BIOORGANIC CHEMISTRY 12, 158–169 (1984)

Kinetic α-Deuterium Isotope Effects for Enzymatic and Acid Hydrolysis of Aryl-β-D-Glycopyranosides

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Received October 24, 1983

The kinetic α-deuterium isotope effect for the enzymatic- and acid-catalyzed hydrolysis of a series of aryl-β-D-[1-^{2}H]glycopyranosides has been measured. The magnitude of the effect indicates a considerable steric hindrance of the anomeric C—H (C—D) bond in an early transition state for both kinds of reactions. Better leaving aryl groups decrease the isotope effect for the acid-catalyzed hydrolysis, as predicted by the predominant A-1 character of the reaction. In contrast, the α-deuterium effect for the enzymatic-catalyzed reactions is increased by better leaving aglycon groups, suggesting a mechanism with considerable S_N2 characteristics. The isotope effect for the acid hydrolysis of 4-methylumbelliferyl- and 4-methylfenyl-β-D-glucopyranoside has been measured over the temperature range 40–85°C. The results indicate a different temperature dependence of the effect for both β-D-glucopyranosides.

INTRODUCTION

Kinetic α-deuterium isotope effects have often been used as a possible means of distinguishing between S_N1 and S_N2 mechanisms. Whereas isotope effects $k_H/k_D > 1$ were expected for S_N1 reactions, effects near unity were believed to be indicative for S_N2 mechanisms.

The kinetic α-deuterium isotope effect is based on a different change in the frequency of the C—H and the C—D bending and stretching vibrations when the C atom rehybridizes from tetrahedral in the ground state to trigonal (S_N1 reaction) or to trigonal bipyramidal (S_N2 reaction) in the transition state (*1*). The main contribution to the isotope effect is the change in the H—C leaving group out-of-plane bending force constant (*2*). Depending on the nature of the transition state and the progress of the reaction in this complex, the C—H (C—D) out-of-plane bending vibration is hindered more or less. When no change of the bending vibration occurs in going to the transition state, k_H/k_D is near unity. For a maximal change the isotope effect approximates 1.38 at 25°C (*2*). Intermediate values between 1 and 1.38 are possible for S_N1 and S_N2 mechanisms. The magnitude of the secondary α-deuterium effect may be calculated from an approximated form of the Bigeleisen (*3*) equation

0045-2068/84 $3.00

$$\frac{k_H}{k_D} = \frac{\pi_i \frac{\mu^{\neq}_{Hi}}{\mu_{Hi}} e^{(\mu_{Hi}-\mu^{\neq}_{Hi})/2}(1 - e^{-\mu_{Hi}})(1 - e^{-\mu^{\neq}_{Hi}})^{-1}}{\pi_i \frac{\mu^{\neq}_{Di}}{\mu_{Di}} e^{(\mu_{Di}-\mu^{\neq}_{Di})/2}(1 - e^{-\mu_{Di}})(1 - e^{-\mu^{\neq}_{Di}})^{-1}} \quad [1]$$

with $\mu_i = \frac{hc\nu'_i}{kT}$, $h = 6.61\ 10^{-34}$ J · sec, $c = 3 \cdot 10^{10}$ cm sec^{-1}, $k = 1.38 \cdot 10^{-23}$ JK^{-1}, and ν'_i frequencies of stretching and bending vibrations (cm^{-1}) for the C—H and C—D bonds.

This equation predicts a temperature-dependent isotope effect. As a consequence, isotope effects may be compared only if they are measured at the same temperature.

A first approximation of Eq. [1] can be made if $\frac{\nu'_{Hi}}{\nu'_{Di}} = \frac{\nu'^{\neq}_{Hi}}{\nu'^{\neq}_{Di}}$ = constant. Streitwieser and co-workers (*2*) calculated from ir data that ν'_{Hi}/ν'_{Di} averages 1.35 for the stretching and bending frequencies of the C—H(C—D) bending. Substitution of $\nu'_{Hi}/\nu'_{Di} = 1.35$ in Eq. [1] leads to

$$\ln \frac{k_H}{k_D} = \sum_i \ln C_i + \frac{0.187}{T} \sum_i (\nu'_{Hi} - \nu'^{\neq}_{Hi}) \quad [2]$$

with

$$C_i = \frac{(1 - e^{-\mu_{Hi}})(1 - e^{-\mu^{\neq}_{Di}})}{(1 - e^{-\mu_{Di}})(1 - e^{-\mu^{\neq}_{Hi}})} \quad [3]$$

The conversion of a tetrahedral carbon to a sp^2 hybridized carbon causes a total net change in vibrational frequencies of about 565 cm^{-1} (*4*). All values of $\sum_i (\nu'_{Hi} - \nu'^{\neq}_{Hi})$ between zero and 565 cm^{-1} are directly related to the degree of bond cleavage in going from the ground state to the transition state. The maximum value of $0.187 \sum_i (\nu'_{Hi} - \nu'^{\neq}_{Hi})$ in Eq. [2] is about 106°K.

