COMBINED CYTOCHEMICAL AND ELECTRON MICROSCOPIC DEMONSTRATION OF β-GLUCURONIDASE ACTIVITY IN RAT LIVER WITH THE USE OF A SIMULTANEOUS COUPLING AZO DYE TECHNIQUE

MASANDO HAYASHI, TSURANOBU SHIRAHAMA, and ALAN S. COHEN

From the Departments of Pathology, Beth Israel Hospital and Harvard Medical School and The Evans Memorial Department of Clinical Research, University Hospital and the Department of Medicine, Boston University School of Medicine, Boston University Medical Center, Boston, Massachusetts 02215

ABSTRACT

Rat liver was fixed in formal-cacodylate-sucrose and frozen sections were incubated in a simultaneous-coupling medium containing naphthol AS-BI glucuronide as substrate and hexazonium pararosanilin as the diazo reagent. By light microscopy, the sections demonstrated β-glucuronidase activity as red discrete granules in the pericanalicular cytoplasm and as a generalized cytoplasmic stain in the parenchymal cells. Brief treatment of sections in cold ethanol prior to incubation markedly enhanced the staining for the enzyme and made it possible to demonstrate sufficient amounts of the reaction product in sections embedded in epoxy resin following dehydration and propylene oxide treatment. Electron microscopy revealed that the reaction product was moderately electron opaque and deposited in greater amounts in the vacuolated dense bodies and occasionally in the dense bodies which did not show obvious vacuoles. In each dense body, the deposits occurred preferentially at the edge as well as in the area surrounding the vacuoles in the matrix. Control sections incubated in the presence of glucosaccharo-1:4-lactone were devoid of the reaction product. No deposits of the reaction product were found in the nucleus, mitochondria, or microbodies. The limitations of the present cytochemical technique for use in electron microscopy are briefly discussed.

INTRODUCTION

The application of dye methods for fine structural localization of enzymes by electron microscopy has been explored of late in several laboratories as an alternative to metal salt precipitation techniques. Efforts have been concentrated on increasing the electron opacity as well as reducing the solubility of the final reaction product, primarily by incorporating the metal in the product. Hanker et

al. (7) introduced certain sulfur-containing dyes which are able to react with osmium tetroxide to form an electron-opaque osmium black pigment, and they applied the principle to several cytochemical methods. Tice and Barrnett (17) developed the technique of using metal-chelated diazophthalocyanins as capture reagents for the demonstration of acid phosphatase activity. Holt and Hicks (11) utilized another principle based on the formation of an insoluble osmium-containing complex by certain azoindoxyls for the demonstration of esterase activity. On the other hand, several nonmetal-containing dyes have been found to possess sufficient electron opacity. Barrnett (2) tested a number of complex dye intermediates as diazonium salts in an effort to demonstrate alkaline phosphatase activity. Davis, Lehrer, and Ornstein (4, 12) demonstrated esterase activity with an unchelated azo dye formed in a medium containing α-naphthyl acetate as substrate and hexazonium pararosanilin as the coupler.

We have been trying to demonstrate β-glucuronidase activity by electron microscopy with a simultaneous-coupling azo dye technique employing naphthol AS-BI glucuronide as the substrate and hexazonium pararosanilin as the diazo reagent (8). The present report concerns (*a*) the effect of treatment of sections in cold ethanol, prior to incubation for the enzyme activity, in accelerating the staining reaction, (*b*) our preliminary observation of the enzyme localization in liver cells, and (*c*) evaluation of the present staining technique for use in electron microscopy.

MATERIALS AND METHODS

Adult male Sprague-Dawley rats were used. Small blocks (3 × 3 × 3 mm) of the liver tissues were fixed for 20 hr at 4°C in a methanol-free formaldehyde fixative (16) containing 4% formaldehyde, 7.5% sucrose, and 0.1 M cacodylate buffer pH 7.4. After fixation, the tissues were washed with three changes of 0.1 M cacodylate buffer pH 7.4 containing 7.5% sucrose for 2 hr at 4°C. Frozen sections were cut at 8 and at 40 μ on a Leitz freezing microtome and kept in cold 7.5% sucrose. Sections were treated in 70, 100, 70% ethanols each for 1 min at −15°C and rinsed in two changes of cold 7.5% sucrose.

