Use of Colloidal Gold Particles in Double-Labeling Immunoelectron Microscopy of Ultrathin Frozen Tissue Sections

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ABSTRACT Complexes of protein-A with 5 and 16 nm colloidal gold particles (PA/Au^5 and PA/Au^{16}) are presented as sensitive and clean immunoprobes for ultrathin frozen sections of slightly fixed tissue. The probes are suitable for indirect labeling and offer the opportunity to mark multiple sites. The best procedure for double labeling was to use the smaller probe first, i.e., antibody 1 – PA/Au^5 – antibody 2 – PA/Au^{16}. When this was done, no significant interference between PA/Au^5 and PA/Au^{16} occurred. Using this double-labeling procedure we made an accurate comparison between the subcellular distributions of amylase as a typical secretory protein and of GP-2 a glycoprotein, characteristic for zymogen granule membrane (ZGM) preparations.

We prepared two rabbit antibodies against GP-2. One antibody (R × ZGM) was obtained by immunizing with native membrane material. The specificity of R × ZGM was achieved by adsorption with the zymogen granule content subfraction. The other, R × GP-2, was raised against the GP-2 band of the SDS polyacrylamide profile of ZGM. We found that the carbohydrate moiety of GP-2 was involved in the antigenic determinant for R × ZGM, while R × GP-2 was most likely directed against GP-2 polypeptide backbone.

The immunocytochemical observations showed that GP-2, on the one hand, exhibited the characteristics of a membrane protein by its occurrence in the cell membrane, the Golgi membranes, and its association with the membranes of the zymogen granules. On the other hand, GP-2 was present in the contents of the zymogen granules and in the acinar and ductal lumina. Also, a GP-2-like glycoprotein was found in the cannulated pancreatic secretion (Scheffer et al., 1980, *Eur. J. Cell Biol.* 23:122–128). Hence, GP-2 should be considered as a membrane-associated secretory protein of the rat pancreas.

Ultrathin frozen sections of mildly fixed cells and tissues are very suitable for localizing intracellular antigens by means of immunoferritin cytochemistry (12, 27, 38). The major advantages of the technique are (*a*) the use of a particulate label, ensuring an accurate positioning of binding sites with high resolution, (*b*) a good accessibility of membrane-enclosed antigens. Recent developments (37) also allow excellent delineation of ultrastructural detail.

As ferritin was the only suitable electron-dense label for frozen sections until now, a limitation was that only one antigen per section could be studied. Recently, however, iron-dextran particles have been introduced. One of these, Imposil, was shown to be useful in combination with ferritin (5). Another electron-dense particle that has come into use for cytochemical purposes recently is colloidal gold (8, 16). Gold particles of several diameters can conveniently be prepared. Romano and Romano (28) were the first to use gold particles complexed with *Staphylococcus aureus* coat protein-A (PA) as immunolabels. PA binds specifically with the Fc portions of IgG molecules (9).

In previous immunoferritin studies, we described both similarities and differences in the subcellular distributions of two zymogens in the rat and the guinea pig pancreas (11, 12). In this article we aimed at a comparison of the localizations of

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amylase as a typical secretory protein, and that of a glycoprotein, designated GP-2 (23), characteristic for zymogen granule membrane preparations. To this end, we explored the possibilities of double labeling with two sizes of colloidal gold particles complexed with PA. We show that GP-2 is present along the whole cell membrane as well as the membrane of the Golgi complex and zymogen granules, but that it also occurs in the contents of the zymogen granules and acinar and ductal lumina.

MATERIALS AND METHODS

Preparation of Zymogen Granule Membranes

Zymogen granule membranes (ZGM) were isolated essentially according to the method of MacDonald and Ronzio (23). Pancreases from overnight-fasted male Wistar rats were immersed in 0.3 M sucrose with 0.25 mg/ml soybean trypsin inhibitor (SBTI) and were homogenized in a Polytron PMT 10 homogenizer (Polytron Corp., Elkhart, Ind.). The homogenates were centrifuged at 500 *g* for 10 min to remove debris. Zymogen granules were sedimented at 1,750 *g* for 30 min and contaminating mitochondria were removed by swirling the pellet gently with the SBTI-containing sucrose 3 × 1 ml (18). The granules were resuspended in 0.15 M NaCl with 0.67 mg/ml SBTI and lysed by the addition of 8 vol of 0.2 M $NaHCO_3$ at pH 8.2. ZGM were further purified on a discontinuous sucrose gradient (24). Briefly, lysed granules were centrifuged at 100,000 *g* for 30 min. This yielded a supernate that was considered to be the zymogen granule content subfraction. The pellet was resuspended in 1 M sucrose, 0.3 M sucrose was layered on top, and the gradient was centrifuged at 150,000 *g* for 60 min. ZGM, banding at the 0.3- to 1-M sucrose interface, were carefully pipetted and treated twice with 0.25 M NaBr to detach residual granule content proteins. Washed ZGM were finally pelleted at 195,000 *g* for 1 h. A typical ZGM preparation from 20 rats contained 1–1.5 mg of protein.

Preparation and Characterization of Antibodies

ANTI-ZGM: A white New Zealand rabbit was immunized by means of three subcutaneous injections of ZGM (each containing 300 μg of protein) in equal volumes of Freund's complete adjuvant. 3 wk after the final boost, the rabbit was bled and the IgG fraction (called R × ZGM) either was precipitated from the serum with $(NH_4)_2SO_4$ or was obtained by DEAE cellulose treatment (17). Antibodies directed against zymogens contaminating the ZGM subfraction were removed by adsorption of R × ZGM with zymogen granule lysate, known to contain an average collection of zymogens (24, 30, 35). To this end, glutaraldehyde-activated AcA-22 ultragel (LKB Produkter, Stockholm, Sweden) (36) was loaded with the zymogen granule content subfraction (defined above). A column with a wet volume of 5–10 ml was packed in a hypodermic syringe. Half-column volumes of R × ZGM in phosphate-buffered saline (PBS) at serum concentrations were led over the column. Contaminant antibodies binding to the column were eluted with 0.1 M HCl (pH 2.2) and R × ZGM was adsorbed two more times. In the first run, 1.5% of the protein of the starting material bound to the column. In the second and third runs, no further protein adsorption occurred.

ANTI-GP-2: In addition to R × ZGM, we raised an antiserum against the major glycoprotein in the ZGM subfraction known as GP-2 (23). GP-2, with an apparent molecular weight of ~75,000, constituted ~30% of the proteins in the ZGM subfraction and stained with periodic acid-Schiff (PAS) (Fig. 1). ZGM polypeptides were separated on a 7–15% linear gradient SDS polyacrylamide slab gel, using the buffer system of Laemmli (20). A small, Coomassie Brilliant Blue (CBB)-stained longitudinal section of the slab was used to localize the position of GP-2. The region corresponding to GP-2 was excised from the main gel, homogenized in a small volume of PBS, and dialyzed against several changes of PBS for 72 h at 4°C. The GP-2/polyacrylamide suspension was used to immunize a rabbit as described above. The collected IgG fraction also contained antibodies against polyacrylamide. It was therefore adsorbed with polyacrylamide suspension in a small volume of PBS. The resulting anti-GP-2 (R × GP-2) was further adsorbed with immobilized zymogen granule-content proteins as described for R × ZGM. No protein could be removed from R × GP-2 by this procedure.

OTHER ANTIBODIES USED: Affinity purified rabbit IgG directed against rat pancreatic α-amylase (R × Am) was prepared and tested as described before (12). Because of our observation that serum or complete IgG preparations from nonimmunized rabbits often exhibit immunocytochemical reactivity with rat tissues, we used an affinity purified rabbit antibody against horseradish peroxidase (R × HRP) (Boehringer, Mannheim, W. Germany) for controls on specificity. Affinity purified goat anti-rabbit IgG (own preparation) was conjugated to PCl_5-activated Lissamine Rhodamine (Polysciences, Inc., Warrington, Pa.) (2).

SPECIFICITY TESTS: The specificity of R × ZGM and R × GP-2 was tested on ~40 μm frozen sections of SDS polyacrylamide gel electrophoresis (SDS PAGE) on which the polypeptides of ZGM or content subfraction had been separated. Immunoreaction was visualized using peroxidase-conjugated goat anti-rabbit IgG (Miles Laboratories, Inc., Elkhart, Ind.). Bound antibody was demonstrated with diaminobenzidine (for details, see reference 12). The tests showed that both R × ZGM and R × GP-2 were directed against GP-2 (Fig. 1). In addition, R × ZGM gave a weak reaction with one other minor PAS-positive but CBB-negative component of ZGM, probably corresponding to GP-1 of Ronzio et al. (30). R × GP-2 failed to react with this or any component other than GP-2.

To test whether carbohydrates contributed to the antigenic determinants recognized by R × ZGM and R × GP-2, vicinal OH groups of ZGM and granule content in SDS PAGE sections as well as in tissue sections were oxidized with 0.5% H_5IO_6 for 0.5 h before adding the antibodies. In the case of R × ZGM, the immunoreaction disappeared in both gels (Fig. 1) and tissue sections (not shown), while that of R × GP-2 was only slightly diminished. Hence, carbohydrates were significantly involved in the antigenic determinants of R × ZGM, but not in the recognition of GP-2 by R × GP-2. This difference between the antibodies is most likely because native material was used to raise R × ZGM and SDS-denatured peptides for R × GP-2.

Preliminary immunocytochemical observations have shown that R × ZGM, in contrast to R × GP-2, reacts very specifically with the acinar cells of the submandibular gland (SM) in a similar fashion as with pancreatic acinar cells. In SDS PAGE of SM homogenate, R × ZGM recognized one PAS-positive component with an apparent molecular weight of ~150,000 (Fig. 2). This component was not recognized by R × GP-2. SM homogenate was then used to adsorb R × ZGM. All reactivity for ZGM gels including that for GP-1 was absorbed out (Fig. 2). In addition, no immunoreaction was left in tissue sections (not shown). R × GP-2 could not be depleted by SM homogenate. Obviously, all reactivity of R × ZGM in pancreas tissue is assembled on one glycoprotein in the SM. This indicates that the R × ZGM reaction with GP-1 and GP-2 in the pancreas is caused by homologous antigenic sites. The fact that R × GP-2 does not react with SM proves that it recognizes a completely different domain of the GP-2 molecule.

Previously, we have found an R × GP-2-binding glycoprotein with a molecular weight slightly lower (~5,000) than that of GP-2, present in cannulated pancreatic secretion. This glycoprotein could be obtained in small amounts from the secretion by centrifugation (200,000 *g* for 30 min) (33). R × GP-2 adsorbed with this secreted GP-2-like glycoprotein did not recognize any component in ZGM gels (Fig. 2) or in tissue sections (not shown) anymore, demonstrating the unlikeliness of R × GP-2 contaminated with antibody against a ZGM component co-banding with GP-2.

