Immunocytochemical Localization of Lysosomal β-Galactosidase in Rat Liver

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ABSTRACT β-galactosidase is a ubiquitous lysosomal hydrolase that specifically cleaves terminal β-galactosyl residues from glycoproteins, glycosaminoglycans, oligosaccharides, and glycolipids. To study the intracellular distribution of this enzyme, we prepared a specific polyclonal antibody to lysosomal β-galactosidase by immunizing rabbits with a highly purified preparation of β-galactosidase from rat liver. Using this antibody we employed an immunocytochemical technique (protein A coupled to horseradish peroxidase and diaminobenzidine cytochemistry) and showed that β-galactosidase is present in all hepatocytes of the rat liver. All types of lysosomes, the rough endoplasmic reticulum, and the specialized region of smooth endoplasmic reticulum known as GERL showed immunoreactivity. This in situ distribution suggests that these organelles are involved in the biosynthesis and intracellular sorting of this lysosomal enzyme.

Biogenesis of lysosomal enzymes is thought to proceed along the same intracellular pathways as described for membrane and secretory glycoproteins (1–3). Lysosomal hydrolases resemble secretory proteins in two respects: they are glycoproteins, and some of the newly synthesized enzymes are exported extracellularly (3). For two lysosomal hydrolases, cathepsin D and β-glucuronidase, the co-translational segregation into intracellular membrane-bound polysomes has been demonstrated (3, 4). This is the first step in the pathway followed by genuine secretory proteins beginning their extracellular transport. However, unlike secretory proteins, these hydrolases are sequestrated into lysosomes apparently through recognition of the phosphomannosyl residues on their oligosaccharide chains (5, 6).

Localization of lysosomal enzymes in various steps of this pathway has been deduced from the study of subcellular fractions or from cytochemical demonstration of enzyme products (7, 8). Neither of these techniques is completely satisfactory since cross-contamination of isolated fractions can rarely be definitively excluded and reliable enzyme cytochemical methods demonstrating the presence of most lysosomal hydrolases are not available. Therefore, we undertook the immunocytochemical localization of one of these hydrolases, β-galactosidase (β-gal)[1], to provide direct evidence for its intracellular localization.

Using a highly specific antibody prepared against β-gal isolated from rat liver, in conjunction with protein A labeled with horseradish peroxidase (pA-HRP) (9), and diaminobenzidine (DAB) cytochemistry (10), we assessed the intracellular distribution of β-gal at both the light and electron microscopic levels. DAB reaction product, revealing the antigenic sites of β-gal, was seen in all types of lysosomes, the rough endoplasmic reticulum, and the specialized region of the smooth endoplasmic reticulum (GERL) (11–13).

MATERIALS AND METHODS

Purification of β-galactosidase: β-gal was purified from the liver of Sprague-Dawley rats (Madison, WI) by modifications of procedures used to purify this glycoprotein from human (14) and mouse liver (15). Briefly, we prepared a tissue fraction enriched in lysosomes by differential centrifugation of homogenates from 54 livers. The lysosomes were disrupted by osmotic shock, and solubilized lysosomal enzymes were separated from membrane fragments by centrifugation. Purification of lysosomal β-gal was performed by

[1] *Abbreviations used in this paper:* β-gal, β-galactosidase; DAB, diaminobenzidine; pA-HRP, protein A-horseradish peroxidase.

ammonium sulfate precipitation and stepwise affinity, anion exchange, and molecular sieve chromatography with concanavalin A (Pharmacia, Inc., Piscataway, NJ), DEAE cellulose (Whatman Chemical Separation, Inc., Clifton, NJ), and Sephadex G-200 (Pharmacia, Inc.) (16). Using this approach, we recovered 10 mg (4% yield) of β-gal purified 3223-fold compared with the homogenate. We demonstrated a single protein band by polyacrylamide gel electrophoresis under nondenaturing conditions that showed β-gal activity. Using polyacrylamide slab gel electrophoresis, we resolved the purified protein into two major and one minor band under denaturing and reducing conditions. The two major bands had molecular weights of 56,000 and 63,000; these bands likely represent multiple forms of the enzyme, as has been demonstrated to exist in human (14) and mouse liver (15). The minor band had a molecular weight of 41,000 and likely represented either a minor contaminant or a proteolytic product of one of the major bands.

Preparation of Antisera to β-Galactosidase: 200 μg of purified β-gal in 1.5 ml of complete Freund's adjuvant was injected intradermally into New Zealand white rabbits (Institute Hill Animal Facility, Rochester, MN) at 6-wk intervals and the rabbits were sequentially bled beginning at 12 wk. The antiserum preparation used in this study gave a single coincident precipitin line on double immunodiffusion and rocket immunoelectrophoresis against purified β-gal and solubilized liver fractions. In addition, the precipitin band formed by rocket immunoelectrophoresis contained β-gal activity. The same antiserum has been shown to precipitate selectively the activity of β-gal from homogenates of rat liver, spleen, kidney, and heart, and to exhibit temperature and pH-dependent binding to radiolabeled β-gal (17). Further characterization of the antiserum is underway and complete details will be published in a separate manuscript now in preparation.

