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TECHNICAL MANUSCRIPT 526

THE ULTRASTRUCTURAL DEMONSTRATION OF β-GALACTOSIDASE USING THE INDIGOGENIC TECHNIQUE

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In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences-National Research Council.

ABSTRACT

The indigogenic technique is useful for histochemical demonstration of the activity of certain enzymes, including glycosidases, β-glucuronidase, and leucine aminopeptidase. To extend the application of this technique to the level of the electron microscope, we have studied β-galactosidase in alveolar macrophages washed from rabbit lungs. This enzyme has not previously been demonstrated at the ultrastructural level. Preliminary studies involved the relevant properties of the indigo reaction product. Subsequently we have attempted to establish optimal conditions of fixation, incubation, embedding, and staining for demonstration of the reaction product in the electron microscope. In a comparison of fixatives, glutaraldehyde provided the best combination of preservation of enzyme activity and cell detail. Incubation times of 1 to 1½ hours provide small but relatively easily identified crystals of reaction product. A very brief exposure of the thin section to conventional heavy-metal stain brings out cell detail without losing too much contrast of reaction product.

Proof of the reliability of the technique was provided by two means: (i) the reaction product was never found over nuclei, and only rarely found in extracellular areas, and (ii) competitive inhibition of β-galactosidase by galactonolactone and nonspecific inhibition of the enzyme by parachloromercuribenzoate have established the specificity of this particular substrate for this enzyme.

The reaction product is not found exclusively in lysosomes, and in fact is not primarily associated with any one group of cytoplasmic organelles. A striking feature of its distribution is a relatively constant localization within the nuclear membrane.

THE ULTRASTRUCTURAL DEMONSTRATION OF β-GALACTOSIDASE USING THE INDIGOGENIC TECHNIQUE*

Following the work of Holt et al.[1] on nonspecific esterase, Pearson and his co-workers[2] have demonstrated the usefulness of the indigogenic technique for histologic demonstration of hydrolytic enzymes. By attaching a specific sugar moiety to the indoxyl group via a glycosidic linkage, one obtains a substrate that is hydrolysed only by the enzyme specific for that particular glycoside. The reaction occurs in two steps. Hydrolysis of the indoxyl glycoside yields an intermediate, probably a radical, of which little is known. In the presence of an oxidizing agent, two of the indoxyl radicals combine rapidly to form indigo. The unsubstituted indigo is blue. Substitution of halogens in various positions may alter this color. More importantly, such substitution can alter the solubility of the indolyl glycoside and its rate of reaction to form indigo. Previous work by Holt[3] and by Pearson and his co-workers[2] has established that the 5-bromo-4-chloro substitution has the most desirable properties for histochemistry. In this fashion β-glucuronidase, β-galactosidase, β-xylosidase, β-fucosidase, and N-acetyl glucosaminidase have been demonstrated in fixed frozen sections of mammalian tissues. The specificity of the technique for the enzyme in question has been demonstrated by the use of both nonspecific enzyme inhibitors and analogous competitive inhibitors.

Previously, the indigogenic technique for demonstrating glycosidases had been confined to the level of light microscopy. Tsou et al.[4] have used the indigogenic principle to demonstrate phosphatase activity in the electron microscope. The present work, however, is the first demonstration of glycosidase activity in the electron microscope by the indigogenic technique. We elected to study β-galactosidase in suspensions of alveolar macrophages washed from rabbit lungs. This preparation was chosen to minimize problems associated with uneven fixation of tissue blocks or uneven penetration of substrate into tissue blocks.

The animals used were adult New Zealand rabbits. In order to obtain a large number of alveolar macrophages for study, the rabbits received 2.5 or 5 mg of killed vaccine (BCG) via the intravenous route 3 to 7 weeks before the cells were harvested. At the time of the harvest, the animals received an air embolus. After removal of the lungs from the chest cavity, the cells were harvested by a modification of the technique described by Myrvick[5] and processed by standard techniques. The cells were fixed for various intervals in the cold. The cells were washed with phosphate-buffered sucrose and suspended at 37 C in indoxyl galactoside substrate solution made according to methods developed in this laboratory. Following centrifugation, those samples to be studied in the electron microscope were suspended in a small volume of 2% agar. Small blocks were then osmicated for 1 hour. They were dehydrated by graded alcohols for a total time of 40 min. The propylene oxide steps of conventional embedding were omitted; the blocks passed from alcohol to Epon via a half-and-half mixture.

