THE BINDING AND CATALYTIC PROPERTIES OF LYSOZYME

Thesis by

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To Marcy--soul mate and friend.

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ABSTRACT

The binding and catalytic properties of hen's egg white lysozyme have been studied by a variety of techniques. These studies show that the enzyme has three contiguous binding subsites, A, B, and C. The application of nuclear magnetic resonance (NMR) spectroscopy to probe the binding environment of several saccharides to lysozyme has demonstrated that the reducing end sugar rings of chitotriose, chitobiose and the β-form of N-acetylglucosamine all bind in subsite C. The central sugar ring of chitotriose and the sugar ring at the nonreducing end of chitobiose were found to bind in subsite B, while the nonreducing end sugar residue of chitotriose occupied subsite A. The dynamics of the binding process has also been investigated by NMR. The formation rate constant of chitobiose--and chitotriose-enzyme complexes were found to be about 4×10^{-6} M^{-1} sec^{-1} with small activation energies.

The stereochemical path of the lysozyme catalyzed hydrolysis of glycosidic bonds has been shown to proceed with at least 99.7% retention of configuration at C-1 of the sugar. The lysozyme catalyzed hydrolysis of glucosidic bonds has been shown to be largely carbonium ion in character by virtue of the α-deuterium kinetic isotope effect ($k_H/k_D = 1.11$) observed for the reaction. It is probable that the mechanism of action of the enzyme involves a carbonium ion intermediate which is stereospecifically quenched by solvent. However, acetamido group participation cannot be ruled out for natural substrates.

TABLE OF CONTENTS

INTRODUCTION

In 1922, Fleming discovered an enzyme which was capable of lysing suspensions certain bacteria. Although the protein is found in extracts from many sources (most mammalian tissue, avian egg white, papaya latex, bacteriophages), the most readily available, and therefore the one most studied is obtained from hen's egg white. The homogeneity of this protein has been established by a variety of physical and chemical methods (Jolles, 1964b), and it is therefore well suited to a detailed structural analysis.

Canfield (1963, 1965) and Jolles (1963, 1964a) have elucidated the sequence of the 129 amino acids as well as the nature of the four disulfide bonds in the molecule by chemical means. These results agree well with each other and the few discrepancies in sequence have been resolved by the x-ray crystallographic analysis studies of Blake et al., (1967a) at 2 Å resolution. The accepted primary structure of the enzyme is shown in Fig. 1.

The tertiary structure of the crystalline enzyme (Blake et al., 1965, 1967a) at 2 Å resolution shows lysozyme to be a generally elliptical molecule with a fairly well defined cleft on one side. The molecule has a comparatively small proportion of α-helix and large stretches of fairly irregular structure. There are several regions of extended conformation which had been seen previously in only the β-form of fibrous proteins. Most of the polar residues are located on the surface of the molecule. The two exceptions are glutamine 57

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	LYS	VAL	PHE	GLY	ARG	CYS	GLU	LEU	ALA	ALA	ALA	MET	LYS	ARG	HIS
16	GLY	LEU	ASP	ASN	TYR	ARG	GLY	TYR	SER	LEU	GLY	ASN	TRP	VAL	CYS
31	ALA	ALA	LYS	PHE	GLU	SER	ASN	PHE	ASN	THR	GLN	ALA	THR	ASN	ARG
46	ASN	THR	ASP	BLY	SER	THR	ASP	TYR	GLY	ILU	LEU	GLN	ILU	ASN	SER
61	ARG	TRP	TRP	CYS	ASN	ASP	GLY	ARG	THR	PRO	GLY	SER	ARG	ASN	LEU
76	CYS	ASN	ILU	PRO	CYS	SER	ALA	LEU	LEU	SER	SER	ASP	ILU	THR	ALA
91	SER	VAL	ASN	CYS	ALA	LYS	LYS	ILU	VAL	SER	ASP	GLY	ASP	GLY	MET
106	ASN	ALA	TRP	VAL	ALA	TRP	ARG	ASN	ARG	CYS	LYS	GLY	THR	ASP	VAL
121	GLN	ALA	TRP	ILU	ARG	GLY	CYS	ARG	LEU	*					

COMPOSITION

12 ALA	8 CYS	8 ASP	2 GLU
3 PHE	12 GLY	1 HIS	6 ILU
6 LYS	8 LEU	2 MET	13 ASN
3 TYR	2 PRO	3 GLN	11 ARG
10 SER	7 THR	6 VAL	6 TRP

Figure 1. The primary structure of hen's egg white lysozyme

*Disulfide bridges are between links 6-127, 30-115, 64-80, and 76-94.

which forms a hydrogen bond to the main peptide chain and serine 91 which seems to hydrogen bond to two water molecules within the enzyme. There are several hydrophobic groups on the surface of the molecule, and a large proportion of these are at the surface of the cleft. These include three tryptophane, one valine and one isoleucine residues. The cleft is of particular interest since substrate molecules bind in that region.

There have been a great number of chemical modifications of the lysozyme molecule but only a few concrete results have been obtained from these studies (for a recent review see Jolles, 1967). These results suggest that one or more tryptophane residue is involved in the binding process or confers structural stability on the enzyme. Aside from reagents which affect tryptophane residues, the enzyme is remarkably stable to a variety of treatments (Parsons, et al., 1968). Parsons and Raftery (1968) have recently shown that esterification of one carboxyl group of an aspartic acid residue results in loss of catalytic activity, while the binding process is only slightly inhibited. This strongly suggests that this aspartic acid residue is involved in the catalytic process.

Despite the ready supply and stability of lysozyme, the nature of the bonds cleaved by the enzyme has been determined only recently. Salton (1952) demonstrated that the substrate is located in the bacterial cell wall. Berger and Weiser (1957) showed that lysozyme degrades chitin, a β (1 $\rightarrow$4) linear polymer of N-acetyl-D-glucosamine (NAG). Salton and Ghuysen (1959) isolated a tetrasaccharide from a lysozyme digest of isolated cell walls of Micrococcus lysodeikticus which

contained equi-molar amounts of NAG and a new sugar, N-acetyl-muramic acid (NAM). Upon incubation with lysozyme, the tetra-saccharide released a disaccharide which contained NAG and NAM. It was concluded that the bond cleaved was a β(1 →4) linkage between the NAG-NAM disaccharides. Jeanloz et al. (1963) synthesized a β(1-6) linked NAG-NAM which was not identical to the naturally occuring material. They suggest that the linkage in the natural dimer is (1-4). Thus, the structure of the substrate for the enzyme in the cell walls seems to be the linear polymer of the disaccharide NAG-NAM with all β(1 →4) linkages. The free carboxyl group of some of the NAM residues appears to form an amide bond to a peptide which is highly cross-linked and lends structural stability to the cell wall (Sharon, 1967). The proposed structure is shown in Fig. 2.

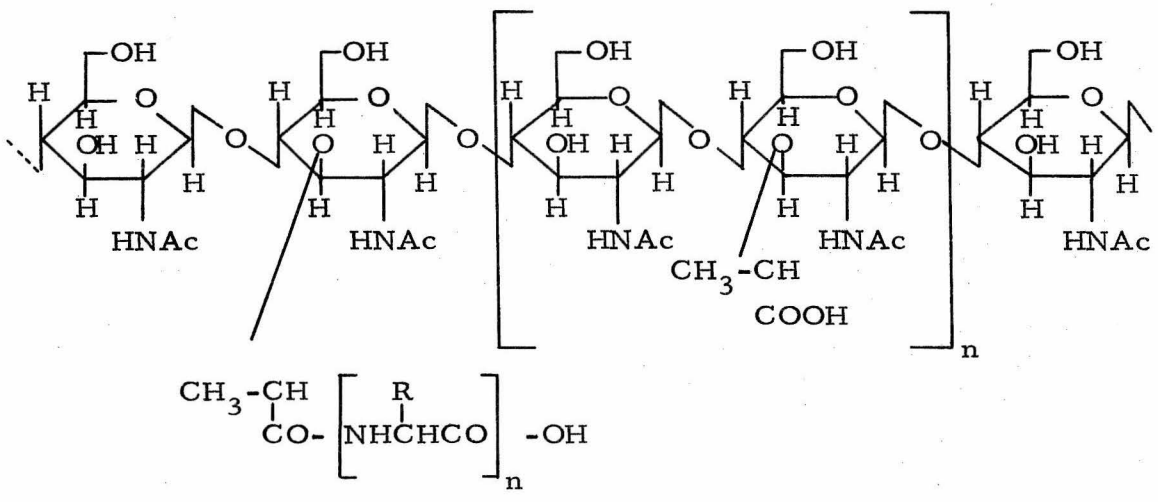

Figure 2. The structure of bacterial cell wall polysaccharide.

The structure of the natural substrate is remarkably similar to that of chitin, since NAM is the 3-0-(D-1-carboxyethyl) ether of NAG.

Because of the great difficulty in obtaining large amounts of the natural substrate, most studies of the catalysis by lysozyme have been concerned with chitin oligosaccharides and their derivatives. Chitobiose, chitotriose, chitoteraose, chitopentose, and chitohexaose are all substrates for lysozyme (Rupley, 1965). Rupley (1965, 1967) has determined the relative rates of hydrolysis of these compounds to be 0.003: 1 : 8 : 4000 : 30000, respectively, for substrate concentrations of 10^{-4} M. Lysozyme is also known to possess transferase activity with chitin oligosaccharide substrates (Dahlquist and Raftery, 1967; Rupley, 1964, 1967) such that high molecular weight, insoluble material is formed in these reactions as well as NAG. Transglycosylation has also been observed with cell wall oligosaccharide substrates (Sharon, 1967).

Blake et al. (1967b) have studied the relative binding orientations of several saccharides to the crystalline enzyme by difference Fourier methods. Such studies are possible because the enzyme-saccaride complexes are isomorphous with the native protein. The binding orientations of NAG, NAM, 6-iodo-α-methyl-NAG, α-benzyl-NAM, chitobiose, NAG-NAM and chitotriose have all been studied at low resolution (6 Å). The results of these studies indicate that all these saccharides are bound to the main cleft in the surface of the enzyme. This cleft is quite extensive and the sugars appear to occupy a number of subsites within the cleft. The authors find that the sugars can bind in six positions within the cleft and sum up their results as follows:

(a) The difference density due to NAG is too extensive to represent one NAG in a definite location.

(b) 6-iodo-α-methyl NAG binds to only one of the NAG sites.

(c) NAM appears to bind to two adjacent sites.

(d) Chitobiose binds in two ways which have not been distinguished.

(e) NAG-NAM binds in a manner similar but not identical to one of the binding modes of chitobiose.

(f) Chitotriose is bound stably covering three apparent binding sites.

(g) α-Benzyl-NAM is bound in a site which is in doubtful contact with the enzyme surface.

The NAG and chitotriose complexes have been studied at high resolution (2 Å). The studies on the NAG complex shows that the α- and β- forms of the sugar do not bind in exactly the same manner. The main difference is that α-NAG forms a hydrogen bond between 0-1 and the peptide NH of residue 109, which the β-compound does not form. The high resolution study of the trisaccharide complex shows that the reducing end sugar residue makes the same contacts as does β-NAG with the other sugar residues running up the cleft. These conclusions are summarized in Fig. 3. The binding subsite for the nonreducing end of the trisaccharide (subsite A) makes nonpolar interactions with the sugar and a hydrogen bond between the NH of the acetamido group of the sugar residue and the side chain of aspartic acid 101. Subsite B, which interacts with the central sugar residue of the trisaccharide, also makes nonpolar contacts with the substrate as well as a hydrogen bond between 0-6 and aspartic acid 101.

From the stability of the trisaccharide complex, it seems clear

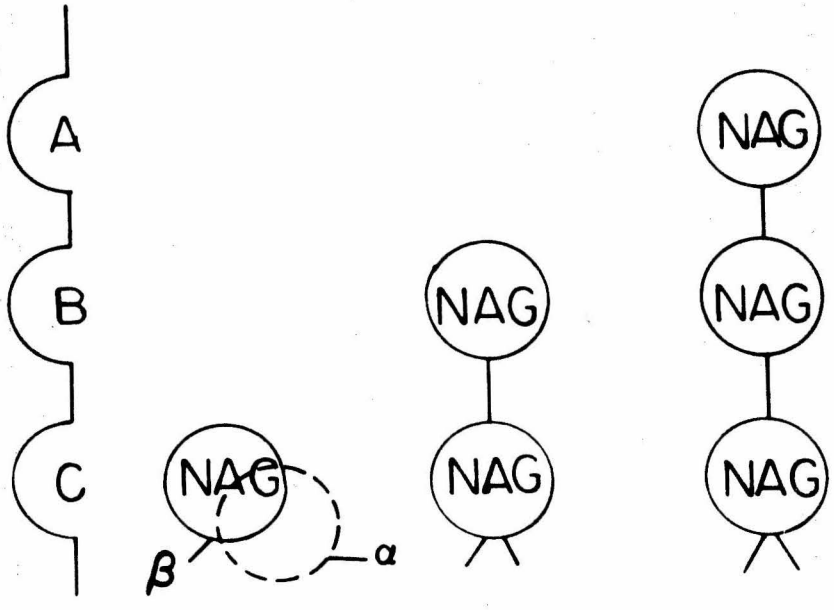

Figure 2. The relative binding orientations of N-acetyl glucosamine, chitobiose and chitotriose.

that the preferred binding mode is unproductive, and does not relate directly to the active site of the enzyme. Blake et al. (1967b) have concluded from careful model building experiments that the binding cleft can accommodate up to six sugar residues. These subsites include A, B, and C which are involved in the strong, unproductive mode of chitotrose binding and three more (D, E, F) which run down from the cleft subsite C. If binding occurs in subsite D, there are steric interactions which would force the saccharide in that subsite out of the normal chair conformation and into a conformation in which C-6 is in an axial position. Sugar rings may bind in subsites E and F without steric interactions of this sort and some favorable interactions are indicated as well, from model building studies.

Although the high resolution study of the chitobiose and NAM lysozyme studies has not been completed, the low resolution studies allow placement of these substrates in the binding area. This places chitobiose in subsites B and C. Because of interactions between the hydroxyl group at position 3 in NAG and subsite C, it is impossible for NAM to bind in that subsite with its bulky lactyl group at C-3. The low resolution studies place NAM in subsite D and perhaps weakly in B. The binding of NAG-NAM appears to be in subsites C and D and another mode which is out of alignment with subsites A-F. The authors argue that because the hydrolysis of the NAG-NAM polymer is at the reducing end of NAM, the active site must be between subsites B and C or D and E. Because chitotriose is stable when bound A-B-C, the active site must be between subsites D and E. These arguments are summarized in Fig. 4. Rupley has found that chitohexaose is cleaved by lysozyme

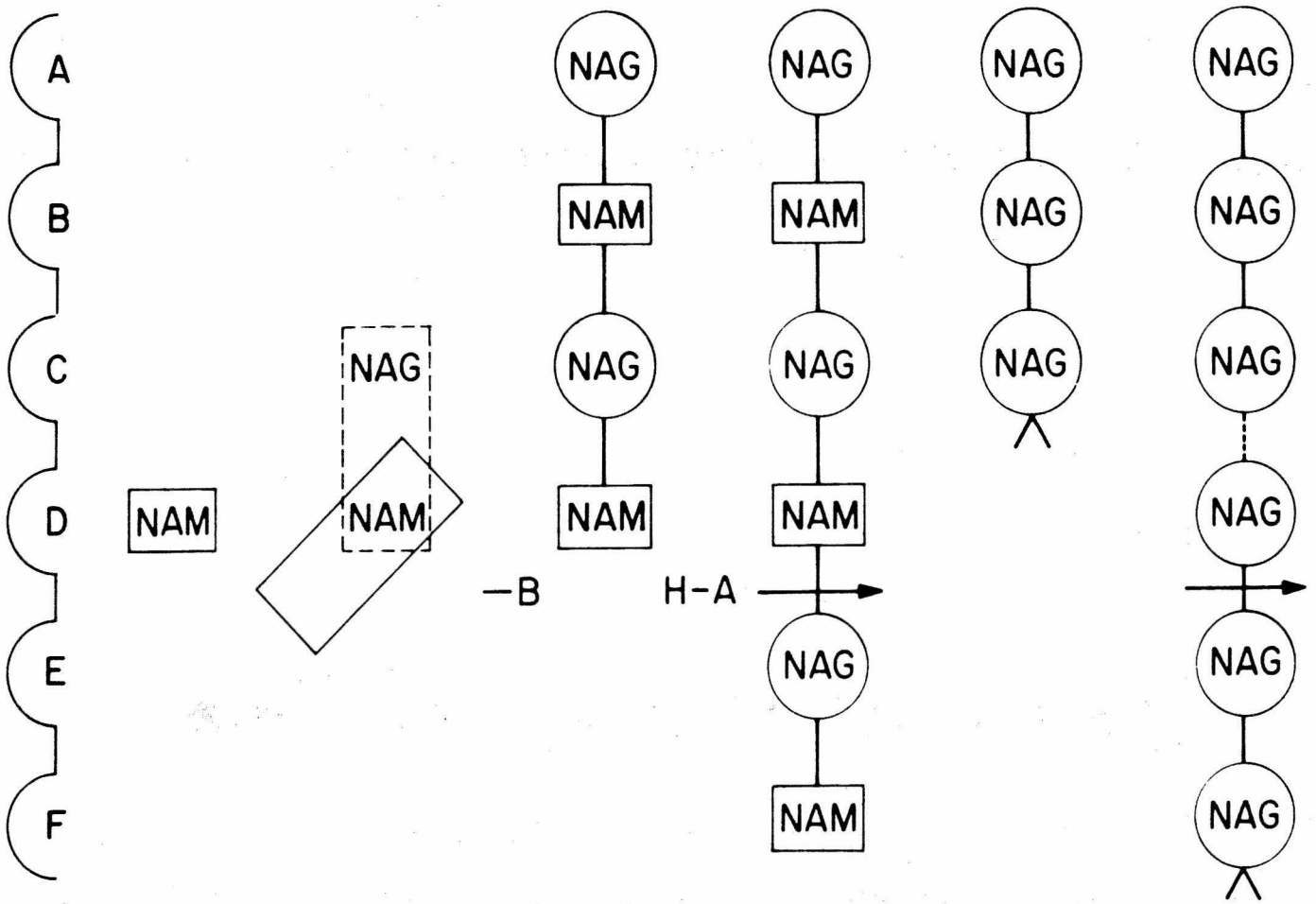

Figure 3. The proposed active site as reduced by Blake et al., (1965, 1967) from model building studies.

at a point two residues from the reducing end of the saccharide. This result can be easily accommodated with the proposed cleavage site in the binding cleft.

In view of the residues situated near residues D and E and the accepted mechanisms for glycoside hydrolysis, Blake et al. (1967b) have proposed a tentative mechanism of action for lysozyme. This theory invokes an ionized carboxyl group in the binding cleft (aspartic acid 52) which facilitates the production of a carbonium ion at C-1 of the sugar residue bound in subsite D. Further, binding in subsite D results in distortion of the sugar ring in that subsite, thus favoring the production of the carbonium ion which relieves this steric interaction. The theory also provides a general acid in glutamic acid residue 35 for protonation of the glycosidic oxygen of the sugar. Similar carbonium ion mechanisms have been suggested by Vernon (1967) for lysozyme and by Mayer and Larner (1958) for glycosidases in general.

Koshland (1953) has pointed out that the hydrolysis of glycosides by certain enzymes may involve the product of covalently bound intermediates. The proposed mechanism involves two bimolecular nucleophilic displacements at C-1 of the sugar. The first of these is attack and displacement by nucleophile on the enzyme to form a covalently bound glycosyl-enzyme intermediate. The enzyme is then displaced by solvent to give the free sugar. This mechanism has been considered by Lowe (1967) in the case of lysozyme.

Piszkiewicz and Bruice (1967, 1968) have demonstrated that the acetamido group of NAG provides anchimeric assistance in the spontaneous hydrolysis of aryl-2-acetamido-2-deoxy-β-D-glycopyranosides.

The authors propose a mechanism involving assistance by the acetamido group of the substrates of lysozyme to be of possible importance for the enzyme catalysed reaction. A mechanism of this sort has also been postulated by Lowe.

A single nucleophilic displacement has also been proposed by Koshland to explain the stereo chemistry of the β-amylase hydrolysis of amylose. This mechanism has been considered by several authors (Lowe, 1967; Piszkiewicz and Bruice, 1967; Bernhard, 1967) to be a possible mechanism for lysozyme.

The fact is that virtually all possible mechanisms have been proposed by various people to explain the action of lysozyme. None of these proposals have been accompanied by any concrete proof of their existance in the case of lysozyme and only in a very few cases for other enzymes.

The work described in this thesis has been divided into two parts. The first of these is concerned with the binding properties of hen's egg white lysozyme. The binding studies have been carried out in solution in an attempt to correlate the results in solution to those in the crystalline state. There has been some controversy over the years as to the relevance of crystallographic descriptions of macromolecules to the solution state. Such binding studies may help answer this question. Lysozyme is a particularly good system in which to study this problem because there are many substrates and inhibitors which are proposed to bind in different relative orientations to the crystalline enzyme and there is reason to believe that relative binding modes may be distinguished in solution.

The second part of this thesis is concerned with the catalytic properties of lysozyme. Mechanistic interpretations of enzyme action have been satisfactory in only a very few cases. As in the case of lysozyme, one may find virtually every conceivable mechanism in the literature, and very little accompanying evidence. The mechanistic work described here has been directed toward results which can reasonably hope to distinguish between the wealth of possible mechanistic paths for lysozyme.

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PART I

THE BINDING PROPERTIES OF LYSOZYME

CHAPTER I

THE BINDING OF CHITIN OLIGOSACCHARIDES TO LYSOZYME BY ULTRAVIOLET DIFFERENCE SPECTROSCOPY

INTRODUCTION

In enzyme reactions the binding of substrates to a specific site on the enzyme surface plays a major role in determining the specificity of the catalytic reaction. This phenomenon of binding is probably the least understood of the complex series of interactions involved in enzymic catalysis. In the Michaelis-Menten (1913) scheme of enzyme kinetics the quantity K_m is considered a measure of the binding process. It is desirable, however, to have independent methods of studying binding of substrates and inhibitors to enzymes. Such a study is possible if interaction between an enzyme and a substrate results in a perturbation which can be quantitatively determined. In this respect it is of interest that Hayashi et al. (1963) have shown that lysozyme (E. C. 3.2.1.17) displays a red-shift in its ultraviolet spectrum on interaction with a substrate (glycol chitin) or an inhibitor (N, N'-diacetyl chitobiose). The spectral red-shift has been associated with a change in the environment of at least one tryptophanyl residue. From solvent perturbation measurements (Hayashi et al., 1963; 1964) it has been proposed that such red-shift in a tryptophane spectrum is consistent with its removal from an exposed, solvent accessible region, to a less polar environment.

There is ample evidence for the occurrence of tryptophanyl residues at the binding site of lysozyme. The recent structure determination for the enzyme in the crystalline state (Blake et al., 1965) and the identification of the region of the enzyme to which inhibitors bind (Johnson and Phillips, 1965) by x-ray analysis methods have shown that three tryptophanes occur at the binding site. Chemical evidence (Hayashi et al., 1965; Hardegan and Rupley, 1964; Parsons et al., 1968) also confirms the presence of tryptophane residues in this region. It is thus a reasonable assumption that the tryptophane(s) responsible for the observed red-shift (Hayashi et al., 1963), on interaction of lysozyme with substrates and inhibitors, is intimately related to the binding site and that a study of this phenomenon should provide some insight into the binding process. In the present communication, we report the application of this spectral change for the quantitative measurement of binding equilibria of substrates and inhibitors to lysozyme.

From initial studies it became clear that the magnitude of the difference spectrum was related to the amount of inhibitor added to a constant amount of enzyme, and was in fact a measure of the concentration of enzyme-substrate complex formed. This observation provides us with a method for calculation of the binding constants for inhibitors and substrates of lysozyme.

MATERIALS AND METHODS

Lysozyme was purchased from Sigma Chemical Company (Lot #105B 8700). Chitin oligosaccharides was prepared by acid hydrolysis of chitin (California Corporation for Biochemical Research) followed by chromatography according to Rupley (1964). These saccharides were further purified by gel filtration on Bio Gel P-2.

All difference spectra were measured in a Cary Model 14 spectrophotometer, by the methods of Williams et al. (1965) using a 0-0.1 absorbance slidewire assembly at 25°C ± 0.1°C. All enzyme concentrations were 0.36 mg/ml (2.48×10^{-5} M) and had an absorbance of 0.95 at 280 mμ. Substrate concentrations were varied from 5×10^{-3} M to 1×10^{-6} M. The phosphate buffers employed were constant in sodium ion concentration (0.1 M) and the total phosphate concentration was constant at 0.01 M. All pH measurements were made with a Sargent Model DR pH meter at 25°C ± 0.1°C.

RESULTS AND DISCUSSION

An example of the difference spectrum obtained on interaction of lysozyme and chitotriose at pH 5.0 is shown in Fig. 1A. The maximum difference was obtained at 293.5 mμ. Figure 1A-E shows how the magnitude of the difference spectrum changed with decreasing chitotriose concentration while keeping the lysozyme concentration constant. This variation of peak height with substrate concentration amounts to a titration curve of enzyme.

The region of high substrate concentration, where the magnitude of the difference spectrum remains constant, corresponds to saturation of the enzyme with substrate. The ratio of peak height (ΔA_0) at saturation to the peak height (ΔA) at some other substrate concentration is a measure of the amount of enzyme-substrate complex, ES, at that substrate concentration.

$\frac{\Delta OD}{\Delta OD^0} = \frac{[ES]}{[E^0]}$, and E^0 is known so the concentration of ES can be calculated. The amount of free substrate present can be estimated since $[S] = [S^0] - [ES]$.

The following scheme was employed for calculation of the binding constant K_s.

$$E + S \rightleftharpoons ES$$

$$K_s = \frac{[E][S]}{[ES]}$$

$$\log K_s = \log \frac{[E]}{[ES]} + \log [S]$$

A plot of log S versus log $\frac{[ES]}{[E]}$ gives a line with an intercept of - log K_s or pK_s. Such a plot for binding of chitotriose to lysozyme at pH 5.5 is protrayed in Fig. 2, which shows that the slope equals one, indicating one molecule of chitotriose binding to the enzyme. The dissociation constant as determined from this plot is 8×10^{-6} M. Table 1 shows the results of similar determinations on a series of chitin oligosaccharides. The results show that the minimal structure necessary for good binding is that of the trisaccharide N,N',N''-triacetylchitotriose.

Since chitotriose and the higher oligosaccharides are substrates for lysozyme it was necessary to estimate the extent of any hydrolysis undergone while the dissociation constants were being determined. We have been able to show by measurement of the increase of saccharide reducing groups (Dische, 1962), that less than 0.2 per cent hydrolysis of chitotriose occurred during the spectral determinations. For the tetrasaccharide approximately 1.0 per cent hydrolysis was observed. In contrast to this negligible degradation, the penta- and hexasaccharides were cleaved to the extent of 20 per cent and 50 per cent, respectively. From these results it is obvious that the dissociation constants for the penta- and hexasaccharides represent values obtained from mixtures of saccharides. It cannot be concluded therefore that the binding strength of the penta- and hexasaccharides is equal to that of the trisaccharide and the tetrasaccharide.

The effects of ionic strength on the binding process were determined at pH 5.0 using tri-N-acetyl chitotriose as a substrate and the

results which are shown in Table 1 indicate no changes on increasing the ionic strength of the buffers used.

The determinations of K_s show that only one substrate molecule per lysozyme molecule is bound at that site which is responsible for the difference spectrum. This does not exclude the possibility of binding at other sites with no change in light absorption. Since the spectral change is believed to be due to a perturbation of the tryptophane spectrum, we conclude that the region of the lysozyme molecule, where the tryptophane(s), whose absorption spectrum has been perturbed occurs, includes only one binding site. Furthermore the stoichiometry of the reaction with all substrates studied requires that this binding site which we are studying be the strongest binding site on the enzyme.

The determination of the dissociation constant using chitotriose as a substrate has been extended to include several pH values and the results plotted as $-\log K_s$ (pK_s) versus pH, as recommended by Dixon (1953, 1958), are shown in Fig. 3. Dixon has given a theoretical treatment to the effects of pH on substrate (or inhibitor) binding to enzymes, and has devised a set of rules for their interpretation. By application of these rules to the data shown in Fig. 3 we can say that two ionizable groups on the enzyme are perturbed by the presence of the trimer substrate. The pK_a values involved are one of 4.2 for a group on the enzyme which is perturbed to a value 3.55 on the enzyme-substrate complex and a second group of pK_a 5.8 on the enzyme which is changed to a value of 6.25 on the enzyme-substrate complex. The values observed suggest that the group of pK_a 4.2 on the enzyme is probably a carboxyl side chain of aspartic or glutamic acids. The group of higher

pK_a value (5.8) is consistent with assignment to the imidazole group of histidine or possibly a carboxyl group of abnormally high pK_a. In this respect it is of interest that the structure of lysozyme in the crystalline state, as deduced by x-ray analysis (Blake et al., 1965), shows that several aspartyl side chains occupy positions at the binding site of the enzyme while the single histidine in the molecule occurs at a position far removed from the binding site. The titration data of Donovan et al. (1960) on lysozyme indicate a carboxyl group of pK_a 6.3, which is abnormally high. These results indicate that the second group on the enzyme, (pK_a = 5.8) which is perturbed on interaction with chitotriose, may also be a carboxyl group. However, configuration changes caused by the ionization of histidinyl imidazole could also give rise to the pH dependency in the pH region 5.5 to 6.5

The theory of Dixon (1953, 1958) states that if one ionic form of an enzyme binds substrate more strongly than another, there will be proportionally more of this form present in the enzyme-substrate complex than in the free enzyme. This amounts to a change in the ionization constant for the ionizable group(s) involved. This does not require that the perturbed group(s) be actually involved in the binding process but rather than the binding is affected by the ionization of these groups. It could be a result of configuration changes which involve ionizable groups some distance from the binding site. Further studies directed toward identification of the nature of the ionizable group of pK_a 5.8 on lysozyme, which is disrupted on binding of the trisaccharide, are described in Chapter IV.

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TABLE 1.--Binding constants of oligosaccharides to lysozyme

Oligosaccharide	K_s, pH 5.0, μ = 0.1 M (dissociation
N-Acetyl Glucosamine	$4 - 6 \times 10^{-2}$ M
Chitobiose	$2.5 \pm 0.5 \times 10^{-4}$ M
Chitotriose (μ = 0.1 M)	$8 \pm 1 \times 10^{-6}$ M
(μ = 0.2 M)	$8 \pm 1 \times 10^{-6}$ M
(μ = 0.3 M)	$9 \pm 1 \times 10^{-6}$ M
Chitotetraose	$8 \pm 1 \times 10^{-6}$ M
Chitopentaose	$10 \pm 1 \times 10^{-6}$ M
Chitohexaose	$8 \pm 1 \times 10^{-6}$ M

Figure 1. Difference spectra of lysozyme in the presence of chitotriose at pH 5.0, 25°C ± 0.1 showing maximum difference (ΔA) at 293.5 mμ. Lysozyme concentration was 2.48×10^{-5} M. Chitotriose concentration was 2.56×10^{-3} M in A, 1.28×10^{-3} M in B, 4×10^{-5} M in C, 2×10^{-5} M in D, 1×10^{-5} M in E.

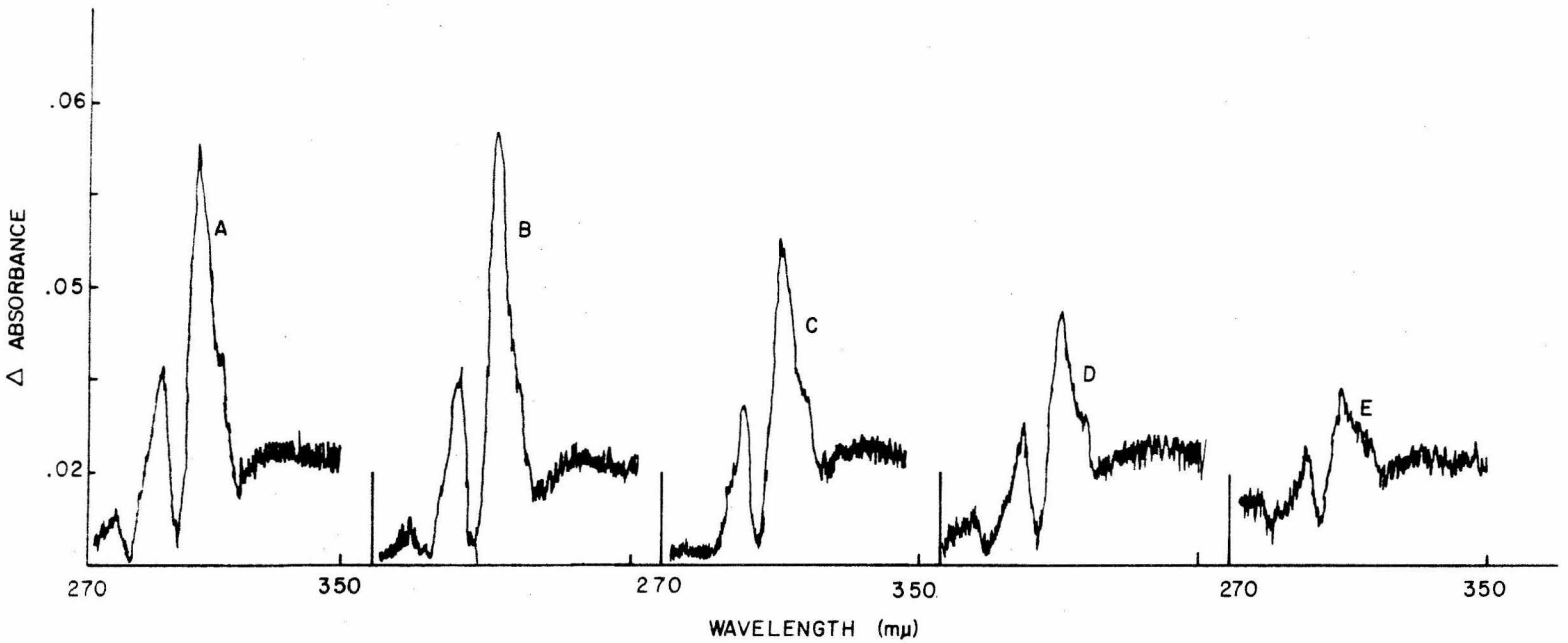

.06
.05
.02
Δ ABSORBANCE
A
B
C
D
E
270
350
270
350
270
350
WAVELENGTH (mμ)

Figure 2. Plot of data obtained from difference spectra of lysozyme in the presence of chitotriose at pH 5.5. Lysozyme concentration was 2.48×10^{-5} M. Chitotriose concentration varied from 2.56×10^{-3} M to 5×10^{-6} M.

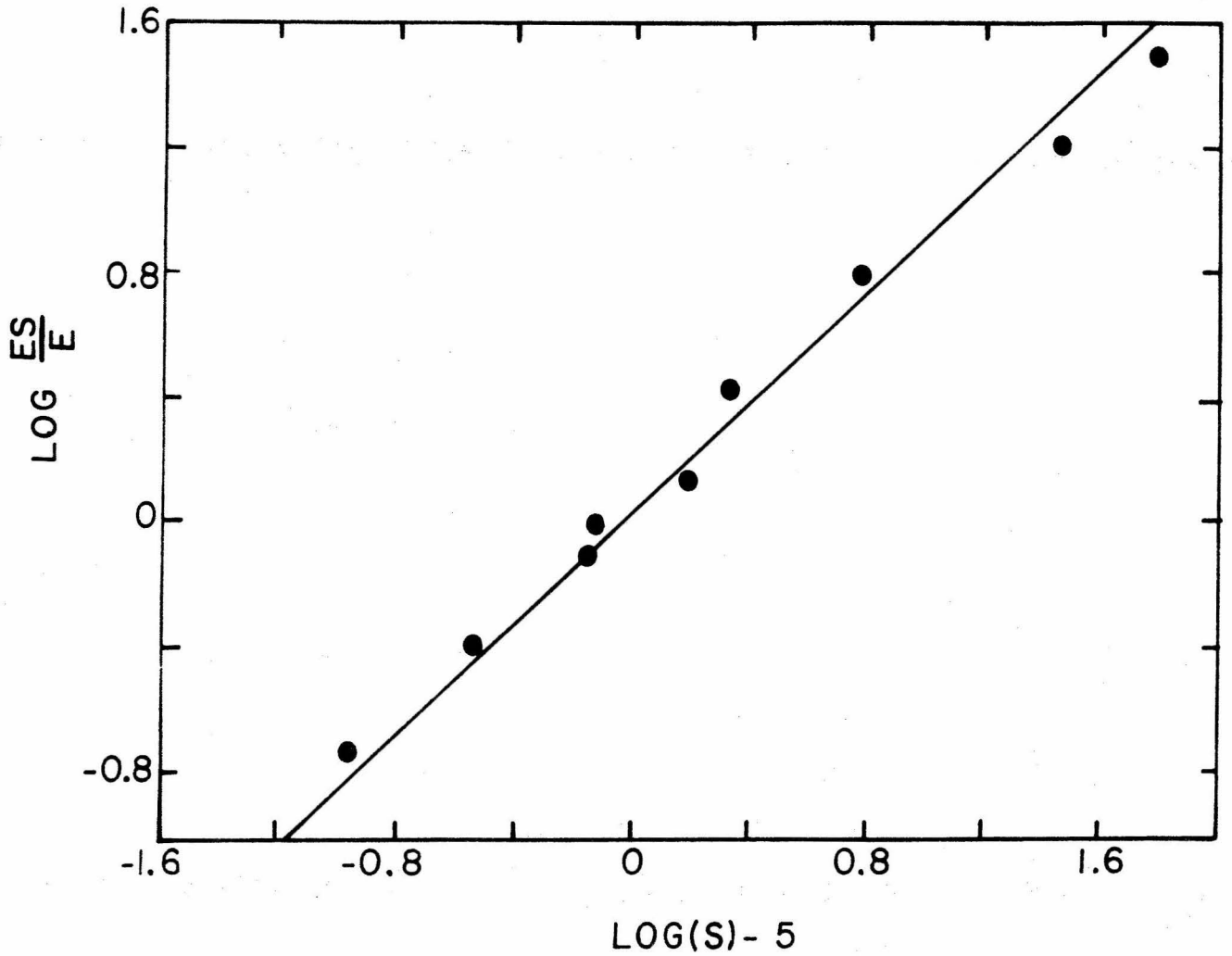
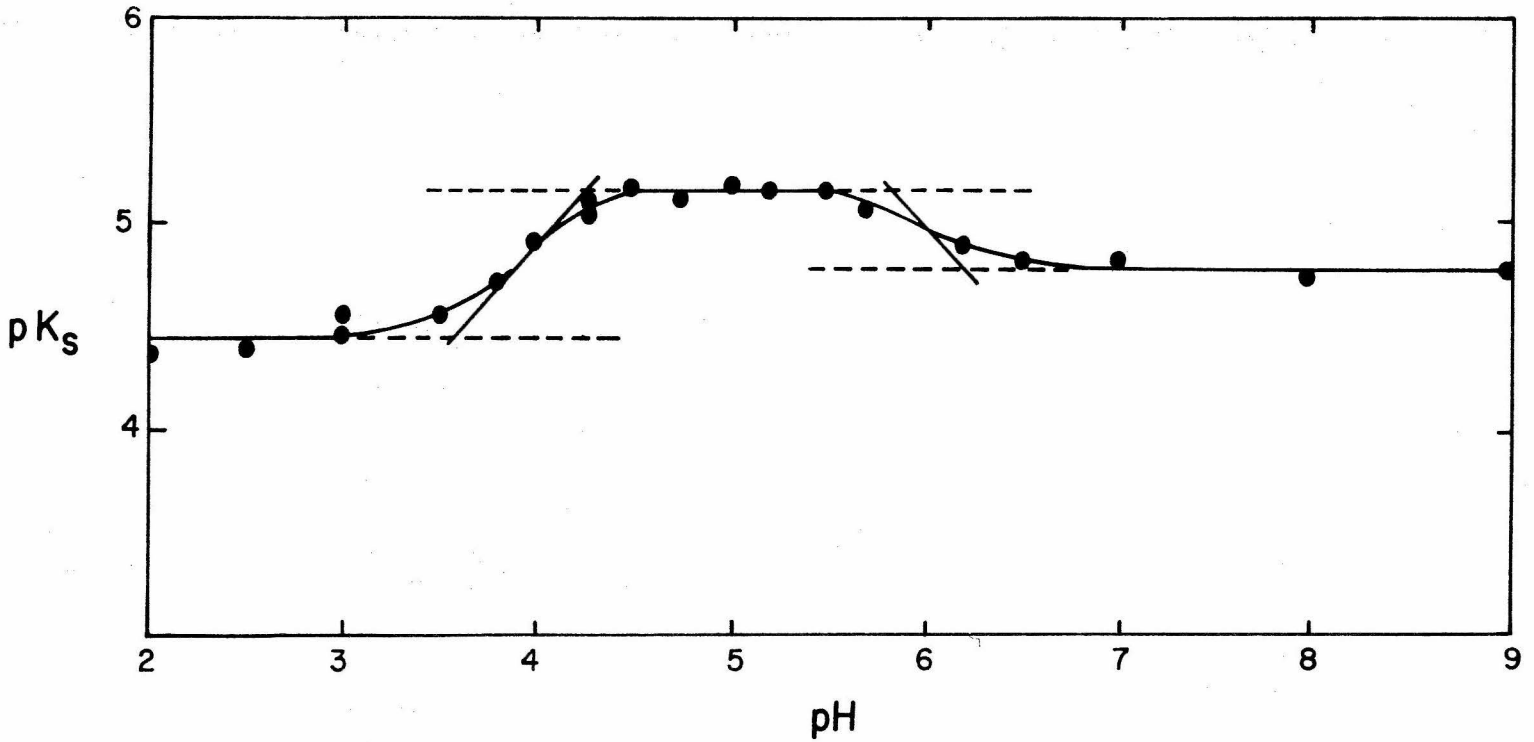

1.6
0.8
0
-0.8
LOG ES/E
-1.6
-0.8
0
0.8
1.6
LOG(S)- 5

Figure 3. Plot of - log K_s (pK_s) for chitotriose and lysozyme with pH.*

*Data of L. Jao.


6
5
4
pK$_S$
2
3
4
5
6
7
8
9
pH

CHAPTER II

THE ASSOCIATION OF MONOSACCHARIDE INHIBITORS WITH LYSOZYME BY PROTON MAGNETIC RESONANCE

INTRODUCTION

Many physical tools have been employed to investigate enzyme-substrate and enzyme-inhibitor interactions in solution. In general, most of these studies do not yield detailed information regarding the nature of such interactions. Nuclear magnetic resonance spectroscopy, however, by virtue of its extreme sensitivity to minute structural and conformational changes within molecules and to environmental effects, promises to be a potentially important spectroscopic method for the study of such processes.

Most of the results reported so far (Zimmerman and Brittin, 1957; Fischer and Jardetsky, 1965) have been concerned with line-width changes of the nuclear resonances of the small molecule upon association with the macromolecule. This method has been used to study the binding of penicillin G to bovine serum albumin (Fischer and Jardetsky, 1965) and the association of ethanol and DPN[1] to an alcohol dehydrogenase (Jardetsky et al., 1963; Hollis, 1967). Selective broadening of some of the proton resonances of the small molecules has been

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[1] Abbreviations used are: methyl-α-NAG, methyl-2-acetamido-2-deoxy-α-D-glucopyranoside; methyl-β-NAG, methyl-2-acetamido-2-deoxy-β-D-glucopyranoside; NAG, 2-acetamido-2-deoxy-D-glucopyranose, DPN, diphosphopyridine nucleotide.

observed and has been interpreted as indicating intimate contact between such protons (or neighboring groups) and the macromolecule.

In general, one also expects chemical shift changes for some nuclei of the inhibitor or substrate molecule when it is bound to the enzyme. If the small molecule exchanges rapidly between the free and complexed environments, the observed spectral positions of the affected resonances will be shifted from their corresponding positions for the unassociated molecule by amounts which depend upon the chemical shifts of these nuclei in the complex as well as on the fraction of the inhibitor or substrate molecules in the complexed state. One might argue that the chemical shift changes are more stereospecific than the linewidth changes in these studies, and hence provide a more sensitive probe of the active site and the nature of the enzyme-substrate or enzyme-inhibitor interactions. However, the chemical shift changes are in general considerably smaller than the accompanying broadening of the resonances and are therefore more difficult to measure. A recent publication of Spotswood, Evans and Richards (1967) describes chemical shifts in the resonances of inhibitor molecules when bound to α-chymotrypsin.

Since the work described in this communication was started a preliminary account of a study of the association of NAG and related compounds with lysozyme as detected by p. m. r. methods has appeared (Thomas, 1966). In addition to line-broadening effects due to binding it was observed that the resonance of the acetamido methyl groups of various NAG derivatives were shifted to higher field in the presence of

lysozyme. This effect was observed for α-NAG, β-NAG, methyl-α-NAG, methyl-β-NAG, and N-acetyl-D-galactosamine.

In this chapter of the thesis, we present the results of an investigation of the chemical shift changes occurring in the association of lysozyme with some specific inhibitors. The emphasis of this work is on the quantitation of such chemical shifts and the interpretation of the results obtained to infer information regarding the microscopic environment experienced by the inhibitor when bound to the enzyme.

MATERIALS AND METHODS

N-acetyl-D-glucosamine (m. p. 203-205°C) was purchased from the California Corporation for Biochemical Research. N-acetyl (d_3)-D-glucosamine was obtained by acetylation of D-glucosamine-HCl (California Biochem.) with acetic anhydride-d_6 (Volk Radiochemical Company) according to published procedures (P. Horton et al., 1966). Recrystallization of the resulting N-acetyl (d_3)-α-D-glucopyranose was effected from aqueous-alcohol mixtures (m. p. 203-205°C). Methyl-2-acetamido-2-deoxy-α-D-glucopyranoside (m. p. 188°C) was synthesized as previously reported (Zillikin et al., 1955) and purified by chromatography on a charcoal-celite column. Methyl-2-acetamido-2-deoxy-β-D-glucopyranoside (m. p. 204-205°C) was synthesized (Sophianpoulos et al., 1962) from D-glucosamine. Lysozyme (Lot #668-8590) was obtained from Sigma Chemical Company.

The 60 M Hz proton magnetic resonance (p. m. r.) spectra were recorded on a Varian A60A spectrometer at a probe temperature of 40°C. A Varian HA-100 spectrometer, operating in frequency sweep mode, was used for the 100 M Hz p. m. r. spectra, which were measured at 30°C. The water resonance was used as a lock signal for the studies in H_2O while a capillary of TMS was employed in the studies conducted in D_2O.

