Enzymic Hydrolysis of the Carbon-Fluorine Bond of α -D-Glucosyl Fluoride by Rat Intestinal Mucosa

LOCALIZATION OF INTESTINAL MALTASE

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1. α -D-Glucosyl fluoride was hydrolysed by an extract of rat intestinal mucosa. The pH optimum was 6.6 and the K_m 0.4 mM at 20°. Activity was assayed by release of either glucose or fluoride.
2. The α -D-glucosyl fluoride-hydrolase activity of the extract was associated with both mutarotase and α -D-glucosidase activities.
3. Tris (5 mM) inhibited both the α -D-glucosidase and α -D-glucosyl fluoride-hydrolase activities by 55% but did not inhibit mutarotase. The K_i of tris for both enzyme activities was 2 mM.
4. The extract did not hydrolyse melibiose and lactose. Mutarotase used both α -D-glucose and β -L-arabinose as substrates but the glucosyl fluoride-hydrolase activity did not extend to β -L-arabinosyl fluoride.
5. The thermal stability of α -D-glucosidase and α -D-glucosyl fluoride hydrolase was identical. Mutarotase was more thermolabile.
6. A preparation of the brush border of intestinal epithelial cells contained both α -D-glucosyl fluoride-hydrolase and α -D-glucosidase activities. In each precipitate and washing the ratio of the two activities was the same. All the mutarotase activity was in the first supernatant.
7. Agidex, a fungal amyloglucosidase, cleaved glucosyl fluoride in addition to maltose. Tris inhibited both activities and in each case the K_i was 3 mM.
8. The probable identity of α -D-glucosyl fluoride hydrolase with α -D-glucosidase is discussed and a possible mechanism for the reaction suggested.
9. Incubation of intestinal slices with α -D-glucosyl fluoride led to complete hydrolysis in 30 min. The glucose rapidly entered the cell and was metabolized, leaving the fluoride in the incubation medium. This constitutes a further proof that the intestinal α -D-glucosidase, although on the brush border, is located outside the site of active transport of sugars.

In an attempt to find a reagent that might form a covalent bond with the intestinal 'carrier' for active sugar transport, α -D-glucosyl fluoride was investigated. It was observed that α -D-glucosyl fluoride was rapidly enzymically hydrolysed to glucose and F^- ion. Most previous reports of carbon-fluorine bond cleavage have been confined to hydrolysis of fluoroacetic acid in bacteria (Goldman, 1965) although *p*-fluorophenylalanine has been shown to be converted by an oxidative mechanism into tyrosine (Kaufman, 1961) in rat and sheep liver. The present paper describes the properties of the α -D-glucosyl fluoride hydrolase, and some tissue accumulation experiments with α -D-glucosyl fluoride. A preliminary account of part of this work has been published (Barnett, Jarvis & Munday, 1966).

MATERIALS AND METHODS

Materials

Chemicals. α -D-Glucosyl fluoride, m.p. 120–130° (decomp.), $[\alpha]_D^{20} + 90 \pm 2^\circ$ (c 1.0 in water) (Helferich, Bauerlein

& Weigand, 1926), and β -L-arabinosyl fluoride, m.p. 92–96° (decomp.), $[\alpha]_D^{20} - 170 \pm 5^\circ$ (c 0.4 in water) (Kent & Barnett, 1964), were prepared by sodium methoxide deacetylation of the respective acetyl fluorides. Purity was estimated to be greater than 99% by paper chromatography (see below). Other sugars, tris, *o*-dianisidine and the enzyme preparations glucose oxidase and peroxidase were obtained from British Drug Houses Ltd. (Poole, Dorset). *p*-Nitrophenyl α -D-glucoside and 3-*O*-methyl-D-glucose were obtained from Koch-Light Laboratories Ltd. (Colnbrook, Bucks.). Agidex was a gift from Glaxo Laboratories Ltd. (Greenford, Middlesex).