β-Glucuronidase activity was demonstrated with a simultaneous-coupling azo dye technique as described previously (8). In short, the sections were incubated in a medium containing 0.25 mM naphthol AS-BI β-glucuronide,[1] 0.1 M acetate buffer, with or without 7.5% sucrose, and 1.8 mM hexazonium pararosanilin at pH 5.2 for 20 min at 37°C in water. Following incubation, 8-μ sections were rinsed in water, mounted on slides, and examined under a light microscope. The 40-μ sections were rinsed in three changes of 7.5% sucrose and fixed in 1% osmium tetroxide buffered at pH 7.2 for 1 hr (14). They were washed, dehydrated through graded alcohols, treated with propylene oxide (10 min in absolute ethanol-propylene oxide mixture 1:1, two changes of propylene oxide each for 10–15 min, and 2 hr to overnight in propylene oxide-epoxy resin mixture 1:1), and embedded in Epon (13). After polymerization at 45°C, thick sections of each specimens of 1–2 μ were studied under a light microscope, and desired areas were trimmed for electron microscopic observation. Thin sections were then cut with a glass knife and

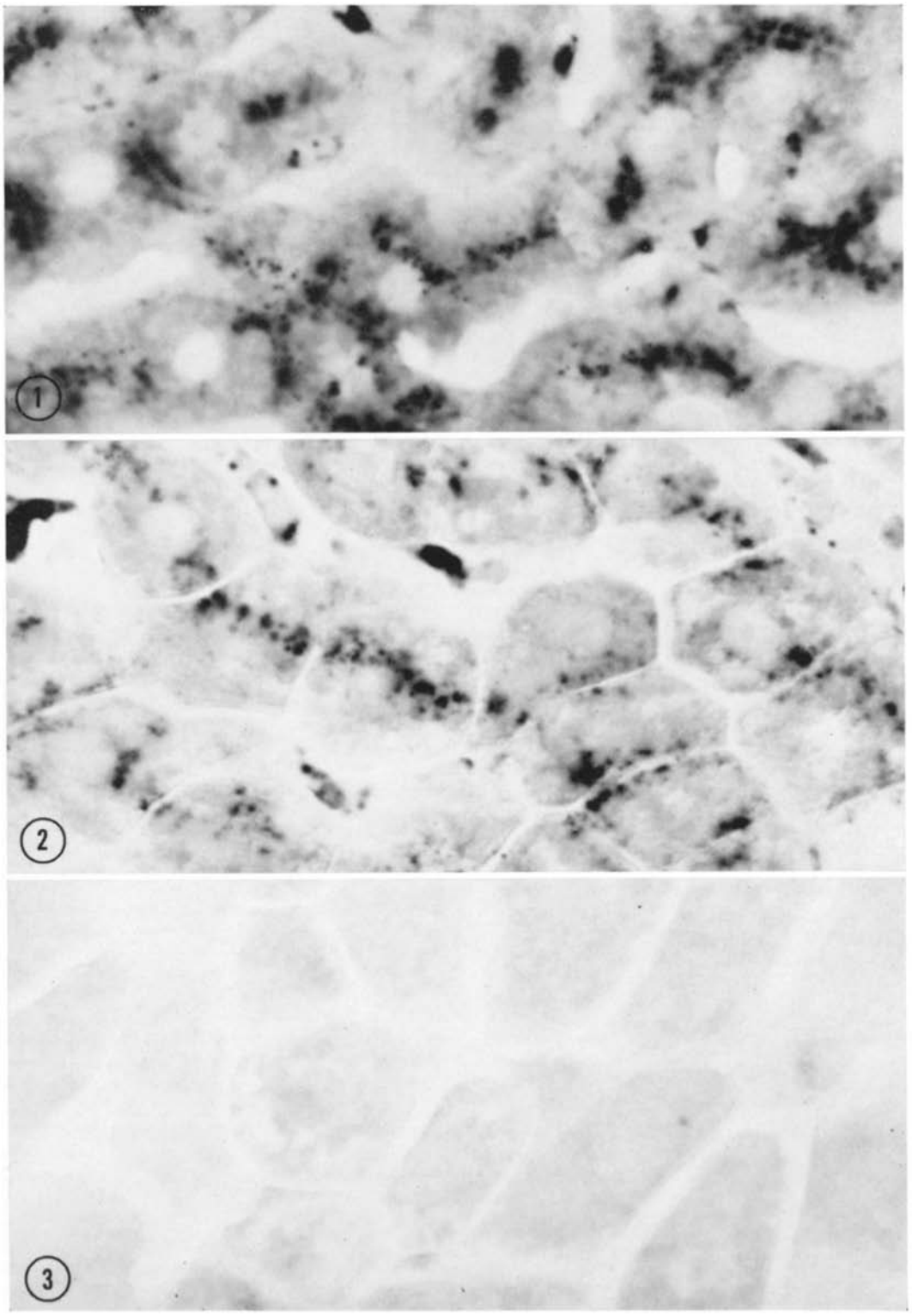
[1] Available from Sigma Chemical Co., St. Louis, Mo

