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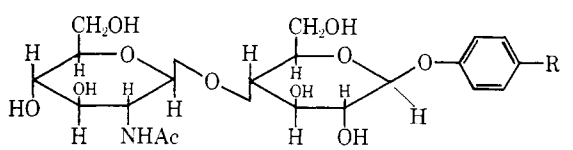
Application of Secondary α-Deuterium Kinetic Isotope Effects to Studies of Enzyme Catalysis. Glycoside Hydrolysis by Lysozyme and β-Glucosidase*

F. W. Dahlquist, T. Rand-Meir, and M. A. Raftery

ABSTRACT: Secondary kinetic isotope effects (α-deuterium) have been explored as a method for delineating the nature of the transition state in enzyme-catalyzed reactions. Hen egg-white lysozyme and almond β-glucosidase were chosen as enzymes for these experiments. Initially, model studies were performed on the cleavage of phenyl glucosidic bonds in acid (SN1 mechanism) and in base (SN2 mechanism). The values obtained (k_H/k_D = 1.13 for SN1 mechanism, k_H/k_D = 1.03 for SN2 mechanism) were in the anticipated range. The value obtained for β-glucosidase-catalyzed hydrolysis of phenyl β-D-glucopyranoside was k_H/k_D = 1.01, and this suggests a displacement mechanism for this enzyme. Such a result is in agreement with recent chemical evidence. For application of the method to studies of the lysozyme mechanism, the disaccharide phenyl 4-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-β-D-glucopyranoside was synthesized by enzymatic transglycosylation using ^{3}H- and ^{14}C-labeled phenyl glucosides (C_1-H and C_1-*d*, respectively). The value of k_H/k_D obtained for the lysozyme-catalyzed hydrolysis of this aryl disaccharide glycoside was determined to be 1.11. This result indicates considerable carbonium ion character in the transition state of the enzyme-catalyzed reaction. It was found that this result held over the pH range 3.1–8.3 as well as close to the pH optimum.

The X-ray analysis studies of Blake *et al.* (1965, 1967a,b) on the binding of various inhibitors and substrates to lysozyme has led to a mechanistic proposal for catalysis (Blake *et al.*, 1967a; Phillips, 1967). This involves catalytic production of a carbonium ion which is given steric and electrostatic stabilization by the enzyme. Recent chemical studies have shown that (a) lysozyme-catalyzed hydrolysis of a glycosidic bond proceeds with retention of configuration to at least 99.7% (Dahlquist *et al.*, 1969); (b) aryl *glucosides* of the type



serve as substrates for the enzyme (Raftery and Rand-Meir, 1968; Rand-Meir *et al.*, 1969) with exclusive cleavage of the aryl glucosidic bond; (c) for such glucosidic substrates the mechanism of hydrolysis involves a carbonium ion intermediate (Dahlquist *et al.*, 1968). Evidence for such an intermediate was obtained by use of secondary α-deuterium kinetic isotope effects to study the enzyme-catalyzed reaction. This communication describes further kinetic isotope effect studies on model glucosides as well as extension of the lysozyme studies to ensure uniformity of mechanism over a wide range of pH. Application of the kinetic isotope method to study the mechanism of β-glucosidase-catalyzed hydrolysis of aryl glucosides is also described.

Experimental Section

All pH measurements were performed with a Radiometer pH Meter 26. The ultraviolet spectrophotometric measurements were carried out with a Gilford 240 spectrophotometer. Proton magnetic resonance spectra were obtained with a

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Varian HR 220 nuclear magnetic resonance spectrometer. Radioactivity measurements were made with a Packard Tri-Carb liquid scintillation spectrometer, Model 3324.

[^{14}C]Phenyl β-D-Glucopyranoside-d_1. The synthesis of the unlabeled compound has been described by Lemieux (1963). For the labeled compound a flask containing 8 ml of acetic anhydride was cooled to 0° and 50 μl of 60% perchloric acid was added. The flask was warmed to room temperature and 2.0 g of glucose-d_1 (Merck Sharp & Dohme of Canada, Ltd., Lot 1318) was added such that the temperature remained between 30 and 40°. The mixture was cooled to 20° and 0.6 g of red phosphorus, 3.6 g of bromine, and 0.75 ml of water were added. After 2 hr at room temperature, 8 ml of chloroform was added and the resulting solution was filtered through glass wool, washed twice with cold water, once with saturated sodium bicarbonate, and was stirred 10 min with dry sililic acid. The chloroform was evaporated and the residue was triturated with 10 ml of ether–petroleum ether (bp 30–60°) (2:1). The solid was filtered and dried to give 2.7 g of the crude acetobromoglucose-d_1 which was used without further purification.

