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The Hydrolysis of Glycosyl Fluorides by Glycosidases

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1. α-D- and β-D-Glucopyranosyl, α-D- and β-D-galactopyranosyl, α-D-mannopyranosyl and α-D-xylopyranosyl fluorides were hydrolysed specifically by the respective glycosidases from several sources. 2. Use of specific inhibitors with a mixture of glycosidases from *Helix pomatia* intestinal juice showed that each glycosyl fluoride was hydrolysed only by the respective glycosidase. α-D-Glucopyranosidase and α-D-xylopyranosidase activities were shown to be due to different enzymes. 3. Partially purified enzyme preparations containing only one of the glycosidase activities hydrolysed only the corresponding glycosyl fluoride. 4. The configuration at C-1 of α-D-mannopyranosyl fluoride was confirmed since it was hydrolysed by an α-D-mannosidase preparation that contained no detectable β-D-mannosidase activity. 5. An attempt to prepare *o*-nitrophenyl β-D-mannopyranoside led only to *o*-nitrophenyl α-D-mannopyranoside.

Mammalian and fungal α-D-glucosidases have been shown to hydrolyse α-D-glucopyranosyl fluoride to glucose and fluoride (Barnett, Jarvis & Munday, 1967). To determine the specificity requirements of the reaction and the extent to which other glycosidases can utilize glycosyl fluorides as substrates, we have prepared some glycosyl fluorides and tested them as substrates for glycosidases from several sources.

MATERIALS AND METHODS

α-D-Galactopyranosyl fluoride. α-D-Galactopyranose penta-acetate (34g.) was dissolved in anhydrous HF (100g.) at −70°. After 1hr. at −15° and 10min. at room temperature the solution was poured into a chloroform–ice mixture, and the chloroform layer was separated, washed with aq. $NaHCO_3$ and water, and dried over $CaCl_2$. Evaporation *in vacuo* gave syrupy tetra-*O*-acetyl-α-D-galactopyranosyl fluoride. A portion (10g.) was dissolved in dry methanol (50ml.) and 2N-sodium methoxide (2ml.) added. After 2hr. at room temperature the solution was deionized with anhydrous methanolic Amberlite MB-1. The methanolic solution was evaporated *in vacuo* to about 5ml., giving crystalline *α-D-galactopyranosyl fluoride* (3·5g.), m.p. 75–76°, and, after two recrystallizations from methanol–ether, decomp. 130–135° and $[\alpha]_D^{20}$ +127±5° (*c* 2·4 in water) (Found: F, 10·3; $C_6H_{11}FO_5$ requires F, 10·4%).

Other glycosyl fluorides. α-D-Glucopyranosyl fluoride, decomp. 120–130° and $[\alpha]_D^{20}$ +90±2° (*c* 1·0 in water) (Helferich, Bäuerlein & Wiegand, 1926), β-D-glucopyranosyl fluoride, m.p. 91–92° (decomp.) and $[\alpha]_D^{20}$ +20±3° (*c* 5·0 in methanol) (Micheel & Klemer, 1952), and α-D-xylopyranosyl fluoride, m.p. 106–110° and $[\alpha]_D^{24}$ +55±10° (*c* 0·2 in water) (Micheel *et al.* 1957), were prepared from the respective tetra-acetates. Deacetylation of tetra-acetyl-α-D-mannopyranosyl fluoride, m.p. 67–68° and $[\alpha]_D^{21}$ +18·4±3° (*c* 6·1 in chloroform), gave syrupy α-D-mannopyranosyl fluoride, $[\alpha]_D^{21}$ +11±4° (*c* 6·4 in water) (Micheel & Borrmann, 1960). β-D-Galactosyl fluoride, $[\alpha]_D^{20}$ +50±5° (*c* 11·6 in water) (Micheel, Klemer, Baum, Ristič & Zumbülte, 1955), was also obtained as a syrup. Exhaustive attempts to crystallize the 1,2-*trans*-glycosyl fluorides were not made since they are very labile.

