THE COLORIMETRIC ESTIMATION OF β-D-GLUCOSIDASE*

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In a previous paper (1) a method for the synthesis of 6-bromo-2-naphthyl-β-D-galactopyranoside and a colorimetric technique for the determination of β-D-galactosidase were described. In this communication the synthesis of 6-bromo-2-naphthyl-β-D-glucopyranoside and its use for the colorimetric determination of the dynamics and distribution in mammalian tissue of β-D-glucosidase are described. This enzyme cleaves the β-D-glucosidic linkage of simple alkyl and aryl glucosides (2). It has been demonstrated by polarimetric or reductimetric methods in sweet almond emulsin, plants belonging to the Rosaceae, in the kernels of a number of higher plants, in yeasts and several molds, in sulfatase bacteria, in the kidney and liver of horses and cattle, and in the small intestine of the pig (2).

![6-Bromo-2-naphthyl-β-D-glucopyranoside](image)

In selecting a suitable chromogenic substrate for β-D-glucosidase, it was observed that 6-bromo-2-naphthyl glucoside was hydrolyzed enzymatically 5 to 10 times faster than the non-bromo analogue. The bromo group facilitated enzymatic hydrolysis similarly in the substrate for β-D-galactosidase (1). An attempt to develop a histochemical method for β-D-glucosidase was not as successful as for β-D-galactosidase for reasons given below.

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**Preparation of Substrate**

6-Bromo-2-naphthyl-β-D-tetraacetyl Glucopyranoside—By the modified Helferich's procedure (3, 4), β-D-glucopyranose pentacetate (5) (10 gm., 0.026 mole), 6-bromo-2-naphthol (6) (16 gm., 0.072 mole), and 50 mg. of p-toluene sulfonic acid were fused *in vacuo* for 30 minutes at 100°. The melt was taken up in 100 cc. of benzene and washed with ice-cold water, 2 per cent sodium hydroxide, again with water, and dried over anhydrous calcium sulfate (Drierite). The filtered, dried solution was evaporated under reduced pressure to give 12 gm. of crude product which was re-crystallized from methanol to give 10.5 gm. (75 per cent) of the purified product, m.p. 142–143°.

C_{25}H_{25}O_{10}Br. Calculated, C 52.09, H 4.55, Br 14.78; found, C 52.26, H 4.45, Br 14.68

6-Bromo-2-naphthyl-β-D-glucopyranoside—Deacetylation was accomplished by the catalytic method of Zemplen and Kung (7). The tetracetate (3.0 gm.) was suspended in anhydrous methanol and a piece of sodium 2 to 3 mm. in diameter was added. It was allowed to stand at 4° for 2 days. The product separated in fine, white needles, m.p. 206–207°; [α]_{D}^{23} = -36° (C 2.2 in pyridine); yield 80 to 85 per cent.

C_{16}H_{15}BrO_{6}. Calculated, C 49.88, H 4.45; found, C 49.68, H 4.44

The product could be crystallized from methanol to contain 1 mole of methanol, m.p. 198–200°. The solvent of crystallization was removed by drying at 100° in a drying pistol over phosphorus pentoxide for 5 hours.

C_{16}H_{17}BrO_{6}+CH_{3}OH. Calculated, C 48.93, H 5.07; found, C 48.59, H 4.82

When an attempt was made to crystallize the product from hot water, a jelly-like mass was formed which yielded a yellow powder when dried in air. Heating with dilute hydrochloric acid gave bromonaphthol, which could be demonstrated by coupling with tetrazotized diorthoanisidine.

**Method**

1. Substrate. A solution (100 cc.) of 6-bromo-2-naphthyl-β-D-glucopyranoside is made by dissolving 10 mg. in 20 cc. of absolute methyl alcohol and 20 cc. of distilled water at the boiling point, followed by addition of 20 cc. of phosphate-citrate buffer, pH 4.95, and 40 cc. of distilled water. No spontaneous hydrolysis occurs at room temperature after several days. The solution is never chilled and is preferably used immedi-

1 Microanalyses by Mrs. Shirley Golden.

2 All the reagents may be obtained from the Dajac Laboratories, Monomer-Polymer, Inc., 511 Lancaster Street, Leominster, Massachusetts.
ately after preparation 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 rapidly 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**

