Effects of deuterium substitution α and β to the reaction centre, ^{18}O substitution in the leaving group, and aglycone acidity on hydrolyses of aryl glucosides and glucosyl pyridinium ions by yeast α -glucosidase

A probable failure of the antiperiplanar-lone-pair hypothesis in glycosidase catalysis

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Neither k_{cat} , nor $k_{\text{cat}}/K_{\text{m}}$ for five aryl α -D-glucopyranosides correlates with aglycone $\text{p}K_{\text{a}}$, and isotope effects, described according to the convention used by Cleland [(1982) *CRC Crit. Rev. Biochem.* 13, 385–428], of $^{18}\text{O}(\text{V}) = 1.002 \pm 0.008$, $\alpha^{\text{D}}(\text{V}) = 1.01 \pm 0.04$ and $\alpha^{\text{D}}(\text{V/K}) = 0.969 \pm 0.035$ are observed for *p*-nitrophenyl, and one of $\beta^{\text{D}}(\text{V}) = 1.02 \pm 0.04$ for phenyl α -D-glucopyranoside; k_{cat} , but not $k_{\text{cat}}/K_{\text{m}}$, correlates with aglycone $\text{p}K_{\text{a}}$ for five α -D-glucopyranosyl pyridinium ions with a Brønsted coefficient of -0.61 ± 0.06 , and isotope effects of $\alpha^{\text{D}}(\text{V}) = 1.22 \pm 0.02$, $\beta^{\text{D}}(\text{V}) = 1.13 \pm 0.01$ and $\alpha^{\text{D}}(\text{V/K}) = 1.018 \pm 0.046$ for the 4-bromoisoquinolinium, and $\alpha^{\text{D}}(\text{V}) = 1.15 \pm 0.02$ and $\beta^{\text{D}}(\text{V}) = 1.085 \pm 0.011$ for the pyridinium salts are observed. These data require that a non-covalent event, fast in the case of the *N*-glycosides but slow in the case of the *O*-glycosides, precedes bond-breaking, and that bond-breaking involves substantial charge development on the glycone and near-perpendicularity of the C2–H bond to the planar oxocarbenium ion system. A model meeting these requirements is that the non-covalent event is a conjoint change of protein and substrate conformation which puts the pyranose ring in the $^2,^5\text{B}$ conformation of the bond-breaking transition state. This model also explains the contrast between the powerful inhibition of the enzyme by deoxynojirimycin ($K_{\text{i}} = 23 \pm 3 \mu\text{M}$) and feeble inhibition by castanospermine [Saul, Chambers, Molyneux & Elbein (1983) *Arch. Biochem. Biophys.* 221, 593–597], but is directly contrary to the predictions of Deslongchamps' 'Theory of Stereoelectronic Control' [Deslongchamps (1975) *Tetrahedron* 31, 2463–2490; (1983) *Stereoelectronic Effects in Organic Chemistry*, p. 39, Pergamon Press, Oxford].

The hydrolysis of glycosides is one of the simplest chemical reactions catalysed by enzymes, yet a detailed understanding of the catalytic mechanisms of glycosidases, other than some β -glycosidases, which operate with retention of the anomeric configuration (reviewed by Sinnott, 1978; Warshel, 1981; Pincus & Scheraga, 1981; Lalégerie *et al.*, 1982), remains elusive. In the present paper we report mechanistic investigations on a 'retaining' α -glucosidase, the α -glucosidase from baker's yeast (*Saccharomyces cerevisiae*) (Barnett, 1971). Kinetic probes of four types, and with two sets of substrates, have been used. The two sets of substrates are aryl α -glucopyranosides and α -D-glucopyranosyl pyridinium salts; these

latter substrates are noteworthy, since the ground-state conformation is not the normal $^4\text{C}_1$ chair but conformations of a twist type, probably $^1\text{S}_3$ (Fig. 1) (Hosie *et al.*, 1984), and acid or electrophilic assistance to aglycone departure is structurally impossible. The kinetic probes are the variation of rate parameters with leaving-group acidity, leaving group ^{18}O isotope effects, and α - and β - ^2H -isotope effects. α - ^2H kinetic isotope effects arise as a result of a weakening of a C–H bending vibration as the hybridization of the carbon atom to which it is attached changes from sp^3 to sp^2 , although effects of departing and incoming groups can alter the observed effects. β - ^2H kinetic isotope effects arise from hyperconjugation from the C– ^1H or C– ^2H bond into an adjacent electron-deficient centre. They are thus geometry-dependent, being at a

Abbreviation used: SDS, sodium dodecyl sulphate.

Fig. 1. The two chemical types of substrates, showing sites of isotopic substitution and ground-state conformation

maximum when the dihedral angle between the C-¹H or C-²H bond and the electron-deficient p-orbital of the reaction centre is 0° or 180°. When the dihedral angle is 90°, small inverse effects are observed, arising from the inductive effect of ²H, which has its origin in the anharmonicity of the C-H bond vibration [for a review of secondary ²H kinetic isotope effects, see Cleland (1985); for one of isotope effects on enzymic reactions, see Cleland (1982)].

