LYSOZYME-SUBSTRATE REACTIONS AND HYDROLYSIS OF GLYCOSIDASE-GLYCOSIDE MODELS

Thesis for the Degree of Ph. D.
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LYSOZYME-SUBSTRATE REACTIONS AND HYDROLYSIS OF GLYCOSIDASE-GLYCOSIDE MODELS

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Ph. D. degree in Biochemistry

John C. Speck, Jr.
Major professor

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ABSTRACT

LYSOZYME-SUBSTRATE REACTIONS AND HYDROLYSIS
OF GLYCOSIDASE-GLYCOSIDE MODELS

By

Hernan Nunez

The catalytic role of the amino acid side chains at the active site of lysozyme was studied. The investigation was carried out with lysozyme itself and with model compounds.

The participation of a protein carboxyl or amide group in the lysozyme-catalyzed reaction would result in an acylal or an imidate glycosyl-enzyme intermediate, respectively. Among the compounds that possibly would react with these intermediates are hydroxylamine, with the acylal, and sodium borohydride, with the imidate. Both of these substances were investigated as trapping reagents during the lysozyme-catalyzed reaction. The results indicated that neither of these reagents trapped these intermediates, if present. In the reaction with the model compounds, hydroxylamine formed five percent hydroxamic acid with tetrahydropyranyl acetate but sodium borohydride did not reduce methyl or ethyl acetimidate.

The amino acid side chain involved in a stable and apparently covalent enzyme-substrate complex was also

investigated. It was found that the marked stability of the enzyme-substrate complex is the result of electrostatic interactions, since high salt concentration, pepsin hydrolysis, and complete enzymatic hydrolysis dissociated the substrate from the enzyme.

The following acetals were synthesized, and their hydrolysis rates in 50% dioxane-water were determined: acetamidoacetaldehyde diethyl acetal, 3-([tetrahydropyran-2-yl)oxy]-propionic acid methyl ester, 3-[(tetrahydropyran-2-yl)oxy]-propionic acid, 3-[(tetrahydropyran-2-yl)oxy]-propionamide, 2-(tetrahydropyran-2-yloxy)-acetic acid ethyl ester, 2-(tetrahydropyran-2-yloxy)-acetic acid, 2-(tetrahydropyran-2-yloxy)-acetamide, 6-ethoxytetrahydropyran-2-carboxylic acid ethyl ester, 6-ethoxytetrahydropyran-2-carboxylic acid, and 6-ethoxytetrahydropyran-2-carboxamide.

These glycoside model compounds contain neighboring groups which resemble either the amino acid side chains at the lysozyme active site or the acetamido group in some lysozyme substrates.

The results indicated that specific acid catalysis governs the hydrolysis of these compounds, i.e., there is no intramolecular catalysis by the acetamido, amide, and carboxyl neighboring group.

The effects of the amide, carboxyl and ester substituents in the rate constants for the hydrolysis of the tetrahydropyran derivatives indicate that the hydrolysis proceeds via

a cyclic carbonium ion mechanism. Since the substituent effects on the hydrolysis rate constants of the tetrahydropyran derivatives resemble the structural effects on the hydrolysis of glucopyranosides, the results suggest that the glucopyranoside hydrolysis also proceeds via a cyclic carbonium ion mechanism.

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By

Hernan Nunez

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TABLE OF CONTENTS

TABLE OF CONTENTS--continued Page

TABLE OF CONTENTS--continued Page

LIST OF TABLES

LIST OF TABLES--continued

LIST OF FIGURES

INTRODUCTION

Approach of the Present Investigation

Lysozyme (3.2.1.17 N-acetylmuramide glycosylhydrolase) is the most thoroughly characterized glycosidase at the present time. Although its three dimensional molecular structure has been well defined by x-ray analysis, its catalytic mechanism, i.e., the means by which it catalyzes cleavage of the polysaccharide molecule, remains hypothetical (1,2). The purpose of this study was to clarify some aspects of this mechanism.

Two approaches were used in this investigation. The first one concerned the lysozyme-catalyzed reaction itself. It involved a) the determination of the nature of an enzyme-substrate complex formed between lysozyme and soluble M. luteus cell wall material discovered by Rynbrandt (3), and b) the search for chemical reactions that would trap a glycosyl-enzyme intermediate possibly formed during the lysozyme-catalyzed cell wall hydrolysis.

In the second approach the rate constants for hydrolysis of acetal model compounds were studied. The acetal model compounds contain neighboring groups which resemble either

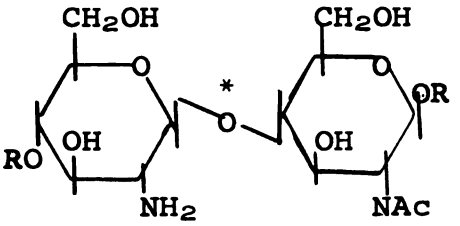
the amino acid side chains at the lysozyme active site or the acetamido group on the substrate molecule. Since all lysozyme substrates are cyclic acetals, mechanistic information may be obtained from these model compounds.

Structure of the Lysozyme Substrates

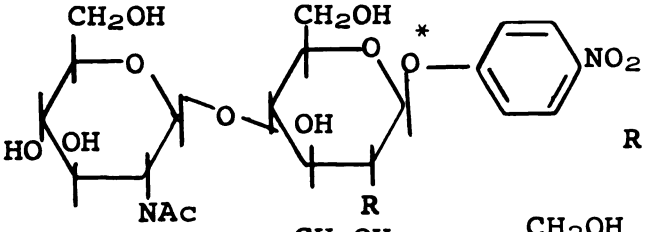
Lysozyme catalyzes the hydrolysis of the β-(1-4) linkage between N-acetylmuramic acid and N-acetylglucosamine residues found in the polysaccharide material of most bacterial cell wall; it also catalyzes hydrolysis of N-acetylglucosamine oligomers derived from chitin (4-8), β-linked benzyl glycosides of di-N-acetylchitobiose (9), β-aryl glycosides of di-N-acetylchitotriose (10,11), and β-aryl glycosides of tri-N-acetylchitotriose (12).

Recently (15-17,22), several compounds with a structure in which the 2-N-acetyl substituent on the sugar ring is absent (Figure 1), have been reported as undergoing lysozyme-catalyzed hydrolysis. It would seem therefore that the substrate acetamido group is not essential for lysozyme activity. Evidence will be reviewed later, however, that shows that the lysozyme-catalyzed hydrolysis seems to go faster when the acetamido group is present than when it is absent from the substrate (13,17).

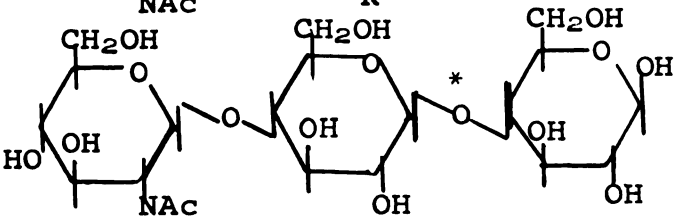
The substrate used in the preparation of the enzyme-substrate complexes studied in the present investigation was



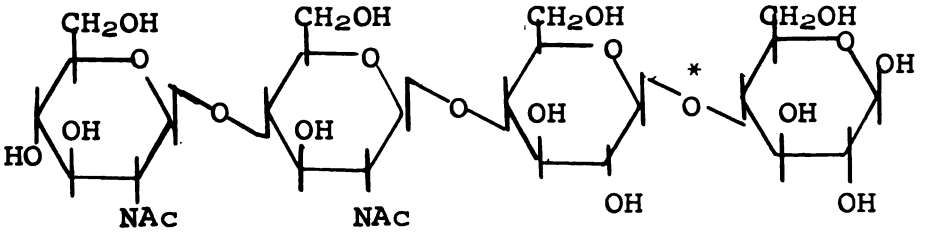
R = partially de-N-acetylated chitin oligomers (Ref. 17)



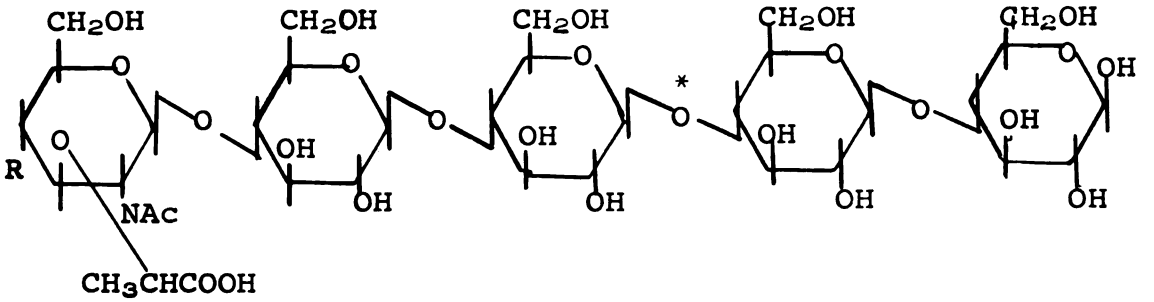
R = OH, NAc (Ref. 13)



(Ref. 16)



(Ref. 16)



R = N-acetylglucosamine (Ref. 16)

* Bond cleaved in the lysozyme-catalyzed reaction

Figure 1. Compounds cleaved by lysozyme.

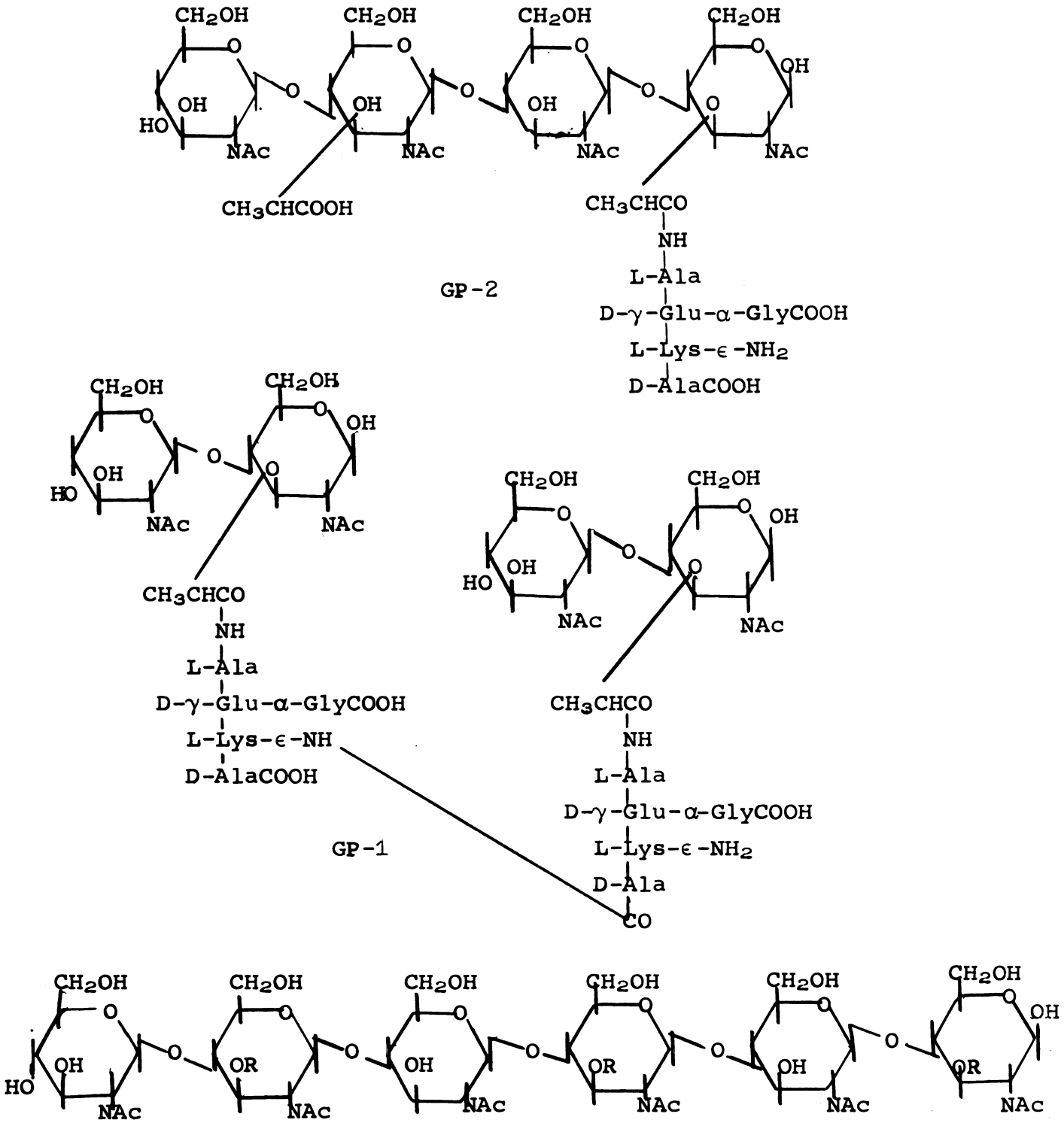


Figure 2. Species isolated from M. luteus cell walls after controlled lysozyme hydrolysis (3,18).

a glycopeptide (GP-2) obtained from M. luteus cell walls by Rynbrandt (3) and Sharon and co-workers (18). Fraction I (3) was also used. Fraction I is a mixture of oligosaccharides (mainly hexasaccharide) and glycopeptides (mainly GP-1). The structure of these species is shown in Figure 2.

Lysozyme Active Site Structure

The three-dimensional structures determined by x-ray studies of lysozyme and lysozyme-inhibitor complexes indicate that the active site of the enzyme is a cleft which is only half filled when tri-N-acetylglucosamine is an inhibitor. A total of six sugar residues can be fitted into this cleft in a satisfactory manner. Several specific interactions between the sugar residues and the protein molecule can be seen in such a structure. Each sugar residue occupies a "subsite" in the cleft, and the six subsites have been designated A, B, C, D, E and F respectively (19,20,21).

By comparing the products formed from the hydrolysis of various chitin and cell wall oligomers, it has been concluded that the cleavage in the lysozyme reaction occurs between the D and E subsites (22,23,24).

Model building indicates that sugar residue D makes reasonable contacts with the atoms of the protein molecule except that its CH_2OH group makes too close a contact with the main chain CO of residue 52, with Trp 108, and with the acetamido group of sugar residue C. This overcrowding can

be relieved by distortion of the ring to a half-chair conformation.

Inspection of the environment of subsite D shows that the side chains of Glu 35 and Asp 52 are disposed on each side of the β-(1-4) linkage between the D and E residues, suggesting that these residues are involved in the cleavage mechanism. The carboxyl group of Glu 35 lies in a predominately nonpolar region which makes it likely to be the carboxyl group postulated to have an abnormally high pK_a (approximately 6.3) in the enzyme (25). The carboxyl group of Asp 52 lies in an essentially polar region and appears involved in a complex network by hydrogen bonds. This situation may hold the residue in an ionized carboxylate state. It is interesting to note that the lysozyme-catalyzed hydrolysis of tri-N-acetylglucosamine (24) and some aryl-glycosides (15) appears to have an absolute dependence of activity on a carboxyl group of normal pK_a which probably is that of Asp 52.

Postulated Reaction Mechanisms

By putting to use the information obtained through this static picture of the lysozyme-substrate complex and the information about the reaction mechanism of the nonenzymatic hydrolysis of acetal and glycoside compounds, it is possible to propose probable mechanisms for the lysozyme-catalyzed reaction (28). All these mechanisms have in common the

following steps:

1.--Attachment of the substrate with simultaneous conformational changes of the sugar residue D. This distortion brings the sugar ring part way to the transition state conformation, thus decreasing the activation energy and consequently accelerating the reaction (29).

2.--Cleavage of the substrate linkage between residues bound at subsites D and E. This is accomplished by participation of the Glu 35 carboxyl group. Since this carboxyl group probably is protonated in the pH region of maximal lysozyme activity it could act as a general acid catalyst to protonate the glycosidic oxygen atom causing heterolysis of the carbon-oxygen bond.

3.--The portion of the substrate lying in the E subsite and beyond is released from the cleft, leaving a glycosyl-enzyme intermediate.

4.--The glycosyl-enzyme intermediate reacts either with water (hydrolysis reaction) or with an acceptor molecule (transfer reaction).

It is necessary to postulate a glycosyl-enzyme intermediate because in all the lysozyme-catalyzed reactions studied so far, the stereochemical result is retention of the configuration at the C-1 atom of the sugar residue located in subsite D (15,16,26,30).

For the cleavage step, which involves acid catalysis by Glu 35, the following mechanisms have been considered (15,28):

1.--The carboxylate anion of Asp 52 may act as a nucleophile, displacing the aglycone portion of the substrate and forming a mixed ester-acetal linkage in the glycosyl-enzyme intermediate. However, inspection of the model shows that the nucleophile and the leaving group cannot assume the correct geometry for the transition state of a bimolecular substitution. Furthermore, the carboxyl group of Asp 52 cannot, without considerable distortion, be located closer than 3 A. This distance would not allow the formation of a covalent bond.