FIGURES 1–3 are light micrographs of sections of rat liver stained for β-glucuronidase activity in a simultaneous-coupling azo dye medium (8) for 20 min at 37°C after formal-cacodylate-sucrose fixation.

FIGURE 1 An 8-μ frozen section incubated for β-glucuronidase activity following treatment in ethanol for 3 min at −15°C (see text). The reaction product is noted as a number of discrete granules in the pericanalicular cytoplasm as well as a faint generalized cytoplasmic stain in the parenchymal cells. Also noted are stained granules in the Kupffer cells. (Usually the staining is more intense in ethanol-treated sections than in untreated sections.) × 1,200.

FIGURE 2 A 2-μ section cut from an Epon-embedded 40-μ section which had been treated in cold ethanol and then stained for β-glucuronidase activity. The pericanalicular localization of stain is preserved following the embedding procedures. × 1,200.

FIGURE 3 A 2-μ section cut from an Epon-embedded 40-μ section which had been stained for β-glucuronidase activity without pretreatment in ethanol. No appreciable stain is visible. × 1,200.



mounted on copper grids without coating. No lead or uranium staining was done on any of the specimens. Grids were examined with a Siemens Elmiskop 1 electron microscope and photographed at magnifications of 4,000–20,000.

CONTROLS: Various controls included incubation (*1*) in the presence of a specific inhibitor, 0.25 mM of glucosaccharo-1:4-lactone; (*2*) in the absence of substrate; (*3*) in buffer alone, following ethanol pretreatment; and (*4*) in the full staining medium without pretreatment in ethanol. Unstained control tissues were fixed in the same formaldehyde fixative and then postfixed in osmium tetroxide. In addition, some blocks were fixed directly in osmium tetroxide for comparison.

RESULTS

Preliminary Studies Related to the Method

The staining reaction for β-glucuronidase was carried out at variable pH values ranging from 4.5–5.5 and during variable periods of incubation from 20–60 min at 37°C with the use of the present simultaneous-coupling technique. Rat liver tissues appeared to show the best localization of the reaction product with respect to the intensity and discreteness following 20-min incubation at pH 5.2 (8, 9).

It was noted that the staining reaction was markedly accelerated and that the localization of the reaction product appeared to be more discrete in sections treated for a few minutes with cold ethanol prior to incubation than in sections not treated in ethanol. The temperature rather than period of treatment seemed to have a more critical effect on the enzyme activity. Treatment of sections in ethanol at room temperature (25°C) for 3 min markedly reduced the enzyme activity. However, when the temperature of ethanol was maintained at around −15°C, marked enhancement in staining occurred following treatment for as long as 60 min. With ethanol pretreatment, an intense reaction was obtained following relatively short incubation such as 20 min at 37°C. Although prolonged incubation increased the staining in sections without ethanol treatment, it tended to cause apparent diffusion of the reaction product. Sections embedded in epoxy resin following staining for the enzyme, and dehydration demonstrated a sufficient amount of the reaction product when they had been treated in ethanol prior to incubation, but demonstrated faint or no staining without ethanol treatment.

