A Mammalian Thioglycosidase

Abstract. A thioglycosidase capable of catalyzing the hydrolysis of 6-purine β-D-glucothiopyranoside and other thioglycosides has been found in a number of mammalian tissues (including tumors) as well as in almond emulsin. The substrate specificity of mammalian thioglycosidase involves both the aglycone and the glycosyl moieties.

In the course of a study of the relationship between chemical structure and antitumor activity among derivatives of 6-mercaptopurine, 6-purine β-D-glucothiopyranoside (MPG) and a number of related thioglycosides were synthesized (*1*). When the thioglycoside was treated with a commercial almond emulsin preparation, enzymatic hydrolysis to 6-mercaptopurine (6-MP) and glucose was observed (*1*). The marked activity of almond emulsin was surprising in view of Pigman's (*2*) report that synthetic thioglycosides do not serve as substrates for this enzyme.

In the course of antitumor trials in mice, MPG when administered intraperitoneally was found to have activity against sarcoma 180 and adenocarcinoma 755, with relatively low toxicity in comparison to its aglycone (*3, 4*). However, when MPG was administered orally, greater antitumor activity as well as increased toxicity were observed. These facts led to a study of the possible occurrence of a thioglycosidase in the digestive tract of the mouse.

Investigation showed the presence of such an enzyme, widely distributed in mammalian species and tissues, with significant activity in various rodent tumors (*5*). This was particularly surprising since enzyme preparations from animal sources, although they have been shown to contain β-glucosidase and β-glucuronidase activity (*6*), attack neither the natural thioglycosides of black mustard (*7*) nor synthetic thioglycosides (*2, 8*). No report of thioglycosidase activity from animal sources has appeared heretofore, whereas plant enzymes capable of splitting the natural thioglycosides of plants have been known for many years. Thus, the mustard thioglycosides sinigrin, sinalbin, and glucocheirolin are readily cleaved by the enzyme myrosin (*9*).

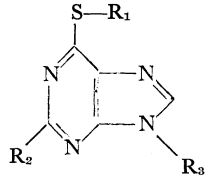
It has now been found that MPG is attacked not only by almond emulsin (*1*) but also by the thioglycosidase of the black mustard, *Brassica nigra* Koch, and by thioglycosidase preparations from a variety of animal species.

Determination of thioglycosidase activity in the studies reported here (*10*) was based on the marked differences between the ultraviolet absorption characteristics of the purinyl thioglycoside substrates and their aglycone cleavage products—6-mercaptopurine (*11*), thioguanine, and others. Tissues are readily assayed for thioglycosidase as follows: A 1:5 dilution of a tissue homogenate is prepared in saline at 0°C. One milliliter of the homogenate is incubated at 37°C for 3 hours with 5 mg of MPG at *p*H 5.8 (the optimum *p*H for thioglycosidase) in acetate buffer in a total volume of 5 ml. The solution is freed of protein by trichloroacetic acid precipitation, and the amount of 6-MP liberated by enzyme action is determined spectrophotometrically.

By means of this procedure, the presence of a thioglycoside-splitting enzyme has been demonstrated in every living species thus far examined, including man, dog, rat, mouse, pigeon, hog, and calf, and in the microorganisms *Tetrahymena pyriformis* and *Escherichia coli* (*12*).

Thioglycosidase activity was demonstrated in a number of transplantable animal tumors as follows (the numbers in parentheses describe enzyme activity in terms of micrograms of 6-MP liberated per gram of fresh tissue): sarcoma 180, solid (840); sarcoma 180, ascites (0); sarcoma T241 (717); sarcoma MA 387 (324); Ehrlich carcinoma, solid (585); Ehrlich carcinoma, ascites (0); Bashford carcinoma 63 (513); mammary adenocarcinoma E0771 (486); Miyono adenocarcinoma (500); bladder carcinoma, C57 black mouse (765); lung carcinoma, C57 black mouse (579); adenocarcinoma 755 (678); Wagner osteogenic sarcoma (500); Mecca lymphosarcoma (1188); Gardner lymphosarcoma (540); Harding-Passey mouse melanoma (500); Flexner-Jobling rat carcinoma (339); Walker carcinosarcoma 256 (1476); Jensen rat sarcoma (432); and Murphy-Sturm rat lymphosarcoma (48).

