SUBSTRATE DISTORTION IN LYSOZYME-SUBSTRATE INTERACTIONS

by

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Abstract

The evidence supporting the hypothesis that substrate distortion towards the transition-state conformation in the lysozyme catalysed hydrolysis of glycosides makes an important contribution towards the rate enhancement observed for the enzymic hydrolysis is reviewed in the light of the currently held views on the mechanism of action of lysozyme and the known interactions of the enzyme with substrates and inhibitors.

A series of oligosaccharides having the general structure $(NAG)_n$-X, where n= 1-3, NAG= N-acetyl-D-glucosamine and X= an aryl glycoside were prepared. When X is an aryl glycoside of N-acetyl-D-xylosamine the effect of substrate distortion in the lysozyme catalysed hydrolysis should be eliminated. Therefore by comparison with other oligosaccharides for which substrate distortion could play a role in the enzymic hydrolysis, an estimation of this factor could be made.

p-Nitrophenyl 2-acetamido-2-deoxy-β-D-xylopyranoside was shown by N.M.R. to exhibit a dissociation constant and bound chemical shift value on association with lysozyme consistent with the sugar residue being bound in subsite C of the active cleft. This is in accord with the results obtained for the binding of other monosaccharide inhibitors having a 2-acetamido group, N-acetyl-D-glucosamine, methyl 2-acetamido-2-deoxy-α-D-glucopyranoside and p-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside, confirming the strong interaction of the 2-acetamido group at subsite C.

NAG–NAX–PNP was shown by N.M.R. to bind to lysozyme with the sugar residue proximal to the aglycone occupying subsite C of the active cleft, predicting that the compound binds to a considerable extent in the binding mode B, C. This binding mode was shown by N.M.R. to be present in the binding of NAG_2, p-nitrophenyl β-NAG_2 and NAG–Glu–PNP to lysozyme.

Within experimental error the same dissociation constant for the binding of NAG–NAX–PNP was obtained from fluorescence and inhibition studies. This compound was shown to bind more strongly to lysozyme than either NAG_2 or p-nitrophenyl β-NAG_2 with a favourable energy difference of approximately 1Kcal/mole.

Similarly from fluorescence and inhibition studies NAG_2–NAX–PNP was shown to bind more strongly to lysozyme than either NAG_3 or p-nitrophenyl β-NAG_3 by about 1Kcal/mole.

The kinetics of the lysozyme catalysed hydrolysis of p-nitrophenyl β-NAX, NAG–NAX–PNP, NAG_2–NAX–PNP and NAG_3–NAX–PNP were studied and compared with the lysozyme catalysed hydrolysis of the corresponding NAG glycosides. The rate of hydrolysis of the NAX compounds was found to be considerably slower than that of the NAG compounds. The upper limit of k_{cat}/K_m (apparent) found for the lysozyme catalysed hydrolysis of NAG_3–NAX–PNP was 1,000 times less than k_{cat}/K_m (apparent) found for p-nitrophenyl β-NAG_4 consistent with the hypothesis that substrate distortion is important in the lysozyme catalysed hydrolysis of glycosides. 3,4-dinitrophenyl β-NAX, NAG–NAX–DNP and NAG_2–NAX–DNP did not show any increase in the rate of hydrolysis in the presence of lysozyme over that found for the spontaneous hydrolysis, confirming the role of substrate distortion in the enzymic catalysis. The account that must be taken of the binding modes and strength of binding observed for the lower NAX containing oligosaccharides in the interpretation of the kinetic results

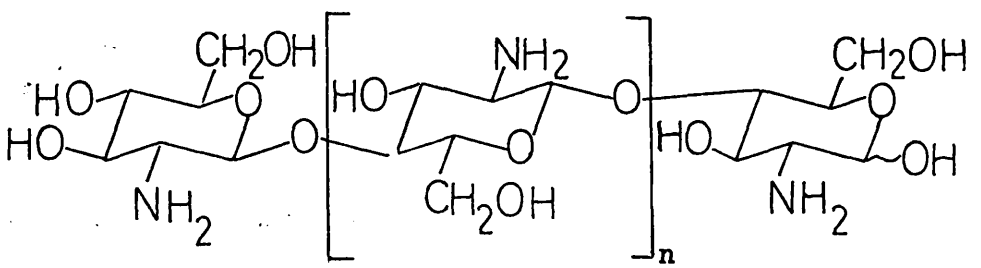
is discussed.

NAG-NAX-PNP, NAG_2-NAX-PNP and NAG-Glu-PNP were shown to be competitive inhibitors of the lysozyme catalysed hydrolysis of 3,4-dinitrophenyl β-NAG_4. The inhibition constant found for NAG-Glu-PNP differed from the dissociation constant for this compound found by N.M.R. The reasons for this difference are discussed in relation to the possible binding modes available for this compound and the ability of the N.M.R. method to distinguish between different binding modes.

p-Nitrophenyl 2-deoxy-β-D-glucopyranoside was synthesized and fully characterised. Attempted transglycosylation reactions of this compound with lysozyme are discussed.

Nomenclature.

The following nomenclature will be used for the compounds shown below.



n = 0 chitobiose,

n = 1 chitotriose,

n = 2 chitotetraose, etc.

The N-acetylated derivatives of the above will be named thus:-

n = 0 Di-N-acetyl chitobiose,

n = 1 Tri-N-acetyl chitotriose etc.,

and abbreviated as NAG_2, NAG_3, etc.

The peracetylated oligasaccharides will be named as:-

n = 0 Di-N-acetyl-hexa-O-acetyl chitobiose,

n = 1 Tri-N-acetyl-octa-O-acetyl chitotriose, etc.,

and abbreviated as acetylated NAG_2 etc.

Glycosides will be abbreviated thus:- Methyl 2-acetamido-2-deoxy-β-D-glucopyranoside, abbreviated as Methyl β-NAG.

Glycosides of the N-acetylated derivatives above will be abbreviated as Methyl β-NAG_2 etc.

Oligosaccharides containing NAG sugar residues and having a different terminal sugar residue will be named thus:-

i.e. 4-O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-β-D-glucose will be abbreviated as NAG-Glu. The p-nitrophenyl β-glycoside will be abbreviated as NAG-Glu-PNP.

Similarly oligosaccharides containing a terminal 2-acetamido-2-deoxy-β-D-xylopyranoside residue will be named as NAG-NAX, NAG_2-NAX, etc., and the corresponding p-nitrophenyl β-glycosides named as NAG-NAX-PNP, etc.

D-Xylose will be abbreviated as Xyl and oligosaccharides containing a xylose residue will be abbreviated as above i.e. NAG-Xyl etc.

2- Deoxy-D-glucose will be abbreviated as Deoxy-Glu in oligosaccharides named as above.

3,4-Dinitrophenyl glycosides of oligosaccharides containing a terminal NAX residue will be abbreviated as NAG-NAX-DNP, etc.

CONTENTS

CONTENTS (Cont'd)

Tables.

Tables (cont'd)

Tables (cont'd)

INTRODUCTION

With customary perception, Pauling[1 ab] in 1948 proposed that, 'enzymes are molecules that are complementary in structure to the activated complexes of the reactions that they catalyse, that is to the molecular configuration that is intermediate between the reacting substances and the products of reaction for these catalysed processes. The attraction of the enzyme molecule for the activated complex would thus lead to a decrease in its energy, and hence to a decrease in the energy of activation of the reaction and to an increase in the rate of the reaction'.

Evidence suggests that binding and strain towards the transition-state complex is a major source of the catalytic power of enzymes, and featuring in such investigations has been the work on the enzyme lysozyme.

Bacteriolytic enzymes termed lysozymes can be conveniently defined as comprising those enzymes of vertebrate origin that are chemically and structurally related to hen egg white lysozyme and have similar lytic properties[2]. The availability, purity and stability of lysozyme from hen egg white has led to extensive studies on this enzyme as a means of gaining wider knowledge of the factors involved in enzyme catalysis, and more recently to studies on lysozymes from widely differing sources, which may help to elucidate the biological function of this group of enzymes[2,4-12]. Especially interesting and important is the investigation into the role of lysozyme in certain types of leukemia [4,5,13,14] and the significance of the enzyme level in human serum and synovial fluids in cases of rheumatoid arthritis [15,16]. If the enzyme is ascribed an important role in these or other human disorders the design and investigation of inhibitors of the enzyme will become especially relevant.

Since this study, and most of the work herein, was concerned with heg egg white lysozyme the name lysozyme will be used for this species unless otherwise specified.

Numerous reviews[2,3,17,68] have dealt with various aspects of lysozyme and hence this introduction will be concerned mainly with those aspects of physical properties, structure and interactions of lysozyme with substrates and inhibitors which are relevant to the elucidation of the method of catalysis employed by the enzyme.

Composition and Structure

The amino acid composition and detailed sequence of lysozyme was extensively studied by Jolles[18] and Canfield[19], and has been upheld, with few changes, by other workers[2,20-22]. The interesting features of this primary structure consisting of a single polypeptide chain of 129 amino acids, (Mol. Wt. 14,388), are the four disulphide bridges, (30-115, 64-80, 76-94, 6-127), the single histidine residue, (15) and the six tryptophans (28, 62, 63, 108, 111, 123).

The three dimensional structure of the enzyme crystal obtained by Philips and his co-workers[23,24], showed that the molecule has roughly the shape of a prolate spheroid with overall dimensions 45 x 30 x 30Å, with a deep cleft at one side which is evident in the course of the main polypeptide chain through the molecule which it divides roughly into two sections, the first of these comprising the two ends of the chain is folded around a central core of hydrophobic residues while the second section, which contains the central region, is sheet like and consists roughly of hydrophilic residues either on the outer surface of the molecule or lining the cleft.

Following the crystal structure determination a major advance in the understanding of the action of lysozyme was made by studying the lysozyme-inhibitor complexes[25,26] formed by co-crystallization of the enzyme with inhibitor molecules, or by soaking enzyme crystals in solutions of inhibitor, both of which formed isomorphous crystals the X-ray structures of which can be relatively easily determined by comparison with the data from crystals of the enzyme alone. The 2Å resolution X-ray study of inhibitor complexes shed great light on the binding of inhibitors to the enzyme, leading to tentative proposals as to the mechanism of catalysis, which in turn led to an almost exponential growth in the study of lysozyme interactions in solution.

The natural substrate for lysozyme is the polysaccharide component of the cell walls of gram-positive bacteria (especially micrococcus lysodeikticus)[27], which consists of alternately β(1-4) linked NAG and NAM residues (fig.1). Hydrolysis by lysozyme occurs causing bond breaking at the C(1) atom of the NAM residue and the glycosidic oxygen linking it to the C(4) atom of the neighbouring NAG residue[28]. Lysozyme also cleaves chitin, the β (1-4) linked NAG polymer and oligosaccharides derived from this were substrates for the enzyme[29]. While studying the hydrolysis of NAG_3[30] it was shown that the enzymic activity was competitively inhibited by the monomer NAG. The manner of this inhibition was studied by Philips and his co-workers[26], who studied the interaction of compounds shown in table 1 with lysozyme at 6Å resolution.

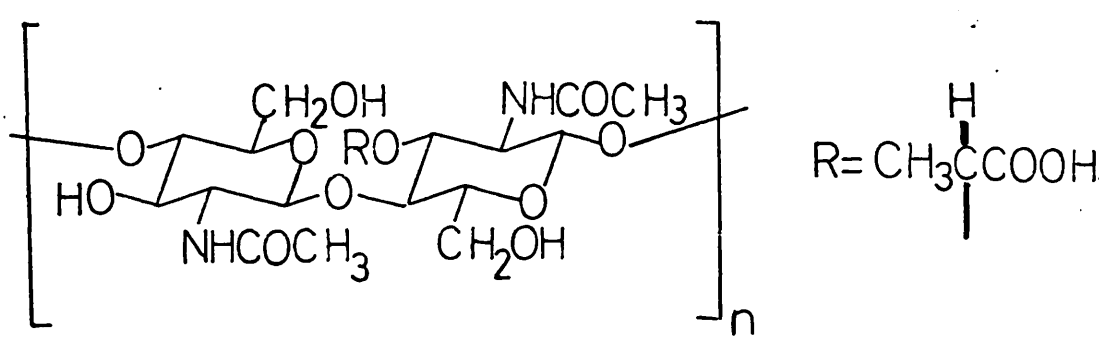


fig 1

Table 1.

Interaction of Inhibitors with Lysozyme at 6Å Resolution

Compound	Binding site
NAG	C, C^1
NAM	C^1
Methyl 6-iodo-α-NAG	C
NAG_2	B+C, C+ out of cleft
NAG-NAM	C+ out of cleft
NAG_3	A+B+C
Benzyl α-NAM	In interstices

Note Binding sites named from model building studies using the 2Å resolution of the NAG_3-enzyme complex.

Site C = site found for β-NAG at 2Å resolution

Site C^1 = site found for α-NAG at 2Å resolution

From these results the possession of an acetamido group on the inhibitor molecule was shown to be a strong requirement for binding to lysozyme. The study of NAG and NAG_3 was extended to 2Å resolution[31] which showed that both anomers of NAG were bound to lysozyme although in distinct ways. For the α-anomer the most specific interactions are those of hydrogen bonds between the NH and CO carbonyl oxygen of the acetamido side group of the amino sugar and the CO and NH groups of the main polypeptide chain belonging to the amino acid residues 107 and 59 respectively. These interactions are also common to the β-anomer. The α-anomer also makes contacts between the α-hydroxyl group and the main chain NH of residue 109, with another possible contact between the C(6)-hydroxyl and the side chain oxygen of aspartic acid 52. The β-anomer lies higher in the cleft making contacts between the C(6) and C(3)-hydroxyls and the side chain indole rings of tryptophans 62 and 63. Although the main requirement for binding is that due to the acetamido group the results show that small changes in the inhibitor molecule can change its mode of binding.

Further information from these studies was that the enzyme cleft was narrowed to some extent upon binding of the inhibitor molecule and that tryptophan 62 moved by about 0.7Å, providing evidence in support of the induced fit theory of Koshland[32].

From the 2Å resolution study of the binding of NAG_3[31] in subsites A, B and C in the unproductive mode, with its reducing end in subsite C bound in a similar way to the β-anomer of NAG, it was suggested from model building that a further three sugar residues might bind in subsites named D, E and F. NAG residues could bind in subsites E and F making satisfactory interactions with the enzyme without any distortion of the sugar residues.

In subsite D however the C(6) and O(6) atoms of the NAG residues are too close to the main chain carbonyl of residue 52, to trytophan 108 and to the acetamido group of the NAG residue bound in subsite C. This overcrowding can be overcome by distortion of the normal chair conformation of the NAG residue in subsite D towards a conformation nearer to the half chair, bringing the C(6) atom into an axial position, making hydrogen bonding between the C(6)-OH and either the carbonyl grouping of residue 52 or residue 35 possible.

These observations in conjunction with the finding that bond cleavage occurred between the C(1) and the bridge oxygen[28] led to the following proposals on the structure of the enzyme substrate complex. That (a) the hexamer of NAG will bind in the active cleft occupying subsites A to F. (b) The (NAG-NAM)n oligosaccharides (n = 3,4 etc.) will bind with the NAM residues in subsites B,D,F since in subsites A,C,E the C(3)-hydroxyl of the sugar residues points into the cleft and the D-lactyl ether at C(3) in NAM could not be accommodated in these subsites. Since the position of cleavage of NAG-NAM oligosaccharides is at C(1) of the NAM residue and the BC linkage is stable (since the X-ray binding mode for NAG_3 in subsites ABC is unproductive), hydrolysis will occur between subsites D and E. This also fits in with the observation that NAG_6 is cleaved to NAG_4 and NAG_2[33], the NAG_2 coming from the reducing end of the hexamer. This rational led to the indentification of glutamic acid residue 35 and aspartic acid residue 52, disposed on either side of the $\beta(1,4)$ linkage involved in catalysis, as being the most likely residues involved, and to mechanistic postulates on their mode of action in catalysing the hydrolysis of the glycosidic bond (see page 30).

It should also be mentioned here that the overcrowding that takes

place on placing a sugar residue in subsite D may also be overcome by changes in the enzyme conformation or by a balance of changes between enzyme and substrate conformation for which no experimental details have been reported. The change in the conformation of the enzyme in binding NAG_3 however is the same as that for NAG, and in view of the results of this study (see discussion), and other related findings[37,69] it seems likely that changes in the conformation of the sugar residue in subsite D at least plays some part in the binding and perhaps the catalysis of oligosaccharides.

Further X-ray work was concerned with low molecular weight synthetic substrates which had only a poor specificity for the enzyme. The X-ray analysis shone considerable light on the solution studies of these compounds (see pages 22,31).

Phenyl β-NAG_2 was shown to bind[31] to lysozyme in the non-productive mode with sugar residues in subsites B and C, the phenyl ring lying near subsite D, but not bound like a sugar residue. Other possible modes of binding for this compound could be in subsites E and F which are not accessible in the lysozyme crystals used in the experiment since they are blocked by contact with an adjacent molecule in the crystal structure.

NAG-Glu the $\beta(1-4)$ linked disaccharide was shown to bind in two modes, the first mode with the NAG residue in subsite C and the Glu residue in subsite D. The orientation of the Glu residue in this binding mode is different from that proposed from the model building using NAG residues. In this mode the Glu residue makes a hydrogen bond through its C(2)-hydroxyl with the aspartic acid residue 52 and the difference density map indicates both some distortion of the sugar residue in subsite D and a change in the enzyme conformation, in that the indole ring of tryptophan 108 has moved slightly back into the cleft. The C(6)-

hydroxymethyl group tends towards an axial position and the sugar residue tends to fit the density better in the half chair than in the chair conformation. This orientation of the Glu residue was thought to be unfavourable for a NAG residue in this position due to steric interaction between the acetamido group and the enzyme near the region of amino acid 46. Preliminary investigations into the binding of NAG_4 lactone* [34] which exists with the terminal sugar residue in the half boat conformation with the C(6)-hydroxymethyl group axial, suggest that this compound binds in subsites A to D. This has prompted the investigation of the binding of NAG_4 itself to lysozyme, and it is proposed that this can also bind in a mode occupying subsites A to D with the sugar residue in subsite D having an axial C(6)-hydroxymethyl group[34]. If these results are confirmed this would substantiate this mode of binding in subsite D which Philips[31] considered was possible when considering the binding of NAG-Glu. Sykes[35] has also observed that the tetrasaccharide $(NAG\text{-}NAM)_2$ can also bind with the NAM residue in subsite D.

The second mode of binding observed for NAG-Glu is one in which the acetamido group of the NAG residue maintains its contacts with the enzyme in subsite C but the inhibitor is rotated upwards out of the cleft with the Glu residue occupying a position near tryptophan 62. These modes of binding would seem relevant to the studies done on NAG-Glu-PNP, and will be considered in relation to its hydrolysis and binding.

NAG-Xyl binds in a similar manner to that proposed for the first mode of binding discussed for NAG-Glu, but there is less evidence that the sugar ring in subsite D is distorted from a chair conformation and the enzyme itself appears to undergo less of a conformational change[31].

* see fig.4

This would be in keeping with the postulate that the C(6)-hydroxymethyl group in sugars having this group would have unfavourable interactions with the enzyme molecule if the sugar residue was bound in subsite D in the normal chair conformation.

The idea that the sugar residue undergoing alkyl oxygen fission at C(1) should be bound in a half chair conformation which would be favourable for stabilization of the carbonium ion formed[36] supports the idea that enzymes can bind their substrates in conformations related to those of intermediates or transition states in the enzyme catalysed reaction[1]. Closely related to the conformation most favourable for a carbonium ion at C(1) in a sugar residue is that of a lactone of the corresponding sugar. The low resolution study of NAG(1-5)-lactone Fig.(2)[31] showed this to bind in subsite C again demonstrating the clear specificity for the N-acetyl group in this site.

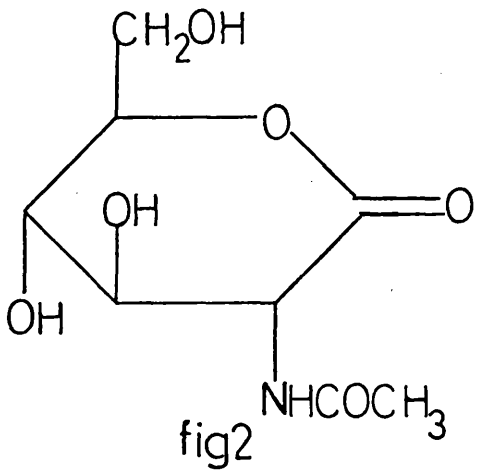


fig2

As mentioned above the NAG_4 lactone would appear to bind in subsites A-D confirming the strong binding found for this compound in solution studies[37].

As well as demonstrating the power of the X-ray method in determining modes of binding in substrate/inhibitor-enzyme complexes in the crystal state these studies clearly indicate that care must be exercised

in interpreting data on similar interactions found from solution studies which do not unambiguously define the specific sites of interaction.

In comparison of studies in solution and in the crystal, the question arises as to whether the enzyme conformation in the crystal phase is perturbed by the effect of lattice forces on the polypeptide chains. For lysozyme considerable evidence suggests that the enzyme conformation is very similar in both the solution and the crystal phase[38]. Among the most convincing of this evidence is the many solution studies using N.M.R[51-56], Fluorescence[74-76] and other methods of measuring enzyme interactions in solution[72,73], which give data on the binding of substrates/inhibitors to lysozyme which are consistent between the different methods used and with the evidence from X-ray studies. Evidence supporting the contacts made by the sugar residues with the enzyme postulated from the X-ray model has also been found. Verhoeven et al[39] have shown by N.M.R that compound I (fig.3) binds to lysozyme in a similar manner to Methyl β-NAG and that there is a charge transfer of the pyridinium-indole type indicating that the complex in solution is similar to that in the crystal with respect to the proximity of the O(6) ligand to the tryptophan 62 residue on the enzyme.

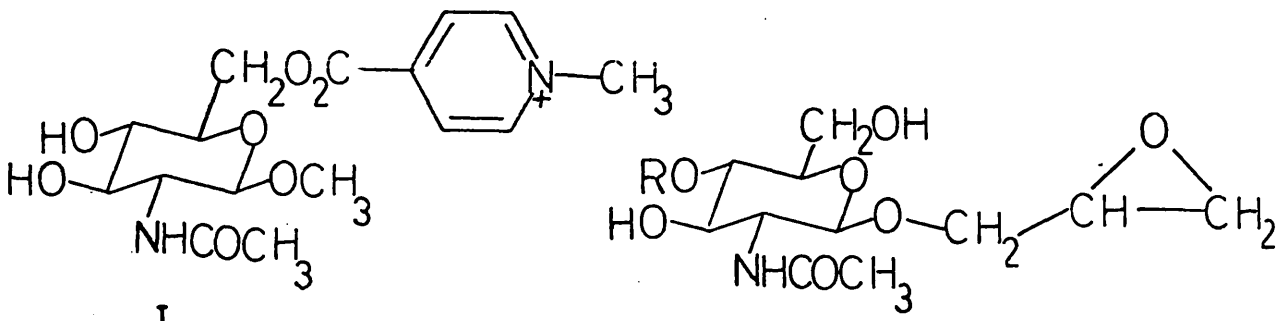


fig 3

Sharon[40] has shown that the compounds II - IV (fig.3) bind with dissociation constants similar to those found for the corresponding NAG oligomers which have been shown by N.M.R to bind with the reducing end sugar residue in subsite C. For compound II - IV this would result in the epoxypropyl group lying near site D. These compounds were also shown to be irreversible inactivators of the enzyme. By isolating the peptide to which the alkylating group was attached and identifying it as consisting of amino acid residues 46-53[22] this strongly suggested that either aspartic acid residues 48 or 52 had been modified. An X-ray analysis[78] of the inhibitor complex formed by compound III showed, by comparison with that found for Phenyl β-NAG_2, that III bound with the sugar residues in subsites B and C with the epoxypropyl group forming a covalent bond to the enzyme through the carboxyl side chain of aspartic acid 52. Since the loss of catalytic activity could be due to blocking of the active site in the alkylated enzyme a specific catalytic role can not be assigned to the modified residue, but it does confirm that the catalytic power of the enzyme is contained near subsite D. It has also been shown that all the carboxyl groups in lysozyme except aspartic acid 52 and glutamic acid 35 can be modified without complete loss of enzymic activity[41].

Such evidence in conjunction with the known composition of the crystal structure, which contains 30% by weight of water and allows contacts between adjacent lysozyme molecules only over a small fraction of their surface area lends support to the idea that there is relatively little difference between the enzyme conformation in the crystal and in solution, allowing comparison of data from both phases to be made with some degree of assurance.

Binding Studies in Solution

Lysozyme - small molecule interactions in solution have been widely studied by N.M.R as this technique offers the only other method, apart from X-ray diffraction studies, of detecting individual atoms in macromolecular systems[42-46], as well as offering the possibility of studying the dynamic aspects of the interaction in enzyme systems[47]. Two approaches have been used in N.M.R studies, that of looking at the spectrum of the enzyme itself and identifying regions of the spectrum with specific sites of interest on the enzyme[48-50], or centering attention on the spectrum of a small molecule, or ion, which interacts with the enzyme at a site of interest. Such interactions can, in favourable cases, cause changes in the line positions of the spectrum of the small molecule and/or the relaxation times.

For a small molecule interacting with an enzyme such that it is in equilibrium,

$$E + I \rightleftharpoons EI \qquad (I)$$

it can be considered that the small molecule exists free in solution (subsite A), or associated with the enzyme (subsite B). If the magnetic environments of the sites differ then there may be a chemical shift difference in the resonance frequencies of certain nuclei of the small molecule, especially if the bound species is proximate to an aromatic system or electric fields due to polar groups or metal ions. The resonance frequencies associated with each site can be assigned a W_A (free) and W_B(bound), and if the rate of exchange (k_{-1}sec^{-1}) between the free and the bound species is small compared to the difference in frequency $(W_A-W_B)/2\pi$ then separate resonances will occur at W_A and W_B. For a system in rapid equilibrium a single resonance appears at a

distance $\delta = P_B \Delta$ from $W_A/2\pi$, where P_B is the fraction of the small molecule bound at a given time and Δ is the chemical shift difference $(W_A - W_B)/2\pi$ between the bound and the unbound species.

From eqn.(I) $$K_D = \frac{[E][I]}{[EI]}$$

in the fast exchange limit we have,

$$\delta = P_B \Delta$$

$$= \frac{[EI]}{[I_o]} \Delta$$

Therefore $$[EI] = [I_o] \frac{\delta}{\Delta}$$

now $$[E] = [E_o] - [EI] = [E_o] - [I_o] \frac{\delta}{\Delta}$$

and $$[I] = [I_o] - [EI] = [I_o] - [I_o] \frac{\delta}{\Delta}$$

where $[I_o]$ = initial concentration of the small molecule

and $[E_o]$ = initial enzyme concentration.

Therefore $$K_D = [E_o] \frac{\Delta}{\delta} - [I_o] - [E_o] + [I_o] \frac{\delta}{\Delta}$$

If $\delta \ll \Delta$ as is usually the case, and K_D is of the order of $[I_o]$, then the term $\frac{\delta}{\Delta} [I_o]$ can be omitted to give,

$$K_D = \frac{[E_o] \Delta}{\delta} - [E_o] - [I_o]$$

or $$[I_o] = \frac{[E_o] \Delta}{\delta} - K_D - [E_o]$$

hence a plot of $\left[I_o\right]$ versus $\frac{1}{\delta}$ affords the chemical shift value Δ and the dissociation constant $K_D(M)$.

For small molecule-lysozyme interactions it has been shown that for monomer sugar residues containing an acetamido group the H^1 resonance of the acetamido group shows a definite upfield shift and line broadening on binding of the sugar to lysozyme. This has been attributed to the proximity of the acetamido methyl protons in the bound sugar to the aromatic group of a tryptophan residue on the enzyme. A number of monomer sugars containing an acetamido group at the C(2) position have been studied by the N.M.R. technique (Table 2).

Before inferences on the binding of these and other compounds to lysozyme are made it should be noted that a lack of a detectable chemical shift or line broadening in the spectrum of a compound in contact with an enzyme does not preclude the possibility of its binding, since (a) the fraction of a compound bound at any time is small and (b) there are several mechanisms for line broadening, that operating for the NAG monomers is primarily exchange broadening which is only a mechanism for broadening if it occurs between sites of different chemical shift, i.e. an observed lack of change in a spectrum could be explained by the compound binding to lysozyme in such a manner that the acetamido group does not experience a very great change in the magnetic field.

Table 2

Binding of Monosaccharide Inhibitors to Lysozyme as Determined by N.M.R.

Compound	T°C	pH	K_D(M)	Δppm	Ref.
Methyl α-NAG	33	5.3	$(3.9\pm0.5)\times10^{-2}$	0.65 ± 0.08	(a)
"	31	5.5	$(5.2\pm0.4)\times10^{-2}$	0.55 ± 0.08	(b)
"	31	5.5	4.4×10^{-2}	0.73	(c)
Methyl β-NAG	33	5.3	$(2.9\pm0.4)\times10^{-2}$	0.69 ± 0.1	(a)
"	31	5.5	$(3.3\pm0.5)\times10^{-2}$	0.54 ± 0.04	(b)
α-NAG	33	5.2	$(2.3\pm0.4)\times10^{-2}$	0.78 ± 0.08	(d)
	31	5.5	1.6×10^{-2}	0.71	(e)
β-NAG	33	5.2	$(2.3\pm0.5)\times10^{-2}$	0.30 ± 0.04	(d)
	31	5.5	3.1×10^{-2}	0.51	(e)
Methyl 6-iodo-α-NAG	31	5.5	3.14×10^{-2}	0.63	(c)
Methyl 6-deoxy-α-NAG	31	5.5	3.7×10^{-2}	0.73	(c)
p-Nitrophenyl β-NAG	31	5.0	$(3.4\pm0.3)\times10^{-2}$	0.37	(f)

(a) ref.51; (b) ref.52; (c) ref.53; (d) ref.54; (e) ref.55; (f) ref.56.

Hence a compound could be binding strongly to lysozyme and yet appear to bind very little, or not at all, from the chemical shift and line width data.

The results shown in Table 2 have been interpreted from observed X-ray diffraction studies which place the NAG monomer in subsite C. By comparison of the results the most important contribution to binding at subsite C arises from interactions of the acetamido group, shown from X-ray to form hydrogen bonds between the NH and CO carbonyl oxygen with the main chain amino acid residues 107 and 59. It has also been suggested that there is a strong non polar association of the acetamido methyl group with the aromatic indole ring of tryptophan 108 which is proposed to account for the large chemical shift value for these protons in saccharides bound at subsite C. Differences in the chemical shift values can be used to determine if the orientations of the sugar residue bound to the enzyme are the same. Thus it can be seen that although α and β-NAG bind to lysozyme with similar affinities ($K_D M$) the magnitude of their chemical shift is different indicating different orientations in the enzyme complex which is in accord with the results from X-ray studies. The methyl glycosides of NAG appear to bind with similar orientations, that for the β compound having been observed by X-ray. Since the chemical shifts are upfield in the bound state this implies that the acetamido methyl protons are near the face of the aromatic residue rather than the edge. The chemical shift values for α and β-NAG also suggest that the acetamido methyl protons are further from tryptophan 108 in the β complex than in the α complex, in accord with the X-ray data.

Comparison of the data on Methyl α-NAG, Methyl 6-iodo-α-NAG and

Methyl 6-deoxy-α-NAG, (table 2), allow an estimation of the importance of the hydrogen bonding contact, postulated from X-ray data for a sugar bound in subsite C in the orientation of Methyl α-NAG, between the O(6) and the NH group of tryptophan 62. From the results in table 2 it can be seen that the three compounds bind to lysozyme with approximately equal affinity. Small differences in the chemical shift value might indicate that the 6-iodo compound is bound in a slightly different environment. The results suggest that either hydrogen bonding via the C(6)-oxygen is unimportant in subsite C or that there is some potential for Van der Waals interactions involving the C(6) region which compensates for the loss of a hydrogen bonding site. It is suggested from X-ray data that saccharides occupying subsite C in the normal fashion have up to thirty Van der Waals contacts which contribute to binding. The p-nitrophenyl glycoside of β-NAG appears to bind in a similar fashion to the other monomers studied. Since the experimental error in values of $K_D(M)$ and $\triangle$p.p.m. found from N.M.R. necessarily have a high error ($\pm$ 10-20%) care must be taken in placing undue emphasis on small measured differences between compounds, i.e. the factor of 2 found by Raftery in the relative dissociation constants for the α and β anomers of NAG, represents only a small energy difference of 0.4Kcal. at 31°C.

The N.M.R. method has also been extended to the study of oligosaccharides containing residues of NAG interacting with lysozyme and the results again show good correlation with those obtained by X-ray. Compounds of interest studied are shown in table3.

Table 3

Binding of Oligosaccharides to Lysozyme as Determined by N.M.R

Compound	T°C	pH	Δ ppm acetamido methyl C	B	A	Ref.
NAG_2	33	5.2	0.41 ± 0.02	0		(a)
"	45	5	0.57 ± 0.04	0		(b)
"	55	9.7	0.77 ± 0.04	0		(b)
Methyl β-NAG_2	35	5	0.60 ± 0.05	0		(b)
"	55	9.7	0.80	0		(b)
NAG_3	65	9.8	0.61	0	-0.08	(b)
Methyl β-NAG_3	65	9.7	0.63	0	-0.08	(b)
NAG-Glu-PNP	31	5.0	0.43 ± 0.03			(c)
p-Nitrophenyl β-NAG_2	31	5.0	0.5—0.6			(c)

(a) Ref.54; (b) Ref.57; (c) Ref.56

For the NAG oligosaccharides the resonance of the acetamido group on the reducing terminal sugar residue was found to shift upfield and broaden on addition of lysozyme, consistent with it being bound in subsite C. The remaining sugar residues were assigned to subsite B, (for NAG_2) and B,A, (for NAG_3) and the chemical shift values for the acetamido methyl group in these sites are shown above. The anomeric forms of the mutorotated mixture of NAG_2 did not resolve over the pH range 2-10 at 31°C and 45°C, suggesting that they both bind at the same site with the same average orientation, in a similar manner to that observed for the Methyl β-glycoside. At 31°C and pH 5.5 the triose showed only broadening of the acetamido methyl resonance possibly due to the fact that the dissociation rate of the enzyme substrate complex was too slow under these conditions to satisfy the conditions necessary to obtain a spectrum representative of the average of the free and enzyme bound substrate species. At 65°C, pH 9.7 the resonance corresponding to the reducing end acetamido group of Tri-N-acetyl chitotriose was shifted to higher field and was well resolved suggesting that the increase in temperature caused an increase in the exchange rate sufficient to cause a sharper resonance, although the exchange rate at this temperature may not be sufficiently fast to satisfy the criteria for using the two site analysis. Tetra-N-acetyl chitotetraose under the same conditions showed only broadening of the acetamido methyl resonances with a slight upfield shift in that of the reducing end sugar resonance and clearly did not satisfy the conditions of fast exchange. Sykes et al[58] in an N.M.R. study of the interaction of α and β-NAG with lysozyme have shown that the association constant and bound chemical shift of the acetamido methyl group of each anomer vary with PH.

