β-D-Galactosidase Activity in Macrophages: Electron Microscopy and Biochemistry[1] (35160)

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The results of biochemical studies and light microscopic examination of intact cells and tissue sections suggest a role for the acid hydrolases in cellular immunity (1, 2). Although the matter is not yet resolved, there is accumulating evidence in the literature that these hydrolases are not limited to a uniform population of cell organelles (3–7). Moreover, the intracellular distribution of the commonly used lysosomal marker, acid phosphatase, may not parallel that of the glycosidases (6, 7). Ide and Fishman, using cytobiochemical techniques, recently have reported that in the gonadotropin-stimulated mouse kidney the distribution of β-glucuronidase is different from that of acid phosphatase (7).

Of the various methods for the *in situ* demonstration of glycosidases (8), the indigogenic technique possesses certain advantages (9, 10). Further, Holt (10) and Pearson *et al.* (11) have shown that the 5-bromo-4-chloro substituted indoxyl substrates are especially well suited for the precise localization of sites of enzyme activity. Holt and Hicks (12) found, however, that the indigo formed from these substrates failed to remain localized during routine dehydration and embedding for electron microscopy. More recently Tsou *et al.* (13) have used an iodo-substituted indoxyl substrate to demonstrate phosphatase activity with the electron microscope (13). The present study describes the first application of the indigogenic principal to the demonstration of β-glycosidase activity at the ultrastructural level.

Materials and Methods. To ascertain the electron density of 5,5′-dibromo-4,4′-dichloroindigo a suspension of this material was prepared by alkaline hydrolysis of 5-bromo-4-chloroindoxyl acetate. A droplet 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 epoxy resin by the procedures outlined below.

The problems associated with uneven fixation of tissue blocks and with uneven penetration of substrate were minimized by the use of cell suspensions. Slight modifications of the technique described by Myvrik *et al.* (14) were used to obtain stimulated, enzyme-rich alveolar macrophages from rabbit lungs. Samples of variously fixed cells and their corresponding homogenates were taken for evaluation of morphology and biochemical activity. The latter activity was compared to unfixed preparations. Fixatives used were glutaraldehyde, or formaldehyde prepared from paraformaldehyde (15). Concentration varied from 0.5 to 5% in phosphate buffer, and duration of fixation varied from 1 to 60 min. The fixed samples were subsequently rapidly washed in phosphate-buffered saline and resuspended in phosphate-buffered sucrose. They were then incubated for varying lengths of time in 5-bromo-4-chloroindol-3-yl-β-D- galactopyranoside (indoxylgalacto-

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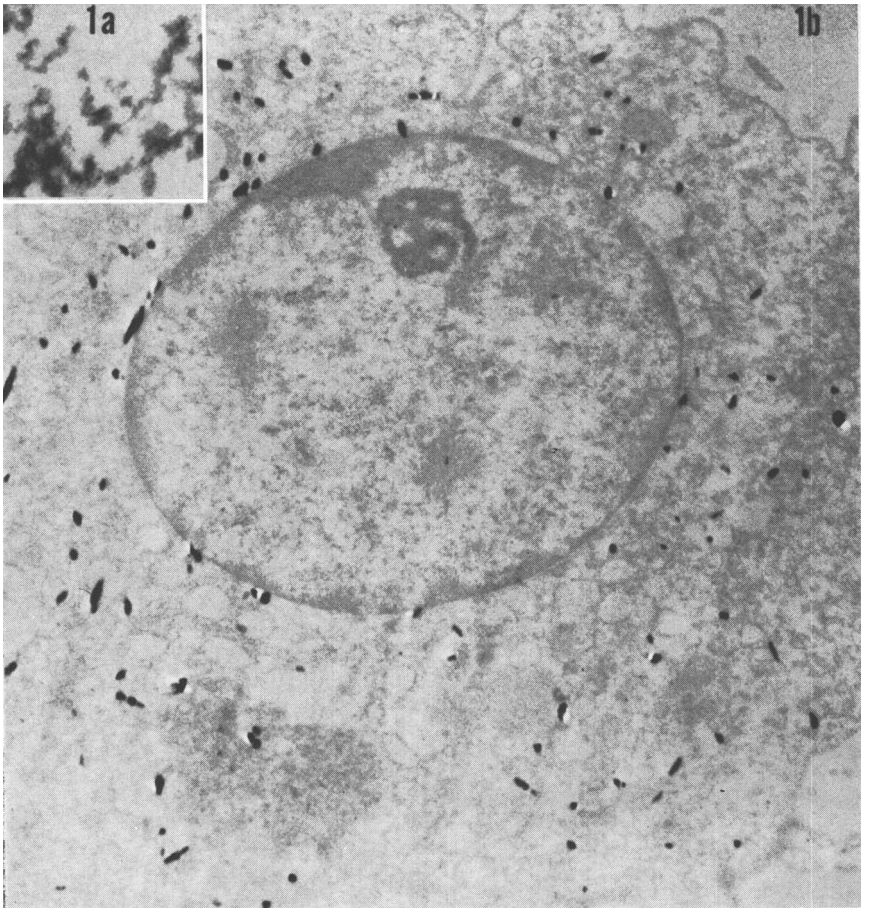