Preparation of mucosal extract. The gut of a hooded rat was removed after anaesthesia with ether and rinsing with ice-cold glucose-free Krebs-Henseleit medium (Krebs & Henseleit, 1932) *in situ*. After eversion of the gut on a glass rod, the mucosal layer was scraped into 5 mM-EDTA, pH 7.4, at 2° to give a 1% suspension (5% for mutarotase assay), homogenized in a hand-operated ground-glass homogenizer, and centrifuged at 2° and 26 000g for 60 min. or until clear. The supernatant was the 'mucosal extract'. In some cases EDTA solution containing 0.05% sodium deoxycholate, which released the hydrolases, was used. Since EDTA interfered with fluoride estimations it was omitted in

assays in which the release of fluoride was measured.

Brush-border preparation. This was made by the method of Gallo & Treadwell (1963). Enzymes were released for assay by treatment with ultrasound for 3 min. in a 60 W MSE Sonicator cooled with ice-water. The solution was centrifuged at 32 000*g* for 60 min. and the supernatant used.

Assay of enzyme activities

α -D-Glucosyl fluoride hydrolase and α -D-glucosidase: glucose-oxidase method A. A 1.0 ml. portion of mucosal extract was added to 1 ml. of 3 mM-glucosyl fluoride or -maltose and 1 ml. of 'glucose oxidase reagent' containing 125 mg. of glucose oxidase, 5 mg. of peroxidase and 1.0 ml. of 1% *o*-dianisidine in 100 ml. of 0.5 M-sodium phosphate buffer, pH 7 (Huggett & Nixon, 1957). The colour developed at 420 $m\mu$ was measured in a Unicam SP. 600 against a blank containing 5 mM-EDTA in place of enzyme extract. After an initial lag period, a steady rate of colour development was obtained (Fig. 1). When the system had reached this steady state (10–50 min.), either the glucose-oxidase system was saturated, in which case neither more glucosyl fluoride hydrolase nor more substrate would have increased the rate, or the following conditions applied. The rate of hydrolysis of glucosyl fluoride, or production of glucose, exactly equalled the rate of utilization of glucose by the glucose-oxidase system. This is proportional to the rate of colour development, which must itself therefore be directly proportional to the rate of hydrolysis of glucosyl fluoride. Under the steady-state conditions, therefore:

$$\begin{aligned} \frac{-d(\text{glucosyl fluoride})}{dt} &= \frac{d(\text{glucose})}{dt} = \frac{-d(\text{glucose})}{dt} \\ &= k \frac{d(\text{colour})}{dt} \end{aligned}$$

Fig. 1. Variation of extinction at 420 $m\mu$ with time for various concentrations of mucosal extract incubated at 25° with 1 mM-glucosyl fluoride and the glucose-oxidase system (method A). Volumes of 5% mucosal extract in 3 ml.: 0.05 ml. (●), 0.10 ml. (○), 0.15 ml. (△), 0.25 ml. (■). The gradients from the straight-line parts of these curves were used to construct Fig. 2.

A standard curve for glucose was constructed by incubating with the 'glucose oxidase reagent' for 2 hr. at 25°; this allowed the colour (as extinction) per mole of glucose to be calculated. The rate of colour production during the hydrolysis of glucosyl fluoride was computed from the straight-line or steady-rate portion of the experimental graphs. The molar rate of oxidation of glucose, and hence the rate of formation of glucose from glucosyl fluoride, could thus be determined. The rate of glucose formation measured by this method was equal to the rate of fluoride formation (Fig. 2). In the range used both were proportional to the enzyme concentration. At high concentrations of substrate the rate of glucose formation fell below the rate of fluoride formation. This was accounted for by the transferase activity of the enzyme at these concentrations leading to products other than glucose. This should have given apparent substrate inhibition in K_m curves determined by this method, but in practice this was negligible.

This method does not allow the initial rate of hydrolysis to be measured owing to the lag period, but in all cases the steady rate was obtained before more than 3% of the glucosyl fluoride had been hydrolysed. The discrepancy between measured rate and true initial rate should therefore not be large and should permit the simple kinetic studies described in this paper.

