Catalysis by β-Glucosidase A_3 of *Aspergillus wentii*

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(1) α-Deuterium kinetic isotope effects on $V_{max.}$ for hydrolysis of both β-D-glucopyranosylpyridinium ions and aryl β-D-glucopyranosides are in the range k_H/k_D 1.08—1.14, indicating that bond-breaking limits the rate of hydrolysis of both sets of substrates. The variation of $k_{cat.}$ with aglycone acidity, expressed by log $k_{cat} = A + \beta_{lg}pK_a$, is governed by a β_{lg} value of −0.96 ± 0.19 for the *N*-glycosides and −0.05 ± 0.05 for the *O*-glycosides. The latter, low value of $|\beta_{lg}|$ is evidence for extensive proton donation to the oxygen at the transition state, even for the departure of acidic aglycones. The dependence of rate on protonation of a group of $pK_a < 6$ required by this idea is indeed observed. (2) Comparison of log k_{cat} values with spontaneous hydrolysis rates indicates that nucleophilic and non-covalent interactions accelerate C–N bond cleavage in the ES complex by a factor of $10^{(8 + 0.3[pK_a]_N)}$ for glucosyl pyridinium salts where $[pK_a]_N$ is the pK_a of the pyridine. On the assumption that these interactions are similar for *O*-glucosides, proton donation to the aglycone oxygen atom can be estimated to contribute a rate-enhancement of $10^{(0.1+0.7[pK_a]_O)}$, where $[pK_a]_O$ is that of the free phenol. (3) D-Glucono-δ-lactone and 5-amino-5-deoxy-D-gluconolactam are bound $10^{2.8}$ and $10^{2.2}$ times, respectively, more tightly than β-D-glucopyranose, because of their analogy to a transition state in which α-deuterium kinetic isotope effects have shown the anomeric carbon atom to have substantial sp^2 character. (4) Cationic inhibitors are bound $10^{2.5}$—$10^{3.5}$ times more tightly than directly comparable neutral ones, if the positive charge resides on the equatorial substituent at C-1, but protonated 2-amino-2-deoxyglucose is bound no more tightly than glucose. This indicates the presence of a negative charge in the EI complex near C-1 on the α face of the pyranose ring (an aspartate residue previously identified by covalent labelling) which is partly compensated by a positive charge near C-2.

THE A_3 isoenzyme of the extracellular β-glucosidases produced by the mould *Aspergillus wentii* is a uniquely well defined β-D-glucopyranosidase. It is a crystalline, monomeric glycoprotein, some 21% carbohydrate, with a molecular weight of 170 000.[1] Affinity labelling with 1,2-anhydro-1,2,3,5/4,6-inositol has indicated that this enzyme, which contains only a single polypeptide chain, has one active site associated with it.[2] The label forms an ester with the side chain carboxylate group of an aspartic acid residue, and the amino-acid sequence of a peptide containing this active-site residue has been determined.[3]

This enzyme is thus a suitable vehicle for investigation of the general phenomenon of glycosidase catalysis. We now report the results of three types of investigation, which together allow us to form a picture of the transition state leading to the glucosyl–enzyme intermediate. This glucosyl–enzyme intermediate is required by the overall retention of anomeric configuration in the enzyme-catalysed reaction.[2] The types of investigation are as follows. (i) The influence of the pK_a of the liberated phenol on the steady-state kinetic parameters for aryl glucosides was studied. Proton donation by some group on the enzyme to the phenolic oxygen is likely and we describe the contrasting behaviour of substrates against which such a process is structurally impossible, the β-D-glucopyranosylpyridinium salts. (ii) α-Deuterium kinetic isotope effects upon $V_{max.}$ for hydrolysis of both types of substrate were measured. Such effects give information about the hybridisation of the reaction centre in the rate-determining transition state; effects near 1.00 indicate no hybridisation change, whereas effects above *ca.* 1.2 indicate a transition state in which the reaction centre has completely changed from tetrahedral to trigonal.[4] (iii) The inhibition of the enzyme by neutral and cationic reversible inhibitors was examined.

EXPERIMENTAL

Substrates.—Commercially unavailable aryl glucosides were made by condensation of the sodium salt of the phenol with 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide, followed by deacetylation with methanol.[5] Physical properties were in agreement with those reported in the references in Table 2. β-D-Glucopyranosylpyridinium salts were made analogously to the β-D-galactopyranosyl compounds.[6]

Characterisation data of new compounds (1)—(10) are reported in Table 1. [1-^{2}H]-D-Glucose was made by reduction of the aldonolactone with sodium amalgam in a D_2O solution kept at constant pD by addition of D_3PO_4.[7] The deuteriated substrates were prepared, *via* the acetylglucosyl bromide, analogously to the protiated ones, the m.p.s being for 4-methylumbelliferyl β-D-glucopyranoside, 204—207° (decomp.) (lit.,[8] 211°) for 4-nitrophenyl β-D-glucopyranoside, 164—166° (lit.,[9] 164—165°), for β-D-glucopyranosyl-4-bromoisquinolinium bromide, 155—158° (decomp.), and for β-D-glucopyranosyl-3-carboxamidopyridinium bromide, 163—165° (decomp.) (lit.,[10] 151—152°). A ^{1}H n.m.r. spectrum of the starting tetra-*O*-acetyl-α-D-glucopyranosyl bromide revealed 3—4% of residual protium at C-1 of the sugar.

