facts characterizing activated cleavages, such as why the "activator" must possess a free C-terminal carboxyl group and the "substrate", a free α-amino group and (3) it demonstrates conclusively that one common mechanism underlies the ability of (a) Z-Ala-Leu to catalyze the *hydrolysis* of Phe-Trp-NH_2, (b) Z-Ala-Leu to catalyze *acyl-transfer* reactions of Leu-Trp-Met-Arg, and (c) Phe-Trp-NH_2 to catalyze *amino-transfer* reactions of Z-Leu-Phe.

If a single explanation encompasses pepsin's hydrolytic, acyl-transfer, and amino-transfer activities, it is meaningless to make mechanistic proposals which rely on distinctions between activator and substrate or between acyl-transfer and amino-transfer reactions, at least where so-called activated cleavages are concerned. Furthermore, not once have we mentioned an acyl-enzyme or amino-enzyme intermediate.

Can the scope of the suggested mechanism be extended? Obvious targets for future investigation are the supposed acyl- and amino-enzyme reactions exhibited by such peptides as Leu-Tyr-Leu, which appear capable of undergoing self-condensation. For example, the mechanistic interpretation developed here is not incompatible with the finding that peptic cleavage of *Leu-Tyr-Leu gives *Leu-*Leu (Newmark & Knowles, 1975):

2*Leu-Tyr-Leu ⟶ *Leu-Tyr-Leu-*Leu-Tyr-Leu ⟶ *Leu-Tyr-Leu-*Leu + Tyr-Leu

*Leu-Tyr-Leu-*Leu + *Leu-Tyr-Leu ⟶⟶ *Leu-Tyr-Leu + [*Leu-*Leu] + Tyr-Leu

References

Anderson, G. W., Zimmerman, J. E., & Callahan, F. M. (1964) *J. Am. Chem. Soc. 86*, 1839–1842.

Chipman, D. M., & Sharon, N. (1969) *Science 165*, 454–465.

Hehre, E. J., Genghof, D. S., & Okada, G. (1971) *Arch. Biochem. Biophys. 142*, 382–393.

Hehre, E. J., Brewer, C. F., & Genghof, D. S. (1979) *J. Biol. Chem. 254*, 5942–5950.

Newmark, A. K., & Knowles, J. R. (1975) *J. Am. Chem. Soc. 97*, 3557–3559.

Silver, M. S., & James, S. L. T. (1981) *Biochemistry* (preceding paper in this issue).

Oxygen-18 Leaving Group Kinetic Isotope Effects on the Hydrolysis of Nitrophenyl Glycosides. 1. β-Galactosidase-Catalyzed Hydrolysis†

Steven Rosenberg and Jack F. Kirsch*

ABSTRACT: Oxygen-18 leaving group kinetic isotope effects (KIEs) have been determined on both V_{max} (V) and V_{max}/K_m (V/K) for the β-galactosidase-catalyzed hydrolysis of *p*-nitrophenyl β-D-galactoside (I) and 2,4-dinitrophenyl β-D-galactoside (II). The former substrate exhibits KIEs of 1.022 ± 0.002 and 1.014 ± 0.003 on V and V/K, respectively, while corresponding KIEs for the latter are 1.002 ± 0.009 and 1.030 ± 0.003. These results indicate that bond scission is largely rate determining for I but not for II at substrate saturation. The first irreversible step for both substrates must involve cleavage of the bond to the nitrophenyl leaving group. The mechanism proposed for this reaction is characterized by two parallel pathways for substrate hydrolysis. The predominant route for all but the most reactive substrates involves a S_N2 nucleophilic displacement of aglycon by the enzyme to yield a covalent galactosyl-enzyme which in turn is hydrolyzed via a nucleophilic attack by water. The most reactive substrates (e.g., II) form transiently an enzyme-bound galactosyl oxocarbonium ion which partitions between enzyme to give the covalent galactosyl-enzyme and H_2O to yield galactose.

β-Galactosidase, the *lacZ* gene product of *Escherichia coli*, has served as the prototypical example of a protein whose synthesis is controlled by the interplay of several proteins which interact with the DNA template, the *lac* repressor, cAMP binding protein, and *E. coli* RNA polymerase (Zabin & Fowler, 1978). Until recently, despite the wealth of detailed information available on the regulation of the synthesis of this enzyme, the mechanistic details by which it catalyzes the hydrolysis of lactose and other O, S, and N β-galactosides were not well understood (Wallenfels & Weil, 1972). Recent work by Sinnott and co-workers [Sinnott (1978) and references therein] utilizing structure–reactivity correlations and α-secondary ^2H KIEs has led to a detailed proposal for the mechanism of this reaction. In addition, the complete amino acid sequence of the monomer has now been determined (Fowler & Zabin, 1978).

This communication describes the application of oxygen-18 leaving group KIEs[1] to probe further the mechanism of this reaction. The application of new general methods to measure KIEs on V and on V/K (Rosenberg & Kirsch, 1979a,b) has yielded unique information on the nature of the rate-determining step and the structure of the transition state for this enzyme-catalyzed reaction. The results of these studies lead to the proposal of a new mechanism of action for the β-galactosidase catalyzed reaction.

Materials and Methods

Materials. Acetone was dried over anhydrous potassium carbonate, distilled, and stored over molecular sieves. Methanol was dried by distillation from 3% (v/v) trimethyl orthoformate. Diglyme was stirred over CaH_2 and $LiAlH_4$ overnight, distilled under reduced pressure, and stored over molecular sieves under N_2. Methanolic HCl (3–5%) was prepared the day it was used by the careful addition of 5 mL of redistilled acetyl chloride to 100 mL of redistilled methanol. The syntheses of oxygen-18-labeled *p*-nitrophenol and 2,4-dinitrophenol have been described (Rosenberg & Kirsch,

† From the Department of Biochemistry, University of California, Berkeley, California 94720. *Received September 16, 1980.* This investigation was supported by National Science Foundation Grant PCM 74-17643A02 and U.S. Public Health Service Predoctoral Traineeship 5-T01 GM00031-20 to S.R. A preliminary version of these results was presented at the 1978 meeting of the American Society of Biological Chemists in Atlanta, GA, June 1978, Abstract 151.

[1] Abbreviations used: KIE, kinetic isotope effect; EIE, equilibrium isotope effect; PNPGal, *p*-nitrophenyl β-D-galactopyranoside; 2,4-DNPGal, 2,4-dinitrophenyl β-D-galactopyranoside; 3,5-DNPGal, 3,5-dinitrophenyl β-D-galactopyranoside; PNPOH, *p*-nitrophenol; DNPOH, 2,4-dinitrophenol.

