A Study of the Action of Lysozymes on Some New Synthetic Substrates and Inhibitors

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Science Faculty of the University of Glasgow

by

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STATEMENT

This project was carried out in the Chemistry Department of the University of Glasgow, with the guidance of Dr. B. Capon. There is no part being submitted concurrently for another degree.

September 1970 - September 1973

Signed

(F. W. Ballardie)

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To Marilyn

ABSTRACT

Hen egg white lysozyme has, in recent years been one of the most widely studied of enzymes, since its three-dimensional structure was determined by X-ray crystallography. From these studies, there have been proposed several mechanisms of action of the enzyme, at least three of which are possible. One object of this work was to determine which is the correct one.

Despite the interest, only a handful of significant papers have appeared regarding the mechanism of action of the enzyme. This is because accurate kinetic investigation on the natural substrate is difficult or impossible, and all the synthetic substrates prepared prior to this work were very poor ones.

The first object of this work was therefore to synthesise a good substrate whose hydrolysis could be followed easily. An obvious candidate was an aryl glycoside of NAG_4, but there were considerable practical problems to overcome, since glycosides of NAG_4 had not been previously prepared. The chromatographic separation, on a preparative scale, of the peracetates of NAG_2 to NAG_6 was achieved, and from these, some 2, 4-dinitro , p-nitro , and 3, 4-dinitrophenyl glycosides were synthesised. The increased specificity of the p - nitrophenyl glycosides and 3, 4-dinitrophenyl glycosides on increasing the sugar chain length from NAG_2 to NAG_4 was observed , as expected.

NAG_4-β-3, 4 dinitrophenyl was a 250-fold better substrate for hen egg-white lysozyme than NAG_2-β-2, 4 dinitrophenyl, the best synthetic substrate prepared prior to this work. NAG_4-β-3, 4-dinitrophenyl was less active than NAG_6, and was used in unsuccessful attempts to detect a covalent intermediate in its hydrolysis.

In an attempt to make even better substrates, the acetylated β-fluorides of NAG_1, NAG_2 and NAG_4 were prepared. The former two were deacetylated, using a new deacetylation technique. These fluorides proved to have high spontaneous rates of hydrolysis. The NAG_2-β-fluoride proved to be a better substrate for lysozyme than NAG_2-β-2, 4 dnp.

The 2 methyl oxazoline derivative, formed by neighbouring group participation of the acetamido group, was shown to be present, by PMR, during the methanolysis of NAG_1-β-fluoride.

A series of inhibitors for the enzyme was studied, using NAG_4-β-3, 4-dinitrophenyl as substrate. These were the reducing sugars, NAG_2 to NAG_4, and their paranitrophenyl glycosides.

Attempts were made to study the lactone inhibitors reported by Secemski.

A comprehensive analysis of a full kinetic model of lysozyme reactions was carried out. The behaviour of many reactions was explained in terms of changes in non-productive complexes, and a distorted productive complex. Some important parameters for individual reactions were determined.

NAG_4-β-3, 4 dinitrophenyl was also found to be a substrate for human milk, duck II and duck III lysozymes, and proved useful in the assay of hen-egg-white lysozymes.

ABBREVIATIONS

Lysozyme refers to the lysozyme from hen-egg white, unless otherwise stated.

The trivial nomenclature, used for the sake of brevity throughout the text, is that recommended by Barker et al. [22]

n	Name	Trivial name
1	N-acetyl D glucosamine	NAG_1
2	Di-N-acetyl chitobiose	NAG_2
3	Tri-N-acetyl chitotriose	NAG_3
4	Tetra-N-acetyl chitotetraose	NAG_4
5	Penta-N-acetyl chitopentaose	NAG_5
6	Hexa-N-acetyl chitohexaose	NAG_6

The per O acetylated derivates have the prefix Ac;
e.g. Ac NAG_2 is chitobiose octaacetate.

Substituents at C 1, the reducing end of the sugar, are written as suffixes to the above.

E.g. $AcNAG_2$-β-2,4 dnp is 2,4 dinitrophenyl-2-acetamido 3,6 di-O-acetyl-2-deoxy-4-O-(2-acetamido - 3,4,6-tri-O-acetyl- 2-deoxy -β-D glucopyranosyl-) - β -D glucopyranoside; or -2,4 dinitrophenyl - di-N-acetyl chitobioside.

-Cl indicates the 1-chloro sugar.

The configuration of the glycosidic bond is shown by α or β inserted between glycone and aglycone

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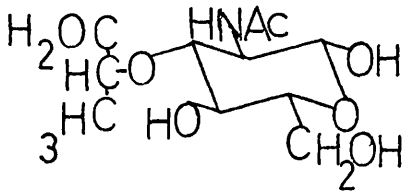
1. INTRODUCTION

1. 1 Background to Lysozymes and their substrates

Since the discovery half a century ago [1] that gram positive bacteria are particularly susceptible to the action of human lysozyme, forty years passed before the enzyme substrate was identified.

The bacterolytic properties of hen egg white were described by Fleming before he obtained evidence that enzymes were present. He termed these, lysozymes, describing their lytic properties. This work will be concerned with the vertebrate lysozymes, as distinct from 'phage lysozymes.

Early experiments [2, 3] showed that NAG was released from the bacterial cell walls, and in 1959, it was shown [4] that hen-egg white lysozyme degrades chitin, the (1 → 4) linked polymer of NAG. In the same year it was shown [5] that the cell wall tetrasaccharide NAG-NAM-NAG-NAM, where NAM = N-acetyl muramic acid (1) gave only one



(1)

product on degradation, namely NAG-NAM, and it was concluded that the tetrasaccharide is (1 → 4) linked.

Jeanloz suggested [7] that all glycosidic linkages are (1 → 4), and that the cell walls are mucopolysaccharides, also containing short polypeptide chains consisting of both D and L amino acids linked to lactyl side chains of some NAM residues, and cross-connecting the polysaccharide chains to form giant "bag shaped" macromolecules.

Work on the saccharides from the cell walls of different bacteria shows that the specificities of different lysozymes may change for different substrates. [133]

Lysozyme does exhibit chitinase activity, but on cell walls acts only as an N-acetyl muramidase.

The Biological Role of Lysozymes

It is not entirely clear what the role of lysozymes in resistance to bacterial infection is. They are not found throughout vertebrate cells and secretions [8], and there appears to be few bacteria pathogenic to vertebrates that are susceptible to lysis by lysozyme alone. Of course, it may be that these bacteria are not pathogenic because they are open to attack.

Many totally different roles have been suggested since it was discovered that lysozyme and α-lactalbumin have very similar structures [10,11,12] and that the latter plays a part in galactose biochemistry. There may be quite novel roles as yet undiscovered, for lysozyme. There are medical implications in the determination of lysozyme levels, and a comprehensive review [13] of the prognostic and diagnostic value in cases of human leukaemia has been written. There is also interest in the measure of urinary and serum lysozyme levels for diagnostic purposes [14], in the case of acute renal failure, since human lysozyme, having a molecular weight of under 15,000, is the first protein to pass through increasing pore sizes in the glomerulae.

An interaction between lysozyme and penicillin has been noted, but the implications are uncertain. [134]

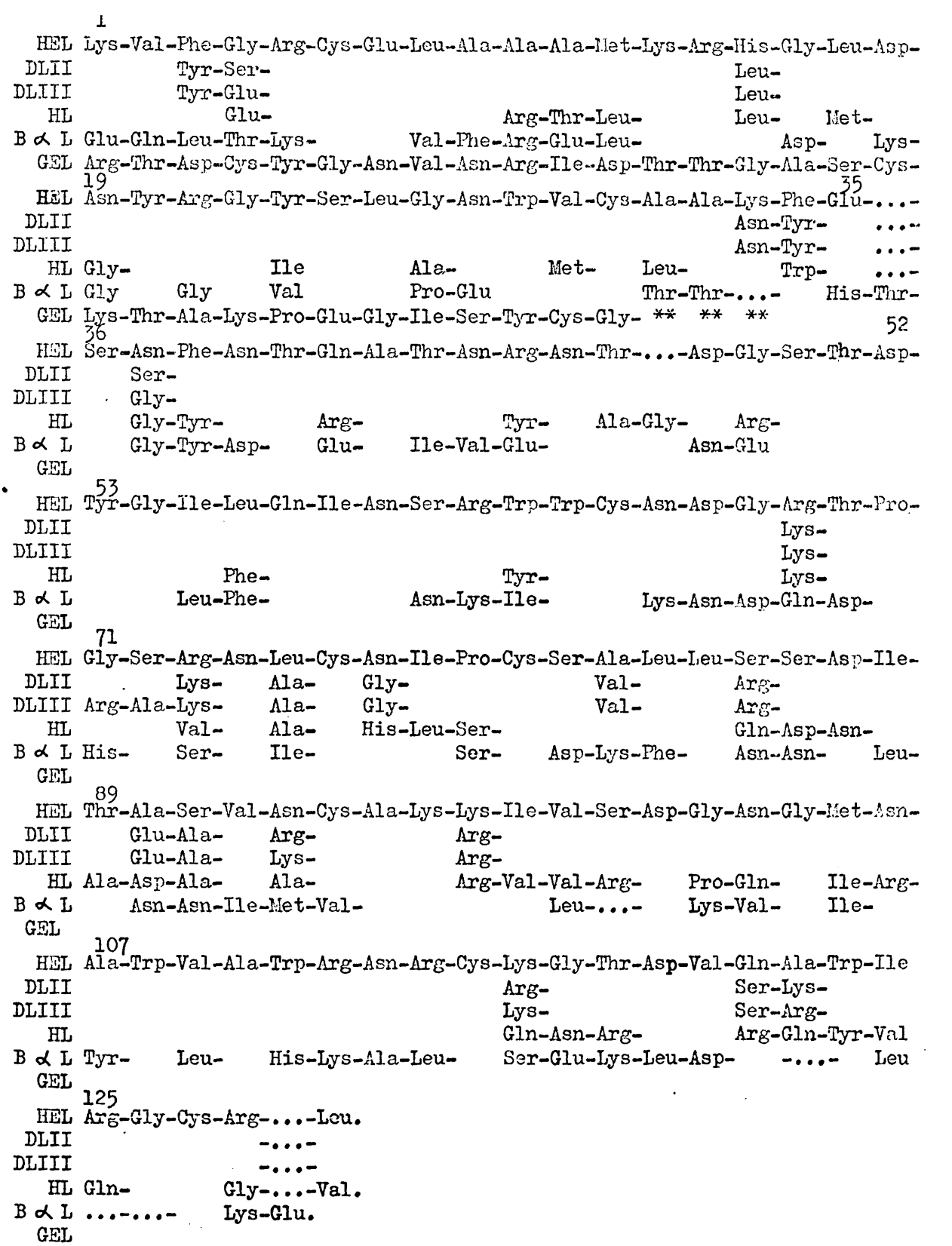


Fig. 1. The full primary structures of hen egg-white lysozyme (HEL), duck egg-white lysozymes II and III (DLII and DLIII), human milk and leukaemic lysozymes (HL), bovine α-lactalbumin (B α L) and the first thirty residues of goose egg-white lysozyme (GL).

Human Lysozymes

The lysozyme from human milk has been isolated, and is reported to have twice the activity for Micrococcus Luteus (formerly Lysodeikticus) that hen-egg white lysozyme has.[15] The enzyme has also been found to have chitinase activity, and forms transglycosylation products.[16] The pH optimum is thought to be 4.2, for human leukaemic lysozyme.[23] The primary structure of the enzyme has been determined,[17,18] and was originally thought to consist of 129 amino acids, as for hen-egg white lysozyme, but it has now been shown [19] to have 130 residues, the extra one being Val.100.

It can be concluded that the catalytic group Glu.35 could be in the same position, but that "Asp 52" is the 53rd residue. The full primary structure is as shown in Fig. 1.

Human milk and human leukaemic lysozymes have been found to have the same sequences [20], and therefore possibly the same tertiary structure, but two different types of lysozyme from humans have been separated by gel filtration [21], and therefore there is some doubt as to the validity of this comparison.

The X-ray structure of human leukaemic lysozyme at 6 Å resolution has been published [22], and the tertiary structure is very similar to that of hen-egg white lysozyme, and it is concluded that all regions have very similar foldings. It was also found that NAG binds in the cleft of human lysozyme in a position corresponding to subsite C of hen-egg white lysozyme, and α and βNAG bind more similarly than they do in the latter.

The active site remains substantially unchanged; Glu 35 and Asp 52 are unchanged, but four substitutions occur among the fifteen residues that make up the extensive binding site proposed for hen-egg white lysozyme.
All four substitutions occur close together and involve residues comprising subsites A, B and C.

It is proposed that subsite C is the strongest and most specific of the three sites, and contains the binding site for N-acetyl group of residue in this site, due to NH and CO groups of residues 59 and 107 which form H bonds with the N-acetyl group, as for hen egg white lysozyme.

Subsite B is equivalent, but not identical to the corresponding hen-egg white lysozyme site. The non-polar contacts of Trp 62 are possibly the same, but Tyr replaces this amino acid.

Subsite A is unlikely to be the same as for hen-egg white. Contacts with the sugar residue are probably tenuous; of the four amino acids that make contact with the sugar, three are substituted:

Leu 75 by Ala;

Trp 62 by Tyr;

Asn 103 by Gln.

Other Lysozymes

There are over twenty known lysozymes, but some of the most recently studied in detail are the lysozymes from other avian egg whites.

Goose egg white lysozyme has a poor chitinase activity which drops off completely at pH's 4.7 and 6.7, [16] and it does not give rise to

transglycosylation products. This enzyme is only slightly inhibited by NAG_4 [94], but in contrast, it is bound strongly to oligosaccharides from bacterial cell walls, although its muramidase activity is less than that of hen-egg white lysozyme. [24] The sequence of the first thirty amino acid residues has been determined [17] Fig. 1, and this NH_2 terminal sequence suggests that the primary structure is distinctly different from hen-egg white, human, duck-egg white and turkey-egg white lysozymes. These results suggest that goose egg-white lysozyme represents a different class of lysozymes.

Duck egg white provides an interesting case, in that chromatography on Amberlite gives two and sometimes three distinct enzymes. The 88th communication on lysozymes by Jollès [25] provides leading references and reports a preliminary X-ray investigation on these Histidine-free enzymes. The duck II and III lysozymes are believed to have chitinase activity greater than that of hen-egg white lysozyme, [16] and all three are active against Micrococcus Luteus. Their behaviour in the presence of inhibitors is slightly different. [18] There are six amino acid differences between the primary structures of II and III, and they differ from hen lysozyme by 19 and 20 and from human lysozyme by 48 and 47 residues respectively.

Fig and papaya lysozymes have been reported to have a greater chitinase activity than hen-egg white and human lysozymes [23], but the reverse is true regarding muramidase activity.

The amino acid sequence of guinea hen-egg white lysozyme has been reported [26]. There are 129 amino acids, and Glu 35 and Asp 52 are present as in quail egg lysozyme.

Synthetic Lysozymes

Synthetic lysozymes with substitutions in the region 51 - 53 amino acid residues showed no activity. [27]

At present, Professor Kenner (Liverpool) is carrying out work on synthetic analogues of lysozyme; variants of an artificial sequence which are more accessible to synthesis than the natural sequence. The comparison of enzymic activities of these analogues should increase the understanding of enzyme action and possibly open the way to simplified enzyme structures. The biosynthesis of lysozyme has been studied and found that the path from N towards C terminal is followed.[28]

Bovine α - Lactalbumin

The possible correlation of the primary structures of hen-egg white lysozyme and bovine α-lactalbumin was first suggested by Brew [11], and the three-dimensional structure is thought to be closely similar to that of hen-egg white lysozyme [29], but direct confirmation of this hypothesis is awaited.

Asp 52 is present, but Glu 35 is not [20], and therefore it might be informative to test this protein for lytic activity.

The physicochemical properties of human milk α-lactalbumin and human milk lysozyme have been compared [31], with striking similarities. The thermal denaturation occurs at a lower temperature for the α-lactalbumin, possibly implying a weaker secondary structure.

These findings raise the possibility that there are close relationships between supposedly distinct enzymes.

The Preparation and Isolation of Lysozymes

The classical procedures using ion exchangers and salt precipitation are well documented. [9] More recently, however, affinity chromatography on dispersed chitin, carboxymethyl chitin or glycol chitin, has played an important part in the separation of rare lysozymes. [65]

The ultra-violet spectra of lysozyme

Physical studies on lysozyme [37] have resulted in the measurement of the extinction coefficient of lysozyme, $E^{1\%}$ as 26.35 $\pm$ 0.18, at 280 nm, which represents a molar extinction coefficient of 37,912, for a molecular weight of 14,388.

1. 2 Synthetic substrates for lysozyme

It appears that only hen-egg white lysozyme has been tested for activity on synthetic substrates. The use of Micrococcus Luteus for assaying lysozyme has disadvantages [45], in that the substrate is not a single chemical entity and therefore the cleavage of a single chemical bond cannot be studied; so any extrapolations of detailed intra-complex actions are difficult to make. However, cell suspensions have been used extensively [85], although the measured rates are dependent on the batch of substrate used. This makes it impossible to quote an absolute activity for any lysozyme solution. Furthermore, since an insoluble substrate was being used, the velocity is dependent on the interfacial area, or the degree of emulsification of substrate as well as the amount present. It is therefore possible for the maximum rate to be determined by the number of enzyme molecules that can be adsorbed on to the surface of the cells. Since a molecule of lysozyme covers about 10^3 Å^2 of surface area [83]; in 1 cm^2 or 10^{16} Å^2 of cell surface area, about 10^{13} molecules or 10^{-10} moles of lysozyme may be adsorbed. Therefore 10^4 cm^2 of surface area of cells must be exposed to allow 10^{-6} moles of lysozyme to be fully adsorbed in order to react. It is possible, therefore, that the "V max" obtained from these assays is limited by the quality of the batch used.

Glycol chitin has been used as a lysozyme substrate, but is also subject to some of these disadvantages [131]. Oligosaccharides of chitin, and from bacterial cell walls have been used extensively as lysozyme

substrates, [32] but suffer from the disadvantages that they are difficult to prepare, and their hydrolyses cannot be continuously and accurately followed. [33]

The first attempt to overcome these problems was by Osawa in 1966 [100], who synthesised paranitrophenyl NAG_2, and found it to be a substrate for lysozyme, albeit a very poor one. This compound allowed the cleavage of the aryl glycosidic bond to be followed spectrophotometrically, although the hydrolysis was very slow. The specificity of this substrate was some 3×10^6 fold lower than that of NAG_6 [82]. Michaelis-Menten kinetics were demonstrated for this substrate, and for other aryl-β- chitobiosides.[124] A ρ value of + 1.2 was calculated, although estimates for this vary [91] to $\rho = 0.55$. However, the major criticism [103] of these substrates is that their hydrolysis is not simple, and that contrary to the first reported findings[100], that no cleavage between the NAG residues occurs, in fact it has been found that under Michaelis Menten conditions, hydrolysis of this bond is twenty times faster than of the aryl glycosidic bond [115]. Induction periods are observable under certain conditions [66], and therefore the complex hydrolysis of this substrate may proceed through a build-up of higher oligomers as with the hydrolysis of NAG_2 [82] and NAG_3 [101], and it is these that are hydrolysed. This problem may be somewhat alleviated by using a better leaving group, assuming that the ρ value of the true catalytic step is positive*, but 2, 4 dinitrophenyl- NAG_2 still has a specificity 3×10^4 times lower than NAG_6 [66]. Unless this condition is

* See Discussion.

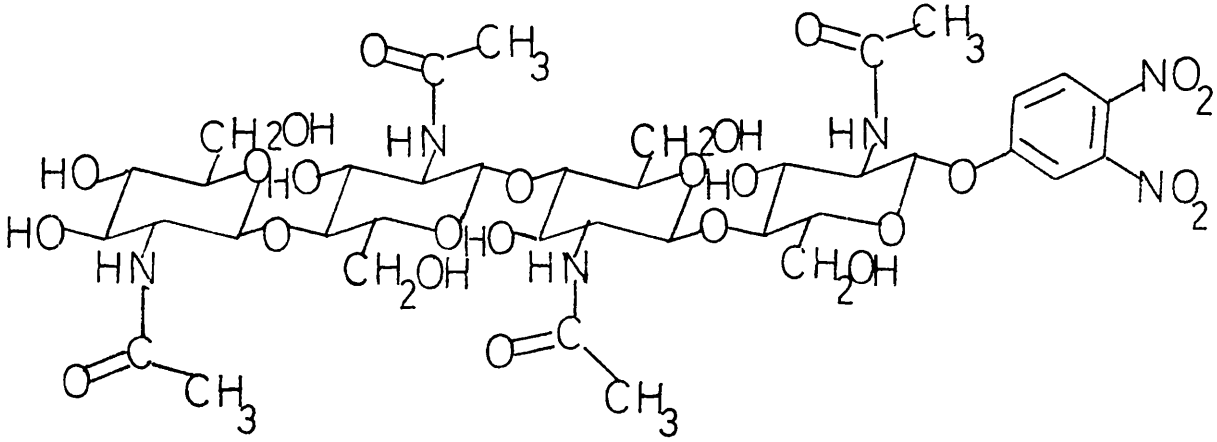


Fig. 2. NAG_4-β-3, 4-dinitrophenyl.

fulfilled, the extensive studies carried out on other aryl -β- chitobiosides are of doubtful validity. 84, 91, 67, 124.

The induced synthesis of aryl glycosides of higher oligomers of NAG has been studied 30, but for similar reasons, and since acceptor specificity is so variable 92, the results can only be considered in a qualitative manner.

The first step taken towards synthesising a pure spectrophotometric substrate of higher specificity was by Osawa 130, who synthesised paranitrophenyl NAG_3, but this was not found to be an improvement over 2, 4 dinitrophenyl NAG_2 66.

This work has been repeated 102, and the corresponding (1 → 4) (1 → 6) and (1 → 3), (1 → 6) linked paranitrophenyl -β- NAG_3 glycosides synthesised, but they are hydrolysed only with marked induction periods.

Aryl -β- glycosides containing glucose and 2-deoxy-glucose as the glycone residue, and NAG, have been synthesised 115, 125, but although the former solved the problem of multiple bond cleavage, ostensibly because it bound in sites C D and E, and the latter was a 7 times better substrate than paranitrophenyl NAG_2, they were still very poor. The first object of this work, therefore, was to synthesise a good chromophoric substrate for hen-egg white lysozyme, and it was thought that from the proposed structure of the lysozyme cleft 83, that aryl -β- glycosides of NAG_4, Fig. 2, might prove to be most rewarding. The need for such a substrate was pointed out in 1967. 147.

Such a substrate would prove useful not only in mechanistic studies, but for assaying solutions for lysozyme content. The measurement of lysozyme activity with a pure chemical compound could result in the determination of absolute activities. It has been pointed out [85] that presently available synthetic substrates require very long assay periods and massive amounts of lysozyme.

1. 3 Kinetics of lysozyme catalysed hydrolyses

A comprehensive kinetic analysis of the reaction of lysozyme with oligosaccharides from bacterial cell walls has been made,[103] but this is not suitable for the analysis of the cleavage of spectrophotometric substrates derived from chitin oligosaccharides, since in these, cleavage of only one bond is productive for phenol release, and cleavage occurs throughout the chain.

A model was set up which would take into account the species present during a lysozyme-catalysed hydrolysis. The effects of transglycosylation are not considered, since they should not affect the initial rates of hydrolysis of substrates which are hydrolysed by a simple mechanism.

Michaelis Menten Kinetics

In a simple two step enzyme catalysed reaction, the rate of reaction is expressed in the equation derived by Michaelis and Menten:-

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

$$\text{Rate, } v = \frac{V_{max}}{1 + K_{m/S}} \quad (1)$$

$$\text{Where } V_{max} = k_2.(E)_o \quad (2)$$

$$\text{and } K_m = \frac{(E).(S)}{(ES)} = \frac{k_2 + k_{-1}}{k_1} = K_s + \frac{k_2}{k_1} \quad \ldots 2\,(a)$$

$$\text{where } K_s = \frac{k_2}{k_1}$$

This may be extended to cover the situation where three steps are present, as suspected in lysozyme catalysed hydrolyses.[54]

In the scheme:

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

the measured Michaelis constants may be expressed in terms of the individual rate constants, as follows:

$$k\ cat = \frac{k_2 k_3}{k_2+k_3} \qquad (3)$$

$$Km\ (app.) = \frac{Km.k_3}{k_2+k_3}$$

$$Km\ (app.) = Ks.\ \frac{k_3}{k_2+k_3} \quad \frac{+k_2k_3}{k_1(k_2+k_3)} \qquad (4)$$

If, as suspected, that $k_3 \gg k_2$ in lysozyme catalysed reactions, [103] then

$$Km\ (app.) = Ks + \frac{k_2}{k_1}$$

which is the same as for the two step mechanism.

Effect of added nucleophiles on the measured rates

Since it is known that lysozyme catalysed hydrolyses proceed with retention of configuration,[30] implying a double displacement mechanism of action, the effect of an added nucleophile to the reaction media can provide information as to the nature of ES'. If a covalent intermediate exists, Fig 4, then the addition of a nucleophile may affect the observed rates as follows:

In the scheme:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} ES' + P_1 \xrightarrow{k_3\,(H_2O)} S\text{-}OH + E$$

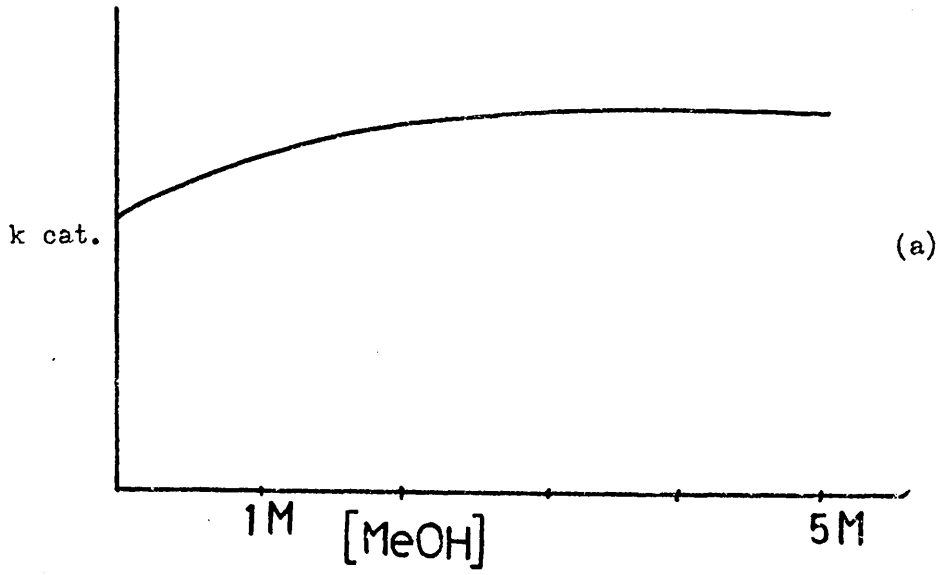
$$\xrightarrow{k_4(N)} S\text{-}N + E$$

which has been studied by Bender for α- chymotrypsin[64] and Viratelle for β-galactosidase,[144, 6]. it can be shown that the presence of the added nucleophile, for example, methanol, increases the rate of the deglycosylation step from $k_3.(H_2O)$ to $(k_3.(H_2O) + k_4(N))$.

This results in an increase in the observed k cat only if the deglycosylation step is rate-determining or if it becomes faster than the glycosylation step.

The observed value of k cat increases with methanol concentration until the last step is no longer rate determining; that is glycosylation is now the slowest step - Fig. 3(a).

On the other hand, if k_2 is the rate determining reaction, that is glycosylation is slowest, then the addition of methanol should not alter k cat. In practice, a small decrease in k cat is often observed, as in Fig 3(b), possibly due to a "medium effect".[149]



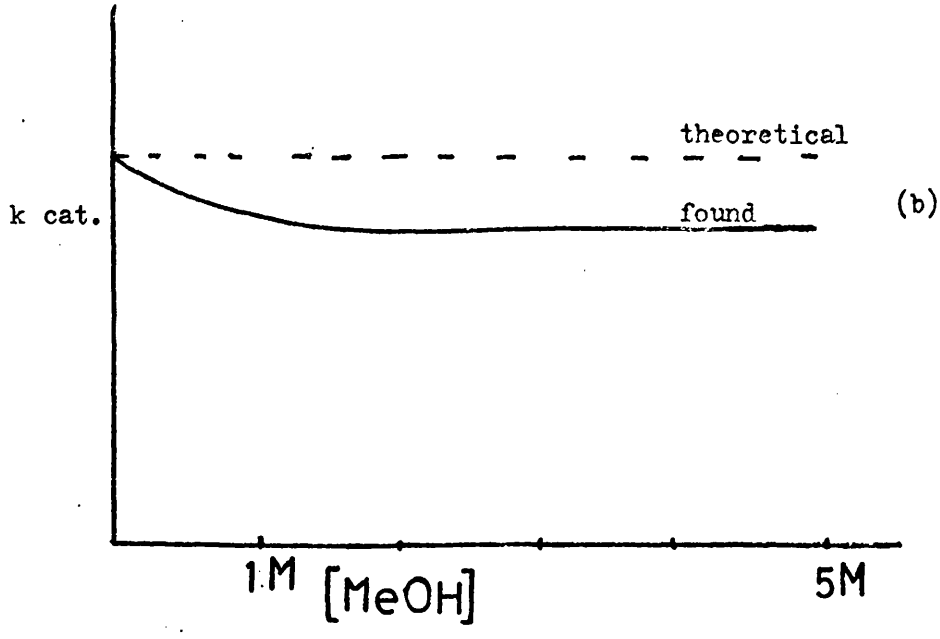


Fig.3- Variation of k cat. with added nucleophiles.

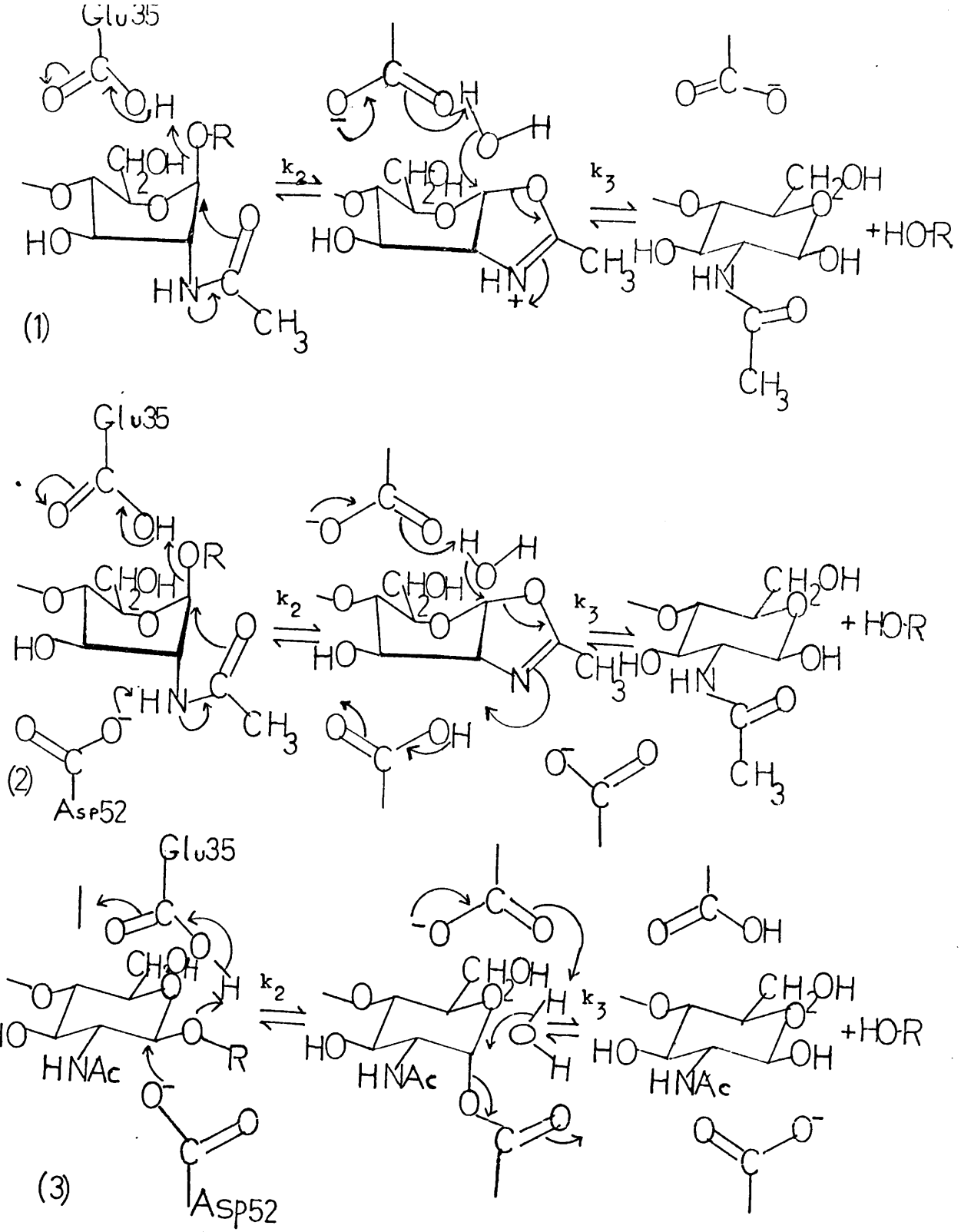


Fig.4 Possible mechanisms of action of lysozyme.

It is therefore possible to obtain a qualitative idea of the ratio of k_2 to k_3, and if situation a) is found, then the presence of a covalent intermediate is unequivocally confirmed.

The relative rates of glycosylation and deglycosylation may alter with the acidity of the aglycone being used, since the former may become faster with better leaving groups, and the effect observed can therefore change. [6]

The relative affinity of a glycosyl-enzyme for water and methanol does not necessarily follow their nucleophilicities to saturated carbon, SN_1 and SN_2, since a 1:121 ratio has been observed for galactosyl -β- galactosidase intermediate. A cationic form of intermediate has been invoked to explain this observation, which is of the same order of magnitude as their relative reactivities to carbonyl carbons. [6]

Effect of non-productive binding and pH on the measured Michaelis Constants

Dixon [56] has treated the pH dependence of an enzyme reaction. Fastrey [57] has given equations for the independent effect of pH and non-productive binding on the measured Michaelis parameters. Lucas [63] has studied the pH dependences of individual rate constants in papain catalysed reactions. Chipman's analysis for lysozyme was for cell wall oligosaccharides and did not include pH dependent terms [103].

A rigorous treatment of the kinetics of lysozyme catalysed reactions may be defined in Fig. 5, involving seventeen inter-dependent equilibria.

For reasons stated in the discussion, the species EHS'p, the intermediate will not be included in the mathematical analysis.

The subscripts p and np attached to the species refer to modes of binding 9 and 1 to 8 respectively, as defined in Fig. 6. Only mode 9 is productive for release of phenol. If the aryl glycoside is being used as an inhibitor, then all modes of binding except 4, which will not be significant, are inhibitory to the hydrolysis of another aryl glycoside of NAG_4.

This scheme differs from that for the hydrolysis of reducing sugars; for example for NAG_5, complexes such as 6, 7, 8 and 9 may be regarded as productive. From experimental results,[82] it would appear that the latter two exist to a significant extent.

The equilibrium constants are dissociation constants and may be defined as follows:

$$Khes1 = \frac{\bar{E}HSp.H^+}{EH_2Sp} \qquad Khes2 = \frac{\bar{\bar{E}}Sp.\ H^+}{\bar{E}HSp}$$

$$Kes1 = \frac{EH_2.Sf}{EH_2Sp} \qquad Kes2 = \frac{\bar{\bar{E}}.Sf}{\bar{E}Sp} \qquad Kes\ 3 = \frac{EH_2.\ Sf}{EH_2\ Snp}$$

$$Kes4 = \frac{EH_2Sp}{EH_2Snp} \qquad Kes5 = \frac{\bar{\bar{E}}.Sf}{\bar{E}Snp} \qquad Kes6 = \frac{\bar{\bar{E}}Sp}{\bar{E}Snp}$$

$$Khe1 = \frac{\bar{E}H.H^+}{EH_2} \qquad Khe2 = \frac{\bar{\bar{E}}.H^+}{\bar{E}H} \qquad Kces1 = \frac{\bar{E}HSp}{\bar{E}HSnpi}$$

$$Ks1 = \frac{\bar{E}H.Sf}{\bar{E}HSp} \qquad Ks2 = \frac{\bar{E}H.Sf}{\bar{E}HSnp} \qquad Khces1 = \frac{\bar{E}HSnpi.H^+}{EH_2\ Snp}$$

$$Khces2 = \frac{\bar{\bar{E}}Snpi.H^+}{\bar{E}HSnpi}$$

The equation of conservation of enzyme is:

$$Eo = \bar{E}H + \bar{\bar{E}} + EH_2 + \bar{E}HSp + \bar{E}HSnp + \bar{\bar{E}}Snp + \bar{\bar{E}}Sp + EH_2Sp + EH_2Snpi$$

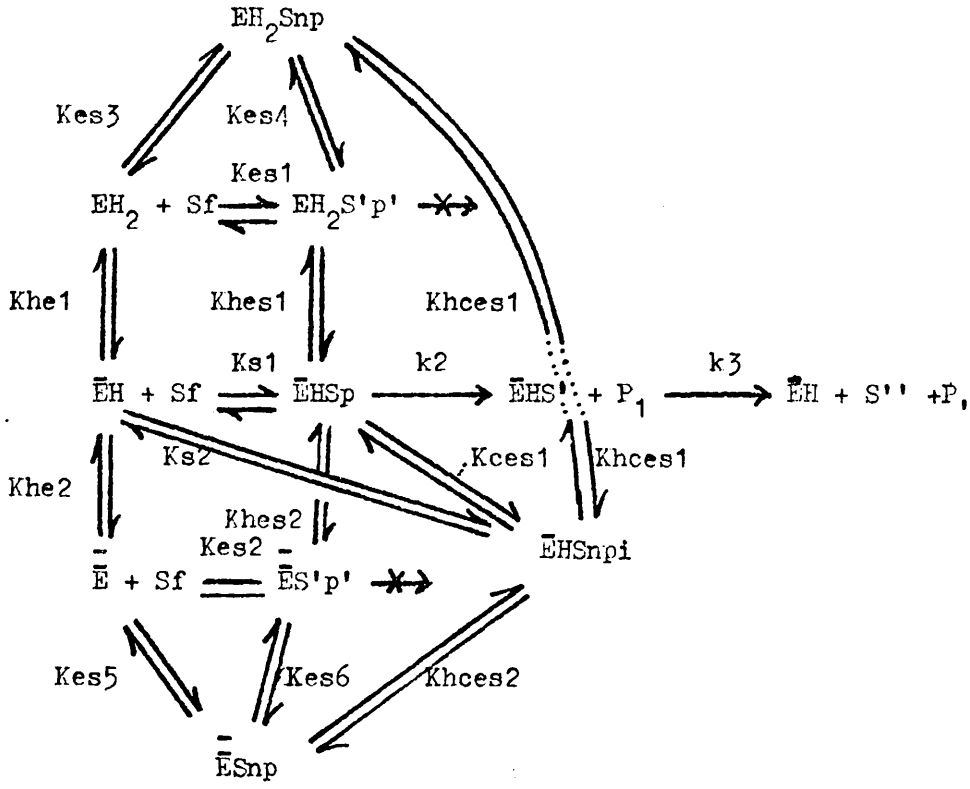


Fig.5 - Model for the kinetic analysis of lysozyme catalysed reactions.

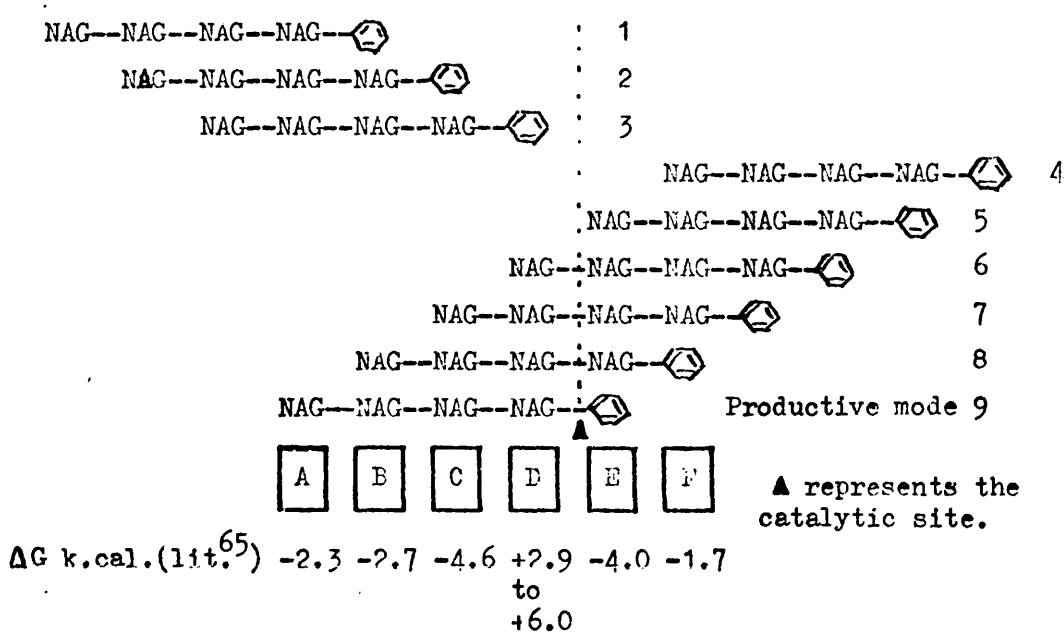


Fig.6 -Productive and Non-Productive modes of binding for an aryl glycoside of NAG 4.