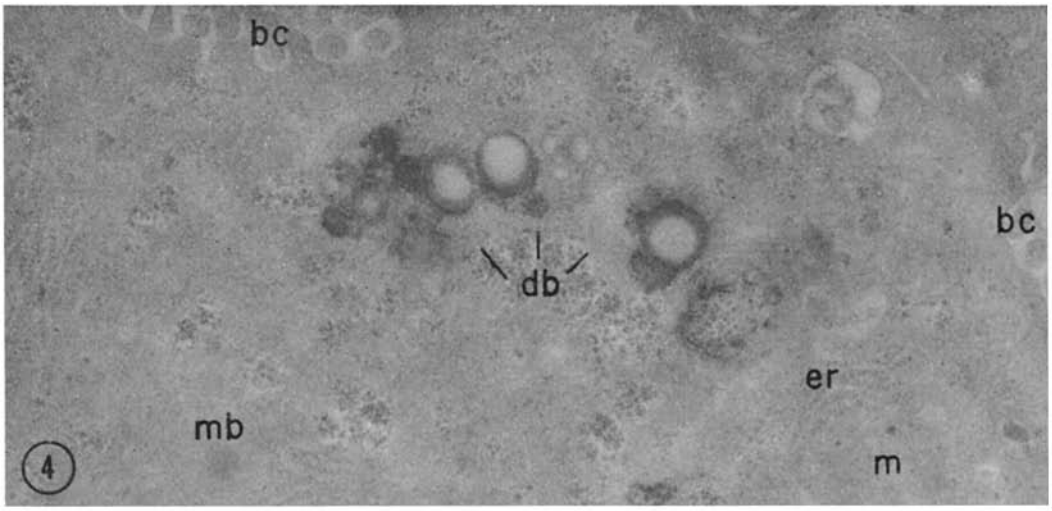
In frozen sections stained for the enzyme for 20 min at pH 5.2, the solubility of the reaction product was tested in reagents used in preparing tissues for electron microscopy. Intensity and discreteness of the staining in sections as well as extraction of the dye in the reagents were examined. The dye was almost insoluble in ethanol, slightly soluble in propylene oxide in the first immersion, but much less soluble in the second immersion, and sparingly soluble in epoxy resin. However, embedding of stained sections in epoxy resin without propylene oxide treatment did not appreciably prevent the loss of dye in final sections. The reason may be that more dye was extracted from such sections by epoxy resin than from those sections previously treated in propylene oxide.

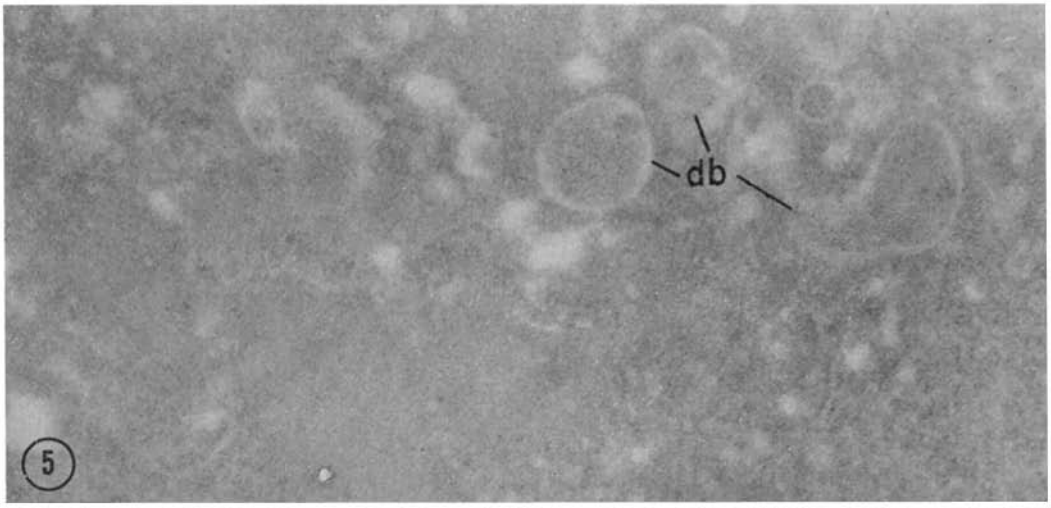
FIGURES 4–9 are electron micrographs of rat liver parenchymal cells from tissue fixed in formal-cacodylate-sucrose.

