CHEMICAL MODIFICATIONS OF LYSOZYME

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CHAPTER 1: INTRODUCTION

Lysozymes, the members of a class of enzymes having a closely defined glycolytic activity, are widely distributed in Nature, but that from hen's egg white, which was discovered by Fleming in 1922,[1] has been by far the most thoroughly studied. The determination of the 3-dimensional structure of hen's egg white lysozyme by X-ray crystallography[2,3] made it the first enzyme to be fully structurally characterised. Since then, in contrast to the situation with most enzymes, where kinetic, binding and model studies in association with specific active site modification work have dominated the understanding of catalytic mechanisms, further X-ray crystallographic work provided a basis on which the mass of data accumulated has been rationalised. This unusual situation is due at least in part to the nature of hen egg white lysozyme crystals, which are such that inhibitor molecules may readily be made to diffuse into the active site, thereby permitting valuable crystallographic studies to be made on enzyme-inhibitor complexes.

Lysozyme* has long been known to possess activity against certain strains of bacteria, and against Micrococcus lysodeikticus in particular,[1] and this was later shown to be due to cleavage of the cell wall mucopolysaccharide.[4] The

* Where not otherwise stated 'lysozyme' henceforth refers to hen egg white lysozyme.

first 'non-bacterial' substrate for lysozyme which was discovered[5] was chitin, the β(1 → 4) linked polymer of N-acetyl glucosamine. The susceptibility of this polymer to the enzyme shows that it also possesses β-glucosaminidase activity. Further investigation of the products of lysis of M. lysodeikticus cell walls resulted in the isolation of a tetrasaccharide containing equal amounts of N-acetylglucosamine (NAG) and N-acetyl muramic acid (NAM).[6] This could be degraded further by lysozyme to a disaccharide NAG-NAM,[7] which was shown by comparison with the synthetic β(1 → 6) linked disaccharide to possess a β(1 → 4) linkage.[8] The glycosidic link between the two disaccharide units in the tetrasaccharide was first assumed, and later proved,[9] also to be β(1 → 4), the proposed structure of the tetrasaccharide thus being as shown in figure 1. Lysozyme can thus hydrolyse β(1 → 4) linkages between NAM and NAG or between two NAG residues.

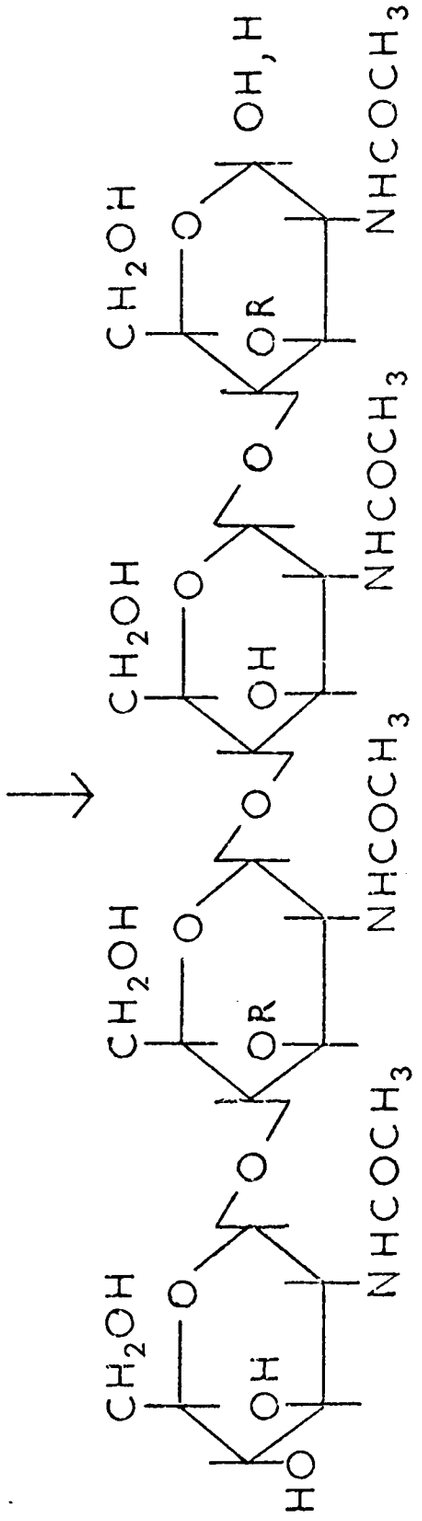
Glycolysis is not however the only reaction catalysed by lysozyme. Transglycosylation is commonly observed with both cell wall[10] and chitin oligosaccharide substrates,[11,12] and the enzyme also possesses the ability to catalyse the hydrolysis of certain carboxylic esters.[13]

The nature of the cell walls

The mucopolysaccharide from which the tetrasaccharide shown in figure 1 is derived is a complex cross-linked macromolecular structure, containing polypeptide chains of both D- and L-amino acids attached to the lactyl groups on some of the

Figure 1

The cell-wall tetrasaccharide from M. lysodeikticus



NAG = N-acetyl glucosamine; NAM = N-acetyl muramic acid

R = $CH_3-\overset{H}{\underset{|}{C}}-COOH$

→ denotes position of cleavage by lysozyme

NAM residues,[9] and having a molecular weight of up to 10^9.[14]

Although many bacterial cell walls are known to possess this type of mucopolysaccharide, in the case of gram-negative bacteria an outer fatty layer protects these species against attack by lysozyme in most cases, unless this layer is damaged in some way.[15] The susceptibility of the cell wall is also strictly dependent on the nature of the saccharide residues; removal of the N-acetyl groups from the amino-sugar residues,[15] or introduction of O-acetyl groups,[16,17] both render the mucopolysaccharide insensitive to lysozyme. It is interesting that resistant strains of M. lysodeikticus, grown in the presence of lysozyme, contain one O-acetyl group per disaccharide unit in the cell wall polysaccharide.[17]

Furthermore, cell walls in which carboxyl groups are protected are less susceptible to attack by lysozyme than normal cell walls[18] which in turn are less good as substrates than cell walls with protected basic groups.[18,19] As lysozyme is a basic protein this effect is attributed to the relative ease with which it may approach the relevant part of the mucopolysaccharide; carboxymethylation of up to three of the lysine residues in lysozyme also enhances its activity against M. lysodeikticus,[20] supporting this theory.

The surprising fact remains however that the natural function of lysozyme is not understood. It appears not to be merely an anti-bacterial agent, as most of the bacteria which are susceptible to lysis by it are relatively harmless to their host; one suggestion which has been put forward is that its function in vivo is to 'clear up' cell wall fragments from bacteria killed in other ways.[21]

The amino-acid sequence of lysozyme

The amino-acid sequence of lysozyme was determined independently by Jolles[22] and by Canfield[23] in 1963, and the positions of the 4 disulphide bridges in the molecule were subsequently determined.[24,25,26] There were a few discrepancies between the two sequences originally published, but these were mostly resolved when the 3-dimensional structure at 2Å resolution was determined. Canfield's sequence,[26] which proved to be the more accurate, is reproduced in figure 2, and shows a single polypeptide chain of 129 residues, (molecular weight ca. 14,500), cross-linked by 4 cystine bridges. It can also be seen that lysozyme is a very basic protein (having in fact 17 basic groups, Lys and Arg, and 10 acidic groups, Asp and Glu). Very recent unpublished evidence has shown that residue 103 is not aspartic acid, as in Canfield's sequence, but is asparagine,[27] and the implications of this resequencing will be discussed later.

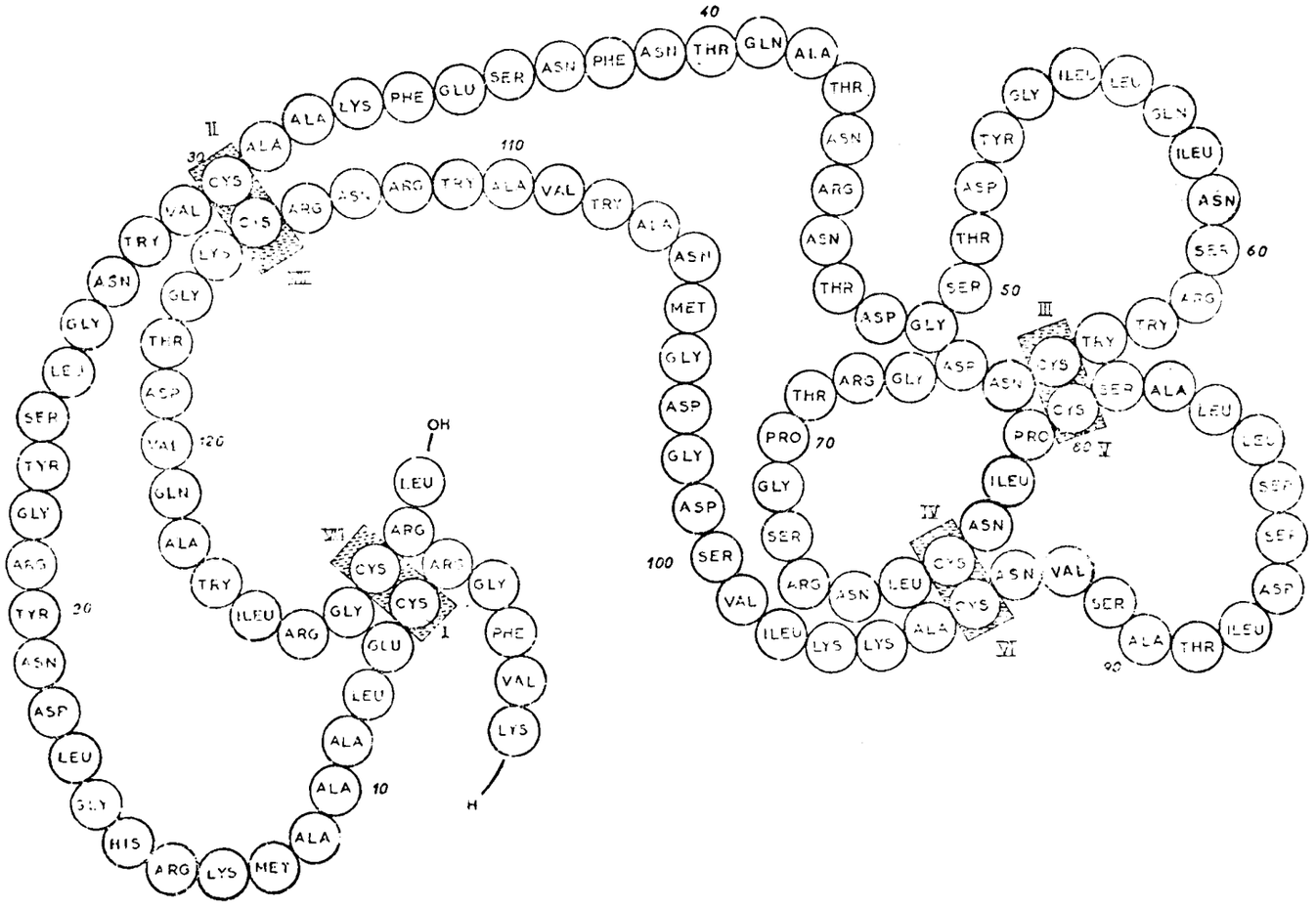
The modification of amino-acid side chains in lysozyme

The wealth of data obtained up to 1967 from experiments involving the specific modification of certain types of amino-acid side chain is well reviewed by Jolles.[28] The most significant results to date are those obtained from lysine, cystine, histidine, tyrosine, tryptophan and carboxyl group modifications.

Lysine residues in lysozyme may be guanidinated with O-methyl isourea,[29,30] the single histidine residue may be alkylated with iodoacetic acid or other

Figure 2

The amino-acid sequence of lysozyme, (Canfield)[26]



(Reproduced from Jollès,[28] with modifications)

reagents,[20,30] two of the tryptophan residues (108 and 111) may be converted to N'-formylkynurenine residues by the action of ozone in formic acid,[31] and up to three tyrosine residues may be acetylated with acetylimidazole[30] (after prior guanidination of the lysines), all without loss of enzyme activity.

However the enzyme is wholly or partially inactivated by acetylation of lysine residues,[29,30] by nitration of two tyrosine residues,[32] by carboxymethylation of more than three lysine residues,[20] and by reduction of the cystine groups,[33,34] (although in this case the process is reversible and activity is regained on oxidation). However most, if not all, of these processes are associated with conformational changes in the enzyme, and more significant is the complete inactivation caused by certain tryptophan modifications (e.g. with ozone,[31] with 2-hydroxy-5-nitrobenzyl bromide,[35] and with N-bromosuccinimide[36]), and by non-specific esterification of carboxyl groups.[30,37,38]

When the crystallographic model revealed the significance of carboxyl groups and tryptophan residues to the mechanism of the enzyme more specific modifications were attempted on these residues. For example the selective modification of a single tryptophan (residue 62) with N-bromosuccinimide results in complete loss of enzyme activity,[39] and the elegant work of Imotu et al.,[40] involving chromatography of lysozyme, and of lysozyme modified at Trp 62, on a column of carboxymethyl chitin illustrates that this tryptophan is essential for the binding of this oligosaccharide.

Specific carboxyl group and tryptophan modifications are discussed later (p. 33 and p. 40).

Lysozymes from different sources

Jolles[28] has described the criteria which allow an enzyme to be described as a lysozyme, and to date at least 25 lysozymes from sources as widely diversified as papaya latex,[41] human tears,[42] and quail eggs[43] have been described. Analytical and sequencing work on these enzymes shows that the primary structure is not conserved in lysozyme from different sources. The absence of any histidine residue in lysozyme from duck egg white, which is nevertheless active against M. lysodeikticus, confirms the inessentiality of this residue to the enzyme's glycolytic function.[44] One cystine residue is similarly unnecessary, although the heat stabilities of lysozymes are dependent on the number of S-S bridges.[28]

It has recently become apparent that lysozymes of plant origin (e.g. papaya[45] and fig[46]) show significant mechanistic differences from hen egg white enzyme. In particular, although their activity towards M. lysodeikticus is low, that observed with chitin oligosaccharides is considerably enhanced.[45,46] Furthermore transglycosylation is not catalysed by the papaya enzyme, and the stereochemical course of glycolysis is different from that obtaining with hen egg white enzyme.[47] These and other factors suggest that a different mechanism of action applies to plant lysozymes.

The 3-dimensional structure of lysozyme

The 3-dimensional structure of lysozyme has been the subject of many reviews,[2,3,48,49] and only a summary of the main features will be given here. The lysozyme molecule is fairly typical among enzymes whose complete structures are now known, polar side chains appearing predominantly on the outside of the molecule, and non-polar or hydrophobic residues being mainly buried inside. The folding of the main chain is partly in α-helices (ca. 35%), partly in the anti-parallel β-pleated sheet conformation, and partly random, and the overall shape of the molecule is roughly ellipsoidal, with a cleft running down one side. Three of the six tryptophan residues are in this cleft, but the single histidine residue is on the far side of the molecule from it. Saccharide inhibitors may be incorporated into the enzyme crystals simply by cocrystallisation or by allowing diffusion of the saccharide into preformed crystals,[50] and these compounds are bound in various positions along the cleft.

$(NAG)_3$,* although hydrolysed slowly by lysozyme in solution forms also a non-productive stable crystalline complex with it. Crystallographic studies of this complex[51] show that $(NAG)_3$ occupies about half of the cleft with its reducing terminal at the centre of the cleft. The sites of the three N-acetylglucosamine

* $(NAG)_n$ refers to the β(1→4) linked polymer of N-acetylglucosamine where n is the number of sugar residues.

residues are known as A, B and C, (reading from the non-reducing terminal), and many polar and non-polar contacts between the trisaccharide and the enzyme are evident. 6 hydrogen bonds, (including 4 to site C), are observed, and there is a total of about 48 Van der Waals contacts of less than 4Å, (including 30 in site C).[52] Trp 62 (and Trp 63) are both involved in binding in site C, and on binding of the trisaccharide the ring of Trp 62 actually moves 0.75Å, narrowing the cleft, and improving the contacts in sites B and C, thus providing an example of the 'induced fit' theory of Koshland.[53] Asp 101 is also important to saccharide binding, forming H-bonds with the O-6 of the sugar residue in site B, and with the N-H of the residue in site A.

The two anomeric forms of N-acetylglucosamine itself also bind in the cleft of lysozyme, in site C.[50,51] The β-form takes up a position very similar to that adopted by the terminal residue of $(NAG)_3$, and similar shifts in the enzyme conformation are seen on binding of this sugar. α-NAG also occupies site C, although in a different conformation from β-NAG. N-acetyl muramic acid (NAM) also binds to this site and comparison with NAG suggests that it binds in the α-form. The α-form of NAG-NAM is also the only one observed when this disaccharide is bound to the enzyme, and this results in the NAG residue lying outside the cleft.

The problem of how higher oligosaccharides than the trimer bind to lysozyme cannot be tackled directly by X-ray crystallography, owing to the fact that these compounds are substrates for the enzyme and are hydrolysed rapidly by it.

The obvious assumption is that the rest of the cleft may be occupied by such saccharides, and that this contains the catalytically important part of the enzyme. (Incidentally, crystallographic studies of this part of the cleft are hindered in any case by the nature of the packing of the molecules in the tetragonal crystals normally used).[21]

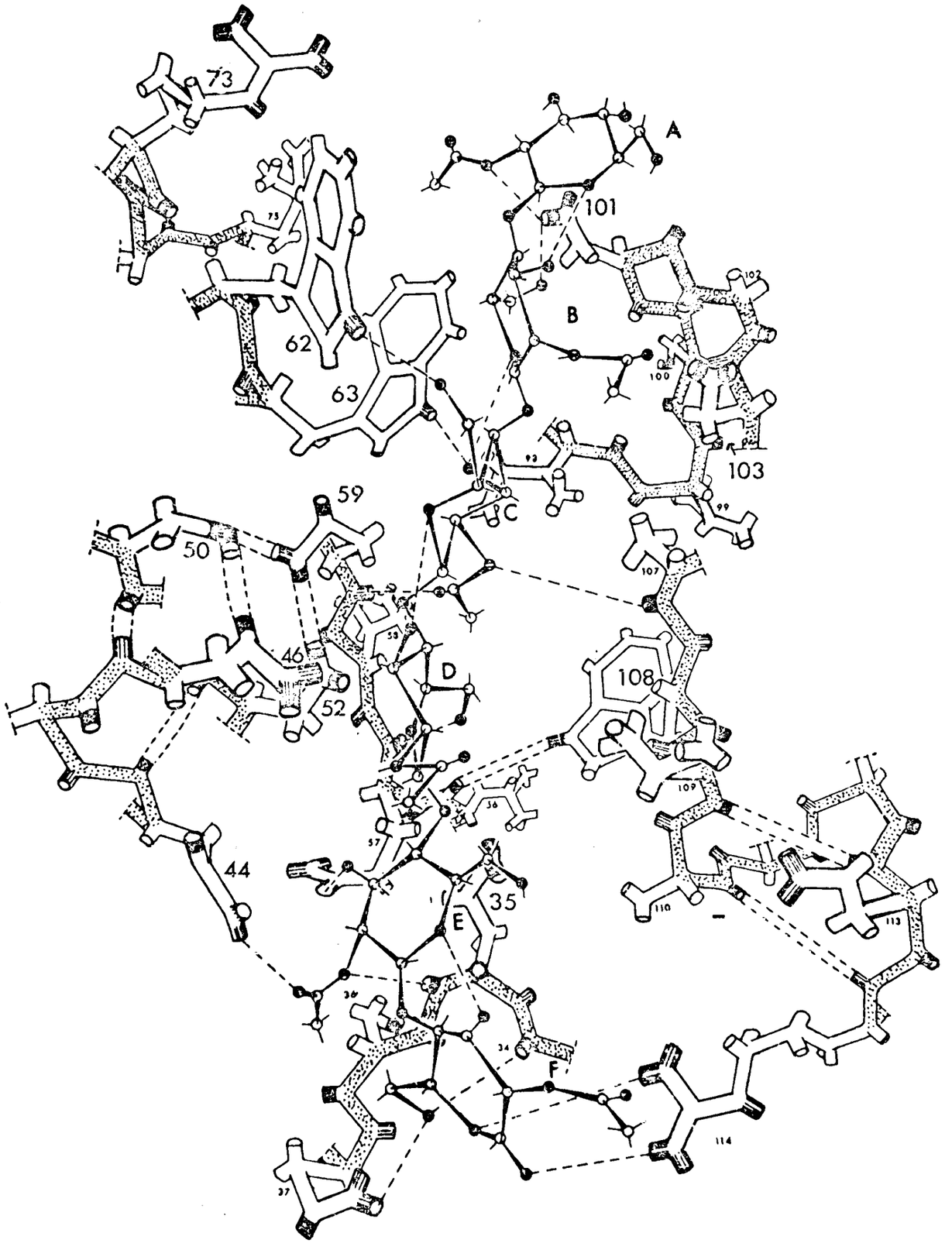
It may be shown by model building that three more NAG residues could be added to $(NAG)_3$, to fill the rest of the cleft. These are said to occupy subsites D, E and F. NAG residues in sites E and F present no problems, there being several possible hydrogen bonds and many non-polar contacts. A NAG residue in site D however would have to be distorted from its normal chair conformation towards a half-chair conformation, as otherwise its CH_2OH group would make unacceptable contacts with the enzyme main chain, with Trp 108, and with the acetamido group on the NAG residue in site C.[48,51] The accepted mode of binding of the hexasaccharide, $(NAG)_6$, when spanning sites A to F, is thus as shown in figure 3.

The catalytic site

The position of the catalytic site may be deduced from the crystallographic evidence by consideration of how a cell wall substrate, -NAG-NAM-NAG-NAM-NAG-NAM-, binds. Sites A, C, and E can only accommodate NAG residues, as in these sites the hydroxyl group on C-3 of the sugar residue points into the

Figure 3

The atomic arrangement in the lysozyme molecule at 2Å resolution, showing the proposed mode of $(NAG)_6$ binding[51]



cleft, and if this hydroxyl group were substituted, as in NAM residues, the situation would be sterically impossible. Therefore sites B, D, and F must be sites for NAM binding. Hydrolysis is known to occur between NAM and NAG residues only; as site F is at the end of the cleft and as the B-C region is known not to be at the catalytic site, (from the observation that $(NAG)_3$ is not hydrolysed when bound in sites A, B, and C), it is deduced that hydrolysis must occur between sites D and E. The only significant groups of the enzyme close to a $\beta(1 \rightarrow 4)$ saccharide linkage from site D to site E are Asp 52 and Glu 35, both of which approach to about 3 Å the oxygen in this linkage, and which are on opposite sides of it. Glu 35 is in a non-polar environment, but Asp 52 is in a polar region and is probably hydrogen-bonded to Asn 59. Other residues of a hydrogen-bonding nature in the vicinity of Asp 52 are Asn 46, Ser 50 and Asn 44.

Although it should not be immediately assumed that the enzyme conformation is the same in solution as in the crystal, there is a growing body of evidence which suggests that this is so. In particular, low resolution crystallographic comparison of the tetragonal and triclinic forms of lysozyme has shown no difference in enzyme conformation, suggesting that it is independent of crystal lattice forces.[106] The association constants of NAG and $(NAG)_2$ with lysozyme have also been shown to be similar for crystalline and dissolved enzyme.[54] In all, the extent to which the observed structural features and physical and chemical properties of the enzyme can be correlated with the crystallographic model is striking.

Cleavage pattern of oligosaccharides by lysozyme

One of the most direct ways of chemically determining the position within the cleft where catalytic action occurs is the study of the relative rates of cleavage of different bonds in oligosaccharide substrates. Although the very slow rate of cleavage of the lower oligosaccharides of NAG results in complex products, possibly arising not from 1:1 [ES] complexes but from [ES_2] complexes,[12] and complicated by concurrent transglycosylation, hydrolysis of the pentamer and hexamer is rapid and shows zero-order kinetics at 10^{-4}M substrate. Hexamer hydrolyses almost uniquely to $(NAG)_4$ and $(NAG)_2$, whereas pentamer gives $(NAG)_4$ + NAG and $(NAG)_3$ + $(NAG)_2$ with relative rates 1.0 and 0.5.[12] Similar indications of the position of cleavage within these oligomers are obtained when transfer products in reaction mixtures containing added labelled monomer are studied,[12,55] and also when transglycosylation products formed during the hydrolysis of cell wall oligosaccharides are investigated.[56] These results strongly suggest that the position of the catalytic site is as predicted from the crystallographic evidence.

The binding of oligosaccharides by lysozyme

The association constants of a great number of saccharides with lysozyme have been determined, using the techniques of U-V difference spectroscopy, fluorescence spectroscopy, p.m.r. spectroscopy, equilibrium dialysis, etc.,

(e.g. references 57, 58, 59, 60). Oligosaccharides of NAG have been particularly well studied and approximate Ka values for these are given below; ($Ka = \frac{[ES]}{[E][S]}$, where [E], [S], and [ES] refer to the concentrations of enzyme, substrate, and enzyme-substrate complex respectively).

Saccharide	Ka (litres.moles^{-1})	Reference
NAG	15-25	57,59,60
$(NAG)_2$	5×10^3	57,59,60
$(NAG)_3$	1×10^5	57,58,59,60
$(NAG)_4$, $(NAG)_5$, $(NAG)_6$	$1\text{-}2 \times 10^5$	57,60

The fact that the observed Ka is constant from $(NAG)_3$ to $(NAG)_6$ has been taken to mean that unproductive binding in sites A, B, and C only is being seen for these oligomers. Productive binding, spanning sites D and E is presumably weak and does not contribute much to the observed saccharide binding.

The nature of binding to sites D, E, and F was therefore investigated with cell wall oligosaccharides, whose NAM residues impose limitations on the number of ways in which binding may occur, (see p. 11). Ka values for the trimer, tetramer, and hexamer are given below:[59,61]

Saccharide	Ka (litres.moles^{-1})
NAG-NAM-NAG	3×10^5
NAG-NAM-NAG-NAM	2×10^3
$(NAG\text{-}NAM)_3$	3.5×10^4

Comparison between the trimer and tetramer indicates that the latter must be occupying sites A to D, (C being unacceptable for NAM), and that D is unfavourable for binding, as predicted from the crystallographic model. As there would be no difference in Ka between tetramer and hexamer if the extra two residues of the latter were projecting out of the cleft beyond A, it follows from the enhanced binding of hexamer that it occupies sites A-F and that E and F are favourable for binding.

By using comparisons of this sort, and assuming that units of an oligosaccharide contribute additively to the total binding energy observed, one may estimate the contributions of individual sub-sites to binding energies. Such comparisons may be confused by the effects of conformational changes in the enzyme, and there will undoubtedly be an entropy effect in favour of the binding of an additional residue when one or more are already bound, but nevertheless the approach provides a useful indication of the sub-site properties. The free energies of association of saccharide residues to sub-sites A to D are given by Chipman et al.[59] as A: -(1.8 to 1.9) Kcal./mole (for NAG); B: -3.5 Kcal./mole (for NAG) and -3.9 Kcal./mole (for NAM); C: -5.7 Kcal./mole (for NAG); D: +2.9 Kcal./mole (for NAM). Rupley[60] uses a correction for the entropy effect mentioned above and quotes values for sites A, B, and C of -2.3, -2.8 and -4.6 Kcal./mole respectively, (all for NAG binding).

The free energies of association of saccharide residues to sites E and F are

more difficult to determine and various values have been quoted for these. Comparison of the binding constants of $(NAG\text{-}NAM)_3$ and $(NAG\text{-}NAM)_2$ (p. 15) suggests that the combined free energies of association to sites E and F is -1.7 Kcal./mole. However comparison of the rates of hydrolysis of $(NAG)_6$ and the rates of the two possible cleavages in $(NAG)_5$, in association with some transglycosylation studies suggest values of -4 Kcal./mole and -(1.5 to 2) Kcal./mole for binding to sites E and F respectively.[12,52]

The latter approach is the more likely to satisfy the requirement that productive complexes only must be studied in any analysis of this kind, although clearly a more positive way of investigating such complexes is required. In this respect the work of Hess,[62,63] using a dye, Biebrich scarlet, which has been shown to bind to lysozyme in the site D-F region, has proved valuable. The study of the competitive binding of this dye with $(NAG)_6$ in the productive mode of binding shows that the productive association constant for this substrate is 2×10^5, i.e. the same as that observed in 'normal' binding studies for $(NAG)_3$ or $(NAG)_6$ (p. 15). This suggests firstly that in the case of $(NAG)_6$ a considerable amount of the binding is productive, in contrast to earlier conclusions, and secondly that the combined effect on binding by sites E and F is approximately equal and opposite to that of site D. The conclusion that the free energy of association of a NAG residue to site D is about +6 Kcal./mole is in accord with the estimated energy required to distort the sugar ring into the conformation suggested by the crystallographic model.[52]

To summarise, sites A, B, and C, (and especially C), are binding sites, and site D is unfavourable for binding. The effect of this anti-bonding site is approximately cancelled out for hexasaccharides in the productive binding mode, (spanning all the sub-sites), by sites E and F which are favourable for binding. The free energy values estimated for the contribution of each site to binding are entirely in accord with the number of polar and non-polar contacts which saccharide residues would make in these sites, as deduced from the crystallographic model.

The contribution of individual structural features on the enzyme to saccharide binding

More detailed studies have now separated individual contributions from certain enzyme structural features to binding.[64] In particular, Trp 62 has been shown, with the help of the specific oxidation of this residue which may be carried out with N-bromosuccinimide,[39] to contribute 2.6 Kcal./mole to binding energies at sites B and C. The acetamido group in site C is responsible for about 3 Kcal./mole, and interactions with Asp 101 in site B add a further 1.3 Kcal./mole at least to binding energies. These three groups thus account for nearly three quarters of the observed binding energy of $(NAG)_3$.

The inhibitory effect on lysozyme of certain saccharides

Binding of oligosaccharides, and the structural features which are necessary

in an oligosaccharide to make binding possible have also been studied by investigating the inhibitory effect of added oligosaccharides on the lysis of M. lysodeikticus cell walls by lysozyme.[14] Cellobiose and D-glucosamine are ineffective as inhibitors, illustrating the requirement of an acetamido group in site C for saccharide binding. This requirement is extremely specific; Neuberger and Wilson have shown that the 2-formamido, 2-propionamido, and 2-butyramido analogues of NAG are inefficient as inhibitors.[65] It is possible however for glucose residues to bind in sites D and E, and to a lesser extent C, when these residues are incorporated into a larger olicosaccharide.[66] Thus for example NAG-NAM-GLUC-GLUC-GLUC-GLUC (where GLUC represents glucose) is hydrolysed to NAG-NAM and $(GLUC)_4$, and, with a much slower rate, to NAG-NAM-GLUC-GLUC and $(GLUC)_2$, reflecting in particular the stronger binding of NAG than GLUC in site C.

The use of p.m.r. spectroscopy in the study of oligosaccharide binding

The extensive use of p.m.r. spectroscopy in the study of binding of chitin oligosaccharides is worth separate mention at this stage. Dahlquist et al. have used the chemical shift of the acetamido methyl protons of NAG residues to probe the binding environments for various compounds ranging from NAG, and the α- and β-methyl glycosides of NAG, to $(NAG)_4$.[67,68,69] The acetamido group resonance shifts upfield when such a group is bound in site C, in accord with it

being near the face of the indole ring of Trp 108, as predicted from the crystallographic model. Considerable line-broadening also results from this binding. Very slight broadening, but no chemical shift, is observed for acetamido groups on NAG residues in site B, and the effect in site A is a slight downfield shift compared with the resonance position in solution.

The results indicate that the α- and β-methyl glycosides of NAG bind competitively in site C, with their acetamido groups in identical environments, though with different binding constants. The same conclusion does not apply to the anomers of NAG, the α- and β-forms again having slightly different binding constants but also differing acetamido group magnetic environments.[67,69] The latter observation probably reflects only a small shift in orientation of the acetamido group relative to the anisotropic tryptophan ring, as the crystallographic model suggests that the acetamido contacts are the same for both anomers. Other oligosaccharides were found to bind as expected; thus $(NAG)_2$, $(NAG)_3$ and $(NAG)_4$ binding was observed in sites B-C, A-B-C, and solution-A-B-C respectively.

The rate constants for association (k_1) and dissociation (k_{-1}) of the anomers of NAG, of the methyl glycoside of NAG and of $(NAG)_2$ have been determined from studies of the line widths of the acetamido methyl resonances.[70,71] For both NAG and its methyl glycoside, k_{-1} is smaller for the β-form than for the α-, indicating that the β-form is more stably bound. k_1 values are fairly constant for these compounds, but increase sharply with $(NAG)_2$. No further increase in k_1 is

seen with $(NAG)_3$ association, (data of Chipman and Schimmel by the temperature jump relaxation method[72]), although the dissociation rate constant k_{-1} drops sharply on going from $(NAG)_2$ to $(NAG)_3$, this accounting entirely for the differing binding constants of these oligosaccharides. This is attributed to the relatively slow attachment of trisaccharide to sites B and C on the enzyme being followed by faster association with site A. Strong interactions with Asp 101 in this site result in dissociation of trisaccharide being more difficult than with disaccharide.

Finally, the study of the p.m.r. spectrum of the enzyme itself, and in particular the resonances of indole N-H protons, has been used to ascertain how many Trp residues are involved in binding of small saccharides. As suggested by the crystallographic model, two Trp residues are involved in binding (Trp 62 and Trp 63), whereas Trp 108, the only other tryptophan near the binding cleft, is not involved.[73]

The transglycosylation reaction catalysed by lysozyme

As well as being a process which often hinders the interpretation of kinetic data for lysozyme, transglycosylation has also provided a means of studying several important aspects of the enzyme's activity. The process has been observed for both chitin[74] and cell wall[10,14] substrates, and under certain conditions the reaction of lysozyme with $(NAG)_4$ even leads to an insoluble chitin-like substance.[74] Hydrolysis of both types of tetrasaccharide proceeds only after an induction period,

which may be abolished by addition of a little higher oligosaccharide[56,74] and this has been attributed to the fact that the observed rate of hydrolysis is that of higher (above tetra-) saccharides, which are initially formed by transglycosylation reactions. This suggests that a glycosyl enzyme intermediate is formed in the slow hydrolysis of, for example, $(NAG\text{-}NAM)_2$ to two disaccharide units, and that reaction with more tetrasaccharide may yield the hexasaccharide, which then hydrolyses rapidly, as it fills the whole cleft on binding.

Transfer reactions are not only known with saccharide acceptors, and the study of the rates, specificity, and stereochemical outcome of transfer reactions to other hydroxyl and thiol acceptors has been used to investigate the nature of sites E and F and the mechanistic course of the reaction, (which is presumed to occur through the same intermediate as hydrolysis, the acceptor then being water).

One of the most important conclusions of this work is the fact that transfer reactions are absolutely stereospecific, both with saccharide acceptors[66,75] and with alcohols such as methanol.[76] The β-configuration of the glycosidic link is retained; for example, in the case of the production of the methyl glycoside of NAG from $(NAG)_2$ cleavage in the presence of methanol, the product was found to show greater than 99.7% retention of configuration.[47] As Rupley has shown,[55] from hydrolysis of glycosides by lysozyme in $H_2{}^{18}O$, that cleavage occurs between the bridge oxygen and the C-1 on the sugar ring, these results indicate that the mechanism must involve either a double displacement on C-1 or the formation of

a carbonium ion, which is tightly held for long enough for the leaving saccharide to diffuse away and for an acceptor to take its place. It is worth noting that although transglycosylation with NAG or NAM saccharide acceptors does give the $\beta(1 \rightarrow 4)$ linkage,[75] transfer products containing $\beta(1 \rightarrow 2)$ and $\beta(1 \rightarrow 3)$ linkages have also been observed with non amino-sugar acceptors.[77] This observation has been attributed to the different steric requirements and orientation taken up by these sugars in site E.

Information about the nature of sites E and F may be gained from the analysis of the products of transfer reactions when two acceptors are competing for the same glycosyl enzyme, although (except in the case of water as an acceptor) experimental difficulties may arise associated with the hydrolysis of the product. Experiments of this sort have confirmed that sites E and F may both bind sugar residues favourably,[75] and that an acetamido group on the residue in site E (but not site F) is particularly advantageous for binding, in excellent accord with the crystallographic model. It is of interest that very approximate calculations of the binding energies at sites E and F based on these results give values of -4 and -1 Kcal./mole respectively,[21] in accord with the conclusions of Rupley,[52] (p. 17).

With simple alcohols as acceptors, the variation of acceptor structure has also led to certain deductions concerning the mechanistic course of transfer, and hence hydrolysis, reactions.[78] This approach overcomes the difficulties which are encountered in overall kinetic studies of hydrolysis due to non-productive

substrate binding. As expected, alcohols such as ethanol are much less effective as acceptors, by a factor of about 500, than NAG. However there does not seem to be any significant variation in acceptor reactivity between alcohols of widely differing pK values. Thus ethanol and phenol for example have the same reactivity as acceptors compared with water. At the pH of the experiments (5.3) phenol is a poorer nucleophile than ethanol, and Rupley[78] has interpreted these results to imply the involvement of general base catalysis by the enzyme, which increases the nucleophilicity of the weaker bases so that all alcohols are roughly equal in reactivity. General acid catalysis would therefore apply to hydrolysis. Rupley has also noted that sulphur acceptors, (H_2S, CH_3SH and SCN^-), are not at all effective in transfer reactions. On the assumption that this is a kinetic comparison, the fact that sulphur is a much better nucleophile than oxygen in both anionic and uncharged species would suggest that nucleophilic attack by the acceptor on a tetrahedral intermediate (such as would be required in any sort of double displacement reaction) does not occur. Sulphur is known however to be acceptable in the site D-E region,[79] and a carbonium ion type of intermediate, for which the acceptor's nucleophilicity is not critical in further reaction, seems likely. Rupley therefore favours a hydrolytic mechanism involving general acid catalysis and the formation of an intermediate of considerable carbonium ion character.

The conditions used in the transglycosylation reactions with sulphur acceptors however, (40°, 24 h , pH 5.3), were such that an equilibrium situation might

exist, in which case the apparent failure of sulphur compounds to act as acceptors may be due to the fact that the equilibrium ES $\rightleftharpoons$ ES' + acceptor may be shifted to the right in the case of sulphur acceptors and to the left in the case of oxygen acceptors. This would be consistent with the observation that a glycoside with a sulphur linkage is hydrolysed with a greater catalytic rate constant than the oxygen analogue,[79] and would not preclude the possibility that the reverse reaction, leading to transglycosylation products, has a greater rate constant for sulphur than for oxygen acceptors.