Glycosidases: glucose-oxidase method B. A 1 ml. sample of mucosal extract was added to 0.5 ml. of 10 mM-disaccharide (maltose, lactose or melibiose) and 3.5 ml. of 0.02 M-sodium phosphate buffer, pH 4–8. After 0 and 30 min., 0.5 ml. samples were withdrawn and added to 1 ml. of 0.5 M-

Fig. 2. Variation of rate of hydrolysis of glucosyl fluoride with enzyme concentration at 25° and pH 7.0 measured by fluoride release (●) and glucose-oxidase method A (□). The independent reaction mixtures contained glucosyl fluoride (2 mM), 5% mucosal extract and sodium maleate buffer, pH 7.0 (50 mM), in 3 ml. In the measurements by fluoride release ethanol (2 ml.) was added to portions of the mixture (1 ml.) measured at suitable time-intervals and the fluoride content assayed. In the glucose-oxidase method the reaction mixture also contained the components of the glucose-oxidase system other than the sodium phosphate buffer. The molar rate of glucose formation was obtained by comparison of the colour developed with that of standard quantities of glucose under identical conditions for an 'infinite' time (2 hr.).

sodium phosphate buffer, pH 7, at 100° and maintained at this temperature for 10 min. Then 1 ml. of cooled solution was added to 2 ml. of 'glucose oxidase reagent', incubated for 60 min. at 37° and the extinction measured at 420 m μ . The glucose oxidase preparation contained a little α -D-glucosidase and α -D-glucosyl fluoride-hydrolase activities that did not significantly interfere with this or the previous assay. This method could not be used with glucosyl fluoride owing to its instability at 100°.

Glucosyl fluoride hydrolase by release of F⁻. A 0.1 ml. sample of EDTA-free mucosal extract was added to 0.5 ml. of 4 mM- α -D-glucosyl fluoride or β -L-arabinosyl fluoride and 0.4 ml. of 0.02 M-sodium maleate, pH 4-7, or sodium hydrogen carbonate buffer, pH 6-8. After 10 min. incubation the reaction was stopped with 2 ml. of ethanol. Portions of the solution containing 1-3 μ g. of released F⁻ ion were estimated by the method of Belcher, Leonard & West (1959) on one-tenth scale.

Assay of α -D-glucosidase with o-nitrophenyl α -D-glucoside. α -D-Glucosidase was assayed by a method similar to that of Kuby & Lardy (1953).

Mutarotase. This was assayed by the method of Bailey & Pentchev (1965) with a 2 dm. jacketed cell in a Hilger and Watts standard polarimeter Mk III at 589 m μ . First-order rate constants were derived from the plot of $\log(\alpha_T - \alpha_E)$ against T , where α_T was the rotation at time T and α_E the rotation at equilibrium. Units of enzyme activity were determined from the expression: units = $(K_{cat} - K_{spont.}) / K_{spont.} \times 10$ where K_{cat} is the rate constant in the presence of enzyme, $K_{spont.}$ is the rate constant in its absence, since 1 unit of enzyme activity is defined as the amount that increases the spontaneous rate by 10%.

Other methods

Chromatography. Thin-layer chromatography was performed on silica gel (Kieselgel G nach Stahl; E. Merck A.-G., Darmstadt, Germany) pre-run in 0.02 M-sodium acetate and oven-dried, developed with ethyl acetate-propan-2-ol-water (26:14:7, by vol.). Spots were detected with anisaldehyde and conc. H₂SO₄. Paper chromatography was performed on Whatman no. 1 paper and developed with butanol-ethanol-water (4:1:5, by vol.; organic layer). Spots were detected with alkaline AgNO₃ (Trevelyan, Procter & Harrison, 1950).

Tissue accumulation experiment. The method used was that of Crane & Mandelstam (1960) for intact strips of everted rat intestine. A 1 mM concentration of sugar was used in all experiments and the tissue was incubated at 37° for 30 min. Reducing sugar was measured by the method of Nelson (1944) and fluorine by the micro-diffusion method of Hall (1960) followed by the fluoride method of Belcher *et al.* (1959). The pH was adjusted to about 4.3 with acetic acid before the estimation.