FIG. 1a. Electron micrograph of 5,5′-dibromo-4,4′-dichloroindigo prepared by alkaline hydrolysis of 5-bromo-4-chloroindoxyl acetate; ×56,000. (b) Alveolar macrophage: In this unstained preparation, the indigo reaction product stands out as dense granules and rods; ×10,500.

side) substrate prepared according to methods developed in this laboratory (9). Samples for morphology were dehydrated in graded ethanol over a total time of 1 hr, followed by transfer directly to unpolymerized epoxy resin. Thick (1 μ) plastic sections were stained with basic fuchsin and examined in the light microscope. Thin sections were subjected to varying heavy metal stains prior to examination in the electron microscope. In a few experiments, the ultrastructural distribution of reaction product was evaluated for cell samples that were exposed to cold ethanol (—15°) for intervals up to 5 min following standardized fixation procedures.

The hydrolysis of the indoxyl galactoside by the cellular homogenates as a function of pH was compared with hydrolysis of lactose and of *ortho*-nitrophenyl galactoside (ONPG) (9). In addition, enzyme activity of sediment and supernatant was assessed following suspension of fixed cells in distilled water, or exposure of these cells to repeated homogenization, sonication or incubation in substrate containing 0.1% final concentration of Triton X-100.

The specific enzymic nature of both cytochemical and biochemical reactions was evaluated by assay of enzyme activity following treatment with heat (56° for 1 min), 2×10^{4} *M para*-chloromercuribenzoate (PCMB), 1% galactonolactone, or 1% glucuronolactone.

Results. Unembedded preparations of 5, 5′-dibromo-4,4′-dichloroindigo and sections

