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TECHNICAL MANUSCRIPT 459

A STUDY OF THE INDIGOGENIC PRINCIPLE
AND IN VITRO
MACROPHAGE DIFFERENTIATION

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TECHNICAL MANUSCRIPT 459

A STUDY OF THE INDIGOGENIC PRINCIPLE
AND IN VITRO MACROPHAGE DIFFERENTIATION

William E. Bennett

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Pathology Division
MEDICAL SCIENCES LABORATORY

Project 1T013001A91A

July 1968

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 5-bromo-4-chloro-substituted indoxyl glycosides were employed in a study of the cytochemistry of macrophages. During short-term (72 hours) culture, these cells demonstrated concomitant increases in (i) the number of organelles identified as lysosomes and (ii) sites that stained for β -galactosidase and β -glucuronidase activities. Moreover, there was a progressive increase in the densities of enzyme reactive centers. Indigo reaction product was not observed over nuclei; lipid droplets and cell background were free from spurious precipitations. Both galactosidase and glucuronidase were optimally active at acid pH values. Enzyme activity was completely inhibited by the aldonolactone corresponding to the sugar moiety of the substrate. Unrelated sugars did not influence hydrolysis, whereas substrate analogs caused various degrees of inhibition.

Biochemical assays of cell homogenates under the conditions employed in the cytochemical studies revealed the semiquantitative potentialities of the indoxyl methods. Optical density measurements demonstrated that the rate of indoxyl galactoside hydrolysis was dependent on both time and enzyme concentration.

Present evidence suggests that the glycosidases reflect and probably contribute to both physiological and pathological processes.

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I. INTRODUCTION

Methods that demonstrate enzymes in tissues are well documented. However, there is a paucity of information regarding the applicability of these methods for investigating enzymes in individual cells. Ideally, histochemical methods should be readily amenable to studies of enzyme activity of cells and their organelles.¹ For example, cytochemical adaptation of these methods revealed that macrophages transform in vitro with increases in their acid phosphatase - positive granules identified as lysosomes.^{2,3} As the functional element of the reticulo-endothelial system (RES), macrophages from stressed and control animals have been examined for their enzymes.⁴⁻⁶ Biochemical comparisons of systemic phagocyte populations have yielded inconsistent results.^{4,5} However, Dannenberg et al.,⁷ in a cytochemical study of phagocytosis, compared the most reactive cells of different phagocyte populations. They observed significant differences between population subsets that could not have been selected out for evaluation with biochemical methods.

Our interest in the RES and the initial chemical lesion(s) underlying clinical pathology has produced several reliable and sensitive markers of enzyme activity.⁸⁻¹⁰ These substrates have been employed at the tissue level, and they appear to satisfy the requisite criteria for markers of cells and appropriate organelles. Several glycosidases and a peptidase have been demonstrated with these substrates.

This study examines the semiquantitative aspects and specificity of two indoxyl glycosides and demonstrates their usefulness as enzyme markers at both cellular and subcellular levels. In addition, this study extends the observations of previous reports on the enzyme cytochemistry of phagocyte transformation in vitro.

II. MATERIALS AND METHODS

A. CELL SAMPLES

Specific-pathogen-free mice weighing between 20 and 30 g were used as a source of peritoneal mononuclear phagocytes. Cells were collected by peritoneal lavage and then maintained in 20% newborn calf serum in medium 199 (NBCS/199) for periods up to 72 hours.⁸ Cover slips and mass cultures were used for cytochemical and quantitative biochemical studies, respectively. Alveolar macrophages from the lungs of BCG-stimulated rabbits also were employed for certain quantitative experiments.¹¹

Aliquots of samples for biochemical assay were stored at -70 C for periods up to 5 days.¹²

B. CYTOCHEMISTRY AND MORPHOLOGY

Following appropriate intervals of in vitro maintenance, cover slip monolayers were processed for demonstration of enzyme activity: Samples were rinsed in saline, fixed in 1.25% buffered glutaraldehyde for 12 minutes, then impregnated with 0.88 M sucrose.³ The substrate conditions employed were those described by Pearson and co-workers.⁸⁻¹⁰ Incubation for 3 and 5 hours at 37 C proved optimal for demonstration of macrophage β -glucuronidase and β -galactosidase activities, respectively. The two substrates were: 5-bromo-4-chloroindol-3-yl- β -D-glucopyruroniside and 5-bromo-4-chloroindol-3-yl- β -D-galactopyranoside.* Heat-inactivated cell samples and medium from which substrate was omitted were regularly included as controls. Following incubation, preparations were rinsed, water-mounted, and viewed under light microscopy.

