Substrate Specificity and Kinetic Properties of α -Galactosidases from *Vicia faba*

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1. The hydrolysis of a variety of galactosides and other glycosides by α -galactosidases I and II of *Vicia faba* was studied. 2. The effect of temperature on kinetic parameters was also examined. 3. Both enzymes are inhibited by excess of substrate (*p*-nitrophenyl α -D-galactoside); with enzyme I this is competitive and is caused by the galactosyl moiety. 4. Enzyme I is inhibited by oligosaccharides possessing terminal non-reducing galactose residues and to a smaller extent by L-arabinose and D-fucose. 5. The effect of pH on K_m and V_{max} values suggests that carboxyl and imidazole groups are involved in the catalytic activity of enzyme I. 6. Photo-oxidation experiments with enzyme I also suggest that an imidazole group is present at the active site.

The presence of different molecular forms of α -galactosidase in broad bean (*Vicia faba*) and other seeds was reported by Dey & Pridham (1968, 1969*a, b*). Forms I and II from dormant broad beans have been purified and shown to have different molecular weights and properties (Dey & Pridham, 1968, 1969*a*). This paper describes a detailed study of the specificities and catalytic actions of the enzymes. The investigation was carried out with a view to understanding the physiological role of α -galactosidases in germinating seeds.

MATERIALS AND METHODS

Preparation of substrates. Alkyl α -D-galactosides were prepared by the method of Dale & Hudson (1930) and the aryl α -D-galactosides by a method reported earlier (Dey, 1967; Malhotra & Dey, 1967). *p*-Nitrophenyl β -L-arabinoside was prepared by the method of Feier & Westphal (1956) and *p*-nitrophenyl α -D- and β -D-glucosides were prepared by the method of Montgomery, Richtmyer & Hudson (1942). *p*-Nitrophenyl α -D-fucoside was a gift from Dr G. A. Levvy, Rowett Research Institute, Bucksburn, Aberdeen. The rest of the substrates were pure products obtained from commercial suppliers.

The physical constants of 6-bromo-2-naphthyl α -D-galactoside were found to be different from those reported by Monis, Tsou & Seligman (1963). The results are compared as follows: 6-Bromo-2-naphthyl tetra-*O*-acetyl- α -D-galactoside, m.p. 155–156°, $[\alpha]_D^{20} + 175^\circ$ (c 1.0 in CHCl_3) (Found: C, 52.0; H, 4.6; Br, 14.5. Calc. for $\text{C}_{24}\text{H}_{25}\text{BrO}_{10}$: C, 52.1; H, 4.5; Br, 14.5%). The values reported by Monis *et al.* (1963) were: m.p. 75–85°, $[\alpha]_D^{24} + 94^\circ$ (c 1.3 in dioxan). 6-Bromo-2-naphthyl α -D-galactoside, m.p. 179–180°, $[\alpha]_D^{20} + 200^\circ$ (c 1.0 in methanol) (Found: C, 50.2; H, 4.5; Br, 21.4. Calc. for $\text{C}_{16}\text{H}_{17}\text{BrO}_6$: C, 49.9; H, 4.5; Br, 20.7%). The

literature values were: m.p. 225°, $[\alpha]_D^{28} + 60^\circ$ (c 1.2 in pyridine). The high optical rotation of the compounds obtained by us is characteristic of the α -isomer and is comparable with those of other aromatic α -D-galactosides (Dey, 1967).

Enzyme preparation. α -Galactosidases I and II were separated and purified as described previously (Dey & Pridham, 1969*a*).

Analytical methods. (a) Substrate specificity. Initial rates of hydrolyses were measured in McIlvaine (1921) buffer, pH 4.0, under the conditions described by Dey & Pridham (1969*a*). In all cases constant rates were observed over the incubation periods used.

Hydrolyses of all nitrophenyl glycosides were followed by determination of the liberated nitrophenol at 405 nm.; the extinction coefficients of the various nitrophenols under the assay conditions used were: *o*-nitrophenol, 4.05×10^3 ; *m*-nitrophenol, 1.56×10^4 ; *p*-nitrophenol, $1.53 \times 10^4 \text{ cm}^2/\text{m-mole}$.

The release of *p*-aminophenol from *p*-aminophenyl α -D-galactoside was measured by the method of Furth & Robinson (1965).