A second approximation can be made under certain conditions of temperature and ν'. At temperatures <300°K and with $\nu' > 3000$ cm^{-1} the $\sum_i \ln C_i$ term reduces to zero ($\pi_i C_i = 1$) and Eq. [2] becomes a linear function of $1/T$. This approximation is also consistent with the findings of Seltzer and Zavitsas (*4, 5*). They investigated the temperature dependence of the kinetic isotope effect for the *cis–trans* isomerization of maleic acid and for the unimolecular formation of methyl radicals at temperatures >300°K. For both organic reactions a linear dependence was observed. The $\sum_i \ln C_i$ term was fairly constant in the investigated temperature range and $\pi_i C_i$ approximated unity. From the slope, $\sum_i (\nu'_{Hi} - \nu'^{\neq}_{Hi})$ was calculated and used as a real measure of the progress of the reaction in the transition complex.

In the present study, kinetic isotope effects for the enzymatic hydrolysis of glycopyranosides by β-D-glucosidase from *Stachybotrys atra* and by β-D-xylosi-

dase from *Bacillus pumilus* will be determined and compared with k_H/k_D values for the acid hydrolysis of the same products.

The magnitude of the isotope effect will be used to characterize the transition state for both kinds of reaction. The influence of different substituents in the leaving group on the kinetic isotope effect may provide some indications about the mechanism of the reactions. Since the acid and the enzymatic hydrolysis will be carried out at 75° and 25° or 30°C, respectively, the temperature effect on k_H/k_D for the acid hydrolysis will be investigated in order to compare the acid and the enzymatic reactions.

MATERIALS AND METHODS

Enzymes. β-D-Xylosidase from *B. pumilus* PRL B12 and β-D-glucosidase from *S. atra* DSL 1 were isolated, purified, and standardized as described (*6–8*).

Synthesis of C-1 deuterated gluco- and xylopyranosides. [1-^{2}H]Glucose[[1-^{2}H]xylose] was prepared by sodium amalgam reduction of gluconolactone [xylonolactone] in ^2H_2O, a reaction described by Murray and Williams (*9*). Gluconolactone [D(+)-gluconic acid-δ-lactone (purc)] was a Koch & Light product. A mixture (syrup) of δ- and γ-xylonolactone (ir detection) was obtained by electrolytic oxidation of D-xylose according to the method of Isbell and Frush (*10*). The crude xylonolactone was not purified prior to use in the next reaction step.

To a solution of 20 g gluconolactone [14 g xylonolactone] in 50 ml ^2H_2O, 100 g 5% sodium amalgam, prepared according to Vogel (*11*), was added slowly. Reaction temperature was 5–10°C. To avoid hydrolysis of the lactone, the reaction mixture was titrated with a solution of $^2H_3PO_4$ (85% in diethyl ether) by means of a Radiometer titrator TTT1, keeping the pH between 3 and 3.5. After reduction, the mercury was removed by filtration and the filtrate was neutralized with $CaCO_3$. The $Ca_3(PO_4)_2$ precipitate was centrifuged at 10,000*g* for 35 min. Sodium hydroxide (2 *N*) was added to the supernatant in order to hydrolyze the unreacted lactone (final pH 8.5). The solution was acidified to pH 6 with H_3PO_4 (85%) and neutralized with solid $CaCO_3$. The $Ca_3(PO_4)_2$ and the calcium gluconate [xylonate] were removed by centrifugation (10,000*g* for 35 min). The supernatant was evaporated to a syrup. The yield, calculated from D-[1-^{2}H]glucose[D-[1-^{2}H]xylose] in the syrup was 35% [25%].