Methods and materials

The material used for kinetic measurements was yeast α -glucosidase (type III, lot no. 12F 0725) purchased as a suspension in (NH₄)₂SO₄ solution from Sigma (Poole, Dorset, U.K.), who inform us that it was isolated from baker's yeast, *Saccharomyces cerevisiae*. The material as supplied was admixed with 25% (w/w) bovine serum albumin for stability. That there was no kinetically significant interaction between the two proteins was confirmed by isoelectric focusing (of a different sample, lot no. 12C-0287) between pH 4.0 and 7.0. Two peaks of protein, pI 5.5 and 5.8₅, and one of activity, pI 5.8₅, were observed; *K_m* values for *p*-nitrophenyl α -D-glucopyranoside were unchanged on removal of the albumin, or its re-addition to the separated enzyme. Isoelectric focusing of a sample of lot no. 12F 0725 gave the same pattern of two protein bands and one band of activity, and SDS/polyacrylamide-slab-gel electrophoresis of

this material gave two bands on staining with alkaline Ag⁽¹⁾ (Wray *et al.*, 1981). Comparison with standards indicated the bands were of *M_r* 67000 (bovine serum albumin) and 44000. A *M_r* value for native baker's yeast α -glucosidase of 44000 was reported by Siro & Lövgren (1978); those authors, however, report many bands of low *M_r* on SDS/polyacrylamide-gel electrophoresis, clearly due to proteolytic degradation.

Substrates

Unlabelled α -D-glucopyranosyl pyridinium bromides are described elsewhere (Hosie *et al.*, 1984); unlabelled 4-methylumbelliferyl and 2-naphthyl α -D-glucopyranosides were purchased from Koch-Light Laboratories, Colnbrook, Bucks., U.K.; unlabelled phenyl and *p*-nitrophenyl α -D-glucopyranosides were purchased from Sigma.

Introduction of ²H at C-1. 1,2,3,4,6-Penta-O-acetyl-[1-²H] β -D-glucopyranose, m.p. 131.5–133°C, was made (in 30% yield from lactone) by reduction of the tetrahydropyran derivative of D-glucono- δ -lactone (Bhattacharjee *et al.*, 1975) with NaB²H₄ in ²H₂O, followed by acetylation of the crystalline [1-²H] β -D-glucose with acetic anhydride in the presence of sodium acetate (Wolfson & Thompson, 1963). This penta-acetyl glucose was converted into 2,3,4,6-tetra-O-acetyl-[1-²H] α -D-glucopyranosyl bromide in 50% yield by reaction with 20% (w/v) HBr in acetic acid. A 15% (w/v) solution of the penta-acetate in this medium was left at 22°C for 20 min, then it was taken up in dichloromethane (1.5 vol.), washed well with ice-cold water, then saturated NaHCO₃ solution, then ice-cold water again, dried (MgSO₄) and evaporated. The crude material, which crystallized from dry diethyl ether/ligroin, was used directly to prepare [1-²H] α -D-glucopyranosyl pyridinium bromide {m.p. 145–147°C, [α]_D²⁵ + 53.8°, Anal.* (C₁₁H₆O₅NBr) C, H, N, Br} and [1-²H] α -D-glucopyranosyl 4-bromoisoquinolinium bromide [m.p. 140–142.5°C, [α]_D²⁵ + 49.3°, Anal. (C₁₅H₁₇O₅NBr₂) C, H, N, Br calcd. 35.35; found 35.93%] in 7% and 9% yields respectively by the method of Hosie *et al.* (1984).

1,2,3,4,6-Penta-O-acetyl-[1-²H] α -D-glucopyranose (m.p. 109–111°C) was made (in 34% yield) by reduction of unprotected D-gluconolactone with sodium amalgam in the presence of ²H₃PO₄ in ²H₂O, as described by Murray & Williams (1958), followed by acetylation of the

* The American Chemical Society (1983) abbreviated system for expressing elemental analysis is used. In this system, routine analyses that agree with calculated values within 0.4% are indicated by a listing of elements without numerical values. In cases where it is necessary to rely on a normally unacceptable analysis, the values are given in full.

syropy product with acetic anhydride in pyridine by the method of Wolfrom & Thompson (1963). This material was used to make *p*-nitrophenyl [1- ^2H]- α -D-glucopyranoside [m.p. 214–217°C, Anal. ($\text{C}_{12}\text{H}_{15}\text{O}_8\text{N}$) C, H, N, in 30% yield] via fusion with *p*-nitrophenol and ZnCl_2 as described by Montgomery *et al.* (1942). From ^1H n.m.r. of both penta-acetyl glucoses, we determined ^2H incorporation to be more than 95%.

Introduction of ^2H at C-2

The Lemieux (Lemieux *et al.*, 1968a) 'oximino-chloride' method for α -glucoside synthesis via phenyl 3,4,6-tri-*O*-acetyl- α -D-arabinoheptopyranosulose, which could be reduced with NaB^2H_4 was the method adopted.