The specificity of the indoxyl glycosides also was examined. Following 30 minutes of pre-incubation at 37 C, monolayer preparations were incubated in substrate containing selected analog inhibitors. The following were employed at 1% final concentration: lactose, galactose, isopropyl- β -D-thiogalactopyranoside, galactono-1,4-lactone, and boiled saccharate.¹³ Controls that contained neither substrate nor inhibitor were included for comparison.

The morphological aspects of phagocyte transformation in vitro were examined by phase contrast illumination of osmium tetroxide - fixed preparations.

C. BIOCHEMISTRY

1. β -Galactosidase (3.2.1.23)**

Assays were performed by a modification of the method of Lederberg.¹⁴ Equal volumes of frozen-thawed cell samples and 5 mM *o*-nitrophenyl- β -D-galactopyranoside (ONPG) in 0.1 M citrate-phosphate buffer, pH 3.9, were incubated at 37 C for 2 hours. The reaction was stopped and deproteinized by addition of trichloroacetic acid to a final concentration of 2.5%. Samples were chilled and filtered, and color was developed at pH 10.4 by

* These substrates are available from The Cyclo Corporation, 1922 E. 64th Street, Los Angeles, California 90001.

** Enzyme Nomenclature, International Union of Biochemistry, Elsevier Publishing Co., Amsterdam, 1965.

mixing equal volumes of filtrate and glycine-NaOH buffer. The extent of hydrolysis was read from a standard curve prepared from dilutions of *o*-nitrophenol. For the conditions of assay, hydrolysis of substrate was linear with respect to both time and concentration of cell sample. Concurrent tubes in which lysed cells or substrate were replaced by water remained colorless.

Another method for the quantitative measurement of galactosidase activity employed the natural substrate lactose. Reaction vessels contained cell samples and half volumes of prepared substrate (0.1 M lactose at pH 3.8 in 0.1 ml acetate buffer containing 3×10^{-3} M KCl). The mixture was incubated at 37 C for 1 hour, and then the reaction was stopped by placing the tubes in boiling water. Coagulated protein was removed by centrifugation, and free glucose was assayed with tris-buffered reagent glucose oxidase.* The amount of free glucose was read from a standard curve.

2. β -Glucuronidase (3.2.1.31)

Quantitative measurements of β -glucuronidase activity were obtained with phenolphthalein glucuronide by a slight modification of the technique adapted by Cohn and Benson.⁸ Equal volumes of cell sample and 6×10^{-3} M substrate, pH 4.5, were incubated at 37 C for 2 hours. The extent of hydrolysis was determined by reference to a standard curve prepared from dilutions of free phenolphthalein.

3. Specific Activity

The protein content of cell samples was assayed by a modification of the method of Lowry.¹⁵ Hydrolysis of substrate was then expressed in terms of specific activity; i.e., micrograms of indicator released per milligram of protein per hour. The pH values employed for the enzyme determinations were previously determined to be the levels of optimal activity.

III. RESULTS

A. MORPHOLOGICAL STUDIES

Mononuclear phagocytes undergo differentiation with development of lysosomes when maintained in short-term culture.^{8,9} In the present study, the morphologic features of this process were observed in individual experiments and related to the glycoside cytochemistry of

* Worthington Biochemicals, Freehold, New Jersey.

companion samples. In vitro, phagocyte attachment to glass and cell spreading become apparent within 2 to 4 hours. Phase microscopy of osmium-fixed 24-hour cultures demonstrated further spreading and increases in the size and number of mitochondria, lipid droplets, and osmiophilic dense bodies identified as lysosomes. These appeared to have mostly a perinuclear distribution. At 72 hours, however, the dense bodies were more generally distributed throughout the cytoplasm, and their numbers were increased. Lipid droplets accumulated in the zone peripheral to the dense bodies. Cultured cells also demonstrated the progressive development of a centrosphere, which was observed with phase microscopy or with light microscopy of stained preparations. In binucleate and multinucleate cells, this zone seemed localized between the nuclei (Fig. 1).