The velocity of reaction is given by:

$$v = k_2 \cdot \bar{E}HSp$$

But

$$\bar{E}HSp = \frac{Eo}{\left(1 + H^+ \frac{(1 + Kes\ 4)}{(Khes1.Kes4)} + \frac{1}{H^+}\left(\frac{(Khes2 + Khes2.Kes6)}{Kes6}\right) + \frac{1}{Kces1} + \frac{Ks1}{Sf}\left(1 + \frac{Khe2}{H^+} + \frac{H^+}{Khe1}\right)\right)}$$

$$v = \frac{k_2.Eo}{\left(1 + H^+ \frac{(Kes4 + 1)}{(Khes1.Kes4)} + \frac{1}{H^+}\left(\frac{(Khes2 + Khes2.Kes6)}{Kes6}\right) + \frac{1}{Kces1} + \frac{Ks1}{Sf}\left(1 + \frac{Khe2}{H^+} + \frac{H^+}{Khe1}\right)\right)}$$

4 (a)

The function $\frac{Ks1}{Sf}\left(1 + \frac{Khe2}{H^+} + \frac{H^+}{Khe1}\right)$ is pH-dependent, and small compared with the other terms in the denominator (see discussion).

Therefore, for So $\gg$ Km (app) $\gg$ Eo
that is under normal Michaelis Menten conditions

$$v = V \text{ max (app.)} = \frac{k_2 E_o}{(1 + H^+.\bar{K}_2 + 1/\bar{K}_3 + \bar{K}4/H^+}$$

where $\bar{K}_2 = \frac{Kes4 + 1}{Khes1\ Kes4}$ an association constant

$\bar{K}_3 = Kces1$ a dissociation constant

$\bar{K}_4 = \frac{Khes2 + Khes2\ Kes6}{Kes6}$

$$V \text{ max (app)} = \frac{V \text{ max}}{(1 + H^+\bar{K}_2 + 1/\bar{K}_3 + \bar{K}4/H^+)} \quad \ldots\ldots \quad 4\ (b)$$

further, if 4 (a) is divided throughout by $(1 + H^+\bar{K}_2 + 1/\bar{K}_3 + \bar{K}_4)$

$$v = \frac{V \text{ max (app.)}}{\left(1 + \frac{Ksl}{So}\left(1 + \frac{Khe2}{H^+} + \frac{H^+}{Khel}\right)\right)} \quad \ldots\ldots \quad 4\ (c)$$
$$(1 + H^+\bar{K}_2 + 1/\bar{K}_3 + \bar{K}_4)$$

Since k cat. Eo = V max (app) and k_2.Eo = V max

$$k \text{ cat} = \frac{k_2}{(1 + H^+\bar{K}_2 + 1/\bar{K}_3 + \bar{K}_4/H^+)} \quad \ldots\ldots \quad (5)$$

For the situation when k_3 is not very much larger than k_2, this will be multiplied by the factor of equation (3).

When this function is fitted to the experimental plot of k cat vs. pH, it is possible to determine the values of the constants $\bar{K}_1$, $\bar{K}_2$, $\bar{K}_3$ and $\bar{K}_4$. An Algol program designed to carry this out is given in the appendix. For the purpose of the program, k_2 is the first constant given, $\bar{K}_1$.

To obtain the experimental data to use in the function, it is necessary to find the effect of pH on V max (app.). It is most important that a sufficiently high substrate concentration is used to saturate the enzyme under all conditions, thus eliminating any effects

of the affinity of the enzyme to substrate. Under these conditions, all the enzyme is complexed, and the velocity of reaction is the rate of breakdown of the only productive complex present, $\bar{E}HSp$. The necessity of being able to determine k_2 for the breakdown of the enzyme-substrate complex in order to get a true value of ρ for a series of aryl glycosides for glycosidases has been stated by Capon.[129]

The equation derived is similar in form to that of Waley,[58] and that of Dixon, but allows for complexing of all states of protonation of the enzyme.

The measured Michaelis equilibrium constant may be related similarly, to these constants.

$$\text{Since Km (app)} = \frac{\Sigma E.\Sigma S}{\Sigma ES}$$

$$= \frac{(\bar{\bar{E}} + \bar{\bar{E}}H + EH_2)\,(Sf)}{(\bar{E}HSp + \bar{E}HSnpi + EH_2Sp + \bar{\bar{E}}Sp + EH_2Snp + \bar{\bar{E}}Snp)}$$

$$= \frac{\bar{E}HSp\left(\frac{Ksl}{Sf} + \frac{Khe2.Ksl}{H^+.\,Sf} + \frac{H^+.Ksl}{Khel.Sf}\right).\,Sf}{\bar{E}HSp\left(1 + \frac{1}{Kcesl} + \frac{H^+}{Khesl} + \frac{Khes2}{H^+} + \frac{H^+}{Kes4.Khesl} + \frac{Khes2}{Kes6.H^+}\right)}$$

$$\text{Km (app)} = \frac{Ksl.\left(1 + \frac{Khe2}{H^+} + \frac{H^+}{Khel}\right)}{\left(1 + H^+.\bar{K}_2 + \frac{1}{\bar{K}_3} + \frac{\bar{K}4}{H^+}\right)} \qquad (6)$$

where $Ksl = \frac{\bar{E}H.Sf}{\bar{E}HSp} = \frac{k - 1 + k_2}{k_1} = Ks + \frac{k_2}{k_1}$ as in 2 (a)

Effect of pH on the ratio of productive to non-productive binding

If every species of complexed enzyme except $\bar{E}HSp$ is regarded as non productive as far as reaction is concerned, then the ratio of productive to non productive binding may be defined as follows:

$$\frac{P}{NP} = \frac{\bar{E}HSP}{(\bar{E}HSnpi + \bar{E}H_2Sp + \bar{E}Sp + \bar{E}H_2Snp + \bar{E}Snp)}$$

$$= \frac{1}{\left(H^+ \left(\frac{Kes4 + 1}{Khes1 . Kes4}\right) + \frac{1}{H^+}\left(\frac{Khes2 + Khes2\ Kes6}{Kes6}\right) + \frac{1}{Kces1}\right)}$$

$$\frac{P}{NP} = \frac{1}{\left(H^+\bar{K}_2 + \frac{1}{\bar{K}_3} + \frac{\bar{K}_4}{H^+}\right)} \qquad (7)$$

$\bar{K}_2$, $\bar{K}_3$ and $\bar{K}_4$ are the constants as defined previously. Once these constants have been determined for a particular substrate, it is then possible to calculate the ratio $\frac{P}{NP}$ for any pH.

Covalent intermediates and the effect of pH and non-productive binding on the size of the observable 'burst'.

It has been proposed [83] that the lysozyme catalysed hydrolysis of oligosaccharides proceeds through a three step mechanism involving concerted acid-base catalysis by Glu 35 and Asp 52, lying between subsites D and E in the enzyme cleft.

One of the possible mechanisms of cleavage of the glycosidic bond gives an intermediate in which there is an ion pair formed between Asp 52 and the oxonium ion, Fig. 4. This may have an infinity of forms - from the fully separated ion pair, to the collapsed covalent form, in which an oxygen of the carboxyl of Asp 52 is covalently bound to carbon 1 of the sugar moeity in site D of the enzyme.

Philips [83] thought that the ion pair would not collapse to a covalently bound intermediate, as it would require too much distortion of the enzyme. However, Capon [129] has suggested that there should be at least 30 k.cal to be gained through neutralisation of the charges, and this energy would be available to pay for this distortion.

Therefore, there is need for an experiment designed to detect this intermediate. In a rapid initial burst experiment, using a spectrophotometric substrate, it may be possible to observe the presence of this intermediate.

A typical observation would appear as in Fig 7. A rapid build up of the covalent intermediate, 9, with consequent release of the aglyone, followed by its slow hydrolysis, C, is unequivocal proof of the three step mechanism, and shows the precise pathway of cleavage of the substrates.

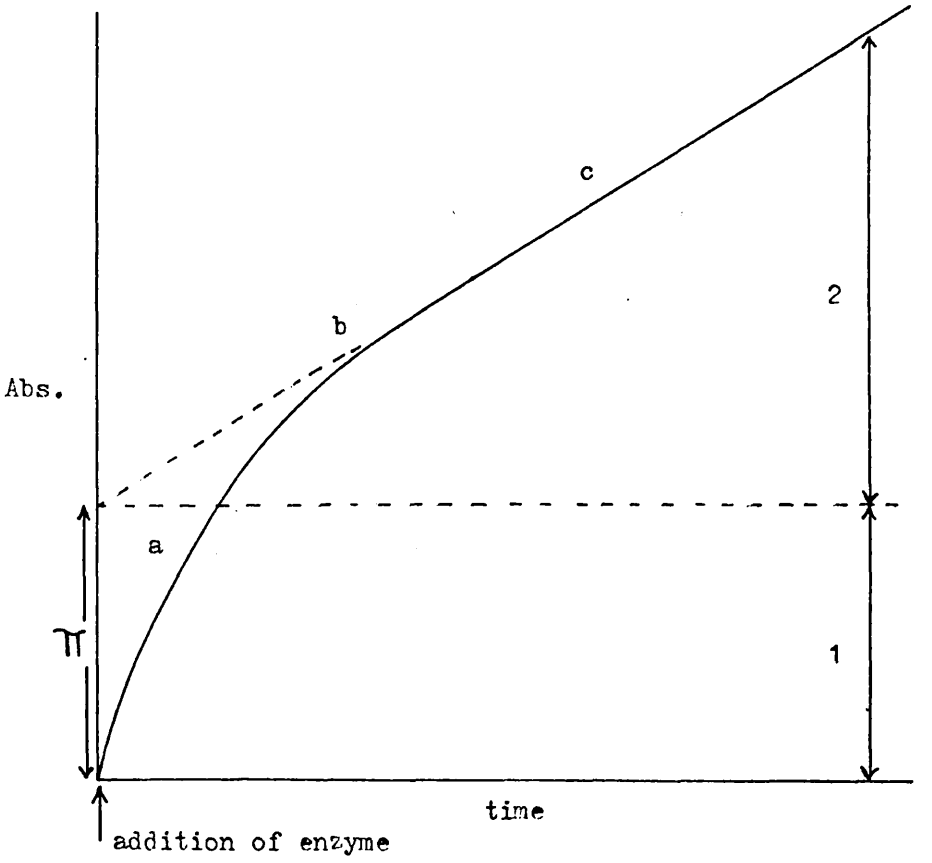


Fig.7 -Detection of a covalent intermediate in a titration experiment.

In the kinetic scheme, Fig. 5, the product P_1 comes from one pathway, that is the formation of EHS'p, the covalent intermediate. This has two phases, as in Fig. 7.

1. When extrapolated to $t = 0$, P_1 comes solely from the formation of $\bar{E}HS'p$; the concentration of this throughout the reaction remains constant after the steady state has been reached and provided So remains constant.

2. A time-dependent production, due to breakdown of $\bar{E}HS'p$ followed by recycling of $\bar{E}H$ and further reaction.

The size of the burst, π , is equal to the concentration of P_1 at $t = 0$, since it is this that is being physically measured, and since (P_1) at $t = 0$ is equal to $(\bar{E}HSp)$, therefore

$\pi = (\bar{E}HSp)$ at steady state.

The rate of build up of $\bar{E}HS'p = k_2.(\bar{E}HSp)$

The rate of breakdown of $\bar{E}HS'p = k_3.(\bar{E}HS'p)$

In the steady state, once point b has been reached, then

$$k_2.(\bar{E}HSp) = k_3.(\bar{E}HS'p)$$

$$\therefore \pi = \bar{E}HS'p = \frac{k_2}{k_3} . \bar{E}HSp$$

$$\therefore \pi = \frac{k_2}{k_3} . \frac{\bar{E}H}{Ks1} . Sf \qquad (8)$$

or

$$\pi = \frac{k_2}{k_3} . \frac{Eo}{(1 + H^+ . K_2 + 1/K3 + K4/H^+)} \qquad \text{from (5)}$$

$$\therefore \pi = Eo.\frac{k\ cat}{k_3} \qquad (9)$$

Hence, in a complex case involving non-productive binding, the observed burst is not simply equal to the concentration of enzyme, but is reduced by the factor $\frac{k\ cat}{k_3}$, that is $\frac{k_2}{k_3} \times \frac{P}{NP}$.

Therefore, for a burst to be observed $\frac{P}{NP}$ must not be small, nor $\frac{k_2}{k_3}$.

The derivation of the equation for a simple case has been done by Ouillet [137] , along with a detailed study of the transient phase, a. It may be feared that the formation of a very slowly hydrolysing covalent intermediate would deplete the concentration of $\bar{E}HSp$, and so disturb the equilibrium between productive and non productive complexes, and therefore the "pool" of complexes would be depleted until all complexes had formed $\bar{E}HS'p$, the covalent intermediate. However, in most cases, the hydrolysis of $\bar{E}HS'p$ has a finite, if not high rate, which allows the release of $\bar{E}H$, and formation of the steady state situation, and in this sense, $\bar{E}HS'p$ is part of an 'equilibrium,' although k_2 and k_3 are irreversible.

The rate of hydrolysis of the covalent intermediate relative to the rate of its formation may be slowed by lowering the pH of the media.

In the free enzyme, the carboxyls of Glu 35 and Asp 52 have pKa's of 6.5 and 4.5 respectively. [54] Perturbation of these values on complex formation has been observed [35, 47, 132]; and although estimates vary, Glu 35 is perturbed by -1.8 to +2.0 pH units.
The diagram in Fig 8 shows, in a qualitative manner, what the effect of pH changes are on the values of the rates of the steps involving

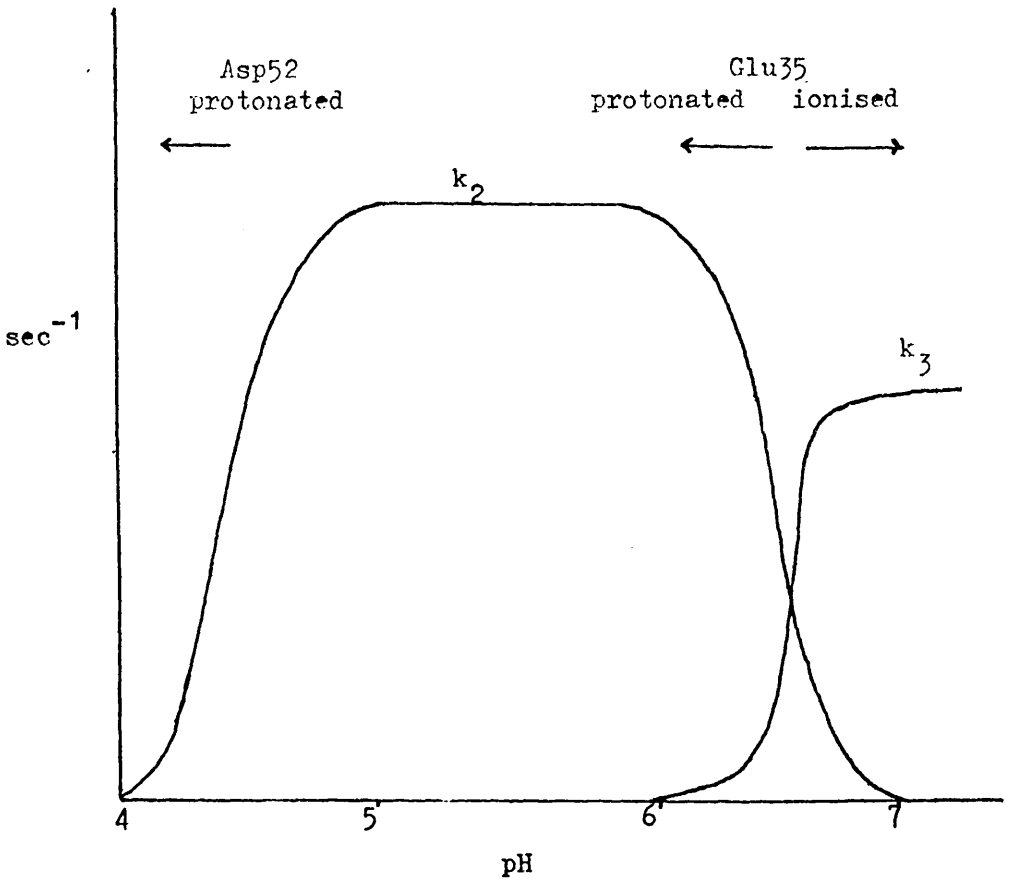


Fig.8 Qualitative effect of pH on k_2 and k_3

k_2 and k_3 is, defined as in Fig 4. The step k_2 involves both Glu 35 and Asp 52 in their various states of protonation, and thus is bell-shaped. The step k_3 involves only the ionisation of Glu 35, and appears as in Fig 8.

The deglycosylation step may be slowed by protonating Glu 35 and preventing it from acting as a general base in the hydrolysis of the covalent intermediate. In order to obtain the most favourable ratio of k_2 to k_3, lowering of pH may help, although too acid a media would also decrease k_3. It is not possible to estimate the best pH for this, since the values of the pKa's of Glu 35 and Asp 52 are perturbed on complex formation. [34]

It may be speculated that it is possible to reduce the rate of the deglycosylation step, by reducing the concentration of water, but this is hardly practicable.

The specificity of substrates for lysozyme

The specificity of any enzyme is the limitation of the action of the enzyme to one substance, or a small number of closely related substances.

The differences between the specificity of an enzyme for each of the members of a group of substrates may manifest itself either in a difference of rate of reaction of the enzyme-substrate complexes or in a difference of affinity.

Only in very few studies has the effect of substrate structure on V max and Km been separately determined, [59, 70] for a homologous series of substrates.

Where there is non productive binding between enzyme and substrate, as is almost certainly the case with most lysozyme substrates, the experimentally measured Michaelis constants k cat and Km (app.) are complex quantities, and may be related to the individual kinetic parameters as has been demonstrated in equations (5) and (6).

When the relative specificities of an enzyme for several substrates are being compared directly, it is possible to eliminate the effects of non productive binding by comparing only the ratio k cat/Km (app.) for each substrate.

Since:

$$\frac{k\ cat}{Km\ (app.)} = \frac{k_2}{Ksl\left(1 + \frac{Khe2}{H^+} + \frac{H^+}{Khel}\right)} \quad .. (9) .. \text{ from (5) and (6)}$$

If the pH is held constant, then specificity comparisons result in a direct comparison of the ratio $\frac{k_2}{Ksl}$ for each substrate.

An increase in the specificity, $\frac{k\ cat}{Km\ (app.)}$ may be realised by one of two methods:

First, by increasing k_2, the catalytic constant for the breakdown of the productive complex. It is known that ρ has a positive value,[124] and therefore more acidic phenols should increase the rate of this step. This has the added bonus that cleavage between sugar residues in an aryl glycoside of higher oligomers of NAG will become less significant and may nullify effects of hydrolysis via a complex mechanism, as suspected for paranitrophenyl glycosides.[115]

Second, by decreasing Km (app.): An increase in specificity could be achieved by designing a substrate which binds more strongly to the enzyme in the productive mode. By increasing the chain length to four sugar residues in order to maximise the favourable binding interactions of this mode, it should become more predominant. The favourable interactions of sites E and F are not used, however, and it cannot be expected, therefore, that this mode is as strong as that for a hexasaccharide. Further, the hexasaccharide has a greater number of strong binding modes, and therefore the expected Km (app.) should be lower.

The quantity defined by equation (9) is equal to the second order rate constant for the lower part of the Michaelis Menten plot, where the rate is given by:

$$\text{Rate} = \frac{k_{cat.}}{Km} \quad (E)_o \cdot (S)_o \cdot$$

$$= \frac{k_2 \cdot (E)_o \cdot (S)_o}{Ksl \left(1 + \frac{Khe2}{H^+} + \frac{H^+}{Khel}\right)}$$

When conditions are chosen so that this region is being studied, it is possible to directly compare the specificities of a series of substrates from the gradient of the plot of rate vs $(S)_o$, if $(E)_o$ is held constant. Or, if the correct conditions of $(E)_o$ and $(S)_o$ can be found, and these are held constant, a direct comparison of the specificities can be taken from the initial rates of hydrolysis.

The effect of an added inhibitor on Km (app).

Any inhibitor, NAG_n - OR, where n = 1 to 5 and R = H or Ar, may be regarded as binding in any of the modes shown in Fig. 6, and all except 4 will competitively inhibit the further binding of a molecule of a substrate which is an aryl glycoside of NAG_4. The resultant formation of a series of complexes, all of which are non productive as far as release of phenol from the substrate is concerned, will raise the Km (app.).

Osawa has studied [130] the effect of inhibitors on the hydrolysis of NAG_3-β-paranitrophenyl , and has stated that non substrates are non competitive inhibitors and substrates are competitive inhibitors. This seems doubtful in view of the proposed modes of binding and the equilibria involved;- the inhibitors are binding reversibly to the same sites as the substrates, and therefore it would be expected that competitive inhibition prevails throughout.

The equation of conservation of enzyme is:

$$Eo = \bar{E}H+\bar{\bar{E}}+EH_2+\bar{E}HSp+\bar{E}HSnp+\bar{\bar{E}}Snp+\bar{\bar{E}}Sp+EH_2Sp+EH_2Snpi+\bar{E}HIi+\bar{\bar{E}}Ii+EH_2Ii$$

for the scheme shown in Fig. 5, which may be modified to Fig. 9. The dissociation constants may be defined:

$$KEI1 = \frac{\bar{\bar{E}}H.If}{\bar{E}HIi} \qquad KEI2 = \frac{EH_2.\ If}{EH_2Ii}$$

$$KEI3 = \frac{\bar{\bar{E}}.If}{\bar{\bar{E}}Ii} \qquad KEI4 = \frac{\bar{E}HI'p'}{\bar{E}HInpi}$$

where 'p' indicates binding of the inhibitor in mode 9, Fig. 5. npi indicates all other modes.

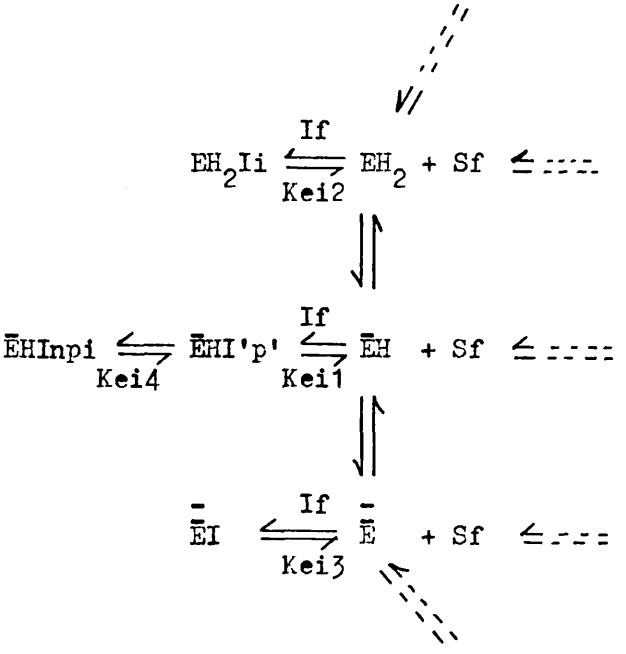


Fig.9 - Model for the binding of an inhibitor to lysozyme.

$$\bar{E}HSp = \frac{Eo}{\left(1 + H^+.\bar{K}_2 + \frac{1}{\bar{K}_3} + \frac{H^+}{\bar{K}_4} + \frac{Ksl}{Sf}\left(1 + \frac{Khe2}{H^+} + \frac{H^+}{Khel}\right) + \frac{If}{Sf} \cdot KSl\left(\frac{H^+}{Khel.Kei2} + \frac{1}{Keil} + \frac{Khe2}{H^+.Kei3} + \frac{1}{Keil.Kei4}\right)\right)}$$

- with no assumptions.

But rate = $v = k_2.\bar{E}HSp$ and since $If \approx Io$; $Sf \approx So$.

$$v = \frac{Vmax}{\left(\left(1 + H^+K_2 + \frac{1}{K_3} + \frac{H^+}{K_4}\right) + \frac{KSl}{So}\left(\left(1 + \frac{Khe2}{H^+} + \frac{H^+}{Khel}\right) + I.\left(\frac{H^+}{Khel.Kei2} + \frac{Kei4+1}{Keil.Kei4} + \frac{Khe2}{H^+.Kei3}\right)\right)\right)} \quad \ldots 11$$

where $Vmax = k_2.Eo$.

Equation (11) has the same form as for the simple case for competitive inhibition.

$$v = \frac{Vmax}{1 + \frac{Km}{S}\left(1 + \frac{I}{Ki}\right)}$$

that is, on addition of an inhibitor Km becomes $Km' = Km\left(1 + \frac{I}{Ki}\right)$

All the quantities in equation (11) may be calculated and used from data obtained from equations (5) and (6), except the inhibitor dissociation constants Kei2, Keil, Kei3 and Kei4, and therefore determination of these parameters allows the binding of the three protonated forms of the

enzyme to the inhibitor to be compared. Further, determination of Keil, the binding constant of an inhibitor with the reducing end sugar, or residue carrying the aglycone moeity, in site D, will allow the calculation of the binding energy of that mode, and ultimately of each subsite, by comparing different inhibitors.

This could be carried out by substituting values of v and So from the second order part of a Michaelis Menten plot.

If (11) is divided throughout by $(1 + H^+.\bar{K}_2 + 1/\bar{K}_3 + K_4/H^+)$, then the form is,

$$v = \frac{Vmax\ (app.)}{\left(\dfrac{\left(1 + KS\left(1 + \dfrac{Khe2}{H^+} + \dfrac{H^+}{Khe1}\right) + I.\left(\dfrac{H^+}{Khe1.Kei2} + \dfrac{Kei4 + 1}{Kei1.Kei4} + \dfrac{Khe2}{H^+.\ Kei3}\right)\right)}{So\ .\ \left(1 + H^+.\bar{K}_2 + \dfrac{1}{\bar{K}_3} + K_4/H^+\right)}\right)}$$

The form is therefore identical to the simple case.

$$\therefore \text{Km (app) becomes Km' (app.)} = \text{Km (app)} \left(1 + \frac{I}{Ki}\right)$$

$$\therefore K'm\ (app) = KSl. \frac{\left(\left(1 + \frac{Khe2}{H^+} + \frac{H^+}{Khel}\right) + I\left(\frac{H^+}{Khel.Kei2} + \frac{Kei4 + 1}{Keil.Kei4} + \frac{Khe2}{H^+.Kei3}\right)\right)}{(1 + H^+.\bar{K}_2 + 1/\bar{K}_3 + K_4/H^+)}$$

$$\therefore Km'\ (app.) = Km\ (app.) \frac{\left(\left(1 + I\left(\frac{H^+}{(Khel.Kei2} + \frac{Kei4 + 1}{Keil.Kei4} + \frac{Khe2}{H^+.Kei3}\right)\right)\right)}{(1 + K\bar{K}ei/H^+ + H^+/Khe1)}$$

Therefore, the Ki (app.) that is measured and calculated from the simple form of the equation is a complex pH-dependent entity, given by the expression:

$$Ki\ (app.) = \frac{\left(1 + \frac{Khe2}{H^+} + \frac{H^+}{Khel}\right)}{\left(\frac{H^+}{(Khel.Kei2} + \frac{Kei4 + 1}{Keil.Kei4} + \frac{Khe2}{H^+.Kei3}\right)} \quad \ldots\ldots\ldots \quad (13)$$

Chipman [32] has published a comprehensive review of work on the binding of oligosaccharides from chitin and from cell walls. The Ki (app.) measured by the early methods is a complex quantity, and more recently, attempts have been made to single out the dissociation constant for one binding mode, which is more meaningful in terms of cleft interactions.

BS dye has been found to be a competitive inhibitor for the binding of chitin oligosaccharides, and the "productive" and "non productive", modes of binding have been separated, [61] and as expected, these have been found to be very different for NAG_3 and NAG_6, but Ki (app.) is almost identical. [65] A study of hydrogen ion release has also been used, [62] as well as temperature jump studies. ^{19}F NMR studies of the binding of N trifluoracetyl glucosamine oligomers to lysozyme has been carried out and an increase in the difference between the pKa's of the catalytic groups has been observed on complex formation. [47]

Extensive NMR studies on the binding of methyl NAG has been carried out. Sykes has stated that the two anomers have the same Ks, [106] but this has been disputed. [111, 139] Dimerisation of lysozyme has been implied to explain the pH dependence of the binding of αmethyl NAG. [105] Although contradictory results exist, using Micrococcus Luteus, [120] ionic strength of the media is believed to have no effect on saccharide binding, [108] although the ionisation of the catalytic groups is affected. [132] Sykes has stated that there is

a specific interaction between acetate and lysozyme, and that the measured Ks for NAG (NMR) is a function of acetate concentration.[104] Indole and imidazole have been found to be competitive inhibitors[53] for lysozyme, using Micrococcus Luteus as substrate, but because of the unreliability of this, the central point still to be answered for inhibitor studies, is:are they competitive or not? Further, more accurate data is required. The best data so far is listed in reference 65.

The preparation of the 2'3'-epoxypropyl glycosides of NAG, NAG_2 and NAG_3 have been reported, and have been shown to be specific and irreversible inhibitors for lysozyme.[141] The inactive product obtained from lysozyme and that derived from labelled NAG_2 was shown by degradation to have the NAG_2 bound to Asp52.[142]

1. 4 The possible mechanisms of action of lysozyme

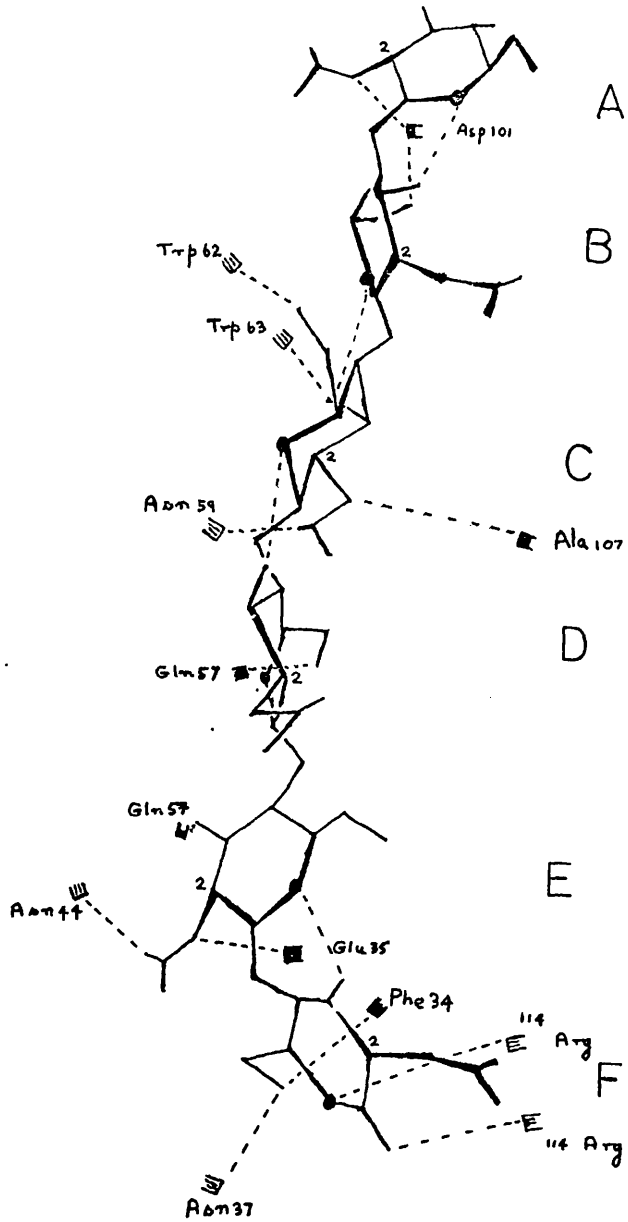
The most likely mechanisms of action of lysozyme have been set out,[83] and these are shown in Fig. 4. None of these have yet been convincingly proven or disproven.

The method of detection of and the implications of a covalent glycosyl-enzyme intermediate have been discussed, but the consequences of the almost equivalent mechanism, where the oxygen of Asp 52 remains ionised and separated from the oxonium ion formed by the sugar, are important to note.

It has been suggested[83] that in the reactive lysozyme-substrate complex, the pyranose ring bound in subsite D is strained from its normal chair conformation towards a half-chair in which carbon atoms 1, 2, 5 and the ring oxygen atom lie in the same plane, and that such distortion is an aid in catalysis, because the unfavourable interactions between the atoms C_6 and O (6) and the polypeptide chain carbonyl of residue Asp 52, with Trp 108 and with the acetamido group of NAG residue C, Fig. 10, are relieved on going to the transition state, which should be a half chair conformation, if a carbonium ion is present.[146]

There are two methods of studying the interactions in subsite D, implicit from this mechanism of action.

The first is to remove the C_6 carbon carrying the hydroxyl suspected of these interactions, and therefore the residue in site D would be expected to bind more strongly, and further, any aglycone attached to this residue would not be as susceptible to catalytic



-indicates N-H of amino acid residue

" -C=O

" ring oxygen

" hydrogen bonds

Fig.10 Atomic arrangement of NAG_6 in the lysozyme cleft (Blake et. al. 83)

cleavage by the enzyme. This has been studied by Chipman,[51] who synthesised oligomers containing N-acetyl-D-xylosamine of the type NAG_n-NAX. It requires at least 10k.cal to distort the ring to a half chair, and if the distortion of the bound substrate has proceeded very far there must be a strong binding interaction to pay for it, and it was thought that NAX could still bind in C site.[129]

The second method is to study the binding of oligosaccharides whose reducing -end residue is already in the half-chair conformation.[44] Since the carbon 1 is sp^2 hybridised in lactones, the end residue can bind in site D without the sacrifice of the free energy necessary to distort this unit into the conformation necessary for favourable binding.

Secemski[35] has synthesised NAG_3-NAGlactone, and has found that it binds more strongly to lysozyme than NAG_4.

However, it has been shown that 1, 5-D- gluconolactone is a distorted chair, and not a half-chair in the solid state.[143] Further, distortion is due to sp_2 hybridised C_1 and the eclipsed interaction of the equatorial group on C_2, and therefore 2-acetamido lactones may have a different conformation from gluconolactones.

Inhibition studies were originally suspect,[52] since the Ki (app) for NAG_4, measured using Micrococcus Luteus, was not measured by the same workers as for the lactone. However, Philips has shown that the lactone does bind ABCD[60], whereas the reducing sugar binds predominantly ABC, and it is thought that

the ABCD mode for the lactone is some 3000-fold stronger than that for the reducing sugar.[52]

Evidence for the carbonium-ion mechanism, using the transferase activity of lysozyme has been put forward by Rupley,[81] since the acceptor reactivity does not follow the pKa's of nucleophiles, nor their nucleophilicities as would be expected if there were a covalent intermediate. However, in the absence of information on the selectivity of the type of intermediates postulated - either a glycosyl enzyme or oxazoline, it is difficult to judge this contention.

Contradictory evidence exists, however, since distortion is not a major factor in the hydrolysis of NAG-Glu-paranitrophenyl,[107] although it is thought to be for NAG_4.

The third possible mechanism involves anchimeric assistance from the 2-acetamido group of the residue whose aglycone is being catalytically removed. This may occur with or without general base catalysis from Asp 52: Fig.4 (ii) or (i) respectively.

There is contradictory evidence concerning these possibilities;

It has been stated from crystallographic data, that Asp 52 is not correctly orientated for (ii) to occur,[83, 118] but Asp 52 has been identified as essential to lysozyme activity,[114] and therefore it is unlikely that (i) is the correct mechanism.

However, participation by the 2-acetamido group in extensive model studies is almost certainly correct, and mechanism (i) has been suggested as being correct,[125,124] although a twist boat conformation would

be required for the residue in site D, to form the oxazoline intermediate. This work has been criticised [115] since NAG-Glu-para-nitrophenyl and NAG_2 paranitrophenyl cannot be compared directly.

The only unequivocal way of clarifying this, would be to synthesise the oxazoline intermediate, and see if lysozyme catalysed its hydrolysis. [121] This is no mean task, since the preparation of these compounds is not easy, [98, 99, 87] and the hydrolysis would be difficult to follow. [97]

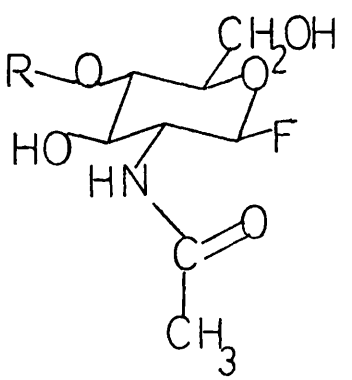
1. 5 Further synthetic substrates for lysozyme

In order to avoid orientation effects of the aryl aglycone moeity, and unfavourable interactions in site E, another easily assayed substrate was sought.

Barnett has found that the carbon-fluorine bond of α -D-glucosyl fluorides is hydrolysed by enzymic extracts of rat intestinal mucosa,[40] and went on to study the hydrolysis of several glycosyl fluorides by the corresponding glycosidases.[41] It was found that in certain cases, the fluoride can have very much greater specificity than the corresponding paranitrophenyl glycoside, and the best example was α -D-glucosyl fluoride which was 240-fold more specific than paranitrophenyl α-D glucoside. It was also shown that some glycosidases do not accept aryl glycosides as substrates,[42] but these substrates are limited to the pH range 5 to 8, owing to instability of the fluorides.

Their hydrolysis may be followed by a fluoride ion activity electrode which, although optimal pH, constant ionic strength, and the masking of heavy metals is necessary, can measure (F^-) down to 0.5 ppm.[36] Alternatively, a pH stat may be used.[42]

However, to synthesise the corresponding substrates for lysozyme, Fig 11, requires the β anomer of 2-deoxy-2 acetamido-glucosyl fluorides to be synthesised, and these are unknown. The α-anomer has been prepared[150] in the deacetylated form, and the acetylated β - NAG 1 and 2 have also been prepared, but removal of the O acetates[148] was not possible without removing the fluorine.



$R = H, NAG_1, NAG_3.$

Fig. 11 2-deoxy-2-acetamido-β-D-glucopyranosyl fluoride oligomers.

The reason for this is almost certainly because the molecule is set up for neighbouring group participation by the 2-acetamido group, resulting in cleavage of the C_1 - F bond. Intra-molecular participation of this type has been noted in β-glucose fluorides: 1, 2 epoxides can be formed during the acid and alkaline catalysed hydrolysis,[43] and with base present, has been used to prepare 1, 6 anhydro glucose in high yield.[39]

This opens the interesting possibility that an oxazoline-type intermediate could be isolated from the reaction of β-fluoro NAG.

Spectro-fluorimetric substrates

The fluorimetric assay of N-acetyl-β-D-glucosaminidase using 4-methyl umbelliferyl-N-acetyl-β-D-glucosaminide as substrate has been studied.[48] The found k cat was about the same as for paranitrophenyl NAG, and the phenyl glycoside was more soluble.

The method has advantages, in that the sensitivity is very high, and very dilute enzyme solutions may be used, and therefore the lower part of a Michaelis Menten plot may be studied in detail whilst preserving the essential condition that $E_o \ll S_o$.

This is only true when the pH of the media allows ionisation of the umbelliferone to occur, otherwise fluorescence is severely suppressed,[50] and an aliquot method may have to be used. This is usually inaccurate, and the application of continuous fluorogenic assays to the determination of enzyme steady state kinetics using conditions of pH and wavelength appropriate to the corresponding

ionised or photo-ionised phenol have been studied.[49] This is limited, however, by the pH max of the enzyme, and therefore the sensitivity advantage may be reduced to that of a spectrophotometric substrate.

Nitro-umbelliferyl aglycones are unlikely to provide an advantage, since, although they are more acidic, their fluorescence is lower.[152] The proposed substrate for lysozyme, therefore was 4-methyl-umbelliferyl - β -NAG_4, which could have an advantage in sensitivity over 3, 4 dinitrophenyl -β - NAG_4 for assaying lysozyme.