Dimeric 3,4,6-tri-*O*-acetyl-2-deoxy-2-nitroso- α -D-glucopyranosyl chloride (16g) (Lemieux *et al.*, 1968a) and phenol (6.7g) in dry dimethyl formamide (50ml) were left for 66h at room temperature. The red solution was diluted with dichloromethane (500ml), extracted with 0.01M-NaOH (5 $\times$ 100ml), dried, and evaporated to a syrup, which crystallized on standing {yield 64%, m.p. 165.5–167°C [lit. (Lemieux *et al.*, 1968b) 165–167°C], Anal. ($\text{C}_{18}\text{H}_{21}\text{O}_9\text{N}$) C, H, N, after recrystallization from aqueous ethanol}. The phenyl 3,4,6-tri-*O*-acetyl 2-oximino- α -D-arabinoheptopyranoside product (10g) was hydrolysed in a mixture of acetonitrile (30ml), in aqueous HCl (24ml) and acetaldehyde (2ml) for 5h at room temperature (Lemieux *et al.*, 1973a), and then extracted with ethyl acetate (2 $\times$ 75ml). The organic layer was washed with water (50ml), dried, and evaporated. The resulting syrupy phenyl 3,4,6-tri-*O*-acetyl- α -D-arabinoheptopyranosulose (7.7g) was dissolved in dry tetrahydrofuran (150ml) and to this solution, cooled to 0°C, was added NaB^2H_4 (3.75g) in cold $^2\text{H}_2\text{O}$ (15ml) (cf. Lemieux *et al.*, 1973b). After 30min at 0°C and 3h at room temperature, the excess NaB^2H_4 was destroyed by the cautious addition of acetic acid, and the solution was evaporated to a syrup, which was washed with methanol to remove H_3BO_3 . The syrup was then taken into acetic anhydride (75ml), cooled, and dry pyridine (90ml) was added. After 18h at room temperature the solution was poured into ice/water (750ml). The resultant solids, filtered off and recrystallized from ethanol yielded phenyl 2,3,4,6-tetra-*O*-acetyl [2- ^2H]- α -D-glucopyranoside {m.p. 110–111°C, $[\alpha]_D^{25} + 180.7^\circ$ (c1 in chloroform), Anal. ($\text{C}_{20}\text{H}_{23}\text{O}_{10}$) C, H, in 63% yield from the oximino compound}. Zemplén deacetylation, and recrystallization from ethanol, gave phenyl [2- ^2H]- α -D-glucopyranoside (m.p. 173–174°C) used in kinetic isotope-effect work.

For preparation of the [2- ^2H]- α -D-glucopyranoside pyridinium salts, phenyl 2,3,4,6-tetra-

O-acetyl [2- ^2H]- α -D-glucopyranoside (3.4g) was first converted into the corresponding 2,3,4,6-tetra-*O*-acetyl [2- ^2H]- α -D-glucopyranosyl bromide by reaction for 4h at 40°C in 20% (w/v) HBr in acetic acid (30ml) containing a few drops of acetic anhydride (to remove traces of water which otherwise led to extensive degradation). The reaction mixture was then taken up into dichloromethane (50ml), washed successively with ice/water (2 $\times$ 30ml), saturated NaHCO_3 solution (3 $\times$ 40ml), 12% (w/v) aqueous FeCl_3 solution (2 $\times$ 20ml, to extract phenol), and water, and then dried and evaporated. The crude material crystallized from a mixture of dry diethyl ether and ligroin, and was used to prepare [2- ^2H]- α -D-glucopyranosyl 4-bromoisoquinolinium bromide {m.p. 139–142°C, $[\alpha]_D^{25} + 46$; Anal. ($\text{C}_{15}\text{H}_{17}\text{O}_5\text{NBr}_2$) C, H, N, Br} and [2- ^2H]- α -D-glucopyranosyl pyridinium bromide [m.p. 144–145°C, Anal. ($\text{C}_{11}\text{H}_{16}\text{O}_5\text{NBr}$) C, H, N, Br], in 2% yields, by the method of Hosie *et al.* (1984).

^1H n.m.r. of the 2,3,4,6-tetra-*O*-acetyl [2- ^2H]- α -D-glucopyranosyl bromide indicated that there was about 5% of residual ^1H at position 2.

m-Tolyl α -D-glucopyranoside {m.p. 163–165°C, $[\alpha]_D^{25} + 180.4$ (c 0.2 in H_2O) Anal. ($\text{C}_{13}\text{H}_{19}\text{O}_6$) C, H} was made by the Lemieux oximino-chloride method, with NaBH_4 reduction of the uloside; its physical properties are in excellent agreement with those reported by Hall *et al.* (1961) (m.p. 159–161°C, $[\alpha]_D^{25} + 178^\circ$) for a material made by the ZnCl_2 -fusion method of Montgomery *et al.* (1942), but differ drastically from those reported by Yamada *et al.* (1975) {m.p. 185–186°C, $[\alpha]_D^{25} + 52.6^\circ$ (H_2O)}, the identity of whose material must now be questioned.

p-Nitrophenyl 2,3,4,6-tetra-*O*-acetyl-[1- ^{18}O]- α -D-glucopyranoside (m.p. 112–113°C) was made by the method of Montgomery *et al.* (1942) from *p*-[1- ^{18}O]-nitrophenol. This latter compound was made by Mr. A. J. Bennet and Mrs. S. Wijesundera in this laboratory, essentially by the method of Rosenberg & Kirsch (1979), except that reaction of lithium [1- ^{18}O]-methoxide with *p*-nitrofluorobenzene was carried out in dimethyl sulphoxide, not hexamethylphosphoric triamide; the isotopic enrichment, estimated by direct mass spectrometry [M^+] was 97%. The acetylated labelled glycoside was subject to Zemplén deacetylation and, after recrystallization from methanol, gave a material of m.p. 216–218°C.

