# INVESTIGATION OF THE CATALYTIC MECHANISM OF LYSOZYME USING SUBSTRATE ANALOGS

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## Abstract

The catalytic mechanism of hen egg-white lysozyme is investigated by comparing the rate of hydrolysis of the trifluoro-N-acetyl analog of chitohexose to that of chitohexose. Since the binding properties of the two hexasaccharides are shown to be similar, differences observed in the rates of hydrolysis of the two oligosaccharides are discussed in terms of recent model studies for and mechanistic studies of lysozyme hydrolysis.

The role of distortion in the catalytic mechanism is investigated by comparing the kinetic parameters of the lysozyme-catalyzed hydrolysis of a xylose substrate analog with that of a glucose substrate analog.

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# Preface

I thank the many people at Caltech from whom I have drawn support these past years--the fellow graduate students, postdoctoral fellows, faculty, administration , employees, and undergraduates, most particularly those of Fleming House. I also thank the many friends and relatives outside the Caltech community for relationships necessary for my understanding of myself. I would especially like to thank Michael Raftery, my thesis advisor, for research ideas, for his patience and for his encouragement during my stay in the Chemistry Division. For typing this manuscript,as well as allowing me to maintain contact with science-society issues by typing numerous letters over the years, I thank Edi Bierce.

I gratefully acknowledge to the people of the United States financial support from the National Institutes of Health. I hope my services will be worth the investment.

Abbreviations

Glc, glucose; NAG, N-acetyl-D-glucosamine; NAM, N-acetyl-D-muramic acid; TFAG, N-trifluoroacetyl-D-glucosamine; Xyl, xylose; Asp, aspartic acid; Trp, tryptophan; Glu, glutamic acid; His, histidine; cpm, counts per minute; kcal/mole, kilocalories per mole; $\phi$, phenyl; O.D., optical density.

## INTRODUCTION

Lysozyme (E.C. 3.2.1.17) is an enzyme which catalyzes the hydrolysis of the bacterial cell wall polysaccharide. Since Gram positive bacteria have no additional membranes to protect their cell wall (1), they are especially susceptible to lysis. The standard assay is to measure the rate at which a cloudy suspension of dead Micrococcus lysodeikticus clears in the presence of the enzyme. Lysozyme is a basic, low molecular weight protein with the name N-acetylmuramide glycanohydrolase (2). It has been isolated from a variety of sources (3).

Unusual blood serum concentrations of lysozymes in humans have been associated with leukemia (4), chronic myeloleukosis (5) and burn patients (6). Gastric lysozyme excretion may be related to stomach diseases (7). Advantage has been taken of the bacteriocide action of lysozyme to treat cariogenic and noncariogenic streptococci (8). Lysozyme is considered by some to be a part of the immunological system of organisms. Since a large number of bacteria are relatively unaffected by lysozyme, an alternative role has been suggested closer to that of a scavenger, digesting cell wall remnants (2).

## Protein Structure

Hen egg-white lysozyme, hereafter abbreviated to lysozyme, comprises about 3% of the protein weight of hen egg white (2). It is easily purified from this source and thus has been extensively studied. The enzyme consists of one polypeptide chain of 129 amino acid residues whose sequence is known (10). There are no free thiol groups and four disulfide bridges. Basic functional groups outnumber acidic groups 18 to 10 (11), and the pI for the enzyme is 11 (12).

The X-ray structure of the enzyme crystallized from 1 M sodium chloride at pH 4.7 indicates the presence of a large cleft region in an ellipsoidal, globular protein (13, 14, 15). Polar residues, especially those positively charged, are concentrated on the surface of the molecule and most of the hydrophobic residues are in the interior. The cleft region also contains a number of hydrophobic side chains.

The polypeptide chain is folded with a large degree of $\alpha$-helix and $\beta$-pleated sheet structure and this structure is stable to great changes in its environment (e.g., temperature (16)). Using spectroscopic techniques in the region of pH 5, no changes have been observed in the structure on changing the concentration of urea from 0 to 8 M (17). It is possible to denature the enzyme reversibly (18).

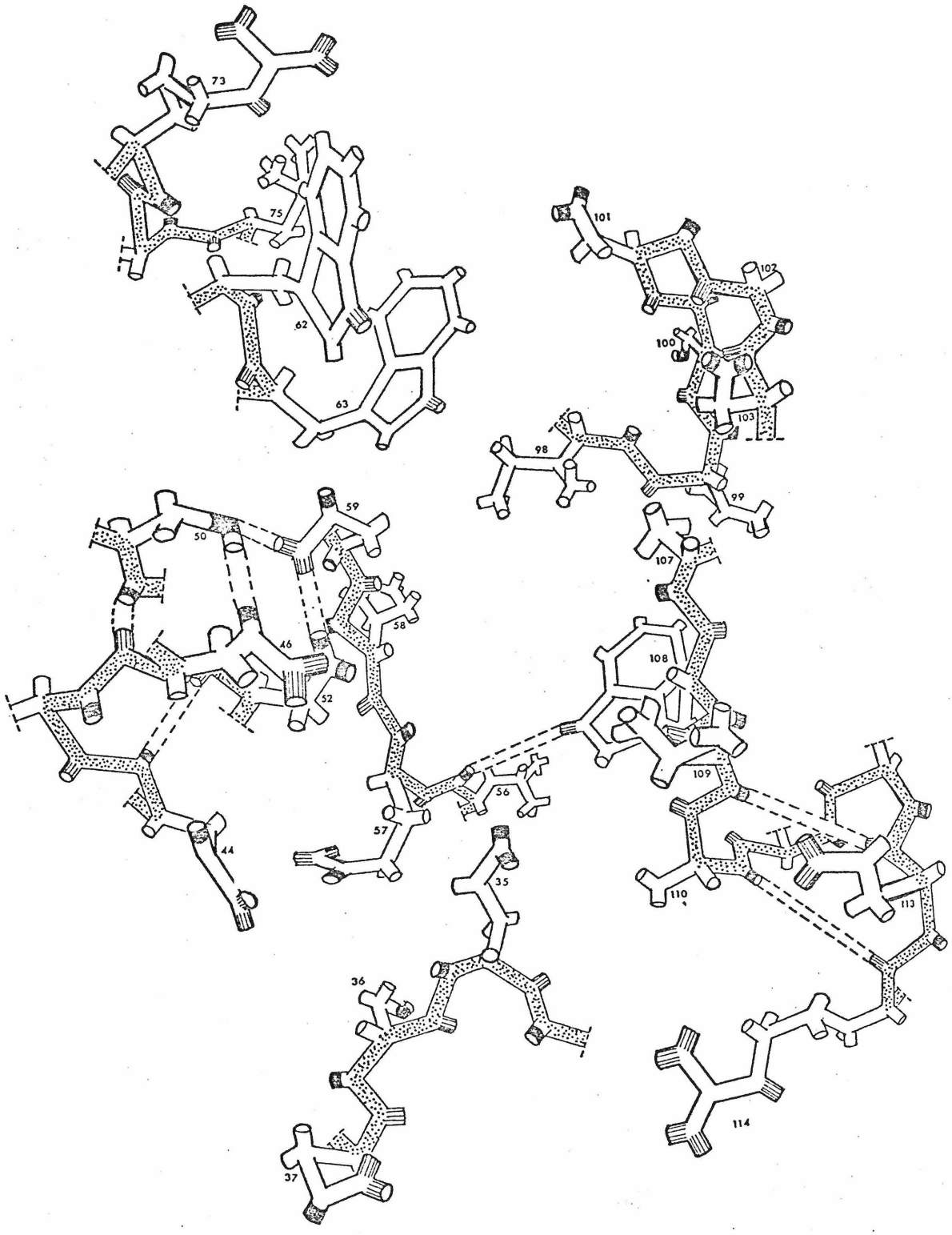



Figure 1. Cleft Region of Lysozyme Crystal Structure (14).

## Lysozyme-Catalyzed Reactions

Lysozyme catalyzes the hydrolysis of $\beta(1 \rightarrow 4)$ linkages in appropriate polysaccharide substrates. During the reaction with the bacterial cell wall mucopolysaccharide (see Figure 2), hydrolysis occurs only after the N-acetylmuramic acid residues (19). Thus, at final equilibrium of this lysozyme-catalyzed hydrolysis, the disaccharide NAG-NAM predominates. Chitin is also a substrate for this enzyme with degradation going completely to the monosaccharide N-acetylglucosamine (20).

Lysozyme also catalyzes transglycosylation. Glycosides attach to the reducing end of acceptor substrates. Thus long chains of chitin and of cell wall polysaccharides are produced during the lysozyme catalyzed hydrolysis of their respective lower molecular weight substrates (21). Experiments with radiolabeled substrates have demonstrated this is a transglycosylation reaction and not reversal of hydrolysis (22). Some alkyl alcohols compete effectively with water as a nucleophile during lysozyme catalysis producing alkyl glycosides as a transfer product (23).

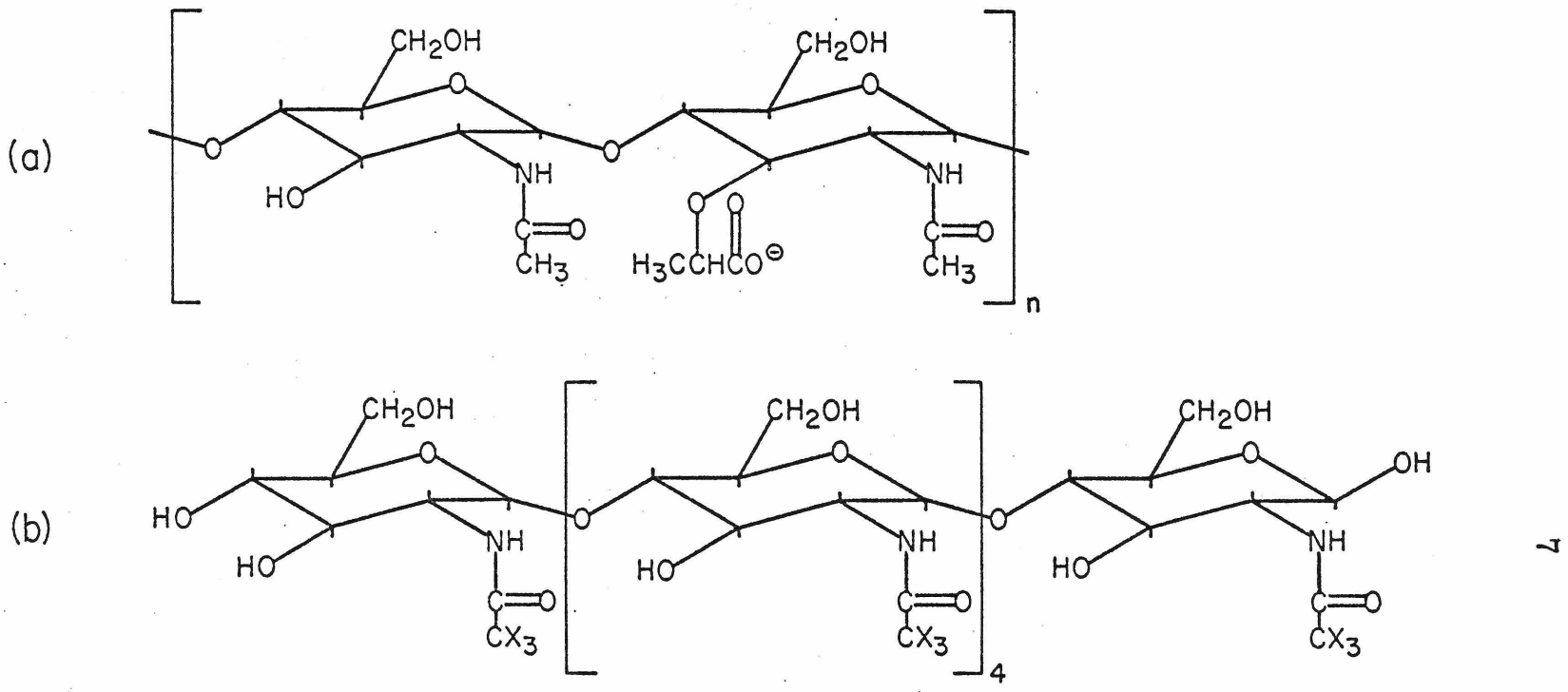



Figure 2. Lysozyme Substrates

a) $(NAG\text{-}NAM)_n$; b) X = H, chitohexaose;
X = F, N-trifluoroacetyl analog of chitohexaose

## ROLES OF AMINO ACID RESIDUES IN LYSOZYME

Modification studies have been used to assign a major function to the lysine residues. All six are easily acetylated (24), therefore are accessible to the solvent. Acetylation of these residues results in an enzyme completely inactive toward bacterial cell wall by the standard assay procedure (25). When the residues are guanidated, however, total enzymatic activity is retained. That lysine is not involved at the catalytic side is demonstrated by the observation that acetylated lysozyme is, in fact, active with glycol chitin as substrate (26), with a slight shift in the pH profile (27). Also, this enzyme derivative is completely active on chitotetraose (28). This is strong evidence that the positive charge of the protonated lysyl amine side chains serve to interact with the negatively charged bacterial cell wall carbohydrate, but that they are not necessary for catalysis.

After these studies were reported, two other works have added exo-cis-3, 6-endoxo-$\Delta^4$-tetrahydrophthalic anhydride (29), tetrafluorosuccinic anhydride (30), maleic anhydride (30) and diketene (30) to the list of reagents which block the lysines resulting in deactivation of the enzyme toward the cell wall assay. Because the guanidyl moiety is difficult to modify under mild conditions, the major positive residues of lysozyme, arginine, have been studied little. Arginine 114 has been implicated in substrate binding from X-ray crystallographic work but no solution evidence has been presented yet (28). Modification of seven of the eleven arginines with

2, 3-butanedione causes no change in enzymatic activity toward chitotetraose (28).

During acetylation of lysozyme, the $\alpha$-amino group is the first nucleophile to react. Thus this protonated amine has been shown to be unnecessary for enzymatic activity (25).

Specific modification of the single histidine residue in lysozyme with iodoacetate (27, 31), with iodoacetamido (27) and with 4-bromoacetamido-2-nitrophenol (27) has been shown to result in a completely active enzyme derivative. This is consistent with the X-ray crystal structure position of the histidine far from the active site. Further, the $pK_a$ of this histidine is not affected by substrate binding (32). The latest reported work on this residue has been interpreted as evidence for histidine involvement (33). It was reported that lysozyme which was iodinated only at the histidine with excess iodine in a potassium iodide solution at pH 4. 7 is only 80% active against cell walls. However, an inactive lysozyme derivative after iodine oxidation was found previously in which no iodine was incorporated (34). Amino acid analysis revealed the presence of one less tryptophan residue than in the native. Presence of this derivative must be ruled out before interpretation of this experiment on the affect of histidine on lysozyme activity can be made.