Kinetic Measurements.—Michaelis–Menten parameters for hydrolysis of glucosylpyridinium salts in 50mM-sodium acetate–HCl buffer, pH 4.0 at 25 °C, were measured by monitoring change of absorbance with a Unicam SP 1800 spectrophotometer fitted with a cell-block through which water maintained at 25.0 ± 0.1 °C by a Tecam Tempunit was circulated. Wavelengths at which the reaction was followed (in nm) and extinction coefficient differences (in l mol^{-1} cm^{-1}) were, for the pyridinium salt, 265, −2 709; for the isoquinolinium salt, 358, −1 218; for the 3-carboxamidopyridinium salt, 278, −1 183; for the 4-bromo-

isoquinolinium salt, 350, −5 020; for the 3-bromopyridinium salt, 285, −3 497.

α-Deuterium kinetic isotope effects were measured spectrophotometrically as described previously [7] except that and β-D-glucopyranose, which mutarotates slowly under these conditions, are given elsewhere.[11]

Rates with other aryl glucosides were measured spectrophotometrically in a Zeiss PMQII/M4 spectrophotometer with

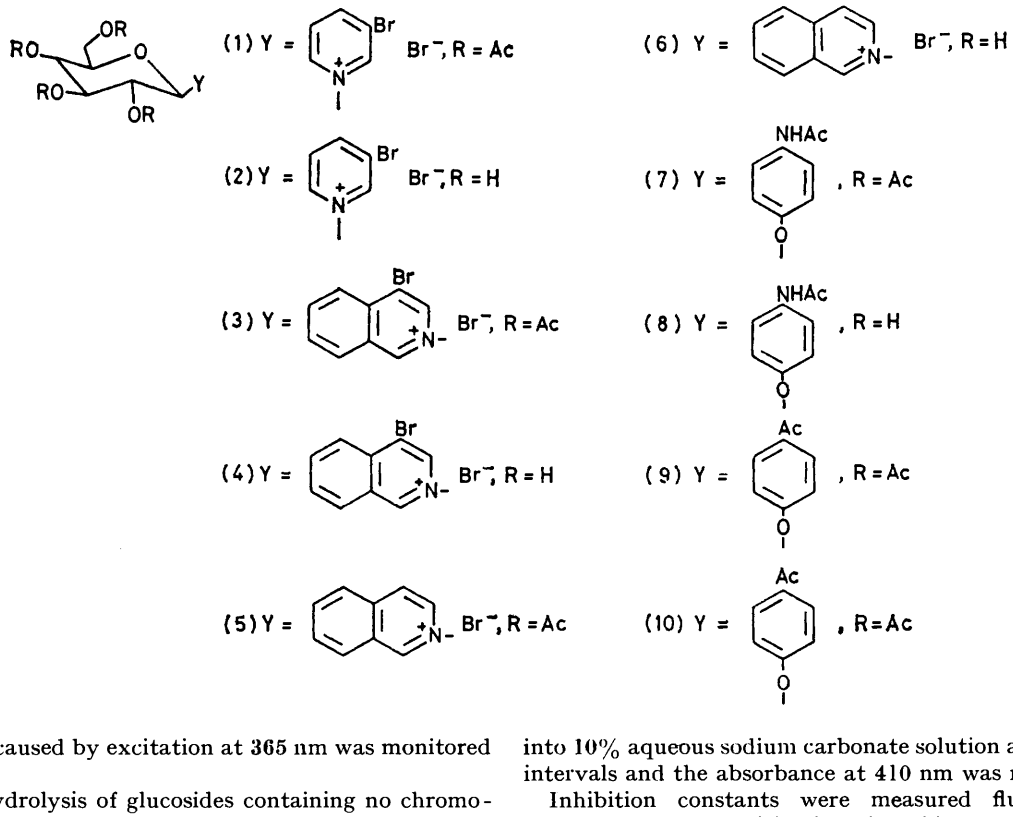
TABLE 1

New substrates (1)—(10)

Compound	M.p. (°C)	Empirical formula	$[\alpha]_{25}^{D}$(°) (*c* 1; $CHCl_3$)	Required (%) C	H	N	Br	Found (%) C	H	N	Br	Anomeric proton: Chemical shift (τ)	Anomeric proton: Splitting *J*/Hz
(1)	162—165	$C_{19}H_{23}Br_2NO_9$	−15	40.1	4.1	2.5	28.1	39.7	4.3	2.5	27.0	2.33 ‡	8.0
(2)	133—134	$C_{11}H_{15}Br_2NO_5$		33.0	3.5	3.8	39.9	32.6	3.8	3.6	40.0		
(3)	153—154	$C_{23}H_{25}Br_2NO_9$	−66	44.6	4.1	2.3	25.8	43.9	4.1	2.2	25.3	2.45 ‡	8.0
(4)	157—158	$C_{15}H_{17}Br_2NO_5$		39.9	3.8	3.1	35.4	39.8	4.0	3.0	35.2		
(5)	190—195	$C_{23}H_{26}BrNO_9$	−21	51.1	4.8	2.6	14.8	50.2	5.1	2.4	15.1		
(6)	159—161	$C_{15}H_{18}BrNO_5 \cdot H_2O$		46.2	5.1	3.6	20.0	45.8	5.1	3.5	20.0	4.03 §	8.0
(7)	126—128												
(8)	211—421	$C_{14}H_{19}NO_7$	−65.2 *	53.6	6.1	4.5		53.0	6.5	4.1			
(9)	173		−27.9										
(10)	194	$C_{14}H_{18}O_7$	−96.5 *	56.4	6.4			56.6	6.6				