To follow the enzymic hydrolysis of 6-bromo-2-naphthyl α -D-galactoside, a 10 mM solution of substrate in methanol was prepared. This was diluted tenfold in McIlvaine buffer, pH 4.0, before assay. This solution (0.05–0.3 ml.) and the enzyme (1 μg .) (total volume of digest 0.4 ml.) were incubated for 30 min. at 30°. The reaction was stopped by adding 0.1 M-NaOH (4 ml.) and the liberated bromonaphthol determined at 296 nm. This method of determination was 10 times as sensitive as that described by Tsou & Su (1964) with this same substrate.

The hydrolysis of melibiose was followed by determining the liberated glucose with glucose oxidase as described by Huggett & Nixon (1957).

Hydrolysis of α -D-galactose 1-phosphate was detected qualitatively by paper chromatography with butan-1-ol-ethanol-water (40:11:19, by vol.) solvent, and the liberated

galactose was located with AgNO_3 reagent (Trevelyan, Procter & Harrison, 1950).

The release of galactose from other galactosides was measured by the micro-cuprimetric method of Nelson (1944) with arsenomolybdate reagent (Hestrin, Feingold & Schramm, 1955).

(b) Effect of temperature. Enzymic hydrolysis of *p*-nitrophenyl α -D-galactoside was measured under the standard assay conditions with various concentrations of substrate (0.15–0.9 mM). The values of K_m and $V_{\max}$ were determined at 30°, 35°, 40° and 45° by the double-reciprocal method of Lineweaver & Burk (1934). There was no thermal inactivation of the enzyme in this temperature range (Dey & Pridham, 1969a).

(c) Effect of pH. McIlvaine buffer, pH 2.5–7.5, was used to determine the K_m and $V_{\max}$ values for the hydrolysis of *p*-nitrophenyl α -D-galactoside and raffinose at various concentrations and for each pH value a Lineweaver–Burk plot was constructed. α -Galactosidase I only was used for this study.

(d) Inhibition. The inhibition studies were carried out with α -galactosidase I in McIlvaine buffer, pH 4.0, with *p*-nitrophenyl α -D-galactoside (0.6 mM final concentration) as substrate.

In the photo-oxidation experiment a test tube (1 cm. $\times$ 5 cm.) containing 2 ml. of a solution consisting of 1 μ g. of α -galactosidase I, 0.02% Methylene Blue and McIlvaine

buffer, pH 6.0, was placed 10 cm. from two Shandon Photol fluorescent lamps. The solution was kept cold with ice-water during irradiation. Samples (0.2 ml.) were withdrawn at various times and assayed for enzyme activity with *p*-nitrophenyl α -D-galactoside as substrate. Parallel experiments were performed while bubbling air through the reaction mixture. In all cases control experiments were carried out in the absence of light.

RESULTS AND DISCUSSION

Substrate specificity. The $V_{\max}$ and K_m values for the hydrolysis of various substrates by α -galactosidases I and II are presented in Table 1. Neither enzyme was able to hydrolyse *p*-nitrophenyl β -D-galactoside and *p*-nitrophenyl α - and β -D-glucosides. This shows that the configuration at C-1 and C-4 of the substrate is important. On the other hand the structure at C-6 of the galactose moiety of an α -D-galactoside seems to be relatively less important with enzyme I, as the change from $-\text{CH}_2\cdot\text{OH}$ to $-\text{CH}_3$ (i.e. α -D-galactoside to α -D-fucoside) does not cause any appreciable decrease in the $V_{\max}$ value. Enzyme II hydrolyses *p*-nitrophenyl α -D-fucoside almost three times as fast as the corresponding