Preparation of Tissue for Immunocytochemistry: Untreated Sprague-Dawley male rats (350–400 g) (Marland Farms, Hewitt, NJ) were anesthetized by ether, and the livers were fixed either by immersion or perfusion in the following fixatives at 4°C: (*a*) 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4, 7.5% sucrose; (*b*) 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4, 0.05 M L-lysine, 0.01 M sodium periodate, 7.5% sucrose (18); and (*c*) 4% paraformaldehyde, 0.05% glutaraldehyde, 0.1 M phosphate buffer, pH 7.4, 7.5% sucrose (19). When perfusion fixation was employed, cold isotonic saline was injected into the portal vein for 30 s, followed by perfusion with the above fixatives for 15 min for a flow rate of 8 ml/min. The livers were removed, thin slices were prepared, and fixation was continued by immersion. The total fixation time was 4 h in cold fixative, as in *a* and *b*, above, and 2 h in cold fixative, as in *c*, with continuous gentle shaking of tissues in a Dubnoff metabolic shaking incubator.

Preparation of Sections: After an overnight rinse in cold 0.1 M phosphate buffer, pH 7.4, 7.5% sucrose (PBS), nonfrozen sections ~30 μm thick was prepared from liver slices from all three fixatives with an Oxford Vibratome (Ted Pella, Tustin, CA), using a slow speed and high amplitude setting, and placed in cold 7.5% sucrose. The sections prepared from liver tissue fixed in fixative, as in *c*, were treated for 30 min in cold lysine, 10 mg/ml (20) in phosphate buffer, pH 7.4, 7.5% sucrose prior to exposure to immunoreagents.

Immunocytochemical Procedures: The Vibratome sections were exposed to either rabbit monospecific rat β-gal antiserum or β-gal IgG (purified as described in reference 21) at 4°C for 20–40 h, with intermittent, mild agitation. To prevent sections from falling on each other, thereby possibly inhibiting penetration of reagents, single sections (~2 mm^2) were placed in Linbro tissue-culture U-shaped multi-well plastic trays that contained 0.2 ml of antiserum or antibody (final concentration of 0.18 mg IgG/ml) diluted with PBS. Antiserum dilutions ranged from 1:10 to 1:100. A total of 10–14 sections were exposed to β-gal antibody. After several rinses in cold PBS the sections were treated with protein A-horseradish peroxidase (0.1 mg/ml) (E. Y. Laboratories, San Mateo, CA) for 1 h at room temperature, in the dark.

After several rinses in PBS and in 7.5% sucrose, the sections were incubated in 3,3′-diaminobenzidine tetrahydrochloride (DAB) (Sigma Chemical Co., St. Louis, MO) at pH 7.6 (10) at room temperature for 10 min in the dark, to reveal peroxidase activity.

The incubating medium preparation follows: 10 mg of DAB was dissolved in 10 ml of 0.1 M Tris HCl buffer that contained 7.5% sucrose. The medium was adjusted to pH 7.6 before adding 0.2 ml of 1% H_2O_2 (freshly prepared) and was filtered before use. The following controls were performed: (*a*) exposure of sections to nonimmune rabbit serum or chromatographically purified IgG (final concentration 0.22 mg/ml of IgG) (Cappel Laboratories, Inc., Cochranville, PA), treatment with pA-HRP and incubation in DAB, as described above; (*b*) treatment with pA-HRP and incubation in DAB, as described above, but without prior exposure to immune or nonimmune antiserum or IgG; (*c*) treatment with unlabeled pA and incubation in DAB, as described above; and (*d*) incubated in DAB, as above, but no treatment with antiserum, IgG, pA-HRP, or pA.

In addition, Vibratome sections of liver were treated as follows: (*a*) pre-exposure to nonimmune rabbit serum before exposure to immune antiserum; and (*b*) treatment with cold 0.02% Triton X-100 for 30 min before exposure to immune antiserum.

Additional rat tissues exposed to β-gal antiserum include the intestine, kidney, and cerebellum. Liver sections were also exposed to rabbit anti-rat β-glucuronidase serum.

Vibratome sections treated in the above procedures were examined by light microscopy after mounting in glycerogel. Vibratome sections were also prepared for electron microscopy after additional fixation in cold 2.5% glutaraldehyde 0.1% M PBS for 30 min before osmication, and electron microscope procedures followed, as described elsewhere (19). EPON sections were examined with and without en bloc uranyl stain (22, 23), and with and without lead stain (24), using a Philips EM-300.

EPON sections 2–3 μm and 500–800 Å thick) were cut and examined in two directions, parallel and perpendicular to the plane of the section. This permitted assessment of the penetration of immunoreagents.

RESULTS

Light Microscope Observations

Fig. 1*b* shows the immunocytochemical localization of β-gal to spherical dot-like structures in all the hepatocytes in a 2-μm EPON section. The antigenic sites of β-gal are found exclusively in lysosomes. The identification of these organelles is based on their distribution along the bile canaliculus. At this level, no other organelles show DAB reaction product. The occasional staining of the sinusoidal areas are nonspecific, most probably resulting from diffusion of hemoglobin and/or leakage of endogenous peroxidase from disrupted Kupffer cells. Fig. 1 is one control, a 20-μm nonfrozen section that shows complete absence of reaction product in the lysosomes of all hepatocytes. This control was incubated with nonimmune rabbit serum. All other controls were also negative.