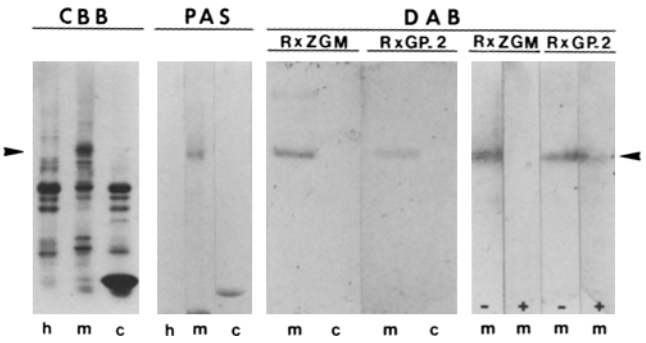


FIGURE 1 Panel of SDS PAGE profiles of pancreas homogenate (*h*), and of the membrane (*m*) and content (*c*) subfractions of zymogen granules, stained with CBB or PAS. The lanes under *DAB* were stained with diaminobenzidine (DAB) after immunoreaction with R × ZGM or R × GP-2. The four lanes at the right are profiles of membrane, without (−) or with (+) oxidation of exposed carbohydrates with 0.5% H_5IO_6 for 30 min before the immunoincubation. The thick bands in the middle of *h*, *m*, and *c* under CBB represent amylase. The arrowheads indicate the level of GP-2 (~75,000 mol wt), which is the major PAS-positive component in membrane. GP-2 is absent from content. The PAS-staining band in content is SBTI that is added during the isolation procedure. The profiles under DAB show that R × GP-2 reacts with GP-2 only, and that R × ZGM in addition recognizes a component of ~120,000 daltons, probably GP-1. GP-1 could occasionally be seen in PAS-stained gels of membrane. In the right four lanes it can be seen that carbohydrate oxidation quenches the reaction of R × ZGM with GP-1 and GP-2 completely, but only slightly affects that of R × GP-2.

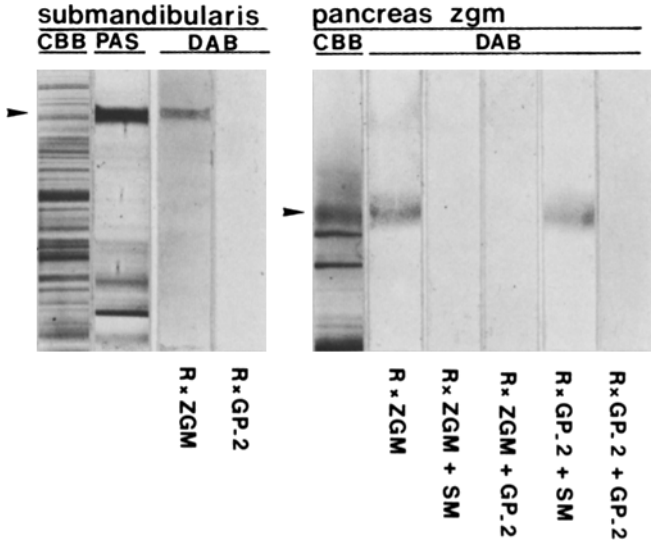


FIGURE 2 Panel of SDS PAGE profiles of SM and of the membrane subfraction of pancreas ZGM, stained with CBB, PAS, or by diaminobenzidine (DAB) after immunoreaction with R × ZGM or R × GP-2. Polyacrylamide gradient is 9–18% in the four lanes at the left and 7–11% in the other lanes. The arrowhead in the SM panel indicates the location of a glycoprotein of ~150,000 mol wt that reacts with R × ZGM but not with R × GP-2. The arrowhead in the ZGM panel indicates the level of GP-2 in ZGM. The profiles under DAB show that pretreatment of R × ZGM with SM (R × ZGM + SM) causes the reaction with GP-2 to disappear, whereas this is not so in the case of R × GP-2 (R × GP-2 + SM). In addition, these profiles show that by pretreatment of R × ZGM and R × GP-2 with a GP-2-like glycoprotein from pancreatic juice (R × ZGM + GP-2 and R × GP-2 + GP-2, respectively), the reaction with GP-2 is totally extinguished.

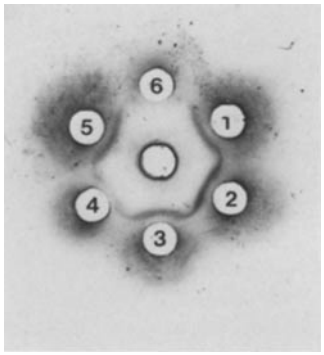


FIGURE 3 Ouchterlony double-diffusion tests of R × ZGM and R × GP-2 against ZGM. Central well, ZGM suspension (1.5 mg/ml protein); wells *2*, *4*, and *6*, R × ZGM undiluted (20 mg/ml) and diluted with PBS 1:2 and 1:4, respectively; wells *1*, *3*, and *5*, R × GP-2 (serum concentration). The plate was stained with CBB. The precipitin lines of R × ZGM and R × GP-2 fuse, which demonstrates that both antibodies recognized the same component of ZGM. A similar pattern was obtained when ZGM were solubilized with 2% Triton X-100.

That R × ZGM and R × GP-2 both recognized the same component of ZGM was also shown in an Ouchterlony double-diffusion test. Fig. 3 shows the precipitin lines of R × ZGM and R × GP-2 with ZGM suspension in the central well. The precipitin lines fused. R × ZGM showed only one line, which supports the idea that GP-2 and GP-1 have identical antigenic determinants. Because of the possibility that antigens in the membrane suspension could not diffuse independent of each other, we also used ZGM solubilized in 2% Triton X-100 (final concentration). This gave similar results.

Tissue Fixation and Cryoultramicrotomy

Rats of 200–300 g had free access to food and water before being killed. Pancreas fragments with natural edges were fixed on melting ice for 1 h in a large volume of a mixture containing 2% formaldehyde and 0.2–0.5% glutaraldehyde (final concentrations) in 0.1 M phosphate buffer (PB) at pH 7.4. The fragments were stored for up to 1 wk in 0.1 M PB with 2.3 M sucrose in liquid nitrogen, or in 0.1 M PB with 1 M sucrose and 2% formaldehyde (final concentration) at 4°C. The latter procedure improved the quality of ultrastructure, but slightly decreased immunoreactivity.

Frozen sections were cut as described previously (12) but 2.3 instead of 1 M sucrose was routinely used as a cryoprotectant and the cutting temperature was lowered to approx. −90°C for getting ~100-nm sections. For electron microscopy, 100-nm sections were put on Formvar-carbon-coated copper grids. For light microscopy, ~200-nm sections were cut at approx. −70°C and were put on degreased microscope slides covered with a film of 1% gelatin in 0.1% $KCr(SO_4)_2$.

Immunolabeling Procedures

IMMUNOFLUORESCENCE: The sections on the slides were stained indirectly with goat anti-rabbit IgG conjugated with rhodamine, enclosed in 70% glycerol in PB, and covered with coverslips (see, for details, reference 11). Control sections were treated with R × HRP instead of R × Am or R × ZGM. Nonspecific fluorescence was very low.

PROTEIN A-COLLOIDAL GOLD COMPLEXES: Two sizes of colloidal gold particles were used as electron-dense immunolabels.[1] The small particles with an average diameter of 5.2 nm (16) called Au^5 were prepared by reducing $HAuCl_4$ with white phosphorus. The larger particles, measuring 16 nm (10) and called Au^{16}, were obtained by reduction of $HAuCl_4$ with sodium citrate. The particles were adsorbed with Staphylococcal protein A (PA; Pharmacia Fine Chemicals AB., Uppsala, Sweden) to produce stabilized suspensions of PA/Au^5 and PA/Au^{16} probes. The preparations were pelleted twice to remove excess PA: PA/Au^5 at 125,000 g_{avg} for 45 min and PA/Au^{16} at 15,000 g_{avg} for 45 min. The pellets were then dissolved in PBS. Because the PA/Au^5 particles varied considerably in size, we centrifuged them at lower speed (at 50,000 g_{avg} for 45 min) before the washings to remove the largest particles.

Taking into account the amount of PA that was bound by the gold sol (~8 μg/ml Au^5 and ~5 μg/ml Au^{16}) and the number of particles per ml gold sol (16), we estimated the number of PA molecules complexed to Au^5 and Au^{16} at ~2 and ~40, respectively.

IMMUNOELECTRON MICROSCOPY: Immunoincubations, washing, staining, and embedding were performed at room temperature (38). The incubation with antibodies and labels lasted 30 min; the washes with PBS in between the immunoincubations and with H_2O before staining and embedding were 3 × 1 min. The washes with PBS after the gold labeling were 3 × 10 min. The following protocols show the sequences of the main immunolabeling steps used:

Single labeling:

(*1*) R × ZGM – PA/Au^5

(*2*) R × GP-2 – PA/Au^5

Double labeling:

(3) R × ZGM – PA/Au^5 – R × Am – PA/Au^{16}

(4) R × Am – PA/Au^5 – R × ZGM – PA/Au^{16}

(5) R × ZGM – PA/Au^{16} – R × Am – PA/Au^5

(6) R × Am – PA/Au^{16} – R × ZGM – PA/Au^5

R × GP-2 occasionally took the place of R × ZGM in double-labeling experiments. Control incubations to test aspecific background labeling and co-labeling of the first by the second two steps were as follows:

(7) R × HRP – PA/Au^5

(8) R × HRP – PA/Au^{16}

(9) R × ZGM – PA/Au^{16} – R × HRP – PA/Au^5

(10) R × ZGM – PA/Au^5 – R × HRP – PA/Au^{16}

To minimize the tendency of gold particles to adhere to the section surfaces, we mixed the probes before use with bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) or goat serum from which IgG was removed by $(NH_4)_2SO_4$ precipitation. The supernatant IgG-less serum was dialysed against PBS. After immunostaining, the sections were postfixed with 0.5% glutaraldehyde. Next, the sections were positively stained with neutral and acidic (pH 4) uranyl acetate and embedded in methyl cellulose (37). A typical sequence of treatments for double labeling would then be: 1st antibody – PBS – 1st probe – 2nd antibody – PBS – 2nd probe – PBS – H_2O – glutaraldehyde – H_2O uranyl (pH 7) – H_2O – uranyl (pH 4) – H_2O – embedding.

[1] Methods to prepare colloidal gold particles of any size between 5 and 16 nm and the procedure to make PA/Au complexes are described in detail in an additional communication by Slot and Geuze.

The protein contents (Lowry) of the antibodies used for cytochemistry were: R × ZGM, 0.2 mg/ml; R × GP-2, 0.3 mg/ml; R × Am, 75 μg/ml; R × HRP, 75 μg/ml.