Table 1. *Specificity of α -galactosidases from Vicia faba*

Substrate	α -Galactosidase I		α -Galactosidase II		$V_{\max}$ ratio (I/II)	K_m ratio (I/II)
	$V_{\max}$. (μ moles min. ⁻¹ mg. ⁻¹)	$10^3 \times K_m$ (M)	$V_{\max}$. (μ moles min. ⁻¹ mg. ⁻¹)	$10^3 \times K_m$ (M)		
Glycone and stereochemical specificity:						
<i>p</i> -Nitrophenyl α -D-galactoside	25.53	0.38	2.39	0.45	10.68	0.84
<i>p</i> -Nitrophenyl α -D-fucoside	24.10	4.76	6.96	5.88	3.46	0.81
<i>p</i> -Nitrophenyl β -L-arabinoside	16.40	14.30	2.39	12.50	6.86	1.14
<i>p</i> -Nitrophenyl α -D-glucoside	Not hydrolysed		Not hydrolysed		—	—
<i>p</i> -Nitrophenyl β -D-glucoside	Not hydrolysed		Not hydrolysed		—	—
<i>p</i> -Nitrophenyl β -D-galactoside	Not hydrolysed		Not hydrolysed		—	—
Sucrose	Not hydrolysed		Not hydrolysed		—	—
Aglycone specificity:						
Methyl α -D-galactoside	1.66	7.13	0.29	14.30	5.72	0.49
Ethyl α -D-galactoside	1.66	8.93	0.28	8.00	5.93	1.11
<i>n</i> -Propyl α -D-galactoside	2.20	6.13	0.27	5.88	8.14	1.04
Phenyl α -D-galactoside	20.30	1.11	4.36	1.25	4.65	0.88
<i>o</i> -Cresyl α -D-galactoside	26.00	1.33	2.90	0.78	8.96	1.70
<i>m</i> -Cresyl α -D-galactoside	24.30	1.38	2.80	2.00	8.68	0.69
<i>p</i> -Cresyl α -D-galactoside	23.00	1.54	2.43	1.00	9.40	1.54
<i>p</i> -Aminophenyl α -D-galactoside	26.60	0.95	2.72	0.87	9.77	1.09
<i>m</i> -Chlorophenyl α -D-galactoside	20.60	0.83	3.30	1.17	6.24	0.79
<i>o</i> -Nitrophenyl α -D-galactoside	42.10	1.14	2.80	0.69	15.03	1.65
<i>m</i> -Nitrophenyl α -D-galactoside	5.86	10.00	0.31	2.50	18.90	4.00
<i>p</i> -Nitrophenyl α -D-galactoside	25.53	0.38	2.39	0.45	10.68	0.84
6-Bromo-2-naphthyl α -D-galactoside	22.70	1.80	1.66	0.62	13.67	2.90
α -D-Galactose 1-phosphate	Hydrolysed		Hydrolysed		—	—
Galactinol	2.15	0.13	0.72	0.69	2.98	0.18
Melibiose	2.54	0.96	0.41	0.77	9.16	1.24
Raffinose	28.40	4.00	4.18	5.00	6.79	0.80
Stachyose	9.00	7.50	1.36	5.26	6.61	1.42

galactoside. However, complete removal of C-6 (as in the β -L-arabinoside) decreases the rate of hydrolysis by enzyme I but has little effect with enzyme II. The affinity ($1/K_m$) of enzyme for substrate is largely dependent on the structural changes in the glycone moiety and for both enzymes follows the order: α -D-galactoside > α -D-fucoside > β -L-arabinoside. This suggests that one of the specific points of binding of the substrate with the enzymes may be through the primary alcohol group of the galactose moiety.

The variation in the structure of the aglycone residues of the α -D-galactosides affected the values of K_m and V_{max} for both enzymes. The hydrolyses of aromatic galactosides proceeded more readily than those of alkyl derivatives. Relationships between K_m and the V_{max} values were highly irregular with changing aglycones. For example, in Table 1 a comparison of the values for raffinose, melibiose and *n*-propyl α -D-galactoside and of phenyl and *o*-nitrophenyl α -D-galactosides shows that a greater affinity of the enzyme for the substrate is not an essential factor for rapid hydrolysis and vice versa.

Among the aromatic α -D-galactosides, a bulky bromonaphthyl aglycone residue does not decrease the affinity or the rate of hydrolysis to a great extent with either enzyme. With the exception of *m*- and *o*-nitrophenyl galactosides, the nature of the substituent in the phenyl ring does not seem to have a large effect on the V_{max} . However, the affinity ($1/K_m$) for both enzymes shows some tendency to increase when electron-attracting substituents are present in the phenyl ring of the substrate. When pK_m is plotted against the Hammett substituent constant, σ (Hammett, 1940; cf. Hall, Hollingshead & Rydon, 1962), there is an approximately linear relationship, although the high degree of scatter shows that factors other than electronic effects play some role in enzyme-substrate binding, for example the orientation and molecular size of the substituent and its degree of hydration.

