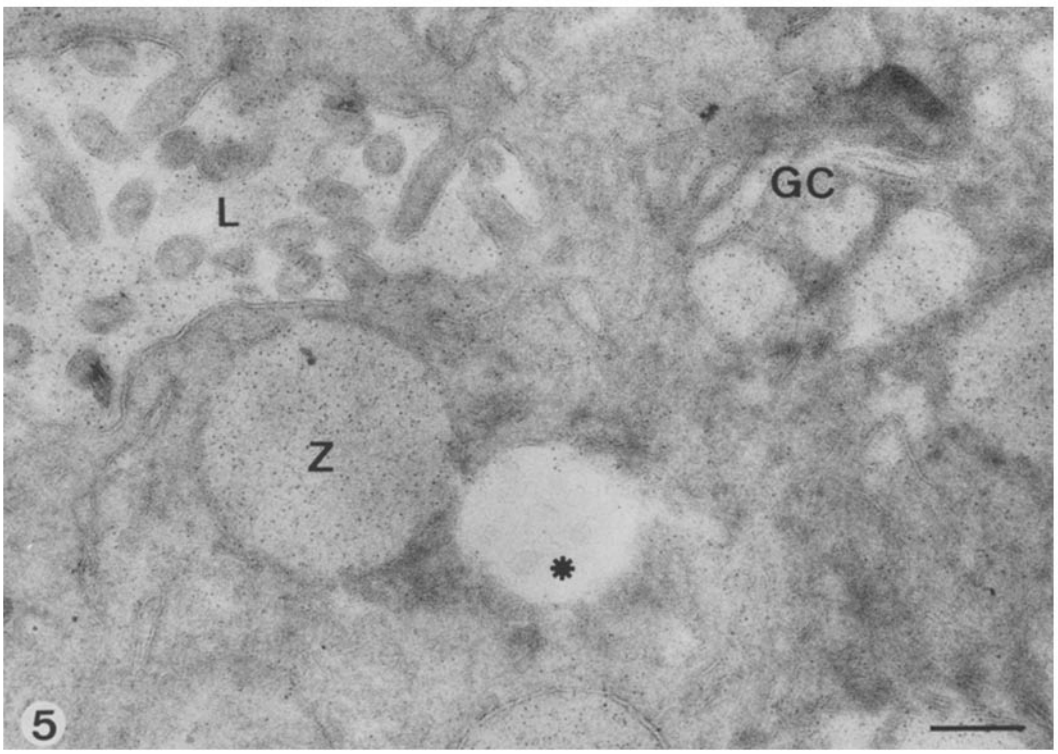


FIGURE 4 Rat; stimulated; amylase. Ferritin particles are present over the RER cisternae and the perinuclear cisterna. The autophagic vacuole (*A*) contains only a few particles. The nucleus (*N*) and the mitochondrion (*M*) are not labeled. × 98,000.

FIGURE 5 Rat; stimulated; amylase. Apex of depleted cell with characteristic proximity of Golgi complex (*GC*) and acinar lumen (*L*). The Golgi complex, zymogen granules (*Z*), and lumen are immunostained. Note the paucity of particles over the endocytic vacuole (asterisk). The particles present over the apical cytoplasm most likely belong to tangentially cut acinar lumen and small apical RER cisternae. × 63,000.

before (e.g., references 2, 10–12, and 15) and will not be given in detail here. In general, the cell morphology in the frozen sections was similar to that in plastic sections. On account of the UA double staining, the bilayered nature of the membranes was apparent, even in the case of the RER (Figs. 2, 4, and 6). The frozen sections showed Golgi areas with clearly distinguishable substructures (Figs. 6–8). The Golgi stacks consisted of three to five cisternae, often with increasing width towards the trans-side of the stack. All Golgi cisternae showed contents of almost equal electron density (Figs. 5–10). In the peripheral area between the RER and the cis-Golgi face occurred smooth-surfaced membranous elements embedded in an electron-dense material (Figs. 8 and 9). These peripheral elements were predominantly located in groups at the cis-faces of the free margins of the Golgi stacks. At the trans-side of the Golgi stack occurred condensing vacuoles in various stages of maturation (Figs. 5–9). In the guinea pig, they often showed flattened extensions (Fig. 11) which probably corresponded to the rigid lamellae of GERL (7, 8, 20, 21). Continuities between RER and condensing vacuoles were not encountered (20). Rigid lamellae in rat were less apparent. The coating at the cytoplasmic aspects of vesicles and condensing vacuoles (4) was not observed in the frozen sections. Multivesicular bodies were only seldomly distinguished, although they are numerous in stimulated cells (4).