The chemical shifts for the 100 M Hz spectra were obtained by counting the frequency difference between the manual oscillator and the sweep oscillator on the HA-100 spectrometer with a Hewlett Packard Model 5212A counter. All chemical shifts were measured relative to

an internal standard acetone (0.5%). In each case, except those for mutarotational studies, the chemical shifts were measured at least three times with a standard deviation of 0.04 cps or less. Data were analyzed by least squares methods.

Enzyme concentrations of samples used for the p.m.r. measurements were determined from ultraviolet absorbance at 280 mμ of a 25 λ aliquot, after dilution to 5 ml. with water, using the known extinction coefficient (Sophianpoulos et al., 1962) for lysozyme.

RESULTS

The p.m.r. spectrum at 60 M Hz of N-acetyl-D-glucosamine exhibits a resonance at τ 8.09, which can be unambiguously assigned to the methyl group protons of the acetamido side-chain (P. Horton et al., 1967). Since it is the most intense resonance in the spectrum and is unsplit, it is best suited for our present study. Figure 1A shows this resonance at 60 M Hz with an acetone internal standard included in the sample. The addition of lysozyme (3×10^{-3} M final concentration) to this same sample of NAG (5×10^{-2} M) resulted in the spectrum shown in Fig. 1B. From the known binding constant (Part I, Chapter II), it was determined that approximately 5% of the total NAG present was bound to the enzyme under these conditions. Two resonances instead of one were observed for these acetamido methyl protons in the presence of the enzyme. Both of these resonances were broadened, and both appeared at higher fields from the position of the original NAG methyl resonance (0.9 Hz and 3.3 Hz, respectively).

As proof that the effects observed in the p.m.r. spectra of NAG in the presence of lysozyme were due to association between the inhibitor and the enzyme rather than to bulk susceptibility effects in the relatively concentrated protein solution, the p.m.r. spectra of NAG in similarly concentrated solutions of ribonuclease A were also recorded. Such a spectrum is displayed in Fig. 2. In addition to the absence of the additional resonance observed in the presence of lysozyme, it was shown that the addition of ribonuclease to a NAG solution did not result in any chemical shift of the acetamido methyl resonance from its

position in free NAG. Neither was the methyl resonance noticeably broadened. It was further substantiated that the concentration of NAG itself did not affect the chemical shift of its acetamido methyl protons. No concentration shifts were observed over the concentration range 10^{-2} M to 10^{-1} M.

To ascertain that the two observed methyl resonances of NAG in the presence of lysozyme correspond to the acetamido methyl groups of the α- and β-anomeric forms of the inhibitor a freshly dissolved sample of α-NAG was used. Figure 3 summarizes the p. m. r. spectra of the NAG methyl protons in the presence of the enzyme obtained at various intervals during the mutarotation of NAG from the α-anomeric form to the equilibrium mixture of α- and β-anomers. Lysozyme was also added to a fresh sample of β-NAG prepared by acetylation of β-D-glucosamine (Kuhn and Haber, 1953). The spectrum for this solution showed at early times only the methyl resonance which we have attributed to the β-anomer in Fig. 3, with subsequent development of the resonance due to the α-anomer.

These observations are in agreement with those previously observed (Thomas, 1967) for NAG in the presence of lysozyme, and they are also in accord with the results obtained from x-ray analysis studies (L. Johnson and D. C. Phillips, 1965) of lysozyme-NAG complexes in the crystalline state which have shown that both anomers of the sugar bind to the enzyme. Earlier studies employing ultraviolet spectroscopic methods allowed estimation of the dissociation constant for the lysozyme-NAG complex (K_s = 4-6 x 10^{-2} M). This value obviously represents the binding of both forms of NAG and is a complex constant.

It is evident from our present observations that the chemical exchange of both anomeric forms of the inhibitor NAG between the free and enzyme bound species is rapid. It is also evident that both anomers of NAG, when bound to lysozyme have their acetamido methyl groups in environments which lead to increased magnetic shielding of these protons, since the resonances observed for both anomers are shifted upfield from their positions in the absence of the enzyme. The fact that we observe separate resonances for the α- and β-anomers in the presence of lysozyme indicates that the two anomeric forms bind to the enzyme either with different affinities, or in a manner such that their acetamido side chains do not occupy the same position on the enzyme or both. These conclusions are possible, whether or not the two anomers bind to different sites or compete for the same sites on the enzyme surface. In addition, the rate of interconversion of NAG between its α- and β-anomers must be slow.

To decide whether the α- and β-anomeric forms of NAG compete for the same sites on the enzyme surface, a sample of crystalline N-acetyl(d_3)-α-D-glucopyranose (m. p. 203-205°C) was added to an equilibrated mixture of NAG and lysozyme and the p. m. r. spectrum was recorded at 60 M Hz within two minutes after mixing. As seen in Fig. 4, the added α-(d_3)-anomer (although not observable) decreased the observed chemical shifts (and therefore the per cent bound) of both α- and β-NAG. Table 1, both anomers were approximately equally affected by the added α-(d_3)-anomer. The conclusion from this experiment is that α-NAG and β-NAG do compete for the same sites on the enzyme.

A quantitative analysis of the chemical shift data obtained for the NAG lysozyme system is complicated by the mutarotation equilibrium between the α- and β-anomers of the inhibitor, since the enzyme would shift this equilibrium slightly if the two anomeric forms bind to the enzyme with different affinities. To facilitate quantitative treatment of the experimental data, identical binding studies were therefore repeated using the methyl glycosides of NAG, where the conformation of each anomer is frozen. Table 2 summarizes the results obtained at 100 M Hz for the association of lysozyme with methyl-N-acetyl-β-D-glucopyranoside and separately with methyl-N-acetyl-α-D-glucopyranoside.

That methyl-α-NAG and methyl-β-NAG compete for the same sites on the enzyme surface was shown by adding N-acetyl-(d_3)-α-D-glucopyranose to a solution of lysozyme containing either methyl-α-NAG or methyl-β-NAG, and showing that the added deuterated compound reduced the amount of bound methyl-α-NAG or methyl-β-NAG. Table 3 gives the quantitative data obtained. It may be seen that methyl-α-NAG and methyl-β-NAG compete for the same sites on the enzyme occupied by α-NAG.

The results summarized in Table 2 can be readily interpreted in terms of the following simple equilibrium between the enzyme (E) and the inhibitor (S),

$$E + S \rightleftharpoons ES . \qquad (1)$$

Under conditions of rapid chemical exchange of the inhibitor between its free and enzyme-bound forms, it is readily shown that

$$K_s \frac{\delta}{\Delta} = \left(E_0 - S_0 \frac{\delta}{\Delta}\right)\left(1 - \frac{\delta}{\Delta}\right) \qquad (2)$$

where δ is the observed chemical shift of the average methyl resonance referred to that of the unbound inhibitor, Δ is the chemical shift difference for the methyl resonance between the bound and unbound species, K_s is the enzyme-inhibitor dissociation constant, and E_0, S_0 denote the total concentrations of the enzyme and inhibitor, respectively. In the limit where $\delta/\Delta \ll 1$, a condition which is fairly well satisfied in our experiments, expression (2) simplified, and may be rearranged to give

$$S_0 = E_0 \frac{\Delta}{\delta} - K_s - E_0 \qquad (3)$$

Thus, if the enzyme-inhibitor binding is studied by varying the inhibitor concentration at a fixed concentration of enzyme, a plot of S_0 versus the observed $1/\delta$'s should yield a straight line with slope $E_0\Delta$ and intercept equal to $-(K_s + E_0)$, provided the above conditions are met.

The chemical shift data obtained for methyl-α- and methyl-β-NAG are plotted in this manner in Fig. 5. In these experiments, E_0 was held fixed at 3.0×10^{-3} M. It is seen that the variation of S_0 with $1/\delta$ is indeed linear for both anomeric methyl glycosides over the range of conditions of our enzyme-inhibitor binding experiments. From an analysis of these plots, we extracted the following dissociation constants and chemical shifts for the two enzyme-glycoside complexes:

$$K_{s\alpha} = 5.2 \pm 0.4 \times 10^{-2} \text{ M}, \qquad \Delta_\alpha = 0.55 \pm 0.02 \text{ ppm}$$

$$K_{s\beta} = 3.3 \pm 0.5 \times 10^{-2} \text{ M}, \qquad \Delta_\beta = 0.54 \pm 0.04 \text{ ppm}$$

Thus, it appears that the two anomeric glycosides bind to lysozyme with slightly different affinities. However, the results indicate that the acetimido methyl groups of both glycosides occupy identical magnetic environments when bound to the enzyme.

The results of the above analysis indicate that only one inhibitor molecule is bound per lysozyme molecule. Thus, if there is more than one comparably strong binding site on the enzyme surface, it is apparent that the formation of one enzyme-inhibitor complex inhibits binding at the remaining sites. As usual, it is not possible to establish the formation of one 1:1 enzyme-inhibitor complex rather than several such complexes, irrespective of whether binding at one or several sites is responsible for the observed spectral changes. The reason for this is that the concentration of the various enzyme-inhibitor complexes are related to one another merely by ratios of their formation constants. In our case, where the various 1:1 enzyme-inhibitor complexes would be in rapid equilibrium with one another, the expression for the observed chemical shift can be reduced to the same form as equation (2), with K_s and Δ replaced by their appropriate weighted averages, namely

$$K_s \longrightarrow 1\Big/\left(\sum_{i=1}^{n} 1\Big/K_{s_i}\right)$$

$$\Delta \longrightarrow \left(\sum_{i=1}^{n} \Delta_i\Big/K_{s_i}\right)\Big/\left(\sum_{i=1}^{n} 1\Big/K_{s_i}\right) \qquad (4)$$

Here, n denotes the number of binding sites, and the subscript i is used to denote the parameters for the i^{th} enzyme-inhibitor complex.

DISCUSSION

The most reasonable explanation of the observed shifts for the acetamido methyl protons on the binding of acetamido sugars to lysozyme is that these protons become more magnetically shielded in the enzyme-bound state because of their environment on the enzyme. The most likely causes for this are proximity of the methyl group to (a) an aromatic side chain of the enzyme (C. E. Johnson and F. A. Bovey, 1958), or (b) the electric field of an ionizable group nearby (Buckingham et al., 1960). There is ample evidence (Hayashi et al., 1963) that the binding of N-acetyl-D-glucosamine and related compounds to lysozyme results in a red-shift in the spectrum of the enzyme, and this observation has been attributed to a tryptophanyl side chain becoming less solvent accessible. In addition, X-ray studies (Thomas, 1967; Blake et al., 1967) of the binding of small sugar molecules such as NAG to crystalline lysozyme suggest that in the enzyme-inhibitor complex, the acetamido methyl group is close to the aromatic ring of tryptophane residue number 108. There is, however, also evidence that ionizable groups are present at the binding site of lysozyme (Rupley et al., 1967).

The results of this work indicate that the acetamido methyl protons of methyl-α-NAG and methyl-β-NAG experience identical magnetic environments in the enzyme-inhibitor complex. This conclusion may not apply in the case of NAG where both binding constants and chemical shifts for the anomeric forms could be different from each other. On the other hand, in the methyl glycosides, the glycosidic methyl groups of methyl-α-NAG and methyl-β-NAG were found to experience different

magnetic environments when bound to the enzyme. In the presence of lysozyme, this methyl resonance was shifted to lower fields in methyl-β-NAG, whereas in the case of methyl-α-NAG, the glycosidic methyl resonance was not noticeably affected. Of the other protons in the NAG molecule, only those of the 6-hydroxy-methyl group were investigated in the presence of lysozyme. No observable chemical shift of these protons was induced by the enzyme, whereas broadening of the resonances was observed, as expected.

Parsons and Raftery (1968) have been able to show that other acetamido sugars bind to the same site as do the various NAG derivatives. Such compounds as N-acetyl-D-galactosamine, N-acetyl-D-mannosamine, acetamidocyclohexanol, and acetamidocyclohexane all interact with the same site on the enzyme and the methyl group of the acetamido side chain undergoes a chemical shift in each case. This is consistent with the idea that the acetamido side chain is necessary for binding to the enzyme to occur, probably through the formation of hydrogen bonds. All the compounds studied probably form the same hydrogen bonds between the enzyme and the acetamido group of the inhibitor, but interactions at other "points of contact" between the enzyme and inhibitor molecules may differ depending on the nature of the substituents and their conformation on each pyranose ring.

It would appear from our results that there is <u>at least</u> one strong binding site on the enzyme for simple acetamido pyranosides, such as NAG. However, it appears that if there is more than one strong binding site on the enzyme surface, the formation of one enzyme-inhibitor complex inhibits binding at the remaining sites, so that only 1:1

complexes result. This conclusion that only one inhibitor molecule is bound strongly per lysozyme molecule agrees with the spectrophotometric studies discussed in this thesis and elsewhere (Lehrer and Fasman, 1966 and 1967; Rupley et al., 1967). However, these spectrophotometric studies also suggest that there are three continguous binding sites for pyranose rings on the enzyme surface, since the binding strength of oligosaccharides to lysozyme was found to increase up to the trisaccharide of NAG (chitotriose).

In conclusion it is felt that the present studies show that the use of p.m.r. spectroscopy offers advantages over methods previously used for the study of enzyme-inhibitor or enzyme-substrate interactions, since information regarding the environment experienced by the small molecule on association with the macromolecule can often be obtained.

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TABLE 1.--Chemical shift data for the acetamido methyl protons of α-NAG and β-NAG association with lysozyme in the absence and presence of α-NAG(d_3). Measurements were made at 60 M Hz in 0.1 M citrate buffer, pD 5.5, at 40°C.

Inhibitor	Inhibitor Conc. (M)	Lysozyme Conc. (mg/ml)	Chemical Shift Relative to Acetone (Hz)	δ(Hz)
α-, β-NAG	0.05	0	10.92	0
α-NAG	0.016	50	14.76	3.84
β-NAG	0.016		12.24	1.32
α-NAG	0.016	50	12.54	1.62
β-NAG	0.016		11.50	0.58
α-NAG(d_3)	0.067		- - -	- -

TABLE 2.--Chemical shift data for the acetamido methyl protons of methyl-α-NAG and methyl-β-NAG association with lysozyme (3.0×10^{-3} M). Measurements were made at 100 M Hz, in 0.1 M citrate buffer, pH 5.5, at 31°C

Methyl-α-NAG $S^0 \times 10^2$	δ(Hz)	$\frac{1}{\delta}$
7.15	1.30	0.769
4.29	1.69	0.592
3.57	1.82	0.549
2.86	1.95	0.513
1.43	2.39	0.418
Methyl-β-NAG $S^0 \times 10^2$	**δ(Hz)**	**$\frac{1}{\delta}$**
7.21	1.45	0.690
5.77	1.71	0.585
4.33	1.95	0.513
3.60	2.20	0.454
2.88	2.43	0.412
1.44	3.13	0.320

TABLE 3.*--Chemical shift data for acetamido methyl protons obtained from the association of methyl-α-NAG and methyl-β-NAG with lysozyme in the absence and presence of α-NAG(d_3). Measurements were made in 0.1 M citrate buffer, pD 5.5, at 40°C, in a Varian A-60A Spectrometer

Inhibitor	Inhibitor Conc. (M)	Lysozyme Conc. (mg/ml)	Chemical Shift Relative to Acetone (Hz)	δ(Hz)
Methyl-α-NAG	0.05	0	11.52	0
Methyl-α-NAG	0.016	50	13.12	1.60
Methyl-α-NAG	0.016	50	12.76	1.24
plus				
α-NAG(d_3)	0.019		- -	- -
Methyl-β-NAG	0.05	0	11.54	0
Methyl-β-NAG	0.016	50	13.84	2.30
Methyl-β-NAG	0.016	50	13.18	1.64
plus				
α-NAG(d_3)	0.023		- -	- -

*Data of S. M. Parsons.

Figure 1. (a) Proton magnetic resonance spectrum at 60 M Hz of the acetamido methyl protons of NAG (5×10^{-2} M) in 0.1 M citrate, pH 5.5. Acetone (0.5%) is used as an internal standard and its resonance appears to lowest field in spectrum. A sweep width of 50 cps was used. (b) Proton magnetic resonance spectrum at 60 M Hz of a similar solution of NAG as shown in Fig. 1a with added lysozyme (3×10^{-3} M).

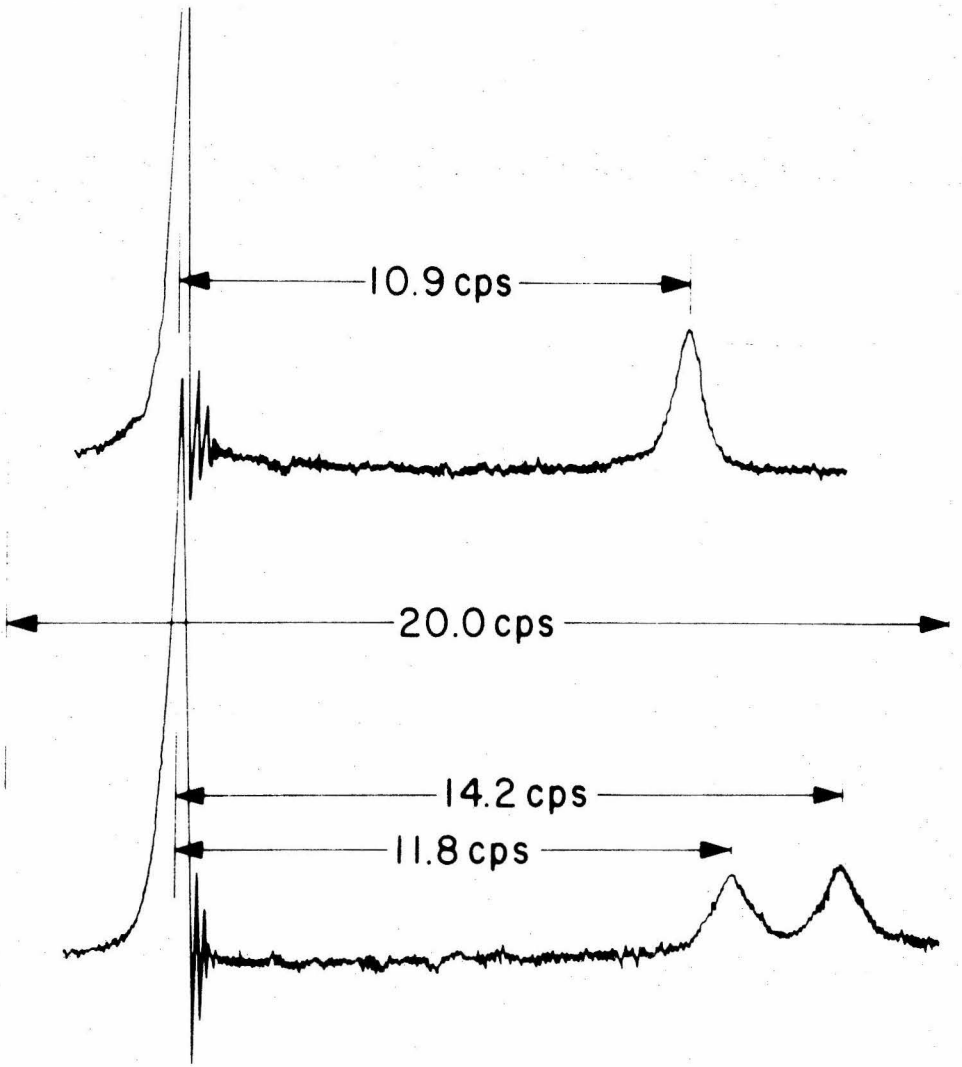

10.9 cps
20.0 cps
14.2 cps
11.8 cps

Figure 2. The acetamido methyl p.m.r. spectrum at 60 M Hz of NAG (5×10^{-2} M) in the absence and in the presence of ribonuclease A (3×10^{-3} M), in 0.1 M citrate, pH 5.5. Acetone (0.5%) was used as an internal reference, and appears to lower field.

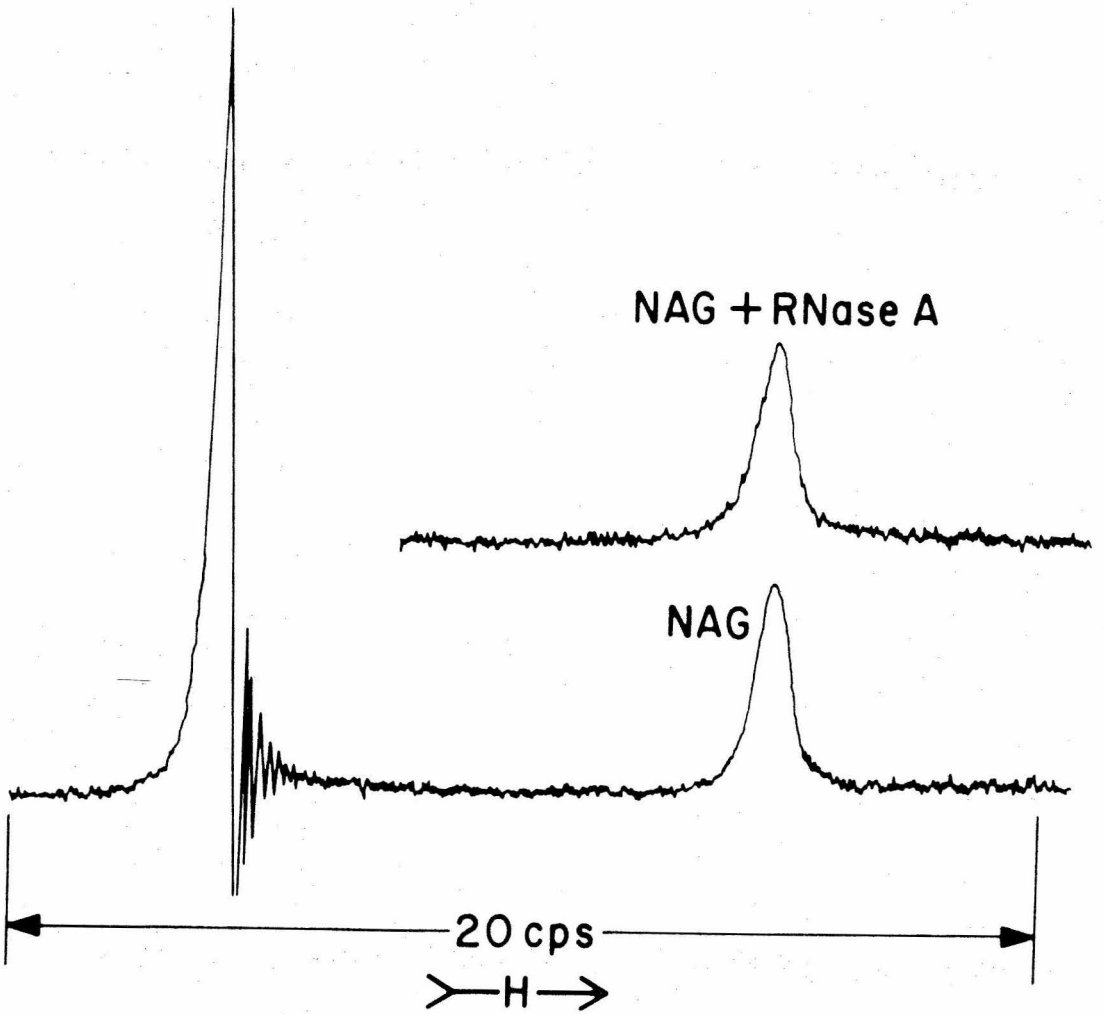

NAG + RNase A
NAG
20 cps
H

Figure 3. Time study of the p.m.r. spectrum of α-NAG (5×10^{-2} M) after addition to lysozyme (3×10^{-3} M) in 0.1 M citrate in D_2O, pD - 5.9. A solution of acetone (0.5%) was used as an internal standard, and its resonance appears to lower field of the NAG resonances.

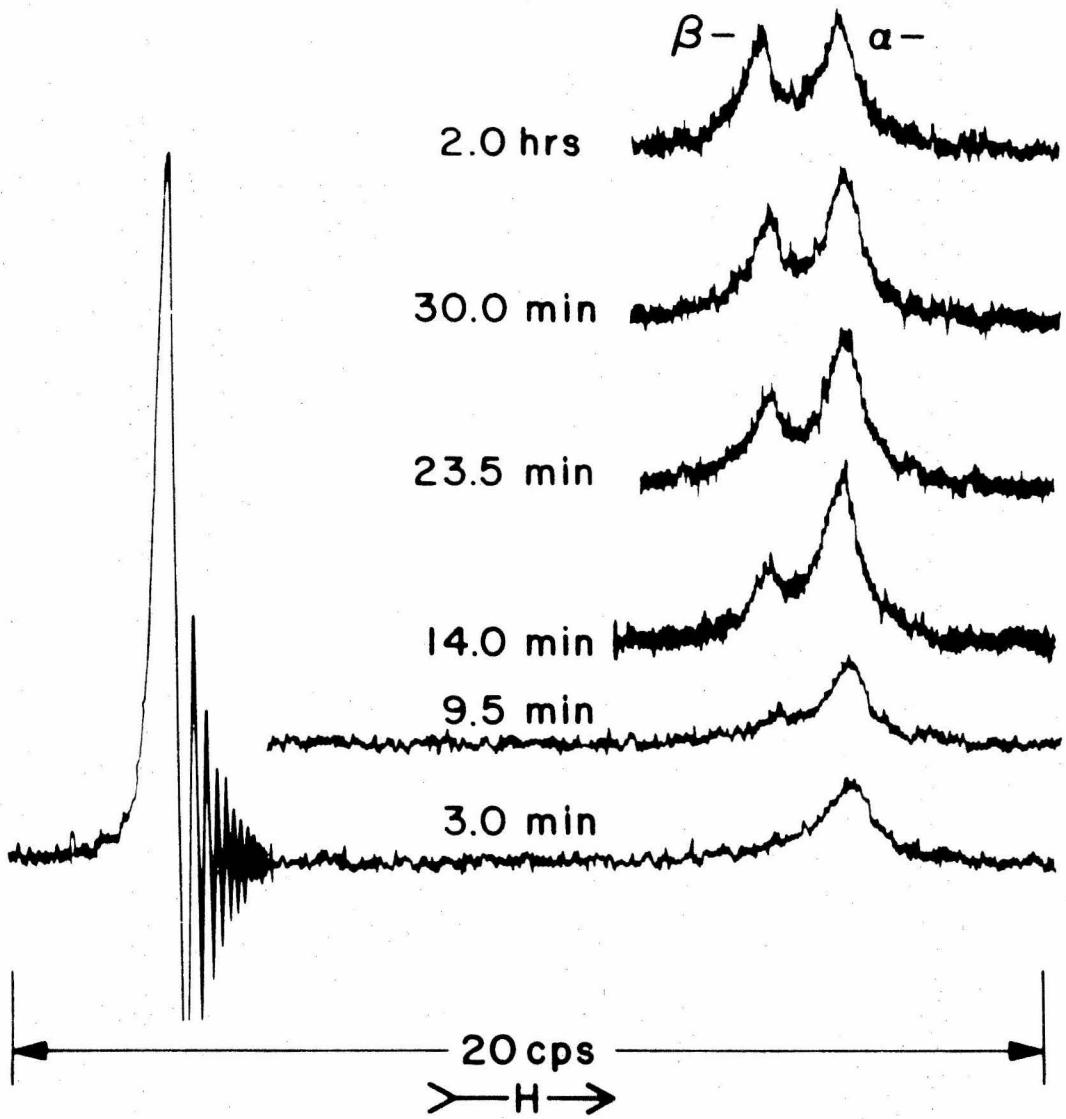

β−
α−
2.0 hrs
30.0 min
23.5 min
14.0 min
9.5 min
3.0 min
20 cps
H

Figure 4. Demonstration of competitive binding to lysozyme of α-NAG and β-NAG. The resonance due to acetone protons is shown at left. Other resonances shown are (a) the acetamido methyl proton resonance of free NAG; (b) NAG (at mutarotation equilibrium, 1.6×10^{-2} M) in the presence of lysozyme; (c) same as in (b) but with added α-NAG(d_3) (6.7×10^{-2} M). In this case the spectrum was recorded immediately after addition of the α-deuterio anomer.*

*Data of S. M. Parsons.

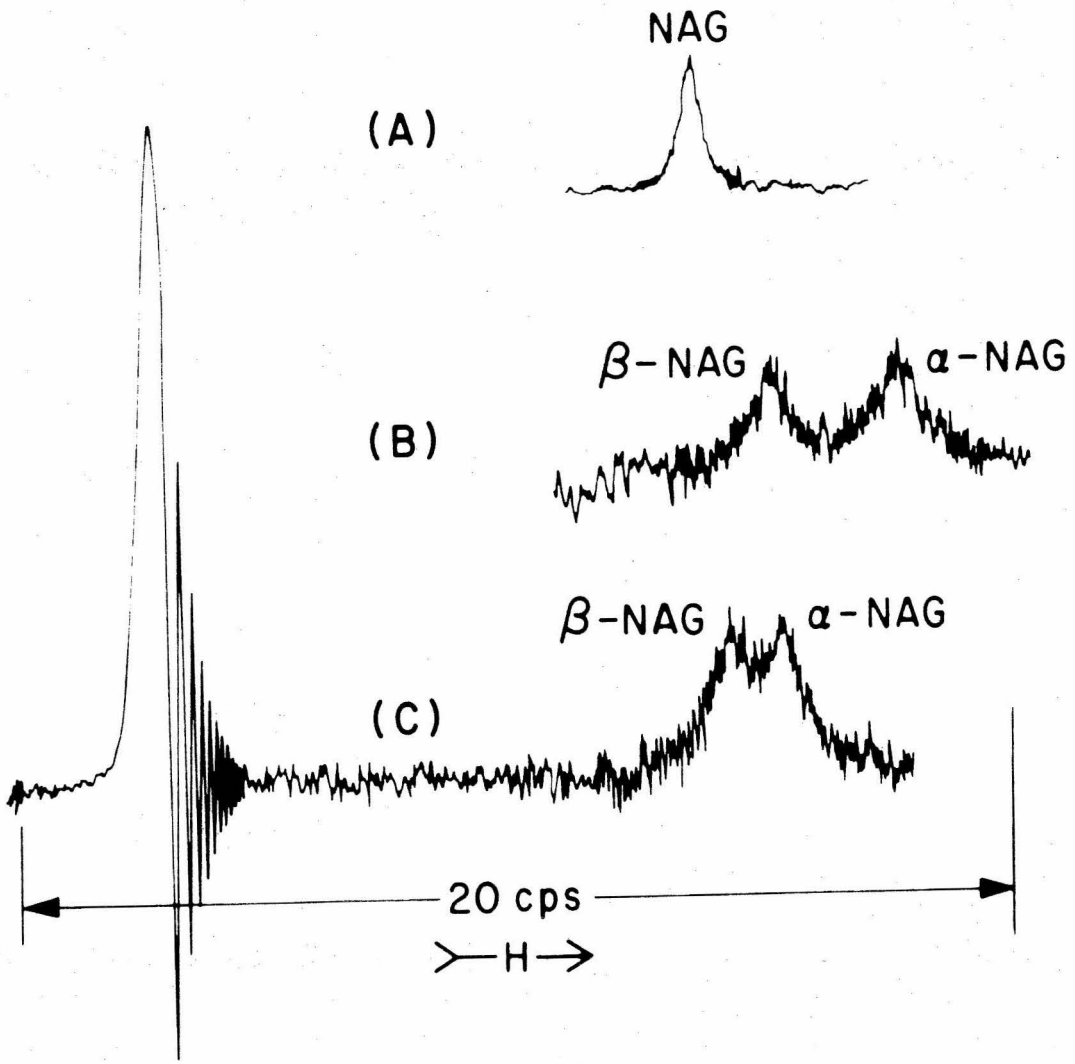
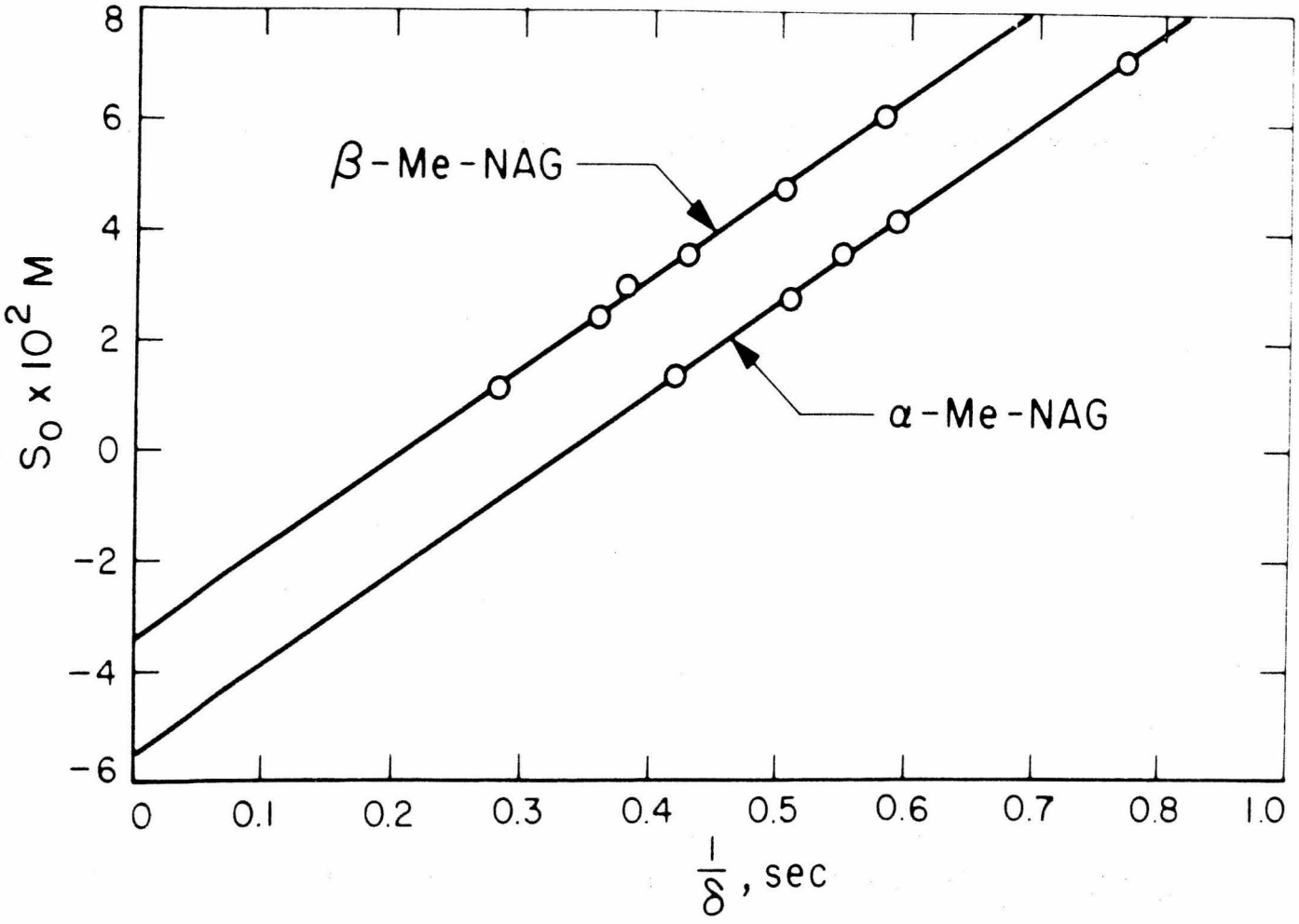

NAG
(A)
β-NAG
α-NAG
(B)
β-NAG
α-NAG
(C)
20 cps
H

Figure 5. Plots of chemical shift (δ) data for the association of methyl-α-NAG and separately methyl-β-NAG with lysozyme. The ordinate showing $S_0 \times 10^2$ refers to the concentration of each inhibitor used (1×10^{-2} to 7×10^{-2} M) in the presence of a constant concentration of the enzyme (3×10^{-3} M).


8
6
4
2
0
−2
−4
−6
$S_0 \times 10^2$ M
β-Me-NAG
α-Me-NAG
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
1.0
$\frac{1}{\delta}$, sec

CHAPTER III

A NUCLEAR MAGNETIC RESONANCE STUDY OF ASSOCIATION EQUILIBRIA AND ENZYME-BOUND ENVIRONMENTS OF N-ACETYL-D-GLUCOSAMINE ANOMERS AND LYSOZYME

INTRODUCTION

The use of nuclear magnetic resonance to study association phenomena of macromolecules has been recognized for some time and the theory dealing with such processes has been outlined by Zimmerman and Brittin (1957) and by Jardetsky (1964). Previous attempts to gain information from such studies by n. m. r. methods have employed linewidth measurements of resonances attributed to nuclei in the associating small molecule (Jardetsky , 1964) ; Fischer and Jardetsky, 1965; Hollis, 1967). It is also possible for a chemical-shift difference to occur for certain nuclei between free and bound species. Such an effect could result from proximity to aromatic systems, electric fields due to polar groups, secondary magnetic-field differences arising from induced magnetic moments in neighboring atoms or indeed from van der Waal effects (Buckingham, 1960; Buckingham et al. , 1960). Such a chemical-shift difference had not been observed until recently in studies of association equilibria involving enzymes and inhibitors or substrates (Spotswood et al. , 1967). The results described in the previous section have described such an effect in p. m. r. studies of the

association between lysozyme and N-acetyl-D-glucosamine.[1] It was further demonstrated in that section that in the presence of lysozyme the acetamido methyl proton resonances of the α- and β-anomeric forms of NAG were resolved and also that the two forms compete for the same binding site(s) on the enzyme.

Our earlier measurements of NAG binding to lysozyme, employing ultraviolet difference spectral techniques (see Part I, Chapter I), showed a dissociation constant (K_s) for NAG of 4-6 $\times 10^{-2}$ M, which was confirmed by the results of other workers (Lehrer and Fasman, 1966, 1967; Rupley et al., 1967; Chipman et al., 1967). It was not possible by the techniques used by any of these authors to determine whether both anomeric forms of NAG were bound to the enzyme, and if so, to estimate dissociation constants of both forms. It was demonstrated however, by use of these spectral techniques that oligosaccharides of chitin showed increases in binding strength to lysozyme up to the trisaccharide (Rupley et al., 1967). Thus it was evident that there are three strong contiguous binding sites for sugar rings on the surface of lysozyme. Although it was shown in these studies that NAG bound stoichiometrically to the enzyme it was not possible to relate this one-to-one binding to association with one of the three strong sites rather than to multiple equilibria with all binding sites, with the dissociation constant obtained being a complex entity.

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[1] Abbreviations used are: p. m. r., proton magnetic resonance; NAG, N-acetyl-D-glucosamine; E. C., Enzyme Commission; c. p. s., cycles per second; n. m. r., nuclear magnetic resonance; p. p. m., parts per million; p. m. r., proton magnetic resonance; α-methyl-NAG, methyl-2-acetamido-2-deoxy-α-D-glucopyranoside; β-methyl-NAG, methyl-2-acetamido-2-deoxy-β-D-glucopyranoside.

The present communication provides a quantitative interpretation of the shifts observed in the NAG methyl group magnetic resonances in the presence of lysozyme in terms of the dissociation constants of α-NAG and β-NAG and of the magnetic environments of their binding sites. It is shown that it is possible to calculate the dissociation constant for each anomer of NAG as well as to probe the environment of each anomer while bound to the enzyme. The results presented show that although α- and β-NAG are competitive for the same overall site on the enzyme and although they have nearly the same free energy of binding, it is possible to distinguish different magnetic environments for the binding sites of the two anomeric forms.

Quantitation of Chemical Shift Data

Calculation of the dissociation constant and the chemical shift of the bound form of the small molecule is possible for a system in rapid equilibrium

$$E + S \rightleftharpoons ES$$

and

$$K_S = \frac{[E] \cdot [S]}{[ES]} \qquad (1)$$

where $[E] = E_0 - [ES]$ and $[S] = S_0 - [ES]$. In this system we regard the chemical shift of the free inhibitor as zero and refer to the observed chemical shift as δ and the chemical shift of the bound inhibitor as Δ. If the exchange lifetime is much less than $\frac{1}{\Delta}$ in seconds then

$$\delta = P_b \Delta$$

where P_b is the fraction of substrate present in the bound form. Therefore

$$\delta = \frac{[ES]}{S_0} \cdot \Delta$$

and

$$[ES] = \frac{\delta S_0}{\Delta} \quad . \tag{2}$$

Substitution of eq. 1 leads to eq. 3

$$K_S = E_0\left[\frac{\Delta-\delta}{\delta}\right] - \frac{\delta}{\Delta} S_0\left[\frac{\Delta-\delta}{\delta}\right] \quad . \tag{3}$$

If the observed shift δ is much smaller than the total shift Δ and K_S is of the order of S_0 then

$$S_0 = \frac{E_0 \Delta}{\delta} - K_S - E_0 \tag{4}$$

A plot of S_0 versus $1/\delta$ gives a line whose intercept is $-(K_S + E_0)$ and whose slope is used to calculate Δ.

Table 1 shows the results obtained from a study of the association of NAG and lysozyme at 100 M Hz. The results are plotted in Fig. 1 and it is seen that the lines representing the α- and β-anomers intersect on the S_0 axis.

It has been shown in the previous section that α-NAG and β-NAG compete with each other for the same site on lysozyme. In view of this competition the chemical-shift data can be quantitated using the simple competitive scheme:

$$E + \alpha \rightleftharpoons E\alpha \quad \text{and} \quad K_\alpha = \frac{[E]\cdot[\alpha]}{[E\alpha]}$$

$$E + \beta \rightleftharpoons E\beta \quad \text{and} \quad K_\beta = \frac{[E]\cdot[\beta]}{[E\beta]} \quad .$$

Substitution similar to that of eq. 1 (see Appendix A) gives

$$S_0 \cdot A = \frac{E_0 \Delta_\alpha}{\delta_\alpha} - K_\alpha - E_0, \qquad \text{where } A = \frac{[\alpha_0]}{S_0} + \frac{K_\alpha}{K_\beta} \cdot \frac{[\beta_0]}{S_0} \tag{5}$$

and

$$S_0 \cdot B = \frac{E_0 \Delta_\beta}{\delta_\beta} - K_\beta - E_0, \qquad \text{where } B = \frac{[\beta_0]}{S_0} + \frac{K_\beta}{K_\alpha} \cdot \frac{[\alpha_0]}{S_0} \tag{6}$$

where S_0 is the total concentration of NAG, E_0 is the total concentration of enzyme, $[\alpha_0]$ is the total concentration of α-NAG, $[\beta_0]$ is the total concentration of β-NAG and δ_α and δ_β refer to the observed chemical shifts of the α- and β-NAG present in mutarotation equilibrium. Equations 5 and 6 allow interpretation of the data plotted in Fig. 1.

The ratio of the slope of the α-NAG data to that of β-NAG (provided $[\alpha_0] = [\beta_0]$) is $K_\beta \Delta_\alpha / K_\alpha \Delta_\beta$ and was found to be 2.7. The intercept for the α-form is

$$\frac{-(K_\alpha K_\beta + K_\beta E_0)}{\frac{[\alpha_0]}{S_0} \cdot K_\beta + \frac{[\beta_0]}{S_0} \cdot K_\alpha} \tag{7a}$$

while for the β-form the intercept is

$$\frac{-(K_\alpha K_\beta + K_\alpha E_0)}{\frac{[\beta_0]}{S_0} \cdot K_\alpha + \frac{[\alpha_0]}{S_0} \cdot K_\beta} \tag{7b}$$

and both were determined to be $(2.5 \pm 0.4) \times 10^{-2}$ M. This figure agrees fairly well with that ($4\text{-}6 \times 10^{-2}$ M) obtained for NAG binding to lysozyme using the ultraviolet difference spectral technique. Theoretically it is possible to determine the values of K_α and K_β from eqs. 7a and 7b. However, the error in the intercept for the β-anomeric form (due to the smaller observed chemical shift associated with this form) is too great to allow this calculation.

Line-Width Measurements

In an attempt to resolve the question of whether K_α and K_β are different or whether Δ_α and Δ_β are different we have employed line-width measurements. If the observed line-width is $1/\pi T_{2\,obs}$, then in the absence of saturation or line broadening due to slow exchange, when the enzyme is present

$$\left(\frac{1}{T_{2\,obs}}\right)_E = P_f \cdot \frac{1}{T_2^f} + P_b \cdot \frac{1}{T_2^b} + I \qquad (8)$$

where P_f and P_b represent fractions of the inhibitor (α-NAG or β-NAG) which are free in solution and bound to the enzyme respectively, and T_2^f and T_2^b represent the spin-spin relaxation times of the free and bound inhibitor species. The term I represents the inhomogeneity of the field. In the absence of enzyme

$$\left(\frac{1}{T_{2\,obs}}\right)_F = \frac{1}{T_2^f} + I \,. \qquad (9)$$

Then the contribution to the line-width due to association ($1/T_2'$)

$$\frac{1}{T_2'} = \left(\frac{1}{T_{2\,obs}}\right)_E - \left(\frac{1}{T_{2\,obs}}\right)_F \tag{10}$$

$$= \left(P_f - 1\right) \frac{1}{T_2^f} + P_b \cdot \frac{1}{T_2^b} \cdot \tag{11}$$

Since $P_f + P_b = 1$

$$\frac{1}{T_2'} = P_b\left(\frac{1}{T_2^b} - \frac{1}{T_2^f}\right) \cdot \tag{12}$$

For the case of small molecules associating with macromoecules

$$\frac{1}{T_2^b} >> \frac{1}{T_2^f}$$

and therefore

$$\frac{1}{T_2'} = P_b \frac{1}{T_2^b} \cdot \tag{13}$$

This has the same form as eq. 2 and from considerations similar to those used to derive eqs. 5 and 6 it can be shown (see Appendix B) that

$$\frac{(T_2')_\beta}{(T_2')_\alpha} = \frac{K_\beta}{K_\alpha} \times \frac{(T_2^b)_\beta}{(T_2^b)_\alpha} \tag{14}$$

where $(T_2')_\beta/(T_2')_\alpha$ is the ratio of the contributions to the line-widths of the α- and β-anomeric forms due to association with the enzyme while $(T_2^b)_\beta$ and $(T_2^b)_\alpha$ represent the relaxation times of the bound species of β-NAG and α-NAG. The ratio $(T_2')_\beta/(T_2')_\alpha$ was determined according to eq. 10 to be 1.3 ± 0.1 from the data shown in Fig. 2. If

the sites occupied by the acetamido side chains of α-NAG and β-NAG are magnetically equivalent, then $\Delta_\alpha = \Delta_\beta$ and $(T_2^b)_\alpha = (T_2^b)_\beta$. We have shown, however, that the ratio of the slopes in Fig. 1 is

$$\frac{K_\beta \cdot \Delta_\alpha}{K_\alpha \cdot \Delta_\beta} = 2.7 \quad \text{and above that} \quad \frac{K_\beta}{K_\alpha} \times \frac{(T_2^b)_\beta}{(T_2^b)_\alpha} = 1.3 \ .$$

Therefore,

$$\frac{\Delta_\alpha}{\Delta_\beta} \times \frac{(T_2^b)_\alpha}{(T_2^b)_\beta} = 2.1 \ . \qquad (15)$$

Since this ratio is not equal to unity this means that the binding sites on the enzyme for the acetamido side chains of α-NAG and β-NAG are not magnetically equivalent. This approach demonstrates that although α-NAG and β-NAG bind to the same site on lysozyme they do not occupy that site in an identical manner.