The syrup was acetylated according to Wolfrom and Thompson (*12*) without further purification. Both 1,2,3,4,6-penta-*O*-acetyl-β-D-[1-^{2}H]glucopyranose [β-[1-^{2}H]Glc*p*(Ac)$_5$][1] and 1,2,3,4-tetra-*O*-acetyl-β-D-[1-^{2}H]xylopyranose [β-[1-^{2}H]Xyl*p*(Ac)$_4$] were crystallized from MeOH. β-[1-^{2}H]Glc*p*(AC)$_5$: yield, 86%; mp 130°C; β-Glc*p*(AC)$_5$, mp 135.5°C. β-[1-^{2}H]Xyl*p*(Ac)$_4$: yield, 92%; mp 122.7–

[1] Abbreviations used: β-[1-^{2}H]Glc*p*(Ac)$_5$, 1,2,3,4,6-penta-*O*-acetyl-β-D-[1-^{2}H]glucopyranose; β-[1-^{2}H]Xyl*p*(Ac)$_4$, 1,2,3,4-tetra-*O*-acetyl-β-D-[1-^{2}H]xylopyranose; β-Glc*p*, β-D-glucopyranoside; β-Xyl*p*, β-D-xylopyranoside; 2-NO_2Ph, *o*-nitrophenyl; 4-NO_2Ph, *p*-nitrophenyl; 4-MeOPh, *p*-methoxyphenyl; 4-MePh, *p*-methylphenyl; 4-MeUmb, 4-methylumbelliferyl.

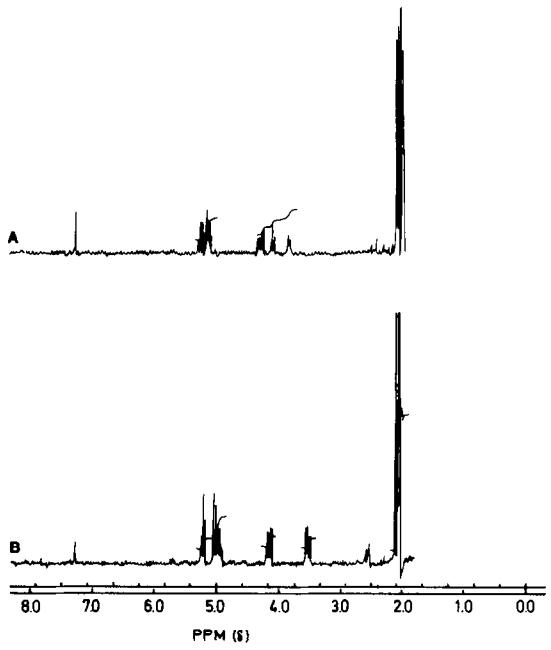

FIG. 1. Pmr spectra of β-[1-^{2}H]Glc*p*(Ac)$_5$ (A) and β-[1-^{2}H]Xyl*p*(Ac)$_4$ (B).

124.4°C; β-Xyl*p*(Ac)$_4$, mp 126°C. Pmr data indicated (Fig. 1 and Table 1) that at least 95% of the products were deuterated at C-1 of the glycon moiety.

Aryl-2,3,4,6-tetra-*O*-acetyl-β-D-[1-^{2}H]glucopyranosides [aryl-2,3,4-tri-*O*-acetyl-β-D-[1-^{2}H]xylopyranosides] were made by condensation of 2,3,4,6-tetra-*O*-acetyl-α-D-[1-^{2}H]glucopyranosylbromide (acetobromoglucose) [2,3,4-tri-*O*-acetyl-α-D-[1-^{2}H]xylopyranosylbromide (acetobromoxylose)] with the potassium salt of the phenol. Acetobromoglucose [acetobromoxylose] was prepared from 15 g glucose pentaacetate [3 g xylose tetraacetate] according to the method of Fisher (*13*). After bromation the acid was removed by repeated evaporation of the solution in the presence of toluene.

The acetobromoglucose residue was dissolved in a minimal volume of *N,N*-dimethylformamide. The potassium salt was prepared by lyophilization of an aqueous solution of KOH and phenol (ratio mol KOH/mol phenol, 1.125/1). The dry powder was dissolved in a minimal volume of *N,N*-dimethylformamide and added to the acetobromoglucose solution (mol phenol/mol carbohydrate, 1/1). The glucoside tetraacetate crystallized by pouring the solution slowly into water with vigorous stirring. The suspension was centrifuged (13,000*g*, 30 min) and the product was recrystallized from MeOH (Yield, 64%).