Carboxyl groups were implicated in enzymatic activity originally by esterification studies using dry methanol and HCl (24). This enzyme mixture was shown to have much decreased activity toward bacterial cell walls. Lysozyme esterified with dry ethanol

and HCl has a decreased affinity for chitotriose (27). Using $NH_2CH_2SO_3H$ as a nucleophile with a water-soluble carbodiimide, modification of all the carboxyl groups except that of glutamic acid residue 35 (Glu 35) results in an enzyme derivative with no activity (35). The same modification in the presence of an inhibitor results in all carboxyls amidated except those of Glu 35 and Asp 52. This derivative has 50% activity, indicating that carboxylic acid residues other than Glu 35 and Asp 52 are not necessary for activity. The crucial involvement in catalytic activity of the Asp 52 region of lysozyme is demonstrated by the fact that an enzyme esterified selectively at Asp 52 with triethyloxonium tetrafluoroborate is inactive (36).

Tyrosine residues are probably not directly involved in enzymatic activity. A diisopropylfluorophosphate treated enzyme is completely active with glycol chitin as a substrate and 80% active on cell walls (37). An enzyme O-acetylated on all tyrosines with acetylimidazole is one hundred per cent active on cell walls compared with native (27). Modification of tyrosine residues has been shown to affect the protein conformation (38, 39).

Tryptophan residues may be involved in lysozyme activity. A 2.85 mole-per-mole-enzyme incorporation of 2-hydroxy-5-nitrobenzyl bromide completely deactivates the enzyme (40). N-Bromosuccinimide deactivates the enzyme by selective modification of Trp 62 (41). Transforming the indolyl moiety of Trp 108 into an oxindole using iodine has been reported to produce an enzyme less

than one per cent active against cell walls and chitin oligomers (42). However, the X-ray crystallographic structure of this derivative at 2.5 Å resolution indicates the presence of a covalent bond between the Glu 35 carboxyl and the δ carbon of Trp 108 (43). This internal ester of Glu 35 could be the reason for loss of activity.

## PROTEIN-SUBSTRATE INTERACTION IN CLEFT

### Binding of ABC

Binding of the chitin oligomer inhibitors to the enzyme has been studied in some detail. The X-ray crystal structure of the chitotriose-lysozyme complex has been done at pH 4.7 in 1 M sodium chloride (44) (see Figure 2). The three sugar residues bind to the macromolecule in what is referred to as three subsites A, B and C. Monomer N-acetyl glucosamine binds in subsite C. The $\alpha$ and $\beta$ anomers bind in slightly different manners and, from nmr studies in solution (45, 46), with somewhat different strengths (47). The acetamido is hydrogen bonded to the protein at the same place in both sugars. The reducing terminal residue of the trisaccharide apparently binds with many of the same hydrogen and hydrophobic bonds as the NAG $\beta$ anomer. Again, evidence consistent with similarity in binding in solution has been obtained by nmr (48). This solution data indicates the reducing terminal residue acetamido group binds to the same protein location whether it is part of the chitin trimer, dimer or monomer. The sugar residue at subsite B interacts with the enzyme through three hydrogen bonds and many nonpolar contacts. Likewise, at subsite A the nonreducing terminal residue is held by nonpolar contacts in addition to a hydrogen bond to the carboxylate of Asp 101.

Modification studies and the X-ray crystal structure both indicate three of lysozymes tryptophan residues are at the active site (49). On binding of substrate, even on binding the monomer,

the cleft narrows slightly and the indole ring of Trp 62 moves closer about 0.75 Å. The indole rings of Trp 62 and Trp 63 form hydrogen bonds with the substrate. It has been shown by fluorescence spectroscopy that two tryptophans apparently change from a hydrophilic environment to a hydrophobic one on substrate binding (50). Two tryptophans are quenched independently by two carboxyls (51). Assignment of the nmr resonances of the indole N-H on lysozyme can be made which are consistent with this description of the tryptophan locations in lysozyme (52).

Solution binding studies (uv difference spectra) at room temperature at pH 5.3 and ionic strength of 0.1 clearly show an increase in binding strength going from monomer to trimer chitin (53). For chitotriose, $\Delta G = -7.2$ kcal/mole, $\Delta H = -14.3$ kcal/mole and $\Delta S = -23$ e.u. Chitotetraose, chitopentaose and chitohexaose bind with a change in free energy similar to chitotriose. At pH 5.0, ionic strength 0.1, by the same technique, the following binding constants were measured (54):

| | |
|---|---|
| NAG | $4\text{-}6 \times 10^{-2}$ M |
| $NAG_2$ | $1.75 \times 10^{-4}$ M |
| $NAG_3$ | $6.58 \times 10^{-6}$ M |
| $NAG_4$ | $9.45 \times 10^{-6}$ M |
| $NAG_5$ | $9.35 \times 10^{-6}$ M |
| $NAG_6$ | $6.15 \times 10^{-6}$ M |

The reason the monomer binding constant is not lower may be because it adsorbs the effect of the conformational change of the enzyme (2).

The activation parameters for the binding of monosaccharide inhibitors indicate the complexation is not a simple bimolecular process (47). One alternative suggestion is a diffusional rate initial complex followed by a conformational change.

The importance of the acetamido group for binding at subsite C is shown by the inability of glucose or glucosamine to bind there (53). Both the nitrogen and oxygen of this group seem to be hydrogen bonded to the peptide backbone. Studies of complex formation and rate of hydrolysis of substituted and deacylated chitin and glycol chitin lead to the conclusion that not only is the acetamido group essential for binding at subsite C, but the acetyls may assist enzyme-complex formation at A and E also (55). Studies on the acceptor specificity of lysozyme catalyzed transglycosylation show actamido group does aid binding at subsite E (56).

The bulky, charged lactate moiety of N-acetyl-muramic acid seems to prevent this sugar residue from binding to subsite C. Cell wall disaccharide binds in subsites BC with about the same free energy as chitobiose (-7.9 kcal/mole and -7.4 kcal/mole, respectively) (2). The cell wall tetrasaccharide binds ABCD with less than -6.9 kcal/mole and the hexasaccharide binds with -8.6 kcal/mole (2). It is of interest that in the cell wall murein, the cross-linking peptides are attached to the carbohydrate chains by a peptide bond at the lactyl moiety, making it bulkier yet (57).

Binding at DEF

The apparent discrepancy of decreased binding strength of cell wall tetramer compared to dimer and of the similarity of binding constants of the chitin oligomers longer than dimer can be rationalized by the crystal structure. By model building it is possible to hypothesize the extension of the inhibitor chitotriose by three additional sugar residues down the cleft (binding to subsites D, E and F) (14, 15, 44). However, reasonable binding interactions in subsites E and F can only be obtained if the residue in subsite D is distorted from the chair to a half-chair configuration. Then the interactions deduced from the acceptor specificity studies mentioned above (56) can be made. Sugar-enzyme bonding energy interactions at all subsites--observed for A, B, C, hypothesized for D, E, F--are shown in Table I.

Lysozyme catalyzed hydrolysis of chitohexaose gives, as initial products, chitotetraose and chitobiose. If the hexamer binds in all six subsites proposed by the crystal structure, this result implies catalysis would occur between residues in subsites D and E. Carboxyl modification studies mentioned earlier have indicated catalysis does occur in this area of the protein. The carboxyl side chains of Asp 52 and Glu 35 are well juxtaposed to participate in hydrolysis of that particular $C_1$-oxygen bond. The fact that N-acetylmuramic acid cannot fit in subsite C, and thus during lysozyme catalyzed hydrolysis of cell wall polysaccharides is always located in D, and that the major hydrolysis product is a NAG-NAM dimer supports this model of enzyme-substrate binding.

Table I

Summary of Subsite Interactions (2)

| Subsite | $\Delta\Delta F^*$ (kcal/mole) | Polar contacts | Total van der Waals contacts |
|---|---|---|---|
| A | -1.8 | (NH)--Asp 101 | 7 |
| B | -2.9 | (O-6)--Asp 101 | 11 |
| C | $\leq$-5.7 | (O-6)--Try 62 | 30 |
| | | (O-3)--Try 63 | |
| | | (NH)--CO 107 | |
| | | (CO)--NH 59 | |
| D | +3 to +6? | (O-6)--CO 57 | 35 |
| | | (O-1)--Glu 35 | |
| E | -4? | (O-3)--Gln 57 | 45 |
| | | (NH)--CO 35 | |
| | | (CO)--Asn 44 | |
| F | -1 | (O-6)--CO 34 | 13 |
| | | (O-6)--Asn 37 | |
| | | (O-5)--Arg 114 | |
| | | (O-1)--Arg 114 | |

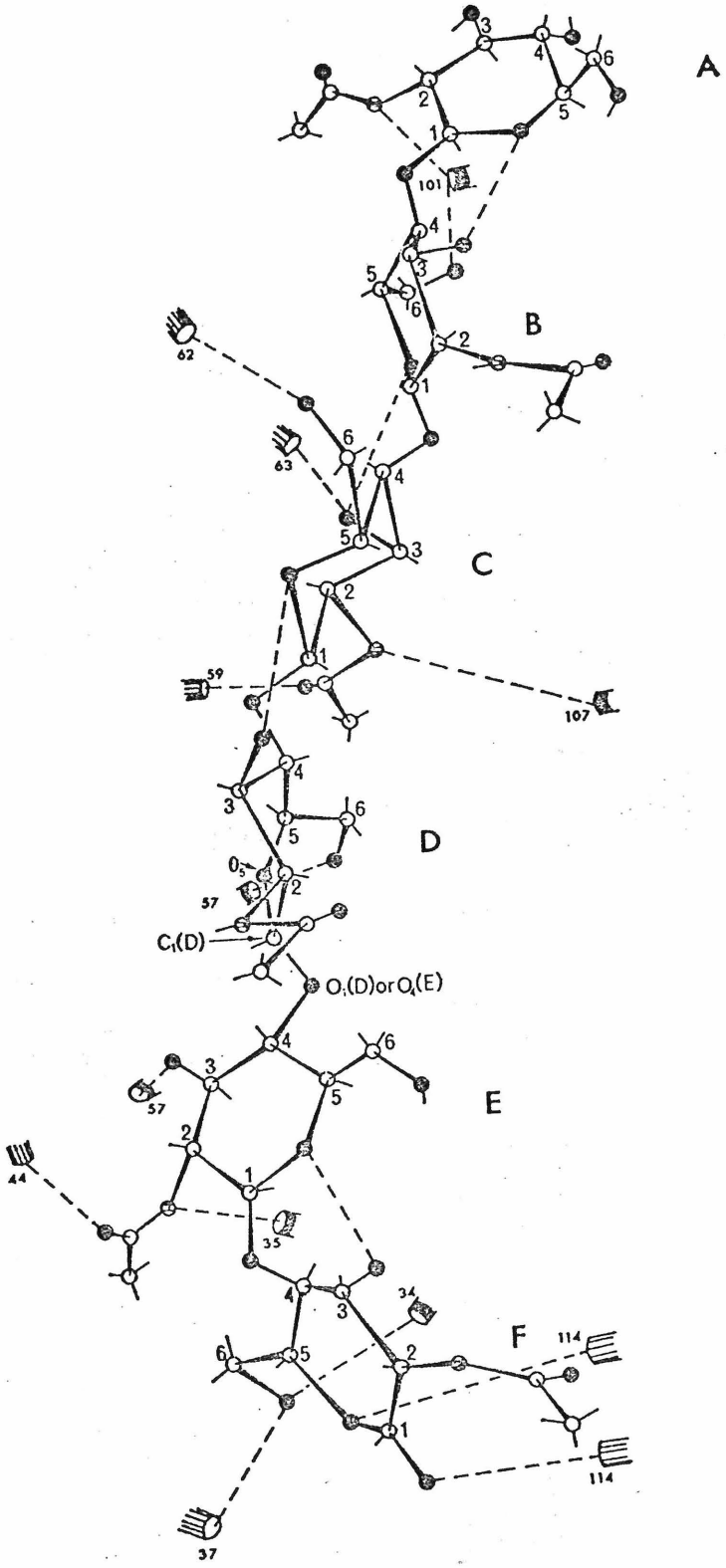
* From various chemical experiments.

The δ-lactone of D-gluconic acid has recently been demonstrated crystallographically to have a half-chair configuration (58). An initial experiment to demonstrate that a half-chair configuration does bind to subsite D used an analogous compound, glucono-(1-5)-lactone. This failed because apparently the compound reacted with water, changing its structure. The new lactone had two hydroxyls which hydrogen bonded the Asp 52 and Glu 35, from the X-ray crystal structure (43). Another analogous compound, the δ-lactone of N-acetylglucosamine was the reducing terminal residue of chitotetraose in another study (59). From pH 6.2 to 7.8 the dissociation constant of its complex with lysozyme is $0.8 \times 10^{-6}$ M. This significant increase over both the binding of chitotriose and chitotetraose was interpreted as the contribution of the half-chair δ-lactone to the binding.

From the crystal model, the $C_6$ carbon and the $C_6$ carbon hydroxyl group of the sugar residue in subsite D appear to be the steric reason for distortion. To test this, the binding strength was determined for a chitin oligomer with an N-acetyl xylosamine residue at this position (60). The $(NAG)_3$-N-acetylxylosamine, different from chitotetraose only in the replacement of the $-CH_2OH$ group with a hydrogen on the reducing terminal residue, complexed with lysozyme four times more strongly than chitotriose. A difference in binding energy of +1.4 kcal/mole for glucose compared to +2.9 kcal/mole for N-acetylmuramic acid has been explained by the fact the glucose can bind with less distortion (61).

Thus the evidence to date is consistent with the chitohexose-lysozyme substrate complex model proposed on the basis of the X-ray structure (shown in Figure 3).

Figure 3. Proposed Binding Interaction of the Chitohexaose-Lysozyme Interaction (14)



The pH dependence of the binding of the inhibitor chitotriose, whose reducing terminal residue binds to subsite C in a mode similar to β-NAG, has also been studied (51, 53, 54). By spectrophotometric techniques, the complex formation was shown dependent on two ionizations. In one study the binding curve indicated the pK of one group changed from 4.2 to 3.6 on trimer binding while the pK of a second group went from 5.6 to 6.3 (54). Results of a second study agree with the values of the first ionization but have slightly higher values for the effect of binding on the second ionization (6.3 to 6.7) (53).