* In water. ‡ In $CDCl_3$. § In D_2O, relative to HOD (τ 5.25).

a small portion of enzyme was added to a large volume of substrate using the same Hamilton syringe for both protiated and deuteriated material. The effects on 4-methylumbelliferylglucoside hydrolysis were measured on a Farrand Mark 1 spectrofluorimeter with a cell-block thermostatted (to within 0.5 °C) by a Grant thermostatted bath. a cell-block thermostatted to within 0.2 °C. Wavelengths used (in nm) and extinction coefficient differences (in l mol^{-1} cm^{-1}) were, for the glucoside of *p*-guanidinophenol, 280, 680; for that of *p*-methoxycarbonylphenol, 235, 4 950; and for that of *p*-acetylphenol, 295, 4 260. With *p*-nitrophenyl glucoside, samples from a reaction mixture were pipetted



Fluorescence caused by excitation at 365 nm was monitored at 450 nm.

Enzymic hydrolysis of glucosides containing no chromophore, and of the glucosides of *p*-methoxy-, *p*-hydroxy-, *p*-methyl-, *p*-acetamido-, *p*-chloro-, and *p*-cyanophenyl-glucosides was monitored polarimetrically in 50mM-glycine–HCl buffer, pH 3.0 at 35 °C in a Perkin-Elmer 141M polarimeter at 578 nm, with substrate concentrations from 1.5 to 20mM. Differences in molar rotation between the glucoside into 10% aqueous sodium carbonate solution at regular time intervals and the absorbance at 410 nm was read.

Inhibition constants were measured fluorimetrically, using 4-methylumbelliferyl β-glucoside as substrate, and were carried out at 25 °C in the following buffers: pH 2.0—3.5, 50mM-glycine–HCl; pH 4.0—6.0, 50mM-sodium acetate–HCl; and pH 6.5—7, 50mM-Na_2HPO_4–HCl. Details are given elsewhere.[12]

The pK_a values of *p*-acetamido- and *p*-guanidino-phenol

cation were measured photometrically at 270 nm in 0.1M-glycine–NaOH buffers.

The non-enzymic hydrolyses of glucosides were followed by withdrawing six ampoules of solutions of the substrate from a constant temperature (±0.5 °C) oil-bath at appropriate intervals (0.5—2 h) and analysing for *p*-nitrophenol by absorbance, 4-methylumbelliferone by fluorescence, or glucose, as appropriate. Glucose was determined with glucose oxidase (Sigma)–peroxidase and 2,2′-azino-di-(3-ethylbenzothiazoline-6-sulphonate) (Boehringer). Glucose oxidase and peroxidase preparations from other suppliers contained interfering amounts of β-glucosidase. The concentration of 4-methylumbelliferyl glucoside was 2mM, that of other substrates 20mM, of buffers 50mM; these were glycine–HCl at pH 3.0; sodium acetate–HCl at pH 5.0; and phosphate at pH 6.0, 7.0, and 8.0.

RESULTS

Kinetic data on the hydrolyses of various D-glucopyranosyl derivatives are given in Table 2. k_{cat} Values are calculated

TABLE 2

Michaelis–Menten parameters for hydrolyses of *O*- and *N*-glucosides by the β-glucosidase A_3 of *Aspergillus wentii*

Aglycone	Reference for synthesis †	$10^3 K_m$/ mol l^{-1}	k_{cat}/ s^{-1} ‡
Measured at pH 3.5 and 35 °			
2,4-Dinitrophenol	13	0.75 *	295 *
4-Nitrophenol		2.1	540
		0.9 *	225
4-Methylumbelliferone	8	0.77 *	540 *
4-Cyanophenol	9	1.2	160
4-Hydroxyacetophenone	Table 1	1.3	910
Methyl 4-hydroxybenzoate	14	1.4	1 150
4-Chlorophenol	9	3.5	190
4-Hydroxyphenylguanidinium ion	15	7.3	310
4-Acetamidophenol	Table 1	1.2	540
Phenol		3.9	310
4-Methylphenol	9	3.0	450
4-Methoxyphenol	9	2.0	600
Hydroquinone	16	2.3	230
4-Glucose		0.15	335
4-Methyl β-glucopyranoside	17	0.3	255
Methanol		14	140
Benzyl alcohol	18	15	165
Cyclohexanol	18	10	100
Benzoic acid	19	48	24
Measured at pH 4.0 and 25 °C			
3-Bromopyridine	Table 1	1.2	4.4
4-Bromoisoquinoline	Table 1	0.069	0.25
Nicotinamide	10	0.070	1.9
Pyridine	20	0.33	0.023
Isoquinoline	Table 1	0.09	0.0066

* Measured at 25 °C. † For compounds not commercially available. ‡ For the purposes of calculating β_{lg}, the activation energy for hydrolysis of all aryl glucosides is assumed to be constant. An inconstancy of ±5 kcal introduces an error of 0.1 log units into the extrapolated k_{cat}.

assuming pure proteon and $E_{278}^{1mg/ml}$ 1.91; the specific activity [30 U mg^{-1} against *p*-nitrophenyl glucoside (20 mM) at pH 4.0 and 35 °C] of a sample of this enzyme kept at 4 °C did not significantly alter over 4 years. With enzyme of this specific activity, 1.09 moles of the affinity label 1,2-anhydro-1,2,3,5/4,6-inositol are incorporated per mole of enzyme protomer inactivated.[2] log k_{cat} Values are displayed in Figure 1 as a function of the pK_a of the departing aglycone. These pK_a values were taken from Kortüm