2.--An alternative mechanism involves heterolysis of the carbon-oxygen bond under the influence of the negative charge of the Asp 52 carboxylate anion. The reaction would proceed as it does in nonenzymic hydrolysis, by the formation of a carbonium ion. In solution the carbonium ion is stabilized by interaction with the dipolar molecules of the solvent, whereas in the lysozyme-catalyzed reaction the carbonium ion is stabilized by the negative charge held at a distance of about 3 A.

3.--The Asp 52 carboxylate anion may act as a general base by abstracting the proton of the substrate acetamido nitrogen with a simultaneous displacement of the aglycone. Examination of the lysozyme-substrate complex indicates that the Asp 52 participation in that fashion is not sterically feasible (2).

4.--It has also been proposed that intramolecular nucleophilic displacement of the aglycone portion of the substrate

by the substrate acetamido group takes place forming a protonated oxazoline (28,40-42). However, it has been shown that the substrate acetamido group is not essential for enzymatic hydrolysis of the substrate.

These possible reaction mechanisms are open to experimental test.

There is the suggestion that the glycosyl-enzyme intermediate may resemble a carbonium ion more than a covalent intermediate (31,87); however, it would be premature to rule out of consideration a covalent mechanism in which Asp 52 may be involved (2). That is, conformational changes in the enzyme may take place in solution which can make sterically possible the Asp 52 carboxylate nucleophilic participation.

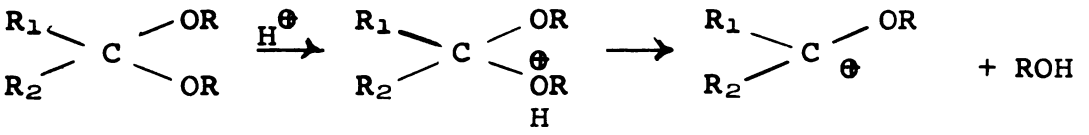
In fact, the following observations may indicate that the lysozyme molecule under the influence of large substrates can undergo conformational changes: a) The chemical modification of Asp 87, Asp 66 and Glu 7 is considerably affected by the presence of substrate. These amino acid residues are not, according to the lysozyme-substrate complex model, in the cleft or in direct contact with the substrate (33). b) The carboxyl group of Asp 101 has been postulated to be bound to the acetamido nitrogen of sugar residue A and the O of C-6 of sugar residue B. In solution, however, the substrate apparently provides little protection for chemical modification of this aspartic acid chain and

moreover its chemical modification does not eliminate enzymatic activity against cell walls (33). c) The Trp 108 has been converted to a N'-formyl-kinurenine residue without any loss of either the catalytic activity or the binding capacity of lysozyme. Since Trp 108 seems to be involved in the binding properties of the enzyme this finding indicates that the N'-formyl-kinurenine residue may act as well as the indole ring in the 108 position (34). Alternatively this result may indicate that Trp 108 is unimportant for binding the substrate in solution.

Chemical Studies with Glycosidase Model Compounds

Although no enzymatic reaction mechanism has been elucidated, it has usually been assumed that enzymes follow the same chemical principles that operate in organic nonenzymatic reactions. In this sense the knowledge of the substrate cleavage position and the knowledge of the amino acid side chain composition in the lysozyme catalytic site, has stimulated basic research on the hydrolytic reaction mechanisms of ketal, acetal and glycoside compounds.

Several independent lines of evidence (35) suggested that the acid-catalyzed hydrolysis of acetals and ketals, in dilute acid solutions, proceeds by a pathway (A-1) involving a protonation step followed by a unimolecular rate determining decomposition to an alcohol and a carbonium ion intermediate.



according to the rate law

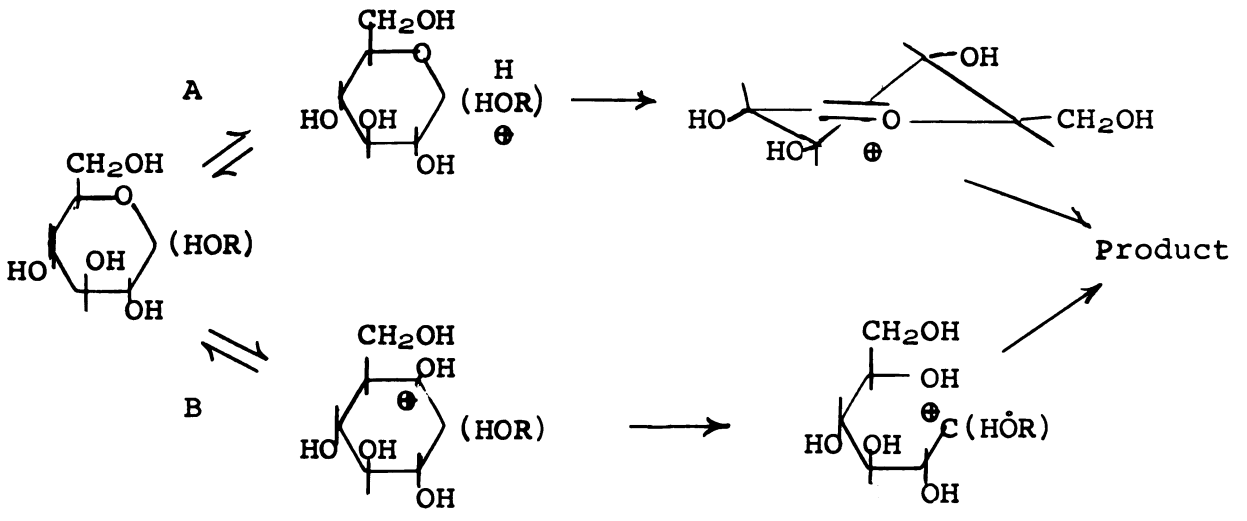
$$k_{obs} = k_{H^+}(H^+) + \Sigma k_{HA_i}(HA_i)$$

in which the terms in the summation on the right hand side of the equation are neglected. That is to say, general acid catalysis is insignificant compared to the specific acid catalysis.

Recent findings, however, suggested that in acetal compounds in which the protonation step is more difficult or in which the A-1 transition state is sterically unfavorable, solvent participation and general acid catalysis can occur in the C-O bond breaking step. These studies involved a series of 2-aryloxytetrahydropyrans (36), and 2-(para-substituted phenyl)-4,4,5,5-tetramethyl-1,3-dioxolanes (37).

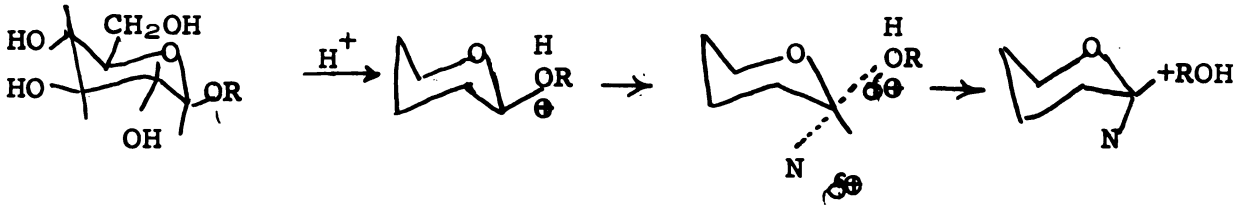
The acid-catalyzed hydrolysis of glycopyranosides has been found to proceed by two reaction mechanisms (27,28).

1.--Nucleophilic unimolecular substitution (A-1) in which, as in simple acetals, a carbonium ion is formed.



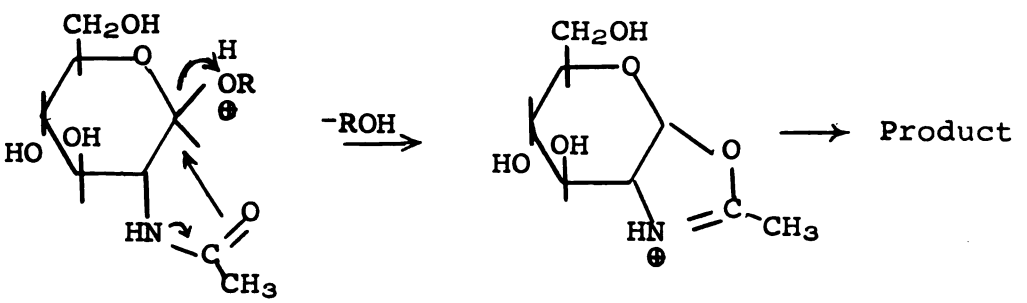
The acid-catalyzed hydrolysis of glycosides usually involves the formation of a cyclic carbonium ion (pathway A). This process requires a conformational change towards the half chain conformation (32) and hydrolysis is, characteristically, slower than in the case of simple acetals and ketals (28).

2.--Nucleophilic bimolecular substitution (A-2) in which the bond making and bond breaking processes occur simultaneously and the net stereochemical result is inversion of the configuration at the reaction center. The nucleophile (N) may be a solvent molecule or an intramolecular neighboring group



It seems probable that lysozyme and other glycolytic enzymes function by extremely facile general acid-general base catalysis and, perhaps, molecular distortion mechanisms in which the participant groups catalyze the reactions near neutrality at rates much greater than can be observed in simple acid- catalyzed reactions at the same pH value. The observation of solvent participation and general acid catalysis, already mentioned, plus the neighboring group participation, summarized in the following paragraphs, seems to be important for understanding the lysozyme-catalyzed reaction.

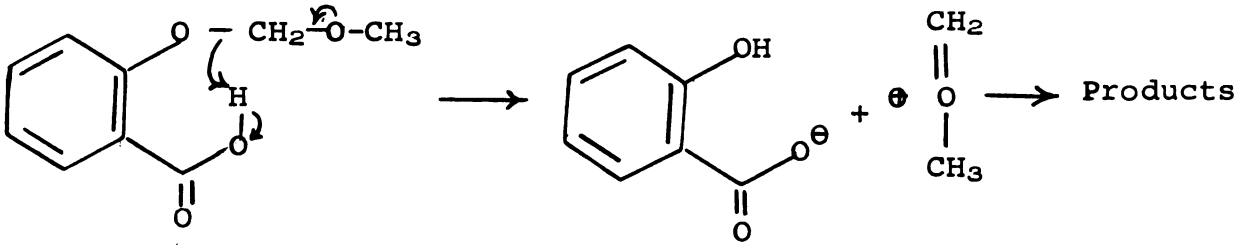
Anchimeric assistance by the acetamido group in the specific acid catalyzed hydrolysis of β-N-acetylglucosaminyl bonds has been reported by Bruice et al. (40-42). This assistance showed a remarkable dependence on the nature of the aglycone. The most probable mechanism was thought to be a nucleophilic displacement by the 2-acetamido oxygen of the protonated aglycone. A similar mechanism had been previously proposed for the hydrolysis of acetylated glycoside halides (28) in which an oxazoline intermediate is formed.



Isolated cases of intramolecular carboxyl group participation has been observed in glycosides. Karrer (44), Helferich

(45), Capon (46), and Bruice (41,47) observed that o-carboxyphenyl-β-D-glucoside is spontaneously hydrolyzed in aqueous solution. Three possible mechanisms have been hypothesized (intramolecular general acid catalysis, intramolecular displacement of the protonated aglycone by carboxylate ion, and intramolecular nucleophilic-electrophilic catalysis) for this process (46,47). The carboxyl group participation in the hydrolysis of poly- and oligouronides has been proposed to involve general acid catalysis (48,49). Evidence for carboxyl group participation in the hydrolysis of methyl-α-D-glucopyranosiduronic acid has also been presented (50).

There is less information for carboxyl group participation in acetal and ketal hydrolysis than that for glycosides. Actually only one case has been reported, that by Capon (51). This investigator interpreted the enhanced hydrolysis rate of o-methoxymethoxybenzoic acid as an intramolecular acid catalysis



Bruice and Piszkiewicz (47) in an attempt to study the carboxyl group participation, found that there is no carboxyl group participation in the hydrolysis of seven 1,3-dioxanes and 1,3-dioxalanes.

Chemical Studies on the Lysozyme-Catalyzed Reaction Mechanism

Several different approaches have been used in the past to determine the participation of individual amino acid side chains in the catalytic activity of lysozyme. Most of these approaches involved chemical modification of the amino acid side chains. Thus, esterification with methanol-hydrochloric acid of carboxyl groups (53), acetylation of amino groups (54), iodination of tryptophan (55), oxidation (53) or reduction (57) of disulfide bonds, photooxidation of histidine and aromatic amino acids (58) and oxidation of tryptophan (56) cause partial or complete inactivation of lysozyme.

The significance of these investigations with respect to the catalytic role of the modified amino acid is difficult to assess since the chemical modifications were not associated with either the binding properties or the catalytic properties of the enzyme. Most recently, however, many of these limitations have been overcome. The availability of soluble, low molecular weight substrates of known molecular structure have permitted the use of techniques that can differentiate between binding properties and catalytic properties. Furthermore, the information about the three dimensional structure of the lysozyme-inhibitor complexes and the conclusions inferred from model building, have directed most of the chemical modification studies to a limited number of amino acid residues.

Carboxylic acid side chains have been the most thoroughly studied, not only because of the postulated role in lysozyme, but also because of the earlier postulation of their catalytic participation in other glycosidases, α-amylase (59) and β-glucosidase (60).

In only one instance, however, has the direct participation of a carboxyl group in lysozyme catalysis been reported (61). A single carboxyl group was esterified with triethyloxonium fluoroborate. The lysozyme derivative was isolated from other esterified lysozyme species and showed to have essentially no enzymatic activity against M. luteus cell walls, whereas it retained good substrate binding properties. The carboxyl group was identified as Asp 52.

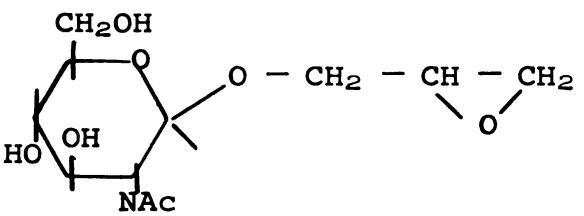
The results from other recent modifications of the carboxyl side chains in lysozyme confirm their necessity for activity but do not prove or disprove their catalytic role. Thus, Kravchenko (62) and Raftery (63) have carried out esterification using methanol-hydrochloric acid. Binding studies (63) indicated that the tertiary structure in the region of the binding site was not seriously disrupted by the esterification process. The activity of the esterified enzyme was, however, 3 to 5% of that of native lysozyme.

Koshland et al. (33), using a newly developed technique have modified in the presence of substrate all the carboxyl groups except those of Glu 35 and Asp 52. The modified lysozyme retained 60% of the native enzymatic activity.

Removal of the substrate led to modification of all the carboxyl groups except that of Glu 35; this resulted in loss of activity. The participation of Asp 52 in binding properties or catalytic properties was not investigated.

Chemical modification of lysine (63), histidine (63), and tyrosine (63) side chains have shown that they are not implicated either in binding properties or catalytic properties in the lysozyme catalyzed reaction. These results agree with the conclusions obtained from x-ray studies.

A different approach for studying the implication of amino acid side chains in the catalytic activity of lysozyme has been undertaken by Sharon and co-workers (64). These investigators designed a specific inhibitor of lysozyme which can react with carboxyl groups.



Incubation of this compound with lysozyme produced a covalently bound enzyme-inhibitor complex. The alkylated amino acid residue was not identified.

Another approach was originated in this Laboratory by Rynbrandt (3). The enzyme was incubated with low molecular weight substrates (Figure 2) obtained from M. luteus cell walls and the reaction was quenched with guanidine hydrochloride. After reduction of the disulfide bonds and alkylation with N-ethylmaleimide a stable lysozyme substrate complex

was obtained. The author suggested that these may be covalently bound complexes formed as intermediates in the lysozyme-substrate reaction.

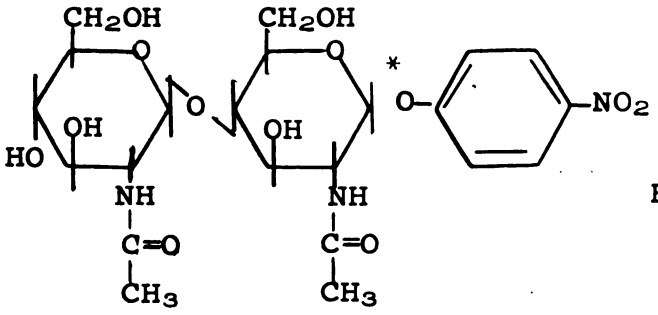
There is also the possibility, however, that these complexes are the result of electrostatic binding of the substrate to the enzyme, since the lysozyme molecule is positively charged and the substrate negatively charged under the conditions used for preparing the complex. In fact, Rynbrandt (3) observed that the complex does not form when the substrate is freed of pentapeptide, i.e., when the negative charge is decreased. Also the complex does not form when the sulfhydryl groups of the reduced enzyme are alkylated with iodoacetate, which increases the negative charge on the protein. Finally, it was found that previously inactivated lysozyme also combines with substrate, though to a less extent than it does when the lysozyme is denatured and chemically modified in the presence of substrate.