Our light microscope studies revealed that the antigenic sites of rat liver β-gal are recognized by the lysosomes of other cell types: rat intestine, kidney, and cerebellum. Fig. 6 illustrates immunoreactive lysosomes in the rat cerebellum: in Purkinje cells, pericytes, and molecular and granular layer neurons.

Electron Microscopic Observations[2]

The localization of antigenic sites of β-gal to numerous lysosomal structures concentrated along the bile canaliculus, as revealed by electron-dense DAB reaction product, is illustrated in Fig. 3*a*. Fig. 3, *b* and *c* show reaction product associated with the endoplasmic reticulum. Longer exposure (40 h) to immune antibody yielded more reaction product in a greater number of endoplasmic reticulum domains than did shorter exposure (20 h). The immunoreactive domains had more reaction product. The reaction product appears to be localized to the membrane and within the cisternae. Fig. 3*b* also shows reaction product in a lysosome connected to smooth-surfaced membranes. The lysosomal membrane appears to be stained (see Discussion regarding membrane staining). An additional antigenic site of β-gal localization is found in structures at the *trans* aspect of the Golgi apparatus. These structures are parts of GERL (Fig. 4*a*). Of importance is the consistent absence of reaction product in all the elements of the Golgi apparatus. The absence of reaction product in the Golgi apparatus was observed under the following conditions:

[2] All electron micrographs in Figs. 3–6 are areas of hepatocytes from nonfrozen sections of liver exposed to rabbit anti-rat β-gal antibody for 20 h, treated with pA-HRP, and then incubated in DAB, except for Fig. 5 which was exposed to rabbit anti-rat β-glucuronidase antibody.

