GLYCOSIDASE-SUBSTRATE INTERACTIONS: GLYCOSIDE MODEL HYDROLYSIS RATES AND LYSOZYME-SUBSTRATE REACTIONS

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ABSTRACT

GLYCOSIDASE-SUBSTRATE INTERACTIONS: GLYCOSIDE MODEL HYDROLYSIS RATES AND LYSOZYME-SUBSTRATE REACTIONS

by Donald Rynbrandt

Several acetal, ketal, and 2-substituted tetrahydropyran glycoside model compounds were synthesized. These included 2-methylthio-, 2-methoxy- and 2-ethoxy-acetaldehyde diethyl acetal, 4-methylthiobutraldehyde diethyl acetal, 5-methylthio- and 5-methoxy-2-pentanone diethyl ketal, and 2-ethoxy-, 2-(2-methylthioethoxy)-, 2-(2-methoxyethoxy)-, and 2-(2-pyridinyl-ethoxy)-tetrahydropyran.

The hydronium ion-catalyzed hydrolysis rates of these glycoside model compounds were determined by spectrophotometric and hydrogen peroxide-oxidative methods.

A comparison of these hydrolysis rates indicates that the sulfur atom of 2-methylthioacetaldehyde diethyl acetal increases the hydrolysis rate of that acetal by anchimeric assistance and sulfonium ion formation. Extrapolation of this mechanism to glycosidase catalysis indicates that a sulfonium ion-linked glycosylated enzyme intermediate is possible.

These results were extended by an investigation of the reaction of hen's egg white lysozyme with soluble low-molecular weight substrates prepared from M. lysodeikticus cell wall. The peptide-containing lysozyme substrates formed a stable enzyme-substrate complex which could be purified and

analyzed when the lysozyme-substrate reaction was quenched with guanidine hydrochloride, dithiothreitol, and N-ethyl maleimide. This complex may be one of the intermediates in lysozyme-substrate reactions.

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By

Donald Rynbrandt

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To Kathy

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INTRODUCTION

The elucidation of glycosidase activity may be approached from two directions; by study of the non-enzymatic hydrolysis of model glycosides or by study of the enzymatic hydrolysis process itself.

Since glycosidase substrates are really cyclic acetals, much mechanistic information may be obtained from the hydrolysis rates of acetals and ketals. Very little has been said about the effect of nucleophiles on the hydrolysis rates of acetals and ketals, even though theoretical considerations indicate that nucleophiles should enhance the hydrolysis rate by stabilizing the carbonium-ion intermediate.

One part of my thesis, then, will relate the preparation and hydrolysis of acetals and ketals substituted with nucleophilic groups. Comparison of the hydrolysis rates will afford a mechanistic interpetation of nucleophilic effects in acetal and ketal hydrolysis, and by extrapolation, in glycosidase-catalyzed reactions.

The second part concerns the problem of glycosidase catalysis in a more classical sense; it involves the preparation of soluble, homogenous, chemically defined substrates for the somewhat atypical glycosidase, lysozyme (muramidase), and a study of the binding of these substrates to this enzyme, which catalyzes the cleavage of the $\beta(1\rightarrow4)$ linkage between

N-acetyl muramic acid and N-acetyl glucosamine in bacterial cell wall polymers and the β(1→4) linkage in N-acetyl glucosamine polymers (chitin).

Investigation of the hydrolysis of acetals and ketals is not a recent phenomonon. In 1908, the hydrolysis of acetals was investigated by Fitzgerald and Lapworth.[1] A decade later, Skrabel and coworkers[2,3,4,5] carried out a systematic investigation of the hydrolysis effects of acyl and aryl group substitutions. The following mechanism was postulated to account for the fact that the rate constants for alkyl-substituted acetal hydrolyses were constant:

$$RCH(OX)_2 + H_2O \xrightarrow[r.d.]{k_1} RCH(OX)(OH) + XOH$$

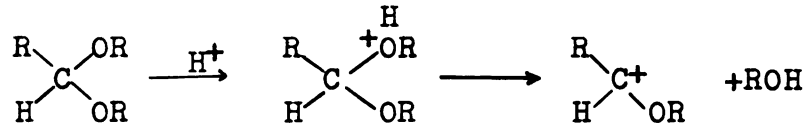
$$RCH(OX)(OH) \xrightarrow{k_2} RCHO + XOH$$

Variations in the carbonyl portion of a series of pentaerythritol acetals were found to produce corresponding variations in the hydrolysis rates.[6] Finally, Skrabel[7] was able to demonstrate that carbonyl moeity alkyl substituent effects were additive. An investigation of the hydrolysis of cyclic acetals and ketals by Leutner[8] demonstrated that acetal stability was a function of ring size; the 7-membered ring was most stable.

Configurational analysis by Hermans[9] of acetal hydrolysis products indicated that the released alcohol retained its original configuration; therefore the carbon-oxygen bond of the carbonyl function, and not that of the alcohol function,

was cleaved. This mechanism was supported by the rate studies of Palomaa and coworkers[10] on alkoxy- and hydroxy-substituted ethyl acetals. Later work also supported this mechanism. Hydrolysis of the acetals of D-(+)-2-butanol[11] and D-(+)-2-octanol[12] produced no change in the final configuration of the alcohols.

This retention of alcohol configuration led O'Gorman and Lucas[12] to modify the alcoholic carbonium ion mechanism proposed by Hammett[13] into a form which is accepted as the correct mechanism for acid-catalyzed acetal hydrolysis. Since the alcohol moeity was not racemized, the carbonyl carbon atom, not the alcohol carbon atom, had to form the carbonium ion:

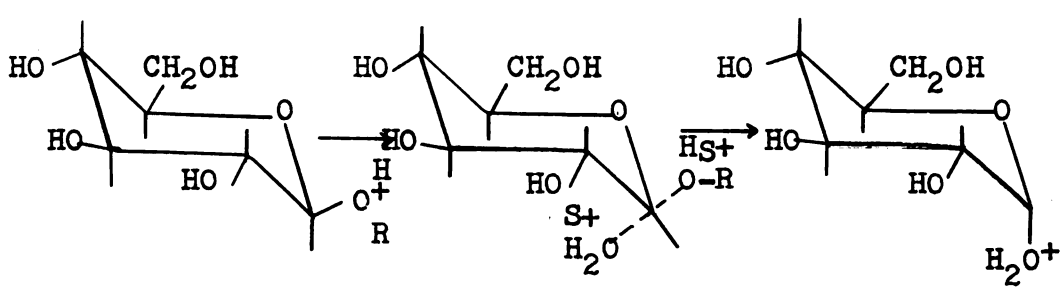


This mechanism was supported by the work of Zucker and Hammett[14] which predicted that specific oxonium ion catalysis should be a function of the Hammett acidity function H_o, rather than the concentration of the oxonium ion. Indeed, McIntyre and Long[15,16] did demonstrate a direct relationship between the Hammett acidity function H_o and the hydrolysis rate of methylal in strong acid solutions. This indicated that the activated complex resembled the conjugate acid of methylal; the reaction was therefore unimolecular (no water molecules bound in the transition state). Support was also drawn from the observation that dimethyl acetal hydrolysis proceeded more rapidly in deuterium oxide than in water;

this was interpreted to indicate that a rapid protonation step was followed by a rate-determining decomposition of the complex.[17] Kreevoy and Taft[18,19,20] correlated inductive, resonance, and steric effects upon acetal hydrolysis and concluded that the transition state resembled a carbonium ion at the carbonyl carbon atom.

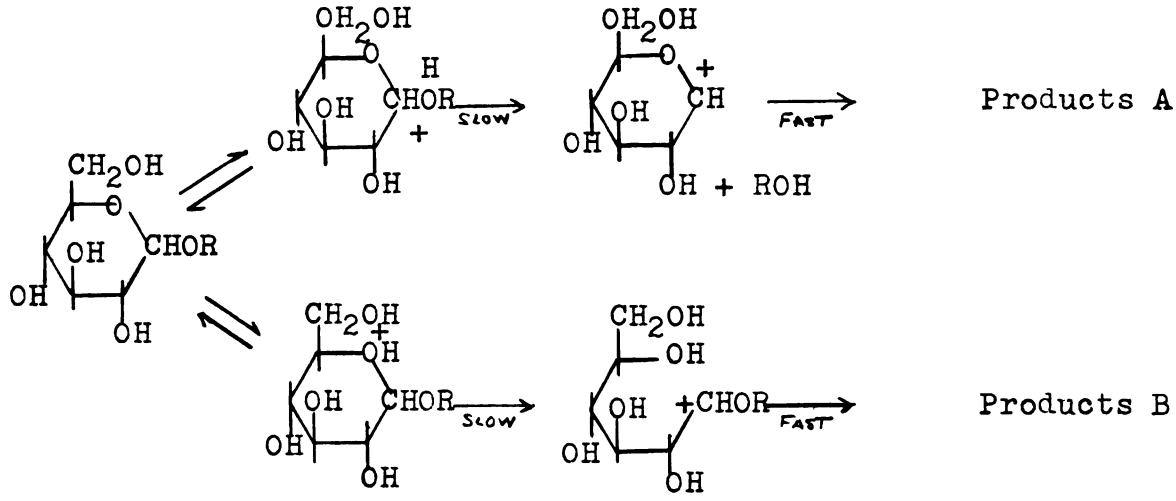
The acid-catalyzed hydrolysis of glycosides presents an analogous situation; hydrolysis of several D-glucopyranosides in O^{18} enriched water indicated that the reaction proceeded by hexose-oxygen bond cleavage. The center of substitution was therefore the C_1 atom of the pyranose ring; the only exception to this rule is the hydrolysis of t-butyl-β-D-glucopyranoside which proceeds by alkyl-oxygen bond cleavage due to the favorable formation of the t-butyl carbonium ion.[21]

However, glycoside hydrolysis is complicated by several possible hydrolytic mechanisms. One of these is a "bimolecular" synchronous mechanism with a linear transition state which leads to inversion of configuration:

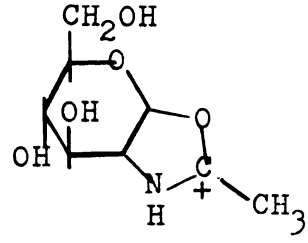


The other mechanisms involve carbonium ion formation as in acetal hydrolysis, although the situation is complicated by the fact that the reaction may proceed with or without ring

opening:[22]



The following transition state has been suggested in the hydrolysis of a substrate containing a nucleophile such as the N-acetyl group:



The actual mechanism of glycoside hydrolyses appears to depend on the substrate and the reaction conditions. Thus the analogous-methanolysis of 2,3,4,6, tetra-O-methyl-α-D-glucopyranosyl chloride was unimolecular by kinetic analysis, but yielded an anomeric mixture predominating in either α- or β- form, depending on whether or not chloride ion was present. On the other hand, the glucopyranosyl chloride reacted with thiophenoxide ion in propanol to yield the β- product in a true bimolecular reaction. The analogous mannose compound underwent methanolysis to yield an anomeric

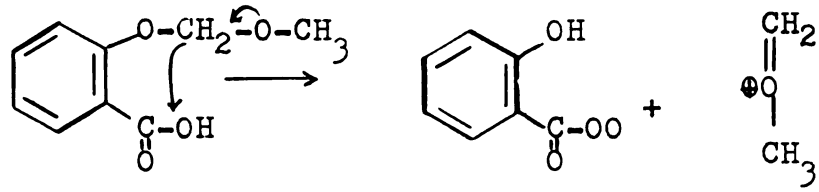
mixture which was unaffected by the presence of chloride ion, and which did not react at all with thiophenoxide ion. It appeared that the glucopyranosyl reaction mechanism could be either uni- or bimolecular; on the other hand the manno-pyranosyl system reaction mechanism appeared to be solely unimolecular due to steric effects.[22]

In general, the hydrolysis of simple glycosides appears to be unimolecular and to involve the formation of a carbonium ion in the rate determining step; that is, the rate appears to be dependent on H_o rather than upon the concentration of oxonium ion.[21] In all probability, these carbonium ions are ring-closed; the hydrolysis of α-methyl-D-glucopyranoside was found to exhibit a primary oxygen isotope effect which was attributed to C_1 carbon-oxygen bond cleavage during the rate-determining step. This is consistent with the formation of a ring-closed carbonium ion since the C_1-oxygen bond in the open-carbonium ion mechanism breaks in a step subsequent to the rate-determining step.[25]

It is notable that the effects of neighboring groups on the hydrolysis of acetals, ketals, and glycosides have been well documented only in the case of the carboxyl group. As early as 1919, Karrer[26] noted that the β-D-glucoside of salycic acid (o-carboxyphenyl-β-D-glucoside) spontaneously hydrolyzed in aqueous solution. This phenomonon was confirmed by Helferich,[27] who noted that spontaneous hydrolysis occurred only in unbuffered solutions and only when the glucoside was in the un-ionized carboxyl form; the salt and

ester forms of this glycoside were inactive. Capon studied the anomalously rapid hydrolysis of o-carboxyphenyl-β-D-glucoside and postulated that the carboxyl group participated as a nucleophilic-specific acid catalyst, a general acid catalyst, or as a combination of the two mechanisms.[28] However, Bruice[29] analyzed the scanty published data and concluded that electronic effects upon simple specific acid catalysis could account for the supposed carboxyl catalytic effect.

Capon[30] also studied the hydrolysis of a simple carboxyl-substituted acetal and observed a rate enhancement over that expected for specific acid catalysis (relative to the para-isomer and the methyl ester) and postulated the following mechanism:



Again Bruice[29] analyzed the results and concluded that in this case, the water-deuterium oxide rate ratio indicated general acid catalysis or specific acid-nucleophilic catalysis in a pH region in which only simple specific acid catalysis should have been observed. Thus a carboxyl group effect may be operative in this instance.

The hydrolysis of oligouronides also seemed to be influenced by intramolecular carboxyl group general acid catalysis.[31]

Bruice[29] conducted a penetrating analysis of carboxyl group participation in ketal hydrolysis. He investigated the hydrolysis kinetics of 19 1,3 dioxanes and 1,3 dioxolanes; 7 of these were carboxyl-substituted. The ketal structures were chosen so that a differentiation between intramolecular general acid catalysis and specific acid-nucleophilic attack could be made if carboxyl group participation was noted. Variations in the pH-log k_{obs} profiles of carboxyl substituted ketals indicated either undissociated carboxyl-group participation or simple specific acid hydrolysis of the anion form of these ketals.

However, the carboxyl-substituted ketals exhibited no significant positive deviation from the Hammett plots of log k_h versus σ, indicating that the reaction mechanism was simple specific acid catalysis. A water-deuterium oxide rate ratio of 0.20 also indicated specific acid catalysis.

Thus the evidence for carboxyl group participation appears to be meager; the only probable instances are the hydrolysis of the simple benzoic acid-substituted acetal[30] and the hydrolysis of oligouronides.[31]

This fact is somewhat disappointing in view of the implication of carboxyl groups in the catalytic activity of α-amylase,[32] β-glucosidase,[33] and lysozyme.[34] Lysozyme was discovered by Fleming in 1922;[35] soon thereafter, a large number of lysozymes were characterized from diverse sources: vertebrate tissues and secretions,[36] invertebrates,[37] insects,[38] bacteria,[39] and even plants.[40] These diverse

lysozymes, although differing slightly, have some common characteristics: They are basic proteins of low molecular weight (about 15,000), are stable at acidic pH (some even at 100° and pH 4.5 for 1-2 minutes), unstable at alkaline pH, and are capable of hydrolyzing M. lysodeikticus suspensions and solutions of N-acetylglucosamine polymers derived from chitin.[36] Therefore lysozyme is also called β-glycosamidase, N-acetyl muramide glycanohydrolase or muramidase.

The most common and readily available lysozyme is that derived from chicken egg white. This lysozyme may be crystallized directly from the egg white[40] and further purified by chromatography on a Dowex-50 column.[41] Hen egg white lysozyme is a single polypeptide chain consisting of 129 amino acid residues whose sequence is known[42,43] and whose structure has been established to 2 Å resolution.[44] The molecule is ellipsoidal, about 45 x 30 x 30 Å, and possesses a marked cleft on one side which has been the object of much speculation. This lysozyme has four disulfide bridges, three 10-residue α-helix runs, several turns of 3^{10} helix and a section of antiparallel pleated sheet conformation; the remainder of the molecule has no well-defined conformation.

Several active site amino acids have been detected by chemical means. Difference titrations of lysozyme versus lysozyme plus N-acetyl glucosamine or N-acetyl glucosamine trimer indicated a pK shift from 6.3 to 6.7 due to saccharide binding; the residue responsible for this perturbation

was thought to be Glu 35, since it apparently resides in a relatively nonpolar environment.[45] N-acetyl glucosamine polymers also perturb the lysozyme U.V. spectrum;[46] this spectrum and that of free lysozyme[47] varies with pH to yield pK values resembling those derived from difference titrations.[45] X-ray diffraction perturbations caused by the binding of N-acetyl glucosamine, tri-N-acetylglucosamine, and N-acetyl glucosaminyl-N-acetyl muramic acid, and model building studies have indicated that the hexasaccharide composed of alternating N-acetyl glucosamine (NAG) and N-acetyl muramic acid (NAM) units is cleaved between residue 4 (NAM) and 5 (NAG).[48] This was confirmed by the finding that the hexamer of NAG is cleaved into dimer and tetramer, this cleavage occurring two residues distant from the reducing end of the oligomer.[34]

The X-ray diffraction work also indicated that substrate binding was confined to the cleft of the molecule; the catalytically important residues appeared to be Glu 35 and Asp 52.[48]

Several catalytic mechanisms have been proposed[22] which involve these residues. The first proposal is basically the nucleophilic glycoside hydrolysis mechanism of Koshland.[49] Glu 35 appears to be in a non-polar environment and to be in a position to protonate the glycosidic oxygen atom; it may therefore assist in glycosidic bond rupture by general acid catalysis. On the other hand, Asp 52 appears to be in a polar environment and is probably

ionized; it may displace the disaccharide by nucleophilic attack on carbon C_1 of the fourth hexasaccharide residue to form a glycosyl-enzyme which then undergoes nucleophilic attack by water (or other acceptor molecules). However, a detailed examination of the X-ray diffraction model of lysozyme made this mechanism an unlikely choice; the nucleophile and the leaving group do not appear to be able to attain a configuration favoring the required bimolecular transition state. This model also does not allow C_1 and Asp 52 the proximity required for a covalent bond.

An alternate mechanism[22] involves the formation of a stabilized carbonium ion in a manner analogous to carbonium ion formation in model system acid-catalyzed hydrolysis reactions. This mechanism invokes the following steps:

1. The substrate is attached to lysozyme and held in place by hydrogen-bond and hydrophobic interactions. The ring of residue 4 is distorted from the normal chair conformation into a half-chair conformation favoring carbonium-ion formation.
2. Glu 35, being un-ionized, protonates the glycosidic oxygen atom.
3. Heterolysis of the residue 4 C_1 carbon-oxygen bond yields a carbonium ion which is promoted and stabilized by the negative charge on Asp 52, which is in an ionic environment 3 Å away.
4. The resulting free disaccharide diffuses out and a water molecule attacks the carbonium ion along the

same path with resultant overall retention of configuration at C_1.

Lowe advanced some interesting mechanisms for the lysozyme-catalyzed hydrolysis of o- and p-nitrophenyl β-D-chitobiosides which involve Glu 35 and Asp 52.[50] One of these mechanisms invokes participation of Glu 35 and the N-acetyl moiety of the terminal N-acetyl glucosamine residue with consequent formation of a cyclic ion. This mechanism again requires that the glucosidic ring undergoing hydrolysis take up a strained configuration, in this case a boat configuration. The other proposed mechanism postulated that Glu 35 acts as a general acid, while the carboxylate ion of Asp 52 acts as a nucleophile, yielding a glycosyl-enzyme intermediate.

It is therefore possible that some type of strong binding situation, either ionic or covalent, obtains in lysozyme catalysis; nor should the possibility of neighboring group assistance be overlooked.

