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TECHNICAL MANUSCRIPT 365

THE HISTOCHEMICAL DEMONSTRATION OF INTESTINAL β-D-FUCOSIDASE WITH 5-BROMO-4-CHLOROINDOLE-3-YL-β-D-FUCOPYRANOSIDE

John R. Esterly
Alfred C. Standen
Bjarne Pearson

FEBRUARY 1967

DEPARTMENT OF THE ARMY
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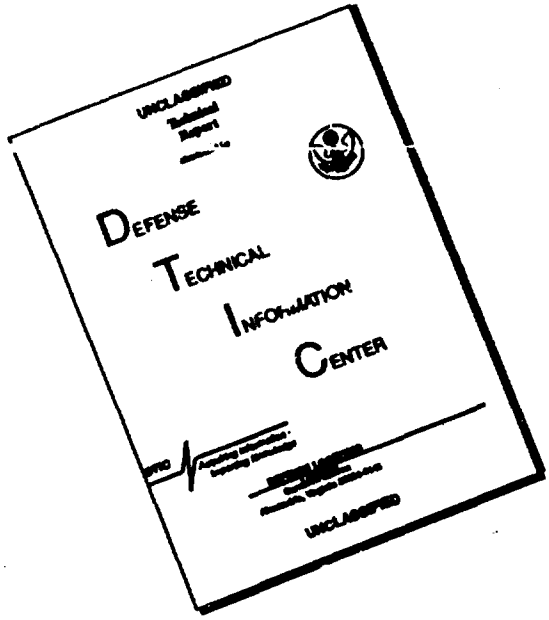

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MEDICAL SCIENCES LABORATORY

Project 1L013001A91A

February 1967

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

A halogen-substituted indolyl substrate for β-D-fucosidase shows strong positive reactions in the interstitial monunuclear cells in the jejunum. This localization and the optimum pH are different from those of the closely analogous substrate for β-D-galactosidase. The results of competitive inhibition of these substrates suggest that β-D-fucosidase and β-D-galactosidase are distinct but similar tissue enzymes.

I. INTRODUCTION

Halogen-substituted indolyl substrates are particularly suitable for the histochemical detection and demonstration of enzymes because the final reaction product is finely granular, distinctively colored, insoluble, and stable. The colorless synthetic substrate is hydrolyzed by the tissue enzyme and forms a substituted indigo. Since this colored reaction product is insoluble, the frozen section can subsequently be dehydrated and preserved in the usual manner. Studies with substrates for mammalian glucosidase, deoxyglucosidase, galactosidase, and glucuronidase have been reported and, in each, enzymatic specificity for the corresponding glycosides was demonstrated.[1-3] The specificity of a substrate for β-D-fucosidase is important because of the similarity in structure between galactose and fucose (6-deoxygalactose).

II. MATERIALS AND METHODS

The substrate solution was prepared by dissolving 8 mg of crystalline 5-bromo-4-chloroindole-3-yl-β-D-fucopyranoside* in 1.0 cc of dimethyl formamide. This was added to 0.5 cc of 0.015 M NaCl and 8 mg spermidine trihydrochloride in 30 cc of 0.1 M buffered acetate. Ferric cyanide was not used. Frozen sections were incubated at room temperature for 1 to 6 hours. The best results were obtained at pH 6.2 after 3 hours. The addition of catalytic quantities of $MgCl_2$ and copper glycinate had no potentiating effect; the reaction occurred satisfactorily in the presence of citrate or phosphate buffers. The incubating solution was always used promptly after preparation, but the dissolved substrate was apparently stable and good reactions were obtained with delays as long as 2 weeks. Prior fixation of sections in dilute cold formalin or glutaraldehyde for up to 1 hour neither inhibited nor enhanced the reaction. After incubation the slides were rinsed in distilled water and mounted in glycerogel or fixed in formalin and cleared in alcohol and xylene. On occasion, sections were counterstained before dehydration.

