THE COLORIMETRIC ESTIMATION AND HISTOCHEMICAL DEMONSTRATION OF $\beta$-D-GALACTOSIDASE*

BY RICHARD B. COHEN,† KWAN-CHUNG TSOU,‡ SELMA H. RUTENBURG,§ AND ARNOLD M. SELIGMAN

(From the Yamin Laboratory for Surgical Research, Beth Israel Hospital, the Department of Surgery, Harvard Medical School, Boston, and the Chemical Laboratories of Harvard University, Cambridge, Massachusetts)

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$\beta$-Galactosidase hydrolyzes $\beta$-d-galactosides, $\alpha$-L-arabinosides, lactose, and heptosides with a $\beta$-d-galactose configuration (1). It occurs with $\beta$-glucosidase in certain plants, bacteria, molds, and in mammalian tissues. It occurs without $\beta$-glucosidase in seeds of alfalfa and coffee, soy beans, and different strains of bacteria ($Lactobacillus delbrueckii$, $Escherichia coli$) (1). Enzymatic activity has been assayed by either polarimetric or reductimetric methods (1). Since it was shown that the specificity of the carbohydrases resides in the sugar moiety rather than the alcoholic moiety of the substrate (1), and that aryl glycosides are hydrolyzed more readily than alkyl glycosides (1), it was possible to develop a colorimetric method for demonstrating $\beta$-galactosidase activity by the synthesis of 6-bromo-2-naphthyl-$\beta$-d-galactopyranoside. The bromonaphthyl analogue was selected because it was found to be hydrolyzed enzymatically 10 times as fast as the simpler 2-naphthyl-$\beta$-d-galactopyranoside. For colorimetric estimations, the azo dyes produced from $\beta$-naphthols are more easily extracted with organic solvents from the proteins of tissue than are the azo dyes produced from $\alpha$-naphthols. Furthermore, it was hoped that 6-bromo-2-naphthol would be sufficiently insoluble in aqueous media to remain at the site of enzymatic hydrolysis in frozen sections of tissue at pH 5 for the long period of incubation which would be required in the

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† Research Fellow of the National Cancer Institute.
‡ Research Fellow in Chemistry, Harvard University.
§ Research Fellow in Surgery, Harvard Medical School.

histochemical demonstration of the enzyme. If this requirement were met satisfactorily, coupling with an appropriate diazonium compound at higher pH would result in formation of an insoluble azo dye. Such a method would differ from the histochemical methods developed previously for the phosphatases (2–4), esterase (5), and serum cholinesterase (6) because of dependence upon the insolubility of the naphtholic hydrolytic product (7).

The colorimetric method is similar in principle to the methods previously described for esterase and lipase (8, 9), the phosphatases (10), carboxypeptidase (11), and serum cholinesterase (6). In the quantitative estimation of enzymatic activity, 2 molecules of the hydrolysis product, 6-bromo-2-naphthol, are coupled with tetrazotized diorthoanisidine to form an azo dye which is extracted into chloroform and the color density is measured with a photoelectric colorimeter (Klett).

The present report includes the synthesis of the substrate, the method of colorimetric estimation of β-D-galactosidase activity in tissue homogenates, the distribution of the enzyme in the organs of a number of mammalian species, and a histochemical procedure with a discussion of its limitations.

### Preparation of Substrate

6-Bromo-β-naphthyl-β-D-tetraacetylgalactopyranoside (12) was prepared from D-galactose (100 gm.), acetic anhydride (540 cc.), and anhydrous sodium acetate (44 gm.) by heating and stirring at 100° for 20 minutes. The mixture was cooled and poured into 1600 cc. of ice water and stirred for 1 hour. The product was extracted with benzene, washed, dried, and evaporated to a syrup from which the pentacetate crystallized. It was recrystallized from 95 per cent alcohol, m.p. 142–143° (corrected); 55 per cent yield.

