STUDIES ON THE CATALYTIC MECHANISM OF LYSOZYME

Thesis

by

Lois Elaine Hodgson Smith

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Abstract

Secondary α-tritium kinetic isotope effects have been utilized to probe the nature of the transition state in the lysozyme catalyzed hydrolysis of chitotriose. A general synthesis of specifically labeled chitin oligomers (in particular 1, 1', 1''-^{3}H-chitotriose, the substrate used in these studies) is described. Injection of Drosophila melanogaster larvae with labeled N-acetyl-D-glucosamine yields chitin, which can be hydrolyzed to give a range of chitin oligomers from chitobiose to chitoheptose. The value of k_H/k_T obtained for the lysozyme catalyzed hydrolysis of chitotriose was found to be 1.19. This result indicates very considerable carbonium ion character in the transition state, and thus the mechanistic alternatives for lysozyme hydrolysis become distinguishable.

There is evidence for the existence of a stabilized intermediate formed after the production of the carbonium ion in a post-rate limiting step. Three alternatives exist for this stabilized intermediate:

1) a carbonium ion stabilized to some degree by asp 52 (an ion pair)

2) an acylal formed with asp 52 (a covalent intermediate)

3) an oxazoline

Substrates of the form NAG-GLU- NO_2 have been found to undergo lysozyme catalyzed transglycosylation. Since these substrates are cleaved specifically between GLU and the aglycone, the possibility of an oxazoline intermediate can be eliminated.

A study of the variation with pH of the ratio $\frac{\text{transfer}}{\text{transfer + hydrolysis}}$ for lysozyme has yielded the titration curve of a group of pKa ~ 6.5. This has been assigned to glu 35. Because its pKa is unchanged from that in the unbound enzyme, the possibility of a highly charged intermediate can be eliminated. Therefore the stabilized intermediate must be an acylal or a tight ion pair.

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Alexander Fleming discovered in 1922 an enzyme which catalyzes the hydrolysis of the cell walls of certain bacteria. This enzyme--lysozyme--can be obtained from many sources including phage, most mamalian tissue, tears, papaya, and the white of birds' eggs.[1,2,3] The most commonly studied lysozyme is that from hen-egg white, and this is the enzyme for which the crystal structure is known.[4,5] Lysozyme from other sources such as bacteriophage varies markedly in sequence from that of hen-egg white but retains much of the same specificity. Other avian egg lysozymes differ only slightly from that of hen. The chemistry of these other enzymes has not been clarified to the extent of that of hen egg-white lysozyme.

Lysozyme is a basic protein (with an isoelectric point of 10.5-11) of molecular weight at pH 5.4 of 14,400 ± 100 (determined by ultracentrifugation). Determined analytically, the molecular weight is found to be 14,388 with the amino acid composition[6]:
Asp(21) Thr(7) Ser(101) Glu(5) Pro(2) Gly(12) Ala(12) Cyss(4) Val(6) Met(2) Ilu(6) Leu(8) Phe(3) Tyr(3) Lys(6) His(1) Arg(11)

Jolles (1963,1964)[7,8] and Canfield (1963, 1965)[9,10] have sequenced the enzyme's single chain of 120 amino acids as well as establishing the positions of the four disulfide bonds through chemical analysis. The sequencing results from the two groups agree well with each other. The few disputed amino acid residues have been resolved by the X-ray crystallographic data at 2Å resolution of

Blake et al. (1967). The primary structure of lysozyme is shown in Figure 1.

The X-ray studies of Blake et al. (1967) have established the tertiary structure to 2Å resolution. The protein is an ellipoid with dimensions of about 35 × 30 × 30Å. There exists a distinct cleft on one side. The acidic and basic side chains and other polar groups are distributed on the surface of the enzyme and the majority of non-polar side chains are located in the interior. The other hydrophobic groups are found on the surface and mainly in the binding cleft. These include one isoleucine, one valine, and three tryptophanes. Figure 2 illustrates the three dimensional structure of lysozyme.

There is a comparatively small portion of α-helix (three regions of ~10 amino acids each and a short piece of only 5 residues). There are several regions of extended conformation and large stretches of fairly irregular structure.

There have been a large number of chemical modification studies done with lysozyme, but little of this work has yielded concrete results. Modification of histidine does not affect the activity of the enzyme,[11] nor essentially does the modification of lysine residues with o-methyl isourea[11,12] The pH profile of the acetylated enzyme[13] shifts to lower pH and shows ~40% of normal activity towards cell walls. This result probably arises from the decreased basicity of the protein and the effects of this basicity change on the association of the negatively charged bacterial cell wall substrate. It was found that acetylated

lysozyme exhibited native activity towards an uncharged substrate-glycol chitin. The reaction of tyrosines with iodine[14] and acetyl imidazole[15] has also yielded fully active enzyme. This is of interest since asp-52 which is involved in the catalytic function of the enzyme is H bonded to tyr-53.

Koshland[15,16] and co-workers used a water soluble carbodiimide to react carboxyl groups with amines. All carbonyls except glutamic acid 35 were modified in this manner to yield an inactive enzyme. When substrate (chitin oligosaccharides) was included in the reaction mixture, aspartic acid 52 was partially shielded from reaction and the enzyme had 50% activity. When the enzyme was reacted a second time in absence of substrate, all activity was lost. These studies lend strong support to the hypothesis that asp-52 and glu-35 are important to the catalytic activity of lysozyme, as does the work done recently using triethyl oxonium fluoroborate as an esterifying reagent. Parsons et al.[11,17] achieved specific esterification of asp-52 with concomitant loss of catalytic activity. The binding of substrate however is only slightly inhibited.

Chemical modification of tryptophan residues has been carried out in a number of laboratories which resulted in loss of activity. The results suggest that at least one of the tryptophans is involved in binding or in maintaining structural integrity of the enzyme.

The effects of ionizable groups on the spectral properties of tryptophans have been reported.[21] There are two carbonyl groups of apparent pKa values of 3.15 and 6.20 which perturb the spectrum

of the tryptophans. Other titration studies show that of 11 titratable carboxyls, three are unavailable except under denaturing condition.[22,23] Some of the carboxyls have abnormal pKa values--two of pKa ~3.2 and one at the high value of 6.3. The amino groups titrate normally and the three tyrosyl phenoxy groups have a pKa (intrinsic) of 10.8--higher than normal.

The nature of the bonds cleaved by lysozyme has been found relatively recently. Salton (1952)[24] found that the substrate was located in the bacterial cell wall. The partial solubilization has been shown to involve degradation of the cell wall mucopolysaccharide whose sugar backbone consists of alternating residues of 2-acetamido-2-deoxy-D-glucose (NAG) and its 3-0-lactyl derivative N-acetyl-D-muramic acid (NAM) which have a β (1-4) linkage (Figure 3). Lysozyme cleaves this polymer by hydrolysis of the NAM C_1 glycosidic bond to give as end product the disaccharide NAG-NAM. The free carbonyl group of some of the NAM residues of the bacterial cell wall seems to form an amide bond to a highly cross linked peptide which adds structural stability to the cell wall[26] (Figure 3). It was also found that chitin, aβ (1-4) linear polymer of N-acetyl-D-glucosamine (GlcNAc) was a substrate for the enzyme.

Chitin digosaccharides have been used as substrates in most studies of the binding and catalysis of lysozyme. Chitiobiose, chitotriose, chitotetrose, chitopentose, and chitohexose are all substrates,[27] and Rupley[28] has found the relative rates of hydrolysis

of these compounds to be 0.003 : 1 : 8 : 4000 : 30,000 respectively, for substrate concentrations of 10^{-4} M.

Transferase activity has been found with chitin oligosaccharide substrates such that high molecular weight insoluble material is found in these reactions as well as NAG.[27,28,29] The transglycosylation reaction has been observed for cell wall oligosaccharide substrates as well.[26]

Blake and co-workers[5] studied the binding of several saccharides to lysozyme with difference Fourier X-ray techniques. The native enzyme and the enzyme inhibitor complexes are isomorphous. The X-ray studies indicate that there exists an extensive binding site in the cleft of lysozyme which can associate with up to six sugar residues. These subsites have been designated ABC DEF. Only sites ABC have been examined extensively with the X-ray analysis of the chitotriose lysozyme complex at 2Å resolution. The non-reducing end of the trisaccharide occupies site A, and the reducing end site C. The $\alpha + \beta$ anomers of GlcNAc also occupy site C though with different orientations. The stability of the chitotriose complex indicates that the binding mode ABC is unproductive and does not involve the enzyme's active site. Blake[5] et al. proposed the existance of sites DEF from careful model building studies and also suggested that the active site of hydrolysis was between D and E. If binding occurs in subsite D steric interaction forces the sugar residue out of its normal chair configuration into a half-chair in which C_6 may assume an axial position. The conclusion was also

reached that NAM cannot bind in subsite C for steric reasons NAG:NAG therefore binds in sites C and D (Figure 4).

Results obtained from nuclear magnetic resonance techniques have confirmed those from the X-ray techniques suggesting that the conformation of the enzyme is very similar in the crystal and in solution. Dahlquist and Raftery measured the dissociation constants for several lysozyme inhibitors and also found a measure of the magnetic environment of the binding subsite. It was found that β NAG, methyl-β NAG, and methyl-α NAG all bound with the same orientation in site C, at least with respect to the N acetyl methyl group, whose resonance was monitored. But α NAG bound in a different magnetic environment. The reducing terminal residue to the di and trisaccharide also binds in the same way as β NAG. Rupley[31] found that glucose, glucosamine, and maltose had no detectable binding indicating an absolute requirement for the acetamido group on the substrate. The C_1 hydroxyl would also seem important since α and β NAG bind with different orientations. Binding studies with the diastereomers of trans-1-acetamido cyclohexanol suggest that the C_3 hydroxyl is indispensible for binding while C_1 hydroxyl is not. The NMR binding data are found in Figure 5.

The binding constants (obtai ned from UV difference spectra at pH 5.3)[31] show an increase in binding strength going from NAG to chitotriose. Chitotetrose, chitopentose, and chitohexose bind similarly to the trisaccharide.

The binding constants are as follows:

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

Cell wall disaccharide binds in subsites BC with about the same free energy as chitobiose (-7.9 kcal/mole and -7.4 kcal/mole, respectively).[33] The cell wall tetrasaccharide binds in sites ABCD with less than -6.9 kcal/mole and the hexasaccharide binds with -8.6 kcal/mole. The decrease in binding strength of the cell wall tetramer compared to dimer can be attributed to the distortion of the sugar residue in site D to the half chain conformation.

The hypothesized distortion of the sugar bound to site D has been supported by recent evidence. The δ-lactone of D-gluconic acid has been shown to have a half-chain configuration.[34] A similar residue, the δ lactone of N-acetyl glucosaminic acid, was synthesized at the reducing end of chitotetrose in a recent study. The binding was found to be significantly greater than that of chitotriose or chitotetrose. This increase in binding was attributed to the half-chair conformation of the residue in site D. The C_6 carbon and C_6 hydroxyl group of the sugar residue in site D appear to be the steric reason for distortion.

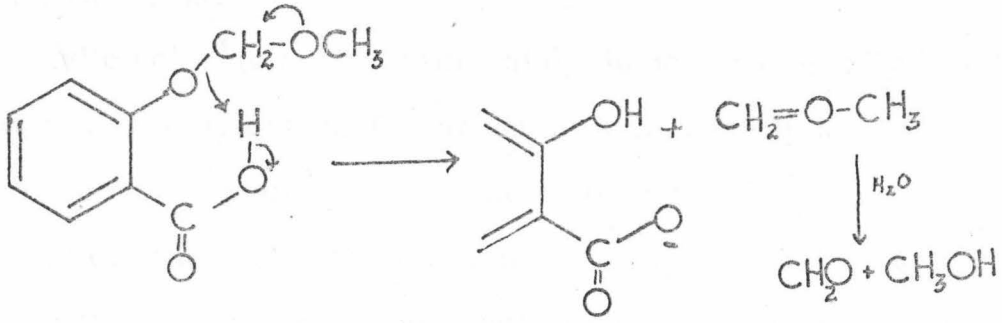
The pH dependence of binding of chitotriose has been studied.[32,35] The formation of the enzyme inhibitor complex was dependent upon two ionizations. One group changed from pK of 4.2 to 3.6 upon binding of trimer and the second group went from 5.6 to 6.3. These ionizations have been assigned to asp 101 and glu 35. The pKa of asp 52 has been measured as 4.5 with a difference titration of native lysozyme vs. the asp 52 ethyl ester.[36] The pKa of asp 52 is also probably observed in the binding study of β-Methyl NAG and in the pH-activity profiles of the lysozyme hydrolysis of some small substrates and inhibitors.[37-39] The ionization of asp 52 seems to be unaffected by substrate binding or inhibitor binding.[36] The pK of glu 35 exhibits a large shift to ~8.5 in the presence of glycolchitin, a high molecular weight substrate. X-ray studies have also suggested that asp 52 carboxylate interacts largely with other amino acids on the protein. The single interaction with the inhibitor through the glucose C-2 hydroxyl is very likely replacing a similar interaction with water in the unbound enzyme. Thus the environment of this residue remains constant during the binding of substrates.

There have been several mechanisms proposed to account for lysozyme catalyzed hydrolysis of glycosidic bonds. These proposals are based on the various mechanisms seen in the hydrolysis (or solvolysis) of acetals and glycosides (Figure 6).

The acid catalyzed solvolysis of acetals and simple glycosides are thought to proceed via a carbonium ion mechanism.[40] The first step involves equilibrium protonation of the acetal oxygen

followed by heterolysis of the carbon oxygen bond to produce a stabilized carbonium ion (Figure 6). Attack by solvent usually follows with the production of the hemiacetal.

There is no hard evidence for intermolecular general acid catalysis in simple ketals and acetals. However, there exists a case of intramolecular general acid catalysis. Capon and Smith[41] have studied the spontaneous hydrolysis of methoxymethyl salicylate at pH 4 and have suggested general acid catalysis is involved.



It has also been shown that o-carboxyphenyl-β-NAG undergoes intramolecular protonation.

These studies are relevant to the mechanism of lysozyme proposed by Blake from model building studies. The catalytic site was proposed to be between sites D and E on the enzyme where there exist two acidic residues glu 35 and asp 52 close to the substrate glycosidic bond. The two residues are in rather different environments and exhibit pKa's of 6.5 and 4.5. Blake suggested that a mechanism based on the formation of a carbonium ion at C_1 of the

sugar bound in subsite D. Electrostatic stabilization is provided by the carboxylic anion of asp 52 and steric stabilization by the configuration of the enzyme in subsite D which binds a sugar in the half chair conformation in preference to the full chair. Glu 35 in this scheme acts as a general acid, protonating the glycosidic oxygen of C_4 of the sugar in subsite E.

Other mechanisms are possible, however, based on different nonenzymatic hydrolysis of acetals and glycosides. Hydrolysis of glycosidic bonds in nonenzymatic systems can occur by acid, base, or nucleophilic catalysis.

In hydroxylic solvents nucleophilic catalysis does not usually occur. Bimolecular attack at the C_1 carbon does not usually take place under these circumstances unless the attacking nucleophile is very powerful or unless the attack is intramolecular. Basic hydrolysis of aryl glycosides probably involves intramolecular nucleophilic attack. Acetylated glycosyl halides also undergo intramolecular nucleophilic catalyzed hydrolysis.[43] The acetamido group at the C_2 carbon of NAG glycoside can also displace the aglycone to form an oxazoline intermediate. And the specific acid catalysed hydrolysis of methyl-β-NAG involves some acetamido group participation (Figure 7). Also the hydrolysis of p-Nitrophenyl-β-NAG occurs at a rate independent of pH between 1.5 and 10.5 while the α compound shows no spontaneous hydrolysis.[42,44,45] Bruice interpreted these results as indicating acetamido participation in the base hydrolysis but not in the acid hydrolysis because of steric hindrance.

Some solvolysis reactions involve acetamido participation in a limited way. The methanolysis of 2,3,4,6 tetra-o-methyl-α-D-glucopyranosyl chloride occurs via an A-1 carbonium ion mechanism with predominantly inversion at the C_1 carbon.[46] When chloride ion is added to the reaction mixture, product with retention of configuration increases. This has been attributed to the existence of an ion pair intermediate between the negative departing chloride and the positive pyranosyl cation. This intermediate is long-lived enough to interact with a solvent molecule resulting in inversion of configuration. Similarly the spontaneous hydrolysis of P-NO_2phenyl β-NAG has been explained in terms of an ion pair intermediate. The C_1 carbon-oxygen bond is broken before the transition state and the acetamido serves to force the p-nitrophenylate anion from the ion pair.[47]

Acetamido group participation has been suggested by Lowe[48] for the mechanism of lysozyme. Nucleophilic participation by a group or groups on the enzyme could also be involved in the mechanism of lysozyme. Koshland[49] postulated single and double SN_2 displacement mechanisms for the enzyme and a single displacement mechanism was proposed by Bernhart.[50] However, any mechanism proposed must take into account the results obtained by Dahlquist et al. in the study of the hydrolysis of chitobiose.[51] The reaction proceeds with at least 99.7% retention of configuration. This observation rules out the possibility of an odd number of nucleophilic displacements.

In base catalyzed hydrolysis of glycosides a hydroxyl at the C_2 carbon which is trans to the aglycone reacts more quickly than a C-2 hydroxyl which is cis.[52] A mechanism for this base catalyzed hydrolysis has been proposed involving initial removal of the C_2 hydroxyl proton followed by intramolecular nucleophilic displacement of the aglycon anion by the C-2 oxygen to yield the 1,2 eopxide.[53]

A summary of proposed mechanisms for lysozyme is given in Figure 8. As mentioned previously, any mechanism must take into account that the reaction proceeds with retention of configuration. This rules out any single displacement mechanism and restricts any suggested carbonium ion mechanism to one involving stereospecific attack by the solvent (or sugar as in trans-glycosylation reactions) on the carbonium ion.

Glucosidic and 2-deoxyglucosidic bonds are susceptible to lysozyme catalyzed hydrolysis. This suggests that the acetamido group is not absolutely necessary for catalysis. However, its contribution cannot be analyzed since there is no way to compare the catalytic constants for the β glucoside and corresponding β glycosaminide since the relative strengths of the catalytic and unproductive binding modes are unknown. But it seems likely that the mechanism for lysozyme glucoside hydrolysis will reflect the glycosaminide mechanism except for the part played by the acetamido group.

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Figure 1 - Primary structure of hen egg white lysozyme from J. Jolles, B. Jauregui-Adell and P. Jolles, C. r. hebd. Acad. Sci. Paris 258 3926 (1963).

