α-SECONDARY TRITIUM ISOTOPE EFFECTS IN THE HYDROLYSIS OF GLYCOPYRANOSIDES OF N-ACETYL-D-GLUCOSAMINE

Thesis by

Lawrence H. Mohr

In Partial Fulfillment of the Requirements

for the Degree of

Master of Science

California Institute of Technology

Pasadena, California

1974

(Submitted September 20, 1973)

ACKNOWLEDGMENTS

I would like to express my thanks to Professor Michael A. Raftery for his interest and support through the many ups and downs of my stay at Caltech.

I would also like to thank the National Institutes of Health and the California Institute of Technology for their financial aid.

ABSTRACT

Part I. Secondary tritium isotope effects were used to study the aqueous hydrolysis of a series of α- and β-glycopyranosides of N-acetyl-D-glucosamine. The magnitude of the secondary tritium isotope effects, and their dependence on the structure of the aglycone, are compatible with the carbonium ion mechanism suggested for the specific acid catalysis of these compounds by Piszkiewicz and Bruice (1, 2, 3). The secondary tritium isotope effect determined for the spontaneous hydrolysis of the p-nitrophenyl-2-acetamido-2-deoxy-β-D-glucopyranoside is not consistent with an intramolecular, nucleophilic displacement mechanism. A mechanism involving the equilibrium formation of a carbonium ion-anion pair is proposed. The relevance of these model studies to hydrolysis of oligosaccharides of N-acetyl-D-glucosamine by lysozyme is discussed.

Part II. Secondary tritium isotope effects are also used to study the enzymatic hydrolysis of two β-aryl glycopyranosides of N-acetyl-D-glucosamine by β-N-acetyl-D-glucosaminidase isolated from jack bean meal. The results obtained indicate a transition state for reaction processing less carbonium ion character than the corresponding hydrolysis catalyzed by aqueous acid or lysozyme. A mechanism involving anchimeric assistance by the acetamido group on the substrate is proposed.

TABLE OF CONTENTS

PART I

INTRODUCTION

Lysozyme hydrolyses chitin, a β-1,4 linked polymer of N-acetyl-D-glucosamine (GlcNAc)* (4). As a chemical model for this process, we have studied the aqueous hydrolysis of a number of β-aryl and β- and α-alkyl glycopyranosides of GlcNAc. We have used α-secondary tritium isotope effects to study the mechanism of these hydrolyses. These effects occur when the rate of a reaction, involving cleavage of a bond to a carbon atom, is affected by the substitution of a tritium atom for a proton attached to the carbon. Most previous work on secondary isotope effects has involved substitution of deuterium for hydrogen (see Refs. 5 and 6 for reviews). Secondary deuterium isotope effects are related to secondary tritium isotope effects by the following expression (7).

$$\log {}^{k_H}/_{k_T} = 1.44 \log {}^{k_H}/_{k_D}$$

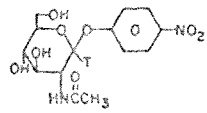
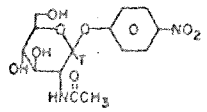
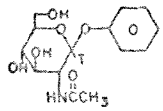
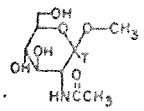
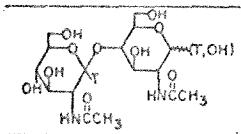
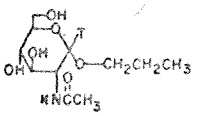
EXPERIMENTAL PROCEDURE

The glycopyranosides synthesized for this study are shown in Table I. The tritiated compounds were made from GlcNAc-1-T (Amersham Searle, specific activity 4.4 Ci/m mole). The ^{14}C-labeled

---

*Abbreviations used are: N-acetyl-D-glucosamine, GlcNAc; p-nitrophenyl 2-acetamido-2-deoxy-β-D-glycopyranoside, p-nitrophenyl β-GlcNAc, phenyl 2-acetamido-2-deoxy-β-D-glucopyranoside, phenyl β-GlcNAc; methyl 2-acetamido-2-deoxy-β-D-glucopyranoside, methyl β-GlcNAc; propyl 2-acetamido-2-deoxy-α-D-glycopyranoside, propyl α-GlcNAc, chitobiose, β-1, 4-$(GlcNAc)_2$.

TABLE I: α-Secondary tritium isotope effects* for α- and β-glycopyranosides of glc-NAc.

Compound	pH	$k_H/k_T \pm$ S.D.	k_H/k_D (Calc.)
 p-nitrophenyl β-glc-NAc	7.1	1.103 ± .016	1.07
 p-nitrophenyl β-glc-NAc	.12	1.038 ± .014	1.03
 phenyl β-glc-NAc	.30	1.070 ± .006	1.05
 methyl β-glc-NAc	.30	1.097 ± .010	1.07
 β-1,4-(glc-NAc)$_2$	.30	1.122 ± .011	1.08
 propyl α-glc-NAc	.30	1.136 ± .013	1.09

*Each isotope effect consisted of at least three separate experiments. S. D. equals one standard deviation. $^{k_H}/_{k_D}$ (calc.) was calculated from the expression $1.44\ (^{k_H}/_{k_D}) = (^{k_H}/_{k_T})$.

compounds were made from GlcNAc-^{14}C, labeled in the acetamido group. The latter compound was synthesized from D-glucosamine and acetic anhydride-1-^{14}C (New England Nuclear, specific activity 10 mCi/m mole) by the method of Horton (8). Methyl β-GlcNAc and propyl α-GlcNAc were prepared and purified by the method of Neuberger and Wilson (9). P-nitrophenyl β-GlcNAc and phenyl β-GlcNAc were prepared by the method of Leaback and Walker (10). β-1, 4-$(GlcNAc)_2$ was prepared by the method of Smith et al. (11). Each glycoside gave a constant $^{3}H/^{14}C$ ratio over the entire peak upon chromatography by the methods discussed below.

