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TECHNICAL MANUSCRIPT 405

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DEPARTMENT OF THE ARMY
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COMPETITIVE INHIBITION OF HISTOCHEMICAL SUBSTRATES FOR GLYCOSIDASES

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August 1967

In conducting the research described in this report, the investigator 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.

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ABSTRACT

Competitive inhibition of glycosidases in the rat jejunum was studied using four halogen-substituted indolyl substrates: 5-bromo-4-chloroindol-3-yl-β-D-galactopyranoside, 5-bromo-4-chloroindol-3-yl-β-D-fucopyranoside, 5-bromo-4-chloroindol-3-yl-β-D-glucopyranoside, and 5-bromo-4-chloroindol-3-yl-β-D glucopyruroniside. These substrates have identical indolyl moieties and differ only in the substitution and/or configuration of the glycon. Inhibition of enzyme activity was studied with sugars and sugar derivatives whose structure was analogous to that of the glycosides. In general, inhibition was noted when the substrate and inhibitor were derived from the same sugar. Aldonolactones were the strongest inhibitors, and, from the limited data, carboxyl substituents appeared to be more important than other end groups or the configuration of the pyranoside ring.

COMPETITIVE INHIBITION OF HISTOCHEMICAL SUBSTRATES FOR GLYCOSIDASES

The valid interpretation of histochemical reactions for enzymes depends upon their specificity. The reaction should be enhanced by the conditions that are optimal for the enzyme and be diminished in the presence of inhibitors. Inhibition of the substrate reaction may be effected either by enzyme inactivation or by competition with analogous compounds. These methods have been used to define the specificity of numerous in vivo and in vitro biochemical systems.

Halogen-substituted indoxyl substrates have been developed for β-D-galactosidase,[1] β-D-glucosidase,[2] β-D-glucuronidase,[3] and β-D-fucosidase,[4] and enzymatic specificity has been demonstrated for each. The substrates contain an identical indolyl moiety (5-bromo-4-chloroindol-3-yl) and glycosides that differ from each other only in the configuration or substitution of C_4 or C_6 (Fig. 1). The availability of these substrates presented a unique opportunity to observe the comparative effect of analogue inhibitors on the reactions of these structurally similar substrates.

Specimens of jejunum were obtained from freshly killed, adult Sprague Dawley rats, frozen in a dry ice and acetone bath, and sectioned in a cryostat at -15 C. The sections were incubated for 3 hours at 37 C in a solution of the substrate and inhibitor. The detailed techniques for these reactions have been reported elsewhere;[1,3,4] the 5-bromo-4-chloroindolyl glucoside was used in the same manner as the 5-bromoindolyl compound described previously.[2] The pH range and optimum for reactions in the jejunum were determined for each substrate. The substrate concentration was adjusted so that reactions of similar intensity were obtained for each by 3 hours. Sections in each experiment were prepared from a single block of tissue and were mounted at the tip of cover slips in order to conserve substrate; final incubation volume was 1 cc. Solution inhibitors were added to each substrate so that the final concentration was 0.01% to 2%. After incubation, the sections were dehydrated, cleared, and mounted in balsam. Inhibition was graded (0 to ++++) by the degree of diminished staining and the inhibitor concentration that was required for this effect.

The staining reaction occurred over a broad range of pH values. The pH optima were: galactosidase, 5.4; fucosidase, 6.2; glucuronidase, 4.8; and glucosidase, 5.4.

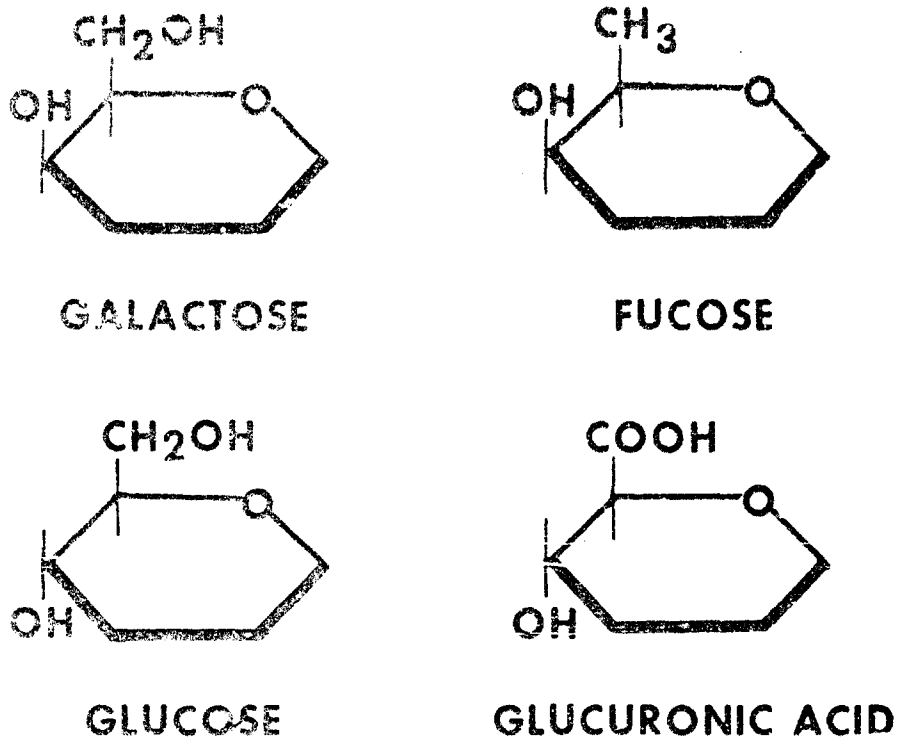

Figure 1. Structural Similarity of Indoxyl Substrates as Illustrated by Comparison of Glycon Moieties. In each case the compound is a β-isomer and the glycoside is in the D-configuration.