The acetobromoxylose residue was dissolved in 50 ml acetone. The potassium salt of the phenol was prepared by adding 11.4 mmol KOH and 12.1 mmol phenol to a mixture of 15 ml methanol and 30 ml acetone. Both solutions were mixed at 25°C. The KBr precipitate was filtered off and the filtrate was evaporated. The

TABLE 1

PMR DATA OF DEUTERATED AND UNDEUTERATED β-Glc*p*(Ac)$_5$ AND β-Xyl*p*(Ac)$_4$

	Chemical shifts (ppm)		Coupling constants (Hz)			
β-Glc*p*(Ac)$_5$		β-[1-^{2}H]Glc*p*(Ac)$_5$	β-Glc*p*(Ac)$_5$		β-[1-^{2}H] Glc*p*(Ac)$_5$	
H-1	5.72		$^2J_{(1,2)}$	8.2		
H-2	5.14	5.14	$^3J_{(2,3)}$	9.6	$^3J_{(2,3)}$	9.6
H-3	5.26	5.26	$^3J_{(3,4)}$	9.2	$^3J_{(3,4)}$	9.0
H-4	5.13	5.13	$^3J_{(4,5)}$	10.0	$^3J_{(4,5)}$	10.5
H-5	3.84	3.85	$^3J_{(5,6A)}$	4.6	$^3J_{(5,6A)}$	4.6
H-6$_A$	4.30	4.30	$^3J_{(5,6B)}$	2.2	$^3J_{(5,6B)}$	2.4
H-6$_B$	4.11	4.11	$^2J_{(6A,6B)}$	12.3	$^2J_{(6A,6B)}$	12.4
CH_3CO	2.00	2.00				
	2.03	2.03				
	2.09	2.09				
	2.09	2.09				
	2.12	2.12				
β-Xyl*p*(Ac)$_4$		β-[1-^{2}H]Xyl*p*(Ac)$_4$	β-Xyl*p*(Ac)$_4$		β-[1-^{2}H] Xyl*p*(Ac)$_4$	
H-1	5.73		$^2J_{(1,2)}$	6.8		
H-2	5.04	5.04	$^3J_{(2,3)}$	8.4	$^3J_{(2,3)}$	8.2
H-3	5.20	5.22	$^3J_{(3,4)}$	8.0	$^3J_{(3,4)}$	8.2
H-4	4.98	4.98	$^3J_{(4,5A)}$	4.9	$^3J_{(4,5A)}$	5.4
H-5$_A$	4.16	4.15	$^3J_{(4,5B)}$	8.0	$^3J_{(4,5B)}$	8.4
H-5$_B$	3.54	3.52	$^2J_{(5A,5B)}$	12.0	$^2J_{(5A,5B)}$	12.0
CH_3CO	2.05	2.05				
	2.10	2.10				

Note. Chemical shift values were obtained at 300 MHz for the compounds in $CDCl_3$, relative to tetramethylsilane.

residue was dissolved in methanol for crystallization. The crystals were deacetylated without further purification. Aryl-2,3,4,6-tetra-*O*-acetyl-β-D-[1-^{2}H]glucopyranosides [aryl-2,3,4-tri-*O*-acetyl-β-D-[1-^{2}H[xylopyranosides] were deacetylated with sodium methoxide in absolute methanol (*14*). The solution was neutralized with Dowex 50W-X8 or acetic acid. After evaporation of the solvent, the glucosides were recrystallized from methanol. The xylosides were crystallized subsequently from water and ethylacetate. The melting points and optical rotation values are given in Table 2.

Characterization of products. Pmr spectra were recorded on a Varian HR 300 MHz spectrometer in $CDCl_3$ as solvent and with tetramethylsilane (TMS) as internal standard. Melting points were determined with a Mettler FP- melting points apparatus. Optical rotations were measured with a Perkin–Elmer Model 141 automatic polarimeter.

TABLE 2

MELTING POINTS AND OPTICAL ROTATIONS $[\alpha]_D$ OF Aryl-β-D-GLUCO- AND XYLOPYRANOSIDES

Aglycon	Melting point (°C)		$[\alpha]_D^{25}$ (°ml g^{-1} dm^{-1})					
	Deuterated	Undeuterated	Deuterated			Undeuterated		
β-D-Glucopyranosides								
4-Nitrophenyl	164	165.8	−96	c 0.1	(H_2O)	−99	c 0.5	(H_2O)
4-Methylphenyl	178 −179.5	180	−70	c 0.1	(H_2O)	−70	c 0.1	(H_2O)
4-Methoxyphenyl	176 −177	176.2–176.5	−70	c 0.1	(H_2O)	−72	c 0.1	(H_2O)
4-Methylumbelliferyl	212	212	−110	c 0.1	(MeOH)	−100	c 0.1	(MeOH)
β-D-Xylopyranosides								
4-Nitrophenyl	154.0–156.4	157.4–158.8	−58.4	c 0.5	(MeOH)	−61.5	c 0.5	(MeOH)
4-Methylphenyl	157.3–161.7	159.0–162.5	−48	c 0.05	(MeOH)	−48	c 0.05	(MeOH)
4-Methoxyphenyl	155.7–156.3	156.6–156.7	−40.8	c 0.5	(MeOH)	−40.8	c 0.5	(MeOH)
2-Nitrophenyl	166.3–168.3	168.8–169.8	−77.4	c 0.5	(MeOH)	−79	c 0.5	(MeOH)