EXPERIMENTAL

Reagents

General

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

Acetal, Ketal, Tetrahydropyran ether model glycoside reagents

a. Benzene, anhydrous

Reagent grade benzene was dried by azeotropic distillation.

b. Bromoacetaldehyde diethyl acetal

Matheson, Coleman and Bell reagent grade.

c. Diethyl ether

Mallinkrodt anhydrous ether.

d. Diethyl malonate

Matheson, Coleman and Bell reagent grade.

e. 3,4 Dihydropyran

Aldrich Chemical Co. practical grade was redistilled on a packed column; the fraction boiling from 83.4-84.5° was collected.

f. Dimethyl formamide

Eastman Kodak reagent grade was redistilled prior to use; the fraction boiling at 152-154° was collected.

g. Dioxane

Dioxane was purified by a modification of the method of Fieser.[51] A solution of 2 l dioxane, 200 ml water, and 27 ml concentrated hydrochloric acid was refluxed for 36 hr 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 sodium borohydride for 24 hr prior to distillation directly from the 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.

h. Ethanol, absolute

Gold Shield 200-proof ethyl alcohol was refluxed with and redistilled from barium oxide.

i. Ethanol assay system

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

j. Ethylene chlorohydrin

Eastman Kodak reagent grade.

k. Ethyl orthoformate

Aldrich Chemical Company reagent grade.

l. 2-Ethoxyacetaldehyde diethyl acetal

Aldrich Chemical Company and Matheson, Coleman and Bell.

m. Methanol, absolute

J. T. Baker absolute methyl alcohol.

n. 2-Methoxyacetaldehyde dimethyl acetal

Aldrich Chemical Company.

o. 2-(2-hydroxyethyl)-pyridine

Aldrich Chemical Company.

p. Thionyl chloride

Matheson, Coleman and Bell.

Lysozyme reagents

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

Rohm and Haas

b. Amberlite CG-120 resin

Amberlite CG-120 type II (200 mesh and finer) purchased from Rohm and Haas had previously been classified for quantitative amino acid chromatography by the method of Hamilton;[52] the fraction used in this work was the coarse residue which did not flow at a wash velocity of 590 ml per min.

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

California Corporation for Biochemical Research and BioRad Laboratories.

d. Biogel P-4, 50-100 mesh

BioRad Laboratories

e. Carboxymethyl cellulose

California Corporation for Biochemical Research.

f. Dialysis tubing

Cellulose "dialyzer tubing" (15/16 in), no. 4465-A2,

was purchased from A. H. Thomas Company and used in several substrate preparation experiments. However, this material did not retain lysozyme; therefore "Visking" sausage casing (16/32 in), purchased from Union Carbide Corporation, was utilized in dialyses when retention of molecules of m.w. greater than 10,000 was important.

g. Dimethylsulfoxide

Aldrich Chemical Company reagent grade.

h. Dithiothreitol

California Corporation for Biochemical Research.

i. 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°.

j. Glycerol, anhydrous

Reagent grade glycerol was distilled under reduced pressure (0.05 mm) and stored over phosphorus pentoxide.

k. Guanidine hydrochloride

Reagent grade guanidine hydrochloride (Mallinkrodt) 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 off-white solid was recrystallized from 95% ethanol.

l. Hydroxylamine hydrochloride

Matheson, Coleman and Bell practical grade.

m. Iodoacetic acid

Eastman Kodak reagent grade.

n. Lysozyme

Worthington Biochemical Corporation twice recrystallized salt-free lysozyme and Pentex, Incorporated crystalline lysozyme.

o. Micrococcus lysodeikticus cells, dried

Sigma Chemical Company pfs grade

p. Polychromatic ninhydrin spray solution

This solution was prepared as described by Litwack.[53] Solution A was a 0.2% ninhydrin solution made up in a mixture of 50 ml anhydrous ethanol, 10 ml glacial acetic acetic acid, and 2 ml 2,4,6 collidine. Solution B was a 1% solution of cupric nitrate trihydrate in redistilled water. Solutions A and B were combined in a 50:3 ratio (v/v) prior to use.

q. Sephadex G-15

Pharmacia Fine Chemicals, Incorporated.

r. Urea

Mallinkrodt reagent grade. Urea solutions were deionized prior to use by storage over Amberlite MB-3 mixed bed resin.

Preparations

2-Methylthioacetaldehyde

The preparation of this aldehyde was a modification of the method of Cope and co-workers.[54]

A mixture of 2-methylthioacetaldehyde dimethyl acetal (20 ml), water (20 ml), and concentrated hydrochloric acid

(0.2 ml) contained in a 100 ml flask fitted with reflux condenser and magnetic stirring bar was stirred for 30 min at room temperature, and then refluxed and stirred for 5 min to yield a homogenous solution. This solution was cooled and saturated with sodium chloride. The resulting organic layer was removed in a separating funnel (upper layer) and the water layer extracted 3 times with ethyl ether (15 ml portions). The ether extracts were combined with the organic layer; the resulting solution was dried over magnesium sulfate. Filtration and distillation through a Vigereux column yielded a light yellow oil which was fractionally distilled on the same still at reduced pressure to yield 2-methylthioacetaldehyde (3.8 g) b.p. 77° (105 mm).

2-Hydroxyethyl Methyl Sulfide

This sulfide was prepared by a modification of the procedure of Windaus and Schildneck.[55]

Sodium (80.5 g) was reacted with absolute ethanol (1500 ml) in a 3-l three-necked round bottom flask fitted with a stirrer, a reflux condenser, and a dropping funnel. When reaction had ceased, methyl mercaptan (supplied from a gas cylinder) was bubbled through the solution until it was saturated. The resulting sodium methyl mercaptide solution was heated to reflux. Heating was discontinued and ethylene chlorohydrin (302 g) was slowly added with vigorous stirring over a 2 hr period. Then 1000 ml of ethanol were

distilled off and the concentrate cooled and filtered. The precipitate was washed three times with the previously removed ethanol. The filtrate was fractionally distilled through a 50-cm packed column under reduced pressure to yield 2-hydroxyethyl methyl sulfide (283 g), b.p. 74° (20 mm).

2-Methylthioacetaldehyde Diethyl Acetal

The procedure employed was a modification of the method described by Cope.[54]

Sodium (6.9 g) was reacted with absolute Methanol (100 ml) in a round bottom flask fitted with a reflux condenser and a drying tube. When the sodium methoxide solution had cooled, a magnetic stirring bar and gas delivery tube were added to the flask. Methyl mercaptan (gas cylinder) was slowly bubbled through the stirred solution to saturation. Then 2-bromoacetaldehyde diethyl acetal (60 g) was added and the resulting solution refluxed for 24 hr. Filtration and concentration by distillation on a Vigeraux column still produced a suspension which was again filtered; the filtered precipitates were washed with ether (10 ml) in each case. The resulting yellow ether solution was fractionally distilled on a Vigereux column still to give 2-methylthioacetaldehyde diethyl acetal (20 g), b.p. 77.5-79.5° (13 mm).

2-Methoxyacetaldehyde Diethyl Acetal

A solution of 2-bromoethanal diethyl acetal (30 g) and sodium methoxide (7.9 g sodium in 60 ml absolute methanol) was sealed into a heavy glass reaction tube and placed in a 105° oil bath for 48 hr. The resulting reaction mixture was filtered and distilled to remove the methanol. Anhydrous ethyl ether (50 ml) was added to the slurry, the resulting suspension was filtered, and the precipitate washed with ether (25 ml).

The filtrate was concentrated by distillation through a Vigereux column to yield a slurry of product and salt. Much of the remaining salt was removed by repetition of the ether-filtration-distillation step. The resultant yellow suspension was taken up in ether (60 ml) and centrifuged. The supernatant was distilled through a Vigereux column to remove the ether; the crude product was then distilled in the same still at reduced pressure to yield 2-methoxyacetaldehyde diethyl acetal (4 g), b.p. 49.5-50.0° (12 mm).

4-Methylthio Butraldehyde Diethyl Acetal

2-Chloroethyl methyl sulfide

A slight modification of the method of Kerner and Windaus[56] was utilized to prepare this compound. A solution of thionyl chloride (68 g) in anhydrous chloroform (65 g) was added dropwise to a stirred solution of 2-hydroxyethyl methyl sulfide (50 g) in anhydrous chloroform (70 g). The

reaction mixture was heated once midway in the addition to maintain gentle chloroform reflux. Stirring was maintained for four hours after completion of the thionyl chloride addition. The chloroform was removed by distillation on a steam bath. The residue was distilled under reduced pressure to yield 2-chloroethyl methyl sulfide (44 g), b.p. 48-50° (22 mm).

Ethyl-2-methylthio-ethyl malonate

The preparation of this compound was a modification of the Organic Synthesis Ethyl-n-butylmalonate preparation.[57]

Diethyl Malonate (58 ml) was added dropwise over a period of one hour to a sodium ethoxide solution prepared by reacting sodium (8.33 g) with anhydrous ethanol (180 ml). The reaction mixture was then heated to 50° to prevent solidification and 2-chloroethyl methyl sulfide was added over a 2 hr period. The reaction mixture was refluxed for 2 hr, then cooled and filtered. The filtrate was distilled to remove the ethanol; the residue was washed once with 10% sodium chloride solution. The organic layer was removed and the water layer was extracted with ethyl ether (100 ml). The organic layer and the ether extract were combined and dried over anhydrous sodium sulfate. The ether was removed by distillation at reduced pressure (22 mm); the residue was fractionally distilled at reduced pressure to yield ethyl-2-methylthio-ethyl malonate (40 g), b.p. 148-150° (22 mm).

4-Methylthio-butyric acid

Again, this preparation is a modification of the method outlined in Organic Synthesis.[58] Ethyl-2-methylthio-ethyl malonate (40 g) was slowly added under nitrogen atmosphere to a hot (90°) solution of potassium hydroxide (40 g) in water (40 ml). This reaction mixture was refluxed for 3 hr; then water (40 ml) was added and 40 ml of liquid was distilled off to remove the ethanol. The remaining liquid was cooled and a cold solution of sulfuric acid (64 g) in water (70 ml) was added over a 1 hour period. The reaction mixture was refluxed for 8 hours and then cooled; the upper organic layer was removed and the water layer was extracted four times with ethyl ether (40 ml portions). The organic layer and ether extracts were combined and dried over anhydrous sodium sulfate. The ether was removed by reduced pressure distillation (15 mm) and the residue fractionally distilled at reduced pressure to yield 4-methylthio butyric acid (15.5 g) b.p. 88-90° (0.60 mm).

4-Methylthiobutyric acid chloride

Thionyl chloride (34.5 g) was slowly added to 4-methylthiobutyric acid (15.5 g). After 6 hours at room temperature, the residual thionyl chloride was evaporated at reduced pressure under nitrogen to yield 4-methylthiobutyric acid (10.5 g) b.p. 86-89° (10 mm).

Elemental analysis:

Calculated:	C, 39.35%	H, 5.91%	Cl, 23.28%	S, 20.98%
Found:	C, 39.49% 39.35%	H, 6.09% 5.94%	Cl, 23.51% 23.44%	S, 20.90%

4-Methylthiobutraldehyde

Rosenmund reduction was utilized to prepare this aldehyde. A solution of 4-methylthiobutyric acid chloride (10.5 g) in anhydrous benzene (50 ml) containing 5% palladium-barium sulfate catalyst (5 g) was heated to 70° and stirred while hydrogen was bubbled through the suspension. The effluent gas was passed through 0.11 N sodium hydroxide solution to monitor the progress of the reaction. When reaction was 80% complete by sodium hydroxide neutralization, the catalyst was filtered off and washed with anhydrous benzene (20 ml). The filtrate and washings were combined and the benzene distilled off at reduced pressure (15 mm) to yield a residue of crude yellow 4-methylthiobutraldehyde (10 g). Elemental analysis:

Calculated:	C, 50.08%	H, 8.48%	S, 27.10%
Found:	C, 50.92%	H, 8.43%	S, 26.45% 26.34%

4-Methylthiobutraldehyde diethyl acetal

The crude 4-methylthiobutraldehyde from the Rosenmund reduction (10 g) was mixed with triethyl orthoformate (25 g), absolute ethanol (10 ml) and ammonium chloride (0.1 g) and refluxed for 18 hours. Low boiling components, primarily ethyl formate, were stripped off during reflux on the spinning band still. The ethanol was then distilled off and the residue fractionally distilled at reduced pressure the yield 4-methylthiobutraldehyde diethyl acetal (4 g) b.p. 102-103° (10 mm).

5-Hydroxyvaleraldehyde

The method of Schniepp and Geller[59] was used to prepare 5-hydroxyvaleraldehyde.

Preparation of Valeraldehyde Diethyl Acetal

A solution of valeraldehyde, (30 g), triethylorthoformate (60 g), absolute ethanol (10 ml) and concentrated sulfuric acid (0.05 ml) was kept at room temperature for 48 hr. The solution was then neutralized with ethanolic sodium ethoxide and fractionally distilled to remove ethyl formate and ethanol. Fractional distillation on a packed column at reduced pressure gave valeraldehyde diethyl acetal (28 g) b.p. 59° (15 mm).

2-Pentanone Diethyl Ketal

A solution of 2-pentanone (43 g), triethylorthoformate (90 g), absolute ethanol (5 ml), and concentrated sulfuric acid (0.03 ml) was allowed to stand at room temperature for 36 hr. The resulting solution was neutralized with ethanolic sodium ethoxide, concentrated by fractional distillation on a Vigereux column, and fractionally distilled in the same still at reduced pressure to yield 2-pentanone diethyl ketal (51 g) b.p. 87-88° (14 mm).

Preparation of 5-Chloro-2-Pentanone

A mixture of water (263 ml), concentrated hydrochloric acid (225 ml) and α-acetyl-γ-butyrolactone was heated to maintain a rapid rate of carbon dioxide evolution. After 25 min, gas evolution had ceased; the heat input was then increased to rapidly distill off the ketone-water mixture. After 450 ml distillate had been collected, 300 ml of water was added and distillation continued. This addition-distillation step was repeated; the distillates were combined and the ketone layer separated. The water layer was extracted three times with ether (100 ml portions) and the extract combined with the ketone layer. After drying over calcium chloride, the ether was removed by distillation; the residue was fractionally distilled at reduced pressure to yield 5-chloro-2-pentanone (124 g) b.p. 59-60° (12 mm). The ketone was stored at 5° to retard decomposition.

Preparation of 5-Chloro-2-Pentanone Diethyl Ketal

A solution of 5-chloro-2-pentanone (62 g), triethyl orthoformate (88 ml), absolute ethanol (5 ml) and concentrated sulfuric acid (0.05 ml) was allowed to stand at room temperature for 24 hr. The red solution was neutralized with sodium ethoxide in ethanol and distilled to remove ethyl formate and ethanol. The residual oil was fractionally distilled at reduced pressure on a 1/2 by 20 inch column packed with 2 mm glass helices to yield 5-chloro-2-pentanone diethyl

ketal (74 g), b.p. 92-93° (13 mm).

Elemental analysis:

Calculated:	C, 55.52%	H,	9.84%
Found:	C, 58.86%	H,	10.06%
	58.63%		9.94%

5-Methylthio-2-Pentanone Diethyl Ketal

A sodium methoxide solution prepared by reacting sodium (1.15 g) with absolute methanol (25 ml) was saturated with methyl mercaptan. The resulting sodium methyl mercaptide solution was mixed with 5-chloro-2-pentanone diethyl ketal (10.7 g), sealed in a glass reaction tube, and placed in a boiling water bath for 24 hr. The reaction mixture was then cooled and filtered; the filtrate was concentrated by distillation, mixed with ether (10 ml) and again filtered. This filtrate was distilled at reduced pressure (13 mm) to yield the crude ketal which was fractionated on the spinning band column at reduced pressure (13 mm) to yield impure ketal, b.p. 80-89°. Refractionation of this material on a packed column at reduced pressure yielded 5-methylthio-2-pentanone diethyl ketal (5 g), b.p. 54° (0.025 mm).

Elemental analysis:

Calculated:	C, 58.20%	H,	10.75%	S,	15.54%
Found:	C, 58.63%	H,	10.90%	S,	16.47%
	58.46%		10.69%		16.62%

5-Methoxy-2-Pentanone Diethyl Ketal

A sodium methoxide solution prepared by adding sodium (1.15 g) to absolute methanol (25 ml) was mixed with 5-chloro-2-pentanone diethyl ketal (10.7 g), sealed in a glass reaction tube and placed in a boiling water bath for 48 hr. The reaction mixture was then cooled, filtered, and concentrated by distillation. Ether (10 ml) was added to the concentrate and the precipitate removed by centrifugation. Removal of the ether by distillation afforded an oil, which when fractionally distilled at reduced pressure, yielded 5-methoxy-2-pentanone diethyl ketal (4.5 g), b.p. 70-71° (13 mm).

Elemental analysis:

Calculated:	C, 63.12%	H, 11.66%
Found:	C, 63.33%	H, 12.07%

2-Ethoxy Tetrahydropyran

To a stirred solution of anhydrous ethanol (30 g) and concentrated sulfuric acid (0.1 ml) was added 3,4 dihydropyran (60 g). After 14 hr at room temperature, the reaction mixture was neutralized with ethanolic sodium ethoxide solution and fractionally distilled at reduced pressure to yield 2-ethoxy tetrahydropyran (32 g), b.p. 40.5-41.0° (11 mm).

2-(2-Methoxyethoxy)-Tetrahydropyran

Sixty grams of 3,4-dihydropyran were slowly added to a

stirred solution of 2-methoxyethanol (48 g) and concentrated hydrochloric acid (0.1 ml). The reaction mixture was kept at room temperature for 12 hr and then fractionally distilled at reduced pressure to yield 2-(2-methoxyethyl)-tetrahydropyran (43.5 g), b.p. 80-82° (15 mm).

2-(2-Methylthioethoxy)-Tetrahydropyran

Thirty-two grams of 3,4-dihydropyran were slowly added to a stirred solution of 2-methylthioethanol (30 g) and concentrated hydrochloric acid (0.1 ml). The reaction mixture was kept at room temperature for 12 hours and then fractionally distilled at reduced pressure to yield 2-(2-methylthioethoxy)-tetrahydropyran (25 g), b.p. 114-116° (15 mm).

2-(2-Pyridinylethoxy)-Tetrahydropran

Hydrogen chloride was bubbled into a solution of 3,4-dihydropyran (16.8 g), 2-(2-hydroxyethyl)-pyridine (24.6 g) and dimethylformamide (30 ml) contained in an ice-cooled round-bottom flask fitted with a drying tube and reflux condenser until 6 g of HCl had been added. The solution was then heated to 90° and held at that temperature for 4 hr, then cooled and held at room temperature for 3 hr. The resulting brown solution was neutralized with barium oxide for 12 hr, suspended in ethyl ether (200 ml) and centrifuged to yield a yellow solution. Concentration by evaporation at

reduced pressure (11 mm), followed by distillation at reduced pressure (11 mm) afforded a brown oil. Fractional distillation at reduced pressure on a Vigereux column still yielded 2-(2-pyridinylethoxy)-tetrahydropyran (9.5 g), b.p. 84-87° (0.75 mm).

Elemental analysis:

Calculated:	C, 69.51%	H, 8.25%	N, 6.76%
Found:	C, 69.07%	H, 8.07%	N, 7.16%

Preparation of Acetate Buffers for Ketal Hydrolysis Rate Determinations

The ionization constant of acetic acid in 49.6% dioxane--50.4% water (by weight), the final composition of the ketal hydrolysis medium, was determined by extrapolation from the acetic acid ionization constant data published by Harned and Owen;[60] it was found to be 2.63×10^{-7}M (pK = 6.58).

Acetate buffers were prepared by weighing the required amounts of glacial acetic acid and sodium bromide (when required to adjust ionic strength) into a volumetric flask. The required amount of 3.030N sodium hydroxide was then pipetted into the flask and the contents diluted to the mark to yield the buffer. A Beckman Model G pH meter was used to determine the pH values of the solutions.