B. CYTOCHEMICAL STUDIES

The glycosidase activity of macrophages maintained in culture for various periods was evaluated with the indolyl glycoside substrates. Hydrolysis of these substrates results in the deposition of a blue-green indigo reaction product.

1. β -Glucuronidase Activity

Macrophages maintained in vitro for 3 to 4 hours and then evaluated for glucuronidase revealed limited reaction product with an essentially perinuclear distribution. Cells evaluated following 48 to 72 hours of culture demonstrated a progressively increased deposition of reaction product. This product appeared as both (i) a diffuse background in the cell cytoplasm and (ii) localized deposits distributed in a pattern that paralleled that of the osmiophilic dense bodies. In binucleate and multinucleate cells, reactivity was highest in zones between nuclei. Extracellular indigo was not observed. No correlation existed between the numbers or distribution of reactive centers and that of lipid droplets characteristic of cells in a particular experiment.

Studies were also performed to determine the pH dependence of macrophage glucuronidase by this cytochemical method. Cover slip monolayers from 72-hour cultures were incubated in indolyl glucuronide buffered at increments of 0.6 unit between pH 2.2 and 6.9. Strong cellular reactivity was observed in the range of pH 3.4 to 5.2 in the cells. However, the substrate solutions buffered at pH values of 3.4 and 4.0 developed minimal indigo color during the incubation of monolayer samples. Control tubes in which cell preparations were omitted did not reveal pH-dependent hydrolysis.



Figure 1. Morphological Features of Phagocytes Differentiating In Vitro. Cells were fixed at refrigerator temperature in 1.25% buffered glutaraldehyde for 7 minutes, then osmium tetroxide for 12 minutes. Phase contrast microscopy, 2500X. A, Typical cells after 2 hours in vitro; occasional dense bodies are apparent. B, Characteristic cell after 24 hours in vitro; the cell has enlarged and reveals the development of refractile lipid droplets and osmiophilic dense bodies, the latter appearing localized to one side of the nucleus. C, A macrophage from a 72-hour culture; cell diameter has increased markedly and numerous dense bodies are apparent; there is an increase in the numbers of mitochondria that reveal some pleiomorphism; the focus is sufficient to demonstrate a few of these in the upper left of the figure.

2. β -Galactosidase

The β -galactosidase activity of short-term phagocyte cultures demonstrated the temporal increase and distribution pattern similar to that observed for β -glucuronidase. In other experiments, we examined the enzyme activity of cell monolayers cultured for 72 hours then placed in galactoside substrates buffered from pH 2.2 to 6.9. Reaction product deposition appeared greatest between pH 3.8 and 5.4. Compared with glucuronidase, however, a greater proportion of cellular reactivity was retained at lower pH values, specifically pH 3.0 and 3.4.

Figures 2 and 3 depict cells incubated for glucuronidase and galactosidase activities following 4, 24, and 72 hours in culture. Their appearance typifies that of the most reactive 10% of the cells for the respective populations.

3. Inhibitor Studies

Because hydrolysis of 5-bromo-4-chloro-substituted indolyl substrates results in the formation of identical molecules of bisindigo, studies with selected inhibitors were conducted to investigate the specificity of the above reactions. The results of these experiments are presented in Table 1. The blue-green indigo was not observed in fixed cells maintained at 75 C for 15 minutes prior to incubation in substrate. Similarly, no reaction product was observed when the incubation medium contained 1% aldonolactone of the configuration corresponding to the sugar moiety of the indolyl glycoside. Galactose, nitrophenyl galactoside, and phenolphthalein glucuronide demonstrated analog inhibition in the presence of the corresponding cytochemical substrate.

C. BIOCHEMICAL STUDIES

Increases in the specific activities of the β -glucuronidase of macrophages maintained in 20% NBS/199 for 72 hours have been demonstrated with quantitative biochemical methods.³ The cytochemical observations above are in agreement with these quantitative results. Therefore, it seemed desirable to obtain some documentation on the sensitivity of the indolyl method. Accordingly, the cytochemical activities of macrophages maintained in culture for various periods were arbitrarily scored, and these values were compared with the biochemical results from companion samples. Figure 4 presents the specific activities of preparations from a typical experiment. Enzyme levels were evaluated with the biochemical substrates nitrophenyl galactoside, lactose, and phenolphthalein glucuronide. Under the conditions of culture and assay, macrophage galactosidase and glucuronidase increased moderately during in vitro maintenance. In individual experiments, a strict parallel existed between the biochemical measurements and the semiquantitative cytochemical estimates of enzyme activity.