0006-2960/81/0420-3189$01.25/0

1979b; Gorenstein, 1972). Unlabeled phenols were purchased from Aldrich and recrystallized before use. *lacZ* β-galactosidase from *E. coli* was obtained from Worthington as a chromatographically purified suspension in saturated ammonium sulfate. All other materials were reagent grade and were used without further purification. Glass-distilled water was used in all isotope effect experiments.

p-Nitrophenyl β-D-Galactoside. *p*-Nitrophenyl β-D-galactoside was prepared by standard methods (Conchie & Levy, 1963; Leaback, 1960). Both the normal substrate and that enriched with oxygen-18 were synthesized from a single lot of 2,3,4,6-tetraacetyl-1-bromo-α-D-galactose (Sigma) and purified by the same methods. The compounds were identical by melting point (178–179 °C) and ultraviolet–visible spectrum. Hydrolysis of the substrate enriched with oxygen-18 and analysis of the resulting *p*-nitrophenol by mass spectrometry showed 74.5 ± 0.5% oxygen-18 enrichment, identical with that of the starting *p*-nitrophenol which was 75% enriched.

2,4-Dinitrophenyl β-D-Galactoside. This substrate was prepared by the methods of Ballardie et al. (1973) and Sinnott & Viratelle (1973). Both the unenriched and enriched substrates were prepared and purified in this manner. The normal substrate and that enriched with oxygen-18 had mp 153–154 °C [lit. mp 150–151 °C (Hengstenberg & Wallenfels, 1969) and 161–163 °C (Sinnott & Viratelle, 1973)].

Methods. Measurement of Isotope Effects on V. The general method used for this procedure has been described in detail elsewhere (Rosenberg & Kirsch, 1979b). All experiments were done in 0.1 M sodium phosphate buffer containing 1 mM $MgCl_2$ so as to be directly comparable with the results of Sinnott & Souchard (1973). Most of the isotope effects were measured at pH 7.0, although that on the reaction of PNPGal was determined over the pH range 6.0–7.9. All spectrophotometric measurements were done on a Cary Model 118C spectrophotometer interfaced via a microprocessor (Claremont Research) to an ASR-33 teletype and tape punch. All KIE determinations were done by monitoring four or five reactions simultaneously, as has been described (Rosenberg & Kirsch, 1979b). The enzyme concentrations were 0.1–0.3 μg/mL. Initial substrate concentrations were 1.9–2.1 mM for DNPGal and 0.3–0.42 mM for PNPGal, greater than 10 times the reported K_m's for these substrates under these conditions (Sinnott & Souchard, 1973). In addition, the K_m's for these substrates were independently determined in this work. Values of 31 ± 4 μM and 165 ± 11 μM for PNPGal and DNPGal were obtained; these are in very close agreement with those reported. Reactions were initiated by the addition of a small amount of a concentrated stock solution of the enriched or unenriched substrate to the enzyme solution. The DNPGal reactions were monitored at 470 nm, and the PNPGal experiments were followed at 440 nm at pH 7.0, at 350 or 360 nm at pH 6, and at 450 nm at pH 7.9. These wavelengths were chosen so that the total absorbance change during the reaction was 0.9–1.2 A units. All experiments were done at 25.0 ± 0.1 °C. The oxygen-18 enrichments were 74% and 49% for PNPGal and DNPGal, respectively.

Data Analysis. Kinetic data were monitored for 30–60 min, and the end points were recorded after the absorbance of the samples had not shown an appreciable increase (less than 0.002 A unit) for 10 min. Typically, 20–30 data points were acquired for each kinetic run. All of the data were analyzed by fitting the progress curves to the integrated form of the Michaelis–Menten equation with V, K, the end point, and the initial substrate concentration as variable parameters (Rosenberg & Kirsch, 1979b). The calculated values of V for both the unenriched and enriched substrate were averaged for each set of four or five kinetic runs, and the KIE for each set and its associated standard error determined by using eq 1 and 2. The

$$\mathrm{KIE} = V_{16}/V_{18} \quad (1)$$

$$\mathrm{SE(KIE)} = [(\mathrm{SE}_{16}/V_{18})^2 + (V_{16}\mathrm{SE}_{18}/V_{18}^2)^2]^{1/2} \quad (2)$$

KIE for an entire experiment comprising 4–6 sets of kinetic runs was then calculated by weighted averaging of the KIEs for each set.

Some of the KIEs on V with PNPGal as substrate had to be corrected for the equilibrium isotope effect on the acidity of *p*-nitrophenol, which has the value $K_{16}/K_{18} = 1.0181 \pm 0.0019$ (Rosenberg, 1978), since the labeled and unlabeled products are ionized to different extents at the pH of the experiment. The details of this correction are complex, but they amounted to less than 1.007 for the KIE reported in this communication (corresponding to less than 30% of the observed KIEs), so they will not be described here. Finally, the KIEs were corrected for incomplete enrichment of the oxygen-18-labeled substrate by using eq 3 (Dahlquist et al., 1969)

$$\mathrm{KIE}_{\mathrm{final}} = (\mathrm{KIE}_{\mathrm{cor}} - 1 + f)/f \quad (3)$$

where $\mathrm{KIE}_{\mathrm{cor}}$ is the observed KIE corrected for the equilibrium isotope effect on *p*-nitrophenol acidity if necessary, f is the fraction of isotopic enrichment in the oxygen-18-enriched substrate, and $\mathrm{KIE}_{\mathrm{final}}$ is the final value of the KIE corrected for both incomplete enrichment and the equilibrium isotope effect. These two corrections, which are in opposite directions, had a combined effect of less than 1.005 on the KIE, corresponding to less than 20% of the final KIEs.

Measurement of KIEs on V/K. All experiments were done at pH 7.0 in 0.1 M sodium phosphate buffer containing 1 mM $MgCl_2$ with approximately 50% enriched substrates. Reactions of PNPGal were initiated by enzyme addition while those with DNPGal were started by the addition of substrate. The initial substrate concentrations were 0.2–0.3 mM for PNPGal and 0.4–0.5 mM for DNPGal. All experiments were performed at 25.0 ± 0.1 °C. The time courses of the reactions were monitored in a Cary 118C spectrophotometer at 440 nm for PNPGal and at 450 nm for DNPGal.

The general experimental procedure used has been described in detail elsewhere (Rosenberg & Kirsch, 1979a). An aliquot of the reaction mixture was quenched at ~10% reaction by the addition of sufficient 5.8 N HCl to bring the pH of the solution to 2, and the remainder of the reaction was allowed to proceed to completion. The product was isolated by the following procedure. The quenched solution (50–100 mL) at pH 2 was extracted twice with 100 mL of ether; the ethereal solutions were combined and taken to dryness by rotary evaporation. The residue was dissolved in ~10 mL of 0.1 M potassium carbonate and acidified to pH 2. The solution was extracted twice with 10 mL of ether. The ethereal solutions were combined, dried with sodium sulfate, and taken to dryness in a vacuum oven at 40–60 °C. The identical procedure was used to isolate the product from the aliquot which had been allowed to react to completion. The method for isolating 2,4-dinitrophenol for the DNPGal experiments was identical with that described above except that the solution was initially quenched to pH 1.5 and was then placed on ice to retard any nonenzymatic hydrolysis of this more labile substrate.