Table 1. Activities of animal and plant thioglycosidases on purinyl thioglycosides.



R_1	R_2	R_3	Activity: Mammalian thioglycosidase	Activity: Almond emulsin	Activity: Myrosin
β-D-Arabinopyranose	H	H	–		
α-L-Arabinopyranose	H	H	+		+
β-D-Galactopyranose	H	H	+	+	+
β-D-Glucopyranose*	H	H	+	+	+
β-D-Glucopyranose	NH_2	H	–		–
β-D-Glucopyranose (sulfone)	H	H	–		
β-D-Glucopyranose	NHG†	H	–		–
β-D-Glucopyranose	H	β-D-Ribofuranose	+		
β-D-Glucopyranose	H	Tri-*O*-acetyl-α-D-ribofuranose	+		
β-D-Glucofuranuronic acid*	H	H	–	–	
β-D-Glucopyranuronic acid*	H	H	–	–	
Lactose	H	H	–		–
β-D-Mannopyranose	H	H	+		+
α-L-Rhamnopyranose	H	H	–		–
β-D-Ribopyranose	H	H	–		?
2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranose	H	H	–		–
2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranose (sulfone)	H	H	–		
β-D-Xylopyranose	H	H	+		+
Miscellaneous thioglycosides					
3-Chloro-6-β-D-glucothiopyranosyl-pyridazine			–		+
2-β-D-Glucothiopyranosyl-4-pyrimidine			+		+
2,4-Dinitrophenyl β-D-glucothiopyranoside			–		+

* These compounds were tested also as substrates for bacterial β-glucuronidase and were found to be inactive. † G = a second β′-D-glucopyranosyl group.

A few samples of human malignancies have also been examined, and here too significant thioglycosidase activity was found.

Some representative values for thioglycosidase activities of normal mouse tissues are: small intestine, 5370; spleen, 2895; liver, 2100; stomach, 1626.

Mammalian thioglycosidase activity is inhibited by delta-gluconolactone at relatively high concentrations. Twenty-five-percent inhibition is produced at $10^{-3}M$, and 50 percent, at $10^{-2}M$. A number of related compounds were studied as inhibitors, including D-glucurono-δ-lactone, D-gluconic acid, D-galacturonic acid, D-galacturono-δ-lactone, D-gulono-δ-lactone, D-arabono-δ-lactone, calcium-D-arabonate, isoascorbic acid, iso-inositol, D-ribono-δ-lactone, and D-glucoheptono-δ-lactone. Of these, all were inactive with the exception of D-galactono-δ-lactone. Various other substances have been examined for activity on mammalian thioglycosidase [enzyme inhibition or no activity are indicated by (+) or (−), respectively]: $HgCl_2$ (+); NaN_3 (−); $CuSO_4$ (+); NaF (+) Na_2SO_3 (+); H_2O_2 (+); 2,4-dinitrophenol (+); 2,4-dinitrothiophenol (+); Na_3AsO_4 (+); glutathione (−); H_2S (−); cysteine (−); EDTA (−).

Further studies of the properties of the enzyme and its substrate specificity were undertaken with acetone powders of mouse intestine, hog liver, and hog pancreas (which are quite similar in thioglycosidase activity). These may be stored for at least 15 weeks at −10°C, after which time the activity slowly declines. The optimum *p*H curve is rather broad, with the maximum activity at about *p*H 5.8. The enzyme is sensitive to elevated temperatures, and heating for 5 minutes at 60°C results in a loss of 50 percent in activity; heating for 5 minutes at 70°C causes almost total inactivation. The rate of hydrolysis decreases with time; after about 3 hours under the conditions given above, the reaction finally ceases entirely, although excess enzyme and substrate are present, as shown by the fact that addition of either more substrate or more enzyme to the reaction mixture causes further hydrolysis. However, the enzymatic hydrolysis is not inhibited by the hydrolysis products.

A number of purine, pyrimidine, and other thioglycosides (*13*) have been examined as substrates for the mammalian thioglycosidase. The results are summarized in Table 1. From these results, certain generalizations may be made. The active substrates include derivatives of D-glucose, D-galactose, L-arabinose, and D-xylose, while the D-arabinoside is virtually inactive. The essential configuration required for activity is that of β-D-glucopyranose or β-D-galactopyranose. The hydroxymethyl group of C-5 may be replaced by —H, but replacement by —COOH (as in the thioglucuronides) results in loss of activity. The 6-purinyl structure is the most active aglycone found to date, but a rather wide range of aglycone variation is permitted, including purines, pyrimidines, and pyridazines.