Another possible explanation is that the rates of exchange of the α and β anomers with the enzyme are not identical and "fast" (of the order of 10^5 lmole^{-1} sec^{-1} for the formation of the enzyme substrate complex). This could lead to differential broadening of the resonance of one anomer relative to that of the other anomer. The results described above lead to line width values in the bound state of 10-20 cps which generally agree with the line widths obtained by McDonald and Phillips (1967) for methyl group resonances of lysozyme itself. This suggests that the exchange rates are at or very near the fast exchange limit and probably no extra broadening is caused by exchange phenomena.

The most satisfactory method of proving this argument would be, of course, to determine K_α, K_β, Δ_α, and Δ_β. As mentioned already, this is not possible using the plot of S_0 versus $1/\delta$ as has been demonstrated for methyl-2-acetamido-2-deoxy-α-D-glucopyranoside (α-methyl-NAG) and its corresponding β-anomer, β-methyl-NAG.

Mutarotation Studies

Our early observations on the use of p. m. r. to study the association equilibria of NAG and lysozyme showed that freshly dissolved α-NAG or β-NAG initially displayed in the presence of lysozyme only one acetamido methyl resonance to higher field of the corresponding resonance for free NAG. After about twenty minutes two separate resonances of almost equal intensity were present, corresponding to the α- and β-anomeric forms of the inhibitor in association with the enzyme after mutarotation equilibrium had been reached. Figure 3 shows the development with time of the resonance due to α-NAG in a solution of lysozyme and β-NAG. This study of the phenomenon at 100 M Hz shows that small but measurable changes in the chemical shifts of each anomer occur during the approach to mutarotation equilibrium. Such changes were not reliably obtained during our earlier studies at 60 M Hz.

The mutarotation of freshly dissolved α- or β-NAG provides a method of varying the relative concentrations of α-NAG to β-NAG. The changes in chemical shift of the resonances of the two forms as their relative concentration changes can be quantitated as follows from eqs. 5 and 6.

$$\frac{E_0 \Delta_\alpha}{\delta_\alpha} - K_\alpha - E_0 = S_0 \left[\frac{[\alpha_0]}{S_0} + \frac{K_\alpha}{K_\beta} \frac{[\beta_0]}{S_0} \right]$$

$$= S_0 \left[\frac{[\alpha_0]}{S_0} + \frac{[\beta_0]}{S_0} + \frac{K_\alpha}{K_\beta} \cdot \frac{[\beta_0]}{S_0} - \frac{[\beta_0]}{S_0} \right]$$

$$= S_0 + [\beta_0] \left[\frac{K_\alpha}{K_\beta} - 1 \right] .$$

$$\frac{E_0 \Delta_\alpha}{\delta_\alpha} = E_0 + K_\alpha + S_0 + [\beta_0] \left[\frac{K_\alpha}{K_\beta} - 1 \right] . \qquad (16)$$

and

$$\frac{E_0 \Delta_\beta}{\delta_\beta} = E_0 + K_\beta + S_0 + [\alpha_0] \left[\frac{K_\beta}{K_\alpha} - 1 \right] . \qquad (17)$$

If the superscript t denotes a measurement before mutarotational equilibrium has been established and e denotes the measurement of mutarotational equilibrium then

$$\frac{\delta_\alpha^t}{\delta_\alpha^e} = \frac{E_0 + K_\alpha + S_0 + [\beta_0^e] \left[\frac{K_\alpha}{K_\beta} - 1 \right]}{E_0 + K_\alpha + S_0 + [\beta_0^t] \left[\frac{K_\alpha}{K_\beta} - 1 \right]} . \qquad (18)$$

Similarly for the β-NAG resonances,

$$\frac{\delta_\beta^t}{\delta_\beta^e} = \frac{E_0 + K_\beta + S_0 + [\alpha_0^e] \left[\frac{K_\beta}{K_\alpha} - 1 \right]}{E_0 + K_\beta + S_0 + [\alpha_0^t] \left[\frac{K_\beta}{K_\alpha} - 1 \right]} . \qquad (19)$$

Since δ_α^t and δ_α^e are the observed chemical shifts of the acetamido methyl resonances of the α-anomeric form of a partially and fully mutarotated sample of NAG in the presence of lysozyme, they are measurable. The magnitudes of $[\beta_0^e]$ and $[\beta_0^t]$ can be obtained from integration measurements relative to the α-anomeric resonances at times e and t. Figure 3 shows the progress with time of the resonances of the acetamido methyl groups of α- and β-NAG following mixing of a sample of pure β-NAG with a solution of lysozyme. It is evident from the data that small but measurable chemical shift differences occur for each anomeric methyl resonance between early times where there is a preponderance of one anomeric form and at mutarotational equilibrium where a steady state concentration of each anomeric form exists. The analogous experiment where pure α-NAG was mixed with a solution of lysozyme and the changes in relative concentrations of the anomeric forms with time recorded by measuring the chemical shifts of the acetamido proton resonances was also performed. The quantitative data obtained from these observations is presented in Table II. Substitution of the values obtained for δ^t/δ^e for either anomer and the concentrations of each anomer present at time t and at equilibrium into eqs. 18 or 19 yields preliminary estimates of K_α/K_β assuming that K_α or K_β is small relative to $E_0 + S_0$. This ratio was then used to obtain K_α and K_β from eq. 7. Such values were resubstituted into eqs. 18 or 19 to yield a better estimate of K_α/K_β. This was done until the value of K_α/K_β did not change upon further iteration. Table III summarises the results obtained in this way from four separate determinations. The magnitudes of the dissociation constants obtained

($K_\alpha = (1.6 \pm 0.1) \times 10^{-2}$ M; $K_\beta = (3.3 \pm 0.2) \times 10^{-2}$ M) show that there is very little difference in the relative binding strengths of the two anomeric forms. More interesting, however, is the significant difference in the chemical shifts of the bound forms of each anomer (Δ_α = (0.68 ± 0.03) p.p.m.; Δ_β = (0.51 ±0.03) p.p.m., both to higher field).

Our previous measurements of the association of α-methyl-NAG and β-methyl-NAG with lysozyme, using p.m.r. techniques, showed that the acetamido methyl group of both glycosides displayed a chemical shift of (0.54 ± 0.02) p.p.m. to higher field in the bound state. It was further demonstrated that both of these glycosides bound to the same site on the enzyme as did α-NAG and that they both were competitive with N-acetyl(d_3)-α-D-glucosamine. Since it was further shown by similar experiments that α- and β-NAG bind competitively to lysozyme it is obvious that α-NAG, β-NAG, α-methyl-NAG, and β-methyl-NAG all bind competitively on the enzyme. The chemical shift of the bound form can be used to compare the microenvironment experienced by each inhibitor. Thus, the value of Δ = (0.51 ± 0.03) p.p.m. obtained for β-NAG (Table II) suggests that its binding orientation is the same as that of α-methyl-NAG and β-methyl-NAG. On the other hand the chemical shift (Δ) for the bound form of α-NAG in the same binding site was found to be (0.68 ± 0.03) p.p.m., which means that a different magnetic environment is experienced by its acetamido methyl group. This suggests that the orientation of this inhibitor is different from α-methyl-NAG, β-methyl-NAG, and β-NAG when bound to the same site on lysozyme.

The factor of two in relative dissociation constants ($K_{\alpha\text{-NAG}}$ = 1.6×10^{-2} M; $K_{\beta\text{-NAG}}$ = 3.3×10^{-2} M) for the two anomers of NAG binding to the enzyme represents only a small energy difference equal to 0.4 kcal at 31°C. This small difference combined with the observation that α- and β-NAG are competitive for the same site on the enzyme (Part I, Chapter II) would ordinarily provide good evidence that the binding of the anomers is identical. However, due to the extreme sensitivity of the magnetic-resonance method to environmental changes expressed as Δ (the chemical shift of selected protons in the enzyme-bound form), it is clearly shown that the bound orientations of α-NAG are different.

Since the only difference between α-NAG and β-NAG lies in the configuration of hydrogen and hydroxyl groups at C_1, it is reasonable to assume that it is this grouping which results in a difference in dissociation constant and in bound orientation of the two anomers. Parsons and Raftery (unpublished, 1968) have shown that the binding energy of β-NAG or β-methyl-NAG can be accounted for in terms of the interactions due to the acetamido side chain and the C-3 hydroxyl group. Since α-NAG has the capability to bind through these two interactions as well as β-NAG and since it binds better than β-NAG it is logical to conclude that α-NAG forms a bond to the enzyme through its C-1 hydroxyl group, such as a hydrogen bond. It can be further suggested that such a hydrogen bond would involve the hydrogen of this hydroxyl group rather than the oxygen, since α-methyl-NAG does not behave like α-NAG. Formation of such a bond, however, leads to a difference in bound orientation compared to β-NAG. Therefore it is likely that

other bonds to the enzyme which are formed by β-NAG are not formed when α-NAG binds, umless conformation changes of the enzyme also occur.

The elegant crystallographic studies of Blake et al. (1967) on association of NAG with lysozyme have shown that in the crystalline enzyme the orientations of α-NAG and β-NAG are different while they bind in an overlapping fashion. The acetamido side chains of both anomers were considered to make the same contacts (probably through hydrogen bonds) with the enzyme but the pyranose rings were orientated in different ways. As a result, the orientation of the acetamido methyl group changed somewhat with respect to a tryptophane residue (number 108 in the amino-acid sequence) on the enzyme. The anisotropy of this aromatic ring could very well be responsible for the change in the magnetic environment of lysozyme-bound α-NAG relative to similarly bound β-NAG.

It is of special interest that our results obtained from use of p. m. r. techniques to study enzyme-inhibitor association in solution yield information which is entirely consistent with the aforementioned X-ray analysis studies on crystalline preparations. Further studies of a similar nature on association of oligometric inhibitors with lysozyme (see Part I, Chapter 5), show further agreement with the X-ray analysis studies of Blake et al. (1967). Such consistency between the binding properties of an enzyme in the crystalline state and in solution are taken by us to indicate structural similarity of the enzyme in the two states.

In conclusion, we feel that the results presented in this section

serve to demonstrate the capability of the n. m. r. technique to serve as a tool in obtaining information of macromolecular association phenomena such as enzyme-substrate or enzyme-inhibitor interactions. The method yields information, in addition to dissociation constants, such as knowledge of the microenvironment on the macromolecule experienced by the associating small molecule. Such data are a direct result of the sensitivity of nuclei to change in environment which is detectable by the n. m. r. method.

EXPERIMENTAL

Lysozyme was obtained from Sigma Chemical Company (Lot 96B-8572). Solutions were made up to approximately 3×10^{-3} M lysozyme in 0.1 M citrate buffer pH 5.5 containing 0.5% acetone as an internal reference. The exact concentration of the enzyme was determined by removing 25 $\mu\ell$, diluting to 5.00 ml with 0.1 M citrate buffer pH 5.5 and measuring the optical density of the solution at 280 mμ with a Cary Model 14 spectrophotometer. The known extinction coefficient was used to estimate lysozyme concentrations (Sophianpoulos et al., 1962).

N-Acetylglucosamine was obtained from California Corporation for Biochemical Research. Recrystallization of this material from ethanol-water gave α-NAG, m.p. 202-204°C. The β-anomer was synthesized by acetylation of β-glucosamine in dimethyl formamide according to the procedure of Kuhn and Haber (1953). The melting point of this material was 181-183°C.

The measurements of proton magnetic resonance spectra were performed on a Varian HA-100 spectrometer in frequency sweep mode. In general, the water resonance was used as the lock signal, although sometimes a capillary of tetramethylsilane was employed for this purpose. Chemical shifts of the acetamido methyl group were measured relative to the internal acetone standard. These shifts were determined by electronic counting of the difference between the sweep frequency and the manual oscillator frequency using a Hewlett-Packard frequency counter. In each case the spectra were measured three

times and the chemical shifts were determined to ±0.03 c.p.s. or less from the mean. For those measurements which required time averaging to increase the signal-to-noise ratio, a Varian C-1024 time-averaging computer was used.

All measurements were carried out at a probe temperature of $31^{\circ} \pm 1^{\circ}$. Every sample was pre-equilibrated at 31° for at least 5 minutes in a water bath before introduction into the probe.

For experiments in which the acetate impurity in lysozyme interfered with the proton resonances of α-NAG a sample of acetate-free enzyme was prepared. This was accomplished by multiple ultrafiltrations of a solution of the enzyme in a Diaflo Ultrafiltration apparatus (Amicon Corporation) using a UM-1 membrane. The solvent for the protein sample was 0.1 M citrate, pH 5.5. Following the multiple ultrafiltrations exhaustive dialysis against distilled water and lyophilisation yielded lysozyme which displayed no p.m.r. spectrum for free acetate.

Mutarotation Studies

A weighted amount of crystalline α- or β-NAG was thermally equilibrated in a 1.00 ml volumetric flask in a water bath at 31°. This material was dissolved in a thermally equilibrated solution which contained enzyme, buffer, and acetone. After the sample dissolved, it was transferred to a n.m.r. tube, equilibrated in the water bath for 1 minute, and placed in the probe. The spectrometer was locked on the water resonance, and the spectrum recorded. A spectrum could be produced in this manner in 3-4 minutes from the time the crystalline NAG was dissolved.

APPENDIX A

Quantitation of chemical shift data for association of an enzyme with an inhibitor which is at equilibrium between two forms.

In the following derivation $[\alpha_0]$ refers to the initial concentration of α-NAG, $[\beta_0]$ to β-NAG, Δ_α and Δ_β to the enzyme bound chemical shift of a nucleus in α-NAG and β-NAG respectively, δ_α and δ_β to observed chemical shift differences of nuclei in α-NAG and β-NAG in the presence of the enzyme compared with the chemical shifts of the same nuclei in the absence of the enzyme, $(P_b)_\alpha$ and $(P_b)_\beta$ to the enzyme bound fractions of the total α-NAG and β-NAG present, Eα and Eβ to lysozyme-α-NAG and lysozyme-β-NAG complexes, K_α and K_β to the dissociation constants of those complexes.

For a system in which α-NAG and β-NAG are binding competitively to lysozyme we can write

$$E + \alpha \quad E\alpha \qquad K_\alpha = \frac{[E][\alpha]}{[E\alpha]} \tag{21a}$$

$$E + \beta \quad E\beta \qquad K_\beta = \frac{[E][\beta]}{[E\beta]} \tag{21b}$$

and

$$\delta_\alpha = (P_b)_\alpha \Delta_\alpha = \frac{[E\alpha]}{[\alpha_0]}\ \Delta_\alpha$$

$$\delta_\beta = (P_b)_\beta \Delta_\beta = \frac{[E\beta]}{[\beta_0]}\ \Delta_\beta$$

$$[E\beta] = [E\alpha] \cdot \frac{[\beta]\, K_\alpha}{[\alpha]\, K_\beta} \tag{22}$$

$$K_\alpha = \frac{(E_0 - [E\alpha] - [E\beta])([\alpha_0] - [E\alpha])}{[E\alpha]} \tag{23}$$

$$K_\alpha = \frac{\left(E_0 - [E\alpha]\left\{1 + \frac{[\beta]}{[\alpha]}\frac{K_\alpha}{K_\beta}\right\}\right)([\alpha_0] - [E\alpha])}{[E\alpha]} \tag{24}$$

$$K_\alpha = \frac{\left(E_0 - \frac{\delta_\alpha}{\Delta_\alpha}[\alpha_0]\left\{1 + \frac{[\beta]}{[\alpha]}\frac{K_\alpha}{K_\beta}\right\}\right)\left(\alpha_0 - \frac{\delta_\alpha}{\Delta_\alpha}[\alpha_0]\right)}{\frac{\delta_\alpha}{\Delta_\alpha}[\alpha_0]} \tag{25}$$

$$\frac{\delta_\alpha}{\Delta_\alpha - \delta_\alpha} K_\alpha = \left(E_0 - \frac{\delta_\alpha}{\Delta_\alpha} S_0 \left\{\frac{[\alpha_0]}{S_0} + \frac{[\beta]}{S_0}\frac{[\alpha_0]}{[\alpha]}\frac{K_\alpha}{K_\beta}\right\}\right) \tag{26}$$

where $S_0 = [\alpha_0] + [\beta_0]$.

Letting

$$A' = \frac{[\alpha_0]}{S_0} + \frac{[\beta]}{S_0}\frac{K_\alpha}{K_\beta}\frac{[\alpha_0]}{[\alpha]} \tag{27}$$

$$K_\alpha \frac{\delta_\alpha}{\Delta_\alpha - \delta_\alpha} = E_0 - \frac{\delta_\alpha}{\Delta_\alpha} S_0 \cdot A' \tag{28}$$

$$S_0 \cdot A' \frac{\Delta_\alpha - \delta_\alpha}{\Delta_\alpha} = \frac{\Delta_\alpha - \delta_\alpha}{\delta_\alpha}(E_0) - K_\alpha \tag{29}$$

Using the approximations

$$[\beta]\frac{[\alpha_0]}{[\alpha]} \sim [\beta_0]$$

and

$$\frac{\Delta_\alpha - \delta_\alpha}{\Delta_\alpha} \sim 1 \ ,$$

$$A' \sim A = \frac{[\alpha_0]}{S_0} + \frac{[\beta_0]}{S_0} \frac{K_\alpha}{K_\beta}$$

and

$$S_0 \cdot A = \frac{\Delta_\alpha}{\delta_\alpha} E_0 - K_\alpha - E_0 \quad . \tag{30a}$$

Similarly $$S_0 \cdot B = \frac{\Delta_\beta}{\delta_\beta} E_0 - K_\beta - E_0$$

where $$B = \frac{[\beta_0]}{S_0} + \frac{[\alpha_0]}{S_0} \frac{K_\beta}{K_\alpha} \quad . \tag{30b}$$

APPENDIX B

Quantitation of line-width measurements for association with an enzyme of an inhibitor which is in mutarotation equilibrium.

In the following derivation $(P_b)_\alpha$ and $(P_b)_\beta$ refer to the fractions of α-NAG and β-NAG which are enzyme bound, $(T_2^b)_\alpha$ and $(T_2^b)_\beta$ to the spin-spin relaxation times of the bound species of α-NAG and β-NAG, $(1/T_2')_\alpha$ and $(1/T_2')_\beta$ to the contributions to the observed line widths due to association with the enzyme of α-NAG and β-NAG (as defined in eq. 10) S_0 to the total concentration of NAG, $[\alpha_0]$ and $[\beta_0]$ to the total concentrations of α-NAG and β-NAG, K_α and K_β to the dissociation constants of Eα and Eβ which are defined as the complexes of lysozyme with α-NAG and with β-NAG.

The contributions to the observed line width of nuclei in each anomeric form due to association with the enzyme may be expressed as

$$\left(\frac{1}{T_2'}\right)_\alpha = (P_b)_\alpha \left(\frac{1}{T_2^b}\right)_\alpha \tag{31a}$$

$$\left(\frac{1}{T_2'}\right)_\beta = (P_b)_\beta \left(\frac{1}{T_2^b}\right)_\beta \quad . \tag{31b}$$

Since eqs. 31a and 31b have the same form as eqs. 21a and 21b in Appendix A, an equation analogous to eq. 29 can be written (assuming $A' = A$; see Appendix A).

$$S_0 \cdot A \frac{\left(\frac{1}{T_2^b}\right)_\alpha - \left(\frac{1}{T_2'}\right)_\alpha}{\left(\frac{1}{T_2^b}\right)_\alpha} = \frac{\left(\frac{1}{T_2^b}\right)_\alpha - \left(\frac{1}{T_2'}\right)_\alpha}{\left(\frac{1}{T_2'}\right)_\alpha} \cdot E_0 - K_\alpha \qquad (32)$$

which may be rearranged to

$$(T_2')_\alpha = \frac{(T_2^b)_\alpha}{E_0}\left[\frac{K_\alpha}{1 - \frac{(T_2^b)_\alpha}{(T_2')_\alpha}} + S_0 \cdot A\right] . \qquad (33)$$

Substitution for A gives

$$(T_2')_\alpha = \frac{(T_2^b)_\alpha}{E_0 K_\beta}\left[\frac{K_\alpha K_\beta}{1 - \frac{(T_2^b)_\alpha}{(T_2')_\alpha}} + [\alpha_0]K_\beta + [\beta_0]K_\alpha\right] . \qquad (34a)$$

Similar arguments for the case of the β anomer give

$$(T_2')_\beta = \frac{(T_2^b)_\beta}{E_0 K_\alpha}\left[\frac{K_\alpha K_\beta}{1 - \frac{(T_2^b)_\beta}{(T_2')_\beta}} + [\beta_0]K_\alpha + [\alpha_0]K_\beta\right] . \qquad (34b)$$

Thus

$$\frac{(T_2')_\beta}{(T_2')_\alpha} = \frac{K_\beta}{K_\alpha}\frac{(T_2^b)_\beta}{(T_2^b)_\alpha}\frac{\left[\frac{K_\alpha K_\beta}{1 - \frac{(T_2^b)_\beta}{(T_2')_\beta}} + [\beta_0]K_\alpha + [\alpha_0]K_\beta\right]}{\left[\frac{K_\alpha K_\beta}{1 - \frac{(T_2^b)_\alpha}{(T_2')_\alpha}} + [\beta_0]K_\alpha + [\alpha_0]K_\beta\right]} . \qquad (35)$$

Under the conditions of these experiments

$$1 > \frac{(T_2^b)_\alpha}{(T_2')_\alpha} \qquad \text{and} \qquad 1 > \frac{(T_2^b)_\beta}{(T_2')_\beta}$$

and to within a 5% error

$$\frac{(T_2')_\beta}{(T_2')_\alpha} = \frac{K_\beta}{K_\alpha} \frac{(T_2^b)_\beta}{(T_2^b)_\alpha} \quad .$$

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TABLE 1. Chemical shift data for the acetamido methyl protons of α-NAG and β-NAG association with lysozyme (2.95×10^{-3} M). Measurements were made at 100 M Hz, in citrate buffer 0.1 M, pH 5.5, 31°C.

NAG $\times 10^2$	δ_α(c.p.s.)	δ_β(c.p.s.)	$\frac{1}{\delta_\alpha}$(sec)	$\frac{1}{\delta_\beta}$ (sec)
9.95	2.59	0.92	0.386	1.09
7.17	3.36	1.17	0.298	0.856
5.98	3.85	1.35	0.260	0.741
4.78	4.37	1.55	0.229	0.645
2.39	6.69	2.39	0.150	0.418

TABLE 2. Quantitation of chemical shift data for the acetamido methyl protons of α-NAG or β-NAG, in the presence of lysozyme, during the time necessary to reach equilibrium. Measurements made at equilibrium are given as δ^e, while those made at time t, before equilibrium was reached are given as δ^t. The concentration of each anomer at times t and e are shown as $[\alpha_0^t]$, $[\alpha_0^e]$, $[\beta_0^t]$, $[\beta_0^e]$ and were determined from peak height measurements.

$S_0 \times 10^2$	Anomer form employed	Methyl resonance measured	$\frac{\delta_\alpha^t}{\delta_\alpha^e}$	$\frac{\delta_\beta^t}{\delta_\beta^e}$	$\frac{[\alpha_0^e]\times 10^2 M}{[\alpha_0^t]\times 10^2 M}$	$\frac{[\beta_0^e]\times 10^2 M}{[\beta_0^t]\times 10^2 M}$
3.65	β-NAG	β-	- -	1.055	- -	$\frac{1.84}{1.35}$
3.65	β-NAG	α-	0.944		$\frac{1.81}{2.30}$	
3.40	α-NAG	α-	0.932		$\frac{1.72}{1.20}$	
6.80	α-NAG	α-	0.945		$\frac{3.44}{2.40}$	

TABLE 3. Summary of chemical shift data for the acetamido methyl protons of α-NAG and β-NAG during mutarotation of each anomer in the presence of lysozyme ($2.8 - 3.0 \times 10^{-3}$ M). Measurements were made in 0.1 M citrate buffer, pH 5.5, at 31°C, at 100 M Hz.

$S_0 \times 10^2$	Anomeric form	K_α / K_β	K_α	K_β	Δ_α(p. p. m.)	Δ_β(p. p. m.)
3.65	β	0.53	1.6×10^{-2}	3.1×10^{-2}	0.71	0.48
3.65	α	0.47	1.5×10^{-2}	3.2×10^{-2}	0.68	0.51
3.40	α	0.40	1.5×10^{-2}	3.6×10^{-2}	0.66	0.55
6.80	α	0.53	1.6×10^{-2}	3.1×10^{-2}	0.71	0.48

Figure 1. Plot of the chemical shift data (from Table 1) for the acetamido methyl resonances of α-NAG and β-NAG (at mutarotation equilibrium) in association with lysozyme. The concentrations plotted are those of α-NAG plus β-NAG (i.e., total NAG). Measurements were made in 0.1 M citrate buffer, pH 5.5, at 31°C, at 100 M Hz.

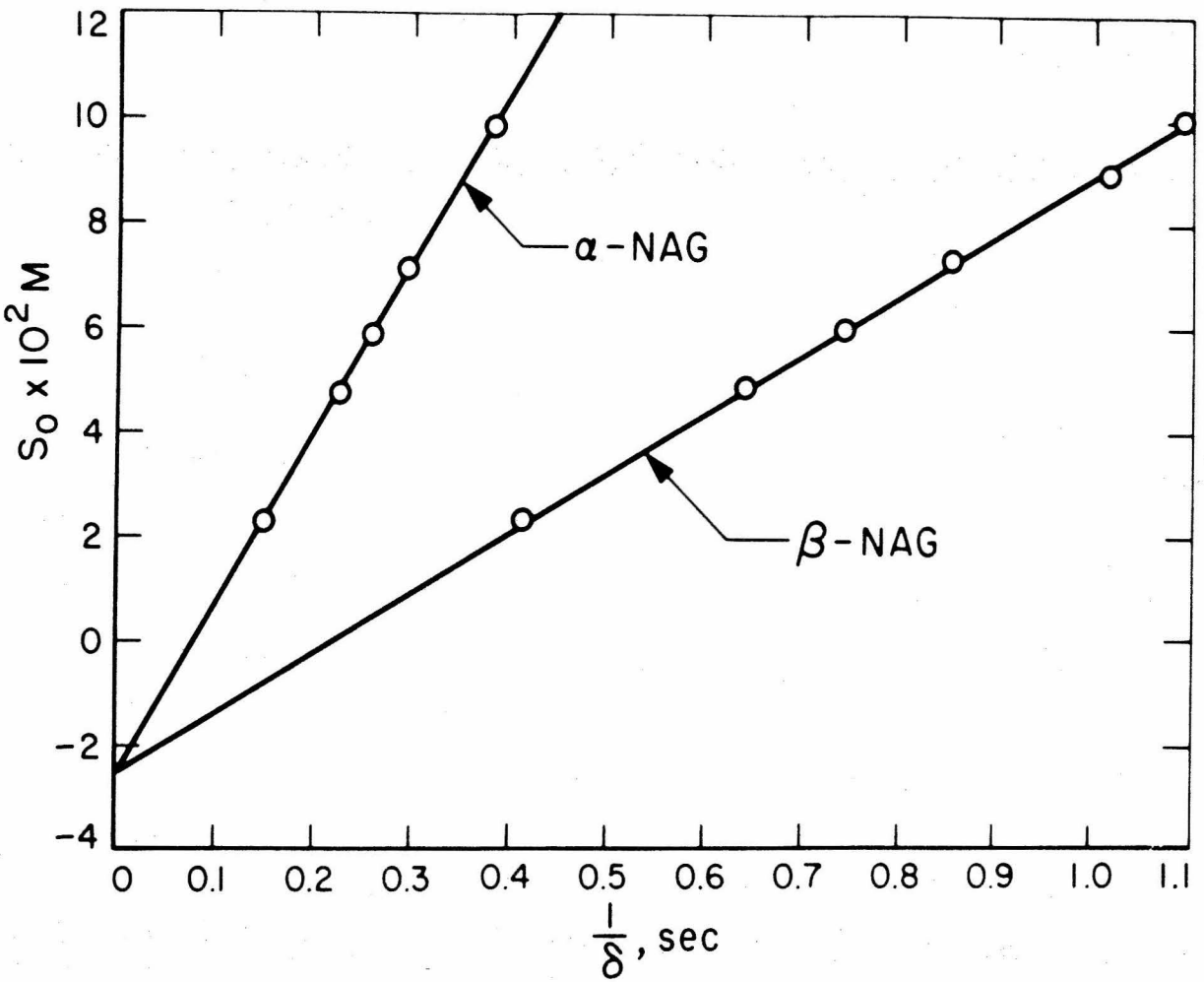

12
10
8
6
4
2
0
-2
-4
$S_0 \times 10^2$ M
α-NAG
β-NAG
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
1.1
$\frac{1}{\delta}$, sec

Figure 2. Time averaged spectrum (15 scans) of the acetamido methyl resonances of α-NAG and β-NAG (at mutarotation equilibrium; total NAG concentration = 1.21×10^{-2} M) in the presence of lysozyme (3.0×10^{-3} M). Measurements were made in 0.1 M citrate buffer, pH 5.5, at 31°C, at 100 M Hz.

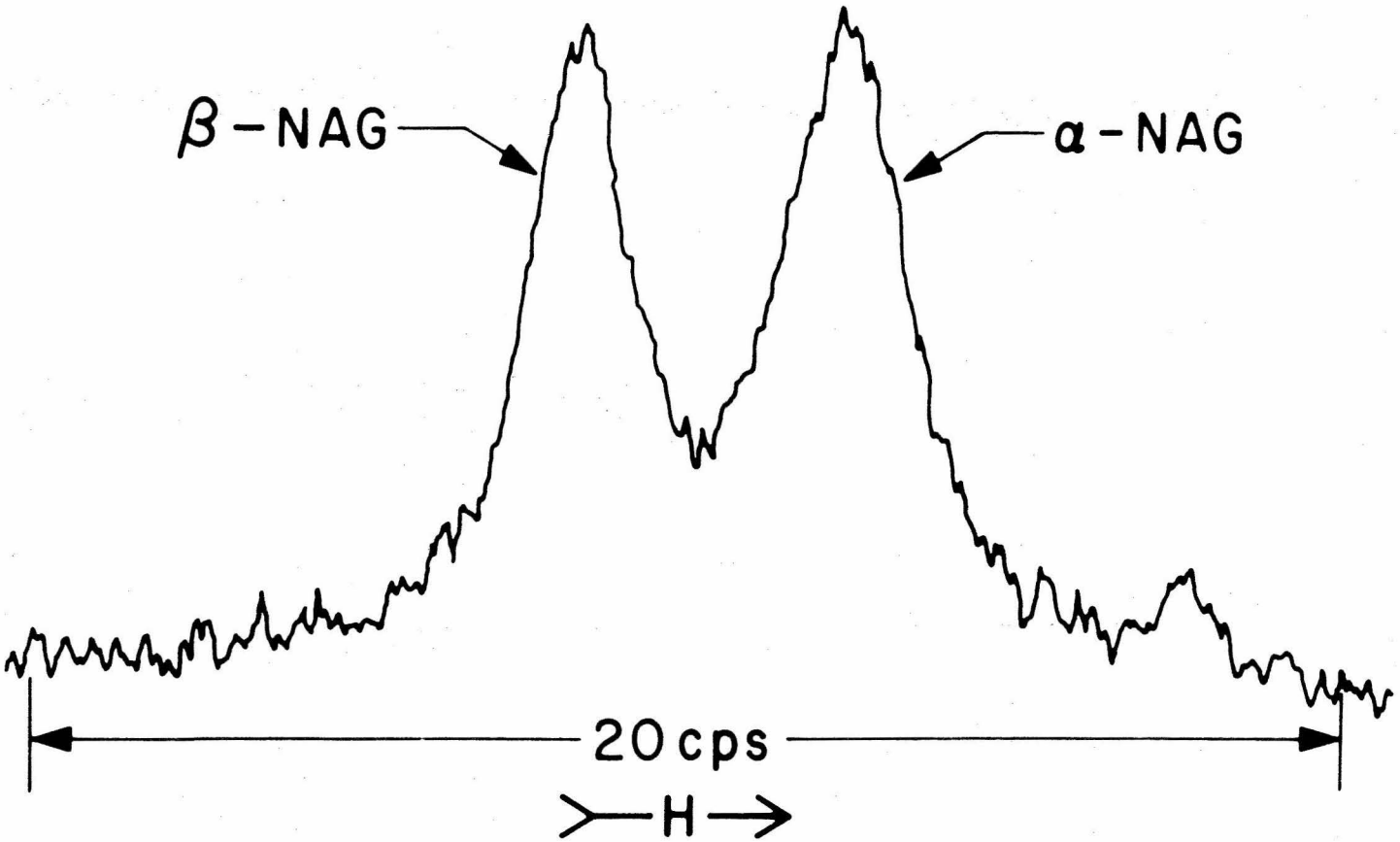
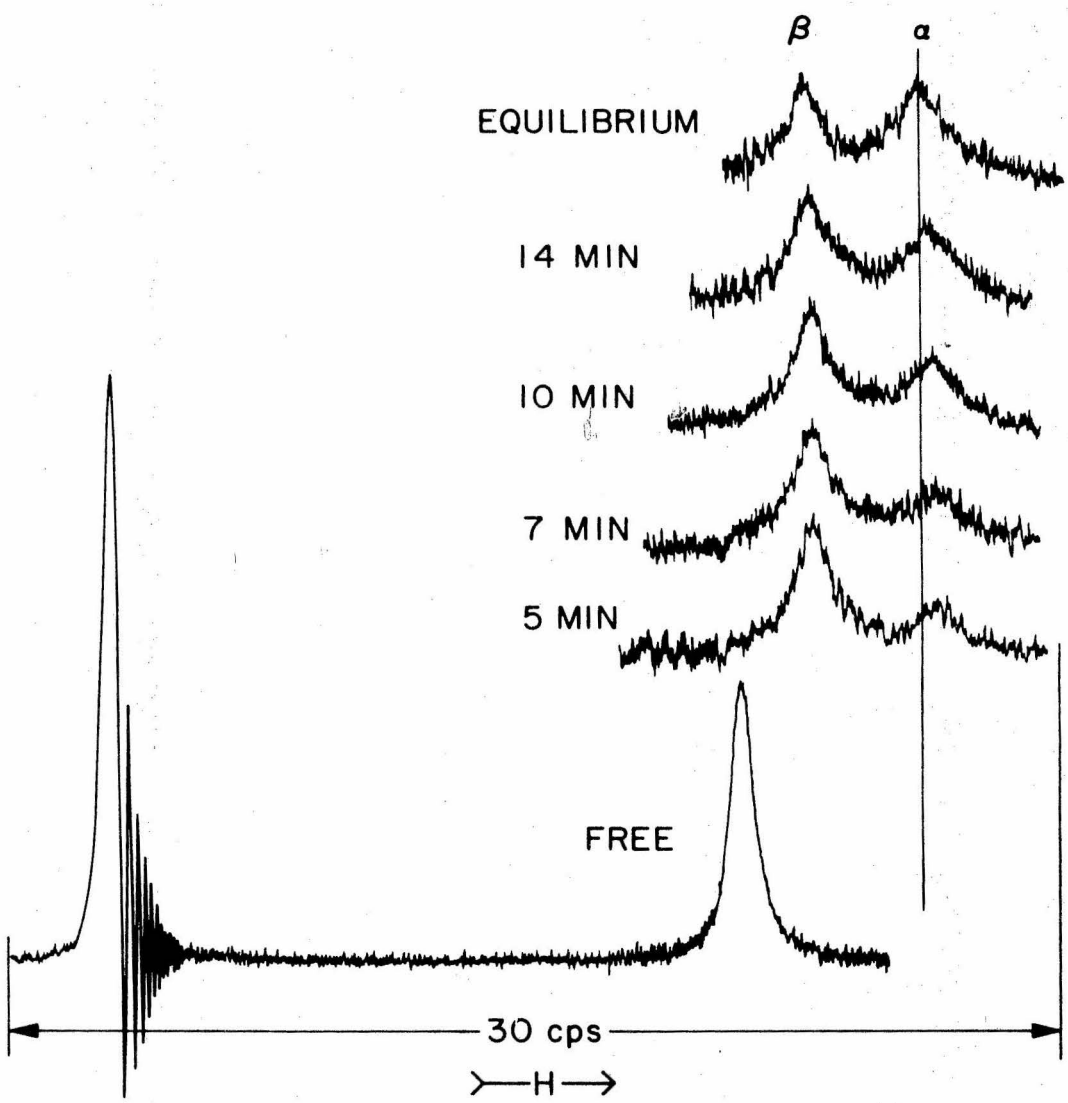

β-NAG
α-NAG
20 cps
H

Figure 3. Proton magnetic resonance spectra of NAG (acetamido methyl protons) free in solution and of β-NAG during mutarotation in the presence of lysozyme (3.0×10^{-3} M). The intense resonance at left is that of the protons of an internal acetone standard. After five minutes in the presence of lysozyme β-NAG was present in excess over α-NAG. The establishment of mutarotation equilibrium was followed with time until it was attained after approximately twenty minutes. Spectra were recorded at 100 M Hz and 31°C. Samples were dissolved in 0.1 M citrate, pH 5.5.


β
α
EQUILIBRIUM
14 MIN
10 MIN
7 MIN
5 MIN
FREE
30 cps
H

CHAPTER IV

A NUCLEAR MAGNETIC RESONANCE STUDY OF ENZYME-INHIBITOR ASSOCIATION. THE USE OF pH AND TEMPERATURE EFFECTS TO PROBE THE BINDING ENVIRONMENT

INTRODUCTION

In the preceding sections we have shown that lysozyme causes a shift in the proton magnetic resonance spectrum of the acetamido methyl group of various N-acetyl pyranosides and analogues of sugars (Parsons and Raftery, unpublished results, 1968). It has been further demonstrated that this observed chemical shift δ can be quantitated for the equilibrium

$$E + S \rightleftharpoons ES \qquad \text{and} \qquad K_s = \frac{E \cdot S}{ES} .$$

If Δ represents the chemical shift of the acetamido methyl group in its binding site on the enzyme and δ represents the observed chemical shift

$$S_0 = \frac{E_0 \Delta}{\delta} - K_s - E_0$$

where E_0 and S_0 represent the total concentrations of enzyme and sugar molecules, respectively. The quantity Δ is a measure of the magnetic environment of the binding site of the enzyme and can be

determined for any or all of the nonequivalent protons of the sugar molecule.

The present section describes a study of the binding of methyl-2-deoxy-2-acetamido-β-D-glucopyranoside to lysozyme, using proton magnetic resonance methods. In this study both the glycosidic and acetamido methyl resonances were observed in the presence of the enzyme and the effects quantitated as a function of temperature and pH. The results obtained show that a substrate or inhibitor molecule may be utilized as an effective probe of its binding site on the enzyme.

EXPERIMENTAL

All solutions contained approximately 3×10^{-3} M lysozyme (Sigma Chemical Co., Lot #96B 8572) and 0.5% each of methanol and acetone as internal p.m.r. standards. The buffers were made by mixing 0.1 M citric acid with either 0.1 M sodium citrate, 0.1 M disodiumphosphate, or 0.1 M trisodiumphosphate. At pH 5.5, identical results were obtained using either a citrate buffer or a citrate-phosphate buffer.

Methyl-2-acetamido-2-deoxy-β-D-glycopyranoside was prepared according to the procedure described by Conchie and Levvy (1963). The melting point of this material was 202-204, and was free of N-acetyl-glucosamine as determined by the Park-Johnson (1949) method.

For measurements at pH 4.5, lysozyme was dialysed against an excess of 10^{-3} M acetic acid-d_3 (Tracerlab, Inc.) to exchange the acetate impurity in the enzyme preparation which interfered at this pH with the acetamido methyl resonance of the substrate, and finally lyophilised.

All spectra were obtained with a Varian HA-100 nuclear magnetic resonance spectrometer, operating in frequency sweep mode. The water resonance was used as a lock signal, and each sample was allowed to come to thermal equilibrium before measurements were taken. For measurements at temperatures other than 31°, the operating temperature of the probe, a Varian V-4341 Variable Temperature accessory was employed. The difference in chemical shift of methanol as a function of temperature was used to determine the probe temperature.

Chemical shifts were determined by electronic counting of the difference between the sweep frequency and manual oscillator frequency using a Hewlett-Packard counter.

The chemical shift of the glycosidic methyl group in the bound state was determined by measurement of the ratio of the glycosidic methyl group shift to the acetamido methyl group shift. This ratio was then multiplied by the value of the chemical shift (Δ) of the bound acetamido group which was determined separately from a concentration study of the observed chemical shift of the acetamido group resonance. This method was used because the glycosidic methyl group displayed only a small observed shift, and the error in a concentration study of its resonance to determine K_s and Δ would have been very large.

RESULTS AND DISCUSSION

The Temperature Dependence of β-MeNAG* Binding

Figure 1 shows the temperature dependence of the dissociation constant (K_s) for β-methyl-NAG and lysozyme. To determine the enthalpy of the binding equilibrium, at pH 5.5 in 0.1 M citrate buffer, pK_s($-\log K_s$) was plotted versus 1/T. A value of - 5.5 kcal was obtained for ΔH under these conditions. Unfortunately, an interpretation of this value in terms of specific types of interaction between the sugar and enzyme is difficult. In this regard it has been demonstrated (Rupley et al., 1967) that the binding of saccharides to lysozyme involves the displacement of protons from the enzyme. Thus, the overall reaction whose enthalpy has been measured involves contributions from the loss of protons by the enzyme and interactions of these protons with the solvent, as well as from the binding interactions of interest. Even if the contributions of interest could be isolated, the binding is most probably a complex combination of a polar and hydrogen bonding interactions, whose separate contributions to the total enthalpy would be difficult to evaluate.

The determined values of Δ for the acetamido methyl protons remained constant within experimental error over the temperature range studied and were found to be 0.51-0.54 p.p.m. This would suggest that there were no significant conformational changes taking place

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*Abbreviations used are: β-methyl-NAG, methyl-2-deoxy-2-acetamido-β-D-glucopyranoside; p.m.r., proton magnetic resonance; di-NAG, chitobiose, tri-NAG, chitotriose.

in the binding site over the temperature range studied. This observation is in agreement with the p. m. r. studies of lysozyme carried out by McDonald and Phillips (1967) at 220 M Hz and Sternlicht and Wilson (1967) at 100 M Hz. These investigators found that at temperatures below 75-80°, the transition temperature for reversible thermal denaturation, the lysozyme p. m. r. spectrum did not appear to undergo any gross changes.

The pH Dependence of the Binding Constant

The pH dependence of the dissociation constant for β-MeNAG and lysozyme at 31° is shown in Fig. 2. The data are presented as pK_s versus pH as recommended by Dixon (1958). Interpretation of this data, according to Dixon's theory concerning the effects of pH on substrate binding, allows us to say that one ionizable group on the enzyme is perturbed by the association with β-MeNAG. This group has a pK_a of 6.1 in the free enzyme, which is perturbed to a value of 6.6 in the enzyme inhibitor complex. Our previous measurements on the binding of tri-N-acetylchitotriose to lysozyme by optical techniques (see Part I, Chapter I) showed that association with the tri-saccharide affected the pK_a's of two ionizable groups on the enzyme. A group of pK_a 4.2 in the free enzyme was changed to a value of 3.6 in the enzyme substrate complex, and a second group of pK_a 5.8 in the free enzyme was perturbed to 6.3 in the complex. The results of Rupley et al. (1967) and Lehrer and Fasman (1966) obtained from similar studies of the binding of tri-N-acetylchitotriose and lysozyme by related optical methods support these earlier findings.

Thus, the binding of β-MeNAG as studied by p. m. r. methods affects the ionization of a group whose determined pK_a is only slightly different from the pK_a of one of the ionizable groups perturbed by tri-NAG binding. It is probable that these are indeed the same ionizable group on the enzyme. The pK_a associated with this ionizable group suggests it is either a histidine residue or a carboxyl of unusually high pK_a. The titration data of Donovan et al. (1960), indicated a carboxyl group of pK_a 6.3 to be present in lysozyme, while Meadows et al. (1967) have demonstrated that the pK_a of the single histidine residue in lysozyme is 5.8 and is not affected by substrate binding. Therefore, it appears reasonable to say that the ionization of a carboxyl group of pK_a 6.1 in lysozyme is affected by the binding of β-MeNAG and also of tri-NAG. The group of lower pK_a (4.2) in lysozyme which is affected by tri-NAG binding shows no effect on the binding of β-MeNAG and it is reasonable to infer that this also is a carboxyl group. This group must interact with a sugar ring(s) of the trisaccharide in binding regions other than the β-MeNAG binding site.

Work from this laboratory (see Part I, Chapter V) concerning the relative binding orientations of NAG, di-NAG, and tri-NAG has shown that the reducing end of each saccharide occupies the same binding site. This observation as well as our demonstration that α-NAG and β-NAG bind with different orientations in a single binding site Part I, Chapter III) are in complete agreement with the X-ray analysis studies of Blake et al. (1967) on lysozyme-saccharide complexes in the crystalline state. It would appear therefore that for lysozyme the binding process is quite similar to the crystal and in solution. We regard

the demonstrations of similar binding properties in the crystalline state and in solution as indirect but nevertheless convincing proof of identical structure in the two states. This is of course restricted, at least in detail, to the region of the molecule to which inhibitors bind. Examinations of a space-filling model of lysozyme built in this laboratory from Corey-Pauling-Koltum (CPK) models using the coordinates obtained from the X-ray analysis date of Blake et al. (1967) allows us to speculate as to the specific amino acid residues whose ionizations are being observed. There are at least two carboxyl residues whose ionizations could obviously affect β-MeNAG binding, aspartic acid 52 and glutamic acid 35, and it would appear that the group of pK_a 6.1 is associated with one of these residues. Since the environment of glutamic acid 35 is nonpolar, Blake et al. (1967) have suggested that it would be expected to have an abnormally high pK_a and we therefore assign the pK_a value of 6.1 to this group.

The Effect of pH on Chemical Shifts of β-MeNAG Methyl Groups

The pH dependency of Δ, for two resonances of β-MeNAG have been measured. Figure 3 shows the effect of pH on the acetamido methyl resonance of NAG, and also similar data for the glycosidic methyl group resonance. The glycosidic methyl group shows a relatively small downfield shift which is pH independent, within experimental error. Such a result enables us to state that no significant conformation changes at the binding site of the enzyme take place as a result of pH effects in the range studied, since chemical shifts are extremely sensitive to changes in distance from the source to the effect causing them (Buckingham et al., 1960).