The pH dependence of oligosaccharide binding

pK values have been assigned to groups of lysozyme and of lysozyme-saccharide complexes by several authors in more than 20 papers. Apart from oligosaccharide binding and hydrolysis, the phenomena studied include denaturation, (both thermal and with guanidine hydrochloride), fluorescence spectra, p.m.r. spectra (of the enzyme and of inhibitors), and association in solution.

Tri-saccharide binding however, to summarise the data available, depends on at least three ionisable groups. Values reported for two of these are 4.2 and 6.1 $\pm$0.4, perturbed to 4.1 $\pm$0.6 and 6.5 $\pm$0.2 respectively in the enzyme-saccharide complex.[55,57,58,60] The third pK, which has only been observed by Rupley,[55,60] is 1.8, perturbed to 1.2 in the [ES] complex.

The interpretation of these pK values is aided by the study of monomer

binding, although the experimental evidence is conflicting. Rupley claims that NAG binding is dependent on a high pK group (pK ca. 6.5), but that it is virtually independent of pH below pH 5.[60] Recent studies of the circular dichroism of lysozyme in the presence of NAG suggest the importance of two ionisable groups (pK values 6.5 and 2.5) in binding of this monosaccharide.[80] Significantly the group of intermediate pK value observed in $(NAG)_3$ binding does not seem to be involved in NAG binding.

This group (pK ca. 4.2) therefore seems likely to be Asp 101, as this is shown by the crystallographic model to be involved in binding of saccharide residues in sites A and B, but not in site C. The high pK (6.0-6.5) observed in NAG and $(NAG)_3$ binding might at first suggest the involvement of a histidine residue. However the single histidine residue in lysozyme has a pK of 5.8, unaffected by saccharide binding,[81] and is thus not involved in the binding process. Many studies have implicated the existence of a carboxyl group with abnormally high pK value in lysozyme, and it is generally accepted that Glu 35, in its non-polar environment could be the residue responsible.

The low pK group (pK ca. 2) is not as easy to identify. Rupley considers that Asp 52 has a pK value of 3 to 3.5 and therefore suggests that Asp 66, (which is in one of the 'wings' of the molecule, on the same side as Asp 52, and appears to be highly hydrogen-bonded), has this pK value.[64] However this assignment was prompted by his failure to observe the ionisation of this group in NAG binding,

as Asp 52 would be likely to be involved in this. The more recent confirmation that the group is involved in NAG binding shows that it is likely to be Asp 52 after all. In support of this there is considerable structural evidence, from the polar nature of the environment of Asp 52, that this group should have an abnormally low pK. Asp 52 is also thought likely to be one of the three carboxylic acids with pK values around 2 which are revealed by denaturation studies,[82] as it is expected to play a role in maintaining the enzyme's structure.

In a more detailed recent study, Rupley[64] has suggested that a fourth group, (of pK 4.3 perturbed to 4.7 in the complex), is involved in $(NAG)_3$ binding. Although this was assigned to Asp 103, the recent structural evidence that 'Asp 103' is in fact asparagine[27] rules out this possibility. Asp 66 also would seem to be unlikely as its polar environment should give it a lower pK than 4.3.

To summarise, it appears that at least three ionisable groups are involved in trisaccharide binding. These are probably Glu 35, Asp 101 and Asp 52 and/or Asp 66.

The pH dependence of the binding of the α- and β-methyl glycosides of NAG shows rather different results. The β-anomer binding is dependent upon a group of pK 6.1 (probably Glu 35 as above) but, in addition to this, a group of pK 4.7, unperturbed by binding, is shown by the pH dependence of the acetamido methyl chemical shift, and is presumably close to the active site.[83] (Although this was assigned to Asp 103, in the light of the recent structural evidence this cannot be so).

In the case of the α-methyl glycoside however, Kowalski and Schimmel,[84] using the technique of U-V difference spectroscopy, observed a bell-shaped pH profile of binding, which could be interpreted in terms of two ionisable groups of pK values 6.5 and 4.6, both perturbed by binding. Again the higher pK group is probably Glu 35, and it is tempting to assume that the other group is the same as was found to be close to the β-anomer binding site, although the striking difference in pH profiles of binding is surprising, and must be due to subtle differences of orientation of the anomers in site C. Such orientational differences have been suggested for the α- and β-anomeric forms of NAG, but not for the methyl glycosides, from studies of the magnetic environments of the acetamido methyl groups (p.20). If the pK of 4.6 to 4.7 is due to a single ionisable group it must by elimination be associated with Asp 101 (apparently far from site C) or Asp 66 (also not close, and expected to have a low pK); although the latter seems slightly more likely, the assignment of this pK to a single group is not satisfactory.

The interpretation of pK values is always difficult, and often may not yield useful results, partly because of the complexity of enzyme systems, and partly because the observed values may not reflect the ionisations of single groups. The possibility of conformational changes in the enzyme, associated with the ionisation of a group distant from the active site, giving rise to apparent pK's for substrate binding is another factor complicating pK value interpretation. This may be particularly relevant in the case of lysozyme as binding is known to cause conformational changes around the cleft region.

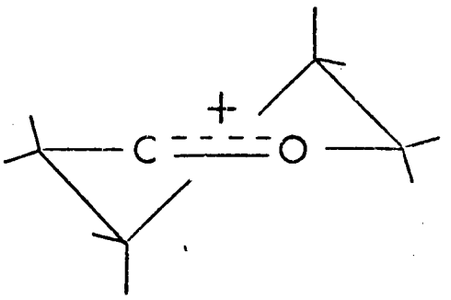
The pH dependence of catalysis by lysozyme

The situation is further complicated in the study of the pH dependence of catalysis by lysozyme, as, in addition to the limitations on interpretation of pK values mentioned above, there is the additional difficulty that observed pK values may be associated with an ionisation which affects the ratio of productive to non-productive binding of the substrate. It is of interest however that the pH rate profiles of hydrolysis of $(NAG)_3$,[55] $(NAG)_5$[12] and some p-nitrophenyl glycosides which have been shown to be lysozyme substrates[76,85] (see p. 36) are bell-shaped with a maximum rate around pH 5. The basic limb may well be due to Glu 35 ionisation, but the interpretation of the acidic limb is open to uncertainty.

The non-enzymic hydrolysis of glycosides

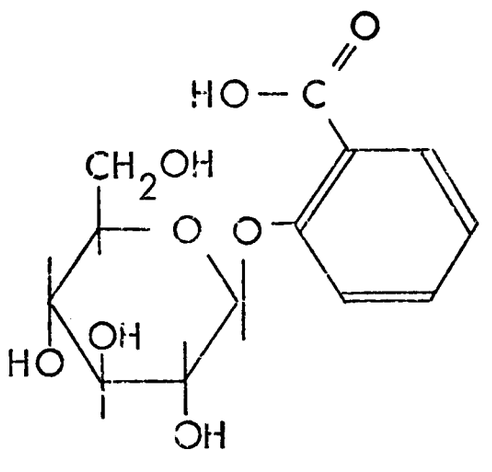
Base catalysed hydrolysis of alkyl glycosides occurs slowly in hot concentrated base, although aryl glycosides are more readily attacked.[86] In the case of the latter the reaction is accelerated by electron-withdrawing substituents in the aromatic ring (ρ = +2.5) suggesting nucleophilic attack in the rate determing step.[87]

Much more common however is the specific acid catalysed hydrolysis of glycosides,[88] for which the corresponding ρ value is -0.66.[87] This is now known to occur via protonation of the exocyclic oxygen atom and formation of a ring closed carbonium ion, which would be stabilised by adoption of the half-chair



conformation. Asymmetric induction results in the predominant inversion of configuration which is observed at C-1 in these reactions.

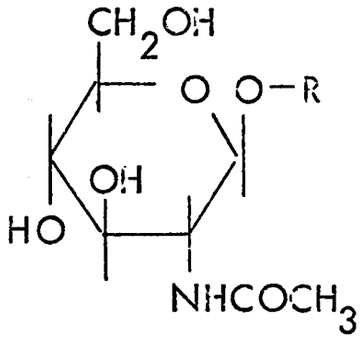
Intramolecular general acid catalysis has also been reported in the case of o-carboxyphenyl-β-D-glucoside,[86,89,90] (I). The pH rate profile for hydrolysis



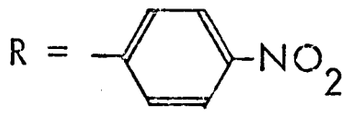
(I)

of this compound shows a plateau below pH 4, the rate of hydrolysis decreasing above this pH value; a rate enhancement of about 10^4 compared with the value predicted simply for specific acid catalysis is observed, and must be due to general acid catalysis by the o-COOH group.[89]

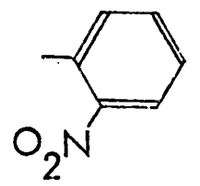
It has also been shown that glycoside hydrolysis may be assisted by neighbouring group participation from a substituent on C-2 of the sugar ring.[89,91] The glycoside is required to be the β-form so that a trans relationship exists between the two groups involved. Of particular interest in connection with the study of catalysis by lysozyme is the observation that the o- and p-nitrophenyl β-glycosides of NAG (II) show rate enhancement due to participation of the 2-acetamido group of a factor of 10^3 compared with the corresponding 2-hydroxy compound, and by a factor of 10^5 compared with the purely specific acid- and specific base-catalysed



(II)



or



hydrolysis. The participation is shown to be nucleophilic in character by the lack of a kinetic deuterium solvent isotope effect.[89]

Catalysis by lysozyme

The crystallographic model shows that hydrolysis of oligosaccharides by lysozyme occurs in the neighbourhood of two carboxylic acid groups, Glu 35 and Asp 52, of unusually high and low pK values respectively. The saccharide residue which upon hydrolysis becomes the new reducing terminal residue is thought to be in a strained conformation tending towards a 'half-chair'. Other data in the foregoing discussion have shown that bond cleavage occurs between C-1 of this strained sugar ring and the bridge oxygen, and that reaction occurs with retention of configuration at C-1. This, in association with the results of transglycosylation experiments, has led to the proposition of a mechanism involving general acid catalysis, and a glycosyl enzyme intermediate of considerable carbonium ion character, (p. 24).

The single histidine residue is known not to be involved in the glycolytic function of the enzyme as it (a) is on the opposite side of the molecule from the cleft,[3] (b) is not present in all lysozymes having glycolytic activity (e.g. duck egg white lysozyme),[44] (c) may be carboxymethylated without loss of enzyme glycolytic activity,[20] and (d) has a pK of 5.8 unaffected by saccharide binding.[81] Bruice and Piszkiewicz[13] have shown that this histidine is involved in the esteratic function of lysozyme, which is completely independent of the glycolytic function.

The involvement of Glu 35 in the catalytic mechanism is however quite clear, apart from the crystallographic deduction that this would be so. Its

abnormally high pK for a carboxylic acid (6.0-6.5), has been observed in binding and hydrolysis studies, and its catalytic importance has also been demonstrated by an interesting specific modification. The oxidation of tryptophan residues to oxindoles with iodine is commonly carried out as a modification reaction for these residues. With lysozyme this treatment gives two products; one contains iodine and is partially active and the other does not and is inactive.[92,93] The latter product however does still possess normal ability to bind saccharides.[40,94] X-ray crystallography has revealed that it contains an intemal ester linkage between Glu 35 and α-C of the indole ring of Trp 108.[95] This linkage is labile and the oxindole of Trp 108 is readily formed by, for example, lyophilisation of the product. Thus removal of the carboxyl group of Glu 35 from the active site results in complete loss of catalytic activity, without loss of substrate binding ability. As Glu 35 would be unionised in the optimum pH range for lysozyme activity, (pH 5-6), it seems clear that it acts as the general acid catalyst whose presence is implied by other studies.

While accepting the existence of general acid catalysis by Glu 35, it seems unlikely that one or both of the other two strucitural features near the site of bond cleavage, namely Asp 52 and the acetamido side chain of the sugar residue in site D, play no part in the reaction.

The aspartic acid will be ionised at the pH of optimal enzyme activity, and could thus theoretically act as a base, or as a nucleophile, or simply as a

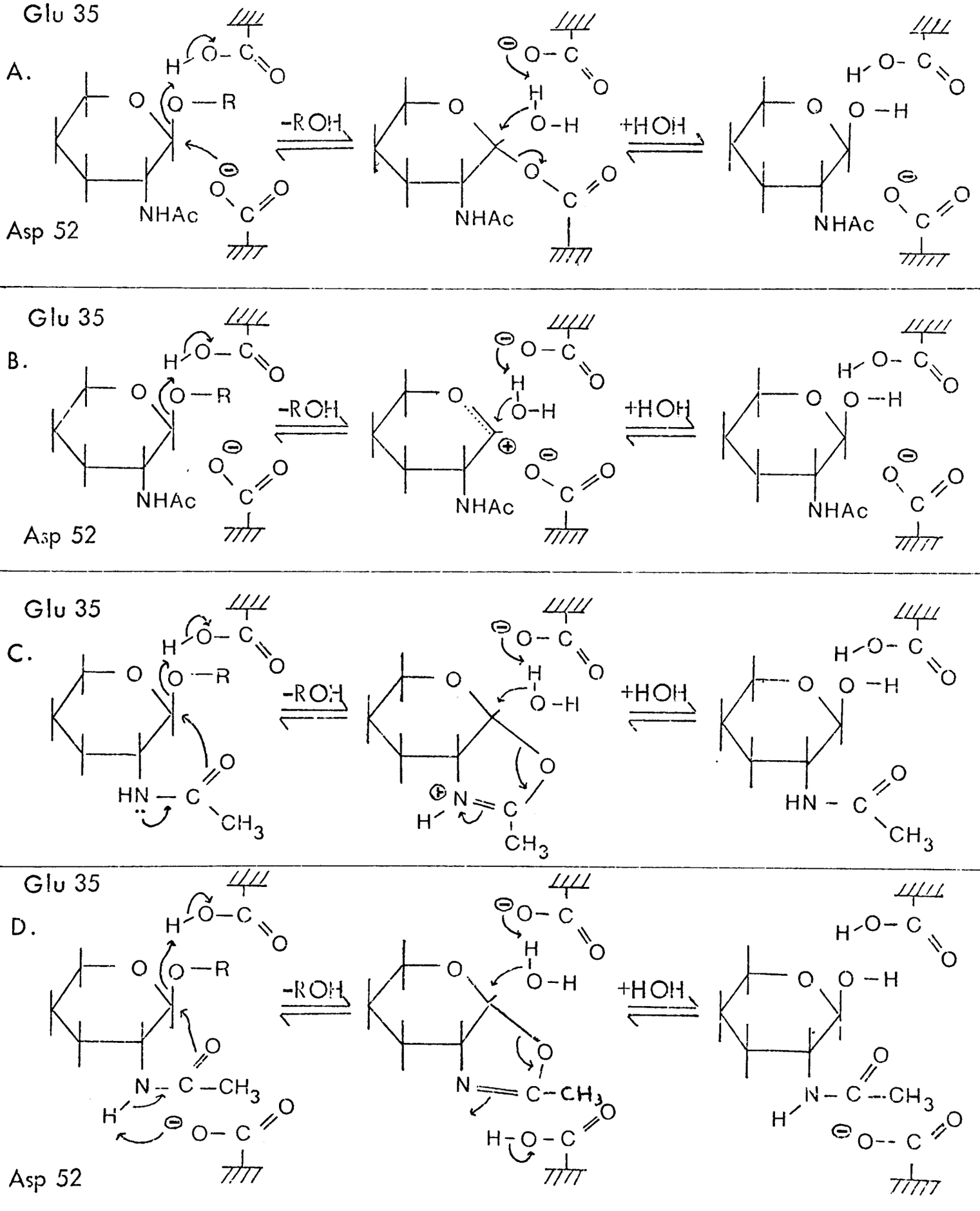
stabilising negative charge for a carbonium ion. Four possible mechanisms involving Asp 52 and/or acetamido group participation are shown in figure 4; in all the mechanisms shown Glu 35 acts as general acid catalyst and overall configuration at C-1 is retained.

In mechanism A, the aspartate ion acts as a nucleophile for direct displacement of the leaving group; mechanism B involves the formation of a carbonium ion which is held in an ion pair by the aspartate ion; nucleophilic attack by the neighbouring acetamido group, again with general acid catalysis from Glu 35, is represented in mechanism C, and in D this process is further assisted by Asp 52 acting as a general base.

If no enzyme conformational changes occur in subsite D when sugar is bound, (which would be surprising in view of the predicted distortion of the sugar ring in this site), Asp 52 would not be suitably placed to assist acetamido group participation, as in mechanism D. Similarly, unless such conformational changes occur in the [ES] complex, mechanism A would also seem sterically unlikely owing to the distance (ca. 3Å) between Asp 52 and the C-1 of the sugar ring. The strained nature of the sugar ring in site D as predicted from model building would favour stabilisation of the carbonium ion in mechanism B or acetamido group participation as in mechanism C, and it is possible therefore that the enzyme's great catalytic efficiency arises at least in part from its ability to distort the substrate on binding into a form more like the transition state in the catalytic reaction, thereby reducing the activation energy in its rate determining step.

Figure 4

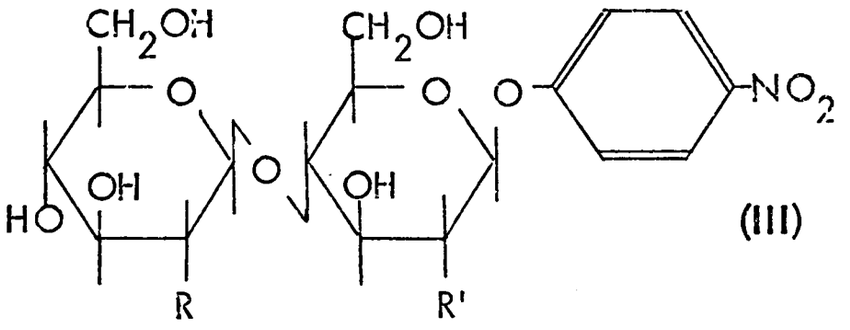
Mechanisms for lysozyme catalysis



Participation of the substrate acetamido group in lysozyme catalysis

Although the data presented earlier in favour of a carbonium ion intermediate would suggest mechanism B to be correct, there is still a considerable body of evidence against this view. However, although participation of a trans-acetamido group in non-enzymic glycoside hydrolysis (p. 31) is well established, convincing evidence for or against similar participation in lysozyme catalysis has proved difficult to obtain.

A detailed kinetic analysis of lysozyme kinetics was long hindered by the absence of a suitable substrate, until the discovery that p-nitrophenyl β-glycosides of disaccharides (III) were hydrolysed, though slowly, by lysozyme with Michaelis-Menten kinetics.[79,96,97]



(III)

a; R = $NHCOCH_3$ = R'
b; R = $NHCOCH_3$, R' = OH
c; R = $NHCOCH_3$, R' = H

The corresponding β(1→6) linked disaccharide to IIIa has been shown not to be a substrate for lysozyme.[98]

Lowe and Sheppard[96] observed a 100-fold rate enhancement of p-nitrophenol production for (IIIa) compared with (IIIb), after corrections had been made for non-productive substrate binding. Rand-Meir et al. found that (IIIa) hydrolysed

20 times more between the NAG residues than at the p-nitrophenyl glycosidic link, and also observed extensive transglycosylation with this substrate, but not with (IIIb).[97] Hydrolysis of higher oligosaccharides produced by transglycosylation in the case of (IIIa) could account for its enhanced rate of p-nitrophenyl production.

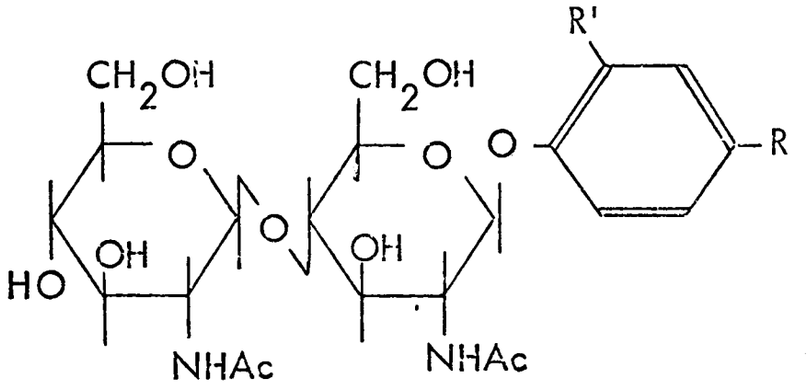
It should be stressed at this stage that comparisons of rates of hydrolysis of compounds, such as (IIIa) and (IIIb), which have differing binding requirements must be viewed with caution, as rate differences could be due to small changes of the substrate orientation in binding, or of the enzyme conformation. It can definitely be stated however that an acetamido group on the substrate is not essential for glycolysis by lysozyme as compound (IIIc) is also cleaved.[97]

Similar conclusions have recently been drawn from the study of the lysozyme catalysed hydrolysis of some p-chlorophenyl glycosides analogous to (III).[107] When $R = R' = NHCOCH_3$, difference spectroscopy reveals non-productive complex formation, but when $R = R' = NH_2$ or NHC_2H_5, productive complex formation predominates, and the compounds are still hydrolysed, although slowly.

It might be thought that the determination of the secondary α-deuterium kinetic isotope effect for a single substrate would be a way of probing the enzyme's mechanism without the complication of possible non-productive binding. This parameter has been determined for (IIIb) as 1.11,[99,100] and comparison with the corresponding values for non-enzymic S_N1 and S_N2 reactions, (1.13 and 1.03 respectively), led the authors to suggest considerable S_N1 character in the enzymic

reaction. However apart from the fact that there was no neighbouring acetamido group in the substrate chosen, (and that therefore no deductions can be made about its involvement in 'normal' enzyme reactions), the interpretation of such kinetic isotope effects is by no means clear, neighbouring group assistance in S_N1 type reactions, for example, not causing any change in the observed k_H/k_D value.[108]

A more satisfactory way of studying the mechanistic pathway however is the comparison of the rates of lysozyme catalysed hydrolysis of β-aryl di-N-acetyl chitobiosides, (IV), as the structure of the aglycone is altered.



(IV)

For a series of compounds in which R and/or R' are of an electron-withdrawing nature the Hammett ρ value for enzyme catalysed hydrolysis has been reported as +1.2.[79] This lies between the values obtained for acid and base catalysed non-enzymic reactions (-0.66 and +2.5 respectively), and may be interpreted to mean the involvement of some basic or nucleophilic catalysis in the enzyme mechanism, as well as general acid catalysis. However Tsai et al.[101] have shown, although with rather different data, that a positive ρ value only applies to electron withdrawing R substituents (R' = H), ($\rho = +0.55$), and that a different mechanism

applies for electron donating substituents ($\rho = -2.96$). The immediate conclusion is that electron-withdrawing substituents in the phenyl ring cause the mechanism of hydrolysis to involve base or nucleophile participation (mechanisms A or C), whereas with electron-donating substituents protonation of the glycosidic oxygen atom, and formation of a carbonium ion, (mechanism B), is more favourable.

β-Phenyl di-N-acetyl chitobioside ((IV), R = R' = H), is expected from this conclusion to show intermediate characteristics in lysozyme-catalysed hydrolysis. The thiochitobioside analogue of this compound (with S replacing O in the phenyl glycosidic link) is shown to bind less strongly to lysozyme but to have a greater catalytic rate constant than the normal glycoside, proving that there is more nucleophilic or basic catalysis in the reaction of this substrate.[79]

To summarise, there is a great deal of conflicting evidence as to the nature of the enzyme catalytic mechanism. Data pertaining to hydrolysis of normal enzyme oligosaccharide substrates is sparse, and the results with model compounds are in favour of a carbonium ion type mechanism (mechanism B) for electron-rich aglycones, and of a nucleophilic or basic catalysis with electron deficient aglycones (mechanism A or C). Oligosaccharide substrates appear to behave more like the former type of model substrate than the latter, although it may well be that the true course of hydrolysis of these substrates follows an intermediate pathway.

Modification of aspartic acid residue 52

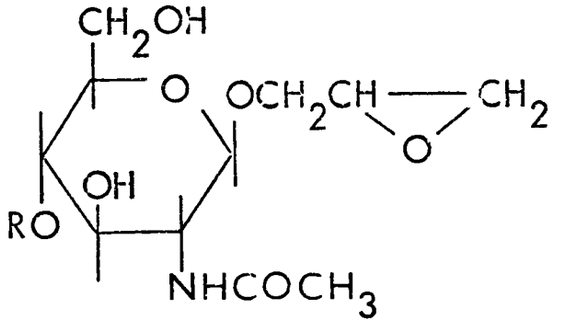
Aspartic acid 52 should be unnecessary if mechanism C is operative, whereas it is absolutely essential to mechanism B, (or A). Specific modification of Asp 52, to remove its ionic charge without seriously affecting its size, followed by kinetic studies on the derivative, would seem to be the only certain way of resolving this last mechanistic problem. Various modifications have been achieved, but in all cases there is the serious criticism that the modified residue had been made much more bulky.

As described earlier, (p. 7), non-specific esterification of all the enzyme's available carboxyl groups, (or the specific modification of Glu 35), result in inactivation. Thus esterification with ethanol/HCl, for example, gives an inactive product containing 6.3 ester residues/mole.[30] However, esterification with triethyloxonium fluoroborate, (a selective carboxyl reagent which preferentially attacks groups of low pK values), yields two single ester derivatives.[102] In one the modification is labile, and the product does not bind substrate well; this is thus a binding-site modification. The other product, which is inactive, but maintains substrate binding ability, contains the ethyl ester of Asp 52.

Carbodiimides, in conjunction with an added nucleophile, (commonly glycine methyl ester or glycinamide), have also been used as modifying reagents for carboxyl groups in lysozyme.[103,104] Asp 52 and Glu 35 can be protected by performing the modification in the presence of oligosaccharides ($(NAG)_3$-$(NAG)_5$),

and all the other carboxyl groups are then modified, in this case to the $CO\text{-}NHCH_2SO_3^-$ derivative, with loss of only 43.5% enzyme activity.[104] After removal of the saccharide, retreatment of the enzyme results in modification of Asp 52, Glu 35 remaining predominantly unattacked. As this causes complete inactivation of the enzyme, it was concluded that Asp 52 is essential for its catalytic activity.[104] However the nature of the modification is such that the active site must be highly crowded, and inactivation is hardly surprising.

The only specifically designed active site directed modifying agent for Asp 52 which has been described to date is the epoxide, (V).



(V)

R = H, NAG, or $(NAG)_2$

A preliminary report on the synthesis and use of this compound[105] states that it is an effective inhibitor, (especially when R = NAG or $(NAG)_2$), and a private communication from the authors has indicated that the inhibitor attaches to Asp 52, the saccharide part of the molecule binding in sites A, B and C, (R = $(NAG)_2$), with the side chain extending down the cleft to the catalytic region. The inhibition however is irreversible, and treatment of the presumed ester product with hydroxylamine has not eliminated the inhibitor and produced the hydroxamic acid.

The purpose of this work

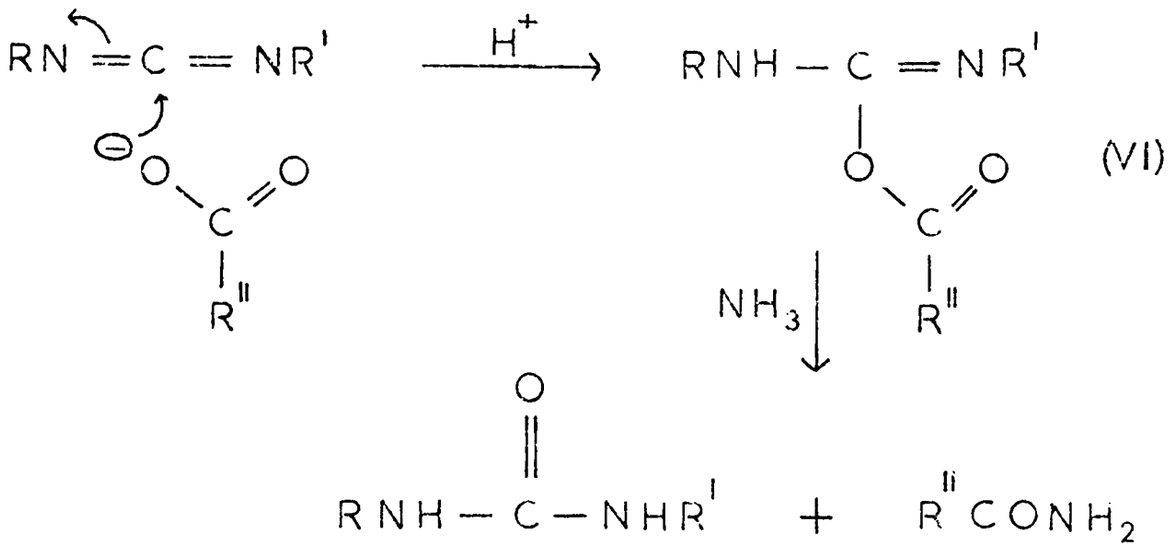
The true nature of lysozyme catalysis could hopefully be understood if acceptable evidence as to the involvement of Asp 52 in the mechanism became available. Modification with an active site directed reagent was therefore planned. In order that the modification should be as conservative as possible it was decided to attempt the conversion of Asp 52 to asparagine, which would be very similar in size but simply have no charge. In order to achieve this conversion under suitably mild conditions, recourse was made to the reaction of carboxylic acids with carbodiimides, (figure 5). A carboxyl group may be activated by a carbodiimide to give the intermediate (VI), (figure 5), and treatment of this with a nucleophile, in this case ammonia, results in the elimination of a urea, and production of an amide of the carboxylic acid.

Model building based on a NAG residue in site C showed that inhibitor A (VII) is suitably shaped for its carbodiimide grouping to come directly over Asp 52, when the NAG residue is anchored in site C. Inhibitors B and C ((VIII) and (IX)) were considered possible alternatives, although the correspondence of the -N=C=N- groupings with the Asp 52 position is not quite as suitable as with (VII).

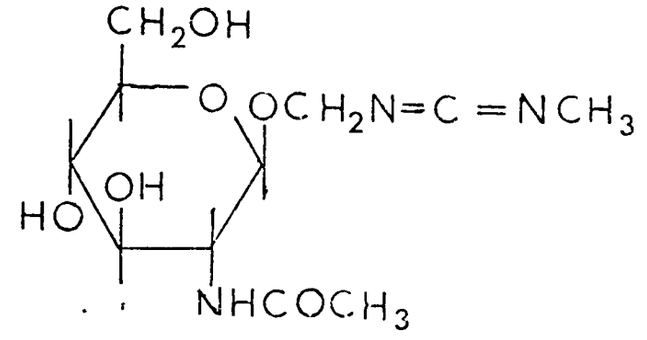
As it was clear that the activated carboxyl group (VI) might react rapidly with any neighbouring nucleophile in the enzyme, it was necessary that inhibitions should be performed in the presence and absence of added nucleophile to determine whether or not this was occurring; preliminary experiments with a radioactively

labelled nucleophile ([1-^{14}C]-glycine methyl ester) were also planned in order to observe how much added nucleophile was being taken up by the enzyme.

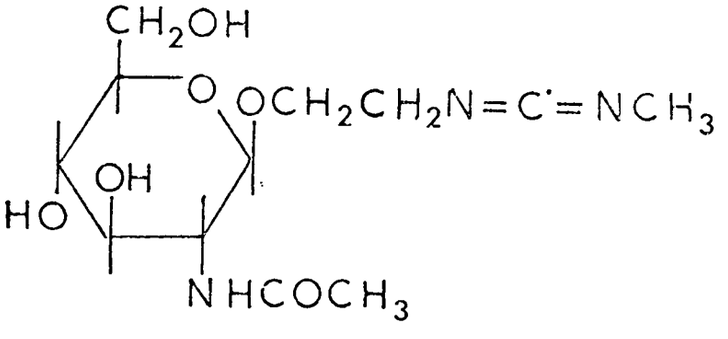
Figure 5



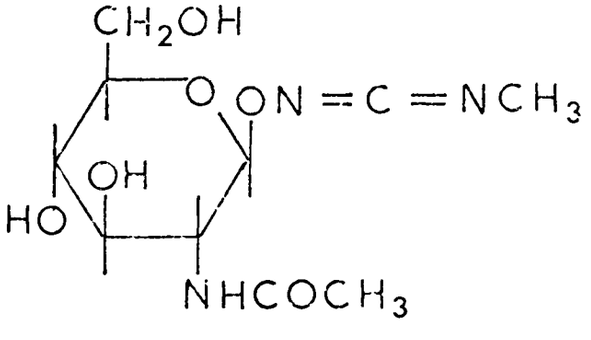
(VI)



(VII) Inhibitor A



(VIII) Inhibitor B

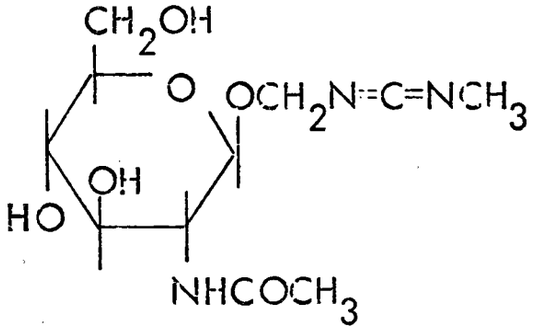


(IX) Inhibitor C

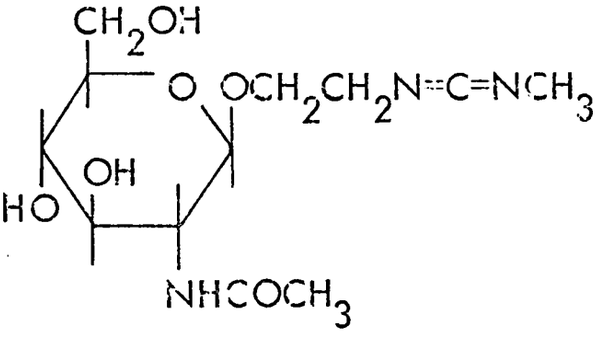
CHAPTER 2: SYNTHESIS OF A LYSOZYME INHIBITOR

Introduction

As explained in chapter 1, inhibitor A was the first choice of reagent designed to modify specifically aspartic acid 52 in lysozyme. Difficulties were encountered in the synthesis of this compound, and inhibitor B (which should be almost as suitable as A) was finally synthesised instead.



Inhibitor A (VII)



Inhibitor B (VIII)

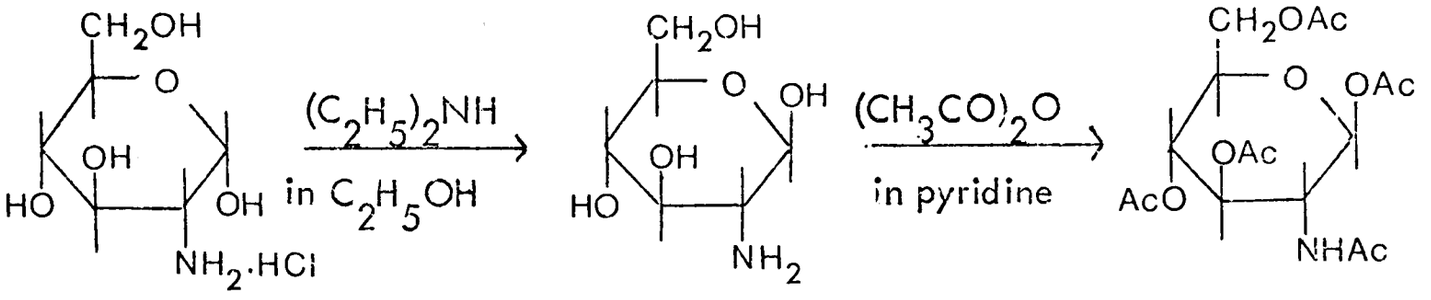
The synthesis of inhibitor A

The synthetic route chosen to this material, outlined in figure 6, involved the preparation of 3,4,6-tri-O-acetyl-2-acetamido-2-deoxy-chloromethyl-β-D-glucoside (XIII) from 1,3,4,6-tetra-O-acetyl-2-acetamido-2-deoxy-β-D-glucose (XII), followed by replacement of the chlorine by an isothiocyanato group, reaction of this with methylamine to form the thiourea, hydrolysis of the O-acetyl groups, and, finally, desulphurisation of the thiourea. Schmidt and Striewsky[125] have used a similar route to alkyl-(methoxymethyl)-carbodiimides.