Table I summarizes the preparation of the acetate buffers. The concentrations listed are those found prior to dilution with dioxane; the actual hydrolysis concentrations are one-half the stated concentrations. The notation (H^+)

TABLE I

Preparation of Acetate Buffers for Ketal Hydrolysis Rate Determinations

The figures in parentheses indicate the grams of glacial acetic acid and sodium bromide per liter buffer or the milliliters of 3.030N sodium hydroxide per liter buffer.

pH	Acetic Acid, M	Sodium Acetate, M	Sodium Bromide, M	Γ	$10^7(H^+)$	$10^7(H^+)_c$
4.18	0.0087 (0.676g)	0.0025 (0.824ml)	0.0000	0.0050	9.14	11.8
4.40	0.0500 (4.502g)	0.0250 (8.24ml)	0.0000	0.0500	5.26	10.3
4.64	0.0500 (6.005g)	0.0500 (16.65ml)	0.0000	0.1000	2.63	6.45
4.22	0.0750 (6.005g)	0.0250 (8.30ml)	0.0250 (2.569g)	0.1000	7.89	19.2
4.34	0.1516	0.0744	0.000	0.1588	5.26	12.0
4.20	0.0750 (6.005g)	0.0250 (8.27ml)	0.0750 (7.730g)	0.2000	7.89	24.3

Dilution of the pH 4.22 and pH 4.64 acetate buffers with 0.0500 M sodium bromide (10.291 g per 2 l) solution by factors of 1:2 and 1:10 produced diluted buffers of identical ratios, ional strengths and hydronium concentrations.

refers to the uncorrected hydronium ion concentration of the dioxane-diluted buffer; $(H^+)_c$ refers to the hydronium ion activity of the dioxane-diluted buffer corrected for secondary salt effects by the use of the following equation:[61]

$$(H^+)_c = \frac{K\ (acid)}{f_{\pm}\ (salt)}$$

$f_{\pm}$ is determined by use of:

$$f_{\pm} = \frac{1.26\sqrt{\Gamma}}{1 + 2.0\sqrt{\Gamma}} \qquad \Gamma = \text{Ional strength}$$

Determination of Hydrolysis Rates

All acetal and ketal and most tetrahydropyranyl ether hydrolysis rate determinations were based on increments in absorbance created by the $n \longrightarrow \pi^*$ transition of the carbonyl group of the aldehyde or ketone produced upon hydrolysis.

Although the aldehyde or ketone produced in the course of hydrolysis becomes hydrated, the equilibrium between non-hydrated and hydrated forms apparently is very rapid since kinetic measurements are not perturbed by this phenomonon.[62] This situation also appears to apply to the equilibrium between 5-hydroxyvaleraldehyde and its cyclic hemiacetal.

All kinetic measurements were carried out in solutions of 50% dioxane - 50% perchloric acid or buffer solution (by volume) this corresponds to a 49.6:50.4 weight:weight ratio.[63] Absorbance was measured in a Beckman D.U. or Cary Model 11 spectrophotometer: In all cases the cell compartment was thermostatted to 25.0 $\pm$ 0.05°.

The concentration of acetal, ketal, or tetrahydropyran ether was adjusted so that the absorbance of the completely hydrolyzed solution was less than 1.0.

In most cases, absorbance measurements were carried out in 1 cm quartz cells. However, the low solubility of several of the compounds necessitated a lower concentration and measurement in a 10 cm quartz cell (Cary 11).

Changes in absorbance (increments) were read at fixed time intervals on the Beckman DU or read off the chart at fixed intervals in the case of the Cary 11. The $\log_{10}$ of these increments was then plotted as a function of time in seconds. The slope of this line multiplied by the natural log conversion factor 2.303 then gave the value of the first-order hydrolysis rate constant k_1. The pseudo-second order hydrolysis rate constant was determined by dividing the first-order rate constant by the acid concentration in the case of perchloric acid-catalyzed hydrolyses, or by the hydronium ion activity in the case of the buffer-catalyzed hydrolysis runs. The hydronium ion activity of the buffer solutions was calculated according to the following formula:[64]

$$(H^+) = \frac{k\ (acid)}{(salt)\ f_{\pm}^2}$$

$f_{\pm}$ is determined from the relationship:

$$f_{\pm} = \frac{1.26\sqrt{\Gamma}}{1 + 2.0\sqrt{\Gamma}}$$

where Γ is the ional strength of the buffer solution.

Certain of the acetals and ketals exhibited a primary salt effect: In these cases the hydrolysis rates were extrapolated to zero ional strength.

Procedure for Determination of Acetal, Ketal, and Tetrahydropyran Hydrolysis Rates

Hydrolysis reaction mixtures were prepared by two methods. The first method employed two flasks immersed in a 25.0° water bath to equilibrate the dioxane (5-15 ml) acetal/ketal/tetrahydropyran solution and the perchloric acid or buffer solution. At zero time an equivalent (5-15 ml) volume of perchloric acid or buffer was rapidly pipetted into the dioxane-acetal/ketal/tetrahydropyran solution and mixed by shaking. The reaction mixture was then transferred to a 1- or 10-cm quartz cell; the filled cell was placed in a thermostatted spectrophotometer compartment (25.0°) and the absorbance determined as a function of time.

The second method employed a reaction flask consisting of an inner well surrounded by a concentric outer well. The appropriate amounts of acetal, ketal or tetrohydropyran and dioxane (5-15 ml) were pipetted into the center well; the perchloric acid or buffer was then carefully pipetted into the outer well. The flask was equilibrated in a 25.0° water bath. The solutions were very rapidly mixed at zero time by violent shaking; the reaction mixture was transferred to a 1 or 10 cm quartz cell. The cell was placed in a thermostatted spectrophotometer compartment (25.0°) and the absorbance

determined as a function of time.

Procedure for Determination of Tetrahydropyran Hydrolysis Rates

Since the high absorbance of the pyridine ring system of 2-(2-pyridinyl-2-ethoxy)-tetrahydropyran effectively blanked out that of the aldehyde produced by acid-catalyzed hydrolysis, rate measurement by absorbance increments was not possible. Therefore the hydrolysis rates of several tetrahydropyran ethers were followed by a modification of the hydrogen peroxide assay of Satterfield and co-workers.[65] The assay is based on alkaline hydrogen peroxide oxidation of liberated aldehyde; the amount of acid formed is determined by back-titration with hydrochloric acid.

The hydrolysis reaction mixture was prepared by weighing 203-217 mg 2-(2-pyridinylethoxy)-tetrahydropyran or 155-167 mg 2-(2-methoxyethoxy)-tetrahydropyran into the center compartment of a reaction flask. Perchloric acid or acetate buffer was pipetted into the outer compartment. The flask was equilibrated in a 25.0° water bath; the contents were mixed at zero time by shaking in the bath. The reaction was allowed to proceed at 25.0°. At 100- or 200-sec intervals a 2 ml portion was withdrawn and quenched in 20 ml of 0.02 N sodium hydroxide solution.

A solution of 3% hydrogen peroxide (2 ml) was added to each flask. The flasks were then placed in a 60° water bath for 5 minutes, then cooled and titrated with 0.01 N hydro-

chloric acid to a phenolpthalein end point (the amount of perchloric acid present in the reaction mixture aliquot was subtracted).

The assay was standardized with known quantities of the tetrahydropyran ether hydrolysis product, 5-hydroxy-valeraldehyde (1×10^{-5}, 5×10^{-5}, and 10×10^{-5} moles).

First order rate constants were determined by two methods. Several constants were determined by plotting aldehyde concentration as a function of time. The half-life of the reaction as determined from this plot then allowed a determination of the first order rate constant k_1 by the following relationship:

$$k_1 = \frac{0.693}{t_{\frac{1}{2}}}$$

Most of the rate constants were determined by plotting the logarithm of the fraction C_t/C_o (C_t is the concentration of unhydrolyzed tetrahydropyran ether at time t and C_o is the concentration at zero time). The slope of this line multiplied by the natural logarithm conversion factor 2.303 then yielded the first-order hydrolysis rate k_1.

In both cases, the pseudo-second-order hydrolysis rate constant k_2 was obtained by dividing k_1 by the acid concentration.

Rate of Ethanol Formation in Hydrolyzing Samples of Methylthioacetals and Ketals

The rate of ethanol formation in hydrolyzing samples

of 2-methylthioacetaldehyde diethyl acetal or 5-methylthio-2-pentanone diethyl ketal was determined by quenching portions of the hydrolysis solution. The alcohol content of the quenched samples was determined by a coupled enzyme system in which the horse liver alcohol dehydrogenase mediated reduction of NADH was coupled to methylene blue reduction (decolorization) by a diaphorase from Clostridium kluyveri (Determatube C-ALK).

2-Methylthioacetaldehyde diethyl acetal

A solution of 9.6-12.3 mg of 2-methylthioacetaldehyde diethyl acetal in 10.0 ml 0.020 M hydrochloric acid was prepared by equilibrating acetal and acid separately in a 25.0° water bath; the two components were mixed at zero time and allowed to hydrolyze at 25.0°.

At appropriate intervals, usually 100 seconds, 1.0 ml samples were withdrawn from the reaction solution and quenched in 10.0 ml 0.02 M sodium bicarbonate solution. The quenched samples were then assayed for ethanol concentration by mixing 0.1 ml of each sample with 3.0 ml of the standard C-ALK assay system in a 1 cm cuvette. The absorbance drop at 600 mμ allowed a determination of the ethanol concentration; a blank consisting of 0.1 ml of a solution of 12 mg 2-methylthioacetaldehyde in 10 ml water and an ethanol standard containing 2.0 μg ethanol per 0.1 ml allowed an accurate determination of the ethanol concentration in the samples. Ethanol evolution was plotted as a function of time (Figure 7). Ethanol release kinetics were determined by plotting ethanol concentration increments as a function of time (Figure 8).

5-Methylthio-2-pentanone diethyl ketal

A solution of 90.7-97.5 mg of 5-methylthio-2-pentanone diethyl ketal in a mixture of 5.0 ml dioxane and 5.0 ml 0.1 M pH 4.34 acetate buffer was prepared by equilibrating ketal and dioxane buffer solution separately in a two-compartment reaction flask in a 25.0° water bath. At zero time the two components were rapidly mixed and the resulting solution allowed to hydrolyze at 25.0°.

At appropriate intervlas, usually 600 seconds, 0.1 ml samples were withdrawn and quenched in 5 ml (first two samples) or 10 ml (remaining samples) of 0.002 M sodium bicarbonate solution. The quenched samples were assayed for ethanol concentration by mixing 0.1 ml of each sample with 3.0 ml of the standard C-ALK assay system in a 1 cm cuvette. The ethanol concentration was derived from the decrease in absorbance at 600 mμ with the aid of an ethanol concentration curve prepared from standard solutions containing 1, 2, and 3 μg ethanol per 0.1 ml. The assay response was found to be linear with alcohol concentration. The assay blank consisted of 0.1 ml of a mixture of 0.1 ml dioxane buffer solution and 10 ml of the bicarbonate solution. Ethanol evolution was plotted as a function of time (Figure 9).

Preparation of Micrococcus Lysodeikticus Cell Walls

Cell walls were prepared from dried *M. lysodeikticus* cells by a modification of the method of Sharon and Jeanloz.[66]

M. lysodeikticus cells (15 g), "Superbrite" 0.1 mm glass beaks (250 g) and redistilled 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 redistilled water and the washings combined with the previously decanted suspension.

The suspension was centrifuged at 3000 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 redistilled water followed by centrifugation at 10,000 rpm for 20 minutes at 0°. The washed cell wall pellet was suspended in redistilled water (400 ml) and centrifuged at 3000 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 redistilled 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 lyophilized to yield

off-white amorphous cell wall material (2.15 g).

Hydrolysis of M. Lysodeikticus Cell Walls

Hydrolysis yielding hexa-, tetra-, and disaccharides and glycopeptide I

This hydrolysis of cell walls is an adaptation of the method of Sharon et al.[67] Dry M. lysodeikticus cell wall (2.1 g) was suspended in 0.05 M ammonium acetate solution (100 ml); Pentex three times recrystallized lysozyme (5 mg) and toluene (0.3 ml) were added and the suspension held at 37° for 24 hr. The resulting mixture was transferred to a 50 cm dialysis bag formed from 18/32 inch Visking sausage casing.

The bag was suspended in a vertical length of 21 mm diameter glass tubing fitted with inlet and outlet tubes; dialysis was carried out by passing 600 ml toluene-saturated redistilled water through the glass tubing over a 12 hr period. Concentration of the dialysate on a rotary evaporator followed by freeze drying yielded 450 mg of yellow solid.

This material was dissolved in 5 ml water and placed on a 3.5 x 20 cm Amberlite CG-120 column in the acid form. The column was then eluted with 500 ml of redistilled water; concentrating the eluate on a rotary evaporator and freeze drying the concentrate produced 210 mg of white solid.

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Hydrolysis yielding hexasaccharide and glycopeptide GP-1

A suspension of M. lysodeikticus cell walls (4.00 g) and toluene (1.0 ml) in an 0.050 M ammonium acetate solution 200 ml) containing Pentex lysozyme (8 mg) was magnetically stirred for 12 hr at 37°. The resulting yellow solution was placed in 15/16 inch dialysis tubing and dialyzed against redistilled water (700 ml) for 24 hr. The dialyzate was lyophilized to yield a yellow powder (350 mg).

The lyophilized powder was dissolved in redistilled water (5 ml) and applied to an Amberlite CG-120 H^+ column. The column was eluted with redistilled water (600 ml) at a rate of 100 ml per hr; the eluate was lyophilized to yield a white amorphous powder (284 mg).

Hydrolysis yielding glycopeptide GP-1a

M. lysodeikticus cell walls (1.7 g) were suspended in 0.05 M ammonium acetate solution (100 ml); Worthington lysozyme (4 mg) and toluene (0.3 ml) were added and the suspension held at 37° for 2.5 hr. Then carboxymethyl cellulose in the sodium form (15 g) and redistilled water were added and the suspension stirred for 1 hour at room temperature; additional carboxymethyl cellulose (15 g) was added and stirring was continued for 2 hr. The thick suspension was diluted by the addition of redistilled water (100 ml) and centrifuged at 10,000 rpm for 20 min; the pellet was washed by resuspension in redistilled water (100 ml) and centrifugation at 10,000 rpm for 20 min. The supernatants

were combined and lyophilized to yield an off-white powder (1.27 g).

Automated Gel Filtration Apparatus

An automated gel filtration apparatus, which is diagrammed in Figure 1, was utilized to fractionate the lyxozyme M. lysodeikticus cell wall digests.

A Milton Roy "Minipump" metering pump pumped boiled redistilled water (via 1 mm id polyethylene tubing) through a 95 cm bed of Sephadex G-15 contained in a 100 x 2.5 cm Kontes chromatography column at a rate of 10 ml per hour. The inner surface of the column was coated with dichlorodimethylsilane to reduce interface effects and improve resolution. The top cap of the column was fitted with a stopcock so that air could be purged from the system prior to use. The bottom cap contained a stopcock so that the system could be closed off when it was not in use.

The column eluate then flowed through 0.05 mm polyethylene tubing to a 1 cm path-length flow cell mounted in the sample compartment of a Beckman DB spectrophotometer. The monitored effluent then passed through 0.05 mm id polyethylene tubing to a Technicon fraction collector, where it was drop-counted into 3 ml fractions.

The spectophotometer output was fed into a Sargent SRL recorder fitted with log conversion gears and a 1 inch per hour chart motor. The recorder was equipped with an event marker connected in series with the fraction collector

Figure 1. Diagram of automated gel filtration apparatus. The system utilized a Sephadex G-15 column (2.5 x 95 cm), a Milton Roy "Minipump" metering pump, a Technicon fraction collector, a Beckman DB spectrophotometer, and a Sargent SRL recorder fitted with an event marker.

AUTOMATED GEL FILTRATION APPARATUS

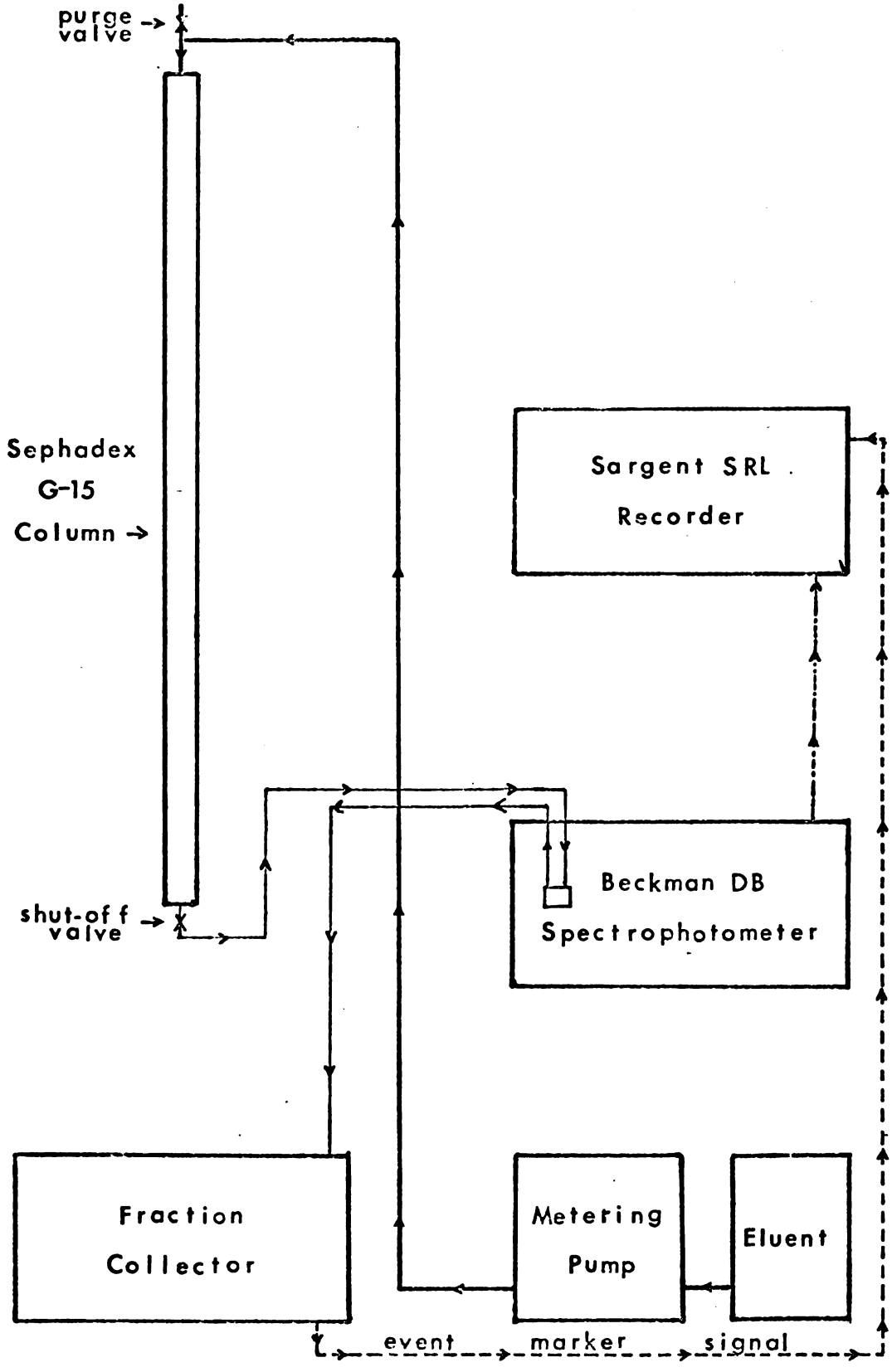


Figure 1

motor so that the absorbance peaks could be correlated with fraction numbers.

Since the M. lysodeikticus cell wall saccharides absorb strongly in the 220-240 mμ region, a plot of $A_{220-240}$ as a function of time as provided by the recorder indicated saccharide elution peaks without recourse to lengthy tube-by-tube analyses.

Preparation of Disaccharide, Tetrasaccharide, and "Fraction 1" Hexasaccharide-Glycopeptide Mixture

A 100 mg sample of M. lysodeikticus cell wall saccharide mixture (24 hr digest) was dissolved in 2 ml of redistilled water and placed on the Sephadex G-15 column of the automated gel filtration apparatus. The column was then eluted with boiled redistilled water at a rate of 10 ml/hr. Absorbance was measured at 230 mμ; the recorder trace showed six distinct peaks (Figure 2). Peaks 1, 2, and 3 when lyophilized yielded useful amounts of material (Table II); fractions 4, 5, and 6 yielded minute quantities of material.