Mass spectrometric analysis of the nitrophenols was performed as described previously by using a Du Pont Model 21-491 mass spectrometer equipped with a Columbia Scientific Model 260/722 data system and digital printer (Rosenberg & Kirsch, 1979a). Typically, three to five samples of the product at both ~10% and 100% reactions were analyzed,

Table I: Kinetic Isotope Effects on V for the β-Galactosidase-Catalyzed Hydrolysis of *p*-Nitrophenyl β-D-Galactoside

expt	KIE_{obsd}[a]	pH[d]	monitored wavelength (nm)	EIE correction[b]	KIE_{cor}[b]	KIE_{final}[c]
1	1.0181 (0.0020)	7.90	450	1.0016	1.0165 (0.0020)	1.0223 (0.0020)
2	1.0200 (0.0007)	7.82	450	1.0019	1.0181 (0.0007)	1.0244 (0.0007)
3	1.0227 (0.0021)	7.02	440	1.0067	1.0159 (0.0021)	1.0215 (0.0021)

[a] KIE_{obsd} is the observed kinetic isotope effect on V. Each value represents the results of an independent experiment consisting of 10–15 kinetic runs each of the unenriched and enriched substrates. Values in parentheses are the propagated standard errors of the observed KIEs. [b] The EIE corrections are determined, and the corrected values of the KIEs are calculated as has been described [see Materials and Methods and Rosenberg (1978)]. [c] The final values of the KIEs are calculated from eq 3 and the known fraction of oxygen-18 in the partially enriched substrate (0.74). [d] Determined at 25 °C in 0.1 M sodium phosphate buffer.

yielding 1000–2000 measurements of the isotope ratios. Only the molecular ion peaks were scanned, and no correction was made for the natural abundance of other stable isotopes, as this correction is smaller than the experimental error with the relatively high isotopic enrichment used.

Data Analysis. The observed isotope ratios ($[^{18}O]/[^{16}O]$) were calculated from the intensities of the parent molecular ions, corresponding to m/e 186 and 184 for 2,4-dinitrophenol and 141 and 139 for *p*-nitrophenol. All of the individual measurements of the isotope ratios were averaged for each sample, yielding a mean and standard error. The final values of the isotope ratio at ~10% reaction and 100% reaction were calculated by weighted averaging of the isotope ratios for each of the three to five samples at these extents of reaction. The exact fraction of reaction at which the initial aliquot was quenched was determined from the absorbance at this time and at the end point of the reaction. The latter was determined by fitting the progress curve of the reaction to the integrated form of the Michaelis–Menten equation. The KIE and its propagated standard error were then calculated from the isotope ratios and the fraction of reaction by using eq 4

$$KIE = \frac{\ln [1 - [f(R_{100} + 1)/(R + 1)]]}{\ln [1 - [fR(R_{100} + 1)/R_{100}(R + 1)]]} \quad (4)$$

(Bigeleisen & Wolfsberg, 1958).

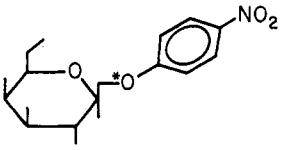
Control Experiments. One of the KIE experiments on V with PNPGal as substrate was run "double blind", a procedure in which the experimenter did not know in advance which substrate solution contained the isotopically enriched substrate. The experiment was run as described above, and at the completion of the reactions, the solutions containing the enriched and unenriched products were each pooled, and the products were isolated and subjected to mass spectrometric analysis to determine the identity of the enriched substrate solution. This analysis indicated that the more slowly reacting substrate contained 73.3 ± 0.2% oxygen-18 where 74.5% was expected.

The kinetic parameters for the enzyme-catalyzed hydrolysis of commercial PNPGal (Sigma) and synthesized PNPGal (^{16}O) were compared. This experiment was performed at pH 7.0 at saturating substrate concentrations under standard conditions. The ratios of V and K for the synthesized substrate to the commercially obtained substrate were 0.998 ± 0.006 and 1.02 ± 0.04, respectively.

A third control experiment (suggested by Professor W. P. Jencks) involved testing whether an impurity which might inactivate some small fraction of the enzyme was present in either the oxygen-18-enriched or unenriched substrate. A set of five aliquots of an enzyme solution at pH 7.85 were placed in five cuvettes to which were added 100 μL of a concentrated solution of either oxygen-18-enriched or natural abundance PNPGal in alternate cuvettes. The kinetics were monitored as described above. The observed KIE comparing the unenriched and enriched substrates was V_{16}/V_{18} = 1.020 ± 0.006. At the completion of these reactions a second 100-μL aliquot of unenriched PNPGal was added to all five cuvettes, and the kinetics of the second reactions were monitored. The ratio of the values of V for cuvettes which contained only natural abundance PNPGal compared to those to which unenriched substrate was added to enriched product as described was 1.002 ± 0.004. This value represents the average from four sets of experiments. Thus, it seems there is no significant preferential inactivation of the enzyme by either substrate which could account for the observed KIE on V with this substrate.

Results

p-Nitrophenyl β-D-Galactoside. Typical measurements of V_{16}/V_{18} for the β-galactosidase-catalyzed hydrolysis of PNPGal (I) at pH 7.9 in the presence of 1 mM $MgCl_2$ are

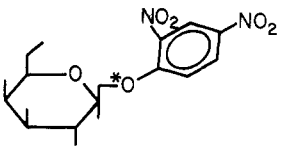


I

shown in Figure 1. The labeled substrate was enriched with 74% oxygen-18 in the galactosyl oxygen atom. A summary of all the measurements of the KIE on V for this substrate along with the corrections (see Materials and Methods) due to the equilibrium isotope effect on *p*-nitrophenol acidity and incomplete labeling of the enriched substrate on the observed KIE is given in Table I. The KIE was also measured at pH 6.0, yielding V_{16}/V_{18} = 1.022 ± 0.012 on the basis of three separate experiments. Data were less reproducible under these conditions due to a smaller absorbance change and the decreased activity of the enzyme. The relatively large, apparently pH-independent KIE observed at substrate saturation indicates that the bond to the isotopically labeled atom is substantially cleaved in the transition state for the overall reaction sequence. Thus, the "chemical step" must significantly contribute to the overall rate-determining process with this substrate.

Typical data for the determination of the KIE on V/K as measured by mass spectrometric analysis of the product *p*-nitrophenol as a function of the extent of reaction are shown in Figure 2. The KIE of 1.014 ± 0.004 indicates that bond scission occurs in the first irreversible step in the reaction sequence. The results of all the KIE measurements with PNPGal as substrate are summarized in Table II.