Effect of temperature. A rise of 10° caused about a twofold increase in both V_{max} and K_m for α -galactosidase I, and about fourfold and threefold increases in these values for α -galactosidase II. The rise in the K_m (i.e. lower affinity) with the rise of V_{max} in an independent way supports the results obtained from the substrate-specificity studies, i.e. the nature of the substrate affects K_m and V_{max} differently. It is therefore evident that K_m is independent of V_{max} , and possibly represents the dissociation of the enzyme-substrate complex only (i.e. $\approx K_s$).

The values of $\log V_{max}$ and pK_m (i.e. $-\log K_m$) at different temperatures were plotted against $1/T$ and straight lines were obtained for both the enzymes (Fig. 1). The linear relationship between the kinetic constants and $1/T$ suggests that K_m and V_{max} are simple constants rather than complex functions of

Fig. 1. Effect of temperature on V_{max} and K_m for the hydrolysis of *p*-nitrophenyl α -D-galactoside by α -galactosidase I (○) and II (●) in McIlvaine buffer, pH 4.0.

several velocity constants, and that with the α -galactosidases the breakdown of a single intermediate complex is rate-determining. The energies of activation calculated from Fig. 1(a) are 15.4 and 27.2 kcal./mole for enzymes I and II respectively, and the corresponding ΔH values (from Fig. 1b) are -13.28 and -16.5 kcal./mole. The ΔS value for enzyme I at 30° is therefore -32.4 and that for enzyme II is -43.6 cal./deg./mole. If the K_m is regarded as the dissociation constant of the enzyme-substrate complex the values of ΔH and ΔS would represent the changes occurring during the formation of the Michaelis complex. The negative entropy values suggest that both enzymes probably undergo considerable conformational changes during the course of the reaction.

Effect of pH on K_m and V_{max} . The effect of pH on initial velocities of *p*-nitrophenyl α -D-galactoside and raffinose hydrolyses by α -galactosidase I has already been reported (Dey & Pridham, 1969a). We have now determined the V_{max} and K_m values for both of these substrates over the pH range 2.5-7.5 at which the enzyme is stable. The results are presented in Figs. 2 and 3 in the form recommended by Dixon (1953). In the pK_m -pH plots the straight line portions (with slopes +1, 0, -1) of the curves intersect when they are extended. The application of Dixon's rules (Dixon, 1953; Dixon & Webb, 1964, p. 134) shows that with both substrates the pK values of the ionizing groups existing in the free enzyme (downward bend of the curve) are 2.9-3.4 and 4.7-4.8 and that in the enzyme-substrate complex (upward bend of the curve) there is a group with

Fig. 2. K_m and V_{max} , as a function of pH for the hydrolysis of *p*-nitrophenyl α -D-galactoside by α -galactosidase I.

Fig. 3. K_m and V_{max} , as a function of pH for the hydrolysis of raffinose by α -galactosidase I.

pK 5.15–5.25. Groups present in the free enzyme with pK values 2.9–3.4 and 4.7–4.8 also appear in the $\log(V_{max}/K_m)$ -pH plots. The group with pK 4.7–4.8 does not seem to take part in the substrate binding, but its pK is shifted (pK 5.15–5.25), presumably owing to the proximity of the substrate to this group in the enzyme-substrate complex. The group corresponds quite closely with the pK value (5.1–5.2) obtained from $\log V_{max}$ -pH curves. The second pK (6.3–6.6) shown in this latter curve also appears to represent a group present in the enzyme-substrate complex; however, it does not appear in the pK_m -pH plots, but is present in the

Fig. 4. Effect of photo-oxidation on α -galactosidase I in the presence of Methylene Blue: (a) un-aerated; (b) aerated. Experimental conditions are described in the Materials and Methods section. ●, In light; ○, in darkness.

$\log(V_{max}/K_m)$ -pH plots (6.35–6.7). This group, therefore, which exists in the free enzyme, can still ionize when present in the enzyme-substrate complex without any change in pK value. It is therefore not involved in substrate binding, although concerned in activity.