RESULTS

Products of enzymic cleavage of α -D-glucosyl fluoride. A 5 ml. sample of 1 mM-glucosyl fluoride in Krebs-Henseleit medium, pH 7.4, was incubated for 30 min. at 37° with about 1 g. of everted rat intestine. Thin-layer chromatography of the supernatant showed only one spot, corresponding to glucose, R_f 0.12. α -D-Glucosyl fluoride (R_f 0.20) was not

detectably hydrolysed under these conditions in the absence of gut. In the absence of glucosyl fluoride no sugars were detected. The activity was destroyed by boiling for 10 min.

Demonstration of transferase activity. A 1.0 ml. sample of 6 mM-glucosyl fluoride, 1.0 ml. of 1.5 mM-glucose containing [U-¹⁴C]glucose (5×10^5 counts/min.), 0.7 ml. of 50 mM-sodium maleate buffer, pH 7.0, and 0.3 ml. of 2% mucosal extract were incubated at 25° for 2 hr., when about 20% of the glucosyl fluoride was hydrolysed. Ethanol (6 ml.) was added to stop the reaction and the products were chromatographed on paper after deionization. Four spots (R_{glc} 2.1, 1.0, 0.80 and 0.36) were obtained that corresponded to glucosyl fluoride, glucose, presumably glucosylglucosyl fluoride and glucosylglucose (maltose has R_{glc} 0.36) were obtained. The ratio of activity in the glucose and glucosylglucose spots was 100:2.1 measured by a Baird and Tatlock Radioactive Chromatogram Counter.

Comparison of substrate specificity of mutarotase and α -D-glucosyl fluoride hydrolase. The sugars previously shown to be mutarotated by mutarotase were D-glucose, L-arabinose, D-galactose and D-xylose (Bailey & Pentchev, 1965). D-Galactosyl fluoride is known only in the unstable β -form in which the fluorine is *trans* to a hydroxyl group. β -L-Arabinosyl fluoride and β -L-arabinose were therefore chosen as the most suitable compounds to compare substrate specificity for the two types of enzyme activity. α -D-Glucose (46.2 units/g. at 37°) and β -L-arabinose (42.1 units/g. at 18°) were mutarotated by the mucosal extract. β -L-Arabinosyl fluoride was not detectably hydrolysed by the extract over the pH range 4-8 at 37°.

Substrate specificity of glucosidase activity. β -D-Galactosidase (lactose as substrate) and α -D-galactosidase (melibiose as substrate) could not be detected in the mucosal extract. At pH 7.0 and 20° 1 mM-o-nitrophenyl α -D-glucoside was a poor substrate (0.032 unit/g.) compared with 1 mM-maltose (0.65 unit/g.), where 1 unit is equivalent to 1 μ mole of substrate hydrolysed/min.

Thermal stability of α -D-glucosidase, α -D-glucosyl fluoride hydrolase and mutarotase. The mucosa extract was pre-incubated for 10 min. at temperatures between 0° and 70°, cooled rapidly at 0° and then assayed for α -D-glucosidase and α -D-glucosyl fluoride-hydrolase activities at 25° by the glucose-oxidase method A, and mutarotase at 37° with α -D-glucose as substrate. Activities were measured as a percentage of the activity of a sample maintained at 0° (Fig. 3).