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* Synthesized by Dr. Herman Plaut and staff at Cyclo Chemical Incorporated, 1922 E. 64th Street, Los Angeles, California 90001.
The elemental analysis of the compound was:

	Expected	Found
Carbon	42.8	42.86
Hydrogen	4.08	3.85
Nitrogen	3.56	3.82
Bromine	20.4	20.59
Chlorine	9.3	8.78

Specimens for frozen sections were prepared from freshly killed Sprague-Dawley rats. Additional material from New Zealand rabbits, Swiss-Webster mice, and guinea pigs was also examined.

III. RESULTS

A. SUBSTRATE REACTION

A strong substrate reaction was found only in interstitial mononuclear cells in the lamina propria of the jejunum (Fig. 1 and 2). Positive staining cells were also present in the duodenum and cecum, but they were not so numerous as in the jejunum. None was identified in the esophagus, stomach, ileum, or colon. Faint reactions were detected in occasional sections of epididymis, but in a survey of rat and rabbit tissues no other organs with reactive cells were found.

The interstitial cell staining could be detected in the pH range of 4.8 to 7.4, but was strongest between 6.0 and 6.6. Light staining of epithelial cytoplasm was also present at pH 6.6. In mouse and guinea pig jejunum, the interstitial cell reaction was similar, but epithelial cytoplasmic staining was more prominent.

B. COMPARISON OF β-D-FUCOSIDASE AND β-D-GALACTOSIDASE

In tissues, 5-bromo-4-chloroindole-3-yl-β-D-fucopyranoside is hydrolyzed to form 5,5'-bromo, 4,4' chlorobisindigo (Fig. 3). The reaction is identical with those of the substituted indolyl glycoside substrates described previously. The substrates for fucosidase and galactosidase (5-bromo,4-chloroindole-3-yl-β-D-galactopyranoside) have identical β linkages and pyranoside rings and differ only at the sixth carbon of the glycon (Fig. 4).

The optimum pH for the β-D-galactosidase reaction was 5.4. The β-D-fucosidase is weakly detected at this pH, but is best demonstrated above pH 6.0. The major difference between the substrates, however, was the distribution of reactive cells in mammalian tissue (Table 1). Numerous organs contained galactosidase-positive cells, but in none was the reaction comparable in intensity with that seen with the indolyl fucoside. Comparable sections are illustrated in Figure 5.

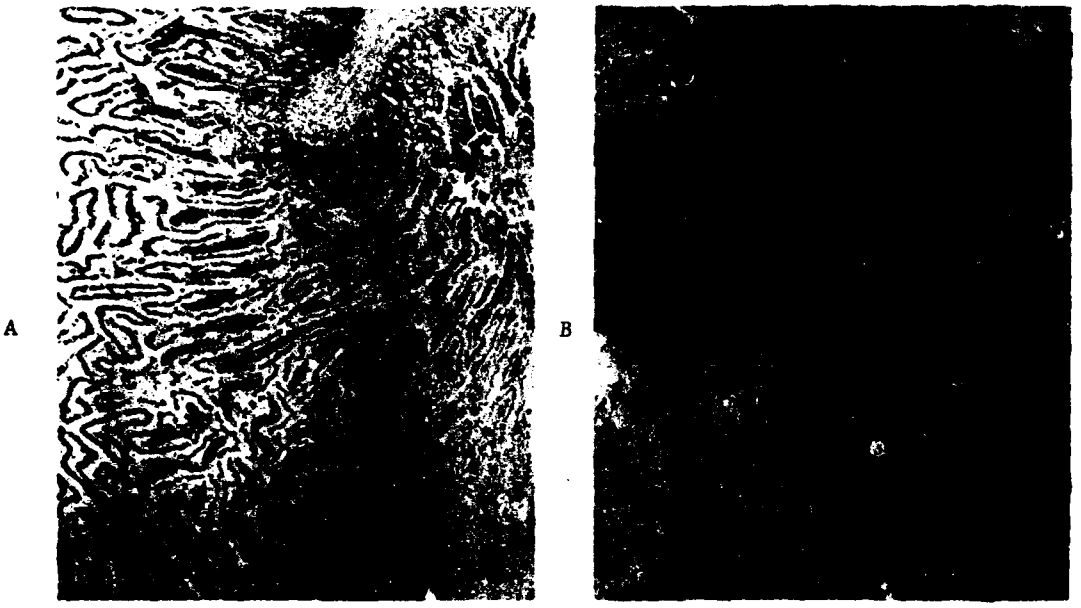


Figure 1. Prominent Staining of the Interstitial Mononuclear Cells of Fresh-Frozen Section of Rat Jejunum Incubated with Halogen-Substituted Indolyl-Fucopyranoside. Dilute eosin was used as a counterstain before dehydration and clearing. A. 50X, B. 195X.