Descending chromatography of the first three fractions was carried out on Whatman No. 1 paper for 50 hr in n-butanol/ acetic acid/ water (25/6/25 v/v, upper phase). N-acetyl glucosamine was used as a standard since the solvent was run off the end of the paper; spot concentrations were 100 μg. Chromatograms were visualized by the method of Sharon and Seifter;[68] chromatograms were dipped into a 0.5 M solution

Figure 2. Automated Gel Filtration of M. lysodeikticus Saccharides. A 100 mg load of 24-hour digested M. lysodeikticus saccharides was placed on the 2.5 x 95 cm Sephadex G-15 column of the automated gel filtration apparatus and eluted with boiled redistilled water at 10 ml/hr. Peaks 1, 2, and 3 were respectively hexasaccharide-glycopeptide mixture, tetrasaccharide and disaccharide. Peaks 4, 5, and 6 yielded amounts too small to be characterized.

AUTOMATED GEL FILTRATION OF M. lysodeikticus SACCHARIDES

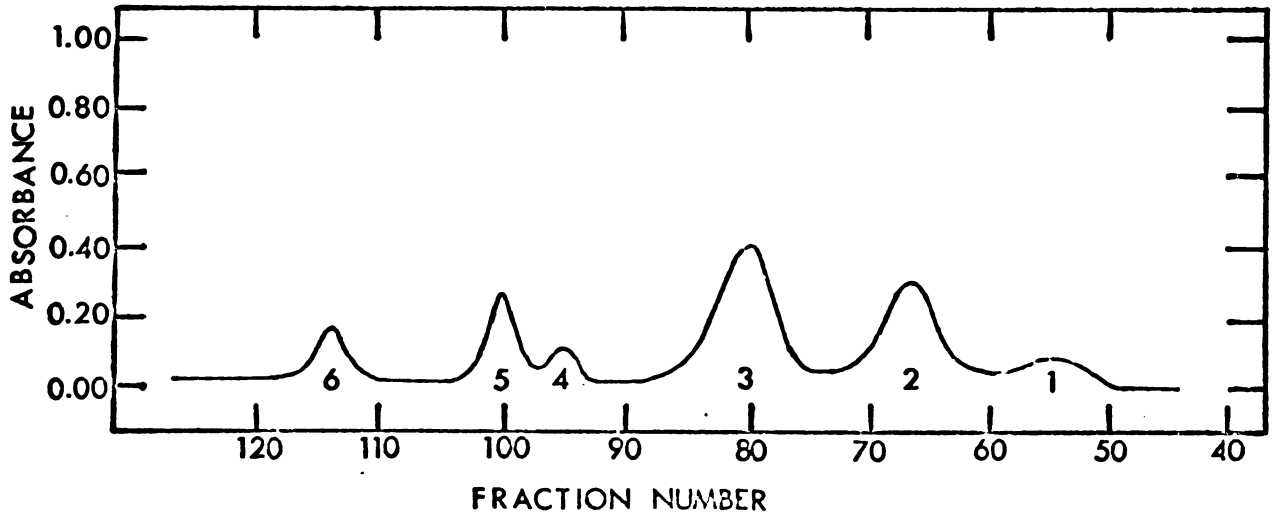


Figure 2

TABLE II

Sephadex G-15 Gel Filtration of M. lysodeikticus Cell Wall Saccharides

The column load was 100 mg of M. lysodeikticus cell wall saccharide mixture; fractions containing a peak were pooled and lyophilized. Peak volume refers to the volume required to elute a peak completely; fraction volume refers to the volume pooled and lyophilized to form a fraction.

Fraction	Peak Volume, ml	Fraction Volume, ml	Lyophilized Weight, mg
1	36	27	7.1
2	36	18	27.5
3	45	24	44.7

of sodium hydroxide in a mixture of ethanol and n-propanol (6/4, v/v) and then were placed in a 120° oven for 5 to 10 minutes. Chromatogram spots could then be detected by their yellow fluorescence under short-wavelength ultraviolet light.

Fraction 1 produced no well-defined spot; some material was retained at the origin, while the remainder formed an ill-defined streak.

Amino acid analysis of Fraction 1 (Table III) indicated that it was a mixture of hexasaccharide and glycopeptide of nearly equal molecular weight.

Fraction 2 produced a well-defined spot of R_g (mobility relative to n-acetyl glucosamine) = 0.47-0.54, which indicated that it was the tetrasaccharide: N-acetyl-β-D-glucosaminyl-(1→4)-N-acetyl-β-muramyl-(1→4)-N-acetyl-β-D-glucosaminyl-(1→4)-N-acetyl muramic acid[1] (muramic acid is the trivial name for 2-amino-3-O-(D-1-carboxyethyl)-2-deoxy-D-glucose). Elemental analysis of fraction 2 and determination of water of hydration by weight loss upon dehydration at 78.5° over phosphorus pentoxide in an evacuated (0.005 mm) Abderhalden drying pistol indicated that the lyophilized product approximated the composition of an octahydrate:

Weight loss: Calc. 12.88%, Found 13.0%.

Elemental analysis:

Calculated:	C, 40.79%	H, 7.03%	N, 5.01%
Found:	C, 40.97%	H, 6.86%	N, 4.91%

Amino acid analysis of fraction 2 hydrolysates (constant boiling HCl, 105°, 24 hr) yielded equimolar quantities of

TABLE III

Amino Acid Analysis of Fraction 1

Samples (3 mg) of Fraction 1 were hydrolyzed (constant boiling HCl, 105°, 24 hr) and subjected to amino acid analysis on the Spinco Automatic Amino Acid Analyzer. Muramic acid and glucosamine values were corrected for loss incurred during 24 hr hydrolysis at 105°. The presence of histidine is an indication of cell wall contamination by cellular components. All values are expressed in μmoles/mg fraction 1.

Expt.	Muramic Acid	Glucosamine	Lys	His	Glu	Gly	Ala
317	1.8	1.7	0.21	0.01	0.25	0.25	0.50
391	1.7	1.5	0.11	0.07	0.14	0.15	0.30
396	1.8	1.7	0.24	0.04	0.27	0.28	0.58

muramic acid and glucosamine (corrected for hydrolysis loss) and very minor quantities (less than 0.01 μmole) of lysine, histidine, glycine, alanine, and glutamic acid.

Digestion of fraction 2 by lysozyme (4 mg Fr. 1 and 0.1 mg Worthington lysozyme in 0.2 ml 0.05 M ammonium acetate solution held at 37° for 24 hr), followed by chromatography as previously described yielded a vivid spot, R_g = 1.0 and a very faint spot, R_g = 0.50, corresponding to the original tetrasaccharide.

Fraction 3, when chromatographed as previously described, produced a well-defined spot, R_g = 0.96-1.0, indicating that this fraction is the disaccharide N-acetyl-β-D-glucosaminyl-(1→4)-N-acetyl-muramic acid (R_g = 1.0). Amino acid analysis of fraction 3 hydrolysates (constant boiling HCl, 105°, 24 hr) yielded equimolar quantities of muramic acid and glucosamine and insignificant quantities of lysine, histidine, glycine, alanine, and glutamic acid (less than 0.01 μmole).

Chromatography of fractions 4, 5 and 6 gave no fluorescent spots; amino acid analysis of hydrolysates of these fractions (constant boiling HCl, 105°, 24 hr) indicated the presence of ammonia and minute quantities of glucosamine, muramic acid, lysine, glycine, alanine, and glutamic acid.

Preparation of M. lysodeikticus Cell Wall Hexasaccharide and Glycopeptide GP-1

Three 100 mg portions of the 12 hour lysozyme-digested

M. lysodeikticus cell wall saccharide preparation were successively fractioned by dissolving the portions in 2 ml redistilled water and placing the resulting solutions on the automated gel filtration apparatus. The column flow rate was 10 ml/hr, the absorbance was measured at 230 mμ, and 3 ml fractions were collected. Fractions 1, 2, and 3 corresponding to the first three peaks, were collected and lyophilized from each run. The lyophilized fraction 1 preparations were then pooled to yield 64 mg of tan powder.

This material was then fractionated by a modification of the glycopeptide separation method of Mirelman and Sharon.[69] The pooled fraction 1 material was dissolved in 2 ml redistilled water, and the pH of the solution adjusted to 2.3 with formic acid. The solution was then placed on a 40 x 1.5 cm column of BioRad 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 50 ml of 0.2 M pH 2.9 pyridine-formic acid buffer at a rate of 50 ml per hr; elution was continued with a 500 ml gradient from 0.2 M, pH 2.9 to 1.0 M pH 5.3 pyridine-formic acid buffer (250 ml of each buffer). The column eluate was collected in 5 ml fractions. Fractions were analyzed by evaporating selected tubes to dryness on the rotary evaporator. Redistilled water (3 ml) was added to each tube and the absorbance of the resulting solutions was measured at 240 mμ.

Two A_{240} peaks were visible; one at an elution volume of 35 ml (A-1), the other at an elution volume of 200 ml

(A-2). Fractions corresponding to these peaks were pooled and lyophilized to yield 25 and 31 mg respectively.

Samples (3 mg) of fractions A-1 and A-2 were subjected to hydrolysis (constant boiling HCl, 105°, 24 hr) and amino acid analysis; the results are summarized in Table IV. Fraction A-1, when chromatographed on Whatman No. 1 chromatography paper in 1-butanol/acetic acid/water (25/6/25, v/v, upper phase) for 48 hr and visualized as previously described, yielded a fluorescent spot, R_g = 0.21 (mobility relative to N-acetyl glucosamine). Fraction A-1 therefore appears to be the hexasaccharide, N-acetyl-β-D-glycosaminyl-(1→4)-N-acetyl-β-muramyl-(1→4)-N-acetyl-β-D-glucosaminyl-(1→4)-N-acetyl-β-muramyl-(1→4)-N-acetyl-β-D-glucosaminyl-(1→4)-N-acetyl muramic acid.

Fraction A-2, when chromatographed on Whatman No. 1 paper in 1-butanol/acetic acid/water (4/1/5, v/v, upper phase) with an alanine standard for 48 hr, formed a well-defined spot, R_{ala} = 0.10 (mobility relative to alanine).

The amino acid composition and chromatographic behavior of this glycopeptide (GP-1) indicated that it was identical with the glycopeptide GP-1 isolated by Mirelman and Sharon.[69]

Preparation of M. lysodeikticus Cell Wall Glycopeptide GP-2

Portions (200 mg) of the carboxymethylcellulose-treated lysozyme M. lysodeikticus cell wall digest were

dissolved in 2 ml portions of water and placed on the column of the automated gel filtration apparatus. The Sephadex column was eluted with boiled redistilled water at a rate of 12 ml per hr; the eluate was monitored at 240 mμ and collected in 5 ml fractions.

The A_{240} trace exhibited one large symetrical peak at an elution volume of 140 ml (gel-filtered fraction 1 consistantly elutes at this volume). Very minor disaccharide and tetrasaccharide peaks were observed in this instance. 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 of fraction 1 pooled from several gel filtration runs was dissolved in 5 ml of redistilled 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 <u>in vacuo</u> (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 one large symmetrical peak at 115 ml elution volume (Figure 3); no other well-defined, large peaks were found. The fractions containing this peak were pooled and lyophilized to yield 90 mg of amorphous white powder.

Hydrolysis (constant boiling HCl, 105°, 24 hr) of a sample (4 mg) of this glycopeptide material (GP-2) followed by amino acid analysis of the hydrolysate yielded results summarized in Table IV.

Chromatography of this glycopeptide fraction, utilizing alanine as a reference, on Whatman No. 1 paper in 1-butanol/acetic acid/water (4/1/5, v/v, upper phase) for 48 hr yielded a single spot of R_{ala} = 0.22. This chromatogram was developed by two successive methods; the first method utilized the chromogenic ninhydrin spray. The chromatogram was sprayed with the solution and then placed in a 110° oven for 15 minutes. A well-defined alanine spot and a very faint glycopeptide spot were noted. The same chromatogram was then dipped in 0.5 M sodium hydroxide solution (6:4 ethanol-propanol) and placed in a 120° oven for 15 minutes. A faintly fluorescent spot was noted under ultraviolet light; it was in the same position as the ninhydrin-glycopeptide spot.

The composition and chromatographic behavior indicate that GP-2 is identical to the glycopeptide GP-1a isolated by

Figure 3. Chromatography of M. lysodeikticus glycopeptide GP-2 on a 2.5 x 75 cm column of BioRad Aminex AG-50W-X2 resin. The column was eluted with 200 ml 0.2 M pH 2.9 pyridine-formate buffer and 1000 ml of a gradient from 0.2 M pH 2.9 to 1.0 M pH 5.3 pyridine-formate buffer. The column eluate was collected in 5 ml fractions and analyzed by evaporating selected tubes to dryness, adding 5 ml water, and reading the absorbance at 240 mμ.

CHROMATOGRAPHY OF M. lysodeikticus GLYCOPEPTIDE GP-2

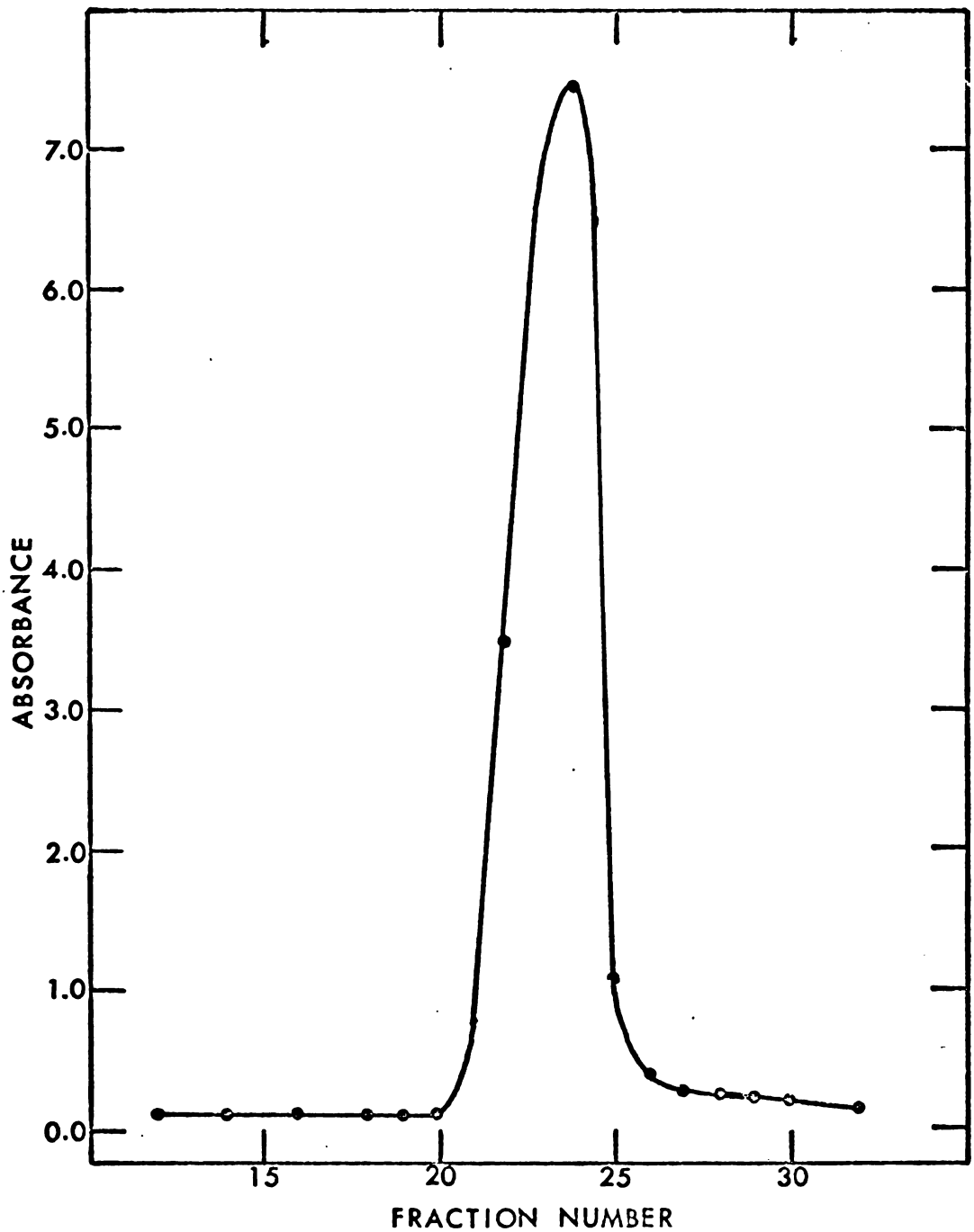


Figure 3

TABLE IV

Amino Acid Analysis of M. lysodeikticus Cell Wall Saccharide A-1 and Glycopeptides GP-1 and GP-2

All values are expressed in μmoles; muramic acid and glucosamine values are corrected for losses incurred during 24 hr hydrolysis. The analyses did not include basic amino acids, so lysine is absent.

Fraction	Muramic Acid	Glucosamine	Glutamic Acid	Glycine	Alanine
A-1	2.9	2.8	0.095	0.099	0.13
GP-1	1.5	1.6	1.5	1.5	3.1
GP-2	1.5	1.6	0.78	0.75	1.6

by Mirelman and Sharon.[69]

Purification of N-Acetyl Glucosamine Tetramer

A 100 mg sample of the tetramer of N-acetyl glucosamine, originally prepared by charcoal-celite column fractionation of a chitin hydrolysate (prepared by G. Stone), was dissolved in a 2 ml redistilled water and placed on the Sephadex G-15 column of the previously described automated gel filtration apparatus. The column was eluted with boiled redistilled water at a rate of 10 ml/hr; the column eluate was monitored at 230 mμ and collected in 3 ml fractions. The A_{230} trace displayed one large symetrical peak at an elution volume of 225 ml; the tetramer is therefore quite similar to M. lysodeikticus tetrasaccharide in that they display very similar elution characteristics on the Sephadex G-15 column. Two small trailing peaks were observed; they were attributed to lower molecular weight impurities. Fractions containing the major peak were pooled and lyophilized to produce 66 mg of N-acetyl glucosamine tetramer.

Purification of N-Acetyl Glucosamine Hexamer

A sample (100 mg) of N-acetyl glucosamine hexamer originally prepared by charcoal-celite column chromatography of a chitin hydrolysate (prepared by G. Stone) was dissolved in 2 ml redistilled water and placed on the Sephadex G-15 column of the automated gel filtration apparatus, and eluted

with boiled redistilled water at a rate of 10 ml/hr. The column eluate was monitored at 230 mμ and collected in 5 ml fractions. The A_{230} trace formed one major peak at 300 ml elution volume: The chromatographic behavior of this N-acetyl glucosamine polymer was therefore very similar to that displayed by Fraction 1, which contained M. lysodeikticus cell wall hexasaccharide. Two minor peaks indicating contamination of the original sample were also present. Fractions containing the major peak were pooled and lyophilized to yield 38 mg of purified hexamer.

Preparation of N-Ethyl Maleimide Inactivated Lysozyme

Worthington lysozyme (100 mg) and dithiothreitol (200 mg) were dissolved in 10 ml of 6 M guanidine-hydrochloride which was 0.06 M in pH 8.2 phosphate buffer. The pH of the resulting solution was adjusted back to 8.2 with 2 N sodium hydroxide solution. The solution was gassed with nitrogen to remove air; the container was then stoppered to exclude air and kept in the dark at room temperature for 6 hours. N-ethyl malemide (440 mg) was added; the pH of the resulting solution was quickly adjusted to pH 6.8 with 2 N hydrochloric acid. The reaction mixture was held at room temperature in the dark for 20 min, then placed in 16/32" sausage casing and dialyzed against two 2 liter volumes of distilled water. The resulting precipitate was washed twice with two 10 ml portions of redistilled water and lyophilized to yield 72 mg of amorphous white powder.

The activity of this N-ethyl maleimide modified lysozyme was checked by suspending 1 mg of the modified lysozyme in 3 ml M. lysodeikticus cell suspension (13 mg M. lysodeikticus cells suspended in a mixture of 90 ml 0.066 M pH 6.5 phosphate buffer and 10 ml 1.0 M sodium chloride solution). The turbidity of the suspension as measured by absorbance at 550 mμ (Beckman Model B spectrophotometer) did not change over a 2 hour period (A_{550} remained constant at 0.62); hence this modified lysozyme was completely inactive.