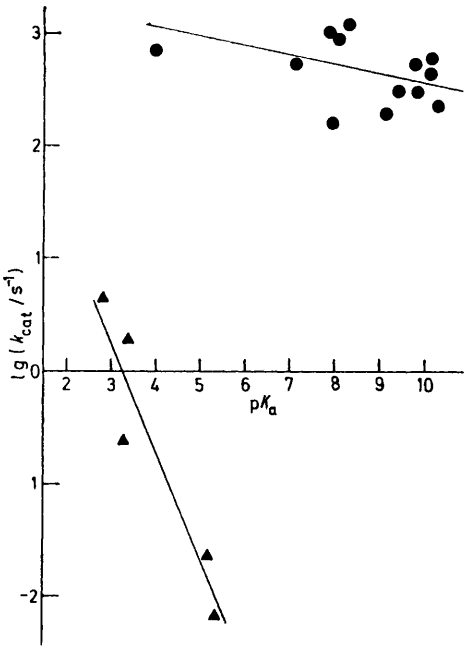


FIGURE 1 Plot of log k_{cat} values for pyridinium salts (▲) and aryl glucosides (●) as a function of leaving group pK_a. The lines are those given by least-squares analysis

et al.[21] for phenols or Perrin [22] for pyridines, with the exception of *p*-$CH_3CONHC_6H_4OH$ and *p*-$H_2\overset{+}{N}{=}C(NH_2)NHC_6H_4OH$, for which we report pK_a values of 9.82 and 9.42, respectively.

With aryl glucosides, pronounced substrate inhibition was observed at pH 4.0, but this was abolished at pH 7.0. Data for 4-nitrophenyl glucoside are illustrated in Figure 2.

The α-deuterium kinetic isotope effect for hydrolysis of the 4-bromoisoquinolinium salt is 1.11 ± 0.03 and for the nicotinamide salt is 1.09 ± 0.02, when measured at a concentration $>10K_m$ under the same conditions as the Michaelis–Menten parameters. Those for 4-methylumbelliferyl and *p*-nitrophenyl glucosides at pH 4.0 and 35 °C were

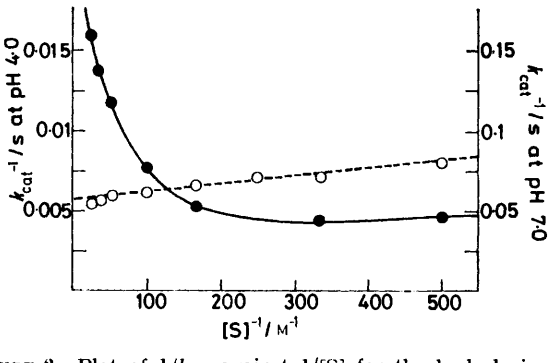


FIGURE 2 Plot of $1/k_{cat}$ against 1/[S] for the hydrolysis of 4-nitrophenyl-β-glucoside by β-glucosidase A_3 at 35° and pH 4.0 (●, left ordinate) and pH 7.0 (○, right ordinate). The kinetic constants, calculated with additional points at lower concentrations, are: at pH 4.0, k_{cat} 510 s^{-1}, K_m 1.6 × 10^{-3} mol l^{-1}, constant for substrate inhibition K_{is} 7.0 × 10^{-3} mol l^{-1}; at pH 7.0, k_{cat} 18 s^{-1}, K_m 0.85 × 10^{-3} l mol^{-1}. A similar concentration dependence was observed with 4-methylumbelliferyl β-glucoside as substrate with the following kinetic constants at 25°: at pH 4.0, k_{cat} 260 s^{-1}, K_m 0.57 × 10^{-3} mol l^{-1}, K 2.3 × 10^{-3} mol l^{-1}; at pH 7.0, k_{cat} 6.9 s^{-1}, K_m 0.059 × 10^{-3} mol l^{-1}, no substrate inhibition.

measured at the substrate concentration that gives maximal velocity ($\sqrt{K_iK_s}$) and are 1.14 ± 0.05 and 1.08 ± 0.02, respectively; at pH 7.0 they become experimentally identical at 1.08 ± 0.04.

Figure 3 shows the variation with pH of the hydrolysis

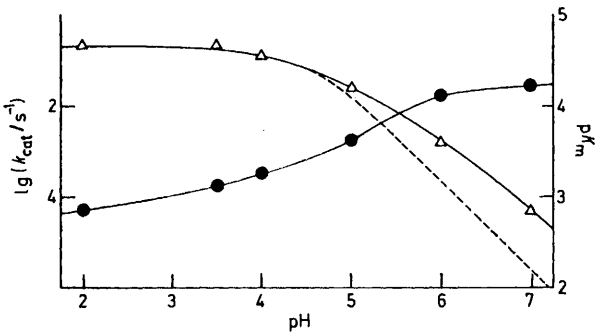


FIGURE 3 pH Dependence of log k_{cat} and pK_m for the hydrolysis of 4-methylumbelliferyl β-glucoside by β-glucosidase A_3, at 25°: log (k_{cat}/s^{-1}) (left ordinate) △; pH dependence of log k_{cat} calculated as depending on a single ionizing group with pK_a from pH of half-maximal activity (pH 4.5) – – – –; pK_m (right ordinate) ●

of 4-methylumbelliferyl glucoside. This shows that the kinetic consequences of a change in pH from 3.5 to 4.0 are comparatively minor, and hence that the conclusions reached from a comparison of the pyridinium salt data (pertaining to pH 4) and aryl glucoside data (pertaining to pH 3.5) will be unaffected by the change in pH. Both k_{cat} and K_m decrease as the pH is increased above pH 4.0, but the variation in k_{cat} cannot be described by a single ionisation; calculation of the variation on this basis and the pH value at which k_{cat} has its half-maximal value produces the dotted line shown.