Thus, the enzyme appears to resemble the thioglycosidase enzymes previously found only in plants and is similar to the classical β-glucosidase found in plants and animals in the structural requirements for the glycosyl residue. Furthermore, both classes of enzymes are inhibited by delta-gluconolactone (*14*).

When the presence of purinethioglycosidase at high levels of activity in tumors was first discovered, it was hoped that MPG might serve as a relatively nontoxic carrier of 6-MP, which thus would be released *in situ* in the tumor, and that the differential effect of 6-MP on tumors might thus be increased. The pharmacological properties of MPG, its rapid clearance, and its poor cellular penetration (*4*) appear to interfere with the realization of this aim. Thus, to explain the significant antitumor effects of MPG and related compounds on the basis of tumor thioglycosidase levels would appear to be premature at this point, for when MPG was administered intraperitoneally to the mouse, no evidence of cleavage to 6-MP was observed in any tissue, in spite of the demonstrated presence of thioglycosidase activity in nearly all mammalian tissues. However, orally administered MPG is rapidly hydrolyzed, liberating 6-MP in the gut.

No clues to the occurrence of natural substrates for this thioglycosidase have yet been found. Its high level of activity and wide distribution are all the more puzzling since MPG appears to possess the optimal substrate configuration.

IRVING GOODMAN*, JAMES R. FOUTS†, EDWARD BRESNICK, ROSALIE MENEGAS, GEORGE H. HITCHINGS

Wellcome Research Laboratories, Tuckahoe, New York

References and Notes

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9. The enzyme thioglycosidase was discovered by Boutron and Fremy [F. Boutron and E. Fremy, *Ann. Chem. Liebigs* 34, 230 (1840)] and has been known variously as myrosin, myrosinase, and sinigrase.
10. We gratefully acknowledge the valuable technical assistance of Mr. Ludwig Salce in these studies.
11. Absorption maxima and molecular extinctions at *p*H 6: for 6-MP; $\lambda_{max.} = 320$ mμ, $E_m = 18.9 \times 10^3$; for MPG, $\lambda_{max.} = 280$ mμ, $E_m = 16.0 \times 10^3$.
12. Resting cells of *Escherichia coli* B showed no thioglycosidase activity, whereas a sonicated cell-free extract of this organism showed marked activity. This observation may have bearing on the cell permease hypothesis of Monod *et al.* [G. N. Cohen and J. Monod, *Bacteriol Revs.* 21, 169 (1958)].
13. A paper describing details of the synthesis and properties of a series of purine thioglycosides is in preparation.
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* Present address: Department of Biochemistry, Columbia University, New York, N.Y.
† Present address: State University of Iowa, Iowa City.

27 March 1959

Learning Differences in Two Separated Lines of Mice

Abstract. Significant differences in conditioning and response topography were obtained with two lines in C57BL/10 mice, suggesting the occurrence of a behavioral mutation. It is suggested that the two lines be classified as substrains. The two substrains would appear to be useful in experiments on the genetics of behavior.

In the summer of 1957 while working at the Behavior Laboratory of the Roscoe B. Jackson Memorial Laboratory (*1*), I found that 150-day-old C57BL/10 mice had a significantly higher conditioning score than did equivalent mice tested the previous year in a different study (*2*). In trying to determine the cause of this difference, I noted that the mice used in 1957 had come from the colony at the Main Laboratory while the mice tested in 1956 had come from the Behavior Laboratory. Mice from the Behavior Laboratory colony which were 150 days old were then tested and found to have a mean conditioning score very similar to the mean obtained the previous year with the Behavior Laboratory animals. A number of 50-day-old mice were available from both laboratories and were also tested. The same phenomenon was obtained: the mean score of the Behavior Laboratory mice in 1957 was not significantly different from that of the Behavior Laboratory mice tested in 1956, while the Main Laboratory animals were again significantly better on the conditioning task.

These findings suggested either that there had been some genetic change or that the environmental rearing conditions in the two laboratories were different enough to affect conditioning ability differentially. To determine whether these differences were simply correlated