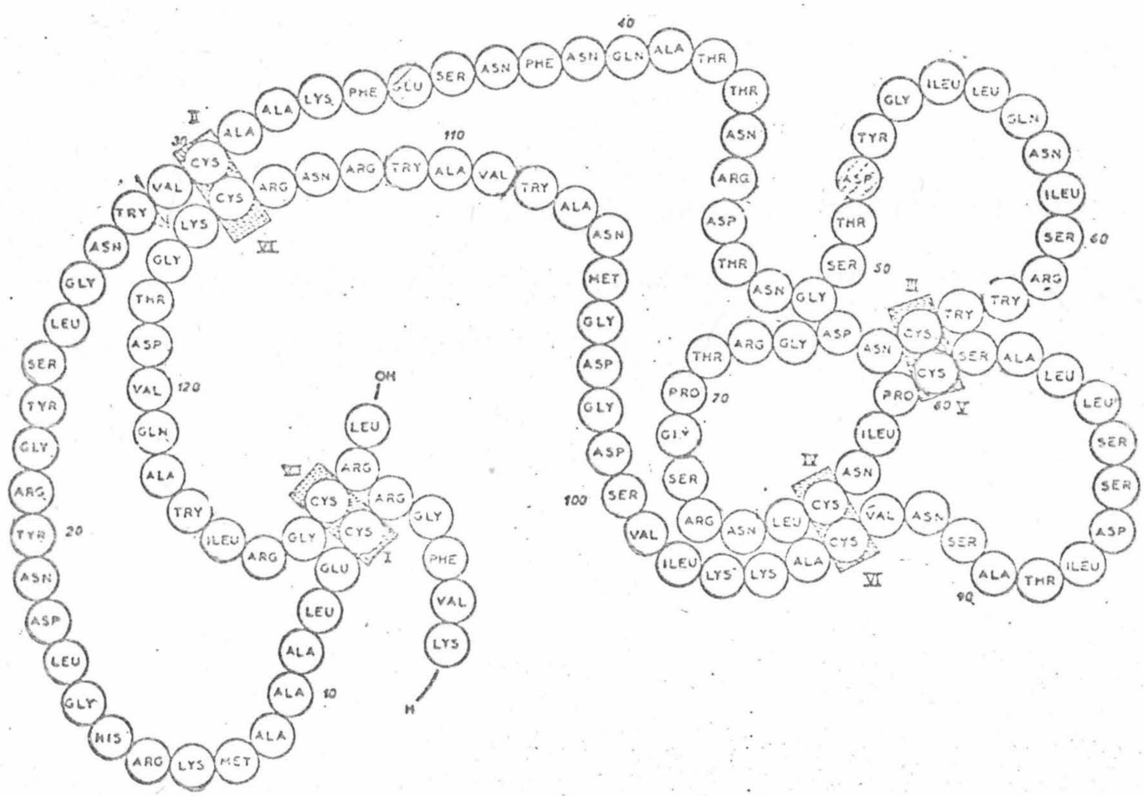
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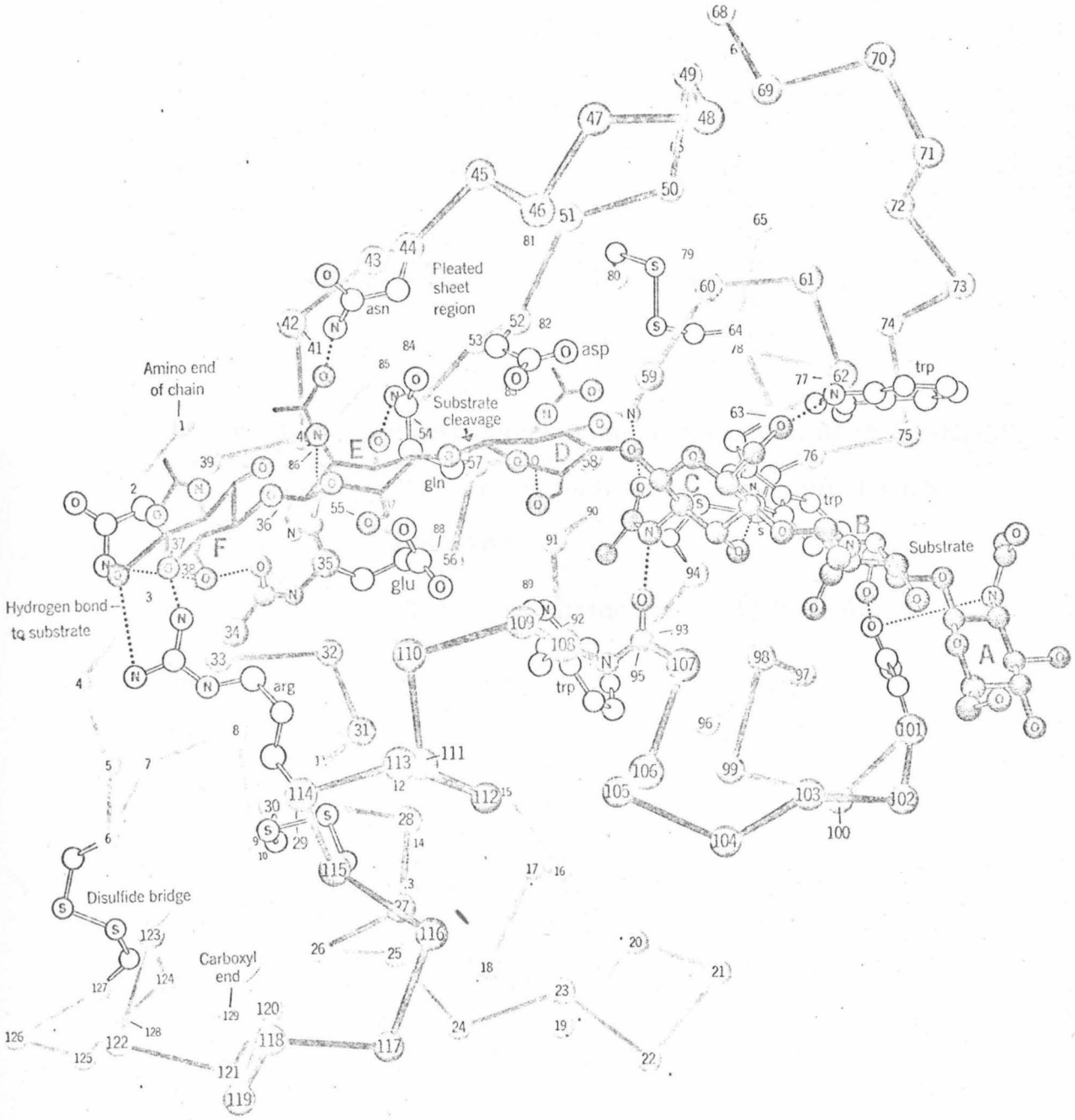
Figure 2 - The three dimentional structure of lysozyme.
from "The Structure and Actions of Proteins"
R. E. Dickerson and I. Geiss, Harper and Row, N. Y. (1969)


Amino end of chain
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Substrate cleavage
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Hydrogen bond to substrate
arg
Disulfide bridge
Carboxyl end
A
B
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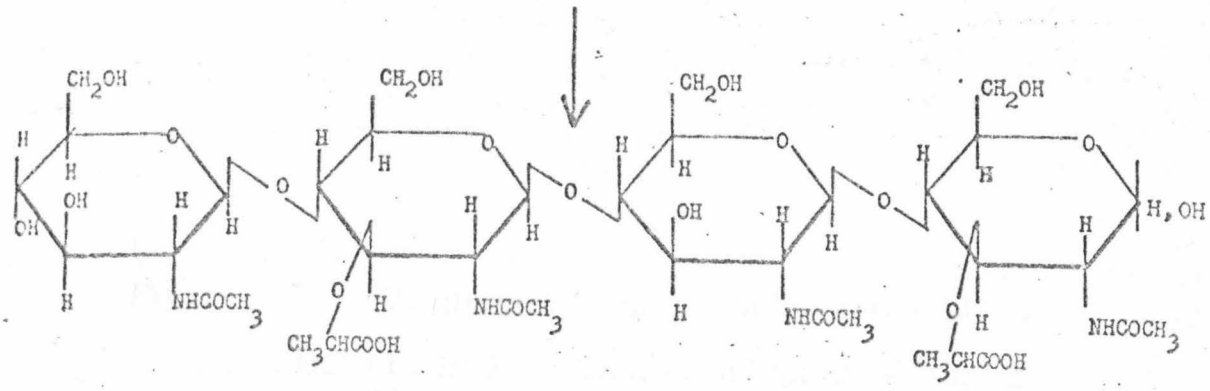
Figure 3 - A) Tetrasaccharide from cell wall $(NAG\text{-}NAM)_2$ The arrow indicatescleavage point with lysozyme.

B) Tetrasaccharide from chitin (NAG_4).



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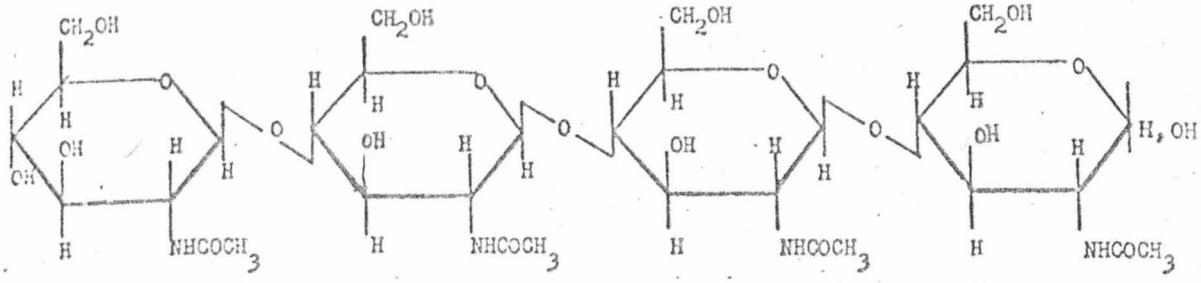

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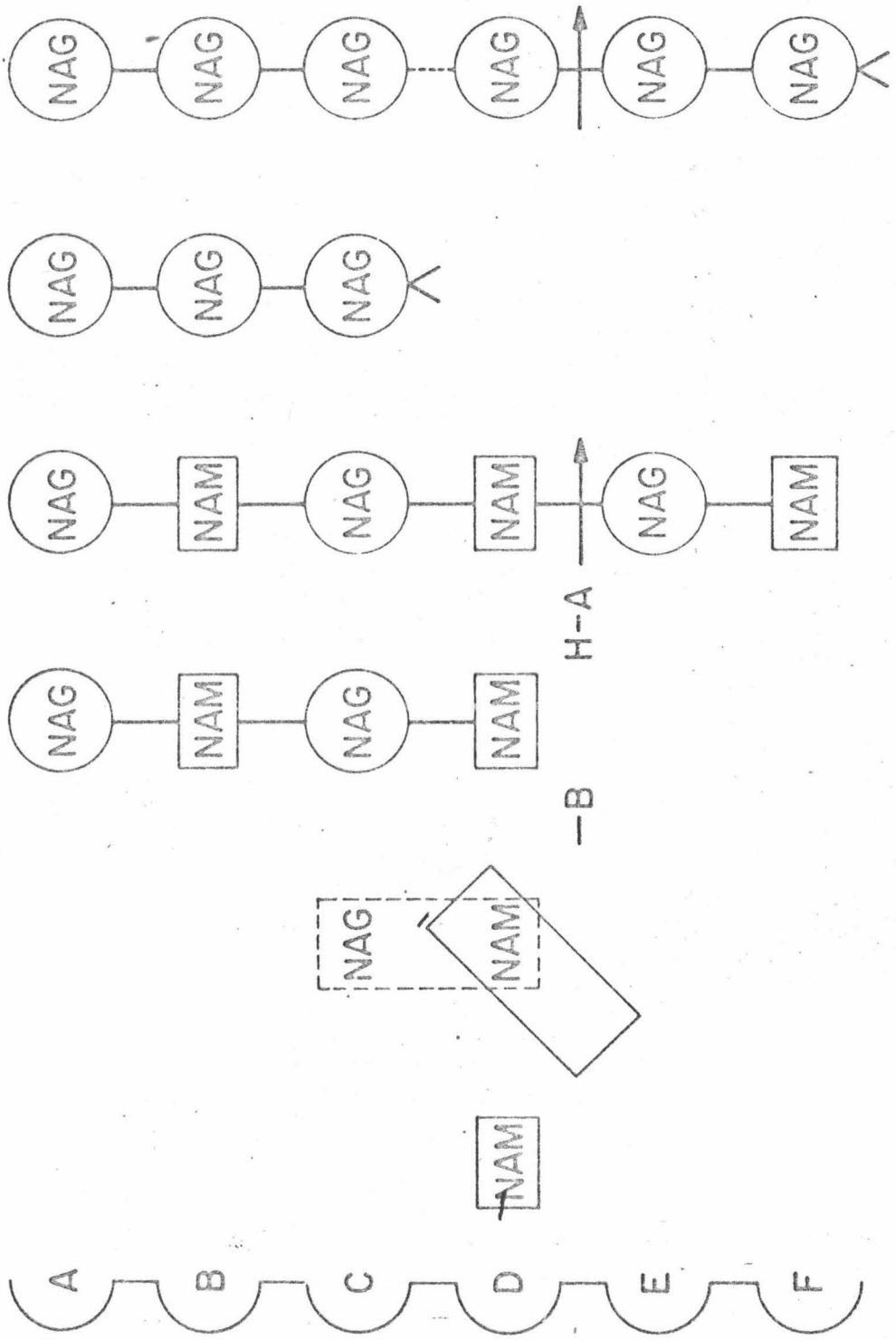
Figure 4 - The proposed binding in the active site as deduced by Blake [4] from model building studies.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B
C
D
E
F

Figure 5 - Scheme for the relative modes of binding with lysozyme of various saccharide inhibitors and substrates.

from F. W. Dahlquist, and M. A. Raftery, Biochemistry 8 713 (1969).

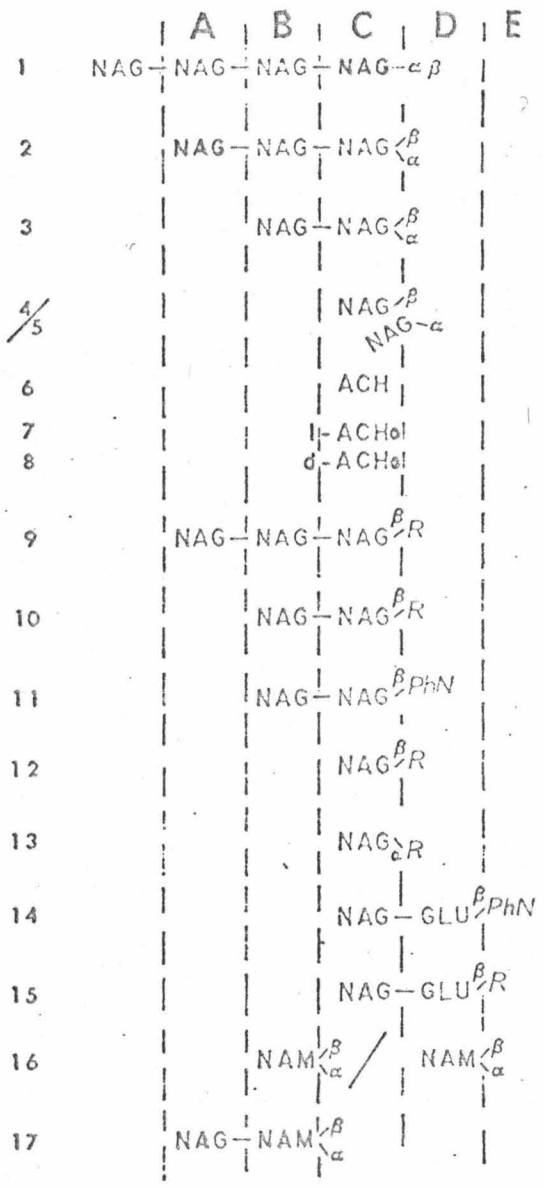

A
B
C
D
E
1
NAG—NAG—NAG—NAG—α β
2
NAG—NAG—NAG
3
NAG—NAG
4/5
NAG
NAG—α
6
ACH
7
l-ACHol
8
d-ACHol
9
NAG—NAG—NAG—R
10
NAG—NAG—R
11
NAG—NAG—PhN
12
NAG—R
13
NAG—R
14
NAG—GLU—PhN
15
NAG—GLU—R
16
NAM
NAM
17
NAG—NAM

Figure 6 - Mechanism for acid catalyzed hydrolysis or solvolysis of acetals.

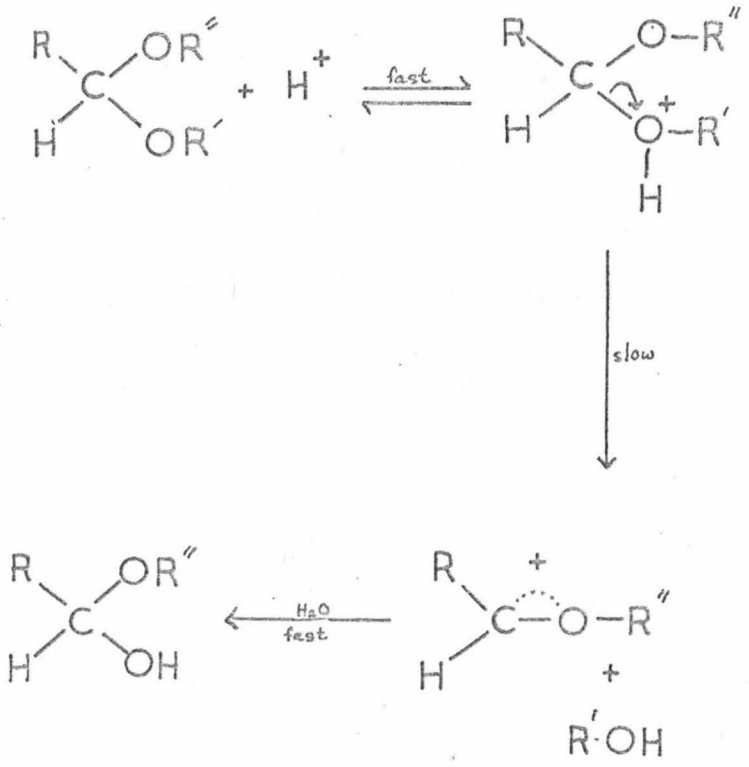

R
OR″
C
H
OR′
+ H+
fast
R
O–R″
C
H
O+
–R′
H
slow
R
OR″
C
H
OH
H₂O
fast
R
+
C
O–R″
H
+
R′OH