Kinetic techniques. The enzymatic reactions were performed, respectively, in 0.1 *M* Na_2HPO_4–NaH_2PO_4 buffer (pH 6.7, 30°C) and in 0.01 *M* Na_2HPO_4–KH_2PO_4 0.001 *M* EDTA buffer (pH 7.15, 25°C) for β-D-glucosidase from *S. atra* and for β-D-xylosidase from *B. pumilus.*

Hydrolysis of substituted phenyl β-D-gluco- and xylopyranosides was followed continuously (*o*- and *p*-nitrophenol) or discontinuously (other phenols, D-glucose or D-xylose) as indicated (*15, 16*). The k_H and k_D values were determined under identical conditions. Each k_H and k_D value is the average of at least five determinations.

Substrate concentrations were at least ten times the K_m values, except for the hydrolysis of *p*-nitrophenyl-β-D-xylopyranoside by β-D-xylosidase from *B. pumilus* (substrate inhibition) (*15*).

The acid hydrolysis of the *para*-substituted phenyl-β-D-gluco- (0.5 or 2 *N* HCl) and xylopyranosides (0.5 *N* HCl) was followed at 436 nm with a Perkin–Elmer Model 141 polarimeter. The hydrolysis of 4-methylumbelliferyl-β-D-glucopyranoside was monitored at 347 nm with a Vitatron MPS-type photometer. The hydrolysis of *o*-nitrophenyl-β-D-xylopyranoside was followed discontinuously (*17*).

The pseudo-first-order rate coefficients (ln *e*; sec^{-1}) were calculated as indicated (*18*).

RESULTS AND DISCUSSION

Analysis of the 300-MHz pmr spectra of β-[1-^{2}H]Glc*p*$(Ac)_5$ and β-[1-^{2}H]Xyl*p*$(Ac)_4$ (Fig. 1 and Table 1) indicated that both products were completely deuterated at C-1: the doublet at 5.72–5.73 δ and coupling of the proton on C-2 with an anomeric proton were absent. The good agreement between the chemical shift values and the coupling constants for all the other protons further proved that the synthesized products were respectively, β-[1-2H]Glc*p*$(Ac)_5$ and β-[1-^{2}H]Xyl*p*$(Ac)_4$.

Acid Hydrolysis of Glycopyranosides

Temperature dependence. The isotope effects for the acid hydrolysis of 4-MePh β-Glc*p* and 4-MeUmb β-Glc*p* were measured between 40 and 80°C (Table 3). For both β-D-glucopyranosides the ln k_H/k_D values decreased linearly with increasing temperature (Figs. 2 and 3). The intercept and the slope of ln k_H/k_D versus $1/T$ were, respectively, -0.796 ± 0.055 and (305 ± 19)°K for 4-MeUmb β-Glc*p* and -0.236 ± 0.018 and (102 ± 6)°K for 4-MePh β-Glc*p*.

From the data, one can observe that the temperature dependence is different for both glucopyranosides. These results suggest that kinetic isotope effects determined at different temperatures must be interpreted with caution. In addition, the values of the slopes for both reactions were larger than or near the maximal value (106°K), as calculated by Seltzer (*4*). The intercepts were significantly different from zero. From these results, the function ln k_H/k_D versus $1/T$ seems more complex than Eq. [2] in which $\sum_i \ln C_i$ was independent of temperature. However, the temperature range was too small to analyse the nonlinearity of the temperature dependence. Analysis of the slopes and the intercepts for 4-MeUmb β-Glc*p* and 4-MePh β-Glc*p* was not possible since spectroscopic data to calculate ν'_{Hi}/ν'_{Di} for glycopyranosides were not available.

Substituent effects. In the investigated temperature range, the isotope effects for aryl-β-D-glycopyranosides were smaller than the maximum value for an extreme A-1 mechanism, but they were larger than unity. This indicates important steric hindrance of the C—H bond in the transition state. The intermediate value of the isotope effect for the acid hydrolysis of aryl-glycopyranosides agrees with the findings of Sinnott *et al.* (*19*), indicating an A-1 mechanism with S_N2 characteristics. This is supported by the shift of the isotope effect caused by the substituent of the leaving aryl group.