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In order to ascertain the electron density of 5,5-dibromo-4,4-dichloroindigo itself, we prepared a suspension of this material by alkaline hydrolysis of 5-bromo-4-chloroindoxyl acetate. A portion of this suspension was placed directly on a carbon-coated grid and allowed to dry. Another portion was mixed in 2% agar and embedded in Epon as outlined above. With no further staining procedure, we were able to phot graph both of these preparations in the electron microscope. The resulting pictures show a rather irregularly granular material with no particular features (Fig. 1).

Having thus established the electron density of indigo, we investigated the optimal conditions of fixation. Janigan's[5] work suggested that paraformaldehyde might offer less destruction of enzyme activity than other fixatives for the electron microscope. In our hands, however, cells fixed in 4% phosphate-buffered paraformaldehyde underwent significant morphologic changes during incubation in substrate. Glutaraldehyde fixation protected the cells against these changes, and thus provided the best overall compromise between loss of enzyme activity and preservation of cell detail.

Fixation for different times revealed no clearly defined difference in amount of reaction product at different intervals up to 60 min. This is consistent with correlated biochemical data, which show an early small drop in enzyme activity followed by a levelling off at about 70 to 80% of the total activity.

Indigo is known to be slightly soluble in certain organic solvents. Therefore, it seemed appropriate to examine the dehydration and embedding procedures. Although morphologic detail was not altered by remaining overnight in 100% ethanol prior to embedding, virtually all visible reaction product disappeared during such treatment. In fact, the less the cells are exposed to alcohol the better. The situation with propylene oxide is not so clear. After incubation the cells are obviously blue, and the agar blocks correspondingly appear blue, even after osmication. Immersion in propylene oxide quickly removes the blue color from these blocks, and indeed removes the blue color from blocks of pure indigo suspended in agar. Surprisingly, however, the overall appearance of the indigo in the electron microscope remains unchanged. In spite of this, we have decided to omit the propylene oxide step of the embedding, if only to facilitate photography of thick Epon sections in the light microscope.

Another potential pitfall in using the indigogenic technique for the electron microscope lies in handling the thin sections, particularly in the method of staining. We found that no reaction product was visible in thin sections stained by conventional techniques with uranyl acetate and lead citrate. Indigo was clearly visible in the light microscope in adjacent thick sections. Further investigation revealed two reasons for this. The staining techniques themselves enhanced the electron density of the cell components enough to render the indigo invisible. In addition, exposure of the thin section to the staining solution sometimes resulted in removal of some of the reaction product. Often, deposition of the reaction product was indicated by holes in the section (Fig. 2). Here again a compromise is necessary; we have arrived at a 2-minute exposure to saturated uranyl aqueous acetate as the best compromise between enhancement of cell detail and effacement of reaction product.