Above pH 6 the association constant and/or bound chemical shift for both anomers fall off sharply. These results imply that caution must be exercised in the interpretation of the data found by Raftery at pH 9.7. The change in the dissociation constant and chemical shift with pH could be related to changes in the binding orientation of the sugar or of the enzyme conformation as the state of ionization of the amino acid residues, aspartic acid 52 and glutamic acid 35, changes.

Sykes[51,54] measured the rates of formation and lifetimes of the lysozyme-inhibitor complexes for the saccharides shown on Table 4 using a transient N.M.R. method. The rate constants for the β anomers ($k_{-1}sec^{-1}$), where $k_{-1}sec^{-1}$ and $k_1M^{-1}sec^{-1}$ represent the enzyme-substrate equilibrium as shown below,

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES$$

imply that the β anomers make more stable contacts with the enzyme than do the α anomers. For di-N-acetyl chitobiose the rate constant for formation of the enzyme substrate complex is significantly different from that of the monomers α and β-NAG indicating that the dimer has a more stable binding.

Table 4

Rates of Formation and Lifetimes of Lysozyme-Inhibitor Complexes

Compound	T°C	pH	$k_1 M^{-1} sec^{-1}$	$k_{-1} sec^{-1}$
Methyl α-NAG	33	5.3	1.5×10^5	5.5×10^3
Methyl β-NAG	33	5.3	1.6×10^5	4.5×10^3
α-NAG	33	5.2	3.5×10^5	8.5×10^3
β-NAG	33	5.2	1.4×10^5	3.3×10^3
* NAG_2	33	6.0	3.6×10^5	720

* Assuming $K_D = 2.1 \times 10^{-4} M$.

The results on the activation parameters for Methyl β-NAG suggest that the binding of monosaccharides to lysozyme is not a simple bi-molecular process[51].

p-Nitrophenyl β-NAG_2 has been shown to bind to lysozyme with the acetamido group proximal to the aglycone undergoing a chemical shift to higher field[56,59], indicating that the mode of binding observed by N.M.R. is that of the non-productive complex with the sugar residues occupying subsites B and C in accord with the results from the X-ray study.

NAG-Glu-PNP[56,59] binds with the NAG residue in subsite C as seen from the N.M.R. results, but the orientation of the Glu residue is not observed. X-ray results showed that there were two predominant modes of binding for this compound only one of which placed the Glu residue in subsite D. Sykes[60] has found from an N.M.R. study of this compound, by following the change in the dihedral angle of the anomeric proton at various $[EI]/[I]$ ratios, calculated from the initial concentrations of enzyme and inhibitor, using the dissociation constant of 1.5×10^{-2} M [57], that any change in the dihedral angle between H_1 and H_2 of the glucose ring in the bound substrate is small, indicating that the glucose ring is not distorted, or that only a small fraction of the bound substrate is distorted. If the latter is the case this could be represented by the following mechanisms.

$$E + S \rightleftharpoons ES \rightleftharpoons ES_2$$

$$K_1 = \frac{[E][S]}{[ES]} M \; ; \; K_2 = \frac{[ES_1]}{[ES_2]} \quad \text{with } K_2 \gg 1$$

$$K_D = \frac{K_1 K_2}{1+K_2} \doteqdot K_1 M = \text{observed value}$$

$$\text{or by} \quad E + S \overset{K_p}{\rightleftharpoons} ES \quad \text{productive}$$

$$E + S \overset{K_u}{\rightleftharpoons} ES \quad \text{non-productive}$$

$$\text{where} \quad K_u \ll K_p \text{ such that } K_D \doteq K_u$$

and presumably the non-productive complex would be that observed by X-ray which places the NAG residue in subsite C but orientated so that the glucose residue points out of the cleft. This result indicates that care must be taken when relating binding constants from N.M.R. work to observed kinetic results and ultimately to proposals on the mechanism of the enzyme catalysis.

More recently the use of the fluorine nucleus as an N.M.R. probe in lysozyme - small molecule interactions has been studied[61-66]. N-trifluoroacetyl-α-D-NAG, was shown to bind to lysozyme in the active cleft with a dissociation constant $K_D \doteq 10^{-2}$M and a chemical shift value Δ= 0.78 p.p.m.[61] These values are similar to those found for α-NAG, but the trifluoroacetyl compound must be situated differently in the active site from its proton analogue since if they both experienced an identical change in the magnetic environment a much larger chemical shift would be expected for the fluoro compound[67]. Lack of a detectable shift in the binding of N-trifluoroacetyl-β-D-NAG and the corresponding α and β Methyl glycosides which were shown by competition experiments to compete with α-NAG for subsite C[62], albeit more weakly than the proton analogues, re-illustrates the caution that must be exercised in interpreting N.M.R. results, especially negative ones. The study of tri-N-trifluoroacetyl chitotriose[65] showed this to bind undergoing a downfield chemical shift of the N-trifluoroacetyl resonance at the reducing end sugar residue in comparison to the monomer and

dimer compounds which experienced an upfield shift. The tri-N-trifluoroacetyl chitotriose was shown to bind with its reducing end in subsite C by using Co^{++} as a paramagnetic probe, and hence its orientation in subsite C must be different from the monomer or dimer. A pH study of its binding and the similarity of the dissociation constant to that of NAG_3 suggest that the two compounds are bound similarly and that for the trimer substitution of H by F at the acetamido methyl groups does not appreciably affect the binding. The change in the chemical shift for the N-trifluoroacetyl group at the non-reducing end of the trimer on the ionisation of the group with pK_a 3.25, which has been assigned to Aspartic acid 101, fits the X-ray data which suggests that this group is involved in the binding at subsite A. It was also observed that the chemical shift value for the reducing end N-trifluoroacetyl group was also changed on passing through this pH range and this can be rationalised if the trimer sugar undergoes translational movement relative to the binding cleft upon the formation of a hydrogen bond between the ionised aspartic acid 101 carboxyl and the terminal reducing sugar residue in site A. The use of paramagnetic probes has also been exploited in the study of the N-monofluoroacetyl-Nag anomers and the methyl glycosides. The chemical shift value for the N-monofluoroacetyl-α-Nag was different from that of the β compound or of either the α or β Methyl glycosides, in accord with the evidence on the binding of the protonated analogues found from X-ray and N.M.R. All the monofluoro compounds showed a downfield chemical shift for the fluorine nucleus on binding to lysozyme. The distances of the fluorinated sugars from the catalytic site was mapped using Gd^{+++} as a paramagnetic probe which showed that the orientation of the N-monofluoroacetyl group was different

from that of N-acetyl group in the proton analogues. While results from fluorine studies with lysozyme have merely confirmed the findings of other studies on the proton analogues the method would seem to be applicable to larger enzymes where the X-ray data would be more difficult to obtain.

Various methods have been used to determine the binding of saccharides to lysozyme in solution, the results of which are summarised in Table 5.

Values obtained for the same compounds by different methods are in good agreement. The wide range of methods applicable to lysozyme binding in solution is fortunate in that certain methods are more appropriate for strongly binding compounds than for weaker complexes.

Deductions on the nature of the active site have been made from the binding studies. The N-acetyl glucosamine oligosaccharides from the monomer through to the trimer show an increase in binding, but then no further increase is observed in going from trimer to hexamer. This could be used as evidence to support the theory of substrate distortion at site D since higher oligosaccharides than the trimer would be expected to bind in subsites A to C with the reducing end residue in C and the remaining sugar residues in B and A with the rest of the molecule lying outside the active cleft.

Since the N-acetyl muramic acid residue can only occupy subsites B, D and F the trisaccharide NAG-NAM-NAG will occupy subsites A, B and C and this compound has a free energy of association 2.9 Kcal/mole more favourable than the tetramer NAG-NAM-NAG-NAM which could be taken as the minimum energy requirement to fill site D. The NAM residue in the tetramer may not fully occupy site D and so the unfavourable energy requirement at site D may be greater.

Table 5

Binding of Saccharides to Lysozyme in Solution

Compound	$K_a(M^{-1})$	pH	T°C	Method	Ref.
α-NAG	63	5.5	31	N.M.R.	55
"	42	5.2	33	N.M.R.	54
"	30-50	4.5-8	22	U.V.	70
β-NAG	30	5.5	31	N.M.R.	55
"	34	6.2	25	Inhibition	71
"	43	5.2	33	N.M.R.	54
NAG_2	$5x10^3$	5-6	25	U.V.Inhibition Fluorescence	72,73, 74,75
NAG_3	$1x10^5$	"	25	U.V.Inhibition Fluorescence	72,73,79, 75,76,77
NAG_4	$1x10^5$	"	"	U.V.Inhibition Fluorescence	72,73 2
NAG_5	$1x10^5$	"	"	U.V.Inhibition Fluorescence	72,73 2
NAG_6	$1x10^5$	"	"	U.V.Inhibition Fluorescence	72,73 2,77
NAG-NAM	20	"	"	Equil.Dialysis Fluorescence	74
NAG-NAM-NAG	$3x10^5$	"	"	Fluorescence	74,75
$(NAG-NAM)_2$	$2x10^3$	"	"	Fluorescence	74,75
NAG-NAX	$2.4x10^4$	5.2	25	Fluorescence	69
NAG_2-NAX	$>1.5x10^5$	5.2	25	Fluorescence	69
NAG_3-NAX	$5.5x10^6$	5.2	25	Fluorescence	69
NAG_4lactone	$3.3x10^6$	6.2-7.8	25	Fluorescence	37

Using the dye Biebrich Scarlet the binding of NAG_3 and NAG_6 to the catalytic site has been estimated[77]. The data indicates that both oligomers bind to the enzyme in the unproductive mode with a dissociation constant of 1×10^{-5}M at pH 6.5–7.5 and 25°C without displacing the dye from the catalytic site. At higher concentrations both oligomers will competitively displace the dye from the catalytic site allowing differentiation between the productive and non-productive binding modes for these two compounds. Spectrophotometric determination of the concentrations of the enzyme-dye complex in the presence of various sugar concentrations gave productive binding dissociation constants for the trimer and hexamer of 2.0×10^{-2}M and 5×10^{-6}M respectively at pH 7.6 and 25°C showing that the hexamer binds in a productive mode to about the same extent that it and smaller polysaccharides bind in the non-productive mode. That the dye and enzyme formed a 1:1 complex was shown from a job plot of absorbance at various ratios of initial dye concentrations $[D_o]$ and enzyme $[E_o]$ concentrations with $D_o + E_o$ = constant. The previous interpretation of the binding of NAG_6 was that at any given time only a small proportion of the sugar was bound to the enzyme productively and was hydrolysed rapidly. Since the dissociation constant for the productive mode is approximately equal to that for the non-productive mode the favourable energy interactions in filling subsites E and F must approximately compensate for the unfavourable interaction at subsite D.

Contributions at subsites E and F to the free energy of binding have been calculated from acceptor reactivity in the transglycosylation reactions with lysozyme[86,87], and in the relative rates of cleavage of NAG_5 in binding modes A to E and B to F[73] to give values of ΔF_u = −4Kcal. and −1.7Kcal. respectively. This suggests that the cost of filling

subsite D is about 6Kcal. An estimate of the favourable interactions available from hydrogen bonding in site D if the sugar adopts a half chair conformation is 3-6 Kcal/mole leading to a total of 6 to 12 Kcal/mole being available for distortion. The amount of energy required to distort a glucopyranose ring has been estimated as 10Kcal/mole and hence distortion of the sugar residue in site D would appear to be possible.[95]

The δ-lactone prepared from NAG_4,(I),(fig.4), was shown to inhibit the lysozyme catalysed hydrolysis of micrococcus lysodeikticus 100 times more effectively than NAG_4 itself[37].

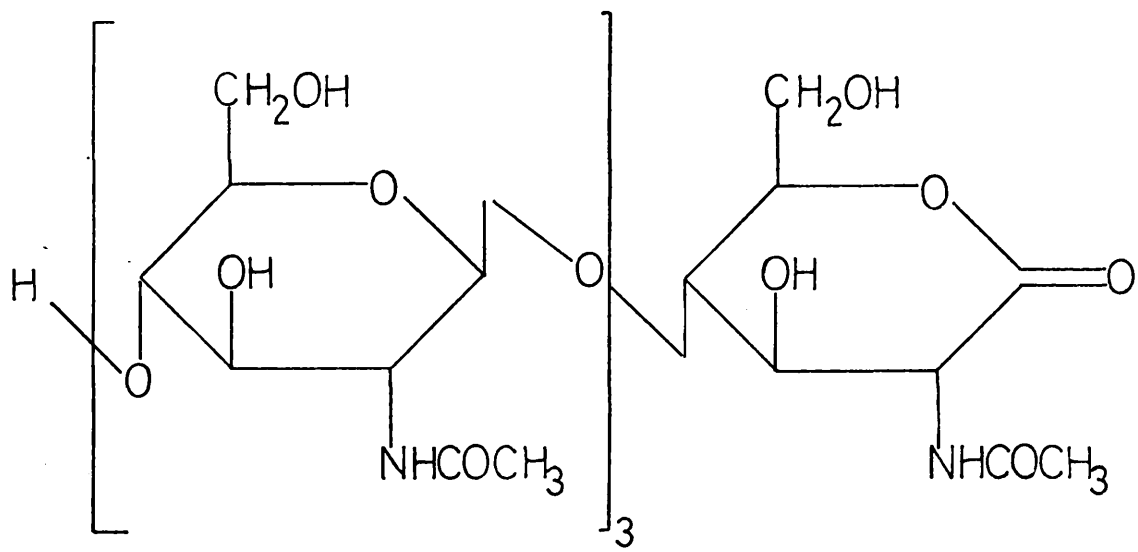


fig 4

Since the δ-lactone ring of compound (I) should exist in the half chair conformation, and if the sugar residue in subsite D binds to lysozyme with distortion towards the half chair conformation, then (I) would be a transition-state analogue for lysozyme. A transition-state analogue should bind much more strongly than the corresponding substrate and in agreement with this (I) was found to bind with an association constant of $3.3 \times 10^6 M^{-1}$ at pH6.2-7.8 and 25°C as measured by fluorescence

spectroscopy, i.e. approximately 32 times greater than that found for the association constant for NAG_4 of $1x10^5M^{-1}$. By estimating the association constant for NAG_4 bound in subsites A to D it was estimated that the affinity of subsite D for the half chair conformation of the sugar residue is 6000 times greater than that for the chair conformation. This factor can be assumed to be an estimate of the contribution to catalysis that can be assigned to the tighter binding of the transition state in the half chair conformation. As previously mentioned preliminary X-ray diffraction studies indicate that this compound can bind to lysozyme in subsites A to D[34].

Further support for the role of substrate distortion in lysozyme catalysis has been presented in the binding study of NAG_3-NAX to lysozyme by fluorescence measurements[69]. As the preparation used in the study was contaminated with NAG_4 this had to be taken into account in considering the results. The association constant found for NAG_3-NAX of $(5.5\pm1.3)x10^6M^{-1}$ is 50 times greater than that found for NAG_3 binding as studied by fluorescence. Hence it appears that the NAX residue is bound to subsite D with a net favourable interaction of 2.2Kcal./mole. The energy difference between a NAX residue and a NAM residue in subsite D is thus approximately 5Kcal./mole.

These studies well illustrate the magnitude of the energy factor available for catalysis due to the strain imposed on fitting a sugar residue into subsite D, they do not unfortunately tell us how much this contributes to the lowering of the activation energy for the cleavage of the glycosidic bond.

Kinetic studies on the hydrolysis of substrates by lysozyme are essential for determining the mechanism of catalysis. Various low molecular weight compounds have been tested as substrates, but

interpretation of the observed results are difficult since they are complicated by unproductive binding and transglycosylation. Multiple binding modes can also lead to more than one bond in the substrate being hydrolysed. Since hydrolysis of glycosides by lysozyme shows a rate enhancement in the order of 2×10^{10} over that for the acid catalysed hydrolysis, factors other than ring strain due to substrate distortion must be playing a major role in the enzymic reaction. For lysozyme the most likely contributions are expected to come from participation by (a) the acetamido group on the sugar residue, or (b) by the carboxyl groups of the side chains of glutamic acid 35 and or aspartic acid 52, or by a mixture of both.

The mechanism suggested for acetamido group participation is shown in figure 5.

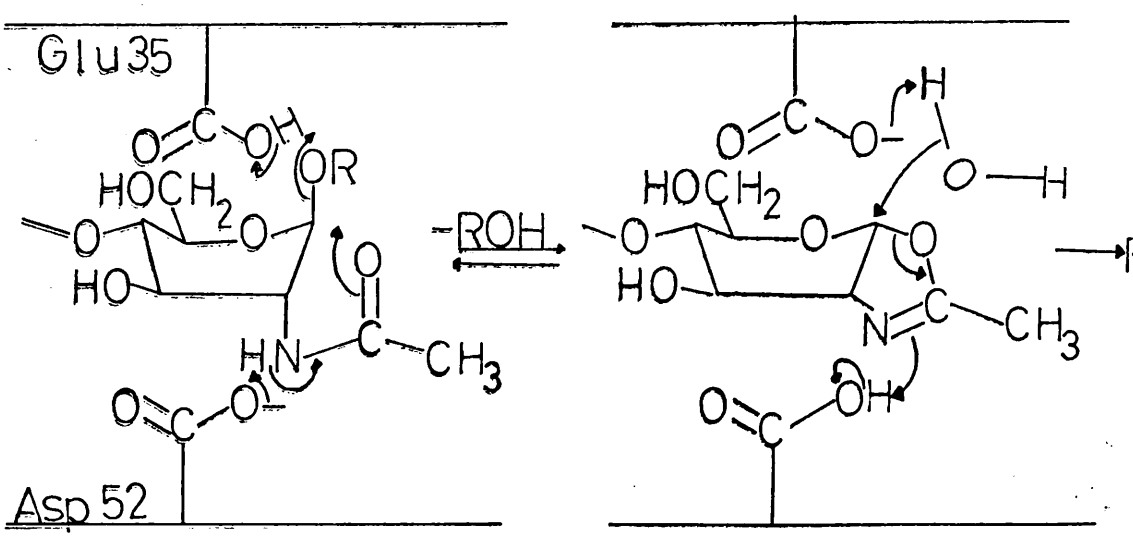


fig5

From figure 5 it can be seen that the leaving group and the acetamido group must assume a 1,2 trans-diaxial conformation for effective intramolecular catalysis. Distortion of the sugar ring in

subsite D towards a half chair conformation would favour participation. Lowe[59] favoured this mechanism from an analysis of the rates of hydrolysis by lysozyme of p-nitrophenyl β-NAG_2 and NAG-Glu-PNP which differed by a factor of 100 in favour of the former. Intramolecular catalysis by the acetamido group in the spontaneous hydrolysis of p-nitrophenyl β-NAG has been observed by Bruice[80], who also observed that the C(2)-hydroxyl group is also capable of participation in a similar manner. The ratio for the rate constants ($k^{spon.}_{NHAC}/k^{spon.}_{OH}$) was 10^3. The Hammett ρ value found for the substituted phenyl β-NAG molecules[81,68] of +2.8 supports nucleophilic catalysis. Lowe[79] found ρ=1.23 for the lysozyme catalysed hydrolysis of substituted aryl di-N-acetyl chitobiosides supporting concerted acid-base or acid-nucleophile catalysis.

The results found from lysozyme hydrolysis may be complicated by hydrolysis of the NAG-NAG bond. Raftery[56] has shown that p-nitrophenyl β-NAG_2 cleaves to give p-nitrophenyl β-NAG as well as NAG_2 and free phenol, leading to transglycosylation products being formed. NAG-DeoxyGlu-PNP is hydrolysed by lysozyme with release of p-nitrophenol[56], but since the stereochemistry of the products from this reaction are not known, and hydrolytic rate constants are sensitive to electronic effects in the C(2) position, these results alone can not discount acetamido participation in lysozyme hydrolysis.

Mechanisms involving the carboxyl groups of aspartic acid 52 and glutamic acid 35 are shown in figures 6 and 7.

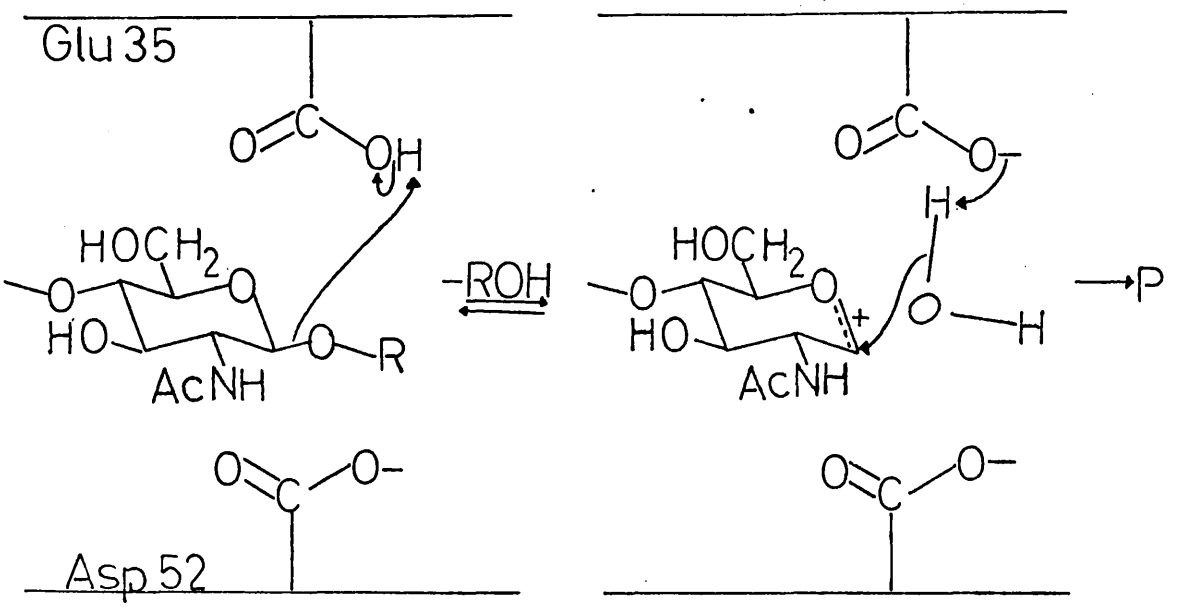

Glu 35
HOCH2
HO
AcNH
-ROH
P
Asp 52

fig 6

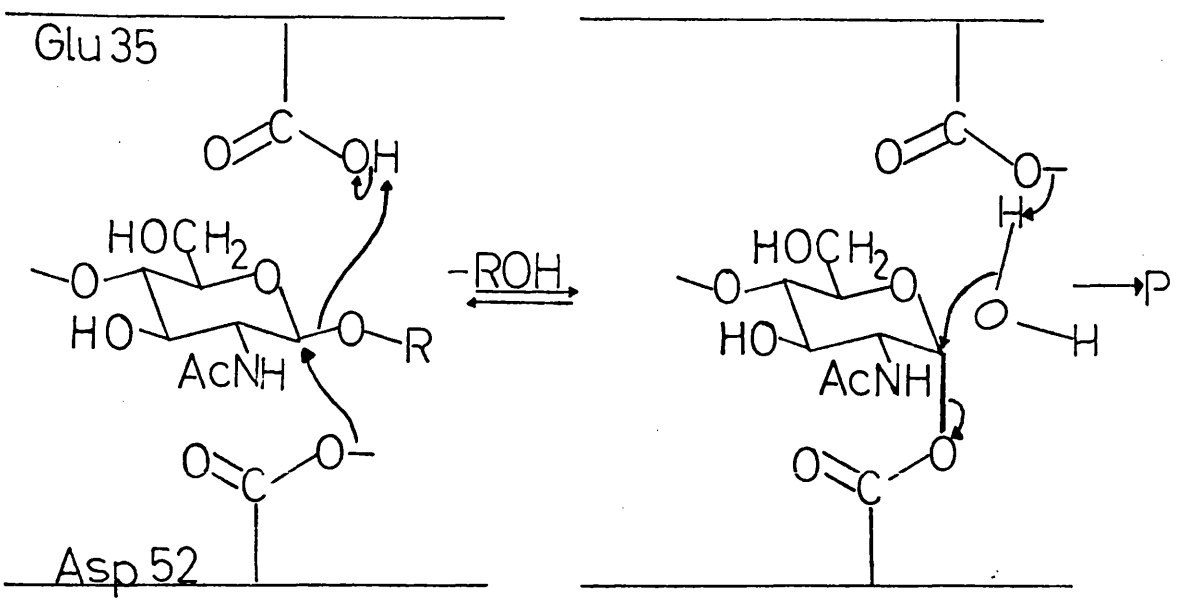

Glu 35
HOCH2
HO
AcNH
-ROH
P
Asp 52

fig 7

The mechanism involving glutamic acid 35 as providing general acid catalysis with the carbonium ion formed in the transition state being electrostatically stabilized by the carboxyl anion of aspartic 52, (fig.6) has received considerable support from the X-ray data[31] and other studies. Participation of the carboxyl group in the hydrolysis of o-carboxyphenyl β-D-glucopyranosides has been shown in the rate enhancement of these compounds over that of the p-carboxyphenyl series[82], and also in the hydrolysis of o-carboxyphenyl 2-acetamido-2-deoxy-β-D-glucopyranoside[81], although the effect of the o-carboxy group and the acetamido group is not additive.

The study of compound I (fig.8)[83] has shown that the rate enhancement of the o-carboxy group is due either to general acid catalysis or an Al mechanism[81]

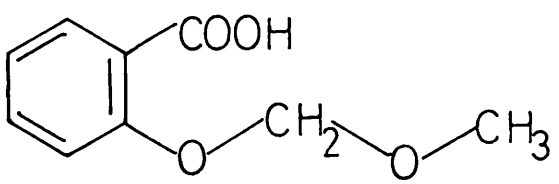


Fig. 8

with the carboxylate anion stabilizing the positively charged protonated substrate.

Model reactions necessarily have a different environment than that pertaining in the enzyme system which makes the transposition of enhancement effects from model studies to enzymic reactions difficult.

Various studies have implicated aspartic acid 52 and glutamic acid 35 as being necessary for catalysis[90,78,41]. The studies on the pH

dependence of binding of inhibitors to lysozyme and hydrolysis of substrates, have assigned pK_a values to these amino acids in the free enzyme and in the complex. The rate of release of p-nitrophenol from p-nitrophenyl β-D-glucopyranoside in the transglycosylation reaction with NAG_4[89] was influenced by groups with pK_a of 3.5 and 6.5. The hydrolysis of NAG-Glu-PNP studied over a wide pH range[56] and the plot of log V_{max}/K_m had inflection points at pH 2-4.5 and pH 6. The binding of Methyl β-NAG as a function of pH[91] showed that a group with pK_a of 6.1 in the free enzyme was shifted to 6.6 in the complex. In the presence of glycol chitin the pK_a value for this group changes to 8.0-8.5[92] consistent with the value found from the study of the hydrolysis of micrococcus lysodeikticus of 8.7. From X-ray data glutamic acid 35 is in a hydrophobic region of the enzyme which would raise its carboxyl group pK_a thus the group with pK_a of 6 has been assigned to glutamic acid 35.

Aspartic acid 52 has been assigned a pK_a of 4.6[94] in the native enzyme which is not seriously perturbed on binding of substrate[92] and this agrees with the pK_a of 4.7 not assigned to any specific residue found by other workers[91,70].

These results are consistent with the role of glutamic acid 35 as a general acid catalyst and aspartic acid 52 being dissociated in the productive enzyme substrate complex and able to stabilize the developing carbonium ion, but they do not give conclusive proof for either of these roles and the mechanism shown in fig.6 still rests largely on the X-ray data.

Other studies on hydrolysis have increased the available information on lysozyme. The hydrolysis of p-nitrophenyl-β-NAG_3 was shown to have a complex break up pattern, and the hydrolysis of NAG(1-4)NAG(1-6)NAG-PNP[85] was very much slower, probably due to complex break up followed by transglycosylation.

The transglycosylation activity of lysozyme has been used to study the compounds which can be accommodated in subsites E and F[86,87], helping to establish the nature of these subsites and their presence in the lysozyme cleft. With p-nitrophenyl 6-deoxy-β-NAG[88] and p-nitrophenyl β-D-xylose[89] as acceptors in the transfer reaction with NAG_4 no release of phenol was found even although transglycosylation was taking place, which could be evidence that substrate distortion is necessary for catalysis or merely that the linkages formed in the transglycosylation reaction were not β(1-4). In such cases the products of the transfer reaction would need to be isolated for study.

Most workers agree that there must be more than one factor responsible for lysozyme catalysis. In this study it has been attempted to isolate one likely factor, substrate distortion, and examine its contribution. It was considered that oligosaccharides containing a reducing end terminal NAX residue could be used to study the effect of substrate interaction between the hydroxymethyl group on the sugar and the enzyme, allowing an estimate of the contribution to the rate enhancement found for the lysozyme catalysed hydrolysis of glycosides due to this effect.

PREPARATIVE EXPERIMENTAL

General

Melting points were determined using a Kofler-Reichert hot stage apparatus and are uncorrected.

Nuclear Magnetic Resonance (N.M.R.) Spectra were run on a Varian T-60 MHz or a Varian HA-100 MHz Spectrometer. Chemical shifts, unless otherwise stated, were measured relative to an internal tetramethylsilane (T.M.S.) reference standard and are expressed in parts per million p.p.m.

Optical rotations were measured using a Perkin-Elmer 141 Polarimeter at 25°C with a 10cm. path length cell using the Sodium D line.

Infrared (I.R.) spectra were obtained using either Unicam SP 1,000 and SP 200 spectrometers, or a Perkin-Elmer 257 Grating Infrared Spectrometer.

Absorption wavelengths are quoted as cm^{-1}, and the abbreviations used are (s), strong; (m), medium; (w), weak.

Extinction coefficients were measured using a Cary 16 spectrometer at 40°C.

Elemental Analysis were obtained by Mr J. Cameron and Miss F. Cowan at the University of Glasgow.

Experimental Route for the Preparation of p-Nitrophenyl 2-acetamido-2-deoxy-β-D-xylopyranoside.

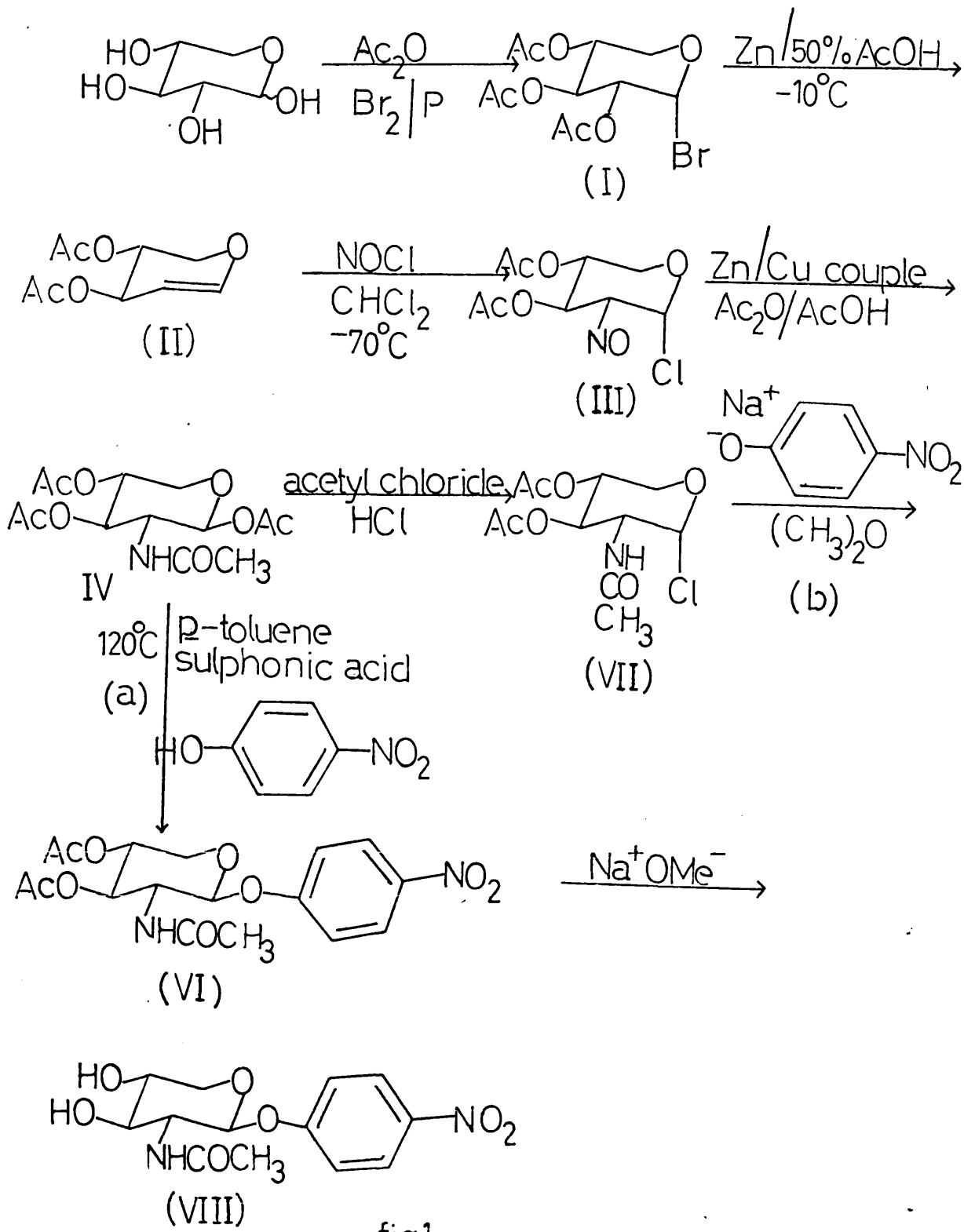


fig1

2,3,4-Tri-O-acetyl-α-D-xylopyranosyl Bromide (I)

This compound was prepared from D-xylose in 56% yield by the method described by Weygand[96]

M.pt. 96-97°C (lit.[96] 102°C)

Compound (I) decomposes readily on standing and was therefore used immediately in the next stage without further purification.