1. 6 Model Studies

The special catalytic power of enzymes can be attributed to several causes such as proximity, strain and orientation effects, and many of these have their analogies in model systems.

Bruice has studied systems which could be of relevance to the understanding of the mechanism of action of lysozyme.

Ortho and paranitro-phenyl - 2 -acetamido - 2 deoxy— β glucopyranosides were looked at to see if the 2-acetamido substituents provided anchimeric assistance in the hydrolytic cleavage of the glycosidic bond.[118]

It was found that the hydrolysis of α-glycosides followed a rate expression of the type: kobs = $k_H aH + k_{oH}$ Kw/aH; that is only specific acid and specific base catalysis.

On the other hand, the β-glycosides followed a rate expression of the type:

$$k\ obs = k_H aH + k_{oH}\ Kw/aH + k_o$$

where k_o is the first order rate constant for spontaneous hydrolysis. Further, the value of k_o was such that the pH rate profile was substantially flat from pH 1 to 10, with a rise in the rate outwith this range. At neutrality, the spontaneous hydrolysis was some 10^5 faster than the specific acid or specific base catalysis. No deuterium isotope effect was noted, and therefore proton transfer was complete in the transition state, or occurs after the transition state. The most likely intermediate was postulated as a protonated oxazoline, Fig. 12, although this has never been observed.[119]

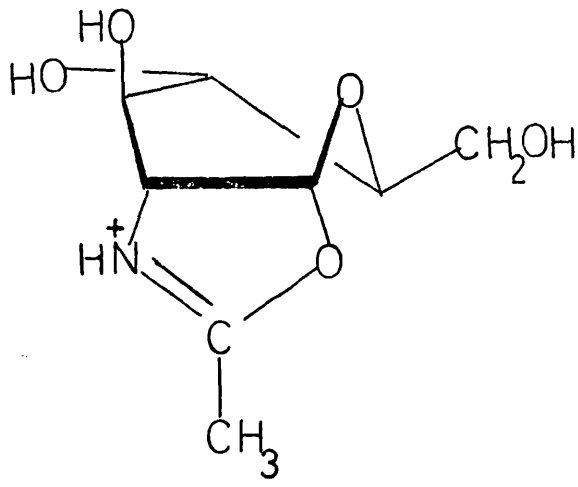


Fig.12 Protonated oxazoline intermediate in β-glycoside cleavage.

The most likely conformation of the pyrananose ring is the skew boat.

The 2-acetamido group was found to be some 300-fold more effective than the corresponding equatorial 2-hydroxy group in glucosides, at promoting catalysis of the β-glycosidic carbon-oxygen bond.

Barnett has studied the acid and alkaline catalysed hydrolysis of glycosyl fluorides, and has found that β-D-glucosyl fluoride reacts 5×10^3 faster than the corresponding α compound, and that they give the same product.[43] The reaction presumably is accelerated by 6 hydroxy or 2 hydroxy participation. The rates of hydrolysis were comparable with the corresponding methyl glycosides.

The hydrolysis of orthocarboxy phenyl - β -D - 2 acetamido-2 deoxy-glucopyranoside has been studied, and intramolecular general acid catalysis, as in glucosides,[123] as well as specific acid catalysis was observed, under suitable conditions.[33,119] The reaction proceeds with retention of configuration.

The structure of a glycoside is analagous to that of acetals, and general acid catalysis in acetals of a suitable structure has been observed.[122]

The hydrolysis of 2-acetamido-2-deoxy-β-D-glycosides is generally thought to proceed with retention of configuration, and this has been postulated for the specific acid catalysed hydrolysis of methyl-β-NAG and of NAG_2.[121]

Attempts have been made to prepare the oxazoline intermediate, using 2-carboxy phenyl - β - NAG,[33] and it was thought that if a more suitable aglycone were used, such as 2, 4 dinitrophenol, this might prove to be more successful.

The effect of the 2-acetamido group on the behaviour of β-fluoro-NAG should also be interesting, since it is almost certainly because of this effect that these compounds have not been previously prepared.

The hydrolysis of 2-methyl oxazoline itself has been studied,[97] and the rate falls off with pKa's of 0 and 5.5, the latter being the pKa of protontation of 2 methyl oxazoline.

2. [illegible]

2.1 Preparative Experimental

GENERAL

Melting points were measured on a Kofler-Reichert hot stage melting point apparatus and all are uncorrected.

I.R. Spectra were run on a Perkin Elmer SP2000 spectrophotometer and were calibrated using a polystyrene film.

P.M.R. spectra were run on a Varian T60 spectrometer for routine analyses, and on a Varian HA100 100 MHz spectrometer. Chemical shifts are quoted in τ values, downfield from external T.M.S.. 220 MHz PMR spectra were obtained from the SRC service.

^{19}F N.M.R. were recorded on an HA100 or Jeol spectrophotometers, and shifts are quoted in cycles relative to external T.F.A.

Mass spectra were recorded on an A.E.I. MS12 at 340°c.

Elemental analyses were determined by Mr. J. Cameron and Miss F. Cowan, University of Glasgow, for C, H and N figures. F analyses were carried out in the laboratories of A. Bernhardt, West Germany.

2. 1. 1.

The Preparation of the acetylated oligomers of 2-acetamido-2 deoxy-D-glucose

2-acetamido-1,3,4,6-tetra-O-acetyl-2-deoxy- β-D-glucose ($AcNAG_1$)

2-amino-2-deoxy-D glucose hydrochloride (Koch-Light Laboratories) was converted to the β-penta acetate by the method of Findlay and Levvy.[68] The use of zinc chloride as Lewis acid normally favours the formation of an α-acetate, but the β is formed here. Mpt. 185° (lit.186°)

Mass spectrum: $(M - 59)^+$ at m/e = 236.

Chitobiose octaacetate ($AcNAG_2$)

Chitin was acetolysed according to the method of Barker,[46] and the product recrystallised from methanol.

Mpt. 305-6° (lit. 308-9°)

Mass spectrum: $(M-59)^+$ at m/e = 617.

Chito-triose, -tetraose and-pentaose peracetates ($AcNAG_{3,4,5}$)

Only the peracetate of chitotriose has been previously prepared pure.[22] Previous attempts at separating the acetylated oligomers of 2-acetamido-2-deoxy-D-glucose have proved fruitless.[33]

The chitin oligosaccharides NAG_2 to NAG_6 have been previously prepared in small quantities, using carbon-celite[22] and gel filtration columns,[55] but this produces difficulties with desalting,[61] and adds an extra stage to the synthesis of glycosides, many of which are low yield.[75]

This prompted the development of a system for continuous production of preparative quantities of these peracetates.

The use of columns in solid/liquid partition chromatrography, approximating to the capabilities of TLC has been reported,[71] using

silica gel 500 to 1000 times the weight of material being separated. After much trial and error, the best solvent system developed was mixtures of methanol and chloroform.

The system developed was as in Fig. 13.

The adsorbent used was Mallinckrodt Silicic Acid, 100 mesh, activated 12 hours at 200°. 1.8 kg were used, and 4.5 g of oligosaccharide mixture could be separated on this.

The column was packed wet, with a thin slurry of the adsorbent and 3% methanol/chloroform.

The mixture of acetylated oligosaccharides was applied in a minimum volume of 10% methanol/chloroform, and the column eluted with a progressively increasing polarity of solvent; polarity gradient = 1% methanol/litre.

Solvent added	Fractions (30ml)	Compound	Typical weight
2 l. 3%; 1.4 l. 4½%			
1.4 l. 6%	130-140	Ac NAG_1	0-0.05 g
1.4 l. 7½%	145-160	Ac NAG_2	0.2 g
1.4 l. 9%	162-175	Ac NAG_3	1.1 g
1.4 l. 10½%	180-210	Ac NAG_4	1.5 g
1.4 l. 12%	215-250	Ac NAG_5	0.6 g
1.4 l. 13½%	255-290	(Ac NAG_6)	0.1 g
1.4 l. 15%	295-325	(Ac NAG_7)	0.05 g

The separation was monitored either by the U.V. $n \longrightarrow \pi^*$ absorption of the acetates (ε of one acetate group = 0.091; λ = 260 nm), or, more regularly, by testing the homogeneity of the fractions by T L C of the eluant - every fifth tube, on silica gel G, 11% methanol/chloroform, Ceric Sulphate developer. The column could be re-used by

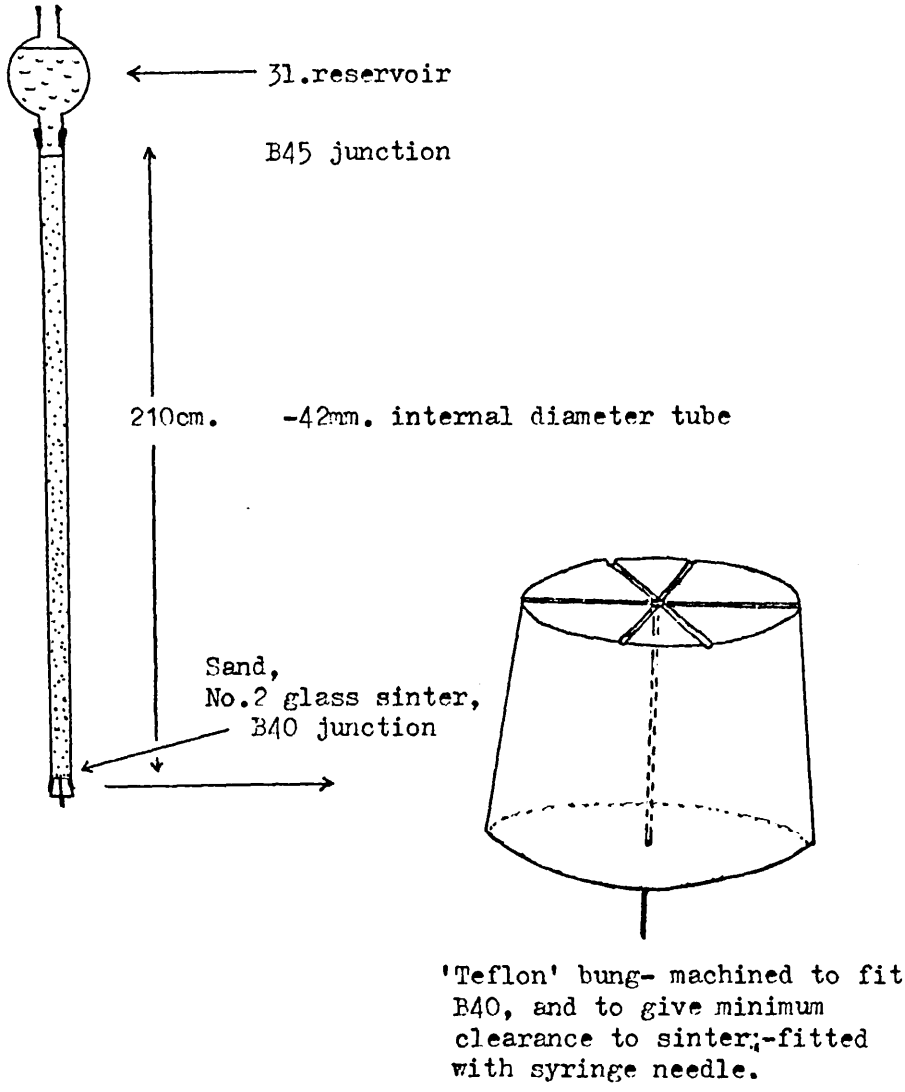
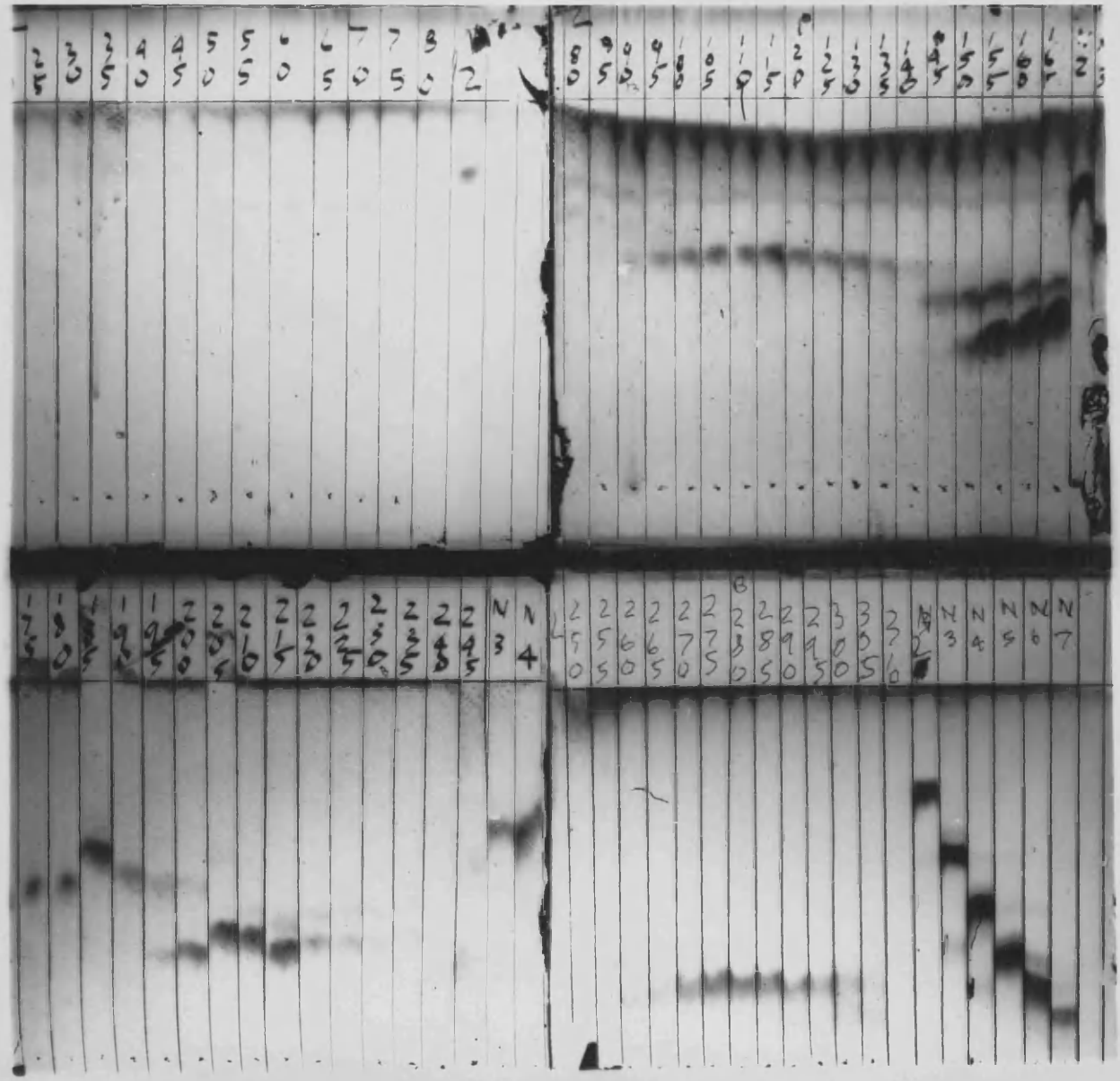


Fig.13 Column design used for the separation of NAG oligomer peracetates.



washing with 2 l. of 3% methanol/chloroform. Homogenous fractions were pooled and evaporated on a Rotovap, and recrystallised from methanol. The solvent system was also used for T.L.C.

Preparation of acetylated oligosaccaride mixture for separation

The acetolysis procedure was a modification of that described by Barker.[46] The conditions varied according to the quality of the chitin used, for example: Chitin (Kodak Laboratories) was ball-milled for two days, then dried at 150° for 1 hour. 50 ml of Analar concentrated sulphuric acid were added slowly to 500 ml of acetic anhydride, with stirring and cooling, in a 1 litre round bottomed flask. The cooled chitin was added to this, and left overnight to soak. The mixture was then incubated at 55° c for 1½ hours with occasional swirling before working up as described in Ref. 22, using up to 8 litres of chloroform for extraction.

The crude mixture contained impurities from the chitin, running between the oligomers,[135] as seen on T,L.C. These were removed by dissolving the mixture in a minimum of methanol, and allowing fractional crystallisation of the acetylated oligomers, at 0° for several days. This also served to enrich the mixture in the higher oligomers. (T.L.C.; 11% methanol/chloroform)

Properties and Analyses of peracetyl chitin oligomers

		C	H	N	O	Molecular weight	Mpt	
Ac NAG_2	Calculated	28	40	2	17	676.63	308°	lit.[46]
Rf = .43		49.704	5.959	4.14	40.197			
	Found	49.69	5.87	4.16			304°	
Ac NAG_3	Calculated	40	57	3	24	963.91	315°	lit.[46]
Rf = .23		49.844	5.961	4.359	39.836			
	Found	49.78	6.09	4.42			317°	(dec.)
Ac NAG_4	Calculated	52	74	4	31	1251.18	-	
Rf = .11		49.919	5.962	4.478	39.641	$(\alpha)_{Ac}^{D}$ = +22.4°		(c = 1, acetic acid)
	Found	50.02	6.00	4.39			321°	(dec.)
Ac NAG_5	Calculated	64	91	5	38	1537	-	
Rf = .07		49.967	5.962	4.552	39.519			
	Found	49.85	6.04	4.36			328°	(dec.)

Ac NAG_6 Rf = .02

Ac NAG_7 Rf = .013

The Rf values are quoted for 10% methanol/chloroform solvent

Preparation of NAG_2, NAG_3, NAG_4 and NAG_5

The peracetylated oligosaccharides could be deacetylated with sodium methoxide, by the addition of 0.1 moles of methoxide to a dry methanol/chloroform solution of the peracetate. Partial evaporation of the solvent, followed by cooling, trituration and the addition of small quantities of ether, were often necessary to induce crystallisation.

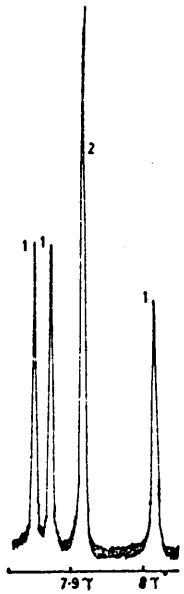
The de-O-acetylated sugars NAG_2 to NAG_5 compared favourably on T L C (n propanol, ammonia, water; 6:3:2) with the reducing sugars prepared independently by Foster,[33] and the analyses were very similar.

220 MHz PMR of the peracetyl chitin oligomers, in $CDCl_3/CD_3OD$.

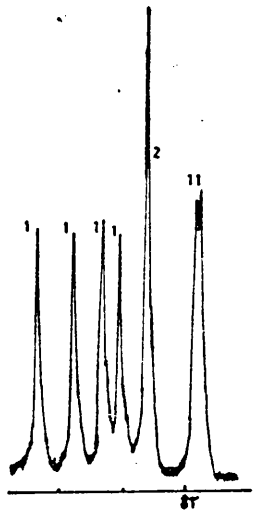
Resolution of the acetate groups and integration provided a means of characterising the peracetyl derivatives of the oligosaccharides and the expanded spectra at 8τ are shown in Fig. 14. The full structure of Ac NAG_4 is given in Fig. 15. The small divisions are 0.1ppm. The highest field signals are those of the N acetate methyls (8.067τ) and the lowest field singlet is of the acetate methyl of the oxygen attached to C 1 of the sugar (7.85τ for a β acetate; 7.76τ for an α acetate.) This is known since it is this signal which disappears on α chloride formation.

The sugars formed from acetolysis of chitin are all α acetates, since this is the thermodynamically more stable isomer and is formed preferentially under acid catalysed conditions.

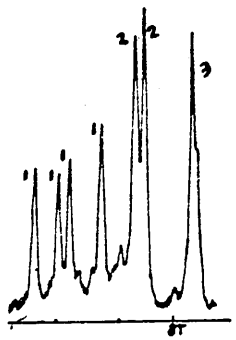
The configuration of the C1 acetate is known from the coupling constant of the C1 proton to the C2 proton. The axial-equatorial



AcNAG$_1$

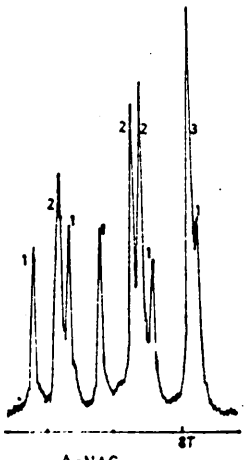


AcNAG$_2$

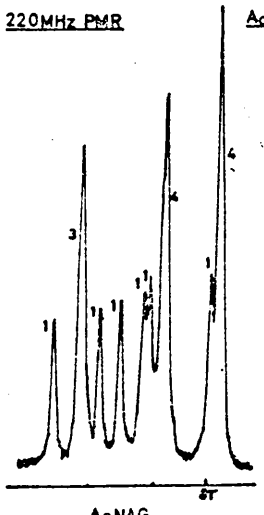


AcNAG$_3$

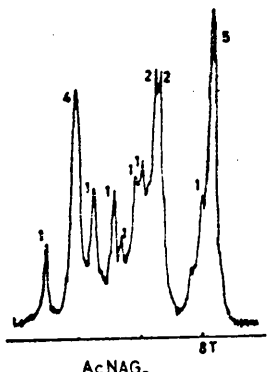
Fig. 14 220MHz PMR



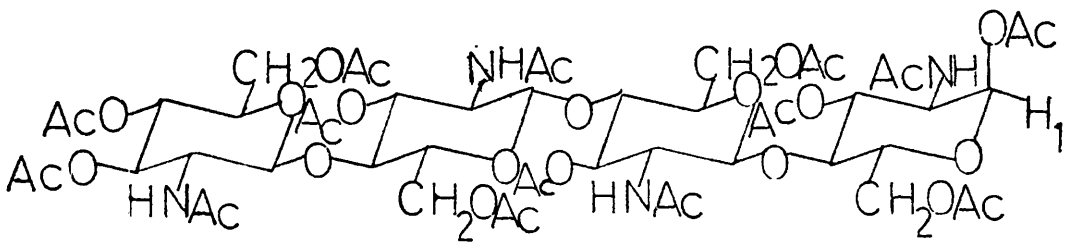
AcNAG$_4$



AcNAG$_5$



AcNAG$_6$



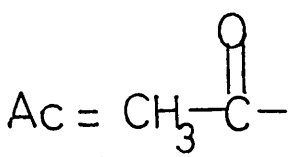


Fig. 15, Ac NAG_4

coupling is indicated by a figure of JH_1H_2 = 2.0 c/s; at 3.80τ.

The β-glucosamine pentaacetate has the C1 proton at 4.18τ; JH_1H_2 = 7.6 c/s. α acetates usually have H_1 at lower field.[175]

The 220 MHz NMR of Ac NAG_1 has been published.[38]

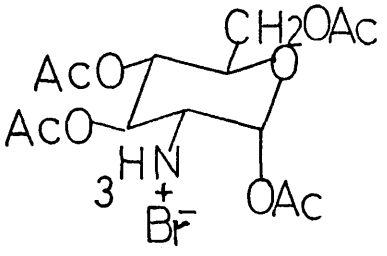
The use of lanthanide shift reagents did not aid resolution, since it was sufficiently good with the lower oligomers, and a mutually suitable solvent could not be found for the higher ones.

2. 1. 2.

The preparation of 2, 4 dinitro phenyl -β- glycosides of NAG_1, NAG_2 & NAG_3

The acetylated derivatives of 2, 4 dinitrophenyl glucosides have been prepared via bromoglucose.[69] A seven stage method for the preparation of the glucosides, using trimethyl silyl ether protecting groups and α-bromoglucose has been published.[72]

Leaback and Walker have reported the failure of α-NAG bromide to form glycosides, due to ease of N-acetyl transfer to C_1.[93] This was confirmed, and (1) was isolated from reaction mixtures.



The use of α-NAG bromide has been described,[77] but it was not isolated. Unsuccessful attempts were made to increase the nucleophilicity of 2, 4 dinitrophenol to the slow reacting α-chloro NAG, by using quaternary ammonium salts of the phenol, or aprotic solvents, such as DMF and DMSO: as in Ref 74.[86, 117] Lewis acids were also uniformily unsuccessful.

The following synthetic scheme gave maximum yield.[75]

2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-α-D glucopyranosyl chloride (α-AcNAG$_1$Cl)

β-D-glucosamine pentaacetate was treated with redistilled acetyl chloride, ether and dry hydrogen chloride, as described in 99, but using a sealed flask as the reaction vessel. After 30 hours, the solution was evaporated on a Rotovap, and the residue a zeotroped three times with sodium dried benzene. The crude chloride was recrystallised from chloroform and ether.

Mpt = 128° (lit. [78] 127-8°) Yield = 91%

2,4 dinitrophenyl-2-acetamido-3,4,6 tri-O-acetyl-2-deoxy-β-D glucopyranoside (Ac NAG$_1$-β-2, 4 dnp)

17.3g of Ac NAG$_1$Cl were dissolved in 500 ml of acetone, 26.2 g of 2, 4 dinitrophenol and 142 ml. of IN sodium hydroxide were added, and the solution left at room temperature for 20 hours.

The acetone was removed on a Rotovap, the precipitate filtered and washed thoroughly with cold sodium bicarbonate solution, then distilled water. The glycoside was dried, and recrystallised from methanol, chloroform and ether to give pale fawn needles.

Mpt. 163-4° Yield = 4.2 g; 17.3%

NMR: $CD_3OD/CDCl_3$

8.17τ 3H amide acetate methyl

7.94-8.0τ 9H 0 acetate methyls

7.4-4.5τ 6H ring protons

4.33τ 1H doublet; JH_1H_2 = 8.3c/s-α anomeric proton indicative of diaxial coupling.

2.5τ 1H doublet J = 9 c/s
1.6τ 1H doublet of doublets
1.37τ 1H doublet
} Aromatic protons

U.V. λ max = 275 nm (methanol)

Analysis: Found: C 46.15%; H 4.39%; N 8.45%

$C_{20} H_{23} O_{13} N_3$ requires: C 46.79%; H 4.52%; N 8.18%

De-O-acetylation of this compound was found to remove the phenol, when methoxide was used, [124,116] but the following method was used.[75]

2,4 dinitrophenyl-2-acetamido-2-deoxy-β-D-glucopyranoside (NAG_1-β-2 4 dnp

Ac NAG_1-β-2, 4 dnp was de-O-acetylated by dissolving 0.5 g in 30 ml. of dry methanol and 20 ml. of dry chloroform, adding 10 ml. of a 16% w/w solution of dry hydrogen chloride in methanol, at room temperature.

The reaction was followed by TLC on silica gel G, using 1:1:1 ethyl acetate/methanol/benzene, as solvent.

Reaction time: 3 hours at room temperature.

Rf = 0.9 Ac NAG_1 2, 4 dnp

Rf = 0.6 NAG_1 2, 4 dnp

The solvent was evaporated at 30° on a Rotovap, and the resulting oil was triturated with cold chloroform to start crystallisation. The solid product was filtered, washed with a little ice cold methanol, and recrystal-lised carefully from aqueous methanol.

Yield = 0.14 g; 38% Mpt. 124-5°

$(\alpha)_D^{20}$ = + 32° (C.1, methanol)

I. R. No O acetates present

Amide I & II: 1650 cm^{-1} and 1560 cm^{-1}.

Aromatics: 1610 cm^{-1} and 1525 cm^{-1}.

broad O-H and N-H 3200-3600 cm^{-1}.

U.V. λ max = 275 (methanol)

Analysis: Found: C 41.15%; H 4.56%; N 10.09%

$C_{14} H_{17} N_3 O_{10} \cdot H_2O$ requires: C 41.49%; H 4.73%; N 10.89%

2-acetamido-4-O-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl) - 3,6 -di-O-acetyl-2-deoxy-α-D glucopyranosyl chloride

(α-Ac NAG_2 Cl)

Chitobiose octaacetate was converted to α-Ac NAG_2 Cl by the method of Zurabyan.[99] Yield 90% Mpt 196° (lit.[99] 195°)

2,4 dinitrophenyl-2-acetamido-4-O-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy- β -D-glucopyranosyl)-3,6-di-O-acetyl-2-deoxy- β -D-glucopyranoside (Ac NAG_2-β-2,4 dnp)

5.5 g of α-Ac NAG_2 Cl and 5 g of dry sodium 2,4 dinitrophenolate were dissolved in 500 ml of dry Analar acetone, and the mixture left at room temperature for 4 days. 100 ml of water were added and the acetone was removed on a Rotovap at 35°. The precipitate was then treated as for Ac NAG_1-β-2,4 dnp.

Yield= 0.76 g; 11.5% Mpt. = 171-2° (lit.[124] 191-2°)

N.M.R. $CD_3OD/CDCl_3$

8.1τ	6H,	amide acetate methyls	
7.94 -8.05τ	15H	O acetate methyls	
7.5 -4.57τ	13H	ring protons	
4.33τ	1H	doublet JH_1H_2 = 8.3 c/s-	anomeric protons
2.5τ	1H	doublet J = 9c/s	aromatic protons
1.6τ	1H	doublet of doublets	aromatic protons
1.3τ	1H	doublet	aromatic protons

Analysis:

Found: C 47.96%; H 5.13%; N 6.80%

$C_{32}H_{40}N_4O_{20}$ requires C 48.00%; H 5.04%; N 6.99%

U.V. λ max = 275 nm (methanol)

2, 4 dinitrophenyl-2-acetamido-4-O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-deoxy-β-D-glucopyranoside (NAG_2-β-2, 4 dnp)

Ac NAG_2-β-2, 4 dnp was deacetylated as for Ac NAG_1-β-2, 4 dnp. Trituration was with methanol.

Yield 62% Mpt. 183-5°

Rf = 0.75 Ac NAG_2-β-2,4 dnp

Rf = 0.31 NAG_2-β-2, 4 dnp

methanol/ethyl acetate/benzene 1:1:1

Estimation of 2, 4 dinitrophenol: A 1×10^{-4} M solution of 2, 4 dinitrophenyl NAG_2 in 1NNaOH was heated to 100° for 15 minutes, and the phenol released estimated from the absorbance at 400 nm.

Absorbance calculated: 1.02 Found: 0.99

I.R. No O acetates present.

3600-3200 cm^{-1} (broad) O-H and N-H

1650 cm^{-1} and 1560 cm^{-1}: Amide I and II

1525 cm^{-1} and 1610 cm^{-1}: Aromatics

The alkaline hydrolysis of aryl glycosides may not give quantitative release of phenol, since migration of the aryl group round the sugar ring may occur, with formation of an aryl ether.[140]

2-acetamido-4-O-/2-acetamido-4-O-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl)-3,6-di-O-acetyl-2-deoxy-β-D-glucopyranosyl)-3,6-di-O-acetyl-2-deoxy-α-D-glucopyranosyl chloride (α-$AcNAG_3$Cl)

was prepared from $AcNAG_3$ as for α-$AcNAG_2$ Cl

Yield: 82% Mpt. = 192-3° (lit.[136] 169°)

2, 4 dinitrophenyl-2-acetamido-4-O-(2-acetamido-4-O-(2-acetamido-3, 4, 6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl)-3, 6-di-O-acetyl-2-deoxy-β-D-glucopyranosyl)-3, 6-di-O-acetyl-2-deoxy-β-D-glucopyranoside

Ac NAG_3-β-2, 4 dnp)

was prepared from Ac NAG_3 Cl as for the conversion of Ac NAG_1 Cl to Ac NAG_1 2, 4 dnp

Yield = 3.4% Mpt. 240-242°

I.R : N - H 3300 cm^{-1}

O-acetate 1745 cm^{-1}

Aromatic 1605 cm^{-1}

Amide I & II 1660 cm^{-1} and 1530 cm^{-1}

Analysis

Found: C 47.11%; H 5.26%; N 6.32%

$C_{44}H_{57}N_5O_{27}$ requires C 48.58%; H 5.28%; N 6.44%

U.V. λmax = 275 nm (methanol)

2, 4 dinitrophenyl-2-acetamido-4-O-(2-acetamido-4-O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-deoxy-β-D-glucopyranosyl)-2-deoxy-β-D-glucopyranoside (NAG_3-β-2, 4 dnp)

Ac NAG_3-β-2, 4 dnp was de-O-acetylated as for Ac NAG_2-β-2, 4 dnp

Yield: 60%

Rf = 0.65 Ac NAG_3-β-2, 4 dnp

Rf = 0.25 NAG_3-β-2, 4 dnp

1:1:1 methanol/ethyl acetate/benzene.

Estimation of 2, 4 dinitrophenol:

Found absorbance: 0.83 Calculated: 0.91; 400 nm

U.V. λmax 275 nm (methanol)

I.R. showed absence of O-acetates.

2. 1. 3 The preparation of the paranitrophenyl - β - glycosides of NAG_2, NAG_3, NAG_4 and NAG_5

Paranitrophenyl glycosides have been prepared directly from the acetate by fusion.[68] This did not succeed either with α acetates because of their lower reactivity, or with more acidic phenols, probably because of their lower nucleophilicity or stability.

Osawa has prepared NAG_2 - β -paranitrophenyl[100] and NAG_3-β-paranitrophenyl[130] from the corresponding α-chlorides, and this method was used with success.

This could not be used for higher oligomers, however, since their chlorides are quite insoluble in acetone. The following method, therefore was used to prepare NAG_4-β-pnp, and could be used with equal success for the high yield preparation of the glycosides of the lower oligomers.

α-Ac NAG_4Cl

4.2 g. of dry, recrystallised chitotetraose peracetate were suspended in 500 ml. of redistilled acetyl chloride and 100 ml. of dry ether in a 1 litre round bottomed flask, and saturated to -20°c with dry hydrogen chloride gas. 2 ml of glacial acetic acid were added to help effect solubility. The flask was sealed, clamped, and allowed to come to room temperature and left for 24 hours. The Ac NAG_4 did not dissolve completely. Opening of the flask was accomplished by cooling to -20° before releasing the pressure.

The acetyl chloride was removed on a Rotovap at 40°, and the syrupy residue aezeotroped four times 50 ml. of dry benzene to remove

the acetic acid. The white, powdery residue was run on T.L.C. in 11½% methanol and chloroform against a standard of Ac NAG_4.

The plate showed at least 70% of α-Ac NAG_4 Cl present (Rf = 0.45), about 20% of starting material (Rf = 0.4), and small quantities of breakdown products.

The mixture was not purified further, and the crude material was used generally for the synthesis of glycosides.

Ac NAG_4-β-paranitrophenyl

1 g of the crude α-Ac NAG_4 Cl was mixed with 0.3 g of dry sodium paranitrophenolate in a 25 ml round bottomed flask. 7 ml of dry DMSO were added, and the mixture shaken at room temperature for 24 hours.

The reaction mixture was poured onto 100 ml of an ice-water mixture. The product crystallised out, was filtered and washed with bicarbonate, then distilled water. Fawn crystals were left, which were dried and recrystallised from methanol/chloroform and ether.

Yield = 180 mg; 17%

Mpt = 266-7°

Rf = 0.41; pure by T L C 11% methanol/chloroform

U.V. λ max = 295 nm (methanol)

Analysis

Found: C 50.31%; H 5.42%; N 6.28%

Calculated for $C_{56}H_{75}N_5O_{32}$: C 50.6%; H 5.65%; N 6.51%

NAG_4-β- paranitrophenyl

70 mg. of Ac NAG_4-β-paranitrophenyl were suspended in 1.75 ml. of dry methanol, and warmed to 40° c. 0.1 ml. of I N sodium methoxide solution was added, and the mixture shaken. The compound dissolved and reprecipitated in a few minutes. The suspension was then cooled to 0° overnight, and the deacetylated product purified by adding 20 ml. of distilled water and passing the resultant solution through a 30 x 2 cm. column of Sephadex G15, followed by elution with distilled water. The eluent was followed by U.V., and homogeneous fractions were pooled and freeze dried, followed by final drying of the product at 40° in vacuo.

Yield = 23 mg = 34% Mpt = 292-3° (dec.)

Analysis

Found: C 43.22%; H 6.08%; N 6.49%;

Calculated for $C_{38}H_{51}N_5O_{23}.6H_2O$: C 43.10%; H 6.52%; N 6.62%

U.V. λmax = 295 nm (water)

Release of phenol after heating at 100°c, 4 hours in 2 N H Cl = 92% of theoretical; measurement at 400 nm after dilution in alkali:

Rf: 6:2:1 n propanol, ammonia, water = 0.33

(Rf: - NAG_4 = 0.20)

Rf: 6:3:2 n propanol, ammonia, water = 0.645

NAG_5-β - paranitrophenyl

Ac NAG_5 was reacted with acetyl chloride, ether and dry hydrogen chloride to form the crude α-chloride, as for the preparation of α-Ac NAG_4 Cl.

1 g. of impure α-Ac NAG_5 Cl was mixed with 0. 4 g. of dry sodium paranitrophenolate and dissolved in 7 ml. of DMF, and left at room temperature for 24 hours. The reaction mixture was poured onto 100 ml. of ice-water. A mixture of 3 compounds precipitated out, and these were separated on P.L.C. (Kieselguhr H; 11½% methanol/ chloroform; elute with 15% methanol/chloroform).

Yield = 8 mg = 0.7% Mpt. = 256-7°

Rf = 0.35 11% methanol/chloroform pure by TLC

U.V. λ max = 294 nm (methanol)

The peracetate was deacetylated as for Ac NAG_4-β-paranitrophenyl

Yield = 3 mg.

U.V. max = 298 nm.

2. 1. 4 The preparation of 3, 4 dinitrophenyl -β-glycosides of NAG_2, NAG_3, NAG_4

3, 4 dinitrophenol

The nitration of m-nitrophenol by a modification of the methods in the literature [96, 79] gave simultaneously the 3, 4; the 2, 3 and 2, 5 isomeric dinitrophenols.

40 g. of m-nitrophenol were shaken at intervals into 40 ml. of a 1: 1 mixture of nitric acid (S.G. 1.4) and water in a 250 ml. round-bottomed flask fitted with an air condenser. After a small

amount had been added, the reaction was started by warming. The phenol dissolved with the evolution of nitrogen dioxide, and the exothermic reaction continued by further additions of phenol. After the reaction had subsided and cooled, the remaining acid was decanted, and the oily product washed with water. This was left for two days to become semi-solid. The remaining water was removed as completely as possible, and 60 ml. of 97% ethanol used to wash the product, which then took on the consistency of a thick pulp. This was triturated under the alcohol and left for several hours. The solid was filtered off and was almost pure 2, 5 dinitrophenol. It was recrystallised twice from 95% ethanol.

Yield = 9 g. Mpt. = 108° (lit.[79] 105.8-106.2°)

The supernatant contained a mixture of 2, 3 and 3, 4 dinitrophenols, and these were separated by fractional crystallisation. The liquid was evaporated to dryness, and the solid dissolved in 200 ml. of benzene containing a little pet. ether (60-80). The solution was boiled for 30 minutes to allow resin to collect, and was decanted, before cooling rapidly in ice with stirring. The 3, 4 dinitrophenol was precipitated and filtered at once, before recrystallisation from benzene.

Yield = 10 g. Mpt = 134°-135° (lit.[79] 135.1-135.5°)

3, 4 dinitrophenol was converted to the dry sodium 3, 4-dinitrophenolate by dissolving it in methanol and adding 95% of the theoretical amount of 1 N sodium hydroxide solution, followed by removal of solvent on a Rotovap and careful drying of the solid, 40°/1 mm. (It is explosive)

P.M.R. of isomeric dinitrophenols $CDCl_3/CD_3OD$

Aromatic substitution patterns are not always easy to interpret,[80] and therefore results are presented here.

2, 5 dinitrophenol

2.06τ; 1H; doublet of doublets; H_4; JH_4H_3 = 8.8 c/s JH_4H_6 = 2.4 c/s

0.5τ; 1H; O - H

1.95τ; 1H; broadened doublet; H_6; JH_6H_4 = 2.4 c/s

1.62τ; 1H ; broadened doublet; H_3; JH_3H_4 = 8.8 c/s

2, 4 dinitrophenol

2.61τ; 1H; doublet; H_6; JH_6H_5 = 9.0 c/s

1.45τ; 1H; doublet of doublets; H5; JH_5H_6 = 9 c/s; JH_5H_3 = 3 c/s

0.8 τ; 1H; doublet; H3; JH_3H_5 = 3 c/s

4.75τ; 1H; O - H.