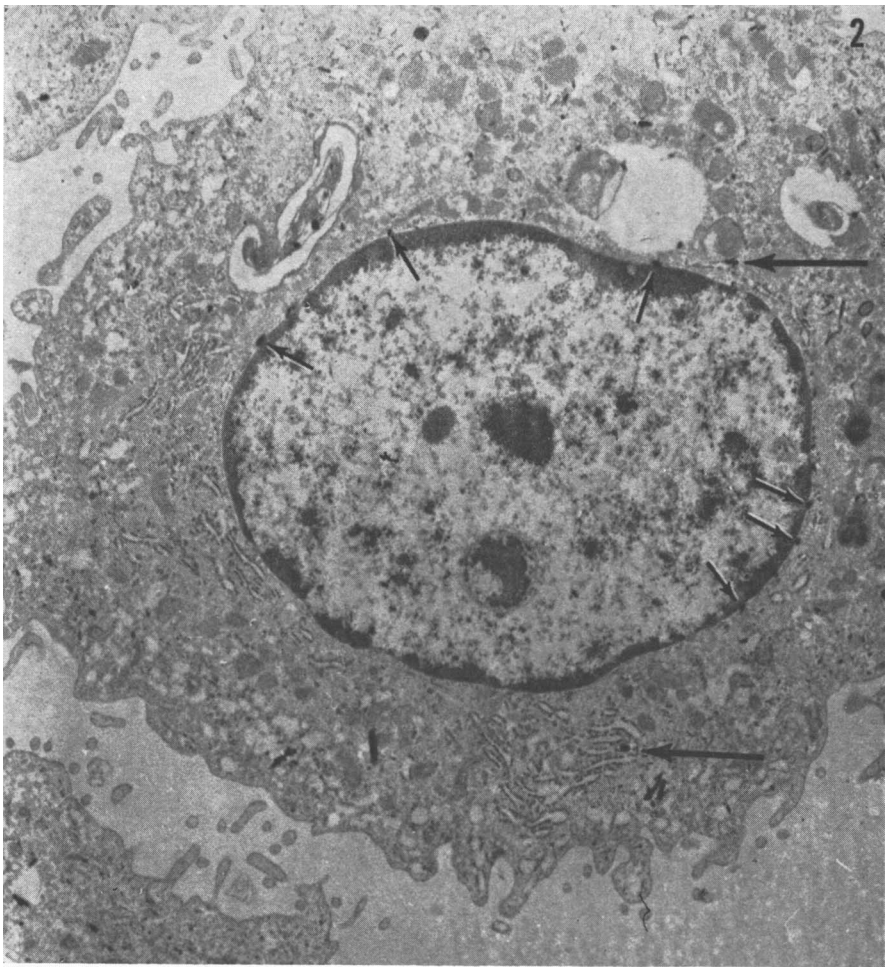

FIG. 2. Alveolar macrophage, uranyl acetate for 2 min: Some of the granules of reaction product within the nuclear membrane are shown by the small arrows. The large arrows indicate reaction product within the cisternae of the rough endoplasmic reticulum; ×9300.

of this material suspended in agar both proved to be electron dense without the addition of heavy metal stains. In both cases the resulting pictures show a rather irregularly granular material with no particular features (Fig. 1a).

Initial studies on samples of cells processed by routine methods of dehydration, embedding, and staining for electron microscopy revealed little discernible reaction product in the electron microscope, although parallel studies in the light microscope indicated that these cells were strongly reactive. Because of the known, though limited, solubility of indigo in certain organic solvents (16), the time of dehydration in ethanol was shortened to 1 hr. For similar reason, propylene oxide was eliminated from the embedding procedure. Following these procedural changes, indigo was readily visible in cells in thick (1 μ) plastic sections examined in the light microscope. Visualization in the electron microscope remained unsatisfactory. In thin sections subjected to conventional double staining techniques with uranyl acetate and lead citrate, holes were observed in the quantity and distribution of indigo seen in the light microscope. Examination of unstained thin plastic sections in the electron microscope revealed abundant reaction product in the same locations (Fig. 1b). Demonstration of cellular detail, however, was not satisfactory.