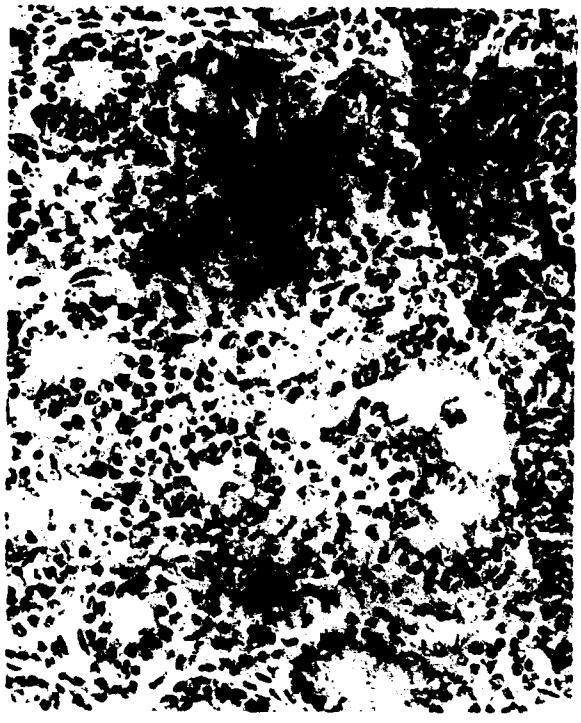


Figure 2. Photomicrograph Illustrating Quality of Intense Staining with Indolyl Substrates. Cryostat section of rat jejunum; hemotoxylin and eosin counterstain. 350X.

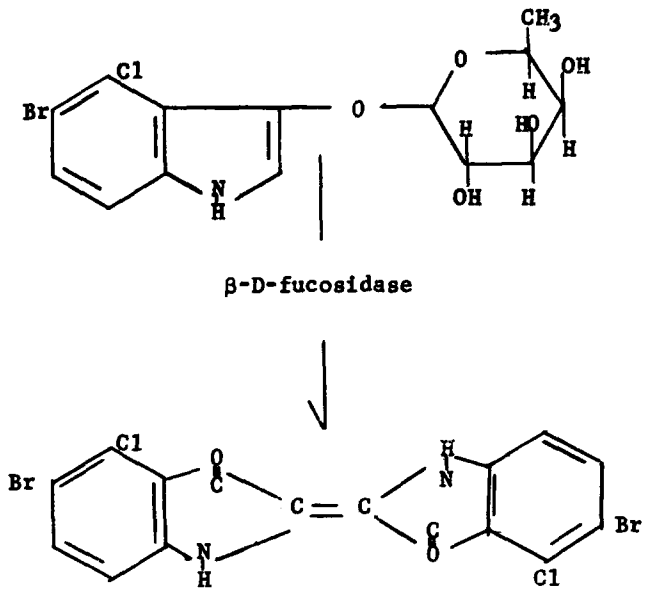
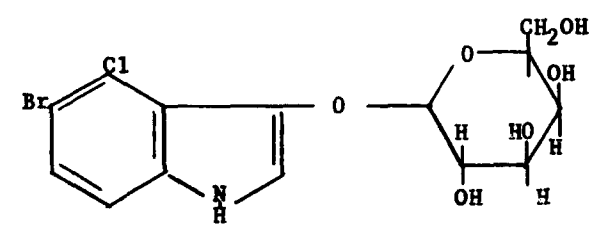
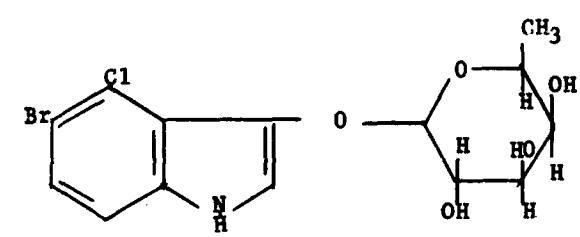


Figure 3. Principle of Reaction for β-D-Fucosidase.