Table 3 gives the K_i values of various cationic and neutral

TABLE 3

Inhibition of β-glucosidase A_3 by cationic and neutral β-glucosyl derivatives at pH 4.0 and 25 °C. Substrate: 4-methylumbelliferyl β-glucoside. K_i^c and K_i^a are dissociation constants for competive and anti-competitive inhibition calculated according to ref. 12

Inhibitor	10^3 K_i^c/mol l^{-1}	K_i^a/K_i^c	$-\Delta\Delta G°$/kJ mol^{-1} [a]
β-Glucose	2.8	0.7	
β-2-Amino-2-deoxyglucose	18	0.5	0
β-Glucosylamine	0.0016	1.0	18.6
N-Bromoacetyl-β-glucosylamine	0.25 [b]	3	6.6
D-Glucono-δ-lactone	0.0096	30	14.6
5-Amino-5-deoxy-D-gluconolactam	0.036	5	11.2
β-Glucosylbenzene	96	30	
N-β-Glucosylpyridinium ion	0.30	3.2	14
N-β-Glucosylpiperidine	0.091	3.2	17
N-β-Glucosylimidazole	5.9	3	7

[a] Additional interaction energy calculated from $\Delta\Delta G° = -RT\ln K_i^c/K_i^c$ (ref) with β-glucose as reference except for the last three inhibitors which are compared with β-glucosylbenzene. [b] An additional time-dependent inhibition was observed with this inhibitor.

inhibitors of the enzyme; the variation with pH of these values for β-D-glucopyranosylamine and β-D-glucopyranosylpyridinium ions is shown in Figure 4. K_i Values for amines are calculated assuming that only the cationic form binds. The bases of this assumption are: (i) the K_i value for β-glucose itself, which is isosteric with β-D-glucosylamine, is high, (ii) the variation with pH of K_i values calculated from the concentration of the cationic form is identical to that for the quaternary β-D-glucopyranosylpyridinium ion, which cannot ionise.

The rates, and activation parameters, for the spontaneous hydrolyses of various glucosides are given in Table 4. The

TABLE 4

Rate constants and activation parameters for the non-enzymatic hydrolysis of β-glucopyranosides at pH 5

Leaving group	k (100°)/s^{-1}	k * (25°)/s^{-1}	$\Delta H‡$/kJ mol^{-1}	$\Delta S‡$/K mol^{-1} K^{-1}
p-Nitrophenol	0.64×10^{-6}	52×10^{-12}	118	−32
4-Methylumbelliferone	0.28×10^{-6}	4.7×10^{-12}	135	−3
Glucose	0.22×10^{-6}	13×10^{-12}	123	−11

* Extrapolated from data at other temperatures.

rate of spontaneous hydrolysis of *p*-nitrophenyl glucoside shows a broad minimum at pH 4—6 with contributions from acid and base catalysis (*ca.* 30% rate increase) at pH 3.0 and 6.5 respectively.[23] With 4-methylumbelliferyl glucoside the minimum was shifted to pH 5 to 6.5 (*ca.* 30% rate increase at pH 4 and 7, respectively). The other substrates were studied only at pH 5. Phenyl glucoside, *o*-hydroxymethylphenyl glucoside (salicin), and methyl glucoside were hydrolysed at <0.03% h^{-1} at 105°, corresponding to rate constants of $< 2 \times 10^{-8}$ s^{-1}. At a higher temperature (120°) decomposition reactions prevented accurate measurements. Cellobiose was hydrolysed with k(100°) 0.22×10^{-6} s^{-1} and

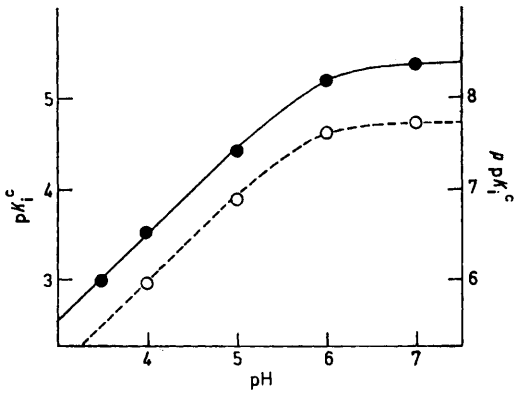


FIGURE 4 pH Dependence of the inhibition of β-glucosidase A_3 by β-glucosylpyridinium ion (●, left ordinate) and β-glucosylammonium ion (○, right ordinate). The concentration of the latter was calculated from the total inhibitor concentration with pK_a 5.6.[12]

$\Delta H*$ 123 kJ mol^{-1}. With methyl β-cellobioside no hydrolysis could be observed [k(100°) $< 2 \times 10^{-8}$ s^{-1}]. We therefore assume that the cleavage of cellobiose is not due to simple hydrolysis but to a series of rearrangements starting at the reducing end as described for the alkaline degradation of polysaccharides.[24]

DISCUSSION

(1) *The Enzymic Transition State.*—In the hydrolysis of aryl glucosides, substrate inhibition is apparent, especially for the *p*-nitrophenyl compound. Analogously, inhibition by various neutral and cationic glucosyl derivatives has both a competitive and an anti-component. In the absence of some assurance that this com-

plex kinetic behaviour is a phenomenon unrelated to catalysis, attempts to investigate this catalysis by steady-state methods will rest on an insecure base. The previous observations that nitration of the enzyme with tetranitromethane results in disappearance of substrate inhibition without substantial change in $V_{max.}$ provides such an assurance.[25]

Breaking of the C–N bond is both the rate-limiting and the first essentially irreversible step in the enzymic hydrolysis of the pyridinium salts, as shown by (i) the existence of an α-deuterium kinetic isotope effect and (ii) the correlation of rate with pK_a of the leaving group.