Optimum pH and K_m values for α -D-glucosidase and α -D-glucosyl fluoride hydrolase. pH-activity curves for each enzyme were measured by the glucose-oxidase method B and fluoride release

Fig. 3. Activity of α -D-glucosyl fluoride hydrolase (●), α -D-glucosidase (○) and mutarotase (□) in the mucosal extract after preincubation for 10 min. at various temperatures, expressed as a percentage of that found after preincubation at 0°. α -D-Glucosyl fluoride hydrolase and α -D-glucosidase were assayed by glucose-oxidase method A at pH 6.5 and 25°. Mutarotase was assayed with glucose as substrate at pH 6.5 and 37°.

respectively. All the curves were identical over the range pH 4–8, giving an optimum at pH 6.5–7.0. Sodium phosphate buffer (0.02M) was used for α -D-glucosidase activity, but this interfered with the fluoride determination and therefore 0.02M-sodium maleate and sodium hydrogen carbonate buffers were used for the α -D-glucosyl fluoride-hydrolase activity. The K_m of α -D-glucosidase for maltose was determined by glucose-oxidase methods A and B to test the assumptions made in method A. The K_m by both methods was 1.3mM at 20°. The K_m of α -D-glucosyl fluoride for the hydrolase measured by release of fluoride was 0.36mM and by glucose-oxidase method A 0.55mM.

Inhibition of enzyme activities by tris. Assays were as described in the Materials and Methods section except that tris, adjusted to the correct pH with hydrochloric acid, was added to give the required concentration. Glucose oxidase was unaffected by tris and the small effect on the blanks due to the α -glucosidase present was negligible.

α -D-Glucosyl fluoride-hydrolase and α -D-glucosidase activities measured by the glucose-oxidase method A at pH 7.0 and 20° showed an inhibition with 5mM-tris of 55% in each case. The K_i values of tris for α -D-glucosyl fluoride-hydrolase and α -D-glucosidase activities were 2.3mM and 1.8mM respectively at 25° (Fig. 4). Mutarotase with α -D-glucose as substrate at pH 7.0 and 37° showed no inhibition with 10mM-tris.

Assay of relative enzyme activities during a preparation of brush borders. A standard preparation of brush borders was carried out and the α -D-glucosyl

Fig. 4. Lineweaver-Burk plots of $1/v$ against $1/s$ for glucosyl fluoride in the presence (●) and absence (○) of 5 mM-tris at pH 6.5 and 25°. Glucosyl fluoride hydrolase was measured by glucose-oxidase method A.

Table 1. *Percentage of total activity in the fractions of a brush-border preparation*

The method used was that of Gallo & Treadwell (1963).

Fraction	α -D-Glucosyl fluoride hydrolase	α -D-Glucosidase	Mutarotase
Homogenate	100	100	100
Supernatant after 10 min. at 1500g	28	26	105
Precipitate after 10 min. at 1500g	73	69	0
Supernatant after washing	19	19	0
Precipitate after washing	57	53	0

sidase (maltase), α -D-glucosyl fluoride-hydrolase and mutarotase activities were determined at each stage. Table 1 shows that the former two activities have a constant ratio at each stage, and the activity is located mainly in the brush border, whereas mutarotase is found in the supernatant fraction.

Activity of Agidex towards α -D-glucosyl fluoride. Agidex hydrolysed both maltose and α -D-glucosyl fluoride. The K_m values at 25° were 1.3mM and 3.1mM respectively.

Both activities were inhibited by 5mM-tris, to the extent of 34% and 50% respectively. The K_i of tris for both α -D-glucosyl fluoride-hydrolase and α -D-glucosidase activities was 3.2mM at 25°. The preparation showed no mutarotase activity.

Tissue accumulation of α -D-glucosyl fluoride. The initial and final medium concentrations, final tissue concentration and tissue/medium ratio of reducing sugar, and, for α -D-glucosyl fluoride, also of fluoride, together with the percentage recoveries,

Table 2. *Tissue accumulation experiments*

Sugar (initial concn. 1 mM)	Final medium concn. (mM)		Final tissue concn. (mM)		Tissue/medium ratio		Recovery (%)	
	Reducing sugar	Fluoride	Reducing sugar	Fluoride	Reducing sugar	Fluoride	Reducing sugar	Fluoride
D-Galactose	0.70	—	2.81	—	4.0	—	83	—
3-O-Methylglucose	0.60	—	2.59	—	4.3	—	94	—
D-Mannose	0.95	—	0.40	—	0.42	—	102	—
D-Glucose	0.22	—	0.27	—	1.2	—	25	—
D-Glucose	0.14	—	0.36	—	2.5	—	25	—
D-Glucose	0.11	—	0.32	—	2.9	—	17	—
α -D-Glucosyl fluoride	0.12	1.0	0.18	0.37	1.5	0.37	14	105
α -D-Glucosyl fluoride	0.0	1.0	1.18	0.16	∞	0.16	18	103
α -D-Glucosyl fluoride	0.0	1.0	1.41	0.17	∞	0.17	22	105