In spite of these observations, the possibility of a covalent complex still exists. Thus, part of the present work was to investigate the nature of this complex.

Substrate Acetamido Group Participation

It is evident from the nature of different substrates lysozyme can hydrolyze that the acetamido group at C-2 of the sugar residue D is not essential for lysozyme activity (Figure 1).

There are, however, two instances in which an enhancement effect of the substrate acetamido group in the relative rate of the lysozyme-catalyzed reaction has been observed. One case involved the comparison of the hydrolysis rate of the indicated bonds of compounds I and II (13). Compound I hydrolyzed 10^2 times faster than compound II. These results however, should be considered tentative since obtaining the relative values involved several assumptions (2).



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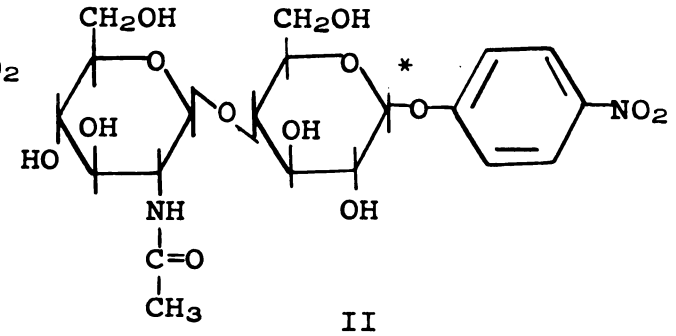

II

Lowe and Sheppard (13) interpreted these results as evidence for a lysozyme mechanism in which general acid catalysis by Glu 35 is assisted by the acetamido group.

Another study was carried out with 50% de-N-acetylated chitin oligomers (17). Although these substrates bind to the enzyme as well as the unmodified chitin oligomers, the hydrolysis rate of the de-N-acetylated ones was only 20% of that of the normal chitin oligomers. Hayashi and coworkers (17) postulated that the positive charge of the amino group left instead of the acetamido group is responsible for the decrease in enzymatic activity.

EXPERIMENTAL

Reagents

General

All concentrated acids, common inorganic salts and organic reagents were reagent grade.

Lysozyme Reagents

1. Amberlite MB-3 mixed bed resin, 20-50 mesh

   Rohm and Haas

2. Amberlite CG-120 resin

   Amberlite CG-120 type II (200 mesh and finer) purchased from Rohm and Haas had previously been classified (3) for quantitative amino acid chromatography by the method of Hamilton (89); the fraction used in this work was the coarse residue which did not flow at a wash velocity of 590 ml per min.

3. Aminex AG 50W-X2, 200-300 mesh, hydrogen form

   California Corporation for Biochemical Research and BioRad Laboratories.

4. Biogel P-4, 50-100 mesh

   BioRad Laboratories

5. Carboxymethyl Cellulose

   California Corporation for Biochemical Research

6. Dialysis tubing

"Visking" sausage casing (18/32 in), purchased from Union Carbide Corporation.

7. Ethanol assay system

Determatube C-ALK ethanol assay system purchased from Worthington Biochemical Company.

8. Glass beads

Minnesota Mining and Manufacturing Company "superbrite" type 130-5005 0.1 mm diameter glass beads were stirred in hot chromic acid cleaning solution for six hours, then exhaustively washed with distilled water and dried at 105°.

9. Guanidine hydrochloride

Reagent grade guanidine hydrochloride (Mallinckrodt) was recrystallized from 95% ethanol or prepared by the addition of concentrated hydrochloric acid to reagent grade guanidine carbonate (Eastman Kodak) to pH 7. The resulting solution was filtered and evaporated to dryness; the white solid was recrystallized from 95% ethanol.

10. Hydroxylamine hydrochloride

Matheson, Coleman and Bell (practical grade) or Mallinckrodt (reagent grade) recrystallized from half of its weight in water-95% ethanol (4:1, v/v).

11. Leucine aminopeptidase

Leucine aminopeptidase was prepared by Dr. J. C. Speck according to the method of Hill *et al*. (90). The enzymatic activity (C_1 = 3) of the preparation used in the present

work was determined by the method of Bryce and Rabin (91).

11. Lysozyme

Worthington Biochemical Corporation twice recrystallized salt-free lysozyme and Sigma Chemical Company three times crystallized salt-free lysozyme.

12. Micrococcus luteus cells, dried

Sigma Chemical Company pfs grade, and Miles Laboratories, Inc.

13. Pepsin

Pentex Incorporated three times crystallized pepsin.

14. Prolidase

Prolidase was purified from swine kidney by the method of Davis and Smith (92). The preparation which is obtained at Step 2 (C_1 = 10 to 20) was used.

15. Papain

Papain, twice crystallized suspension (33 mg/ml, 15 u/mg). Worthington Biochemical Corporation.

16. Sephadex G-15

Pharmacia Fine Chemicals, Incorporated.

17. Trypsin

Worthington Biochemical Corporation twice recrystallized salt-free trypsin.

18. Urea

Mallinckrodt reagent grade. Urea solutions were deionized with Amberlite MB-3 mixed bed resin, filtered and recrystallized from the concentrated water solutions.

Acetal Reagents

1. Acetonitrile

Fisher Scientific Company reagent grade. Redistilled.

2. Aminoacetaldehyde diethyl acetal

Aldrich Chemical Company, Inc.

3. Benzene

Reagent grade benzene was dried by azeotropic distillation.

4. Diethyl ether

Mallinckrodt anhydrous ether

5. 3,4-Dihydropyran

Aldrich Chemical Company practical grade was redistilled on a Vigreux column; the fraction boiling at 84.0-84.5^{o} was collected.

6. Dioxane

Dioxane was purified by a modification of the method of Fieser (93). A solution of 2 l dioxane, 200 ml of water, and 27 ml of concentrated hydrochloric acid was refluxed for 36 hours under nitrogen. The solution was cooled and shaken with potassium hydroxide pellets to saturation; the resulting upper dioxane layer was decanted and dried over potassium hydroxide pellets. The dried dioxane was refluxed with 2 g of sodium borohydride for 24 hours prior to distillation directly from the sodium borohydride. This purified dioxane was then refluxed with and redistilled from sodium borohydride immediately prior to use to remove any traces of peroxide formed during storage.

7. Ethanol, absolute

   Gold Shield 200-proof ethyl alcohol

8. Methanol, absolute

   J. T. Baker absolute methyl alcohol.

9. β-Propiolactone

   Aldrich Chemical Company reagent grade.

10. Sodium borohydride

    Alfa Inorganics, Incorporated sodium borohydride powder

11. Sodium 3,4-dihydro-2H-pyran carboxylate

    Shell Development Company practical grade.

Preparations

Acetamidoacetaldehyde Diethyl Acetal

The method of Eiter and Sackl (65) was utilized to prepare this compound.

Acetic anhydride (17 g) was added dropwise with stirring to aminoacetaldehyde diethyl acetal (20 g.). The reaction mixture was allowed to stand at room temperature (23°) for 15 hours. Absolute methanol (5 ml) was added to eliminate the excess of acetic anhydride and after 2 hours at room temperature it was fractionally distilled to yield acetamidoacetaldehyde diethyl acetal (18 g), b.p. 94.0-94.5°(0.3 mm).

2-(Tetrahydropyran-2-yloxy)-acetic Acid Ethyl Ester

A slight modification of the method of Haynes and Plimer (66) was utilized to prepare this compound.

A mixture of ethyl glycolate (19.5 g), 2,3-dihydropyran (29.5 g) was added with concentrated hydrochloric acid (0.1 ml). The temperature rose rapidly but was kept below 40° by cooling. The mixture was allowed to stand at room temperature for 3 hours. Pellets of potassium hydroxide (1.6 g) were added to remove the acid catalyst. The liquid was decanted and distilled at reduced pressure to yield 2-(tetrahydropyran-2-yloxy)-acetic acid ethyl ester (42 g), b.p. 110° (10 mm).

The amide was prepared by treatment of the ester (20 g) with an excess of 9 M ammonia solution (300 ml) for 18 hours. The excess of ammonia was partially eliminated with a rotary evaporator. The concentrated solution was freeze-dried. The white crystals were recrystallized from benzene to yield the amide (12 g), m.p. 65°.

Methylhydroxypropionate

The procedure employed was a modification of the methods described by Gresham et al. (67).

β-Propiolactone (72 g) was dropwise added with stirring to a solution of sodium hydroxide (2 g) in dry methanol (190 ml). The reaction was exothermic and a solid carbon dioxide-acetone bath was employed to maintain the temperature at 0° with a 15-minute addition time. The sodium hydroxide catalyst was neutralized with an equivalent of concentrated sulfuric acid and the solution filtered from the salt. Low boiling material was eliminated and the residue fractionally distilled

at reduced pressure to yield methylhydroxypropionate (55 g), b.p. 74° (10.5 mm).

3-[(Tetrahydropyran-2-yl)oxy]-propionic Acid Methyl Ester

The general method of Woods and Kramer (68) was utilized to prepare this compound.

To a stirred solution of methylhydroxypropionate (52 g) and concentrated hydrochloric acid (0.1 ml) dihydropyran (63 g) was added. After 4 hours at room temperature the acid catalyst was neutralized and the resulting solution distilled at reduced pressure to yield 3-[(tetrahydropyran-2-yl)oxy]-propionic acid, methyl ester (76 g), b.p. 112°(10 mm) (88).

To prepare the amide, the ester (37 g) was added to an excess of 15 M ammonia solution (350 ml) in a 500 ml round-bottomed flask. The suspension was stirred for 12 hours and the excess of ammonia partially eliminated with a rotary evaporator. The concentrated solution was freeze dried. The white crystals were recrystallized from benzene and sublimed at controlled temperature (40°) and reduced pressure (1 mm) to yield the amide (31 g), m.p. 43-45°.

6-Ethoxytetrahydropyran-2-carboxylic Acid Ethyl Ester

This compound was prepared by a modification of the procedure of Sax (69).

A suspension of sodium 3,4-dihydro-2H-pyran carboxylate (45 g) in ethanol (150 ml) was mixed with dry, 2.3 M solution of hydrogen chloride in ethanol until the reaction mixture

was slightly acidic. The suspension was filtered to eliminate the sodium chloride and the filtrate mixed with dry benzene (200 ml). The reaction mixture was refluxed until water formation stopped. After elimination of low boiling material the reaction mixture was fractionally distilled at reduced pressure to yield 6-ethoxytetrahydropyran-2-carboxylic acid ethyl ester (40 g), b.p. 97° (3.5 mm).

The amide was prepared by mixing the ester (10 g) with excess of 14 M ammonia solution (140 ml). The suspension was stirred for 15 hours at room temperature. The crystals were filtered and dried over barium oxide to yield the amide (8.7 g), m.p. 126°.

Ethyl Acetimidate

This compound was prepared according to the method outlined in Organic Syntheses (70).

A mixture of ethanol (46.1 g), acetonitrile (41 g) and ether (50 ml) was cooled with an ice-water bath. Dry hydrogen chloride (40 g) was added in 2.5 hours. The resulting solution was allowed to stand at 5° for 12 hours and at room temperature for an additional 24 hours. The ethyl acetimidate hydrochloride crystals (45 g) were filtered and the dry crystals used for preparing the ethyl acetimidate free base.

For preparing the free base, ethyl acetimidate hydrochloride (34.8 g) was rapidly added to a vigorously stirred solution, precooled to 0°, containing water (200 ml), potassium carbonate (55.3 g) and ethylene chloride (70 ml).

After seven minutes, the bottom layer of ethylene chloride was separated. A second extraction with ethylene chloride (50 ml) was carried out and the extracts combined. The extract was dried with potassium carbonate (10 g) and low boiling material evaporated. Fractional distillation of the residue yielded ethyl acetimidate (10 g), b.p. 90° (atmospheric pressure).

Methyl Acetimidate Hydrochloride

The preparation of this compound was also carried out according to the method outlined in Organic Syntheses (70).

A mixture of methanol (32 g), acetonitrile (41 g) and ether (50 ml) was cooled with an ice-water bath. Dry hydrogen chloride (39 g) was slowly added in 2 hours. The resulting solution was allowed to stand 2 hours at 5° and 12 additional hours at room temperature. The methyl acetimidate hydrochloride crystals (30 g) were filtered and washed with ether, m.p. 94° (decomposition) in a sealed tube (71).

Tetrahydropyranyl Acetate

The method of Bowman (72) was utilized in the preparation of this compound.

Acetic acid (30 g) was added dropwise to stirred dihydropyran (84 g) containing catalytic amounts (5 mg) of p-xylene sulfonic acid. The temperature of the reaction mixture rose to 40°. The resulting solution was allowed to stand at room temperature for 30 minutes. It was neutralized with potassium

carbonate (2 g) and after decantation it was distilled at reduced pressure to yield tetrahydropyranyl acetate (45 g), b.p. 49°(2 mm).

Determination of Rate Constants for Hydrolysis of Acetals and Tetrahydropyran Derivatives

The rates of hydrolysis were measured in solutions of 50% dioxane-50% perchloric acid or buffer solution (by volume). This dioxane water solution has been analytically found to correspond to 49.6% dioxane and 50.4% water by weight (39). All the components were brought to 25.0 ± 0.1° before mixing and the reaction mixture maintained at this temperature in a water-jacketed cell compartment for the course of the reaction.

In a typical experiment 2 mmoles of the acetal or 4 mmoles of the tetrahydropyran derivative was placed in a stoppered 50-ml Erlenmeyer flask and brought to the bath temperature. The dioxane-acid solution was prepared by mixing 10.0 ml of dioxane and 10.0 ml of perchloric acid or buffer solution and also brought to the bath temperature. At zero time the contents of both Erlenmeyer flasks were mixed and an aliquot of the reaction mixture was transferred to the spectrophotometric cell.

The rate determinations, by the Guggenheim method (74), were based on the increase of absorbance at 280 mμ. The increments were obtained by reading the absorbance of the system at fixed time intervals. The log of these increments

was then plotted as a function of time. The value of the first-order hydrolysis rate constant, k_1, was obtained by multiplying the slope of the plot by 2.303, the natural log conversion factor. The pseudo second-order hydrolysis rate constant, k_2, was then obtained by dividing k_1 by the acid concentration.

The change in absorbance of the aliphatic acetal was always measured in a 1-cm path cell in a Beckman DU spectrophotometer. The change in absorbance of the tetrahydropyran derivatives was measured in both a 5-cm and a 1-cm path cell in a Cary Model 11 spectrometer and a Beckman DU spectrophotometer equipped with a Gilford model 2000 recorder, respectively.

Reactions of Hydroxylamine with Tetrahydropyranyl Acetate

In an aqueous solution of hydroxylamine and acylal at least three different reaction processes may take place. These are spontaneous hydrolysis of the acylal, attack of the hydroxylamine nucleophile reagent at the ester center, and attack of the hydroxylamine reagent at the acetal center. The product of these processes are aldehyde and acid, aldehyde and hydroxamic acid, and oxime and acid, respectively. Analysis of the products in the first two processes is complicated by the oximation reaction that eventually occurs with the free aldehyde product. However this difficulty could be overcome by measuring the kinetics of the appearance of

the free acid since the formation of hydroxamic acid is negligible, measured as described below.

Reaction of Hydroxylamine at the Ester Center of Tetrahydropyranyl Acetate

Tetrahydropyranyl acetate (14.4 mg) was added to 10 ml of 0.5 M pH 7.0 hydroxylamine solution. The solution was mixed and allowed to react. Aliquots (0.5 and 1.0 ml) were taken at 10 and 15 minutes and analyzed for hydroxamic acid content (Table VII) (52).

Kinetic Measurements of the Spontaneous Hydrolysis and the Reaction of Hydroxylamine at the Acetal Center of Tetrahydropyranyl Acetate

All the kinetic measurements were carried out at 6.0 ± 0.1°. Constant temperature was maintained by circulating water from a Forma constant temperature bath through a jacketed vessel. The temperature and the concentration of the reacting species were chosen as the best compromise between the response of the instrument and the velocity of the reaction.

The rates were measured titrimetrically at constant pH with a Radiometer TTT-1 autotitrator and a Radiometer Titrigraph utilizing a Radiometer GK2321C electrode. The instrument was equipped with a Radiometer temperature compensator. The constant pH for the kinetic measurement was 6.55 which is the experimentally determined pK_a for hydroxylamine hydrochloride under these conditions of temperature and ionic strength.