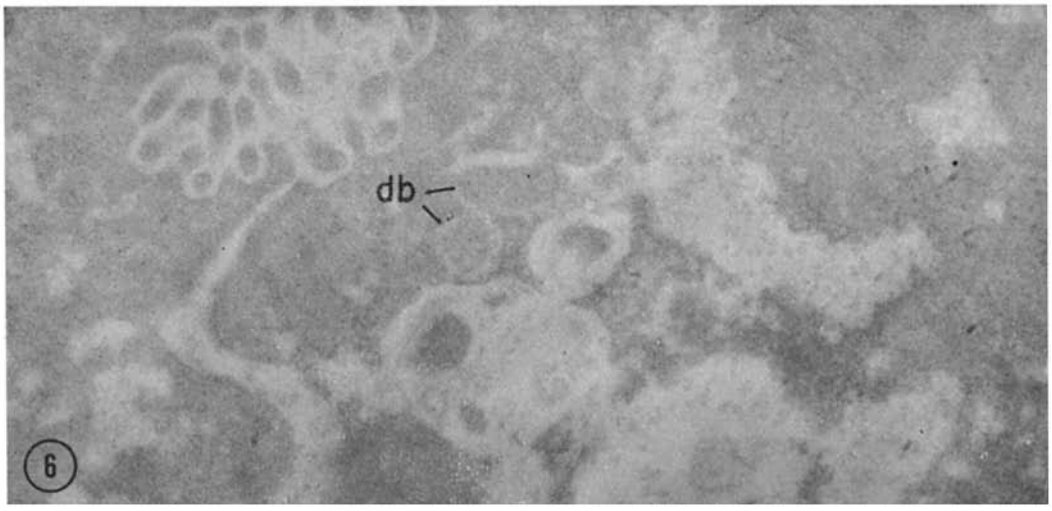
FIGURE 4 Unstained control. A portion of the liver cell which includes several dense bodies (*db*) as well as mitochondria (*m*), microbody (*mb*), and endoplasmic reticulum (*er*). Portions of two bile canaliculi (*bc*) are shown. × 30,000.

FIGURE 5 A control for stained preparations which has been incubated in the staining medium for β-glucuronidase activity in the presence of 0.25 mM glucosaccharo-1:4-lactone. Features like those in unstained control sections are noted, except that most of the endogenous dense material in the dense bodies (*db*) is lost during the preparation. Dense bodies contain ferritin-like materials in the matrix, but include no appreciable reaction product. × 30,000.

FIGURE 6 Preparation incubated in the full staining medium without pretreatment in ethanol. No reaction product is visible in the dense bodies (*db*). × 30,000.







Light Microscopic Localization of β-Glucuronidase Activity in Rat Liver

Frozen sections cut at 8 μ from rat liver fixed in formal-cacodylate-sucrose and incubated in the present simultaneous-coupling medium for β-glucuronidase activity have demonstrated the final reaction product as a number of red discrete granules in the pericanalicular cytoplasm of the parenchymal cells under the light microscope (Fig. 1). The stained granules were greater in number in the parenchymal cells of the periportal zone than in those of the central area, where a generalized cytoplasmic staining was obvious. In the Kuffper cells, granules of varying sizes were noted; some were larger than those found in the parenchymal cells. Usually, the staining was more intense in ethanol-treated sections than in untreated sections. No staining was noted in control sections incubated either in the presence of glucosaccharo-1:4-lactone or in the absence of substrate.

The same enzyme localization was noted in Epon sections cut at 1–2 μ from 40-μ sections embedded in the resin following incubation for β-glucuronidase activity. The azo dye was localized in discrete granules of the parenchymal cells in sections which had been treated in cold ethanol prior to incubation (Fig. 2). Sections not treated with ethanol showed faint or no staining following the process of Epon embedding (Fig. 3).

Electron Microscopy

MORPHOLOGY OF UNSTAINED CONTROL LIVER (FIG. 4): Most of the structures normally seen in rat liver parenchymal cells in osmium tetroxide–fixed material (3) were well preserved in tissues fixed in formal-cacodylate-sucrose followed by postfixation in osmium tetroxide, except that in the formaldehyde-fixed tissues the membrane structures were less obvious. In the cytoplasm close to the bile canaliculi, several dense bodies were noted, some of which contained dense materials in both matrix and vacuoles. In the matrix, ferritin-like particles were noted. In contrast to the moderately dense limiting membranes of the mitochondria and microbodies, the limiting membranes of the dense bodies were usually not obvious in either unstained or stained preparations. Instead, the dense bodies were surrounded by a clear, narrow area, probably at or in the neighborhood of the membrane structures.