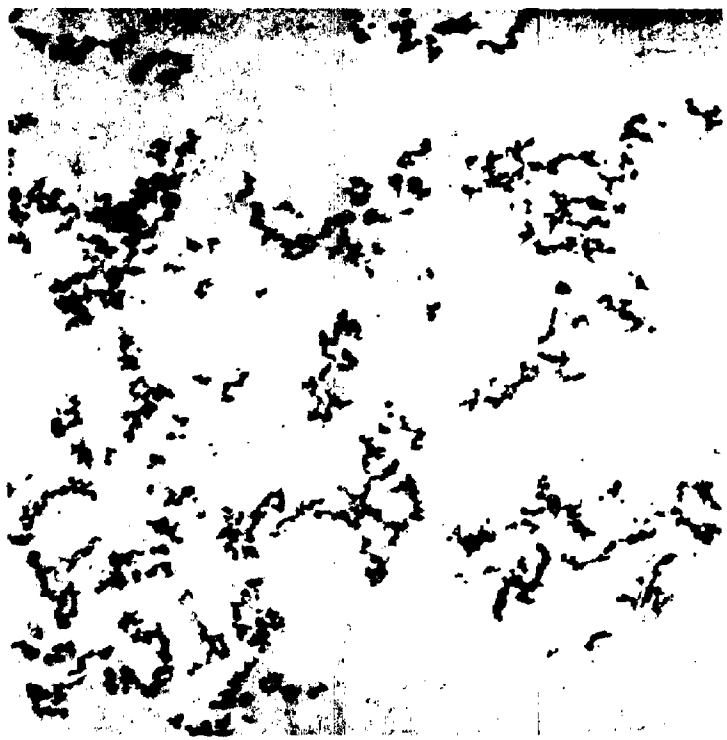

FIGURE 1. 5,5'-Dibromo-4,4'-Dichloroindigo Prepared by Alkaline Hydrolysis of 5-Bromo-4-Chloroindoxyl Acetate. Suspended in agar, embedded in Epon by the techniques described. 23,500X

FIGURE 2. Alveolar Macrophage Section Stained with Uranyl Acetate to Demonstrate Effect of Staining Technique in Removing Indigo Reaction Product from Section. 8,400X

We also have studied incubation time with substrate. These have shown the expected increase in reaction product with increasing time of incubation in substrate. For the purposes of the electron microscope the best incubation times lie between 1 and 3 hours. Before this, the amount of reaction product formed is rather small; after this, the crystals become larger than is desirable for localization.

With the above techniques we have been able to demonstrate galactosidase activity satisfactorily not only in rabbit alveolar cells, but also in rabbit peritoneal cells, including neutrophils, macrophages, lymphocytes, and eosinophils (Fig. 3 through 7). These photographs demonstrate the sites where we have found the reaction product. They also provide some of the proof of the specificity of the reaction. Of fundamental importance is the fact that no reaction product is present over nuclei or outside of cells. This provides evidence that diffusion and nonspecific hydrolysis of substrate are not significant problems in our system. To further prove the specificity of this reaction for this particular enzyme, we have done a series of inhibition experiments. Nonspecific inhibition of enzyme activity with heat or with parachloromercuribenzoate provides complete inhibition of hydrolysis of substrate. Moving to specific inhibitors, galactonolactone competes with galactoside for the active site on the enzyme galactosidase. The presence of galactonolactone (1%) in the substrate brings about almost complete inhibition of the hydrolysis of the substrate. Glucuronolactone, on the other hand, does not inhibit the reaction at all, thus proving that the inhibition by galactonolactone is based on the similarity of structure with the substrate.

Having thus proven the specificity of the reaction, we can then comment on the localization of the reaction product. In our hands the localization has been quite constant in the different inflammatory cell types mentioned previously. Much of the reaction product is present in the cytoplasm without association with specific organelles. Occasionally the reaction product appears in dilated sacs of endoplasmic reticulum, and occasionally it appears in lysosomes. Most of the lysosomes seen do not contain reaction product. A very constant feature of localization in our material is the appearance of reaction product between the layers of the nuclear membrane. At present we are performing cellular fractionation studies in an effort to further define the localization of the enzyme.

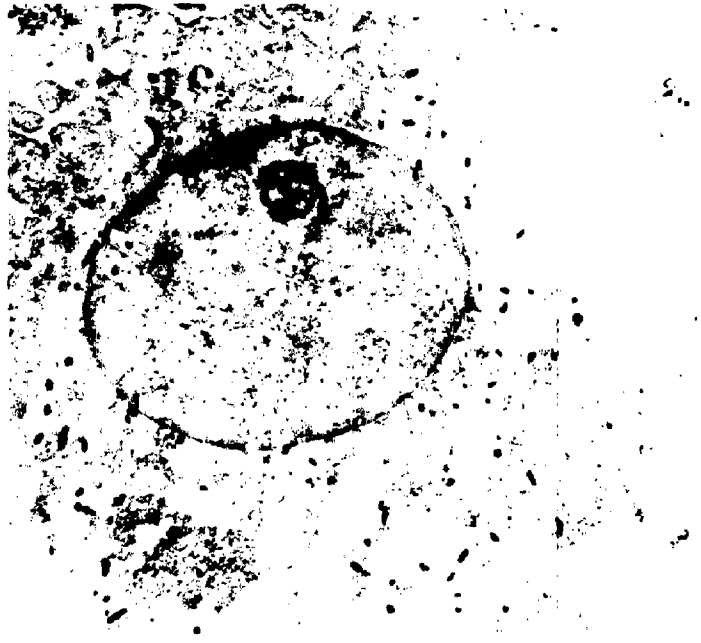

FIGURE 3. Alveolar Macrophage Section Unstained to Enhance Visibility of Indigo Reaction Product. Note perinuclear localization. 8,400X