In a typical experiment, 10.0 ml of 0.01 M hydroxylamine hydrochloride in 0.25 M potassium chloride solution, was continuously stirred and brought to 6.0 ± 0.1° in a jacketed vessel. Then the pH was adjusted to 6.55 with 0.13 M sodium hydroxide solution delivered from the pH-stat syringe. Tetrahydropyranyl acetate (7.2 mg) was added from a 10-μl syringe previously calibrated.

The rate determinations, by the Guggenheim method (74), were based on the rate at which sodium hydroxide solution was delivered from the pH-stat syringe in order to maintain the pH constant. The increments were obtained by reading off the chart the amount of base delivered at fixed times. The log of these increments was then plotted as a function of time. The pseudo first-order constants, k_{obs}, were obtained from the slopes of these plots.

Reduction of Ethyl Acetimidate with Sodium Borohydride

Ethyl acetimidate (435 mg) was added to water (10 ml) precooled and stirred at 0 to 3° in a 20 ml beaker equipped with the electrode and syringe of the pH-stat. Immediately sodium borohydride (25 mg) was added. The pH of the solution was maintained constant at pH 7 or 8 by the addition of 6 M hydrochloric acid. A total of 200 mg of sodium borohydride was added in portions of 25 mg in 20 minutes. After the addition of the reducing agent the solution was allowed to stand 40 minutes at 0 to 3°. Aliquots (2 ml) of the reaction

mixture were analyzed for the presence of aldehyde with 2,4-dinitrophenylhydrazine reagent.

Reduction of Methyl Acetimidate Hydrochloride with Sodium Borohydride

Methyl acetimidate hydrochloride (700 mg) was added to 10 ml of 1 M, pH 7.0 sodium phosphate buffer precooled to 0 to 3° in a beaker equipped with the pH-stat electrode and syringe. Immediately the pH was readjusted and sodium borohydride (25 mg) added. The pH was maintained constant at 7 by adding 6 M hydrochloric acid. A total of 250 mg of sodium borohydride was added in 30 minutes. After the addition of the reducing agent, the solution was allowed to stand 30 minutes at 0 to 3°. Aliquots (2 ml) of the reacted mixture were tested for the presence of aldehyde with 2,4-dinitrophenylhydrazine reagent. For the ethanol test with the Determatube C-ALC enzymatic system, aliquots (0.1, 1.0, and 3.0 ml) of the reacted solution were used.

Determination of Ethanol Formation in the Sodium Borohydride Reduction of Methyl Acetimidate Hydrochloride

The ethanol formation in the reduction of methyl acetimidate hydrochloride with sodium borohydride was determined by an enzymatic system. In this system an alcohol dehydrogenase-catalyzed reduction of NADH is coupled to a methylene blue reduction (decolorization) mediated by a diaphorase. The commercially available assay system Determatube C-ALC was used.

Determination of Aldehyde Formation in the Sodium Borohydride Reduction of Imidates

The aldehyde formation in the sodium borohydride reduction of methyl acetimidate hydrochloride and ethyl acetimidate was determined with the 2,4-dinitrophenylhydrazine reagent.

Lysozyme Preparations

Preparation of *M. luteus* Cell Walls

The procedure of Rynbrandt (3) was used. This procedure is a modification of the method of Sharon and Jeanloz (73). *M. luteus* cells (15 g), "Superbrite" 0.1 mm glass beads (250 g) and distilled water (250 ml) were placed in a 400 ml stainless steel Sorvall Omni-mixer assembly. The assembly was immersed in an ice-salt bath in the cold room to equilibrate. The cold mixture was then homogenized at full mixer speed for 50 minutes. The resulting suspension was decanted from the glass beads into an ice-cooled 2-l beaker. The beads were washed with five 200-ml portions of ice-cold distilled water and the wash combined with the previously decanted suspension.

The suspension was centrifuged at 3,000 rpm for 20 minutes at 0°. All centrifugations were carried out in a Sorvall RC-2B centrifuge equipped with a GSA head. The precipitate was discarded; the supernatant was centrifuged at 10,000 rpm for 20 minutes at 0° to sediment the cell walls.

The cell walls were washed three times by suspension in 400-ml portions of ice-cold distilled water followed by centrifugation at 10,000 rpm for 20 minutes at 0°. The washed cell wall pellet was suspended in distilled water (400 ml) and centrifuged at 3,000 rpm for 20 minutes at 0°; the pellet, consisting of residual heavy contaminants, was discarded, the supernatant was centrifuged at 10,000 rpm for 20 minutes at 0°.

The cell wall pellet was suspended in distilled water (250 ml) and held at 100° for 20 minutes in a boiling water bath. The boiled suspension was centrifuged at 10,000 rpm for 20 minutes; the pellet was freeze dried to yield off-white amorphous material (2.0 g).

Preparation of M. luteus Glycopeptide GP-2

The method of Rynbrandt (3) was used for preparing this glycopeptide. M. luteus cell walls (1.7 g) were suspended in 0.05 M ammonium acetate solution (100 ml); lysozyme (4 mg) and toluene (0.3 mg) were added and the suspension held at 37° for 2.5 hours. Then carboxymethyl cellulose in the sodium form (15 g) and distilled water were added and the suspension stirred for 1 hour at room temperature (23°); additional carboxymethyl cellulose (15 g) was added and stirring was continued for 2 hours. The thick suspension was diluted by the addition of distilled water (100 ml) and centrifuged at 10,000 rpm for 20 minutes; the pellet was washed by resuspension in distilled water (100 ml) followed

by centrifugation at 10,000 rpm for 20 minutes. The supernatant fractions were combined and freeze dried to yield an off-white powder (1.2 g).

Portions (200 mg) of the carboxymethylcellulose-treated lysozyme-*M. luteus* cell wall digest were dissolved in portions (2 ml) of distilled water and placed on the automated gel filtration apparatus (3). The 2.5 x 90-cm Sephadex G-15 column was eluted with boiled distilled water at a rate of 12 ml per hour; the eluate was monitored at 240 mμ and collected in 5-ml fractions.

The A_{240} trace exhibited one large symmetrical peak at an elution volume of 140 ml. This peak was collected by pooling the fractions under the peak; freeze drying this solution yielded 70-90 mg of white amorphous powder.

A 250 mg sample pooled from several gel filtration runs was dissolved in 5 ml of distilled water. The pH of the solution was adjusted to 2.2 with formic acid; the solution was then placed on a 75 x 2.5 cm column of Aminex AG 50W-X2 resin which had previously been equilibrated with 0.2 M, pH 2.9 pyridine-formic acid buffer. The column was first eluted with 200 ml of 0.2 M pH 2.9 pyridine-formic acid buffer and then switched to a 1000 ml gradient from 0.2 M pH 2.9 to 1.0 M pH 5.3 pyridine-formic acid buffer (500 ml of each buffer). The column eluate was collected in 5-ml fractions.

The fractions were analyzed by concentrating selected tubes on a rotary evaporator; residual pyridine was removed by evaporation *in vacuo* (0.025 mm) over concentrated sulfuric acid.

Redistilled water (5 ml) was added to each of these tubes and the absorbance of the resulting solutions was measured at 240 mμ. The A_{240} plot formed a large symmetrical peak at 115 ml elution volume. The fractions containing this peak were pooled and freeze dried to yield 80-90 mg of amorphous white powder.

Preparation of Reduced, N-Ethylmaleimide-alkylated Lysozyme

Ten ml of 6 M guanidine hydrochloride which was 0.06 M in pH 8.2 sodium phosphate buffer was bubbled with nitrogen for 30 minutes. This solution was added to the lysozyme (100 mg) and, when the protein was completely dissolved, thioglycerol (310 mg) was added. The pH of the resulting solution was adjusted back to 8.2 with 2 M sodium hydroxide solution. The solution was swept with nitrogen to remove air; the container was then stoppered to exclude air and kept in the dark at room temperature (23°) for 5 hours. The pH of this solution was quickly adjusted to pH 6.8 with 1 M hydrochloric acid and immediately N-ethylmaleimide (450 mg) was added. The reaction mixture was held at room temperature in the dark for 20 minutes, then placed in 18/32-inch sausage casing and dialyzed against two 2-liter volumes of distilled

water. The resulting precipitate was washed twice with two 10-ml portions of distilled water and freeze dried to yield 96 mg of amorphous white powder.

Preparation of Fraction I

Fraction I is a mixture of oligosaccharides (mainly hexasaccharide) and small glycopeptides (mainly GP-1) (3). Fraction I was prepared according to the method of Rynbrandt (3).

A suspension of M. luteus cell walls (4.0 g) and toluene (1.0 ml) in an 0.05 M ammonium acetate solution (200 ml) containing lysozyme (8 mg) was stirred for 12 hours at 37°. The resulting suspension was placed in a 15/16 inch dialysis tubing and dialyzed against distilled water (700 ml) for 24 hours. The dialyzate was freeze dried to yield a yellowish powder.

The freeze dried powder was dissolved in distilled water (5 ml) and applied to an Amberlite CG-120 H^+ column. The column was eluted with distilled water (600 ml) at a rate of 100 ml per hour; the eluate was freeze dried to yield a white amorphous powder (280 mg).

This powder (100 mg) was dissolved in water (2 ml) and gel filtered on the 2.5 x 90-cm Sephadex G-15 column of the automated system (3). The column was then eluted with distilled water at a rate of 10 ml per hour and the absorbance recorded at 240 mμ. The first peak fractions were collected and freeze dried to yield a tan powder (20 mg).

Preparation of the Enzyme-substrate Complex

The method of Rynbrandt (3) was used for preparing the enzyme-substrate complex. Lysozyme (100 mg) and substrate (GP-2 or Fraction I) (25 mg) were dissolved in 10 ml of 0.1 M pH 8.2 sodium phosphate buffer. The resulting reaction mixture was incubated at room temperature (23°) for 15 minutes; then granular guanidine hydrochloride (15 g) was added to stop the reaction. After 30 minutes at room temperature the pH of the quenched reaction mixture was adjusted to 8.2 by addition of 2 M sodium hydroxide solution. Thioglycerol (400 mg) was added and the solution pH was again adjusted to 8.2. The reaction mixture was allowed to stand at room temperature in the dark for 5 hours. The pH was then adjusted to 6.8 with 2 M hydrochloric acid and N-ethylmaleimide (600 mg) was added. After reaction for 20 minutes at room temperature in the dark, a further amount of thioglycerol (25 mg) was added to react with the excess of N-ethylmaleimide. Then it was placed in a 18/32-inch sausage casing bag and was dialyzed against two 2-liter volumes of distilled water. The resulting precipitate was centrifuged (clinical centrifuge), washed three times with 10-ml portions of redistilled water, and freeze dried.

The freeze dried material was dissolved in 5 ml of 5% formic acid and placed on a 1.1 x 35-cm Biogel P-4 column. Elution was carried out with 5% formic acid at a rate 25 ml per hour. The eluate was collected in 2-ml fractions and

analyzed by measuring the A_{280} of each fraction. A single peak at 18 ml elution volume was observed. The peak fractions were freeze dried to yield 80 mg of white material.

For amino acid analysis 2 mg of the freeze dried material were hydrolyzed (2 ml, constant boiling HCl, 105°, 24 hours) and placed on the amino acid analyzer.

Detection of Muramic Acid and Glucosamine in the Enzyme-substrate Complexes

The acid hydrolyzate of the substrates utilized in the investigation contains muramic acid and glucosamine; since these compounds yield discrete peaks on the amino acid analyzer, their presence in the hydrolyzate of enzyme-substrate complex could be quantitatively determined.

Since both muramic acid and glucosamine were extensively degraded during the hydrolysis process, a correction factor for this loss was determined (3). The corrected amino acid analyzer constant for muramic acid and glucosamine are respectively 9.43 and 16.1 area units/mg.

Application of these correction factors allowed a quantitative determination of N-acetylglucosamine and N-acetylmuramic acid as present in the enzyme-substrate complex prior to hydrolysis.

Tryptic Hydrolysis of the Enzyme-substrate Complex

The tryptic hydrolysis of the enzyme-substrate complex was carried out by the method of Kimmel *et al*. (43). The enzyme-substrate complex (75 mg) was dissolved in 8 M urea

solution (2 ml) by stirring 18 hours at 4°. Then a 1% solution of trypsin in 8 M urea was prepared and the pH adjusted to 4.1. This solution was allowed to stand at room temperature (23°) for 30 minutes. This procedure irreversibly denatures chymotrypsin (43). The enzyme-substrate complex solution and 0.2 ml of the tryptic solution were mixed and placed in the pH-stat. The hydrolysis was initiated by diluting the solution to 2 M in urea by adding 5.8 ml of distilled water. The precipitate formed on dilution almost disappeared at the end of the hydrolysis period (1.5 hours, pH 8.0, 23°). The resulting suspension was centrifuged (clinical centrifuge) and the pellet discarded.

The clear solution (5 ml) containing the peptide mixture was placed on the 2.5 x 90-cm Sephadex G-15 column of the automated system (3). The column was then eluted with distilled water at a rate of 10 ml per hour and the absorbance recorded at 240 mμ. The material from the first peaks were freeze dried. This freeze dried material (2.5 mg) was hydrolyzed (2 ml constant boiling HCl, 105°, 24 hours) and placed on the amino acid analyzer (Table IV).

As a control for the gel filtration process, a tryptic hydrolyzate (5 ml) of reduced, N-ethylmaleimide-alkylated lysozyme was mixed with substrate GP-2 (1 mg) and the resulting solution placed on the 2.5 x 90-cm Sephadex G-15 column. The material from the first peaks were freeze dried, hydrolyzed and analyzed as indicated above (Table IV).

Pepsin Hydrolysis of the Enzyme-substrate Complex

The enzyme-substrate complex (50 mg) dissolved in 5% formic acid (5 ml) was mixed with pepsin (0.2 ml of 3.5 mg/ml pepsin solution in 0.5 M sodium chloride). The reaction mixture was allowed to stand three hours at room temperature (23°) and the reaction stopped by freeze drying. Half of the dry material (25 mg) was dissolved in water (3 ml). The undissolved material (1 mg after freeze drying) was discarded and the soluble material gel filtered on a 2.5 x 90-cm Sephadex G-15 column previously equilibrated with water. The column was eluted with water at a rate of 10 ml per hour and the absorbance measured at 230 mμ with the automated system. The material from the first peaks were freeze dried. The dry material (2.5 mg) was hydrolyzed (2 ml constant boiling HCl, 105°, 24 hours) and placed on the amino acid analyzer (Table II).

Complete Enzymatic Hydrolysis of the Enzyme-substrate Complex

Complete enzymatic hydrolysis of the enzyme-substrate complex was carried out essentially as described by Hill and Schmidt (75). The enzyme-substrate complex (80 mg) was added to water (20 ml) and stirred in order to make a suspension. Sodium acetate (7.5 ml of 0.2 M solution adjusted to pH 5.2) and sodium cyanide (3.0 ml of 0.1 M solution adjusted to pH 7), then were added. The resulting suspension was placed in a water bath at 40° and papain (1.2 mg) was added. This mixture

was incubated in a stoppered flask for 2 hours. During this time it was gently stirred. Thymol was added in order to eliminate growth of microorganisms. At the end of the digestion the pH was adjusted to 2 in order to inactivate the papain. The precipitate formed was eliminated by centrifugation and the supernatant freeze dried.

The freeze dried material was dissolved in water (2 ml) and adjusted to pH 8.5. Forty microliters of 0.5 M, pH 8.5 Tris buffer and 0.025 M manganese chloride (0.5 ml) were added to this solution. This solution was then mixed with the leucine amino peptidase preparation (0.240 ml) and with prolidase preparation (0.025 ml). The mixture was incubated at 40° for 19 to 24 hours. The enzymes were inactivated by adjusting the pH to 2. The precipitate formed was eliminated by centrifugation. An aliquot (0.2 ml) of the supernatant was analyzed on the amino acid analyzer (Table III). The supernatant was gel filtered on a 2.5 x 90-cm Sephadex G-15 column previously equilibrated with water. The column was then eluted with water at a rate of 10 ml per hour and the absorbance measured at 230 mμ with the automated system. The first peaks were freeze dried. The dry material (2.5 mg) was hydrolyzed (2 ml constant boiling HCl, 105°, 24 hours) and placed on the amino acid analyzer (Table III).

Sodium Chloride Effect on the Enzyme-substrate Complex

Sodium acetate buffer (0.001 M, pH 4.0), 0.001 M, pH 7.0 sodium phosphate buffer and 0.001 M, pH 9.0 Tris buffer

were prepared in 1 M sodium chloride solution.