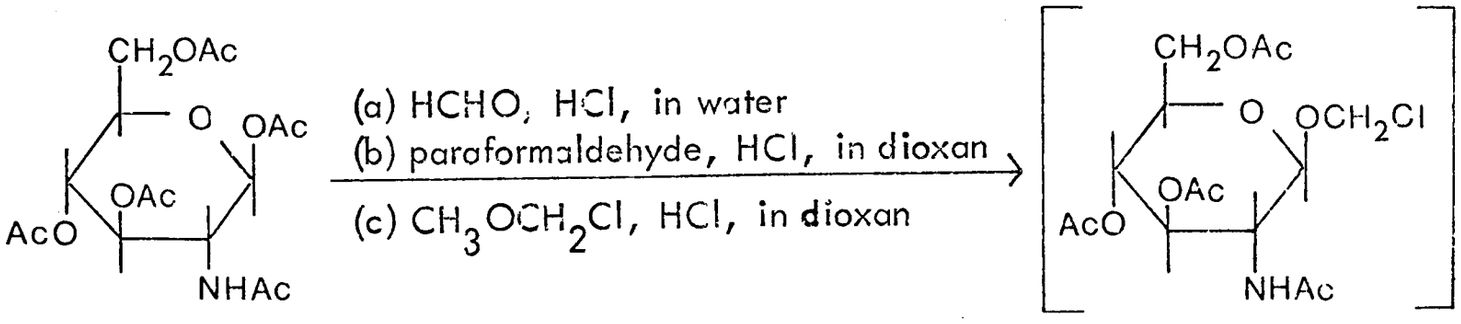
The β-D-glucosamine pentaacetate (XII) was successfully prepared by the

Figure 6

The synthesis of Inhibitor A

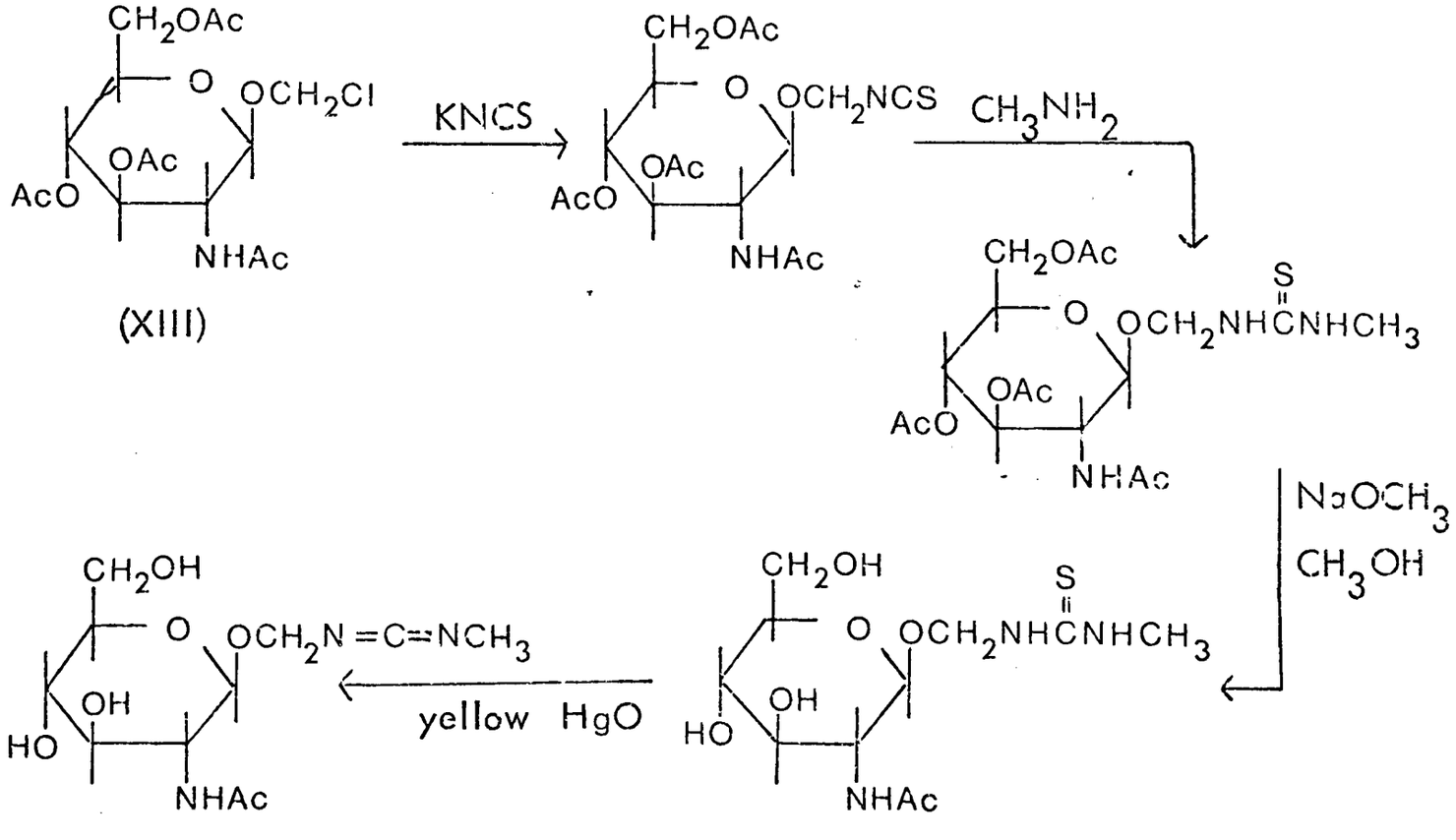


(X) (XI) (XII)



(XII) (XIII)

The remaining proposed synthetic route was:



Inhibitor A (VII)

method of Westphal and Holzmann,[109] after an attempted preparation by the method of Lobry de Bruyn and Van Ekenstein[110] had failed (in accord with the observations of Westphal and Holzmann[109] and of Baker et al.[111]).

Attempts to prepare the chloromethyl glycoside (XIII) from (XII) met with little success. Chloromethylation with formaldehyde and hydrogen chloride in water gave rise to hydrolysis products, and when the reaction was performed in dry dioxan with paraformaldehyde or methyl chloromethyl ether and hydrogen chloride, complex mixtures of products were formed, including one crystalline one which had lost the amide grouping, but whose structure could not be determined.

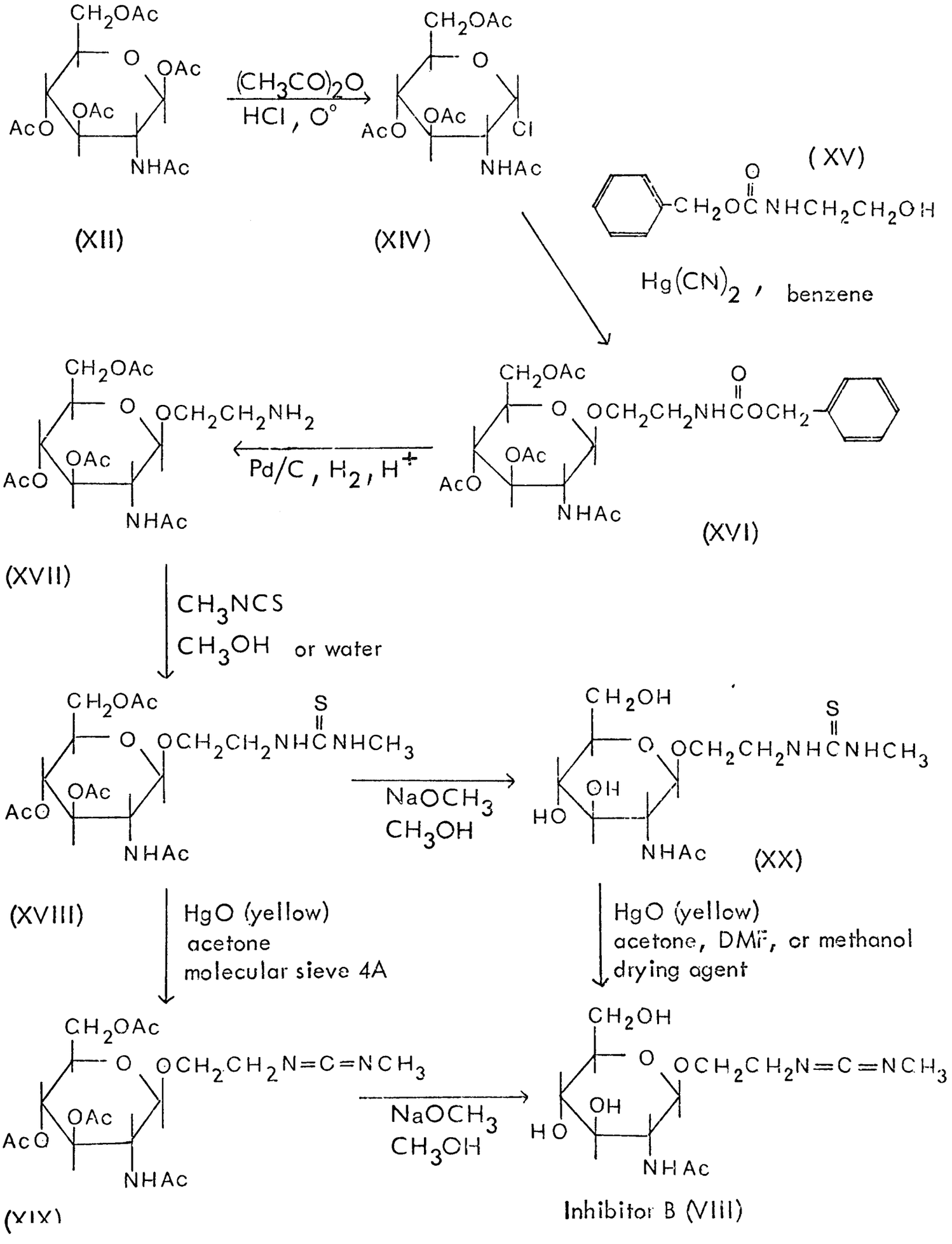
The presence of the OCH_2Cl grouping was suspected in one of the oils separated on p.l.c., but it was decided to proceed with the synthesis of inhibitor B, which should involve more easily isolatable intermediates.

The synthesis of inhibitor B

The reaction scheme shown in figure 7 was followed. Attention was paid to the choice of mild non-acidic reaction conditions in order to avoid the possibility of anomerisation of the glycosidic linkage. 1-α-Chloro-3,4,6-tri-O-acetyl-2-acetamido-2-deoxy-D-glucose (XIV) was prepared from β-D-glucosamine pentaacetate (XII) by the method of Leaback and Walker[112] after an attempt by the method of Baker et al.[111] had failed. (Baker[113] has observed that such reactions are difficult in ethereal solution owing to the presence of the weakly basic N-acetyl group which tends to form an ether-insoluble hydrochloride).

Figure 7

The synthesis of Inhibitor B



Although the analogous bromo-compound to (XIV) might be thought to be more suitable for the subsequent formation of the glycosidic bond, as in (XVI), this compound undergoes a ready transformation to the hydrobromide of α-1,3,4,6-tetra-O-acetyl-D-glucosamine.[112,114] Kuhn and Kirschenlohr[115] have succeeded in using acetobromoglucosamine in glycoside synthesis, in the presence of mercuric cyanide catalyst, but many other workers have reported difficulties.[111,112,114] The chloro-compound (XIV) is more stable but may undergo rearrangement to the tetraacetylglucosamine hydrochloride when water is present.[112,114,116]

2-(Benzyloxycarbonylamino)ethanol (XV) was prepared as described by Graham and Neuberger.[117] (XIV) and (XV) were then coupled in benzene with a mercuric cyanide catalyst.[115,118,119] The analogous glucose derivative to (XVI) has been prepared in this way,[117] though with the use of silver carbonate, silver perchlorate and a drying agent in nitromethane in the coupling reaction. Heyns[118] and Zervas[120] both report the advantage of blocking the nitrogen of the glucosamine with the diphenyl phosphoryl group, in order to prevent possible neighbouring group effects of the N-acetyl group, but the coupling reaction proceeded well without recourse to this method.

The benzyloxycarbonyl protecting group was then removed by catalytic hydrogenolysis, to give (XVII); this compound was never prepared completely free of amine hydrochloride, but a combination of infra-red, mass, and p.m.r. spectra, as well as the reconversion of a portion to compound (XVI) in 71% yield confirmed its structure.

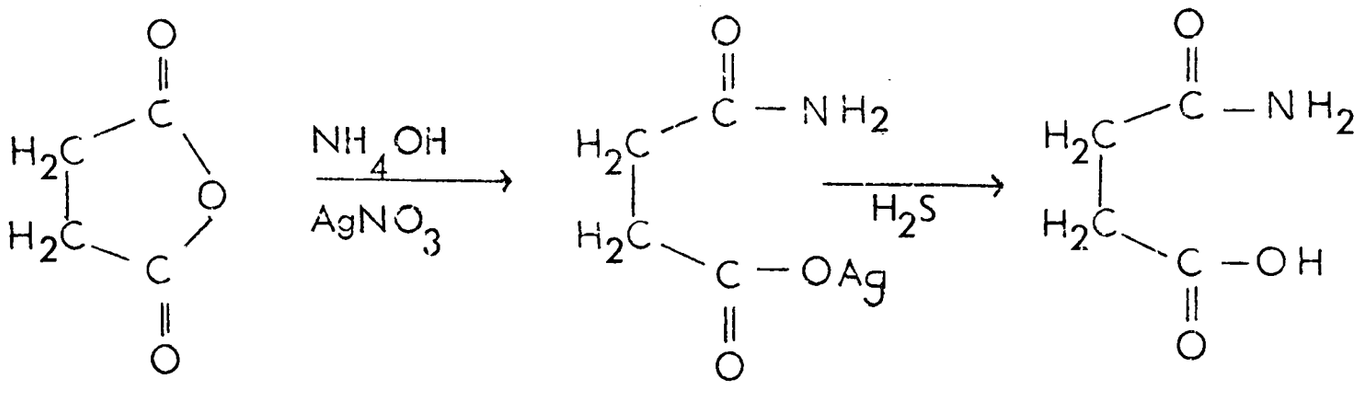
The thiourea (XVIII) was then prepared by treatment of (XVII) with methyl isothiocyanate. The ultra-violet spectrum of the product (λ_{max} = 236 nm. in water, unchanged on addition of hydrochloric acid) confirmed[121] that the possible rearrangement to the S-alkyl isothiourea had not taken place.

Removal of the O-acetyl groups of (XVIII) in sodium methoxide,[122,123] followed by desulphurisation of the thiourea with yellow mercuric oxide[124,125,126] showed some evidence of the formation of inhibitor B, (VIII), but the performance of these reactions in the reverse order was found to be much more satisfactory. Jochims[127] has used yellow mercuric oxide to desulphurise some 1,3,4,6-tetra-O-acetyl-β-D-glucos-2-yl thioureas to the corresponding carbodiimides.

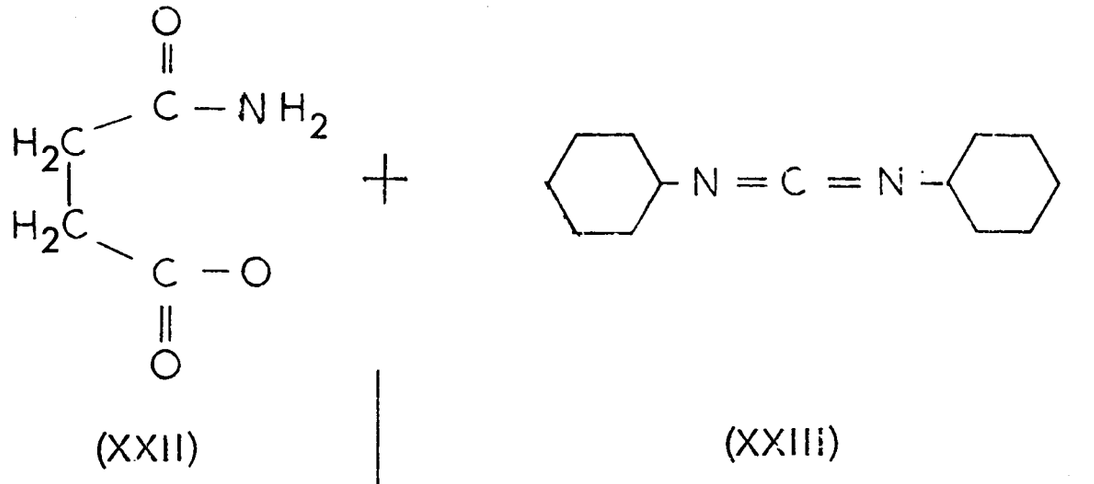
Model reaction

As it was of interest to determine whether carbodiimides could cause intramolecular coupling of an amide and a carboxylic acid, succinamic acid (XXII) was prepared from succinic anhydride (XXI) by treatment with ammonia,[128,129] (figure 8). Reaction of this with dicyclohexyl carbodiimide (XXIII) in ethanol yielded a mixture of products from which succinimide (XXIV) could be isolated in 10% yield; p.m.r. studies of the crude reaction product suggested that it contained about 20% succinimide, 50% unchanged succinamic acid, and 30% succinic anhydride.

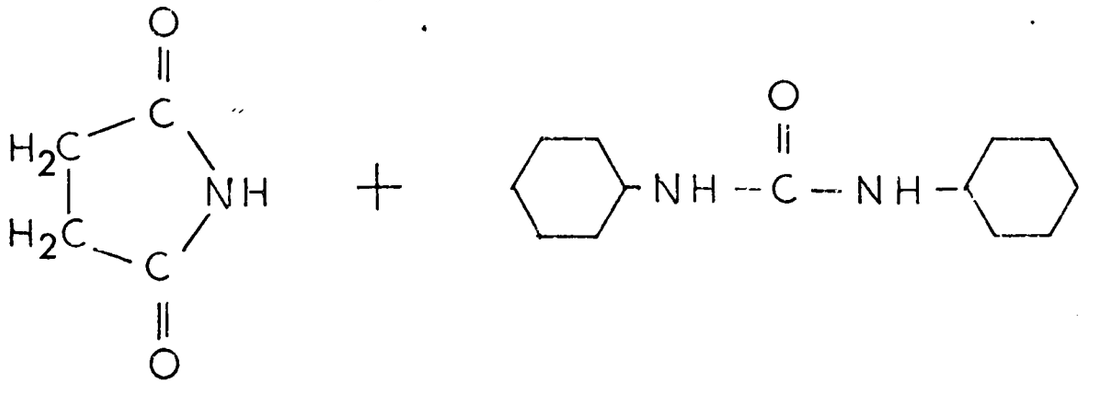
Figure 8



(XXI) (XXII)



(XXII) (XXIII)



(XXIV)

EXPERIMENTAL

Melting points were determined on a Kofler block and are uncorrected.

Infra-red spectra were determined using a Unicam S.P.200 spectrophotometer, and ultra-violet spectra using a Unicam S.P.800 spectrophotometer. Optical densities of solutions in the ultra-violet were measured on a Unicam S.P.500 spectrophotometer.

Rotations were measured using a Perkin-Elmer 141 Polarimeter at the D-line, at 20°, in a 1 dm. cell. Low resolution mass spectra were determined using an A.E.I. M.S.9 instrument or a Varian C.H.7 instrument, by Dr. R. Aplin. Samples were introduced by direct insertion. Except where indicated otherwise the base peak was that due to the $COCH_3$ fragment (m/e = 43). Peak intensities are given as a percentage of the base peak intensity.

Proton magnetic resonance spectra were determined at 100 Mc/s using a Perkin-Elmer R.14 instrument by Mrs. E. Richards in this department. All p.m.r. signals are quoted as τ values, relative to the sodium salt of 3-(trimethylsilyl)-propane sulphonic acid (TSS = 10 τ), in D_2O solutions, or relative to tetramethyl-silane (TMS = 10 τ), in other solvents.

Microanalyses were performed by Dr. Strauss in this department.

Analytical (0.1 mm.) and preparative (1 mm.) thin layer chromatoplates were made in this department from Stahl silica and were unbaked. The positions of spots on these plates were determined by development in iodine vapour, or, with

fluorescent plates, by viewing at 254 nm. or 366 nm.

Scintillation counting was carried out with a Beckman DPM-100 Liquid Scintillation Counter. Samples for this instrument were prepared by mixing an aliquot (usually 0.05 ml.) of the solution to be counted with 7 ml. of the scintillant solution in a small bottle, which was then sealed. The scintillant used was a solution of 2,5-diphenyloxazole (PPO) (6 g.) and 1,4-bis-(5-phenyl-oxazol-2-yl)benzene (POPOP) (60 mg.) in methanol (300 ml.) and toluene A.R. (700 ml.).

The preparation of 1,3,4,6-tetra-O-acetyl-2-acetamido-2-deoxy-β-D-glucose (XII)

(a) The method of Lobry de Bruyn and Van Ekenstein[110] was used. Anhydrous sodium acetate (71.6 g., 0.87 moles) was stirred at 100° with acetic anhydride (658 ml., 710 g., 6.96 moles) for 1¼ h. The sodium acetate did not completely dissolve in this time. α-D-Glucosamine hydrochloride, (X), (Koch-Light, Puriss, Batch no. G 026 h) (53.75 g., 0.25 moles) was added to the suspension; heat was evolved, and the mixture was kept at 100° for 2 h. The brownish solution was then poured into ice water (2 l.) and after decomposition of the excess acetic anhydride the pH was adjusted to just under 7 with sodium hydroxide. This was followed by extraction with chloroform, and the extract, after drying, was evaporated to a light brown tar (12 g.). Crystallisations were attempted from ethanol and aqueous ethanol, but these yielded only a few milligrams of crystalline material.

(b) The method of Westphal and Holzmann[109] was then used. β-D-Glucosamine (XI) was first prepared by stirring α-D-glucosamine hydrochloride (X), (29 g., 0.135 moles), for 2 days, at 18°, in a mixture of diethylamine (21 ml., 14.9 g., 0.204 moles) and ethanol (330 ml.). The supernatant was sucked off and the residual white powder washed with ethanol. Yield 22.94 g., m.p. 127-128°, (Lit: 110° dec.).[130]

This material was purified by suspending it, (22.3 g., 0.124 moles) in ethanol (100 ml.) at 37-38° for 15 h. The product was washed with ethanol and ether; yield 22.1 g., (94%), m.p. 127-128°.

A subsequent preparation from α-D-glucosamine hydrochloride (100 g., 0.475 moles) yielded 82.6 g. (97%), m.p. 129-132° of this material. $[\alpha]_D^{20}$ = +38.0° after 15 min., and +50.1° after 3 h., (C = 1 in water). (Lit.: +28.0° initially, +47.5° at equilibrium).[109]

The β-D-glucosamine preparation, (22 g., 0.123 moles if not contaminated with the hydrochloride), was added to a mixture of acetic anhydride (137.5 ml.) and pyridine (229 ml.); a small amount of white powder which immediately separated was removed by decantation of the supernatant. The reaction evolved heat, and was cooled in ice water. Crystallisation of the product began after 10 h., and after 2 days the crystals were filtered off, washed with water and dried. Further crystals were obtained after partial evaporation of the supernatant. Yield 24 g., (50%), m.p. 186-188°, (Lit: 187-189°).[130] The product was pure by t.l.c. (R_F = 0.26 in ethyl acetate).

A larger scale preparation was carried out similarly, except that the white solid which remained undissolved in the acetylation mixture, and which was assumed to be glucosamine hydrochloride, (giving a positive silver nitrate test), was retreated with alcoholic diethylamine, as described earlier. The product from this was then acetylated successfully. The total yield from an original 100 g., (0.465 moles), of α-D-glucosamine hydrochloride was 100.4 g., (56%), m.p. 187--188°.

$[\alpha]_D^{20}$ = -0.91°, (C = 1 in chloroform), (Lit: +1.2°).[130]

(Calculated for $C_{16}H_{23}O_{10}N$: C, 49.35; H, 5.95; N, 3.60. Found: C, 49.47; H, 5.79; N, 3.61%).

ν_{max} ($CHCl_3$): 3420 (NH), 1740 ($OCOCH_3$), 1680 ($NHCOCH_3$), 1530 $cm.^{-1}$ ($NHCOCH_3$ II).

The mass spectrum (220°) showed a molecular ion peak, m/e = 389, (0.1%), and also: 346 (4.8%) ($M-COCH_3$); 330 (1%) ($M-OCOCH_3$); 287 (2.3%) ($M-COCH_3-OCOCH_3$); 228 (2.8%) ($M-COCH_3-2 \times OCOCH_3$), and other peaks due to loss of acetyl fragments.

The p.m.r. spectrum, (100 Mc/s), showed the following signals: τ ($CDCl_3$): 4.14 (1H, broad d , J = 9.5 c/s, N-H), 4.26 (1H, d, $J_{1,2}$ = 8.5 c/s, H-1), 4.60-5.00 (2H, m, H-3, H-4), 5.40-6.00 (3H, m, H-2, H-6, H-6'), 6.00-6.30 (1H, m, H-5), 7.89, 7.91, 7.95 (12H, 3s, $4 \times OCOCH_3$), 8.08 (3H, s, $NHCOCH_3$).[131]

Attempted preparations of 3,4,6-tri-O-acetyl-2-acetamido-2-decxy-chloromethyl-β-D-glucoside (XIII)

(a) β-D-Glucosamine pentaacetate, (XII), (1 g., 2.57 mmoles) was stirred with formaldehyde solution (37-41%, 10 ml., 0.13 moles) at 60-70°, and dry hydrogen chloride was passed into the suspension, which cleared within 5 min. Charring occurred and after 2 h. the brown solution was cooled and extracted with methylene chloride. The extract was washed with water, dried, and concentrated. T.l.c. in ethyl acetate showed no mobile material, and an infra-red spectrum in methylene chloride bore little resemblance to that of starting material, suggesting considerable decomposition. (O-acetyl absorption at 1745 $cm.^{-1}$ and amide absorption at 1685 $cm.^{-1}$ were both considerably diminished in intensity, and the 900-1200 $cm.^{-1}$ region showed many new peaks (O-H or C-O-C)).

(b) The procedure described in (a) was followed exactly except that the reaction was kept at 15°. T.l.c. of the methylene chloride extract showed some starting material (R_F = 0.24 in ethyl acetate), and the infra-red spectrum showed peaks due to starting material as well as extra absorption in the 3500-3800 $cm.^{-1}$ region (O-H) and 900-1200 $cm.^{-1}$ region (O-H or C-O-C).

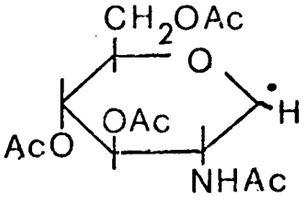
(c) Paraformaldehyde (0.7 g., 23.3 mmoles) and β-D-glucosamine pentaacetate (XII) (1 g., 2.57 mmoles) were stirred together in dry dioxan at 10°, and dry hydrogen chloride was passed into the suspension, which cleared after 5 min. After a further 45 min. the solution was evaporated to dryness at 15°, and the

product was taken up in methylene chloride. This solution was washed with water, dried, and evaporated to about 10 ml. T.l.c. in ethyl acetate revealed three components, of R_F values 0.58, 0.41, and 0.20, none of which was at the position of starting material. One third of the product was chromatographed on a 100 x 20 cm. preparative thin layer plate, using 2 elutions with chloroform. Iodine staining of the edges of the plate showed 4 bands, which were scraped off and the products eluted with ethyl acetate.

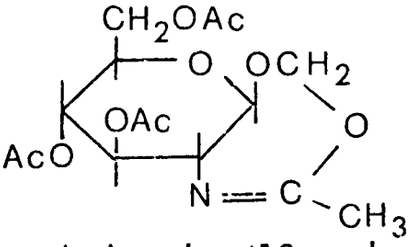
The fastest moving band (A) yielded 40 mg. of crystalline material, (m.p. 145°). T.l.c. in ethyl acetate showed a major component at R_F 0.52 and a minor one at R_F 0.13.

ν_{max} ($CHCl_3$): 1740 $cm.^{-1}$ ($OCOCH_3$).

The p.m.r. spectrum, (100 Mc/s), showed τ ($CDCl_3$): 7.80–8.00 (12H, m, 4 x $OCOCH_3$), and was complex below 7 τ (14H). No evidence of $NHCOCH_3$ was seen.

The mass spectrum (165°) showed no evidence of the presence of chlorine in the molecule. The highest peak was at m/e = 418 (3%), and other important peaks were at 360 (5%); 359 (12%) (? 418 - $OCOCH_3$); 330 (1.5%) (ring residue  = 330); 300 (3.4%) (? 359 - $OCOCH_3$); 299 (5%) (? 359 - CH_3COOH). Mass spectra of peracetylated glucosamine derivatives, (e.g. other spectra in this chapter), show a strong M-43 peak due to loss of the

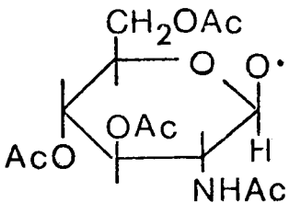
$COCH_3$ grouping from the amide nitrogen. This feature is absent from this spectrum. It is interesting that one of the possible reaction products,



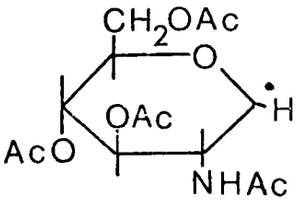
would give a molecular ion peak at m/e = 359, (observed here), but the 418 peak is unexplained.

The remaining three bands (B, C, and D) from the p.l.c. plate yielded a few milligrams of oil each. All were impure by t.l.c., and their infra-red spectra were very similar to that of starting material, except for extra absorption at 1050 cm.$^{-1}$ (? C-O-C).

The mass spectrum of the fastest moving of these three bands (B) was run at 165° and showed no clear molecular ion. The highest molecular weight peaks were at m/e = 346 (0.3%), and 330 (0.4%), probably due to the fragments



and

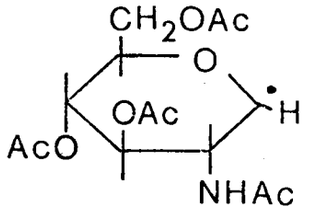


. The rest of the spectrum was very similar to one of β-D-glucosamine pentaacetate, run for comparison. HCl peaks at m/e = 36 and 38 (of intensities 11.5% and 4.9% respectively) were suspected.

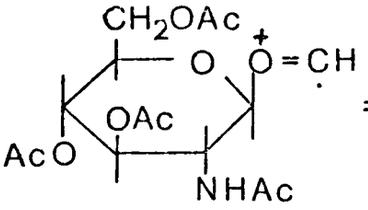
(d) Methylchloromethyl ether (0.4 g., 4.96 mmoles) was added to a suspension of β-D-glucosamine pentaacetate (XII) (1 g., 2.57 mmoles) in dioxan (15 ml.) and dry hydrogen chloride was passed for 1 h. at 10°. T.l.c. of the product, worked up as described in (c), showed mostly starting material (R_F 0.26 in ethyl acetate) and a minor component at R_F 0.43. The reaction was continued,

in dioxan (20 ml.), with fresh methyl chloromethyl ether (1 ml., 13.2 mmoles) for $3\frac{1}{2}$ h. at 25°. The product was again worked up as before, and t.l.c. in ethyl acetate now showed a series of spots of R_F values 0.04, 0.10, 0.18, and 0.43, but no starting material (R_F 0.26).

P.l.c. was carried out as before on two 100 x 20 cm. plates. Apart from a band near the solvent front, which was shown not to contain carbohydrate, only 2 bands could be detected. The faster moving one showed no amide absorption in the infra-red (as band A, attempt (c)); the slower moving band did show amide absorption and its overall infra-red and mass spectra were similar to those of band B, attempt (c). The mass spectrum was run at 120° and much of it could be rationalised on the basis of fragmentations from



= 330 (0.1%), and



= 359 (0.12%).

The preparation of 1-α-chloro-3,4,6-tri-O-acetyl-2-acetamido-2-deoxy-D-glucose (XIV)

(a) The method of Baker et al.[111] was used. This involves the treatment of β-D-glucosamine pentaacetate, (XII), with a saturated solution of hydrogen chloride in a mixture of dry ether and acetic anhydride at 0°. The sugar did not dissolve, even with added acetic acid[132] and extra solvent. After 4 days the

reaction mixture was worked up as described in reference 111, and this yielded crystalline material with identical spectra and melting point (186°) to those of starting material.

(b) The method of Leaback and Walker[112] was then used. Dry β-D-glucosamine pentaacetate, (XII), (10 g., 0.026 moles), was stirred with acetic anhydride (30 ml.) saturated at 0° with dry hydrogen chloride. After 2 h. the solid had dissolved, and the yellowish solution was kept at 18° for 16 h. before working it up as described in reference 112. Yield of crude product: 6.5 g., (69%), m.p. 122-124° dec. This was recrystallised from ethyl acetate/pet. ether (80-100). Yield 6 g. (64%), m.p. 128° dec., (Lit: 125-126°,[111] and 133-134° dec.[112]).

Two subsequent preparations from β-D-glucosamine pentaacetate, (45.7 g., 0.118 moles, and 35.5 g., 0.091 moles), yielded 28.45 g., (66%), and 24.26 g., (73%), respectively of the crude product. These products were combined and recrystallised. Yield 48.8 g., m.p. 128-129° dec.

$[\alpha]_D^{20}$ = +120.9°, (C = 1 in chloroform), (Lit: +118°).[112]

(Calculated for $C_{14}H_{20}O_8NCl$: C, 45.97; H, 5.51; N, 3.83; Cl, 9.69. Found: C, 45.96; H, 5.58; N, 3.88; Cl, 9.88%).

The mass spectrum at 165° showed a molecular ion peak at m/e = 365 (0.2%), and many peaks due to losses of Cl, HCl, and acetyl fragments.

The p.m.r. spectrum, (100 Mc/s), showed the following peaks:

τ ($CDCl_3$): 3.82 (1H, d, $J_{1,2}$ = 3.5 c/s, H-1), 3.97 (1H, broad d, J = 8 c/s, N-H),

4.53-4.96 (2H, m, H-3,H-4), 5.30-6.04 (4H, m, H-2,H-5,H-6,H-6'), 7.92 (3H, s, $OCOCH_3$), 7.97 (6H, s, 2 x $OCOCH_3$), 8.04 (3H, s, $NHCOCH_3$).[133]

The preparation of 2-(benzyloxycarbonylamino)ethanol (XV)

The procedure of Graham and Neuberger[117] was used. Benzyl chloroformate, (B.D.H.), (12 g., 0.07 moles), was added in portions during 30 min. to a stirred solution of ethanolamine (B.D.H., redistilled, b.p. 168-170°), (3.7 g., 0.061 moles) in sodium bicarbonate solution (75 ml., containing 12.6 g., 0.15 moles) at 0°. After $3\frac{1}{2}$ h. the product, a white suspension, was acidified with concentrated hydrochloric acid, extracted with chloroform and worked up as described in reference 117. The product was recrystallised from chloroform/pet. ether (40-60). It was found that the effectiveness of stirring of the reaction mixture greatly influenced the yield of product, which was also increased by allowing most of the reaction to occur at room temperature. Thus the first reaction attempted, before these factors were taken into account, yielded less than 10% of the product, whereas the second attempt with vigorous stirring and shaking at room temperature gave 6.7 g., (56%), of recrystallised product, m.p. 56-59°, (Lit: 62-62.5°).[134] A further recrystallisation raised the m.p. to 59-61°.

In a later larger scale preparation, from ethanolamine (30.5 g., 0.50 moles) and benzyl chloroformate (85.3 g., 0.50 moles), traces of the chloroformate which tended to contaminate the crystalline product were removed by extensive washing

with pet. ether (40–60). Yield 45.3 g., (46.5%), m.p. 63.5–64.5°, pure by t.l.c. (R_F = 0.40 in ethyl acetate). The carbonyl absorption in the infra-red was at 1710 $cm.^{-1}$.

(Calculated for $C_{10}H_{13}O_3N$: C, 61.53; H, 6.71; N, 7.17. Found: C, 61.37; H, 6.77; N, 7.15%).

The preparation of 1-β-(2-(benzyloxycarbonylamino)ethyl)-3,4,6-tri-O-acetyl-2-acetamido-2-deoxy-D-glucoside (XVI)

The conditions described by Kuhn and Kirschenlohr[115] for the preparation of the 1-β-ethyl analogue of (XVI) were used in this preparation.

The N-protected ethanolamine, (XV), (1.1 g., 5.64 mmoles) was stirred with powdered mercuric cyanide (4 g., 15.9 mmoles) in benzene (5 ml.), and (XIV) (1.38 g., 3.8 mmoles) was added. Stirring was continued for 3½ h. at 20° and for a further 1½ h. at 35°, after which the mixture was diluted with chloroform (20 ml.) and was washed repeatedly with water. The organic layer was dried, and t.l.c. in ethyl acetate revealed three components of R_F values 0.38 (reference R_F value of (XV) = 0.38), 0.42 (reference R_F value of (XIV) = 0.44), and 0.23. The sample was applied to three 100 x 20 cm. p.l.c. plates (HF 254/366). Three elutions were made with 5% ethyl acetate in chloroform, followed by one with 10% and one with 15% ethyl acetate in chloroform. Four bands separated and were scraped off and eluted completely with ethyl acetate (bands 1–4 in order of

decreasing R_F value).

Band 1: Fluoresced at 366 nm., and yielded yellow oil, (157 mg.). This was expected to be compound (XIV), (of R_F 0.42 in ethyl acetate), but in fact t.l.c. showed it to have an R_F value of 0.18 in this solvent. Its infra-red spectrum was similar to that of (XIV) except that extra absorption was seen at 3450 and 1050 $cm.^{-1}$. It appears that some hydrolysis had occurred in the separation procedure.

Band 2: Fluoresced at 366 nm., and yielded an oil, (64 mg.). T.l.c. in ethyl acetate showed components at R_F 0.26 and 0.19, and the infra-red spectrum was similar to that of band 1.

Band 3: Fluoresced at 254 nm., and yielded white needles, (240 mg.). The spectral and t.l.c. properties of this material were identical to those of 2-(benzyloxycarbonylamino)ethanol, (XV), except that it was contaminated with a small amount of material with R_F 0.19.

Band 4: Fluoresced most at 254 nm., and yielded white crystals, (400 mg.). These were recrystallised from chloroform/pet. ether (60-80). M.p. 170°, $[\alpha]_D^{20} = -16.0°$, (C = 1 in chloroform).

(Found: C, 55.28; H, 6.15; N, 5.16. $C_{24}H_{32}O_{11}N_2$ requires: C, 54.96; H, 6.15; N, 5.34%).

The carbonyl region of the infra-red spectrum showed three overlapping peaks: ν_{max} ($CHCl_3$): 1745 ($OCOCH_3$), 1710 ($-NHCOOCH_2-$), 1685 $cm.^{-1}$ ($NHCOCH_3$).

The p.m.r. spectrum (100 Mc/s) showed the following peaks:

τ ($CDCl_3$): 2.70 (5.5H, broad s, C_6H_5 and $CHCl_3$), 3.89 (1H, broad d, J = 8.5 c/s, $N\underline{H}COCH_3$), 4.43-5.05 (5H, m, $OC\underline{H}_2C_6H_5$, $N\underline{H}COO$, H-3, H-4), 5.30 (1H, d, J = 7.5 c/s, H-1), 5.60-6.80 (8H, m, $-OC\underline{H}_2C\underline{H}_2NH-$, H-2, H-5, H-6, H-6'), 7.80-8.00 (9H, 2s, 3 x $OCOC\underline{H}_3$), 8.10 (3H, s, $NHCOC\underline{H}_3$).

The mass spectrum (250°) showed a molecular ion peak at m/e = 524 (0.2%). Other significant peaks were at 465 (0.8%) ($M-OCOCH_3$); 357 (2%) ($M-OCOCH_3-C_6H_5CH_2OH$); 330 (6.5%) ($M-OCH_2CH_2NHCOOCH_2C_6H_5$); 108 (39%) ($C_6H_5CH_2OH$); 91 (54%) ($C_6H_5CH_2.$). Peaks at m/e = 108 and m/e = 91 are characteristic in the mass spectra of benzyloxycarbonyl derivatives.[135]

In a repeat preparation, from chloroacetylglucosamine, (XIV), (4.05 g., 11.1 mmoles), 2-(benzyloxycarbonylamino)ethanol, (XV), (3.23 g., 16.6 mmoles), and mercuric cyanide, (3.0 g.), in benzene, (15 ml.), reaction was allowed to proceed at 18° for 3½ h. and at 35° for 3 h., after which the product crystallised out from the mixture. Subsequent treatment of the mixture was as before, except that the product was isolated by careful crystallisation of the crude mixture from ethyl acetate/pet. ether (40-60). After recrystallisation the yield of pure product was 2.16 g., (37%), m.p. 169-170°.