Using the Asp 52 ethyl ester derivative of lysozyme the pK of Asp 52 was specifically measured as 4.5 (64). As mentioned above, Asp 52 is near the catalytic site and therefore its ionization probably is the one observed in the binding study of βMeNAG. It may also be responsible for the ionization in the region of pH 4.4 indicated in pH-activity profiles of small substrates with the native enzyme (65, 66, 67, 68). Its ionization seems unaffected by binding of a variety of substrates and inhibitors. This has been shown to be consistent with the X-ray crystal structure of the NAG-glucose-lysozyme complex in which the Asp 52 carboxylate was largely hydrogen bonded to other amino acid residues on the protein. Its one interaction with the inhibitor, through the glucose C-2 hydroxyl, is probably replacing a similar interaction with a single water molecule (69). Thus the environment of this residue remains quite constant during binding of the saccharides.

## LYSOZYME CATALYSIS

### Effect of pH

Both the rates of cleavage of polysaccharide substrates and the inhibition of lysozyme are pH dependent. Description of this dependence in terms of ionizable groups on the protein is quite detailed. The methyl protons of β-methyl-N-acetylglucosamine bound to lysozyme exhibit no change in nmr chemical shift through the pH range of 2.5 to 10 (62). Thus there is probably no significant conformational change in the binding site of this compound. The chemical shifts of the acetyl methyl protons are affected by two ionizing groups. Since there is probably no conformational change, the two groups must be nearby to have such an effect. One pK was measured as 4.7, the other 7.0. It was also determined that one and only one ionizable group on the enzyme is perturbed by the association with β-methyl NAG--a group of $pK_a$ 6.1 changes to $pK_a$ 6.6 in the complex (62).

The binding of the α-anomer of N-acetyl-D-glucosamine is affected by at least two ionizable groups (63). Although neither studies using the uv difference technique (63) nor the nmr (46) approach were able to assign precise values to these groups, the data is in accord with a group of pK 6 which affected the binding constant of β-methyl NAG and with a group of pK 4.7 that was determined to be near the binding site of its acetamido methyl.

The ionization at pK 6 has been assigned to glutamic acid 35. This residue is located in a hydrophobic region in the crystal structure--the only carboxyl other than Asp 52 at subsites C and D (44). The carboxyl is not hydrogen bonded (11). As mentioned above, the pK of this group increases on binding of a saccharide in subsite C. No further increase is observed, however, when the inhibitor occupies both C and D (65, 70). When the whole cleft is complexed with glycol chitin, the pK of Glu 35 is raised to 8 or 8.5 (70). The dependence on an apparent pK of 8.7 for cell wall lysis under saturating conditions can be attributed to Glu 35. The anomalously high pK even in the uncomplexed enzyme can be explained by stabilization from the ionized Asp 52 carboxyl nearby (64).

Aspartic acid 101 is the other ionizable side chain in the cleft. A pK of 4.5 has been assigned to it on the basis of the pH dependence of trisaccharide binding (53). β-Methyl NAG binding does not affect this ionization, as expected from its location in subsite A. $NAG_2\beta Me$, on the other hand, has been shown to hydrogen bond to Asp 101. The pK of the acid is lowered to 3.9 (70). Chitotriose binds to the carboxylate anion even more tightly, dropping the pK to 3.7.

An interesting effect was observed with Asp 101 on binding of trifluoro-N-acetyl-D-glucosamine trimer (71). The fluorines of this analog of chitotriose can be observed directly by F-nmr. A change in chemical shift is noted in fluorines on residues at both ends of the trimer as a result of ionization of Asp 101. Together with information already presented, this has been interpreted as a translation of the

trisaccharide inhibitor relative to the lysozyme cleft.

Effect of Ionic Strength of Buffer

In complete absence of salt, lysozyme has no activity on bacterial cell walls (66), glycol chitin (72) or chitin (73). The optimum pH for lytic activity varies with buffer cationic concentration and charge (74). Maximal lysozyme activity on cell walls is greater at pH 9.2 than the maximum activity at pH 6.2, unlike the bell-shaped curve around neutral pH for chitotetraose (66). Salt concentrations above 0.2 M are inhibitory to lytic activity at any pH (74).

The effect of ionic strength on the ionization of Asp 101, Asp 52 and Glu 35 is opposite to that of simple carboxylic acids (64). This can be explained by the overall positive electrostatic charge of the protein. Considering the whole protein, ionization of a carboxyl results in a loss of charge rather than in a gain as for a simple carboxylic acid. Thus the behavior of the ionization is what would be expected from a cationic acid.

Nonenzymatic Hydrolysis of Glycosidic Bonds

Hydrolysis of glycosidic bonds in nonenzymic systems can occur by acid, base or nucleophilic catalysis (Figure 4). In hydroxylic solvents, nucleophilic catalysis is much less a factor than the other two because bimolecular attack at the C-1 carbon usually does not occur unless the nucleophile is very powerful or is intramolecular (2). In this mechanism, inversion of configuration of the product is obtained.

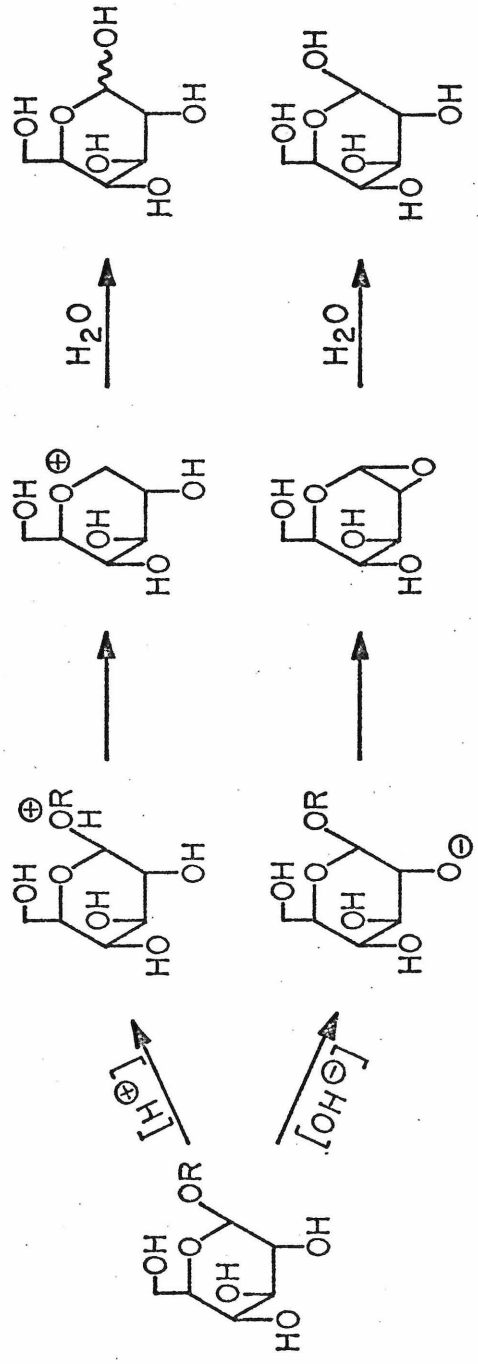
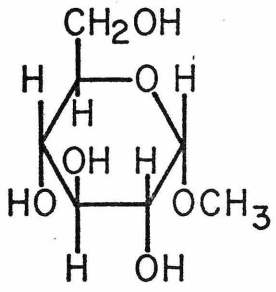



Figure 4. Acid and Base Glucosidic Catalysis

In base catalyzed hydrolysis it has been noted glycosides with a hydroxyl at the C-2 carbon trans to the aglycan react more rapidly than their corresponding cis isomers (75). In the case of the $\beta$ glycosides one of the products identified is the 1, 6 anhydride. Thus a mechanism has been proposed involving the removal of the $C_2$ hydroxyl proton. The molecule then undergoes intramolecular nucleophilic attack to yield the 1, 2 epoxide with release of the aglycan anion. In base, intramolecular displacement is the major pathway (76-78). Base catalysis of aryl glycosides probably involves nucleophilic substitution at the aromatic moiety (79).

Acid catalysis of glycoside hydrolysis has been established to occur by a carbonium ion mechanism (80). Tracer studies with $^{18}O$ have shown fission of the glycoside-oxygen bond in almost all compounds studied (79, 81). An exception, t-butyl-$\beta$-D-glucopyranoside, hydrolyzes under acid conditions with cleavage of the t-butyl-oxygen bond. This is best explained by the high stability of the t-butyl cation (82).

The ring oxygen adjacent to the $C_1$ carbon probably stabilizes the positive charge of the transition state during acid catalysis. In other words, the transition state has partial oxonium character. The oxonium ion character of this bond would increase with proper orientation of the oxygen and $C_1$ carbon. The stabilization is maximized when the $C_1$, $C_2$, $C_5$ and ring oxygen are all in the same plane. For a glycopyranoside, this involves distortion from the stable chair configuration to the half-chair (75). The energy required

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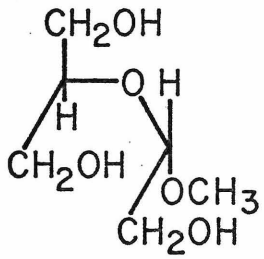



II

Figure 5. Hydrolysis of the Pyranoside is $10^6$-fold Slower than Open Chair Compound

for this distortion (80) probably contributes to the significantly slower rate of acid catalysis for this family of compounds compared to the furanosides (83). At 25°, the glucopyranoside (I) is cleaved $10^6$ times slower than the corresponding open chain compound (II), shown in Figure 5. Similarly, the transition states of $\alpha$-glucopyranosides would be expected to have more carbonium ion character than the $\beta$-anomers (84). The poorer the leaving group (and glycosyls are poor leaving groups) the greater carbonium ion character in the transition state (75).

Two different types of mechanism for acid catalyzed hydrolysis have been described (Figure 6). In one, bimolecular nucleophilic rate determining step is involved, in the other, nucleophilic attack is a post-rate-determining step (79). In other words, a water molecule is considered to participate in the transition state in the first mechanism but is not in the second (85). In the first mechanism, proton transfer to the glycosidic bonding oxygen occurs in a fast step. A slow bimolecular nucleophilic substitution step completes the reaction, resulting in inversion of configuration at the $C_1$ carbon. This is called an A-2 process.

The alternative mechanism for acid catalyzed nucleophilic substitution at the $C_1$ carbon involves the slow formation of the carbonium ion followed by a rapid nucleophilic attack (79). This mechanism is designated A-1. It can occur in two ways in glycopyranoside reactions: with ring opening, which results in loss of optical activity in the product, or with the ring remaining closed

(A-1) $S + H^{\oplus} \rightleftharpoons SH^{\oplus}$; $SH^{\oplus} \xrightarrow{\text{slow}}$ products

(A-2) $S + H^{\oplus} \rightleftharpoons SH^{\oplus}$; $SH^{\oplus} + H_2O \xrightarrow{\text{slow}}$ products

Figure 6. A-1 and A-2 Acid Catalysis

during the reaction. In the latter case, if the lifetime of the transition state is short, significant inversion of configuration could occur in the product.

Since the transition states in the two processes, A-1 and A-2, involve different number of molecules, the entropies of activation would be expected to be quite different. In the A-2 mechanism, there would be a loss of translational and rotational freedom of the water molecule which would contribute to a lower entropy of activation relative to that of the unimolecular transition state of the A-1 process. And this is the tendency in reactions which have been examined (85). For ester hydrolysis, typical values for an A-2 process are -15 to -30 e. u. compared to values of 0 to +10 e. u. for A-1. Most acetal hydrolyses proceed with entropies of activation which are positive or slightly negative, strongly indicating an A-1 mechanism for these reactions. Because of large structure and solvation effects, care must be used in applying the entropy criterion to glycosides. However, the entropies of activation of many pyranosides are consistent with an A-1 mechanism for acid-catalyzed glycoside hydrolysis (113).

General acid catalysis, which is important in enzyme catalysis, is not a predominant mechanism in model studies. It is of importance generally only when the general acid is attached to the substrate (75). Systems such as o-carboxyphenyl-$\beta$-D-glucopyranoside (86) and o-carboxyphenyl-2-acetamido-2-deoxy-$\beta$-D-glucopyranoside (87) have demonstrated general acid hydrolysis.

As mentioned above, one of the conditions under which nucleophilic catalysis can be an important mechanism in glycosidic hydrolysis in hydroxylic solvents occurs when it is attached to the substrate. An example is the reaction of acetylated glycosyl halides (88). In addition, it is possible for an acetamido group at the $C_2$ carbon to displace the aglycan forming an oxazoline intermediate. It has been suggested the specific acid catalyzed hydrolysis of Me$\beta$NAG involves some acetamido group participation. p-Nitrophenyl-$\beta$-NAG hydrolyzes at a rate independent of pH between 1.5 and 10.5, while the $\alpha$-anomer shows no such spontaneous hydrolysis (78, 87, 89). These results have been interpreted on the basis that acetamido participation, while sterically hindered in the $\alpha$ compound, occurs in the hydrolysis reaction of p$NO_2\beta$NAG.

In some reactions, the mechanism of the intramolecular nucleophilic participation contribution to the hydrolysis has been shown to be a bit more complicated. Methanolysis of 2, 3, 4, 6-tetra-o-methyl-$\alpha$-D-glucopyranosyl chloride (with no intramolecular catalysis) has been shown to occur by A-1 carbonium ion process with predominantly inversion at the $C_1$ carbon (79). Adding chloride ion to the reaction mixture has little effect on the rate of methanolysis, but markedly decreases the proportion of the inversion product. The simplest explanation for this effect is that an ion-pair intermediate between the leaving chlorine atom and the pyranosyl cation exists long enough to interact with a solvent molecule, resulting in inversion of configuration in the product, or with a chloride ion,

yielding the anomeric pyranosyl chloride (79). This ion-pair concept has been used to explain secondary tritium isotope effects for the spontaneous hydrolysis of p-$NO_2$-phenyl-β-NAG. These effects are much greater than would be expected for the intramolecular nucleophilic displacement mechanism discussed above but are consistent with a stereospecific ion-pair. Thus the $C_1$ carbon-oxygen bond is broken before the transition state and the acetamido serves to force the p-nitrophenolate anion from the ion-pair (90).