3, 4 dinitrophenol

2.9τ; 1H; doublet of doublets; H_6; JH_6H_5 = 8.9 c/s; JH_6H_2 = 2.6

2.8τ; 1H; irresolved; H_2;

1.95τ; 1H doublet of doublets; H_5; JH_5H_6 = 8.9 c/s; JH_5H_2 = 1.2 c/s

4.4τ; 1H; O - H

The pKa's of the phenols[76] are:

	P-NO_2	2, 4 dinitro	3, 4 dinitro	2, 5 dinitro	3, 5 dinitro	2, 3 dinitro
pKa:	7.20	4.00	5.42	5.21	6.69	4.95

Ac NAG_4-β-3, 4 dinitrophenyl

4.3 g of crude α-Ac NAG_4 Cl was mixed with 3 g of the dried sodium salt of 3, 4 dinitrophenol, and 15 ml. of standard dimethyl formamide were added. The mixture was shaken for 24 hours at room temperature and then added slowly to 120 ml of an ice-water mixture. The product precipitated immediately and was filtered and washed with sodium bicarbonate solution, then distilled water. The product was purified by dissolving it in 15% methanol in chloroform and applying it to a chromatographic grade silica column, followed by elution with this solvent. The product was recrystallised from 1:1 methanol and chloroform, with ether added.

Yield = 735 mg = 18% Mpt. = 241-2°.

Rf = 0.38 in 11% methanol-chloroform.

Rf of peracetate = 0.40; Rf of chloride = 0.45.

U.V. λmax = 280 nm. (methanol)

Analysis:

Found: C 48.18%; H 5.43%; N 5.90%;

Calculated for $C_{56}H_{74}N_6O_{34}$: C 48.91%; H 5.42%; N 6.11%

NAG_4-β-3, 4 dinitrophenyl

The acetate could be de-O-acetylated as for NAG_4-β-paranitrophenyl, but a more efficient method was using dry methanol and chloroform with sodium methoxide, as for the de-O-acetylation of fluorides (2.1.7). The solvent was partly removed at 20° on a Rotovap, and water added. After purification on Sephadex G15, the fractions, monitored by U.V., were immediately cooled to avoid hydrolysis of the product, and mixed

with Amberlite MB-1 to remove ionic material. The solution was filtered and freeze dried, to give a white amorphous powder.

Yield = 75%, Mpt. = 248° (dec.)

Rf = 0.58 6:3:2 n propanol, ammonia, water

With care, the compound could be recrystallised from methanol/water.

Analysis

Found: C 42.08; H 5.75; N 6.99

Calculated for $C_{36}H_{56}N_6O_{25}.4H_2O$: C42.70; H 6.04; N 7.86

U.V. λmax = 283 nm. (water) ϵ = 6550

I.R. Showed absence of O acetates;

Amide I and II: 1650 cm^{-1} and 1560 cm^{-1}

Aromatics 1610 cm^{-1} and 1525 cm^{-1}

broad O-H and N-H 3200-3600 cm^{-1}

The maximum concentration of compound obtainable in D_2O was 2×10^{-3} M, and therefore CAT was used to obtain a spectrum. The four amide acetate methyls were observed as peaks of the ratio 1:2:1, centred at 7.34 τ.

Ac NAG_3-β-3,4 dinitrophenyl

Ac NAG_3-β-3, 4 dinitrophenyl was prepared from α-Ac NAG_3 Cl 0.8 g, as for the corresponding tetrameric glycoside.

Yield = 0.36 g = 39% Mpt. = 251-2°

U.V. λmax = 283 nm. (methanol)

Rf = 0.53, 11% methanol/chloroform

Analysis

Found: C 48.77%; H 5.51%; N 5.99%

Calculated for $C_{44}H_{57}N_5O_{27}$: C 48.58%; H 5.28%; N 6.44%

I.R. 3400 cm^{-1} - N-H; 1755 cm^{-1} - O acetate; 1670 cm^{-1} and 1560 cm^{-1} - Amide I and II; 1610 cm^{-1} - aromatic

NAG_3-β-3, 4 dinitrophenyl

De-O-acetylation was effected as for the tetrameric glycoside.

U.V. λ max = 283 nm (water) ε = 6500

I.R. Showed absence of O acetates

Amide I and II: 1650 cm^{-1} and 1560 cm^{-1};

Aromatics: 1610 cm^{-1} and 1525 cm^{-1}

broad NH and O-H 3200 - 3600 cm^{-1}

Analysis

Found: C 38.98 %; H 5.53%; N 7.12 %

Calculated for $C_{30}H_{43}N_5O_{20}.7H_2O$: C 39.78%; H 6.32% ; N 7.62%

Ac NAG_2-β-3, 4 dinitrophenyl

Ac NAG_2-β-3, 4 dinitrophenyl was prepared from 1 g. of α-Ac NAG_2 Cl as for the corresponding tetrameric glycoside

Yield = 0.6 g; 53% Mpt. = 216 -217°

Rf = 0.65 11% methanol/chloroform Ac NAG_2; Rf = 0.7

I.R. 3400 cm^{-1} N-H;

1755 cm^{-1} O acetate

1670 cm^{-1} and 1560 cm^{-1}: amide I and II

1610 cm^{-1} aromatics

U.V. λ max = 280 nm (methanol)

Analysis

Found C 48.47%; H 5.16%; N 6.81%

Calculated for $C_{32}H_{40}N_4O_{20}$: C 48.00 %; H 5.04 %; N 6.99 %

NAG_2-β-3, 4 dinitrophenyl

De-O-acetylation was effected as for AcNAG_1-β-F. Partial evaporation of the solution caused crystallisation of the product. Ether was added to the cooled mixture to aid crystallisation, and the product filtered and washed with ether. Recrystallisation was from aqueous methanol.

Yield = 0.31 g; 75% Mpt. = 197-9°.

I.R. Showed absence of O acetates

3600 cm^{-1} to 3200 cm^{-1} O-H and N-H

1665 cm^{-1} and 1560 cm^{-1} amide I and II

1610 cm^{-1} aromatics.

U.V. λmax = 283 nm (water) ε = 6560

Analysis

Found: C 40.93%; H 4.83%; N 8.79%

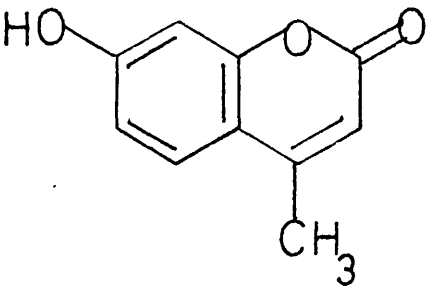
Calculated for $C_{22}H_{30}N_4O_{15}\cdot 3H_2O$: C 40.99%; H 5.63%; N 8.69%

2. 1. 5 The synthesis of NAG_4 -β- 4 methyl umbelliferyl.

Ac NAG_4-β-4 methyl umbelliferyl

4 methyl umbelliferone (1) was obtained from Koch Light Laboratories. The sodium salt was prepared by dissolving 17.6 g of 4 methyl umbelliferone in methanol and adding 95 ml of 1 N sodium hydroxide.

(1)



The solution was evaporated on a Rotovap, and the solid dried in a pistol.

1.5 g. of crude α-Ac NAG_4 Cl were mixed with 1.5 g. of sodium 4-methyl umbelliferate and 5 ml. of standard dimethyl formamide were added. The mixture was shaken overnight at room temperature, and then added slowly to 40 ml. of an ice-distilled water mixture. The product was precipitated and washed with sodium carbonate solution, then distilled water. Recrystallisation was from methanol/chloroform

with ether added.

Yield = 0.3 g = 17% Mpt = 275° (dec)

I.R. 3400 cm^{-1} N -H

1755 cm^{-1} O acetates

1720 cm^{-1} lactone

1665 cm^{-1} amide I

1660 cm^{-1} to 1600 cm^{-1} aromatics

1560 cm^{-1} amide II

U.V. λ max = 320 nm (methanol) glycoside

λ max = 324 nm (methanol) free phenol

λ max = 368 nm (methanol) sodium umbelliferate

Analysis

Found: C 50.74%; H 5.61%; N 3.60%

Calculated for $C_{60}H_{78}N_4O_{32}$: C 52.71%; H 5.75%; N 4.09%

NAG_4-β-4 methyl umbelliferyl

0.2 g Ac NAG_4-β-4 methyl umbelliferyl was de-O-acetylated as for Ac NAG_1-β-F, and purified on Sephadex G15 before freeze drying.

Yield = 80 mg = 60% Mpt. = 303°-306°.

U.V. λ max = 323 nm (water)

R.F. = 0.58 n propanol/ammonia/water 6:3:2

(Rf NAG_4 = 0.45; Rf NAG_4-β- 3, 4 dnp = 0.65)

I.R. 3600 cm^{-1} - 3200 cm^{-1} (broad) O - H and N - H

No O acetates

1720 cm^{-1} lactone C = O

1665 cm^{-1} amide I

1660 cm^{-1} to 1600 cm^{-1} aromatics

1560 cm-1 amide II

2. 1. 6 The Synthesis of NAG$_3$-β- 2, 4 dinitro-5-acetamido-phenyl

2, 4-dinitro-5-acetamido phenol

m amino phenol was acetylated with acetic anhydride and sodium acetate, according to the method of Reverdin,[89] and Ibuta.[116]

m-acetamido phenyl acetate Mpt = 76-8° (lit.[89] 78°).

Mono-nitration of the diacetate was effected by the method of Meldola,[90] to give 4-nitro-3-acetamido -phenol, Mpt. 268° (lit.[90] 266°), and 2-nitro-5-acetamido-phenol, Mpt. 221° (lit.[90] 220°)

The two isomers, whose O acetate groups are lost during the first nitration, yield the same dinitro acetamido phenol on further nitration, namely 2, 4 dinitro-5-acetamido-phenol, Mpt. 167-9° (lit.[90] 168°)

Ac NAG$_3$-β-2, 4 dinitro-5-acetamido phenyl

1.1 g. of α-Ac NAG$_3$ Cl were dissolved in 60 ml. of acetone, and 0.48 g. of 2, 4 dinitro-5-acetamidophenyl, with 3.8 ml of 0.5N sodium hydroxide were added. The solution was kept at 18° for 20 hours, and the acetone removed on a Rotovap. A syrupy product was precipitated, which proved to be a mixture of two compounds when examined on T.L.C.

Rf_1 = 0.7

Rf_2 = 0.36

Solvent system 11% methanol/chloroform.

The mixture was separated by P.L.C., Silica Gel H.

The faster running spot proved to be the glycoside, which was recrystallised from methanol-chloroform and ether.

Yield = 10 mg = 0.9% Mpt = 221-2°

I.R. 3360 cm^{-1} N-H

1735 cm^{-1} O acetates

1660 cm^{-1} amide I

1545 cm^{-1} amide II

U.V. λmax 275 nm (methanol); free phenol = 285 nm; phenolate = 286 and 402 nm.

NAG_3-β-2, 4 dinitro-5-acetamido-phenyl.

$AcNAG_3$-β-2,4 -dinitro-5-acetamido-phenyl was de-O-acetylated as for Ac NAG_2-β-2, 4 dnp.

Yield = 3 mg; 40%

Rf acetate = 1 2:1:1 methanol, ethyl acetate, benzene

Rf product = 0.85

I.R. 3600-3200 cm^{-1} N-H and O-H

1660 cm^{-1} amide I

1550 cm^{-1} amide II

UV. λmax = 276 nm (water)

2. 1. 7 The synthesis of the β-fluorides of NAG_1, NAG_2 and NAG_4

The method used was a modification of that of Helferich[95], for the preparation of β-D-glucopyranosyl fluoride.

Ac NAG_1-β-F

4 g. of α-Ac NAG_1 Cl. Mpt. 127° prepared by the method of Horton[78] was mixed with 8 g. of sulphuric acid-dried silver monofluoride (ROC/RIC), and 15 ml. of dry acetonitrile were added. The flask was sealed and protected from the light, then shaken at room temperature for 48 hours.

The reaction was followed by T.L.C. 8% methanol-chloroform. Rf chloride = 0.7 Rf product 0.55. The reaction mixture was filtered through celite 535, and the acetonitrile removed on a Rotovap. The solid residue was dissolved in 15% methanol-chloroform and applied to a 3 cm. x 40 cm. column of silica, chromatrography grade, in order to adsorb dissolved silver salts. The column was eluted with this solvent system, and fractions containing fluoride evaporated to a small volume, about 15 ml. 10 ml. of ether was added, and crystallisation commenced. The product was recrystallised from methanol-chloroform, to give very highly crystalline white prisms.

Yield = 3.6 g; 95% Mpt. = 156.5°

P.M.R. $CDCl_3/CD_3OD$

7.9τ to 8.06τ	12H,	acetate methyls
6τ to 7τ	7H,	multiplet Ring protons
4.61τ	1H,	doublet of doublets. H_1
	JH_1H_2	= 6.8 c/s diaxial coupling
	JH_1F	= 53 c/s
3.85τ	N-H:	disappears on D_2O addition

The identity of the H_1 F coupling was confirmed by ^{19}F decoupling.

Irradiation of the low field half of the ^{19}F quartet, centred at 56.444554 MHz caused the lower half of the H_1 spectrum, at 4.17 to collapse to a singlet. Irradiation of the high field half of the ^{19}F quartet, centred at 56.444546 MHz, caused the higher half of the H_1 spectrum, at 5.05τ to collapse to a singlet. Irradiation of the complete ^{19}F spectrum caused the quartet to collapse to a singlet, at 4.61τ.

^{19}F N.M.R.

$\delta^{19}F$ = +5644.5c/s (TFA); doublet of doublets

JH_1F = 58.0 c/s

JH_2F = 13 c/s

Analysis:

Found: C 49.14 %; H 5.97 %; N 3.45 %; F 4.88 %

Calculated for $C_{14}H_{20}FNO_8$: C 48.14 %; H 5.77 %; N 4.01%; F 5.44 %

NAG_1-β-F

0.4 g. of dry Ac NAG_1-β-F was dissolved in 4 ml. of dry methanol and 4 ml. of dry chloroform in a dry round-bottomed flask. 0.3 ml. of 1 N sodium methoxide was added with shaking. After 1 minute at room temperature, the solution was evaporated on a Rotovap to 2 ml volume, not using a water bath, causing cooling of the solution. Trituration and the addition of a few drops of dry ether effected crystallisation of the product, which was filtered and washed with ether.

Yield = 0.2 g; 65% Mpt. = 199-201°.

From addition of methoxide to crystallisation of product took no more than two minutes.

I.R. 3600 cm^{-1} - 3200 cm^{-1} O-H and N-H

No O acetates

1660 cm^{-1} amide I

1565 cm^{-1} amide II

P.M.R. CD_3OD; spectrum run immediately after solution obtained

7.983τ singlet 3 H N acetate methyl

6τ to 7τ multiplet 7 H ring protons

4.82 τ doublet of doublets 1H

JH_1F = 54 c/s JH_1H_2 = 7.8 c/s

$(\alpha)_{25}^{578}$ = - 0.00024° (C.0·4, methanol)

^{19}F NMR $\delta^{19}F$ = + 5780 c/s (TFA); JH_1F = 11 c/s; JH_2F = 52 c/s

Analysis

Found: C 41.02%; H 6.04%; N 5.81%; F 8.89%

Calculated for $C_8 H_{14} FNO_5$: C 43.05%; H 6.32%; N 6.28%; F 8.51%

Ac NAG_2-β-F

α-Ac NAG_2 Cl was converted to the β-fluoride as for the synthesis of Ac NAG_1-β-F. The product was white, highly crystalline material.

Yield = 72% Mpt. = 210-211°.

P.M.R.

$CDCl_3/CD_3OD$

7.8 - 8.06 τ	21 H	acetate methyls	
5.8 - 7 τ	15 H	ring protons	
4.68 τ	1 H	carbon 1 proton;	doublet of doublets
		JH_1H_2 = 5.5 c/s	
		JH_1F = 57.0 c/s	

^{19}F NMR

δ = + 5610 c/s (TFA); doublet of doublets

JH_1F = 50.4 c/s JH_2F = 11.3 c/s

Rf. = 0.7 9% methanol-chloroform; Rf chloride = .65

Rf Ac NAG_2 = 0.48

Analysis

Found: C 49.67%; H 6.00%; N 3.88%; F 2.75%

Calculated for $C_{26} H_{37} FN_2 O_{15}$: C 49.21%; H 5.56%; N 4.415%; F 2.994%

NAG_2-β-F

Ac NAG -β-F was de-O-acetylated as for Ac NAG_1-β-F.

Yield = 53% Mpt. = 238-240°

I.R.

3600 cm^{-1} - 3200 cm^{-1} O-H and N-H

No O acetates

1660 cm^{-1} amide I

1565 cm^{-1} amide II

Analysis

Found: C 41.13%; H 5.92%; N 5.92%; F 2.28 %

Calculated for $C_{16}H_{27}FN_2O_{10}$: C 45.07%; H 6.38%; N 6.57%; F 4.46%

Ac NAG_4-β-F

2 g. of crude α-Ac NAG_4Cl and 2 g. of dry silver monofluoride (ROC/RIC) were mixed with 25 ml of dry acetonitrile in a 50 ml. round-bottomed flask. The flask was sealed and shielded from the light, and shaken for 30 hours. Examination of the reaction mixture by TLC showed that all chloride had been converted to fluoride, but some acetate was present from the crude starting material.

Rf fluoride = 0.65 Rf chloride = 0.7 Rf acetate = 0.6, in 11% methanol-chloroform. The mixture was filtered through celite 535, and silver salts removed by adsorption on silica. The product was purified by crystallising out the fluoride from 1:1 methanol chloroform. This was achieved by partial evaporation, and cooling of the solution, followed by several evaporations with methanol to yield crops of white crystals.

Yield = 0.4 g; 20% Mpt = 270-271°

P.M.R.

$CDCl_3/CD_3OD$

7.86τ to 8.06τ,	39H acetate methyls
5.7τ to 7τ	31H ring protons
4.6 τ	1H doublet of doublets
	JH_1H_2 = 6.0 c/s
	JH_1F = 40 c/s

The Anomeric proton could only be seen after 49 scans CAT. The lowest field O acetate, the anomeric acetate at 7.76 τ was not present, as expected.

Analysis

Found: C 49.13%; H 5.99%; N 3.66%; F 0.61%

Calculated for $C_{50}H_{71}FN_4O_{29}$: C 49.7%; H 5.89%; N 4.65%; F 1.57%

The analyses of the fluorides NAG_2-β-F and $AcNAG_4$-β-F indicate that these two compounds are unlikely to be pure; analyses of de-O-acetylated NAGs are notoriously inaccurate.[33]

2. 2. Kinetic Experimental

The majority of the kinetic runs using aryl glycosides were followed on a Cary 16 recording spectrophotometer, on line to a Digico Micro 16 P computer, with appropriate hardware.

The programs used were based on 'Mathchat', with Glasgow University extensions. The first-order rate constant and the initial slope programs, written by Dr. B. Capon, and the Michaelis Menten program given in the appendix, were based on the generalised least squares procedure of Wentworth[169] for obtaining the best fit of experimental data to a theoretical curve.

A few runs were performed on a Cary 14 recording spectrophotometer, in which case the data was logged and calculated on the KDF9 using appropriate Algol programs.

The cell compartment was thermostatted at the appropriate temperature, and the kinetic procedure developed was generally: equilibration of buffer in 10 mm cells, followed by the addition of stock substrate solution, stock inhibitor solution, if any, and finally stock enzyme solution to a total of 2.50 ml.

The stock solutions of NAG_4-β-3, 4 dnp were generally 2×10^{-3} M; 2.14 mg./ml; and stock enzyme solution was 2.5×10^{-4} M; 3.59 mg./ml. Accuracy of weighings was checked by measuring absorbances of diluted samples at 283 nm and 280 nm, respectively, using the appropriate extinction coefficients.

When reducing sugars were used as inhibitors, they were allowed to mutarotate to equilibrium during 24 hours. The concentration of paranitrophenyl glycosides, when used as inhibitors

and substrates was checked by measuring the absorbance at 295 nm, ϵ= 10,000.

If the concentration of 3, 4 dinitrophenyl glycoside substrate was taken above 1 x 10^{-4} M, then allowance was made for their spontaneous hydrolysis.

The extinction coefficient of 3, 4 dinitrophenol at 400 nm was determined by dilution of a 5 x 10^{-3} M stock solution of the phenol, in water, into the buffer, and measuring its absorbance manually, on the Cary 16.

Rapid initial burst experiments followed the same general method, except that care was exercised in filtering the more concentrated stock enzyme solutions, and allowing for dilution effects caused by the larger amounts of solution added if the substrate had any initial absorbance. This was the case with paranitrophenyl glycosides, and with 3, 4 dinitrophenyl glycosides at pH's less than 4.

pH's were measured on a Radiometer pH meter 26, standardised using E I L standard buffers, at 25°c. Initially, all standardisations and measurements were carried out at 40°c, but this did not appear to alter values by more than 0.01 pH units.

The hydrolysis of the β fluorides and of the lactones were followed by titrating the released acids with 0·1ON sodium hydroxide on a pH stat:

Radiometer titrator type TTT1c;

Radiometer titrigraph type SBR2c

and Radiometer Autoburette type ABU12.

The samples were weighed out to give 4/5 of full scale uptake

of alkali at infinity (0.02 m. moles of compound), the boiled-out distilled water brought to the correct pH after standardising the meter, then the samples dissolved in this water, with rapid shaking and replacement of the solution in the titrator

3. RESULTS

TABLE 1

Lysozyme catalysed hydrolysis of NAG_2 -β-2,4 dnp.

37°. pH 5.1; I = 0.1 acetate; Cary 14; 410 nm ε = 9600;

2 mm. cells

$(E)_o = 5 \times 10^{-4}$ m/l; Samples weighed into cells.

Run	(S) m/l.	Initial rate m/l/s (enzyme only)
20	3×10^{-3}	4.05×10^{-7}
21	5×10^{-3}	6.1×10^{-7}
22	6×10^{-3}	6.2×10^{-7}
23	7.5×10^{-3}	8.0×10^{-7}
24	9×10^{-3}	7.1×10^{-7}
25	1×10^{-2}	7.0×10^{-7}
26	2×10^{-2}	7.0×10^{-7}

Km = 2.1×10^{-3} m/l

Vmax = 6.8×10^{-7} m/l/s k cat = $1.3 \times 10^{-3} s^{-1}$

k spont = 1.4×10^{-4} s^{-1}

TABLE 2

Lysozyme catalysed hydrolysis of NAG_3 -β- 2,4 dnp

37°; pH 5.1 I = 0.1 acetate. 410 nm ϵ = 9600.2 mm cells.

$(E)_o = 1 \times 10^{-4}$ m/l; Stock substrate = 1×10^{-2} m/l

Run	(S) m/l.	Initial rate m/l/s (enzyme only)
50	1×10^{-4}	9.27×10^{-8}
51	2×10^{-4}	8.86×10^{-8}
52	3×10^{-4}	9.32×10^{-8}
53	4×10^{-4}	10.42×10^{-8}
54	5×10^{-4}	13.78×10^{-8}
55	7×10^{-4}	15.7×10^{-8}
56	1×10^{-3}	15.11×10^{-8}
57	2×10^{-3}	13.18×10^{-8}

Km = 1.2×10^{-4} m/l/s

Vmax = 1.5×10^{-7} m/l/s

k cat = 1.5×10^{-3} s^{-1}

TABLE 3

Lysozyme catalysed hydrolysis of NAG_3 -β- paranitrophenyl

Second order conditions. Cary 16

$(E)_o = 2.5 \times 10^{-4}$ m/l 40°, pH 5.08 citrate. 350 nm $\Delta\varepsilon = 2680$

Stock substrate = 5×10^{-4} m/l

Run	(S) m/l	Initial rate m/l/s
191	2.5×10^{-5}	1.138×10^{-9}
192	1×10^{-5}	5.58×10^{-10}
193	5×10^{-6}	2.075×10^{-10}
194	2.5×10^{-6}	1.055×10^{-10}

Slope of graph of rate vs[S] = $\frac{k\ cat}{Km\ (app.)}$. $(E)_o = 4.59 \times 10^{-5}$

$\frac{k\ cat}{Km} = 0.196$ $lm^{-1}s^{-1}$

TABLE 4

Lysozyme catalysed hydrolysis of NAG_4-β-paranitrophenyl Cary 16

40°, pH 5.08 citrate. 350 nm. $\Delta\varepsilon = 2680$

$(E_o) = 1 \times 10^{-5}$ m/l Stock substrate = 5×10^{-3} m/l

Run	(S) m/l	Initial rate m/l/s
196	1×10^{-3}	2.695×10^{-9}
197	7.5×10^{-4}	2.57×10^{-9}
198	5×10^{-4}	2.065×10^{-9}
199	4×10^{-4}	1.95×10^{-9}
200	2.5×10^{-4}	1.55×10^{-9}
201	1×10^{-4}	7.02×10^{-10}

			St.Dev.	
Km	$= 4.49 \times 10^{-4}$	m/l	1.07×10^{-4}	m/l
Vmax	$= 4.02 \times 10^{-9}$	m/ls	$.53 \times 10^{-9}$	m/l/s

k cat = 4.02×10^{-4} s^{-1}

TABLE 5

Lysozyme catalysed hydrolysis of NAG_2 -β- paranitrophenyl

40°. pH 5.08 citrate 350 nm $\Delta\varepsilon = 2680$ Cary 16

$(E)_o = 2.5 \times 10^{-4}$ m/l Stock substrate = 1×10^{-2} m/l

Run	(S) m/l	Initial rate m/l/s	Induction period
218	2.5×10^{-4}	2.67×10^{-10}	20 minutes
219	1×10^{-4}	8.8×10^{-11}	30 minutes
221	5×10^{-5}	5.22×10^{-11}	40 minutes
222	2×10^{-5}	2.07×10^{-11}	80 minutes

$\frac{k\ cat}{Km} = 3.9 \times 10^{-3}$ $lm^{-1}s^{-1}$

All these curves exhibited an induction period.

TABLE 6

A direct comparison of the specificities of NAG_2-, NAG_3-, NAG_4 and NAG5 - -paranitrophenyl glycosides.

40°. pH 5.08 citrate, 350 nm $\Delta\varepsilon = 2680$.

$(E)_o = 2.5 \times 10^{-4}$ m/l $(S)_o = 1 \times 10^{-4}$ m/l

Run	Substrate m/l	Initial rate m/l/s	Induction period
224	NAG_3-β-pnp	1.24×10^{-9}	nil
225	NAG_4-β-pnp	1.02×10^{-8}	nil
226	NAG_2-β-pnp	8.8×10^{-11}	190 minutes
223	NAG_5-β-pnp	1.9×10^{-10}	nil

TABLE 7

Lysozyme catalysed hydrolysis of NAG_4-β-3,4 dnp.

40°; pH 5.08 citrate; Boehringer enzyme; batch 7371222, Oct. 1973

400 nm. $\varepsilon = 5690$. Cary 16.

$(E)_o = 5 \times 10^{-7}$ m/l

Run	(S) m/l	Initial rate m/l/s
293	5×10^{-6}	2.815×10^{-10}
294	6×10^{-6}	4.14×10^{-10}
295	7×10^{-6}	4.13×10^{-10}
296	8×10^{-6}	4.46×10^{-10}
297	9×10^{-6}	4.53×10^{-10}
298	1×10^{-5}	5.06×10^{-10}
299	1.1×10^{-5}	5.42×10^{-10}
302	1.4×10^{-5}	6.29×10^{-10}
303	1.5×10^{-5}	6.0×10^{-10}
304	1.75×10^{-5}	6.6×10^{-10}
305	2×10^{-5}	7.08×10^{-10}
306	3×10^{-5}	6.74×10^{-10}
307	4×10^{-5}	6.65×10^{-10}
308	5×10^{-5}	6.65×10^{-10}
309	6×10^{-5}	6.74×10^{-10}

Km $= 7.1 \times 10^{-6}$ m/l

Vmax $= 8.3 \times 10^{-10}$ m/l/s

k cat $= 1.65 \times 10^{-3}$ s^{-1}

TABLE 8

Lysozyme catalysed hydrolysis of NAG_4-β-3,4 dnp.

40^{o} pH 6.08 citrate Cary 16 on-line

400 nm. ε = 11,450

$(E)_o = 2.05 \times 10^{-6}$ m/l

Run	(S) m/l	Initial rate m/l/s	St.dev %
368	2×10^{-5}	1.595×10^{-9}	1.02
369	2.5×10^{-5}	1.550×10^{-9}	2.67
370	3×10^{-5}	1.796×10^{-9}	1.46
371	3.5×10^{-5}	1.797×10^{-9}	2.05
372	4×10^{-5}	1.941×10^{-9}	1.77
373	5×10^{-5}	1.931×10^{-9}	1.11
374	6×10^{-5}	2.138×10^{-9}	2.9
375	7×10^{-5}	1.999×10^{-9}	1.49
376	8×10^{-5}	2.143×10^{-9}	1.26
377	9×10^{-5}	2.173×10^{-9}	1.98

Km = 9.92×10^{-6} m/l

Vmax = 2.36×10^{-9} m/l/s

k cat = 1.15×10^{-3} s^{-1}

TABLE 9

Lysozyme catalysed hydrolysis of NAG_4-β-3,4 dnp

40°. pH 7.03 I = 0.1 phosphate. ε = 13,050; 400 nm. Cary 16 on line

$(E)_o = 2.1 \times 10^{-6}$ m/l.

Run	(S) m/l	Initial rate m/l/s	St.dev %
395	2×10^{-5}	4.07×10^{-10}	19.1
385	3×10^{-5}	5.662×10^{-10}	6.58
386	4×10^{-5}	6.80×10^{-10}	1.02
389	7×10^{-5}	7.546×10^{-10}	2.27
390	8×10^{-5}	7.804×10^{-10}	4.68
391	9×10^{-5}	7.329×10^{-10}	4.56
392	1×10^{-4}	7.132×10^{-10}	5.25
393	1.2×10^{-4}	7.921×10^{-10}	6.83

Km = 1.113×10^{-5} m/l $\pm$ $.22 \times 10^{-5}$

Vmax = 8.63×10^{-10} m/l/s $\pm$ $.33 \times 10^{-10}$

k cat = 4.11×10^{-4} s^{-1}

TABLE 10

Lysozyme catalysed hydrolysis of NAG_4-β- 3,4 dnp.

40° pH 4.02 citrate; ε = 1025. 400 nm.

$(E)_o = 2 \times 10^{-6}$ m/l Cary 16 on line.

Run	(S) m/l	Initial rate m/l/s	St.dev.%
420	2×10^{-5}	1.225×10^{-9}	8.29
421	3×10^{-5}	2.284×10^{-9}	7.46
422	4×10^{-5}	2.516×10^{-9}	6.84
423	5×10^{-5}	2.432×10^{-9}	6.41
425	7×10^{-5}	2.603×10^{-9}	5.20
428	1×10^{-4}	2.700×10^{-9}	4.81
430	2.5×10^{-5}	1.167×10^{-9}	9.29

Km = 4.9×10^{-5} m/l

Vmax = 3.28×10^{-9} m/l/s

k cat = 1.64×10^{-3} s^{-1}

TABLE 11

Lysozyme catalysed hydrolysis of NAG_4-β-3,4 dnp.

40°. pH = 5.71 I = 0.1 acetate buffer ϵ = 10380. 400 nm

$(E)_o = 5.1 \times 10^{-7}$ m/l Cary 16 on line.

Run	(S) m/l	Initial rate	St.dev.%
525	5×10^{-6}	3.526×10^{-10}	3.06
527	7×10^{-6}	4.125×10^{-10}	2.76
528	8×10^{-6}	4.859×10^{-10}	2.39
531	1.2×10^{-5}	4.754×10^{-10}	2.07
533	1.6×10^{-5}	5.080×10^{-10}	2.10
534	1.8×10^{-5}	4.787×10^{-10}	1.91
535	2×10^{-5}	5.081×10^{-10}	1.96
536	3×10^{-5}	5.058×10^{-10}	2.52
537	4×10^{-5}	5.771×10^{-10}	2.45
539	6×10^{-5}	7.064×10^{-10}	1.74
540	7×10^{-5}	7.614×10^{-10}	2.76

Km = $4.78 \times 10^{-6} \pm 1.39 \times 10^{-6}$ m/l

Vmax = $6.69 \times 10^{-10} \pm 0.46 \times 10^{-10}$ m/l/s

k cat = $1.31 \times 10^{-3} \pm 0.09 \times 10^{-3}$ s^{-1}

TABLE 12

Lysozyme catalysed hydrolysis of NAG_4-β-3,4 dnp

40^{o}. pH 5.35. I = 0.10 acetate buffer ε = 7900. 400 nm

$(E)_o = 5.1 \times 10^{-7}$ m/l Cary 16 on line

Run	(S) m/l	Initial rate m/l/s	St.dev. %
541	5×10^{-6}	5.192×10^{-10}	2.93
542	6×10^{-6}	3.311×10^{-10}	5.08
543	7×10^{-6}	5.063×10^{-10}	5.70
544	8×10^{-6}	4.655×10^{-10}	2.93
545	9×10^{-6}	5.803×10^{-10}	2.93
547	1.2×10^{-5}	5.93×10^{-10}	2.26
549	1.6×10^{-5}	5.76×10^{-10}	2.13
550	1.8×10^{-5}	6.023×10^{-10}	1.93
551	2×10^{-5}	6.748×10^{-10}	3.54
552	3×10^{-5}	6.252×10^{-10}	4.62
553	4×10^{-5}	7.044×10^{-10}	3.55
554	5×10^{-5}	7.388×10^{-10}	3.58

Km = $5.41 \times 10^{-6} \pm 1.45 \times 10^{-6}$ m/l

Vmax = $8.03 \times 10^{-10} \pm 0.62 \times 10^{-10}$ m/l/s

k cat = $1.58 \times 10^{-3} \pm 0.12 \times 10^{-3}$ s^{-1}

TABLE 13

Lysozyme catalysed hydrolysis of NAG_4-β-3,4 dnp.

40^{o}. pH = 5.19. I = 0.1 acetate buffer. ϵ = 6760. 400 nm.

$(E)_o = 5.1 \times 10^{-7}$ m/l Cary 16 on line.

Run	(S) m/l	Initial rate m/l/s	St.dev. %
557	5×10^{-6}	2.658×10^{-10}	9.43
558	6×10^{-6}	4.043×10^{-10}	5.62
559	7×10^{-6}	5.866×10^{-10}	3.19
560	8×10^{-6}	6.547×10^{-10}	4.26
561	9×10^{-6}	6.357×10^{-10}	2.63
563	1.2×10^{-5}	6.479×10^{-10}	2.75
564	1.4×10^{-5}	6.947×10^{-10}	2.63
566	1.8×10^{-5}	6.712×10^{-10}	2.88
567	2×10^{-5}	6.593×10^{-10}	2.85
568	3×10^{-5}	7.09×10^{-10}	4.46
569	4×10^{-5}	7.241×10^{-10}	3.47
571	6×10^{-5}	8.386×10^{-10}	3.72

Km = $4.45 \times 10^{-6} \pm 1.4 \times 10^{-6}$ m/l

Vmax = $8.6 \times 10^{-10} \pm 0.75 \times 10^{-10}$ m/l/s

k cat = $1.69 \times 10^{-3} \pm 0.15 \times 10^{-3}$ s^{-1}

TABLE 14

Lysozyme catalysed hydrolysis of NAG_4 -β-3,4 dnp

40° pH 5.08. I = 0.1 acetate buffer. ϵ = 5820. 400 nm.

$(E)_o = 5.0 \times 10^{-7}$ m/l Cary 16 on line.

Run	(S) m/l	Initial rate m/l/s	St. dev.%
589	5×10^{-6}	1.342×10^{-10}	46.6
590	6×10^{-6}	1.645×10^{-10}	20.6
592	8×10^{-6}	4.02×10^{-10}	6.77
593	9×10^{-6}	5.307×10^{-10}	4.54
594	1×10^{-5}	6.954×10^{-10}	3.37
595	1.2×10^{-5}	6.581×10^{-10}	4.39
597	1.6×10^{-5}	6.654×10^{-10}	3.56
600	3×10^{-5}	8.172×10^{-10}	4.38
601	4×10^{-5}	8.281×10^{-10}	2.83
602	5×10^{-5}	8.283×10^{-10}	4.70
603	6×10^{-5}	9.335×10^{-10}	2.69

Km = $9.83 \times 10^{-6} \pm 2.9 \times 10^{-6}$ m/l

Vmax = $1.07 \times 10^{-9} \pm 0.11 \times 10^{-9}$ m/l/s

k cat = $2.014 \times 10^{-3} \pm 0.22 \times 10^{-3}$ s^{-1}

TABLE 15

Lysozyme catalysed hydrolysis of NAG_4 -β-3,4 dnp

40^{o}. pH = 4.88. I = 0.1 acetate buffer ε = 4440. 400 nm

$(E)_o = 4.94 \times 10^{-7}$ m/l. Cary 16 on line.

Run	(S) m/l	Initial rate m/l/s	St. dev. %
605	6×10^{-6}	3.202×10^{-10}	8.5
606	7×10^{-6}	3.669×10^{-10}	6.79
607	8×10^{-6}	5.858×10^{-10}	4.78
609	1×10^{-5}	6.762×10^{-10}	3.46
610	1.2×10^{-5}	8.20×10^{-10}	3.99
612	1.6×10^{-5}	8.693×10^{-10}	3.94
620	3×10^{-5}	8.304×10^{-10}	5.41
617	5×10^{-5}	9.038×10^{-10}	3.02
619	7×10^{-5}	8.758×10^{-10}	4.74

$Km = 7.87 \times 10^{-6} \pm 2.75 \times 10^{-6}$ m/l

$Vmax = 1.08 \times 10^{-9} \pm 0.14 \times 10^{-9}$ m/l/s

$k\ cat = 2.19 \times 10^{-3} \pm 0.28 \times 10^{-3}\ s^{-1}$

TABLE 16

Lysozyme catalysed hydrolysis of NAG_4 -β-3,4 dnp

40°. pH = 4.40 I = 0.10 acetate buffer. ϵ = 2010 400 nm

$(E)_o = 9.88 \times 10^{-7}$ m/l Cary 16 on line

Run	(S) m/l	Initial rate m/l/s	St. dev. %
640	1×10^{-5}	8.52×10^{-10}	10.3
642	1.4×10^{-5}	1.153×10^{-9}	6.98
643	1.6×10^{-5}	1.012×10^{-9}	9.09
644	1.8×10^{-5}	1.118×10^{-9}	6.51
645	2×10^{-5}	1.229×10^{-9}	6.42
647	4×10^{-5}	1.182×10^{-9}	5.22
649	6×10^{-5}	1.464×10^{-9}	4.63
650	7×10^{-5}	1.449×10^{-9}	4.34

$Km = 7.04 \times 10^{-6} \pm 1.99 \times 10^{-6}$ m/l

$Vmax = 1.56 \times 10^{-9} \pm 0.09 \times 10^{-9}$ m/l/s.

$k\ cat = 1.58 \times 10^{-3} \pm .09 \times 10^{-3} s^{-1}$

TABLE 17

Lysozyme catalysed hydrolysis of NAG_4-β-3,4 dnp

40^{o} pH = 5.04 I = 0.1 acetate ε = 5560. 400 nm

$(E)_o = 5 \times 10^{-7}$ m/l Cary 16 on line

Run	(S) m/l	Initial rate m/l/s	St.dev.%
472	5×10^{-6}	3.691×10^{-10}	11.33
473	6×10^{-6}	4.568×10^{-10}	4.85
474	7×10^{-6}	4.275×10^{-10}	6.38
475	8×10^{-6}	4.787×10^{-10}	5.03
476	9×10^{-6}	5.471×10^{-10}	4.87
477	1×10^{-5}	5.161×10^{-10}	5.14
479	1.4×10^{-5}	6.991×10^{-10}	3.56
480	1.6×10^{-5}	7.003×10^{-10}	6.02
482	2×10^{-5}	8.710×10^{-10}	3.45
483	3×10^{-5}	9.148×10^{-10}	3.63
485	5×10^{-5}	9.798×10^{-10}	2.98
486	6×10^{-5}	9.447×10^{-10}	4.24

$Km = 1.11 \times 10^{-5} \pm .09 \times 10^{-5}$ m/l

$Vmax = 1.21 \times 10^{-9} \pm .04 \times 10^{-9}$ m/l/s

$k\ cat = 2.14 \times 10^{-3} \pm .01 \times 10^{-3}\ s^{-1}$

TABLE 18

Lysozyme catalysed hydrolysis of NAG_4-β-3,4 dnp

40^{o}. pH = 5.03 I = 0.10 acetate. ε = 5660. 400 nm

$(E)_o = 4.47 \times 10^{-7}$ m/l. Cary 16 on line

Run	(S) m/l	Initial rate m/l/s	St. dev. %
505	5×10^{-6}	3.458×10^{-10}	9.21
506	6×10^{-6}	3.796×10^{-10}	5.08
507	7×10^{-6}	3.952×10^{-10}	4.35
508	8×10^{-6}	5.525×10^{-10}	2.88
509	9×10^{-6}	7.662×10^{-10}	2.24
521	1.2×10^{-5}	6.235×10^{-10}	3.38
524	2×10^{-5}	7.687×10^{-10}	1.40
517	4×10^{-5}	8.58×10^{-10}	3.30
518	5×10^{-5}	8.82×10^{-10}	2.75

Km $= 7.61 \times 10^{-6} \pm 2.75 \times 10^{-6}$ m/l

Vmax $= 1.04 \times 10^{-9} \pm 0.15 \times 10^{-9}$ m/l/s

k cat $= 2.33 \times 10^{-3} \pm 0.03 \times 10^{-3} s^{-1}$.