1-Deoxynojirimycin was generously given by Professor Dr. G. Legler, Institut für Biochemie, Universität zu Köln, Köln, Germany.

Kinetic methods

All kinetic data pertain to 50mM-sodium phosphate buffer, pH6.80, at 25.0°C. The buffer

solution was made by mixing 50mM-solutions of AnalaR NaH_2PO_4 and AnalaR Na_2HPO_4 in h.p.l.c.-grade water from a Millipore Milli-Q water-purification system, until the required pH was registered on a Radiometer PHM 62 pH-meter calibrated with BDH standard buffer. Enzyme was dialysed against buffer before use.

Michaelis-Menten parameters. These were measured, for all substrates except the 4-methylumbelliferyl glucoside, from initial changes in absorbance monitored with either a Pye-Unicam SP.8-200 kinetic system or a Unicam SP.1800 fitted with a cell block through which water, maintained at $25.0 \pm 0.1^\circ\text{C}$, was circulated by a Techne Tempunit. Wavelengths (in nm) and molar-absorption-coefficient differences ($\text{M}^{-1}\cdot\text{cm}^{-1}$) are as follows: *p*-nitrophenol, 400, 6.41×10^3 ; 2-naphthol, 330, 1.15×10^3 ; phenol, 277, 0.84×10^3 ; *m*-cresol, 279, 1.019×10^3 ; 3-bromopyridine, 280, -3.73×10^3 ; 4-bromoisquinoline, 344, -6.11×10^3 ; pyridine, 265, -2.58×10^3 ; isoquinoline, 340, -3.84×10^3 ; 4-methylpyridine, 265, -2.745×10^3 . Michaelis-Menten parameters for the 4-methylumbelliferyl glucoside were obtained on a Perkin-Elmer 3000 fluorescence spectrometer, with excitation at 360nm and emission at 450nm. Fluorescence of aglycone was calibrated at various substrate concentrations to allow for quenching. Thermostatic control of temperature at $25.0 \pm 0.1^\circ\text{C}$ was achieved by the use of a jacketed cell through which water was passed from a bath by a Tempunit.

V_{max} and K_m values were determined from linear least-squares treatment of v against $v/[S]$ plots, $[S]$ varying from 0.7 to $7K_m$ or until substrate inhibition became detectable. V_{max} values were standardized against *p*-nitrophenyl glucoside, and were converted into absolute k_{cat} values on the assumptions that (a) the most active enzyme sample was 100% active, (b) the enzyme contained 25% bovine serum albumin, (c) protein could be estimated from $A_{1\text{cm}}^{1\text{mg/ml}}$ 1.91, and (d) the M_r per active site was 44000. The scientific argument is dependent on the correctness of these assumptions only if they result in errors of a couple of orders of magnitude, so that k_{cat}/K_m values approach the diffusion limit; relative k_{cat} values are probably good to $\pm 10\%$.

Kinetic isotope effects. These were all measured in the Pye-Unicam SP.8-200 spectrometer system.

^{18}O for *p*-nitrophenyl glucoside and ^{13}C for phenyl glucoside were measured by addition of enzyme (15 μl) to stock solutions (0.3ml) of ^1H - and ^2H -glycosides [2.22mM and 4.54mM respectively, the concentrations that give maximal rates ($\sqrt{K_m K_{\text{is}}}$), in 1mm-path-length cells, and absorbance changes were recorded. Ten consecutive pairs of readings were taken, runs being in the order ^1H

^2H ^2H ^1H ^1H ^2H ^2H etc., so as to minimize any effects of loss of enzyme activity. Quoted effects are means $\pm$ s.d. for the ten comparisons.

Measurements carried out as above are subject to large random errors arising from the proportionality of rate and enzyme concentration. With dialysed enzyme, the superior technique, of adding a concentrated solution of substrate to the same solution of dilute enzyme, did not seem to be practicable, because of the rapid loss of activity of dilute enzyme. However, subsequent to the secondary ^2H kinetic-isotope-effect measurements on *O*-glycosides, we found that additional bovine serum albumin (1mg/ml) stabilized even dilute enzyme. If the u.v. cells used in the isotope-effect measurements are 'scrambled' between runs, then spectrophotometric, temperature, and path-length errors all appear in the s.d.; the only possible systematic error is the purity of the substrate.

Secondary ^2H kinetic isotope effects on V_{max} for the pyridinium salts were thus measured by addition of concentrated substrate solution (50 μl) to stabilized enzyme solution (450 μl) in 2mm-path-length cells, to give a final substrate concentration of $6\text{--}7K_m$; successive pairs of runs were compared, but runs were performed in the order ^1H ^2H ^2H ^1H ^1H ^2H , as before. Quoted effects, the first values in Table 2 (below), are the means $\pm$ s.d. for 10–15 such runs. The substrate-purity problem was then addressed by repeating the measurements with a final concentration of $12\text{--}14K_m$; these are the second values for each effect in Table 2.