Preparation of Reduced, Carboxymethylated Lysozyme

Reduced, carboxymethylated lysozyme was prepared by a modification of the method described by Crestfieild, Stein and Moore.[70]

Lysozyme (Worthington, 50 mg) was dissolved in 12 ml of an 8 M urea solution prepared by mixing deionized urea (5.78 g), EDTA (24 mg), and pH 8.6 Tris buffer (5.3 g Tris and 9 ml 1.0 N HCl made up to 30 ml with redistilled water) with sufficient distilled water to make a final volume of 12 ml. After 20 min at room temperature, 200 mg of dithiothreitol was added; the resulting solution was kept in the dark at room temperature for 4 hr. A solution of 630 mg of iodoacetic acid in 3.4 ml 0.95 M sodium hydroxide solution was then added; after 20 minutes reaction in the dark at room temperature, the solution was placed in 16/32" sausage casing and dialyzed against 5 changes of redistilled water in the dark. The resulting white precipitate was washed

three times in 10 ml portions of redistilled water by suspension followed by centrifugation (clinical centrifuge) and then lyophilized to produce 35 mg of white, amorphous powder.

Amino acid analysis of a hydrolysate (5 mg, constant boiling HCl, 105°, 24 hr) of this modified lysozyme indicated that no cysteine or cystine was present.

Enzymatic activity of the reduced, carboxymethylated lysozyme preparation, as measured by turbidity (A_{550}) changes in a M. lysodeikticus cell suspension (1 mg of modified lysozyme added to 3 ml of a suspension of 15 mg cells in a mixture of 90 ml 0.06 M pH 6.5 phosphate buffer and 10 ml 1.0 M sodium chloride solution) was undetectable.

Detection of Muramic Acid and Glucosamine in Lysozyme-Substrate Reaction Mixtures

The hydrolysis mixtures of the substrates utilized in lysozyme-substrate reactions contain muramic acid and glucosamine; since these compounds yield discrete peaks on the amino acid analyzer, their presence in hydrolysates of lysozyme-substrate reaction mixtures 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 by hydrolyzing (2 ml constant boiling HCl, 105°, 24 hr) 1 μmole amounts of muramic acid and glucosamine and subjecting the resulting hydrolysate to amino acid analysis. The corrected amino acid analyzer

constants for muramic acid and glucosamine were respectively 9.43 and 16.1 area units/mg (normal amino acid constants lie in a range between 24 and 26 area units/mg).

Application of this correction factor allowed a quantitative determination of N-acetyl muramic acid and N-acetyl glucosamine as present in the lysozyme-substrate reaction mixture prior to hydrolysis. These values in turn allowed a determination of the amount of substrate present in the lysozyme-substrate reaction mixtures.

In each case, the amount of substrate present was expressed as the μmolar ratio of glucosamine to phenylalanine; since hen's egg white lysozyme contains 3 phenylalanine residues, a ratio of 0.33 (1 μmole of glucosamine/3 μmoles phenylalanine) indicated that one N-acetyl glucosamine residue per mole of lysozyme was present.

Non-Aqueous Lysozyme-Substrate Reactions: Dimethyl Sulfoxide Quenching

Worthington lysozyme (10 mg) and substrate (5 mg of *M. lysodeikticus* "Fraction 1" hexasaccharide-glycopeptide mixture or tetrasaccharide) were suspended in 1 ml anhydrous glycerol and stored at room temperature (23°) in a dessicator over phosphorus pentaoxide for a determined incubation time. The reaction mixture was then quenched with dimethyl sulfoxide (3 ml), held at room temperature for 2 hr, and dialyzed against 6 changes (50 ml) of 8 M urea solution (the

final 2 changes were 0.1 M, pH 8.2 in phosphate buffer), or against 6 changes of 6 M guanidine hydrochloride solution (in which case the dialysis bag liquid was made up to 0.1 M pH 8.2 phosphate by the addition of a solid buffer system).

Dithiothreitol (100-200 mg) was added to the pH 8.2 dialysis bag liquid, and the solution pH adjusted back to 8.2 by addition of 2 N sodium hydroxide solution. After 4 hr at room temperature, 200-600 mg of N-ethyl maleimide was added. This resolution was dialyzed (16/32" sausage casing) against 6 changes (50 ml) of 8 M urea or 6 M guanidine hydrochloride after 15 minutes reaction time. Dialysis was continued against 6 changes of redistilled water. The resulting precipitate was centrifuged and washed 10 times with 1 ml portions of redistilled water, lyophilized, hydrolyzed (2 ml constant boiling HCl, 105°, 24 hr) and subjected to amino acid analysis (Table XIX).

Blank runs consisted of a suspension of reduced, N-ethyl-maleimide-treated lysozyme or reduced, iodoacetate-treated lysozyme (10 mg) and substrate (5 mg) in 1 ml of anhydrous glycerol. This suspension was subjected to the dialysis-washing procedure described in the "live run" experiment, but a change of the dialysis sac was substituted for the inactivation step (Tables XX and XXI).

Non-Aqueous Lysozyme-Substrate Reactions Utilizing Urea Quenching

Lysozyme (Worthington, 10 mg) and *M. lysodeikticus*

cell wall Fraction 1 (mixture of hexasaccharide and glycopeptide, 5 mg) were suspended in 1 ml anhydrous glycerol and stored in a desicator over phosphorus pentoxide for the stated incubation time. The reaction mixtures were quenched by the addition of 100 mg of solid urea and 2 ml of 8 M urea solution. The resulting solutions were treated by one of two procedures: (a) dialysis (16/32" sausage casing) against pH 8.2 8 M urea solution (0.25 M in sodium bicarbonate) or (b) dialysis against 6 changes of unbuffered 8 M urea solution, in which case the dialysis sac liquid was lyophilized and dissolved in pH 8.2 8 M urea (0.17 M phosphate buffer) to bring it to pH 8.2.

Dithiothreitol (200 mg) was added to the pH 8.2 dialysis sac liquid in both cases; after 5 hours at room temperature, a solution of 630 mg iodoacetic acid in 3.4 ml 1 N sodium hydroxide solution was added. When 15 minutes had elapsed, the reaction mixture was successively dialyzed against 6 changes of 8 M urea and 3 changes of distilled water.

The precipitates were centrifuged and washed 10 times (clinical centrifuge), and then hydrolyzed (2 ml constant boiling HCl, 105°, 24 hr) and subjected to amino acid analysis (Table XXII).

Blank runs were prepared by two procedures: (a) Reduced, carboxymethylated lysozyme (10 mg) and Fraction 1 (5 mg) were suspended in 1 ml anhydrous glycerol. When the required reaction period had elapsed, 100 mg of solid urea

and 2 ml 8 M urea solution were added to the glycerol solution.

This solution was treated as detailed in the description of the "live" run experiments with the exception of the iodoacetate treatment; this was replaced by a dialysis sac change (Table XXIII); (b) reduced, carboxymethylated (RCM-) lysozyme or reduced, N-ethyl maleimide treated (RNEM-) lysozyme (10 mg) and M. lysodeikticus cell wall Fraction 1 were dissolved in 1 ml 6 M guanidine hydrochloride or 8 M urea solution. This solution was dialyzed (16/32 sausage casing) against 12 changes (50 ml) of 6 M guanidine hydrochloride or 8 M urea solution and 6 changes (50 ml) of redistilled water. The resulting precipitate was centrifuged (clinical centrifuge) and washed 10 times with 1 ml portions of redistilled water. The washed precipitate was lyophilized, hydrolyzed (2 ml constant boiling HCl, 105°, 24 hr), and analyzed for amino acid content (Table XXIV).

Aqueous Lysozyme-Substrate Reactions

Lysozyme (Worthington, 10 mg) and substrate (5 mg of M. lysodeikticus Fraction 1 hexasaccharide-glycopeptide mixture, hexasaccharide, glycopeptide GP-1, glycopeptide GP-2 or tetrasaccharide, or N-acetyl glucosamine hexamer or tetramer) were dissolved in 1 ml pH 8.2 0.1 M phosphate buffer. The resulting reaction mixture was incubated at room temperature (23°) for 15 minutes; then 1.5 g granular

guanidine hydrochloride 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 N sodium hydroxide solution. Dithiothreitol (100 mg) was added and the solution pH was again adjusted to 8.2. The reduction mixture was allowed to stand at room temperature in the dark for 5 hr. The pH was then adjusted to 6.8 with 2 N hydrochloric acid and 220 mg of N-ethyl maleimide was added. After 15 minutes reaction at room temperature in the dark, the solution was placed in a 16/32" sausage casing bag and was dialyzed against two 2-1 changes of distilled water.

The resulting precipitate was treated by one of two methods: (a) The precipitate was dissolved in 6 ml 6 M guanidine hydrochloride solution and dialyzed against two 2-1 changes of distilled water. This step was repeated three times. The precipitate was centrifuged and washed ten times with 2 ml portions of redistilled water, hydrolyzed (2 ml constant boiling HCl, 105°, 24 hr), and placed on the amino acid analyzer (Table XXV). (b) The precipitate was reprecipitated three times as in (a) and lyophilized. The lyophilized material was dissolved in 2 ml 5% formic acid.

The formic acid solution was placed on a 20 x 1 cm Biogel P-4 column and eluted with 5% formic acid at a rate of 25 ml/hr. The eluate was collected in 3 ml fractions and analyzed by measuring the A_{280} of each fraction. A single peak at 9 ml elution volume was observed. The peak fractions were lyophilized to yield 3 mg of white solid which was

hydrolyzed (2 ml constant boiling HCl, 105°, 24 hr), and placed on the amino acid analyzer (Table XXV).

Blanks were prepared by dissolving 10 mg of reduced, carboxymethylated lysozyme or reduced; N-ethyl maleimide treated lysozyme and 5 mg of substrate in 1 ml 6 M guanidine hydrochloride solution. This solution was dialyzed (16/32 sausage casing bag) against two 2-1 changes of distilled water. The resulting precipitate was then treated by either method (a) or (b) as previously described (Table XXVI).

Several glycopeptide GP-2 blank preparations utilized gel filtration exclusively. Glycopeptide GP-2 (5 mg) and 10 mg of either reduced, carboxymethylated lysozyme or reduced, N-ethyl maleimide treated lysozyme were dissolved in 1 ml 6 M guanidine hydrochloride and dialyzed (16/32 sausage casing bag) against two 2-1 changes of distilled water. The resulting precipitate was centrifuged, washed twice with 2 ml portions of redistilled water, and dissolved in 2 ml 5% formic acid. This solution was chromatographed on a 1 x 20 cm BioGel P-4 column with 5% formic acid at an elution rate of 25 ml/hr. The A_{280} peak from this column was lyophilized and rechromatographed on the same P-4 column with 5% formic acid. The A_{280} peak was lyophilized and hydrolyzed (2 ml constant boiling HCl, 105°, 24 hr), and then subjected to amino acid analysis (Table XXVI).

Effect of Hydroxylamine on the Lysozyme-Glycopeptide GP-2 Reaction

Lysozyme (Worthington, 20 mg) and glycopeptide GP-2 (10 mg) were dissolved in 2.0 ml pH 8.2 0.1 M phosphate buffer and allowed to stand at room temperature (23°) for 15 minutes. The reaction was quenched by adding 3.0 g of solid guanidine hydrochloride. The pH of this solution was adjusted to 8.3 with 2 N sodium hydroxide solution and 400 mg of dithiothreitol was added. After adjusting to pH 8.3, the reaction mixture was kept under nitrogen atmosphere in the dark for 5 hr. N-ethyl maleimide (840 mg) was added and the solution pH adjusted to 6.8 with 2 N hydrochloric acid. When 15 minutes had elapsed, the reaction mixture was dialyzed (16/32" sausage casing bag) against two 2-1 changes of distilled water.

The resulting precipitate was centrifuged, washed twice, and then lyophilized. The lyophilized white solid was dissolved in 3 ml 5% formic acid, placed on a 20 x 1 cm BioGel P-4 column, and eluted with 5% formic acid at a rate of 25 ml/hr. The eluate A_{280} peak fractions were lyophilized and rechromatographed on the same P-4 column. The eluate A_{280} peak was lyophilized and divided into two portions.

The first portion was hydrolyzed (constant boiling HCl, 105°, 24 hr) and analyzed for amino acid content (Table XXVII).

The remaining portion of lyophilized material was dissolved in 2 ml 6 M guanidine hydrochloride solution 1.0 M in

hydroxylamine and 0.06 M in phosphate buffer. This solution was kept at room temperature for 12 hr and then dialyzed (16/32 sausage casing bag) against two 2-1 changes of distilled water.

The precipitate was centrifuged and washed twice with 2 ml portions of distilled water. The washed precipitate was dissolved in 2 ml 5% formic acid and chromatographed on a 20 x 1 cm BioGel P-4 column with 5% formic acid at an elution rate of 25 ml/hr. The eluate A_{280} peak was lyophilized, hydrolyzed (constant boiling HCl, 105°, 24 hr) and subjected to amino acid analysis (Table XXVII).

RESULTS

TABLE VI

Hydrolysis Rate of 2-Methylthioacetaldehyde Diethyl Acetal in Dioxane-Water

The reaction mixture consisted of 10.8 to 14.4 mg 2-methylthioacetaldehyde diethyl acetal, 5 ml of dioxane, and 5 ml of perchloric acid. Absorbance was measured at 275 mμ in a 1-cm cell. Absorption increment time intervals ranged from 150-300 seconds depending on the hydrolysis rate.

Perchloric Acid, M	Γ	$10^4 k_1$	$10^2 k_2$
0.005	0.010	1.22	2.44
0.010	0.020	2.19	2.19
0.020	0.040	4.93	2.46
0.020	0.040	4.93	2.46
0.025	0.050	5.21	2.10
0.025	0.050	5.10	2.05
		Average =	2.28

TABLE VII

Hydrolysis Rate of 2-Methoxyacetaldehyde Diethyl Acetal in Dioxane-Water

The reaction mixture contained 45 to 99 mg 2-methoxyacetaldehyde diethyl acetal, 10 ml of dioxane and 10 ml of perchloric acid. Absorbance was measured at 270 mμ in a 10-cm cell. Absorption increment time intervals ranges from 100-300 seconds, depending on hydrolysis rate. Hammet acidity values (H_o) were obtained from data cited by Kreevoy and Taft.[72]

Perchloric Acid, M	H_o	$10^4 k_1$	$10^4 k_2$	Log k_1 + H_o	Log k_2
0.543	0.10	4.90	9.04	-3.21	-3.04
0.543	0.10	4.80	8.85	-3.22	-3.05
0.990	-0.13	21.2	21.6	-2.80	-2.61

The value of k_2 obtained by extrapolation of Log k_2 ($k_1/HClO_4$ conc) to zero ional strength was 2.63×10^{-4} sec^{-1} $mole^{-1}$ (Figure 4).

TABLE VIII

Hydrolysis Rate of 2-Ethoxyacetaldehyde Diethyl Acetal in Dioxane-Water

The reaction mixture contained 27 to 97 mg 2-ethoxyacetaldehyde diethyl acetal, 10 ml of dioxane, and 10 ml of perchloric acid. Absorbance was measured at 270 mμ in a 10-cm cell. Absorption increment time intervals ranged from 100-300 seconds.

Perchloric Acid, M	H_o	$10^4 k_1$	$10^4 k_2$	Log k_1 + H_o	Log k_2
0.290	0.22	1.41	4.90	-3.63	-3.31
0.290	0.22	1.91	6.70	-3.50	-3.17
0.543	0.10	6.92	12.8	-3.10	-2.81
0.543	0.10	7.05	13.0	-3.10	-2.81
0.990	-0.13	62.0	62.7	-3.34	-2.20
0.990	-0.13	35.1	35.5	-3.59	-3.45

The value of k_2 obtained by extrapolation of Log k_2 to zero ional strength was 1.70×10^{-4} sec^{-1} $mole^{-1}$ (Figure 4).

Figure 4. Extrapolation of Hydrolysis Rates of 2-Methoxy- and 2-Ethoxy- Diethyl Acetal. The hydrolysis rates of 2-methoxy- (x----x) and 2-ethoxy- (•——•) acetaldehyde diethyl acetal were extrapolated to zero perchloric acid concentration. The vertical lines indicate the experimental error for each set of determinations.

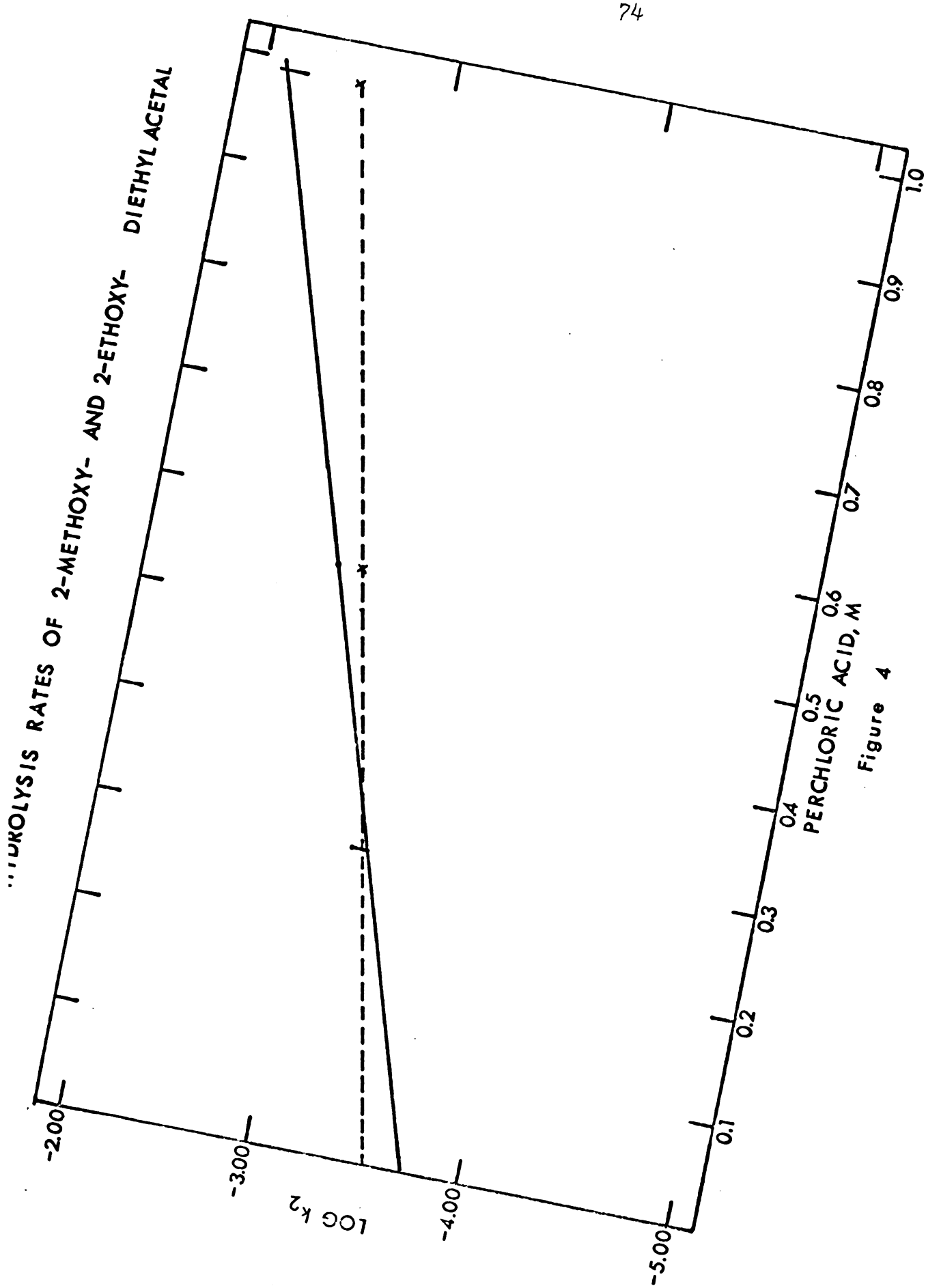

...UROLYSIS RATES OF 2-METHOXY- AND 2-ETHOXY- DIETHYL ACETAL
log k_2
-2.00
-3.00
-4.00
-5.00
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
PERCHLORIC ACID, M

Figure 4

TABLE IX

Hydrolysis Rate of 4-Methylthiobutraldehyde Diethyl Acetal in Dioxane-Water

A stock solution of 39.2 mg 4-methylthiobutraldehyde diethyl acetal in 15 ml of dioxane was prepared; 5 ml portions of this solution were mixed with 5 ml of perchloric acid to form the reaction mixture. Absorbance was measured at 270 mμ in a 1-cm cell. The absorption increment time interval was 300 seconds.