Figure 7 - Acetamido group participation in hydrolysis

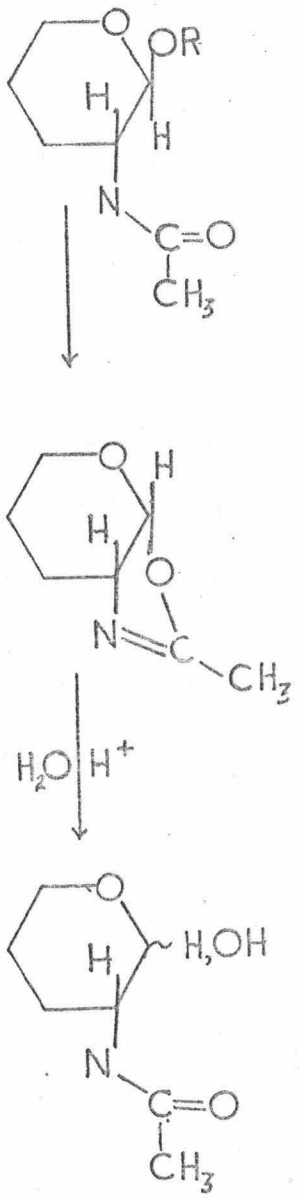
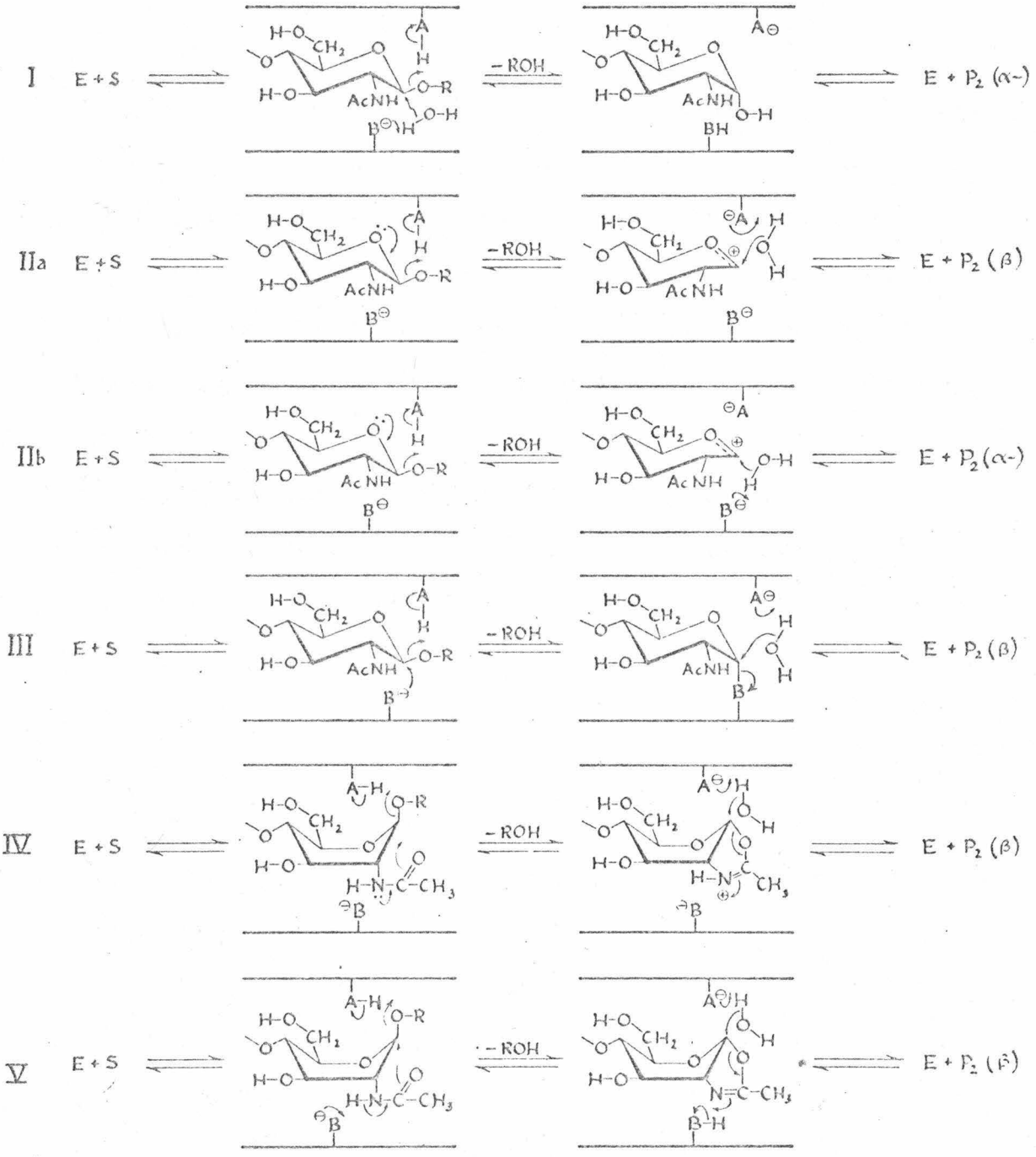

O
OR
H
H
N
C=O
CH3
H
O
H
N
C
CH3
H2O
H+
O
H
H,OH
N
C=O
CH3

Figure 8 - Possible mechanistic pathways for substrate during lysozyme catalyzed cleavage of glycosidic bonds from M. A. Raftery and T. Rand-Meir Biochemistry 7 3281 (1968).


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

Introduction

The synthesis of specifically labeled substrates for lysozyme that bind and cleave in a known pattern has not been a simple problem. One complication arises from the formation of non-productive complexes involving three strongly binding subsites, A, B, C, for small substrates. Further difficulties arise from trans-glycosylation reactions which occur in addition to hydrolysis under most circumstances,[1,2,3] so that scrambling of labels can occur.

Aryl glycosides have been examined as substrates and p-nitrophenyl-4-0-(2-acetamido-2-deoxy-β-D glucopyranosyl)β-D-glucopyranoside (NAG-glucose-φNO_2) labeled with deuterium on the C_1 of the glucose has been studied via kinetic isotope effects, since it is cleaved specifically between the glucose and aglycone. However, it is not a "natural" substrate and lacks an acetamido group. The analogous NAG-NAG-φNO_2 compound's hydrolysis has not been examined with kinetic isotope effects because the major bond cleavage occurs between the NAG residues.[4]

The best possible substrate would be a chitin oligomer uniformly labeled--in the case for the study of isotope effect uniformly labeled in the C1 position.

The total synthesis of chitobiose specifically labeled in the C1 position proved unworkable, the main difficulty being the formation

of the $\beta(1-4)$ linkage between the two sugars. The biological synthesis of chitin provides uniform labeling and the possibility of a wide range of chitin oligosaccharides.

Insect cuticle is a very good source of chitin and the physical, chemical, and morphological changes in the cuticle during the life cycle of the insect from egg to larva to pupa to adult have been studied in detail.[5] This is especially true of drosophila melanogaster (fruit fly).[6] The molting process and aspects of puparium formation have been examined autoradiographically. The results show alternate phases of production of enzymes involved in cuticle synthesis and enzymes involved in degradation. The synthesis was found to begin four hours before each molt and continued three hours after puparium formation. Thus flies leave the puparium 134 hours after the egg was laid.

The puparium contains about 57% chitin, the remainder being pigments and protein. Chitin exhibits great resistance to chemical reagents and so it is possible to remove substances associated in the empty puparium.

Incorporation of a specifically labeled sugar into drosophila was first attempted with feeding. This proved unsuccessful. Direct injection of the chitin precursor just prior to chitin synthesis in the formation of the puparium gave good results. This system has many fortuitous advantages--there is known to be no degradation of chitin during or after the time chosen for injection of labeled sugar and

therefore there should be little or no scrambling of the label. The main disadvantage in the use of this insect system is that a large number of animals were needed and each one must be injected (in this case 20,000 were required).

Experimental

Materials

The Oregon-R-stock of Drosophila melanogaster (kindly supplied by H. K. Mitchell) was used in all experiments. The mass culture technique described by Mitchell and Mitchell (1964)[6] with two hour egg collection was used. About one thousand eggs were collected at a time (per feeding tray) to ensure synchronous animals. Good feeding under non-crowded conditions was necessary for synchrony. The larvae were injected under a microscope 74 hours from egg laying.

All radioactive measurements were done on a Packard Tri Carb liquid scintillation spectrometer (Model 3375/3380) using a modified Bray's solution (Mohr et al., 1972).[7]

Methods

(N-acetyl-[D-glucosamine-1-^{3}H]). Tritiated Glc-NAc (N-acetyl-[D-glucosamine-1-^{3}H]) specific activity 4.4 Ci/mmole was obtained from Amersham/Searle (code TRK · 376 batch 1) (1 mCi/ml). The ^{3}H-Glc-NAc was injected into 74 hr (from egg laying) Drosophila larvae at a concentration of 1.0 mCi/ml using 0.1 μl per animal.

A total of 20,000 animals were injected. For injection, larvae were washed from feeding trays with water; the animals were washed again in water to remove yeast and food particles, then in 1% trichloroacetic acid for 2-3 minutes followed by a thorough washing in a dilute streptomycin and penicillin solution. The animals were then etherized and mounted on double sticky Scotch Taped microscope slides for injection. Injections under a microscope were made into the haemolymph, three segments from the end of the animal. The larvae were allowed to remain on the tape at least ten minutes after injection and were then washed off the slide with a wet sterile brush and transferred to a thick yeast/cornmeal suspension for subsequent growth.

After the larvae had all pupated (> 140 hours from egg laying) they were removed from the feeding box, washed thoroughly to remove food particles and placed in a ventilated box without food until emergence (> 200 hours). The empty pupal cases were collected, washed several times in water, then ground in a mortar and pestle with liquid nitrogen to a fine powder.

Purification of ^{3}H-chitin (N-acetyl-[D-glucosamine-1-^{3}H]$_n$).

The purification of ^{3}H chitin was achieved by KOH (5%) extraction at 100° for 2 days (Fraenkel, 1940) of the ground pupal cases. After extraction the chitin was centrifuged, washed eight times with water, twice with ethanol and twice with ether to yield an off-white powder (484 mg of pupal cases yielded 255 mg purified chitin).

Acid Hydrolysis of ^{3}H-Chitin. ^{3}H-Chitin (255 mg) was dissolved in 3.5 ml of conc. HCl at 0° for 2 hours, then hydrolyzed at 40° for 1 hour. The mixture was neutralized with a slight excess of lead carbonate and filtered over celite. The salt ($PbCl_2$) was washed several times with water. The combined filtrate was reduced in volume by lyophilization and run on a column of Bio-Gel P-4 (as before) in water. Two hundred 10 ml fractions were collected over a period of 36 hours (Figure 1). A series of oligomers of ^{3}H-Glc-NAc were isolated. (^{3}H-Glc-NAc)$_3$ centered at fraction 103. The fractions for each oligomer were pooled, lyophilized and rerun on Bio-Gel P-4.

Specificity of Labeling. ^{3}H NAG isolated from the acid hydrolysis of ^{3}H chitin was oxidized to N-acetyl glucosaminic acid with mercuric oxide[8] according to a published procedure to effect release of the ^{3}H labeled at C-1. Any scrambling of the label should show up as ^{3}H incorporation in the sugar ring. No scrambling was observed.

Results

Detailed work by Mitchell (1971)[6] on the biochemistry of the cuticle formation in Drosophila melanogaster was of very great help in the utilization of the insect larvae for chitin synthesis from specifically labeled N-acetyl-glucosamine monomer. The events in cuticle formation are illustrated in Figure 2 . The injection method proved very efficient, yielding $>50\%$ incorporation of label in the final NAG oligomers. The apparatus for injection was devised by H. K. Mitchell. It consisted of an automatic pump controlled by a

foot lever which could alternately pump into the needle or eject from the needle quantities of fluid in the 0.1 μl range. The needles were hand made by drawing out capillary tubes after heating in a flame. The very small thin wall tubes were cut and bevelled under a microscope to produce a miniature hypodermic needle, appropriate for size of the larvae injected (the animals are $\sim$0.2 cm long and weigh $\sim$0.8 mg). About 0.05 mg of chitin could be maximally obtained from each pupal case.

The acid hydrolysis of chitin (Figure 1) yielded a wide array of oligomers from NAG VIII to NAG I.

Discussion

This method of chitin synthesis allows for specific and uniform labeling of chitin in any portion of the N-acetyl-glucosamine sugar ring, simply by injecting the appropriately labeled NAG monomer, into enough larvae. The uniformity of label makes studies with lysozyme much simpler since nonspecific cleavage is a major difficulty in studying the products of lysozyme hydrolysis.

References

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3. J. A. Rupley, Proc. Roy. Soc. (London), 167B, 416 (1967).
4. T. Rand-Meir, F. W. Dahlquist, and M. A. Raftery, Biochem., 8, 4206 (1969).
5. G. Fraenkel and K. M. Rudall, Proc. Roy. Soc. (London), Ser. B, 129, p1 (1940).
6. H. K. Mitchell, U. M. Weber-Tracy, and G. Schaar, J. Exp. Zool., 176, 4 (1971).
7. L. H. Mohr, L. E. H. Smith, and M. A. Raftery, Biochim. Biophys. Acta, in press.
8. J. Findlay, G. A. Levy, and C. A. Marsh, J. Amer. Chem. Soc., 69, 467 (1958).

Figure 1 - Chromatographic separation on Bio Gel P 4 of the products of the acid hydrolysis of ^{3}H chitin. The peaks I - VIII were identified as chitin oligomers NAG to NAG_8 .

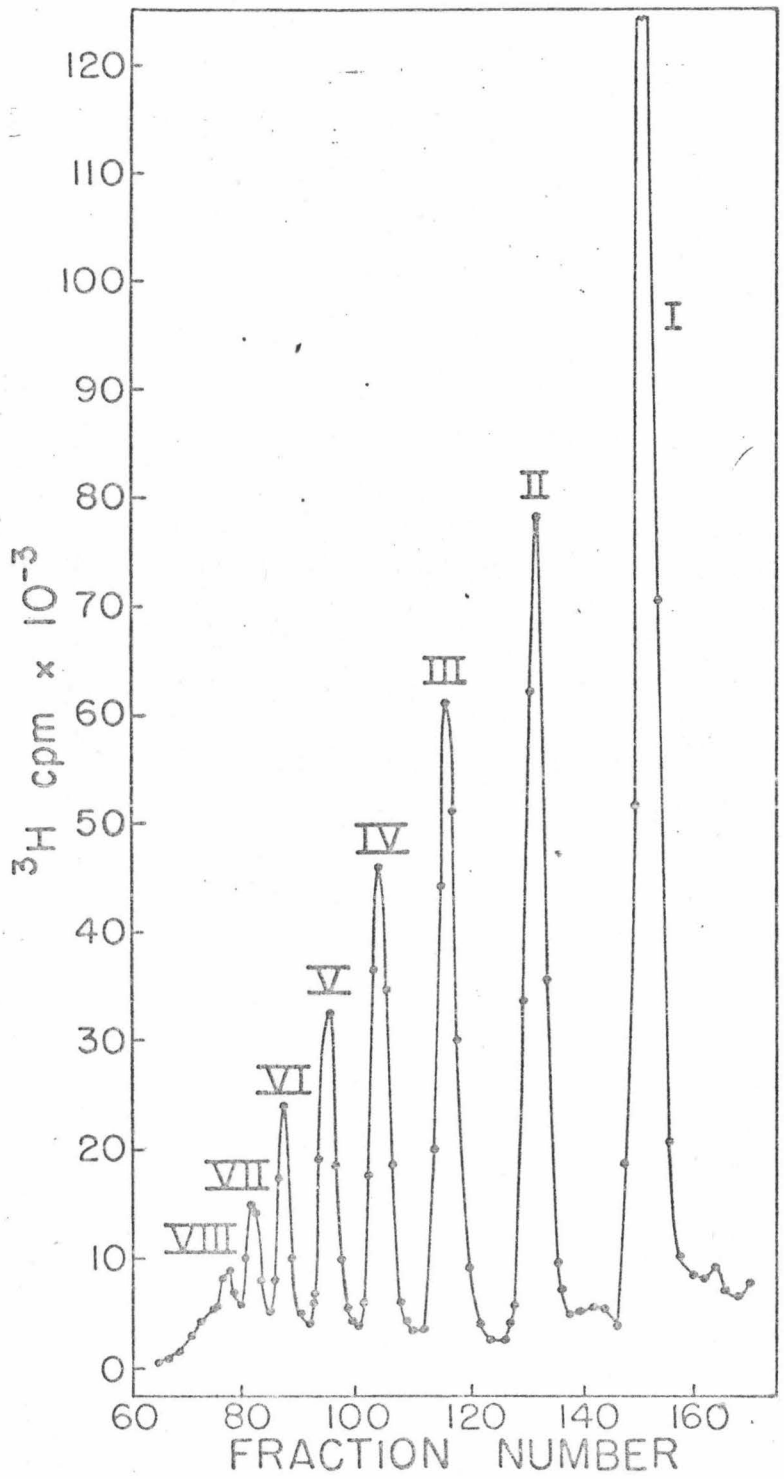
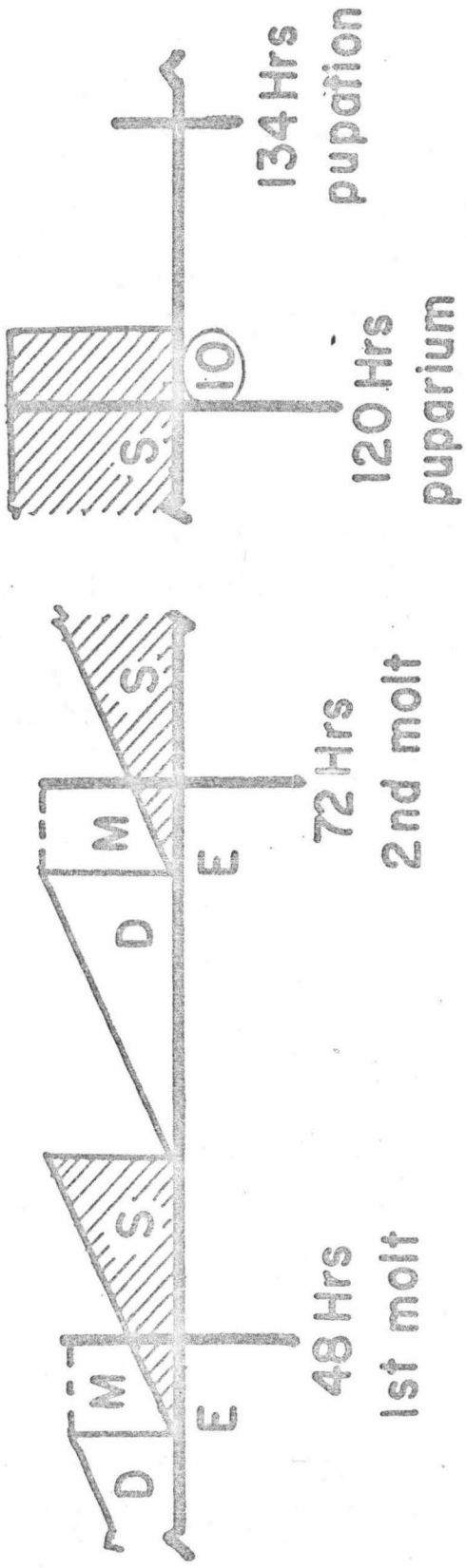

120
110
100
90
80
70
60
50
40
30
20
10
0
^3H cpm x 10^{-3}
I
II
III
IV
V
VI
VII
VIII
60
80
100
120
140
160
FRACTION NUMBER

Figure 2 - The pattern of chitin synthesis in Drosophila melanogaster. S refers to chitin synthesis and D to chitin degradation.