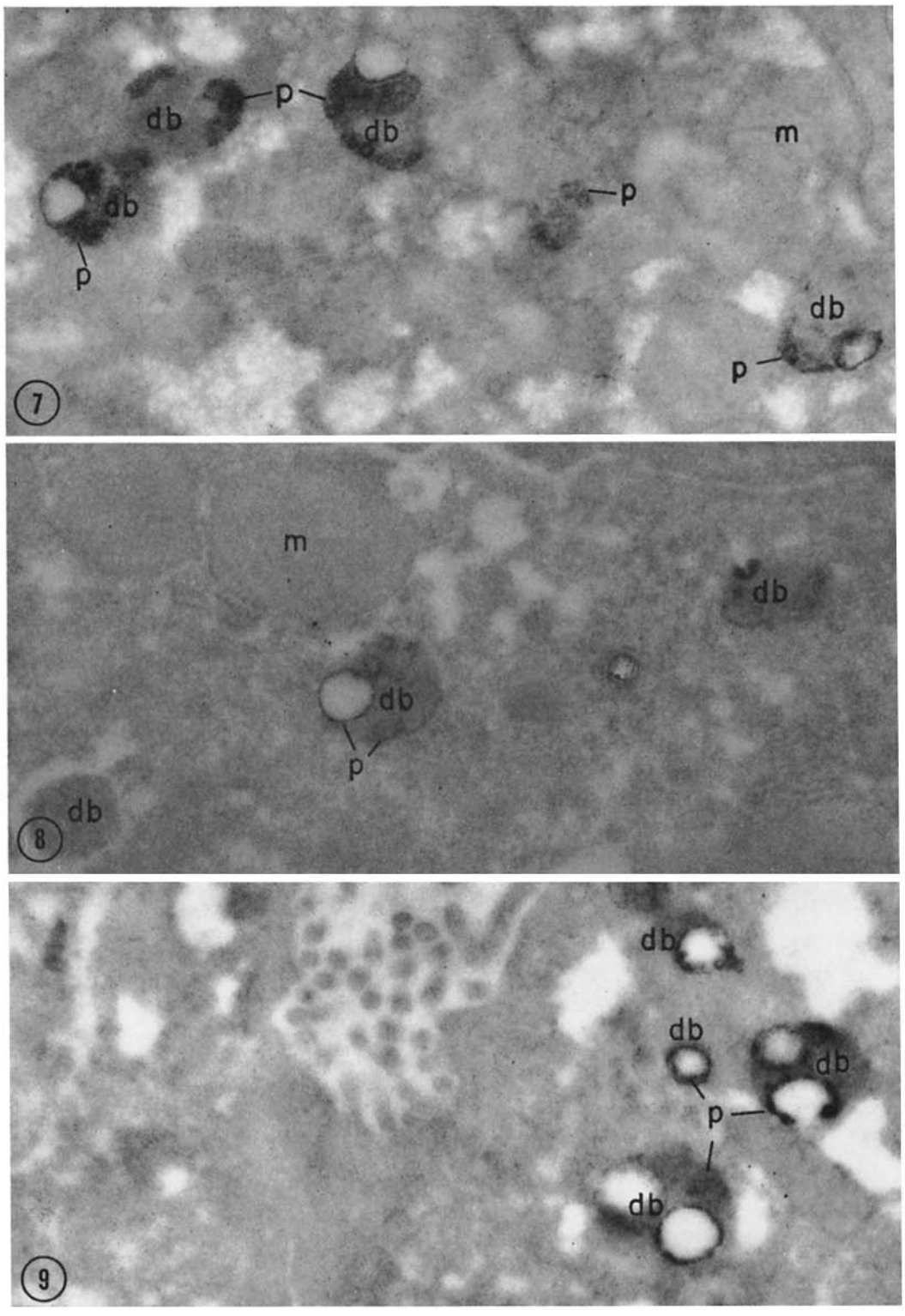
CONTROLS FOR STAINED PREPARATIONS: Sections incubated in the staining medium for β-glucuronidase activity in the presence of 0.25 mM glucosaccharo 1:4 lactone (Fig. 5), in the absence of substrate, or in buffer alone showed features comparable to those of unstained control liver sections, except that most of the endogenous dense materials in the dense bodies was lost during the preparation. Similarly, preparations incubated in the full staining medium without treatment in ethanol prior to incubation (Fig. 6) demonstrated features almost identical to those obtained in the above control specimens, and no appreciable opaque deposits were noted in the dense bodies.