3,4-Di-O-acetyl-D-xylal (II)

This was prepared according to the method given in reference (96) and isolated as a clear syrup, B.pt. 84°C at 0.6mm. (lit.[96] B.pt. 95-110°C at 1.5-1.8mm), in 44.5% yield.

N.M.R. ($CDCl_3$)

δ = 2.12 p.p.m., (6 protons), singlet, acetyl group methyls.

δ = 4.13 p.p.m., (2 protons), multiplet, H-3, H-4.

δ = 5.00 p.p.m., (3 protons), broad doublet, H_2-5, H-2

δ = 6.60 p.p.m., (1 proton), doublet, H-1, $J_{1,2}$ = 5cps.

I.R. (neat)

3040(m), 2970(m), 2900(w), 1750(s), 1645(s), 1450(m), 1378(s), 1240(s), 1110(s), 1030(s), 975(s), 855(m), 775(m).

3,4-Di-O-acetyl-2-deoxy-2-nitroso-α-D-xylopyranosyl Chloride (III)

This compound was prepared according to the method of Lemieux[97] by the action of nitrosyl chloride on (II) in methylene chloride at -75°C, affording a viscous blue syrup in 90% yield which was immediately used

in the next stage. The labile nature of (III) made recrystallisation and characterisation inadvisable, but in view of the poor yields obtained in the following stage, the observation by Lemieux[97] that (III) converted readily to 3,4-di-O-acetyl-2-nitro-D-xylal and that 3,4,6-tri-O-acetyl-2-nitro-D-glucal was sometimes obtained as a product of the nitrosyl chloride addition to 3,4,6-tri-O-acetyl-D-glucal, various modifications of the reaction conditions were tried, i.e. changes in the rate of addition of nitrosyl chloride, time of addition, temperature, addition of nitrosyl chloride in a methylene chloride solution[98], all of which gave a blue syrup on solvent removal and did not lead to improved yields of (IV).

2-Acetamido-1,3,4-tri-O-acetyl-2-deoxy-β-D-xylopyranose (IV)

This compound was prepared from (III) by the method described by Lemieux[97] with the following modifications.

A zinc-copper couple, prepared as in Fieser & Fieser[99], was added to a solution of (III) in an acetic acid, acetic anhydride mixture and the mixture was stirred for two days at room temperature after which it was filtered, the solids washed with acetic acid, acetic anhydride mixture and a freshly prepared zinc-copper couple was added to the combined filtrates and the stirring continued. This was repeated every two days until the amount of (IV) formed was at a maximum as shown by T.L.C (1 : 1 v/v benzene, ethyl acetate). The solution was filtered and the solids collected were washed with chloroform (500 ml.). The combined filtrates were diluted with one litre of chloroform and then washed with water, saturated sodium bicarbonate and again with water. The chloroform extract was dried over magnesium sulphate and then evaporated under reduced

pressure to give a syrup which smelled of acetic anhydride. This was allowed to stand with absolute methanol for two hours at room temperature and then stored overnight at 0°C.

The first crop of crystals obtained (1-2g.) did not have the characteristic amide absorption bands in the I.R. spectrum and was identified as tetra-O-acetyl-2-oximino-D-xylo-pentopyranose (V), M.pt.225°C (phase change, 190°C to needles). Recrystallised from methanol, chloroform.

N.M.R. ($CDCl_3$)

δ = 2.03 p.p.m., 2.06 p.p.m., 2.10 p.p.m., (12 protons), poorly resolved triplet.

δ = 5.6-6.2 p.p.m., 4.7-5.4 p.p.m., 3.3-4.6 p.p.m., (5 protons), complicated multiplets.

I.R. (nujol).

1760(s), 1645(w) broad, 1510(s), 1310(m), 1250(s), 1240(s), 1160(m), 1130(m), 1090(s), 1060(s), 910(m), 890(m), 780(w), 700(m).

Analysis

Calculated for $C_{13}H_{17}O_9N$; %C,47.13; %H, 5.17; %N,4.23; Found,%C, 47.35; %H, 5.26; %N, 4.43

On further standing at 0°C the solution yielded further crops of crystals identified as 2-acetamido-1,3,4-tri-O-acetyl-2-deoxy-β-D-xylopyranose (IV). Recrystallisation from methanol, chloroform gave 2g. of material, M.Pt. 213-214°C (lit.[100] 214-215°C)

N.M.R. ($CDCl_3$)

δ= 6.13 p.p.m., (1 proton), doublet, N-H

δ= 5.73 p.p.m., (1 proton), doublet, H-1, $J_{1,2}$ = 6cps.

δ= 5.03 p.p.m., (2 protons), multiplet

δ= 4.23 p.p.m., (2 protons), multiplet

δ= 3.60 p.p.m., (1 proton), multiplet

δ= 2.10 p.p.m., (9 protons), O-acetyl methyls

δ= 2.00 p.p.m., (3 protons), N-acetyl methyls

See study of conformation of this compound by N.M.R. (Experimental discussion).

I.R. (nujol)

3320(s), 1750(s), 1740(s), 1665(s), 1525(s), 1315(w), 1300(m), 1220(s), 1110(m), 1075(s), 1040(s), 1010(m), 912(m), 895(w).

Analysis

Calculated for $C_{13}H_{19}O_8N$; %C, 49.21; %H, 6.04; %N, 4.41; Found, %C, 49.57; %H, 6.25; %N,4.21.

In a separate experiment 10g. of the tetra-O-acetyl-2-oximino-D-xylo-pentopyranose (V), accumulated from several experiments, was reacted with 40g. of zinc-copper couple in a mixture of 210ml. of acetic acid and 30ml. of acetic anhydride, fresh zinc-copper couple being added every two days as described above. The reaction was followed by T.L.C., and after 8 days, work up by an analogous method as above yielded 2g. of pure 2-acetamido-1,3,4-tri-O-acetyl-2-deoxy-β-D-xylose (IV) and 3g. of unreacted (V).

A commercially prepared zinc-copper couple (Ventron Corp., Massachusetts) was used in the above experiments, but the yield of (IV) was not increased.

The dark viscous syrup remaining after two or three successive crops of compound (IV) had been removed was applied to a column of silica (5x120cm.) and eluted with an increasing polarity gradient of methanol in chloroform. Fractions, 30 minutes, were collected and monitored by T.L.C. (1 : 1 v/v benzene, ethyl acetate). From 10g. of syrup only 0.54g. of compound (IV) was isolated plus a small amount of compound (V). The majority of the material eluted from the column did not run on T.L.C. using the solvent system above. Therefore the low yield from the reaction was not due to a failure to separate the components of the product mixture.

p-Nitrophenyl 2-acetamido-3,4-di-O-acetyl-2-deoxy-β-D-xylopyranoside (VI)

This compound was prepared either by the method described by Findlay et al[100], involving fusion of p-nitrophenol with 2-acetamido-3,4-di-O-acetyl-2-deoxy-β-D-xylose (IV) at 120°C. with p-toluene sulphonic acid as catalyst, or by the method described by Osawa[103] via the 2-acetamido-3,4-di-O-acetyl-α-D-xylopyranosyl chloride. The latter method gave slightly improved yields, (25%), over those obtained by the former method, (17%). Both methods gave identical material on recrystallisation from ethanol, chloroform.

2-acetamido-3,4-di-O-acetyl-α-D-xylopyranosyl chloride (VII)

This compound was prepared according to the method of Khorlin et al.[102] 0.9g. of compound (IV) was dissolved in 25ml. of redistilled acetyl chloride and saturated with dry hydrogen chloride gas at -10°C. The reaction

mixture was left overnight at room temperature in a tightly sealed flask then evaporated to dryness under vacuum. The solid obtained was evaporated three times with dry benzene to remove traces of acetyl chloride, then dried in vacuum. This gave a fawn coloured product, M.pt. 120-121°C, pure by T.L.C., (3 : 1 v/v ethyl acetate, benzene), which was used without further purification.

The crude chloride was reacted with p-nitrophenol according to the method of Osawa[103]. Recrystallisation of the solid product from ethanol, chloroform gave p-nitrophenyl 2-acetamido-3,4-di-O-acetyl-2-deoxy-β-D-xylopyranoside M.pt. 232°C.

N.M.R. ($CDCl_3$)

δ = 8.15 p.p.m., (2 protons), doublet, aromatic protons J = 9cps.

δ = 7.20 p.p.m., (2 protons), doublet, aromatic protons J = 9cps.

δ = 7.01 p.p.m., (1 proton), singlet, N-H.

δ = 6.09 p.p.m., (1 proton), doublet, H-1, $J_{1,2}$ = 4cps.

δ = 4.7-5.5 p.p.m., (2 protons), multiplet.

δ = 3.3-4.5 p.p.m., (3 protons), multiplet.

δ = 2.13 p.p.m., (6 protons), singlet, O-acetyl methyls.

δ = 2.00 p.p.m., (3 protons), singlet, N-acetyl methyl.

Further N.M.R. study of this compound is discussed in the Experimental discussion.

I.R. (K Br.)

3340(s), 3080(w), 2920(m), 2860(m), 1735(s), 1665(s), 1610(m), 1593(s), 1510(s), 1498)s), 1375(s), 1352(s), 1245(s), 1223(s), 1110(m), 1080(s), 1070(s), 1045(s), 900(w), 865(w), 850(m), 750(m).

Analysis

Calculated for $C_{17}H_{20}O_9N_2$; %C, 51.52; %H, 5.09; %N, 7.07; Found, %C, 52.14; %H, 5.24; %N, 7.21.

P-Nitrophenyl 2-acetamido-2-deoxy-β-D-xylopyranoside (VIII)

This compound was prepared by de-O-acetylation of compound (VII) using sodium methoxide according to the method of Zemplen[104]. Recrystallisation from methanol gave compound (VIII) in 63% yield. M.pt.207-208°C.

N.M.R. (d_5 Pyridine)

δ= 8.20 p.p.m., (2 protons),doublet, aromatic protons, J = 9cps.

δ= 7.30 p.p.m., (2 protons), doublet, aromatic protons, J = 9cps.

The resonance due to the N-H proton appeared under this doublet.

δ= 5.87 p.p.m., (1 proton), doublet, H-1, $J_{1,2}$ = 6cps.

δ= 3.8-5 p.p.m., multiplet, ring protons.

δ= 2.07 p.p.m., (3 protons), singlet, N-acetyl methyl.

I.R. (nujol)

3450(s), 3300(s), 3120(w), 1630(s), 1610(m), 1595(s), 1525(s), 1498(m), 1380(s), 1260(s), 1245(s), 1210(m), 1120(s), 1082(s), 1068(s), 1010(s), 965(s), 865(s), 765(s), 700(s).

Analysis

Calculated for $C_{13}H_{16}O_7N_2CH_3OH$; %C, 48.84; %H, 5.85; %N, 8.14. Found; %C, 48.70; %H, 5.25; %N, 8.15.

U.V. compound showed maximum absorbance at 300nm. with an extinction coefficient of 10^4 in aqueous solution. (See experimental discussion).

3,4-Dinitrophenol (IX)

This compound was prepared by a modified procedure of the method of Hollerman[105] and Sidgwick[106], developed in this laboratory[107], and recrystallised from benzene. M.pt. 137°C. (lit.139°C).

3,4-Dinitrophenyl 2-acetamido-3,4-di-O-acetyl-2-deoxy-β-D-xylopyranoside (X)

This compound was prepared using 2-acetamido-3,4-di-O-acetyl-2-deoxy-α-D-xylopyranosyl chloride (VII) and the sodium salt of compound (IX). To 1.7g of compound (VII) and 1.3g of the sodium salt of compound (IX) was added 4ml. of dry dimethylformamide. The mixture was shaken overnight at room temperature and then poured into a mixture of ice and water. The solid precipitate was filtered, washed with water to remove excess sodium 3,4-dinitrophenolate and dried under vacuum at 40°C. Recrystallisation from ethanol, chloroform gave material M.pt. 175-178°C.

N.M.R. ($CDCl_3$ + CD_3OD)

δ = 8.06 p.p.m., (1 proton), doublet, aromatic proton, J = 8.5cps.

δ = 7.47 p.p.m., (1 proton), doublet, aromatic proton, J = 2.5cps.

δ = 7.34 p.p.m., (1 proton), doublet of doublets, aromatic proton, J = 8.5 and 2.5cps.

The N-H proton signal resonates under this signal.

δ = 5.51 p.p.m., (1 proton), doublet, H-1, $J_{1,2}$ = 6.5cps.

δ = 5.14 p.p.m., (1 proton), multiplet, H-3.

δ = 4.98 p.p.m., (1 proton), multiplet, H-4.

δ= 4.33 p.p.m., (1 proton), multiplet, H-2

δ= 4.21 p.p.m., (1 proton), doublet of doublets H-5,

$J_{5,5'}$ = -12.5, $J_{4,5}$ = 4cps.

δ= 3.71 p.p.m., (1 proton), doublet of doublets, H-5,

$J_{4,5'}$ = 5.5, $J_{5,5'}$ = -12.5cps.

δ = 2.16 p.p.m., (6 protons), singlet, O-acetyl methyls.

δ= 2.01 p.p.m., (3 protons), singlet, N-acetyl methyl

I.R.(nujol)

3340(s), 1745(s), 1665(s), 1610(m), 1560(s), 1510(m), 1290(m), 1240(s), 1120(m), 1100(s), 1080(s), 1065(s), 1005(m), 900(m), 875(m), 825(m), 755(s), 725(m), 705(s).

Analysis

Calculated for $C_{17}H_{19}O_{11}N_3$; %C, 46.26; %H, 4.34; %N, 9.52; Found, %C, 47.25; %H, 4.53; %N, 9.42.

3,4-Dinitrophenyl 2-acetamido-2-deoxy-β-D-xylopyranoside (XI)

(X) was de-O-acetylated using sodium methoxide according to the method of Zemplen[104]. The reaction mixture was applied to a column of Sephadex LH20 (30x1.5cm.) and eluted with water. Fractions were monitored for glycoside by ultra violet absorbance at 284nm , pooled and freeze dried to give a micro-crystalline solid. M.pt. 175°C.

N.M.R. (d_5 Pyridine)

δ = 8.88 p.p.m., (1 proton), doublet, N-H, J = 9cps.

δ = 8.08 p.p.m., (1 proton), doublet, aromatic proton, J = 8.5cps.

δ = 7.90 p.p.m., (1 proton), doublet, aromatic proton, J = 2.5cps.

δ = 7.50 p.p.m., (1 proton), doublet of doublets, aromatic proton, J = 8.5 and 2.5cps.

δ = 5.98 p.p.m., (1 proton), doublet, H-1, $J_{1,2}$ = 6.5cps.

δ = 4.62 - 4.9 p.p.m., 4.14 - 4.58 p.p.m., 4.06 - 3.68 p.p.m., multiplets.

δ = 2.12 p.p.m., (3 protons), singlet, N-acetyl methyl.

I.R. (nujol)

3420(s), 3320(s), 3120(s), 1630(s), 1610(m), 1595(s), 1380(s), 1288(s), 1250(s), 1120(s), 1080(s), 1065(s), 1005(s), 965(m), 893(m), 855(m), 840(w), 820(m), 755(m), 725(m), 700(m).

Analysis

Calculated for $C_{13}H_{15}O_9N_3$; %C, 43.70; %H, 4.232; %N, 11.761; Found, %C, 42.79; %H, 4.46; %N, 11.58.

U.V. Extinction coefficient 5,550 in aqueous solution at λ max. 284nm.

p-Nitrophenyl 3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranoside (XII)

1,3,4,6-Tetra-O-acetyl-2-deoxy-D-glucopyranoside, a mixture of the α and β-anomers was supplied by Dr. B. Capon. This was reacted with acetyl chloride, and dry hydrogen chloride gas as previously described to give 3,4,6-tetra-O-acetyl-2-deoxy-D-glucopyranosyl chloride. The configuration at the anomeric carbon atom was not identified. The crude chloride was one spot on T.L.C. (3 : 1 v/v ethyl acetate, benzene). It was dissolved in dry acetone and reacted with p-nitrophenol in the presence of potassium carbonate. Compound (XII) was obtained in 41% overall yield from the tetra-acetate and recrystallised from 70% ethanol, water. M.pt. 154°C.

N.M.R. ($CDCl_3$)

δ = 8.2 p.p.m., (2 protons), doublet, aromatic protons, J = 9cps.

δ = 7.07 p.p.m., (2 protons), doublet, aromatic protons, J = 9cps.

δ = 5.23 p.p.m., (1 proton), doublet of doublets, $J_{1,2a}$ = 9cps, $J_{1,2e}$ = 3cps.

δ = 5.10 p.p.m., (1 proton), triplet, H-4, J = 9cps.

δ = 3.7 - 4.3 p.p.m., (4 protons), multiplet, H-3, H-5, H_2-6.

δ = 2.3 - 2.9 p.p.m., (2 protons), multiplet, H_2-2.

δ = 2.13 p.p.m., δ = 2.07 p.p.m., (9 protons) two singlets, O-acetyl methyls.

Resonances were assigned by decoupling experiments as described in the experimental discussion.

I.R. (nujol)

1765(s), 1750(s), 1610(m), 1595(s), 1525(s), 1510(m), 1385(s), 1250(s), 1225(s), 1125(m), 1080(s), 1060(m), 980(m), 865(m), 760(m).

Analysis.

Calculated for $C_{18}H_{21}O_{10}N$, H_2O; %C, 50.35; %H, 5.39; %N, 3.26; Found, %C, 49.83; %H, 4.64; %N, 3.70.

p-Nitrophenyl 2-deoxy-β-D-glucopyranoside (XIII)

Compound (XII) was de-O-acetylated according to Zemplen's method[104], to give compound (XIII). Recrystallisation from ethanol, water gave material with M.pt. 164°C (lit.[56] 167-169°C)

N.M.R. (d_5 pyridine)

δ = 8.2 p.p.m., (2 protons), doublet, aromatic protons, J = 9cps.

δ = 7.26 p.p.m., (2 protons), doublet, aromatic protons, J = 9cps.

δ = 5.63 p.p.m., (1 proton), doublet of doublets, H-1, $J_{1,2a}$ = 9cps. $J_{1,2e}$ = 3cps.

δ = 3.9 - 5.0 p.p.m., (7 protons), multiplet, O-H_2 plus ring protons.

δ = 2.2 - 2.9 p.p.m., (2 protons), multiplet, ring protons.

I.R. (nujol)

3320(s), 3220(s), 3100(m), 1615(m), 1595(m), 1523(s), 1498(s), 1350(s), 1250(s), 1180(s), 1153(m), 1105(s), 1082(s), 1070(s), 1045(s), 990(s), 925(m), 868(s), 825(m), 780(m), 760(s), 730(m), 700(m), 668(s), 652(m).

Analysis.

Calculated for $C_{12}H_{15}O_7N$, H_2O; %C, 47.53; %H, 5.65; %N, 4.62; Found, %C, 47.00; %H, 4.78; %N, 4.24.

p-Nitrophenyl 2-acetamido-6-O-toluene-p-sulphonyl-2-deoxy-β-D-glucopyranoside (XIV).

p-Nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (0.51g.) was dissolved in 8ml. of dry pyridine and cooled to -10°C. 1.1 mole equivalents of p-toluene sulphonic acid in 2ml. of dry pyridine were added over a period of twenty minutes. The mixture was allowed to stand 48 hours at room temperature. Removal of the pyridine under reduced pressure at 40°C left an oil which was dissolved in chloroform. The chloroform solution was washed at 50°C with saturated potassium bicarbonate solution, dried and evaporated under reduced pressure to yield a solid, M.pt. 110°C. (When extraction was carried out at room temperature an oil is produced in much reduced yield.) Recrystallisation from refluxing toluene yielded 0.370g., (50% yield) of solid, M.pt. 154°C which was pure by T.L.C. (11% methanol in chloroform).

I.R. (nujol)

3450-3300(s), 3100(m), 1660(s), 1630(m), 1610(m), 1595(s), 1515(s), 1495(s), 1250(s), 1195(s), 1175(s), 1080(s), 1000(m), 975(m), 870(m), 820(m), 760(s), 700(m), 680(s).

p-Nitrophenyl 2-acetamido-6-iodo-2,6-dideoxy-β-D-glucopyranoside (XV)

0.35g. of (XIV) was dissolved in 10ml. of dry acetone. 0.38g. of sodium iodide was added and the mixture refluxed for five hours by which time all of the starting material had reacted (T.L.C. 11% methanol in chloroform). The sodium p-toluene sulphonate precipitated was filtered, suspended in dry acetone, re-filtered and washed with dry acetone. The combined acetone filtrates were evaporated under reduced pressure and the solid obtained was recrystallised from water. 0.3g. (95% yield), M.pt. 197-198°C.

The weight of sodium p-toluene sulphonate precipitated was 0.127g. Theoretical release was 0.145g. showing that one primary hydroxyl group in compound (XIV) had been tosylated.

N.M.R. (CD_3OD.)

δ= 8.2 p.p.m., (2 protons), doublet, aromatic protons, J = 9cps.

δ= 7.26 p.p.m., (2 protons), doublet, aromatic protons, J = 9cps.

δ= 2.33 p.p.m., (2 protons), singlet, H_2-6.

δ= 8.00 p.p.m., (3 protons), singlet, N-acetyl methyls.

Di-N-acetyl-hexa-O-acetyl chitobiose (XVI)

This compound was prepared according to the method described by Barker[108]. Chitin as supplied by Eastman-Kodak was found to be the most satisfactory yielding 9.5g. of (XVI) per 100g. Recrystallised from methanol M.pt. 308°C (lit.[109] 308-309°C).

I.R. spectrum showed bands corresponding to the amide I and II and to the O-acetates.

Peracetylated oligosaccharides from chitin.

Chitin was hydrolysed according to the method of Barker[109] reducing the time of incubation at 55°C to one hour fifteen minutes. Work up by the described method and recrystallisation from methanol yielded 5g. of a solid mixture of the peracetylated oligosaccharides from the dimer through to the hexamer as shown by T.L.C. (1 : 9 v/v methanol, chloroform).

4.5g. of the mixture was separated by the method developed in this laboratory[107] using a (4x210cm.) column of Mallinckrodt Silicic acid (100 mesh) employing a gradient elution of methanol in chloroform (3-15%). Fractions, 30 minutes, were collected using an L.K.B. Ultrorac fraction collector and monitored by T.L.C. (1 : 9 v/v methanol, chloroform).

Routine yields of the higher peracetylated oligosaccharides (tetrameter and pentamer) of 1-1.5g. each were obtained from acetolysis mixtures which had been recrystallised two or more times from methanol.

The peracetylated oligosaccharides were recrystallised from methanol and ran as one spot on T.L.C. in the solvent system above.

I.R. spectra showed bands corresponding to the amide I and II and to the O-acetates.

M.Pts.

(a) Acetylated NAG_3 300°C (dec.)

(b) Acetylated NAG_4 320°C (dec.)

(c) Acetylated NAG_5 300°-305°C (dec.)

Analysis

Calculated for (a) $C_{40}H_{57}O_{24}N_3$; %C, 49.84; %H, 5.961; %N, 4.36; Found, %C, 49.56; %H, 6.08; %N, 4.13.

Calculated for (b) $C_{52}H_{74}O_{31}N_4$; %C, 49.92; %H, 5.96; %N, 4.48; Found, %C, 48.65; %H, 6.11; %N, 4.30.

Calculated for (c) $C_{64}H_{91}O_{38}N_5$; %C, 49.97; %H, 5.96; %N, 4.55; Found, %C, 49.20; %H, 6.07; %N, 4.26.

De-O-acetylation of the peracetylated oligosaccharides

De-O-acetylation was accomplished by the method of Zemplen[104]. The compounds were purified by chromatography using a (1.5x15cm.) column of Sephadex G-15 eluting with water. Fractions were monitored by T.L.C. (3 : 6 : 2 v/v/v ammonia, n-propanol, water). Those containing the de-O-acetylated material were combined, freeze dried and then dried in vacuum.

I.R. spectra showed amide I and II bands at 1660-1620cm^{-1} and 1565-1550cm^{-1} respectively.

The oligosaccharides were shown to be pure by T.L.C. in the solvent system above and had identical Rf. values to authentic oligomers prepared by Capon and Foster[109]. The molecular weight of the tetramer, NAG_4, was determined using a Mecrolab vapour pressure osmometer, model 301A, and was estimated as 870 (calc. 830).

M.Pts.

NAG_2 205-208°C (lit. 206-212°C)

NAG_3 305°C (dec.) (lit. 309-311°C)

NAG_4 285-295°C (lit. 290-300°C)

NAG_5 280-290°C (lit. 285-295°C)

Optical Rotations.

$NAG_2 \left[\alpha\right]_D^{25} = +16.1$ (lit. $\left[\alpha\right]_D$ 14.9 to 17.2)

$NAG_3 \left[\alpha\right]_D^{25} = +2.0$ (lit. $\left[\alpha\right]_D$ 1.8 to 2.2)

$NAG_4 \left[\alpha\right]_D^{25} = -2.5$ (lit. $\left[\alpha\right]_D$ -1.9 to -4.1)

$NAG_5 \left[\alpha\right]_D^{25} = -7.2$ (lit. $\left[\alpha\right]_D$ -6.1 to -9.1)

2-Acetamido-1,3,4,6-tetra-O-acetyl-2-deoxy-β-D-glucopyranose (XVII)

This compound was prepared according to the method of Findlay et al[101]. Recrystallisation from methanol gave compound (XVII) in 28% yield. M.pt. 186°C (lit.[101] 185-186°C)

2-Acetamido-3,4,6-tri-O-acetyl-2-deoxy-α-D-glucopyranosyl Chloride (XVIII)

This compound was prepared in 69% yield from compound (XVII) according to the method of Leaback[113]. Recrystallisation from ethyl acetate-hexane gave material, M.pt. 127°C $[\alpha]_D^{25}$ + 115° (C, 1, $CHCl_3$) [lit.[113] 133°C $[\alpha]_D^{25}$ + 118° (C, 1, $CHCl_3$)] which was used without further purification.

2-Methyl-4,5-(3,4,6-tri-O-acetyl-2-deoxy-D-glucopyrano)-Δ^2-oxazoline (IXX)

This compound was prepared from compound (XVIII) by the method of Khorlin et al[102] and isolated as a glass. A modification of the procedure used in reference (102), namely washing the reaction mixture with saturated aqueous cadmium chloride solution, proved helpful in removing the collidine from the reaction solution.

I.R. (thin film).

3020(m), 2960(m), 1740(s), 1675(s), 1432(m), 1370(s), 1315(m), 1220(s), 1125(m), 1030(s), 938(s), 750(s).

N.M.R. ($CDCl_3$)

δ = 5.93 p.p.m., (1 proton), doublet, H-1, $J_{1,2}$ = 7cps.

δ = 5.26 p.p.m., (1 proton), triplet

δ = 4.93 p.p.m., (1 proton), doublet

δ = 4.20 p.p.m., (3 protons), doublet

δ = 3.2-3.8 p.p.m., (1 proton), multiplet

δ = 2.03 p.p.m., (12 protons), broad singlet.

Identical material to the above was prepared from 2-acetamido-2-deoxy-D-glucopyranose by the method of Pravdic et al[114].

Attempted saponification of compound (IXX)

De-O-acetylation of (IXX) using triethylamine according to the method of Khorlin et al[102] proved unsuccessful. An oil was obtained which had the characteristic amide I and II bands at 1650cm^{-1} and 1540cm^{-1}, and hence the oxazoline ring had been opened during saponification.

2-Methyl-4,5-[4-O-(3,4,6-tri-O-acetyl-2-acetamido-2-deoxy-β-D-glucopyranosyl)-3,6,-di-O-acetyl-2-deoxy-D-glucopyrano]-Δ^2-oxazoline (XX)

Compound (XX) was prepared according to the method of Khorlin et al[102] with the modification in the work up procedure mentioned for compound (IXX). M.pt. 185°C (lit.[102] 189-190°C)

I.R. (nujol).

3240(m), 1745(s), 1673(s), 1655(s), 1560(s), 1310(m), 1240(s), 1160(w), 1130(m), 1035(s), 935(m), 900(w), 885(w).

N.M.R. ($CDCl_3$).

δ = 6.20 p.p.m., (1 proton), doublet N-H, J = 9cps.

δ = 5.92 p.p.m., (1 proton), doublet, J =7cps.

δ = 5.66 p.p.m., (1 proton), doublet, J = 3cps.

δ = 5.14 p.p.m., (1 proton), quintet

δ = 4.78 p.p.m., (1 proton), doublet J = 8cps.

δ = 3.3-4.5 p.p.m., (9 protons), multiplets.

δ = 2.08 p.p.m., (3 protons), singlet.

δ = 2.05 p.p.m., (9 protons), broad doublet.

δ = 1.99 p.p.m., (6 protons), broad doublet.

δ = 1.92 p.p.m., (3 protons), singlet.

Analysis.

Calculated for $C_{26}H_{36}O_{15}N_2$; %C, 50.64; %H, 5.88; %N, 4.59; Found, %C, 49.94; %H, 5.65; %N, 4.26.

Attempted saponification of compound (XX)

De-O-acetylation was attempted via the method of Khorlin et al[102]. 0.6g. of the acetylated oxazoline (XX) was dissolved in 30ml. of dry methanol. 5ml. of triethylamine was added and the solution allowed to stand for 3 days at room temperature after which the solvent was removed by evaporation under reduced pressure at 30°C. The solid product obtained was recrystallised from methanol/chloroform/ether, M.pt. 180 - 190°C. The infrared spectrum of this solid showed that is was not fully de-O-acetylated, and the amide I band was not split into two peaks as for the starting material. Further attempts to fully de-O-

acetylate this material using methanol and triethylamine resulted in the formation of NAG_2 as was confirmed by the melting point of 210°C found for the resulting solid (c.f. NAG_2 M.pt. 210°C), by comparison of the I.R. spectra for the two compounds and the T.L.C. characteristics in (2, 1, 1, v/v/v, methanol, ethyl acetate, benzene).

3,4-Dinitrophenyl β-NAG_4.

This compound was supplied by F. W. Ballardie[107] to whom the author acknowledges his gratitude.

Source of other compounds used in this study.

The compounds shown below were purchased from Koch-Light Laboratories Ltd. Prior to use they were recrystallised from aqueous ethanol and dried under vacuum.

p-Nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside. M.pt. 205-208°C.

p-Nitrophenyl β-D-xylopyranoside. M.pt. 157-158°C.

p-Nitrophenyl β-D-glucopyranoside. M.pt. 161-165°C.

Methyl 2-acetamido-2-deoxy-α-D-glucopyranoside. M.pt. 192°C.

N-acetyl-D-glucosamine. M.pt. 205°C.

Formation of oligosaccharides by transglycosylation.

Small scale experiments using p-nitrophenyl 2-acetamido-2-deoxy-β-D-xylopyranoside incubated with lysozyme in the presence of NAG_4, NAG_5 or NAG_6 showed the presence of higher molecular weight compounds containing a p-nitrophenyl glycoside as determined by T.L.C. (3 : 6 : 2, v/v/v, ammonia, n-propanol, water). The T.L.C. plate shown in fig. 2 illustrates a typical result for the incubation of 1.5mg. of p-nitrophenyl 2-acetamido-2-deoxy-β-D-xylopyranoside with 0.62mg. of NAG_5 and 0.54mg. of lysozyme in 250 ul of citrate buffer[110] pH 5.1 and 25 μl of spectroscopic dioxan at 40°C. From the T.L.C. it was estimated that the optimum time of incubation was 30 minutes.

Similar small scale experiments were performed using NAG_4 and NAG_6. In the presence of NAG_4 the optimum time of incubation for formation of transglycosylation products was 20 hours. With NAG_6 transglycosylation products were observed on T.L.C. after incubation for one minute. Fig. 3.

Preparative scale reactions, allowing separation of the transglycosylation products by column chromatography, established that on incubation of p-nitrophenyl 2-acetamido-2-deoxy-β-D-xylopyranoside with NAG_4 and lysozyme a significant amount of NAG-NAX-PNP was formed. Incubation using NAG_5 was preferred for formation of NAG_2-NAX-PNP and NAG_3-NAX-PNP. Preparative scale reactions also established that when using NAG_4 in the transglycosylation reaction larger amounts of products were formed by using an equal amount by weight of reducing sugar and monomer glycoside.

For each of the transglycosylation reactions described below small scale T.L.C. experiments were performed to estimate the optimum conditions.

