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# The Hydrolysis of Glycosyl Fluorides by Glycosidases

## DETERMINATION OF THE ANOMERIC CONFIGURATION OF THE PRODUCTS OF GLYCOSIDASE ACTION

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The enzymic hydrolysis of glycosyl fluorides is conveniently followed by using a pH-stat. Reactions involving glucosyl or galactosyl fluorides can also be followed by using glucose oxidase or galactose oxidase respectively. The pH-stat allows the rapid assay of intestinal α-glucosidase in crude homogenates. Use of glycosyl fluorides as substrates for glycosidases facilitates the polarimetric or g.l.c. determination of the anomeric nature of the initial product of hydrolysis. Hydrolysis by fungal amyloglucosidase proceeds with inversion of configuration whereas that by yeast and rat intestinal α-glucosidase, coffee-bean α-galactosidase and almond emulsin β-glucosidase proceeds with retention of configuration. β-D-Glucopyranosyl azide was not a detectable substrate for almond emulsin β-D-glucosidase.

Glycosyl fluorides have been shown to be excellent substrates for the corresponding glycosidase (Barnett, Jarvis & Munday, 1967*a*,*b*) and recently for the enzyme sucrose phosphorylase (Gold & Osber, 1971). The reaction has been followed either by determination of the fluoride liberated colorimetrically, or, in the special case of glucosyl fluorides, by the determination of free glucose by using glucose oxidase. In this paper an easy general assay for glycosidases is described, using glycosyl fluorides as substrates and titration of the hydrogen fluoride liberated in a pH-stat.

Full understanding of the mechanism of glycosidase action requires elucidation of the anomeric nature of the initial hydrolysis product. Amylases have been classified as α or β depending on the nature of the product (Kuhn, 1925), but the initial product of glycosidase action is not often determined. Three methods have been used to infer the nature of the initial product. One is isolation of a product that does not mutarotate, such as a methyl glycoside formed by solvolysis in methanol (Hash & King, 1958) or a known oligosaccharide formed in a transfer reaction (Rupley & Gates, 1967). This method suffers from the objection that it is not true hydrolysis and such transfer reactions cannot always be observed. Alternatively, normal aqueous hydrolysis is allowed to proceed under conditions in which the rate of mutarotation is minimized, but the rate of hydrolysis is fast. The anomeric configuration is then determined either by g.l.c. (Parrish & Reese, 1967) or polarimetrically (see Swain & Dekker, 1966). Suitable substrates for this purpose must be rapidly hydrolysed by the enzyme and in the latter case should preferably have an optically inactive aglycone. The paper explores the suitability of glycosyl fluorides as substrates for these determinations. A preliminary account of some of this work has been published (Barnett & Jarvis, 1967).

## EXPERIMENTAL

*Compounds.* α- and β-D-Glucopyranosyl fluoride, α-D-galactopyranosyl fluoride, rat intestinal α-D-glucopyranosidase and coffee-bean α-D-galactopyranosidase were prepared by the methods previously described (see Barnett *et al.* 1967*b*) as was β-D-glucopyranosyl azide (Micheel, Klemer, Baum, Ristic & Zumbulte, 1955). Yeast α-D-glucopyranosidase, and α-glucosidase-free glucose oxidase were obtained from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K.; galactose dehydrogenase was from The Boehringer Corporation (London) Ltd., London W.5, U.K.; *Aspergillus* glucoamylase was from Koch–Light Ltd., Colnbrook, Bucks., U.K., and almond β-glucosidase was from BDH Chemicals Ltd., Poole, Dorset, U.K.

*Enzyme units.* The unit of glycosidase activity used in this paper is defined as that amount of enzyme required to hydrolyse 1 mM-glycosyl fluoride at the rate of 1 μmol/min at 25°C in the glucose oxidase or galactose dehydrogenase assay system unless otherwise stated. The relative rates of hydrolysis of the fluorides compared with nitrophenyl glycosides or natural substrates are given elsewhere (Barnett *et al.* 1967*b*), except for yeast α-D-glucopyranoside, which hydrolysed α-D-glucopyranosyl fluoride at

0.51 times the rate of *p*-nitrophenyl α-D-glucopyranoside at 25°C and pH 7.0.