2,4-Dinitrophenyl β-D-Galactoside. The effects of oxygen-18 substitution of the galactosyl oxygen of DNPGal (II)



II

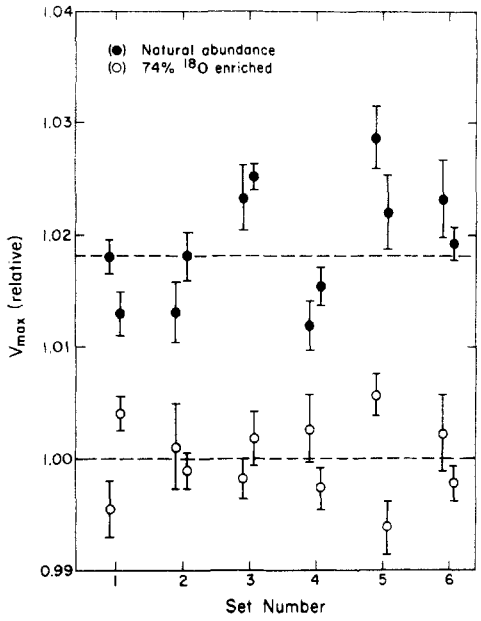


FIGURE 1: Typical data representing the oxygen-18 kinetic isotope effect on V with p-nitrophenyl β-D-galactoside as substrate. The relative values of V observed for the β-galactosidase catalyzed cleavage of unlabeled PNPGal (●) and 74% oxygen-18-enriched PNPGal (○) for a typical experiment at pH 7.90 are shown. The average value of V for the labeled substrate for each set was assigned an arbitrary value of 1.00. The error bars represent the standard errors in V for the individual kinetic runs as calculated by nonlinear regression analysis (see Materials and Methods). The measured isotope effect, V_{16}/V_{18} = 1.0181 ± 0.0021, indicated by the upper dashed line, represents the weighted mean of all the isotope effect determinations in this experiment (12 enriched and 12 unenriched), calculated as described in the text. This result is reported as experiment 1 in Table I.

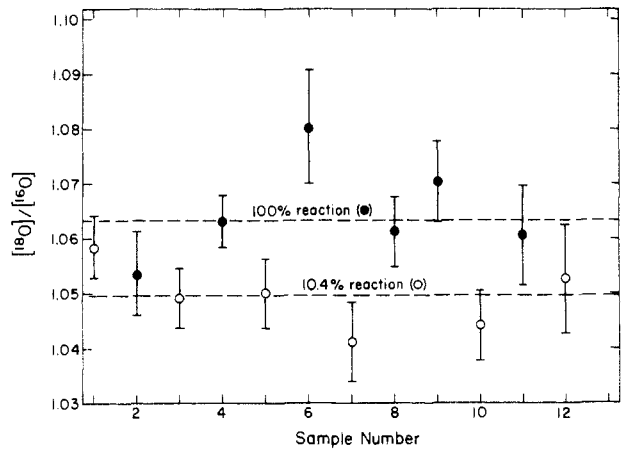


FIGURE 2: Typical determination of the kinetic isotope effect on V/K with p-nitrophenyl β-D-galactoside as substrate. The observed isotope ratios for a competitive experiment with PNPGal (51.5% enriched with oxygen-18 in the galactosyl oxygen atom) as substrate at pH 7.0 are shown. The isotope ratios at 100% reaction (●) and 10.4% reaction (○) were measured by direct mass spectrometric analysis of the PNPOH product. Each point represents between 50 and 250 measurements of the isotope ratio of a given sample. The weighted means of the isotope ratios at the two extents of reaction are indicated by the dashed lines. The isotope effect calculated from these data is $(V/K)_{16}/(V/K)_{18}$ = 1.014 ± 0.004.

on V and V/K for the β-galactosidase-catalyzed hydrolysis of this substrate differ significantly from those observed with PNPGal. Some typical data obtained at substrate saturation with DNPGal are shown in Figure 3. In contrast to the results obtained with PNPGal, no significant KIE on V for DNPGal was found, indicating that some step other than the scission of the bond to the isotopically labeled atom is the rate-determining step at substrate saturation. The results shown in

Table II: Summary of the Oxygen-18 Kinetic Isotope Effects for the β-Galactosidase Catalyzed Hydrolysis of p-Nitrophenyl β-D-Galactoside

pH	KIE on V^a	KIE on V/K^b
7.0	1.023 (0.002)	1.014 (0.003)
7.9	1.023 (0.001)	
6.0	1.022 (0.012)	

[a] Determined directly by the difference in rates at saturating substrate concentrations. Each experiment consisted of 10–15 kinetic runs each of the enriched and unenriched substrates. These data are the weighted averages of the experiments reported in Table I, or the text; values in parentheses are the propagated standard errors of the isotope effects. [b] Measured by the competitive technique, comparing the isotope ratio of partially enriched product at ca. 10% and 100% reaction. The value shown here is the mean of the KIEs measured in two independent experiments, the individual values of which were 1.014 ± 0.004 (data of Figure 2) and 1.015 ± 0.005.

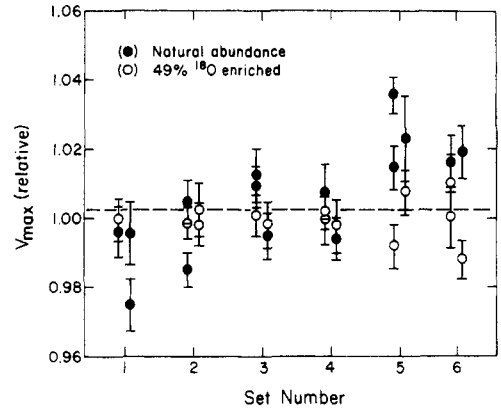


FIGURE 3: Typical data for the determination of the kinetic isotope effect on V with 2,4-dinitrophenyl β-D-galactoside as substrate. The relative values of V observed for the β-galactosidase catalyzed hydrolysis of natural abundance DNPGal (●) and 49% oxygen-18-enriched DNPGal (○) are shown. These values were normalized as described in the legend to Figure 1. The error bars represent the standard errors on V calculated by nonlinear regression analysis (see Materials and Methods). The *observed* kinetic isotope effect, indicated by the dashed line, is V_{16}/V_{18} = 1.003 ± 0.006, without correction for 50% isotopic enrichment.

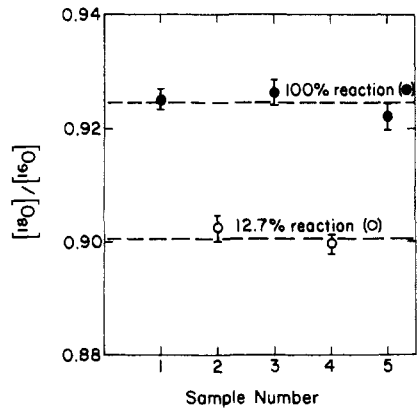


FIGURE 4: Determination of the kinetic isotope effect on V/K for 2,4-dinitrophenyl β-D-galactoside as substrate. The observed isotope ratios at 100% reaction (●) and 12.7% reaction (○) were measured by direct mass spectrometric analysis of the DNPOH product for an experiment with DNPGal as substrate at pH 7.0. Each point represents 120–240 measurements of the isotope ratio for a given sample. The weighted means of the isotope ratios are indicated by the dashed lines. The isotope effect calculated from these data is $(V/K)_{16}/(V/K)_{18}$ = 1.029 ± 0.002.