A solution containing 0.95 g of phenol and 0.5 mCi of [^{14}C]phenol, uniformly labeled (Nuclear-Chicago, Code CFA 125 batch 25, specific activity 25 mCi/mmole), and 0.5 g of sodium hydroxide in 12.5 ml of water was added to a solution of 2.7 g of acetobromoglucose-d_1 in 19 ml of acetone. After 4 hr at room temperature the solution was placed in the refrigerator and left overnight. The acetone was evaporated under reduced pressure and the water solution was extracted twice with an equal volume of chloroform. The chloroform layer was washed with water, dilute sodium hydroxide, twice more with water, and was dried over anhydrous magnesium sulfate. The chloroform solution was evaporated under reduced pressure, 12 ml of ethanol was added, and the solution was stored for 1 day at −10°. The resulting crystals were filtered, washed with cold ethanol, and dried under vacuum to give 483 mg of tetra-*O*-acetyl β-phenylglucoside. This material was suspended in 27 ml of absolute methanol and 0.55 ml of 0.4 N barium methoxide was added. The resulting solution was kept at 0° for 24 hr. The solution was evaporated to a small volume and 250 mg of the desired [^{14}C]phenyl β-D-glucopyranoside-d_1 (mp 170°) was obtained. This material was chromatographed on Sephadex LH-20 in methanol before use in kinetic studies.

[^{3}H]Phenyl D-Glucopyranoside. The synthesis was carried out on the same scale as for the deuterated and ^{14}C-labeled compound. In this case glucose was used and the final specific activity of the sodium [^{3}H]phenoxide used (generally labeled Nuclear Chicago Co.) was 0.5 mCi/mmole. The yield was 270 mg of phenyl glucoside melting at 171–172°. This material was also chromatographed on Sephadex LH-20 in methanol before use in kinetic studies. A mixture of the ^{14}C and ^{3}H material in this chromatographic system gave a single peak with constant $^{3}H/^{14}C$ ratio throughout.

Phenyl 4-O-(2-Acetamido-2-deoxy-β-D-glucopyranosyl)-β-D-glucopyranoside. This material was synthesized using the transferase activity of lysozyme. A sample of 170 mg of GP[1] which contained 2.3×10^7 cpm of ^{14}C and 9.4×10^7 cpm of ^{3}H was incubated with 880 mg of chitobiose (prepared by gel filtration), 1.0 g of lysozyme, 4 ml of dioxane, and 20 ml of 0.1 M citrate buffer (pH 5.5) at 40° for 20 hr. Several drops of toluene were added to inhibit bacterial and fungal contamination. The mixture was filtered and applied to a 150 × 4 cm column of Bio-Gel P-2 (100–200 mesh) using 0.1 M sodium chloride as solvent. Fractions of 12.8 ml were collected and 100-μl samples were removed for scintillation counting. The fractions corresponding to the desired compound (115–130) were pooled and the solution was applied to a 100-ml column of Amberlite MB-1 mixed-bed ion-exchange resin. This was eluted with 2 l. of distilled water. The eluent was concentrated under reduced pressure to a glass. This material was taken up in 1.0 ml of methanol–water (1:1) and applied to a 50 × 2 cm column of Sephadex LH-20 which had been equilibrated with methanol–water (1:1) as solvent. The material chromatographed as a single peak with constant $^{3}H/^{14}C$ ratio. The peak was concentrated under vacuum to a glass which crystallized upon standing. The yield of the desired NPG was 15 mg.

Model Studies. The acid-catalyzed hydrolysis of GP was carried out at 50° in 2.00 N HCl (P-H Tamm Laboratories, Sweden). The concentration of GP in the solutions ranged from 5×10^{-3} to 2×10^{-2} M. One-milliliter samples were removed, neutralized by addition of 1.00 ml of 2.00 N NaOH (P-H Tamm Laboratories, Sweden), and brought to pH 11.00 with 8.0 ml of 0.1 M dimethylamine hydrochloride buffer (pH 11.00). The absorbance at 290 mμ of this solution was then determined. After about 1 half-life at 50°, the temperature was raised to 70° for 24 hr in order to obtain the infinity reading for the reaction. The data were then analyzed by plotting log $(A_\infty - A)/A_\infty$ *vs.* time. The slope of the line was determined by least-square methods.

The methoxide-catalyzed methanolysis of GP was carried out at 70° in 3.0 M sodium methoxide which was prepared by the reaction of metallic sodium with methanol dried over magnesium. The concentration of the methoxide was determined by titration with standard acid. In this case, the per cent reaction was determined by chromatography of the reaction, after neutralization, on Sephadex LH-20 using methanol–water (1:1) as solvent and reading the absorbance at 265 mμ.

Enzyme Studies. The almond β-glucosidase- (Worthington Biochemical Corp.) catalyzed hydrolysis was carried out at pH 5.0 in 0.1 M acetate buffer and 25°. The concentration of enzyme was 1 mg/ml. Aliquots of 0.1 ml were removed and added to 1.0 ml of 0.2 N NaOH solution, and the absorbance at 290 mμ was determined using a 1.0-ml cuvet with a 1-cm path length (Scientific Cell Co., N. Y.).

The lysozyme-catalyzed hydrolysis of NGP was carried out at 40° in 0.1 M citrate buffer (pH 5.5), 0.1 M citrate (pH 3.1), and citrate phosphate buffer (pH 8.3). The fraction of reaction was determined by chromatography on Bio-Gel P-2 (200–400 mesh) using 0.1 M sodium chloride as solvent. The concentration of substrate used was 10^{-2} M while the enzyme concentration was 50 mg/ml in each case.