According to the modified Helferich procedure (13, 14), the pentacetate (5.0 gm., 0.013 mole), 6-bromo-2-naphthol (15) (8.0 gm., 0.036 mole), and 20 mg. of p-toluenesulfonic acid were fused in vacuo for 30 minutes at 100°. The melt was taken up in benzene and the solution was washed with water, cold 2 per cent sodium hydroxide, again with water, and dried over anhydrous calcium sulfate (Drierite). After removing the solvent under reduced pressure a syrup was obtained (6.3 gm.), which was dissolved in 75 per cent methanol (25 cc.). An amorphous solid separated on standing in the cold. It was separated by decantation and dried in a vacuum desiccator over phosphorus pentoxide; yield 5.0 gm. (66 per cent);

2 Microanalyses by Mrs. Shirley Golden. The substrate may be purchased from Dajac Laboratories, Monomer-Polymer, Inc., 511 Lancaster Street, Leominster, Massachusetts.
(Y):~ = -35.1° (C, 2.15 in chloroform). Attempts to crystallize this solid were unsuccessful. It sintered at 68-70° (uncorrected).

C₆H₅OBr. Calculated, C 52.09, H 4.55; found, C 52.22, H 4.78

Infra-red absorption spectra showed an ester band at 5.72 µ, and the characteristic 6.10 and 6.22 µ bands of the 6-bromo-2-naphthyl group.¹

6-Bromo-2-naphthyl-β-D-galactopyranoside—Deacetylation was accomplished by Zemplen’s catalytic method (16). The pentacetate (3.0 gm.) was dissolved in 30 cc. of methanol and a piece of sodium 2 to 3 mm. in diameter was added to the solution. It was allowed to stand at 4° overnight. The product (2.0 gm.) was collected and recrystallized from methanol as a white powder; yield 1.8 gm. (85 per cent). It was found to sinter at 198° and melt at 205°; [α]°D = -51.8° (C, 3.0 in pyridine). Its solubility in water was less than 0.1 mg. per cc.

C₁₆H₁₁₀₆Br. Calculated, C 49.88, H 4.55; found, C 49.60, H 4.39

Method

Reagents—

1. Substrate.² A solution (100 cc.) of 6-bromo-2-naphthyl-β-D-galactopyranoside is made by dissolving 15 mg. in 10 cc. of absolute methyl alcohol and 15 cc. of distilled water at the boiling point, followed by the addition of 55 cc. of distilled water and 20 cc. of phosphate-citrate buffer, pH 4.95. No spontaneous hydrolysis occurs at room temperature after 2 weeks. The solution is never chilled and is used within a few hours because of precipitation of the poorly soluble substrate.

2. Phosphate-citrate buffer (pH 4.95) is prepared by mixing 500 cc. of 0.1 M citric acid and 500 cc. of 0.2 M disodium phosphate. Toluene (20 cc.) is added as a preservative.

3. Tetrazotized diorthoanisidine.³ The powder (1 mg. per cc.) is dissolved in cold water immediately before use. In solution, the diazonium compound decomposes extensively on standing at room temperature for 20 to 30 minutes.

4. Trisodium phosphate (0.2 M).

5. Trichloroacetic acid solution (80 per cent).

6. Anhydrous chloroform.

Procedure

Tissues, obtained from freshly killed animals and from humans as soon after death as possible, were homogenized in distilled water at a concen-

³ Available in powder form containing 20 per cent tetrazotized diorthoanisidine, 5 per cent zinc chloride, and 20 per cent aluminum sulfate. Trade name, du Pont naphthanil diazo blue B, E. I. du Pont de Nemours and Company, Inc.
tration of 5 mg. per cc. with a motor-driven ground glass homogenizer for 2 minutes and centrifuged for 2 minutes at 2500 r.p.m. The supernatant solution was used for estimation of enzymatic activity. No loss of activity was observed after standing at 4° for several days.