Figure 4 indicate that bond cleavage must occur in the first irreversible step in the reaction sequence for this substrate since a large KIE is observed on V/K. The results of all the KIE

Table III: Summary of the Oxygen-18 Kinetic Isotope Effects for the β-Galactosidase-Catalyzed Hydrolysis of 2,4-Dinitrophenyl β-D-Galactoside[a]

expt	KIE on V[b]	KIE on V/K[c]
1	1.006 (0.011)	1.039 (0.006)
2	0.994 (0.015)	1.029 (0.002)
mean[d]	1.002 (0.009)	1.030 (0.003)

[a] All KIEs were measured at pH 7.0 in 0.1 M sodium phosphate buffer containing 1.0 mM $MgCl_2$ at 25.0 °C. Values in parentheses are the propagated standard errors of the observed KIEs. [b] Measured directly by the difference in rates between unenriched and 49% oxygen-18-enriched DNPGal at saturating substrate concentrations. Reported values are corrected by eq 3 for incomplete labeling. The data of Figure 3 are experiment 1. [c] Measured by the competitive technique, comparing the isotope ratio of product (2,4-dinitrophenol) at ~10% and 100% reaction. The data of Figure 4 are experiment 2. [d] The weighted mean of the independent determinations of the KIEs.

measurements with DNPGal as substrate are summarized in Table III.

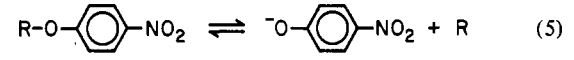
Discussion

The utility of the kinetic isotope effect method in probing enzyme reaction mechanism and transition-state structure is based upon the fact that isotopic substitution does not perturb the potential-energy surface for a reaction (Van Hook, 1970). Thus, it is the most subtle form of structural perturbation available to the enzymologist interested in the detailed mechanism of enzymatic catalysis. Kinetic isotope effects for elements heavier than hydrogen have been investigated to a very limited extent in enzyme-catalyzed reactions because of the necessity of measuring rates or isotopic compositions to a precision of ~0.2%. The information gained is unique, however, since only with the KIE method can a quantitative analysis of transition-state structure be achieved.

The KIEs reported in this communication reflect the changes in force constants to the galactosyl oxygen atom between the ground state and transition state for the β-galactosidase-catalyzed hydrolysis of nitrophenyl β-D-galactosides. A KIE significantly greater than 1.00 thus indicates a substantial weakening of the force constants to the galactosyl oxygen atom in the transition state for either the overall rate-determining step in the reaction sequence (V KIEs) or the first irreversible step (V/K KIEs) (O'Leary, 1978).

The Maximum KIE for Aryl Galactoside Hydrolysis. Previous work from our laboratory (Sawyer & Kirsch, 1973, 1975) has utilized the study of KIEs on model reactions to ascertain the likely maximum KIE due to scission of a bond to the isotopically labeled atom. Two items of information are necessary for the quantitative analysis of leaving group KIEs in terms of transition-state structure in enzyme-catalyzed reactions, i.e., the KIE for complete scission of the bond to the isotopically labeled atom which is the EIE for the reaction and the extent to which this step in the reaction sequence in an enzyme-catalyzed reaction is rate determining.

Recently, we have measured equilibrium oxygen-18 isotope effects on the acidities of *p*-nitrophenol and 2,4-dinitrophenol (Rosenberg, 1978). Complete in-plane force fields for these molecules and their anions have been constructed from the measurement of the IR frequency shifts due to isotopic substitution and from force fields for related compounds. Theoretical values for these EIEs in excellent agreement with those experimentally determined have been calculated (G. Burton, M. Liskin, and J. F. Kirsch, unpublished results). These force fields have been used in conjunction with additional IR measurements of oxygen-18-enriched nitrophenyl acetates, phosphates, and galactosides to yield estimates of the EIEs for equilibria of the type



(5)

where R = CH_3CO^+, O_3P^-, CH_3^+, and galactosylium. The results of such a calculation for *p*-nitrophenyl β-D-galactoside and *p*-nitrophenoxide yield an EIE of K_{16}/K_{18} = 1.0425 (G. Burton, M. Liskin, and J. F. Kirsch, unpublished results). This EIE is equivalent to the maximum KIE expected for a reaction in which the bond between the anomeric carbon of the galactoside and the galactosyl oxygen atom is completely broken in the transition state. This maximum KIE would be reduced somewhat if the transition state were to include partial proton transfer to the leaving group. We have obtained experimental confirmation of this calculated EIE for reactions of glycosides in which C–O bond cleavage is thought to be nearly complete in the transition state. These reactions yield KIEs between 1.04 and 1.05 (Rosenberg & Kirsch, 1981). Reactions characterized by substantially smaller KIEs require either that some step other than bond scission is at least partially rate determining or that the structure of the transition state does not include complete C–O bond cleavage. Thus, because it is rarely possible to determine accurately the extent to which the bond cleavage step is rate determining, only lower limits to this parameter can be obtained from KIE measurements in the absence of additional data. This analysis assumes that only the step involving bond cleavage yields a substantial KIE.

It has been shown by computer calculations (Saunders, 1975) that the leaving group KIE increases essentially linearly with the fraction of bond cleavage for N, S, and Cl leaving groups. With the assumption that a similar linear relationship holds for oxygen as the departing group (Hogg et al., 1980), an observed KIE can be translated into a minimum estimate of the extent to which bond scission is rate determining by comparison with the maximum theoretical KIE calculated for complete bond cleavage (Bilkadi et al., 1975). The result of such a calculation is a lower limit, because a transition state with less than complete bond cleavage would result in a smaller maximum KIE for completely rate-determining bond scission. A KIE close to the maximum can be interpreted unambiguously; i.e., bond cleavage must be rate determining, and the transition state for that step is characterized by close to complete C–O bond scission. The above discussion provides a framework within which the KIEs determined in this work can be interpreted.

KIE on V for p-Nitrophenyl β-D-Galactoside. The observed oxygen-18 leaving group KIE of 1.022 ± 0.002 at saturating concentrations of this substrate requires that bond scission occurs in the overall rate-determining step in the reaction sequence under these conditions. Quantitatively, the step involving bond scission is at least 52 ± 5% rate determining, assuming a maximum KIE of 1.0425. This observation is consistent with transition states generated by either a S_N2 or S_N1 mechanism of C–O bond cleavage, the former requiring nucleophilic participation in the rate-determining step by enzyme or water and the latter, the generation of a carbonium ion such as has been postulated for lysozyme. A method based on α-deuterium secondary isotope effects to distinguish these possibilities is discussed below.

KIE on V/K for p-Nitrophenyl β-D-Galactoside. The oxygen-18 leaving group KIE on V/K for PNPGal as substrate was determined by a competitive technique involving mass spectrometric analysis of the product as a function of the extent of reaction (see Materials and Methods). Such a measurement determines whether the steps up to the first irreversible step

in the reaction sequence are isotopically sensitive (O'Leary, 1978). The observed KIE $(V/K)_{16}/(V/K)_{18} = 1.014 \pm 0.003$ indicates that the glycosidic bond is partially broken in the first irreversible step. Quantitatively, bond scission must be at least 33 ± 7% complete in the transition state for this step.