RESULTS

As judged from survey views of immunofluorescent sections, R × Am, R × ZGM, and R × GP-2 labeled acinar cells only.[2] Twins of phase-contrast and fluorescence micrographs for R × Am and R × ZGM are presented in Figs. 4 and 5. Fig. 4 shows that R × Am stains the cytoplasm, the zymogen granules, and the lumina of the acini. R × GP-2 gave a faint but distinct reaction over the cytoplasm (data not shown; see reference 33), where R × ZGM was negative (Fig. 5). For the rest, the fluorescence patterns for R × ZGM and R × GP-2 were similar, so that the following description for R × ZGM is also valid for R × GP-2. Fig. 5 shows that R × ZGM stained the zymogen granules as intensely fluorescing rings round weakly reactive centers. Fluorescence also occurred along all faces of the acinar cells. At boundaries with nonexocrine tissue components, the reaction was only faint, possibly because only the acinar cell faces contributed to the fluorescence. The spots of relatively intense fluorescence along the lateral cell sides, most likely represented the local interdigitations of the cell membranes between neighboring acinar cells. A strong positive reaction was seen over the acinar and ductal lumina. This reaction was even more intense than that over the centers of zymogen granules. Nonspecific fluorescence, as judged from R × HRP-treated sections, was negligible.

The results of labeling ultrathin sections with gold confirmed and refined those obtained by fluorescence microscopy. GP-2 labeling was identical for both R × ZGM and R × GP-2.[3] However, the R × GP-2 reaction was weaker. For this reason we used R × ZGM for the demonstration of double labeling. R × GP-2 single labeling is illustrated in Figs. 12 and 16. For the sake of brevity and to facilitate comparison of R × ZGM and R × Am, their labeling patterns are mainly shown in micrographs of double-labeled sections (Figs. 6–11 and 13). The labeling for amylase with the gold probes was similar to that described before with ferritin (11, 12). Reaction occurred throughout the rough endoplasmic reticulum (RER), including the perinuclear cisterna and the transitional elements, the peripheral vesicles and tubules at the cis-face of the Golgi-complex, and in all Golgi cisternae, condensing vacuoles, and zymogen granules (Figs. 6–11 and 13). As shown previously (12), there was a steep increase in intensity of labeling from the RER to the cis-Golgi cisternae, whereas only a slight increase occurred across the stack of cisternae and condensing vacuoles towards the zymogen granules (Figs. 9–11 and 13). Label was uniformly distributed over the contents of all these structures.

The R × ZGM labeling pattern was quite different from that of R × Am as could easily be appreciated from double-labeled sections. R × ZGM did not react with the RER (Figs. 6–8 and 13). The opportunity to compare the reaction pattern of R × ZGM with that of R × Am as a reference marker for zymogens was particularly advantageous in the case of a complicated structure such as the Golgi complex (Figs. 9–11 and 13). Unlike R × Am, R × GP-2 (Fig. 12), and R × ZGM (Figs. 10, 11, and 13), labeling increased sharply from the cis- towards the trans-Golgi cisternae. Furthermore, labeling with the small gold particles revealed that the reaction was mainly confined to the membranes of the Golgi complex (Figs. 11 and 12). We got the impression that the label was associated with the content-facing surface of the Golgi membranes, rather than being located over the membranes themselves (Figs. 10, 12, and 13). The secretory contents were progressively co-labeled from the trans-Golgi cisternae towards the condensing vacuoles and zymogen granules. Again, membrane labeling of condensing vacuoles and zymogen granules was mainly associated with the inner faces (Figs. 10, 12, and 13). In the majority of apical zymogen granules, no difference was seen in labeling intensity of the membranes and the contents. However, in the thicker fluorescent sections such a difference was indeed clearly demonstrated. R × ZGM and R × GP-2 also labeled the contents of acinar and ductal lumina (Figs. 15 and 16).

Both R × ZGM and R × GP-2 reacted with lysosomal structures, presumably autophagic vacuoles, and with the cell membrane. In the rat exocrine pancreatic cells, autophagic vacuoles were mostly located at the cis-side of the Golgi complex (Fig. 13). Developing autophagic vacuoles contained recognizable cell structures and were often surrounded by two concentric membranes (Fig. 13). The label of these developing vacuoles was mainly present over their enveloping membranes, while in older, more electron-dense residual bodies the contents were also labeled (Fig. 14). The cell membrane of all cell sides was labeled. Reaction occurred predominantly over the outer aspects of the membranes (Figs. 7, 8, and 14–16). Centro-acinar cells and ductal cells were not labeled (Figs. 7, 15, and 16). Small vesicles beneath the apical cell membrane, possibly of endocytotic nature, were often positive for R × ZGM (Fig. 15, upper acinar cell), but lacked R × Am labeling. At the lateral cell sides, the gold particles accurately followed the meandering

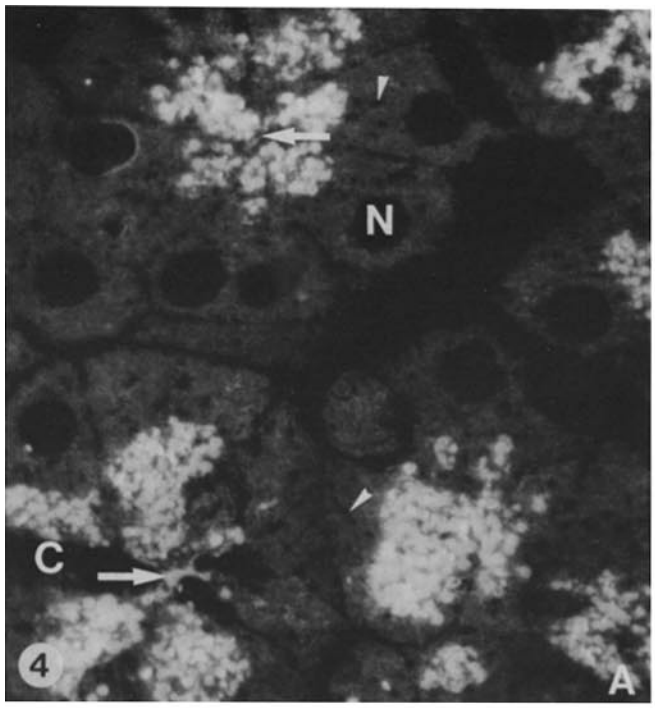
[2] Preliminary immunofluorescence observations showed that, in contrast to R × Am, both R × ZGM and R × GP-2 labeled parts of the brush borders but not the rest of the cell membrane and interiors of intestinal cells. Because R × GP-2 does not react with salivary gland cells and saliva, intestinal GP-2 sticking to the inner surface of the intestine was most likely derived from pancreatic secretion (33).

[3] Of course we have to bear in mind that R × ZGM also reacted with GP-1. The fact that both R × ZGM and R × GP-2 gave the same labeling pattern indicates that either GP-2 and GP-1 exhibit the same distribution in the pancreas or that GP-1 contributes only to a negligible extent in the R × ZGM reaction.

FIGURES 4 and 5 Twins of fluorescence (*A*) and phase-contrast (*B*) micrographs of thin (~200 nm) frozen sections, indirectly immunostained with rhodamine.

FIGURE 4 R × Am. Fluorescence can be seen in the cytoplasm, the zymogen granules, and the acinar lumina (arrows). Mitochondria (arrowheads, compare *A* and *B*), nuclei (*N*), centroacinar cells (*C*), connective tissue, and blood vessels are negative. Bar, 10 μm. × 1,540.