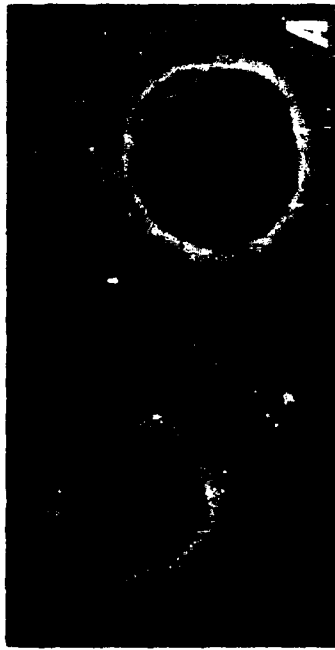


Figure 2. Beta-Glucuronidase Activity of Mouse Peritoneal Phagocytes Following Various Intervals of In Vitro Maintenance. 2500X.
A, Four hours in vitro; diffuse-appearing reaction product in the "hof" of the nucleus. B, Glucuronidase activity of a cell following 24 hours in vitro; increased reaction product with a juxtannuclear distribution is apparent.
C, Typical cell from the most reactive 10% of population following 72 hours in vitro; reaction product is markedly increased and more generally distributed throughout cytoplasm than at 48 hours; background and nucleus are unreactive.



Figure 3. Beta-Galactosidase Activity of Mouse Peritoneal Phagocytes Maintained In Vitro. 2500X. A, Preparation from a 4-hour culture; minimal reaction product discernible in cytoplasm; nuclei unreactive. B, Galactosidase activity of cell following 24 hours in vitro; cell spreading is apparent with few positive reaction sites. C, Phagocyte following 72 hours in vitro; note the many discrete and intensely stained sites of reaction product; nucleus and background reveal no diffused indigo.