The purity of each glycosyl fluoride was tested by thin-layer chromatography of the fluoride and its hydrolysis products, and by analysis of free and bound fluorine (see below). The crystalline fluorides were 98% pure.

o-Nitrophenyl α-D-mannopyranoside. Tetra-*O*-acetyl-α-D-mannopyranosyl bromide (10g.), $[\alpha]_D^{24}$ +95±5° (*c* 20 in acetone), in acetone (64ml.), was added to *o*-nitrophenol (4·2g.) and NaOH (1·7g.) in water (42ml.). After 5hr. at room temperature the acetone was removed, the aqueous mixture extracted with chloroform, and the chloroform layer washed with NaOH solution and water and dried over $CaCl_2$. The solvent was removed *in vacuo* to give a syrup (2·8g.) of *o*-nitrophenyl tetra-*O*-acetylmannopyranosides, $[\alpha]_D^{24}$ +40±1° (*c* 28 in methanol). This was dissolved in methanol (10ml.) and N-sodium methoxide (1ml.) was added. After 1hr. the solution was deionized with methanolic Amberlite MB-1. The methanol was removed *in vacuo* to give *o-nitrophenyl α-D-mannopyranoside* (0·9g., yield 50%) after two recrystallizations from methanol–water, m.p. 182–183° and $[\alpha]_D^{24}$ +84±4° (*c* 0·69 in water). A small second crop (15mg.), m.p. 174°, was obtained and the final mother liquor (0·4g.) had $[\alpha]_D^{20}$ +20±1° (*c* 8·0 in methanol) (Found: C, 48·3; H, 4·9; N, 4·9; $C_{12}H_{15}NO_8$ requires C, 48·0; H, 5·0; N, 4·7%).

p-Nitrophenyl α-D-mannopyranoside. This was made by the procedure described above for *o*-nitrophenyl α-D-mannopyranoside. The syrupy mixture of *p*-nitrophenyl tetra-*O*-acetyl-D-mannopyranosides, $[\alpha]_D^{22}$ +48±1° (*c* 5 in methanol), on deacetylation gave *p*-nitrophenyl α-D-mannopyranoside, m.p. 177–178°, which gave no depression

of m.p. with authentic material made by the method of Westphal & Feier (1956).

Other nitrophenyl glycosides. *o*-Nitrophenyl α-D-galactopyranoside, m.p. 144–145°, was made by the method of Porter, Holmes & Crocker (1953). *p*-Nitrophenyl α-D-glucoside was obtained from Koch–Light Laboratories Ltd. (Colnbrook, Bucks.) and *o*-nitrophenyl β-D-glucoside and *o*-nitrophenyl β-D-galactoside were from British Drug Houses Ltd. (Poole, Dorset). *p*-Nitrophenyl α-D-xylopyranoside was kindly given by Dr P. W. Kent and Mr M. H. Higham (Department of Biochemistry, University of Oxford).

Other chemicals. Phenyl β-D-mannopyranoside was kindly given by Dr J. Conchie (Rowett Research Institute, Bucksburn, Aberdeen). Methyl α- and β-D-galactopyranosides were made by the action of methanolic HCl on galactose and the methanolic displacement of tetra-acetyl-α-D-galactosyl bromide, followed by deacetylation, respectively, and all other chemicals were obtained from British Drug Houses Ltd. A solution containing galactono-(1→5)-lactone was prepared by the method of Levvy, McAllan & Hay (1962).

Enzymes. α-D-Glucopyranosidase was obtained from rat intestinal mucosa as previously described (Barnett *et al.* 1967) or from a fungal preparation, Agidex, a gift from Glaxo Laboratories, Greenford, Middlesex. β-D-Glucopyranosidase from almond emulsin was obtained from British Drug Houses Ltd. α-D-Galactopyranosidase from Santos green coffee beans (Importers, Retail Saleroom Ltd., Southampton) was a partially purified preparation obtained by the method of Courtois & Patek (1966). A partially purified preparation of α-D-mannopyranosidase was prepared from jack beans (British Drug Houses Ltd.) (Li, 1966).