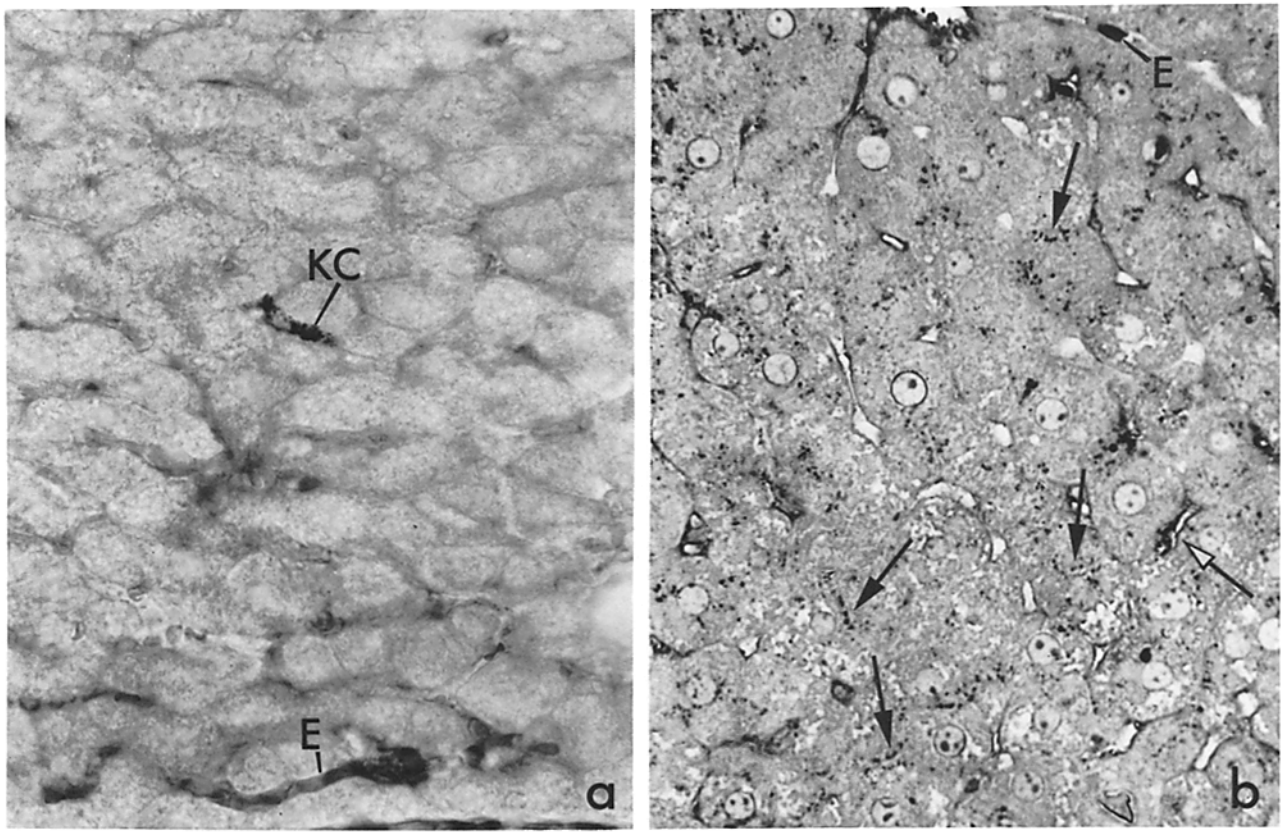

FIGURE 1 Light microscope preparations; liver fixed in fixative a. (*a*) Control. Vibratome section (≃20 μm) was exposed to normal rabbit serum for 20 h, treated with pA-HRP, and incubated in DAB. All hepatocytes are totally without reaction product. Only the erythrocytes (*E*) (due to their hemoglobin content) and Kupffer cells (*KC*) (due to their endogenous peroxidase activity) show reaction product. (*b*) A 2-μm section from an EPON bloc of a nonfrozen section exposed to rabbit anti-rat β-gal antiserum for 20 h, treated with pA-HRP, and incubated in DAB. DAB reaction product is seen in the lysosomes of all hepatocytes. Four linear arrays of lysosomes are indicated by black arrows. The white arrow indicates sinusoidal staining, probably due to absorption of hemoglobin released from erythrocytes (*E*) during fixation. Section stained with toluidine blue. × 425.

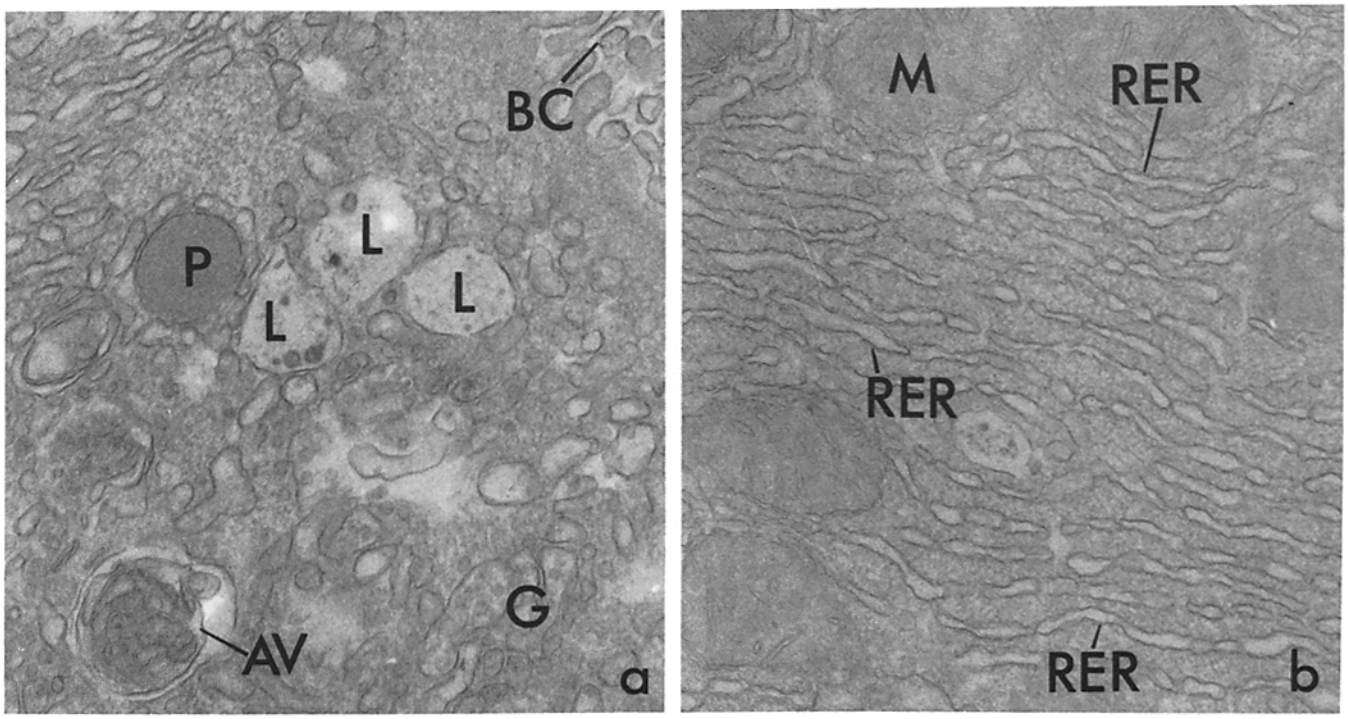

FIGURE 2 Electron micrographs of control sections of hepatocytes from rat liver exposed to normal rabbit serum for 20 h, treated with pA-HRP, and incubated with DAB. Reaction product is not seen in any of the sections. Labeled in the figures are a cluster of lysosomes, *L*, including an autophagic vacuole, *AV*; bile canaliculus, *BC*; Golgi apparatus *G*, including very low density lipoprotein particles; rough endoplasmic reticulum, *RER*; mitochondria (*M*); and peroxisome, (*P*). It is evident that reaction product is not present anywhere. Fixation in fixative a. × 36,000.