*Assay of glycosidase activity with the* pH-*stat.* The Radiometer pH-stat was adjusted to titrate to the constant pH required with 0.01 M-NaOH. Usually the 0.25 ml burette was used with a jacketed (25°C) micro-electrode assembly (TTA 31) with a maximum capacity of about 10 ml. The initial volume was 5.0 ml and during titration this did not increase by more than 4% (0.2 ml). The glycosyl fluoride was dissolved in water and the pH adjusted on the pH-stat to the required value (usually pH 6.0). The volume of the solution was then adjusted with water to give a concentration of 50 mM. The enzyme was added to 5% KCl (0.5 ml) and water (2 ml). The pH was adjusted to the required value with the pH-stat and then water was added to give a total volume of 4.5 ml. The system was left for at least 5 min to equilibrate to 25°C and 50 mM-glycosyl fluoride (0.5 ml) at 25°C was added. The rate was recorded as soon as the system had settled down, which was almost immediately. For determinations of $K_m$, different quantities of glycosyl fluoride were added and the preincubated volume was adjusted accordingly. The convenient sensitivity of the method was about 5 nmol of glycosyl fluoride hydrolysed/min (0.5 μl added/min). The method is limited to the pH range 5–8 owing to the instability of the fluorides in acid and alkali (Barnett, 1969). At 25°C the spontaneous hydrolysis of 5 mM-α-D-glucopyranosyl fluoride is about 2–3 nmol/min between pH 5 and pH 7. Outside this region more care is needed with controls. The rate of spontaneous hydrolysis increases more slowly at alkaline pH values with C-2 *cis*-glycosyl fluorides and is about 10 nmol/min at pH 4.5 and 30 nmol/min at pH 10 for α-D-glucopyranosyl fluoride. The $K_m$ of α-D-glucopyranosyl fluoride for rat intestinal α-D-glucosidase was 1.0 mM at 25°C and pH 6.0.

*Measurement of rat intestinal α-glucosidase in crude homogenates.* Pieces (10 mg) of washed (0.9% KCl) rat intestine were homogenized in 0.9% KCl. Homogenate (0.1 ml; 1 mg) was added to the standard pH-stat assay system and the enzyme activity measured: 1 mg contained about 0.02 unit of enzyme.

*Assay of glycosyl fluoride hydrolysis by titration.* In the absence of a pH-stat, measurement of glycosidases by hydrogen fluoride release can be successfully accomplished by using a micrometer syringe previously calibrated with mercury, and a Pye model 290 pH-meter. The procedure was identical with that described above except that the pH on the expanded scale was corrected manually to the required value, either at 2 min intervals or when it had drifted by 0.2 unit. The rate of addition was recorded. The $K_m$ of α-D-glucopyranosyl fluoride for rat intestinal α-D-glucosidase was 1.0 mM at pH 6.1 and 22°C.

*Assay of α-galactosidase with galactose dehydrogenase.* Coffee-bean α-galactosidase (0–0.1 unit) was added to α-D-galactopyranosyl fluoride (3 μmol), NAD (500 μg), sodium phosphate buffer, pH 6.2 (25 μmol), galactose dehydrogenase (40 μg), in a total volume of 3 ml. The $E_{340}$ was measured. After an initial lag period of up to 15 min a constant steady-state rate was obtained. This corresponded to the rate of the galactosidase reaction and was proportional to the amount of enzyme added. The kinetics of this system are identical with those for the measurement of α-D-glucosidase activity by glucose oxidase and the lag period corresponds to the time taken to produce the steady-state concentration of D-galactose utilized by the second enzyme (for a kinetic derivation see Barnett *et al.* 1967*a*). The $K_m$ by this method was 0.5 mM at pH 6.2 and 25°C.

*Assay of α-glucosidase activity with glucose oxidase.* The method of Barnett *et al.* (1967*a*) was used, α-glucosidase-free glucose oxidase being used.