The enzyme-substrate complex (10 mg) was dissolved in 6 M guanidine hydrochloride prepared in buffered 1 M sodium chloride (1.0 ml). The resulting solution was allowed to stand for 90 minutes at room temperature (23°). Then it was dialyzed against the corresponding buffer in 1 M sodium chloride. The precipitate was centrifuged and washed five times with the appropriate buffered sodium chloride (3 ml). The precipitate was redissolved in 6 M guanidine hydrochloride and the process repeated once more. Finally the precipitate was washed three times with distilled water (3 ml) and freeze dried. The dry material (2.5 mg) was hydrolyzed (2 ml constant boiling HCl, 105°, 24 hours) and placed on the amino acid analyzer (Table V). As a control, the enzyme-substrate complex was washed and redissolved as described above in buffer solution that did not contain sodium chloride (Table V).

Effect of Sodium Borohydride on the Lysozyme Activity

Lysozyme (120 mg) was dissolved in 5 ml of 0.066 M, pH 6.0 sodium phosphate buffer. The resulting solution was mixed with M. luteus cell walls (12 mg) and stirred at 0 to 3°. As soon as the cell wall suspension was formed, sodium borohydride (53 mg) in 4.3 ml of precooled water was continuously added in 30 minutes. The pH of the reaction mixture was maintained constant at 6.0 with 2 M acetic acid delivered by a Radiometer TTT-1 autotitrator and a Radiometer Titrigraph utilizing a Radiometer GK2321C electrode. The reaction mixture

as well as the sodium borohydride solution was maintained at 0 to 3° with an ice-water bath.

Thirty minutes after the final addition of sodium borohydride solution an aliquot (0.5 ml) of the reaction mixture was diluted to 5.0 ml with 0.066 M, pH 6.0 sodium phosphate buffer. This lysozyme solution was immediately assayed for enzymatic activity against M. luteus cells (Figure 4).

In the control for this reaction the M. luteus cell walls was omitted from the reaction mixture.

Effect of Hydroxylamine on the Lysozyme Activity

Lysozyme (5 mg) was added to 5 ml of 1.0 M, pH 5.8 hydroxylamine hydrochloride solution. The resulting solution was mixed with M. luteus cell walls (10 mg) while being gently stirred. Further additions of M. luteus cell walls (10 mg) were made at 50 and 75 minutes. The reaction mixture was incubated at room temperature for a total period of 150 minutes. During this period most of the cell wall material was dissolved. Aliquots (5 µl) were taken at 0, 50, 110 and 140 minutes for assaying the enzymatic activity against M. luteus cells (Figure 3).

In the control for this reaction the M. luteus cell walls were omitted from the reaction mixture.

RESULTS

In Tables I and V are given the results of the experiments carried out with the enzyme-substrate complexes. Table I shows that practically all the theoretical lysozyme amino acid residues are present in the hydrolyzate from the complete enzymatic hydrolysis, indicating that completeness of the hydrolytic process was achieved. Tables II and III show that the amino acid and carbohydrate composition of peak I of the gel-filtered hydrolyzates from the peptic hydrolysis and from the complete enzymatic hydrolysis, respectively, is similar to the amino acid and carbohydrate composition of the substrate, GP-2.[1] Table IV shows that peak I of the gel-filtered tryptic hydrolyzate of the enzyme-substrate complex has an amino acid and carbohydrate composition similar to that of the control. Table V indicates that the enzyme-substrate complexes lose the substrate after the sodium chloride-guanidine hydrochloride treatment.

The results obtained from the trapping experiments using the live enzyme are given in Figures 3 and 4. These results

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[1]It is known that pepsin, trypsin, and papain do not catalyze the hydrolysis of the pentapeptide present in the substrate. The complete enzymatic hydrolysis, however, involves the use of a mixture of enzymes (leucine amino peptidase and prolidase) which might cleave the substrate pentapeptide. This could explain the lower than theoretical values obtained for glucosamine and muramic acid in Table III.

indicate that lysozyme does not decrease its activity when it catalyzes the hydrolysis of M. luteus cell walls in the presence of hydroxylamine or sodium borohydride.

The results obtained with the model compounds designed to investigate the possibility of a reaction between the postulated glycosyl-enzyme intermediate and hydroxylamine or sodium borohydride are given in Table VII and page 55, respectively. These results indicate that sodium borohydride does not react with the imidate model compound under the test conditions, whereas hydroxylamine reacts with the acylal model compound only to a small extent (5%).

In Tables VIII to XVII are given the rate constants for the acid hydrolysis of the acetal and tetrahydropyran model compounds. These results show that the second-order rate constants for the hydrolysis of all the model compounds vary as functions of the inductive effects of the substituents, as shown in Figures 5 to 7.

Lysozyme-substrate Reactions

Table I. Amino Acid Composition of the Enzyme-substrate Complex after Complete Enzymatic Hydrolysis

An aliquot of the hydrolyzate was analyzed with the amino acid analyzer. The relative number of amino acid residues was calculated using leucine as the reference.

Amino Acid	Amino acid residues per mole of lysozyme	
	Found	Theoretical
Lysine	5.1	6.0
Histidine	0.9	1.0
Arginine	9.7	11.0
Aspartic acid	6.5	8.0
Threonine	6.1	7.0
Glutamic acid	2.2	2.0
Proline	1.6	2.0
Glycine	8.6	12.0
Alanine	11.8	12.0
Valine	6.6	6.0
Methionine	1.2	2.0
Isoleucine	5.8	8.0
Leucine	8.0	8.0
Tyrosine	2.9	3.0
Phenylalanine	2.9	3.0

Table II. Amino Acid and Carbohydrate Composition of Peak I of the Gel-filtered Peptic Hydrolyzate of Enzyme-substrate Complex

The dried material from peak I was hydrolyzed in constant boiling hydrochloric acid and analyzed with the amino acid analyzer. The relative number of amino acid and amino sugar residues was calculated by using glycine as the reference.

Residue	Relative number of amino acid and sugar residues
Glutamic acid	1.0
Glycine	1.0
Alanine	1.7
Glucosamine	2.0
Muramic acid	2.0

Table III. Amino Acid and Carbohydrate Composition of Peak I of the Gel-filtered Hydrolyzate from the Complete Enzymatic Hydrolysis of the Enzyme-substrate Complex

The dried material from peak I was hydrolyzed in constant boiling hydrochloric acid and analyzed with the amino acid analyzer. The relative number of amino acid and amino sugar residues was calculated by using glycine as the reference.

Residue	Relative number of amino acid and sugar residues
Glutamic Acid	1.1
Glycine	1.0
Alanine	1.9
Glucosamine	1.3
Muramic Acid	1.7

Table IV. Amino Acid and Carbohydrate Composition of Peak I of the Gel-filtered Tryptic Hydrolyzate of the Enzyme-substrate Complex

The dried material from peak I was hydrolyzed in constant boiling hydrochloric acid and analyzed with the amino acid analyzer. The relative number of amino acid and amino sugar residues was calculated by using isoleucine as the reference.

Residue	Relative number of amino acid and sugar residues	
	Peak I	Control*
Aspartic acid	1.5	2.2
Serine	3.2	1.7
Glutamic acid	2.9	1.4
Glycine	3.7	1.5
Alanine	4.3	3.8
Isoleucine	1.0	1.0
Leucine	1.1	1.4
Glucosamine	2.3	2.4
Muramic acid	2.4	2.4

*As a control the tryptic hydrolyzate of reduced, N-ethylmaleimide alkylated lysozyme was mixed with substrate just before gel filtration.

Table V. Amino Acid Composition of the Enzyme-Substrate Complex after Sodium Chloride-Guanidine Hydrochloride Treatment

The enzyme-substrate complex was dissolved in 6 M guanidine hydrochloride prepared in 1 M sodium chloride solution already buffered with acetate, phosphate, or Tris at pH 4, 7, or 9 respectively. The solution was dialyzed against the corresponding 1 M sodium chloride buffered solution. The precipitate formed was then centrifuged and washed. This process was repeated once more. The precipitate was hydrolyzed and analyzed with the amino acid analyzer from which results the glucosamine/phenylalanine ratio was calculated.

Treatment	Glucosamine/Phenylalanine
None	0.40
pH 4	0.00
pH 7	0.00
pH 9	0.00
pH 4 *	0.30
pH 9 *	0.08
None **	0.45
pH 7 **	0.00

*Control experiments in which sodium chloride was excluded.

**Fraction I (3) was used in the preparation of this particular enzyme-substrate complex. In all the other experiments the enzyme substrate complex was prepared using GP-2 as the substrate.

Table VI. Rate Constants for Hydrolysis of Tetrahydropyranyl Acetate in the Presence of and in the Absence of Hydroxylamine

Hydroxylamine hydrochloride in 0.25 M potassium chloride solution was mixed with tetrahydropyranyl acetate. The pH was maintained constant by the addition of sodium hydroxide solution from the pH-stat syringe. The rate constants were evaluated by the Guggenheim method (74) from the increments of the addition of sodium hydroxide solution recorded on the pH-stat chart paper.

Treatment	$10^2 k_{obs}$ min^{-1}		
	Experiment		Average
	1	2	
In the presence of hydroxylamine, pH 6.55	5.8	6.3	6.1
In the absence of hydroxylamine, pH 6.55	6.6	8.4	7.5

Table VII. Hydroxamic Acid Formation in the Reaction of Tetrahydropyranyl Acetate with Hydroxylamine

Ten ml of a 0.5 M, pH 7.0 hydroxylamine solution was reacted with 14.4 mg of tetrahydropyranyl acetate. Aliquots of 0.5 and 1.0 ml were taken at the end of the reaction and analyzed for hydroxamic acid (52)

Aliquot (ml)	Hydroxamic Acid (μmoles) Found	Theoretical	Hydroxamic Acid (Percent formed)
0.5	0.25	5.0	5
0.5	0.25	5.0	5
1.0	0.50	10.0	5
1.0	0.50	10.0	5

Reduction of Imidates with Sodium Borohydride

Methyl acetimidate hydrochloride and ethyl acetimidate were reacted with sodium borohydride as described in the Experimental section.

Low temperature and high pH are the most appropriate conditions for achieving sodium borohydride reduction of cyclic imidates in a medium of low water content (76). Sodium borohydride is, however, unable to reduce the non-cyclic imidates tested here under the present conditions. Even when large excess of reacted mixtures were analyzed no traces of reduction products could be found.

Figure 3. Lysozyme activity after reacting with M. luteus cell walls in the presence of hydroxylamine.

Each assay contained 3.0 ml of M. luteus cell suspension (15 mg of dried cells suspended in 90 ml of 0.067 M, pH 6.8 sodium phosphate buffer, and 10 ml of 1 percent sodium chloride solution) and 5 μg of lysozyme treated as indicated in A and B, below:

A.--Lysozyme dissolved in hydroxylamine solution was added with M. luteus cell walls and the reaction allowed to proceed for 150 minutes. Aliquots were taken at 0, 50, 110 and 140, min and assayed for lysozyme activity ($\frac{\Delta A}{180 \text{ sec}}$).

B.--As a control, lysozyme was dissolved in hydroxylamine solution and aliquots were taken for the activity assay as indicated in A, above, and in the Experimental section.

The changes in absorbance were measured at 550 mμ with a Beckman B spectrophotometer at 23°.

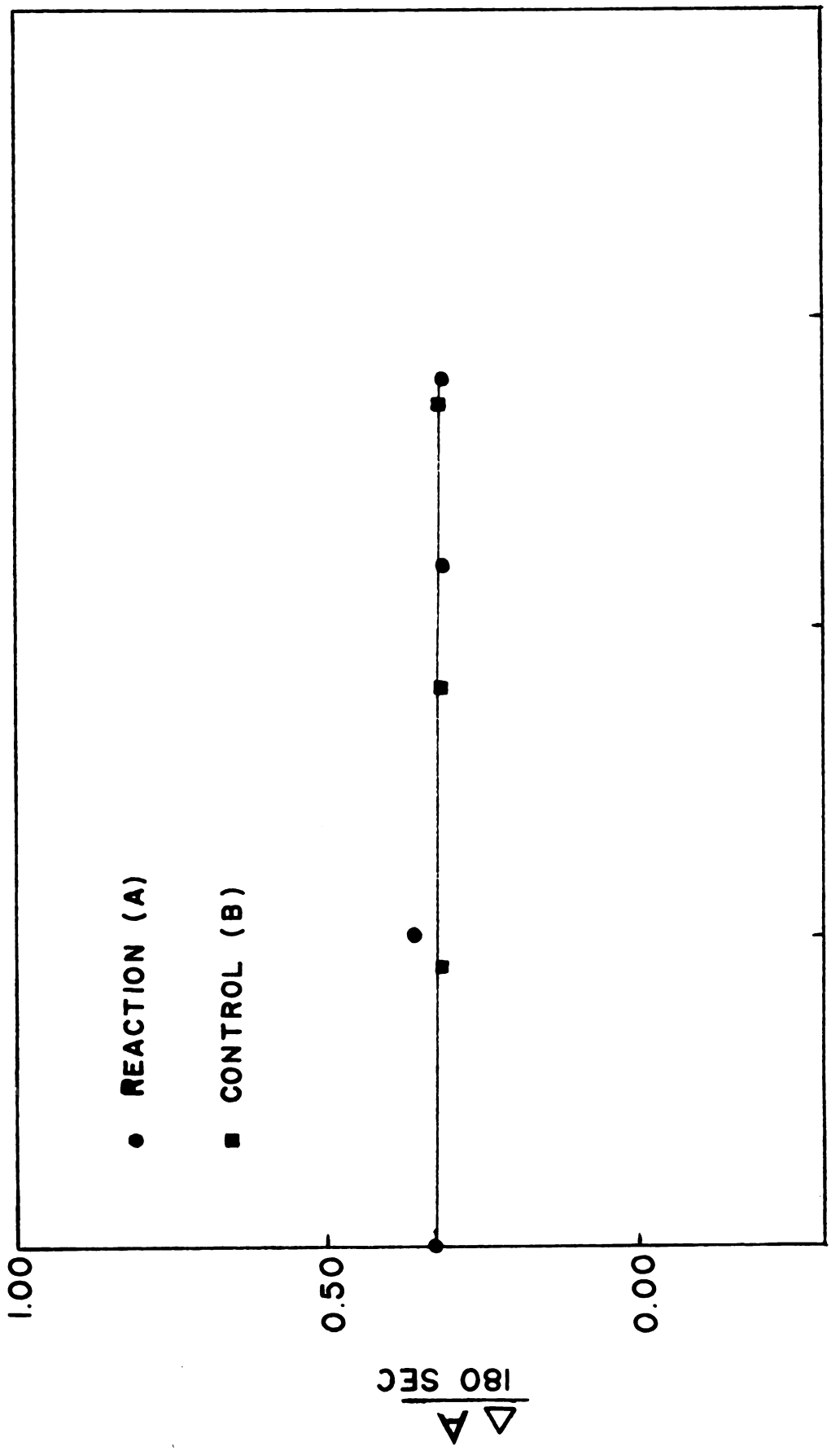


Figure 3

Figure 4. Lysozyme activity after reacting with M. luteus cell walls in the presence of and in the absence of sodium borohydride.

Each assay mixture contained 3.0 ml of M. luteus cell suspension (15 mg of dried cells suspended in 90 ml of 0.067 M, pH 6.8 sodium phosphate buffer, and 10 ml of 1 percent sodium chloride solution) and 5 μg of lysozyme treated as indicated in A and B, below:

A.--Lysozyme dissolved in sodium phosphate buffer was added with M. luteus cell walls and sodium borohydride. The reaction was allowed to proceed for 60 min. At the beginning and at the end of the reaction time, aliquots were taken and assayed for lysozyme activity ($\frac{\Delta A}{180 \text{ sec}}$).

B.--As a control, a solution of the same lysozyme concentration as the reaction mixture A was treated with sodium borohydride as indicated in the Experimental section.

The changes in absorbance were measured at 550 mμ with a Beckman B spectrophotometer.