- from H.K. Mitchell, U.M. Tracy-Weber, and G. Schaar, J. Exp. Zool. 176 4 (1971)


48 Hrs
1st molt
72 Hrs
2nd molt
120 Hrs
puparium
134 Hrs
pupation
D
M
E
S
I

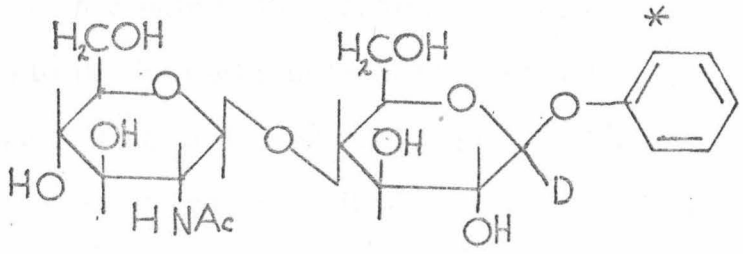
Introduction

X-ray analysis studies of Blake et al.[1,2] on the association of various inhibitors and substrates with lysozyme have led to a proposed mechanism of catalysis.[1-3] The suggested mechanism involves the catalytic production of a carbonium ion which is electrostatically and sterically stabilized by groups on the enzyme. The alternate mechanistic possibilities for a substrate of the chitin oligomer (NAG_n) type include: 1) any mechanism involving an even number of displacements at C-1 of glycoside being cleaved e.g., a double displacement mechanism proposed by Koshland[4] which implies a covalent enzyme substrate intermediate; 2) a displacement mechanism in which the acetamido carbonyl group of the glycoside displaces the aglycone forming an oxazoline intermediate.[5-9] Evidence for the mechanism has been obtained from model studies of the spontaneous hydrolysis of aryl-2-acetamido-2-deoxy-β-D-glucopyranoside derivatives.[5,6] Any mechanistic possibilities involving single displacement have been eliminated by the results of Dahlquist, et al.[10] indicating that hen egg white lysozyme catalyzed hydrolysis of a glycosidic bond proceeds with quantitative retention of configuration (>99.7%).

These mechanistic alternatives were resolved for the case of the enzyme catalyzed hydrolysis of the substrate phenyl-4-0-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-β-D-glucopyranoside

(NAG-GLU-φ) labeled with deuterium in the C-1 position of the glucose ring and labeled with ^3H in the phenyl ring, through the use of secondary kinetic isotope effects.

It has been shown that substrates of the type NAG-GLU-φ act as substrates for lysozyme and are cleaved exclusively between the glucose residue and the phenyl ring.



The isotope effect for the lysozyme catalyzed hydrolysis was found to be kH/kD = 1.11.[19,20] This result suggested that the enzyme catalyzed reaction proceeded through an intermediate with considerable carbonium ion character. (Model studies on phenyl-β-D-glucopyranoside in acid catalyzed hydrolysis which is known to proceed via a carbonium ion mechanism revealed an isotope effect of 1.13.)

The α-secondary tritium or deuterium secondary isotope effects are primarily due to a net change between the ground and transition states of the zero point energy of the bending vibrational frequencies of the carbon-tritium and carbon-hydrogen bonds. A secondary isotope effect greater than 1 (i.e., kH/kT >1) should

arise from any reaction in which the difference in zero point energies between the hydrogen-carbon and tritium-carbon bond bending frequencies is less in the transition state than in the ground state. Streitweiser[11] found that reactions involving the rate determining formation of a carbonium ion from a tetrahedral carbon atom fell into this category. The decrease in the zero point energy of the carbon-tritium out of plane bending vibration in the transition state can be attributed to the change from sp^3 to sp^2 hybridization and also to the increase in bond length between the carbon atom and the leaving group in the transition state. A transition state which resembles the product (if the reaction goes via a carbonium ion mechanism) will have an isotope effect greater than 1 and ranging as high as 1.25 (for tritium). A transition state which resembles the starting materials will have an isotope effect close to 1. If a reaction proceeds via an Sn2 nucleophilic displacement, the incoming group with its partial bond formation in the transition state compensates for the partial loss of the bond (and out of plane bending frequency) of the leaving group.[12]

Thus the geometry of the transition state of a reaction will govern the α-secondary isotope effect. Any uncompensated weakening in the transition state of the carbon hydrogen bond of the reacting carbon will tend to cause an increase in kH/kT. Therefore, secondary isotope effects can be utilized to determine the degree of covalent bond formation in the transition state.

The results of Dahlquist and co-workers strongly suggested that lysozyme catalysis occurred with the rate determining production of a carbonium ion intermediate for the substrate (NAG-GLUφ) studied. However, the possibility still remained that a more natural substrate, that is, one with an N-acetyl moiety on the 2-position of the sugar might hydrolyze via a different mechanism. (figure 1)

The study of the α-secondary tritium isotope effects of the lysozyme catalyzed hydrolysis of NAG_3 were undertaken in order to distinguish among the catalytic alternatives for a natural substrate.

Experimental

Materials

Both chitin (lot 80-CH 60) and hen egg white lysozyme (lot L6876) were purchased from Sigma Chemical Company. The tritiated NAG_3 (N-acetyl-[D-glucosamine-1-^{3}H]$_3$) was prepared according to the method mentioned in the previous chapter.

Methods

N,N′,N″-triacetyl-^{14}C-chitotriose [(^{14}C-Glc-NAc)$_3$]. The synthesis of the unlabeled compound has been described (Barker, et al., 1958).[13] A modification of this procedure was employed for the labeled material. Chitosan was prepared from chitin by de-N-acetylation and was then subjected to acid hydrolysis as described. The acid hydrosylate (500 mg) in water (12.5 ml) was treated at 0° with methanol (1.25 ml), Amberlite IRA-400 (CO_3^{2-} form) (15 ml) and acetic-1-^{14}C-anhydride (0.33 ml, 0.5 mCi)

purchased from New England Nuclear, lot No. 555-241 (specific activity 10 mc/mM). The stirred mixture was stored at 0-5° for 1.5 hours. Thereafter the resin was removed and replaced by Amberlite IR-120 (H^+ form) (1 ml) to remove any free amino sugars. The deionized solution was concentrated to 10 ml, insoluble material removed and the supernatant concentrated by lyophilization. The material was then applied to a Bio-gel P-4 (200-400 mesh) column (5 cm × 150 cm) in water with 10 ml fractions collected. The separated oligosaccharides ($glc\text{-}NAc_1$ to $(glc\text{-}NAc)_7$) (Figure 2) were then rechromatographed on Bio-Gel P-4 in water.

Purification of a Mixture of $(^3H\text{-}Glc\text{-}NAc)_3$ and $(^{14}C\text{-}Glc\text{-}NAC)_3$. The ^3H and ^{14}C trisaccharides were pooled and spotted on Watman No. 3 MM paper in an 8 inch strip for descending chromatography (Jeanes et al., 1951).[14] The paper was developed with pyridine, 2-pentanol and water (1:1:1 by volume) and run against the machine direction for 12 hours.

The product was visualized on the paper in a Varian Aerograph thin layer scanner (model 6000). The paper was cut perpendicular to the direction of development in 1/2 to 1 cm wide strips; each strip was eluted with water. An aliquot was counted and the $^3H/^{14}C$ ratio recorded. The eluents from strips with a constant ratio were pooled, lyophylized and rerun on a Bio-Gel P-4 column in H_2O. Again the fractions were analyzed for $^3H/^{14}C$ ratio and fractions with a constant ratio were pooled and lyophilized (see Figure 3).

Lysozyme Hydrolysis of Chitotriose [(^{3}H-Glc-NAc)$_3$ and (^{14}C-Glc-NAc)$_3$]. The enzyme hydrolysis of labeled chitotriose was conducted at 40° at pH 5.5 in 0.1 M citrate buffer. The enzyme concentration was 2.5×10^{-3} M; that of the substrate was 5.1×10^{-3}M. The hydrolysis was allowed to proceed for 5 minutes at 40° (< 5% of completion), then quenched with 100 μl of 10% ammonia. The reaction mixture (total volume of 0.5 ml) was run on a P-4 column (0.9 cm × 90 cm) in 0.05 ammonium formate buffer pH 9.5. The fraction size was 0.3 ml. The runs were first analyzed by checking the ^{3}H/^{14}C ratio for an aliquot from each fraction. When it had been determined that the ratio was again constant across each peak, aliquots were taken from fractions in each peak (I, II, III of Figure 4) to make up 1 ml of solution each of which was counted in 15 ml of modified Bray's (Mohr, et al., 1972).[15] An aliquot of starting material was also made up to 1 ml with 0.05 M ammonium formate buffer and counted as described.

Calculation of Isotope Effects. In order to account for any possible non-uniformity of label in the reducing end of the substrate (^{3}H chitotriose) caused by an isotope effect in the acid hydrolysis of the ^{3}H chitin the following scheme was observed for calculation of isotope effects.

X = $^{3}H/^{14}C$ ratio of each monosaccharide subunit before acid hydrolysis.

Y = $^{3}H/^{14}C$ ratio of the monosaccharide subunit of product monosaccharide affected by the isotope effect of enzyme hydrolysis.

Z = $^{3}H/^{14}C$ ratio of the reducing end NAG subunit of the substrate trisaccharide affected by acid hydrolysis.

Therefore, assuming cleavage NAG-NAG-NAG → NAG + NAG-NAG

III I II

$$\text{starting material (III) ratio} = \frac{2X + Z}{3}$$

$$\text{product } NAG_2 \text{ (II) ratio} = \frac{X + Z}{2}$$

$$\text{product NAG (I) ratio} = Y$$

$$kH/kT = \frac{X}{Y} = \frac{3(III) - 2(II)}{I}$$

Although Dahlquist, et al., have shown the assumed cleavage to be the major mode of catalyzed hydrolysis, it is not necessary to assume this specific cleavage since calculations based on fission of the bond closest to the reducing end yields the same isotope effect, as do calculations based on the $^{3}H/^{14}C$ ratios of starting trimer and the ratio of the sum of the products

$$\text{i.e.} \quad kH/kT = \frac{3(III)}{2(II) + I}$$

Results

Detailed work by Mitchell (1971)[16] on the biochemistry of cuticle formation in Drosophila melanogaster enabled us to utilize the insect larvae for chitin synthesis from a specifically labeled N-acetyl-glucosamine monomer. See previous chapter.

The purity of the substrates was judged primarily by the constancy of the $^{3}H/^{14}C$ ratio on paper and on gel filtration columns. This method is very sensitive to radioactive impurities. Also the monosaccharide Glc-NAc isolated from the hydrolysis of ^{3}H chitin (Figure 5) was oxidized to the N-acetyl glucosaminic acid with mercuric oxide with release of the ^{3}H label at the C-1 position to show any scrambling of label during the biological synthesis. No scrambling was observed. The purity of the sample was also checked by isolating the starting material from one enzymatic hydrolysis for use as substrate in a subsequent run. Using trisaccharide obtained in this way an isotope effect within the error of previous determinations was obtained.

The pattern of hydrolysis of the substrate used in these studies, $(Glc\text{-}NAc)_3$, has been examined by Dahlquist et al. (1967) and by Rupley (1967). The cleavage pattern we observed is clean, yielding dimer and monomer products in the ratio of ~ 2:1 (Figure 4). There was little or no transfer observed [$(GlC\text{-}NAc)_4$ would be the main transfer product] and none expected since the reaction proceeded

for less than one turnover. The hydrolysis was expected to be specific (based on the results of Dahlquist and Rupley) for the bond nearest the reducing end. The ratio of $(Glc\text{-}NAc)_3$ to lysozyme was 2:1, allowing binding of one mole of trisaccharide to the nonproductive sites (A, B, C) on the enzyme and thus leaving an enzyme substrate ratio of 1:1 for catalysis.

The isotope effect for $(Glc\text{-}NAc)_3$ was 1.19 ± 0.01 calculated from three measurements. The observed counts of ^{3}H and ^{14}C were converted to disintegrations and to isotopic ratios by the methods described by Dahlquist et al. (1969).[19]

Discussion

The tritium isotope effect of 19% for lysozyme catalyzed hydrolysis of Glc-NAc trimer suggests a transition state with considerable carbonium ion character. An isotope effect of this size would appear to rule out both single and double displacement mechanisms involving attack by solvent or by the enzyme itself, at least in any pre-rate-determining step.

Dahlquist, et al. (1968, 1969)[19,20] have previously shown that the lysozyme catalyzed hydrolyses of aryl glucoside substrates exhibit secondary α-deuterium isotope effects of 11% (equivalent to ~ 15% tritium effect). From this evidence it was concluded that for these substrates the enzyme proceeded via a carbonium ion mechanism. However the possibility still remained that for cell wall substrates or for Glc-NAc oligomer substrates the N-acetyl group (missing in

the aryl glucosides) could provide anchimeric assistance. Also the aromatic leaving group could have an influence on the isotope effect.

Although it is possible to conclude from our result that the enzyme hydrolysis does not proceed via anchimeric assistance, it is difficult to judge to a fine degree just how much the transition state resembles a carbonium ion since the isotope effect for the enzyme catalysis is greater than any effect seen in the related model carbonium ion reactions (Mohr et al., 1972).[15] However, our result (a 19% tritium isotope is equivalent to ~ 14% deuterium effect) is typical of most reactions classified as S_N1. We can conclude only that the carbonium ion character of the transition state of the enzymatic reaction is greater than that of the model systems. This may be attributable to the large differences in solvation between the two systems.

The carbonium ion-like transition state is almost certainly stabilized electrostatically (Blake et al., 1967)[21] (probably by the β-carboxylate ion of Asp 52) (Parsons et al., 1969)[22] and also stabilized by binding in subsite D which favors a half-chair conformation over a normal chair (Phillips, 1967).[23] The negative charge of Asp 52 is held at a distance of ~ 3 Å from both the C_1 carbon of residue D and from the ring oxygen (Vernon, 1967)[24] which will share the charge. There would be some covalent character associated with this ion pair (Jencks, 1969)[25] which would tend to decrease the observed isotope effect from a maximum possible value.

Protonation of the leaving group before bond cleavage would tend to decrease the isotope effect since the leaving group would then be stabilized making the transition state more reactant-like (and less carbonium-like) according to Hammond's postulate. A concerted protonation would stabilize the leaving group less and the tendency would be towards a larger isotope effect.

The isotope effect observed for the $(Glc\text{-}NAc)_3$ substrate is slightly larger than that for the aryl glucoside substrate (Dahlquist et al., 1969).[19] The difference could be attributed to the change in leaving group from aromatic (phenyl) and slightly electron withdrawing to a sugar (electron-donating) or possibly to the presence of the N-acetyl moiety. From studies of hydrolysis of Glc-NAc glycosides in solution (Mohr et al., 1972)[15] an increase in isotope effect would be predicted in going from an electron-withdrawing to an electron-donating group. However this may or may not be applicable to enzyme hydrolysis. It is impossible to say whether or not the N-acetyl group influences the isotope effect for the enzyme reaction one way or another.

The isotope method has inherent limitations common to all measurements of reaction rates. We can know for certain only about the energy of the transition state corresponding to the rate determining step. It is inherently impossible to draw any conclusions about the mechanism of its formation or its breakdown. And, in fact, it is not always possible to find out what is happening in the whole of the rate

determining step itself. The weakening of the carbon-hydrogen (tritium) bond may not be significantly advanced in the transition state but may still be completed further along the same reaction step. It is well to keep these limitations in mind.

However, the isotope method has the very great advantage of utilizing substrates which have identical orientations with respect to the catalytic groups on the enzyme. This method has large advantages over the use of substrate analogues. Analogues may bind to the enzyme in slightly different orientation or may in fact react with different mechanisms making interpretation of results very difficult. Isotopic substitution is a much subtler change than the introduction of a substituent. The important feature of isotope effects is that there is no difference in the potential energy surfaces for reactions involving isotopic substitution--in this case tritium and hydrogen. The rate differences observed arise from changes only in the minimum energy levels which are affected by the different vibrational frequencies of hydrogen and tritium containing molecules.

In conclusion, the α-secondary isotope effect observed strongly suggests a carbonium ion mechanism for lysozyme. There still exists the possibility of post-rate determining step collapse of the carbonium ion to a covalent intermediate, either via attack by the anion Asp 52 or by the carbonyl oxygen of the N-acetyl group to give an oxazoline intermediate (Beranek and Raftery, 1972).[26] In fact the observed half life of the intermediate, which must be long enough to

allow for the observed transfer reaction to other saccharide, would suggest some such stabilization.

References

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Figure 1 - The substrates used in the lysozyme catalyzed hydrolysis studies - 1, 1', 1''-(^{3}H)chitotriose and N, N', N'' - triacetyl (^{14}C)chitotriose.

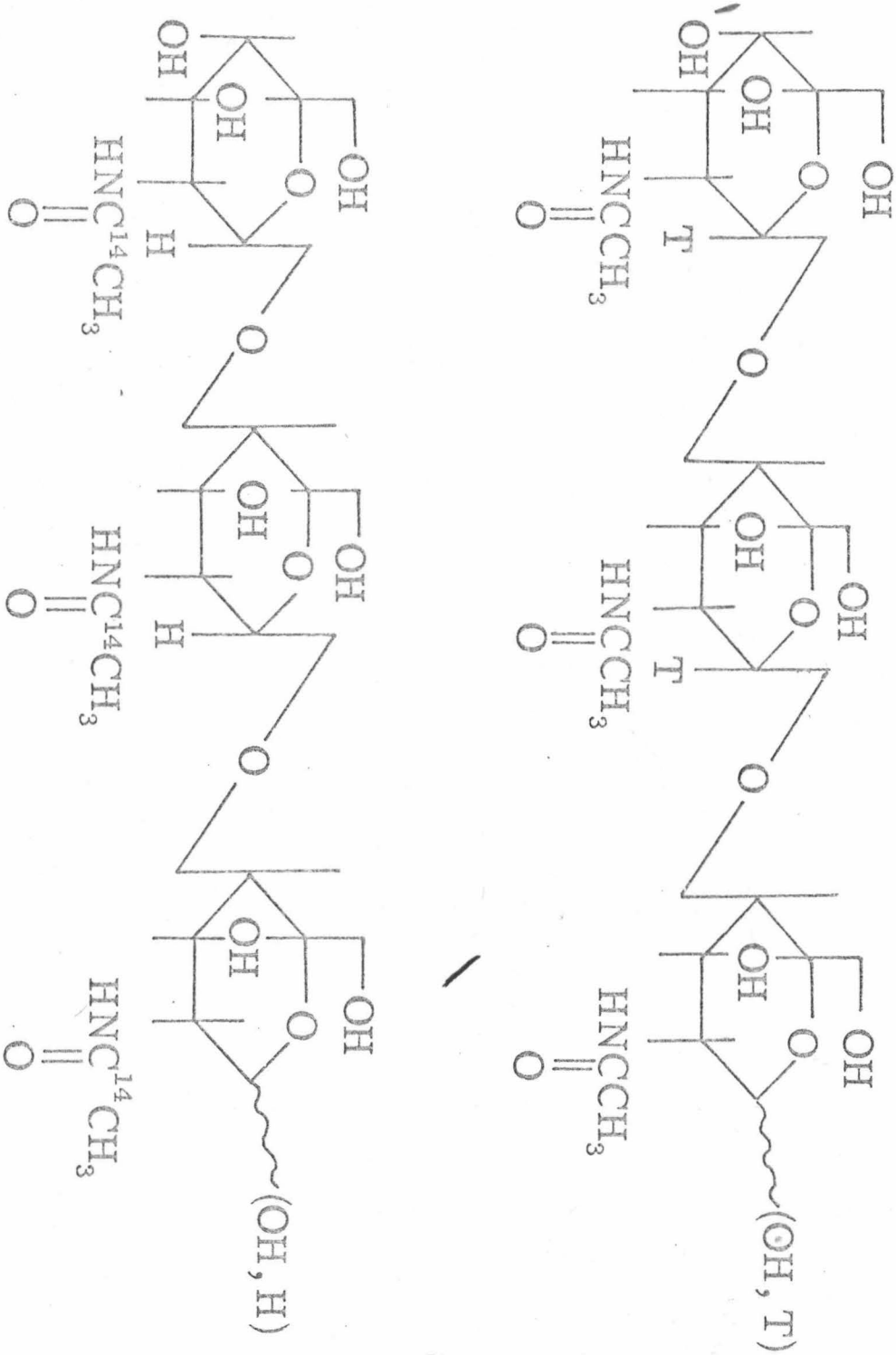

OH
OH
OH
O
T
HNCCH3
O
(OH, T)
OH
OH
OH
O
T
HNCCH3
O
OH
OH
OH
O
T
HNCCH3
O
OH
OH
OH
O
H
HNC14CH3
O
OH
OH
OH
O
H
HNC14CH3
O
OH
OH
OH
O
H
HNC14CH3
O
(OH, H)

Figure 2 - The chromatographic separation on Bio Gel P 4 in H_2O of the reaction mixture from the synthesis of ^{14}C labeled chitin oligomers. The various peaks were identified as NAG(I), NAG_2(II), and so on up to NAG_7(VII).