β-D-galactoside



β-D- fucoside

Figure 4. Structural Formulas of Substrates for β-D-Fucosidase and β-D-Galactosidase. The single difference is at the sixth carbon of the glycon moiety.

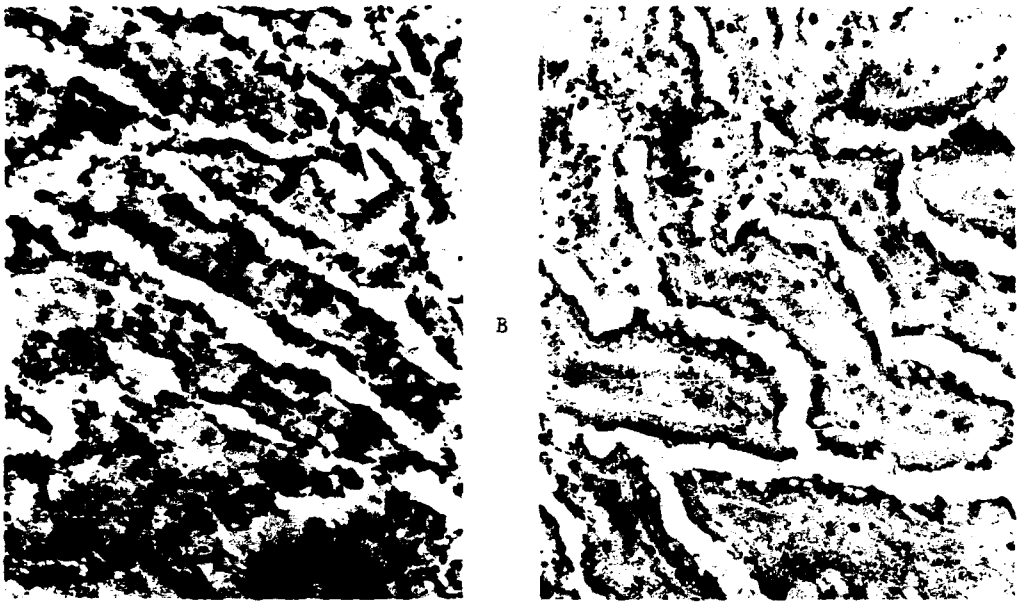


Figure 5. Adjacent Cryostat Sections of Rat Jejunum Incubated with Indolyl Substrates for β-D-Fucosidase (A) and β-D-Galactosidase (B). 190X.

TABLE 1. DISTRIBUTION OF REACTIVE CELLS IN MAMMALIAN TISSUE

Galactosidase	Fucosidase
Ileum	
Jejunum	Jejunum
Cecum	
Colon	
Adrenal	
Liver	
Kidney	
Spleen	
Lymph nodes, etc.	

Distinction between these substrates was demonstrated by competitive inhibition. Tissue sections were incubated for both β-D-fucosidase and β-D-galactosidase reactions. Analogue compounds of the substrate glycon were added to the substrate solutions. The data listed in Table 2 show that some overlapping occurs between β-D-fucosidase and β-D-galactosidase inhibition but that fucose and fuconolactone inhibit galactosidase activity less than fucosidase activity. In contrast, galactose and galactonolactone show a pronounced effect on the galactosidase reaction.