Secondary tritium isotope effects were determined as follows. A combined sample of ^{14}C and ^{3}H labeled glycopyranosides containing 1-3 mg (3×10^{-3} - 10^{-2} mmoles) in 1 ml of solution was hydrolysed at 50°C until 2-5% of the glycopyranoside had reacted as judged by the appearance of the GlcNAc peak upon chromatography. The reaction mixture was then neutralized and eluted from a Dower 50 (H^{+}) column (0.2 × 2 cm) with 3 ml of distilled water to remove amines. The eluate was taken to dryness three times (with 2 additions of 10 ml portions of 80% aqueous HOAc) on a rotary evaporator at 40°C under vacuum in order to remove labeled acetic acid. GlcNAc was then separated from the remaining glycopyranosides chromatographically and the ^{3}H to ^{14}C ratio of the two materials compared.

P-nitrophenyl β-GlcNAc was separated from GlcNAc on a Sephadex LH-20 column (0.9 × 50 cm) in 50% aqueous MeOH. Twenty fractions of 3.1 ml were taken. Phenyl β-GlcNAc was separated from

GlcNAc on Dowex 50 (K^+) (1.5 × 25 cm) with water as eluent. Seven ml fractions were collected. Methyl β-GlcNAc and propyl α-GlcNAc were separated from GlcNAc on Watman 3 MM paper in butanol/ethanol/water (4:1:5). The sugars were applied in a strip to a 13 × 50 cm sheet of paper and run (descending chromatography) for 6 hours after the eluent reached the bottom of the paper. The sugars were located by cutting a strip from the edge of the paper into 2 cm sections, eluting with water and counting radioactivity. β-1, 4-$(GlcNAc)_2$ was separated from GlcNAc on Bio-Gel P-2 in water. The columns (0.9 × 50 cm) was eluted in 1 ml fractions.

Scintillation counting was performed in a Packard Model 3375 Tri-Carb Liquid Scintillation Spectrometer. Efficiencies of quenched samples were determined by the external standard method. The scintillation solution contained 19.2 grams Permablend III (Packard) and 420 grams Naphthalene in 8 liters of 1, 4-Dioxane.

RESULTS

Each of the five pairs of glycopyranosides was hydrolysed in aqueous hydrochloric acid at 50°C. Under these conditions, amide hydrolysis is competitive with glycoside hydrolysis (12). The reaction sequence is shown in Figure 1. Removal of amines by Dowex 50 (H^+) chromatography, and acetic acid by rotary evaporation, left GlcNAc and the starting material as the only labeled compounds in the reaction mixture. These were separated chromatographically, and the tritium to ^{14}C ratio determined for each by scintillation counting. The secondary isotope

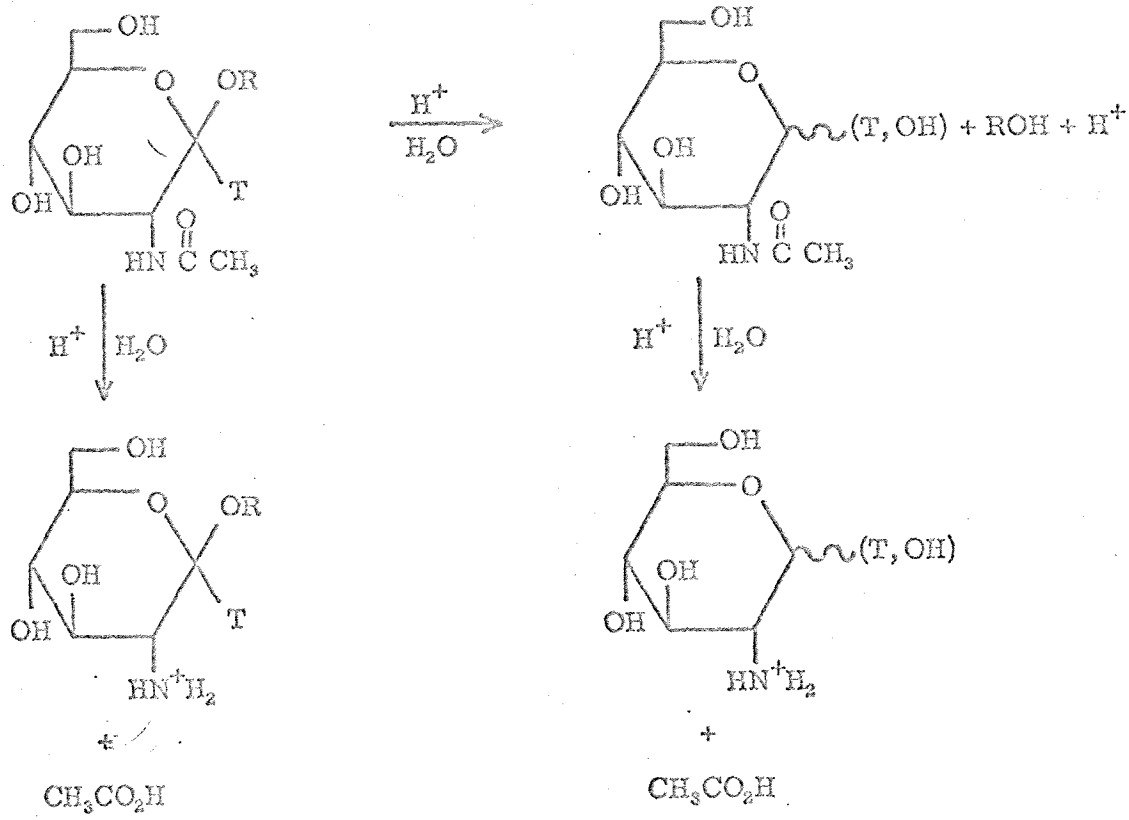


Figure 1: The reaction sequence for the hydrolysis of glycopyranosides of GlcNAc in aqueous hydrochloric acid.

effect, ($^{k}H/^{k}T$), was determined by dividing the tritium to ^{14}C ratio of the starting material by the tritium to ^{14}C ratio of the GlcNAc produced. The secondary tritium isotope effects determined are shown in Table I.