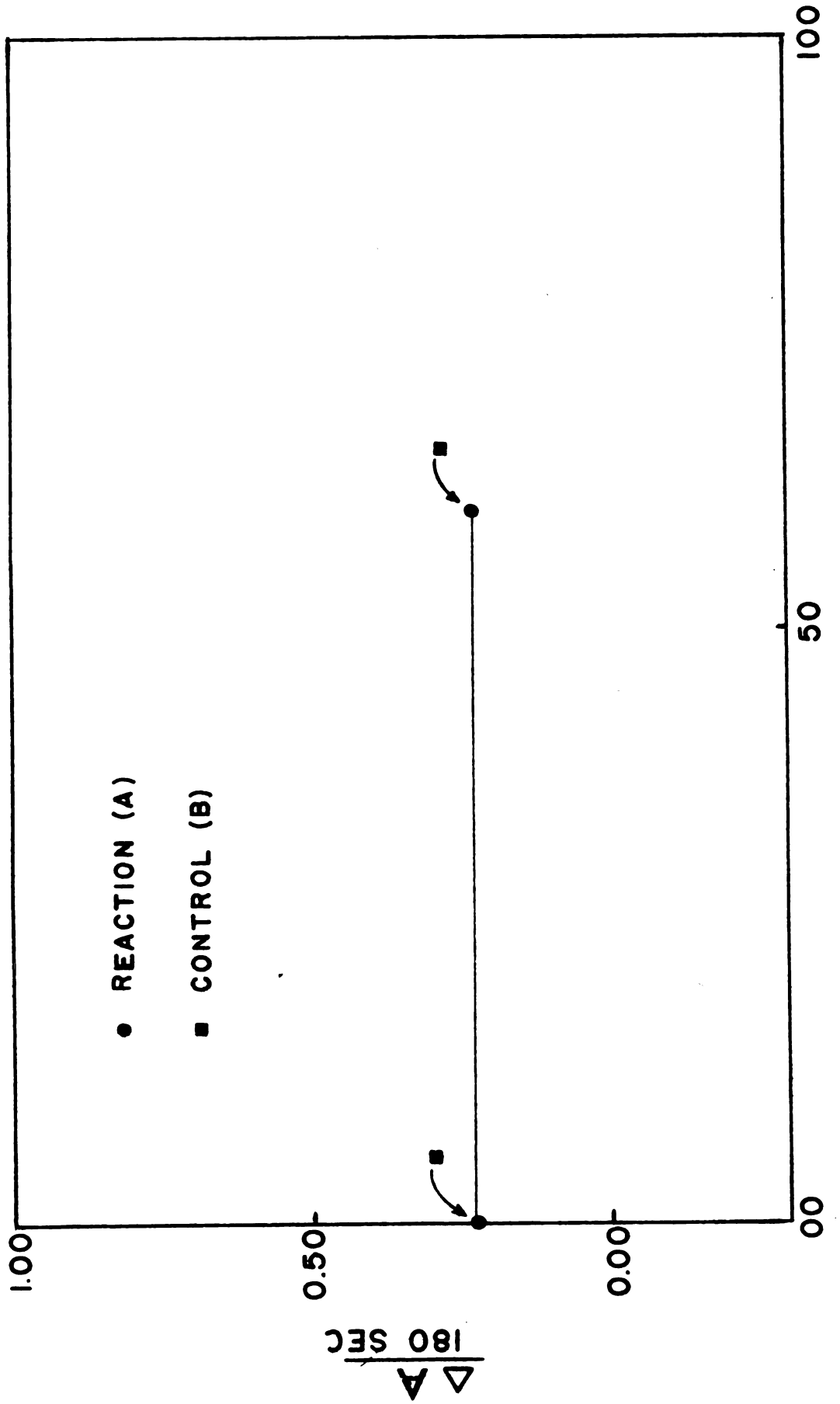


Figure 4

Hydrolysis Rates of Acetals

Table VIII. Rate Constants for Hydrolysis of Acetamidoacetaldehyde Diethyl Acetal

The reaction mixture contained 2 mmoles of acetamidoacetaldehyde diethyl acetal, 10.0 ml of dioxane and 10.0 ml of perchloric acid or buffer solution. The change in absorbance was measured at 280 mμ.

Perchloric Acid, M		10^4k_1	10^4k_2
0.082	0.164	0.78	9.4
0.082	0.328*	0.78	9.4
0.10	0.20	0.77	7.7
0.10	0.20	0.77	7.7
0.165	0.330	1.7	10.0
0.165	0.330	1.7	10.0
0.1 M, pH 4.0 acetate buffer		0.00	
0.2 M, pH 8.9 Tris buffer		0.00	

*Ionic strength adjusted with potassium bromide.

Table IX. Rate Constants for Hydrolysis of 3-[(Tetrahydropyran-2-yl)oxy]-propionamide

The reaction mixture contained 4 mmoles of the amide, 10 ml of dioxane, and 10 ml of perchloric acid. The change in absorbance was measured at 280 mμ.

Perchloric Acid, M	10^3k_1	10^2k_2
0.025	0.44	1.75
0.012	0.22	1.75
0.098	2.2	2.2
0.10	1.95	1.95
0.10	2.05	2.05
	Average	1.94

Table X. Rate Constants for Hydrolysis of 3-[(Tetrahydropyran-2-yl)oxy]-propionic Acid

To prepare the free acid form of this compound, 0.01 mole of the corresponding methyl ester was weighed in a 25 ml volumetric flask. Then, 0.01 mole of sodium hydroxide in solution and an equal volume of dioxane were added. After the saponification reaction had been completed it was diluted to 25 ml with 50% dioxane-water (v/v). For the kinetic runs 10.0 ml of this solution and 10.0 ml of perchloric acid in 50% dioxane-water, previously equilibrated at 25.0 ± 0.1°, were mixed and transferred to the spectrophotometric cells.

Perchloric Acid, M	10^3k_1	10^2k_2
0.10	1.95	1.95
0.10	1.95	1.95
0.10	2.05	2.05
0.10	2.05	2.05
	Average	2.0
pH 4.8*	0.00	0.00

*This pH value was obtained by half neutralization of the salt formed in the saponification reaction.

Table XI. Rate Constants for Hydrolysis of 3-[(Tetrahydropyran-2-yl)oxy]-propionic Acid Methyl Ester

The reaction mixture contained 4 mmoles of the ester, 10.0 ml of dioxane, and 10.0 ml of perchloric acid. The change in absorbance was measured at 280 mμ.

Perchloric Acid, M	10^4k_1	10^2k_2
0.05	4.93	0.99
0.05	4.93	0.99
0.05	4.90	0.99
0.05	4.90	0.99
	Average	0.99

Table XII. Rate Constants for Hydrolysis of 2-(Tetrahydropyran-2-yloxy)-acetamide

The reaction mixture contained 4 mmoles of the amide, 10.0 ml of dioxane, and 10.0 ml of perchloric acid. The change in absorbance was measured at 280 mμ.

Perchloric Acid, M	10^3k_1	10^2k_2
0.10	3.0	3.0
0.10	2.9	2.9
0.10	3.0	3.0
	Average	3.0

Table XIII. Rate Constants for Hydrolysis of 2-(Tetrahydropyran-2-yloxy)-acetic Acid

To prepare the free acid form of this compound, 0.01 mole of the corresponding ethyl ester was weighed in a 25 ml volumetric flask. Then, 0.01 mole of sodium hydroxide in solution and an equal volume of dioxane were added. After the saponification reaction had been completed it was diluted to 25 ml with 50% dioxane-water (v/v). For the kinetic run 10.0 ml of this solution and 10.0 ml of perchloric acid in 50% dioxane-water, previously equilibrated at 25.0 ± 0.1°, were mixed and transferred to the spectrophotometric cell.

Perchloric Acid, M	10^3k_1	10^2k_2
0.10	6.1	6.1
0.11	6.5	5.9
0.11	7.0	6.4
0.11	7.6	6.9
0.11	7.7	7.0
0.10	5.9	5.9
	Average	6.4
pH 4.8*	0.00	0.0

*This pH value was obtained by half neutralization of the salt formed in the saponification reaction.

Table XIV. Rate Constants for Hydrolysis of 2-(Tetrahydropyran-2-yloxy)-acetic Acid Ethyl Ester

The reaction mixture contained 4 mmoles of the ester, 10.0 ml of dioxane, and 10.0 ml of perchloric acid. The change in absorbance was measured at 280 mμ.

Perchloric Acid, M	10^3k_1	10^2k_2
0.097	3.3	3.4
0.097	3.2	3.3
0.097	3.4	3.5
0.10	3.8	3.8
	Average	3.5

Table XV. Rate Constants for Hydrolysis of 6-Ethoxytetrahydropyran-2-carboxamide

The reaction mixture contained 4 mmoles of the amide, 10.0 ml of dioxane and 10.0 ml of perchloric acid. The change in absorbance was measured at 280 mμ.

Perchloric Acid, M	10^5k_1	10^4k_2
0.05	2.4	4.8
0.10	5.0	5.0
0.10	6.0	6.0
	Average	5.3

Table XVI. Rate Constants for Hydrolysis of 6-Ethoxytetrahydropyran-2-carboxylic Acid

To prepare the free acid form of this compound, 0.01 mole of the corresponding ethyl ester was weighed in a 25 ml volumetric flask. Then, 0.01 mole of sodium hydroxide in solution and an equal volume of dioxane were added. After the saponification reaction had been completed it was diluted to 25 ml with 50% dioxane-water (v/v). For the kinetic run 10.0 ml of this solution and 10.0 ml of perchoric acid in 50% dioxane-water, previously equilibrated at 25.0 ± 0.1°, were mixed and transferred to the spectrophotometric cell.

Perchloric Acid, M	$10^5 k_1$	$10^4 k_2$
0.10	3.6	3.6
0.10	3.2	3.2
0.10	3.3	3.3
	Average	3.4
pH 4.8*	0.00	0.00

*This pH value was obtained by half neutralization of the salt formed in the saponification reaction.

Table XVII. Rate Constants for Hydrolysis of 6-Ethoxy-tetrahydropyran-2-carboxylic Acid Ethyl Ester

The reaction mixture contained 4 mmoles of the ester, 10.0 ml of dioxane, and 10.0 ml of perchloric acid. The change in absorbance was measured at 280 mμ.

Perchloric Acid, M	10^5k_1	10^4k_2
0.10	5.3	5.3
0.10	4.8	4.8
0.10	4.8	4.8
	Average	5.0

Figure 5. Plot of the log of the second-order rate constant for hydrolysis of 2-substituted tetrahydropyran derivatives against σ_I (79).

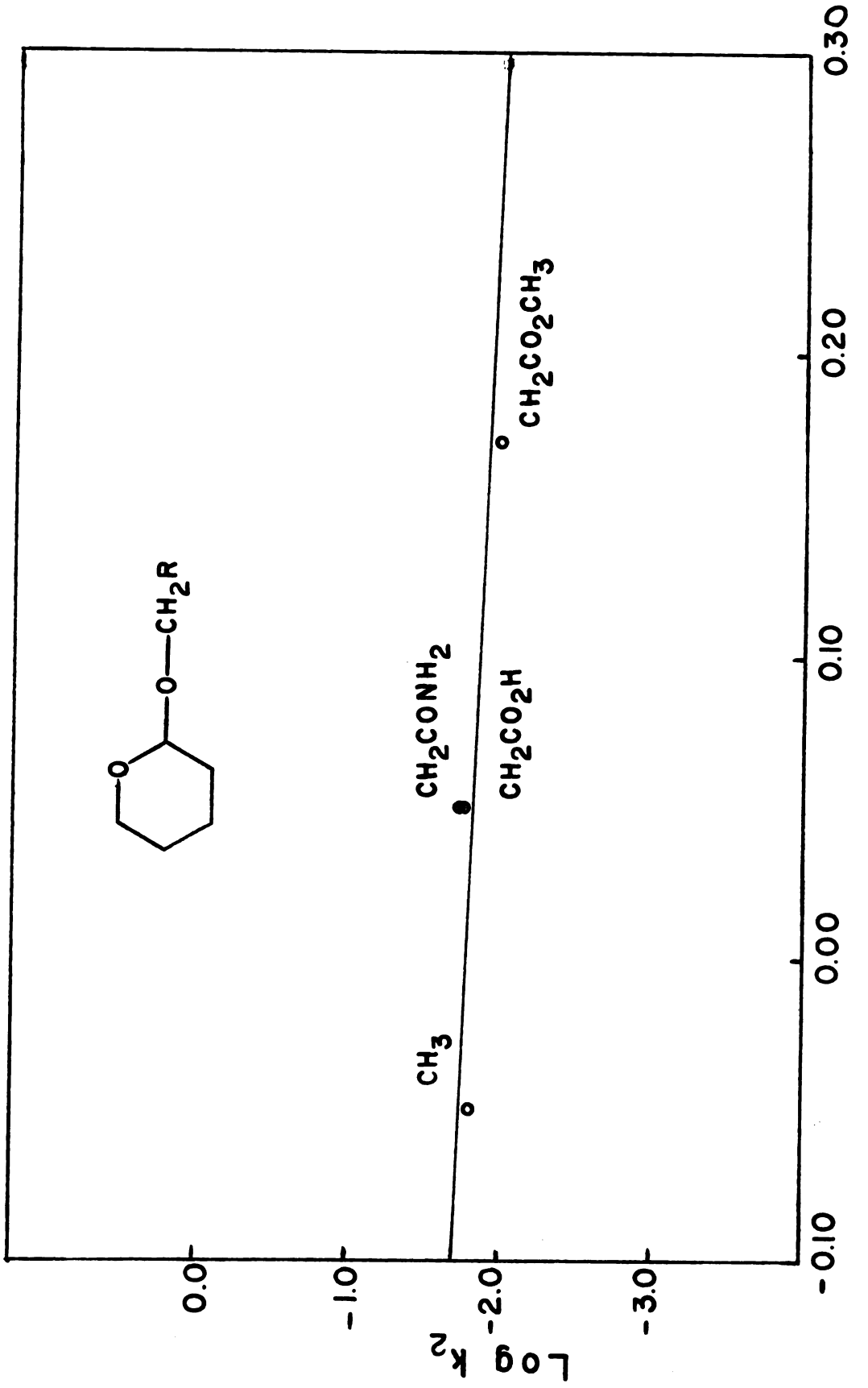

Log k_2
0.0
-1.0
-2.0
-3.0
-0.10
0.00
0.10
0.20
0.30
σ
CH_3
CH_2CONH_2
CH_2CO_2H
$CH_2CO_2CH_3$
O
O
CH_2R
O

Figure 5

Figure 6. Plot of the log of the second-order rate constant for hydrolysis of 2-substituted tetrahydropyran derivatives against σ_I (79).

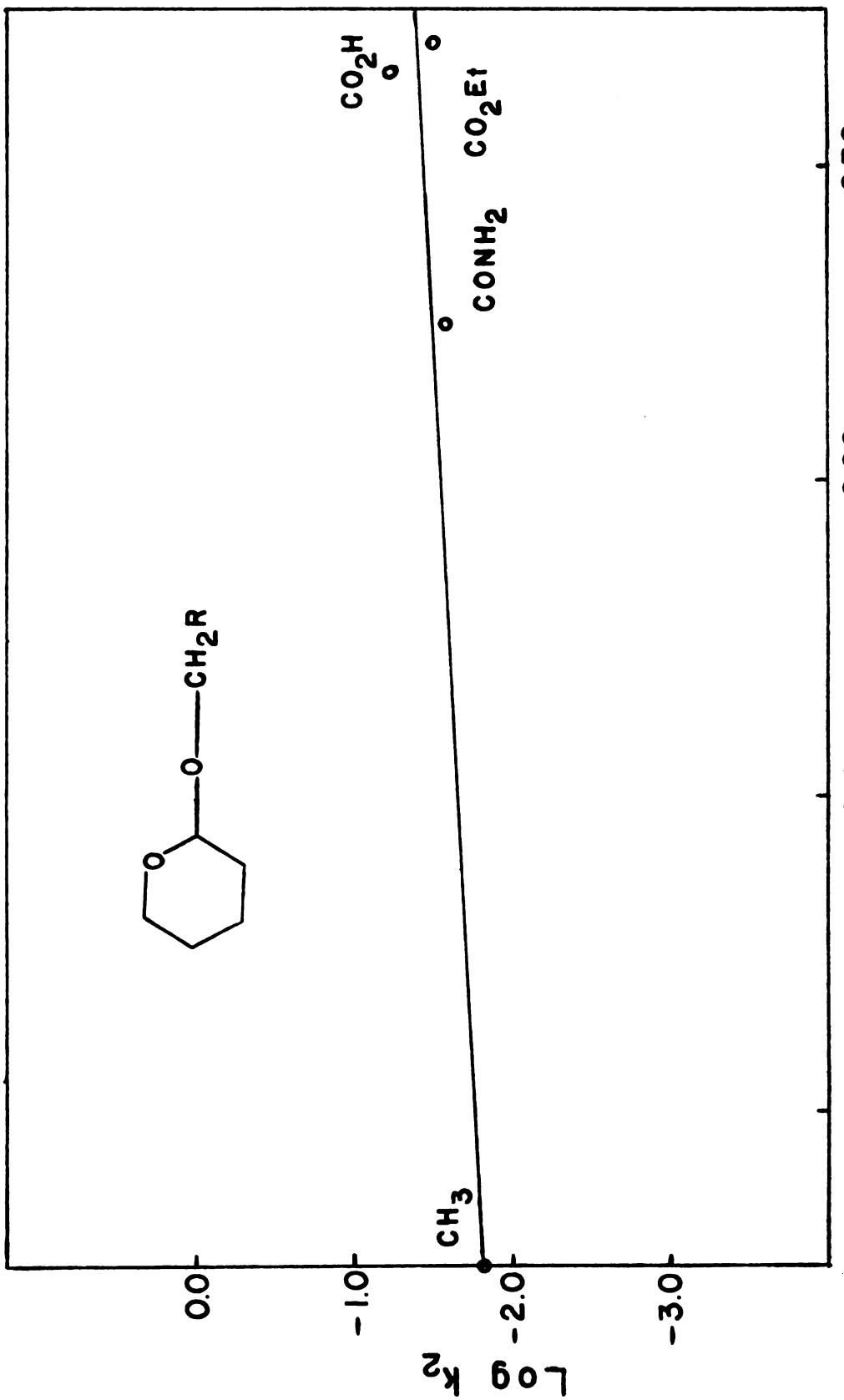

Log k_2
0.0
-1.0
-2.0
-3.0
-0.05
0.00
0.10
0.20
0.30
σ
CO_2H
CO_2Et
$CONH_2$
CH_3
CH_2R
O
O
O

Figure 6

Figure 7. Plot of the log of the second-order rate constant for hydrolysis of 6-ethoxy-2-substituted tetrahydropyran derivatives against σ_I (79).