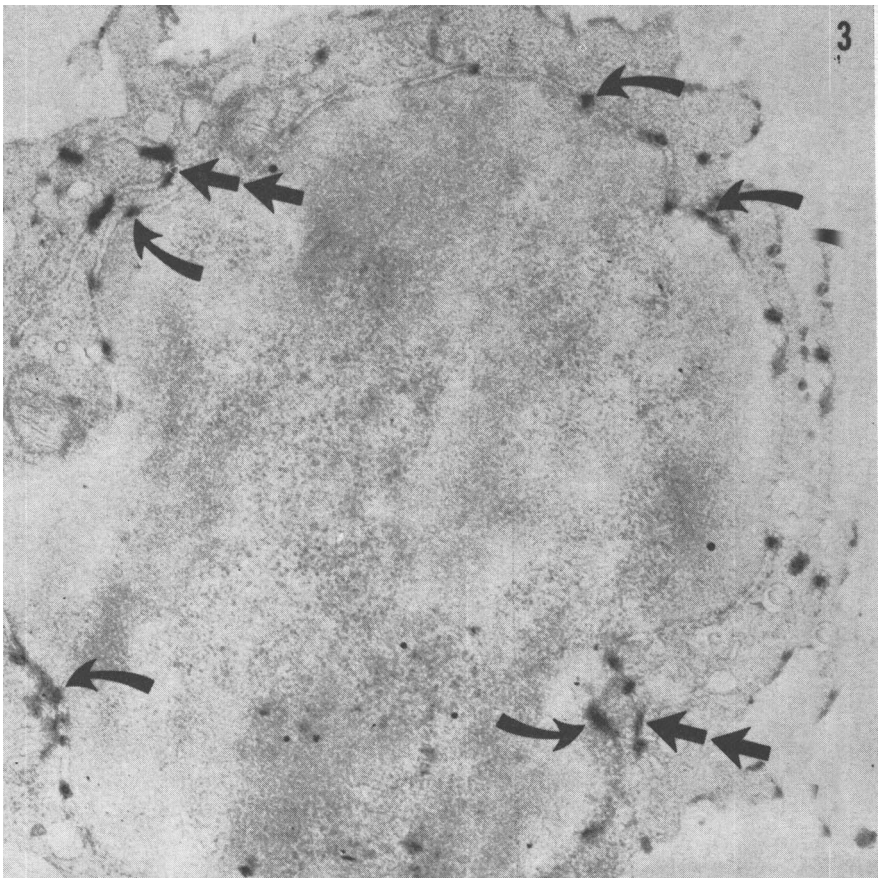

FIG. 3. Lymphocyte, lead citrate for 20 min: Some of the granules of reaction product within the nuclear membrane are shown by the curved arrows. The double straight arrows indicate reaction product within the cisternae of the endoplasmic reticulum; ×28,600.

Visualization of both indigo reaction product and cellular morphology was achieved best following exposure of the sections to uranyl acetate for 2 min (Fig. 2). Alternatively, a 10- to 20-min exposure to lead citrate has occasionally provided desirable staining of cytoplasmic features (Fig. 3).

Varying fixation procedures also affected the appearance of the cells and the amount of reaction product observed. At all time intervals and concentrations used, formaldehyde preserved some enzyme activity, but failed to protect the cells against shrinkage and other artifacts. Similarly, low concentrations of glutaraldehyde provided unsatisfactory preservation of morphology, and difficulties were experienced in achieving adequate settling rates of the blocks in epoxy resin. Two and one-half and 5% concentrations of glutaraldehyde were associated with good retention of cell detail and enzyme activity at all durations of fixation.

Studies of the duration of incubation of cells with substrate have shown increases in reaction product with increasing time of incubation. For the purposes of the electron microscope, the best incubation times lie between 1 and 3 hr. Before this the amount of reaction product formed is rather small; after this the crystals become larger than is desirable for purposes of localization.

The results of the application of this technique are seen in various cell types in Figs. 1b, 2, 3, and 4. In the unstained phagocyte the reaction product stands out clearly (Fig. 1b). The presence of reaction product within the two layers of the nuclear membrane is of particular interest. This localization of reac-