The hydrolysis of p-nitrophenyl β-GlcNAc in 0.1 M sodium phosphate, pH 7.1, was also studied. After hydrolysis, the reaction mixture was chromatographed on Sephadex LH-20 in 50% aqueous methanol. Two peaks of radioactivity were obtained, corresponding to GlcNAc and p-nitrophenyl β-GlcNAc. The GlcNAc peak contained another component, seen as a shoulder on the leading edge of the peak. When this material was isolated and incubated at pH 2 and 50°C for one hour, and rechromatographed, it was eluted at exactly the same position as GlcNAc. It was assumed that this contaminant material was, therefore, an oxazoline intermediate, produced by the displacement of p-nitrophenolate by the acetamido group of GlcNAc (see Discussion). The reaction sequence is shown in Figure 2. Simple oxazoline systems have been shown to hydrolyze rapidly in aqueous solution between pH 0 and 6 (13). For the purpose of determining the isotope effect for the first step of the sequence shown in Figure 2, the GlcNAc peak plus shoulder were combined, and the isotopic ratio determined. The secondary tritium isotope effect for the spontaneous hydrolysis of p-nitrophenyl β-GlcNAc is shown in Table I.

DISCUSSION

The α-secondary tritium isotope effects are primarily due to the net change, between initial and transition states, of the zero point

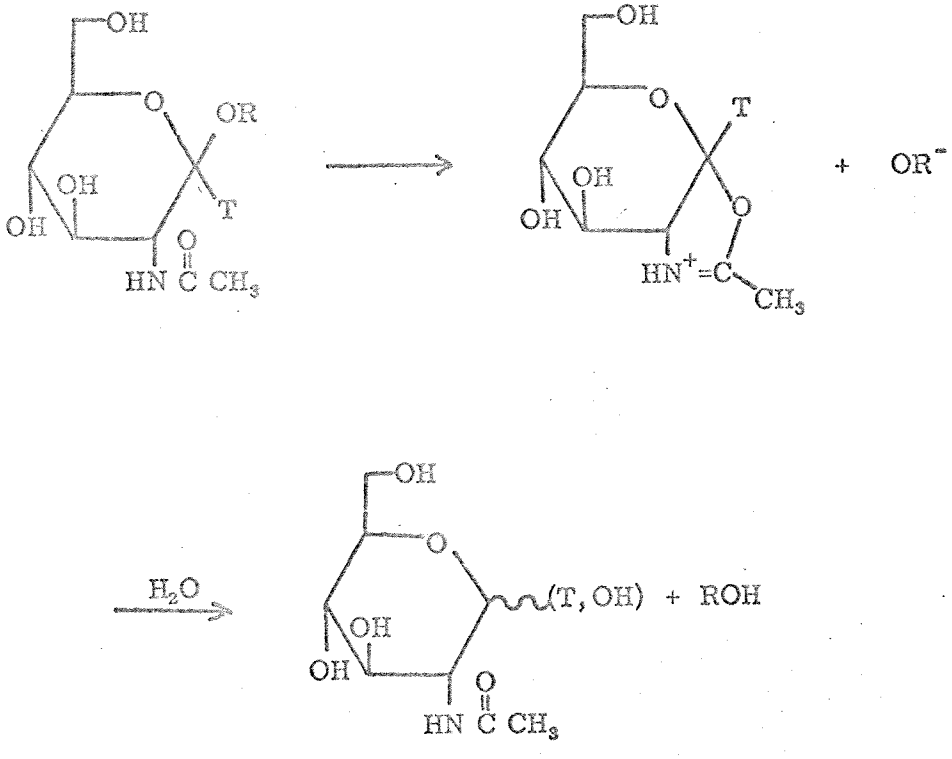


Figure 2: The reaction sequence for the hydrolysis of p-nitrophenyl β-GlcNAc in 0.1 M sodium phosphate, pH 7.1.

bending vibrational frequencies of carbon-tritium and carbon-hydrogen bonds (7). Any reaction which involves a lowering of the α-carbon-tritium zero point bending vibrational energy upon going from the starting material to the transition state, should give a secondary isotope effect of greater than 1 ($k_H/k_T > 1$). Reactions involving rate determining formation of a carbonium ion from a tetrahedrally bound carbon atom fit in this category (14). The lowering of a zero point energy of the carbon-tritium bending vibration is due both to the partial sp^2 hybridization of the carbon atom, and to the increased distance between the carbon atom and the leaving group in the transition state. A transition state that closely resembles the original tetrahedral compound in structure will, of course, have a secondary isotope effect relatively close to 1. In general, reactions which by various criteria are judged to involve rate determining carbonium ion formation, have been found to display α-secondary deuterium isotope effects between 1 - 1.25 (15, 16, 17, 18).

In reactions involving rate determining nucleophilic displacement, the close proximity of the entering and leaving groups in the transition state tends to prevent loss of zero point energy in the α-carbon-tritium bending vibrations (6). In a very tightly packed transition state, the carbon-tritium zero point vibrational energy may even be higher than in the ground state, leading to α-secondary deuterium isotope effects of less than 1 (19). Many reactions, considered to involve nucleophilic displacements, have been found to have α-secondary deuterium isotope effects of between 0.95 and 1.05 (see Refs. 20 and 21).

The acid catalyzed hydrolysis of α- and β-glucopyranosides has been intensively studied, using various techniques (see Ref. 22 for review). It has been shown that primary alkyl and aryl glucopyranosides react exclusively with glucosyl carbon-oxygen bond breakage. Evidence also points to specific acid catalysis, involving reversible protonation of the glucopyranoside, followed by rate limiting, unimolecular, decomposition to a carbonium ion. In the hydrolysis of methyl α-D-glucopyranoside, it has been shown that the glucosyl carbon-methyl oxygen bond is the bond cleaved in the rate determining step (23). It is expected that this same bond is cleaved in the acid catalyzed hydrolysis of other glycopyranosides.

The mechanism of the aqueous hydrolysis of glycopyranosides of GlcNAc has been studied by Piszkiewicz and Bruice (1, 2, 3), employing kinetic methods. All of the alkyl and aryl glycopyranosides studied underwent specific acid catalysis. If the log of the rate constants for specific acid catalysis of a series of β-glucopyranosides of GlcNAc were plotted against the log of the rate constants of the corresponding series of β-glucopyranosides, a straight line was obtained, with only data for methyl β-glycopyranosides not falling on the line. From this and other evidence, Piszkiewicz and Bruice argued that the β-glycopyranosides of GlcNAc, with the exception of the methyl β-glycopyranoside, were hydrolyzed by a mechanism identical to that of the β-glucopyranosides. Furthermore, they suggested that the specific acid hydrolysis of methyl β-GlcNAc involved attack of the acetamido group on the protonated glycopyranoside.