Isotope Effect Measurements. In general, the reaction was allowed to proceed to 2–5% completion and adjusted with 2 N NaOH or 2 M HCl and 0.1 M citrate buffer (pH 5.5 to 5–6). The mixture was then extracted twice with 1.0 ml of ether. This removed all the phenol produced by the reaction. The ethereal solution was washed with an equal volume of

[1] GP, phenyl β-D-glucoside; NGP, phenyl 4-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-β-D-glucopyranoside.

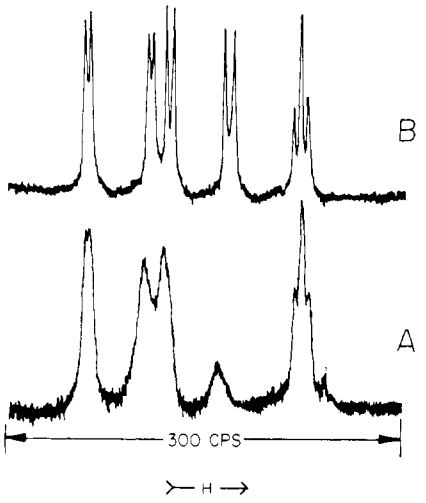

FIGURE 1: The proton magnetic resonance spectra of phenyl β-D-glucopyranoside-d_1 and phenyl β-D-glucopyranoside at 220 MHz. The spectra are of the region 3.5–5 ppm downfield of tetramethylsilane. Spectrum A is GP-d_1. Spectrum B is GP.

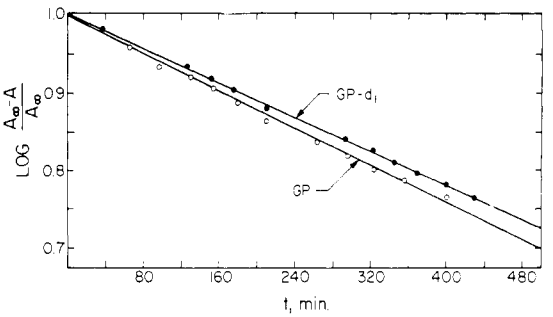

FIGURE 2: Pseudo-first-order plot of hydrolysis of GP and GP-d_1 in 2.0 N HCl at 50°. The points represented as ○ are the data for GP while those represented as ● are the data for GP-d_1.

the pH 5.5 citrate buffer and an equal volume of saturated sodium chloride solution and was then passed over a small column of anhydrous sodium sulfate in a pasteur pipet. One milliliter of this dried ethereal solution was mixed in a polyethylene vial with 15 ml of Bray's solution (Bray, 1960); the relative $^3H/^{14}C$ ratio of the product was determined and compared with that of the starting material. A very small aliquot of the starting material (5–10 μl) was removed from its stock solution and was mixed with 1.0 ml of dry ether and 15 ml of Bray's (1960) solution in order to determine its relative $^3H/^{14}C$ ratio.

Results

The degree of deuterium substitution at C-1 of the GP with deuterium and ^{14}C label was determined by nuclear magnetic resonance. The spectra and integrals which were obtained for the unlabeled and labeled compounds at 220 MHz in deuterated dimethyl sulfoxide are shown in Figure 1. These data show that the fraction of deuterium substitution in the sample was 0.65 ± 0.05 at the C-1 position.

The rate constants for the acid-catalyzed hydrolysis of GP and for GP-d_1 at 50° in 2 N HCl were determined from the data shown in Figure 2. The unlabeled material displayed a first-order rate constant of 2.56 ± 0.01 × 10^{-3} min^{-1} while the deuterated material showed a rate constant of 2.34 ± 0.02 × 10^{-3} min^{-1}. The deuterated material should not show exact first-order kinetics since it is a mixture of the deuterated and protiated material. However, the discrepancies should be small over the first half-life if the isotope effect is not very large. The ratio of the observed rates for the protiated material to that of the deuterated material $(k_H/k_D)_{obsd}$ is 1.09 ± 0.02. The correction of this observed ratio to give the actual isotope effect is complex but (see eq 5 in the Appendix) gives approximately $k_H/k_D = 1.14$ for the acid-catalyzed reaction isotope effect.