Table 18 (b)

The lysozyme catalysed hydrolysis of NAG_4-β-3, 4 dnp

40° pH = 5.03 I = 0.1 acetate buffer

Cary 16 . 400 nm ε = 6040

(methanol) = 2.0 m/l; 202 μl in 2.5 ml

$(E)_o = 1.0 \times 10^{-6}$ m/l

Run	(S) m/l	Initial rate m/l/s	St. dev. %
731	1×10^{-5}	1.037×10^{-9}	1.74
732	1.2×10^{-5}	1.074×10^{-9}	2.17
733	1.4×10^{-5}	1.316×10^{-9}	1.48
734	1.6×10^{-5}	1.239×10^{-9}	1.84
735	1.8×10^{-5}	1.152×10^{-9}	2.38
736	2×10^{-5}	1.202×10^{-9}	2.04
737	3×10^{-5}	1.541×10^{-9}	1.82
738	4×10^{-5}	1.528×10^{-9}	1.73
739	5×10^{-5}	1.429×10^{-9}	1.73
740	6×10^{-5}	1.611×10^{-9}	1.64
741	7×10^{-5}	1.684×10^{-9}	2.32
742	8×10^{-5}	1.650×10^{-9}	1.51
743	1.0×10^{-4}	1.948×10^{-9}	1.37

Km = $8.21 \times 10^{-6} \pm 1.47 \times 10^{-6}$ m/l

V max = $1.863 \times 10^{-9} \pm 0.08 \times 10^{-9}$ m/l/s

k cat = 1.86×10^{-3} s^{-1}

TABLE 19

Lysozyme catalysed hydrolysis of NAG_4-β-3,4 dnp

40°. pH = 5.03 I = 0.1; (acetate) = 0.02M; (KCl) = 0.08 M.

ε = 5660. 400 nm. Cary 16 on line

$(E)_o = 4.47 \times 10^{-7}$ m/l

Run	(S) m/l	Initial rate m/l/s	St. Dev. %
488	5×10^{-6}	3.146×10^{-10}	5.59
490	7×10^{-6}	4.51×10^{-10}	4.0
491	8×10^{-6}	5.152×10^{-10}	3.64
492	9×10^{-6}	4.119×10^{-10}	3.76
493	1×10^{-5}	4.522×10^{-10}	5.28
494	1.2×10^{-5}	5.589×10^{-10}	2.59
495	1.4×10^{-5}	6.749×10^{-10}	2.47
497	1.8×10^{-5}	7.527×10^{-10}	2.0
499	3×10^{-5}	7.774×10^{-10}	3.39
500	4×10^{-5}	7.576×10^{-10}	2.22
501	5×10^{-5}	8.129×10^{-10}	3.13
502	6×10^{-5}	8.846×10^{-10}	3.28
503	7×10^{-5}	7.47×10^{-10}	2.27

Km = $7.55 \times 10^{-6} \pm 1.56 \times 10^{-6}$ m/l

Vmax = $9.23 \times 10^{-10} \pm .60 \times 10^{-10}$ m/l/s

k cat = 2.07×10^{-3} s^{-1}.

TABLE 20

Lysozyme catalysed hydrolysis of NAG_4-β-3,4 dnp

40°. pH = 5.04 I = 0.02 acetate buffer ε = 5660. 400 nm

Cary 16 on line

$(E)_o = 9 \times 10^{-7}$ m/l

Run	(S) m/l	Initial rate m/l/s	St. Dev. %
670	1×10^{-5}	0.997×10^{-9}	1.82
671	1.2×10^{-5}	0.969×10^{-9}	1.65
672	1.4×10^{-5}	1.116×10^{-9}	1.33
673	1.6×10^{-5}	1.115×10^{-9}	1.57
674	1.8×10^{-5}	1.115×10^{-9}	1.83
669	2×10^{-5}	1.133×10^{-9}	1.55
675	3×10^{-5}	1.30×10^{-9}	1.38
676	5×10^{-5}	1.475×10^{-9}	1.33
677	7×10^{-5}	1.447×10^{-9}	2.06
678	8×10^{-5}	1.671×10^{-9}	1.21
679	1×10^{-4}	1.60×10^{-9}	1.36

Km = $8.90 \times 10^{-6} \pm 0.32 \times 10^{-6}$ r/l

Vmax = $1.74 \times 10^{-9} \pm 0.02 \times 10^{-9}$ m/l/s

k cat = $1.93 \times 10^{-3} \pm 0.02 \times 10^{-3}$ s^{-1}

TABLE 21

Lysozyme catalysed hydrolysis of NAG_4-β-3,4 d n p, with α - methyl NAG as inhibitor.

40° pH = 5.08 citrate.

$(E)_o = 1.2 \times 10^{-6}$ m/l $\quad (I) = 8 \times 10^{-2}$ m/l.

Run	(S) m/l	Initial rate m/l/s	St. Dev. %
336	1×10^{-5}	5.507×10^{-10}	4.58
347	1.1×10^{-5}	5.679×10^{-10}	3.86
338	1.5×10^{-5}	7.486×10^{-10}	2.21
339	1.75×10^{-5}	9.261×10^{-10}	2.34
340	2×10^{-5}	9.841×10^{-10}	0.8
341	2.5×10^{-5}	1.166×10^{-9}	2.09
343	3.5×10^{-5}	1.387×10^{-9}	2.12
352	5.5×10^{-5}	1.469×10^{-9}	1.83
351	8×10^{-5}	1.469×10^{-9}	1.17

Km $= 2.25 \times 10^{-5} \pm 0.1 \times 10^{-6}$ m/l

Vmax $= 2.04 \times 10^{-9} \pm 0.05 \times 10^{-9}$ m/l/s

k cat $= 1.7 \times 10^{-3}$ s^{-1}.

TABLE 22

Lysozyme catalysed hydrolysis of NAG_4-β- 3,4 dnp, with NAG_3 as inhibitor.

40^{o}. pH = 5.03 I = 0.1 acetate buffer

ε = 5660 400 nm

$(E)_o = 1 \times 10^{-6}$ m/l (I) = 4×10^{-5} m/l

Run	(S) m/l	Initial rate m/l/s	St. Dev. %
718	1.2×10^{-5}	6.75×10^{-10}	3.75
719	1.4×10^{-5}	6.35×10^{-10}	4.21
720	1.6×10^{-5}	9.49×10^{-10}	3.65
721	1.8×10^{-5}	7.742×10^{-10}	4.61
722	2×10^{-5}	8.928×10^{-10}	5.08
724	5×10^{-5}	1.324×10^{-9}	2.74
725	5×10^{-5}	1.373×10^{-9}	2.60
726	6×10^{-5}	1.328×10^{-9}	4.83
727	7×10^{-5}	1.353×10^{-9}	3.16
728	8×10^{-5}	1.59×10^{-9}	6.59
730	1×10^{-4}	1.498×10^{-9}	7.62

Km = $2.151 \times 10^{-5} \pm 0.51 \times 10^{-5}$ m/l

Vmax = $1.90 \times 10^{-9} \pm 0.19 \times 10^{-9}$ m/l/s

k cat = $1.90 \times 10^{-3} \pm 0.19 \times 10^{-9}$ s^{-1}.

TABLE 23

Lysozyme catalysed hydrolysis of NAG_4 -β- 3,4 dnp with NAG_3-β- paranitrophenyl as inhibitor.

40°. pH 5.03 I = 0.1 acetate buffer. ε = 5660. 400 nm

$(E)_o = 1 \times 10^{-6}$ m/l (I) = 2.65×10^{-5} m/l.

Run	(S) m/l	Initial rate m/l/s	St.Dev. %
703	1×10^{-5}	6.749×10^{-10}	5.83
704	1.2×10^{-5}	7.146×10^{-10}	4.16
707	1.8×10^{-5}	9.227×10^{-10}	3.01
708	2×10^{-5}	8.249×10^{-10}	2.64
709	3×10^{-5}	1.264×10^{-9}	2.44
710	4×10^{-5}	1.286×10^{-9}	1.88
711	5×10^{-5}	1.364×10^{-9}	2.07
714	8×10^{-5}	1.554×10^{-9}	2.34
715	9×10^{-5}	1.526×10^{-9}	2.10
716	1×10^{-4}	1.798×10^{-9}	2.36

Km = $2.34 \times 10^{-5} \pm .38 \times 10^{-5}$ m/l

Vmax = $2.03 \times 10^{-9} \pm .13 \times 10^{-9}$ m/l/s

k cat = $2.03 \times 10^{-3} \pm .13 \times 10^{-3}$ s^{-1}

TABLE 24

Lysozyme catalysed hydrolysis of NAG_4-β-3,4 dnp, with NAG_4 as inhibitor.

40^{o} pH = 5.03 I = 0.1 acetate buffer. ϵ = 5660. 400 nm

$(E)_o = 9 \times 10^{-7}$ m/l $(I) = 2 \times 10^{-5}$ m/l

Run	(S) m/l	Initial rate m/l/s	St.Dev. %
744	1×10^{-5}	3.603×10^{-10}	6.11
745	1.2×10^{-5}	5.114×10^{-10}	4.81
760	1.6×10^{-5}	7.323×10^{-10}	12.3
758	1.8×10^{-5}	7.453×10^{-10}	5.91
749	2×10^{-5}	8.441×10^{-10}	3.15
750	3×10^{-5}	1.179×10^{-9}	1.95
751	4×10^{-5}	1.141×10^{-9}	1.95
752	5×10^{-5}	1.445×10^{-9}	1.94
753	6×10^{-5}	1.119×10^{-9}	3.38
754	7×10^{-5}	1.168×10^{-9}	3.03
759	8×10^{-5}	1.376×10^{-9}	3.93
757	1×10^{-4}	1.498×10^{-9}	2.26

Km = $2.81 \times 10^{-5} \pm .81 \times 10^{-5}$ m/l

Vmax = $1.94 \times 10^{-9} \pm .24 \times 10^{-9}$ m/l/s

k cat = $2.15 \times 10^{-3} \pm .21 \times 10^{-3}$ s^{-1}

TABLE 25

Lysozyme catalysed hydrolysis of NAG_4-β-3,4 dnp with NAG_4-β-paranitrophenyl as inhibitor

40^{o}. pH = 5.03 I = 0.1 acetate ε = 5660 400 nm.

$(E)_o = 8 \times 10^{-7}$ m/l $(I) = 8.2 \times 10^{-6}$ m/l

Run	(S) m/l	Initial rate m/l/s	St. Dev.%
848	1×10^{-5}	4.476×10^{-10}	5.60
849	1.2×10^{-5}	4.428×10^{-10}	5.76
850	1.4×10^{-5}	5.29×10^{-10}	7.72
851	1.6×10^{-5}	5.474×10^{-10}	4.07
853	2×10^{-5}	6.142×10^{-10}	3.04
854	3×10^{-5}	7.335×10^{-10}	4.65
855	4×10^{-5}	8.612×10^{-10}	3.26
857	6×10^{-5}	9.602×10^{-10}	2.94
858	7×10^{-5}	1.035×10^{-9}	2.35
859	8×10^{-5}	9.725×10^{-10}	5.76
861	1×10^{-4}	1.09×10^{-9}	3.64
862	1.2×10^{-4}	1.00×10^{-9}	2.88

Km = $2.13 \times 10^{-5} \pm 0.17 \times 10^{-5}$ m/l

Vmax = $1.30 \times 10^{-9} \pm 0.03 \times 10^{-9}$ m/l/s

k cat = $1.63 \times 10^{-3} \pm 0.004$ s^{-1}

TABLE 26

Lysozyme catalysed hydrolysis of NAG_4-β-3,4 dnp with NAG_2 as inhibitor.

40^{o}. pH = 5.08 I = 0.1 acetate ε = 5820 400 nm.

$(E)_o = 1 \times 10^{-6}$ m/l $(I) = 2 \times 10^{-4}$ m/l

Run	(S) m/l	Initial rate m/l/s	St.Dev.%
889	1.8×10^{-5}	1.091×10^{-9}	2.38
890	2×10^{-5}	0.931×10^{-9}	3.35
891	3×10^{-5}	1.23×10^{-9}	1.98
892	4×10^{-5}	1.502×10^{-9}	2.06
894	6×10^{-5}	1.401×10^{-9}	1.88
895	7×10^{-5}	1.641×10^{-9}	1.65
897	9×10^{-5}	1.56×10^{-9}	1.43
898	1×10^{-4}	1.31×10^{-9}	1.54

Km = $1.06 \times 10^{-5} \pm 0.46 \times 10^{-5}$ m/l

Vmax = $1.68 \times 10^{-9} \pm 0.15 \times 10^{-9}$ m/l/s

k cat = $1.68 \times 10^{-3} \pm 0.15 \times 10^{-3}$ s^{-1}

TABLE 27

Lysozyme catalysed hydrolysis of NAG_4-β-3,4 dnp, with NAG_2-β- paranitrophenyl as inhibitor.

40^{o}. pH 5.08 I = 0.1 acetate buffer $\varepsilon = .5820$ 400 nm

$(E)_o = 1 \times 10^{-6}$ m/l $(I) = 3.72 \times 10^{-4}$ m/l

Run	(S) m/l	Initial rate m/l/s	St. Dev.%
1061	1.2×10^{-5}	8.88×10^{-10}	3.83
1062	1.6×10^{-5}	8.488×10^{-10}	9.27
1063	1.8×10^{-5}	9.39×10^{-10}	10.6
1065	2×10^{-5}	1.011×10^{-9}	3.49
1066	3×10^{-5}	1.088×10^{-9}	4.52
1067	4×10^{-5}	1.472×10^{-9}	4.04
1068	5×10^{-5}	1.631×10^{-9}	4.10
1071	8×10^{-5}	1.335×10^{-9}	5.60
1075	1.2×10^{-4}	1.737×10^{-9}	4.62

$Km = 1.53 \times 10^{-5} \pm .38 \times 10^{-5}$ m/l

$Vmax = 1.86 \times 10^{-9} \pm .17 \times 10^{-9}$ m/l/s

$k\ cat = 1.86 \times 10^{-3} \pm .17 \times 10^{-3}\ s^{-1}$

TABLE 28

Human milk lysozyme (Koch Light) catalysed hydrolysis of NAG_4 -β- 3,4 dnp

40^{o}. pH = 5.08 I = 0.1 acetate buffer

$(E)_o$ = 8.6×10^{-7} m/l by U.V.; 3.20 mg/ml 10µl.

Run	(S) m/l	Initial rate m/l/s - Enzyme only	St.Dev.%
863	7.8×10^{-6}	6.37×10^{-10}	4.98
864	1.56×10^{-5}	7.92×10^{-10}	3.40
865	4.68×10^{-5}	7.94×10^{-10}	3.06
866	9.4×10^{-5}	9.44×10^{-10}	3.16
867	1.25×10^{-4}	1.053×10^{-9}	1.9
868	1.56×10^{-4}	1.01×10^{-9}	2.36
869	3.12×10^{-4}	1.15×10^{-9}	2.02
870	3.68×10^{-4}	0.96×10^{-9}	2.3
871	5.9×10^{-4}	1.34×10^{-9}	2.0
872	5.52×10^{-4}	1.36×10^{-9}	1.99
873	7.36×10^{-4}	1.03×10^{-9}	1.76
874	9.2×10^{-4}	1.255×10^{-9}	1.8
875	1.10×10^{-3}	1.04×10^{-9}	2.40

Km = $7.51 \times 10^{-6} \pm 0.39 \times 10^{-6}$ m/l

Vmax = $1.16 \times 10^{-9} \pm 0.05 \times 10^{-9}$ m/l/s

k cat = $1.35 \times 10^{-3} \pm 0.01 \times 10^{-3}$ s^{-1}

TABLE 29

Human milk lysozyme (Jollès) catalysed hydrolysis of NAG_4 -β-3,4 dnp.

40°. pH = 5.08 I = 0.1 acetate buffer

$(E)_o = 9.25 \times 10^{-7}$ m/l by U.V. 10µl 1.018mg/ml.

Run	(S) m/l	Initial rate m/l/s - Enzyme only	St.Dev.%
900	1×10^{-5}	5.968×10^{-10}	3.15
901	1.2×10^{-5}	7.392×10^{-10}	4.87
902	1.4×10^{-5}	4.582×10^{-10}	9.37
903	1.6×10^{-5}	5.372×10^{-10}	5.08
904	2×10^{-5}	5.826×10^{-10}	5.49
905	3×10^{-5}	9.45×10^{-10}	3.38
906	6×10^{-5}	7.89×10^{-10}	4.71
907	7×10^{-5}	8.13×10^{-10}	2.57
908	8×10^{-5}	9.24×10^{-10}	2.28
909	1×10^{-4}	9.75×10^{-10}	2.52
910	1.4×10^{-4}	8.73×10^{-10}	2.51
911	2×10^{-4}	8.52×10^{-10}	2.77

Km = $6.8 \times 10^{-6} \pm 2.0 \times 10^{-6}$ m/l

Vmax = $9.4 \times 10^{-10} \pm 0.5 \times 10^{-10}$ m/l/s

k cat = $1.01 \times 10^{-3} \pm 0.05 \times 10^{-3}$ s^{-1}

TABLE 30

Duck-egg white lysozyme II catalysed hydrolysis of

NAG_4-β-3,4 dnp

40° pH 5.08 I = 0.1 acetate buffer

$(E)_o$: 2.75 mg/ml 10 µl 7.3×10^{-7} by U.V.

Run	(S) m/l	Initial rate m/l/s	St.dev.%
946	1×10^{-5}	5.70×10^{-10}	11.02
947	1.2×10^{-5}	6.54×10^{-10}	9.79
948	1.4×10^{-5}	1.043×10^{-9}	6.72
949	1.6×10^{-5}	1.004×10^{-9}	7.41
950	2×10^{-5}	1.129×10^{-9}	6.70
951	3×10^{-5}	1.78×10^{-9}	6.13
952	5×10^{-5}	1.03×10^{-9}	3.30
954	6×10^{-5}	1.09×10^{-9}	4.04
956	8×10^{-5}	1.082×10^{-9}	3.51
957	1×10^{-4}	1.198×10^{-9}	3.30
958	1.2×10^{-4}	1.272×10^{-9}	3.10

Km = $7.12 \times 10^{-6} \pm 2.48 \times 10^{-6}$ m/l

Vmax = $1.26 \times 10^{-9} \pm 0.07 \times 10^{-9}$ m/l/s

k cat = $1.70 \times 10^{-3} \pm 0.01 \times 10^{-3}$ s^{-1}

TABLE 31

Duck-egg lysozyme III catalysed hydrolysis of NAG_4 -β- 3,4 dnp

40° pH5.08 I = 0.1 acetate

$(E)_o$: 6×10^{-7} m/l by U,V. 10µl 2.43 mg/ml

Run	(S) m/l	Initial rate m/l/s	St.Dev.%
965	1×10^{-5}	3.124×10^{-10}	14.2
966	1.2×10^{-5}	3.766×10^{-10}	7.01
968	1.6×10^{-5}	4.525×10^{-10}	7.37
970	2×10^{-5}	5.546×10^{-10}	4.16
971	3×10^{-5}	5.699×10^{-10}	5.23
972	4×10^{-5}	7.41×10^{-10}	4.33
973	5×10^{-5}	7.63×10^{-10}	4.10
974	6×10^{-5}	7.96×10^{-10}	4.69
975	7×10^{-5}	9.62×10^{-10}	3.86
976	8×10^{-5}	8.39×10^{-10}	3.45
978	1×10^{-4}	1.142×10^{-9}	2.47
979	1.2×10^{-4}	1.02×10^{-9}	3.52
980	1.8×10^{-5}	4.816×10^{-10}	6.10

Km = $3.29 \times 10^{-5} \pm 0.71 \times 10^{-5}$ m/l

Vmax = $1.35 \times 10^{-9} \pm 0.12 \times 10^{-9}$ m/l/s

k cat = $2.26 \times 10^{-3} \pm 0.02 \times 10^{-3}$ s^{-1}

TABLE 32

Lysozyme catalysed hydrolysis of NAG_3-β-3,4 dnp.

40^{o}. pH 5.08 I = 0.1 acetate buffer. ϵ = 5820 400 nm.

$(E)_o = 5 \times 10^{-6}$ m/l

Run	(S)m/l	Initial rate m/l/s	St.Dev.%
805	5×10^{-5}	1.199×10^{-9}	2.84
807	7×10^{-5}	1.25×10^{-9}	2.62
808	8×10^{-5}	1.414×10^{-9}	2.01
809	9×10^{-5}	1.317×10^{-9}	3.15
810	1×10^{-4}	1.641×10^{-9}	1.57
811	2×10^{-4}	1.958×10^{-9}	1.97
813	4×10^{-4}	2.248×10^{-9}	1.11
815	6×10^{-4}	2.295×10^{-9}	1.77
816	7×10^{-4}	2.17×10^{-9}	6.59

Km = $6.62 \times 10^{-5} \pm 0.62 \times 10^{-5}$ m/l

Vmax = $2.60 \times 10^{-9} \pm 0.07 \times 10^{-9}$ m/l

k cat = $5.2 \times 10^{-4} \pm 0.01 \times 10^{-4}$ s^{-1}

TABLE 33

Lysozyme catalysed hydrolysis of NAG_2 -β-3,4 dnp, under Michaelis Menten conditions.

40^{o} pH 5.08 I = 0.1 acetate ε = 5820 400 nm

$(E)_o = 5 \times 10^{-5}$ m/l.

k spont = 1.4×10^{-6} s^{-1}

Run	(S) m/l	Initial rate m/l/s - Enzyme only	St.Dev.%
1105	5×10^{-4}	1.48×10^{-9}	2.44
1104	6×10^{-4}	1.80×10^{-9}	1.9
1103	7×10^{-4}	1.89×10^{-9}	1.66
1102	8×10^{-4}	2.62×10^{-9}	1.89
1101	9×10^{-4}	4.66×10^{-9}	1.65
1106	1×10^{-3}	4.94×10^{-9}	1.83
1107	4×10^{-3}	5.67×10^{-9}	1.41

Km = $2.54 \times 10^{-3} \pm 2.2 \times 10^{-3}$ m/l

V max = $1.05 \times 10^{-8} \pm 0.67 \times 10^{-8}$ m/l/s

k cat = $2.01 \times 10^{-3} \pm 1.3 \times 10^{-3}$ s^{-1}

TABLE 34

Lysozyme catalysed hydrolysis of NAG_2-β-3,4 dnp under second order conditions.

40°. pH 5.08 I = 0.1 acetate. ε = 5820 400 nm

$(E)_o = 2 \times 10^{-4}$ m/l.

Run	(S) m/l	Initial rate m/l/s	St.dev.%
1088	2.5×10^{-4}	3.28×10^{-9}	1.32
1090	7.5×10^{-5}	1.07×10^{-9}	2.62
1091	5×10^{-5}	0.997×10^{-9}	3.11
1092	2.5×10^{-5}	0.361×10^{-9}	10.4

$\frac{k\ cat}{Km}$ = 0.064 l/m/s

TABLE 35

Lysozyme catalysed hydrolysis of NAG_3-β-5-acetamido-2,4-dinitrophenyl under second order conditions.

40° pH 5.08 I = 0.1 acetate ε = 5820 400 nm

$(E)_o = 1 \times 10^{-4}$ m/l.

Run	(S) m/l	Initial rate m/l/s	St.dev.%
639	1.5×10^{-4}	6.0×10^{-8}	4.4

$\frac{k\ cat}{Km}$ = 4 l/m/s

Table 35 (b)

The lysozyme catalysed hydrolysis of NAG_2 -β-2, 4 dnp

37° I = 0.1 acetate buffers

$(E)_o = 4.6 \times 10^{-4}$ m/l $(S)_o = 6 \times 10^{-3}$ m/l

Cary 14 . 410 nm

Run	pH	ε 2, 4 dnp.	Enzyme + spontaneous rate m/l/s	Enzyme only m/l/s
35	5.71	9600	1.62×10^{-6}	7.5×10^{-7}
36	5.53	9600	1.62×10^{-6}	7.5×10^{-7}
37	5.35	9600	1.58×10^{-6}	7.1×10^{-7}
38	5.20	9600	1.62×10^{-6}	7.5×10^{-7}
39	4.98	9600	1.67×10^{-6}	8.0×10^{-7}
40	4.78	8950	1.69×10^{-6}	8.2×10^{-7}
41	4.56	8300	1.70×10^{-6}	8.3×10^{-7}
42	4.40	7600	1.70×10^{-6}	8.3×10^{-7}
43	4.21	6700	1.78×10^{-6}	9.1×10^{-7}
44	4.00	5800	1.83×10^{-6}	9.6×10^{-7}

Table 36

The hydrolyses of NAG_4 -β-3, 4 dnp by various hen egg-white lysozymes

(a) 40° pH 5.08 citrate buffer ε = 5820 400 nm

Cary 16 $(E)_o = 1 \times 10^{-6}$ m/l by weight

$(S)_o = 1 \times 10^{-5}$ m/l by weight

Run	Initial rate m/l/s	Batch	Activity found Units/mg. 110
288	1.17×10^{-9}	Boehringer 7371222 Oct.73	1.95
289	9.7×10^{-10}	" 6289418	1.62
290	9.4×10^{-10}	Worthington 10,900 F.O.C.C.	1.57
291	9.8×10^{-10}	Miles Grade II 110	1.64
292	9.7×10^{-10}	Miles Grade I 7102	1.62

(b) 40° pH 5.08 I = 0.1 acetate buffer ε = 5820 400 nm

Cary 16 on-line $(E)_o = 1 \times 10^{-5}$ m/l by weight

$(S)_o = 1 \times 10^{-4}$ m/l by weight

Run	Initial rate m/l/s	St. dev.%	Batch	Activity found Units/mg. 110
930	1.284×10^{-8}	0.37	Miles I 7102	
931	1.231×10^{-9}	0.37	"	2.04
932	1.259×10^{-8}	0.31	"	
933	1.206×10^{-8}	0.51	"	
934	1.244×10^{-8}	0.50	Miles II 110	
935	1.249×10^{-8}	0.42	"	2.03
936	1.236×10^{-8}	0.58	"	
937	1.212×10^{-8}	0.69	Boehringer 6459319 April 1972	

Table 36 (cont.)

Run	Initial rate m/l/s	St dev.%	Batch	Activity found Units/mg. 110
938	1.139×10^{-8}	0.47	Boehringer 6459319, April 1972	1.89
939	1.135×10^{-8}	0.64	"	
940	1.242×10^{-8}	0.53	Boehringer 7471123/1 Dec. 1973	
941	1.229×10^{-8}	0.44	"	1.97
944	1.179×10^{-8}	0.66	"	
942	1.0295×10^{-8}	0.48	Worthington F.O.C.C.	
943	1.034×10^{-8}	0.57	"	1.67

(c) Analyses of lysozymes

Lysozyme	N % 109 Calculated	* U.V.	C%	H%	N%	Ash% Found
Miles I	18.3	0.362	49.81	7.04	18.41	0.02
Miles II	"	0.364	45.01	6.87	16.48	0.7
Worthington	"	0.350	47.12	6.89	16.28	4.5
Boehringer (April 1972)	"	0.355	43.95	6.52	16.40	2.5
Boehringer (Dec. 1973)	"	0.363	45.22	6.87	16.26	4.4

*$(E)_o$ = 1 x 10^{-5} m/l by weight; absorbances of these solutions at 280 nm, Cary 16

Calculated absorbance = 0.379

The batch dates given are expiry dates.

Table 37

Model studies.

(a) NAG_1 -β-2, 4 dnp hydrolysis 25° Cary 14 410 nm

Run	pH	I	Buffer	k obs s^{-1}
96	1.18	0.1	HCl	8.95×10^{-5}
87	1.18	"	"	9.78×10^{-5}
97	1.8	0.1	Cl Ac	9.04×10^{-5}
85	1.8	"	"	10.08×10^{-5}
99	3.73	0.1	Ac OH	8.78×10^{-5}
83	3.73	"	"	9.07×10^{-5}
100	4.83	"	"	9.46×10^{-5}
101	5.71	"	"	9.41×10^{-5}
102	7.27	"	tris	7.77×10^{-5}
103	9.12	"	"	8.23×10^{-5}
104	10.41	"	phosphate	7.61×10^{-5}
105	11.29	"	"	9.99×10^{-5}
108	13	"	Na OH	29.94×10^{-5}
109	13	"	"	45.8×10^{-5}

Methanolysis:

106	spectrophotometric	354 nm.	methanol	24.79×10^{-5}
107	"	"	"	24.81×10^{-5}
113	polarimetric			20.9×10^{-5}

Table 37 (cont.)

(a)

Run	pH	I	(KCl)	(Ac OH)	(AcO^-)	k obs. s^{-1}	St. Dev %
88	5.08	0.1		0.0364	0.1	8.03×10^{-5}	
89	5.08	0.1	0.02	0.0291	0.08	8.12×10^{-5}	
90	5.08	0.1	0.04	0.0218	0.06	7.56×10^{-5}	
91	5.08	0.1	0.06	0.01445	0.04	7.77×10^{-5}	
92	5.08	0.1	0.08	0.0073	0.02	6.75×10^{-5}	
1270	3.96	0.1	-	0.454	0.1	6.96×10^{-5}	0.31
1271	"	"	"	"	"	6.55×10^{-5}	0.74
1272	"	"	"	"	"	6.97×10^{-5}	1.00
1273	"	"	"	"	"	7.06×10^{-5}	0.36
1274	"	"	"	"	"	6.91×10^{-5}	0.30
1286	3.96	0.1	-	0.454	0.1	6.78×10^{-5}	1.60
1287	3.96	"	0.02	0.363	0.08	6.69×10^{-5}	1.42
1288	3.96	"	0.04	0.273	0.06	6.64×10^{-5}	1.20
1289	3.96	"	0.06	0.182	0.04	6.32×10^{-5}	2.10
1290	3.96	"	0.08	0.091	0.02	6.37×10^{-5}	1.44

(b) Table 37 (contd)

NAG_1-β-F hydrolysis in distilled water at 25° pH stat

Titration with 0.1CN sodium hydroxide; 4.46 mgs used.

Run	pH	k obs s^{-1}	St.dev.%
1205	4.0	7.224×10^{-3}	2.99
1206	5.0	7.638×10^{-3}	1.44
1207	6.0	7.322×10^{-3}	3.17
1208	6.0	7.15×10^{-3}	7.0
1209	7.0	7.05×10^{-3}	3.2
1210	8.0	7.30×10^{-3}	5.75
1211	8.80	5.753×10^{-3}	2.55
1212	10.0	5.429×10^{-9}	5.01
1213	11.0	2.937×10^{-3}	7.18

NAG_1-β-F hydrolysis in 0.10 M sodium perchlorate at 25° pH stat

1089	4.0	7.56×10^{-3}	3.81
1081	4.0	6.82×10^{-3}	2.99
1078	4.5	8.10×10^{-3}	2.26
1086	4.5	7.73×10^{-3}	2.35
1083	4.5	7.61×10^{-3}	3.22
1079	5.0	7.56×10^{-3}	1.98
1077	6.0	6.85×10^{-3}	1.26
1076	7.0	6.84×10^{-3}	0.96
1075	8.0	6.64×10^{-3}	0.78
1080	9.0	6.59×10^{-3}	0.92
1082	9.5	6.74×10^{-3}	0.86
1087	10.0	6.4×10^{-3}	2.92

(Table 37 cont.)

Average uptake of alkali = 98% of theoretical

NAC_2-β-T:

8.52 mgs used; hydrolysis at 25°. distilled water

1084 5.0 3.2×10^{-3} 2.18

Uptake of alkali = 66% of theoretical.

4. DISCUSSION

4. Kinetic Discussion

The purpose of this analysis was to relate the observed parameters to those expected from the kinetic model, to determine their significance, and to explain any anomalies in terms of mechanistic changes.

4. 1. 1. The variation of k cat with pH

The plot of k cat vs. pH, from tables 8 to 18, is given in Fig. 16 for the lysozyme catalysed hydrolysis of NAG_4-β-3,4 dnp. The plot (2) gives the best fit near the pH optimum for the enzyme, which is pH = 5.04. The fit was obtained by feeding reasonable values for the constants as defined in Fig. 1, into the Algol program given in the Appendix, as follows:

Khes1 = 10^{-4} - a pKa_1 of 4 for the complex $\bar{E}HSp$

Khes2 = 10^{-6} - a pKa_2 of 6 " " "

Kces2 = 10^{-2} - At equilibrium 1% of the productive complex $\bar{E}HSp$ is in equilibrium with 99% of the non productive complexes $\bar{E}HSnpi$.

Kes4 = 10^{-2} Similarly for the diprotonated complexes EH_2S

Kes6 = 10^{-2} " for the dianionic species $\bar{\bar{E}}S$

k_2, the true catalytic constant = 10^{-2} sec^{-1} - fed in as $\bar{K}_1 = 10^{-2}$;

Hence $\bar{K}_2 = 10^{+6}$

$\bar{K}_3 = 10^{-2}$

$\bar{K}_4 = 10^{-4}$

$\bar{K}_1 = 10^{-2}$

The calculated values for these parameters for the best fit are:-

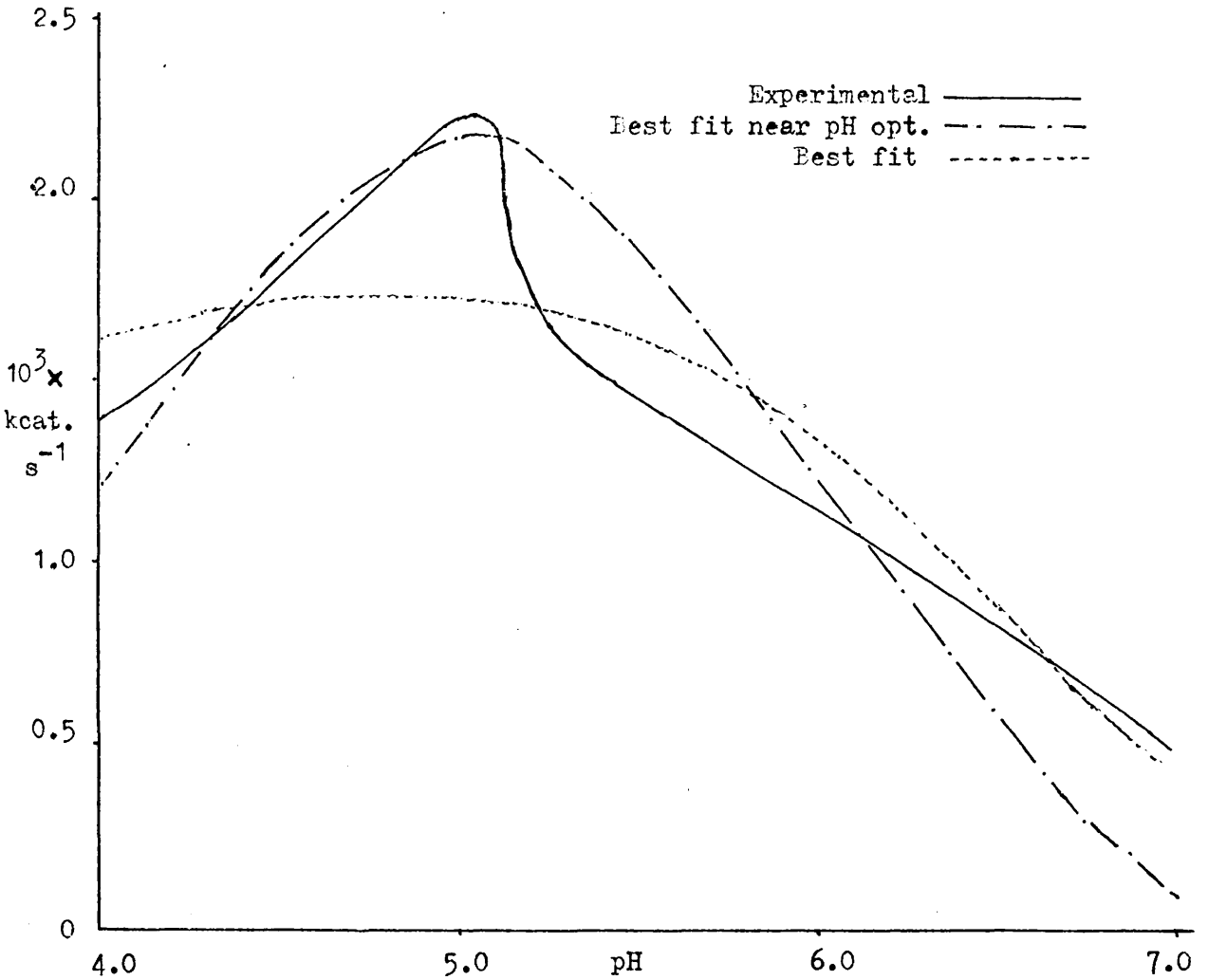


Fig.16 kcat. vs. pH for lysozyme catalysed hydrolysis of NAG_4-β-3,4dnp.

$\bar{K}_1 = 0.633 \text{ sec}^{-1} = k_2$

$\bar{K}_2 = 3.062 \times 10^{6}$

$\bar{K}_3 = 4.313 \times 10^{6}$

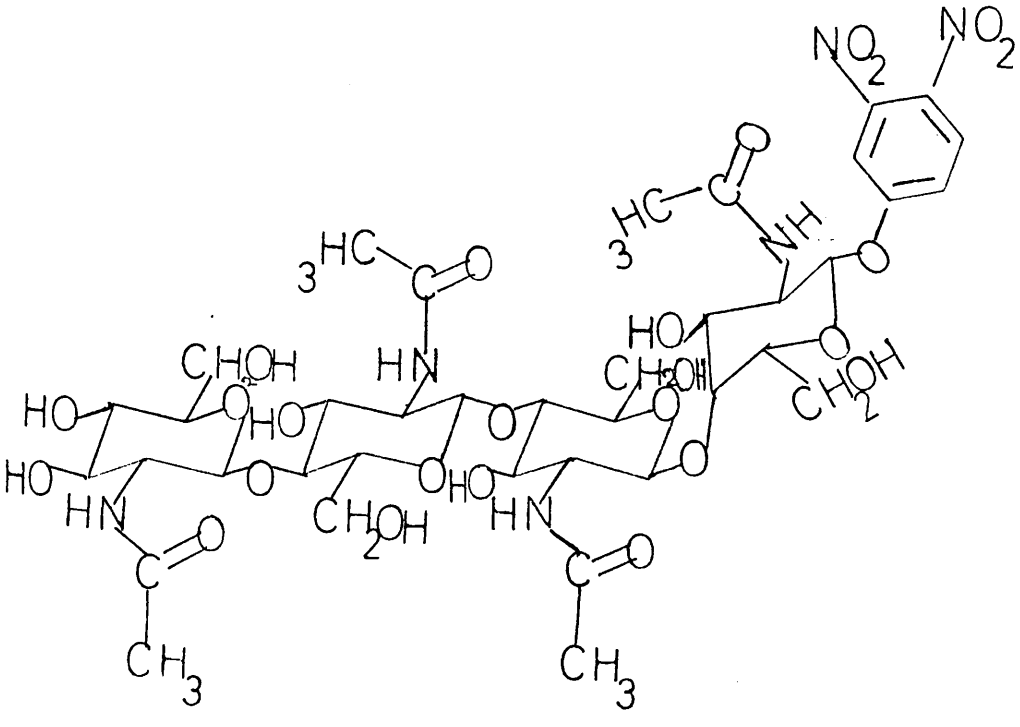
$\bar{K}_4 = 2.928 \times 10^{-4}$

Reasonable values for these constants can therefore be obtained from the pH rate profile.

These constants are a combination of equilibrium constants and are not resolvable under this scheme into individual components.

One of the main problems when studying the glycosidase-catalysed hydrolysis of a series of aryl glycosides is to obtain the ρ value for the breakdown of the enzyme substrate complex,[129] and thereby obtain a measure of the electronic requirements of the transition state. Using this method of obtaining data, k_2 can be correlated with the pKa 's of the aryl group. This is only possible if k_2 is the rate determining step, and it has been suggested that this is so,[124] with ρ estimated as + 1.2 to + .55.[91]

From molecular orbital calculations,[55] and in a kinetic model for cell wall hydrolyses, Chipman[103] has calculated a value of 1.75 sec^{-1} for k_2, and therefore it might appear that there is some anomaly between these figures; since 3, 4 dinitrophenol is a very much better leaving group. This may be rationalised, though, since the productive complexes are defined differently. The lysozyme - $(\text{NAG} - \text{NAM})_3$ complex almost certainly contains a closely bound hexasaccharide residue because of the binding of the sugars in sites E and F, which should counteract the unfavourable interactions of site D.



A B C D E F

Enzyme subsites.

Fig.18. Productive complex for NAG_4-β-3,4dinitrophenyl.