The homogenate supernatant (0.6 cc.) and 5 cc. of buffered substrate solution were incubated at 37° for 2 hours. Duplicate determinations checked within 10 per cent. The control consisted of a single tube containing the same reagents and homogenate which had been heated to 100° for a few minutes. At the termination of the period of incubation, 0.5 cc. of 0.2 M trisodium phosphate was added to each tube in order to raise the pH to 7.5 to 7.8, which is optimum for coupling. A cold solution of tetrazotized diorthoanisidine (1 cc.) was added to each tube and mixed thoroughly. The reaction was permitted to proceed for about 1 minute. 80 per cent trichloroacetic acid (2 cc.) was added to each tube in order to precipitate protein and favor release of dye from the protein complex. Chloroform (10 cc.) was added from a burette and the tubes were shaken vigorously. The tubes were allowed to stand until the chloroform had separated. A pipette was introduced to the bottom of each tube, while a positive pressure was maintained in the pipette by blowing gently. Chloroform was then aspirated into the pipette and 5 cc. were transferred to a Klett tube. In order to clarify the solution, the tube was centrifuged at 2500 r.p.m. for 5 minutes. The color density was measured with a photoelectric colorimeter (Klett) through a green filter.

**Fig. 1.** Calibration curve. 6-Bromo-2-naphthol plotted against color density (Klett photoelectric colorimeter).
The readings were converted to micrograms of 6-bromo-2-naphthol with a calibration curve prepared from 10 to 80 \( \gamma \) of 6-bromo-2-naphthol (Fig. 1). The blue azo dye was bluish purple in chloroform solution and did not fade on standing, whereas in ethyl acetate the color was red and faded badly in 10 to 20 minutes. This azo dye sticks to protein more tenaciously than the azo dye formed with \( \beta \)-naphthol (8–11), but the little protein contained in 0.6 cc. of the supernatant of homogenate offered no special problem.

**Results**

*Enzyme Kinetics*

With homogenates of rat kidney as a source of \( \beta \)-n-galactosidase, the temperature of optimum activity was 37° and the optimum pH was 5, with considerable activity between pH 4 and 6 and very little activity above pH 7 (Fig. 2).

At pH 5 and 37°, the hydrolysis of the substrate proceeded according to zero order kinetics for the first 3 to 4 hours and then leveled off rather suddenly (Fig. 3). That this was not due to thermal inactivation was shown in experiments in which homogenates were stored at 37° for 6 hours. These preparations behaved exactly like homogenates stored for a similar period at 4°.

Hydrolysis of the substrate as a function of the concentration of the enzyme was determined over the range of 0.1 to 1.4 cc. of the supernatant of homogenate of rat kidney (Fig. 4). In the range of concentration from 0.1 to 0.8 cc. there was no change in the specific activity of the whole extract. Because the azo dye adheres to protein and because the amount of protein was increased with increasing volumes of extract, it was important to determine the efficiency with which the dye was extracted over this range of protein concentration. This was accomplished by adding a fixed quantity of 6-bromo-2-naphthol to each tube of homogenate over the same range of concentration (0.1 to 1.4 cc.) as above. Several concentrations of 6-bromo-2-naphthol were used in separate experiments covering the range of 0.01 to 0.08 mg. Over a 10-fold difference in protein concentration, only 1 to 2 per cent less dye was extracted when higher concentrations of protein were used. The results were no better when more trichloroacetic acid was used. With low concentrations of 6-bromo-2-naphthol the amount of dye extracted was independent of protein concentration within these limits. It was, therefore, concluded that error from this cause was insignificant.

It was shown that the decline in reaction rate after 3 to 4 hours (Fig. 3), and in specific activity of the homogenates above 0.8 cc. (Fig. 4), was due to inhibition from accumulation of 6-bromo-2-naphthol. Addition of 0.02
mg. per cc. of 6-bromo-2-naphthol inhibited enzymatic hydrolysis 50 to 60 per cent when 0.6 cc. of the supernatant of homogenate was used. This corresponds closely to the quantity of bromonaphthol formed at the break

\[ \text{COLOR DENSITY} \]

\[ \text{PH} \]

\[ \text{COLOR DENSITY} \]

\[ \text{HOURS} \]

\[ \text{COLOR DENSITY} \]

\[ \text{HOURS} \]