Another method for measuring this isotope effect depends upon the fact that the initial rate of release of products of the deuterated compound will be somewhat slower than that of the unsubstituted compound (see Collins, 1964, for a review). To exploit this phenomenon, the deuterated material was synthesized with ^{14}C-labeled phenol while the protiated material had ^3H in its phenyl moiety. For an acid-catalyzed hydrolysis of a mixture of the deuterated and undeuterated material, the $^3H/^{14}C$ ratio of the phenol isolated after a small amount of reaction should be greater than the $^3H/^{14}C$ ratio of the starting material. If the reaction is allowed to proceed to the extent of 2–3% or less, the observed isotope effect $(k_H/k_D)_{obsd}$ is given by $[S_0]^*P/[S_0]P^*$ (see eq 4 of the Appendix), where $[S_0]^*/[S_0]$ refers to the $^3H/^{14}C$ ratio of the starting material and P/P^* refers to the $^{14}C/^3H$ ratio of the products. The observed isotope effect must then be corrected for the deuterium content of the substrate by eq 6 in the Appendix. The experimental data for a typical experiment for the acid-catalyzed reaction is shown in Table I. The conditions used were 2 N HCl at 50° for 15 min, which corresponds to about 3% hydrolysis. Four similar experiments gave $(k_H/k_D)_{obsd} = 1.080 \pm 0.004$. To test for small amounts of impurities, the acid-catalyzed reaction was allowed to proceed for 1 half-life, and the starting material was reisolated. Using the GP obtained in this way for determination of the acid-catalyzed isotope effect gave $(k_H/k_D)_{obsd}$ of 1.078. This result suggests that impurities were releasing phenol neither in any significant amount nor at a rate which seriously affected the desired answer. It should be noted that phenol is known to exchange its ring protons under strongly acidic conditions. Because of this it was not possible to obtain an infinity $^3H/^{14}C$ ratio of the phenol released. A control experiment, in which a mixture of ^3H- and ^{14}C-labeled phenol was treated at 50° in 2 N HCl for 30 min, showed no change in the $^3H/^{14}C$ ratio of the phenol after the treatment. Thus, no significant exchange occurred in the short times required for isotope effect measurements at 3% completion of reaction.

The determination of the isotope effect for the base-catalyzed reaction was more difficult than for the acid-catalyzed reaction. Because of the slowness of the base-catalyzed reaction, determination of the isotope effect by absolute rates was not attempted. Treatment of GP with 3.0 N sodium methoxide in anhydrous methanol at 70° for 1 hr and subsequent isolation of the product gave the data summarized in Table I. Four similar determinations gave an observed isotope effect of 1.021 ± 0.002. Up to this point, the quoted reproducibility errors have also been the expected statistical errors associated with the counts accumulated. In the case of the methoxide-catalyzed reactions, somewhat more material was used and counting was carried out to four times the counts accumulated in the acid-catalyzed experiments. This resulted in the lowered reproducibility error quoted. Unfortunately, insufficient ma-

TABLE I: Examples of Counting Data for Determination of the Observed Isotope Effect.

	Counts in Channel 1[a]	Counts in Channel 2[a]	Time (min)	Rel $^3H/^{14}C$	$(k_H/k_D)_{obsd}$
2.0 N HCl, 50°					
GP	500,000	135,079	12.9	3.639	
Phenol	256,210	65,705	100.0	3.930	1.080
3.0 N $NaOCH_3$, 70°					
GP	2×10^6	539,482	57.9	3.752	
Phenol	2×10^6	238,993	121.6	3.712	1.020
β-Glucosidase					
GP	657,990	175,850	20.0	3.686	
Phenol	900,000	238,993	80.0	3.742	1.010
Lysozyme					
NGP	500,000	134,448	18.1	3.659	
Phenol	500,000	127,610	81.7	3.914	1.070

[a] Background channel 1, 8 cpm; background channel 2, 12 cpm; ^3H relative efficiency in channel 2 compared with channel 1: 0.020; ^{14}C relative efficiency in channel 1 compared with channel 2: 0.323. Note: The absolute efficiency is quite sensitive to the window settings and it is difficult to reset the window exactly. However, the relative efficiency does not change appreciably. This explains the apparent lack of constancy of the GP ratios.

terial was available to do this for each measurement in the other systems.

The β-glucosidase-catalyzed hydrolysis of GP showed only a very small observed isotope effect of 1.010 ± 0.004. In order to determine if the measured effect was real and to further test the purity of the GP, the entire time course of the observed isotope effect was followed and the results of this study are shown in Figure 3. The solid line shows the theoretical curve obtained by applying eq 3 of the Appendix with an initial observed isotope effect of 1.010. The data fit this theoretical curve reasonably well. At 90% reaction, an aliquot was removed from the reaction mixture and the residual GP was reisolated. The $^3H/^{14}C$ ratio of this material was compared to that of the starting material before reaction. The amount of ^{14}C in the reisolated GP was found to have increased by a factor of 1.018. Application of eq 2 in the Appendix gives a theoretical value for an increase of 1.020 for an initial observed isotope effect of 1.010.

The substrate for the lysozyme-catalyzed reaction was made by treatment of chitobiose with lysozyme in the presence of GP. The resulting NGP was isolated by chromatography on Bio-Gel P-2 and the chromatogram obtained is shown in Figure 4. The peak corresponding to fractions 115–130 was pooled. Rand-Meir *et al.* (1969) have shown by degradative procedures that the transferase activity of lysozyme forms only β-(1–4) linkages when aryl glucosides are used as acceptors.