Perchloric Acid, M	Γ	$10^3 k_1$	$10^1 k_2$
0.010	0.020	1.30	1.30
0.010	0.020	1.30	1.30
		Average =	1.30

TABLE X

Hydrolysis Rate of Valeraldehyde Diethyl Acetal in Dioxane-Water

Two stock solutions were prepared containing 36 and 83 mg valeraldehyde diethyl acetal per 15 ml of dioxane; 5 ml portions of these solutions were mixed with 5 ml of perchloric acid to form the reaction mixture. Absorbance was measured at 270 mμ in a 1-cm cell. The absorbance increment time interval was 300 seconds.

Perchloric Acid, M	Γ	$10^3 k_1$	$10^1 k_2$
0.010	0.020	1.85	1.85
0.010	0.020	1.87	1.87
0.010	0.020	1.81	1.81
		Average =	1.84

TABLE XI

Hydrolysis Rate of 5-Methoxy-2-Pentanone Diethyl Ketal in Dioxane-Water

Stock solutions containing 174 to 177 mg of 5-methoxy-2-pentanone diethyl ketal per 20 ml of dioxane were prepared; 5 ml portions of these solutions were mixed with 5 ml of acetate buffer to form the reaction mixture. Absorbance was measured at 270 mμ in a 1-cm cell. The absorption increment time interval ranged from 300-600 seconds depending on the rate of hydrolysis. The notation (corr.) indicates that the hydrolysis rate was corrected for secondary salt effects.

Acetic Acid, M	Acetate, M	Γ	$10^4 k_1$	$10^{-2} k_2$ (corr.)	$10^{-2} k_2$
0.00375	0.00125	0.0025	4.34	3.65	4.77
0.00375	0.00125	0.0025	4.26	3.57	4.66
0.0250	0.0125	0.025	3.69	3.58	7.02
0.0250	0.0125	0.025	3.68	3.57	7.00
0.0375	0.0125	0.050	6.58	3.41	8.34
0.0375	0.0125	0.050	6.83	3.54	8.77
0.0375	0.0125	0.100	9.66	3.97	12.25
0.0375	0.0125	0.100	9.48	3.91	12.05

Value of $10^{-2} k_2$ extrapolated to zero ional strength = 4.20 (Figure 5).

Value of $10^{-2} k_2$ (corr.) extrapolated to zero ional strength = 3.55 (Figure 5).

Figure 5. Extrapolation of 5-Methoxy-2-Pentanone Diethyl Ketal Hydrolysis Rate. Both uncorrected (----) and secondary-salt effect-corrected (•——•) 5-methoxy-2-pentanone diethyl ketal hydrolysis rates were extrapolated to zero ional strength. The vertical lines indicate the experimental error for each set of determinations.

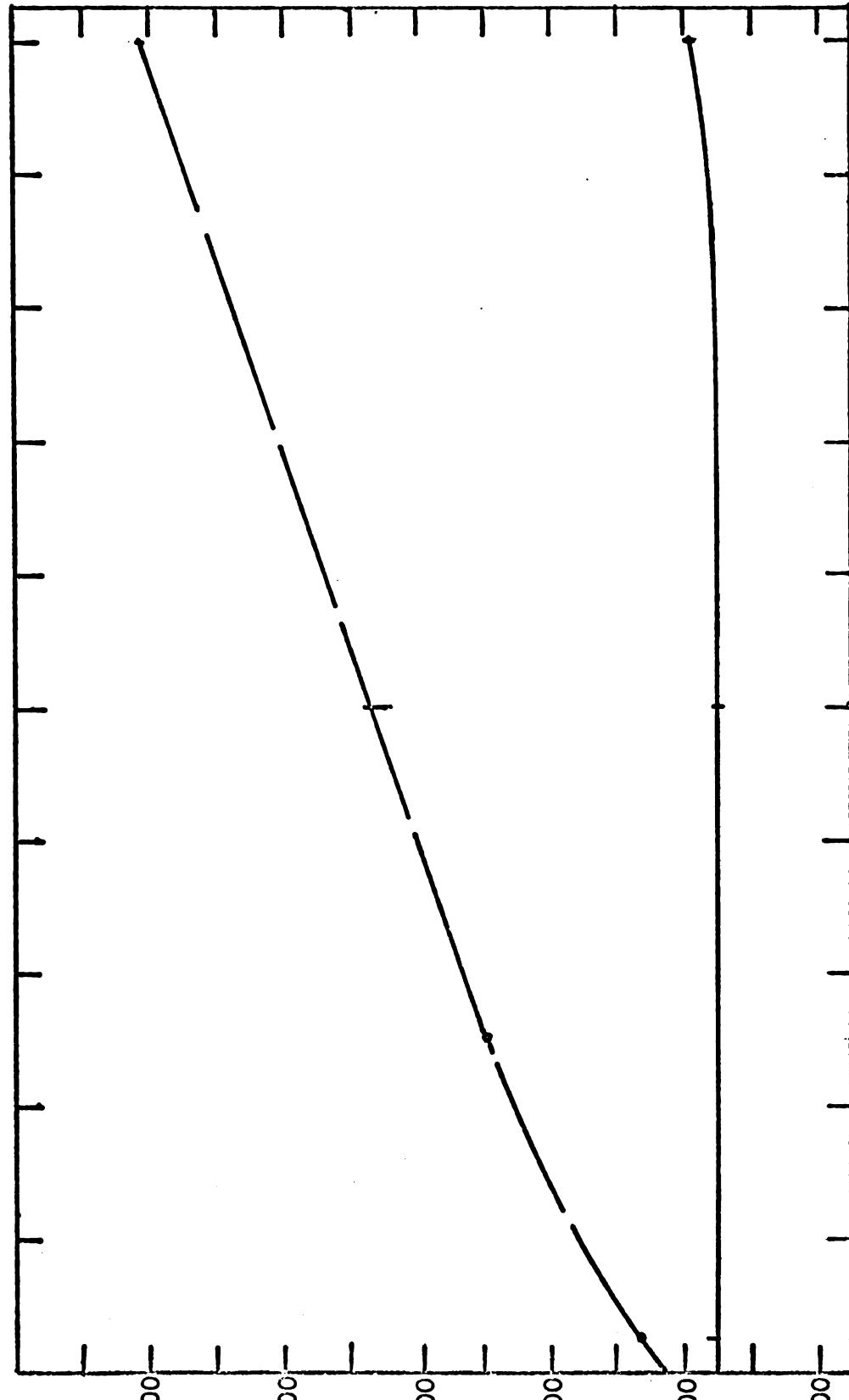

EXTRAPOLATION OF 5-METHOXY-2-PENTANONE DIETHYL KETAL HYDROLYSIS RATE
$10^2\ k_2$
12.00
10.00
8.00
6.00
4.00
2.00
0.01
0.02
0.03
0.04
0.05
0.06
0.07
0.08
0.09
0.10
IONAL STRENGTH

Figure 5

TABLE XII

Hydrolysis Rate of 5-Methylthio-2-Pentanone Diethyl Ketal in Dioxane-Water

Stock solutions containing 180 to 188 mg of 5-methylthio-2-pentanone diethyl ketal in 20 ml of dioxane were prepared; the reaction mixture contained 5 ml of this stock solution and 5 ml of acetate buffer. Absorbance was measured at 275 mμ in a 1-cm cell. The absorbance increment time interval was 600 seconds. The notation (corr.) denotes hydrolysis values which have been corrected for primary salt effects.

Acetic Acid, M	Acetate, M	Γ	10^4 k_1	10^{-2} k_2 (corr.)	10^{-2} k_2
0.00435	0.00125	0.0025	3.06	2.57	3.36
0.00435	0.00125	0.0025	3.10	2.60	3.40
0.025	0.0125	0.025	2.77	2.67	5.26
0.025	0.0125	0.025	2.77	2.67	5.26
0.025	0.0125	0.025	2.67	2.59	5.08
0.0025	0.0025	0.050	1.71	2.67	6.75
0.0025	0.0025	0.050	1.95	3.03	7.42
0.00375	0.00125	0.050	5.74	3.00	7.29
0.0125	0.0125	0.050	1.91	2.96	7.28
0.0125	0.0125	0.050	1.92	2.98	7.32
0.0188	0.0065	0.050	5.72	2.99	7.26
0.025	0.025	0.050	1.89	2.94	7.19

TABLE XII (Continued)

Acetic Acid, M	Acetate, M	Γ	$10^4 k_1$	$10^{-2} k_2$ (corr.)	$10^{-2} k_2$
0.025	0.025	0.050	1.94	3.01	7.38
0.0375	0.0125	0.050	5.77	3.01	7.32
0.0758	0.0372	0.0744	3.81	3.18	7.25
0.0758	0.0372	0.0744	3.79	3.16	7.21
0.0345	0.0125	0.100	7.62	3.14	9.67
0.0345	0.0125	0.100	7.67	3.16	9.72

Value of $10^{-2} k_2$ extrapolated to zero ional strength = 3.16 (Figure 6).

Value of $10^{-2} k_2$ (corr.) extrapolated to zero ional strength = 2.60 (Figure 6).

Figure 6. Extrapolation of 5-Methylthio-2-Pentanone Diethyl Ketal Hydrolysis Rate. Both uncorrected (•———•) and secondary-salt effect-corrected (----) 5-methylthio-2-pentanone diethyl ketal hydrolysis rates were extrapolated to zero ional strength. The vertical lines indicate the experimental error for each set of determinations.

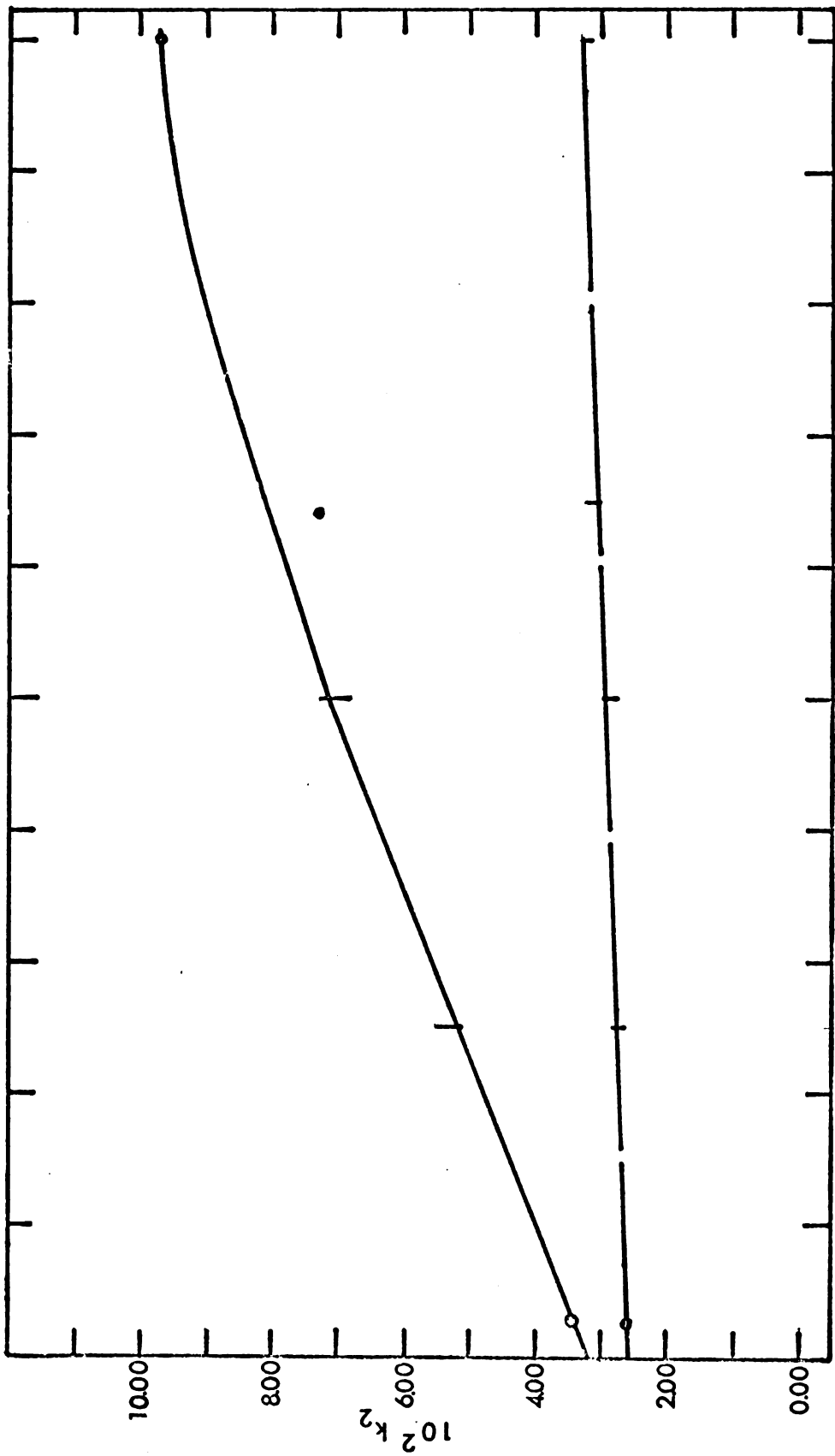

EXTRAPOLATION OF 5-METHYLTHIO-2-PENTANONE DIETHYL KETAL HYDROLYSIS RATE
$10^2\ k_2$
10.00
8.00
6.00
4.00
2.00
0.00
0.01
0.02
0.03
0.04
0.05
0.06
0.07
0.08
0.09
0.10
IONAL STRENGTH

Figure 6

TABLE XIII

Hydrolysis Rate of 2-Pentanone Diethyl Ketal in Dioxane-Water

The reaction mixture contained 41 mg of 2-pentanone diethyl ketal, 5 ml dioxane and 5 ml of acetate buffer. Absorbance was measured at 270 mμ in a 1-cm cell. The absorbance increment time interval was 300 seconds.

Acetic Acid, M	Acetate, M	Γ	$10^4 k_1$	$10^{-2} k_2$ (corr.)	$10^{-2} k_2$
0.025	0.0125	0.025	9.07	8.82	17.2
0.025	0.0125	0.025	8.75	8.50	16.7
0.025	0.0125	0.025	8.70	8.46	16.5
			Average =	8.59	

TABLE XIV

Hydrolysis Rate of 2-Ethoxytetrahydropyran in Dioxane-Water

The reaction mixture consisted of 55 to 58 mg of 2-ethoxytetrahydropyran, 10 ml of dioxane, and 10 ml of perchloric acid. Absorption was measured at 284 mμ in a 10-cm cell. The time increment was 120 seconds.

Perchloric Acid, M	Γ	$10^3 k_1$	$10^2 k_2$
0.077	0.154	1.07	1.39
0.077	0.154	1.22	1.59
0.077	0.154	1.22	1.59
0.077	0.154	1.21	1.58
	Average =	1.18	1.53

TABLE XV

Hydrolysis Rate of 2-(2-Methylthioethoxy)-Tetrahydropyran in Dioxane-Water

The reaction mixture consisted of 75 to 77 mg of 2-(2-methylthioethyoxy)-tetrahydropyran, 10 ml of dioxane, and 10 ml of perchloric acid. The absorption was measured at 284 mμ in a 10-cm cell. The time increment was 120 seconds.

Perchloric Acid, M	Γ	$10^3 k_1$	$10^2 k_2$
0.077	0.154	1.21	1.58
0.077	0.154	1.27	1.65
	Average =	1.24	1.61

TABLE XVI

Hydrolysis Rates of 2-(2-Methoxyethoxy)-Tetrahydropyran in Dioxane-Water

The reaction mixture consisted of 75 to 76 mg of 2-(2-methoxyethoxy)-tetrahydropyran, 10 ml of dioxane, and 10 ml of perchloric acid. The absorbance was measured at 284 mμ in a 10-cm cell. The time increment was 120 seconds.

Perchloric Acid, M	Γ	$10^3 k_1$	$10^2 k_2$
0.060	0.120	1.23	2.05
0.060	0.120	1.23	2.05
0.060	0.120	1.23	2.05
	Average =	1.23	2.05

TABLE XVII

Hydrolysis Rate of 2-(2-Pyridinylethoxy)-Tetrahydropyran in Water

Reaction conditions are given in the Experimental Section.

Perchloric Acid, M	Γ	$10^3 k_1$	$10^2 k_2$
0.109	0.218	2.86	2.63
0.109	0.218	1.30	1.20
0.119	0.238	2.00	1.68
0.119	0.238	2.52	2.12
		Average =	1.91
0.1 M pH 4.64 acetate buffer		0.00	

TABLE XVIII

Hydrolysis Rate of 2-(2-Methoxyethoxy)-Tetrahydropyran in Water

Reaction conditions are given in the Experimental Section. Most of the rates were determined by plotting log C_t/C_o vs. time.

Perchloric Acid, M		$10^3 k_1$	$10^2 k_2$
0.040	0.080	*9.23	2.31
0.109	0.218	7.72	0.82
0.021	0.042	*5.54 3.06	2.66 1.57
0.021	0.042	*5.54 3.26	2.66 1.57
0.021	0.042	**3.94	1.90
0.021	0.042	2.52	1.21
0.021	0.042	1.86	0.90

* Denotes rates determined by $t_{\frac{1}{2}}$.

**Denotes rates determined by absorption increments at 270 mμ.

Figure 7. Ethanol Release by Hydrolyzing 2-Methylthioacetaldehyde Diethyl Acetal. Ethanol release by hydrolyzing 2-methylthioacetaldehyde diethyl acetal was determined by assay of quenched samples of the hydrolysis mixture. The symbols (x, o, •) represent the three runs made to determine the kinetics of ethanol release.

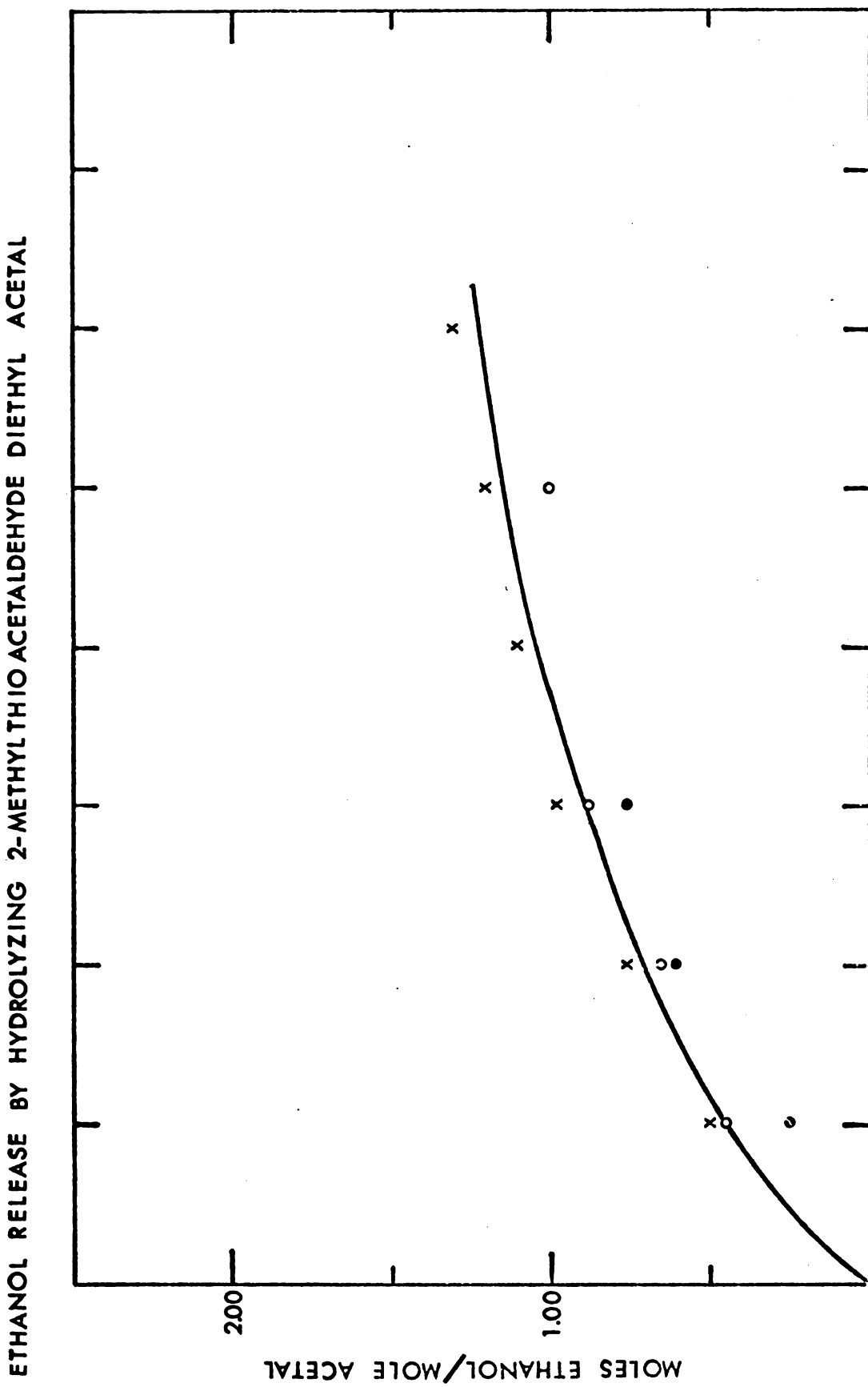

ETHANOL RELEASE BY HYDROLYZING 2-METHYLTHIOACETALDEHYDE DIETHYL ACETAL
MOLES ETHANOL/MOLE ACETAL
2.00
1.00
100
200
300
400
500
600
700
SECONDS

Figure 7

Figure 8. Ethanol Release Kinetics in the Hydrolysis of 2-Methylthioacetaldehyde Diethyl Acetal. Increments in ethanol concentration were obtained from the data used to plot Figure 7. The natural logarithms of these increment values, when plotted against time, yielded a straight line. This indicates that ethanol release follows first-order kinetics.