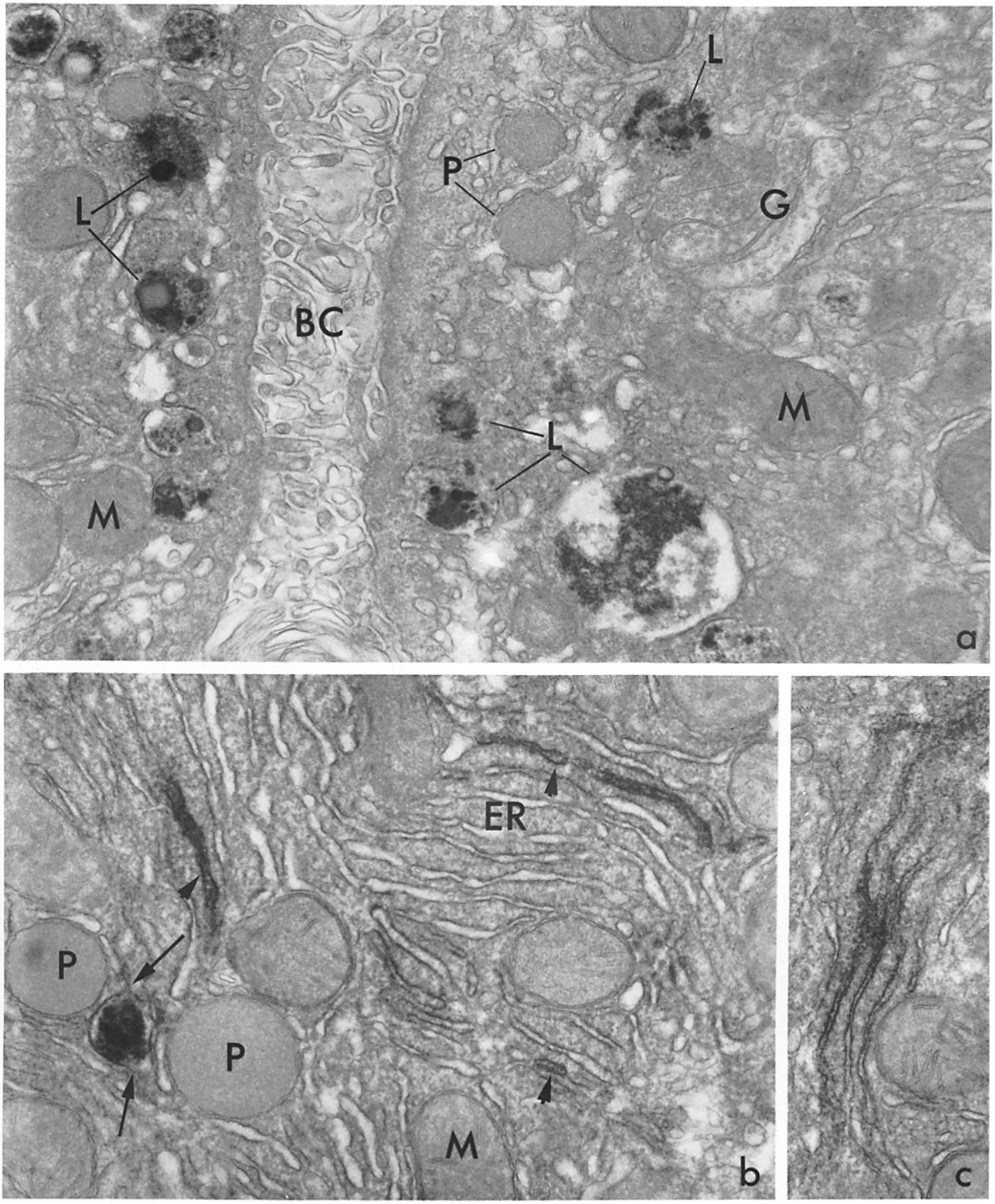

FIGURE 3 (*a*) The section passes along the length of a bile canaliculus (*BC*). Note the numerous lysosomes (*L*) with reaction product on both sides of the canaliculus. Golgi apparatus (G), mitochondria (*M*), and peroxisomes (*P*) are unreactive. Note the absence of reaction product in the ribosomes of this domain of rough endoplasmic reticulum. (*b*) The main interest of this section is an immunoreactive lysosome with proximity to smooth membrane, possibly endoplasmic reticulum (arrows), which is also reactive. Note the extensive endoplasmic reticulum with unreactive ribosomes in this domain (*ER*). In some domains the ribosomes are barely evident. Arrowheads are directed towards areas of endoplasmic reticulum membrane with reaction product. Reaction product is also present within the cisternae. (c) A region of a parallel array of endoplasmic reticulum is seen. Note the reaction product associated with the rough endoplasmic reticulum in this domain. Fig. 3, a–c has not been stained with lead; *a* is fixed in fixative a, and *b* and c in fixative b. × 28,000 (*a*); × 32,000 (*b*); × 34,000 (c).