Separation of the glycosides formed by transglycosylation was achieved

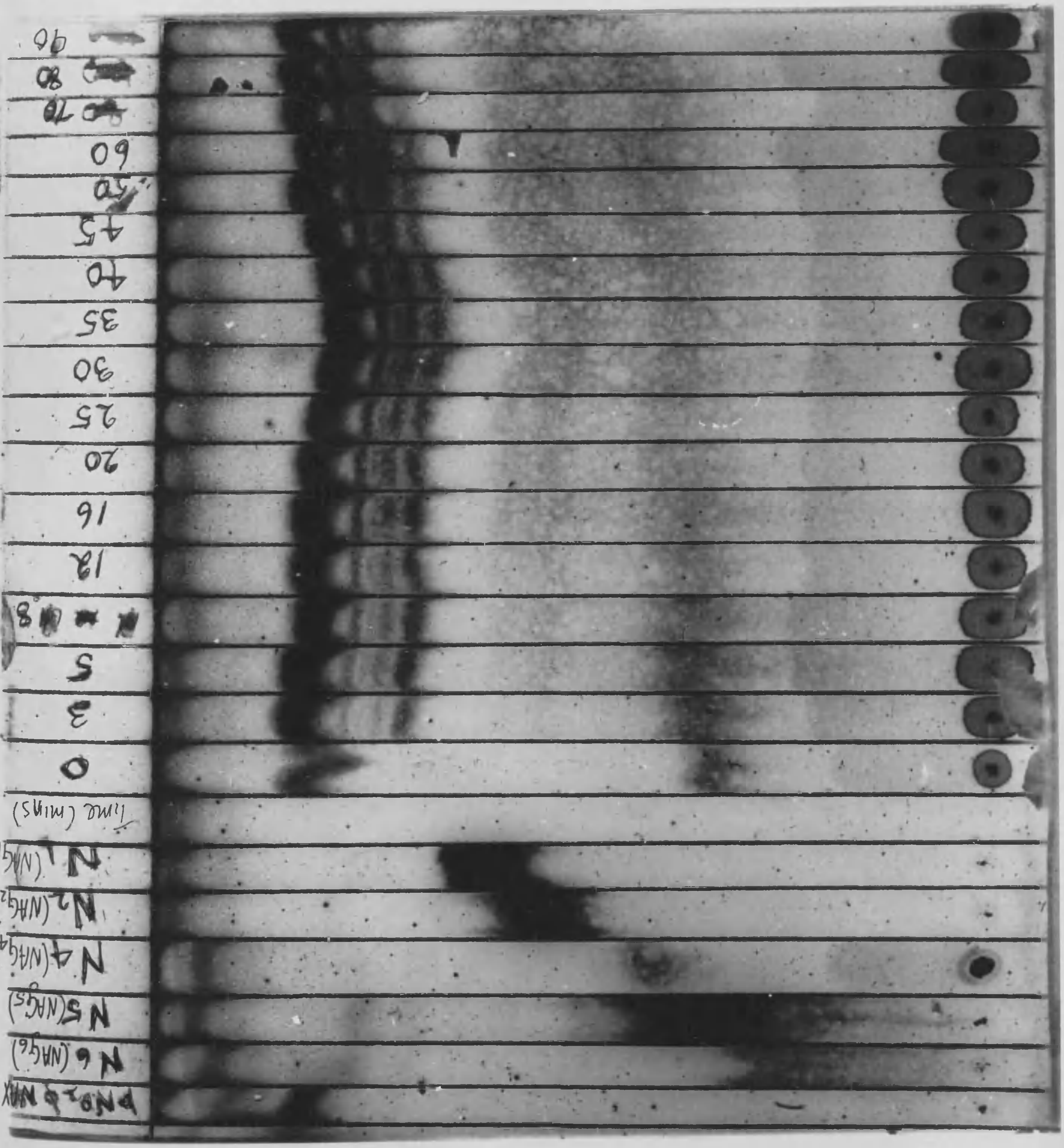

90
80
70
60
50
45
40
35
30
25
20
16
12
8
5
3
0
time (mins)
N1 (NAG1)
N2 (NAG2)
N4 (NAG4)
N5 (NAG5)
N6 (NAG6)
PNO2 φ NAX

Fig 2

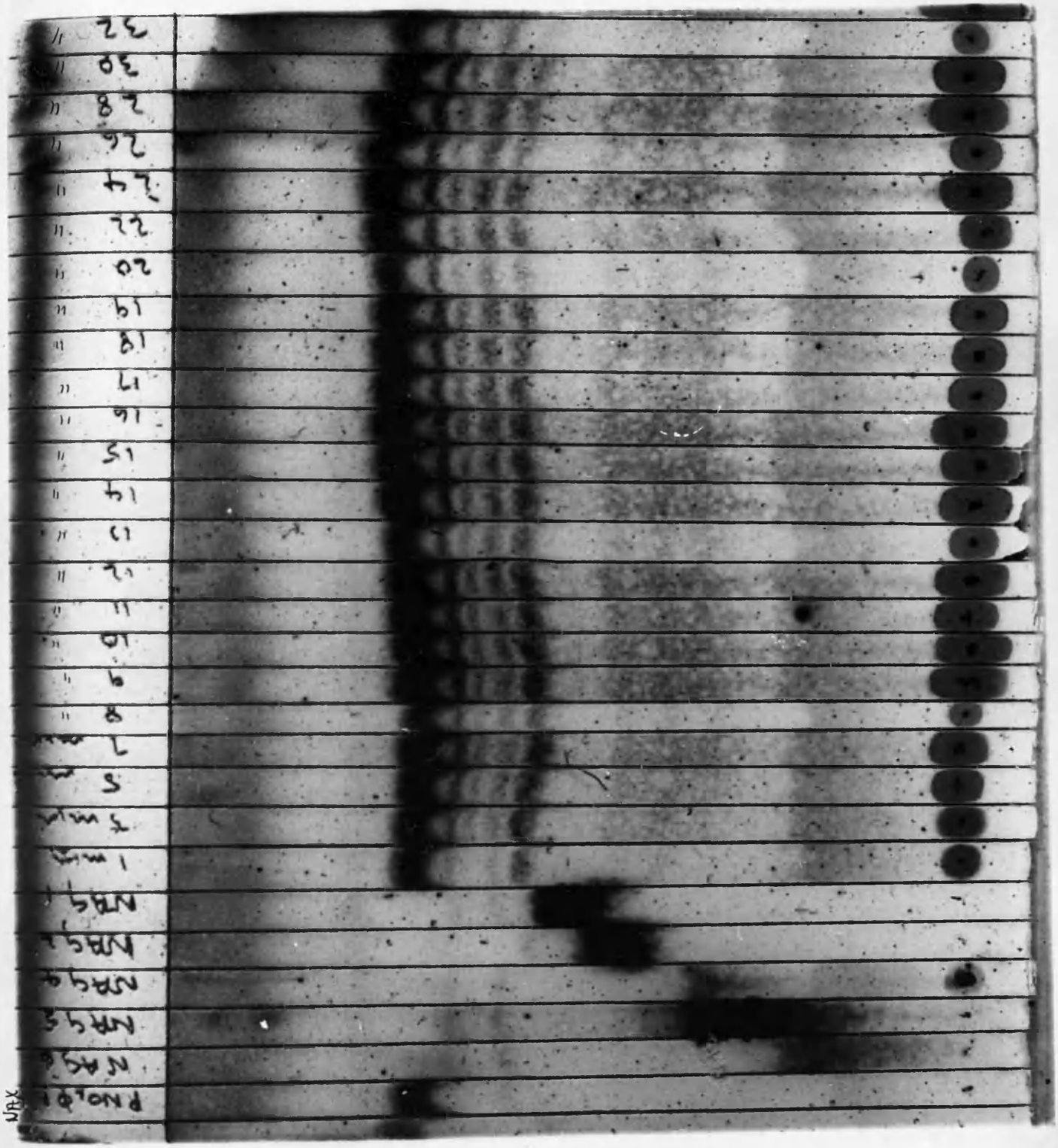


Fig 3

by chromatography on a (1.5x210cm.) column of Sephadex LH20 eluting with water at a flow rate of 12ml. per hour. The effluent was monitored for glycosides using a Cecil Instruments C.E.212 Variable Wavelength Ultraviolet Monitor set at the wavelength for maximum absorption of the glycoside. The monitor was linked to a Servoscribe potentiometric recorder, providing a continuous trace of the effluent. Fractions containing a glycoside were combined, stirred with Amberlite M.B.1. resin to remove any traces of reducing sugars, filtered and freeze dried. After drying in vacuo the extinction coefficient of the glycoside was measured and compared with that of the monomer glycoside. If the extinction coefficient was less than expected the glycoside was dissolved in the minimum amount of water and rechromatographed on a (1.5x60cm.) column of Sephadex G-15. Boiled out distilled water was used when eluting from Sephadex columns.

Transglycosylation reactions with p-Nitrophenyl 2-acetamido-2-deoxy-β-D-xylopyranoside

0.15g. of p-nitrophenyl 2-acetamido-2-deoxy-β-D-xylopyranoside, 0.15g. of NAG_4 and 0.024g. of lysozyme were dissolved in 30ml. of citrate buffer pH 5.1 and 3ml. of spectroscopic dioxan. After incubation at 40°C for 20 hours the mixture was chromatographed as described above. 12mg. of NAG-NAX-PNP, 4mg. of NAG_2-NAX-PNP and 2mgs. of NAG_3-NAX-PNP were isolated.

0.3g. of p-nitrophenyl 2-acetamido-2-deoxy-β-D-xylopyranoside, 0.15g. of NAG_5 and 0.11g. of lysozyme were dissolved in 50ml. of citrate buffer pH 5.1 and 5ml. of spectroscopic dioxan. After incubation at 40°C for 30 minutes the reaction was terminated by the addition of di-

sodium tetraborate buffer pH 9.7. After chromatography 5mg. of NAG-NAX-PNP, 6mg. of NAG_2-NAX-PNP and 4mg. of NAG_3-NAX-PNP were isolated.

The glycosides isolated were pure by T.L.C. (3 : 6 : 2, v/v/v, ammonia, n-propanol, water).

Characterisation of the transglycosylation products.

NAG-NAX-PNP

M.pt. 206-212°C (dec.)

N.M.R. (D_2O) (8mg. in 0.5ml. C.A.T. 16 times).

The spectrum showed characteristic resonance signals for the aromatic protons with a coupling constant J = 9cps., the anomeric proton, a doublet, with coupling constant $J_{1,2}$ = 6cps, and two sharp signals corresponding to the N-acetyl methyl protons a distance of 4cps. apart.

I.R. (nujol)

The spectrum showed characteristic bands at 1635-1660cm^{-1}, amide I; 1540-1570cm^{-1}, amide II, and bands due to the aromatic group at 1610, 1595, 1520 and 1498cm^{-1}.

U.V.

8.08mg. of NAG-NAX-PNP was dissolved in 1.5ml. of distilled water. 50 µl. of this solution made up to 2.5ml. with water had an absorbance of 1.67 a.u. at 300nm. giving a concentration by U.V. of 8.35×10^{-3}M for

the original solution. 500 µl. of N/1 sodium hydroxide was added to the solution. After incubation of the sealed solution at 80°C for three hours the solution had an absorbance of 2.56 a.u. at 400nm. leading to a concentration by U.V. of 8.52×10^{-3}M for the original solution. Concentration of the original solution by weight was 10.4×10^{-3}M.

NAG_2-NAX-PNP.

M.pt. 255-260°C

N.M.R. (D_2O)

The anomeric proton could not be clearly distinguished. Three sharp signals in the region expected for N-acetyl methyl groups (approximately δ= 2 p.p.m.) were present.

U.V.

1.47mg. of NAG_2-NAX-PNP was dissolved in 1ml. of distilled water. 0.2ml. of this solution made up to 2.5ml. with water had an absorbance of 0.13 au at 300nm.. Expected absorbance for this solution calculated on the molecular weight for NAG_2-NAX-PNP was 0.15 au.

NAG_3-NAX-PNP.

1.33mg. was dissolved in 1ml. of acetate buffer pH 5.2. 0.1ml. of this solution in 2.5ml. of buffer had an absorbance of 0.463 au. at 300nm. Expected absorbance for this solution calculated on the molecular weight for NAG_3-NAX-PNP was 0.57.

Transglycosylation reaction with 3,4-Dinitrophenyl 2-acetamido-2-deoxy-β-D-xylopyranoside.

0.15g. of 3,4-dinitrophenyl 2-acetamido-2-deoxy-β-D-xylopyranoside, 0.15g. of NAG_5 and 0.024g. of lysozyme were dissolved in 30ml. of citrate buffer pH 5.1 and 3ml. of spectroscopic dioxan. After incubation at 40°C for 2 hours the mixture was chromatographed on a (1.5x60 cm) column of sephadex G-15 and the effluent was monitored at 284nm. This small Sephadex column was used due to the high spontaneous rate of hydrolysis of the 3,4-dinitrophenyl glycosides which would have resulted in appreciable hydrolysis of the transglycosylation products during the time required for separation using the larger Sephadex column. The small column did not however give a complete separation of the transglycosylation products. Three fractions were taken consisting mainly of trimer, monomer and dimer glycosides respectively. These were stirred for ten minutes with Amberlite M.B.1. resin to remove reducing sugars, filtered and freeze dried. The fractions were then each dissolved in the minimum amount of water and rechromatographed separately on the column of Sephadex G-15, monitoring for glycosides as before at 284nm. This second separation gave the pure dimer and trimer after further treatment of the respective solutions eluted from the column with Amberlite M.B.1. resin and freeze drying.

The compounds separated were pure by T.L.C. (6, 3, 2, v/v/v, n-propanol, ammonia, water). The fastest running compound was identified as the monomer, 3,4-dinitrophenyl 2-acetamido-2-deoxy-β-D-xylopyranoside by comparison with an authentic standard.

NAG-NAX-DNP. 1.4mg. was dissolved in 0.1M acetate buffer pH 5.23. 0.010ml. of this solution made up to 2.5ml. with buffer had an absorbance of 0.04 au. at 284nm. Expected absorbance at this wavelength, 0.05 au.

NAG_2-NAX-DNP. 1.9mg. was dissolved in 0.1M acetate buffer pH 5.23. 0.010ml. of this solution made up to 2.5ml. with buffer had an absorbance of 0.04 au. at 284nm. Expected absorbance at this wavelength, 0.05 au.

Transglycosylation reaction with p-Nitrophenyl β-D-glucopyranoside.

0.8g. of p-nitrophenyl β-D-glucopyranoside, 0.35g. of NAG_4 and 0.35g. of lysozyme were dissolved in 60ml. of citrate buffer pH 5.1 and 6ml. of spectroscopic dioxan. After incubation at 40°C for 20 hours the mixture was chromatographed. Three glycosides were separated and shown to be pure by T.L.C. (6, 3, 2, v/v/v, n-propanol, ammonia, water). The last glycoside to be eluted from the column was the monomer, p-nitrophenyl β-D-glucopyranoside. 20mg. of NAG-Glu-PNP was isolated and characterized as shown below. The first glycoside eluted from the column, (3mg.), appeared from T.L.C. to be NAG_2-Glu-PNP.

NAG-Glu-PNP.

M.pt. 253-260°C (dec.) (lit.[57] 256-258°C (dec.))

U.V.

1.18mg. of NAG-Glu-PNP was dissolved in 2ml. of water and hydrolysed by adding 2ml. of 3N hydrochloric acid and heating at 100°C for 30 minutes. 1ml. of the solution was neutralised by the addition of 5N sodium hydroxide solution and the resulting solution was made up to 10ml. with 0.1N sodium hydroxide solution. This solution had an absorbance of 0.11 au. at 400nm. The calculated absorbance for this solution was 0.104 au.

In a separate estimation 1.26mg. of NAG-Glu-PNP was dissolved in 1ml. of citrate buffer pH 5.2. 0.1ml. of this solution diluted to 2.5ml. with citrate buffer pH 5.2 had an absorbance of 1.1 au. at 300nm. The calculated absorbance for this solution was 1.2 au.

N.M.R. (H_2O)

The N.M.R. spectrum of NAG-Glu-PNP showed one *N*-acetyl methyl resonance at the expected frequency.

I.R. (nujol).

The I.R. spectrum showed bands at 1625 cm^{-1} and 1570 cm^{-1} corresponding to amide I and II frequencies, as well as strong aromatic bands at 1610 cm^{-1}, 1595 cm^{-1} and 1505 cm^{-1}.

Transglycosylation reactions with p-Nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside.

0.48g. of p-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside, 0.13g. of NAG_4 and 0.11g. of lysozyme were dissolved in 50ml. of citrate buffer pH 5.1 and 5ml. of spectroscopic dioxan. After incubation at 40° for 20 hours the mixture was chromatographed. 15mg. of p-nitrophenyl β-NAG_2 and 2mg. of p-nitrophenyl β-NAG_3 were isolated and characterised by comparison with chemically synthesized material prepared according to the method of Osawa[103,111]. Due to the ease of preparation of acetylated NAG_3, previously described, it was more convenient to prepare p-nitrophenyl β-NAG_3 chemically than enzymatically.

p-Nitrophenyl β-NAG_2

	Enzymatic synthesis	Chemical synthesis
M.pt.	220°C (dec.)	222°C (dec.)(lit.[103] 226-227°C)

N.M.R.(D_2O)

The spectrum showed characteristic resonance signals for the N-acetyl methyl protons a distance of 7cps. apart.

I.R.(nujol)

The spectrum showed characteristic bands at 1630-1660cm^{-1}, amide 1; 1540-1570cm^{-1}, amide 11, and bands due to the aromatic group at 1605, 1595,1515, and 1500cm^{-1}

U.V.

10µl of a 4.13×10^{-3}M solution of p-nitrophenyl β-NAG_2 added to 3ml. of citrate buffer pH 5.2 had an absorbance of 0.13 au. at 300nm. Expected absorbance was 0.137au.

p-Nitrophenyl β -NAG_3

M.pt. 236-238°C (lit.[111] 238-240°C)

The material prepared chemically by the method of Osawa was identical by T.L.C. (6, 3, 2, v/v/v, n-propanol, ammonia, water) to the material prepared enzymatically.

U.V.

0.1ml. of a stock solution of 5×10^{-4}M in 2.5ml. of water had an absorbance of 0.18 au. at 300nm. Calculated absorbance for this solution was 0.20 au.

Transglycosylation reaction with p-Nitrophenyl β-D-xylopyranoside.

0.9g. of p-Nitrophenyl β-D-xylopyranoside, 0.48g. of NAG_4 and 0.2g. of lysozyme were dissolved in 75ml. of citrate buffer pH 5.1 and 7.5ml. of spectroscopic dioxan. After incubation at 40°C for 20 hours the mixture was chromatographed. The effluent showed the presence of four glycosides as separate peaks from the U.V. monitor. The separated glycosides were treated with Amberlite and freeze dried. T.L.C. (3 : 6 : 2, v/v/v, ammonia, n-propanol, water) showed that the first two glycosides eluted from the column each consisted of two compounds which ran very close together in the solvent system used. The third glycoside ran as one spot and the fourth glycoside was shown by T.L.C. to be p-nitrophenyl β-D-xylopyranoside. By comparison with T.L.C. data from transglycosylation reactions with other monomer glycosides the glycoside which was eluted third from Sephadex LH20 was suspected to be the dimer NAG-XYL-PNP, the configuration of the glycosidic linkage between the sugar residues being unknown. The first three glycosides eluted from the Sephadex LH20 column were rechromatographed separately on Sephadex G-15. The first and second glycosides from the LH20 column were not resolved into their separate components and still showed two spots each on T.L.C. Since separation by Sephadex is by molecular size it was considered that the fractions consisting of two spots on T.L.C. involved the same number of sugar residues, but consisted of two compounds differing in the position of linkage between the NAG and the XYL residues.

The dimer suspected to be NAG-Xyl-PNP had a melting point of 230-235^{o}C (dec.). 2.3mg. of this compound was dissolved in 0.1M acetate buffer pH 5.23. 0.1ml. of this solution in 2.45ml. had an absorbance of 0.325 au. at 300nm., leading to an estimated value of 0.8×10^{-3}M for the concentration of the original solution. The expected concentration of the original solution calculated for the molecular weight of NAG-Xyl-PNP was 0.97×10^{-3}M.

Transglycosylation reaction with p-nitrophenyl 2-deoxy-β-D-glucopyranoside.

0.4g. of p-nitrophenyl 2-deoxy-β-D-glucopyranoside, 0.2g. of NAG_4 and 0.2g. of lysozyme were dissolved in 200ml. of citrate buffer pH 5.1 and 20ml. of spectroscopic dioxan. Complete solubility of the glycoside was not achieved. After incubation at 40°C for 16 hours no higher glycosides were observed by T.L.C. Separation by chromatography on Sephadex LH20 yielded only the monomer glycoside.

In a separate experiment 0.14g. of p-nitrophenyl 2-deoxy-β-D-glucopyranoside, 0.14g. of NAG_4 and 0.020g. of lysozyme, were dissolved in 30ml. of a 10 : 4 v/v mixture of acetate buffer pH 5.25 and spectroscopic dioxan. After incubation at 40°C for three hours the mixture was freeze dried. The solid obtained showed no evidence of transglycosylation products on T.L.C. It was dissolved in di-sodium tetraborate buffer pH 9.7 and chromatographed on Sephadex LH20 yielding only the monomer glycoside. The T.L.C. of the reaction mixture showed evidence of hydrolysis of the NAG_4 hence the enzyme was active.

EXPERIMENTAL DISCUSSION.

Lysozyme's ability to catalyse transglycosylation reactions with acceptor molecules other than water has been used to study the nature of subsites E and F of the enzyme[86,87], and by using various aryl glycosides as acceptor molecules to determine if the products of the transglycosylation reaction are substrates for the enzyme[88,89].

Isolation of the products formed from transglycosylation reactions with aryl glycoside acceptors provides a source of oligosaccharides containing 2-acetamido-2-deoxy sugar residues with differing terminal sugar residues which would be difficult to synthesize chemically by a Koenigs-Knorr type of reaction, or via the 2-methyl-glyco [1',2': 4,5] -2-oxazoline. Utilizing the transglycosylase activity of lysozyme for the preparation of oligosaccharides does however have two major limitations in that, (a) the position of the linkage formed between the sugar residues and its stereochemistry is not unambiguously defined unless chemical tests or comparisons with chemically synthesized material are performed with the isolated oligosaccharide, and, (b) isolation, on a preparative scale, is only feasible for those oligosaccharides which are poor substrates for lysozyme.

Natural substrates for lysozyme have the oligosaccharide sugar residues β (1-4) linked, and for valid comparisons of the enzymic activity to be made this linkage pattern must be shown in the products of the transglycosylation reaction. Raftery[89] and Dahlquist[112] have shown that for the hydrolysis of di-N-acetyl chitobiose by lysozyme in the presence of methanol, the β configuration is retained in the product methyl β-NAG to at least 99.9% confirming the observation of other workers. Indirect evidence that the linkage formed in the transglycosylation reaction has the β (1-4) configuration was presented by the finding of Chipman that

the higher molecular weight oligosaccharides isolated from the reaction of $(NAG\text{-}NAM)_2$ with lysozyme were also substrates for lysozyme. p-nitrophenyl β-NAG_2 chemically synthesised from di-N-acetyl chitobiose has been shown to be identical to the disaccharide glycoside isolated from the transglycosylation reaction using p-nitrophenyl β-NAG and NAG_4[56]. The linkage between the sugar residues in enzymatically synthesized NAG-Glu-PNP has also been shown to be a (1-4) linkage.

Even if retention of configuration obtains in the lysozyme catalysed transglycosylation reaction the position of the linkage may depend on the nature of the acceptor molecule and the orientation with which it is bound in the active cleft. The observation that p-nitrophenyl β-D-xylose forms higher molecular weight aryl glycosides by the transfer reaction without release of p-nitrophenol was confirmed by the results from this study in agreement with the results of others. It seems also possible that the mixture of products obtained from transglycosylation could consist of homologues having different linkages at the terminal sugar residue.

That the isolation of the oligosaccharides formed by the transfer reaction is only possible if their rate of hydrolysis by lysozyme is relatively slow has been shown by the observation that p-nitrophenyl β-NAG_4 was not obtained in any appreciable amounts from the transglycosylation reaction with p-nitrophenyl β-NAG and NAG_4. p-Nitrophenyl β-NAG_4, which would be expected to be a product of the reaction, has a specificity,[107] k_{cat}/K_m, of $0.95 l Mol^{-1} sec^{-1}$ for hydrolysis by lysozyme which could thus be taken as the upper limit of specificity at which substrates for the enzyme can be isolated from the transfer reaction. Even p-nitrophenyl β-NAG_3 which has a poor specificity for lysozyme[107], $k_{cat}/K_m = 0.20 l Mol^{-1} sec^{-1}$, was not isolated in significant quantities from the transglycosylation reaction.

Using transglycosylation reactions for the synthesis of aryl glycosides is also difficult if the glycoside has a high spontaneous rate of hydrolysis under the conditions used for the transglycosylation reaction. Thus the amounts of the higher oligosaccharides isolated from the transglycosylation reaction with 3,4-dinitrophenyl β-NAX was much less than that isolated using p-nitrophenyl β-NAX. This difficulty could be overcome by using NAG_6 instead of NAG_4 or NAG_5 in the transglycosylation reaction which would lead to the formation of the higher oligosaccharides by transfer at a much faster rate.

Unless comparisons of the oligosaccharides can be made with chemically synthesized material characterisation becomes difficult since oligosaccharides in general do not give good analytical data due to strongly bound water molecules being present. Other methods of characterisation can be tedious, i.e. periodate oxidation of NAG-Glu-PNP[56], and do not always give unambiguous assignments of structure.

In spite of these limitations however the transglycosylase activity of lysozyme has allowed investigations to be made of some of the properties of the enzyme which would have been difficult using other methods.

Extinction Coefficients.

Extinction coefficients for the p-nitrophenyl glycosides studied were measured at 40°C using a Cary 16 spectrometer. Stock solutions of the sugars were variously prepared using distilled water, 0.1M acetate buffer pH 5.1 to 5.5, and 0.1M citrate buffer pH 5.5. Aliquots of these solutions were added to 2.5ml. of the same solvent in 1cm. spectrosil quartz U.V. cells and the absorbance taken. p-Nitrophenyl β-NAG recrystallised from aqueous ethanol and dried at 40°C under vacuum had a

measured extinction coefficient of 10,000 at 300nm. for an aqueous solution. Other p-nitrophenyl glycosides studied were also found to have an extinction coefficient of 10,000 at 300nm. under the above conditions. The normally accepted value for the extinction coefficient of a p-nitrophenyl glycoside at 300nm. is 12,000[56]. Since sugars are hygroscopic and the tightly bound water molecules can only be removed by fairly strong heating under vacuum, the difference found in the value for the extinction coefficient was more than likely due to the presence of one or more molecules of water bound to the sugar molecule. Strong heating of the glycosides was avoided and the extinction coefficient of 10,000 at 300nm. was used for the estimation of solutions containing p-nitrophenyl glycosides.

2-Methyl-Δ^2-oxazolines.

The preparation of the oxazolines, 2-methyl-4,5-(2-deoxy-D-gluco-pyrano)-Δ^2-oxazoline and 2-methyl-4,5- 4-O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-deoxy-D-glucopyrano -Δ^2-oxazoline, was attempted as it was considered possible that the interaction of these compounds, and more possibly the higher oligomers, with lysozyme could have provided information on the mechanism of action of lysozyme. Participation by the acetamido group of the sugar residue undergoing glucosyl-oxygen fission has been postulated as a possible mechanism for the lysozyme catalysed hydrolysis of glycosides and since the oxazoline intermediate formed by this pathway would be expected to exist with the sugar residue in a conformation nearer to the half chair than the normal chair conformation, a chemically synthesized oxazoline of the type shown above could be considered to be a transition-state analogue for lysozyme hydrolysis by this mechanism. It was hoped to test these postulates by studying the binding of these compounds to lysozyme and by measuring the rate of hydrolysis of the oxazoline ring in the presence of enzyme. Unfortunately it was not found possible to prepare the compounds as saponification of the corresponding O-acetylated derivatives resulted in the opening of the oxazoline ring.(see preparative experimental).

The N.M.R. spectrum of the O-acetylated oxazoline derivative prepared from NAG shows a doublet at δ = 5.93 p.p.m. having a coupling constant J = 7cps.

From the chemical shift value this doublet is most likely due to the H-1 resonance. The most likely conformation for the sugar ring of this compound is the half chair conformation. This would result in the protons attached to C(1) and C(2) being in an eclipsed conformation, or

nearly so. The Karplus equation predicts a coupling constant of approximately 8cps. for cis-coplaner vicinal protons. The exact magnitude of this coupling constant has been shown[132-134] to be dependent on the electronegativities of the substituents attached to the carbon atoms. The value found for $J_{1,2}$ = 7cps. for the O-acetylated oxazoline derivative of NAG would therefore seem consistent with the sugar residue existing in the half chair conformation. Similarly the O-acetylated oxazoline derivative of NAG_2 would also appear to exist with the oxazoline sugar residue in the half chair conformation from the coupling constant J = 7cps. found for the doublet at δ = 5.92 p.p.m. which was assigned to H-1 of the oxazoline sugar residue.

In order to determine the configuration and obtain some idea of the conformation of the aryl glycosides of NAX and 2-deoxy-D-glucose the N.M.R. spectra of the O-acetylated derivatives were studied. The N.M.R. spectrum of 2-acetamido-1,3,4-tri-O-acetyl-2-deoxy-β-D-xylopyranose was also studied for comparison with the aryl glycosides and to provide further proof of its structure. The O-acetylated compounds were chosen for study as the N.M.R. spectra of the de-O-acetylated compounds are unsuitable since the ring protons, with the exception of H-1, resonate close together. The O-acetylated derivatives of sugars generally have the ring protons spread over a wider energy range and their solubility in organic solvents aids the recording and interpretation of the spectra. Numerous O-acetylated sugars have been studied by this method and their conformations in the solvents used determined[115-117]. Comparison of the coupling constants $J_{1,2}$ cps. for the O-acetylated and de-O-acetylated derivatives enables comparisons to be made about their conformations.

The N.M.R. spectra for the compounds shown in the table were recorded on a Varian HA-100 spectrometer. The ring protons were assigned by decoupling experiments and the N-H resonance by D_2O exchange which took up to two hours for complete exchange. The accurate coupling constants for the NAX compounds were determined from the 220MHz spectra which spread out the ring protons.

N.M.R. Data for O-Acetylated Glycosides.

Chemical Shifts, δ p.p.m.

Compound	N-H	H-1	H-2	H-3	H-4	H-5	H-5'
A	5.94	5.72	4.25	5.0	4.95	4.15	3.75
B	6.16	5.43	4.38	5.06	4.93	4.20	3.69

	H-1	H_a-2	H_e-2	H-3	H-4	H-5	H_2-6
C	5.21	2.37	2.60	3.97	5.04	3.97	4.17

Coupling Constants, Jcps.

Compound	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$J_{4,5'}$	$J_{5,5'}$
A	6	8	8	4	6.25	-12.25
B	4	4	6	2	3	-13

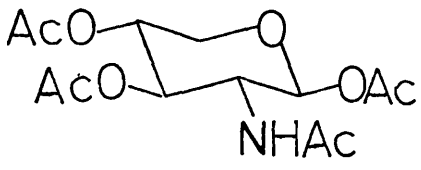
	$J_{1,2a}$	$J_{1,2e}$	$J_{2e,2a}$	$J_{2e,3}$	$J_{2a,3}$	$J_{4,5}$
C	8	2	-13	5	12	9

Note A = 2-Acetamido -1,3,4-tri-O-acetyl-2-deoxy-β-D-xylopyranose

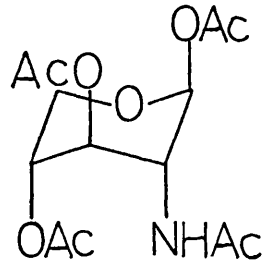
B = p-nitrophenyl 2-acetamido-3,4-di-O-acetyl-2-deoxy-β-D-xylopyranoside.

C = p-nitrophenyl 3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranoside.

From the data found for the coupling constants 2-acetamido-1,3,4-tri-O-acetyl-2-deoxy-β-D-xylopyranose appears to exist in the CI conformation in $CDCl_3$ solution.



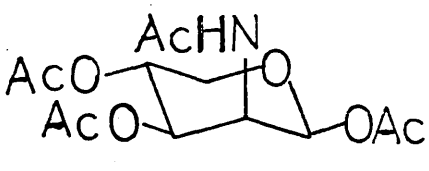
C I



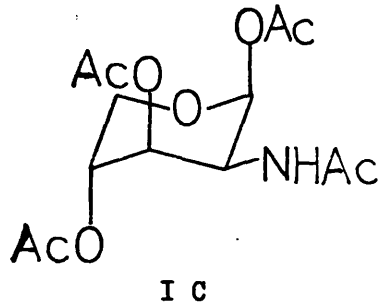
I C

β-D-xylo

The N.M.R. results confirm that the compound indeed has the β-D-xylo configuration. It was felt necessary to unambiguously confirm this, even although the observed melting point for the compound corresponded to that previously reported (see experimental), since the addition of nitrosyl chloride to unsaturated sugars and the subsequent reduction of the acetylated oxime has been reported[97] as giving rise to sugars having different configurations at the carbon atom carrying the N-acetyl group. Had this occurred during the course of this experimental work it could have given rise to a sugar having the β-D-lyxo configuration which could exist in the conformations shown below.



C I



I C

β-D-lyxo

Approximate coupling constants expected for the possible conformations that a β-D-xylo and a β-D-lyxo sugar residue could adopt are shown below. Comparison of these values with the observed coupling constants for the sugar ring protons of 2-acetamido-1,3,4-tri-O-acetyl-2-deoxy-β-D-xylopyranose confirms its conformation.

		$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$J_{5,5'}$
β-D-xylo	CI	7	7	7	3	7
	IC	3	3	3	3	3
β-D-lyxo	CI	3	3	7	3	7
	IC	3	3	3	3	3

In contrast to the above finding, the coupling constants found for p-nitrophenyl 2-acetamido-3,4-di-O-acetyl-2-deoxy-β-D-xylopyranoside suggest that this compound exists predominately in the IC conformation with all the groups on the sugar ring adopting an axial conformation. This is somewhat surprising since for all the β-D-xylopyranose derivatives which have been studied by N.M.R.[116] only the β-chloride has been shown to favour the IC conformation.

The coupling constant $J_{1,2}$ measured for the de-O-acetylated glycoside, p-nitrophenyl 2-acetamido-2-deoxy-β-D-xylopyranoside, in d_5 pyridine was 6cps., consistent with the compound existing to a larger extent in the CI conformation.

The coupling constants found for p-nitrophenyl 3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranoside are consistent with this compound existing predominately in the CI conformation as expected for a hexopyranose sugar due to the preference of the hydroxymethyl group for the equatorial conformation. The N.M.R. study also confirms that this compound is the β-glycoside.

RESULTS

Kinetic Experimental

The rates of hydrolysis of the aryl glycosides were determined by following the release of p-nitrophenol or 3,4-dinitrophenol by the following methods.