FIGURE 5 R × ZGM. Only the exocrine cells show reaction. Endocrine tissue (*End*), connective tissue, and blood vessels are negative. The acinar cells show fluorescence over the cell membranes (with dots where the membranes interdigitate) and over the zymogen granules and acinar lumina (arrows). The zymogen granules stain as rings with a less-stained center. At the boundaries with nonexocrine tissue where only one cell membrane is reactive, fluorescence is weak (arrowheads). Bar, 10 μm. × 1,300.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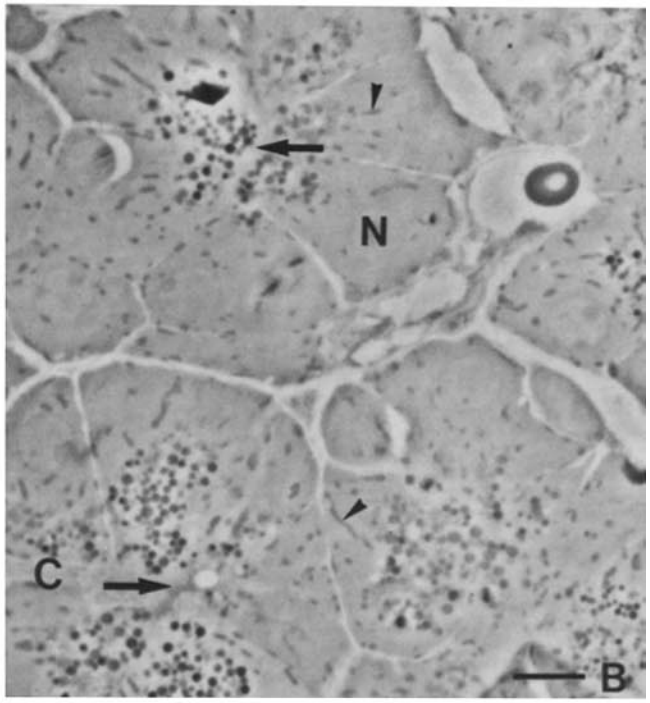
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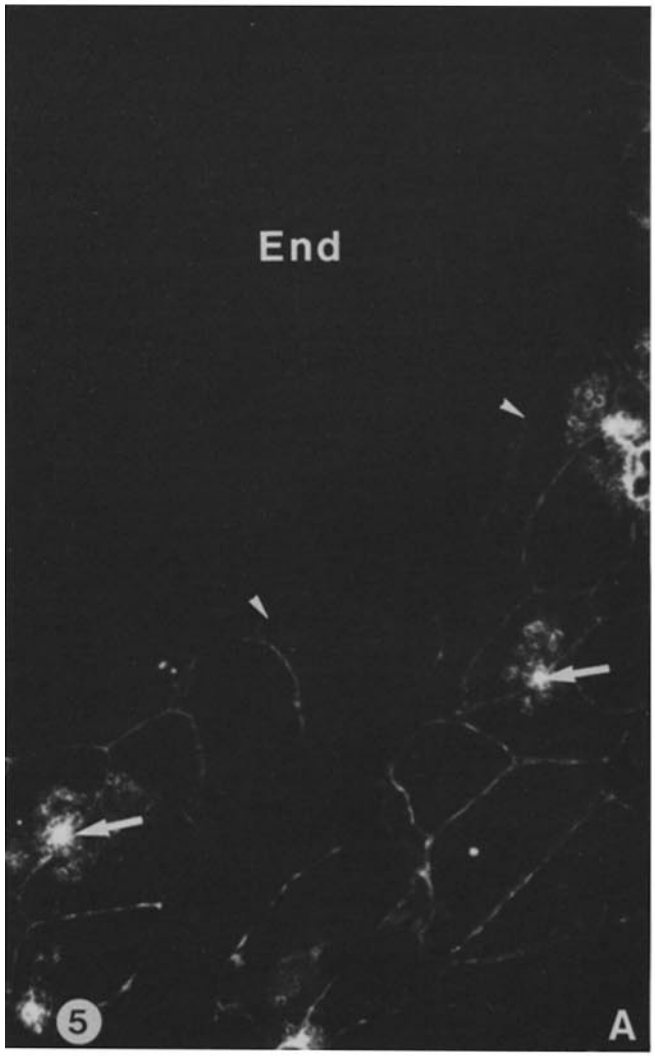
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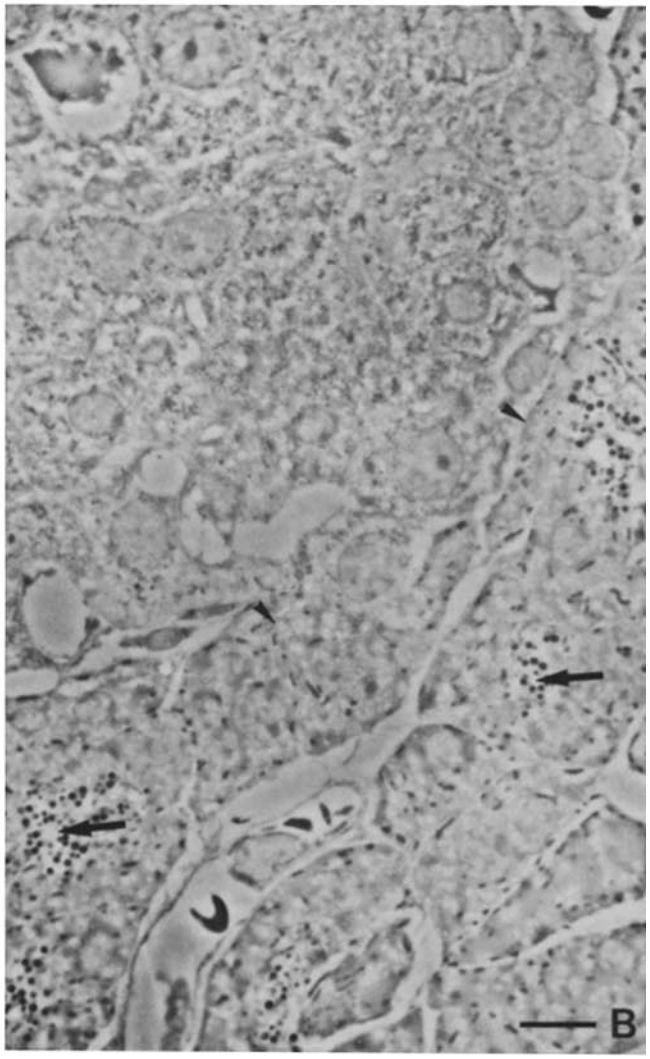
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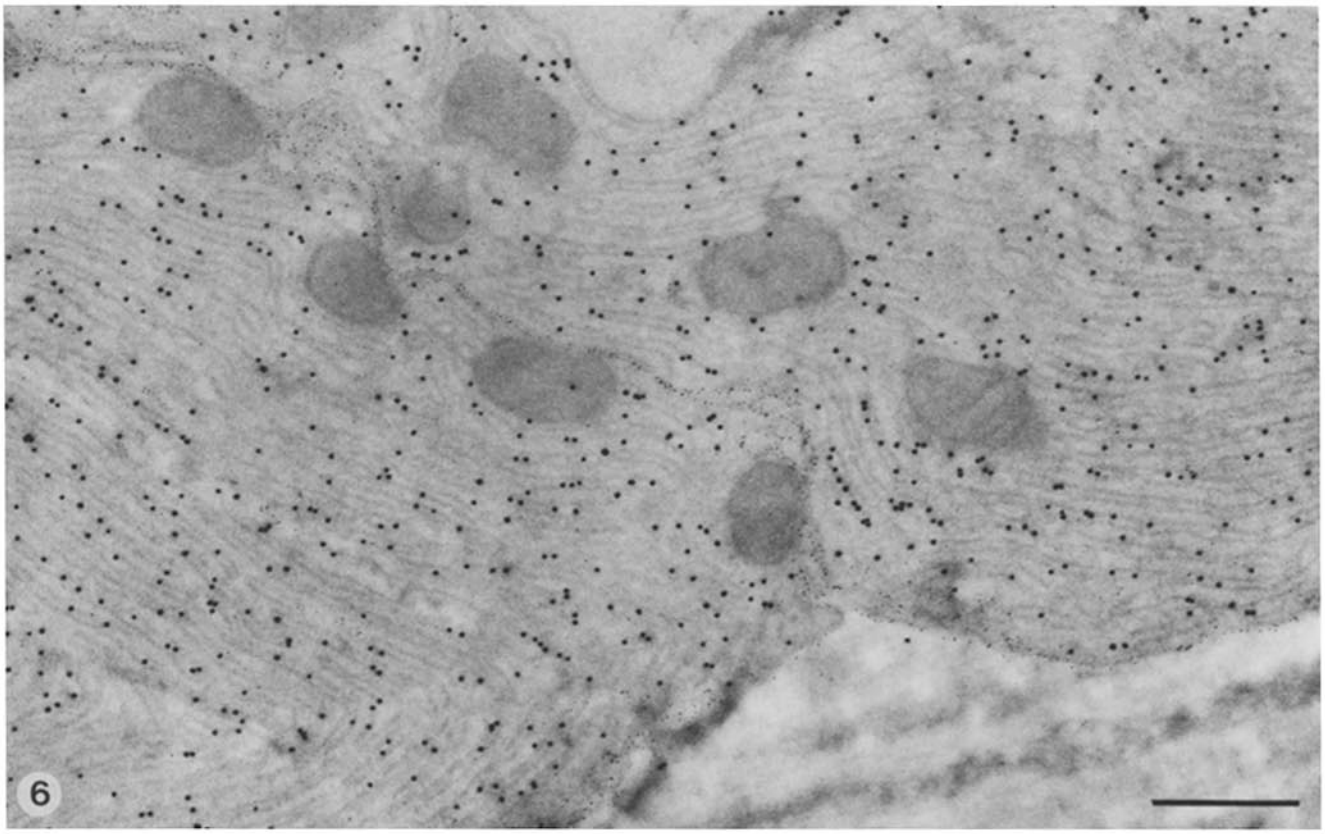
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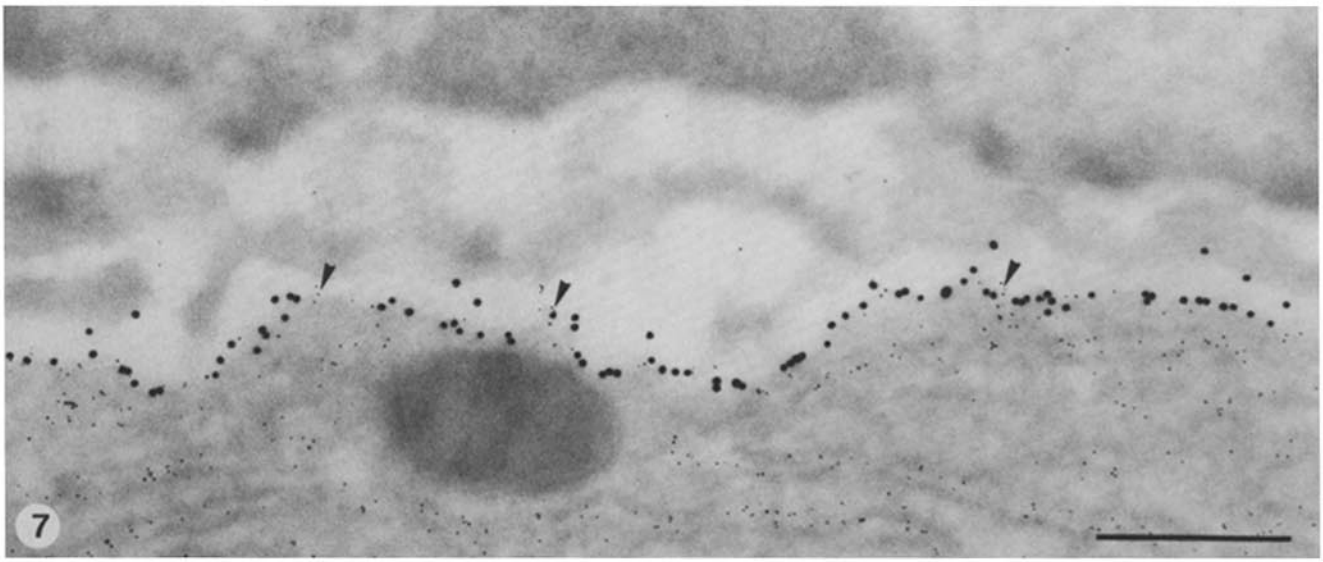
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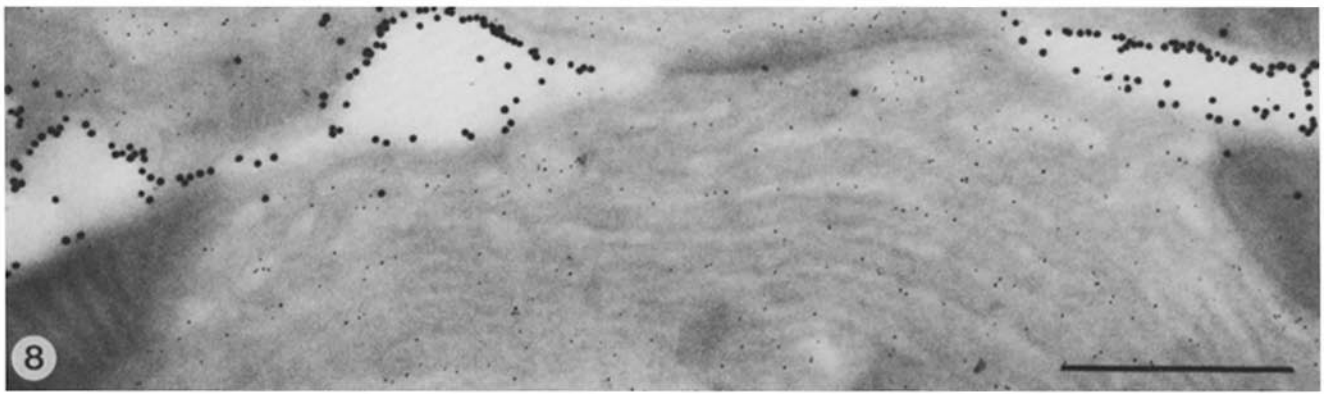
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course of the cell membrane. This was especially apparent at places where the membranes of neighboring cells interdigitated (Fig. 6). Occasionally, R × ZGM-positive but R × Am-negative vesicles were seen adjacent to the lateral cell membrane. Junctional complexes and sites of intimate contact along the lateral cell membranes lacked label (Fig. 8) or showed only a few gold particles (Fig. 15). Label was also absent from the nuclei and mitochondria (e.g., Figs. 6, 12, and 16).