A comparison of the above pK values with those listed by Dixon & Webb (1964, p. 144) indicates the possible participation of a carboxyl group (pK 3.0–4.7) and a histidine imidazolium group (pK 5.6–7.0) in the enzyme-catalysed hydrolysis of both *p*-nitrophenyl α -D-galactoside and raffinose.

Effect of photo-oxidation. The pH studies suggest the presence of a histidyl group in the active site of the enzyme. This possibility was further examined by subjecting the enzyme to photo-oxidation in the presence of Methylene Blue (cf. Weil, James & Burchert, 1953; Weil & Seibles, 1955; Mountler, Alexander, Tuck & Dien, 1957; Nakatani, 1960). As shown in Fig. 4, irradiation of the enzyme in the presence of this dye results in some loss of activity and this is markedly enhanced when air is bubbled through the mixture. However, the possible destruction of other residues, such as tryptophan and tyrosine, by photo-oxidation cannot be ruled out (Weil & Burchert, 1951; Barnard & Stein, 1958; Koshland, 1960). In conclusion, it seems highly probable that histidine and carboxyl groups are involved in α -galactosidase I-catalysed hydrolyses.

Inhibition by substrate. It has been shown that the hydrolysis of *p*-nitrophenyl α -D-galactoside by α -galactosidases I and II is inhibited at high substrate concentration whereas the simple Michaelis law is obeyed when raffinose is used as the substrate (Dey & Pridham, 1969a). We have now examined α -

Fig. 5. Inhibition of α -galactosidase I at high concentrations of *p*-nitrophenyl α -D-galactoside and the effect of galactose and glucose. ○, *p*-Nitrophenyl α -D-galactoside (data from Dey & Pridham, 1969a); ●, galactose; ×, glucose. Details are described in the text.

galactosidase I-catalysed hydrolysis in more detail in an attempt to explain this behaviour. One possible explanation of substrate inhibition (cf. Laidler, 1958, p. 71) may be that for the formation of an effective enzyme-substrate complex a single substrate molecule must associate with a binding site on the enzyme. At higher substrate concentration a second molecule may associate with the enzyme to form an inactive complex, i.e.:

Hence at very high substrate concentration the rate of formation of product would approach zero. Haldane (1930, p. 84) has treated this possibility theoretically, on the model that two substrate molecules may be bound by the different parts of a single site that should normally bind different parts of the same substrate molecule. Neither molecule is therefore correctly bound, so the complex is inactive. In the present study, a plot of the reaction rate against $\log[p\text{-nitrophenyl } \alpha\text{-D-galactoside}]$ (Fig. 5) resulted in a symmetrical 'bell-shaped' curve (open circles) (cf. Laidler, 1958, p. 72) with little inhibition occurring until the substrate concentration reached 0.75 mM (vertical arrow in Fig. 5). While $[S]$ was held at this value galactose was added in increasing concentrations and this resulted in an inhibition curve (closed circles)

Fig. 6. Inhibition of α -galactosidase I-catalysed hydrolysis of *p*-nitrophenyl α -D-galactoside by D-galactose. Conditions of assay and procedure were as described by Dey & Pridham (1969a). Enzyme activities were determined at different galactose concentrations (●, nil; ○, 1.25 mM; ×, 3.75 mM).

paralleling inhibition by *p*-nitrophenyl α -D-galactoside. In a separate set of experiments galactose inhibited the enzyme competitively without pre-incubation (Fig. 6; $K_i 1.41 \pm 0.05$ mM). Glucose did not act as an inhibitor under the same conditions. It is probable therefore that *p*-nitrophenyl α -D-galactoside inhibition is a competitive substrate inhibition. Liberated galactose is unlikely to have a marked effect as it would only be present in low concentrations during the measurement of initial rates.

Methyl α -D-galactoside, which exhibits a low $V_{\max}$ with enzyme I, also competitively inhibits the hydrolysis of *p*-nitrophenyl α -D-galactoside. There was no detectable hydrolysis of the inhibitor itself under the experimental conditions used (Fig. 6). The inhibitor concentration range was 0–20 mM. The K_i for methyl α -D-galactoside (7.6 ± 0.2 mM) is very similar to the Michaelis constant (7.13 mM), which suggests that both the galactosides (and possibly the whole range of substrates for α -galactosidase reported in Table 1) are bound to the same active site on the enzyme molecule and that the K_m is almost a true representation of the dissociation constant, K_s , of the enzyme-substrate complex.