Using the same relative quantities of reagents a later larger scale preparation from (XIV), (48 g., 0.131 moles), which was allowed to react for 3 h. at 35° and for 12 h. at room temperature, yielded 54.2 g., (79%), of recrystallised product, m.p. 170-172°.

The preparation of 1-β-(2-aminoethyl)-3,4,6-tri-O-acetyl-2-acetamido-2-deoxy-D-glucoside (XVII)

An attempt to hydrogenolyse compound (XVI) in dry ethyl acetate (K_2CO_3 washed) in the presence of palladium on charcoal catalyst was unsuccessful, but a repeat attempt in ethyl acetate containing a trace of acetic acid resulted in a product whose infra-red spectrum showed removal of the protecting group. Methanol and ethanol, which were better solvents for the starting material, were used as solvents for subsequent larger scale preparations.

(a) (XVI), (2.16 g., 4.1 mmoles) was dissolved in ethanol (185 ml.), and 5% palladium on charcoal (220 mg.) was added, followed by 3 drops of concentrated hydrochloric acid. The mixture was shaken under hydrogen at atmospheric pressure; hydrogen uptake was rapid and ceased after $2\frac{1}{2}$ h., when 110 ml. had been taken up. The theoretical uptake is 94 ml., not including that taken up by the catalyst itself, (and assuming that the carbon dioxide liberated is all absorbed by the water in the system). Filtration and evaporation gave an oil which later crystallised (1.5 g., 93% crude yield). Recrystallisation was found to be difficult, the product usually coming out of solution as an oil. The only successful recrystallisation solvent used was ethanol/pet. ether (60-80). A hot solution of the crude product was decolourised, filtered, and cooled. The first batch of crystals to appear (short needles, 45 mg.) had a sharp melting point (148-149°), but the next two batches (400 mg. and 115 mg.) had lower melting ranges and showed some

decomposition (144-146° dec., and 143-144°). The final two batches, totalling 346 mg. were similarly less pure. T.l.c. in ethyl acetate showed all the batches to consist only of immobile material, and infra-red spectra of the first three batches revealed no differences between them. A p.m.r. spectrum in D_2O showed that the protecting group had been completely removed; a fuller analysis of the p.m.r. spectrum appears later.

(b) When methanol was used as the solvent for the hydrogenolysis, on a larger scale, the starting material showed some tendency to crystallise out from the solution. However it rapidly redissolved as the methanol-soluble product was formed.

The protected amine (XVI) (53 g., 0.101 moles) was hydrogenolysed in four batches, in methanol (5% solution), with 2% w/w 5% palladium on charcoal as catalyst. With one batch the reaction proceeded smoothly in the absence of added acid, but in the other three cases a trace of concentrated hydrochloric acid was added. Fresh catalyst was added halfway through two of the reactions (which took 6 to 7 h. to reach completion) as they were proceeding rather slowly. The uptake of hydrogen was normally about 5-10% above the theoretical quantity.

The products were filtered through a celite packed sinker, azeotroped with benzene, and evaporated to oils, from which it was found difficult to remove all traces of solvent. The yields at this stage were a little over 100% for this reason. A portion was crystallised as before, after several attempts, for characterisation.

This product will obviously contain some hydrochloride, and this was confirmed by a silver nitrate test on an aqueous solution. Characterisation data is thus not absolutely meaningful.

The product was sparingly soluble in chloroform and methylene chloride, more soluble in ethyl acetate and ethanol, and readily soluble in water.

m.p. 136-140°, $[\alpha]_D^{20}$ = -18.25° (C = 1 in ethanol).

The infra-red spectrum ($CHCl_3$) showed no benzyloxycarbonyl absorption at 1710 $cm.^{-1}$, but showed extra absorption (compared with starting material) at 3300-3400 $cm.^{-1}$ and ca. 1560 $cm.^{-1}$ (NH_2). No evidence of NH_3^+ was seen in the infra-red.

The p.m.r. spectrum, (100 Mc/s), was complex below τ 6.5, but showed peaks above this as follows:

τ (D_2O): 6.94 (2H, broad m, CH_2NH_2), 7.88, 7.93, 7.96 (9H, 3s, 3 x $OCOCH_3$), 8.03 (3H, s, $NHCOCH_3$). No evidence of aromatic protons was seen.

The mass spectrum (185°) showed a molecular ion peak at m/e = 390 (0.2%) and no HCl peaks. Other significant peaks were at 347 (1.0%) ($M-COCH_3$); 332 (1.2%) ($M-NHCOCH_3$); 331 (7.0%) ($M-OCOCH_3$); 330 (6.5%) ($M-OCH_2CH_2NH_2$); 304 (2.7%) ($M-2 \times COCH_3$); 288 (1.2%) ($M-COCH_3-OCOCH_3$); 272 (2.5%) ($M-2 \times OCOCH_3$).

Reconversion of (XVII) to (XVI)

As a further check on the nature of the product, a portion was treated with benzyl chloroformate. The product (XVII) (195 mg., 0.5 mmoles if pure amine) was dissolved in chloroform (10 ml.) and triethylamine (0.1 ml., 73 mg., 0.72 mmoles) was added. Benzyl chloroformate (85 mg., 0.5 mmoles) was stirred with the solution for 12 h. at room temperature. T.l.c. (in ethyl acetate) was used to monitor the reaction, and showed the production of material with an R_F value of 0.23; (R_F of compound (XVI) = 0.23). A little of the immobile starting material did not react in this time. The product was evaporated to dryness, washed with pet. ether, (to remove benzyl chloroformate), taken up in chloroform and washed thoroughly with water, (to remove excess starting material). After drying, the chloroform layer yielded crystalline product, (187 mg., 71%). A recrystallised sample had a melting point of 167-168° and identical infra-red and p.m.r. spectra to those of the compound (XVI) prepared previously.

Attempted purifications of (XVII)

An attempt was made to remove all traces of hydrochloride from the product (XVII). 1 g., (2.5 mmoles), was stirred overnight in a solution of sodium bicarbonate (0.4 g., 46 mmoles) in water (20 ml.). The product was extracted, first into ethyl acetate, and then into chloroform, but the extracts still gave positive silver nitrate tests.

However, dry hydrogen chloride passed into a dry chloroform solution of the crude amine, (0.537 g., 1.38 mmoles), failed to precipitate the hydrochloride, which is presumably therefore soluble in chioroform. Attempts to crystallise the product from the oil obtained after evaporation of the solution were unsuccessful.

Similarly the picrate of (XVII) was not precipitated at room temperature or at 0° when 1 ml. of a saturated solution of picric acid (containing 140 mg., 0.61 mmoles) was added to a solution of the crude amine (178 mg., 0.46 mmoles) in water (5 ml.).

Preparations of 1-β-(2-(N'-methyl)thioureido ethyl)3,4,6-tri-O-acetyl-2-acetamido-2-deoxy-D-glucoside (XVIII)

Many preparations of this material were carried out, the first being in methanol, and later ones in water, (when it was found that the work-up procedure was simpler). Examples of reactions in each solvent are given here.

(a) In methanol.

The amine (XVII), (840 mg., 2.16 mmoles) was stirred with methyl isothiocyanate (Aldrich) (157 mg., 2.16 mmoles) in methanol (25 ml.) at 20°. T.l.c. in 10% ethanol in ethyl acetate showed the formation of a major product of R_F 0.34, and of minor products of lower R_F values. Heating the reaction mixture to 40° for 1 h., and refluxing it for 45 min. did not alter the relative intensities of the major product spot and the small remaining spot at the position of starting

material, (which was immobile in this solvent). The product was then chromatographed on a single 100 x 20 cm. p.l.c. plate using 2 elutions with 10% ethanol in ethyl acetate. The main band was removed and eluted with 20% ethanol in ethyl acetate, yielding an oil, (420 mg., 42%). Crystallisation was attempted from several solvents without success, although the product was later obtained as a powder with ill-defined melting point. It was pure by t.l.c.

λ_{max} (C_2H_5OH): 241 nm., $\epsilon = 1.6 \times 10^4$; λ_{max} (H_2O): 236 nm., unchanged on addition of hydrochloric acid. This is consistent with the compound being an N,N' disubstituted thiourea (λ_{max} (H_2O): 230-240 nm., ϵ = ca. 10^4).[121]

The p.m.r. spectrum (100 Mc/s; D_2O) was virtually identical to that of starting material (p. 67) except for an extra 3 proton singlet at τ 7.11 (due to $NHCH_3$). The mass spectrum at 240° showed as highest molecular weight fragment that with m/e = 404 (2.5%), i.e. $M-OCOCH_3$. Other significant peaks were at 374 (0.9%) ($M-NHCSNHCH_3$ or $M-OCOCH_3-NHCH_3$); 373 (4.9%) ($M-NH_2CSNHCH_3$ or $M-OCOCH_3-NHCH_3-H$); 330 (9.0%) ($M-OCH_2CH_2NHCSNHCH_3$).

(b) In water.

The amine, (XVII), (19.11 g., 49 mmoles) was stirred with methyl isothiocyanate (3.82 g., 52.4 mmoles) in water (60 ml.) at room temperature. The reaction was followed by t.l.c. in 10% ethanol in ethyl acetate. The major product had an R_F value of 0.32 in this solvent and there were several minor products of greater

and smaller R_F values. After 5 h. the mixture, which still contained a little undissolved methyl isothiocyanate, was extracted with pet. ether (40-60) (200 ml.). This extract was shown to contain only methyl isothiocyanate (R_F 0.77 in 10% ethanol in ethyl acetate). The aqueous layer was then extracted 5 times with 200 ml. portions of ethyl acetate. This extracted the major product almost uniquely, (as determined from t.l.c. and from the ultra-violet spectra of the two layers). The product was an oil, (4.7 g., 21%).

In experiments in which triethylamine, (1-1.5 molar equivalents), was added in order to ensure that the free amine (XVII) was present, the mixture of products obtained was more complex, and the yield of crude product was only about one half of that from experiments in which triethylamine was omitted.

The product was purified by elution from a column (40 x 3 cm.) of silica M.60 (Harrington) using a gradient of eluting solvent from pure ethyl acetate to 30% ethanol in ethyl acetate. Crystallisation of the product was attempted from several solvents without success.

$[\alpha]_D^{20}$ = -26.7° (C = 1 in methanol).

ν_{max} ($CHCl_3$): 3600-3200 (NH), 1740 ($OCOCH_3$), 1680 $cm.^{-1}$ ($NHCOCH_3$).

The p.m.r. spectrum (100 Mc/s) showed the following peaks:

τ ($CDCl_3$): 3.05-3.53 (3H, m, 3 x N-H), 4.65-5.10 (2H, m, H-3, H-4), 5.25 (1H, d, J = 9 c/s, H-1), 5.60-6.65 (8H, m, OCH_2CH_2NH, H-2, H-5, H-6, H-6'), 6.95 (3H, d, J = 5 c/s, $NHCH_3$), 7.91 (3H, s, $OCOCH_3$), 7.97 (6H, s,

2 x $OCOCH_3$), 8.05 (3H, s, $NHCOCH_3$).

On addition of D_2O the spectrum was unchanged except for the following peaks: τ 3.10-3.40 (0.9H, m, incompletely exchanged N-H), τ 6.98 (3.0H, broad s, $NH-CH_3$). Some simplification of the τ 6.00-6.40 region was also seen (H-2 and OCH_2CH_2NH).

The mass spectrum (255°) showed a molecular ion peak at m/e = 463 (0.6%). Other significant peaks were at 404 (0.8%) ($M-OCOCH_3$); 373 (4.0%) ($M-NH_2CSNHCH_3$ or $M-OCOCH_3-CH_3NH-H$); 346 (1.5%) ($M-CH_2CH_2NHCSNHCH_3$); 330 (3.7%) ($M-OCH_2CH_2NHCSNHCH_3$). A peak at m/e = 475 (0.9%) was also present in the spectrum, together with one at m/e = 416 (0.5%) possibly due to ($475-OCOCH_3$).

In a later preparation the product was successfully crystallised from an ethanol/ethyl acetate mixture. The crystals softened at 123-130° and melted sharply at 134-135°. The spectral properties of this material were very similar to those of the non-crystalline product, except that the mass spectrum (at 155°) now showed virtually none of the 475 peak (m/e = 463 (4.9%); m/e = 475 (0.7%)).

The preparation of 1-β-(2-(N'-methyl)thioureido ethyl)-2-acetamido-2-deoxy-D-glucoside (XX)

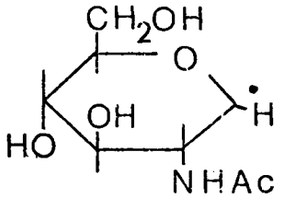
The fully acetylated thiourea (XVIII) (200 mg., 0.43 mmoles) was dissolved in methanol (10 ml.) and a small pellet of sodium (90 mg.) added. The reaction mixture was stirred overnight. T.l.c. in 10% ethanol in ethyl acetate showed that the starting material had completely reacted.

Amberlite cation-exchange resin IR 120 (H), (1.3 g.), was stirred with the solution for $4\frac{1}{2}$ h. to remove sodium methoxide, and, after filtration, the solution was evaporated to a small volume and applied to a 100 x 20 cm. p.l.c. plate. Two elutions with 30% ethanol in ethyl acetate separated the product, (of R_F 0.15 on analytical plates in this solvent), from contaminating immobile material. The product band was eluted with 30% ethanol/ethyl acetate, and yielded an oil (102 mg.). This was taken up in water and centrifuged to remove a little silica (15 mg.). Water was removed, giving the product as a sticky whitish powder (87 mg., 60%).

The infra-red spectrum confirmed that the O-acetyl groups had been removed and that the N-acetyl group (1660 $cm.^{-1}$) was intact. The ultra-violet spectrum in ethanol showed λ_{max} = 240 nm., $\epsilon = 1.06 \times 10^4$.

The p.m.r. spectrum, (100 Mc/s), was complex below τ 7 but also showed the following signals:

τ (D_2O): 7.08 (3H, s, $NHC\underline{H}_3$), 7.97 (3H, s, $NHCOC\underline{H}_3$).

The mass spectrum was run (at 240°) although the compound was not expected to be suitable for mass spectroscopy. The spectrum did show however a peak at m/e = 204 (2.7% relative to the base peak, m/e = 104), probably due to the fragment 

. Other significant peaks were at m/e = 220 (1%), possibly due to ($M-CH_2CH_2NHCSNHCH_3$), and at m/e = 247 (0.5%), possibly due to ($M-NH_2CSNHCH_3$).

In a later larger scale preparation the product was obtained in a pure state by direct extraction of the crude product, first with pet. ether (40-60) to remove non-polar impurities, and then with ethanol/ethyl acetate mixtures. In all preparations carried out, attempts were made to crystallise the product, but these met with no success. The spectral properties of the product made in this way were very similar to those of the earlier product.

$[\alpha]_D^{20} = -6.6°$, (C = 1 in ethanol).

Attempted preparations of 1-β-(2-(N'-methyl)carbodiimido ethyl)2-acetamido-2-deoxy-D-glucoside (VIII)

The desulphurisation of the thiourea (XX) with yellow mercuric oxide was attempted in dimethylformamide, acetone, and methanol. Magnesium sulphate, sodium sulphate and molecular sieve (Union Carbide, Type 4A) respectively were present as drying agents in these reactions. The mercuric oxide used was either prepared freshly by adding sodium hydroxide to mercuric nitrate solution, or was a commercial (B.D.H.) sample. The two forms appeared to be similar in reactivity.

In a typical experiment the thiourea (XX) (50 mg., 1.5 mmoles) was stirred with a 2-3 molar excess of yellow mercuric oxide in the presence of drying agent, (see above). Reactions were followed in the infra-red, especially in the 2100-2200 $cm.^{-1}$ region, (where carbodiimides absorb strongly); samples for these spectra were 'liquid film' or were prepared by allowing a drop of the reaction mixture to evaporate (in the case of volatile solvents) on a rock-salt plate.

It was found that reactions in acetone and methanol gave rise to a product which absorbed at 2130 $cm.^{-1}$ in the infra-red, (whereas reaction in dimethyl-formamide did not give rise to any of this product in 30 h.). The intensity of this absorption was very low in comparison with that of the amide absorption at 1660 $cm.^{-1}$, even after reaction for 1 day at room temperature. Furthermore when the product was isolated (by filtration and evaporation of the reaction mixture) it was found that the 2130 $cm.^{-1}$ absorption completely disappeared within $1\frac{1}{2}$ h. at -20°.

It appears that the carbodiimide product is being formed in this reaction, but that it is unstable.

The preparation of 1-β-(2-(N'-methyl)carbodiimido ethyl)3,4,6-tri-O-acetyl-2-acetamido-2-deoxy-D-glucoside (XIX)

This material was prepared several times, as shortly as possible before its use for preparation of compound (VIII). In a typical preparation the purified thiourea (XVIII) (895 mg., 1.94 mmoles) (dried in vacuo), was dissolved in acetone (A.R.) (25 ml.) and stirred with molecular sieve (Union Carbide, type 4A) and yellow mercuric oxide (B.D.H.) (1.7 g., 7.85 mmoles) at room temperature. The reaction was followed in the infra-red ('evaporated film' spectra), which showed the formation of the carbodiimide (absorbing at 2140 cm.$^{-1}$) and in the ultra-violet which showed the loss of the thiourea function (absorbing at 240 nm. in ethanol). Carbodiimide absorption in the infra-red steadily increased up to 5 days of reaction, after which time the thiourea absorption in the ultra-violet had completely disappeared. No further increase in the carbodiimide peak was seen after this time, and its height was 0.91 of that of the O-acetyl peak at 1740 cm.$^{-1}$ (In an equimolar mixture of dicyclohexylcarbodiimide and β-D-glucosamine pentaacetate (XII) this peak height ratio was 1.1).

After centrifugation, solvent was removed from the supernatant, giving an oil, (740 mg., 89% if pure); this product was stable for several weeks at -20°.

$[\alpha]_D^{20}$ = -27.6°, (C = 1 in methanol).

ν_{max} ($CHCl_3$): 3350 (N-H), 2140 (-N=C=N-), 1740 ($OCOCH_3$), 1680 ($NHCOCH_3$), 1530 $cm.^{-1}$ ($NHCOCH_3$ II).

The p.m.r. spectrum, (100 Mc/s), was complex in the τ 4-7 region but also showed the following peaks:

τ ($CDCl_3$): 3.80 (1H, d J = 9 c/s, $N\underline{H}COCH_3$), 7.00 (3H, s, $N-C\underline{H}_3$), 7.90, 7.95, 7.97 (9H, 3s, 3 x $OCOC\underline{H}_3$), 8.01 (3H, s, $NHCOC\underline{H}_3$). The integration of the τ 4-7 region corresponded to 12 protons. The calculated number is 11 ($OC\underline{H}_2C\underline{H}_2N$, H-1, H-2, H-3, H-4, H-5, H-6 and H-6').

The mass spectrum (170°) showed a molecular ion peak at m/e = 429 (2.0%). Other significant peaks were at 386 (3.2%) ($M-COCH_3$); 370 (16%) ($M-OCOCH_3$); 346 (7%) ($M-CH_2CH_2N=C=NCH_3$); 330 (10%) ($M-OCH_2CH_2N=C=NCH_3$); 83 (95%) ($CH_2CH_2N=C=NCH_3$).

The preparation of 1-β-(2-(N'-methyl)carbodiimidoethyl)-2-acetoamido-2-deoxy-D-glucoside (VIII)

This was prepared immediately before each reaction with enzyme. The fully acetylated carbodiimide product of the last reaction (XIX), (25-300 mg., 0.058-0.7 mmoles) was dissolved in dry methanol (1-5 ml.) and stirred at -10° to -15°. A very small pea of sodium metal was added. Infra-red spectra of 'evaporated film' were run at 5 minute intervals, and showed the removal of the O-acetyl groups

(1740 cm.$^{-1}$). The diimide absorption did not decrease in intensity. Reaction was normally complete in 25-30 min., (a second addition of sodium being made if the reaction was slower than this), and methanol-washed ion-exchange resin (Dowex 50 x 10 - 50 'H' form or Amberlite MB-1) was added, with vigorous stirring, to remove methoxide.

Again the process could conveniently be followed in the infra-red; the decreases in intensities of the methoxide peaks at 1635 cm.$^{-1}$ and 830 cm.$^{-1}$ were used as a guide to the extent of removal of methoxide, which was normally complete within 15 min. at -10°. In the case of the more concentrated reaction mixtures it was not always easy to see the methoxide absorption, and its removal was assumed to be complete after 20 min.

The reaction mixture was then filtered rapidly into a receiving tube at -60°, and the product used immediately for inhibition purposes. An excess of starting material over the amount of product required was always used, to allow for losses in filtration, etc.

The product varied considerably in stability. If methoxide was not rigorously removed, the diimide decomposed completely within half an hour of its preparation. Desalting with MB-1 resin was found to give rise to a more stable product than with the Dowex resin; this product showed virtually no decomposition after 1 h. at -60°, and in one case still showed about 50% of the original diimide absorption intensity after 12 h.

ν_{max} ('evaporated film'): 3600-3100 (OH), 2140 (-N=C=N-), 1655 ($NHCOCH_3$), 1565 $cm.^{-1}$ ($NHCOCH_3$ II).

A separate preparation of (VIII) was carried out from (XIX), (39 mg., 0.091 mmoles), in order to determine the p.m.r. spectrum of the product. The methanolic solution of the product was evaporated at 20° to an oil, which was taken up in d_6-dimethylsulphoxide (0.5 ml.). The p.m.r. spectrum (100 Mc/s) was very complex in the τ 4-7 region, but also showed the following peaks:

τ (d_6-DMSO): 2.29 (0.8H, broad d, J = 9 c/s, $N\underline{H}COCH_3$), 7.10 (1.6H, s, $=N-CH_3$), 8.17 (3.0H, s, $NHCOC\underline{H}_3$).

The $=N-CH_3$ to $NHCOCH_3$ methyl integration ratio is rather low in this spectrum (1.6:3.0). However the starting material used in this particular preparation had been kept for some time, and a p.m.r. spectrum of it revealed exactly the same ratio of diimide to acetyl-methyl integration, (1.6:12.0).

The solution of (VIII) used for the p.m.r. spectrum, (which should contain 27.6 mg. of (VIII) if the yield and recovery in the preparation had been complete), was diluted with dimethyl sulphoxide (1.5 ml.), giving a solution of approximate concentration 13.8 mg./ml. The optical rotation of this was measured, giving a very approximate value for $[\alpha]_D^{20}$ of -19°.

The preparation of succinamic acid (XXII)

Two preparations of succinamic acid from succinic anhydride were carried out. Both involved the treatment of succinic anhydride with aqueous ammonia; in the first, an attempt was made to isolate the succinamic acid directly, and in the second, the product was isolated *via* the silver salt.

(a) Succinic anhydride, (XXI), (B.D.H.), (20 g., 0.2 moles), was dissolved in 0.880 ammonia, (31 ml.), at 0-5°. The mixture was stirred for 2 h.; dissolution was complete after 45 min., and an ultra-violet spectrum after 1½ h. showed that the succinic anhydride, (λ_{max} = 224 nm.), had completely reacted. Excess ammonia was removed from the reaction mixture by warming it under vacuum, and the mixture was then acidified with dilute hydrochloric acid, and evaporated to dryness. The white product was triturated with acetone, to extract product; the extract was partially evaporated and ether added, which caused crystallisation of the product. Yield 4.0 g., (17%), m.p. 157-158° dec., (Lit:- 157°).[128,129] A further 7.86 g. was extracted from the crude product by vigorous trituration with methanol, but crystallisation of this gave a product which rapidly discoloured. The product from the acetone extraction gave the following characterisation data. ν_{max} (Nujol): 3380 and 3220 ($CONH_2$), 2700-2450 (COOH), 1705 (COOH), 1650 $cm.^{-1}$ ($CONH_2$).

(Calculated for $C_4H_7O_3N$: C, 41.02; H, 5.98; N, 11.94. Found: C, 40.88; H, 6.05; N, 11.97%).

A low yield results in this reaction because of the difficulty of extracting the product from the solid reaction product.

(b) The method of Hoogewerff and Van Dorp[128,129] was then followed. Succinic anhydride, (20 g., 0.2 moles), was dissolved in 0.880 ammonia (30 ml., 26.4 g., 0.75 moles) and water, (250 ml.), at 0-5°, over 1½ h. Excess ammonia was then removed by warming the reaction mixture under vacuum, and silver nitrate solution was then added until no further white precipitate was formed. The silver succinamate was filtered and washed with water, and then suspended in water and decomposed by passage of hydrogen sulphide. Crude product was isolated, after filtration, by evaporation of the solution. Only 2.9 g. was obtained initially, but after retreatment of the filtered material with hydrogen sulphide a further 13.3 g. was isolated. Crystallisation from an acetone/methanol/ether mixture yielded a total of 9.2 g., (40%), m.p. 156.5-158.5°, (Lit: 156-157°).[128]

The reaction of succinamic acid (XXII) with dicyclohexyl carbodiimide (XXIII)

(a) Succinamic acid (0.5 g., 4.27 mmoles) and dicyclohexyl carbodiimide (0.83 g., 4.02 mmoles) were stirred together in ethanol (40 ml.) at room temperature for 24 h., with production of a white precipitate. An equal volume of water was added, which precipitated more material, which was then filtered off. The filtrate was evaporated nearly to dryness, diluted again with water, and extracted with ethyl acetate. The extract was dried over magnesium sulphate; t.l.c. in ether

showed spots with R_F values 0.23 and 0.52. (The R_F value of succinimide in this solvent was 0.23, and of dicyclohexylcarbodiimide, 0.52). After evaporation of the ethyl acetate the product (300 mg. oil) was taken up in ether, filtered, and eluted from a column (30 x 1.5 cm.) of silica M.60, using a gradient of eluting solvent from pet. ether (40-60) through ether to ethyl acetate. The final fractions contained product, pure by t.l.c. (R_F 0.21), and were combined and evaporated to a white crystalline solid (41 mg., 10%). A sample was recrystallised from chloroform/pet. ether; m.p. 124-125.5°; (Lit. succinimide: 126°).[130] The infra-red and p.m.r. spectra were identical to those of authentic succinimide, (XXIV).

(b) The reaction was repeated with a 100 mg. sample of succinamic acid. After 14 h. at room temperature and 1 h. at 50° the reaction mixture was worked up as before. After precipitation and removal of the white solid, (dicyclohexyl urea), the product was treated with normal hydrochloric acid, (to decompose any remaining diimide), refiltered, and evaporated to an oil, (121 mg.). A p.m.r. spectrum (100 Mc/s) was run on a sample in D_2O.

τ value	Splitting pattern	Area
7.20	singlet	4.6
7.31	singlet	6.5
7.35-7.45	multiplet	9.5

P.m.r. spectra in D_2O were also run on samples of succinamic acid, succinimide, succinic acid, and succinic anhydride.

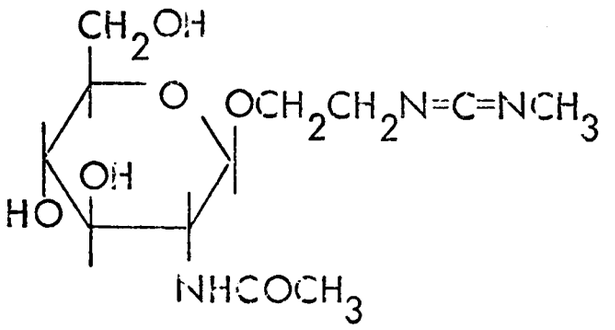
Compound	τ value	Splitting pattern
Succinimide	7.21	singlet
Succinic acid	7.30	singlet
Succinic anhydride	7.30	singlet
Succinamic acid	7.35-7.45	multiplet

On the basis of these results, the product contains succinimide (22%), succinamic acid (46%), and succinic acid or succinic anhydride (32%). The third product is more likely to be the anhydride than the acid as succinamic acid is known[129] to undergo a ready elimination of ammonia on warming.

CHAPTER 3: INHIBITION OF LYSOZYME

A. Introduction

Inhibitions of lysozyme with 1-β-(2-(N'-methyl)carbodiimido ethyl)-2-acetamido-2-deoxy-D-glucoside, (VIII), inhibitor B, were found to lead to products with widely varying degrees of inactivation.



(VIII) Inhibitor B

Studies were initially carried out on the crude products of inhibition; it was later found by ion-exchange chromatography that these products contained several components, and later studies were therefore performed on pure modified enzymes, separated by chromatography. The implication of this observation on the earlier results is discussed in section D of this chapter, but experiments are described in chronological order. Throughout the chapter, crude modified enzymes are denoted by numerals, (which correspond to the inhibitions summarised in table 1, p.110), and pure modified enzymes are denoted by capital letters.

B. Methods and Materials

1. Physical Methods

(i) Ultra-violet

Ultra-violet spectra were determined using a Unicam S.P.800 spectrophotometer. The S.P.800, with a constant wavelength unit attached to an A.E.I. recorder, was also used to monitor absorbance changes at a fixed wavelength, as in the lysozyme assay technique (see p. 88). Protein concentrations were determined from optical density measurements at 280 nm., using a Unicam S.P.500 spectrophotometer; and the molar extinction coefficient used for lysozyme was 36,500.[136]

(ii) pH

The pH of solutions of volume greater than 1 ml. was measured using a Radiometer type TTT 1 c instrument with a scale expander, type PHA 630T.

For solutions of volume less than 1 ml. a Radiometer type PHM 4C pH meter, fitted with micro-electrodes, was used.

A Radiometer pH-stat was used to follow reactions at constant pH.

(iii) Amino-acid analysis

Amino-acid analyses were carried out on a Technicon Autoanalyser, according to the method of Piez and Morris[137] with a column temperature of 65°, except where otherwise mentioned. The buffer system of Thomson and Miles[138] was used. The duration of a complete analysis was 8 h.

Protein and peptide samples were prepared for analysis by hydrolysis in redistilled constant boiling hydrochloric acid, in vacuo, for 24 h. at 110°, in sealed pyrex tubes. Amino-acid samples (0.05-0.3 μmoles) were applied to the column in 0.1N hydrochloric acid. Smaller samples (0.01 μmoles) were analysed on a Beckman instrument, with 10 x scale expansion, in the Biochemistry Department.

(iv) Scintillation counting

Scintillation counting of radioactive samples was carried out on a Beckman DPM-100 Liquid Scintillation Counter as described in Chapter 2, p. 53.

(v) Fraction collection

Automatic fraction collecting of column effluents was carried out on a Beaumaris instrument, or, when available, an L.K.B. Ultrorac fraction collector. With this instrument column effluents were monitored continuously at 280 nm. in a 3 mm. flow cell of an L.K.B. Uvicord II absorptiometer unit, connected to a Servoscribe recorder.

(vi) P.m.r. spectra

P.m.r. spectra were determined at 100 Mc./s on a Perkin-Elmer R.14 instrument by Mrs. E. Richards in this department. A computer of average transience (C.A.T.) was used for accumulation of weak signals. P.m.r. spectra of proteins were determined at 60 Mc./s on a Jeol C 60 HL instrument by K. Morallee in the Inorganic Chemistry Laboratory, Oxford.

(vii) Fluorescence spectra

Fluorescence spectra were determined on an Aminco-Bowman spectrophotofluorometer. Tryptophan fluorescence of 1 ml. samples in 1 cm. cells was studied, using an exciting wavelength of 285 nm. The cell compartment was thermostatted at 25° or 30°, and samples were equilibrated for at least 10 min. at this temperature before spectra were run. λ_{max} values were uncorrected for instrument calibration errors.

(viii) Miscellaneous

Conductivity measurements were made with a Radiometer conductivity meter, type CDM 2e.

The resolution of overlapping peaks on chromatograms was carried out with a Du Pont 310 curve resolver.

A Hewlett-Packard 9100a programmable desk calculator was used for least squares plots of linear graphical data.

2. Protein and Peptide Methods

(i) Enzyme assays

Enzyme activity was determined by measuring the rate of lysis of Micrococcus lysodeikticus cell walls.[139] A suspension of the dried cells, (Koch Light, Batch No 31716), (0.3 mg./ml.), was made up in 0.1M phosphate buffer, pH 7.0, and this was equilibrated at 25°. 2.9 ml. was mixed with 0.1 ml. of the lysozyme solution, (normally containing about 0.004 mg. protein), in a 1 cm. cell, and the decrease in absorption at 450 nm., at 25°, was followed in the S.P.800 (against a water blank and with a scale expansion of 10). The decrease in A_{450} in the first two minutes of each assay, (performed in duplicate for each sample), was used to calculate the enzyme activity from the formula:

$$\text{Activity (units/mg.)} = \frac{\Delta A_{450}/\text{min.} \times 10^3}{\text{enzyme concentration in assay mixture (mg./ml.)}}$$

Native Seravac lysozyme possessed an activity of around 25,000 units/mg., and Worthington lysozyme, 20,000 units/mg.

The first batch of M. lysodeikticus cell walls used (Koch Light, Batch No.31716) was unsatisfactory for lysozyme assay, as a suspension of it had an appreciable settling rate, causing a decrease in A_{450}, with no enzyme present. Another batch (Sigma: Lot 74B-1730) was found to be free from this difficulty, and activity values down to about 2% of native enzyme activity could be measured accurately. Values below 2% are unreliable.

(ii) Inhibitions

Lysozyme (3-400 mg., 0.21-27.6 μmoles) was dissolved in water (3-20 ml.) and adjusted to pH 4.75 with 0.02N sodium hydroxide. (In the first inhibition the nucleophile, glycine methyl ester, was also present in an 80-fold molar excess).

The inhibitor, (VIII), prepared in methanolic solution as described in Chap. 2, p. 77, was added, with stirring, in 5-10 portions over several minutes. The molar excess used varied from 2 to 90 in different inhibitions. The volume of the inhibitor solution was kept as low as the preparation would allow and was normally less than 25% of the total volume of the reaction mixture.

A pH rise of the enzyme solution was observed on addition of the inhibitor, and hydrochloric acid of an appropriate concentration was added from the pH stat syringe to keep the pH at 4.75. The amount of acid added is not significant, as the inhibitor solution was normally slightly alkaline, (about pH 8), (owing to traces of methoxide).

The mixture was stirred for 1 h. at 20°; no further pH changes occurred after the first few minutes. The product was then eluted from a Sephadex G.25 (fine) column; three column sizes were used: 30 x 1.5 cm. (3-10 mg. protein), 37 x 2.5 cm. (10-200 mg.), and 78 x 3.5 cm. (>200 mg. protein). In early inhibitions the eluting solvent was 0.1M phosphate buffer, pH 7.0, but when the protein was required salt-free it was eluted with 0.5% ammonium bicarbonate

solution which was then removed by lyophilisation.

A small amount of the inhibitor solution, still kept at ~60°, was normally retained in order to check, after most had been used for inhibition purposes, that the diimide function was still intact. Infra-red spectra suggested that no decomposition occurred during the time involved in starting an inhibition.

Control experiments were also performed, with lysozyme and methanol, and with lysozyme and glycine methyl ester; the conditions for the incubation and subsequent isolation of the protein were the same as those described above.

Fuller details of all the inhibitions carried out are to be found in the 'results' section of this chapter, p. 109.

(iii) Reduction and carboxymethylation of lysozyme

Although Jolles et al.[22] have reported reduction and carboxymethylation of lysozyme using a 400 x molar excess of thioglycollic acid, followed by the addition of a 700 x molar excess of iodoacetic acid, an overall excess of iodoacetic acid is inadvisable as there is a danger of oxidation of I^- to I_2 with concomitant side reactions.[140] The procedure outlined by Hirs[140] was therefore followed.

Guanidine hydrochloride, (B.D.H. Biochemicals), was recrystallised from methanol, and iodoacetic acid, (B.D.H.), was recrystallised from pet. ether (40/60).

In a typical experiment a 5 mg. (0.345 μmoles) sample of the protein was dissolved in 5.5M guanidine hydrochloride solution (5 ml.), the pH was adjusted

to 8.5 with methylamine (0.2%), and nitrogen was bubbled through at room temperature. A solution of mercaptoethanol, (Koch Light), containing 108 mg./ml. was made up in water, and 0.10 ml. of this was added to the enzyme solution, which was stirred, with nitrogen bubbling, for 6 h., methylamine being added by the pH stat to bring the pH to 8.5. The amount of mercaptoethanol added was thus 10.8 mg., (0.138 mmoles, or a 400 x molar excess over enzyme). Iodoacetic acid, twice crystallised, (24.2 mg., 0.130 mmoles, or a 376 x molar excess over enzyme) was dissolved in 0.25N sodium hydroxide (0.4 ml., 0.100 mmoles), and added carefully to the reduced protein solution, in the dark. Great care was needed at this stage to avoid pH fluctuations with which the pH stat could not easily cope, (despite the prior partial neutralisation of the iodoacetic acid), but with caution the pH could be kept at 8.5 by addition of 0.2% methylamine from the pH stat.

The mixture was stirred for 2 h. in the dark, acidified to pH 4 by addition of 1 drop of 50% glacial acetic acid, concentrated to 4 ml. on a rotary evaporator at 25°, and eluted from a column (30 x 1.5 cm.) of Sephadex G25 (fine) with degassed 50% acetic acid. These operations were performed as much as possible in the dark to avoid photo-oxidation of iodide to iodine. The reduced, carboxymethylated protein was obtained from the relevant column fractions by evaporation and then by lyophilisation.

Other samples (4-100 mg. protein) were reduced and carboxymethylated similarly, in 5 or 6 ml. of solution.

(iv) Trypsin digestion

The recommended procedure for trypsin digestion[140,141] employs a 1% protein concentration. With small protein samples, (less than 25 mg.) such a concentration was impracticable with the pH stat used, as 2.5 ml. of solution was required to cover the electrodes; lower concentrations and longer reaction times were therefore used.

In a typical reaction the reduced, carboxymethylated protein (4.2 mg.) was suspended in water (3 ml.) in a pH stat cell thermostatted at 36°. The pH was adjusted to 7.8 with 0.02N sodium hydroxide. Trypsin (Seravac, bovine, 1 x cryst., diphenylcarbamyl chloride treated, batch no. 289L) (0.5 mg.) was dissolved in water, (1.0 ml.), and 0.05 ml. (i.e. 25 μg., or 0.6% of protein to be digested) was added to the suspension. 0.02N sodium hydroxide was added to keep the pH at 7.8. After 3 h. a second equal portion of trypsin was added, giving a final trypsin:protein ratio of 1:84 weight for weight. The reaction was stopped after a total of 10½ h. by addition of 1 drop of 50% acetic acid, and the product lyophilised. The reaction was still proceeding very slowly at this time, and 0.217 ml. of 0.02N sodium hydroxide had been taken up, corresponding to cleavage of 15 bonds per lysozyme molecule. (There are in fact 17 labile bonds).