The tendency of gold particles to adhere to the sections (mainly mitochondria, nuclei, and erythrocytes) apparently was eliminated when the PA/Au probes were mixed with albumin or IgG-less goat serum before use (Fig. 17). Gold particles are highly electron-dense. Compared to ferritin (11, 12), Au^5 particles could be discovered more easily, especially in the case of relatively thick sections or of structures with a high inherent density. Furthermore, the PA/Au^5 complex in particular was extremely immunosensitive on frozen sections. The separate localization of amylase and GP-2 on the lateral cell side allowed a close examination of the properties of the double-labeling technique. This situation permitted judgment of possible interference between the various steps of the indirect labeling procedure used. It appeared that interference was acceptably low in cases where the larger probe was applied first (Figs. 7 and 8) and was almost absent when PA/Au^5 was the first (Fig. 6). Protocols 9 and 10, which served as further controls on interference, also showed that interference was extremely low.

DISCUSSION

The aim of this study was twofold. We wished to localize a secretory granule membrane antigen at the ultrastructural level and compare its distribution with the well-known subcellular distribution of amylase (11, 12) as an example of a secretory protein. To this end, we investigated the usefulness of PA/Au probes on frozen sections and their suitability for the simultaneous demonstration of the antigens concerned.

Colloidal Gold Labeling of Frozen Sections

Until recently, ferritin was the only reliable label for high-resolution immunocytochemistry and was used almost exclusively in combination with frozen sections (27, 38). Dutton et al. (5) introduced iron dextrans as electron-dense labels and elaborated on the application of one particular particle, Imposil, with overall dimensions of 12 × 21 nm. These authors showed that this particle was a very clean immunocytochemical label and by its oblong shape was distinguishable from ferritin used simultaneously. Another promising group of particulate labels that came into the scope recently are gold colloids (8, 10, 16). Gold particles can be obtained in different sizes and are pluripotent cytochemical markers because they can be associated with various "bridging" proteins such as antigens (22), lectins (15, 16, 32), immunoglobulins (8, 29), and PA (1, 8, 28, 31). For the immunocytochemical localization of antigens, PA/Au probes have already been used as surface labels of resin sections (1, 31), but their usefulness on frozen sections to our knowledge has not yet been shown.

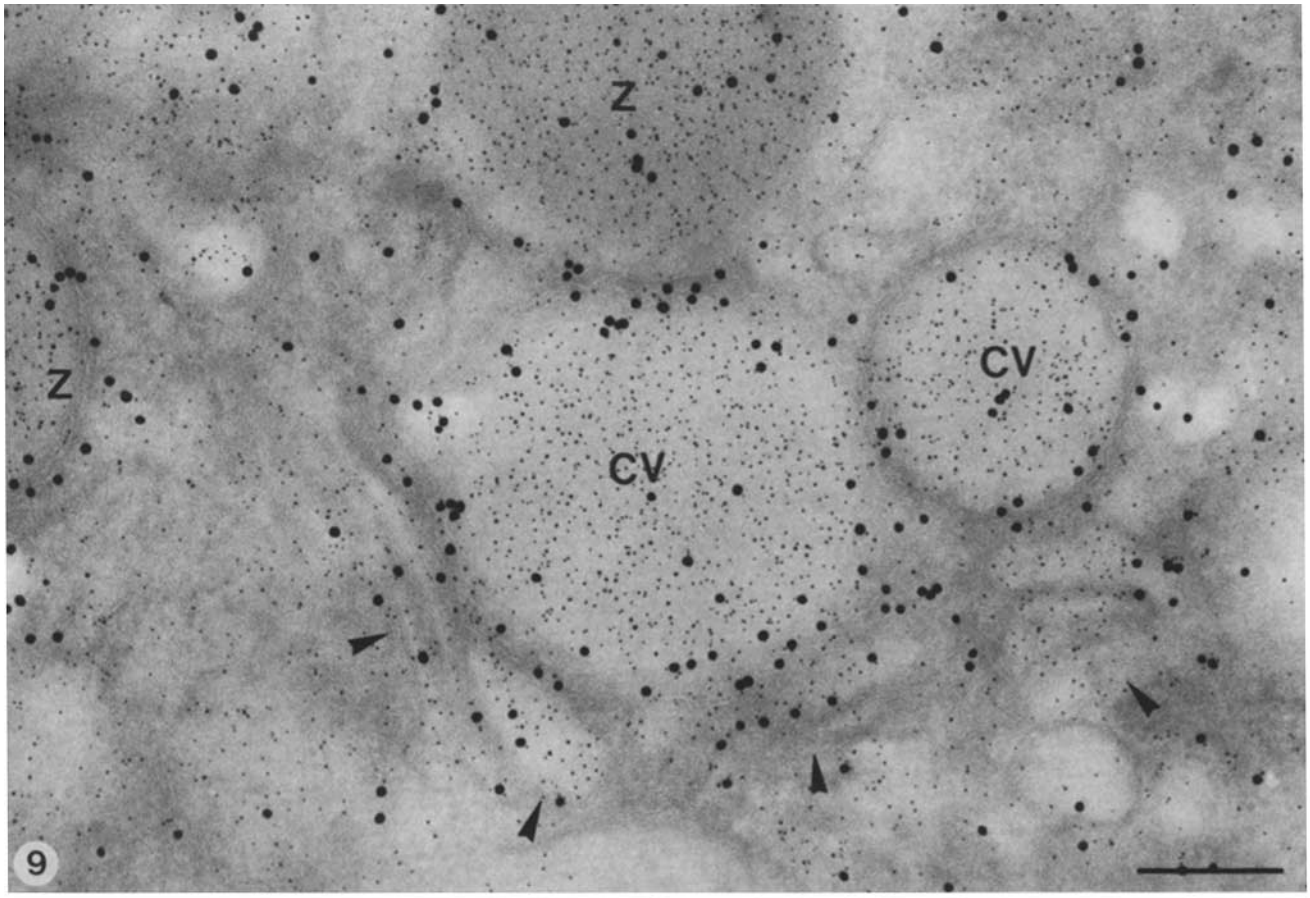
In this study we have demonstrated that PA/Au probes are suitable for frozen sections, provided they are mixed with protein (albumin, IgG-less serum) to reduce aspecific adherence of particles to the sections. In our opinion, the combination of ultrathin frozen sections (prepared by the Tokuyasu [27] method) and PA/Au probes as surface labels offers one of the most powerful immunocytochemical tools available for the demonstration of intracellular antigen. There are several advantages. Firstly, colloidal gold particles are highly electron dense, which makes them easy to perceive even at relatively low magnifications and on frozen sections with a high intrinsic density. Secondly, the coating of the gold particles with PA is rapid and uncomplicated, yielding a probe that is suitable to label IgG of most mammals, except goat and sheep (19). Thirdly, various sizes of gold particles can be made for high-resolution studies (13).[1] This opens up the field of multiple labeling of antigens.

Multiple labeling with gold has already been demonstrated in combination with lectins (14, 32). Two sizes of antigen-coated gold particles were used by Larsson (22) for the simultaneous demonstration of two peptides in resin sections of endocrine tissue. In that study the antibodies were applied to the sections at the same time, followed by a gold label bound to antigen. Indeed, a simultaneous administration of antibodies to the sections seems preferable for multiple labeling as it allows for free competition for binding sites. In the case of PA/Au probes, such a simultaneous labeling would have been possible if, for example, R × ZGM had been bound to PA/Au^5 and R × Am to PA/Au^{16} before application to the section. Had the probes been saturated with antibody, the immunoreaction would have been done in one step and the additional advantage of minimal interference would have been achieved. However, preliminary experiments showed that an indirect labeling procedure yielded considerably more label than when antibody/PA/Au complexes were used. A second reason for choosing an indirect procedure was that is is more practical to

FIGURE 6 All the following micrographs were taken from ultrathin (~100 nm) frozen sections indirectly immunolabeled with PA/Au probes. The sections were stained with alkaline and acidic uranyl acetate, and were embedded in methyl cellulose. Figs. 6–11 and 13 show immuno-double-labeling. Figs. 12 and 14–16 are labeled with PA/Au^5 only. Fig. 6: R × ZGM – PA/Au^5 – R × Am – PA/Au^{16}. Small gold particles indicating R × ZGM binding sites are present over the basal and lateral cell membranes. Note that the label accurately follows the nodes of interdigitating lateral cell membranes, which probably correspond to the fluorescent dots of Fig. 5A. The larger particles, labeling amylase, are located over the RER. Aspecific labeling of mitochondria, the nucleus, and connective tissue is low. The two sizes of particles exhibit sharply separated distributions, which demonstrates that interference between the steps of the labeling procedure is negligible. Bar, 0.5 μm. × 38,500.

FIGURE 7 R × ZGM – PA/Au^{16} – R × Am – PA/Au^5. In this case, the small particles label amylase sites in the RER cisternae, while the larger particles mark GP-2. The centro-acinar cell in the upper half of the figure is negative for both antibodies. The second difference with the protocol of the previous figure is that PA/Au^{16} was used before PA/Au^5. This caused some interference between the labels (arrowheads). Bar, 0.25 μm. × 52,500.

FIGURE 8 Labeling sequence and particle distribution as in Fig. 7. The junction, probably a gap junction, between the two apposed lateral cell membranes is not reactive for R × ZGM. Bar, 0.5 μm. × 66,000.