The method differed from that described for the determination of β-D-galactosidase only in an increase of the period of incubation from 2 to 5 hours. The low order of enzymatic activity in most tissues made this increase necessary. Tissues, obtained from freshly killed animals and from humans as soon after death as possible, were homogenized in distilled water at a concentration 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 was used for demonstration 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 5 hours. This was then alkalized with trisodium phosphate (0.5 cc.), coupled with the tetrazotized diorthoanisidine (1 cc.), acidified with trichloroacetic acid (2 cc.), extracted with chloroform (10 cc.), and its color density measured with a photoelectric colorimeter (Klett), as previously described (1). The readings were converted to micrograms of 6-bromo-2-naphthol, by means of the calibration curve in Fig. 1 of the previous paper (1).

**Results**

**Enzyme Kinetics**

With homogenates of rat kidney as a source of β-D-glucosidase the temperature of optimum activity was found to be 37° and the optimum pH was 5, with considerable activity between pH 4 and 6 and very little activity above pH 7 (Fig. 1).

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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.
At pH 5 and 37° the hydrolysis of the substrate proceeded according to zero order kinetics for the first 14 hours. The rate of enzymatic hydrolysis declined thereafter (Fig. 2).

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 supernatant of rat kidney homogenate. In the range of concentration from 0.1 to 1.2 cc. there was no change in the specific activity of the whole extract (Fig. 3). No significant error resulted from the tendency of the azo dye to adhere to protein (1). The decline in reaction rate after 14 hours and in specific activity of the homogenate above 1.2 cc. was due largely to the accumulation of 6-bromo-2-naphthol. Addition of 0.06 mg. of 6-bromo-2-naphthol inhibited enzymatic hydrolysis 50 to 60 per cent when 0.6 cc. of

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**Fig. 1.** β-D-Glucosidase activity of rat kidney at various pH values.

**Fig. 2.** Rate of hydrolysis by β-D-glucosidase of rat kidney.

**Fig. 3.** Specific activity of β-D-glucosidase. A supernatant of a homogenate of rat kidney was used.
homogenate and 5 cc. of substrate were used. That this decline was not
due to thermal inactivation was shown in experiments in which homogen-
ates incubated at 37° for 6 hours behaved exactly like homogenates stored
at 4° for a similar period. No inhibition was noted when d-glucose was
added to the substrate-enzyme mixture in equivalent molar concentrations.

The addition of more methyl alcohol than was used in preparing the
substrate solution produced inhibition of enzymatic activity. Heavy

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\text{Table I }
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\text{\textbf{\textit{\beta-d-Glucosidase Activity of Organs of Five Species}}} 
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Enzymatic activity is given in micrograms of 6-bromo-2-naphthol produced per
hour at 37°. Each organ from three individuals of each species was homogenized
(5 mg. per cc.) and the supernatant (0.6 cc.) of each was used. The period of incuba-
tion was 5 hours.

<table>
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<tr>
<th>Organ</th>
<th>Rat</th>
<th>Guinea pig</th>
<th>Mouse</th>
<th>Dog</th>
<th>Human</th>
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<td>3-6</td>
<td>0-0.6</td>
<td>0-2</td>
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<td>Lymph node</td>
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</table>

metals, including iron, zinc, and mercury, inhibited the enzyme. Diazo-
nium salts also inhibited this enzyme.

Prior fixation of rat kidney for 24 hours at 4° inhibited enzymatic ac-
tivity as follows: acetone 90 to 100 per cent, absolute methanol or ethanol
100 per cent, propylene glycol dimethyl ether 100 per cent, and 10 per
cent neutral formalin 80 per cent.

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\text{Distribution of Enzyme in Tissues} 
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The distribution and activity of \(\beta\)-d-glucosidase, in micrograms of 6-
bromo-2-naphthol produced per hour at 37° with 0.6 cc. of homogenate
supernatant, are given for the organs of five species in Table I. Each organ from three individuals of each species was used. An average figure is given, unless the range was wide, in which case the two extremes are noted. When a zero reading was obtained with a single specimen, it is included in Table I. All tissues were incubated for 5 hours. With few exceptions the glucoside was hydrolyzed less readily than the galactoside (1).