TABLE 3

KINETIC α-DEUTERIUM ISOTOPE EFFECTS AT DIFFERENT TEMPERATURES

T (°C)	$\frac{1}{T} \cdot 10^3$ (°K^{-1})	4-MePh β-Glc*p*		4-MeUmb β-Glc*p*	
		$10^6 k_H$(sec^{-1})	k_H/k_D	$10^6 k_H$(sec^{-1})	k_H/k_D
40	3.193	2.28 ± 0.08	1.096 ± 0.028		
45	3.143	4.40 ± 0.05	1.091 ± 0.008		
50	3.094	9.90 ± 0.20	1.080 ± 0.014	11.5 ± 0.3	1.157 ± 0.019
55	3.047			27.5 ± 0.5	1.143 ± 0.023
60	3.002	39.7 ± 0.3	1.072 ± 0.009	54.8 ± 0.3	1.132 ± 0.015
65	2.957			93 ± 2	1.111 ± 0.021
70	2.914	144.3 ± 0.3	1.060 ± 0.014	177 ± 3	1.103 ± 0.025
75	2.872	281.3 ± 0.5	1.059 ± 0.003	303 ± 6	1.086 ± 0.019
80	2.832	505 ± 5	1.056 ± 0.012	541 ± 9	1.071 ± 0.010
85	2.792	837 ± 4	1.053 ± 0.005	1040 ± 30	1.049 ± 0.025

Note. Acid hydrolysis of $3 \cdot 10^{-2}$ *M* 4-methylphenyl- and $1.8 \cdot 10^{-4}$ *M* 4-methylumbelliferyl β-D-glucopyranoside in 2 *N* HCl.

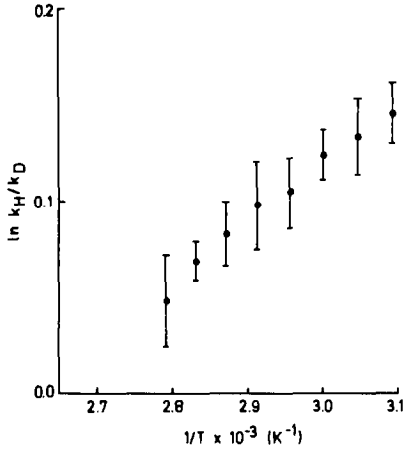

FIG. 2. Temperature dependence of the kinetic α-deuterium isotope effect. Acid hydrolysis of $1.8 \cdot 10^{-4}$ *M* 4-MeUmb β-Glc*p* in 2 *N* HCl.

From Table 4, the secondary α-deuterium effect decreased by substituting the 4-methoxy group with a nitro group. This proves that the transition state of a substrate with a better leaving group is more akin to reactants, in agreement with the experimental data of Mohr and co-workers (*20*) and of Bull and co-workers (*21*) for A-1-like mechanisms. According to the authors, the ease of rupture of the C—O bond increases with electron withdrawing substituents on the phenyl group. As a consequence, the carbenium ion character in the transition state and the secondary isotope effect decrease.

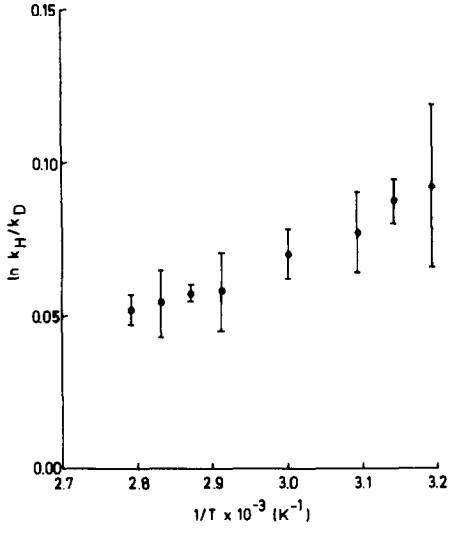

FIG. 3. Temperature dependence of the kinetic α-deuterium isotope effect. Acid hydrolysis of $3 \cdot 10^{-2}$ *M* 4-MePh β-Glc*p* in 2 *N* HCl.