*Polarimetric determination of the anomeric product of glycosidase action.* Optical rotations were measured with a Perkin–Elmer 141 polarimeter with a Servoscribe potentiometric recorder, by using 10 cm path-length cells which required 5 ml to fill the optical path but could hold 6.5 ml. Samples of 10 mM-α- or β-D-glucopyranosyl fluoride or α-D-galactopyranosyl fluoride (4.5 ml, 45 μmol) and 0.2 M-sodium maleate buffer, pH 6.0, (0.5 ml) were added to the cell and the rotation was measured. It was perfectly stable. The enzyme (at least 0.5 unit) was added at zero time and the hydrolysis followed polarimetrically for about 15 min. If the glycosidase formed the anomer with the same configuration only a small change in rotation occurred. After 15 min 1 M-$Na_2CO_3$ (0.2 ml) was added changing the pH to 10 and causing rapid mutarotation to the equilibrium mixture of the sugars. This concentration of alkali did not cause decomposition of any remaining glycosyl fluoride. Blanks lacked either glycosyl fluoride or the enzyme. Addition of alkali had no effect on the glycosyl fluorides, but in some cases caused a slight change in the rotation of the enzyme which was subtracted from the experimental value. To correct for the different dilutions used, specific rotations based on the initial weight of glycosyl fluoride were calculated.

*Determination by g.l.c. of the anomeric product of glycosidase action.* α- or β-D-Glucopyranosyl fluoride (0.9 mg, 5 μmol), sodium acetate buffer (20 μmol), pH 6.0, and enzyme (0.5 unit) in 0.5 ml total volume were incubated at 20°C. A 0.2 ml portion was withdrawn at 10 min and quickly freeze-dried. When it was completely dry 0.25 ml of a solution of pyridine–hexamethyldisilazane–trimethylchlorosilane (1:1:2, by vol.) was added and the solution stoppered. After standing overnight at room temperature the samples were centrifuged. The mixture of trimethylsilyl ethers was investigated by g.l.c. on a Pye 104 Gas Chromatograph, in a 60 in glass column of 3% E. 30 silicone oil as stationary phase on a 100–120 mesh Diatomite CQ support and with $N_2$ as carrier (flow rate 50 ml/min) and temperature 180°C. The trimethylsilyl ether of α- or β-D-glucopyranosyl fluoride was detected first followed by that of α- and then β-D-glucopyranose. Retention times were respectively 4 min for each of the fluorides and 5.1 and 7.8 min for the glucose derivatives. In the absence of enzyme no hydrolysis of α-D-glucopyranosyl fluoride was detected. The β-D-glucopyranosyl fluoride used contained a trace of α- and β-glucose impurities. This did not increase in the absence of enzyme and could be neglected. The ratio of β- to α-D-glucose was determined by assuming that the detector response to both derivatives was identical, and compared with standard equilibrated D-glucose. The results for yeast α-glucosidase and *Aspergillus* glucoamylase with α-D-glucopyranosyl fluoride and almond emulsin β-glucosidase with β-D-glucopyranosyl fluoride are shown in Table 1.

## RESULTS AND DISCUSSION

Glycosidases have been assayed by using aryl glycosides as substrates, since these substrates are often rapidly hydrolysed and can be easily detected spectrophotometrically. Alternatively they have been measured by using the increase in reducing power associated with hydrolysis of the glycosidic linkage, or by specific enzyme assays for one of the products, such as glucose. Although these methods are useful for many enzymes there can be difficulties in their use. Many glycosidases, including mammalian intestinal α-glucosidases, do not accept aryl glycoside as substrates, precluding the use of this method. Methods based on the assay of reducing power often require the removal of the protein on termination of the reaction. Assays using glucose oxidase can be complicated by the presence of α-glucosidase in many commercial preparations. An alternative to these methods is the titrimetric determination of the hydrogen fluoride liberated from glycosyl fluorides.

The method was shown to be simple and easy to use for α-D-glucosidases and α-D-galactosidases. Measurement of rat intestinal α-glucosidase required little preparation of tissue and was rapid and continuous. The validity of the method was

Table 1. *Ratio of β- to α-D-glucose in hydrolysates of α- and β-D-glucopyranosyl fluorides by glycosidases*

The β/α anomeric ratio was determined after 10min incubation of the enzyme with the corresponding glucosyl fluoride, by using g.l.c. of the trimethylsilyl ethers. A lower ratio than the equilibrium ratio indicates the release of the α-anomer, a higher ratio the release of the β-anomer.