The secondary tritium isotope effects obtained in this study for acid hydrolysis range from about 1.04 for p-nitrophenyl β-GlcNAc to 1.14 for propyl α-GlcNAc. The magnitude, and the change of the isotope effect with structure of the leaving group, are consistent with a mechanism involving rate determining formation of a carbonium ion from the protonated glycopyranoside. As the structure of the leaving group changes upon going from alkyl to phenyl to p-nitrophenyl GlcNAc, the ease of rupture of the carbon-oxygen bond increases. This is paralleled by a decrease in the carbonium ion character of the transition state, and hence, a decrease in the secondary isotope effect. The mechanism is shown in Figure 3.

Bull et al. (24) studied the secondary deuterium isotope effects for the acid catalyzed hydrolysis of 2-phenoxytetrahydrofurans. These compounds are known to hydrolyse by a carbonium ion mechanism. The secondary isotope effects were found to decrease with increasing electron withdrawal due to the substituents in the phenyl group. Thus these two studies complement each other.

The isotope effect of approximately 1.10 found for methyl β-GlcNAc is significantly smaller than the isotope effect of 1.14 found for propyl α-GlcNAc. This is in keeping with Piszkiewicz and Bruice's suggestion that the specific acid hydrolysis of methyl β-GlcNAc involves some acetamido group participation. This would also result if the carbonium ion character is more highly developed in the α-glycopyranosides of GlcNAc, than in the β-series. This latter supposition has been shown likely for the α- and β-glucopyranosides (22).

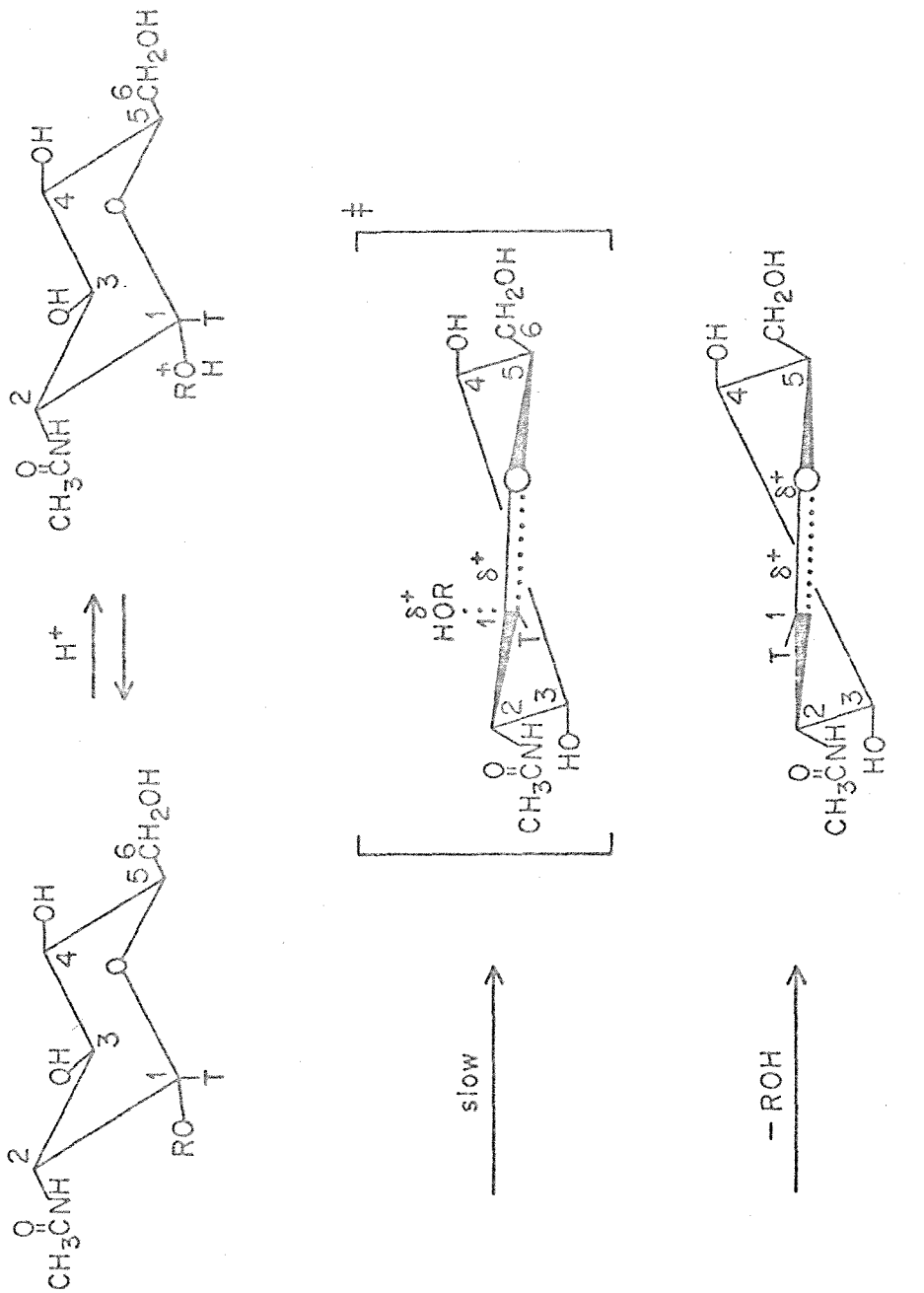


Figure 3: The mechanism suggested for the specific acid catalyzed hydrolysis of glycopyranosides of GlcNAc.

Piszkiewicz and Bruice also found that p-nitrophenyl β-GlcNAc was hydrolyzed at a rate independent of pH, between pH 1.5 and 10.5. They suggested that the mechanism for this spontaneous hydrolysis involved nucleophilic, anchimeric assistance by the acetamido group, with formation of an intermediate oxazoline. The hydrolysis of p-nitrophenyl α-GlcNAc, where assistance by the acetamido group is impossible, showed no pH independent hydrolysis.