Enzymatic Catalysis of Glycosidic Bonds

Of the class of enzymes which cleave glycosidic bonds, several have been purified enough to study the mechanism of catalysis. Almond emulsin β-glucosidase has rather low substrate specificity (75). It can cleave bonds of glucofuranosides as well as glucopyranosides. The endoenzyme α-amylase (E.C. 3.2.1.1) catalyzes hydrolysis of the α 1→4 linkage of amylose with retention of configuration in the product while the β-amylase (E.C. 3.2.1.2), an exoenzyme at the nonreducing end, hydrolyzes the same bond to give β-maltose (75). The two glucamylases most extensively studied catalyze the hydrolysis of maltose to glucose with inversion of configuration. β-Galactosidases probably cleave with retention of configuration.

Generally, the activity of these enzymes is affected by the ionization of two groups. The pK of the lower group is usually about two to three pH units below that of the higher group. Glucamylase

reportedly has two carboxyl groups of pK 3 and 6 which affect activity (75). β-Glucuronidase also is claimed to have two carboxyls involved, in this case with pK values 4 and 6 (91). An imidazole and a sulfhydryl, respectively, have been proposed to be responsible for the pH dependence of β-galactosidase at 6.8 and 9 (75). Identification of the cation state of a histidine residue as being involved in catalytic mechanism of barley β-amylase has been made from its heat of ionization (92).

Thus far, a covalent intermediate has been isolated in only one reaction catalyzed by a saccharide hydrolase. This intermediate was a glucosyl-enzyme isolated from the sucrose phosphorase catalyzed reaction (93). Two methods were used to obtain the intermediate: precipitation at acid pH after addition of substrate to enzyme and, also in the presence of substrate, chemical modification of the enzyme to reduce the rate of acceptor reaction with the intermediate. Consequently, a double displacement mechanism has been proposed for sucrose phosphorylase. The difficulty in obtaining evidence for a covalent intermediate in this class of enzymes as a whole, however, is in marked contrast to the studies of other hydrolases. Hydrolyses catalyzed by many proteases, esterases and phosphatases, for example, have been shown to proceed with a covalent intermediate (94).

Thus only a few generalizations can be made at this time about the catalytic mechanism of oligosaccharide hydrolases. They seem to require a negatively charged carboxyl. This can function as a general base, a nucleophile or to provide electrostatic stabilization

of a positive activated state in the reaction. Also necessary seems to be a proton donor. Significant covalent character for the reaction intermediate appears to occur only in the exceptional case. In most cases, retention of configuration can be explained by electrostatic shielding or ion pairing by an ionized group on the enzyme which prevents attack on the intermediate glycosyl cation (75, 79, 95, 96, 97).

A mechanism for lysozyme catalysis was proposed on the basis of model building studies on the X-ray crystal structure of the lysozyme-inhibitor complex (14). According to this mechanism, the $\gamma$ carboxyl group of Glu 35 donates its proton to the glycosidic bridging oxygen. The $C_1$–O bond breaks leaving a carbonium ion intermediate at $C_1$. This intermediate is stabilized by electrostatic interaction with the Asp 52 carboxylate. Distortion of the sugar residue on binding to subsite D further stabilizes the intermediate by positioning the ring oxygen in a better position to share the positive charge of the adjacent $C_1$ carbon.

Glu 35 and Distortion at Subsite D

Chemical evidence is consistent with the proposed role of Glu 35 and of subsite D. Definitive information has been difficult to obtain as kinetic studies are hindered by transglycosylation side reactions and by availability of several binding modes to substrates. Substrate derivatives have been studied designed to circumvent these complications. Although the data obtained is much clearer than with the natural substrates, its relation to the enzyme catalysis of these

substrates is often ambiguous. For example, strain probably plays a much larger role in enzyme catalysis of cell wall than of phenyl glucosides (98, 99).

Binding of a substrate with the appropriate residue in a half-chair configuration seems to be a good illustration of an enzyme binding preferentially the intermediate of the reaction it is catalyzing, rather than the reactants or the products. More particularly, in older terminology, it is an example of the rack mechanism of enzyme catalysis (100).

The actual energy involved in this distortion of the sugar ring is not known. Distortion of a cyclohexane ring to this configuration has been estimated to involve a change in free energy of 10 kcal/mole (101). The best estimate for pyranose ring distortion seems to be about the same (102). A value for the distortion of the lysozyme-substrate complex has been deduced indirectly. A comparison of the binding strengths of NAG-NAM-NAG with $(NAG\text{-}NAM)_3$ indicates a contribution of +1 kcal/mole for binding to subsites DEF, including distortion (2). An estimate of -1 kcal/mole for the binding energy of interaction at F has been made from transglycosylation experiments (see Table I). With different assumptions about the energy at subsite E, the free energy of binding at D, with distortion, seems to be between +3 (103) and +6 kcal/mole (104). If, in fact, this energy is used to bring the substrate closer to the transition state configuration, a $10^2$- to $10^4$-fold acceleration in rate of cleavage may result (103).

Lactone derivatives of sugars, which have a configuration similar to the proposed transition state of this hydrolysis reaction, do seem to bind to subsite D with a negative change in free energy (59, 60) (see p. 17). Glucose binds to subsite D with less (positive) energy than NAM. The favorable energy for glucose binding relative to NAM binding has been proposed due to the relatively smaller distortion involved in the glucose binding (61). The additional energy required to distort the NAM residue must contribute to its greater rate of lysozyme catalyzed hydrolysis.

The $\gamma$-carboxyl of Glu 35 was assigned the role of general acid in the lysozyme mechanism on the basis of its location to the glycosidic bond cleaved and of its hydropholic environment. Chemical evidence is in accord with this assignment. The Glu 35 $\gamma$-carboxyl has been shown to have a pK of 6 in the native enzyme (p. 21), probably much higher in the lysozyme-substrate complex (p. 22) (64). This is an appropriate value for a proton donor during catalysis under physiological conditions. It is the only group on lysozyme other than the histidine imidazole and possibly the $\alpha$ amino group, neither of which are necessary for catalysis (p. 9 ), to have a pK in this region. Furthermore, this is one of two carboxyls on lysozyme which have not been eliminated by modification studies from a role in the catalytic mechanism (p. 10).

The possibility that this catalysis does in fact proceed with acid catalysis is also supported by chemical studies. First of all, acid catalysis is well-established for nonenzymatic hydrolysis of glycosides

(p. 27). Isotope labeling studies with $^{18}O$ has shown it is the $C_1$–O bond cleaved in the enzyme reaction (104). The relative rates of lysozyme catalyzed hydrolysis of isologs of p-methylphenyl chitobiose replacing the aryl glycosidic bridging oxygen with sulfur and with selenium supports the theory of acid catalysis for the enzyme mechanism (105). The secondary $\alpha$-deuterium isotope effect of the enzyme catalyzed hydrolysis of NAG-glucosephenyl indicates considerable carbonium ion character in the transition state in a pH range of 3.1 to 8.3 (106). More definitively, tritium isotope studies were used with chitotriose tritiated at each $C_1$ carbon as substrate for lysozyme (107). The transition state for this hydrolysis reaction also showed significant carbonium ion character.

Aspartic Acid 52 and Substrate Acetamido

The role of aspartic acid 52 suggested with the original mechanism (14) or of the acetamido group on the saccharide residue in subsite D suggested shortly afterward (108-110) have been somewhat more difficult to study than the role of Glu 35 or of distortion. Asp 52 is definitely at the active site (p. 10). If it has a catalytic role, it could act as a nucleophile, as a general base, or, as in the originally proposed mechanism, to stabilize an intermediate carbonium ion. Evidence for a concerted acid-base or acid-nucleophilic catalysis has been presented from study of $k_{cat}$ of the lysozyme catalyzed hydrolysis of several $\beta$ aryl chitobiosides (108). The fact that products of transglycosylation and transfer reactions retain their

configuration is consistent with nucleophilic attack only if it involves an even number of displacement reactions. A covalent intermediate would explain this observation. That a greater than 99.7% retention of configuration in the products was observed during a lysozyme catalyzed transfer reaction involving chitobiose and methanol is an argument for an intermediate shielded from backside attack with a covalent bond (23). However, much evidence for a carbonium ion intermediate has been presented. Qualitatively, this discrepancy can be explained by the efficient steric hindrance of the protein structure toward attack on a carbonium ion intermediate from a direction leading to inversion of configuration. This is consistent with a good deal of Asp 52 carboxylate character in the transition state, whether carbonium ion or not. Separating the relative importance of each of the possible roles for Asp 52 is difficult.

Participation of the saccharide acetamido group in the hydrolysis mechanism has been demonstrated in nonenzymic catalysis of some $\beta$ glycosides (p. 30). A similar role has been proposed for the enzyme reaction, as all natural substrates of lysozyme do contain $C_2$ acetamido groups on each sugar residue (61, 110, 111). Several experiments have indicated that lysozyme catalyzes the cleavage of glycosidic bonds at N-acetylhexosamine residues from two to over twenty times faster than at glucose residues. The fact that the acetamido compound is hydrolyzed faster has been used to argue for its involvement. The fact that the glucose compound undergoes significant catalysis at all has been used to argue against the need

for acetamido (111, 112) participation. The rate of hydrolysis of a pseudosubstrate with 2-deoxyglucose at subsite D is faster than the rate of the analogous NAG compound, indicating the acetamido is certainly not necessary for catalysis with this substrate (111). Experiments involving NAG binding to subsite D are always complicated by multiple binding sites on the enzyme. In addition, it is impossible in these types of experiments to distinguish the effect the acetamido group contributes by anchiomeric assistance from the effect it has by changing the distortion of the residue at subsite D. As glucose does have a different binding strength to lysozyme than NAM (61), it is probably less distorted which would contribute to a comparatively lower rate of cleavage.

Thus there is much evidence for lysozyme catalysis with Glu 35 acting as a general acid catalyst and with the transition state having much carbonium ion character. The limited evidence seems consistent with proposed distortion at subsite D to stabilize a carbonium ion intermediate. The intermediate exists long enough for the alcohol product to diffuse away and a nucleophile to replace it. The effects of the acetamido group and of Asp 52 on catalysis have thus far proven difficult to ascertain.

## EXPERIMENT I

### N-Trifluoroacetyl-D-Glucosamine Derivatives as Substrates for Lysozyme

This study was designed to differentiate between a carbonium ion mechanism and a mechanism involving anchiomeric assistance of the acetamido group of the substrate's C-2 carbon. The rate of hydrolysis of the chitin substrate was compared to that of a substrate analog with the same steric properties but differing in the electron donating ability of the acetamido oxygen. Since a trifluoromethyl group is the same size as a methyl but has a large electron withdrawing effect (the same $\sigma$ value on an $sp^3$ hybridized carbon as Iodine (114)), N-trifluoroacetyl glucosamine oligomers were used as substrate analogs.

The nonenzymatic hydrolysis of p-nitrophenyl-2-acetamido-2-deoxy-$\beta$-D-glucopyranoside ($pNO_2$-phenyl-NAG) and of its trifluoroacetyl analog ($pNO_2$-phenyl-TFAG) were studied at pH 0.5 and at pH 5.5 (see Figure 7). In the acid solution an A1 mechanism should hold (89), thus any difference in rate should reflect the inductive effect on the reaction transition state without anchiomeric assistance. At pH 5.5, spontaneous hydrolysis occurs (89). The rates of hydrolyses of these model compounds were compared with the rates of the lysozyme-catalyzed hydrolyses at pH 5.5 of the hexasaccharides $(NAG)_6$ and $(TFAG)_6$.

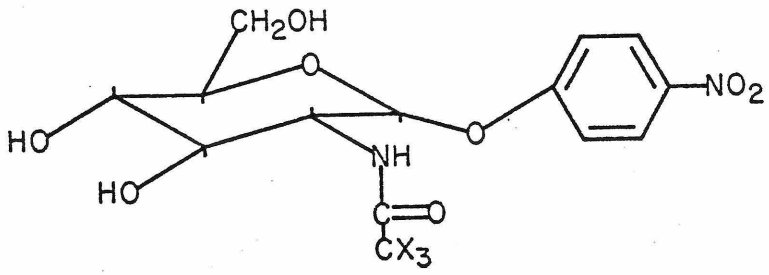



Figure 7. X = H, $pNO_2\phi NAG$ X = F, $pNO_2\phi TFAG$

## Materials

Hen egg-white lysozyme, recrystallized three times, and chitin were obtained from Sigma Chemical Company. The glucosamine hydrochloride used was A-grade reagent from Calbiochem. Trifluoroacetic anhydride was from Matheson Coleman and Bell Co. CM-Sephadex (C-25-120, particle size 40-120 $\mu$) and LH-20 Sephadex were from Pharmacia. Bio-Rad Laboratories supplied the Bio-Gel P-2 and P-4 gels.

The $^{14}$C-acetic anhydride used was Lot 1175-20 from New England Nuclear. The $^{14}$C-N-acetyl-D-glucosamine was prepared by the Horton adoption (115) of the method of Inouye (116) and purified by Bio-Gel P-4 chromatography. Chitin oligosaccharides were prepared by the method of Rupley (117). Chitosan oligosaccharides were prepared by a KOH fusion reaction of chitin followed by hydrolysis of chitosan with 4 N HCl for 24 hr at 100°, adapted from Barker (118).

Triethyloxonium tetrafluoroborate reagent was synthesized from boron trifluoride etherate and epichlorohydrin (both from Matheson Coleman and Bell Co.) according to the procedure of Meerwein (119) and stored under ether with desiccation at 0°.

## Techniques

A quantitative ninhydrin test (120) was used to determine the concentration of amines. A ferro-ferricyanide test (121) was used to determine the reducing sugar concentration.

Visible and ultraviolet absorbances were determined with a Gilford Model 240 spectrophotometer. Scintillation counting of the $^{14}C$ derivatives was performed in 15 ml of Bray's solution (122) in a Packard Model 3375 scintillation spectrometer.

Analytical Methods

a) Model study conditions: Buffer solutions used were 0.01 M sodium citrate and 0.12 M sodium chloride at pH 5.50 at 78°. The Beckman electrodes were standardized at 80° with Beckman standard buffers. The p-nitrophenyl glycoside was added such that its final concentration was about 0.1 mg per ml.

One ml aliquots were sealed under nitrogen in thick walled hydrolysis tubes (6 mm ID). The reaction tubes were then heated at 78°. The short-term experiments were performed in a mineral oil bath, the oil kept circulating by a magnetic stir bar. For the long-term experiments the reaction tubes were suspended in the vapors of refluxing 95% ethanol.

The initial sample ($t_0$) was withdrawn after an incubation period (15 min for the p-nitrophenyl-N-acetyl glucosamine and 12 hr for the trifluoroacetyl analog). Each reaction tube withdrawn was placed immediately in an ice bath, stopping the reaction at that time. All tubes were stored at 0° and analyzed at the same time.