In general, inhibition of the reaction was most marked when both the substrate and the inhibitor were derived from the same sugar. Results for the four substrates are listed in Table 1. A variable degree of inhibition resulted from addition of the sugar in the substrate to the incubating solution. Staining was diminished in the glucuronidase reaction by 0.1% glucuronic acid (+++). In contrast, 2% glucose had no effect on the glucosidase reaction. Aldonolactones were the strongest inhibitors, but overlapping occurred between galactosidase and fucosidase, and between glucosidase and fucosidase.

Glycoside derivatives demonstrated the specificity of carboxyl group location. Gluconic acid (C_1carboxyglucose) inhibited glucosidase activity but had no effect on glucuronidase activity. Saccharic, galacturonic, and glucuronic acids (C_6 carboxyl groups) all inhibited the glucuronidase reaction.

TABLE 1. COMPETITIVE INHIBITION OF GLYCOSIDASES[a/]

Inhibitor[b/]	Galactosidase	Fucosidase	Glucuronidase	Glucosidase
Galactose	++	0	0	0
Fucose	0	+	0	0
Glucuronic acid	0	0	+++	0
Glucose	0	0	0	0
Galactonolactone	+++	+	0	0
Fuconolactone	+	+++	0	0
Glucuronolactone	0	0	++	0
Saccharolactone	0	0	++++	0
Gluconolactone	0	+	0	+++
Gluconic acid	0	0	0	+++
Saccharic acid	0	0	++++	0
Mucic (galactaric) acid	0	0	++	+
Galacturonic acid	0	0	++	0
Polygalacturonic acid	0	0	++	+++
Xylose	0	0	0	+
Lactose	+	0	0	0
Cellobiose	0	+	0	0
Melibiose	0	0	0	+

a. 0 = No effect on tissue staining.
+ = Diminished staining, 2%; inconsistent or no effect at lower concentrations.
++ = Complete inhibition, 2%; diminished staining, 1%.
+++ = Complete inhibition, 1%; diminished staining, 0.1%.
++++ = Complete inhibition, 0.1%; diminished staining, 0.01%.

b. These glycosides and glycoside derivatives were all in the D-form.

Disaccharides were weak inhibitors. Lactose (glucosido-β-galactose) was a consistent inhibitor of galactosidase only at a concentration of 2%. Cellobiose (4-glucosido-β-glucose) had no effect on glucosidase but weakly inhibited the fucosidase reaction. In addition to the compounds listed in Table 1, no inhibition was noted with the following commercially available sugars and sugar derivatives: L-fucose, 2-deoxyglucose, mannose, rhamnose (6-deoxymannose), sorbose, ribose, L-arabinose, lyxose, fructose, inulin, stachyose (lupeose), sucrose (4-fructosido-α-glucose), maltose (4-glucosido-α-glucose), trehalose (1-glucosido-α-glucose), raffinose (melitriose), dulcitol (galactitol), sorbitol (glucitol), inositol, adonitol, N-acetylglucosamine, and phosphoglucuronic acid.

The substrate hydrolysis is identical in these four indolyl glycosidase methods, and the rate and intensity of the staining reactions were similar. The overlapping inhibition between galactosidase and fucosidase activities in this study has been noted previously in biochemical assays.[5] Likewise, the inhibition of glucosidase activity by xylose is probably the result of similarities between glucosidase and xylosidase and/or the similar configuration of glucose and xylose.[6]

These data confirm enzyme specificity for glycoside configuration and linkage.[7,8] Neither L-fucose nor α-linked glycosides had the inhibitory effect of D-fucose and β-linked glycosides. Carboxyl substitutents appeared to be more important than other end groups, or the configuration of the pyranoside ring. Rhamnose (6-deoxymannose) did not inhibit fucosidase, but all compounds with C_6 carboxyl groups were strong inhibitors of glucuronidase. In contrast, glucose and galactose derivatives did not necessarily inhibit glucosidase and galactosidase. There is no obvious explanation for the absence of glucosidase inhibition by glucose. Likewise, the reason for the weak inhibition of fucosidase activity by gluconolactone and cellobiose or the similar effect of melibiose (6-galactosido-α-glucose) on glucosidase is unknown.

Dannenberg and co-workers have used histochemical methods to study the conditions of activation and inhibition of several hydrolytic enzymes in macrophages.* Fishman et al.[9] have studied the inhibition of glucuronidase. The present data confirm their findings with compounds analogous to the galactoside and glucuronide. These studies demonstrate that analogue and end product inhibition are useful techniques for defining the specificity of histochemical methods for enzyme detection.

* Unpublished data.

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