The secondary tritium isotope effect of 1.10, determined here for the spontaneous hydrolysis of p-nitrophenyl β-GlcNAc, is much higher than would be expected for an intramolecular, nucleophilic, displacement mechanism. An isotope effect of this size indicates considerable loss of zero point energy in the carbon-tritium vibration in the transition state. A mechanism consistent with this isotope effect involves equilibrium formation of a stereospecific ion pair, followed by expulsion of p-nitrophenolate by the neighboring acetamido group. Thus, the carbon-oxygen bond is broken prior to the transition state for acetamido attack. The scheme for this proposed mechanism is shown in Figure 4.

Support for this proposal of unassisted cleavage of the carbon-oxygen bond comes from the study by Fife and Jao (25) of the hydrolysis of 2-(p-nitrophenoxy)-tetrahydropyran. They found a pH independent hydrolysis of this compound at 30°C, in 50% aqueous dioxane. The rate constant found for spontaneous hydrolysis was of the same magnitude as the rate constant found by Piszkiewicz and Bruice for the spontaneous hydrolysis of p-nitrophenyl β-GlcNAc at 78°C in aqueous solution.

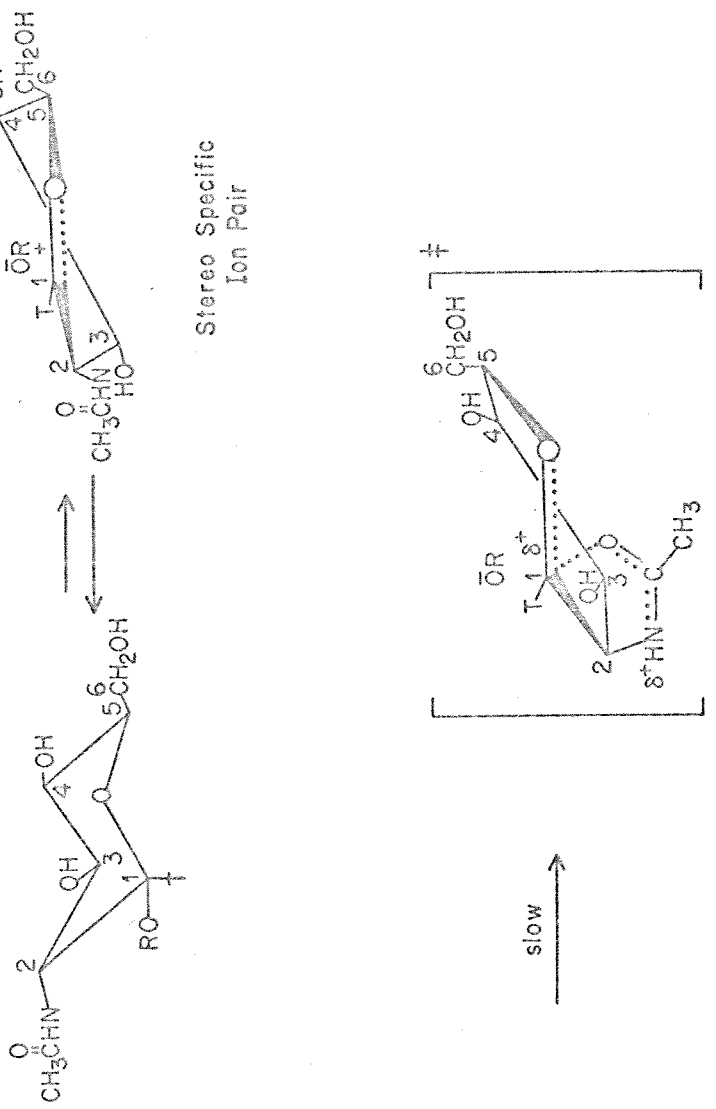


Figure 4: The proposed mechanism for the pH independent hydrolysis of p-nitrophenyl β-GlcNAc.

In summary it is clear that under all conditions investigated for hydrolysis of glycosides of GlcNAc, the transition states involve carbonium ion formation to a greater or lesser extent. With poor leaving groups, such as aliphatic or glycosyl residues, the carbonium ion character of the transition is largest. These results then establish a chemical basis for investigation, by similar kinetic isotope effect approaches, of lysozyme catalyzed hydrolysis of substrates with poor leaving groups, such as oligosaccharides (11).

REFERENCES

1. Piszkiewicz, D., and Bruice, T. C. (1967), J. Amer. Chem. Soc. 89, 6237.
2. Piszkiewicz, D., and Bruice, T. C. (1968), J. Amer. Chem. Soc. 90, 2156.
3. Piszkiewicz, D., and Bruice, T. C. (1968), J. Amer. Chem. Soc. 90, 5844.
4. Berger, L. R., and Weiser, R. S. (1957), Biochim. Biophys. Acta 26, 517.
5. Havelie, E. A. (1963), Prog. Phys. Org. Chem. 1, 109.
6. Saunders, W. H., Jr. (1966), Survey of Progress in Chemistry 3, 109.
7. Jencks, W. P. (1969), "Catalysis in Chemistry and Enzymology", McGraw-Hill, Inc., New York, N.Y., p. 248 and p. 254.
8. Horton, D. (1966), Biochem, Prep. 11, 1.
9. Neuberger, A., and Wilson, B. M. (1971), Carbohyd. Res. 17, 89.
10. Leaback, D. H., and Walker, P. G. (1957), J. Chem. Soc., 4754.
11. Smith, L. E. H., Mohr, L. H., and Raftery, M. A. (1973), J. Amer. Chem. Soc., in press.
12. Foster, A. B., Horton, D., and Stacey, M. (1957), J. Chem. Soc., 81.
13. Martin, R. B., and Parcell, A. (1961), J. Amer. Chem. Soc. 83, 4835.
14. Streitwieser, A., Jr., Jagow, R. H., Fahey, R. C., and Suzuki, S. (1958), J. Amer. Chem. Soc. 80, 2326.