Samples of 0.85 ml were withdrawn from each tube, made alkaline with 100 $\mu$l of 0.2 M sodium borate (pH 10) and the absorbance at 400 nm was measured at room temperature. For the pH

0.53 study, HCl was the only solute in the buffer solution and 300 $\mu$l of 7:1 solution of saturated sodium tetraborate and 2 N NaOH was used to make the solution alkaline.

The observed rate constant, $k_{obsd}$, was calculated by multiplying by 2.303 the slope of a plot of time vs. $\log\left[\frac{(OD_\infty - OD_0)}{(OD_\infty - OD_t)}\right]$.

b) Enzyme hydrolysis conditions: 500 $\mu$l of a solution 0.25 M in $^{14}$C-NAG and 500 $\mu$l of a solution $2.5 \times 10^{-3}$ M in hexasaccharide were added to 1.25 ml of a solution 0.02 M in sodium citrate and 0.24 M in sodium chloride, pH 5.50. This solution was shaken and allowed to equilibrate to 25° in a circulating water bath. The enzyme reaction was initiated by addition of 250 $\mu$l of a $1 \times 10^{-5}$ M lysozyme solution. Thus the final concentrations in the reaction were: $5 \times 10^{-2}$ M NAG, $5 \times 10^{-4}$ M hexasaccharide, $1 \times 10^{-6}$ M lysozyme, 0.01 M sodium citrate and 0.12 M sodium chloride.

At certain time intervals 500 $\mu$l aliquots were withdrawn and immediately acidified with 2 drops of 0.1 M sodium phosphate buffer, pH 2, at 0°. To remove the basic lysozyme protein this solution was passed through 1.5 ml of CM-Sephadex equilibrated with $10^{-2}$ HCl and $10^{-3}$ M NAG. The column was rinsed once with 0.4 ml of the $10^{-3}$ M NAG solution and this rinse was combined with the reaction mixture. The solution was frozen and stored for analysis.

Analysis of the reaction products was performed by separating the reaction mixture on a Bio-Gel P-4 column (2 × 80 cm), eluting with water. 70 $\mu$l fractions were collected and analyzed for ratioactivity. A typical elution pattern is shown in Figure 8. Two peaks

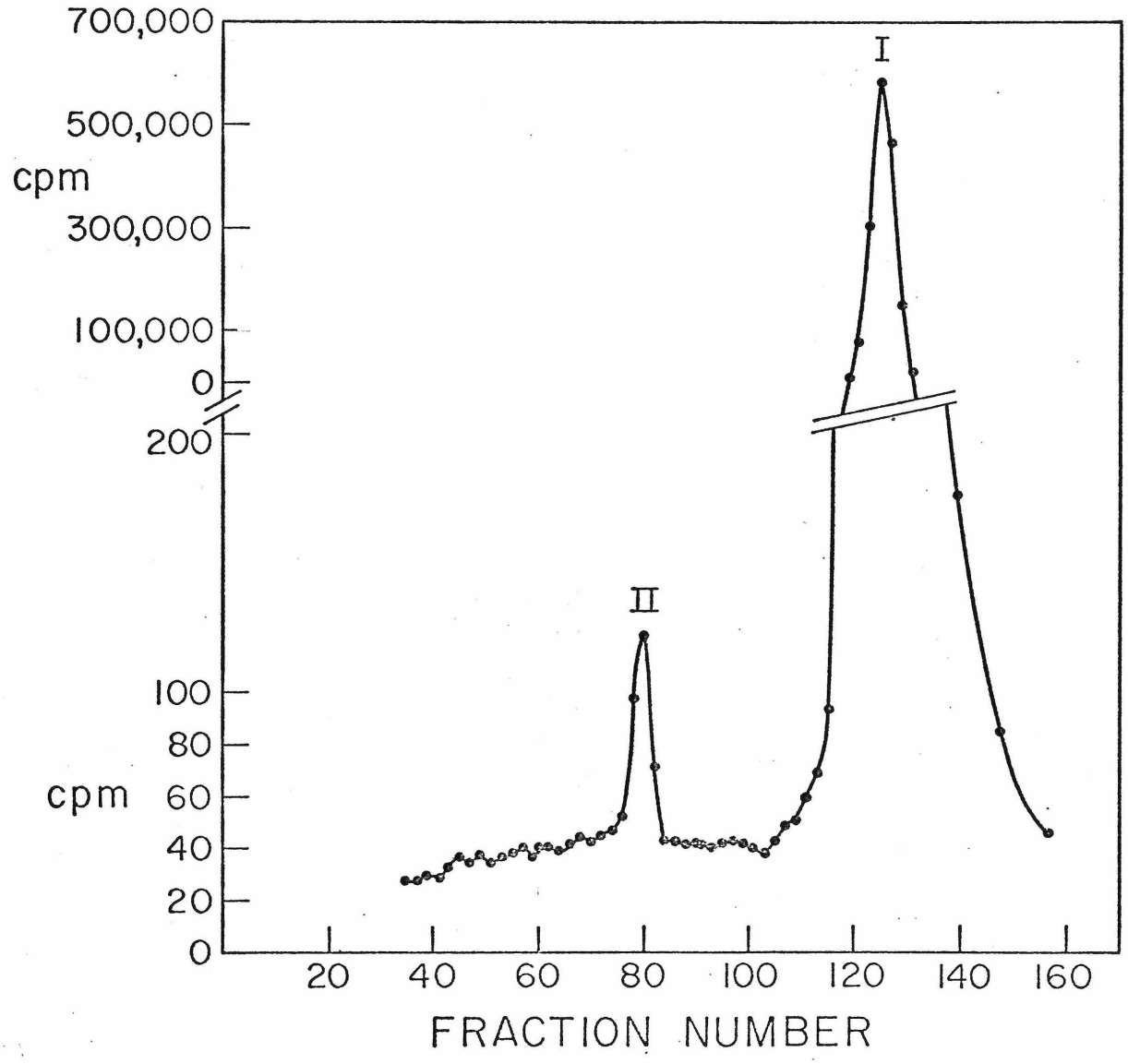



Figure 8. Typical Analytical P-4 Gel Chromatographic Separation of Transfer Reaction $^{14}$C-NAG + $(NAG)_6$ + Lysozyme.
I -- monosaccharide; II -- pentasaccharide

of radioactivity were obtained. One (I) was the excess $^{14}C$-NAG. The other peak (II) was the $^{14}C$ pentasaccharide, a direct measure of the transglycosylation.

Preparative Methods

a) Enzyme substrates: Hexa-N-acetyl glucosamine was separated from a mixture of chitin oligosaccharides by polyacrylamide gel chromatography, as previously described (123). A Bio-Gel P-4 column (5 × 180 cm) was loaded with several grams of the oligosaccharide mixture and eluted with water. The fractions collected were analyzed by reducing sugar content (121). The fractions thus determined to contain the hexamer were pooled, concentrated and rechromatographed. The fractions containing the major peak off the second run, chromatographically pure hexamer, were pooled and lyophilized.

Hexa-N-trifluoroacetyl glucosamine was prepared from chitosan oligomers. The oligomers (3 g), dried in a vacuum desiccator, were suspended in 200 ml of acetonitrile (distilled from $P_2O_5$) and stirred. Trifluoroacetic anhydride (15 ml) was added. After several minutes the suspension cleared. After stirring for one hr at room temperature, the solvent was removed at 40° in vacuo. Traces of trifluoroacetic acid were removed by repeated addition and evaporation of carbon tetrachloride.

The saccharide mixture was gently de-O-trifluoroacetylated by standing in dry methanol for several days. At the end of this time the methanol was evaporated and the mixture dissolved in 200 ml of 90% methanol. This was evaporated and the mixture dissolved in 300 ml of water and lyophilized. The hexamer was separated by gel chromatography by the same procedure used for hexa-N-acetyl glucosamine. A typical chromatographic separation is shown in Figure 9.

b) Model study compounds: p-Nitrophenyl-β-NAG was synthesized from glucosamine hydrochloride by the method of Leaback (124). The acetochloroglucosamine prepared from glucosamine was reacted under alkaline conditions with p-nitrophenol. The p-nitrophenyl 2, 3, 4, 6-tetraacetyl-β-D-glucosaminide thus obtained was de-O-acetylation with sodium methoxide in methanol to form the product.

p-Nitrophenyl-N-trifluoroacetyl-β-D-glucosamine was prepared from p-nitrophenyl NAG. Five grams of dried p-$NO_2$ phenyl NAG was solubilized in 100 ml of dry acetonitrile with several milliliters of trifluoroacetic anhydride. After stirring for 45 min, the solvent was evaporated at 40° in vacuo. The trifluoroacetic acid and anhydride traces were removed by repeated addition and evaporation of carbon tetrachloride.

The glycoside was again dissolved in dry acetonitrile. A 1.2 molar excess to amide of triethyloxonium tetrafluoroborate salt dissolved in acetonitrile was added dropwise. This reagent was put

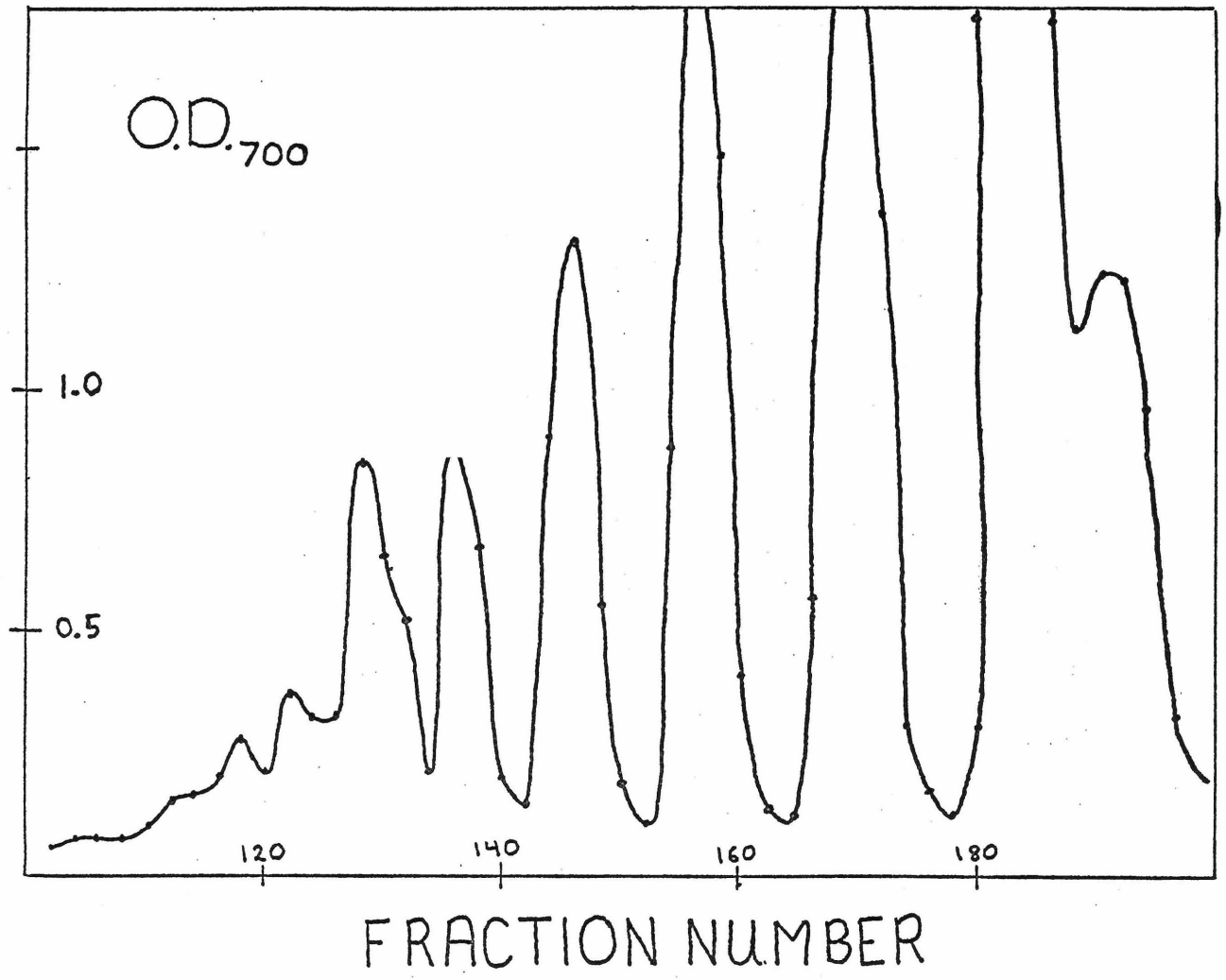



Figure 9. Separation of TFAG oligomers (1.04 g) by P-4 chromatography (column 5 × 180 cm, eluted with water). Fraction size was 16 ml. Plots is of $OD_{700}$ from reducing sugar test (200 $\mu$l sample).

to a similar use by Hanessian (125). After being stirred for one and one-half hr, the reaction was stopped by evaporating the solvent at 40° in vacuo. The product was de-O-trifluoroacetylated by treatment for several days with dry methanol.

The de-O-trifluoroacetylation was completed and imide bond hydrolyzed by evaporating the methanol and lyophilizing the mixture with water. The p-$NO_2$ phenyl glucosamine product was separated from unreacted starting material chromatographically on a 2 × 100 cm Sephadex LH-20 column and eluting with a solution of methanol and water (1:1). The elution pattern was analyzed by ninhydrin (120), reducing sugar test (121) (which is positive for the p$NO_2$ phenyl glycoside) and hydrolysis with concentrated sodium hydroxide solution.

Fractions containing the p-$NO_2$ phenyl glucosamine were pooled, lyophilized and rechromatographed. The trifluoroacetylation procedure in acetonitrile was repeated on the lyophilized, rechromagraphed glycoside, followed by the same de-O-trifluoroacetylation procedure. As before, the compound was chromatographed on the LH-20 column. The new peak in the p-$NO_2$ phenyl acetamido range of the elution pattern was a clear indication of p-nitrophenyl-N-trifluoroacetylglucosamine. The ninhydrin test results and the F-nmr of this compound were consistent with this assignment.