For measurement of ^{18}O for *p*-nitrophenyl glucoside hydrolysis, it was not possible to alter the substrate concentration because of the occurrence of product inhibition. The quoted effects (Table 2 below) are therefore results obtained with 2.22mM substrates after one, two and three successive recrystallizations, 10–15 comparisons of pairs of runs, in the order ^{16}O , ^{18}O , ^{18}O , ^{16}O , ^{16}O , etc. being made. Perhaps the most telling argument for the soundness of our measurement of this (potentially) very small effect is that we had formulated the model described in the Discussion section before performing this experiment, and were disconcerted to find a significant ^{18}O isotope effect on the rate of increase of absorbance at 400nm (1.011 ± 0.012 , 1.014 ± 0.18 and 1.014 ± 0.008). Only after the failure of our best efforts to achieve an effect of 1.00 by successive recrystallizations of the substrates did we appreciate that the A_{400} of a solution of pH 6.80 would be affected by the equilibrium isotope effect on the acid dissociation constant of *p*-nitrophenol ($\text{p}K_a$ 7.15). The effects in Table 2 were corrected for this by using a value of $^{16}K_a/^{18}K_a = 1.018$ (Rosenberg & Kirsch, 1981a).

^{13}C (V/K) values were obtained by linear least-

squares treatment of $1/v$ -against- $1/[S]$ plots for $[^1\text{H}]$ - and $[^2\text{H}]$ -substrate, $[S]$ being calculated from the absorbance of the reaction cell (at 310 nm in the case of the *p*-nitrophenyl compound), rather than from dilutions.

Results and discussion

Results and necessary conclusions

Michaelis-Menten parameters for various substrates, at pH 6.8 and 25.0°C, are given in Table 1, and kinetic isotope effects, described by the Cleland (1982) convention, in Table 2. The results with aryl glucosides show no systematic dependence of either $\log k_{\text{cat}}$ or $\log(k_{\text{cat}}/K_m)$ on the $\text{p}K_a$ of the departing phenol, correlation coefficients of 0.62 and 0.19 respectively being obtained; the five data points are adequate to confirm that the random variation of kinetic parameters with substrate structure observed with ten substrates by Hall *et al.* (1962) was not a consequence of catalysis by several isoenzymes not separable by the techniques of the time.

There is no ^{18}O kinetic isotope effect on V_{max} for

the hydrolysis of *p*-nitrophenyl glucoside; the C–O bond is not being broken in the rate-determining transition state, since the error on the measurement is less than the effect anticipated for bond-breaking. Thus, with the same leaving group and the appropriate sugar, values of $^{18}(\text{V})$ of 1.022 ± 0.002 for *lacZ* β -galactosidase of *E. coli* (Rosenberg & Kirsch, 1981*a*) and 1.030 ± 0.007 for α -L-arabinofuranosidase III of *Monilia fructigena* (M. Herrchen, M. L. Sinnott & S. Wijesundera, unpublished work) have been found, as have values of $^{18}(\text{V}/K)$ of 1.014 ± 0.003 for *lacZ* β -galactosidase, 1.04687 ± 0.0015 for lysozyme and 1.0377 ± 0.0061 for β -glucosidase A of sweet almonds (Rosenberg & Kirsch, 1981*b*). Proton transfer will reduce the observed effect, but not below that observed for acid-catalysed hydrolysis of methyl α -glucopyranoside (1.025 in 1.0M-HClO₄ at 80°C; A. J. Bennet & M. L. Sinnott, unpublished work).

Without additional *ad hoc* hypotheses, k_{cat} for nitrophenyl glucoside cannot be taken as representing hydrolysis of a glucosyl-enzyme, since it is not the highest k_{cat} value observed. A combination

Table 1. Michaelis-Menten parameters for hydrolysis of α -D-glucopyranosyl derivatives in 50 mM-sodium phosphate buffer, pH 6.8 at 25.0°C

The substrate inhibition constant, K_{is} , was calculated from:

$$v = \frac{V_{\text{max}} [S]}{K_m + [S] + \frac{[S]^2}{K_{\text{is}}}}$$

Leaving group	k_{cat} (s ⁻¹)	K_m (mM)	K_{is} (mM)
<i>p</i> -Nitrophenol	20	0.30 ± 0.03	16
4-Methylumbelliferone	8	0.17 ± 0.01	
2-Naphthol	19	0.17 ± 0.02	
<i>m</i> -Cresol	77	0.73 ± 0.05	
Phenol	28	0.94 ± 0.10	28
3-Bromopyridine	29	0.71 ± 0.05	
4-Bromoisquinoline	11	0.131 ± 0.014	
Pyridine	1.69	0.066 ± 0.006	
Isoquinoline	0.52	0.0066 ± 0.0005	
4-Methyl pyridine	0.32	0.019 ± 0.002	

Table 2. Kinetic isotope effects on yeast α -glucosidase-catalysed hydrolyses (pH 6.8, 25.0°C)