With larger protein samples (greater than 25 mg.) a 1% concentration of protein was used. A 1:100 w/w ratio of trypsin to protein was again used, and reaction took about 6-7 h. either to reach completion or to reach the point when

calculation indicated cleavage of 17 bonds per lysozyme molecule. At this point reactions were stopped as above.

(v) Separation of peptides on paper

(a) Detection

For the general detection of peptides and amino-acids on paper, the chromatogram was soaked in a ninhydrin/cadmium solution.[142] Spots, ranging in colour from bright yellow to deep red, appeared after a few hours at room temperature. After marking these, the chromatogram was stained for tyrosine-containing peptides by soaking it first in 0.1% α-nitroso-β-naphthol in acetone, and, after drying, in 10% nitric acid in acetone.[143] Heating the paper in front of an electric fire caused tyrosine peptides to show up as red-purple spots. Tryptophan-containing peptides give a duller grey-brown colour.

(b) Electrophoresis

Whatman 3MC or 3MM paper was used for this and all paper chromatographic work. Electrophoresis was at 3.0 or 3.5 Kv., and was carried out in large tanks in an apparatus similar to that described by Michl.[144] The tanks were filled with water-cooled white spirit, (containing about 10% pyridine in the case of electrophoreses at pH 6.5). Paper strips, pre-wetted with buffer, were hung in the tanks with the top (-ve) and bottom (+ve) ends dipping in buffer. The buffers used were pH 6.5 (pyridine:acetic acid:water, 25:1:225) and pH 1.9 (formic acid 2% : acetic acid 8%).

A mixture of amino acids and/or various dyes was applied to the edge of each sheet of paper used to serve as a marker.

(c) Paper chromatography

Descending paper chromatography was carried out with a solvent consisting of n-butanol:acetic acid:water:pyridine in the ratio 30:6:24:20.

(d) Peptide maps

Peptide maps of lysozyme and modified lysozyme tryptic peptides were produced as follows. The peptide mixture (3-5 mg.) was taken up in 0.05M ammonia (20-50 μl.) and applied in a 2 cm. streak to a 57 cm. strip of Whatman 3MC paper, along a line 23 cm. from the bottom. A spot of methyl green cationic indicator was applied next to the streak. Electrophoresis, with the bottom of the paper at the positive electrode, was at pH 6.5, 3 Kv., for 40 min.

After drying the paper, the edges of the peptide strip were cut off and stained, which located the extent of the separation. The strip containing the peptides, normally about 42 cm. long, was then sewn onto a second sheet of paper along a line 10 cm. from the top. Descending paper chromatography for 16 h. in the solvent described in (c), followed by staining, resulted in a map showing good separation of most of the peptides.

A combination of electrophoresis and paper chromatography was used similarly in preparative isolation of peptides. Peptides were washed off the paper by downward elution of strips with 10% acetic acid.

(vi) Peptide sequencing techniques

(a) Carboxypeptidase A digestion

Carboxypeptidase A-DFP was obtained from Sigma Chemical Co. (dialysed, recrystallised, DFP treated, lot 18B-2693) as an aqueous suspension containing 20 mg./ml. It was solubilised by a method[145] adapted from that of Harris[146] which involved centrifugation, suspension of the residue in 1% sodium bicarbonate A.R., dissolution by gradual addition of 0.1N sodium hydroxide, adjustment to pH 8 to 9 with 0.1N hydrochloric acid, and dilution with 0.2M N-ethylmorpholine acetate, pH 8.5. In this way a solution containing 1 mg./ml. carboxypeptidase A was prepared.

The enzyme's activity was checked on a known tri-peptide, Ala-Gly-Val, (supplied by Dr. G. T. Young); 0.2 μmoles of this peptide was dissolved in 0.2M N-ethyl morpholine acetate, pH 8.5, (0.4 ml.) and 50 μl. of the enzyme solution (containing 50 μg. carboxypeptidase A) was added. After incubation at 37° for 6 h. the mixture was lyophilised and analysed. This analysis showed valine, a trace of glycine and alanine, and a peak between the normal positions of methionine and isoleucine, (probably Ala-Gly). This is the result to be expected, as carboxypeptidase A is known to cleave C-terminal bonds to valine readily, but to cleave those to glycine very slowly.[145] A control, containing no peptide, showed no peaks on analysis.

Unknown peptides were treated in exactly the same way.

(b) Leucine amino-peptidase digestion

Leucine amino-peptidase (LAP), (Seravac, batch 17B), was used without any of the suggested pretreatments,[147] as it was required to be as non-specific as possible. In later work LAP supplied by Worthington Biochemical Corporation, as a suspension in 75% saturated ammonium sulphate, 0.1M tris, 0.005M $MgCl_2$, pH 8.0, was used.

Its activity was checked on the same tri-peptide as was used with carboxy-peptidase A, (Ala-Gly-Val). This peptide (0.1 μmole) was dissolved in 0.05M tris buffer, pH 8.5, containing also 0.005M $MgCl_2.6H_2O$ (A.R.) (0.5 ml.). LAP (0.32 mg.) was dissolved in this same buffer (0.1 ml.), and 50 μl. was added to the peptide solution, which was incubated at 37-39° for 36 h. The remaining 50 μl. of the enzyme solution was added to 0.5 ml. of buffer alone, as a control experiment. After 36 h. the solutions were acidified with 1.0N hydrochloric acid (0.1 ml.), and analysed directly.

The control showed a small peak at about the position of phenylalanine; the tripeptide digest also showed this peak and also Ala (1.00), Gly (1.03), and Val (0.99).

LAP digestions of unknown peptides were performed in an exactly similar fashion.

(c) Edman degradation

The method used for Edman degradations was that described by Gray[148]

and by Husain.[149] Pyridine (A.R.) was refluxed over sodium hydroxide and distilled twice (b.p. 117.2°). Phenylisothiocyanate (Koch Light, 'pure') was redistilled (b.p. 97-98°/14 mm.).

The peptide to be degraded (0.05-0.1 μmoles) was dissolved in 50% aqueous pyridine (0.4 ml.) in a small centrifuge tube. Triethylamine (10 μl.) and phenylisothiocyanate (10 μl.) were added, and the tube was flushed with nitrogen, stoppered, and kept at 45° for 1 h. The yellow solution was then evaporated to dryness in vacuo over sodium hydroxide and phosphorus pentoxide in a desiccator placed in an oven at 60°.

After about ½ h. the sticky residue was treated with anhydrous redistilled trifluoracetic acid (0.3 ml.), and the solution incubated, under nitrogen, at 45° for 30 min. Solvent was removed as before, giving a pale solid, adhering to the tube walls. Water (0.3 ml.) was added and the phenyl thiocarbamyl amino acid was extracted with ether (peroxide free) (3 x 1.0 ml.). The aqueous layer was dried as before and was then used for the next cycle of the degradation. The ethereal layer was dried ($MgSO_4$), evaporated, and incubated with 1.0N hydrochloric acid (1.0 ml.) at 30° for 30 min., to complete conversion of the amino acid derivative to the phenylthiohydantoin (PTH-amino acid). The product was extracted with n-butyl acetate (3 x 1.0 ml.), and the extract was dried ($MgSO_4$) and evaporated to a very small volume.

(d) Thin layer chromatography of PTH-amino acids

20 x 20 cm. silica thin layer (0.1 mm.) plates (HF 254) were used. Elution was first with chloroform:methanol 9:1 (Randerath, 'solvent 2')[150] and then in a direction at right angles with chloroform:formic acid 20:1 (Randerath, 'solvent 3').[150] Spots were detected in the ultra-violet, or developed by placing the plate in a chlorine tank for 25 min., aerating for 3 min., and then spraying with a 1:1 mixture of 0.05M potassium iodide and a saturated solution of o-tolidine (A.R.) in 2% acetic acid.[151]

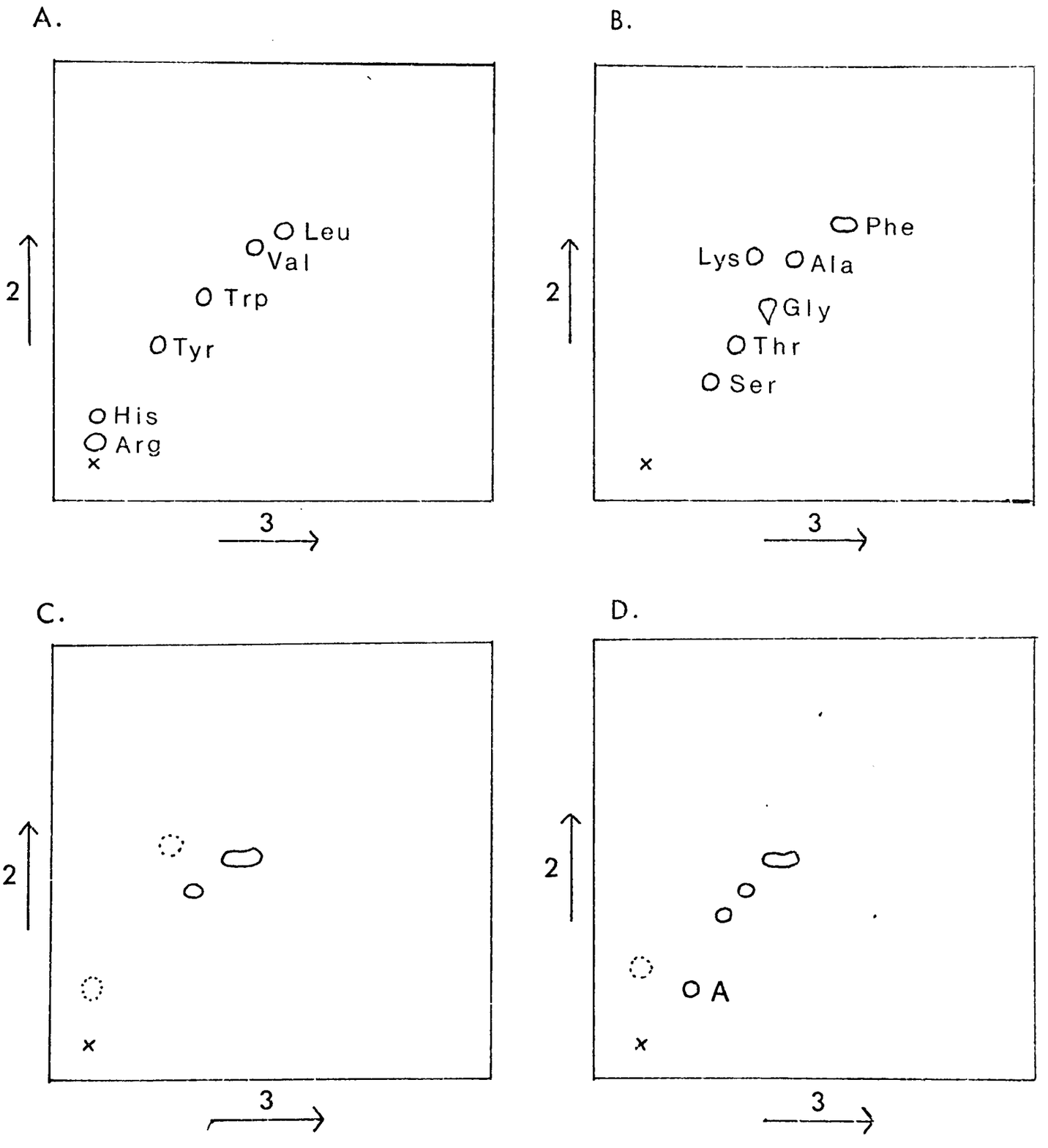
Mixtures of known PTH amino acids were separated well under these conditions. Examples of two chromatograms are shown in figure 9, A and B. Although the R_F values, especially in solvent 3, were rather different from those quoted by Randerath[150] assignments were easy to make in most cases, although individual PTH's were sometimes chromatographed in order to check these.

Attempts to use the method to identify PTH's prepared via Edman degradations were not very satisfactory. A control Edman cycle and PTH preparation was carried out with no sample present and the product gave a 2-dimensional chromatogram showing several spots (figure 9, C).

The known peptides Asn-Ser-Trp, (isolated by S. S. Husain from papain and Ala-Gly-Val were both subjected to 3 cycles of the Edman degradation, and the resulting PTH's subjected to this chromatographic procedure. The chromatograms all showed the 'background' spots seen in the control, and extra spots were very

Figure 9

Thin-layer chromatography of PTH-amino acids



(2 and 3 refer to solvents described by Randerath[150])

difficult to see and identify. For example, figure 9 D shows the product derived from the first Edman cycle on Asn-Ser-Trp. The spot marked A is at the PTH-Asn position as given by Randerath.[150]

The method was used similarly on unknown peptides, which in fact gave slightly more easily interpretable results, but the method was never entirely satisfactory, and sequence data was much more successfully obtained by analytical and electrophoretic studies on the residual peptide after an Edman cycle, ('subtractive' Edman method). The 'dansyl' method for peptide end-group analysis[152] is more widely used than that involving the PTH isolation and identification, but does not distinguish Asp and Asn, which was a necessary requirement in this work.

(vii) Identification of asparagine

(a) By amino-acid analysis

Asparagine normally runs with serine or threonine in most analyser buffer systems. It gave rise to an easily recognisable peak on the Technicon analyser as its 440:590 nm. colour value ratio was higher than that of all other amino acids except proline, and was approximately equal to that of cystine. Conditions which separated asparagine were found, after running a series of analyses, at various temperatures and with various buffer systems, of a standard mixture containing differing amounts of Asp, Asn, Thr, Ser and Glu. Identification of the peaks was confused until it was discovered by analysis, paper chromatography, and electrophoresis at pH 6.5, that the asparagine used (B.D.H.) contained about 5% aspartic acid.

The best conditions found, although they did leave Ser and Asp unresolved were: column temperature 25° for 3 h., and then raised to 65°; eluting buffer pH 2.90 citrate/methanol (10:1). Under these conditions elution times were: Thr 151 min., Ser/Asp 171 min., Asn 184 min., Glu 242 min. For complete analyses, a normal autograd mixture was connected to the column after Glu had been eluted. This did not resolve Gly and Ala, but all other peaks were well resolved.

(b) On paper

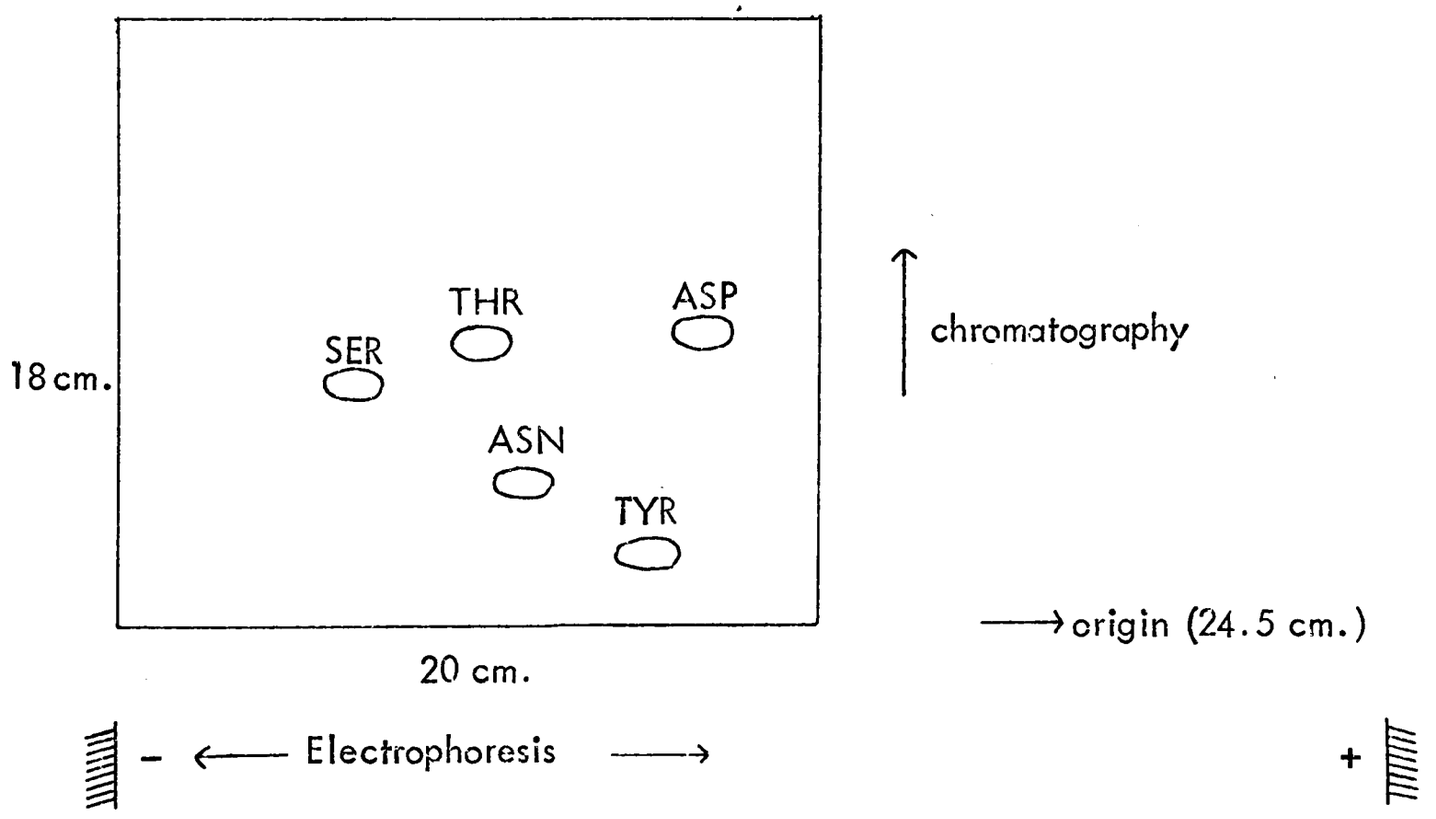
Methods of separating amino acids on paper are well documented,[153] and the method of Rockland and Underwood[154] was first tried. A mixture of the amino-acids Asp, Asn, Thr, Ser, Gly, and Tyr, (these being chosen because of their relevance to an unknown peptide to be studied) was subjected to ascending chromatography on Whatman 541 paper, first with a t-butanol/formic acid/water (695:10:295) mixture, and then in a direction at right angles with a phenol solution, as described in reference 154. Staining with ninhydrin/collidine[155] gave rise to spots which varied in colour from orange (Asn) to grey (Asp).

Asn and Asp were well separated but Ser, Thr, Tyr, and Gly were not completely resolved, and it was decided to attempt to use a combination of electrophoresis and paper chromatography to separate the mixture.

Electrophoresis of the mixture at pH 1.9, 3 Kv., for 35 min. separated glycine, whose mobility was greater than that of the remaining amino-acids

(unresolved). When electrophoresis was run for 2 ¼ h., glycine ran off the paper and three spots remained. In order of decreasing mobility these were Ser, Thr/Asn, and Tyr/Asp.

The electrophoresis was repeated, with the sample on one edge of a sheet of Whatman 3MC paper (57 x 20 cm.). The only marker which remained on the paper after electrophoresis for 2 ¼ h. was the yellow component of the methyl green dye, and this was used to locate the section of the sheet containing the amino acids, which was cut out. Ascending chromatography at right angles to the direction of electrophoresis was carried out on the same sheet of paper with the t-butanol/formic acid/water (695:10:295) solvent of Rockland.[154] Staining with ninhydrin/collidine showed an excellent separation of all components.



3. Materials

(i) The preparation of [1-^{14}C]-glycine methyl ester hydrochloride

A sealed tube containing [1-^{14}C]-glycine, (Radiochemical Centre CFA 30, Batch 51), (0.1 mC, 552 μC/mg.), was broken open and the contents washed out as well as possible with dry methanol (20 ml.) into a flask containing 'cold' glycine (49.7 mg., 0.662 mmoles). The suspension was cooled to 0° and dry hydrogen chloride was passed to saturation. The reaction mixture was allowed to warm to room temperature, and, after 18 h., was evaporated to dryness; methanol was added again and evaporated twice more, to remove traces of hydrogen chloride.

The product was crystallised from methanol/ether, giving white needles. Yield 65 mg., (102%), m.p. 173.5-175.5°, (Lit: 175°).[130] A solution of 3.8 mg. in 2 ml. water had a total count of 4.680×10^6 c.p.m., and the activity of the product is thus 1.231×10^6 c.p.m./mg.

Unlabelled glycine (100 mg., 1.33 mmoles) was treated in exactly the same way giving the product (142 mg.) in 85% yield, m.p. 175-177°.

τ (D_2O):6.04 (2H, s, CH_2), 6.14 (3H, s, OCH_3).

(ii) The preparation of chitin oligosaccharides[156]

Hydrolysis of chitin

Chitin, (10 g., flakes), from shrimp shells, (from a batch prepared by A. Williams), was digested with concentrated hydrochloric acid (100 ml.) at 40° for 3¼ h., with vigorous stirring. The flakes gradually dissolved, giving a very dark viscous liquid which was cooled in ice and diluted with an equal volume of water. The solution was neutralised, between 5° and 10°, with sodium bicarbonate (A.R.) (63 g.), and was filtered to remove brown solid. (In these operations the addition of a couple of drops of n-octanol was found to be very effective in reducing the otherwise uncontrollable amount of froth). The filtrate was evaporated to 40 ml., refiltered to remove a large amount of sodium chloride, diluted to 200 ml. with water, and stirred with Amberlite M.B-3 self-indicating ion-exchange resin. Two batches, totalling 250 g., were used, the first bringing the conductivity down to 16 mmho, and the second to 0.02 mmho.

The mixture was filtered and evaporated at 40° to a syrup, which was dried in vacuo over solid sodium hydroxide, giving a white powder (1.84 g.) (2.89 g. in a second preparation).

Separation of oligosaccharides

A column (140 x 2 cm.) of Biogel P4, (Biorad Laboratories, 200-400 mesh, batch no. 41864), was set up as described by the manufacturers of the gel. The

crude oligosaccharide mixture was taken up in water (15 ml.) and the very viscous liquid was filtered and eluted in two 7.5 ml. portions from the Biogel column, which was pumped at 10.2 ml./h. with a peristaltic pump. The eluting solvent was water, degassed, and saturated with chloroform as a bacteriostat. Fractions of 3 ml. (18 min.) were collected and their absorbance at 220 nm. was measured in 1 mm. silica cells. A typical elution profile is shown in figure 10, and this clearly shows a separation of the oligosaccharides from the hexamer (first peak) to the monomer (last peak). Fractions under each peak were pooled and combined with the similar products from the second chromatography. After lyophilisation, the yields of crude oligosaccharides were:

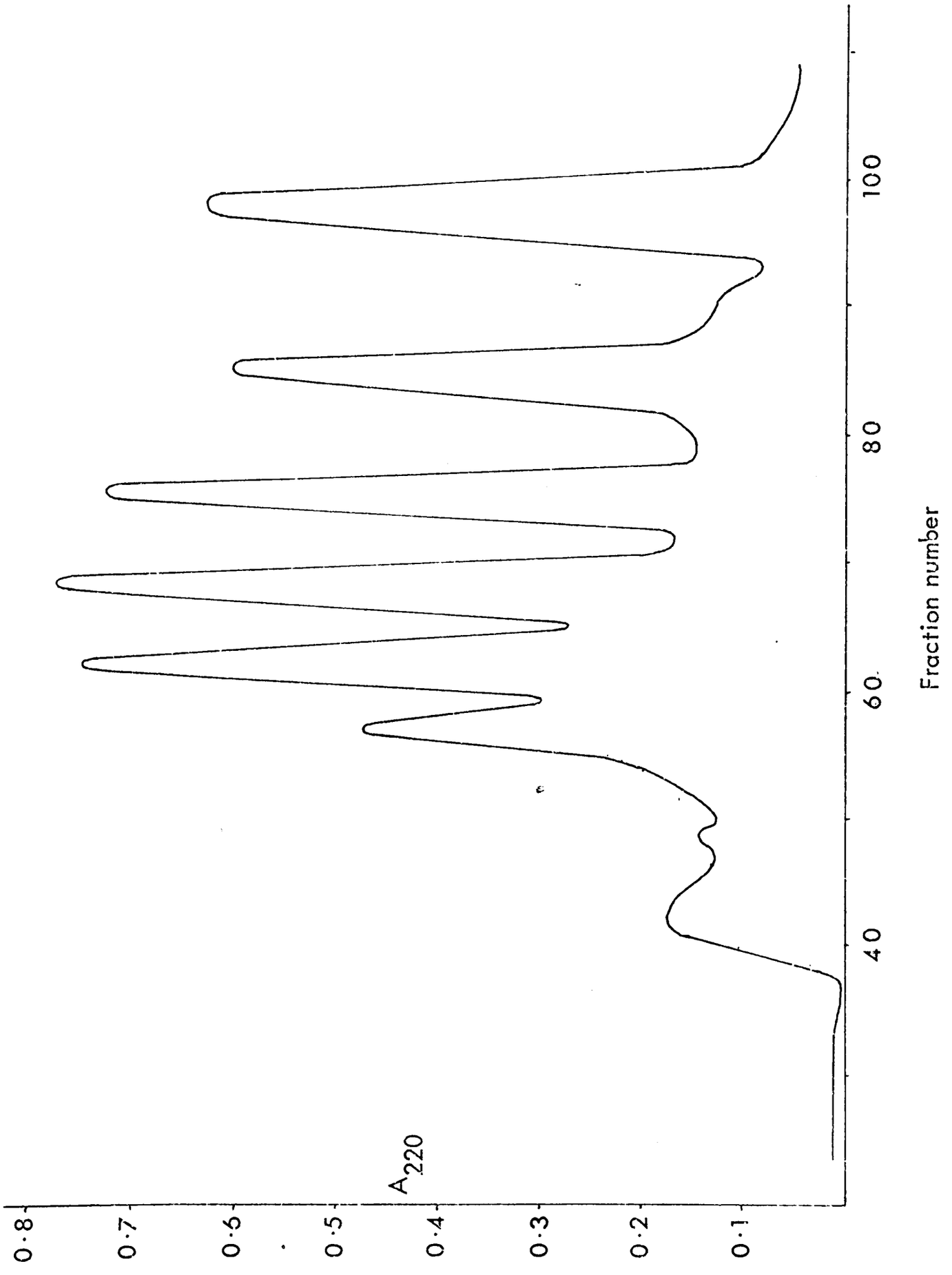
$(NAG)_6$:*	102 mg.	(Lit: 59 mg.)[156]
$(NAG)_5$:	181 mg.	(Lit: 152 mg.)
$(NAG)_4$:	255 mg.	(Lit: 231 mg.)
$(NAG)_3$:	220 mg.	(Lit: 345 mg.)
$(NAG)_2$:	225 mg.	(Lit: 327 mg.)

These products, as well as the NAG product and authentic NAG, were subjected to descending paper chromatography on Whatman 3MM paper with n-butanol: ethanol:water:0.880 ammonia (40:15:49:1) as solvent. Spots were detected by staining the paper in aniline hydrogen phthalate solution;[157] heating the

* See footnote on p. 9.

Figure 10

Chromatography of chitin oligosaccharides on Biogel P.4



chromatogram for 15 min. at 105° caused spots to show up (dark brown on a light brown background). Authentic NAG and that produced by hydrolysis of chitin had identical R_F values, (0.50). Other R_F values were $(NAG)_2$ 0.44; $(NAG)_3$ 0.37; $(NAG)_4$ 0.26; $(NAG)_5$ 0.21; $(NAG)_6$ 0.11. The higher oligosaccharides, $((NAG)_4$-$(NAG)_6)$, tended to migrate as streaks instead of discrete spots unless very little material was applied to the paper. A plot of $\log\left(\frac{1}{R_F} - 1\right)$ against n, where n is the degree of polymerisation of the $(NAG)_n$ oligomer should be a straight line for members of a homologous series.[158] This plot, (figure 11), was however a smooth curve, as has previously been observed in some cases.[156,159] It is possible that the use of chromatography paper which has been pre-washed with the solvent might alter the R_F values so that they bear the theoretical relationship to each other.

$(NAG)_2$, $(NAG)_3$, $(NAG)_4$ and $(NAG)_6$ were purified by rechromatography on the Biogel P4 column. The purity of the tetramer and hexamer was checked by rotation measurements.

$(NAG)_4$: $[\alpha]_D^{20}$ = -5.47° after 10 min. (C = 0.9 in water), (Lit: -5.4°)[156]

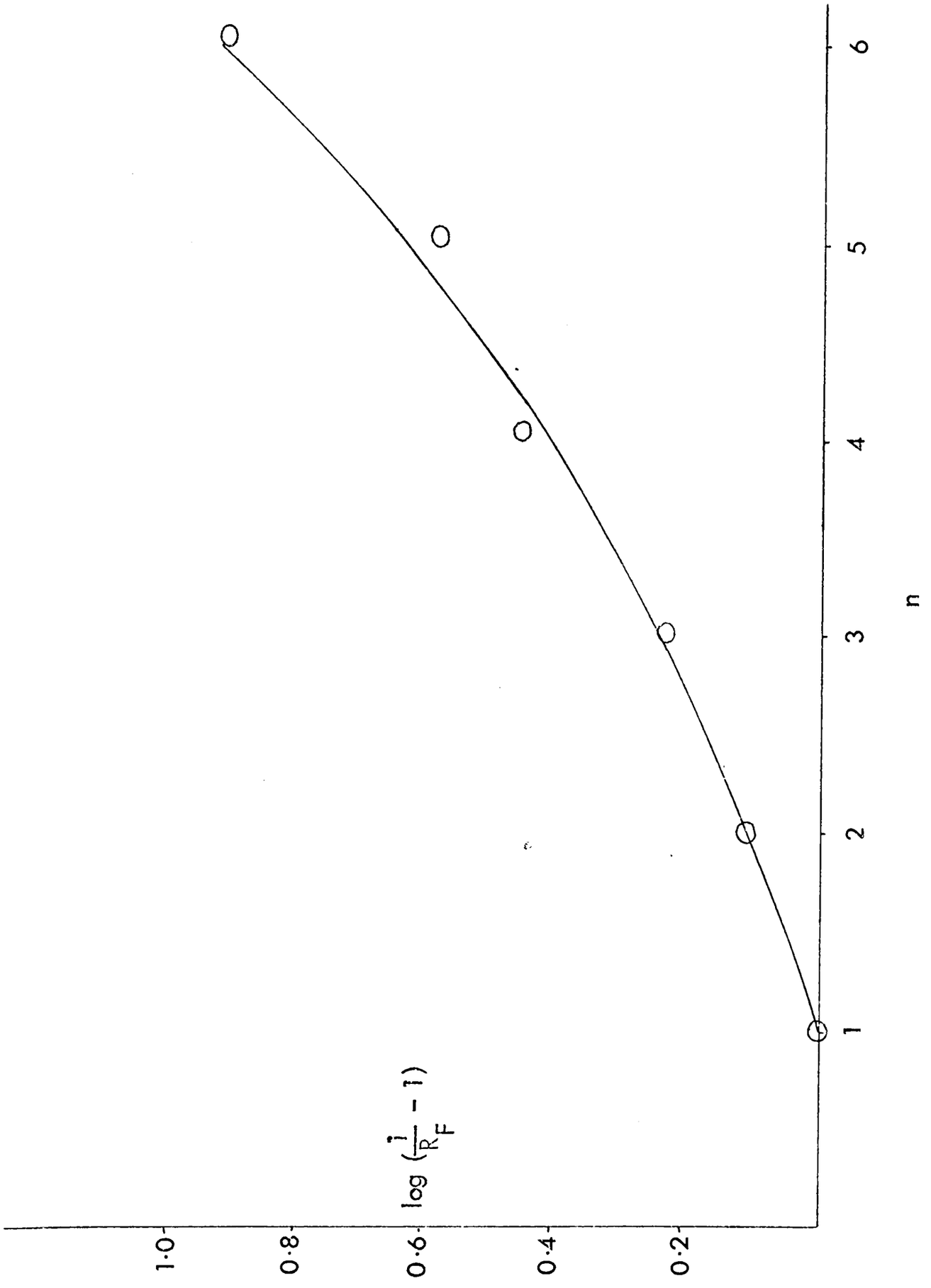
$[\alpha]_D^{20}$ = -4.80° after 18 h., (Lit: -4.8°,[156] -4.1°[159])

$(NAG)_6$: $[\alpha]_D^{20}$ = -12.7° after 10 min. (C = 1.0 in water)

$[\alpha]_D^{20}$ = -9.5° after 18 h., (Lit: -11.7°,[156] -11.4°[159])

Figure 11

R_F values of chitin oligosaccharides, $(NAG)_n$



C. Results

1. Inhibitions

(i) Summary

The inhibitions performed are summarised in Table 1, and were all carried out at 20-25° and at pH 4.75 as described earlier (Chap.3, B.2(ii)). Products were assayed against M. lysodeikticus, and in the case of inhibitions 1:1 and 1:3 (see table 1), their ^{14}C-content was determined by scintillation counting.

Although conclusions may be drawn from all the activity values in the table, inhibitions 1, 3, and 4 were performed with a special view to understanding the factors involved in a successful inhibition. Peptide studies were carried out on the products from inhibitions 1:2, 2:1, 3:1, and in the case of 2:1 the active site peptide was isolated and investigated. Binding studies (with NAG and $(NAG)_3$)* were carried out with 2:1, using the technique of fluorescence spectroscopy. $(NAG)_2$ binding studies were performed by p.m.r. spectroscopy on the products from inhibitions 2:1, 3:1, 3:2, 3:3, and 4, as well as with native enzyme, and further p.m.r. experiments involving the use of Co^{++} as an active site probe were attempted.

When it was found that ion-exchange chromatography could be used to purify modified enzymes, the products 2:1, and 4 were studied in this way.

* see footnote on p. 9.

Table 1

Inhibition	Amount of lysozyme mg. (μmoles)	Vol. of lysozyme solution (ml.)	Amount of inhibitor μmoles (molar excess)	Vol. of methanol (ml.)	Glycine methyl ester hydrochloride mg. (molar excess)	Percentage native activity
1:1	10[a] (0.69)	3.0	25.6 (37)	0.4	6.9 (80)	30
1:2	10[a] (0.69)	3.0	25.6 (37)	0.4	0 (0)	30
1:3	10[a] (0.69)	3.0	0 (0)	0	6.9 (80)	71
1:2:2	3.3[b] (0.23)	4.0	11.7 (51)	0.4	0 (0)	23
2:1	200[c] (13.8)	5.0	610 (44)	3.0	0	4.2
2:2	10[c] (0.69)	4.0	0 (0)	3.0	0	86
2:1:2	6.7[d] (0.46)	5.0	23 (50)	0.5	0	2.4
3:1	29[c] (2.0)	5.0	20 (10)	1.0	0	86
3:2	29[c] (2.0)	5.0	10 (5)	0.5	0	72
3:3	29[c] (2.0)	5.0	4 (2)	0.2	0	81
3:4	29[c] (2.0)	5.0	0 (0)	0.5	0	87
4	14.5[c] (1.0)	5.0	93 (93)	1.0	0	35
5	400[e] (27.6)	20	755 (27)	3.3	0	63
6	300[e] (20.6)	12	730 (35)	4.0	0	56
7	300[e] (20.6)	10	614 (30)	4.0	0	71

Notes: a. Seravac, 3 x cryst., batch no.36. b. Product of inhibition 1:2. c. Seravac, batch no.58Z.
d. Product of inhibition 2:1. e. Worthington, batch no.LY9FA. f. A mixture of [1-^{14}C]- and cold glycine methyl ester hydrochloride in the ratio 3.8:10 (section B.3(i)).

Inhibitions 5, 6 and 7 were carried out on a larger scale for preparative separation of pure modified enzymes, which were subjected to fluorescence binding studies and active site investigation by p.m.r. spectroscopy (using Ho^{++} as an active site probe). Finally, crystallisation of one of the pure modified enzymes was carried out.

(ii) Uptake of ^{14}C in inhibitions 1:1 and 1:3

The mixture of [1-^{14}C]- and cold glycine methyl ester hydrochloride, in the ratio 3.8:10 (section B.3(i)), used in inhibitions 1:1 and 1:3 showed activity of 339,000 c.p.m./mg. A 1:1 molar incorporation of this into 10 mg. of lysozyme would result in a total activity of 29,600 c.p.m. in the protein. The observed total count in sample 1:1 was 2,960 c.p.m., corresponding to an uptake of 10%. The count of sample 1:3 was zero.

(iii) Tests for the presence of N-acetyl glucosamine in product 2:1

The method of Zuckerhandl and Messiner-Klebermass[160] was first used with modifications which were found to make it more sensitive. The sample was dissolved in 50% ethanol/N sodium hydroxide (2 ml.) and the solution was boiled for 20 sec., cooled, and Ehrlich reagent (1 ml.) added. (This reagent was prepared by dissolving p-dimethylaminobenzaldehyde (80 mg.) in 3N hydrochloric acid (9 ml.)). The optical density at 565 nm. was measured after 20 min. against water.

Sample	A_{565}
β-D-glucosamine pentaacetate (1.5 mg.)	0.232
Native lysozyme (1.5 mg.)	0.006
2:1 (1.5 mg.)	0.008
Control (no sample)	0.001

A rough calculation indicates that the expected A_{565} difference between native and 2:1 lysozyme if inhibitor were bound to the latter in a 1:1 molar ratio is only about 0.006, i.e. only just within the accuracy of the experiment.

The method of Aminoff, Morgan and Watkins[161] as modified by Reissig et al.[162] and later by Sharon,[14] which is more sensitive, was also used and showed no evidence of the presence of N-acetyl glucosamine in 2:1, although the conditions of the test caused the protein to precipitate initially.

Sample	A_{585}
β-D-glucosamine pentaacetate (0.2 μmoles)	0.043
Native lysozyme (0.2 μmoles)	0.000
2:1 (0.2 μmoles)	0.000
Control	0.000

(iv) Attempted reactivations of 2:1 with acid and base

In order to determine whether the modification was labile to mild acid or base treatment, samples of 2:1 (2 mg.) were incubated at pH 4.75 and pH 10.0. Controls with native enzyme were run simultaneously. Samples were readjusted to near neutrality after incubation.

Acidic treatment was for 19 h. at 20°, pH 4.75 in water.

Sample	Activity (% of native)
Control (native)	86
2:1	4.7
2:1 without treatment	5.0

Basic treatment was for 20 min. at 20°, pH 10.0 in water.