Z
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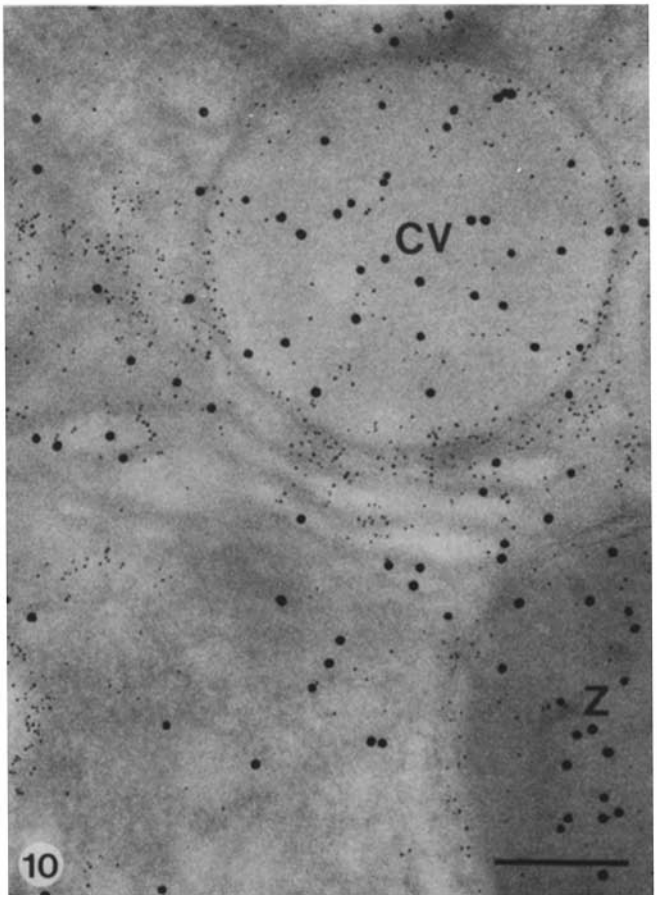
9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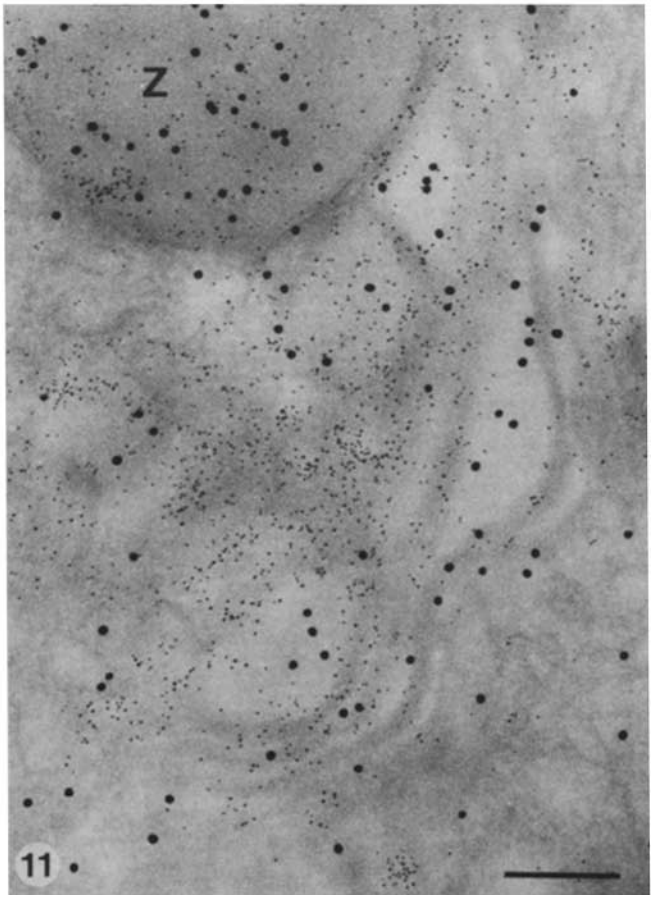
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11

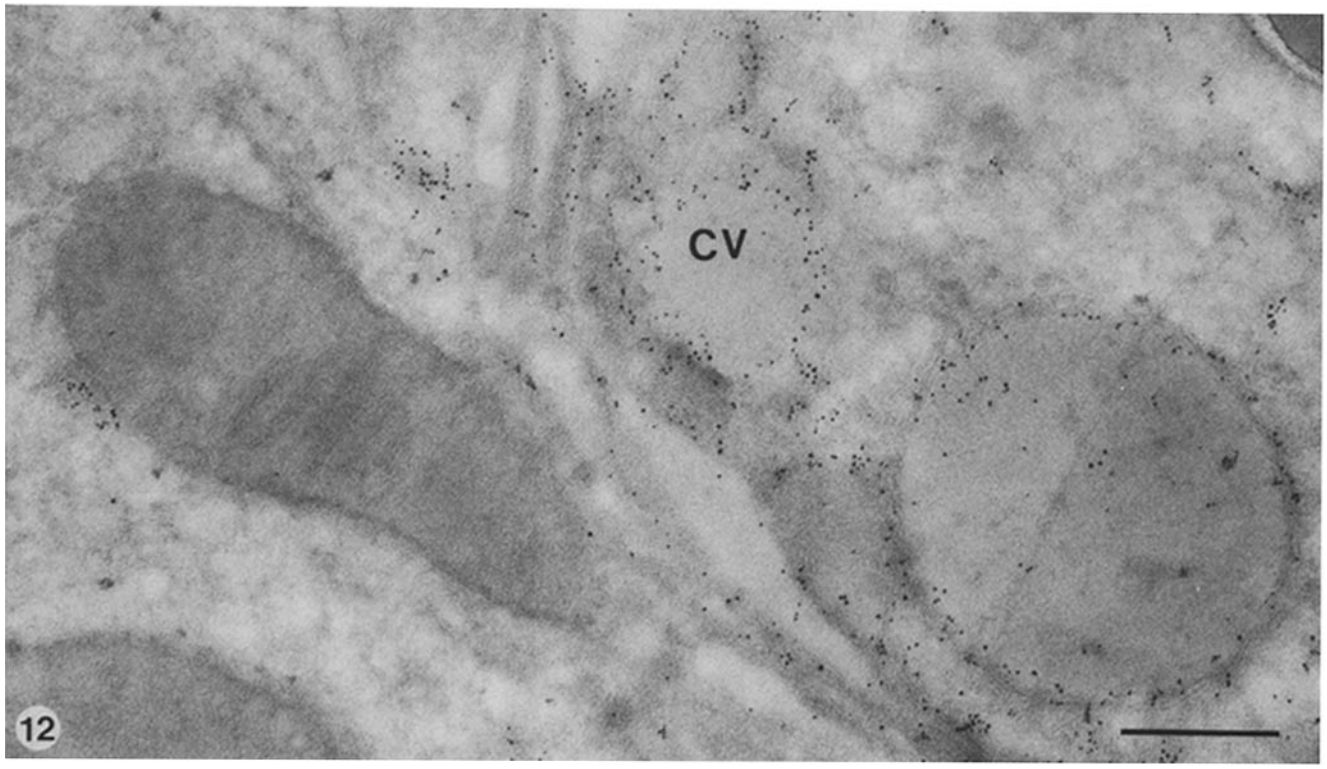


FIGURE 12 Single labeling with R × GP-2 and PA/Au^5. Like R × ZGM, R × GP-2 reacts predominantly with the membranes of the trans-Golgi cisternae and condensing vacuoles (*CV*). Bar, 0.25 μm. × 90,000.

work with unconjugated probes that can be used for other purposes as well.

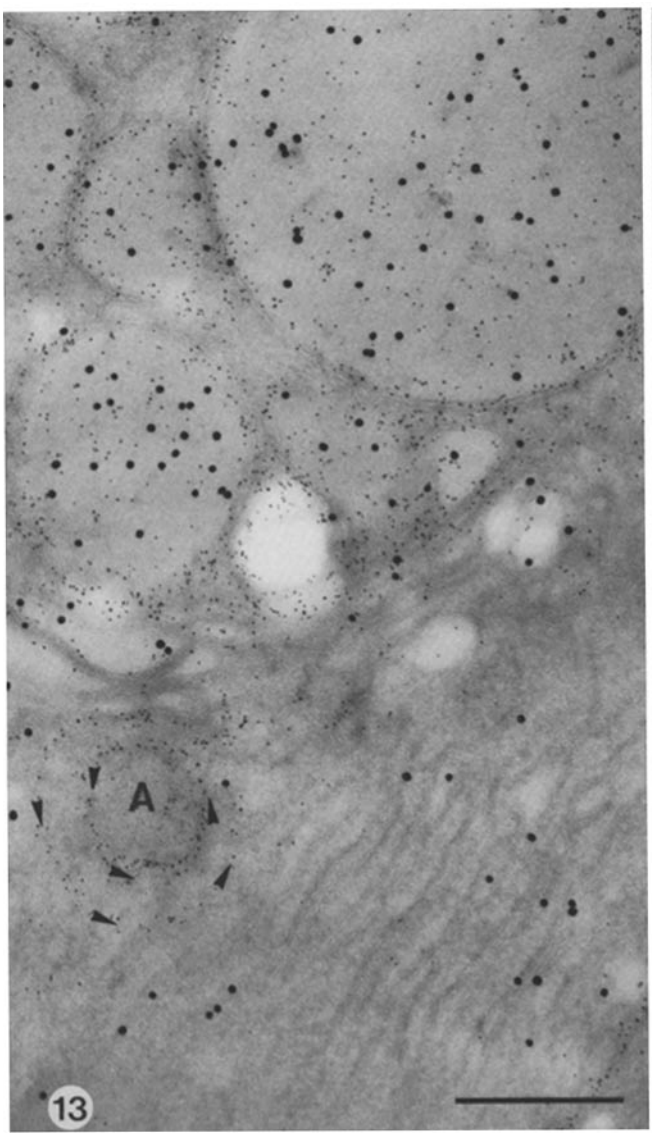
For a proper judgment of possible interference between the various steps in our incubation sequences, it was advantageous that our antibodies labeled, at least at some places, sharply separated sites. As we showed already with fluorescence microscopy, such a situation existed at the lateral cell sides, where R × ZGM and R × GP-2 reaction was confined to the cell boundary, while R × Am stained the cytoplasm. As can be seen in the micrographs of the lateral cell sides, the antibody-probe-antibody-probe sequence results in acceptably low levels of co-labeling of the first-step antibody-binding sites with the second probe, irrespective of whether R × ZGM or R × Am was administered first. When Au^{16} was the first label, some interference of varying intensity with Au^5 was noticed. Probably the larger particles are more subject to mutual hindrance and, when applied first, leave binding sites free to be approached by the smaller second probe.

In practice, sequences 3 and 4 (see Materials and Methods) were best. The apparent inability of PA/Au^{16} as second probe to mark sites of the first antibody as well may be caused by the properties of the gold particles and/or the reaction mechanism of IgG and PA. As for the particles, a comparable lack of interference was observed by Horrisberger and Vonlanthen working with lectin-coated gold (14, 15). These authors suggest that repulsive electrostatic forces of the charged gold particles prevent potential interaction. In addition, the reaction between PA and IgG may be of importance. It is now clearly established that PA reacts with the Fc fragment of IgG (9), to wit, the intact combination of the constant domains 2 and 3 (6, 34). PA has at least two binding sites for IgG and is capable of producing $(IgG)_2PA$ when the reaction is carried out in solution (21, 25). Soluble complexes of $(IgG)_2PA$ fail to interact further with PA, indicating that possible other PA-binding sites of the IgG molecule are blocked by the binding of one PA (25). Thus, IgG seems to be monovalent for PA. This monovalency may underlie the lack of interference in our labeling sequence and would explain why the second antibody, that had most likely reacted with free IgG-binding sites of the first probe as well, was not visualized by the second probe.

FIGURE 9 R × Am – PA/Au^5 – R × ZGM – PA/Au^{16}. R × Am (Au^5) labeled the contents of the Golgi cisternae, the condensing vacuoles (*CV*), and the zymogen granules (*Z*). R × ZGM labeling (Au^{16}) is present over the Golgi complex (arrowheads) and is associated with the periphery of the condensing vacuoles. Bar, 0.25 μm. × 76,000.