Localization of Zymogens

Labeling patterns for amylase and chymotrypsinogen were similar. The labeling intensity for chymotrypsinogen was less than for amylase and appeared more variable from cell to cell. Correspondingly, the labeling of rat and guinea pig tissue, and of unstimulated and stimulated rat cells, was qualitatively alike. Therefore, the ferritin localization described below applies for both antigens in stimulated as well as in unstimulated rat cells and in guinea pig cells.

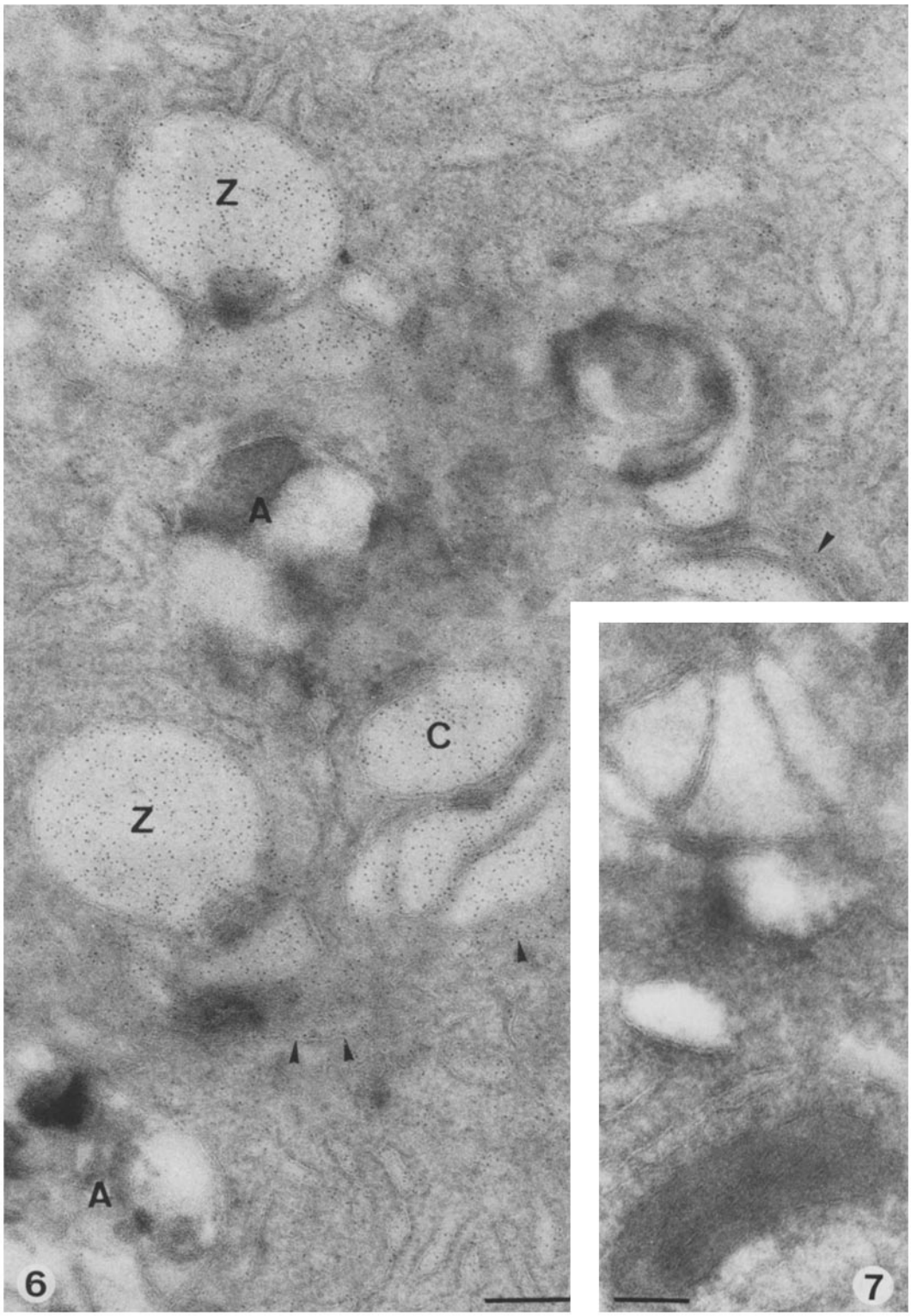
All RER cisternae including that surrounding the nucleus (Fig. 4), and the transitional RER cisternae at the cis-side of the Golgi complex (Fig. 9), were labeled for both zymogens. The distribution of the RER label could properly be studied in cross-sectioned cisternae. It then appeared that the majority of label was present over the cisternal space, but label occurred also over the RER membranes and attached ribosomes (Figs. 2–4 and 6). The cytoplasmic matrix showed no significant labeling. Occasionally, some ferritin particles were observed over the cytoplasm adjacent to the apical cell membrane in stimulated cells (Fig. 5). These particles most likely represented label of obliquely sectioned small structures such as vesicles and RER profiles.