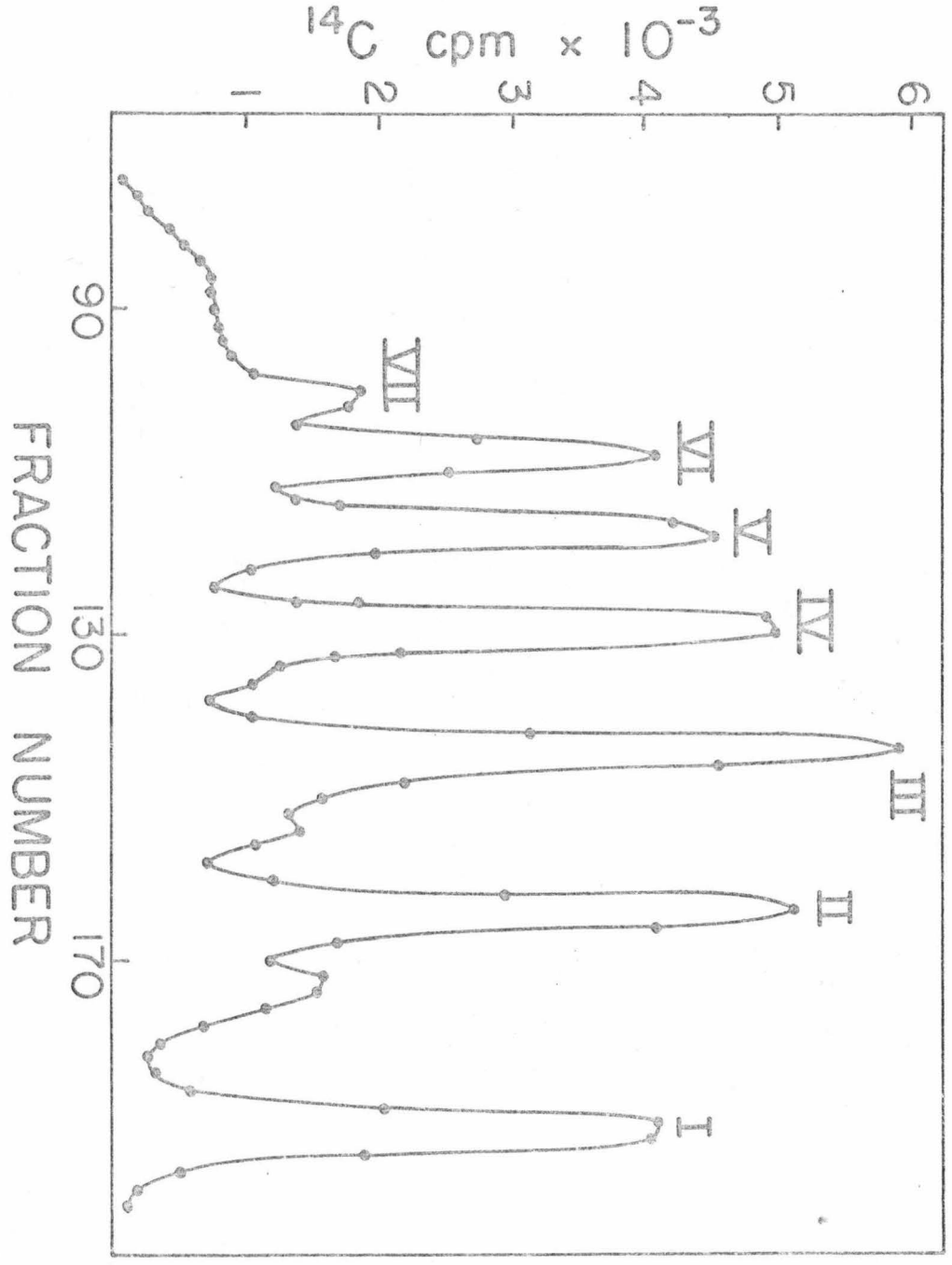

^{14}C cpm x 10^{-3}
1
2
3
4
5
6
90
130
170
FRACTION NUMBER
VII
VI
V
IV
III
II
I

Figure 3 - The chromatography on Bio Gel P 4 of the mixture of (^{3}H) and (^{14}C) chitotriose to check for purity. The ratio observed was constant within the counting error.

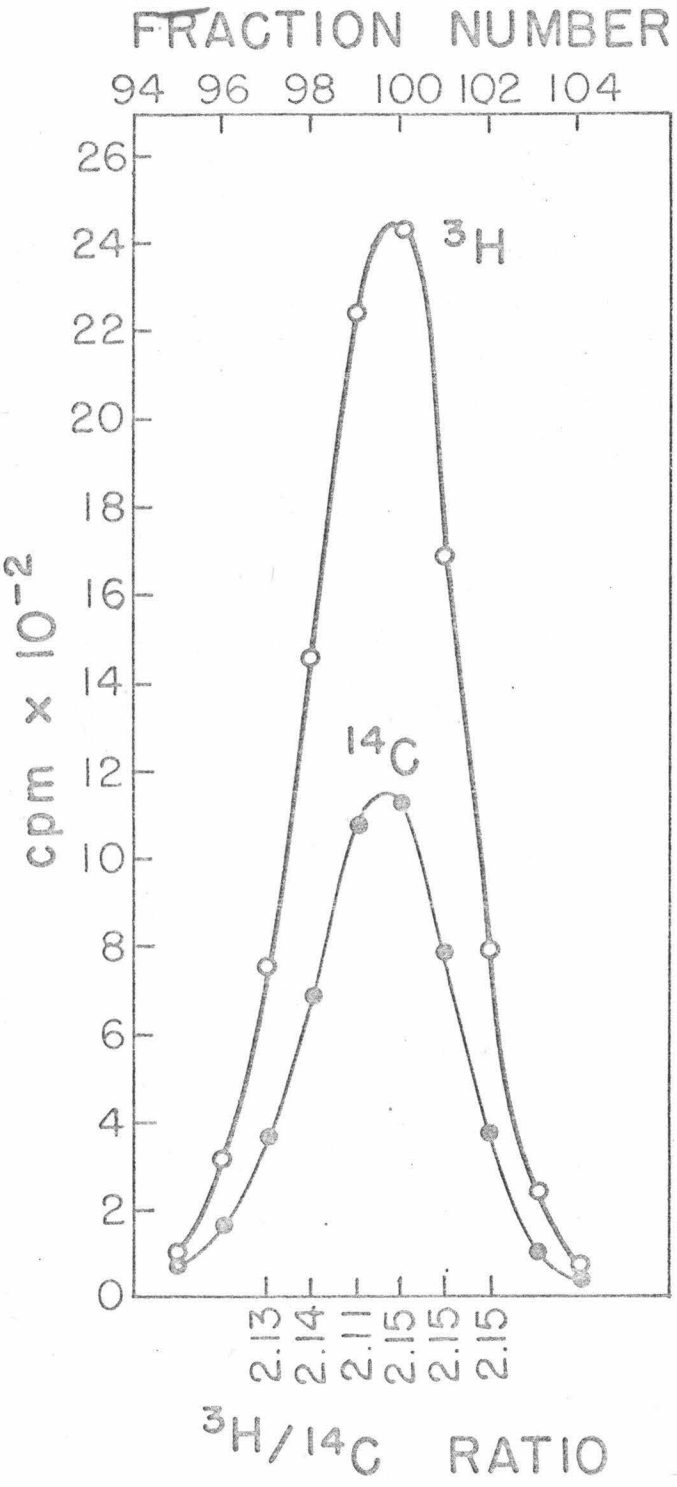

FRACTION NUMBER
94 96 98 100 102 104
26
24
22
20
18
16
14
12
10
8
6
4
2
0
cpm x 10⁻²
³H
¹⁴C
2.13
2.14
2.11
2.15
2.15
2.15
³H/¹⁴C RATIO

Figure 4 - The chromatographic separation on Bio Gel P 4 in 0.05 M ammonium formate of the products of the lysozyme catalyzed hydrolysis of chitotriose. The peaks were identified as NAG(I), NAG_2(II), NAG_3(III), and a mixture of NAG_3 and lysozyme.

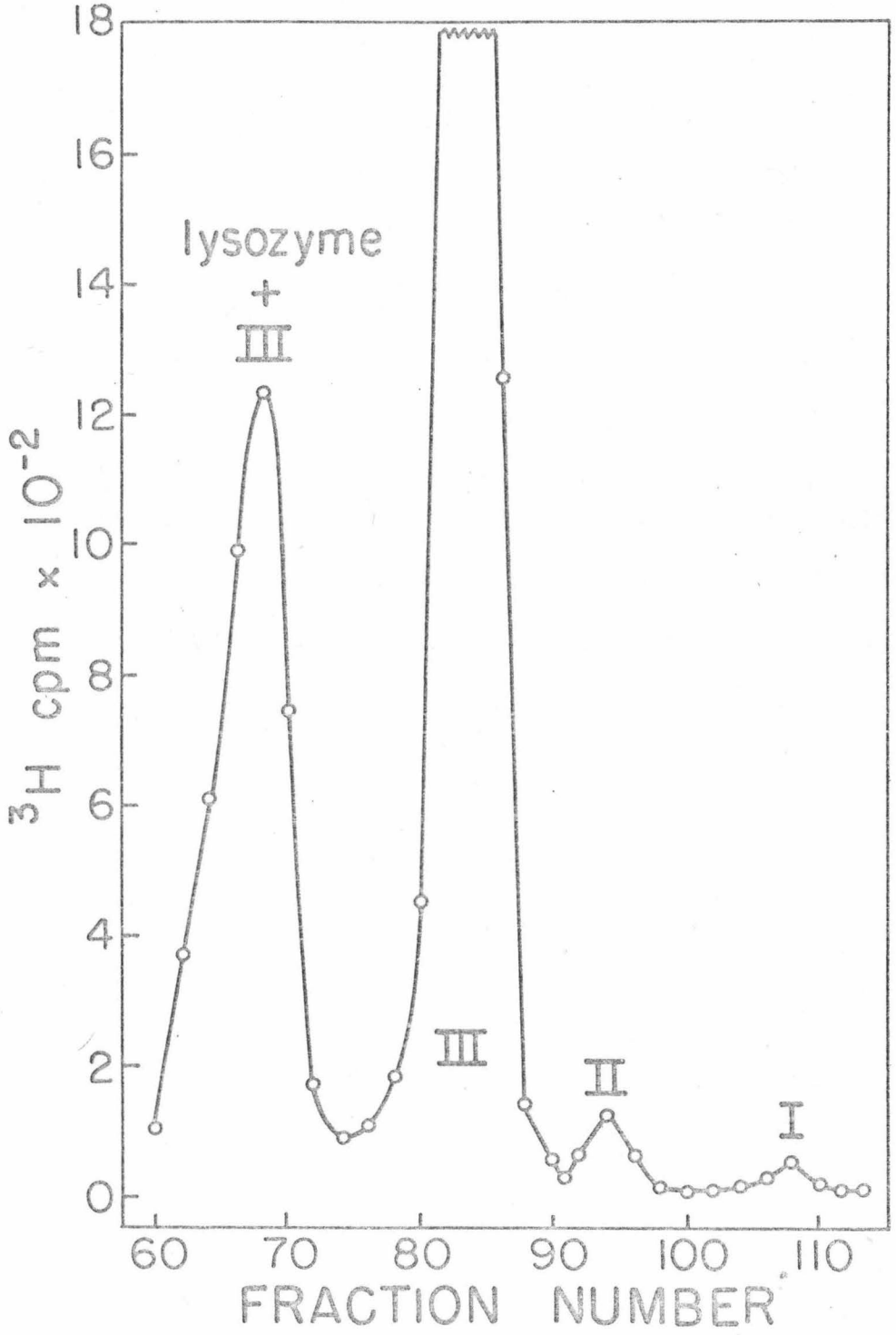
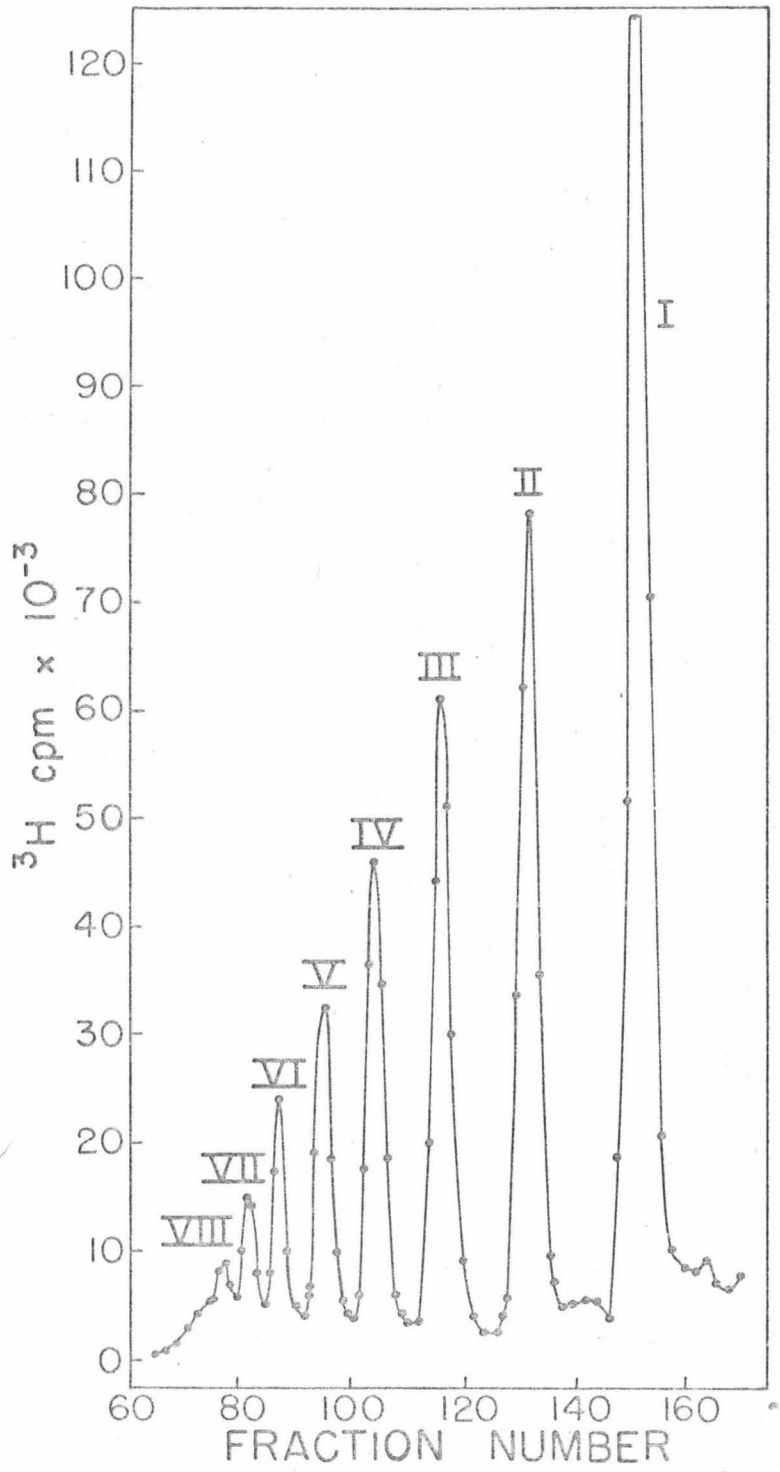

18
16
14
12
10
8
6
4
2
0
^3H cpm x 10^{-2}
lysozyme
+
III
III
II
I
60
70
80
90
100
110
FRACTION NUMBER

Figure 5 - Chromatographic separation on Bio Gel P 4 of the products of the acid hydrolysis of ^{3}H chitin. The peaks I - VIII were identified as chitin oligomers NAG to NAG_8 .


120
110
100
90
80
70
60
50
40
30
20
10
0
^3H cpm x 10^{-3}
I
II
III
IV
V
VI
VII
VIII
60
80
100
120
140
160
FRACTION NUMBER

Introduction

There has been a considerable amount of work done to elucidate the catalytic mechanism of lysozyme hydrolysis. Most of this work has dealt with binding and the steps leading to the transition state (now known to be a carbonium ion like structure). However, in order to understand enzyme catalysis more fully, it is necessary to understand what happens in post-rate-determining steps in the hydrolysis.

It is reasonable to assume that a stabilized intermediate exists after the carbonium ion since a free and unstabilized carbonium ion would have too high an energy to permit the catalyzed reaction to take place at a reasonable rate. It would also not explain the relative rates of trans-glycosylation and hydrolysis. The intermediate seen by either water or an incoming saccharide must be stable enough to allow time for release of the portion of the substrate bound in site E and also for subsequent binding of the acceptor. A very unstable intermediate would presumably react only with the substrate in site E before it diffused away or with water which undoubtedly diffuses into the reactive site more readily than a sugar residue. So the carbonium ion must be followed by a stabilized intermediate in the reaction pathway. And this intermediate must not be too stable to undergo rapid hydrolysis.

It is known from the work of Parsons and Raftery that asp 52 is necessary for catalysis, but its role has not been well defined. Asp 52 plays little part in binding. It has been postulated that the carboxylate anion of 52 (pKa = 4.5) stabilizes the incipient carbonium ion in the rate determining step. This ion pair might then collapse to form a covalent acylal intermediate in a post-rate determining step.

It is also possible that the oxygen of the acetamido group on C_2 of the sugar residue in site D could attack the positively charged C_1 to form an oxazoline intermediate. Asp 52 could stabilize the positively charged nitrogen of the acetamido group.

The last possibility for the post-rate determining step in catalysis is simply the production of a carbonium ion stabilized by asp 52.