Treatment of NGP with lysozyme at pH 5.5 in 0.1 M citrate buffer for 12 hr at 40° resulted in a 2% release of phenol. The radioactivity measurements for this reaction are summarized in Table I. Three other measurements under these conditions gave an observed isotope effect of 1.068 ± 0.004. The isotope effect was also determined at pH 3.1 and 8.3. This range covers almost the entire pH profile for the lysozyme-catalyzed hydrolysis of substrates of this nature (Raftery and Rand-Meir, 1968; Rand-Meir *et al.*, 1969). These reactions required long periods of time (days) to proceed 2–3% and both gave the same observed isotope effect as the pH 5.5 reactions. While the rate of the lysozyme-catalyzed hydrolysis of NGP at these pH extremes is slow, the substrate was quite stable in the absence of the enzyme. These blanks showed 0.02 and 0.08% hydrolysis at pH 3.1 and 8.3, respectively.

The isotope effect measurements are summarized in Table II. The observed isotope effect measurements have been corrected for deuterium content by use of eq 6 in the Appendix. The error limits of the corrected isotope effects are estimated to be ±0.01.

Discussion

It has been determined that α-deuterium kinetic isotope effects are a useful criterion for determining the degree of nu-

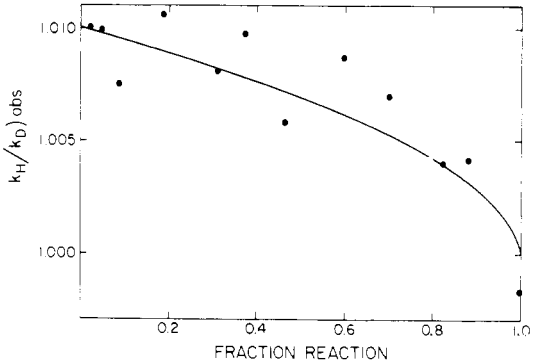

FIGURE 3: A plot of $(k_H/k_D)_{obsd}$ *vs.* fraction reaction for the hydrolysis of GP by β-glucosidase. The term $(k_H/k_D)_{obsd}$ is the $^3H/^{14}C$ ratio of the total phenol produced at that point relative to the $^3H/^{14}C$ ratio of GP before reaction. The solid points represent the observed data while the line is the theoretical curve for an initial $(k_H/k_D)_{obsd}$ of 1.010.

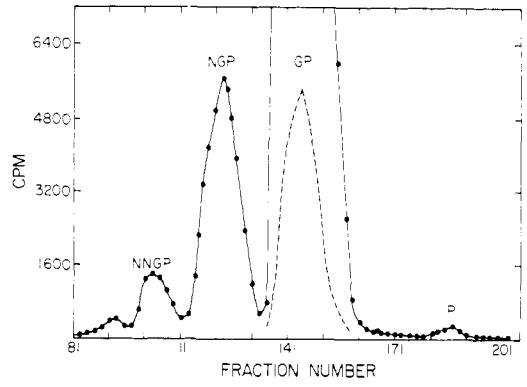

FIGURE 4: Chromatography on Bio-Gel P-2 of the reaction mixture for the synthesis of NGP utilizing the transferase activity of lysozyme. The various peaks were identified as phenol (P), phenyl β-glucoside (GP), NGP, and the β-(1–4)-linked trisaccharide NAG-NAG-glucose-β-phenyl glucoside (NNGP).

cleophilic participation of solvent in the rate-determining steps of many solvolytic reactions (see Halevi, 1963, for a review). A typical SN2 reaction shows an effect near unity while SN1 reactions display an isotope effect yielding k_H/k_D of about 1.14. The explanation of this decrease in rate is that substitution of deuterium for the α-hydrogen results in a loss in the HCX bending force constant in the transition state accompanying the breaking of the C–X bond. In the case of nucleophilic attack, some of this effect is compensated for as the new carbon–nucleophile bond is being formed.

The acid-catalyzed hydrolysis of glycosides is thought to proceed primarily through an A1 carbonium ion reaction (Cordes, 1967; Vernon, 1967, for recent reviews). There is very little evidence for an A2 mechanism (protonation, followed by nucleophilic attack) in any hydrolyses of glucosides in aqueous solution (Cordes, 1967). The α-deuterium isotope effect of 1.13 which we have observed for the acid-catalyzed hydrolysis of GP agrees with the accepted value of around 1.14 for typical SN1 reactions (see Halevi, 1963, for a review of secondary isotope effects). Jones and Thornton (1967) found k_H/k_D of 1.24 ± 0.08 per deuterium atom for the solvolysis of methyl chloromethyl-d_2 ether as compared with the undeuterated compound. The proposed mechanism for hydrolysis of this compound is quite similar to that proposed for glucoside hydrolysis and the α-deuterium isotope effect would be expected to have the same value. The rather large error in the data of Jones and Thornton makes a quantitative comparison impossible but the effects are at least qualitatively the same. Thus it appears that the acid-catalyzed hydrolysis of GP does involve a carbonium ion intermediate and the deuterium isotope effect for the reaction yields a good model value for studies of a carbonium ion intermediate in the enzymatic hydrolysis of glucosides.