All these enzyme preparations had no detectable quantities of any of the other glycosidases under the experimental conditions used except the last, which contained some α-D-galactopyranosidase. A mixture of glycosidases was obtained from *Helix pomatia* (Haig and Son, Beambrook, Newdigate, Surrey) intestinal juice (Myers & Northcote, 1958).

Assay of enzyme activity. The enzyme preparation (0·2ml.) was added to 0·1M-sodium citrate–phosphate, sodium acetate or sodium maleate buffer (0·5ml.), 10mM-substrate (0·1ml.) and either water (0·2ml.) or 50mM-inhibitor (0·2ml.). The citrate–phosphate buffer could not be used with glycosyl fluorides, since it interfered with the fluoride analysis. With nitrophenyl derivatives the reaction was stopped by the addition of M-Na_2CO_3 (1·0ml.) and the colour read in a Unicam SP. 600 at 400mμ for *p*-nitrophenol and 420mμ for *o*-nitrophenol. With glycosyl fluorides, the reaction was stopped with ethanol (2·0ml.) and the fluoride that had been released was measured by the method of Belcher, Leonard & West (1959) on one-tenth scale. With the 1,2-*trans*-glycosyl fluorides, which were unstable at pH4·3, the time of colour development was reduced to 17min. Since the fluoride method is very sensitive to impurities, blanks and standard fluoride curves were prepared containing inhibitor, enzyme and buffer, and also substrate and buffer. In some cases (e.g. with snail α-D-mannosidase) the interference was too great for accurate measurement. Phenol released from phenyl β-D-mannoside was measured by the method described by Levvy, Hay & Conchie (1964).

Assay of purity of glycosyl fluorides. The fluorides were chromatographed on thin-layer plates as described previously (Barnett *et al.* 1967) before and after boiling in water to convert the glycosyl fluorides into the free sugar. Methyl glycosides, possible impurities in the syrupy fluorides, were not hydrolysed by this procedure. All the fluorides gave a single spot, which was converted on hydrolysis into a single spot of hexose or pentose. With the 1,2-*trans*-glycosyl fluorides, bound fluorine was almost quantitatively released by boiling in 0·1N-KOH.

Other methods. Melting points are uncorrected; rotations were measured on a Hilger and Watts standard polarimeter Mk. III.

Microanalyses were performed by Weiler and Strauss (Oxford).

RESULTS

The percentage inhibition produced by a variety of inhibitors on the glycosidases with both nitrophenyl glycosides and glycosyl fluorides as substrates is shown in Table 1. The results were obtained at the pH optimum for the enzyme, except for the β-D-glucopyranosidase with a pH optimum of 5·5, where, owing to the acid-instability of β-D-glucopyranosyl fluoride, the reaction was studied at pH6·1. In each case the activity of glycosyl fluoride hydrolysis was diminished in about the same proportion as that of the corresponding nitrophenyl glycoside. With α-D-galactosidase, the inhibition appeared to be greater for the fluoride than for *o*-nitrophenyl α-D-galactoside. The results indicate that the activity of glycosidases towards glycosyl fluorides is specific both for the glycosidic moiety and for the configuration at C-1, as with more usual substrates. The α-D-mannosidase activity of the intestinal juice was too weak, and the pH optimum of the β-galactosidase was too acid, to give reliable results with the respective glycosyl fluorides.

Glucono-(1→5)-lactone inhibited α-D-glucosidase but not α-D-xylosidase activity, clearly indicating the presence of two separate enzymes in *Helix pomatia* intestinal juice.

Specificity of partially purified glycosidases from various sources towards glycosyl fluorides. Partially purified enzymes from various sources were tested for the ability to hydrolyse glycosyl fluorides other than that corresponding to the known glycosidase activity. In each case only the glycosyl fluoride of the correct configuration was hydrolysed. Some kinetic parameters of the enzymes are given in Table 2.