Anti-amylase labeled the peripheral elements clearly (Fig. 9), but with anti-chymotrypsinogen the label was sparse (Fig. 8). Both antibodies labeled all cisternae of all Golgi stacks, condensing vacuoles, and zymogen granules (Figs. 6 and 8–10), and labeling over these structures was constantly much more intense than over the RER and peripheral elements. At the trans-side of the Golgi stack, the labeling was confined to locally occurring RER cisternae and to the condensing vacuoles (Figs. 6 and 8–11). Rigid lamellae, whether or not connected with the condensing vacuoles and vesicles, were not labeled (Fig. 11).

Ferritin was also present over all the zymogen granules and the acinar lumina (Fig. 5), but was sparse over endocytotic vacuoles (Fig. 5) and vesicles, multivesicular bodies, and autophagic vacuoles (Figs. 4 and 6). Mitochondria (Figs. 2–4) and nuclei (Fig. 4) were unlabeled. Nonspecific background as judged from control sections was negligible (Fig. 7).

FIGURE 6 Amylase. Survey of a Golgi area in a stimulated rat cell showing ferritin labeling of the Golgi cisternae including those at the cis-side of the stacks (arrowheads), the condensing vacuole (*C*), and the young zymogen granules (*Z*). Note that the density of ferritin labeling is higher in the Golgi cisternae than in the RER cisternae. There is no great difference between the Golgi cisternae, the condensing vacuole, and the zymogen granules in the level of ferritin labeling. Probably, the dense packing of the proteins in the granules has diminished the accessibility of the antigenic sites. The autophagic vacuoles (*A*) show only a few ferritin particles. × 65,000.