Correlation coefficients of 0.95 and 0.90 are calculated for the relation between log k_{cat} and log k_{cat}/K_m, respectively, with aglycone pK_a. However, the deviation of experimental points from the least squares line is large even for k_{cat} (root mean square deviation 0.35 log units) and it is only because of the steep slope in Figure 1 (-0.96 ± 0.19) that dependence on aglycone pK_a is observable. The scatter to be expected from secondary interactions with aglycone can however be gauged from these measurements.

If the log k_{cat} values for aryl glucosides are considered, the scatter of the points from a line with β_{lg} −0.1 is less (root mean square deviation 0.25 log units) than the scatter of the points for pyridines from a line with β_{lg} −0.96 (0.35 log units). A distinction between a random dependence of log k_{cat} on aglycone acidity caused by a rate-limiting conformation change (*cf.* the situation which apparently obtains for the *lac*-(*Z*)-β-galactosidase of *Escherichia coli* [26]) and an apparently random dependence caused by a low β_{lg} value and adventitious secondary interactions with aglycone can be made on the basis of the presence or absence of an isotope effect. In this case a definite secondary isotope effect is observed for two aryl glucoside substrates.

There remains the possibility that the secondary isotope effect which has been observed for *O*-glycosides is a consequence of the hydrolysis of a glucosyl–enzyme intermediate being rate limiting. However, if this were the case, all k_{cat} values for oxygen glycosides would be accurately equal, whereas they do in fact vary over a factor of 7. This possibility can therefore be disregarded.

The transition state leading to the glucosyl–enzyme in hydrolysis by this enzyme can now be described in some detail. The α-deuterium kinetic isotope effects for *N*-glycoside hydrolysis are in the intermediate range for nucleophilic substitution at this type of saturated carbon centre, and appreciably less than those for the acid-catalysed hydrolyses of inosine and adenosine (k_H/k_D *ca.* 1.2),[27] and less than that for the spontaneous hydrolysis of a glycopyranosylpyridinium salt (1.16).[28] There are two possible reasons for this, significant nucleophilic participation by the ionised aspartate residue identified by affinity labelling, or an early transition-state in an enzyme-catalysed S_N1 reaction. The high β_{lg} value argues against the latter; the value of *ca.* 1 indicates that the change in charge on nitrogen on going from the ES complex to the enzymic transition state is similar to that brought about on deprotonation of freee pyridinium ion in aqueous solution. The β_{lg} value is lower than that observed for the spontaneous hydrolysis of β-D-galactopyranosylpyridinium salts (1.26 [28]) but is large enough to indicate that the C–N bond is largely broken at the transition state. Therefore we are (reluctantly) forced to the conclusion that the medium value isotope effect, significantly lower than those found for *lac*-(*Z*)-β-galactosidase hydrolysing pyridinium salts,[6] is a reflection of weak nucleophilic participation by the aspartate residue. The very S_N1-like bimolecular reactions of methoxymethyl derivatives show similar isotope effects, one of 1.14 (per D) being observed for the attack of acetate on methoxymethyl 2,4-dinitrophenolate [29] and one of 1.07 (per D) for attack of acetate on *NN*-dimethyl-*N*-methoxymethyl-*m*-nitroanilinium ion.[30]

Essentially the same α-deuterium kinetic isotope effects are observed in the hydrolysis of aryl glucosides as pyridinium salts. If, as seems reasonable, the degree of nucleophilic assistance, in any event weak, is the same for departure of pyridines as of phenols, then the observation of similar isotope effects for both sets of substrates means that rupture of the glycone–aglycone bond at the transition state is about the same for both sets of substrates.

However, experimentally the β_{lg} value for aryl glucoside hydrolysis is zero (-0.055 ± 0.045). This means that there is essentially no change in the charge on oxygen on going from the ES complex to the transition state of the enzyme-catalysed reaction. With C–O bond cleavage far advanced in this transition state, such absence of charge on the phenolic oxygen is only possible if proton-donation to it is also far advanced in the transition state. Complete protonation, as in the *A*-1 hydrolysis of aryl glycopyranosides [9,31] would however give rise to a definitely positive β_{lg} value (0.2—0.3).

The correlation line with β_{lg} *ca.* 0 describes the behaviour of alkyl glucosides (aglycone pK_a in the 14.5—15.5 region [32]) as well as aryl glucosides (Table 2). It also extends into regions of pK_a where acidic aglycones (4-nitro- and 2,4-dinitro-phenols, 4-methylumbelliferone) are being liberated. Extensive proton-transfer in the transition state to the oxygen of phenols of such low pK_a requires that the catalysing acid be of pK_a lower than *ca.* 6. If this is indeed the case, and if C–O bond breaking is rate limiting, then the ionisation of the acid catalytic group should influence pH–rate profiles of aryl glucoside hydrolysis at pH values above 6.