Method (a)

Reaction mixtures containing a drop of toluene to inhibit bacterial growth were incubated at 40°C in stoppered vials. 100µl. aliquots of the reaction mixture were removed at various time intervals, added to 3ml. of 0.1M di-sodium tetraborate buffer, pH9.35, in 10mm. spectrosil quartz cells and the absorbance of the resulting solution was measured at 400nm using either a Zeiss PMQ11 spectrophotometer or a Cary Model 14 spectrophotometer. The extinction coefficient of 18,000 was used for the quantitative estimation of the p-nitrophenol released.

Method (b)

All other rate measurements were performed using a Cary Model 16 spectrophotometer. The temperature of the cell block was maintained constant at 40°C by means of a thermostatting bath fitted with an electronic relay system and was measured using an N.P.L. calibrated thermometer.

10mm. and 2mm. spectrosil quartz cells were used. In the case of experiments where 10mm. cells were employed the cell plus the buffer solution was allowed to equilibrate in the cell compartment for 30 minutes before addition of the enzyme and substrate solutions. After addition the cell was shaken, replaced in the cell compartment and allowed to re-equilibrate for a short time (the length of time varied with the substrate, but for the p-nitrophenyl glycosides was of the order of a few minutes). In the cases where 2mm. cells were used the buffer solution plus the substrate was allowed to equilibrate for 30 minutes before addition of the

enzyme, then allowed to re-equilibrate for one minute.

The absorbance data output from the Cary 16 was led directly into a digico micro 16P computer linked on line to the spectrometer. The data was analysed using a program written by Dr. B. Capon which fitted the absorbance readings to the equation; $y=a+bx+cx^2$ using a generalised least squares method according to the method of Wentworth[118], allowing calculation of the initial reaction rates.

For the p-nitrophenyl glycosides an extinction coefficient of 2680 for the change in absorbance at 350nm. was used. For the 3,4-dinitrophenyl glycosides the extinction coefficient for 3,4-dinitrophenol at 400nm. at the buffer pH used in the experiment was determined prior to the experiment and over the buffer pH range used was ca. 5,500-6160.

Solutions and Buffers

'Analar' grade reagents were used for the preparation of buffer solutions. Solutions were prepared using degassed, distilled water. Merck 'spectrograde' dioxan was used for solutions containing dioxan.

pH Measurements

The pH of the buffer solutions was measured at the temperature of the experiment using a Radiometer Model 26 pH Meter having an external temperature compensator. The pH meter incorporated a Radiometer type G202C glass electrode and a type K401 calomel electrode. The meter was standardised against commercial standard buffers complying to BS 1647, 1961.

Lysozyme Cleavage of p-Nitrophenyl pyranosides

Stock solutions of lysozyme, NAG_4 and aryl pyranoside were freshly made up prior to use in 0.1M citrate buffer pH 5.3 containing 10% v/v

of spectroscopic dioxan. Aliquots of the stock solutions were mixed and made up to a final volume of 1.6 ml. with buffer solution to give the final concentrations shown in table 1. The rate of release of p-nitrophenol was followed by method (a). The results are shown graphically in figures 1-5 and the graphically estimated initial rates ($M\ sec^{-1}$) and rate constants are shown in table 1.

Stock solutions were prepared at the following concentrations:

Lysozyme	1.6×10^{-3} M
NAG_4	1.2×10^{-2} M
p-Nitrophenyl β-NAG	1.25×10^{-2} M and 1.0×10^{-1} M
p-Nitrophenyl β-NAX	1.25×10^{-2} M and 1.0×10^{-1} M
p-Nitrophenyl β-Glu	1.25×10^{-2} M and 1.0×10^{-1} M

Table 1.

Hydrolysis of p-Nitrophenyl pyranosides

Run	Compound	$[S_o]$ M	$[E_o]$ M	$[NAG_4]$ M	Rate Msec^{-1}	k_{obs} sec^{-1}
1	p-Nitrophenyl β-NAG	3.12×10^{-3}	4×10^{-4}	6×10^{-3}	4.1×10^{-9}	1.3×10^{-6}
2	"	3.12×10^{-3}	4×10^{-4}	6×10^{-3}	3.3×10^{-9}	1.1×10^{-6}
3	"	3.12×10^{-3}	4×10^{-4}	6×10^{-3}	3.9×10^{-9}	1.2×10^{-6}
4	"	3.12×10^{-3}	4×10^{-4}	-	4.6×10^{-10}	1.5×10^{-7}
5	"	2.5×10^{-2}	4×10^{-4}	6×10^{-3}	3.5×10^{-8}	1.1×10^{-6}
6	p-Nitrophenyl β-NAX	3.12×10^{-3}	4×10^{-4}	6×10^{-3}	0	
7	"	3.12×10^{-3}	4×10^{-4}	6×10^{-3}	3.3×10^{-10}	1×10^{-7}
8	"	3.12×10^{-3}	4×10^{-4}	6×10^{-3}	6.8×10^{-10}	2.2×10^{-7}
9	"	3.12×10^{-3}	4×10^{-4}	-	7.1×10^{-10}	2.3×10^{-7}
10	"	3.12×10^{-3}	-	6×10^{-3}	8.3×10^{-10}	2.5×10^{-7}
11	"	2.5×10^{-2}	4×10^{-4}	6×10^{-3}	2.6×10^{-9}	1.0×10^{-7}
12	"	2.5×10^{-2}	4×10^{-4}	6×10^{-3}	3.7×10^{-9}	1.2×10^{-7}
13	p-Nitrophenyl β-Glu	3.12×10^{-3}	4×10^{-4}	6×10^{-3}	1.6×10^{-10}	0.5×10^{-7}
14	"	3.12×10^{-3}	4×10^{-4}	-	-	
15	"	3.12×10^{-3}	-	6×10^{-3}	-	
16	"	2.5×10^{-2}	4×10^{-4}	6×10^{-3}	2.6×10^{-9}	1×10^{-7}
17	"	2.5×10^{-2}	4×10^{-4}	6×10^{-3}	2.6×10^{-9}	1×10^{-7}
18	"	2.5×10^{-2}	4×10^{-4}	6×10^{-3}	2.9×10^{-9}	1.1×10^{-7}
19	"	2.5×10^{-2}	4×10^{-4}	-	-	

Note The rates were measured from the graphical data and represent the maximum rate observed for the cases where an induction period was found. The graphical representation provides a better illustration of the relative hydrolysis rates and the above data can only be considered semi-quantitative.

Although expressed as k_{obs} sec^{-1} the rate constants for hydrolysis

in these systems are not simple first order rate constants since the hydrolysis is complicated by transglycosylation reactions. The above representation merely allows some comparison to be made between different concentrations of the aryl glycosides used in the study.

Table 2

Hydrolysis of p-Nitrophenyl pyranosides

Run	Compound	$[S_o]$ M	$[E_o]$ M	$[NAG_4]$ M	Rate $Msec^{-1}$
1	p-Nitrophenyl β-NAG	3.12×10^{-3}	-	-	3.6×10^{-10}
2	"	3.12×10^{-3}	4×10^{-4}	6×10^{-3}	3.5×10^{-9}
3	p-Nitrophenyl β-NAX	3.12×10^{-3}	4×10^{-4}	6×10^{-3}	3.0×10^{-10}
4	"	3.12×10^{-3}	4×10^{-4}	-	8.0×10^{-10}
5	"	3.12×10^{-3}	-	-	6.9×10^{-10}

Note The rates were measured by method (b). Runs 2 and 3 had long induction periods of one and six hours respectively hence the rates measured necessarily have a high error.

Hydrolysis of p-Nitrophenyl pyranosides from Table 1

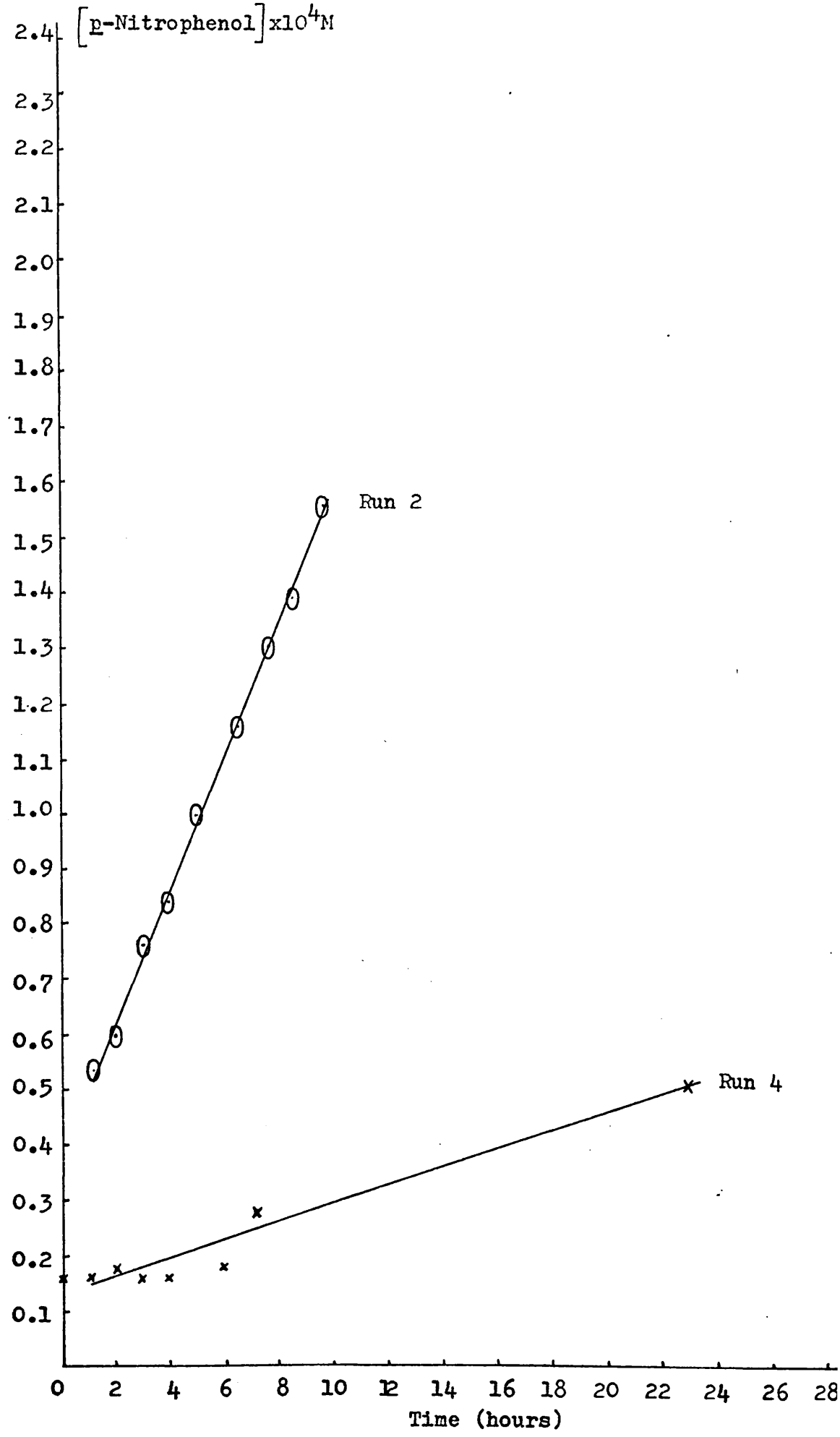


Fig 1.

Hydrolysis of p-Nitrophenyl pyranosides from Table 1

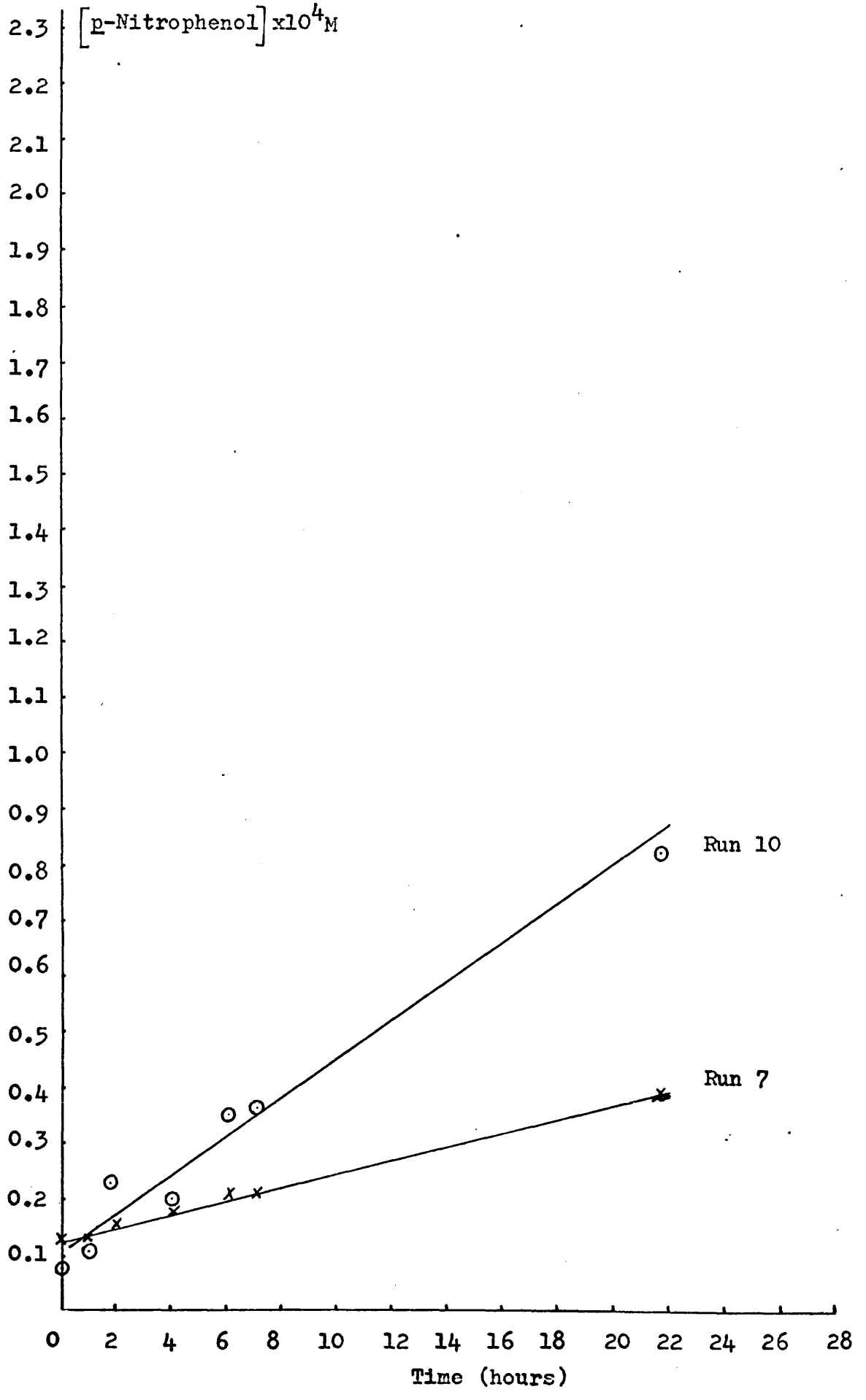


Fig 2.

Hydrolysis of p-Nitrophenyl pyranosides from Table 1

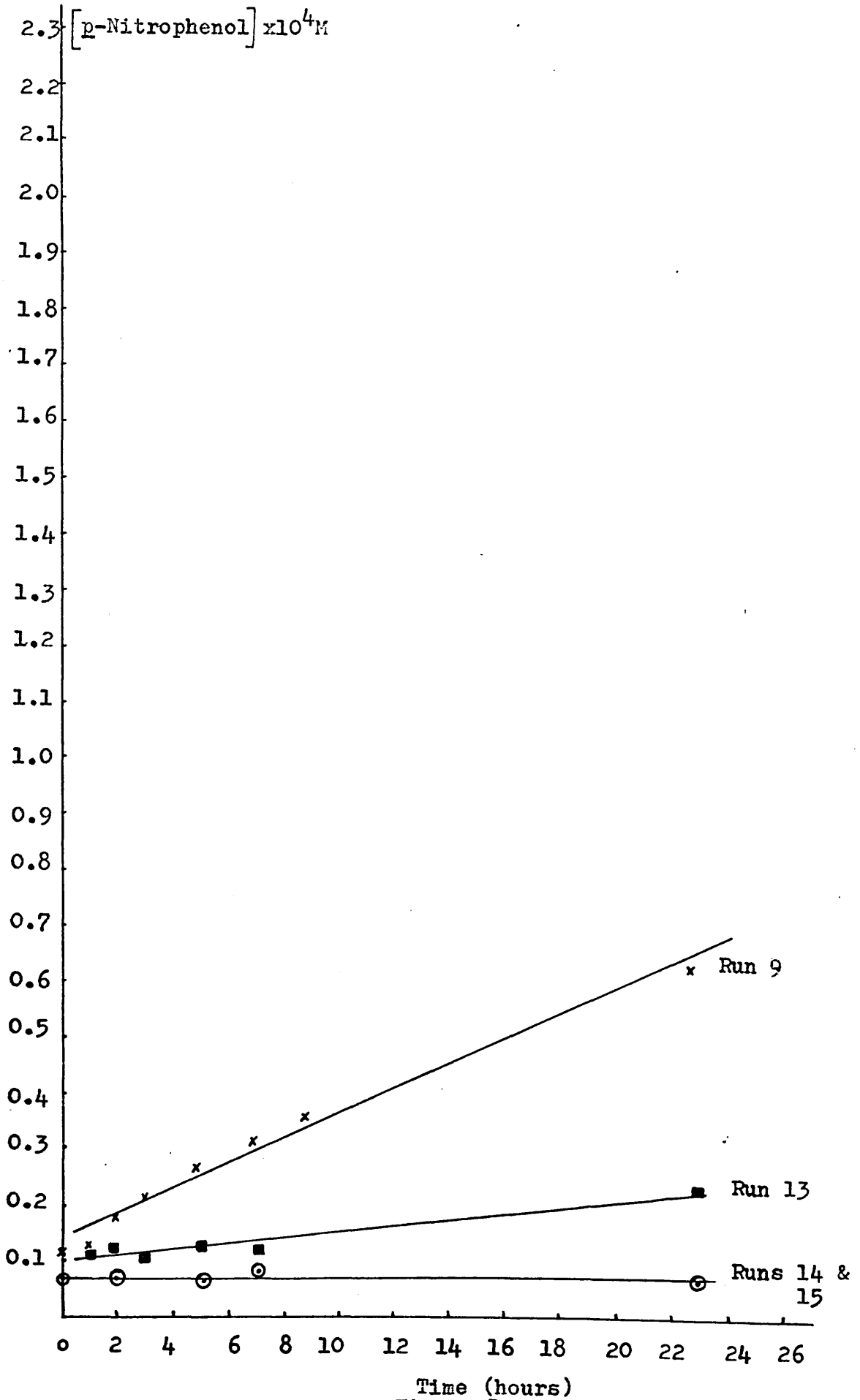


Figure 3

Hydrolysis of p-Nitrophenyl pyranosides from Table 1

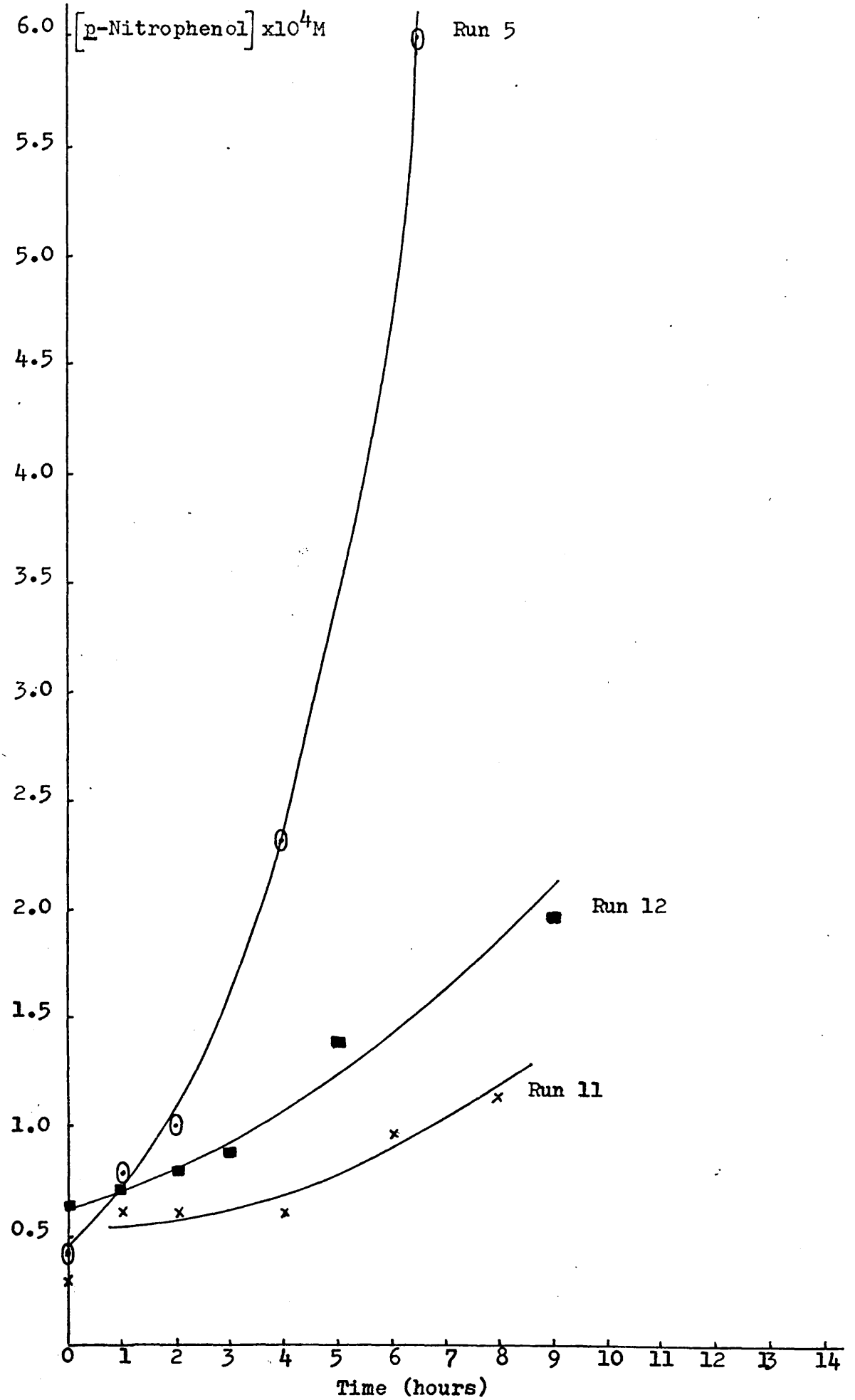


Figure 4

Hydrolysis of p-Nitrophenyl pyranosides from Table 1

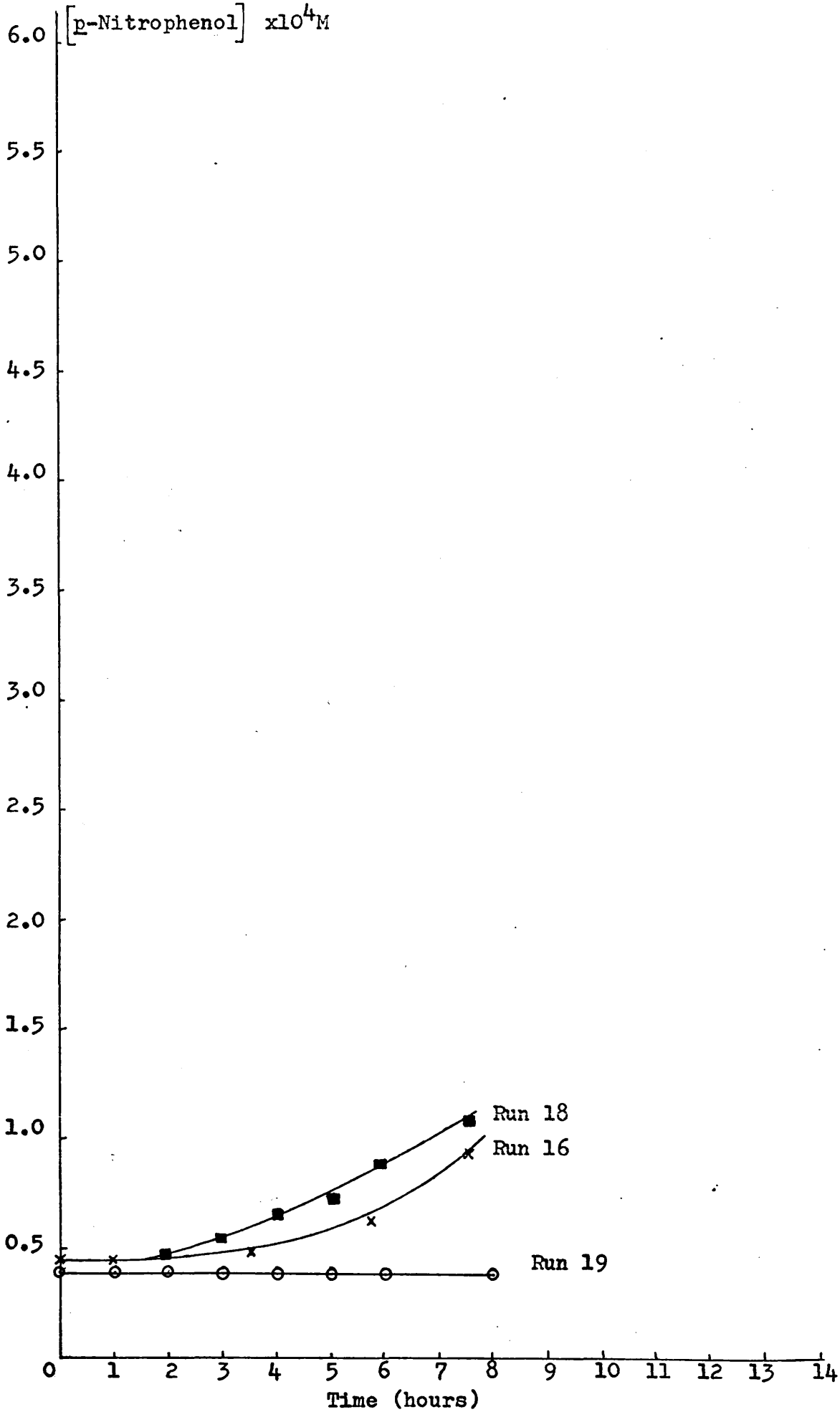


Figure 5

Hydrolysis of NAG-NAX-PNP

The lysozyme catalysed hydrolysis of NAG-NAX-PNP was studied under the conditions listed in table 3. For runs 1 to 8 there was no observable hydrolysis, and a finite rate could not be computed. The reactions were followed at 350nm by method (b).

Table 3

Run	$[S_o]$ M	$[E_o]$ M	Initial Rate.Msec^{-1}	Buffer
1	1.09×10^{-5}	2.5×10^{-4}	-	0.1M citrate pH5.1
2	2.25×10^{-5}	2.5×10^{-4}	-	"
3	6.7×10^{-5}	2.5×10^{-4}	-	"
4	8.4×10^{-5}	2.5×10^{-4}	-	"
5	1.0×10^{-4}	2.5×10^{-4}	-	"
6	1.0×10^{-4}	2.5×10^{-4}	-	"
7	1.0×10^{-4}	2.5×10^{-4}	-	"
8	2.5×10^{-4}	2.5×10^{-4}	-	"
9	7.5×10^{-4}	1.0×10^{-4}	3.6×10^{-11}	0.1M acetate pH5.23
10	1.0×10^{-3}	1.5×10^{-3}	4.17×10^{-11}	"

For run 9 the initial rate was calculated from the chart and represents the maximum possible rate.

For run 10 the computed rate shown had a standard deviation of 20%.

The hydrolysis of p-nitrophenyl β-NAG_2 was performed under enzyme/substrate conditions used in run 10 giving an initial rate of 3.26×10^{-10} Msec^{-1} and a standard deviation of 1.9%. The solutions used in runs 1 to 4 were run on T.L.C. (using n-propanol, ammonia, water, 6 : 3 : 2, v : v : v) against NAG and p-nitrophenyl β-NAG standards. After 24 hours no detectable hydrolysis of NAG-NAX-PNP was observed.

Hydrolysis of NAG_2-NAX-PNP

The lysozyme catalysed hydrolysis of NAG_2-NAX-PNP was studied under the conditions listed in table 4. For runs 1 to 4 a finite rate could not be computed and the rates shown were estimated from the chart recording at 350nm.

Table 4.

Run	$[S_o]$ M	$[E_o]$ M	Initial Rate $Msec^{-1}$	Buffer
1	$2.5x10^{-5}$	$2.5x10^{-4}$	$0.6x10^{-11}$	0.1M acetate pH5.23
2	$1.0x10^{-4}$	$1.0x10^{-4}$	$1.5x10^{-11}$	"
3	$1.0x10^{-3}$	$1.0x10^{-4}$	$8\ x10^{-11}$	"
4	$1.0x10^{-4}$	-	$2\ x10^{-11}$	"
5	$2.4x10^{-3}$	-	$1.5x10^{-10}$	"
6	$1.8x10^{-3}$	$1.25x10^{-3}$	$1.2x10^{-10}$	"

For the conditions studied in run 5 an aliquot method (method (a)) was performed giving a maximum initial rate of $1x10^{-10}Msec^{-1}$.

A repeat scan over 13 hours for $[S_o]=5x10^{-4}M$, $[E_o]=2x10^{-3}M$ was performed on a SP800 U.V. spectrometer. No hydrolysis was observed.

Hydrolysis of NAG_3-NAX-PNP

The lysozyme catalysed hydrolysis of NAG_3-NAX-PNP was studied under the conditions listed in table 5. For runs 1 to 4 there was no observable hydrolysis at 350nm using 2mm. cells and a finite rate could not be computed.

Table 5.

Run	$[S_o]$ M	$[E_o]$ M	Initial Rate $Msec^{-1}$	Buffer
1	3.7×10^{-5}	1×10^{-4}	-	0.1M acetate pH5.23
2	5.0×10^{-4}	1×10^{-5}	-	"
3	5.0×10^{-4}	1×10^{-5}	-	"
4	1.0×10^{-4}	2.5×10^{-4}	-	"
5	2.6×10^{-3}	5.8×10^{-3}	1.8×10^{-8}	"

Hydrolysis of 3,4-Dinitrophenyl glycosides

Hydrolysis was followed at 400nm. in 0.1M acetate buffer pH5.23 by method (b), using an extinction coefficient of 6160 for 3,4-dinitrophenol.

Table 6. Hydrolysis of 3,4-Dinitrophenyl β-NAX.

Run	$[S_o]$ M	$[E_o]$ M	Initial Rate Msec^{-1}	% Standard Dev.
1	3.1×10^{-3}	-	3.3×10^{-8}	0.6
2	1.56×10^{-4}	-	1.06×10^{-9}	13.0
3	1.56×10^{-4}	4×10^{-5}	1.38×10^{-9}	9.0
4	1.56×10^{-4}	4×10^{-5}	1.47×10^{-9}	8.3
5	1.56×10^{-4}	0.8×10^{-5}	1.07×10^{-9}	12.7
6	1.56×10^{-4}	4×10^{-4}	1.7×10^{-9}	8.9

Under the conditions used in run 4 a run containing $[NAG_4] = 1.2\times10^{-5}$M was performed, giving an initial rate = 1.69×10^{-9} (% Standard deviation = 9.1)

Due to the high spontaneous rate of hydrolysis of 3,4-dinitrophenyl β-NAX the rates were computed from a reaction time of 20 mirutes, hence appreciable transglycosylation would not be expected in the presence of NAG_4 during this time interval. From a run with $[S_o] = 4.7\times10^{-4}$M, $[E_o] = 4\times10^{-5}$M and $[NAG_4] = 3\times10^{-4}$M there appeared to be a slight increase in rate after two hours probably due to the formation of transglycosylation products.

Table 7.

Hydrolysis of NAG-NAX-DNP

Run	$[S_o]$ M	$[E_o]$ M	Initial Rate $Msec^{-1}$	% Standard Dev.
1	$1x10^{-4}$	-	$2.19x10^{-10}$	19.2
2	$1x10^{-4}$	-	$2.20x10^{-10}$	13.3
3	$1x10^{-4}$	$1x10^{-4}$	$2.87x10^{-10}$	13.4
4	$1x10^{-4}$	$1x10^{-4}$	$1.65x10^{-10}$	14.9

Table 8.

Hydrolysis of NAG_2-NAX-DNP

Run	$[S_o]$ M	$[E_o]$ M	Initial Rate $Msec^{-1}$	% Standard Dev.
1	$1x10^{-4}$	-	$1.47x10^{-10}$	14.0
2	$1x10^{-4}$	-	$1.60x10^{-10}$	15.7
3	$1x10^{-4}$	$1x10^{-5}$	$1.88x10^{-10}$	15.4
4	$1x10^{-4}$	$1x10^{-4}$	$1.90x10^{-10}$	17.8
5	$1x10^{-4}$	$1x10^{-4}$	$1.80x10^{-10}$	15.5

The Interaction of Inhibitors with Lysozyme as Studied by N.M.R. Experimental Method.

(a) The proton N.M.R. spectra were measured using a Varian HA-100 spectrometer operating in the frequency sweep mode. The water resonance was used for the field-frequency lock. Chemical shifts were determined using a Hewlett Packard electronic counter.

(b) Spectra were recorded at $33.5^{\circ} \pm 0.5^{\circ}C$, unless otherwise stated. Samples were equilibrated at $35^{\circ}C$ in a thermostatted bath before being transferred to the probe.

(c) All measurements were made using 0.1M citrate buffer[110] pH5.5. To achieve solubility of the aryl glycosides 15% v/v of spectroscopic dioxan was added to the buffer solution. Tertiary butanol, 0.2%, was added to samples as an internal reference standard, except for NAG.

(d) Boehringer lysozyme (batch no. 7471123/1) was used. The enzyme solutions were prepared immediately prior to use, filtered through cotton wool to remove any insoluble material, and the concentration measured on a Cary 16 U.V. spectrometer using the extinction coefficient of 36,000 for lysozyme absorbance at 280nm.

(e) Weighed samples of the sugar were dissolved in 0.5ml. of enzyme solution using a microlitre syringe, transferred to the N.M.R. sample tubes and equilibrated as above. Concentrations of the p-nitrophenyl glycosides, before and after the spectra were recorded, were measured by

removing 10µl of the N.M.R. sample, diluting to 2.5ml. and measuring the absorbance of this solution at 350nm. using an extinction coefficient of 1760. Results before and after the experiment were invariant.