Sample	Activity (% of native)
Control (native)	102
2:1	4.6
2:1 without treatment	4.5

2. Peptide studies

(i) 1:2

Samples of native Seravac lysozyme (3 x cryst., batch no.36), and of the product 1:2 (29 mg.) were reduced, carboxymethylated, and digested with trypsin, as described in sections B.2(iii) and B.2(iv) of this chapter. Peptide mapping of these samples was then carried out (section B.2(v)(d)); the maps obtained showed slight differences between native and modified enzyme. The same differences were more clearly seen in the peptide map of 2:1 (see sub-section (iii)) and are discussed fully later.

(ii) 3:1

A sample of 3:1 (0.25 μmoles) was reduced, carboxymethylated, and digested with trypsin. A peptide map of the resulting mixture showed no differences from a native map.

(iii) 2:1

Samples (29 mg., 2 μmoles) of native Seravac lysozyme (Batch no.58Z) and of 2:1 were reduced and carboxymethylated, and then digested with trypsin. 1.0 mg. of each of the tryptic digests was hydrolysed and analysed (section B.1(iii)), in order to check the completeness of carboxymethylation of cysteine. Cystine peaks were absent from these analyses, and although the carboxymethyl cystine peaks were slightly lower than the theoretical 8 residues, (6.84 and 7.04 respectively), the accuracy of 'large' amino-acid analyses on the Technicon instrument is rather low, and carboxymethylation was assumed to be complete. Peptide maps of samples (2.5 mg.) were then prepared, and, as shown in figure 12, revealed significant differences between native and modified enzymes.

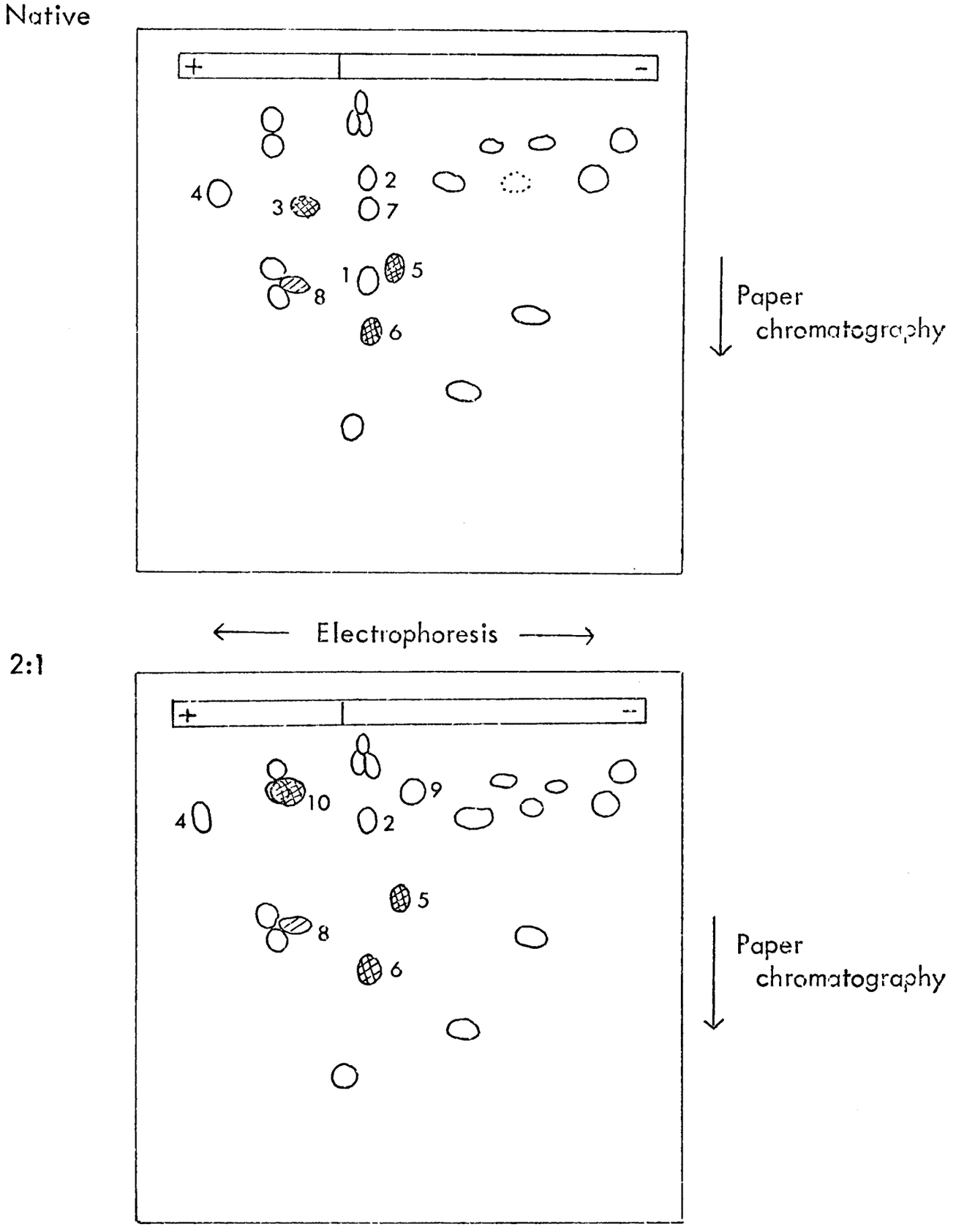
Several spots were identified without recourse to preparative separation and analysis of the peptides.[163] On the native map, assignments were made as follows: (the colour of the spot after ninhydrin/cadmium development, and its reaction to the tyrosine stain, are given in brackets for each spot).

Spot 1: (yellow, -ve); Gly 117 - Arg 125

2: (red, -ve), or possibly 7: (pink, -ve); Phe 34 - Arg 45

Figure 12

Tryptic peptide maps



Open circles represent spots shown by the ninhydrin/cadmium reagent, and shaded circles show spots positive to the tyrosine stain.

Spot 3: (yellow, +ve); Asn 46 - Arg 61

4: (orange, -ve); Asn 74 - Lys 96

5: (pink, +ve), and 6: (yellow, +ve); His 15 - Arg 21 and Gly 22 - Lys 33

8: (faint yellow-pink, faintly +ve); unknown, but probably a chymotryptic peptide.

Apart from the unexplained absence of spot 1, (which was incidentally not seen in other native peptide maps), in the peptide map of the 2:1 tryptic digest, and the appearance of a new spot 9 (red, -ve), the major significant difference in this map is the complete loss of the Asp 52 peptide spot 3, and the appearance of a new tyrosine peptide spot 10 (? yellow, +ve), overlapping with one of the peptide spots seen in the native map, on the 'acidic' side of the electrophoretic origin.

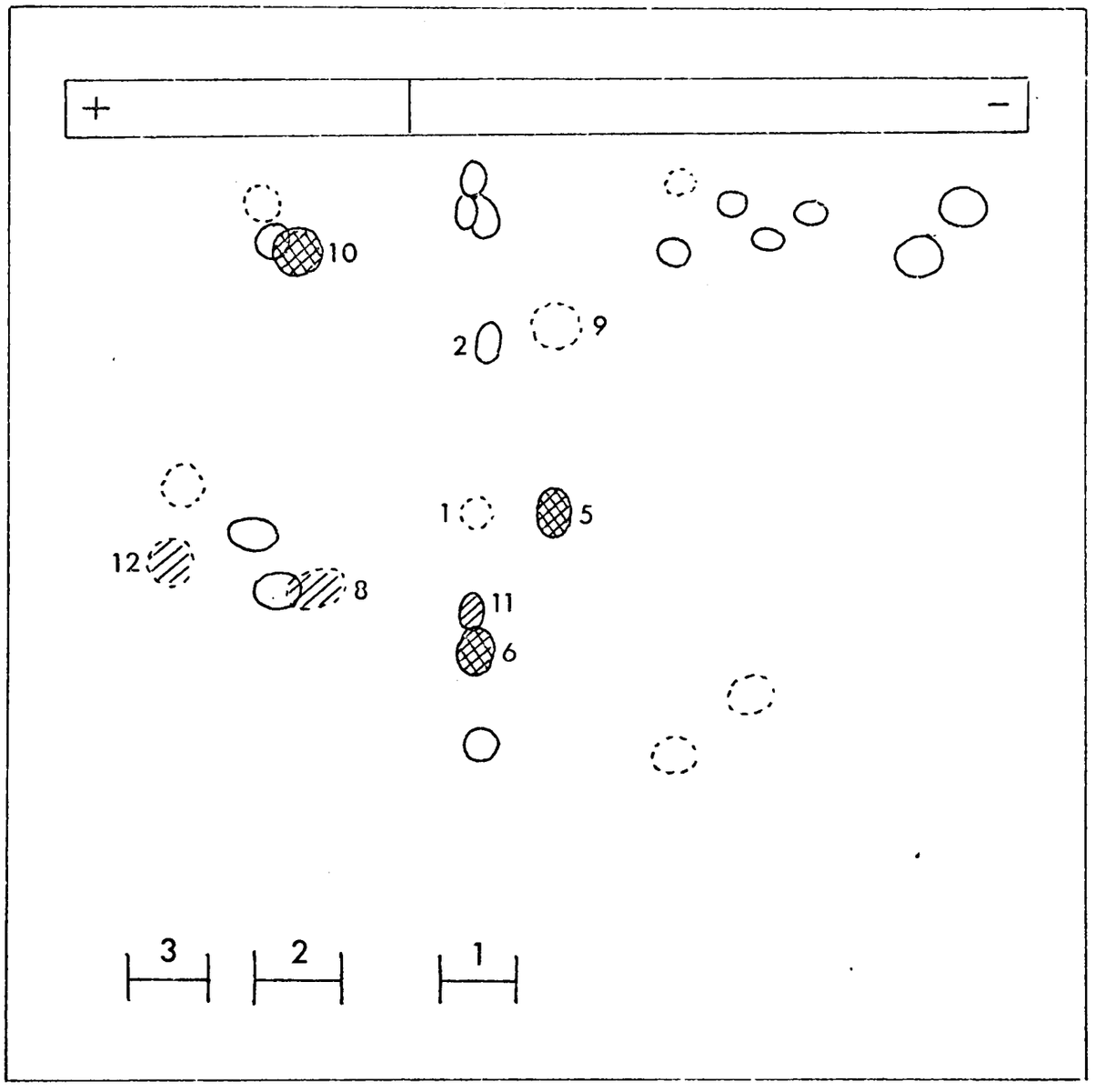
(The peptide map of modified enzyme 1:2, (see sub-section (i)), showed the Asp 52 peptide spot 3, and also a trace of the new spot 10).

A larger scale reduction, carboxymethylation and trypsin digestion was then carried out on product 2:1 (100 mg., 6.9 μmoles). A peptide map was prepared from a 4.0 mg. sample of this digest to check the positions of the tyrosine peptides (figure 13). This was very similar to the earlier map prepared (figure 12), but the larger sample used in this case revealed traces of peptide 1, and also showed small amounts of two new tyrosine peptides, spot 11, (overlapping with spot 6) and spot 12.

The remainder of the tryptic digest was then applied in a 38 cm. streak

Figure 13

Tryptic peptide map of modified enzyme 2:1



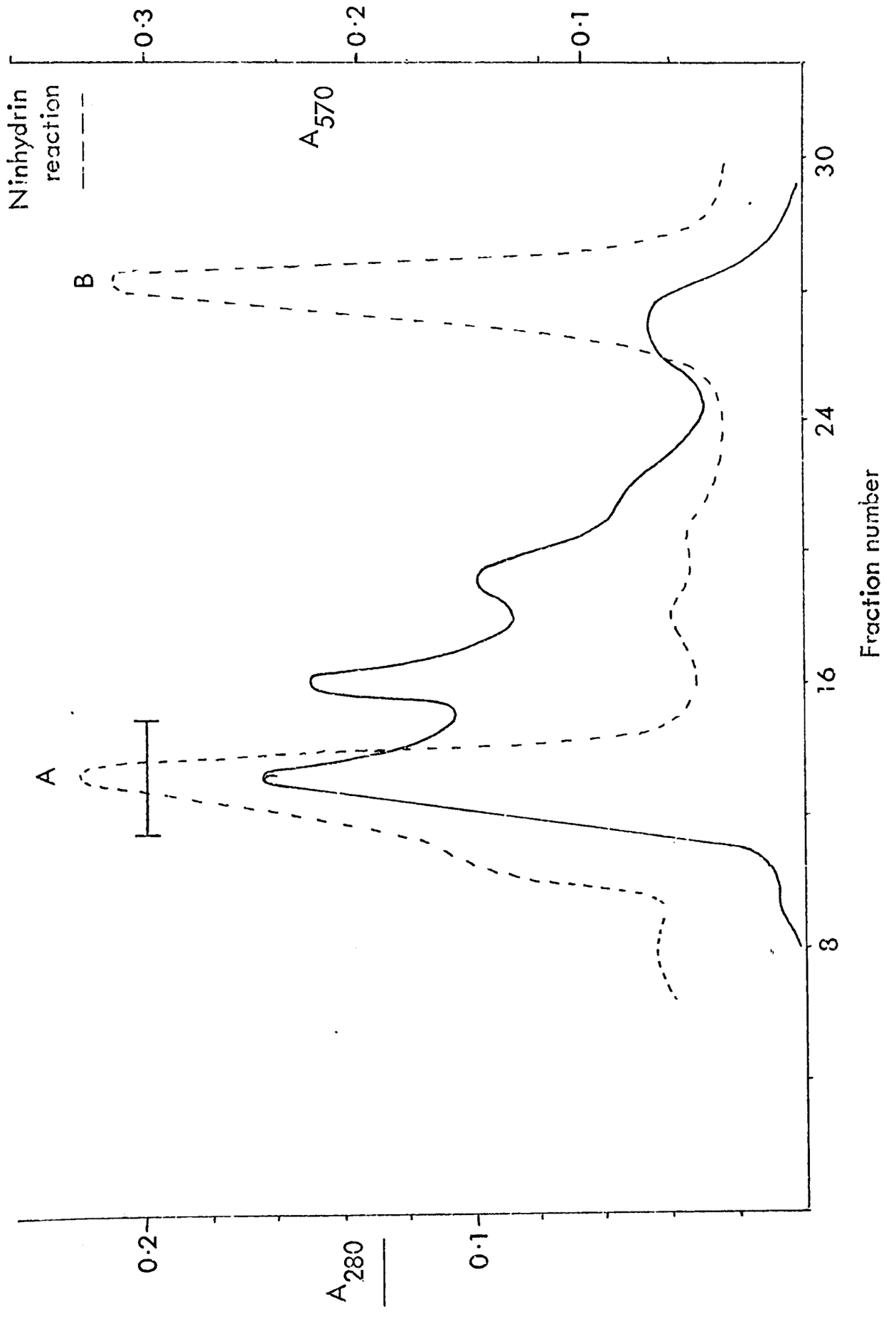
to Whatman 3MC paper and subjected to electrophoresis at pH 6.5, 3.0 Kv. for 35 min., and at 2.5 Kv. for a further 15 min. Three tyrosine containing bands were located and cut out. (In order of increasing acidity these were named bands 1, 2 and 3, corresponding to the regions marked on figure 13).

Band 3, which only showed a faint indication of the presence of tyrosine, was eluted from the paper with 10% acetic acid. Bands 1 and 2 were subjected to descending paper chromatography, after sewing their strips onto new sheets of paper, and the regions containing the tyrosine peptides corresponding to spots 11 + 6 and 10 respectively (figure 13) were located and cut out. The peptides were completely eluted with 10% acetic acid (10 ml.) and, after some evaporation, these solutions were applied to a column (30 x 1.5 cm.) of Sephadex G.25 (fine) and eluted with 10% acetic acid. Fractions of 2.75 ml. were collected and were studied at 280 nm., and for peptide content by ninhydrin tests on aliquots. The method of Hirs[164] was used for this except that aliquots, (0.2 ml.), were first neutralised with sodium hydroxide before alkaline hydrolysis and treatment with ninhydrin. The ninhydrin solution used was that used in the amino-acid analyser, which is essentially a more dilute form of that described by Moore and Stein.[165]

The peptide mixture derived from band 1 was shown to be very complex, containing several tyrosine peptides in unresolved peaks. The mixture derived from band 2 was somewhat simpler, and gave the elution profile shown in figure 14. Spots of peptides A and B were placed on paper and stained for tyrosine. Peptide A

Figure 14

G.25 Chromatography of the crude peptide 10 product



did contain tyrosine, but peptide B gave no colo ration with the tyrosine reagent. Peptide A thus corresponds to peptide 10 on the maps, and the fractions indicated on figure 14 were taken as this material. The volume of this solution was 12 ml. and its optical density at 277.5 nm. was 0.160. It was assumed that the peptide contained a single tyrosine residue (and no tryptophan or phenylalanine), and with the use of the molar extinction coefficient for tyrosine of 1300,[166] the amount of peptide present is calculated as 1.48 μmoles.

0.1 μmole was hydrolysed and analysed: Asp 2.7, Thr 2.0, Ser 1.0, Glu 0.4, Gly 1.2, Ala 0.7, Met 0.1, Leu 0.2, Tyr 1.0, Lys 0.3, Arg 0.2.

The analysis is clearly non-integral and the peptide impure.

The peptide was again eluted from Sephadex G.25. A_{280} measurements and ninhydrin tests revealed a single peak with a small shoulder on the left hand side. Again the later part of the peak was taken as 'pure' peptide (0.86 μmoles).

A portion (0.1 μmoles) was hydrolysed and analysed, and still showed the same analysis: Asp 2.8, Thr 2.0, Ser 1.0, Glu 0.4, Gly 1.1, Ala 0.8, Val 0.1, Met 0.1, Leu 0.3, Tyr 0.9, Lys 0.3.

A further portion (0.06 μmoles) was digested with carboxypeptidase A for 24 h. at 37° as described in section B.2 (vi)(a). The analysis of the product showed tyrosine as the largest peak, and smaller amounts of Ala (? Gly) (50% of Tyr), and Leu (20% of Tyr). Leucine aminopeptidase digestion (section B.2 (vi)(b)) was carried out for 36 h. at 35° on a portion of the peptide (0.06 μmoles).

The product analysis showed: Asp 2.1, Thr 2.0, Ser 1.2, Glu 0.4, Gly 1.4, Ala 1.1, Val 0.2, Leu 0.2, Tyr 0.9.

Peptide A (0.09 μmoles) was then subjected to 3 cycles of the Edman degradation (section B.2 (vi)(c)) and PTH derivatives prepared. Two-dimensional thin layer chromatography of the three PTH's suggested that the sequence was Asn, Ser or Thr, Asp, but as explained earlier these assignments are tentative. The peptide residue after 3 Edman cycles was digested with LAP and gave the analysis: Asp 1.1, Thr 1.0, Ser 1.0, Gly 0.9, Ala 0.8, (Tyr 1.4). The Tyr analysis is inaccurate as the peak was unresolved from an LAP-derived peak. The degradation had clearly removed 1 Asp, 1 Thr, and probably 1 Asn, as this cannot be seen on these analyses, (giving a peak of low colour value unresolved from Thr).

Analyses using the buffer system designed to show the presence of Asn, (Section B.2 (vii)(a)) were then carried out on (a) a fresh LAP digest of the peptide, and (b) an LAP digest of the peptide after one Edman cycle. These showed:

(a) Thr 2.0, Asp + Ser 2.3, Asn 0.8, Glu 0.2, Gly + Ala 1.6, Leu (? Ileu) 0.3, Tyr 0.7, Lys 0.6, Arg 0.3.

The low Tyr value suggests incomplete LAP digestion, as carboxypeptidise A digestion has shown it to be the C-terminal residue.

(b) Thr 2.0, Asp + Ser 0.9, Gly + Ala 2.0, Arg ? 1.0.

The complete absence of Tyr suggests only partial digestion of the peptide by LAP. However the absence of Asn suggests that there is no Asn residue in the N-terminal region.

A sample of peptide A (0.01 μmoles) was subjected to electrophoresis, alongside a sample of Asp, at pH 6.5, 3.5 Kv., for 35 min. The ratio of the mobilities of the peptide and aspartic acid was 0.251. A small amount of impurity was also seen on the electrophoretogram with mobility with respect to Asp 0.14.

The remaining peptide (ca. 0.11 μmoles) was degraded three times by the Edman method, and small samples, (ca. 0.015 μmoles) were subjected to electrophoresis after each cycle. Results were as follows:

	Mobility w.r.t. Asp	Ninhydrin colour	Tyrosine stain
After 1 Edman cycle	0.29	yellow	+
	0.25	pink	+
After 2 Edman cycles	0.31 (major)	pink/orange	+
	0.35 (minor)	yellow	±
	0.49 (very minor)	-	±
After 3 Edman cycles	-0.02 (major)	orange	+
	0.37 (very minor)	pink	±

After the first Edman cycle the product clearly showed some starting material (mobility 0.25), but in the other products the major component was quite unmistakable.

The unused degraded peptide was subjected to preparative electrophoresis at pH 6.5. The neutral band was cut out and eluted with 10% acetic acid, and the product further purified on a column (21 x 0.8 cm.) of Sephadex G.25 (fine)

in the same solvent. The single peak absorbing at 280 nm. was isolated and, after lyophilisation of the solution, digested with LAP for 57 h. Great care was taken to avoid contamination in all operations as the amount of peptide was extremely small (ca. 0.02 μmoles). A control was run with LAP and no sample. Half of the peptide digest (ca. 0.01 μmole) was analysed on the modified Beckman instrument (section B.1 (iii)), and gave the following peak areas (corrected for colour values obtained from a standard analysis):

Asp 10.32, Thr 10.31, Ser 13.5, Glu 1.44, Gly 14.10, Ala 6.93, Ileu 0.87, Leu 1.61, Tyr 6.05.

Asn runs exactly under Thr on this instrument and has a similar colour value to it. The control analysis showed relatively large amounts of some amino-acids. Integrated peak areas on the same scale as above were:

Asp 3.17, Ser + Thr (unresolved) 48.5, Gly 13.5, Ala 12.5, Ileu 1.22, Leu 1.91.

It appears that the control product contained an impurity not present in the peptide product, whose analysis may therefore tentatively be considered valid.

The other half of the digest (0.01 μmoles) was subjected to electrophoretic and paper chromatographic separation of its component amino-acids (section B.2 (vii)(b)), but the chromatogram was partially spoiled during staining by the presence of an amine in the oven used. The Asn region was not obscured however and the chromatogram showed no evidence of its presence.

3. Fluorimetric binding studies with 2:1

The method of Chipman et al.[59] was followed with some modifications. Enzyme solutions of twice the concentration required in the experiment were made up in McIlvaine buffer,[167] pH 5.45: saccharide solutions were made up similarly, in the same buffer or in water. 0.5 ml. portions of enzyme and saccharide solutions were mixed, equilibrated to 25°, and the fluorescence spectra run, with an exciting wavelength of 285 nm. Native enzyme showed an emission maximum at 359 nm., and 2:1 gave a maximum at 350 nm. This shift in the λ_{max} value is in the same direction as when $(NAG)_3$ or NAG is bound to native enzyme; binding was studied by measurements of the fluorescence intensity decrease at 350 nm. for $(NAG)_3$, or at 360 nm. for NAG binding, as saccharide concentration was increased. (Chipman et al.[59] used the intensity variations at 325 nm., but the 350-370 nm. region was found here to be more satisfactory as greater changes were apparent at this higher wavelength). Corrections were made for the fluorescence of solutions of saccharide alone at the wavelength chosen. In the case of $(NAG)_3$ these corrections were less than 10% of the observed intensity, but in the case of NAG, where concentrations as high as 0.6M were used, the saccharide had a fluorescence intensity of up to 30% of the observed intensity in the presence of enzyme.

The relative fluorescence intensity of lysozyme saturated with the saccharide (F_∞) was extrapolated from the data from a plot of $\frac{1}{F_o - F}$ against $\frac{1}{[S]}$, where

F is the observed fluorescence of a solution containing free saccharide at concentration [S], and F_o is the measured fluorescence of a solution of enzyme alone. [S] is assumed in this treatment to equal S, the total saccharide concentration.

A plot of $\log \frac{F_o - F}{F - F_\infty}$ against log S should then give a straight line (of slope unity, because of the 1:1 complex formation), which gives a value for the binding constant Ka, ($Ka = \frac{[ES]}{[E][S]}$, where [E], [S] and [ES] are the concentrations of enzyme, saccharide, and enzyme-saccharide complex respectively), from the relationship $\log [S] = pKa$ at $\log \frac{F_o - F}{F - F_\infty} = 0$ (see appendix 1). It should be pointed out that the [S] values used should be corrected for the amount of saccharide which is bound to the enzyme and is thus not free in solution. The method of Rupley, as used on later experiments (section C.6 (i)) takes account of this correction, which however is unnecessary in the NAG binding experiments described here.

(i) Native lysozyme and NAG

N-acetyl glucosamine (NAG) was obtained from Koch-Light Laboratories (Pure, Batch no.3899). The enzyme solution was 0.3 mg./ml., and the saccharide solutions ranged from 1.33M down to 0.0105M. (The final concentrations on mixing equal portions of these were of course one half of these values). In this, and all fluorescence experiments it was found that the instrument was not stable enough to give reproducible results. Variations of up to 10% in fluorescence

intensities were common (even with the same sample run twice). For this reason F_o values were difficult to obtain accurately, and three F_o values observed were used in the data analysis. The 'best' final straight line was given by the mean F_o value (slope 1.01 by a least squares fit), and this gave a log Ka value of +0.85. The other 2 F_o values, which can approximately be considered as the extremes of error, gave log Ka values of +0.75 and +0.91. (Lit. values of log Ka are 1.18-1.30, pH 5.4;[59] 1.22-1.40, pH 5.0[57]).

(ii) Modified lysozyme (2:1) and NAG

The enzyme solution made up contained 0.27 mg./ml., and the log Ka value obtained as in (i) was 0.87.

(iii) Native lysozyme and $(NAG)_3$

The enzyme solution contained 0.30 mg./ml., and the $(NAG)_3$ solutions ranged from 10^{-3}M to 1.95×10^{-6}M. The log Ka value obtained was +4.70 (uncorrected). (Lit. values: 5.09, pH 5.0;[64] 5.04, pH 5.4;[59] 4.97-5.18, pH 5.0[57]).

(iv) Modified lysozyme (2:1) and $(NAG)_3$

The enzyme solution contained 0.27 mg./ml., and the $(NAG)_3$ solutions were as in (iii). Only a very small effect was seen on the 350 nm. intensity (about 4% of that observed for native enzyme). This meant that the variations in 350 nm. intensity were only just within the instrumental error and the final graph was a very poor straight line. A meaningful binding constant could not be

obtained from the data but it may be pointed out that, though very small, the fluorescence intensity changes appeared to occur over the same concentration range as with native.

4. P.m.r. studies of $(NAG)_2$ binding to modified enzymes

$(NAG)_2$ was used as a probe for some of the modified enzymes prepared, initially in a rather qualitative way to see whether native and modified enzymes had similar effects on the methyl resonances; it was then hoped to investigate the effect of added cobalt (Co^{++}) on the resonance positions and line widths, but this presented unexpected problems.

(i) $(NAG)_2$ binding

0.1M $(NAG)_2$ was made up in pD 5.9* citrate buffer (0.1M, in D_2O, and containing 0.1% acetone). Solutions of native lysozyme (Seravac, batch 58Z), and modified enzymes 2:1, 3:1–3:3, and 4, containing approximately 8 mg./0.25 ml. were made up in the same buffer. Accurate protein concentrations were determined from optical density measurements at 280 nm. of solutions made by diluting 10 µl. aliquots in 1 ml. of pH 5.5 citrate buffer (0.1M), and all the solutions were adjusted to exactly the same concentration (1.82 mM) by dilution with calculated quantities of buffer.

* 'pH' values of deuterium oxide solutions are given as pD values, obtained by adding 0.4 units to the value measured with a glass electrode.[174]

Samples were prepared for p.m.r. spectroscopy by mixing 0.25 ml. of enzyme solution with 0.25 ml. of disaccharide solution, final concentrations thus being [E] = 0.91×10^{-3}M, [S] = 5.0×10^{-2}M. Centrifugation was necessary with the modified enzyme solutions to remove small amounts of denatured material. A spectrum of disaccharide (0.25 ml.) and buffer (0.25 ml.) was also run. Sample temperature was 35.1° and 100 Mc./s spectra were recorded in triplicate at 1 c/s per chart division. Peak positions were measured relative to the acetone signal, and were in the region of 15-20 c/s upfield from it.

$(NAG)_2$ alone gave sharp well-resolved singlets at 15.56 and 18.58 c/s upfield from acetone, and when native lysozyme was present, the higher field signal (corresponding to the CH_3 of the NAG residue at the reducing end of the disaccharide, CH_3-(2)) broadened considerably and shifted upfield, to 19.13 c/s from acetone, as observed by Dahlquist and Raftery.[68] Line-widths and resonance positions are summarised below:

Sample	Activity (% of native)	Resonance position (c/s upfield from acetone)		Line width at half height (mm.)	
		CH_3-(1)	CH_3-(2)	CH_3-(1)	CH_3-(2)
$(NAG)_2$ alone	-	15.56	18.58	4.8	6.8
+ native	100	15.65	19.13	4.9	6.8
+ 2:1	4	15.43	18.70	5.2	6.0
+ 3:1	86	15.47	19.12	5.1	6.9
+ 3:2	72	15.54	19.10	5.0	6.5
+ 3:3	81	15.69	19.26	5.2	6.6

The experiment with inhibited enzyme 4 was performed on another occasion, and a slightly different adjustment of the instrument resulted in narrower signals.

Sample	Activity (% of native)	Resonance position (c/s)		Line width (mm.)	
		CH_3-(1)	CH_3-(2)	CH_3-(1)	CH_3-(2)
$(NAG)_2$ alone	-	15.66	18.62	3.9	3.7
+ 4	35	15.48	18.94	4.1	4.8

(ii) Co^{++} binding

Small accurately known molar quantities of cobalt(II) chloride were obtained by lyophilisation of aliquots of an aqueous solution of $CoCl_2.6H_2O$. A preliminary experiment showed that $[Co^{++}]$ up to 10^{-1}M had no effect on the pH of a 10^{-3}M solution of lysozyme.

Experiment 1

$(NAG)_2$, 5×10^{-2}M; $[Co^{++}]$, O, 10^{-2}M, and 10^{-1}M in pD 6.40 acetate buffer (0.03M) in D_2O. The buffer concentration was chosen so that the acetate signal could be used as an internal reference. With no cobalt present the $(NAG)_2$ methyl resonances were 15.2 and 12.3 c/s downfield from acetate. 10^{-2}M cobalt caused considerable broadening of the $(NAG)_2$ resonances, and complete disappearance of the acetate peak. 10^{-1}M cobalt caused complete collapse of the $(NAG)_2$ resonances.

Experiment 2

$(NAG)_2$, 5×10^{-2}M; lysozyme, 0.72×10^{-3}M; $[Co^{++}]$, O, 1×10^{-4},

3×10^{-4}, 1×10^{-3}, 2×10^{-3}, 1×10^{-2}, 1.88×10^{-2}M; all solutions were made up in D_2O, containing 0.1% acetone (as an internal reference), and adjusted to pD 6.40. No effects were seen on the $(NAG)_2$ methyl resonances, apart from small (± 0.2 c/s) random positional variations, up to 10^{-2}M cobalt. At and above this concentration general broadening of the $(NAG)_2$ and acetone peaks occurred.

Experiment 3

The $(NAG)_2$ concentration was reduced by a factor of 10, which necessitated the use of a CAT to observe the methyl resonances. $(NAG)_2$, 5×10^{-3}M; lysozyme, 0.755×10^{-3}M; $[Co^{++}]$, O, 0.47×10^{-3}M, 1.82×10^{-3}M; acetone 0.05%; solutions were all made up in D_2O adjusted to pD 6.40. 25, 135, and 68 spectrum accumulations respectively were used.

With no cobalt present the $(NAG)_2$ resonances were seen as one sharp peak (ca. 14 c/s upfield from acetone) and one broad peak (ca. 30 c/s upfield from acetone). When cobalt was present in the concentrations given, only one resonance was seen (14-16 c/s from acetone). As some enzyme absorption was evident in this spectral region after spectrum accumulation it is possible that a broadened methyl resonance could be hidden under this.

5. Ion-exchange chromatography of modified enzymes

(i) Analytical

Suitable conditions for the chromatography of lysozyme and its derivatives

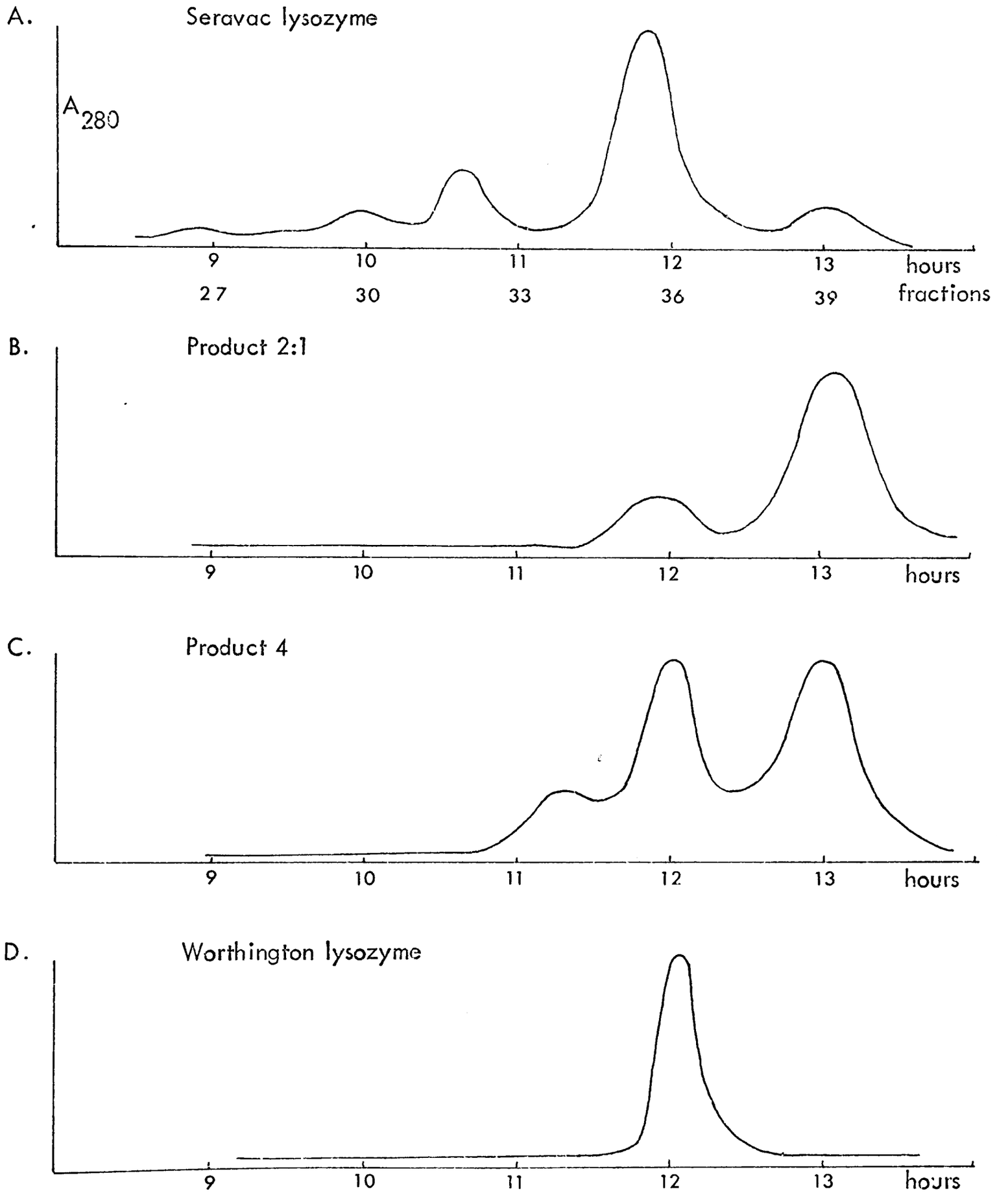
on the cation-exchange resin Biorex 70 (Batch no.6218) were found to be elution with pH 10 borate buffer (0.0125M), with a sodium chloride salt gradient up to 0.4M. Protein was eluted in the region corresponding to 0.15-0.25M eluent. For analytical work, samples (ca. 5 mg.) were prepared by dissolving them in pH 10 borate buffer (0.0062M) (2-3 ml.). The conductivity was checked to be below that of the starting buffer, (2.1 mmho), and the pH was readjusted to 10.0 if necessary. Elution was from a small (60 x 1 cm.) column of Biorex 70, equilibrated in pH 10 borate buffer (0.0125M), and eluted at 22 ml./h., with the aid of a peristaltic pump. 250 ml. each of this borate buffer with no extra salt and that with 0.4M sodium chloride present were used to provide the eluting buffer gradient. 20 min. (7.3 ml.) fractions were collected, and fractions containing protein assayed in the normal way against M. lysodeikticus. The column was regenerated after each run by elution of starting buffer until the conductivity of the effluent buffer was the same as that applied to the column. Buffers were degassed rigorously to prevent the formation of air bubbles in the tightly packed column, which nevertheless had to be repacked several times.

(a) Native Seravac enzyme, (Batch no.58Z). (Figure 15A)

This was shown to contain 5 components. The major component (58% of total eluted) was eluted after 12 h., and had 122% of 'native' activity. Another large peak (24% of total eluted) at 10½ h. had 43% native activity. Small peaks seen at 8¾ h. and 13¼ h. (2% and 7% of total eluted) had activities 43% and

Figure 15

Ion-exchange chromatography on Biorex 70



109% respectively, and there was a final small peak at 10 h., (9% of total).

(b) Inhibited enzyme 2:1. (Figure 15B).

The major component eluted (71% of total) was after the normal native position, at 13¼ h., and had 1.2% native activity. A minor peak was seen at the native position (12 h., 29% of total) but only had 5% activity. Recovery of 2:1 in this chromatography was an estimated 85%, the remainder presumably being denatured.

(c) Inhibited enzyme 4. (Figure 15C)

After dialysis, a sample of product 4 (5 mg.) was eluted in the normal way, and was shown to contain 17% of a component at 11 h., 41% at 12 h. (87% activity) and 42% at 13 h. (9.7% activity). The central peak is certainly native enzyme, and the modified enzyme peak at 13 h. is probably the same as that in sample 2:1, its higher activity being due to contamination from the 'tail' of the native peak.