FIGURE 10 R × ZGM – PA/Au^5 – R × Am – PA/Au^{16}. Because the small particles now label GP-2 sites, the difference between the labeling patterns of R × Am and R × ZGM is more marked than in Fig. 9. R × ZGM activity is present at the trans-side of the Golgi complex, and at the periphery of the condensing vacuole (*CV*) and the zymogen granule (*Z*). Bar, 0.25 μm. × 73,500.

FIGURE 11 Labeling sequence as in Fig. 10. The small particles belonging to R × ZGM located over the membranes of the Golgi cisternae, mainly of the trans-side. In contrast, R × Am labeled the contents of the Golgi cisternae, including those at the cis-side. Both antibodies reacted with the contents of the zymogen granule (*Z*), while R × ZGM label is also associated with the granule membrane. Bar, 0.25 μm. × 66,000.



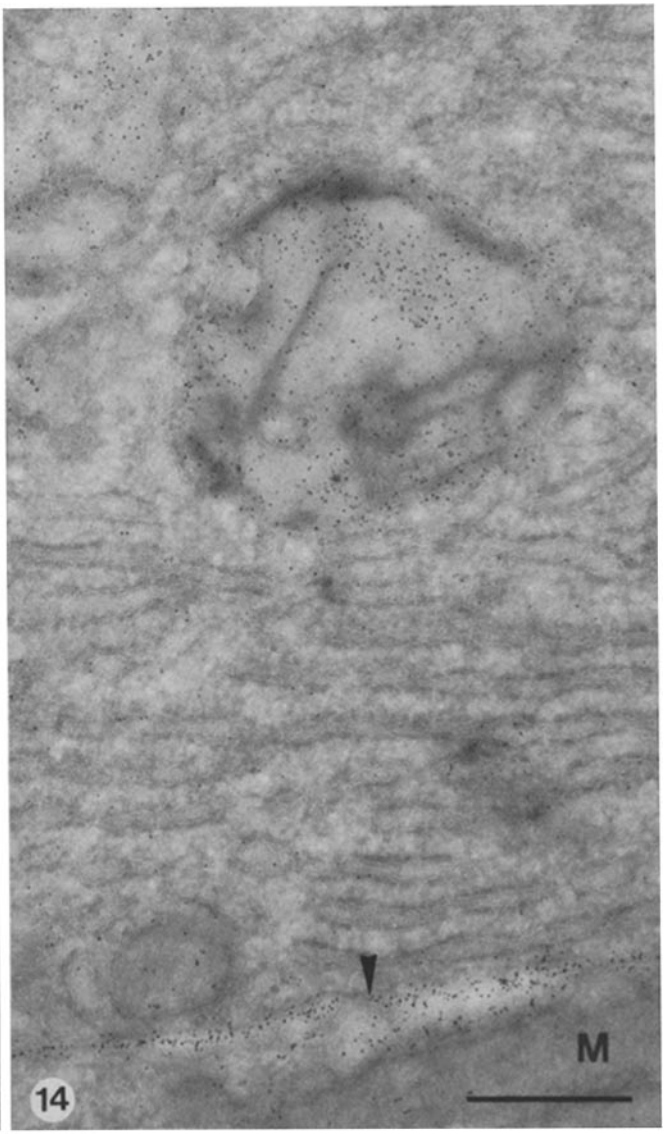


FIGURE 13 R × ZGM – PA/Au[5] – R × Am – PA/Au[16]. Only R × ZGM labeled the autophagic vacuole (*A*). The developing autophagic vacuole is located at the cis-side of the Golgi complex and is enveloped by two concentric membranes (arrowheads) with a labeling intensity corresponding to that of the trans-Golgi membranes, but different from that of the cis-Golgi membranes, which show only a few particles. Bar, 0.5 μm. × 43,500.

FIGURE 14 Single labeling with R × ZGM and PA/Au[5]. Label is present over the residual body and the lateral cell membranes (arrowhead). *M*, mitochondrion. Bar, 0.5 μm. × 43,000.

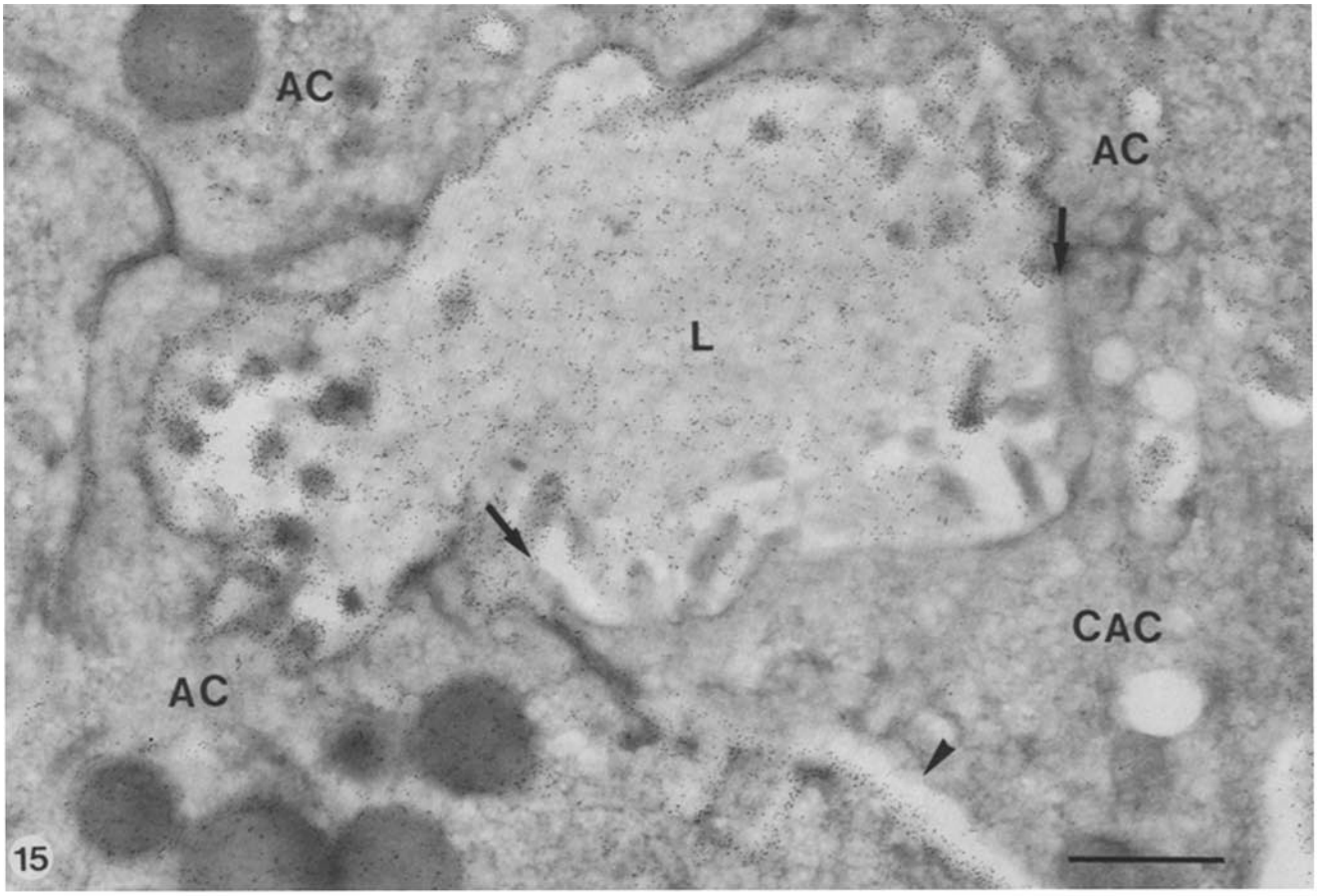
Immunocytochemical Observations

We worked with two antibody preparations directed against zymogen granule components that co-purified with ZGM. Both antibodies reacted with GP-2, while R × ZGM was also slightly reactive with GP-1. Adsorption of R × ZGM and R × GP-2 with GP-2-like protein quenched the reaction with gels and tissue sections completely. We have obtained two indications that the antibodies recognized different parts of the molecule.

(*a*) R × ZGM affinity was dependent on the carbohydrates of GP-2 and the immunoreaction was quenched by periodate oxidation of carbohydrates in SDS PAGE and tissue sections. In contrast, oxidation of carbohydrates only slightly affected R × GP-2 reactivity. Probably, R × GP-2 was directed against

FIGURE 15 Single labeling with R × ZGM and PA/Au[5]. Section through termination of duct system with three acinar cells (*AC*) and one centroacinar cell (*CAC*) around a duct lumen (*L*). Reaction can be seen in the lumen and along the outer faces of the apical and lateral cell membranes of AC. The apical membrane (between arrows) and the lateral cell membrane (arrowhead) of CAC are negative. Bar, 0.5 μm. × 42,000.