This result does not exclude the possibility of a conformation change in the enzyme resulting in poor binding of the glycoside. It does, however, suggest that such a conformation change, if it occurs, is independent of pH. The acetamido methyl resonance, however, shows two breaks due to ionizations on the enzyme substrate complex which affect the magnetic environment of the acetamido group while bound. The pK_a of a group whose ionization affects the magnetic environment of a particular nucleus may be obtained in the following manner. If for the scheme

$$ESH \rightleftharpoons ES + H \qquad \text{and} \qquad K_a = \frac{[ES][H]}{[ESH]}$$

Δ_1 is a chemical shift associated with ESH and Δ_2 a chemical shift similarly associated with the unprotonated form ES, then (neglecting charges) the observed chemical shift, Δ, will be

$$\Delta = \frac{[ESH]}{[ES] + [ESH]} \cdot \Delta_1 + \frac{[ES]}{[ES] + [ESH]} \cdot \Delta_2$$

$$\Delta = \frac{\frac{[ESH]}{[ES]}\ \Delta_1 + \Delta_2}{([ESH]/[ES]) + 1}$$

$$\Delta = \frac{\left(\frac{[H]}{K_a}\right)\Delta_1 + \Delta_2}{\left(\frac{H}{K_a}\right) + 1}$$

when $K_a = [H]$ or $pK_a = pH$,

$$\Delta = \frac{\Delta_1 + \Delta_2}{2} \quad .$$

Taking the plateau values of Δ for particular states of ionization of the enzyme-substrate complex, as shown in Fig. 3, pK_a's of 4.7 ± 0.1 and 7.0 ± 0.5 are obtained for the ionizable groups in the enzyme-substrate complex. The higher pK_a is consistent with the idea that the same ionizable group which affects the binding of β-MeNAG also has an effect on the magnetic environment of the β-MeNAG acetamido methyl protons. The lower value of 4.7 is interesting in that the ionization of the group involved has no noticeable affect on binding. This implies that the pK_a of this group remains unperturbed in the presence of β-MeNAG.

The most probable causes for pH dependent changes in the magnetic environment of a binding site are changes in the ionization state of a group on the enzyme which affects (1) the electric field in the binding site, (2) the magnetic anisotropy of the group in question, (3) the conformation of the macromolecule, or (4) the state of aggregation of the enzyme, which can be regarded as a conformational change. A gross conformational change should have some affect on the glycosidic methyl resonance as well as the acetamido methyl group resonance. However, the chemical shift of the glycosidic methyl group is pH independent. Furthermore, in the pH region 3.0-5.5, the dissociation constant of β-MeNAG does not change, which would not be expected if the conformation of the binding site were changing. It is, therefore, unlikely that the group of pK_a 4.7 which affects the chemical shift of the acetamido methyl group when bound is associated with conformational changes in the enzyme. The effect of this group is most likely manifested as an electric field or magnetic anisotropy change which affects the magnetic environment of the binding site. Such electric field effects

have been shown (Buckingham et al., 1960) to fall off as the square of the distance to the nuclei being observed, while anisotropy effects fall off as the cube of the distance. Therefore, this group must be near the binding site of the acetamido methyl group of β-MeNAG. The determined pK_a value of 4.7 furthermore suggests that it is a carboxyl group.

Our present results therefore indicate two groups (pK_a = 4.7 and 6.1) in the enzyme which are close to the binding site of β-MeNAG and our previous results (Part I, Chapter I) show two groups are affected by the binding of tri-NAG. Examination of the space-filling model, shows a total of four carboxyl residues near the β-MeNAG binding site. These are the carboxyl side-drains of glutamic acid 35 and aspartic acids 103, 101, and 52. We have already assigned to glutamic acid residue 35 a pK_a value of 6.1. We can, on the basis of the X-ray analysis results of Blake et al. (1967) when compared with our previous studies (Part I, Chapter I) assign a pK_a value of 4.2 to aspartic acid residue number 101, since the tri-saccharide interacts with it, while monosaccharides do not. Aspartic acid 52 is involved in structural stabilization of the enzyme through hydrogen bonding and should have a low pK_a. It has been shown (Sophianpoulos and Weiss, 1964) that the thermal stability of lysozyme is affected by the ionization of groups on the enzyme of low pK_a, approximately pK_a = 2. Therefore aspartic acid 52 is possibly associated with such a low pK_a. The only remaining acidic group near the binding cleft is aspartic acid 103. This is solvent accessible and should have a normal pK_a. This residue is, we feel, most probably associated with the pK_a value of 4.7.

At present, two derivatives of lysozyme have been prepared in this laboratory (Parsons, unpublished results, 1968) both of which are mono-ethyl esters of lysozyme. These derivatives have binding properties differing from those of the native enzyme, and further investigation of these binding properties should hopefully provide support for our present assignment of the various pK_a's of the acidic residues which occupy positions at the binding site of lysozyme.

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Figure 1. Plot of the temperature dependence of the dissociation constant (K_s) for β-methyl-NAG and lysozyme.

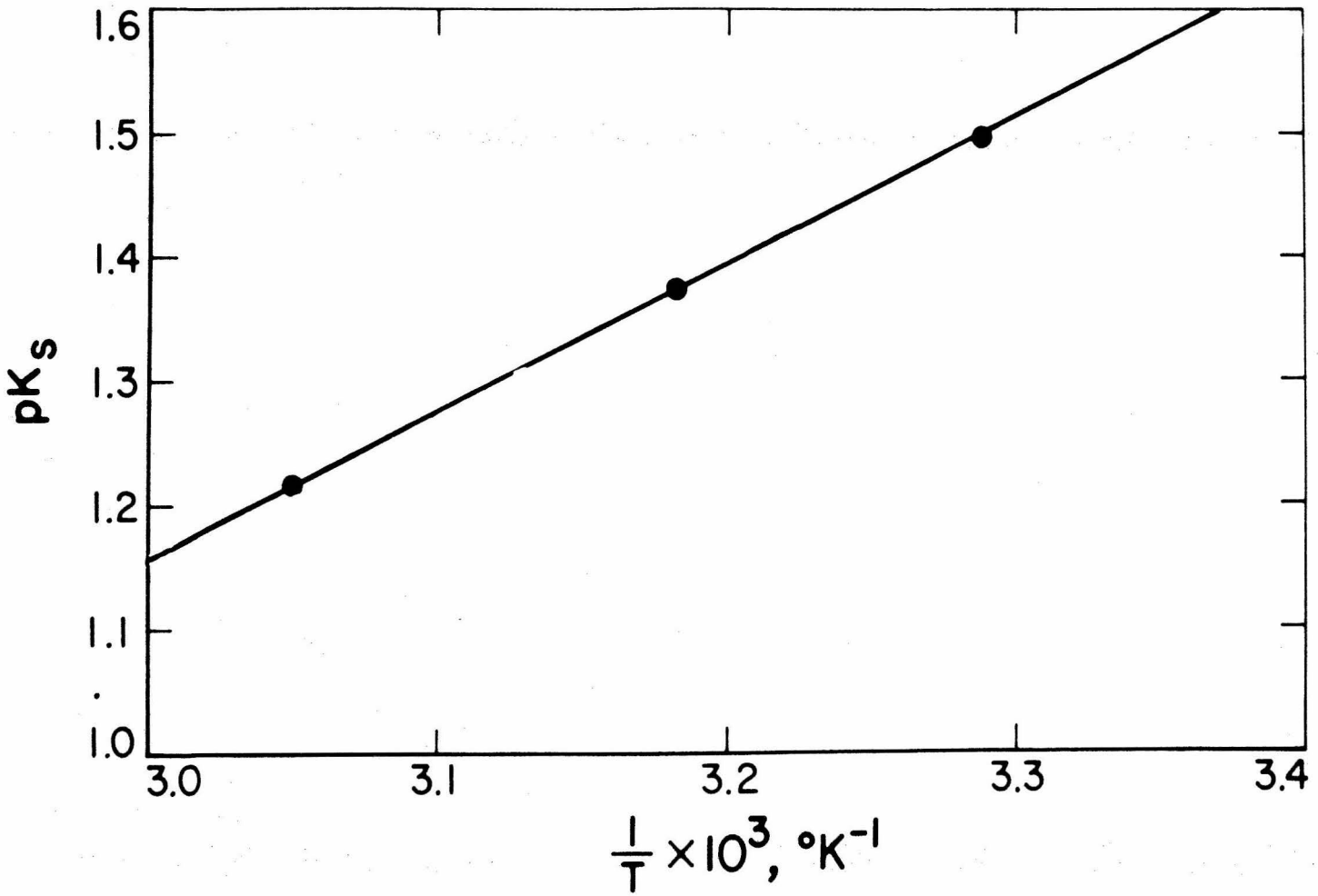

1.6
1.5
1.4
1.3
1.2
1.1
1.0
pK_s
3.0
3.1
3.2
3.3
3.4
$\frac{1}{T} \times 10^3$, °K^{-1}

Figure 2. The effect of pH on the dissociation constant (K_s) for β-methyl-NAG and lysozyme. To determine the pK_a values of ionizable groups on the enzyme and the enzyme-inhibitor complex – log K_s (pK_s) was plotted with pH.

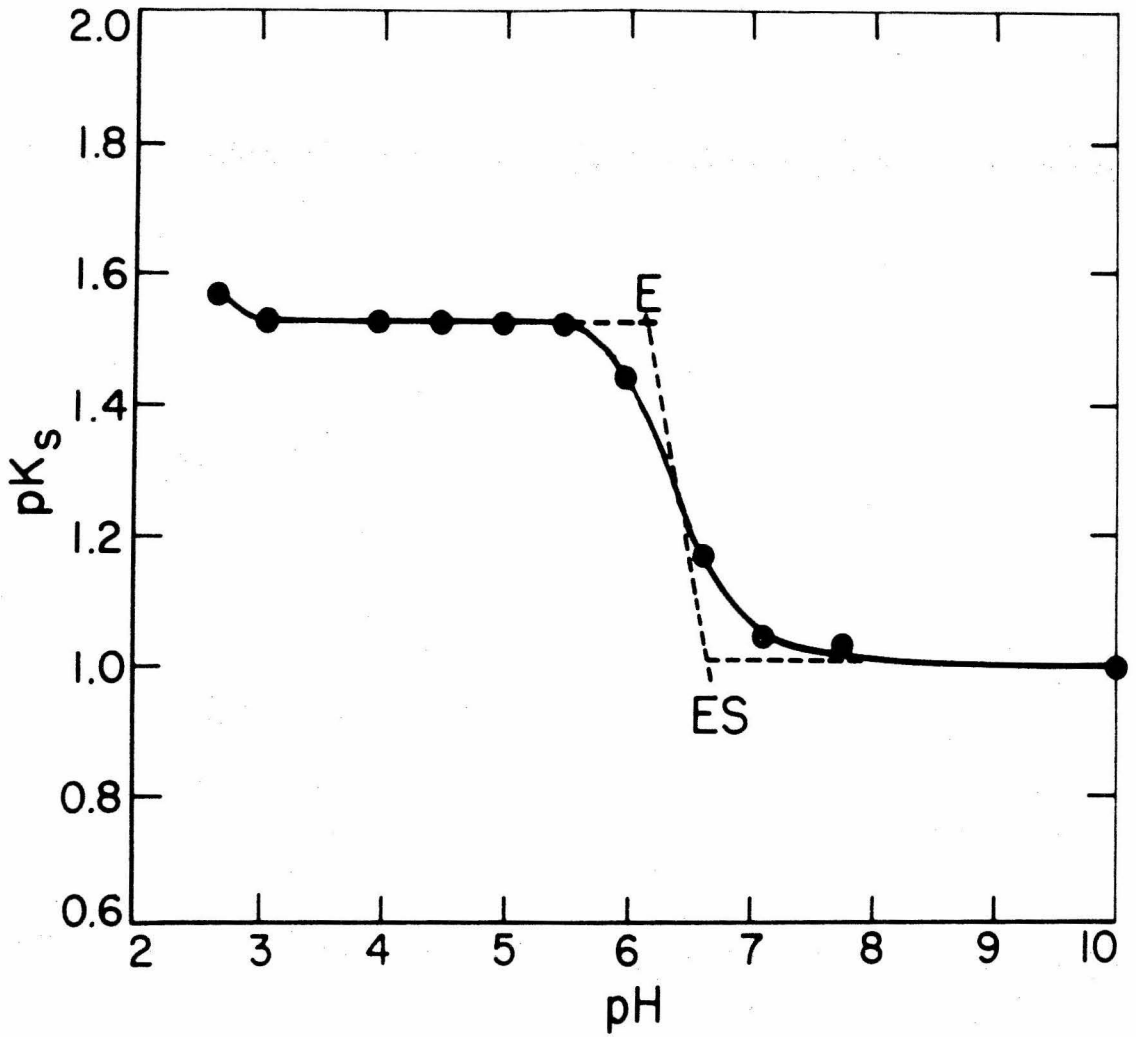
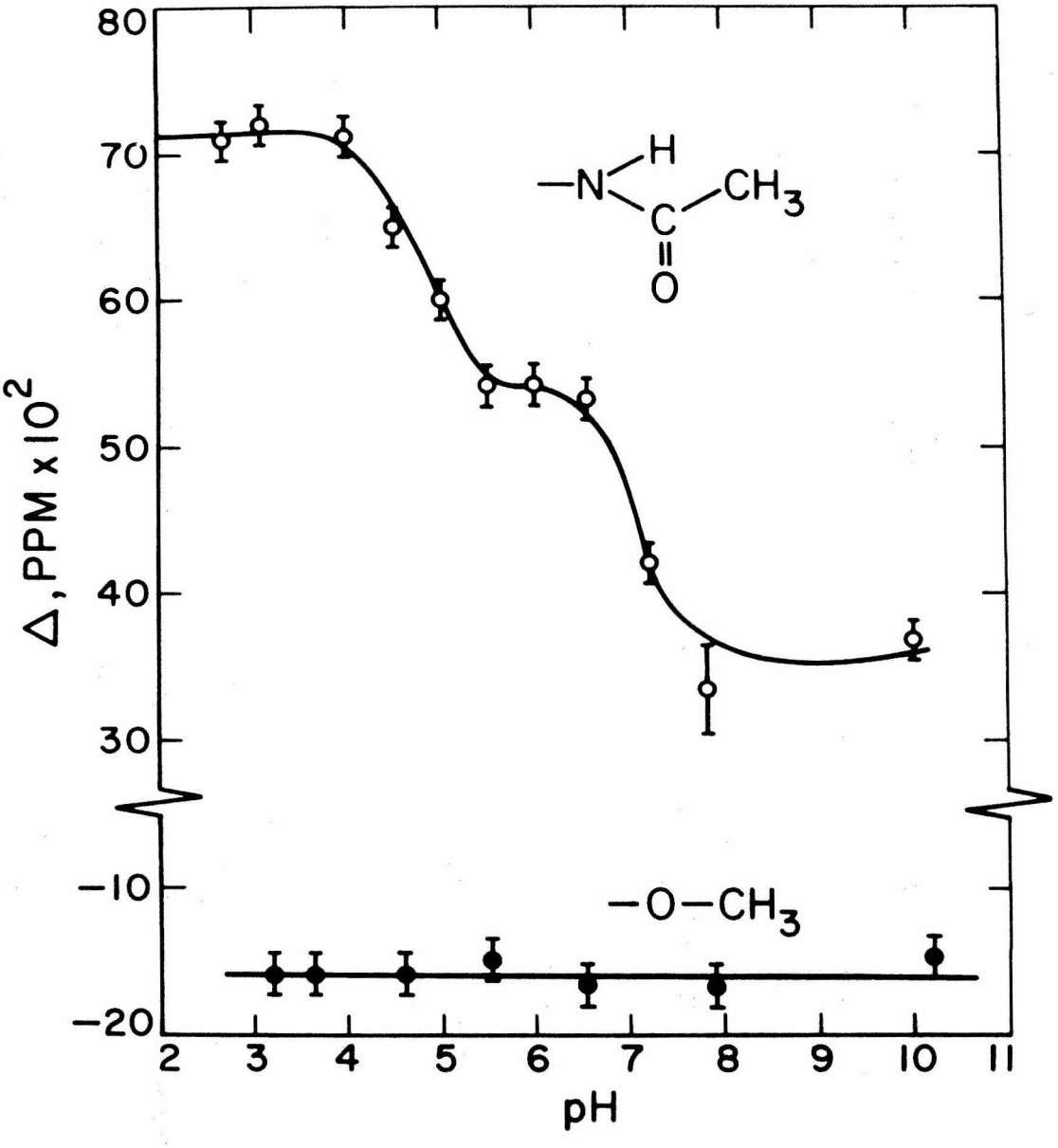

2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
pK$_s$
E
ES
2
3
4
5
6
7
8
9
10
pH

Figure 3. The pH dependency of the chemical shifts (Δ) of the glycosidic methyl proton — ● — and the acetamido methyl protons — O — of β-methyl NAG when bound to lysozyme.


80
70
60
50
40
30
−10
−20
Δ, PPM x 10²
2
3
4
5
6
7
8
9
10
11
pH
H
N
CH₃
C
O
−O−CH₃

CHAPTER V

SOME PROPERTIES OF CONTIGUOUS BINDING SUB-SITES ON LYSOZYME AS DETERMINED BY PROTON MAGNETIC RESONANCE SPECTROSCOPY

INTRODUCTION

Recent use of proton magnetic resonance techniques as a probe of the association between lysozyme and some of its inhibitors has demonstrated the potential of the method for studies of enzyme-inhibitor or enzyme-substrate interactions. Based on quantitation of chemical shifts induced in selected nuclei of the inhibitors, as a result of macromolecular association, it has been possible to gain information regarding the environment experienced by these nuclei in the complexed state. One stringent requirement is that the exchange rate between free and bound species of the inhibitor be rapid. It has been shown in Chapter III that the p. m. r. method can be used to determine whether the enzyme-bound orientations of competitive inhibitors are identical or not through a comparison of the magnetic environment(s) experienced by certain nuclei in the inhibitors when bound to the enzyme. In this manner it was shown that although α-NAG[1] and β-NAG bind

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[1] Abbreviations used are: NAG, 2-acetamido-2-deoxy-D-glucopyranose; methyl-β-NAG, methyl-2-acetamido-2-deoxy-β-D-glucopyranoside; methyl-α-NAG, methyl-2-acetamido-2-deoxy-α-D-glucopyranoside; p. m. r., proton magnetic resonance; n. m. r., nuclear magnetic resonance; p. p. m., parts per million; c. p. s., cycles per second; IR, infrared spectroscopy.

competitively to lysozyme with almost the same binding constant (K_s α-NAG = 1.6×10^{-2} M; K_s β-NAG = 3.3×10^{-2} M), their average orientations in the complexed state are different. This conclusion was arrived at through determination of the chemical shift induced in the acetamido methyl protons of the two anomeric forms upon association with tne enzyme. Thus the availability of a second parameter, the chemical shift, as an index of association allows additional information to be gleaned regarding the nature of a macromolecular binding site.

Previous studies, described in Chapter I, employing ultraviolet spectroscopic methods, on the association of β-(1-4) linked oligosaccharides of NAG have allowed calculation of the dissociation constants for the monosaccharide through the hexasaccharide in association with the enzyme. In our studies, as well as those of Rupley et al., (1967), it was shown that binding strength increased, with increasing chain length, up to the trisaccharide but that the tetra-, penta-, and hexasaccharides did not appear to bind any more strongly than did the trisaccharide. These results indicated that lysozyme contains three contiguous subsites to which acetamido-pyranose rings bind strongly. This interpretation is in agreement with findings (Johnson and Phillips, 1965; Blake et al., 1967) employing X-ray analysis techniques to study the association of crystalline lysozyme with NAG, chitobiose, and chitotriose. It should be noted that dissociation constants obtained by the ultraviolet spectroscopic method which we employed and by others (Rupley et al., 1967), as well as fluorescent spectroscopic techniques further used (Lehrer and Fasman, 1967 ; Chipman et al., 1967) to study association of various inhibitors with lysozyme, are composed

of mean values for both anomeric forms of the saccharides under investigation. It is not possible by these techniques to determine whether both anomeric forms bind or to estimate individual binding constants for the α- and β-forms.

The use of p.m.r. techniques can, however, allow distinction between the different anomeric forms in association with the enzyme. Although it could be shown by such an approach in Chapter III that one-one complexes were formed between the enzyme and the various monosaccharide inhibitors, it was not possible to relate this to association with only one of the three contiguous subsites rather than to multiple equilibria with all three sites. If this latter possibility were the case the dissociation constants and chemical shifts obtained would represent complex entities. The present study was undertaken in an attempt to resolve this question as well as that of the relative ways in which the mono-, di-, and trisaccharides of NAG and their methyl glycosides bind to the enzyme.

EXPERIMENTAL

Materials

NAG was obtained from the California Corporation for Biochemical Research. The oligosaccharides chitobiose, chitotriose, and chitotetraose were prepared from partial acid hydrolysates of chitin by a gel-filtration method on Bio Gel P-2 (200-400 mesh) and were shown to be homogeneous by chromatography on paper. The staining procedure used has been described by Powning and Irzykiewicz (1965). Crystallization of chitobiose prepared in this manner was effected from methanol-water and yielded α-chitobiose (m. p. 262-264°C, d.)

Synthesis of Chitobiose-N_1-d_3

This material was prepared from chitobiose by the following procedure. Treatment of chitobiose (1. 9 gr) with 80 ml pyridine-acetic anhydride (1 : 2 v/v) for 16 hours at room temperature followed by dilution with chloroform (50 ml), extraction with cold water (3 x 100 ml), cold dilute sodium bicarbonate (3 x 100 ml), and cold water (2 x 50 ml) gave a solution which on drying over sodium sulfate, evaporation to a syrup, and trituration with diethyl ether yielded a crystalline product. This was characterized as octaacetylchitobiose (m. p. 300-305°C).

Treatment of octaacetylchitobiose (5. 4 gr) with acetic anhydride (100 ml), which had been saturated with dry HCl at 0°C, for two days at room temperature followed by dilution with chloroform (100 ml), neutralisation by extraction with saturated sodium bicarbonate solution at 0-5°C, washing the chloroform extract with cold water, drying over

anhydrous sodium sulfate, evaporation to a syrup at 30°C, and trituration with diethyl ether yielded a semicrystalline product. Following recrystallization from acetonepetroleum ether (30-60°) this material characterized by n. m. r. and IR as acetochlorochitobiose, had a melting point of 185-187°C. Yield 2.4 gr.

To obtain heptaacetylchitobiose hydrochloride a solution of acetochlorochitobiose (1.0 gr) in dry nitromethane (30 ml) was left at room temperature for 1 day after addition of 50 λ 0.1N HCl and 50 λ water. The crystallized hydrochloride was collected, washed with ether, and air dried. The supernatant was again treated with 50 λ 0.1N HCl and 50 λ water and left overnight. Four such treatments gave 0.7 gr of the hydrochloride, m.p. 218°C, d.

Octaacetylchitobiose-N_1-d_3 was obtained by acetylation of 0.7 gr heptaacetylchitobiose hydrochloride with a mixture of pyridine (5 gr) and acetic anhydride-d_6 (1.0 gr) for 4 hours at room temperature. The product was isolated by the procedures already outlined. Yield 0.5 gr, m.p. 300-305°C. Deacetylation of this material (0.4 gr) in methanol containing sodium methoxide (0.1 M) at 40°C for 3 hours followed by evaporation of the solvent, dissolution in water, neutralization with dilute sulfuric acid, and gel filtration on a column (2.5 x 90 cm) of Bio-Gel P-2 gave chitobiose-N_1-d_3. Yield 0.15 gr, m.p. 260-.262°C,d.

Synthesis of Methyl-β-chitobioside

Acetochlorochitobiose was prepared as already described. This material (0.4 gr) was reacted in anhydrous methanol (25 ml) containing 0.1 gr silver carbonate and 1.0 gr anhydrous calcium sulfate for 16

hours at room temperature. It was then filtered, evaporated to a syrup, dissolved in chloroform (50 ml), extracted twice with 50 ml dilute aqueous ammonia and with dilute bicarbonate, dried over anhydrous sodium sulfate, and evaporated to dryness. Deacetylation was effected in 0.1 M sodium methoxide in methanol (30 ml) at 40°C for 3 hours. After evaporation of the solvent the residue was dissolved in water (50 ml) and quickly neutralized. The inorganic salts and sugars which contained free reducing termini were quantitatively removed by stirring the aqueous solution with 5 gr Amberlite MB-1 (mixed-bed ion exchanger) for 1 hour. The supernatant was lyophilised. Yield 0.120 gr. Final purification was effected by dissolving this material in water (2 ml) and subjecting it to gel filtration on a column (1.5 x 90 cm) of Bio-Gel P-2 using water as the eluting solvent. The glycoside was detected by reading the absorbance of the eluted fractions at 225 mμ. The requisite tubes were pooled and lyophilised to give 100 mg of methyl-β-chitobioside, m.p. 287-288°C.

Synthesis of Methyl-β-Chitotrioside

Chitotriose (4.5 gr) was converted to its peracetyl derivative by heating under reflux in acetic anhydride (180 ml) containing 3.1 gr anhydrous sodium acetate for 1 hour with vigorous stirring. 1967). The product was isolated by methods described for the isolation of octaacetylchitobiose. Yield 5.0 gr, m.p. 315°C. Reaction in acetic anhydride-dry HCl for 48 hours as described for the preparation of acetochlorochitobiose yielded, after working up, .7 gr of acetochlorochitotriose. This material (0.5 gr) was reacted with dry

methanol (25 ml) in the presence of silver carbonate (1 gr) and anhydrous calcium sulfate (1.0 gr) for 24 hours with stirring in the dark. The reaction mixture was filtered, evaporated to a syrup, and dissolved in chloroform (100 ml). The chloroform solution was extracted twice with an equal volume of 5% ammonia solution, once with saturated NaCl solution and dried over anhydrous sodium sulfate. The chloroform was evaporated and the resulting amorphous solid dried in vacuo. This material was deacylated in 0.1 N sodium methoxide at 40°C for 3 hours and left overnight at room temperature. The sodium methoxide was neutralized with acetic acid and the solution was then evaporated to dryness. The residue was dissolved in water (100 ml) and deionized by treatment with the mixed-bed ion exchanger Amberlite MB-1. This treatment also removed any reducing sugars present. The residual solution was lyophilised. Fractionation on a column (1.5 x 70 cm) of Bio-Gel P-2 using water as eluting solvent gave two compounds, which were characterized as methyl-β-chitobioside (50 mg, m.p. 287-8°C) and the methyl-β-chitotrioside (20 mg, m.p. 306-310°C, d.) by virtue of their n.m.r. spectra. They were found to be homogeneous compounds on paper chromatography in pyridine-water-2 pentanol (1 : 1 : 1).

Lysozyme (Lot #96B-8572) was purchased from Sigma Chemical Company, Amberlite MB-1 mixed-bed ion exchanger was obtained from Mallinkrodt.

Methods

Enzyme solutions contained approximately 3×10^{-3} M lysozyme (Sigma Chemical Company, Lot #96B-8572) and 0.5% each of methanol

and acetone as internal p.m.r. standards. The buffers were made by mixing 0.1 M citric acid with either 0.1 M sodium citrate, 0.1 M disodiumphosphate, or 0.1 M trisodiumphosphate. The exact concentration of the enzyme was determined by removing 25 μℓ, diluting to 5.00 ml with 0.1 M citrate buffer pH 5.5 and measuring the optical density of the solution at 280 mμ with a Cary Model 14 spectrophotometer. The known extinction coefficient was used to estimate lysozyme concentrations (Sophianpoulos et al., 1962).

All spectra were obtained with a Varian HA-100 nuclear magnetic resonance spectrometer, operating in frequency sweep mode. The water resonance was used as a lock signal, and each sample was allowed to come to thermal equilibrium before measurements were taken. For measurements at temperatures other than 31°, the operating temperature of the probe, a Varian V-4341 Variable Temperature accessory was employed. The difference in chemical shift of methanol or ethylene glycol as a function of temperature was used to determine the probe temperature.

Chemical shifts were determined by electronic counting of the difference between the sweep frequency and manual oscillator frequency using a Hewlet-Packard counter.

The chemical shift of the glycosidic methyl group in the bound state was determined by measurements of the ratio of the blycosidic methyl group shift to the acetamido methyl group shift. This ratio was then multiplied by the value of the chemical shift (Δ) of the bound acetamido group which was determined separately from a concentration study of the observed chemical shift of the acetamido group resonance.

This method was used because the glycosidic methyl group displayed only a small observed shift, and the error in a concentration study of its resonance would have been very large.

All data were analysed by least-squares methods. The error limits quoted are standard deviations (σ) from the mean.

Mutarotation Studies

A weighted amount of crystalline α-chitobiose was thermally equilibrated in a 1.00 ml volumetric flask in a water bath at 31°. This material was dissolved in a thermally equilibrated solution which contained enzyme, buffer, and acetone. After the sample dissolved, it was transferred to a n.m.r. tube, equilibrated in the water bath for 1 minute, and placed in the probe. The spectrometer was locked on the water resonance, and the spectrum recorded. A spectrum could be produced in this manner in 3-4 minutes from the time the crystalline inhibitor was dissolved.

RESULTS

Assignment of Methyl Group Proton Resonances in Chitin Oligosaccharides

The various chitin oligosaccharides have magnetically non-equivalent acetamido methyl group resonances. The p.m.r. spectra, due to these methyl groups, of the mono-, di-, tri-, and tetrasaccharides are shown in Fig. 1. The resonances for chitobiose cannot be unambiguously assigned to its two acetamido methyl groups without specific deuteration of one of them. This was accomplished by use of an N → O acyl shift previously employed in this Laboratory for preparation of specifically labelled chitotriose (Dahlquist and Raftery, 1967). The p.m.r. spectra of chitobiose and chitobiose-N_1-acetyl-d_3 are compared in Fig. 2. The deuterated compound lacks the acetamido resonance to higher field and therefore this resonance in chitobiose can be attributed to the acetamido group on the pyranose ring at the reducing end of the disaccharide.

The p.m.r. spectrum for the acetamido methyl resonances of chitotriose is shown in Fig. 1. The chemical shift of the resonance to higher field corresponds closely to that due to the acetamido methyl group at the reducing end of chitobiose (see Table 1) and has therefore been assigned to the methyl group at the reducing end of chitotriose. The acetamido methyl resonances to lower field consist of a poorly resolved doublet of unequal line width with the broader resonance occurring about 0.45 Hz upfield from the sharper one. It was not possible to directly assign these two resonances specifically to each of the acetamido methyl groups on the non-reducing pyranose rings of the

trisaccharide. Such an assignment was possible, however, based on a comparison with the p. m. r. spectrum of the tetrasaccharide, chitotetraose. Examination of the acetamido methyl group p. m. r. spectrum of chitotetraose revealed a resonance to higher field which could again be assigned to the methyl group on the pyranose ring at the reducing end of the molecule. In addition a poorly resolved doublet was observed as in the case of the trisaccharide. However, the peak heights of the components of the doublet were nearly equal indicating that the broad resonance was due to two methyl groups and the sharp resonance due to a single methyl group. On this basis the broad component of the doublet (occurring to higher field) was assigned in one case to the middle acetamido methyl group of chitotriose and in the other case to the two interior acetamido methyl groups of chitotetraose. The sharp resonance in the doublet, occurring to lower field, was in each case assigned to the acetamido methyl group on the nonreducing end of chitotriose and chitotetraose. The chemical shift of this resonance also corresponds closely to that for the acetamido methyl group on the nonreducing end of chitobiose (see Table 1). It would appear from the present results that the interior acetamido methyl groups of chitotriose and chitotetraose are somewhat restricted in their environment and that as a result their resonances have greater intrinsic line widths.

We have also studied the association of the methyl glycosides of chitobiose and chitotriose with lysozyme. Previously we have shown that methyl-β-NAG and methyl-α-NAG are well suited for such studies since their binding to the enzyme is not complicated by attendant

mutarotation as in the case of the free saccharides. The p. m. r. spectrum (at 60 M Hz) of methyl-β-chitobioside revealed three methyl groups as singlets. The one furthest downfield (τ = 6.49) was, by analogy with methyl-β-NAG, due to the glycosidic methyl group. The other two methyl resonances occurred very close to the positions occupied by the acetamido methyl resonances of NAG, chitobiose, and methyl-β-NAG and were, on this basis assigned to the two acetamido methyl groups of methyl-β-chitobioside. The p. m. r. spectrum at 100 M Hz of these two groups is shown in Fig. 3 and compared with the acetamido methyl group spectra of chitobiose, NAG, and methyl-β-NAG. The acetamido methyl resonance to lower field corresponds closely to that which has already been assigned to the nonreducing end of chitobiose and on this basis can be assigned to the sugar residue distal from the glycosidic methyl group of methyl-β-chitobioside. Table 1 shows the chemical shifts of these resonances relative to acetone. That the resonance to higher field (19.51 Hz relative to acetone) represents the acetamido methyl group at the glycosidic end of methyl-β-chitobioside may be inferred from the comparison with the acetamido methyl resonances of NAG, methyl-β-NAG, and chitobiose, as shown in Fig. 3. The upfield shift caused in the acetamido methyl resonance of NAG on glycosidation to form methyl-β-NAG (1.08 Hz) was also seen on glycosidation of chitobiose. The change in chemical shift in this case was 0.96 Hz.

The proton magnetic resonance spectra due to the acetamido methyl groups of chitotriose and methyl-β-chitotriose are shown in Figs. 3E and 3F, respectively. It is evident that the resonance to

higher field in Fig. 3F corresponds to the methyl group at the glycosidic end of the molecule. It has been shifted to higher field upon glycosidation when compared with the resonance due to the acetamido methyl group at the reducing end of chitotriose. The increase in chemical shift to higher field on glycosidation was 1.21 Hz. The acetamido methyl resonances to lower field in Fig. 3F are barely resolved. Their chemical shifts relative to acetone are given in Table 1. It can be seen that the component to higher field has the same chemical shift as that which we have previously ascribed to the internal acetamido methyl group of chitotriose. Thus we also assign this resonance to the internal acetamido group of methyl-β-chitotrioside and the resonance to the lower field to the acetamido methyl group at the non-glycosidic end of the trisaccharide glycoside.

The Association of Chitobiose with Lysozyme

The effect of binding to lysozyme on the acetamido methyl resonances of chitobiose at pH 5.5 and 31°C is shown in Fig. 4. The most pronounced effect observed was that the reducing end acetamido methyl resonance was shifted to higher field and was at the same time broadened considerably when compared to the spectrum obtained in the absence of enzyme. The nonreducing end acetamido methyl resonance, on the other hand, was not broadened nearly so much and displayed no chemical shift.

We have previously shown (see Chapters II - IV) that for a single binding site the observed shift, δ, is related to the total substrate or inhibitor concentration, S_0, by the following equation

$$S_0 = \frac{\Delta E_0}{\delta} - K_s - (E_0 - ES) \ ,$$

where Δ is the chemical shift associated with the binding site environment, the term E_0 represents the total enzyme concentration and K_s is the dissociation constant of the enzyme-substrate or enzyme-inhibitor complex. This equation can be simplified to $S_0 = \Delta E_0/\delta - K_s$. From a plot of S_0 versus $\frac{1}{\delta}$ values for K_s and Δ can be obtained. Figure 5 shows such a plot for chitobiose in association with lysozyme at pH 5.0 and 45°C. The value of K_s under these conditions was found to be equal to zero, within the error limits. This is in accord with the value of $K_s = 2.5 \times 10^{-4}$ M determined by ultraviolet spectroscopic methods since this value is essentially zero relative to the concentration range of chitobiose used ($2\text{-}10 \times 10^{-2}$ M) for the p.m.r. measurements. The value of Δ calculated from this plot was 0.57 ± 0.04 p.p.m. to higher field. Since the α- and β-anomeric forms of the disaccharide were present at mutarotation equilibrium in the sample of chitobiose used, this value of Δ is not a true measure of the binding environment but is equal to

$$\frac{\Delta_\alpha}{\frac{[\alpha]}{S_0} + \frac{K_\alpha}{K_\beta} \times \frac{[\beta]}{S_0}} \quad \text{or} \quad \frac{\Delta_\beta}{\frac{[\beta]}{S_0} + \frac{K_\beta}{K_\alpha} \times \frac{[\alpha]}{S_0}} \ ,$$

where S_0 refers to the total inhibitor or substrate concentration, $[\alpha]$ and $[\beta]$ to the concentrations of the α- and β-anomeric forms at mutarotation equilibrium, K_α and K_β to the dissociation constants of the enzyme-α and enzyme-β complexes and Δ_α and Δ_β to the chemical

shifts associated with the binding sites of the α- and β-anomeric forms.*

We have shown earlier that the value K_α/K_β can be determined when the α- and β-forms of the associating saccharide show resolved resonances in the presence of the enzyme. However, the anomeric forms of the disaccharide did not resolve over the pH range 2-10, at both 31°C and 45°C. This strongly suggests that the enzyme does not differentiate between the two anomeric forms. Furthermore, at 31° the spectrum of chitobiose in the presence of lysozyme did not appear to undergo any change in shape or chemical shift when a sample of the disaccharide which was predominantly α-chitobiose was dissolved in enzyme solution and the acetamido methyl resonances observed as a function of time. At 45°C and pH 5.5, conditions under which the reducing end acetamido methyl resonances are sharper because of increased exchange rates, a freshly dissolved sample of α-chitobiose showed the same shift as a sample which had attained mutarotation equilibrium. Unfortunately, it was not possible to perform experiments where the resonances of the freshly dissolved disaccharide were observed directly as a function of time at the higher temperature because sufficient hydrolysis and transglycosylation occurred in the 30-40 minutes required for the experiment. Thus the disaccharide concentration was changed and sufficient trisaccharide was produced to compete effectively for the disaccharide binding site. The results

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*for a complete discussion of this question relating to determination of values of K_s and Δ for such anomeric mixtures see Part I, Chapter III.

obtained clearly demonstrate that both anomeric forms of the saccharide occupy magnetically equivalent positions on the enzyme surface with the same affinity, and that these equivalent positions are competitive for both anomers. These results can be most reasonably accommodated if the equivalent positions are actually the same binding site, with both anomers having the same average orientation in that binding site.

The pH dependency of the magnetic environment of the reducing end acetamido methyl group while bound was measured over the pH range 2.5 to 10.0 at 45°C and is shown in Fig. 6. The nonreducing end showed no observable change in chemical shift upon association with the enzyme at any of the pH's studied. The results discussed in Part I, Chapter IV show that such a study can provide pK_a values for groups located near the binding site or for groups which affect the conformation of the binding site or orientation of the substrate molecule in the binding site. The data obtained for the chitobiose binding site show that there are at least three ionizable groups which effect the environment of the reducing end acetamido methyl group. These correspond to approximate pK_a values of 3.2, 4.7, and 7.5. Thus for the scheme;

$$ESH_3 \rightleftharpoons ESH_2 + H^+ \qquad K_3 = \frac{[ESH_2][H]}{[ESH_3]}$$

$$ESH_2 \rightleftharpoons ESH + H^+ \qquad K_2 = \frac{[ESH][H]}{[ESH_2]}$$

$$ESH \rightleftharpoons ES + H^+ \qquad K_1 = \frac{[ES][H]}{ESH}$$

if Δ_0, Δ_1, Δ_2, and Δ_3 correspond to the chemical shifts of the non-,

mono-, di-, and tri-protonated species of the enzyme substrate complex, the observed value of the chemical shift at a particular pH is given by

$$\Delta_{obs} = \frac{\Delta_3}{1 + \frac{K_3}{H} + \frac{K_3K_2}{H^2} + \frac{K_3K_2K_1}{H^3}} + \frac{\Delta_2}{\frac{H}{K_3} + 1 + \frac{K_2}{H} + \frac{K_2K_1}{H^2}}$$

$$+ \frac{\Delta_1}{\frac{H^2}{K_3K_2} + \frac{H}{K_2} + \frac{K_1}{H} + 1} + \frac{\Delta_0}{\frac{H_3}{K_2K_1K_3} + \frac{H_2}{K_2K_1} + \frac{H}{K_1} + 1}$$

Using values of 3.2, 4.7, and 7.2 for pK_3, pK_2, and pK_3 respectively as well as values of Δ_3, Δ_2, Δ_1, and Δ_0 of 0.52, 0.65, 0.59, and 0.74 p.p.m. upfield, respectively in eq. 1, the solid line shown in Fig. 6 was obtained. While the fit to the observed values is quite good, there is a fairly large range of values for the parameters which will fit the data about as well as the ones given above.

Binding studies were also carried out at pH 2.5 and 9.7 and 45°C by UV difference spectroscopy and in each case the value of K_s was found to decrease only about a factor of two from its value at pH 5.0· of 5.5×10^{-4} M. A more complete study of the effect of pH on K_s for chitobiose was not attempted since there was not a sufficient change in the value to obtain significant data by this method.

The Association of Methyl-β-Chitobioside with Lysozyme

Our previous results on the association with lysozyme of methyl-α-NAG, methyl-β-NAG and the α and β forms of NAG, using p. m. r. methods, have shown that all these monosaccharides bind with the same average orientation on the enzyme with the exception of α-NAG. This last saccharide binds competitively with the β-anomer but does not enter precisely the same environment as judged from the chemical shift undergone by its acetamido methyl group. In addition, it was shown that the glycosidic methyl group of methyl-β-NAG undergoes a chemical shift to lower field due to association with the enzyme whereas the glycosidic methyl group of methyl-α-NAG did not appear to undergo any change in chemical shift due to binding. In the work presented here the association with lysozyme of methyl-β-chitobioside at pH 5.5, 31°C, was studied using both the two acetamido methyl resonances and the glycosidic methyl resonance to probe the magnetic environment of the site on the enzyme to which it binds. Figure 7 shows the effects of binding to the enzyme on the acetamido methyl resonances. The most pronounced effects observed were that the acetamido methyl resonance of the methyl glycosidic end was shifted to higher field and also was broadened considerably. The resonance due to the acetamido methyl group distal from the glycosidic methyl group did not appear to undergo any change in chemical shift. This resonance was broadened but by an amount less than that observed for the acetamido methyl resonance at the glycosidic end of the molecule. The glycosidic methyl group was also found to undergo a chemical shift due to association with the enzyme as shown in Fig. 8. As in the

case of methyl-β-NAG this shift was to lower field. Qualitatively, these observed shifts are in agreement with the behaviour of the monosaccharide, the monosaccharide β-methyl-glycoside and the disaccharide chitobiose. Calculation of the chemical shift, Δ, for the acetamido methyl protons proximal to the glycosidic end of the disaccharide glycoside yielded a value of 0.60 ± 0.05 p.p.m. to higher field. Similarly a value of 0.20 ± 0.03 p.p.m. to lower field was obtained for the glycoside methyl group. Table 2 lists these values and compares them to the chemical shifts observed for the various monosaccharides and monosaccharide glycosides previously studied.

A study of the association of methyl-β-chitobioside with lysozyme at pH 9.7 and 55°C was also conducted. The effects of adding varying amounts of the glycoside to a constant amount of enzyme on the acetamido methyl group resonances of the small molecule are shown in Fig. 9. It was seen that the resonance due to the acetamido methyl group proximal to the glycosidic group was shifted to higher field and that this shift increased as the concentration of the glycoside was decreased. The calculated chemical shift of this resonance for the bound form of the associating small molecule was 0.80 ± 0.04 p.p.m. to higher field. The acetamido methyl group distal from the glycosidic end did not undergo any change in chemical shift as a result of association with lysozyme. As expected, a slight broadening of the resonance was observed as a result of macromolecular association. The glycosidic methyl group was found to undergo a chemical shift to lower field due to binding to the enzyme. The value obtained for Δ was 0.16 ± 0.02 p.p.m. to lower field.

The Association of Chitotriose with Lysozyme

At pH 5.5 and 31°C the acetamido methyl group p.m.r. spectrum of chitotriose was not affected very much by the presence of lysozyme. A slight broadening of the resonances was observed but no detectable changes in chemical shift were seen. Under the conditions used it was known that the enzyme (3×10^{-3} M) was saturated with the trisaccharide ($2\text{-}8 \times 10^{-2}$ M) since the dissociation constant had previously been shown to be 8×10^{-6} M. Due to this strong binding it was possible that the dissociation rate of the lysozyme-chitotriose complex was too slow to satisfy the condition necessary to obtain a spectrum representative of the average of free and enzyme-bound chitotriose species. In an attempt to approach this condition the experiment was repeated but an elevated temperature was employed. At 55°C, twenty degrees below the thermal denaturation transition temperature for the enzyme, a more profound effect on the p.m.r. spectrum of the acetamido methyl groups was observed. Unfortunately chitotriose was found to be fairly rapidly hydrolysed by lysozyme under these conditions. To circumvent this complication the high temperature binding studies were carried out at pH 9.7. A separate study was conducted to show that hydrolysis of the trisaccharide by the enzyme did not occur at any detectable rate at pH 9.7 and 65°C. The effect of binding to lysozyme at this pH and 55°C on the acetamido methyl resonances of chitotriose is shown in Fig. 10B. Under these conditions broadening of the resonances was observed, and it was also evident that a chemical shift to higher field was experienced by the resonance corresponding to the acetamido methyl group at the reducing end of the trisaccharide.

However, this resonance appeared to be very broad and it seemed as though the enzyme-substrate complex was still too long-lived to satisfy the exchange rate necessary to obtain a spectrum representative of the weighted average of bound and unbound species. Upon raising the temperature to 65°C, a profound effect was seen as shown in Fig. 10C. The resonance corresponding to the acetamido methyl group at the reducing end of the trisaccharide was clearly resolved and was further shifted to higher field. Thus the increase in temperature to 65° caused an increase in the exchange rate sufficient to cause a sharper resonance for the reducing end acetamido methyl group. It is possible, however, that the observed spectrum still was not the weighted average of the free and bound species. Figure 11 shows the effects of adding varying concentrations of the trisaccharide to a constant amount of enzyme at pH 9.7 and 65°C. No separate effects attributable to the α- and β- anomeric forms of the trisaccharide were observed in contrast to those seen for α- and β-NAG. This is in agreement with the results obtained for α- and β-chitobiose during the present study. Therefore it is evident that the α- and β-anomeric forms of the trisaccharide both bind to the enzyme and the acetamido methyl protons at the reducing ends of both anomeric forms undergo a chemical shift to higher field due to the magnetic environment experienced in the complexed state. The value of Δ calculated for the shift of these protons was 0.61 ± 0.12 p.p.m. to higher field. However, there is reason to doubt that this determination was made under conditions which allow "averaging" of the observed chemical shift between the free and bound forms. Since the dissociation constant of the trisaccharide-enzyme complex is small

under the conditions used for the chemical shift measurements, the observed chemical shift, at constant enzyme concentration, should be linear with respect to substrate concentration. However, the observed shift increased proportionately more than the substrate concentration was decreased. This suggests that at low S_0/E_0 ratios, the exchange rate was more rapid and a more nearly "averaged" spectrum was obtained. Therefore, the measurements taken at the lowest S_0/E_0 ratio should be the most correct. The value of Δ for the acetamido methyl resonance of the reducing-end acetamido methyl resonance at the lowest S_0/E_0 ratio was 0.70 p.p.m. upfield. Further, in those instances where incomplete exchange was observed, the best theoretical fit to the observed spectra was obtained with a value of 0.77 p.p.m. upfield (see Part I, Chapter VI).