15. Lewis, E. S., Johnson, R. R., and Coppinger, G. M. (1959), J. Amer. Chem. Soc. 81, 3140.

16. Seltzer, S. (1961), J. Amer. Chem. Soc. 83, 2625.

17. Shiner, V. J., Jr., Buddenbaum, W. E., Murr, B. L., and Lamity, G. (1968), J. Amer. Chem. Soc. 90, 418.

18. Bull, H. G., Pletcher, T. C., and Cordes, E. H. (1970), Chem. Commun., 527.

19. Johnson, R. R., and Lewis, E. S. (1958), Proc. Chem. Soc. 52.

20. Llewellyn, J. A., Robertson, R. E., and Scott, J. M. (1960), Can. J. Chem. 38, 222.

21. Shiner, V. J., Jr., Rapp, M. W., and Pinnick, H. R., Jr. (1970), J. Amer. Chem. Soc. 92, 232.

22. Capon, B. (1969), Chem. Reviews 69, 407.

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

24. Bull, H. G., Kockler, K., Pletcher, T. C., Ortiz, J. J., and Cordes, E. H. (1971), J. Amer. Chem. Soc. 93, 3002.

25. Fife, T. H., and Jao, L. K. (1968), J. Amer. Chem. Soc. 90, 4081.

PART II

INTRODUCTION

β-N-acetyl-D-glucosaminidase is a glycosidase that hydrolyzes glycosides of β-D-GlcNAc. This enzyme has been found in numerous mammalian tissues (1, 2). It has also been found in plant tissue (3). Evidence points to its metabolic role being the degradation of hyaluronic acid, chondroitin, and mucopolysaccharides, and the cleavage of certain mucoproteins (4, 5).

Li and Li (3) have obtained the first pure β-N-acetyl-D-glucosaminidase from jack bean meal. The enzyme hydrolyses substrates with retention of configuration (6). It is also strongly inhibited by 2-acetamido-2-deoxy-D-glucono-(1→5)-lactone. In these two respects it is notably similar to lysozyme, and it has been speculated that the mechanism of catalysis of the two enzymes are similar (7). The determination of α-tritium secondary isotope effects provides a sensitive method for comparing the transition states for the two enzymatic reactions.

RESULTS

β-N-acetyl-D-glucosaminidase was isolated from jack bean meal (3), and the specific activity of the enzyme preparation used in the isotope effect experiments was identical to the specific activity of the crystalline enzyme obtained by Li and Li. Secondary tritium isotope effects for the enzymatic hydrolysis of β-D-nitrophenyl and

β-phenyl-D-GlcNAc were determined at pH 5.0. At this pH no significant side reactions take place and the apparent isotope effect can be plotted as a function of the percent hydrolysis. This should follow the expression shown below (8):

$$\text{Apparent Isotope Effect} = \frac{{}^{3}H/{}^{14}C \text{ Original Reactant}}{{}^{3}H/{}^{14}C \text{ Product}} = \frac{1}{X}\left[1 - (1 - X)^{k_H/k_T}\right]$$

where k_H/k_T = actual isotope effect

X = fraction reactant hydrolysed.

The α-secondary tritium isotope effects can be determined by extrapolating the apparent isotope effect to zero percent hydrolysis. The apparent isotope effect versus percent hydrolysis plots are shown in Figure 1 for the β-aryl glycosides. The determined α-secondary tritium isotope effects are shown in Table I.

DISCUSSION

It can be seen that β-N-acetyl-D-glucosaminidase catalyses the hydrolysis of the model compounds with an α-secondary tritium isotope effect greater than one, but smaller than half the size of the corresponding aqueous acid hydrolysis. The size of the effect and the change of the effect with the structure of the aglycone indicate some carbonium ion character to the transition state for enzymatic hydrolysis. But the extent of carbonium ion formation is small compared to the aqueous acid hydrolysis or hydrolysis by lysozyme, where an α-secondary tritium

TABLE I: Secondary isotope effects during catalysis by aqueous acid and N-acetyl-D-glucosiminidase.

Compound	Aqueous Acid Hydrolysis $K_H/K_T \pm$ S.D.	β-N-Acetyl-D-Glucosaminidase Hydrolysis K_H/K_T
p-nitrophenyl β-glcNAc	1.038 ± .014	1.003
phenyl β-glcNAc	1.070 ± .008	1.034