The smaller KIE observed on V/K as compared with V for this substrate is unusual and can be rationalized in two ways. First, it should be noted that the two KIEs were measured by completely independent techniques. Although the reproducibility of these techniques is very good (ca. ±0.3% in separate experiments), it is possible that there is a small deterministic error in one of the measurements. The difference between the KIEs is small (less than 1%), and although the propagated standard errors of the measurements indicate that the KIEs are significantly different (by a t test), this does not reflect the possible contribution of some small deterministic artifact. We have measured KIEs on nonenzymatic organic reactions by both techniques where the complication of the effect of substrate concentration is not a factor. In these examples, the same value of the KIE has been observed within 0.5% (Rosenberg & Kirsch, 1979a,b). Since, as shown in Table I, the KIE on V has been determined to be ~1.02 in several experiments under a variety of conditions, we believe this value is more likely to be correct.

A second possible explanation (suggested by Professor J. P. Klinman) for a smaller KIE on V/K than on V is that the reaction of PNPGal with β-galactosidase is partially diffusion limited. That is, the substrate is somewhat "sticky", and the formation of the Michaelis complex is partially irreversible. The value of V/K for this substrate at pH 7 in the presence of 1 mM $MgCl_2$ is close to 10^7 M^{-1} s^{-1} (Sinnott & Souchard, 1973).

Whether the small difference in the KIEs on V/K and V for PNPGal is due to a subtle methodological artifact or to partially rate-determining diffusion of the substrate into the active site in the productive orientation for catalysis to occur, the observation of an oxygen-18 leaving group KIE significantly greater than 1.00 on both V and V/K requires that the bond cleavage step contributes significantly to both the overall rate-determining step and the first irreversible step for this substrate.

KIE on V for 2,4-Dinitrophenyl β-D-Galactoside. The KIE for DNPGal at substrate saturation, $V_{16}/V_{18} = 1.002 \pm 0.009$, is not significantly different from 1.00, which requires that scission of the bond to the aglycon does not occur in the overall rate-determining step for this substrate. A substantial body of evidence exists which suggests that hydrolysis of some form of a galactosyl enzyme intermediate, formed after aglycon departure, is rate determining for DNPGal and related substrates (Sinnott & Viratelle, 1973; Sinnott & Souchard, 1973). The present observation of a negligible oxygen-18 leaving group KIE on V for this substrate is consistent with the rate-determining hydrolysis of such an intermediate.

KIE on V/K for 2,4-Dinitrophenyl β-D-Galactoside. The KIE for DNPGal measured by the competitive mass spectrometric technique is $(V/K)_{16}/(V/K)_{18} = 1.030 \pm 0.003$, requiring that C–O bond scission be close to completion in the first irreversible step in the reaction sequence for this substrate. Quantitatively, the C–O bond is at least 71 ± 7% broken in the transition state for the first irreversible step, assuming that the maximum oxygen-18 leaving group KIE of 1.0425 can be used for 2,4-dinitrophenoxide as well as for p-nitrophenoxide. This is likely to be a good assumption since the oxygen-18 equilibrium isotope effects on the acidities of these nitrophenols are very similar (Rosenberg, 1978).

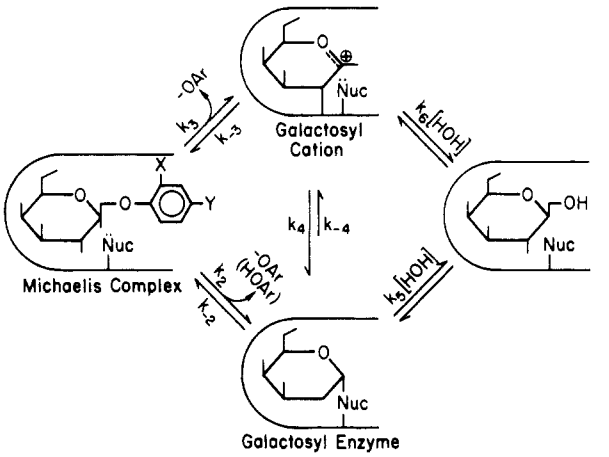
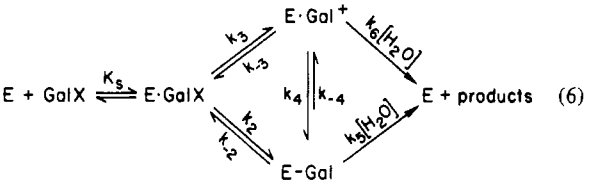


FIGURE 5: Alternate pathways for β-galactoside hydrolysis. Substrate hydrolysis is proposed to occur via two pathways whose relative fluxes are dependent upon substrate structure and chemical reactivity. Less reactive aryl galactoside substrates such as PNPGal are hydrolyzed via nucleophilic attack by the enzyme (k_2), whereas the most reactive substrates (i.e., 2,4-DNPGal) react (see text) via the k_3 pathway to form an enzyme-bound galactosyl cation. This intermediate is sometimes trapped to yield the galactosyl enzyme (k_4). Hydrolysis of this covalent intermediate occurs rarely via the intermediacy of the galactosyl cation (k_{-4}, k_6) or most often directly via k_5.

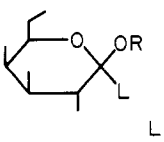
A Mechanism for β-Galactosidase. We have previously presented preliminary evidence that the β-galactosidase-catalyzed hydrolysis of β-galactosides occurs through two parallel pathways, as shown in eq 6 (Rosenberg & Kirsch, 1978). A



(6)

more detailed presentation of this mechanism is shown in Figure 5. The major feature of this mechanism is that the two pathways have relative fluxes which are dependent upon substrate structure and chemical reactivity. Substrates with more basic leaving groups are proposed to react via nucleophilic attack of the enzyme in a S_N2 (k_2) process to yield *directly* a covalent galactosyl enzyme. Hydrolysis of very reactive substrates with acidic leaving groups (e.g., DNPGal) proceeds via a S_N1 step (k_3) to form a transient enzyme-bound galactosyl oxocarbonium ion which partitions between the nucleophilic residue on the enzyme (k_4) and solvent (k_6). The hydrolysis of the covalent galactosyl enzyme occurs predominantly through nucleophilic attack of water (k_5), although a minor pathway involving the galactosyl oxocarbonium ion (k_{-4}, k_6) cannot be excluded. The key feature which regulates the relative fluxes through the two pathways is that the thermodynamically unstable oxocarbonium ion is only accessible from the less stable substrates.

A commonly used criterion for distinguishing between the S_N1 and S_N2 mechanisms shown as the two pathways in eq 6 is the value of the observed α-secondary deuterium isotope effect (Kirsch, 1977; Hogg, 1978).