The resonance corresponding to the central acetamido group of the trisaccharide did not appear to undergo any chemical shift due to association with lysozyme. A small broadening effect, as expected, was observed.

The resonance corresponding to the acetamido methyl group on the nonreducing end of chitotriose displayed a downfield shift in the presence of lysozyme. This is in contrast to the upfield shifts previously observed for the other acetamido methyl resonances of chitotriose as well as for chitobiose, methyl-β-chitobioside and various derivatives of NAG. It allows distinction of the magnetic environment associated with the site on the enzyme occupied by the nonreducing end acetamido methyl group of chitotriose. The magnitude of the downfield shift observed for this resonance was $\Delta = 0.08 \pm 0.01$ p.p.m.

The Association of Methyl-β-Chitotrioside with Lysozyme

The association of the trisaccharide glycoside with the enzyme was studied only at pH 9.7 and at elevated temperatures. Results similar to those obtained for chitotriose binding to lysozyme as a function of temperature were observed. In Fig. 12B the result of adding lysozyme to a solution of the glycoside at 55°C is shown clearly to be that all methyl group resonances were broadened and that the resonance corresponding to the acetamido methyl group proximal to the glycosidic group appeared also to be shifted to higher field. However, the spectrum obtained was reminiscent of those previously observed for chitobiose, methyl-β-chitobioside and chitotriose when the rate of the dissociation of the enzyme-substrate or enzyme-inhibitor complex was too slow to obtain a spectrum representing a weighted average of enzyme-bound and free forms of the small molecule. Raising the temperature to 65° decreased the line width of the reducing end acetamido methyl resonance. Again, it is probable that the observed spectrum is still not representative of the fast exchange limit. The type of spectrum seen is shown in Fig. 11C. Clearly the resonance corresponding to the acetamido methyl group proximal to the glycosidic methyl group was shifted to higher field due to association with the enzyme. The calculated chemical shift for this group was $\Delta = 0.63$ p.p.m. to higher field. This value is probably somewhat low because of incomplete exchange.

The resonance due to the middle acetamido methyl group of the glycoside did not appear to undergo any change in chemical shift due to macromolecular association whereas the acetamido methyl group

distal from the glycosidic end of the molecule displayed a small downfield shift of its p. m. r. resonance upon association with the enzyme. The calculated value of Δ was 0.08 $\pm$ 0.02 p. p. m. to lower field. This result agrees well with the downfield shift obtained for the acetamido methyl group at the nonreducing end of chitotriose in association with lysozyme.

The glycosidic methyl group of methyl-β-chitotrioside was also found to undergo a downfield shift in its p. m. r. spectrum in the presence of lysozyme. The value of Δ obtained was 0.19 p. p. m. to lower field. This value agrees quite well with those obtained for the glycosidic methyl groups of methyl-β-NAG and methyl-β-chitobioside. The chemical shift results for all of the mono-, di and trisaccharides and their glycosides are summarized in Tables 2 and 3.

The Association of Chitotetraose with Lysozyme

A study of the effects of added lysozyme on the p. m. r. spectrum of the acetamido methyl groups of chitotetraose was carried out at pH 9.7 and 65°C. It was shown that using these conditions no detectable hydrolysis of the tetrasaccharide occurred over a period of an hour. The results obtained are shown in Fig. 13. It was observed that in comparison with the spectrum of the free tetrasaccharide the presence of enzyme caused broadening of all the methyl resonances and appeared to shift that resonance corresponding to the acetamido methyl group at the reducing end of the tetrasaccharide to higher field. The spectrum was reminiscent of those obtained for chitotriose and methyl-β-chitotrioside in the presence of lysozyme when the rate of

dissociation of the enzyme inhibitor complex was not sufficiently fast to result in sharpening of the observed resonances. Upon raising the temperature at 70°C, irreversible denaturation of the protein was observed. Therefore it was not possible to obtain more clear-cut results for the tetrasaccharide in association with the enzyme. It was possible, however, from the results seen at 65°C to say qualitatively that the acetamido methyl group at the reducing end of chitotetraose can occupy the environment experienced by the acetamido methyl groups at the reducing ends of NAG, chitobiose, and chitotriose and proximal to the glycosidic methyl group of methyl-β-NAG, methyl-α-NAG, methyl-β-chitobioside and methyl-β-chitotrioside.

DISCUSSION

In general two types of information may be obtained from an n. m. r. experiment of the type described in this investigation: (a) the position or chemical shift of the resonance which is controlled by the magnetic environment and (b) the shape or width of the observed resonance, which is controlled by the relaxation times. Earlier work described in this thesis on inhibitor binding to lysozyme has shown that the chemical shifts of selected nuclei in the inhibitors are very sensitive to small changes in their enzyme-bound environments. The information obtainable from such changes in chemical shifts of selected nuclei in various inhibitor molecules is more valuable, for this reason, than information gleaned from line width changes due to macromolecular association.

While the work reported here was in progress a brief account of the effect of lysozyme on the n. m. r. spectra of chitobiose and methyl-β-chitobioside (Thomas, 1968) has appeared. The only effect which was observed was an enzyme induced broadening of the reducing-end acetamido methyl proton resonance in chitobiose and a similar broadening of the resonance due to the acetamido methyl group oroximal to the glycosidic end of methyl-β-chitobioside. The results reported here on the association of these inhibitors with lysozyme as well as other molecules such as chitotriose, methyl-β-chitotrioside and chitotetraose show this type of "selective" line-broadening at low temperatures. Extrapolated line widths for enzyme-bound forms of the inhibitors from such observed "selective" broadening are of the order of 150-250 c. p. s. In view of the line widths normally observed for methyl groups on proteins

(McDonald and Phillips, 1967) such values are clearly unrealistic. There are several possible explanations for the broadening effects published by Thomas (1968) and observed in the initial stages of our investigations. One such explanation is the phenomenon of exchange broadening, which occurs when the rate of exchange of a nucleus between two (or more) environments is of the order of $\frac{1}{2\pi(\Delta\nu)}$, where $\Delta\nu$ is the chemical shift difference for the nucleus in one site relative to the other site(s). Another explanation is the presence of paramagnetic material at or near one of the environments.

Jardetsky (1964) has suggested that the cause of differential broadening in the nuclear magnetic resonance spectrum of small molecules in the presence of macromolecules to which they bind is the restricted movement of the small molecule in the binding site. This restricted movement gives rise to large dipolar fields which are efficient in causing relaxation and therefore results in broadened lines. The more strongly a particular portion of the molecule is held in place on the macromolecule, the more broadened its resonance will appear. This explanation was suggested by Thomas (1968) to account for the large increase in the line width of the reducing-end acetamido methyl group resonance of chitobiose and the resonance due to the acetamido methyl group proximal to the glycosidic end of methyl-β-chitobioside in the presence of lysozyme.

It is interesting to note that the exceptionally broadened resonances are also those which, in the present investigation, show measurable chemical shifts upon association with lysozyme. The line widths are dramatically affected by temperature as are the chemical

shifts of the broadened resonances. This is shown in Figs. 4, 9, 10, and 11. Separate measurements of the binding constants for the various chitin oligomers used showed that there was no significant change in the fraction of the oligomer which was bound to the enzyme as the temperature was varied. These facts strongly suggest that the broadening is caused by slow exchange of the saccharide between the free and bound state. In Chapter VI we describe the use of such line-width measurements on the acetamido methyl resonances of chitobiose and chitotriose to measure the exchange rates of the free and bound species. The observed spectra are completely explainable using line widths for the bound state of 8-15 Hz and formation rate constants for the enzyme substrate complex of 10^6-10^7 l mole^{-1} sec^{-1}. While there appears to be slightly different line widths associated with the various subsites in the binding region of lysozyme, these are within the range of 8-15 Hz and do not account for the large differential broadening effects mentioned earlier. The results of curve fitting to match the observed spectra for the exchange of chitobiose and chitotriose are summarized in Table 4.

The data presented in Tables 2, 3, and 4 suggest that the various subsites associated with the binding of chitobiose and chitotriose may be assigned specific magnetic parameters. At pH 9.7, the reducing-end acetamido methyl group resonances of chitobiose and chitotriose both displayed chemical shifts of 0.77 ± 0.04 p.p.m. upfield with a half-width of 15 Hz while bound. Thus, subsite C may be assigned these values for the acetamido methyl group resonances of sugar units bound in the subsite at pH 9.7.

The acetamido methyl resonances corresponding to the nonreducing sugar ring of chitobiose and the central sugar residue of chitotriose both displayed no measurable chemical shift and a half-width of 10 Hz while associated with subsite B. The nonreducing-end acetamido methyl resonance of chitotriose showed a chemical shift of 0.08 p.p.m. to lower field and a half-width of 8 Hz while bound to the enzyme at subsite A.

The methyl-β-glycosides of chitobiose and chitotriose also fit nicely into the general pattern. At pH 9.7 the glycosidic methyl group resonance of each had a chemical shift of 0.18 ± 0.02 p.p.m. to lower field. The acetamido methyl resonances proximal to the glycosidic methyl groups of each glycoside also showed values of Δ which would place that sugar residue in each case in subsite C. Again the central sugar residue of the trisaccharide and the sugar residue distal from the glycosidic end of the disaccharide showed no measureable chemical shifts in the appropriate acetamido methyl resonances, thereby placing them in subsite B. The acetamido methyl resonance distal from the glycosidic methyl group of methyl-β-chitotrioside showed the small downfield shift (0.08 p.p.m.) characteristic of subsite A.

The binding orientation of chitotetraose is more difficult to define quantitatively because the exchange rate of the saccharide-enzyme complex was too slow to allow more than a qualitative measure of chemical shifts. The spectra obtained for chitotetraose were reminiscent of the chitotriose spectra obtained at temperatures about 10^{o} lower. This corresponds to about a factor of two in exchange rate. The reducing-end acetamido methyl resonance underwent changes

similar to the reducing-end resonance of chitotriose. Thus, the reducing end of the tetrasaccharide most probably occupies subsite C, with the other sugar residues being in subsited B and A, and either in solution or in a weak subsite beyond subsite A.

Our earlier measurements on the binding to lysozyme of methyl-β-NAG at pH 5 gave a chemical shift of 0.54 ± 0.04 p.p.m. upfield for the acetamido methyl resonance and 0.18 ± 0.02 p.p.m. downfield for the glycosidic methyl resonance. These values are within experimental error of those obtained for the acetamido methyl group resonance proximal to the glycosidic methyl group and the glycoside methyl group resonance respectively of methyl-β-chitobioside, at the same pH. This places methyl-β-NAG in subsite C at pH 5. However at pH 9.7, the acetamido methyl chemical shift of methyl-β-NAG was 0.36 ± 0.04 p.p.m. upfield while the glycosidic methyl group did not change from its value of 0.18 p.p.m. downfield. The glycosidic methyl shift still agreed with the value postulated for subsite C, but the acetamido methyl resonance shift was a factor of two different from its postulated value using methyl-β-chitobiose, methyl-β-chitotriose, chitobiose and chitotriose as references under these conditions. The most reasonable explanation of this apparent anomoly is that binding in subsite B has some effect on the chemical shift for the acetamido methyl resonance in subsite C. The earlier work on methyl-β-NAG binding showed that the glycosidic methyl group resonance was somehow isolated from the effects of ionizable groups at the binding site while the acetamido methyl resonance was strongly affected. This result suggests that binding in subsite B may affect the orientation of a particular ionizable

group with respect to the acetamido methyl group bound in subsite C.

All these results are summarized in Fig. 14 which depicts the lysozyme molecule to have three contiguous binding subsites to which acetamido pyranosides bind strongly. These subsites are labelled A, B, and C and have been assigned particular magnetic properties on the basis of the effects observed. The various oligomers of NAG bind with their reducing ends occupying subsite C. The α- and β-anomeric forms of the di- and trisaccharides bind identically, while insufficient information is available to judge whether both anomeric forms of the tetrasaccharide bind identically. The anomeric forms of the monosaccharide, NAG, have been shown previously (see Chapter III) to bind with different orientations in subsite C. On the other hand, the corresponding α- and β-methyl glycosides of NAG bind identically in subsite C (Raftery et al., 1968), and it has been further shown that the orientations of both glycosides are identical with the orientation of β-NAG in the same subsite. The present work has shown that methyl-β-chitobioside and methyl-β-chitotrioside bind with their glycosidic pyranoside rings in subsite C on the basis of the chemical shifts of their glycosidic methyl groups and their acetamido methyl groups.

These observations of the relative binding orientations of the saccharides are in general agreement with X-ray analysis studies of Blake et al. (1967) on crystalline lysozyme-saccharide complexes. These crystal studies have shown an extensive binding area on the enzyme's surface containing at least three binding subsites: A, B, and C. It was found that the reducing ends of β-NAG, and chitotriose at low resolution (6 Å) bind in subsite C, while subsite B binds the

nonreducing end of chitobiose and the central sugar ring of chitotriose. Subsite A was found to be occupied only by the nonreducing end of chitotriose. The X-ray studies also demonstrated that chitobiose is bound in an anomolous way.

The authors had not, however, determined the conditions which distinguished between the two binding modes of chitobiose and lysozyme. Our results show clearly that lysozyme binds both anomeric forms of the disaccharide equally well and with the same orientation at pH 5. Furthermore, because of the equality of the acetamido methyl group shifts of methyl-β-chitobioside and chitobiose, it appears that α- and β-chitobiose and methyl-β-chitobioside bind identically to lysozyme at 9.7. The fact that there was no resolution of resonances due to the α- and β-anomeric forms also supports this hypothesis. These facts suggest that the anomolus binding of chitobiose observed in the crystalline enzyme is not due to differential binding of the anomeric forms of chitobiose, assuming identical binding properties for the enzyme in solution and in the crystalline state.

While the crystallographic studies did not allow distinction of the anomeric form(s) of chitotriose which bound to the enzyme, we can say from our studies that both anomeric forms of the trisaccharide bind equally well and in an identical manner.

The general agreement, however, on the relative modes of association of a homologous series of inhibitors with an enzyme as determined by difference Fourier analysis for crystalline preparations and by n. m. r. methods for solutions is encouraging.

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TABLE 1.--Chemical shift data (apparent maxima) for methyl groups in chitin oligosaccharides and their methyl-glycosides.

Inhibitor	Resonance[a]				
	CH_3-N_1[b]	CH_3-N_2[b]	CH_3-N_3[b]	CH_3-N_4[b]	OCH_3[c]
NAG	17.71				
Methyl-β-NAG	18.79				14.72
Chitobiose	18.35	15.63			
Methyl-β-chitobioside	19.51	15.33			13.94
Chitotriose	18.32	16.28	15.83		
Methyl-β-chitotrioside	19.53	16.28	15.90		13.33
Chitotetraose	18.35	16.54	16.54	15.98	

All chemical shifts are Hz at 100 M Hz.

[a]The acetamido methyl groups are numbered 1, 2, 3, 4 beginning at the reducing or glycosidic termini of the molecules.

[b]Values relative to acetone; all chemical shifts to higher field.

[c]Values relative to methanol; all chemical shifts to lower field.

TABLE 2.--Chemical shift data for inhibitors and substrates complexed with lysozyme at pH 4.9-5.4 and at various temperatures.

Compound	Temp. (°C)	Δ(p.p.m.)[a]		
		N_1-CH_3[b]	N_2-CH_3[b]	OCH_3[c]
α-NAG	31	0.68 ± 0.02	-	
β-NAG	31	0.51 ± 0.03	-	
Methyl-α-NAG	31	0.55 ± 0.02	-	0
Methyl-β-NAG	31	0.54 ± 0.04	-	0.17 ± 0.03
	55	0.51 ± 0.03	-	0.16 ± 0.05
Chitobiose	45	0.57 ± 0.04	0	-
Methyl-β-chitobiose	35	0.60 ± 0.05	0	0.20 ± 0.05

[a]The acetamido methyl groups are numbered 1, 2 beginning at the reducing or glycosidic termini of the inhibitor molecules.

[b]Values relative to acetone; all chemical shifts to higher field.

[c]Values relative to methanol; all chemical shifts to lower field.

TABLE 3.--Chemical shift data for inhibitors and substrates complexed with lysozyme at pH 9.7, at various temperatures.

Compound	Temp. (°C)	Δ(p.p.m.)[a]			
		CH_3-N_1[b]	CH_3-N_2[b]	CH_3 N_3[b]	-OCH_3[c]
Methyl-β-NAG	31	0.36	-		0.16 ± 0.02
Chitobiose	55	0.77 ± 0.04	0		
Methyl-β-chitobioside	55	0.80 ± 0.04	0		0.16 ± 0.02
Chitotriose*	65	0.61 ± 0.12	0	0.08	
Methyl-β-chitotrioside*	65	0.63	0	0.08	0.19

*Not as fast exchange limit.

[a]The acetamido methyl groups are numbered 1, 2, 3 beginning at the reducing or glycosidic termini of the inhibitor molecules.

[b]Values relative to acetone; all chemical shifts to higher field.

[c]Values relative to methanol; all chemical shifts to lower field.

TABLE 4.--Magnetic parameters and rate constants for formation of enzyme-substrate complex which gave the best theoretical fit to the observed spectra

Inhibitor	Temp. range (°C)	Δ(p. p. m.)[a]			Halfwidth (Hz)			k_f[c] (range) (ℓ mole^{-1} sec^{-1})
		CH_3-N_1[b]	CH_3-N_2[b]	CH_3-N_3[b]	CH_3-N_1[b]	CH_3-N_2[b]	CH_3-N_3[b]	
Chitobiose, pH 5	10 - 50	0.57	0	-	15	10	-	10^6 - 10^7
Chitobiose, pH 9.7	10 - 50	0.77	0	-	15	10	-	10^6 - 10^7
Chitotriose, pH 9.7	10 - 65	0.77	0	-0.09	15	10	8	5×10^6 - 10^7

[a]The acetamido methyl groups are numbered beginning at the reducing termini of the inhibitor molecules.

[b]Values relative to acetone; all chemical shifts are to higher field.

[c]Second order rate constant for formation of the enzyme-inhibitor complex.

Figure 1. P.m.r. spectra of the acetamido methyl groups of: A, chitotetraose; B, chitotriose; C, chitobiose; D. NAG. The sharp and intense resonance to lowest field is that for the methyl protons of an acetone internal standard.

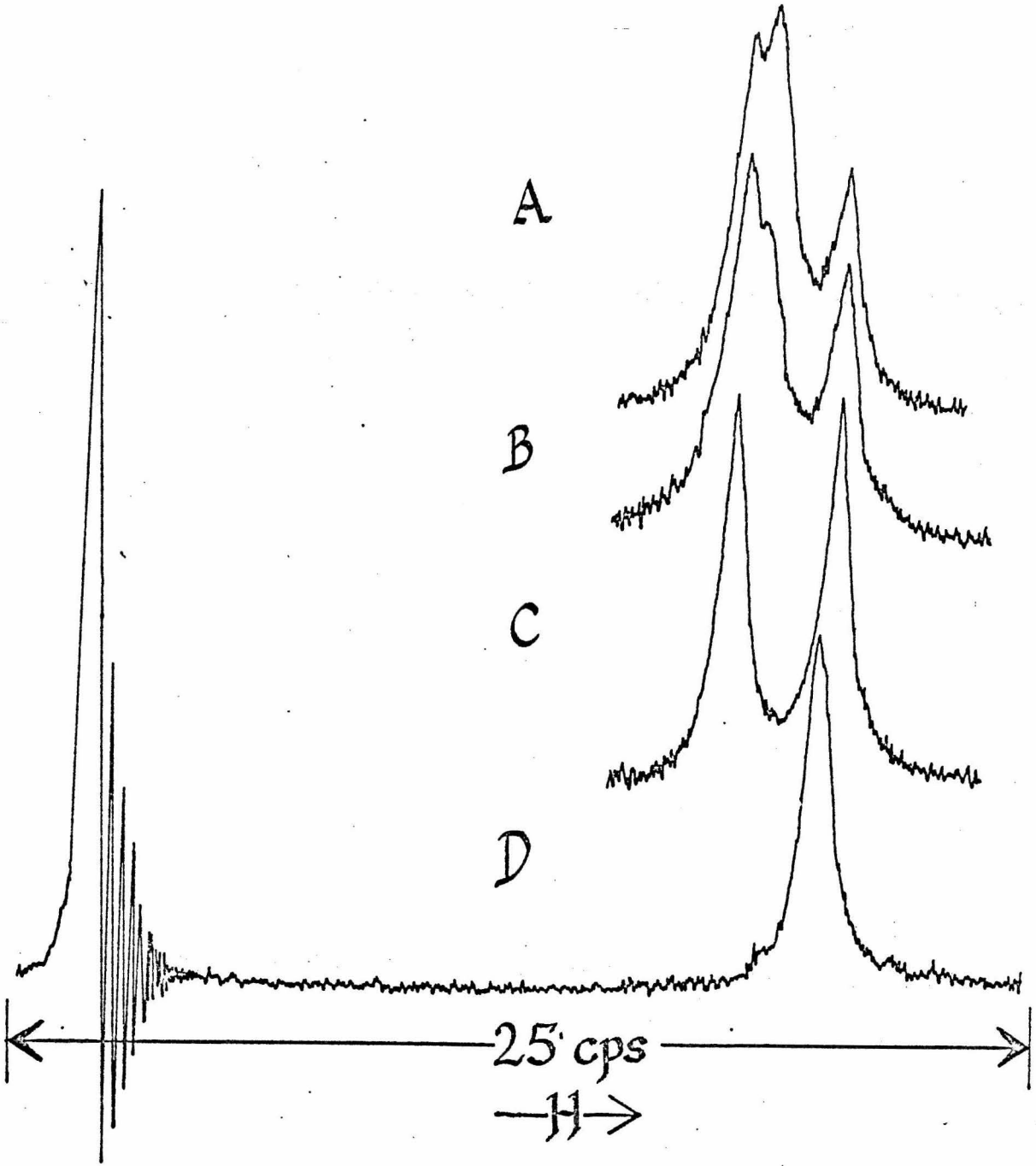

A
B
C
D
25 cps
H

Figure 2. P. m. r. spectra of the acetamido methyl protons of: A, chitobiose-N_1-d_3; B, chitobiose, relative to an acetone standard (to lowest field).

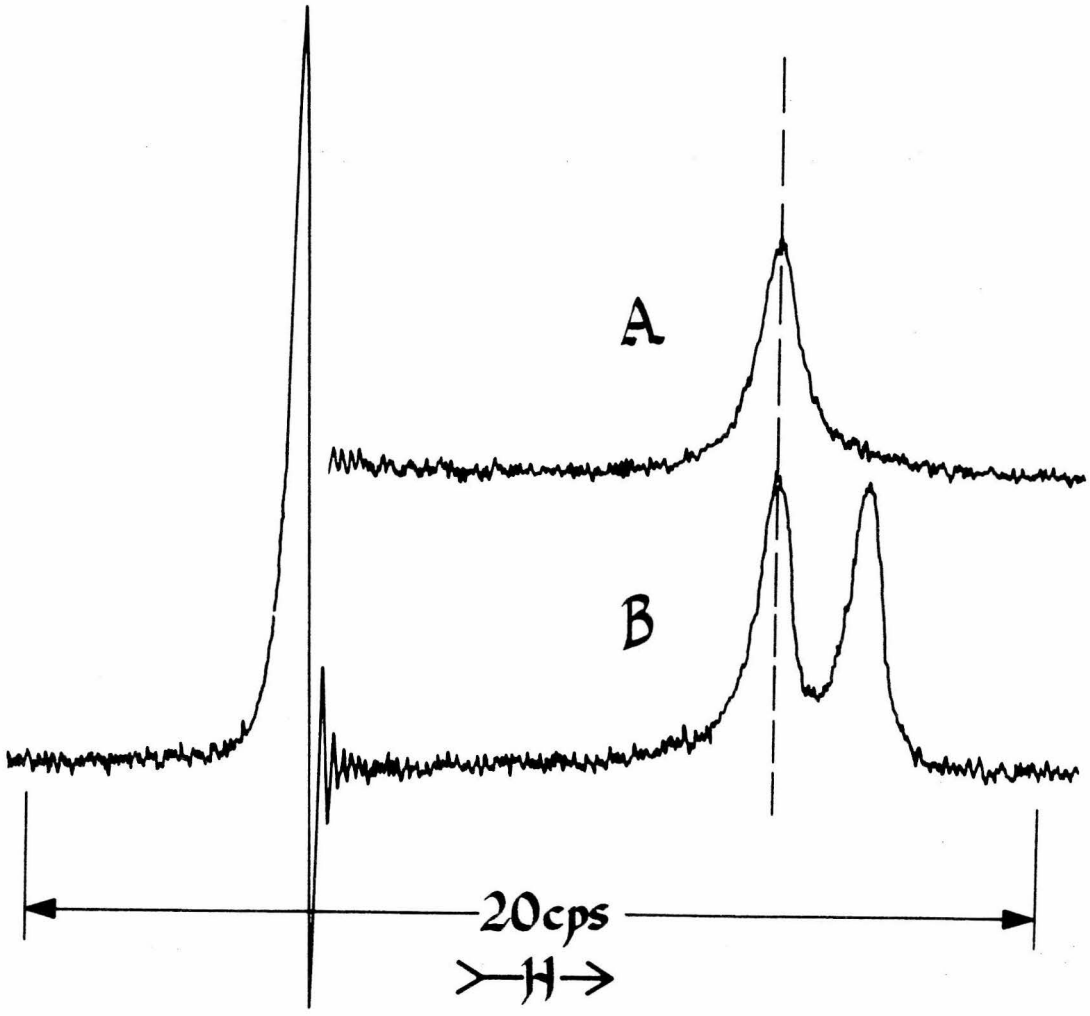

A
B
20cps
H

Figure 3. P.m.r. spectra of the acetamido methyl protons of: A, NAG; B, methyl-β-NAG; C, chitobiose; D, methyl-β-chitobioside; E, chitotriose; F, methyl-β-chitotrioside. All spectra were measured relative to an internal acetone standard, shown to lower field.

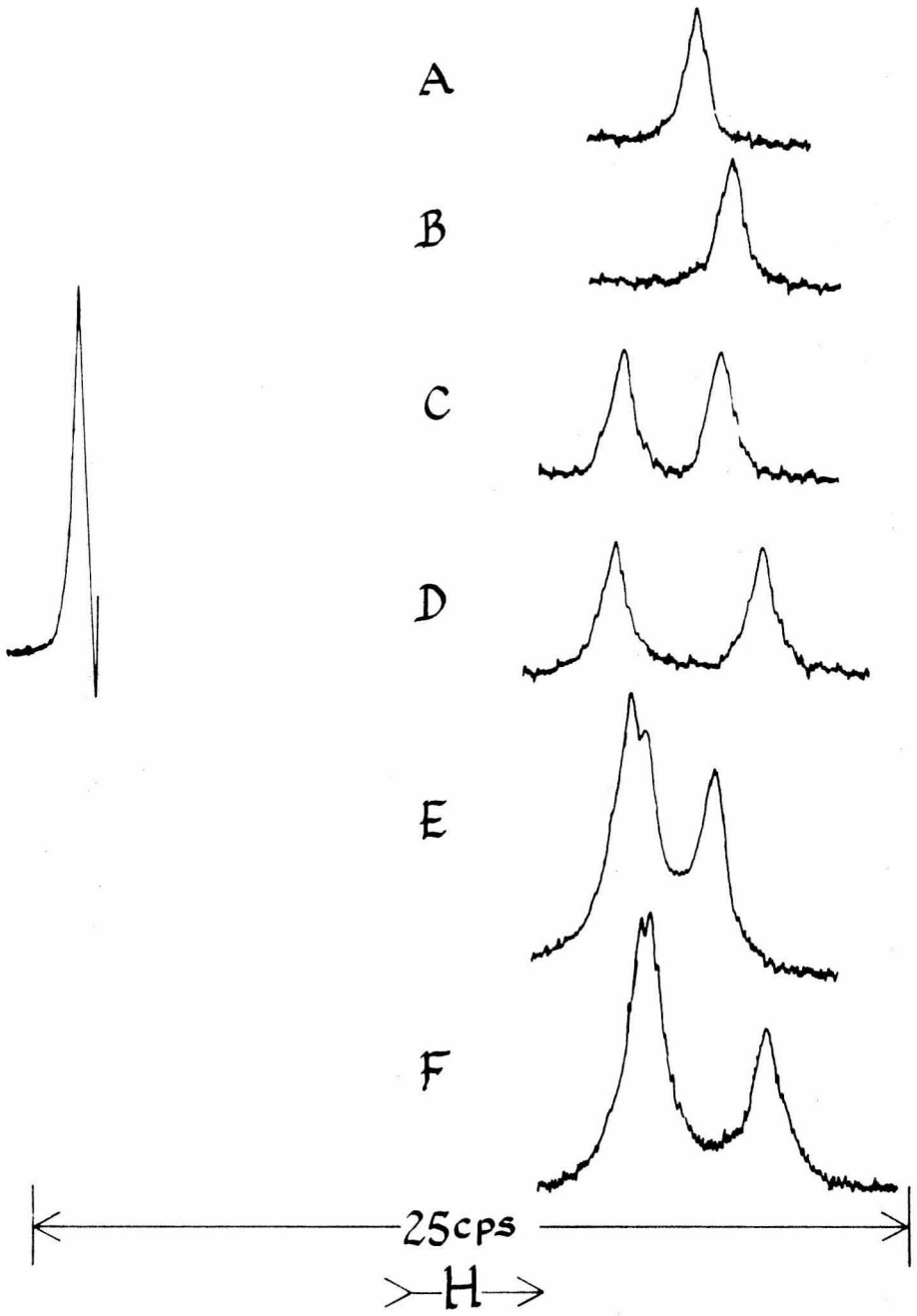

A
B
C
D
E
F
25cps
H

Figure 4. P. m. r. spectra of the acetamido methyl protons of: A, chitobiose; B, chitobiose ($\sim 5 \times 10^{-2}$ M) in the presence of lysozyme (3×10^{-3} M) in 0.1 M citrate buffer, pH 5.5 at 31°C. Spectra were measured relative to an internal acetone standard which is shown to lower field of the acetamido methyl resonances.

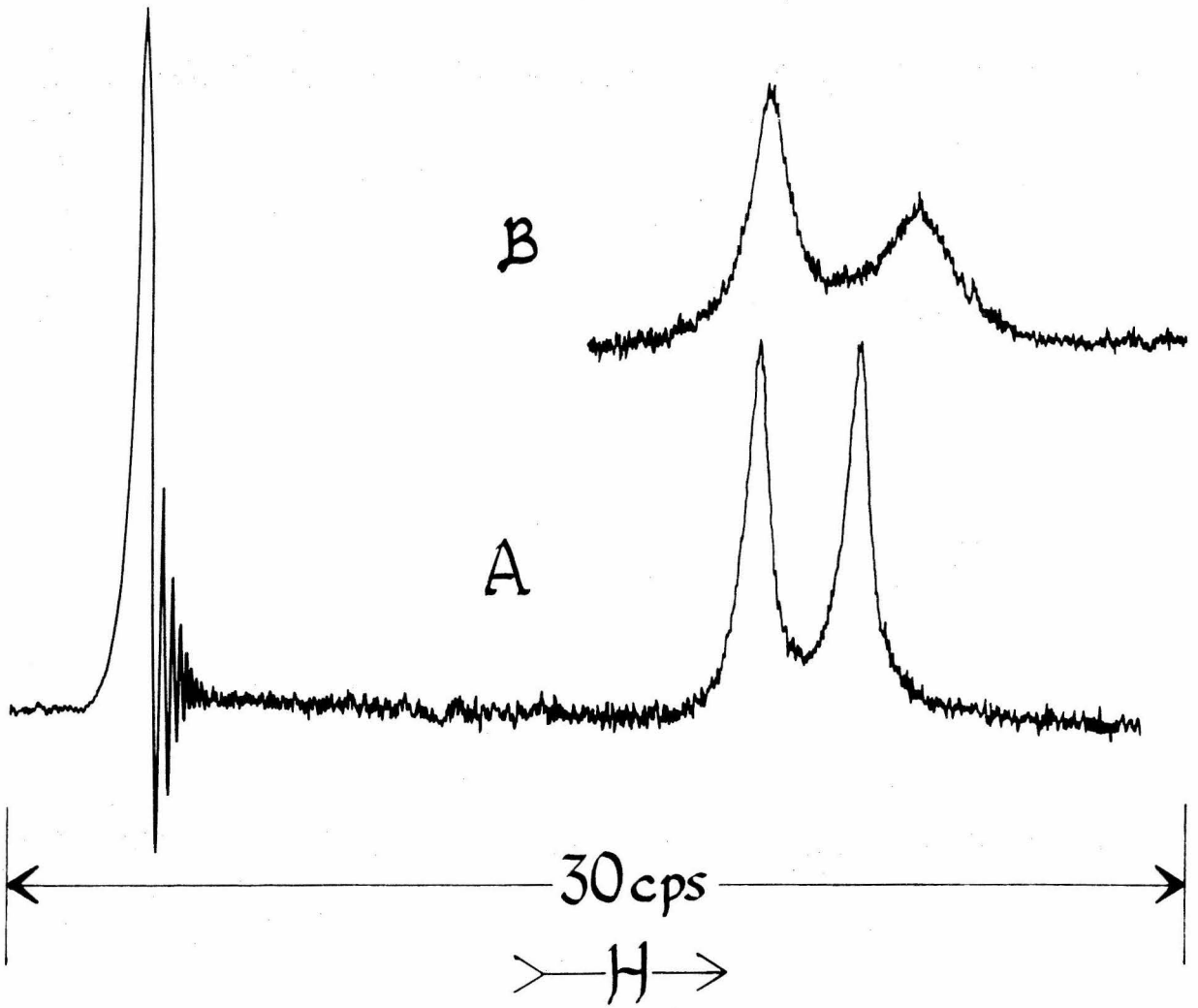

B
A
30 cps
H

Figure 5. Plot of the reciprocal of the observed chemical shift (δ) for the reducing-end acetamido methyl group resonance of chitobiose versus varying chitobiose concentrations (S_0) in the presence of a constant concentration of lysozyme (3×10^{-3} M).

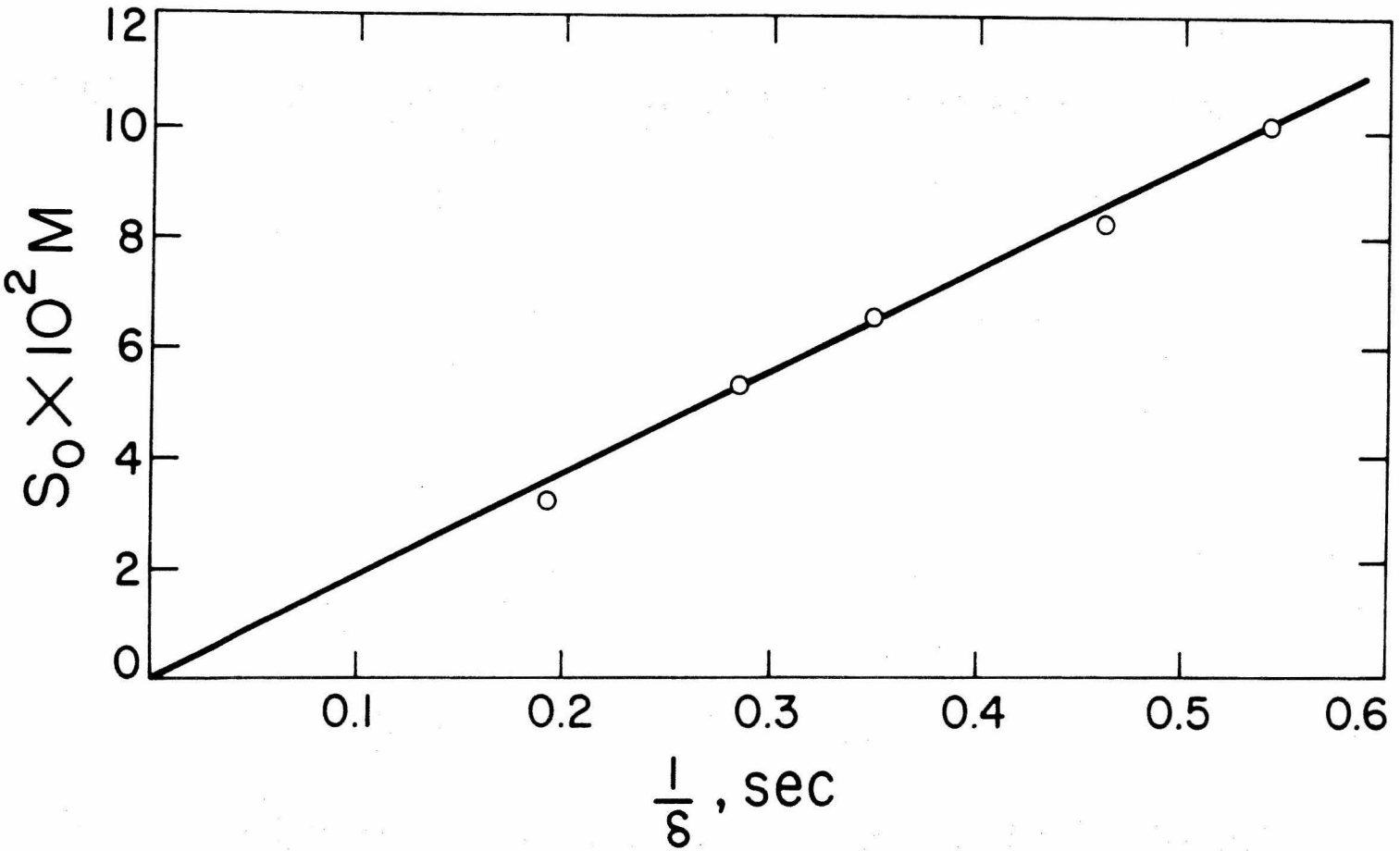

12
10
8
6
4
2
0
$S_0 \times 10^2$ M
0.1
0.2
0.3
0.4
0.5
0.6
$\frac{1}{\delta}$, sec

Figure 6. The pH dependence of Δ for the reducing end acetamido methyl resonance of chitobiose at 45°C. The solid line is a theoretical curve discussed in the text.

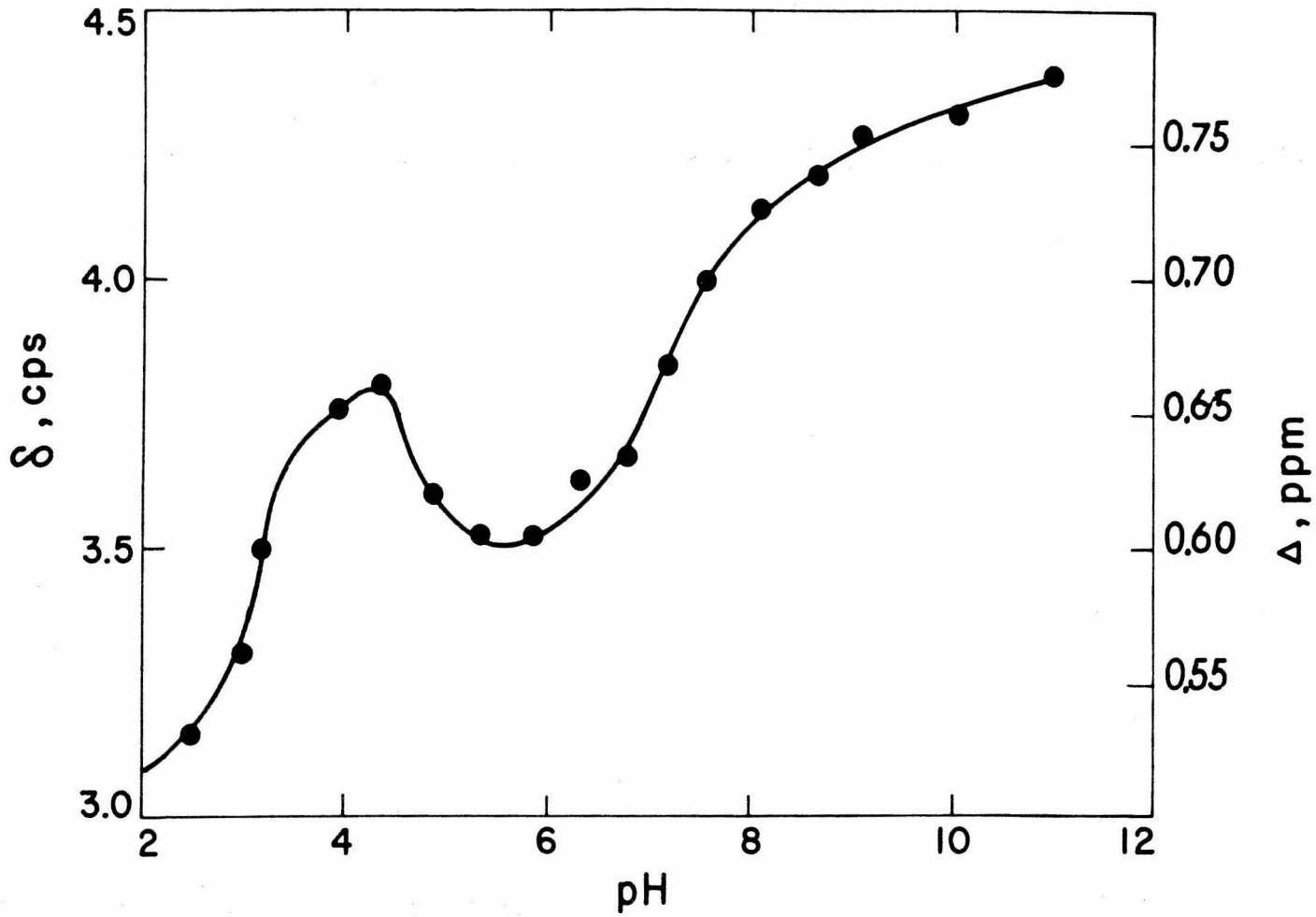

4.5
4.0
3.5
3.0
δ, cps
2
4
6
8
10
12
pH
0.75
0.70
0.65
0.60
0.55
Δ, ppm

Figure 7. P. m. r. spectra of the acetamido methyl resonances of methyl-β-chitobioside in the absence and presence of lysozyme in 0.1 M citrate buffer, pH 5.5, 31°C. An acetone internal standard is shown at lower field.

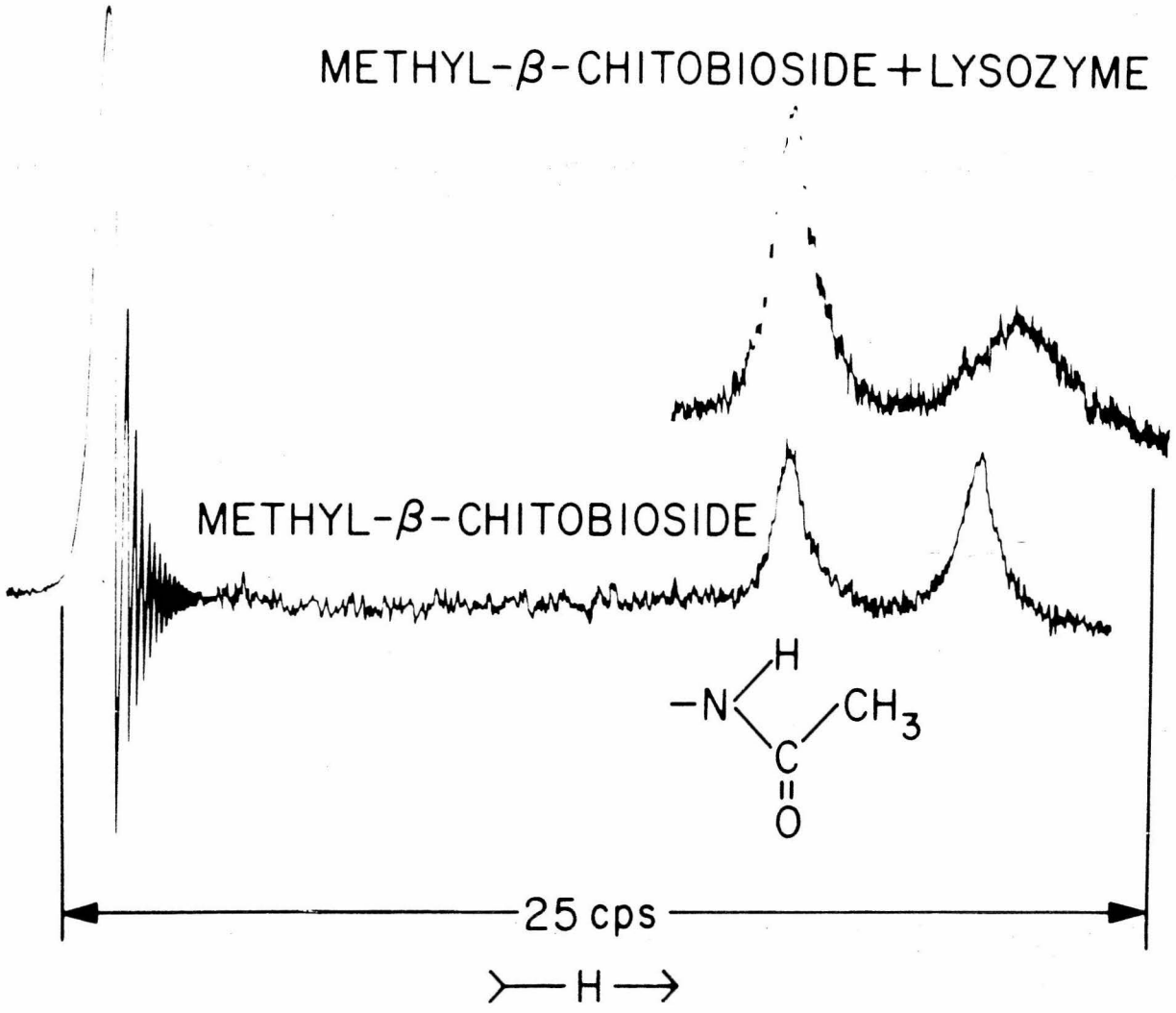

METHYL-β-CHITOBIOSIDE + LYSOZYME
METHYL-β-CHITOBIOSIDE
H
N
CH3
C
O
25 cps
H

Figure 8. P. m. r. spectra of the glycosidic methyl protons of methyl-β-chitobioside in the absence and presence of lysozyme in 0.1 M citrate buffer, pH 5.5, 31°C. A methanol internal standard is shown at higher field.