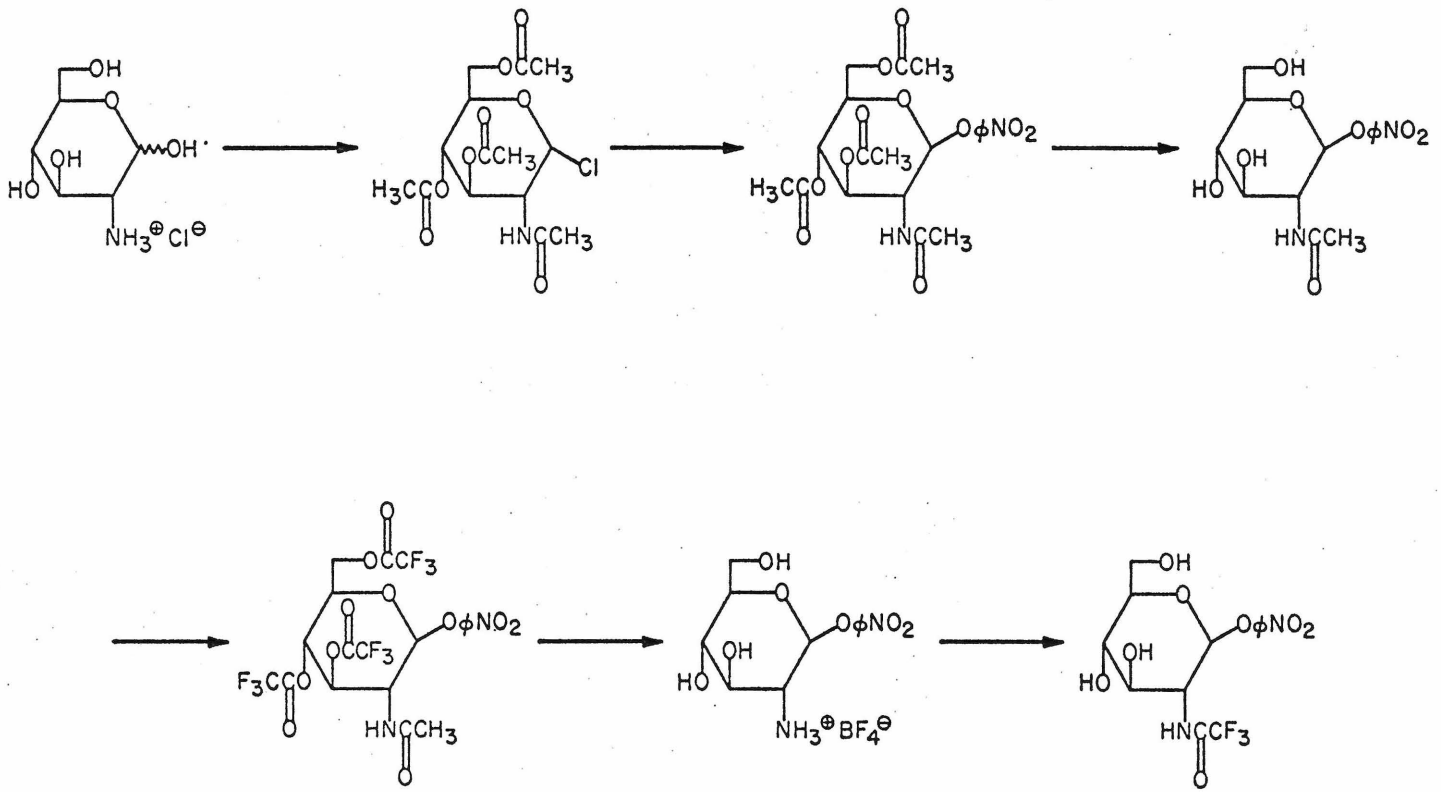



Figure 10. Outline of Synthesis of p-$NO_2\phi$TFAG

Results

Acid catalyzed hydrolysis of p-nitrophenyl-NAG and p-nitrophenyl-TFAG at pH 0.53 indicated that the former compound was hydrolyzed ten times faster (Figure 11). The rate constants and their ratio are shown in Table II. At pH 5.5 the observed difference in spontaneous hydrolysis was somewhat greater than at the lower pH. The results are shown in Figure 12 and the ratio of rate contants in Table II. Under these conditions the ratio of the rates is greater than at acid pH but still not of the magnitude expected for a mechanism dependent on rate limiting nucleophilic participation by the acetamido or trifluoroacetamido group.

The lysozyme catalyzed hydrolysis of $(NAG)_6$ and $(TFAG)_6$ was monitored not by detection of reducing groups produced but by transglycosylation with $^{14}$C-NAG. The hydrolysis of $(NAG)_6$ has been shown to be essentially specific for the glycosidic bond between the second and third residues from the reducing end of the hexasaccharide (104). The transglycosylation with $^{14}$C-NAG yields $(NAG)_4$-$\beta$-(1-4)-($^{14}$C-NAG), a $^{14}$C-labeled chitopentaose. This was the only $^{14}$C-labeled chitooligosaccharide observed on Bio-Gel P-4 chromatography of the products of the lysozyme reaction (Figure 8).

Similarly, hydrolysis catalyzed by the enzyme of $(TFAG)_6$ in the presence of $^{14}$C-NAG yielded $(TFAG)_4$-$\beta$-(1-4)-($^{14}$C-NAG) as the only radio-labeled product. Results by this method were much more accurate than those from attempts to follow hydrolysis by monitoring the increase in sugar reducing groups. The $(NAG)_6$ hydrolysis was

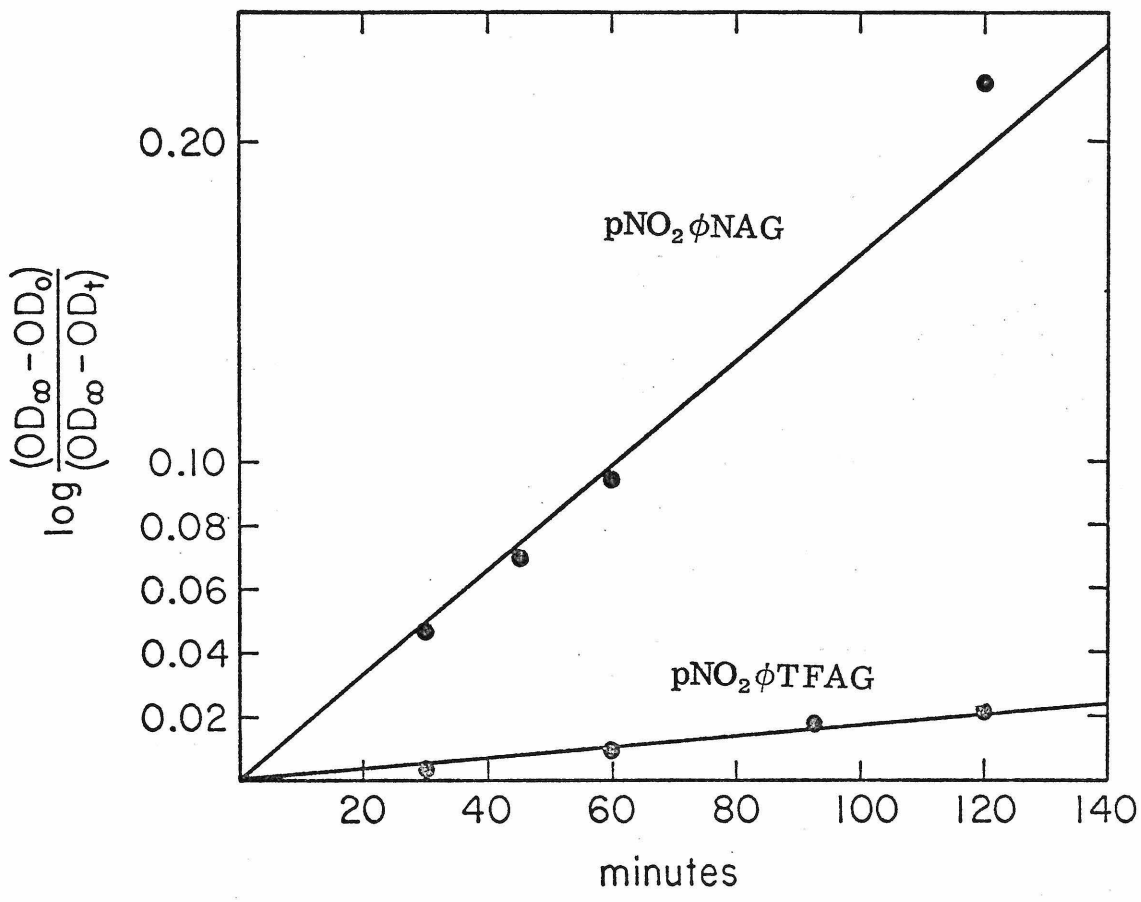



Figure 11. pH 0.53 Hydrolysis

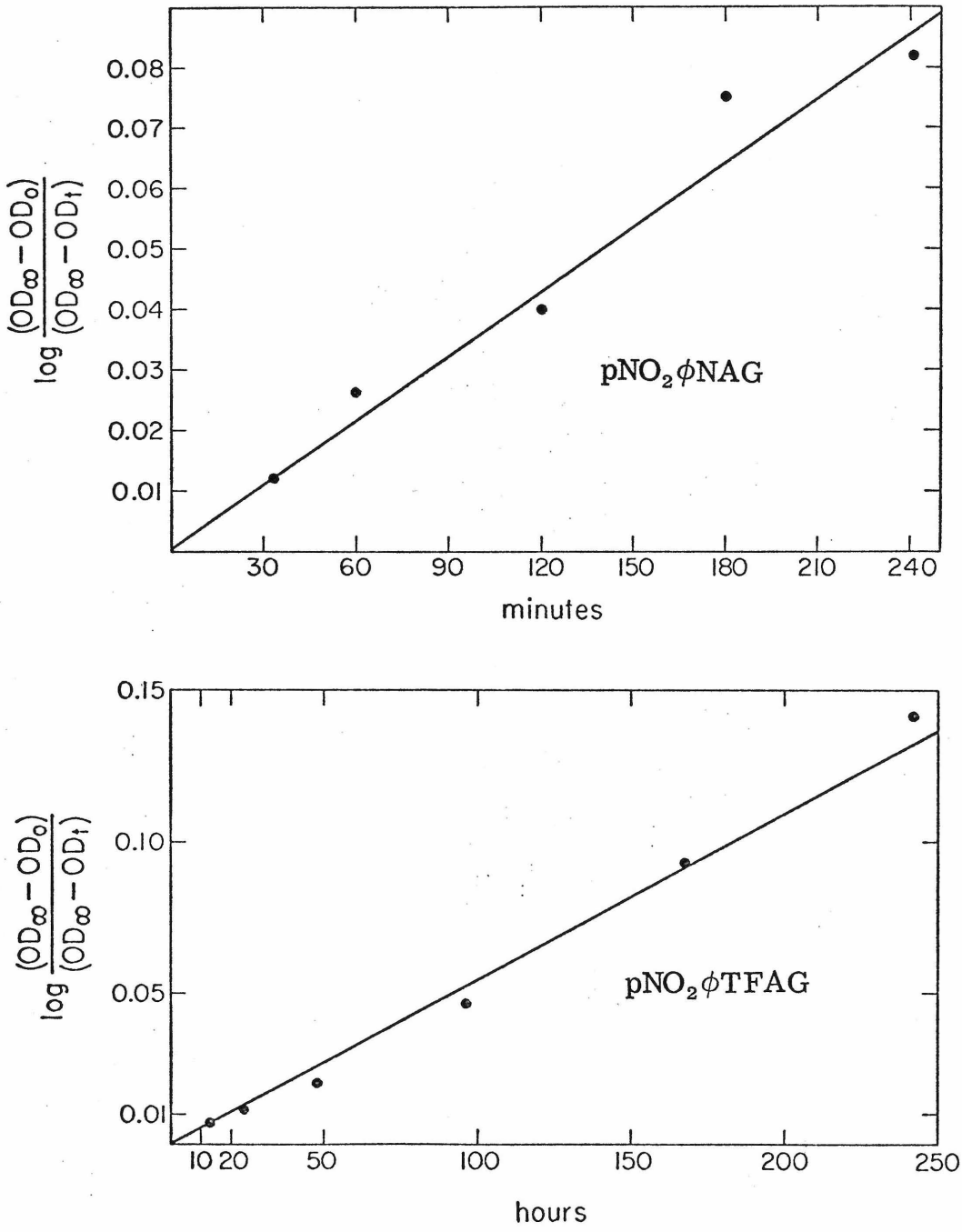



Figure 12. Hydrolysis at pH 5.5

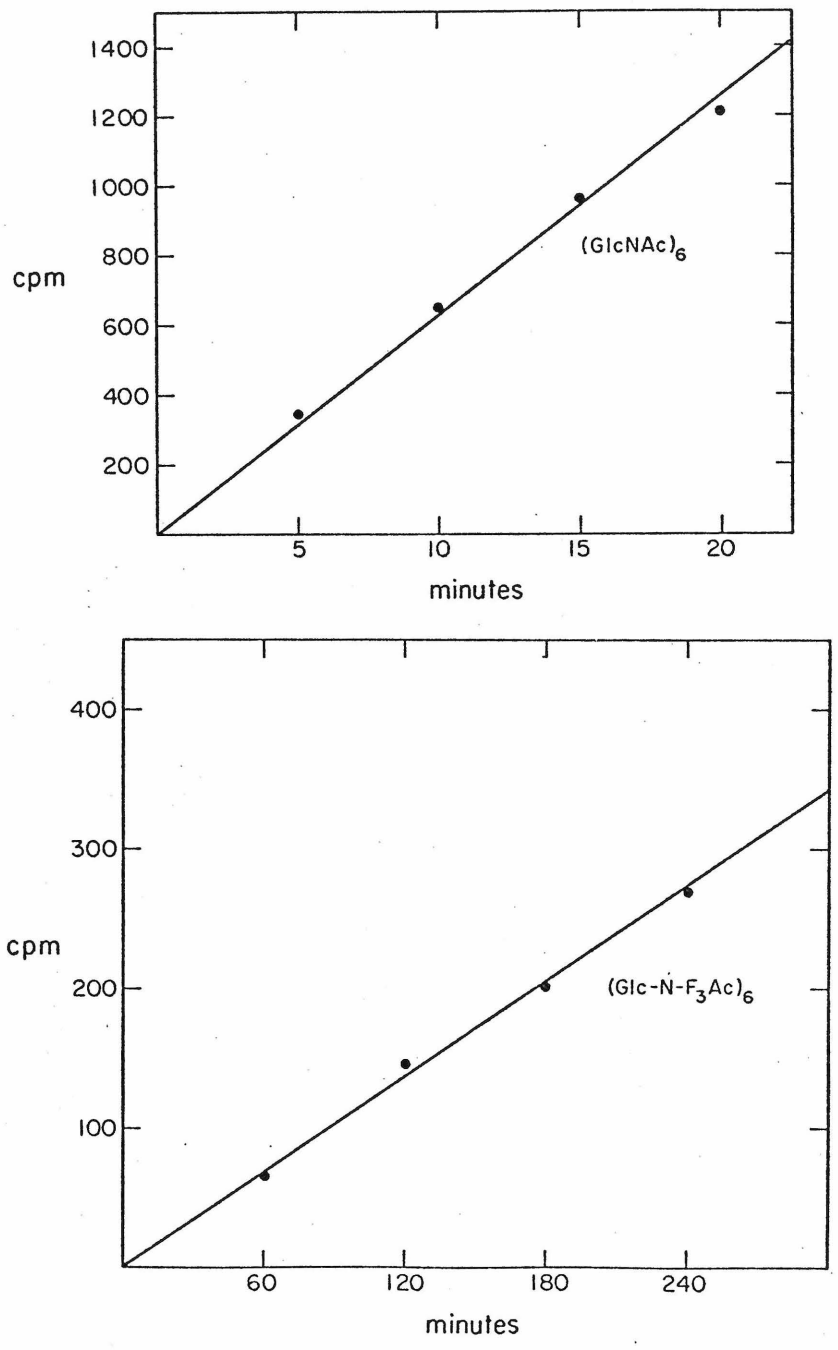



Figure 13. Lysozyme-Catalyzed Hydrolysis at pH 5.5 of $(NAG)_6$ and $(TFAG)_6$

observed to have a rate constant 56 times that of the $(TFAG)_6$, as seen in Table II.