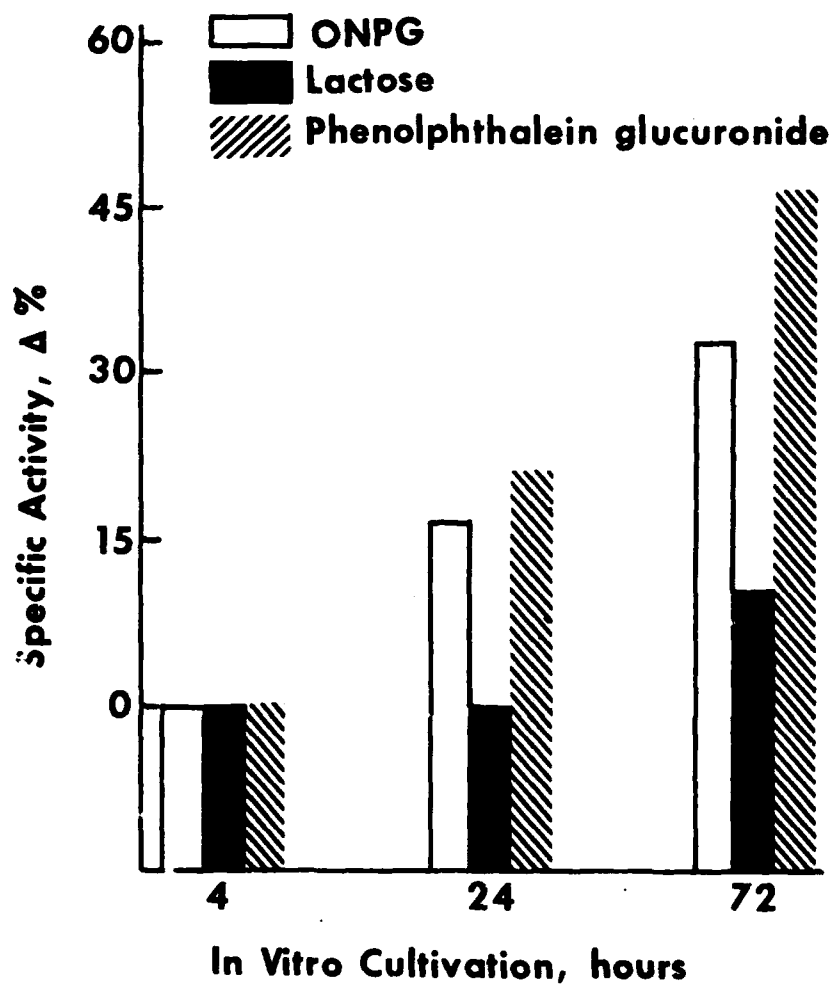


Figure 4. Hydrolysis of Nitrophenyl Galactoside, Lactose, and Phenolphthalein Glucuronide by Macrophages in Culture for Various Periods.

TABLE 1. SEMIQUANTITATIVE CYTOCHEMICAL ESTIMATE
OF ANALOG INHIBITION

Condition	Relative Inhibition ^{a/}	
	β -Galactosidase	β -Glucuronidase
75 C	+++	+++
Lactose 1%	+	-
Galactose 1%	+++	-
Galactonolactone 1%	+++	-
Glucuronolactone 1%	-	+++
Control	-	-

a. Reactions are scored from ++++ for complete, or almost complete, inhibition to - for the absence of an inhibitor effect.

Other biochemical experiments examined the quantitative aspects of β -galactosidase inhibition. The substrate analogs employed and their concentrations were identical to those used in the cytochemical method. Data from these experiments are presented in Table 2. It is apparent that the inhibitors had comparable effects on β -galactosidase in both the cytochemical and biochemical systems. Galactose markedly inhibited hydrolysis of substrates with galactose specificity.

TABLE 2. QUANTITATIVE BIOCHEMICAL MEASUREMENT
OF THE ANALOG INHIBITION OF β -GALACTOSIDASE

Condition	Inhibition, % ^{a/}
75 C	100
Lactose	16
Galactose	72
Galactonolactone	83
Glucuronolactone	0
Control	0

a. Per cent inhibition is calculated from specific activities of experimental samples compared with the control.

A comparison of the relative sensitivity of the indoxyl and nitrophenyl glycosides to enzymatic hydrolysis was obtained by the following method. Frozen-thawed samples of rabbit alveolar macrophages were cleared of gross debris by centrifugation, and dilutions were dispensed to tubes containing indolyl galactoside, nitrophenyl galactoside, or water. Following incubation, optical densities were read at the wavelength of maximum absorption, 650 μ . The indolyl-containing samples were not deproteinized because attempts to extract indigo from protein were unsuccessful. Therefore, samples were removed from the water bath, inverted, and immediately read against their corresponding water blanks. Turbidity corrections were made by subtracting the optical densities at 546 μ from the corresponding experimental values. In other experiments, a comparison was made of the hydrolysis of the two substrates as a function of time. It proved advantageous to mix attemperated solutions of indolyl substrates and enzyme in timed sequences so that optical density readings could be made immediately following incubation.

The results of these experiments are presented in Figures 5 and 6. It is evident that indolyl galactoside is a sensitive indicator of β -galactosidase activity. Optical density measurements at 650 μ demonstrate the linear formation of bisindigo with respect to both protein concentration and incubation time. Indeed, color development was visible within minutes following mixture of microgram quantities of cell homogenate and substrate. Samples centrifuged following 3 minutes of boiling revealed that the indigo was attached to protein.

IV. DISCUSSION

Progress in the area of enzyme cytochemistry has been complicated by problems of substrate insolubility and poor specificity, diffusion of reaction product or its intermediates, and spurious precipitations. Moreover, quantitative or semiquantitative potentialities demand that the rate of stain deposition bear a correspondence to both the concentration of enzyme and incubation time.

In the present study, the rate of formation of reaction product from 5-bromo-4-chloroindol-3-yl- β -D-galactopyranoside was evaluated biochemically. The development of color was followed photometrically and changes were evident within minutes. Increases in optical density were linear with respect to the enzyme concentration and incubation time. Because identical molecules of indigo product result from the hydrolysis of both the galactoside and glucuronide substrates, identical kinetics should apply for the formation of indigo following their hydrolysis.