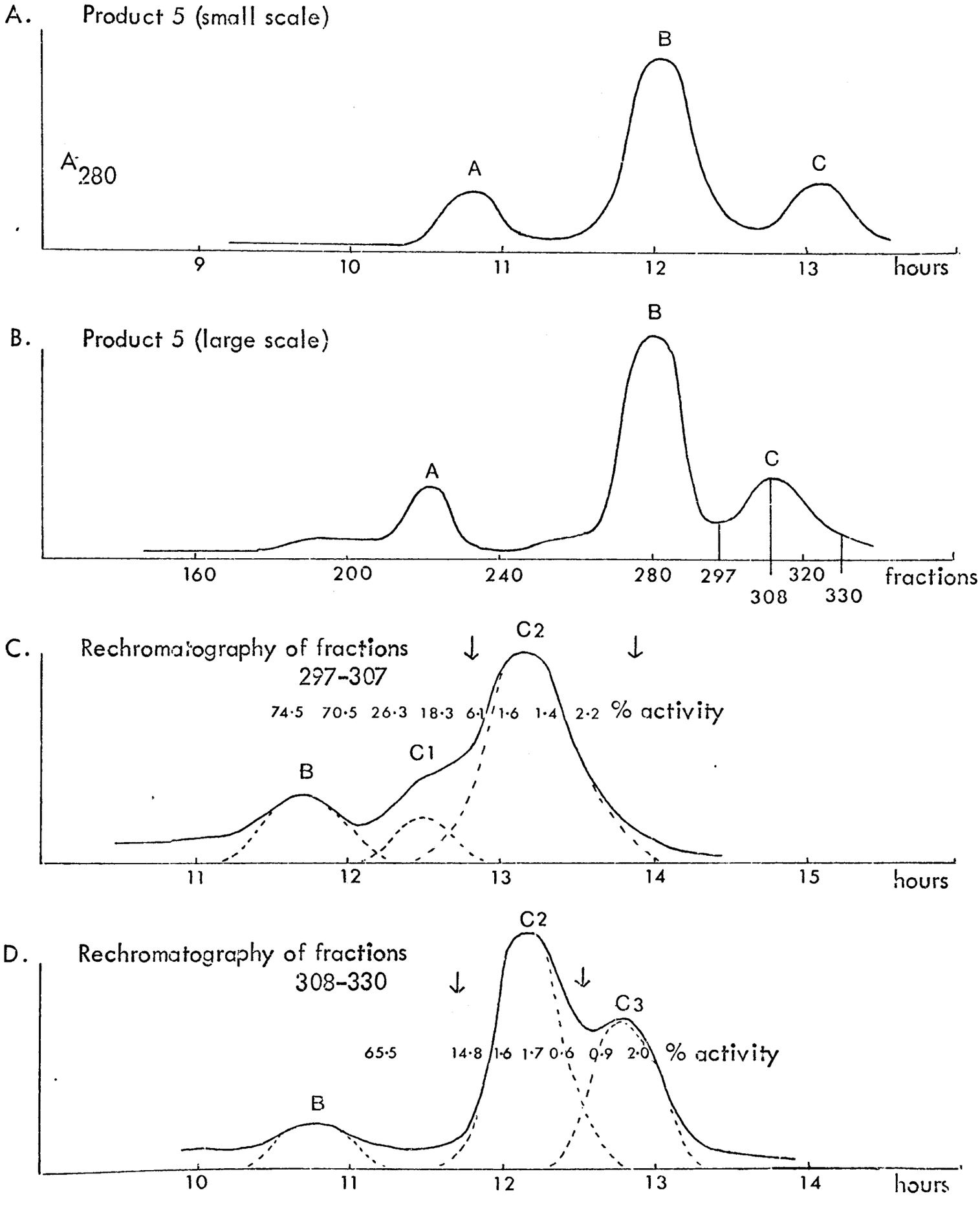
(d) Native Worthington enzyme, (batch no.LY9FA). (Figure 15D)

This was eluted as a single peak after 12 h.

(e) Inhibited enzyme 5. (Figure 16A)

This contained 15% of the peak before the native position, (10¾ h., 49% activity), 67% of native enzyme, (12 h., 70% activity), and 18% of the peak at 13 h. (10% activity). The suggestion that activity in the last peak was at least partly due to contamination with native enzyme was supported by the fact

Figure 16


A.
Product 5 (small scale)
A280
A
B
C
9
10
11
12
13
hours
B.
Product 5 (large scale)
A
B
C
160
200
240
280
297
308
320
330
fractions
C.
Rechromatography of fractions
297-307
74·5 70·5 26·3 18·3 6·1 1·6 1·4 2·2 % activity
B
C1
C2
11
12
13
14
15
hours
D.
Rechromatography of fractions
308-330
65·5 14·8 1·6 1·7 0·6 0·9 2·0 % activity
B
C2
C3
10
11
12
13
14
hours

that an earlier fraction in the peak when assayed showed 18% activity. As shown in figure 16A the three peaks are named A, B, and C in order of elution, and this notation will be used henceforth.

(f) Inhibited enzyme 6

This contained 15% of peak A (56% activity), 71% of peak B (native) (90% activity), and 14% of peak C (5.7% activity). Inhibited enzyme 7 gave a virtually identical chromatogram.

(ii) Preparative

For preparative separation of modified enzymes a large column (86 x 5.6 cm.) of Biorex 70 was used. The starting buffer for elution was pH 10 borate, 0.1M in sodium chloride, and the second buffer was as before, pH 10 borate, 0.4M in sodium chloride. 7 or 8 litres of each of these buffers was used. 400 fractions of 250 drops (about 17 ml.) were collected in each chromatography.

Products 5, and 6 and 7 (together) were eluted in this way, samples being applied in 20-25 ml. solution, adjusted to pH 10.00 with sodium hydroxide. Good separation of the A, B, and C peaks was achieved in each case.

(a) Inhibited enzyme 5. (Figure 16B)

The A peak had activity 42% of native, B 87.5%, and representative fractions through the C peak 13.2, 7.6, 4.8, 4.0, 5.6, and 7.6% activity. Peak C was split into 2 halves; after dialysis, evaporation and lyophilisation the products were rechromatographed on the analytical Biorex column.

The frontal half (fractions 297-307 in the large scale chromatography) was eluted with a buffer gradient from 400 ml. each of two pH 10 borate buffers, 0.1 and 0.4M in Na^+. This showed (figure 16C) a small native peak followed by a large modified enzyme peak which clearly showed a shoulder (C1) on the frontal side. Activity measurements were carried out on all fractions, and these suggested (see figure 16C) that the native peak had 75% activity, C1 about 15-20%, and the main modified enzyme, C2, about 1.5% of native activity. Curve resolution with a Du Pont instrument showed that C2 accounted for 91% of the modified enzyme peak, and indicated that the fractions between the arrows in figure 16C contained C2 in at least 99% purity.

The second half of the C peak (fractions 308-330 in the large scale chromatography) was rechromatographed similarly, though with 350 ml. each of the two buffers. It showed (figure 16D) a minor component, C3, in addition to the main modified enzyme C2, which accounted for 61% of the total modified material. Activity measurements through the C peak showed a small amount of native tailing at the frontal edge, and a fairly constant activity around 1% for the rest of the peak. Curve resolution was again used to indicate the fractions which contained the main peak (C2) in greatest purity (>95%), and C3 was also isolated.

The combined C2 products from the two separations were desalted by elution from a column (35 x 2.5 cm.) of Sephadex G.25 (fine) with 0.5% ammonium

bicarbonate, which was removed from the protein by lyophilisation. This product was then rechromatographed on Biorex 70, and was eluted as a single peak, with a slight amount of active contaminant on the frontal side. (Activity values of every third fraction through the peak were 5.7, 1.6, 0.2, 0.2 and 0.0%). A central cut of this peak yielded 10.6 mg. of pure C2 modified enzyme after desalting as before. The A, C1 and C3 components were also obtained salt-free in a similar fashion and yielded 32, 3, and 15.5 mg. respectively.

(b) Inhibited enzymes 6 and 7

The chromatography of the combined products of these inhibitions gave very similar results to that of product 5, except that the A peak showed a shoulder on the frontal side, which had not been apparent in the previous chromatography. Rechromatography of A, in two halves, resulted in a partial separation of the components A1 and A2, and the major one (A2) was isolated as usual. C1, C2, and C3 were isolated as before. C2 was purified by rechromatography, and, after desalting the fractions chosen as containing pure material, a yield of 14.5 mg. C2 was obtained.

6. Studies on the pure modified enzymes

(i) Fluorimetric binding studies

These were carried out by the method of Rupley.[64] The enzyme concentration was kept as low as possible, (ca. 0.02 mg./ml.), so that corrections

in the value of the saccharide concentration for the amount of the saccharide bound in an [ES] complex would be minimised. The buffer used for all solutions was pH 5.00 and ionic strength 0.1, prepared as described by Coch Frugoni.[168] All enzyme solutions were adjusted to approximately the same concentration (1.3 μmolar). The temperature of all determinations was 30°; excitation was at 285 nm., and readings were taken at 370 nm., for both NAG and $(NAG)_3$ binding studies.

The results obtained were analysed by the method of Rupley,[64] (see appendix 1), which involves plotting ΔF against $\frac{\Delta F}{S}$, (where ΔF is the observed change in fluorescence intensity at the chosen wavelength in the presence of saccharide at a total concentration S). The slope of this plot is -1/Ka to a first approximation and the intercept on the y-axis ΔF_{max}, i.e. the value of ΔF when the enzyme is saturated. Using this value of ΔF_{max} the proportion of S in the form of an [ES] complex may be estimated and corrected for, and a final plot of ΔF against $\frac{\Delta F}{[S]}$, where [S] is the concentration of unbound saccharide, gives a more accurate Ka value. This correction in the value of S in unnecessary in the case of NAG binding.

(a) $(NAG)_3$ binding

Two determinations of the $(NAG)_3$ binding constant to native (Worthington) enzyme were carried out using a range of $(NAG)_3$ concentrations from 10^{-4}M to 1.56×10^{-6}M. The binding constant obtained (log Ka = 5.05) agrees well with

the known value under these conditions (5.09).[64] Figure 17 shows typical fluorescence spectra and a final plot for native enzyme binding $(NAG)_3$.

Two attempted determinations of this binding constant for modified enzyme C2 were made using $(NAG)_3$ concentrations up to 2×10^{-3}M, but binding could not be observed.

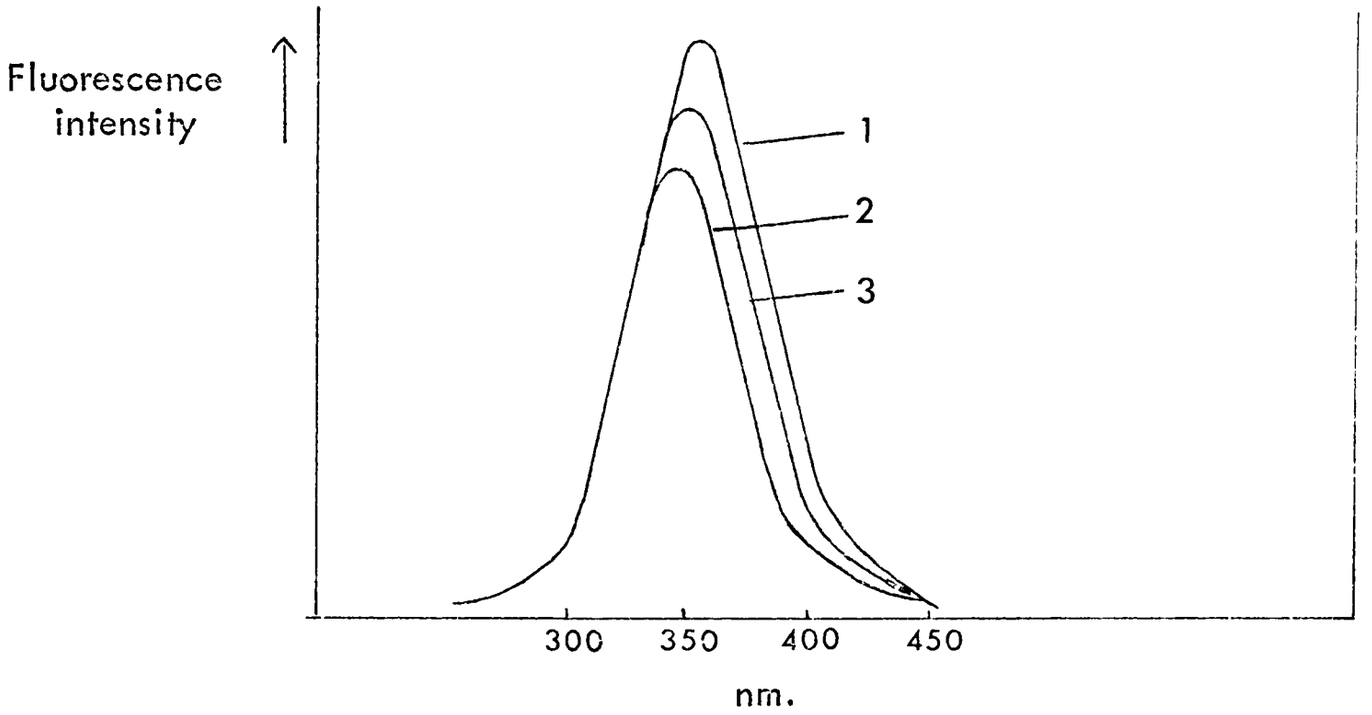
$(NAG)_3$ concentrations up to 4×10^{-4}M were used in the single experiments done with A, C1 and C3. Modified enzyme A clearly showed $(NAG)_3$ binding with a log Ka value of 4.54. The fluorescence spectra of C1 however showed only very slight effects on addition of $(NAG)_3$, which corresponded to a rather dubious log Ka value of 5.3. C3 similarly showed slight effects, ΔF_{max} being only 7% of the F value with no saccharide present (F_o), compared to 38% in the case of native enzyme. A binding constant (log Ka = 5.09) was calculated for C3 however. The data obtained are summarised below:

Sample	log Ka	λ_{max} (nm.)	λ_{max} on saturation (nm.)
Native	5.05	355	349
A	4.54	359	351
C1	(? 5.3)	359	350
C2	-	350	-
C3	(5.09)	354	352

The intensity of the 370 nm. emission of C2 was 69% of that of equally concentrated native enzyme, and saturated native enzyme has a corresponding value of 62%

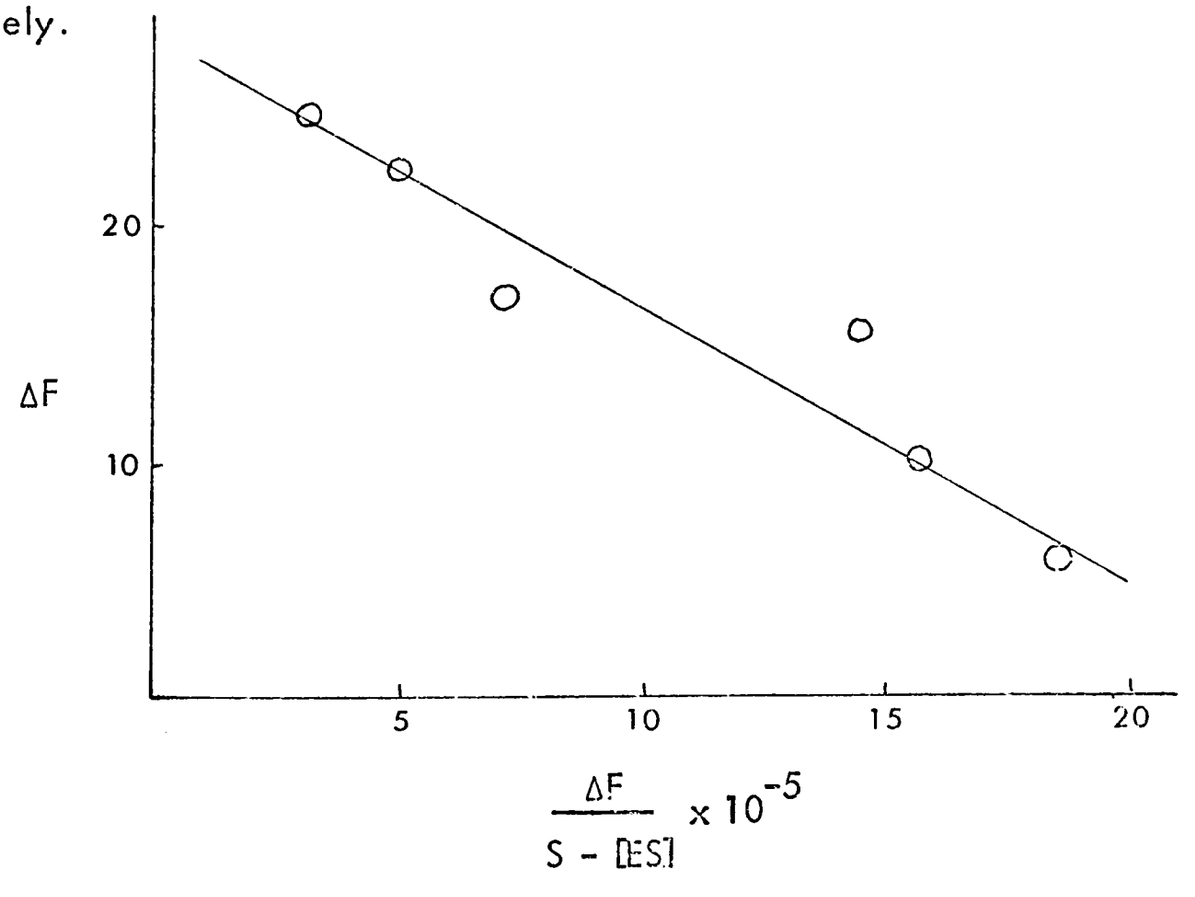
Figure 17

The binding of $(NAG)_3$ to native lysozyme



pH 5.0, $\mu = 0.1$, 30°. Spectrum 1: $[E] = 1.3 \times 10^{-6}$M.

Spectra 2 and 3: $[E] = 1.3 \times 10^{-6}$M, $[(NAG)_3] = 5.0 \times 10^{-5}$M and 6.3×10^{-6}M respectively.



(b) NAG binding

N-acetylglucosamine obtained from Koch-Light Laboratories, (Puriss, batch 43642) was found to be unsuitable for use in determinations of binding constants by fluorescence measurements as it contained an impurity (which was not removed by recrystallisation) which fluoresced strongly in the region to be studied. Fluka NAG (batch 418923) was found to be satisfactorily purer and was therefore used, and concentrations up to 0.5M could be used without the fluorescence of the sugar at 370 nm. reaching more than 25% of the enzyme fluorescence.

With a different instrument calibration than was used for the $(NAG)_3$ binding studies, native enzyme showed λ_{max} = 348 nm., shifted to ca. 343 nm. on saturation with NAG. The log Ka value determined was 1.20 (Lit: 1.18-1.30, pH 5.4;[59] 1.22-1.40, pH 5.0[57]).

C2, (λ_{max} = 341 nm.), showed apparently random and small fluorescence intensity variations in the presence of NAG concentrations from 3.9×10^{-3}M to 5.0×10^{-1}M and no shift in λ_{max} was seen. These variations were mostly within the instrumental variations observed when several spectra of the same sample were run, although spectra were always run in duplicate. The four most concentrated solutions of NAG used however (5.0×10^{-1}, 2.5×10^{-1}, 1.25×10^{-1}, and 0.625×10^{-1}M) did show some evidence of quenching of the enzyme fluorescence, in accord with a very approximate association constant of 15, (log Ka ca. 1.18). The 370 nm. intensity of a solution of C2 was 56%

of that of an equally concentrated solution of native enzyme, and the corresponding value for native enzyme 88% saturated with NAG was 61%.

(ii) Holmium binding studies by p.m.r. spectroscopy

(a) Modified enzyme A

A solution of A (9.5 mg.) in D_2O (0.4 ml.) was adjusted to pD 6.95 with a solution of DCl in D_2O. A single scan enzyme spectrum was recorded, with sample temperature 40°, on the 60 Mc./s Jeol instrument. The solution was then made 17, 33, and 60 mM in holmium (II) nitrate and a spectrum was run at each concentration. These showed exactly the same sort of changes in the high field region of the spectrum as occur with native enzyme,[169] namely a progressive loss of a signal or signals about 1 τ downfield from the position of the DSS signal. Although it was not possible to calculate a binding constant for Ho^{++} from the spectra run it would appear that A binds Ho^{++} similarly to native enzyme.

(b) Modified enzymes C2 and C3

C2 (9.0 mg./0.4 ml., pD 7.2) was studied similarly with holmium nitrate concentrations of 1.5, 6.2, 25 and 57 mM. No changes were seen in the aromatic or high field regions of the spectrum, apart from a little general broadening of resonances at the highest Ho^{++} concentration used.

C3 (9.5 mg./0.4 ml., pD 6.9) also showed no changes in its p.m.r. spectrum up to a holmium nitrate concentration of 60 mM.

(iii) Crystallisation of modified enzymes

Conditions for lysozyme crystallisation are 2-5% protein, 5% sodium chloride, and pH 4.7 acetate buffer (0.02-0.2M). Preliminary trials were carried out on native enzyme in 5% solution. Samples (6-25 mg.) of lysozyme were dissolved in pH 4.7 acetate buffer (0.4M) and an equal volume of 10% sodium chloride added to give a final concentration of 5% protein. After centrifugation to remove denatured material the supernatant was transferred to glass or polythene tubes of internal diameter 3-10 mm. which were then sealed and left at 20°. The first batch of enzyme used (Worthington batch no.LY9FA) contained denatured material and invariably gave small tetragonal crystals (about 0.2 mm. or less in length). A second batch (Worthington, salt-free, no.LYSF9FM) contained no denatured protein, and gave larger crystals (0.6 mm.), especially in the larger vessels used. Crystal size did not seem to depend on whether glass or polythene tubes were used, the advantage of the latter being that the crystals did not stick to the tube walls.

Prior to committing all of the remaining C2 protein to crystallisation small scale crystallisations were carried out in 'microslides'. 3% protein solutions (0.6 mg./20 μl.) were crystallised at pH 4.0 to 5.6. 5% sodium chloride was present in all cases. All these solutions produced very small crystals; those grown at pH 4.0 and 5.6 appeared to be needles, and those grown at pH 4.7 appeared to be more like the normal tetragonal shape. The standard conditions were

considered the most satisfactory, and 14.5 mg. C2 was crystallised at 20°, from a pH 4.7 solution (400 µl.) containing 5% sodium chloride in a 10 mm. internal diameter glass tube. A mixture of small (<0.2 mm.) needles and tetragonal crystals was formed. These were too small for crystallography, and various other attempts were made to grow larger crystals.

Gel filtration on Sephadex G.25 in 0.5% ammonium bicarbonate, followed by lyophilisation, or vacuum dialysis, with successive washings of the sample with pH 4.7 acetate buffer (0.2M), were both used as methods for removing sodium chloride and bringing the sample back to a form from which crystallisation could be repeated. Crystallisations in 4.0% and 4.5% sodium chloride were unsuccessful; the best crystals which were obtained were derived from a solution which contained 3.2% protein and 5% sodium chloride at pH 4.7, and which was allowed to cool slowly from 37°, immersed in water in a thermos flask. Crystals obtained in this way were needles (1.0 mm. long, 0.1 mm. wide).

Attempts were made to obtain reasonable X-ray diffraction patterns from one of these crystals, (by S. Banyard, Dept. of Molecular Biophysics), but although a few spots could be seen, diffraction was extremely weak, and the crystal could not be correctly oriented for this reason. Further attempts are to be made on these or fresh crystals.

As it was hoped that the presence of some native lysozyme would promote crystallisation of C2 in the tetragonal form, all the fractions from earlier column

chromatography which contained mixtures of C1, C2, and native lysozyme were combined and desalted. Under the standard conditions for crystallisation however this material also yielded 1.0 mm. needles. Modified enzyme A did not crystallise under conditions of native crystallisation.

D. Discussion

1. Factors involved in inhibition

Although at first sight the degree of inactivation of the enzyme seems to bear only a random relationship to the inhibition conditions used, several important conclusions may be drawn from the data presented in table 1.

In inhibition 1:1 the activity of the enzyme after treatment with a 37-fold molar excess of inhibitor in the presence of an 80-fold molar excess of glycine methyl ester, (30% of native activity), was the same as that of the product of a parallel reaction, 1:2, carried out with no nucleophile added. The product of a control reaction (1:3) with glycine methyl ester present but no inhibitor had 71% activity, and later control experiments (2:2 and 3:4) in which methanol alone was added to the enzyme solution gave products of 86% activity, the inactivation in these presumably being due to some denaturation on lycphilisation, etc.

Further confirmation of the fact that the presence of glycine methyl ester had no effect on the course of inhibition came from ^{14}C determinations in the products. Although the degree of inactivation in product 1:1 was 70%, the ^{14}C content indicated only 10% incorporation of the nucleophile. The control product 1:3 contained no ^{14}C.

As the main course of inactivation of the enzyme thus does not involve the presence of an added nucleophile, further studies were carried out on the simpler product, as prepared in the absence of added nucleophile. It is clear then

that intermolecular amide formation, as outlined in figure 5, p. 44, is not occurring in the inhibition to any great extent. Model studies with succinamic acid (Chap. 2, p. 81), have shown however that intramolecular imide formation between a carboxylic acid and an amide may occur in the presence of a carbodiimide, and this was considered at this stage to be a possible pathway in the enzyme reaction, especially as Asp 52 is known to be very close to Asn 59 and within reach of Asn 44 and Asn 46. No reactivation of inhibited enzyme was observed however after prolonged incubation at pH 4.75, or brief incubation at pH 10.0 (at which the enzyme is less stable),[170] showing that the modification is not labile to mild acid or base treatment.

It is possible that the inhibitor may have caused a gross conformational change in the enzyme, but more likely causes of inactivation are (a) an intramolecular reaction leading to blocking of the catalytic site or a binding site, and (b) irreversible attachment of the inhibitor in the active site, blocking the catalytic or binding site, or both. Tests for the presence of the N-acetyl glucosamine residue in the modified enzyme proved negative, but owing to the high sensitivity required of these tests and the possibility of interference by the enzyme, pathway (b) cannot be ruled out.

An indication that the inhibition was not a simple reaction leading specifically to one product was obtained from the reinhibition of two of the products 1:2 and 2:1, both with a 50-fold molar excess of inhibitor. This treatment

reduced the activities to 77% (1:2:2) and 57% (2:1:2) respectively of the values after one inhibition, and even after two inhibitions the sample activities differed by a factor of 10.

It was noticed however that the degree of inactivation in an inhibition showed a certain dependence on enzyme concentration, the least active product (2:1) resulting from the inhibition involving the most concentrated solution. This is probably due to the fact that the inhibitor can only exist transiently in acidic aqueous solution. It can also be seen that the inhibitor:enzyme molar ratio has to be greater than about 20 if any inactivation is to result; above this value the ratio does not seem to be as important, little activity difference being seen between the products of experiments with 37-fold and 93-fold molar excesses of inhibitor (1:2 and 4) which were similar in enzyme concentration. However Worthington enzyme, as used in inhibitions 5, 6 and 7, appeared to be generally less susceptible to the action of the inhibitor than Seravac enzyme, (inhibitions 1, 2, 3 and 4), although it was later (section C.5(i), p. 130) shown to be purer.

2. Peptide studies

As might be expected, the magnitude of the differences observed in peptide maps of tryptic digests of the reduced and carboxymethylated inhibited enzymes correlated with the extent of inactivation in these products. Thus 3:1 (86% active) showed no peptide differences from native enzyme, 1:2 (30% active) showed an

indication of some differences, and 2:1 (4% active) clearly showed differences, including in particular the loss of the normal Asp 52 peptide and the appearance of a new tyrosine-containing peptide.

This peptide was isolated by a combination of electrophoresis, paper chromatography and gel filtration, and a portion was hydrolysed. The amino-acid analysis of this hydrolysate was not integral, suggesting that the peptide was impure. Refiltration on Sephadex G.25 failed to purify the peptide further. The analysis significantly showed however only very small amounts of Lys and Arg, suggesting that tryptic cleavage had not taken place next to either of these residues. This analysis suggests that the peptide is mostly the Asx 46*–Tyr 53 active site peptide, (Asx-Thr-Asx-Gly-Ser-Thr-Asx-Tyr); in the native enzyme residues 46, 48 and 52 are Asn, Asp and Asp respectively, (p. 6). A likely contaminant would seem to be the genuine tryptic peptide, CMCys 6-Lys 13, (CMCys-Glu-Leu-Ala-Ala-Ala-Met-Lys).

Digestion with carboxypeptidase A released Tyr as the main product, suggesting that this is the C-terminal residue. The trypsin used in the digestion which led to this peptide, (Seravac, batch no.289L) was of a diphenyl carbamyl chloride treated batch, and the manufacturers claim only 0.24% chymotryptic activity for it. The fact that cleavage of the modified lysozyme by this trypsin

* Asx refers to a derivative of Asp or to aspartic acid, unspecified.

appears to have occurred at Tyr 53 suggests that this residue may be in an unusual environment, which in turn would suggest that the adjacent Asp 52 may be modified.

A leucine aminopeptidase digestion of the peptide showed the presence of two aspartic acid residues. Although this indicates that two of the Asx residues in the Asx 46-Tyr 53 peptide are aspartic acid (as in the native enzyme) it is possible that a modified aspartic acid may have released Asp on leucine aminopeptidase digestion of the peptide. In view also of the impure nature of the peptide, and the fact that an Asp 52 modification was implicated by the position of the tryptic cleavage, examination of the Asx residues in more detail was necessary.

The N-terminal sequence of the peptide was examined by chromatographic study of the PTH-amino acids prepared via Edman degradations; this method was not found to be very satisfactory owing to the interference of by-products in the chromatography (section B.2 (vi) d, p. 98), although a tentative assignment of the sequence Asn-Thr-Asp could be made. This was supported by the analysis of an LAP digest of the residual peptide after three Edman degradation, which clearly showed the removal of 1 Asp, 1 Thr, and probably 1 Asn. The assignment of Asn to the N-terminal residue was also suggested by amino-acid analyses (using a buffer system designed to resolve Asn), of leucine aminopeptidase digests of the whole peptide, and of the peptide after one Edman degradation.

The electrophoretic mobilities of peptides are closely related to their charges and molecular weights.[171] The electrophoretic mobility at pH 6.5 relative to that of Asp was determined for the whole peptide and for the peptide after one, two, and three Edman degradations. Values obtained for this ratio were 0.25, 0.29, 0.31, and -0.02 respectively, (positive values signifying mobility in the same direction as Asp). It is quite clear from these results that the residual peptide after three residues had been removed from the N-terminal end has no net charge at pH 6.5, and thus Asp 52 must be modified. With this deduction it is not possible to assign an accurate molecular weight to the whole peptide, but if residue 52 were for example asparagine, this molecular weight would be 870. From the data of Offord,[171] such a peptide should have a mobility relative to aspartic acid at pH 6.5 of about 0.29 if it contains one net charge, and of about 0.55 for a net charge of two. It is also clear therefore that the whole peptide has a net charge of one, i.e. contains only one aspartic acid. Similar remarks apply for the peptide after one and two residues have been removed from the N-terminus (predicted mobilities for singly charged species 0.33 and 0.36 respectively). This interpretation of the electrophoretic data is not altered if one assumes that an inhibitor residue is bound to Asp 52. If anything, the larger molecular weight which would result from this would make the mobility data agree even more closely with that expected.

A very small sample of the residual pentapeptide, after three Edman

degradations of the whole peptide, was purified by electrophoresis and gel filtration, digested with leucine aminopeptidase, and analysed. This analysis cannot strictly be considered meaningful, as a control product showed many larger peaks, but it would seem that the control contained an impurity not present in the peptide sample. The presence of one residue of aspartic acid in the LAP digest of the peptide, (not seen in the control), suggests that it was liberated from a modified Asp 52 during digestion, and no Asn was seen either in this analysis, if the presence of 1 Thr residue is assumed, or in a paper separation of the amino-acids in the digest.

Although acceptably small amounts of amino-acid impurities were seen in most analyses, (especially of the whole peptide), the presence of 0.6 to 0.8 residues of alanine in all the analyses performed is surprising, as it is not a component of the active-site octapeptide isolated; the possibility that this 'alanine' might be for example an aspartic acid derivative may not be ruled out however.

3. Binding studies

(a) NAG and $(NAG)_3$ binding, by fluorimetry

The binding of NAG and of $(NAG)_3$ to native and modified enzyme (2:1) was studied by the method of Chipman et al. (appendix 1).[59] As explained earlier, the analysis of results does not include a correction in the saccharide concentration for the amount bound to the enzyme. The log Ka value for $(NAG)_3$ binding to native enzyme was determined as 4.7 (Lit. 5.09, pH 5.0;[64] 5.04, pH 5.4;[59] 4.97-5.18, pH 5.0[57]).

Modified enzyme 2:1 showed a fluorescence emission maximum 9 nm. lower than that of native enzyme. This indicates immediately that the tryptophan residues are in a more hydrophobic environment in 2:1 than in native enzyme.[58,172,173] 2:1 showed only small fluorescence intensity changes in the presence of $(NAG)_3$ which did not permit the calculation of a binding constant. However they appeared consistent with the possibility that the modified material did not bind $(NAG)_3$ but was contaminated with about 4% native enzyme. This would account entirely for the observed activity of 2:1 which is also 4%. (An alternative possible explanation, ruled out by later results with the pure modified enzyme, is that the latter has intrinsic low activity and that its nature is such that different and smaller fluorescence changes occur on binding $(NAG)_3$, compared with native enzyme).

NAG did however appear to bind to modified enzyme 2:1 with approximately the same binding constant, (log Ka ca. 0.8 to 0.9) as native enzyme. This observation, though apparently inconsistent with the $(NAG)_3$ binding result, may reflect a different mode of NAG binding to the enzyme.

(b) $(NAG)_2$ binding, by p.m.r. spectroscopy

The effect that is observed on the acetamido methyl p.m.r. signals of $(NAG)_2$ when the disaccharide is bound to lysozyme has been studied by Dahlquist and Raftery.[68] $(NAG)_3$, although possessing a higher binding constant, is less suitable for such studies as its rate of exchange between enzyme and solution is slow at 30°. In order to observe true 'time-averaged' resonances for the methyl

groups in $(NAG)_3$ a higher temperature (65°) has to be used, and then, in order to slow the otherwise rapid hydrolysis of this substrate, a high pH (9.7) is required.[68] $(NAG)_2$ was therefore used as a probe for some of the modified enzymes.

As might be expected, the samples of least activity against M. lysodeikticus show the most difference from native enzyme in this study. Indeed the samples 3:1, 3:2 and 3:3, (see table 1, p. 110), which showed virtually no inactivation on inhibition perturbed the $(NAG)_2$ methyl resonances similarly to native enzyme. In conjunction with the results of the peptide studies on 3:1 (p. 114) this shows that 3:1 (and presumably 3:2 and 3:3 which were treated with a lower molar excess of inhibitor) are effectively unmodified, having lytic activity and binding and structural properties all very similar to the corresponding properties of native enzyme.

Samples 4 and 2:1 on the other hand showed less evidence of binding of $(NAG)_2$, from the line width and position of the CH_3 signal associated with the reducing terminal NAG residue; this was more apparent with 2:1, which also showed less lytic activity than 4. It would thus appear, in agreement with the results discussed in the previous section, that loss of activity is associated with a loss of ability to bind oligosaccharides.

(c) Co^{++} binding, by p.m.r. spectroscopy

The work of McDonald and Phillips[175] has shown that Co^{++} binds to the Asp 52-Glu 35 region (site D) of the lysozyme active site, with an association

constant Ka = 57 l./mole. If $(NAG)_2$, binding to sites B and C in lysozyme were also added, large shifts and line-broadening effects should be observed for the methyl resonances of the disaccharide. A study of these, in solutions of varying Co^{++} concentrations, should enable both the determination of the exact cobalt position, and the redetermination of the binding constant of Co^{++} to the enzyme. The method could then be applied to the modified enzymes. On the assumption that these consist of simple mixtures of native and modified enzyme it can be shown (Appendix 2) that, if $[Co^{++}] < K_{Ni}$, (where K_{Ni} is the dissociation constant of Co^{++} and native enzyme), the fraction of the mixture that is modified may be calculated from the slope and intercept of a graph of $\frac{1}{\tau_{obs} - \tau_{enz}}$ against $\frac{1}{[Co^{++}]}$, where τ_{obs} is the observed chemical shift of an active site probe in the presence of cobalt at concentration $[Co^{++}]$, and τ_{enz} is the chemical shift of this probe in the presence of the same concentration of enzyme, but in the absence of cobalt.

The probe chosen was $(NAG)_2$, for the reasons explained in the previous section. As low a concentration as was acceptable for the p.m.r. work was considered advisable, as the effect hoped for will be greater at high ratios of bound:unbound disaccharide. The pH chosen was 6.0, as the data of McDonald and Phillips[175] show suitably large effects due to Co^{++} at this pH.

A buffered solution was used at first, although it was found that $[Co^{++}]$ up to 10^{-1} M had no effect on the pH of a 10^{-3} M solution of lysozyme. However acetate, which was considered the most suitable buffer salt, was found to be

unsatisfactory in the experiment as the presence of 10^{-2}M Co^{++} caused complete disappearance of the acetate resonance, presumably owing to binding of the metal ion. The $(NAG)_2$ methyl resonances were also considerably broadened at 10^{-2}M Co^{++}. As McDonald and Phillips[175] however used Co^{++} concentrations up to 1.5×10^{-1}M without serious line broadening effects on the p.m.r. signals it seems possible that Co^{++} also binds to the disaccharide.

Unbuffered solutions were therefore used, at pD 6.40, with $(NAG)_2$, (5.0×10^{-2}M), and native lysozyme, (0.72×10^{-3}M), no effect was seen on the $(NAG)_2$ methyl resonances with added Co^{++} up to 1.9×10^{-2}M, except the general broadening referred to above. At this $(NAG)_2$ concentration the enzyme is more than 99% saturated, but the ratio of bound to free disaccharide is 1:68. In order to magnify any possible effects on the methyl resonances a lower $(NAG)_2$ concentration, 5×10^{-3}M, was then used. With this, although the enzyme is still 98% saturated, the ratio of bound to free $(NAG)_2$ was 1:5.6. Although, with the aid of spectrum accumulations, the methyl resonances in the presence of 0.76×10^{-3}M lysozyme could still be seen in the absence of Co^{++}, addition of as little as 0.5×10^{-3}M Co^{++} caused disappearance of the higher field signal, (due to the acetamido methyl group on the reducing terminal sugar residue); this signal was almost certainly lost by line broadening.