Figure 3 shows that this is so, a marked fall in k_{cat} being observed above pH 5. That this fall is not accurately governed by one single ionisation is not surprising: the minor kinetic consequences of the myriad ionisations available to a polyelectrolyte such as a protein are intrinsically uninterpretable.[33]

However, the absence of a variation in k_{cat}/K_m with pH below pH 6, in contrast to the compensating variations in k_{cat} and K_m individually, deserves comment. In

principle, this could arise from a perturbation of a pK_a from *ca.* 6.2 in the free enzyme to *ca.* 5 in the ES complex, or from a ' sticky substrate ' for which K_m is governed by the bond-breaking step [*i.e.* with reaction (1) where k_{-1}

$$E + Glu\ X \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E.Glu\ X \xrightarrow{k_2} E.Glu \xrightarrow{k_3} E + Glu\ OH \quad (1)$$

$\ll k_2$ as well as $k_2 \ll k_3$, so that $K_m = k_1/k_2$]. The latter cause is unlikely for two reasons. Firstly, with k_2 values *ca.* $10^{2.5}$ s^{-1}, and K_m values *ca.* $10^{-3.5}$ mol l^{-1}, k_1 values would have to be *ca.* 10^6 l mol^{-1} s^{-1}, well below the diffusion controlled rate. Secondly, the much lower values of k_{cat} for the *N*-glycosides studied mean that if all substrates were truly ' sticky ', then K_m values for these compounds would be proportionately lower than those for aryl glucosides; this is not the case.

(2) *Contributions to Catalysis.*—Any form of proton donation to the aglycone in the transition state for their enzymic hydrolysis is structurally impossible for the glycosylpyridinium salts. Therefore the whole of the catalytic activity of the enzyme against them must arise from nucleophilic and noncovalent interactions. A knowledge of the rates of the S_N1 reactions of glycosylpyridinium salts in water would enable the kinetic consequences of these nucleophilic and non-covalent interactions in the active site to be quantified.

The rates of the apparently S_N1 reactions of β-D-galactopyranosyl salts in aqueous 1.0M-KCl have been measured.[28] Recent measurements of the lifetimes of oxocarbonium ions by indirect [34] and direct [35] methods have indicated that the anticipated lifetimes of glycopyranosyl cations in water are too short for them to have a real existence. Nonetheless, studies of the solvolysis of the β-D-glucopyranosyl derivatives with various leaving groups in 1 : 1 ethanol–trifluoroethanol have indicated that any ' push ' from the solvent to leaving group departure occurs as much from the front as from the back side of the reaction centre, and is consequently probably electrostatic in nature.[36] Therefore, specific covalent contributions from water to the departure of leaving groups in free solution are probably small, and so the rates of spontaneous hydrolyses of pyridinium salts can be used as a rough estimate of the ' unassisted ' solvolysis rate. On the assumption that the *gluco*-derivatives solvolyse slower than the *galacto*-derivatives by the usual factor of $10^{0.5}$,[37] the spontaneous hydrolyses of the

$$\log k_{spon} = -4.84 - 1.26\ (pK_a)_N \quad (2)$$

pyridinium salt substrates are given by equation (2). Since the enzymic rates are given by equation (3), the

$$\log k_{cat} = 3.2 - 0.96\ (pK_a)_N \quad (3)$$

rate enhancement (T) attributable to nucleophilic and non-covalent interactions is given by equation (4). It is possible to estimate the spontaneous rates of hydrolysis of *O*-glycosides also. Combination of the data for *p*-nitrophenyl and 4-methylumbelliferyl glucosides in Table 4 and an estimate of the spontaneous rate

$$\log T = 8 + 0.3\ (pK_a)_N \quad (4)$$

of hydrolysis of 3,4-dinitrophenyl glucoside based on a three-fold reduction in the measured rate for the C-4 epimer [38] gives equation (5). The β_{lg} value

$$\log k_{spon} = -2.7 - 1.1\ (pK_a)_O \quad (5)$$

being in excellent agreement with that (1.18) obtained for spontaneous hydrolyses of 2-aryloxytetrahydropyrans.[39] k_{cat} Values at 35° for enzymic hydrolysis of *O*-glycosides are given by equation (6) and so the

$$\log k_{cat} = 3.14 - 0.055\ (pK_a)_O \quad (6)$$

total rate enhancement (Ω) brought about by the enzyme is given by equation (7), assuming k_{cat} is two-fold lower at 25°.

$$\log \Omega = 5.5 + 1.0\ (pK_a)_O \quad (7)$$

To estimate the contribution of acid catalysis to this total rate enhancement it is necessary to take into account the differing leaving abilities of oxygen and nitrogen leaving groups of the same pK_a. In an apparently unassisted S_N1 reaction, 3,4-dinitrophenolate (pK_a 5.4) leaves from the β-position of a galactopyranose ring at the same rate as β-chloropyridine (pK_a 2.8).[28] The β_{lg} values for the spontaneous hydrolysis of both *N*- and *O*-β-galactosyl derivatives are the same within experimental error, although this error could well disguise a difference of β_{lg} values of *ca.* 0.2. If we assume that the nucleophilic and non-colvalent contributions to the departure of 3-chloropyridine and 3,4-dinitrophenol are equal, we have equation (8). On this assumption the

$$\log T = 5.4 + 0.3\ (pK_a)_O \quad (8)$$

contribution of proton donation to catalysis, a rate-enhancement of Ξ-fold, is given by equation (9). This

$$\log \Xi = 0.1 + 0.7\ (pK_a)_O \quad (9)$$

gives proton-activating factors [40] in the range 10^3—10^7 for the aryl glucosides and *ca.* 10^9 for alkyl glucosides. These are larger than those estimated for *lac*-(*Z*)-β-galactosidase (10^2—10^5),[38] which however has to work in neutral or slightly alkaline medium and therefore cannot use a highly acidic acid catalytic group.