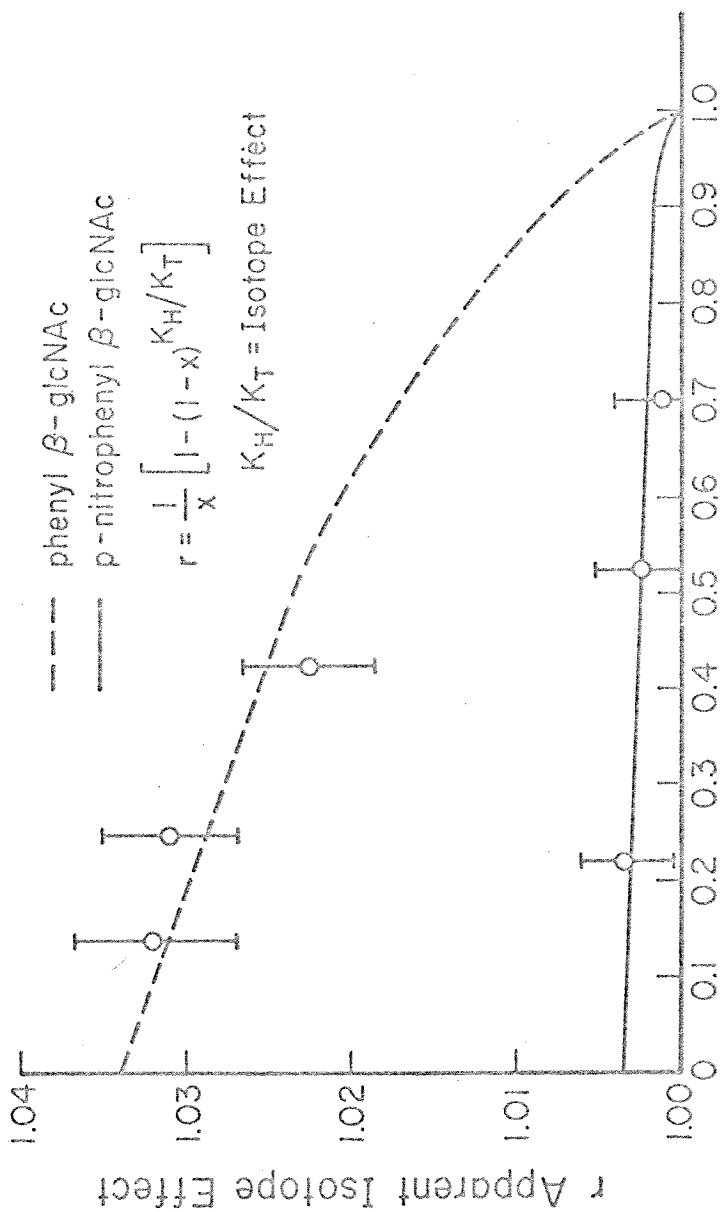


Figure 1: Plot of apparent isotope effect versus fraction hydrolysed.

isotope effect of 19% has been determined for oligomeric GlcNAc substrates (9).

One explanation for the small isotope effects involves a carbonium ion mechanism with a reactant like or product like transition state. Such a transition state, only partially distorted from tetrahedral geometry, would be of lower energy than the half chain transition state suggested for lysozyme (9). It has been estimated that about 10 kilocalories are required to distort a pyranose ring to its half chair conformation (10). This is consistent with the fact that for lysozyme hydrolysis of oligomeric GlcNAc substrates, binding of sugar residues neighboring the residue hydrolysed help force the latter residue into a hign energy conformation. On the other hand, β-N-acetyl-D-glucosaminidase has a binding site for only one sugar residue. It is doubtful that the half chain form of this sugar could form enough non-covalent interactions with the enzyme to stabilize such a transition state.

Arguing against a carbonium ion mechanism with a reactant like or product like transition state is the fact that 2-acetamido-2-deoxy-D-glucono-(1→5)-lactone inhibits the enzyme 10^4 times more effectively than β-D-GlcNAc. This lactone is in a half chair conformation (7), and it is expected that this compound is a reasonably good structural analogue of the transition state (11). Hence, the transition state is not reactant like or product like. This argument, plus the argument discussed above concerning the high energy nature of the half chair conformation, point to some covalent catalysis being involved in the formation of the transition state.

A second possibility for explaining the small magnitude of the α-secondary tritium isotope effects is that one step in the reaction sequence involves a carbonium ion like transition state, but that this step is only partially rate determining. This seems unlikely when one considers the high energy nature of such a transition state. The only other obvious candidates for a partially rate determining step involve binding of reactant or release of product from the enzyme, and these steps should be quite fast. It is always possible, however, that a conformation change of the enzyme is partially rate determining.

A third possibility is that the attack of a nucleophile is important in the transition state for the enzymatic reaction. The nucleophile could be a function group on the enzyme or the acetamido group present on the substrate.

The maximum velocity versus pH plot for β-N-acetyl-D-glucosaminidase indicates a constant maximum velocity between the lowest pH studied, pH 3, and pH 6, after which the maximum velocity decreases (7). This makes it unlikely that a group on the enzyme that ionizes between pH 3 and pH 6 could be the nucleophile involved. This eliminates a carboxylic acid unless it ionizes at an abnormally low pH.

There is precedent for the acetamido group on the substract acting as an intramolecular nucleophile. Bruice and Piszkiewicz have shown that the spontaneous hydrolysis of β-D-nitrophenyl-D-GlcNAc involves nucleophilic participation of the acetamido group, and have speculated that this group may act in a similar way in the specific acid catalyzed hydrolysis of β-methyl GlcNAc (12, 13).

In the most stable $^4C_1$ chair conformation of β-glycosides of D-GlcNAc, the acetamido group and the aglycone occupy neighboring equatorial positions (14). In order for anchimeric assistance to take place the acetamido group must be able to displace the aglycone from the back side of carbon 1. This necessitates the two groups having a trans axial relationship to one another. Formation of the $^{1,4}B$ boat conformation would make anchimaeric assistance possible and the formation of this boat conformation would involve a $^4H_3$ half chair transition state (14). These relationships are shown in Figure 2. Thus intramolecular catalysis of the acetamido group may be concerted with enzymatic catalysis stabilizing a half chair conformation of the sugar residue.

The reduction of the maximum velocity between pH 6 and pH 7 indicates the participation of a protonated group on the enzyme in catalysis. The most likely explanation involves general acid catalysis by a function group on the enzyme. This would be similar to the mechanism proposed for lysozyme catalysis. This is very difficult to prove, however.

The ρ value, determined by a Hammett plot, for the hydrolysis of a group of β-aryl glycosides of D-GlcNAc by β-N-acetyl-D-glucosaminidase, is +0.3 (7). The acid and base catalyzed hydrolyses of β-aryl glycosides of D-glucose show ρ values of -0.66 and +2.48, respectively (15). The acid catalyzed reaction involves rate determining carbonium ion formation following specific acid catalysis (16). Base catalysis takes place by nucleophilic attack of the ionized 2-hydroxyl group (17). The intermediate ρ value determined for the enzymatic