Asp & Dahlqvist (1968), working with rat intestinal β -galactosidases, have shown that lactose acts as a competitive inhibitor when phenyl β -D-galactoside is used as a substrate and vice versa. The K_i values are approximately equal to the K_m values for both compounds. It was suggested that the β -galactosidases hydrolysed lactose and phenyl β -D-galactoside at the same active site.

Table 2. *Inhibition of α -galactosidase I by heavy-metal ions and thiol-specific reagents in McIlvaine buffer at 30°*

The experimental conditions are described in the Materials and Methods section.

Inhibitor	Concn. of inhibitor (mM)	Inhibition (%)
Ag ⁺	(pH 4.0) 0.0250	76
	0.0125	32
	(pH 6.0) 0.0025	88
	0.0010	23
Hg ²⁺	(pH 4.0) 1.00	55
	0.060	39
	(pH 6.0) 0.0003	79
	0.0001	43
Cu ²⁺	(pH 6.0) 25.0	46
	50.0	33
<i>p</i> -Hydroxymercuribenzenesulphonic acid	(pH 4.0) 0.0050	57
	0.0010	30
	(pH 6.0) 0.020	68
	0.010	39
<i>N</i> -Ethylmaleimide	(pH 4.0) 70.0	0
	(pH 6.0) 70.0	0
Iodoacetamide	(pH 4.0) 25.0	5
	(pH 6.0) 25.0	2
Iodoacetic acid	(pH 4.0) 25.0	2
	(pH 6.0) 25.0	2
5,5'-Dithiobis-(2-nitrobenzoic acid)	(pH 6.0) 25.0	6

Table 3. *Protective effect of galactose on the inhibition of α -galactosidase I by Ag⁺*

Purified enzyme (1 μ g. of protein) was incubated for 5 min. with D-galactose in McIlvaine buffer, pH 6.0, at 30° and then AgNO₃ was added (final concn. 1 μ M) to the solution (0.4 ml.). Enzyme activity was determined with *p*-nitrophenyl α -D-galactoside as substrate (Dey & Pridham, 1969a). Control experiments contained galactose but no AgNO₃.

Concn. of galactose (mM)	Inhibition (%)
0.5	45
1.0	45
2.0	40
4.0	23
5.0	18

Inhibition by thiol-specific reagents and heavy-metal ions. The results presented in Table 2 show that, without preincubation, α -galactosidase I is highly sensitive to heavy-metal ions such as Ag⁺ and Hg²⁺ but much less sensitive to Cu²⁺. Although the high sensitivity to these metal ions suggests the

participation of a thiol group in the enzyme catalysis, typical thiol-specific reagents (Table 2) failed to inhibit the activity even after a 20 min. preincubation. In addition, when the enzyme was titrated with 5,5'-dithiobis-(2-nitrobenzoic acid) (Ellman, 1959) thiol groups could not be detected. If thiol groups are present in the protein they must therefore be highly inaccessible.

In the absence of a thiol group at the active site the inhibition by Ag⁺ may possibly be attributed to its combination with carboxyl or histidine residues or both. This possibility has been suggested by Dixon & Webb (1964, p. 346) and Myrbäck (1957). The Ag⁺ inhibition was competitive at both pH 4.0 (*K_i* 4.0 μ M) and pH 6.0 (*K_i* 0.59 μ M). The substrate concentration ranges at both pH values were the same as those shown in Fig. 6 and the inhibitor concentration range was 0–12.5 μ M at pH 4.0 and 0–1 μ M at pH 6.0. The large decrease in *K_i* with the rise of pH suggested that the ions were binding with a group that lost protons over this range, i.e. one that was not overwhelmingly unprotonated at pH 4. However, the fact that the decrease was much less than 100-fold suggested that the group did not have a p*K* greater than 6. Ag⁺-inactivated enzyme at pH 6.0 regained 83% of its activity when it was dialysed against McIlvaine buffer, pH 6.0. Dialysis against a buffer containing 0.1M-cysteine resulted in 98% restoration of the enzyme activity. A similar result was obtained when inactivation followed by dialysis was carried out at pH 4.0.