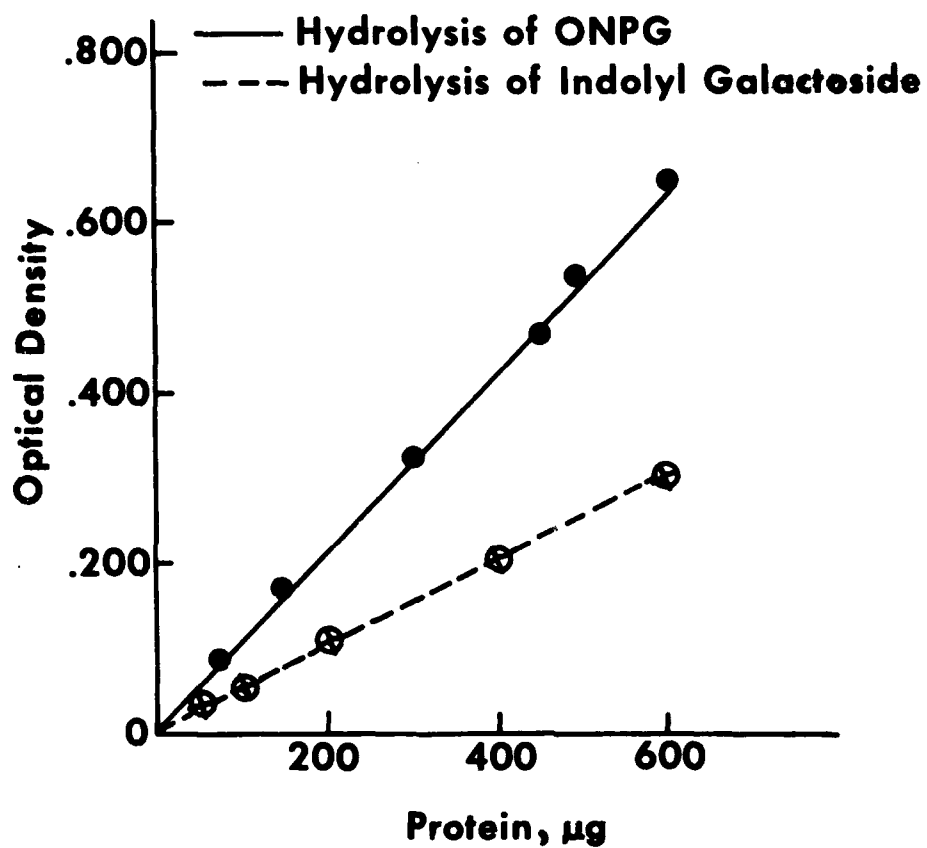


Figure 5. Hydrolysis of Substrates as a Function of Cell Enzyme Concentration. Incubation for 60 minutes at 37 C.