Kidney, thyroid, and small intestine were most active in the rat. The pancreas showed consistently low activity. The pancreas of the guinea pig, on the other hand, had the greatest activity of any mammalian tissue so far examined. Pancreas of a 6 week-old pig also had high glucosidase activity (30 \( \gamma \) per hour). The liver of the guinea pig was comparatively active. The tissues of mice, except for liver and small intestine, were consistently low in enzymatic activity. Dog and human tissues showed low enzymatic activity. Kidney of one human subject had high activity (10 \( \gamma \) per hour). A number of samples of human urine, saliva, and duodenal washings were moderately active, whereas the sera from several subjects were devoid of enzymatic activity.

**DISCUSSION**

As in the case of the galactoside (1), the specificity of the substrate is inherent in its structure. It was hydrolyzed readily by emulsin prepared from sweet almonds (8).

A controversy exists as to whether \( \beta \)-galactosidase and \( \beta \)-glucosidase are the same or distinct enzymes (2). The fact that different methods of inactivation and purification of the two enzymes from almond emulsin have produced no variation in the rate of enzymatic hydrolysis has led to the assertion that they are one and the same enzyme (2). On the other hand, the finding that there are sources which contain one enzyme and not the other has been taken as evidence that they are not the same (2). It has been suggested that the \( \beta \)-glycosidases are a class of related enzymes, each with an absolute specificity for one glycoside, but all possessing some relative activity for other members of its class (2). The work concerning the specificity of these enzymes has been done largely with preparations extracted from the higher plants. In the present study, the evidence suggests that the \( \beta \)-d-galactosidase and \( \beta \)-d-glucosidase of mammalian tissue are actually distinct enzymes. Thus, acetone fixation of rat kidney tissue for 24 hours at 4° completely inhibits the \( \beta \)-d-glucosidase, while \( \beta \)-d-galactosidase loses at most not more than 15 per cent of its activity. The activity of glucosidase is inhibited by salts in concentrations which do not affect the activity of galactosidase. In general the activity of \( \beta \)-d-glucosidase in mammalian tissue is considerably lower than that of \( \beta \)-d-galactosi-
dase. Finally, in two instances, a human kidney and a sample of urine from a normal child, β-D-glucosidase activity was relatively high, while β-D-galactosidase activity was absent.

**Histochemical Demonstration of β-D-Glucosidase**

In the development of a histochemical method for β-D-glucosidase, the difficulties encountered were similar to those for β-D-galactosidase. Additional difficulty was imposed by the low order of activity of this enzyme in mammalian tissue and by the ease of its diffusibility. The latter could not be prevented by the use of salts, because enzymatic activity is inhibited by sodium chloride (0.5 N), sodium sulfate (0.5 N), and ammonium sulfate (0.5 N). It is activated by 1,4-naphthoquinone and 2-methyl-1,4-naphthoquinone (10 γ per cc.) and by sodium bisulfate (0.27 mg. per cc.), but only enough to shorten the period of incubation by half. An entirely satisfactory method for its histochemical demonstration was not achieved.

In fresh, frozen sections of the pancreas of the guinea pig, enzymatic activity is sufficiently high to demonstrate its presence histochemically in the cytoplasm of acinar cells after 2 hours of incubation. Diffusion of the enzyme was demonstrated after as little as 1 hour of incubation by the identification of bromonaphthol (coupling to an azo dye) in the supernatant fluid and in fat droplets. While the localization of the enzyme within the cytoplasm of these acinar cells is not precise, it is totally absent in cell nuclei and in the cells of the islands of Langerhans.

**SUMMARY**

6-Bromo-2-naphthyl-β-D-glucopyranoside is hydrolyzed by a carbohydrate in mammalian tissue. A colorimetric method for the determination of β-D-glucosidase with this substrate is described. Some of the enzyme kinetics are given.

A survey of the organs of five species shows considerable species variation in distribution. The activity of β-D-glucosidase is uniformly lower than that of β-D-galactosidase. The enzyme activity in the pancreas of guinea pig is much higher than in any other organ. The histochemical demonstration is possible only in pancreas of guinea pig.

Evidence is presented that, at least under certain circumstances, β-D-galactosidase and β-D-glucosidase in mammalian tissue are distinct enzymes.

**BIBLIOGRAPHY**

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