| Hydrolysate | β/α ratio |
|---|---|
| Equilibrium D-glucose | 1.45 |
| Yeast α-glucosidase | 0.29 |
| *Aspergillus* α-glucosidase | 3.22 |
| Almond emulsin β-glucosidase | 3.18 |

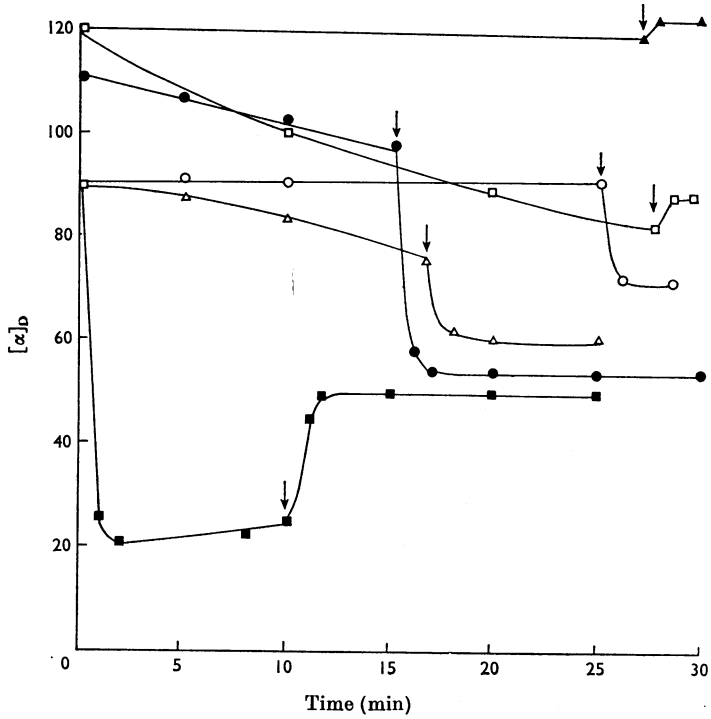


Fig. 1. Specific rotational changes during the hydrolysis of α-D-glucopyranosyl fluoride or maltose by α-D-glucosidases at pH6.0 and 25°C. Polarimeter traces were continuous, but not corrected for dilution changes. The points shown were taken at suitable times from the trace and rotations were converted into specific rotations by using the initial weight of α-D-glucopyranosyl fluoride or maltose. ■, *Aspergillus* glucoamylase and α-D-glucopyranosyl fluoride; ○, rat intestinal α-glucosidase and α-D-glucopyranosyl fluoride; △, yeast α-D-glucosidase and α-D-glucopyranosyl fluoride; □, *Aspergillus* glucoamylase and maltose; ▲, maltose alone; ●, D-glucose alone. The enzyme was added at zero time and 0.2ml of 1M-sodium carbonate at the time indicated by the arrow. α-D-Glucose was dissolved at zero time.

shown by comparison of the $K_m$ values determined by this method with those determined by spectrophotometric methods. They were identical within experimental error. It suffers from the disadvantage of a slightly decreased sensitivity compared with a glucose oxidase method and a limitation on the pH range which can be used without careful controls. α-D-Glucosyl fluoride, α-D-galactosyl fluoride, α-D-xylosyl fluoride and β-D-arabinosyl fluoride are stable in the pH range 5–8 (Barnett, 1969). The utility of the method for other glycosidases in which the corresponding fluoride has a *trans*-hydroxyl group at C-2 is limited by the instability of the fluorides except for β-D-glucopyranosyl fluoride, which crystallizes well and when stored dry at −14°C, keeps for several months.

For a full understanding of the mechanism of action of glycosidase activity, the initial product of hydrolysis must be known. In the direct methods of determination in which the free sugar is produced, this immediately begins to mutarotate. Substrates for such determinations should therefore be very rapidly hydrolysed. The polarimetric analysis of the nature of the initial product is complicated if the substrate is a disaccharide, since the second sugar can also mutarotate, and this is a complication in the g.l.c. method if the disaccharide contains only one sugar as in maltose. Glycosyl fluorides therefore seem ideal substrates for this type of determination since the reaction rates are always fast and the aglycone is fluoride.