For each compound a set of samples covering the concentration range studied was prepared without enzyme present.

For NAG-NAX-PNP and p-nitrophenyl β-NAG_2 a sample corresponding to the highest sugar concentration studied was prepared. This was then used to obtain the concentration range studied using a series dilution. For the enzyme experiments the lysozyme concentration was kept constant by using a stock enzyme solution for the series dilution.

(f) Spectra were recorded immediately after insertion in the probe and at five minute intervals until reproducible results were obtained. The chemical shift of the standard was calibrated followed by that of the sugar resonance of interest. Chemical shift values are expressed in Hz.

Treatment of Results.

For each compound studied the chemical shifts relative to the standard of the acetamido methyl resonance (s) of interest in the absence of enzyme were plotted against sugar concentration to give a straight line graph which was used to calculate the chemical shift value in the absence of lysozyme at the sugar concentrations used in the enzyme experiments. As can be seen from the following tables some compounds showed a small concentration dependence for the chemical shift values of the acetamido methyl resonances.

For those compounds for which the acetamido methyl resonance (s) experienced a concentration dependent chemical shift in the presence of

lysozyme, the change in the chemical shift δHz in the presence of enzyme relative to the internal standard was measured over the concentration range studied and analysed, according to the method of Dahlquist and Raftery[55], discussed in the introduction, to give the bound chemical shift value Δp.p.m. and the dissociation constant K_DM from the graph of initial sugar concentration, $[I_o]$ M, vs. $\frac{1}{\delta}$ (Hz^{-1}).

For the disaccharides which showed a change in chemical shift of the acetamido methyl resonances in the presence of enzyme but for which the value of K_DM was such that the enzyme was saturated with inhibitor over the concentration range studied, the bound chemical shift value Δp.p.m. was calculated from the unmodified equation:-

$$K_D = [E_o]\frac{\Delta}{\delta} - [I_o] - [E_o] + [I_o]\frac{\delta}{\Delta}$$

A Linear Least Squares program (L.L.SQ.) written by Dr.B. Capon was used to calculate the slope of the graph and the intercept. Standard deviations on the computed slope of the graph were c.a. 10% and for the intercept c.a. 20%.

Table 9

Chemical Shift Data for the Interaction of the Acetamido Methyl Protons of p-Nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside with Lysozyme.

Sugar Concentration $[I_o] \times 10^3 M$	Enzyme Concentration $[E_o] \times 10^3 M$	Chemical Shift from Tertiary Butanol (Hz)	Change in Shift δ (Hz)	$\frac{1}{\delta}$ (Hz^{-1})
3.9	3	74.6	2.7	0.371
5.0	3	74.3	3.0	0.333
7.0	3	74.6	2.8	0.357
14.4	3	75.4	2.2	0.455
20.0	3	75.7	2.0	0.500
25.0	3	75.9	1.9	0.527
28.3	3	76.1	1.8	0.555
31.8	3	76.3	1.6	0.625
34.5	3	76.9	1.4	0.715
50.0	3	76.9	1.4	0.715
8.4	-	77.4		
15.8	-	77.6		
20.2	-	77.7		
29.7	-	77.9		
41.0	-	78.3		
50.0	-	78.3		

Table 10

Chemical Shift Data for the Interaction of the Acetamido Methyl Protons of p-Nitrophenyl 2-acetamido-2-deoxy-β-D-xylopyranoside with Lysozyme.

Sugar Concentration $[I_o] \times 10^{-3}M$	Enzyme Concentration $[E_o] \times 10^{-3}M$	Chemical Shift from Tertiary Butanol (Hz)	Change in Shift δ (Hz)	$\frac{1}{\delta}$ (Hz^{-1})
6.9	3	71.8	5.5	0.183
7.2	3	72.2	5.1	0.196
9.8	3	72.5	4.8	0.208
14.4	3	73.2	4.2	0.238
18.6	3	73.2	4.4	0.227
19.6	3	73.3	4.3	0.233
21.5	3	73.1	4.5	0.222
24.8	3	73.5	4.1	0.244
29.8	3	74.2	3.5	0.286
38.7	3	74.5	3.4	0.294
49.5	3	74.6	3.4	0.294
15	-	77.4		
18	-	77.6		
25	-	77.6		
30	-	77.8		

Table 11

Chemical Shift Data for the Interaction of the Acetamido Methyl Protons of Methyl 2-acetamido-2-deoxy-α-D-glucopyranoside with Lysozyme.

Sugar Concentration $[I_o] \times 10^{-2}$M	Enzyme Concentration $[E_o] \times 10^{-3}$M	Chemical Shift from Tertiary Butanol (Hz)	Change in Shift δ(Hz)	$\frac{1}{\delta}$(Hz^{-1})
1.86	3	76.4	3.2	0.3125
3.06	3	77.2	2.4	0.4167
4.03	3	77.3	2.3	0.4355
4.99	3	77.4	2.2	0.4545
5.99	3	77.7	1.9	0.5263
6.99	3	77.7	1.9	0.5263
8.98	3	78.0	1.6	0.6250
2.01	-	79.7		
2.94	-	79.4		
3.97	-	79.7		
5.06	-	79.7		
6.00	-	79.6		
7.04	-	79.8		
7.91	-	79.5		
9.04	-	79.6		

Table 12

Chemical Shift Data for the Interaction of the Acetamido Methyl Protons of 2-Acetamido-2-deoxy-D-glucopyranoside with Lysozyme

Sugar Concentration $[I_o] \times 10^2 M$	Enzyme Concentration $[E_o] \times 10^3 M$	Chemical Shift from Lock Signal (Hz)		Change in Shift δ(Hz)		$\frac{1}{\delta}$(Hz^{-1})	
		βanomer	αanomer	β	α	β	α
1.1	2	269.7	272.9	2.3	5.5	0.435	0.182
2.2	2	269.5	271.7	1.9	4.1	0.527	0.244
3.1	2	269.4	271.2	1.7	3.5	0.589	0.286
4.0	2	269.5	270.9	1.8	3.2	0.555	0.312
5.9	2	269.4	270.5	1.6	2.7	0.625	0.372
8	2	269.1	269.9	1.0	1.8	1	0.55
1.1		267.4					
2.2		267.6					
3.1		267.7					
3.9		267.7					
5.9		267.8					
8.0		268.1					
10.4		268.6					

Table 13

Chemical Shift Data for the Interaction of the Acetamido Methyl Protons of NAG-Glu-PNP with Lysozyme.

Sugar Concentration $[I_o] \times 10^3 M$	Enzyme Concentration $[E_o] \times 10^3 M$	Chemical Shift from Tertiary Butanol (Hz)	Change in Shift δ (Hz)	$\frac{1}{\delta}(Hz^{-1})$
5.1	3	79.7	3.4	0.294
9.6	3	80.2	3.0	0.334
15.6	3	81.1	2.2	0.455
20.3	3	81.1	2.3	0.435
24.4	3	81.5	2.1	0.477
30.6	3	81.7	2.0	0.50
35.4	3	82.1	1.7	0.59
10.5	-	83.2		
18.3	-	83.4		
34.3	-	83.8		

Table 14

Chemical Shift Data for the Interaction of the Acetamido Methyl Protons of NAG-NAX-PNP with Lysozyme.

Temperature $33.5 \pm 0.5^{\circ}C$

Sugar Concentration $[I_o] \times 10^2 M$	Enzyme Concentration $[E_o] \times 10^3 M$	Chemical Shift from Tertiary Butanol (Hz) A^1	B^1
1.6	-	84.3	79.8
1.8	3	82.8	d
2.0	3	82.7	d
2.3	3	83.0	d
2.7	3	82.9	80.2
3.2	3	84.5	80.3
4.0	3	83.7	80.6
4.13[c]	-	83.6	80.1
4.13[c]	3	83.3	80.0

Temperature $55 \pm 1^{\circ}C$

4.13	-	83.0	79.8
4.13	3	82.5[f]	74.1[e]

Note: (1) A and B refer to the acetamido methyl resonances of the sugar residues distal and proximal to the aglycone respectively.

(c) Results from a separate experiment

(d) Both acetamido methyl resonances broadened on the addition of enzyme. The resonance of the acetamido methyl group of the NAX residue suffered the most broadening, and at these concentrations it was not possible to measure the centre of the resonance with any great accuracy.

(e) Difficult to assess the centre of the peak due to its breadth.

(f) From the spectrum possible that another peak is present 2 c.p.s. upfield from A.

Table 15

Chemical Shift Data for the Interaction of the Acetamido Methyl Protons of p-Nitrophenyl β-NAG_2 with Lysozyme.

Sugar Concentration $[I_o] \times 10^2 M$	Enzyme Concentration $[E_o] \times 10^3 M$	Chemical Shift from Tertiary Butanol (Hz) A^1	B^1
$33.5 \pm 0.5^{\circ}C$			
3.55	-	84.5	77.8[d]
3.55	3	83.7	73.8[a,c]
$55 \pm 1^{\circ}C$			
3.55	-	84.3	77.2
3.55	3	84.2	77.8[b]

Note

(a) The acetamido methyl resonance proximal to the aglycon broadens considerably on addition of enzyme. There was very little broadening of the distal acetamido methyl resonance.

(b) The distal acetamido resonance was broadened considerably and there appears to be slight splitting in the resonance at 77.8Hz.

(1) A and B refer to the acetamido methyl resonances of the sugar residues distal and proximal to the aglycone respectively.

(c) Changes in $[I_o]$ over the concentration range $(3.55-2.2) \times 10^{-2} M$ did not change the resonance positions.

(d) Changes in $[I_o]$ over the concentration range $(3.55-1.0) \times 10^{-2} M$ did not change the resonance positions.

Table 16

Chemical Shift Data for the Interaction of the Acetamido Methyl Protons of Di-N-acetyl chitobiose

Sugar Concentration $[I_0] \times 10^2 M$	Enzyme Concentration $[E_0] \times 10^3 M$	Chemical Shift from Tertiary Butanol	
		A^1	B^1
1.02	-	81.5	79.0
2.04	-	81.9	79.1
3.55	-	82.2	79.4
4.25	-	82.0	79.6
6.1	-	82.0	80.2
2.14	3	81.5	72.8
2.75	3	81.4	72.3
4.05	3	81.6	72.3
4.82	3	81.1	72.2
5.94	3	81.9	72.8

Note: (1) A and B refer to the acetamido methyl resonances of the sugar residues at the non-reducing end and reducing end respectively.

Table 17

Dissociation Constants, K_DM, and Bound Chemical Shift Values, Δp.p.m., of Lysozyme Inhibitors.

Compound	K_DM	Δp.p.m.
p-Nitrophenyl β-D-NAG	2.76×10^{-2}	0.34
p-Nitrophenyl β-D-NAX	5.21×10^{-2}	0.11
Methyl α-D-NAG	5.78×10^{-2}	0.79
NAG-Glu-PNP	2.33×10^{-2}	0.35
Di-N-acetyl Chitobiose	-	0.51
NAG-NAX-PNP	-	0.83[a]
p-Nitrophenyl β-NAG_2	-	0.51[b]

Note: (a) From the results at $55\pm1^{\circ}C$. This may represent an upper limit using the concentration of inhibitor $[I_o]=4.13\times10^{-2}$M. Also possible that even at this temperature the system is not in the fast exchange limit.

(b) From the results at $33.5\pm0.5^{\circ}C$. Difficult to measure accurately due to the width of the resonance signal.

The Interaction of Inhibitors with Lysozyme as Studied by Fluorescence Spectroscopy

Experimental Method

(a) Measurements were performed using a Perkin-Elmer MPF-2A fluorescence spectrophotometer.

(b) Boehringer lysozyme (batch no. 7482325/1) was used; solutions were prepared and concentrations determined as for N.M.R. experiments.

(c) All measurements were made using 0.1M acetate buffer pH 5.25.

(d) Excitation wavelength was 285nm. The fluorescence emission intensity was measured at the wavelengths quoted in the tables of results.

(e) Stock solutions of enzyme and inhibitor were prepared in 0.1M acetate buffer. Measurements were made on 2.5ml. samples in 1cm. square quartz fluorescence cells prepared by taking aliquots of the stock solutions using a microlitre syringe and making the final volume up to 2.5ml. with buffer.

(f) The fluorescence emission intensity of a solution containing enzyme at the concentration used in the experiment was measured at the emission wavelength used in the experiment, which was previously determined, from preliminary experiments using enzyme in the presence and absence of inhibitor, as the wavelength giving the largest change in the relative fluorescence emission.

The fluorescence emission intensity for solutions containing both inhibitor and enzyme was then measured over the concentration range studied, and corrected for an enzyme emission intensity of 100%.

Treatment of Results

The data was analysed using the treatment described by Lehrer and Fasman[76] as follows:-

For the fluorescence emission intensity of an enzyme/inhibitor solution to be proportional to the concentration of free enzyme present in the solution, the absorbance of the solution at the excitation and emission wavelengths must be less than 0.1a.u. If this holds then the degree of association β can be expressed as,

$$\beta = \frac{F-F_E}{F_{ES}-F_E}$$

and the association constant K_aM^{-1} as,

$$K_a = \frac{\beta}{1-\beta} \cdot \frac{1}{[I]}$$

where $[I] = [I_o] - \beta[E_o]$

i.e. $[I]$ = free substrate where

$[I_o]$ = total substrate present,

$[E_o]$ = total enzyme present,

F = fluorescence of the mixture,

F_E = fluorescence of the uncomplexed enzyme,

and F_{ES} = fluorescence of the enzyme-substrate complex.

A plot of $\frac{1}{F-F_E}$ versus $\frac{1}{[I_o]}$ was analysed using a linear Least Squares computer program to find $F_{ES}-F_E$ at $\frac{1}{[I_o]} = 0$. K_aM^{-1} was calculated from,

$$K_a = \frac{1}{[I_o] - \frac{1}{2}[E_o]}, \text{ when } \beta = \tfrac{1}{2},$$

from a knowledge of the original enzyme concentration and the concentration of substrate at $\beta = \frac{1}{2}$.

The data was also analysed from a computer fit of F_E-F versus $[I_o]$ which provided $\Delta(F_E-F)$ maximum and K_DM by an analagous method to that used to determine Michaelis Menten parameters, thus avoiding the errors involved in dealing with reciprocal plots. Surprisingly good agreement was found for the results determined by both methods.

Table 18

Interaction of p-Nitrophenyl β-NAG_2 with Lysozyme as Studied by Fluorescence

$[E_o] = 4 \times 10^{-7}$M. Fluorescence emission wavelength 325 nm.

$[I_o] \times 10^6$M	$F_E - F$
2	1.6
4	4.8
6	8.0
8	11.4
10	14.5
12	17.8
14	19.4
16	22.6
18	24.1
20	25.8

L.L.SQ. analysis of $\frac{1}{F_E - F}$ versus $\frac{1}{[I_o]}$ gives a negative intercept and the graph of $F_E - F$ versus $[I_o]$ shows that saturation of the enzyme by the inhibitor was not nearly achieved. Hence the value of K_DM for p-nitrophenyl β-NAG_2 is such that it cannot be determined by fluorescence due to the absorbance of the inhibitor at the concentration range required for its determination.

Table 19

Interaction of NAG-NAX-PNP with Lysozyme as Studied by Fluorescence

$[E_o] = 4 \times 10^{-7} M$ Fluorescence emission wavelength 325 nm.

Run 1		Run 2		Run 3		Run 4	
$[I_o] \times 10^6 M$	$F_E - F$	$[I_o] \times 10^6 M$	$F_E - F$	$[I_o] \times 10^6 M$	$F_E - F$	$[I_o] \times 10^6 M$	$F_E - F$
2	10.0	2	9.4	2	7	2	7
4	18.5	4	15.6	4	15	4	15
5	22.5	6	21.9	6	19	6	20
6	26.5	8	28.1	8	26	8	26
8	30.6	10	33.8	10	32	10	31
10	36.7	12	37.5	12	36	12	36
12	39.8	14	41.6	14	40	14	40
14	42.8	16	44.5	16	43	16	44
16	47.0	18	48.5	18	45	18	47
18	51.0	20	50.0	20	48	20	51
20	53.0	22	53.2			22	53
		24	56.4			24	55
		26	59.4			26	58

L.L.SQ. $\frac{1}{F_E - F}$ versus $\frac{1}{[I_o]}$

Run	Intercept	Standard Deviation	$K_a M^{-1}$
1	9.67×10^{-3}	4.3%	5.62×10^4
2	10.4×10^{-3}	6.7%	5.62×10^4
3	5.64×10^{-3}	23.0%	$\simeq 5 \times 10^4$
4	5.7×10^{-3}	15.0%	$\simeq 5.3 \times 10^4$

$F_E - F$ versus $[I_o]$

Run	$K_D M$	Standard Deviation
1	1.7×10^{-5}	8.5×10^{-7}
2	2.3×10^{-5}	1.2×10^{-6}
3	3.3×10^{-5}	4.7×10^{-6}
4	3.4×10^{-5}	2.3×10^{-6}

Table 20

Interaction of NAG_2-NAX-PNP with Lysozyme as Studied by Fluorescence

$[E_o] = 1 \times 10^{-7} M$ Fluorescence emission measured at 325 nm.

Run 1		Run 2		Run 3	
$[I_o] \times 10^7 M$	$F_E - F$	$[I_o] \times 10^7 M$	$F_E - F$	$[I_o] \times 10^7 M$	$F_E - F$
3.2	4.3	3.2	5.7	3.2	5.0
6.4	7.2	6.4	9.3	6.4	12.5
9.6	11.6	9.6	11.4	9.6	18.6
12.8	15.2	16.0	17.1	12.8	23.7
16.0	16.6	22.4	20.0	16.0	26.2
19.2	18.8	28.8	22.1	19.2	30.0
22.4	20.3	35.2	25.0	22.4	32.5
25.6	22.4	41.6	27.2	25.6	35.0
28.8	25.4	48.0	29.2	28.8	36.2
32.0	27.6	64.0	32.1	32.0	37.5
35.2	28.3	80.0	34.2	41.6	40.0
41.6	30.4	96.0	36.4	48.0	43.7
48.0	29.7			64.0	47.5
56.0	33.3				
64.0	35.5				
80.0	37.7				
96.0	39.1				
112.0	42.0				

L.L.SQ. $\frac{1}{F_E - F}$ versus $\frac{1}{[I_o]}$

Run	Intercept	Standard Deviation	$K_a M^{-1}$
1	0.0169	6.97%	2.53×10^5
2	0.0262	6.24%	4.5×10^5
3		graphically	5.5×10^5

$F_E - F$ versus $[I_o]$

Run	$K_D M$	Standard Deviation
1	3.66×10^{-6}	1.78×10^{-7}
2	2.51×10^{-6}	2.05×10^{-7}
3	2.79×10^{-6}	3.28×10^{-7}

Table 21

Interaction of p-Nitrophenyl β-NAG_3 with Lysozyme as Studied by Fluorescence

$[E_o] = 8 \times 10^{-8} M$ Fluorescence emission wavelength 325 nm.

Run 1		Run 2	
$[I_o] \times 10^7 M$	$F_E - F$	$[I_o] \times 10^7 M$	$F_E - F$
4	2.9	4	4.4
8	5.2	8	6.7
12	8.9	12	10.5
16	10.5	16	12.6
20	13.5	20	14.8
24	16.4	24	15.6
28	17.8	28	18.6
32	19.4	32	20.1
36	21.6	36	21.6
40	24.6	40	24.6
50	26.2	50	27.6
60	29.8	60	29.8
70	31.3	70	32.1
80	34.3	80	34.3
90	37.3	90	35.8
100	37.3	100	37.3
120	39.6	120	41.3

L.L.SQ. $\frac{1}{F_E - F}$ versus $\frac{1}{[I_o]}$

Run	Intercept	Standard Deviation	$K_a M^{-1}$
1	0.0103	16%	$> 8.3 \times 10^4$
2	0.0214	9.62%	2.66×10^5

$F_E - F$ versus $[I_o]$

Run	$K_D M$	Standard Deviation
1	9.1×10^{-6}	8.7×10^{-7}
2	6.1×10^{-6}	3.5×10^{-7}

Table 22

Interaction of NAG_3 with Lysozyme as Studied by Fluorescence

$[E_o] = 5 \times 10^{-7} M$ Fluorescence emission wavelength 340nm.

Run 1		Run 2	
$[I_o] \times 10^6 M$	$F_E - F$	$[I_o] \times 10^6 M$	$F_E - F$
4	3.3	2	1.2
6	4.1	4	2.4
8	5.0	6	3.6
10	5.8	8	4.9
12	6.6	12	6.2
16	7.5	16	7.3
31	11.6	24	8.6
40	13.3	32	9.7
70	14.1	38.6	11.1
		56.7	12.4

L.L.SQ. $\frac{1}{F_E - F}$ versus $\frac{1}{[I_o]}$

Run	Intercept	Standard Deviation	$K_a M^{-1}$
1	0.0610	9.9%	0.4×10^5
2	0.0397	18.0%	0.2×10^5

$F_E - F$ versus $[I_o]$

Run	$K_D M$	Standard Deviation
1	2.1×10^{-5}	2.2×10^{-6}
2	2.5×10^{-5}	2.3×10^{-6}

Table 23

Interaction of NAG_4 with Lysozyme as Studied by Fluorescence

$[E_o] = 5 \times 10^{-7} M$ Fluorescence emission wavelength 340 nm.

Run 1		Run 2		Run 3	
$[I_o] \times 10^6 M$	$F_E - F$	$[I_o] \times 10^6 M$	$F_E - F$	$[I_o] \times 10^6 M$	$F_E - F$
2	5	1	2.8	2	2.2
4	11	2	4.3	4	5.0
6	12	3	6.4	6	6.5
8	15	4	7.1	7	8.6
10	16	5	8.6	9	8.6
12	16	7	8.6	14	9.3
18	18	10	10.0	20	10.0
25	20	15	12.8	30	10.6
35	20	20	14.2	40	11.5
45	21	25	14.2	50	12.2
55	22	35	14.2	60	12.2

L.L.SQ. $\frac{1}{F_E - F}$ versus $\frac{1}{[I_o]}$

Run	Intercept	Standard Deviation	$K_a M^{-1}$
1	0.035	10%	1.3×10^5
2	0.062	6%	1.5×10^5
3	0.052	20%	5.6×10^5

F_E-F versus $[I_o]$

Run	$K_D M$	Standard Deviation
1	6.5×10^{-6}	7.2×10^{-7}
2	5.5×10^{-6}	5.1×10^{-7}
3	7.8×10^{-6}	1.2×10^{-6}

Table 24

Interaction of NAG_5 with Lysozyme as studied by Fluorescence

$[E_o] = 5 \times 10^{-7} M$ Fluorescence emission wavelength 320 nm.

Run 1		Run 2	
$[I_o] \times 10^6 M$	$F-F_E$	$[I_o] \times 10^6 M$	$F-F_E$
3	11.9	0.6	2.8
6	17.9	1.2	4.6
9	21.3	3.0	7.5
12	22.7	4.2	9.3
15	25.3	5.4	10.9
18	22.7	7.2	12.2
48	22.7	9.0	14.3
		12.0	14.8
		15.0	16.1
		18.0	17.5
		21.0	17.5

L.L.SQ. $\frac{1}{F-F_E}$ versus $\frac{1}{[I_o]}$

Run	Intercept	Standard Deviation	$K_a M^{-1}$
1	0.029	2.5%	5.89×10^5
2	0.056	10.0%	2.67×10^5

$F-F_E$ versus $[I_o]$

Run	$K_D M$	Standard Deviation
1	5.5×10^{-6}	0.37×10^{-6}
2	5.8×10^{-6}	0.98×10^{-6}

Table 25

Interaction of NAG_6 with Lysozyme as Studied by Fluorescence

$[E_o] = 5 \times 10^{-7} M$ Fluorescence emission wavelength 320 nm.

Run 1		Run 2	
$[I_o] \times 10^6 M$	$F-F_E$	$[I_o] \times 10^6 M$	$F-F_E$
0.4	1.3	0.8	2.7
0.8	2.7	1.6	3.9
1.6	4.8	2.4	6.7
2.4	6.7	3.2	7.9
3.2	9.2	4.0	9.2
4.0	10.6	6.0	10.6
6.0	12.8	8.0	11.8
8.0	14.7	10.0	14.4
10.0	18.7	12.0	13.2
12.0	18.7	14.0	13.2
34.0	22.8	16.0	13.2

L.L.SQ. $\frac{1}{F-F_E}$ versus $\frac{1}{[I_o]}$

Run	Intercept	Standard Deviation	$K_a M^{-1}$
1	0.019	36%	-
2	0.049	16%	1.6×10^5

$F-F_E$ versus $[I_o]$

Run	$K_D M$	Standard Deviation
1	8.0×10^{-6}	7×10^{-7}
2	5.8×10^{-6}	9×10^{-7}

Interaction of Inhibitors with Lysozyme as Studied by the Inhibition of the hydrolysis of 3,4-Dinitrophenyl β-NAG_4

The inhibition of the lysozyme hydrolysis of 3,4-dinitrophenyl β-NAG_4 was studied by measuring the rate of release of 3,4-dinitrophenol from the glycoside under Michaelis Menten conditions in the presence and absence of inhibitor.

The rates of hydrolysis were measured at 40°C in 0.1M acetate buffer by following the change in absorbance of the solution at 400nm using a Cary 16 spectrometer.

The extinction coefficient for 3,4-dinitrophenol at 400nm, at the buffer pH used in the experiment, was determined prior to each experiment. The data was analysed by an initial slopes computer program using a Digico Micro 16P computer on line to the Cary 16 as previously described. The Michaelis Menten parameters were determined by a computer fit of the observed initial rates $Msec^{-1}$ versus the substrate concentration $[S_o]M$.

The concentrations of the solutions used were determined by measurement of their absorbance at the appropriate wavelength as previously described.

All the compounds studied were found to be competitive inhibitors for the lysozyme hydrolysis of 3,4-dinitrophenyl β-NAG_4, allowing the data to be analysed according to the following treatment.

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \xrightarrow{k_{+2}} E + \text{products.}$$

Applying the Briggs Haldane analysis for the system shown above in the steady state,

$$\frac{d[ES]}{dt} = 0 = k_{+1}([E] - [ES])[S] - (k_{-1}+k_{+2})[ES]$$

Therefore $$[ES] = \frac{[E][S]}{\frac{k_{-1}+k_{+2}}{k_{+1}} + [S]},$$

and $$v = k_{+2}[ES],$$

$$= \frac{k_{+2}[E][S]}{\frac{k_{-1}+k_{+2}}{k_{+1}} + [S]},$$

$$Vmax = k_{+2}[E],$$

Therefore $$v = \frac{V}{1 + \frac{Km}{[S]}}$$

where $$K_m = \frac{k_{-1}+k_{+2}}{k_{+1}}$$

Applying the Briggs Haldane analysis to the case of competitive inhibition we have,

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES$$

$$E + I \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} EI$$

$$ES \xrightarrow{k_3} E + \text{products},$$

and for the system in the steady state

$$k_{+1}\left[S\right]\left(\left[E\right]-\left[ES\right]-\left[EI\right]\right)=\left(k_{-1}+k_{+3}\right)\left[ES\right],$$

$$k_{+2}\left[I\right]\left(\left[E\right]-\left[ES\right]-\left[EI\right]\right)=k_{-2}\left[EI\right]$$

$$v = k_{+3}\left[ES\right]$$

$$= \frac{k_{+3}\left[E\right]}{1+\frac{k_{-1}+k_{+3}}{k_{+1}\left[S\right]}\left[1+\frac{k_{+2}\left[I\right]}{k_{-2}}\right]}$$

substituting

$$K_m \text{ for } \frac{k_{-1}+k_{+3}}{k_{+1}}, \text{ and } K_i \text{ for } \frac{k_{-2}}{k_{+2}}$$

$$v = \frac{V}{1+\frac{K_m}{\left[S\right]}\left[1+\frac{\left[I\right]}{K_i}\right]}$$

Thus for a competitive inhibitor there is an apparent increase of K_m by the factor $1+\frac{\left[I\right]}{K_i}$ and hence the apparent K_m increases without limit as $\left[I\right]$ increases. For a finite inhibitor concentration the limiting velocity is equal to V_{max} for the uninhibited reaction.

Substituting K_p for $K_m\left(1+\frac{\left[I\right]}{K_i}\right)$

where K_p is the apparent Michaelis Menten parameter measured for the inhibited hydrolysis then,

$$K_i = \frac{\left[I\right]}{\frac{K_p}{K_m}-1}$$

Table 26

Hydrolysis of 3,4-Dinitrophenyl β-NAG_4

$[E] = 1 \times 10^{-6} M$ pH 5.23

$[S] \times 10^{5} M$	Initial Rate $\times 10^{10} M sec^{-1}$	% Standard Dev
0.8	2.91	12.6
1.2	6.89	4.6
1.4	7.62	4.7
1.6	7.42	2.9
2.0	7.33	2.4
2.4	8.14	2.5
4.0	9.61	4.4
6.0	10.53	4.1
8.0	10.92	4.7
12.0	10.27	4.9
16.0	11.27	4.4
20.0	11.17	3.7

	V_{max} $M sec^{-1}_{10}$	$K_m M$
Calculated	11.58×10^{-10}	0.94×10^{-5}
Standard Deviation	0.84×10	0.23×10^{-5}

Table 27

Hydrolysis of 3,4-Dinitrophenyl β-NAG_4

$[E] = 5 \times 10^{-7} M$ pH 5.23

$[S_o] \times 10^6 M$	Initial Rate x $10^{10} M sec^{-1}$	% Standard Dev.
5	2.53	10.8
6	3.26	8.1
7	3.06	8.4
8	3.37	6.7
9	3.40	8.7
10	2.62	10.5
12	3.25	10.3
14	4.31	5.7
16	4.65	5.4
18	5.26	4.7
20	5.30	5.8
30	5.72	4.8
40	5.35	5.2
50	5.21	6.1
60	5.93	4.2
70	6.60	3.2
100	6.83	4.5
120	5.58	5.3

	$V_{max} M sec^{-1}$	$K_m M$
Calculated	6.7×10^{-10}	8.0×10^{-6}
Standard Deviation	0.5×10^{-10}	1.1×10^{-6}

Table 28

Inhibition by NAG-NAX-PNP

$[I] = 1 \times 10^{-4} M$ $[E] = 5 \times 10^{-7} M$ pH 5.23

$[S_o] \times 10^{6} M$	Initial Rate $\times 10^{10} M sec^{-1}$	% Standard Dev.
7	1.88	12.3
8	2.49	10.2
9	3.12	8.8
12	3.26	7.0
14	3.72	5.5
16	4.22	4.7
18	4.02	5.0
20	4.83	4.0
30	5.96	3.8
40	5.72	3.7
50	6.00	3.9
60	6.94	3.9

	V_{max} $M sec^{-1}$	$K_p M$
Calculated	9.3×10^{-10}	2.2×10^{-5}
Standard Deviation	0.87×10^{-10}	0.43×10^{-5}

Table 29

Inhibition by NAG_2-NAX-PNP

$[I] = 1 \times 10^{-4} M$ $[E] = 5 \times 10^{-7} M$ pH 5.23

$[S_o] \times 10^5 M$	Initial Rate $\times 10^{10} M sec^{-1}$	% Standard Dev.
1.0	1.49	9.3
1.4	1.15	10.6
1.6	1.65	9.5
1.8	0.83	17.2
3.0	1.69	8.8
4.0	2.92	4.3
5.0	3.23	6.6
6.0	5.25	3.8
7.0	4.90	2.9
8.0	5.97	2.2
16.0	7.74	2.4
24.0	8.94	1.9

	V_{max} $M sec^{-1}$	$K_p M$
calculated	14×10^{-10}	1.5×10^{-4}
standard deviation	3×10^{-10}	0.5×10^{-4}

Table 30

Inhibition by NAG_2-NAX-PNP

$[E_o] = 1 \times 10^{-6}M$ $[I] = 1.19 \times 10^{-5}M$ pH 5.23

$[S_o] \times 10^5 M$	Initial Rate $\times 10^{10} Msec^{-1}$	% Standard Dev.
0.8	2.81	5.1
1.2	5.12	4.2
1.6	5.37	4.6
2.0	6.67	3.2
2.4	6.56	4.4
3.2	6.64	3.9
4.0	8.51	7.0
6.0	9.53	4.7
8.0	10.64	4.3
10.0	10.92	3.8
12.0	10.56	4.9
16.0	11.83	4.6

	V_{max} $Msec^{-1}$	K_pM
Calculated	13.5×10^{-10}	2.5×10^{-5}
Standard Deviation	0.69×10^{-10}	0.3×10^{-5}

Table 31

Inhibition by NAG-Glu-PNP

$[E] = 5 \times 10^{-7} M$ $[I] = 1 \times 10^{-4} M$ pH 5.23

$[S_o] \times 10^{6} M$	Initial Rate $\times 10^{10} M sec^{-1}$	% Standard Dev.
8	2.46	18.0
10	3.30	9.6
12	3.29	9.9
16	3.46	7.3
30	3.78	12.5
40	4.92	6.0
50	4.46	6.8
60	5.47	7.3
70	5.34	5.7
80	5.50	4.8
100	6.73	3.4

	V_{max} $M sec^{-1}$	$K_p M$
calculated	6.8×10^{-10}	1.5×10^{-5}
standard deviation	0.85×10^{-10}	0.3×10^{-5}

Table 32

Inhibition by NAG-Glu-PNP

$[E] = 1 \times 10^{-6} M$ $[I] = 7.7 \times 10^{-5} M$ pH 5.23

$[S_o] \times 10^5 M$	Initial Rate $\times 10^{10} M sec^{-1}$	% Standard Dev.
1.2	6.47	4.3
2.0	7.54	5.6
3.2	8.92	3.0
4.0	8.33	2.8
4.8	9.63	3.5
6.0	9.68	1.8
8.0	10.77	2.3
12.0	10.45	2.7
16.0	11.45	2.7

	V_{max} $M sec^{-1}$	$K_p M$
Calculated	11.68×10^{-10}	1.12×10^{-5}
Standard Deviation	0.42×10^{-10}	0.21×10^{-5}

Inhibition by NAG-Xyl-PNP*

$[E] = 1 \times 10^{-6} M \quad [I] = 1.6 \times 10^{-4} M \quad pH\ 5.23$

Under these conditions the hydrolysis of 3,4-dinitrophenol β-NAG_4 was not inhibited by this compound. For $[S_o] = 6.0 \times 10^{-5} M$ the rate of hydrolysis was $10.93 \times 10^{-10} M sec^{-1}$ in the absence of inhibitor and $10.53 \times 10^{-10} M sec^{-1}$ with inhibitor present at the concentration shown above.