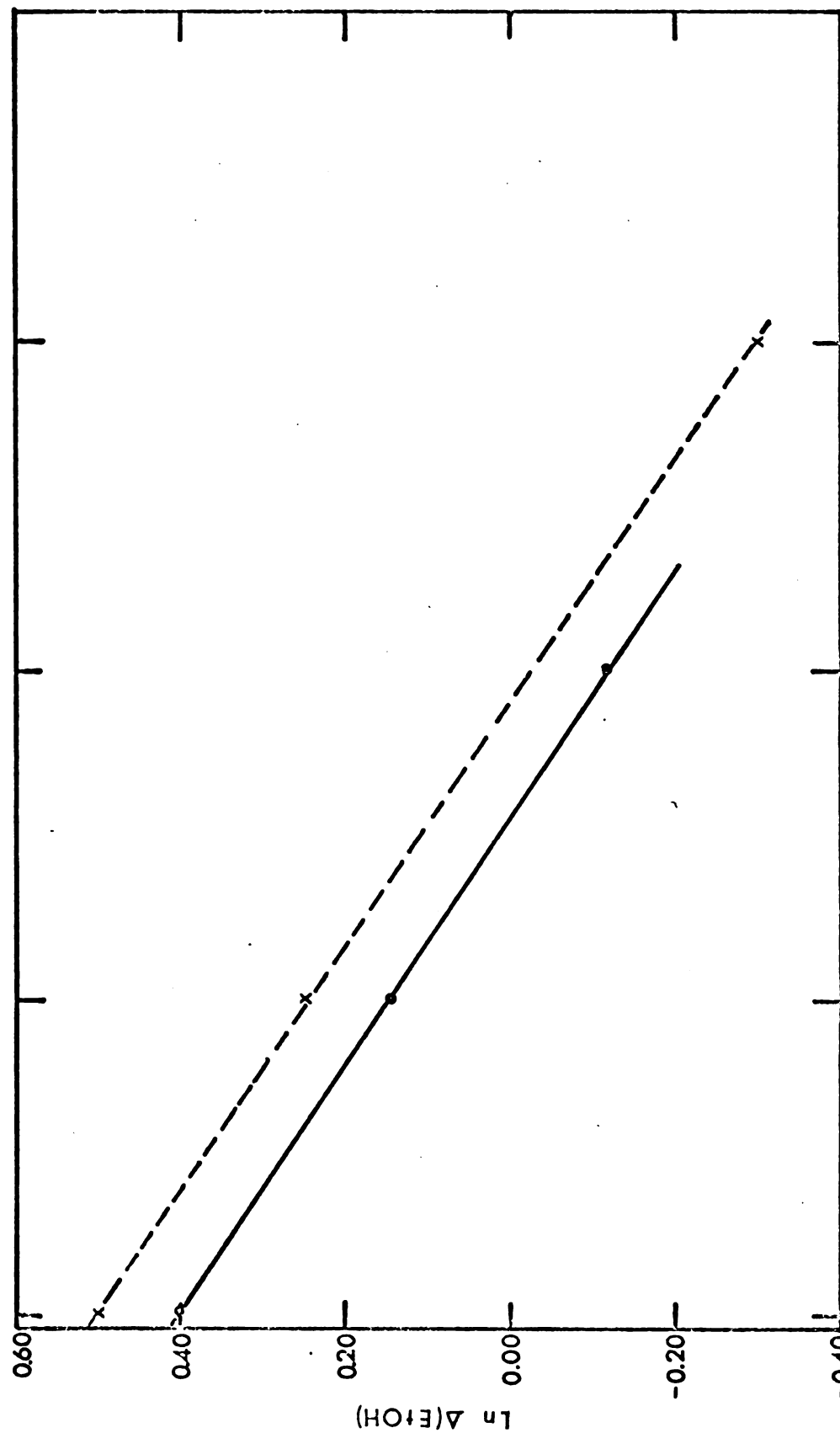

ETHANOL RELEASE KINETICS IN THE HYDROLYSIS OF 2-METHYLTHIO ACETALDEHYDE DIETHYL ACETAL
ln Δ(EtOH)
0.60
0.40
0.20
0.00
-0.20
-0.40
0
100
200
300
SECONDS

Figure 8

Figure 9. Ethanol Release by Hydrolyzing 5-Methylthio-2-Pentanone Diethyl Ketal. Ethanol release by hydrolyzing 5-methylthio-2-pentanone was determined by assay of quenched samples of the hydrolysis mixture.

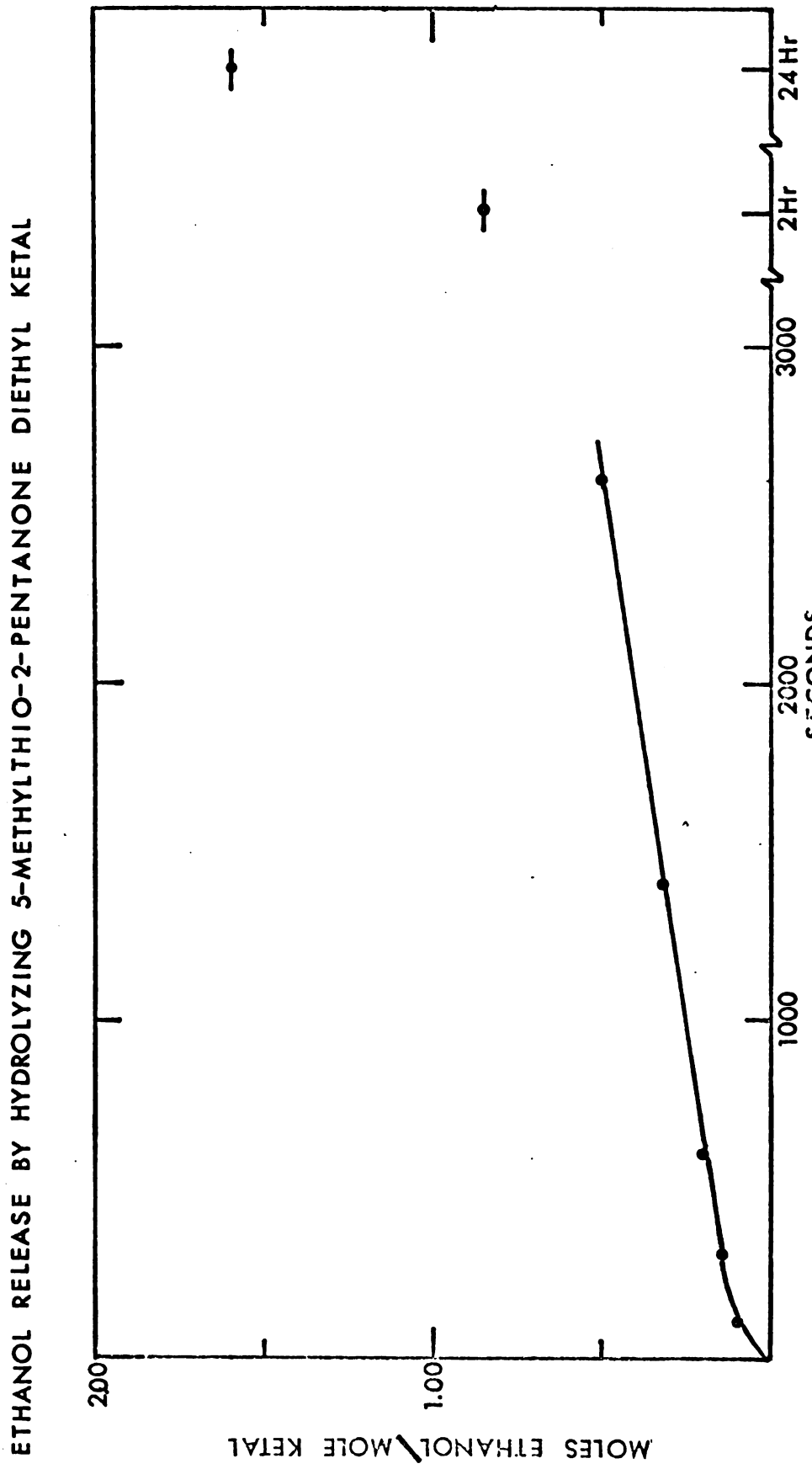

ETHANOL RELEASE BY HYDROLYZING 5-METHYLTHIO-2-PENTANONE DIETHYL KETAL
MOLES ETHANOL \ MOLE KETAL
2.00
1.00
1000
2000
SECONDS
3000
2Hr
24Hr

Figure 9

TABLE XIX

Substrate Concentrations in Non-Aqueous Lysozyme-Substrate Reaction Mixtures which were Dimethyl Sulfoxide Quenched

Substrate	Incubation Period	Dialysis Medium	Glucosamine/ Phenylalanine ratio
M. l. Tetra-saccharide	12 hr.	urea	0.000
M. l. Tetra-saccharide	3 days	urea	0.000
M. l. Fr. 1	2 days	urea	0.07
M. l. Fr. 1	3 days	urea	0.11
M. l. Fr. 1	4 days	urea	0.13
M. l. Fr. 1	12 days	GuCl	0.29
M. l. Fr. 1	21 days	GuCl	0.30

M. l. is an abbreviation for M. lysodeikticus.

TABLE XX

Substrate Concentrations in Non-Aqueous Lysozyme-Substrate Reaction Mixtures

Blank runs using reduced, N-ethyl maleimide treated lysozyme.

Substrate	Incubation Period	Dialysis Medium	Glucosamine/ Phenylalanine ratio
M. l. Tetra-saccharide	5 hr	GuCl	0.00
M. l. Tetra-saccharide	18 days	GuCl	0.00
M. l. Fr. 1	5 hr	GuCl	0.01
M. l. Fr. 1	1 day	urea	0.00
M. l. Fr. 1	7 days	urea	0.00
M. l. Fr. 1	5 hr	urea	0.02*
M. l. Fr. 1	1 day	urea	0.06*
M. l. Fr. 1	3 days	urea	0.02*

*Runs made with incompletely inactivated lysozyme preparations (0.1% of original activity by M. lysodeikticus cell wall turbidity measurements).

TABLE XXI

Substrate Concentrations in Non-Aqueous Lysozyme-Substrate Reaction Mixtures

Blank runs using reduced, carboxymethylated lysozyme suspended in glycerol.

Substrate	Incubation Period	Dialysis Medium	Glucosamine/ Phenylalanine ratio
M. l. Fr. 1	2 days	urea	0.00
M. l. Fr. 1	7 days	urea	0.00

M. l. is an abbreviation for M. lysodeikticus cell wall.

TABLE XXII

Substrate Concentrations in Non-Aqueous

Lysozyme-Substrate Reactions Utilizing Urea Quenching

Substrate	Incubation Time	Buffering Medium	Glucosamine/ Phenylalanine Ratio
M. l. Fr. 1	2 days	Bicarbonate	0.09
M. l. Fr. 1	2 days	Phosphate	0.09

M. l. is an abbreviation for M. lysodeikticus cell wall.

TABLE XXIII

Substrate Concentrations in Non-Aqueous Lysozyme-Substrate Reactions

Blank runs utilizing glycerol.

Substrate	Lysozyme Preparation	Dialysis Medium	Glucosamine/ Phenylalanine Ratio
M. l. Fr. 1	RCM	urea	0.15
M. l. Fr. 1	RNEM	urea	0.16

M. l. is an abbreviation for M. lysodeikticus cell wall.

TABLE XXIV

Substrate Concentrations in Non-Aqueous Lysozyme-Substrate Reactions

Blank runs without glycerol.

Substrate	Lysozyme Preparation	Dialysis Medium	Glucosamine/ Phenylalanine Ratio
M. l. Fr. 1	RCM	GuCl	0.13
M. l. Fr. 1	RNEM	GuCl	0.06
M. l. Fr. 1	RNEM	GuCl	0.06
M. l. Fr. 1	RNEM	urea	0.13

M. l. is an abbreviation for M. lysodeikticus cell wall, RCM refers to reduced carboxymethylated lysozyme, and RNEM refers to reduced N-ethyl maleimide treated lysozyme.

TABLE XXV

Substrate Concentrations in

Aqueous Lysozyme-Substrate Reactions

Substrate	Treatment	Glucosamine/ Phenylalanine Ratio
NAG Tetramer	Reprecipitation	0.02
NAG Hexamer	Reprecipitation	0.02
M. l. Fr. 1	Reprecipitation	0.30
M. l. Hexa-saccharide	Reprecipitation	0.00
M. l. Hexa-saccharide	Reprecipitation	0.00
M. l. GP-1	Reprecipitation	0.15
M. l. GP-1	Reprecipitation	0.29
M. l. GP-2	Reprecipitation	0.29
M. l. GP-2	Reprecipitation-P-4 Gel Filtration	0.38

M. l. is an abbreviation for M. lysodeikticus cell wall, NAG is an abbreviation for N-acetyl glucosamine.

TABLE XXVI

Substrate Concentrations in

Aqueous Lysozyme-Substrate Reactions

Blank runs.

Substrate	Treatment	Glucosamine/ Pehnylalanine Ratio
M. l. GP-1	Reprecipitation	0.03
M. l. GP-2	Reprecipitation	0.14
M. l. GP-2	Reprecipitation-P-4 Gel Filtration	0.05
M. l. GP-2	P-4 Gel Filtration RNEM Lysozyme	0.14
M. l. SP-2	P-4 Gel Filtration RCM Lysozyme	0.00

M. l. is an abbreviation for M. lysodeiktious cell wall.

TABLE XXVII

Effect of Hydroxylamine

on the Lysozyme-Glycopeptide GP-2 Reaction

Substrate	Treatment	Glucosamine/ Phenylalanine Ratio
M. l. GP-2	P-4 Gel Filtration	0.26
M. l. GP-2	P-4 Gel Filtration NH_2OH P-4 Gel Filtration	0.18

M. l. is an abbreviation for M. lysodeikticus cell wall.

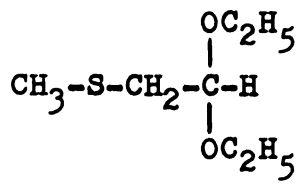
DISCUSSION

Acetal, Ketal and Tetrahydropyran Ether Hydrolysis Rates

Acetal hydrolysis rates

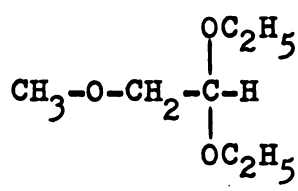
The second-order hydrolysis rates of acetals in concentrated perchloric acid solutions (greater than 0.1 M) were corrected according to the method of Kreevoy.[73]

Comparison of the corrected second-order hydrolysis rates of the following substituted acetals with that of the parent compound, diethyl acetal, demonstrates that all three substituted acetals hydrolyze more slowly than the parent acetal.

VI 

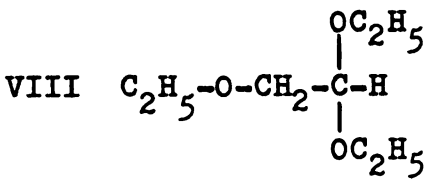
2-Methylthioacetaldehyde Diethyl Acetal

$k_2 = 228 \times 10^{-2}$ sec^{-1} mole^{-1}

VII 

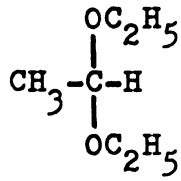
2-Methoxyacetaldehyde Diethyl Acetal

$k_2 = 2.63 \times 10^{-4}$ sec^{-1} mole^{-1}

VIII 

2-Ethoxyacetaldehyde Diethyl Acetal

$k_2 = 1.70 \times 10^{-4}$ sec^{-1} mole^{-1}



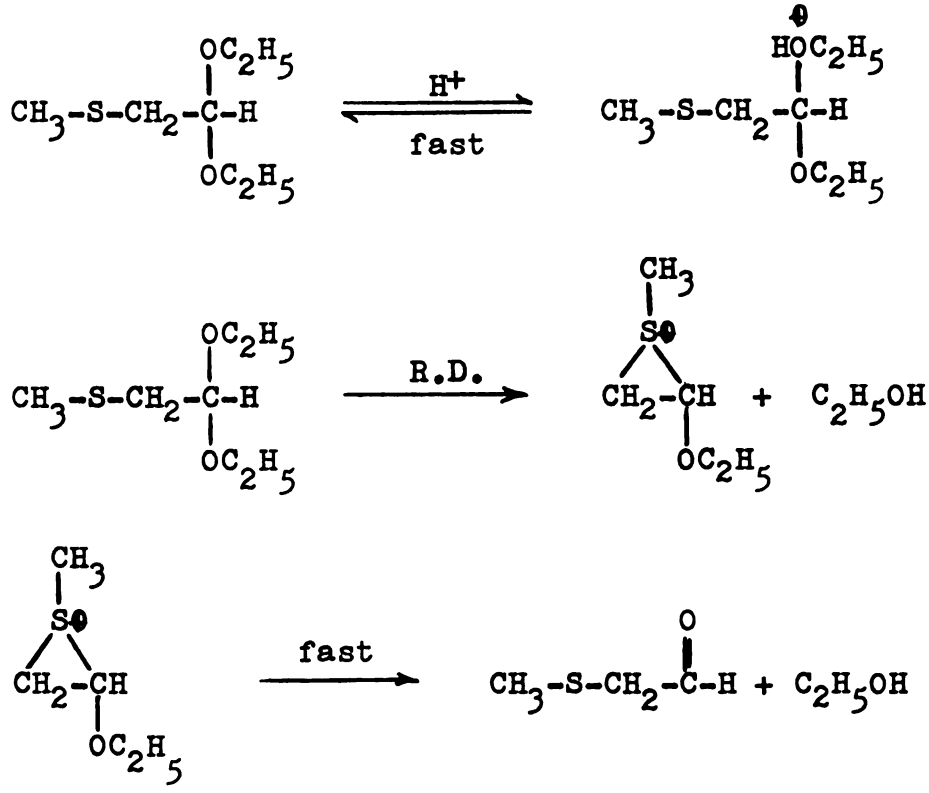
Acetaldehyde Diethyl Acetal

$k_2 = 0.254$ sec^{-1} mole^{-1}

The rate retardation observed in the hydrolysis of the substituted acetals is due to the inductive effect of the methylthio-, methoxy-, and ethoxy- groups.