Leaving group	$^{18}(\text{V})$	$\alpha\text{D}(\text{V})$	$\alpha\text{D}(\text{V}/K)$	$\beta\text{D}(\text{V})$
<i>p</i> -Nitrophenol	0.999 ± 0.012 1.002 ± 0.018 1.002 ± 0.008	1.014 ± 0.04	$0.969 \pm 0.03_5$	—
Phenol	—	—	—	1.02 ± 0.04
4-Bromoisquinoline	—	$1.204 \pm 0.03_3$ $1.224 \pm 0.02_0$	$1.018 \pm 0.04_6$	$1.132 \pm 0.03_0$ 1.133 ± 0.009
Pyridine	—	$1.153 \pm 0.01_8$ $1.155 \pm 0.01_8$	—	$1.086 \pm 0.02_1$ $1.084 \pm 0.01_1$

of non-productive binding and rate-limiting deglucosylation could explain the absence of an ^{18}O kinetic isotope effect and detectable correlation of $k_{\text{cat.}}$ with $\text{p}K_{\text{a}}$, but not the absence of such a correlation with $k_{\text{cat.}}/K_{\text{m}}$. Moreover, the absence of detectable α - or β - ^2H kinetic isotope effects on $k_{\text{cat.}}$, and of an α - ^2H kinetic isotope effect on $k_{\text{cat.}}/K_{\text{m}}$ for *p*-nitrophenyl glucoside, make the non-productive-binding-plus-deglucosylation hypothesis untenable without yet further *ad hoc* hypotheses (e.g. a change in chemical mechanism between the aryl glucosides and the glucosyl pyridinium ions).

The simplest explanation for the results with aryl glucosides is that bond-breaking is preceded by a rate-limiting non-covalent event that depends in a random way on aglycone structure.

The data on the pyridinium salts indicate that this event is rapid and irreversible for these substrates. Although $\log(k_{\text{cat.}}/K_{\text{m}})$ does not correlate with aglycone $\text{p}K_{\text{a}}$ (correlation coefficient, r ,

the $^4\text{C}_1$ conformation, and $k_{-1} \ll k_{+2}$. However, work by Finch and co-workers (Finch & Nagpurkar, 1976; Bourne *et al.*, 1973) on α -D-glucopyranosyl imidazole makes this model untenable; the reverse anomeric effect of the half positive charge attached to C-1 in the protonated form of this compound is insufficient to flip the pyranose ring to a boat (Finch & Nagpurkar, 1976), but the compound is bound to α -glucosidase with the modest K_{i} of 0.3 mM (Bourne *et al.*, 1973). The "sticky" $^4\text{C}_1$ pyridinium ion' hypothesis can therefore be discarded; it could not in any event explain the aryl glucoside results.

Our data therefore *require* that the bond-breaking step in catalysis by this enzyme be preceded by a kinetically discrete non-covalent transformation of the first-formed E·S complex. If we take the observed overall stereochemical retention as sufficient evidence for a double-displacement reaction, then we must have a four-step mechanism (Scheme 2):

$$\text{in which } k_{\text{cat.}} = 1/[(1/k_{+2}) + (1/k_{+3}) + (1/k_{+4})]$$

$$K_{\text{m}} = \frac{k_{-1} + k_{+2}}{k_{+2}k_{+1}} \left/ \left(\frac{1}{k_{+2}} + \frac{1}{k_{+3}} + \frac{1}{k_{+4}} \right) \right.$$

Scheme 2

$-0.48) \log k_{\text{cat.}}$ does, with $r = -0.985$ and a Brønsted coefficient (β_{lg}) of -0.61 ± 0.06 . There are both α - and β - ^2H kinetic isotope effects on $V_{\text{max.}}$ for two substrates, but there is no α - ^2H kinetic isotope effect on $V_{\text{max.}}/K_{\text{m}}$ for the substrate that has the largest such effect on $V_{\text{max.}}$. Standing by themselves, these results with the pyridinium salts could be rationalized by the proposal that the enzyme binds the small proportion of pyridinium salt in the $^4\text{C}_1$ conformation at the diffusion-controlled rate, and that positively charged substrates in this conformation are 'sticky'. The observed random dependence of $k_{\text{cat.}}/K_{\text{m}}$, but not $k_{\text{cat.}}$, would be observed on Scheme 1 below,

Nature of the bond-breaking step

The bond-breaking step is only kinetically accessible in the case of the pyridinium salts. From the data of Arnett & Reich (1980) it is possible to calculate a $\beta_{\text{eq.}}$ value of 1.47 ± 0.22 for quaternization of pyridines by methyl iodide. If a similar $\beta_{\text{eq.}}$ value governs displacement reactions of α -glucosylpyridinium salts, then the value of β_{lg} for these substrates indicates that the C-N bond is roughly half-broken at the transition state.

The value of α - ^2H kinetic isotope effects (Table 2) are about half the equilibrium effect for hydration of acetaldehyde $\{K[\text{CH}_3\text{CHO}]/$

$$k_{\text{cat.}}/K_{\text{m}} = \frac{k_{+1}k_{+2}}{k_{-1} + k_{+2}}$$

Scheme 1

if k_{+1} were the diffusion-controlled rate constant (say $10^8 \cdot \text{M}^{-1} \text{s}^{-1}$) times the fraction of substrate in

$K[\text{CH}_3\text{O}(\text{HOH})_2] = 1.37$; Lewis & Wolfenden, 1977). This probe of transition-state structure thus

indicates that the reaction centre is about half-way to sp^2 hybridization. Since the degree of C–N cleavage, as measured by the β_{1g} value, and the degree of rehybridization of C-1 are the same, little nucleophilic assistance to aglycone departure must be taking place, and therefore there must be substantial charge build-up on the C-1–O-5 region of the pyranose ring. Results with deoxynojirimycin (see below), and values of β - 2H kinetic isotope effects confirm this.