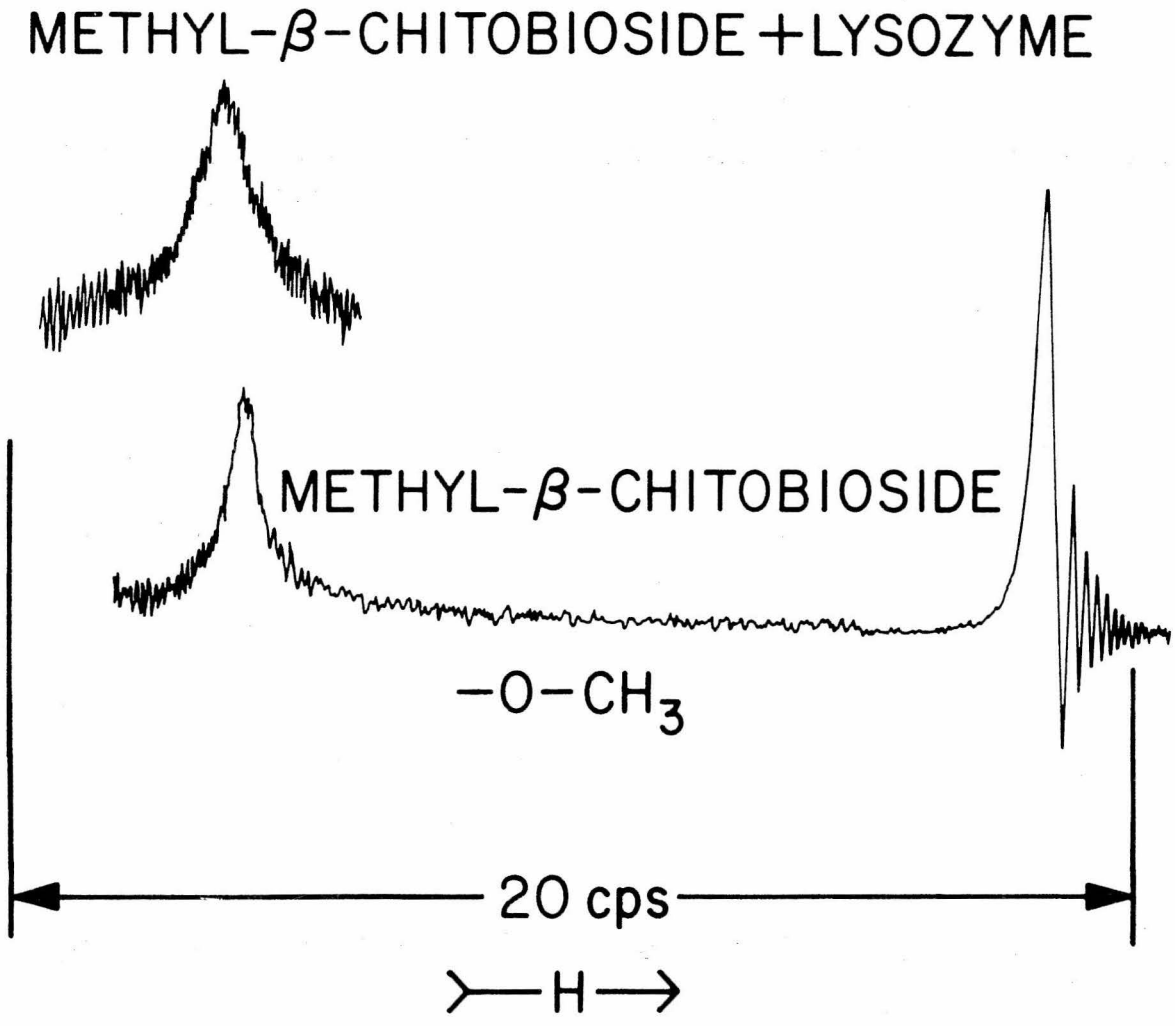

METHYL-β-CHITOBIOSIDE + LYSOZYME
METHYL-β-CHITOBIOSIDE
−O−CH3
20 cps
H

Figure 9. P.m.r. spectra of the acetamido methyl protons of methyl-β-chitobioside in the absence and presence of lysozyme (3.12×10^{-3} M), in citrate-phosphate buffer, pH 9.7, A, (4.18×10^{-2} M) with enzyme; B, (7.85×10^{-2} M) with enzyme; C, (9.70×10^{-2} M) with enzyme; D, (5×10^{-2} M) without enzyme.

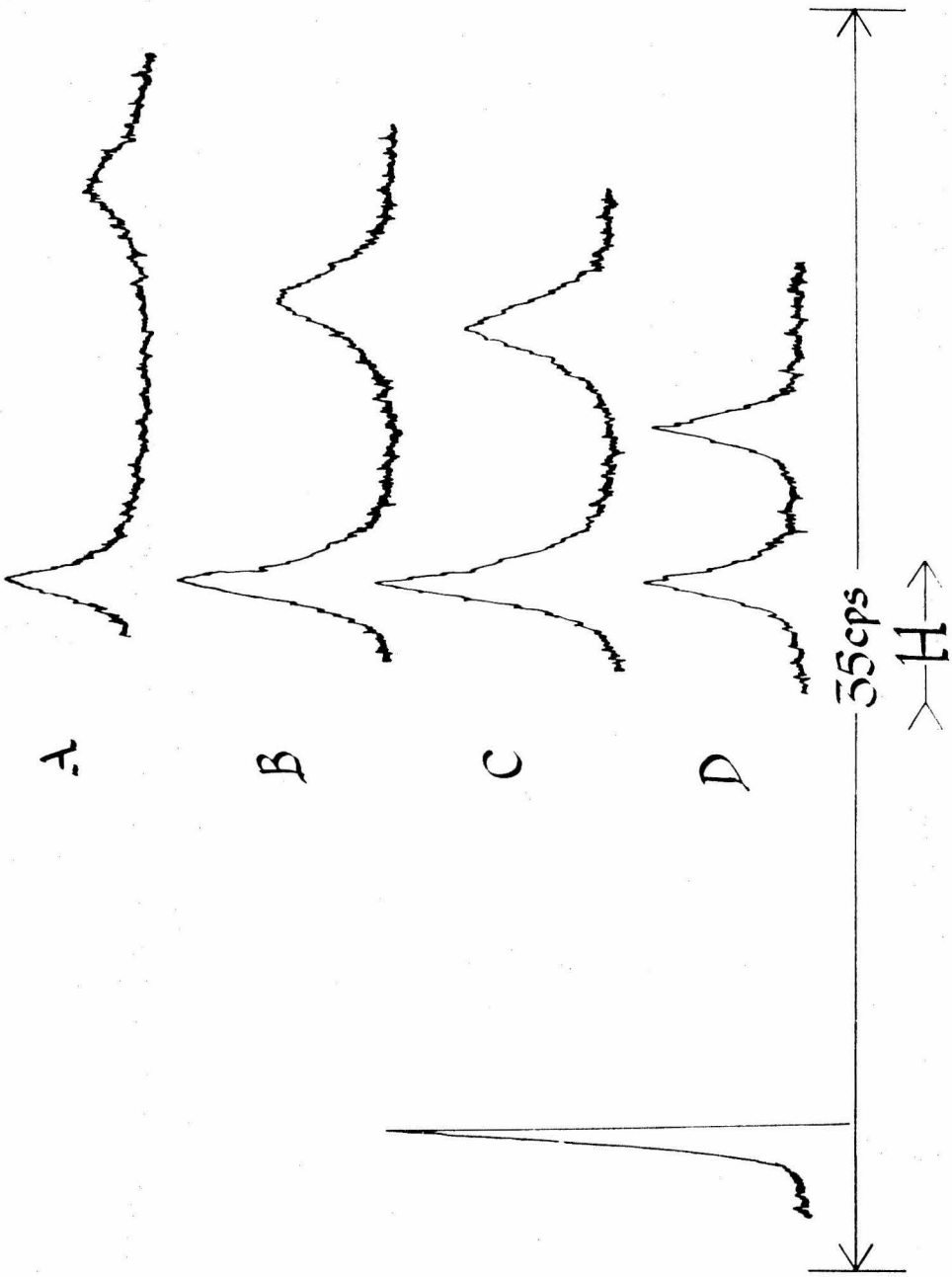

A
B
C
D
55cps
H

Figure 10. P.m.r. spectra of the acetamido methyl protons of chitotriose in the absence and presence of lysozyme (3.12×10^{-3} M), in citrate-phosphate buffer, pH 9.7: A, chitotriose; B, chitotriose (0.62×10^{-2} M) plus lysozyme at 55°C; C, chitotriose (9.62×10^{-2} M) plus lysozyme at 65°C. An internal standard of acetone is shown at lowest field.

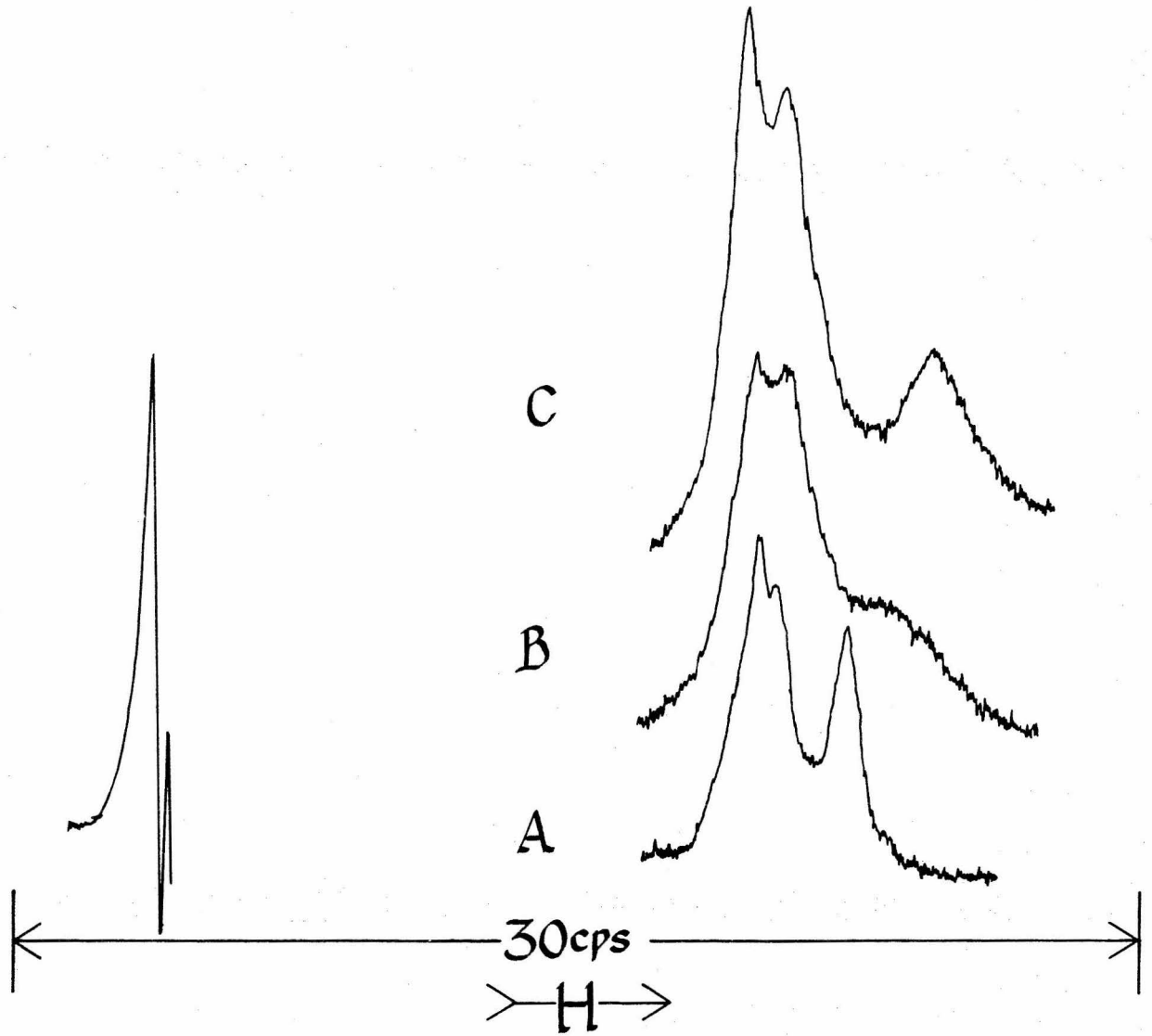

C
B
A
30cps
H

Figure 11. P.m.r. spectra of the acetamido methyl groups of chitotriose in the absence and presence of lysozyme (3.1×10^{-3} M) at pH 9.7, 65°C in citrate-phosphate buffer: A, chitotriose (5.48×10^{-2} M) plus lysozyme; B, chitotriose (7.71×10^{-2} M) plus lysozyme; C, chitotriose (9.62×10^{-2} M) plus lysozyme; D, chitotriose without added lysozyme. An internal standard of acetone is included and shown at lower field.

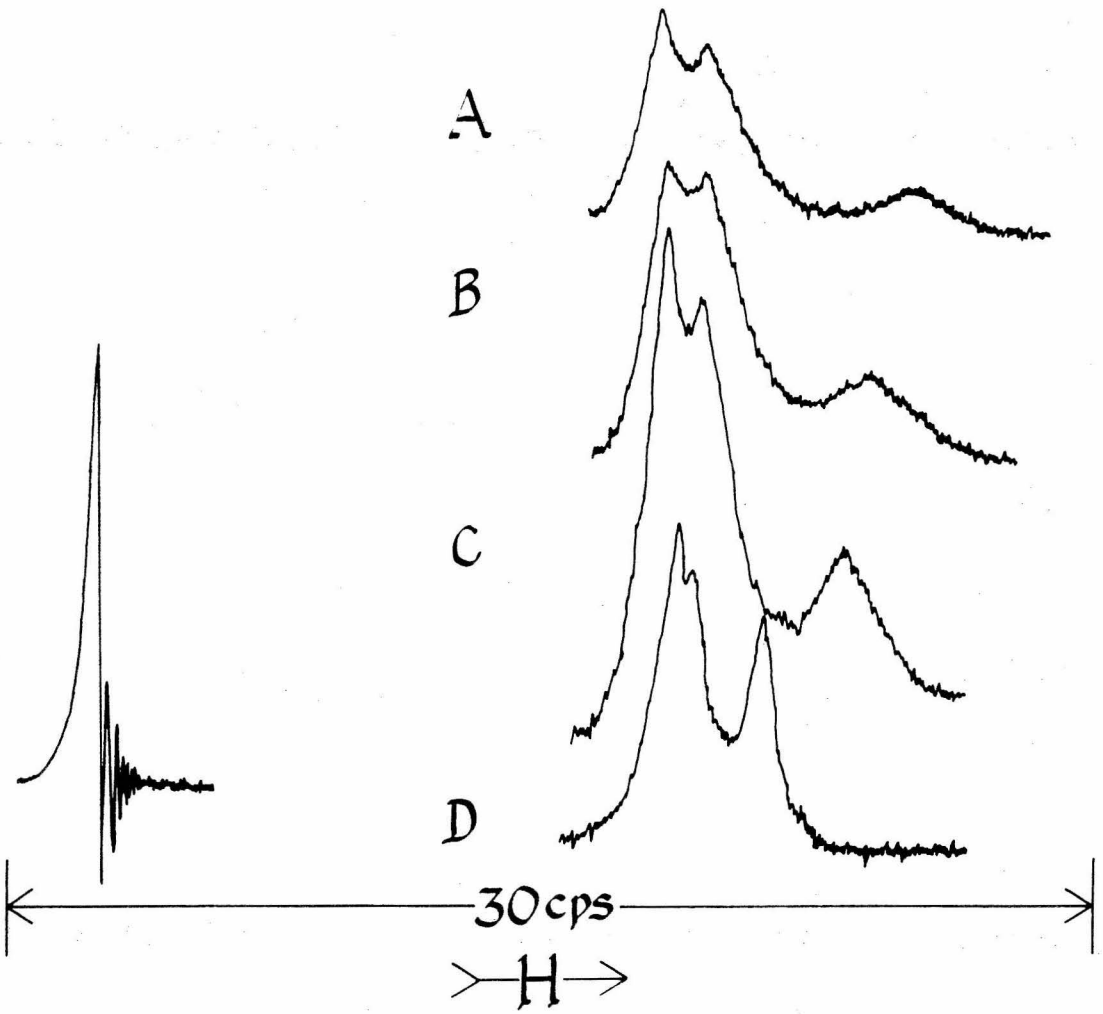

A
B
C
D
30 cps
H

Figure 12. P.m.r. spectra of the acetamido methyl groups of methyl-β-chitotrioside at pH 9.7, in the presence and absence of lysozyme (1.56×10^{-3} M): A, methyl-β-chitotrioside; B, methyl-β-chitotrioside (2.36×10^{-2} M) plus lysozyme at 55°C; C, methyl-β-chitotrioside (2.36×10^{-2} M) plus lysozyme at 65°C. An internal standard of acetone was included and its resonance is shown at lower field.

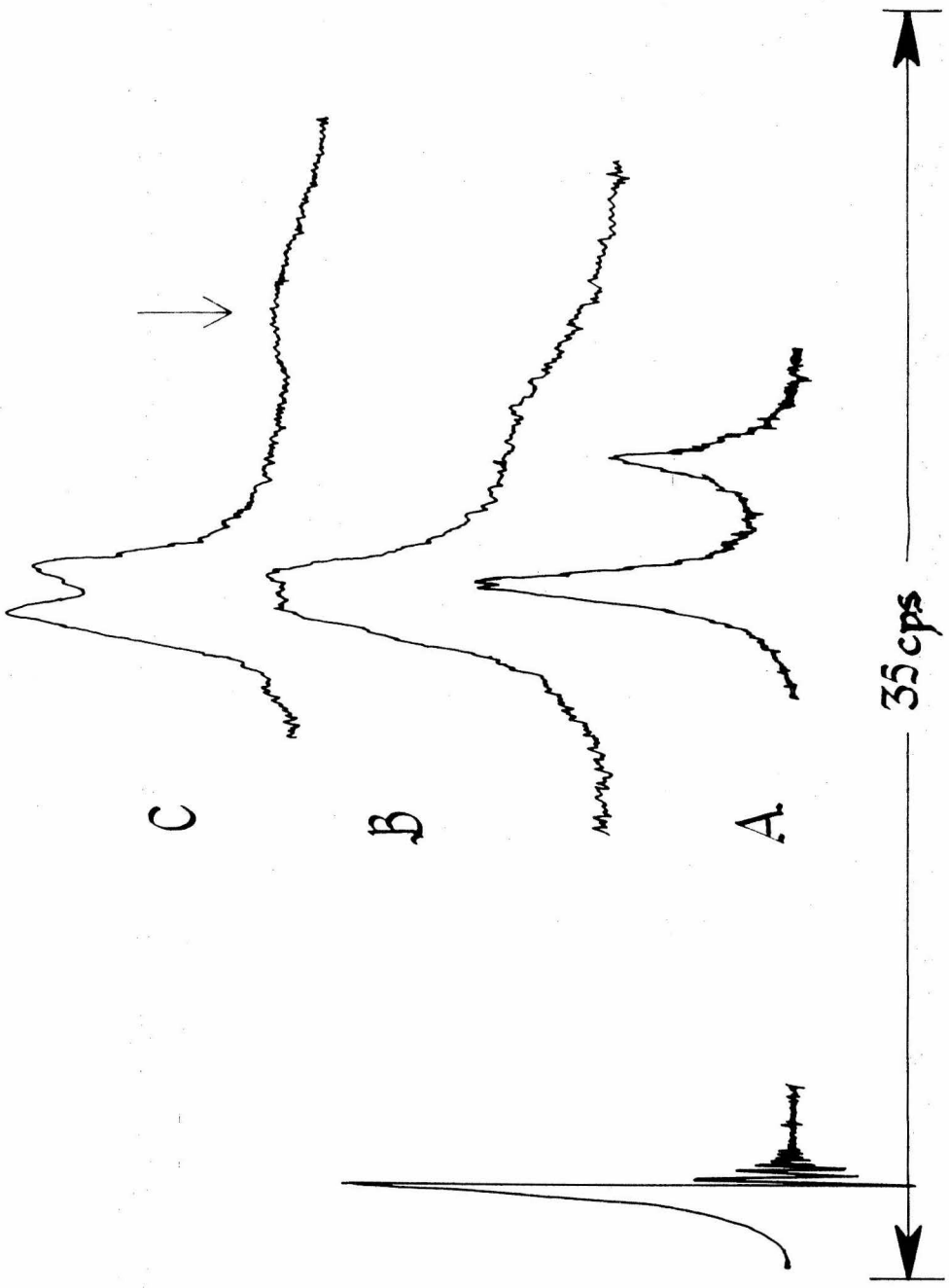

C
B
A
35 cps

Figure 13. P.m.r. spectra of the acetamido methyl groups of chitotetraose in the absence and presence of lysozyme (3.1×10^{-3} M) at pH 9.7 and 65°C: A, chitotetraose (6.09×10^{-2} M) plus lysozyme; B, chitotetraose (5.0×10^{-2} M) without lysozyme. An internal standard of acetone was included and its resonance is shown to lower field.

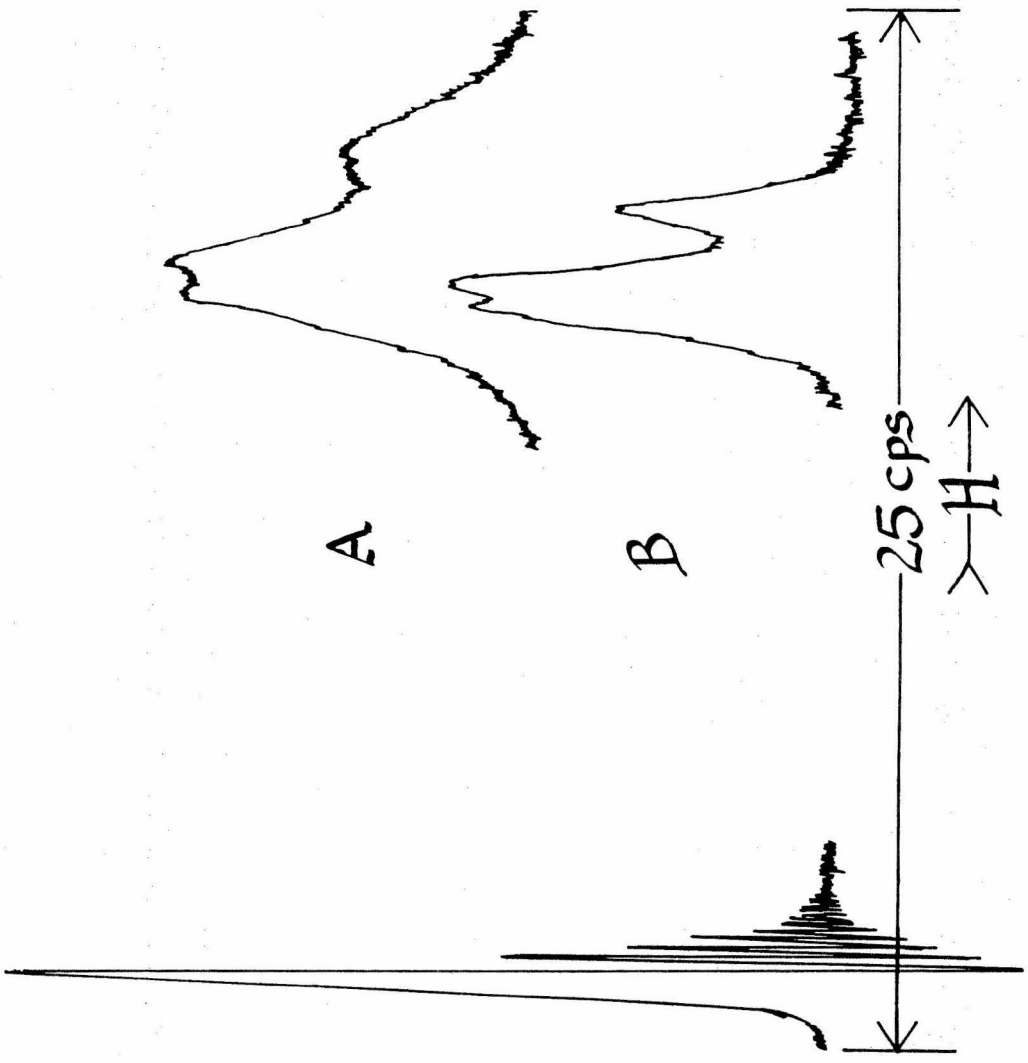
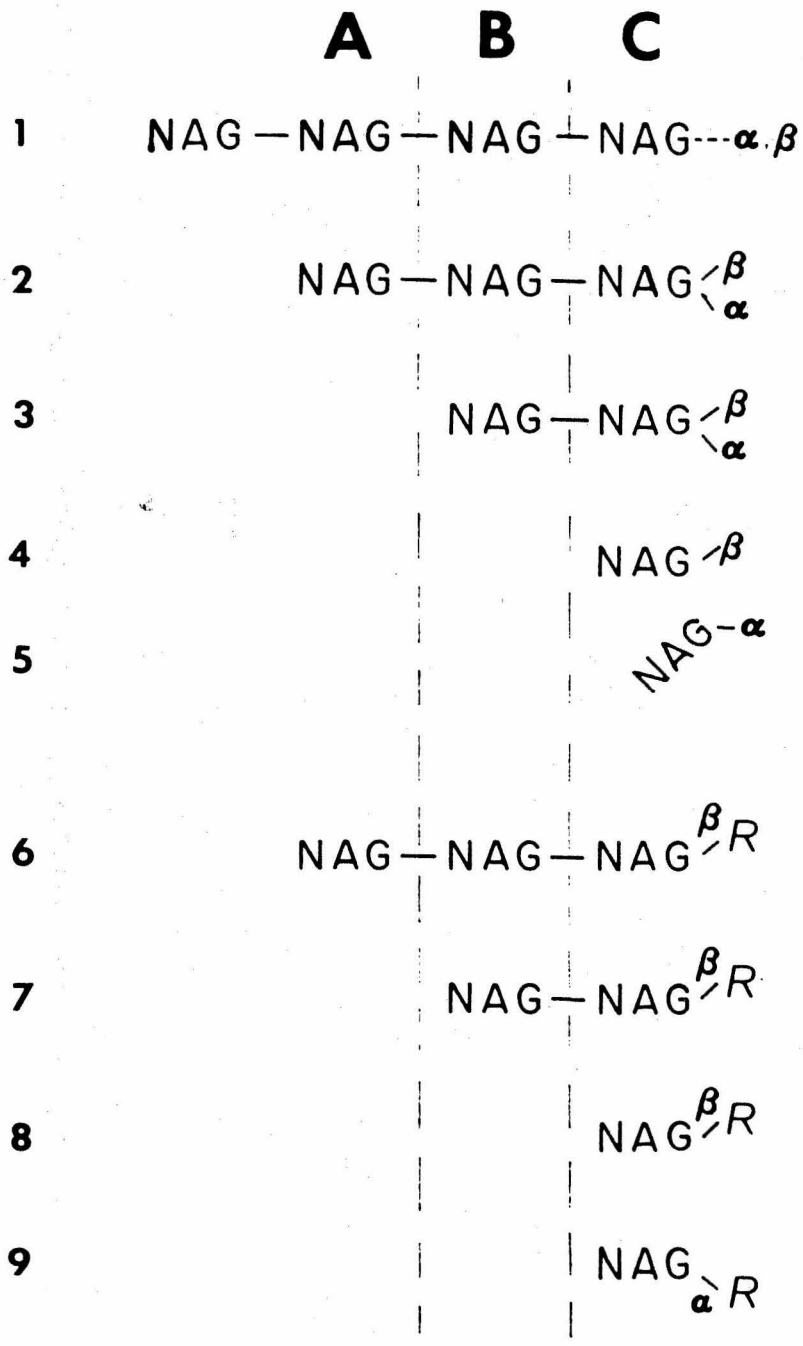

A
B
25 cps
H

Figure 14. Scheme for the relative modes of association with lysozyme of various saccharide inhibitors. Where α- and β-anomeric forms of the free saccharides are shown on a single line (as with chitotetraose) no information on relative binding modes was obtained. Where α- and β-forms are depicted separately (as with α-NAG and β-NAG) different binding modes for the anomers were elucidated. Where α- and β-forms are shown (as with chitotriose and chitobiose) on the same molecule on two levels it was shown that both anomeric forms bind identically. In the glycoside series methyl groups are depicted by R, and the anomeric form of the acethyl glycoside is indicated.


A
B
C
1
NAG—NAG—NAG—NAG---α,β
2
NAG—NAG—NAG
β
α
3
NAG—NAG
β
α
4
NAG
β
5
NAG-α
6
NAG—NAG—NAG
β
R
7
NAG—NAG
β
R
8
NAG
β
R
9
NAG
α
R

CHAPTER VI

THE DYNAMICS OF LYSOZYME-SACCHARIDE INTERACTIONS BY NUCLEAR MAGNETIC RESONANCE

INTRODUCTION

We have shown in the preceding chapters that derivatives of N-acetyl-D-glucosamine show changes in their nuclear magnetic resonance spectra upon association with hen's egg white lysozyme. These chemical shifts may be quantitated using the relationship

$$S_0 = \frac{E_0 \Delta}{\delta} = K_s$$

where δ is the observed chemical shift of the nucleus under consideration, S_0 and E_0 are the total concentration of substrate and enzyme respectively, K_s is the dissociation constant of the enzyme substrate complex and Δ is the chemical shift of the nucleus when bound to the enzyme. We have been able to assign values of Δ for the acetamido group binding regions for the three strong contiguous binding subsites A, B, and C of the enzyme.

One stringent requirement of the method is that the rate of exchange of the substrate molecule between the solution environment and the enzyme surface be "rapid" on the n. m. r. time scale. Under these conditions the observed spectrum is the average of the free and

bound environments. For very slow exchange rates the observed spectrum consists of separate absorptions for the free and bound state. However, if the rate of exchange is of the order of the difference in precessional frequency of the nucleus, the spectrum is more complex. Alexander (1962) has presented a quantum mechanical treatment which describes the absorption for various exchange rates. For intermediate exchange rates, the apparent absorption maximum lies between the free and averaged positions and changes as a function of the exchange rate. Furthermore, for intermediate rates the observed spectrum is significantly broadened and the extent of this broadening is also a function of the exchange rate. Thus the exchange rate may be obtained from the line shape and position. This work describes exchange rate measurements for chitobiose and chitotriose association with lysozyme.

MATERIALS AND METHODS

Lysozyme was purchased from Sigma Chemical Co. (Lot #77B-8040). Chitin oligosaccharides were prepared by acid hydrolysis of chitin (California Corporation for Biochemical Research) followed by gel filtration on Bio-Gel P-2, 200-400 mesh.

All difference spectra were measured on a Cary Model 14 spectrophotometer using a 0-0.1 absorbance slidewire. Temperatures were controlled to $\pm 0.5^{\circ}C$ with a Cary model thermostated cuvette holder. Enzyme concentrations were 0.6 mg/ml and gave a total difference spectrum amplitude of 0.06-0.08 OD when saturated with substrate. The data were treated by least square methods according to the equations described in Chapter I.

The buffers were made up by mixing 0.1 M citric acid with 0.1 M sodium citrate to pH 5.0 or with 0.1 M trisodium phosphate to pH 9.7. All pH measurements were made with a Radiometer pH meter 26.

For the p.m.r. measurements, enzyme solutions contained approximately 3×10^{-3} M lysozyme (Sigma Chemical Co., Lot #77B-8040) and 0.5% each of methanol and acetone as internal p.m.r. standards. The buffers were made by mixing 0.1 M citric acid with either 0.1 M sodium citrate, 0.1 M disodiumphosphate, or 0.1 M trisodiumphosphate. The exact concentration of the enzyme was determined by removing 25 $\mu\ell$, diluting to 5.00 ml with 0.1 M citrate buffer pH 5.5 and measuring the optical density of the solution at 280 mμ with a Cary Model 14 spectrophotometer. The known extinction coefficient was used to estimate lysozyme concentrations (Sophianpoulos et al., 1962).

All spectra were obtained with a Varian HA-100 nuclear magnetic resonance spectrometer, operating in frequency sweep mode. The water resonance was used as a lock signal, and each sample was allowed to come to thermal equilibrium before measurements were taken. For measurements at temperatures other than 31°, the operating temperature of the probe, a Varian V-4341 Variable Temperature accessory was employed. The difference in chemical shift of methanol or ethylene glycol as a function of temperature was used to determine the probe temperature.

Chemical shifts were determined by electronic counting of the difference between the sweep frequency and manual oscillator frequency using a Hewlett-Packard counter.

The theoretical curves were calculated using an IBM 7094 program based on the treatment of Alexander (1962). This program was written by J. Beauchamp and modified by T. Gehrig and F. Weigert.

All data were treated by least squares analysis.

RESULTS AND DISCUSSION

An example of the effect of temperature on the acetamido methyl group absorptions of chitobiose in the presence of lysozyme at pH 5.0 is shown in Fig. 1. In the preceding chapter, we have assigned the upfield methyl resonance to the reducing end acetamido methyl group of chitobiose. The observed resonances show marked changes in position and shape as the temperature increases, although there is very little change in the fraction of saccharide which is bound to the enzyme over this temperature range at this concentration. Chitobiose alone shows no such temperature effect over this range of temperature. Further, the upfield resonance appears to show the largest effect and this resonance also displays a large chemical shift upon association with the enzyme, while the downfield methyl group shows no chemical shift in the presence of lysozyme. This clearly suggests that chitobiose is exchanging between solution and the enzyme surface at an intermediate rate of exchange and that therefore exchange rate information may be obtained in this system.

Gutowsky and Saika (1953, see also Gutowsky et al., 1953) have considered the exchange of a nucleus between two environments, sites A and B. The authors derived an expression for the total magnetization of the system in the xy plane, G (the applied field is in the z direction).

$$G = \frac{-i\omega_1 M_0 \ (\tau_A + \tau_B) + \tau_A \tau_B(\alpha_A p_A + \alpha_B p_B)}{(1+\alpha_A \tau_A)(1+\alpha_B \tau_B) - 1}$$

where $\omega_1 = \gamma H_1$ (γ is the gyromagnetic ratio of the nucleus under discussion and H_1 is the amplitude of the radiofrequency field), τ_A and τ_B

are the pseudo-first order lifetimes of the nucleus in sites A and B, p_A and p_B refer to the fractional populations in sites A and B, $\alpha_A = \frac{1}{T_{2A}} - i(\omega_A - \omega)$ and $\alpha_B = \frac{1}{T_{2B}} - i(\omega_B - \omega)$ (T_{2A} and T_{2B} are the transverse relaxation times of sites A and B respectively while ω_A and ω_B are the chemical shifts of site A and B).

The imaginary part of G gives the absorption mode signal. Thus, for a system in which the chemical shifts, transverse relaxation times and populations of the two sites are known, the shape of the absorption (the magnitude of the imaginary part of G as a function of ω) can be calculated for various lifetimes. The lifetime is defined as the mean time a particular molecule can spend in a given state without exchanging and is given by

$$\tau_i = \frac{\text{number of molecules in state i}}{\text{rate of removal of molecules from state i}}$$

For an equilibrium of the type

$$E + S = ES$$

where the individual steps may be written

$$E + S \xrightarrow{k_1} ES \qquad \text{and} \qquad ES \xrightarrow{k_2} E + S$$

$\tau_{ES} = \frac{1}{k_2}$ and $\tau_S = \frac{1}{k_1 E}$. Thus the value of k_2 may be determined directly from the line shape measurements, while determination of k_1 reauires an independent measurement of the dissociation constant of the enzyme-substrate complex, K_s.

These measurements of K_s were carried out using the ultraviolet difference spectral techniques described in Chapter I. Figure 4 shows the effect of temperature on the dissociation constant of the chitotriose-lysozyme complex determined in this manner at pH 9.7. The data are plotted as pK_s ($-\log K_s$) versus $1/T$ and the slope of the line gives ΔH for binding of -13 kcal/mole. It should be mentioned that the K_s determined in this buffer system (0.1 M citrate-phosphate) is nearly three times smaller than the value obtained in Chapter I in 0.01 M phosphate and 0.1 M sodium chloride. This is consistent with the observed effects of citrate on catalysis by the enzyme, but we have no specific explanation for the phenomenon. Similar treatment of the binding of chitobiose at pH 9.7 gave ΔH of -8.4 kcal/mole and $K_s = 4.3 \times 10^{-4}$ M at 25°C while the value of K_s decreased to 1.5×10^{-4} M and ΔH became -11 kcal/mole for the enzyme-disaccharide complex formation.

Determination of the Chemical Shifts and Transverse Relaxation Times of the Bound State

Table 1 summarizes our previous results concerning the chemical shifts of chitobiose and chitotriose while bound to lysozyme. Most of these values were determined under conditions which were at or very near the fast exchange limit of the enzyme saccharide complexes and should therefore be fairly good measures of the chemical shifts associated with the bound environment. The only exception to this is the chemical shift for the reducing end acetamido methyl resonance of chitotriose. The large error in the experimental determination of the chemical shift suggests that the exchange rate is still in the intermediate

range. A value of 0.77 p.p.m. upfield was chosen for the bound chemical shift of this resonance. This value gives the best theoretical fit to the observed spectra. Further, a change in this value to 0.63 p.p.m. results in only a small change in the value of the lifetime (about 20%) and no change in relative values of the lifetimes at various temperatures. Similarly, under conditions of fast exchange, the values of T_2 are also the average of the free and bound state. It is somewhat more difficult to estimate the line width on the enzyme however, because the increases in the width in the observed spectrum are small and the observed line width is very susceptible to field inhomogeneity, the presence of paramagnetic material, etc. The line widths of the acetamido methyl resonances of methyl-α and methyl-β glucosaminide when bound to subsite C seem to be 13-15 Hz. This value also seems to fit the β form of N-acetyl-D-glucosamine, while the α form width seems to be about 10 Hz (see Chapter III), but its binding orientation is different from the others. The reducing end acetamido methyl resonance of chitobiose also displays a width of about 15 Hz while bound, and it appears that the enzyme does not discriminate between the α and β forms of the disaccharide. The nonreducing end acetamido methyl resonance is somewhat sharper than the reducing end and we estimate a value of 10 Hz for the subsite B acetamido methyl binding site. Since we have established that chitotriose binds with its nonreducing end in subsite C and central sugar residue in subsite B, in complete agreement with the x-ray crystallographic analysis of Blake et al. (1965, 1967), it seems reasonable to assign values of 10 and 15 Hz to the bound environment of the central and

reducing end acetamido methyl group resonances of chitotriose. The nonreducing end acetamido methyl group resonance appears to be somewhat sharper than the central acetamido methyl resonance in the presence of the enzyme and has been estimated a value of 8 Hz. While the error in these estimates is probably fairly high (about 20%) the qualitative trend in the line width values associated with subsites A, B, and C is clear.

This trend in line width seems to correlate reasonably well to the binding strength of the subsites A, B, and C. Thus subsite C which is the strongest of the three since the monosaccharide binds there, appears to have the shortest relaxation time; subsite B can be assigned an intermediate value; while subsite A has the longest relaxation time. This correlates to the binding studies of these saccharides and lysozyme discussed in Chapter I. These studies showed that chitobiose bound about 100 times better than the monosaccharide while chitotriose bound only about 30 times better than the disaccharide. Since chitobiose binds to subsites B and C and chitotriose binds to subsites A, B, and C the relative values of 100 and 30 may be assigned to subsites B and A respectively. Jardetzky (1964) has suggested that the preferential broadening observed in certain resonances of small molecules in the presence of macromolecules may be due to the relative strength of interaction of nuclei associated with the broadened resonance and the macromolecule binding site. Our results agree with this suggestion, however the magnitude of the effect is much smaller than that observed by Jardetzky. It would appear therefore that care must be used in application of Jardetzky's preferential broadening

arguments to enzyme substrate interactions.

Calculation of Theoretical Spectra

The theoretical spectra are calculated by a computer program based on the treatment of Alexander (1962) which describes the effect of exchange on a spin coupled system. For a weakly coupled system, this treatment reduces to that of Gutowski and Holm (1956) which describes the imaginary part of the Gutowski and Saika equation discussed above.

The computer program allows the calculation of a theoretical absorption for each of several spectral lines. These individual lines are then weighted and summed to give the composite spectrum. Thus in the case of chitotriose three separate absorptions are calculated, all with the same fraction bound and the same lifetimes, each is given a relative weight of 1.00, and then summed to give the composite spectrum.

The use of an internal standard of acetone in the sample allows an estimate of the increase in line width associated with the viscosity of the enzyme solution at lower temperatures, paramagnetic material in the enzyme sample, and most other effects not associated with the binding or exchange processes. After a good agreement for peak positions of the observed and theoretical spectra had been obtained, small increments of line width were added to the theoretical spectrum corresponding to the increase in line width of the acetone resonance for that particular sample. The results of the curve for chitobiose at pH 5.0, chitobiose at pH 9.7, and chitotriose at pH 9.7 fitting are shown

in Figs. 1, 2, and 3, respectively. In general the agreement is quite good. In Fig. 1, at 52°, the upfield acetamido methyl resonance appears to be shifting back toward its position in the absence of lysozyme. This is because sufficient hydrolysis and transglycosylation of chitobiose had occurred at pH 5.0, near the pH maximum for catalysis by lysozyme, to produce chitotriose which competes effectively for the strong binding site on the enzyme surface. In the case of chitotriose which is a better substrate for lysozyme than the disaccharide, the experiments could not be carried out at pH 5.0 because of extensive hydrolysis of the sugar. These studies were therefore carried out at pH 9.7, where the enzyme is catalytically inactive but is still stable and binds chitotriose reasonably well.

The conversion of the lifetime measurements shown in Figs. 1, 2, and 3 to rate constants are summarized in Table 2. The probable errors in the determinations of k_1 were estimated to be 40-50%. This includes an error of about 30% in the lifetime determinations, an error of 10-15% in the dissociation constant measurements and takes into account the +1°C variation of the temperature in the probe of the spectrometer.

A recent publication by Chipman and Schimmel (1968) describes temperature jump relaxation study of the rates of association and dissociation of chitotriose and chitobiose to lysozyme at pH 6.00 and approximately 28.7°C. They found an association rate constant for both chitobiose and chitotriose of 4.5×10^6 l. $mole^{-1} sec^{-1}$. This value agrees well with our determination of the association rate constant of the chitobiose lysozyme complex at pH 5.0 and 30°C of

3.8×10^6 1. $mole^{-1} sec^{-1}$. The association constant for chitobiose and lysozyme at pH 5.0 shows very little temperature dependence over the range 9-52°C. An Arrenhius plot of the data shown in Table 2 gives an activation energy (E_a) of 0.4 ± 0.3 kcal $mole^{-1}$ for the process. The disaccharide lysozyme complex at pH 9.7 shows an E_a of 7.3 ± 0.9 kcal $mole^{-1}$ for its formation. Interestingly, the trisaccharide enzyme complex at pH 9.7 shows very little if any temperature dependence in its formation rate constant, corresponding to 1.1 ± 0.7 kcal $mole^{-1}$. In view of the estimated error in the determinations of the rate constants, only an E_a greater than 2 kcal $mole^{-1}$ should be considered significant. Thus for chitobiose at pH 5.0 and chitotriose at pH 9.7 the formation of the enzyme substrate complex displays a rate constant of about 4×10^6 1 $mole^{-1} sec^{-1}$, considerably below the accepted value for the diffusion controlled biomecular rate constant for such processes of 10^8 1 $mole^{-1} sec^{-1}$ (Amdur and Hammes, 1966) and yet has virtually no energy of activation. This suggests that the binding of the saccharides involves a transistion state with a very unfavorable entropy of activation.

Increasing the pH from 5.0 to 9.7 produces a marked effect on the activation energy for the formation of the disaccharide enzyme complex. The most reasonable explanation for the increase is that the ionization of some group on the enzyme surface produces a potential energy barrier to the formation of the complex. This could occur if binding to the basic form of the enzyme resulted in solvent reorganization, if different bonds were formed or broken during the transition state in the basic form of the protein than in the acidic form or perhaps

Some sort of short range repulsive force set up by the ionization to the basic form of the enzyme. If this is indeed the case, then the binding of chitotriose under the same conditions must nullify a good deal of the effect of the ionization. There are two possible explanations for the decrease in the activation energy. The first is that the trisaccharide enzyme transition state does not involve the same interactions in subsites B and C as the dissacharide enzyme complex. This seems somewhat unlikely in view of the great similarity of the orientation of the di- and trisaccharides while bound to the enzyme. If indeed the transition states for the two complexes are similar to those subsites, the additional bonds being formed in subsite A by the trisaccharide must somehow counteract the unfavorable interactions due to the ionization to the basic form of the enzyme.

It is interesting to speculate about the nature of the ionizable group responsible for the change in activation energy for the disaccharide. An ionizable group of pK_a 6.5-7.0 has been implicated in the binding to lysozyme of the monosaccharide (Chapter IV), the disaccharide (Chapter V) and the trisaccharide (Chapter I). It appears that one group is responsible for all the effects and no group of different pK_a has been shown to be important in the binding process in the basic pH range. This group therefore is most probably also responsible for the change in activation energy. Since it affects the binding of the mono-, di- and trisaccharides, it would appear to be associated with subsite C. We have suggested in Chapter IV that this group is glutamic acid residue 35 since it occupies a rather hydrophobic environment and might therefore be expected to have a high pK_a.

These measurements are the first to consider the activation energy of enzyme substrate formation. This would seem to give more information than the measurements of the rate constants alone. While an interpretation of the small activation energies observed in these experiments is difficult, these small values do suggest that the discrepancies between the observed rate constants and the theoretical diffusion controlled rate constants are caused by the statistically unfavorable nature of the transition state of the complex. This is most probably a reflection of the rather specific nature of the complex, such that only a few of the possible collisions between the enzyme and substrate have the proper orientation to result in the formation of the complex. The application of activation energy measurements for complex formation to other enzymes, particularly those with charged substrates, should prove interesting.