It is more difficult to study post-rate determining steps than it is to look at steps which proceed or include a rate-determining event. However, it is possible to distinguish among the three alternatives mentioned above and to distinguish the role that asp 52 plays in catalysis by utilizing the fact that the acceptor molecule for the lysis reaction (water or a saccharide) is affected by events only after the production of the common intermediate for transfer or hydrolysis, that is, after the overall rate limiting step.

So the three cases to be considered for the stabilized intermediate are:

1. an oxazoline formed by attack of the acetamido group in a post-rate limiting step;

2. an acylal formed by attack of asp 52 in a post-rate limiting step;

3. a stabilized carbonium ion, an ion pair, formed with asp 52.

Experimental

Materials

Chitin oligosaccharides were prepared by partial acid hydrolysis of chitin[1] (Sigma) followed by gel filtration on Biogel P-2.[2] The p-nitrophenyl-4-0-(2-acetamido-2-deoxy-β-D glucopyranosyl)-β-D glucopyranoside (NAG-GLU-ϕNO_2) was prepared utilizing the transferase activity of lysozyme[3] as was p-nitrophenyl-4-0-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-β-D-2-deoxy glucopyranoside (NAG-deoxyGLU-φNO_2). Lysozyme was obtained from Sigma (3× crystallized). The p-nitrophenyl-β-D-2-deoxy glucose (deoxyGLU-φNO_2) and p-nitrophenyl-β-D-2-deoxy-2-acetamido glucose were prepared from published procedures.[4] The NAG_6 (3H) used in the transfer/hydrolysis ratio experiment was obtained via the method of Smith, et al.[5] The phenyl-β-D-2-deoxy-2-acetamido-glucopyranoside was prepared from a published procedure.[4] CM sephadex was obtained from Pharmacia. Dowex 50 (Na^+) × 2 ion exchange resin was purchased from Dow Chemical Company.

Methods

Transfer reaction with NAG-GLU-φNO_2

Lysozyme (25 mg) plus 2 mg NAG-GLU-φNO_2 in 100 μl dioxane were incubated at pH 5.5 in 1 ml of 0.1 M citrate buffer (0.1 M NaCl) for 20 hours. The reaction mixture was added to ~10 mg of CM-50 sephadex swollen in 0.1 M citrate pH 5.5 and filtered through cotton on to a P-2 column (0.9 × 100 cm) and eluted with water. Fractions of 72 drops (~ 2 ml) were collected, a total of 50 fractions. (see figure 1)

Transfer reaction of NAG-deoxy GLU-φNO_2

NAG-deoxy-GLU-φNO_2 (5 mg) was taken up in 100λ dioxane and 300λ H_2O then 100λ of this solution was added to 25 mg of lysozyme in 250λ of 0.1 M citrate pH 5.5 and also 650λ of 0.1 M citrate buffer pH 5.5. The reaction was allowed to proceed at 40°C for two hours, then run on a column of P-2 as above.

Transfer/total reaction ratio experiment

300λ of 0.02M Na phosphate buffer pH 8 (0.24M Na Cl) 50λ of a 0.7 mg/ml solution of lysozyme, 50λ of NAG (3H) solution (2.9 mg/ml) and 5λ φNAG (66.4 mg/2 ml) were combined and left at room temperature for 25 minutes, at which time the reaction was quenched with the addition of ~ 10 mg CM50sephadex in 1 ml of 0.1 M Na phosphate buffer at pH 8.5 (shaken for five minutes) then filtered through cotton in a pasteur pipette onto a Dowex 50 Wcolumn (1.3 × 180 cm).. The Dowex 50 (Na^+) was prepared by equilibrating

the resin with 5% NaCl on a filter, then washing with 2 × distilled water.

The Dowex-50W column was eluted with water with 80 drop (2.6 ml) fractions collected. Samples of NAG_6, NAG_4, NAG_2, and NAG were run separately under the same conditions to mark their elution position. (see figure 2)

The transfer reaction was run at various pH's ranging from pH 3 to pH 9 and at varying times (so that reaction went ~ 3%).

The Dowex-50 column could not separate NAG_6 from NAG_4 but there was a good separation between NAG_6 and NAG_4 together and NAG_2 and $NAG_5\varphi$. The tubes containing NAG_2 and $NAG_5\varphi$ were combined and lyophilized, then loaded on to a P-4 column (0.9 × 100 cm) with 50 drop (~ 1 ml) fractions (eluted with water). The P-4 column fractionation achieved a separation of NAG_2 and $NAG_5\varphi$, the ratio of which gives a measure of transfer to total reaction--transfer plus hydrolysis. The fractions were counted (^3H) in Bray's scintillation cocktail[7] (15 ml) in a Packard Tri Carb scintillation counter. (figure 3)

Results

The reaction of NAG-GLU-φNO_2 with lysozyme is shown in Figure 1. The absorbance at 280 nm of the fractions was recorded, and the peaks identified as indicated. Starting material coincided with peak I. Peak II was eluted coincidentally with $NAG_4\varphi NO_2$. Peak III was identified as lysozyme with Lowry's reagent. Similar results were obtained for the reaction of NAG-deoxy-GLU-φNO_2 with lysozyme. In both cases peak II increased with time and peak I decreased. It is possible that even higher oligomers than NAG-GLU-NAG-GLU-φNO_2 were present in very low yields. This chromatographic procedure was not capable of distinctly separating higher oligomers.

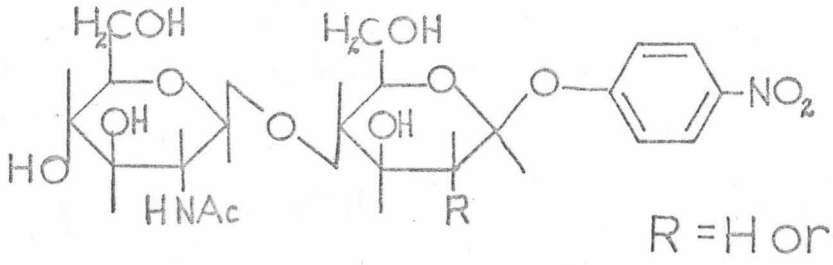
The experiments designed to monitor the percentage of transfer with respect to pH was based on the scheme in Figure 4. Under well chosen conditions, NAG_6 will cleave exclusively into NAG_4 and NAG_2.[8] The intermediate, NAG_4 bound to lysozyme ABCD, will be partitioned into products NAG_4 and $NAG_5\varphi$ in the presence of the acceptor $NAG\varphi$ (Figure 2). This acceptor saccharide was chosen for convenience of separation of the products. It was present in a concentration much greater than that of NAG_6 so as to minimize the reaction of NAG_6 as an acceptor to produce NAG_{10}. Also the reaction was allowed to proceed only to ~ 3% completion so that the total amount of trans-glycosylation product plus hydrolysis product was only a small percentage of NAG_6 initially present. Thus $NAG_5\varphi$ did not compete significantly with NAG_6 for further reaction with lysozyme.

It was not possible to completely separate all the compounds of the reaction mixture by gel filtration or ion exchange chromatography, mainly because of the very large excess of NAG_6 present over NAG_5 and NAG_4. However, NAG_2 and $NAG_5\varphi$ could be separated from NAG_6 and NAG_4 (which were eluted very close together) on Dowex-50W resin (Figure 2). The polystyrene backbone bound the aromatic aglycone of $NAG_5\varphi$ enough to retard its elution. The fractions were monitored for ^3H. The separation between NAG_2 (a measure of total reaction) and $NAG_5\varphi$ (a measure of transfer) was not always complete so the fractions containing both these products were lyophilized and eluted from a P-4 column (Figure 3) which separated them distinctly. The ratio of transfer/total reaction was calculated from the sums of all the counts under the appropriate peaks.

The results of the partitioning vs. pH study are shown in Figure 5 . The effect of pH on the fraction of transfer was really very small; the fraction of transfer varied from 25 to just over 30% between pH 3 and 9. The errors from such a slight effect are quite large and there is considerable scatter in the data. The errors arise from incomplete separation in the first chromatography (Dowex-50) with loss of some NAG_2 and the random error associated with handling of the material during two chromatographic separations. Small contaminations in NAG_6 also proved to be a considerable nuisance. The data do however clearly show that there is a pH dependence of the ratio of transfer/total reaction, on a group of pKa about 6.5 (between 6 and 7).

Discussion

From the work of T. Rand-Meir, et al.[6] it is known that lysozyme cleaves only the aryl glucosidic bond of the synthetic substrates with the general structure



Therefore any oligosaccharides found in the reaction between lysozyme and NAG-GLU-φNO_2 (or NAG-deoxGLU-φNO_2) which are larger than the starting material must necessarily have been formed from the transfer reaction of lysozyme with NAG-GLU as the intermediate (and NAG-GLU-φNO_2 acting as the acceptor). The presence of a higher oligosaccharide (identified as NAG-GLU-NAG-GLU-φNO_2) in the lysozyme reaction of NAG GLU-φNO_2 proves that transfer does take place when the sugar residing in site D (whose C_1 oxygen bond is broken) does not have an acetamido group at C-2. This result strongly suggests that collapse to an oxazoline intermediate does not take place in the post rate-limiting step. It seems likely that if the acetamido group were necessary for the stability of the intermediate which goes on to react with an acceptor molecule (water or sugar) then denied its normal stabilization the intermediate would react only with water, which is present in very much larger concentrations than any sugar acceptor

The results obtained from the reaction of NAG-deoxy-GLU-φNO_2 again indicate that transfer does occur. Therefore, it is clear that in the case of NAG-GLU-φNO_2, the C-2 hydroxyl is not participating in the formation of an epoxide intermediate (via attack of the C-2 hydroxyl oxygen on C_1) to stabilize the carbonium ion formed in the rate limiting step.

The remaining possible structures for the stabilized intermediate are: 1) a carbonium ion stabilized to some degree electrostatically; or 2) an acyl formed by reaction of the carbonium ion with asp 52. These two can be distinguished if some group on the enzyme close to the active site with a known pKa reacts in a differential manner to transfer and hydrolysis (the group most likely to be affected is glu 35).

The results of the study of the affect of pH on the fraction of transfer (Figure 5) indicate that a group of pKa $\sim$ 6.5 is affecting the post-rate limiting step(s) of the lysozyme reaction, and in particular this group is affecting the ratio of the concentrations of the lysozyme sugar intermediates with water and with sugar bound in site E. The rate of transglycosylation is proportional to the concentration of the lysozyme intermediate with acceptor sugar bound in site E (E-T M) The hydrolysis rate is similarly proportional to the concentration of the lysozyme intermediate with water in site E.(E-T) (figure 6)

The only way the fraction of transglycosylation can be affected by pH is if the group seen at pKa ~ 6.5 differentially affects the binding of acceptor molecules in site E in some way. Virtually the only candidates for a direct binding effect are glu 35 and asp 52, situated between sites D and E. Asp 52 is unlikely to be the group affecting the transfer/hydrolysis ratio for several reasons. First of all its pKa is 4.5 in the unbound enzyme and does not change when site C is filled. Although a change in dielectric constant when sites D and E are occupied could raise its pKa, it is unlikely that such a large change would be seen (glu 35 changes only from pKa ~ 5.9 to 6.3 when site D is bound). Also, since asp 52 must be involved in the stabilization of the intermediate either electrostatically or covalently, it would seem that the intermediate would be destabilized when asp 52 was protonated (at least for the electrostatic case). Then the fraction of hydrolysis (vs. transfer) should increase with pH. The opposite effect is seen. If there exists an acylal intermediate then asp 52 would not show up at all in the titration. So it must be concluded that glu 35 is the group seen if direct binding is affected by pH. If the pKa of a group, through a conformation change, indirectly affects binding, a group other than glu 35 might be involved. However, there are no groups in sites E-F that are likely candidates and the only group in the upper binding sites A-D that might affect a conformation change would be asp 101. But its pKa is ~ 3.7. The only other residue whose intrinsic pKa is in the range of 6.5 is a lone histidine far removed from the active cleft. So the pKa of 6.5 can be assigned to glu 35.

Given the assumption that glu 35 is necessary for reaction in its ionized form, (this follows from microscopic reversibility considerations) then in order for glu 35 to have an effect on the ratio of transfer to total reaction (percent transfer) the species E-T and E-T-M cannot be in equilibrium. A slow step between the two species (step 3) would be sufficient to produce such a situation.

Nominally one would expect to see two pKa's - that of glu 35 in the stabilized intermediate with water in site E and that of glu 35 in the stabilized intermediate with sugar bound in site E. (steps 1 and 4 in figure 6) But only a single titration rather than the bell shaped curve predicted from the kinetic scheme of figure 6 is observed.

The most satisfying explanation is that glu 35 is not available to solvent for titration when sugar is bound in site E. Acceptor sugar will probably bind productively only when glu 35 is ionized. The proton on the C4 hydroxyl of the acceptor sugar is very likely to hydrogen bond to the carboxyl oxygen of ionized glu 35 since glu 35 must pick up this same proton in the next productive step (step 8). This hydrogen bond would effectively prevent the second titration from being seen.

In summary this postulated mechanism predicts that a single titration will be seen - that of glu 35 in the enzyme intermediate E-T with water bound in site E. The pKa of glu 35 will be perturbed from its pKa in the native enzyme by any charges or dielectric changes arising from the stabilized intermediate bound in sites A-D.

Since the pKa of glu 35 is 6.3 in the Michaelis-Menton complex with substrate bound in sites A-D, we can say that the pKa has not altered significantly in the presence of the stabilized intermediate E-T M . (figure 6) If there existed a fully charged carbonium ion next to glu 35 (the model building studies of Blake indicated about a 3Å separation between the carboxyl of glu 35 and the C_1 carbon of the carbonium ion intermediate) it would almost certainly affect its pKa substantially, shifting it to a lower value. Since the pKa remains at ~ 6.5 it must be assumed that there is little positive charge near the carboxyl of glu 35 and therefore the post rate limiting intermediate must either be a covalent acylal or a fairly tight ion pair formed with asp 52 carboxylate anion.

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7. L. M. Mohr, L. E. H. Smith, and M. A. Raftery, Biochim. Biophys. Acta, in press.

8. J. A. Rupley Proc. Roy. Soc. (London), B167, 416 (1967).

Figure 1 - Transglycosylation reaction of lysozyme with NAG- GLUϕNO_2.

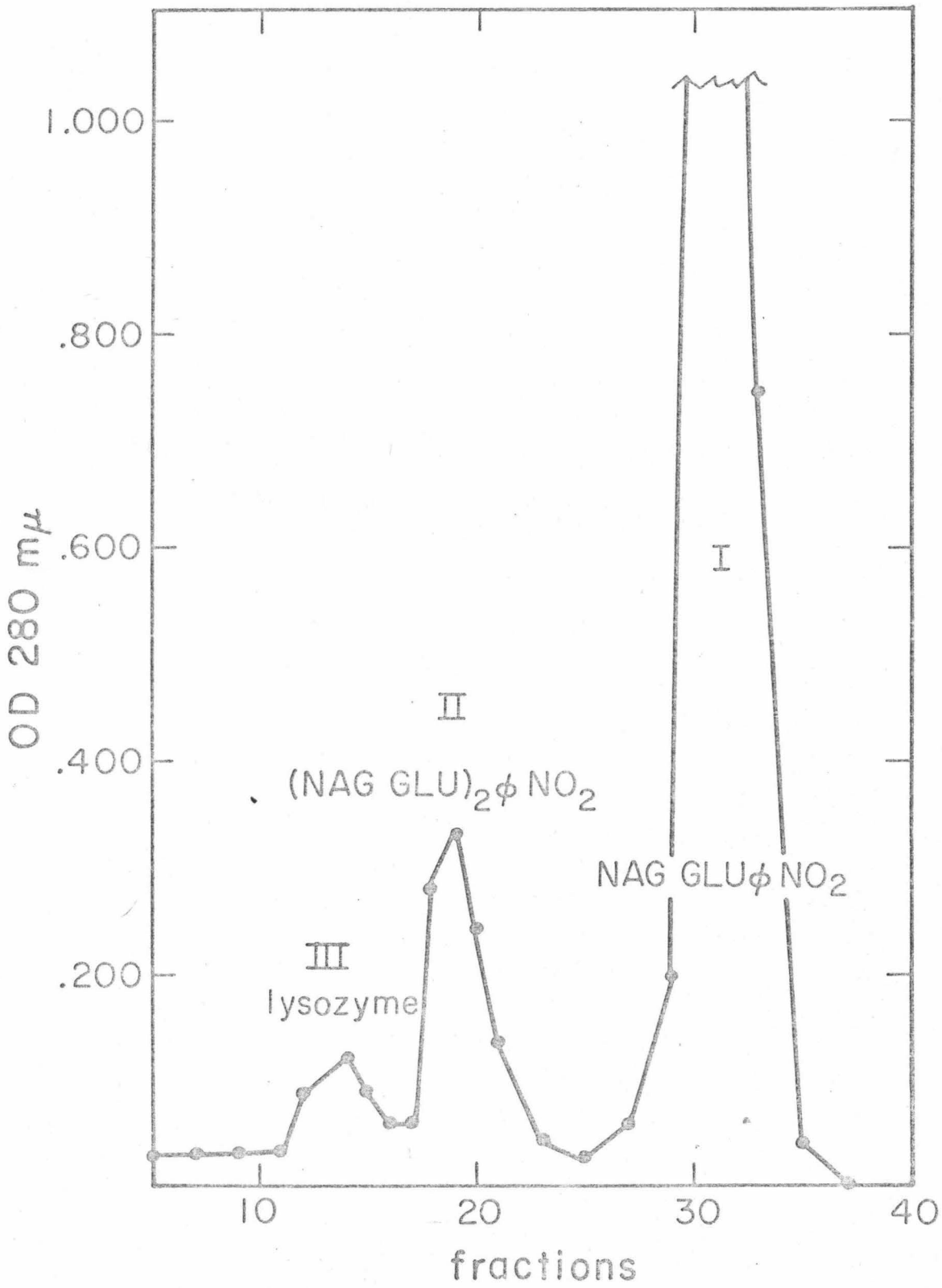

1.000
.800
.600
.400
.200
OD 280 mμ
I
II
(NAG GLU)2φ NO2
NAG GLUφ NO2
III
lysozyme
10
20
30
40
fractions

Figure 4 - Scheme for the reaction of lysozyme with NAG_6 and $NAG\phi$.