L = H,D

An α-secondary ^2H KIE close to 1.00 suggests nucleophilic participation, whereas a KIE in the range 1.10–1.34 has been

taken to implicate a carbonium ion-like transition state (Dahlquist et al., 1969; Sinnott & Souchard, 1973). Recent work has shown that α-secondary ^{2}H KIEs of up to 1.20 are also consistent with a borderline S_N2 mechanism (Craze et al., 1978; Knier & Jencks, 1980). The likelihood of a glycosidic carbonium ion as a free, solvent-equilibrated intermediate has been further cast in doubt because of the extrapolated extremely short lifetime of such an intermediate (10^{-15} s; Young & Jencks, 1977). Small values of α-secondary ^{2}H KIEs still seem to provide good evidence for a S_N2 mechanism, if it can be shown that the "chemical step" in an enzyme-catalyzed reaction is rate determining.

Sinnott & Souchard (1973) measured the α-secondary ^{2}H KIE on V and obtained $V_H/V_{^2H} = 1.04 \pm 0.02$ and 1.25 ± 0.02 for PNPGal and 2,4-DNPGal, respectively. The large α-secondary ^{2}H KIE on V for 2,4-DNPGal represents hydrolysis of some form of the galactosyl enzyme, as shown by methanol competition. This value increased to 1.34 ± 0.04 in the presence of 1.5 M methanol (Sinnott & Viratelle, 1973). This, in accord with the above model, represents more efficient trapping of the oxocarbonium ion by solvent (methanol and water) relative to capture by the nucleophilic element on the enzyme. The figure of 1.34 ± 0.04 is close to the limit expected in reactions involving $sp^2 \rightleftharpoons sp^3$ interconversion, which is ~1.40 (Bilkadi et al., 1975; Palmer & Jencks, 1980). Thus, the transition state for this step must have very close to sp^2 hybridization. Since the trapping of the oxocarbonium ion is rate determining for this substrate, the α-secondary ^{2}H KIEs provide no information with respect to the mechanism of its formation.

The oxygen-18 leaving group KIE on V/K for 2,4-DNPGal observed in this study of 1.030 ± 0.003 requires that no step prior to bond scission be irreversible, but this KIE by itself does not differentiate between S_N1 and S_N2 mechanisms. Transition states for reactions of glycosides by either mechanism are characterized by a large degree of bond breaking to the leaving group; i.e., values of β_{lg} for acid-catalyzed hydrolysis of glycosides are ~0 and for alkaline hydrolysis are −1.1 [Young & Jencks (1977) and references therein]. In addition, both these reactions yield large oxygen-18 leaving group KIEs in the range $k_{16}/k_{18} = 1.03$–1.04 (Rosenberg & Kirsch, 1981; Banks et al., 1961).

The likely mechanism of galactosyl enzyme formation for all but the most reactive aryl galactosides can be deduced from the isotope effect results with PNPGal for which formation of the galactosyl enzyme *is* rate determining. The α-secondary ^{2}H KIE on V of 1.04 ± 0.02 is characteristic of either a S_N2 mechanism or a rate-determining step in which no covalent changes occur (e.g., an enzyme conformation change; Sinnott, 1978). The latter explanation, consistent with all the earlier data, is eliminated by the large oxygen-18 leaving group KIEs on V and V/K. Further evidence has recently been advanced in support of the proposal that the β-galactosidase mechanism involves intramolecular attack of the C-2 hydroxyl group of the substrate on C-1 (Brockhaus et al., 1979). This mechanism cannot be differentiated from one involving nucleophilic attack by the enzyme on the basis of the KIE data.

A reaction thought to be a S_N2 attack of a nucleophile on a glycoside is methoxide-catalyzed methanolysis for which $k_H/k_D = 1.03$ for phenyl glucoside (Dahlquist et al., 1969). In the same study, the sweet almond β-glucosidase catalyzed hydrolysis of phenyl β-D-glucoside was shown to have a KIE on V/K of 1.015 ± 0.004. A large oxygen-18 leaving group KIE for *p*-nitrophenyl β-D-glucoside as substrate has been observed with the purified β-glucosidase A, making it likely that this reaction also proceeds via a S_N2 mechanism (Rosenberg & Kirsch, 1981), assuming that the earlier result for the α-secondary ^{2}H KIE on V/K was not influenced by the heterogeneity of the commercial enzyme preparation used. Thus, there is evidence for another enzyme-catalyzed hydrolysis of a glycoside to proceed via a S_N2 mechanism, and the present results suggest such a mechanism obtains for the β-galactosidase catalyzed hydrolysis of the less reactive aryl galactosides.

Sinnott & Souchard (1973) have pointed out that such a mechanism is unlikely, despite the observation of small α-secondary ^{2}H KIEs for less reactive aryl galactosides, because there is little correlation of k_{cat} and k_{cat}/K_m with leaving group pK_a. This is in contrast to the behavior of the galactosylpyridinium salts for which a good correlation for both k_{cat} and k_{cat}/K_m is observed (Sinnott & Withers, 1974). The behavior of the aryl galactosides is more complex for two reasons: (1) degalactosylation—this becomes increasingly more rate determining with increasing substrate reactivity; (2) steric effects—we find that a reasonably good correlation of k_{cat}/K_m with aglycon pK_a is obtained with $\beta_{lg} = -0.3$ to -0.4 if only the data for para-substituted substrates are considered [data of Sinnott & Souchard (1973)], suggesting that the full impact of the original correlation was obscured by the ortho and meta substituents in the aglycons.

In view of the results of Young & Jencks (1977) and of Craze et al. (1978) (see above), it is clear that the glycosidic oxocarbonium ion must be substantially stabilized by the enzyme, and the above discussion argues that it can only be formed in a kinetically significant quantity from the most reactive aryl galactosides, e.g., 2,4-DNPGal and 3,5-DNPGal (k_3, eq 6). What is mandated by the present and previous results showing a combination of a large oxygen-18 leaving group KIE together with a small α-secondary ^{2}H KIE (Sinnott & Souchard, 1973) on the same step is that the formation of the galactosyl enzyme proceeds through a S_N2 pathway for PNPGal and presumably for related substrates.

References

Ballardie, F., Capon, B., Sutherland, J. D. G., Cocker, D., & Sinnott, M. L. (1973) *J. Chem. Soc., Perkin Trans. 1*, 2418.

Banks, B. E. C., Meinwald, Y., Rhind-Tutt, A. J., Sheft, I., & Vernon, C. A. (1961) *J. Chem. Soc.*, 3240.

Bigeleisen, J., & Wolfsberg, M. (1958) *Adv. Chem. Phys. 15*, 261.

Bilkadi, Z., deLorimer, R., & Kirsch, J. F. (1975) *J. Am. Chem. Soc. 97*, 4317.

Brockhaus, M., Dettinger, H., Kurz, G., Lehmann, J., & Wallenfels, K. (1979) *Carbohydr. Res. 69*, 264.

Conchie, J., & Levy, G. A. (1963) *Methods Carbohydr. Chem. 2*, 335.

Craze, G. A., Kirby, A. J., & Osborne, R. (1978) *J. Chem. Soc., Perkin Trans. 2*, 357.

Dahlquist, F. W., Rand-Meir, T., & Raftery, M. A. (1969) *Biochemistry 8*, 4214.

Fowler, A. V., & Zabin, I. (1978) *J. Biol. Chem. 253*, 5521.

Gorenstein, D. G. (1972) *J. Am. Chem. Soc. 94*, 2523.