By using the polarimetric method, the enzyme was allowed to react with substrate under conditions which minimized mutarotation, while the rotation was followed continuously. After about 15min alkali was added, which rapidly mutarotated the free sugar, leaving any remaining glycosyl fluoride unaffected. Two clear types of plot emerged in which the rotation approached the equilibrium value either from a lower or a higher value, indicating approach from either the β- or α-isomer respectively in the D-series (Figs. 1 and 2). Comparison of the method using maltose as a substrate for glucoamylase (Fig. 1) shows the superiority of α-D-glucosyl fluoride over maltose as a substrate for the enzyme. α-D-Glucose and α-D-galactose controls showed exactly the same behaviour towards alkali as the product of the enzyme reactions leading to the α-sugars, whereas the corresponding fluorides were unaffected by this concentration of alkali. Determination of the product of almond emulsin β-glucosidase action on β-D-glucopyranosyl fluoride was hampered by the high rotation of the crude enzyme preparation used. However, rotational changes indicated that β-D-glucopyranose was the initial product. In this case and in other similar cases, the g.l.c. method would be the method of choice.

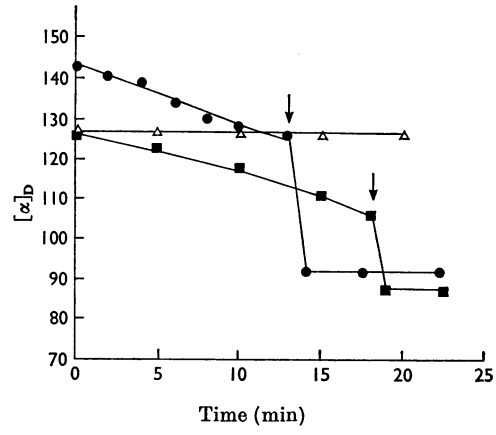


Fig. 2. Specific rotation changes during the hydrolysis of α-D-galactopyranosyl fluoride by α-D-galactosidase at pH6.0 and 25°C. ■, Coffee-bean α-galactosidase and α-D-galactopyranosyl fluoride; △, α-D-galactopyranosyl fluoride alone; ●, α-D-galactose alone. Results were corrected as described for Fig. 1. Enzyme was added at zero time and 0.2ml of 1M-sodium carbonate at the time indicated by the arrow (alkali was not added to the α-D-galactopyranosyl fluoride alone in this experiment; however, addition of alkali causes no change in rotation after 10min).

Analysis by g.l.c. of the products of glycosyl fluoride hydrolysis showed results very similar to those obtained by using oligosaccharide substrates (Parrish & Reese, 1967). Both α- and β-D-glucopyranosyl fluoride trimethylsilyl derivatives had an identical retention time, which was lower than that of the derivatives of α- and β-D-glucose. They produce no high-molecular-weight products, thus decreasing the time for each chromatographic run compared with oligosaccharide substrates. The reactions were also performed at room temperature, rather than the elevated temperature (40°C) previously used. The results (Table 1) clearly show the predominance of one anomer over the other after 10min incubation and, with both α- and β-glucopyranosyl fluoride, compare favourably with those of Parrish & Reese (1967) who comment on the difficulty of finding a suitable substrate for β-glucosidases. Despite its instability to prolonged storage, β-D-glucopyranosyl fluoride must be regarded as a suitable general substrate.

The g.l.c. method has the advantage over the polarimetric method that it is unaffected by impurities (other than glucose) in the enzyme preparation and can be used with a much lower percentage hydrolysis than that required by the polarimetric method.

The initial product of glucoamylase action as

previously reported (Barnett & Jarvis, 1967; Parrish & Reese, 1967) is β-D-glucose. The other glycosidases, yeast and rat intestinal α-D-glucosidase, and coffee-bean α-D-galactosidase, are shown to hydrolyse with retention of configuration like the previously described (Parrish & Reese, 1967) almond emulsin β-glucosidase. These results support the hypothesis (Parrish & Reese, 1967) that the exoglucanases hydrolyse with inversion of configuration, but true glycosidases hydrolyse with retention of configuration.

Glucopyranosyl azides have a very similar structure both to glycosides and glycosyl fluorides. β-D-Glucopyranosyl azide was not, however, a detectable substrate for 1.0 unit of almond emulsin β-galactosidase at pH 5.5 in the standard pH-stat assay (rate <0.1% of that of β-D-glucopyranosyl fluoride).

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