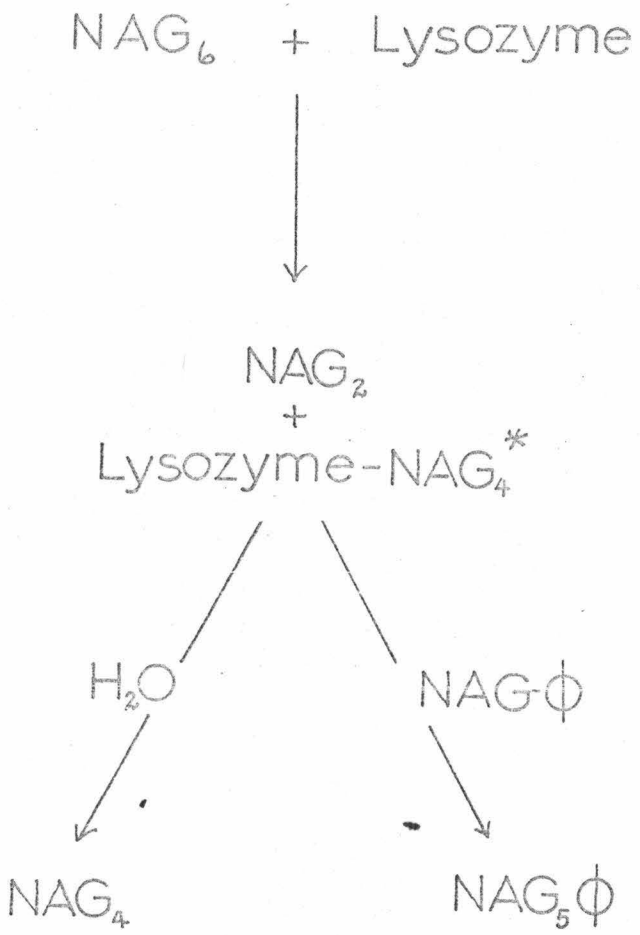

NAG6 + Lysozyme
NAG2
+
Lysozyme-NAG4*
H2O
NAG-ϕ
NAG4
NAG5ϕ

Figure 2 - Chromatography on Dowex 50 W of the reaction mixture from the lysozyme catalyzed hydrolysis and transglycosylation reaction of NAG_6 and $NAG\phi$. The peaks were identified as $NAG_5\phi$ ($V\phi$), NAG_2 (II), and NAG_6 and NAG_4 (VI + IV)

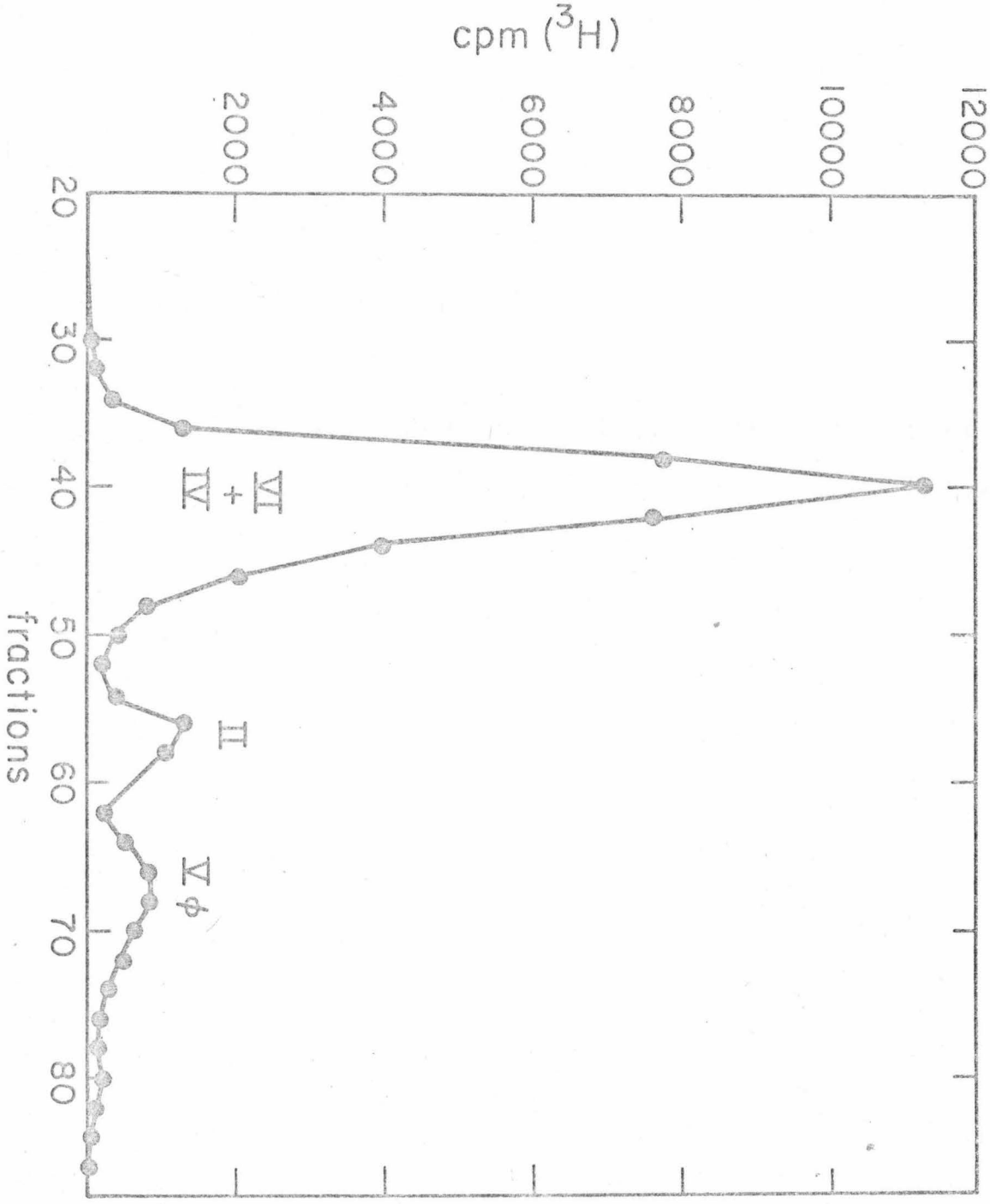

cpm (^{3}H)
2000
4000
6000
8000
10000
12000
20
30
40
50
60
70
80
fractions
IV + VI
II
V φ

Figure 3 - An example (at pH 9) of the chromatographic separation on Bio Gel P4 of NAG_2 (II) and $NAG_5\phi$ (I)

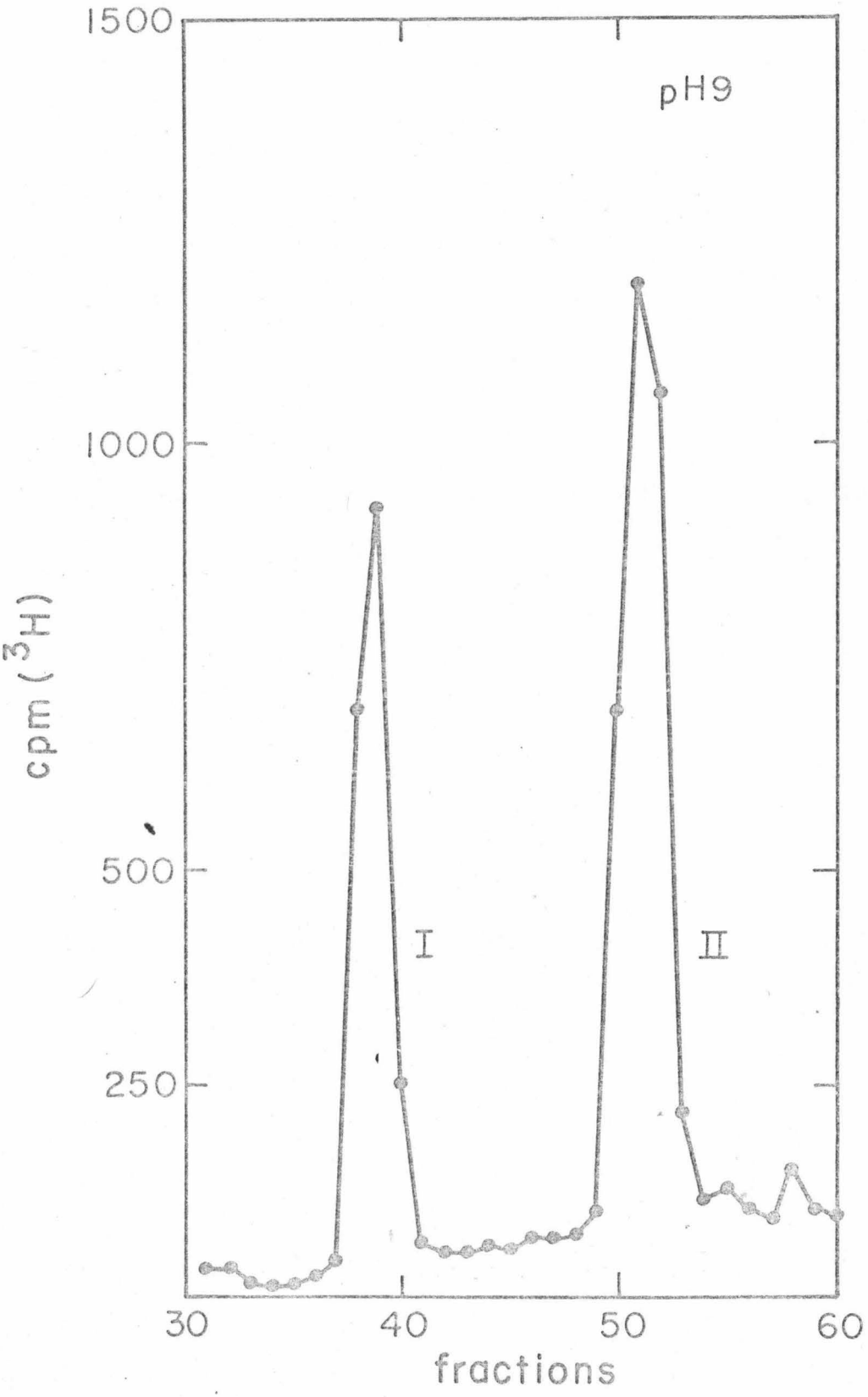

1500
1000
500
250
pH9
cpm (^{3}H)
I
II
30
40
50
60
fractions

Figure 5 - A study of the variation with pH of the ratio of transglycosylation to total reaction (transfer plus hydrolysis).

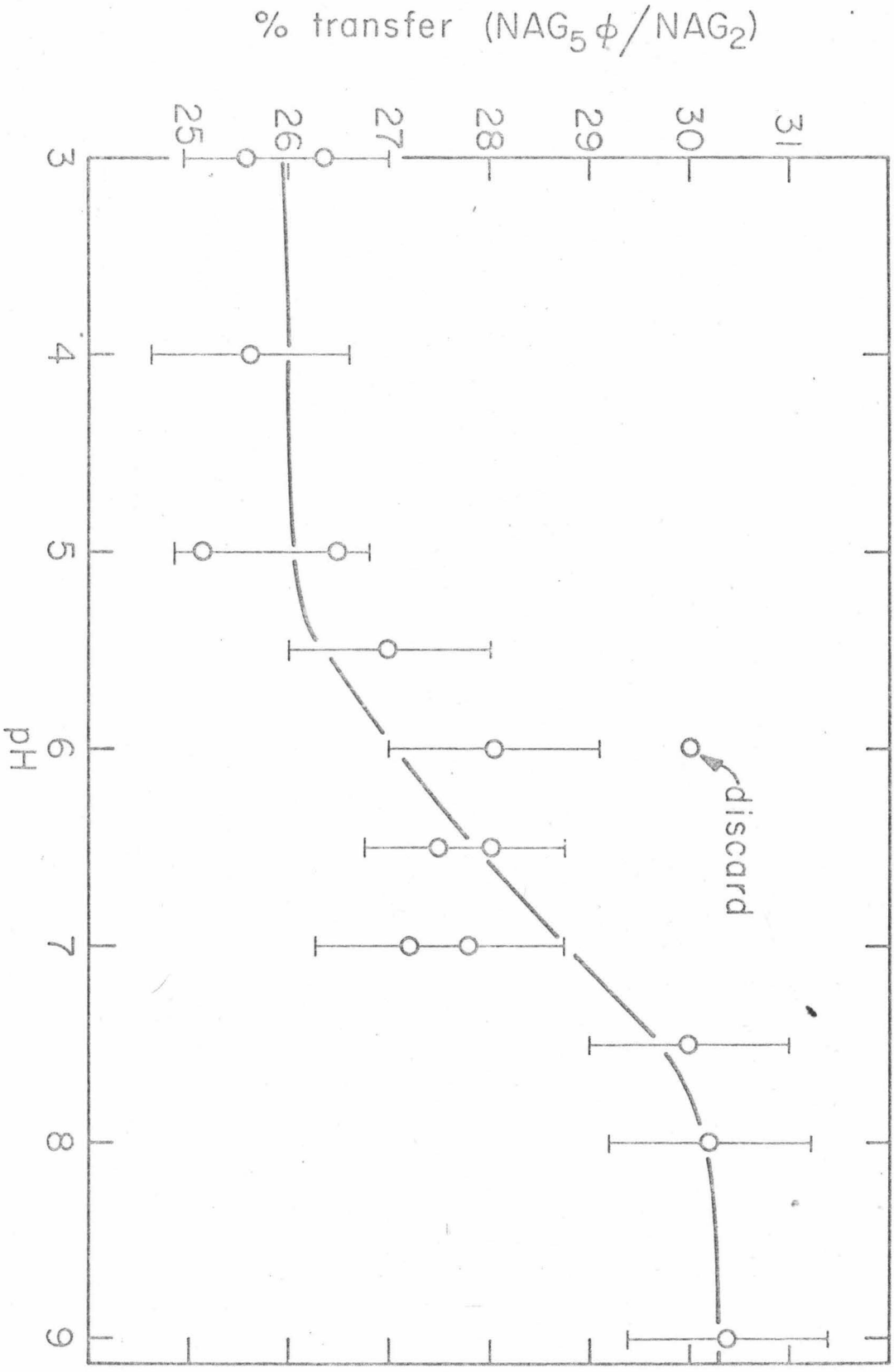
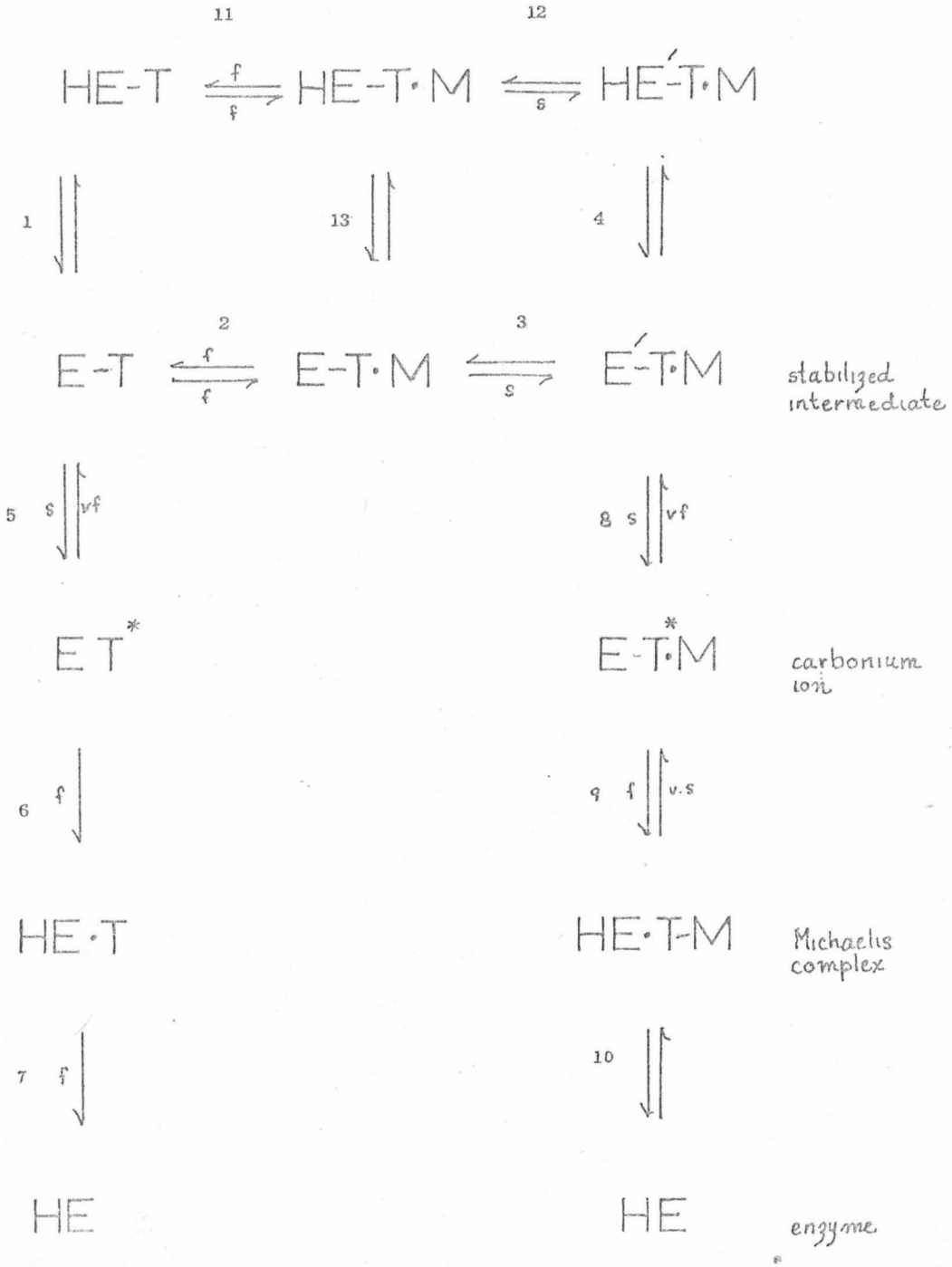

% transfer (NAG5 ϕ/NAG2)
25
26
27
28
29
30
31
3
4
5
6
7
8
9
pH
discard

Figure 6 - A proposed kinetic scheme for lysozyme catalyzed transglycosylation and hydrolysis of NAG_6. The species with H (such as HE and HE-T) indicate that glu 35 is protonated. The species with T (such as E-T) indicate that NAG_4 is bound in sites ABCD. M indicates that an acceptor sugar is bound in site E (and perhaps in F as well)


11
12
HE-T
f
f
HE-T·M
s
HE´-T·M
1
13
4
E-T
2
f
f
E-T·M
3
s
E´-T·M
stabilized intermediate
5
s
vf
8
s
vf
E T*
E-T·M*
carbonium ion
6
f
9
f
v.s
HE·T
HE·T-M
Michaelis complex
7
f
10
HE
HE
enzyme

Propositions

Proposed: A direct method to prove the theory of assymetry of lipid membrane structure of erthyrocytes.

Lipids are an important component of mammalian cell plasma membrane, constituting about half of their mass. This important class of compounds falls into three categories--phospholipids, the major component of most cells, neutral lipids such as cholesterol and glycolipid.[1] The lipids are thought to be arranged in a bilayer structure[2] with the hydrophobic fatty acid chains in the center of the sheet and the polar head groups on the outside.

Phospholipids in a membrane structure exhibit a melting transition at a particular temperature which is very dependent upon the nature of the fatty acid side chain of the phospholipid. Below the melting temperature the lipids are rigid; above the melting temperature the phospholipids are free to exchange with neighbors, as quickly as 10^{-6} sec.[3,4] However, a "flip-flop" motion--that is exchange of lipids between two sides of the bilayer is very much less frequent, the half life to flip-flop for a phospholipid being about 6 hours at 30°.[5]

Of the major classes of phospholipids, phosphatidyl choline (PC) sphingomyelin, phosphatidylserine (PS) and phosphatidylethanolamine (PE) are most commonly found in mammalian cells (Figure 1). The proportion of sphingomyelin to PC varies from 0.25 to 60 (and in

the erythrocytes of herbivores PC is essentially absent). However sphingomyelin appears to replace PC. The sum of PC plus sphingomyelin remains fairly constant (0.55 to 1)[6,7] and accounts in most species for about 60% of the total phospholipid.