FIGURE 16 Single labeling with R × GP-2 and PA/Au[5]. The *inset* of the figure shows an intercalated duct in survey. × 2,450. Part of the section enclosed in the rectangle is shown in the main figure. GP-2 reactivity is present in the ductal lumen and on the cell membrane of the acinar cell at the left. Bar, 0.5 μm. × 42,000.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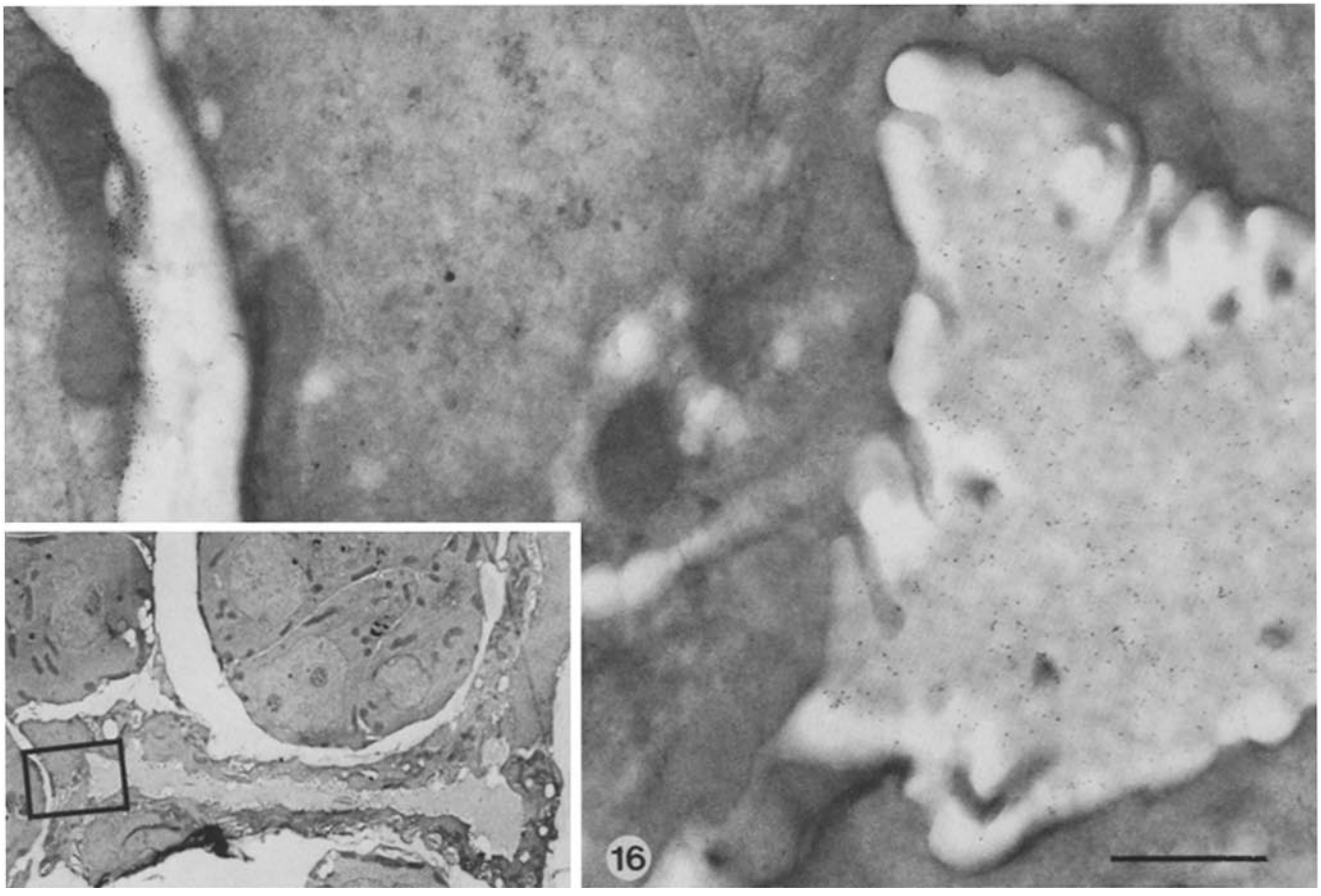
15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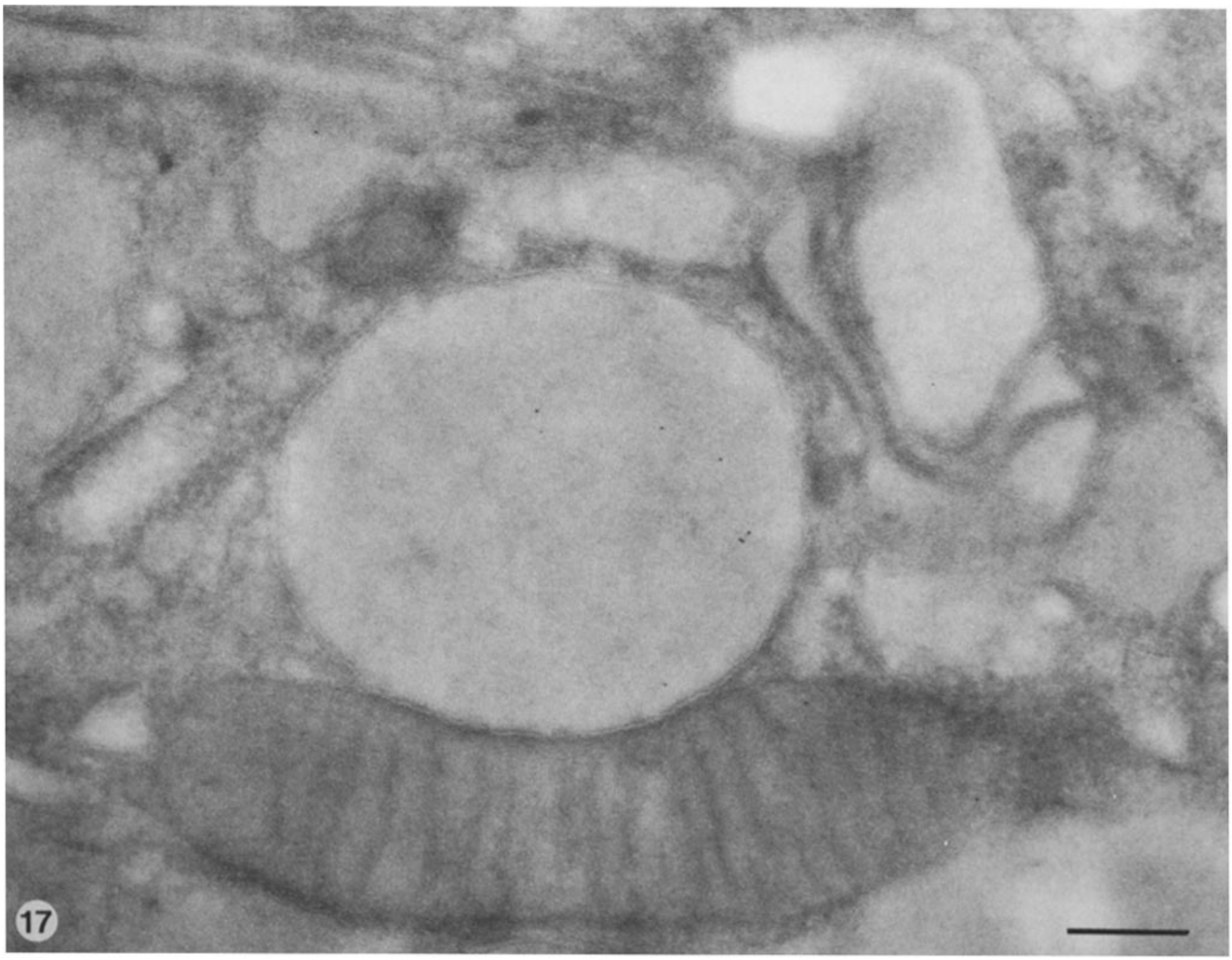

FIGURE 17 Control on aspecific adherence of the gold probes. R × HRP – PA/Au^5. Bar, 0.25 μm. × 75,000.

the protein part of GP-2. This would agree with the weak but distinct immunoreaction of R × GP-2 with the cytoplasm (RER) in immunofluorescence (33).

(*b*) Only R × ZGM reacted with submandibular gland cells in the same fashion as with the exocrine pancreas and recognized one glycoprotein in SDS PAGE of submandibular homogenate. Probably, R × ZGM determinant carbohydrates are also present in the submandibular gland, be it on a glycoprotein of higher molecular weight that is not recognized by R × GP-2.

Both antibodies labeled the Golgi complex, the zymogen granules, the entire cell membrane except for the junctional regions, the autophagic vacuoles, and the secretory lumina. Double labeling was especially advantageous for the interpretation of the labeling patterns in the Golgi complex and the zymogen granules. As described previously (12), R × Am labeled the contents of all Golgi cisternae and zymogen granules. However, R × ZGM and R × GP-2 labeling was markedly different. In the Golgi complex, label was mainly confined to the membranes of the trans-cisternae. Both reactions increased in density from the cis- towards the trans-Golgi side with an even steeper gradient than observed for zymogens. So far, R × ZGM and R × GP-2 labeling patterns coincide with the position of a Golgi membrane glycoprotein.

Along the line trans-Golgi side – condensing vacuoles – zymogen granules – acinar lumen, the labeling patterns progressively acquired the characteristics of a secretory protein. However, unlike amylase and chymotrypsinogen (12), GP-2 antigenicity, besides being present in the contents of granules and lumina, also retained a membrane-associated position. This shift again shows that the labeling is indicative of GP-2 rather than representing a mixed demonstration of GP-2 and zymogen. Apparently, GP-2 starts as a membrane-associated protein in the Golgi complex and expands into the contents of the granules during condensation and maturation. The presence of a GP-2-like substance in pancreatic juice (33) and the GP-2 reactivity over the brush borders of intestinal cells indicates that GP-2 at least partially becomes discharged from the cells together with the "normal" secretory proteins.

The finding of GP-2 in the zymogen granule content makes it puzzling that GP-2 was not present in granule bicarbonate lysate. This has also been found by others (30). The situation is reminiscent of that in the parotid gland of the rabbit, where SDS PAGE profiles of secretory granule membranes and duct collection were shown to share three glycoproteins that copurified with the granule membranes and were almost lacking from the granule content subfraction (3). These proteins were nevertheless considered as secretory contaminants, because they were present in salivary juice and could selectively be removed from the membranes with saponin and Na_2SO_4 (4). In the case of the rat, however, we were not able to dissociate GP-2 from the membranes by a similar treatment (33). We

therefore conclude that GP-2 exhibits a firm association with the granule membrane, withstanding strong chaotropic agents. Because the distance of the gold label from the membrane was often much too large for single GP-2 molecules sticking in the membranes, two possibilities with respect to the nature of GP-2 can be envisaged. Firstly, GP-2 may constitute a coherent system of polymeric molecules inserted into the inner face of the granule membrane. Upon secretion, this conglomerate would remain intact and can be removed from the secretion by centrifugation (33). A second, alternative, possibility is that GP-2 is indeed a real membrane component that to some extent detaches from the inner surface of the granule membrane and the apical cell membrane and appears in the secretion. If so, such free GP-2 is only a minor component in the granule content because it was not detected in gels and did not influence immunoreactivity of R × ZGM and R × GP-2 after absorption with the granule content subfraction. Further work will be needed to elucidate this problem. It would also be of interest to investigate whether the protein is engaged in the process of zymogen packaging and condensation, or whether it plays some role in the internal structure of the zymogen granules (7).

Both R × ZGM and R × GP-2 also labeled the lateral and basal cell membranes and autophagic vacuoles. Low levels of GP-2 have indeed been found in the plasma membrane preparation of the rat pancreas (30). It can be questioned whether GP-2 originates from exocytosis-derived apical GP-2 or whether it is added directly to the latero-basal cell membrane by means of vesicles coming from the Golgi complex. The presence of small GP-2-positive vesicles along the lateral cell membrane may support the latter possibility.

Another unexpected phenomenon was the labeling of lysosomal structures with both R × ZGM and R × GP-2. These structures were most likely autophagic in nature, as they often contained recognizable exocrine cell components such as RER and mitochondria. In developing autophagic vacuoles, label occurred predominantly over the enveloping membranes. This observation probably contributes to the question of the origin of the autophagic vacuole envelope. Although the vacuoles were mostly located at the cis-side of the Golgi complex, their envelope labeling was similar in density to that of the trans-Golgi membranes. This might support the notion that lysosomes in the pancreas originate from the trans-Golgi face (26).

In conclusion, GP-2 is not a distinctive feature of the membranes of pancreatic zymogen granules (23), as it is present not only in the zymogen granule membranes but in all structures of the secretory pathway and in the cell membrane and lysosomes. Until details on its relationship to membranes are known, GP-2 might equally be considered a membrane-associated secretory protein.

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