**Fig. 2.** \( \beta \)-d-Galactosidase activity of rat kidney at various pH values. Period of incubation, 2 hours.

**Fig. 3.** Rate of hydrolysis by \( \beta \)-d-galactosidase of rat kidney in the curves (0.1 mg. in 5 cc.). No inhibition was observed with \( \alpha \)-galactose in equivalent molar concentration. The addition of more methyl alcohol than was used in preparing the substrate solution produced extensive inhibition of enzymatic activity. Iron, zinc, and mercury salts inhibited the enzyme, and magnesium produced slight activation. Diazonium salts also inhibited this enzyme.
Prior fixation of rat kidney for 24 hours at 4° inhibited enzymatic activity as follows: acetone 0 to 15 per cent, absolute methanol or ethanol 90 per cent, propylene glycol dimethyl ether 100 per cent, dioxane 100 per cent, and 10 per cent neutral formalin 75 per cent. The inhibition by formalin fixation was similar to the inhibition of alkaline phosphatase of rat liver, and greater than the inhibition of four other enzymes (17).

Distribution of Enzyme in Tissues

The specificity of the substrate is inherent in its structure. Since most sources of β-D-galactosidase also contain β-D-glucosidase, experiments with mammalian tissues do not contribute evidence on the question of whether or not these are separate enzymes (1). This substrate, as well as 6-bromo-2-naphthyl-β-D-glucopyranoside, was readily hydrolyzed by emulsin prepared from sweet almonds, and both substrates were hydrolyzed by the tissues reported in Table I to have galactosidase activity. With few exceptions, however, the glucoside was hydrolyzed less readily than the galactoside.

The distribution and activity of β-D-galactosidase, in micrograms of 6-bromo-2-naphthol produced per hour at 37° per 0.6 cc. of homogenate supernatant, are given for the organs of five species in Table I. The human tissues were incubated for 5 hours; the others for 2 hours. Each organ represented three or more specimens of each species. An average figure is given in Table I unless the range was wide, in which case the two

extremes are noted. Whenever a zero reading was obtained with a single specimen, it is included.

In the rat, the kidney was particularly active and the adrenal and thyroid glands possessed nearly the same high enzymatic activity. The activity of pancreas was low, whereas the small and large intestine showed moderate activity. In the guinea pig, on the other hand, there was very low activity in the thyroid, adrenal gland, and kidney, but uniformly high activity in the pancreas. The uteri of rats and mice showed moderate activity. Compared to other organs of the mouse, the activity of uterus was particularly great. The salivary glands of the dog contained moderate enzymatic activity, but the pancreas was low. The presence of the enzyme in organs unrelated to the digestive tract, such as adrenal, thyroid, uterus, and spleen, raises the question of what function the enzyme serves. Equally perplexing is the striking difference in distribution of the enzyme in various species. The enzyme was found in variable quantity in 1 cc. each of urine, saliva, and duodenal fluid. It was not

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</table>
infrequently entirely absent from these fluids. No activity was noted in several samples of human serum.

**Histochemical Demonstration of β-D-Galactosidase**

Several factors combined to make the development of a histochemical means of demonstrating this enzyme particularly difficult. Enzymatic activity was so low, even in the most active organs (rat kidney and guinea pig pancreas), as to require long incubation with the substrate (hours). Diazonium salts inhibited enzymatic activity, and coupling to an azo dye could be performed only after enzymatic hydrolysis was complete. It was, therefore, necessary to prepare a substrate which would yield a naphthol sufficiently insoluble to remain at the site of enzymatic activity until coupling to a very insoluble azo dye could be accomplished. The naphthol could not be too insoluble, however, since the galactose moiety conferred little water solubility on the substrate. Although acetone as a fixative was not very destructive of enzymatic activity (15 per cent), frozen sections prepared from acetone-fixed tissue behaved poorly on long incubation in aqueous media. Frozen sections of formalin-fixed tissue (2 to 24 hours at 4° in 10 per cent neutral formalin) survived prolonged incubation, but only 25 per cent of the enzymatic activity remained. This necessitated a 4-fold increase in the period of incubation with substrate. Fresh frozen sections disintegrated on incubation in aqueous media at pH 5 for many hours, unless the sections were first mounted on microscope slides. The enzyme was readily diffusible from the sections during the period of incubation, unless the substrate medium was made hypertonic with 1M sodium chloride. In order to preserve histologic structure of sections of fresh tissue, it was necessary to bring the sections to this tonicity by brief immersion in solutions of sodium chloride containing progressively increasing concentration of the salt. Although we found that enzymatic activity of fresh tissue (not of fixed tissue) could be enhanced with 1,4-naphthoquinone or 2-methyl-1,4-naphthoquinone (10 γ per cc.) or with sodium bisulfite (0.27 mg. per cc.), the increase in enzymatic activity was not great enough to shorten the incubation period significantly (2 hours to 1 hour).