are shown in Table 2. Good recoveries are obtained for the sugars D-galactose and 3-O-methylglucose, which are actively transported but are not extensively metabolized, and for D-mannose, which is not actively transported. Measured by the reducing-sugar method, D-glucose and α -D-glucosyl fluoride appear to be actively transported but extensively metabolized, and the recovery of fluoride is complete. Chromatography showed that α -D-glucosyl fluoride was completely converted into glucose and fluoride after 30min. incubation with everted rat intestine.

DISCUSSION

Of the enzymes previously known to occur in the rat intestine, those that might hydrolyse α -D-glucosyl fluoride were mutarotase (Bailey & Pentchev, 1965) and α -glucosidase (Ruttloff, Noack, Friese & Schenk, 1964). The mucosal extract contained both of these activities. The following observations indicate that the α -D-glucosyl fluoride hydrolase is α -D-glucosidase.

The glucosidase activity was specific for α -D-glucosidase whereas the mutarotase could use β -L-arabinose as a substrate. β -L-Arabinosyl fluoride was not a substrate for the fluoride hydrolase, which therefore resembled the α -glucosidase rather than mutarotase as far as specificity could be tested.

Previous reports indicated that α -D-glucosidase was located in the brush borders (Miller & Crane, 1961) whereas mutarotase was not (Bailey & Pentchev, 1965). Both these observations were confirmed and at each stage of the preparation the ratio of glucosidase to glucosyl fluoride hydrolase was identical (Table 1).

α -D-Glucosidase has previously been shown to be inhibited by tris (Larner & Gillespie, 1956). This observation was confirmed. The inhibition constants (K_i) of tris for both α -D-glucosidase and α -D-glucosyl fluoride-hydrolase activities were,

within experimental error, identical, both for the intestinal enzyme and for Agidex, although the Michaelis constants (K_m) were not. This is as predicted for a single enzyme activity on two different substrates. Mutarotase activity was not significantly inhibited by tris.

The thermal stabilities of the α -D-glucosidase and α -D-glucosyl fluoride-hydrolase activities were identical. Mutarotase was more thermolabile (Fig. 3).

Previously, fluorinated analogues of metabolites have been observed both to inhibit and to be utilized by enzyme systems with the fluorine intact. Only rarely have there been reports of enzymic carbon-fluorine bond cleavage (Goldman, 1965). There is no other case known in which the fluorine acts as an analogue of the alkoxy radical in this way.

The mechanism of glycosidases is believed to proceed in most cases via a specific enzyme-glycoside complex in which the alkoxide ion is initially displaced after protonation, the nature of the leaving ion being unimportant. This mechanism would allow the expulsion of the readily protonated fluorine atom (Scheme 1). The non-enzymic hydrolysis of glycosides and glucosyl fluorides is also believed to proceed by the same mechanism (Kent & Barnett, 1964).

α -D-Glucosyl fluoride might have been expected to be actively transported by intestine since it has the correct structural requirements (Crane, 1960). However, the tissue accumulation experiments (Table 2) indicate that it is first hydrolysed to glucose and fluoride. The glucose is transported into the cell and metabolized, whereas the fluoride remains in the mucosal medium. Since the glucosyl fluoride-hydrolase activity is due to the intestinal maltase, which is located on the brush border of the epithelial cell, it follows that the site of intestinal maltase is on the surface of the cell, but outside the active transport system. This result was previously deduced by Miller & Crane (1961) from their

Scheme 1.

observations on the action of the inhibitor phlorrhizin, which selectively blocks active transport but not intestinal maltase.

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