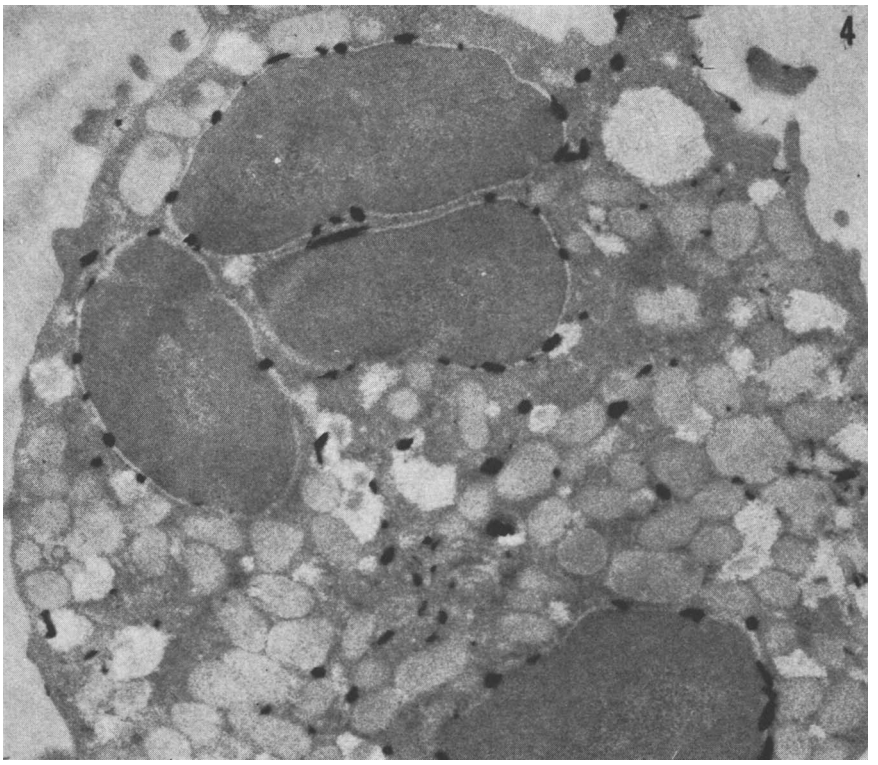

FIG. 4. Heterophile, unstained: Reaction product is present within the nuclear membrane and at the periphery of some of the granules; ×17,600.

tion product has been observed more consistently than any other (Figs. 1b, 2, 3, 4). The next most consistent localization is in the cisternae of the endoplasmic reticulum (Fig. 2, 3). In heterophiles (Fig. 4) and eosinophiles, the reaction product is not found within granules, but rather consistently appears to localize at the periphery of some granules. In alveolar macrophages, however, lysosomes only rarely show reaction product at their edges; it is found even less often in the interior of the lysosomes. Indigo reaction product is not found over nuclei or mitochondria, and is seen extremely rarely outside of cells. The same distribution of reaction product was observed for cells that were exposed to cold ethanol over brief intervals of time prior to incubation in substrate.

The enzyme dependence and specificity of reaction product deposition was established through inhibition studies. Heat and PCMB, both nonspecific enzyme inhibitors, brought about almost complete loss of enzyme activity. Galactonolactone, a specific inhibitor of β-D-galactosidase, also caused complete loss of enzyme activity, whereas an analogue, glucuronolactone, had no visible effect (Table I).

Biochemical data from parallel homogenized samples were fully consistent with the data obtained with the electron microscope. Fixation in glutaraldehyde caused a rapid reduction in enzyme activity to 70–80% of the unfixed level, with little further decrease in activity at longer fixation times. Homogenates of formaldehyde-fixed samples, however, hydrolyzed substrate less readily than corresponding samples fixed in glutaraldehyde. Repeated homogenization, sonication or incubation in substrate containing Triton X-100 failed to provide an increased in enzyme activity in the formaldehyde-fixed samples.

Binding of enzyme activity to the cell occurred during fixation. After 10 or more minutes of treatment in glutaraldehyde, saline

TABLE I. Inhibition Studies.

	% Inhibition as measured biochemically	Amount of inhibition seen visually in intact cells
Normal substrate	0	0
Galactose, 1%	72	+++
Galactonolactone, 1%	83	+++-++++
Glucuronolactone, 1%	0	0
p-Chloromercuribenzoate $2 \times 10^{-4} M$	>95	++++
Heat (56° for 1 min)	>95	++++

wash solutions and a final distilled water supernatant of cells were devoid of enzyme activity.