TABLE 2. ENZYME INHIBITORS FOR FUCOSIDASE AND GALACTOSIDASE

Inhibitor	Fucosidase	Galactosidase
Galactonolactone, 0.1%	+	0
Galactose, 1%	+	Trace
Fuconolactone, 0.1%	Trace	+
Fucose, 1%	Trace	+

IV. DISCUSSION

Limitations common in histochemical reactions have frequently restricted the usefulness of techniques for enzyme detection. Diffusion both of the enzyme and of reaction intermediates and products may be retarded by prior fixation and incubation at low temperatures. Nevertheless, rapid examination is necessary if the color indicator is either soluble or unstable. The suitability of halogen-substituted indolyl substrates lies in an intensely colored end product that requires no further coupling. It is insoluble, binds to protein, and is stable over many years. Furthermore, synthetic modification of glycon moiety has made it possible to demonstrate the corresponding specific glycosidase.

Both the dextro- and levo-rotary forms of fucose are widely distributed in nature. The immunologic significance of this glycoside has been suggested by Springer and Williamson.[4] L-fucose has been found in mucins and blood-group antigens.[5] β-D-fucosidases were characterized by Levvy and McAllan. They used p-nitrophenyl-β-D-fucoside to demonstrate activity in homogenates of rat epididymus, ox liver, and the visceral hump of the limpet, Patella vulgata.[6] With the use of fuconolactone and galactonolactone, these investigators found a greater difference between tissue sources than between β-D-fucosidase and β-D-galactosidase activities in the same specimen.[7]

The present study is the first attempt to demonstrate β-D-fucosidase activity in tissue sections. Interstitial mononuclear cells in the lamina propria gave strong reactions in the rat, rabbit, mouse, and guinea pig. The epithelial mucosa also stained at a somewhat higher pH. Repeated attempts under varied conditions failed to demonstrate activity in the rat liver, and only on occasion was a trace reaction found in the epididymis.

Our evidence for distinguishing between β-D-fucosidase and β-D-galactosidase activities is based on differences in distribution, optimal pH, and the effect of analogue inhibitors. Galactosidase activity is widespread in mammalian tissues, but fucosidase was consistently present only in the jejunum. The indolyl fucoside reacts strongly over a wider and higher pH range than the similar substrate for galactosidase. Estimates by Holt[8] of enzyme activity at different pH values, using the indigo system, have been criticized because indolyls may form both indigo and dehydroindigos (colorless), the proportion of the latter increasing at alkaline pH values. Holt's studies were done in the presence of ferric cyanide, an oxidant not used in our study; furthermore, the comparative data show an increased rather than decreased dye accumulation at the higher pH. It seems reasonable, therefore, to assume that the differences in reactions reflect a difference in enzymatic activity.

Finally, the data on inhibition summarized in Table 2 show a distinction between these glycosidases. Overlapping does occur, but there are separable differences in the inhibitory effect of fucose- and galactose-like analogues.

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Unclassified
Security Classification

DOCUMENT CONTROL DATA - R&D

(Security classification of title, body of abstract and indexing annotation must be entered when the overall report is classified)

1. ORIGINATING ACTIVITY *(Corporate author)*
Department of the Army
Fort Detrick, Frederick, Maryland, 21701

2a. REPORT SECURITY CLASSIFICATION
Unclassified

2b. GROUP

3. REPORT TITLE
THE HISTOCHEMICAL DEMONSTRATION OF INTESTINAL β-D-FUCOSIDASE WITH 5-BROMO-4-CHLOROINDOLE-3-YL-β-D-FUCOPYRANOSIDE

4. DESCRIPTIVE NOTES *(Type of report and inclusive dates)*

5. AUTHOR(S) *(Last name, first name, initial)*
Esterly, John R.
Standen, Alfred C.
Pearson, Bjarne (NMI)

6. REPORT DATE	7a. TOTAL NO. OF PAGES	7b. NO. OF REFS
February 1967	14	8

8a. CONTRACT OR GRANT NO.	9a. ORIGINATOR'S REPORT NUMBER(S)
b. PROJECT NO. 1L013001A91A	Technical Manuscript 365
c.	9b. OTHER REPORT NO(S) *(Any other numbers that may be assigned this report)*
d.	

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11. SUPPLEMENTARY NOTES	12. SPONSORING MILITARY ACTIVITY
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14. Key Words
*Histochemistry
*Fucosidase
Galactosidase
Detection
Tissues
Jejunum

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Unclassified
Security Classification