However it is probable that the lysozyme $-NAG_4-\beta$-3,4dinitrophenyl productive complex exists most of the time as in Fig. 18, and only occasionally will the phenyl glycosidic bond be correctly oriented in the cleft for rapid catalytic cleavage.

Rees[174] has studied polysaccharide conformations in detail and has found that distortion is more likely to be via rotation about the glycosidic bond than opening of the bond angle of 117°, associated with the glycosidic linkage.

The approximate rotation of the C_1-H and C_4-H planes about the glycosidic bond are each about 40° away from planarity, giving a most stable conformation several k cal below the planar case . The C_3-OH to C_5-O hydrogen bonding would have to be broken for rotation to occur.

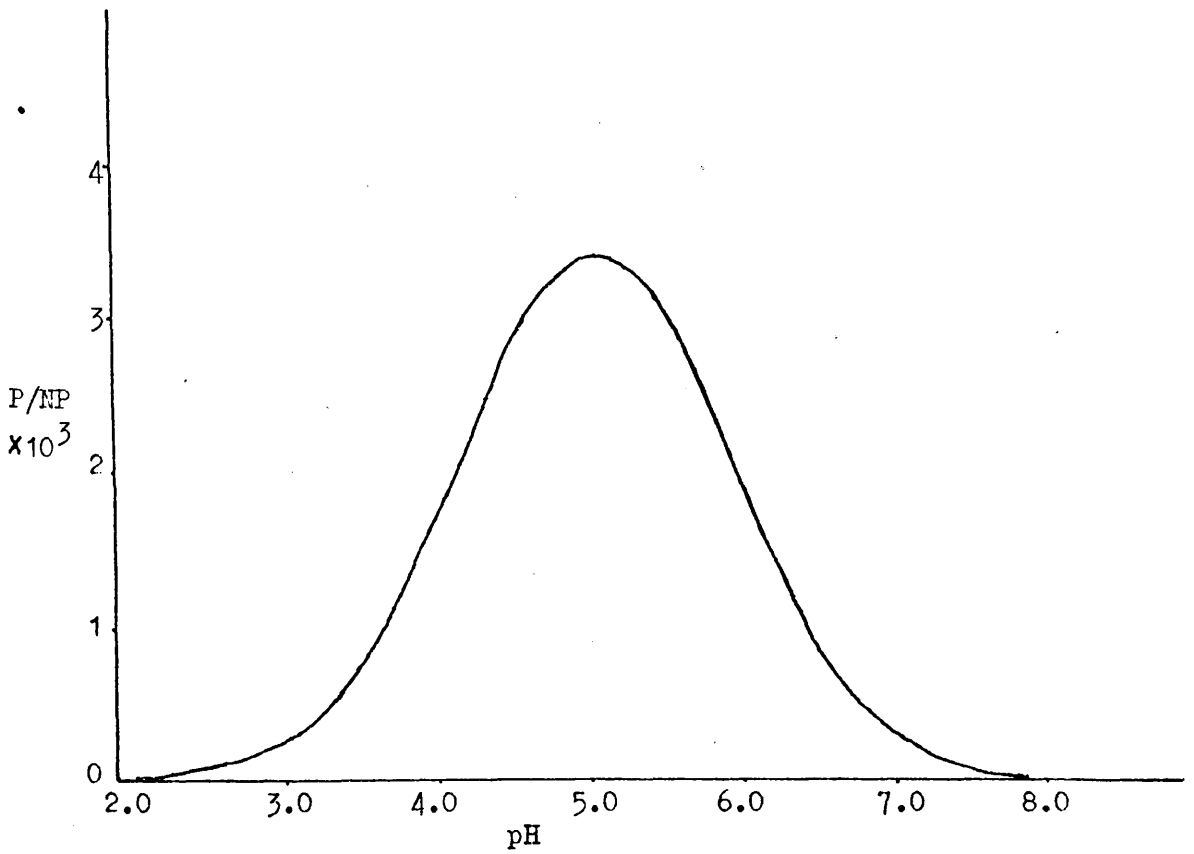


Fig.17 Ratio productive/non-productive binding for the lysozyme catalysed hydrolysis of NAG_4-β-3,4dnp.

4. 1. 2 The resultant deduction of the variation of P/NP with pH

Using these values for the parameters, the variation of the ratio of productive to non-productive complex with pH can be determined and is plotted in Fig. 17.

This bell-shaped profile very nearly mimics that of the pH - k cat plot, since at least one of the terms H^+K_2, $1/K_3$ and $\bar{K}_4/H^+$ is always very much larger than 1. (table 1)

In equation (7)

i.e. $$P/NP = \frac{1}{(H^+ (\frac{Kes4 + 1}{Khes1Kes4}) + \frac{1}{H^+} (\frac{Khes2 + Khes2Kes6}{Kes6}) + \frac{1}{Kcesl}}$$

the H^+ term is dominated by 1/Khes1Kes4, (since Kes4 is small), resulting from the presence of the non productive complex EH_2Snp.

Similarly, the $1/H^+$ term is dominated by the $(\frac{Khes2}{Kes6})$ term which results from $\bar{\bar{E}}Snp$. Therefore, the shape of the pH-rate profile is dominated by the way in which the concentration of the species EH_2Snp and $\bar{\bar{E}}Snp$ vary with pH, and not as might be expected, by the variation of concentration of $\bar{E}HSp$ with pH.

The reciprocal of the parameter $\bar{K}_3$ is equal to $\frac{\bar{E}HSnpi}{\bar{E}HSp}$ and this is 242 in value.

Validity of this analysis:

Therefore, having made the basic assumption that non-productive complexes are more favourable for this substrate, first of all because of their degeneracy, and secondly because of the proposed energetically unfavourable interactions present in the productive complex,(Ref: 83), a theoretical model can be used to explain the available experimental data. If this

assumption is not made, then the estimated equilibrium constants may still give a minimum value for the residuals, although very much more computer time is required, before ultimately arriving at the same values of the parameters.

Sensitivity of function to variations and errors in parameters $\bar{K}_1$ $\bar{K}_2$ $\bar{K}_3$ $\bar{K}_4$

The value of k cat is, of course, directly proportional to that of $k_2 = \bar{K}_1$ - equation (5). The relative sensitivity of the function to the changes in the values of $\bar{K}_2$ $\bar{K}_3$ and $\bar{K}_4$ varies with pH, as shown in table 1. At the pH optimum, the $\bar{K}_2$ and $\bar{K}_4$ terms only constitute some 20% of the total value of the function, the most important contribution coming from $\bar{K}_3$.

A decrease of one pH unit implies that the $H^+.\bar{K}_2$ term contributes over 50% to the value; similarly an increase of pH by 1 implies that $\bar{K}4/H^+$ contributes this amount.

Table 38: Values of components in function $(H^+.K_2 + 1/K_3 + H^+/K_4)$

pH	3	4	5	6	7
$H^+.\bar{K}_2$	3000	300	30	3	0.3
$\frac{1}{\bar{K}_3} = \frac{Khces1}{Kes4.Khes1} = \frac{Khes2}{Khces2.Kes6}$	242	242	242	242	242
$\bar{K}_4/H^+$	0.293	2.93	29.3	293	2930
Total value of function	3242	545	301.3	538	3172.3

Terms $\bar{E}HISnpi$, EH_2Snp, $\bar{\bar{E}}Snp$ involving true non-productive complexes 1 to 8 in Fig. 1a - not diprotated or dianionic forms of complex 9, again dominate the ratio P/N P as pH is varied.

It is thus most important to have allowed for complexing of

all forms of the enzyme in the original model, since it is the formation and depletion of these species which partly determines the shape of the pH-rate profile.

The value of P/NP is approximately halved by a change in pH of one unit. This is as expected for a simple scheme:

$$pH = pKa + \log \frac{(\text{Ionised})}{(\text{Unionised})}$$

		I/Total	U/Total
If pH = pKa	I = U	0.5	0.5
pH = pKa + 1	I = 10U	.91	.09
pH = pKa + 2	I = 100U	.99	.01

The pKa's that are deduced directly from the shape of the pH - k cat plot, therefore are complex quantities, and are a combination of thepKa's for each individual non-productive mode and the productive mode, but therefore reflect mainly the pKa of the major non-productive mode(s) present.

This arises from an examination of the model; the quantity 1/Kcesl, the reciprocal of the equilibrium constant for the equilibrium $\overline{EHS}p \rightleftharpoons \overline{EHS}$ npi may be replaced by $\frac{Khcesl}{Kes4Khesl}$ or $\frac{Khes2}{Khces2\ Kes6}$ a pH-independent term which must be equal in value to 1/Kcesl and is thus significant at all pH's from 4 to 6. Qualitatively, this situation may be viewed by considering a variation of true productive binding (complexes EHsp EH_2Sp $\overline{E}Sp$) relative to true non-productive binding - an increase of the ratio as pH is altered results in the observed k cat not changing as much as the pKa of the productive complex would dictate.

Therefore, the pH dependence of k cat gives a pKa value for this which is artificially low.

The predominance of non-productive complexes also implies that any study of the effect of pH on Km (app.), even though the figures are related to the parameter Ksl through equation (6), will result in the observation of the effect of the state of protonation of non-catalytic groups on Ksl. The data obtained in this study was not considered sufficiently accurate to make any detailed extrapolations from the Km (app.) values, tables 8 to 18.

4. 2. The evaluation of the dissociation constant, Ksl, for the productive enzyme-substrate complex, and the problem of unfavourable aglycone interactions.

The use of several different physico-chemical techniques -45 gives leading references - has given estimates of the pka's of the catalytic groups of lysozyme

as : Glu 35 pKa 6.5

Asp 52 pKa 4.5

Equation (9) gives the dependence of Km (app.) on pH, the constants $\overline{K}_2$, $\overline{K}_3$, $\overline{K}_4$, Ksl and these pKa's. The function $(1 + Khe2/H^+ + H^+/Khe1)$ has therefore, its minimum value at pH 5.5, of 1.3. At pH 5, the pH optimum of the enzyme, the value becomes 1.36, and using this and the found value of Km (app.) of 9.3×10^{-6} for NAG_4-β-3, 4 dnp at pH 5.03, 40°, gives $Ksl = 1.94 \times 10^{-3}$ m/l.

This is a sensible value for Ksl, since Chipman [103] has quoted a value of 5×10^{-4} m/l for the dissociation constant for binding of $(NAG\text{-}NAM)_2$ in subsites A to D.

This is important, since it is the dissociation constant for a single enzyme substrate complex, No. 9 in Fig. 6, and using the relationship :

$-\Delta G = RT\ln K$ - where K is an association constant, the total binding energy for that single mode of binding becomes immediately accessible.

The value for NAG_4-β-3, 4 dnp, calculated in this manner is $+\Delta G = -3.90$ k cal.

From the available data for the binding of N-acetyl-D-glucosamine residues in sites A B C D, it may be calculated that the free energy of the binding of NAG_4 in this mode would give + ΔG of from-6.7 to-3.6 k cal.

Therefore, it may be deduced that the aglycone, 3, 4 dinitrophenol may contribute by means of unfavourable interactions in site E, some (-0.3to) +2.8 k cal of free energy to impair the overall binding of the glycoside.

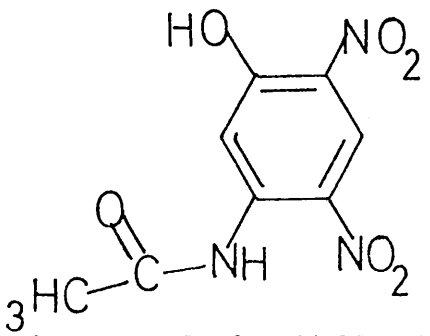
Since site D is believed to also have unfavourable interactions, it may be speculated that the mode of binding 9, may be as in Fig. 18, with the aglycone and end sugar residue projecting into the solvent media, away from the catalytic groups, situated in the enzyme cleft. A similar case has, in fact, been observed by X-ray, for the binding of NAG_2-β -pnp to lysozyme, where the sugars bind in sites B and C, and the phenol over, but not in site D.[125]

This may account for the fact that the ratio k cat/Km (app.) of this substrate is only some 232 $lm^{-1}s^{-1}$, some five fold lower than that of the reducing sugar NAG_5[66] notwithstanding the better leaving group of the former. However, it is also possible that the parameters $\bar{K}_2$, $\bar{K}_3$ and $\bar{K}_4$ are quite different for NAG_5, and this may account for the larger value of k cat with this substrate, even though k_2 should be lower.

Since the major interaction of a sugar residue binding in site E is thought to be due to the acetamido group hydrogen bonding to (1) N-H is H bonded to C = O of Glu 35 (2) C = O of amide is

H bonded to NH_2 of Asn 44,[92] and up to about $\Delta G = -4$ k cal;[83] extrapolated from X-ray model, and acceptor specificity studies, then, using an aglycone bearing a favourably oriented N-acetyl group, it may be possible to overcome this.

Using the aglycone 2, 4 dinitro, 5-acetamido phenol, (1)



(1)

the only such compound of suitable pKa, conferring adequate leaving group ability, and ease of preparation, the glycoside of NAG_3 was studied under conditions expected to be second order, from estimates of the Michaelis Menten parameters from the work on 2, 4 dinitro and 3, 4 dinitro phenyl NAG_3.

A value of k cat/Km (app.) of 4 1/m/s at 40°, pH5, I = 0.1 acetate, table 35 was obtained, and therefore it may be concluded that:

(a) The nitro groups still prevent, through steric hindrance; and/or:

(b) The site D interactions are still too unfavourable, or

(c) The acetamido group of the aglycone is not correctly orientated - model studies show that it is fairly close; or

(d) That acetamido binding in site E is not a major factor to consider.[65]

From the best figures available, it cannot be determined

whether sites D or E have the more powerful interactions, unfavourable and favourable respectively. From the value of ΔG for site D of + 4.38 k cal calculated in this work (see 4.5) as against the estimated value of - 4 k cal for binding of a NAG residue in site E, it would appear that the former "wins" and that the productive complex is still as defined in Fig. 18, apart from the considerations a to d.

4. 3. The evaluation of the individual contributions of sites A, B and C + D to the total binding energy of NAG_4-β-3,4 dnp

It is, of course, possible to study the pH-k cat profiles of the series NAG_n-β-3,4 dnp, where n = 2 to 4 to obtain the corresponding values of $\overline{K}_2$, $\overline{K}_3$ and $\overline{K}_4$, and derive values of K_{s1} for each of these substrates, and consequently the free energy of association for the productive complex. A simple subtraction would then yield the individual contributions of sites A, B and C + D.

However, the problem is very much easier, if we make the assumption that k_2, the catalytic constant for the cleavage of the productive complex to free aglycone and enzyme-sugar residue, is the same for each of these glycosides.

If, then the ratios k cat/Km (app.) of each substrate are compared, using equation (9),

$$\frac{k\ cat}{Km\ (app.)} = \frac{k_2}{Ksl\ (1 + \frac{Khe2}{H^+} + \frac{H^+}{Khel})}$$

the value of k_1 may be determined for each member of the homologous series. The individual values of k cat and Km (app.) can be determined from a Michaelis Menten plot, or the ratio k cat/Km (app.) can be determined under second order conditions. The results of these studies are given in table 39.

It can be seen therefore that site A has a favourable binding interaction of -2.2 k cal, which compares well with the quoted literature value[65] of -2.3 k cal.

Table 39

Substrate	k cat/Km (app.) l/m/s	Table	k_2 sec^{-1}	Ksl	ΔG for productive mode of binding in subsites	
NAG_2-β-3,4 dnp	0.064	34	0.63	10	+0.72 k cal	C + D
NAG_3-β-3,4 dnp	7.1	32	0.63	6.53×10^{-2}	- 1.7 k cal	B + C + D
NAG_4-β-3,4 dnp	232	14,7	0.63	2.6×10^{-3}	- 3.9 k cal	A + B + C + D

Binding Site	Calc. Δ G	Lit.[65] Δ G
A	-2.2 k cal	- 2.3 k cal
B	-2.42 k cal	- 2.7 k cal.
C + D	+0.72 k cal	- 1.7 to + 1.4 k cal.
C		- 4.6 k cal.
D	+4.38 k cal	+2.9 to + 6 k cal
E		- 4 k cal.
F		- 1.7 k cal

The total interactions of site B may be obtained from a comparison of the values of Ksl of the trimeric and dimeric glycosides. The value of Δ G for binding of a NAG residue in site B is - 2.42 k cal., which is slightly more than that of site A, as expected from the literature value of - 2.7 k cal.

The major saccharide-chain interactions of site A have been observed in the X-ray model, and are mainly due to a hydrogen bond formed between the N of the acetamido group in the cleft and the carboxyl side chain of aspartate 101, as well as non-polar bonds. The unit bound in site B is stabilised by non-polar bonds of Trp 62, and the side chain of aspartate 101, which hydrogen bonds to the oxygen at C - 6.

The interactions between a NAG residue in site C and the amino acids of the polypeptide chain have been studied the most extensively. There is strong hydrogen bonding between the N of the acetamido group in the cleft and the carbonyl of Ala 107, and between the carbonyl of the acetamido group and the N - H of Ile 59, as well as two hydrogen bonds between the oxygens at C - 6 and C - 3 and the N of tryptophans 62 and 63. There are also non-polar interactions.

Site C is therefore expected to be the strongest binding site, and this is indeed found; forming the basis for extensive N.M.R. studies.[106, 109]

The binding of residues in site C is thus the best documented, and the data gives the most reliable of all the binding site interaction estimates.

This is convenient, since it is not possible to single out the interactions of either site C or site D, but only to obtain a combined value for C + D from the value of Ksl for NAG_2-β-3,4 dnp, of + 0.72 k cal.

If it is accepted that the ΔG for site C is - 4.6 k cal, then it is possible to obtain a reliable estimate of the binding of a NAG residue in site D. This is found to be + 4.38 k cal, as compared with the estimates of from + 2.9 k cal to + 6 k cal. This figure is an estimate for the binding of a NAG residue in site D, even though the productive complex may be defined as in Fig. 18, which adversely affects the value of k_2.

Validity of this analysis

The only assumption made in the derivation of the equations from the model is that the function $\frac{Ksl}{Sf}\left(1 + \frac{Khe2}{H^+} + \frac{H^+}{Khel}\right)$ is small compared with the other terms in the denominator, equation 4 (a). This is allowable, since, using Khel = 3.16×10^{-5} m/l and Khe2 = 3.16×10^{-7} m/l, from the estimates of the pKa's of Asp 52 and Glu 35, respectively,[54] the function has a minimum value at the pH max. of the enzyme of 1.3. The function

$(1 + H^{+}.\overline{K}_2 + \frac{1}{\overline{K}_3} + \frac{H^{+}}{\overline{K}_4})$ has a value of 302.

Secondly, the assumption that k_2 is identical for each of the productive complexes of the homologous series of glycosides, depends on the ρ values being identical, which will be so, since the same leaving group is being used, namely 3, 4 dinitrophenol, but also depends on the productive complexes being defined similarly, Fig. 18.

This may be allowable, but depends on a comparison of the mobility of a NAG residue bound in site C, allowing that above site D to move freely between solution and poor binding near the catalytic site for NAG_2-β-3,4 dnp, and the flexibility of a NAG residue bound similarly in site C, but with the restrictions imposed by a further sugar residue in site B, linked to it through the 4 - O glycosidic bond. This may, but not necessarily, result in some difference between the productive complexes, although it may be small. (see 4.6 (2))

Third, it is assumed that all compounds hydrolyse by a simple mechanism. This is discussed further in the examination of their specificities (4.5)

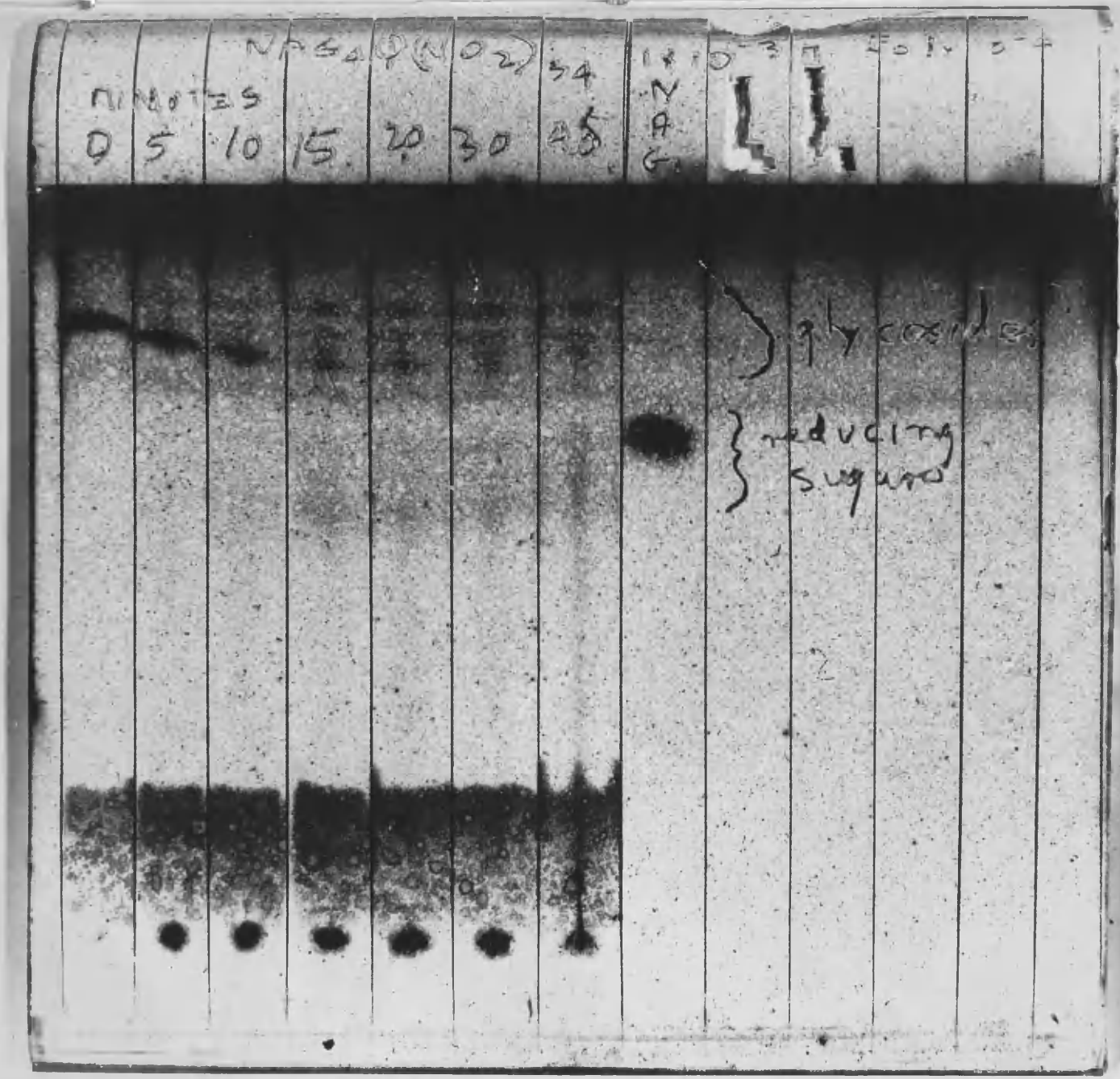

NaSO4(NO2)34
minutes
0 5 10 15 20 30 45
NAG
glycosides
reducing sugars

Fig. 19

4. 4. The problem of multiple bond cleavages in NAG_4 - β -3, 4 dinitrophenyl

Fig. 19 shows the result of incubating 1 x 10^{-3} M NAG_4 - β-3, 4 dinitrophenyl; 1 x 10^{-4} M HEW lysozyme at 40°, pH5 acetate buffer. It can be seen that by comparing the production of the reducing sugar chitotetraose with the formation of the monomer, dimer and trimer glycosides, that cleavage down the NAG chain, between sugar residues occurs at approximately the same rate as the release of phenol, which was measured spectrophotometrically. The TLC plate was silica gel G, run in 6:3:2 ammonia/propanol/water and developed with ceric sulphate.

From the figures computed, Kcesl = 4 x 10^{-3}; therefore

$$\frac{(\overline{EHSp})}{(\overline{EHSnpi})} = \frac{1}{250}$$

There are three sites down the NAG_4 chain which can cleave to give the free sugars, NAG_1 to NAG_3 and glycosides 3, 4 dinitrophenyl NAG_3 to 3, 4 dinitrophenyl NAG_1, and only one site of cleavage which can result in direct release of 3, 4 dinitrophenol. It would be expected that, since the ratio of these complexes P/NP is 1/250, then phenol release would be about 80 times slower than cleavage between the sugar residues. Since this is clearly not the case, then NAG -NAG bonds could be cleaving about 80 times more slowly than aryl glycoside-sugar fission occurs, or most likely, from an expected binding energy of at least -9.6 k cal for mode 3, that this binding, which is non-productive for all bond cleavages, would dominate the binding, and lower Kcesl.

Further, the complexity of the reaction and transglycosylation products which build up later on do not affect the initial rates of phenol release measured, since there is only one substrate present from which free phenol may be produced at the start of the reaction.

It can also be seen that, although NAG_4-β-3, 4 dnp is the best spectrophotometric substrate synthesised so far, that non-productive binding is still a major problem, and that only $\frac{1}{4}\%$ of the substrate is bound productively to $\bar{E}H$. Attempts to further improve the specificity of the substrate manifested themselves in the work on β-fluorides and the ideas in 4.12.5.

4. 5. The relative specificities of substrates for lysozyme.

4. 5. 1. k cat/Km (app.) - ratios from initial rates of hydrolysis

The direct comparison of the k cat/Km (app.) ratio of the series of paranitrophenyl - β- glycosides of NAG_2, NAG_3, NAG_4 and NAG_5 is given in table 6 and Fig. 20. The conditions $(E)_o = 2.5 \times 10^{-4}$ m/l; $(S)_o = 1 \times 10^{-4}$ m/l were chosen in order that each hydrolysis exhibits second order kinetics. The substrate NAG_2-β -pnp was hydrolysed only after an induction period of some 3 hours, and therefore its hydrolysis is certainly complex, but serves to illustrate the effect of alteration of substrate structure.

A 14-fold increase in initial rate on lengthening the sugar chain from NAG_2 to NAG_3. and an 8-fold increase for NAG_3 to NAG_4 was observed.

The initial rate of hydrolysis of the NAG_5 glycoside was only twice that of the NAG_2 glycoside, and this may be due to the pentameric compound cleaving preferentially to give NAG_4 and NAG- β- paranitrophenyl, which is not observed, rather than release phenol. The comparison of the NAG_5 glycoside with the tetrameric compound may be rationalised by looking at the subsite binding energies for NAG residues, Fig. 6, and table 39. The modes with the phenyl residues situated as in 8 and 9, give binding energies of -9.2 k cal and -5.2 k cal respectively, which gives dissociation constants of 4×10^{-7} m/l and 2.5×10^{-4} m/l. This implies that binding in mode 8, which gives chain cleavage is some 1000 fold stronger than the mode giving phenol release. Further, the former is more likely to have a "held down" productive

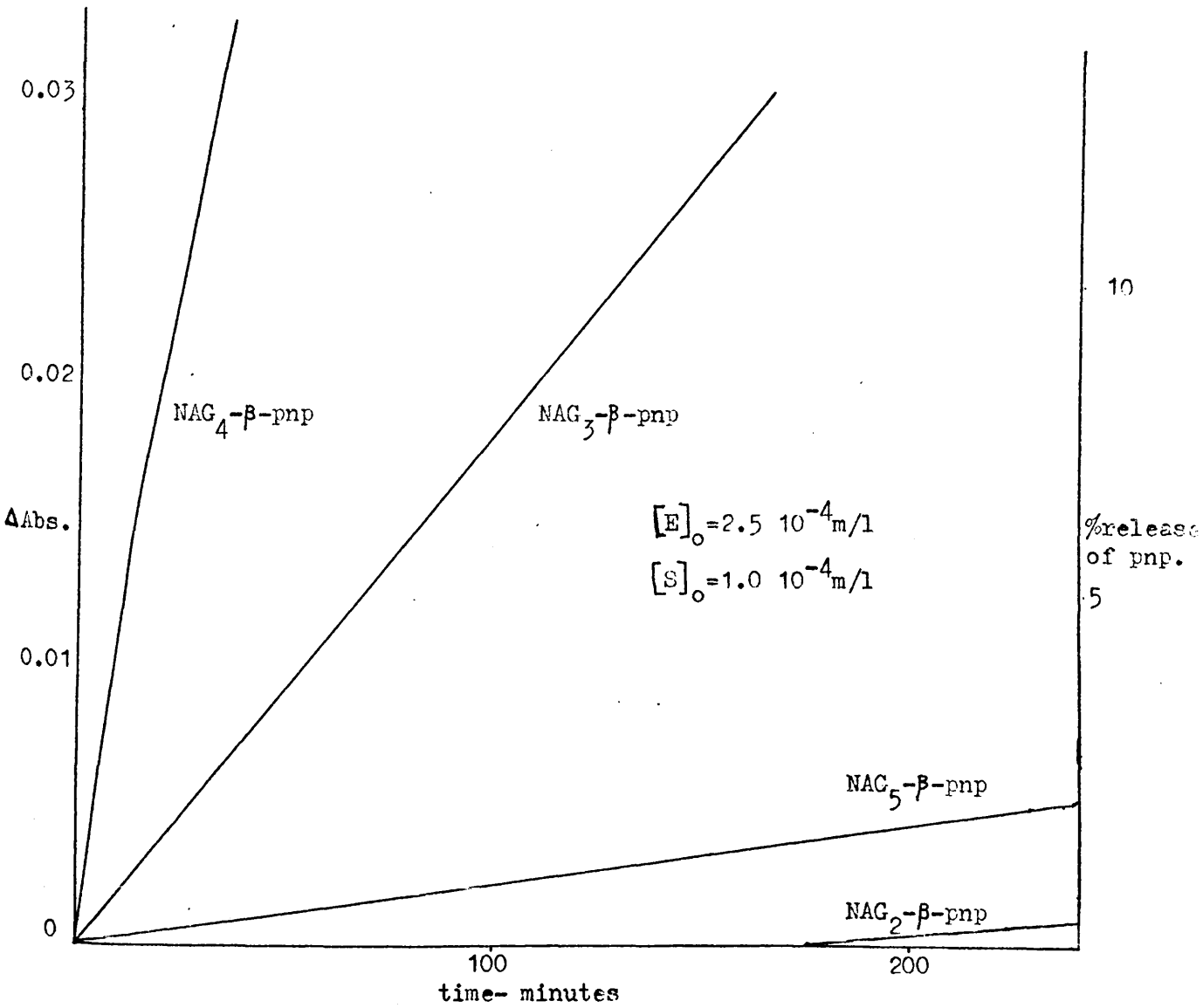


Fig.20 - Lysozyme catalysed hydrolysis of paranitrophenyl glycosides

complex whereas the latter could be as in Fig. 18.

The strongest mode of binding for the NAG_4 glycoside, mode 3, Fig. 6, is similarly non-productive as far as phenol release is concerned, but the essential difference is that it is also non-productive for chain cleavage, and therefore the substrate remains intact and still available for binding in mode 9.

The 8-fold increase in rate on lengthening the chain length from NAG_3 to NAG_4 is almost certainly due to a more favourable value of Ksl, the binding constant for the productive mode, 9, which alters the specificity according to equation 9.

$$\text{Specificity} \propto \frac{k2}{Ksl\left(1 + \frac{Khe2}{H^+} + \frac{H^+}{Khel}\right)} \qquad (9)$$

The values of Table 39, predict a 25 fold decrease in Ksl of from 5.2×10^{-2} m/l to 2×10^{-3} m/l, assuming k_2 does not alter, and that the mechanisms are the same. This, unfortunately, is by no means certain, even at these relatively low substrate concentrations.

4. 5. 2 Synthetic substrate specificity comparisons from first and second order kinetics

A comparison of the relative specificities of the series of oligosaccharides with different aglycones, by considering the ratio k cat/Km (app.) is more easily understood if the data of table40 is presented as in table 41, and absolute values are not quoted.

k cat/Km (app.) ratio studies are the most significant comparisons between substrates, since the effects of non-productive binding are eliminated, and are more reliable for the smaller substrates, since for these, because of solubility limits and high spontaneous rates, it is often difficult to obtain, independently, values of k cat and Km (app.), whereas the ratio k cat/Km can be found from studies where second order kinetics are displayed. There is also the advantage that at the low substrate concentrations involved, the cleavage between NAG residues should be minimised, relative to release of phenol. This could be up to 50-fold for NAG_2-β-pnp.[115]

Taking a broad view of the results, the following points may be noted:

1. On increasing the acidity of the aglycone from 3, 4 to 2, 4 dinitrophenyl NAG_2 glycosides, the increase in specificity is about $^1/_4$ of the increase in Ka. This implies that the ρ value is about 0.25, if it is assumed that Ksl is the same for each substrate. As there is no induction period for either of these compounds, and conditions are not favourable for NAG-NAG hydrolysis, they are probably hydrolysed by a simple mechanism. Furthermore, initial rates of hydrolysis should be independent of transglycosilation effects.

Table 40

$\frac{k\ cat}{Km\ (app.)}$ ratios of substrates for lysozyme

Substrate	pH	temp	k cat s^{-1}	Km (app.) m/l	$\frac{k\ cat\ l/m/s}{Km(app)lit.}$	k cat s^{-1}	Km (app.) m/l	$\frac{k\ cat\ l/m/s}{Km\ (app.)}$ found
NAG_5	5	40°	0.012	1×10^{-5}	1200 [82]	-	-	-
NAG_6	5	40°	0.11	1×10^{-5}	11,000 [82]	-	-	-
NAG-Glu-pnp	5.0	40°	9.5×10^{-6}	2.8×10^{-2}	0.00041 [115]	-	-	-
NAG_2-pnp	5.0	40°	8.4×10^{-5}	2.9×10^{-2}	0.0029 [115]	-	-	-
"	5.2	35°	5×10^{-6}	4×10^{-3}	0.0013 [124]			
"	5.08	40°	-	-	-			0.0039
NAG-2 deoxy Glu-pnp	5.0	40°	1.22×10^{-4}	8×10^{-3}	0.0167 [115]	-	-	-
NAG_2-3,4 dnp	5.08	40°				1×10^{-3} (c) 2×10^{-3}	8×10^{-4} (c) 2.5×10^{-3}	0.064 (a) 0.8 (b)
NAG_2-2,4 dnp	5.0	35°	5.1×10^{-4}	1.7×10^{-3}	0.3 [124]	-	-	-
"	5.1	40°	-	-	-	1.3×10^{-3}	2.1×10^{-3}	0.6
NAG_3-pnp	5.08	40°	1×10^{-4}	5×10^{-4}	-	-	-	0.20
NAG_3-3,4 dnp	5.08	40°	-	-	-	5.2×10^{-4}	6.6×10^{-5}	7.1
NAG_3-2,4 dnp	5.08	40°	-	-	-	1.5×10^{-3}	1.2×10^{-4}	13
NAG_4-pnp	5.08	40°	-	-	-	4×10^{-4}	4.5×10^{-4}	0.95
NAG_4-3, 4 dnp	5.08	40°	-	-	-	2.16×10^{-3}	9.3×10^{-6}	232

(a) determined under second order conditions

(b) from Michaelis Menten plot - calculated

(c) - estimate - since few velocities were obtained near V max due tc insolubility of the substrate

Table 41

k cat/Km (app.) ratio changes for synthetic substrates

Aglycone:	pnp		3, 4 dnp		2, 4 dnp
ka		-41x-		-26x-	
pKa	7.2		5.42		4.0
Glycone					
NAG_2 -		-15x-		-10x-	
	50x		120x		25x
NAG_3 -		-35x-		-2x-	
	8x		30x		
NAG_4-		-150x-		-	

2. With the NAG_3 glycosides, there is a very much greater difference between paranitrophenyl and 3, 4 dinitrophenyl than between 3, 4 and 2, 4 dinitrophenyl glycosides. Again, if Ksl is assumed to be not too different, then k_2 is not increasing as the pKa of the leaving group increases, tending to suggest that the rate-determining step is no longer purely glycosylation, but is altered according to equation (3). Km (app.) is also lowered if this happens, but this is not observed, within the limits of the accuracy of the experimental data, table 40.

3. The fifty-fold increase in specificity, as indicated by the ratio k cat/Km (app.) of the NAG_3 relative to the NAG_2 paranitrophenyl glycoside is four times greater than the difference in initial rates, Fig. 20, probably due to the different conditions under which the runs were conducted, with differing amounts of transglycosylation, which is very fast compared with hydrolysis of an enzyme-substrate intermediate, [103] occurring.

4. There is also about a five-fold greater increase in the ratio k cat/Km (app.) on increasing sugar chain length from NAG_2 to NAG_3 than NAG_3 to NAG_4, both with paranitro- and 3, 4 dinitrophenyl glycosides. This may be explained from similar conclusions to the difference in the initial rate studies, although the change is more marked.

3, 4 dinitrophenyl glycosides seem more susceptible to this change than paranitrophenyl glycosides, although this comparison may not be entirely valid, since the mechanism of the paranitrophenyl glycoside hydrolyses may be altering.

5. The susceptibility of the oligomers NAG_4:NAG_3:NAG_2 is about 10:2:1; to increase in acidity of leaving group, from paranitrophenol to 3, 4 dinitrophenol. This could mean that the productive complex is changing, Fig. 18, in increasing chain length, that is, effectively, ρ is more positive for NAG_4 compounds, which is not so from the values of k cat, table 40, or, most likely, that the mechanism changes. (4. 6. (2))

6. Even in a reaction which is aided by transglycosylation, that is, the velocity increases with time, provided there is no visible induction period, the initial rate should be unaffected, although the polynomial terms will differ. This, in fact, was observed at high concentrations of NAG_2-β-3, 4 dnp. A k cat/Km (app) determination under second order conditions may give a different value from that obtained from individual estimates of k cat and Km (app.) and calculating the quotient, since the mechanism of hydrolysis of the one substrate may alter as concentration changes.[115] This was observed for NAG_2-β-3, 4 dnp, table 40.

TLC examination of the reaction mixture of NAG_2-β-2, 4 dnp showed transglycosylation products after incubation for 15 hours at 40°, but none initially, although less than 5% could not be detected.

The hydrolysis of NAG_4-β-pnp showed more NAG_2 and NAG_2-β-pnp than other sugars and glycosides, and comparing this with Fig. 19, where cleavage is more evenly distributed, gives further evidence for the different mechanisms of hydrolysis of NAG_4-β-pnp and NAG_4-β-3, 4 dnp.

7. A full Michaelis-Menten study on NAG_4-β-4 methyl-umbelliferyl could not be carried out, due to difficulties experienced both with solubility and instrumentation. The former has been noted in a comparison of paranitrophenyl and 4-methyl umbelliferyl glucosides.[48]

However, preliminary studies suggested, that due to suppression of the fluorescence of the phenol, because of the pH of the media, at 5, being very much less than the pKa of 8, there was no advantage in sensitivity over spectrophotometric substrates, in particular NAG_4-β-3, 4 dnp. For these reasons, the studies were not continued.

8. The highest specificity chromophoric substrate synthesised so far, namely NAG_4-β-3, 4 dnp has unfortunately proved to be some five fold lower in specificity than NAG_5, and forty-five fold lower than NAG_6, despite the better leaving group. This is a result of lower k cat values, and not higher Km (app.) values. This can only be explained in terms of the form of the productive complex, Fig. 18. Possibilities for overcoming this problem are discussed in section 4. 12.

4.6 A comparison of the k cat values for synthetic substrates

The values of k cat cannot be as accurately determined for NAG_2 glycosides as can the ratios k cat/Km (app.), but consideration of these can still give information, provided that too much emphasis is not placed on small changes.

The solution of the kinetic model led to equation (5), expressing k cat:

$$k\ cat = \frac{k_2}{(1 + H^+\bar{K}_2 + 1/\bar{K}_3 + \bar{K}4/_{H^+})} \quad \ldots \quad (5)$$

An examination of table 42 raises the following points:

1. On changing the aglycone from 3, 4 dinitrophenol to 2, 4 dinitrophenol for both the NAG_2 and NAG_3 glycosides, there is little alteration in the value of k cat. It is therefore probable that, since the values of $\bar{K}_2$, $\bar{K}_3$ and $\bar{K}_4$ will most likely be similar within each of the pairs of glycosides, that glycosylation could no longer be the rate determining step, and that k cat is more nearly given by:

$$k\ cat = \frac{k_2\ k_3}{(k_2 + k_3)\ (1 + H^+\bar{K}_2 + 1/\bar{K}_3 + \bar{K}_4/H^+)}$$

- from (3) and (5)

The approach to the limiting condition where $k_2 \gg k_3$ implies that:

$$k\ cat = \frac{k_3}{(1 + H^+\bar{K}_2 + 1/\bar{K}_3 + \bar{K}_4/H^+)}$$

The most likely situation is that k cat is not very sensitive to changes in pKa of the leaving group - a 26 fold increase in the

Ka of the phenol gives only a three-fold increase in k cat, implying a small positive ρ value.