LOCALIZATION OF β-GLUCURONIDASE ACTIVITY BY ELECTRON MICROSCOPY (FIGS. 7–9): Sections stained for β-glucuronidase activity in the present staining medium following ethanol pretreatment showed the reaction product as amorphous deposits of moderate electron opacity in the dense bodies in the pericanalicular cytoplasm of the parenchymal liver cells. The reaction product was noted in variable amounts and was present in greater amounts in the vacuolated dense bodies. Occasionally, the dense bodies which did not show obvious vacuoles also contained the reaction product. In each dense body, the reaction product was localized preferentially at the edge as well as in the area close to vacuoles in the matrix. The nucleus, mitochondria, and microbodies were usually free of the reaction product.

DISCUSSION

It has generally been stated that in order for an enzyme histochemical staining method to be applicable to electron microscopy, the method should provide critically precise localization of the reaction product. The final product must be electron opaque and insoluble in the reagents used in the preparation of tissues for electron microscopy (1). In practice, therefore, each specific application of staining methods requires individual consideration.

The present study has shown that a brief treatment of sections in cold ethanol prior to incubation markedly enhanced the staining for β-glucuronidase activity and made it possible to demonstrate the reaction product by electron microscopy. The enhanced staining in ethanol-treated sections may be due to the fact that ethanol removed lipid, at least partially, from cellular membranes and that penetration of the ingredients of incubation



FIGURES 7–9 refer to preparations stained for β-glucuronidase activity following treatment in cold ethanol. Moderately opaque reaction product (*p*) is noted in variable amounts in the dense bodies (*db*). It is present in greater amounts in vacuolated dense bodies and occasionally in those dense bodies which do not show obvious vacuoles, but many of the latter bodies do not demonstrate reaction product. The product in the dense bodies is localized preferentially at the edge as well as in the area close to vacuoles in the matrix. Mitochondria (*m*) and microbodies are free of the reaction product. × 30,000.

medium was considerably facilitated, especially in the initial stage of reaction. Oil red 0 staining demonstrated that the ethanol treatment used in the present study, that is, 70, 100, 70% ethanols each for 1 min at −15°C, removed appreciable amounts of lipid from the tissue sections. With this pretreatment, the incubation period could be shortened and diffusion of the reaction product was considerably reduced. The greater amount of the reaction product in ethanol-treated sections than in untreated sections may be the major cause of positive staining in Epon sections of the former tissues. However, differences in the intensity of staining between ethanol-treated and untreated sections appeared to be greater in sections which had been postfixed in osmium tetroxide, dehydrated, and embedded in epoxy resin than in original stained sections not processed for dehydration and embedding. Although no quantitative evidence is available, it was our impression that the azo dye deposited in ethanol-treated sections contained more such dye component which is less soluble in propylene oxide and epoxy resin than did the azo dye deposited in untreated sections.

Identity of the red stain in pericanalicular granules demonstrated by light microscopy and the opaque deposits in the dense bodies demonstrated by electron microscopy may be justified not only by their identical localization in the cell in positively stained preparations, but also by the absence of electron-opaque deposits in preparations incubated in the staining medium in the presence of glucosaccharo-1:4-lactone, in the absence of substrate, and in buffer alone. Much of the endogenous opaque materials in the dense bodies normally seen in unstained (unincubated) controls appeared to be extracted during the preparation. Thus, opaque deposits noted in stained preparations are considered to be the reaction product. The product was deposited in variable amounts among the dense bodies; it was present in greater amounts in the vacuolated bodies, but was absent from some bodies. The general distribution is similar to that reported for acid phosphatase in liver cells (6, 10, 15).

According to de Duve and associates (5), the major part of rat liver β-glucuronidase is confined within the lysosomes, but part of the enzyme is obviously nonlysosomal and associated with microsomes. A generalized cytoplasmic staining in the parenchymal cells noted by light microscopy in β-glucuronidase preparations appeared to indicate the presence of nonlysosomal enzyme. Present results with electron microscopy did not demonstrate convincingly the reaction product in the endoplasmic reticulum, possibly because the reaction product was not sufficiently electron opaque and because it was partially lost during the process of embedding. The present observations were limited, therefore, to the major sites of enzyme activity in the cell. The results, however, may provide useful evidence for the interpretation of the light microscopic localization of β-glucuronidase activity in tissues (9) as demonstrated by the present simultaneous-coupling azo dye technique.

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