Inhibition by p-Nitrophenyl β-NAX

$[E] = 1 \times 10^{-6} M \quad [I] = 3.5 \times 10^{-3} M \quad pH\ 5.23$

Under these conditions the hydrolysis of 3,4-Dinitrophenyl β-NAG_4 was not inhibited by this compound. The rate of hydrolysis for $[S_o] = 12 \times 10^{-5} M$ was $12.69 \times 10^{-10} M sec^{-1}$ in the presence of inhibitor and $12.73 \times 10^{-10} M sec^{-1}$ with no inhibitor present.

* See experimental section for a discussion of this compound.

Inhibition by p-Nitrophenyl 2-acetamido-6-iodo-2,6-dideoxy-β-D-glucopyranoside

$[E] = 1 \times 10^{-6} M$ $[I] = 2.0 \times 10^{-4} M$ pH 5.23

Under these conditions the hydrolysis of 3,4-dinitrophenyl β-NAG_4 was not inhibited by this compound. The rate of hydrolysis for $[S_o] = 12 \times 10^{-5} M$ was $12.29 \times 10^{-10} M sec^{-1}$ in the presence of inhibitor and $12.73 \times 10^{-10} M sec^{-1}$ with no inhibitor present.

D I S C U S S I O N

Possible Binding Modes for a NAX Residue

The binding of a sugar residue to lysozyme in subsite D has been directly observed by X-ray crystallography for the D-glucose residue in NAG-Glu, for the D-xylose residue in NAG-Xyl and has been inferred for a NAG residue from the model building studies as discussed in the introduction to this work. That a NAG residue might bind to lysozyme in subsite D in a similar orientation to the one found for the D-glucose residue in one of the binding modes observed for NAG-Glu may also be possible although from the X-ray model this mode appears to lead to appreciable steric interaction between the acetamido group of the NAG residue in subsite D and the enzyme.

A NAX residue bound in subsite D could thus be expected to bind in either of two ways, (a) in a similar orientation to that proposed for a NAG residue in subsite D from the model building studies and, since the unfavourable steric interaction between the C(6)-hydroxymethyl group and the enzyme would be absent, the NAX residue could exist in the usual undistorted chair conformation, or, (b) the NAX residue could bind in a similar orientation to that observed for a D-glucose residue bound in subsite D but again with less distortion from the chair conformation than was found for the D-glucose residue. A D-xylose residue in this binding mode appears to undergo less distortion from the chair conformation than was found for D-glucose. It may also be possible that the absence of the C(6)-hydroxymethyl group could cause the NAX residue to bind in a similar manner to the D-xylose residue with slight changes in the orientation to reduce any unfavourable interactions which might arise between the acetamido group and the enzyme. Hopefully the X-ray analysis of the lysozyme-inhibitor complex with inhibitor molecules containing a terminal

NAX residue will resolve which binding modes are possible for these compounds. At present however, it would appear reasonable to propose that distortion of a NAX residue bound in subsite D towards the half chair conformation is less likely to be a requisite for binding than it would be for a NAG residue bound in a similar orientation, and that a NAX residue should be able to be accommodated in subsite D at least as favourably as a NAG residue in any of the binding orientations available for the latter at this subsite. Diagrams showing possible binding modes for a NAG and a NAX residue in subsite D are shown in figures 1 - 4.

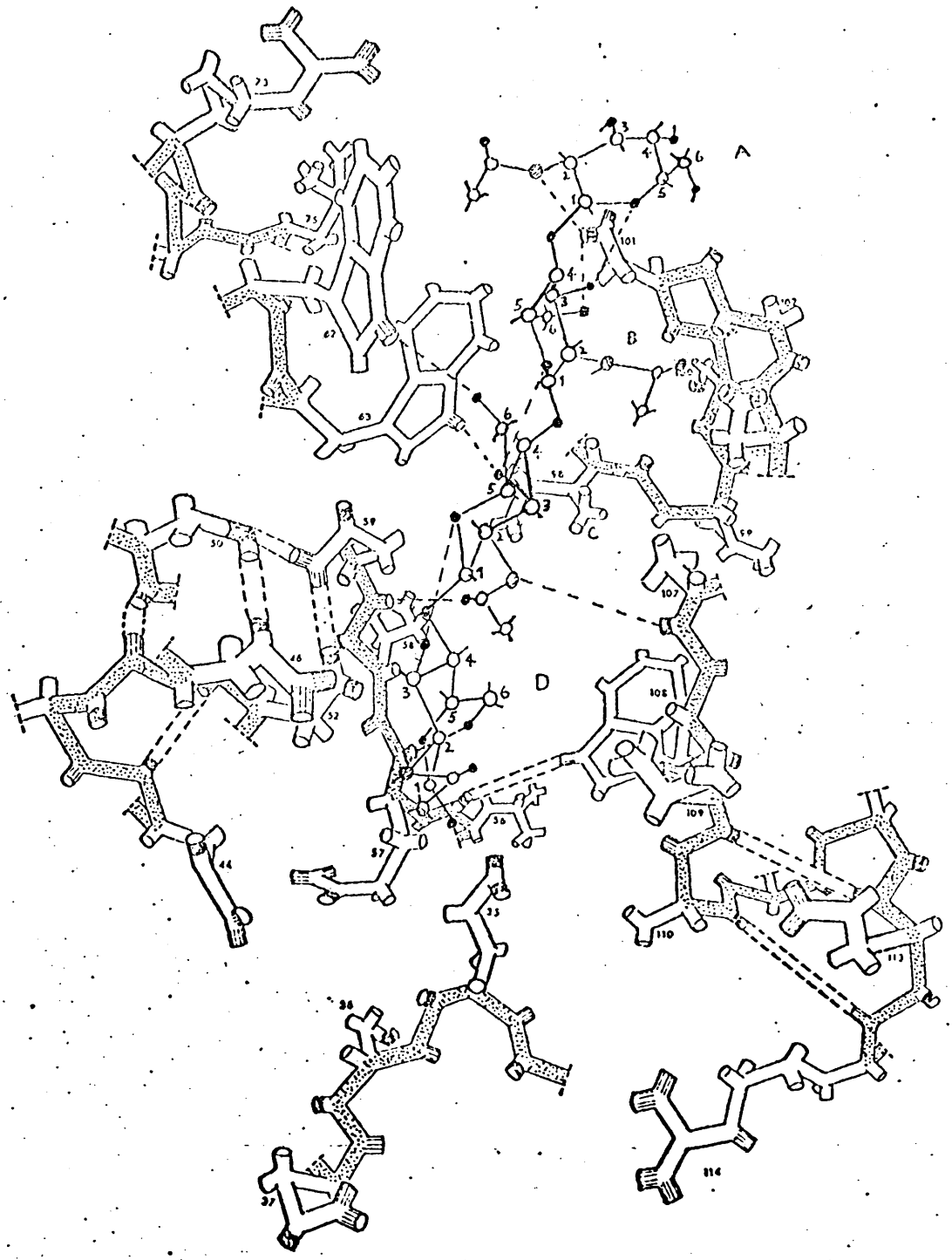


Figure 1

Binding of NAG_4 to lysozyme. The binding of sugar residues A,B and C is that observed by X-ray for the lysozyme-NAG_3 complex. Residue D is shown bound in the position inferred from the model building studies.

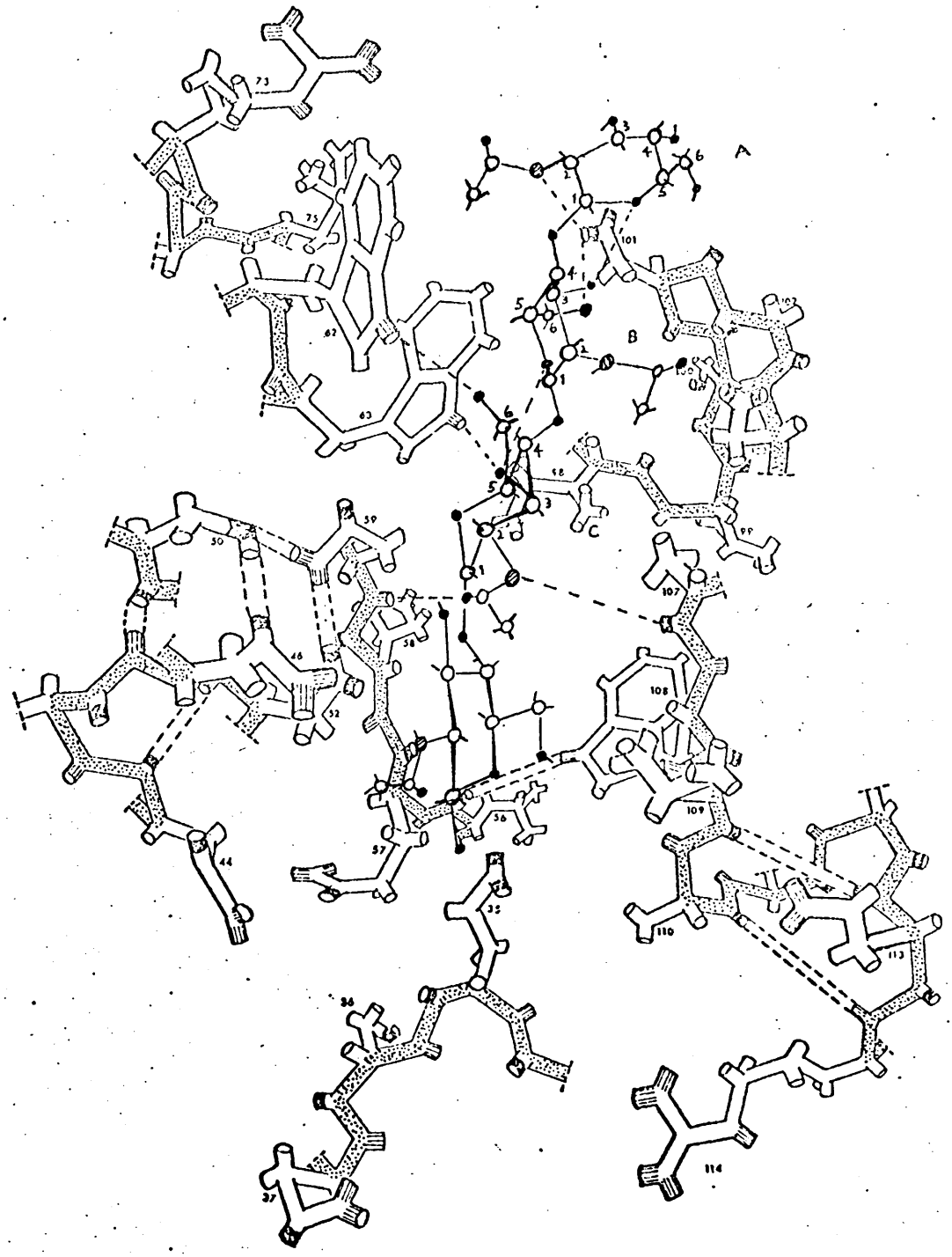


Figure 2.

Binding of NAG_4 to lysozyme. The binding of sugar residues A,B and C is that observed by X-ray for the lysozyme-NAG_3 complex. Residue D is shown bound in a similar orientation to that observed by X-ray for a D-glucose residue bound in subsite D.

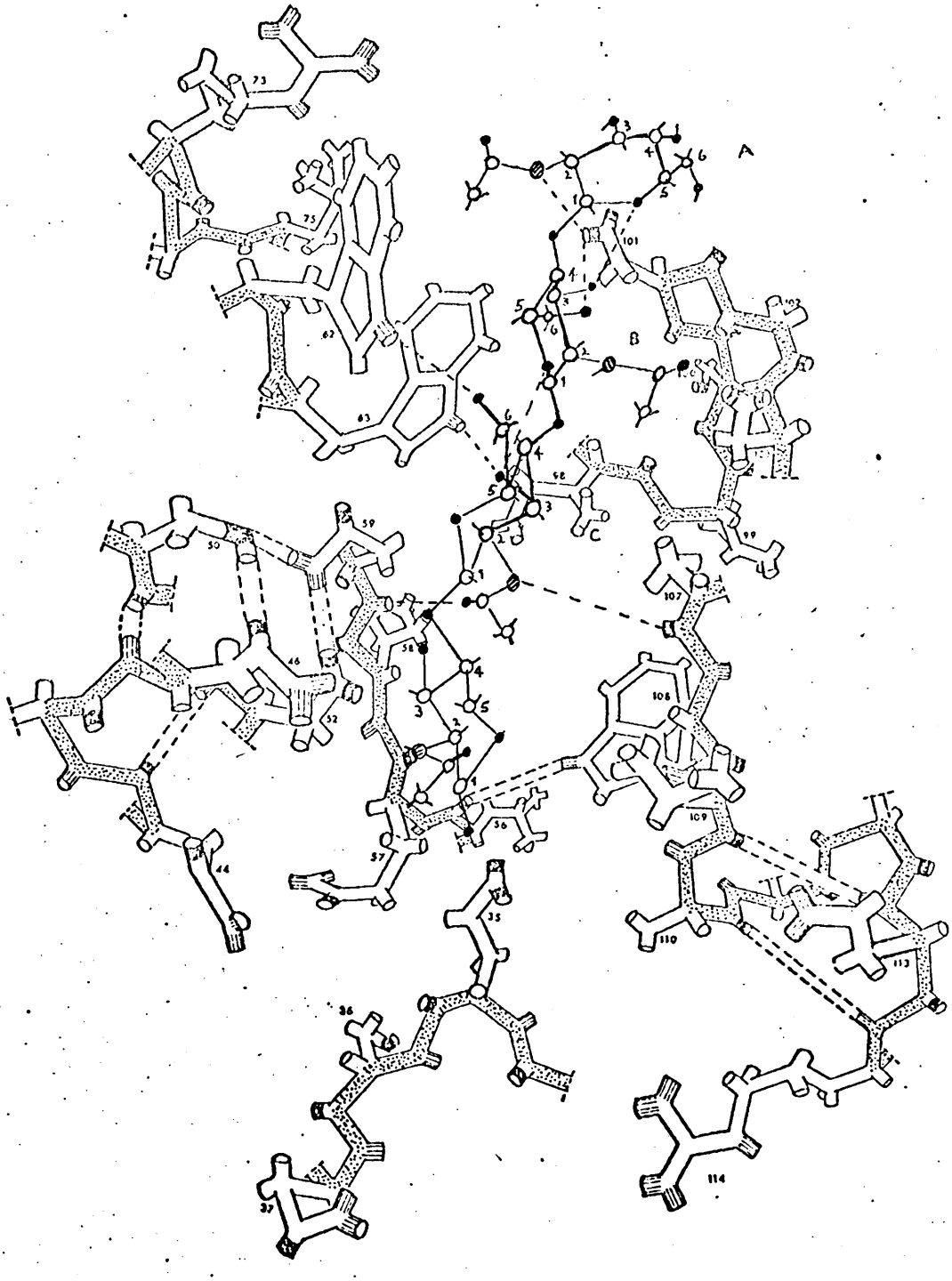


Figure 3.

Binding of NAG_3-NAX to lysozyme. The binding of sugar residues A,B and C is that observed by X-ray for the lysozyme -NAG_3 complex. Residue D is shown bound in a similar orientation to that inferred from the model building studies for a NAG residue bound in subsite D but without any distortion of the NAX residue from the chair conformation.

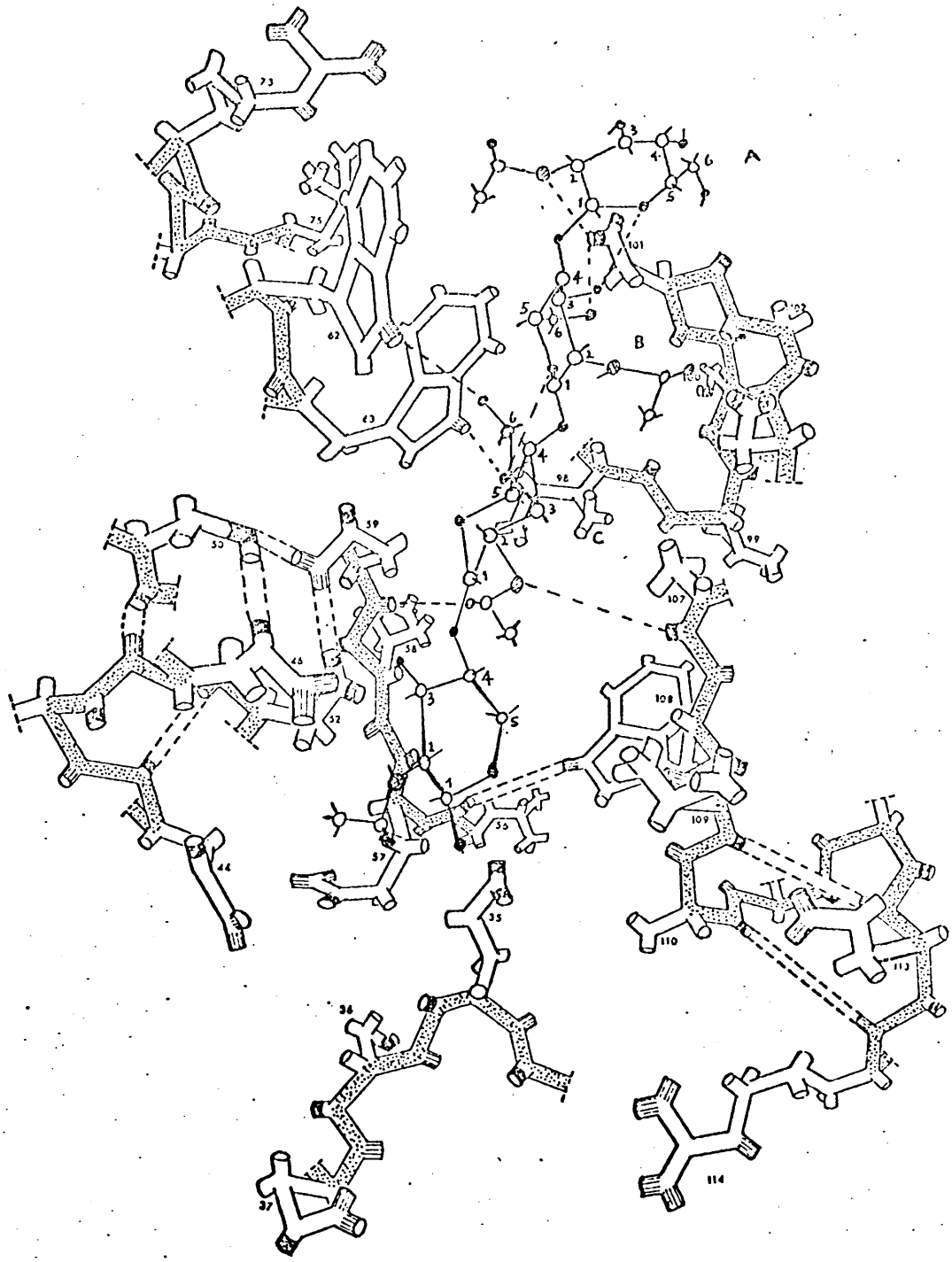


Figure 4.

Binding of NAG_3-NAX to lysozyme. The binding of sugar residues A,B and C is that observed by X-ray for the lysozyme -NAG_3 complex. Residue D is shown bound in a similar orientation to that observed by X-ray for a D-xylose residue bound in subsite D.

N.M.R. Binding Studies

From the N.M.R. results (table 3.17) it would appear that p-nitrophenyl β-NAX conforms to the binding to lysozyme observed by other monosaccharide inhibitors having a 2-acetamido group, namely binding in subsite C, as would be expected from the known affinity of this subsite for sugar residues having a 2-acetamido group. The need for caution in placing undue emphasis on what are relatively small changes in the values found for dissociation constants and bound chemical shift values from the N.M.R. method was emphasized in the introduction to this work, but in view of the good agreement found for the bound chemical shift values for p-nitrophenyl β-NAG, methyl α-NAG and NAG-Glu-PNP in this study (table 3.17) with the values found for these compounds by other workers (tables 1.2 and 1.3) it appears that p-nitrophenyl β-NAX binds in subsite D with a somewhat different orientation to that of methyl α-NAG, for whilst both compounds exhibit similar dissociation constants, $K_D \doteqdot (5\text{-}6)\times10^{-2}$M the bound chemical shift value of $\triangle$ = 0.11 p.p.m. found for p-nitrophenyl β-NAX differs from that found for methyl α-NAG, $\triangle$ = 0.79 p.p.m. The difference in the bound chemical shift values found for α and β-NAG (table 1.2) has been interpreted as being due to the acetamido methyl protons of the latter being further away from the tryptophan 108 amino acid residue of the enzyme, which is in accord with the orientations observed for these compounds in subsite C from the X-ray analysis of their binding to lysozyme. Hence the data suggests that whilst p-nitrophenyl β-NAX binds to lysozyme in subsite C with an affinity approximately equal to that of methyl α-NAG its orientation of binding in subsite C is different such that its acetamido methyl protons are further from tryptophan 108.

From the results found for the compounds listed in table 1.2 it was

suggested that either hydrogen bonding to lysozyme via the C(6)-oxygen was unimportant for sugar residues bound in subsite C or that any reduction in the binding due to the removal of the C(6)-oxygen was compensated for by Van der Waals interactions involving the C(6) region. From the results observed for the binding of p-nitrophenyl β-NAX it appears that hydrogen bonding via the C(6)-oxygen is unimportant for sugar residues having a 2 -acetamido group, confirming the strong interaction of this group with the enzyme at subsite C.

As with other sugar residues the binding observed for p-nitrophenyl β-NAX at subsite C does not preclude its binding at other subsites which may also be accessible to the sugar but where the acetamido methyl resonance does not experience a large bound chemical shift value due to the enzyme environment of the bound sugar.

The bound chemical shift values found for NAG_2 and p-nitrophenyl β-NAG_2 at 33.5°C in this study (table 3.17) agree with the results for these compounds found by other workers (table 1.3). These results have been interpreted as being consistent with the binding of the NAG residues of these compounds in subsites BC. From the results found for the binding of p-nitrophenyl β-NAG_2 to lysozyme at 55°C, which have not been previously reported, it appears that at the higher temperature the fraction bound in subsites BC is less than at 33.5°C. At the higher temperature the acetamido methyl resonance distal from the aglycone was considerably broadened in the manner usually observed for a NAG residue bound in subsite C, and hence it appears that at this temperature the distal NAG residue of p-nitrophenyl β -NAG_2 is bound in subsite C. From the N.M.R. results it is not possible to infer the binding mode of the NAG residue proximal to the aglycone although the possibility that this could represent p-nitrophenyl β-NAG_2 bound CD i.e. in a productive mode of binding suggests itself. The fact that the distal acetamido resonance did not experience an upfield shift as well as line

broadening could be due to the condition of fast exchange between the bound and unbound forms of the inhibitor necessary for observing a chemical shift change not being satisfied under the experimental conditions. As stated in the section on results, there was no observable hydrolysis of the p-nitrophenyl β-NAG_2 during the time taken to record the results, and on cooling the sample to $33.5\pm0.5^{o}C$ and repeating the experiment the sample gave the same result as was found for freshly dissolved sample at this temperature. The slight splitting observed for the acetamido methyl resonance proximal to the aglycone could be due to different modes of binding being observed, possibly as a result of different orientations of the bound sugar residue.

The binding of NAG-NAX-PNP to lysozyme as observed by N.M.R. followed a more predictable pattern. At $33.5\pm0.5^{o}C$ the resonance signal corresponding to the acetamido methyl group proximal to the aglycone was broadened considerably on addition of lysozyme. At $55\pm1^{o}C$ this resonance experienced an upfield chemical shift and line broadening similar to that observed for disaccharides bound in subsites B and C. This result was consistent with NAG-NAX-PNP being bound to lysozyme in subsites B and C. At $33.5\pm0.5^{o}C$ the condition of fast exchange between bound and unbound forms of the compound was not attained resulting in line broadening of the acetamido methyl resonance signal similar to that observed for NAG_3[57] binding to lysozyme at $35^{o}C$. From the results at $55\pm1^{o}C$ the upfield chemical shift observed allowed the bound chemical shift value Δ = 0.83 p.p.m. to be calculated. This value is consistent with the sugar residues being bound in subsites B and C, although it must be noted that even at the higher temperature the condition of fast exchange between free and bound forms of the compound, which is necessary to justify use of the two site analysis, may not have been fully realised. As mentioned in table 3.

the acetamido methyl resonance corresponding to the NAG residue was also slightly broadened on addition of lysozyme and the possibility that multiple binding modes are being observed under the experimental conditions of the N.M.R. method suggests itself. Qualitatively the results indicate that NAG-NAX-PNP binds more strongly to lysozyme than p-nitrophenyl β-NAG_2.

It was considered possible that the binding of NAG-NAX-PNP to lysozyme in subsites C and D might have been observed by N.M.R. had this been a favourable binding mode for this compound. From the result for the dissociation constant for NAG_3-NAX binding to lysozyme as measured by fluorescence spectroscopy Chipman[69] has postulated that there is a net favourable interaction of 2.2 Kcal/mole for the binding of a NAX residue to lysozyme in subsite D. The N.M.R. results for the binding of NAG-NAX-PNP to lysozyme indicate that binding of the sugar residues in subsites C and D was not a major binding mode under the experimental conditions used as no appreciable upfield chemical shift was observed for the acetamido methyl resonance of the NAG residue at either $33.5\pm0.5^{\circ}C$ or $55\pm1^{\circ}C$ although as previously mentioned some line broadening of the resonance did occur. For the aryl glycosides studied the effect, if any, that the p-nitrophenyl group could have on the binding of the compound must be taken into account. From the results of the hydrolysis studies on p-nitrophenyl β-NAG_2[56,59] and p-nitrophenyl β-NAG_3[85] the lysozyme catalysed release of free p-nitrophenol observed for both these compounds suggests that the aryl group can be accommodated in subsite E although for both of these compounds the major linkage hydrolysed was that between NAG residues which would place the p-nitrophenyl group either in subsite F or out of the active cleft.

Fluorescence Binding Studies

The dissociation constants K_DM and association constants K_aM^{-1} found for the interaction with lysozyme of the compounds studied by fluorescence spectroscopy (tables 3.18-3.25) are shown in table 4.1

Table 1.

Compound	K_DM	K_aM^{-1}
NAG-NAX-PNP	2.6×10^{-5}	3.8×10^{4}
NAG_2-NAX-PNP	2.8×10^{-6}	3.6×10^{5}
p-Nitrophenyl β-NAG_3	7.5×10^{-6}	1.3×10^{5}
NAG_3	2.3×10^{-5}	4.3×10^{4}
NAG_4	6.6×10^{-6}	1.5×10^{5}
NAG_5	5.7×10^{-6}	1.8×10^{5}
NAG_6	6.9×10^{-6}	1.5×10^{5}

For the free sugars NAG_3 to NAG_6 the fluorescence emission spectrum of lysozyme was enhanced on binding of the sugars and the wavelength of maximum emission was shifted 10nm. to lower wavelength in accord with the observations of other workers[2,76,119,120].

In the experiments using NAG_5 and NAG_6 binding to lysozyme the percentage hydrolysis of these compounds occurring during the time required to record the results was estimated from the specificities determined for the lysozyme catalysed hydrolysis of these compounds[2] and was calculated as not being greater than 1.5% and 8% respectively.

For the analysis used for the calculation of the binding constants from the fluorescence measurements the criteria that at both the emission

and excitation wavelengths used in the experiment the absorbance of the solutions used should not exceed 0.1 a.u. resulted in the binding constants for p-nitrophenyl β-NAG_2 not being able to be determined as saturation of the enzyme could not be attained without exceeding the absorbance limit for the solution. For NAG-NAX-PNP a plot of F_E-F versus $[I_o]$ showed that complete saturation of the enzyme was not attained under the experimental conditions, but sufficient data points were obtained to allow the results to be computed with an acceptable standard deviation. Comparison of the results for these two compounds indicates that NAG-NAX-PNP was bound more strongly to lysozyme than p-nitrophenyl β-NAG_2.

For the aryl glycosides studied the fluorescence emission spectrum of lysozyme was decreased and the maximum emission wavelength was shifted 5nm. to lower wavelength on binding of the sugar.

The changes observed in the fluorescence emission spectrum of lysozyme on the binding of NAG oligosaccharides have been rationalised as involving a change in the average tryptophyl environment to a less aqueous state[76,120]. The main contribution to the lysozyme fluorescence spectrum comes from the six tryptophan residues in the enzyme, a small contribution coming from tyrosine. Three of the tryptophan residues in the enzyme have been implicated as being involved in the active cleft, and, since the emission of the indole chromophore of tryptophan is sensitive to its environment, fluorescence studies of the binding of substrates and inhibitors to lysozyme have provided both details of the binding constants and allowed postulates to be made as to the position of binding of these sugars in the active cleft. The positions of the three tryptophans, 62, 63 and 108, located near the active site can be seen from the X-ray structure, figure 4.1.

For the free enzyme the fluorescence emission shows very little

change with pH up to the basic region whereas the lysozyme - NAG_3 complex pH profile shows changes between the pH regions 2.5 to 4.5 and 5.5 to 7.5 consistent with carboxyl groups having pK_a values of 3.5 and 6.3 in the complex being involved in quenching the fluorescence emission. The change in the fluorescence spectrum due to the carboxyl group with pK_a = 6.3 in the complex has been shown to be due to the interaction of this group with tryptophan residue 108 and from the X-ray structure of lysozyme showing the relative positions of the amino acid residues and other evidence bearing on the value of the pK_a for the carboxyl group of glutamic acid 35 in the enzyme-NAG_3 complex[92], it appears likely that this group is the one with a pK_a = 6.3 involved. The carboxyl group having a pK_a = 3.5 in the enzyme-NAG_3 complex has not been unambiguously assigned but from the X-ray structure and the study[92] done on the ionization behavious of the cleft carboxyls of lysozyme it appears that aspartic acid 101 could be the residue involved. The studies on the changes in the fluorescence emission spectra of the free enzyme and the enzyme-NAG_3 complex with varying pH also indicate that the enzyme undergoes a conformational change on binding NAG oligosaccharides in accord with the results of the X-ray binding studies[26,31].

Comparison of the data of tables 4.1 and 1.5 shows that good agreement between the results found for the oligosaccharides NAG_4 to NAG_6 from this study with those found by other workers was attained. The value found for the dissociation constant for the binding of NAG_3 to lysozyme $K_D = 2.3 \times 10^{-5}$M differs slightly from the value of 1×10^{-5}M found by other workers. The difference in these values does not appear to be wholly due to experimental error and implies that NAG_3 is bound somewhat less strongly than the higher oligosaccharides. The actual energy difference between the binding constants found is however small, and is of the order of

o.5 Kcal/mole.

The binding constants found for NAG-NAX-PNP and NAG_2-NAX-PNP are similar to those found by Chipman for the corresponding reducing sugars (table 1.5) suggesting that the p-nitrophenyl group does not enhance nor reduce the binding of these sugars to lysozyme, assuming that the aryl glycosides and the corresponding reducing sugars bind to lysozyme in the same manner. The dissociation constant for the binding of NAG_2 to lysozyme has been measured by fluorescence spectroscopy, ultraviolet difference spectroscopy and inhibition of the hydrolysis of micrococcus lysodeikticus, (table 1.5) and has been found to be $2x10^{-4}M$. From the N.M.R. results the dissociation constant for the binding of p-nitrophenyl β-NAG_2 to lysozyme was estimated as being less than $1x10^{-3}M$. The interaction of this compound with lysozyme has been measured by ultraviolet difference spectroscopy[123] giving a dissociation constant $K_D = 5x10^{-4}M$ and also by the inhibition of the lysozyme hydrolysis of 3,4-dinitrophenyl β-NAG_4[124] giving a dissociation constant $K_D = 5.7x10^{-4}M$. From these results it appears that NAG_2 and p-nitrophenyl β-NAG_2 bind to lysozyme with similar affinities. The small difference between the values for the dissociation constants found for the two compounds could be due to the aryl glycoside being bound to lysozyme slightly less strongly than the reducing sugar although such a small difference could be accounted for by differences in the experimental conditions used and by experimental error. Both NAG_2 and p-nitrophenyl β-NAG_2 have been shown from the N.M.R. results to bind to lysozyme in subsites B and C. The N.M.R. results also show that NAG-NAX-PNP binds in subsites B and C and comparison of the dissociation constants shows that NAG-NAX-PNP binds more strongly to lysozyme than either NAG_2 or p-nitrophenyl β-NAG_2. The energy difference between the binding of NAG-NAX-PNP and NAG_2 in subsites B and C is thus of the order of

1.2 Kcal/mole, and between the binding of NAG-NAX-PNP and p-nitrophenyl β-NAG_2 is approximately 1.7 Kcal/mole. Thus a NAX residue can bind in subsite C with a more favourable energy of interaction than that for a NAG residue. Similarly the results indicate that NAG_2-NAX-PNP binds to lysozyme more strongly than NAG_3 with a favourable energy difference of about 1.3 Kcal/mole and comparison between the binding of NAG_2-NAX-PNP and p-nitrophenyl β-NAG_3 shows that the former binds more strongly to lysozyme by about 0.7 Kcal/mole.

X-ray studies of the binding of small oligosaccharides to lysozyme illustrate that caution must be observed when interpreting the binding modes of these small molecules from results from solution studies, however it does appear likely that there is a small favourable energy interaction of approximately 1 Kcal/mole for the binding of a NAX residue to lysozyme in place of a NAG residue.

Chipman[69] has postulated that from the binding constants found for NAG_3-NAX and NAG_3 interacting with lysozyme that a NAX residue could bind in subsite D with a net favourable interaction of 2.2 Kcal/mole. Comparison of Chipman's results for the binding of NAG_2 and NAG-NAX to lysozyme shows that the latter is bound more favourably by 1 Kcal/mole and hence the favourable interaction of 2.2 Kcal/mole found for the NAX residue of NAG_3-NAX may not be wholly due to the NAX residue being bound in subsite D.