Discussion

The ten-fold rate difference in acid hydrolysis between p-nitrophenyl-NAG and p-nitrophenyl-TFAG, is best explained by an inductive effect of the trifluoroacetamido group. The mechanism has been shown most likely to involve a carbonium ion intermediate with the transition state resembling the starting material to a considerable extent due to the nature of the leaving group (see p. 27 ). If acetamido participation were important in the rate limiting step, then a factor of at least $10^3$ decrease in rate on substitution with a trifluoroacetamido group would be expected.

Similarly, at pH 5.5 the spontaneous hydrolysis of p-nitrophenyl-TFAG relative to p-nitrophenyl-NAG (1:40) would be expected to be slowed down to a much greater extent if anchimeric assistance were the sole rate-limiting step in the mechanism. The result obtained is in agreement with the mechanism involving equilibrium formation of a stereospecific ion pair, followed by expulsion of the leaving group by the neighboring acetamido or trifluoroacetamido group. However, at pH 5.5 this mechanism implies the eventual formation of an oxazoline intermediate. This would also occur with the trifluoroacetyl analog whose intermediate would react more readily with water.

Table II

| Substrate | pH | Temp °C | $k_{obsd}$ ($min^{-1}$) | Ratio $k_{obsd\,H_3}/k_{obsd\,F_3}$ |
|---|---|---|---|---|
| <u>with enzyme</u> | | | | |
| $(NAG)_6$ | 5.5 | 25 | | 56 ± 6 |
| $(TFAG)_6$ | | | | |
| <u>without enzyme</u> | | | | |
| $pNO_2\phi NAG$ | 5.5 | 78 | $(8.2 \pm 1.2) \times 10^{-4}$ | 40 ± 10 |
| $pNO_2\phi TFAG$ | | | $(2.0 \pm 0.2) \times 10^{-5}$ | |
| $pNO_2\phi NAG$ | 0.53 | 78 | $(1.6 \pm 0.2) \times 10^{-3}$ | 10 ± 2.5 |
| $pNO_2\phi TFAG$ | | | $(1.6 \pm 0.2) \times 10^{-4}$ | |

The hydrolysis of $(TFAG)_6$ by lysozyme, with isolation of $(TFAG)_4$-β-(1-4)-($^{14}$C-NAG) in the presence of $^{14}$C-NAG shows that the bond cleaved in this enzymic reaction is the same as that hydrolyzed in $(NAG)_6$. This shows that the occupancy of the six lysozyme binding subsites (A-F) is the same for the two hexasaccharides. The trisaccharide $(TFAG)_3$ has already been shown to have binding properties to lysozyme similar to chitotriose (71).

The extent that information from model studies can be applied to enzyme catalyzed reactions can only be speculated but it is of interest that at pH 5.5 the rate ratio of enzyme hydrolysis of $(NAG)_6$ to that of $(TFAG)_6$ (56:1) is, within the errors of this determination, the same as that found for the spontaneous hydrolysis of p-nitrophenyl-NAG and p-nitrophenyl-TFAG.

Previous model studies (90) have shown that acid catalyzed and spontaneous hydrolysis of alkyl- and aryl-glycopyranosides, respectively, involve transition states with considerable carbonium ion character. The model studies conducted here, on substitution of trifluoroacetamido for acetamido side chains in NAG glycosides, show that anchimeric assistance does not appear to be important in the rate limiting step of glycoside hydrolysis under the conditions studied.

As mentioned (p. 36 ) lysozyme catalyzed hydrolysis of chitotriose probably involves a rate-limiting step whose transition state resembles a carbonium ion structure. It is unlikely a substrate like $(TFAG)_6$ would have a different transition state from chitin oligomers since side chain participation would be more difficult.

It is therefore considered that the results obtained in this study of model and enzyme catalyzed reaction, comparing the effects of acetamido and trifluoroacetamido groups, show that nucleophilic anchimeric attack does not appear to be an important factor in the transition state of either model reaction for lysozyme catalyzed hydrolysis.

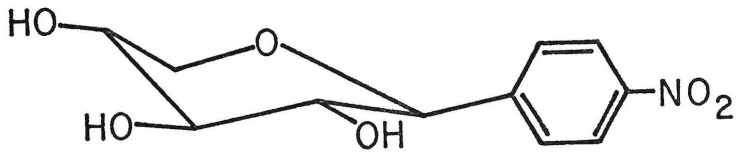
## EXPERIMENT II

### A Kinetic Study of Distortion in Lysozyme Catalysis

From the model building experiments with the crystal structure of the lysozyme-chitotriose complex, the cause of the saccharide distortion in subsite D appears to be the steric interaction of the $C_6$ carbon and its hydroxyl group with the enzyme. As mentioned above (p. 17), chitotetraose does bind more strongly to lysozyme when its reducing terminal residue has been replaced by an N-acetyl-D-xylosamine (60). This is consistent with the proposal that this residue, identical to N-acetyl-D-glucosamine but for the $C_6$ carbon and hydroxyl, can bind to subsite D without distortion and thus actually contribute to the overall binding strength of the complex.

To investigate the effect of distortion on the catalytic mechanism, the lysozyme-catalyzed hydrolysis of a xylose derivative was compared to that of a glucose derivative. Since an N-acetylglucosamine binds much more strongly to subsite C than to any other subsite, the compounds p-nitrophenyl-4-O-(2-deoxy-2-acetamido-β-D-glucopyranosyl)-β-D-glucopyranoside (NAG-Glc-$\phi NO_2$) and p-nitrophenyl-4-O-(2-deoxy-2-acetamido-β-D-glucopyranosyl)-β-D-xylopyranoside (NAG-xyl-$\phi NO_2$) were used. In the dominant binding mode of these compounds, the nonreducing terminal residue is complexed to subsite C, with the adjacent sugar at subsite D. The nitrophenyl group would not be expected to have the same interaction with subsite E as an N-acetylglucosamine residue and probably not contribute much.

In the reaction, a carbonium ion intermediate of the lysozyme-catalyzed hydrolysis would be expected to be distorted toward a half-chair configuration. If distortion does play a role in the catalytic mechanism for these substrates, a portion of the binding energy of the glucose-lysozyme complex would go to distort the glucose ring. Binding of the xylose compound to lysozyme in a productive mode would not involve this distortion. Thus the energy to reach the transition state for xylose would include a much larger component for distorting the pyranose configuration from the chair form than would that of the glucose. Hence, if this factor is the dominant difference between these two compounds, the xylose derivative would be expected to be hydrolyzed slower in a lysozyme-catalyzed reaction. The structure of p-nitrophenyl-D-xylopyranoside is shown:




Materials

p-Nitrophenyl-β-D-xylopyranoside was purchased from Gallard-Schlesinger Chemical Mfg. Corp. Hen egg-white lysozyme, recrystallized three times, and chitin were obtained from Sigma Chemical Co. Bio-Gel P-2 and P-4 gels were supplied by Bio-Rad

Laboratories. p-Nitrophenyl-4-O-(2-deoxyacetamido-β-D-glucopyranosyl)-β-D-glucopyranoside has been prepared according to a published procedure (65). The Amberlite MB-1 used was analytical grade from Mallinckrodt.

Techniques

A quantitative ninhydrin test (120) was used to determine the concentration of amines. A ferro-ferricyanide test (121) was used to determine the reducing sugar concentration.

Visible and ultraviolet absorbances were determined with a Gilford Model 240 spectrometer. The pH measurements were made on a Radiometer pH meter. A thermostated water bath was used to maintain constant 40°. The lab bench centrifuge was an Adams Safe-guard centrifuge and the larger centrifuge was a Sorvall Superspeed RC-2B. The lyophilizer was a Virtis product.

Preparative Methods

N-Acetylglucosamine-xylose-p-nitrophenol was prepared using conditions of a similar reaction reported earlier (65). p-Nitrophenyl xylose (2.0 g), lysozyme (763 mg) and chitotetraose (763 mg) were added to a solution of 164 ml sodium citrate buffer (0.1 M, pH 5.5) and 16 ml dioxane in a 250 ml volumetric flask. Several drops of toluene were added as a bacteriocide. The reaction was allowed to stand for 18 hr. It was then concentrated in vacuo to about 70 ml and loaded on a Bio-Gel P-4 column (5 × 180 cm). Separation is shown in Figure 14.

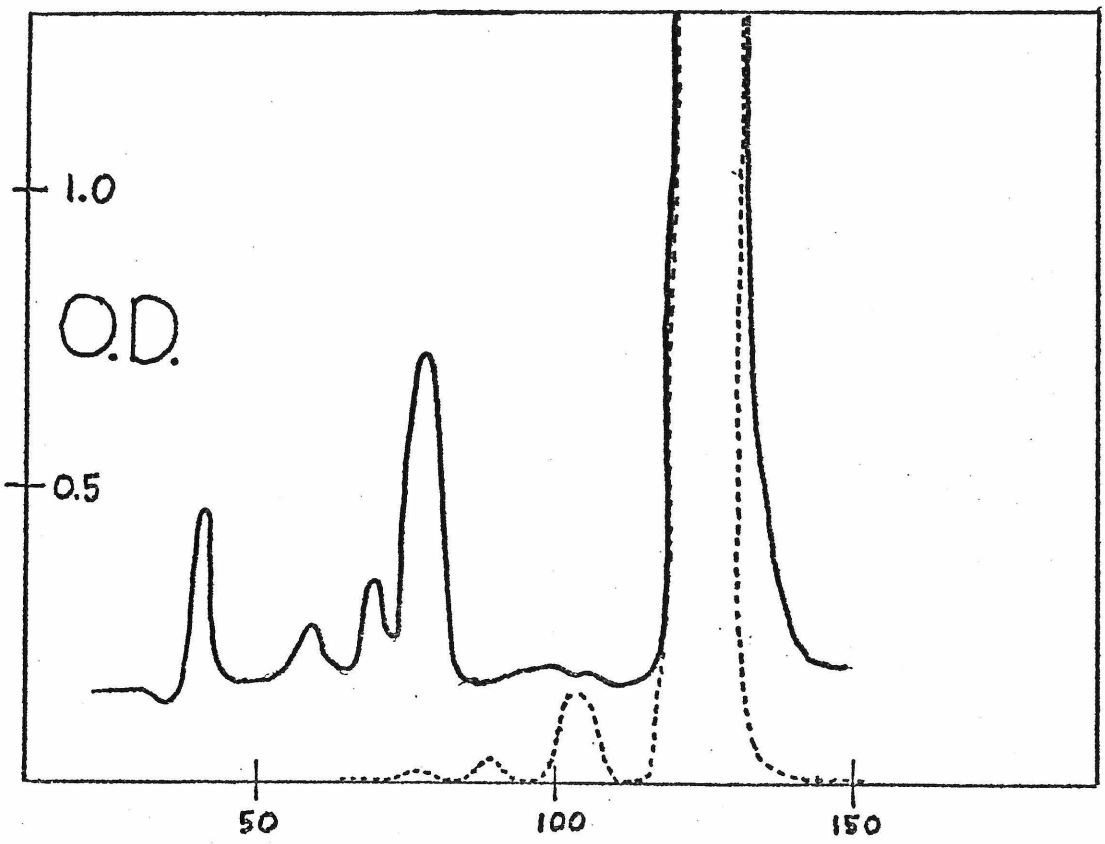



Figure 14. Separation by P-4 gel chromatography (5 × 180 cm, eluted with water) of the reaction mixture of 2.0 g $pNO_2\phi$Xyl, 763 mg lysozyme and 763 mg chitotetraose in 180 ml of 10% u/v dioxane-sodium citrate buffer (0.1 M, pH 5.5) after incubation 18 hours at 40°. Fraction size was 20 ml. Solid line is plot of absorbance readings at 700 nm of the reducing sugar test (5 μl samples). The broken line is a plot of absorbance at 280 nm of the sample dilute 100 fold. The second peak from the right on the broken line plot was interpreted to be due to NAG-Xyl-$\phi NO_2$.

Fractions 96 through 111 were pooled and 80 ml of Amberlite MB-1 resin was added. This solution was shaken vigorously for 90 seconds and filtered immediately through a sintered glass funnel. The absence of chloride was determined by $AgNO_3$ test. The solution was lyophilized and 140 mg of product was recovered. Two additional desalting steps were required, each using approximately the same milliequivalent of mixed bed resin as of salt impurity in the sample. Purity of sample was determined by measuring the absorbance at 400 nm in alkaline solution of a sample of the acid hydrolysate of a weighed amount of material. An extinction coefficient of 18,000 was used for the p-nitrophenolate anion.

Chitotetraose was prepared from chitin. The chitin was acid hydrolyzed (114) and chitotetraose was separated (123) by methods described earlier (p. 45 ).