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TABLE 1.--Magnetic parameters and rate constants for formation of enzyme-substrate complex which gave the best theoretical fit to the observed spectra

Inhibitor	Temp. range (°C)	Δ(p. p. m.)[a]			Halfwidth (Hz)			k_f[c] (range) (1 mole^{-1} sec^{-1})
		CH_3-N_1[b]	CH_3-N_2[b]	CH_3-N_3[b]	CH_3-N_1[b]	CH_3-N_2[b]	CH_3-N_3[b]	
Chitobiose, pH 5	10 - 50	0.57	0	-	15	10	-	10^6 - 10^7
Chitobiose, pH 9.7	10 - 50	0.77	0	-	15	10	-	10^6 - 10^7
Chitotriose, pH 9.7	10 - 65	0.77	0	-0.09	15	10	8	5×10^6 - 10^7

[a]The acetamido methyl groups are numbered beginning at the reducing termini of the inhibitor molecules.

[b]Values relative to acetone; all chemical shifts are to higher field.

[c]Second order rate constant for formation of the enzyme-inhibitor comples.

TABLE 2.--Summary of rate constant measurements for lysozyme saccharid complexes

CHITOBIOSE pH 5.0

T(°C)	$K_s \times 10^5$ moles/l	$\frac{1}{k_2} \times 10^4$ sec	$k_1 \times 10^{-6}$ l mole^{-1} sec^{-1}
52°	82.	3.3	3.7
40°	41.	6.5	3.8
30°	20.	13.	3.8
20°	10.	26.	3.8
9°	5.0	60.	3.3

CHITOBIOSE pH 9.7

T(°C)	$K_s \times 10^4$ moles/l	$\frac{1}{k_2} \times 10^4$ sec	$k_1 \times 10^{-6}$ l mole^{-1} sec^{-1}
50°	12.	1.1	7.6
40°	8.2	2.8	4.4
30°	5.2	7.3	2.6
20°	3.1	16.	2.0
10°	1.9	36.	1.5

CHITOTRIOSE pH 9.7

T(°C)	$K_s \times 10^6$ moles/l	$\frac{1}{k_2} \times 10^3$ sec	$k_1 \times 10^6$ l mole^{-1} sec^{-1}
67°	160.	0.96	6.5
54°	78.	2.2	5.8
40°	31.	7.7	4.2
30°	16.	15.	4.2
20°	7.6	32.	4.1
9°	3.0	64.	5.1

Figure 1. Observed and theoretical spectra for the interaction of chitobiose (3.5×10^{-2} M) and lysozyme (3.0×10^{-3} M) at pH 5.0 as a function of temperature at 100 M Hz.

Temp. (^{o}C)	τ_S(sec)	τ_{ES}(sec)
2		
9	0.069	0.0060
20	0.030	0.00261
30	0.015	0.00130
40	0.075	0.00653
52	0.00453	0.000330

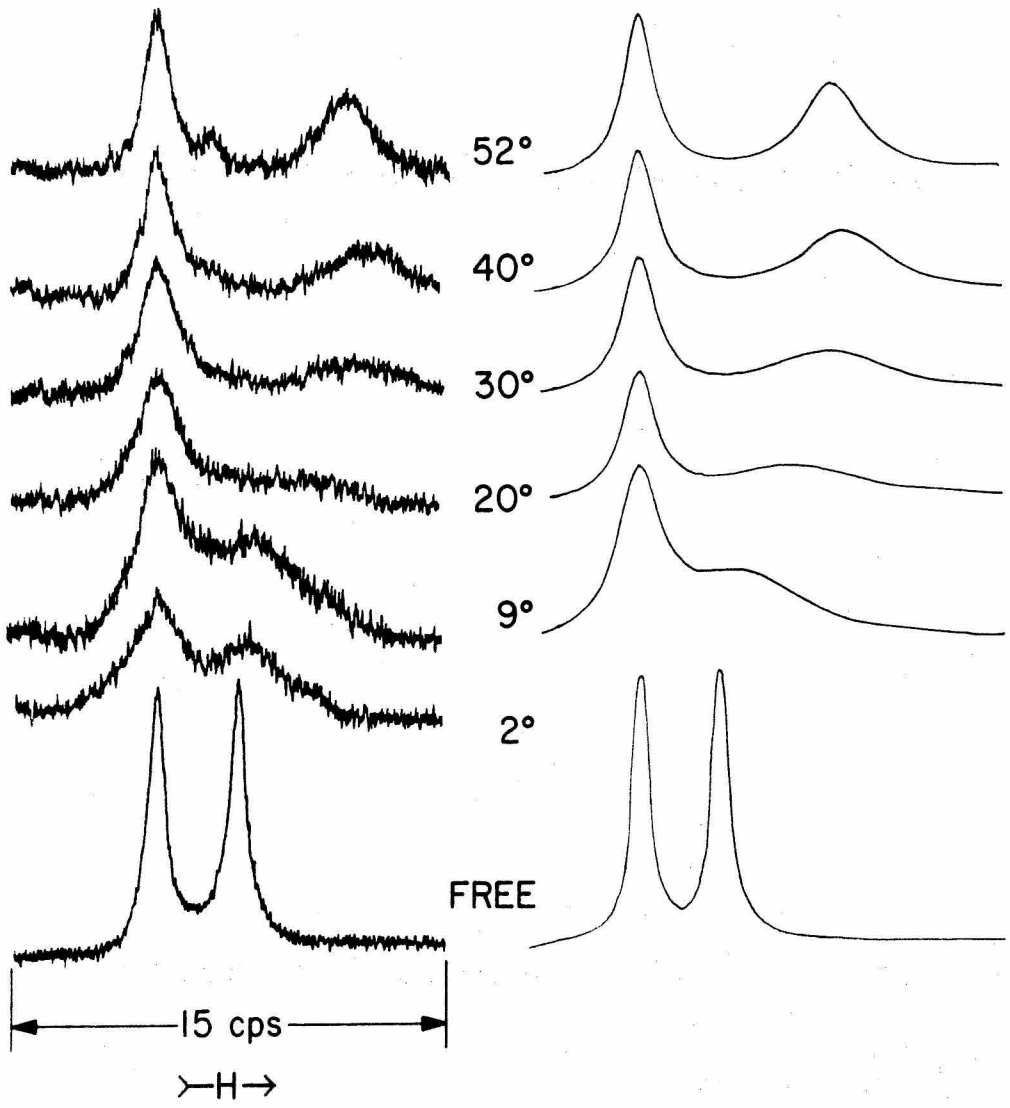

52°
40°
30°
20°
9°
2°
FREE
15 cps
H

Figure 2. Observed and theoretical spectra for the interaction of chitobiose (4.1×10^{-2} M) and lysozyme (3.0×10^{-3} M) at pH 9.7 as a function of temperature at 100 M Hz.

Temp. (^{o}C)	τ_S(sec)	τ_{ES}(sec)
10	0.0493	0.0036
20	0.0220	0.0016
30	0.010	0.00073
40	0.00384	0.000280
50	0.00100	0.000073

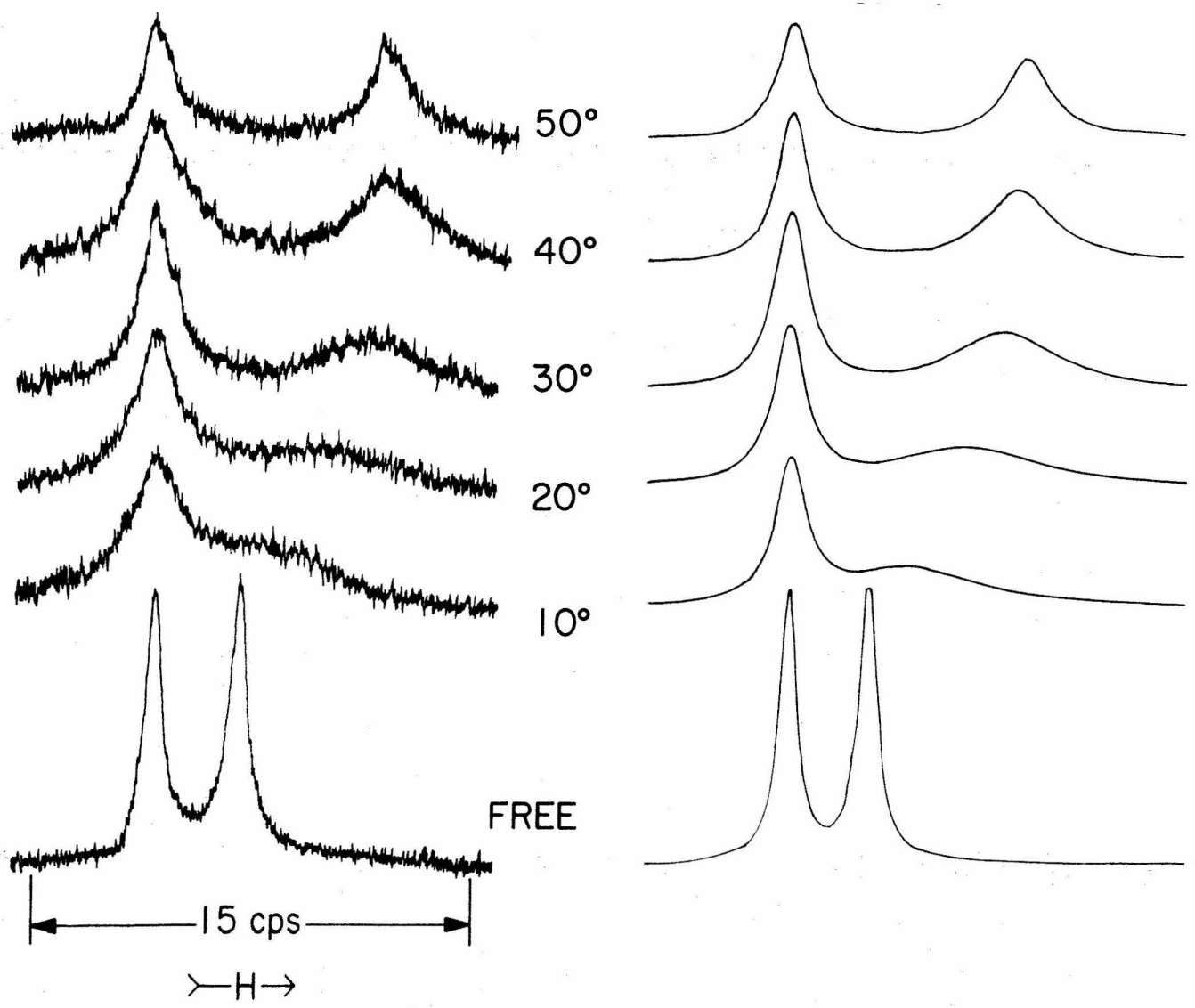

50°
40°
30°
20°
10°
FREE
15 cps
H

Figure 3. Observed and theoretical spectra for the interaction of chitotriose (4.7×10^{-2} M) and lysozyme (3.0×10^{-3} M) at pH 9.7 as a function of temperature at 100 M Hz.

Temp. (^{o}C)	τ_S(sec)	τ_{ES}(sec)
9	1.00	0.0640
20	0.50	0.0320
30	0.24	0.0153
40	0.12	0.00766
54	0.035	0.00224
67	0.015	0.00096

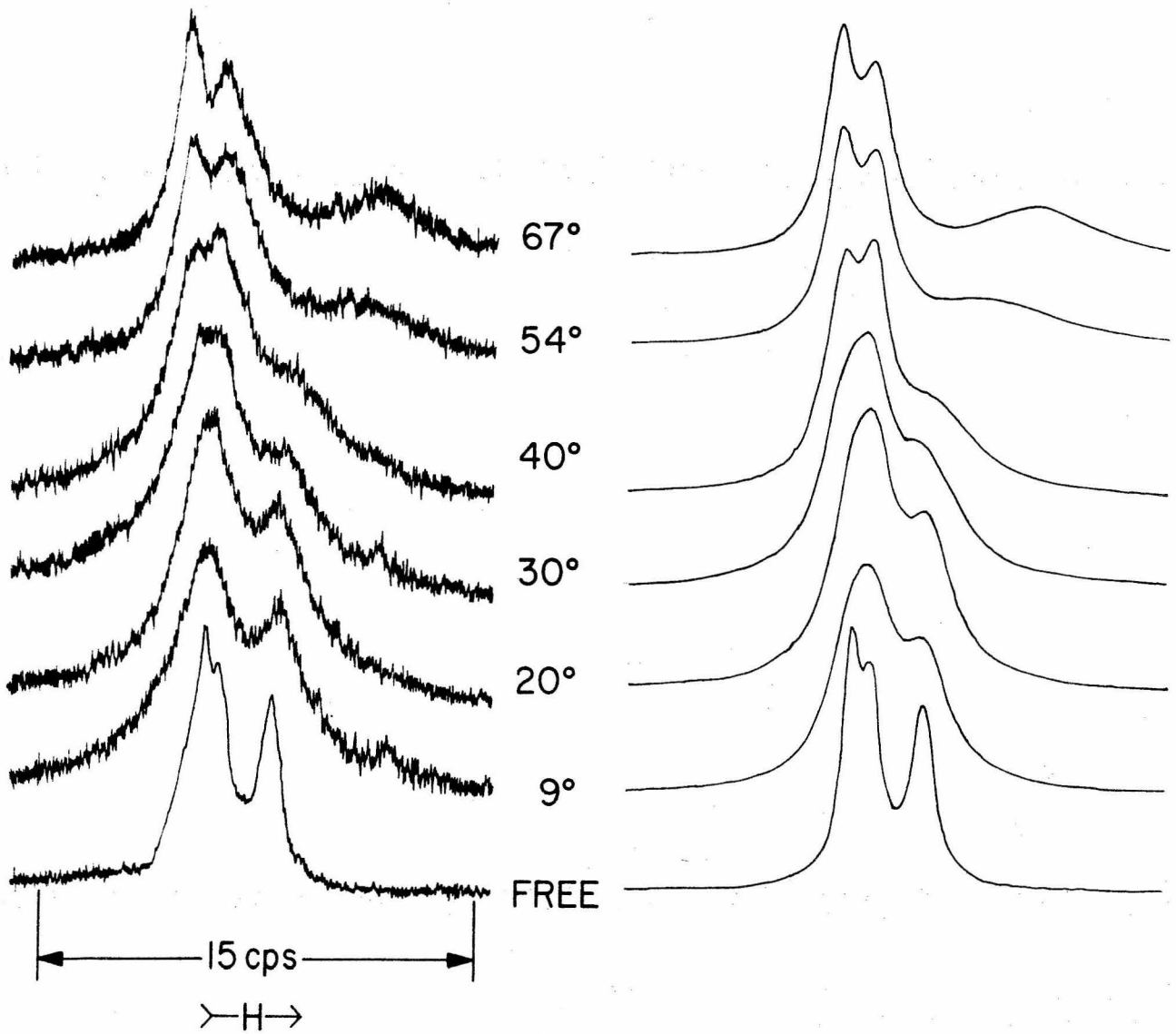
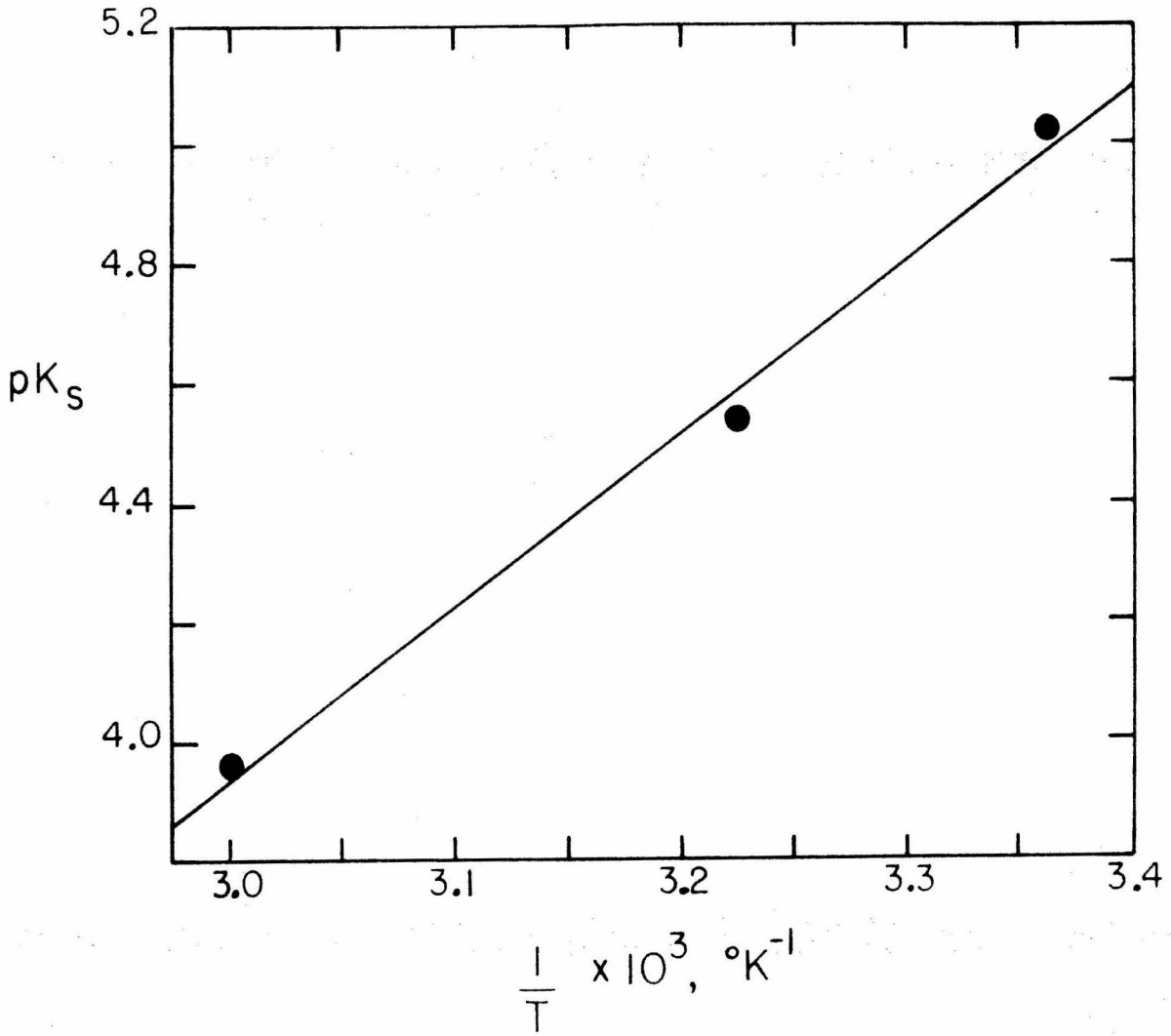

67°
54°
40°
30°
20°
9°
FREE
15 cps
H

Figure 4. The temperature dependence of the dissociation constant of the chitotriose-enzyme complex at pH 9.7.


5.2
4.8
4.4
4.0
pK_S
3.0
3.1
3.2
3.3
3.4
1/T x 10^3, °K^-1

PART II

THE CATALYTIC PROPERTIES OF LYSOZYME

CHAPTER VII

STEREOSPECIFICITY OF LYSOZYME-CATALYZED CLEAVAGE OF GLYCOSIDIC BONDS

Hen egg-white lysozyme is the enzyme which, from a structural point of view, is at present best understood. Although several studies of the action of the enzyme on cell-wall oligosaccharides (Salton, 1964; Sharon, 1967; Chipman et al., 1968) and on chitin oligosaccharides (Wenzel et al., 1957; Rupley, 1964; Maksimov et al., 1965; Dahlquist and Raftery, 1967; Rupley and Gates, 1967) have been reported little work has been done on the detailed mechanism of the bond cleavage. From crystallographic studies involving lysozyme-saccharide complexes Blake et al., 1965 1967 have outlined a tentative scheme for the hydrolytic mechanism. This proposal involves catalytic production of a carbonium-ion which is given steric and electrostatic stabilization by the enzyme. Other mechanistic schemes are also plausible (Raftery and Rand-Meir, 1968) for such an enzyme-catalyzed reaction and in this respect a knowledge of the stereochemistry of the products of lysozyme action is of importance.

Since we had already shown (Dahlquist and Raftery, 1967) that hydrolysis of chitotriose by lysozyme involves the formation of an intermediate capable of glycosyl transfer to yield chitotetraose it was felt that this transfer reaction afforded a means of investigating the stereochemistry of the enzymic reaction.

The ability of lysozyme to act as a transferase has been previously recognized. Maksimov (1965) has shown that lysozyme-catalyzed hydrolysis of chitotetraose resulted in production of insoluble chitin-like polymers. Rupley (1964) has also shown that glycosyl units could be transferred by lysozyme from chitotriose to NAG.[1] The action of lysozyme on a cell-wall tetrasaccharide was shown to result in glycosyl transfer with production of higher molecular weight compounds, as judged by paper chromatography (Sharon, 1967; Chipman et al., 1968) of reaction mixtures.

Since such higher molecular weight compounds formed by transglycosylation of cell-wall oligosaccharides have been shown to be susceptible to degradation by lysozyme it has been suggested that all of the glycosidic bonds in the compounds have the same stereochemistry as in the initial substrates (Sharon and Seifter, 1964; Sharon, 1964, 1967; Pollock et al., 1967; Chipman et al., 1968). There is, however, no direct chemical evidence bearing on the stereochemistry of the glycosidic bonds in these compounds. Furthermore, in the absence of evidence that lysozyme cannot hydrolyze α-linked glycosidic bonds or form them (to a greater or lesser extent) by transglycosylation, it is not clear that the enzymatic reaction proceeds with complete or partial retention or inversion of configuration.

---

[1] Abbreviations used are α-methyl-NAG, methyl-2-acetamido-2-deoxy-α-D-glucopyranoside; β-methyl-NAG, methyl-2-acetamido-2-deoxy-β-D-glucopyranoside; NAG, 2-acetamido-2-deoxy-D-glucopyranose.

The purpose of the present investigation was to carry out a quantitative study of the stereochemistry of the lysozyme-catalyzed hydrolysis of β-(1-4) linked glycosidic bonds. This is the first description of the stereochemical course of a glycosidase catalyzed reaction to the one per cent level.

EXPERIMENTAL

Materials

Hen egg-white lysozyme was purchased from Sigma Chemical Company (Lot #96B-8572). Chitobiose, chitotriose, and chitotetraose were isolated from acid hydrolysates of chitin by gel-filtration. Chitobiose octaacetate was prepared by acetylation of chitobiose with pyridine-acetic anhydride (1 : 2 v/v) as described by Zechmeister and Toth (1931).

The synthesis of N,N'-diacetyl-^{14}C-chitobiose was accomplished, as outlined in Fig. 1, through first treating chitobiose octaacetate (I) with trithyloxonium fluoroborate (Hanessian, 1967) to obtain [3,4,6-tri-O-acetyl-2-deoxy-2-amino-D-glucopyranosyl-β-(1-4)-1', 3',6',-tri-O-acetyl-2-deoxy-2-amino-D-glucopyranose, hydrofluoroborate (II).] Acetylation of this material in pyridine and acetyl chloride-1-^{14}C (Volk Radiochemical Company, Lot #MA 1093) gave octaacetyl-chitobiose (III), with ^{14}C labelled acetamido groups. Deacetylation in methanol (containing 0.1 M sodium methoxide) gave the desired N,N'-diacetyl-^{14}C-chitobiose with a specific activity of 50 μc/mmole. This material was characterized by its melting point of 260-262°C(7) and its proton magnetic resonance spectrum.

A mixture of α- and β-methyl-N-acetylglucosaminides was prepared by acetylation (Horton, 1966) of glucosamine with acetic anhydride-^{3}H (Volk Radiochemical Company, Lot #1314-31-39), followed by reaction with methanol, as previously described (Zilliken et al., 1955). The specific activity of the glycoside mixture was 1 mc/mmole. The relative concentration of α- to β-methyl-NAG in

this mixture was determined by integration of the methoxyl resonances obtained in a 60 M Hz proton magnetic resonance spectrum of the sample. The ratio of α- to β-methyl-NAG was found to be 1.9 : 1.0.

Methods

Hydrolysis of chitotriose by hen egg-white lysozyme was conducted under the following conditions. The trisaccharide (10^{-2} M) was incubated with the enzyme (3×10^{-3} M) in 0.1 M citrate buffer, pH 5.5 for 1.5 hours at 40°C. Total volume of such incubation mixtures was 250 λ. Following incubation the mixture was subjected to gel-filtration on a column (0.9 x 100 cm) of Bio-Gel-P-2 (200-400 mesh) which was equilibrated with 25% acetic acid. The eluting solvent was 25% acetic acid and fractions of 1 to 2 ml were collected at a flow rate of 30 ml per hour. Reducing sugars in the eluted fractions were determined by analysis of an aliquot (200 λ) by the ferricyanide method for reducing sugars.

Hydrolysis of chitobiose was attempted using a solution which contained the disaccharide (1.2×10^{-1} M) and lysozyme (3×10^{-3} M) in the buffer used for hydrolysis of the trisaccharide. The mixture was incubated at 40°C for 15 hours and then chromatographed on Bio-Gel-P-2 as already described.

Hydrolysis of Chitobiose in the Presence of Methanol

A solution containing 40 mg of lysozyme and 40 mg of N,N'-diacetyl-^{14}C-chitobiose in 800 microliters of 0.1 M citrate buffer, pH 5.5, which was 8M in methanol was incubated for 20 hours at 38°C. A drop of toluene was added to inhibit fungal and bacterial contamination.

After incubation the solution was diluted to 50 ml with water. To this was added 0.4 mg each of α- and β-methyl-NAG, and 5×10^5 dpm of the mixture of α- and β-methyl-NAG-^{3}H. This solution was passed through an Aminex UM-1 ultrafiltration membrane (Amicon Corporation) to remove the enzyme. The filtrate was treated with Amberlite MB-1 ion-exchange resin to remove salts and most of the free sugars (the hemiacetal is a weak acid). The sample was lyophilized, taken up in a minimum of water, streaked on Whatman 3 MM paper as a 7 cm-wide band and developed in descending chromatography for a distance of 60 inches using the upper phase of pyridine, ethyl acetate, water (1 : 2 : 2). Following development, the chromatogram was cut in half lengthwise. The sugars were located on one strip using a modification (W. J. Dreyer, personal communication) of the peptide-bond spray (Mazur et al., 1962). Two-centimeter strips were cut out from the second half of the chromatogram and these were eluted with water. An aliquot of each eluted fraction was removed, and after addition to 15.0 ml of Bray's solution (Bray, 1960), the ratio of ^{3}H to ^{14}C was determined by liquid scintillation in a Packard Model 3324 Tri Carb liquid scintillation spectrometer.

RESULTS AND DISCUSSION

Hydrolysis of Chitobiose

Treatment of the disaccharide with lysozyme for extended time periods (15 hours) resulted in extensive degradation to form the monosaccharide, NAG. The results obtained from product analysis are shown in Fig. 2. It was evident that transglycosylation also occurred in this reaction to form products with molecular weights corresponding to the tri- and tetrasaccharides of N-acetylglucosamine. This result confirmed the suitability of this system for study of the transglucosylation reaction.

Methanol Partitioning during Hydrolysis of Chitobiose by Hen Egg-White Lysozyme

Figure 3 shows the results obtained. Good yields of β-methyl-NAG were achieved. There was a small amount of a compound which chromatographed with an R_f value almost equal to that of α-methyl-NAG, and was found to appear in each of three separate experiments. However, this compound was not α-MeNAG since its peak at fractions 20 and 21 did not coincide with the tritiated α-MeNAG carrier peak. It is possible, however, that this apparent impurity could mask a low level of α-MeNAG. Table 1 shows the $^{3}H/^{14}C$ ratios of the fractions corresponding to the β-methyl-NAG and α-methyl-NAG peaks. These figures allow a comparison of both the constancy of the ratios across each peak and also of the relative amounts of β-methyl-NAG to α-methyl-NAG formed during the transglycosylation. By comparing the $^{3}H/^{14}C$ ratio of the α-MeNAG to that of the β-MeNAG peak, it was

found that the ^{14}C content of the α-methyl-NAG area was 0.3% that of the β-methyl-NAG peak. At least some, if not all, of this ^{14}C was due to the faster-running impurity. These results indicate that the transfer reaction catalyzed by hen egg-white lysozyme forms the β product with a very high level of stereospecificity. Since the glycosidic linkage in N,N'-diacetyl chitobiose is β-(1-4) the reaction therefore involves retention of configuration.

There have been a great variety of mechanisms suggested for the lysozyme catalyzed hydrolysis of glycosidic bonds. The most reasonable of these are summarized in Fig. 4. Rupley and Gates (1967) have shown that lysozyme cleaves the bond between C-1 of one pyranose ring and the glycosidic oxygen joining it to C-4 of an adjacent sugar ring. All the pathways shown can involve catalysis by both acidic and basic residues on the enzyme surface, in agreement with the observed (Rupley and Gates, 1967) bell shaped pH-activity curve for the lysozyme catalyzed reaction. The mechanisms are shown as concerted ones, however some steps could occur as pre-equilibria, such as protonation of the glycosidic oxygen of the sugar residue.

In view of the differences in the predicted stereochemical course of the various mechanisms, the observed retention of configuration in hydrolysis of chitobiose allows rejection of certain of the proposed mechanisms. Thus, the single displacement mechanism (I) may be eliminated. Mechanism IIb in which a carbonium ion intermediate is stereospecifically quenched by solvent to give the α-product may also be eliminated. A small but significant amount of the α-methyl glycoside in addition to the β-linked material formed in relatively large

amounts would have constituted direct proof of the carbonium ion mechanism. However, the relative amount of the α-linked material is at best very small and cannot be proved to be present at all because of the small amount of contaminant near its chromotographic position.

The "double displacement" (Koshland, 1953) mechanism (III) is consistent with the observed retention of configuration. This is also true of a variation of the carbonium ion mechanism suggested by Blake et al. (1965, 1967) in which the attack of solvent on the stabilized carbonium ion gives exclusively the β-product (mechanism IIa). Anchimeric assistance by the acetamido group of the substrate (mechanism IV and V) would also result in retention of the configuration at C-1. This mechanism has been shown to be important in the spontaneous hydrolysis of some aryl glycosides of N-acetylglucosamine by Piszkiewicz and Bruice (1967, 1968) and has been suggested by these authors as well as Lowe (1967) as the mechanism of action for lysozyme.

While the results described here do not distinguish between these three mechanistic possibilities, they do allow some other mechanisms to be eliminated. The quantitative description of the stereochemical course of the lysozyme catalyzed hydrolysis of chitobiose is the first such description for a glycosidase at the one per cent level.

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TABLE 1

Compound	Fract. No.	dpm ratio [$^{3}H/^{14}C$]
β-methyl-NAG	14	0.104
	15	0.107
	16	0.089
α-methyl-NAG	18	63.4
	19	60.8
	20	6.47

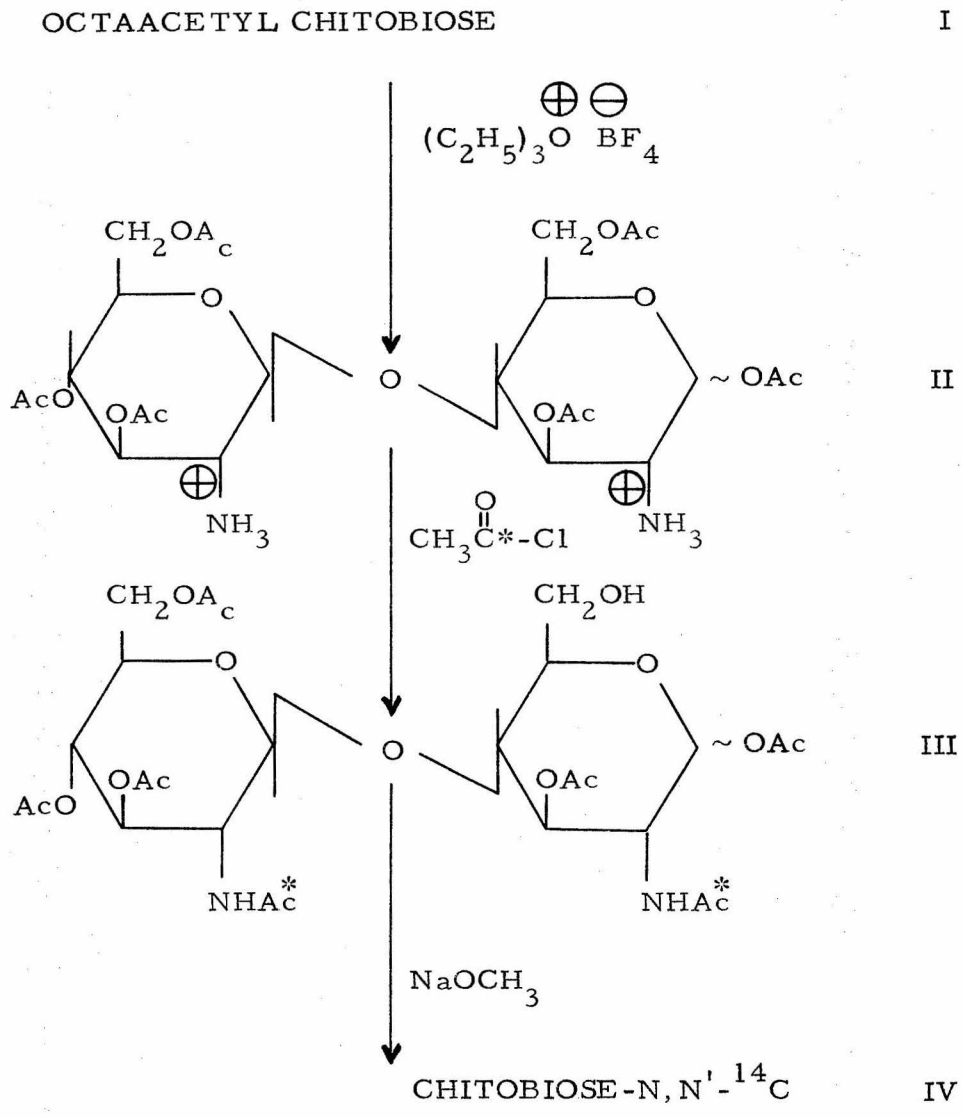


Figure 1. Outline of scheme for the synthesis of chitobiose-N,N'-^{14}C from octaacetyl chitobiose.

Figure 2. Gel-filtration patterns obtained from mixtures of chitobiose and lysozyme. Numerals I to IV represent NAG, chitobiose, chitotriose, and chitotetraose.

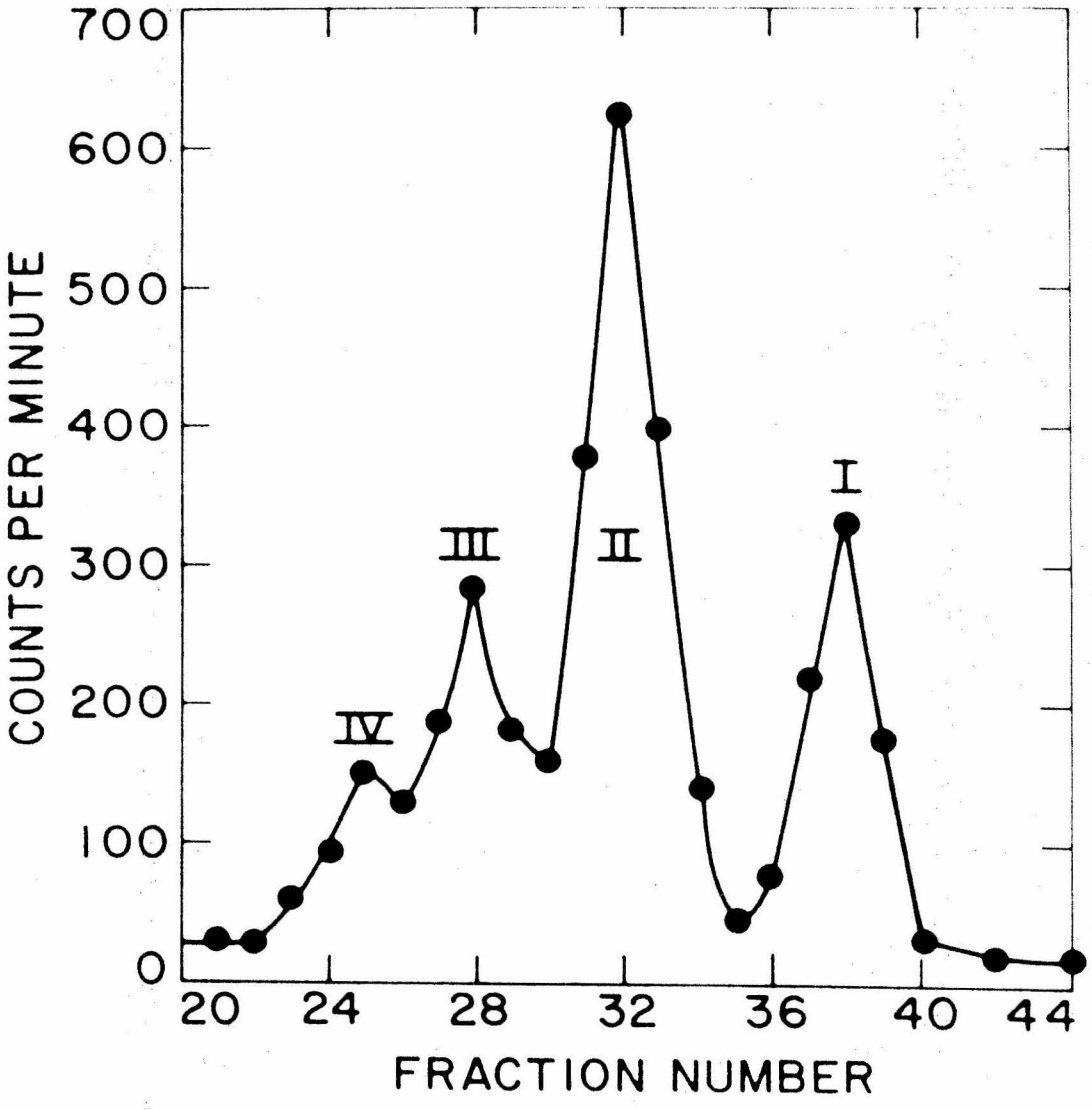
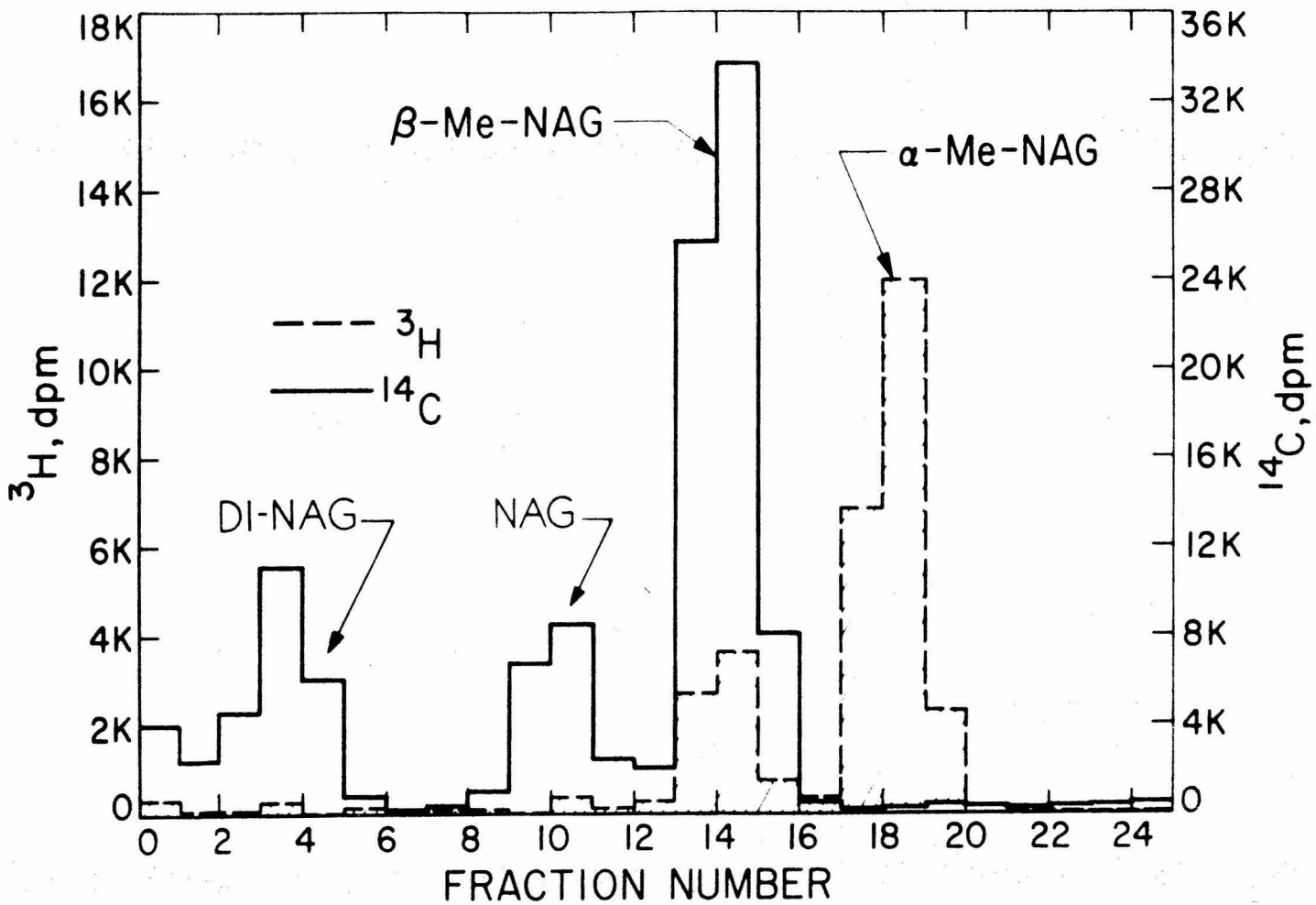

700
600
500
400
300
200
100
0
COUNTS PER MINUTE
20
24
28
32
36
40
44
FRACTION NUMBER
I
II
III
IV

Figure 3. Nomogram of radioactivity measurements of fractions eluted from paper chromatogram employed to separate products of lysozyme reaction of chitobiose. Solid lines (——) denote ^{14}C disintegrations of residual chitobiose-N,N'-^{14}C and of product of hydrolysis and transglycosylation catalyzed by lysozyme. Dashed lines (---) (enclosing shaded area) denote ^{3}H disintegrations of added methyl-β-NAG and methyl-α-NAG.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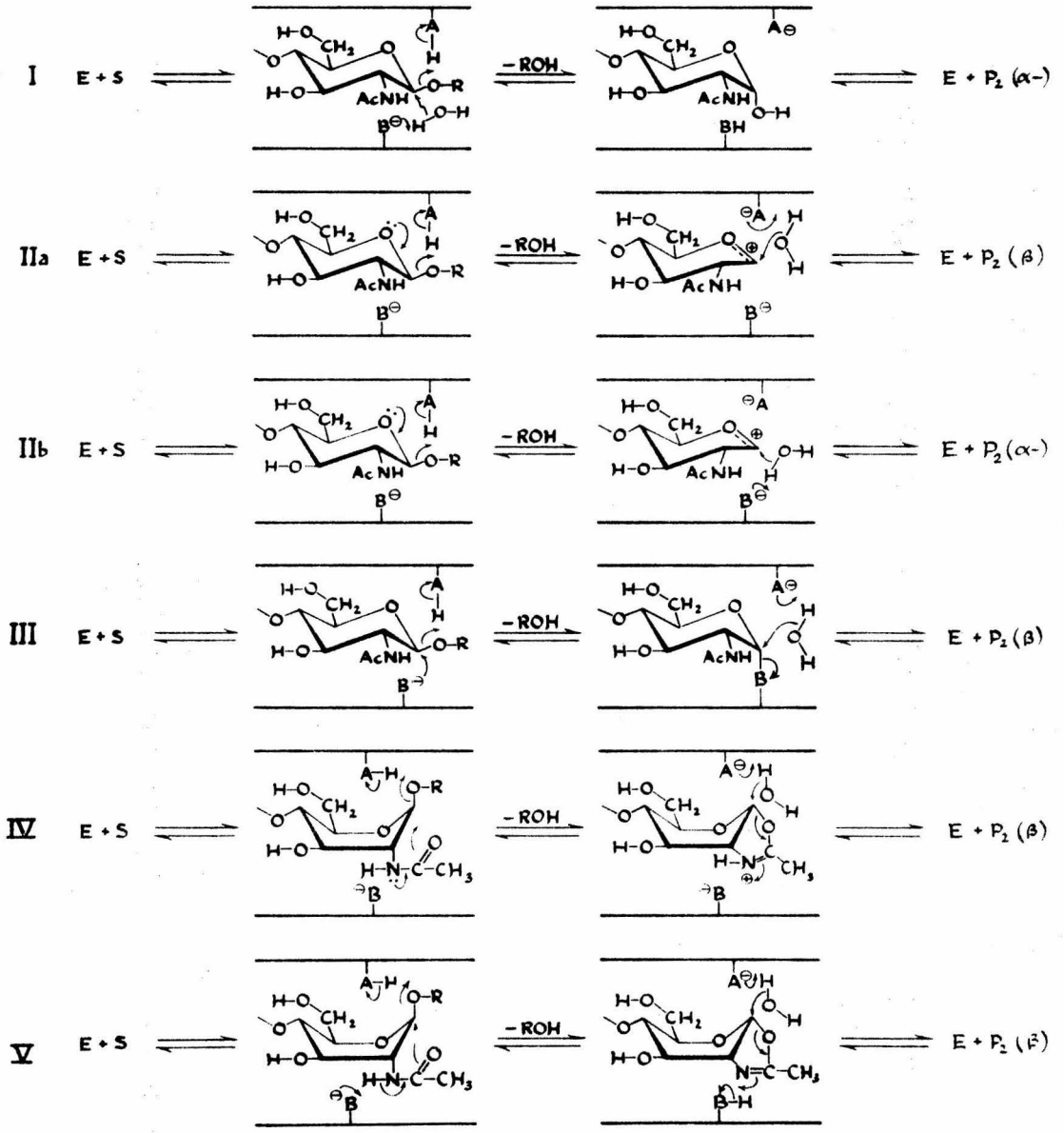
12K
10K
8K
6K
4K
2K
0
36K
32K
28K
24K
20K
16K
12K
8K
4K
0
β-Me-NAG
α-Me-NAG
3H
14C
DI-NAG
NAG
3H, dpm
14C, dpm
0 2 4 6 8 10 12 14 16 18 20 22 24
FRACTION NUMBER

Figure 4. Depiction of mechanistic pathways possible for substrate during lysozyme catalyzed cleavage of glycosidic bonds. AH and B^{O} denote an acid and a base respectively which are situated at the catalytic site of the enzyme. Further explanations are given in the text.