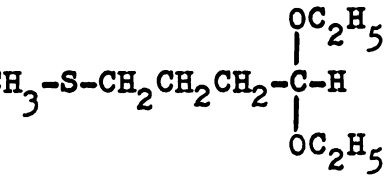
The inductive effects of these substituents as measured by the σ* values are very similar;[74,75] since these substituted acetals are structually very similar, the hydrolysis rates of these acetals should be very similar.

However, it is evident that this is not the case: 2-Methylthioacetaldehyde diethyl acetal hydrolyzes 100 times faster than the corresponding methoxy- and ethoxy- acetals. This large disparity in hydrolysis rates is best explained by assuming that the sulfur atom of the methylthio group is acting as a nucleophile and enhancing the hydrolysis rate of 2-methylthioacetaldehyde diethyl acetal by anchimeric assistance. The most probable mechanism involves the rate-determining formation of a cyclic sulfonium ion by the following mechanism:



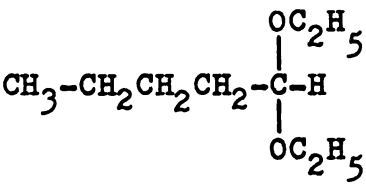
Enzymatic assays of 2-methylthioacetaldehyde diethyl acetal hydrolysis mixtures indicate that ethanol is released in a manner consistant with first order kinetics (Figure 7-8). No initial rapid release of ethanol is noted. This indicates that if an intermediate cyclic sulfonium ion is present during the hydrolysis of this acetal, its formation is the rate determining step of the reaction. If cyclic sulfonium ion decomposition were rate determining, a rapid release of one mole of ethanol per mole of acetal concomitant with the rapid formation of the ion would be apparent.

The hydrolysis rate of 4-methylthiobutraldehyde diethyl acetal differs very little from that of the parent acetal, valeraldehyde diethyl acetal:

IX 

4-Methylthiobutraldehyde Diethyl Acetal

$k_2 = 0.130$ sec^{-1} mole^{-1}

X 

Valeraldehyde Diethyl Acetal

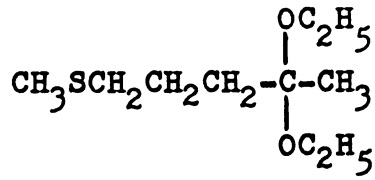
$k_2 = 0.184$ sec^{-1} mole^{-1}

In this case inductive effects are negated by the distance of the inductive group from the reactive site. Apparently no anchimeric assistance is operative in this case even though the formation of a 6-membered cyclic sulfonium ion would appear to be more favorable than the formation of a 3-membered cyclic sulfonium ion.

Ketal hydrolysis rates

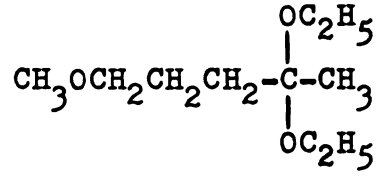
The hydrolysis rates of the ketals of 5-methoxy-2-pentanone and 5-methylthio-2-pentanone exhibit a small salt effect even when the second-order hydrolysis rates are corrected for secondary salt effects (Figures 4 and 5); therefore the hydrolysis rates of these ketals were extrapolated to zero ional strength. The magnitude of this residual salt effect is so small that no mechanistic implications may be attached to it.

The corrected hydrolysis rates of the substituted ketals are slightly slower than that of the parent ketal:

XI 

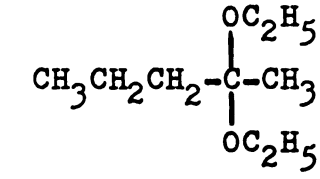
5-Methylthio-2-Pentanone Diethyl Ketal

$k_2 = 3.55 \times 10^{-2}$ sec^{-1} mole^{-1}

XII 

5-Methoxy-2-Pentanone Diethyl Ketal

$k_2 = 2.60 \times 10^{-2}$ sec^{-1} mole^{-1}

XIII 

2-Pentanone Diethyl Ketal

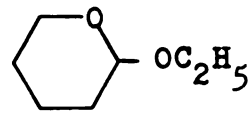
$k_2 = 8.59 \times 10^{-2}$ sec^{-1} mole^{-1}

Again, the disparity in hydrolysis rate is small when the substituted ketals are compared with the parent ketal and can be accounted for by small magnitude inductive and field effects. The rate of evolution of ethanol from 5-methylthio-2-pentanone diethyl ketal hydrolysis reaction mixtures is consistant with first-order kinetics and does not indicate the formation of a cyclic sulfonium ion (Figure 9).

Hydrolysis of tetrahydropyran ethers

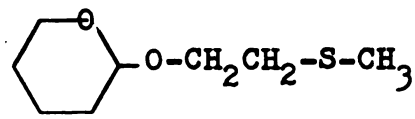
The 2-alkoxy substituted tetrahydropyran system bears a marked resemblance to structures commonly associated with glycosides. As such, the alkoxy-tetrahydropyrans would appear to be ideal model compounds in the study of glycoside hydrolysis since they allow direct observation of C_1-O bond hydrolysis free from aberrations created by adjacent hydroxyl groups. These ethers are therefore ideally suited for a demonstration of anchimeric assistance in glycoside model hydrolysis.

Unfortunately, the hydrolysis rates of all the alkoxy-substituted tetrahydropyrans which were investigated were quite similar:

XIV 

2-Ethoxytetrahydropyran

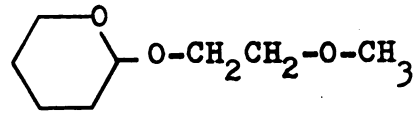
$k_2 = 1.53 \times 10^{-2}$ sec^{-1} mole^{-1}



XV

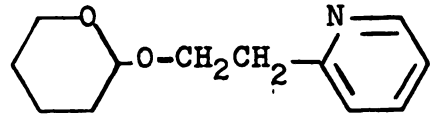
2-(2-Methylthioethoxy)-Tetrahydropyran

$k_2 = 1.61 \times 10^{-2}$ sec^{-1} mole^{-1}

XVI 

2-(2-Methoxyethoxy)-Tetrahydropyran

$k_2 = 2.05 \times 10^{-2}$ sec^{-1} mole^{-1}
1.73×10^{-2} sec^{-1} mole^{-1} (XVIII)

XVII 

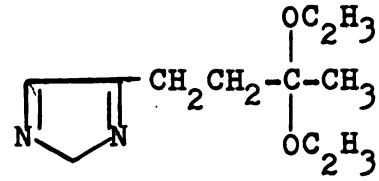
2-(2-Pyridinylethoxy)-Tetrahydropyran

$k_2 = 1.91 \times 10^{-2}$ sec^{-1} mole^{-1}
0.00 in acetate buffer

No evidence for anchimeric assistance is noted in the case of the hydrolysis of the tetrahydropyran ethers: XIV, XV, and XVI exhibit remarkably similar hydrolysis rates in dioxane-water. The hydrolysis rates of XVI and XVII in water as determined by hydrogen peroxide assay also are very similar. The latter results indicate that protonated heterocyclic nitrogen does not participate in anchimeric assistance. Since XVII did not hydrolyze at a measurable rate in acetate buffer of pH 4.64, a pH at which the pyridine nitrogen is only partially protonated, the possibility that unprotonated heterocylcic nitrogen participates in anchimeric assistance is also remote.

The lack of evidence for anchimeric assistance by a nitrogen-containing heterocycle is disappointing since previous experiments in this laboratory indicated that the

following imidazole-substituted ketal is capable of anchimeric assistance:[76]



4(5)-(γ-Oxobutyl)-Imidazole Diethyl Ketal

It should be noted that repeated attempts to synthesize a imidazole-substituted tetrahydropyran failed to yield a nitrogen-containing product.

It is apparent that the only probable instance of anchimeric assistance in the hydrolysis rate of the model glycosides discussed is that which occurs during the hydrolysis of 2-methylthioacetaldehyde diethyl acetal. This demonstration of anchimeric assistance by the sulfur atom of the methylthio group becomes more important when it is related to glycosidase catalysis. It is entirely possible that the methionine methylthio group participates in glycosidase catalysis with consequent formation of a covalent glycosyl-sulfonium ion-enzyme intermediate.

Lysozyme-Substrate Reactions

Non-aqueous reactions

Non-aqueous lysozyme-substrate reactions were carried out in nearly anhydrous glycerol (the small amount of water contributed by the water of hydration of lysozyme and substrate was impossible to exclude). Glycerol was chosen as a

solvent for several reasons: (a) glycerol does not appreciably perturb the native configuration of lysozyme,[77] (b) both lysozyme and substrate are slowly soluble in glycerol, and (c) lysozyme retains its enzymatic activity in glycerol as demonstrated by its ability to slowly solubilize M. lysodeikticus cell walls suspended in glycerol.

The very low concentration of water in the lysozyme-substrate reactions conducted in glycerol should decelerate the reaction between enzyme and substrate and allow a demonstration of the existence of a stable enzyme-substrate intermediate if one indeed exists.

Quenching of glycerol-solvated lysozyme-substrate reactions with dimethyl sulfoxide, followed by exhaustive washing procedures, produced positive results in the case of M. lysodeikticus Fr. 1 hexasaccharide-glycopeptide mixture (Table XIX). Hydrolysates of lysozyme Fr. 1 reaction mixtures yielded sufficient glucosamine and muramic acid to give glucosamine/phenylalanine ratios which ranged up to 0.30 (approximately one mole of glucosamine per mole of lysozyme).

Hydrolysates of lysozyme-M. lysodeikticus tetrasaccharide prepared under the same conditions yielded no detectable muramic acid or glucosamine. This set of experiments also indicated that 6 M guanidine hydrochloride solution is a much more effective denaturing agent and dialyzing agent than 8 M urea solution. The secondary and tertiary structure of lysozyme is completely disrupted by even 5 M guanidine

hydrochloride solutions;[78] this is not the case in urea solutions, even at 8 M concentrations.[79] Indeed, lysozyme displays a low level of lytic activity in 8 M urea as measured by lysis of bacteria[80] and solubilization of M. lysodeikticus cell walls.[81] This behavior probably accounts for the low level of binding observed when 8 M urea solutions were utilized as a dialysis medium; the lysozyme may have recovered some activity subsequent to quenching and prior to reduction and alkylation.

In contrast to the binding observed in "live" runs, glycerol-solvated blank runs utilizing reduced, N-ethylmaleimide-treated lysozyme and M. lysodeikticus Fr. 1 give no evidence for binding (Table XX) when the N-ethylmaleimide-treated lysozyme is completely inactive; the low level of binding observed in some of the blank runs can be attributed to the low level of lytic activity retained by these preparations (after this incident, the lytic activity of all blank lysozyme preparations was carefully checked).

Glycerol-solvated blank runs utilizing reduced, carboxymethylated lysozyme and M. lysodeikticus Fr. 1 also give no evidence for binding (Table XXI).

On the other hand, glycerol-solvated lysozyme-substrate reactions utilizing urea quenching gave equivocal results. "Live" runs utilizing urea quenching and carboxymethylation resulted in reaction mixture hydrolysates which contained substantial amounts of muramic acid and glucosamine (Table XXII). However, blank runs utilizing either reduced,

carboxymethyl lysozyme or reduced N-ethylmaleimide treated lysozyme had glucosamine/phenylalanine ratios of the same magnitude (Table XXIII).

Lysozyme-substrate blanks prepared without glycerol also gave hydrolysates containing substantial amounts of muramic acid and glucosamine (Table XXIV). Much of the difficulty encountered in these urea-quenched glycerol-mediated lysozyme-substrate reactions may have been due to the previously mentioned inadequacies of urea as a lysozyme denaturant; quenching will not be effective if it is not rapid and complete.

In summary, glycerol-mediated lysozyme-substrate reactions indicate that the M. lysodeikticus Fr. 1 hexasaccharide-glycopeptide mixture is the only substrate which demonstrates active binding under the conditions employed in these experiments. The highest glucosamine/phenylalanine ratio in the runs with active lysozyme was 0.30; even when this value is compared with the highest blank value of 0.16 (this blank is not analogous because it was prepared under conditions which were not identical with the high-labeling runs with active enzyme) a preferential binding of M. lysodeikticus cell wall hydrolysis products is indicated. These results are especially striking when compared with the negative labeling results encountered with M. lysodeiktucus tetrasaccharide.

Aqueous lysozyme-substrate reactions

Short-time lysozyme-substrate reactions carried out by quenching, washing and hydrolyzing 15-minute reaction mixtures confirmed and extended the observations carried out in glycerol (Table XXV).

N-acetylglucosamine tetramer and hexamer did not bind under aqueous reaction conditions (the observed glucosamine/phenylalanine ratio of 0.02 observed in several cases is the detection limit of the assay system; lower muramic acid and glucosamine concentrations yield no visible chromatogram peaks).

As observed in the glycerol-mediated lysozyme-substrate reactions, Fraction 1 from lysozyme hydrolysates of M. lysodeikticus cell walls strongly binds to reduced, alkylated lysozyme even after exhaustive reprecipitation.

The phenonomon of Fraction 1-lysozyme binding was clarified when Fraction 1 was separated into its component glycopeptides and hexasaccharide. Purified M. lysodeikticus hexasaccharide does not bind at all under the conditions employed in the aqueous labeling experiments (Table XXV). On the other hand, both M. lysodeikticus glycopeptide GP-1 and glycopeptide GP-2 appear to bind to lysozyme very strongly, as evidenced by the high glucosamine/phenylalanine ratio (0.15-0.29).

Inactivated lysozyme-glycopeptide blanks in some cases also exhibited apparent incorporation of substrate, but the level was always less than half that found in the

experiments with active lysozyme (Table XXVI).

It is now apparent that substrate binding in both non-aqueous and aqueous lysozyme-substrate reactions is observed only when M. lysodeikticus glycopeptide GP-1 or GP-2 is utilized as substrate.

In accord with the results obtained in the non-aqueous lysozyme-substrate reactions, the blank runs in the aqueous lysozyme-substrate reactions evidence substrate incorporation, (Table XXVII). However, the high level of incorporation demonstrated by M. lysodeikticus glycopeptides GP-1 and GP-2 indicates that they preferentially bind to native lysozyme under the conditions of these experiments. The interactions between lysozyme and glycopeptides GP-1 and GP-2 are very strong; the complex survives washing, reprecipitation, and gel filtration. The rigorous isolation methods, especially the gel filtrations, indicate that this complex may be covalent.

The nature of the binding observed with these glycopeptides was investigated by treating a lysozyme GP-2 complex of known substrate concentration with a solution of hydroxylamine. If the primary binding interaction is a covalent bond between lysozyme and glycopeptide created by the formation of an ester linkage between a substrate carboxyl group and an enzyme hydroxyl group or between a substrate hydroxyl group and an enzyme carboxyl group, then the hydroxylamine should liberate the substrate by cleaving the ester linkage and forming a hydroxamic acid. In this instance, hydroxy-

lamine did not appreciably lower lysozyme-glycopeptide GP-2 binding as evidenced by the glucosamine/phenylalanine ratio (Table XXVI). It appears that the primary binding force in the lysozyme GP-2 complex is not a covalent ester linkage.

A comparison of the structures of the binding- and non-binding-substrates indicates that the pentapeptide side chains of the glycopeptides are the distinguishing features of the binding substrates (Figure 10).

It should be noted that although glycopeptide GP-1 binds to lysozyme, it is the lysozyme-hydrolyzed analogue of GP-2.

Since all natural lysozyme substrates (bacterial cell walls) contain peptide chains, it is tempting to speculate that these peptide chains play an important part in the binding of substrate to lysozyme during the catalytic process. These peptide chains may play an important part in lysozyme catalysis by enhancing the enzymatically productive substrate orientations.

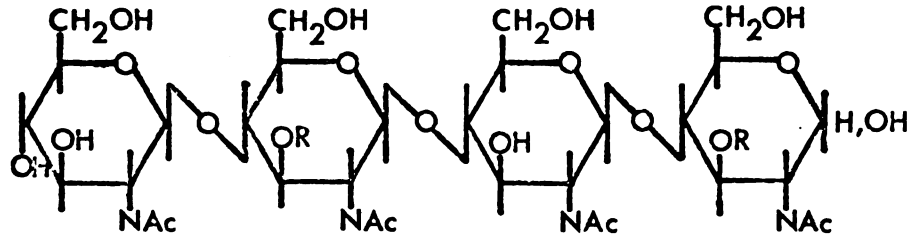
Hydrogen bonding,[82] hydrophobic bonding, and ionic bonding may be important in this type of substrate bonding phenomonon, but no definite assessment of their relative contributions may be made.

In summary, then, the glycoside model compound experiments indicate that stable covalent intermediates, especially those of the sulfonium ion type, may be of importance in glycosidase-transglycosidase catalysis. The lysozyme-substrate experiments indicate that a stable intermediate may be

Figure 10. Lysozyme substrates utilized in lysozyme-substrate reactions.

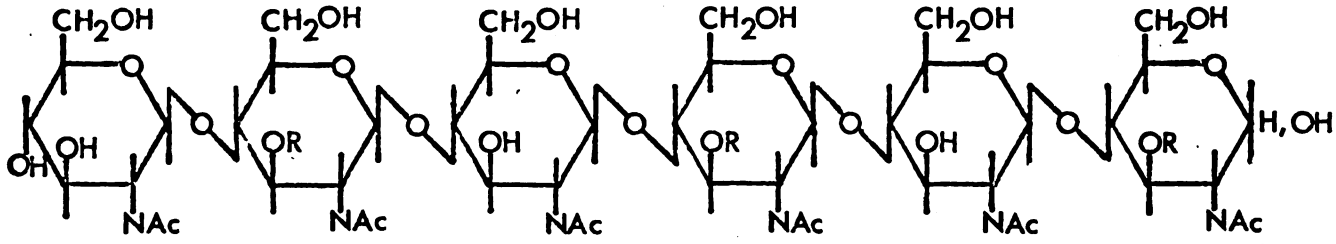
Figure 10. LYSOZYME SUBSTRATES

Non-Binding Saccharides



N-Acetyl glucosamine Tetramer: R=H

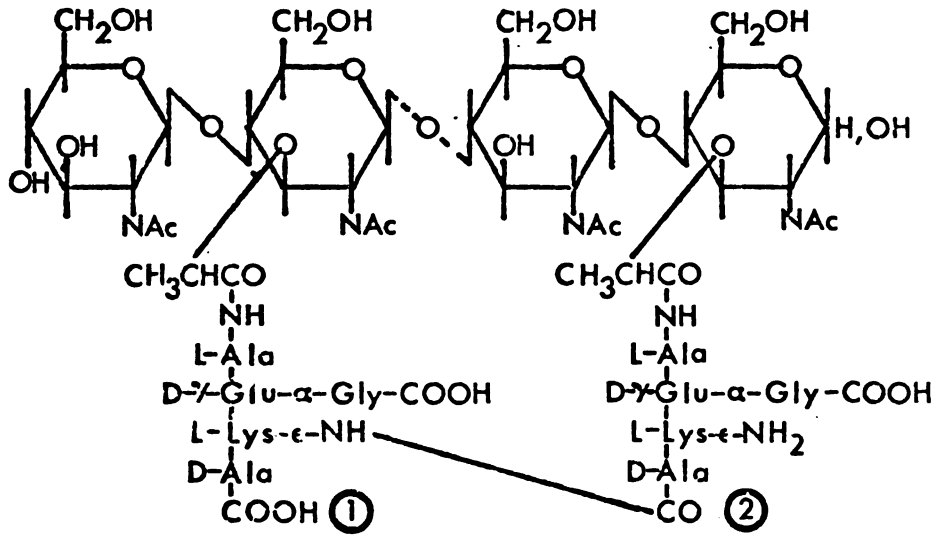
M. lysodeikticus Tetrasaccharide: R=$CH_3\dot{C}HCOOH$



N-Acetyl glucosamine Hexamer: R=H

M. lysodeikticus Hexasaccharide: R=$CH_3\dot{C}HCOOH$

Binding Glycopeptides



M. lysodeikticus Glycopeptide GP-1: dashed glycosidic linkage is absent (disaccharide-pentapeptide dimer)

M. lysodeikticus Glycopeptide GP-2: pentapeptide ① is absent (tetrasaccharide-monopentapeptide)

present in the reaction between lysozyme and M. lysodeikticus glycopeptides. The presence of this intermediate is not due to ester formation, and in all probability, it is not due to sulfonium ion formation.

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