As shown in Table 3, the inhibitory effect of Ag⁺ was decreased when low concentrations of galactose were present. The protection was observed only when galactose was added before the addition of Ag⁺. This confirms that Ag⁺ reacts with the active site.

Inhibition by Hg²⁺ usually suggests reaction with thiol groups. However, Webb (1966, p. 737) discussed the co-ordination of Hg²⁺ between carboxyl and amino groups. Hg²⁺ is also known to react with amino and imidazolium groups of histidine (Simpson, 1961) and with peptide linkages (Haarmann, 1943). In the present study the degree of inhibition by Hg²⁺ was less than that of Ag⁺ at pH 4.0 (Table 2) and it was non-competitive (*K_i* 75 μ M). This discounts the possibility of Hg²⁺ forming complexes with carboxyl or histidine residues at the active site of the enzyme at pH 4.0. Combination of Hg²⁺ with these residues at centres other than the reactive site are of course possible. Inhibition by Hg²⁺ at pH 6.0 on the other hand was much greater and competitive (*K_i* 0.035 μ M). The substrate concentration ranges at both pH values were the same as those shown in Fig. 6 and the inhibitor concentration range used was 0–1 mM at pH 4.0 and 0–0.15 μ M at pH 6.0. Presumably reaction is occurring with the histidine at the active site in this instance. The activity of the

Fig. 7. Dependence of inhibition on preincubation time of α -galactosidase I with various concentrations of *p*-hydroxymercuribenzenesulphonic acid (○, 1 μ M; ●, 5 μ M; □, 10 μ M; ■, 50 μ M) in McIlvaine buffer, pH 4.0.

Table 4. Inhibition of α -galactosidase I-catalysed hydrolysis of *p*-nitrophenyl α -D-galactoside by sugars and related compounds at 30°

Experimental conditions are described in the Materials and Methods section.

Inhibitor	Concn. of inhibitor (mM)	Inhibition (%)
L-Arabinose	25.0	16
	6.25	0
D-Arabinose	25.0	0
	6.25	0
D-Fructose	25.0	0
D-Fucose	25.00	14
	6.25	4
L-Fucose	25.0	0
Galactinol	25.0	74
	6.25	44
D-Galactose	25.0	90
	6.25	70
α -D-Galactose 1-phosphate	25.0	8
	6.25	0
D-Galactose 6-phosphate	25.0	4
	6.25	0
D-Glucose	25.0	8
	6.25	0
α -D-Glucose 1-phosphate	25.0	8
	6.25	0
Melibiose	3.75	39
	1.50	21
<i>myo</i> -Inositol	25.0	44
	6.25	16
Raffinose	8.0	57
	2.0	34
Stachyose	50.0	34
	25.0	14
Sucrose	25.0	8

Hg²⁺-inhibited enzyme (at pH 6.0) was restored to 42% on dialysis against McIlvaine buffer, pH 6.0, containing 0.1M-cysteine.

The inhibition by *p*-hydroxymercuribenzenesulphononic acid was not instantaneous and a preincubation (20 min.) with the enzyme was required for completion of inhibition (Fig. 7). The inhibition was non-competitive both at pH 4.0 (K_i 6 μ M) and pH 6.0 (K_i 10 μ M). The substrate concentration ranges at both pH values were the same as those shown in Fig. 6 and the inhibitor concentration range was 0–10 μ M.

Inhibition by sugars and related compounds. The inhibitory effects are summarized in Table 4. The inhibition by galactosides follows the order: galactinol > raffinose > melibiose > stachyose. D-Galactose is a powerful inhibitor. The formation of this latter hexose during germination of *Vicia faba* seeds by α -galactosidase-catalysed hydrolysis of galactosylsucrose derivatives would be expected to eventually inhibit the further utilization of the oligosaccharides. The seed can, however, convert galactose into galactose 1-phosphate (Pridham, Walter & Worth, 1969) and this is a very weak inhibitor of α -galactosidase.

The structural analogues of D-galactose, L-arabinose and D-fucose, also inhibit the hydrolysis of *p*-nitrophenyl α -D-galactoside by enzyme I whereas their enantiomers are ineffective. This is in accordance with the results of the substrate specificity experiments (Table 1), i.e. that *p*-nitrophenyl β -L-arabinoside and α -D-fucoside served as substrates.

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