Hengstenberg, W., & Wallenfels, K. (1969) *Carbohydr. Res. 11*, 85.

Hogg, J. L. (1978) in *Transition States in Biochemical Processes* (Gandour, R. D., & Schowen, R. L., Eds.) pp 201–224, Plenum Press, New York.

Hogg, J. L., Rodgers, J., Kovach, I., & Schowen, R. L. (1980) *J. Am. Chem. Soc. 102*, 79.

Kirsch, J. F. (1977) in *Isotope Effects in Enzyme Catalyzed Reactions* (Cleland, W. W., O'Leary, M. H., & Northrop,

D. B., Eds.) pp 100-121, University Park Press.
Knier, B. L., & Jencks, W. P. (1980) *J. Am. Chem. Soc. 102*, 6789.
Leaback, D. H. (1960) *J. Chem. Soc.*, 3166.
O'Leary, M. H. (1978) in *Transition States in Biochemical Processes* (Gandour, R. D., & Schowen, R. L., Eds.) pp 285-316, Plenum Press, New York.
Palmer, J. L., & Jencks, W. P. (1980) *J. Am. Chem. Soc. 102*, 6472.
Rosenberg, S. (1978) Ph.D. Thesis, University of California, Berkeley, CA.
Rosenberg, S., & Kirsch, J. F. (1978) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 37*, Abstr. 151.
Rosenberg, S., & Kirsch, J. F. (1979a) *Anal. Chem. 51*, 1375.
Rosenberg, S., & Kirsch, J. F. (1979b) *Anal. Chem. 51*, 1379.
Rosenberg, S., & Kirsch, J. F. (1981) *Biochemistry* (following paper in this issue).
Saunders, W. H. (1975) *Chem. Scr. 8*, 27.
Sawyer, C. B., & Kirsch, J. F. (1973) *J. Am. Chem. Soc. 95*, 7375.
Sawyer, C. B., & Kirsch, J. F. (1975) *J. Am. Chem. Soc. 97*, 1963.
Sinnott, M. L. (1978) *FEBS Lett. 94*, 1.
Sinnott, M. L., & Souchard, I. J. L. (1973) *Biochem. J. 133*, 89.
Sinnott, M. L., & Viratelle, O. M. (1973) *Biochem. J. 133*, 81.
Sinnott, M. L., & Withers, S. G. (1974) *Biochem. J. 143*, 751.
Van Hook, W. A. (1970) *ACS Monogr. No. 167*, 1-84.
Wallenfels, K., & Weil, R. (1972) *Enzymes, 3rd Ed. 7*, 617-663.
Young, P. R., & Jencks, W. P. (1977) *J. Am. Chem. Soc. 99*, 8238.
Zabin, I., & Fowler, A. V. (1978) in *The Operon* (Miller, J. H., & Reznikoff, W. S., Eds.) pp 89-122, Cold Spring Harbor Monographs, Cold Spring Harbor, NY.

Oxygen-18 Leaving Group Kinetic Isotope Effects on the Hydrolysis of Nitrophenyl Glycosides. 2. Lysozyme and β-Glucosidase: Acid and Alkaline Hydrolysis†

Steven Rosenberg and Jack F. Kirsch*

ABSTRACT: Oxygen-18 leaving group kinetic isotope effects (KIEs) have been measured for a set of glycosyl transfer reactions with *p*-nitrophenyl β-D-glycosides as substrates. Acid-catalyzed hydrolysis and alkaline hydrolysis exhibit KIEs of k_{16}/k_{18} = 1.0355 ± 0.0015 and 1.0386 ± 0.0032, respectively. Lysozyme and β-glucosidase A show KIEs on V_{max}/K_m (V/K) of $(V/K)_{16}/(V/K)_{18}$ = 1.0467 ± 0.0015 and 1.0377 ± 0.0061, respectively. The large magnitude of these KIEs requires that carbon–oxygen bond scission be far advanced in the transition states for these reactions; therefore in the transition states for the first irreversible steps in these reaction sequences, scission of the glycosidic bond must be essentially complete for the reactions catalyzed by lysozyme and β-glucosidase A, which are thought to proceed via S_N1 and S_N2 mechanisms, respectively. Acid-catalyzed hydrolysis is shown to proceed through a transition state involving at least 80% C–O bond cleavage and only partial proton transfer to the leaving *p*-nitrophenyl oxygen atom.

The mechanisms of enzyme-catalyzed hydrolysis of glycosides and oligosaccharides are among the most studied and best understood in enzymology. Two limiting mechanisms of catalysis have been proposed for these reactions. The first, involving formation of an enzyme-bound oxocarbonium ion with the assistance of general acid catalysis, is typified by the reaction catalyzed by hen egg-white lysozyme (Blake et al., 1967). The second mechanism, that of double displacement first proposed by Koshland (1953), involves nucleophilic attack by the enzyme to form a covalent glycosyl-enzyme intermediate followed by its hydrolysis. More complex schemes involving a combination of these mechanisms, kinetically significant protein conformation changes, and possible intramolecular attack by the C-2 oxygen atom of the substrate have been proposed for β-galactosidase and other enzymes such as β-glucosidase (Sinnott & Souchard, 1973; Brockhaus et al., 1979; Hehre et al., 1977).

Secondary deuterium KIEs[1] have been utilized to study these reactions and to attempt to differentiate between the two mechanisms outlined above (Dahlquist et al., 1969; Sinnott & Souchard, 1973; Sinnott, 1978). However, recent work has led to some question being cast upon the validity of such measurements to differentiate between S_N1 and S_N2 mechanisms (Craze et al., 1978; Knier & Jencks, 1980). The previous paper describes the application of new techniques for the measurement of small kinetic isotope effects to the determination of oxygen-18 leaving group KIEs for reactions catalyzed by *Escherichia coli lacZ* β-galactosidase (Rosenberg & Kirsch, 1981). The measurement of the KIEs due to a second isotope near the reaction center is useful in resolving mechanistic ambiguities, as shown, for example, by Goitein et al. (1978) by using ^{14}C KIEs. This communication extends our studies on β-galactosidase to reactions catalyzed by lysozyme and β-glucosidase and to some relevant nonenzymatic reactions. The results obtained yield unique information on transition-state structure and the nature of the rate-determining step in these reactions.

† From the Department of Biochemistry, University of California, Berkeley, California 94720. *Received September 16, 1980.* This investigation was supported by National Science Foundation Grant PCM 74-17643A02 and U.S. Public Health Service Predoctoral Traineeship 5-T01 GM00031-20 to S.R.

[1] Abbreviations used: KIE, kinetic isotope effect; EIE, equilibrium isotope effect; GlcNAc, 2-acetamido-2-deoxy β-D-glucopyranoside; PNPGlu, *p*-nitrophenyl β-D-glucopyranoside; PNPGal, *p*-nitrophenyl β-D-galactopyranoside; GlcNAcGluPNP, *p*-nitrophenyl 4-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-β-D-glucopyranoside.

0006-2960/81/0420-3196$01.25/0