TABLE 4

KINETIC α-DEUTERIUM ISOTOPE EFFECTS OF Aryl-β-D-GLYCOPYRANOSIDES

Substrate	Acid hydrolysis (75°C)		β-D-Xylosidase from *B. pumilus* (25°C)		β-D-Glucosidase from *S. atra* (30°C)	
	$10^5 k_H$(sec^{-1})	k_H/k_D	k_H(sec^{-1})	k_H/k_D	k_H(sec^{-1})	k_H/k_D
2-NO_2Ph β-Xyl*p*	130.8 ± 0.2	1.063 ± 0.009	13.0 ± 0.1	1.039 ± 0.005	13.3 ± 0.3	1.101 ± 0.015
4-MeOPh β-Xyl*p*	102.4 ± 0.5	1.113 ± 0.009	0.53 ± 0.01	1.034 ± 0.006	3.3 ± 0.3	1.116 ± 0.011
4-MePh β-Xyl*p*	104.0 ± 0.3	1.064 ± 0.005	1.07 ± 0.02	1.069 ± 0.003	5.0 ± 1.0	1.112 ± 0.015
4-NO_2Ph β-Xyl*p*	46.6 ± 0.2	1.064 ± 0.008	7.83 ± 0.13	1.087 ± 0.004	9.0 ± 0.7	1.154 ± 0.009
4-MeOPh β-Glc*p*	9.27 ± 0.07	1.075 ± 0.011			36 ± 2	1.122 ± 0.015
4-MePh β-Glc*p*	9.13 ± 0.01	1.067 ± 0.008			36 ± 2	1.114 ± 0.017
4-NO_2Ph β-Glc*p*	2.53 ± 0.02	1.052 ± 0.008			36 ± 2	1.121 ± 0.015
4-MeUmb β-Glc*p*	5.54 ± 0.03	1.093 ± 0.009			36 ± 2	1.091 ± 0.016

Note. Acid hydrolysis of gluco- and xylopyranosides (0.5 *N* HCl). Enzymatic hydrolysis of glycopyranosides by β-D-glucosidase (0.1 *M* phosphate buffer, pH 6.7) and by β-D-xylosidase (10 m*M* phosphate buffer, 1 m*M* EDTA, pH 7.15).

For 2-NO_2Ph β-Xyl*p* a k_H/k_D value near unity was expected; the electron withdrawing capacity of the *ortho* substituent is the same as for the *para* substituent, and *ortho* substitution increases the steric crowding at the C—H bond. The unexpected, rather large isotope effect for the *ortho* derivative (Table 4) indicates a mechanism different from *para* substituted aryl-β-D-Xyl*p*, in agreement with earlier results obtained for the acid hydrolysis of a series of xylopyranosides (*17*).

Enzymatic Hydrolysis

The kinetic isotope effects for β-D-xylosidase and β-D-glucosidase-catalyzed reactions are summarized in Table 4.

β-D-Xylosidase from B. pumilus. The small isotope effects (1.034–1.087) obtained with this β-D-xylosidase indicate a large steric crowding around the C—H bond in the transition state, and do not allow us to discriminate between an S_N1 or an S_N2 mechanism for the enzymatic reaction. However, several experimental data (e.g., inversion of anomeric configuration (*22*), negative $\Delta S^{\neq}$ values (*15*), weak inhibition of D-xylal (*23*)) suggest, in contrast to most glycosidases, a one-step mechanism with predominant S_N2 characteristics. The rate-determining heterolysis of the glycosidic bond probably occurs simultaneously with the attack of water on C-1 of the glycon moiety (S_N2 mechanism) (*15*). The proposed S_N2-like mechanism is supported by the influence of the substituent of the leaving aryl groups upon the isotope effects. In contrast with the acid-catalyzed reactions, a larger isotope effect was obtained for the 4-nitro than for the 4-methoxy substituent. As reported for S_N2 reactions by Westaway and Ali (*24*), substituents distant from the reaction center will not affect the steric crowding around the C—H bond in the substrate. According to the authors, the frequencies of the vibrations in the ground state for the C—H and C—D out-of-plane bending vibrations will be independent of the substituent on the leaving group. As a consequence the difference in the isotope effects may be explained by a difference in steric crowding around the C—H bond in the transition state (difference either in carbon-leaving

group bond or in nucleophile–carbon bond or in both of them) (*24, 25*). From a theoretical study, Thornton and co-workers (*26–28*) concluded that replacing an electron-donating substituent with an electron-withdrawing substituent in the leaving group hardly changes the carbon-leaving group distance in an S_N2 transition state, although the nucleophile–carbon bond distance increases. This means that a better leaving group requires less push from the nucleophile for bond breaking. From this the steric crowding decreases for a better leaving group and the isotope effect increases, in agreement with our results. Since the magnitude of the isotope effect increases by replacing 4-methoxy by 4-nitro, the nucleophilic push of water is less pronounced and the nucleophile–carbon is larger for 4-NO_2Ph β-Xyl*p* than for 4-MeOPh β-Xyl*p*.