Bretscher[8] has proposed that the reason for the constancy of choline and amino phospholipids is that the choline phospholipids constitute the outer half of the bilayer leaflet and the amino phospholipids constitute the inner cytoplasmic half of the membrane bilayer.

There exists indirect evidence to support such a proposal:

1) Erythrocytes from most mammals are lysed by crude preparations of phospholipase A_2 which cleaves the fatty acyl ester bound on any phospholipid except sphingomyelin. Phospholipase A_2 will not attack sheep erythrocytes which have no PC.[6]

2) A crude phospholipase from sea snake degrades some PC from intact erythrocytes. When added to erythrocyte ghosts (lysed cells) the enzyme degrades PS and PE as well.[9]

3) Reagents known to react with amino groups do not react with the surface of intact erythrocytes and seem to be more reactive to cell ghosts.[10]

However, the evidence is indirect and it could be argued that there are difficulties with accessibility especially for the phospholipase experiments.

A much more direct approach would be to label PC with radioactive choline in vivo then measure the amount of label on each side of the bilayer.[1] Normally this could prove exceedingly difficult since a mammal would have to fed labeled choline for some time and the label would certainly be incorporated nonspecifically.

It is proposed that the use of immature red blood cells--reticulocytes--will provide a simple way of radioactively labeling PC in an erythrocyte.

It has been discovered recently[11] that reticulocytes isolated from rabbits with phenylhydrazine induced reticulocytosis are found to contain an enzyme which catalyzes the synthesis of PC from CDP-choline and 1,2-digyceride In contrast, erythrocytes are devoid of this enzyme. The specific incorporation of CDP choline into PC according to thin layer chromatographic analysis was >90%.

Once radioactive PC is incorporated into the reticulocyte membrane it is necessary to measure the amount of radioactive lipid on each side of the membrane bilayer. Freeze fracture has been utilized to separate a bilayer of sodium stearate, labeled on one side only. The freeze fracture method specifically cleaved the bilayer in the plane of the hydrocarbon tails. This same freeze fracture technique could be applied to reticulocyte membranes. This procedure in essence consists of allowing bilayers to adhere to a glass plate, freezing the plate and a thin layer of water on it with dry ice, then separating the ice from the glass. Only the outer half of the bilayer remains on the glass, which can then be counted in a thin

window gas flow geiger counter. The lipids could also be washed off the plate and tested for choline, phosphate and counted (C^{14}). The specific activity of the freeze fractured lipids could be compared to the specific activity of lipids in the whole reticulocyte and in the starting material.

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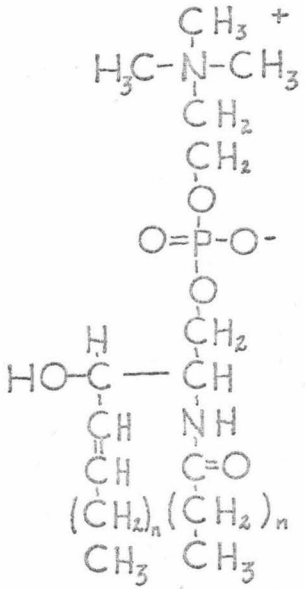
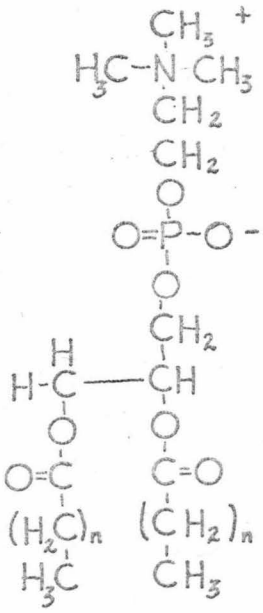
Figure 1 - Common phospholipids

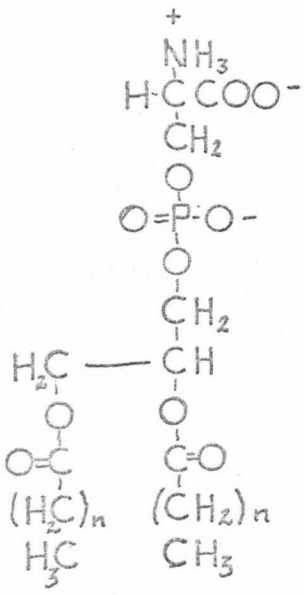
sphingomyelin



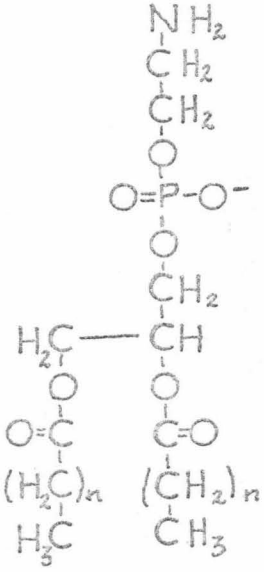
phosphotidyl choline



phosphatidyl serine



phosphatidyl ethanolamine



Proposed: Labeling lysozyme's asp 52 by hydrolysis of the β ethyl ester with $H_2{}^{18}O$. Isotope effects would indicate any participation of asp 52 in the formation of the carbonium ion.

The glycosidic activity of hen egg white lysozyme has been attributed to the side chains of glu 35 acting as a general acid and asp 52 acting as a general base. The protonated carbonyl group of glu 35 donates a proton to the C_4 oxygen of the sugar residue in site E, with subsequent formation of a carbonium ion intermediate in site D. It has been suggested that asp 52 in its ionized form stabilizes this positively charged intermediate. It has also been suggested that the carbonium ion collapses with asp 52 in a post-rate determining step to form an acylal intermediate. The difference between a covalent bond between C_1 of the sugar residue in site D and the O of asp 52 carbonyl group and ion pair formation is only in the degree of bond formation.

The carboxyl oxygen of asp 52 is a suitable site for isotopic labeling for a complementary isotope effect study. The other atom ivolved in the proposed acylal formation--the C-1 carbon of the sugar residue in site D has effectively been studied, via the secondary isotope effects arising from changes in the C-1 hydrogen bond during catalysis. There was found to be considerable carbonium ion

character in the transition state (k_H/k_T = 19%). But secondary tritium isotope effects of >30% are known for reactions involving an unstabilized carbonium ion in the transition state. So there may well be some degree of bond formation between asp 52's carboxyl oxygen and C-1 carbon in the transition state if asp 52 does indeed stabilize the carbonium ion as has been suggested. There could be a larger isotope effect if reaction of the acceptor molecule in site E (saccharide or water) with the intermediate is partially rate determining.

It is possible to distinguish whether or not the formation of the carbonium ion is wholly rate determining. If the formation of the intermediate totally governs the rate of reaction, then if the concentration tion of the two acceptors for the lysozyme reaction (water and sugar) is varied, the partitioning of the intermediate into products may vary but the sum of the rates of production of hydrolysis and transfer products will be the same, and will correspond to the rate of formation of the intermediate. If the production of the carbonium ion is not entirely rate limiting, then there might well be a larger isotope effect for asp 52 than that expected from the previous isotope studies on C-1.

The specific labeling of asp 52 can be accomplished utilizing the asp 52 ethyl ester derivative of lysozyme prepared by Parsons and Raftery.[1] The derivative can be hydrolyzed at pH 2 to regenerate active lysozyme. If this derivative were hydrolyzed in

$H_2{}^{18}O$, specific incorporation of ^{18}O into the enzyme could be achieved (or any other oxygen isotope such as ^{17}O or even S could be incorporated specifically). The maximum labeling would be 50% since only one of the two asp 52 carboxyl oxygens would be incorporated from the solvent.

The maximum $^{16}O/^{18}O$ isotope effect has been calculated to be 19%.[2] Few isotope effect studies have been reported with ^{18}O, but the effects range from ~1% to 10%.[3]

The proposed isotope effect study should indicate how much bond formation there is between asp 52 and C-1 of the reacting substrate in site D in the transition state. This information would complement what is known about the carbonium ion character of the transition state.

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Proposed: A scheme to identify rhodopsin as a Ca^{++} channel in a ROS disc and "map" its ionic pore

Vertebrate rod photoreceptor cells found in the retina of the eye consist of three parts: 1) a synaptic terminal leading eventually to the optic nerve and then to the brain; 2) a cell body containing the nucleus and metabolic organelles; and 3) a large outer segment containing the light sensitive system. The rod outer segment consists of stacks of enclosed membranes--disc-like sacs--enclosed by an outer segment membrane.[1] The rod outer segment discs (ROS) are free-floating membranous intracellular organelles with an interior space which is separate and distinct from the photoreceptor intracellular space.

Rhodopsin, the visual pigment of the photoreceptor cells is a structural component of the ROS disc membrane[3] and constitutes about 35% of the dry weight of the discs (and 80-90% of the protein) the rest being mostly phospholipids.[4] The initial step in the process resulting in visual sensation is the absorption of a photon by rhodopsin followed by cis-trans isomerization of retinal, the carotenoid chromophore bound to the apoprotein opsin. The protein changes conformation concomitantly.[5,6]

The mechanism of the visual process is not completely understood but these important properties of vertebrate rods are known.

There is a dark current across the ROS plasma membrane which is suppressed by light.[11]

Sodium ions must be present in the bathing solution for photocurrents to be obtained. The dark current and photocurrent are largest in solutions containing less than 10^{-5} M Ca^{++}. As the Ca^{++} concentration is raised to 20 mM, both currents become smaller and then disappear. The effects of external Ca^{++} are rapid and reversible. Despite their physical separation from the plasma membrane nearly all the rhodopsin bearing internal discs of rods are capable when illuminated of suppressing the dark current through the plasma membrane.[11]

These observations have been explained by Hagins with a scheme employing three assumptions[8]:

1) $[Ca^{++}]$ is maintained by pumps at a much lower level in the cytoplasm of the ROS than in intradisc spaces

2) the Na^{+} conductance of ROS membrane decreases as cytoplasmic $[Ca^{++}]$ increases

3) Light transiently increases the permeability of the disc membrane to Ca^{++}, allowing many Ca^{++} ions to enter the cytoplasmic space per photon absorbed.

The third assumption has been shown to be valid. Mason and co-workers[7] have found direct experimental evidence that Ca^{++} is released by the ROS discs upon light exposure. The discs were isolated and allowed to accumulate radioactive Ca^{++}. The disc

membranes were then bleached for various time periods. It was found that Ca^{++} was released from the discs in direct response to the amount of photopigment bleached.

Although rhodopsin changes conformation upon bleaching and bleaching produces a Ca^{++} flux from the ROS discs it is not clear that rhodopsin acts alone or in concert with another protein(s) which then acts as an ion pore or channel.

The most direct proof that rhodopsin alone is the Ca^{++} ionophore as well as the photoreceptor is to isolate rhodopsin[12] from isolated ROS discs[13] as well as ROS disc lipids.[12] These are all known procedures. The rhodopsin and ROS disc lipids could then be recombined so that closed vesicles were formed. The same Ca^{++} flux studies performed with whole ROS discs could then be repeated with the vesicles containing rhodopsin as the sole protein. Recombination of purified rhodopsin and phosphatidyl choline into bilayers has been achieved by Hubbell,[14] using a detergent to solubilize and purify the photopigment with subsequent dialysis of the rhodopsin, lipid, detergent mixture to remove detergent. The formation of closed bilayers (vesicles) rather than multilamellar sheets can usually be achieved by varying conditions during dialysis[15] or by sonication.

A repetition of the result found with whole ROS discs, that is a linear release of Ca^{++} with bleaching, would indicate that rhodopsin acts as both photoreceptor and ionophore.

If rhodopsin does indeed function as a pore, then further studies to elucidate the pore mechanism are warranted. It is proposed that the amine acids in the pore can be identified and "mapped".

Rhodopsin can be synthesized in vitro using intact bovine retinas bathed in a solution of amino acids and other nutrients.[16] ROS discs labeled with ^{14}C leucine have been prepared so it should be possible to incorporate sufficient quantities of a ^{13}C (or other NMR sensitive isotope such as ^{19}F or ^{15}N) labeled amino acid into rhodopsin to obtain an NMR spectrum (a solution labeled ~ 1 mM in rhodopsin would suffice). Each amino acid in rhodopsin could be present in more than one position, but the polar ones are likely to occur mostly in the pore or in the part of the molecule exposed to an aqueous environment. Most of rhodopsin is surrounded by a phospholipid membrane. It is difficult to say how broad specific resonances might be, but rhodopsin is known to be in a very fluid lipid environment. Amino acid side chains exposed to water would very likely be sharp.

So the amino acids of the pore could be mapped in the following way:

1) prepare labeled ROS discs with a specific amino acid
2) look at NMR spectrum in the bleached and unbleached state --in the bleached state the resonances belonging to amino acids in the pore may sharpen since they are now exposed to water

3) look at NMR spectrum in the presence of Mn^{++} in the unbleached states Mn^{++} should act as Ca^{++} normally does having the same charge and nearly the same ionic radius. Mn^{++} is a paramagnetic ion, however, and it should now broaden the resonances belonging to groups in the pore itself when rhodopsin is bleached. When it is unbleached, Mn^{++} will broaden only those resonances exposed to solvent on the outside of the protein.

In this manner, it may be possible to identify specific amino acids in the pore (and those exposed to solvent on the interior of the disc and on the disc's exterior).

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Proposed: A method for isolation of the botulinum toxin receptor.

Although in the past few years much progress has been made in the elucidation of the mechanisms of nerve function, most achievements have been in the understanding of the functions of the post-synaptic side of the neural junction. Very little is known about the mechanisms of acetylcholine (ACh) release or movement on the presynaptic membrane except that

1) an action potential travelling down a nerve axon will cause release of ACh into the synaptic cleft;

2) there exist vesicular structures which contain ACh from which release is most likely to occur;

3) some ACh is released spontaneously (miniature end plate potentials)(mepp) ;

4) Ca^{++} is involved in release of ACh.

Specific toxins have proven to be a very useful tool in solving the problems of isolation, purification, and elucidation of the role of specific parts of the neuromuscular junction. There exists a paradox in dealing with some neurotoxins. Investigators are attempting to decipher pathophysiology nearer the molecular level than the knowledge of physiology will support. It is known that botulinum toxin suppresses the release or movement of ACh but almost nothing is known about the mechanisms of ACh release or movement. It would

seem that the best approach to this difficulty would be isolation (and subsequent study of) the specific molecules which interact with the toxin in order to elucidate their physiological function.

Present evidence indicates botulinum toxin acts presynaptically to block both mepp and end plate potentials.[2] This effect is not a consequence of interference with impulse conduction in the motor nerve or of inhibition of synthesis or storage of ACh.[2,3] The toxin must evidently affect the mechanism by which quanta of ACh are released from the nerve endings. It has been shown that this blockage does not occur via interference with Ca^{++} uptake which is known to be coupled to quantal ACh release.[5] Electron microscopy studies utilizing conjugates of botulinum toxin and ferritin confirmed that toxin specifically situates itself within the cholinergic synaptic interspace.[6]

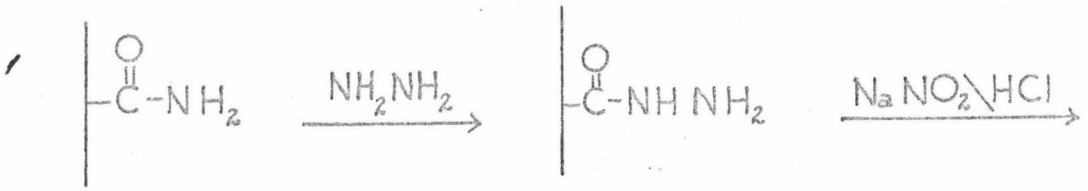
Botulinum neurotoxin is a protein of molecular weight $\sim 130,000$[7] and is the most toxic substance known. An estimate of 0.5-5 μg toxin per human oral lethal dose has been made.[7] A 5 ml volume of ingested fluid need be only 10^{-9} M in toxin to be lethal. The binding of neurotoxin seems to be specific, rapid, and very strong.[8] The experiments of Bülbring indicate that antiserum cannot free bound toxin on nerve diaphragms nor can extensive washing.

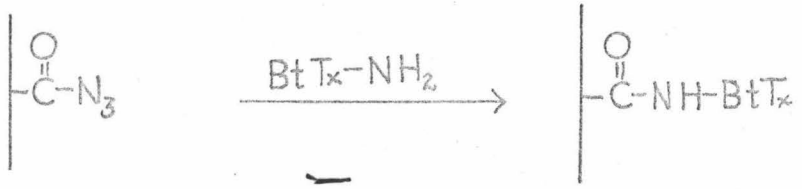
The botulinum neurotoxin receptor must then possess some properties which will facilitate its isolation--specificity of binding and strength of binding to the toxin. The most difficult problem to be surmounted in the isolation of the BtTx receptor rests on the

probability of there being a very low concentration since so little toxin is required for cessation of function. This last difficulty can be overcome with the multiple use of affinity chromatography. The unique properties of BtTx and the low concentration of its receptor preclude a direct and simple approach to the isolation. BtTX cannot be used directly as an affinity resin since the receptor undoubtedly binds irreversibly. Nor can toxin be used as an antigen for the production of antibodies (Ab) to be used to isolate a labeled BtTx-receptor complex since the rabbit would die before Ab production could begin.

The following scheme is proposed:

1) use of BtTx bound to polyacrylamide gel (or agarose)





--this resin would selectively bind BtTx receptor from a crude preparation of electroplax.

2) preparation of Ab against whole affinity resin-receptor complex--injection of the total washed resin into rabbits for

production of Ab against BtTx receptor and BtTx. The advantage of this method is that the active sites of both receptor and toxin are blocked by each other and the rabbit will not die from toxin poisoning or from Ab binding to its own receptor. (Hartman and co-workers have shown that polyacrylamide gels can be injected into rabbits for Ab production).[9]

3) purification of Ab

a) removal of non-specific serum protein by elution of rabbit serum from BtTx-receptor column of step 1

b) take Ab bound to resin in step a, containing Ab to BtTx and to receptor and elute from Bt-Tx affinity-resin. The eluent will contain Ab to receptor alone.

4) preparation of a reversibly binding affinity resin with Ab--using the same procedure as step 1 the specific Ab could be attached to polyacrylamide beads. There could be a preselection of more weakly binding Ab by precipitation of the more strongly binding ones with a subequimolar quantity of receptor before Ab are bound to the affinity resin. This would ensure that the receptor bound reversibly under nondenaturing conditions.

If there still exist multiple bands on SDS polyacrylamide gels (after electro phoresis) the individual bands can again be injected into rabbits for production of specific antibodies. (From the work of Elgin and Stumpf (unpublished) it is known that SDS treated proteins will produce Ab.)

The best source of BtTx receptor is probably electroplax from electric fish.

It is interesting to speculate on the possible function of the BtTx receptor. It would seem likely that Botulinum neurotoxin blocks some step preceding an amplification step since the toxin is effective in such low concentration. Perhaps there exists a vesicle release site which is blocked by the toxin. With isolated BtTx receptor this and other possible roles can be examined experimentally.

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