The literature data available to 'scale' our β - 2H kinetic isotope effects are limited. The extensive data on solvolysis reactions of saturated alkyl

elaborate hypotheses on the basis of these small differences, but it is probably worth noting that 'retaining' glycosidases seem to possess active-site carboxylate groups in all cases so far examined [see Fowler & Smith (1983) and Legler & Herrchen (1984) for recent examples], that in spontaneous hydrolyses of glucopyranosyl derivatives, β -carboxylate nucleofugacities lie between those of α -4-bromoisoquinoline and α -pyridine (Brown & Bruice, 1973; Hosie *et al.*, 1984), and that therefore, if k_{+3} of Scheme 2 is complex (as set out in Scheme 3), then we could be observing a change in the rate-determining step.

Scheme 3

systems are not really applicable, since such reactions experience elimination and hydride-shift which are subject to *primary* kinetic isotope effects. A lower limit can be obtained from the value of $\beta_D(K)$ of 0.81 obtained by Cook *et al.* (1980) for the oxidation of [2,2,6,6- 2H]cyclohexanol by NAD^+ ; this effect will arise overwhelmingly from the two axial 2H atoms (which make a dihedral angle of about 30° with the electron-deficient p-orbital) (the two equatorial ones nearly eclipse the C=O bond), so a lower limit of 10% per 2H atom can be placed on the equilibrium effect for an ideally placed C–H or C- 2H bond hyperconjugating into $>\text{C}=\text{O}$; if it hyperconjugates into $>\text{C}=\text{O}^+-\text{R}$ the effect will of course be much bigger. A relevant kinetic effect, uncomplicated by elimination or hydride-shift, is one of (maximally) 1.51 for the dediazonium of [2,4,6- 2H]benzene diazonium tetrafluoroborate; (Szele & Zollinger, 1981), this effect will arise largely from the two 2H atoms *ortho* and aligned with the empty sp^2 orbital of the developing phenyl cation.

From these limited data it thus appears that observed β - 2H kinetic isotope effects are compatible with the C–N bond being about half-broken, and the anomeric carbon being about half-rehybridized, if the C2–H bond is well placed to hyperconjugate with the electron-deficient centre.

Both α and β secondary kinetic isotope effects are different for the two pyridinium salts, the difference being in the sense of more carbonium ion character for the faster leaving group for both types of effect. We are unwilling to construct

Such a change in the rate-determining step could be the cause of the β_{1g} value, which is low compared with that obtained for a number of β -glycosidases (Sinnott & Withers, 1974; Legler *et al.*, 1980; Burton & Sinnott, 1983), despite α - 2H kinetic isotope effects being the same as those observed with the *lacZ* β -galactosidase of *E. coli* (Sinnott & Withers, 1974).

Model, and further evidence for it

Oxocarbenium ions have to be planar; studies of $\text{CH}_2=\text{O}^+-\text{CH}_3$ under stable ion conditions have, for example, indicated that conformation (II) lies at least 50 kJ (11.9 kcal)/mol above conformation (I) (Farcaşiu *et al.*, 1979) (Fig. 2). The lactone function likewise is generally planar. Therefore it is reasonable to use the conformational preferences of δ -lactones as a guide to the conformational preferences of pyranosyl cations. Available data is that the classical boat conformation is comparable in energy with the half-chair conformation; in the gas phase the classical boat conformation of δ -valerolactone lies only 2.5 kJ (0.6 kcal)/mol above the half chair (Philip *et al.*, 1981). In the sugar series, as expected from this small difference in the unsubstituted system, conformational preferences vary with substituents, for example, tri-*O*-acetyl-xylono- δ -lactone is in the $^{2.5}B$ conformation (Nelson, 1979); mannono- δ -lactone is in the $B_{2.5}$ conformation, and, importantly for the present considerations, although glucono- δ -lactone is predominantly in the 4H_3 half-chair conformation, the classical boat $^{2.5}B$ conformation can be

Fig. 2. Planar (I) and perpendicular (II) conformations of the methoxymethyl cation and permissible conformations of the D-glucopyranosyl cation

detected by ¹H n.m.r. (Walaśzek *et al.*, 1982). Thus ⁴H₃, ^{2,5}B, and B_{2,5} conformations (Fig. 2) are possible candidates for the conformation of the glucopyranosyl cation intermediate (or cation-like transition state) in the α-glucosidase catalytic mechanism.