Identity of α-D-galactopyranosyl fluoride hydrolase with α-D-galactopyranosidase. The partially purified coffee-bean α-D-galactosidase was preincubated at temperatures between 0° and 80° for 10min. and then cooled to 0°, and the α-D-galactopyranosyl fluoride hydrolase and α-D-galactosidase activities were measured at 25°. The two curves of activity plotted against preincubation temperatures were

Table 1. *Effect of inhibitors on the nitrophenyl glycosidase and glycosyl fluoride hydrolase activities of* Helix pomatia *intestinal juice*

The substrate (1mM) and inhibitor (10mM) were incubated with intestinal juice at the pH optimum. The numbers in parentheses were obtained with substrate (0·5mM) and inhibitor (5mM). Substrates: *A*, *p*-nitrophenyl α-D-glucoside; *B*, α-D-glucosyl fluoride; *C*, *o*-nitrophenyl β-D-glucoside; *D*, β-D-glucosyl fluoride; *E*, *p*-nitrophenyl α-D-xyloside; *F*, α-D-xylosyl fluoride; *G*, *o*-nitrophenyl α-D-galactoside; *H*, α-D-galactosyl fluoride; *I*, *o*-nitrophenyl β-D-galactoside.

	Inhibition (%)								
Substrate	*A*	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*
Dilution of intestinal juice ...	100	100	100	20	10	10	5	10	20
pH optimum	5·5	5·5	5·5*	5·5*	4·5	4·5	3·5	3·5	5·0
Inhibitor									
Methyl α-D-glucoside	54 (26)	47	0	0	—	—	15	30	0
Methyl α-D-galactoside	8	0	—	—	—	—	15	30	0
Methyl β-D-galactoside	5	0	—	—	—	—	14	30	33
Melibiose	0	0	0	0	—	—	41	48	0
'Galactono-(1→5)-lactone'	8	0	—	—	—	—	35	32	79
Glucono-(1→5)-lactone	100 (94)	84	100	100	0	0	13	37	74
Xylose	0	—	—	—	(15)	25	—	—	—
Glucose	47 (32)	58	—	—	0	0	15	29	62

* Observations of inhibition at pH 6·1.

Table 2. *Kinetic data for glycosidases at* 25°

Enzyme	Source	pH optimum	pH of incubation medium	Substrate	K_m (mM)	Specific activity (μmoles produced/ min./g. dry wt.)
α-D-Glucosidase	Rat intestine	6·6	7·0	*p*-Nitrophenyl α-D-glucoside	1·4	0·025*
				α-D-Glucosyl fluoride	0·4	0·9*
α-D-Glucosidase	Agidex	4·7	7·0	*p*-Nitrophenyl α-D-glucoside	3·6	0·07
				α-D-Glucosyl fluoride	3·0	17·0
β-D-Glucosidase	Almond emulsin	5·4	6·1	*o*-Nitrophenyl β-D-glucoside	10·0	227
				β-D-Glucosyl fluoride	1·0	95
α-D-Galactosidase	Coffee bean	6·1	6·1	*o*-Nitrophenyl α-D-galactoside	0·5	22
				α-D-Galactosyl fluoride	0·4	24
α-D-Mannosidase	Jack bean	4·5	4·5	*o*-Nitrophenyl α-D-mannoside	2·5	0·15†
				p-Nitrophenyl α-D-mannoside	1·6	0·21†
				α-D-Mannosyl fluoride‡	3·6	0·14†

* Per g. wet wt. † Per g. of original jack bean. ‡ Syrupy.

identical and the temperature giving 50% of the original activity was 61·5°.

The competitive inhibition constant K_i of melibiose for each activity was identical, K_i 11mM at 25° and pH 6·1.

Identity of α-D-mannopyranosyl fluoride hydrolase with α-D-mannopyranosidase. Partially purified jack-bean α-D-mannosidase was preincubated as described above for α-D-galactosidase. The curves of activity plotted against preincubation temperature were identical with either α-D-mannopyranosyl fluoride or *o*-nitrophenyl α-D-mannopyranoside as substrate and the temperature giving 50% of the original activity was 70·5°.