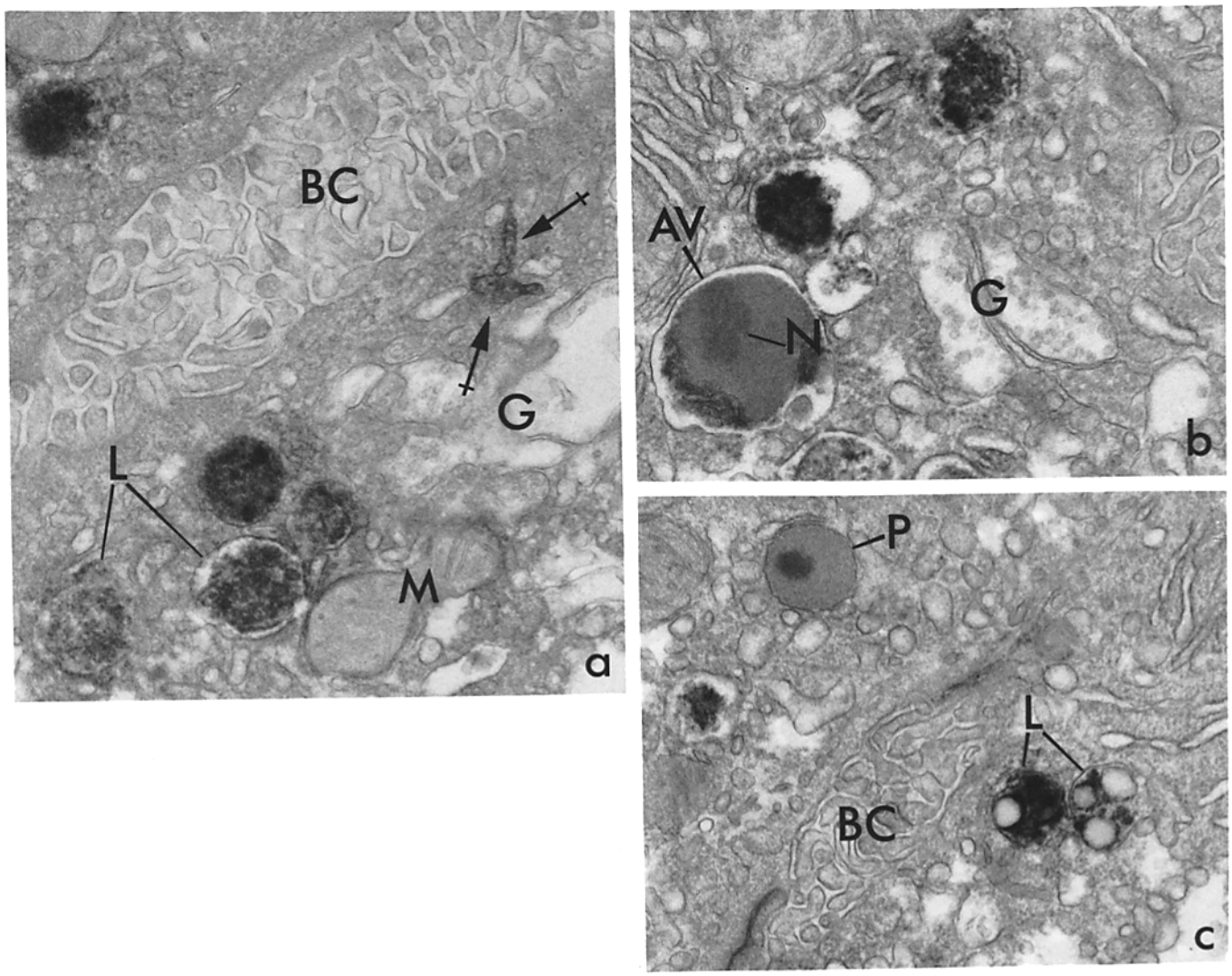

FIGURE 4 (*a*) The section passes along the length of a bile canaliculus (*BC*). Note, the cluster of positive lysosomes at the lower left, that the *lysosome* indicated by *L* shows reaction product in the membrane. Note reaction product in portions of endoplasmic reticulum referred to as GERL (crossed arrows). A portion of the Golgi apparatus is seen at *G*; it is dilated artifactually. Such dilatation may be taken as a severe test for immunoreagent penetration. *M*, mitochondria. (*b*) A region is seen where dilated cisternae of the Golgi apparatus (*G*) are seen. Also seen are very low density lipoprotein particles within the Golgi apparatus, bile canaliculus, and three immunoreactive lysosomes. Note the autophagic vacuole (*AV*) within which is a nucleoid-containing peroxisome (*N*) surrounded by reaction product. (c) A region is shown with a bile canaliculus (*BC*). A peroxisome (*P*) is sectioned through its nucleoid. Two residual bodies (*L*) show reaction product and contain lipid-like electron-lucent spheres. Fig. 4, *a–c* have not been lead stained. × 35,000 (*a*); × 34,000 (*b*); × 28,000 (*c*).

(*a*) in sections treated with Triton X-100 before exposure to immune antiserum; (*b*) in sections exposed to immune antiserum for 40 h; and (*c*) in disrupted or poorly preserved Golgi apparatus (Fig. 4*a*). Mitochondria, peroxisomes, plasma membrane and its specializations (microvilli at the bile canalicular and sinusoidal surfaces), and ribosomes are also devoid of reaction product.