I
E + S
-ROH
E + P2 (α-)
IIa
E + S
-ROH
E + P2 (β)
IIb
E + S
-ROH
E + P2 (α-)
III
E + S
-ROH
E + P2 (β)
IV
E + S
-ROH
E + P2 (β)
V
E + S
-ROH
E + P2 (β)

CHAPTER VIII

ON DISTINGUISHING POSSIBLE MECHANISTIC PATHS FOR LYSOZYME BY THE USE OF α-DEUTERIUM KINETIC ISOTOPE EFFECT

INTRODUCTION

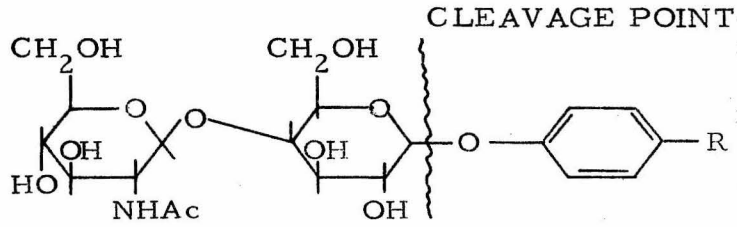
The X-ray crystallographic analysis studies of Blake et al., (1965, 1967) on the binding of various inhibitors and substrates to lysozyme has led to a theory of the mechanism of catalysis by the enzyme. This involves catalytic production of a carbonium ion which is given steric and electrostatic stabilization by the enzyme. We have shown in the previous section that the lysozyme catalyzed hydrolysis of chitobiose procedes with retention of configuration to at least 99.7%. If the carbonium ion mechanism is important for lysozyme, the stereochemistry of the reaction must be controlled by the accessability of solvent to only one side of the enzyme bound carbonium ion.

Since the lysozyme catalyzed hydrolysis of glycosidic bonds procedes with retention of configuration, the double displacement mechanism of Koshland (1953) is also a likely candidate for lysozyme. This involves displacement of the aglycon by some nucleophile on the enzyme followed by displacement of the nucleophilic group by solvent. The work of Legler (1968) on β-glucosidase from several sources suggests that this mechanism may be important for that enzyme.

A variation to the displacement mechanism has been shown to

be important in model studies by Piszkiewicz and Bruice (1967, 1968) involving the spontaneous hydrolysis of aryl glycosides of N-acetyl-glucosamine. This mechanism involves nucleophilic displacement of the aglycone by the acetamido carbonyl group of the sugar. This mechanism has been suggested for lysozyme by the authors as well as Lowe (1967).

Recently Raftery and Rand-Meir (1968) have shown that lysozyme will catalyse the hydrolysis of aryl-4-0-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-β-D-glucopyranosides (I). The catalytic rate constants are of the same order of magnitude as those for the corresponding aryl-β-chitobiosides, but a direct comparison is made difficult because of the many non-productive binding modes of the substrates



(I)

which may or may not compete with the productive mode for substrate molecules (Rand-Meir et al., 1968). This suggests that while lysozyme can act without participation by the acetamido group of the substrate, the acetamido group may assist the hydrolysis when it is present.

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

participation of solvent in the rate determining steps of many solvolytic reactions (see Halevi, 1963 for a review). A typical S_N2 reaction shows an effect near unity while S_N1 reactions display an isotope effect yielding k_H/k_D of about 1.14. The explanation of this decrease in rate is that substitution of deuterium for the α-hydrogen results in a loss in the HCX bending mode which accompanies the breaking of the C-X bond. In the case of nucleophilic attack, some of this effect is compensated for as the neq carbon-nucleophile bond is being formed.

This work describes the application of α-secondary kinetic isotope studies to the mechanism of the lysozyme catalyzed hydrolysis of phenyl-4-0-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-β-D-glucopyranoside (NGP). This reaction was chosen because the mechanism cannot involve anchimeric assistance by an acetamido group of the substrate. Thus any evidence of nucleophilic attack should be associated with nucleophiles at the enzyme surface or groups other than an acetamido group of the substrate. The β-glucosidase catalyzed hydrolysis of phenyl-β-D-glucopyranoside (GP) has been studied since there is some evidence (Legler, 1968) for nucleophilic attack in its mode of action. The acid catalyzed hydrolysis has been studied as a model carbonium ion reaction (see Cordes, 1967 for a recent review of the mechanism of acetal hydrolysis). The base catalyzed hydrolysis of GP has been studied as a model S_N2 reaction.

EXPERIMENTAL

All pH measurements were performed on a Radiometer pH Meter 26. The ultraviolet spectrophotometric measurements were carried out on a Gilford 240 spectrophotometer. Proton magnetic resonance spectra were obtained with a Varian HR 220 NMR spectrometer. Radioactivity measurements were made with a Packard Tri-Carb Liquid Scintillation Spectrometer Model 3324.

Phenyl-^{14}C-β-D-glucopyranoside-d_1

The synthesis of the unlabelled compound has been described by Lemieux (1963). For the labelled compound a flask containing 8 ml of acetic anhydride was cooled to 0°C and 50 ul of 60% perchloric acid was added. The flask was warmed to room temperature and 2.0 gm of glucose-d_1 (Merck, Sharp, and Dohme of Canada, Ltd., Lot 1318) was added such that the temperature remained between 30 and 40°C. The mixture was cooled to 20° and 0.6 gm of red phosphorous, 3.6 gm of bromine and 0.75 ml of water were added. After 2 hours at room temperature, 8 ml of chloroform were added and the resulting solution was filtered through glass wool, washed twice with cold water, once with saturated sodium bicarbonate, and was stirred 10 minutes with dry salicylic acid. The chloroform was evaporated and the residue was triturated with 10 ml of ether-petroleum ether 2:1. The solid was filtered and dried to give 2.7 gm of the crude acetobromoglucose-d_1 which was used without further purification.

A solution containing 0.95 gm phenol and 0.5 mc of phenol-^{14}C (Nuclear Chicago, Code CFA 125 batch 25, specific activity

25 mc/mmole) and 0.5 gm sodium hydroxide in 12.5 ml of water was added to a solution of 2.7 gm of acetobromoglucose-d_1 in 19 ml of acetone. After 4 hours at room temperature the solution was placed in the refrigerator and left overnight. The acetone was evaporated under reduced pressure and the water solution extracted twice with an equal volume of chloroform. The chloroform layer was washed with water, dilute sodium hydroxide, twice more with water and was dried over anhydrous magnesium sulfate. The chloroform solution was evaporated under reduced pressure and 12 ml of ethanol was added and the solution was stored for one day at -10°C. The resulting crystals were filtered, washed with cold ethanol and dried under vacuum to give 483 mg of tetra-O-acetyl-β-phenylglucoside. This material was suspended in 27 ml of absolute methanol and 0.55 ml of 0.4 N barium methoxide was added. The resulting solution was kept at 0°C for 24 hours. The solution was evaporated to a small volume and 250 mg of the desired phenyl-^{14}C-β-D-glucopyranoside-d_1 (mp 170°C) were obtained. This material was chromatographed on Spehadex LH-20 in methanol before use in kinetic runs.

Phenyl-^{3}H-D-glucopyranoside

The synthesis was carried out on the same scale as for the deuterated and ^{14}C labelled compound. In this case glucose was used and the final specific activity of the sodium phenoxide -^{3}H was 0.5 mc/mmole. The yield was 270 mg which melted at 171-2°C. This material was also chromatographed on Sephadex LH-20 in methanol before use in kinetic runs. A mixture of the ^{14}C and ^{3}H material on

this chromatographic system gave a single peak with constant $^{3}H/^{14}C$ ratio throughout the peak.

Phenyl-4-0-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-β-D-glucopyranoside

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

The acid catalyzed hydrolysis of GP was carried out at 50°C in 2.00 N hydrochloric acid (P-H Tamm Laboratories, Sweden). The

concentration of GP in the solutions ranged from 5×10^{-3} M to 2×10^{-2} M. One milliliter samples were removed, neutralized by addition of 1.00 ml of 2.00 N sodium hydroxide (P-H Tamm Laboratories, Sweden) and brought to pH 11.00 with 8.0 ml of 0.1 M dimethyl amine hydrochloride buffer pH 11.00. The absorbance at 290 mu of this solution was then determined. After about one half life at 50°C, the temperature was raised to 70°C for 24 hours in order to obtain the infinity reading for the reaction. The data were then analyzed by plotting $\log \frac{A_\infty - A}{A_\infty}$ vs. time. The slope of the line was determined by least square methods.

The aqueous base catalyzed reaction was carried out at 70°C in 2.00 N sodium hydroxide. Again the concentration of glycoside was near 10^{-2} M for all the reactions studied. In this case samples were removed, cooled to 25°C and the absorbance at 290 mu was determined directly. The total concentration of the glycoside at zero time was determined by reading the absorbance at 265 mu. Because of the slow rates observed under these conditions, only initial rates were determined. It was very difficult to obtain reproducible data (to less than 0%) in this system.

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

The almond beta glucosidase (Worthington Biochemical Co.) catalyzed hydrolysis was carried out at pH 5.0 in 0.1 M acetate buffer and 25°C. The concentration of enzyme was 1 mg/ml. Aliquots of 0.1 ml were removed and added to 1.0 ml of 0.2 N sodium hydroxide solution, and the absorbance at 290 mu was determined using a 1.0 ml cuvette with a ten centimeter path length (Scientific Cell Co., N.Y.).

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

Isotope Effect Measurements

In general, the reaction was allowed to proceed to 2-5% completion and adjusted with 2 N sodium hydroxide or 2 HCl and 0.1 M citrate buffer pH 5.5 to pH 5-6. The mixture was then extracted with 1.0 ml of ether twice. This removed all the phenol produced by the reaction. The etheral solution was then washed with an equal volume of the pH 5.5 citrate buffer, an equal volume of saturated sodium chloride solution and the etheral solution was then passed over a small column of anhydrous sodium sulfate in a pasteur pipette. One milliliter of this dried etheral solution was mixed in a polyethglene vial with 15 ml of Brays solution (Packard Instrument Co.), the relative $^{3}H/^{14}C$ ratio of the product was determined and compared to that of the starting

material. A very small aliquot of the starting material (5-10 ul) was removed from its stock solution and was mixed with 1.0 ml of dry ether and 15 ml of Bray's (1960) solution in order to determine its relative $^{3}H/^{14}C$ ratio.

RESULTS

The degree of deuterium substitution at C-1 of the GP with deuterium and ^{14}C label was determined by n. m. r. The spectra and integrals which were obtained for the unlabelled and labelled compounds at 220 M Hz in deuterated dimethylsulfoxide are shown in Fig. 1. These data show that the fraction of deuterium substitution in the sample was 0.65 ± 0.05 at the C-1 position. An assignment of the spectrum aside from C-5 (the triplet occurring furthest upfield) and C-1 was not attempted.

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

Another method for measuring this isotope effect depends on the fact that the initial rate of release of products of the deuterated compound will be somewhat slower than that of the unsubstituted compound (see Collins, 1964 for a review). To exploit this phenomenon,

the deuterated material was synthesized with ^{14}C labelled phenol while the protiated material had ^{3}H in its phenyl moiety. For an acid catalyzed hydrolysis of a mixture of the deuterated and undeuterated material, the $^{3}H/^{14}C$ ratio of the phenol isolated after a small amount of reaction should be greater than the $^{3}H/^{14}C$ ratio of the starting material. If the reaction is allowed to proceed to the extent of two to three percent or less, the observed isotope effect $k_H/k_D)_{obs}$ is given by $S_0^* P/S_0 P^*$ (see eq. 4), where S_0^*/S_0 refers to the $^{3}H/^{14}C$ ratio of the starting material and P/P^* refers to the $^{14}C/^{3}H$ ratio of the products. The observed isotope effect must then be corrected for the deuterium content of the substrate by eq. 6 in the Appendix. The data for a typical experiment for acid catalyzed reaction are shown in Table 1. The conditions were 2 N HCl at 50°C for 15 minutes which corresponds to about 3% of reaction. Four similar experiments gave $k_H/k_D)_{obs}$ = 1.080 ±0.004. To test for small amounts of impurities, the acid catalyzed reaction was allowed to proceed for one half life, and the starting material was reisolated. Using the GP obtained in this way gave $k_H/k_D)_{obs}$ of 1.078. This suggests that impurities are not releasing phenol in any amount, or at a rate which seriously affects the desired answer. It should be noted that phenol is known to exchange its ring protons under strongly acidic conditions. In fact, any attempts to obtain an infinity $^{3}H/^{14}C$ ratio of the phenol released, were hampered by this phenomenon. A control experiment, in which a mixture of ^{3}H and ^{14}C labelled phenol was treated at 50°C in 2 N HCl for 30 minutes, showed no change in the $^{3}H/^{14}C$ ratio of the phenol after the treatment.

The determination of the isotope effect for the base catalyzed reaction was somewhat more difficult than for the acid catalyzed reaction. It was impossible to obtain rate data using small amounts of material for the aqueous base catalyzed reaction which was reproducible to more than ±10%. For this reason, determination of the isotope effect by absolute rates was not attempted. At 70°C in 2 N NaOH for 90 minutes, the observed isotope effect as determined by the enrichment method was 1.056 ± 0.004. The reaction was also carried out in 3 N methoxide. Treatment of GP with 3.0 N sodium methoxide in anhydrous methanol at 70°C for one hour and subsequent isolation of the product gave the data summarized in Table 1. Four similar determinations gave an observed isotope effect of 1.021 ± 0.002. Up to this point, the quoted reproducibility errors have also been the expected statistical errors associated with the counts accumulated. In the case of the methoxide catalyzed reactions, somewhat more material was used and counting was carried out to four times the accumulated counts as in the other experiments. This resulted in the lowered reproducibility error quoted. Unfortunately, there was not sufficient material to do this for each measurement in the other systems.

The β-glucosidase catalyzed hydrolysis of GP had only a very small observed isotope effect of 1.01 ± 0.004. In order to determine if the measured effect was real and to further test the purity of the GP, the entire time course of the observed isotope effect was followed and the results of this study are shown in Fig. 2. The solid line is the theoretical curve obtained by applying equation 3 of the Appendix with an initial observed isotope effect of 1.010. The data fit this theoretical

curve reasonably well. At 95% reaction, an aliquot was removed from the reaction mixture and the residual GP was reisolated. The $^{3}H/^{14}C$ ratio of this material was compared to that of the starting material before reaction. The amount of ^{14}C in the reisolated GP was found to have increased by a factor of 1.018. Application of eq. 2 in the Appendix gives a theoretical value for the increase of 1.020 for an initial observed isotope effect of 1.010.

The substrate for the lysozyme catalyzed reaction was made by treatment of chitobiose with lysozyme in the presence of GP. The resulting NGP was isolated by chromatograph on Bio-Gel P-2 and that chromatogram is shown in Fig. 3. The peak corresponding to fractions 115-130 are pooled. Raftery and Rand-Meir (unpublished, 1968) have shown by degradative procedures that the transferase activity of lysozyme forms only β (1-4) linkages when aryl glucosides are used as acceptors.

Treatment of NGP with lysozyme at pH 5.5 in 0.1 M citrate buffer for 12 hours at 40°C resulted in a two percent release of phenol. The radioactivity measurements for this reaction are summarized in Table 1. Three other measurements under these conditions gave an observed isotope effect of 1.068 ± 0.004. The isotope effect was also determined at pH 3.1 and 8.3. These reactions required 4 and 8 days respectively to proceed 3% and both gave the same observed isotope effect as the pH 5.5 reactions.

The isotope effect measurements are summarized in Table 2. The observed isotope effect measurements have been corrected to the real values by use of eq. 6 in the Appendix. The error limits of the corrected isotope effects are estimated to be ± 0.01.

DISCUSSION

The acid catalyzed hydrolysis of glycosides is thought to proceed primarily through an A1, carbonium ion reaction (Cordes, 1967; Vernon, 1967 for recent reviews). There is very little evidence for an A2 mechanism (protonation, followed by nucleophilic attack) in any hydrolyses of glucosides in aqueous solution. The α-deuterium isotope effect of 1.13 for the acid catalyzed hydrolysis of GP agrees with the accepted value of around 1.14 for typical S_N1 reactions (Halevi, 1963 for a critical review of secondary isotope effects). Jones and Thornton (1967) found k_H/k_D of 1.24 ± 0.08 per deuterium atom for the solvolysis of methyl chloromethyl-d_2 ether as compared to the undeuteratee compound. The proposed mechanism for hydrolysis of this compound is quite similar to that proposed for glucoside hydrolysis and the α-deuterium isotope effect would be expected to have the same value. The rather large error in the data of Jones and Thornton makes a quantitative comparison impossible but the effects are at least qualitatively the same. Thus it appears that the acid catalyzed hydrolysis of GP does involve a carbonium ion intermediate and the deuterium isotope effect for the reaction is a good model value for a carbonium ion intermediate in the enzymatic hydrolysis of glucosides.

Studies (Ballou, 1954; Whistler and BeMiller, 1958) on the reaction of aryl glucosides in basic solution have shown that compounds with their C-2 hydroxyl group trans to the aglycone react much faster than the ones with a cis orientation. It appears that the trans compounds react by a mechanism involving participation of the C-2 oxyanion. This scheme is summarized in Fig. 5. More recently,

Gasman and Johnson (1966) have shown that cleavage of nitrophenyl-2-O-methyl-β-glucosides are much slower than that of the corresponding unmethylated compounds. The methylated compounds appeared to proceed by a mechanism involving bimolecular nucleophilic aromatic substitution, while the unmethylated compounds involved the C-2 oxyanion pathway. The α-deuterium isotope effect for the aqueous base catalyzed hydrolysis of GP of 1.09 suggest a mechanism involving considerable carbonium ion character rather than pure nucleophilic attack by the C-2 oxyanion. McClosky and Coleman (1945) and Gasman and Johnson (1966) have found that the yield of 1,6-anhydro-β-D-glucopyranose in the basic hydrolysis of aryl-β-glucosides decreases as the electron withdrawing character of the aglycone is increased. This is consistent with the supposition that the basic hydrolysis of GP involves partial carbonium ion character and that the extent of this is a function of the leaving group. Such a conclusion is similar to that of Shiner et al. (1968) in which a trend toward more carbonium ion character (as measured by α- and β-deuterium isotope effects) was seen in the solvolysis of meta and para substituted 1-phenylethyl halides, as the electron withdrawing character of the substituents was decreased.

If the mechanism of the aqueous base catalyzed hydrolysis of GP is indeed intermediate between S_N1 and nucleophilic attack, then a decrease in the "ionizing power" of the solvent should decrease the amount of carbonium ion character in the transition state. The solvent which was chosen was methanol since it is of similar acidity to water and some work has been done on the methoxide catalyzed cleavage of aryl glycosides (Gasman and Johnson, 1966). The deuterium isotope

effect measured in 3 N sodium methoxide in methanol gave k_H/k_D in 1.03. This result suggests that there indeed has been a decrease in the carbonium ion character of the transition state, and that nucleophilic attack is now the predominant mechanism. This system should give a good model value for the isotope effect of an enzyme mechanism involving nucleophilic attack.

It should be mentioned that enzymes may activate the substrate through distortion. It is possible to conceive a mechanism to give sort which might have rather unusual vibration and rotational modes in the transition state. Since the isotope effect measurements are really probes of the vibrational nature of the transition state it is possible that causes other than bond forming and breaking are responsible for isotope effects in these cases.

The isotope effect for the β-glucosidase catalyzed hydrolysis of GP was found to be $k_H/k_D = 1.015$. A value of this magnitude strongly suggests a mechanism involving nucleophilic attack. The possible sources of the nucleophile are the enzyme, solvent, or possibly an hydroxyl group of the substrate itself. Legler (1968) has recently shown that the substrate analogue conduritol B epoxide (3,5/4,6-cyclohexenetetrol oxide) inactivates glucosidases from many sources. The inactivation involves the formation of a covalent bond with what appears to be a carboxyl group on the enzyme surface. Treatment of the inactivated enzyme with hydroxylamine releases one molecule of (+)-inositol per molecule of enzyme. The stereochemistry of the product suggests a trans ring opening of the epoxide by a carboxylate anion. Thus a carboxyl group on the enzyme is the most likely

candidate for the attacking nucleophile in the hydrolysis of GP by β-glucosidase. The mechanism of this enzyme would seem to be a classic example of the displacement mechanism suggested by Koshland (1953).

Some care must be taken in the interpretation of such a small isotope effect since factors other than nucleophilic attack could produce the observed small effect. It is possible a pre-rate determining equilibrium could occur which involves the isotopically substituted center. The secondary isotope effect for such a process would be expected to be very small. An example of this for the β-glucosidase case is the formation of an enzyme glucosyl intermediate which reacts with the phenol in solution to reform starting material much more often than it reacts with water to form products. This can be ruled out since the residual GP isolated after 95% reaction had a different $^{3}H/^{14}C$ ratio than the phenol isolated at that point. The difference was a small but measureable factor of 1.016. It is also possible that the rate determining step precedes the reaction at the isotopically substituted center, and that the observed isotope effect is a reflection of the difference in the binding characteristics of the deuterated-^{14}C material relative to the ^{3}H material. Such a possibility is unlikely but cannot be ruled out.

From the results of the base catalyzed and β-glucosidase catalyzed hydrolysis of GP one would expect a displacement reaction in the case of the lysozyme catalyzed hydrolysis of NGP to have a very small α-deuterium isotope effect. In fact, the isotope effect was 1.11, a large effect. This suggests that the enzyme mechanism proceeds through an intermediate with considerable carbonium ion character. It

is interesting to note that the isotope effect did not change over the pH range 3.1 to 8.3 and thus no change in mechanism occurs over this range of pH, which includes nearly all those pH's at which the enzyme is even slightly active.

This evidence for carbonium ion character would seem to rule out both the single and double displacement mechanisms which involve either attack by solvent or by nucleophiles of the enzyme itself. The evidence is consistent with the carbonium ion mechanism proposed by Blake et al. (1967) from model building studies. It is possible, however, that for natural substrates an acetamido group may provide anchimeric assistance as proposed by Piszkiewicz and Bruice (1967, 1968) and Lowe (1967), since that group is not adjacent to the reactive center in the case of NGP.

Studies on the relative rates of hydrolysis of p-nitrophenyl-2-deoxy-4-0-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-β-D-glucopyranoside and NGP by lysozyme (Rand-Mier and Raftery, 1968) have shown that the deoxy compound displays a rate constant about 16 times greater than NGP. However, the acid catalyzed rate of hydrolysis of p-nitrophenyl-2-deoxy-β-D-glucopyranoside is about 10^3 times greater than the rate of GP under the same conditions. This result suggests that some of the factors which are responsible for the increase in rate for the acid catalyzed hydrolysis of the deoxy compound are also important for the enzyme catalyzed reaction. It is very difficult however to discover just what these factors are. There are problems associated with these and most other model studies arising from binding orientations near catalytic groups, conformational changes in the substrate,

steric interactions with the active site, etc. which accompany structural changes in the substrate.

The use of isotopically substituted substrates provides a means of circumventing these problems, since they would be expected to bind with identical orientations to the active site. Further, there is a wealth of information concerning the magnitude of isotope effects for various reactions and the relation of the isotope effect to the reaction mechanism. While not all enzymatic reactions lend themselves to study by these methods, the use of secondary isotope effects should provide a useful tool for describing some enzyme mechanisms.

APPENDIX

I. Enrichment in Starting Material and Products

A. Simple First Order Reactions

For a reaction of the type

$$A \xrightarrow{k} B \qquad A^* \xrightarrow{\Delta k} B^* \qquad \text{where } \Delta = \frac{k_D}{k_H}$$

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

and

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

* refers to deuterated material,

or

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

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

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

when $\frac{B^*}{A_0^*}$ and $\frac{B}{A_0}$ are $\ll 1$

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

B. Enzymatic Reactions

For a Michelis-Menten scheme

$$E + A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EA \xrightarrow{k_3} E + B$$

and

$$E + A^* \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EA^* \xrightarrow{\Delta k_3} E + B$$

$$dA/dt = - k_3 \frac{E \cdot A}{K_M}$$

$$dA^*/dt = - \Delta k_3 \frac{E \cdot A^*}{K_M}$$

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

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

II. Correction for Deuterium Composition

From eq. 1

$$A_0 - B = A_0 e^{-kt} \qquad \text{and} \qquad A_0^* - B^* = A_0^* e^{-\Delta kt}$$

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

$$x = \text{fraction deuterated} = \frac{A_0^*}{A_0 + A_0^*}$$

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

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

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

then

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

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

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

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

or

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

III. Equations for Converting Raw Counting Data to Observed Isotope Effect

If a = counts/minute in Channel 1

b = counts/minute in Channel 2

E = disintegrations/minute ^3H

F = disintegrations/minute ^{14}C
c_1 = ^{3}H efficiency in Channel 1
d_1 = ^{14}C efficiency in Channel 2
c_2 = ^{3}H efficiency in Channel 4
d_2 = ^{14}C efficiency in Channel 2

then

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

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

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

from eq. 4 $\frac{B}{B^*}\frac{A_0^*}{A_0} = \left(\frac{k_H}{k_D}\right)_{obs}$.

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TABLE 1.--Examples of counting data for determination of the observed isotope effect

	Counts in Channel 1	Counts in Channel 2	Time (min)	Relative $^3H/^{14}C$	$\frac{k_H}{k_D}$ obs
2.0 N HCl, 50°					
GP	500,000	135,079	12.9	3.639	
phenol	256,210	65,705	100.0	3.930	1.080
2.0 N N OH, 70°					
GP	462,451	123,057	20.0	3.7073	
phenol	268,588	68,181	20.0	3.9234	1.058
3.0 N Na CH_3, 70°					
GP	2×10^6	539,482	57.9	3.752	
phenol	2×10^6	238,993	121.6	3.712	1.020
β-glucosidase					
GP	657,990	175,850	20.0	3.686	
phenol	900,000	238,993	80.0	3.724	1.010
Lysozyme					
NGP	500,000	134,488	18.1	3.659	
phenol	500,000	127,610	81.7	3.914	1.070

Background Channel 1: 8 counts/minute
Background Channel 2: 12 counts/minute

Relative efficiency ^3H in Channel 2 compared to Channel 1: 0.020
Relative efficiency ^{14}C in Channel 1 compared to Channel 2: 0.323

Note: The absolute efficiency is quite sensitive to the window settings and it is difficult to reset the window exactly. However, the relative efficiency does not change appreciably. This explains the apparent lack of constancy of the GP ratios.

TABLE 2. --Summary of observed isotope effects

	$\left(\frac{k_H}{k_D}\right)_{obs}$	$\frac{k_H}{k_D}$
2 N Hcl, 50°	1.080 ± 0.004	1.13
2 N NaOH, 70°	1.056 ± 0.004	1.09
3 N $NaOCH_3$ in mesh 70°	1.021 ± 0.002	1.03
β-glucosidase	1.010 ± 0.004	1.015
Lysozyme pH 5.5	1.068 ± 0.004	1.11
pH 3.1	1.070	1.11
pH 8.3	1.068	1.11

Figure 1. The p. m. r. spectra of phenyl-β-D-glucopyranoside-d_1 (GP-d_1) and phenyl-β-D-glucopyranosice (GP) at 220 MHz. The spectra are of the region 3. 5 to 5 p. p. m. downfield of tetramethylsilane. Spectrum A is GP-d_1. Spectrum B is GP.

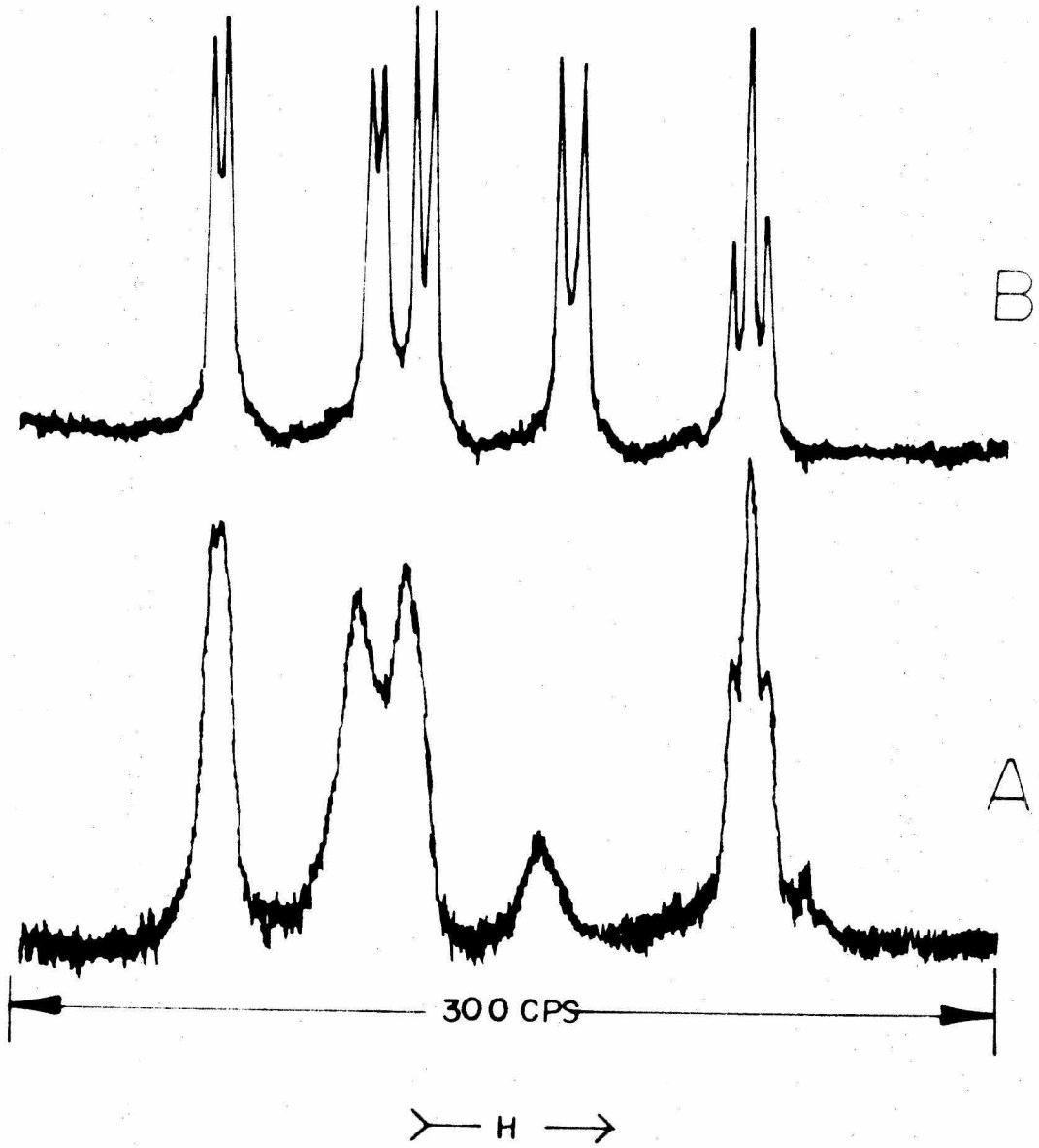

B
A
300 CPS
H

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

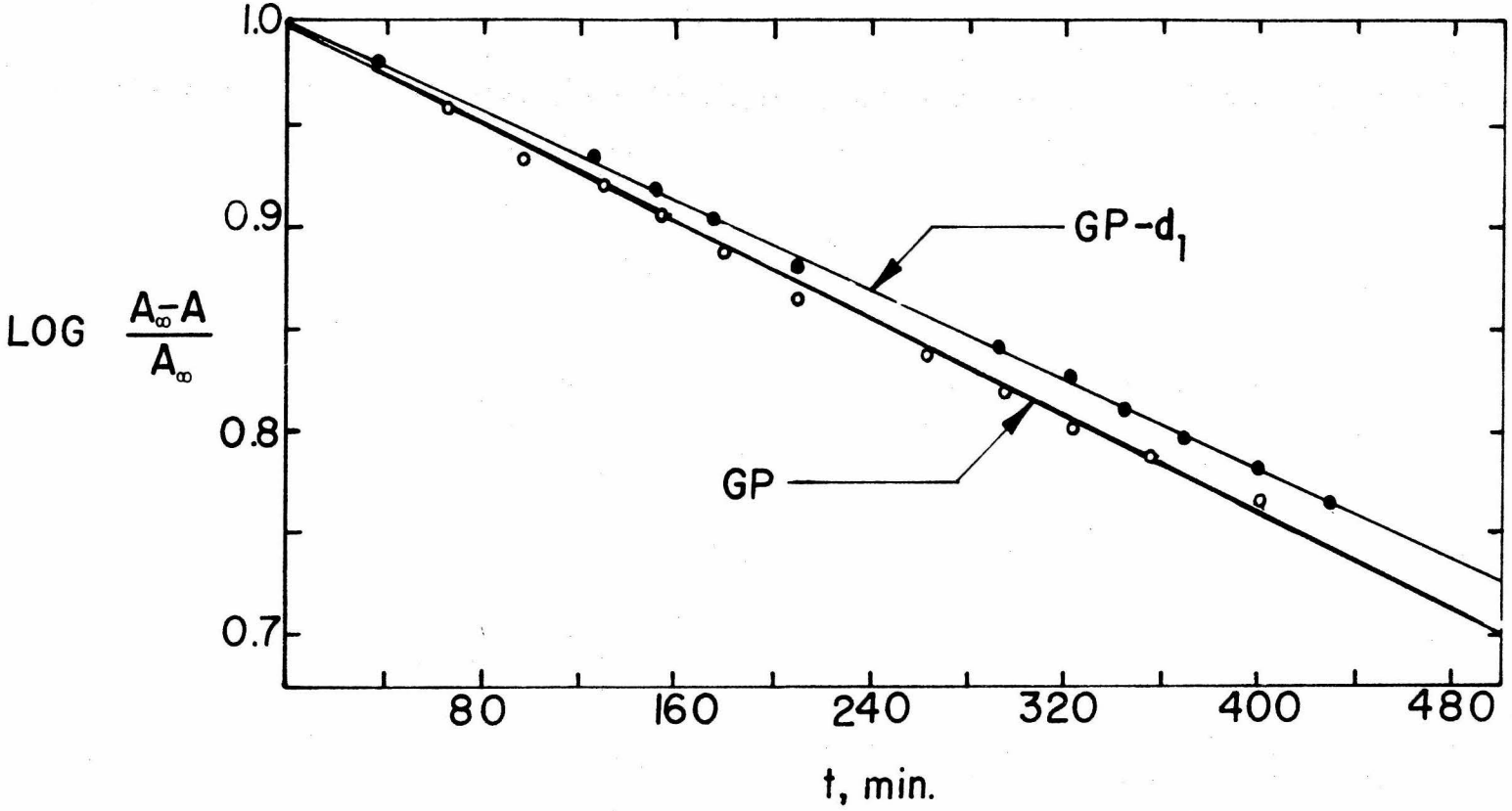

1.0
0.9
0.8
0.7
LOG $\frac{A_\infty - A}{A_\infty}$
GP-d_1
GP
80
160
240
320
400
480
t, min.

Figure 3. Chromatography on Bio-Gel P-2 of the reaction mixture for the synthesis of NGP utilizing the transferase activity of lysozyme. The various peaks were identified as phenol (P), phenyl-β-glucoside (GP), NGP, and the β(1-4) linked trisaccharide NAG-NAG-glucose-β-phenyl glucoside (NNGP).

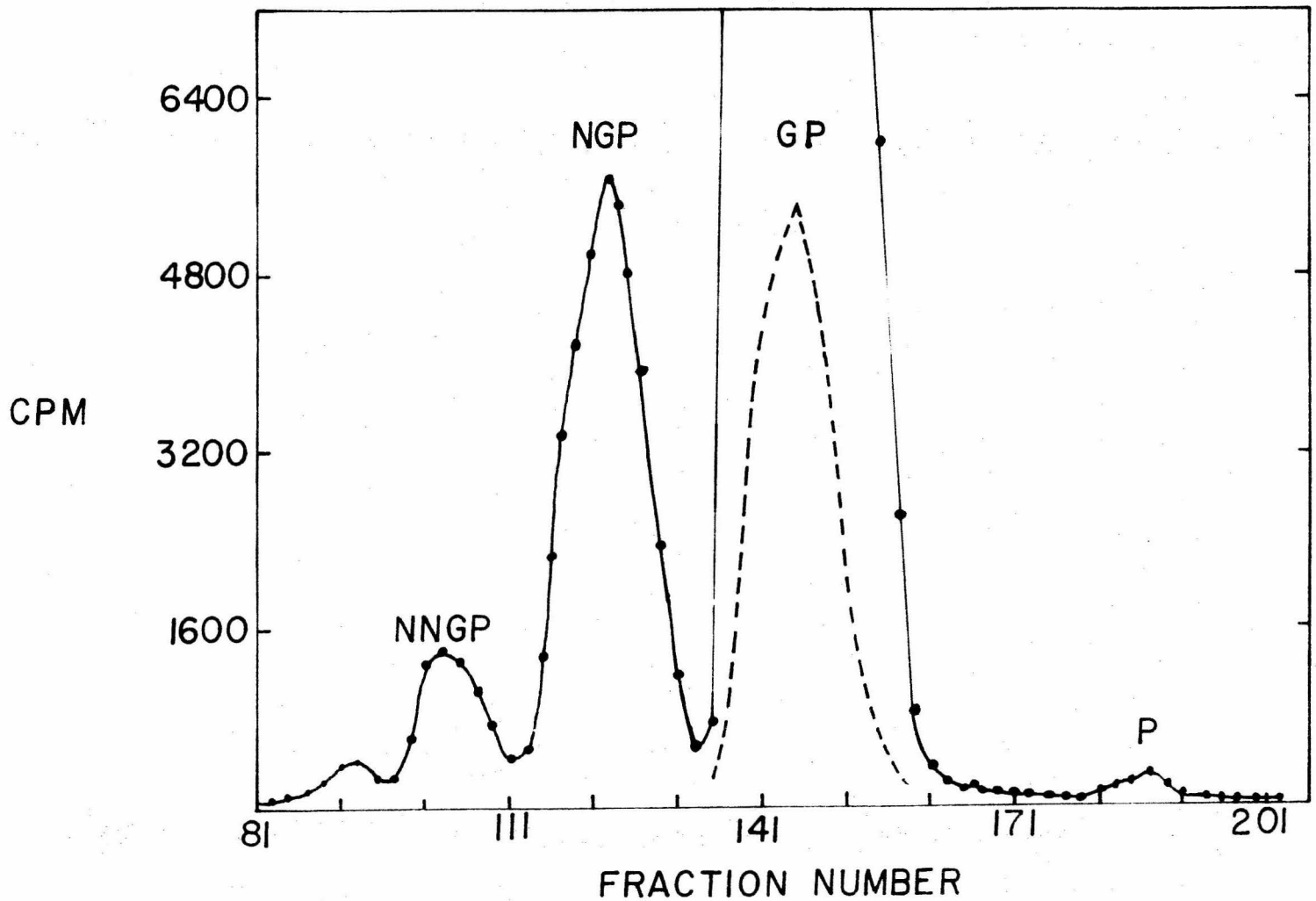
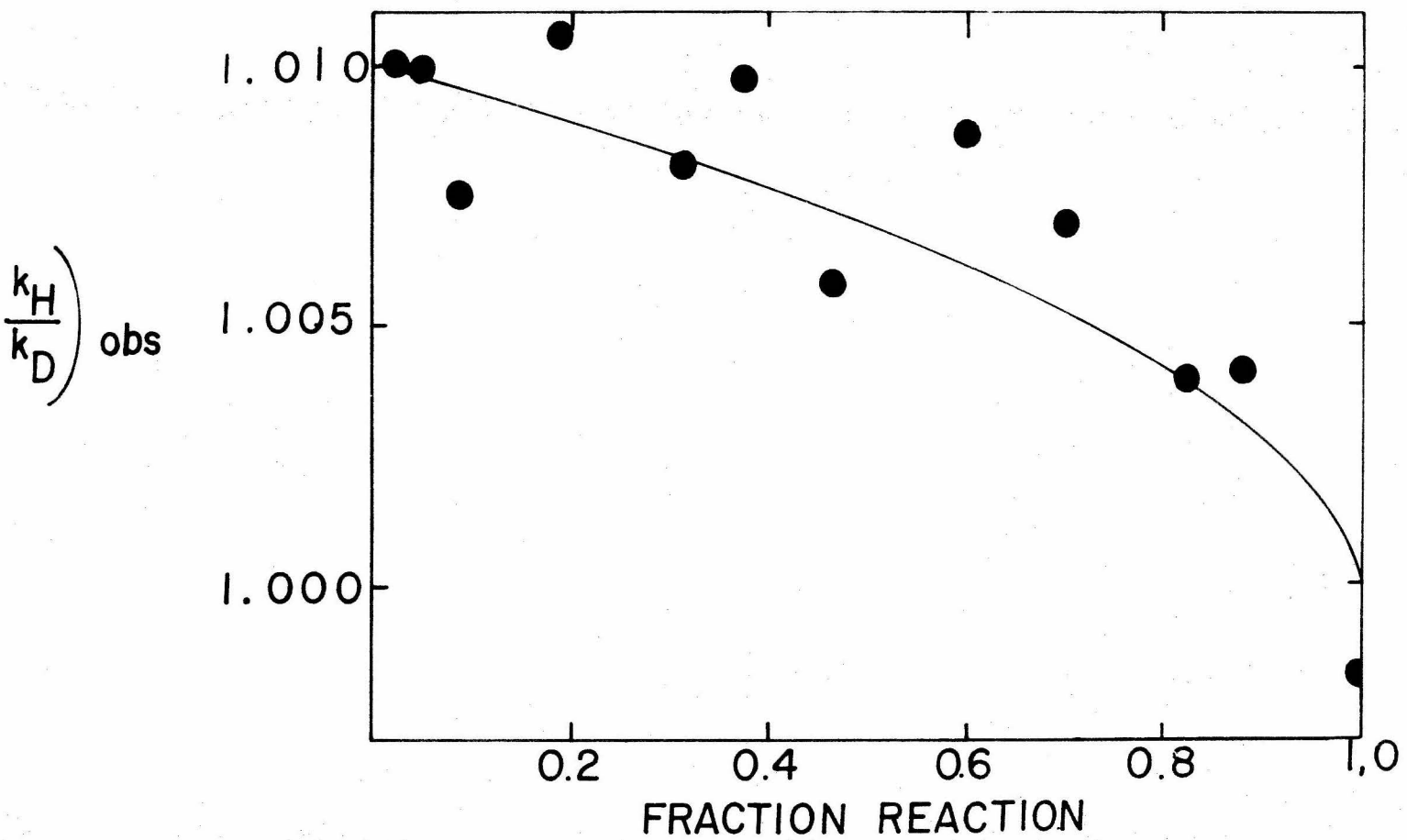

6400
4800
3200
1600
CPM
NGP
GP
NNGP
P
81
111
141
171
201
FRACTION NUMBER

Figure 4. A plot of $k_H/k_D)_{obs}$ versus fraction reaction for the hydrolysis of GP by β-glucosidase. The term $k_H/k_D)_{obs}$ is the $^3H/^{14}C$ ratio of the total phenol produced at that point relative to the $^3H/^{14}C$ ratio of GP before reaction. The solid points represent the observed data while the line is the theoretical curve for an initial $k_H/k_D)_{obs}$ of 1.010.


$\left(\frac{k_H}{k_D}\right)_{obs}$
1.010
1.005
1.000
0.2
0.4
0.6
0.8
1.0
FRACTION REACTION

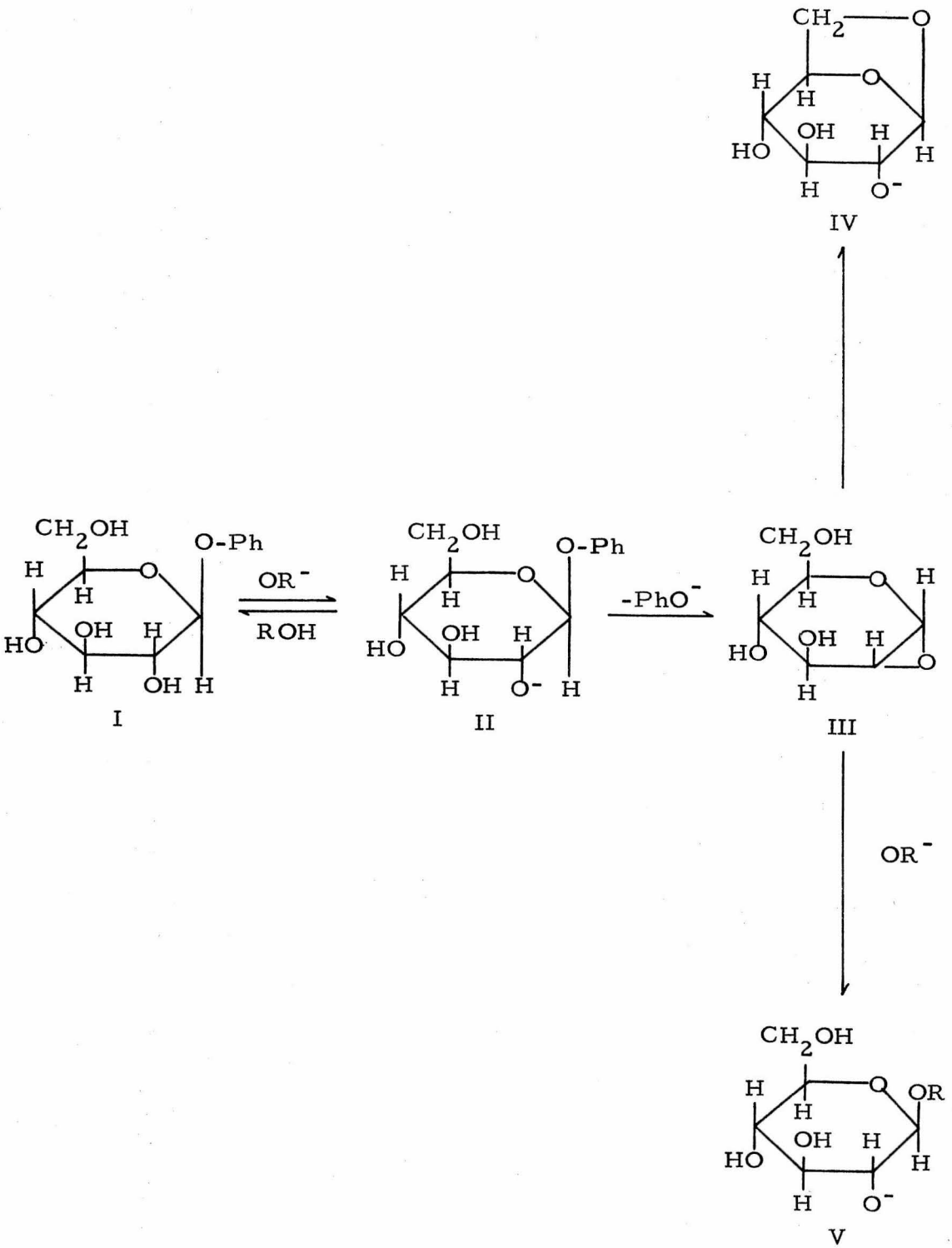


Figure 5