Analytical Methods

One milliliter of dioxane was diluted to 10 ml with sodium citrate buffer (0.1 M, pH 5.00, room temperature) to make a standard buffer. (Dioxane solubilizes the substrate while does not change lysozyme conformation at this per cent solution (126).) The buffer for the enzyme solution was prepared by first mixing a pH 5.00 sodium citrate buffer (0.1 M) $3.6 \times 10^{-3}$ M in lysozyme. This buffer was filtered with glass wool and centrifuged on the Sorvall to remove insoluble material. One milliliter of dioxane was diluted to 10 ml with this solution for the standard enzyme buffer used in the kinetic study.

Saccharide samples were carefully weighed to an accuracy of ±0.02 mg and placed in vials. Reaction was initiated by adding the appropriate (with or without enzyme) standard buffer. The vial caps were screwed on using sheet paraffin as a seal. The vials were incubated at 40° in a water bath. The samples withdrawn (100 μl) at certain time intervals were diluted with 1.00 ml 0.1 M potassium tetraborate buffer (pH 9.2). The solution was cleared of any suspension using a desk top centrifuge and the absorbance was measured at 400 nm.

## Results

The initial rate of reaction, corrected for nonenzymatic hydrolysis, was measured for four different substrate concentrations for NAG-Glc-$\phi NO_2$ and for NAG-Xyl-$\phi NO_2$. This data was plotted as $\frac{1}{V}$ against $\frac{1}{S}$ in order to use the Lineweaver-Burk expression to obtain $K_m$ and $V_{max}$ values (128). The plot for each compound is shown in Figure 15.

The maximal velocities and the Michaelis equilibrium constants for the compounds are presented in Table III. The $K_m$ values are similar but the $V_{max}$ of NAG-Glc-$\phi NO_2$ is 400-fold greater that of NAG-Xyl-$\phi NO_2$.

## Discussion

The data presented seems to indicate that the two compounds NAG-Glc-$\phi NO_2$ and NAG-Xyl-$\phi NO_2$ bind to lysozyme with equal strengths but are hydrolyzed at different rates. NAG binding to

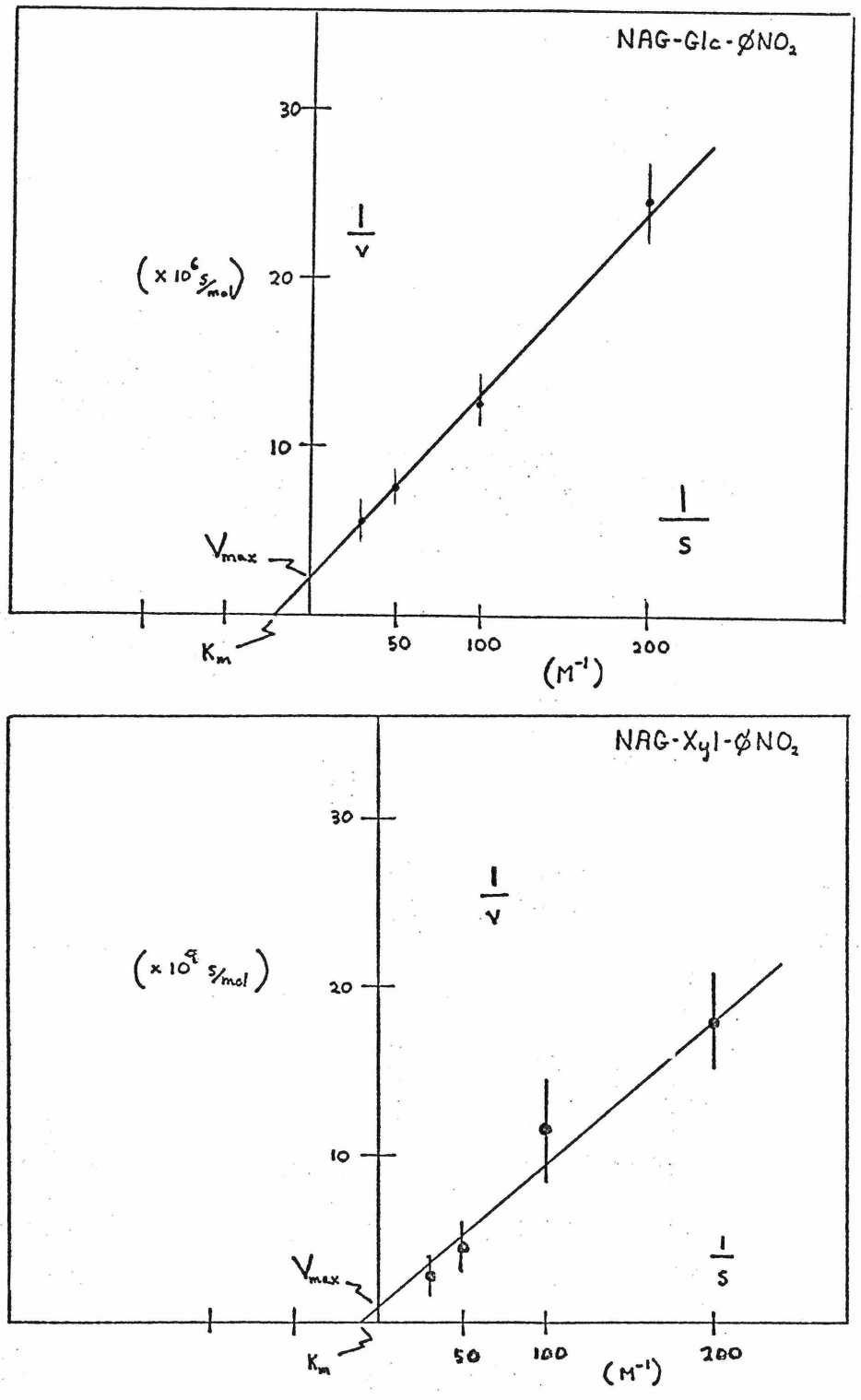



Figure 15. Lineweaver-Burk Plot of Hydrolysis Data (pH 5.0, 40°).

Table III

| | | |
|---|---|---|
| NAG Glc $\phi NO_2$ | | |
| | $V_{max}$ | $(4.2 \pm .8) \times 10^{-7}$ mol/sec |
| | $K_m$ | $(4.6 \pm .8) \times 10^{-2}$ M |
| NAG Xyl $\phi NO_2$ | | |
| | $V_{max}$ | $(1 \pm .5) \times 10^{-9}$ mol/sec |
| | $K_m$ | $(7.1 \pm 1.5) \times 10^{-2}$ M |

For the reaction: 1 ml $3.6 \times 10^{-3}$ M lysozyme, pH 5.0, 40°

subsite C is a likely reason for the similarity of binding of the two compounds. In both cases, the observed $K_m$ may possibly be a $K_s$ for a nonproductive binding mode in the proper subsites but not with proper orientation for catalysis. If, however, the slower rate of hydrolysis of the xylose compound is caused by the catalytic step rather than the binding step, the interaction in subsite D is the cause of the difference. Evidence is strong that the xylose residue is distorted little on binding in a productive mode with lysozyme while the glucose residue is distorted significantly. Since the distortion is toward a configuration which would be of lower energy in the transition state of the lysozyme general acid catalyzed reaction, it would tend to increase the rate of that reaction. The fact that the $V_{max}$ for the enzyme-catalyzed hydrolysis of the compound which binds with distortion is four hundred times that of the non-distorted compound, supports the interpretation that distortion is important in the mechanism of lysozyme catalysis of these two compounds.

EXPERIMENT III

Synthesis of Glc-Glc-NAG

A compound was designed to be attached covalently to subsites ABC through a peptide bond with Asp 101. This compound, Glc-Glc-NAG, would probably interact with the lysozyme cleft in a configuration similar to chitotriose due to the binding of NAG to subsite C. Where in chitotriose the acetamido on the subsite A sugar residue seems to share a hydrogen bond with the Asp 101 carboxylate group (Figure 3), in this new compound the amine replacing the acetamido would probably form this bond. Activation of the carboxyl with a carbodiimide reagent (127) would make the formation of an amide bond at this position highly likely.

Glc-Glc-NAG was synthesized.

Materials

Chitin and hen egg-white lysozyme were obtained from Sigma Chemical Co. Trifluoroacetic anhydride was supplied by Matheson Coleman and Bell Co. and the Bio-Gel P-4 by Bio-Rad Laboratories. Radiolabeled compounds were prepared with acetic anhydride-1-$^{14}$C from Lot No. 1175-21 of Volk Radiochemical Co.

Techniques

Techniques were the same as in Experiment I (p. 41 ).

Results

The trisaccharide was prepared in the following manner:

Chitin was deacetylated by a KOH fusion reaction at 170° (118). The chitosan obtained was hydrolyzed in 4 M HCl at 100° for about 26 hr (118). The glucosamine oligomers were trifluoroacetylated with trifluoroacetic anhydride in dry acetonitrile for 12 hr. The oligomers were de-O acetylated by treatment with methanol for several days, and recovered by lyophilization from water.

The TFAG oligomers were separated by P-4 polyacrylamide gel filtration, eluting with water (see Figure 9). Some of the oligomers were used in fluorine-nmr experiments investigating the lysozyme-substrate analog complex. The longer chains (longer than trisaccharides) were reacted with lysozyme and $^{14}$C-NAG in pH 5.5 citrate buffer 0.1 M NaCl for 16 hr. The radiolabeled NAG was prepared according to the method of Horton (115). The reaction mixture was separated on the same P-4 column and the transfer products were observed by $^{14}$C measurement (Figure 16).

The fractions containing the trimer TFAG-TFAG-NAG were pooled and desalted using P-4 gel chromatography. These fractions were, of course, contaminated with TFAG-TFAG-TFAG. The mixture was de-trifluoroacetylated by treatment with pH 12 LiOH solution for 8 hr.

The Glc-Glc-NAG was separated from Glc-Glc-Glc by carboxymethyl cellulose ion-exchange chromatography using a 0.05 M to 1.0 M LiCl gradient. This is adapted from a previously published

procedure (129). Monitoring the separation by radioactivity, ninhydrin and reducing sugar tests, the fractions with Glc-Glc-NAG were identified. The compound was desalted with P-4 gel chromatography. This chromatographic system, eluting with water, was capable of separating the mono-, di- and triamine trisaccharides, thereby serving as a further indication of the homogeneity of the compound synthesized.

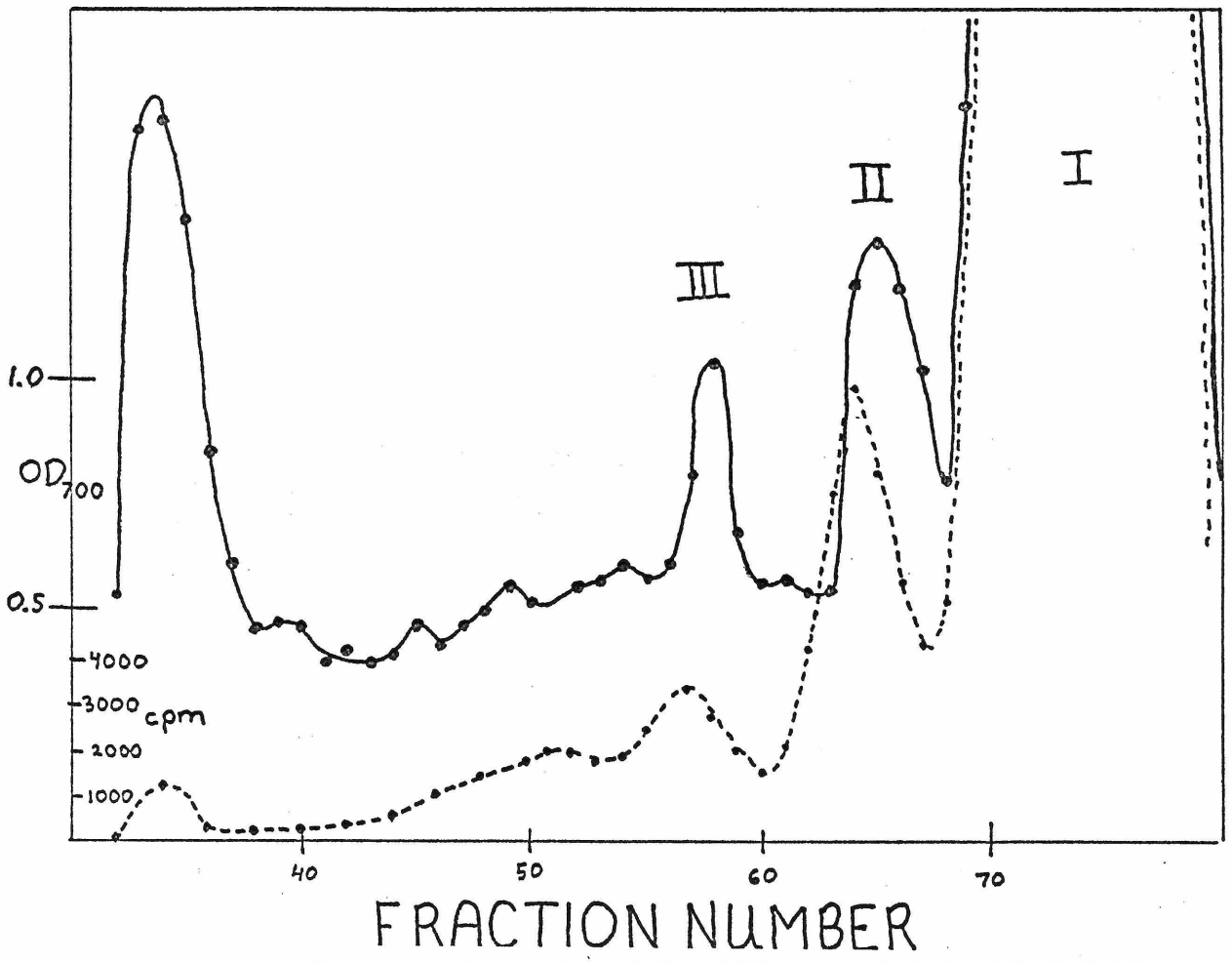



Figure 16. Separation by P-2 chromatography (4.0 × 180 cm, eluted with 0.1 M ammonium formate buffer) of the reaction mixture of 263 mg TFAG oligomers longer than tetramer, 400 mg lysozyme and 643 mg $^{14}$C-NAG (about $2 \times 10^7$ cpm) in 25 ml 0.1 M sodium citrate buffer, pH 5.5 for 15 hr at 37°. Fraction size is 15 ml. Solid line is a plot of absorption from reducing sugar test (100 μl sample). Broken line is plot of radioactivity measurement on 250 μl samples. Broken line peak in trisaccharide region (III) was assigned to TFAG-TFAG-NAG.

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