It should however be emphasised that the foregoing analysis is dependent on the assumption of similar transition states for hydrolyses of pyridinium salts and aryl glucosides, and that therefore its conclusions are probably only good to at best an order of magnitude.

(3) *Binding of Inhibitors.*—Results of the inhibition studies at pH 4.0 are given in Table 3. All inhibitors except D-glucono-δ-lactone showed mixed competitive–anti-competitive inhibition: plots of reciprocal velocity against inhibitor concentration were linear, indicating that only a single inhibitor molecule needed to be bound to cause complete inhibition.

Two features deserve comment. Firstly, conversion of

D-glucose to the lactone, a process involving merely the removal of two hydrogen atoms, increases the binding energy by 16 kJ mol^{-1}. This high additional binding energy could arise by reversible addition of the active site carboxylate,[41] or because the lactone, which is in a half-chair conformation, is an analogue of a transition state with a considerable amount of oxocarbonium ion character.[42]

The strong inhibition (compared to β-glucose) by 5-amino-5-deoxy-D-gluconolactam is in support of the latter explanation. In the lactam the conformation will still be the half-chair, but the susceptibility of the carbonyl group to nucleophilic addition will be much reduced. The former explanation must be favoured, however, for the β-glucosidase B from sweet almonds for which a carboxylate group has also been identified at the active site, but where the lactam inhibits only slightly better than glucose.[43]

Secondly, inhibitors bearing a positive charge on the exocyclic atom attached to C-1 are bound much more tightly than neutral ones, the K_i ratios for the isosteric pairs β-D-glucopyranose/β-D-glucopyransylammonium ion and β-D-glucopyranosylbenzene/β-D-gluocpyranosyl pyridinium ion being 4 000 and 320, respectively. The ratio for the primary amine could be increased by additional hydrogen bonding but the ratio for the quaternary salt, which cannot form hydrogen bonds, is still large. It indicates an effective charge on the active site equivalent to a single, point negative charge a distance $97/\varepsilon$ Å away, where ε is the effective dielectric constant. Evidence that this effective charge does in reality arise from a localized region of high charge (rather than from the aggregate effect of a number of distant charges) comes from the data on the β-D-glucopyranosylimidazolium ion. Its value of $\Delta\Delta G^0$ is what is anticipated from the half-charge borne by the nitrogen attached to the sugar, with no contribution from the charge on N-3.

Further support for the idea of a localized charge near C-1 comes from the observation that the 2-amino-2-deoxy-β-D-glucopyranose cation is bound no more tightly than β-D-glucopyranose. The contrasting behaviour of protonated glucosylamine and protonated glucosamine can be interpreted in terms of two alternative modes of localisation of the charges at the active site. There could either be a net negative charge on the β-face of the bound inhibitor in addition to the negative charge on the aspartate residue on the α-face,[3] or there could be a positively charged group on close proximity to the aspartate, but away from the aglycone, on the α-face. With this second mode of localisation of the charge, a positive charge attached to C-1 would experience largely the negative charge of the aspartate, while a positive charge attached to C-2 would experience approximate electrical neutrality. We favour the second proposal for the following reasons.

(1) k_{cat} Values of *O*-glucosides do not fall at low pH, indicating that the pK_a of the catalytic aspartate residue is lowered significantly from 'normal' values, as it would be by the proximity of a positive charge.

(2) Inspection of molecular models reveals that a negative charge on the β-face would have to be implausibly close to N-1 of the bound glucosylamine cation for the binding of the glucosamine cation to be so different.

Conclusions.—Figure 5 represents our picture of the

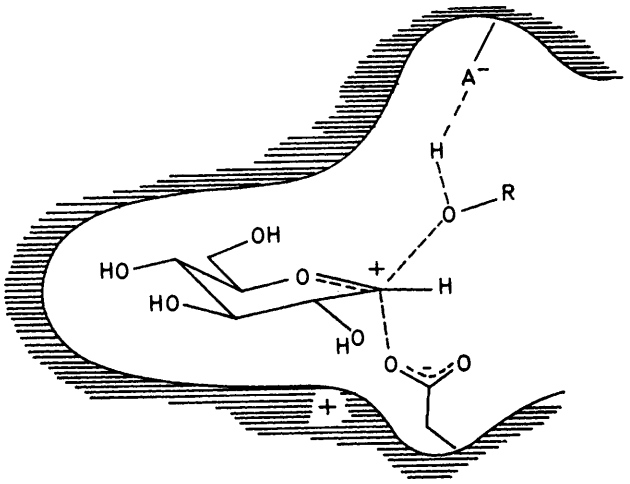


FIGURE 5

first transition state for the hydrolysis of *O*-glycosides by this enzyme. It predicts that incorporation of ^{13}C at C-1 of the glycone [or ^{18}O in the leaving group of an *O*-glycoside] will not give rise to a large isotope effect, but that incorporation of ^{15}N into the leaving group of an *N*-glycoside], will result in a (relatively) large heavy atom isotope effect.

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