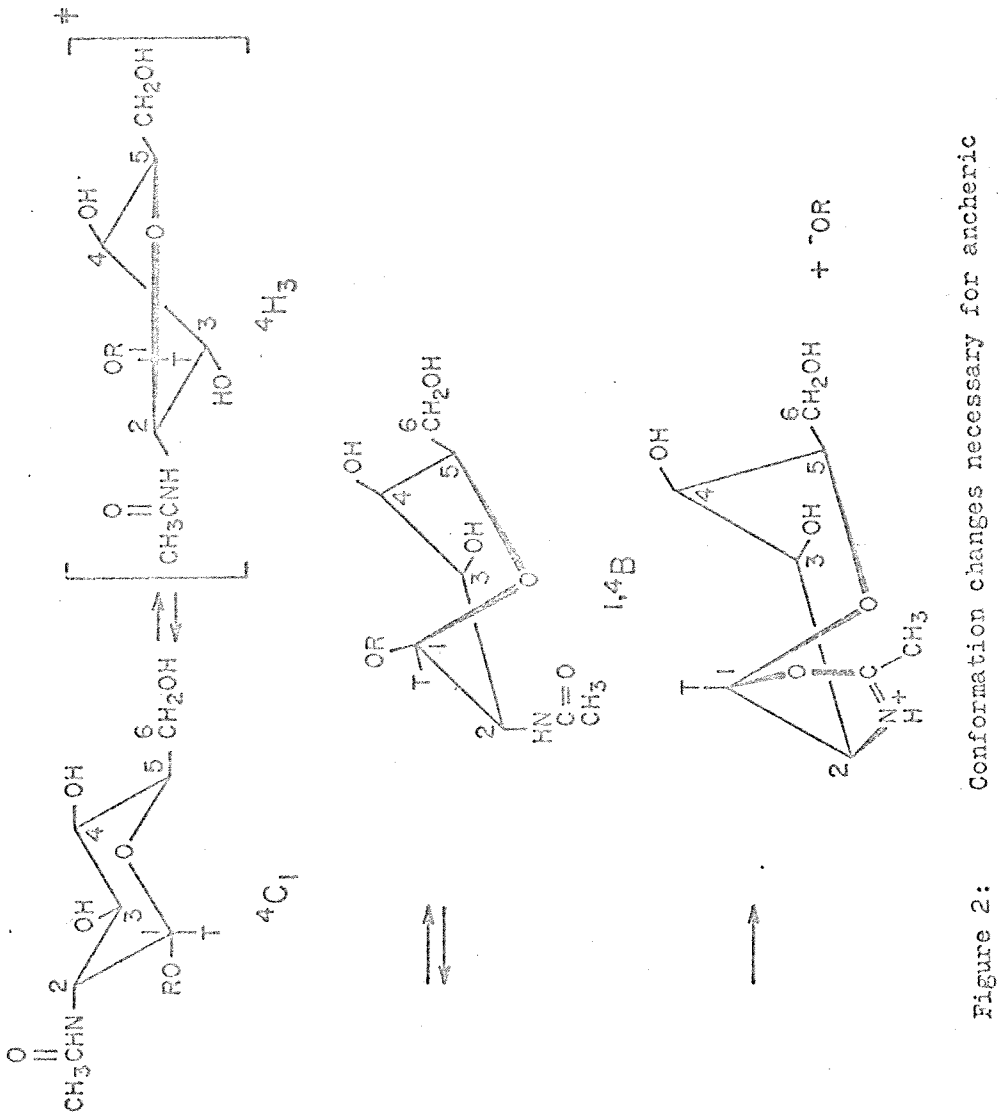


Figure 2: Conformation changes necessary for ancheric assistance to occur.

catalysis is compatible with concerted general acid-nucleophilic catalysis (16).

EXPERIMENTAL PROCEDURE

Combined samples of ^{3}H and ^{14}C labeled β-glycosides of D-GlcNAc containing approximately 3 mg (10^{-2} mmoles) were hydrolyzed at 25 °C in 2.0 ml of solution 0.1 M in sodium citrate, pH 5.0, and containing 0.02 units of β-N-acetyl-D-glucosaminidase. A 50 μl sample was taken to determine the $^{3}H/^{14}C$ ratio of the original reactant and the total amount of radioactive material at the start of the experiment. Thereafter 200 μl samples were taken sequentially and the glycosides were separated from hydrolysis products using the same chromatographic methods used for the corresponding aqueous acid hydrolysis, following destruction of enzymatic activity.

For the hydrolysis of β-phenyl-D-GlcNAc, the enzyme was destroyed by heating the 200 μl samples in a 70 °C water bath for forty minutes and then filtering the reaction mixture through cotton before chromatography (3).

For the hydrolysis of β-D-nitrophenyl-D-GlcNAc, the enzyme was removed by filtration through a PM-10 ultrafilter (Amicon). The ultrafilter was washed with 2 ml of water and the total filtrate chromatographed.

The enzyme was prepared by the method of Li and Li (3), except that crystallization was not attempted due to the small amount of enzyme obtained (200 μg). The specific activity was equal to that of the crystalline material obtained by Li and Li, using their assay method with β-p-nitrophenyl-D-GlcNAc as substrate. Protein was determined by the Lowry method (17).

REFERENCES

1. Conchie, J., Findlay, J., and Levy, G. A. (1959), Biochem. J. 71, 318.
2. Conchie, J., Findlay, J., and Levy, G. A. (1959), Nature 183, 615.
3. Li, S., and Li, Y. (1970), J. Biol. Chem. 245, 5153.
4. Conchie, J., Findlay, J., and Levy, G. A. (1956), Nature 178, 1469.
5. Linker, A., Meyer, K., and Weissman, B. (1955), J. Biol. Chem. 213, 237.
6. Leaback, D. H., and Walker, D. G. (1967), Biochem. J. 104, 70P.
7. Leaback, D. H. (1968), Biochem. Biophys. Res. Commun. 32, 1025.
8. Melander, L. (1960), "Isotope Effects on Reaction Rates", The Ronald Press, New York, N.Y., p. 53.
9. Smith, L. E. H., Mohr, L. H., and Raftery, M. A. (1973), J. Amer. Chem. Soc., in press.
10. Chipman, D. M., and Sharon, N. (1969), Science 165, 454.
11. Lienhard, G. E. (1973), Science 180, 149.
12. Piszkiewicz, D., and Bruice, T. C. (1967), J. Amer. Chem. Soc. 89, 6237.
13. Piszkiewicz, D., and Bruice, T. C. (1968), J. Amer. Chem. Soc. 90, 5844.
14. Stoddart, J. F. (1971), "Stereochemistry of Carbohydrates", Wiley-Interscience, New York, N.Y., p. 58.
15. Nath, R. L., and Rydon, H. N. (1954), Biochem. J. 57, 1.

16. Capon B. (1969), Chem. Reviews 69, 407.

17. Lowry, O. H., Rosebrough, N. J., Farr, A., and Randall, R. J. (1951), J. Biol. Chem. 193, 265.