FIGURE 7 Rat; stimulated; control. Representative micrograph of a section incubated with normal rabbit IgG instead of rabbit anti-zymogen IgG. Few ferritin particles are seen. × 65,000.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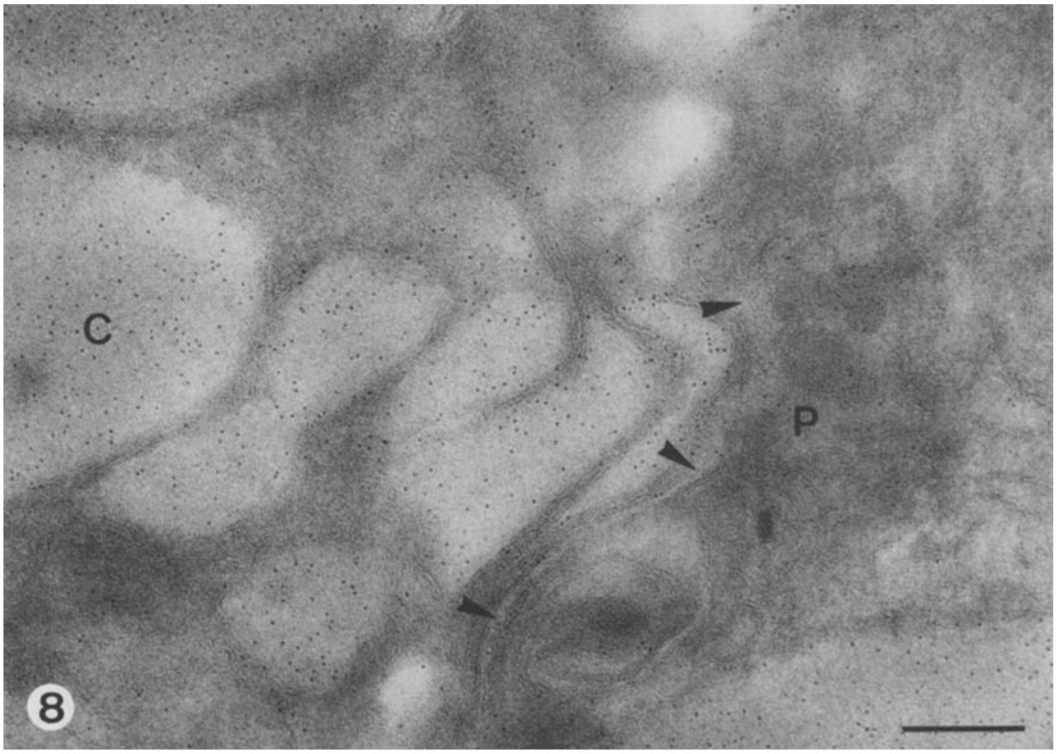