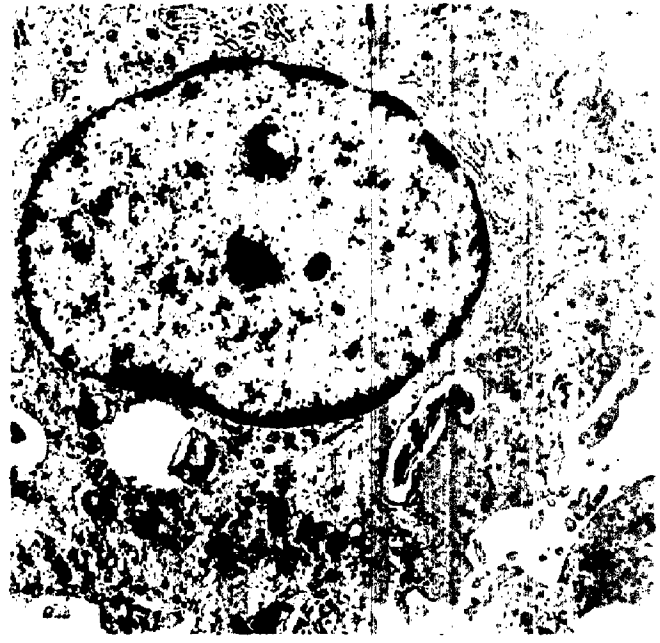

FIGURE 4. Alveolar Macrophage Section Exposed to Uranyl Acetate for 2 Minutes. Reaction product is found in the rough endoplasmic reticulum in addition to the nuclear envelope. 8,400X

FIGURE 5. Alveolar Lymphocyte Section Stained for 20 Minutes with Lead Citrate. Reaction product is clearly seen within the layers of the nuclear membrane and within the endoplasmic reticulum. 23,500X

FIGURE 6. Alveolar Neutrophil Section Stained for 2 Minutes with Uranyl Acetate. Note lack of lysosomal localization of reaction product. 17,000X

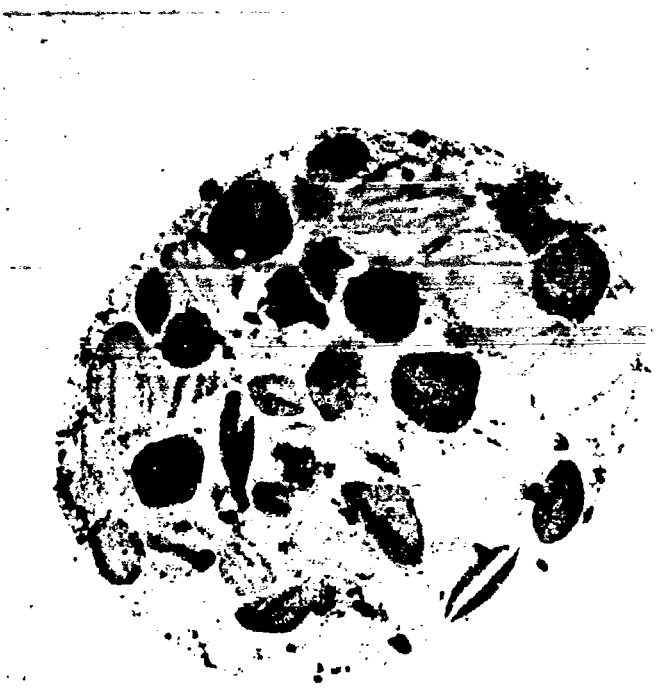

FIGURE 7. Peritoneal Eosinophile Section Stained for 2 Minutes with Uranyl Acetate. Reaction product is located at the edge of several granules. 14,000X

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14. Key Words			
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