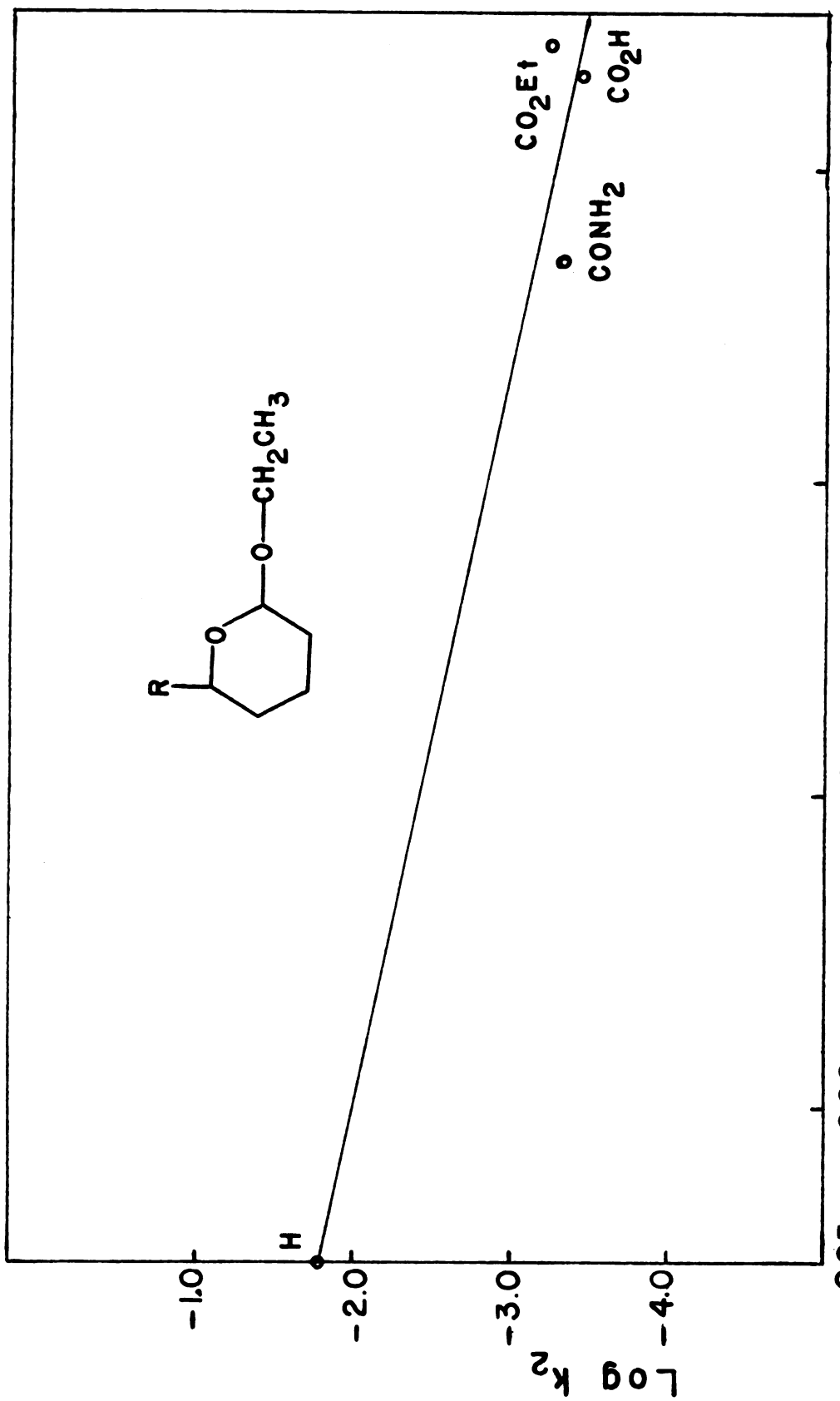

Log k_2
-1.0
-2.0
-3.0
-4.0
-0.05
0.00
0.10
0.20
0.30
σ
H
CO_2Et
CO_2H
$CONH_2$
R
O
O
CH_2CH_3

Figure 7

DISCUSSION AND CONCLUSIONS

Lysozyme-substrate Complex

The lysozyme-substrate complex discovered by Rynbrandt (3) was investigated to determine the nature of the binding between the enzyme and the substrate.

The preparation of the enzyme-substrate complex involved: 1) incubation of the substrate with lysozyme for 15 minutes, 2) quenching the reaction by disrupting the three-dimensional structure of lysozyme with guanidine hydrochloride, 3) reduction of the disulfide bridges and 4) permanent lysozyme inactivation by alkylation of the liberated thiol groups with N-ethylmaleimide. The presence of substrate in the complex was verified by amino acid analysis.

The dialyzed and washed complex was subjected to peptic hydrolysis, and the hydrolyzate then analyzed by gel filtration. A peak which contained only substrate material was found (Table II). The same result was obtained when the complete enzymatic hydrolysis procedure of Hill and Schmidt (75) was used (Table III). These observations were interpreted as evidence that the substrate was not covalently bound to the enzyme since it becomes free after the protein

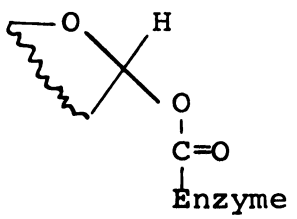
is destroyed. An alternative explanation, however, is that the enzymatic treatment may have cleaved the covalently bound substrate. This alternative explanation can be valid for both the peptic and the complete enzymatic hydrolysis, since the specificity of these enzymes is broad.

As a milder procedure for isolating a peptide-substrate complex, tryptic digestion of the enzyme-substrate complex was carried out. Gel filtration of this enzymatic hydrolyzate gave a peak which contained both substrate and a mixture of peptides. However, a control consisting of a mixture of substrate and tryptic peptides from reduced and N-ethylmaleimide—treated lysozyme yielded a similar peak upon gel filtration (Table IV). Therefore, it is evident that the peptide-substrate complex, if any, was mixed with other peptides and therefore required further separation studies.

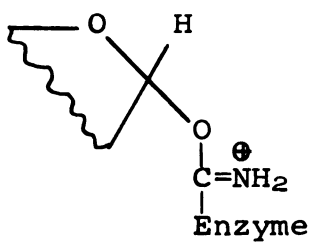
A different approach, however, confirmed the supposition that the complex was formed through electrostatic interactions and not through covalent binding. It was discovered that washing the enzyme-substrate complex (dissolved in 6 M guanidine hydrochloride) with 1 M sodium chloride completely eliminated the substrate from the protein. This was accomplished at pH 4, 7 and 9 (Table V). These results indicate that the substrate is not covalently bound to the enzyme.

Trapping Experiments

According to postulated mechanisms, Asp 52 may be involved in the glycosyl-enzyme intermediate (a) in the lysozyme-catalyzed reaction. Another (77), but so far unexplored possibility is the participation of the Gln 57 amido group (b).



(a)



(b)

Since hydroxylamine is a good trapping reagent for esters an attack on the acylal intermediate at the carboxyl carbon by hydroxylamine is reasonable. During the course of this investigation, however, it was observed that lysozyme is not inactivated during the reaction with M. luteus cell walls in the presence of 1 M hydroxylamine (Figure 1). This lack of inactivation was nevertheless inconclusive, since the reaction of hydroxylamine with acylals had not been investigated.

Tetrahydropyranyl acetate was used as a model compound for studying the hydroxylamine reaction with acylals. The results of these experiments (Table VII) show that the

reaction with hydroxylamine forms only about 5 percent of hydroxamic acid during the time required for tetrahydropyranyl acetate to undergo complete hydrolysis. On the other hand, the kinetic experiments designed to determine the reactivity of hydroxylamine towards the acetal center of this model compound (Table VI) show that the rate constant for hydrolysis has the same value with or without hydroxylamine in the reaction mixture. Thus, it can be concluded that the low reactivity of hydroxylamine towards the ester center is not due to a preferential reactivity towards the acetal center but rather to a competition between hydrolysis of the acetal bond and nucleophilic attack at the ester carbonyl carbon by hydroxylamine.

Since the glycosyl-enzyme intermediate (a) is expected to be rapidly converted into the carboxyl form, hydroxylamine attack at the ester center would not presumably be favored during the lysozyme-catalyzed reaction.

It was also of interest to study the effect of sodium borohydride on the catalytic activity of lysozyme. Recent studies have shown that cyclic imidates can be reduced to aldehydes with sodium borohydride (76). If an imidate glycosyl-enzyme intermediate (b) were present in the lysozyme-catalyzed reaction and if reduction of this intermediate could be accomplished, then inactivation of the enzyme would occur. Inactivation, however did not happen when lysozyme was reacted with M. luteus cell walls in the presence of sodium borohydride (Figure 4).

To see if an acyclic imidate can actually be reduced by sodium borohydride, two model compounds were studied, methyl acetimidate and ethyl acetimidate. The results of these studies show that neither one is reduced under the conditions employed (page 55).

In summary, then, the experiments with the lysozyme itself indicate that the investigated enzyme-substrate complex is not a covalently bound complex. Electrostatic forces seem to be responsible for the strong association phenomenon. However, if a covalently bound glycosyl-enzyme intermediate is formed during the lysozyme-catalyzed reaction, an acylal or an imidate intermediate are not excluded by the results obtained with model systems.

Hydrolysis of Acetals and Tetrahydropyran Derivatives

In the design of both the acetals and the tetrahydropyran derivatives studied here, the substituents were chosen such that each series of model compounds would allow a differentiation between intramolecular catalysis and inductive effects on the rate constant of the acid-catalyzed hydrolysis, if intramolecular catalysis was indeed present.

The tetrahydropyran derivatives bear a marked resemblance to structures commonly associated with glycosides. It is known that substitution in both the aglycone and the sugar ring usually influences the hydrolytic rate constant of

glycosides in a manner dependent on the inductive effect of the substituent (78). In this respect, the tetrahydropyran derivatives would appear to be ideal model compounds with which to study the effect of the substituents in the glycoside hydrolysis, free from complications created by adjacent hydroxyl groups.

Hydrolysis of the Acetal Derivative

The inductive constant, σ_I, is similar for both the ethoxy and the acetamido group (79). Therefore the second-order hydrolysis rate constant for 2-ethoxyacetaldehyde diethyl acetal and acetamidoacetaldehyde diethyl acetal should be similar if other polar effects, resonance effects, and steric effects are similar, as seems to be the case.

Comparison of the second-order hydrolysis rate constants of the acetamidoacetaldehyde diethyl acetal (Table VIII) with that of the 2-ethoxyacetaldehyde diethyl acetal, shows that, at the most, acetamidoacetaldehyde diethyl acetal hydrolyzes five times as fast if 2.0×10^{-4} is taken as the unit value or at the same rate if 8.6×10^{-4} is taken as the unit value.

1 $CH_3CH_2\text{-}O\text{-}CH_2CH(OCH_2CH_3)_2$

2-ethoxyacetaldehyde diethyl acetal

$k_2 = 2.0 \times 10^{-4}$ l. mole^{-1} sec^{-1} (3)

$k_2 = 8.6 \times 10^{-4}$ l. mole^{-1} sec^{-1} (80)

2 $CH_3CO\text{-}NH\text{-}CH_2CH(OCH_2CH_3)_2$

2-acetamidoacetaldehyde diethyl acetal

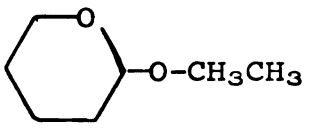
$k_2 = 9.0 \times 10^{-4}$ l. $mole^{-1}$ sec^{-1}

The structural position of the acetamido group in the acetamidoacetaldehyde diethyl acetal is similar to that of the acetamido group in the β-N-acetylglucosaminyl derivatives studied by Bruice. Since the rate constants for the hydrolysis of these glycosides are higher than expected, Piszkiewicz and Bruice (40-42) have proposed that acetamido group participation can account for the rate-augmenting effect observed.

The results obtained with acetamidoacetaldehyde diethyl acetal, however, indicate that there is little or no participation by the acetamido group in the hydrolysis of this compound. This lack of participation may be due to entropy factors and is discussed later in connection with the tetrahydropyran derivatives.

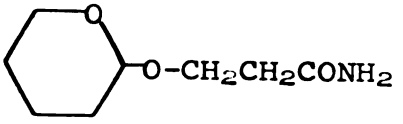
Rate Constants for Hydrolysis of the Tetrahydropyran Derivatives

All of the propyloxytetrahydropyran-related derivatives hydrolyze at about the same rate:

3 

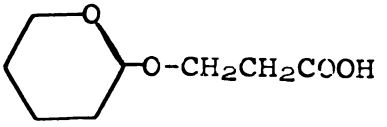
2-ethoxytetrahydropyran

$k_2 = 1.6 \times 10^{-2}$ l. $mole^{-1}$ sec^{-1}

4 

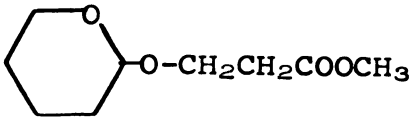
3-[(tetrahydropyran-2-yl)oxy]-propionamide

$k_2 = 1.9 \times 10^{-2}$ l. mole^{-1} sec^{-1}

5 

3-[(tetrahydropyran-2-yl)oxy]-propionic acid

$k_2 = 2.0 \times 10^{-2}$ l. mole^{-1} sec^{-1}

6 

3-[(tetrahydropyran-2-yl)oxy]-propionic acid methyl ester

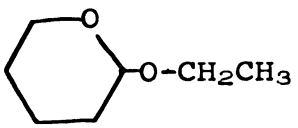
$k_2 = 1.0 \times 10^{-2}$ l. mole^{-1} sec^{-1}

Since the amide, carboxyl and ester substituents are three atoms away from the reactive center their inductive effects have only a small influence in the hydrolysis process. Furthermore, there appears to be no intramolecular catalysis from any of the substituents since all their inductive constants (79) agree very closely with the log of the respective rate constant value when a linear free-energy relationship of the type described by the Hammett equation

$$\log k = \rho\sigma$$

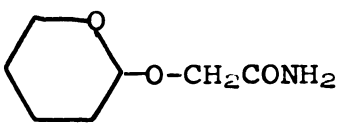
is applied to the hydrolysis rate constants of this series of compounds (Figure 5).

When the amide, carboxyl and ester substituents are positioned two atoms away from the center of hydrolysis, as in the case of the ethoxytetrahydropyran-related derivatives, the rate constants are slightly affected:

3 

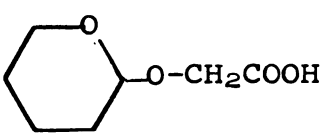
2-ethoxytetrahydropyran

$k_2 = 1.6 \times 10^{-2}$ l. mole^{-1} sec^{-1}

7 

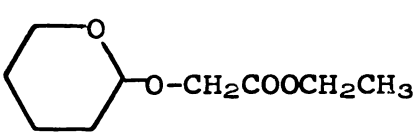
2-(tetrahydropyran-2-yloxy)-acetamide

$k_2 = 3.0 \times 10^{-2}$ l. mole^{-1} sec^{-1}

8 

2-(tetrahydropyran-2-yloxy)-acetic acid

$k_2 = 6.4 \times 10^{-2}$ l. mole^{-1} sec^{-1}

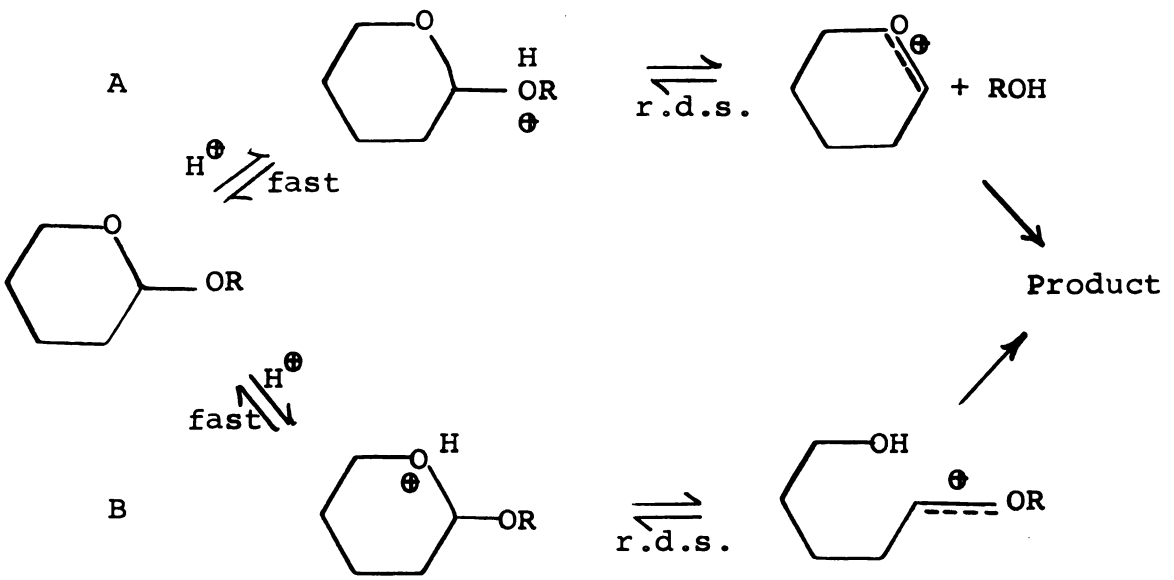
9 

2-(tetrahydropyran-2-yloxy)-acetic acid ethyl ester

$k_2 = 3.5 \times 10^{-2}$ l. mole^{-1} sec^{-1}

All of the rate constants are higher than that of the parent compound, 2-ethoxytetrahydropyran. These rate constants could not be explained on the basis of an intramolecular participation of the amide and carboxyl groups, since the ester substituted compound, which apparently cannot assist in the hydrolysis process, undergoes hydrolysis at about the same rate. Moreover, the rate constants for hydrolysis of all the compounds in the series appropriately fit on a straight line after a Hammett type free-energy relationship is applied (Figure 6).