This is the first study to compare two such acidic aglycones. Previously, 2, 4 dinitrophenyl glycosides had only been compared with glycosides using poor leaving groups, whose hydrolysis was probably complex, and therefore it could not be determined whether or not the limiting condition was being approached.[124]

2. There are small increases in k cat on increasing the sugar chain length from NAG_2 to NAG_3 and NAG_3 to NAG_4, for paranitrophenyl, 3, 4 dinitrophenyl and 2, 4 dinitrophenyl glycosides. This may be fortuitous, since the parameters $\overline{K}_2$, $\overline{K}_3$ and $\overline{K}_4$ differ for different oligomers (4. 7. (3)), resulting in about a four-fold difference in the function $(1 + H^+.\overline{K}_2 + 1/\overline{K}_3 + \overline{K}_4/H^+)$.

The other parameter involved in determining the value of k cat for each substrate is k_2, and this depends on the productive complexes, Fig. 18.

It may be deduced, therefore, that the productive complexes are not changing, and that the increases in k cat as sugar chain length is increased are due only to the reduction in the function $(1 + H^+.\overline{K}_2 + 1/\overline{K}_3 + \overline{K}_4/H^+)$

Further weight is given to this conclusion by the observation that there is little change in susceptibility of each oligomer to increase in the acidity of the leaving group, implying that the productive complexes and ρ are not changing, and therefore the comparisons of section 4.3, where Ksl was deduced, are valid.

3. There is a larger difference between NAG_2-β-pnp and NAG_2-β-3,4 dnp than between either NAG_3-β-pnp and NAG_3-β-3,4dnp

or NAG_4-β-pnp and NAG_4-β-3, 4 dnp. This suggests that there is a more important change of mechanism between NAG_2-β-pnp and NAG_2-β-3,4 dnp than between the members of the other pairs. This again suggests that results involving paranitrophenyl glycosides must be viewed with some scepticism, because of their complex hydrolysis.

4. On changing the aglycone from paranitrophenol to 3, 4 dinitrophenol for both the NAG_3 and NAG_4 glycosides, there is a five-fold change in k cat for each, suggesting that there is little difference between the complexity of the hydrolyses of the paranitrophenyl glycosides, although, again, this may be fortuitous.

5. There is a relatively smaller increase in k cat on increasing sugar chain length from NAG_2 to NAG_3 than NAG_3 to NAG_4, both with paranitrophenyl and 3, 4 dinitrophenyl glycosides. This contrasts with the changes in specificities, where the largest increase is from NAG_2 to NAG_3 (4. 5. 2. (4)).

A comparison of equations (5) and (9) implies that this is due to the function $(1 + H^+.\overline{K}_2 + 1/\overline{K}_3 + \overline{K}_4/H^+)$ affecting the former and Ksl influencing the latter.

Table 42

k cat changes for synthetic substrates

Aglycone:	pnp	3, 4	2, 4
Ka	-41x-	-26x-	
Glycone			
NAG_2-	-10x-	-1x-	
	2x	1x	1x
NAG_3-	-5x-	-3x-	
	4x	4x	
NAG_4-	-5x-	-	

Table 43

Km(app.) changes for synthetic substrates

Aglycone:	pnp	3, 4 dnp	2, 4 dnp
Glycone			
NAG_2-	-2 to 30 x-	-1x-	
	5 to 50x	40x	18x
NAG_3-	-10x-	-2x-	
	2x	7x	
NAG_4-	-50x-		

4. 7 The Km (app.) values for synthetic substrates

Similar limitations are imposed on any comparison of the Km (app.) values, as on the k cat values. The most reliable results for a series of glycosides were for 3, 4 dinitrophenyl glycosides, and their relative magnitudes are given in table 43.

The solution to the kinetic model gave:

$$Km\ (app.) = \frac{Ksl\ \left(1 + \frac{Khe2}{H^+} + \frac{H^+}{Khe1}\right)}{\left(1 + H^+ \bar{K}_2 + \frac{1}{\bar{K}_3} + \bar{K}_{4/H^+}\right)} \quad \ldots\ldots \quad (6)$$

The following may be deduced:

1. For all the glycosides, there is approximately the same relative change in Km (app.) on increasing sugar chain length, implying the aglycone is not having a great deal of effect (see 4. 5. 2. (1)); but all the paranitrophenyl glycosides have very much higher Km (app.) values than either the corresponding 3, 4 dinitrophenyl or 2, 4 dinitrophenyl glycosides. This is almost certainly because of the complex mechanism involved in their hydrolysis. The Km (app.) is raised because the rates are dependent on transglycosylation, which increases on raising the concentration of substrate

2. There is a larger effect seen by increasing sugar chain length from NAG_2 to NAG_3 than NAG_3 to NAG_4. This trend is the same direction as for specificity increases, but is of even larger magnitude, since both a decrease in Ksl and an increase in the

function $(1 + H^+.\overline{K}_4 + 1/\overline{K}_3 + \overline{K}_{4/H^+})$ contribute to the change, equation (9)

Table 44

	Ksl (table 39)	Km (app.)	$(1 + H^+.\overline{K}_2 + 1/\overline{K}_3 + \overline{K}_{4/H^+})$
NAG_2-β-3, 4 dnp	10	8×10^{-4}	$\sim 1 \times 10^4$
NAG_3-β-3,4 dnp	0.065	6.6×10^{-5}	1.3×10^3
NAG_4-β-3,4 dnp	0.002	9.3×10^{-6}	3×10^2

3. Using the data of the 3, 4 dinitrophenyl glycosides, it is possible, from the values of K s l deduced (4.3), to estimate values of the function $(1 + H^+.\overline{K}_2 + 1/\overline{K}_3 + \overline{K}_4/H^+)$ for each of the homologous series NAG_2, NAG_3 and NAG_4 glycosides, table 44 - compare table 38.

There are approximately 8-fold and 4-fold decreases in the value of the functions for each higher oligomer, NAG_2 to NAG_3 and NAG_3 to NAG_4 glycosides, implying that, since the H^+, the $1/H^+$ and non-pH dependent terms are dominated by non-productive species (4.1.2) that the total value of the function is also.

Therefore the decrease implies that there is, relative to productive binding, 8 and 4 times as much non-productive binding when V max is reached, with NAG_2-β-3, 4 dnp against NAG_3-β-3, 4 dnp and with NAG_3-β-3,4 dnp against NAG_4-β-3, 4 dnp.

The initial proposal in this work was to increase the amount of productive complex present relative to non-productive,

by using glycosides of NH_4^+, and from these results, it can be seen that there is a 30-fold improvement in this ratio.

4. 8. The use of NAG_4-β-3, 4 dnp to study saccharide inhibition

From an examination of the possible modes of binding of any saccharide, Fig. 6, whether it plays the role of a substrate or of an inhibitor, it is apparent that Km (app.) should be the same as Ki (app.) except for mode 4 contributing to the former, and not the latter, but this should be insignificant. This is provided that the former is based on a simple mechanism for hydrolysis. Mathematically, these two quantities are expressed by equations (6) and (13) respectively.

From the results of tables 21 to 27, it can be seen that, as expected, the k cat values do not alter in the presence of an inhibitor, within experimental error, and that contrary to the findings of Osawa, non substrates, such as α-methyl NAG, are still competitive inhibitors, as are substrates.

An examination of the Km (app.) values, table 40, and the Ki (app.) values, table 45, yields the following points:

1. The most reliable Ki (app.) values were obtained for paranitrophenyl glycosides and for reducing sugars. All the paranitrophenyl glycosides showed a lower Ki (app.) value than the corresponding reducing sugars, about 3-fold for the NAG_2, 2-fold for the NAG_3, and 1.5-fold for the NAG_4 compounds. This is qualitatively in agreement with the findings of Otson[67], but the difference found in these studies, for NAG_2 compounds, is about 10-fold less.

This result is at first, surprising, since kinetic studies (4. 2) have shown that there could be unfavourable interactions

of a phenolic aglycone in the cleft. This may be rationalised by the realisation that a different mode of binding is being studied; that is, in the consideration of Ksl, it is mode 9, Fig. 18, and for Ki (app.), the non-productive mode 3, predominates and substantially determines the value of Ki (app.) It is implicit, therefore, that the phenol, being hydrophobic, binds favourably in site D, with ΔG about -0.3 k cal, although this may be otherwise, in site E, or at least, in the latter, the orientation of the phenolic group required for catalytic action implies unfavourable interactions.

The smaller differences between the Ki (app.) values for the glycoside and reducing sugar for the higher oligomers are because the contribution of the phenol is a lower proportion of the total binding energy.

2. There is a thirty-fold decrease in Ki (app.) on increasing sugar chain length from NAG_2 to NAG_3, but only a three-fold decrease on increasing it from NAG_3 to NAG_4, for both reducing sugars and for paranitrophenyl glycosides. In view of the modes of binding which would be expected to predominate, that is, B C for NAG_2 residues, and ABC for NAG_3 residues. This seems reasonable, but contrary to literature results, NAG_4 compounds, whose major mode of binding, 3, Fig. 6, would be expected to have the end residue out of the cleft, past site A, in solution, do have lower values for Ki (app.) than the corresponding NAG_3 compounds.

This is probably because other strong binding modes, 7 and 8, Fig. 6, make a significant contribution to the total value of Ki (app.), equation 13, and that the degeneracy is greater for tetrameric compounds:[59] there are six possible inhibitory modes of binding for NAG_2, seven for NAG_3, and eight for NAG_4.

3. The value of Km (app.) for NAG_4-β-3, 4 dnp is approximately the same as the Ki (app.) for NAG_4-β-pnp, as would be expected from the kinetic analysis, but the value of Km (app.) for the latter is 100-fold greater. Similarly, the Km (app.) values for NAG_2 and NAG_3 paranitrophenyl glycosides are higher than their Ki (app.) values. This can only be construed as further evidence for their complex modes of hydrolysis.[115]

4. It would appear that the values of Ki (app.) are approximately equal to the corresponding values of Km (app.) for NAG_2 and NAG_3 3, 4 dinitrophenyl glycosides. However, these values of Ki (app.) cannot be taken as reliable, since there was no change in the observed value of Km (app.) for the substrate, NAG_4-β-3, 4 dnp, when the inhibitor concentrations were 4×10^{-4} m/l and 2×10^{-4} m/l, respectively, substrate concentrations being around 10^{-5} m/l. This is because, on the second order part of the Michaelis Menten curve, where both inhibitor and substrate are binding to enzyme, some of the former will be binding productively for release of 3, 4 dinitrophenol, and therefore the total amount of 3, 4 dinitrophenol released over the curve will be the same as for the uninhibited reaction, and therefore, inhibition, although it occurs, will not be observed.

The point may be raised, however, that although substrate and inhibitor are both binding to enzyme, there is a lower proportion of the latter in the productive mode. This follows from section 4.7.3.

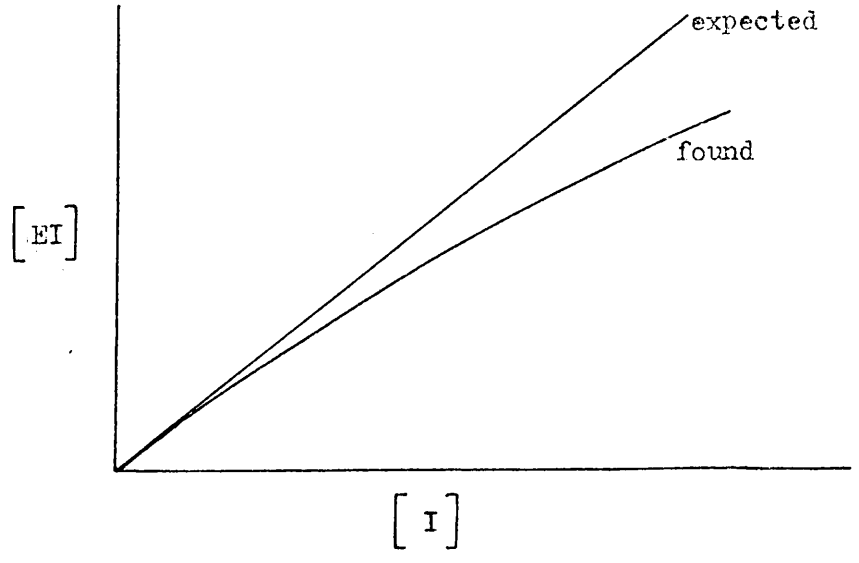
However, the binding of the substrate may affect different inhibitor binding modes in dissimilar ways, and vice versa. For example, NAG_4-β-3, 4 dnp binding in mode 5, Fig. 6, would prevent a molecule of NAG_2-β-3, 4 dnp binding productively but would still allow binding of the NAG residues of the latter in sites B and C.

5. It is unfortunate, that for practical reasons, the inhibitor concentration has to be chosen to be approximately equal to the expected value of Ki (app.), causing a doubling of Km (app.) for the substrate.

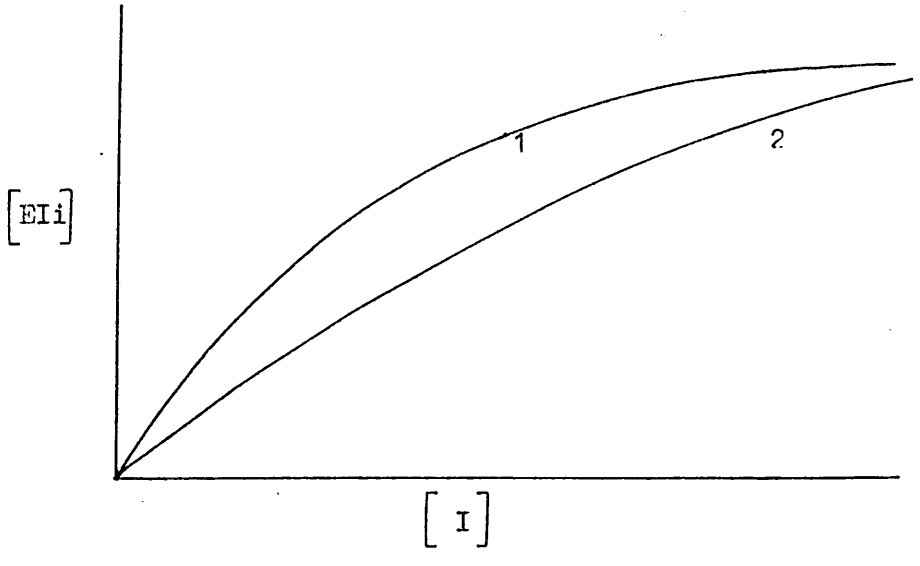
The reason for this is that, at the particular substrate concentration chosen, certain binding modes may predominate, and this may change with alteration of inhibitor concentration: viz: Each binding mode, Fig. 6, has a free energy of binding, and a dissociation constant:

$$Kd = \frac{(E).(I)}{(EI)}$$

This is not an ordinary equilibrium, since normally, $(E)_o \ll (I)_o$, and therefore, although $(I) \simeq (I)_o$, $(E) \neq (E)_o$, and so, as (EI) increases as (I) increases, (E) will decrease substantially, and hence to keep Kd constant, (EI) will not increase as much as expected, Fig. 21 (a)



(a)



(b)

Fig.21

Therefore, each binding mode has a Michaelis-Menten type association, and it is possible to have the situation as in Fig. 21 (b), where a different ratio of the amounts of two binding modes occurs at various inhibitor concentrations, and this is almost certainly what occurs in the hydrolysis of NAG_2-β-pnp, since the ratio of NAG-NAG cleavage to NAG_2-paranitrophenyl cleavage varies some 50-fold with concentration.[115]

This argument invalidates the results of Otson[67], where he compared the release of phenol with NAG-NAG cleavage when the studies of the former were conducted under conditions of 1500-fold higher concentrations of substrate than the latter.

6. It was not possible, using the available data to calculate separate values of Keil, 2, 3 and 4, although in principle this could be done.

7. Inhibition by saccharides larger than NAG_4 was not studied, since their specificities would be higher, for example, NAG_5 has a specificity 5 times greater than for the substrate, and under some of the conditions used, there would be only about 25% of the inhibitor intact at the end of a kinetic run.

4. 9 The use of NAG_4-β-3, 4 dnp for assaying lysozyme

The use of NAG_4-β-3, 4 dinitrophenyl has provided a convenient and accurate method for assaying solutions for lysozyme levels, and since human milk lysozyme also catalyses the hydrolysis of this substrate, tables 28 and 29, there is clinical importance in this result.

The activity of the batches may be more conveniently described by expressing the rates of hydrolyses, under identical conditions, in terms of units of activity per milligram of enzyme, as defined by the International Union of Biochemistry:[110]
"One unit is that amount of enzyme which causes the transformation of one micromole of substrate in one minute, under defined conditions."
This is contrasted with units of activity defined by Shugar,[45] for reaction with Micrococcus Luteus:
"One unit is that amount of enzyme which causes a decrease in optical density of 0.001/minute under assay conditions."

The former was used to calculate the activities of the batches, table 36 (a), and it can immediately be seen that the least active batch of enzyme, Worthington 2.x crystallised, is only some 19% less active than fresh Boehringer, 3.x crystallised. This contrasts with two-fold difference in their quoted activities of 10,900 units/mg; and 20,000 units/mg., respectively, measured in different laboratories using the method of Shugar. This illustrates the dependence of Shugar's method on the cell wall preparation used.

It is interesting to note that Miles Grade I, 6 x crystallised

is little, if any, more active than Boehringer 3 times crystallised enzyme, table 36 (b), especially since the former has a nitrogen analysis equivalent to the theoretical [109], and with a very much lower salt, or ash content.

It may be speculated that activity is therefore dependent on salt concentration, or ionic strength, as has been found for hen egg-white and human milk lysozymes with Micrococcus Luteus, [120, 88] but a comparison of the results of tables 18, 19 and 20 disprove this, for the synthetic substrate, NAG_4-β-3, 4 dinitrophenyl. Possibly the mechanisms are not the same, since cell-wall hydrolysis exhibits a rate maximum at pH of about 7, whereas, synthetic substrates have theirs at pH = 5.

The only explanation of this, then, is that the Miles I enzyme contains inactive protein, and this is borne out by the finding that this batch contained insoluble, possibly denatured material, which had to be removed by filtration before solutions could be used.

The question raised by Sykes that there is specific interaction between acetate buffer and lysozyme, has also been disproved, or at least any binding is so weak that it does not alter the kinetic parameters for NAG_4-β-3, 4 dinitrophenyl in either citrate buffer, table 7, or acetate buffer, table 14, at pH 5.08, within experimental errors. Altering acetate concentration 5-fold, at constant ionic strength has no effect either, table 18 against table 19.

Although NAG_4-β-3, 4 dinitrophenyl is a good substrate for

directly comparing the activities of enzyme batches, its absolute sensitivity is much less than that of Micrococcus Luteus. A short calculation shows that equal weights of enzyme would cause absorbance changes of 1000 times greater with the cell wall preparation, and although the noise would probably be greater, the sensitivity of this method would almost certainly be about 10^2 better.

Some hydrolyses were performed under conditions more appropriate to spectrometers less sensitive than the Cary 16, table 36 (b), and this also gives an indication of the reproduceability of the kinetic runs. This is only about 90%, although the standard deviations are about 0.5%.

With the Cary 16 - on line system, it was possible to obtain kinetic results down to $(E)_o = 1 \times 10^{-7}$ m/l.

In conclusion, therefore, NAG_4-β-3, 4 -dinitrophenyl is accurate for measuring enzyme concentrations down to 5×10^{-7} m/l on an absolute basis, and for comparing the activities of several solutions. It is not sufficiently sensitive to measure very low enzyme concentrations, for example 10^{-8} to 10^{-9} m/l.

4. 10 The catalytic action of human, duck II, and duck III lysozymes on NAG_4-β-3, 4 dnp

Two samples of human milk lysozyme were studied for activity on NAG_4-β-3, 4 dnp, tables 28 and 29. There was no way that the absolute concentrations of enzyme could be determined, since insufficient material was available for analysis, and samples were probably impure. The values of V max, therefore, were a result of the action of 10 microlitres of solution of the concentrations by weight, as stated. Values for k cat were calculated by assuming an extinction coefficient similar to that for hen egg-white lysozyme, and measuring a concentration from the U.V. absorbance, which allows for non-absorbing impurities in the sample.

It can be seen that there is very little difference in the Km (app.) values for both the samples, and that they are similar to that for hen egg-white lysozyme.

If human milk lysozyme has the same tertiary structure as human leukaemic lysozyme, this result is not surprising, because of the similarity of the cleft.

Since Km (app.) is substantially a measure of binding of the substrate in subsites A, B and C, it may be concluded that this is very similar to that for hen egg-white lysozyme, and although binding in subsite C for NAG_1 is thought to be slightly greater for human milk lysozyme[18], this could be compensated for by weaker binding in subsite A.

Human milk lysozyme also forms transglycosylation products,[16] but this should be subject to the same restrictions as hen egg-white lysozyme.

The k cat values appear to be slightly lower than for the latter, but this is probably due to inactive protein present. However, it has been found that human milk lysozyme is twice as active towards Micrococcus Luteus as is hen egg-white lysozyme,[15] and it seems unlikely that this relative reactivity is the same for the hydrolysis of NAG_4-β -3, 4 dnp.

The pH of maximum activity of human leukaemic lysozyme has been shown[23] to be 4.2, on NAG_5, but this is certainly not the case for human milk lysozyme on NAG_4-β-3, 4 dnp. Rates of hydrolysis were both much lower, and erratic at this pH, and a full Michaelis Menten plot could not be obtained.

Duck lysozyme II, table 30, had much the same effect on the substrate as hen egg-white lysozyme, although it differs by 19 amino acid residues. The same problem was found in determining the absolute concentration, but contrary to the published findings[16] that its action on NAG_4 and NAG_5 is more rapid than both human milk and hen egg-white lysozymes, this was not found to be so. NAG_1 is believed to bind slightly less strongly to this enzyme than human milk and hen-egg-white lysozymes,[18] but this had no measurable effect on the Km (app.).

Duck lysozyme III, table 31, provided a more interesting case, in that, although the k cat value was similar to that of hen

egg-white lysozyme, again contrary to the findings of Jolles,[16] the Km (app.) value was almost five times that of Duck II lysozyme. This does substantiate the finding that NAG_1 binds more weakly to duck III lysozyme,[18] but implies that the change in the cleft must be fairly marked, although there are only six replacements[25] between their primary structures, Fig. 1. None of these are involved in the binding interactions known to occur in subsites A, B and C of hen egg-white lysozyme, Fig. 10. Although the duck III enzyme differs from hen egg-white lysozyme by 20 residues, presumably the duck II enzyme is fairly similar to the latter in cleft structure, and therefore the duck III lysozyme must differ considerably, possibly in conformation, from the duck II lysozyme, and not allowing favourable binding of the substrate.

Further extrapolations cannot be made until the full X-ray structures are published.

NAG_4-β-3, 4 dnp, either because of there being no "Glu 35",

NAG_4-β-3, 4 dnp, either because of there being no "Glu 35",

or because of its structure. Difficulties were also experienced with solubility of this protein.

4.11 Model Studies

Two systems were studied in detail:

NAG_1-β-2, 4 dinitrophenyl, and

NAG_1-β-fluoride, Figure 11.

4. 11. 1. NAG_1-β-2, 4 dinitrophenyl

A preliminary investigation of the methanolysis of this compound by T L C showed that the glycoside NAG_1-β-O Me was the sole product of reaction:

System 1:1:1 methanol/ethyl acetate/benzene.

Rf values

NAG_1-β-2, 4 dnp	0.55
product	0.3
NAG_1-α- O Me	0.35
NAG_1-β- O Me	0.3

P.M.R. investigation of the dried product, in d5 pyridine, showed greater than 95% of the product was NAG_1-β-O Me; no α - product was observed.

NAG_1-α-O Me

-O-C$\underline{H}_3$		6.72 τ	
$\underline{H}_1$		4.86 τ	JH_1H_2 = 3.5 c/s
-C$\underline{H}_3$	acetamido	7.92 τ	

NAG_1-β-O Me

-O-C$\underline{H}_3$		6.50 τ	
$\underline{H}_1$		5.14 τ	JH_1H_2 = 7.8 c/s
- C$\underline{H}_3$	acetamido	7.92 τ	
H-O-C$\underline{H}_3$	methanol	6.43 τ	

The results of the hydrolysis of this β-glycoside are given in table 37 (a), and Fig. 22. The pH-rate profile is substantially flat from pH 1.18 to 11.29. This corresponds to the spontaneous hydrolysis, which is pH-independent. The k obs may then be taken as ko, the first order rate constant for spontaneous hydrolysis, which is 9.2×10^{-5} s^{-1}. Allowing for the temperature difference, this is several hundred fold greater than for the spontaneous hydrolysis of NAG_1-β-pnp (1.66×10^{-5} s^{-1} at 78.2°).[118]

This implies that the aglycone is being lost in a rate-determining step, with a positive ρ value. Piszkiewicz gives an accurate value of +2.6.[119] It is not unreasonable, therefore, to suppose that the mechanism of spontaneous hydrolysis differs little from that proposed by these workers, that is, intramolecular participation by the 2-acetamido group, to give an oxazoline-type intermediate.

This gives no information about the rate of build-up, with loss of phenol, of an intermediate, relative to breakdown. This was resolved by comparing the rates of methanolysis, as observed spectrophotometrically, against that observed polarimetrically. These were found to be close, within experimental error, runs 106, 107 and 113, and no build up of an intermediate was observed from the polarimetric trace. The final product was, as the NMR confirmed, NAG_1-β-O Me, $(\alpha)_{25}^{D}$ = -16.8° (c = 1), methanol); lit.[73]: $(\alpha)_{20}^{D}$ = -47.1° (c = 1.5, water).

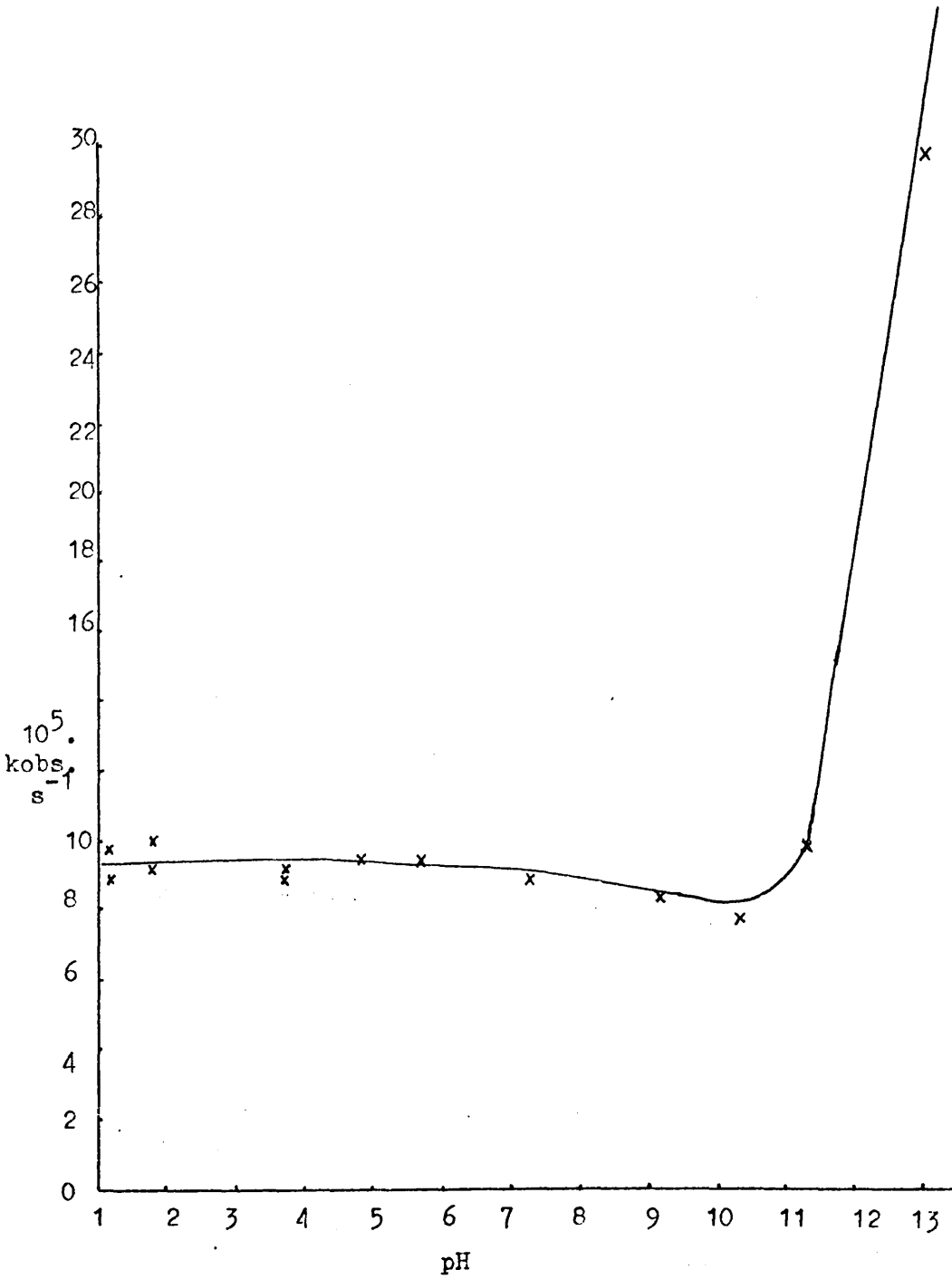


Fig.22 - pH vs. kobs for NAC$_1$-β-2,4dnp.

Therefore, breakdown of intermediate with retention of configuration to give the β-methyl glycoside was faster than build up, and no steady state concentration was observed.

In hydrolysis, specific base catalysis was observed above pH = 11.29, but results were difficult to obtain without large errors in this region, and a value for k_{OH^-} is not quoted.

No specific acid catalysis was observed down to the lowest pH obtainable with ionic strength 0.1 buffers, that is pH = 1.18.

This is to be expected, since the aglycone, 2, 4 dinitrophenol, is acidic, with a pKa of 4.0, and formation of the conjugate acid, with the glycosidic oxygen protonated, would not be favourable.

Specific base catalysis and specific acid catalysis, if it exists, are pushed to more extreme pH's than for the corresponding paranitrophenyl glycosides,[118] and this effect has been observed previously.[119]

This is because of the even greater dominance of the spontaneous hydrolysis rate over the specific acid and specific base catalysed pathways, and these would not be observed until they become very large, at extreme pH's.

An analysis of simple free energy versus reaction co-ordinate suggests that acetals can be susceptible to general acid catalysis in their hydrolysis, if the acetal oxygen is made less basic, and/or the carbonium ion, which could result from cleavage of a glycosidic oxygen-carbon bond, is made more stable.[112]

This results in unfavourable specific acid catalysis, and the enhancement of general acid catalysis, which sometimes can be observed.

The reproduceability of the hydrolysis of NAG_1-β-2, 4 dnp is shown in runs 1270 to 1274, table 37 (a). Five runs under identical conditions gave four runs within 1.2% error, and one run 6% low. Solutions were from a stock, since equal concentrations aided reproduceability - machine noise is greater at extremes of absorbances.

The general acid catalysis was studied under high concentrations of acetic acid 0.45 to 0.09 M, runs 1286 to 1290, in order to show any slight effect, whilst not being masked by the spontaneous rate.

Within experimental error, no general acid catalysis was observed, although intramolecular general acid catalysis has been observed in the hydrolysis of 2-carboxy phenyl-β-NAG [119], in which the effective concentration of the carboxylic acid is very much larger.

4. 11. 2 NAG_1-β- fluoride

The hydrolysis of NAG_1-β- fluoride was followed in a pH stat. The results of the hydrolyses are given in table 37 (b), and the first runs in distilled water, 1205 to 1213 showed a fall in rate above pH 8. This was attributed to an ionic strength effect, and repetition of these runs in 0.10 M sodium perchlorate showed that the rates were invariant, within experimental error, in the pH range 4 to 10. The limits of this range were defined by the dissociation constants of hydrofluoric acid, which has pKa's of 3.17 and 3.41 at 25°, [113] and the titrator scale, respectively.

Within this pH range, spontaneous hydrolysis was observed, and was some 100 fold faster than for NAG_1-β-2, 4 dnp.

Galactosyl -β-2, 4 dnp [75] was found to have a rate constant of $1 \times 10^{-5} s^{-1}$, pH 3.96, 25°, and the corresponding glucoside would be expected to be slightly lower.

The spontaneous rate of hydrolysis of β-glucosyl fluoride is low, [43] cf. [166], and an upper limit of a first order rate constant at pH5 is $1 \times 10^{-4} s^{-1}$.

This indicates the superiority of the 2-acetamido group over the hydroxide-ion catalysed participation by the 2-hydroxyl, in providing anchimeric assistance for the reaction, but also implies that fluorides are more susceptible to acetamido participation than 2, 4 dinitrophenyl glycosides. This is discussed further in terms of the anomeric effect, 4. 12. 4.

Although the hydrolysis of NAG_2-β-fluoride was less than half as fast, presumably because the carbon 4 substituent hinders

conformational changes in the glycone ring, the half life of this fluoride in water was still only about two minutes, and so the use of these higher oligomer β-fluorides as lysozyme substrates was not pursued in depth, 4.12.4.

P.M.R. showed that the methanolysis of NAG_1-β-fluoride gave NAG_1-β-O Me as sole product. On dissolving this fluoride in deutero methanol, the slow conversion of the β-fluoride to the β-methyl glycoside product could be followed, from the P.M.R. spectral changes, as observed on the Varian H A 100 spectrometer. Two N-acetate methyl peaks and two anomeric protons could be observed, with characteristics:

NAG_1-β-F:

7.983 τ 3H singlet. N acetate methyl

4.82 τ 1H doublet of doublets

JH_1H_2 = 7.8 c/s; JH_1F = 54 c/s

NAG_1-β-OCD_3:

8.005 τ 3H singlet. N acetate methyl

5.68 τ 1H doublet; JH_1H_2 = 8.0 c/s

Retention of configuration was occurring, again greater than 95%. This was determined by the addition of 5% of α-methyl NAG to the β-anomer: the spectrum, in d_4 pyridine, gave an observable α-methoxyl peak, at 6.72τ, and a barely observable anomeric proton, at 4.86τ. An indication of the sensitivity of the spectrometer to impurities was indicated by this.

The methanolysis had a half-life of about 800 seconds; some 10-fold slower than spontaneous hydrolysis, whereas the

2, 4 dinitrophenyl glycoside methanolysed three times faster than its spontaneous hydrolysis. A protonated oxazoline-type intermediate, Fig. 12, was proposed.

Since the analagous 2-methyl oxazoline has a pKa of 5.5,[97] the addition of a strong, non-nucleophilic base should slow the reaction by deprotonation.

On the addition of 1.5 moles of collidine, pKa = 7.5, the overall reaction was very much slower. After forty minutes, three methyl peaks near 8τ could be seen. The two highest field signals corresponded to NAG_1-β-fluoride and NAG_1-β-O Me, Fig. 23 (a).

The three peaks altered as follows:

(2) was decreasing at about the same rate as

(3) was increasing.

(1) was approximately steady,

so that, after 105 minutes, the expansion appeared as in Fig.23 (b); about 20% of peak (1) was present. After four hours, the spectrum was as in Fig. 23 (c), and left overnight, peaks 1 and 2 disappeared, and the product was β-methyl NAG.

Examination of the anomeric region showed a new anomeric proton appearing, corresponding to the lowest field methyl, and therefore an intermediate with the following spectral characteristics was present:

7.967 τ ; 3H; singlet: methyl protons

3.96 τ ; 1H; doublet, sharp; anomeric proton

$J_{H_1H_2}$ = 7.0 c/s

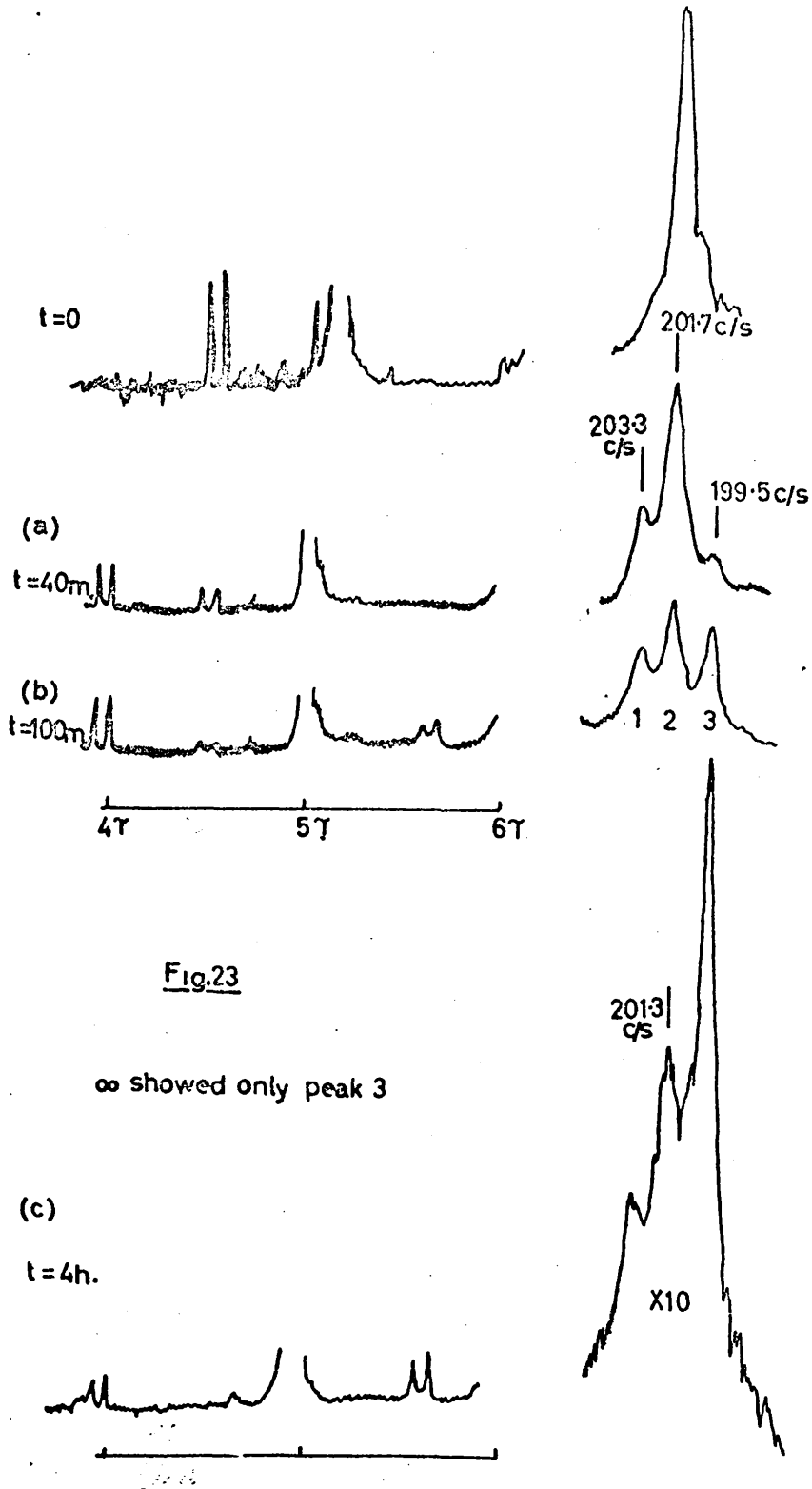


Fig.23

∞ showed only peak 3

The use of a stronger non-nucleophilic base, "proton sponge", which has a pKa of 12.4, gave similar spectral changes, even more slowly, but if the base was dissolved in the deutero-methanol first, then after 90 minutes at 35°, no β -methyl glycoside could be seen. The only anomeric proton observable was the very sharp doublet corresponding to the intermediate, Fig. 24 (a). The β -fluoride anomeric, since half is hidden under the hydroxyl peak, and the line width of the other half of the doublet of doublets, is about twice that of the intermediate, could not be seen. Expansion of the methyls showed that intermediate to fluoride was about 1:1. After 24 hours, there was still intermediate present, although some product had formed, about 30%, Fig. 24 (b).

The solution I.R. of this reaction, run in 0.04 mm. barium fluoride cells, in deutero-methanol showed the amide I of the fluoride at 1647 cm^{-1}, fairly broad, changing to the C = N of the intermediate at 1664 cm^{-1}, sharp, Fig.24(c).

The amide II bands, which are usually lowered in hydrophilic solvents, did not show distinctly from the aromatics in either solution.

On following this reaction by U.V., using tri-n-butylamine the side of the amide $n \rightarrow \pi^*$ peak, at 240 nm showed a decrease of 0.1 AU, in a 10^{-2} molar solution of the fluoride, to a minimum after 2 hours. The absorbance slowly increased then to less than the original value after 36 hours.