TABLE II: Summary of Observed Isotope Effects.

	$(k_H/k_D)_{obsd}$	k_H/k_D
2 N HCl, 50°	1.080 ± 0.004	1.13
3 N $NaOCH_3$ in mesh, 70°	1.021 ± 0.002	1.03
β-Glucosidase	1.010 ± 0.004	1.015
Lysozyme		
pH 5.5	1.068 ± 0.004	1.11
pH 3.1	1.070	1.11
pH 8.3	1.068	1.11

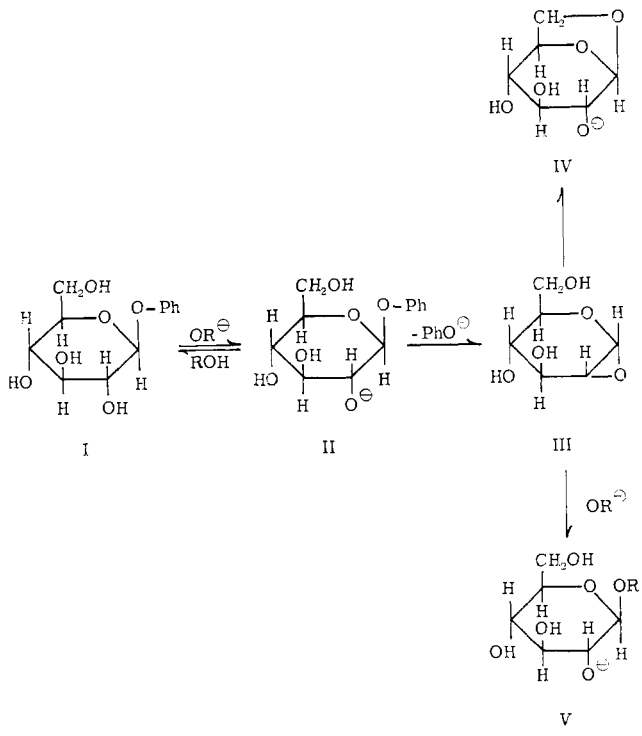

FIGURE 5: Proposed scheme for base-catalyzed hydrolysis of phenyl β-D-glucopyranoside (Ballou, 1954).

Studies (Ballou, 1954; Whistler and BeMiller, 1958) on the reaction of aryl glucosides in basic solution have shown that compounds with their C-2 hydroxyl group *trans* to the aglycone react much faster than those with a *cis* orientation. It appears that the *trans* compounds react by a mechanism involving participation of the C-2 oxyanion. This scheme is summarized in Figure 5. More recently, Gasman and Johnson (1966) have shown that cleavage of nitrophenyl 2-*O*-methyl-β-glucosides is much slower than that of the corresponding unmethylated compounds. Hydrolysis of the methylated compounds appeared to proceed by a mechanism involving bimolecular nucleophilic aromatic substitution, while the unmethylated compounds utilized the C-2 oxyanion pathway. The α-deuterium isotope effect of the solvolysis of GP in 3 N sodium methoxide in methanol was k_H/k_D = 1.03. This suggests that nucleophilic attack is the predominant mechanism for this reaction under these conditions. This value for the isotope effect is somewhat larger than that accepted for a purely SN2 reaction and probably reflects a small amount of carbonium ion character in the transition state. McClosky and Coleman (1945) and Gasman and Johnson (1966) have found that the yield of 1,6-anhydro-β-D-glucopyranose in the basic hydrolysis of aryl-β-glucosides decreases as the electron-withdrawing character of the aglycone is increased. This is consistent with the supposition that the basic solvolysis of GP involves partial carbonium ion character and that the extent of this is a function of the leaving group. Such a conclusion is

similar to that of Shiner *et al.* (1968) in which a trend toward more carbonium ion character (as measured by α- and β-deuterium isotope effects) was seen in the solvolysis of *meta*- and *para*-substituted 1-phenylethyl halides, as the electron-withdrawing character of the substituents was decreased.

Thus, the acid- and base-catalyzed solvolyses of GP represent good model systems for the carbonium ion and displacement mechanisms, respectively. The isotope effects associated with these solvolyses provide good theoretical values for these extremes of mechanism. Isotope effect measurements for enzyme-catalyzed solvolyses of glucosides should then provide a significant indication of the mechanism of action of these enzymes.

The isotope effect for the β-glucosidase-catalyzed hydrolysis of GP was found to be $k_H/k_D = 1.010$. A value of this magnitude strongly suggests a mechanism involving nucleophilic attack. The possible sources of the nucleophile are the enzyme, solvent, or possibly an hydroxyl group of the substrate itself. Legler (1968) has recently shown that the substrate analog conduritol B epoxide (3,5/4, 6-cyclohexenetetrol oxide) inactivates glucosidases from many sources. The inactivation involves the formation of a covalent bond with what would appear to be a carboxyl group on the enzyme surface. Treatment of the inactivated enzyme with hydroxylamine releases one molecule of (+)-inositol per molecule of enzyme. The stereochemistry of the product suggests a *trans* ring opening of the epoxide by the enzyme. A carboxyl group on the enzyme was suggested to be the attacking nucleophile in the hydrolysis of GP by β-glucosidase. The mechanism of this enzyme would seem to be a classic example of the displacement mechanism suggested by Koshland (1953).