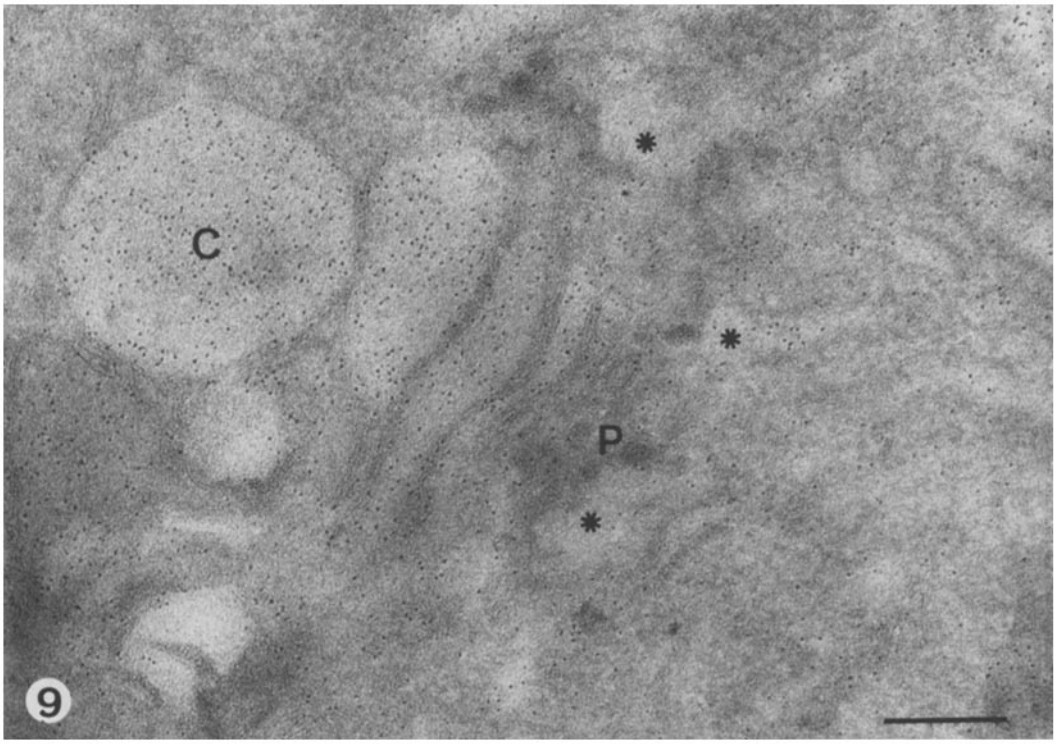
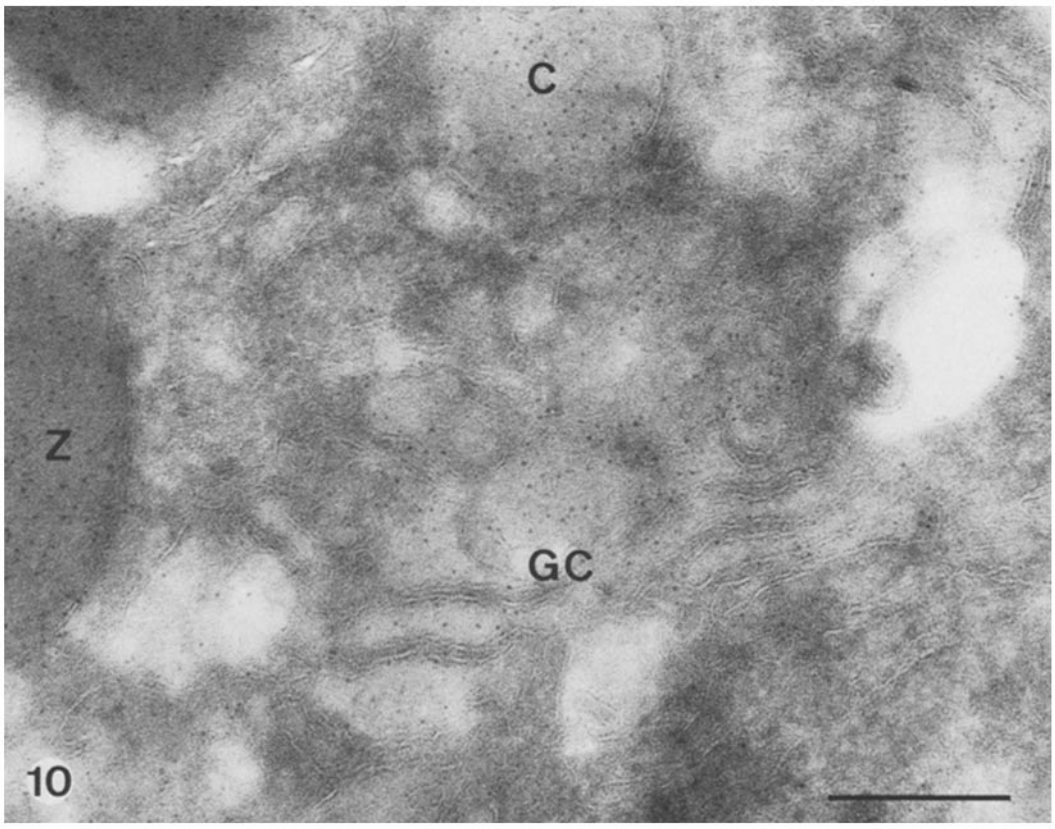


FIGURE 8 Rat; unstimulated; chymotrypsinogen. All of the Golgi cisternae are labeled. The trans-cisterna at the right of the condensing vacuole (*C*) shows slightly more label than the cis-one (arrowheads). Note that the peripheral elements (*P*) and the intervening cytoplasmic matrix are relatively electron dense. × 82,000.