The competitive inhibition constants, K_i, of mannose for α-D-mannosidase with both substrates were identical, K_i 21mM at 25° and pH 4·5. The jack-bean extract contained no detectable β-D-mannopyranosidase.

DISCUSSION

The results in Tables 1 and 2 indicate that the glycosyl fluorides obey the normal specificity rules for glycosidase activity in that the enzyme is specific for both the glycosyl moiety and the configuration at C-1. In a previous paper (Barnett *et al.* 1967) we suggested that the initial step in glucosidase hydrolysis of α-D-glucosyl fluoride involved the protonation of the fluorine atom and the elimination of

hydrogen fluoride to leave either a carbonium ion or an enzyme–glycoside covalent complex. The theoretical mechanism, in which fluorine takes the place of oxygen in the normal glycoside substrate, requires that the specificity requirements of glycosidases with glycosyl fluorides should be the same as with glycosides as substrates, and that all glycosidases that use protonation as the initial step should hydrolyse the respective glycosyl fluoride. The present results confirm our suggestion.

The enzymic hydrolysis of α- and β-D-galactosyl fluorides had previously been observed (Hofsten, 1961), but in that investigation neither of the fluorides was characterized, nor were kinetic parameters given. Indeed there appears to be no previous record of the properties of α-D-galactopyranosyl fluoride.

To confirm that α-D-galactosyl and α-D-mannosyl fluorides were hydrolysed by the respective glycosidases, the temperature–stability curves were compared, and the K_i values of a competitive inhibitor determined, with the fluorides and nitrophenyl glycosides as substrates for the enzymes. As with α-D-glucosidase (Barnett *et al.* 1967), these were identical for the glycoside and the fluoride.

Although it was only considered valid to estimate the K_m for the crystalline fluorides the K_m values of the glycosyl fluorides for the enzymes were very similar to those of the nitrophenyl glycosides. The K_m of the fluoride for β-D-glucosidase was 1mM compared with 10mM for *o*-nitrophenyl β-D-glucosides, possibly indicating the superior binding of the fluoride at the enzyme site. The relative rates of hydrolysis of the glycosyl fluorides were of the same order as the nitrophenyl glycosides, or higher, with the exception of β-D-glucosidase (Table 2).

Recently some doubt has been cast on the configuration at C-1 of tetra-*O*-acetyl-α-D-mannopyranosyl fluoride since the chemical shift of its fluorine magnetic-resonance spectrum is more similar to that of the derivatives of β-D-glycopyranosyl and β-D-galactopyranosyl fluorides than that of the α-isomers (Hall & Manville, 1965). The present enzymic evidence supports the rotational and kinetic evidence for the α-configuration of this fluoride, since the compound was hydrolysed by a jack-bean α-D-mannosidase, which contained no β-D-mannosidase activity.

In an attempt to make a nitrophenyl β-D-mannopyranoside to assay β-D-mannosidase, the reaction of tetra-*O*-acetyl-α-D-mannopyranosyl bromide with *o*-nitrophenoxide ion led mainly to retention of configuration at C-1 in the product, as indicated by the rotation. Deacetylation led to only one isolatable product, the *o*-nitrophenyl α-D-mannopyranoside (50%), to which the α-configuration was assigned since it had a high positive rotation comparable with those of other phenyl α-D-mannopyranosides. The mother liquor (25%) also had a positive rotation, indicating that only a low proportion of any β-isomer was present. Similar results were obtained with *p*-nitrophenoxide ion. Retention of configuration is presumably due to the intermediate formation of an orthoester or a 1,2-acetonium ion, since, using silver carbonate as catalyst, Isbell & Frush (1949) have shown that the Koenigs–Knorr reaction of tetra-*O*-acetyl-α-D-mannopyranosyl bromide with methanol leads to mixtures of methyl tetra-*O*-acetyl-α- and -β-D-mannopyranosides and tri-*O*-acetyl-(1,2-methyl orthoacetyl)-D-mannopyranose. Although this procedure is therefore of little use for the preparation of β-D-mannopyranosides, it gives a convenient preparation of the α-D-mannopyranosides.

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