Similarly, polarimetric studies showed very small rotation changes, but indicated a two-stage reaction.

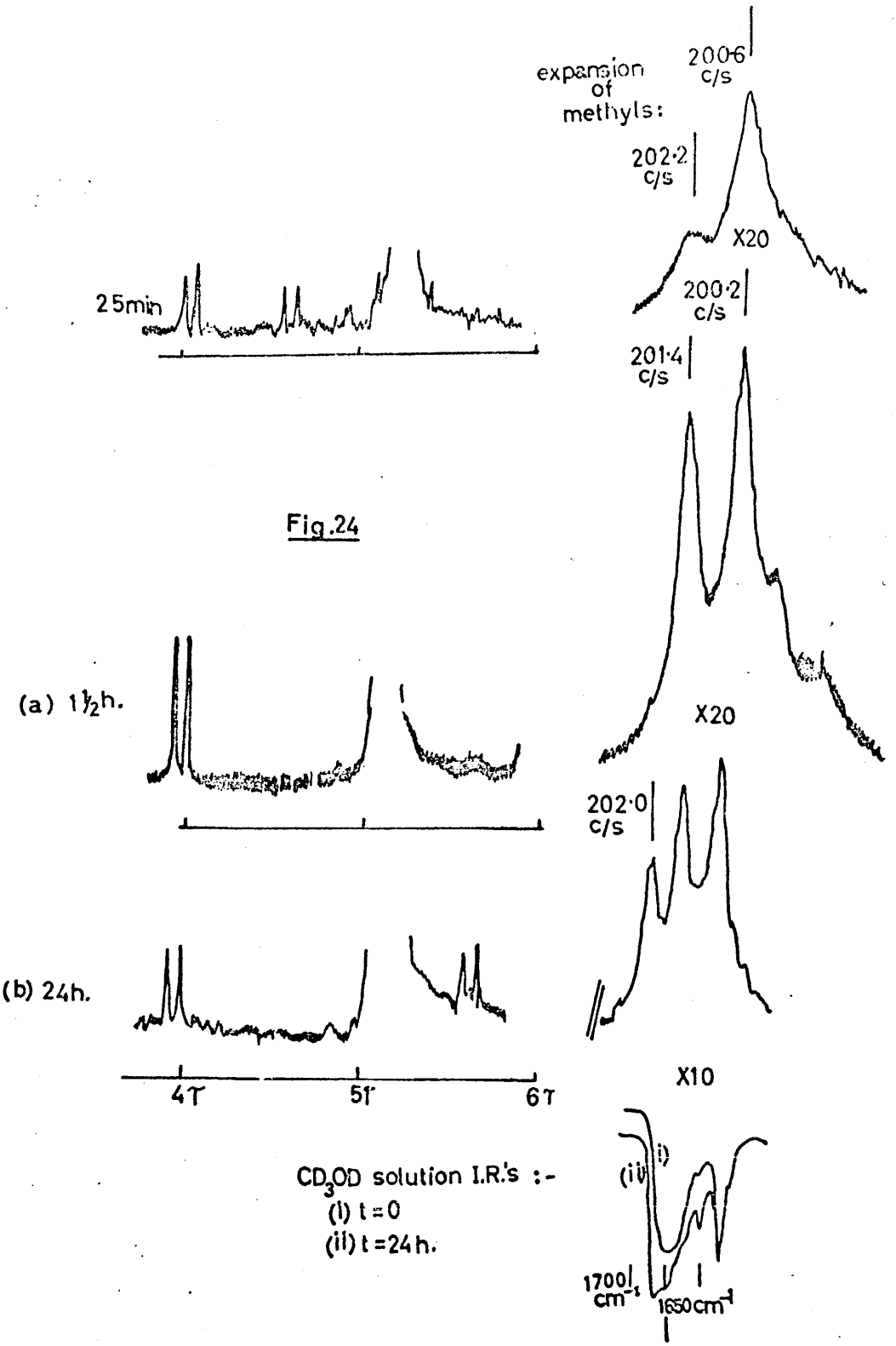


Fig.24

CD_3OD solution I.R.'s :-
(i) t = 0
(ii) t = 24h.

This evidence indicates that the intermediate was the non-protonated oxazoline, Fig. 12, which has been postulated as an intermediate in reactions of many 2-acetamido glycosides, but never observed. It is formed by participation of the 2-acetamido group, and stabilised by the removal of the N-H proton, which is more efficient with stronger bases.

The conformation of this oxazoline can be deduced from the P.M.R.

Applications of PMR spectroscopy to the determination of carbohydrate conformations have been made by many workers, 126 gives leading references. I.R. has been used with pyranosides.[173]

The Karplus relationship[154], when applied to carbohydrates,[153,170] gives calculated coupling constants higher than those observed. This is because of the reduced electron density between vicinal protons, caused by electronegative substituents, and some attempts have been made to rationalise electronegativity effects with orientation.[155, 156, 157] The maximum electronegativity effect is observed when the substituent and a proton are trans diaxial[155]. For example, replacement of a C_1 - OH by fluorine would cause a greater decrease in the H_1 H_2 coupling constant when these substituents are α, than when they are β.

Abraham's Jo parameters, used in the Karplus equation,[151, 153] give calculated coupling constants higher than observed, contrary to experimental evidence.[158]

The observed chemical shift of a proton can be related to the electronegativity of a substituent.[159]

Electronegativity = 0.0114 δ internal + 1.78 - where δ is in cps at 60 MHz, for the difference in shift between the α and β protons of an ethyl derivative. This does not take into account magnetic anisotropy effects, almost certainly present in carbohydrates.

The electronegativity can be related to coupling constant.[158]

A method is apparent, therefore, to relate observed chemical shifts to φ, the dihedral angle of the Karplus relationship:
Shift $\longrightarrow$ electronegativity $\longrightarrow$ J obs. $\longrightarrow$ Jo for $180^{\circ} \leqslant \varphi \geqslant 90^{\circ}$
Once Jo for φ in this range has been determined, Jo for $90^{\circ} \leqslant \varphi \geqslant 0^{\circ}$ may be calculated, and also a value of φ, assumed to be 180° for β-glycosides, and calculated for the oxazoline. This method avoids magnetic anisotropy changes, since the comparison of shift and coupling constant is being made within the one molecule, and any change in environment on changing from one molecule to another is avoided, and effective electronegativity is constant in one compound.

Since electronegativity, as defined by Dailey,[159] cannot be determined absolutely, in this system, it is still reasonable to assume that a coupling constant and a shift are proportional to effective electronegativity, within the one system, as found by Williamson[158].

Assuming φ is 180° for both the β-fluoride and the β-methyl glycoside,[170] this gives effective Jo's of 7.52 c/s, and 7.72 c/s respectively, from the Karplus relationship. The value of Jo, for the oxazoline would then be expected to be (7.38 - 1.09) c/s = 6.29 c/s, taking into account both the effective electronegativity,

Table 46

	δH_1	c/s	Relative effective electronegativity	J obs c/s	Jo c/s	
β-fluoride	5.18	310.8	5.32	7.8	7.52	180°
β-methylglycoside	4.32	259.2	4.73	8.0	7.72	180°
oxazoline	6.04	362.4	5.80	7.0	6.3	0°

and the range of φ , between 0^{o} and 90^{o} for the oxazoline.

The found value of J for the oxazoline is 7.0 c/s, table 46, giving a value of $\cos^{2}\varphi$ of 1.1, from the Karplus equation: $J = Jo \cos^{2}\varphi - 0.28$.

It can only be assumed that within experimental error, and the limitations of this method, that the dihedral angle between protons H_1 and H_2 is 0^{o}, that is they are eclipsed.

The use of transition metals to assist the solvolysis of fluorides has provided rate increases of up to 10^{7} with zirconium,[128,136,145] but the use of these salts with NAG_1-β-fluoride in methanol, gave immediate conversion of the fluoride to β-methyl NAG. This followed from catalysis of the oxazoline opening by released acid from the zirconium nitrate. When base was added, complexing of the base to the metal ions occurred, and intractable precipitates were formed.

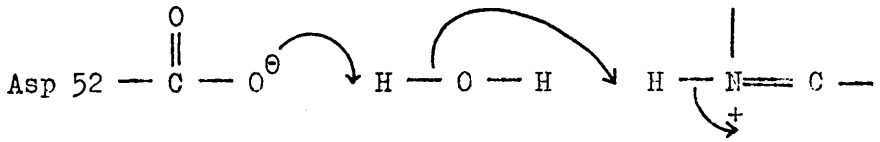
It is interesting to note that the breakdown of the oxazoline intermediate occurs with attack at C_1 only, whereas the hydrolysis of 2-methyl oxazoline proceeds with attack at C_2, probably with formation of a tetrahedral intermediate which breaks down to form O-acetyl ethanolamine as initial product, as deduced from acid inhibition.[27] The difference in the mode of opening is because C_1 has substituents equivalent to an acetal, accounting for its high reactivity.

Attempts to isolate the oxazoline from the reaction media resulted in co-precipitation of sugar and proton sponge, but this should be possible by other methods.

4. 12 Conclusions on the mechanism of action of lysozyme

4. 12. 1. Model studies and acetamido participation.

Model studies have shown that 2-acetamido participation occurs under suitable conditions. This, of course, is not necessarily the case in the lysozyme catalysed hydrolysis of natural substrates, particularly since Asp 52 is not correctly orientated for mechanism (2) Fig. 4, to occur. It is conceivable, however, that Asp 52, when ionised, could act as a general base, catalysing the deprotonation of an oxazoline-type intermediate with the intervention of a water molecule between the amino acid and the substrate:[168]



The consequences of this type of mechanism, if it were to occur, on the pH dependence of a reaction are as follows:

Asp 52 has a pKa of about 4.5, and therefore, at low pH's, the carboxyl would be unionised and unable to act as a general base. The oxazoline intermediate would remain protonated, thereby facilitating the final k_3 step, involving water, Fig. 4.

If k_3 plays a part in determining the value of k cat, which this work has shown it might (4. 6. 1), then the observed rates, as determined by k cat, should increase at pH's below 4.5.

Since model studies have shown that acetamido participation is most likely with good leaving groups, then for both reasons, the most probable place to find this pH dependence is with 2, 4 dinitrophenyl glycosides.

Table 35 (b) shows the results obtained with NAG_2-β-2, 4 dnp, hydrolysis being studied from pH 4.0 to 5.71, showing an enzyme only, rate increase of almost 30% at the lowest pH.

NAG_4-β-3, 4 dinitrophenyl showed a 30% decrease in k cat over the same pH range, tables 10 and 18.

The NAG_2-β-2, 4 dnp substrate concentration, 6×10^{-3} m/l was chosen to be as high as practical considerations allowed, three times the Km (app.) value, at which concentration the enzyme is 87½% saturated. If the Km (app.) changed when the pH was altered, the change would almost certainly be an increase, resulting in an even larger decrease in the observed rates, and not an increase.

There is evidence, therefore for acetamido participation as one of the pathways by which 2, 4 dinitrophenyl glycosides may be hydrolysed by lysozyme. Further weight is given to this argument in (4. 12. 4). It has previously been suggested that electron withdrawing aglycones may enhance the favourability of this mechanism, although the effects of a complex mechanism may obscure results with aglycones no more acidic than paranitrophenol.[91]

It is unlikely that this mechanism is significant with 3, 4 dinitrophenyl glycosides or with natural substrates, since they do not exhibit this pH dependence. Similarly, the hydrolysis of Micrococcus Luteus exhibits a pH optimum at pH 7, and again, some difference in mechanism may be speculated.

4. 12. 2. Titrations and covalent intermediates

The following substrates for lysozyme were studied under rapid initial burst conditions, in order to attempt to detect a covalent intermediate:

NAG_2-β-2, 4 dnp

NAG_3-β-2, 4 dnp

NAG_4-β-pnp

NAG_4-β-3, 4 dnp

NAG_2-β-fluoride

In order to observe a burst, the magnitude of which is given by equation (9):

$$\Upsilon = (E)_o \times \frac{k_2}{k_3} \times \frac{P}{NP}$$

if the essential conditions $(S)_o \geqslant$ Km (app.) and $(S)_o \gg (E)_o$, are preserved.

The experiments were conducted at pH's of 5.0, 4.5, 4.0 whilst monitoring for phenol release at wave lengths appropriate to the absorbance of the ionised phenol, for 2, 4 dinitro and 3, 4 dinitrophenols, and at pH 3.0, whilst monitoring at wave lengths of maximum extinction coefficient difference between glycoside and unionised phenol for the dinitrophenols, and similarly at all pH's for paranitrophenol. These wavelengths were 330 nm 3, 4 dinitrophenol and 350 nm for paranitrophenol. The measured $\Delta\epsilon$ values were 1640 and 2680 respectively.

In no experiment was a titration observed.

The titration of NAG_2-β-fluoride, followed in a pH stat,

and results corrected for lysozyme addition, did not show any rapid initial burst. π , the observed burst size, must be reduced by at least the ratio of productive to non-productive binding.

The maximum value of this factor is 0.003 at pH 5, and 0.0003 at pH 3, for NAG_4 compounds (4.2); and 0.0001 at pH5 for NAG_2 compounds (4. 7. 3)

The concentration of any covalent intermediate, if it did exist, would be negligible, and therefore it is justified not to include this species in the equations of enzyme conservation (1.3)

The effect of methanol on the rate of hydrolysis of NAG_4-β-3, 4 dnp is presented in table 18 (b).

There is a 20% reduction in the k cat value with 2 M methanol; compare table 18.

This is larger than the experimental error and may be due to small pH changes on addition of methanol, as well as other effects.[149] This is in contrast to the four-fold increase in k cat observed for the β-galactosidase catalysed hydrolysis of 2, 4 - dinitrophenyl-β - galactopyranoside.[149]

It would perhaps have been more pertinent to have studied this effect with NAG_3-β-2, 4 dinitrophenyl, but accurate results were difficult to obtain with this substrate.

With both these methods of investigation, only a positive result is meaningful; that is the negative results obtained here do not preclude the existence of a covalent intermediate.

4. 12. 3. The carbonium ion mechanism and lactones

The preparation of NAG_4 lactone by the method of Secemski [35] was attempted. One mole of iodine was consumed, and a product was obtained which had a weak carbonyl absorption at 1735 cm^{-1}. It was recrystallised from DMSO/acetone.

This material hydrolysed at pH = 5, 25°c. with a half life of 11 hours, with an initial uptake of 15% and hydrolysed to 98% of theoretical, based on a molecular weight of 830. At pH = 7, the hydrolysis was more rapid. The hydrolysed product exhibited a pKa of 3.0, but was not pure by T L C. Lysozyme did not catalyse its hydrolysis, and the ferric chloride-hydroxylamine test was negative. [171, 172]

The material gave a Ki (app.) of 4×10^{-5} m/l, using NAG_4-β-3, 4 dnp as substrate.

Secemski quotes $t\ \frac{1}{2}$ = 105 minutes under these conditions, a positive ferric chloride test, with the 23% lactone present, and a 20-fold acceleration in hydrolysis rate by 10^{-3} m/l lysozyme at pH 5.

Other attempts were made to prepare $NAG_1\gamma$, and NAG_2, and $NAG_3\delta$ lactones by the method of Findlay, [68] using mercuric oxide.

Compounds with the following characteristics were obtained, all of them non-crystalline and with a syrupy consistency:

	$t_{\frac{1}{2}}$ - seconds; pH = 5, 25°.	ν c = 0, cm^{-1}
NAG_1 γ lactone	120	strong, 1786
NAG_2 δ ''	180	'' 1740
NAG_3 δ ''	180	'' 1740
cf: glucono γ ''		'' 1790
glucono δ ''		'' 1730

None of the NAG lactones prepared gave ferric chloride tests, although Couling has found that NAG_1 γ lactone does.[172] They were impure by T.L.C. Spectrofluorimetric binding studies with these materials did not show any decrease in the Ki (app.) values over the reducing sugars.

This work was not continued, but the following points may be raised about the binding of transition state analogues to lysozyme.

NAG_4 lactone will only give a lower Ki (app.) if the ABCD mode becomes of greater strength than the ABC mode for NAG_4,[32] which dominates its binding and almost entirely accounts for its Ki (app.) value.

Phillips has shown that NAG_4 lactone does bind ABCD, implying that this mode does overwhelm the ABC mode of NAG_4 lactone; - but ABC may not be a favourable mode for the lactone, if the end residue did not bind well in site C. Therefore, only a positive result, that is a decrease in the Ki (app.) is meaningful.

Further, it could be that site D is not a strong binding site, even if the unfavourable interactions were removed. This is because the strongest binding sites , C and E, rely on hydrogen bonding to the amide group, which sits in the cleft, for their

higher ΔG values.[65] In site D, the amide group does not bind in the cleft.

Any decrease in Ki (app.), and Secemski has observed this, using Micrococcus Luteus as substrate, does not necessarily imply that this factor is "the contribution to catalysis", but that the ABCD for the lactone is more favourable that the ABC mode of the reducing sugar. These two factors may fortuitously coincide, since distortion in the productive complex, Fig. 18, may not be as pronounced.

Further, any meaningful interpretation in relation to a carbonium ion mechanism, involving a half chair conformation of the residue in site D, must be based on the lactone being in this conformation. Phillips has found,[60] by X-ray, that the lactone is in a boat conformation with C_6-OH 'axial' , that does not allow overlap of the ring oxygen orbitals with the sp^2 hybridised C_1 orbitals, which is required for stabilisation during a carbonium ion mechanism.

A better measure of the contribution to catalysis of distortion would be an analysis, based on 1.3.7, of the binding of oligomers containing N-acetyl xylosamine in their end residue.[51]

If the carbonium ion mechanism is the correct one, then the hydrolysis of their glycosides should still be catalysed when the N-acetyl-xylosamine residue binds in site D, because the catalytic groups can still provide general acid and base catalysis, and although there is no C_6 hydroxyl interaction aiding attainment of the half-chair conformation, there is unlikely to be anything preventing its distortion, and so a truer measure of the site D

contribution to catalysis should be obtained.

Unfortunately if the $\underline{C_6}$ hydroxyl is removed, then the productive complex distortion, Fig. 18, is likely to be altered, along with the total favourable binding which could exist after distorting, through hydrogen bonding to the $\underline{C_6}$ hydroxyl. If it were removed from the unfavourable interaction, as with the lactones, this would have to be taken into account in the estimation of the contribution to catalysis from site D interactions, as distinct from general acid catalysis and general base catalysis.

The beneficial effect that the removal of these interactions would have, would manifest itself in a decrease in the binding constant for the productive mode, Ks1, (4.2). This contributes 0.25% to the value of Km(app.) for NAG_4-β-3,4 dnp, and similarly to a Ki(app.). Chipman has quoted a 40 to 50-fold decrease in Ki(app.), when comparing NAG_3-NAX and NAG_3[51] On extension of this to NAG_3-NAX-glycosides, these factors would have to be taken into account.

4. 12. 4. The lysozyme catalysed hydrolysis of NAG_2-β-fluoride

The hydrolysis of NAG_2-β-fluoride was studied under conditions which were expected to be second order, from the parameters for NAG_2 glycosides, table 40. The results are shown in table 47, and the ratio k cat/Km (app.) may be estimated by the methods of (4. 5. 2). The value of k cat/Km (app.) is about 15 to 20 l/m/s at 25°. At 40°, this would probably be about 45 to 60 l/m/s, that is a factor of 100 higher than the k cat/Km (app.) ratio for NAG_2-β-2, 4 dnp, table 40, and some 1000-fold greater than NAG_2-β-3, 4 dnp.

From these results, a lower limit can be placed on the value of k cat for NAG_2-β-fluoride. Run 1291, approximately Michaelis Menten conditions, indicates that k cat $\geqslant$ 0.03 s^{-1}.

The result that the k cat value for the fluoride is at least 20-fold higher than that for NAG_2-β-2, 4 dnp, most likely implies that the productive complex is no longer distorted, as in Fig. 18, but that the fluorine atom does not cause steric hindrance, with the result that the NAG residue above site D can 'fit in' more easily. This does not allow for any unfavourable interactions in site D. Another explanation would be that the mechanism is changing.

It is most interesting to note that the β - fluorides react 100-fold faster than the 2, 4 dinitrophenyl glycosides, in their hydrolyses, both in model systems, 4. 11. 2, and in their catalysis by lysozyme, although the fluoride ion is only 0.6 pKa units more acidic than the phenol ;- table 48.

A probable cause of this phenomenon is the higher ground state energy of the fluoride, caused by the so called "anomeric effect."

Table 47

The lysozyme catalysed hydrolysis of NAG_2-β-fluoride

25°c. pH 5.0. distilled water. pH stat

$(S)_o = 2 \times 10^{-3}$ m/l; 8.52 mgs in 10 ml.

		Initial rates m/l/s		
Run	$(E)_o$ m/l	Enzyme + spontaneous	spontaneous	enzyme
1290	-	-	1.8×10^{-6}	-
1291	2×10^{-4}	7.5×10^{-6}	1.8×10^{-6}	5.7×10^{-6}
1292	4×10^{-4}	18.6×10^{-6}	1.8×10^{-6}	16.8×10^{-6}
1293	5×10^{-3}	too fast to follow		

Table 48

Glycoside	k_o s^{-1} at 25°	
NAG_1-β-F	7×10^{-3}	(a)
NAG_1-β-2, 4 dnp	7×10^{-5}	(b)
NAG_1-β-pnp	$\simeq 2 \times 10^{-7}$	(c)
Glucosyl-β-F	$\leqslant 1 \times 10^{-4}$	(d)
Glucosyl-β-2, 4 dnp	$\simeq 6 \times 10^{-6}$	(e)
Glucosyl-β-pnp	$\simeq 6 \times 10^{-10}$	(c)

(a) Table 37 (a)

(b) Table 37 (b)

(c) Estimated from 118, since experiments carried out at 78.2°c.

(d) Estimated from 43.

(e) Estimated from the figure obtained for galactosyl-β-2, 4 dnp, of 1×10^{-5} s^{-1}, 25°, I = 0.1 .

This effect makes an equatorially attached group on the anomeric carbon atom less stable than it would be at other positions on the ring. The original explanation was that unfavourable dipole-dipole interactions occurred between the carbon-oxygen bond of the ring and the bond from C1 to the aglycone,[163] and that these were at a maximum when the effective charge density on the substituent atom was highest,[164] as occurs in the fluoride. This explanation has been criticised,[165] gives leading references, and the more favoured explanation is that α anomers are stabilised by back donation of lone pair electrons from the ring oxygen into antibonding orbitals of the α substituent.

The anomeric effect amounts to some 0.55 k cal for β-glucose in water,[165] but this is increased in non polar solvents, and with electronegative substituents. The former explanation, however, is more attractive here, since the latter does not explain the high reactivity of NAG β -fluorides.

This high reactivity cannot be attributed to the anomeric effect only, since β glucosyl fluoride is slower in its enzyme catalysed hydrolysis than even a mono nitro-phenyl glucoside,[41] and the rate of its spontaneous hydrolysis is low.

There is probably also an effect due to the steric hindrance, afforded by an aryl aglycone, to ring conformational changes during hydrolysis, avoided with the fluoride.

The 2-acetamido group must jointly play a part in the relative reactivity of the β-fluoride and the 2, 4 dinitrophenyl glycoside, since it does appear that the fluoride is very much more susceptible

to anchimeric assistance by the amide carbonyl than is the aryl glycoside.

Since tri-O-acetyl-β-D-Xylopyranosyl chloride completely inverts in solution, to assume the $^{1}C_{4}$ conformation, rather than the normally more stable $^{4}C_{1}$ conformation, [167] again because of the anomeric effect, then the energy barrier to forming the oxazoline intermediate is reduced, and hydrolysis would be more rapid.

Since acetamido participation has been confirmed during methanolysis, 4. 11. 2, and almost certainly occurs during spontaneous hydrolysis, it may be speculated that, since the acceleration of both enzymic and spontaneous rates is approximately similar with NAG β-fluorides, similar mechanisms are occurring in spontaneous and lysozyme catalysed reactions.

This adds further weight to the hypothesis that acetamido participation occurs during the lysozyme catalysed hydrolysis of glycosides with good leaving groups, 4. 12. 1.

4.12.5. Possible future methods of investigating lysozyme mechanisms

The β-glycosides of NAG_4 were found to be poorer substrates than expected, due to the form of the productive complex, Fig. 18. In order to make use of the binding interactions of sites E and F, to overcome this, estimated at some -4 k cal. and -1.4 k cal. respectively[65] whilst still retaining the advantages of a spectrophotometric substrate, the glycoside, Fig. 25 is suggested as a likely candidate.

Sites A, C and E are strong binding sites, because the acetamido groups are orientated into the cleft, and B and F are sites of weaker interactions, mainly hydroxyl-side chain hydrogen bonds, but perhaps the interactions would be sufficiently favourable to "hold down" the aglycone.

The synthetic problem is not as formidable as it would first appear. The use of α-Ac NAG_4 Cl as the glycone and the appropriate aglycone, comprising NAG substituted at C4 with 2-acetamido-5-methyl-hydroxy-4-hydroxy-phenol with possibly a nitro group at the 6 position to confer adequate spectroscopic properties, could be coupled by the methods described in Chapter 2.

The synthesis of the aglycone could be achieved by the method described by Whitehouse,[160] and Heyns.[161] These papers describe the preparation of C-4 substituted NAG, the most difficult position to selectively act on.

As well as the possibility of increasing the specificity to that of NAG_6 and greatly enhancing the ratio of productive to non-productive binding, as far as phenol release is concerned, the problems of multiple bond cleavage would almost certainly be

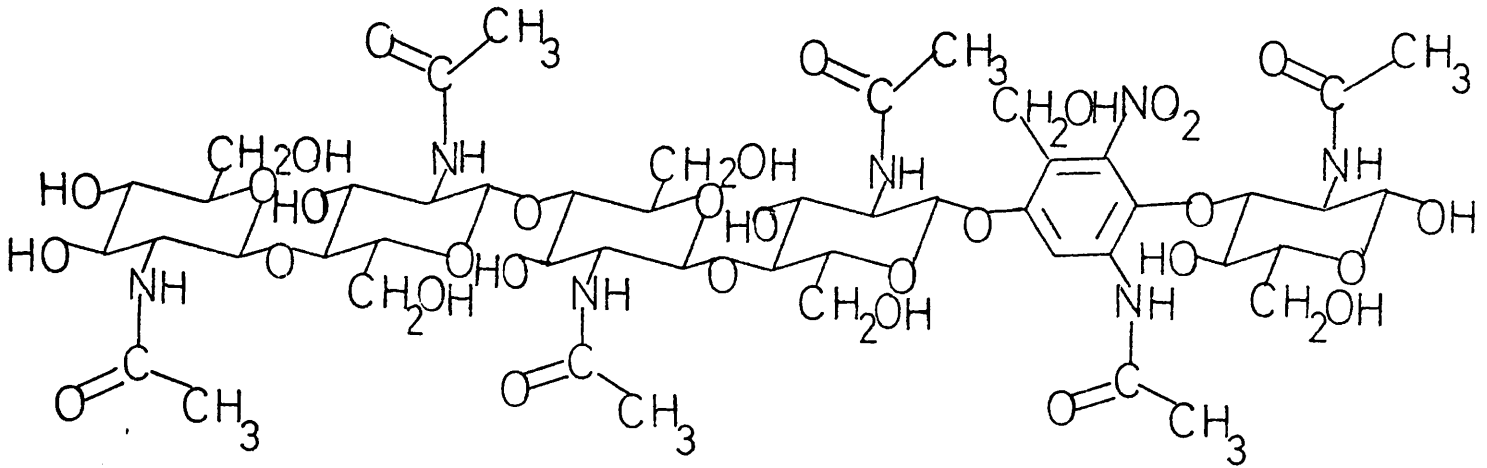


Fig.25

eliminated, since NAG_6 cleaves almost exclusively to NAG_4 and NAG_2.

The acetylated fluoride, $AcNAG_4$-β-F has been prepared (2.1.7). Difficulty was experienced in achieving complete de-O-acetylation, but this should be able to be overcome, given sufficient material.

The high spontaneous rate of hydrolysis to be expected of NAG_4-β-fluoride, table 37 (b), run 1084, would preclude the use of all but relatively high enzyme concentrations, in order to observe an enzymic rate of hydrolysis.

It is unlikely that conditions could be found where any parameter but the k cat value could be studied.

No titration was observed with NAG_2-β-fluoride. Since there is only a 32-fold increase in the productive to non-productive binding ratio under conditions of saturation, with NAG_4 glycosides (4. 7. 3) it still therefore seems most unlikely that a rapid initial burst could be observed with NAG_4-β-fluoride, although it might be with the synthetic substrate suggested, Fig. 25, since the proportion of productive binding would be expected to be greater.

NAG_4-β-fluoride would most likely still be subject to large amounts of non-productive binding, since although the fluorine atom should have no orientation effects, the most favourable modes of binding would still be equivalent to those of the reducing sugar, NAG_4; that is mode ABC would predominate.

The observation of NAG_4 oxazoline, which might be prepared from the fluoride, is unlikely, by P.M.R.; first, since the spontaneous hydrolysis could be very fast compared with any effect

that lysozyme might have on it, and second, it is unlikly that sufficient concentration could be obtained to obtain a spectrum :- NAG_4-β-3,4dnp in D_2O required 49 scans of CAT to observe the four acetamido methyl signals. However, Fourier transform might overcome this difficulty.

Most important, is the realisation that the amino acid carboxyls will generally be in the wrong state of protonation for catalysis of the hydrolysis of this proposed intermediate; see Fig.4, mechanism (2) that is Asp52 is ionised, and Glu35 protonated at the pH optimum- the opposite states to those required for the k_3 step to occur.

Despite its disadvantages, it is possible that the specificity of NAG_4-β-fluoride could approach or exceed that of NAG_6, because of a high k cat value, (4.12.4), probably exceeding 1 second^{-1}. This is estimated from the k cat for NAG_2- -fluoride, of 0.03 s^{-1}, and the 30-fold increase in the productive to non-productive complexes with the NAG_4 compound - table 44. It is doubtful if the ratio k cat/Km(app.) could be determined, since second order conditions could not be used.

In conclusion, some, but not all of the questions raised from literature studies have been answered, but many important new routes of attack have been opened for final and unequivocal determination of the mechanism of action of lysozyme.

5. APPENDIX

```
 5COMMENT MICHAELIS MENTEN PROGRAM
 6T !"TYPE RUN NUMBER"
 7A RN
10T !"TYPE NUMBER OF SUBSTRATE CONCENTRATIONS"
20A N
30
35T !
40T !"TYPE POWER FACTOR FOR RATES FORMAT 1E-X"
45A PR
47T !"TYPE POWER FACTOR FOR SUBSTRATE CONCS. FORMAT 1E-Y"
48A PS
49T !"TYPE INITIAL RATES(M/L/S),%ST.DEVS.,SUBSTRATE CONCS.(M/L)"
50T !!
52F I=1,N;D055/64
53GO 65
55A V(I),EV(I),S(I);T "  //",!
60S V(I)=V(I)*PR
62S EV(I)=EV(I)*V(I)/100
64S S(I)=S(I)*PS
65T !
70T !"TYPE ESTIMATED K-M"
80A A(1,1)
81S A(1,1)=A(1,1)*PS
85T !"TYPE ESTIMATED V-MAX"
90A A(2,1)
91S A(2,1)=A(2,1)*PR
95S P=0
100S P=P+1
110F R=1,3;F Q=1,3;S B(Q,R)=0
120F I=1,N;DO140/270
130GO 280
140
150S DF(3)=V(I)-(A(2,P)*S(I)/(A(1,P)+S(I)))
160S DF(2)=-S(I)/(A(1,P)+S(I))
170S DF(1)=A(2,P)*S(I)/((A(1,P)+S(I))↑2)
180S DX=A(2,P)*S(I)/((A(1,P)+S(I))↑2)-A(2,P)/(A(1,P)+S(I))
190S DY=1
200S WY=1/(EV(I)↑2)
210S WX=1/(S(I)*.01)↑2
220S L=DX↑2/WX +DY↑2/WY
230S Q=1
240F R=1,3;S B(Q,R)=B(Q,R)+DF(Q)*DF(R)/L
250S Q=Q+1
260IF(Q-4),240,270,240
270
280S BD=-B(1,1)*B(2,2)+B(1,2)↑2
290S DA(1)=(B(1,2)*B(2,3)-B(2,2)*B(1,3))/BD
291
300S DA(2)=(B(1,2)*B(1,3)-B(1,1)*B(2,3))/BD
301
310S Q=P+1
315F R=1,2;S A(R,Q)=A(R,P)-DA(R)
320IF(P-5),100,330,100
```

```
330
340
350S SO=B(3,3)-B(3,1)*DA(1)-B(3,2)*DA(2)
351S SO=-SO
355S SD(1)=FSQT(B(2,2)*SO/BO*(N-2))
357S SD(2)=FSQT(B(1,1)*SO/BO*(N-2))
360
365T !"F.BALLARDIE RUN NUMBER",%4.00,RN,"     M.M.PROGRAM"
370T !!!"SUBSTRATE CONC.       EXPERIMENTAL RATE          CALC.RATE'
371T !"  MOLES/LITRE          MOLES/LITRE/SEC           M/L/S"
380F I=1,N;DO400/410
385S P=P+1
390GO420
400S BA(I)=A(2,P)*S(I)/(A(1,P)+S(I))
410T !,%,S(I),"    ",V(I),"     ",BA(I)
415
416
420T !"  VMAX                          K-M          ITERATED"
430F I=1,P;T !A(2,I),"    ",A(1,I)
440T !"                VMAX                    K-M"
450T !"CALCULATED",A(2,P),"    ",A(1,P)
460T !"ESTIMATED ",A(2,1),"    ",A(1,1)
470T !"STAND.DEV.",SD(2),"    ",SD(1)
500T !!!"FULL DATA LIST YES TYPE 1 /NO TYPE -1/"
502A Z
503IF(Z),550,550,505
505T !"RESIDUALS      VEXPT-VCALC."
510F I=1,N;DO520/530
515GO540
520S BB(I)=V(I)-BA(I)
530T !,BB(I)
540
550QUIT
```

(i): A program to calculate Michaelis Menten parameters from initial rates and substrate concentrations.

15.52.25

```
1/CRD-A/000012/000020/000045

*ALGOL
'BEGIN''REAL'PER;'INTEGER'F1,F2,N,M,Q,O,I,J,NO,K,CO,L;
'ARRAY'PH,H,K,KO,KI,OH[1:20],SUM[1:61];
OPEN(10);OPEN(20);Q=4;
F1=FORMAT('['NE'DD.DD']');F2=FORMAT('['4S'NE'D.DDDD@'NE'ND']');
START:'FOR'M=1'STEP'1'UNTIL'Q'DO'K[M]=READ(20);
'IF'K[1]'LT'0'THEN''GOTO'ULT;
NO=READ(20);K=1;
'IF'NO'LT'0'THEN''GOTO'CALC;
'IF'NO'EQ'9999'THEN''GOTO'AUT;
N=READ(20);
'FOR'M=1'STEP'1'UNTIL'N'DO''BEGIN'PH[M]=READ(20);
OH[M]=EXP(2.3026*(PH[M]-13.2617));H[M]=EXP(-2.3026*PH[M]);
KO[M]=READ(20);'END';J=1;
CALC:SUM[J]=0;K=K+1;'IF'K'GT'51'THEN''GOTO'CONT;
OG:'FOR'M=1'STEP'1'UNTIL'N'DO''BEGIN'
KI[M]=K[1]/(1+H[M]*K[2]+1/K[3]+K[4]/H[M]);
SUM[J]=SUM[J]+((KO[M]-KI[M])/KO[M])**2;'END';
'IF'NO'EQ'9999'THEN''GOTO'TEST;
OUT:OUTPUT(10,J);'FOR'M=1'STEP'1'UNTIL'Q'DO'OUTPUT(10,K[M]);
WRITETEXT(10,'['''['C']'SUM'*'OF'*'SQUARES'*'OF'*'RESIDUALS='']');
'IF'J'EQ'1'THEN''GOTO'DUCK;'IF'NO'EQ'9999'THEN'J=J-1;
DUCK:CHAROUT(10,61);OUTPUT(10,SUM[J]);OUTPUT(10,K);
WRITETEXT(10,'['PH'['8S']'KOBS'['11S']'KCALC'['10S']'RESIDUALS']');
'FOR'M=1'STEP'1'UNTIL'N'DO'
'BEGIN'NEWLINE(10,1);WRITE(10,F1,PH[M]);WRITE(10,F2,KO[M]);
WRITE(10,F2,KI[M]);WRITE(10,F2,KO[M]-KI[M]);
'END';CHAROUT(10,61);'GOTO'START;
CONT:WRITETEXT(10,'['CONTINUE']');CHAROUT(10,61);
CO=READ(20);'IF'CO'LT'0'THEN''GOTO'OUT;J=1;K=1;'GOTO'OG;
AUT:PER=READ(20);
RES:I=1;L=1;O=1;
SOK:J=1;'GOTO'CALC;
REP:K[I]=K[I]+0.01*PER*K[I]*L;'GOTO'CALC;
TEST:J=J+1;
'IF'J'EQ'2'THEN''GOTO'REP;'IF'SUM[J-1]'LT'SUM[J-2]'THEN''GOTO'REP;
K[I]=K[I]-0.01*K[I]*PER*L;
L=-L;'IF'J'EQ'3'THEN''GOTO'DOK;
ADV:I=I+1;L=1;
'IF'I'LT'Q+1'THEN''GOTO'SOK;'GOTO'RES;
DOK:'IF'L'LT'0'THEN''GOTO'SOK;
'IF'O'EQ'Q'THEN''GOTO'OUT;O=O+1;'GOTO'ADV;
ULT:CLOSE(10);CLOSE(20);
END'
ENDJOB

**
```

```
NOT ENOUGH DATA.  TYPE MORE TO RESUME? 6.270-1;3.670+6;4.770-3;5.040-4;
? 9999;
? 1;
?
CONTINUE
NOT ENOUGH DATA,  TYPE MORE TO RESUME? 1;
CONTINUE
NOT ENOUGH DATA,  TYPE MORE TO RESUME? -1;
+2.0000 0000 000 @+ 0;
+6.3308 0037 998 @- 1;
+3.0620 5300 205 @+ 6;
+4.3130 3976 042 @- 3;
+2.9284 9294 067 @- 4;

SUM OF SQUARES OF RESIDUALS=+6.3578 8678 879 @- 1;
+5.2000 0000 000 @+ 1;
PH          KOBS              KCALC             RESIDUALS
+04.02      +1.6400@- 3       +1.2104@- 3       +4.2963@- 4
+04.40      +1.5800@- 3       +1.7660@- 3       -1.8599@- 4
+04.88      +2.1900@- 3       +2.1645@- 3       +2.5476@- 5
+05.04      +2.2400@- 3       +2.1833@- 3       +5.6687@- 5
+05.08      +2.0100@- 3       +2.1785@- 3       -1.6854@- 4
+05.19      +1.6900@- 3       +2.1460@- 3       -4.5600@- 4
+05.35      +1.5750@- 3       +2.0490@- 3       -4.7396@- 4
+05.71      +1.3100@- 3       +1.6438@- 3       -3.3377@- 4
+06.08      +1.1700@- 3       +1.0884@- 3       +8.1562@- 5
+07.03      +4.3000@- 4       +1.8968@- 4       +2.4032@- 4
```

(ii) : A program to fit kcat to pH ; and the best fit output near the pHmax for NAG_4-β-3,4 dnp.

(iii)
```
 1T !"TYPE NUMBER OF C H N O F"
10A C,H,N,O,R
20S T=12.01115*C+1.00797*H+14.0067*N+15.9994*O+19.00*R
30T !!"PERCENT CARBON",%6.04,100*(12.01115*C)/T
40T !"PERCENT HYDROGEN",100*(1.00797*H)/T
50T !"PERCENT NITROGEN",100*14.0067*N/T
60T !"PERCENT OXYGEN",100*(15.9994*O)/T
70T !"PERCENT FLUORINE",100*(19.00*R)/T
80T !"MOLECULAR WEIGHT",%9.04,T
90QUIT
```

(iv)
```
WRITE
10COMMENT NON PRODUCTIVE BINDING PROGRAM
20T !"TYPE K2,K3,K4."
25
27
30FOR J=2,4;A K(J)
35T !"TYPE INCREMENTS OF PH"
37A N
38S Q=1/N
39S R=10/N
40FORI=Q,R;DO60/78
50GO79
60S H(I)=FEXP(-2.3026*I*N)
70S R(I)=(1/K(3)+H(I)*K(2)+K(4)/H(I))
75
78S R(I)=1/(R(I))
79T !"PRODUCTIVE TO NON PRODUCTIVE BINDING RATIO"
80T !"   PH     P/NP RATIO"
90FOR I=Q,R;T !,%4.02,I*N,"  ",%,R(I)
```

(iii) : A program to calculate percentage compositions

(iv) : A program to calculate P/NP ratios.

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