The fact that the lower field methyl resonance, due to the methyl group on the non-reducing terminal NAG residue, is not affected by Co^{++} nearly as much

as the higher field signal in this experiment suggests that the desired effect is being observed, as the methyl group responsible for the higher field signal would be much closer to the Co^{++} in an enzyme-Co^{++}-$(NAG)_2$ complex. However the operation of opposing effects results in experimental difficulties; high Co^{++} concentrations, with suitably large perturbation of the $(NAG)_2$ resonances, may not be used, owing to the general line broadening effect. Because therefore only small effects may be studied, the $(NAG)_2$ concentration must be kept very low, (but not below the value of 1/Ka), to maximise the ratio of bound to unbound material, and then the enzyme spectrum obscures the resonance to be studied.

4. Ion-exchange chromatography

The cation-exchange resin Biorex 70 has been used successfully in the separation of modified forms of lysozyme from the native enzyme by several workers,[13,92,102,156] and chromatography on this resin, in pH 10 borate buffer, with a gradient of salt concentration, was found to be a very satisfactory way of purifying the modified enzymes prepared here. Native Worthington enzyme was eluted as a single peak in this system, but the native Seravac enzyme used was found to contain at least five components, the major one (58% of total) having similar chromatographic properties to Worthington enzyme.

The products of inhibition showed a component (C) which was eluted later than native enzyme (B), i.e. was of a more 'cationic' nature than native lysozyme;

in most cases there was also a third peak (A) which was eluted earlier than native enzyme. Attention was paid in particular to the less acidic modified enzyme (C), as it was hoped that this would be the material modified at Asp 52. Rechromatography of this component, after its large scale separation, revealed that it too contained three components, (C1, C2 and C3), and the major one, C2, (ca. 76% of the total) was separated and purified. Although this component showed apparent very low activity against M. lysodeikticus it is considered to be an inactive derivative as the assay method is not reliable below 2% activity, and in any case a very small amount of contamination by native enzyme is still possible. The A component however had approximately 50% of native activity.

The chromatography of the product 2:1 deserves special mention, as this material had been used extensively in earlier peptide and binding studies. This showed that 2:1 consisted mostly of the C component (71%), contaminated with a product which was similar to native enzyme in chromatographic behaviour but which only had 5% of native activity. Earlier results obtained with the product 2:1 therefore reflect quite closely the nature of the modified material C.

5. Binding of NAG and $(NAG)_3$ to pure modified enzymes

The fluorimetric method of Rupley,[64] which takes into account the correction necessary in the free saccharide concentration, (used in the data analysis), for the amount bound to the enzyme, was used. The binding constant determined

for $(NAG)_3$ and native Worthington enzyme was found to be in close accord with the literature value under these conditions (log Ka = 5.05; Lit: 5.09[64]).

Modified enzyme A bound $(NAG)_3$ less strongly than native enzyme, (log Ka = 4.54), and its λ_{max} of emission was 4 nm. higher, but C2 did not bind the trisaccharide at all, and showed fluorescence properties similar to those of native enzyme when saturated with $(NAG)_3$ or NAG, namely a shift of λ_{max} to a shorter wavelength and a decrease in fluorescence intensity, which were quantitatively similar in these two species. Modified enzymes C1 and C3 clearly did not bind $(NAG)_3$ like native enzyme, although small fluorescence perturbations were observed when $(NAG)_3$ was added to these materials, corresponding to binding constants of approximately the same magnitude as with native enzyme. These probably reflect contamination by native enzyme in the case of C1, but such contamination is unlikely in the case of C3 from its chromatographic behaviour, and it is possible that true binding, associated with smaller fluorescence changes than with native enzyme, is being observed. However it should be kept in mind that the fluorescence measurements were made at the limit of the instrument's sensitivity.

Studies of the binding of NAG to native lysozyme gave a log Ka value of 1.20, (Lit: 1.18-1.30, pH 5.4;[59] 1.22-1.40, pH 5.0[57]); modified enzyme C2 provided another example of the situation where the series of spectra ran differed from each other by little more than the instrumental variation on several spectra

of the same sample. One can only say that there was a suspicion of binding, (log Ka ca. 1.2), between NAG and C2, although this was not associated with a change in λ_{max} of emission. As such a shift was observed when NAG bound to native enzyme, binding to C2 if it does occur must be taking place in an unusual way.

To summarise, modified enzyme A contains tryptophan residues in a more hydrophilic environment than in the native enzyme, and binds $(NAG)_3$ less well than native. Modified enzyme C2 contains a modification which results in tryptophan residues being shifted to a more hydrophobic environment, as occurs when saccharides bind to native enzyme. The fluorescence properties of C2 would be adequately explained by the presence of a NAG residue, from the inhibitor, irreversibly attached in site C of this modified enzyme.

6. Holmium binding studies with pure modified enzymes

It has recently been shown by X-ray crystallography that gadolinium, (Gd^{++}), binds to lysozyme quite specifically in site D.[176] The metal ion binds between Asp 52 and Glu 35, and is shifted slightly towards Asn 44 and Asn 46. The latter residues may contribute slightly to the binding strength but the main interactions are clearly with the two carboxylic acid residues.

The binding of divalent gadolinium, holmium, and europium ions is at present being studied by p.m.r. spectroscopy,[169] and it has been shown that when

these bind to the active site large changes occur in the aromatic and high field regions of the enzyme p.m.r. spectrum. Although individual resonances cannot be assigned, the changes are such that binding constants may be estimated. These were found to be quite similar for the three rare earth ions studied, (the Ka value for Ho^{++} being 80 ± 10 l./mole at pD 5.5-5.9).

This perturbation of the enzyme p.m.r. spectrum by Ho^{++}, Gd^{++}, or Eu^{++} provides a ready method for screening the pure modified enzymes prepared here for modification of carboxylate groups in site D. Modified enzyme A was shown in this way to possess similar Ho^{++} binding properties to native enzyme, and thus Asp 52 and Glu 35 are unmodified. However modified enzyme C2 and the minor component C3 possessed no Ho^{++} binding ability, confirming that the C component represents the Asp 52 modified material studied earlier.

7. Crystallisation of pure modified enzyme C2

The technique of X-ray crystallography would seem to be very suitable for active site study of C2 and attempts were therefore made to grow crystals of this modified enzyme in a form suitable for diffraction work. C2 was successfully crystallised many times, but either gave very small tetragonal crystals or larger needles. It was hoped that the needles (1.0 x 0.1 mm.) might be large enough to determine crystallographically at least whether they were of the same space group as the tetragonal or triclinic native crystals which have been studied in

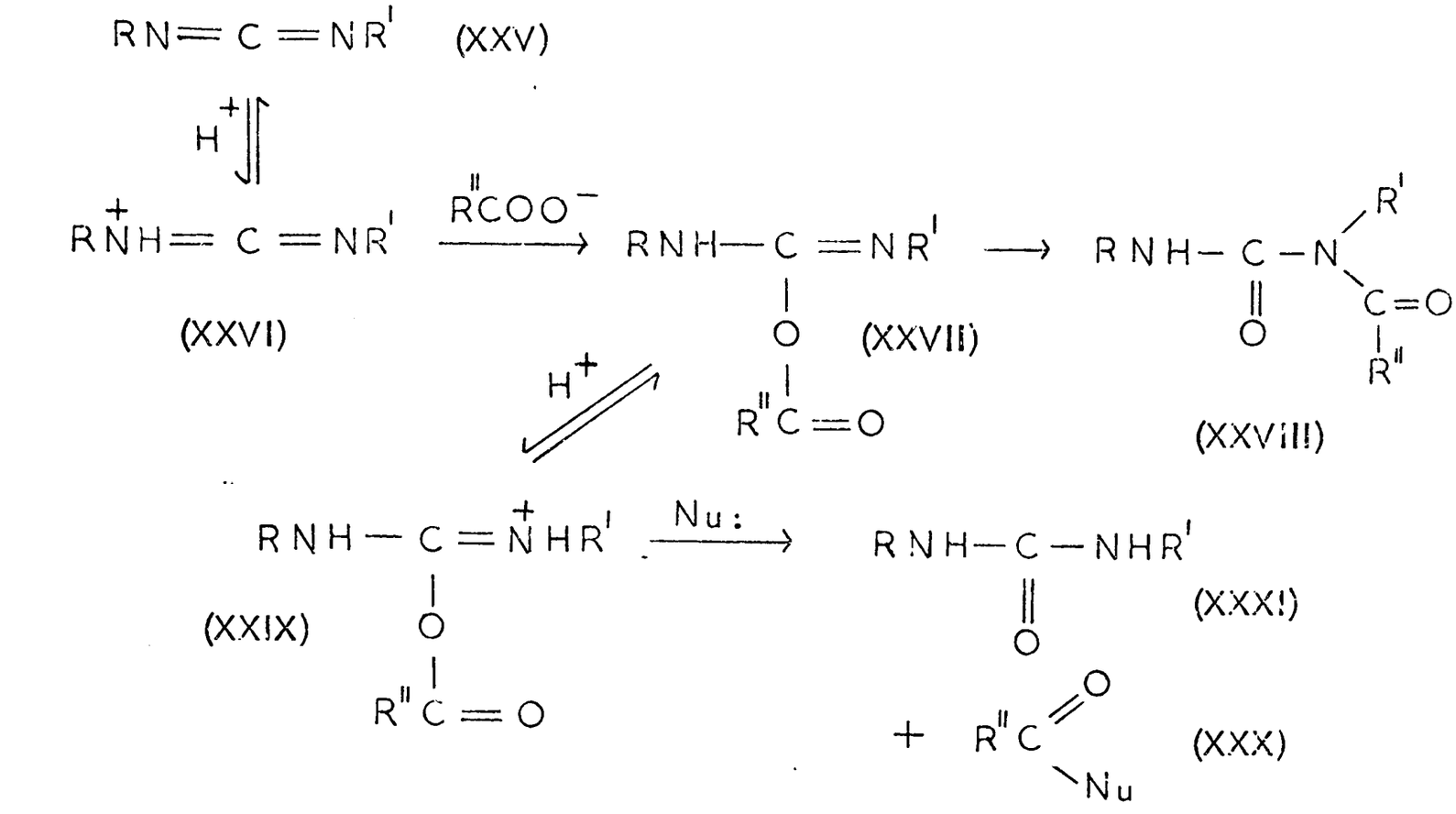
detail, in which case it would be a relatively simple problem to obtain a difference Fourier map of modified compared with native enzyme. In a preliminary study however the X-ray diffraction spots observed from one of the needles were so weak that the correct orientation of the crystal for data collection could not be determined. Further attempts are to be made to obtain data from these crystals.

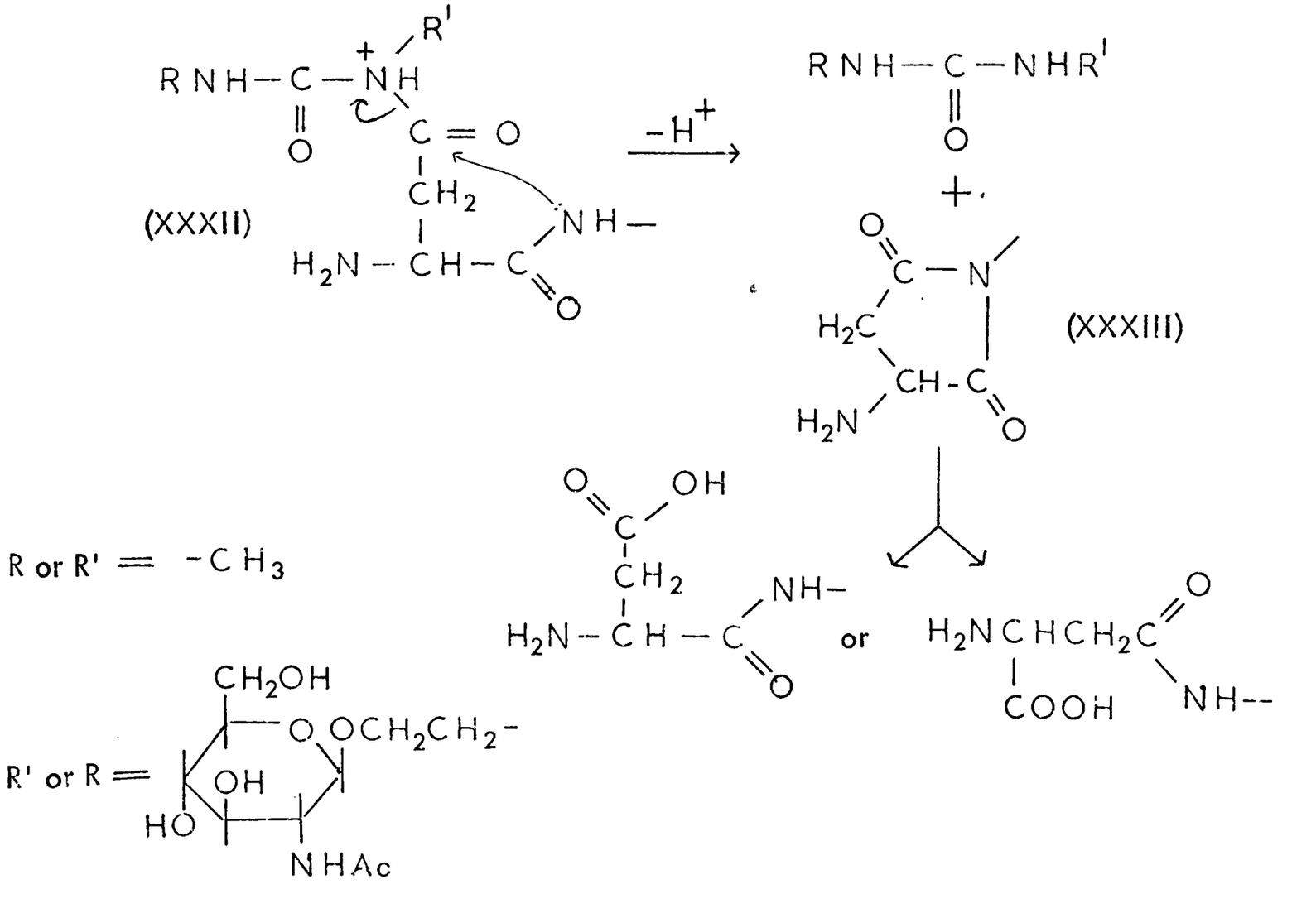
CHAPTER 4: THE MODE OF INHIBITION OF LYSOZYME BY THE ACTIVE-SITE DIRECTED CARBODIIMIDE

The reaction of carbodiimides with carboxylic acids[124] (figure 18) is thought to be initiated by protonation of the diimide, (XXV) $\longrightarrow$ (XXVI), followed by attack by the anion of the acid to give the O-acyl isourea (XXVII). This may then rearrange *via* a cyclic transition state to the N--acyl urea (XXVIII), or, if the intermediate (XXVII) is again protonated, to (XXIX), this may react with a nucleophile Nu:, to give a carboxylic acid derivative (XXX) and the N,N'-disubstituted urea (XXXI). In basic conditions the equilibria (XXV) $\rightleftharpoons$ (XXVI) and (XXVII) $\rightleftharpoons$ (XXIX) are shifted to the left and thus overall reaction is slower and the formation of the derivative (XXX) is further inhibited; the competition between the reactions leading to (XXVIII) and (XXX) is also affected by the nature of the substituents R, R', and R".[124]

A variety of products (XXX) is however possible, according to the nature of the nucleophile, Nu:. In the case of reactions of carbodiimides with mono-carboxylic acids alone, the carboxylate ion may take the part of the nucleophile, but of more interest here are reactions in the presence of other nucleophiles. When ammonia or an amine is present, primary and substituted amides may result, and alcohols yield ester derivatives of the acid. Intramolecular reactions are also commonly observed with carboxylic acids containing other functional groups; for example hydroxy-acids may yield lactones. Enolic OH groups may also act

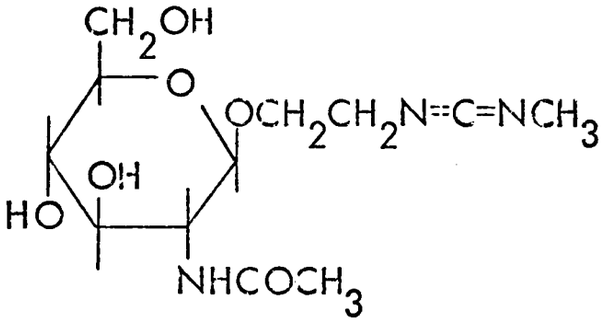
Figure 18


RN=C=NR'
(XXV)
H+
RNH=C=NR'
(XXVI)
RCOO−
RNH—C=NR'
O
R"C=O
(XXVII)
R'
RNH—C—N
C=O
R"
(XXVIII)
H+
RNH—C=NHR'
O
R"C=O
(XXIX)
Nu:
RNH—C—NHR'
O
(XXXI)
+ R"C
O
Nu
(XXX)


R'
RNH—C—NH
O
C=O
CH2
NH—
H2N—CH—C
O
(XXXII)
−H+
RNH—C—NHR'
O
+
O
C—N
H2C
CH-C
H2N
O
(XXXIII)
R or R' = −CH3
O
OH
C
CH2
NH—
H2N—CH—C
O
or
O
H2NCHCH2C
COOH
NH—
CH2OH
O
OCH2CH2−
R' or R =
OH
HO
NHAc

as nucleophiles in the reaction displacing the urea, and it is even possible for the carbonyl oxygen of an amide to take the part of the nucleophile, although this requires the presence of a strong base. It has now also been shown, (p. 81), that a suitably oriented amide group may couple intramolecularly with a carboxylic acid under non-basic conditions to give the imide, (figure 8, p. 51).

The inhibition of lysozyme by the carbodiimide (VIII) has been shown to



(VIII)

be virtually independent of the presence of added nucleophile, and therefore some sort of intramolecular reaction must occur in this process. Possible causes of inactivation of the enzyme on inhibition may thus be summarised as

(a) irreversible attachment of the inhibitor, probably via an O-- to N-acyl migration, and

(b) blocking of the catalytic or binding sites, or both, of the enzyme, by covalent linkage of carboxylic acid residue(s) with neighbouring nucleophile(s). If the inhibitor binds as expected, with the sugar residue in site C and the carbodiimide side chain extending down the cleft to the Asp 52/Glu 35 region, the initial protonation (XXV) ⟶ (XXVI) and formation of the intermediate (XXVII)

may be assisted by Glu 35, which would be unionised at the pH of the experiment, (4.75).

The speed of a second protonation, (XXVII) ⟶ (XXIX), promoting attack by a neighbouring nucleophile on the activated carboxyl group would then largely determine whether subsequent reaction led to a rearranged product or not. It is clearly impossible to predict which route would predominate, although it may be pointed out that, if Glu 35 is responsible for the initial protonation of the carobdiimide, the presence of a now ionised carboxylate group might reduce the effect of the second protonation, (from the solvent), to such an extent that two protonations, one of Glu 35 and one of (XXVII), may be needed to promote nucleophilic attack on the latter. There are however in the catalytic region of the enzyme several groups which could possibly act as nucleophiles. In particular Asn 59 (which is thought to hydrogen-bond to Asp 52) and two other asparagine residues, Asn 44 and Asn 46, are close to Asp 52, and the possibility of participation by a main chain amide grouping may also not be ruled out.

Although the inhibition was found in some cases to give rise to a product or products (A) of more acidic behaviour than the native enzyme (B) on ion-exchange chromatography, attention was confined mostly to the less acidic modified enzymes (C) which were invariably formed in the reactions. The isolation of an active site peptide containing a modified Asp 52 residue from a mixture of C (71%) and other material (29%) in 25% overall yield, after elution from paper, confirmed that at

least the major component of C (C2) contained this modified aspartic acid; the failure of C2 and C3 to bind holmium (II) also confirmed a modification in the Asp 52/Glu 35 region for these modified enzymes.[169]

Furthermore, the degree of inactivation of the enzyme on inhibition was found to be associated with a loss of ability to bind small oligosaccharides. C2, which was completely inactive, did not bind $(NAG)_3$ at all, and showed fluorescence properties similar to those of native enzyme when saturated with NAG or $(NAG)_3$; this, in association with the knowledge that Asp 52 is modified, is strong evidence for the irreversible attachment of the inhibitor in C2. There are however two ways in which intramolecular reactions alone could account for the observed properties of this modified enzyme, without the product containing such an attached inhibitor residue. These possibilities are firstly, that both Asp 52 and a carboxyl group in the binding site are intramolecularly modified by a neighbouring nucleophile, and secondly, that modification of Asp 52 alone in this way causes a conformational change in the A-B-C sites resembling that induced by substrate and preventing $(NAG)_3$ from binding. The peptide studies carried out suggest that the former of these possibilities is unlikely, and the latter would also seem improbable from consideration of the position of Asp 52 in relation to the A B C binding sites.

It may thus be concluded with some assurance that C2 contains an inhibitor residue bound in some way at Asp 52. Although the peptide isolated which

contained this modified aspartic acid possessed a C-terminal tyrosine residue, despite being formed by tryptic digestion of the modified enzyme, the presence of other chymotryptic peptides was suggested in the peptide maps prepared, and it is possible that the rate of chymotryptic cleavage at Tyr 53 might be increased by the presence of a bulky side chain on the neighbouring Asp 52 residue. It was also surprising to find that amino-acid analyses of acid-hydrolysed and LAP-digested samples of the peptide invariably showed Asp contents in accord with the liberation of Asp from the modified form, although it had been conclusively proved that this residue was modified before digestion. This is readily acceptable in the case of acid hydrolysis, but in the case of enzymic digestion is more unexpected. N-Terminal asparaginyl peptides are known however to undergo a rearrangement to β-aspartyl peptides,[177] and it is possible that the same sort of neighbouring group displacement, (figure 18, (XXXII) ⟶ (XXXIII)), may lead to the free acid during the course of LAP digestion of the peptide. The leaving group, an N,N'-disubstituted urea, is in this case a good one, but if the imide (XXXIII) is formed this would be expected to cleave equally in both possible directions, in which case the dipeptide βAsp-Tyr should also be formed in the digestion. It is conceivable however that the unexplained peak in the analyses at the position of alanine could be due to βAsp-Tyr. Other types of intramolecularly assisted expulsion of the leaving group which would not involve the formation of a β-aspartyl linkage, (for example involving the phenolic group of the neighbouring

tyrosine residue) may also be postulated, but are less likely.

If indeed an O ⟶ N-acyl migration has led to irreversible attachment of the inhibitor in modified enzyme C2, there are two possible nitrogen atoms to which such a migration may occur, and it might be expected that two products would result. Migration is known to occur preferentially to the less basic nitrogen atom,[124] and although in the case of inhibitor (VIII) little difference would be predicted between the two N-atoms, that attached to the methyl group should be slightly less basic and thus be the preferred acceptor for the acyl group. Although the two possible derivatives may have identical ion-exchange chromatographic properties, it seems more likely that one of the modified enzymes C1 or C3 represents the second isomer. C1 is more likely, although formed in very low yield, as C3 showed more evidence of $(NAG)_3$ binding ability, and may contain a modification not involving the irreversible attachment of the inhibitor. However the low yield of this material prevented more detailed studies.

The modification in product A, though not involving Asp 52 or Glu 35, remains undetermined although it is tentatively suggested to be at a binding site. From the $(NAG)_3$ binding constant determined it may be calculated that the binding energy of $(NAG)_3$ is ca. 0.6 Kcal./mole less for A than for native enzyme. If such binding energy difference is the same in productive enzyme-substrate complexes it would account entirely for the observed lower rate of lysis of M. lysodeikticus cell-walls, (40-50% of native).

To summarise, modification of Asp 52 with the active site directed inhibitor (VIII) leads to a product which in all probability contains the inhibitor irreversibly attached through an N-aspartyl linkage. As such a modification also blocks binding sub-site C, and as no ready method is available for the removal of the inhibitor molecule, the derivative is unsuitable for the study of the function of Asp 52 in the catalytic mechanism of lysozyme.

However X-ray crystallographic studies of the derivative (C2) should be of interest, and may provide insight into the degree of flexibility of the Asp 52/ Glu 35 region. For example if it is shown that there is a possible conformation of this region which is quite different from that predicted by model building of productive enzyme-substrate complexes,[51] catalytic mechanisms which may be ruled out on the basis of the inflexibility of this region may again become acceptable.

Furthermore it would be of great interest to use the derivative in studies of the binding properties of sub-sites A, B, E and F. It is expected from the enzyme fluorescence properties reported here that the Asp 52-modified enzyme C2 is in the 'induced-fit' conformation revealed by X-ray crystallographic work on the lysozyme-NAG complex.[51] Sites E and F may not be appreciably conformationally altered however. There is some evidence that site E binds NAG residues almost as well as site C,[12,55] and it is significant that an indication of the binding of NAG to the modified enzyme C2 with approximately the same

association constant as with native enzyme has been obtained here. Further study of this binding, which might not be observable for lysozyme containing a free site C if the latter possesses even only a slightly greater affinity for NAG, would be valuable. It is also of interest to note that NAG binding to site E could not have been observed under the conditions of the X-ray crystallographic work on the lysozyme-NAG complex, as the crystal packing of the tetragonal crystals used causes this region to be inaccessible to NAG molecules diffused into the crystals.[21] Such binding studies could profitably be extended to other mono- and di-saccharides in order to explore more fully the binding properties of sites E and F, whose predicted combined affinity for NAG residues is in excess of that of the only other two adjacent sites available for disaccharide binding (A and B), when sites C and D are blocked. A more accurate knowledge of these binding properties, in association with the known productive binding constant for $(NAG)_6$ should enable a better estimate to be obtained of the negative contribution to binding of site D and hence of the degree of distortion of a sugar residue bound in this site.

APPENDIX 1

The determination of binding constants from fluorescence data

Symbols: E, S refer to the total concentration of enzyme and saccharide.

[E], [S] and [ES] refer to the concentrations of free enzyme, free saccharide and enzyme-saccharide complex respectively

Ka is the association constant and equals $\frac{[ES]}{[E][S]}$.

F_o, F, and F_∞ refer to the fluorescence intensities at a particular wavelength of the enzyme alone, the enzyme in the presence of saccharide at concentration [S], and the enzyme when saturated with saccharide respectively.

(n.b. The symbols ΔF and ΔF_{max}, representing $(F_o - F)$ and $(F_o - F_\infty)$ respectively, have also been used in the text for the sake of simplification).

(a) Method of Chipman et al.[59]

$$Ka = \frac{[ES]}{[E][S]} = \frac{[ES]}{(E - [ES])[S]} \quad \text{and hence} \quad [ES] = \frac{EKa[S]}{1 + Ka[S]}$$

If $[ES] \ll S$, $[S] \approx S$ and $[ES] = \frac{EKaS}{1 + KaS}$

$$\frac{(F_o - F)}{(F_o - F_\infty)} = \frac{[ES]}{E} = \frac{KaS}{1 + KaS}$$

and $$\frac{1}{(F_o - F)} = \frac{1}{(F_o - F_\infty)} \cdot \frac{1}{KaS} + \frac{1}{(F_o - F_\infty)}$$

Thus the intercept on the ordinate of a plot of $\frac{1}{(F_o - F)}$ against $\frac{1}{S}$ gives $\frac{1}{(F_o - F_\infty)}$ and hence F_∞. Ka may be determined from the gradient of this plot or alternatively from a plot of $\log \frac{(F_o - F)}{(F - F_\infty)}$ against log S as:

$$\frac{(F_o - F)}{(F - F_\infty)} = \frac{[ES]}{[E]} = KaS$$

and $$\log \frac{(F_o - F)}{(F - F_\infty)} = \log Ka + \log S$$

The slope of such a plot is unity for 1:1 complex formation and the value of $\log S = pKa$ at $\log \frac{(F_o - F)}{(F - F_\infty)} = 0$.

(b) Method of Rupley[64]

This method is essentially the same as that above.

$$\frac{(F_o - F)}{(F_o - F_\infty)} = \frac{Ka[S]}{1 + Ka[S]} \quad \text{if } S \neq [S]$$

$$(F_o - F) = (F_o - F_\infty) - \frac{(F_o - F)}{Ka[S]}$$

A plot of $(F_o - F)$ against $\frac{(F_o - F)}{[S]}$ would give an accurate value of Ka from the gradient. To a first approximation if S values are used instead of [S] values, the

intercept on the ordinate gives a quite accurate value of $(F_o - F_\infty)$, and this is used to correct S for the amount of saccharide bound in an [ES] complex.

$$\frac{[ES]}{E} = \frac{(F_o - F)}{(F_o - F_\infty)}, \text{ and hence } [ES] = E\,\frac{(F_o - F)}{(F_o - F_\infty)}$$

A second plot of $(F_o - F)$ against $\frac{(F_o - F)}{(S - [ES])}$ then gives a more accurate Ka value from the slope.

APPENDIX 2

The determination of Co^{++} binding constants from p.m.r. data and of the composition of simple mixtures of native and modified enzymes

The chemical shift of a probe, e.g. an acetamido methyl group on a saccharide residue bound in site C, is measured in the presence of a fixed concentration of lysozyme and of varying concentrations of cobalt, $[Co^{++}]$.

Symbols: τ_{obs} = the observed chemical shift of the probe in the presence of cobalt (II) at concentration $[Co^{++}]$.

τ_{max} = the chemical shift of the probe which would be observed if the enzyme were fully saturated with Co^{++}.

τ_{enz} = the chemical shift of the probe in the presence of the same enzyme concentration [E], but no Co^{++}.

$K_i = \dfrac{[E][Co^{++}]}{[ECo^{++}]}$, where $[ECo^{++}]$ is the concentration of the enzyme-cobalt complex.

Native enzyme

$$\text{As } K_i = \frac{[E][Co^{++}]}{[ECo^{++}]} = \frac{(E - [ECo^{++}])[Co^{++}]}{[ECo^{++}]},$$

$$\frac{(\tau_{obs} - \tau_{enz})}{(\tau_{max} - \tau_{enz})} = \frac{[ECo^{++}]}{E} = \frac{[Co^{++}]}{K_i + [Co^{++}]}$$

$$\text{and } \frac{1}{(\tau_{obs} - \tau_{enz})} = \frac{K_i}{[Co^{++}](\tau_{max} - \tau_{enz})} + \frac{1}{(\tau_{max} - \tau_{enz})}$$

As in the analysis of fluorescence data (appendix 1), when $[ECo^{++}] \ll Co^{++}$, $[Co^{++}] \approx Co^{++}$. This approximation is valid with inhibitors which bind as weakly as Co^{++}, ($K_i = 1.75 \times 10^{-2} M$). Hence a plot of $\frac{1}{(\tau_{obs} - \tau_{enz})}$ against $\frac{1}{[Co^{++}]}$ gives K_i and $(\tau_{max} - \tau_{enz})$ from the gradient and intercept.

Modified enzymes

If the crude modified enzyme consists of a simple mixture of native and modified enzyme, where Y is the fraction of modified:

$$(\tau_{obs} - \tau_{enz}) = \frac{(1 - Y)(\tau_{Nmax} - \tau_{Nenz})[Co^{++}]}{K_{Ni} + [Co^{++}]} + \frac{Y(\tau_{Mmax} - \tau_{Menz})[Co^{++}]}{K_{Mi} + [Co^{++}]}$$

where subscripts N and M refer to native and pure modified enzyme respectively. If, as expected, $K_{Mi} \gg K_{Ni}$, i.e. native enzyme binds Co^{++} much better than modified, and if $[Co^{++}] < K_{Ni}$,

$$(\tau_{obs} - \tau_{enz}) = \frac{(1 - Y)(\tau_{Nmax} - \tau_{Nenz})[Co^{++}]}{K_{Ni} + [Co^{++}]}$$

$$\text{and } \frac{1}{(\tau_{obs} - \tau_{enz})} = \frac{K_{Ni}}{(1 - Y)(\tau_{Nmax} - \tau_{Nenz})[Co^{++}]} + \frac{1}{(1 - Y)(\tau_{Nmax} - \tau_{Nenz})}$$

Knowing K_{Ni} and $(\tau_{Nmax} - \tau_{Nenz})$ the gradient or intercept of a plot of $\frac{1}{(\tau_{obs} - \tau_{enz})}$ against $\frac{1}{[Co^{++}]}$ gives Y.

If $[Co^{++}] \gg K_{Ni}$ however, i.e. native enzyme is saturated,

$$(\tau_{obs} - \tau_{enz}) = (1 - Y)(\tau_{Nmax} - \tau_{Nenz}) + \frac{Y(\tau_{Mmax} - \tau_{Menz})[Co^{++}]}{K_{Mi} + [Co^{++}]}$$

and if $(\tau_{Nmax} - \tau_{Nenz}) = (\tau_{Mmax} - \tau_{Menz})$

$$(\tau_{obs} - \tau_{enz}) = \frac{(\tau_{Nmax} - \tau_{Nenz})(K_{Mi} + [Co^{++}] + Y[Co^{++}] - YK_{Mi} - Y[Co^{++}])}{K_{Mi} + [Co^{++}]}$$

$$= \frac{(\tau_{Nmax} - \tau_{Nenz})([Co^{++}] + K_{Mi}(1 - Y))}{K_{Mi} + [Co^{++}]}$$

and $$\frac{1}{(\tau_{obs} - \tau_{enz})} = \frac{K_{Mi} + [Co^{++}]}{(\tau_{Nmax} - \tau_{Nenz})([Co^{++}] + K_{Mi}(1 - Y))}$$

which is a much more complicated expression than that obtained when $[Co^{++}] < K_{Ni}$, i.e. when native enzyme is unsaturated; $[Co^{++}]$ values less than K_{Ni} should therefore be used.

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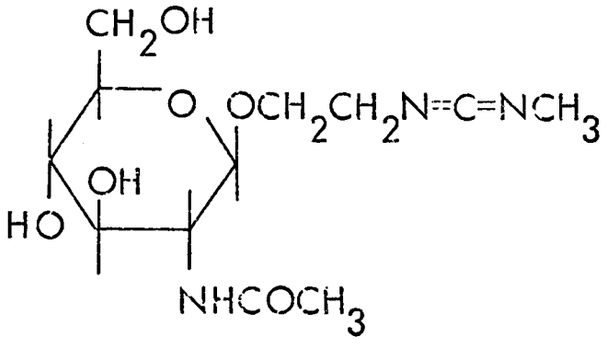
ABSTRACT

The three-dimensional structure of the glycosidase, hen egg white lysozyme, has been determined by X-ray crystallography;[1] the molecule is roughly ellipsoidal, with a cleft running down one side, and this cleft, which is capable of accommodating up to six sugar residues (in sub-sites A-F) is known to be the site of catalytic action, glycolysis occurring between sites D and E. The only significant enzyme functional groups in this region are the carboxylic acids Asp 52 and Glu 35, and it is generally accepted that Glu 35, which is in a hydrophobic environment and which would be expected to be unionised at the optimum pH for lysozyme action, acts as a general acid catalyst in the cleavage of the glycoside.

General acid catalysis alone is however not sufficient to account for the catalytic properties of lysozyme, and controversy exists over the source of the extra catalysis required. Asp 52, which has a low pK value and which would be ionised at the optimum pH for lysozyme activity, is an obvious choice of a residue which could participate in the mechanism, either by stabilising an intermediate carbonium ion by ion-pair formation, or by intramolecularly assisting expulsion of the aglycone, both after protonation of the glycosidic oxygen atom by Glu 35.[2,3] However, as the best substrates for lysozyme are polysaccharides of N-acetyl glucosamine and its derivatives, it is also possible that, with these substrates, the neighbouring acetamido group could intramolecularly assist glycolysis.[2,4]

Although detailed studies of the glycolysis and transglycosylation reactions catalysed by lysozyme have been made, the question of whether neighbouring group participation assists in the catalytic mechanism remains unresolved. The kinetic analysis of hydrolysis of normal substrates, (for example $\beta(1 \longrightarrow 4)$ linked polymers of N-acetyl glucosamine), is complicated by non-productive binding and transglycosylation, and use has to be made of model substrates such as aryl glycosides of N-acetyl glucosamine oligomers; although poor substrates these are hydrolysed with Michaelis-Menten Kinetics, but results obtained from them are subject to the criticism that one cannot be certain that they bind exactly as, or even are subject to the same mechanism of hydrolysis as normal substrates. Modification of the enzyme rather than the substrate is therefore a more satisfactory approach, and this thesis describes the attempted conversion of aspartic acid 52 to asparagine, a modification which should not affect the accessibility of the active site, but which should enable a more definite conclusion to be made as to the degree of involvement of this residue in the catalytic mechanism.

The reaction chosen for effecting this modification was that of the acid with a carbodiimide in the presence of ammonia, and in order that the modification would be specific for Asp 52 the diimide was incorporated into the compound (I) below, which was synthesised in eight stages. (I) should bind to lysozyme with its sugar residue in site C and with its C-1 side chain extending down the cleft to the Asp 52 region.



(I)

Reaction of lysozyme with this compound has been shown to be complex; inactivation of the enzyme was found to be independent of added nucleophile (and must therefore involve some intramolecular reaction or the irreversible attachment of the inhibitor), and a variety of products of more or less acidic nature than native enzyme was formed. The separation and purification of these modified enzymes by cation-exchange chromatography is described. The major product which was eluted before native enzyme in such a separation was unmodified at Asp 52 or Glu 35, and, as its lower rate of lysis of M. lysodeikticus cell walls than native enzyme (40-50%) could be accounted for entirely by a loss of binding ability, a binding site modification is suggested for this material.

The major modified enzyme of less acidic nature than native enzyme was shown to contain a catalytic site modification, and an active site peptide containing residue 52 was isolated. This residue was shown not to be aspartic acid by electrophoresis at pH 6.5, although the modification was labile to acid hydrolysis and leucine aminopeptidase digestion, both of which released aspartic acid. New methods for the separation and identification of asparagine by amino-acid analysis

and on paper are described, and the modified residue 52 was shown not to be asparagine by these methods.

The modification of aspartic acid 52 in lysozyme by the diimide I was shown to be associated with a loss of binding ability for small oligosaccharides, the product being completely inactive and showing no ability to bind tri-(N-acetyl)-glucosamine. Possible intramolecular reactions and modes of irreversible attachment of the inhibitor to Asp 52 are considered, and it is concluded that the latter is in all probability responsible for the inactivation of the enzyme.

The Asp 52-modified enzyme has been crystallised and it is hoped that X-ray diffraction data can be obtained from the crystals, in which case the nature of the modification should be confirmed. Although the fact that the derivative does not bind substrate makes it unsuitable for investigation of the function of Asp 52 in the catalytic mechanism the fact that it contains a blocked sub-site C (which is normally the strongest binding site) means that it could be used in valuable studies of the binding of saccharide residues to other sites, and in particular to sites E and F. Preliminary evidence as to the strong binding of N-acetyl glucosamine in site E has been obtained.

References (Abstract)

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