Some care must be taken in the interpretation of such a small isotope effect as we observed for the β-glucosidase-catalyzed reaction since factors other than nucleophilic attack could produce small effects. It is possible that a pre-rate-determining equilibrium could occur which involves the isotopically substituted center. The secondary isotope effect for such a process would be expected to be very small. An example of this for the β-glucosidase case is the formation of an enzyme–glucosyl intermediate which reacts with the phenol in solution to re-form starting material much more often than it reacts with water to form products. This can be ruled out since the residual GP isolated after 90% reaction had a different $^3H/^{14}C$ ratio than the phenol isolated at that point. The difference was a small but measurable factor of 1.018 as compared with a theoretical value of 1.020. This measurement, as well as the time course of $(k_H/k_D)_{obsd}$ shown in Figure 3, both show a very satisfactory (to within 0.2%) fit to the theoretical values for a $k_H/k_D = 1.010$. The results also suggest that it is *very unlikely* that there is an impurity in the GP which would lead to an error in the isotope effect measurements.

It has been assumed that the enzyme-catalyzed reaction displays Michaelis–Menten kinetics. For the simple scheme

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES \xrightarrow{k_2} E + P$$

our derivation requires a steady-state approximation on the concentration of ES, that is $k_{-1} \gg k_2$. If this does not hold, then the measured value of the isotope effect will be smaller than the actual value. In the extreme case, when $k_{-1} \ll k_2$, the isotope effect for the catalytic step will not be observable since the sampling of the deuterio and protio material has preceded the catalytic step. While such possibilities are unlikely, it is well to keep them in mind.

From the results of the base-catalyzed and β-glucosidase-catalyzed hydrolysis of GP one would expect a displacement reaction in the case of the lysozyme-catalyzed hydrolysis of NGP to have a very small α-deuterium isotope effect. In fact, the isotope effect was 1.11, a large effect. This suggests that the lysozyme-catalyzed reaction proceeds through an intermediate with considerable carbonium ion character. It is interesting to note that the isotope effect did not change over the pH range 3.1–8.3 and thus no change in mechanism occurs over this range of pH, which includes nearly all those pH values at which the enzyme is even slightly active.

This evidence for carbonium ion character would seem to rule out both the single- and double-displacement mechanisms which involve either attack by solvent or by nucleophiles of the enzyme itself. The evidence is consistent with the carbonium ion mechanism proposed by Blake *et al.* (1967a) from model building studies. It is possible, however, that for cell wall substrates an acetamido group may provide anchimeric assistance as proposed by Piszkiewicz and Bruice (1967, 1968) and Lowe (1967), since no such group is adjacent to the reactive center in the case of NGP.

While our results show little indication of nucleophilic attack at C-1 of the substrate in the lysozyme-catalyzed reaction of NGP, this mechanism could be dependent upon the nature of the leaving group. At present we are attempting to answer this question. One would almost surely expect some sort of electrostatic effect such as an ion pair to stabilize the developing carbonium ion on the enzyme surface. Such an ion pair could collapse to a covalently bonded intermediate in a post-rate-determining step and subsequent breakdown of this could govern the stereochemistry of the product. Based on the crystallographic results of Blake *et al.* (1967b) and the chemical modification studies of Parsons and Raftery (1969) the group most likely to be involved in such a manner is the β-carboxylate of Asp-52.

The usual method of determining enzyme mechanisms has been by analogy to various nonenzymatic "model" reactions which occur in solution. In general a few such model reactions may apply to the enzymatic reaction. The approach has been to then modify the substrate in a way which should allow distinction between the proposed mechanisms by means of rate measurements. There are problems associated with such effects of substrate modifications on the rate. It is very difficult to determine the effect of the modification on the binding phenomenon. While values of K_m (or more directly K_s) may be compared, it is impossible to compare the binding *orientations* of the modified and unmodified substrate. This is particularly true in the case of lysozyme since there seems to be a strong nonproductive mode of binding competing with a peak productive binding mode for some substrates. Thus, while physical measurements of the binding environment employing nuclear magnetic resonance (Raftery *et al.*, 1968) have shown some substrates and inhibitors to bind in nearly the same orientation in the *strong* binding mode, even these methods cannot compare the catalytic binding modes of various substrates to the enzyme.

The use of isotopically substituted substrates provides a means of circumventing these problems, since they would be expected to bind with identical orientations to the active site.

Further, there is a wealth of information concerning the magnitude of isotope effects for various reactions and the relation of the isotope effect to the reaction mechanism. While not all enzymatic reactions lend themselves to study by these methods, the use of secondary isotope effects should provide a useful tool for describing some enzyme mechanisms.