**Histochemical Procedure**

Frozen sections, 10 μ thick, were prepared according to Coons' modification of the Linderstrøm-Lang technique (18). The sections were affixed to microscope slides previously coated with egg albumin (gelatin blocked the reaction). The slides were placed for 2 minutes in each of six solutions of sodium chloride, increasing in concentration by 1 per cent from 1 to 6 per cent. They were then incubated in the buffered substrate solution

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(described above), to which had been added 5.8 gm. of NaCl per 100 cc. (1 M), for 2 hours at 37°. The sections were washed for 1 minute in 1 per cent sodium chloride and transferred to a cold coupling solution prepared with 20 cc. of 0.1 M phosphate buffer (pH 7.5), 80 cc. of cold water, and 100 mg. of tetrazotized diorthoanisidine. Maximum color developed in 1 to 2 minutes. The sections were washed with water and mounted in glycerogel or glycerol.

All the organs of the rat listed in Table I were examined. Only the organs with significant activity by homogenate assay showed enzymatic activity histochemically. The stain was confined to the cytoplasm of epithelial cells. Cell nuclei, skeletal muscle, smooth muscle, and connective tissue were unstained.

6-Bromo-2-naphthol is a stronger acid than β-naphthol and has greater affinity for protein. It is also more soluble in lipide than β-naphthol and has particular affinity for myelin. Incubation of sections in a saturated solution of 6-bromo-2-naphthol for 2 hours, followed by washing and coupling, resulted in diffuse staining of all structures, although staining was a little darker in acidophilic structures, suggestive of the distribution of eosin. However, it was shown that diffusion of bromonaphthol or of the enzyme from one section to another under the conditions recommended in the procedure did not occur. This was demonstrated in experiments in which heat-inactivated sections of kidney or sections of tissues which contained traces or no enzyme, such as brain, were incubated in the same flask with active sections, such as rat kidney. Increased staining due to adjacent kidney did not occur. The affinity of bromonaphthol for protein in the region of enzymatic activity probably helped localize the dye to those areas. Diffusion was demonstrated when the incubation medium did not contain 1 M sodium chloride. The supernatant in the latter experiment contained considerable bromonaphthol, in contrast to the former. Diffusion of enzyme from the sections presumably was responsible for the high content of bromonaphthol in the supernatant in the case of experiments without sodium chloride. Detailed studies of tissue sections stained by this method will be published elsewhere.

SUMMARY

6-Bromo-2-naphthyl-β-D-galactopyranoside was synthesized and was readily hydrolyzed by a carbohydrase in mammalian tissue. A colorimetric method for the determination of β-D-galactosidase with this substrate has been described. Some of the enzyme kinetics are given and a survey of enzymatic activity of the organs of five species, including man, shows considerable species variation in distribution. The enzymatic activity was high in kidney, adrenal, thyroid, spleen, and pancreas of different species. The intestinal tract showed variable activity.
The difficulties involved in the development of a histochemical method for this enzyme are presented, together with a brief description of a histochemical procedure and its limitations. Enzymatic activity, when present, was situated in the cytoplasm of epithelial cells and was absent from nuclei, connective tissue, and muscle.

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