FIGURE 9 Rat; stimulated; amylase. Similar view as in Fig. 8, but of a thinner section. As in Fig. 8, there is a steep increase of ferritin labeling from the RER and peripheral elements (*P*) to the cis-Golgi cisterna, indicating that the concentration of zymogens starts at the interface of the Golgi complex and the RER. The asterisks indicate transitional RER cisternae which are identifiable by their swollen appearance. Condensing vacuole, *C*. × 86,000.



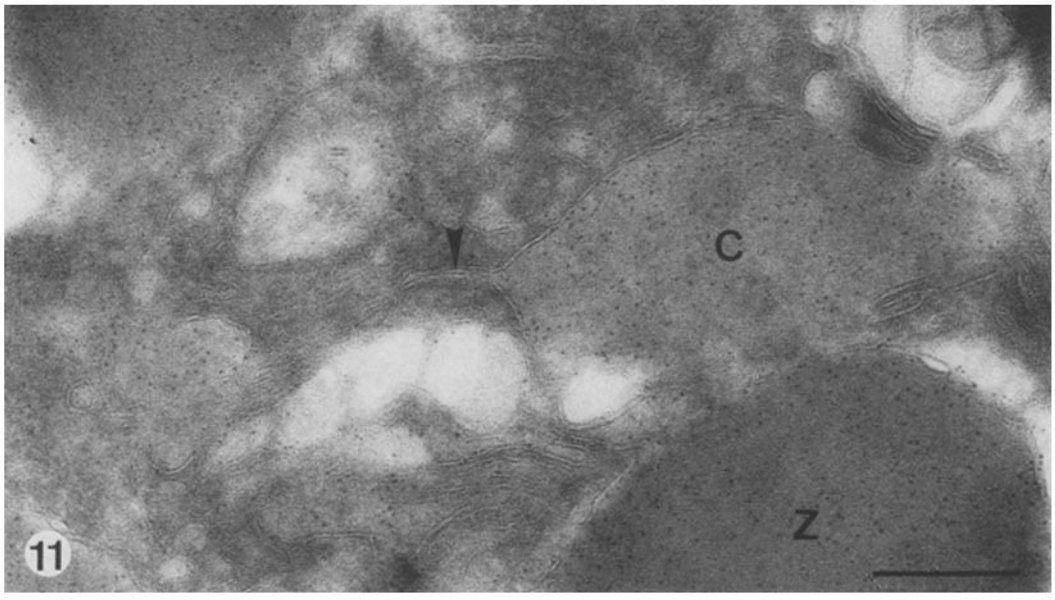


FIGURE 10 Guinea pig; unstimulated; amylase. Immunolabeling can be seen in all Golgi cisternae (*GC*), in a condensing vacuole (*C*), and in zymogen granules (*Z*). × 120,000.

FIGURE 11 Guinea pig; unstimulated; amylase. The figure shows a labeled condensing vacuole (*C*) attached to an unlabeled rigid lamella (arrowhead). Note that the difference in labeling intensity between condensing vacuole and zymogen granule (*Z*) is not proportional to the difference in electron density of their content. For a possible explanation, see text. × 91,000.

DISCUSSION

We found that anti-amylase labeled the tissue sections more heavily than anti-chymotrypsinogen, presumably because the first antibody was directed against rat pancreatic amylase and the immunolabeling for chymotrypsinogen was based on cross-reaction. Furthermore, amylase is a major pancreatic protein in the rat (between 10 and 20% of the total protein, own estimation). This made amylase suitable for a study of the occurrence of a secretory protein in structures such as the RER in which it is believed to be present in a nonconcentrated form.

Our observations support and extend those of Kraehenbuhl et al. (14) on the localization of secretory proteins in bovine exocrine pancreatic cells. These authors described immunolabeling for five proteins in all secretory granules and in the Golgi complexes of each cell. However, the RER was unlabeled and Golgi substructures were not differentially identified.

We detected both anti-amylase and anti-chymotrypsinogen labeling in the RER cisternae including the perinuclear cisterna. A similar localization was described for RNase in bovine pancreatic cells (22). We did not observe significant labeling of the cytoplasmic matrix. The RER in all secretory cells was labeled, and labeling occurred throughout the RER. A systematic search of subsequent sections labeled with different antibodies would be necessary to determine whether individual RER cisternae or parts of them are specialized in the production of particular zymogens.