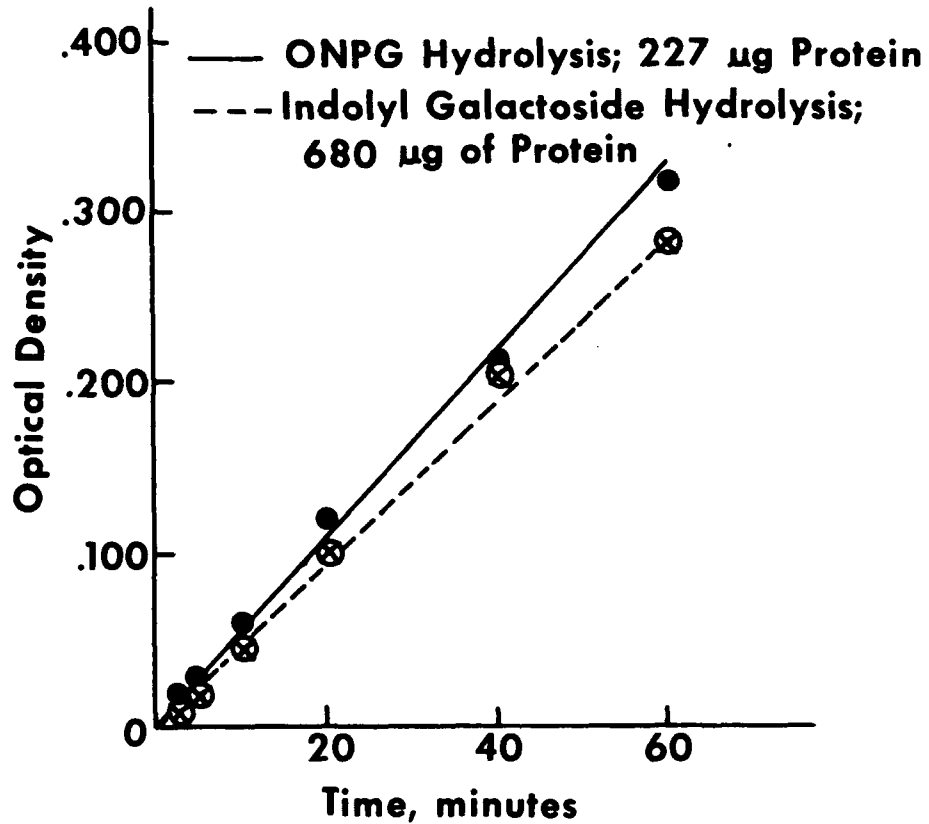


Figure 6. Hydrolysis of Substrates as a Function of Time. Cleared lysates of alveolar macrophage were employed at 227 μg and 680 μg total protein with *o*-nitrophenol- β -D-galactopyranoside (ONPG) and 5-bromo-4-chloro indol-3-yl- β -D-galactopyranoside, respectively.

Tsou and Su¹⁶ investigated in more detail the kinetics of the hydrolysis of the unsubstituted indoxyl phosphate by purified alkaline phosphatase. The results were compared with those for hydrolysis of glycerol- and phenylphosphates under similar conditions. Their findings supported the histochemical use of indoxyl substrate except for the random crystallization of reaction product. Crystallization artifacts also occurred in lipid-rich tissues when 5-iodoindoxyl phosphate was employed in a subsequent study.¹⁷

However, results from this laboratory and other laboratories¹⁸ demonstrated that spurious crystallization does not follow hydrolysis of the 5-bromo-4-chloro-substituted indoxyl substrates. Accordingly, it seemed desirable to investigate the indoxyl glycosides as enzyme markers of single cells and their organelles. The mononuclear phagocyte of the mouse maintained in vitro provided a model system for this evaluation. In cell culture, these phagocytes undergo transformation with the progressive development of osmiophilic dense bodies that stain for acid phosphatase. Moreover, biochemical measurements of maintained phagocytes have revealed progressive increases in other acid hydrolases.

The cytochemical aspects of the development of two such enzymes (β -glucuronidase and β -galactosidase) now have been demonstrated with the appropriate dihalogen-substituted indoxyl substrates. Semiquantitative estimates of activity in cultured phagocyte populations revealed a time-dependent increase in the cells reactive for both hydrolases. Occasional cells were frequently more or less reactive than the average for the population. Within individual cells, reaction product deposition increased with time in culture. A comparative study of companion samples revealed similar distribution patterns for indigo product and osmiophilic dense bodies identified as lysosomes. Both reaction product and dense bodies have a perinuclear distribution in cells during the first 24 hours of culture. In binucleate cells, granules were found between nuclei, and, in cells with a deep nuclear indentation, localization in the "hof" of the nucleus was predominant. These areas are associated with the Golgi apparatus, as revealed in electron microscopy. Maintenance of cells in culture beyond 24 hours results in the more general distribution of dense bodies and enzyme-reactive sites. From these observations, it appears that a correspondence exists among the patterns of development of β -galactosidase, β -glucuronidase, and osmiophilic dense bodies. That this is true for acid phosphatase and dense bodies has been reported at both the levels of light and electron microscopy.¹⁹

Observations of our cytochemical preparations incubated in the indoxyl substrates revealed no extracellular reaction product, and nuclei were unreactive. No correspondence was found between the rather typical pattern of lipid droplet distribution in cultured cells and that of reaction product. These findings are consistent with (i) the lack of affinity between fat droplets and the 5-bromo-4-chloro indoxyls and (ii) the substantivity of both this intermediate product and the derived bisindigo.¹⁰