The results with compounds 4 to 9 can best be explained by assuming that the hydrolysis proceeds via a cyclic carbonium ion (pathway A), rather than an acyclic carbonium ion (pathway B) as follows:

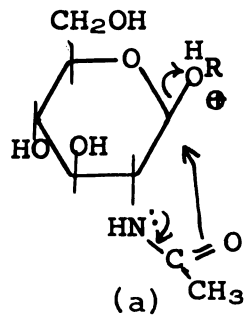


The inductive effect of the amide, carboxyl, and ester substituents would decrease the basicity of the exocyclic

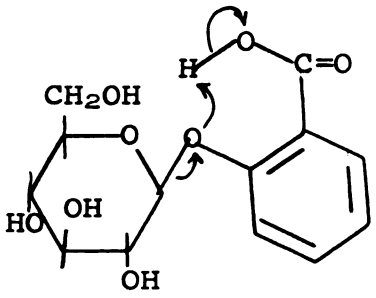
oxygen and consequently decrease the equilibrium concentration of the protonated intermediate. But at the same time they increase the ease of carbon-oxygen cleavage. Partial cancellation of the opposing effects should have small influence on the hydrolytic rate constants. For the same reasons a ρ of small magnitude should be observed in a Hammett type free-energy relationship plot. This actually was found (Figure 5 and 6). This would not be expected if an acyclic carbonium ion (pathway B) were involved, since it would be destabilized by an aglycone having the inductive characteristics of the amide, carboxyl, or ester substituents. This destabilization would produce a decrease in the rate constants relative to the parent compound 2-ethoxytetrahydropyran. A similar situation is well documented for acyclic acetals (84) and for 2-alkoxytetrahydropyrans (85).

Intramolecular catalysis in the hydrolysis of compounds 2, 4, 5, 7, and 8 was expected since the structural position of their acetamido, amide, and carboxylate groups are similar to the corresponding acetamido and carboxyl groups in β-N-acetyl-glucosaminyl derivatives (40-42) and o-carboxyphenyl-β-D-glucoside (46). The rate constants for the hydrolysis of these glycosides are higher than expected. Piszkiewicz and Bruice (40-42) have proposed acetamido group participation (a), and Piszkiewicz and Bruice (41) and Capon (46) have proposed general acid catalysis (b) to account for the difference observed. As alternative mechanisms Capon (46) has

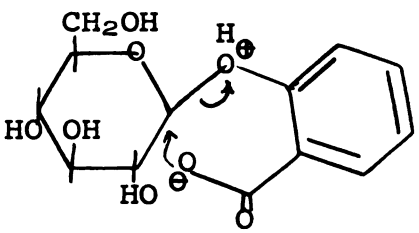
proposed nucleophilic displacement by the carboxylate group (c) or a combination of nucleophilic and electrophilic mechanisms (d):



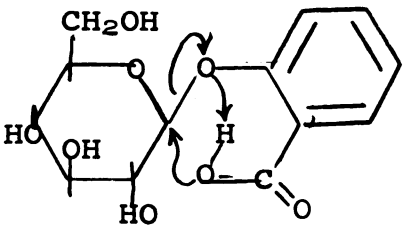
(a)



(b)



(c)

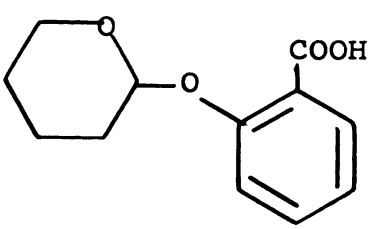


(d)

However, because of steric effects, a nucleophilic displacement by the amide or the carboxylate groups in compounds 4, 5, 7, and 8 appears to be unlikely in the hydrolysis via a cyclic carbonium ion, as postulated here.

In the o-carboxyphenyl-β-D-glucoside (41,46), as well as in all the compounds in which intramolecular catalysis by the carboxyl group has been reported, i.e., methyl-α-D-glucopyranosiduronic acid (86) and polysaccharides containing 1,4-linked hexuronic acids (48,49), the carboxyl group position is relatively well defined with respect to the center of hydrolysis. The same can be said about the acetamido group in β-N-acetylglucosaminyl derivatives (40-42) and in

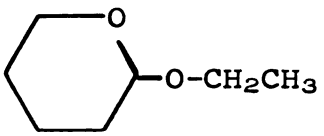
the acetylated glycoside halides (28). It would seem therefore that for the acetamido group of compound 2 to participate in a nucleophilic displacement and for the carboxylic group of compounds 5 and 8 to participate as a general acid in the hydrolysis of these compounds, restrictions in the spatial characteristics of the neighboring group and the reaction center are also required. In compound 2, 5 and 8, however, a restriction of this type does not occur since the aliphatic carbon chains allow a relatively large degree of freedom for the movements of the substituent groups. This conclusion appears to be supported by preliminary results obtained in this Laboratory with 2-(o-carboxyphenoxy)tetrahydropyran, a compound in which the carboxyl group is in a restricted position similar to that of the carboxyl group of o-carboxyphenyl-β-D-glucoside (46). 2-(o-Carboxyphenoxyl)-tetrahydropyran (I) has been found to hydrolyze at pH 7.4 with $k_{obs} = 1.8 \times 10^{-3}$ sec^{-1}.



(I)

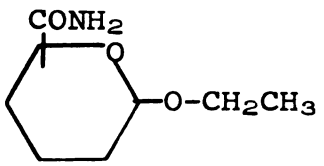
The results obtained with the 6-ethoxy-2-substituted tetrahydropyrans, compounds 10 to 12, indicate that the electron withdrawing character of the amide, carboxyl, and

ester groups decrease the rate constants of the acid-catalyzed hydrolysis relative to the parent acetal 2-ethoxy-tetrahydropyran, by two orders of magnitude:

3 

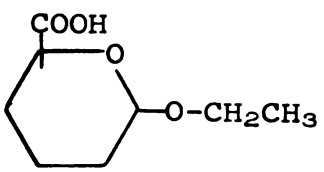
2-ethoxytetrahydropyran

$k_2 = 1.6 \times 10^{-2}$ l. mole^{-1} sec^{-1}

10 

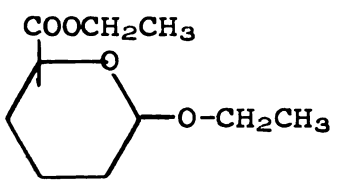
6-ethoxytetrahydropyran-2-carboxamide

$k_2 = 5.3 \times 10^{-4}$ l. mole^{-1} sec^{-1}

11 

6-ethoxytetrahydropyran-2-carboxylic acid

$k_2 = 3.4 \times 10^{-4}$ l. mole^{-1} sec^{-1}

12 

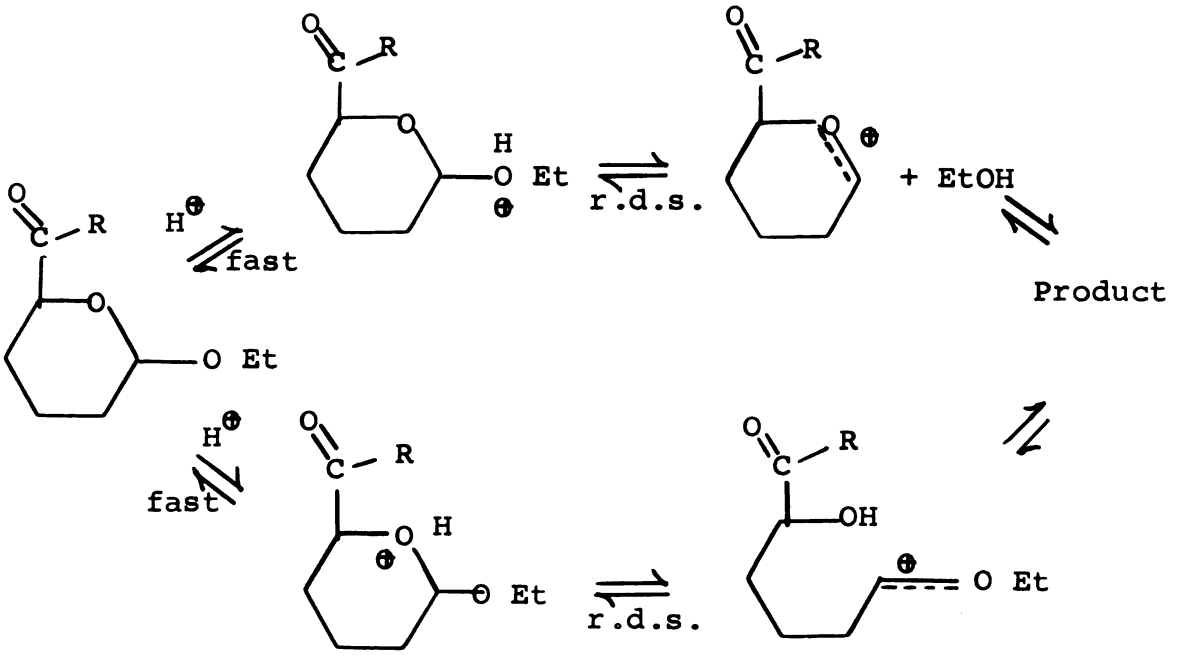
6-ethoxytetrahydropyran-2-carboxylic acid ethyl ester

$k_2 = 5.0 \times 10^{-4}$ l. mole^{-1} sec^{-1}

These results are to be expected from previous studies on structurally similar compounds. For example, there is a

decrease, though to a smaller extent, in the acid-catalyzed hydrolysis rate constant of glucopyranosiduronides relative to the corresponding glucopyranosides (81). Also in some cyclic acetals [e.g., 1,3-dioxolanes (47), and tetrahydropyran derivatives (83)], and glycosides (82) it has been shown that the more electron-attracting is the ring substituent the more difficult is the hydrolysis of the acetal or the glycoside linkage.

Again these results can best be explained by assuming that the hydrolysis proceeds via a cyclic carbonium ion (pathway A), rather than by an acyclic carbonium ion (pathway B) as follows:

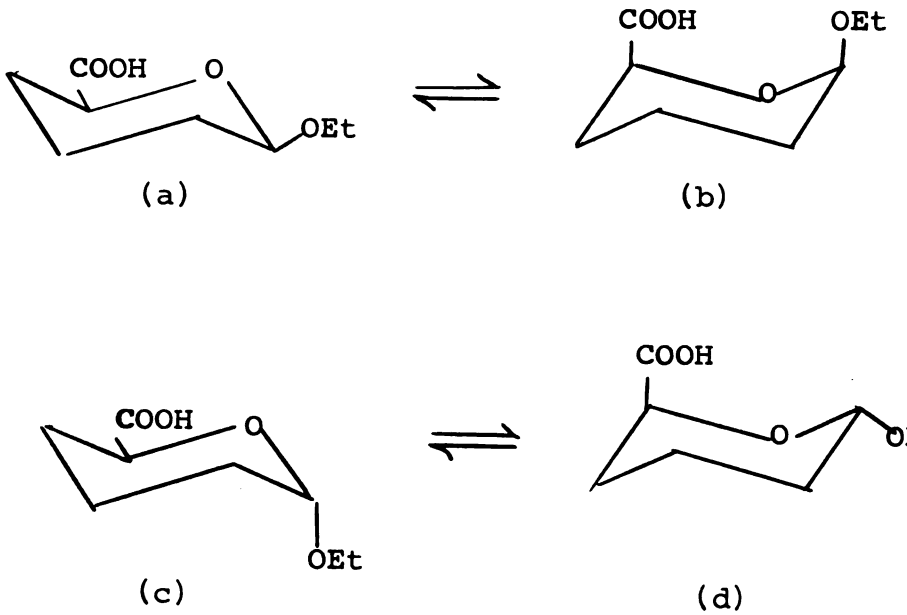


The inductive effect of the ring amide, carboxyl, and ester substituents can produce a destabilization of the

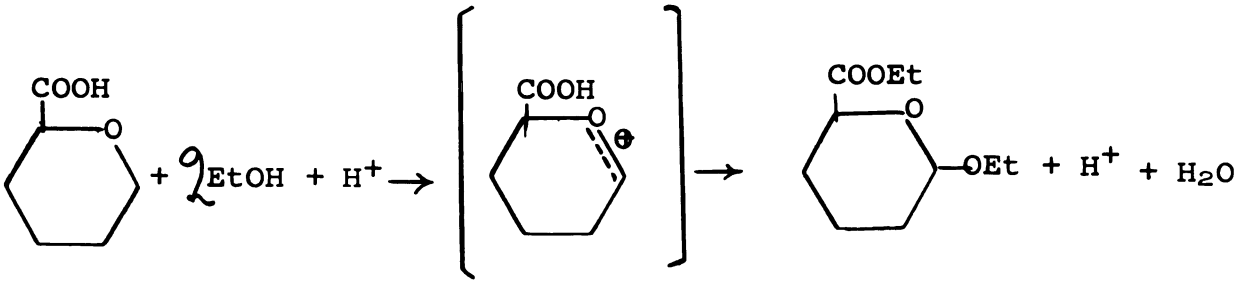
carbonium ion as well as a decrease in the ease of heterolysis. These two factors would decrease the hydrolysis rate constant, as observed.

The rate-retarding effect of the amide, carboxyl, and ester substituents in the 6-ethoxy-2-substituted tetrahydropyrans is, nevertheless, in accordance with the inductive constants of the substituents as shown in Figure 7. This lack of carboxyl group participation is interesting since it has been suggested that a carboxyl group at the same relative position assists the hydrolysis of methyl-α-D-glucopyranosiduronic acid (86).

It is possible that the conformation of the 6-ethoxy-2-substituted tetrahydropyran is responsible for the observed lack of carboxyl group participation. A theoretical conformational analysis indicates that four conformational species may be present in the compound being studied, two cis and two trans:



The carboxylate group could participate in an intramolecular nucleophilic displacement of the "aglycone" only in the (d) conformation, whereas the protonated carboxyl group could participate as a general acid only in the (b) conformation. Since none of this anchimeric assistance is actually observed, the straightforward conclusion is that either the carboxyl group does not participate or the actual conformation of the species present in the compound does not allow such participation. From purely theoretical considerations it would appear that the (a) configuration, which apparently cannot allow carboxyl group participation, is the most abundant because of its conformational stability. That is, during the synthesis of the compound



the cis conformation with both substituents in the equatorial position could be favored.

From the results obtained with the acetal and the three series of tetrahydropyran derivatives it can be concluded that these compounds undergo a specific acid-catalyzed hydrolysis. Their corresponding hydrolytic rate constants can be predicted from the σ value of the respective substituent. That is, no indication of intramolecular catalysis

by the acetamido, amide, and carboxyl groups has been observed in the hydrolysis of these compounds. The results also indicate that hydrolysis of the tetrahydropyran derivatives involves a cyclic carbonium ion intermediate. Since the structural effects in the rate constants for hydrolysis of the tetrahydropyran derivatives resemble the structural effects in the hydrolysis of glucopyranosides the results suggest that the pyranoside hydrolysis also proceeds via a cyclic carbonium ion.

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