At present we lack evidence on how secretory proteins gain access to the Golgi complex. For the guinea pig pancreas, it has been postulated that peripheral vesicles derived from transitional RER cisternae transfer their proteinaceous content either directly to the condensing vacuoles in unstimulated cells (11) or to the Golgi cisternae in stimulated cells (12). The latter route also appeared from our electron microscope autoradiographical studies on the frog pancreas (25, 26). Alternatively, Novikoff et al. (20, 21) suggested from cytochemical work on acid phosphatase and thyamine pyrophosphatase, and from morphological observations, that secretory proteins enter the Golgi area at the trans-side by traveling from the RER either directly or via the rigid lamellae of GERL into the condensing vacuoles. In this view, Golgi cisternae would not play an important role in secretory protein processing.

Our work indicates the presence of zymogens in the peripheral area at the cis-Golgi face and in all Golgi cisternae in both unstimulated and stimulated cells. These observations substantiate the supposition that all these Golgi substructures are involved in the transport of secretory proteins (9, 12, 25, 26). Of course, it cannot be excluded that the Golgi cisternae are in fact "blind sacs" which are bypassed by the bulk of proteins on their way to the secretory granules. However, autoradiographically demonstrated shifts of newly formed proteins across the Golgi stack make this unlikely (12, 15, 25, 26). The observation that in all Golgi complexes all the cisternae labeled for both zymogens suggests that no specialization occurred within the stack of Golgi cisternae for the transfer of one of the zymogens studied.

Golgi membranes are known to contain glycosyl transferases which attach sugar residues to nascent glycoprotein molecules. Indeed, secretory glycoproteins in many cell types have been demonstrated to occur in the Golgi cisternae, often showing a gradient of increasing density from the cis- towards the trans-cisternae (e.g., references 5, 24, 28, and 33). A corresponding gradient across the Golgi stack has been described for secretory peroxidase (1, 7, 9, 19), which can be detected in ultrathin sections by enzyme cytochemistry.

In this immunocytochemical study on amylase and chymotrypsinogen, we observed an obvious and steep increase of labeling intensities from the RER and peripheral elements to the Golgi stack (Figs. 6, 8, and 9). The use of ferritin as an immunolabel in principle permits quantification of antigen by relating the number of ferritin particles and the volume of the compartments concerned. There are, however, several factors which complicate such an estimation. A major one is the penetrability of the immunoreagents into the frozen sections. Differences in the macromolecular density of structures lead to differences in penetrability of the relatively large IgG-ferritin conjugates into the frozen section, and thus seriously affect comparisons of labeling intensities between structures with different densities.[1] A situation of increasing structural density can be expected to occur along the line RER—Golgi complex—secretory granules. If a hindrance to labeling had occurred, however, its effect would have increased

[1] Stereoscopic observations of ferritin-labeled thin sections showed that, for example, zymogen granules labeled only at the section surface, while the labeling depths of less dense structures were different.

in parallel with the increase of the labeling intensity observed, and thus the differences between the RER and the Golgi cisternae would have been even larger than observed. Therefore, we feel justified to conclude from our ferritin density patterns that the Golgi cisternae contain a higher zymogen concentration than the RER cisternae.

The observations that the peripheral area at the cis-face of the Golgi complex and the Golgi cisternae are immunolabeled, and that the Golgi cisternae are more heavily labeled than the RER, are compatible with the view that secretory proteins enter the Golgi complex at the cis-side. An active role of Golgi cisternae in the processing of nonglycosylated secretory proteins may probably be related to their concentration. Concentration already begins at the boundary between the RER and the Golgi complex. This view is supported by immunofluorescence observations made on thin sections (32). Our results support the supposition that condensing vacuoles arise by conversion of Golgi cisternae. With respect to protein packaging, Golgi cisternae and condensing vacuoles can therefore be considered as a functional unity.

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