Fig. 4*b* illustrates reaction product in an autophagic vacuole, which is presumably in an early stage of digestion because of the recognizable nucleoid-containing peroxisome. Another type of lysosome, a residual body with electron-lucent lipid spheres, is seen with reaction product in Fig. 4*c*. Thus far, coated vesicles (50–80 nm) in the Golgi-GERL region have not been observed with reaction product. However, the interpretation of this finding is unclear.

Fig. 2, *a* and *b* are control sections incubated in nonimmune antiserum. The lysosomes (residual bodies and autophagic vacuoles) and the endoplasmic reticulum are without reaction product. Other cell types that show β-gal immunoreactive lysosomes are endothelial cells and Kupffer cells (not illustrated); the lysosomes of these cell types were unreactive with nonimmune serum.

A preliminary experiment on the immunolocalization of another lysosomal enzyme, β-glucuronidase, revealed that this enzyme has the same intracellular distribution as β-gal. These observations will be reported in a separate manuscript now in preparation. Fig. 5 illustrates the localization of β-glucuronidase to lysosomal structures near the apical portion of the plasma membrane.

All three fixatives gave good but not excellent ultrastructural preservation. Each fixative showed localization of β-gal to the endoplasmic reticulum, lysosomes, and GERL. However, when glutaraldehyde was present in the fixative, variability in the distribution of reaction product was observed at

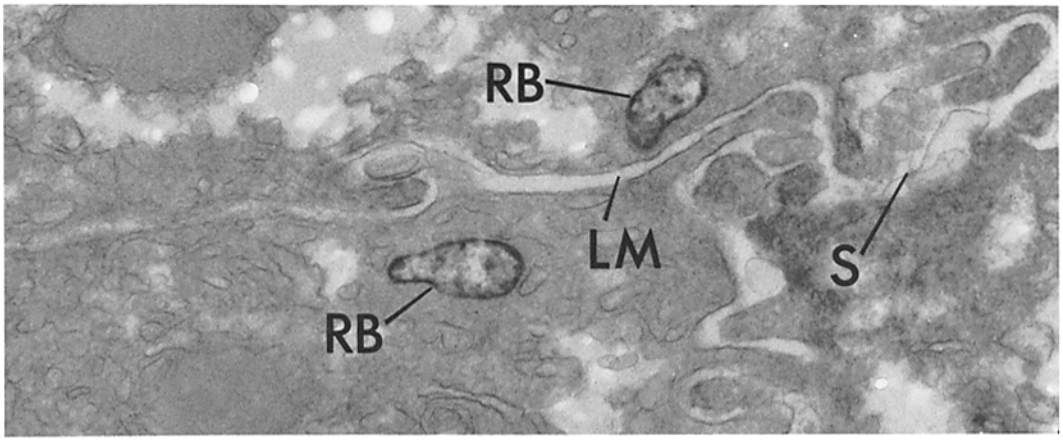

FIGURE 5 Portions of two hepatocytes, from tissue exposed to rabbit anti-β-glucuronidase antibody for 20 h. DAB reaction product is localized to only the residual bodies (*RB*) close to the apical portion of the lateral plasma membrane (*LM*). A portion of a sinusoid (*S*) is evident × 44,000.