The results found for the binding of the δ-lactone derived from NAG_4 (figure 1.4), compare favourably with the results of Chipman discussed above as this lactone binds to lysozyme with an energy of interaction 2.1 Kcal/mole more favourable than that found for NAG_4. As discussed in the introduction to this work the lactone derived from NAG_4 could be expected to bind to lysozyme with the lactone moeity occupying subsite D in a similar conformation to that expected for a NAG residue bound in this

subsite with the sugar ring distorted towards a half chair conformation, and preliminary X-ray studies[34] indicate that this mode of binding is observed in the lysozyme complex of this compound. Secemski et al[37] also estimated that the association constant for NAG_4 bound in subsites A to D would be $2 \times 10^3\ M^{-1}$, and if this were so then the binding of the δ-lactone derived from NAG_4 in subsites A to D would be more favourable than the binding of NAG_4 by approximately 5 Kcal/mole. This value also correlates with the estimated energy difference of 5 Kcal/mole between the binding of a NAX residue and a NAG residue in subsite D calculated from the results of Chipman[69] using the difference between the binding constants found for $(NAG\text{-}NAM)_2$ and NAG-NAM-NAG as a measure of the unfavourable interaction for the binding of a sugar residue with a 2-acetamido and a C(6)-hydroxymethyl group in subsite D.

In addition to the postulates based on the observed binding constants found for the lactone, Secemski et al also interpreted the changes observed for the emission spectrum of the enzyme-lactone complex with pH as indicating that the binding mode for this compound was in subsites A to D. The fluorescence emission spectrum for the complex differed from that found for the lysozyme-NAG_3 complex between the pH range 3 to 7.5 and the change indicated that the pK_a of glutamic acid 35 was altered from the value of 6.3 normally associated with this amino acid residue in the lysozyme-NAG_3 complex. The values for the dissociation constant for the lactone complex with differing pH showed an increase in the binding over the pH range 3 to 6.2 and was thereafter constant up to pH 8. The pH profile suggested that glutamic acid 35 had a pK_a of 4.7 in the complex and that ionization of the carboxyl group caused an increase in the strength of binding for the complex which was the converse of that observed for the lysozyme-NAG_3 complex. These observations suggest

either that there is an interaction between the lactone and glutamic acid 35 which, from the available X-ray data, would presumably be due to the binding of the lactone ring in subsite D, or, that in the lysozyme-lactone complex ionization of the carboxyl group of glutamic acid 35 leads to a change in the enzyme conformation causing stronger binding of the sugar molecule.

Inhibition of the Lysozyme Catalysed Hydrolysis of 3,4-Dinitrophenyl β-NAG_4

The inhibition constants, K_iM, found for the interaction with lysozyme of the compounds studied were determined by the method described in the results section (chapter 3), and are shown in table 4.2. Those compounds for which the inhibition constants could be determined under the experimental conditions used were shown to be competitive inhibitors of the lysozyme catalysed hydrolysis of 3,4-dinitrophenyl β-NAG_4 causing an increase in the apparent K_m value for the hydrolysis without affecting the value of V_{max}, as was seen from the graph of initial rate ($Msec^{-1}$) versus substrate concentration for the lysozyme catalysed hydrolysis of 3,4-dinitrophenyl β-NAG_4 in the presence of a constant inhibitor concentration, and also from the Lineweaver-Burk plot of the reciprocal of the initial rate versus the reciprocal of the substrate concentration which gave straight lines having a common intercept on the vertical axis = $^1/V_{max}$ for both the inhibited and uninhibited hydrolysis.

Those compounds for which there was no apparent increase in the value of K_m for the lysozyme catalysed hydrolysis of 3,4-dinitrophenyl β-NAG_4 at the inhibitor concentrations used, NAG-Xyl-PNP, p-nitrophenyl β-NAX, p-nitrophenyl 6-iodo-6-deoxy-NAG, did not cause any change in the value of V_{max} from that found for the uninhibited hydrolysis.

Table 2.

Inhibition Constants Found From The Inhibition of the Lysozyme Catalysed Hydrolysis of 3,4-Dinitrophenyl β-NAG_4.

Results from hydrolysis in 0.1M acetate buffer pH 5.23 at 40°C.

Compound	K_iM
NAG-NAX-PNP	6×10^{-5}
NAG_2-NAX-PNP	6×10^{-6}
NAG-Glu-PNP	$(1\text{-}4)\times10^{-4}$
NAG-Xyl-PNP	$>3\times10^{-4}$
p-Nitrophenyl β-NAX	$>7\times10^{-3}$
p-Nitrophenyl 6-iodo-6-deoxy-NAG *	$>2\times10^{-4}$

* p-Nitrophenyl 2-acetamido-6-iodo-2,6-dideoxy-β-D-glucopyranoside.

In order to compare the inhibition constants for NAG-NAX-PNP and NAG_2-NAX-PNP determined at 40°C, with the dissociation constants found for the interaction of these compounds with lysozyme as measured by fluorescence spectroscopy at 25°C (table 4.1.), the effect of the different temperatures used in the two methods must be allowed for in comparison of the results from the two methods. The standard free energy change $\Delta F^\circ = -RT \log_e \frac{k_{+1}}{k_{-1}}$ for the equilibrium, $E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES$ between enzyme and substrate or inhibitor has been shown[125] to be constant with respect to temperature for most enzyme systems up until a certain critical temperature at which a change takes place probably due to denaturation of the enzyme. Comparison of the standard free energy for NAG-NAX-PNP interacting with lysozyme at 25°C (fluorescence measurement)

ΔF° = -6.26 Kcal/mole with the value found at 40° (inhibition studies) of ΔF° = -5.81 Kcal/mole and similarly for NAG_2-NAX-PNP for which ΔF° = -7.62 Kcal/mole at 25°C and -7.54 Kcal/mole at 40°C, suggests that the interaction of these compounds with lysozyme conforms to that found for most enzyme systems. The effect of temperature on the enzyme/substrate equilibrium is also related to the enthalpy change for the process[122,131] by,

$$\Delta H^{\circ} = 2.303RT^2 \frac{d \log \frac{k_{+1}}{k_{-1}}}{dT},$$

hence a plot of $\log k_{+1}/k_{-1}$ versus $\frac{1}{T}$ gives a straight line of slope $= -\Delta H^{\circ}/2.303R$. Thermodynamic parameters for binding of NAG_2 and NAG_3 to lysozyme at pH 5.3 have been calculated[73,2], giving values of $\Delta H^{\circ} = -11.6 \pm 0.6$ Kcal/mole for NAG_2 and $\Delta H^{\circ} = -14.3 \pm 0.6$ Kcal/mole for NAG_3. The results for the binding of NAG-NAX-PNP and NAG_2-NAX-PNP from fluorescence and inhibition studies give values for ΔH° for these compounds of -12.2 Kcal/mole and -10.9 Kcal/mole respectively.

Inhibition studies on NAG-NAX-PNP were performed using an enzyme concentration $[E_o] = 5 \times 10^{-7}$M and inhibitor concentration $[I_o] = 1 \times 10^{-4}$M. For the fluorescence measurements on this compound the enzyme concentration $[E_o]$ was 4×10^{-7}M and the inhibitor concentration was $2 \times 10^{-6} - 2 \times 10^{-5}$M. Comparison of the dissociation constant measured by fluorescence spectroscopy with the measured inhibition constant for this compound suggests that increasing the inhibitor concentration from approximately 10^{-5} to 10^{-6}M does not cause any large change in the binding of NAG-NAX-PNP to lysozyme, the difference in the values measured being most likely due to the different temperatures employed in the two methods. Thus either the binding modes observed by both experimental methods are very similar, or,

that since $\frac{1}{K_D} = \sum_n \frac{1}{K_b}$, where K_b represents the dissociation constant for one of several binding modes, the dissociation constant measured under both conditions is dominated by one strong binding mode.

Similarly the results from the fluorescence measurements for NAG_2-NAX-PNP under conditions of $[E_o] = 1x10^{-7}M$ and $[I_o] = 3x10^{-7} - 1x10^{-5}M$ compared with the results from the inhibition studies on this compound under conditions $[E_o] = 1x10^{-6}M$ and $[I_o] = 1x10^{-5}M$ or $1x10^{-4}M$ suggest that similar binding modes are being observed by both methods.

The competitive inhibition of the lysozyme catalysed hydrolysis of 3,4-dinitrophenyl β-NAG_4 would be expected to be due to the binding of inhibitor molecules in subsites A to E of the active cleft of lysozyme. Binding in these subsites would also be expected to have an effect on the fluorescence emission spectrum of lysozyme and hence be measurable by fluorescence spectroscopy.

The lysozyme catalysed hydrolysis of 3,4-dinitrophenyl β-NAG_4 was not inhibited by NAG-Xyl-PNP at a concentration of $1.6x10^{-4}M$. The dissociation constant for NAG-Xyl-PNP must therefore be greater than $3x10^{-4}M$ if this compound can act as a competitive inhibitor of lysozyme hydrolysis.

The inhibition results indicate that the inhibition constant for p-nitrophenyl β-NAX must be greater than $7x10^{-3}M$. From the N.M.R. results the dissociation constant found for this compound was $5.2x10^{-2}M$.

From the results of the inhibition studies the dissociation constant for p-nitrophenyl 6-iodo-6-deoxy-NAG must be greater than $2x10^{-4}M$ as would be expected for a monomer sugar. The sparingly soluble nature of this compound in D_2O did not allow its dissociation constant to be measured by N.M.R.

The inhibition constant of $(1-4)x10^{-4}M$ found for NAG-Glu-PNP differs

from the dissociation constant of 2.33×10^{-2}M found for the interaction of this compound with lysozyme by N.M.R. (table 3.), suggesting that the compound binds more strongly to lysozyme than the N.M.R. results indicate.

For the equilibrium :-

$$E + S \rightleftharpoons ES$$

the N.M.R. method is based on the assumption that,

$$\tau_E = N_{ES}\tau_{ES} + N_S\tau_S,$$

where τ_E = the observed chemical shift value of the resonance of interest in the presence of enzyme,

τ_{ES} = the chemical shift value of the resonance for the compound in the bound state,

τ_S = the chemical shift value of the resonance for the compound free in solution,

N_S and N_{ES} = the mole fractions of the compound in the free and bound state respectively.

For such a system the dissociation constant measured by the N.M.R. method is given by,

$$K_D = \frac{N_S}{N_{ES}} \times [E],$$

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

the true dissociation constant for the equilibrium.

If however, there are more than one enzyme-substrate complexes possible i.e.

$$ES \rightleftharpoons E + S \rightleftharpoons ES_1$$

then the observed chemical shift in the presence of enzyme of the resonance of interest will be :-

$$\tau_E = N_{ES}\tau_{ES} + N_S\tau_S + N_{ES_1}\tau_{ES_1}$$

If the resonance has the same chemical shift value for the compound bound in the complex ES_1 as for the compound free in solution i.e. $\tau_S = \tau_{ES_1}$, as has been observed for a NAG residue binding to certain of the subsites of the active cleft of lysozyme[57], then the N.M.R. method will not distinguish between the compound free in solution and bound in the complex ES_1. For such a system the dissociation constant measured by the N.M.R. method will be given by,

$$K = \frac{N_S + N_{ES_1}}{N_{ES}} \times [E]$$

$$= \frac{N_S}{N_{ES}}[E] + \frac{N_{ES_1}}{N_{ES}}[E]$$

$$= K_{ES} + \frac{K_{ES}}{K_{ES_1}}[E]$$

where $K_{ES} = \frac{[E][S]}{[ES]}$ and $K_{ES_1} = \frac{[E][S]}{[ES_1]}$

Thus the measured dissociation constant will differ from that found for the system where only one enzyme-substrate complex is present. Thus depending on the relative values of K_{ES}, K_{ES_1} and $[E]$ the dissociation constant measured by N.M.R. could differ from that for the equilibrium,

$$E + S \rightleftharpoons ES$$

This analysis places some doubt as to the dissociation constants measured by N.M.R. representing true dissociation constants for the enzyme-substrate binding.

Another possible cause of the discrepancy between the results found for the binding of NAG-Glu-PNP by N.M.R. and inhibition measurements could be the relative concentrations of the enzyme and inhibitor used in the two methods. For the N.M.R. measurements the enzyme concentration was 1×10^{-3}M and the inhibitor concentration $(5-34) \times 10^{-3}$M, whilst for the inhibition studies for an enzyme concentration of 5×10^{-7}M or 1×10^{-6}M the inhibitor concentration was 1×10^{-4}M and 7.7×10^{-5}M respectively.

The binding constants found by N.M.R. for the binding of NAG-Glu-PNP have been used as a measure of the productive binding for this compound[56,59] in comparisons of the rate of hydrolysis of lysozyme found for this compound compared to that found for other aryl glycosides. Skyes[60] however found that there was little change in the dihedral angle between the H_1 and H_2 protons of the β-D-glucose residue of NAG-Glu-PNP on binding of the compound to lysozyme. Since a β-D-glucose residue bound in subsite D would be expected to exist with the sugar ring distorted towards the half chair conformation, if the N.M.R. method was measuring the productive binding of NAG-Glu-PNP to lysozyme the dissociation constant found from this method would predict a change in the coupling constant between the

H_1 and H_2 protons of the β-D-glucose residue for the free and the bound substrate. X-ray analysis of NAG-Glu binding to lysozyme has also shown that the binding of a NAG residue in subsite C does not necessarily mean that the Glu residue will be bound in subsite D (see introduction to this work).

Raftery[56] used the similarity between the dissociation constant for NAG-Glu-PNP found by N.M.R. with the value of K_m apparent found for the lysozyme catalysed hydrolysis of this compound to postulate that the binding observed by N.M.R. was at least competitive with the productive mode of binding for NAG-Glu-PNP. Since however K_m apparent will reflect the strongest binding modes be they productive or non-productive this postulate does not hold since the productive mode of binding could be much weaker than the mode of binding observed by N.M.R. The N.M.R. method may also not reflect the strongest possible binding mode for the compound.

Lysozyme Catalysed Hydrolysis of Glycosides.

No definite mechanism has as yet been proven for any enzyme system capable of catalysing the hydrolysis of glycosidic bonds, but the study of the kinetics of the enzyme catalysed process would appear to be one of the most likely methods to yield information on the factors involved in the catalysis.

The energy contribution to the lysozyme catalysed hydrolysis of glycosides arising from the proposed distortion of a NAG residue in subsite D towards the half chair conformation has been variously estimated as being between 6 to 12 Kcal./mole, and it was hoped that the results of the hydrolysis of the aryl β-glycosides of NAG oligosaccharides having a terminal NAX residue could confirm this contribution to the rate enhancement observed for the enzyme catalysed hydrolysis of NAG oligosaccharides.

The hydrolysis of p-nitrophenyl β-NAG, p-nitrophenyl β-D-glucoside and p-nitrophenyl β-NAX was studied in 0.1M citrate buffer pH 5.3 containing 10% v/v of spectroscopic dioxan at 40°C, the results of which are shown in tables 3.1 and 3.2, and presented graphically in figures 3.1 - 3.5. Apart from the rates measured for the spontaneous hydrolysis of these compounds the other estimated rates represent complex hydrolysis patterns due to the formation of higher molecular weight oligosaccharides by transglycosylation and the existence of multiple binding modes for the glycosides formed. Hence the graphical representation gives a clearer indication of the differences between the compounds.

Comparison of the hydrolysis of p-nitrophenyl β-NAG in the presence and absence of lysozyme (tables 3.1 and 3.2) showed that under the experimental conditions studied the presence of lysozyme alone did not cause an increase in the rate of hydrolysis over that of the spontaneous rate.

In the combined presence of lysozyme and NAG_4 the rate of release of p-nitrophenol increased after an induction period of one hour by a factor of about ten. This apparent rate increase was due to the formation of higher molecular weight p-nitrophenyl glycosides by transglycosylation reactions between NAG_4 and p-nitrophenyl β-NAG, the induction period being due to the time required for the build up of the transglycosylation products. p-Nitrophenyl β-D-glucoside also showed a slight increase in the rate of release of p-nitrophenol when it was incubated with lysozyme and NAG_4. The rate of release of free p-nitrophenol was slower than that found for p-nitrophenyl β-NAG by about a factor of 10 but it still represented an increase in the spontaneous rate which was so small that it could not be estimated.

p-Nitrophenyl β-NAX exhibited a slightly faster spontaneous rate of hydrolysis than p-nitrophenyl β-NAG. Unlike p-nitrophenyl β-NAG however the rate of release of p-nitrophenol did not appear to be increased by the presence of lysozyme and or NAG_4. The graphical representation of the hydrolysis, figure 3.5, does however indicate that at the higher sugar concentration studied the release of p-nitrophenol in the presence of enzyme and NAG_4 showed an induction period longer than that observed for p-nitrophenyl β-NAG under the same conditions.

The reaction mixtures all showed the formation of higher molecular weight aryl glycosides by T.L.C. (6 : 3 : 2, v/v/v, n-propanol, ammonia, water).

Raftery[89] has also studied the release of p-nitrophenol from p-nitrophenyl β-NAG and p-nitrophenyl β-D-glucoside under similar conditions to those used in this study obtaining similar results to the above.

Due to the complex nature of the hydrolysis process under the conditions used it is difficult to infer any strong conclusions from the results obtained, however the fact that the hydrolysis of p-nitrophenyl β-NAX in the presence of NAG_4 and lysozyme showed an induction period suggests that the higher molecular weight aryl glycosides formed by transglycosylation were being hydrolysed, albeit more slowly than the corresponding products from the transglycosylation reaction with p-nitrophenyl β-NAG.

Hydrolysis of $(NAG)_n$-NAX-PNP oligosaccharides.

Tables (3.3 - 3.5) show the results found for the hydrolysis of the oligosaccharides prepared from transglycosylation reactions with lysozyme and p-nitrophenyl β-NAX.

The spontaneous hydrolysis of p-nitrophenyl β-NAX had a first order rate constant $k_{obs} = 2.2 \times 10^{-7} sec^{-1}$ at 40°C and pH 5.3. The spontaneous hydrolysis of the higher oligosaccharides would be expected to be equally slow if not slower.

The attempted hydrolysis of NAG-NAX-PNP at 40°C is shown in table 3.3. For a substrate concentration $[S_o] = 1.09 \times 10^{-5}$M to 2.5×10^{-4}M in the presence of an enzyme concentration $[E_o] = 2.5 \times 10^{-4}$M there was no detectable release of free p-nitrophenol. The highest rate recorded was that where $[S_o]$ was equal to 1×10^{-3}M and $[E_o] = 1.5 \times 10^{-3}$M which gave an observed rate of $4.17 \times 10^{-11} Msec^{-1}$ for the hydrolysis over a period of 24 hours. Under exactly similar conditions and at the same enzyme and substrate concentrations p-nitrophenyl β-NAG_2 was hydrolysed giving an initial rate of $3.26 \times 10^{-10} Msec^{-1}$, and at pH 5.2 and 35°C the specificity k_{cat}/K_m for the lysozyme catalysed hydrolysis of this compound has been

measured[79] as being $1.2 \times 10^{-3} M^{-1} sec^{-1}$. From the spontaneous rate of hydrolysis found for p-nitrophenyl β-NAX it appears that the hydrolysis of NAG-NAX-PNP is not catalysed by lysozyme, or at most the lysozyme catalysed hydrolysis is much slower than for the NAG analogue. T.L.C. of the hydrolysis solutions showed no evidence of hydrolysis of the glycosidic bond or of the NAG-NAX linkage.

NAG_2-NAX-PNP showed a measurable spontaneous rate of hydrolysis at pH 5.23 and 40°C (table 3.4), but no increase in rate was observed in the presence of lysozyme. An upper limit for the value of k_{cat}/K_m for this compound would thus be $5 \times 10^{-4} M^{-1} sec^{-1}$ which is considerably less than the value of $0.196 M^{-1} sec^{-1}$ found for the lysozyme catalysed hydrolysis of p-nitrophenyl β-NAG_3[107].

NAG_3-NAX-PNP showed no observable hydrolysis in 0.1M acetate buffer pH 5.23 at 40°C for a substrate concentration of $3.7 \times 10^{-5} M$ to $1.0 \times 10^{-4} M$ and an enzyme concentration of $1.0 \times 10^{-5} M$ to $2.5 \times 10^{-4} M$. At a substrate concentration of $2.6 \times 10^{-3} M$ and an enzyme concentration of $5.8 \times 10^{-3} M$, at the same conditions of temperature and pH as above, an initial rate of hydrolysis of $1.8 \times 10^{-8} M sec^{-1}$ was observed. This represents an increase over the spontaneous rate of hydrolysis of this compound, expected for this substrate concentration, of approximately $1 \times 10^{-10} M sec^{-1}$, leading to an estimated specificity for this compound of $10^{-3} M^{-1} sec^{-1}$ again very much less than the value of $0.95 M^{-1} sec^{-1}$ found for the specificity of p-nitrophenyl β-NAG_4 hydrolysis by lysozyme[107] under similar conditions of pH and temperature.

Due to the slow enzymic rate of hydrolysis of the p-nitrophenyl β-glycosides of NAX and oligosaccharides having a terminal NAX residue the hydrolysis of the 3,4-dinitrophenyl β-glycosides were studied.

The results for the hydrolysis of 3,4-dinitrophenyl β-NAX are shown

in table 3.6. The compound had a rate constant $k_{obs} = 1x10^{-5}sec^{-1}$ for the spontaneous hydrolysis at pH 5.23 and 40°C. No increase in the spontaneous rate was observed on addition of lysozyme. The high spontaneous rate of hydrolysis for 3,4-dinitrophenyl β-NAX allowed computation of the initial rates of hydrolysis over a period of twenty minutes and as expected over this time interval the compound showed no increase in the initial rate of hydrolysis, over that found for the spontaneous rate, in the presence of NAG_4 and lysozyme. However after two hours there appeared to be a slight increase in the rate of hydrolysis probably due to the formation of transglycosylation products which were hydrolysed by the enzyme.

NAG-NAX-DNP did not show any increase in the rate of hydrolysis, over that found for the spontaneous rate, for a substrate concentration of $1x10^{-4}M$ in the presence of lysozyme at a concentration of $1x10^{-4}M$. Similarly NAG_2-NAX-DNP showed no observable increase in the rate of hydrolysis for a substrate concentration of $1x10^{-4}M$ in the presence of lysozyme at a concentration of $1x10^{-4}M$. Both these compounds had a first order rate constant $k_{obs} = 10^{-6}sec^{-1}$ for spontaneous hydrolysis at pH 5.23 and 40°C.

The detailed interpretation of these results for the aryl glycosides of NAX is hampered by the presence of non-productive modes of binding for these compounds which can cause self-competitive inhibition by the substrate. If one complex is formed more strongly than the others then the properties of that complex will dominate the observed reaction of the compound with the enzyme[28]. Thus the association constant K_aM^{-1} observed will be the sum of the association constants for the productive and non-productive modes of binding, i.e.

$$K_a = \Sigma_i K_a' + \Sigma_i K_a''$$

The observed rate of hydrolysis of the compound will also be affected by the non-productive binding

$$k_{obs} = k_{cat} \frac{\Sigma K_a'}{\Sigma K_a' + \Sigma K_a''}$$

where K_a' = association constant for binding in a productive mode

and K_a'' = association constant for binding in a non-productive mode.

This productive and non-productive association is however present for the binding of all the small molecular weight substrates the hydrolysis of which have been studied.

The kinetics of enzymic hydrolysis have been analysed over a wide range of experimental conditions[126,127] in order to determine the relevant parameters defining the hydrolysis.

For the system:-

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} \underset{+P_1}{ES'} \xrightarrow{k_3} E + P_2$$

The simplest analysis under conditions $[S_o] \gg [E_o]$ gives the Michaelis-Menten relationship:-

$$\text{Rate of hydrolysis } \frac{dP_1}{dt} = v = \frac{k_{cat}[E_o][S_o]}{K_m + [S_o]}$$

$$\text{where } k_{cat} = \frac{k_2 k_3}{(k_2+k_3)} \text{ and } K_m = \frac{(k_{-1}+k_2)k_3}{(k_2+k_3)k_1}$$

For the same system under $[E_o] \gg [S_o]$ conditions the rate of hydrolysis can be expressed as:-

$$\frac{dP_1}{dt} = v = \frac{k_{cat}[E_o][S_o]}{K_m + [E_o]},$$

which is equivalent to the expression developed by Bender[126] assuming that only a small amount of hydrolysis has occurred and hence $([S_o] - P_1) = [S_o]$. This expression is similar to the one used by Lowe et al[79] when studying the lysozyme catalysed hydrolysis of phenyl β-NAG_2 and the corresponding thiol analogue.

For both of these expressions, providing that $[S_o]/K_m \ll 1$ or $[E_o]/K_m \ll 1$, then the specificity of the hydrolysis k_{cat}/K_m can be found from the observed rate of hydrolysis from:-

$$\text{Rate} = \frac{k_{cat}}{K_m}[E_o][S_o]$$

From this expression the upper limit of k_{cat}/K_m for the lysozyme catalysed hydrolysis of the aryl glycosides of NAX could be estimated. For NAG_3-NAX-PNP, which could be expected to bind to lysozyme in a productive mode better than any of the other NAX compounds studied, the value found for $k_{cat}/K_m = 10^{-3}M^{-1}sec^{-1}$ is 1,000 times less than that found for p-nitrophenyl β-NAG_4. For the smaller oligosaccharides fluorescence and inhibition studies have shown that those compounds having a NAX residue bind more strongly to lysozyme than the corresponding NAG analogues. Since this binding is presumably non-productive then the apparent inability of lysozyme to catalyse the hydrolysis of the aryl glycosides of NAX could be due in part to the presence of strong non-

productive modes. The energy difference of approximately 1Kcal/mole found for the binding of NAG-NAX-PNP and NAG_2-NAX-PNP compared to the corresponding NAG analogues is however not in itself large enough to account for the large difference in the rates of hydrolysis found for these compounds.

p-Nitrophenyl 2-deoxy-β-D-Glucopyranoside

The only other known report of this compound was made by Raftery[56]. The observations made in this study on this compound differ from those previously reported.

The compound was synthesized as described in the experimental section from 1,3,4,6-tetra-O-acetyl-2-deoxy-β-D-glucopyranoside via the 3,4,6-tri-O-acetyl-2-deoxy-D-glucopyranosyl chloride. The N.M.R. of the acetylated glycoside, p-nitrophenyl 3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranoside was studied (see experimental discussion) and the results confirmed that the compound had the β-D configuration, and that in $CDCl_3$ solution it existed mainly in the C1 conformation. The deacetylated glycoside, p-nitrophenyl 2-deoxy-β-D-glucopyranoside showed the same magnitudes for the coupling constants $J_{1,2a}$ and $J_{1,2e}$ in d_5-pyridine as were found for the O-acetylated glycoside.

Raftery[56] reported the synthesis of p-nitrophenyl 2-deoxy-β-D-glucopyranoside using 3,4,6-tri-O-benzoyl-2-deoxy-α-D-glucosyl bromide with the sodium salt of p-nitrophenol in aqueous acetone. The authors did not quote any physical properties of the de-O-benzoylated compound other than a melting point of 167 - 169°C (uncorr.) and a molar release of p-nitrophenol on acid hydrolysis of the compound.

Previous attempts[128,129] at the synthesis of aryl 2-deoxy-β-D-

glucopyranosides using 3,4,6-tri-O-benzoyl-2-deoxy-α-D-glucosyl bromide and phenol in quinoline in the presence of silver oxide did not afford any crystalline glycoside. Treatment of pure α or β-tetra-O-acetyl-2-deoxy-D-glucose and a phenol with either zinc chloride or toluene-p-sulphonic acid[128] resulted only in the isolation of a crystalline aryl 2-deoxy-α-D-glucopyranoside 3,4,6-triacetate.

The melting point found for p-nitrophenyl 2-deoxy-β-D-glucopyranoside in this study was 164°C (uncorr.) (c.f. Raftery[56] 167 - 169°C). The p-nitrophenyl 2-deoxy-α-D-glucopyranosides have been studied by Shafizadeh and Stacey[130]. For the tri-acetate they reported a melting point of 140 - 141°C. The corresponding β-glycoside was found from this work to have a melting point of 154°C. p-nitrophenyl 2-deoxy-α-D-glucopyranoside was quoted[130] as having a melting point of 173 - 174°C.

The investigation of the lysozyme catalysed hydrolysis of p-nitrophenyl 2-deoxy-β-D-glucopyranoside was considered to be important since Raftery[56,89] has concluded from studies of this hydrolysis that anchimeric assistance by acetamido side chain at C_2 of the sugar ring undergoing enzymatic glycosyl rupture was not a likely mechanism for the lysozyme catalysed hydrolysis of glycosidic linkages.

The synthesis of NAG-Deoxy-Glu-PNP by means of a transglycosylation reaction between p-nitrophenyl 2-deoxy-β-D-glucopyranoside and NAG_2 in the presence of lysozyme was reported[56] and the dimer glycoside separated was found to be hydrolysed by lysozyme giving a value of $3 \times 10^{-6} sec^{-1}$ for V_{max}/K_m in comparison to the value of $5.2 \times 10^{-7} sec^{-1}$ found for the hydrolysis of p-nitrophenyl β-NAG_2.

In an earlier publication Raftery[89] found for p-nitrophenyl 2-deoxy-β-D-glucopyranoside in the presence of lysozyme and NAG_4 that the rate of release of p-nitrophenol at pH 5.5 and 40°C was sixteen times greater

than that found for p-nitrophenyl β-D-glucopyranoside under the same conditions. The authors reported achieving a solubility of 2.0×10^{-2}M for p-nitrophenyl 2-deoxy-β-D-glucopyranoside in 0.1M citrate buffer pH 5.5 containing 10% v/v of dioxan. It was attempted to repeat these results but it was not found possible to achieve solubility of the glycoside at a concentration greater than 3×10^{-3}M under the same conditions as above. At this concentration in the presence of lysozyme and NAG_4 no release of p-nitrophenol was observed under the experimental conditions reported. The insolubility of aryl 2-deoxy-D-glucopyranosides was commented on by Shafizadeh[130].

As reported in the experimental section attempted transglycosylation reactions with p-nitrophenyl 2-deoxy-β-D-glucopyranoside showed no evidence for the formation of higher molecular weight aryl glycosides.

In view of the conflict between these results and those of Raftery and of the importance of the proposals based on the latter's observations as to the mechanism of the lysozyme catalysed hydrolysis of glycosides further investigation of this compound seems advisable.

Conclusions.

The energy available for distortion of a pyranose ring in subsite D of lysozyme, estimated as about 6Kcal/mole, could be expected to lead to a rate enhancement of up to 10^4-10^5 depending on whether or not the distortion is towards a conformation directly on the path of the reaction towards the transition state intermediate involving the formation of a carbonium ion. The factor which substrate distortion could contribute to the enzymic rate enhancement could also be dependent on the energy required to cause the pyranose ring to adopt the half chair conformation.

The best estimate for a rate enhancement due to substrate distortion from the results of this study comes from a comparison of the specificity found for the lysozyme catalysed hydrolysis of p-nitrophenyl β-NAG_4 which was at least 1,000 times greater than that found for the hydrolysis of NAG_3-NAX-PNP. The specificity however represents a complex mixture of interactions between enzyme and substrate which means that the factor of 1,000 can not be taken as a direct measure of the rate enhancement due to distortion of a NAG residue in subsite D towards a half chair conformation.

Other factors which could affect the hydrolysis of an oligosaccharide containing a NAX residue compared with the corresponding NAG analogue are, different orientation of binding to lysozyme, different conformation of the NAX residue and differences in binding strengths between a NAX and a NAG residue leading to different relative proportions of the compounds in productive and non-productive modes of binding.

Differences between the orientation of binding of a NAX and a NAG residue in subsite D could cause a difference in the enzyme rate enhancement due to the relative positions of the catalytic groups on the enzyme with respect to the glycosidic linkage undergoing hydrolysis. Thus if

the orientation of a NAX residue in subsite D was such that there was less exposure of the glycosidic bond to the catalytic groups on the enzyme this could lead to a decrease in the rate of hydrolysis which would not be directly related to the bound conformation of the sugar ring.

Similarly if the NAX residue existed in a different conformation to that of the NAG residue the catalytic effect of the enzyme might be reduced. This is especially worth noting in the case of a NAX residue since the N.M.R. study of p-nitrophenyl 2-acetamido-3,4-di-O-acetyl-2-deoxy-β-D-xylopyranoside showed that this compound existed mainly in the 1C conformation which has all the groups on the sugar ring in an axial conformation. The de-O-acetylated glycoside however appeared to exist to a greater extent in the normal C1 conformation. The anomeric proton on the NAX residue of NAG-NAX-PNP had a coupling constant $J_{1,2}$ = 6 cps in aqueous solution and thus it would also be expected to exist mainly with the NAX residue having a C1 conformation. The relative equilibria between C1 and 1C conformations for a NAX and a NAG sugar residue may however be different as the latter is expected to have a strong preference for the C1 conformation due to the C(6)-hydroxymethyl group.

Differences in the strength of binding to lysozyme could also affect the rate of hydrolysis of oligosaccharides having a NAX residue compared to the NAG analogues. The results of this study have shown that the dissociation constants for the NAX compounds are smaller than those for the corresponding NAG compounds showing that the former bind more strongly to lysozyme. This binding is however for the small oligosaccharides studied most likely to involve non-productive complexes. Thus strong non-productive binding could also cause a decrease in the observed rate of hydrolysis for the NAX compounds.

The above discussion shows that the observed results for the

hydrolysis of the oligosaccharides having a NAX residue in place of a terminal NAG residue whilst consistent with the proposal that substrate distortion in the lysozyme catalysed hydrolysis of glycosides contributes to the enzymic rate enhancement do not conclusively prove the proposal.

The complexity of a substrate which would be necessary to conclusively show that substrate distortion on the lysozyme catalysed hydrolysis of glycosides contributes to the enzymic rate enhancement would make it a daunting prospect from the point of view of chemical synthesis. It appears however that this effect could never be satisfactorily explained by any conceivable model studies and hence its confirmation or otherwise will depend on suitable substrates being obtained.

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