Identification of *k*₂ in Scheme 2 with a conjoint change in the conformation of the protein and substrate, in which the sugar ring was converted into either the ^{2,5}B or B_{2,5} conformation, would immediately explain why the change is fast with pyridinium salt substrates and slow with aryl glucosides, despite overlapping ranges of absolute *k*_{cat} values. With the sugar ring already on the twist-boat pseudorotational itinerary, less energy is required to attain the boat conformations than with substrates such as aryl glucosides, in which the ⁴C₁ chair conformation is strongly preferred. In both the B_{2,5} and ^{2,5}B conformations of the pyridinium salts the reverse anomeric effect is adequately expressed; indeed the B_{2,5} conformation is the preferred conformation of the tetra-*O*-acetylated compounds in [²H]chloroform (Lemieux, 1971). If the preferred conformation were ⁴H₃, and the *k*₊₂ step took place so as to distort the ⁴C₁ chair to this conformation before bond-breaking, this too would be accelerated if the ground state were not the ⁴C₁ chair but a twist

boat. However, it is difficult to see how the protein conformational changes associated with the change in substrate ring conformation, from ⁴C₁ to ⁴H₃, which involves only small atomic displacements (Warshel & Levitt, 1976), could give rise to a discrete kinetic event which can become rate-limiting. Recent molecular dynamics simulations of protein structure (McCammon & Karplus, 1983) have indicated that motions of surface atoms of the protein occur with an r.m.s. displacement and time constant on the order of 0.1 nm (1 Å) and 10⁻¹² s respectively.

Moreover a number of β-glycosidases, considered to work via glycosyl-cation-like transition states, show no sign of the introduction of conformational distortion in a separate, kinetically significant step. These are the β-glucosidases from *Aspergillus wentii* (Legler *et al.*, 1980), the A (Rosenberg & Kirsch, 1981*b*) and B (M. L. Sinnott and I. J. L. Souchard, unpublished work) isoenzymes from sweet almonds, the *lacZ* (Sinnott, 1978) and *ebg* (Burton & Sinnott, 1983) β-galactosidases from *Escherichia coli*, and lysozyme (Rosenberg & Kirsch, 1981*b*).

Therefore, if the non-covalent event required by our kinetic data is a conjoint change in the conformation of substrate and protein, it is only likely to be kinetically significant if the associated change

in substrate conformation is substantial, to a boat. Of the two classical boat conformations, $^{2,5}B$ and $B_{2,5}$ (Fig. 2) in which the oxocarbenium ion can be planar, only the $^{2,5}B$ conformation is compatible with the significant β - 2H kinetic isotope effects; in the $B_{2,5}$ conformation of the oxocarbenium ion the electron-deficient p-orbital is orthogonal to the C2-H bond. The $^{2,5}B$ conformation is adopted to some extent by D-glucono- δ -lactone (Walażek *et al.*, 1982).

The identification of the reactive conformation of bound substrate as $^{2,5}B$ is made much firmer because this conformation alone rationalizes rather puzzling results with competitive inhibitors of the 'transition-state analogue' type. Piperidines with the appropriate substitution pattern are powerful inhibitors of the corresponding glycosidases, because of the resemblance of the protonated molecule to the oxocarbenium-ion-like transition states occurring during catalysis (Lalegerie *et al.*, 1982). Thus deoxynojirimycin is a powerful inhibitor of α -glucosidases (Legler & Jülich, 1984) (Fig. 3) and has a K_i value of $23 \pm 3 \mu M$ measured against our sample of yeast α -glucosidase. However, with yeast enzyme from the same supplier, Saul *et al.* (1983) found that castanospermine (Fig. 3) was not a powerful inhibitor. (For the structure and relative stereochemistry of castanospermine, see Hohenschutz *et al.*, 1981; for the absolute stereochemistry, Bernotas & Ganem, 1984). The

only difference between the two inhibitors is the two bridging methylene groups in castanospermine, which rigorously preclude it from adopting the $^{2,5}B$ conformation, but are as compatible with the 4H_3 conformation as deoxynojirimycin. If the nitrogen atom of castanospermine is pyramidalized so that the ring junction is *trans*, in the equivalent of the $^{2,5}B$ conformation the ring junction has to be *trans* axial-flagstaff, which is impossible; if it is pyramidalized *cis*, so that the ring junction is equatorial-flagstaff in the equivalent of the $^{2,5}B$ conformation the hydroxy group on the five-membered ring has to occupy the same as the flagstaff hydrogen atom (Fig. 3).

We conclude that there is a strong probability that k_{+2} (Scheme 3) represents a conformational change of the E-S complex to place the pyranose ring in the $^{2,5}B$ conformation.

Deslongchamps (1975) has put forward the idea that departure of a leaving group from a tetrahedral carbon atom, substituted with one or more heteroatoms, is favoured in an additive way by the presence of antiperiplanar lone pairs of electrons on these heteroatoms. The idea is clearly applicable to glycosyl transfer, both enzyme and non-enzymic, and indeed Deslongchamps (1983) has stated " α -glycosides must hydrolyse *via* their ground state conformation whereas β -glycosides must first assume a boat conformation in order to fulfill the stereo-electronic requirement". Our

Fig. 3. Deoxynojirimycin (III) and castanospermine (IV), illustrating how the former, but not the latter, can adopt the equivalent of the $^{2,5}B$ conformation

model for α -glucosidase catalysis directly contradicts this idea; we propose that, presented with a substrate which, in its ground state has an antiperiplanar lone pair, the enzyme converts it into the $^{2,5}\text{B}$ conformation, where no such lone pair is available, before performing bond cleavage. The general problems arising from the application of the 'theory of stereoelectronic control' have been discussed elsewhere (Sinnott, 1984).

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