Our semiquantitative estimates of the increases in reaction product deposition in cultured phagocytes were further substantiated by quantitative biochemical studies. There was a small but consistent increase in the hydrolysis of both phenolphthalein glucuronide and *o*-nitrophenyl galactoside between 2 and 24 hours of culture. Samples maintained in vitro for 72 hours demonstrated further increases in the hydrolysis of these two substrates. However, there was not an exact parallel between the hydrolysis of lactose and the analogous substrate, nitrophenyl galactoside. Isozymes of galactosidase have been reported for mammalian tissues.²⁰ Moreover, homogenates of the mouse intestine preferentially hydrolyze certain substrates of galactoside specificity.²¹ Preliminary studies of homogenous preparations of macrophages from both rabbit and mouse suggest the existence of galactosidases separable by their electrophoretic mobilities.*

The rate of optical density increase concomitant with the hydrolysis of indoxyl galactoside was less rapid than that associated with the hydrolysis of *o*-nitrophenyl galactoside. As cytochemical markers, however, the substituted indoxyls are hydrolyzed at local sites of enzyme activity. These sites possess high specific activity, thus permitting enzyme identification and localization within cells. In addition, observation of intracellular architecture and enzyme reactive centers lends direct support to certain functional relationships that otherwise could escape detection.²²

The broad pH range over which good enzyme activity was demonstrated with the indoxyl substrates is in contrast to the sharp optima obtained with the biochemical methods. Janigan¹⁸ has reported that tissue fixation results in the flattening of the pH activity curve of several glycosidases. Further, the extent of flattening was found to be both pH- and time-dependent. Tissues in glutaraldehyde for 7 hours revealed only small differences in the activity of β -glucuronidase between pH 3.5 and 6.0. However, the macrophages used for the present report were exposed to glutaraldehyde for only 12 minutes. Whether or not such short exposures alter the pH dependence of indoxyl glycoside hydrolysis is under study. In addition, the observation of generalized cytoplasmic staining in cultured cells might result from diffusion and/or extra-lysosomal enzyme.^{1,20,22}

The glycosidases are rather specific enzymes, and extensive studies have been made of their activity in the presence of analog inhibitors. Certain of these were employed to evaluate the indoxyl glycosides as specific markers at the cellular and subcellular levels. The aldonolactones were markedly inhibitory for enzyme activity in the presence of the corresponding substrates. Galactose inhibited β -galactosidase activity to a greater extent than did the disaccharide lactose. These semiquantitative estimates paralleled the results obtained from biochemical measurements of

* Bennett, unpublished data.

the effects of the selected inhibitors. Significantly, the indoxyl-substituted substrates of glucoside and fucoside specificity are not hydrolyzed by fixed mononuclear phagocytes.²⁴ However, the extreme sensitivity of certain glycosidases to glutaraldehyde fixation is worthy of note.

Evidence for a role of the lysosomal glycosidases in the economy of the host is compelling but inconclusive. In Gaucher's disease, assays of spleen homogenates revealed that the β -glucuronidase and β -galactosidase activities were elevated and markedly reduced, respectively.²⁵ These enzymes are localized in splenic reticular cells that belong to the RES.²⁶ In a less clearly defined example, pyelonephritis, the urines of patients contain increased amounts of β -glucuronidase.²⁷ Macrophage glycosidases also probably play an active role in altering the infectivity and immunogenicity of microbial agents.²⁸ Direct evidence pertaining to the processing of virus by lysosomes has been reported.²⁹

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<p>The 5-bromo-4-chloro-substituted indoxyl glycosides were employed in a study of the cytochemistry of macrophages. During short-term (72 hours) culture, these cells demonstrated concomitant increases in (1) the number of organelles identified as lysosomes and (2) sites that stained for β-galactosidase and β-glucuronidase activities. Moreover, there was a progressive increase in the densities of enzyme reactive centers. Indigo reaction product was not observed over nuclei; lipid droplets and cell background were free from spurious precipitations. Both galactosidase and glucuronidase were optimally active at acid pH values. Enzyme activity was completely inhibited by the aldono-lactone corresponding to the sugar moiety of the substrate. Unrelated sugars did not influence hydrolysis, whereas substrate analogs caused various degrees of inhibition.</p> <p>Biochemical assays of cell homogenates under the conditions employed in the cytochemical studies revealed the semiquantitative potentialities of the indoxyl methods. Optical density measurements demonstrated that the rate of indoxyl galactoside hydrolysis was dependent on both time and enzyme concentration.</p> <p>Present evidence suggests that the glycosidases reflect and probably contribute to both physiological and pathological processes.</p>		
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