Changing identical groups from the *para* to the *ortho* position may considerably increase the steric crowding at the C atom of the substrate. The magnitude of the isotope effect will be smaller for *ortho* substituents; 1.04 versus 1.08 for the *ortho-para*-nitro groups, respectively (Table 4).

β-D-Glucosidase from S. atra. In contrast to the *B. pumilus* enzyme, the isotope effects are larger (1.091–1.154) for the β-D-glucosidase from *S. atra*. Since the reaction proceeds with retention of configuration (*16*), according to most glycosidases, a two-step mechanism has been proposed. The aglycon is split off in the glycosylation step with formation of an enzyme–glycosyl intermediate, whereas in the second, or deglycosylation step, the glycon moiety is liberated. As for the β-D-xylosidase enzyme, the isotope effects are intermediate and do not reflect an extreme S_N1 or S_N2 mechanism for the rate-limiting step(s). However, they agree with the experimental evidence for a mechanism with predominant S_N2 character in both the glycosylation and deglycosylation step (e.g., negative $\Delta S^{\neq}$ values for both steps (*29*), weak inhibition of D-glucal (*29*)).

Identical kinetic α-deuterium effects were obtained for the glucopyranosides and for 2-NO_2Ph β-Xyl*p*. Since for these substrates the reaction of water with the common enzyme–glycosyl intermediate (deglycosylation step) is rate limiting (*29*), the absence of influence of the aglycon group is evident.

Different isotope effects, however, were obtained for 4-MePh- and 4-NO_2Ph β-Xyl*p*. In contrast with the glycopyranosides and with 2-NO_2Ph β-Xyl*p*, both the heterolysis of the glycosidic bond and the hydrolysis of the enzyme–xylosyl complex are rate determining (*29*). As a consequence, the difference in the k_H/k_D values must be caused by a difference in the heterolysis step. The same trend as with the β-D-xylosidase-catalyzed reactions is noticeable. A larger isotope effect is observed for 4-NO_2Ph β-Xyl*p* as compared to 4-MePh β-Xyl*p*, which refers to a larger nucleophile–carbon distance in the transition state for the 4-nitro derivative.

para-Methoxyphenyl β-Xyl*p* cannot be compared with 4-NO_2Ph β-Xyl*p* and 4-MePh β-Xyl*p*, since for this substrate the heterolysis of the glycosidic bond is rate limiting (*29*).

Acid and Enzymatic Hydrolysis

Comparison of the isotope effect for the acid and enzymatic hydrolysis is only

possible when they are determined at the same temperature. Because of this, comparison is only allowed for 4-MeUmb β-Glc*p* and for 4-MePh β-Glc*p* (see Temperature Dependence). For the acid hydrolysis at 30°C the extrapolated value are respectively, 1.23 and 1.11 for 4-MeUmb β-Glc*p* and 4-MePh β-Glc*p*. The isotope effects for the enzymatic hydrolysis are, respectively, 1.09 and 1.11 (Table 4). The α-deuterium effect for the acid and enzymatic hydrolysis of 4-MePh β-Glc*p* differs, which can be explained by the respective mechanisms. In contrast, the extrapolated isotope effect for the acid hydrolysis of 4-MePh β-Glc*p* at 30°C is the same as for the enzymatic hydrolysis in spite of a different mechanism. From this, the hindrance of the H-C leaving group out-of-plane bending vibration in the transition state must be the same, which can be explained by a rather short leaving group distance for the acid hydrolysis (predominant A-1 mechanism) and a relatively large nucleophile-leaving group distance for the enzymatic reaction (predominant S_N2 mechanism). The experimental data illustrate that the magnitude of the isotope effect is not always a useful criterion to distinguish between different types of mechanisms. The effect of the substituents in the leaving group on the k_H/k_D values seems to be more indicative. According to our results, better leaving aryl groups decrease the isotope effect for an S_N1-like mechanism but increase it for an S_N2-like reaction. The same explanation may be valid for both phenomenons; an early transition state for good leaving groups and a late transition state for leaving groups with electron-donating substituents. This implicates, for mechanisms with predominant S_N1 characteristics, less bond breaking of the carbon-leaving group bond for electron-withdrawing groups than for electron-donating substituents, which results in smaller isotope effects. On the other hand, it implies, for mechanisms with predominant S_N2 character, less bond formation for electron-withdrawing groups as compared with electron-donating groups. This results in larger isotope effects. Further investigation, however, is necessary to prove this hypothesis.

ACKNOWLEDGMENTS

We thank Professor M. Anteunis and Dr. A. De Bruyn (Laboratory for nmr spectroscopy) for the nmr facilities and assistance in interpreting the spectra.

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