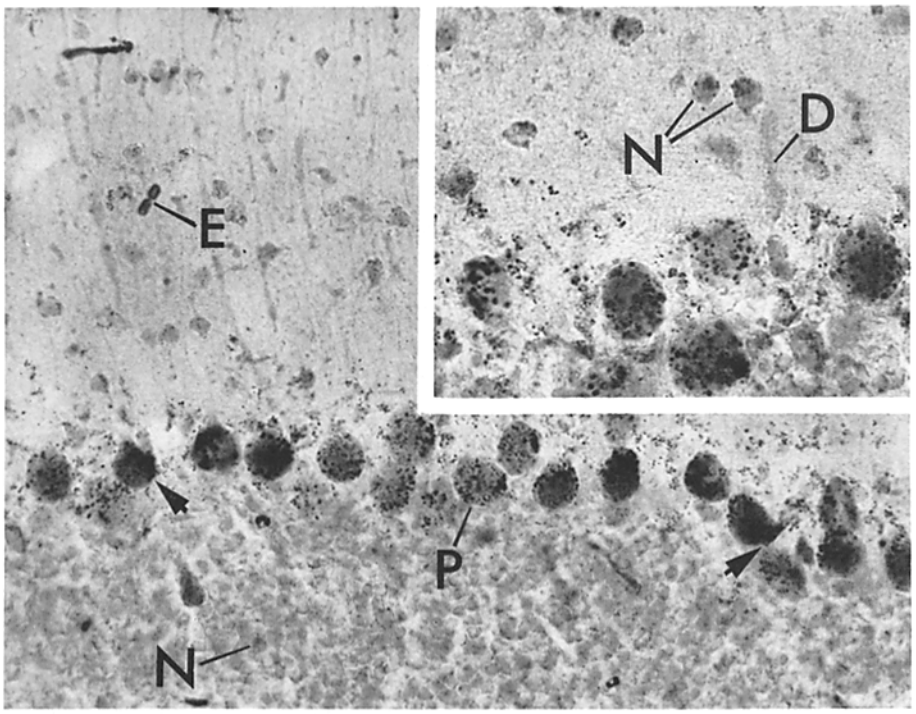

FIGURE 6 Section of cerebellum from tissue exposed to β-gal antibody. The major portion of the micrograph shows DAB reaction product in the lysosomes of Purkinje cells (*P*) with their dendrites oriented upwards (*D*). *E*, erythrocytes. The *inset* is an enlargement of an area of the lower power. Evident at low magnification, but more obvious at the high magnification, are the lysosomes of neurons of the molecular layer, the granular layer (*N*), and the Purkinje cells. Arrowheads indicate pericytes with intensely reactive lysosomes. × 250; *inset*, × 400.

both light and electron microscopic levels.

This indirect immunocytochemical method yielded identical localizations whether immune whole antiserum or purified immune anti-IgG was used. The advantage in using purified IgG is the complete absence of both background staining and surface adsorption to the sections. However, these can be reduced or eliminated by prior exposure of sections to nonimmune serum before exposure to immune antiserum, by adding nonimmune serum to the immune antiserum, or by using higher dilutions of the immune antiserum.

DISCUSSION

The indirect immunocytochemical method used in this study involved three major steps: exposure of aldehyde-fixed tissue to rabbit anti-rat β-gal antiserum or purified IgG, subsequent exposure to protein A linked to horseradish peroxidase, and incubation in diaminobenzidine to reveal sites of peroxidase activity. By the use of this method we have localized the antigenic sites of β-gal, independent of its enzymatic activity, to all types of lysosomes. Thus far, the only exception appears to be coated vesicles in the Golgi-GERL zone, which are considered to be primary lysosomes (25). Reaction product was also seen in the endoplasmic reticulum and in the specialized acid phosphatase-rich region of the smooth endoplasmic reticulum (GERL) of rat hepatocytes. In no other organelles was reaction product seen.

From our electron micrographs, it is impossible to differentiate whether the electron-dense reaction product seen in the rough endoplasmic reticulum resulted from the presence of β-gal associated with membrane, ribosomes, or lumina. Not all of the rough endoplasmic reticulum within any given hepatocyte was immunoreactive for β-gal, suggesting that this method may be capable of localizing regions of the rough endoplasmic reticulum actively synthesizing the enzyme (3). However, these observations must be interpreted with caution because of the possibility of diffusion and adsorption artifacts in DAB cytochemistry (26).

The presence of β-gal in GERL is consistent with its involvement in the biogenesis of lysosomes (11–13). Earlier studies, in which the endocytic pathways for galactose-germinated glycoproteins labeled with an electron-dense tracer

(horseradish peroxidase) were followed, suggested that GERL was part of an extensive lysosomal compartment (27–29). GERL may be involved in the final stage of lysosomal enzyme sorting, by recognition of its phosphomannosyl residues (5, 6) or by a noncarbohydrate directed pathway (30). However, this cannot be determined from a static study such as ours. The presence of β-gal in all kinds of lysosomes (25) indicates that there is no enzymatic heterogeneity, or selective sorting of lysosomal hydrolases during their compartmentalization.

Of particular interest is the absence of reaction product in the entire Golgi apparatus. Studies on the immunocytochemical localization of two other lysosomal hydrolases, "acid" α-D-mannosidase isolated from rat epididymis (31) and β-glucuronidase isolated from rat liver (21, 32), confirm this unexpected finding. This is true even when procedures to optimize access of antibody to antigenic sites (e.g., Triton X-100 treatment and prolonged exposure to immune antiserum) are employed. These observations suggest either that β-gal, a known glycoprotein (1) that presumably undergoes terminal sugar modification similar to other lysosomal glycosidases (33), may not pass through the Golgi apparatus on its way to lysosomes, or that β-gal is present in only sublimiting concentrations, indicating that the Golgi apparatus does not function as a condensing organelle. We have also visualized the lysosomes, but not the Golgi apparatus, by β-gal in Vibratome sections of Purkinje cells and other neurons of the cerebellum and in kidney and intestinal epithelia.

α-D-mannosidase II, isolated from rat liver Golgi membranes, was detected in the Golgi apparatus but not in GERL or lysosomes of rat hepatocytes (21), nephron, intestinal absorptive and crypt cells, and neurons in the cerebellum (preliminary studies). These observations validate the specificity of the reported observations.

In situ procedures, in contrast to methods employing isolation of fractions (34), are more likely to identify accurately those organelles that participate in the biogenesis of enzymes since cells are not disrupted and contamination of fractions is not a concern. Localization of other lysosomal enzymes (35) for which there are no enzyme cytochemical methods should be possible with immunocytochemical procedures, helping to identify the cell organelles involved in their intracellular traffic.

The significance of reaction product seen in lysosomal membranes and endoplasmic reticulum membranes and cisternae will require further study, as will the possible role of GERL in processing lysosomal enzymes in rat hepatocytes.

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