Appendix

Enrichment in Starting Material and Products. SIMPLE FIRST-ORDER REACTIONS. For a reaction of the type

$$A \xrightarrow{k} B \text{ or } A^* \xrightarrow{\Delta k} B^*$$

where A* and B* refer to deuterated materials and where $\Delta = k_D/k_H$

$$A = A_0e^{-kt} \quad A^* = A_0^*e^{-\Delta kt} \tag{1}$$

and

$$\frac{A}{A^*}\frac{A_0^*}{A_0} = e^{k(\Delta-1)t} \tag{2}$$

or

$$\ln \frac{A}{A_0} = -kt \qquad \ln \frac{A_0^* - B^*}{A_0^*} = -\Delta kt$$

$$\ln\left(1 - \frac{B}{A_0}\right) = -kt \qquad \ln\left(1 - \frac{B^*}{A_0^*}\right) = -\Delta kt$$

$$\ln\left(1 - \frac{B^*}{A_0^*}\right) = \Delta \ln 1 - \frac{B}{A_0} \tag{3}$$

when A_0^*/B^* and B/A_0 are $\ll 1$

$$\frac{B^*}{B}\frac{A_0}{A_0^*} = \Delta = \frac{k_D}{k_H} \tag{4}$$

ENZYMATIC REACTIONS. For Michaelis–Menten scheme

$$E + A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EA \xrightarrow{k_2} E + B$$

and

$$E + A^* \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EA^* \xrightarrow{\Delta k_2} E + B$$

$$dA/dt = -k_2\frac{E \cdot A}{K_M}$$

$$dA^*/dt = \Delta k_2\frac{E \cdot A^*}{K_M}$$

$$dA/dA^* = \frac{1}{\Delta}\frac{A}{A^*}$$

which reduces to eq 3. This assumes no isotope effect on the value of K_M.

Correction for Deuterium Composition. From eq 1, $A_0 - B = A_0e^{-kt}$ and $A_0^* - B^* = A_0^*e^{-\Delta kt}$. Let C = fraction completion of reaction; then

$$C = \frac{A_0 + A_0^* - (B + B^*)}{A_0 + A_0^*} = \frac{A_0}{A_0 + A_0^*}e^{-kt} + \frac{A_0^*}{A_0 + A_0^*}e^{-\Delta kt}$$

x = fraction deuterated = $A_0^*/(A_0 + A_0^*)$

$$C = (1 - x)e^{-kt}\left[1 + \frac{x}{1 - x}e^{k(1-\Delta)t}\right]$$

$$\ln C = \ln(1 - x) - kt + \ln\left[1 + \frac{x}{1 - x}e^{k(1-\Delta)t}\right]$$

for the first few per cent of reaction

$$d \ln C/dt = -k + k(1 - \Delta)\frac{\frac{x}{1 - x}e^{k(1-\Delta)t}}{1 + \frac{x}{1 - x}e^{k(1-\Delta)t}}$$

then

$$\left(\frac{k_D}{k_H}\right)_{obsd} = 1 - (1 - \Delta)\frac{\frac{x}{1 - x}e^{k(1-\Delta)t}}{1 + \frac{x}{1 - x}e^{k(1-\Delta)t}}$$

$$\left(\frac{k_D}{k_H}\right)_{obsd} = \frac{1 + \frac{x}{1 - x}e^{k(1-\Delta)t}[\Delta]}{1 + \frac{x}{1 - x}e^{k(1-\Delta)t}} \tag{5}$$

in the the case of the observed isotope effect for the reactions studied by enrichment $k(1 - \Delta)t \cong 0$ and eq 5 reduces to

$$\left(\frac{k_D}{k_H}\right)_{obsd} = 1 - x - \Delta x$$

or

$$\frac{k_H}{k_D} = \frac{x}{\left(\frac{k_D}{k_H}\right)_{obsd} - 1 + x} \tag{6}$$

Equations for Converting Counting Data to Observed Isotope Effect. If a = counts per minute in channel 1, b = counts per minute in channel 2, E = disintegrations per minute ^3H, F = disintegrations per minute ^{14}C, c_1 = ^3H efficiency in channel 1, d_1 = ^{14}C efficiency in channel 1, c_2 = ^3H efficiency in channel 2, and d_2 = ^{14}C efficiency in channel 2, then

$$\frac{E}{F} = \frac{d_2\left(a - b\frac{d_1}{d_2}\right)}{c_1\left(b - a\frac{c_2}{c_1}\right)}$$

if subscripts p and s denote product and starting material, then

$$\frac{E_p}{F_p}\frac{F_s}{E_s} = \left(\frac{a_p - b\dfrac{d_1}{-d_2}}{b_p - a_p\dfrac{c_2}{c_1}}\right)\left(\frac{b_s - a_s\dfrac{c_2}{c_1}}{a_s - b_s\dfrac{d_1}{d_2}}\right) = \frac{B}{B^*}\frac{A_0^*}{A_0} \quad (7)$$

from eq 4

$$\frac{B}{B^*}\frac{A_0^*}{A_0} = \left(\frac{k_H}{k_D}\right)_{obsd}$$

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