Data from inhibition studies at the biochemical level were exactly parallel to the observations made in the electron microscope as depicted in Table I. Consistency between electron microscopic and biochemical data was also found when examining the pH dependency of β-D-galactosidase activity. The indoxyl substrate is hydrolyzed maximally over a relatively broad range of pH. The peak activity occurs at pH 5, and is 1 unit higher than the peaks of enzyme activity as measured by the other two substrates (Fig. 5).

Discussion. The advantages of the indigogenic technique for the demonstration of

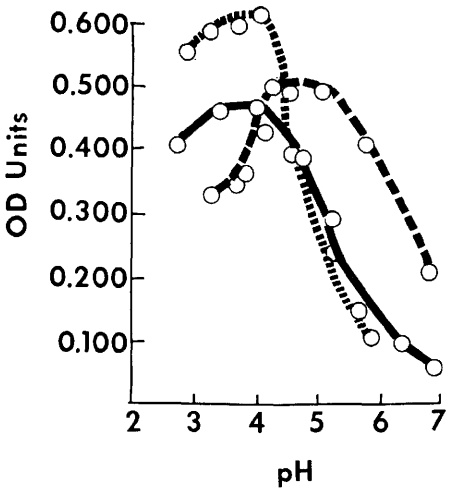

FIG. 5. pH dependence of β-D-galactosidase activity: (—) ONPG; (....) lactose; (- -) indoxyl galactoside. Maximal hydrolysis of the latter substrate occurs at a pH approximately 1 unit higher than that of the other two.

hydrolytic enzyme activity in the light microscope are well known (9–11). Our observations indicate that the desirable properties of the 5-bromo-4-chloro substituted substrates, including visibility, localization, substantivity and specificity, are also applicable in the electron microscope. Only slight modifications of normal processing techniques for the electron microscope are required for demonstration of enzyme activity at this level. Reaction product is slightly soluble in organic solvents and is masked, or removed, in cells subjected to conventional double staining techniques. These effects are minimized by shortening the duration of these treatments.

In these circumstances glutaraldehyde provides certain advantages over formaldehyde. The former gives better perservation of cellular detail, while causing less inhibition of enzyme activity. The present data indicate that formaldehyde may directly inactivate the enzyme rather than seal it in sites which are less accessible to substrate.

Under the conditions employed at this study, reaction product was localized most consistently between the layers of the nuclear membrane. It was also observed in endoplasmic reticulum and at the edges of lysosomes, but only rarely within lysosomes. In an earlier light microscopic study of macrophage glycosidase activity, it was not possible to determine whether or not the observed diffuse cytoplasmic staining represented an artifact of diffusion (9). The results of the present study support the extralysosomal distribution of β-galactosidase. They also are consistent with the findings of Ide and Fishman (7) who observed a dual localization for β-glucuronidase. The localization of enzyme reactivity in both nuclear envelope and endo-

plasmic reticulum is not surprising in view of the known communication between these structures. Synthesis of enzyme at these sites may explain these observations (17).

Nonspecificity and artifactual deposition of reaction product are common problems in enzyme cytochemistry. The data from the inhibition studies reported herein, and the absence of reaction product over nuclei, mitochondria, and extracellular regions, provide substantial evidence of the specificity and validity of the indigogenic technique for the demonstration of β-D-galactosidase.

The ability to attach various moieties to the indoxyl group provides the opportunity for studying a number of hydrolytic enzymes using techniques based on a common principle. This permits a semiquantitative comparison of the activity of various hydrolytic enzymes within a single tissue or cell system.

Summary. The indigogenic technique, a simple, specific, and reproducible method for demonstrating hydrolytic enzymes, has been adapted for electron microscopy. β-D-Galactosidase has been demonstrated in rabbit alveolar macrophages and other cell types with the same halogenated indoxyl substrate used in light microscopy. The reaction product is substantive and is readily identified in the electron microscope. The method is applicable to other hydrolytic enzymes.

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